Basic Elements of Toxicology

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1 INTRODUCTION

Toxicology, essentially addressing the potentially harmful effects of chemicals to living organisms, is now a universally recognized scientific and medical discipline devoted to a large and widespread number of basic and applied issues. Although only generally accepted as a specific and defined area of knowledge and investigation since the early part of the twentieth century, its principles and implication have been appreciated for aeons. Thus, the harmful and lethal effects of certain substances, plants, fruits, insect bites, animal venoms and minerals, have been known since prehistoric times. Indeed, the Greek, Roman and subsequent civilizations knowingly used certain substances and extracts for lethality in hunting, protection, warfare, suicide and homicide. Currently, activities in toxicology are mainly centred around, though not exclusively, determining the potential for chemicals, both naturally occurring and synthetic, to produce adverse effects, and as a consequence to assess hazard and risk from such chemicals to humans and lower animal forms, thus allowing the development of appropriate precautionary, protective, restrictive and therapeutic measures. For example, substances used, or of potential use, in commerce, the home, the environment and medical practice may present variable types of harmful effects, whose nature is determined by many factors, including particularly the physicochemical characteristics of the material, its potential to interact with biological materials and the pattern of exposure.

Toxicology investigations can have far-reaching implications for health-related issues in the workplace, commerce, home and general environment. For man-made and man-used materials, a balanced critical approach may be necessary in order to assess the risk–benefit ratio for their employment in specific circumstances, and to determine what precautionary and protective measures are needed for safe use. Indeed with drugs, pesticides, industrial chemicals, food additive and cosmetic/personal care preparations, mandatory toxicology testing and regulations exist. In the UK, safety evaluation toxicology has been closely associated with pathology and experimental pathology, but in many other countries, including the USA, toxicology has been regarded as a component or branch of pharmacology. However, with the growth of toxicology it has become a major multidisciplinary science with significant overlap into other health-related science.

2 HISTORICAL DEVELOPMENT OF TOXICOLOGY

As noted in Section 1 above, toxicology in formal terms has been regarded as a relatively young science. However, the origins of toxicology are ancient, and it is likely that man, unknowingly, undertook the first experiments in toxicology in searches for an acceptable diet on moving out of the habitat in which he evolved. It is likely that many of these early excursions had an unfortunate outcome. In Greek and Roman periods, poisons, generally of plant origin, were used for murder and suicide, whilst the potential danger of medicinal products and their adulterants has been recognized since Babylonian times. Poisoning for nefarious purposes has remained a problem ever since, and much of the earlier impetus to the development of toxicology had been primarily forensic. Another motivation for the development of toxicology was the careful description of adverse reactions to medicinal products that began to appear in the eighteenth century. Thus William Withering described digitalis toxicity in 1785, and around 1790 Hahnemann, the founder of homoeopathy, carried out toxicological experiments on himself and his healthy friends with therapeutic agents of his time, including cinchona, aconite, belladonna,

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ipecacuanha and mercury. The introduction of anaesthesia was followed by formal enquiries into sudden deaths during chloroform anaesthesia in the closing years of the nineteenth century.

During World War I, a variety of poisonous chemicals were used in the battlefields of northern France and Belgium. This was the stimulus for much work on mechanisms of toxicity, as well as medical countermeasures to poisoning, In fact war, or the prospect of war, played as great a part in the development of toxicology as of many other sciences. Much of the basic work on organophosphates (OPs) was stimulated by the discovery of these compounds by the Germans in the 1930s. Although defence considerations stimulated this work, much of it, particularly related to treatment, is applicable to the use in industry and agriculture of OP pesticides. Similarly, chelation therapy, initially studied in relation to organic arsenicals, is now used in the treatment of poisoning by many metals.

Occupational toxicology (see **Occupational Toxicology** and **Occupational Toxicology and Occupational Hygiene within the European Union (EU) Chemicals Regulation**) originated in the late 1600s and early 1700s with the writings of Bernardino Ramazzini, a professor of medicine in the Universities of Modena and Padua, and referred to as the father of occupational medicine. It gained impetus in the nineteenth century as a product of the industrial revolution, with early descriptions of occupational diseases induced by chemicals, such as cancer of the scrotum in chimney sweeps. Although in theory, but much less in practice, affected workers in the past had some remedies at law, major advances in the control of occupationally related diseases of chemical origin came in the period after 1960 with the setting of threshed limit values (TLVs) and occupational exposure limits (OELs). Additionally, in Western countries the increasing wealth of workers and the activities of their Unions have enabled them to make use of existing legal remedies and make representation in respect of needed improvements in occupational litigation. This has resulted in many companies having to take care of their workers and devote greater resources to industrial hygiene and occupational medicine. However, there are still clear needs for improvement in underdeveloped countries and with a few employers in Western countries.

Regulatory toxicology (see **Regulatory Toxicology**) has its origins in the development of the chemical and pharmaceutical industries in the nineteenth and twentieth centuries. The first toxicology regulation is often said to be the Alkali Act of 1863 (UK, 1863), which was intended to control pollution by heavy industry. Regulatory toxicology now accounts for a very large majority of expenditure on toxicology testing and administration. Toxicology has only come of age as a defined scientific discipline over the past few decades, as concern for worker and consumer health, and for the adverse environmental impact of xenobiotics, increased. Additionally, the growth of toxicology has been fuelled by a series of disasters such as Seveso, Bhopal, tri-*ortho*-cresyl phosphate (TOCP) poisoning incidents, methyl mercury incidents and the thalidomide tragedy, which threw up lacunae in knowledge concerning the toxic effects of substances, as well as the inadequacy of testing procedures. One of the earliest of such disasters occurred in the USA in 1937 and resulted in the deaths of 105 individuals from poisoning by an elixir of sulfanilamide containing the solvent diethylene glycol (DEG) (Calvery and Klumpp, 1939). This led to the passing of legislation in the form of the US Federal Food, Drug and Cosmetic Act, forbidding the marketing of new drugs until cleared for safety by the US Food and Drug Administration (FDA). Regulations have been elaborated at national, continental (European Union (EU) and North Atlantic Free Trade Area (NAFTA) and international levels. It is therefore disappointing and frustrating to note that contamination of medicinal formulations with DEG and resultant large-scale outbreaks of poisoning with acute renal failure, often with mortalities, has occurred on several occasions up to the present time, since the original poisoning outbreak in 1937. Most of these have been recorded in children from countries having generally lower standards of human care, and have included the following: Cape Town, 1969 (seven deaths from DEG-contaminated sedative formulations; Bowie and McKenzie, 1972); Bombay, India, 1986 (14 deaths from DEG-contaminated glycerine: Pandya, 1988); Nigeria, 1990 (47 deaths from DEG-adulterated paracetamol (acetaminophen) syrup: Okuonghae *et al*., 1992); Dhaka, Bangladesh, 1990–1992 (236 deaths of 339 children having acute renal failure from DEG-containing paracetamol (acetaminophen) elixir: Hanif *et al*., 1995); Haiti, 1996 (85 deaths in a follow-up group of 87 cases of acute renal failure from DEG-contaminated paracetamol (acetaminophen) syrup: O'Brien *et al*., 1998); Gurgaon, India 1998 (acute renal failure with 33 deaths from contaminated cough expectorant: Singh *et al*., 2001); New Delhi, India (encephalopathy and renal failure in 11 children from DEG-contaminated paracetamol elixir; Hari *et al*., 2006). In Argentina, during 1992, there were 15 deaths of 29 victims of poisoning from the use of DEG-containing propolis syrup (for upper respiratory infection) (Ferrari and Giannuzzi, 2005). A series of 64 Chinese adult patients (Guangdong Province) with severe liver disease received intravenous (iv) Armillarisin-A (3-acetyl-5-hydroxylmethyl-7-hydroxycoumarin) that contained DEG as solvent. Of these, 15 had confirmed DEG poisoning, of whom 12 died; findings in the poisoned patients included metabolic acidosis, acute renal failure and acute renal tubular necrosis with interstitial nephritis on renal biopsy (Lin *et al*., 2008). Subsequently five employees of the Qiqihar No. 2 Pharmaceutical Company of Heilongjiang Province, who

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were responsible for allowing DEG to be used in the production of Armillarisin A, were jailed for between four and seven years (EAASM, 2008). With respect to all these cases of lethal human DEG poisoning the lessons are clearly there for all, but unfortunately unlearned by many. In some cases the DEG contamination was accidental, but in others was financially driven. DEG, an inexpensive solvent, was more profitable to use than the more expensive propylene glycol or glycerine. Stricter pharmaceutical manufacturing oversight and enforcement is required on a worldwide basis (Wax, 1996).

The main international organizations currently regulating chemicals are the Codex Alimentarious Commission (CAC), and its committees, for food standards, and the Organization for Economic Cooperation and Development (OECD) for the standardization of test methods. The International Conferences on Harmonization (ICH) and the Veterinary International Conferences on Harmonization (VICH) have attempted to harmonize test requirements for pharmaceuticals, both human and veterinary. Because of the tendency for new test methods to be introduced, often without eliminating older tests, regulations have been inclined to become ever more complex, with the result that the cost of laboratory toxicology testing has become a significantly large administrative and economic segment of product development. However, more recently introduced test methods do not always imply added costs; thus the introduction of *in vitro* genotoxicity studies may permit the avoidance of costly and time-consuming long-term *in vivo* carcinogenicity bioassays. However, the complexity of toxicological regulations may imply, not only an effect on the profits of companies developing the chemical or drug, but also loss of potentially useful substances. In some cases this has led to sufficient disquiet for legislative action to be taken. Examples of this are the 'orphan drug' procedure in the USA, and the clinical trials exemption in the UK. Industrial effects of the increasing complexity of regulatory activity have been the need to establish regulatory departments within chemical and pharmaceutical organizations for the interpretation of, adherence to, and oversight of regulations, and to establish discussion channels with appropriate government departments on safety issues related to company products. Also, for reasons of size and costs, many companies have chosen not to develop their own toxicology testing facilities, or abandon or significantly reduce their own 'in-house' facilities. This has resulted in the significant growth of the contract toxicology testing industry.

Organochlorine insecticides probably averted an epidemic of typhus at the end of World War II, but it was the persistence of these compounds in the environment that was probably the greatest stimulus to the evolution of environmental toxicology. A major landmark in the evolution of this branch of toxicology was the publication by Rachel Carson of *Silent Spring* (Carson, 1962).

The past decades have seen a gradual shift in the emphasis of toxicology from its origins in acute toxicity, particularly human, to long-term and nontarget species toxicity. More or less in parallel, stress has changed from studies of natural, usually plant, compounds to products of chemical synthesis. Additionally, in recent years considerable resources have gone into testing for carcinogenic potential, whilst there have been extensive and intensive investigations into *in vitro* alternatives to animal toxicology testing.

Clinical toxicology, concerned with the causation, diagnosis and management of poisoning was originally under the control of general physicians in general hospitals. Much of the impetus for the subsequent development of clinical toxicology came from the activities of government defence research establishments. For example, chelation therapy for heavy-metal poisoning was discovered during searches for a method to treat organic arsenical poisoning during World War II, while oximes for OP poisoning were developed during the 'cold war' in the 1950s and 1960s (see above). As a distinct specialization, clinical toxicology is relatively new domain, having developed as a consequence of the fact that general physicians may not necessarily have access to the information required to treat their patients. Currently clinical toxicology has its own certification requirements, specialized societies and journals specifically devoted to the specialization. Poison information services developed during the 1950s in the USA and UK, and the concept has since spread throughout the world. Thus poisons information centres, which have access to information on thousands of drugs and chemicals that people may become accidentally or deliberately poisoned with, are to be found in major cities in most developed countries. In many cases, units exist, not only to back up clinicians with information and to act as a general advisory service to the general population, but also to carry out hands-on management of poisoning (see also **Susceptibility of Children to Environmental Xenobiotics**; **Ethical, Legal, Social and Professional Issues in Toxicology**).

A recent development was the recognition that differing toxicology requirements may be a barrier to free trade. Within major trading blocks such as NAFTA and the EU it has been necessary to elaborate common toxicological requirements for clearance of materials, while the agreement on the application of sanitary and phytosanitary methods ('SPS agreement', WTO, 1994), achieved after tortuous negotiations in 1994, requires that, in most circumstances, CAC maximum residue levels (MRLs) for food additives, contaminants and pesticides, be accepted for world trade purposes. This inevitably raised the profile of the international expert committees, such as the Joint Expert Committee on Food

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Table 1 Major driving forces for the development and increasing specialization of the scientific basis and applications of toxicology

- Exponential increase in the number of synthetically produced industrial and domestic chemicals
- Major increase in the numbers and nature of new drugs, pharmaceutical preparations, tissue-implantable materials and medical devices
- Increase in the number and nature of pesticides and related products
- Growing concern about the number of food-additive materials
- Increasing concern that environmental agents, including pesticides, chemical pollutants and naturally occurring toxins, are contributing to the causation and pathogenesis of diseases, in particular cardiovascular, neurological, pulmonary and neoplastic.
- Mandatory testing and regulation of chemicals and drugs used commercially, domestically and medicinally
- Enhanced public awareness of the potential for adverse effects from xenobiotics and naturally occurring chemicals to man, animals and the general environment
- The potential for interactions between occupational, domestic and environmental exposures and its influence on adverse effects
- Litigation, principally as a consequence of occupationally related illness, unrecognized or poorly documented product safety concerns and environmental harm. Recently, certain lawyers (of the ambulance-chasing type) are inserting advertisements on television to draw attention of the general public to adverse drug effects and offering their services for litigation against companies and prescribers (a practice that will have detrimental implications for future drug development)
- Activities of public awareness and 'watchdog' groups

Additives (JECFA) and the Joint Meeting on Pesticide Residues (JMPR).

Major, extensive and rapid developments in the scientific basis of toxicology and its practical applications have been obvious since the early 1950s. These developments have been a consequence of a variety of reasons, the major elements of which are listed in **Table 1**. Reflecting these developments has been a major proliferation in the number of textbooks and journals devoted to general and special aspects of toxicology; a proliferation of abstracting and information services; the provision of undergraduate and graduate courses and of certification requirements in general and applied toxicology; and the establishment of an industry devoted to toxicology testing and consultation. Along with these has been an increase in the number of professional organizations and certification boards specifically devoted to toxicology. As a consequence of the markedly expanded scope of toxicology, a number of circumscribed differing subdisciplines have emerged, and several major areas of subspecialization have been defined (**Table 2**).

An excellent informative demonstration on milestones in the history of toxicology has been prepared by Dr. Steven W. Gilbert and Dr. Antoinette Hayes, and is reproduced with their kind permission in **Figure 1**.

3 DEFINITION AND SCOPE OF TOXICOLOGY

Definitions of toxicology vary according to the relative importance that the defining author or authority ascribe to the component elements. Most toxicology considerations, and hence definitions, are concerned with the effects from natural or synthetic (man-made) substances, including biological toxins and a wide range of commercial and domestic chemicals. Thus, many definitions hinge around chemical toxicology. The central consideration in toxicology is the potential for the agent(s) under investigation to cause adverse (harmful) effects. Thus, in definitions of toxicology, adverse effects imply those that are detrimental to the survival or normal functioning of living organisms (Ballantyne, 1989). In view of this, some authorities and organizations have suggested that an overall definition should include 'catch all' causative agents that may produce adverse effects in biological materials. This has led to the inclusion of biological and physical agents in some overview definitions. The core of chemical toxicology is that of a discipline concerned with investigating the potential of chemicals, or mixtures of them, to produce harmful effects in living organisms and determining the implications of these effects; in this chemical-limited concept, chemicals cover naturally occurring substances (from plants, animals and microorganisms) and synthetic (man-made) materials, the latter often being referred to as xenobiotics. One overview definition covering the various elements of toxicology is as follows: 'Toxicology is a study of the interaction between chemical, biological and physical agents in biological organisms in order to quantitatively determine the potential for these agents to produce morphological and/or functional injury that results in adverse effects in living organisms, and to investigate the nature, incidence, mechanism of production, factors influencing

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Table 2 Major subspecialties of toxicology

Specialty	Major functional components
Laboratory	Design and conduct of *in vivo* and *in vitro* toxicology testing programmes
Regulatory	Administrative function concerned with the development and interpretation of mandatory toxicology testing programmes, and with particular reference to ensuring the safe use, handling and transportation of substances used commercially, domestically and therapeutically, and with the development of product-safety literature and labels
Clinical	The causation, diagnosis and management of poisoning in humans
Veterinary	The causation, diagnosis and management of poisoning in domestic and wild animals
Forensic	Establishing the cause for death or intoxication in humans, by analytical procedures, and with particular reference to legal processes
Occupational	Determining the potential for adverse effects from chemicals and other agents in the occupational environment and the development of appropriate screening procedures and precautionary and protective measures
Product	Assessing the potential for adverse effects from commercially produced chemicals and formulations, and development of recommendations on safe patterns, protective and precautionary measures, and development of relevant literature for users
Pharmacological	Assessing the toxicity of therapeutic agents
Aquatic	Assessing the toxicity to aquatic organisms of chemicals discharged into marine and fresh waters
Environmental	Determining the adverse effects of toxic pollutants, usually at low concentrations, released from commercial, industrial, domestic and natural sources into the immediate environment and subsequently widely distributed by air and water current and by diffusion through soil. It differs from ecotoxicology (see below) in focussing on the effects on individuals
Ecotoxicology	Determining adverse effects and impact from synthetic or natural pollutants on populations, communities, and terrestrial, freshwater and marine animal, vegetable and microbial ecosystems. If differs from environmental toxicology (see above) in that the aim is to integrate the effects at all levels of biological organization from molecular to whole communities, and therefore is a broader discipline.
Toxicology	Determining the toxicity of substances of biological origin, including plants, animals and pathogenic microorganisms

their development, and reversibility of such adverse effects'.

Within the scope of this definition, adverse effects are those that are detrimental to either the survival or the normal functioning of a living organism. Inherent in this definition are the following key elements in toxicology, particularly with respect to chemical toxicology:

1. Chemicals, or their conversion products, are required to come into close structural and/or functional contact with tissue(s) or organ(s) for which they have a potential to cause injury.
2. When possible, the observed toxicity (or an end point reflecting it) should be quantitatively related to the degree of exposure to the chemical (the exposure dose). Ideally, the influence of differing exposure doses on the magnitude and/or incidence of the toxic effect(s) should be investigated. Such dose–response relationships are of prime importance in confirming a causal relationship between chemical exposure and toxic effect, in assessing relevance of the observed toxicity to practical (in-use) exposure conditions, and to allow hazard evaluations and risk assessment.
3. The primary aim of most toxicology studies is to determine the potential for harmful effects in the intact living organism, in many cases (and often by extrapolation) to man.
4. Toxicological investigations should ideally permit the following characterization of toxicity to be evaluated:
   (a) The basic structural, functional or biochemical injury produced
   (b) The dose–response relationships
   (c) The mechanism(s) of toxicity, that is, the fundamental chemical and biological interactions and resultant aberrations that are responsible for the genesis and maintenance of the toxic response
   (d) The factors that may influence the toxic response, for example, route of exposure, species, gender, age, formulation of test chemical and environmental conditions
   (e) The development of approaches and methods for the recognition of specific toxic responses
   (f) The reversibility of toxic effects, either spontaneously or with treatment procedures.

The word 'toxicity' is used to imply the induction of adverse effects and to describe the nature of adverse effects produced and the conditions necessary for their induction, that is, toxicity is the potential for a material to produce injury in biological systems. In some related or subdisciples of toxicology, specific terminology may be used to describe adverse effects; for example, with pharmacologically active and therapeutic agents ('drugs')

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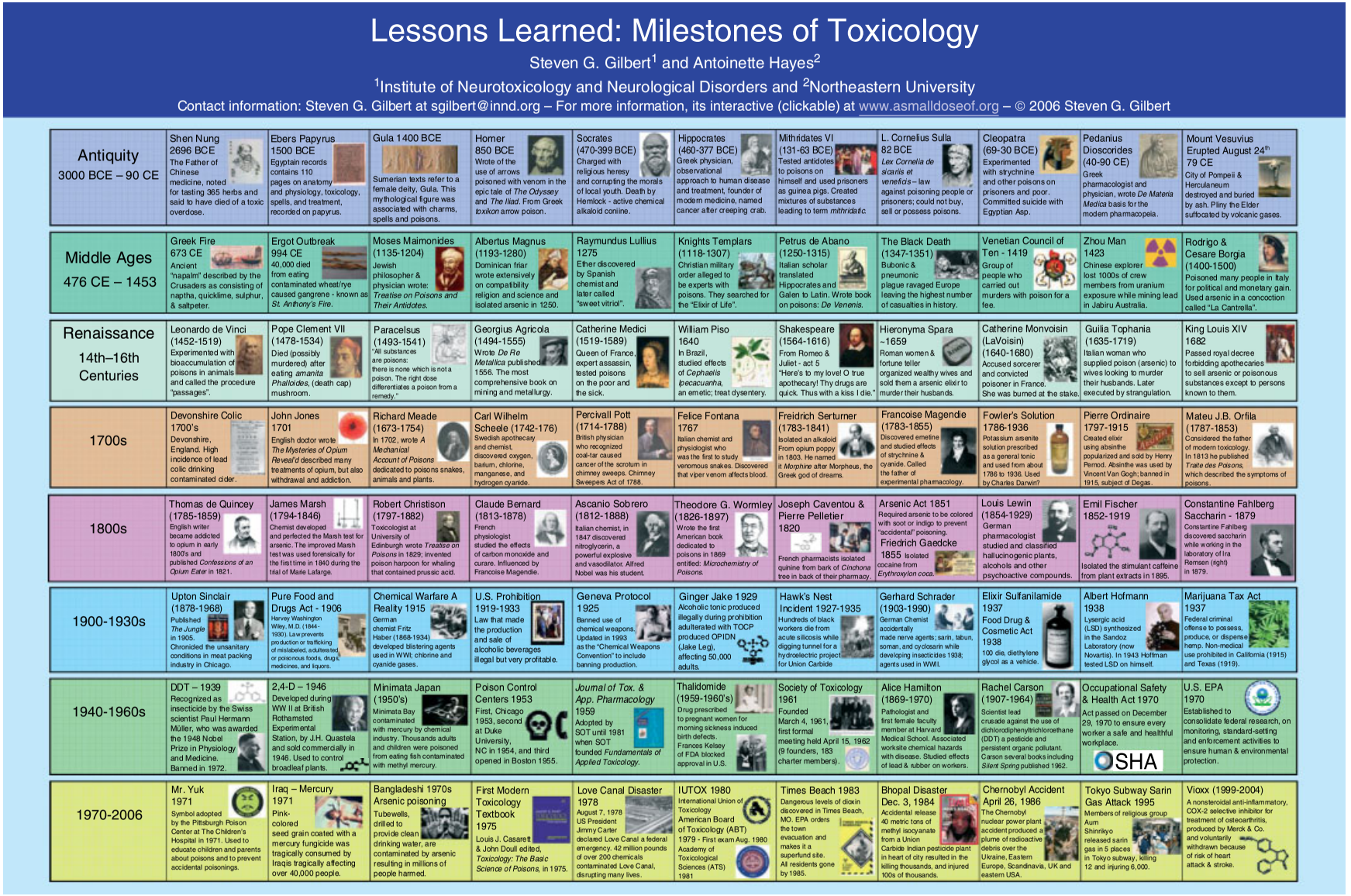

Figure 1 Milestones of Toxicology. Prepared by Dr. Steven W. Gilbert (Institute of Neurotoxicology and Neurological Disorders, Seattle and Dr. Antoinette Hayes (Northeastern University). Originally published in Toxipedia (*www.asmalldoseof.org*). (Reproduced with permission from Gilbert, Institute of Neurotoxicology and Neurological Disorders.)

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description of adverse or nondesired effects is most appropriately undertaken using certain specific terms, as discussed in Section 11.

Toxicity (i.e. the potential to injure) investigations require clear differentiation from the process of hazard evaluation, which determines the likelihood that a given material will exhibit its known toxicity under particular conditions of use.

4 DESCRIPTIVE TERMINOLOGY OF TOXIC EFFECTS

Precision in communication depends on a clear understanding of the definitions of technical and scientific terms in the context of their intended use. This section discusses the derivation and meanings of frequently used expressions in toxicology. A schematic representation of the basis for the general classification of toxic effects is given in **Figure 2**. Before toxicity can develop, a substance must come into contact with a body surface, such as skin, eye or mucosa of the alimentary or respiratory tract; these are, respectively, the cutaneous, ocular, peroral (po) and inhalation routes of exposure. Other routes of exposure, notably in experimental or therapeutic situations, are subcutaneous (sc), iv, intramuscular (im) and intraperitoneal (ip). Harmful effects that occur at the sites where a substance comes into initial contact with the body are referred to as local effects. If substances are absorbed from the sites of contact, they, or products of their bioconversion, may produce toxic effects in cells, tissues or organs remote from the site of exposure; these remote responses are referred to as systemic effects. Many substances may produce both local and systemic toxicity. Also, since the nature and probability of toxicity developing depends on the number of exposures, this forms an additional general means for classifying toxic effects into those developing after a single (acute) exposure or multiple (repeated) exposures. Repeated exposure toxicity can cover a wide timespan; however, it is descriptively convenient to refer to short-term repeated (not more than 5% of lifespan), subchronic (5–20% of lifespan) and chronic (entire lifespan or the major portion of it). Examples of toxic effects classified according to location and to number of exposures are given in **Table 3**.

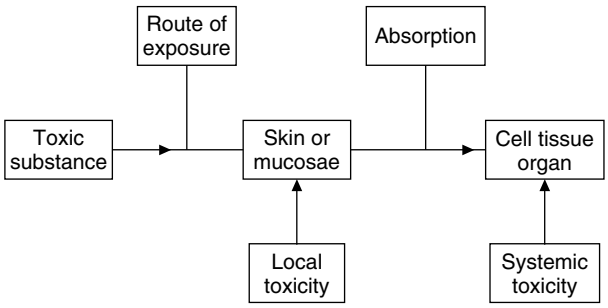

Figure 2 Schematic basis for the general classification of toxic effects.

Additional descriptions of toxicity are by the time to development and the duration of induced effects. Thus they may be described as temporary (reversible or transient) or permanent (persistent). Latent (delayed-onset) toxicity exists when there is a period free from signs following (usually) an acute exposure. Latent toxicity is of particular importance in clinical toxicology since individuals exposed to chemicals of known latency in toxicity should be kept under review in order that any delayed adverse effects may be both promptly recognized and treated. Cumulative toxicity involves progressive injury produced by summation of incremental injury resulting from successive exposures. Examples of toxicity according to the timescale for development and duration of effect are given in **Table 4**. Effects may also be classified, and described, according to the primary tissue or organ forming the target for toxicity, for example, hepatotoxic, nephrotoxic, neurotoxic, genotoxic, ototoxic, immunologic. A description of toxicity from a material requires inclusion of the following: if effects are local, systemic or mixed; their nature and (if known) mechanism of toxicity; organs and tissues affected and condition of exposure resulting in toxicity (including species, route and number or magnitude of exposure).

5 MORPHOLOGICAL AND FUNCTIONAL NATURE OF TOXIC EFFECTS

The nature and magnitude of a toxic effect depend on many factors, amongst which are the physicochemical properties of the substance, its bioconversion, the conditions of exposure, and the presence of bioprotective mechanisms. The last factor includes physiological mechanisms, such as adaptive enzyme induction, DNA repair mechanisms and phagocytosis. Some of the frequently encountered types of morphological and biochemical injury constituting a toxic response are listed below. They may take the form of tissue pathology, aberrant growth processes, altered or aberrant biochemical pathways, or extreme physiological responses.

Inflammation: This is a frequent local response to irritant chemicals or may be a component of systemic tissue injury. The inflammatory response may be acute with irritant or tissue-damaging materials, or chronic with repetitive exposure to irritants or the presence of insoluble particulate material.

Fibrosis: This production and accumulation of fibrous connective tissue may occur as a consequence of the inflammatory process.

Necrosis: Pathologically used to describe circumscribed death of tissues or cells, necrosis may result from

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Table 3 Examples of toxic effects classified according to timescale and location

Exposure time	Location	Effect	Substance
Acute	Local	Skin corrosion	Methylamine
		Lung injury	Hydrogen chloride
	Systemic	Kidney injury	Phenacetin
		Haemolysis	Arsine
	Mixed	Lung injury and methaemoglobinaemia	Oxides of nitrogen
Short-term	Local	Skin sensitization	Ethylenediamine
Repeated	Local	Lung sensitization	Toluene di-isocyanate
		Nasal septum ulceration	Chromates
	Systemic	Neurotoxicity	Acrylamide
		Liver injury	Arsenic
	Mixed	Respiratory irritation and neurobehavioural	Pyridine
Chronic	Local	Bronchitis	Sulphur dioxide
		Laryngeal carcinoma	Nitrogen mustard
	Systemic	Leukaemia	Benzene
		Angiosarcoma (liver)	Vinyl chloride
	Mixed	Emphysema and kidney injury	Cadmium
		Pneumonitis and neurotoxicity	Manganese

Table 4 Examples of toxic effects classified according to the time for development or duration of the lesion

Timescale	Effect	Substance
Persistent	Testicular injury	Dibromochloropropane
	Scarring (skin/eye)	Corrosives
	Pleural mesothelioma	Asbestos
Transient	Narcosis	Organic solvents
	Sensory irritation	Acetaldehyde
Cumulative	Squamous metaplasia	Formaldehyde
	Liver fibrosis	Ethanol
Latent	Pulmonary oedema	Phosgene
	Peripheral neuropathy	Anticholinesterase organophosphates
	Pulmonary fibrosis	Paraquat

a variety of pathological processes induced by chemical injury, for example, corrosion, severe hypoxia, membrane damage, reactive metabolite binding, inhibition of protein synthesis and chromosome injury. With certain substances, differing patterns of zonal necrosis may be seen. In the liver, for example, galactosamine produces diffuse necrosis of the lobules (Mehendale, 1987), acetaminophen (paracetamol) mainly centrilobular necrosis (Goldfrank *et al*., 1990) and certain organic arsenicals peripheral lobular necrosis (Ballantyne, 1978).

Enzyme inhibition: This may decrease biological activity in biologically vital pathways, producing impairment of normal function. The induction of toxicity from enzyme inhibition may be due to accumulation of substrate or to deficiency of product or function. For example, OP anticholinesterases produce toxicity by accumulation of acetylcholine at cholinergic synapses and neuromuscular junctions (Ellenhorn and Barceloux, 1988). Cyanide inhibits cytochrome oxidase and interferes with mitochondrial oxygen transport, producing cytotoxic hypoxia (Ballantyne, 1987).

Biochemical uncoupling: Agents capable of biochemical uncoupling interfere with the synthesis of high-energy phosphate molecules, but electron transport continues resulting in excess liberation of energy as heat. Thus, uncoupling produces increased oxygen consumption and hyperthermia. Examples of uncoupling agents are dinitrophenol and pentachlorophenol (Williams, 1982; Kurt *et al*., 1988).

Lethal synthesis: This occurs when foreign substances of close structural similarity to normal biological substrates become incorporated into biochemical pathways and are then metabolized to a toxic product. A classical example is that of fluoroacetate, which becomes incorporated in the Krebs cycle as fluoroacetyl coenzyme A, which combines with oxaloacetate to form fluorocitrate. The latter inhibits aconitase, blocking the tricarboxylic acid cycle and results, particularly, in cardiac and nervous system toxicity (Albert, 1979).

Lipid peroxidation: In biological membranes free radicals start a chain of events causing cellular dysfunction and death. The complex series of events includes

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oxidation of fatty acids to lipid hydroperoxides which undergo degradation to various products, including toxic aldehydes. The generation of organic radicals during peroxidation results in a self-propagating reaction (Horton and Fairhurst, 1987). Carbon tetrachloride, for example, is activated by a hepatic cytochrome P450-dependent mono-oxygenase system to the trichloromethyl and trichloromethyl peroxy radicals; the former radical probably covalently binds with macromolecules and the latter initiates the process of lipid peroxidation leading to hepatic centrilobular necrosis. The zonal necrosis is possibly related to high cytochrome P450 activity in centrilobular hepatocytes (Albano *et al*., 1982).

Covalent binding: Electrophilic reactive metabolites covalently binding to nucleophilic macromolecules may have a role in certain genotoxic, carcinogenic, teratogenic and immunosuppressive events. Important cellular defence mechanisms exist to moderate these reactions, and toxicity may not be initiated until these mechanisms are saturated.

Oxidative stress: Oxidative stress induces injury to cells by excessive production of reactive oxygen species (ROS) having high reactivity against DNA, lipids and proteins (Pérez *et al*., 2006).

Receptor interaction: Occurs at a cellular or macromolecular level and involves specific chemical structures modifying the normal biological effect mediated by the receptor; these may be excitatory or inhibitory. An important example is effects on Ca^{2+} channels (Braunwald, 1982).

Endocrine disruption: A number of xenobiotics found in both human community and wildlife environments have been shown to have a potential to disrupt endocrine functions. Endocrine disrupting effects of chemicals have a potential to cause reproductive problems and increase the risk for development of endocrine-dependent cancers. Interference with steroid biosynthesis may have adverse effects on sexual differentiation, growth and development (Sanderson, 2006). Additionally, there have been studies on the interactions between xenobiotics and hormone receptors, particularly thyroid hormone, oestrogen and androgen receptors.

Immune-mediated hypersensitivity: Such reactions stimulated by antigenic materials are particularly important considerations for skin and lung resulting in allergic contact dermatitis, respiratory sensitization and asthma (Cronin, 1980; Brooks, 1983; Bardana *et al*., 1992; Isola *et al*., 2008; see also **Cytogenetics**; **Allergic Asthma and Rhinitis: Toxicological Considerations**; **Assessing Impacts of Environmental Contaminants on Wildlife**). Skin-sensitizing potential, a Type VI hypersensitivity reaction, can be investigated by standard *in vivo* studies in laboratory animals, such as occluded patch tests (Buehler, 1965) and the guinea-pig maximization procedure (Magnusson and Kligman, 1969; Magnusson and Kligman, 1970), and by induction of the response by the local lymph node proliferation assay (Gerberick *et al*., 2000; Hilton *et al*., 1998; Kimber *et al*., 1994; 2001; Ryan *et al*., 2008), and where considered necessary confirmed by the human repeat insult patch test. Recently, various changes in epidermal Langerhans cells have been suggested as possibly forming the basis for the development of *in vitro* assays for assessing skin sensitization potential (Ryan *et al*., 2005). Respiratory sensitization, mediated by IgE- and Th2-cell responses, cannot be predicted by conventional repeated inhalation exposure studies, but can addressed by studies such as the mouse IgE test and cytokine fingerprinting (Dearman and Kimber, 2001; Dearman *et al*., 2003; Kimber *et al*., 2002; Holsapple *et al*., 2006).

Immunosuppression: Suppression of the immune system by xenobiotics may have important repercussions with respect to increased susceptibility to infective agents and certain aspects of tumorigenesis.

Neoplasia: Agents stimulating neoplasia cause an aberration of tissue growth and control mechanisms of cell division, and result in abnormal proliferation and growth. This is a major consideration in repeated exposure to xenobiotics. The terms tumorigenesis and oncogenesis are general expressions used to describe overall the development of neoplasms; the word carcinogenesis should be restricted specifically to malignant neoplasms. In experimental and epidemiological situations, oncogenesis may be exhibited as an increase in the total number of neoplasms, an increase in specific types of neoplasm, the occurrence of 'rare' or 'unique' neoplasms or a decreased latency to detection of neoplasms.

Chemical carcinogenesis is a multistage process. Simplistically, the first, and critical, stage is a genotoxic event followed by other processes leading to the pathological, functional and clinical expression of neoplasia. One multistep model that has received much attention is the initiator–promoter scheme (**Figure 3**). The first stage, that of initiation, requires a brief exposure to a genotoxically active material which results in binding of the initiator or reactive metabolite to cellular DNA; there is a low, or no, threshold for this initiation stage. The second stage, that of promotion, permits the expression of the carcinogenic potential of the initiated cell. Promoting agents have the following characteristics:

- They need not be genotoxic
- Pepeated exposure is required after initiation
- They show some evidence for reversibility
- They may have a threshold for promoting activity.

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Scheme	Initiator–promoter relations	Neoplasm
A	I	No
B	I P P P P P P P P P	Yes
C	I P P P P P P P P P P P	Yes
D	I P P P P P P P P P P	Low/no
E	P P P P P P P P P P P P P P P P	No
F	P P P P P P P P P P P I	No
G	I I I I I I I I I I	Yes

Figure 3 Schematic representation of inter-relationships between initiator and promoter in the two-stage mechanism of carcinogenesis: (A) an initiating dose of a genotoxic carcinogen is not by itself oncogenic; (B) if the initiating dose is followed by multiple applications of an epigenetic promoter, neoplasia results (the classical initiator–promoter relationship); (C) if promotion is delayed following initiation, a neoplastic response occurs indicating a persisting initiating effect; (D) if promoter dosing is infrequent or doses are small, there may not be a neoplastic response or a low tumour incidence, indicating a threshold for the the promoting effect; (E) multiple applications of an epigenetic promoter alone due not result in neoplasia; (F) initiation must precede promotion; (G) a genotoxic carcinogen may act as both initiator and promoter.

Genotoxic initiators may also act in a promotional manner following initiation. Substances causing or enhancing a carcinogenic process may be conveniently described as genotoxic and epigenetic carcinogens; the former are capable of causing DNA injury and the latter exert onocogenic effects by mechanisms (epigenetic) other than direct genotoxicity. Genotoxic materials acting directly with DNA are referred to as primary carcinogens; those requiring metabolic activation are procarcinogens, with the metabolically active electrophile being the ultimate carcinogen. Examples of primary carcinogens include alkylene epoxides, sulfate esters and nitrosoureas; procarcinogens include polycyclic aromatic hydrocarbons, aromatic amines, azo dyes and nitrosamines.

Epigenetic carcinogens include the following differing functional classes: promoters, cocarcinogens, hormones, immunosuppressives and solid-state materials. Cocarcinogens, when applied just before or with genotoxic carcinogens, enhance the oncogenic effect. Various mechanisms may cause enhancement, including increased absorption, increased metabolic activation of procarcinogen, decreased detoxification or inhibition of DNA repair. One group of epigenetic carcinogens are the peroxisome proliferators, which induce liver tumours in experimental rodents. These materials produce hepatomegaly and hepatocyte peroxisome proliferation and induce several liver enzymes, including those of the peroxisomal fatty acid β-oxidation system. Phthalate esters are one class of compound producing peroxisomal proliferation and experimental hepatocarcinogenesis (Rao and Reddy, 1987). A threshold may exist with epigenetic carcinogens, but there is disagreement about the way in which data from studies with epigenetic carcinogens should be analysed for risk-assessment purposes.

5.1 Genotoxicity

Genotoxic chemicals, which interact with DNA and possibly lead to heritable changes, may be conveniently classified as clastogenic or mutagenic.

Clastogenic effects occur at the chromosomal level and are usually visible by light microscopy. They may involve simple breaks, rearrangement of segments, or gross destruction of chromosomes. Severe clastogenic effects may be incompatible with normal function, and cell death occurs. The relevance of chemically induced sublethal cytogenetic effects is not totally understood, but could lead to dysfunction of the reproductive system and other tissues with rapid cell turnover rates (see **Genetic Toxicology Testing and its Relevance to Human Risk and Safety Evaluation**; **Carcinogenesis and Carcinogens that are also Genotoxic**).

Mutagenic effects are focal molecular events in the DNA molecule, which involve either substitution of a base pair or deletion or addition of a base. Base-pair transformations ('point mutations') may occur by direct chemical transformation, incorporation of abnormal base analogues or alkylation. Addition or deletion of a base will result in a disturbance of the triplet code and hence alteration of the codon sequence distal to the addition or deletion ('frameshift mutation'). Intracellular DNA repair enzymes are present, but if the capacity of repair mechanisms is exceeded then abnormal coding will be transcribed into RNA and expressed as altered protein structure and possibly function, depending on the molecular segment affected. As noted above, it is considered that in genotoxic carcinogenesis the molecular DNA event of initiation is fundamental to multistage oncogenesis. There is now considerable evidence showing a good correlation (with some test systems) between carcinogenic and mutagenic potential. Thus, the use of certain mutagenicity tests procedures has become widely accepted as a means of screening chemicals for their carcinogenic potential (Krisch-Volders, 1984; Brusick, 1988; see **Genetic Toxicology Testing and its Relevance to Human Risk and Safety Evaluation**).

Over the past decade there has been considerable interest and major developments in the field of toxicogenomics (Borlak, 2005; Hamadeh and Afshari, 2004; see **Reactive Oxygen Species in the Induction of Toxicity**). Toxicogenomics is concerned with a study of

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the complex interactions between the cellular genome, toxic agents, organ dysfunction and the disease state (Gatzidou *et al*., 2007). Following exposure of an organism to a toxic agent there is a cellular response exhibited as an alteration of the gene expression, which through transcription into messenger RNA (mRNA) results in altered protein synthesis and function. A major technical advance relating to defining molecular events in toxicogenomics is that of microarray technology, which permits the simultaneous analysis of the transcriptional expression level for thousands of individual genes under various physiological conditions (de Longueville *et al*., 2004). As a molecular complement, post-transcriptional and post-translational events can be investigated using high-throughput proteomics or wider metabolic aspects by metabolomics (Robertson, 2005). These approaches permit a detailed molecular perspective on how an organism responds to stress or xenobiotics. The applications of toxicogenomics have been broadly classified into two overlapping areas: mechanistic research and predictive toxicology (Cunningham, 2006; Cunningham *et al*., 2003; Gatzidou *et al*., 2007; Pennie *et al*., 2000). Transcript profiling is a major factor for understanding a mode of action and the detection of the induction of patterns of gene expression changes, and is the basis for predictive toxicology. Studies indicate that short-term gene expression profiles are likely to have significant potential for predicting carcinogenicity (Ellinger-Ziegelbauer *et al*., 2004; Fielden *et al*., 2008). Microarray technology has become a powerful tool to explore the expression levels of thousands of genes or even complete genomes after exposure to xenobiotics, but a major current challenge in toxicogenomics is functional interpretation, linking potentially inter-related alterations in gene expression to conventional toxicological end points (Moggs, 2005; Yu *et al*., 2006).

5.2 Reproductive and Developmental Toxic Effects

These aspects of toxicology are concerned with, respectively, adverse effects on the ability to conceive and adverse effects on the structural and functional integrity of the conceptus up to and around parturition.

Adverse effects on reproduction may result from a variety of differing mechanisms on reproductive organs and their neural and endocrine control mechanisms (Barlow and Sullivan, 1982). Developmental toxicity deals with adverse effects on the conceptus from the stage of zygote formation, through the stages of implantation, germ layer differentiation, organ formation and growth processes during intrauterine development and the neonatal period. The most extreme toxicity, death, may occur as preimplantation loss, embryo resorption, foetal death or abortion. Nonlethal foetotoxicity may be expressed as delayed maturation, including decreased body weight and retarded ossification. Structural malformations (morphological teratogenic effects) may be external, skeletal or visceral. The preferential susceptibility of the conceptus to chemical (and other environmental) insults in comparison with the adult state is related to: (i) the small numbers of cells and their rapid proliferation rates; (ii) a large number of nondifferentiated cells lacking defence capabilities; (iii) requirements for precise spatial and temporal interactions of cells; (iv) limited metabolic capacity and (v) immaturity of the immunosurveillance system (Tyl, 1988). There is now considerable awareness that functional, in addition to structural, malformations of development may occur, and this is reflected in the increased monitoring in developmental toxicity studies. Malformation from chemical exposure may result from, amongst other mechanisms: (i) genotoxic injury; (ii) interference with nucleic acid replication, transcription or translation; (iii) essential nutrient deficiency and (iv) enzyme inhibition. The most sensitive period for induction of structural malformations is during organogenesis. Functional teratogenic effects may be induced at later stages, particularly neurobehavioural disturbances (Rodier, 1980).

5.3 Pharmacological Effects

These may be induced by drugs and chemicals generally and usually involve interaction between causative agent and cell receptors resulting in suppression or exaggeration of physiological functions expressed normally by the involved receptor(s). This differentiates pharmacological effects from toxic effects, with the latter being, by definition, adverse in nature. The receptor–agent complex is reversible and thus the induced effects are transient. However, marked (potent) pharmacological effects may cause significant physiological hyper- or hypofunction, and in these cases there are borderline or overlapping considerations with toxicity. Pharmacological effects can be a cause of temporary incapacitation or inconvenience in the occupational environment, as well as side-effects of medication. For example, narcosis from acute overexposure to an organic solvent may clearly be of relevance in safe workplace considerations; such a reversible narcosis needs to be differentiated from central nervous system injury resulting from long-term, low-concentration solvent exposure (World Health Organization, 1985). Another important pharmacological effort, particularly from airborne materials in the workplace, is peripheral sensory irritation. Materials having such effects interact with sensory nerve receptors in skin or mucosae, producing local discomfort and related reflex effects. For example, with the eye there is pain or discomfort, excess lacrimation and blepharospasm. Although such effects are

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warning and protective in nature, they are also distracting and thus likely to predispose to accidents. For this reason, peripheral sensory irritant effects are widely used in defining, along with other considerations, exposure guidelines for workplace environments (Ballantyne, 1984; see **The Influence of Temperature on Toxicity**).

6 DOSAGE–RESPONSE RELATIONSHIPS

6.1 General Considerations

A fundamental concept in biology is that of variability. Individual members of the same grouped species and strain differ to variable degrees with respect to their biochemical, cellular, tissue, organ and overall characteristics. Additionally, within a given individual there is a spectrum of variability in certain features, for example, cell size and biochemical function within a particular cell series. The differences between individuals are, at least in part, a consequence of genetic factors and age. Since toxicity is the result of adverse effects on biological systems, or modifications of defence mechanisms, it is not unexpected that the majority of toxic responses will also show variability between individuals of a given strain. Also, because of genetic and biochemical variability, even larger discrepancies in response will be observed between species. It is axiomatic to the toxicologist that, within certain limits and under controlled conditions, there is a positive relationship between the amount of material to which given groups of animals are exposed and the toxic response, and that the response of a given animal may differ quantitatively from that of other animals in the same dosage group. As the amount of material given to a group of animals is increased, so does the magnitude of the effect and/or the number who are affected. For example, a specific amount of a potentially lethal material given to a group of animals may not kill all of them; however, as the amount of material is increased, so the proportion dying increases. This reflects the variability in the susceptibility of the population studied to the lethal toxicity of the test substance. Likewise, if an irritant material is applied to the skin, as the amount is increased over a given area this is associated with: (i) an increase in the number of the population affected and (ii) an increase in the severity of the inflammation. For the two examples given above, death is an 'all-or-none' response (a quantal response), whereas inflammation may be considered from a dose–response viewpoint as having two elements, that is, its presence or otherwise, and the degree of inflammation which represents a continuous (or graded) response. The above considerations, which reflect variability in biological systems, form the basis for the fundamental concept of dose–response relationships in both pharmacology and toxicology, there usually being a positive relationship between dose and response *in vivo* and in many *in vitro* test systems.

It follows from the above discussion that the amount of material to which an organism is exposed is one prime determinant of toxicity. The dose–response relationships for differing toxic effects produced by a given material in a particular species may vary. Thus, as discussed later, dose–response relationships have to be carefully interpreted in the context of the effect studied and the particular conditions under which the information was collected.

The word 'dose' is most frequently used to denote the total amount of material to which an organism or test system is exposed, and 'dosage' defines the amount of material given in relation to a recipient characteristic (e.g. weight). Dosage allows a more meaningful and comparative indicator of exposure. For example, 500 mg of a material given as a po dose to a 250 g rat or a 2000 g rabbit will result in dosages of 2 and 0.25 mg (kg body weight)$^{-1}$, respectively. It follows that comparative dosing studies should be expressed in dosage units. Dose in most reports usually implies the exposure dose, that is, the total amount of material that is given to an organism by the particular route of exposure, or the amount incorporated into a test system. Another expression of dose is absorbed dose, which is the amount of material penetrating into the organism through the route of exposure. Absorbed dose may show a closer quantitative relationship with systemic toxicity than exposure dose, since it represents the amount of material directly available for metabolic interactions and systemic toxicity. A further expression of dose is target-organ dose, which is the amount of material (parent or metabolite) received at the organ or tissue exhibiting a specific toxic effect of interest. This should be expressed (if possible) in terms of the mechanistically causative molecule (parent chemical or reactive metabolite). Clearly, target-organ dose is a more precise quantitative indication of toxicity than exposure dose, since it is a measure of the amount of material at the site of toxicity, whereas exposure dose is total dose to the organism and only a proportion of this (or a metabolite) will ultimately gain access to the target site(s) for the toxic response. However the estimation of target or organ-tissue dose requires a detailed knowledge of the pharmacokinetics and metabolism of the material. For this reason, most information relates to the exposure dose.

The exposure dose is of practical importance since it reflects the amount of material to which the organism is actually exposed and its relationship to the likelihood of the development of a particular toxic end point, and therefore is of particular use for hazard-evaluation purposes. Absolute target-organ doses, on the other hand, allow a more detailed biological evaluation of toxicity in relation to bioavailable chemical, and when related to

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exposure dose may be used for rational risk-assessment procedures.

If a material is capable of inducing several differing types of toxicity, the dose (or dosage) of material required to cause the individual effects may differ, with the more sensitive toxic effect appearing at the lower dosages. The first distinct toxicity, at lower dosages, may not necessarily be the most logically significant effect. For example, with epicutaneously applied materials, local inflammation may appear before more sinister systemic toxicity. Conversely, if the most significant toxicity occurs at lower dosages, then other toxicity at higher dosages may be of lesser interpretive significance.

6.2 Expression of Dosage–Response Relationships

As discussed above, with a given population there is a quantitative variability in susceptibility to a chemical by individual members of that population. Thus, with a genetically homogeneous population of animals of the same species and strain, the proportion exhibiting a particular toxic effect will increase as the dosage increases. This is shown schematically in **Figure 4** as a cumulative frequency distribution curve, where the number of animals responding (as a proportion of the total in the group) is plotted as a function of the dosage given (as a $\log_{10}$ function). In many instances there is a sigmoid curve, with a log-normal distribution and being symmetrical about the mid-point. This is a typical dosage–response relationship, often loosely referred to as a dose–response relationship. There are several important elements to this curve that require consideration when interpreting its toxicological significance:

- The majority of individuals responding do so symmetrically around the mid-point (i.e. the 50% response value). The position of the major portion of the dosage–response curve around its mid-point is sometimes referred to as the potency.
- The mid-point of the curve (50% response point) is a convenient description of the average response, and is referred to as the median effective dosage (ED_{50}) for the effect being considered. If mortality is the end point, then this is specifically referred to as the median lethal dosage (LD_{50}). The ED_{50} is used for the following reasons: (i) it is at the mid-point of a log-normally distributed curve and (ii) the 95% confidence limits are narrowest at this point.
- A small proportion of the population, at the left-hand side of the dosage–response curve, respond to low dosages; they constitute a hypersusceptible or hyper-reactive group.
- Another small proportion of the population, at the right-hand side of the curve, do not respond until higher dosages are given; they constitute a hyposusceptible or hyporeactive group.
- The slope of the dosage–response curve, particularly around the median value, gives an indication of the range of doses producing an effect. It indicates how greatly the response will be changed when the dosage is altered. A steep slope indicates that a majority of the population will respond over a narrow dosage range, and a shallower slope indicates that a much wider range of dosages is required to affect the majority of the population.

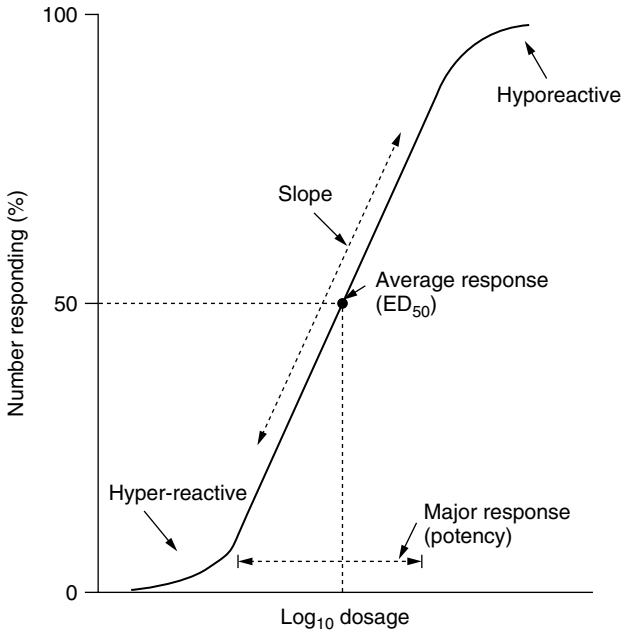

Figure 4 Sigmoid dosage–response curve for a toxic effect plotted as proportionate response against $\log_{10}$ dosage. The curve is typically symmetrical about the average (50% response) point. The major response (potency) occurs around the average response. The slope of the curve is is determined by the increase in response as a function of incremental increases in dosage. Hyper-reactive and hyporeactive individuals in the group are at the extreme left- and right-hand sides of the curve, respectively.

The shape of the dosage–response curve, and its extreme portions, depend on a variety of endogenous and exogenous factors; the former may include cellular defence mechanisms and reserves of biochemical function. Thus, toxicity may not be initiated until cellular defence mechanisms are exhausted, or a biochemical detoxification path is near saturation. Also, saturation of a biochemical process that produces toxic metabolites may result in a plateau for toxicity.

An important variant of the sigmoid dosage–response curve may be seen with genetically heterogeneous populations, where the presence of an usually high incidence in the hypersusceptible area could indicate the existence

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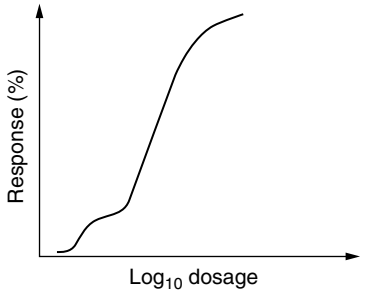

Figure 5 Variant of the sigmoid cumulative dosage–response curve resulting from an enhanced hyper-reactive response. This can represent a genetic variant in a proportion of the population causing an enhanced sensitivity to the toxic effect.

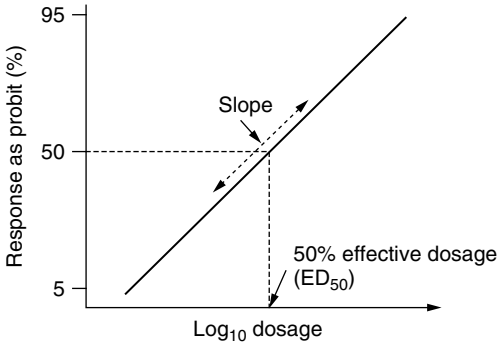

Figure 6 Linear transformation of dosage–response data by log-probit plot.

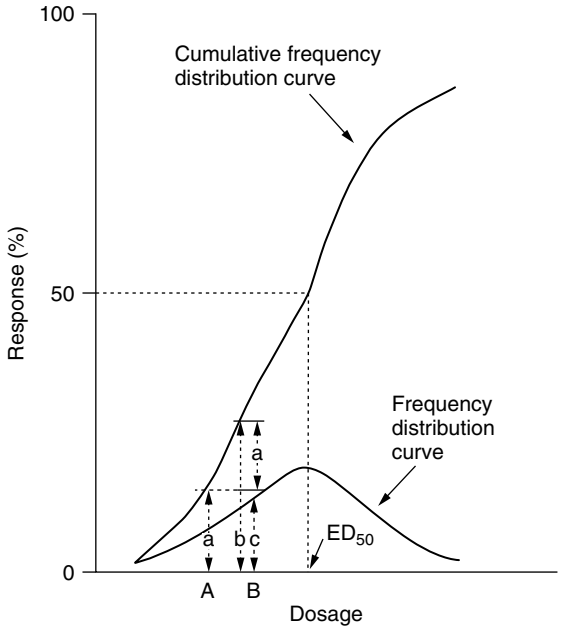

Figure 7 Relationships between a cumulative frequency distribution curve and a normal frequency distribution curve for quantal data. The cumulative frequency distribution curve shows the proportionate response for each dosage, and the expected total response for any given dosage. The normal frequency distribution curve shows the response specific for a given dosage compared with lower dosages. For the normal frequency distribution curve, the response (c) at any given dosage (e.g. at B) is obtained by taking the total response at that dosage (b) and subtracting the response (a) at the immediately lower dosage.

of a special subpopulation that have a genetically determined hypersusceptibility to the substance being tested (**Figure 5**).

Data plotted on a dosage–response basis may be quantal or continuous. The quantal response is 'all-or-none', for example, death. The graded, or variable, response is one involving a continual change in effect with increasing dosage, for example, enzyme inhibition, degree of inflammation or physiological function, such as heart rate. The dosage–response curve is often linearly transformed into a log-probit plot ($\log_{10}$ dose vs. probit response) because it permits the examination of data over a wide range of dosages, and allows certain mathematical procedures (e.g. calculation of confidence limits and slope of response) (**Figure 6**). Quantal data can also be plotted as a frequency histogram or frequency distribution curve; this is done by plotting the percentage response at a given dose minus the percentage response at the immediately lower dose (i.e. response specific for the dosage). This procedure usually results in a Gaussian distribution (**Figure 7**), reflecting the differential biological susceptibility of the test organism to the treatment. In such a normal frequency distribution curve the mean ± 1 standard deviation (SD) represents 68.3% of the population, the mean ± 2 SD represents 95.5% and the mean ± 3 SD is 99.7% of the population. It is important to stress that not only will the incidence of the effect of interest vary with dosage, and determine the dosage–response relationship, but also the severity or magnitude of the effect will change with varying dosage. Thus, for any given dosage producing a particular response incidence, those responding may show a difference in the magnitude of the effect.

The absence of a clear dosage–response relationship in a controlled experiment may indicate a nontoxic or nonpharmacological action of the material. For example, an aminoalkyltrialkoxydisilane given by gavage to rats resulted in the following mortalities (expressed as (number dying/number dosed)): $16\,g\,kg^{-1}$ (4/5), $8\,g\,kg^{-1}$ (0/5), $4\,g\,kg^{-1}$ (3/5) and $2\,g\,kg^{-1}$ (0/5). Clearly, there was no dosage–response relationship in this study. Necropsy of dying rats showed that polymerization of the material had occurred in the stomach, producing a hard, opalescent, solid mass completely occluding the stomach. Hence, the cause of death was a consequence of mechanical obstruction and nutritional deprivation, rather than intrinsic toxicity.

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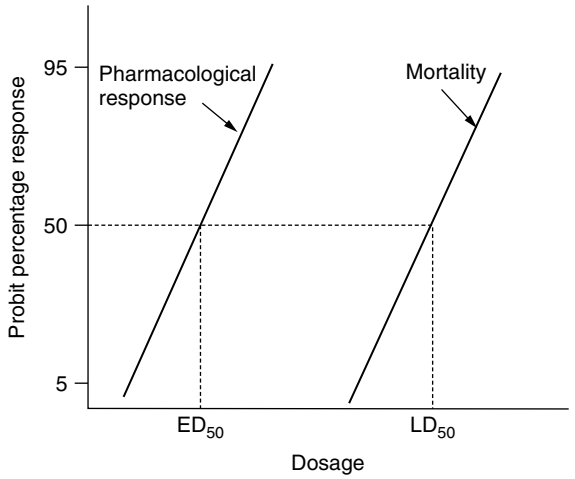

Figure 8 One simplistic method to assess 'safety ratios' for drugs is by comparing the ratio of the therapeutically effective dosage (ED_{50}) to that causing mortality (LD_{50}); the ratio LD_{50}/ED_{50} is referred to as the 'therapeutic index' (TI). For parallel pharmacological effect and lethality dosage–response lines, the TI will be similar over a wide range of dosages, as shown in the figure above. However, nonparallel lines may give misleading conclusions if the TI_{50} is calculated (see **Figure 9**).

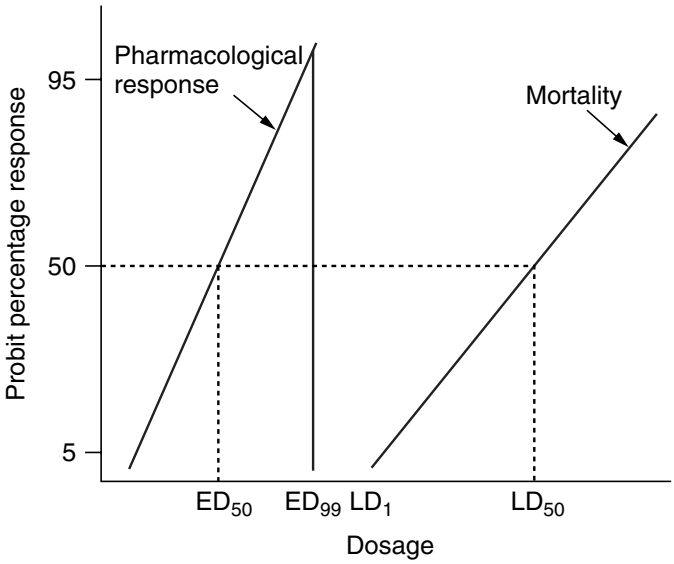

Figure 9 The TI_{50} may give misleading information if the dosage–response lines for pharmacological and lethal effects are not parallel. In the example shown here, there may be a reasonable safety margin based on the LD_{50} and ED_{50} considerations. However, due to the shallower slope of the mortality dosage–response line, the TI will be significantly lower at the 1 and 5% levels, and thus the hyper-reactive group may be at greater risk. In this case a better index of safety will be the ratio of LD_1/ED_{99}, which is referred to as the 'margin of safety'.

For drugs, one convenient indication of 'safety' often used is the ratio of the median effective dose causing death to that producing the desired therapeutic response (i.e. LD_{50}/ED_{50}); this is frequently referred to as the therapeutic index (TI_{50}). In general, the higher this ratio, the greater is the degree of safety with respect to lethality. However, very considerable caution is needed in applying this information. For example, if the slopes of the dosage–response curves for drug effectiveness and lethality are parallel, then the assumption of an equal therapeutic ratio over a range of dosages and to a majority of the population is justified (**Figure 8**). If, however, the dosage–response curve for lethality is shallower than that for the therapeutic response (**Figure 9**), then there will be a decreasing TI at the lower dosages, and the hyperreactive groups may be at greater risk. One approach which can be used to take into account differences in slopes is to calculate the ratio between the dosage causing a 1% mortality (LD_1) and that producing near maximum therapeutic efficacy (ED_{99}). This ratio, LD_1/ED_{99} is referred to as the 'margin of safety' (**Figure 9**). A complete appraisal of safety-in-use, of course, also requires considerations on sublethal and long-term toxicity, and at therapeutic dosages the likelihood of side effects and idiosyncratic reactions.

The slope of the dosage–response relationship, particularly around the mid-point, can be of value for more precisely assessing hazard or potential for overdose situations. Thus, for example, in considering lethality, a steep slope indicates that a large proportion of the population will be at risk over a small range of doses. Likewise, with a material producing central nervous system depression, a steep slope implies that a small incremental increase of dosage may result in coma rather than sedation.

In most cases of acute lethal toxicity, the dosage–response curve is a log-normal cumulative frequency distribution or Gaussian frequency distribution. In a few cases, however, there may be two definite peaks in the frequency-distribution curve; this is distinct from an increase in hyper-reactive groups at the left-hand side of the dosage–response curve and is known as a bimodal distribution; it may reflect different modes of toxicity possibly with differing latencies. Such a bimodal distribution may reflect different modes of lethality toxicity possibly with differing latency. Earlier deaths at the lower dosages, producing the first phase of the bimodal distribution, represent a quantitatively more potent toxicity; those surviving the first phase toxicity may succumb to the higher dosage latent toxicity. For example, with op anticholinesterase, the first deaths are due to the cholinergic crisis resulting from acetylcholinesterase inhibition, and late toxicity may result from delayed-onset peripheral neuropathy. In some cases, log-probit plots will allow the determination of ED_{50} values for each subgroup in the bimodal distribution.

For many toxic effects, except genotoxic carcinogenesis, there is a dose below which no effect or

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response can be elicited; this corresponds to the extreme left-hand side of the dosage–response curve. This dosage, below which no effect occurs, is referred to as the 'threshold dosage'. The threshold concept, a corollary of the dosage–response relationship, is important in that it implies that it is possible to determine a 'no-observable effect level' (NOEL), which can be used as a basis for assigning 'safe levels' for exposure.

6.3 Applications of Dosage–Response Information

It is important to reiterate that conclusions drawn from dosage–response studies are valid only for the specific conditions under which the information was collected. Within this constraint, dosage–response information allows at least the following:

1. Confirmation that the effect under consideration is a toxic (or pharmacological) response to the chemical or therapeutic agent. Thus, a positive dosage–response relationship is good evidence for a causal relationship between exposure and the development of toxicity or pharmacological effects.
2. Quantitative dose–response information allows the determination of an average (median) response, gives the range of susceptibility in the population studied, and indicates where the dosage for hypersusceptible groups is expected.
3. The slope of the dosage–response curve gives information on the range of effective dosages and the differential proportion of the population affected for incremental increases in dosage. With a shallow slope the range of effective doses is widespread; the proportion of the population additionally affected by incremental increases in dosage is small. In contrast, a steep slope implies that the effective dose for the majority of the population is over a narrow range, and there will be a significant increase in the proportion of the population affected for small incremental increases in dosage.
4. The shape of the left-hand side of the dosage–response curve may indicate the existence of an unusually high hypersusceptible proportion of the population. This may, for example, indicate a genetically determined increased susceptibility to the chemical or pharmacologically active substance studied.
5. Quantitative comparison for a specific end point may be made between different materials with respect to average and range of response, particularly if the information has been collected under similar conditions.
6. The data may allow conclusions on 'threshold' or 'no-effect' dosages for the response or the determination of those concentrations or doses that do not result in the appearance of any effects due to exposure to the substance tested (NOEL) or do not cause the development of effects regarded as potentially biologically harmful (no observed adverse effect level (NOAEL)). Recently there has been the development of a concept of benchmark dose (BMD) as an alternative to the NOAEL, or compromise between the NOAEL and the lowest observable adverse effect level (LOAEL). The BMD concept, introduced by Crump (1984), is the dose of a material that is required to achieve a predetermined response of a toxicological effect. For quantal data, the BMD involves fitting a dose–response curve to bioassay data, and the dose (or lower bound on the dose) corresponding to a given low-level response on the fitted curve (e.g. a 5 or 10% elevation in incidence over background: BMD_{05}, BMD_{10}) selected as a characterization of the dose level at which a detectable increase in the measured effect of interest occurs (Sand *et al*., 2008; Rhomberg, 2005). Also cited are the lower 95% confidence limits on the BMD; for example, $BMDL_{05}$.

For continuous data, it has been standard to define the BMD as corresponding to a percentage change in response relative to background (Sand *et al*., 2008). Also, for continuous dose–response data, Sand *et al*. (2006) proposed an approach that defines the BMD as the dose at which the slope of an S-shaped dose–response relationship changes the most in the low dose region. The dose is in a transition region where the sensitivity to chemical exposure may start to change noticeably. After the application of appropriate uncertainty factors (UFs), the BMD is used for the determinations of certain safe dose values, such as acceptable intakes or reference doses (RfDs). The fact that the BMD approach involves uncertainty analysis has been cited as a major improvement over the NOAEL approach (Sand *et al*., 2008). Another frequently cited dose is the reference dose (RfD), which is an estimate of a daily exposure to the human population (including sensitive subgroups) that is likely to be without appreciable risk of deleterious effects during a lifetime (Barnes, 2000). In general, the RfD is calculated from the NOAEL, derived from animal studies, with the application of UFs and a modifying factor (MF):

$$\text{RfD} = \text{NOAEL}/\text{UFs} \times \text{MF} \quad (1)$$

In the simplest case, the NOAEL is divided by two Ufs of 10 each, in order to address extrapolation from animal to human and the sensitivity within the human population respectively; in such cases an MF of 1 is used:

$$\text{RfD} = \text{NOAEL}/10 \times 10 \times 1 \quad (2)$$

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As noted above, the derivation has generally involved the application of a 100-fold safety factor to a measure of the threshold for toxicity, such as the NOAEL or BMD. This 100-fold factor is composed of two 10-fold factors allowing for human variability and species differences. It has been proposed that the 10-fold factors can be refined with a chemical-specific adjustment factor (CSAF) when suitable data are available (WHO, 2001). Such refinements would emphasize pathway-related factors associated with variability in kinetics, and derived from databases that quantify interspecies differences and human variability in Phase I metabolism, Phase II metabolism and renal excretion (Dorne and Renwick, 2005).

The above considerations are briefly illustrated in the following section for acute lethal toxicity.

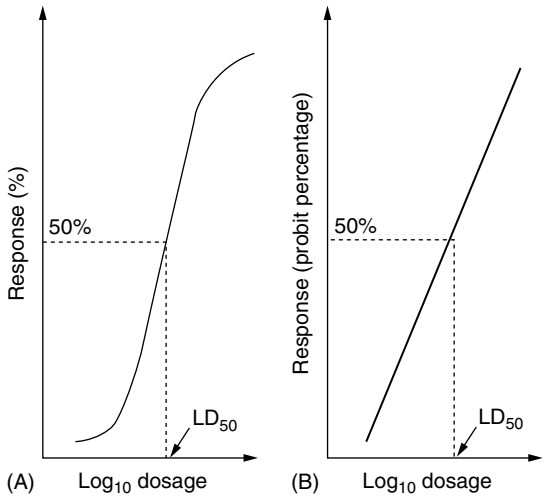

Figure 10 Dosage–mortality data plotted (A) as a cumulative frequency distribution curve (% response versus $\log_{10}$ dosage) and (B) linearly transformed by log-probit plot.

6.4 Dosage–Response Considerations for Acute Lethal Toxicity

Death, a quantal response, is an end point incorporated in many acute toxicity studies, and often used for the calculation of LD_{50} values. Acute lethal toxicity studies involve giving differing dosages of the test material to groups of laboratory animals of the same strain by a specific route of exposure and under controlled experimental conditions, e.g. with respect to diet, caging, temperature, relative humidity and time of dosing. Mortalities at each dosage are recorded over a specified period of time, usually 14 days. By epicutaneous or respiratory exposure, the exposure time should be stated, since the degree of local injury and the potential for systemic toxicity are a function of this time, as well as the exposure dosage. For routes other than inhalation, the exposure dosage is usually expressed as mass (or volume) of test material given per unit of body weight, for example, ml (kg body weight)$^{-1}$ or mg (kg body weight)$^{-1}$. For inhalation, the exposure dose is expressed as the amount of test material present per unit volume of exposure atmosphere: mg m^{-3} or ppm. Dose–response information collected for differing concentrations of an atmospherically disposed material should be over similar periods of time in order to allow the most meaningful comparisons to be made. Alternatively, the effect of differing inhalation exposure doses can be made by exposing different groups to the same concentration of test substance for various exposure periods; this may allow the calculation of a median time to death (50% response rate) for the population exposed to a specific atmospheric concentration of test material (LT_{50}). By using both of these approaches it is possible to reach conclusions on the differential sensitivity of a population to varying concentrations for a specified period of time, or to differing exposure periods for a given concentration.

Dosage–mortality data usually conforms to the sigmoid cumulative frequency distribution curve (**Figure 10A**), which may be converted to a linear form using a log-probit plot (**Figure 10B**). Lethal toxicity is usually initially calculated and compared at a specific mortality level; most frequently used is that causing 50% mortality in the population studied, since this represents the midpoint of the dosage range about which the majority of deaths occur and usually with a symmetrical distribution. This is the median lethal dose for 50% of the population studied (LD_{50}), that is, that dose, calculated from the dosage–mortality data, which causes death of half of the population dosed under the specific conditions of the test. This concept of the LD_{50} was introduced by Trevan (1927). By inhalation, the reference is the lethal concentration$_{50}$ (LC_{50}) for a specified period of time (i.e. x h LC_{50}). Other values calculated include the LD_5 and LD_{95}, which give statistical indications of near-threshold and near-maximum lethal toxicity, respectively, and the range of doses over which a lethal response may occur.

Since the LD_{50}, for economical and ethical reasons, is usually conducted with only small numbers of animals, there is a UF associated with the calculation of the LD_{50} (or LC_{50} or LT_{50}). This is estimated from the 95% confidence limits; that is, the dosage range for which there is only a 5% chance that the LD_{50} (or other LD value) lies outside. The 95% confidence limits are narrowest at the LD_{50} (**Figure 11**), which is a further reason why this is an appropriate point for the comparison of acute lethal toxicity.

The LD_{50}, by itself, is an insufficient index of lethal toxicity, particularly if comparisons are to be made between different materials. The whole of the dosage–response information should be examined,

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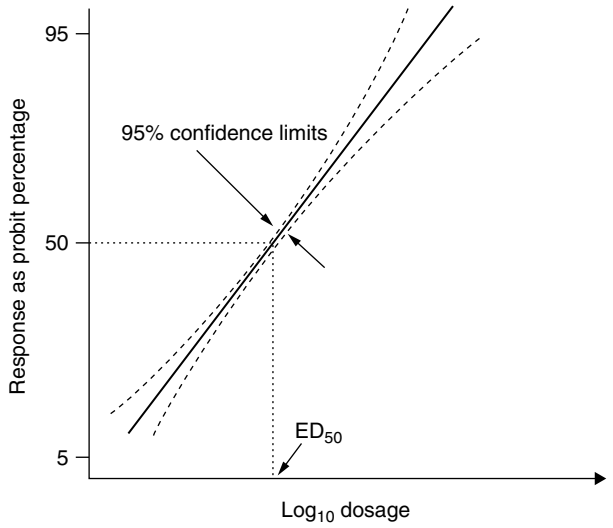

Figure 11 Dosage–mortality curve fitted with 95% confidence limits. The limits are closest at the ED_{50} and diverge at the extremes of the dosage response.

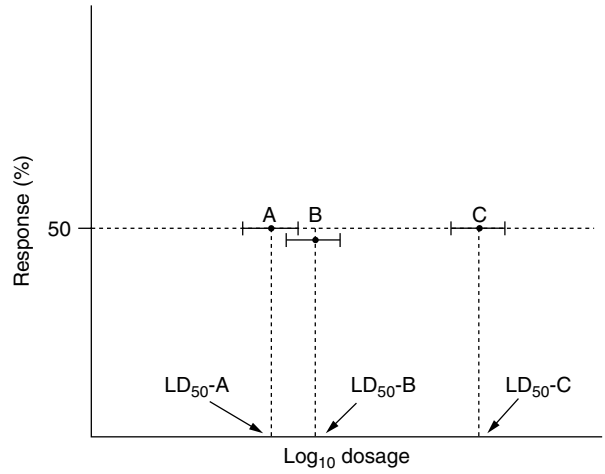

Figure 12 Comparison of the acute lethal toxicity for three compounds based on LD_{50} data alone. Compounds A and B have overlapping 95% confidence limits, and therefore have comparable acute lethal toxicities. Compound C has 95% confidence limits that are numerically separate from those of A and B, and is thus significantly less lethally toxic than either A or B, based on LD_{50} considerations.

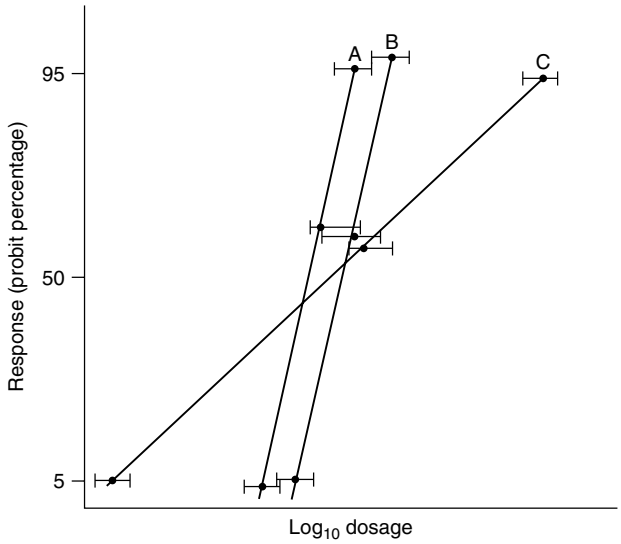

Figure 13 The influence of the slopes of dosage-mortality plots on the interpretation of LD_{50} data. All three illustrative materials (A, B and C) have overlapping 95% confidence limits at the 50% response level, and are therefore of comparable LD_{50}. Materials A and B have parallel dosage–response lines and overlapping 95% confidence limits at the 5 and 95% levels; therefore, these two materials are of comparable acute lethal toxicity over a wide range of dosages. Material C, in contrast, has a shallower slope and significantly different LD_5 and LD_{95} values, and therefore over a wide range of dosages C has a differing lethal toxicity to A and B. With materials A and B, because of the steep slope of the dosage–mortality line, a much larger proportion of the population will be affected by small incremental increases in dosage. With material C, there may be a greater risk for the hyper-reactive group since the LD_5 lies at a much lower dosage than for A and B.

including the slope of the dosage–response line and 95% confidence limits. For example, two materials with differing LD_{50} values, but overlapping 95% confidence limits are not regarded as being of significantly different lethal toxicity, since there is a statistical probability that the LD_{50} of one material will be within the 95% confidence limits of the other. However, when there is no overlap of 95% confidence limits, then the materials are considered to have significantly different lethal toxicity at the LD_{50} level (**Figure 12**). A particularly important consideration is that of the slope of the dosage–response curve (**Figure 13**). For example, if two materials have similar LD_{50} values with overlapping 95% confidence limits and identical slopes on the dosage–response lines (and therefore statistically similar LD_{10} and LD_{90} values), they are lethally equitoxic over a wide dosage range (A and B, **Figure 12**). However, materials having similar LD_{50} values but differing slopes (and hence significantly different LD_{10} and LD_{90} values) may not be considered to be lethally equitoxic over a wide dosage range (A or B versus C, **Figure 13**). Thus, materials having a steep slope (A or B, **Figure 13**) may affect a much larger proportion of the population by incremental increases in dosages than is the case with materials having a shallow slope; thus, acute overdose may be a more serious problem affecting the majority population for materials with steeper slopes. In contrast, materials having a shallower slope

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(C, **Figure 13**) may present problems for the hyper-reactive groups at the left-hand side of the dosage–response curve, and may occur at significantly lower dosages than for hyper-reactive individuals associated with the steep slope group. It follows from the above that a proper interpretation of acute lethal toxicity information should include examination of LD_{50}, 95% confidence limits, slope and extremes of the dosage–response curve.

It needs to be stressed that dosage–response information requires to be interpreted in terms of the conditions by which it was obtained; the following few examples are used to illustrate the care necessary:

1. The numerical precision of the LD_{50} lies only in the statistical procedures by which it is calculated. If an experiment to determine LD_{50} is repeated at a later time, slightly different dosage–response data may be obtained because of biological and environmental variability, resulting in a different numerical value for the LD_{50}. Therefore, LD_{50} values should be regarded as representing an order of lethal toxicity under the specific circumstances by which the information was collected.
2. An important consideration in interpreting the acute hazard from a chemical is the time to toxic effect. Thus, materials of similar LD_{50}, but differing times to death may present different hazards. For example, with substances having similar LD_{50} and slope values, those having more rapid times to death can be considered as presenting a greater acute hazard. However, those substances with longer latency to effect may have a potential to produce cumulative toxicity by repeated exposure. For example, the acute po LD_{50} of 2,4-pentanedione in the rat is $0.58\,g\,kg^{-1}$ and that of 2,2-bis(4-aminophenoxyphenyl)propane (BAPP) is $0.31\,g\,kg^{-1}$, with respective times to death of 2–5 hours and 13–14 days; on this basis, 2,4-pentanedione would be regarded as presenting a greater acute potential hazard than BAPP (Tyler and Ballantyne, 1988).
3. A more complete interpretation of the significance of LD_{50} data may require consideration of the cause of death. If differing potentially lethal toxic effects are produced, it is important to know if this can lead to a multimodal dosage–response curve, and thus to differing hazards by immediate or latent mortality or morbidity. Clearly, latency is of importance in clinical toxicology for decisions on immediate medical management and observations and treatment for latent toxicity. For example, *tert*-butyl nitrite given by acute ip injection to mice has a 30 minute LD_{50} of $613\,mg\,kg^{-1}$ and a seven day LD_{50} of $187\,mg\,kg^{-1}$. The earlier deaths were probably related to cardiovascular collapse and methaemoglobin formation, whereas later deaths were due to liver injury (Maickel and McFadden, 1979).
4. Acute LD_{50} data may not be a direct guide to defining lethal toxicity by multiple exposures. Thus, with a material producing significant cumulative toxicity, the acute lethal dose (and dosage) may be significantly higher than that producing death by multiple smaller exposures. For example, the four hour LC_{50} for trimethoxysilane is 47 ppm (rat); however, for rats given 20 exposures, each of seven hours over four weeks, the LC_{50} was 5.5 ppm for that time period (Ballantyne *et al.*, 1988). Thus, the potentially lethal vapour concentration of trimethoxysilane for repeated exposure is significantly less than that by acute exposure.

Any investigation into lethal toxicity should attempt to allow the maximum amount of usable information to be obtained. For this reason, acute toxicity studies should be designed not only to determine lethal toxicity, but also to monitor for sublethal and target organ toxicity; this is possible by incorporating into the protocol observations for signs, body weight, haematology, clinical chemistry, urinalysis, gross and microscopic pathology and other specialized procedures as considered appropriate for the material under test. In this way a significantly greater amount of relevant information can be obtained, and the most useful and meaningful information collected to allow a comparative evaluation of acute toxicity and potential hazards, and the potential for cumulative toxicity (Zbinden and Flury-Roversi, 1981).

Detailed discussions on dosage–response relationships and their toxicological and pharmacological relevance have been written by Sperling (1984), Tallarida and Jacob (1979) and Timbrell (1982).

7 FACTORS INFLUENCING TOXICITY

With animal studies and human poisoning, the nature, severity, incidence and probable induction of toxicity depend on a large number of exogenous and endogenous factors. Some of the more important are as summarized as follows.

7.1 Species and Strain

Species and strain differences in susceptibility to chemical-induced toxicity may be due, to variable extents, to differences in rates of absorption, metabolic conversions, detoxification mechanisms and excretion. In some cases animal studies may give underestimates,

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and in other instances overestimates, for acute po toxicity to humans. For example, the acute po LD_{50} of ethylene glycol has been determined in several laboratory mammals to range from 4.7 to 7.5 g kg^{-1} (Sweet, 1985–1986a) and that for methanol to range from 5.63 to 7.50 g kg^{-1} (Sweet, 1985–1986b); however, these chemicals are significantly more lethally toxic to humans, with both having a minimal lethal dosage in humans around 0.5–1.0 g (kg body weight)$^{-1}$.

7.2 Age

With some substances age may significantly affect toxicity, probably mainly due to relative differences in metabolizing and excretory capacities. In one extensive compilation of LD_{50} values for drugs to neonatal and adult mammals (Goldenthal, 1971), the ratio (LD_{50} adult) /(LD_{50} neonate) varied from <0.02 (for amidephrine) to 750 (for digitoxin).

7.3 Nutritional Status

Nutritional status may significantly influence the level of cofactors and biotransformation mechanisms important for the expression of toxicity, and in this way diet can affect toxicity (Rao and Knapka, 1998). Moreover, diet may markedly influence the natural tumour incidence in animals and modulate carcinogen-induced tumour incidence (Grasso, 1988). Khanna *et al.* (1988) studied the effect of protein deficiency on the neurobehavioural effects of acrylamide in rat pups exposed during the intrauterine and early postnatal stages. They found acrylamide to be more toxic in protein-deficient hosts, owing to a significant decrease in dopamine and benzodiazepine receptor binding. Based on a two-year study, Hubert *et al.* (2000) found that a dietary restriction of around 25% is appropriate for Sprague–Dawley rats in toxicity and carcinogenicity assays to improve survival without an impairment of growth and routine clinical chemistry monitors. Feeding is an important factor in the design and interpretation of acute po toxicity studies. For example, Kast and Nishikawa (1981) compared the acute po toxicity of several anti-gastric-ulcer drugs and β-adrenoceptor agonists and blockers; the ratio (LD_{50} fed)/(LD_{50} fasted) for rats and mice ranged from 1.3 to 1.47, indicating a higher toxicity in the starved animals. The authors concluded that the greater acute toxicity in the starved animals was due to accelerated gastric emptying and intestinal absorption. The importance of dietary factors in toxicity has been reviewed by Angeli-Greaves and McLean (1981) and Grasso (1988).

7.4 Time of Dosing

Diurnal and seasonal variations in toxicity may relate to similar variations in biochemical, physiological and hormonal profiles. Examples of temporal variations in biological activity include circadian dependence of metabolic adverse effects of cyclosporin (Malmary *et al.*, 1988), toxicity of methotrexate (Marks *et al.*, 1985) and seasonal variations in gentamicin nephrotoxicity (Pariat *et al.*, 1988). Circadian toxicology is discussed in **Chronotoxicology**.

7.5 Environmental Factors

A variety of environmental factors are known to influence the development of toxicity, including temperature, relative humidity and photoperiod. The influence of temperature may vary between differing chemicals and the effects investigated. For example, colchicine and digitalis are more toxic the higher the temperature (Lu, 1985); in contrast, studies on the behavioural toxicity of the anticholinesterase soman suggest that the lower the temperature, the greater the susceptibility (Wheeler, 1987). The influence of temperature on toxicity is clearly an important consideration for materials used in arctic and tropical areas. The influence of temperature on toxicity is discussed in **The Influence of Temperature on Toxicity**.

7.6 Dosing Characteristics

The nature, severity and likelihood of inducing toxicity are influenced by the magnitude, number, frequency and profiling of dosing. Thus, local or systemic toxicity produced by acute exposure may also occur by a cumulative process with repeated lower-dosage exposures; also, additional toxicity may be seen with the repeated exposure situations. For example, acute exposure to formaldehyde vapour causes peripheral sensory irritant effects and (with sufficiently high concentrations) injury and inflammatory change in the respiratory tract; short-term repeated vapour exposure can result in the development of respiratory sensitization; longer-term vapour exposure may cause squamous metaplasia and nasal tumours (Wartew, 1983). The relationships for cumulative toxicity by repetitive exposure compared with acute exposure toxicity may be complex, and the potential for repeated exposure cumulative toxicity from acutely subthreshold doses may not be quantitatively predictable. For example, the LC_{50} for a four hour exposure to trimethoxysilane vapour is 47 ppm; by repeated exposure over a four week period (seven hours a day, five days a week) the LC_{50} is 5.5 ppm (Ballantyne *et al.*, 1988). In contrast, acute

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exposure to benzene vapour for 26 hours (95 ppm) or 96 hours (21 ppm) produced severe bone marrow cytotoxicity, whilst a similar exposure dose given over a longer period of time (95 ppm for 2 hours a day for two weeks) produced little toxicity (Toft *et al*., 1982).

For repeated exposure toxicity, the precise profiling of doses may significantly influence toxicity. For example, with formaldehyde in a four week vapour inhalation study, it was determined that exposure of rats to 10 or 20 ppm by interrupted exposure over eight exposure periods produced more nasal mucosal cytotoxicity than did continual exposures (Wilmer *et al*., 1987). In a four-week inhalation study with carbon tetrachloride, it was found that interruption of a daily six hour exposure by 1–5 hour periods of nonexposure caused more severe hepatotoxicity than with continuous exposures, but five minute peak loads superimposed on a steady background only slightly aggravated the hepatotoxic effect of carbon tetrachloride vapour (Bogers *et al*., 1977).

7.7 Formulation and Presentation

For chemicals given perorally or applied to the skin, toxicity may be modified by the presence of materials in formulations that facilitate or retard the absorption of the chemicals. With respiratory exposure to aerosols, particle size significantly determines the depth of penetration and deposition in the respiratory tract (see **Neurotoxicology**).

7.8 Miscellaneous

A variety of factors in addition to the above may influence the nature and exhibition of toxicity, depending on the conditions of the study; for example, housing conditions, handling and dosing volume. Variability in test conditions and procedures may result in significant interlaboratory variability in results of otherwise standard procedures; for example, LD_{50} determination (Griffith, 1964; Hunter *et al*., 1979).

All the above factors need to be taken into consideration during risk assessments for xenobiotic exposures in order to more accurately predict human responses (Aldridge *et al*., 2003).

8 BIOHANDLING AS A DETERMINANT OF SYSTEMIC TOXICITY

The induction of systemic toxicity results from a complex inter-relationship between absorbed parent material and conversion products formed in tissues, their distribution in body fluids and tissues, binding and storage characteristics and their excretion. Some of these factors are considered below (see also **Figure 14**).

8.1 Absorption

The absorption of a substance from the site of exposure may result from passive diffusion, facilitated diffusion, active transport or the formation of transport vesicles (pinocytosis and phagocytosis). The process of absorption may be facilitated or retarded by a variety of factors, which include elevated temperature, that increases percutaneous (pc) absorption by cutaneous vasodilation, and surface-active materials, that facilitate penetration. The integrity of the absorbing surface is important; for example, the acute PC LD_{50} for HCN (solution) is 6.89 mg kg^{-1} for rabbits with intact skin, and 2.34 mg kg^{-1} if the skin is abraded (Ballantyne, 1987).

8.2 Biodistribution

Following absorption, materials circulate either free or bound to plasma protein or blood cells; the degree of binding, and factors influencing the equilibrium with the free form, may influence availability for metabolism, storage or excretion. Within tissues there may be binding, storage, metabolic activation or detoxification; binding may produce a high tissue/plasma partition and be a source for slow titration into the circulation following the cessation of environmental exposure. Examples of storage sites include fat for lipophilic materials (e.g. chlorinated pesticides) and bone for fluoride, lead and strontium. The relationship between exposure dose and release rate may be complex; for example, volatile lipophilic materials are generally more rapidly desorbed than nonvolatile lipophilic substances. Permeability of tissues may be modified by tissue-specific barriers; for example, the blood–brain barrier and placenta. This may affect differential toxicity within classes of compounds, for example, neurobehavioural effects produced by organomercurials and, to a lesser degree, with inorganic mercury compounds (Lu, 1985).

8.3 Biotransformation

Metabolism of substances is conveniently classified under the following two major headings (Williams, 1959):

Phase I reactions: A functional group is introduced into the molecule by oxidation, reduction or hydrolysis.
Phase II reactions: There is conjugation of an absorbed material or its metabolite with an endogenous substrate.

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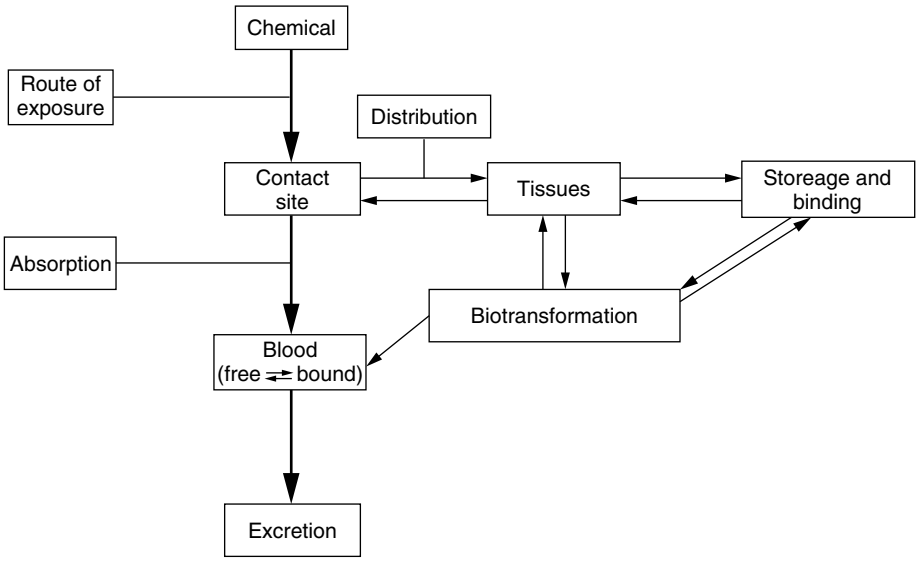

Figure 14 Possible pathways for the fate of a chemical absorbed from the route of exposure.

For many materials there is an initial Phase I reaction to produce materials which are conjugated by Phase II processes. In other instances, only a Phase II process may be utilized. Reactions of a Phase I type include oxidation, reduction and enzymatic hydrolysis; Phase II reactions include conjugation with glucuronic acid, sulfate, glycine and glutathione, and acetylation and methylation. Phase I reactions, particularly, may result in the formation of toxic metabolites from relatively innocuous precursors, that is, metabolic activation. Phase II conjugates are generally more water-soluble than the parent compound or Phase I metabolites and hence usually more readily excreted. With toxic parent compounds, or toxic metabolites, there may be conversion to less toxic products; that is, detoxification has occurred. Examples of metabolic activation and detoxification are given in **Table 5**. Many activation reactions are catalysed by a cytochrome-P450-dependent mono-oxygenase system, which is particularly active in the liver. Clearly, a major determinant of the likelihood of toxicity developing, and its severity, is the overall balance between the absorption rate of a chemical, its metabolic activation and detoxification, and the excretion of toxic species (see **Toxicokinetics**).

8.4 Excretion

Substances may be excreted as parent compound, metabolites and/or Phase II conjugates. A major route of excretion is by the kidney, and in some cases the urinary elimination of parent compound, metabolite or conjugate, may be used as a means for assessing absorbed dose. Some materials may be excreted in bile and thence in faeces; in such cases there may also be enterohepatic cycling. Certain volatile materials and metabolites may be eliminated in expired air. The excretion of materials in sweat, hair, nails and saliva is usually quantitatively insignificant, but these routes may be of importance for a forensic or industrial diagnosis or confirmation of intoxication (Paschal *et al*., 1989; Randall and Gibson, 1989). Materials excreted in milk may be transferred to the neonate.

Table 5 Examples of the metabolic transformation of chemicals

Biotransformation	Chemical	Conversion
Detoxification	Cyanide	Enzymatic conversion to less toxic thiocyanate
	Benzoic acid	Conjugation with glycine to produce hippuric acid
	Bromobenzene	3,4-Epoxide reactive metabolite is enzymatically hydrated to the 3,4-dihydrodiol or conjugated with glutathione
Activation	Carbon tetrachloride	Microsomal enzyme metabolic activation to hepatotoxic trichloromethylperoxy radicals
	2-Acetylaminofluorene	*N*-Hydroxylation to more potent carcinogen *N*-Hydroxyacetylaminofluorene
	Parathion	Oxidative desulfuration to the potent cholinesterase inhibitor paraoxon

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8.5 Biohandling Interactions

The probability of adverse effects developing in response to chemical exposure depends particularly on the magnitude, duration, frequency and route of exposure. These will determine the amount of material to which an organism is exposed (the exposure dose), and hence to the amount of material which can be absorbed (the absorbed dose). The latter determines the amount of material available for distribution and toxic metabolite formation, and hence the likelihood of inducing a toxic effect. Opposing absorption and metabolite accumulation is elimination. Hence, for a given environmental exposure situation, the probability of inducing toxicity, and its magnitude, depend on the relationship between rate of absorption, metabolism (activation and detoxification) and elimination of parent material and metabolites.

The amount of a material in contact with the absorbing surface is one of the principal determinants of absorbed dose. In general, the higher the concentration, the greater is the absorbed dose. However, if mechanisms other than simple diffusion across a concentration gradient are operating, a simple proportionate relationship between concentration and absorbed dose may not be present. In such instances, a rate-limiting factor could result in proportionately smaller increases in absorbed dose for incremental increases in concentration at the absorption site. Also, and in particular when there is absorption by active transport, there may be saturation of the absorption process and a ceiling value.

When there is repeated exposure, the relative amounts of biotransformation products, and the distribution and elimination of metabolites and parent compound, may be different from that following an acute exposure. For example, repeated exposure may induce and enhance mechanisms responsible for the biotransformation of the absorbed material, and thus alter the relative proportions of parent molecules and metabolites (activation and detoxification), and hence the probability for target-organ toxicity. Also, if there is slow detoxification, storage and/or slow excretion, repeated exposures may lead to the accumulation of toxic species and hence a potential for cumulative toxicity.

9 ROUTES OF EXPOSURE

The primary tissue or system by which a material comes into contact with the body, and from where it may be absorbed in order to exert systemic toxicity, is the route of exposure. The usual circumstances of environmental exposure are by ingestion (po), inhalation and skin (epicutaneous/pc) or eye contact. Also, for investigational, therapeutic and certain forensic purposes, im, iv and sc injections may be routes of exposure.

The relationship between route of exposure, biotransformation and potential for toxicity may be complex and also influenced by the magnitude and duration of dosing. Materials that undergo hepatic activation are likely to exhibit greater toxicity when given po than if absorbed across the lung or skin, owing to the high proportion of material passing directly via the portal vein following po dosing. In contrast, materials that undergo hepatic detoxification are likely to be less toxic perorally than when absorbed pc or across the respiratory tract. However, in determining the relevance of route to biotransformation and toxicity, both the magnitude and timescale for dosing should be considered. Thus, when a single, large dose (bolus) of a metabolically activated material is given po, its rapid metabolism may result in the immediate development of a severe acute toxicity. However, if the same material is given po at much lower rates (e.g. by dietary inclusion), then there will be slow and sustained absorption, and in such circumstances the rate of generation of the toxic species may approach that resulting from continuous exposure by other routes. With materials that are detoxified by the liver, a slow continuous alimentary absorption will result in an anticipated low toxicity, compared with other routes of exposure. However, a po bolus may result in the detoxifying capacity of the liver being overwhelmed, and unmetabolized material may enter the circulation to initiate. A few comments on specific routes of exposure follow.

9.1 Peroral

If a material is sufficiently irritant or corrosive, it will cause local inflammatory or corrosive effects on the upper alimentary tract. This may lead to, for example, fibrosis, dysphagia and perforation with mediastinitis and/or peritonitis and the complications thereof. Additionally, carcinogenic materials may induce tumour formation in the alimentary tract. The gastrointestinal tract is an important route by which systemically toxic materials may be absorbed (see **Peroral Toxicity**).

9.2 Cutaneous Contact and Percutaneous Absorption

Skin contact is an important route of exposure in the occupational and domestic environments, and can result in local and/or systemic toxicity. Local effects may include acute inflammation and corrosion, chronic inflammatory responses, immune-mediated reactions and neoplasia. The PC absorption of materials can be a significant route for the absorption of systemically toxic materials (Billingham, 1977; Bronough and Maibach, 1985; see **Cutaneous Toxicology**), and indeed is now a

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means for the systemic titration of pharmacologically active materials (Woodford and Barry, 1986). Factors influencing the PC absorption of substances include skin site, integrity of skin, temperature, formulation and physicochemical characteristics, including charge, molecular weight and hydrophilic and lipophilic characteristics (Billingham, 1977; Dugard, 1977; Stuttgen *et al*., 1982; Kemppainen and Reifenrath, 1990). In addition to enhancing pc absorption by topical epicutaneous contact, some materials may also do so following systemic absorption. For example, ethanol can act as a topical penetration enhancer through solubility and skin-disrupting actions (Cornwell and Barry, 1995; Walters *et al*., 1997). Also, Brand *et al*. (2006) demonstrated that ethanol dosed acutely to rats by gavage increased the pc absorption of epicutaneously applied paraquat, *N,N*-dimethylformamide, and *N,N*-diethyl-*m*-tolbutamide.

Although it is well appreciated that pc absorption of materials may occur when they contaminate the skin as liquid or solid, it has been shown that pc absorption can also result from exposure of the skin to vapour. In the majority of situations, absorption by the inhalation route is generally regarded as being significantly greater than by pc absorption from the vapour phase (McDougal *et al*., 1986; Jacobs and Phanprasik, 1993). When controlled studies are conducted in humans, difference of conduct and interpretive opinion may exist, particularly with the need for artefact-free techniques. For example, Johanson and Boman (1991) conducted a comparative study on human volunteers who were exposed for two hours mouth-only to 50 ppm 2-butoxyethanol, followed by one hour of no exposure, followed by a further two hours of skin-only exposure to 50 ppm 2-butoxyethanol. Using areas under the curve for concentrations of 2-butoxyethanol in finger-prick blood samples, they calculated that approximately 75% of the total uptake of 2-butoxyethanol vapour was absorbed through the skin. Subsequently, Corley *et al*. (1997) criticized this approach, mainly on the basis that the finger-prick sampling was compounded by locally high concentrations of 2-butoxyethanol at the site of absorption. They conducted a study involving exposure of one arm of human volunteers subjected to 50 ppm [$^{13}C_2$]-2-butoxyethanol vapour (50 ppm) for two hours. Blood samples were taken by finger-prick from the exposed arm and by catheter from the unexposed arm, and analysed for 2-butoxyethanol and its major haemolytic metabolite, butoxyacetic acid. They found the concentration of 2-butoxyethanol in the finger-prick blood samples to be almost 1500 times those taken by catheter from the unexposed contralateral arm. Blood butoxyacetic acid concentrations were found to be within a factor of four of each other for the two sampling techniques, and it was considered that the metabolite was a better indicator of absorption into the systemic circulation. In a physiologically based pharmacokinetic model of a 'worst case' scenario (100% body exposure) of an eight hour exposure to 25 ppm vapour, they calculated that only 15–27% of the total uptake of 2-butoxyethanol would be by pc absorption. In contrast, with styrene, measurement of urinary metabolites in workers showed no significant pc absorption of styrene (Limasset *et al*., 1999).

9.3 Inhalation

The likelihood of toxicity from atmospherically dispersed materials depends on a number of factors, the most important of which include physical state, physicochemical properties, and concentration, time and frequency of exposure. The water solubility of a gas or vapour influences the depth of penetration of a material into the respiratory tract. As water solubility decreases, and lipid solubility increases, there is more effective penetration towards the alveoli. Water-soluble molecules, such as formaldehyde, are more effectively scavenged by the upper respiratory tract.

The penetration and distribution of fibres and particulates in the respiratory tract are determined principally by their size. Thus, in general, particles having a mass medium aerodynamic diameter (MMAD) greater than 50 μm do not enter the respiratory tract; those of diameter >10 μm are deposited in the upper respiratory tract; those having a range of 2–10 μm are deposited in the trachea, bronchi and bronchioles; and only particles whose diameter is <1 μm reach the alveoli. Thus, larger insoluble particles are more likely to cause local reactions in the upper respiratory tract, and the potential for alveolar injury is greater with smaller diameter particles. Fibres have aerodynamic characteristics such that those having diameters >3 μm are unlikely to penetrate the lung. In general, fibres having a diameter <3 μm and length <200 μm will enter the lung. Fibres of diameter >10 μm may not be removed by normal clearance mechanisms. Several studies have indicated that fibres of diameter >1.5 μm and length <8 μm have maximum biological activity (Asher and McGrath, 1976; Stanton *et al*., 1981). Dust may be a significant cause of lung disease (Conference, 1990).

The likelihood that inhaled substances will produce local effects in the respiratory tract depends on their physical and chemical characteristics (particularly volatility), reactivity with lining fluids, reactivity with tissue components and site of deposition. Depending on the nature of the material, conditions of exposure and biological reactivity, the types of response produced include acute inflammation and injury, chronic inflammation, immune-mediated hypersensitivity reactions and neoplasia. The degree to which inhaled gases, vapours and particulates are absorbed and, hence, their potential to produce systemic toxicity, depend

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mainly on molecular weight, solubility in tissue fluids, metabolism by lung tissue, diffusion rate and equilibrium state.

A relatively recent, but rapidly expanding area relating mainly, though not exclusively, to the inhalation route of exposure is nanotoxicology. This subject area is concerned with assessing toxicity related to particles in the 10^{-9} m size. More specific definitions indicate that the size is $\leq$100 nm in one dimension, or a range of 1–100 nm (Borm and Kreyling, 2004). Nanoparticles currently in use have been made from transition metals, silicon, carbon and metal oxides (zinc dioxide and titanium dioxide) (Dreher, 2004), and in many cases engineered nanoparticles exist as nanocrystals (Murray *et al*., 2000). Nanoparticles are of technological interest because, for a given particle-type, as the particle size is decreased within the nanoscale range, certain fundamental physicochemical characteristics change, resulting in the development of new and different properties; for example, change from electrically insulating to conducting or insoluble materials becoming soluble (Warheit *et al*., 2000). Manufactured nanoparticles have physicochemical properties that result in their having unique electrical, mechanical, thermal and imaging properties that are highly desirable in the commercial, medical and environmental areas (Dreher, 2004; EPA, 2003). There are indications from pulmonary toxicology studies that nanoparticles cause enhanced toxicity in comparison with larger-sized particles of similar chemical composition (Donaldson *et al*., 2001; Lam *et al*., 2004; Oberdörster, 2000). A substantial proportion of inhaled nanoparticles are likely to deposit in the respiratory tract; ~30% to >90%, depending on breathing rate and particle size. They readily reach the deep lung, and the alveolar region is the primary site of deposition for nanoparticles in the range 10–100 nm. In addition to compartmentalization within the respiratory tract, dissolution is probably another relevant factor in determining the fate and effects of nanoparticles (Borm *et al*., 2006). There are indications that these materials may pose a hazard locally to the lung through oxidative stress, inflammation, and tumorigenesis, and also cause systemic effects by redistribution to other organs following pulmonary deposition (Donaldson *et al*., 2005; 2006). Translocation from lungs has been shown for brain, liver, kidney and spleen (Elder *et al*., 2006; Geiser *et al*., 2005), and the rate depends on the physicochemical properties of the nanoparticles, including size, chemical nature, shape and charge (Oberdörster *et al*., 2002; Kreyling *et al*., 2006). Studies with nanoparticles require that the material tested has detailed characterization (Murdock *et al*., 2008), and a suggested prioritized list for physicochemical characteristics is as follows (Warheit, 2008): particle size and distribution (wet state) and surface area (dry state); crystal structure; aggregation status; composition/surface coatings; surface reactivity; method of nanomaterial synthesis; sample purity. Currently many resources, particularly in the USA, Europe and Japan, are being devoted to the development of risk and safety evaluations of nanomaterials, environmental fate, human health effects, workplace and environmental monitoring, and precautionary measures (Stern and McNeil, 2008; Thomas and Sayre, 2005; Thomas *et al*., 2006a; 2006b). A detailed review of nanotoxicology is given in **Nanotoxicology-The Toxicology of Nanomaterials**.

9.4 Eye

Local and systemic adverse effects may be produced by ocular contamination with liquids, solids and atmospherically dispersed materials. Local effects include transient inflammation, permanent injury and hypersensitivity reactions. Penetration may lead to iritis, glaucoma and cataract. Also, with pharmacologically or toxicologically potent materials, systemically active amounts of material may be absorbed from periocular blood vessels and/or nasal mucosa following passage down the nasolachrymal duct (Shell, 1982; Ballantyne, 1983; see **Ophthalmic Toxicology**).

10 EXPOSURE TO MIXTURES OF CHEMICALS

Circumstances involving exposure to several xenobiotics can result in prior, coincidental or successive exposure to these chemicals, and the nature of the toxicity may vary considerably depending on the conditions of exposure. Thus, an evaluation of the hazards from exposure to multiple chemicals can be much more demanding than is the case for a single chemical. In assessing toxicity from mixtures it is important to consider: (i) chemical and/or physical interactions of the individual materials, (ii) the effect that one chemical may have on the absorption, metabolism and pharmacokinetic characteristics of another and (iii) the possibility for interaction between parent compound and metabolites (Ballantyne, 1985). One longstanding descriptive classification for effects produced by binary mixtures of chemicals is as follows:

Independent effects: Substances qualitatively and quantitatively exert their own toxicity independently of each other.

Additive effects: Materials with similar qualitative toxicity produce a response that is quantitatively equal

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to the sum of the effects produced by the individual constituents.

Antagonistic effects: Materials oppose the toxicity of each other, or one interferes with the toxicity of another; a particular example is that of antidotal action.

Potentiating effects: One material, usually of low toxicity, enhances the expression of toxicity by another; the result is more severe injury than that produced by the toxic species alone.

Synergistic effects: Two materials, given simultaneously, produce toxicity significantly greater than anticipated from that of either material alone; the effect differs from potentiation in that each substance contributes to toxicity, and the net effect is always greater than additive.

In assessing the toxicity of mixtures, the following need to be taken into consideration:

- Possible physical and chemical interaction, which may result in the formation of new substances or groupings, or influence bioavailability
- Time relationships of the exposure for the various components
- Route and conditions of exposure
- Physical and physiological factors affecting absorption
- Mutual influence of materials and metabolites on biotransformation, pharmacokinetic characteristics and target organ doses of toxic species
- Relative affinities of the target sites
- Potential for independent, additive, antagonistic and interactive processes between the various chemical species.

Mixtures may be complex and contain unreacted parent materials, major reaction and degradation products, contaminants and trace additives. It is important to be aware that small quantities of high-toxicity materials may have equal, or greater, significance with respect to adverse health effects than major components. For example, serious consideration needs to be given to repeated exposure toxicity from small quantities of monomer residuals in polymeric materials; for example, ethylene oxide, propylene oxide, vinyl chloride and formaldehyde (Ballantyne, 1989). The contribution to toxicity by trace materials is well illustrated by, for example, the presence of trialkyl phosphorothioate or phosphorothionate impurities in op anticholinesterases (Hollingshaus *et al*., 1981) such as malathion (JMPR, 1998), and the presence of 2,3,7,8-tetrachlorodibenzodioxin in chlorophenols (Kimbrough *et al*., 1984).

Many instances of enhancement of toxicity by specific routes are known. Thus, by skin contact, the systemic toxicity of a material may be enhanced by other materials that facilitate pc absorption. For example, the presence of a surface-active material may result in a carrier function, and the presence of a primary irritant may produce local erythema resulting in increased skin blood flow. If the viscosity of a material is increased, this may enhance local or systemic toxicity due to persistence on the skin.

The inhalation exposure dosage of chemicals may be modified by, for example, the presence of sensory irritants or HCN, which can alter the rate and depth of breathing. Some substances may cause anosmia and hence remove an olfactory warning for other inhaled materials. Particulates may absorb other materials that, if inhaled, cause an increased local burden. When trace quantities of highly volatile and toxic materials are present in a substance, they may, depending on the condition of air movement, have a significant influence on toxicity and hazard. For example, if materials containing trace amounts of acrolein are handled in stagnant air conditions, then potentially toxic vapour concentrations of acrolein may develop; in contrast, when there is free airflow, the acrolein vapour concentration may remain low (Ballantyne *et al*., 1989a). Thus, the degree of ventilation of a space may significantly influence the toxicity of the atmosphere resulting from vapourization of the individual constituents of a liquid mixture.

The endogenous determinants of overall toxicity resulting from exposure to a mixture of chemicals can be very complex. For example, toxicity may be modulated by prior or simultaneous exposure, resulting in enhancement or suppression of metabolic activation or detoxification pathways. The potential for toxicity may depend on the equilibrium state, although this may be continually fluctuating. Modification of toxicity can also result from modulation of pharmacokinetic characteristics, variation in the biodistribution of absorbed materials and metabolites, modifying elimination of the toxic species, and competition for binding sites or receptors. All the above factors will influence the relative and absolute concentration of toxic species at target sites for toxicity. For complex mixture situations, such as environmental considerations, the extremely large number of potential chemical combinations (constituents and concentrations) limit the usefulness of standard toxicity testing for establishing hazard, and modelling approaches have been employed. Most of these models have been constructed on no-interaction assumptions, and rely on concentration-addition or response-addition approaches. Concentration addition is based on an assumption that mixture components contribute to toxicity through a common mechanism of action and an additive effect is obtained from the individual components of the mixture, and thus is regarded as a 'toxic equivalency' approach (Altenburger *et al*., 2000; Safe, 1990). Response addition models are used when mixture constituents have different mechanisms of action, and the combined effects of the constituents are based on the probability that individual constituents

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will affect the exposed organism (Backhaus *et al*., 2000; Walter *et al*., 2002). Combined concentration addition and response addition models can be built into a comprehensive model for noninteracting mixtures (Altenburger *et al*., 2005; Olmstead and LeBlanc, 2005; Teuschler *et al*., 2004). However, the concentration-addition and response-addition models have limited application to complex mixtures, since they do not address the problem of component interactions. Toxicokinetic interactions between chemicals result in one chemical altering the effective concentration of another, and toxicodynamic interactions can occur in which one chemical influences the response of the organism to another chemical (Anderson and Dennison, 2004). In order to integrate interactions into models for mixture toxicity, Rider and LeBlanc (2005) developed a mathematical model that combines concentration addition, response addition and toxicokinetic interaction into the toxicity assessment of chemical mixtures. Haddad *et al*. (2001) developed a physiologically based pharmacokinetic (PBPK) model for the assessment of health risks from chemical mixtures based on an approach to take into account the influence of multichemical pharmacokinetic interactions at a quantitative level.

Detailed discussions on the toxicity and hazard evaluation of mixtures of substances have been presented by the World Health Organization (1981), Murphy (1983), Ballantyne (1985), Bhat and Ahangar (2007) and the National Research Council (1988) (see also **Evaluation of Toxicological Interactions for the Dose-Response Assessment of Chemical Mixtures**; **Toxicology of Chemical Mixtures**).

11 TOXICOLOGY OF DRUGS

11.1 Undesirable Effects

It has been estimated that in the USA adverse dug reactions affect somewhere in the region of two million patients, and are lethal to about 100 000 individuals annually (Nakamura, 2008). Drugs and medicinal products can produce adverse effects in the usually defined manner of toxicity and, additionally, they can cause prolongation or exaggeration of the known pharmacological effect(s) of the substance and in this respect cause the adverse inconvenience of pharmacological activity. Undesirable and unwanted effects can be classified and described according to the following schemes:

1. One system divides them into two types: *Type A*, which are the results of an exaggerated, but otherwise normal, pharmacological action of a drug, such as bleeding induced by anticoagulant drug or hypoglycaemia induced by diabetic drugs; *Type B,* which are totally aberrant effects not expected from the known pharmacological mode of actions of a drug, such as deafness from streptomycin.
2. A second system divides them into three groups: *dose dependent*, as in Type A above, *dose independent*, which are of an allergic nature involving antigen–antibody reactions, and *pseudoallergic* reactions, where allergic reactions are mimicked by mediator release due to direct action of the drug or its metabolite on mast cells.
3. In this approach, specific terms are used to describe grouped effects of an apparently common aetiological factor. They are usually characterized as follows:
   - Side effects: undesirable effects which result from the normal pharmacological actions of the drug.
   - Overdosage: implies that toxicity will occur by dosing in excess of that recommended for the desired pharmacological effect.
   - Intolerance: implies that the threshold dose to produce a pharmacological effect is lowered; this may be a consequence of a genetic abnormality.
   - Idiosyncrasy: an abnormal reaction to a drug due to an inherent, frequently genetic, anomaly.
   - Secondary effects: those arising as an indirect consequence of the pharmacological action of a drug.
   - Adverse drug interactions: adverse effects produced by a combination of drugs, but not seen when the drugs are given separately at the same dose.

Some of the terms used in this third scheme are difficult to define, and particular reactions may difficult to classify into a single one of them. Also, there is clearly bridging with some descriptive classes, notably intolerance and iodiosyncrasy with respect to genetic influences. Amongst the potential causes for adverse drug reactions, genetic variants are a likely large reservoir, and this has given rise to the study of pharmacogenomics (Huang *et al*., 2006; Nakamura, 2008). This specialization has as its main objectives investigation of the influence of genetic variability on bioresponse to drugs, identification of the gene loci responsible for adverse drug reactions and the design of screening procedures for identifying susceptible individuals. Genes currently known to be associated with adverse drug reactions have been classified into three major categories: drug-metabolizing enzymes, drug transporters and human leucocyte antigens (HLAs); those in the first two categories influence pharmacokinetics and those in the latter may possibly influence cellular immune response (Wilke *et al*., 2007). Illustrative examples are as follows. Antituberculosis drugs, including isoniazid, are known to be hepatotoxic, and it has been proposed that the production and elimination of toxic metabolites is dependant on the activities of several enzymes, including *N*-acetyl transferase 2, cytochrome P450 oxidase (CYP2E1) and

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glutathione S-transferase. There is evidence that polymorphisms at the loci for these enzymes could modify the activities, and hence the risk for hepatotoxicity. The prevalence of polymorphisms varies geographically, and correspondingly the risk for hepatotoxicity differs in populations; thus, knowledge of the polymorphisms at these loci might be useful in evaluations for, and in controlling, hepatotoxicity (Roy *et al*., 2008). Decreased drug metabolism and clearance can increase the body load of a drug or metabolite to a toxic level, and thus result in an adverse reaction. For example, genetic variants in the genes encoding thiopurine-*S*-methyl transferase and uridine diphosphate glucuronosyl transferase 1A1 are known to reduce the enzymatic conversion of azothioprine and irinotecan, respectively, and increase the risk for myelotoxicity associated with therapy (Nakamura, 2008). HLA molecules may involve interaction between a certain HLA molecule and a drug, or metabolite, and trigger a cellular immune repose. For example, there is a strong association in Han Chinese between HLA-B 1502 and the Stevens–Johnson syndrome induced by carbamazepine (Chung *et al*., 2004).

11.2 Factors Influencing Drug Toxicity

Factors influencing dose-dependent drug toxicity include formulation, route of administration, pregnancy, age, genetic polymorphism of metabolism, environmental influences on metabolism, renal and hepatic excretion, disease, drug interactions and patient compliance. Genetic factors have been considered above in Section 11.1 Nutritional status can affect both dosing efficacy and toxicity (Thomas *et al*., 1998). Additionally, certain common foods or drinks may interact with drugs or metabolites or modify their metabolism. One well-known example is the interaction of monoamine oxidase inhibitors with food rich in tyramine, causing systemic accumulation of amines and hypertensive crises (Lloyd, 1991). Another, but as yet not as widely appreciated, finding is drug interactions with grapefruit juice (Fuhr, 1998). A major factor in this interaction is suppression of the cytochrome P450 enzyme CYP3A4 in the wall of the small intestine, resulting in diminished first-pass metabolism with higher bioavailability and increased maximum plasma concentrations of the drug substrates. The P-glycoprotein pump, found in the brush border of the intestinal wall, transports many of the cytochrome P450 3A4 substrates, has also been noted to be inhibited by grapefruit juice. A consequence of inhibiting these enzyme systems is an alteration in the pharmacokinetics of several drugs leading to an elevation of their plasma concentrations (Kiani and Imam, 2007). The inhibition of intestinal CYP3A4 was, apparently, was first noted in an interaction study of felodipine with ethanol, where grapefruit juice was used to 'blind' for the administration of ethanol (Bailey *et al*., 1989). It was noted that felodipine concentrations were considerably higher than those previously reported for the dose used. In addition to increasing the bioavailability of drugs, grapefruit juice may prolong the metabolic elimination of some drugs (Fuhr *et al*., 1993; Bailey *et al*., 1994). The interaction between drugs and grapefruit juice is marked for felodipine, nimodipine and saquinavir, for which there are marked increases in the area under the curve (AUC) and/or maximum plasma concentrations greater than 70% of controls (Fuhr, 1998). Major drugs implicated in grapefruit interactions include dihydropyridine calcium antagonists, verapamil, terfenadine, cyclosporin, ethinyloestradiol, 17β-oestradiol, prednisone, midazolam, triazolam, quinidine and saguinavir; all these materials share the property of having significant first-pass metabolism by cytochrome P450 AL/5, predominantly in the gut wall, resulting in Phase I metabolites (Fuhr, 1998). It has been suggested that psoralens, principally 6′,7′-dihydrobergamottin, are major inhibitors, with a possible contribution from naringenin (Edwards *et al*., 1996; Runkell *et al*., 1997). It is proposed that patients be advised to refrain from drinking grapefruit juice when taking a drug that is extensively metabolized, unless a lack of interaction has been demonstrated (Fuhr, 1998).

In many countries, evidence of adverse drug reactions is sought in normal volunteers and patients in all phases of clinical trial leading up to licensing and marketing of a new product. Postmarketing surveillance (PMS) is then carried out to assess long-term safety in thousands of patients in order to detect low-frequency reactions that were not recognized in the relatively small number of patients studied in premarketing trials. There are a variety of PMS schemes, including voluntary reporting of possible cases of drug reaction, while standard epidemiological techniques, such as retrospective studies, prospective cohort studies and case–control studies may be useful.

12 NATURE, DESIGN AND CONDUCT OF TOXICOLOGY STUDIES

12.1 General Considerations

Toxicology studies should permit, within the constraints of the time period studied, a quantitative determination of the potential for a chemical, or mixture of chemicals, to produce local and systemic adverse effects and allow a determination of factors that may influence the nature, severity and possible reversibility of effects. Specific features that any toxicology testing programme should allow are as follows:

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- The nature of any adverse effects
- Relationship of the adverse effects to in-use and practical situations
- Dose–response relationships (average, range, hyper-reactive groups, no-effects and minimum-effects concentrations or dosages)
- Modifying factors
- Effects of gross acute overexposure
- Effects of repeated exposure (short and long term)
- Definition of allowable and nonallowable exposures
- Definition of monitoring procedures
- Guidance on protective and restrictive procedures
- Guidance on first aid and medical management
- Definition of 'at-risk' populations (e.g. by sex, pre-existing disease, or genetic susceptibility).

Information necessary for the above purposes can be obtained only from carefully designed and conducted studies. In some cases, it may not be economically possible to undertake a complete range of toxicology studies, and in such circumstances it is necessary to carefully consider the most appropriate investigational approaches based on known physiochemical properties, existing and suspected toxicology, and anticipated conditions of use. The relevance and credibility of a toxicology study can be no better than its design and conduct permit. For the purposes of hazard evaluation, there is a need to emphasize exposure conditions that may exist under practical conditions of use.

Toxicology testing programmes generally start with single-exposure *in vivo* or *in vitro* studies and progress to investigations evaluating the effects of long-term repeated exposures. Studies having specific end points, such as teratology and reproductive effects, are conducted as the emerging toxicology profile and end-use exposure patterns dictate. Toxicology testing procedures can be conveniently subdivided into general and specific. General toxicology studies are those in which animals are exposed to a test material under appropriate conditions, and are monitored for all types of toxicity that the monitoring procedures permit. Specific toxicology studies are those in which exposed animals, or *in vitro* test systems, are monitored for defined end point(s).

12.2 General Toxicology Studies

These are usually conducted as a programme in the sequence of acute, short-term repeated, subchronic and chronic. Ideally, the protocol for general studies should include provision for some animals to be kept for a period after the end of dosing in order to determine latency and reversibility, or otherwise, of toxic effects. Acute studies give information on toxicity produced by a single exposure, including the effects of massive overexposure; they also give information of use for setting exposure conditions for short-term repeated-exposure studies. The type of monitoring employed in general toxicology studies will be determined by several considerations, including the chemistry of the test material, its known or suspect toxicology, degree of exposure, and the rationale for conducting the investigation. In general, since multiple-exposure studies are most likely to produce the widest range of toxicity, it is usual to employ the most extensive monitoring in these studies. The monitoring employed to detect functional toxicity and toxicological pathology includes the following:

- Inspection, on a regular basis, for signs of toxic and/or pharmacological effects
- Body weight before dosing and at appropriate intervals during the dosing phase
- Food and water consumption
- Haematology for assessment of peripheral blood and haematopoietic tissue responses
- Clinical (blood) chemistry of various substances and of specific enzyme activities, and appropriate urinalysis
- Gross and microscopic pathology with organ weight measurement
- Special pathological or functional tests may be required on a case-by-case basis.

12.3 Specific Toxicology Studies

Many of these procedures are directed at determining a specific toxic or pathological effect for hazard-evaluation and risk-assessment purposes, but others are employed as 'screening' or 'short-term' tests to assess the potential of a substance to induce chronic effects or toxicity with a long latency. Some of the most frequently employed special toxicology methods are listed below.

12.3.1 Primary Irritation

These studies are designed to determine the potential of substances to cause local inflammatory effects, notably in skin and eye (see **Ophthalmic Toxicology**; **Cutaneous Toxicology**). In order to reduce the use of animals for eye irritancy testing, a variety of alternative procedures have been proposed, which include the use of enucleated eyes, various *in vitro* cell or tissue cultures, and the noninvasive measurement of corneal thickness (Nardone and Bradlaw, 1983; Shopsis and Sathe, 1984; Ballantyne, 1986; Borenfreund and Borrero, 1984).

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12.3.2 Peripheral Sensory Irritation

Methods to assess the potential to cause eye or respiratory tract discomfort with associated reflexes are particularly useful to perform, notably with respect to occupational toxicology, since such effects may be distracting (Owens and Punte, 1963; Ballantyne *et al*., 1977; Ballantyne, 1984; see **Peripheral Chemosensory Irritation: Fundamentals, Investigation and Applied Considerations**).

12.3.3 Immune-Mediated Hypersensitivity

Allergenic materials may produce hypersensitivity reactions by skin contact or inhalation, and several methods are available to determine the potential for chemicals to produce allergic contact dermatitis or respiratory sensitization (Goodwin *et al*., 1981; Maurer *et al*., 1984; Karol *et al*., 1985).

12.3.4 Neurological and Behavioural Toxicity

To confirm the existence, nature, site and mechanism of toxic injury to the central and/or peripheral nervous system, a variety of approaches with varying degrees of sophistication are available (see **Neurotoxicology**; **The Role of Behavioural Toxicity in Risk Assessment**). These include observational test batteries (Gad, 1982), light and electron microscopy (Spencer *et al*., 1980), selective biochemical procedures (Abou-Donia *et al*., 1987), electrophysiological, pharmacological, tissue culture and metabolism techniques (Dewar, 1981; Mitchell, 1982). The potential to produce delayed polyneuropathy, carried out in hens and sometimes in rodents, and used in the toxicological assessment of OPs and some other neurotoxic substances, has been increasingly refined (Veronesi, 1992; OECD, 1995).

12.3.5 Developmental Toxicity

Most studies are directed at assessing the potential for chemicals to induce structural defects of development, and essentially involve administering the test material to the pregnant animal during the period of maximum organogenesis (Tuchmann-Duplessis, 1980; Beckman and Brent, 1984; Tyl, 1988). However, there has been increasing interest in the development of test methods to assess possible adverse functional effects resulting from exposure of the foetus both during gestation and in the early neonatal period; for example, developmental neurobehavioural toxicology and immunotoxicology (Zbinden, 1981; Vorhees, 1983; see **Developmental Toxicology**; **Developmental Neurotoxicity**).

12.3.6 Reproductive Toxicity

Reproductive studies are conducted to assess the potential for adverse structural and functional effects on gonads, fertility, gestation, foetuses, lactation and general reproductive performance. Exposure to the chemical may be over one or several generations. In view of the necessarily comparatively low doses used during these long-term studies, they may not be sufficiently sensitive to detect most potentially teratogenic materials. The basis for these reproductive studies has been reviewed (Mattison, 1983; Baeder *et al*., 1985; Rao *et al*., 1987; see **Reproductive Toxicology**).

12.3.7 Metabolism and Pharmacokinetics

These studies may be of very considerable importance in the interpretation of conventional toxicology studies, in helping determine the mechanism of toxicity, in assessing the relationship between environmental exposure concentration and target organ toxicity, and in the design of additional studies to elucidate mechanisms of toxicity. Metabolic studies should yield information on the biotransformation of a material and the nature of the products, the sites at which this occurs and the mechanism of biotransformation. Pharmacokinetic studies should allow a quantitative determination of the rate of uptake, the absorbed dose, the biodistribution, tissue binding and storage, and the routes and rates of excretion of test material and metabolites (Oehme, 1980; Gibaldi and Perrier, 1982).

12.3.8 Genotoxicity

A number of tests, both *in vitro* and *in vivo*, are available to assess the mutagenic or clastogenic potential of chemicals (see **Mutagenesis**; **Cytogenetics**; **Genetic Toxicology Testing and its Relevance to Human Risk and Safety Evaluation**; **Short-term Tests for the Determination of Genotoxic and Carcinogenic Potential of Xenobiotics**). A positive genotoxic result is not necessarily a directly usable end point *per se*, but may assist in defining a potential for adverse health effects or be used in screening for potential longer-term toxicity. Thus, materials with clear mutagenic activity may be suspected of being genotoxic carcinogens, and appropriate further studies may be required; clastogenic materials may be suspect of reproductive or haematological toxicity.

The most widely used *in vitro* mutagenicity test has probably been that described by Ames (1982) and which utilizes histidine-dependent mutants of *Salmonella typhimurium*. The bacteria are incubated in a medium deficient in histidine; if the added test chemical is genotoxic it causes a reverse mutation to the histidine-independent state, which permits bacterial growth. Since the introduction of this bacterial assay, there have been a host of other mutagenic tests developed, including the use of other strains of bacteria and a variety of *in vitro* cell-line cultures. For example, a commonly used test system is a forward gene mutation assay in Chinese hamster ovary (CHO)

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cells with a strain which is deficient in the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT), which confers resistance to toxic purine analogues such as 6-thioguanine and permits growth of the cells in a medium containing such substrates. The presence of a mutant chemical will restore sensitivity to the presence of purine analogues, and this may be used to assess mutagenic potential quantitatively. Clastogenic potential can be assessed *in vitro* by exposing cultured cells and subsequently examining them by light microscopy for chromosome damage. *In vivo* tests for clastogenicity involve direct approaches by examining cell preparations from dosed animals for chromosomal damage (cytogenetic studies) or indirect approaches of detecting products from damaged cells such as micronuclei in erythrocytes.

It is usual to conduct *in vitro* genotoxicity studies in the presence and absence of a metabolic activation system in order to assess the possible influence of metabolism on the genetoxic potential of the test chemical. Frequently employed is a homogenate of liver from animals given the polychlorinated biphenyl, arochlor, which induces a broad range of hepatic P450-metabolizing enzymes.

In vivo genotoxicity studies can be conducted in a variety of ways. For example, the specific locus test in mice involves exposure of nonmutant mice to the test substance and subsequently mating them with multiple-recessive stock. Mutant offspring have altered phenotypes such as hair or eye colour, ear length or hair structure. As noted above, clastogenic potential can be assessed *in vivo* by exposure to the test chemical and subsequently examining mitotically active tissue, such as bone marrow, for chromosome injury.

12.3.9 Combustion Toxicology

It has been estimated that 50–75% of deaths occurring within a few hours of being exposed to a fire are the result of inhalation injuries and systemic toxicity (Ballantyne, 1981). The primary aim of combustion toxicology is to determine the adverse effects produced as a result of exposure to heated or burning materials. Although considerable emphasis has been placed on acute effects, there is increasing concern about the long-term consequences of repeated exposure to the products of combustion in occupationally exposed individuals, such as fire fighters. The design and interpretation of appropriate studies may be difficult because of the large number of variables that may affect the nature, concentration and temporal variations of combustion products. The major, though not exclusive, factors that influence the toxicity and hazard from a fire atmosphere include the nature of the material available for heating or burning, the phase of the combustion process, temperature, air flow and oxygen availability, and potential for interaction between the combustion materials generated. All of these factors may be required to be investigated and considered in evaluating the continually changing hazard from a fire. Principal lines of investigation and sources of information about toxicity and hazards from fire atmospheres are as follows:

1. Physicochemical studies to determine the nature of the products of combustion generated under differing conditions of temperature and oxygen availability
2. Animal exposure studies
3. Clinical and forensic observations on fire casualties to determine the nature and cause of morbidity and mortality from exposure to a fire atmosphere.

Although investigations designed to investigate the nature and determinants for materials producing local respiratory or systemic toxicity are of clear importance, it is also necessary to be aware of the presence of materials that may produce sensory irritant or central nervous system depressant effects. Clearly, irritant effects on the eye or narcosis may impede escape from a potentially hazardous situation. Polymers, which constitute a major component of commercial and domestic buildings, provide good examples of the generation of toxic, irritant and neurobehavioural chemical species on combustion (Ballantyne, 1989; see **Combustion Toxicology and Implications for Adverse Human Health Effects**).

12.3.10 Antidotal Studies

In addition to being aware of the likelihood of spontaneous reversibility of toxic injury (i.e. biochemical, physiological and morphological healing), it is of clear practical importance to investigate the induction of reversibility of toxicity by antidotal procedures (Marrs, 1988; see **Antidotal Studies**). Indications for such studies include high acute toxicity (including dose and time to onset of effects); serious (but potentially reversible) repeated exposure toxicity; where there are indications that early treatment may reduce or abolish latent toxicity; suspicions of a potential for antidotal effectiveness based on considerations of mechanism of toxicity and confirmation that antidotal treatment is effective for a new member of a chemical series for which a generic antidote has been established. Examples of chemicals for which specific antidotal treatment has been investigated include cyanides (Marrs, 1987; Meredith *et al*., 1993; Ballantyne *et al*., 2007), ethylene glycol and DEG (Baud *et al*., 1988; Brent *et al*., 1999; Buchanan *et al*., 2008; Velez *et al*., 2007) and OP anticholinesterases (Arena and Drew, 1986; Ellenhorn and Barceloux, 1988; Bismuth *et al*., 1992).

In addition to investigating specific antidotal therapy, it may also be necessary to confirm, or otherwise, if standard methods of management and support are appropriate for particular substances or groups of materials.

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This may include, for example, potential for aspiration hazards, influence of dilution (by giving fluid to drink) and potential for adverse interaction with drugs used to maintain cardiovascular or respiratory homeostasis.

12.3.11 Human Studies

Information resulting from human exposures may be available for certain chemicals, and can be divided into:

1. That generated experimentally, including volunteer studies and clinical trials
2. That resulting from and observing exposed populations using epidemiological techniques.

Investigational exposures of human subjects have been carried out to variable extents with a heterogeneous collection of substances, including medicinal products, cosmetics and body-care products, certain pesticides (JMPR, 1999) and industrial chemicals, food additives and chemical warfare agents (Marrs *et al*., 1996). The types of studies conducted include efficacy determinations with careful detailed monitoring for potential averse effects (clinical studies), pharmacokinetic and metabolism studies, confirmatory (to laboratory animal findings) irritancy and sensitization studies, monitoring of epidemiological studies. Carefully controlled volunteer studies are conducted for the following basic reasons:

1. To confirm the absence of potentially adverse health effects with substances that by the nature of their use patterns are deliberately brought into contact with specific routes of exposure. These may include, as examples, skin irritation or sensitization tests with cosmetic formulations, or phototoxicty/photoallergy studies with materials dosed orally or epicutaneously.
2. To investigate the metabolism and/or pharmacokinetics of a material. This may be important where prior studies have shown species variability in the pharmacokinetic or metabolic behaviour, which can be correlated with the development, or otherwise, of a particular toxic end point. In such cases, human volunteer studies can aid in determining which animal model may best represent the human situation, and hence allow a comparative risk assessment for the use of the particular material for the human population (Wilks and Weston, 1990).
3. To study quantifiable dose–exposure relationships, where a detailed hazard evaluation or risk assessment is required for the in-use application in the human population. One example is with OPs, where an assessment of dose–response and NOEL values are required with respect to cholinesterase inhibition.
4. To meet national and international governmental regulations where specified.

The conduct of human studies has implications for ethical and potential litigation considerations. Although the objective of a human volunteer study is to obtain information relative to human safety evaluation, the prime concern should be for the volunteers and to ensure that they are not exposed to unacceptable risks (van Gelderen *et al*., 1990). Clearly, detailed forethought is required to avoid the production of adverse effects that could lead to harm and possible subsequent litigation. Occasionally, clinical drugs trials in human volunteer subjects have been associated with severe reactions; for example, in a small trial involving the testing of an anti-inflammatory agent, the adverse effects were critical and volunteers required treatment in an intensive care unit (BBC, 2006). In addition to study design in order to obtain the most relevant and meaningful information, detailed attention should be given to professional, ethical, regulatory and legal factors. Sass and Needleman (2004) have noted that human studies with low statistical power are not useful for determining the presence or magnitude of adverse effects from xenobiotics, and that industry-sponsored studies may (knowingly or unknowingly) lead to bias in study design, analysis and interpretation. In 2003, the US National Research Council noted that because of potential bias in industry-sponsored and conducted studies they should receive careful scrutiny. At an expert workshop held during 2002 in New York it was noted that even at that time there were no general ethical guidelines for studies of pesticide toxicity conducted in humans and no governmental oversight. The participants developed several ethical and public policy recommendations regarding human testing of pesticides (Oleskey *et al*., 2004).

In selecting volunteers for human studies it is necessary to ensure their good health, but constraints may be necessary on certain sectors of the population: for example, women of child-bearing age, atopics and those with genetically determined biochemical disorders, such as glucose-6-phosphate dehyrogenase deficiency. Proposals for human volunteer studies should receive the approval of an independent 'ethics committee' or 'institutional review board' (IRB). They hold responsibility for deciding that the proposals are justified, possible to conduct, that the procedures are acceptable and that the study carries the minimum of risk for the participants. The IRB should be subsequently informed of any changes in the project, if there are any early indications of adverse effects or any other significant untoward events. In general, IRB approval mainly centres on risk–benefit relationships and the adequacy of the informed consent processes (Goldman and Links, 2004). The ethical committee or IRB should be totally independent of the institution or organization conducting the study. It has been proposed that the traditional IRB should be supplemented with an environmental

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health and community review board that would have an extended ethical construct of dignity, veracity, sustainability, justice and community-based issues (Gilbert, 2006).

The major guidelines under which human volunteer studies are permissible are as follows:

1. The reasons for conducting the study have been clearly defined, including an understanding of why the study will give information relevant to hazards evaluation and/or risk assessment in the human that may not be possible by nonhuman studies.
2. The concept in 1 above should be clearly stated in the protocol, which should also contain all technical details, the rationale for the route and mode of exposure, number of volunteer subjects required and monitoring procedures. The protocol should be reviewed and approved by the IRB.
3. A risk–benefit analysis should ideally be carried out in advance of the study; the smaller the direct benefit to the community, the lower should be the risk to individual volunteers.
4. The investigators conducting the study should be appropriately qualified, and this is documented.
5. Compensation to volunteers for participating in a study should be limited to inconveniences and not be an inducement.
6. Informed consent should be obtained from volunteers who must sign an appropriate consent form that should embrace the following: (i) the purpose of the study, the procedures involved and any potential risks have been comprehensively explained to the volunteers (Melnick and Huff, 2004), (ii) there is a right to refuse to participate without penalty and (iii) it is clearly stated that the volunteer can withdraw at any stage of the study.

It is of importance that the publication policies of medical and scientific journals that accept papers dealing with toxicological research involving the participation of human volunteer subjects should stress compliance with ethical, legal and human rights protection aspects. This accords with the clear intent of Article 27 of the Helsinki Declaration: 'Reports of experimentation not in accordance with the principles laid down in the declaration should not be accepted for publication'. This qualification on the publication of toxicological research involving human volunteer participation has been adopted by several professional societies who require documentation of compliance with ethical and regulatory guidelines on submission of manuscripts for publication; for example, the Society of Toxicology (Schwetz *et al*., 2005).

13 REVIEW OF TOXICOLOGY STUDIES

A critical review of toxicology studies requires detailed case-by-case considerations, but attention should be generally directed to at least the following:

- That the laboratory or institution reporting the study has the necessary scientific and/or medical credibility, capabilities, experience and expertise in the areas being investigated.
- The objectives of the investigation should be precisely stated, and the study protocol should reflect this in detail.
- The work should be reported in a clear and unambiguous manner, with all the necessary detail to allow the reader to undertake his/her own assessment and conclusions about the study.
- There should have been adequate quality control procedures, and standards appropriate to good laboratory practices (GLPs) should have been followed.
- The material tested should be precisely specified, including stability and the nature and amounts of any impurities, conversion products or additives.
- It should be confirmed that the methodology that is used for exposure and to monitor the *in vivo* or *in vitro* studies is sufficiently specific and sensitive to allow the various objectives and end points to be determined.
- Studies should be designed to allow a determination of the significance of the results and permit hazard and risk assessment procedures. For example, the number of test and control animals should be sufficient to allow for the detection of biological variability in response to exposure, to allow trends to be appreciated and to permit statistical analyses. There should be sufficient dose–response information to allow decisions on causal relationships and the magnitude of dosages which produce definite and threshold effects and those not producing toxicity.
- Monitoring should allow a determination of whether any injury produced is a direct consequence of toxicity or an effect that is secondary to toxicity at another site. A primary effect is one produced as a result of a direct toxic effect of a chemical, or metabolite(s), on a target organ or tissue. Secondary effects are those occurring, often at another, but nontarget, site, as a consequence of toxicity in the primary tissue or organ. For example, primary pulmonary injury produced by inhaled potent irritant materials may result in significant hypoxaemia and secondary hypoxic injury to other organs, including liver, kidney or brain. Ideally the study should be carefully assessed to allow a conclusion as to whether the toxicity induced is a consequence of the action of parent material or metabolite, for example,

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comparison of routes involving and not involving first-pass effects.

- Detailed assessment is required to determine if the numerical data have been appropriately and correctly evaluated. Thus, although there may be a statistically significant difference between a test group and the controls, this may not be of biological or toxicological significance. Conversely, changes or trends, not of statistical significance, may be of biological and toxicological relevance. Quantitative information should be viewed against the study as a whole, normal biological variability, quantitative changes which imply pathological processes and the magnitude of any changes as they may relate to an adverse effect.

The above considerations demand the careful design of toxicology studies, taking into account all factors that are inherent in the defined and inferred objectives of the investigation. To illustrate the care required in the interpretation of toxicology studies, a few examples are given below of different specific factors that need attention in particular studies:

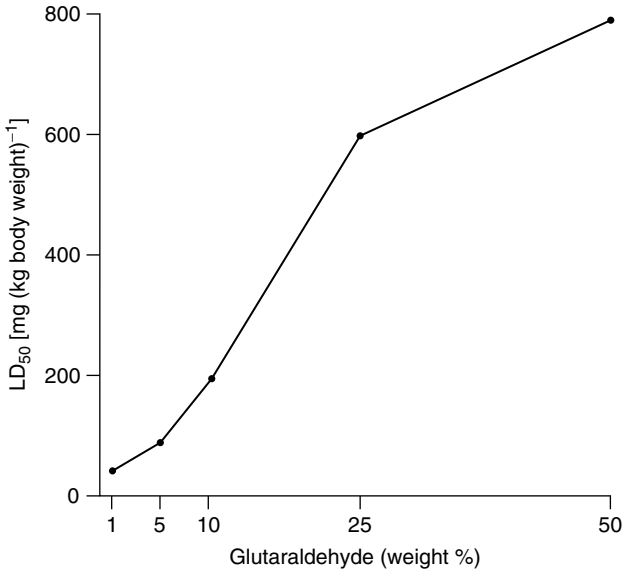

Figure 15 Effect of aqueous dilution on the acute peroral lethal toxicity (as LD_{50}) of glutaraldehyde to rats. As dilution increases the LD_{50} becomes smaller; that is, greater toxicity. (Data after Ballantyne and Jordan, 2001.)

- The acute po LD_{50} of the undiluted diethylamine is $<0.25\,ml\,kg^{-1}$, whereas with a 10% aqueous solution the acute po LD_{50} is $1.41\,ml\,kg^{-1}$, illustrating the influence of dilution of the test material on toxicity. A reciprocal relationship has been demonstrated with glutaraldehyde (**Figure 15**); in this case, acute po toxicity (as mg active material per kg body weight) increases with dilution within the confines of the study.
- Materials with similar LD_{50} values may have differences in acute toxicity shown by other monitors of their toxicity. For example, 2,4-pentanedione has an acute po LD_{50} (rat) of $0.58\,g\,kg^{-1}$, similar to that of BAPP at $0.31\,g\,kg^{-1}$; however, times to death were 2–5 hours with 2,4-pentanedione and three to four days with BAPP, indicating a more serious potential hazard with the former.
- With inhalation studies, the method of generation of the test material in the atmosphere may be a highly important consideration, as indicated by the following three illustrative examples:
  - For acute vapour inhalation studies, and in tests concerned with defining the effects of saturated vapour atmospheres, the vapour may be generated by static or dynamic methods. Static methods involve placing a sample of the test material in the exposure chamber and allowing the atmosphere to equilibrate for an appropriate period of time; thus, all volatile components accumulate to vapour saturation in the chamber. Dynamically generated atmospheres are produced by passing air through the test material and transferring the atmosphere so generated into and through the chamber in a continuous manner; this results in components of the test material being present in the atmosphere in proportion to both their concentration in the test material and volatility. Thus, trace contaminants of highly volatile toxic materials will be present in much higher concentrations with static, as opposed to dynamic, conditions. For example, methoxydihydropyran (MDP), containing 0.037% acrolein, when generated dynamically did not produce mortalities with rats for a four hour exposure period (MDP vapour concentration 7748 ppm; acrolein, trace). However, when the same material was used in a static exposure system there were mortalities due to the accumulation of acrolein vapour in the atmosphere (MDP vapour concentration 8044 ppm; acrolein, 240 ppm); acrolein has a four hour LC_{50} of 8.3 ppm (Ballantyne *et al.*, 1989a).
  - The relative humidity of the chamber atmosphere may influence inhalation toxicity with hydrolysable materials. For example, when tris(dimethylamino) silane (TDMAS) was generated as a vapour with moistened air, a four hour LC_{50} of 734 ppm was determined (female rat), which accords stoichiometrically with toxicity due to dimethylamine formed by hydrolysis of TDMAS. However, when the vapour was generated under dry air conditions, a four hour LC_{50} of 38 ppm was calculated from the exposure–mortality data, indicating

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a highly significantly greater intrinsic toxicity for the TDMAS molecule (Ballantyne *et al*., 1989b).
- A marked difference in toxicity may be obtained for the same material generated in different modes of exposure or different physical states. For example, short-term repeated exposure to vapour from 2-methacryloxypropyltrimethoxysilane does not produce any respiratory tract injury. However, when generated as an aqueous respirable aerosol it produces laryngeal granulomas (Klonne *et al*., 1987).
- The use conditions of the test material may influence toxicity. Thus, the potential for cutting oil to induce cutaneous neoplasms is significantly enhanced after its industrial use, possibly owing to the generation of polycyclic aromatic hydrocarbons (Agarwal *et al*., 1986).

14 HAZARD EVALUATION AND RISK ASSESSMENT

Toxicology is concerned with determining the potential for materials to produce adverse effects, whereas hazard evaluation is a process to determine if any of the known potential adverse effects will develop under specific conditions of use. Thus, toxicology is but one of the many considerations to be taken into account in the hazard evaluation process. The following are some of the other factors that need to be considered in defining whether a specific use of a material will be hazardous, and are discussed in detail by Tyler and Ballantyne (1988):

- Physicochemical properties of the material
- Use pattern
- Characteristics of the handling procedure
- Source of exposure and route of exposure, both normal and possible misuse
- Control measures
- Magnitude, duration and frequency of exposure
- Physical nature of exposure conditions (e.g. solid, liquid, vapour, gas, aerosol)
- Variability in exposure conditions
- Population exposed (e.g. number, sex, age, health status)
- Any experience and information derived from exposed human populations.

The general approach used to assess hazards is as follows:

1. A search for all available health-related information on the substance and, if appropriate, substances of close chemical structure. This may include information on physicochemical properties, *in vivo* and *in vitro* toxicology, epidemiology, known occupational and domestic incidents, case reports, monitoring and use patterns. Mention should be made of the use of structure–activity relationships (SAR) for the prediction of toxicity. Basically this generally involves a computer-based modelling approach to relate chemical structure to (adverse) biological activity and, where sufficient information exists, to develop quantitative structure–activity relationships (QSAR) of biological potency. These approaches may yield valuable information that can be used in the planning of toxicology studies and, within the limits of the methodology and its assumptions, supply interim predictive statements on potential hazards. When used for hazard-evaluation purposes, it should clearly be stated that the information has been derived by SAR methods. Although a useful approach in the absence of 'hard' information, it has been noted that given the large range and variability of possible interactions of chemicals in biological systems, it is highly unlikely that SAR models will ever achieve absolute certainty in predicting toxicity, particularly with respect to the whole-animal system (McKinney *et al*., 2000).
2. Detailed impartial review of all information obtained, emphasizing those studies conducted by credible scientific standards and by relevant routes of exposure.
3. Interpretation of the credible and relevant literature in order to define toxicity and, if possible, mechanism, dose–response relationships and factors influencing toxicity (endogenous and exogenous).
4. Conclusions regarding potential adverse effects from the substance under specific conditions of use.
5. Determination of acceptable handling or in-use conditions and acceptable exposure to the substance with respect to immediate and long-term conditions of use.
6. Determination of the management of overexposure situations.

The process and understanding of hazard evaluation, and its scientific basis, are now at a level where reliable interpretation and prediction can be made. A less reliable and scientifically limited evaluation process is that of risk assessment, which is an important developing component of regulatory and occupational toxicology. It is the objective of risk-assessment processes to assess the probability that adverse health effects will develop from known, or suspect, xenobiotics in the environment (e.g. drinking water or air) or workplace. Such quantitative risk assessments are most frequently conducted for work-time or lifetime exposure to low concentrations of xenobiotic. They are based on extrapolating dose–response relationships from animal studies, or occasionally human

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epidemiological data to: (i) determine risk at known or anticipated ranges of occupational or environmental exposure dosages or (ii) to assess 'risk-free' dosages. The approaches most frequently employed are to assess risk from carcinogens, teratogens, reproductively active substances and genotoxic materials.

With most materials there is usually insufficient information on mechanisms of toxicity for particular materials to allow scientifically valid, appropriate mathematical models to be developed for a specific toxic effect. The methods of extrapolation often make many, often biologically unreasonable, assumptions which include: (i) the existence (or otherwise) of thresholds for specific toxic end points; (ii) linearity of dose–response relationships; (iii) comparability of metabolism and pharmacokinetic parameters between species; (iv) the interaction between xenobiotics and biological systems at low concentrations and (v) the statistical reliability and biological variability resulting from the relatively small numbers of animals that may technically and ethically be incorporated into animal studies. Thus, with current mathematical approaches to data extrapolation, quantitative risk assessments should probably be regarded as 'best guesses' for environmentally safe exposure dosages. The findings from quantitative risk assessment may result in risk-management measures being undertaken (Hallenbeck and Cunningham, 1986). This involves the development and implementation of regulatory action, taking into account additional factors such as available control measures, cost–benefit analyses, 'acceptable' levels of risk, and taking note of various policy, social and political issues. More specific and mechanistically reliable estimates can be obtained from the process of 'biological risk assessment', which allows a more rational risk analysis based upon incorporation of metabolism and pharmacokinetic findings, including interspecies differences, mechanisms of toxicity and influence of physiological variables (Clayson, 1987; National Research Council, 1987).

15 SPECIAL CONSIDERATIONS IN HUMAN HAZARD EVALUATION

By the very nature their intended design, laboratory toxicology studies are conducted under highly controlled conditions using healthy animals often of a particular weight range. The extrapolation of such information to a heterogeneous human population, with differing lifestyles and variable states of health, needs to be undertaken with considerable caution, taking into account all possible known and predictable variables. The possible interactions of multiple exposures to a variety of chemicals or drugs has been discussed earlier in this chapter. Other illustrative examples are presented below.

15.1 Personal Habits

Many personal habits, including diet and the taking of medicinal products, may influence the response to a toxic chemical. Two factors that have received special attention are cigarette smoking and excessive alcohol consumption. Cigarette smoking may lead to increased body burdens of many of the combustion products found in smoke, in particular carbon monoxide. Owing to the significantly increased carboxyhaemoglobin concentrations in smokers, they may be at greater risk in the occupational environment from carbon monoxide and carbon monoxide-generating materials, such as methylene chloride. Other materials in cigarette smoke that may increase the exposure burden include hydrogen cyanide, hydrogen sulfide, acrolein and polycyclic aromatic hydrocarbons. In some instances, there are clear indications of significantly enhanced toxicity, for example, synergism between cigarette smoking and asbestos (Hammond and Selikoff, 1973) or radon (Archer *et al*., 1972). Heavy alcohol consumption may lead to chronic progressive liver injury and fibrosis, and thus increase susceptibility to hepatotoxic substances and impair detoxification pathways.

15.2 Coexisting Disease

Individuals with certain illnesses may be at greater risk from particular drugs or industrial chemicals. For example, those with established cardiovascular disease may be at increased risk from exposure to carbon monoxide or methaemoglobin-generating substances, since both may compromise the available oxygen supply to the myocardium. Inhalation of irritant materials may aggravate chronic progressive pulmonary disease.

15.3 Genetically Susceptible Subpopulations

Individuals with genetically determined biochemical variants may be at greater risk from certain drugs and chemicals than those with normal biochemical features. Some examples are as follows:

- Individuals with hereditary methaemoglobinaemia may generate significant amounts of methaemoglobin at exposure doses of nitrites or aromatic amines that cause only minor methaemoglobin concentrations in the normal population.
- It is well known that slow acetylators are significantly more susceptible to the neurotoxic potential of isoniazid, whereas fast acetylators are more

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likely to develop liver injury, since hepatotoxicity is caused by the metabolite acetylhydrazine (Breckenridge and Orme, 1987). Another aspect of acetylator status relates to the potential of arylamines to induce bladder cancer. Slow acetylators may be more susceptible to arylamine-induced bladder cancer (Cartwright *et al*., 1982), possibly related to a higher urinary excretion of free arylamine (Derwan *et al*., 1986).

- Individuals with glucose-6-phosphate dehydrogenase-deficient erythrocytes may be at increased risk from haemolytic effects of oxidants because of the inability of the erythrocyte to generate sufficient NADPH and maintain an adequate concentration of reduced glutathione, resulting in haemolysis (Calabrese *et al*., 1987). However, animal studies suggest that haemolytic effects occur only on exposure to otherwise toxic concentrations (Amoruso *et al*., 1986).
- Exposure of persons with inherited uroporphyrinogen decarboxylase deficiency to dioxin can cause latent chronic hepatic porphyria to develop into porphyria cutanea tarda (Doss and Columbi, 1987).

Other genetically determined variants that have been implicated as causing increased susceptibility to chemicals include α1-antitrypsin deficiency (emphysema), aryl hydrocarbon hydroxylase deficiency (lung cancer), pseudocholinesterase variants (anticholinesterase toxicity) and thalassaemia (lead). An interaction between environmental factors and specific genotypes has been suggested as being involved in the aetiology of a number of diseases, including parkinsonism (Menegon *et al*., 1998; Cummings, 1999). Genetic screening of workers or prescreening for the identification of susceptible subpopulations to occupational exposure has raised several issues of ethical and social concerns (Christiani *et al*., 2008), and it has been proposed that workers and/or their representatives should be involved in deciding when and where genetic testing in the workplace is done (Holtzman, 2003).

16 PROFESSIONAL AND ETHICAL ISSUES

The right to search for truth implies also a duty. One must conceal any part of what one has recognised as true.

(Albert Einstein)

16.1 General Comments and Principles

There is no utopia with respect to the protection of the human population and the environment against xenobiotics, and it is thus of prime importance that the toxicologist and professional colleagues should be acutely and constantly aware and certain of the ethical and professional foundations of their work and its potential implications, and that they should give opinions in a credible, transparent and professional manner. All professional branches of science and technology have in common the objectives of high standards of learning, practice, personal and professional conduct, and visibility, in addition to more specific considerations relevant to the individual specialization. As a consequence many have developed codes of conduct and guidance note on ethical practices; for example, the Institute of Biology (2005) and the Royal Society of Chemistry (1995). However, codes of conduct and ethics should allow for constructive communication between scientists and the community. This sometimes falls short of expectations, as did the UK Council for Science and Technology 'Universal Ethical Code for Scientists' which was a series of awkwardly worded exhortations and platitudinous rules (Editorial, 2006). Members of the profession of toxicology should also follow the demands of ethical integrity as dictated by their individual conscience and the guidance of representative professional organizations. By their very nature and intended applications, toxicology investigations can have far reaching implications for health-related issues in the workplace, commerce, home and general environment. The same consideration applies to the application and expression of specialist knowledge by the toxicologist in his/her professional capacity. There are, as a consequence, many ethical issues associated with the practice of the science of toxicology and the practical applications of toxicology information. Whereas morality often involves a distinction between what is right and what is wrong, professional ethical considerations may not have such a clear distinction and individual experience, lifestyle, mental orientation and professional outlook are contributing factors. Against these considerations, various competing factors may have an opposing and possibly detrimental effect on the ethical values, and the value of ethical practices, by an individual; these include biases, motivations and potentially conflicting attitudes within a group organization. Thus, toxicologists working in commerce/industry and government service may find themselves in the midst of situations with the potential for group or organization self interest and expectations for bias in attitude and interpretation which could conflict with professional and personal ethical standards, and lead to difficult, strained and possibly inharmonious relationships. Such situations may lead to problems associated with community values, where the behaviours that govern moral and ethical conduct depend on a multiplicity of considerations, principal amongst

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which are majority viewpoints of the general population, the existing background of accepted general concepts and principles of moral and ethical values, and what are considered by the majority of a democratic society to the advantage of society relative to health, welfare and harmonious coexistence. Professionals often find that their ability to maintain and adhere to ethical standards is often undermined and enforceably questioned by others with self interests outside the professional field; these may include financial, commercial, economic and politically motivated considerations. All of these may result in significant influences to bias or attempt to manipulate the interpretation and implications of scientific observations, including iconoclasm of credible findings. Some professional society codes of conduct clearly recognize these potential employer–employee conflicts. For example, the Institute of Biology advises employees that—'In serving the interests of their employer in good faith and to the best of their ability, always keep in mind the obligation to serve the public interest and maintain and enhance the reputation of the profession' (Institute of Biology, 2005, p. 5).

Current society is experiencing an unfortunate, disturbing and widening gap between democratic societal moral and ethical values and what constitutes legal dictates. This is particularly noticeable in the adversarial society, notably in the USA; in dictatorial truth denials by commercial and political organizations; as a consequence of the decreasing separation of government and dictatorial religious dogma and in the distorted and smear tactics employed by corporations and government against those with ethical, reasoned arguments for causation.

From a practical standpoint and on the basis of the above considerations it is not unexpected to find in some situations that the design, conduct and reporting of toxicology studies as well as the interpretation of the findings and their implications with respect to human health and the environment, may be adversely influenced by considerations of a political, economic, personal, security or other self-interest nature. In many circumstances such factors may result in biased interpretation (in some cases deliberate) of otherwise credible experimental findings and thus a distortion or even suppression of findings and of the truth. Additionally, as noted by Golberg (1982), it is not unusual to see uncritical acceptance of adverse conclusions drawn from poor data. It follows that toxicologists should not only focus on the credibility and scientific significance of their work, but also within ethical and professional perspectives on the practical use(s) to which to which scientific discovery and knowledge can (or will) be used and for its general societal repercussions or misapplications. This is not, and should not be, the prerogative, as claimed, of those having hierarchical wider political and commercial interests. There is no universal scheme or consensus approach to ensure that all health-related information be made generally available to the scientific, medical and other interested communities, but this is not only a highly desirable, but indeed regarded by many of us as a necessary feature of all research and development activities having implications with respect to human health and the environment. However, self-interest by various individuals, groups, organizations, on a national or global basis, implies that no such utopia of knowledge is ever likely to exist.

Toxicologists can and do, at various times, become involved in legally orientated situations and their consequences. In cases involving litigation, the professional toxicologist should anticipate that his/her credibility will be questioned and should be appropriately prepared for critical evaluation (Furst, 1997; Furst and Reidy, 1999). On a more routine work basis, other major areas where the toxicologist may become involved in processes interfacing with national and international laws and regulations include the following: animal welfare; conduct and reporting of laboratory studies; regulatory test requirements; mandatory reporting of potential adverse health effects, as, for example, under Section 8(e) of the Toxic Substances Control Act (TSCA); human volunteer studies. Ethical aspects of human volunteer studies has been reviewed above (see Section 12.3.11), and several other aspects of ethical–legal interactions in toxicology are briefly discussed below.

16.2 Conduct of Laboratory Studies

Before there was formal regulatory control of the conduct of toxicology laboratory studies in the USA, some commercial and industrial in-house laboratories conducted studies without due regard to the development and adherence to specific protocols, kept poor records of findings and often only reported selected findings in a study. Although such badly conducted studies were rampant in only a few laboratories, instances of lesser unacceptable procedures and practices were not infrequent in many laboratories. Following enquiries by the FDA into deficiencies, inaccuracies and fraudulent data contained in reports, legislation was introduced in 1979 to ensure integrity and reproducibility of studies, in the form of GLP. Following introduction of the FDA GLP regulations, several other countries recognized the need for such legislation, and now most countries have programmes with a GLP core in operation that deal with laboratory facilities and personnel, protocols, reports and archives (summarized by Ballantyne, 2005).

16.3 Animal Welfare

The use of animals can be an emotive subject, and has been the subject of deep and extensive augments,

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public demonstrations, the use of violence by activists, legislation and the introduction of alternative methodologies (Ballantyne, 2005; Edmunds, 2005). Legal controls on the use of animals in biomedical research lie with government. The most extensive and demanding legislation has been in the UK, where a bill was passed through the British parliament in the nineteenth century (Animal Cruelty Act of 1876), and was updated in 1986 by the Animals (Scientific Procedures) Act. The legislation requires detailed licensing of research facilities and of research scientists, annual returns on animal usage and random audits by the Home Office inspectorate. In the USA, animal welfare became an integral component of conducting sound studies with the adoption of the EPA GLP regulations. As a consequence of the activities of credible animal rights groups and concerns by ethically motivated scientists, there have been considerable efforts to reduce animal discomfort and use *in vitro* toxicology research. These have included reduction in the numbers of animals used, refinement of *in vivo* procedures including improved and noninvasive monitoring procedures, and the development of *in vitro* alternatives. Partly because of the increased activities of animal welfare groups, and associated media reporting, several professional organizations have issued guidance documents for good animal welfare practices; for example, the (American College of Toxicology, 1988, Academy of Medical Sciences, 2007, Society of Toxicology, 2005). These, however, should only be regarded as itemized guidance notes, and not be used as a replacement for a carefully developed individual institutional programme. Continual individual and institutional vigilance is necessary in order to remain within the ethical and legal boundaries of animal care and handling. In-depth reviews on the legal and ethical aspects of animal usage in are to be found in **Chemical Terrorism**.

16.4 Regulatory Activity

The professional activities of many toxicologists will be influenced by the requirements of governmental regulatory agencies. Also, the toxicology community and the general population should be aware of the biased, and possibly misleading, influence that extensive lobbying of political organizations by industry for economic reasons (Loewenberg, 2006). Regulatory activities are to be found principally in relation to the production and in-use aspects of pesticides, industrial chemicals, pharmaceutical preparations, veterinary drugs, food additives, food contact materials, medical devices and cosmetics. The structure of regulations, their precise requirements, and whether statutory or voluntary, varies with the nature of the material regulated and the geographical location of the authority.

Toxicologists in commerce should have responsibility for advising on the scientific aspects of conforming to regulatory requirements, and on study design, conduct and the interpretation of findings. Regulatory agencies themselves have had impact on these aspects of toxicology studies, being obvious from the introduction of GLP principles. There have been phases when the attitude has been 'studies conducted to meet regulatory requirements', and during these phases this has resulted in a fragmentation of intellectual approaches to regulatory-based toxicology. Thus, some toxicology testing requirements that have been proposed by regulatory agencies are too specific for the intended purpose(s) and may contain fine detail that does not permit the flexibility necessary to undertake a vital hazard evaluation procedure. This has, at times, led to an attitude that some mandated regulatory toxicology testing is conducted merely to 'satisfy regulations' rather than to undertake a well-designed study to determine toxicity and permit objective evaluation of hazards. Clearly such attitudes may be conducive to inappropriate toxicology practices, excess use of *in vivo* procedures, and result in documentation that does not allow reasonable assessments of in-use hazards. Such a less than satisfactory and scientifically delinquent approach has resulted in reference to 'cook book' regulatory practices. A rationalized toxicology testing programme requires that for any particular material there is an in-depth consideration of a multitude of factors that may be unique to the material being tested and to its intended use. In no way can a generic protocol substitute for a scientifically reasoned approach to study design. In many cases, however, a scientifically relevant testing programme can be developed in collaborative discussions with regulatory agencies. The current complexities, tardiness and almost incomprehensibility of regulatory toxicology activities, including attempts at global harmonization, has been, to a not inconsiderable extent, a consequence of increasing bureaucracy with excesses of committee structures, permanent and advisory staffing, with personnel lacking the necessary educational and experience backgrounds, infiltration with legislative language and the uncontrolled misuse of meaningless synthetic management and legal language. Attempts at international harmonization, often marred by national interests, have on occasion resulted in flagrant disregard of credible scientific truths, concepts and criteria (Ballantyne, 2005). It is important that competent regulatory agencies who review data for classification and regulatory purposes should have access to credible independent and professionally qualified advisors and advisory committees that do not have vested interests in the economics of any submitting commercial organizations. Advisors should have documented national and international respect for their professional knowledge and integrity (Ballantyne and Marrs, 2004).

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16.5 Publications

It is a core belief and value of many of us that the privilege of being allowed to conduct medical and scientific research, and to search for truth, is inseparable from the duty that no part of the truth is withheld. Implicit in this belief is that knowledge is the property of civilization and as such should not be subject to suppression by political agendas or commercial interests. In addition to the manipulation and suppression of the truth for political, commercial, economic and other limited interest reasons, it is clear in some cases that unethical and unprofessional practices in the conduct of basic and applied research are often associated with willful aberrations in the communication of scientific and medical information. Whilst the majority of full-length publications in respected scientific, medical and technical journals are of the highest credibility and significantly add to our knowledge and understanding, some are less desirable. The reasons for manipulating and/or fabricating information in publications are usually the same as those for deviant conduct in the research process. Malpractices have included the publication of fictitious data (Lock, 1995), manipulation of data to support a predetermined viewpoint, data modified to achieve a desired statistical significance and misquoting others (often cited as 'unpublished data'). Editors and referees should be constantly vigilant to detect unprofessional activity in submitted manuscripts. A further unfortunate trend is for some workers to duplicate publication, in which the same, or a marginally altered, paper is published in different journals. It is very unlikely that this is a desire on the part of the author(s) to ensure that the widest audience has access to the information, but more likely the motivation is related to publication proliferation in research-restricted or competitive environments. Those who see such unethical duplication of information, often against the stated objective of the journals, should make this known to the appropriate editor(s). With the objective of preventing, or minimizing, publication of papers containing biased protocols and containing falsified or manipulated data, many journals and scientific societies have developed guidelines and codes of practice for the publication and presentation of scientific information. Editors should ensure that their referees act with professional scientific integrity and impartiality when reviewing manuscripts, and do not attempt to impose their opinions on the subjective interpretation of the findings. Indeed it is reprehensible for a referee to insist that the author's interpretation of findings be amended to accord with the viewpoints of the referee, a situation which some of us have encountered on occasion. In such circumstances the author(s) should formally complain to the journal editor and publisher. The major functions of journal editors and referees should be to ensure, as far as is possible, that experimental design is appropriate for the stated objective of the study, and the information submitted in the manuscript represents a true, complete and unbiased record of the methods and findings.

As a component in the process of ensuring credible reporting and interpretation of information, most toxicology-orientated journals now require that the author(s) include a disclosure statement, for publication, of any possible conflicts of interest that could result in misrepresentation of the facts and opinions expressed. Also, many journals also require that participants in the peer review process must disclose, to the editor, any conflict of interest(s) that could result in reviewer bias of the manuscript (Lehman-McKeeman and Peterson, 2003; Maurissen *et al*., 2005).

Peer review of manuscripts by credible scientific journals is an integral component of codes of practice to ensure quality and integrity of publications. It involves the scrutiny of an author's manuscripts by others who are recognized experts in the same field, and to render an opinion as to the suitability of the submitted paper for publication in a particular journal. In an international survey of 3040 academics with respect to their attitudes to the peer-review process, it was generally concluded (90% of those surveyed) that the process helps scientific communication and, in particular, improves the quality of published papers (Ware, 2008). The major findings were: peer review is widely supported by academics; it improves the quality of published papers; double-blind review is preferred and most effective and postpublication review may be a useful supplement to formal peer review. Although peer review of manuscripts submitted to scientific and medical journals is a well-established and accepted process, there have been attempts to question the value and integrity of this standard practice. The motivations of these moves against the standard practice of peer review are variable, but in many cases have a commercial interest. For example, in May 2007 the *New England Journal of Medicine* was served with a subpoena from Pfizer's lawyers demanding that the *Journal* produce peer review and other editorial documents concerning several of their products. Apparently, Pfizer required the documentation not only for defence in product-liability litigation, but also to discover 'flaws in methodology' in the research that might have been noted by the *Journal*'s peer reviewers. The request was refused, and in January 2008 Pfizer's attorneys filed a motion to compel the production of the required documents. The journal again refused and filed with the court an Opposition and Motion for Protective Order, on the basis that reviewers had been promised confidentiality and that to provide the requested information would hamper the *Journal*'s ability to serve the medical community. The motions were heard in federal court on 13 March 2008, where the judge concluded that 'the *New England Journal of Medicine's* interest in maintaining the confidentiality of the peer-review process is a very significant one, especially in the light of its non-party status, and tips the scale in favour of the *New England of Medicine*'. He also noted that 'the batch

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or wholesale disclosure by the *New England Journal of Medicine* of the peer review comments communicated to authors will be harmful to the *New England Journal of Medicine's* ability to fulfil both its journalistic and scholarly missions' (Curfman *et al*., 2008b). Pfizer also issued similar subpoenas on the *Journal of the American Medical Association* and *Archives of Internal Medicine*, who both argued the sanctity of the peer-review process, and both had similar progression to that of the *New England Journal of Medicine*. In a 14 March 2008 ruling in federal court in Chicago, the judge concluded, '... given the strong policy behind preserving confidentiality in the peer review process, the Court finds any probative value would be outweighed by the burden imposed on the Journals in invading the sanctity of that process' (DeAngelis and Thornton, 2008; Keys, 2008). Also relevant to the peer-review process is the possibility for breach of reviewer confidentiality by the reviewer communicating review-related information to parties other than the journal for whom a review was conducted (Editorial, 2008). There should be provision for declaration of any conflicts of interest by reviewers that might prohibit confidentiality of the process.

There have, from time-to-time, been proposals from some societies and journals that research sponsored by certain commercial organizations should not be accepted for publication because of possible self-interest bias and possible effect of this on credible reporting and interpretation of information. For example, the American Thoracic Society decided that no longer would it accept medical research sponsored by the tobacco industry in its peer-reviewed journals (Rutter, 1995). Decisions of this nature could be interpreted as journals or societies having a political agenda and exhibiting censorship (Roberts and Smith, 1996), a practice that, if uncontrolled, could extend into research sponsored by other organizations such as pharmaceutical and other industries, or even in-house research activities. One effective control approach is by drawing the attention of the reader to potential ethical and professional issues through the mechanism of disclosure of interests, with particular reference to sources of funding (Landrigan, 1995). Disclosure of interest would be by the inclusion in the published paper of a statement recording source(s) of funding and any vested interest(s) of the author(s) in the work conducted for the sponsoring organization (Lehman-McKeeman and Peterson, 2003). The need for disclosure has been stressed by the recently created nonprofit foundations housed in academic institutions, but organized for the benefits of individual investigators and funded by industry sponsors (Schwartz *et al*., 2008). Although such foundations may be helpful in providing needed research funds, the foundations may not be required to publically disclose details of their funding sources. Funding disclosure in published papers is one critical factor in transparency of biomedical research and its implications for the public health. Perhaps the extreme of industry funding and its potential implications with respect to totally credible and unbiased medical science is afforded by the fact that bioethicists and academic bioethics centres have sought and accepted funding, particularly from pharmaceutical and biotechnology industries (Elliott, 2005).

16.6 General Legal–Ethical–Professional–Employer Interfaces

16.6.1 Overview

By the very nature of their activities, professional toxicologists may find themselves in conflict situations resulting from pressures exerted by others (individuals and organizations) to engage in, or condone, decisions and/or actions that do not accord with personal and professional integrity and ethical principles. Whilst the majority of toxicologists have a high level of ethical and professional standards in the practice of their specialization, a small proportion have lower standards with respect to these qualities; the proportion is about the same as for any other profession (Ballantyne, 1988). In some instances the actions and consequences of lower standards may clearly be illegal and unethical, but in other cases whilst there may not be a direct conflict with the law there is clearly professional misconduct; there is a spectrum of malpractices between these two extremes. Although the majority of toxicological research is undertaken and published against a background of scientific impartiality and integrity, some investigations are conducted with preconceived considerations for the outcome, and in this respect the investigators may design, conduct and interpret the study towards obtaining the desired outcome. The reasons for such professional dishonesty may be multiple, and extend from an individual investigator to a whole organization. They include, but are not limited to, economic considerations, including profit margins, competitive activity within a given production line, product registration, attraction of funding, enforced direction from management, political motivation and career objectives. Malpractices include biased protocol development, nonstated deviations from the protocol, data falsification and fabrication, and omission of data that do not further a preconceived bias. On an organizational basis, instances of lack of scientific integrity, malpractices and professional dishonesty have been noted at times with industry, government and academia, and in some disgraceful distances there have been indications of collusion. For example, in the UK, the Institute of Professionals, Managers and Specialists obtained evidence for concern amongst scientists working for government quangos or for newly privatized laboratories who reported that they were asked to adjust their research

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conclusions and/or resulting advice. This included 17% who were asked to change their conclusions to suit the customer's preferred conclusions, in some cases this was to obtain further contract work (Ballantyne, 2005). Other examples are almost beyond understanding, since it is clear that the deceit will almost certainly become known. One reprehensible instance in the USA was that of the EPA giving misleading information about health effects to New York citizens from debris, dust and smoke in the air around the World Trade Center following the terrorist attacks on 11 September, 2001. Another example of possible government-department withholding of health-related information is by the Centers for Disease Control (CDC) delaying a report indicating greater health risks for people living in some areas around the US Great Lakes areas (Bristol, 2008). The adverse influences on public health issues by some dishonest business organizations, lobbyists and corrupt political administrations are transparently evident on a continuing basis. At the other end of the spectrum of professional dishonesty is misconduct by individuals or small groups, whose motivations include pressure to publish, the need for funding, financial gain and promotion. In order to limit biased or fraudulent activities in studies conducted for commercial organizations by contact, there should be a clear recorded formal agreement that should include the following: ownership and access to data, study design according to credible scientific principles, conduct, auditing, publication rights and indications for project termination (Paris, 1996). One reason for apparent professional and organizational misconduct may stem from the unfortunate trend, seen in both industry and government service, to appoint 'managers', not on the basis of qualifications, knowledge and relevant experience, but on their tenacious 'team player' attitude for the organization, often in the face of obvious truth and credibility. This has resulted in the direction of work being steered towards what is politically or economically desirable by senior organizational management. It has been 'reasoned' that such an organizational structure of lay management with side lining of experienced professionals permits 'the various factors to be viewed in their widest perspective', whilst in fact it is planned to obtain the 'desired interests' of the organization. The ramifications of lay control of professional activity and expression argue for independence of expression without repercussions (see Section 16.6.5). Under research activities, professional malpractice may include the undertaking of work in a clandestine manner for 'reasons' of national security or commercial advantages, and for similar reasons the suppression or manipulation of data. For example, studies may be undertaken confidentially and with no intentions to make the findings publicly known for commercial (monetary) and deceptive business practices, as identified with the tobacco industry, who have studied the relationship between smoking behaviour and product design (Hammond *et al*., 2006). Strategies resulting from these confidential internal studies relate to exploiting the limitations of toxicology testing and to concealing from consumers and regulatory agencies the potential health dangers of products. This underlines the greed and blatant disregard for pubic health by the tobacco industry, and their current advantage in the USA that there is no (at the time of writing) regulatory activity by the FDA with regard to tobacco products (Curfman *et al*., 2008a).

Toxicologists need to be continually vigilant in order to maintain a credible, ethical and professional approach to their work and its implications. This covers work practices and inter-relationships with others, including advisory functions and employers. Some major areas where caution is necessary are as follows.

16.6.2 Professional Conduct

To ensure that scientific investigations are conducted and communicated in publications in accordance with the highest scientific and ethical standards, several professional societies and journals have developed codes of practice for their membership and contributors. Such codes require thoughtful development against a myriad of challenges (Goodman, 1996). They should not be regarded as static documents for display, but as guidelines that require periodic revision against the changing background of investigational procedures and evolving knowledge. It has been proposed that there are three broad approaches to preventing scientific fraud and misconduct: education, standards and training (Evered and Lazar, 1995). These should be conducted by, demonstrated by and practised by the staff of research and development organizations, academic institutions, government organizations, professional organizations and their society members. When there are accusations of professional misconduct and/or behaviour, these should be dealt with promptly, impartially, by transparent methods and with subsequent access to arbitration and appeal if considered necessary.

16.6.3 Personal Integrity and Denigration

Toxicologists may encounter situations in which there are attempts to degrade their personal integrity and credibility, sometimes due to jealousy, on occasion for financial gain, or because of differing competitive scientific opinion. This may be intradepartmental, conflict between organizations or part of the armamentarium of the legal business. This may be seen at its most unpleasant in some court of law proceedings with so-called expert witnesses making attorney-stimulated derogatory remarks, often with financial incentive, at the opposing expert witnesses, and for which there is often little chance for open rebuttal (Hernberg, 1995).

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16.6.4 Expert Witness

Appearing in court proceedings as an expert witness is, in most cases, a voluntary activity, sometimes carried out for sociopolitical reasons, but usually for financial gain. Acting as an 'expert' witness is now a career with some qualified toxicologists in contract organizations or private practice, and their specialist areas can be found in advertising services online. The toxicologist should be aware that as a voluntary expert witness he will be subject to extensive questioning by opposing lawyers concerning his/her qualifications, professional integrity and motivations, and there will be attempts made to destroy credibility as an expert witness. Before accepting an invitation to become an expert witness, the credible toxicologist should make it clear that he/she will act in an ethical and professional manner and not distort the truth for legal augment.

The toxicologist should be aware of, and wary of, the growing nauseating variant on the 'ambulance chasing' activity of the legal business, who seek to further their finances by taking advantage of adverse effects from drugs and medicinal products. In this practice, most noticeable in the USA, the legal business seeks clients, by the medium of television advertisements, who 'might' have developed averse health effects as a result of being exposed to certain industrial chemicals or having been legally prescribed certain specific and named medications. Clients are invited to have a free legal consultation to determine if they have a case against the manufacturer of the named chemical or drug. These advertisements are seen daily and cover, in the main, asbestos, analgesics, antidepressants, cholesterol-lowering drugs and anti-inflammatory agents. Presumably the legal business conduct regular searches of the medical and pharmacological literature for newly reported adverse reactions to drugs. This practice has recently extended into client litigation for general medical and surgical complications; for example, abdominal aortic surgery, haemodialysis, cardiac pacemakers. This practice will ultimately result in significant suppression of new drug developments.

16.6.5 Conflict and Hounding

Leading a credible and ethical professional lifestyle can, if the employee discovers malpractices by his management and/or fellow workers, result in hounding, unfair treatment, dismissal or antisocial activities against the employee, if he/she draws attention to unprofessional or unethical behaviour, including suppression or misrepresentation of health-related information. Discrimination against employees based on disclosure of unsafe and/or unethical practices has sometimes been classed as 'whistleblower' activity, based on the employee making public statements about the suppression or misrepresentation of health-related information. Such unethical employer activity has been recorded to occur in industry, government and academia. In the USA, employees in some states are protected by the so-called 'whistleblower' laws. In New Jersey, for example, this legislation is called the 'Conscientious Employee Protection Act' and is intended to protect employees who act in the public interest, possibly against business interests (Soskolne, 1999). In the UK, protection is provided by the Public Interest Disclosures Act, 1998, which became law on 1 January 1999. It provides that where employees disclose certain types of wrongdoing inside or outside the company or organization they are protected from any form of discrimination or dismissal by their employer (Faculty of Occupational Medicine, 1999). The Act covers situations where a worker reasonably believes that he/she has credible and supportable information and evidence that there has been, or continues to be: (i) the commission of a criminal offence; (ii) failure to comply with a legal obligation; (iii) a miscarriage of justice; (iv) a danger to the heath and safety of any individual; (v) damage to the environment and (vi) the deliberate concealment of information supporting a conclusion of the preceding. 'Whistleblowers' (a demeaning term for ethically and professionally motivated individuals), should be aware that the vicious Mafia-like tactics adopted by industrial and government organizations are likely to persist, and that they will continue to face continued career suicide, economic and emotional deprivation, victimization and personal abuse (Yancy, 2000). Thus, in a survey of 87 'whistleblowers' from public service and industrial organizations in the USA, all but one experienced retaliation (Sockon and Sockon, 1987). There is doubt as to whether protection by legislation is supported by some political administrations; this includes the US Federal Executive Branch from 2000 to 2008 who had a clear industry/commerce financial-motivated approach. The public community should be grateful to those who risk their careers and well-earned reputations by drawing attention to employing organizations who covertly undertake malpractices and misinformation for political, commercial and financial motivations, particularly if the issues involved are health-related matters. The increasing recognition of the value of 'whistleblower' activities and the protection they should be afforded is emphasized by the formation of a National Whistleblowers Center based in Washington DC, which has an extensive informational web site (*http://whistleblower.org*) and several publications, such as Kohn *et al.* (2004).

There is a clear need for greater emphasis to be placed on the utmost importance of professionalism and ethical approaches in toxicology, particularly because of its wide implications for public health. These need to be infused into the early stages in the training of toxicologists at graduate and postgraduate levels. Educationalists need to draw attention to the social responsibility of the toxicologist and to audits of the practice and standards

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of the profession in academia, government service and industrial organizations.

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Acute Toxicity

David J. Andrew

CONTENTS

1 INTRODUCTION

Historically, the study of toxicology was largely limited to investigations of acute toxicity; the induction of effects within a short time period following the administration of a single dose of an acutely toxic substance having obvious advantages for the investigator and some of the fundamental principles of modern toxicology are based on these investigations. The basic principles of acute toxicity testing have changed little, although testing on unwitting or unwilling humans has been replaced by the use of animal models, and studies are now performed primarily for the purposes of ensuring human safety rather than the converse. However more recently, advances, both in our understanding of the mechanisms of acute toxicity and in scientific techniques, have enabled a further move towards the use of 'alternative' models.

1.1 Definition

Acute toxicity can be defined as the adverse effects resulting from a single (or short) exposure to a chemical substance or repeated exposures within a short period of time (less than 24 hours), and which also occur within a short period of time following exposure. Acute toxicity is typically apparent within a few hours of exposure to the substance, however for the effects of some substances may be manifested within a much shorter period of time (seconds or minutes) or may be much more delayed. While some definitions of acute toxicity refer to effects that occur within 14 days of exposure, this time period probably reflects the standard period over which studies of acute toxicity are performed and it is possible that, for some substances, effects may be delayed beyond this period. The time between exposure and toxicity is

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obviously strongly influenced not only by the level of exposure, but also by the kinetic parameters, which are dependent on the route of exposure.

As is also the case for other types of toxicity, acute toxicity results from a combination of the inherent toxicity of the substance and systemic exposure of the tested organism. Obvious exceptions to this are those local effects such as irritation or corrosion which do not require systemic exposure. Systemic exposure can only occur if the substance is absorbed from the gastrointestinal tract, through the skin or from the respiratory tract; all of which provide barriers to absorption of varying effectiveness. Administration of the substance by another, parenteral route (intravenous or intraperitoneal), effectively avoids these physiological barriers to absorption and therefore gives more information on the mechanism of toxicity, although these routes of administration are of limited relevance to most classes of substance. A combination of acute toxicity studies using a parenteral and another route of administration can be used to indicate the extent of absorption (bioavailability) by this route, however it is important to note that other factors such as metabolic deactivation (such as the first pass effect following oral exposure) may also have a major effect on toxicity.

For a large proportion of substances, the toxic effects resulting from a single exposure are distinct from those seen following repeated exposures over a longer period of time. This may simply reflect the fact that acute toxicity studies generally use much higher doses than those used for repeated dose (sub-acute, sub-chronic or chronic) toxicity studies. Thus acute toxicity studies performed with benzene will identify depression of the nervous system typical of hydrocarbon solvents as the major toxic effect, whereas longer-term studies will identify adverse effects on the bone marrow. Those substances that are rapidly absorbed will tend to be more acutely toxic, however distinct, delayed toxic effects may also be seen. The toxicity of rapidly absorbed and eliminated substances which cause rapidly reversible effects is likely to be similar following acute and chronic administration: an example of this is the toxicity of carbamate compounds which cause the inhibition of acetylcholinestesterase activity in the peripheral and central nervous system (see **Toxicology of Pesticides**). Recovery from sub-lethal doses of carbamates is essentially complete due to the rapid reversibility of the critical effect and further exposures are tolerated without enhanced sensitivity. The repeated dose toxicity of these compounds can therefore be compared to a series of acute doses. Conversely, for substances whose toxicities are not totally reversible within a short period of time, the toxicity following repeated dosing will be greater (and potentially much greater) than that seen following acute administration.

While it is possible for teratogenic or carcinogenic effects to result from a single exposure to a substance, the longer latency periods mean that these types of effect are not classified as acute toxicity. Delayed toxicity following an acute exposure is also known for some compounds, one of the best-known examples being the organophosphate tri-orthocresyl phosphate (TOCP), where the characteristic clinical and histopathological effects of neuropathy are typically not apparent until 10–20 days following administration (see **Neurotoxicology**).

1.1.1 Threshold of Toxicity

For all chemicals—even the most potently toxic—there is a level below which adverse effects will not occur or will not be apparent. This concept of the threshold of toxicity is based on the assumption that adverse effects will not occur until a sufficient number of molecules of the toxic substance have interacted with their target within the body, or that at low levels of exposure endogenous defence mechanisms will deactivate the substance or otherwise prevent it interacting with its target.

The acute toxicity of carbon monoxide results from the critical interaction of the carbon monoxide molecule with haemoglobin to form carboxyhaemoglobin which effectively prevents the release of oxygen from the erythrocyte into the tissues. However, there is a threshold for carbon monoxide toxicity equivalent to approximately 10% carboxyhaemoglobin, below which there are no apparent acute toxic effects. The minor paracetamol metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI) is highly reactive and is capable of causing damage by binding to hepatocyte macromolecules. At therapeutic levels of paracetamol, NAPQI is effectively deactivated by binding to glutathione, however at high dose levels, increased NAPQI production results in a depletion of glutathione levels. Glutathione levels may drop to a critical point at which NAPQI is not deactivated and it is able to cause hepatocellular damage.

2 HISTORY

The toxic actions of naturally occurring substances have been studied for millennia; the earliest observations recorded approximately 5000 years ago by Shennong, reputed to be the father of traditional Chinese medicine. The ancient Egyptian Ebers papyrus (circa 1550 BC) and Sumerian texts (circa 1400 BC) also include references to poisonous substances. However it was probably the ancient Greek philosophers and physicians (including Homer, Socrates, Theophrastus, Aristotle, Hippocrates and Mithridates) who first systematically studied the action of toxins and their antidotes—often on prisoners—a practice which was continued by Cleopatra (69–30 BC). Paracelsus (1493–1541) defined the dose-response relationship, one of the basic principles of

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modern toxicology at a time when others (including Rodrigo and Cesare Borgia, and Catherine de'Medici) utilized poisons primarily for political and monetary gain. Again the more acutely toxic substances were used to produce the desired effect and it is reputed that Medici also conducted experiments on the poor and sick in order to refine her assassination techniques.

2.1 The LD_{50}

The formal testing of substances for acute toxicity has been carried out for nearly a century: the important concept of the LD_{50} (the median lethal dose) was first defined by Trevan (1927), and was used as a biological assay for measuring and standardizing the content of digitalis extracts, diphtheria toxin and insulin. Intra-individual differences in susceptibility to a given substance result in a range of sensitivity within a population and plotting the frequency of a quantal response, such as death, against dose gives a bell-shaped curve; plotting the cumulative frequency against dose results in a characteristic sigmoidal dose-response curve characteristic for the compound administered and the species investigated. These dose-response curves were used to estimate the dose lethal to 50% of the animals tested, which was designated the LD_{50}. Mathematical transformation of the sigmoidal curve into a linear function allows more accurate derivation of the LD_{50} and its associated confidence intervals. The use of probit values was introduced later to allow for uncertainty resulting from the use of fewer animals and dose levels (Bliss, 1935), however this method is less effective for studies including groups with 0 or 100% responses. A simplified graphical procedure of calculating LD_{50} values and their associated confidence limits has been defined by Litchfield and Wilcoxon (1949); more recently, computer programs for calculating LD_{50} values have been developed.

The median lethal dose is therefore the statistically derived amount of a substance found or estimated to be lethal to 50% of a tested group of animals. The acute oral LD_{50} of a substance is the most frequently used indication of comparative toxicity and is conventionally expressed in terms of milligrams of substance per kilogram bodyweight: this also enables some estimation of the lethal dose for species other than that tested. Other less commonly used measures of acute toxicity include the LD_1 (the dose level of a substance calculated to be lethal to 1% of a population), the LD_{99} (the dose calculated to be lethal to 99% of a population) and the LD_{lo} (the lowest dose level causing lethality). LD_{50} values are relevant to exposure routes (e.g. dermal, intravenous) other than the inhalation route, for which the analogous measurement LC_{50} (the atmospheric concentration of a substance estimated to be lethal to a group of animals) is used.

2.1.1 Measurement of the LD_{50}

The LD_{50} value is conventionally expressed with its 95% confidence limits to give an indication the uncertainty in its derivation. Separate values may be expressed for males and females and for the sexes combined. While slight differences in LD_{50} values calculated for the two sexes are common and most often to reflect biological variation, more marked differences between the two sexes may reflect a greater sensitivity of one sex.

Considerable effort has, in the past, been expended in precisely measuring the LD_{50} value for substances in an individual study. This approach necessitates the use of a large number of experimental animals: a classical LD_{50} study can use as many as 200 animals (10 animals per sex at 10 different dose levels). However such precise measurements are of limited value as biological variation and other factors can have major influences on the measured LD_{50}, meaning that the results of these studies are very difficult to replicate. This is particularly the case for compounds of moderate or low toxicity. Parameters that can influence the LD_{50} include the age, strain, sex, nutritional status and housing conditions of the animals. It has been recognized for some time that LD_{50} values have poor reproducibility (Griffith, 1964), and multilaboratory trials have showed variation in the LD_{50} value of 3–11 times for the same substances (Hunter *et al.*, 1979).

2.1.2 Use of the LD_{50}

With a few exceptions, such as the use of the mouse intraperitoneal LD_{50} to standardize preparations of very toxic substances such as botulinum toxin, LD_{50} values are primarily used to compare or rank the toxicity of a group of chemicals for purposes such as hazard classification. The use of studies designed to precisely define the LD_{50} have therefore received considerable criticism both for scientific reasons and for reasons of animal welfare. Over recent years, a number of alternative methods to estimating the LD_{50} have been developed, which both reduce the numbers of animals used and also reduce the reliance on lethality as an endpoint.

3 ACUTE TOXIC EFFECTS

Other than death—the most obvious and most frequently measured effect in acute toxicity studies—a single exposure to a toxic substance can potentially have any number of acute effects. Toxicity may occur as a result of an effect at the organ, tissue, cellular or biochemical level and may be reversible or irreversible. The reversibility of a given effect will be governed by its precise nature as well as its severity: for example, the length of time required to synthesize an irreversibly inhibited enzyme or for the cells of a particular organ or tissue to regenerate to replace those lost through cytotoxicity. Irreversible toxicity may result from damage to cells with little or no

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regenerative capacity. The severity of an effect may also be influenced by factors such as species, age, sex and genetic susceptibility.

The acute toxicity of a chemical may be highly specific in that it targets a single enzyme, cell type or organ; or it may be nonspecific and targets a large number of organ or tissue types. Specific toxicity can result from the inherently greater sensitivity of an organ or tissue or from relatively high exposure. An example of the former is the toxicity to the nervous system of sarin; an example of the latter is the toxicity of paraquat to the lung. The liver is a common target of acute toxicity for orally ingested substances as a consequence of physiology: the hepatic portal vein ensures that the absorbed substance is transported effectively to the liver, thereby achieving relatively high tissue levels, which can exceed the detoxification, cellular defence or repair capacities of the organ. Similarly, high dose levels of substances excreted predominantly in the urine may cause acute renal toxicity as a consequence of their concentration by the kidney.

The acute toxicity of chemical substances covers a very large range, with LD_{50} values reported over many orders of magnitude from the essentially nontoxic to the exquisitely toxic (**Table 1**). The substances most acutely toxic to humans are naturally occurring; the reported LD_{50} value for botulinum toxin is in the order of 1 ng kg^{-1} bw. Among the most acutely toxic synthetic substances are those used as chemical weapons including the nerve agents such as sarin and VX (for which the human lethal dose is estimated to be 10 mg by dermal exposure). It is notable that some of the most acutely toxic compounds known are naturally occurring rather than manmade: this runs counter to the common public misconception that natural substances are less harmful than synthetic chemicals.

3.1 Routes of Toxicity

The potential of a substance to cause acute toxicity following different routes of exposure not only depends on its inherent toxicity and toxicokinetic factors, but also its physical form. Acute toxicity via the inhalation route is clearly only relevant for substances where exposure by this route is possible; the respiratory tract can be considered to be a relatively poor barrier to absorption, particularly for those substances reaching the deep lung. Acute dermal toxicity is generally lower than acute oral toxicity as the skin provides a much more effective barrier to absorption, however it is also possible that detoxification may be not be as rapid following dermal administration in the absence of a first-pass effect.

3.2 Acute Toxicity in Humans

Acute poisoning incidents resulting from dietary exposure to food contaminants and from the deliberate or accidental administration of plant or mineral toxins have occurred throughout history. In modern societies, the production of synthetic chemicals has resulted in a large increase in the exposure of populations to toxic chemicals. Whilst exposure to most of these substances is tightly regulated to ensure the absence or to minimize

Table 1 The acute oral toxicity of selected substances

Chemical	Acute oral LD_{50} value (mg kg^{-1}bw)	Chemical	Acute oral LD_{50} value (mg kg^{-1}bw)
Ascorbic acid	11900	Paraquat	150
Ethanol	7060	Methyl isocyanate	69
Cimetidine	6000	Amphetamine	60.5
Paracetamol	3700	Nicotine	53
Sodium chloride	3000	Strychnine	16.2
Ibuprofen	1600	Arsenic pentoxide	8
Aspirin	1500	Sodium cyanide	6.44
Morphine	905	Fluoroacetic acid	2.5
Lithium chloride	751	Mercury (II) chloride	1
Phenobarbital	660	Sarin	0.165
Codeine	542	2,3,7,8-TCDD	0.02
Amitriptyline	530	Tetrodotoxin	0.01
Caffeine	200–400	Polonium 210	0.00001
Warfarin	323	Botulinum toxin	0.000001

Values taken from Sax (1979); Derelanko and Hollinger (1995).

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the likelihood of acute toxicity, it is perhaps inevitable that incidents of acute toxicity continue to occur.

Figures from US Poison Centres for 2006 (Bronstein *et al.*, 2007) show a total of over 2.4 million reported poisoning incidents, of which just over 50% were defined as paediatric (i.e. those involving children of $\leq$5 years old). The majority of these reported exposures resulted in no effect or only minimal toxicity; 'major effects' were reported for less than 1% and death for 0.1% of the cases. The most commonly involved substances were analgesics (11.9% of the reported incidents), with cosmetics (8.9%) and household cleaning substances (8.9%) also important causes. It is perhaps of note that the large majority of the reported incidents (92.6%) occurred within the home, with only 2.1% occurring in the workplace. Figures for the UK (HES, 2007) show a similar pattern, with analgesic poisonings [particularly involving paracetamol and nonsteroidal anti-inflammatory drugs (NSAIDs)] accounting for a large proportion of cases and hospital admissions. The most recent mortality statistics available for the UK are for 2005 [Office for National Statistics (ONS), 2007] and show that, while poisoning deaths account for only a very small proportion (<0.5%) of the total mortalities, the deaths of over 2000 people in that year were attributed by the coroner to poisoning **(Table 2)**. The majority (approximately 75%) of the deaths attributed to poisoning were due to drugs (pharmaceuticals and drugs of abuse), whereas a smaller proportion resulted from exposure to 'nonmedicinal' substances. A larger proportion of deaths were classed as 'accidental' (of which most were overdoses of prescribed pharmaceuticals or drugs of abuse) rather than 'deliberate' and only a very small proportion of the intentional mortalities were classed as homicidal.

Mass human poisoning incidents are relatively rare, but have occurred following the catastrophic failure of chemical manufacturing plants with the release of large amounts of chemical at Seveso (1976) and, with far more severe consequences, at Bhopal (1984). While occupational exposure to chemicals in the developed world may be controlled by the use of protective equipment, exposure limits and other industrial hygiene measures, relatively minor health effects such as headache, nausea and local irritation may still occur. Fortunately, fatal acute poisonings are rare, such as the death due to cyanide poisoning of five employees of an electroplating plant in Auburn, Indiana in 1988.

Mass poisonings resulting from the contamination of food are also well documented and include Minamata disease (Japan, 1956 and 1965); amnesic shellfish poisoning (first reported in Eastern Canada in 1987); toxic oil syndrome (Spain in 1981) and the deliberate adulteration of wine with diethylene glycol in Austria in 1985. The outcomes of such incidents vary widely from minimal acute toxicity to large numbers of fatalities.

Acute toxic effects following the correct therapeutic administration of a pharmaceutical agent can be classified as either an exaggerated pharmacological response, a recognized side effect or an idiosyncratic reaction with an immune system involvement. The potential for acute toxicity following the correct use of any given pharmaceutical will be influenced by its therapeutic index; severe effects (including death) occur with a much greater frequency following accidental or deliberate (suicidal) overdose.

Acute toxicity resulting from exposure to pesticides occurs more frequently in those applying products in the field, as the exposure levels encountered by this group are generally higher, however poisoning incidents account for only a relatively small proportion of the total incidents reported (4% in the USA; Bronstein *et al.*, 2007). There is a risk of acute toxic effects in the consumer resulting from the consumption of treated produce where the crop or produce has been treated with an unlicensed product or treated inappropriately (for example at a higher application rate or

Table 2 UK poisoning deaths (2005)

Category			Number of deaths		
			M	F	Total
Deaths due to poisoning	Drugs, medicinal substances	Heroin	118	20	138
		Antidepressants	104	119	223
		Paracetamol	69	65	134
		NSAIDs	18	18	36
		Total	1098	685	1783
	Nonmedicinal substances	Alcohol	113	51	164
		Carbon monoxide	216	41	257
		Total	452	158	610
	Accidental poisoning deaths		730	345	1075
	Suicidal poisoning deaths		512	283	795
	Homicidal poisoning deaths		13	7	20
				Total poisoning deaths	2393
				Total deaths	512 692

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closer to harvest) with a licensed product. Incidents are more frequently reported in countries where pesticide use is less tightly regulated and often involve the more acutely toxic organophosphate and carbamate compounds.

Deliberate poisoning incidents in humans (suicidal or homicidal) most commonly involve chemicals known to be acutely toxic; paraquat, methanol, ethylene glycol and paracetamol are examples. Homicidal poisonings have become relatively rare in recent times, possibly as a result of increasingly sophisticated and powerful chemical analytical techniques, and are consequently well-publicized when they do occur. Recent cases include the 1994–1995 Japanese terrorist attacks using the nerve agent sarin, the alleged dioxin poisoning of Viktor Yuschenko and the assassination of Alexander Litvinenko with polonium 210.

4 ACUTE TOXICITY AND REGULATION

The safety testing of drugs in experimental animals became mandatory following the introduction of the USA Federal Food, Drug and Cosmetic Act in 1938 following the deaths of more than 100 patients (mostly children) following the use of diethylene glycol in a sulfanilamide preparation. Similar requirements were subsequently introduced in other countries and for other groups of substances, including industrial chemicals, food additives and pesticides.

The various pieces of legislation require the manufacturers, suppliers or importers of chemical substances to appropriately classify the substance to reflect the acute toxic hazard and to show this classification on the packaging of the substance. The precise classification and terminology used to reflect the toxic hazard varies according to the country, the group of substance and the relevant legislation; however, the general principles are similar and follow the assignment of the substance to a particular category of toxicity. For acute oral, dermal and inhalation toxicity, the classification category to which the substance is assigned depends on the estimated LD_{50}/LC_{50} value. The most acutely toxic substances are variously classified as 'Very Toxic', 'Highly Toxic' or 'Extremely Toxic'; substances are typically assigned to this category if the acute oral LD_{50} value is less than $50\,mg\,kg^{-1}$ bw. Less acutely toxic substances (for example those with oral LD_{50} values of between 50 and $200\,mg\,kg^{-1}$ bw) are assigned a classification such as 'Toxic' or 'Hazardous'; those less acutely toxic still (for example those with oral LD_{50} values of between 200 and $2000\,mg\,kg^{-1}$ bw) are assigned a less severe classification such as 'Harmful' or 'Slightly Hazardous'. Substances of little or no acute toxic hazard (generally considered to be those with an oral LD_{50} value of $>2000\,mg\,kg^{-1}$ bw) do not generally require labelling. The classification category to which a substance is assigned also governs the hazard symbol to be used on the label.

Companies are therefore obliged to perform assessments of acute toxicity in order to establish the toxic hazard of their products, to meet legislative requirements regarding safe transportation and to ensure (as far as possible) the safe use of the substance. The results of the acute toxicity studies also define the risk phrases, safety phrases, instructions for safe use and appropriate medical/first aid treatment which appear on the material safety data sheet (MSDS), an important component of product stewardship and occupational safety and a legal requirement under most legislation.

4.1 Industrial Chemicals

The 1976 USA Toxic Substances Control Act (TSCA) was enacted in order to give the Environmental Protection Agency (EPA) the ability to assess the estimated 75 000 industrial chemicals used in the USA. The act requires manufacturers to provide toxicity data for new chemicals and also gives the EPA the authority to require chemical manufacturers to test existing chemicals. The toxicity data requirements are governed by the nature of the chemical and the quantity produced, however the basic toxicity data requirements include acute toxicity studies. In the European Union (EU) there is a harmonized approach to the classification and labelling of industrial chemicals under the Dangerous Substances Directive (Directive 67/548/EEC). The 'base-set' toxicity data requirements for any new chemical produced in quantities of $\leq 100\,kg\,yr^{-1}$ is acute toxicity via the oral and one further exposure route (the choice of second route being governed by the physicochemical properties of the substance and its intended use), skin and eye irritancy, and skin sensitization. Similar data are required for existing chemicals produced or imported at levels of 10 tonnes yr^{-1} or greater. More recent EU legislation on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) has updated the data requirements, but testing for acute toxic effects remains an important part of the hazard characterization (see **Occupational Toxicology and Occupational Hygiene within the European Union (EU) Chemicals Regulation**, **Regulatory Toxicology**, **Risk Assessment of Chemicals**, **Basic Toxicological Issues in Product-Safety Evaluations**).

4.2 Pesticides

Acute toxicity studies (acute oral, dermal and inhalation toxicity studies, skin and eye irritation studies, skin sensitization study) form part of the basic data

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Table 3 USA, EU pesticide and GHS classification categories for acute oral toxicity

USA			EU		GHS		
Category	Signal word	Cut off (mg kg^{-1} bw)	Category	Indication of danger	Category	Cut off (mg kg^{-1} bw)	Signal word
I: Highly toxic	Danger	≤50	Very toxic	≤25	Category 1	≤5	Danger: fatal
II: Moderately toxic	Warning	>50; ≤500	Toxic	>25, ≤200	Category 2	≤50	Danger: fatal
III: Slightly toxic	Caution	>500; ≤5000	Harmful	>200, ≤2000	Category 3	≤300	Danger: toxic
IV: Practically nontoxic	—	>5000	—	>2000 mg kg^{-1} bw	Category 4	≤2000	Warning: harmful
					Category 5	≤5000	Warning: may be harmful

requirements for pesticide active substances under the USA Federal Insecticide, Fungicide and Rodenticide Act (FIFRA, 1947), the Canadian Pest Control Products Act (PCPA, 1985) and (in the EU) Directive 91/414/EEC. Acute toxicity studies are also required for pesticide products under the relevant legislation, however in the EU pesticide products are covered by the Dangerous Preparations Directive (see below) and studies may not be necessary. In the USA the results of the acute toxicity studies are used to categorize the product for general or restricted use depending on its acute toxic hazard; the hazard is indicated on the product label using the words 'Danger', 'Warning' or 'Caution'. In the EU, the indicators of acute toxic hazard are 'Very Toxic', 'Toxic', 'Harmful' or 'Irritant'; the severity of the classification influences the protective equipment and medical information on the product label and certain classifications for acute toxic effects may preclude the use of the product in a domestic situation.

The different classification categories for pesticide products in the USA and EU are shown in **Table 3**; there is a risk that the use of different categories may result in studies of acute toxicity being repeated to satisfy the requirements of the different regulatory agencies. One of the aims of the United Nations (UN) Globally Harmonised System (GHS) of Classification and Labelling of Chemicals, implemented during 2008, is to eliminate such duplication.

4.3 Pharmaceuticals

The USA Food and Drug Administration (FDA) requires that the acute toxicity of a pharmaceutical be tested in two mammalian species (rodent and nonrodent) prior to the first human exposure in clinical trials. Studies are usually performed using two routes of exposure: the route of intended administration to humans and intravenously. Similar data requirements apply in the EU under Directive 2001/83/EC. While acute toxicity studies performed with pharmaceuticals are currently a regulatory requirement, a recent report from a workshop involving the pharmaceutical industry and regulatory agencies highlights that the data from these studies is little used. The report highlights the fact that findings from acute toxicity studies are never used to identify target organs or to set the starting dose for studies in man (Chapman and Robinson, 2007). It was also highlighted that the studies would not be performed if they were not a regulatory requirement.

5 ACUTE TOXICITY TESTING

Acute toxicity studies are, in most cases, the first studies performed with a substance of unknown toxicity. The results of these studies are used in hazard identification, to provide information on the mechanism of toxicity and therefore the likely treatment regimes for accidental overexposure to the substance when in use. The results of the acute toxicity studies are also used to inform the dose selection for longer-term toxicity studies, where these are required by the relevant regulatory regime.

As is the case for other types of toxicity studies, the choice of animal model should ideally be informed by its relevance to humans both in terms of general physiological and biochemical comparability and also in terms of the pharmacokinetics and pharmacodynamics of the substance being investigated. The use of a representative animal model enables the experimental response to be extrapolated to humans with a greater degree of confidence. The species most commonly used for acute toxicity testing—the rat—is widely accepted as a relevant model, however the response of the animal model may be more or less than in humans and, in some cases, the model may have a completely different response. A general principle of chemical risk assessment is the precautionary assumption that humans will respond in the same way as the most sensitive animal species tested. Following this assumption, therefore, testing for acute toxicity in a number of different animal species is preferable and may also reduce some of the uncertainty

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associated with interspecies extrapolation, as observing a common response in a number of different animal models increases the likelihood that the response will also be seen in humans. Despite the potential advantages, acute toxicity testing is most commonly performed in a single species for reasons of convenience, ethics, economics and the extensive background data. While interindividual variation in the test species is minimized by the use of inbred strains and by minimizing variations in age, bodyweight and general health, the potential remains that relying on a single species may considerably under- or overestimate the acute toxicity of a compound.

As the extent of absorption of any given substance appears to be relatively consistent between species, differences in sensitivity are therefore due largely to other toxicokinetic parameters such as metabolism, distribution and excretion, and toxicodynamic factors, which show more marked interspecies variability. Although the expression of the LD_{50} value in terms of $mg\,kg^{-1}$ bw enables some estimation of toxicity in other species, this is not entirely reliable. Better estimates can be made using the linear relationship between the log of the bodyweight and the log of the LD_{50} value, both within a species and between different species (Morrison *et al.*, 1968) or using allometric scaling, however these approaches assume that the kinetics of the substance and the toxic response are the same. More recently, more accurate interspecies extrapolation has been made possible by physiologically based pharmacokinetic (PBPK) modelling, which takes into account differences in kinetic parameters.

5.1 Principles of Acute Toxicity Testing

Most acute toxicity tests are conducted for hazard identification purposes, therefore the principles of study design are relatively unsophisticated. A single large dose of the chemical is administered to a group of animals, which are then observed for a relatively short period of time—typically up to 14 days. The results of an acute toxicity study can indicate the critical toxic effect, the target organ or tissue, can inform the choice of dose levels for longer-term, repeated-dose toxicity studies and indicate the likely effects of accidental or deliberate oral exposure in humans. Well-designed acute toxicity studies are unusual in that groups of animals are dosed sequentially with increasing dose levels of the substance; the time interval between administration of the dose levels should be sufficient for the full effects of the previous dose level to be manifested.

5.1.1 The Limit Dose

The fundamental concept of toxicology—first stated by Paracelsus—that all chemicals are toxic if administered in high enough quantities, clearly has the potential result that test animals are administered unreasonably large dose levels which have no relevance to human exposure scenarios. This potential for the use of very high dose levels of chemicals of limited toxicity is clearly undesirable, not least for reasons of animal welfare. The use of excessive doses may be scientifically unreliable, as very large quantities of a foreign compound may cause purely physical effects which may be misinterpreted as direct toxicity. There is therefore a point at which it should be concluded that a test material is of intrinsically low toxicity and does not pose any hazard with regard to acute lethality. This point is defined as the limit dose and, depending on the relevant legislation is defined as a dose level of 2000 or 5000 $mg\,kg^{-1}$ bw.

5.1.2 Observations

The observations and measurements made in a study of acute toxicity are typically limited and will include periodic observations of clinical signs and behaviour (most frequently during the 24 hours immediately following dosing), and measurement of bodyweight. The observations should therefore provide information on the time course of the acute toxic response, including the rate of recovery and, where more than one dose level is used, information on the dose-response relationship. Terminal investigations are also limited to macroscopic observations of major organs and tissues. Experience indicates that acute toxicity is, in the majority of cases, due to a general biochemical effect rather than any specific effect and is not associated with any visible change in tissue or cell structures (Gad and Chengelis, 1988). The most common causes of death in acute toxicity studies generally involve the nervous, cardiovascular or respiratory systems (DiPasquale and Hayes, 2001) and may not be readily identified by gross necropsy or histopathology. Histopathological investigations may be useful in identifying the smaller proportion of chemicals causing death as a result of specific toxicity to other organs such as the kidney or liver, but are rarely performed as part of a regulatory acute toxicity study. However, if acute toxicity studies are to provide the primary safety data for a pharmaceutical substance supporting single-dose safety and/or kinetic studies in humans, the acute toxicity studies should incorporate investigations of clinical pathology and histopathology. These parameters are assessed at an early time point (i.e. at maximum effect) and at termination (to assess recovery) [Centre for Drug Evaluation and Research (CDER), 1996].

Modern test methods place increasing emphasis on clinical signs, with less reliance on death as an endpoint. However, while some clinical signs (or combinations of signs) are characteristic of a mode of toxicity (e.g. interaction with a specific receptor, inhibition of a particular enzyme) or target organ, most are nonspecific and it is clear that most chemicals cause death by nonspecific actions following acute administration. Clinical

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signs—for example, those associated with the inhibition of a specific enzyme—may be rapidly reversible; others—for example, those associated with functional or structural damage to an organ or tissue—may be less rapidly reversible or irreversible within the period of the study.

The liver is a common target of acute toxicity as a consequence of its role in the metabolism and excretion of xenobiotics. For chemicals administered orally and transported by the hepatic portal system, the liver is often the primary target of toxicity. Clinical signs of hepatotoxicity are largely nonspecific and difficult to distinguish by cage-side observation. Early detection of liver damage requires clinical chemistry investigations, which are not performed as part of acute toxicity studies, however gross changes to the organ at necropsy may indicate hepatotoxicity. The kidneys are a common target of acutely toxic chemicals as they receive a large proportion (circa 25%) of the cardiac output. The role of the kidney in xenobiotic excretion may also result in the concentration of toxic chemicals and/or toxic metabolites in this organ. Clinical signs, such as polyuria and haematuria, seen in acute toxicity studies may indicate nephrotoxicity, however prolonged or repeated exposure may be required for renal effects to be lethal. Pulmonary injury is clearly much more frequent in acute inhalation toxicity studies and may result from local irritation or tissue damage. Clinical signs of pulmonary injury include various effects on the rate, depth and regularity of breathing and are readily detected. However, certain compounds, such as paraquat, may target the respiratory tract regardless of the route of administration. The nervous system is a common target for acute toxicity due to its complexity, high metabolic activity and relatively low regenerative capacity. Relatively nonspecific clinical signs may result from a general stimulation or depression of the nervous system, however the activation or inhibition of a neurotransmitter receptor or the inhibition of a specific enzyme may result in a characteristic effect (Straub tail) or syndrome (cholinesterase inhibition). Chemicals causing effects on the cardiovascular system may not produce any signs other than general nonspecific signs. Chemicals causing effects on the haematopoietic system may cause pallor or cyanosis; haematuria may also be cause by haemolytic agents.

Table 4 illustrates clinical signs observed in acute toxicity studies; it is apparent that many of the clinical signs are nonspecific and are of little value in indicating a target or mechanism of toxicity.

5.2 Acute Oral Toxicity

Investigations of acute oral toxicity are the most fundamental and therefore the most commonly performed toxicological studies *in vivo*. The study forms part of the

Table 4 Signs of toxicity

Clinical sign	Target of toxicity	Clinical sign	Target of toxicity
Piloerection Hypoactivity Ataxia Prostration Loss of coordination Abnormal gait Chromodacryorrhoea Exophthalmos	Non-specific	Lacrimation Salivation Rhinorrhoea Aggression Passivity Resistance to handling Vocalization Hyperactivity	Autonomic nervous system Nervous system/ nonspecific
Straub tail Paraesthesia Tremors Convulsions Opisthotonus Writhing Fasciculations Stereotypy	Nervous system	Abnormal response to stimuli Ptosis Miosis Mydriasis Absent/few faeces Diarrhoea Rapid breathing Shallow breathing	Gastrointestinal tract
Polyuria Haematuria Anuria	Kidney	Noisy breathing Irregular breathing Gasping	Respiratory tract
Pallor Hypothermia Cyanosis	Haematopoietic	Emaciation	Nonspecific

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basic data requirements for most (if not all) groups of substances and the results may be used: (i) for hazard identification purposes; (ii) for classification and labelling purposes and (iii) to inform the dose levels to be used in subsequent animal studies or in clinical studies with pharmaceuticals.

Administration of the test substance is most commonly by gavage dosing, although capsule administration may be used in some species. The rat is the most commonly used species, although the testing of acute oral toxicity in additional rodent or nonrodent species may be required under some regulatory regimes. If rats are used they are fasted overnight, which allows a larger volume of the dosing vehicle to be introduced into the stomach and also eliminates any variation in absorption resulting from the presence of food in the stomach, or variation in response resulting from differences in nutritional status. Shorter periods of fasting are recommended for smaller species such as the mouse as these have greater energy requirements and prolonged fasting can influence the result of the study.

Liquid substances are administered either undiluted or diluted in a suitable dosing vehicle such as water or corn oil. If more than one dose level is used, either the volume of the test material will vary (if it is administered undiluted) or will be constant for each dose level (if a dosing vehicle is used). The vehicle itself should be of known low toxicity and should not directly influence the toxicity of the test compound. Solid substances are either dissolved in a suitable solvent or suspended homogeneously in an aqueous vehicle; suspending agents such as methyl cellulose may be used to ensure homogeneity. The nature of the dosing vehicle and the physical state of the substance in the vehicle can influence the rate and/or extent of absorption from the gastrointestinal tract and hence its systemic availability and toxicity. The acute oral toxicity of lipophilic substances has been shown to be higher when dissolved in a suitable solvent compared to suspension in an aqueous dosing vehicle. Toxicity is also known to be influenced by the size of particles administered as a suspension: fine milling of a solid substance to produce small particles has been shown to increase toxicity compared to coarser particles, presumably due to the increased rate or extent of oral absorption. Corn oil, a vehicle commonly used for substances insoluble in aqueous vehicles, is of low toxicity, but can increase gastrointestinal motility and may result in reduced absorption of the test substance.

The Organisation for Economic Cooperation and Development (OECD) guideline 401 was deleted in 2002, but was the standard international method for many years. This guideline described a more limited version of the classical acute toxicity test and included the concept of the limit dose. Thus it became possible for chemicals of relatively low toxicity to be administered to a single group of five animals at the limit dose of $2000\,mg\,kg^{-1}$ bw if no mortality resulted. If mortality was seen at the limit dose, then testing proceeded in a sequential manner at set dose levels of 500 and $50\,mg\,kg^{-1}$ bw. The results of the study gave an approximation of the LD_{50}, but provided sufficient information to allow the substance to be assigned to a classification category. A number of alternative methods are now available to assess acute oral toxicity.

5.2.1 Fixed-Dose Procedure

This method, proposed by the British Toxicology Society, was validated in an international study involving 20 reference chemicals (Van den Heuvel *et al.*, 1990), and subsequently adopted as OECD guideline 420. The concept behind the design of the method is again that the results of the test are used simply to assign the chemical to a classification category.

The objective of the study is to identify 'evident toxicity', without requiring mortality as an endpoint. Groups of a single sex are administered the test material at one of four fixed dose levels (5, 50, 500 and $2000\,mg\,kg^{-1}$ bw). If mortality occurs, the test is repeated at a lower dose level; if there is no evident toxicity, the test is repeated at the next higher dose level. Testing is not required at dose levels below $5\,mg\,kg^{-1}$ bw or greater than $2000\,mg\,kg^{-1}$ bw and the choice of initial dose level should be informed by any data available on the acute oral toxicity of related compounds in order to reduce the number of animals used. The use of females is preferred, as, if there are marked differences in sex sensitivity, females are generally shown to be the more sensitive sex (Lipnick *et al.*, 1995).

5.2.2 Acute Toxic Class Method

Unlike the fixed-dose procedure, the acute toxic class method (Roll *et al.*, 1986) uses lethality as the primary endpoint, however it does potentially use fewer animals. The method is based on a sequential dosing procedure with the use of a minimum number of animals (three) in each step. The substance is administered sequentially to groups animals at four defined dose levels (5, 50, 300 and $2000\,mg\,kg^{-1}$ bw). The absence or presence of compound-related mortality is used to determine the next step: either no further testing is needed, three additional animals are administered the same dose or dosing proceeds to the next higher or the next lower dose level. This method is reproducible and gives sufficient information on the acute toxicity of the test substance to assign it to an appropriate classification category.

5.2.3 The Up and Down Method

This method (Bruce, 1985), which has been adopted as OECD guideline 425, again uses mortality as an endpoint, but uses fewer animals. LD_{50} values derived from this method have been shown to correlate well with

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more conventionally derived values. The test involves the administration of a dose, estimated to be a step below the LD_{50}, to a single animal, with the choice of subsequent dose level (usually a factor of 3.2 lower or higher) informed by the mortality or survival of this animal at 48 hours. The method is therefore most appropriate for those chemicals causing relatively rapid death and is less suitable for those causing more delayed effects. The LD_{50} and its confidence interval are calculated based on the status of all dosed animals at termination.

5.3 Acute Dermal Toxicity Testing

Dermal exposure is the major route of exposure for most chemicals encountered in the workplace; the assessment of the toxic hazard is therefore a regulatory requirement for this group. In common with studies of acute oral toxicity, the species of choice is the rat, however studies in rabbits may also be encountered. Although the pig is increasingly being used for investigations of dermal toxicity due to the close structural similarity of pig skin to human skin, this species is not used for acute dermal toxicity studies where the primary aim is hazard characterization and assignment of a substance to a classification category.

The general design of the study is that the test substance is applied to the skin of five animals of each sex per dose level used. Prior to application, the skin of the test animals is shorn to allow the test substance to come into close contact with the surface of the epidermis. The applied substance is covered with an occlusive or semi-occlusive dressing in order to maximize contact over the exposure period; the dressing also prevents any loss by run-off, evaporation or oral ingestion by the test animal. Solid substances are commonly ground to a fine powder and applied to moistened skin in order to maximize contact and therefore the potential for dermal penetration and systemic exposure. Older study designs may include investigations of comparative toxicity following application to abraded and intact skin; however dermal abrasion is of less relevance and is additionally now considered to be undesirable for reasons of animal welfare. Modern studies therefore investigate toxicity using intact skin only. Test guidelines specify an exposure period of 24 hours; exposure is terminated by washing the exposure site with water, an aqueous solution of mild detergent or (less frequently) another solvent. Following the termination of exposure, animals are observed for a period of time—typically 14 days. Signs of toxicity will be assessed at regular intervals on the day of exposure and daily during the remainder of the study; the site of exposure is also assessed for local irritant effects. Other investigations are typically limited to weekly measurement of bodyweight and gross necropsy at the end of the study; in some instances more detailed histopathology may be performed on the exposed skin, however this is not a standard guideline requirement.

Mild clinical signs such as chromodacryorrhoea and reduced weight gain observed in the initial stages of an acute dermal toxicity study may be a consequence of the dressing used to apply the test material and not to direct toxicity of the substance, however it can be difficult to definitively identify the cause. A number of dose levels may be used in order to provide an estimation of the LD_{50}; a limit dose of 2000 mg kg^{-1} bw can be used for substances suspected to be of low toxicity (OECD guideline 402).

When compared to acute oral toxicity, it is notable that there are no alternative methods for the assessment of acute dermal toxicity which would reduce the numbers of animals used and/or reduce reliance on lethality as an endpoint. However, a draft method (OECD guideline 434) analogous to the fixed-dose procedure for acute oral toxicity has been proposed. There is also increasing criticism of the routine requirement in some regulatory regimes for an acute dermal toxicity study. Analysis of a large number of studies performed in support of applications for pesticide approval in the EU has shown that the acute dermal toxicity study almost never provides any useful additional information (Thomas and Dewhurst, 2006). Instead it has been proposed that the requirement for an acute dermal toxicity study is not a default, but is triggered by specific criteria, such as marked acute oral toxicity and/or evidence for significant dermal absorption. Similar criteria are applied for other regulatory systems such as the EU biocides system, recognizing that significant toxicity by the dermal route is unlikely in the absence of inherent toxicity of the test material and/or significant systemic exposure.

5.4 Acute Inhalation Toxicity Testing

Acute toxicity may result from the inhalation of a substance in the form of a gas, vapour, aerosol or small solid particle (dust). The assessment of acute inhalation toxicity is therefore a regulatory requirement for a number of substance types, where exposure via this route is possible. Inhalation exposure will only occur if the substance is encountered in an inhalable form, which is determined primarily by particle size. For substances in the form of a solid or nonvolatile liquid, this is generally accepted to mean particles of size $\leq 5-6\,\mu m$ and extensive technical measures are undertaken in the test laboratory in order to generate atmospheres containing particles within this range. Exposure to particles of between 1 and 4 μm is generally considered to be most appropriate in an acute toxicity study, as this is likely to ensure the deposition of particles in all areas of the rat respiratory tract. In the absence of any information on the area of the respiratory tract likely to be most sensitive to the test material, this approach ensures most effective testing. OECD guidance (OECD, 2004) states that

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testing should be performed if it is likely that exposure to particles of respirable size will be encountered under the normal conditions of use. Additionally, exposure may not be expected to occur (and testing is not required) if particles of respirable size cannot be generated under laboratory conditions.

The generally accepted 'acute' exposure period for inhalation toxicity is four hours, and the species most commonly used is the rat. Five animals of each sex per concentration level are exposed to artificially generated atmospheres containing the test material in an inhalable form, such as a vapour or aerosol (for a test material in the form of a liquid) or as a solid aerosol or dust (for solid test materials). Concentration levels are expressed in terms of $mg\,l^{-1}$, or less commonly $mg\,m^{-3}$. A number of concentration levels may be used in order to provide an estimation of the LC_{50} value; a limit concentration of $5\,mg\,l^{-1}$ can be used for substances suspected to be of low toxicity by this route. A maximum concentration of below $5\ mg\,l^{-1}$ may also be used for those substances for which it is technically not possible to generate atmospheres containing a concentration as high as $5\,mg\,l^{-1}$. The test animals are generally restrained during exposure, with only the snout in the exposure chamber (nose/snout-only exposure). Whole body exposure—where the animals are present in the exposure chamber—has been used in the past; this method is less desirable, as exposure of the animals to the test substance results from a combination of inhalation, dermal and oral (resulting from grooming behaviour) and is consequently less representative.

Observations are made during the exposure period (although these may be limited for restrained animals), immediately following exposure, at regular intervals on the day of exposure and daily for the remainder of the study period. Clinical signs may indicate systemic toxicity or local irritation of the respiratory tract, however restraint of the animals during snout-only exposure can result in mild clinical signs that may be mistaken for toxicity. Terminal investigations are generally limited to gross necropsy, although more detailed investigation of the respiratory tract including measurement of lung weight may be made. A comparison of 'wet' and 'dry' lung weights can identify those compounds causing pulmonary oedema. In some laboratories, measurement of core (rectal) temperature is made during or immediately following exposure. While this effect is thought to be specific to the rat, it can give an indication of the potential of the test material to cause respiratory irritation in exposed humans (Pauluhn and Mohr, 2000). This response also has the potential to markedly reduce the systemic exposure of the tested animals to highly irritant chemicals. An alternative fixed-concentration procedure (OECD 433) has been proposed for acute inhalation toxicity testing which is analogous to the oral fixed-dose procedure.

5.5 Dermal Irritation and Corrosion

In addition to acute systemic toxicity, dermal exposure to chemical substances also has the potential to cause local effects (irritation or corrosion) at the site of exposure. Dermal irritation refers to the production of reversible local damage to skin at the site of exposure, Effects appear shortly after exposure and are characterized by the some or all of the signs (erythema, oedema, pain and swelling) typical of an inflammatory response. Irritant chemicals are able to penetrate the stratum corneum and are cytotoxic to the underlying layers of the epidermis. Dermal corrosion is a more severe effect involving full thickness destruction of skin tissue, which may be irreversible. In contrast to skin sensitization (discussed below), dermal irritation and corrosion are nonimmunogenic responses.

Substances of a number of classes, including cosmetics, household products, pesticide products and industrial chemicals, clearly have the potential to come into contact with the skin as a result of deliberate application or accidental exposure. Assessment of the potential for local irritant effects at the site of contact is therefore an integral part of the hazard assessment for these groups. Substances with the potential to cause severe dermal irritation or corrosivity should be identified on the basis of structure–activity relationship (SAR) analysis or physicochemical properties such as pH, and not tested *in vivo*. Regulatory guidelines also specify sequential testing using *in vitro* methods prior to investigation *in vivo*, for reasons of animal welfare. The irritant potential of a substance may also be predicted from data available for related substances or from the findings of the acute dermal toxicity study, and additional testing is not required.

Studies of dermal irritation *in vivo* are performed in the rabbit—the use of albino strains facilitates the observation of irritant effects. Regulatory guidelines specify the use of either three or six animals. A single animal is initially exposed and the study discontinued if severe reactions are observed. A standard exposure time of four hours is used and dermal contact with the test material is maintained by covering the application site with a dressing. Exposure to the test material is terminated by washing the application site either with water or with a solution of mild detergent. Local dermal reactions at the application site are assessed at specified time points, generally within one hour of application and subsequently at 24 hour intervals. Observations are continued for 72 hours or until the irritant reactions have resolved if these are still present at 72 hours. Irritant reactions (erythema and oedema) are scored using standardized scales. Assignment of the substance to a classification category depends on the regulatory regime and is dictated by a combination of the severity and reversibility of the

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reaction. The testing of coloured substances can be problematic, as staining of the skin at the application site may interfere with the assessment of erythema.

5.6 Eye Irritation and Corrosion

Eye irritation is defined as the production of changes in the eye following the application of a test substance to the anterior surface of the eye; corrosion is defined as the production of irreversible tissue damage to the eye. In common with the strategy for dermal irritation/corrosion testing, investigations *in vivo* should not be undertaken until relevant information, such as physicochemical properties (e.g. pH) and data from related substances have been considered. Testing should then be performed sequentially, with an initial screen *in vitro* included to detect those substances likely to be corrosive or severe irritants *in vivo*.

Studies of ocular irritation *in vivo* are performed in the rabbit; this species is preferred for a number of reasons, despite some concerns regarding the reliability of the extrapolation of irritant effects to man due to structural and functional differences. The response of the rabbit eye to a chemical is often more severe than the primate eye (Buehler and Newmann, 1964) and alternative methods, such as the low volume eye test (LVET) have been shown to produce a degree of irritation more consistent with that following human exposure.

The substance (0.1 ml of a liquid; 0.1 ml or 100 mg of a solid) is instilled into the inferior conjunctival sac of one eye of three or six rabbits (following initial testing in one animal); the other eye of each animal remains untreated and acts as a control. It is commonly recommended that solid test materials are ground to a fine powder prior to testing. The test material may be washed from the treated eye with saline or water. The treated eyes are examined at intervals up to 72 hours following instillation of the test material; observations may continue for up to 21 days (or until the reversibility of effects is demonstrated). Ocular reactions (including conjunctival erythema and oedema, iritis and conjunctival opacity) are graded using various schemes, such as that defined by Draize *et al.* (1944). The irritation seen for solid substances may result from a combination of physical and chemical effects. The test substance is assigned to a classification category; the category is governed by a combination of the severity and reversibility of the reaction.

5.7 Skin Sensitization

For regulatory purposes, testing for skin sensitization—the ability of a substance to cause delayed contact hypersensitivity—is usually grouped with other aspects of acute toxicity. Although a sensitization response can be induced by one exposure to a substance, prior exposure to that substance is required for the establishment of the immune memory required to elicit the response (see **Immunotoxicology**). The immune system of the mouse has been better characterized than that of other species, however it is the guinea pig that has been the species of choice for skin sensitization studies for several decades. Guinea pig tests for sensitization can be divided into two general categories: those using injection of an adjuvant to potentiate sensitization and test that do not use adjuvant. Of the numerous tests developed, the Buehler test (Buehler, 1965)—a nonadjuvant method, and the maximization test (Magnusson and Kligman, 1969)—an adjuvant method, are the methods specified by regulatory guidelines. The guinea pig tests are similar in that they consist of two phases: an induction phase, during which an immune response to the test substance may develop, and a challenge phase, during which the immune response is expressed as an allergic dermal reaction. In order to increase the sensitivity of the studies, the concentration of the test substance used for induction should be the minimum irritant concentration; the concentration used for challenge should be the maximum nonirritant concentration. It is important in this type of study for the investigator to be able to distinguish between irritant and hypersensitivity reactions, therefore the study designs include control animals which are only exposed to the test substance during the challenge phase. The incidence of sensitization reactions following challenge exposure is used to assign the chemical to a classification category.

More recently, the mouse local lymph node assay (LLNA) has been validated as an alternative to the guinea pig methods (Basketter *et al.*, 1996), and is the preferred method under some regulatory regimes. The principle underlying the LLNA is that skin sensitizers cause lymphocyte proliferation in the lymph nodes adjacent to the site of application. The test substance is applied in a suitable solvent to the ears of groups of female mice at three concentrations; groups of positive and negative control animals are also used. The immune response to the application of the test substance is assessed by the incorporation of tritiated thymidine in the lymphocytes of the local draining (auricular) lymph nodes compared to control animals, and is expressed as the stimulation index (SI). An SI value of three or greater is considered to represent a positive response for purposes of classification.

6 ALTERNATIVE METHODS IN ACUTE TOXICITY TESTING

In the UK in 2006, approximately 420 000 animals were used in studies performed for toxicological purposes; a relatively large proportion (circa 34%) of these animals were used in tests of acute toxicity (Home Office, 2007). Acute toxicity testing is an easy target for those opposed

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to animal testing and it is increasingly being argued by those within toxicology that the existing *in vivo* studies of acute toxicity are outdated and unsophisticated, and while the more recently developed methods for acute oral toxicity study methods use fewer animals and do not all depend on mortality as an endpoint, similar refinements have not yet been adopted for acute dermal and inhalation toxicity.

From a regulatory standpoint, the primary use of acute toxicity data is for the classification and labelling of chemicals to allow their safe transport and to ensure the protection of the end-user. The relevance of the results of animal studies to humans can be criticized due to the potential for marked species differences in response, and, while it is claimed that similar information could be obtained from studies *in vitro*, these alternative methods also have their own limitations; until alternative procedures have been validated and accepted, conventional *in vivo* methods will continue to be used.

It may be possible to estimate the acute toxicity of a substance by using information available for a structural analogue or analogues; this approach (also known as read-across) is mainly used for the prioritization of untested substances. The EU Dangerous Preparations Directive (Directive 99/45/EEC), which applies to products containing mixtures of substances, adopts a pragmatic approach with regard to acute toxicity testing. Classification of the product can be addressed by a method involving calculation based on the levels and classification of substances within the product. The aim is therefore to reduce the acute toxicity testing required for the large number of products covered by this Directive.

Quantitative structure–activity relationship (QSAR) analysis for predicting acute toxicity is being used increasingly and may be useful for the prioritization of chemicals within a group, but is limited in its ability to predict the response of an animal which is a complex system of numerous different cell and tissue types. The Danish Environmental Protection Agency has made extensive use of QSARs to predict the toxicity (including the acute toxicity) of chemicals; QSAR models are also by the US EPA and other US regulatory agencies to predict acute toxicity for regulatory purposes (Cronin *et al.*, 2003). Available models are used to predict acute oral LD_{50}, skin irritation/corrosivity, eye irritation/corrosivity and skin sensitization. Progress in terms of the training sets and improved predictability is being made constantly.

6.1 *In Vitro* Alternatives

In addition to the refinements to *in vivo* tests described above, significant research has also been carried out into *in vitro* alternatives to animal studies in all areas of acute toxicity testing (see **Alternatives to In Vivo Studies in Toxicology**).

6.1.1 Acute Toxicity

In vitro models of acute toxicity should ideally use human rather than animals cells in order to eliminate interspecies variations in those cellular factors potentially affecting toxicity. The theory that cytotoxicity *in vitro* may be correlated to acute toxicity to an intact animal *in vivo* is based on the premise that the mechanism of toxicity for most chemicals is relatively nonspecific and involves those biochemical processes that are common to all cells; however this is clearly not the case for all substances. The MEIC (multicentre evaluation of *in vitro* cytotoxicity) programme was designed to assess the potential application of *in vitro* assays based on cultured mammalian cells in predicting acute toxicity and, for the chemicals selected, a good correlation was found between cytotoxic concentrations *in vitro* and lethal human blood concentrations. However, simple cell culture models do not take into account the rate or extent of absorption of the substance, for example from the gastrointestinal tract or through the skin. The complete assessment of the acute toxicity of a chemical *in vitro* is therefore dependent on an understanding of its toxicokinetics, including factors such as the rate and extent of absorption, tissue distribution, metabolism and the rate and route(s) of excretion. Subsequent initiatives have therefore also investigated the development of additional *in vitro* tests capable of detecting additional (more specific) mechanisms of toxicity and the incorporation of toxicokinetic parameters. A tiered approach to *in vitro* testing for acute toxicity has also been recommended (Seibert *et al.*, 1996; Botham, 2004). The first tier of this approach involves a combination of basal cytotoxicity tests and physicochemical data on the chemical in question, the second tier involves metabolism *in vitro* in cultured liver cells and the third tier involves the assessment of toxicity to target organs or tissues using (for example) cells of renal, hepatic or nervous system origin. Of relevance to acute oral toxicity testing is the ability to predict the absorption of a chemical from the gastrointestinal tract using physiochemical properties (primarily the octanol/water partition coefficient) or by using cultured human cells such as Caco-2. Validated *in vitro* models using human skin (removed during surgical procedures) have been developed to assess the rate and extent of dermal penetration, however the values obtained from this model are generally higher than those expected *in vivo*. Other *in vitro* assays are available to assess the influence of plasma protein binding on toxicity. Alternatively, kinetic parameters may be modelled using PBPK models, however the majority of these techniques have similarly not been validated to regulatory standards.

6.1.2 Dermal Irritation and Corrosion

The mechanisms by which chemical substances cause dermal irritation are well characterized and therefore

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significant progress has been made in this area over a number of years. Organotypic models consisting of epidermal keratinocytes of human origin, cultured to form multilayered and highly differentiated models of the epidermis are routinely used as screens for corrosive substances, prior to testing *in vivo*. The SkinEthic™ and Epiderm™ models measure cell viability [as assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction] and have been validated by the OECD for the assessment of dermal corrosion. Other OECD-validated methods are the transcutaneous electrical resistance (TER) assay, an ex vivo method which measures the integrity of the stratum corneum and the Corrositex™ assay which measures the time taken for the test substance to degrade a barrier consisting of a hydrated collagen matrix (Fentem *et al.*, 1998). In the EU, the *in vitro* EPISKIN™ model has recently been validated as a stand-alone alternative to the rabbit skin irritation test (Botham, 2007). This human skin model is constructed using multiple layers of viable human keratinocytes, and has a functional stratum corneum supported on a collagen matrix. Test substances applied to the epidermal surface are considered to have the potential to cause dermal irritation if they are able to penetrate the stratum corneum and cause toxicity to underlying cells. Cytotoxicity is assessed by a simple MTT assay and by measuring the release of the inflammatory mediator interleukin-1α.

6.1.3 Eye Irritation and Corrosion

There are currently no validated *in vitro* test systems for the assessment of eye irritation. However, a number of *in vitro* models are available and are used routinely as part of a sequential testing strategy to screen chemicals for severe ocular irritation and corrosivity, and to eliminate the need for the assessment of ocular irritation *in vivo*. The rabbit enucleated eye test (REET) assesses effects on the cornea including thickness, opacity and integrity; similar endpoints are assessed in the chicken enucleated eye test (CEET) or isolated chicken eye test (ICET). The hen's egg test-chorioallantoic membrane (HET-CAM) can also be used as a model of mucous membrane irritation.

6.1.4 Skin Sensitization

There are currently no validated *in vitro* tests for the assessment of skin sensitization. However, the mechanisms of sensitization *in vivo* are relatively well characterized and therefore significant efforts have been made towards the development of sufficiently sensitive *in vitro* assays. Current research is focussing on the expression of surface markers and the release of cytokines by dendritic-like cells (Basketter and Maxwell, 2007). Concurrent progress in the development of QSARs for skin sensitization and the prediction of protein binding means that it is likely that future approaches to testing will involve a combination of *in vitro* and in silico methods (Patlewicz *et al.*, 2007).

7 CONCLUSION

Investigations of acute oral toxicity are the most fundamental and therefore the most commonly performed studies *in vivo*: acute toxicity has been investigated for centuries and formal testing is a regulatory requirement for many classes of substances in order to ensure human safety. The relatively simple nature of acute toxicity studies makes them candidates for replacement by alternative methods and, while progress continues to be made in this area, regulatory acceptance of these methods is presently limited. The assessment of acute toxicity should follow an intelligent, integrated approach utilizing all available methods including (where available) read-across from chemical analogues, QSAR analysis and *in vitro* data. *In vivo* testing should only be performed when necessary and, where studies are required, study design should be considered with regard to the relevance of the endpoint being investigated, and also the principles of reduction and refinement.

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Repeated Exposure Toxicity

Bryan Ballantyne

CONTENTS

1 BACKGROUND

The amount of detail and usable information on potential adverse health effects that can be obtained from general toxicology studies depends on a multiplicity of factors including, although not limited to, route of exposure, species, total dosage, formulation and purity of test material, the general and specific monitors employed and the timescale for exposure. In order to appreciate the importance of repeated-exposure studies in overall health hazard assessments, it is useful to compare the differences in approaches and resultant findings for acute studies with those for repeated-exposure investigations. This is discussed in outline below.

1.1 Acute Studies as Preliminary to Repeated Exposure

Acute (single-exposure) studies are conducted along the following defined lines for the usual routes of exposure. Perorally (po), the dose is given by bolus gavage or by divided doses over 24 hours; percutaneously (pc), the dose is by skin contact (usually occluded) for a specified period (often 4 or 24 hours); respiratory exposure is by a single inhalation dose for a specified period (to various atmospheric concentrations), or for different variable periods of time to a specific test agent concentration. Other routes of dosing, such as intravenous (iv), intraperitoneal (ip) and intramuscular (im), usually employ bolus dosing. Observations are often conducted over a 14-day postdosing period. Acute studies include provision for observation for signs of toxic and/or pharmacological effects. A major end point is often for mortality, with subsequent calculation of the dose that kills half (50%) of the animals tested (LD_{50}) or the timed concentration that does so (LC_{50}). Most studies also incorporate observations for nonlethal signs, body weights and gross pathology by necropsy of animals that die and survivors sacrificed at the end of the 14-day postdosing observation period. Many acute studies are conducted as screens for toxicity, to obtain information on potential overexposure situations and/or to satisfy regulatory requirements. Because of this, and sometimes for reasons of economy, most acute studies do not include provision for physiological, biochemical or histological monitoring. Although

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adding cost, in selected cases much more information related to potential adverse health effects can be obtained by the use of limited haematology, clinical chemistry and histology. Within the constraints of the limited monitoring usually conducted, acute toxicology studies are useful with respect to the following:

1. Give information on potential local and systemic toxicity (and thus permit hazard assessments) by acute overexposure to the material tested.
2. Give information which may be important in determining the acute lethal toxicity, or lack thereof, of the material. In the absence of other information, this is often used as a basis for assuming lethal toxicity in humans, in spite of the difficulties associated with the extrapolation of such data. In a number of cases, acute mortality data obtained from small laboratory animals may be significantly misleading, because of species differences in absorption, pharmacokinetics and metabolism. A notable case is with ethylene glycol: the rat acute peroral LD_{50} is $8.54\,ml\,kg^{-1}$, whereas estimates from human lethal poisoning cases suggests a lethal dose around $1.0\,ml\,kg^{-1}$ (Tyler and Ballantyne, 1988).
3. Permits a comparative evaluation, often based on LD_{50} or LC_{50} data, of the toxicity of materials. However, comparison of such data should not be based solely on the LD_{50} or LC_{50} values, since they may result in misleading conclusions with respect to population hazards. An evaluation of all data, including 95% confidence limits, slope on the dose–mortality regression lines, and times to death, is required.
4. Acute studies, if adequately monitored, may give information on the potential for long-term adverse health effects; for example, delayed-onset peripheral neuropathy from overexposure to organophosphates (Johnson, 1992).
5. Give information needed to meet the requirements of regulatory authorities with respect to classification into categories for use in various activities such as handling, transportation and labelling. Although there are geographical differences in the definition of the various classification bands, there is much activity to attempt to reach harmonization of classification standards on an international basis.

Additionally, and when the available information is sufficient, acute exposure studies may give a limited indication of the potential of a substance to cause local and/or systemic toxicity by repeated exposure. For example, local cutaneous or respiratory tract irritant (inflammatory) effects caused by acute exposure may be anticipated to occur also by repeated exposure to lower concentrations. However, the potential for materials to produce systemic toxicity by acute exposure must be carefully interpreted, as also must the extrapolation of such findings to repeated-exposure conditions. For example, a dose–response relationship for liver injury when the test substance is given perorally may strongly suggest a potential for cumulative hepatotoxicity by lower dose repeated exposure, for example with organic arsenicals (Ballantyne, 1978). In other instances, however, specific acute organ injury may be a secondary effect; for example, renal tubular necrosis in acute inhalation studies with irritant materials may be a consequence of hypoxaemia secondary to lung injury (Ballantyne and Callaway, 1972). A proper evaluation of the potential for toxicity by repeated exposure requires that repeated-exposure studies be conducted. Only in this way can reliable no-observed-effect levels (NOELs) and no-observed-adverse-effect levels (NOAELs) be established. For hazard evaluation purposes it is also useful to have information on the threshold for induction of adverse effects (lowest observed adverse effect level: LOAEL).

1.2 The Need for Repeated-Exposure Studies

Although, as noted above (Section 1.1), in a limited number of cases acute studies may give an indication of the potential for substances to produce toxicity by repeated exposures, this is generally limited to inflammatory effects and clear dosage-related direct organ toxicity. Since many potential toxic effects are not detected in acute studies because of the need for cumulative toxicity to develop or because of a long latency to exhibition of toxicity, reliable evaluations of the potential for toxicity by repeated-exposure conditions requires that studies be conducted by an appropriate route for a sufficient number of exposures. It is generally necessary to conduct repeated-exposure studies initially over a limited period of time in order to determine the development of toxicity as a function of exposure concentration or dose. The initial concentrations or doses chosen will be determined by findings from acute studies, when these permit the detection of toxicity. When acute studies do not exhibit toxic effects then doses may sometimes be chosen on the basis of comparison with findings from the same chemical class of material, or failing this from short-term high-dose studies. The findings from well-conducted and appropriately monitored short-term repeated-exposure studies are used to determine concentrations or doses for longer-term studies. Such longer-term repeated studies may ultimately need chronic (lifetime) studies if malignant or extreme and potent toxicity are suspected. The major goals of repeated-exposure studies are as follows, particularly when used for hazard evaluation and safety confirmation purposes:

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1. To determine the incidence and nature of potentially adverse biological effects by recurrent exposures, and their dose/concentration dependency, including characterization of the shape of the dose–response curve. In general, the longer the period over which exposures are conducted, the lower the individual doses/concentrations that are needed to induce toxicity.
2. To determine the dependency and severity of development of toxicity as a function of the exposure conditions.
3. For the determination of those concentrations or doses which do not result in the appearance of any effects due to exposure to the substance tested (NOEL) or do not cause the development of effects regarded as potentially biologically harmful (NOAEL). Recently there has been the development of a concept of benchmark dose (BMD) as an alternative to the NOAEL, or compromise between the NOAEL and the LOAEL. The BMD involves fitting a dose–response curve to all bioassay data, and the dose (or lower bound on the dose) corresponding to a given low-level response on the fitted curve (e.g. a 5% elevation in incidence over background: BMD05) is selected as a characterization of the dose level at which a detectable increase in the measured effect of interest occurs (Rhomberg, 2005). After the application of appropriate uncertainty factors, the BMD is used in determinations of certain safe dose values such as acceptable intakes or reference doses.
4. To determine the threshold for induction of potentially adverse effects based on observable toxicity; the LOAEL.
5. With the shorter-term repeated-exposure studies, to give sufficient reliable information to permit the design and dosage/concentration choices for more expensive longer-term studies.

2 DETERMINANTS FOR REPEATED-EXPOSURE TOXICITY

As noted previously (Section 1.1), acute studies may show a potential for repeated-exposure local and systemic toxicity, providing the effects are not secondary to some marked disturbance of normal physiology and if they are dose-related. However, in most acute studies the test material is given by bolus dosing, which results in the rapid development of high circulating concentrations of parent material and metabolites if the dosed material is readily absorbed by the route of exposure. In these circumstances, the threshold to induce toxicity may be exceeded and toxic effects exhibited. With repeated-exposure conditions, however, the individual doses given are usually much smaller than those employed in acute studies, and probably subthreshold for the induction of toxicity by acute dosing. In such circumstances there may be an incremental increase in body load of the material (and/or metabolite) with increasing sequential cumulative exposures, and there may be an associated latency to the onset of toxicity by repeated-dose procedures (Ballantyne, 1989). Many factors are associated with the likelihood for toxicity by repeated exposures, and its nature, the most important of these being as follows:

1. **Period of repeated exposures:** As noted above, there may be latency to the development of body loading with parent material (or metabolite) that is sufficient to initiate the toxic effect. Clearly, in addition to the absolute individual doses, the number of exposures will be a determinant of the likelihood of reaching the threshold body load of toxic material and thus for the development of toxicity.
2. **Dosing characteristics:** The precise profiling of exposure doses may have a significant influence on the development of toxicity, which may not be predictable from acute toxicity information. For example, with benzene vapour a 24 hour exposure to 95 ppm or a 96 hour exposure to 21 ppm both produced severe bone marrow toxicity, but in a repeated-exposure study involving exposure to 95 ppm vapour for 2 h day^{-1} for two weeks there was little toxicity (Toft *et al*., 1982). The precise influence of profiling of dosing may vary depending on chemical nature of the test material. For example, in a four-week inhalation study with formaldehyde, 8 hours of continuous exposures were compared with 8 hour intermittent exposures (Wilmer *et al*., 1987). The findings suggested that concentration rather than total dose was the major determinant of toxicity. Thus, interrupted exposures of rats to 10 or 20 ppm formaldehyde vapour induced more cytotoxicity in the nasal mucosa than did continuous exposure to 5 or 10 ppm. In a four-week inhalation study with carbon tetrachloride, interruption of a daily 6 hour exposure by 1–5 hour periods free of carbon tetrachloride produced a slightly more severe hepatotoxicity, whereas 5 minute peak loads superimposed on a fixed background exposure only slightly enhanced the hepatotoxicity of carbon tetrachloride (Bogers *et al*., 1987).
3. **Pharmacokinetics and metabolism:** Given by repeated exposure, the time available for compensatory biological responses is longer, in particular for the induction of biotransformation mechanisms, including metabolic activation and detoxification. Depending on the route of exposure (including any first-pass involvement), the development of toxicity and its severity will depend on the relative balance of the induction of activation and detoxification processes.

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3 DURATION OF REPEATED-EXPOSURE STUDIES

Repeated-exposure studies are usually conducted for a variable combination of the following reasons:

1. To determine the potential for non-oncogenic cumulative or long-term toxic effects.
2. To determine the *in vivo* oncogenic potential of a material.
3. To determine threshold or no-observed-adverse-effect dosages, particularly when the results are to be used to protect populations, for example, establishment of workplace exposure guidelines or acceptable daily intakes.
4. To meet the requirements of regulatory authorities for activities such as product registration.

Depending on the reason(s) for conducting the investigations, and the stage in the assessment of toxicity, repeated-exposure studies vary in length from a few days to a lifetime of exposure. It is convenient to subdivide these into short-term, subchronic and chronic studies, as follows:

3.1 Short-term Repeated Studies

These vary in length from a few (5–9) days to 28 days. The shorter studies, around 7 days, have sometimes been referred to as subacute studies. Although the individual daily dosages employed may be less than acute doses, the total duration and total number of exposures is greater, and not below (sub) that for the acute exposures. The term 'subacute' is not a preferred descriptor. The choice of duration for a short-term repeated-exposure study will depend on a number of factors, including the reason for conducting the study, but they are often conduced as preliminaries to subchronic studies to determine appropriate exposure doses/concentrations.

3.2 Subchronic Studies

These studies involve exposing the test species for 15–20% of their lifespan. Thus, for most laboratory species used in subchronic tests (e.g. rats and mice), exposures will extend over a period of about 90 days. These studies are usually conducted in order to determine the potential for non-oncogenic adverse effects, and their threshold LOAEL and NOAEL. Additionally, and to ensure an appropriate choice of dosing, subchronic studies are also conducted in advance of chronic toxicology studies.

3.3 Chronic Studies

Such studies involve exposing the test species to the test material for their lifespan, or the greater part thereof. Although a primary aim of many chronic studies is to investigate the *in vivo* potential for oncogenicity, the majority are usually conducted as combined chronic toxicity/oncogenicity studies. Because of the high cost of such studies, in the region of, or more than, a million dollars, depending on the route of exposure, decisions on the need for, and design of, such studies requires very careful consideration.

It is clear that repeated-exposure studies of varying duration should be carried out in sequence, with the shorter studies being conducted initially to give guidance on the types of adverse effects to be anticipated (and monitors for them), and also to allow for decisions on appropriate dosing for the longer studies.

4 GENERAL DESIGN FEATURES

Details concerning animal use and care and statistical considerations for toxicological studies are given in **Statistics for Toxicology** and **Animal Welfare in the Toxicology Laboratory**, respectively. The following comments give an overview of the major considerations in the development of protocols for repeated-exposure studies.

4.1 Generic Issues in Experimental Design

During the design of any repeated-exposure toxicity study, a multiplicity of differing factors needs to be taken into account in developing a protocol. Failure to give adequate thought with respect to all, including minor, details might result in the conduct of an unsatisfactory study, or cause the introduction of artefacts, which can complicate interpretation of the findings. Some of the more important relevant considerations are briefly discussed below.

4.1.1 Animals

In order to ensure comparability of groups, and to avoid complications from outlier animals, those that are used in a study are often selected to be of closely comparable age at the start of the study and within a restricted weight range, usually within two standard deviations of the batch mean weight for each sex. To ensure that the animals to be used in a particular repeated-exposure toxicity study are healthy, a randomly selected subgroup from the test batch of animals should be checked for health status before the start of the study; this procedure

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involves, at least, examination by a clinical veterinarian, blood sampling for limited clinical chemistry and viral antibodies, examination for faecal parasites, necropsy for gross pathology, and histology of selected tissues and organs (to include respiratory tract, gastrointestinal tract, liver, kidney, lymph node and spleen).

4.1.2 Environmental Conditions

To minimize the possible effect of environmental influences on both animal welfare and induced toxicity, factors such as environmental temperature and relative humidity should be maintained within accepted optimum conditions. Significant variations from the recommended range of animal-house temperatures can increase susceptibility to infection, impair general health and cause a variation in response to toxic chemicals (WHO, 1978). Changes in temperature may elicit homeostatic changes in various physiological and biochemical systems, and alter rate-determining physiological processes for the absorption and action of toxic chemicals. Also, because of the influence of circadian factors on toxicity (see **Chronotoxicology**), a constant photoperiod should be used.

The positioning of animals in holding rooms can influence certain physiological and pathological processes, and hence the requirement for randomization and periodic rotation of the position of individual cages at least weekly. It has also been advised (Ecobichon, 1992) that 'sentinel' animals be included in the longer-term studies. These animals are not part of the definitive study, but placed in the same room as the test and control animals and periodically monitored for the appearance of bacterial or viral infections, test agent-induced toxic changes due to contamination of the facility, or changes in environmental conditions. These animals should be subjected to periodic haematology, clinical chemistry and bacteriology/virology checks.

4.1.3 Caging

Toxicity can be influenced by a number of factors related to the manner of caging, including type of caging, grouping and bedding material. For example, the toxicity of isoprenaline (isoproterenol) was greater in rats caged singly for more than three weeks than in rats caged in groups (Hatch *et al*., 1965). In a study of the influence of housing conditions in skin oncogenicity bioassays, DePass *et al*. (1986a) showed that differences in housing conditions can influence both the latency and incidence of local neoplasms resulting from chronic cutaneous application of a carcinogen. Multiple caging can result in unique identification problems, and trauma from bites. Additionally, with multiple housing, the susceptibility to transmissible diseases is increased, and if mortalities occur there may be cannibalism. Also, multiple caging does not permit individual animal food consumption values to be obtained, and thus does not allow any correlations between individual weight changes and food consumption to be established. When dosing is by incorporation of test material into the diet, multiple caging does not permit the calculation of individual animal test-agent consumption. For these reasons, it is generally recommended that for repeated-dose toxicity studies animals should be caged singly (Wilson and Hayes, 1994).

The type of cage employed, metal or plastic, solid floor or suspended, requires careful consideration during protocol design (Stevens and Mylecraine, 1994). For example, solid floor caging requires bedding which may create dust, and certain sawdust and chips may induce hepatic mixed function oxidase activity. Suspended wire cages, on the other hand, may result in trauma, especially to the feet and legs. Shoebox-type cages may cause problems associated with stasis of the air in the cage.

Most repeated-exposure studies with rodents are generally conducted using suspended wire cages with the animals housed singly. This eases clinical examinations and allows information to be obtained on food and water consumption.

4.1.4 Diet

It is well appreciated that both the degree and type of nutrition may affect laboratory animal lifespan, expression of toxicity and the pattern of carcinogenicity in control and test-agent animals (Allaben and Hart, 1998; Haseman, 1998; Leakey *et al*., 1998; Turturro *et al*., 1998). Of particular importance in chronic studies is the marked influence that diet may have on natural tumour incidences, and also on modifying carcinogen-induced tumour incidence (Grasso, 1988). For example, Tucker (1979) demonstrated that Charles River mice on a 20% dietary restriction developed fewer liver tumours than mice fed diet *ad libitum*. Conybeare (1980) found that the incidence of liver tumours in mice was reduced from 47 to 12% by restricting food intake to 75% of the normal amount. Benson *et al*. (1956) found that a higher incidence of spontaneous mammary adenocarcinomas developed in rats when olive oil was added to the diet, and Chan and Dao (1981) noted a higher incidence of mammary tumours in rats on a semisynthetic diet containing 16% corn oil. Both the protein and fat content of diets may influence the induction of tumours by carcinogens. High-fat diets enhance mammary tumour incidence caused by dimethylbenzanthracene in rats (Carroll and Khor, 1975), hepatocellular carcinomas in rats caused by *p*-dimethylaminoazobenzene (Miller and Miller, 1953), colon cancer by 1,2-dimethylhydrazine (Reddy *et al*., 1976) and pancreatic adenocarcinomas by azaserine (Roebuck *et al*., 1981). In the rat, a high protein diet enhanced hepatocellular carcinoma development from aflatoxin (Madhavan and Gopalan, 1968),

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and colon carcinoma from 1,2-dimethylhydrazine (Reddy *et al*., 1976).

Diet should ideally be kept constant over the study period and be of known composition and nutritional status. Diets should be used that have been assayed for contaminants such as pesticides, heavy metals and mycotoxins. They should not be used beyond expiratory dates, and stored to prevent contamination.

4.1.5 Test Material

The material to be tested should be of known purity and its chemical identity confirmed. Test material purity, and possible degradation products, should be measured before the start and at the end of the study to ensure stability. With the longer repeated-exposure studies, interim analyses may be required. If a dosing matrix, such as drinking water or diet, is used, then stability of the test material and homogeneity of distribution in the matrix are required. Ideally a single batch (lot) of the test material should be used over the study period to ensure an absence of effects due to possible changes and inconsistencies from batch to batch. This, of course, may not be possible with chronic studies, where there should be detailed attention to analyses and to documentation. Also, with materials to be used commercially, the test material should be as close as possible to that intended to be used or marketed.

4.2 Choice of Species

Most repeated-exposure studies are conducted using rats or mice, with some regulatory agencies also requiring the (additional) use of a larger species (e.g. dog), particularly for subchronic studies. The choice of test species is an important consideration, particularly where there is reason to suspect that species variation in the toxic response may occur, qualitatively or quantitatively. An example is that of butadiene, where the toxic (carcinogenic) potential is greater in the mouse than the rat (Melnick *et al*., 1990). Since it has been demonstrated that the metabolism of butadiene in the rat is more like that in the human than in the mouse, the rat appears to be a more appropriate model for repeated-exposure studies (Bond *et al*., 1995; Himmelstein *et al*., 1997; Anderson, 1998). Also, there may be significant differences between different strains for a given species. For example, Wistar rats are more sensitive to ethylene glycol renal toxicity than Fischer 344 rats; the respective NOAELs were $200\,mg\,kg^{-1}\,day^{-1}$ for male F-344 rats (DePass *et al*., 1986b) and $71\,mg\,kg^{-1}\,day^{-1}$ for male Wistar rats (Gaunt *et al*., 1974). Subsequent toxicokinetic studies (Cruzan *et al*., 2004) showed that the strain differences in renal toxicity were related to differences in the ability to clear ethylene glycol and its metabolites, notably oxalate. Criteria for the selection of a species include:

1. The requirements of regulatory agencies.
2. The material should have similar pharmacokinetic and metabolic features in both the test species and humans. For the majority of substances, with the possible exception of therapeutic agents, this information is either sparse or not available. Therefore, from a practical viewpoint, this criterion can seldom be applied.
3. Use of the most sensitive species (and strain) to the known or suspect toxicity of the test material, on the basis that this represents the most conservative approach in extrapolating toxicity in the test species to humans. Again, such information may not be available at the start of repeated-exposure testing, and acute toxicity information may not be a reliable guide to cumulative or long-term toxicity.
4. Experience of the laboratory and the existence of a good historical control database. Although concurrent control animals should be used in repeated-exposure studies, in some instances comparison with the treatment groups may yield equivocal results, and in such cases reference to the historical database may be of use.
5. The use of primates, even if considered appropriate on investigational scientific grounds, is limited by cost considerations and animal welfare legislation.

In view of the fact that there is species variability in the response to toxic chemicals, and since no one species is a perfect surrogate for the human, several regulatory agencies require the use of two species, often citing a rodent and a nonrodent. Often the choice of a species is based on considerations that include experience with that species, animal size, ease of handling, databases, accommodation and economics. However, from the viewpoint of obtaining relevant useable information from a well-designed study, attention should be paid to information on physicochemical characteristics, known toxicity, and any available pharmacokinetic and metabolism data of the test material.

4.3 Dosing Procedures

The route of exposure will be determined principally by the intended use of the test material and the likely route by which the user will be exposed. In some instances there could be a need for study by more than one route of exposure. For example, with pesticides the applicator may be exposed cutaneously or by inhalation, whereas the consumers of treated crops will be exposed through the peroral route. In most cases the appropriate route can be used in repeated-exposure studies, but in a very

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few instances because of physical or technical problems an alternative route may be required. An example is provided by ethylenediamine (EDA), for which the respiratory tract is a potential route of exposure. However, generation of the vapour for a long-term study presents difficulties, as do the sampling and analyses of the chamber atmosphere; also, EDA reacts with water and carbon dioxide. These problems hamper the conduct of a chronic inhalation study with EDA. In a comparative pharmacokinetic and metabolism study, EDA was given by oral, iv and endotracheal dosing. The equivalency of the fate of EDA by the peroral and endotracheal routes was demonstrated (Yang and Tallant, 1982). Based on these findings, a chronic toxicity/carcinogenicity study was successfully completed in the rat by incorporation of EDA into the diet (Hermansky *et al*., 1999).

By the peroral route, a multiplicity of dosing procedures are available, the choice of which will depend on the intended use of the test material, physical and chemical characteristics, likely exposure patterns by the user, stability of test material, and the test species. In some instances the material may be given by gavage. This procedure ensures that relatively precise daily pulses of chemical are given, but carries the disadvantage that intubation trauma and dose misplacement may occur. Many peroral repeated-dosage studies are conducted by incorporating the test material into diet or drinking water, resulting in a slower titration of the material into the gastrointestinal tract. There may well be differences, therefore, between the absorption pharmacokinetics of test material depending on whether it is given by gavage or dietary/drinking-water incorporation. This factor needs consideration in the design and interpretation of studies. When given by inclusion in the diet, it is essential to conduct tests to ensure homogeneity of distribution and stability in the diet, and also to conduct extraction procedures to preclude binding to the diet that will significantly reduce bioavailability. When test material is incorporated into the diet for dosing, it is common practice to adjust the dietary concentration of test material periodically (usually weekly) to achieve constancy of the target daily doses against a progressively changing body weight. Mean projected dietary concentration is calculated from the preceding body weight gain, and the dietary test material concentration is adjusted to achieve constancy of dosing. Feeding at a constant concentration during a study has also been conducted, but this results in loss of control of constant dosage and may modify toxic response and interpretation of the findings from the study (Wilson and Hayes, 1994). As with dietary incorporation studies, when test material is dissolved in drinking water, homogeneity of distribution and stability needs to be confirmed. Also, the test material should not be so unpalatable as to reduce food or water consumption significantly. In special circumstances the test material may require to be dosed using capsules or by an encapsulation procedure; this is particularly useful for highly volatile materials or to mask objectionable taste. Microencapsulation involves uniformly coating the test material with a degradable but impervious material (Melnick *et al*., 1987; Yuan *et al*., 1991).

By inhalation, the study design will depend on whether exposure is to gas, vapour or aerosol. The principles of ensuring constancy of exposure dose and avoiding artefacts are similar to those by other routes of exposure. The details of generation, distribution, sampling and analysis of the atmosphere should follow the guidance given in **Inhalation Toxicity**. If whole body exposures are undertaken then the possibility for some percutaneous absorption, and, more significantly, swallowing of material due to grooming, should be kept in mind, particularly during interpretation of the findings. This can be avoided by the use of nose-only exposures. For example, in a developmental toxicity study with an ethylene glycol aerosol in which rats and mice received daily 6-hour exposures by whole body exposure over gestational days 6–15 inclusive, maternal toxicity and terata were produced in mice (Tyl *et al*., 1989; 1995a). In simultaneously exposed mice, ethylene glycol was analytically detected on fur after exposure, which could have accounted for between 65 and 95% of the total dose, and be available for absorption percutaneously or orally following grooming. When mice were exposed to ethylene glycol aerosol by a nose-only procedure this prevented most of the maternal and developmental toxicity observed as a result of whole body exposure (Tyl *et al*., 1995b). Measures should be in place to avoid exposure of the laboratory worker or contamination of the animal facility after the end of an exposure period. This will include keeping the animals in a stream of fresh air for a period after the exposure has been completed. The direct instillation of test material into the trachea has been used in some studies as a procedure to investigate toxic and toxicokinetic aspects of pulmonary exposure. Intratracheal dosing has been employed for a variety of differing reasons including its comparatively low cost, simplicity, to ensure the delivery of a well-defined dose, or technical problems with generation of an appropriate test atmosphere for standard inhalation studies (Driscoll *et al*., 2000). Whilst this can be a useful and cost-effective dosing method in certain circumstances, the relevance of the procedure and the findings need to be carefully considered against the background of the reason for the study and significance relevant to in-use situations. The uses and limitations of intratracheal instillation as an exposure route for the evaluation of respiratory tract toxicity have been reviewed by Driscoll *et al*. (2000) and Porter and Hubbs (2006).

Repeated-exposure skin contact studies are labour intensive. Again, prevention of secondary route of exposure by licking the test site should be avoided by choosing an appropriate remote application site and/or covering the application site. Based on screening or prior definitive studies, care should be taken to avoid the

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development of cumulative local skin irritation or injury that could lead to a premature termination of the study.

Repeated-exposure studies have been conducted using parenteral routes such as intravenous, intramuscular, intraperitoneal and subcutaneous. Such labour-intensive studies require special careful attention to the dosing techniques in order to avoid trauma associated with the injection process. Alternative parenteral dosing procedures for continuous dosing by slow release have included the use of implanted material or implantable osmotic minipumps (Dey *et al*., 1982; Ray and Theeuwes, 1987).

4.4 Exposure Groups

A prime consideration in the design of repeated-exposure studies is the number of groups of animals to be used. Although group size should be dictated primarily by the desired statistical power, it is often decided by the guideline protocol which is being adhered to for studies used for regulatory submission. A minimum of three groups should be used for exposure to differing concentrations or dosages of the test material. This allows the demonstration of a dose–response relationship, essential for confirmation of a toxic process. Also, and by careful choice of dosages, by using three groups this should allow for one which exhibits toxicity (top dose), one showing a marginal effect or LOAEL, which should be the intermediate dose, and a NOAEL or NOEL at the lowest dose. This information is used principally in two ways: first, in using the dose–response information for the purposes of defining safety margins in hazard evaluations and assigning permissible exposures (e.g. the threshold limit value (TLV) or acceptable daily intake (ADI)), and second, in setting exposure concentrations or doses for longer-term repeated-exposure studies. In the latter case, and particularly when using information derived from a subchronic study conducted prior to a chronic study, one prime determinant is the establishment of a maximum tolerated dose (MTD). This is a dose which is usually defined as causing no more than a 10% decrease in body weight, does not produce mortality, and produces effects that will not shorten the lifespan and jeopardize the study, but nevertheless produce some toxicity (Sontag *et al*., 1976; Haseman, 1985). The MTD is mostly used as a basis for assigning the highest dose for a chronic study.

The numbers of animals used per treatment group requires careful planning. There should be sufficient to allow for valid statistical analyses, but not in excess of the numbers dictated by good animal welfare practices. Depending on the length of the study, basic group size (from start to end of dosing) often ranges from 5 to 20 animals per sex. For subchronic and chronic studies, 20 per sex per group is a reasonable basic group size. Additional animals may be included if 'recovery' groups are required to assess reversibility, or otherwise, of induced toxicity after the end of the dosing period. This is a useful group to have for interpretive purposes, particularly with subchronic studies. The use of 'recovery' animals in the shorter-term repeated-exposure studies should also be considered. These 'recovery' animals are most frequently added to the high-dose and control groups. Additionally, and in order to study the onset and progression of induced toxicity, many studies also add extra animals for interim sacrifice at predetermined times during the exposure period. These groups also may yield valuable information useful in assessing dose–response relationships.

An untreated control group, equal in size to a dosing group (including interim sacrifice and 'recovery' animals), is of course necessary for primary comparative purposes against the treatment groups. Such a concurrent control group not only serves for the comparison of statistical and dose–response relationships of induced lesions, but may also indicate the presence of any nontreatment and environmental effects. Where the test material has to be given in a vehicle (solvent or suspending agent), some investigators suggest the need to confirm an absence of effects by the vehicle by inclusion of a vehicle control group. It will have been previously confirmed that the inert vehicle does not react with the test agent and the preparation has stability. The requirement for a vehicle control group will depend on the length of the study, physicochemical properties of the vehicle, and the extent to which it has been investigated toxicologically.

An important consideration in planning and protocol development is the development of criteria for decisions on when, and how, to euthanize animals that become moribund during the dosing period. These criteria should be in accord with Good Laboratory Practice (GLP) (see **Quality Assurance in Toxicology Studies**), the practices of the laboratory conducting the study, and national/federal, and/or state animal welfare regulations (see **Animal Welfare in the Toxicology Laboratory**).

4.5 Monitoring for Toxicity

Repeated-exposure toxicity studies are costly, in part because of their labour-intensive nature. It therefore follows that the maximum amount of toxicological and usable information should be obtained from them by appropriate extensive monitoring for toxic and other possible treatment-related effects. In this way, a repeat of the study can be avoided because of unexpected secondary findings indicating a primary toxicity not detected *per se*. Extensive monitoring is employed in the longer-term (subchronic and chronic) studies in order to define, as far as possible, evidence for structural and functional injury to tissues and organs. Whilst the

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shorter-term (five to nine day) studies are often conducted as screening (preliminary) for the longer-term studies, and used to set dosages for such longer-duration studies, it is wise to undertake some specialized monitoring to assess potential target organs and tissues by short-term repeated exposure, and also aid in the planning of detailed monitoring in the longer-term study. The 28-day studies should certainly be well monitored, as for a subchronic study, particularly if they are to be submitted to a regulatory agency. For the longer-term studies (subchronic and chronic), very detailed monitoring should be planned to include, as a basis, well-recognized monitors for the general detection of potentially adverse effects, and also specific techniques to assess sites of toxic response in particular organs and tissues.

Routinely, and discussed in more detail with subchronic studies (Section 6), the major overall monitors should include at least the following:

- Clinical observations for signs of toxic and/or pharmacological effects.
- Periodic measurement of body weight.
- Food and water consumption.
- Peripheral blood haematology.
- Blood biochemistry (clinical chemistry).
- Urinalysis.
- Necropsy for signs of gross pathology.
- Organ weights.
- Processing of tissues and organs for histological examination.

It is important to compare clinical pathology findings from treatment groups to those of the concurrent control groups, for which blood has been collected under identical conditions. For example, Walter (1999) compared clinical pathology values for blood collected from the orbital sinus of rats anaesthetized with either pure (100%) carbon dioxide or an oxygen/carbon dioxide mixture (34, 66%) with the values for blood from unanaesthetized rats. Small, but statistically significant, differences were found for several values; thus, for the carbon dioxide groups there were smaller values for calcium, and higher values for leukocyte counts, lymphocyte counts and glucose. A larger proportionate difference was found for creatine kinase and aspartate aminotransferase activities, with values being highest in the unanaesthetized group. This was attributed to the physical activity in this group of animals. When it is necessary to review study findings by comparing with laboratory historical control values, as for example in studies where the chemical pathology findings are borderline, equivocal, or inexplicable, then the comparisons should be made with great care because of the influence of various factors on measured values; for example, mode and site of collection of blood samples, whether they are taken from anaesthetized, unanaesthetized or sacrificed animals, and the analytical methods employed.

For haematology, clinical chemistry and urinalysis, the choice of individual measurements may vary from laboratory to laboratory, often based on their experience, but certain core measurements are necessary in all studies (see **Clinical Chemistry in Toxicity Testing: Scope and Methods** and **Haematology and Toxicology**). In some studies, viewed on a case-by-case basis, additional specialized monitoring may be considered necessary based on information already available or suspect toxicology resulting, for example, from structure–activity considerations. Thus, in the planning of the protocol, there is a clear need to consider the requirement for additional ('nonstandard') monitors. For example, with 5-ethylidene-2-norbornene (ENB), and based on older studies (Kinkead *et al*., 1971), there was some evidence to suggest a possible effect of ENB on the thyroid gland. Thus, in more recently conducted studies on the repeated-exposure toxicity of ENB, provision was made for measurement of thyroid hormones and TSH in serum, and for detailed histology of the thyroid gland including morphometry (Ballantyne *et al*., 1997). In a National Toxicology Program (NTP) study on the subchronic inhalation toxicology of glutaraldehyde, additional monitors included sperm morphology, vaginal cytology and histoautoradiographic evaluation of respiratory tract cell replication rates in the nasal mucosal epithelium (NTP, 1993). These are but two examples, of many, in which additional monitoring was added to clarify any suspect toxicity. It is becoming more common for additional monitoring to be added to repeated-exposure studies to detect effects, some not anticipated, in order to take advantage of the exposure conditions. For example, some regulatory agencies now advise adding monitors for male fertility to repeated-exposure studies (e.g. sperm morphology, counts, motility). Thought should be given to the use of histochemical, ultrastructural, neurotoxic, respiratory function and genetic toxicology methods in repeated-exposure studies when considered appropriate and based on known or suspect toxicology.

Noninvasive procedures (e.g. observations for clinical signs, body weights, food and water consumption) should be conducted before exposure and then sequentially during the treatment and recovery periods. This allows for the detection of any general factors that may indicate the onset of toxicity, or effects secondary to factors not related to test-material dosing. Haematology, clinical chemistry and urinalysis need to be conducted as a minimum at the end of the dosing period. Where there are provisions for interim sacrifices, then chemical pathology should be undertaken at these times in order to detect any early changes. It may also be possible to collect blood and urine samples at interim periods during the dosing phase even if interim sacrifices are not planned. Decisions on whether to conduct chemical pathology on 'recovery' animals in the post-treatment survival groups may depend on whether there are definitive or equivocal changes in the immediate post-treatment

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sacrificed animals. Because of the automated nature of most chemical pathology investigations, it is recommended that measurements be undertaken on all control and treatment groups, and in the same period of time, to allow valid statistical comparisons. Observations for gross pathology at necropsy and removal of organs for weighing should be conducted on all animals. Organs and tissues showing gross pathological changes, together with those from a predetermined list, should be removed and processed for histological examination. The number of tissues removed will be determined, in part, by the reason for conducting the study and regulatory requirements, but in most cases it is advisable to remove as many as possible and at least keep those not to be examined in fixative in case of the need for examination of them in the future. It is usual in both subchronic and chronic studies to undertake light microscopic examination of all removed tissues from the high-dose and control groups. Decisions on whether to examine tissues from the lower-dose and recovery groups will depend on the findings from the control and high-dose groups.

5 SHORT-TERM REPEATED-EXPOSURE STUDIES

As noted previously, these studies extend over a period of 5–28 days. They are conducted to obtain an initial indication of the potential for adverse effects by recurrent exposure to the test material, and also as a preliminary to obtain guidance on appropriate doses for a subchronic study. For the latter purpose, it is common to use studies of 7–9 days duration. Since there is a greater likelihood for toxicity to be manifested with increasing time of exposure, 28-day studies may be accepted by some regulatory agencies as meeting the requirements for assessing non-oncogenic toxicity by repeated exposure. Detailed monitoring, equivalent to that for a subchronic study, should be incorporated into the 28-day protocol. However, even with the shorter periods, monitoring should ideally be more than signs, body weight, food and water consumption and gross pathology. There should, at least, be some limited haematology, clinical chemistry and histology. In this way a better indication of the potential for toxicity will be obtained, and permit a more reliable guidance for the design of subchronic studies. Detailed biochemical monitoring even of 7-day studies can yield significant information of relevance to target organ toxicity; for example, chloral hydrate in drinking water (Poon *et al*., 2000).

6 SUBCHRONIC STUDIES

These are usually carried out by exposure for 15–20% of the lifespan of the test species. Thus for most common laboratory animals they will be of about three months duration with respect to the dosing period. Such studies allow a determination of the potential non-oncogenic toxicity of the test material, allow the determination of definitive, threshold and no-effects dosages, including a MTD, and are essential for the successful planning of a chronic study. Many aspects of the design of these studies (species, housing conditions, mode of dosing, etc.) have been discussed earlier in this chapter.

Subchronic studies should have at least three test chemical treatment groups, together with untreated controls and, if necessary, vehicle controls. It is always advisable to add a recovery group of animals to determine the reversibility, or otherwise, of induced toxicity. The need for interim sacrifice or monitoring groups will be determined by the nature of the test agent and its known toxicology.

It is necessary to incorporate into the study design procedures that may permit the detection of unsuspected toxicity, latency to onset, and reversibility. Monitoring procedures should allow for the sensitive detection of early indications of toxicity, latency to toxicity, and reversibility of induced toxicity. They should allow for the detection of structural and functional indications of general toxic effects and of specific organ and/or tissue lesions. Standard lists of recommended procedures for monitoring are available in published texts and regulatory guidelines. However, investigators should add, as routine, methods usually conducted in follow-up tests to confirm specific toxic effects; for example, the inclusion of α_{2u}-globulin measurements in urine to biochemically confirm a hyaline droplet nephropathy.

Monitoring for subchronic studies should include the following to ensure the optimum conditions to detect non-oncogenic toxicity:

1. **Clinical observations:** Animals should be examined twice daily for overt signs of toxicity and for mortality, and examined in detail on a weekly basis with particular respect to skin and fur condition, eyes, mucosae, respiratory, circulatory and nervous system functions and general behaviour. These are standard to most profiles. Many subchronic studies do not have provision for detailed examination of the eye, and some ophthalmic toxicology may be overlooked. It is useful to have, as a minimum, expert examination of the eyes by an ophthalmic veterinarian using biomicroscopy at the start and end of dosing. Other detailed ophthalmic investigations are discussed in **Ophthalmic Toxicology** and by Peiffer *et al.* (2000). There is also frequent use of a functional observational battery (FOB) as a screen for the potential of a chemical to produce neurotoxicity (Eapen *et al*., 2007; see also **Neurotoxicology**, **The Role of Behavioural Toxicity in Risk Assessment**).

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2. **Body weight:** Body weights should be measured before dosing and at least weekly during the dosing and recovery periods. Body weight changes for the interweighing periods should be calculated. Body weight and body weight changes are sensitive indicators of the condition of the animals. Changes (usually loss) can be a consequence of alterations in food or water consumption, specific toxic effects or disease processes.
3. **Food consumption:** This is a useful monitor if measured over three-day or weekly intervals. Appropriate containers are needed to limit errors due to spillage. Food consumption data may be useful in the interpretation of body weight changes, and is of course essential if dosing is by incorporation in the diet in order to calculate consumed dosages.
4. **Water consumption:** This measurement, over three- or seven-day intervals, requires the use of individual water bottles for its determination. The information can be of considerable use in assessing body weight or urine volume changes.
5. **Haematology:** Details of the use and application of peripheral blood haematology tests are given in **Haematology and Toxicology**. The following are typical measurements undertaken:
   - haemoglobin concentration;
   - erythrocyte count;
   - packed red blood cell volume (PCV);
   - calculated values: mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC);
   - leukocyte count—total and differential;
   - platelet count;
   - reticulocyte count;
   - activated partial thromboplastin time.
6. **Biochemical disturbances:** For detecting general biochemical disturbances (e.g. electrolytes) or to indicate specific organ/tissue damage, a multiplicity of tests is available (see **Clinical Chemistry in Toxicity Testing: Scope and Methods**). The following measurements are typical for subchronic studies:
   - glucose
   - urea nitrogen
   - creatinine
   - total protein, albumin and globulin (often with protein electrophoresis)
   - bilirubin (total, conjugated and unconjugated)
   - phosphorus
   - Ca^{2+}, Na^{+}, K^{+}, Cl^{-}, HCO^{-}
   - aspartate aminotransferase
   - alanine aminotransferase
   - creatine kinase
   - sorbitol dehydrogenase
   - γ-glutamyl transferase
   - alkaline phosphatase
   - glutamate dehydrogenase
   - ornithine carbamyltransferase
   - special studies (e.g. with organophosphates, measurement of acetylcholinesterase and butyrylcholinesterase).
7. **Urinalysis:** Analysis of urine can yield valuable information not only on renal and urinary tract integrity, but also on acid–base balance and certain organ dysfunctions. Specimens should be collected separately, ideally using metabolic cages, over a specifically timed period (usually 16 hours). Typical measurements and observations include the following:
   - volume
   - appearance
   - microscopy
   - pH
   - osmolality
   - protein
   - occult blood
   - glucose
   - ketones
   - bilirubin and urobilinogen
   - creatinine
   - α_{2u}-globulin
   - N-acetyl-β-D-glucosaminidase.
8. **Necropsy and organ weights:** Necropsy examination with detailed inspection for gross pathology is required on all animals; this includes those dying on study and sacrificed survivors at the interim, immediate post-treatment and recovery periods. Organs removed for weighing usually include brain, liver, kidneys, lungs, heart, spleen, thymus, adrenal glands and gonads. Whilst organ weights relative to body weight are often calculated, interpretation of the results should be undertaken cautiously since if changes in body weights have occurred these relative organ weight values may be misleading. For this reason it is also advisable to calculate organ weights relative to brain weight, since this is less variable and test materials that lead to a change in body weight do not generally affect brain weight.
9. **Histology:** Light microscopic examination of all tissues and organs showing gross pathology should be undertaken. Many studies examine other (no gross pathology) tissues only from the high-dose and control groups. Should histopathology be seen in the high-dose group, it is then necessary that tissues from the other treatment groups should be examined. Decisions on the need to examine tissues from recovery group animals will depend on the findings from the immediate postdosing sacrifice group. It is wise to remove as many tissues and organs as possible and keep them in fixative. Those to be examined are listed in standard texts and regulatory guidelines, but investigators

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should carefully consider the needs for histology based on these and the nature of the study. Tissues not prepared for histology should be kept in fixative in case a subsequent need arises for their examination. Special staining techniques may be required based on the findings of the study overall (see **Pathological Techniques in Toxicology**); for example, myelin stain with suspect neurotoxicity, and Mallory Heidenhain stain for hyaline droplet nephropathy (Kim *et al*., 1997).

10. **Special studies:** Based on findings from prior well-conducted, short-term repeated-exposure studies, from other investigations on the test chemical, or structure–activity relationship (SAR) considerations, there may be a requirement for special investigations related to detection, quantitation, and mechanisms of morphological and/or functional elements of a specific nature (e.g. ocular toxicity) or of a system (e.g. neurotoxicity, immunotoxicity). Special investigations may also be determined by the exposure nature of the test material, such as route and/or physical nature. An example of the latter is in studies of the potential for adverse effects from inhalation exposure to particulates, which require not only special monitoring of the atmosphere for concentration and particle size, but also biological considerations such as pulmonary retention, determination of induced pulmonary injury, and functional changes in pulmonary physiology (Ballantyne, 2006). Retention and elimination of particulates can be undertaken chemically by sequential removal of lungs at various times and analysis for the specific material; this permits estimation of cumulative processes and elimination rates (Sun *et al*., 1987). Lung injury and inflammation can be estimated by biochemical and cytological studies on bronchoalveolar lavage (BAL) specimens (Duniho *et al*., 2002; Sciuto, 2006). Cytology includes macrophage and neutrophil quantitation, and biochemical measurements include lactate dehydrogenase, acid and alkaline phosphatase, β-D-glucuronidase, glutathione reductase, cathepsins B and D, and total protein. Developing pulmonary fibrosis can be followed histologically and/or by measurement of total lung tissue collagen and of BAL fluid hydroxyproline (Pickrell *et al*., 1983). Components of respiratory function tests that may be studied include compliance, total lung capacity (TLC), functional residual capacity (FRC), forced vital capacity (FVC), resistance, CO diffusion capacity, and intrapleural pressure (Kimmel, 2001; Sun *et al*., 1987). The selection of special studies required depends on the nature of the known or suspect toxicity, and can vary from the addition of a single chemical or physical monitor to a multiple series of additional procedures.

7 CHRONIC TOXICITY STUDIES

A chronic study is conducted with the objective of exposure to the test material for the lifespan of the test species, or a significant proportion of it. Therefore, with the small laboratory animals conventionally used, the dosing extends over a period of about two years. The majority of these studies are conducted with the objective of determining the carcinogenic potential of the test material, and only in a relatively few cases is it necessary to extend a subchronic study into a chronic study based on the non-oncogenic toxicity potential of the test material. Nevertheless, most chronic studies are carried as combined chronic toxicity and carcinogenicity studies, since this allows a more definitive assessment of threshold and no-effects levels for known non-oncogenic effects. In this respect they give a more reliable estimate of safety margins in risk assessment procedures and the assignment of permissible exposure levels where there is long-term human, but controllable, exposure; for example industrial chemicals, pesticides, food additives and food contaminants. With respect to determining the carcinogenic potential of a material, this may be exhibited in one of several ways, or a combination of them. These include:

- a direct comparison with the controls
- a decrease in latency to occurrence of tumours also found in the controls
- an increased incidence of tumours of the types seen in the controls
- the development of tumours not seen in controls
- an increase in the multiplicity of tumours in individual animals.

Dose–response relationships and statistical significance compared with the untreated controls are important interpretive considerations. Based on the results of long-term carcinogenicity bioassays there is evidence that developmental combined with adult exposure to carcinogens produces an overall increase in the incidence of malignant neoplasms and an increased incidence of specific neoplasms related to exposures to given carcinogen (Soffritti *et al*., 2008). This has led to proposals that carcinogen bioassays should start with prenatal exposures. Additionally, it has been noted that in conventional chronic oncogenicity bioassays there is a progressive decrease in survival and that this can result in too few surviving animals at terminal sacrifice to permit adequate pathological evaluations. There have thus been proposals that the duration of chronic studies should be shortened to allow a larger number of surviving animals for pathological and other evaluations. However, investigations by Kodell *et al*. (2000) have shown that stopping chronic studies at 18 months is associated with a substantial and unacceptable reduction in the statistical power to detect chemical carcinogens. The majority of combined

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oncogenicity/chronic toxicity studies are still of two years duration with terminal sacrifices at 24 months.

A decision to conduct a chronic study clearly requires a detailed analysis of the need for, and advantages of, the study. This will include considerations on the use and exposure pattern of the test material, findings from other toxicology studies, known dose–response relationships and their relevance to in-use situations, and the demands of regulatory agencies. For example, a material of well-established non-oncogenic toxicity potential with positive findings in genetic toxicology studies will have a higher priority for chronic testing than a material of known non-oncogenic toxicity potential but shown not to be mutagenic or clastogenic in a number of genetic toxicology studies. In view of the duration, labour-intensive nature and high cost of chronic toxicology studies, it is of the greatest importance that they be planned in fine detail to ensure that the objectives for conducting the study are met, including monitoring procedures, and that there is the maximum likelihood for survival over the test period of those animals not affected by known toxicity (e.g. detailed considerations on animal welfare standards and the exclusion of factors that would cause morbidity and mortality for reasons unconnected with the test material). The widest representation of views should be obtained in the planning phase, including those of pathologists and statisticians, and with possible discussions involving the relevant regulatory agencies (national and international). As stated by Stevens and Mylecraine (1994): 'Doing the study right therefore requires that we concern ourselves with the demands of science and society—they need not be mutually exclusive.'

Detailed considerations for the conduct of chronic toxicology studies have been given (WHO, 1978; Stevens and Mylecraine, 1994). There should be at least three treatment groups and a control group. The control group should be at least equal in size to that of one of the treatment groups. Some investigators have used two (separate) control groups against which statistical comparisons can be made. In many cases the use of two control groups, with separate statistical evaluation, has led to interpretive complications due to, at least in part, random differences in tumour and non-oncogenic pathology incidence between the two control groups. It may be better, for comparative statistical purposes, to have a large-size single control group, possibly equal in numbers to the combined treatment groups. Essential to a study of latency is the use of several interim sacrifice groups; as a minimum these should be at 6, 12 and 18 months. However, others suggest a larger number of interim sacrifices, starting at 3 months. In this manner a comparison can be made between the first interim sacrifice and the findings from a previously conducted subchronic study, with respect to the quantitative aspects of the toxicity potential of the test material. Group size should be adequate for both survival considerations and subsequent detailed statistical analyses. At least 20 per sex per group should be used for the final sacrifice (two year) group. Dose selection is usually based on an MTD defined by the subchronic study, which requires careful interpretation against the objectives and conditions for the chronic study. Monitoring for non-oncogenic end points is normally at least the same as for the subchronic study, but with additional procedures based on the findings from the subchronic study, and other known toxicology.

The results from repeated-exposure studies clearly require to be interpreted in terms of metabolic and pharmacokinetic considerations, such as incremental increases in body or organ/tissue load required to initiate a toxic process, biostorage of parent material and/or metabolites and their rate of release from storage sites, development of repair processes, and the balance of induced metabolic activation and detoxification mechanisms. The mode of dosing may influence the toxic response, including route of exposure which will determine the overall rate of absorption and the potential for relative and absolute metabolic activation and detoxification. Also, the pharmacokinetic characteristics and potential for toxicity may depend on whether dosing is by repeated boluses (e.g. gavage) or continuous administration (e.g. in drinking water or food). With the former, intermittent fluctuating high body loading is likely to be attained, and retention of parent material and metabolites will depend on their route(s) and rate of excretion. With sustained dosing, the attainment of a body and tissue/organ load to initiate and sustain a toxic process will be determined by the dynamic equilibrium between absorption, biodistribution, storage and excretion. The same daily dose delivered by bolus or alternatively by sustained dosing may result in differences in the quantitative expression of toxicity, and in some cases the nature of the toxic response. Additionally, and in contrast with acute studies, with the longer-term repeated-exposure studies there may be changing tissue sensitivity with increasing age, altered physiological and metabolic capacities, and the development of spontaneous disease processes. Thus, the interpretation of the findings from long-term repeated-exposure studies should be against not only the apparent toxicity produced, but also against the possible influence of various changing biological parameters.

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Biotransformation of Xenobiotics

John A. Timbrell and Timothy C. Marrs

CONTENTS

1 INTRODUCTION

Xenobiotics which are absorbed into biological systems by passive diffusion across membranes are usually lipid soluble and consequently not ideally suited for excretion. For example, very lipophilic substances, such as the polychlorinated biphenyls are very poorly excreted and, hence, may remain in the mammalian body for many years. After a xenobiotic has been absorbed into a biological system, it may undergo biotransformation to products which are rapidly excreted and therefore elimination of the compound from the animal is facilitated. However, biotransformation may also change the biological activity of the substance. Hence, the metabolic fate of the compound can have an important bearing on its toxic potential, the disposition of the compound in the body and the excretion of the compound.

The products of metabolism are usually more water soluble than the original compound. Indeed, in animals, biotransformation seems directed at increasing water solubility and, hence, excretion. For example, the analgesic drug paracetamol (acetaminophen) has a renal clearance value of $12\,ml\,min^{-1}$, whereas one of its major metabolites, the sulfate conjugate, is cleared at the rate of $170\,ml\,min^{-1}$. Facilitating the excretion

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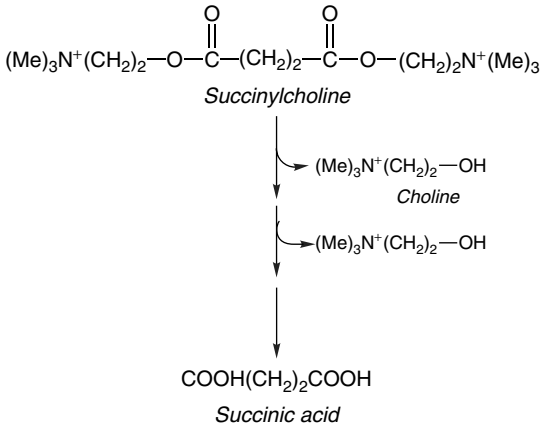

Figure 1 Metabolic hydrolysis of succinylcholine.

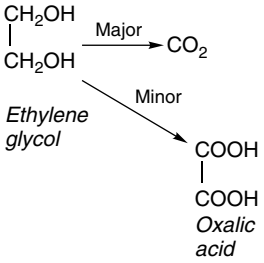

Figure 2 Metabolism of ethylene glycol.

Table 1 The major biotransformation reactions

Phase 1		Phase 2
Oxidation	Aromatic	Sulfation
	Aliphatic	
	Heterocyclic	Glucuronidation
	Alicyclic	
	Of nitrogen	Glutathione conjugation
	Of sulphur	
	N-Hydroxylation	Acetylation
	Dealkylation	Amino acid conjugation
Reduction	Azo	Methylation
	Nitro	
Hydrolysis	Ester	
	Amide	
	Hydrazide	
	Carbamate	
Hydration		
Dehalogenation		

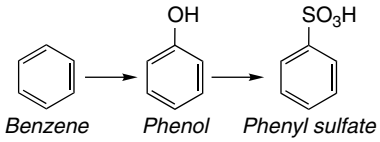

Figure 3 Benzene metabolism.

of a compound means that its biological half-life is reduced and, hence, potential for toxicity is kept to a minimum. Metabolism may also affect the biological activity of a foreign compound. For example, the drug succinylcholine causes muscle relaxation, but its action lasts only a few minutes because metabolism cleaves the molecule to yield inactive products (**Figure 1**). However, in some cases metabolism increases the toxicity of a compound. There are many examples of this (see below) which have been documented, but a relatively simple case is ethylene glycol. This is metabolized to oxalic acid, which is partly responsible for several of the toxic effects (**Figure 2**). Biotransformation is therefore an extremely important phase of disposition, as it may have a major effect on the biological activity of the compound, and by increasing polarity and so water solubility, thereby increase excretion.

Rarely, metabolism may decrease water solubility and so reduce excretion. For example, acetylation decreases the solubility of some sulfonamides in urine and this may lead to crystallization of the metabolite in the renal tubules, causing tissue necrosis.

Very many different reactions and pathways can be involved in biotransformation (**Table 1**). The processes can generally be simply divided into Phases 1 and 2, although further metabolism of conjugates has been termed Phase 3. However, this terminology is becoming unfashionable amongst some toxicologists. Phase 1 is the alteration of the original foreign molecule so as to produce a functional group which can then be conjugated in Phase 2. This can best be understood by examining the example in **Figure 3**. The xenobiotic is benzene, a highly lipophilic molecule which is not readily excreted from the animal except, as it is volatile, in the expired air. Phase 1 metabolism converts benzene into a variety of metabolites, but the major one is phenol. The insertion of a hydroxyl group allows a Phase 2 conjugation reaction to take place with the polar sulfate group being added. Phenyl sulfate, the final metabolite, is very water soluble and is readily excreted in the urine. Some foreign molecules, such as phenol, already possess functional groups suitable for Phase 2 reactions and therefore simply undergo a Phase 2 reaction.

Biotransformation is almost always catalysed by enzymes and these are usually, but not always, found most abundantly in the liver in animals. The reason for this location is that most foreign compounds enter the body via the gastrointestinal tract and the portal blood supply from this organ goes directly to the liver (**Figure 4**). However, it is important to remember that: (i) the enzymes involved with the metabolism of foreign compounds may be found in many other tissues as well as the liver (Krishna and Klotz, 1994); (ii) the enzymes may be localized in one particular cell type in an

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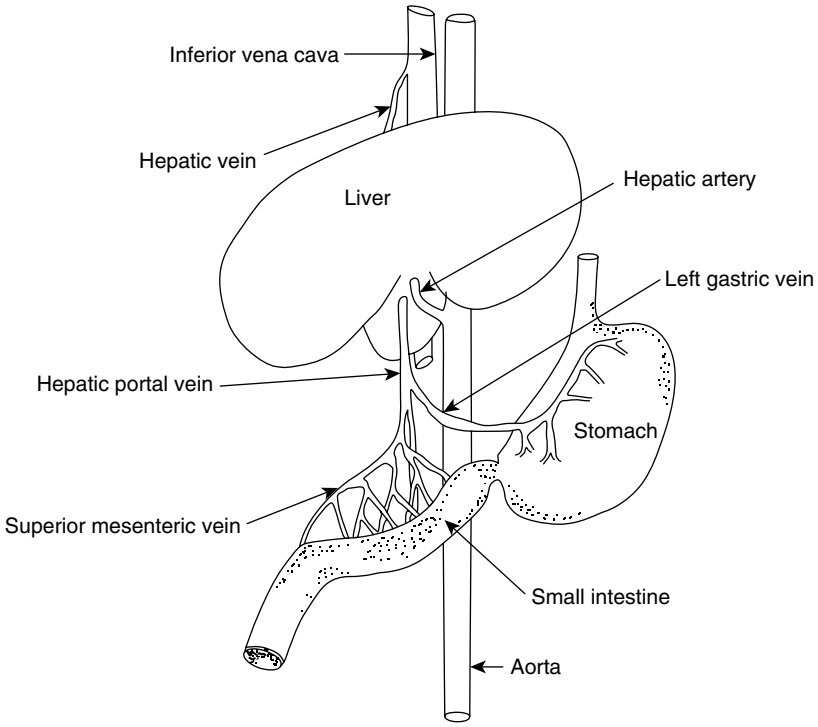

Figure 4 Blood supply to the liver. (Drawn by C. J. Waterfield.)

organ; (iii) unlike the enzymes involved in intermediary metabolism, those involved in the biotransformation of xenobiotics are generally nonspecific and consequently are not always very efficient; (iv) enzymes normally involved in intermediary metabolism may catalyse the biotransformation of a xenobiotic if the chemical structure happens to be suitable and (v) a xenobiotic may undergo many different biotransformations, and the relative importance of each of these may be affected by many factors.

The enzymes involved in biotransformation also have a particular subcellular localization: many are found in the endoplasmic reticulum, some are located in the cytosol and a few are found in other organelles such as the mitochondria.

This chapter focusses on biotransformation in mammals, with particular reference to humans. However, it is important to remember that humans can be exposed to the products of biotransformation in other organisms. For example, in products derived from plants to which agrochemicals, especially pesticides, have been applied, plant metabolites of these products may have been generated and these may be found in food. The same is true of veterinary medicines and animal products: in the case of mammals, metabolism is often, but not always, similar to that in humans. In poultry and fish products, consideration of avian and piscine biotransformation products may be important. In environmental toxicology, metabolism in a wide array of organisms is important and may result in human exposure and, of course, bacterial metabolism in bacteria is important in the mammalian gut.

2 PHASE 1 REACTIONS

2.1 Oxidation Reactions

The majority of oxidation reactions which xenobiotics undergo are catalysed by one enzyme system, the cytochrome P450 mono-oxygenase system (Guengerich, 1991; 1994; 2007; 2008). However, there are a number of other oxidative enzyme systems whose importance in the biotransformation of xenobiotics is increasingly being recognized, and these will be discussed later in this chapter.

2.1.1 Cytochrome P450

Cytochrome P450 is a membrane-bound enzyme system located primarily in the smooth endoplasmic reticulum of the cell, although some forms of cytochrome P450 may be located in other organelles such as the mitochondria. These enzyme systems are called cytochromes P450 because they are coloured proteins, which absorb light at wavelengths near 450 nm when the haem iron is reduced (usually with sodium dithionite) and complexed with carbon monoxide. After homogenization and fractionation of the cell, the enzyme system is isolated in the microsomal fraction. Frequently nowadays, cytochromes

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P450 are known as CYPs and they are found in all forms of life. For a discussion of the evolutionary aspects of CYPs see Lewis and Ito (2008).

In mammals, the liver has the highest concentration of CYPs, although they can be found in kidney, lung, adrenal, gonads, brain, skin and most other tissues and organs (Seliskar and Rozman, 2007). The reactions catalysed by cytochromes P450 also require NADPH and molecular oxygen and the overall reaction is

$$SH + O_2 + NADPH + H^+ \longrightarrow SOH + H_2O + NADP^+$$

where S is the substrate.

The sequence of metabolic reactions is shown in **Figure 5** and involves six distinct steps: (1) addition of substrate to the enzyme; (2) donation of an electron; (3) addition of oxygen and rearrangement; (4) donation of a second electron and (5) loss of water. These steps are followed by (6) loss of the oxidized substrate.

The cytochrome P450 system is actually a collection of enzymes, all of which possess an iron atom in a porphyrin complex. These catalyse different types of oxidation reactions and under certain circumstances may catalyse other types of reaction, such as reduction. There are three main gene families important in xenobiotic metabolism: CYP1, CYP2 and CYP3. CYP4 is involved in fatty acid metabolism. Within these there are subfamilies. These may be further divided into genes coding for single distinct enzyme proteins such as CYP1A1 and CYP1A2 (Nelson *et al*., 1993). Thus with CYP1, there are three subfamilies and three genes and one pseudogene (CYP1A1, CYP1A2, CYP1B1) and with CYP2, 13 subfamilies, 16 genes, 16 pseudogenes (CYP2A6, CYP2A7, CYP2A13, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP2F1, CYP2J2, CYP2R1, CYP2S1, CYP2U1, CYP2W1). With CYP3, there are one subfamily, four genes, two pseudogenes (CYP3A4, CYP3A5, CYP3A7, CYP3A4). For a list of CYPs see Nelson (2007).

One important feature of the cytochrome P450 enzyme system is its broad and overlapping substrate specificities, which reflect the enormous variety of chemicals that may be potential substrates. Furthermore, one substrate may be metabolized to more than one product by different

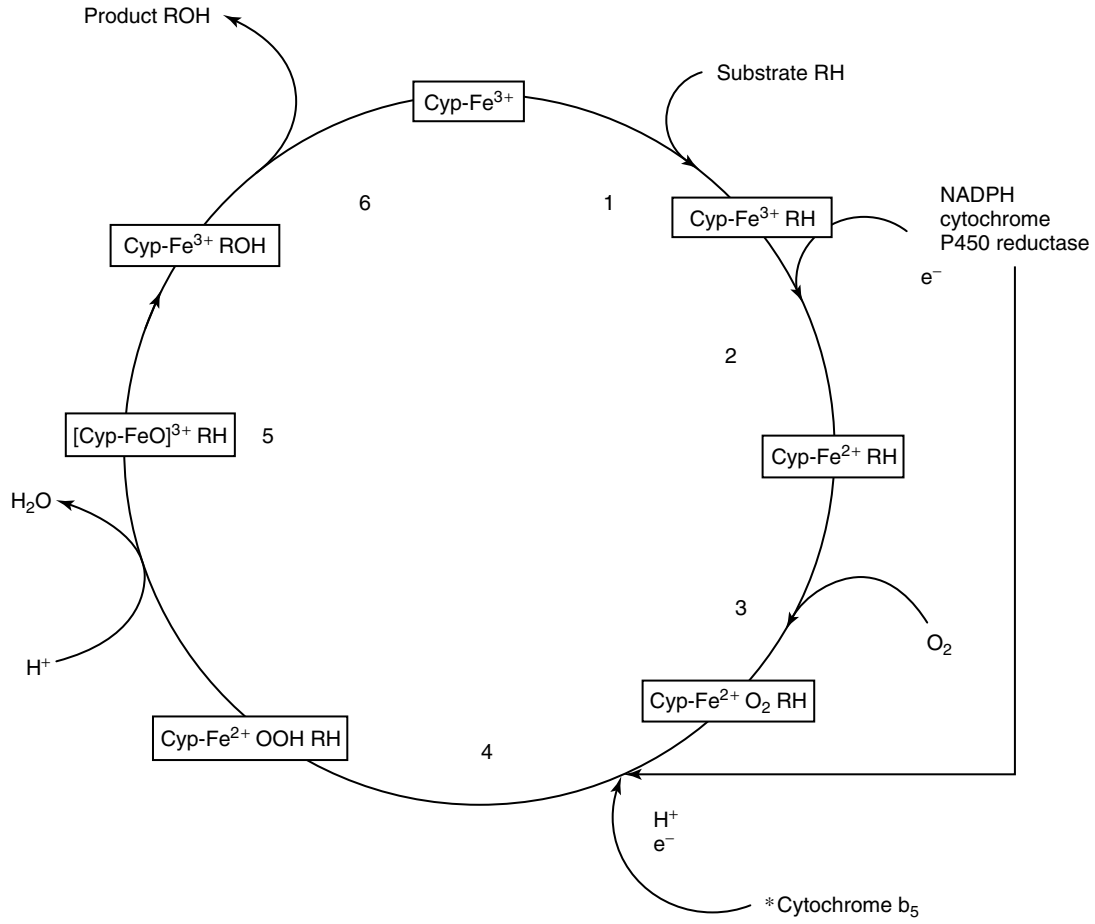

Figure 5 The catalytic cycle of the cytochrome(s) P-450 mono-oxygenase (mixed function oxidase) system. (Adapted from Parkinson A. (2001). Biotransformation of xenobiotics in Klaassen, C. D. (Ed). Cassarett and Doull's Toxicology, 6th Edition, McGraw Hill, Chapter 6. Cyp:- cytochrome P450.)

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forms of cytochrome P450. For example, the drug propranolol can be metabolized by CYP2D6 and CYP2C19 to 4-hydroxypropranolol and naphthoxylacetic acid, respectively. Sometimes the same form of cytochrome P450 may metabolize one drug to more than one product. For example, the drug methoxyphenamine can be metabolized by CYP2D6 either by *O*-demethylation or hydroxylation at the 5-position.

Another important feature of the CYP enzyme system is its inducibility (Okey *et al*., 1986; Whitlock, 1989; Okey, 1990; Batt *et al*., 1992; Pelkonen *et al*., 2008). Thus, treatment of an animal with certain substances may lead to an increase in the synthesis of one or more isoenzymes of cytochrome P450, leading to an apparent increase in overall activity with respect to a particular substrate. There are now a large number of known inducers of different isoenzymes of cytochrome P450. Exposure of an animal to these substances clearly may have an effect on the metabolism of a compound and can influence its toxicity (this is discussed in more detail later in the chapter).

The major types of oxidation reaction catalysed by the cytochrome P450 system may be subdivided into aromatic hydroxylation of carbon, aliphatic hydroxylation of carbon, alicyclic hydroxylation of carbon, heterocyclic hydroxylation, epoxidation, N-, S- and O-dealkylation, N-oxidation, N-hydroxylation, S-oxidation, desulfuration, dehydrogenation, cleavage of esters and deamination.

2.1.1.1 Aromatic Hydroxylations

Aromatic hydroxylation, such as occurs with benzene (**Figure 6**), is a very common reaction for compounds containing an unsaturated ring. The initial products are phenols, but catechols, quinols and further hydroxylated products may be formed. One of the toxic effects of benzene is aplastic anaemia. This is believed to be due to an intermediate metabolite, possibly the hydroquinone, which may be formed in the bone marrow, the target site. Aromatic hydroxylation usually proceeds via an epoxide intermediate, also called an oxirane ring or arene oxide (**Figure 7**), which involves the addition of oxygen across the unsaturated double bond. The formation of this intermediate may have important toxicological implications, for example the hepatotoxicity of bromobenzene (discussed in detail later in the chapter). Epoxides are often chemically reactive and fairly unstable. They may give rise to positively charged intermediates (electrophiles). The products formed *in vivo* will depend on the reactivity of the particular epoxide and they therefore may form a number of other metabolites, either phenols by chemical rearrangement or, following further metabolism, dihydrodiols, glutathione conjugates and catechols. Destabilized epoxides, for example, will tend to form phenols by chemical rearrangement.

The reactivity of metabolic intermediates may well determine the toxicity of the compound in question.

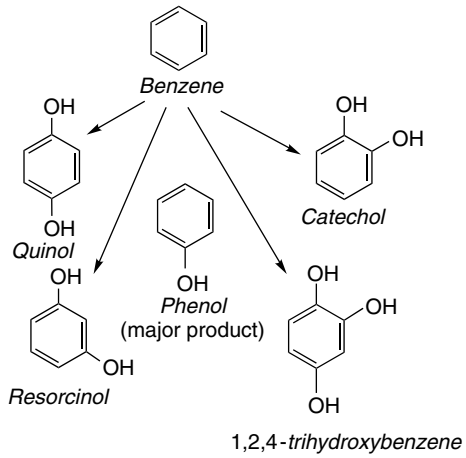

Figure 6 Routes of hydroxylation of benzene.

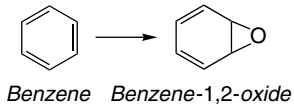

Figure 7 Oxidation of benzene to an epoxide.

However, extremely reactive intermediates are likely to react with many cellular constituents in the close proximity of their formation. Less reactive intermediates may travel to distant sites within or even outside the metabolizing cell and react with more critical cellular targets (Monks and Lau, 1988; Nelson and Pearson, 1990) (see below). Also, aromatic hydroxylation is influenced by the substituents on the ring. Thus, a nitro or other electron-withdrawing group will tend to direct hydroxylation to the *meta* and *para* positions, whereas an electron-donating group such as an amino group will be *ortho*- and *para*-directing.

2.1.1.2 Aliphatic Hydroxylations

Hydroxylation of unsaturated aliphatic compounds may also proceed with the formation of an epoxide across the unsaturated bond. For example, the toxic industrial intermediate vinyl chloride (**Figure 8**) undergoes such a biotransformation catalysed by CYP2E1 to yield chloroacetaldehyde. When the epoxide opens, there may be a shift in the chlorine atom to the adjacent carbon (Hathway, 1984). The active intermediate chloroethylene oxide, which reacts with nucleic acid bases, is thought to be the cause of the mutagenic and carcinogenic potential of vinyl chloride (Bolt, 2005; Bolt and Roos, 2008). Hydroxylation of a saturated aliphatic moiety, such as that in propylbenzene may occur at one of three positions (**Figure 9**). Further metabolism will yield the aldehyde and then the acid from the 3-phenylpropan-l-ol. These further biotransformations may be catalysed by enzymes other than cytochrome P450. Fatty acids such as lauric

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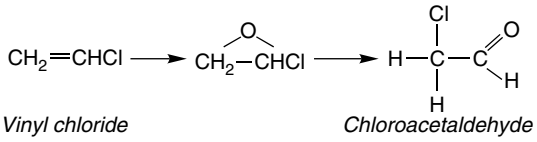

Figure 8 Oxidation of vinyl chloride to an epoxide.

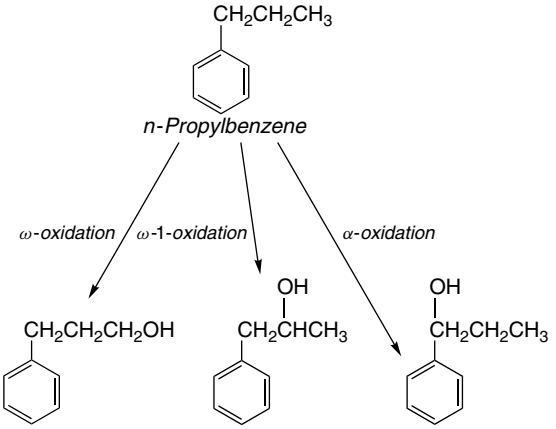

Figure 9 Hydroxylation of propylbenzene.

acid are hydroxylated at the terminal (ω) and penultimate (ω-1) carbon (Shet *et al*., 1996).

2.1.1.3 Alicyclic Hydroxylations

Alicyclic rings may also undergo hydroxylation catalysed by cytochrome P450. For example, cyclohexane may be oxidized to cyclohexanol and then further to the *trans*-l,2-diol. Similarly, heterocyclic rings, such as that in the drug hydralazine may be hydroxylated (see **Figure 19** below). However, other enzymes may also be involved in this type of oxidation, such as the xanthine oxidases (see below).

2.1.1.4 N-, S- and O-dealkylations

Alkyl groups attached to N, O or S atoms are removed by dealkylation reactions, which involve oxidation of the alkyl group. The intermediate hydroxyalkyl compound may be unstable and rearrange with loss of the respective aldehyde (**Figure 10**). Sometimes stable hydroxyalkyl products are produced, for example when dimethylformamide undergoes metabolism (**Figure 11**). S-dealkylation may also involve a factor from the cytosol and so may not be a straightforward microsomal reaction. The chain length will have a bearing on whether dealkylation or oxidation of the alkyl group occurs. The longer the chain length, the more likely is oxidation of the terminal carbon atom to occur.

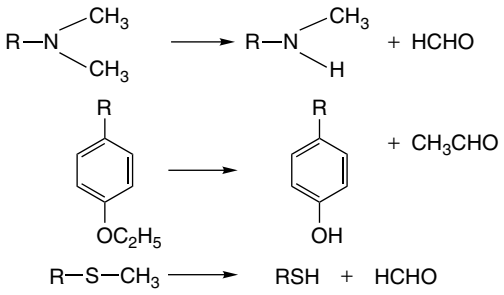

Figure 10 Dealkylation reactions.

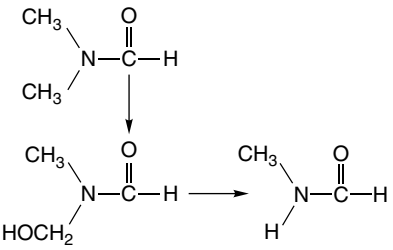

Figure 11 Dealkylation of dimethylformamide via a stable hydroxymethyl intermediate.

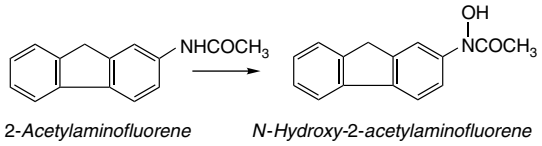

Figure 12 N-Hydroxylation of acetylaminofluorene.

2.1.1.5 N-hydroxylation, S-oxidation, Deamination and so on

Nitrogen atoms in primary arylamines, arylamides and hydrazines may undergo hydroxylation, which is catalysed by cytochrome P450 (**Figure 12**). This reaction may result in a metabolic activation and so be responsible for toxicity (see also below). Nitrogen atoms in xenobiotics may also be oxidized by other oxidases (see below).

Sulphur atoms can also be oxidized by microsomal mono-oxygenases to yield S-oxides and sulfones, such as in the pesticide aldicarb (**Figure 13**). Sulphur atoms may also be removed oxidatively and replaced by oxygen, such as in the metabolism of the insecticide parathion (**Figure 14**). This is a very important general activating pathway for insecticides containing a P=S bond (phosphorothionates); the phosphorothionates have very low anticholinesterase activity, whereas the equivalent phosphates (oxons) are very toxic. Good examples include malathion (FAO/WHO, 1998), chlorpyrifos and fenitrothion (FAO/WHO, 2000), which are converted to their oxons, respectively malaoxon, chlorpyrifos oxon and fenitrooxon. Buratti *et al*. (2005) found that malaoxon formation was catalysed by CYP1A2 and, to a lesser

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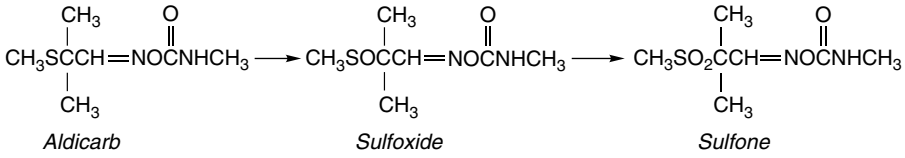

Figure 13 Oxidation of the pesticide aldicarb (Temik) to sulfoxide and sulfone metabolites.

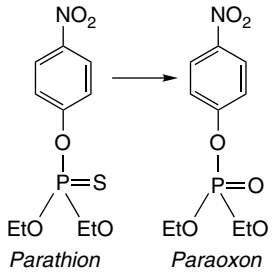

Figure 14 Oxidative desulfuration of parathion.

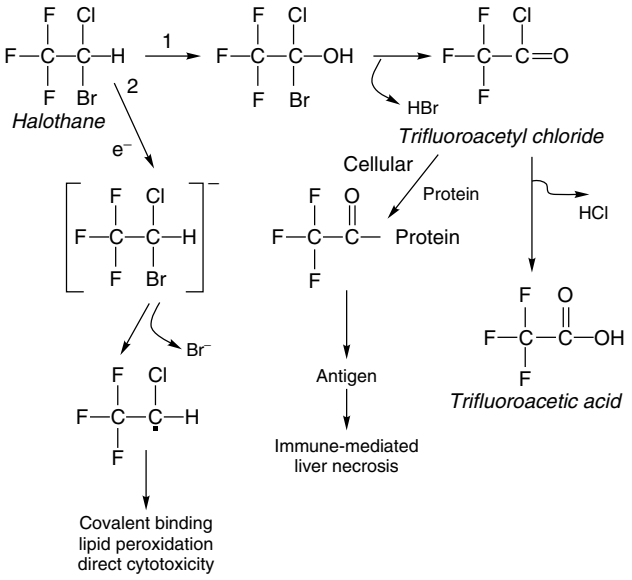

Figure 15 Metabolism of the anaesthetic halothane showing both oxidative (1) and reductive (2) pathways and their role in toxicity.

extent, CYP2B6, whereas CYP3A4 was only important at high malathion levels. Similarly, halogen atoms may be removed via oxidative reactions, as shown for the metabolism of the anaesthetic halothane to trifluoroacetic acid (**Figure 15**).

Amine groups can also be removed oxidatively via a deamination reaction, which may be catalysed by cytochrome P450. For example, in the rabbit, amphetamine is metabolized in this way to phenylacetone (**Figure 16**). However, the initial attack is probably on the carbon atom to yield a carbinolamine, which can rearrange to the ketone with loss of ammonia, as

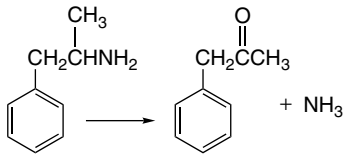

Figure 16 Deamination of amphetamine.

shown. Alternatively, the reaction may proceed via phenylacetone oxime, which has been isolated as a metabolite and for which there are several possible routes of formation. The phenylacetone oxime is hydrolysed to phenylacetone. N-Hydroxylation of amphetamine is also believed to take place and may also give rise to the observed metabolite, phenylacetone. The mechanism underlying the oxidative deamination of amphetamine has been a source of speculation and controversy, but it illustrates that there may be several routes of biotransformation to yield a particular metabolite (Gorrod and Raman, 1989; Kraemer and Maurer, 2002).

2.1.2 Non-Cytochrome P450-Dependent Oxidations

Certain oxidation reactions are catalysed by enzymes other than the cytochrome P450 mono-oxygenase system, such as the microsomal flavin, flavin adenine dinucleotide (FAD)-containing mono-oxygenases (Damani, 1988; Ziegler, 1993; Benedetti and Dostert, 1994; Cashman, 1995). This is responsible for the N-oxidation of tertiary amines such as dimethylaniline and trimethylamine (**Figure 17**). Another example is the N-oxidation of the pyrrolizidine alkaloid senecionine, found in common ragwort (*Senecio jacobaea*). In guinea-pig liver, lung and kidney, oxidation of senecionine is carried out largely by FAD-containing mono-oxygenase (Miranda *et al.*, 1991). The enzyme requires NADPH and molecular oxygen, and proceeds in six steps. Substrate specificity also includes secon-

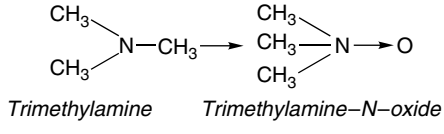

Figure 17 Oxidation of trimethylamine.

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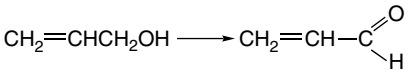

Figure 18 Oxidation of allyl alcohol.

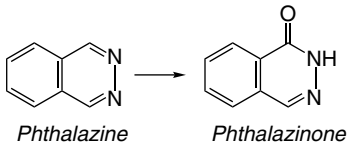

Figure 19 Oxidation of the hydralazine metabolite phthalazine.

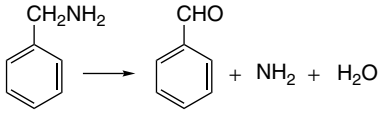

Figure 20 Oxidation of benzylamine by monoamine oxidase.

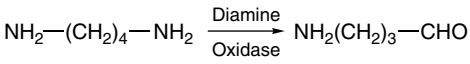

Figure 21 Oxidation of putrescine by diamine oxidase.

dary amines and sulfides, thioethers, thiols and thiocarbamates, and even organophosphates (OPs).

Alcohols, both aliphatic and aromatic, may be oxidized by alcohol dehydrogenase (ADH) (**Figure 18**). This cytosolic enzyme requires NADH. The products from primary alcohols are aldehydes, whereas ketones are formed, more slowly, from secondary alcohols. The oxidation of an alcohol may result in a toxic metabolite such as that from allyl alcohol, where acrolein is formed (**Figure 18**). Increased ADH activity is thus associated with enhancement of allyl alcohol hepatotoxicity (Rikans and Moore, 1987). A microsomal ethanol-metabolizing system has also been demonstrated. This is known to be involved in the metabolism of ethanol and methanol. Aldehydes may be further oxidized to acids by aldehyde dehydrogenase (ALDH), another enzyme which requires NAD (see review by Marchitti *et al*., 2008). There are both cytosolic and mitochondrial ALDHs, and some people of eastern Asiatic descent have a dominant mutation in one of their gene for the mitochondrial ALDH2, making the enzyme less effective. In those affected, acetaldehyde accumulates after drinking alcohol, leading to symptoms such as flushing of the skin, and increased heart and respiration rates (Xiao *et al*., 1996).

Other enzymes may also be involved in the oxidation of aldehydes, such as aldehyde oxidase and xanthine oxidase. These are cytosolic enzymes which contain molybdenum and require flavoprotein cofactors (Beedham, 1988; Garattini *et al*., 2008). There is some evidence that aldehyde oxidase (EC 1.2.3.1) is the same as the 'neonicotinoid nitroreductase' which reduces the neonicotinoid insecticide imidacloprid, at its nitroguanidine moiety (Dick *et al*., 2005; 2007). Xanthine oxidase also catalyses the oxidation of nitrogen heterocycles, such as the purine, hypoxanthine, and also of phthalazine to phthalazinone (**Figure 19**). Aldehyde oxidase will also catalyse the latter reaction. Some amines, such as tyramine, are substrates for the monoamine oxidases, which are mitochondrial enzymes found in a variety of tissues, including the liver. The oxidative deamination of amines via the monoamine oxidases yields an aldehyde product (**Figure 20**). The reaction to cheese with monoamine oxidase inhibitor therapy is discussed in Section 5.2. There are other circumstances whereby the action of monoamine oxidases may give rise to toxic products, such as acrolein in heart tissue produced by the oxidation of allylamine (Biagini *et al*., 1990; Sklar *et al*., 1991). Diamines, such as putrescine and histamine, are metabolized by the soluble enzyme diamine oxidase to dialdehyde and monoaldehyde products (**Figure 21**). Secondary and tertiary amines are less readily oxidized by these enzymes, dealkylation to the primary amine being a preferred pathway (see review by Sessa and Perin, 1994).

Another group of enzymes which are involved in the oxidation of xenobiotics are the peroxidases (Eling *et al*., 1983; Larsson *et al*., 1988). There are a number of these enzymes in mammalian tissues: prostaglandin synthase, found in kidney, lung, intestine and spleen; lactoperoxidase, found in mammary glands; myeloperoxidase, found in the liver Kupffer cells and neutrophils, where it is thought to be responsible for the oxidation of drugs such as hydralazine and procainamide (Uetrecht, 1992). The enzyme is also found in bone-marrow cells, where it may be involved in the metabolic activation of benzene metabolites and therefore in the toxicity of benzene (see reviews by Smith, 1996; Ross, 1996). Uterine peroxidase has been suggested as being involved in the metabolic activation and toxicity of diethylstilbestrol (Metzler, 1984). The overall peroxidase-catalysed reaction may be summarized as follows:

$$\text{peroxidase} + H_2O_2 \longrightarrow \text{compound I}$$

$$\text{compound I} + RH_2 \longrightarrow \text{compound II} + \bullet RH_2^+$$

$$\text{compound II} + RH_2 \longrightarrow \text{peroxidase} + \bullet RH_2^+$$

Among the most important of these enzymes are the prostaglandin synthases (see review by Degen *et al*., 2002). These are known to catalyse the oxidation of *p*-phenetidine, a metabolite of the drug phenacetin, a process which may be involved in the nephrotoxicity of the drug. The prostaglandin-synthase-catalysed oxidation of this compound gives rise to free radicals which may be

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responsible for binding to DNA (Andersson *et al*., 1982). Horseradish peroxidase will also catalyse the oxidation of this compound (Fischer *et al*., 1986).

2.2 Reduction

Reduction may be catalysed by either mammalian microsomal or cytosolic reductases and by the gut bacteria, which also possess reductases. The latter are clearly important for substances taken orally, where contact with gut microflora is likely. Apart from the gut flora, azo and nitro reduction are catalysed by enzymes in the liver, cytochrome P450 or NAD(P)H quinone oxidoreductase (DT diaphorase). Reduction takes place under anaerobic conditions and utilizes NADH or NADPH. FAD may also be involved, possibly as a nonenzymic electron donor. The most commonly encountered types of reductive reaction are the reduction of nitro and azo groups (see review by Zbaida, 2002). However, aldehydes, ketones, disulfides, sulfoxides, quinones, N-oxides, alkenes, halogenated hydrocarbons and certain metals may also be reduced *in vivo*. The reduction of azo groups such as those present in the drug prontosil (**Figure 22**), often in gut bacteria, is a two-step reaction involving first reduction to a substituted hydrazine, followed by a second reduction and cleavage to yield the amine (Gingell and Bridges, 1973). Reduction of azo dyes can give rise to aromatic amines of increased toxicity.

Reduction of nitro groups is an important route of biotransformation, such as for the compound nitrobenzene (**Figure 23**). Again this involves several steps, producing first a nitroso derivative, then a hydroxylamine and finally an amine. Reduction of nitro groups may be associated with toxicity. Nitrobenzene is haematotoxic in rats when given orally, causing methaemoglobinaemia and haemolysis (see review by Beauchamp *et al*., 1982), whereas given intraperitoneally or to rats without gut flora it is devoid of this toxicity (Reddy *et al*., 1976). This is due to reduction of the compound in the gut to yield the nitroso and hydroxylamine metabolites, which are responsible for toxicity to haemoglobin. Another example is nitroquinoline N-oxide, which is reduced to a hydroxylamine (**Figure 24**), which is believed

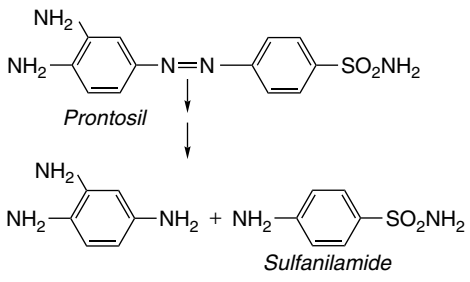

Figure 22 Reduction of the azo bond in prontosil.

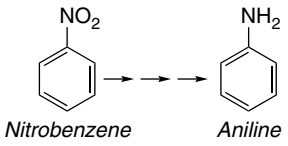

Figure 23 Reduction of nitrobenzene.

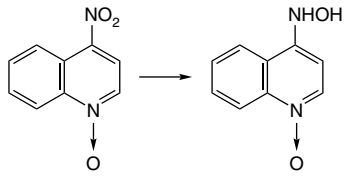

Figure 24 Reduction of the carcinogen nitroqinoline-*N*-oxide.

to be carcinogenic (see review by Bartsch, 1981). Less common reduction reactions include reduction of aldehyde and keto groups, epoxides and double bonds, and may involve other enzymes such as ADH and carbonyl reductase, both of which are found in the liver cytosol, as well as other tissues. Reduction of quinones involves DT diaphorase (quinone reductase Type 1) and disulfides, glutathione reductase and glutathione (see Dinkova-Kostova and Talalay, 2000; Watanabe *et al*., 2004).

Reductive dehalogenation, catalysed by the microsomal enzymes, may also occur in the metabolism of halogenated compounds, such as carbon tetrachloride and the anaesthetic halothane (see **Figure 40** below and **Figure 15**) (Raucy *et al*., 1993). The reductive route is believed to be responsible for the acute toxicity of halothane in rats when the oxygen tension is low. The reactive radical metabolites produced by the reductive pathway will bind covalently to liver protein and this may lead to toxicity. However, the oxidative pathway is probably more important in man under normal conditions of anaesthesia. This also gives rise to a reactive metabolite, trifluoroacetyl chloride (Kenna *et al*., 1988). Both the oxidative and reductive pathways involve cytochrome P450. In the reductive mode, the enzyme donates an electron to the substrate, which then loses a bromide ion, and a second electron is donated, producing a radical species, which may either react with protein or rearrange to other metabolic products (see review by Gut *et al*., 1993). Similarly, reductive dechlorination is also involved in the toxicity of carbon tetrachloride. Here, again, cytochrome P450 donates an electron under reductive conditions and the products are a chloride ion and the trichloromethyl radical (see **Figure 40**, below). This may then react further with oxygen to form the trichloromethylperoxy radical species, which is believed to cause damage to lipids in cell membranes (Mico and

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Pohl, 1983). Alternatively, the trichloromethyl radical may abstract a hydrogen atom from any of a variety of sources (such as glutathione), resulting in the production of chloroform and another radical (such as the glutathionyl radical).

2.3 Hydrolysis

Esterases such as those which hydrolyse carboxylic acid esters and thioesters are seen in a variety of tissues. Esterases are usually found in the cytosol of cells, but microsomal esterases and amidases have also been described; some are also found in the plasma and carboxylesterases are also present in red blood cells. Unfortunately the nomenclature of esterases is confusing, so the Enzyme Commission (EC) numbers are given below. Esterases have been classified as type A, B or C on the basis of activity towards phosphate triesters, the terms A and B esterases having been introduced by Aldridge ((1953a; 1953b), see also Aldridge, 1993c) on the basis of inhibition characteristics. A esterases can hydrolyse OPs without being inhibited (see Walker and Mackness, 1987) and include paraoxonase (PON). B-type esterases are all inhibited by paraoxon (the active metabolite of parathion, an insecticide) and they have a serine residue at the active site; consequently they are also known as serine hydrolases. However, there are a number of different enzymes within this group with different substrate specificities and inhibition characteristics, including the cholinesterases, neuropathy target esterase and the carboxylesterases.

Esterases have been intensively studied in relation to OP esters, which include the OP insecticides and the chemical-warfare agents (see **Toxicology of Chemical Warfare Agents**; **Toxicology of Pesticides**). The phosphohydrolases, (fluorohydrolases, DFPase, EC 3.1.8.2) hydrolyse diisopropyl phosphorofluoridate (DFP), which technically is not an ester but an acid anhydride (see review by Ahmad and Forgash, 1976), as well as fluorine-containing G-type nerve agents, and possibly tabun (see Kasai *et al*., 1992; Noort *et al*., 2002; Benschop and de Jong, 2001; Marrs, 2007). The products of the reaction are hydrofluoric acid and a phosphonate, isopropylmethylphosphonic acid (IMPA), and methylphosphonic acid in the case of sarin. Phosphotriester hydrolases include enzymes that hydrolyse paraoxon to diethylphosphate and 4-nitrophenol; since this involves the loss of the leaving group, the reaction is a detoxication (Edwards, 2001). These enzymes, known as paraoxonases (EC 3.1.8.1) (see also above), can hydrolyse OP esters other than paraoxon, including both insecticides and chemical-warfare agents (see reviews by Dauterman, 1982; Kasai *et al*., 1992; Tang *et al*., 2006; Costa *et al*., 2006). PON is a high density lipoprotein (HDL)-associated enzyme, which seems

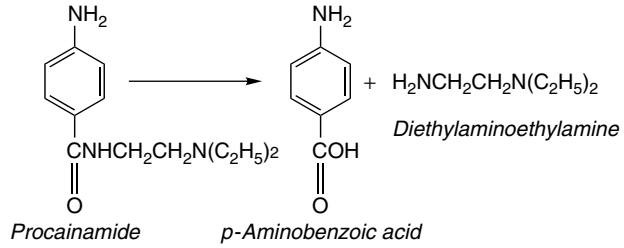

Figure 25 Hydrolysis of an amide, procainamide.

only to be found in mammals (Zech and Chemnitius, 2002). The enzyme exhibits several polymorphisms (Geldmacher-von Mallinckrodt *et al*., 1973), which have been shown to affect the expression and/or the activity level of the enzyme (Costa *et al*., 2003). PON1 exhibits a substrate-dependent activity polymorphism, as well as a large variability in plasma levels among individuals. The PON1 activity polymorphism is determined mainly by a glutamine(Q)/arginine(R) substitution at position 192 of PON1. There is one additional polymorphism in the coding region at L55 M and others in the regulatory and untranslated regions (Furlong *et al*., 2002). Many have sought to connect PON1 polymorphisms and sensitivity to OPs, but it should be remembered that PON has substrates other than OPs (Draganov *et al*., 2005), has important roles in lipid metabolism (Mackness *et al*., 2002) and is implicated in the prevalence of vascular disease (Reddy *et al*., 2008), and that is there is heterogeneity of health outcomes, even without exposure to OPs. Carboxylesterases (EC 3.1.1.1) comprise another important group of esterases. As discussed above, they are B esterases with a broad range of substrate specificity, including many xenobiotics (see review by Satoh and Hosokawa, 1998). There are a number of isozymes which have been characterized with varying substrate specificities (Hosokawa and Satoh, 2006).

A typical amidase reaction is shown in **Figure 25**. Amidases hydrolyse amides; further, hydrazides and carbamates may also be hydrolysed by amidases. Amidases have an important role in the toxicity of the drugs isoniazid (Thomas *et al*., 1981) and phenacetin, where hydrolysis is an important step in the metabolic activation (see **Figure 44** below). Esterases may also have amidase activity and vice versa, and so these two activities may be part of the same overall activity. Amides are generally hydrolysed more slowly than esters. However, electron-withdrawing substituents will weaken the amide bond, making it more susceptible to hydrolysis.

2.4 Hydration

Alkene epoxides and arene oxides, which are three-membered rings containing an oxygen atom, may be

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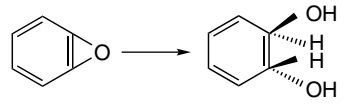


Benzene-1,2-*oxide* *Benzene*-trans-1,2-*dihydrodiol*

Figure 26 Hydration of an epoxide.

reactive metabolic intermediates, although this is not always the case, as stable epoxides exist. They may undergo hydration catalysed by the enzyme epoxide hydrolase. There are several forms of this enzyme, two of which are located in the smooth endoplasmic reticulum, conveniently near to the cytochrome P450 system which produces the epoxide, and one cytosolic enzyme. Indeed, most tissues contain enzyme activity and in the liver and lung, for example, this may parallel the distribution of cytochrome P450 activity. Like the latter enzyme, epoxide hydrolase is also inducible. The reaction can normally be regarded as a detoxication reaction, as the dihydrodiol products are usually much less chemically reactive than the epoxide (**Figure 26**). The products are *trans*-diols. However, there are examples where the diol is further metabolized to a more toxic metabolite, such as benzpyrene-7,8-dihydrodiol. Both aromatic and aliphatic epoxides may be substrates for the enzyme (see Seidegård and Ekström, 1997; Arand *et al*., 2003).

2.5 Other Phase 1 Reactions

Sometimes the enzyme responsible for observed biotransformations is not known or has not been investigated. An example is the N-hydroxylation of aminophenones. 4-Aminopropiophenone is thought to be metabolized to 4-(N-hydroxy)aminopropiophenone, which is a powerful methaemoglobin producer (Marrs *et al*., 1991; Wood *et al*., 1991) and has been considered for use as a cyanide antidote.

3 PHASE 2 REACTIONS

Phase 2 reactions, also known as conjugation reactions, involve the addition of a readily available, polar endogenous substance to the foreign molecule. This polar moiety is conjugated either to an existing group or to one added in a Phase 1 reaction. The polar moiety renders the xenobiotic molecule more water soluble and therefore more readily cleared from the body, and less likely to exert a toxic effect. The endogenous compounds used in Phase 2 reactions are numerous and include carbohydrate derivatives, amino acids, glutathione and sulfate. The mechanism involves formation of a high-energy intermediate: either the endogenous compound is activated as a high-energy derivative (Type 1) or the substrate is activated (Type 2).

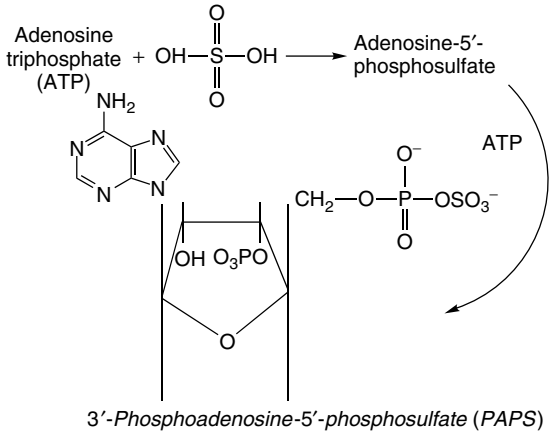
3.1 Conjugation Reactions

3.1.1 Sulfation

The addition of the sulfate moiety to a hydroxyl group is a major route of conjugation for foreign compounds, and also endogenous compounds, such as steroids, may also undergo sulfation. The reaction is catalysed by cytosolic sulfotransferase enzymes found particularly in the liver, gastrointestinal mucosa and kidney. The reaction also requires the coenzyme 3′-phosphoadenosine-5′-phosphosulfate (PAPS). This coenzyme is produced from inorganic sulfate ions and adenosine triphosphate (ATP) in a two-stage reaction (**Figure 27**). Other anions may replace sulfate in the first reaction, but the products are unstable. This may lead to toxicity by the depletion of ATP. The available inorganic sulfate in the body may also be depleted by large doses of compounds such as paracetamol (acetaminophen) which are conjugated with sulfate (see below). There are a number of different sulfotransferases, classified by the particular type of substrate, and some of these exist in multiple forms. The product of sulfate conjugation is an ester which is very polar and water soluble. Both aromatic and aliphatic hydroxyl groups may be conjugated with sulfate, as may N-hydroxy groups and amino groups (**Figure 28**) (see review by Glatt, 2002). Sulfate conjugation may be involved in the metabolic activation of compounds such as the carcinogen acetylaminofluorene (Mulder *et al*., 1988) (see below).

3.1.2 Glucuronidation

In the mammal, glucuronidation is one of the most important Phase 2 metabolic pathways. The addition of



3′-*Phosphoadenosine*-5′-*phosphosulfate* (*PAPS*)

Figure 27 Formation of the sulfate donor PAPS.

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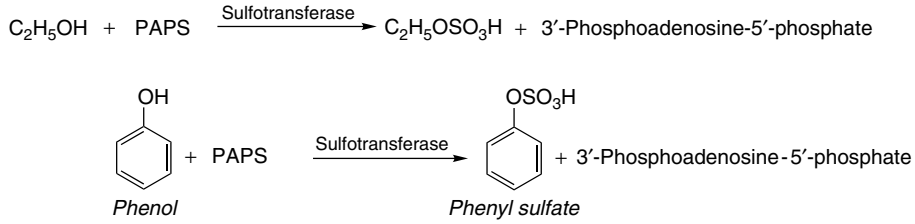

Figure 28 Conjugation of aromatic and aliphatic hydroxyl groups with sulfate.

glucuronic acid, a polar and water-soluble carbohydrate molecule, to hydroxyl groups, carboxylic acid groups, amino groups and thiols is a major route of Phase 2 metabolism. Uridine diphosphate glucuronic acid (UDP glucuronic acid) is the cofactor which donates glucuronic acid and, as with sulfation, the moiety added, glucuronic acid, is in a high-energy form as UDP glucuronic acid. UDP glucuronic acid is synthesized from glucose-1-phosphate in the cytosol in a two-step reaction (**Figure 29**). The addition of the glucuronic acid to the xenobiotic molecule is catalysed by one of a number of glucuronosyl transferases, which are present in the membrane of the mammalian endoplasmic reticulum. Glucuronosyl transferase activity is also seen in creatures other than mammals, such as fish, birds and insects. Additionally, glucuronosyl transferases are found in bacteria, yeast and plants. In humans there are many isoforms, which are grouped into two subfamilies, UGT1A and UGT2B. The enzymes are inducible in animals treated with compounds such as phenobarbital (phenobarbitone) (Burchill and Coughtrie, 1992).

The reaction involves nucleophilic attack by the recipient atom (oxygen, sulphur or nitrogen) at the C-l carbon atom of the glucuronic acid. This displacement reaction involves an inversion of configuration resulting in the product being in the β-configuration (**Figure 30**). As with sulfation, glucuronidation, although generally a detoxication reaction, may occasionally be involved in increasing toxicity, as with the conjugation of a metabolite of acetylaminofluorene (see below) (see review by Bock, 2002). Many xenobiotics are glucuronidated, examples being the pesticides tecnazene (FAO/WHO, 1995) and fenhexamid (FAO/WHO, 2006). Abnormalities in the glucuronosyl transferase system may cause hyperbilirubinaemias such as Crigler–Najjar syndrome and Gilbert's disease (Costa, 2006). Affected individuals may show abnormalities in xenobiotic metabolism, including that of drugs (Maruo *et al*., 2005).

3.1.3 Conjugation with Glucose and Other Sugars

Other carbohydrates may also be involved in conjugation, such as glucose, which is utilized by insects to form glucosides (Yu, 2008). Ribose and xylose may also be used in conjugation reactions. Conjugation with carbohydrates other than glucuronic acid seems uncommon in mammals, but does exist (e.g. Chmela *et al*., 2001). Savage *et al*. (2008) identified a glucosylsulfate as a metabolite of 3,4-dihydro-2,2-dimethyl-2H-naphtho[1,2-b]pyran-5,6-dione (ARQ 501, β-lapachone) in humans.

3.1.4 Glutathione Conjugation

This group of reactions involves the addition of glutathione to a molecule, usually with the subsequent removal of two amino acids to leave a cysteine conjugate. This is then acetylated to yield a mercapturic

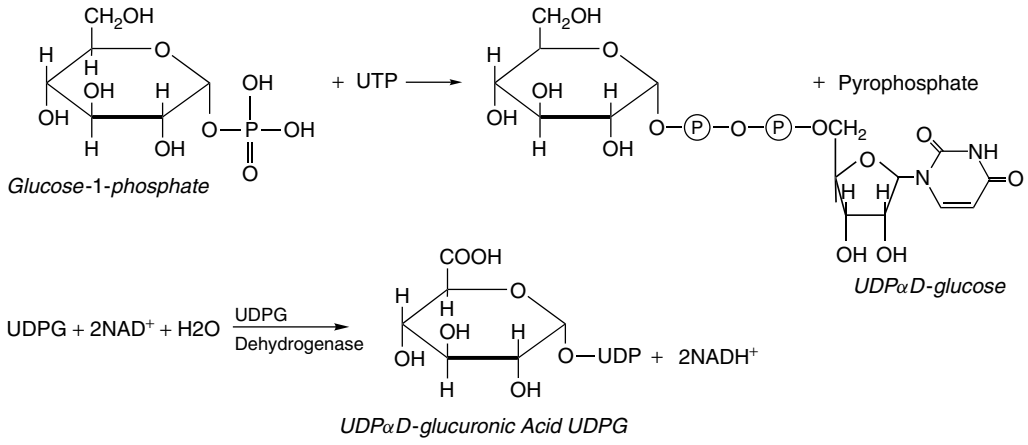

Figure 29 Formation of the glucuronic acid donor UDPG (uridine diphosphoglucose).

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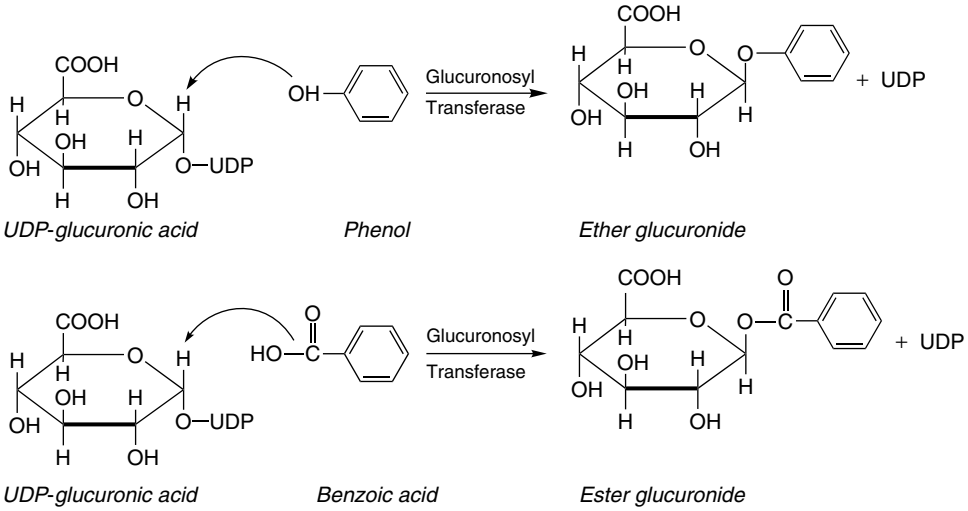

Figure 30 Formation of ether and ester glucuronide conjugates.

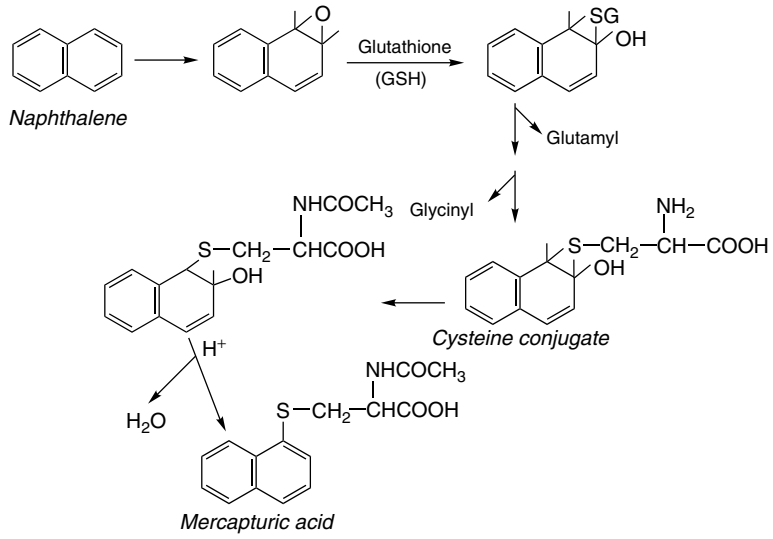

Figure 31 Metabolism of naphthalene to an *N*-acetylcysteine conjugate.

acid or *N*-acetylcysteine conjugate (**Figure 31**). Glutathione is a tripeptide (glutamylcysteinylglycine) found in most mammalian tissues, but especially the liver. Glutathione has a major protective role in the body, as it is a scavenger for reactive compounds of various types (Reed and Beatty, 1980) (see **Glutathione and Glutathione-S-Transferase in Detoxification Mechanisms**). The sulfydryl (SH) group reacts with the reactive part of the foreign compound. Glutathione conjugation is a particularly important route of Phase 2 metabolism from the toxicological point of view, as it is often involved in the removal of reactive intermediates. But this route has also been shown to be the cause of some toxic reactions (Van Bladeren *et al*., 1988; Monks *et al*., 1990; Dekant and Vamvakas, 1993) (see below).

Normally the SH group of glutathione acts as a nucleophile and either displaces another atom (e.g. Cl) or group (e.g. nitro), or attacks an electrophilic site (**Figure 31**). Consequently, glutathione may react either chemically or in enzyme-catalysed reactions with a variety of compounds which are reactive/electrophilic metabolites produced in Phase 1 reactions (Ketterer, 1982). The reactions may be catalysed by one of a group of glutathione transferases (Armstrong, 1997; Sherratt and Hayes, 2002). These are widely distributed enzymes in both the animal and plant kingdoms, and their phylogeny has been studied (Pearson, 2005). In mammals there are two superfamilies of glutathione transferases. Those located in the soluble fraction of the cell are divided into a number of families designated by nonsequential Greek letters. Glutathione transferases that are present in the microsomal fraction are also known as membrane associated proteins in eicosanoid and glutathione metabolisms (MAPEGs) (Jakobsson *et al*., 2000). In recent years there have been major advances in the understanding of the structure of MAPEGs

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(Hebert and Jegerschöld, 2007). The substrates include aromatic, heterocyclic, alicyclic and aliphatic epoxides, aromatic halogen and nitro compounds, alkyl halides and unsaturated aliphatic compounds. Although the specificity is not high for the xenobiotic, there is high specificity for glutathione. The glutathione conjugate which is produced typically undergoes further metabolism, which involves first a removal of the glutamyl residue, catalysed by γ-glutamyl transferase, then loss of glycine, catalysed by cysteinyl glycine dipeptidase and finally the cysteine moiety is acetylated to give an *N*-acetylcysteine conjugate or mercapturic acid (**Figure 31**). The *N*-acetyltransferase (NAT) which carries out this reaction is a microsomal enzyme found in liver and kidney, but is not the same as the NAT which catalyses the acetylation of xenobiotic amine groups (see below). This further metabolism of conjugates, such as illustrated for glutathione conjugates and cysteine conjugates (see below) has been termed Phase 3 metabolism. Glutathione conjugates, or the cysteinylglycine conjugate which results from them, may be excreted directly into the bile and further metabolism may take place in the gastrointestinal tract.

There are numerous examples of xenobiotics being conjugated with glutathione, the earliest to be described being 1,2-dichloro-4-nitrobenzene (Booth *et al*., 1961) and bromosulfophthalein (Combes and Stakelum, 1960). A more recent example is the mammalian detoxication of 1,3-dichloropropene (Stott *et al*., 2001), a substance used to control parasitic plant nematodes.

3.1.5 Cysteine Conjugate β-lyase

This enzyme is responsible for the further metabolism of cysteine conjugates before they are acetylated. Only nonacetylated cysteine conjugates are substrates. The result is a thiol conjugate of the xenobiotic, pyruvic acid and ammonia. The thiol conjugate which results may, in some cases, prove to be toxic (see below) (see review by Cooper and Pinto, 2006).

3.1.6 Acetylation

Acetylation is an important route of metabolism for aromatic amino compounds, sulfonamides, hydrazines and hydrazides (**Figure 32**). The enzymes involved are acetyltransferases and are found in the cytosol of cells in the liver, gastric mucosa and white blood cells. The enzymes utilize acetyl CoA as cofactor. The mechanism of the acetylation reaction involves first an acetylation of the enzyme, followed by addition of the substrate and then transfer of the acetyl group to the substrate. There are two enzymes (NAT 1 and NAT 2) in humans, rabbits and hamsters which differ markedly in activity and substrate specificity. NAT 1 is located in the liver and gut, whereas NAT 2 is found in most tissues (see review by Levy and Weber, 2002). In humans, the possession of a particular mutant isoenzyme in NAT 2 is genetically determined and gives rise to two distinct phenotypes known as 'rapid' and 'slow' acetylators. The acetylator phenotype has an important role in the toxicity of a number of drugs such as hydralazine, isoniazid and procainamide. These examples illustrate the importance of genetic factors in toxicology (Evans, 1992; 1993; Grant *et al*., 1992; Kalow, 1992; Sim *et al*., 2008).

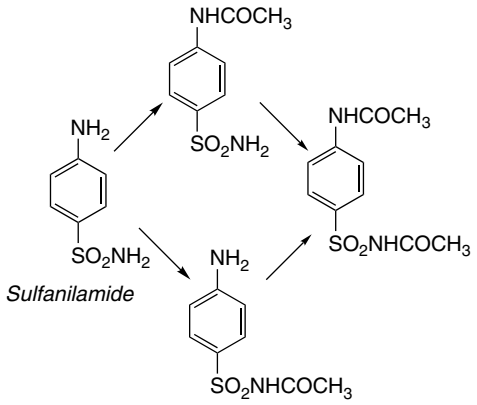

Figure 32 Acetylation of amino and sulfonamido groups.

A related reaction is N,O-transacetylation. This reaction applies to arylamines, which first undergo N-hydroxylation and then the hydroxylamine group is acetylated to yield an arylhydroxamic acid (see **Figure 45** below). This may transfer the acetyl group to another amine molecule or to the hydroxy group, to yield a highly reactive acyloxyarylamine which is capable of reacting, after a rearrangement, with proteins and nucleic acids (King and Weber, 1981) (see below). The enzyme involved, an N,O-acyltransferase, is a cytosolic enzyme.

3.1.7 Amino Acid Conjugation

This is the second type of acylation reaction, but in this type the xenobiotic itself is activated. Organic acids are the usual substrates for this reaction, with conjugation with an endogenous amino acid, or similar substances such as taurine (1-aminoethane sulfonic acid). The first amino-acid conjugation reaction to be described was the reaction that produces hippuric acid (benzoylglycine). This was found in horse urine in 1829 by Liebig and human urine in 1842 by Keller, in both cases the hippuric acid being derived from benzoic acid (Conti and Bickel, 1977). The production of benzoylglycine results from the conjugation of benzoic acid with glycine. However, the particular amino acid utilized depends on the organic acid and the species concerned, and possibly also the availability of amino acids and therefore the diet. Species within a similar evolutionary group tend to utilize the same amino acid. In mammals, glycine

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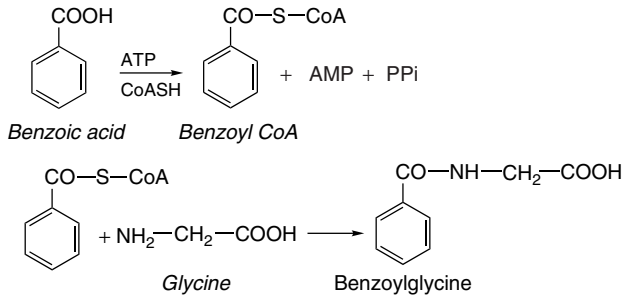

Figure 33 Conjugation of an aromatic acid with glycine.

is the most common amino acid used, but taurine, glutamine, serine, alanine and aspartic acid also take part in conjugation reactions. Ornithine is important in some avian species (Idle *et al*., 1976) and a variety of reptiles (Smith, 1958). The foreign carboxylic acid group is first activated by reaction with coenzyme A in a reaction which requires ATP and is catalysed by a mitochondrial ligase enzyme. The S–CoA derivative then reacts with the particular amino acid (**Figure 33**). This second reaction is catalysed by an acyltransferase enzyme which is found in the mitochondria (see review by Steventon and Hutt, 2002).

3.1.8 Methylation

Hydroxyl, amino and thiol groups in both exogenous and endogenous compounds may be methylated by one of a large number of methyltransferases (**Figure 34**). These enzymes are normally found in the cytosol, although a microsomal O-methyltransferase and an S-methyltransferase have been described. For virtually all methyltransferases, the cofactor required is S-adenosylmethionine, which is the methyl donor, the S-adenosylmethionine being formed from ATP and L-methionine by condensation catalysed by ATP:L-methionine S-adenosyltransferase (see review by Creveling, 2002). As with acetylation, the methylation reaction tends to decrease rather than increase the water solubility of the molecule. A number of metals, such as mercury may also be methylated by micro-organisms, a reaction which changes both the toxicity of mercury and its physicochemical characteristics and, hence, the environmental behaviour of the metal (see review by Boening, 2000).

4 OTHER BIOTRANSFORMATION REACTIONS

There are other reactions which a foreign molecule may undergo. An important point, however, is that although a molecule is foreign to a living organism, it may still be a substrate for an enzyme involved in normal metabolic

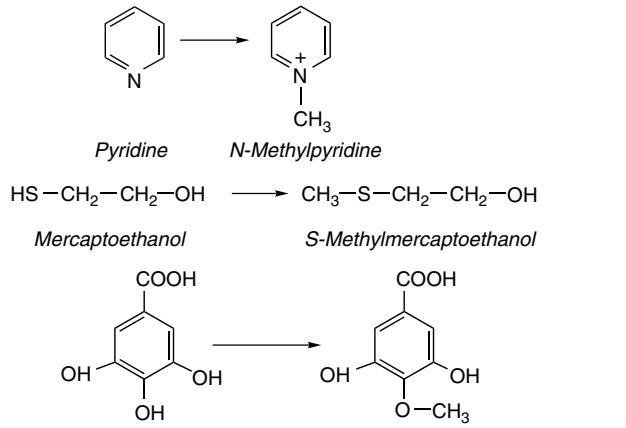

Figure 34 Methylation reactions.

pathways, provided that its chemical structure is appropriate. For example, a foreign compound which is a halogenated fatty-acid derivative may be metabolized by the β-oxidation pathway, but might potentially interfere with that pathway. The possible involvement of enzymes of intermediary metabolism therefore widens the scope of potential metabolic reactions. Foreign compounds can be metabolized by a number of different enzymes simultaneously in the same animal and so there may be many different metabolic routes and metabolites. The balance between these routes can often determine the toxicity of the compound. Some detoxication reactions, for example formation of thiocyanate from cyanide by sulphur transferases *inter alia* rhodanese (cyanide: thiosulfate sulphur transferase; E.C. 2.8.1.1) do not really fall into the classification into Phases 1 and 2 (Isom and Johnson, 1987; Bhatt and Linnell, 1987; Saidu, 2004).

For more information on the metabolism of foreign compounds, the reader should consult the more detailed texts given in the Further Reading section.

5 FACTORS AFFECTING METABOLISM

Factors which affect metabolism may often affect the toxicity of a compound, by changing the rate of removal of the parent compound, by changing the rate of activation or by altering the pattern of metabolism. Different species will often metabolize compounds differently, and so show differences in toxicity. Environmental factors, such as dietary constituents and drugs taken by humans may influence the metabolism, and so alter the toxicity of a particular substance (Shimada *et al*., 1994). In man, genetic factors may play an important role in drug effects (Meyer, 1998) and in determining which metabolic pathway is utilized and therefore whether a compound is toxic or not (Weber, 1987; Sitar, 1989; Alván, 1991; Evans, 1992; Kalow, 1992; Meyer, 1994; Testai, 2001).

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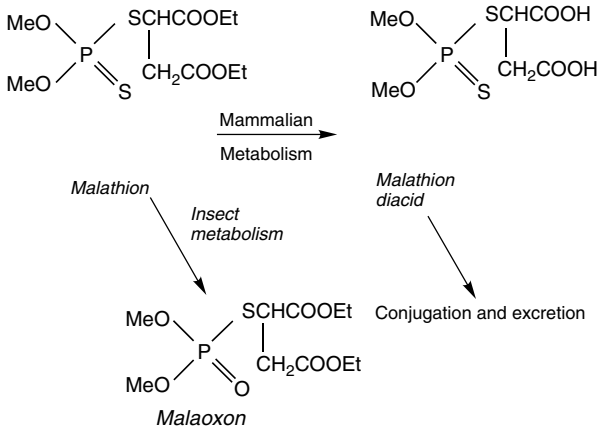

Figure 35 Metabolism of malathion.

The multiple forms of cytochrome P450 may underly many of the observed species differences in metabolism (Berthou *et al*., 1992; Martignoni *et al*., 2006), and may be relevant to the effects of age, sex, nutrition, strain and genetic differences, although any of these factors can affect virtually any Phase 1 or 2 reaction. Different tissues may contain different isoenzymes and, hence, possess different susceptibility to toxic compounds.

5.1 Species

Different species are utilized in the safety evaluation of chemicals and, in the environment, widely different species may all be exposed to a chemical. These species may react very differently to xenobiotics. Indeed, this difference in sensitivity is exploited in pesticides. Insecticides such as organophosphorus compounds and dichlorodipheyltrichloroethane (DDT) are often much more toxic to insects than to humans and other mammals. In the case of malathion and some other phosphorothionates this is due to a metabolic difference (see also above) (**Figure 35**). There are many species differences in metabolism which have been documented (Caldwell, 1980), but this section will mainly concentrate on those which are significant as far as toxicity is concerned. In general, the more closely related the species, the more likely it is that metabolism, including biotransformation, will be similar: thus Smith and Caldwell (1977) found that the rhesus monkey (*Macaca mulatta*) was a good metabolic model for man in about three quarters of the cases they studied, whereas rats and other nonprimates were good models in a far smaller proportion of cases.

5.1.1 Phase 1 Reactions

There are often quantitative differences between species in oxidation reactions, but qualitative differences are perhaps less common. It is difficult to find a species pattern in these differences. These differences may often be the result of differences in cytochrome P450 enzymes. The aromatic hydroxylation of aniline has been shown to vary between various species (**Table 2**), and those species such as the cat (*Felis catus*), which produce more *o*- as opposed to *p*-aminophenol, are more susceptible to the toxicity. The trend in this case is for carnivores to favour *ortho*- rather than *para*-hydroxylation. Another example of quantitative differences in metabolism between species is in the metabolism of ethylene glycol to oxalate (**Figure 2**). The toxicity is partly due to the oxalic acid produced by the oxidative pathway and the toxicity correlates with the production of oxalate. Here again the cat is the species most susceptible to the toxicity, producing the most oxalate, followed by the rat, with the rabbit producing the least (see review by Beasley and Buck, 1980).

Table 2 Species differences in the hydroxylation of aniline

Species	% of dose excreted	
	o-Aminophenol	*p*-Aminophenol
Gerbil	3	48
Guinea pig	4	46
Golden hamster	6	53
Chicken	11	44
Rat	19	48
Ferret	26	28
Dog	18	9
Cat	32	14

Data from Parke (1968).

The N-oxidation of paracetamol (see below) also shows quantitative differences in metabolism between species, which accounts for species differences in hepatotoxicity. Thus, the rat (*Rattus* sp.) is relatively resistant to the toxicity and metabolizes less via the toxic pathway, whereas the hamster (*Mesocricetus auratus*) is very susceptible (Tee *et al*., 1987; Miller *et al*., 1993). In contrast, the rat is well able to carry out N-hydroxylation of acetylaminofluorene (**Figure 12**), a step which is necessary for the compound to exhibit carcinogenicity (Verna *et al*., 1996) (see also **Figure 45** below).

In the metabolism of the drug hexobarbital (hexobarbitone) there are striking differences between species, which correlate with the pharmacological effect (**Table 3**). The overall metabolic rate tends to decrease as the size of the species increases, and this would be expected to have some bearing on drug metabolism. In this example this is approximately true, apart from the rat and rabbit being transposed.

Another Phase 1 reaction which shows a striking species difference that is a cause of selective toxicity is hydrolysis. Thus, the insecticide malathion, which has nugatory acetylcholinesterase activity *per se,* is readily hydrolysed in mammals, but in the insect the carboxylesterase enzyme is absent and oxidative metabolism is the major route (**Figure 35**). This route

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Table 3 Species differences in the metabolism and duration of action of hexobarbitone (hexobarbital)

Species	Duration of action (min)	Plasma half -life (min)	Relative enzyme activity ($\mu g\,g^{-1}\,h^{-1}$)	Plasma level on awakening ($\mu g\,ml^{-1}$)
Mouse	12	19	598	89
Rabbit	49	60	196	57
Rat	90	140	135	64
Dog[a]	315	260	36	19

Data from Quinn *et al*. (1958).
[a]Dose in dogs, 50 mg kg^{-1}; other species, 100 mg kg^{-1}.

leads to the production of malaoxon, which is very toxic because it is a powerful anticholinesterase. The net effect is that malathion is an effective insecticide with low mammalian acute toxicity (FAO/WHO, 1998). This also seems to be true of fenitrothion (FAO/WHO, 2001).

5.1.2 Phase 2 Reactions

A number of Phase 2 reactions also show well-characterized species differences. For example, the metabolism of phenols in most mammals, and also birds, amphibians and reptiles, but not fish, usually involves conjugation with either glucuronic acid or sulfate, depending on the species, but most species utilize a mixture of these two routes. An example is umbelliferone (7-hydroxy coumarin), where differences have been identified between humans, monkeys, dogs and rats in glucuronidation and sulfation reactions (Wang *et al*., 2005; 2006). The domestic cat, however, does not readily conjugate some compounds with glucuronic acid that are conjugated in other species. This phenomenon is important because it renders cats very sensitive to certain xenobiotics, including paracetamol and means that, with certain drugs, the therapeutic dose in cats is much lower than in other pet animals and, indeed, humans. The evolutionary basis of this has been studied; whereas UGTIAI is expressed in the liver, UGT1A6 in cats has stop codons and deletions, which make it unlikely to function as an enzyme. Thus UGT1A6 is a pseudogene in the domestic cat and it has been suggested that the cat can tolerate this because it is exposed to fewer toxins than many animals because of its totally carnivorous diet (Hirom *et al*., 1977; Court and Greenblatt, 2000; Bock, 2002). Like cats, the ferret (*Mustela putorius furo*) liver glucuronidates paracetamol relatively slowly. However, unlike cats, there are no defects in the ferret UGT1A6 gene which could account for the low activity (Court, 2001). It should be noted that cats can glucuronidate some xenobiotics, including apparently nonsteroidal anti-inflammatory drugs (Magdalou *et al*., 1990). Pigs (*Sus scrofa scrofa*) do not conjugate some substrates with sulfate which are so conjugated by other mammalian species (Capel *et al*., 1974; Guhe *et al*., 1996). There are also clear differences in the conjugation of organic acids. Carnivores favour glucuronic acid conjugation, herbivores favour amino-acid conjugation, whereas omnivores utilize both (Calabrese, 1983). Which amino acid is utilized also varies, and although glycine is the most common, glutamine and taurine may be used, and reptiles and some birds utilize ornithine, whereas insects utilize arginine (see also Section 3.1.7).

5.2 Strain of Animal

Different inbred strains of the same animal may show variations in metabolism, just as different species may vary in their response to toxic compounds and in the way they metabolize them. For example, different strains of mice vary widely in their ability to metabolize barbiturates and consequently the magnitude of the pharmacological effect varies between these strains (**Table 4**). Differences can extend to both Phase 1 and Phase 2 metabolism (Stott *et al*., 2004). Similarly, variations between human individuals may also occur, but will be considered separately in this chapter (see below).

5.3 Sex

There can also be variation in the responses between males and females due to metabolic and hormonal differences. Males in some species metabolize compounds more rapidly than females, although this difference is not found in all species. The difference in susceptibility to chloroform-induced liver damage between male and

Table 4 Strain differences in the duration of action of hexobarbitone (hexobarbital) in mice

Strain	Numbers of animals	Mean sleeping time ± SD (min)
A/NL	25	48 ± 4
BALB/cAnN	63	41 ± 2
C57L/HeN	29	33 ± 3
C3HfB/HeN	30	22 ± 3
SWR/HeN	38	18 ± 4
Swiss (non-inbred)	47	43 ± 15

Data from Jay (1955).

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female rats is an example of a sex difference which has a metabolic and hormonal basis, thought to be due to the effects of testosterone on liver microsomal enzyme activity. Thus, treatment of females with testosterone decreases the median lethal dose (LD_{50}) and treatment of males with oestradiol increases the LD_{50} of chloroform. Similarly, there is a sex difference in the nephrotoxicity of chloroform in mice, with male mice being more susceptible. This difference can be removed by castration and restored by administration of androgens to the males. It may be that testosterone is increasing the microsomal enzyme-mediated metabolism of chloroform to toxic metabolites (Pohl, 1979). Other examples are the metabolism of ethylmorphine and hexobarbitone (hexobarbital), which are clearly under hormonal control in the male rat. Castration of the animals significantly reduces the metabolism of both compounds. However, the normal rate of metabolism can be restored by the administration of androgens to the castrated animals. Another example is the methaemoglobin-forming cyanide antidote 4-aminopropiophenone, which is active only after metabolic activation *in vivo*, and exhibits a sex difference in (male) beagle dogs and bitches. Bitches produced more methaemoglobin for a given dose of *p*-aminopropiophenone (PAPP) than male dogs. The probable reason for this difference was a lower rate of N-hydroxylation in male dogs (Bright *et al*., 1987). For a review of sex differences in relation to drugs see Wang and Huang (2007).

5.4 Genetic Factors

Genetic variation in metabolism within the human population probably underlies much of the variability in response of that population to the toxic effects of foreign compounds (Meyer, 1994; 1998; Tucker, 1994) and polymorphisms of PON have been briefly discussed in Section 2.3. There are now many examples of toxic drug reactions which occur particularly in individuals who have a genetic defect or genetic difference in metabolism. Many of these are due to nonsynonymous single nucleotide polymorphisms (SNPs). Perhaps the best known example of genetic variability in man is the acetylator phenotype (Weber, 1987; Evans, 1992; Grant *et al*., 1992). In this example the acetylation reaction (see Section 3.1.6) shows genetic variations which are due to the presence of mutations in the gene coding for NAT 2. These mutations give rise to enzymes with differing activities and result in two distinct populations, the rapid and slow acetylator phenotypes, and evidence suggests that NAT 1 may also show bimodal variation. The slow acetylator phenotype, which occurs to a variable extent in the population, depending on the racial origin (**Table 5**), is an important factor in a number of adverse drug reactions (**Table 6**). For example, the lupus syndrome induced by the drug hydralazine only occurs in slow acetylators. The metabolism of the drug is influenced by the acetylator phenotype, with more being metabolized by an oxidative pathway in slow acetylators (Timbrell *et al*., 1984). It is not yet known whether this is responsible for the toxic effect, but Uetrecht (1992) speculated that oxidation by myeloperoxidase in neutrophils was involved (see reviews by Sim *et al*., 2008; Ladero, 2008).

Table 5 Acetylator phenotype distribution in various ethnic groups

Ethnic group	Rapid acetylators (%)	Drug
Eskimos	95–100	INH
Japanese	88	INH
Latin Americans	70	INH
Black Americans	52	INH
White Americans	48	INH
Africans	43	SMZ
South Indians	39	INH
Britons	38	SMZ
Egyptians	18	INH

INH: isoniazid; SMZ: sulfamethazine
Data from Lunde *et al*. (1977).

The acetylator phenotype is also believed to be a factor in isoniazid toxicity (see below) and bladder cancer, which occurs in workers exposed to aromatic amines. Thus, there is an increased incidence of the cancer in slow acetylators. This is postulated to be due to the decreased ability of slow acetylators to detoxify the aromatic amines by acetylation (Cartwright *et al*., 1982; Mommsen *et al*., 1985; Kadlubar, 1994). However, one study did not find any evidence that the slow acetylator phenotype was more susceptible to benzidine-induced bladder cancer (Hayes *et al*., 1993), and there is some evidence with benzidine of a protective effect from slow-acetylator status (Carreón *et al*., 2006) (see review by Sanderson *et al*., 2007).

Another genetic factor in metabolism is polymorphism in the hydroxylation of debrisoquine. This was discovered by Mahgoub *et al*. (1977) during a human volunteer study (**Figure 36**). This was further characterized and the heterogeneity in oxidation was shown for a number of other drugs, such as phenytoin, sparteine and phenformin (Gonzalez *et al*., 1988; Eichelbaum and Gross, 1990; Llerena *et al*., 2009). Again there are two phenotypes, designated poor metabolizers and extensive metabolizers. Unlike the acetylator phenotype, however, the poor metabolizer phenotype is relatively uncommon, only occurring in about 5–10% of a Caucasian population. In some cases, toxic reactions are associated with the poor-metabolizer status. The difference between poor metabolizers and extensive metabolizers is believed to be due to differences in the isozymes of cytochrome P450 present in the particular subject. It has been shown

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Table 6 Toxicities related to the acetylator phenotype

Xenobiotic	Adverse effect	Incidence
Isoniazid	Peripheral neuropathy	Higher in slow acetylators
Isoniazid	Hepatic damage	Higher in slow acetylators
Procainamide Hydralazine	Lupus erythematosus	Higher in slow acetylators
Phenelzine	Drowsiness/nausea	Higher in slow acetylators
Aromatic amines	Bladder cancer	Higher in slow acetylators

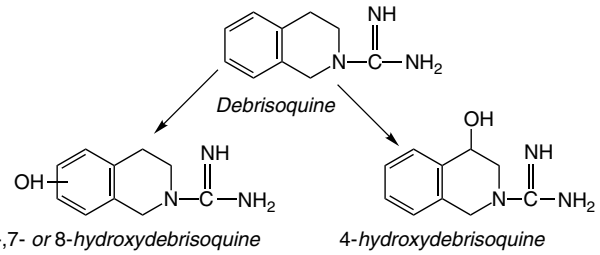

Figure 36 Metabolism of debrisoquine.

(Gonzalez and Meyer, 1991) that poor-metabolizer individuals have a deficiency in activity of liver cytochrome P450 2D6 and this results from various mutations in the CYP 2D6 gene. The poor metabolizer phenotype is known to be caused by two null alleles leading to absence of functional CYP 2D6 protein. There are also CYP 2D6 alleles with low, but definite, activity which produce intermediate metabolizers and individuals with multiple copies of CYP 2D6, who have high activity (see review by Zanger, 2008). It is interesting that there is some evidence that this polymorphism may influence personality type (González *et al*., 2008).

5.5 Environmental Factors

Exposure of animals to chemical substances via the environment, such as in the diet, air or water, may influence the metabolism and therefore the toxic response to the chemical of interest. Humans may be receiving medication with several drugs when exposure to an industrial chemical occurs, for instance. The intake of one drug may affect the metabolism of another. For example, overdoses of paracetamol are more likely to cause serious liver damage if the victim is also exposed to large amounts of alcohol or barbiturates. Both of these drugs influence drug-metabolizing enzymes and thereby increase the metabolism and, in turn, the toxicity of paracetamol (see below).

The diet contains many substances, such as the naphthoflavones found in certain vegetables, which may influence the enzymes involved in drug metabolism. Cigarette smoking is also known to affect drug metabolism.

5.5.1 Induction

One way in which a particular substance may influence the metabolism of another is by increasing the apparent activity of the drug-metabolizing enzymes. This is known as induction. The induction of the microsomal mono-oxygenases, as well as a number of other enzymes, is now a well-known phenomenon and is caused by a large variety of compounds, including many phytochemicals (Batt *et al*., 1992; Murray, 2008). The phenomenon occurs in many species. The different inducers may induce different cytochrome P450 enzymes and are therefore of importance for different types of substrates.

The first type of microsomal enzyme inducer to be described was the barbiturate phenobarbital. When animals are exposed to repeated doses of this compound, there are a number of changes which can be observed. The liver of the animal exposed increases in weight, there is an increase in liver blood flow and the smooth endoplasmic reticulum proliferates. These changes are accompanied by an increase in the total amount of cytochrome P450 in the liver. The activity of other enzymes found in the endoplasmic reticulum also increases.

The effect of enzyme induction is that the metabolism of certain foreign compounds is increased. At the molecular level there is an increase in protein synthesis and underlying this an increase in messenger RNA (mRNA) synthesis. Since the initial discovery that phenobarbital induced the enzymes responsible for its own metabolism, many other inducers have been discovered and studied, and some of these have been found to be different types of inducer. These different inducers induce different isoenzymes of cytochrome P450 and do not necessarily cause all the changes observed with phenobarbital induction, such as the increase in liver weight and blood flow. The inducers currently known can be divided into five types: (i) the barbiturate type; (ii) the polycyclic aromatic hydrocarbon type; (iii) the isoniazid type; (iv) the steroid type, typified by pregnenolone carbonitrile and (v) the clofibrate type. Each of these induces a different form or forms of the enzyme. These can, in some cases, be distinguished and detected on the basis of catalytic activity *in vivo* or *in vitro*, as well as increased amounts of protein. There are also inducers which have a broader inducing ability, such as the compound arochlor 1254, which may be both a barbiturate type and a

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polycyclic hydrocarbon type of inducer. Some of the enzymes induced may be constitutive, whereas others induced by some of these compounds may represent only a small proportion of the total cytochrome P450 present in the uninduced animal. In this case, the induction may cause a large shift in the metabolic profile rather than a simple increase in the overall rate of metabolism. Thus, for the polycyclic hydrocarbon type of inducers, the form of cytochrome P450 induced normally only represents about 5% of the total enzyme, whereas after induction, the amount may be increased by a factor of 16. Furthermore, induction is not confined to the hepatic enzymes, those in other tissues may also be induced. The effect of different inducers on the metabolism of different compounds can be seen from the data in **Table 7**. Clearly, although all these compounds are inducers of cytochrome P450, the effects on different substrates for the enzyme are different. These differential effects even extend to different isomers (**Table 8**). This can be rationalized by the fact that there are many different isoenzymes and that these are induced by different compounds.

In addition to influencing the metabolism of foreign compounds, enzyme induction may have effects on the metabolism of endogenous compounds and so disrupt normal physiological processes.

The mechanisms underlying induction are not all entirely clear but it is known to be a cellular response, which can be studied, for example in isolated hepatocytes. Indeed, the mechanisms seem to vary between the different types and may involve transcriptional activation (e.g. CYP 1A1) or enzyme stabilization (CYP 2E1). The synthesis of new protein is involved, as the induction process can be prevented by inhibitors of protein synthesis. Synthesis of RNA is required, but not of DNA, and so it seems that the effect is at the level of transcription. For the polycyclic hydrocarbon type of inducer, studied using tetrachlorodibenzodioxin (dioxin), which is an exquisitely potent inducer, it seems that there is a cytosolic receptor which binds the inducer. The inducer–receptor complex is then transported to the nucleus and there enhances transcription of the CYP 1A1 gene and other genes such as CYP 1A2, glutathione transferase and glucuronosyl transferase. This causes mRNA coding for cytochrome P450 1A1 and other enzymes to be synthesized. This then allows increased synthesis of the particular cytochrome P450 enzymes for which the mRNA is coded. This involves inducer-dependent derepression and activation of gene expression (Okey, 1990; Waxman and Azaroff, 1992; Whitlock, 1993). However, to date, no receptor for the barbiturate type of inducer has been found, although evidence suggests that the induction may involve inducer-dependent depression and activation of gene expression as for the polycyclic hydrocarbon type. The peroxisome-proliferator (clofibrate) type of induction (CYP 4A) also involves a receptor (Muerhoff *et al*., 1992).

5.5.1.1 *Consequences of Induction*

Induction of drug-metabolizing enzymes may lead to an alteration in the metabolism of a compound, which may then result in the toxicity being either increased or

Table 7 Effects of different inducers on the metabolism of various substrates (units: nmol product per min per nmol cytochrome P450)

Substrate	Inducer[a]				
	Control	Pb	PCN	3MC	ARO
Ethylmorphine	13.7 ± 0.8	16.8 ± 4.3	24.9 ± 3.5	6.4 ± 0.5	9.5 ± 1.2
Aminopyrine	9.9 ± 0.8	13.9 ± 1.7	9.7 ± 1.3	7.6 ± 1.8	13.7 ± 1.2
Benzphetamine	12.5 ± 1.2	45.7 ± 14.0	6.6 ± 0.7	5.7 ± 1.1	15.8 ± 2.7
Caffeine	0.5 ± 0.1	0.7 ± 0.1	—	0.5 ± 0.1	0.6 ± 0.1
Benzo[a]pyrene	0.1	0.1	0.1	0.3	—

Data from Powis *et al*. (1977).
[a]Pb, phenobarbitone; PCN, pregnenolone-16α-carbonitrile; 3MC, 3-methylcholanthrene; ARO, arochlor 1254.

Table 8 Differential effect of cytochrome P450 inducers on the hydroxylation of warfarin isomers (units: nmol metabolite formed per nmol cytochrome P450)

	Hydroxylated warfarin metabolites			
	R-Isomer		*S*-Isomer	
	7-OH	8-OH	7-OH	8-OH
Control	0.22	0.04	0.04	0.01
Phenobarbitone	0.36	0.07	0.09	0.02
3-Methylcholanthrene	0.08	0.50	0.04	0.04

Data from Table 3.6 in Gibson and Skett (1986).

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decreased. Prediction of which will occur is possible only with a knowledge of the metabolism and mechanism of toxicity of the compound in question. In some of the examples at the end of this chapter the effect of inducers will also be discussed.

5.5.2 Inhibition of Metabolism

Just as some of the enzymes involved in biotransformation may be induced, so these enzymes may also be inhibited, and this can have major consequences for toxicity (Netter, 1987; Halpert *et al*., 1994). Such inhibitions are sometimes of major importance clinically, with the interaction between drugs probably more important than induction effects. Inhibition generally requires only a single dose of a compound rather than the repeated doses which are required for induction. The environmental impact of inhibition is, however, probably less than that of induction. Inhibition also may be relevant to the toxic effects of substances encountered in the workplace. For example, workers exposed to the solvent dimethylformamide seem more likely to suffer alcohol-induced flushes than those not exposed, possibly owing to the inhibition of alcohol metabolism (Lyle *et al*., 1979).

There are many different types of inhibitor of the microsomal mono-oxygenase system. Thus, there are inhibitors which appear to bind as substrates and are competitive inhibitors, such as dichlorobiphenyl, which inhibits the O-demethylation of *p*-nitroanisole. There are those inhibitors which are metabolized to compounds which bind strongly to the active site of the enzyme, such as piperonyl butoxide, which probably acts by forming an inactive complex with cytochrome P450, which becomes irreversibly inhibited (Franklin, 1976). There are indeed many compounds which form such inhibitory complexes with cytochrome P450, including some commonly used drugs, such as triacetyloleandomycin. Some inhibitors destroy cytochrome P450, such as carbon tetrachloride, cyclophosphamide, carbon disulfide and allylisopropylacetamide. Finally, there are those which interfere with the synthesis of the enzyme, such as cobalt chloride, which inhibits cytochrome P450 *in vivo* by interfering with the synthesis of the haem portion of the enzyme.

Other toxicologically relevant enzymes involved in biotransformation which also may be inhibited are certain esterases, which are inhibited by organophosphorus compounds (see Section 2.3). Inhibition of monoamine oxidases by drugs such as iproniazid used to treat depression is important clinically, because such treatment results in a decreased metabolism of naturally occurring amines such as tyramine, typically found in cheese, which may be ingested in the diet. These amines may thereby accumulate and can have profound physiological effects such as causing an increase in blood pressure, which in some cases may be fatal (see Strolin Benedetti and Tipton, 1998).

5.5.3 Pathological State

The influence of disease states on metabolism and toxicity has not been well explored. Diseases of the liver might be expected to affect metabolism, but in practice, different liver diseases can influence metabolism differently (Hoyumpa and Schenker, 1982; Howden *et al*., 1989; Kraul *et al*., 1991). Indeed, some metabolic pathways are unaffected by liver damage. For example, the glucuronidation of paracetamol, morphine and oxazepam was found not to be affected by liver cirrhosis in human subjects. Conversely, the oxidation of a number of drugs such as barbiturates, antipyrine and methadone, and the conjugation of salicylates with glycine were all depressed by cirrhosis. Disease states such as influenza are also known to affect drug-metabolizing enzymes, possibly via the production of interferon in response to the infection (Azri and Renton, 1991). Drugs, including complimentary medicines, especially herbal medicines, used to treat disease states, may affect the metabolism of other xenobiotics (see Section 5.5.5).

5.5.4 Age

In general, animals at the extremes of age—neonates and geriatrics—are less able to metabolize foreign compounds than are adult animals between these extremes (Besunder *et al*., 1988; Kinirons and Crome, 1997). However, the development of drug-metabolizing ability is complex and depends on the particular substrate and species, and is also influenced by sex (Horbach *et al*., 1992). For example, in the rat, Phase 1 metabolic activity may develop only after weaning for some demethylation reactions, but the N-hydroxylation of aniline develops from birth. With Phase 2 reactions, again, some are low at birth, such as glucuronidation, whereas acetylation and sulfation are at adult levels and, in the guinea pig, the former is present even in the foetus (Sonawane, 1982). In rats, mono-oxygenase activity begins to decline when the animals reach one year of age. These effects on drug metabolism may be translated into differences in toxicity, but it is not always the young animal which is more susceptible. For example, paracetamol is less hepatotoxic in young mice than in adults. This may be due to the fact that the development of the cytochrome P450 system required to activate paracetamol (see below) reaches maximum, adult levels more slowly than hepatic glutathione levels (Hart and Timbrell, 1979).

5.5.5 Diet

In Section (5.5.1), it was mentioned that phytochemicals can affect induction of metabolic enzymes, and they can also act as inhibitors. This means that constituents of the diet may affect metabolism. Although there is a paucity of information in this area, it is clear that dietary deficiencies can affect the metabolism of foreign

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compounds by altering the enzymes involved (Gibson and Skett, 1994). Thus, a low-protein diet will generally decrease the activity of the monoxygenases and decrease the content of cytochrome P450. For example, rats fed a low-protein (5%) diet show 50% of the *in vitro* microsomal enzyme activity of rats fed a normal diet (20% protein). The effect occurs within 24 hours and the enzyme activity is minimal after four days. *In vivo* findings are in agreement with these observations. The decrease in microsomal enzyme activity due to a low-protein diet may result in reduced toxicity. For example, carbon tetrachloride hepatotoxicity is less in protein-deficient rats than normal animals. However, other changes may occur which have the opposite effect, and so paracetamol is more hepatotoxic in protein-deficient animals, possibly owing to decreased hepatic glutathione levels. As with protein deficiency, a dietary deficiency in lipids, such as linoleic acid, also tends to decrease levels of cytochrome P450. Changes in carbohydrate seem to have few effects on drug metabolism, although an increase in glucose intake seems to decrease hepatic cytochrome P450 and inhibit barbiturate metabolism. The effects of starvation seem to be variable, with some microsomal enzyme activities being increased and others decreased. Deficiencies in vitamins in general also reduce the activity of the mono-oxygenases, although there are exceptions to this. The effects of malnutrition on drugs, *inter alia* their metabolism, has been reviewed (Mehta, 1990).

A notable example of the interaction between a particular dietary constituent and drugs is grapefruit juice. Grapefruit juice can markedly augment oral drug bioavailability by reducing presystemic metabolism, the mechanism being down-regulation of cytochrome CYP3 A4 expression in the intestinal wall; grapefruit juice does not appear to affect hepatic CYP 3A4 expression. This phenomenon can affect a number of drugs, including the calcium-channel antagonist, felodipine, and terfenadine, the latter being a formerly widely used antihistamine (Benton *et al*., 1996; Bailey *et al*., 1998; Bressler, 2006).

A related problem is herbal medicine/drug interactions. A number of these are known and the subject has been reviewed (Mills *et al*., 2004; Hu *et al*., 2005; Nekvindová and Anzenbacher, 2007). Examples include preparations made from St John's wort (*Hypericum perforatum*) (Di *et al*., 2008).

5.5.6 Chiral Factors in Metabolism

The importance of chiral factors in metabolism and toxicity has been recognized only relatively recently. The presence of a chiral centre in a molecule, giving rise to isomers, may influence the routes of metabolism and toxicity of that compound. Alternatively, metabolism may yield a specific isomer as a product from a molecule without a chiral centre. For example, it has now been found that only the glutaminic acid derived from the *S*-(−)-enantiomer of thalidomide is embryotoxic, and not that formed from the *R*-(+) enantiomer (Blaschke *et al*., 1979; Heger *et al*., 1994) (see **Effects of Chirality on Toxicity** for *inter alia* a discussion of nomenclature in relation to chirality).

Benzo[a]pyrene is metabolized stereoselectively, by a particular cytochrome P450 isozyme, to the (+)-(7*R*,8*S*)-oxide, which, in turn, is metabolized by epoxide hydrolase to the (−)-(7*R*,8*R*)-dihydrodiol. This metabolite is further metabolized to (+)-benzo[a]-pyrene-(7*R*,8*S*)-dihydrodiol-(9*S*,10*R*)-epoxide, in which the hydroxyl group and epoxide are *trans* and which is more mutagenic than are other enantiomers. This diol-epoxide metabolite is not a substrate for epoxide hydrolase and consequently is not detoxified by this route, and is highly mutagenic (see Levin *et al*., 1980; Jernström and Gräslund, 1994). The (−)-(7*R*,8*R*)-dihydrodiol of benzo[a]pyrene is significantly more tumorigenic than is the (+)-(7*S*,8*S*)-dihydrodiol. It was felt that in this case the configuration was more important for tumorigenicity than the chemical reactivity.

The hydroxylation of the drug bufuralol (**Figure 37**) in the 1-position only occurs with the (+)-isomer, whereas for hydroxylation in the 4- and 6-positions, the (−)-isomer is the substrate. Glucuronidation of the side-chain hydroxyl group is specific for the (+)-isomer (Francis *et al*., 1982). A further complication in human subjects is that the 1-hydroxylation is under genetic control, being dependent on the debrisoquine hydroxylator status. The selectivity for the isomers in respect of the hydroxylations is virtually abolished in poor metabolizers (Dayer *et al*., 1986). In addition to cytochrome P450, other enzymes are specific for or form specific isomers. Thus, epoxide hydrolase forms *trans*-dihydrodiols from cyclic epoxides and glutathione transferases are also stereospecific enzymes. It should be noted that stereospecific metabolism can have

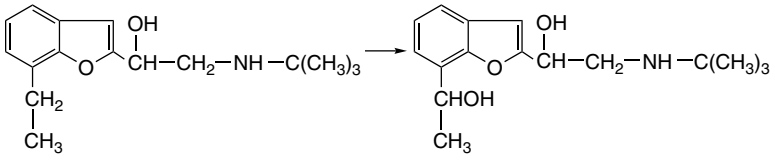

Figure 37 Stereoselective hydroxylation of bufuralol.

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regulatory implications (Srinivas *et al*., 2001; Battershill *et al*., 2004).

6 TOXICATION VS. DETOXICATION

The biotransformation of foreign compounds is often regarded as detoxication because it usually converts compounds into more water-soluble, readily excreted substances. This tends to decrease the exposure of the animal to the compound and so tends to decrease the toxicity. However, in some cases the reverse occurs and a metabolite is produced which is more toxic than the parent compound. There are many factors which affect this, such as the dose, availability of cofactors and the relative activity of the various drug-metabolizing enzymes. There may also be several, competing pathways of metabolism—some leading to detoxication, others to toxicity. Factors which alter the balance between these competing pathways will alter the eventual toxicity. This balance between toxication and detoxication pathways (**Figure 38**) is very important in toxicology and underlies some of the factors which affect toxicity (Nelson and Harvison, 1987; Monks and Lau, 1988; Nelson, 1995). Although in many cases the toxic metabolites are generated by the enzymes involved in Phase 1 pathways, there are now a number of examples where Phase 2 conjugation reactions are involved in toxication, as opposed to detoxication, processes. Thus glutathione conjugation of the cyanide antidote 4-dimethylaminophenol (DMAP) does not necessarily lead to inactive Phase 2 metabolites, but reactive glutathione conjugates of DMAP can generate methaemoglobin (Ludwig and Eyer, 1995).

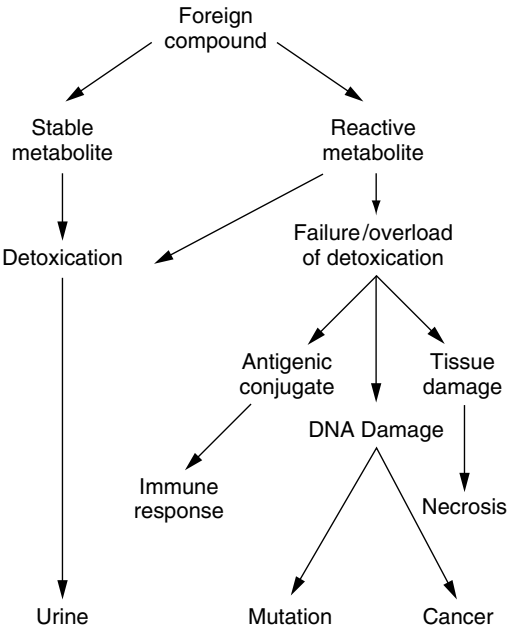

Figure 38 Some of the various consequences of biotransformation.

7 METABOLISM OF SPECIFIC SUBSTANCES

7.1 Paracetamol

A prime example of the role of competing metabolic pathways in toxicity and the importance of endogenous cofactors is afforded by the drug paracetamol (acetaminophen) (Monks and Lau, 1988). This widely used drug is sometimes taken in overdose, generally with suicidal intent. Such overdoses cause centrilobular hepatic necrosis in man and experimental animals, and a wealth of research has revealed that this toxicity is due in part to metabolism of the drug.

There are three pathways of metabolism for paracetamol (**Figure 39**), of which the two most important, quantitatively, are sulfate and glucuronic acid conjugation. The third, resulting in conjugation with glutathione, only represents a few per cent of the dose in humans. This last pathway involves an initial reaction catalysed by cytochrome P450, producing a reactive metabolite which is normally detoxified by conjugation with glutathione. The resulting glutathione conjugate is then further metabolized to a cysteine conjugate

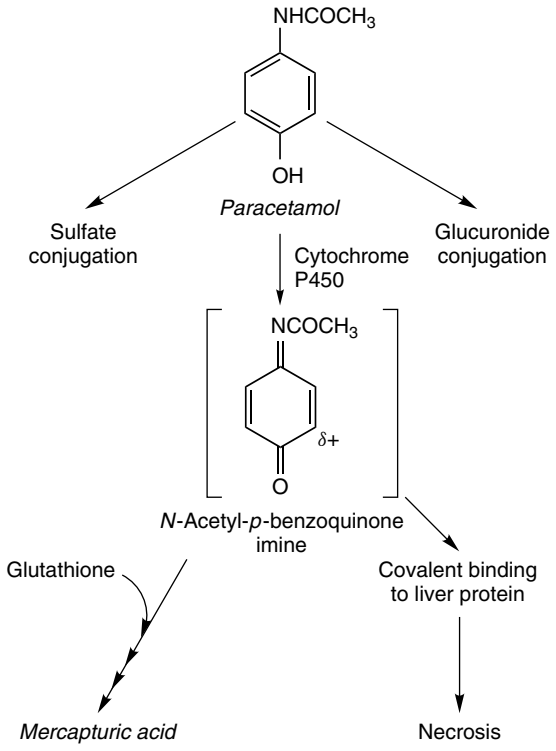

Figure 39 Metabolism of paracetamol.

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which is acetylated and excreted as an *N*-acetylcysteine conjugate or mercapturic acid.

Hepatotoxicity ensues when a sufficiently large dose of paracetamol is taken to deplete the liver of the majority of the glutathione (to 20% or less in experimental animals). This means that the reactive metabolite is able to react with cellular macromolecules, such as proteins, covalently bind to these macromolecules and cause hepatic necrosis. The nature of the reactive metabolite has received much attention, but seems to be a quinoneimine (*N*-acetyl-*p*-benzoquinoneimine (NAPQI)), although the exact mechanism of formation is not certain (Nelson, 1995) (see review by James *et al*., 2003).

In liver microsomal incubations both *in vitro* and *in vivo,* radiolabelled paracetamol binds covalently to protein, and the major adduct isolated from these protein conjugates is 3-cystein-5-yl-4-hydroxyaniline. A number of protein targets have been isolated, in particular a 55–58 kDa cytosolic protein and a 100 kDa protein, which is *N*-10-formyltetrahydrofolate dehydrogenase. Arylation of this protein correlates with loss of enzyme activity (Pumford *et al*., 1997). Mitochondrial protein targets such as glutamate dehydrogenase have also been identified. Again, arylation is associated with loss of enzyme activity (Pumford *et al*., 1997). The loss of enzyme activity may contribute to the development of toxicity, such as the detoxication of ammonia. However, as yet unidentified targets such as a 58 kDa protein may also play a role in the development of the toxicity and it is likely that a number of separate events are involved (Cohen and Khairallah, 1997).

It seems, however, that an electrophilic metabolite is involved in some way in the hepatotoxicity. It is clear that the NAPQI is a cytotoxic, reactive metabolite of paracetamol, produced via an oxidation reaction catalysed by one of several cytochrome P450 enzymes, including CYP 1A1, CYP 1A2, CYP 2E1, CYP 3A1 and CYP 3A2. Evidence suggests that Kupffer cells and nitric oxide (NO) are also involved in the metabolic activation (Pike *et al*., 1998; Ito *et al*., 2004). The reactive metabolite produced will react with glutathione. It reacts with glutathione in two ways: (i) by forming a conjugate and (ii) by oxidizing glutathione and being itself reduced back to paracetamol. NADPH can also reduce NAPQI back to paracetamol in a reaction which may involve glutathione reductase. The reactive metabolite can also react with cysteine residues in proteins via either arylation or oxidation of the cysteine SH.

Deacetylation of paracetamol can also occur, giving rise to *p*-aminophenol and subsequent metabolites; *p*-aminophenol is a known nephrotoxin, and this might account for the nephrotoxicity seen after overdoses of paracetamol and occasionally reported with chronic administration.

There are two main detoxication pathways for paracetamol: conjugation with (i) glucuronic acid and (ii) sulfate. The oxidative pathway(s) leading to mercapturic acid accounts for about 5‰in man. The deacetylation pathway is also presumably minor. However, this balance may be altered in a number of ways. Large doses of paracetamol may deplete animals of sulfate, as well as glutathione. Metabolism may thereby be diverted through the oxidative pathway catalysed by the cytochrome P450 enzymes and more will be conjugated with glucuronic acid (**Figure 39**). Factors which affect the activity of the microsomal enzymes, such as inducing agents will also alter this balance. For example, pretreatment of animals with phenobarbital will increase the hepatotoxicity in some species (rats) by increasing the amount metabolized via the cytochrome P450 pathway. However, this pretreatment will also increase the activity of glucuronosyl transferase and in some species (hamsters) this effect will decrease the hepatotoxicity. In the hamster the oxidative pathway is quantitatively of greater importance than in the rat, a factor which underlies the large difference in susceptibility to the hepatotoxicity between these two species.

7.2 Carbon Tetrachloride

Carbon tetrachloride is a potent hepatotoxin which has been studied extensively. The major toxic effect carbon tetrachloride causes is centrilobular hepatic necrosis, which is dependent upon metabolism via the cytochrome P450 system (CYP 2E1, CYP 2B1 and/or CYP 2B2). However, the enzyme system is acting as a reductase in this instance (reductive dehalogenation). Cytochrome P450 donates an electron to the carbon tetrachloride molecule and thereby allows the homolytic cleavage of a carbon–chlorine bond (**Figure 40**). This yields the trichloromethyl radical and a chloride ion. The trichloromethyl radical may then react with oxygen to give the trichloromethylperoxy radical. Alternatively, the trichloromethyl radical can abstract a hydrogen atom from polyunsaturated lipids and thereby form a lipid radical and a stable product, chloroform. The lipid radical

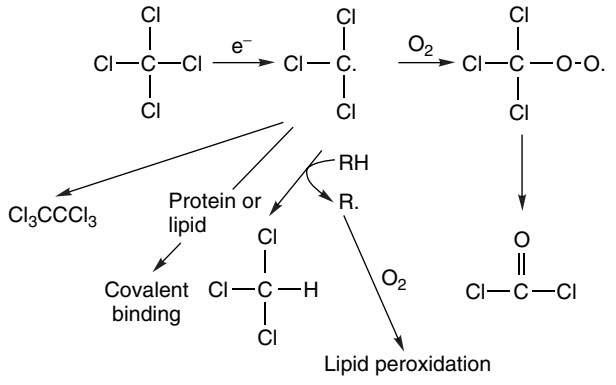

Figure 40 Metabolism of carbon tetrachloride.

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can then proceed to react with other cellular constituents and cause a cascade of disturbances within the cell. Tumour necrosis factor α, NO and transforming growth factors α and β are activated. Also, the trichloromethyl radical can react covalently with lipids and proteins and it is believed that the trichloromethylperoxy radical is the reactive metabolite responsible for lipid peroxidation (see reviews by Weber *et al*., 2003; Manibusan *et al*., 2007).

The oxygen concentration is important for the formation of the trichloromethylperoxy radical and lipid peroxidation. Elevated oxygen concentrations *in vitro* and *in vivo* reduce lipid peroxidation and hepatotoxicity. Burkhart *et al*. (1991) suggested that oxygen, including hyperbaric oxygen, might have a role in carbon tetrachloride poisoning. As carbon tetrachloride is metabolically activated by cytochrome P450 in the smooth endoplasmic reticulum, the reactive radicals formed are able to react with the enzyme itself and the lipids of the endoplasmic reticulum. The result is that cytochrome P450 is destroyed by carbon tetrachloride. Consequently, if animals are given a small dose of carbon tetrachloride ($0.05\,\mathrm{ml\,kg^{-1}}$), a subsequent larger dose is less toxic than the same dose administered to a control animal (Glende, 1972). This is because the form of cytochrome P450 which activates carbon tetrachloride is destroyed. Carbon tetrachloride is thus hepatotoxic only when it can be metabolically activated (Sesardic *et al*., 1989).

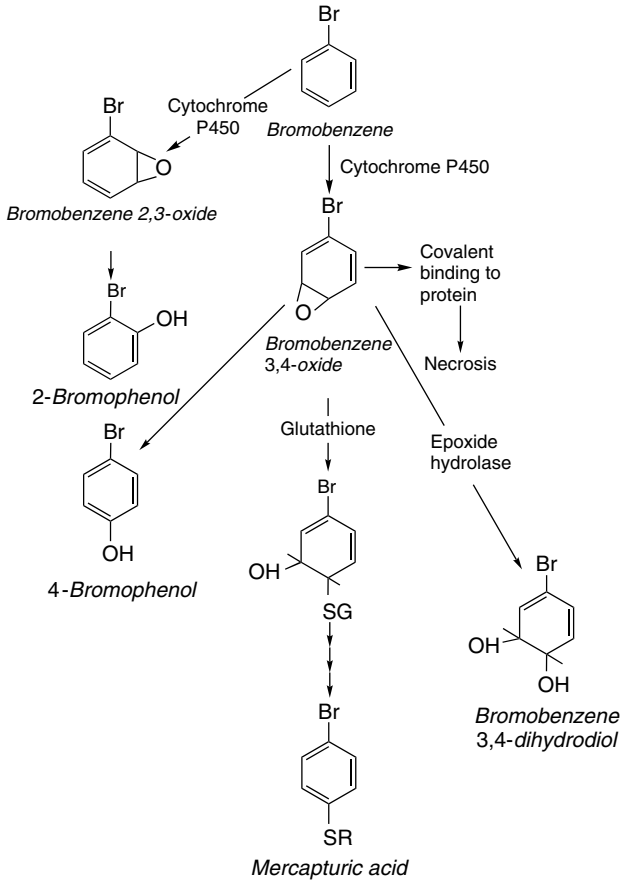

Figure 41 Some of the metabolic pathways for bromobenzene.

7.3 Bromobenzene

Bromobenzene is another hepatotoxic compound, but one which may also damage the kidneys, and the involvement of metabolism in these two toxic effects is different. Bromobenzene metabolism affords an interesting example of the importance of competing pathways of detoxication vs. toxication and of the way in which metabolic pathways may be switched by inducers. The metabolism of bromobenzene is complex, proceeding via two different epoxides and involving multiple reactive intermediates (Lau and Zannoni 1979; 1981; Beyhl and Mayer, 1980; Lau *et al*., 1984).

Bromobenzene is metabolically activated by oxidation, catalysed by cytochrome(s) P450 (CYP 2B) (Harauchi and Hirata, 1994), to yield an intermediate 3,4-epoxide (**Figure 41**). This epoxide is chemically reactive but may be detoxified in two ways. The first involves conjugation with glutathione (**Figure 41**), eventually giving rise to a mercapturic acid conjugate which is excreted in the urine (Thor *et al*., 1979). Cytosolic glutathione transferases seem to be involved in the conjugation of the epoxide with glutathione rather than the microsomal glutathione transferases or a direct chemical reaction with glutathione; moreover bromobenzene adducts to cytosolic glutathione transferases have been isolated (Aniya *et al*., 1988; Koen *et al*., 2006). The second route of detoxication is metabolism to the dihydrodiol mediated by epoxide hydrolase (Monks *et al*., 1984). Just as with paracetamol, a sufficiently large dose of bromobenzene will deplete the liver of glutathione and, hence, the reactive epoxide will bind to tissue macromolecules, a process which seems to underly the liver necrosis.

There is another oxidation pathway, however, also catalysed by cytochrome(s) P450, which gives rise to another epoxide, the 2,3-epoxide, which is believed to be nontoxic. The evidence for this comes from the effect of inducers on the toxicity and metabolism, and illustrates switching of metabolic pathways. Pretreatment of animals with phenobarbitone increases metabolism via the 3,4-epoxide pathway and increases the toxicity. Conversely, pretreatment with 3-methylcholanthrene increases metabolism via the 2,3-epoxide pathway, as indicated by an increased excretion of *o*-bromophenol, and decreases the toxicity (**Table 9**). Also, mice that have the cytochrome P450 isoenzyme, which catalyses the formation of the 3,4-epoxide, show greater hepatic damage after doses of bromobenzene than do those that who do not. *In vitro*, the 3,4-epoxide binds to microsomal protein and seems to prefer to bind to histidine residues, whereas the 2,3-epoxide binds to

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Table 9 Effect of induction with 3-methylchlolanthrene on the metabolism of bromobenzene in rats

Metabolite	% Total urinary metabolites	
	Control	3-MC treated
4-Bromophenylmercapturic acid	72	31
4-Bromophenol	14	20
4-Bromocatechol	6	10
4-Bromophenyldihydrodiol	3	17
2-Bromophenol	4	21

Data from Zampaglione *et al*. (1973).
Dose of bromobenzene 10 mmol kg^{-1}.

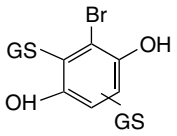

Figure 42 Diglutathione conjugate of bromohydroquinone.

haemoglobin and prefers to bind to the cysteine residues. It is suggested that the 2,3-epoxide is less reactive and more stable than the 3,4-epoxide, and therefore reacts with a different target protein (Lau and Zannoni, 1981). However, the 3,4-epoxide was detected in both blood *in vivo* and hepatocyte incubation medium *in vitro*, when animals or hepatocytes were exposed to bromobenzene. Clearly the reactive intermediate has sufficient stability to move within the hepatocyte and even leave the cell. There would seem to be an optimum reactivity for the epoxide in order for it to be toxic and react with critical sites on macromolecules. Reactive metabolites which are chemically too reactive will tend to interact with biological molecules indiscriminately and may never reach critical target molecules. Further metabolism of the bromophenols may also occur, to give bromoquinols and bromocatechols. 2-Bromohydroquinone will deplete glutathione in both liver and kidney, but only causes pathological damage in the kidney. It is believed that the diglutathione conjugate of bromohydroquinone (**Figure 42**) is the nephrotoxic agent (Monks *et al*., 1991). The metabolism of halogenated benzenes has been reviewed (den Besten *et al*., 1994).

7.4 Methanol and Allyl Alcohol

Methanol is a widely used and readily available solvent, which is often found in combination with ethanol. Consequently, it sometimes features in poisoning cases (see reviews: International Programme on Chemical Safety, 1997; International Programme on Chemical Safety, 2001; Barceloux *et al*., 2002). The toxicity of methanol illustrates the role of metabolism in toxicity where a

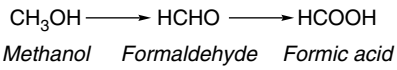

Figure 43 Metabolism of methanol.

chemically reactive metabolite does not seem to be involved. Also, a species difference in detoxication is revealed. It also illustrates the importance of understanding the mechanism of toxicity in order to treat the poisoned patient in a rational way. Methanol is toxic mainly as a result of metabolism to formic acid (**Figure 43**). This is a two-step reaction, with metabolism to formaldehyde being catalysed by either ADH or catalase. The second metabolic step to give formic acid is catalysed by either ALDH or formaldehyde dehydrogenase, an enzyme which requires glutathione.

The formic acid damages the optic nerve, seemingly by inhibiting the mitochondrial enzyme cytochrome oxidase and, hence, reducing the level of ATP available to the nerves (see **Ophthalmic Toxicology**). Humans and certain other primates are much more susceptible than are rodents to the toxicity of methanol; consequently nonhuman primates have been used as a model for human poisoning with methanol (Martin-Amat *et al*., 1977), and indeed are probably the only animal models of poisoning in man that can be used to study antidotes (Blomstrand and Ingemansson, 1984). The differences between primate and rodent susceptibility seems due, at least in part, to the accumulation of formic acid in the former to a greater degree than the latter. Because the metabolism is clearly important in the toxicity, treatment of the poisoning involves blocking the first step in the metabolic pathway by using ethanol as a competitive inhibitor of ADH. An alternative inhibitor of alcohol oxidation, 4-methyl pyrazole (fomepizole) has also been studied as an antidote to methanol (McMartin *et al*., 1980; Blomstrand and Ingemansson, 1984; Flanagan and Jones, 2001).

Another alcohol which is metabolized via ADH, leading to toxicity is allyl alcohol. This compound causes periportal liver necrosis when administered to animals (Badr, 1991; Atzori *et al*., 1989). This is believed to be due to oxidation to allyl aldehyde (acrolein, 2-propenal) catalysed by ADH (**Figure 18**). Allyl aldehyde is reactive and may cause toxicity by reacting with critical macromolecules in the cell. Glutathione conjugation is likely, as an *N*-acetylcysteine conjugate is excreted in the urine. Elegant work using deuterium-labelled allyl alcohol showed that oxidation was necessary for the toxicity (Patel *et al*., 1983).

7.5 Isoniazid

Isoniazid is a first line therapy for tuberculosis, usually given with rifampicin. Isoniazid is a drug which may

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cause hepatic damage in some patients (see review by Tostmann *et al*., 2008). There are several routes of metabolism, but the most important route is the acetylation reaction (**Figure 44**) (see Section 5.4). The acetyl-isoniazid that results from this is further metabolized by hydrolysis to yield acetylhydrazine and isonicotinic acid. Acetylhydrazine has been shown to be hepatotoxic in experimental animals and this is due to further metabolism via a cytochrome P450-mediated pathway (**Figure 44**). The product is suggested to be an N-hydroxylated metabolite, which, on loss of water, would yield a diazene, which can fragment to a reactive intermediate. This may be either a radical or a carbonium ion, which may react with proteins and cause hepatic necrosis. This pathway is induced by phenobarbital, which also increases the covalent binding of the acetyl group to protein and the hepatotoxicity of acetylhydrazine (Lauterburg *et al*., 1985a; 1985b). However, acetylhydrazine may also be further metabolized by a different route, a second acetylation step, of which the effect is detoxication. Both the primary acetylation step, giving acetylisoniazid, and this second step are influenced by the acetylator phenotype (see above). Therefore, although rapid acetylators produce more acetylisoniazid and therefore more acetylhydrazine, this is then more extensively removed by acetylation to diacetylhydrazine in the rapid acetylator. When the plasma level of acetylhydrazine in human subjects after a dose of isoniazid was determined, it was found that the slow acetylator has a greater exposure to acetylhydrazine. This example illustrates both the importance of competing detoxication and toxication routes of metabolism, and also the influence which genetic factors may have on toxicity (Timbrell *et al*., 1980; Lauterburg *et al*., 1985a; 1985b; Timbrell, 1988).

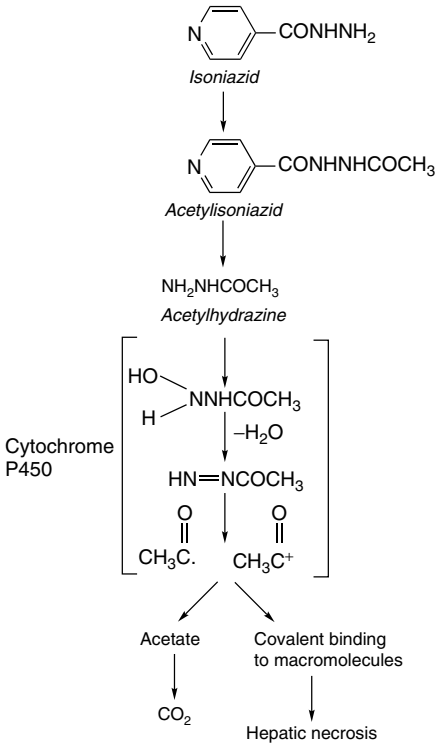

Figure 44 Part of the biotransformation of isoniazid.

7.6 Aromatic Amines

Another example in which acetylation features is the carcinogenicity of aromatic amines (see review by Vineis, 1994). For some aromatic amines, acetylation is a detoxication reaction. For example, 2-naphthylamine and benzidine both cause bladder cancer in man and it seems likely that the acetylation reaction reduces the carcinogenicity. Acetylated derivatives of benzidine are less reactive towards DNA and cause less damage to this macromolecule than does the parent compound. Several studies have shown that the slow acetylator is more at risk than is the fast acetylator from developing bladder cancer, especially those whose work may expose them to aromatic amines, although not all such studies have an increased risk (Evans *et al*., 1983; Miller and Cosgriff, 1983; Ladero *et al*., 1985; Hayes *et al*., 1993; Weistenhofer *et al*., 2008). It has been suggested that aromatic amines and heterocyclic compounds in tobacco smoke may contribute to the elevated risk of bladder cancer in cigarette smokers (Gu *et al*., 2005; Sanderson *et al*., 2007).

However, acetylation may be an activation step, involved in the toxic pathway for some compounds, such as 2-aminofluorene. There are several metabolic pathways involving acetylation and N-hydroxylation yielding *N*-acetoxyaminofluorene (**Figure 45**) and some of the metabolites are mutagenic (Heflich and Neft, 1994). The acetylation steps seem to involve similar, but possibly distinct enzymes, either NAT (see Levy and Weber, 2002), arylhydroxamic acid acetyl transferase (AHAT) and/or N-hydroxy-O-acetyl transferase (NHOAT). 2-Aminofluorene can also be oxidized by prostaglandin H synthase (Degen *et al*., 2002). The *N*-acetoxyaminofluorene may rearrange to yield a reactive nitrenium ion which can react with DNA to give covalent adducts such as *N*-(deoxyguanosin-8-yl)-2-aminofluorene (**Figure 45**). This has been shown both *in vitro* and *in vivo.* Alternatively, *N*-hydroxyacetylaminofluorene may be sulfated on the hydroxyl group and this has also been shown to be an activation step and may be involved in hepatocarcinogenesis, at least in mice. However, using hepatocytes from rabbits which were phenotyped as either rapid or slow acetylators, it was found that the rapid acetylators were more susceptible to DNA damage from aminofluorene, which suggests a role for acetylation. It seems likely that there are several routes for the metabolic activation of acetylaminofluorene in

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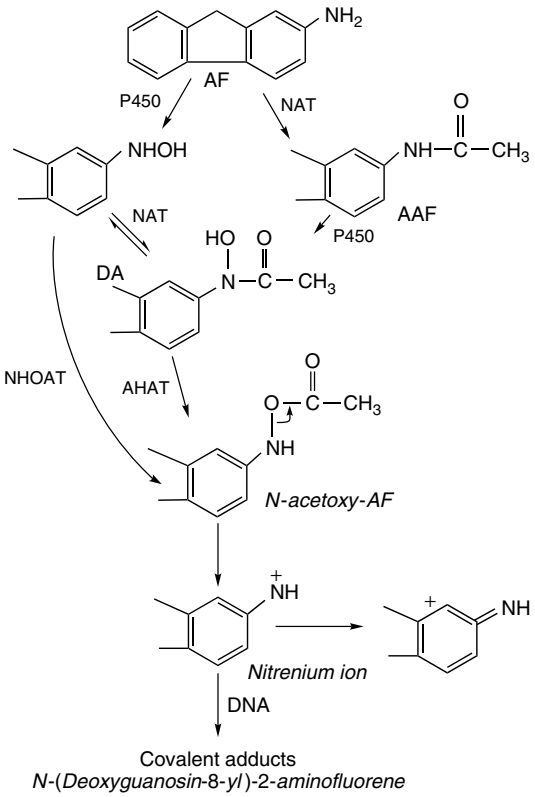

Figure 45 Pathways of metabolic activation of aminofluorene. AF: aminofluorene; AAF: acetylaminofluorene; NAT: N-acetyltransferase; DA: diacetylase; NHOAT: N-hydroxy-O-acetyl transferase; AHAT: arylhydroxamic acid acetyl transferase.

which acetylation, sulfation and glucuronidation may play a part, with variation in the predominant pathways between organs and species. This example, however, illustrates the role that Phase 2 metabolic pathways may have in metabolic activation.

7.7 Haloalkanes

The toxicity of haloalkanes illustrates the role of glutathione conjugation in metabolic activation, as opposed to detoxication. Several different haloalkanes are known to be metabolically activated by conjugation with glutathione. For example, the compounds 1,2-dichloroethane and 1,2-dibromoethane are both conjugated with glutathione in a reaction catalysed by glutathione transferase (**Figure 46**). The resultant haloethylglutathione conjugate can undergo loss of the halogen and rearrangement to yield a charged episulfonium ion which can react with DNA. Incubation of 1,2-dibromothane with DNA and glutathione transferase gives the adduct *S*-[2-(-*N*-guanyl)ethyl]glutathione. This interaction is believed to be responsible for the mutagenicity of these compounds (see review by Anders, 2004).

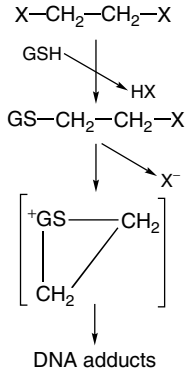

Figure 46 Formation of a reactive metabolite of a haloalkane from a glutathione conjugate.

Some haloalkanes are nephrotoxic and glutathione conjugation has been shown to mediate this toxic effect also. For example, hexachlorobutadiene is conjugated with glutathione, which is then further metabolized to a cysteine conjugate (**Figure 47**). This cysteine conjugate may then undergo further metabolism, either acetylation, deamination or cleavage by the action of the enzyme cysteine conjugate β-lyase. This gives rise to a thiol conjugate which has been shown to be nephrotoxic and is able to bind covalently to protein (Dekant and Vamvakas, 1996; Dekant, 1996).

7.8 Valproic Acid

In addition to enzymes commonly associated with drug metabolism, sometimes enzymes normally involved in intermediary metabolism may be responsible for metabolic activation. Thus, a metabolite of valproic acid (VPA; 2-*n*-propylpent-4-enoic acid) is believed to be involved in the hepatotoxicity sometimes caused by this drug. This metabolite, Δ^4-VPA, irreversibly inhibits enzymes of the β-oxidation system and destroys cytochrome P450. This reactive metabolite may be involved in the hepatotoxicity of the drug. Further metabolism, gives 3-oxo-Δ^4-VPA, which inhibits the enzyme 3-ketoacyl-CoA thiolase, the terminal enzyme of the fatty-acid oxidation system (Li *et al*., 1991; Baillie and Sheffels, 1995). However, recent *in vitro* evidence suggests that reactive oxygen species may also be involved in the toxicity (Tabatabaei *et al*., 1998).

8 CONCLUSION

It can be seen that biotransformation is often a crucial aspect of the toxicity of a compound and may, in a variety of circumstances, be the cause of toxicity rather than a

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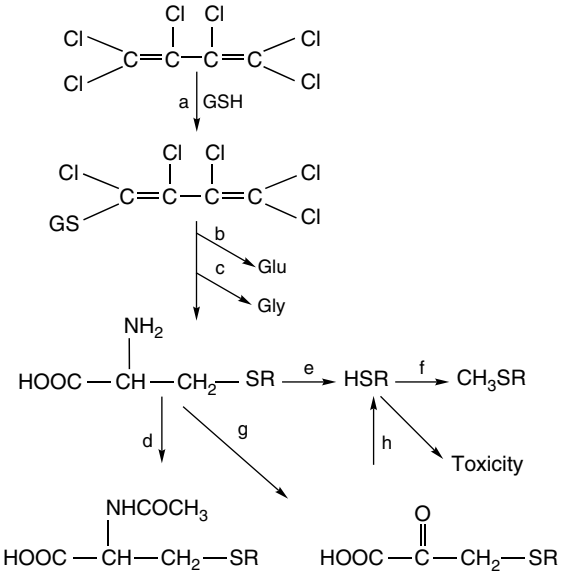

Figure 47 Metabolic activation of hexachlorobutadiene involving the action of β-lyase. The toxicity indicated is nephrotoxicity. R represents the 1,2,3,4,4-pentachlorobutadienyl moiety. GSH, glutathione; Glu, glutamate; Gly, glycine. the letters a–h represent the enzymes involved in the particular reactions: a, glutathione transferase; b, glutamyl transferase; c, dipeptidase; d, N-acetyltransferase; e, β-lyase; f, thiol S-methyltransferase; g, deaminase; h, thiopyruvate lyase.

process of detoxication. It is thus important that the toxicologist study the biotransformation of compounds under study both to understand the nature of the proximate toxicant and to understand what other factors (coadministration of other compounds, the state of the subject, etc.) may affect activation or detoxifying biotransformation pathways. Moreover, for regulated substances such as drugs and pesticides, it is normally required that biotransformation studies be undertaken. With pesticides, such studies are usually done in the rat using radiolabelled (most often using ^{14}C) compounds, as part of studies of absorption, distribution, metabolism and excretion. Such studies can be very useful in the understanding of the toxicity of the test compound. Where metabolism studies are done in more than one species, the studies may help identify which species will most resemble humans in toxic response. A further role of biotransformation studies is in the study of the combined actions of chemicals (Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment, 2002).

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Toxicokinetics of Xenobiotic and Metabolite Distribution and Excretion

Mario A. Inchiosa, Jr. and Mario E. Inchiosa

CONTENTS

1 INTRODUCTION

This chapter will focus principally on factors that influence the distribution of xenobiotics and xenobiotic metabolites in the human or animal body, the kinetics of the accumulation of such substances from acute and chronic exposures and the kinetics of elimination from depots that develop with chronic exposure. As part of this chapter, emphasis will be placed on the physicochemical characteristics of xenobiotics that permit them to penetrate potential physical barriers to their absorption into the plasma and their further distribution into anatomic compartments and tissues. Such barriers include, for example, the cellular membranes that constitute the mucosal surface of the digestive tract, the cellular barrier between the pulmonary alveolar space and the pulmonary interstitium and the cell walls of muscle and organs. The potential barrier to absorption and diffusion represented by the structures noted above is primarily related to the lipid constituents of cell membranes. The epidermis is another important barrier to absorption of xenobiotics. However, this represents only a relative barrier because the penetrability of chemicals through the skin can be affected by the degree of hydration of the skin.

2 CELL MEMBRANE BARRIERS

The bimolecular layer of lipids that make up the structure of cell membranes constitutes the principal barrier to the absorption of xenobiotics into the body, as well as the diffusion and distribution within the animal organism. Thus, the degree of lipid solubility of xenobiotics, whether drugs or toxins, substantially determines the ease of penetration of these chemicals through cell membranes. The entire length of the gastrointestinal tract, from the oral cavity to the anus, represents a continuous sheet of cell membrane surfaces, without any effective pores that would allow absorption of xenobiotics into the plasma. There are channels that permit equilibration of water and electrolytes, but their molecular dimensions are too small for significant diffusion of essentially all xenobiotic molecules through pores (Hogben *et al.*, 1959; Creasey, 1979; Bowman and Rand, 1980). Thus, it is known that highly polar drugs such as d-tubocurarine,

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which contains two fixed quaternary nitrogen atoms, and the sugar alcohol, mannitol, are not absorbed when ingested orally. In their clinical use, such drugs must be injected intravenously, and their further distribution in the body is largely limited to extracellular fluid spaces (Bowman and Rand, 1980). The cellular membranes of the epithelial layer in the respiratory alveoli represent the barrier to absorption of polar compounds following inhalation (Taylor and Gaar, 1970; Burton and Schanker, 1974a; 1974b). Likewise, the trophoblastic membrane of the placenta repels the diffusion of polar substances from the maternal to the foetal circulation (Creasey, 1979; Bowman and Rand, 1980; Pratt, 1990).

2.1 Diffusion Across Cell Membranes

It is the intrinsic lipid solubility of some xenobiotics that permits them to diffuse and distribute across cell membranes. However, since the animal body represents a mostly aqueous environment, drugs and other xenobiotics that gain access to the plasma and distribute further are characterized as having both lipid and water solubility. A particularly good example of this is a substance such as ethanol, which is readily soluble in water and distributes through cell membranes to effect an essentially equal concentration in all of the body water.

Although many endogenous mediators, substrates and metabolites are absorbed or distributed in the body by active transport or facilitated diffusion mechanisms, most drugs and other xenobiotics are absorbed and distribute according to the principles of simple diffusion. The important differences among these mechanisms is that active transport and facilitated diffusion overcome the apparent constraints related to lipid solubility for cell membrane transport and, in the case of active transport, may develop concentration gradients for the solute across membrane barriers. Thus, with simple diffusion, a substance will dissolve in one aqueous medium, for example, the interstitial fluid, possess enough intrinsic lipid solubility to dissolve in cell membrane lipids, and then equilibrate across the cell membrane to reach an equal concentration in another aqueous medium, the cell cytoplasm (**Figure 1**).

Another important characteristic of simple diffusion, as compared with active and facilitated diffusions, is that the latter mechanisms show saturation kinetics. That is, as increasing concentrations of solute are presented to one side of a cellular barrier, a point is reached where there is no further increase in the initial rate of diffusion of solute; in this case, all available transport sites are occupied at any given instant. With simple diffusion, there is a linear relationship between solute concentration presented to the diffusion barrier and the initial rate of diffusion (**Figure 2**). This feature is frequently exploited in clinical situations, where it is desirable to have rapid onset of a pharmacological effect, by loading the plasma concentration in order to obtain a rapid rise in an effect compartment, such as the cerebrospinal fluid (CSF).

2.2 Factors Influencing Simple Diffusion

2.2.1 Partition Coefficient

When a drug or a toxicant is a neutral (i.e. nonionizable) chemical, the relative rate of diffusion of this substance across cell membranes can be predicted by its lipid–water partition coefficient (Larson *et al.*, 1962; Pratt, 1990). Various organic solvents, such as chloroform and hexane, have been substituted for actual lipid solutions in the determination of partition coefficients,

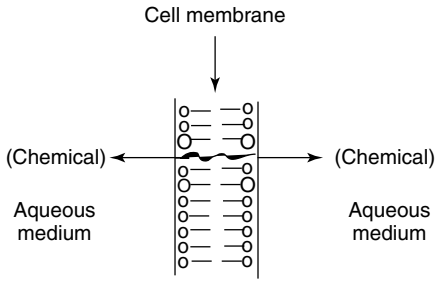

Figure 1 The lipoid barrier presented by the cell membrane.

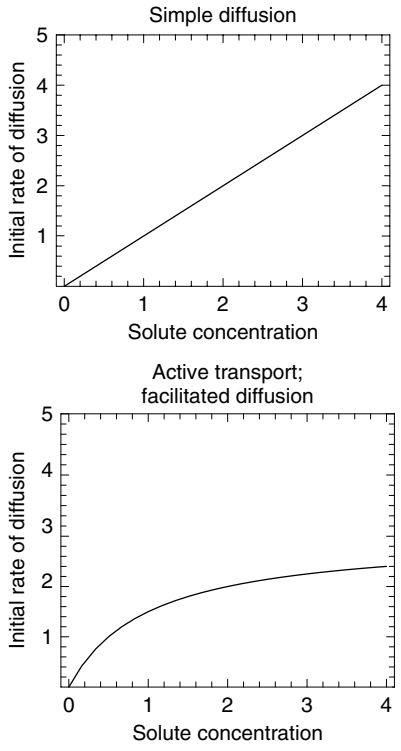

Figure 2 A comparison of the relationship between the solute concentration at the interface with a cell membrane and the initial rate of diffusion, for simple diffusion, and active transport and facilitated diffusion.

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although olive oil was commonly used as the organic phase in earlier studies. In fact, the term oil–water partition coefficient is still used. The common standard adopted by most drug and environmental regulatory agencies today is the *n*-octanol–water partition coefficient. It was demonstrated much earlier that the ease of diffusion across the cell membrane did not show a strong dependence on the molecular radius of the solute, but showed a primary correlation with the partition coefficient (Goldstein *et al.*, 1974) (presented in **Figure 3**). This is reasonable, since the solute is expected to dissolve and diffuse through a bimolecular layer of lipid. The contribution of molecular size to the rate of diffusion across the cell membrane would be the relative friction encountered in the lipid medium as size increases. Perhaps this does not become an important factor because of the small path length across this layer. Estimates have placed this in the range of 35–40 Å (Creasey, 1979).

2.2.2 Degree of Solute Ionization

Prediction of the ease and rate of diffusion across cell membranes is more complex when the solute is a weak acid or weak base. And this would represent the vast majority of drugs and toxicants of interest in this discussion. There is an interaction between two principal physicochemical factors that can be used to approximate the rate of diffusion across cell membranes, and the association with the rate of onset of pharmacological or toxic effects. One factor is the partition coefficient of the nonionized fraction of the weak acid or base; the second factor is the percentage of the drug or toxin that is nonionized at the pH of the body fluid at its interface with cell membranes. Since there is a wide variation in the pH of body fluids, the ease of diffusion of a chemical across cell membranes varies widely. If one is considering absorption from the stomach, the pH of stomach contents may be 1.4, and this will influence the state of ionization of a weak acid or weak base. In what is probably the other extreme, anticipating the absorption of a chemical from saliva through the membranes of the oral cavity may require consideration of the degree of ionization at pH 7.8. An example of consideration of an intermediate pH range may relate to the importance of reabsorption of a drug or toxin from renal tubular urine. Although there may be obligatory filtration of a chemical from plasma into tubular urine, as blood perfuses the renal glomeruli, the potential for the reabsorption of the solute into the efferent renal vessels is a function of the percentage of the drug that is nonionized at the pH of the tubular urine (perhaps 5.5) and the partition coefficient of the nonionized molecules. The extent of reabsorption of solute from tubular urine influences the persistence of the drug or toxin in the body and, therefore, its half-time ($t_{1/2}$) of elimination.

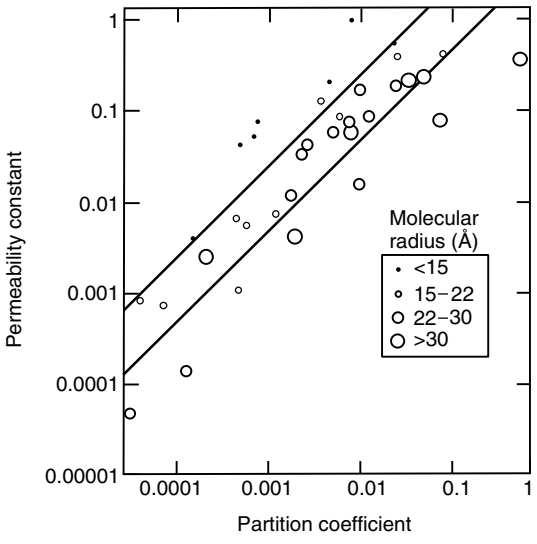

Figure 3 The correlation between the oil-water partition coefficient of a solute and membrane permeability. The lack of an appreciable contribution of molecular size to the relationship is illustrated; relative molecular radii are depicted by the size of the circles. (Reproduced from Goldstein *et al.*, 1974 © John Wiley & Sons, Inc.)

As noted above, a linear relationship between the rate of diffusion of a solute across cell membranes and the partition coefficient of the nonionized form of the solute is expected. The second factor, the percentage of the solute that is nonionized in a particular body medium, contributes substantially to the rate of diffusion that is observed. This nonionized fraction of a weak acid or a weak base, at a particular pH, can be calculated with the Henderson-Hasselbalch equations presented in Appendix A.

The ratios of the concentration of nonionized to ionized forms for weak acids and weak bases, in relation to the differences between the pH and pK_a of the solutes, is presented in **Figure 4**. It is noted that at the pH where there is no difference between the pH of the medium and the ionization constant (pK_a) of the solute, the ratio of the nonionized to the ionized form is 1. In fact, this is the definition of the ionization constant, that is, it is equal to the pH, where there are equal concentrations of the non-ionized and ionized forms of the solute. The figure also shows that this is true for both weak acids and weak bases.

The magnitude of the nonionized fraction is of importance in relation to the ease of establishment of an equilibrium across cell membranes. The nonionized form of the weak acid or weak base is the only form with any degree of lipid solubility. The chemical structure of the solute, that is, the presence of ring structures, long carbon chains, and so on, determines the relative degree of lipid solubility. The ionized fraction of the solute is considered to be completely hydrophilic, and unable to diffuse across cell membranes.

The importance of the ratio between the nonionized and ionized fractions of the solutes at the pH values

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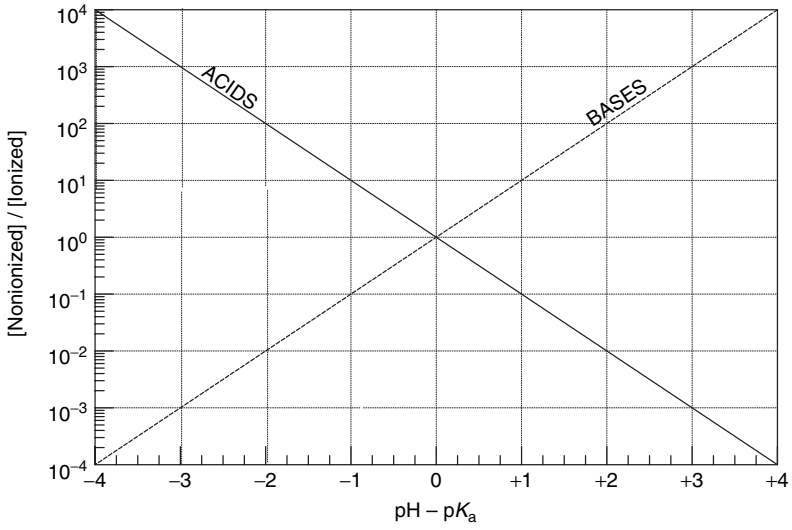

Figure 4 The effect of pH on the state of ionization of weak bases and weak acids. The relationships are only shown for a maximum difference of four units between pH and pK_a, but they can be projected to larger differences.

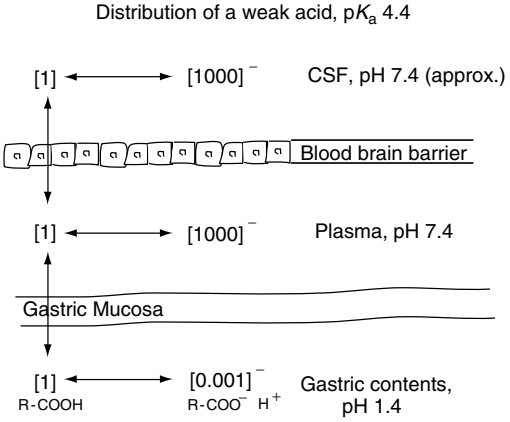

Figure 5 The influence of the pH on the equilibrium distribution of a weak acid, pK_a 4.4, among several anatomic compartments.

in different anatomic compartments can be illustrated by the influence of this ratio on the distribution of the solute across these compartments. **Figure 5** presents a simple example of the contribution of the pH in different anatomic compartments on the final equilibrium distribution of a solute, in this case a weak carboxylic acid with a pK_a of 4.4. (For illustration purposes, only the influence of pH and pK_a are considered in this example.) The nonionized form (the diffusible form) establishes the equilibrium across compartmental barriers. Equilibrium is reached when the diffusible form is at equal concentration in all compartments, and the concentration of the ionized form is appropriate to the pH of the compartment. **Figure 5** demonstrates that, in this example, the drug is at equal total concentration (nonionized + ionized forms) in CSF and plasma, while the total solute concentration in plasma is approximately 1000 times as great as that in the gastric contents. Also, this equilibrium would occur whether the solute had been administered orally or intravenously. It is obvious that even when only a small fraction of the solute is in the nonionized form, in this example approximately 1/10 of 1%, with adequate time this small fraction leads to the drawing of a high concentration of solute into the peripheral anatomic compartment represented by CSF. A therapeutic example where this is important would be in the use of drugs during pregnancy. From the principles that are described, drugs that must be used chronically during pregnancy can be assumed to reach the same concentration in foetal plasma as in maternal plasma. If one considers that the dosing regimen is often directed to maintain a steady-state therapeutic target concentration in the maternal plasma, then there would be ample time to reach equilibrium conditions in the entire body, including foetal plasma and tissues.

The issues of drug treatment during pregnancy also bring up how the state of ionization of drugs and the differences in pH between maternal and foetal plasma influence the risk to the foetus. Some of these considerations are depicted schematically in **Figure 6**. Ethanol would be expected to equilibrate with ease across the trophoblastic membrane of the placenta. This membrane consists of tightly opposed cellular elements that have no effective pores for molecules that are the size of most drugs. The highly polar skeletal muscle relaxant, atracurium, does not diffuse to any extent across the placental barrier. Lidocaine is commonly used as an epidural local anaesthetic for pain relief for both vaginal and caesarean delivery of the foetus. This weak base can show greater concentrations in foetal plasma compared to maternal plasma in conjunction with the relatively more acid conditions that may develop in the foetus in the near-parturient state. This has been termed the 'acid trapping'

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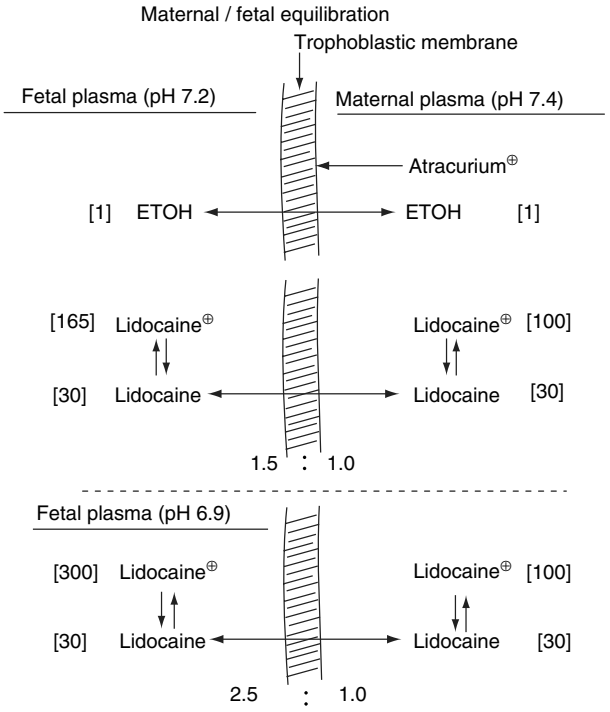

Figure 6 The effects of the pK_a values of several drugs and pH environments on the distribution of drugs between maternal and foetal plasma.

phenomenon of weak bases in acid environments in the body (Millot *et al.*, 1997).

2.2.3 Combined Effects of Partition Coefficient and Ionization

An approximation of the interaction between the partition coefficient of the nonionized form of a solute and the fraction of solute that is nonionized at a particular pH has been termed the 'effective partition coefficient'. Goldstein *et al.* (1974) suggested this term for the arithmetic product of the value for the partition coefficient of one of a series of compounds in a particular immiscible organic solvent-water mixture and the fraction of the drug that is nonionized at a pH of interest. This approach proved to be adequate to establish a rank order of the expected rates of diffusion of a series of weak acids and bases from plasma into CSF (data compiled by Goldstein *et al.*, 1974). A small sample of values from these data compares the effective partition of two clinically important intravenous barbiturates, thiopental and pentobarbital (**Table 1**). These calculations suggest that thiopental would be expected to demonstrate the onset of central nervous system depression approximately 40 times more rapidly than pentobarbital. In fact, these drugs do demonstrate a marked difference in onset of effect when the doses are adjusted to demonstrate their physicochemical properties. We demonstrate this comparison in the medical pharmacology course at New York Medical College. Intravenous injection of 12.5 mg kg^{-1} of thiopental in New Zealand White rabbits results in an immediate surgical anaesthetic state. Injection of the same dose of pentobarbital results initially in only slight impairment of motor function with no loss in consciousness. The motor instability progresses over approximately the next 15 minutes, but the animal never losses consciousness.

Table 1 Effective partition coefficient (expresses the interaction between pK_a and pH). Can be used to rank drugs in relation to onset of effect

	pK_a	Fraction nonionized at pH 7.4 × partition coefficient of non-ionized drug	Effective partition coefficient
Thiopental	7.6	0.61 × 3.3	2.01
Pentobarbital	8.1	0.83 × 0.05	0.04

Another example of the application of the effective partition concept is presented in **Table 2**, which lists the data used in the calculation of the effective partition coefficients in plasma of three narcotic analgesic drugs, and the time for their peak analgesic effects following intravenous bolus injection (Stoelting, 1987). The time to peak analgesic effect ranks inversely with the magnitude of the effective partition coefficient.

The effective partition coefficient was only intended as an approach to rank drugs in order of their expected rates of production of pharmacological or toxicological effects. A more direct approach to predict relative rates of diffusion across cell membranes has been termed the 'overall partition coefficient' (Bowman and Rand, 1980). In this approach, the partition coefficient is determined with the aqueous phase buffered at the pH of interest, that is, pH 1.4 for stomach contents, pH 7.4 for plasma, and so on. For prediction of absorption by inhalation, for example, a relatively neutral pH of the aqueous phase (approximately 7.0) may be chosen to simulate that of the surfactant layer of the pulmonary alveoli (Mason *et al.*, 1977).

Table 2 Effective partition coefficient used to rank time to peak analgesia

	Fraction non-ionized at pH 7.4 × Octanol : H_2O partition coefficient	Effective partition coefficient	Peak analgesic effect (minutes)
Alfentanil	0.89 × 129	115	2
Fentanyl	0.08 × 816	65	6
Morphine	0.23 × 1.4	0.3	20

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An equation has been proposed for estimation of the overall partition coefficient, P_0, as follows (Bowman and Rand, 1980):

$$P_0 = \frac{P_n}{\text{antilog } (\text{pH} - \text{p}K_a) + 1} \quad (1)$$

where P_n is the intrinsic partition coefficient for the nonionized form of the solute.

Equation 1 is in the form relevant to calculations for weak acids; the term $(\text{pH} - \text{p}K_a)$ is reversed to $(\text{p}K_a - \text{pH})$ when the equation applies to weak bases.

3 COMPARTMENTS OF BODY WATER

Although this chapter will primarily focus on a discussion of pharmacokinetic 'compartments' in relation to distribution and excretion of xenobiotics, it may be useful to begin the discussion with the dimensions of body water. Although the volume of distribution (V_d) of a solute is always referred to as an apparent V_d, chemicals with clearly defined physicochemical properties may sometimes be accurately predicted to distribute in relationship to anatomic compartments of body water. A classical presentation of these compartments is presented in **Figure 7**. The cross-hatched lines represent the lipoid barriers represented by cell membranes. The interrupted line between the plasma and interstitial spaces relates to the porous nature of this boundary. The importance of binding to plasma proteins on the distribution of xenobiotics, as well as sequestration ('storage') of solutes within tissues, is also illustrated. Filtration of solute from the plasma into the renal tubules is depicted, as well as reabsorption of lipid soluble solute from the renal tubules. It may be noted that all distributions, with the exception of the direction of formation of products of metabolism, are presented as double arrows to represent the equilibration of solute throughout the body.

The relative compartment volumes depicted in **Figure 7** have been found to apply to man and other mammalian species. Ethanol, which has both lipid and water solubility and shows little or no binding to macromolecules, distributes uniformly in all of the body water. It essentially marks the total volume of body water; it has a V_d of approximately 60% of body weight, or 42 l in a 70 kg subject. In another example, inulin is a highly polar substance that is not absorbed by oral administration, must be injected in order to gain access to the plasma, and is found to mark the extracellular fluid compartments, that is, plasma and interstitial fluid. Inulin is completely eliminated by the kidney, and is not metabolized or taken up into cells. It demonstrates a V_d of approximately 20% of body weight, or 14 l per 70 kg of body weight (Guadino and Levitt, 1949). These same properties are shared by the sugar alcohol, mannitol (Bowman and Rand, 1980). Of note, several of the neuromuscular blocking drugs, such as atracurium and pancuronium, which are also polar molecules with fixed positive charges related to their quaternary nitrogen groups, also equilibrate exclusively in extracellular fluid and demonstrate apparent volumes of distribution that approximate 20% of body weight (Shanks, 1986).

As noted above, for illustration purposes, the boundary between the plasma and interstitial fluid compartments in **Figure 7** is presented as a fenestrated line, to represent the porous barrier between these two spaces. These pores are considered to be spaces at the junctions between the endothelial cells at the capillary level. The pores in skeletal muscle capillaries have been estimated to have a radius of approximately 30 Å (Renkin, 1964). Water, electrolytes and smaller water-soluble molecules are able to filter through these pores and equilibrate

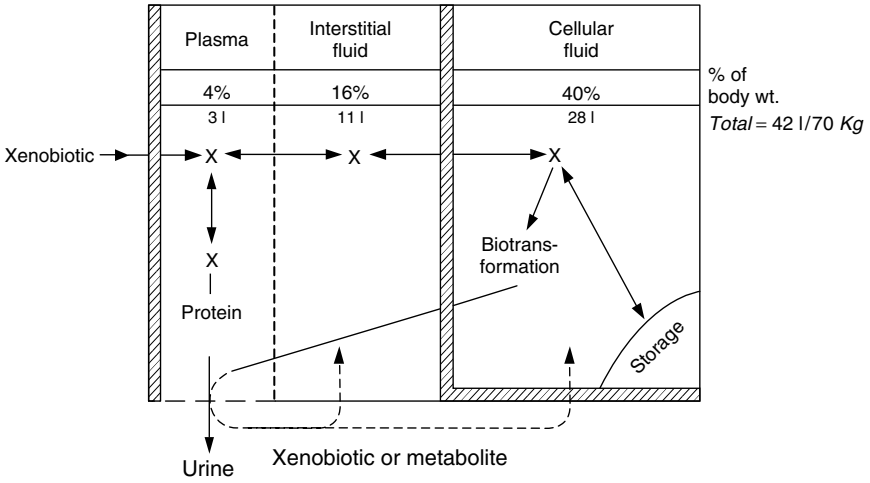

Figure 7 The major anatomical compartments of body water, expressed as both percentages of body weight and as litres in a 70 kg individual. (Reproduced with permission from Inchiosa, 2006. © Taylor and Francis Group.)

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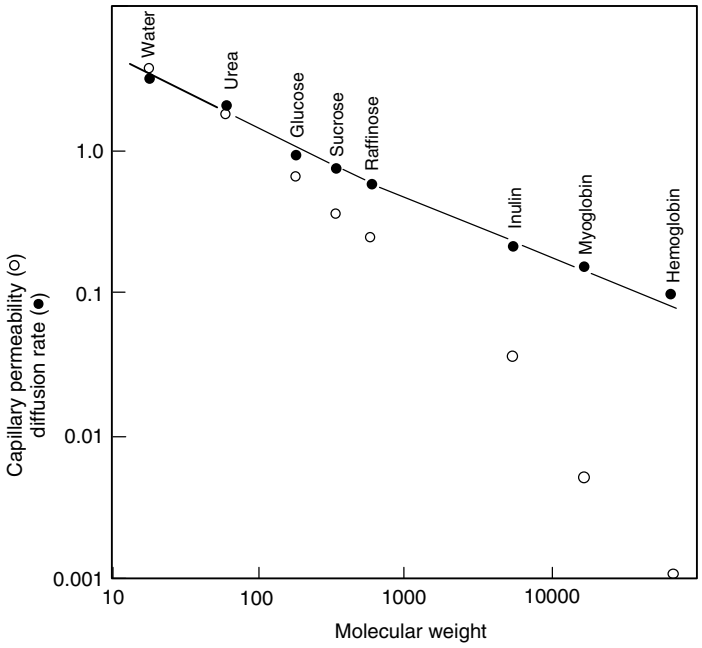

Figure 8 Relationship between molecular weights of several water soluble solutes and their diffusion rates. The filled circles represent diffusion rates ($cm^2\ s^{-1}$) in water at 37 °C, and the open circles indicate the permeability coefficients ($cm^3\ s^{-1}100\ g^{-1}$) across skeletal muscle capillaries. (Reproduced with permission from Inchiosa, 2006. © Taylor and Francis Group.)

between the plasma and interstitial fluid compartments. The relative rate of equilibration is demonstrated by the data in **Figure 8**. The rate of diffusion of raffinose (molecular weight (mol. wt) 594) is markedly more rapid that that of inulin (mol. wt 5500). Large protein molecules are effectively restricted from diffusion across the capillary membrane. Although inulin, with an effective molecular radius of 14.8 Å (Creasey, 1979), is somewhat slowed in its diffusion across the muscle capillary membrane, it is freely filtered from plasma into the kidney tubules across the more porous capillaries of the kidney glomeruli.

It was noted that highly polar substances must be injected in order to gain access to the plasma. Although this is accomplished most directly by an intravenous injection, injections into the interstitial fluid spaces also provide routes to the plasma, since they allow absorption through the capillary membranes into the plasma, in the tissues being perfused (**Figure 7**). Intramuscular, subcutaneous and intraperitoneal routes are examples of injections into interstitial spaces that present no lipoid barriers to the absorption of polar solutes into the plasma.

As noted above, the associations that are possible with certain chemicals between their defined physicochemical properties and their volumes of distribution are almost the exception. For many drugs and other xenobiotics, the influence of macromolecular binding peripheral to the plasma does not permit identification of an anatomic volume in which the solute distributes. The pharmacokinetic 'V_d' of a solute is always designated as an apparent V_d. In the simplest case of a one-compartment pharmacokinetic model, where the solute in the body behaves as though there are no barriers to its distribution and redistribution as the concentration in plasma changes, the formula for calculation of the pharmacokinetic V_d is:

$$V_d = \frac{\text{Dose (mg)}}{\begin{array}{c}\text{Plasma concentration after an initial dose}\\ \text{(or change in concentration from baseline)}\\ (\text{mg l}^{-1})\end{array}} \qquad (2)$$

The calculation simply indicates the 'apparent volume' that the drug must occupy if the concentration throughout its distribution is the same as that in the plasma.

4 EFFECTS OF MACROMOLECULAR BINDING ON DISTRIBUTION

The discussion of xenobiotic distribution in the body to this point has not considered the contribution of the binding of xenobiotics to proteins and other macromolecules to the final equilibrium distribution of the solute. The relative strength of affinity of binding to macromolecules determines the final equilibrium concentration, bound + free, of the solute in plasma and other tissue compartments. These binding forces may greatly influence what is calculated to be the apparent V_d of the solute.

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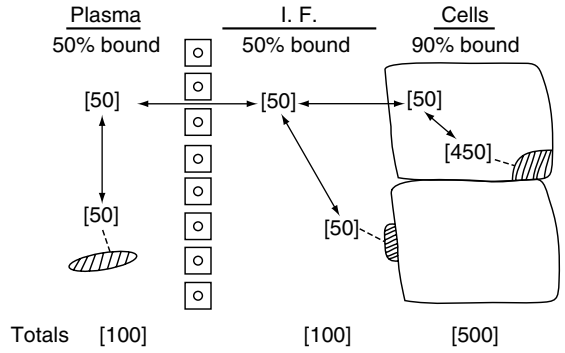

Figure 9 Schematic depiction of the influence of the percentage binding of a solute to soluble proteins, structural entities, organelles and so on, on the equilibrium distribution of the solute in the major anatomical compartments.

The percentage of drug binding to plasma proteins is one of the pharmacokinetic parameters included in most drug compendia. The fraction bound in other anatomic compartments is usually not known. **Figure 9** is presented to illustrate the effect of the competition between plasma protein binding and peripheral binding on the distribution at equilibrium. Reversible equilibrium binding to macromolecules usually represents relatively weak binding forces, such as electrostatic interaction, hydrogen bonding, van der Waals forces, binding of lipophilic molecules to hydrophobic side chains of amino acids in proteins, as well as the partitioning of such molecules into lipid depots. Electrostatic interactions are of particular importance in the interaction between the ionized forms of weak acids and weak bases and their counterions, represented by the charged groups on the side chains of amino acid residues of proteins. For example, the $-COO^-$ groups of aspartic and glutamic acids are particularly plentiful in protein molecules, as well as the $-NH_3^+$ group of lysine (Tanford *et al.*, 1955; Goldstein *et al.*, 1974); these groups will attract the counterions of weak bases and weak acids, respectively.

The number of potential binding sites and their relative affinities contribute importantly to the distribution of solute under equilibrium conditions. Although the percentage of drug binding to plasma proteins is a fundamental pharmacokinetic parameter, it would be of limited application if the percentage of binding varied considerably over the range of therapeutic plasma drug concentrations. In fact, the binding percentage is expected to remain quite constant for most xenobiotic entities. The relationships among the number of potential binding sites, the binding affinity and the percentage binding are presented in **Figure 10**. It can be seen that as the number of binding sites per protein molecule increases, plasma drug concentrations may vary several orders of magnitude without a change in the percentage bound.

It was noted above that the negatively charged side chains of aspartic and glutamic acid are important in the electrostatic interaction with the non-ionized fractions of weak bases. The relationships in **Figure 10** can be placed in perspective by the fact that there is a total of 101 aspartic and glutamic acid residues per molecule of bovine serum albumin. Similarly, there are 57 positively charged side chains of lysine residues per molecule of protein that are available for binding the non-ionized fractions of weak acids (Tanford *et al.*, 1955; Goldstein *et al.*, 1974). As noted above, however, non-ionic binding also takes place between xenobiotic molecules and macromolecules.

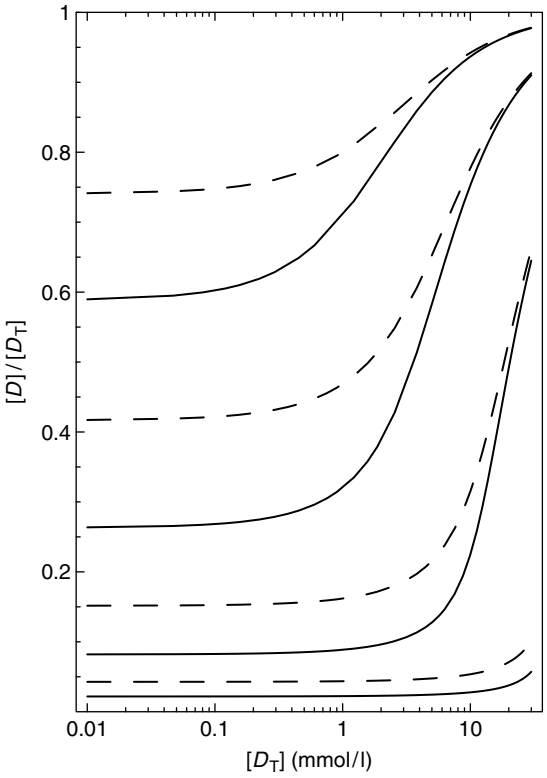

Figure 10 The influence of the potential number of binding sites (n) per molecule of albumin and the dissociation constant for the ligand (K), on the ratio of the unbound concentration [D] to the total solute concentration. The abscissa presents the total drug concentration [D_T] and the ordinate expresses the ratio of unbound concentration to total concentration of drug. The solid lines represent the theoretical results with a K of 1 mmol/l; the broken lines are for a K of 2 mmol/l. The pairs of curves from top to bottom are for n of 1, 4, 16 and 64 binding sites per molecule of albumin. The albumin concentration [P_T] was taken as 0.7 mmol/l. (Modified from Figure 40.9, Bowman and Rand, 1980.) The curves were calculated according to the following formula: $[D] = \frac{1}{2}\left([D_T] - K - nP_T + \sqrt{4D_TK + (-D_T + K + nP_T)^2}\right)$.

Although therapeutic drug concentrations rarely vary over even one order of magnitude, there are clinical situations where patients receiving a large number of drugs may show a competition for available binding sites.

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Plasma concentrations of free solute may increase as a result of such competition, and since the free drug may be responsible for receptor interaction, there may be an excessive pharmacologic or toxic effect. This scenario may also occur or be intensified in a patient with hypo-albuminaemia, which may occur in the aged or malnourished patient.

Figure 9 is designed to provide an example of the influence of macromolecular binding peripheral to the plasma on the final equilibrium distribution, that is the V_d of a xenobiotic. The diagram is schematic; the porous cellular barrier depicted between plasma and the interstitial fluid space represents the capillary endothelium. The size of cells of tissues or organs is not drawn to scale relative to endothelial cells; the size was convenient to depict details of xenobiotic concentrations. Macromolecular structures are represented by hatched lines. As shown, this includes soluble plasma proteins, cell membrane structures in the interstitial fluid compartment, and intracellular structures, or perhaps, lipid depots that sequester lipophilic molecules. The presentation in **Figure 9** is only meant to be illustrative of the many possible interactions of xenobiotic molecules that contribute to the measure of the apparent V_d of a solute, and to demonstrate how such binding forces result in unequal concentrations in different body environments.

It was noted above that competition among solutes for available binding sites can result in displacement of solute and increases in the free concentration. If the competition or displacement primarily affects the binding to plasma proteins, thereby allowing more free solute to leave the plasma, the total plasma concentration (bound + free) will decrease. The new equilibrium will show an increase in the apparent V_d of the solute. In comparison, if the competition or displacement primarily results in an increase in the concentration of free solute peripheral to the plasma, this will allow more solute to equilibrate back into the plasma, thereby raising the plasma concentration and causing a decrease in the apparent V_d. This follows from the fact that the calculation of the apparent V_d is determined mathematically from the division of the total amount of the xenobiotic in the body by the concentration in the plasma.

Units of concentration in **Figure 9** are not indicated, since the diagram is only intended to demonstrate relative concentrations as a result of different binding affinities. If we indicate, for example, that the units are $\mu g\,ml^{-1}$, then the free, diffusible form of the solute everywhere in the body would be expected to be $50\,\mu g\,ml^{-1}$. **Figure 9** is intentionally presented to include only free diffusible solute, and solute bound to macromolecules and structural entities, to limit the complexity. The presentation must be expanded to allow for the fact that there may be concentrations of free nondiffusible solute, as well. This may represent, for example, the polar ionized fractions of weak acids and weak bases. **Figure 11**

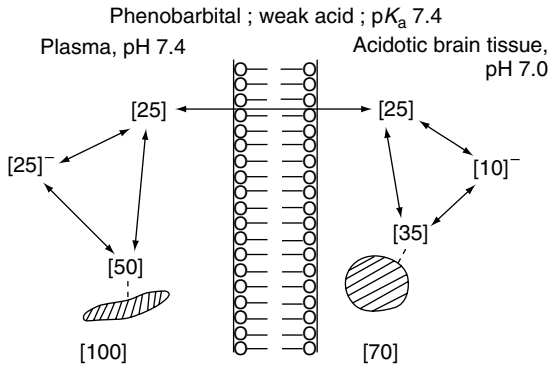

Figure 11 Schematic representation of the equilibrium distribution of a weak acid, phenobarbital, pK_a 7.4, which shows 50% total binding to macromolecular proteins and other structures in both plasma and the central nervous system.

suggests how the equilibrium would be influenced by circumstances where there may be insufficient binding capacity for all of the ionized solute. At equilibrium, the degree of ionization would influence the concentration of solute that is in the diffusible form, but it would be expected to be the same in all compartments. Obviously, different pH environments represented across the lipoid barrier, together with different affinities for macromolecular binding in the compartments, influence the final equilibrium. Phenobarbital was chosen to illustrate these interactions (**Figure 11**). Phenobarbital, a weak acid, provides a convenient starting point for this example, because its pK_a value is the same as the pH of plasma, that is, 7.4 (Holford, 2007). Therefore, phenobarbital would exist in equal concentrations of the ionized and nonionized forms in plasma. Also, the population value for its percentage binding to plasma proteins under normal circumstances is 51% (Holford, 2007). For convenience, the value was taken as 50% binding in both plasma and the tissue compartment, as presented in **Figure 11**. This diagram also suggests, schematically, that there may be binding of the nonionized fraction of phenobarbital to both plasma protein and to structures (organelles) in the tissue compartment. The concentration ratio of ionized to nonionized fractions of phenobarbital in the tissue compartment is calculated with the appropriate Henderson-Hasselbalch equation, noted above. In approximating the various concentrations of ionized and nonionized drug, the bound drug was considered to be in another phase, and not contributing to the proportion of ionized and nonionized molecules. Under these conditions, the concentration ratio of total drug in brain tissue to that in plasma is expected to be 0.7. Of course, it is not common that details of the percentage binding to macromolecules within tissues would be known. However, all of these factors become resolved in the final determination of the apparent V_d of the solute. Determination of V_d in multicompartmental models will be returned to, below.

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Table 3 Lack of consistent relationship between the percentage of binding of several drugs to plasma proteins and their apparent volumes of distribution

Drug	Percentage bound to plasma proteins	Apparent V_d per 70 kg body weight (l)
Gentamicin	10	18
Metronidazole	10	52
Metoprolol	11	290
Sulfamethoxazole	62	15
Cloroquine	61	13 000
Indomethacin	90	18
Imipramine	90	1600

The specific contribution of active and facilitated transport mechanisms to the final distribution of xenobiotics, including P-glycoprotein drug efflux pumps (Kurzawski *et al.*, 2007), is beyond the scope of this chapter. These active and facilitated processes may offset or enhance, to varying degrees, the forces associated with simple diffusion, and result in concentrations at equilibrium that are the net result of these forces. Again, however, all equilibrium forces will be encompassed in the final determination of V_d, and the implications of this V_d for the pharmacokinetics of the xenobiotic will be preserved.

The discussion thus far should provide a framework to appreciate why the single pharmacokinetic parameter of percentage binding to plasma protein for xenobiotics is not adequate to predict whether a chemical will have a small or large V_d relative to body mass. Some examples that show the inconsistencies between the apparent V_d of several drugs and their percentages of binding to plasma proteins (Holford, 2007) are presented in **Table 3**. It is obvious that the binding affinities of the solutes to macromolecules and membranes and organelles peripheral to the plasma, in combination with the states of ionization of the solutes and the partition coefficients of their nonionized fractions, result in a distribution at equilibrium that may have little or no relationship to the percentage of binding to plasma proteins.

5 DIFFUSION INTO SALIVA

Some of the principles described above also apply to the distribution of xenobiotics and their metabolites into saliva. Saliva may represent a convenient noninvasive access to monitoring xenobiotic exposure. It has been found that a fairly constant ratio may exist between the concentration of a xenobiotic in saliva and its concentration in plasma. For this reason, saliva measurements have been applied to studies of drug bioavailability, elimination pharmacokinetics and therapeutic monitoring (Matin *et al.*, 1974; Breckenridge *et al.*, 1977; Parsons and Neims, 1978; Inchiosa *et al.*, 1981; Newton *et al.*, 1981; Poland and Rubin, 1982). Matin *et al.* (1974) have expressed the factors involved in predicting the saliva to plasma ratio R in the following equation:

$$R = \frac{1 + 10^{(\mathrm{pHs} - \mathrm{p}K_a)} f_p}{1 + 10^{(\mathrm{pHp} - \mathrm{p}K_a)} f_s} \tag{3}$$

where
R = saliva/plasma ratio
pHs = saliva pH
pHp = plasma pH
f_p = fraction of drug unbound in plasma
f_s = fraction of drug unbound in saliva.

Equation 3 is in the form for solutes that represent weak acids. In calculations for weak bases, the exponent in the numerator is reversed to $\mathrm{p}K_a - \mathrm{pHs}$, and to $\mathrm{p}K_a - \mathrm{pHp}$ in the denominator. Throughout this chapter, it is assumed that the pharmacokinetic principles of distribution and elimination will generally apply to the metabolites of xenobiotic molecules, as well. With knowledge or prediction of the ratio between the saliva and plasma concentrations of a xenobiotic metabolite, saliva measurements may provide a convenient approach to monitoring xenobiotic exposure.

The conceptual utility of Equation 3 can be seen in that if it is applied to the data of **Figure 11**, and the brain tissue compartment is substituted for the saliva compartment in the equation, the ratio of brain concentration to that in plasma is 0.7, as predicted from first principles.

6 ACCUMULATION OF XENOBIOTICS AND THEIR METABOLITES

6.1 Agents with Dose-Independent Pharmacokinetics

With chronic administration or chronic exposure to xenobiotics that are eliminated by first-order kinetics, that is, as a constant percentage per unit of time, one can predict the time course of accumulation in the plasma. In the simplest presentation of this relationship, it is assumed that the elimination half-life ($t_{1/2}$) of the xenobiotic remains unchanged over the range of body burden of the xenobiotic. The same criterion would apply if one were following the rate of accumulation of a xenobiotic metabolite, that is, the metabolite was the entity of interest and its accumulation was being measured. The principle in this simplest presentation is that the solute of interest shows, 'dose-independent pharmacokinetics'. It is observed, then, that the accumulation in plasma proceeds such that in each time interval equal to the terminal $t_{1/2}$ of the solute, the solute attains a concentration that is half the remaining concentration interval to the projected maximum concentration. The maximum concentration, or plateau concentration is conveniently

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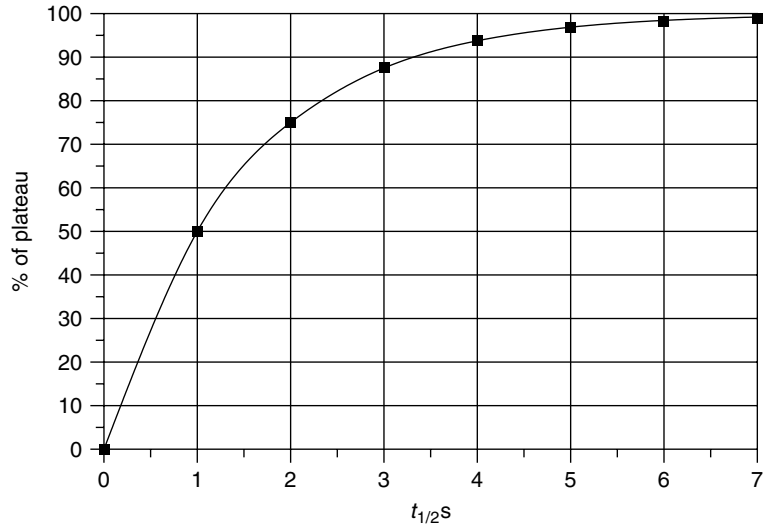

Figure 12 The plasma concentration of a xenobiotic, expressed as a percentage of the projected steady-state plateau concentration, when the xenobiotic is being administered at a constant rate (zero-order rate of administration). The time units of the abscissa are $t_{1/2}$s of elimination of the xenobiotic.

termed the concentration steady state (C_{SS}). The progression of accumulation is presented in **Figure 12**. Note that the legends of the graph are presented in relative units, that is, % of the plateau concentration and $t_{1/2}$s of elimination. The plasma plateau, or C_{SS}, is reached when the rate of elimination of the xenobiotic is exactly equal to the rate of administration or exposure to the xenobiotic. Therefore, the relationship is applicable to all xenobiotics that demonstrate first-order rates of elimination in association with a zero-order rate of administration or intake. The plateau principle is of wide application because the overwhelming majority of xenobiotics usually show first-order rates of elimination in either their therapeutic range for drugs, or from their unintended or unavoidable exposure. The profile of the plasma concentration vs time (normalized to $t_{1/2}$s) in **Figure 12** is typical when there is a zero-order rate of administration, as in the case of a constant intravenous infusion. If the drug, or other xenobiotic, is administered or exposed episodically, the plasma concentration curve will consist of peaks and nadirs in the concentration that relate to the timing of the episodic doses or exposures. However, the 'average' plasma concentration curve will be the same as that associated with the zero-order continuous infusion, provided that the total dose per hour, per day and so on, is the same by both modes of administration or exposure (Buxton, 2006).

6.2 Agents with Dose-Dependent Pharmacokinetics

A therapeutic entity that shows a zero-order rate of elimination (a constant amount of drug per unit of time) would pose an unacceptable risk of continuous accumulation to the extent that the dose exceeds the maximum rate at which the drug can be eliminated. By definition, a half-time of elimination cannot be described for such an entity. There are examples of drugs that may show a transition to dose-dependent kinetics of elimination at the upper limit of their therapeutic plasma concentration, and this may lead to a prolongation of the $t_{1/2}$ of elimination. The antiseizure drug, phenytoin, is a prominent example of this phenomenon (Holford, 2007). This is of most risk for a xenobiotic that has only one route of elimination. This is true of phenytoin, which only demonstrates approximately 2% excretion of the unchanged drug in the urine; 98% undergoes biotransformation before elimination (Holford, 2007). Ethanol is another example of a drug that shows a transition from a first-order to a zero-order rate of metabolism. The rate of metabolism of ethanol is first-order rate at low, essentially noninebriating plasma concentrations, but at blood alcohol levels associated with even moderate consumption, metabolism is functionally saturated and shows a zero-order rate (Fleming *et al.*, 2006).

When the body burden increases to the point where all routes of elimination are saturated, zero-order elimination will take place and the plasma concentration will accumulate linearly. This is presented schematically in **Figure 13**. It can be seen in the figure that if administration/exposure to the xenobiotic is terminated, the plasma concentration will decrease in a linear trend until the biotransformation mechanisms are no longer saturated, and plasma concentrations will again decrease exponentially. Again, it is not expected that any therapeutic entity would be intentionally administered at doses that would result in zero-order rates of elimination.

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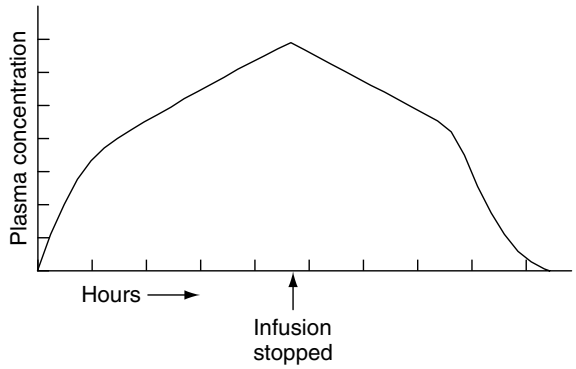

Figure 13 Theoretical depiction of the plasma concentration change during the period when a xenobiotic is administered at a zero-order rate. The xenobiotic shows a progressive transition from exponential rates of elimination to a zero-order rate of elimination until the infusion is stopped. The decrease in plasma concentration reverses in similar phases when the administration is stopped. This schematic presentation would apply particularly to a one-compartment pharmacokinetic model. (See text for explanation.)

Likewise, in view of the extensive repertoire of biotransformation capacities in human and animal species, it is unlikely that exposure to toxicants would be at 'doses' that reach such levels. Therefore, for the purpose of this chapter, we will concentrate on the kinetics of xenobiotics and their metabolites that demonstrate half-times of elimination.

6.3 Application of the Plateau Principle

The plateau principle (**Figure 12**) has wide applications for relationships between the effective 'dose' of a xenobiotic and the C_{SS}, and between the clearance of the drug and the C_{SS}. For a given xenobiotic or metabolite, the C_{SS} will vary in direct proportion to the dose or to the total body clearance (Cl) of the substance. This follows from the formula:

$$C_{ss} = \frac{\text{Dose}}{\text{Cl}} \tag{4}$$

If the xenobiotic demonstrates a one-compartment pharmacokinetic behaviour, the C_{ss} will also vary in proportion to the $t_{1/2}$ of elimination, assuming that changes in $t_{1/2}$ take place in the absence of any change in V_d. The discussion that will follow will return to the relationship between C_{ss} and dose in relation to proposals for the determination of the systemic exposure ('dose') of a xenobiotic, when this cannot be otherwise readily determined. This could include, for example, inhalation, or dermal or oral exposure.

7 ELIMINATION

The time course of elimination of xenobiotics that demonstrate one-compartment and two-compartment pharmacokinetic systems are discussed in further detail elsewhere in these volumes (see **Toxicokinetics**). One may also consult earlier publications for elaboration on these principles (Gibaldi and Perrier, 1975; Wagner, 1975; Ritschel, 1976; Renwick, 1982; Inchiosa, 2006). A summary of the kinetic principles for one- and two-compartment open models will be included here only to provide continuity of the discussion.

7.1 One-Compartment Open Model

The designation of a system as being an 'open' model is based on the knowledge or presumption that there is input and output (excretion) of the xenobiotic. That is, it is not functioning as a closed, or contained, system. The one-compartment designation implies that, initially, the xenobiotic distributes and equilibrates rapidly into the final 'V_d' to which the substance has access. Also, the kinetics of elimination appear to indicate that the equilibrium concentration ratios among various compartments (both anatomic and pharmacokinetic compartments) re-equilibrate rapidly as xenobiotic is eliminated from the body. The one-compartment open model is depicted schematically in **Figure 14**. The elimination rate constant is designated as k_{el}; it may also be simply termed k in the one-compartment model.

7.1.1 *Determination of Apparent V_d*

Because of the relative simplicity of the one-compartment system, the V_d of the xenobiotic can usually be determined graphically from the plasma concentration vs time data following intravenous administration of the substance. (see **Toxicokinetics** for a graphical presentation of such a system.) Then, extrapolation of the terminal portion of the first-order disappearance curve to the ordinate yields the concentration that would have been present at time '0' (the time of the intravenous administration) if there had been instantaneous distribution and equilibration. This value can then be used in the following equation to calculate the apparent V_d:

$$V_d = \frac{\text{Dose}}{\text{Plasma conc. at time '0'}} \tag{5}$$

Figure 14 Diagram of a one-compartment open model.

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A more precise method of determining V_d involves measurement of the entire area under the plasma concentration-time course (AUC) following intravenous injection of a known amount of a xenobiotic. The formula is as follows:

$$V_d = \frac{\text{Dose}}{\text{AUC (0 time to infinity)} \times k} \quad (6)$$

7.1.2 Half-time of Elimination ($t_{1/2}$)

The $t_{1/2}$ of elimination in the one-compartment system can frequently be estimated by inspection of the plasma concentration vs time data. It can also be determined by linear regression analysis of the first-order elimination phase ($\log_n$ plasma concentration vs time; regression coefficient $= k$; $t_{1/2}$ of elimination $= 0.693/k$).

Once the drug has equilibrated in its V_d, the plasma concentration at a time (t) after administration can be calculated as follows:

$$\text{Plasma concentration at time '}t\text{'} = \frac{\text{Dose}}{V_d} \times e^{-kt} \quad (7)$$

7.1.3 Total Body Clearance (Cl)

Total body clearance represents the sum total of all organ and tissue elimination mechanisms. It actually represents the capacity for elimination. This is obvious in the units of clearance, which are in volume (ml, l) per unit of time. It measures the percentage of the total body V_d (expressed in a volume unit) that is cleared of a solute per unit of time. A simple form of the first-order function for a one-compartment system is expressed as follows:

$$Cl = V_d k_{el} \quad (8)$$

A more precise approach, again, requires measurement of the AUC; then:

$$\text{Cl} = \frac{\text{Dose}}{\text{AUC (0 time to infinity)}} \quad (9)$$

7.1.4 Application of Pharmacokinetic Parameters for Estimation of 'Dose'

In a previous publication, approaches were suggested for the determination of a systemic dose (exposure) under conditions where this could not be easily quantified (Inchiosa, 2006). Examples of this would include inhalation, or dermal or oral exposure to a xenobiotic in an occupational or environmental setting. In the case of inhalation exposure, for example, attempts have been made to semiquantify exposure through use of the Ct product, that is, the product of the concentration in the atmosphere for a defined period of exposure. It is obvious that the Ct product provides no information about the *amount* of a possibly noxious agent that has been absorbed systemically.

Inhalation exposure, which represents an example of zero-order rate of administration, together with a first-order rate of elimination for the xenobiotic, results in accumulation of xenobiotic in the body that follows the plateau principle discussed above (**Figure 12**). However, essentially all modes of occupational or environmental exposure, inhalation, dermal, oral and so on, constitute zero-order rates of 'dosing' if one considers the chronic (average) amount per day, for example. A steady-state concentration will be reached in five to seven elimination half-times for the xenobiotic, or a metabolite, of interest. Several options may then be available to determine the systemic 'dose' (Inchiosa, 2006).

7.1.4.1 Option 1

In the case of animal experimentation with xenobiotics, where a constant intravenous dosing regimen can be used to develop the plasma C_{ss}, the systemic dose by another route (e.g. inhalation) can be calculated as follows:

$$\frac{\text{Dose (by inhalation)}}{\text{Dose (i.v. infusion)}} = \frac{C_{ss}\text{(from inhalation)}}{C_{ss}\text{(i.v. infusion)}} \quad (10)$$

The calculation produces a dose (amount per unit of time) by inhalation with the same units as that in the intravenous infusion regimen. It should be noted that the periods of intravenous infusion and exposure by another route do not have to be the same; the only requirement is that there has been sufficient time to reach steady-state conditions.

7.1.4.2 Option 2

In relation to animal experimentation, if the $t_{1/2}$ of elimination of the xenobiotic is very long, or is unknown, it may be impractical to carry out exposures for the time required to reach a plasma C_{ss}. In these cases the AUC of the plasma concentration-time curves can be compared from both intravenous infusion and inhalation (or other) exposures over the same time period. The dose in this comparison would be:

$$\frac{\text{Dose (by inhalation)}}{\text{Dose (i.v. infusion)}} = \frac{\text{AUC (from inhalation)}}{\text{AUC (i.v. infusion)}} \quad (11)$$

7.1.4.3 Option 3

In the case of environmental or occupational exposure to xenobiotics, perhaps the most convenient approach for determination of the systemic 'dose' applies knowledge of the clearance parameter to the plasma concentration data. A requirement in this approach is that the period of exposure to the xenobiotic has been long enough to have produced a plasma C_{ss} (five to seven half-times of elimination). For the one-compartment model, clearance may be calculated from Equations 8 or 9. Then, with a sampling of the plasma at an

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expected time of steady state, the 'dose' from an environmental or occupational exposure can be calculated as follows:

$$\text{Dose per unit of time} = C_{ss} \times \text{Cl} \qquad (12)$$

It is possible that the Cl value may already be known for the xenobiotic of interest, in a species of interest. If the clearance value is not known, and it concerns a farm animal or experimental animal species, it can be determined by measuring the plasma AUC following an intravenous bolus injection, and applying Equation 9. If the Cl value is needed for human considerations, it may be possible to determine the plasma AUC in man with the use of a low, nontoxic dose of the xenobiotic, relying on the assumption, or knowledge, that it follows dose-independent pharmacokinetics. If toxicity remains a concern, perhaps a still lower dose of the substance can be used that contains a stable (nonradioactive) isotope that can be quantified.

This same approach can be used to quantify exposure to a metabolite that is formed from a xenobiotic of interest. Again, it is only necessary to know the clearance value for the metabolite, from prior studies, or as determined for the parent xenobiotic, as discussed immediately above. A plasma concentration of the metabolite, taken at a time when the entire system is at steady state, including a steady-state concentration of the metabolite, will yield the rate of exposure (dose, in $\mu g\,h^{-1}$, etc.) of the subject to the metabolite, when incorporated in Equation 12.

7.2 Two-Compartment Open Model

7.2.1 Calculation and Application of Pharmacokinetic Parameters in the Two-Compartment Model

The two-compartment open model is presented diagrammatically in **Figure 15**. Some of the basic pharmacokinetic principles must be modified when a xenobiotic does not demonstrate rapid equilibration in its ultimate V_d when first administered, and shows perhaps an even greater delay in re-equilibration as the concentration of the substance is lowered in the body as a result of excretion. In this model, it is usually possible to fit the plasma concentration vs time data following an intravenous administration into a distribution phase and an elimination phase, a two-compartment model.

The plasma concentrations following a bolus intravenous administration of a xenobiotic can be determined at any time following the administration, with the formula:

$$\text{Plasma conc. at time '}t\text{'} = Ae^{-\alpha t} + Be^{-\beta t} \qquad (13)$$

In the generalized application of this equation, A and B are constants for a unit intravenous bolus injection (1 mg, 1 μg, etc.) of the xenobiotic. They represent the extrapolated intercepts on the ordinate of the plasma concentration vs time curve for the two exponential terms in the equation, α and β, respectively. The α term is largely viewed as a first-order distribution rate constant, and β represents the first-order elimination rate constant. The elimination half-time of the xenobiotic from the two-compartment system is: $t_{1/2} = 0.693/\beta$. The α and β constants can be derived from the data representing their first-order rates of change by linear regression analysis, as described above for determining the half-time of elimination in the one-compartment model. It should be noted that the α and β constants are hybrid rate constants that are dependent upon the interaction of all of the compartmental constants.

The intercompartmental distribution constants, k_{12} and k_{21}, and the elimination rate constant from the central compartment, k_{10}, (**Figure 15**) can be calculated as follows:

$$k_{21} = \frac{\alpha B + \beta A}{A + B} \qquad (14)$$

$$k_{10} = \frac{\alpha\beta}{k_{21}} \qquad (15)$$

$$k_{12} = \alpha + \beta - k_{21} - k_{10} \qquad (16)$$

(Wagner (1975) presents the derivation of these formulas.)

Using the AUC of the plasma concentration vs time data following an intravenous bolus injection, the

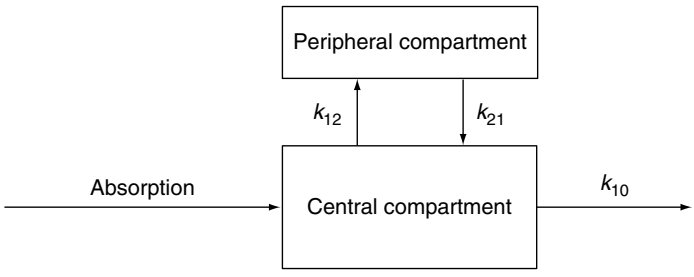

Figure 15 Diagram of a two-compartment open model.

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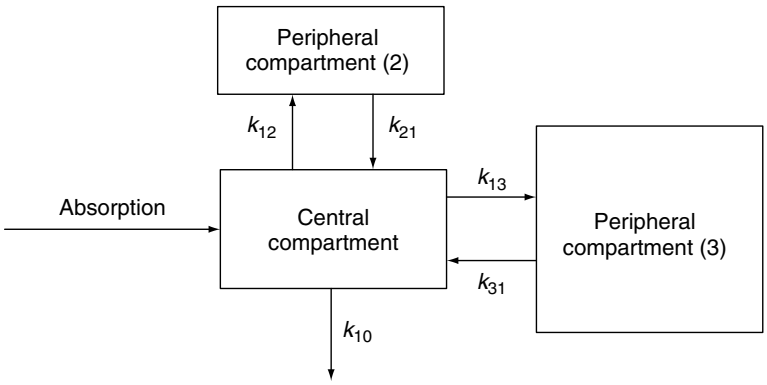

Figure 16 Diagram of a three-compartment open model.

apparent V_d in the two-compartment model is calculated with the formula:

$$V_d = \frac{\text{Dose}}{\text{AUC (0 time to infinity)} \times \beta} \quad (17)$$

Total body clearance is calculated with Equation 9.

7.2.2 Estimation of 'Dose' in the Two-Compartment Model

The same options discussed for the one-compartment model above may be applied for determination of the rate of exposure per unit of time for a xenobiotic or xenobiotic metabolite. As noted before, knowledge of the value for Cl of the xenobiotic (or a metabolite) greatly facilitates the ability to quantify the exposure that has taken place in a subject. If the clearance value is not known, it may be possible to determine it experimentally according to Equation 9. The 'dose' of the exposure can then be calculated with Equation 12. The only requirement is that steady-state conditions are prevailing at the time that the plasma is sampled for the concentration of the xenobiotic or metabolite.

7.3 Three-Compartment Open Model

7.3.1 Calculation and Application of Pharmacokinetic Parameters in the Three-Compartment Model

The complexity of calculations in the three-compartment model increases substantially over the simpler models. This model commonly provides the best fit of the plasma elimination time course for many therapeutic and nontherapeutic xenobiotics. In this model, the distribution and elimination kinetics are found to have the smallest least square deviations when the data are fit with three first-order rates (α, β, γ). The model is presented schematically in **Figure 16**. The formula for calculation of plasma concentration at any time after an intravenous bolus administration of a xenobiotic is as follows:

$$\text{Plasma conc. at time '}t\text{'} = \text{Ae}^{-\alpha t} + \text{Be}^{-\beta t} + \text{Ce}^{-\gamma t} \quad (18)$$

A, B and C are the intercepts on the ordinate for the extrapolated first order rate constants (α, β, γ), which are frequently presented as plasma concentrations (or fractional units; Shafer and Varvel, 1991) resulting from the intravenous bolus injection of a unit of xenobiotic (1 mg, 1μg, etc.).

A schematic depiction of the plasma concentration vs time course for the three-compartment system is presented in **Figure 17**. The calculation of the pharmacokinetic parameters, compartmental volumes and constants for this model requires software for nonlinear regression analysis that is supported by a Fortran compiler. NONMEM[a] is one product that has been used extensively for the determination of the pharmacokinetic parameters of many xenobiotics. STANPUMP[b] is a useful software module that can be used to simulate or predict changes in plasma concentration of a

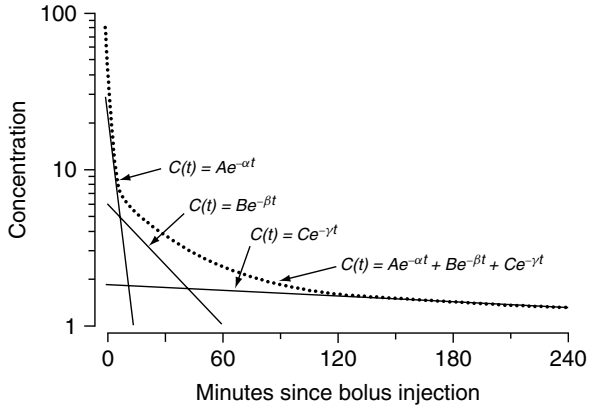

Figure 17 Representation of distribution and elimination constants for a three-compartment pharmacokinetic model. (Reproduced with permission from Schwinn and Shafer, 2000. © Elsevier.)

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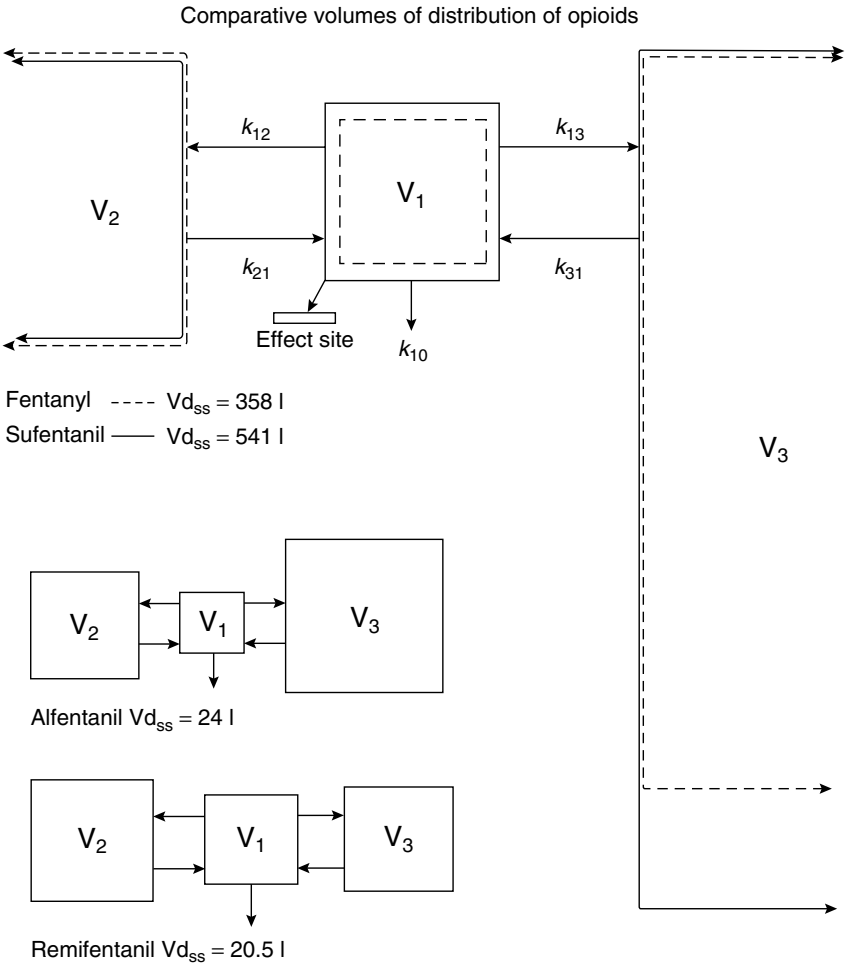

Figure 18 Schematic comparison of the compartmental volume sizes of four opioid analgesic drugs, drawn to approximate scale. Vd_{ss}, steady-state volume of distribution, that is, total volume of distribution. Data for Vd_{ss} values are from Shafer and Varvel, 1991; Minto *et al.*, 1997. (Reproduced with permission from Inchiosa, 2006. © Taylor and Francis Group.)

xenobiotic as a function of any combination of bolus doses or periods of continuous infusion. This software contains preloaded pharmacokinetic constants for a number of intravenous anaesthetic agents. We have used this software extensively in our own studies on the pharmacokinetics of the opioid analgesic, fentanyl (Shibutani *et al.*, 2004; 2005). In addition, if one has knowledge of the pharmacokinetic parameters of a multicompartmental xenobiotic other than those that are preloaded, one can insert those constants in a separate routine in STANPUMP, and calculate plasma concentrations as a function of dose and time.

The volumes of distribution and pharmacokinetic constants of multicompartmental models can vary widely. Compartmental volumes and intercompartmental diffusion constants of several intravenous anaesthetic agents are presented schematically in **Figure 18**. The drawings are made to scale for the various compartmental volumes (data from Hughes *et al.*, 1992; reproduced from Inchiosa, 2006).

7.3.2 Estimation of 'Dose' in the Three-Compartment Model

The same options discussed above for the one- and two-compartment models may be used for determination of the rate of exposure per unit of time for a xenobiotic or xenobiotic metabolite. Again, knowledge of the clearance value of the xenobiotic or a metabolite, and assurance that steady-state conditions are prevailing at the time of sampling of the plasma, permit quantification of the exposure. The 'dose' of the exposure can then be calculated according to Equation 12.

7.3.3 Context-Sensitive Half-time

The studies of Hughes *et al.* (1992), represented a milestone in a new appreciation of the contribution of compartmental volumes and intercompartmental diffusion constants on the 'recovery' of subjects from intravenous anaesthetic agents; however, their observations are applicable to the rates at which the plasma is progressively cleared of xenobiotic, and how this may vary

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greatly from the rate at which the body is cleared of drug or toxin. The rate at which the body is cleared is the common half-time of elimination; Hughes *et al.* introduced the concept of the 'context-sensitive half-time' for a measure of the rate at which the plasma is cleared of xenobiotic. The 'context' in this concept is the period of time over which the xenobiotic has been administered, before administration is stopped and one observes the change in plasma concentration. It would be difficult to overestimate the importance of this concept to appreciation of the pharmacokinetic behaviour of multicompartmental systems. Four years after the publication of this concept, an editorial in another leading anaesthesiology journal concerned with its implications was titled, '(Almost) everything you learned about pharmacokinetics was (somewhat) wrong!' (Fisher, 1996).

As noted above, the context-sensitive half-time of interest to the authors was the time required for the plasma concentration to be reduced by 50% from a level that was being maintained by a constant infusion. Their studies demonstrated that the time required for this 50% reduction could be greatly increased in relation to the preceding duration of drug administration. In the clinical anaesthesia setting, a decrease in concentration by 50% of a drug may be adequate to permit the patient to largely recover from its effects. For example, a patient may no longer demonstrate apnoea if there is a 50% reduction in the plasma concentration of an opioid analgesic agent. Also, a 50% reduction in plasma concentration of a nondepolarizing neuromuscular antagonist may equate with a return of muscle strength and spontaneous respiration. The plasma concentration data is of prime relevance in both of these examples, because the plasma interfaces closely with the receptors of importance for analgesic action in the central nervous system for the opioid (depicted as the effect site in **Figure 18**), or nicotinic receptors at the neuromuscular junction, in relation to the muscle relaxant. However, even when the site of action of a drug or toxin is more remote from the plasma, plasma concentrations are a guide to progressive accumulation or clearing of xenobiotic, since they would be expected to exist in equilibrium with deeper depots, as discussed earlier in this chapter. It should be noted that the central compartment, V_1, is not the volume of plasma, per se, but is considered to include the plasma. Also, intravenous administration is viewed as being directed to the central compartment. The context-sensitive half-time, then, is the hybrid result of the clearance rate of xenobiotic from $V_1(k_{10} \times V_1)$ and the rate of replenishment of V_1 by $k_{21} \times V_2$ and $k_{31} \times V_3$.

Some of the data from Hughes *et al.* (1992), for the relationship between duration of infusion and the context-sensitive half-times of several anaesthetic agents are presented schematically in **Figure 19**. A comparison of the magnitude of the 'context-effect' (i.e. duration of drug administration) on the 50% clearing time following termination of drug administration for fentanyl and sufentanil, demonstrates some of the implications

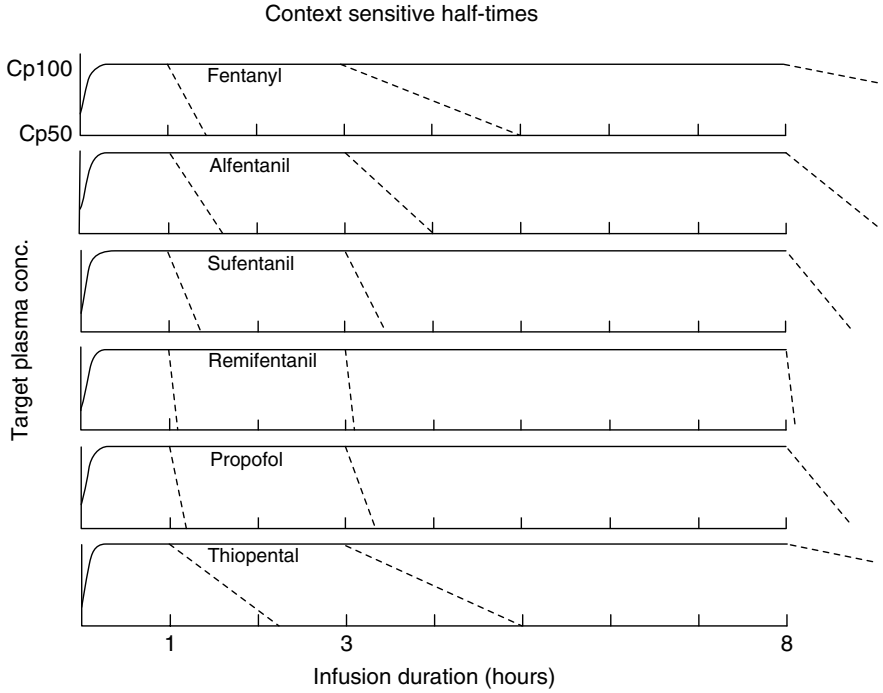

Figure 19 Schematic representation of the context-sensitive half-times of several drugs, following the termination of infusion after 1, 3, and 8 hours. See text for explanation. Data from Hughes *et al.*, 1992; Westmoreland *et al.*, 1993. (Reproduced with permission from Inchiosa, 2006. © Taylor and Francis Group.)

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of this concept. As the duration of infusion increases, sufentanil maintains a more rapid clearing of the central compartment than fentanyl. After eight hours of infusion, the patient would show signs of more rapid recovery from the central nervous system effects of sufentanil, than would be seen for fentanyl. The interesting paradox, however, is that the more rapid pharmacodynamic recovery from sufentanil is because the return of the drug from the peripheral compartments is more delayed than with fentanyl, so the central compartment clears more rapidly. There is actually a greater total fraction of the original body burden of sufentanil remaining in the body when the patient recovers, than the fraction that would be remaining for fentanyl at the same time; at that time the patient may still be showing marked sedation and apnoea from fentanyl.

Formulations provided by Hughes *et al.* (1992) allow application of this concept to a variety of therapeutic and non-therapeutic conditions of exposure. It should be noted that the results of Hughes *et al.* that are presented schematically in **Figure 19** are based on a typical therapeutic setting where a bolus of drug is given to establish an immediate therapeutic level, and this is followed by a graded infusion to maintain a constant plasma concentration. This sequence is commonly referred to as the BET regimen, for bolus, elimination and transfer in multicompartmental systems (Glass *et al.*, 1994). The procedure for this regimen, as presented by Hughes *et al.*, consists of a bolus of size $V_1 C\mathrm{p}_\mathrm{d}$ immediately followed by a graded maintenance infusion, Equation 19 (Hughes *et al.* formula has been expanded, for ease of application, to the following form):

$$\text{Maintenance infusion} = V_1 C\mathrm{p}_\mathrm{d}\left(k_{10} + k_{12}\mathrm{e}^{-k_{21}t} + k_{13}\mathrm{e}^{-k_{31}t}\right) \tag{19}$$

In the term $V_1 C\mathrm{p}_\mathrm{d}$ represents the volume of the central compartment, and $C\mathrm{p}_\mathrm{d}$ is the target concentration for the central compartment; since the plasma is considered to be part of the central compartment, it also represents the plasma concentration.

In the setting where this infusion regimen is being applied, Hughes *et al.* (1992) have presented the formulation for calculating the plasma concentration of the infused drug at any combination of duration of infusion and time after the infusion has been terminated. They posed, as a typical question, calculation of the plasma concentration that would exist 10 minutes after termination of a bolus and infusion regimen that had maintained a constant target concentration for a period of one hour. Their equation is presented below in an expanded form, to solve this question; t would be 10 minutes and T would be 60 minutes in this example. (In the formulas, we have preserved the authors use of A_1, A_2 and A_3 for the extrapolated intercepts of the three exponentials of the three compartment model, Equation 18, in lieu of A, B and C, respectively; they also used λ_1, λ_2 and λ_3, in place of α, β and γ, respectively.)

$$\begin{aligned} c(t) = V_1 C\mathrm{p}_\mathrm{d}\Bigg\{ & \mathrm{A}_1\mathrm{e}^{-\lambda_1 t}\Bigg[\mathrm{e}^{-\lambda_1 T} + \frac{k_{10}}{\lambda_1}\left(1-\mathrm{e}^{-\lambda_1 T}\right) \\ & + \frac{k_{12}}{k_{21}-\lambda_1}\left(\mathrm{e}^{-\lambda_1 T} - \mathrm{e}^{-k_{21}T}\right) \\ & + \frac{k_{13}}{k_{31}-\lambda_1}\left(\mathrm{e}^{-\lambda_1 T} - \mathrm{e}^{-k_{31}T}\right)\Bigg] \\ & + \mathrm{A}_2\mathrm{e}^{-\lambda_2 t}\Bigg[\mathrm{e}^{-\lambda_2 T} + \frac{k_{10}}{\lambda_2}\left(1-\mathrm{e}^{-\lambda_2 T}\right) \\ & + \frac{k_{12}}{k_{21}-\lambda_2}\left(\mathrm{e}^{-\lambda_2 T} - \mathrm{e}^{-k_{21}T}\right) \\ & + \frac{k_{13}}{k_{31}-\lambda_2}\left(\mathrm{e}^{-\lambda_2 T} - \mathrm{e}^{-k_{31}T}\right)\Bigg] \\ & + A_3\mathrm{e}^{-\lambda_3 t}\Bigg[\mathrm{e}^{-\lambda_3 T} + \frac{k_{10}}{\lambda_3}\left(1-\mathrm{e}^{-\lambda_3 T}\right) \\ & + \frac{k_{12}}{k_{21}-\lambda_3}\left(\mathrm{e}^{-\lambda_3 T} - \mathrm{e}^{-k_{21}T}\right) \\ & + \frac{k_{13}}{k_{31}-\lambda_3}\left(\mathrm{e}^{-\lambda_3 T} - \mathrm{e}^{-k_{31}T}\right)\Bigg]\Bigg\} \end{aligned} \tag{20}$$

The question may be reversed to ask how long it would take for the plasma concentration to be reduced to some percentage of the constant level that had been maintained for one hour, but has now been terminated. This can be calculated using a numerical solver such as the 'Solver' feature of Microsoft Excel[c] or the 'FindRoot' command of Mathematica.[d] These programs utilize an iterative algorithm to search for the desired result, that is, the time required for a specific percentage change from the plasma concentration at the time that the infusion (exposure) was terminated.

One of the most useful applications of the context-sensitive concept may relate to questions of dissipation of plasma concentrations and body burdens of toxicants following environmental and occupational exposures. It is reasonable to assume that such exposures may have taken place for periods of time that would have produced steady-state conditions for the xenobiotic and its metabolite(s). Hughes *et al.* (1992) provided the formulation for predicting the plasma concentration that would exist for a toxicant at some specific time after the individual had been removed from exposure; the assumption is that steady-state conditions existed at the termination of exposure. Their condensed equation has been expanded, as follows:

$$c(t) = V_1 k_{10} C\mathrm{p}_\mathrm{d}\left\{\frac{\mathrm{A}_1}{\lambda_1}\mathrm{e}^{-\lambda_1 t} + \frac{\mathrm{A}_2}{\lambda_2}\mathrm{e}^{-\lambda_2 t} + \frac{\mathrm{A}_3}{\lambda_3}\mathrm{e}^{-\lambda_3 t}\right\} \tag{21}$$

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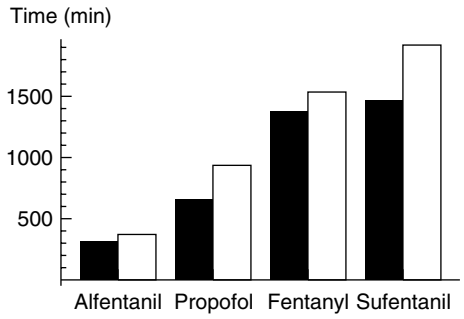


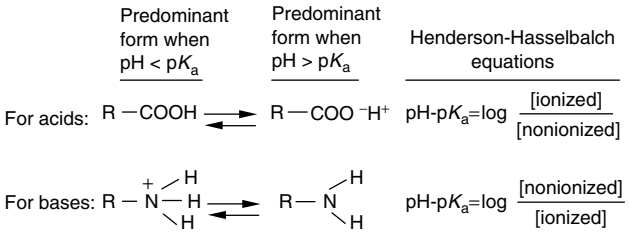
Figure 20 Times required for the decrease in plasma concentration and total body burden from steady-state conditions for four intravenous anaesthetic agents. The black bars indicate the times required for plasma concentrations to decrease by 90% from steady-state concentrations. The white bars indicate the times required for the body burden to decrease by 90% (approximately 3.3 half-times of elimination).

The question may also be posed as to the time it would take for the plasma concentration to be reduced by some specific percentage of the steady state concentration. Again, the 'Solver' feature of Microsoft Excel or the 'FindRoot' command in Mathematica can be used to solve this question. **Figure 20** presents the results for the time required for plasma concentrations to decrease by 90% from steady-state concentrations, for several of the anaesthetic agents for which Hughes *et al.* provided pharmacokinetic data. For comparison, the times that would be required for the body burden of the substance to decrease by 90% (approximately 3.3 half-times of elimination) are also presented.

8 SUMMARY

The objectives for this chapter included a presentation of the physicochemical characteristics of therapeutic and nontherapeutic xenobiotics that have a major influence in determining the rate of onset of pharmacodynamic or toxicodynamic effects. That objective was also expanded to include the role of factors that affect the extent of distribution and accumulation of xenobiotic and xenobiotic metabolites in the animal or human body. The kinetics of excretion of xenobiotics and metabolites were reviewed first for relatively simple one-compartment kinetics, followed by the principles that apply to multicompartmental systems. In all cases, procedures were proposed that would allow quantification of the systemic 'dose' of a xenobiotic, when individuals had been exposed in an environmental or occupational setting that provided no quantitative information on the actual exposure. Finally, kinetic models were proposed that would permit determination of the amount of time that it would take for the plasma to be cleared of the xenobiotic, or a metabolite, and how this might differ from the time for the total body burden to be reduced of the same substances.

APPENDIX A



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Toxicokinetics

Ashley Roberts and Andrew G. Renwick

CONTENTS

1 INTRODUCTION

The generation of *in vivo* toxic effects, distal to the site of administration, may be divided into two distinct aspects: delivery to the target site for toxicity, and cellular events mediated by the chemical at the target organ or cell(s). Thus, the production of toxicity can be subdivided into **toxicokinetics** (the movement of the toxic chemical from the site of administration into the general circulation and subsequently around the body) and **toxicodynamics** (the actions of the chemical within the target organ) (**Figure 1**).

In recent years there has been an increasing emphasis on the development of *in vitro* tests for specific organ toxicity, in order to reduce the numbers of animals given toxic doses of chemicals. However, the interpretation of the results of *in vitro* tests increases the requirement for definition of the *in vivo* concentrations of the chemical that could be present in the target organ, the time course of exposure and the potential for accumulation during repeated dosing. Such information can only be obtained from appropriate *in vivo* toxicokinetic studies on the fate of the chemical within the body.

Toxicokinetics are also of importance in extrapolating both *in vitro* and *in vivo* animal data to humans. The techniques used in toxicokinetics, such as the collection of timed blood and urine samples, can also be investigated in human volunteers, providing that it is ethical to administer the chemical to humans. The basic processes involved in absorption, distribution and elimination, and their mathematical descriptions given in this chapter, are common to all chemicals which are foreign to the body, irrespective of their biological activity; for example toxins, medicines, pesticides, food additives and so on. The mathematical descriptions were developed in order to describe the fate of medicines in humans, and are usually described as *pharmacokinetics*. The use of a separate term, *toxicokinetics*, is of some value because of the implications of analysing data related to high-dose animal studies, but it should be appreciated that many of the best sources of background information

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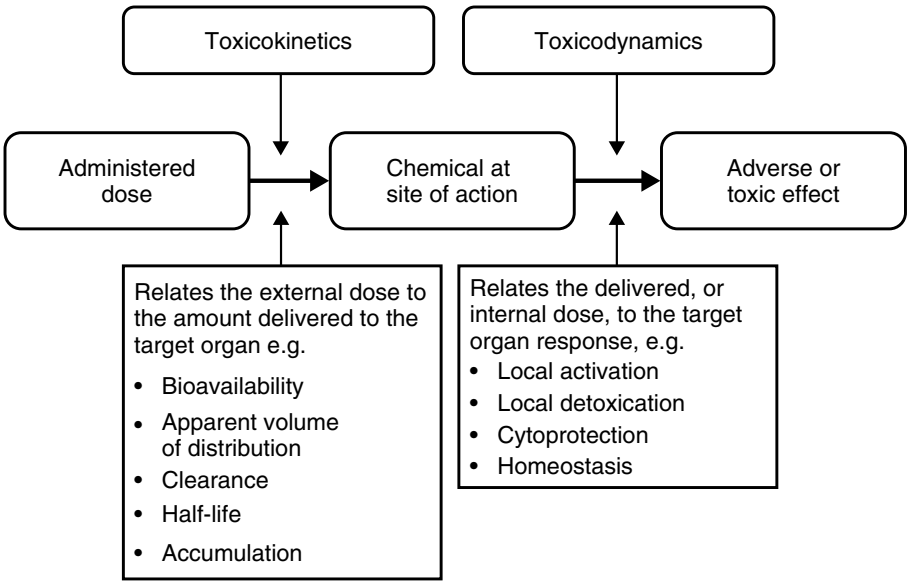

Figure 1 The relationship between toxicokinetics, toxicodynamics and toxic effects.

on 'toxicokinetics' are to be found in the 'pharmacokinetics' literature.

2 ABSORPTION, DISTRIBUTION, METABOLISM AND EXCRETION (ADME) STUDIES

The delivery of the chemical to the site of toxicity (**Figure 2**) depends on the processes of *absorption* from the site of administration into the general circulation and *distribution* via the blood to the site of action and all body tissues. Following absorption, the duration of exposure depends on the rate of *elimination* from the body, which may be by either *metabolism* or *excretion*. Absorption, distribution, metabolism and excretion (ADME) studies form an essential first step in the evaluation of the fate of the chemical in the body (Glocklin, 1982; Barton *et al*., 2006). Usually ADME studies involve following the fate of a radiolabelled dose of the chemical by measuring the total radioactivity in the excreta, and also the tissue distribution of total radioactivity by autoradiography at various times after dosing. Such studies clearly provide important information on the fate of the chemical in the body, but they provide only a part of the total investigation of the toxicokinetics of the compound. A radiolabelled compound is used to provide information on the total balance of all compound-related products whether or not they have been identified and characterized. Thus the strength of radiolabelled ADME studies is the use of *nonspecific detection methods,* which allow the detection of all compound-related products. ADME studies also provide essential data on the extent of absorption and the routes of elimination. However, this strength is also a weakness because it provides limited data on exposure of the body to the parent chemical itself or to specific identified metabolites. Identification of metabolites of the radiolabelled compound in excreta (and the circulation) using chromatographic techniques allows definition of the pathways of metabolism; such information is essential for interspecies comparisons, for the establishment of specific analytical procedures and for indications of possible metabolic bioactivation.

Investigation of the kinetics of the parent molecule itself requires the use of a *chemical-specific detection method*, which not only separates the chemical from the normal (endogenous) constituents of the sample being analysed, but also from any metabolites of the chemical that are formed *in vivo*. In cases where the toxicity of a chemical is due to a specific metabolite, then toxicokinetic studies will need to define the extent of absorption of the parent chemical, its delivery to the site(s) of metabolism, the extent of formation of the metabolite and its delivery to the target organ (**Figure 2**). Additional information essential to the interpretation of toxicity studies includes the influence of dose on the fate of the chemical, and the extent of accumulation of the parent compound and/or active metabolites on repeated administration of the compound.

Toxicokinetics represent an important part of the safety evaluation of chemicals, but because the subject is perceived as being mathematically based it tends to be regarded as difficult and rather unapproachable by biologists. This chapter will relate the underlying physiological and biochemical processes to the basic pharmacokinetic parameters and constants. This is followed by a consideration of the types of experimental data that are needed in order to determine the various parameters. The chapter will conclude with a consideration of specific

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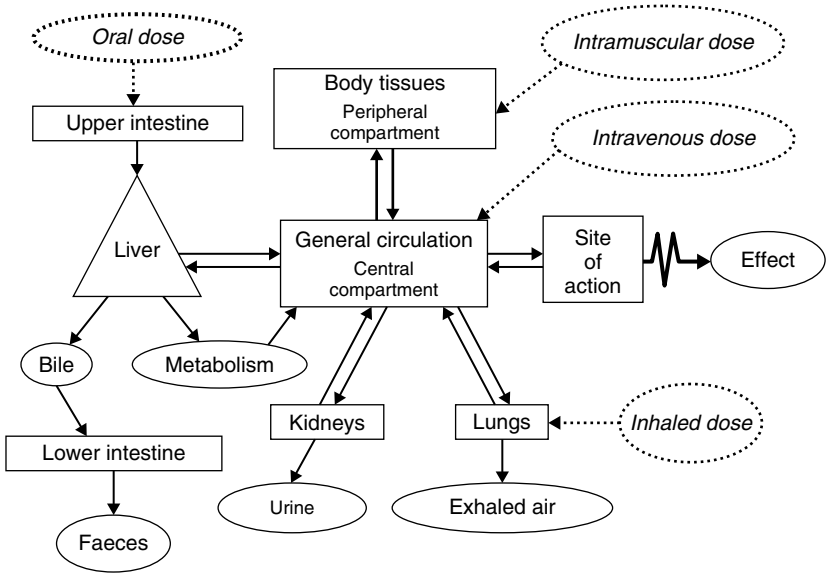

Figure 2 Delivery of chemicals to their site of action. This scheme assumes that the site of toxicity is not locally at the site of administration. Compounds absorbed from the gastrointestinal tract are transported via the hepatic portal vein to the liver before they enter the general circulation. Highly lipid-soluble compounds may be absorbed via the lacteal system.

aspects related to the interpretation of high-dose, chronic animal studies.

3 PHARMACOKINETIC PROCESSES, PARAMETERS AND CONSTANTS

Each of the basic processes involved in pharmacokinetics, that is, absorption, distribution and elimination, may be described by parameters which define the *extent* to which the process occurs and the *rate* at which it occurs. These parameters are usually calculated from the concentrations of the chemical and/or its metabolites in biological fluids, such as whole blood, plasma or urine, measured at known times after the administration of known doses.

3.1 Absorption

Absorption is the process of transfer of the chemical from the site of administration into the general circulation. Absorption from the gastrointestinal tract is studied most frequently because this is the route of exposure of experimental animals via gavage dosing or by incorporation of the chemical into the diet. Oral administration is also the most common route by which humans are exposed to chemicals either as drugs or as intentional, incidental or accidental components of the diet. The process of absorption occurs whenever the compound is given by a route other than direct intravascular injection; for example across the skin, from the airways, from subcutaneous sites and also from the peritoneal cavity.

3.1.1 Rate of Absorption

The rate of absorption depends on both the nature of the chemical and the site of administration. There are five main processes by which chemicals can transfer across cell membranes (**Figure 3**).

1. **Passive diffusion through the cell membrane:** Lipid-soluble chemicals can readily dissolve in membranes and therefore diffuse across cell walls. In contrast, ionized molecules do not readily enter the lipid membrane in the ionized form, and therefore only the unionized form freely diffuses across membranes. For strong acids and bases at physiological pH, the unionized form on each side of the membrane is in equilibrium with the ionized form; the concentrations of the ionized form may be orders of magnitude higher than the concentration of the unionized, lipid-soluble form. Thus the transfer of highly ionized chemicals across cell membranes is slow. For very strong acids and bases, and also quarternary amines (which have a fixed positive charge), the absorption rate across the intestinal wall may be slower than the rate of transfer along the bowel by peristalsis; under such circumstances a major fraction of the dose may be lost in the faeces without ever being absorbed or available to produce systemic toxicity.
2. **Diffusion through open ion channel:** Water-soluble compounds may be able to pass down

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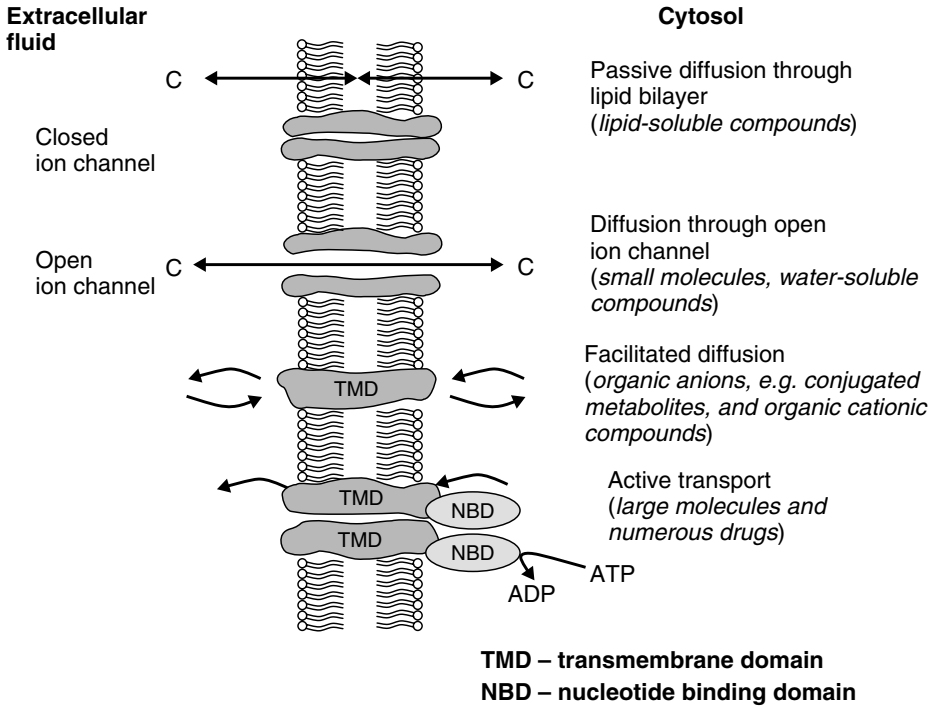

Figure 3 Processes involved in the transfer of chemicals across cell membranes. Passive diffusion is the major pathway for low-molecular-weight lipid-soluble compounds. C = chemical.

ion channels without having to enter the lipid environment of the membrane. Because ion channels are involved in the transfer of specific cations, such as sodium and potassium, this is only applicable to very small water-soluble compounds and inorganic ions.

3. **Facilitated diffusion:** This involves a membrane-bound protein, known as a solute carrier (SLC) transporter (**Table 1**) that shows substrate specificity. The process does not utilize energy, and chemicals are transported only down their concentration gradients.
4. **Active transport:** This process utilizes energy and can transport chemicals into or out of cells against a concentration gradient. It is performed by a family of nonspecific carriers termed the ATP-binding cassette (ABC) superfamily of membrane transporters (**Table 1**).
5. **Pinocytosis:** In this process the cell membrane forms a pocket containing extracellular fluid, containing the chemical, which then fuses into a sphere that is internalized. This is of negligible quantitative importance, except in the case of macromolecules which cannot otherwise cross membranes intact.

Absorption from the gut is often complex because chemicals are absorbed less effectively from the stomach, with its smaller surface area and lower pH, than from the duodenum and jejunum. In consequence there can be a delay before any appreciable absorption occurs, while the compound is in the stomach lumen. However, once the dose has left the stomach, it reaches the large surface area of well-perfused intestine, which can allow very rapid absorption.

The rate of absorption also depends on the site of administration; for example, absorption across the skin is usually extremely slow, because it involves transfer across the stratum corneum—the main permeability barrier of the body. In contrast, absorption from the airways tends to be rapid, because it involves transfer across a thin membrane which has a large surface area and a good blood supply.

In some cases absorption is so rapid that the absorption rate constant cannot be calculated, and the plasma concentration–time curve resembles an intravenous dose (**Figure 4**). A low, almost flat, profile is obtained when absorption is very slow; for example across the skin (**Figure 4**). In many cases the plasma concentration–time profiles after oral dosage are more complex than shown in this figure and often involve a lag phase prior to significant absorption.

3.1.2 Extent of Absorption

There are a number of reasons why all of the dose of a chemical introduced into the gut lumen may not be able to pass into the general circulation as the parent compound (**Table 2**).

The parameter which describes the extent of absorption is the bioavailability (F), which is defined as: *the*

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Table 1 Transporters involved in cellular absorption and efflux of exogenous and endogenous compounds

Transporter	Typical substrates	Sites in the body
ABC superfamily	ATP-binding cassette superfamily of transport proteins; utilize ATP for active transport	
MDR1 or P-glycoprotein (ABCB1)	Hydrophobic and cationic (basic) molecules	Apical surface of membranes of epithelial cells of intestine, liver, kidney, blood—brain barrier, testis, placenta and lung
MRP1 (ABCC1)	Numerous drugs and glucuronide and glutathione conjugates	Basolateral surface of membranes of most cell types with high levels in lung, testes and kidney and in blood—tissue barriers
MRP2 (ABCC2)	Numerous drugs and glucuronide and glutathione conjugates	Apical surface of membranes; mainly in liver, intestine and kidney tubules
BRCP (ABCG2) Breast cancer resistance protein	Various drugs and flavonoids	Apical surface of breast ducts and lobules, small intestine, colon epithelium, liver, placenta, brain barrier and lungs
SLC superfamily	Solute carrier (SLC) superfamily of transporters. Comprises organic anion transporters (OATs) and organic cation transporters (OCTs)	
OAT1 (SLC22A6)	Numerous acids and conjugated metabolites	Kidney (basolateral), brain, placenta, smooth muscle
OAT2 (SLC22A7)	Various anions and dicarboxylic acids	Kidney (basolateral), liver
OAT3 (SLC22A8)	Similar to OAT1	Kidney (basolateral), liver, brain, smooth muscle
OAT4 (SLC22A11)	Steroid sulfate conjugates	Kidney (apical), placenta
OCT1 (SLC22A1)	Various basic molecules	Mainly in the liver, but also in kidney, small intestine, heart, skeletal muscle and placenta
OCT2 (SLC22A2)	Various basic molecules	Mainly in the kidney, but is also in placenta, adrenal gland, neurons and choroid plexus
OCT3 (SLC22A3)	Various basic molecules	Liver, kidney, intestine, skeletal and smooth muscle, heart, lung, spleen, neurons, placenta and the choroid plexus

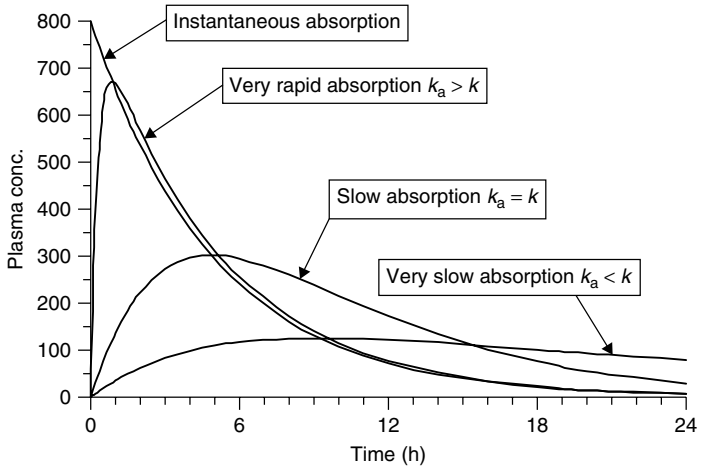

Figure 4 Plasma concentration–time curves of compounds showing different rates of absorption from the site of administration (but the same rate of elimination). k_a = absorption rate constant; k = elimination rate constant (see text).

fraction of the dose which is transferred from the site of administration into the general circulation as the parent compound.

Bioavailability is without doubt the most misused/abused toxicokinetic term; it is often used nonmathematically as a generic term for 'delivery to the site of action', which actually involves both absorption and tissue distribution, or as 'the concentration at the target for toxicity' which involves absorption, distribution and elimination.

Each of the processes in **Table 2** can cause a decrease in the amount of parent chemical able to reach the

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Table 2 Reasons for incomplete absorption from the site of administration

Reason	Example
Incomplete dissolution	The compound given in the diet or as a suspension is not completely dissolved during transit through the intestinal tract.
Incomplete passage across absorptive epithelium	The compound is too polar to be absorbed completely before it is voided in the faeces (oral), or the unabsorbed dose is removed (transdermal). The rate of loss in exhaled air exceeds the rates of dissolution and absorption (inhaled gases).
Metabolism at the site of administration	The compound is metabolized or decomposes due to the pH or enzymes present in the gut lumen (oral or rectal), or inactivation by lung enzymes (inhalation).
Metabolism between site of administration and the general circulation	The compound is metabolized by the gut lumen, gut wall, or liver prior to entering the general circulation (oral dosage).

general circulation. Incomplete absorption ($F < 1.0$) may be due to:

1. an inability of the chemical to cross the lipid barrier of the epithelial membrane or transfer back from the enterocyte into the gut lumen by an efflux transporter (**Table 1**; **Figure 3**), so that the parent compound is unabsorbed, and eliminated in the faeces (indicated by ADME data), or
2. metabolism in the gut lumen, gut wall or liver prior to reaching the general circulation. This is referred to as *first-pass metabolism* since it usually occurs on the first passage through the liver during the absorption process. First-pass metabolism can result in an apparent discrepancy between radiolabelled studies and toxicokinetic data. The former may demonstrate that all of a radiolabelled dose is absorbed and eliminated in urine, but the bioavailability of the parent compound measured by a chemical-specific method may be considerably less than 1.0 (see later for method of measurement). The discrepancy arises from the nonspecific nature of radiochemical measurements which measure parent compound plus metabolites.

3.2 Distribution

Distribution is the process of reversible transfer of the chemical from the general circulation into the body tissues. The process may be characterized by both rate and extent; the corresponding parameters are the *distribution rate constant*(s) (α, k_{12} and k_{21}) and the *apparent volume of distribution* (V) (see later). Measurement of these parameters requires knowledge of the amount of compound that enters the general circulation, and therefore distribution parameters can be defined only when the chemical has been given by direct intravascular injection. Thus intravenous dosage is essential for a full description of the toxicokinetics of any compound, including food additives, for which there would never be parenteral dosage and for which parenteral toxicity studies are not necessary or useful.

3.2.1 Rate of Distribution

Because distribution is usually rapid, the distribution rate constants are measured following the administration of a single rapid (bolus) intravenous dose (**Figure 5**). The rate of distribution into tissues may be slow for two possible reasons. Firstly, distribution will be slow if the chemical has a high affinity for, and accumulates in, a tissue or organ which is only slowly perfused, for example fat or muscle. For such a compound the rate at which the tissues and blood can reach equilibrium will be limited by the blood flow to the tissues. Secondly, for highly polar chemicals the rate of entry into the intracellular fluid of all tissues may be limited by its rate of transfer across the cell membrane.

3.2.2 Extent of Distribution

The tissues into which a chemical (and/or its metabolites) distributes can be identified by autoradiography, but this technique cannot determine the overall extent to which a chemical has left the blood or plasma and entered the tissues. In addition, autoradiography cannot differentiate between parent compound and metabolites, and cannot be used to provide data in humans.

The extent of distribution of a chemical from the blood into tissues depends on a number of variables:

1. **Water solubility:** Water-soluble compounds, for example ethanol and caffeine, show limited uptake into adipose tissue or the central nervous system and are distributed to total body water.
2. **Plasma protein binding:** Chemicals which bind reversibly to plasma proteins with a high affinity show reduced tissue distribution and are retained to a greater extent in the circulation. An equilibrium is established between the concentration unbound in plasma and that (unbound) in tissues (**Figure 6**).
3. **Tissue protein binding:** Chemicals with a high affinity for reversible binding to tissue proteins will

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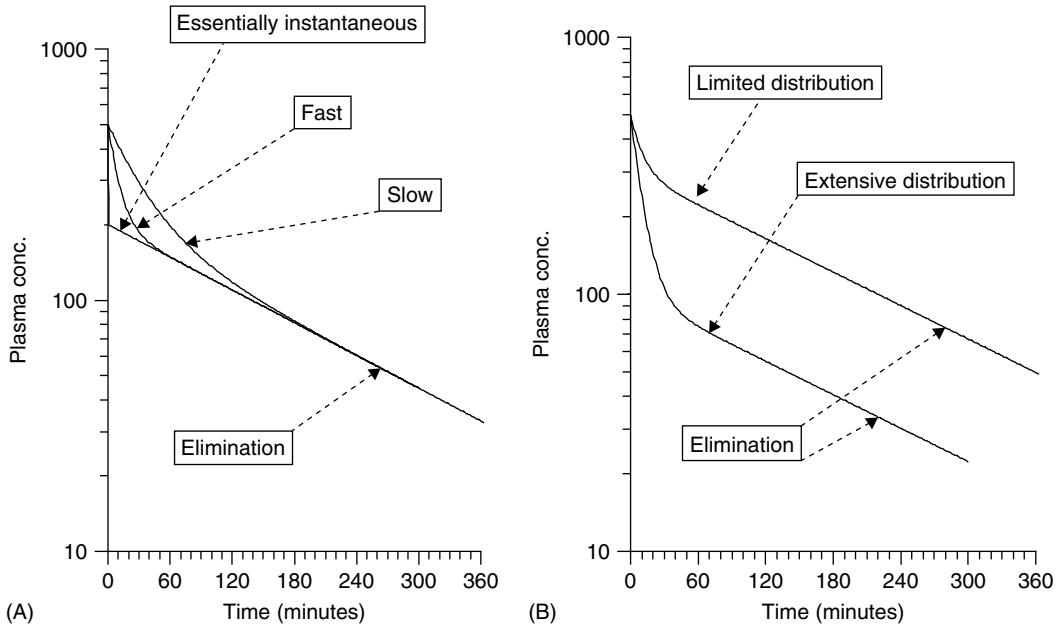

Figure 5 Plasma concentration–time curves following intravenous bolus doses of chemicals, showing different rates of distribution from the blood into peripheral tissues (A) and different extents with the same rates (B).

show more extensive distribution. The blood: tissue equilibrium relates to the unbound chemical in each compartment (**Figure 6**), and therefore the overall extent of distribution depends on the relative binding in tissues and blood.

4. **Lipid solubility:** Lipid soluble compounds are concentrated in adipose tissue, organs that have a high lipid content (such as the central nervous system), and the cytoplasmic membranes and endoplasmic reticulum of all cells in the body.
5. **Efflux transporters:** The transporters listed in **Table 1** show different tissue distributions and these, especially P-glycoprotein, can affect the tissue : plasma concentration ratio for compounds that are substrates.

Information on the concentration of a compound in a specific tissue can be determined only by analysis of that tissue. Cyclohexylamine, a metabolite of the intense sweetener cyclamate, rapidly enters the testes (Roberts and Renwick, 1989), which are the target for toxicity (Bopp *et al*., 1986). The concentrations in the testes are about four times higher than those in plasma. In contrast the hydroxylated metabolites (3- and 4-aminocyclohexanols), which are more polar detoxication products (Roberts *et al*., 1989), enter the testes less readily and there is not a high testes : plasma ratio even after chronic administration. These data illustrate that minor changes in molecular structure, such as the increase in polarity resulting from the hydroxylation of cyclohexylamine, can alter considerably the uptake into tissues, especially organs such as the brain and testes which have an efficient permeability barrier (blood–organ barrier) and facilitated uptake processes or active efflux transporters.

3.3 Elimination

There are two principal mechanisms of elimination of foreign compounds from the body.

Metabolism eliminates the chemical from the body by converting it into a metabolite, which is a different chemical species. The resulting metabolite may itself undergo further metabolism or it may be removed from the body by an excretory process. However, from a toxicokinetic perspective, the parent compound has been eliminated as soon as it has been changed into the initial metabolite. Thus, in the analysis of the kinetics of the parent compound it is essential that the analytical method separates the compound from all of its metabolites. The metabolism of foreign compounds has been the subject of numerous reviews and is discussed in detail in **Biotransformation of Xenobiotics**, **Toxicokinetics of Xenobiotic and Metabolite Distribution and Excretion**, **A Metabolomic Perspective of Small Molecule Toxicity**. The cytochrome P450 isoenzymes in the endoplasmic reticulum are important in the metabolism and elimination of lipid-soluble foreign compounds. Sometimes the initial metabolic step results in the generation of a toxic metabolite which is released into the circulation (see **A Metabolomic Perspective of Small Molecule Toxicity**), in which case measurements of the concentrations of parent compound provide information on the amount of substrate available for

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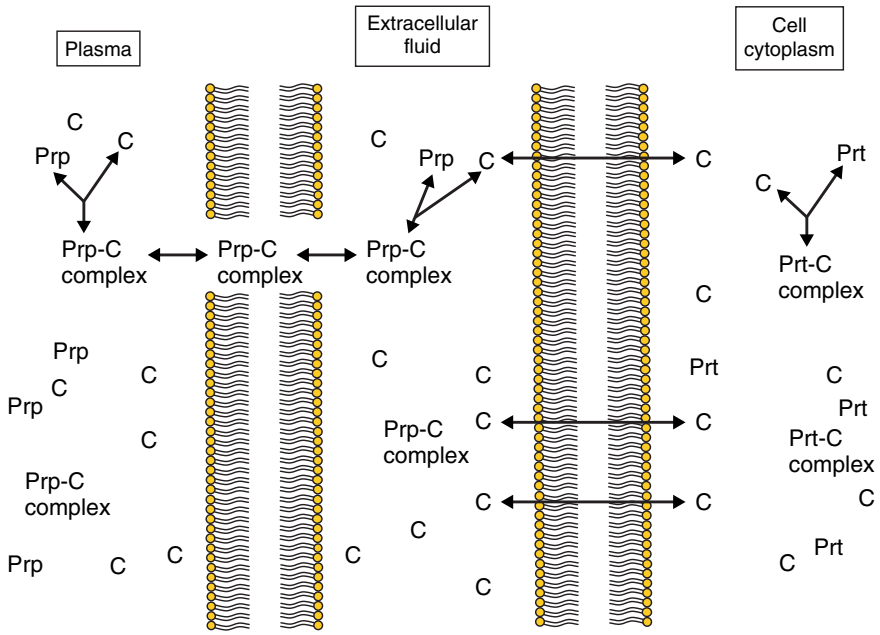

Figure 6 Plasma and tissue protein uptake and reversible protein binding of chemicals. The chemical (C) may bind as the ionized or unionized form to the plasma proteins (Prp) and tissue proteins (Prt). The total tissue : plasma ratio depends on the relative affinity of plasma and tissue protein binding.

activation, but may not be the best estimate of body exposure to the toxic moiety. Under such circumstances toxicokinetics should concentrate on measurement of the toxic moiety itself in plasma and tissues, especially the target organ. When toxicity arises from the formation of an unstable reactive metabolite, exposure to the toxicant may be assessed by a biomarker, such as measuring the products formed from the unstable metabolite either in plasma, tissues or urine. For example, the extent of formation of toxic metabolites that react with glutathione (GSH) can be assessed by measurement of the urinary or biliary excretion of the thio conjugates, that is, the GSH, cysteine or mercapturic acid metabolites. Any bioactivation processes, or covalent binding to proteins or cellular macromolecules *within the target organ*, are part of the toxicodynamics, because it is not part of the movement of the chemical around the body (kinetics).

Excretion can occur via the body's waste products, for example urine, faeces and expired air; the route of importance is determined largely by the physicochemical properties of the compound (**Table 3**) and whether the compound is a substrate for an efflux transporter in the kidney, intestine or liver (**Table 1**). When considering the toxicokinetics of a chemical, confusion can arise when trying to relate studies with radiolabelled compounds to toxicokinetic data. For example, although all of the radioactivity may be eliminated in the urine, it is possible that this is all as metabolites and not as parent compound. Under such circumstances the renal clearance (see later) of the chemical itself will be negligible, and the kidneys will not be of importance in the elimination of the chemical *per se*.

The elimination of a chemical can be divided into the rate and extent of elimination.

3.3.1 Rate of Elimination

The rate of elimination is limited by two biological processes.

1. The ability of the organs of elimination to extract the chemical from the circulation and to remove it from the body by metabolism or excretion.
2. The fraction of the body burden that is in the circulation and is available for elimination. If the compound has entered the body tissues to a major extent, so that at any time only a very small fraction of the total body burden remains in the blood and is available for elimination, then the rate at which it is transferred from the tissues back into the circulation may become the rate-limiting step in elimination of the chemical.

3.3.2 Extent of Elimination

The extent of elimination is of less importance than the rate. Following a single dose the extent of elimination will eventually be 100% of the dose. This is even true for chemicals which bind covalently to tissue macromolecules, because formation of an adduct represents elimination of the parent compound. During chronic

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Table 3 Routes of elimination of foreign compounds

Route	Type of chemical
Expired air	Volatile compounds, for example, gaseous anaesthetics, solvents, aerosol propellants.
Saliva	Many low-molecular-weight compounds, but reabsorption occurs on passage down the gut.
Bile	Large-molecular-weight compounds, usually conjugated metabolites rather than the parent compound. Wide species differences exist in the molecular weight threshold for significant biliary excretion. Reabsorption and/or bacterial metabolism may occur in the lower bowel.
Faeces	An important route for the elimination of compounds not absorbed from the gut, and for compounds excreted in the bile. Some chemicals can pass from the circulation into the gut lumen by diffusion or active transport and thereby undergo elimination in the faeces.
Urine	The major route of elimination for low-molecular-weight, polar compounds. Lipid-soluble compounds are filtered at the glomerulus, but reabsorbed on passage down the renal tubule, and such compounds are eliminated by metabolism and their metabolites removed in the urine and/or bile.
Milk	Both water-soluble and lipid-soluble compounds are present in milk. This route is usually of limited significance with respect to elimination from the mother but may be of critical importance with respect to exposure of the neonate.
Hair	Quantitatively unimportant, but the slow and directional growth of hair can allow an 'exposure history' to be determined based on the position of the chemical along the hair.

intake the body burden increases until the extent of elimination per day equals the daily intake of the chemical (see later).

4 DERIVATION OF PHARMACOKINETIC CONSTANTS

Most pharmacokinetic parameters are based on the measurement of the chemical in plasma samples collected at various times after dosing. In some cases the concentrations in whole blood or urine are used.

4.1 Basic Concepts

Before it is possible to describe the measurement of specific pharmacokinetic parameters it is necessary to define certain basic concepts.

4.1.1 Order of Reaction

At low doses, most processes involved in toxicokinetics can be described as *first-order reactions* with respect to substrate. That is, the rate of reaction is proportional to the amount of substrate present. Examples include passive diffusion, facilitated or active transport, metabolism, protein binding and excretion, in which an increase in concentration will increase the amount of chemical which crosses a membrane, undergoes metabolism, and so on. The equation for such reactions is:

$$\frac{dC}{dt} = kC \qquad (1)$$

where dC/dt is the rate of change in concentration, k is the rate constant and C is the concentration.

The term k is the rate constant for the process being described by the change dC/dt and may be absorption, distribution or elimination; the greater the value of k, the greater the rate of the process. The units of k are time^{-1}, and k can be regarded as the natural log of the proportion of the chemical that is changed within one unit of time. For example, if $k = 0.693\,h^{-1}$ then the concentration will change by a factor of 2 (the natural antilog of 0.693—see below) each hour.

Processes which involve an interaction of the chemical with a cellular protein, such as enzyme-catalysed metabolism or active transport, may be saturated at very high concentrations of the chemical. Under these conditions the rate of change in concentration is not proportional to the concentration of substrate available but is a constant amount per unit time. This is known as a *zero-order reaction*, which can be described by the equation:

$$\frac{dC}{dt} = k \qquad (2)$$

The rate constant (k) of a zero-order reaction can be described in terms of mass (or concentration) per unit time (e.g. $\mu g\ min^{-1}$), and this value is a constant at all concentrations.

A straight line is obtained when the data for a zero-order decrease are plotted on a linear axis (**Figure 7A**). In contrast, for a first-order decrease the rate of change is proportional to the concentration; the rate of change is high at high concentrations (i.e. a steep slope) and the slope decreases with decrease in concentration. Thus first-order reactions result in exponential decreases, and can be described by exponential equations.

$$C_t = C_0 e^{-kt} \qquad (3)$$

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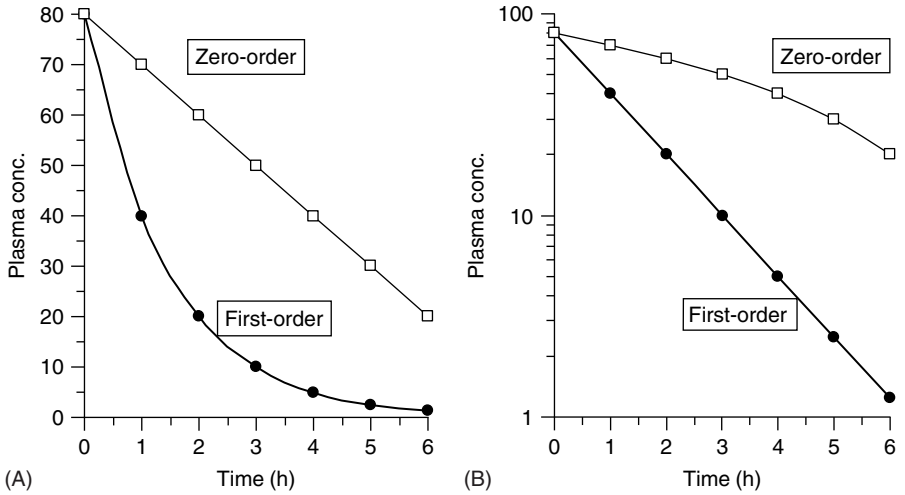

Figure 7 Graphical representations of first-order and zero-order decreases in concentrations, plotted on (A) linear and (B) logarithmic axes.

where C_t is the concentration at time t, C_0 is the initial concentration, and t is the time after dosage; or, taking natural logs,

$$\ln C_t = \ln C_0 - kt \tag{4}$$

Plotting the natural logarithm of the concentration ($\ln C_t$) against time (t) will give a straight line with an intercept of $\ln C_0$ and a slope of $-k$ (**Figure 7B**). If $\log_{10}$ is used then the intercept is $\log_{10} C_0$ and the slope is $-k/2.303$. A zero-order decrease becomes nonlinear when plotted on a logarithmic axis.

4.1.2 Half-Lives

For first-order reactions the rate constant has units of time^{-1} (e.g. min^{-1}) which is difficult to visualize. Therefore the rate of a first-order reaction is normally described by a parameter which is a reciprocal of k, and which therefore has units of time. A property of exponential decreases is that the time taken for any concentration on the curve to decrease by a factor of 2 (i.e. to halve) is a constant, and independent of concentration. This parameter is the half-life of the decrease and is related to k by the equation

$$t_{1/2} = \frac{0.693}{k} \tag{5}$$

This can be seen graphically for the first-order reaction in **Figure 7**, where the concentrations decrease by a factor of 2 between successive hourly intervals, that is, 80, 40, 20, 10, 5, 2.5, 1.25 so that the half-life in this case is 1 hour. The half-life is usually calculated from the slope of the decrease, which can be calculated by a least-squares regression analysis of the ln concentration–time curve data.

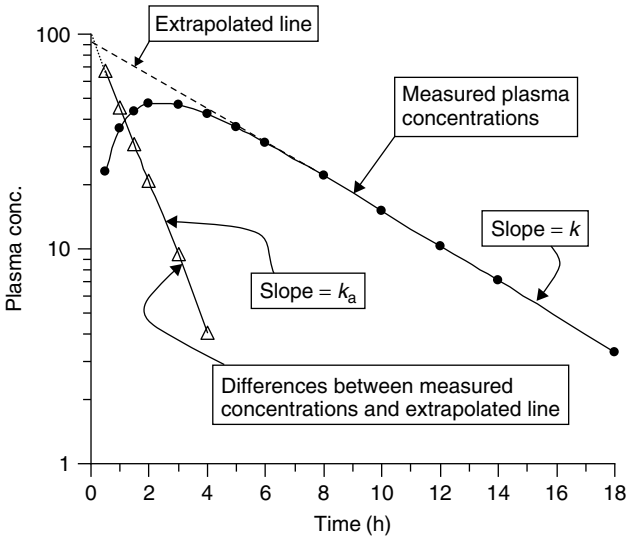

Figure 8 Plasma concentration–time curve following oral administration of a compound showing simple first-order absorption. The absorption rate constant (k_a) is calculated from the difference between the measured values and the extrapolated values (residuals).

4.2 Absorption

4.2.1 Rate of Absorption

Measurement of the rate of absorption requires information on the plasma (or blood) concentrations of the compound at frequent time intervals following a single dose administered to the site of absorption. The resulting data can be analysed in a number of ways. In the simple example shown in **Figure 8**, the absorption occurs rapidly as a first-order process, and the total concentration–time curve is dependent on two exponential terms—one for

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absorption (k_a) and one for elimination (k), that is

$$C_t = \frac{F \times \text{dose} \times k_a(e^{-kt} - e^{-k_a t})}{V \times (k_a - k)} \quad (6)$$

where

F = bioavailability (see later) and
V = apparent volume of distribution (see later).

For rapidly absorbed compounds k_a exceeds k, and therefore the term $e^{-k_a t}$ approaches zero at late time points, and the concentration–time curve is determined by a single exponential term that describes elimination (e^{-kt}). This is shown in **Figure 8** by the extrapolated line of the terminal data. If the whole dose had entered the circulation at time zero, then the concentration–time curve would have resembled this extrapolation line. The *difference* between the actual data obtained and the extrapolated line is due to the absorption of the chemical into the circulation. The differences (or *residuals*) between the actual data and the extrapolation line are then plotted (**Figure 8**) in order to derive the absorption rate constant.

The absorption and elimination of chemicals rarely show this simple pattern and more complex equations may be necessary. For example, if there is a lag time (t_{lag}) between dosing and measurable concentrations, then the time t in the equation given above has to be replaced by ($t - t_{lag}$). In addition if there is a clear distribution phase (see later) then a more complex equation involving three exponential terms may be required; in some cases the absorption rate can only be determined by comparison of oral and intravenous data. Readers are referred to Chapter 4 of Gibaldi and Perrier (1982) for further information. Characterization of the rate of absorption requires the collection of a number of plasma or blood samples (at least three or four) during the absorption phase.

In some cases the rate of absorption does not resemble either a first- or zero-order input and no model can be fitted to the data. Under such circumstances the best estimate of the rate of absorption is the mean absorption time (MAT). This is calculated from plasma concentration–time curve data after *both* oral and intravenous dosing using statistical moments analysis (see Gibaldi and Perrier, 1982; Renwick, 2008).

$$\text{MAT} = \text{MRT}_{\text{oral}} - \text{MRT}_{\text{iv}} \quad (7)$$

where

MRT = mean residence time.

The MRT is the ratio of the area under the first moment of the concentration–time curve (AUMC) divided by the area under the concentration–time curve (AUC).

$$\text{MRT} = \frac{\text{AUMC}}{\text{AUC}} \quad (8)$$

The AUMC is derived by the trapezoidal rule applied to a graph of (concentration × time) ($C \times t$), for each sample, plotted against time (t) for that sample, and therefore does not require fitting of any specific models to the data. Both the AUC and the AUMC data calculated between time zero and the last sample by the trapezoidal rule must be extrapolated to infinity for the above equation to be valid. Extrapolation of the AUC is by addition of C_{last}/β to the AUC measured by the trapezoidal rule. AUMC from the last concentration–time point to infinity is achieved by the addition of

$$\frac{t_{last} \times C_{last}}{\beta} + \frac{C_{last}}{\beta^2} \quad (9)$$

where β is the terminal slope—see later.

In the brief description above it is assumed that the compound is absorbed rapidly but eliminated slowly, for example, a lipid-soluble compound requiring metabolism. For highly polar molecules, absorption from the gut may be slow while elimination from the blood, via the kidneys, may be rapid. However, the concentration–time curve still resembles that shown in **Figure 8**. The reason for this is that the exponential function that defines the later time points is always the lower rate constant, whether it is k or k_a. Therefore, for slowly absorbed compounds the value of ($k_a - k$) will be negative when $k > k_a$, and at late time points e^{-kt} will approach zero and the concentration will be determined by ($-e^{-k_a t}$). Thus for slowly absorbed/rapidly eliminated compounds the terminal slope is determined by k_a and the more rapid increase by k—a situation described as *flip-flop kinetics*. This type of profile is the usual pattern shown by transdermal absorption.

Both the rate and extent of absorption from the gastrointestinal tract can be influenced by a number of variables such as the nature and volume of the vehicle, the dose of compound (especially in relation to its solubility) and the presence of food in the gut lumen. Toxicokinetic studies should be performed under conditions which reflect the dosing regimen used in animal toxicity studies, for example, gavage in corn oil or administration in the diet.

4.2.2 Extent of Absorption

The fraction of the dose absorbed as the parent compound (or bioavailability, F) can be determined only by comparison to conditions in which it is known that all of the dose enters the general circulation *as the parent compound*, that is, by reference to an intravenous dose given as a bolus or as a slow infusion. Because of the different shapes of the concentration–time curves following oral and intravenous doses (**Figure 9**), comparisons of the concentrations at single time points are meaningless.

The bioavailability can be calculated from the area under the plasma concentration–time curve (AUC)

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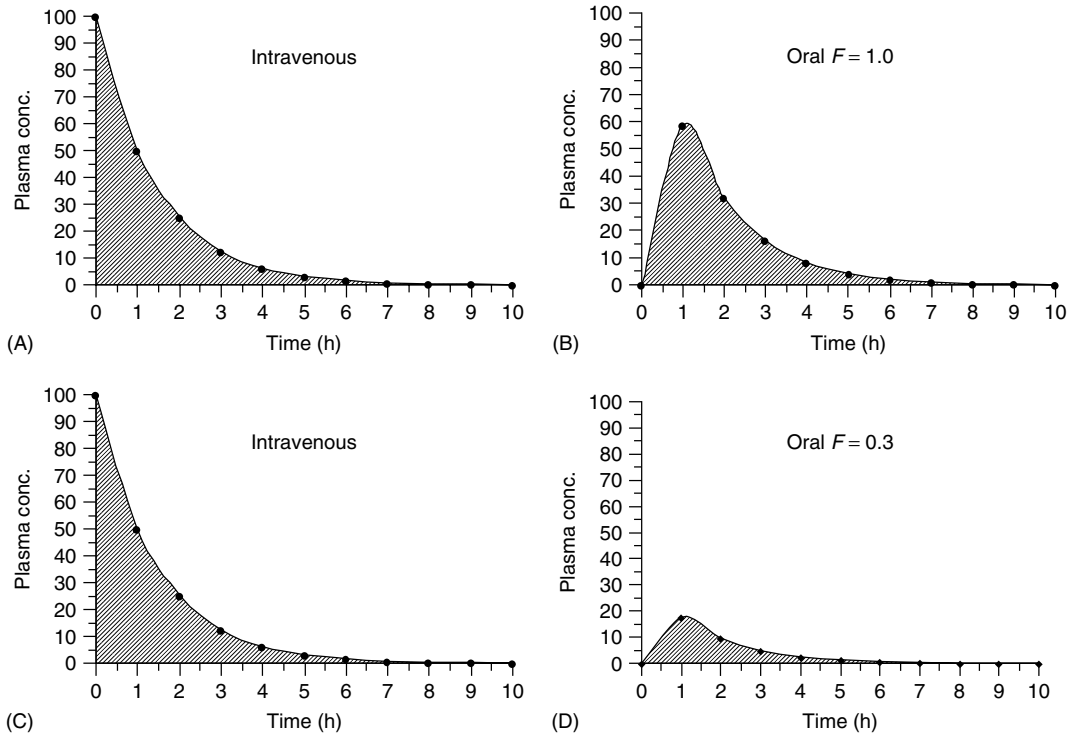

Figure 9 Comparison of areas under the plasma concentration–time curves for a compound showing complete absorption (F = 1.0; (A), (B)) and a compound showing incomplete absorption (F = 0.3; (C), (D)). The reason for the incomplete bioavailability, for example, first-pass metabolism or poor absorption from the gut lumen, cannot be determined without additional information.

between administration and infinity following both routes of administration (**Figure 9**). If the doses are identical:

$$F = \frac{\mathrm{AUC_{oral}}}{\mathrm{AUC_{iv}}} \tag{10}$$

The AUC values must be extrapolated to infinity (see above) for this equation to be valid, and therefore it is important that the concentration–time curves are followed for sufficient time to allow accurate measurement of the terminal elimination rate constant.

This is illustrated in **Figure 9**. In many cases acute toxicity may prevent the administration of a high intravenous dose, while a high oral dose may be essential to obtain measurable plasma concentrations. Under such circumstances different doses may be given by oral and intravenous routes and F is calculated as:

$$F = \frac{\mathrm{AUC_{oral}} \times \mathrm{dose_{iv}}}{\mathrm{AUC_{iv}} \times \mathrm{dose_{oral}}} \tag{11}$$

The basis for this calculation is that plasma clearance (CL—see later) is the same after each of the two separate doses (oral and intravenous). In clinical studies this is ensured by studying the same subjects on two separate occasions. For animal toxicity studies it may be necessary to study different animals for each route, in which case the age and sex of the animals should be comparable.

The use of AUC data assumes that there is no saturation of elimination and that the intravenous dose, with its possibly higher peak plasma concentrations, does not produce cardiovascular, renal or metabolic effects which could alter the plasma clearance of the compound.

The bioavailability (F) can also be calculated from urinary data. The percentage of the dose which is excreted in the urine *as the parent compound* is dependent on the amount of parent compound presented to the kidneys via the circulation, that is, the percentage of the dose excreted in the urine to time t is proportional to the plasma AUC to time t. Thus bioavailability (F) can be calculated as:

$$F = \frac{\%\text{ oral dose in urine as the parent compound}}{\%\text{ intravenous dose in urine as the parent compound}} \tag{12}$$

where the percentage dose is measured until no more parent drug is excreted (i.e. t = infinity).

The extent of absorption for different formulations or vehicles given by the same route can be compared in the absence of information from an intravenous study using the above equations. However the AUC ratio of one to the other is simply a comparison of the internal dose via each mode of administration and is not a measure of the true absolute amount of chemical that reaches the general

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circulation. Such comparative ratios are sometimes called the *relative bioavailability*. To provide a clear separation of such data from the true bioavailability (F as defined above), F is often termed the *absolute bioavailability*.

4.3 Distribution

4.3.1 Rate of Distribution

The rate at which a compound moves out of the blood into body tissues is usually rapid and can only be measured when the dose enters the circulation at a rate considerably greater than the rate of distribution. In effect, this means that the rate of distribution is usually measured following an intravenous bolus dose. The plasma concentration–time curve following a bolus dose depends on the rate and extent of distribution as well as the rate of elimination (**Figure 5**).

In the simplest case (**Figure 5**; bottom line on A), essentially complete distribution will occur between dosing and the collection of the first blood sample, so that distribution will appear to be instantaneous. Thus the concentration–time curve can be described by a single exponential term (k) that is dependent on elimination processes (e.g. metabolism and excretion), and the compound achieves instantaneous distribution within a single compartment (**Figure 10**). The rate constant for a one-compartment model is usually given as k (as used above), rather than k_{10}.

However, normally a distinct distribution phase can be detected (**Figure 5**; top 2 lines on A), during which the chemical is undergoing two processes at the same time: distribution and elimination. The tissues and blood reach equilibrium when the distribution phase is complete, and the concentration–time curve becomes a simple monoexponential decrease determined by the elimination rate constant (k). For such compounds the body can be regarded as comprising two compartments with the chemical achieving instantaneous equilibrium in Compartment 1 (the central compartment), but taking a finite time for equilibration between Compartment 1 and Compartment 2 (the peripheral compartment) (**Figure 10**). Thus two exponential terms are necessary—a rapid distribution rate (α) and the slower elimination rate (β). A more correct nomenclature for such multiple exponential rate constants is λ_1, λ_2, and so on, and λ_z, where these represent the rate constants for the fastest, next fastest and slowest rate, respectively. This nomenclature is receiving increasingly wide usage. The overall elimination rate (β or λ_z) or terminal half-life ($0.693/\beta$) is *not* equivalent to k_{10}; it is a composite term involving k_{10}, k_{21} and k_{12}.

The concentration in the peripheral compartment is zero initially, and then rises during the distribution phase until equilibrium is reached, following which the concentrations decrease in parallel with those in the general

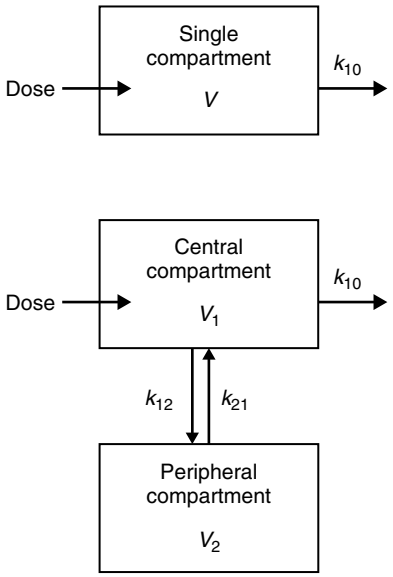

Figure 10 Simple one-compartment and two-compartment models of distribution and elimination. More complex models involving three or more compartments with elimination from both central and peripheral compartments are sometimes necessary to provide a mathematical description of the plasma concentration–time curves. (The relationships between α, β, k_{10}, k_{12} and k_{21} are given in the text; for a one-compartment system $k_{10} = k$).

circulation (**Figure 11**). The absolute concentration in the tissue will depend on the relative affinity of the blood and tissue for the compound (**Figure 6**). Tissues with different relative affinities may be part of the same peripheral compartment because this is a mathematical concept, and the only parameter that is shared is the rate of equilibrium between blood and tissue (**Figure 11**). Thus calculation of a mean concentration for the peripheral compartment is rarely of value. However, concentrations in the peripheral compartment can be predicted from a single postequilibrium measurement and knowledge of the terminal rate constant (β) in plasma.

The rate constants, α and β, and the intercepts, A and B, can be calculated by the method of residuals (**Figure 12**). The 'micro'-rate constants k_{10}, k_{12} and k_{21} can be calculated as:

$$k_{21} = \frac{A\beta + B\alpha}{A + B} \tag{13}$$

$$k_{10} = \frac{\alpha\beta}{k_{21}} \tag{14}$$

$$k_{12} = \alpha + \beta - k_{21} - k_{10} \tag{15}$$

It should be appreciated that such manipulations of derived data exaggerate any errors in the measurements. Accurate parameter estimates require that at least three to four blood samples are collected during each separate phase (e.g. α and β) and that the terminal phase is

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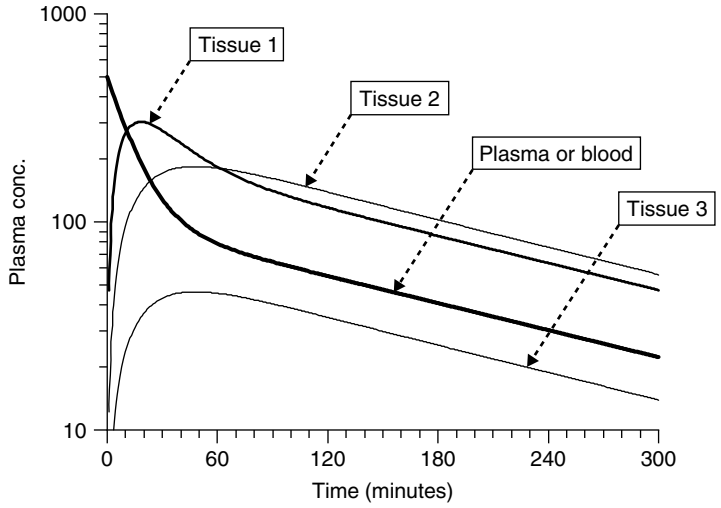

Figure 11 The concentration–time curve for a chemical in plasma (or blood) and in tissues that equilibrate rapidly (Tissue 1) or more slowly (Tissues 2 and 3) with plasma or blood. Tissue 2 shows a higher affinity than Tissue 3 but shares the same distribution rate constants; an alternative explanation for the difference is that Tissue 3 may have a highly active efflux pump on the cell membrane.

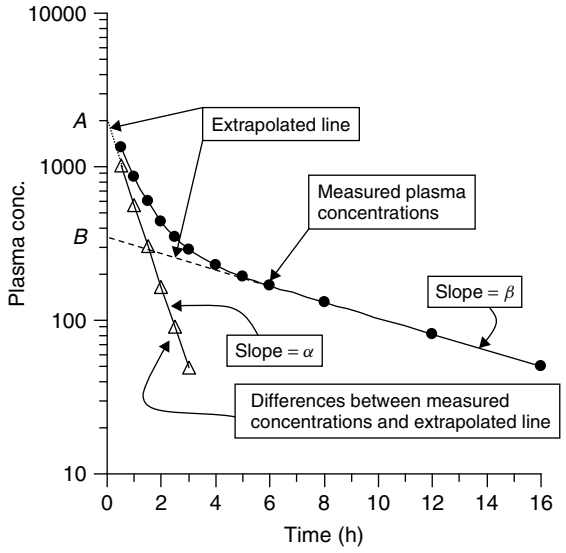

Figure 12 The use of the method of residuals to calculate distribution parameters. The terminal phase is given by $Be^{-\beta t}$ and the differences between the plasma concentrations and this line (the residuals) give the distribution phase $Ae^{-\alpha t}$.

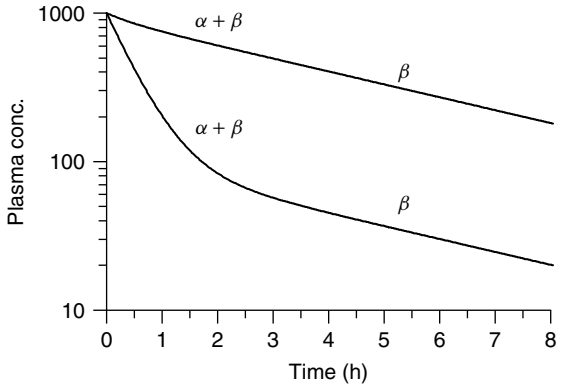

Figure 13 Plasma concentration–time curves for two compounds given as single bolus doses that have the same rate constants (α and β), but with different proportions of the dose undergoing distribution; that is, different apparent volumes of distribution. (Note—normally a higher apparent volume of distribution would result in slower elimination; see text).

followed for as long as possible and for at least two half-lives. Some compounds may require three or more exponential terms (and compartments) to provide an adequate model.

4.3.2 Extent of Distribution

The shape of the concentration–time curve following a single bolus intravenous dose is dependent on both the rate of distribution and the extent to which the dose is retained in the central compartment or distributed to the tissues (**Figure 5**). This is illustrated in **Figure 13**. In the upper line ($C_t = 100e^{-2t} + 1000e^{-0.2t}$), only one-tenth of the material is associated with distribution to the peripheral tissues ($\alpha + \beta$), and the concentration–time curve almost resembles a monoexponential decrease of slope β; the distribution phase could have been missed if the first sample had been taken at one hour, or if the assay method had a coefficient of variation greater than about 5%. In the lower line ($C_t = 1000e^{-2t} + 100e^{-0.2t}$), most of the dose undergoes distribution to the peripheral tissues (Compartment 2) and two phases are apparent,

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with distribution clearly continuing until about three hours after the dose.

The extent of distribution is characterized by the *apparent volume of distribution* (V), which can be calculated by a number of different methods. V is the parameter which relates the concentration in plasma (C) to the total body burden (Ab) with which it is in equilibrium; that is,

$$V = \frac{Ab}{C} \tag{16}$$

Thus for a one-compartment system which shows instantaneous equilibrium:

$$V = \frac{\text{dose}}{C_0} \tag{17}$$

where C_0 is the concentration at $t = 0$.

The apparent volume of distribution is the parameter which relates the total amount of chemical in the body to the plasma concentration. It can be regarded as a dilution factor and represents the volume of plasma into which the chemical appears to have dissolved.

For a two-compartment system (see **Figures 10** and **12**) the situation is more complex. dose/$(A + B)$ gives the volume of the central compartment only, because the constant A is derived prior to distribution and the attainment of equilibrium. The volume of distribution can be calculated as dose/B, but this estimate does not take into account the volume of, or amount of chemical, in the central compartment. The apparent volume of distribution usually calculated for two-compartment systems is V_β:

$$V_\beta = \frac{\text{dose}}{\text{AUC} \times \beta} \tag{18}$$

where AUC is the area under the plasma concentration–time curve between zero and infinity.

It must be emphasized that the volume of distribution is not a real volume, but is the volume of plasma (or blood if the concentration is measured in whole blood) in which the body load/burden *appears* to have dissolved. If the concentration in plasma after equilibration with all the tissues is extremely low then the apparent volume of distribution (Ab/C or dose/C_0) will be extremely high. For example, highly lipid-soluble compounds may have an apparent volume of distribution of 50 litres per kg body weight—a physiological impossibility. Thus all that the apparent volume of distribution really represents is the dilution factor between plasma concentration and body burden. It provides an indication of the extent to which the compound has left plasma and entered the tissues, but it cannot provide information on uptake into specific tissues.

If a chemical enters a tissue but is eliminated without re-entering the general circulation as the chemical itself, this is an elimination or clearance process and *does not contribute to the distribution parameters*.

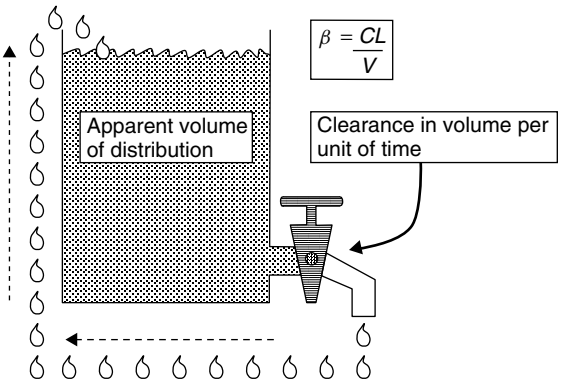

Figure 14 The relationship between apparent volume of distribution (V), clearance (CL) and overall elimination. The elimination rate constant is proportional to CL/V.

4.4 Elimination

4.4.1 Rate of Elimination

The overall rate of elimination and its associated rate constant (k or β or λ_z) depend on two independent variables. The apparent volume of distribution (V—see above) and the clearance (CL). This is illustrated in **Figure 14** by analogy to the emptying of a container of water. The rate at which the level falls in the container will be proportional to the removal (flow) through the tap (analogous to the clearance) but inversely proportional to the volume of the container (analogous to the apparent volume of distribution); that is

$$\text{rate} \propto \frac{CL}{V} \tag{19}$$

4.4.2 Clearance

A clearance process is one in which the chemical is removed permanently from the circulation, that is, by metabolism or excretion. Clearance is defined as: *the volume of blood (or plasma) cleared of chemical per unit time*. Thus, on passage through an organ of elimination the blood can be cleared of the compound, either totally or partially. The extent of uptake and removal is indicated by the decrease in concentration in the blood leaving the organ via the vein (C_v) compared with that entering via the artery (C_a). (The decrease must be due to removal, not simply distribution, which is reversible, and therefore such measurements are usually made at steady state—see later). The removal by the organ is usually expressed as

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the *extraction ratio* (*ER*).

$$ER = \frac{(C_a - C_v)}{C_a} \tag{20}$$

If all of the chemical is removed then $C_v = 0$ and $ER = 1.0$, whereas if only 10% of the arterial concentration is removed $C_v = 0.9$ and $ER = 0.1$. If *ER* approaches 1.0 then all of the blood that flows through the organ of elimination will be cleared of the chemical, and the clearance (*CL*) will equal the blood (or plasma) flow through the organ (Q). If the extraction ratio is 0.1 then only one-tenth of the organ blood flow will be cleared of the chemical, which results in the relationship:

$$CL = Q \times ER \tag{21}$$

The physiological significance of this is that the most important toxicokinetic parameter (*CL*) can be related to the blood flow to the organ of elimination (Q) and the capacity of the organs to extract the chemical (*ER*) and to remove it from the body (e.g. by metabolism). These processes are the basis for developing physiologically based pharmacokinetic (PBPK) models (see **Physiologically Based Pharmacokinetic Modelling**).

If a compound has a very high extraction ratio, then changes in organ blood flow will not significantly affect the extraction efficiency of the organ, and consequently clearance will be dependent on, and directly proportional to, the organ blood flow. If the extraction ratio is low then uptake or extraction by the organ is dependent on the time available for uptake, that is, the slower the organ blood flow (Q), the greater will be the extraction ratio (*ER*). Thus, a change in Q results in an opposite change in *ER* so that *CL* remains relatively constant and independent of organ blood flow.

There are mathematical models which characterize the relationship between *CL*, Q and *ER* (see Wilkinson, 1976). In the context of the present chapter, the importance of this relationship is that if the doses of the chemical given to animals are sufficient to alter organ perfusion, then this could influence the clearance of the chemical if it has a high extraction ratio.

The clearance (*CL*) of a chemical is perhaps the single most important pharmacokinetic parameter because it relates to the ability of the organs of elimination to remove the chemical from the body. It is defined as:

$$CL = \frac{\text{rate of elimination of the chemical}}{\text{plasma concentration}}$$

$$\text{e.g.} \quad \frac{\mu\text{g min}^{-1}}{\mu\text{g ml}^{-1}} \quad \text{or} \quad \text{ml min}^{-1} \tag{22}$$

For first-order reactions the rate of elimination at any point in the plasma concentration–time curve (see **Figure 7**) is directly proportional to the plasma concentration because *CL* is constant. The units of *CL* are volume × time^{-1} (e.g. ml min^{-1}), and clearance represents the volume of plasma (or blood if concentrations are measured in whole blood) cleared of compound per unit time. The plasma clearance, *CL,* represents the sum of all individual clearance processes such as metabolism (CL_M), renal excretion (CL_R), biliary excretion (CL_B), and so on.

$$CL = CL_M + CL_R + CL_B + \ldots \tag{23}$$

Plasma clearance is usually calculated from the dose that enters the systemic circulation and the area under the plasma concentration–time curve (AUC):

$$CL = \frac{\text{dose}}{\text{AUC}} \tag{24}$$

For this equation to be valid both the dose and AUC need to be defined properly.

4.4.2.1 Dose

Because *CL* relates to the capacity of the body to eliminate the chemical, the dose has to be fully available to the organs of elimination. Thus *CL* is calculated from the data following an *intravenous dose*, which can be given either as a bolus or as a slow infusion. If the dose is given orally then the true dose available to the organs of elimination is ($F \times$ dose administered) where F is the bioavailability, that is

$$CL = \frac{\text{dose}_{iv}}{\text{AUC}_{iv}} = \frac{\text{dose}_{oral} \times F}{\text{AUC}_{oral}} \tag{25}$$

Rearrangement of the above equation shows how the bioavailability can be calculated from AUC data, and also explains why this calculation assumes that *CL* is a constant for the two different routes:

$$F = \frac{\text{AUC}_{oral} \times \text{dose}_{iv}}{\text{dose}_{oral} \times \text{AUC}_{iv}} \tag{26}$$

It is common in papers to see a parameter termed 'oral clearance', which is $\text{dose}_{oral}/\text{AUC}_{oral}$: in reality this parameter is *CL/F*, and is of limited value because it depends on two variables, either of which could be influenced by physiological changes.

4.4.2.2 AUC

The AUC can be calculated by the trapezoidal rule between time zero and the last measured concentration, but needs to be extrapolated to infinity (see above) if the calculation of *CL* is to be valid. Consequently, it is essential for the samples to be collected until a loglinear decrease is defined clearly. The AUC can also be derived by fitting a suitable model to the data and calculating the AUC from the derived parameters; for example, $\text{AUC} = C_0/k$ or $\text{AUC} = A/\alpha + B/\beta$.

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4.4.3 Relationship of Clearance to Half-Life

The relationship between clearance, the terminal slope and the apparent volume of distribution can be derived from the simple definition of clearance. *CL* is the rate of elimination from the body divided by the plasma concentration; the rate of elimination from the body at any time (t) is the elimination rate constant (k) times the body burden ($V \times C_t$) and therefore:

$$CL = \frac{k \times V \times C_t}{C_t} = kV \tag{27}$$

or

$$k = \frac{CL}{V} \quad \text{and} \quad t_{1/2} = \frac{0.693V}{CL} \tag{28}$$

For a two-compartment system:

$$\beta = \frac{CL}{V_\beta} \quad \text{and} \quad t_{1/2} = \frac{0.693V_\beta}{CL} \tag{29}$$

This equation shows that the terminal elimination rate constant (k or β) is a composite parameter which is dependent on *two independent, physiological variables*; that is, *CL*, which reflects the rate of extraction and removal from blood, and $1/V$, which reflects the amount of chemical remaining in the blood and available for clearance. This inter-relationship between *CL*, *V* and $t_{1/2}$ assumes that first-order kinetics apply, that is, that the chemical does not show dose-dependent kinetics.

Renal clearance (CL_R) is the only specific clearance term that can be measured easily. CL_R may be defined as:

$$CL_R = \frac{\text{rate of elimination in urine (as the parent compound)}}{\text{concentration in plasma}} \tag{30}$$

The rate of elimination in urine can be calculated by measuring the concentration of the chemical (C_u) in the volume of urine (V_u) produced in a known time interval (Δt). If the concentration in plasma at the midpoint of the urine collection (C_{mid}) is known, then CL_R can be calculated as:

$$CL_R = \frac{C_u \times V_u}{\Delta t} \times \frac{1}{C_{mid}} \tag{31}$$

This equation can be rearranged because ($C_u \times V_u$) equals the total amount excreted (A_{ex}) over the time interval Δt, and the product ($\delta t \times C_{mid}$) is the AUC of the plasma concentration–time curve for the time interval Δt. Therefore CL_R may be calculated also from plasma AUC data and the amount excreted unchanged (A_{ex}) over the same time interval.

$$CL_R = \frac{A_{ex0-t}}{AUC_{0-t}} \tag{32}$$

CL_R is a constant for first-order reactions, and therefore the rate of excretion at any time will be proportional to the plasma concentration. Therefore the decrease in excretion rate with time will mirror the decrease in plasma concentration. Thus a graph of the natural log of the excretion rate vs. time will give a rate constant and half-life the same as those derived from plasma; therefore the terminal half-life can be calculated from serial timed urine collections without taking blood samples. Alternatively, the half-life can be calculated from the amount remaining to be excreted using the sigma-minus method (see under trans-species comparisons, below). (It is not possible to derive total plasma clearance (*CL*), renal clearance (CL_R) or the apparent volume of distribution (V) without taking blood samples.)

Metabolic clearance (CL_M) cannot be measured readily, but metabolism and renal excretion are the two principal routes of total clearance (*CL*) in many cases, and it can be assumed that:

$$CL = CL_M + CL_R \tag{33}$$

$$CL_M = CL - CL_R \tag{34}$$

$$CL_M = \frac{\text{dose}}{\text{AUC}} - \frac{\text{amount excretion unchanged}}{\text{AUC}} \tag{35}$$

$$CL_M = \frac{(\text{dose} - \text{amount excreted unchanged})}{\text{AUC}} \tag{36}$$

5 THE INTERPRETATION OF CHRONIC HIGH-DOSE ANIMAL FEEDING STUDIES

In many cases regulatory decisions use data from chronic high-dose animal feeding studies to predict possible risk for humans. However, chronic high-dose treatments can lead to changes in clearance and accumulation that would not be anticipated from single-dose studies.

5.1 Chronic Intake

During chronic intake the concentration of the chemical rises in the plasma and tissues as illustrated in **Figure 15**. Both of the hypothetical compounds shown in **Figure 15** exhibit rapid absorption and one-compartment kinetics with similar apparent volumes of distribution; therefore, the 12-fold difference in the elimination rate constant arises from a 12-fold difference in clearance. The compound in **Figure 15** with a half-life of 3 hours shows negligible concentrations remaining at 24 hours when the next dose is given. In contrast, at 24 hours the concentration of the compound with a half-life of 36 hours is about 60% of the peak value, so that there is marked accumulation with successive doses. Eventually a steady

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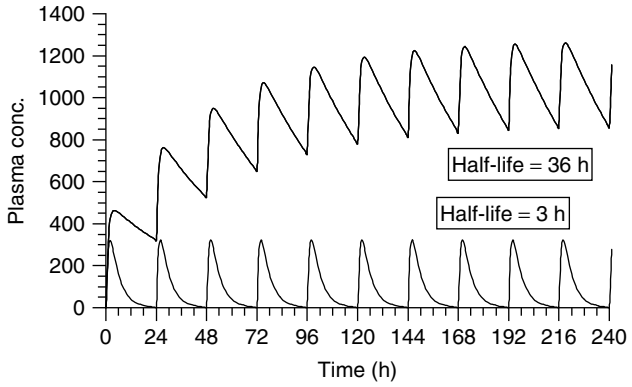

Figure 15 The accumulation of chemicals during chronic intake. In the two examples shown the compounds are given as single oral doses every 24 hours. Both compounds exhibit rapid absorption and one-compartment characteristics with monoexponential decreases (i.e. $C_t = C_0 e^{-kt}$).

state will be reached in which the rate of elimination per 24 hours equals the rate of administration, that is

$$\text{rate of input} = \text{rate of elimination} \tag{37}$$

$$\frac{D \times F}{T} = CL \times C_{ss} \quad \text{or} \quad C_{ss} = \frac{D \times F}{T \times CL} \tag{38}$$

where

D = dose administered
F = bioavailability
T = dose interval
CL = clearance, and
C_{ss} = average plasma concentration at steady state.

These determinants of C_{ss} can be deduced intuitively because C_{ss} is directly proportional to dose given and the fraction absorbed, and inversely proportional to the interval between successive doses or the ability of the organism to eliminate the chemical. It is important to note that the apparent volume of distribution (V) is not included in the equation for the average plasma concentration at steady state because at steady state the tissues and plasma are in equilibrium (see the discussion of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) later).

This equation is based on the definition of clearance (CL = rate of elimination/C) applied to the steady-state condition when the rate of elimination exactly equals the rate of administration. Thus steady-state data can be used to calculate the value of CL for intravenous data or CL/F for oral data, that is

$$CL = \frac{D \times F}{T \times C_{ss}} \tag{39}$$

$T \times C_{ss}$ is the AUC for a dose interval at steady state ($0-T$), so that this equation can also be written as:

$$CL = \frac{D \times F}{\text{AUC}_{0-T}} \tag{40}$$

This can be compared with the measurement of CL after a single dose using the AUC from time zero to infinity, where

$$CL = \frac{D \times F}{\text{AUC}_{0-\infty}} \tag{41}$$

These relationships allow the AUC_{0-T} at steady state, and the average steady-state concentration (C_{ss}) to be calculated from the $\text{AUC}_{0-\infty}$ for a single dose:

$$\text{AUC}_{0-T}(\text{steady state}) = \text{AUC}_{0-\infty}(\text{single dose}) \tag{42}$$

$$C_{ss} = \frac{\text{AUC}_{0-\infty}}{T}(\text{single dose}) \tag{43}$$

The relationship between $\text{AUC}_{0-\infty}$ (single dose) and AUC_{0-T} (steady state) is illustrated in **Figure 16**. *The AUC for a dose interval at steady state is the best estimate of chronic exposure to the chemical* and can be determined without fitting any specific model to the data. If AUC_{0-T} (steady state) is measured and does not equal $\text{AUC}_{0-\infty}$ (single dose), then the most likely explanation is a change in clearance on repeated dosage (see later).

The increase to steady state for a compound which shows a simple monoexponential decline after an intravenous bolus dose is an inversion of the elimination phase, that is, the percentage of steady state achieved after administration for 1, 2, 3, 4 and 5 half-lives is 50, 75, 87.5, 93.75, 96.875% of the steady-state concentration (C_{ss}) or body burden ($C_{ss} \times V$). Thus it takes approximately four to five times the elimination (terminal) half-life to approach steady state. **Figure 17** shows that the rate of accumulation is directly proportional to the elimination half-life and also that the plasma concentration and therefore body burden is directly proportional to the daily dose.

The octachloro analogue of TCDD (OCDD, octachlorodibenzo-*p*-dioxin) is 100–1000 times less potent than TCDD (Couture *et al.*, 1988), but its elimination half-life in rats of three to five months is about four times higher than that of TCDD, so that even greater accumulation is possible (Birnbaum and Couture, 1988). For compounds with such long half-lives, steady state will not have been reached at the end of a 13 week study (**Figure 17**). In addition, if there

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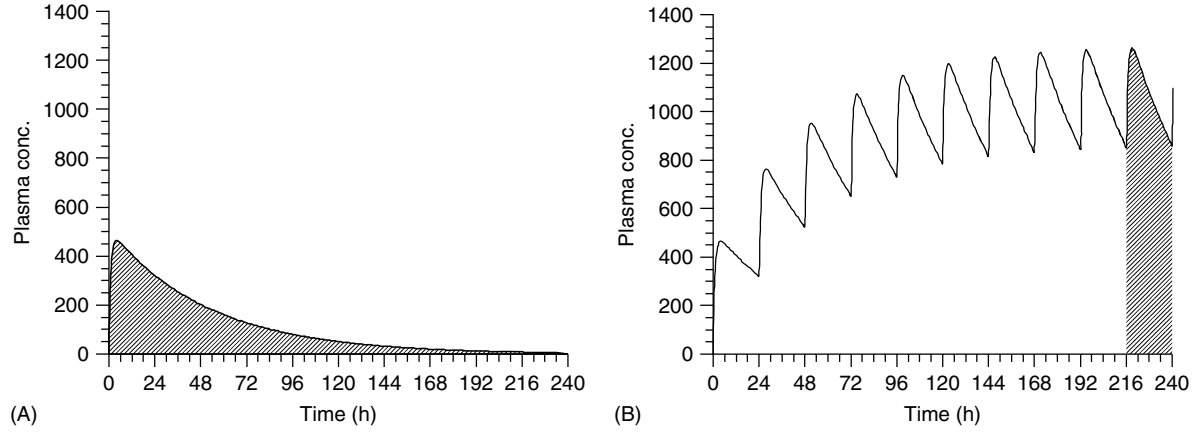

Figure 16 The relationship between $AUC_{0-\infty}$ for a single dose (A) and AUC_{0-T} for a dose interval at steady state (B). The AUC values are the same unless bioavailability or clearance of the chemical changes with repeated dosage.

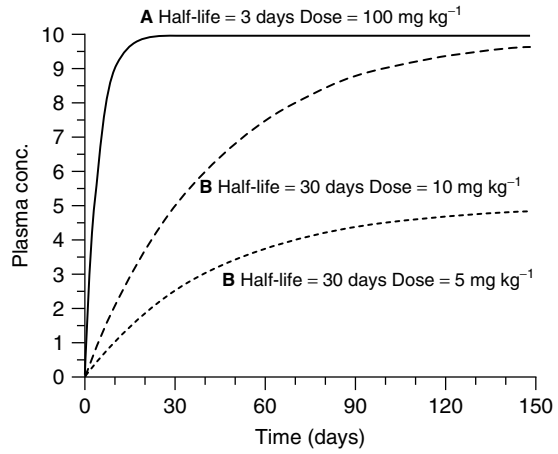

Figure 17 The effect of elimination half-life and dosage on the accumulation of a chemical during repeated dosage. (For clarity each compound is shown as being given by a continuous intravenous infusion.) The compounds have similar apparent volumes of distribution but the clearance of A is 10 times greater than that of B, so that its half-life is 10 times lower. Thus, daily intakes of 100 mg kg^{-1} of A and 10 mg kg^{-1} of B will give similar average steady-state concentrations, while a dose of 5 mg kg^{-1} of B will give an average steady-state concentration one-half of that at a dose of 10 mg kg^{-1} day^{-1}.

Table 4 The use of single-dose data to predict the time for development of steady state

Time (t) (h)	C (μg ml^{-1})	AUC_{0-t} (μg ml^{-1} h)	% ss
0	0	—	—
0.5	11.03	2.76	1.91
1	16.32	9.60	6.66
1.5	15.41	17.52	12.15
2	16.69	25.55	17.71
3	13.89	40.80	28.28
4	13.45	54.47	37.76
5	8.82	65.44	45.37
6	5.82	72.66	50.37
7	4.85	77.98	54.06
8	4.63	82.71	57.34
12	2.41	96.52	66.91
14	2.26	101.26	70.20
24	1.23	118.19	81.93
28	0.93	122.48	84.91
32	0.92	126.18	87.47
48	0.39	136.07	94.33
56	0.35	139.02	96.37
72	0.12	142.46	98.76
∞	—	144.25	100.00

The plasma concentration–time curve data are for a single dose of saccharin given orally to a human volunteer (data from Sweatman *et al*., 1981).
C—concentration in plasma, AUC_{0-t}—area under concentration–time curve to the time of the plasma sample, and % ss—percentage of steady state $AUC_{0-t}/AUC_{0-\infty} \times 100$.

is a minimum body burden or plasma concentration necessary for toxicity this may have been reached only a short while before sacrifice. Clearly the interpretation of data from 'short-term' studies should take into account the half-life of the compound, since a 90 day study may be a steady-state study for some compounds but not for others.

For a compound which shows a more complex plasma concentration–time curve (**Table 4**), for example, oral administration of a slowly absorbed compound like saccharin, the percentage of steady state at any time (t) can be calculated from single-dose data as ($AUC_{0-t}/AUC_{0-\infty} \times 100$). Thus the time to steady state can be assessed without the fitting of any model (**Figure 18**).

'Steady state' will be reached within the first 24 hours of regular dosing for compounds which have a half-life of 6 hours or less. However, there will be large diurnal variations in concentration as illustrated in **Figure 15**,

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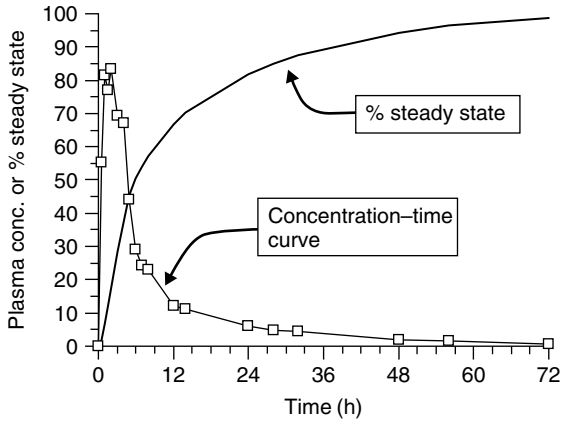

Figure 18 The relationship between single-dose kinetics and the development of steady state during chronic administration. The percentage of steady state at any time is: the AUC to that time, divided by the AUC to infinity times 100 (data from **Table 4**—except that the plasma concentrations have been multiplied by 5 to fit on the same axis as the % steady state).

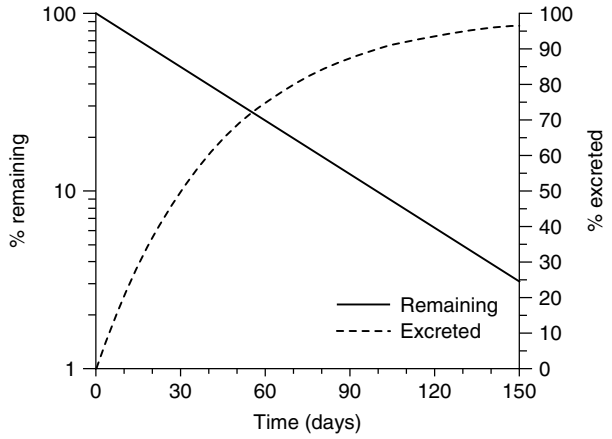

Figure 19 The calculation of the elimination rate constant from the cumulative elimination of the compound (see **Table 5** for data). This analysis assumes that either the parent compound is excreted unchanged or that the formation of metabolites is the rate-limiting step.

where the half-life of one of the compounds is only three hours. Compounds which have long half-lives will accumulate during chronic intake but show less interdose fluctuation.

The slow elimination of compounds such as TCDD allows the half-life to be calculated from the total amount of radioactivity recovered in urine and faeces each day using an adaptation of the *sigma minus method* (Gibaldi and Perrier, 1982). This method is based on measurements of the excretion of the parent compound until no more can be detected in the urine (and/or faeces). For each collection time interval the amount remaining to be excreted is calculated as: the amount excreted as parent compound to infinity minus the amount excreted up to that time; a plot of the natural log of the amount remaining to be excreted against the time at the end of the collection interval will have a slope of $-\beta$ (or $-\lambda_z$), with an intercept of the natural log of the total amount excreted unchanged. This method is applicable even in cases where excretion of the parent compound is not the major route of elimination, because the amount of parent compound excreted at any time is proportional to the plasma concentration (C) and therefore the body burden (V times C) of parent compound at that time. In the case of TCDD, the 'body burden' of TCDD remaining after each day was calculated as: dose administered minus total ^{14}C recovered in excreta to that time (**Table 5**). A plot of the natural log of the body burden vs. time was used to calculate the elimination half-life (**Figure 19**). This approach is particularly useful for calculating the elimination kinetics of compounds that are eliminated very slowly, and also has the advantage of being noninvasive. It should be appreciated that in studies on slowly eliminated lipid-soluble compounds, the radioactivity detected in the excreta may be present partly as metabolites. Under such circumstances the sigma-minus method using excretion of total ^{14}C is only valid if the body burden is as parent compound, and the metabolites, once formed, are eliminated rapidly; that is, the rate-limiting step is the initial metabolism of the parent compound.

5.2 Dose-Dependent Kinetics

A nonlinear relationship between dose and area under the plasma concentration–time curve is indicative of *dose-dependent* or *nonlinear* kinetics; in other words the compound does not obey first-order kinetics at high doses. Nonlinear kinetics can arise whenever an interaction between the chemical and a body constituent is saturated by the presence of excess chemical (**Table 6**).

Nonlinear kinetics can be detected by studying a range of single doses, but frequently nonlinearity is detected during chronic administration when the accumulation of the compound causes saturation. If saturation of elimination occurs during chronic administration, then the clearance will be reduced and the area under curve (AUC) for a dose interval will be increased. Evidence of saturation of elimination is that the AUC for a dose interval at steady state exceeds the AUC to infinity of a single dose.

Sometimes chronic administration results in the induction of the enzymes which are responsible for foreign-compound elimination. When this happens, the clearance will be increased during chronic administration, and therefore the AUC for a dose interval will be reduced, compared with the AUC to infinity of a single dose.

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Table 5 Calculation of elimination rate constant by the sigma-minus method

	% dose excreted (as parent compound plus metabolites)				
Time (d)	Urine	Faeces	Total	Cumulative total	Amount remaining
0	0.0	0.0	0.0	0.0	100.0
2	1.8	2.7	4.5	4.5	95.5
4	1.7	2.6	4.3	8.8	91.2
6	1.6	2.5	4.1	12.9	87.1
8	1.6	2.4	4.0	16.9	83.1
10	1.5	2.2	3.7	20.6	79.4
15	3.5	5.2	8.7	29.3	70.7
20	3.1	4.6	7.7	37.0	63.0
25	2.8	4.1	6.9	43.9	56.1
30	2.4	3.7	6.1	50.0	50.0
40	4.1	6.2	10.3	60.3	39.7
50	3.3	4.9	8.2	68.5	31.5
60	2.6	3.9	6.5	75.0	25.0
70	2.0	3.1	5.1	80.1	19.8
80	1.6	2.5	4.1	84.2	15.8
100	2.4	3.5	5.9	90.1	9.9
120	1.4	2.2	3.6	93.7	6.3
150	1.3	1.9	3.2	96.9	3.1

The data are for the excretion of a compound which is eliminated slowly by both urine and faeces with approximately 60% of the dose excreted in faeces and 40% in urine. The method assumes that the elimination of metabolites is formation rate limited and that there is no accumulation of metabolites.
Time—time at the end of the sequential collection intervals (e.g. 2 = 0–2; 30 = 25–30 and 150 = 120–150).

Table 6 Possible sources of dose-dependent or nonlinear kinetics

Site	Mechanism	Consequences at high dose
Absorption	Dissolution	Elimination of undissolved chemical in faeces; decrease in *F*.
	Active uptake	Saturation of transport; delayed uptake, decrease in *F*.
	First-pass metabolism	Increase in *F*.
	Efflux transporter	Increase in *F*.
Distribution	Plasma protein binding	Increased availability to tissues; increase in *V*; increase in $t_{1/2}$.
	Tissue protein binding	Increased retention in plasma; decrease in *V*; decrease in $t_{1/2}$.
Metabolism	Saturation by substrate or cofactor depletion or product inhibition	Increase in AUC; decrease in clearance; increase in terminal half-life possible (e.g. cofactor depletion); increased renal excretion of parent compound; increased metabolism by alternative unsaturated pathways.
Excretion	Saturation of renal tubular secretion	Increase in AUC; decrease in renal clearance; terminal half-life often increased; increased metabolism.
Cardiac output	Decreased organ perfusion due to cardiovascular toxicity	Slower distribution; increase in AUC and decrease in clearance for compounds showing high renal or hepatic (metabolic) clearance, that is, high extraction ratio.

It should be appreciated that age-related changes in physiological processes, such as hepatic and renal blood flow, will occur during lifetime feeding studies. Dietary intake in g per kg body weight changes during the lifetime of an animal, and this will also affect the plasma concentrations and body burdens of chemicals given in the diet. Therefore it is possible that steady-state concentrations during a chronic bioassay may be age dependent. The basic equation for average steady-state concentration (C_{ss}) still applies; that is

$$C_{ss} = \frac{\text{dose} \times F}{CL \times T} \tag{44}$$

Metabolic and physiological processes, such as renal blood flow, are immature in neonatal animals and humans (Blumer, 1990; Dorne *et al.*, 2004; 2005) and

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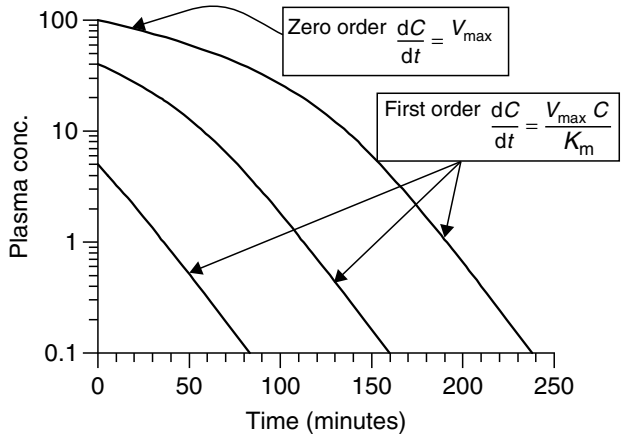

Figure 20 Plasma concentration–time curve for a compound showing saturation kinetics, given at doses sufficient to give initial plasma concentrations of 5, 40 and 100 units. The compound is eliminated by a single saturable pathway, which has K_m = 20 units and V_{max} = 1 unit per minute.

clearances may be lower than in mature individuals. The neonatal phase of a two-generation protocol may result in excessively high plasma concentrations of compounds fed in the diet due to the lower clearance, and also because of enhanced intake due to the higher food consumption in neonates (per g body weight). In ageing animals, C_{ss} may increase due to an age-related decrease in clearance, but in dietary studies to some extent this will be compensated for by a decrease in food consumption.

If saturation of elimination is detected on giving increasing single doses, this is usually seen as delayed elimination at high plasma concentrations, but with the normal rate of elimination once the concentration decreases and first-order kinetics apply (**Figure 20**). Under such circumstances the compound is said to obey Michaelis–Menten kinetics, that is:

$$\frac{-dC}{dt} = \frac{V_{max} \times C}{(K_m + C)}$$

$$\text{where} \quad \frac{dC}{dt} = \text{rate of change of concentration} \tag{45}$$

V_{max} = maximum rate of the enzyme (or transport), K_m = Michaelis constant of the enzyme (affinity constant), and C = concentration available to the enzyme.

At low concentrations $K_m \gg C, \therefore (K_m + C)$ approximates to K_m

$$\frac{-dC}{dt} = \frac{V_{max} \times C}{K_m} = \text{constant} \times C$$
$$= \text{first-order reaction} \tag{46}$$

At high concentrations $C \gg K_m, \therefore (K_m + C)$ approximates to C

$$\frac{-dC}{dt} = \frac{V_{max} \times C}{C} = V_{max} = \text{zero-order reaction} \tag{47}$$

(see **Figure 7**).

The true elimination half-life is measured when concentrations are very low, and therefore is not dose dependent (see **Figure 20**). However, the measured half-life may indicate the possibility of saturation of elimination if the concentration–time curve is not followed for long enough; for example, if measurements had been made for only 50 minutes after each dose in **Figure 20**.

The best indication of dose-dependent elimination is calculation of the clearance as the ratio of dose/AUC to infinity. This is illustrated in **Figure 20**. The initial plasma concentration (C_0) is dependent only on the dose and the apparent volume of distribution (V) (C_0 = dose/V). If linear kinetics had applied to all three doses then there would have been parallel decreases from time zero. It is clear that the slower initial elimination of the higher doses shifts these curves to the right and therefore results in higher AUCs, despite the similar terminal slopes.

A compound which shows nonlinear toxicokinetics similar to those depicted in **Figure 20** is the solvent 1,4-dioxane (Dietz *et al.*, 1982), for which an increase in intravenous dose in rats from 3 mg kg^{-1} to 1000 mg kg^{-1} (333-fold) caused a 4439-fold increase in AUC. The disproportionate increase in AUC of dioxane at high doses was associated with a decrease in the urinary excretion of the metabolite β-hydroxyethoxyacetic acid and an increase in the elimination of the solvent *per se* in the expired air.

Dose-dependent kinetics can arise from the saturation of a number of protein–chemical interactions (**Table 6**). Therefore nonlinear relationships can produce a range of different changes to the plasma concentration–time profile. A number of different approaches can be taken to demonstrate dose-dependent kinetics, for example

1. Divide the plasma concentration for each time point by the dose and plot the resulting dose-adjusted data. For purely linear and first-order processes the adjusted data will be superimposable. A consistent dose-dependent deviation indicates that nonlinearity is present but does not indicate the reason.
2. Fit a common model to the data for each dose and evaluate systematic changes in parameter estimates.
3. Measure metabolite formation either in plasma or urine. A dose-dependent change in the ratio of the AUC for the parent compound to the AUC of the metabolite (or % dose in urine) would indicate saturation of metabolism.
4. Measure tissue to plasma ratios for a range of doses to detect saturation of protein binding in either plasma or tissue.

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6 THE USE OF TOXICOKINETICS IN RISK ASSESSMENT

6.1 Trans-Species Comparisons

The use of animal data to predict potential risks to humans, or to establish safe exposure levels, is based on the assumption that both the toxicodynamics and the toxicokinetics in animals are relevant to humans. There are wide differences between different animal species and between animals and humans in physiological processes, such as organ perfusion rates and in biochemical processes such as the enzymes of foreign-compound metabolism. In consequence, all extrapolations from animals to man must take into account differences in toxicokinetics.

There are two principal methods by which interspecies extrapolations may be made—*allometry* and *PBPK models*. The basis of both methods is that there are underlying similarities between mammalian species in the various processes involved in toxicokinetics. For example, the intestine contains similar digestive enzymes and provides a large surface area for absorption via microvilli, the compositions of blood and body tissues are similar across species with respect to fat and protein content so that tissue distribution should be similar, and the perfusion of the organs of elimination and the basic metabolic and excretory processes within these organs are similar. Thus, reasonable estimates of human pharmacokinetic parameters should be derived from animal data after allowing for differences in body weight, cardiac output, and so on (Poggesi, 2004). It should be emphasized that there are bound to be examples where major species differences exist, for example, in specific pathways of metabolism, which mean that chemical-specific toxicokinetic data are essential (see risk assessment below).

6.1.1 Allometry

Allometry is a mathematical extrapolation based on the body weight of an animal (Calder, 1981). Thus the value of any parameter (P) is related to the average body weight (W) of the species by an equation with two unknown variables:

$$P = aW^x \tag{48}$$

If the parameter estimate P is known in two or more species, then its value in a third species can be deduced from a simple regression equation of log P against log W. Interspecies comparisons of caffeine pharmacokinetics (Bonati *et al*., 1985) showed that the apparent volume of distribution was linearly related to body weight (i.e. $V = 0.79\ W^{1.00}$), whereas the plasma clearance (CL) showed a loglinear relationship (i.e. $CL = 6.26W^{0.739}$); the power term for clearance was similar to that for liver weight (L) (i.e. $L = 0.037W^{0.845}$).

Boxenbaum (1984) provided useful background information on the origins of allometry in pharmacokinetic scaling and extended this method by the use of Dedrick plots in which plasma concentration–time curves in different species can be made superimposable if the concentration is corrected for dose (e.g. $\mu g\ ml^{-1}$ per mg kg^{-1}) and the time is corrected for body weight (e.g. min $kg^{-0.25}$). The idea behind this latter correction is conversion to 'physiological time' which in effect relates body weight to the lifespan of the animal.

Bachmann (1989) applied allometry to the pharmacokinetics for a number of drugs in laboratory animals and humans; for each drug there was a linear relationship between the log of the apparent volumes of distribution and the log of body weights, with correlation coefficients >0.95. The log of the clearance was not as closely related to the log of the body weight unless multiplied by the maximum lifespan potential (MLP) for each species.

6.1.2 Physiologically Based Pharmacokinetic (PBPK) or Physiologically Based Toxicokinetic (PBTK) Modelling

An alternative and more scientifically sound approach is the use of physiologically based pharmacokinetic (PBPK) or toxicokinetic (PBTK) modelling (Gerlowski and Jain, 1983; Chiu *et al*., 2007; Clewell and Clewell, 2008). The basis of the method is that each major organ system, plus any specialized sites such as targets for toxicity, is taken as representing a physiological compartment. Each separate compartment has its own blood flow, tissue volume, uptake process, affinity for the compound (i.e. partition coefficient) and elimination process as appropriate (**Figure 21**). The kinetics of the compound are described by a series of flow-related equations, which can be solved following the incorporation of known physiological values (blood flows and tissue volume/mass) and experimental estimates (partition coefficients and protein binding). The main application of the technique is that species differences can be predicted based on known differences in physiological parameters. In cases where an important physiological value is not known for a species, the missing information can be derived by allometry (see above) from species in which the values are known. Additional advantages of this technique are that it allows an assessment of the impact of altered physiology, such as ageing (Ginsberg *et al*., 2004) and renal function, on the toxicokinetics and target organ exposure, and that data from human studies, such as enzyme activities, including information on human variability (Price *et al*., 2003) can be incorporated directly into the model.

A distinct advantage of PBTK modelling is its ability to incorporate both *in vitro* enzyme activity data and *in*

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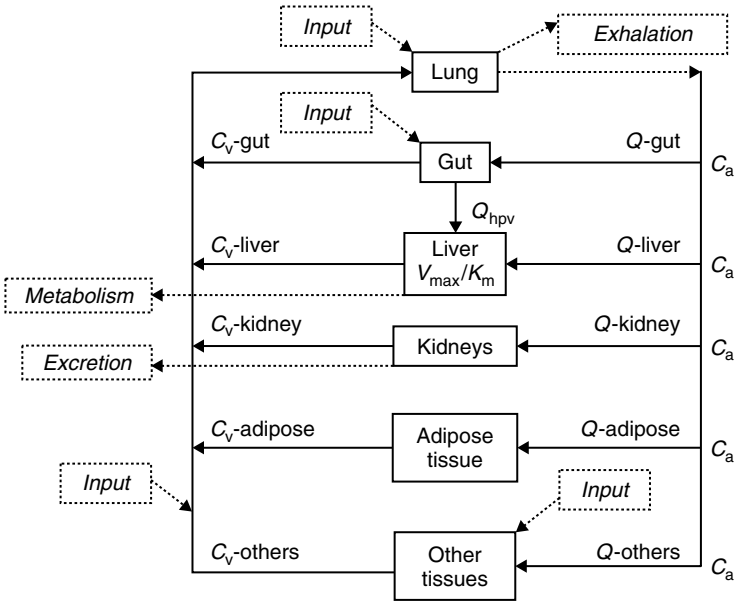

Figure 21 A physiologically based pharmacokinetic model. The rate of delivery to an organ such as muscle is given by the blood flow (Q) times the arterial concentration (C_a). The rate of removal from the organ is blood flow (Q) times the venous concentration (C_v). Input functions and output functions can be first-order or Michaelis–Menten reactions. More complex models have been used, incorporating specific targets, such as bone marrow, or additional routes such as transdermal, in which case the skin becomes a discrete compartment. hpv—hepatic portal vein.

vivo organ blood flows to produce a composite model, which can avoid misleading conclusions. For example, the rate of bioactivation of furan was similar and very high in both mice and humans; but these high rates of metabolism would not be apparent *in vivo*, because they greatly exceeded the rate of liver perfusion (Kedderis and Held, 1996). In consequence the species difference in bioactivation in the PBPK model was determined largely by liver blood flow and not the difference in enzyme activity.

Both physiologically based models and allometry can be of value to regulatory safety assessments (Scheuplein *et al*., 1990; Thompson *et al*., 2008). For example, PBTK models have been applied to the regulatory risk assessment of trichloroethene (Bogen, 1988), tetrachloroethene (Clewell *et al*., 2005) and 1,1,1-trichloroethane (Bogen and Hall, 1989), while both allometry (Beliles and Totman, 1989) and physiological models (Travis *et al*., 1990) have been used to extrapolate data on benzene from rodents to humans. Physiologically based models are far more flexible than allometry, and more complex situations, such as exposure during lactation (Fisher *et al*., 1990), can be taken into account. A more comprehensive risk assessment has been made for acrylamide that incorporated exposure and physiologically based toxicokinetic–toxicodynamic modelling (Doerge *et al*., 2008).

6.2 Approaches to Risk Assessment

Assessments of the risks for humans of exposure to chemicals are usually based on hazards identified in animal experiments, and the associated dose–response curves for the effect. There are various approaches adopted by regulatory bodies for establishing 'acceptable', 'tolerable' or 'virtually safe' exposures for humans.

6.2.1 Low-Dose Risk Extrapolation

This approach is usually restricted to effects for which there may not be a biological threshold in the dose–response relationship, such as genotoxic carcinogenicity. For other effects it is generally considered that there is no significant adverse biological response until homeostatic mechanisms have been exceeded, so that low exposure does not provoke any effect.

The method is based on extrapolation from the incidence in the experimental dose–response range in animals down to a predefined negligible risk (10^{-5} or 10^{-6}), which is also known as the virtually safe dose. This process essentially defines a negligible risk in animals, unless interspecies differences are taken into account. The approach can be improved by the incorporation of toxicokinetic data, usually in the form of PBPK modelling (Krishnan and Johanson, 2005), to correct the external dose in the experimental animals to a target-organ dose, and then to estimate the target-organ dose in humans in relation to exposure. However, such

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toxicokinetic refinements have not altered the vast uncertainties inherent in extrapolating over at least four orders of magnitude away from the dose–response data. The outcomes of such extrapolations are determined largely by the model selected rather than the biological data or its toxicokinetic refinement.

6.2.2 Margin of Exposure (MOE)

This approach compares the doses in animals associated with toxicity, and those in humans, and is usually applied to occupational and environmental exposures. It is the ratio of a reference point or point of departure on the animal dose–response relationship, such as the benchmark dose lower limit (BMDL10) (European Food Safety Authority, 2005) with the estimated human exposure. The adequacy of the margin of exposure (MOE) is then assessed based on the nature of the exposed human groups, the duration of exposure and the nature of the hazard.

The MOE does not provide an estimate of the risk; the only conclusion possible is that the greater the MOE, the lower the risk (Barlow *et al*., 2006); but it is useful for comparisons of the risks associated with human exposures to different chemicals—providing that the reference point and exposure estimates are based on the same methods. Again PBTK modelling can be used to refine the interspecies comparison by the calculation of internal or target-organ doses in animals and exposed humans, but this would prevent comparisons of the derived MOE with the MOEs for compounds for which a PBTK model had not been used.

The MOE can also be compared with the safety factor of 100, which is used traditionally for noncancer end points (see below), although any difference between the reference point and the traditional no-observed-adverse-effect level (NOAEL) (see below) would have to be taken into account.

6.2.3 Safety Assurance Procedures

This approach is generally adopted for nongenotoxic compounds which have undergone toxicity testing before approval and prior to human exposures (so that an MOE cannot be calculated). For some chemicals, such as medicines, the approval can be based on a risk–benefit analysis because of the clear benefit to the 'consumer'. However, for other chemicals such as food additives and pesticides, there are no direct health benefits to the consumer to offset against any possible health risk. Therefore, the establishment of a 'safe dose' in animals, and interspecies extrapolation to give a 'safe dose' in humans is of critical importance. (It should be recognized that there is no such thing as absolute safety, and it is impossible to prove the absence of any phenomenon—including toxicity. Hence there are established guidelines for the conduct of toxicity studies which are designed to ensure that the absence of any adverse affects does not arise from inadequacies in the study.)

The 'safe dose' in animals is the NOAEL (expressed as mg kg^{-1} body weight per day), and this is converted to the 'safe dose' for humans by dividing by a large safety (uncertainty) factor. The 'safe dose' for humans is usually described as the acceptable daily intake (ADI), reference dose (RfD), tolerable daily intake (TDI) or provisional tolerable weekly intake (PTWI). This is the approach adopted by bodies such as the WHO/FAO (World Health Organization and Food and Agriculture Organization) Joint Expert Committee on Food Additives (JECFA; see WHO, 1987), the EFSA (European Food Safety Authority) and the US EPA (US Environmental Protection Agency).

Normally a 100-fold factor is used when the NOAEL is based on animal data, and a 10-fold factor is used when the NOAEL is derived from a study in humans (WHO, 1987). In reality the so-called safety factor is an uncertainty factor which has to allow for possible differences between test species and humans and for possible human variability (**Figure 22**). Each 10-fold factor (interspecies and interindividual) has to allow for toxicokinetic and toxicodynamic differences (Renwick, 1991). In recent refinements to this approach, the 100-fold factor is considered as a four-way box (**Figure 23**), with each 10-fold factor subdivided to allow for toxicokinetic and toxicodynamic aspects

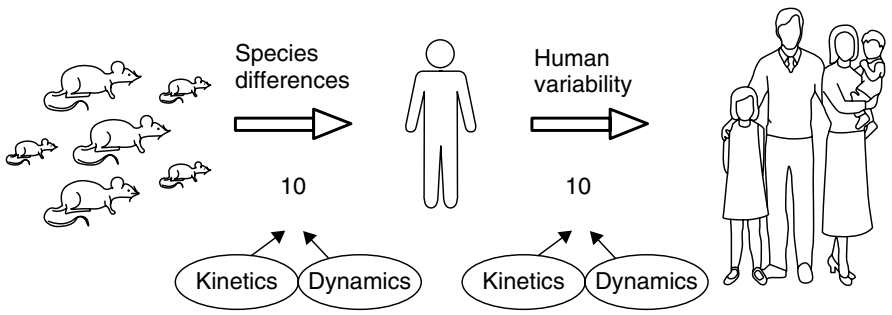

Figure 22 The use of a 100-fold safety or uncertainty factor to convert the no-observed-adverse-effect level (NOAEL) in animals into an acceptable daily intake (ADI) for humans (from Renwick, 1998).

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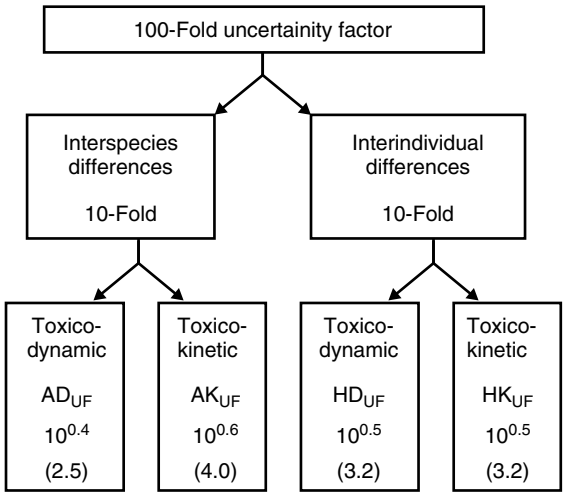

Figure 23 The subdivision of the 100-fold uncertainty factor for the introduction of chemical-specific adjustment factors (based on WHO, 1994; 2005).

(Renwick, 1993) which when multiplied together give the original factors of 10×10. The rationale for this proposal was to allow relevant data on a chemical to replace part of the 'uncertainty', and thereby provide a more scientific basis for the determination of the 'safe' human dose. The values proposed were evaluated at a WHO task group, which considered the approach valuable but revised slightly the proposed default values (WHO, 1994), which reflected the similar variation in toxicokinetics and toxicodynamics in humans (based on pharmacological data (Renwick and Lazarus, 1998)).

The issues critical to the replacement of a default value in **Figure 23** by a chemical-specific adjustment factor (CSAF) are presented in WHO (2005). These include:

1. **Separation of toxicokinetic and toxicodynamic aspects:** Human *in vivo* studies are usually limited to the determination of circulating concentrations of the chemical or its metabolites, and therefore toxicokinetics stops at the delivery of chemical to the target sites; any process occurring within the target organ should be regarded as part of toxicodynamics (including local metabolic bioactivation).
2. **Identification of toxic moiety:** It is important that the activity of the parent compound and metabolites is known. Toxicokinetic data used to calculate a CSAF should relate to the delivery of the toxic moiety (which may be a metabolite) to the target organ in relation to the external dose of the parent compound.
3. **Selection of the appropriate toxicokinetic parameters:** In most cases this would be the AUC of the toxic moiety, but peak concentration may be more appropriate, especially for acute effects.
4. **Selection of appropriate doses:** Although toxicokinetic processes are essentially first-order at low doses, it is possible that saturation may occur at the high doses used in animal studies. The correct comparison is between the toxicokinetics of the chemical in the test species at the NOAEL and in humans at the ADI/TDI that would be calculated in the absence of the toxicokinetic data.
5. **Dose metric:** The 100-fold factor and therefore the defaults shown in **Figure 23** are applied to the external dose expressed in mg kg^{-1} body weight. If the dose is calculated on the basis of the body surface area, then this will be a form of allometry (see above). In humans, body weight and surface area are related by the formula

$$SA = W^{0.425} \times H^{0.725} \times 71.84 \qquad (49)$$

where

SA = surface area
W = weight, and
H = height.

Interspecies comparisons often use simple equations in which the surface area is related to body weight$^{0.66}$ or body weight$^{0.75}$. The use of surface area rather than body weight therefore appears to allow for species differences. The difference between body weight and surface area is equivalent to a factor of about eight for mice, four for rats and two for dogs; that is, using toxicity data from rats, the 'safe dose' for humans based on surface area would be four-fold lower than that expressed on the basis of body weight.

The adequacy of the default 3.2-fold factor for human variations in toxicokinetics has been analysed for most of the major pathways of foreign-compound elimination (Dorne *et al.*, 2005). In general, the default value in **Figure 23** covers over 99% of the human population, but would not allow for situations where the compound was eliminated by a single metabolic pathway that showed a genetic polymorphism. In addition, a significant proportion of certain age groups such as neonates and the elderly would show greater variation than allowed for by the human variability factor; but such age-related differences would not need to be covered by the human variability factor if they had been studied as part of the animal database.

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6.3 Examples of the Importance of Toxicokinetic Data in Risk Assessment

The various issues discussed above are illustrated by data on cyclamate and its metabolite cyclohexylamine (cyclohexanamine), chlorinated dibenzo-*p*-dioxins, and the intense sweeteners stevioside and rebaudioside A.

6.3.1 Cyclamate and Cyclohexylamine

Cyclohexylamine is a metabolite of the intense sweetener cyclamate (Renwick, 1986), which produces testicular atrophy when administered to rats but not mice at doses of 200 mg kg^{-1} per day or more in a 90 day study (see Bopp *et al*., 1986). The terminal half-life in rats is 4.6 hours following a single oral dose of 200 mg kg^{-1} (Roberts and Renwick, 1989), and therefore negligible accumulation would be expected during chronic administration via the diet, although large diurnal variations in plasma concentration would be expected. The concentration–time profile over 24 hours during chronic dietary administration was found to contain only a single broad peak each 24 hours, which arose from the nocturnal feeding habits of the rat. Very wide diurnal fluctuations would have occurred if the compound had been administered by a single daily gavage dose, illustrating that the mode of administration can have important influence on the concentration–time curve. The concentrations of cyclohexylamine in the testes increased rapidly following a single oral dose, and the testes represent part of the rapidly equilibrating central compartment. Therefore the diurnal fluctuations detected in plasma would occur also in the target organ for toxicity.

The area under the concentration–time curve is the best estimate of exposure of the whole body, (if based on plasma concentrations) or the target organ (if based on tissue concentrations). For cyclohexylamine, the AUC values for both plasma and testes were about two to three times higher in rats than in mice given the same dose in mg kg^{-1} body weight. This species difference in toxicokinetics will contribute to the marked difference in sensitivity between rats and mice fed the same dietary concentration of cyclohexylamine (Roberts *et al*., 1989). The species difference was supported by data on the relationship between the concentration in testes at steady state and the daily intake in the test animals (**Figure 24**). For example, the concentration in the rat testes at the minimally effective intake of 200 mg kg^{-1} day^{-1} was approximately 30 µg g^{-1}, while at the clearly toxic daily intake of 400 mg kg^{-1} per day (Roberts *et al*., 1989), the concentration in the testes was approximately 100 µg g^{-1}. In contrast, an intake of 400 mg kg^{-1} day^{-1}, which is nontoxic in mice (Roberts *et al*., 1989), produced concentrations of only about 25 µg g^{-1} in the mouse testes. The concentration–intake data in the rat indicated

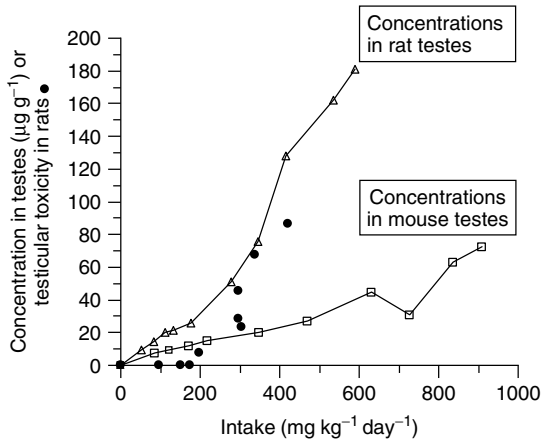

Figure 24 Concentrations of cyclohexylamine in the testes of rats and mice measured at 6 a.m. (from Roberts and Renwick, 1989) in relation to the daily intake, and the corresponding dose–response for testicular atrophy in the rat. (Data from Roberts *et al*., 1989 and Bopp *et al*., 1986.)

nonlinearity at intakes greater than about 200 mg kg^{-1} day^{-1}. This nonlinearity was also detected in plasma and probably contributed to the steep dose–response relationship for testicular atrophy in the rat (**Figure 24**).

Data from studies in humans have shown very wide person-to-person variability in the extent of metabolism of cyclamate to cyclohexylamine during regular cyclamate intake. The majority of the population (about 80%) does not form any detectable cyclohexylamine, while 2–3% of the general population can metabolize 20% or more of a daily dose (Buss *et al*., 1992). The metabolism is performed by the intestinal microflora in the lower bowel, and the metabolic process requires a period of repeated exposure to induce the metabolic activity. Therefore the rate of appearance of cyclohexylamine is slow and there are almost no fluctuations over an eight hour dose interval at steady state (Buss *et al*., 1992). In addition to the extremely wide interindividual variation, there is day-to-day intra-individual variation in subjects who have developed a high metabolic capacity (**Figure 25**) (Renwick *et al*., 2004).

The ADI for cyclamate is based on the NOAEL of 100 mg kg^{-1} body weight for testicular toxicity in rats given cyclohexylamine in a 90-day study. Clearly the default uncertainty factor for human variability (3.2 in **Figure 23**) would be totally inadequate for this example. The risk assessment by the EU SCF (Scientific Committee on Food; SCF, 2000a) used a CSAF for human variability based on the highest percentage metabolism found in the study of Renwick *et al*. (2004). The Committee took the worst-case value of 85% conversion of cyclamate to cyclohexylamine and applied a CSAF of 1.0 for human variability in toxicokinetics and a total composite uncertainty factor of 32.

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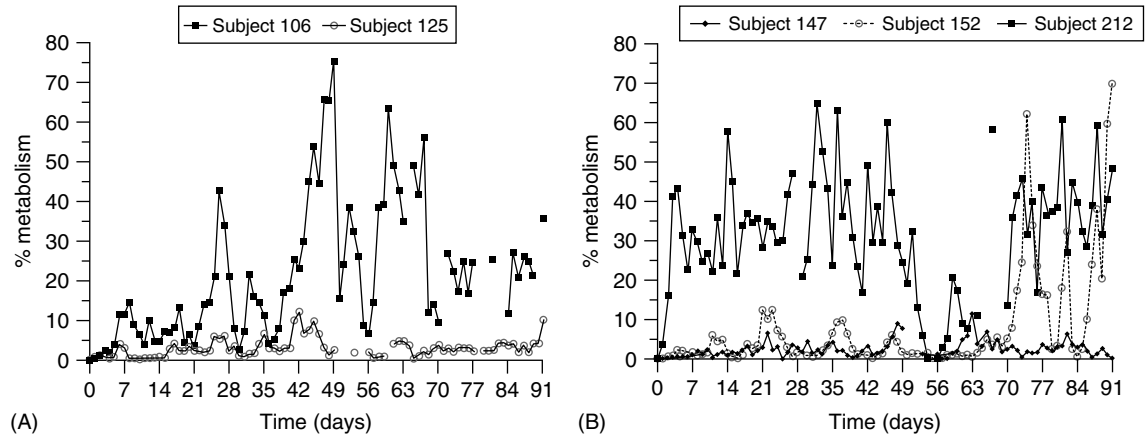

Figure 25 The daily excretion of cyclohexylamine in individuals selected from a screening study for their high metabolizing ability during the administration of 3 g cyclamate daily. (Subject 212 had a short period without cyclamate intake on days 53–55.) The data illustrate the delay prior to extensive conversion of cyclamate to cyclohexylamine and the wide day-to-day fluctuations despite repeated daily intake. (Data from Renwick *et al*., 2004.)

However, this analysis did not take into account two important variables:

1. The highest extent of metabolism on a single day in humans was applied to a NOAEL derived from a 90-day study in rats; the worst-case average extent of cyclohexylamine excretion was about two-fold lower at 40%.
2. Human variability in renal excretion, which would be the major determinant of internal dose, was not allowed for, but variation in renal tubular secretion in humans is low (Dorne *et al*., 2004) compared with other pathways (Dorne *et al*., 2005).

In reality these two deficiencies would cancel each other out.

6.3.2 Chlorinated Dibenzo-p-dioxins

Chlorinated dibenzo-*p*-dioxins, such as TCDD, are among the most toxic chemicals known, and produce acnegenic, carcinogenic, foetotoxic, immunosuppressive and teratogenic effects (JECFA, 2002). There are wide interspecies differences in acute toxicity of TCDD, with the guinea pig about 10–20 times more sensitive than the rat (Kociba *et al*., 1976) or mouse (McConnell *et al*., 1978; Beatty *et al*., 1978), while the hamster appears to be the least sensitive species (Olson *et al*., 1980a). The elimination half-life in guinea pigs (30 ± 6 days; Gasiewicz and Neal, 1979) is similar to that in rats (Rose *et al*., 1976), and only about twice that in hamsters (15 ± 3 days; Olson *et al*., 1980b). Thus the species differences in acute toxicity are not related to the ability to eliminate the compound. Major species differences in elimination half-life would not be expected because TCDD is eliminated essentially unchanged, mostly in the faeces.

There are problems with extrapolation of the data for TCDD in rodents to possible human intakes. The half-life of TCDD in humans based on the elimination by occupationally exposed individuals is about seven years, which is almost 100 times that in rats. Using a value of 7 years it would take about 30–35 years of exposure for humans to reach steady state during constant intake. The four-fold default uncertainty factor for interspecies differences in toxicokinetics (**Figure 23**) would be totally inadequate, and a different approach based on body burdens was adopted in recent evaluations by the EU SCF (SCF, 2000b; SCF, 2001), the JECFA (JECFA, 2002) and the US EPA (see NRC, 2006). The adverse effects that were the focus of the EU SCF and JECFA (2002) evaluations were developmental effects in male rats seen following *in utero* exposure to a single dose given on day 15 of gestation, or a loading dose and weekly injections (Mably *et al*., 1992; Gray *et al*., 1997a; 1997b; Faqi *et al*., 1998; Ohsako *et al*., 2001). The dose–response data in rats could be related to target organ concentrations by special toxicokinetic studies (Hurst *et al*., 2000a; 2000b), so that the safety evaluation was based on internal dose. The evaluations by JECFA (2002) and SCF (2001) allowed for the distribution phase of the bolus doses (Ohsako *et al*., 2001) and weekly subcutaneous injections (Faqi *et al*., 1998) given to rats in order to calculate the foetal tissue exposures on day 16 of gestation and the corresponding maternal body burden. The maternal body burdens in rats were then converted to human equivalent body burdens based on the species difference in half-lives.

$$\text{Body burden at steady state} = \frac{\text{daily dose} \times \text{bioavailability } (F) \times t_{1/2}}{0.693} \quad (50)$$

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The daily intake that would result in the human equivalent body burden was then calculated and used to derive a tolerable weekly intake (SCF, 2001) or a provisional tolerable monthly intake (JECFA, 2002). The usual 100-fold uncertainty factor was reduced to 3.2, because human variability in toxicokinetics was the only area of uncertainty (see **Figure 23**) not replaced by a CSAF. Both interspecies and human variability factors for toxicodynamics were reduced to 1 because at a tissue level the most sensitive human individual would be no more sensitive than the rat strains used in the studies; the interspecies kinetic factor was also 1 because the use of body burden had taken species differences into account.

However, the use of body burden would not allow for species differences in the extent of distribution (NRC, 2006). Ideally the interspecies extrapolation should have been based on the concentration in the plasma (or better still the tissues), but this would have required data on the plasma clearance of TCDD in humans because

$$C_{ss}(\text{steady-state plasma concentration}) = (\text{daily dose} \times F)/CL \quad (51)$$

The use of half-life instead of clearance required an implicit assumption that the difference in half-life between animal species and humans was solely due to differences in clearance; that is, there are no significant differences in the apparent volume of distribution.

$$t_{1/2} = \frac{0.693 V_\beta}{CL} \quad \text{or} \quad CL = \frac{0.693 V_\beta}{t_{1/2}} \quad \text{so that}$$

$$C_{SS} = \frac{\text{daily dose} \times F \times t_{1/2}}{0.693 V_\beta} \quad (52)$$

Because the body load or burden equals the plasma concentration times the apparent volume of distribution ($C \times V_\beta$), this equation can be rewritten to give the relationship between body burden and half-life:

$$C_{SS} \times V_\beta = \frac{\text{daily dose} \times F \times t_{1/2}}{0.693} \quad \text{or}$$

$$\textit{Body burden at steady state} = \frac{\text{daily dose} \times F \times t_{1/2}}{0.693} \quad (53)$$

Similar tissue concentrations in both species would occur if the apparent volume of distribution and the pattern of distribution of the body burden were the same in rats and humans. However, the extent of hepatic sequestration is higher in rats than humans; more importantly the proportion of the body mass present as fat (10% of body weight in rats and about 25% in humans—NRC, 2006) would result in two to three-fold lower concentrations in humans than in rats *for the same total body burden*. Therefore, the recent evaluations by the SCF (2001) and JECFA (2002) have overestimated the tissue exposure in humans at the body burdens that would arise from the established tolerable weekly or monthly intakes.

The SCF (2001) discussed the use of an uncertainty factor of 3.2 for human kinetic variability, but failed to recognize that major influences on half-life were body weight and body fat; as discussed above for the species differences, *a longer half-life* would arise from a higher proportion of body fat and therefore greater sequestration of the body burden in adipose tissue and therefore *lower than predicted concentrations in other nonadipose tissues*.

The interpretation of the human epidemiological data on occupational exposures to TCDD is complex, in relation to comparisons between occupational and environmental exposures. The body burdens arising from occupational exposures have usually been derived by back-extrapolation through time, based on a concentration measured some years after a period of high exposure (NRC, 2006). Occupational exposure usually occurred at some time during the working life, which established a body burden; this body burden would then increase, be maintained, or decrease depending on whether it was below, equal to or greater than the steady-state body burden associated with the subsequent environmental exposure.

This detailed analysis of the case of TCDD illustrates the critical role that toxicokinetics can play in the design and interpretation of toxicity studies, especially when the compound has a very long half-life. In addition it should be appreciated that if as a result of the animal toxicity data it were decided that the exposure of humans should be reduced immediately, it would take about seven years before the existing body burdens decreased by 50%, and about 30–40 years before they become negligible, even if input were to cease immediately and completely. In reality the persistence of TCDD in the environment (DiDomenico and Zapponi, 1986), indicates that this would be rate limiting in any attempted reduction of human body burdens.

6.3.3 Stevioside and Rebaudioside A

The leaves of the plant *Stevia rebaudiana* (Bertoni) have traditionally been used to sweeten food and beverages in South America (Soejarto *et al*., 1982; Gardana *et al*., 2003). The sweet constituents include stevioside and rebaudioside A, which are glycosides of the diterpene steviol (Carakostas *et al*., 2008). The glycosides are hydrolysed to the steviol *in vivo* in rats, pigs and humans (Nakayama *et al*., 1986; Cardoso *et al*., 1996; Geuns *et al*., 2003; 2007), and by rat caecal bacteria and human intestinal microflora *in vitro* (Wingard *et al*., 1980; Gardana *et al*., 2003; Koyama *et al*., 2003). Hydrolysis by the intestinal flora is the essential first step in the metabolism of both stevioside and rebaudioside A (Renwick and Tarka, 2008) (**Figure 26**).

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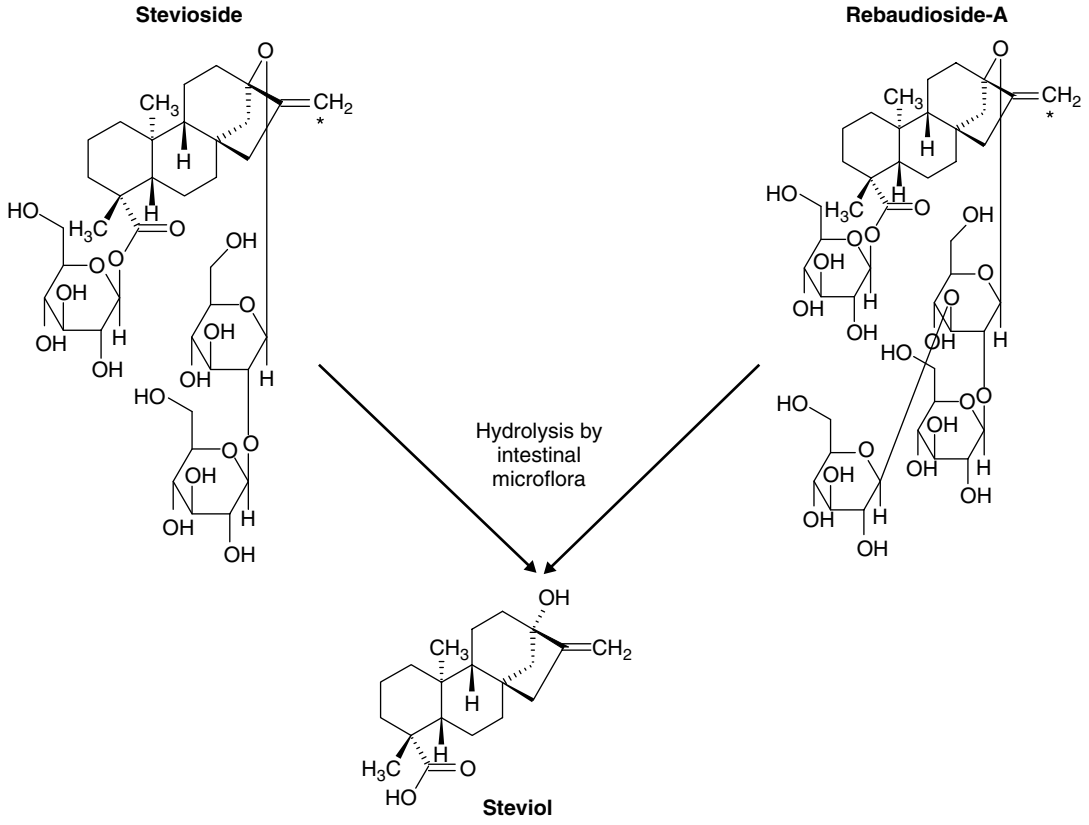

Figure 26 The metabolism of steviol glycosides.

The majority of an oral dose of the radiolabelled steviol given to rats was eliminated in the faeces and bile, mostly as steviol glucuronide, with only a low percentage of the dose eliminated in the urine (Roberts and Renwick, 2008). The authors concluded that the main route of elimination of steviol in rats is as steviol glucuronide in bile, which is hydrolysed by the gut flora to steviol and excreted in the faeces. The glycosides are extensively hydrolysed by the gut flora to steviol, although incomplete hydrolysis results in a small amount of the radiolabel in faeces as the parent glycoside (Roberts and Renwick, 2008). The major constituent in plasma after equimolar doses of steviol, stevioside or rebaudioside A was the aglycone steviol, with lower levels of steviol glucuronide and very low levels of other metabolites.

The kinetics of an oral dose of unlabelled stevioside or rebaudioside A in humans (Wheeler *et al*., 2008) were similar to those in rats except for two important differences:

1. The major route of elimination was as steviol glucuronide in the urine rather than the bile; this difference arises from the different molecular weight thresholds for significant biliary excretion of anions in rats (325) and humans (above 500), since the molecular weight of steviol glucuronide is just under 500.
2. Steviol was detected in a single plasma sample of only one subject after either stevioside or rebaudioside A. In all subjects the major circulating compound was steviol glucuronide.

Steviol glycosides are of low toxicity (Curry and Roberts, 2008). The 63rd meeting of the JECFA (JECFA, 2005) established a temporary ADI of 0–2 mg kg^{-1} body weight for steviol glycosides (expressed as steviol) based on the NOAEL of 970 mg stevioside per kg body weight per day (or 383 mg kg^{-1} body weight, expressed as steviol) in a two-year study in rats, and a safety factor of 200. The larger than normal safety factor was because of unresolved questions at that time. These questions were addressed in the recent risk assessment (JECFA, 2008) and a safety factor of 100 was applied to the NOAEL to give an ADI of 0–4 mg per kg body weight for steviol glycosides (expressed as steviol). However, the use of the standard 100-fold uncertainty factor does not take into account the species differences outlined above.

Any effects of steviol glycosides would arise from their metabolism to steviol. Almost complete intestinal hydrolysis occurs in both rats and humans. Steviol *per se* is the major circulating compound in rats given oral doses of the glycosides; the peak plasma concentrations as steviol were about 150 ng ml^{-1} after oral doses of 4.2 mg kg^{-1} of stevioside or 5 mg kg^{-1} of

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rebaudioside A. The same doses of the glycosides were essentially completely hydrolysed in humans, but the absorbed steviol appeared to undergo complete first-pass conjugation with glucuronic acid; the plasma levels of steviol were below the limit of detection (100 ng ml^{-1}) in all but two samples. Although it would be difficult to derive a quantitative CSAF for interspecies differences in toxicokinetics (**Figure 23**), because the AUC for plasma steviol could not be measured in humans, it is clear from the available data that the default factor of 3.2 is excessive and a value of 1 would seem more than adequate.

7 ADDITIONAL SOURCES OF INFORMATION

It has not been possible to cover the vast subject of toxicokinetics adequately within the confines of a single chapter. There are a number of text books and review articles to which readers are referred for further information, for the interpretation of more complex situations and for sampling techniques.

7.1 General Texts

Benet (1976)—rather specialized but good background reading with respect to possible problems in geriatric animals.

Gibaldi and Perrier (1982)—a definitive text.

Benet *et al*. (1984)—based on a symposium, and therefore lacks the structure of Gibaldi and Perrier or Rowland and Tucker but contains some useful information.

Rowland and Tucker (1986)—a more advanced text with useful chapters on interspecies scaling, dose-dependent and time-dependent kinetics and response modelling.

Gabrielsson and Weiner (1997)—an excellent text book—clearly explained with large numbers of helpful diagrams.

Lipscomb and Ohanian (2007)—a useful book with many valuable chapters.

Renwick (2008)—similar to the present chapter but with an expanded section on dose-dependent kinetics, metabolite kinetics and worked examples.

7.2 Review Articles

Gehring and Young (1978)—contains interesting examples from the work of the pioneers in the application of pharmacokinetics to problems of animal toxicology.

Wilkinson (1984)—a clearly written article which deals with first pass metabolism, dose-dependent kinetics and introduces physiological approaches.

O'Flaherty (1985)—a consideration of dose-dependent metabolism.

Wilkinson (1987)—a comprehensive review of the application of clearance concepts to the elimination of foreign components; not toxicology orientated, but an excellent source of references ($n = 525$).

Scheuplein *et al*. (1990)—an interesting article on the value of toxicokinetic data to regulatory agencies such as the FDA.

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Dixit *et al*. (2003)—a useful overview of recent developments.

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Physiologically Based Pharmacokinetic Modelling

Hon-Wing Leung

CONTENTS

1 INTRODUCTION

Pharmacokinetic models are used to make a rational prediction of the disposition of a chemical throughout the body. Pharmacokinetic modelling has evolved over the past several decades. An early approach assumes that data on the internal environment of a chemical, for example, tissue concentrations, cannot be obtained without employing invasive techniques. These pharmacokinetic models are developed to predict concentrations of chemicals in readily accessible media, such as blood and excreta. The plasma or blood concentration is assumed to mimic the profile in the entire body. Evidently this kind of approach cannot provide information on the concentration-time course of a chemical at the target tissue, which is not necessarily reflected by the blood concentration.

In recent years biologically based models which apply first principles, such as material balance, and incorporate physiological parameters are being developed, initially to describe the kinetics of therapeutic drugs (Himmelstein and Lutz, 1979), then environmental chemicals (Leung, 1991). These models include the exact physiology and anatomy of the animal species being described, as well as parameters such as blood flow, ventilation rates, metabolic constants, rates of absorption and excretion, tissue solubilities and binding to macromolecules (D'Souza and Boxenbaum, 1988). These models are commonly known as physiologically based pharmacokinetic (PBPK) models. **Table 1** presents a listing of PBPK models that have been developed for environmental chemicals.

1.1 Classical Versus Physiological Pharmacokinetics

In simple pharmacokinetic modelling the whole body is treated as a single compartment. More sophisticated models are created by the addition of peripheral compartments (see **Toxicokinetics**). In a multicompartment model the concentration-time course in the central compartment is typically curvilinear with a terminal linear portion. By the method of residuals or feathering, this kinetic behaviour is mathematically resolved into

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decaying exponential terms to account for the curvature of the data. The number of exponential terms corresponds to the number of compartments in the model, each representing an exchange between a peripheral tissue or organ with the central compartment. Obviously compartmentalization by such a rigid curve-stripping process is a rather abstract mathematical construct and lacks physiological relevance.

In recent years physiological modelling has emerged as a pre-eminent approach to pharmacokinetic modelling. However, it must be emphasized that classical and physiological pharmacokinetics are not fundamentally incompatible; in fact, they share a common connection. The difference between them lies in the kinds of parameters on which the models are developed, and consequently they differ in their applications. In classical pharmacokinetic modelling, no attempt is made to assign physiological correlates to model parameters. A compartment is simply considered as a kinetically homogeneous volume with transfer constants in and out of the compartment. In PBPK modelling, compartments are treated as individual organs or tissues arranged in a precise anatomical configuration connected by the cardiovascular system. The transfer of chemicals between compartments is governed by actual blood flow rates and tissue solubilities (partition coefficients).

Because of a lack of biological constraints with conventional pharmacokinetic modelling, empirical data can be fitted by freely varying the model parameters. The best estimates of parameter values can then be statistically compared across experimental conditions, treatments or chemicals to establish whether apparent differences are significant. In contrast, any major discrepancies between the physiological model prediction and experimental data in PBPK modelling will necessitate the reformulation of the model to account for the observed behaviour. Since classical models are constructed without conformity to anatomical reality, they cannot account for physiological or biochemical alterations such as body and organ weight changes (tissue growth or atrophy) or enzyme induction and inhibition. While both classical and physiological models are capable of predicting tissue doses, albeit to somewhat different extents, classical models do not lend themselves to interspecies extrapolation of such dose-effect data (Monro, 1994).

2 THEORY AND PRINCIPLES OF PHYSIOLOGICAL MODELLING

The transfer of a chemical out of a single compartment follows Fick's law of simple diffusion, which states that the flux of a chemical is proportional to its concentration gradient. The differential rate equation describing this first-order process can be written as follows:

$$\frac{\mathrm{d}C}{\mathrm{d}t} = K \cdot \frac{\delta C}{V} \tag{1}$$

where C is the concentration of chemical in the compartment; K is the transfer constant; V is the volume of the compartment and δC is the concentration gradient.

If the transfer is perfusion- or flow-limited, then the transfer constant is the rate of blood flow (Q) to the compartment. It follows, therefore:

$$\frac{\mathrm{d}C}{\mathrm{d}t} = \frac{Q(C_\mathrm{a} - C_\mathrm{v})}{V} \tag{2}$$

where C_a is the concentration of the chemical in the arterial blood entering the compartment and C_v is the concentration of the chemical in the venous blood leaving the compartment.

Since chemicals do not equilibrate freely in body fluids, but depending on their physicochemical properties, may be sequestered in tissue lipids, the concentration determined experimentally from a tissue sample is a composite of both the free and the sequestered form. As the transfer of a chemical in a tissue compartment is assumed to be flow-limited, the chemical concentration in the venous blood exiting from a tissue is equal to the concentration in the tissue fluid, that is, the so-called free form. The partitioning of the chemical between the body fluid and tissue lipids is governed by tissue solubility or partition coefficient P, as follows:

$$P = \frac{C}{C_\mathrm{v}} \tag{3}$$

Substituting this into Equation 2, one obtains:

$$\frac{\mathrm{d}C}{\mathrm{d}t} = \frac{Q\left(C_\mathrm{a} - \dfrac{C}{P}\right)}{V} \tag{4}$$

Equation 4 represents the fundamental relationship on which all PBPK models are constructed. The expression for all nonmetabolizing, noneliminating and nonbinding tissue compartments will have this same mathematical form. The expressions for blood and other eliminating tissues such as liver, kidney and lung are more complex, but are based on the same principles of flow, mass conservation and partitioning.

3 DEVELOPMENT OF PHYSIOLOGICAL PHARMACOKINETIC MODELS

The development of a PBPK model is a highly integrative process. **Figure 1** depicts the flow processes in the development of a PBPK model. The first step involves

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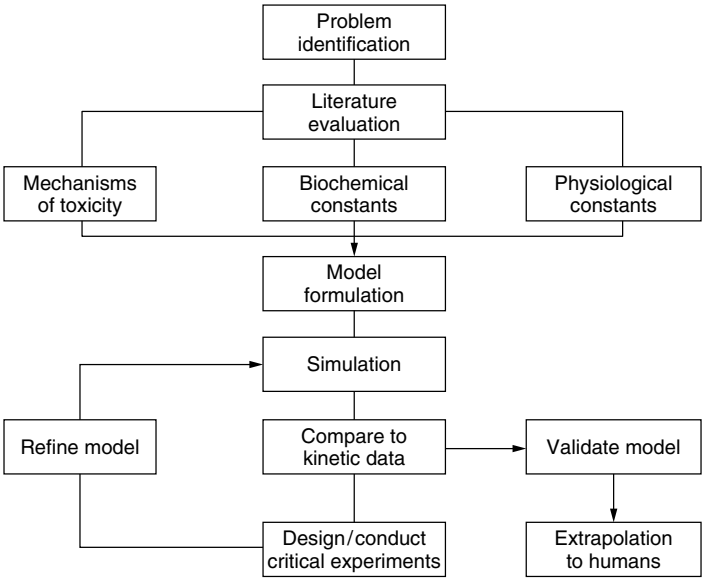

Figure 1 Flow diagram of the development of a physiologically based pharmacokinetic model. Problem identification: the finding of a particular toxicity, in a particular organ, in a particular species. Literature evaluation: the integration of available information about the mechanism of toxicity, the pathways of chemical metabolism, the nature of the toxic chemical species, the tissue-binding characteristics and the physiological parameters of the target species. From these data a model is developed to estimate the appropriate measure of tissue exposure for a wide variety of exposure conditions.

defining the nature of the problem and reviewing the literature to assess the impact of mechanism on the choice of tissue dosimetrics. The actual model formulation is divided into four interwoven steps, as follows:

3.1 Selection of Appropriate Tissue Compartments

The most natural approach to the choice of tissue compartments in PBPK modelling is to model a whole body by describing every organ and tissue. However, such detailed models, aside from the prohibitive labour and expenses to develop them, are not required in most circumstances. The selection should be governed by the degree of detail necessary to provide a satisfactory depiction of the events. A knowledge of the chemical's mode of action and its physicochemical properties will help make such a judgement. For instance, if a substance is known to accumulate in, bind to, be metabolized by or be toxic to specific organs or tissues, these should form the integral compartments of the model that are described in detail. If a chemical is highly lipophilic, then the adipose tissues should be described as a separate compartment. Other 'nontarget' organs and tissues may be lumped together with respect to similar kinds or properties, for example, richly perfused tissues representing kidneys and other visceral organs, slowly perfused tissues representing skin and muscle. The tissues or organs in the same body group are considered to have common kinetic behaviour and can be described by a single concentration profile. Naturally, if the grouping is carried out to the extreme, it degenerates to the classical one-compartment model, where the entire body is assumed to have uniform concentration. This phenomenon is most likely only if a chemical is relatively slow-acting, is hydrophilic and has no complex biochemical mode of action, such as active transport or macromolecular binding.

3.2 Formulation of the Mathematical Relationship

After the model structure has been decided, differential rate equations are written to describe the transport, metabolism, binding and clearance for each compartment. The derivation of the basic form of the equation for nonmetabolizing, noneliminating tissues has previously been described (Equation 4).

For blood, the efferent blood from each tissue compartment is assumed to combine simultaneously to yield a mixed venous blood concentration (C_b) returning to the lungs at a flow rate equal to the cardiac output (Q_b), as follows:

$$\frac{dC_b}{dt} = \sum \left(\frac{Q_i \cdot C_{vi}}{Q_b} \right) \quad (5)$$

where i = name of tissue compartment.

For metabolizing tissues such as the liver, depending on the number and type of metabolic pathways, the basic differential equation is modified by the inclusion of terms describing a first-order metabolic process (K_f)

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or a saturable Michaelis–Menten type enzyme kinetics (K_m and V_{max}) or both:

$$V \cdot \left(\frac{dC}{dt}\right) = Q\left(C_a - \frac{C}{P}\right) - K_f\left(\frac{C}{P}\right) - \frac{\left(V_{max} \cdot \frac{C}{P}\right)}{\left(K_m + \frac{C}{P}\right)} \quad (6)$$

For tissues which exhibit specific binding, in addition to the amount partitioned by solubility, the total tissue concentration will also include the portion in bound form, as follows:

$$\frac{V \cdot \left(\frac{dC}{dt}\right)}{Q} = C_a - \left\{\frac{(C \cdot V)}{(V \cdot P) + \left[\frac{B}{(K_b + C_v)}\right]}\right\} \quad (7)$$

where B = binding capacity and K_b = equilibrium dissociation constant.

Thus, the formulation of the mathematical equations is rather simple. The key consideration is to maintain mass balance within each tissue compartment as well as the entire model by carefully accounting for the inputs and outputs of the chemical.

3.3 Determining Model Parameter Values

The next step in the development of a PBPK model involves obtaining or determining the necessary model parameter values. Three classes of parameters are required: (i) anatomic and physiological variables such as organ and tissue volumes, and blood flow rates; (ii) thermodynamic parameters such as tissue solubility (partition coefficients) and binding constants and (iii) biochemical parameters such as absorption, excretion and metabolic constants.

Values for organ/tissue volumes, blood flows and ventilation rates can be readily obtained from the literature (Arms and Travis, 1988). Values not available may be scaled allometrically (Rowland, 1985). Analysis of organ weights and other physiological parameters have led to numerous equations of the type

$$X = \alpha W^{\beta} \quad (8)$$

where X = the parameter of interest; W = body weight; and α and β are numerical constants.

In general, organ size is directly proportional to body weight, and $\beta \approx 1$. For body surface area, flow rates, metabolic and clearance rates, they tend to vary to a fractional power of the body weight, and $\beta \approx 2/3 - 3/4$. Like all procedures concocted to substitute for missing information, they only provide a best guess in the absence of data. When specific information becomes available, it should be used to adjust or to supplant the procedure (Kirman *et al.*, 2003a).

Tissue:air partition coefficients for volatile chemicals can be determined by head space analysis with the vial equilibration technique, using tissue preparations or homogenates (Fiserova-Bergerova and Diaz, 1986; Gargas *et al.*, 1989; Mattie *et al.*, 1994). As a rough approximation a simple correlation approach based on other physicochemical properties such as water solubility and vapour pressure (Paterson and Mackay, 1989) or *n*-octanol:water partition coefficient (DeJongh *et al.*, 1997) may be used. Tissue:blood partition coefficients are calculated from the tissue:air partition coefficients by dividing by the corresponding blood:air partition coefficient:

$$P_i = \frac{P_{Ai}}{P_{Ab}} \quad (9)$$

where P_{Ai} = tissue:air partition coefficient and P_{Ab} = blood:air partition coefficient.

The metabolic constants V_{max} and K_m can also be estimated using a similar vial equilibration technique with tissue homogenates *in vitro* (Sato and Nakajima, 1979) or with an *in vivo* technique by measuring gas uptake (Gargas *et al.*, 1986; Filser, 1992) or the exhalation rates of animals in an exposure chamber (Gargas and Andersen, 1989). In the gas uptake study a closed recirculated exposure system is used to generate a series of uptake curves at a range of initial concentrations. The shapes of these curves are a function of P_i, V_{max} and K_m. Tissue partition coefficients are experimentally determined by the vial equilibration technique and incorporated into a PBPK model, which is then used to simulate the uptake process. An optimal fit of the family of uptake curves is then obtained by adjusting the biochemical constants for metabolism of the chemical. For materials of low vapour pressure that exhibit increasing blood and tissue solubilities, animals are first exposed by inhalation to a constant concentration of the chemical and then placed in an exhaled breath chamber supplied with fresh air flow. The chemical concentration in the exhaled breath chamber is serially analysed. The metabolic constants are estimated with the PBPK model by optimizing the fit of the elimination curves.

For nonvolatile chemicals, tissue:blood partition coefficients may be estimated by using a vial equilibration (Murphy *et al.*, 1995), an equilibrium dialysis (Lin *et al.*, 1982) or a filtration/extraction technique (Jepson *et al.*, 1994) *in vitro*, by single-pass perfusion of isolated organs in situ (Sultatos *et al.*, 1990), by bolus intravenous injection (Lam *et al.*, 1982) or by whole-body constant intravenous infusion *in vivo* (Chen and Gross, 1979). Metabolic constants for nonvolatiles are determined with traditional enzyme assays by measuring the rate of disappearance of the substrate or the formation of product in tissue homogenates *in vitro* or in exposed animals.

In general, *in vivo* metabolic constants are quite difficult to determine empirically, and for ethical reasons it is nearly impossible to determine them for humans. In situations where only *in vivo* metabolic constants are

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available for the laboratory animal, V_{max} for humans may be scaled allometrically according to the fractional power rule. Alternatively, when *in vitro* data are available, the human *in vivo* V_{max} may be estimated using a parallelogram approach (Reitz *et al.*, 1989; Kedderis and Lipscomb, 2001). The Michaelis constant K_m generally is considered to be invariant among animal species.

Absorption rate constants are estimated from the rising portion of the blood concentration-time curve, and bioavailability is determined from the area under the blood curve following intravenous and other routes of administration.

3.4 Model Calibration, Validation and Reformulation

Once the PBPK model is configured and the requisite model parameters are collected, the model is subject to calibration and validation against kinetic, metabolic and toxicity information. This is accomplished by comparing the model predictions with experimental results. These exercises can suggest additional experiments to collect crucial data for verifying or improving model performance. When the model fails to accurately simulate known kinetic and toxicity behaviour, despite modification of the model parameters consistent with physiological limits, it suggests that there may be additional mode(s) of action unaccounted for by the present model formulation. In such an instance, the model structure will need to be reformulated to justify the discrepancies. Obviously there are multiple ways to restructure a PBPK model if the objective is simply to improve the goodness of fit to the experimental results. However, model reformulation should be guided by plausible biological mechanisms, which can be verified experimentally. A model is considered to be validated when it is successful in simulating the empirical results. The more extensive the database a PBPK model is validated against, the more robust it becomes. A well-validated model can be used to make predictions of responses for a variety of exposure conditions, including ones which are difficult to perform experimentally. It also provides a means of predicting human kinetic behaviour when the biochemical constants and tissue-binding characteristics of the chemical have been determined in human tissues.

4 APPLICATIONS OF PBPK MODELLING IN TOXICOLOGY

4.1 Improving the Scientific Basis for Health Risk Assessment

The most common application of PBPK modelling in toxicology is dosimetric scaling in human health risk assessment (Leung and Paustenbach, 1995; Andersen, 2003). Because of ethical reasons, most toxicological data traditionally are derived from experiments with laboratory animals. High exposure levels are also frequently employed to maximize the likelihood of observing effects. In order to assess the human health risk from exposure to a chemical from the animal toxicity data, it will be necessary to make extrapolations of the toxic response from: (i) the test species to human; (ii) high to low exposure levels and (iii) the test route to another route of exposure.

Historically, exposure is expressed as the dose administered in proportion to body weight. This dosimetric method assumes that the response of the biological system is directly proportional to the initial concentration of the test material, which in turn correlates with the body volume. Interspecies dose adjustment is scaled according to an animal's body mass. A variation based on a similar concept of initial whole-body concentration is to scale according to body surface area (Freireich *et al.*, 1966). Despite its popularity, this form of dosimetric scaling is only marginally accurate for intraspecies extrapolation, and is rarely acceptable for interspecies extrapolation. The apparent unreliability of this approach is due to its failure to consider pharmacokinetic differences between species. The premise for this form of scaling assumes that the intensity of the toxic response correlates with the external exposure concentration or the amount of chemical administered. However, toxicity is not caused simply by the amount of chemical administered, but by the concentration of the chemical reaching the target tissues. Owing to the modifying effects of absorption, distribution, metabolism and excretion processes, target tissue dose is not always directly related to the amount of chemical administered. Another area where pharmacokinetics is important is the extrapolation of biological response from high dose to low dose. At low exposure levels typically associated with environmental conditions, pharmacokinetic processes generally proceed at rates directly proportional to the chemical concentration. However, at the high doses used in toxicity studies, many pharmacokinetic processes, especially metabolism, have a finite capacity and may become saturated. One other important application of PBPK modelling in toxicological risk assessment is extrapolation from one route of exposure to another (Chiu and White, 2006). Inter-route extrapolation is necessary because the bulk of toxicity testing has been conducted with the oral route, whereas environmental and occupational exposures typically occur by inhalation or skin contact. The general aspects of route-to-route extrapolation using PBPK modelling has been described (Gillette, 1987). Specific examples include the dermal to inhalation extrapolation of organic chemical vapours (McDougal *et al.*, 1986), inhalation to oral extrapolation of trichloroethylene (Fisher, 1990) and methylene chloride (Angelo and Pritchard, 1987), and oral to dermal extrapolation of ethyl acrylate (Frederick, 1992).

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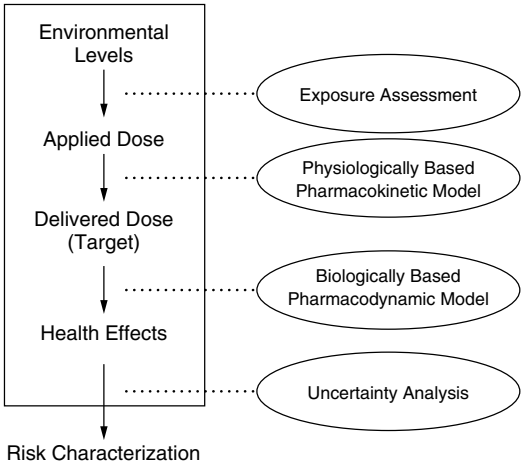

Figure 2 The roles of physiologically based pharmacokinetic and biologically based pharmacodynamic modelling in a refined health risk assessment paradigm.

PBPK modelling provides a means of estimating the tissue doses of chemicals and their metabolites over a wide range of exposure conditions in different animal species. It can provide a biologically based means of extrapolating from the animal results to predict effects in human populations. These techniques have been applied to the human cancer risk assessment of methylene chloride (Andersen *et al.*, 1987a), ethylene dichloride (D'Souza *et al.* 1987), perchloroethylene (Chen and Blancato, 1987), trichloroethylene (Bogen, 1988), 1,4-dioxane (Leung and Paustenbach, 1990), chloroform (Reitz *et al.*, 1990), ethyl acrylate (Frederick *et al.*, 1992) and vinyl chloride (Reitz *et al.*, 1996).

In a refined risk assessment paradigm **(Figure 2)**, a risk assessor conducts an evaluation of the environmental levels and the extent of exposure (frequency and duration) that provides an estimate of the administered dose (see **Risk Assessment of Chemicals**). From the administered dose, a PBPK model is then used to estimate the delivered dose. In the next step, a biologically based pharmacodynamic model is used to provide the connection between the biological effective dose and the toxic response, ultimately yielding a risk estimate. And finally an uncertainty analysis of the model parameters completes the health assessment by providing the confidence limits around the risk estimates.

4.2 Refining Experimental Designs in Toxicity Testing

Metabolism plays a salient role in regulating the toxicity of a multitude of chemicals. Almost all metabolic and many excretory processes utilize specific enzymes or binding proteins that have limited capacity and may become saturated at high substrate concentrations. When these processes are saturated, internal dose parameters, such as area under the tissue curve or the amount of metabolite formed during inhalation exposure, are not linearly related to the externally administered dose or inspired concentration. PBPK analysis of the dose-dependent processes provides an understanding of the relationship between external and internal dosimetrics under various exposure conditions. Recognition of these complex kinetic behaviours is essential to the proper design of toxicological experiments. It is particularly relevant for proper dose selection in contemporary cancer bioassays, which emphasize the use of a maximum tolerated dose. The development of a comprehensive pharmacokinetic description to examine the influence of saturable processes on the delivery of a chemical to target tissues will aid in the correct selection of dosing regimen and test species. While some *in vivo* animal experiments will always be necessary to test the accuracy of the predicted behaviour by the PBPK model, this limited work requires fewer animals than conventional experiments for assessing pharmacokinetic behaviour. Thus, a PBPK guided study design for chronic toxicity testing will enhance the information content of the experiment, while reducing the number of laboratory animals used. By the same token, it has been advocated that PBPK models can be a useful tool to facilitate the selection of drug discovery candidates in pharmaceutical development (Theil *et al.*, 2003).

4.3 Analysing Metabolic Interactions in Chemical Mixtures

In addition to uses for improving toxicity study design, PBPK models have been used to examine chemical interactions (Krishnan *et al.*, 2002; Isaacs *et al.*, 2004). An earliest example of this is the work of Andersen (1987) with a dichloroethylene and trichloroethylene mixture. In this approach a PBPK model is first constructed for the two chemicals individually, then linked via a mass-balanced differential rate equation for the liver compartment that has been generalized to account for the various modes of metabolic interaction. The PBPK models are then tested by optimizing the fit to a series of uptake data in a closed-chamber inhalation exposure to the chemical mixture. These kinetic analyses, coupled with *in vitro* interaction data, can help to delineate the correct mechanistic possibility. The advantage of this PBPK approach is that analysis of the gas uptake data instead of the hepatic metabolism data greatly simplifies the evaluation of the kinetic interactions. Furthermore, a validated metabolic interaction model can be used to readily predict the outcomes of exposures to chemical mixtures of various proportions. This would have been almost impossible if it were to be tested *in vivo*, considering the large number of permutations. Other examples of analyses of metabolic interaction utilizing PBPK modelling include the binary mixtures of

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hexane with methyl-*n*-butyl ketone or 2,5-hexanedione (Mumtaz *et al.*, 1993), trichloroethylene/ethanol (Sato *et al.*, 1991b), benzene/toluene (Purcell *et al.*, 1990), toluene/*m*-xylene (Tardif *et al.*, 1993), carbon tetrachloride/kepone (El-Masri *et al.*, 1996), and carbon tetrachloride/methanol (Evans and Simmons, 1996), and the ternary mixtures of alkyl benzenes (Tardif *et al.*, 1997), vinyl chloride/trichloroethylene/1,2-dichloroethylene (Barton *et al.*, 1995), and trichloroethylene in combination with tetrachloroethylene and 1,1,1-trichloroethane (Dobrev *et al.*, 2001).

4.4 Evaluating Dosimetry During Early Life Stage Development

Recent initiatives with regards to children's health have raised the question of whether differences in pharmacokinetic processes in children may result in a different internal dose compared with adults following a given chemical exposure. Consequently there is a keen interest in the use of PBPK models to predict the dosimetry in the developing embryo, foetus, or neonate. PBPK models are well suited for this purpose because they can more readily accommodate the rapidly changing growth dynamics, changes in the state of differentiation of developing tissues, and unusual routes of exposure (e.g. maternal-mediated placental transfer and lactation). Fisher *et al.* (1989) developed the first PBPK model of pregnancy for an environmental chemical, trichloroethylene. Subsequently, models of increasing complexity have been constructed for a number of putative developmental toxicants, notably those for the glycol ethers (Clarke *et al.*, 1993; Gargas *et al.*, 2000), methyl mercury (Gray, 1995), methanol (Ward *et al.*, 1997), perchlorate (Clewell *et al.*, 2003a; 2003b), bisphenol A (Kawamoto *et al.*, 2007), acrylamide (Walker *et al.*, 2007), polychlorinated biphenyls (PCBs) (Lee *et al.*, 2007), and phthalates (Clewell *et al.*, 2008). Some of these models have focussed on a very short duration of gestation, around the critical window for teratogenesis. Under these conditions, the timescale for pharmacokinetic simulations can be shorter than the timescale for growth, which can then be conveniently ignored. Ideally, however, PBPK models of pregnancy should be validated across a wider range of conditions to permit a more reliable extrapolation to other lengths of gestation and exposure conditions. Some of these models also attempted to extrapolate internal dose in animals to human. For practical reasons, the internal dose extrapolations were limited to maternal blood or milk levels of the chemical or its metabolite. Since it is quite difficult, if not impossible, to collect controlled exposure data for environmental chemicals in pregnant women or newborn children for model validation, the reliability of extrapolations for human in utero or lactational exposures should be carefully assessed.

4.5 Establishing and Adjusting Occupational Exposure Limits

PBPK modelling has also been applied to evaluate the hazards of chemicals in the occupational setting (Sweeney *et al.*, 2001). Occupational exposures to industrial chemicals traditionally have been evaluated by monitoring the airborne concentration of the chemicals in the workplace. However, air monitoring does not represent the absorbed dose, since it ignores modifying processes such as bioavailability and metabolism. In addition, routes of exposure other than inhalation may contribute to the total body burden. In order to determine accurately the actual received dose, biological monitoring techniques can be used (Droz *et al.*, 1999). Reference standards known as biological exposure indices (BEIs) defining the acceptable levels of chemical substances in biological media have been established. PBPK models are well suited for setting BEIs, because they can readily be used to estimate chemical concentrations in a variety of body fluids or tissues corresponding to the airborne exposure concentrations. This is achieved by exercising the PBPK models at an exposure scenario corresponding to an eight hour inhalation at the airborne exposure limit (Leung and Paustenbach, 1988; Leung, 1992). Most occupational exposure limits (OELs) are established for the average or standard worker. PBPK models, however, can accommodate individual physiological variations such as body weight, body fat content, metabolic rate and so on. Theoretically, with the aid of PBPK modelling, one can develop custom exposure limits specifically tailored to a particular worker (Loizou, 2001).

While biomonitoring is a useful tool to provide evidence of exposure, it provides little information about the nature of the exposure. It is also difficult to assess health risks based on biomonitoring data, since most health-based guidelines are expressed as external exposure concentrations in air or water. As discussed earlier, PBPK models have been used successfully to establish BEIs based on external exposure in a process referred to as forward dosimetry. The inverse of the process, called reverse dosimetry, utilizes PBPK modelling to estimate external exposures based on the measured internal dose (Tan *et al.*, 2007). This reverse dosimetry method converts human biomonitoring data to the corresponding external exposure, which can then be compared with health-based guidelines (Liao *et al.*, 2007).

PBPK modelling has also been applied to setting OELs for work shifts of longer duration than the standard eight hours per day, five days per week work schedule (Andersen *et al.*, 1987b). The rationale for adjusting OELs for unusual work shifts is to ensure that workers are not placed at greater risk than those working a standard shift. Therefore, central to the development of a PBPK model for adjusting OELs for non-conventional

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work shifts is the quantification of the degree of risk associated with the standard work shift such that the calculated OEL for the longer schedule poses no more than an equivalent risk. The selection of an appropriate risk index depends on a chemical's mechanism of toxicity. For most systemic toxicants, the risk index is the integrated tissue dose, that is, the concentration and time cross-product. An important advantage of PBPK models is their ability to track the areas under the tissue or metabolism curves. Assuming that the area under the blood curve is the proper risk index associated with styrene exposure, Andersen *et al.* (1987b) determined that a 12 hour exposure to 64 ppm was equivalent to an eight hour exposure to 100 ppm. In another example, blood carboxyhaemoglobin level was identified as the appropriate risk index for methylene chloride exposure. The OEL for nonstandard work shifts is determined by maintaining the end-of-shift blood carboxyhaemoglobin concentration at a level no greater than those observed after exposure to the eight hour time-weighted average OEL.

4.6 Assessing the Performance of Personal Protective Equipment

Traditionally, the performance of personal protective equipment such as a respirator is evaluated by measuring simultaneously the chemical concentration inside and outside the respirator. There are several problems with this approach: (i) it is rather cumbersome for the worker to carry around two air sampling trains; (ii) air sampling over the work shift only gives a snapshot of the concentration, but does not provide a concentration-time profile; (iii) since the exposure concentration rather than the absorbed dose is measured, it will underestimate the overall protection (e.g. skin absorption) offered by the protective equipment. PBPK modelling may provide a means to overcome some of these problems. The approach (Crank and Vinegar, 1992) involves measuring the post-expired breath at the end of the work shift, and using a PBPK model to estimate retrospectively the exposure concentration (i.e. the concentration leaking through the protective equipment). The PBPK model is exercised to identify combinations of exposure duration and concentration that will result in an accurate simulation of the expired breath data. Comparison of the model-predicted exposure concentration and the ambient exposure concentration will yield the protection factor of the protective equipment.

5 UNCERTAINTIES AND LIMITATIONS IN PBPK MODELLING

Since modelling is based on experimental data that have inherent errors, any model will have a certain degree of uncertainty associated with it. There are two important areas of uncertainties in the development of PBPK models. The first concerns the selection of the proper model, and the second deals with the model parameters estimated (Sato *et al.*, 1991a). When a PBPK model is used to make predictions, it should reflect these uncertainties in terms of confidence limits in the values predicted by the model, and the confidence regions around the estimated parameters.

As shown in **Figure 1**, the first step in PBPK modelling is defining the problem to be solved. The next step is to postulate several plausible physiological mechanisms to describe the data set. The third step is to use the data to discriminate between candidate models. The selection of the best model and the estimation of model parameters can be guided by statistical analysis, often with the aid of computer programs. Since small changes in input data or in the goodness of fit to a set of experimental data may significantly affect the predicted output, and, in turn, the risk estimate when the PBPK model is used to support quantitative risk assessment, the uncertainties associated with PBPK model parameters should be carefully analysed. The systematic testing of the effects of the model parameters on the model predictions in PBPK modelling is called sensitivity/variability analysis. It is typically quite difficult to obtain uncertainty estimates based on a deterministic statistical method for a highly parameterized complex model such as a PBPK model. Instead, a Bayesian approach called Markov chain Monte Carlo analysis based on a stochastic selection scheme can be used to generate a distribution of model outputs including measurements of their uncertainties (Thomas *et al.*, 1996; Hack, 2006). An example of this uncertainty evaluation in input parameters is shown in a PBPK model for carbon tetrachloride (Evans *et al.*, 1994). Finally, a PBPK model is developed to provide insight into specific questions, and should never be used for extrapolation beyond its intended purpose. Furthermore, its use for prediction should come only after a thorough validation process.

6 BEST PRACTICES FOR PBPK MODELLING

PBPK modelling has grown considerably since the very first model for an environmental chemical (dieldrin) was developed (Lindstrom *et al.*, 1974). Of the 105 chemicals shown in **Table 1**, about half of them have two or more published PBPK models. The proliferation of models, and the increasing use of these models for human health risk assessment, has called for a need to develop more systematic methods for evaluating and comparing different models, particularly for exploring the relative importance of uncertainties in model structure, dosimetrics and model parameters along with different sources of variability. In order to provide some guidance for the

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Table 1 Physiologically based pharmacokinetic models for environmental toxicants

Chemical	References	Chemical	References
Acetone[a]	Morris *et al.* (1993)	Estradiol	Plowchalk and Teeguarden (2002)
Acrylamide[a]	Kirman *et al.* (2003b)	Ethanol[a]	Pastino *et al.* (1997)
Acrylic acid	Andersen *et al.* (2000)	Ethyl acrylate	Frederick *et al.* (1992)
Acrylonitrile[a]	Gargas *et al.* (1995)	Ethyl acetate	Morris *et al.* (1993)
t-Amyl methyl ether	Collins *et al.* (1999)	Ethyl *t*-butyl ether	Nihlen and Johanson (1999)
Arsenic[a]	Mann *et al.* (1996)	Ethylene dibromide	Hissink *et al.* (2000)
Benzene[a]	Medinsky *et al.* (1989)	Ethylene glycol	Corley *et al.* (2005a)
Benzoic acid	Macpherson *et al.* (1996)	Ethylene glycol ethyl ether acetate	Gargas *et al.* (2000)
Benzo(a)pyrene	Roth and Vinegar (1990)	Ethylene oxide[a]	Krishnan *et al.* (1992)
Bisphenol A	Kawamoto *et al.* (2007)	Fluoride	Rao *et al.* (1995)
n-Butanol	Teeguarden *et al.* (2005)	Fluazifop-butyl[a]	Auton *et al.* (1993)
Bromobenzene	Morris *et al.* (1993)	Furan[a]	Kedderis *et al.* (1993a)
Bromodichloromethane	Lilly *et al.* (1997)	Gasoline	Dennison *et al.* (2003)
1,3-Butadiene[a]	Johanson and Filser (1993)	Glycyrrhizic acid	Ploeger *et al.* (2001)
2-Butoxyethanol[a]	Johanson (1986)	2,2', 4,4', 5,5'-Hexabromobiphenyl	Tuey and Matthews (1980)
n-Butyl acetate	Teeguarden *et al.* (2005)	Hexachlorobenzene[a]	Roth *et al.* (1993)
Caffeine	Ginsberg *et al.* (2004)	Hexane[a]	Perbellini *et al.* (1986)
Carbofuran	Zhang *et al.* (2007)	Hydroquinone	Corley *et al.* (2000)
Carbon tetrachloride[a]	Paustenbach *et al.* (1988)	2-Iodo-3,7,8-trichlorodibenzo-*p*-dioxin	Leung *et al.* (1990)
Chlorfenvinphos	Ikeda *et al.* (1992)	Isoamyl alcohol	Morris *et al.* (1993)
Chloroalkanes[a]	Gargas and Clewell (1990)	Isofenphos	Knaak *et al.* (1990)
Chloroform[a]	Corley *et al.* (1990)	Isoprene	Bogaards *et al.* (2001)
Chloropentafluoro-benzene[a]	Vinegar *et al.* (1990)	Isopropanol	Clewell *et al.* (2001)
β-Chloroprene	Himmelstein *et al.* (2004)	Kepone[a]	Bungay *et al.* (1981)
Chlorpyrifos[a]	Timchalk *et al.* (2002)	Lead[a]	Dalley *et al.* (1990)
Chromium[a]	O'Flaherty (1996)	Lindane	DeJongh and Blaauboer (1997)
Decane	Perleberg *et al.* (2004)	Melamine	Buur *et al.* (2008)
Deltamethrin	Mirfazaelian *et al.* (2006)	Methanol[a]	Horton *et al.* (1992)
Diazinon	Poet *et al.* (2004)	2-Methoxyethanol	Clarke *et al.* (1993)
Di-*n*-butylphthalate[a]	Keys *et al.* (2000)	Methoxyacetic acid[a]	Terry *et al.* (1995)
Dichloroacetic acid	Li *et al.* (2008)	Methyl chloride	Jonsson *et al.* (2001)
1,2-Dichlorobenzene	Hissink *et al.* (1997)	Methylmercury[a]	Farris *et al.* (1993)
1,2-Dichloroethane[a]	D'Souza *et al.* (1987)	Methylethylketone[a]	Liira *et al.* (1990)
1,1-Dichloroethylene[a]	Andersen (1987)	Methyl *t*-butyl ether[a]	Borghoff *et al.* (1996)
1,2-Dichloroethylene	Barton *et al.* (1995)	Methyl methacrylate	Andersen *et al.* (1999)
Dichloromethane[a]	Andersen *et al.* (1987a)	Naphthalene	Willems *et al.* (2001)
2,4-Dichlorophenoxyacetic acid[a]	Kim *et al.* (1994)	Nickel[a]	Menzel (1988)
2,2-Dichloro-1,1,1-trifluoroethane[a]	Vinegar *et al.* (1994)	Nicotine[a]	Plowchalk *et al.* (1992)
Dieldrin	Lindstrom *et al.* (1974)	Octamethylcyclotetra-siloxane	Andersen *et al.* (2001)
Diisopropylfluoro-phosphate	Gearhart *et al.* (1990)	Parathion[a]	Sultatos (1990)
5,5'-Dimethyloxazolidine 2,4-dione[a]	O'Flaherty *et al.* (1992)	Pentachloroethane	Nichols *et al.* (1994)
Dimethyl sulfate	Sarangapani *et al.* (2004)	Perchlorate[a]	Fisher *et al.* (2000)
1,4-Dioxane[a]	Leung and Paustenbach (1990)	Physostigmine	Somani *et al.* (1991)

(continued overleaf)

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Table 1 (*continued*)

Chemical	References	Chemical	References
Polychlorinated biphenyls[a]	Lutz *et al.* (1984)	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin[a]	Leung *et al.* (1988)
Propylene glycol monomethyl ether	Corley *et al.* (2005b)	Tetrachloroethylene[a]	Chen and Blancato (1987)
Soman[a]	Maxwell *et al.* (1988)	Tetrahydrofuran	Droz *et al.* (1999)
Styrene[a]	Ramsey and Andersen (1984)	1,1,1,2-Tetrafluoroethane (HFC-134a)	Gunnare *et al.* (2006)
Toluene[a]	Tardif *et al.* (1993)	1,1,1-Trichloroethane[a]	Reitz *et al.* (1988)
2,4-Toluenediamine	Do Luu *et al.* (1998)	Trichloroethylene[a]	Andersen (1987)
Tetrachlorobenzyltoluenes	Kramer *et al.* (2001)	1,1,2-Trichloro-1,2,2-trifluoroethane	Auton and Woollen (1991)
2,3,7,8-Tetrabromodibenzo-*p*-dioxin[a]	Kedderis *et al.* (1993b)	Vinyl chloride[a]	Barton *et al.* (1995)
2,3,7,8-Tetrachlorodibenzofuran[a]	King *et al.* (1983)	Vinylidene fluoride	Medinsky *et al.* (1990)
		Xylene[a]	Morris *et al.* (1993)

[a]These chemicals have more than one model. The reference identifies the model appearing the earliest in the published literature.

modelling community, there has been the suggestion to establish some internationally recognized good modelling practices (Loizou *et al.*, 2008). These practices can facilitate sharing of models and model evaluations and consistent applications in risk assessments. Best practices allow the efficient evaluation of models through standardization, documentation and transparency. The seven-step process of model assessment for the quality of its purpose, structure, mathematical representation, computer implementation, parameter analysis, validation/calibration and documentation, as described by Clark *et al.* (2004) provides a useful framework. While establishing good modelling practice is a logical beginning, it is presently not regarded to be prudent to set any rigid acceptance criteria for a PBPK model to be used for risk assessment or regulatory purposes. This is because each chemical will need a specific model and it depends on the amount of available data to calibrate and validate the model. In addition, strict regulatory criteria have the tendency to freeze the science in time. Until PBPK modelling has reached the point of relative maturity, it is best to be flexible to take into account the emerging science in this rapidly developing field.

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Evaluation of Toxicological Interactions for the Dose-Response Assessment of Chemical Mixtures

Kannan Krishnan, Sastry Isukapalli and Jonathan Boyd

CONTENTS

1 INTRODUCTION

Dose-response assessment for individual chemicals and chemical mixtures involves the characterization of the relationship between administered dose (or more appropriately target tissue dose) and tissue response, in order to facilitate the determination of safe exposure levels for humans (NRC, 1983; Health Canada, 1994; WHO, 1999; U.S. EPA, 2000). As the mixture dose-response data are often generated in laboratory studies, the extrapolation of responses observed at high doses to low exposure doses, as well as extrapolation of toxicologically equivalent doses from test animals to humans is necessary (Calabrese, 1991; Krishnan and Brodeur, 1991; Krishnan and Brodeur, 1994). Additionally, route-to-route extrapolation, duration extrapolation and evaluation of intraspecies variability in kinetics, dynamics and interactions may be required.

The science of dose-response modelling has evolved significantly over the years for individual chemicals. Models facilitating the conduct of interspecies, intraspecies, route-to-route and high dose to low dose extrapolations in the context of human health risk assessment for individual chemicals have been developed (Gehring *et al.*, 1978; Clewell and Andersen, 1985; 1987; Jarabek, 1995; Chiu *et al.*, 2007). The dose-response assessment for chemical mixtures is essentially similar to that of individual chemicals when the data are obtained for the 'whole' mixture of relevance to human exposure situation [U.S. EPA, 1986; 2000; Agency for toxic substances and disease registry division of toxicology (ATSDR), 2004; Lotz *et al.*, 2008; Meyer-Baron *et al.*, 2008]. During a component-based dose-response assessment, however, focus should be on how the slope or threshold of the components changes. Thus, if the slope of the dose-response curve or threshold of one chemical does not change in the presence of other chemicals, then there is no interaction between the first chemical and the others (Gennings *et al.*, 2005). Conversely, if the rate of change in the response with respect to the dose of the first chemical changes in the presence of other chemicals, then an interaction has occurred (Gennings *et al.*, 2005).

When interactions among chemicals occur, the consideration of mechanisms would be necessary for the conduct of scientifically sound dose-response assessment for mixtures (Haddad *et al.*, 1998; 2001; Krishnan *et al.*, 2002). Thus, when toxicity data for individual chemicals are used to predict or assess the health risk associated with mixtures of chemicals, it becomes relevant to assess how the tissue dose or the tissue response of individual chemicals is altered during combined exposures (**Figure 1**). Toxicokinetic interactions are those that lead to a change in target tissue dose per unit exposure concentration during mixed exposures compared to individual chemical exposures (Krishnan *et al.*, 1994). Interactions

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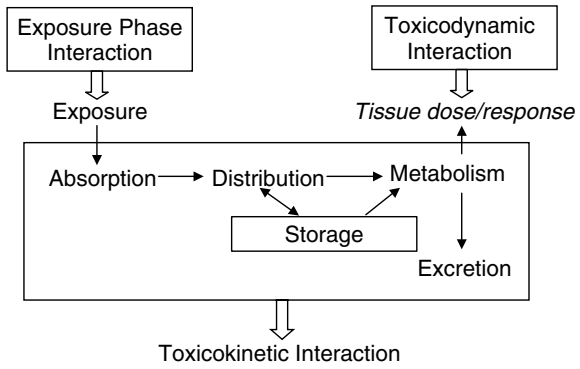

Figure 1 Potential sites or phases where interactions among chemicals occur during combined exposures.

at the toxicokinetic level imply a modulation of absorption, distribution, metabolism and/or excretion of one chemical by another **(Figure 1)**. On the other hand, toxicodynamic interactions are said to result when the tissue response per unit tissue dose differs between mixed exposures and single chemical exposures (Krishnan *et al.*, 1994). Interactions at the toxicodynamic level might involve a competition between two chemicals for binding to a receptor or an alteration by one chemical of the susceptibility, repair and compensatory effects induced by another chemical. **Table 1** lists examples of toxicological interactions observed in humans following combined exposure to environmental pollutants.

Even though there are numerous reports of the occurrence of interactions between chemicals (Calabrese, 1991; Krishnan and Brodeur, 1991; 1994), analysis of such data to determine their relevance to occupational or environmental health risk assessment of mixtures remains to be accomplished in most cases (Vyskocil *et al.*, 2007). In this regard, it is critical that the mechanisms of toxicokinetic and toxicodynamic interactions be evaluated and integrated within quantitative models to facilitate scientifically sound dose-response assessments for chemical mixtures. Such an approach will not only reduce uncertainty relating to mixture risk assessment, but also identify key data gaps that need to be addressed by focussed research programs.

The evaluation and modelling of toxicological data on interactions have been conducted either using statistical approaches (Berenbaum, 1989; Bailer and Bailer, 1999; Hamm *et al.*, 2005; Gennings *et al.*, 2005) or mechanistic approaches (e.g. Mumtaz and Durkin, 1992; Tardif *et al.*, 1993; 1995; 1997; Pelekis and Krishnan, 1997; Haddad *et al.*, 1999; 2000a; 2000b; 2001; Jonker *et al.*, 2005; Yang and Dennison, 2007). Whereas the characterization of the nature and extent of interaction is feasible with the statistical models, their use is somewhat limited with regard to extrapolations (route, species, dose, mixture complexity, etc.) required for the conduct of dose-response assessment of mixtures. However, they are critically useful in facilitating the determination of departure from additivity, as discussed in detail in **Toxicology of Chemical Mixtures**. The present chapter focusses specifically on the current approaches for evaluating toxicological interactions for the dose-response assessment of chemical mixtures. The approaches described in this chapter are: (i) interaction matrix method, (ii) interaction weighting ratio (IWR) method and (iii) physiological modelling.

Table 1 Examples of toxicological interactions among environmental pollutants reported to occur in humans (Krishnan and Brodeur, 1994)

Pollutant type	Supra-additive	Infra-additive(*)
Gases and particulates	• Particulates and sulphur dioxide • Ozone and sulphur dioxide	• Ammonia and sulphur dioxide • Carbon dust and sulphur dioxide • Selenium and mercury • Iron and mercury • Iron and cadmium • Zinc and cadmium • Zinc and lead • Lead and iron
Pesticides	• Malathion and isomalathion	—
Solvents	• Methanol and dichloromethane	• Trichloroethylene and 1,1,1-trichloroethane
	• Methyl ethyl ketone and methyl n-butyl ketone/*n*-hexane	• Trichloroethylene and tetrachloroethylene
	• Isopropanol and carbon tetrachloride	• Ethyl benzene and xylene • Toluene and xylene • Xylene and methyl ethyl ketone • Xylene and isobutanol

(*) Includes reports of metabolic inhibition.

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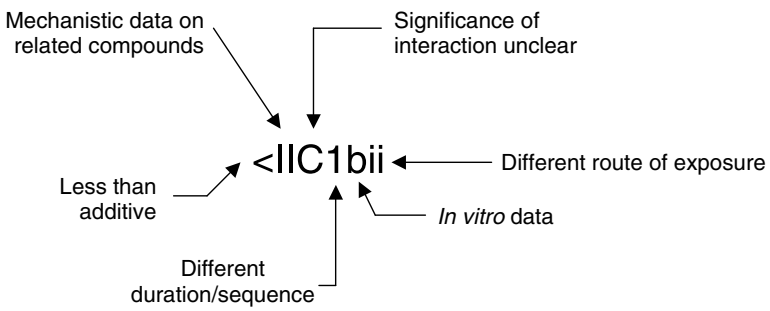

Figure 2 Example of a binary WOE classification scheme (Mumtaz and Durkin, 1992). < = infraadditive toxicity, II = inferred based on mechanistic data on related compounds, C = significance of interaction unclear, 1 = exposure duration/sequence different from that of relevance to risk assessment, b = *in vitro* data and ii = route of exposure different from that of relevance to risk assessment.

2 INTERACTION MATRIX METHOD

This approach, applied to systemically acting mixtures, involves collection and organization of the data on binary chemical interactions, which are then expressed in terms of an alphanumeric code representing the overall weight-of-evidence of the possible occurrence (Mumtaz and Durkin, 1992). This approach focusses to group the quality of interaction data into two biological categories (mechanistic understanding and toxicological significance), and three modifying categories (duration/sequence of exposure, *in vivo* and *in vitro* data, and route of exposure). The two biological categories form the basis of the assessment, while the modifying categories are used to alter the rating of the assessment to reflect the extent of relevant available data. This procedure generates an alphanumeric rating of the weight-of-evidence of binary chemical interactions (BINWOE) in mixtures (**Figure 2**), and presents them in a matrix form for any given mixture. **Table 2** presents the matrix of BINWOE scores for interactions in a mixture of 1,1,1-trichloroethane, 1,1-dichloroethane, trichloroethylene and tetrachloroethylene (Pohl *et al.*, 2003; 2004). This summary table indicates that most of the joint action of these mixture components would result in additivity, except for trichloroethylene + tetrachloroethylene, for which infraadditivity is implied based on available data. The alphanumeric expressions shown in **Table 2** can, in turn, be collapsed to yield a single number, referred to as the interaction factor for use in mixture dose-response assessment (Mumtaz and Durkin, 1992; Mumtaz *et al.*, 1994; Hertzberg and MacDonell, 2002). The interaction factor for each chemical pair within a mixture (e.g. components i and j) is calculated using the following algorithm:

$$\mathrm{IF}_{i,j} = \mathrm{HQ}_i \mathrm{BINWOE}_{i,j} \cdot (\mathrm{HQ}_i \cdot \mathrm{HQ}_j)^{0.5} \qquad (1)$$

Table 2 Matrix of BINWOE determination for simultaneous exposure to chemicals of concern

Effect of \ on toxicity of	1,1,1-Trichloroethane	1,1-Dichloroethane	Trichloroethylene	Tetrachloroethylene
1,1,1-Trichloroethane	—	Nervous system = IIC Liver and kidney = IIC	Nervous system = IIC Liver and kidney = IIB	Nervous system = IIC Liver and kidney = IIB
1,1-Dichloroethane	Nervous system = IIC Liver and kidney = IIC	—	Nervous system = IIC Liver and kidney = IIC	Nervous system = IIC Liver and kidney = IIC
Trichloroethylene	Nervous system = IIC Liver and kidney = IIB	Nervous system = IIC Liver and kidney = IIC	—	Nervous system = IIC Liver and kidney = IIB
Tetrachloroethylene	Nervous system = IIC Liver and kidney = IIB	Nervous system = IIC Liver and kidney = IIC	Nervous system = IIC Liver and kidney < IIB	—

Reproduced from Pohl *et al.*, 2003. © Elsevier. BINWOE, binary weight-of-evidence; =, additivity; <, infra-additivity; C, toxicological significance unknown; B, toxicological significance inferred based on similar chemicals.

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where $IF_{i,j}$ refers to the interaction factor for the effect of i on the toxicity of j, HQ is the hazard coefficient of each mixture component (e.g. $HQ_i = 0.25$ means that component i is present at 25% of its guideline value), and BINWOE is the interaction term, a quantification of concern of interaction for the chemical pair i, j. The BINWOE scores range from 0 to 1.

The basis of the interaction matrix approach is that the level of concern associated with a mixture increases with increasing incidences of supra-additive interactions, and the opposite is likely to be the case with the occurrence of infra-additive interactions among mixture components. The BINWOE interaction matrix essentially provides a complete picture of the current state of knowledge of binary chemical interactions in chemical mixtures **(Table 2)**. Further, using this approach, the available data on interactions can be used to adjust the conventional hazard index (HI) for noncarcinogenic end points for a mixture as follows (Mumtaz and Durkin, 1992; Hertzberg and MacDonell, 2002):

$$HI_A = HI.UF_i^{WOE_N} \tag{2}$$

where HI_A is the hazard index calculated by adjusting for the potential occurrence of interactions among mixture components, HI is the conventional hazard index for a mixture calculated based on additivity assumption, UF_i corresponds to the uncertainty factor accounting for the potential occurrence of interactions among mixture components, and WOE (weight of evidence) is the ratio of the sum of the IF_i calculated as per Equation 1 to the maximal possible WOE score for a given mixture (Mumtaz and Durkin, 1992). The US Environmental Protection Agency has further modified this approach to facilitate the computation of interaction magnitude based on consideration of exposure levels of component chemicals, and the interaction effect on each chemical rather than on the whole mixture (Hertzberg and MacDonell, 2002).

The BINWOE approach, unlike an arbitrarily determined synergistic safety factor, provides a scientifically sound basis for determining the interaction factor for use in the dose-response assessment of mixtures. Further, it reflects the current state of knowledge on toxicological interactions (supra- and infra-additive) among components of a mixture for modifying the HI for systemic toxicity. For evaluating the interactions among mixture components with regard to their impact on cancer outcome, an IWR is useful.

3 INTERACTION WEIGHTING RATIO METHOD

The IWR method integrates available information on interactions among the carcinogenic components of a mixture to facilitate the calculation of an exponent index. The exponent index is a scale of the cancer risk associated with the mixture, based on the assumption of independent joint action. The product of IWR and exponent index is then used to classify the carcinogenic mixture according to its level of concern as low, marginal, moderate, high-moderate or high (Woo *et al.*, 1994):

The IWR is calculated as follows:

$$IWR = \frac{1 + \left(p \cdot H_{syn}\right) + \left(q \cdot H_{pro}\right)}{1 + \left(r \cdot H_{ant}\right) + \left(s \cdot H_{inh}\right)} \tag{3}$$

where H terms represent the hazard-modification scores for interactions (synergism (syn), promotion (pro), antagonism (ant) and inhibition (inh)). The terms p, q, r and s refer to weighting factors that reflect the relative importance of each form of interaction. The number 1 in both the numerator and denominator of this equation (representing additivity) keeps the lower limit of the IWR above zero, thus preventing division by zero when the terms in the denominator (H_{ant} and H_{inh}) are equal to zero. The calculation of H requires systematic identification of all possible binary pairs (interactions) within a given mixture and the use of weighting factors as detailed in Woo *et al.* (1994).

The basis of this approach is that the level of concern associated with a mixture increases with increasing incidences of supra-additive interactions, and the opposite is likely to be the case with the occurrence of infra-additive interactions. Since the IWR is calculated as a ratio of the 'hazard-amplifying interactions' to the 'hazard-reducing interactions' in a mixture, this approach provides a balanced way of considering interaction data in cancer risk assessments for chemical mixtures. Accordingly, as the weighted information indicating synergism or promotion increases relative to antagonism or inhibition, the IWR and the overall assessment of hazard increase. Conversely, as the weighted information reflective of synergism or promotion decreases relative to the information indicating antagonism or inhibition, the overall assessment of the cancer hazard of the mixture decreases (Woo *et al.*, 1994).

The IWR method, similar to the BINWOE method for systemic toxicants, is essentially data based and therefore the quantitative inferences for low-level human exposures are limited. When the magnitude and impact of pharmacokinetic and pharmacodynamic (PD) interactions on the dose-response relationship of mixture components are to be evaluated for various doses, scenarios, complexities, routes and species, mechanism-based modelling approaches are relevant. In this regard, physiologically based models are useful.

4 PHYSIOLOGICALLY BASED MODELLING

Physiologically based modelling refers to the process of reconstructing mathematically the key physiological

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characteristics of an organism, and describing the quantitative inter-relationships among the critical determinants of pharmacokinetics, pharmacodynamics and toxic interactions (Krishnan *et al.*, 1994; Krishnan and Andersen, 2007). This method is unique in permitting the simulation of the potential modulation of tissue dose and response due to pharmacokinetic and PD interactions within multicomponent mixtures (Andersen *et al.*, 1994; Simmons, 1996; Haddad *et al.*, 1998; 2001; Krishnan *et al.*, 2002; Yang and Dennison, 2007). Whereas physiologically based pharmacokinetic (PBPK) models are available for a number of chemicals and chemical mixtures (Simmons, 1996; Reddy *et al.*, 2005; Krishnan and Andersen, 2007; Lipscomb and Ohanian, 2007), such models for simulating the PD process in mixtures are very limited (Timchalk and Poet, 2008). The feasibility of using PBPK models to describe, predict and extrapolate the occurrence and magnitude of interactions as a function of dose levels, exposure scenarios, species, route and mixture complexities arises from the mechanistic nature and biological basis of these models.

PBPK models of mixtures correspond to a set of individual chemical models interconnected via the mechanism of interaction (Krishnan and Brodeur, 1994a) (**Figure 3**). In these models, the organism is represented as a network of tissue compartments (e.g. liver, fat, slowly perfused tissues and rapidly perfused tissues) interconnected by systemic circulation, and the rate of change in chemical concentration in the various model compartments is determined by solving mass balance differential equations (Krishnan *et al.*, 1994). To solve the model equations, the numerical values of physiological (e.g. cardiac output, breathing rates, tissue volumes, tissue blood flow rates), physicochemical (e.g. blood:air and tissue:blood partition coefficients) and biochemical (e.g. metabolic velocity for metabolism, Michaelis affinity constant, inhibition constant) parameters should be known. If one or more mechanistic determinants of chemical uptake, metabolism and disposition is altered during co-exposure (**Table 3**), then a pharmacokinetic interaction results. **Table 4** summarizes the PBPK models for mixtures of environmental contaminants available in the literature. These models facilitate the simulation of the consequence of pharmacokinetic interactions, which is often a change (increase or decrease) in the tissue dose of interacting chemicals. PBPK models, by accounting for interaction mechanisms, permit the prediction of the change in tissue dose of mixture components as a function of dose, route, scenario and mixture complexity as discussed below.

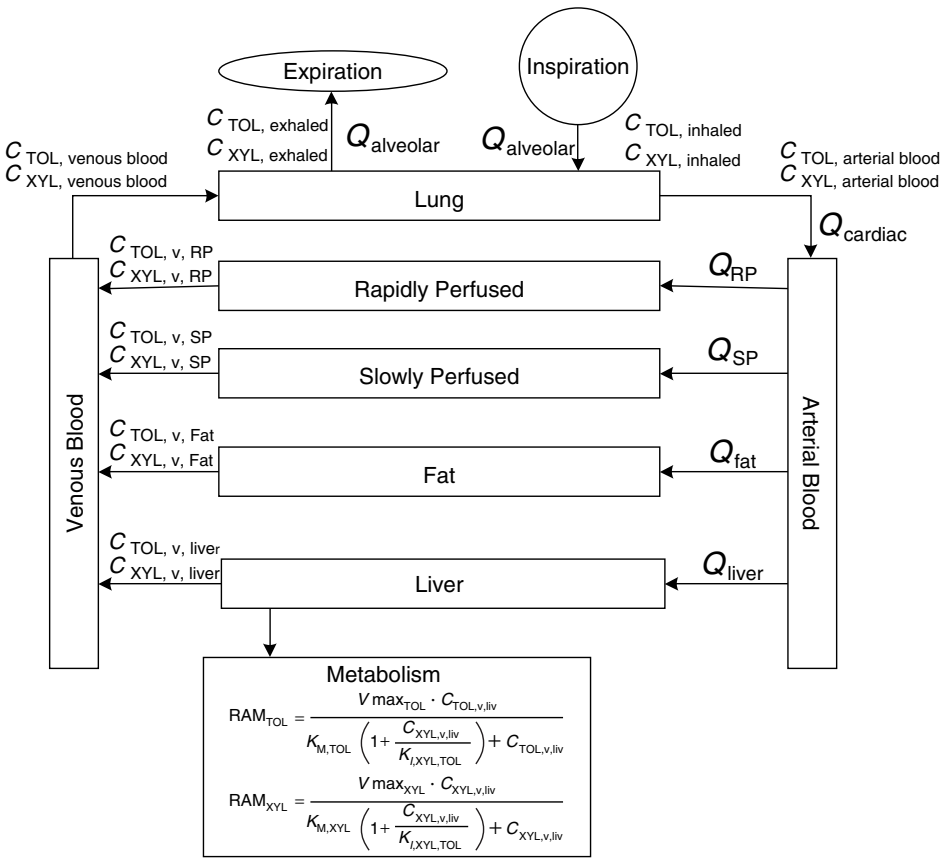

Figure 3 Schematic of a binary mixture PBPK model for inhalation exposures of toluene (TOL) and xylene (XYL) with competitive inhibition between them.

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Table 3 Examples of interaction among environmental pollutants at the absorption, distribution, metabolism and excretion levels

Mechanism/level of interaction	Example of interactions	Reference(s)
Pulmonary absorption	Hydrogen cyanide can enhance the uptake of certain air-borne contaminants by increasing the pulmonary ventilation rate	Dreisbach (1977)
Dermal absorption	Enhanced dermal absorption of pesticides and metals in the presence of organic solvents (e.g. triazines in the presence of trichloroethylene, *N,N*-diethyl *-m*-toluamide in the presence of ethanol, arsenic and nickel in organic solvent mixtures)	Marjukka Suhonen *et al.* (1999), Ross and Shah (2000), Turkall *et al.* (2003), Baynes *et al.* (2005)
Oral absorption/ transportation	Low essential element status, by increasing the expression of DMT1 (divalent metal transporter 1) increases the absorption of toxic metals (Cd, Co, Cu, Fe, Mn, Pb and Zn)	Gunshin *et al.* (1997), Picard *et al.* (2000), Rolfs and Hediger (2001), Roth *et al.* (2003), Bressler *et al.* (2004), Bressler *et al.* (2007), Yokel *et al.* (2006), Madden (2003), Kwong *et al.* (2004), Cory-Slechta (2005)
Tissue distribution	Various dithiocarbamates by forming lipophilic complexes with inorganic lead, lead to greater accumulation in the lipid-rich brain compartments	Tandon *et al.* (1990), Weiss *et al.* (1990)
Tissue distribution	Competition or induction of metallothionein (a low molecular weight protein containing -SH groups) leads to toxicological interactions among metals as well as between metals and nutrients	Telisman (1995), Brzóska and Moniuszko-Jakoniuk (2001), Telisman (1995), Bobillier-Chaumont *et al.* (2006)
Metabolism	Inhibition of specific isoforms of cytochrome P450 by metals have also been reported: CYP1A1 by mercury, CYP2E and CYP3A by cadmium, and CYP1A1 by arsenic.	Ke *et al.* (2002), Alexidis *et al.* (1994), Albores *et al.* (1995)
Metabolism	Lead, by diminishing haem synthesis and changing the rate of haem degradation, leads to change in P450 levels and activities	Moore (2004), Kadiiska and Stoytchev (1980)
Metabolism	Chronic ethanol treatment enhances hepatic CYP2E1 activity, but after an acute administration, it acts as an inhibitor	Phillips *et al.* (1977), Garro *et al.* (1981), Pelkonen and Sotaniemi (1982), Peng *et al.* (1982), Miller and Yang (1984), Swann *et al.* (1984)

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Table 3 (*continued*)

Mechanism/level of interaction	Example of interactions	Reference(s)
Excretion	Arsenic and selenium, during combined exposures, increase the biliary excretion of each other following the formation of the seleno bis-(*S*-glutathionyl) arsinium ion, which is subsequently excreted in bile	Zeng *et al.* (2005)
Excretion	The hepatic injury caused by dioxin leads to an impairment of biliary excretion and concomitant change in copper in tissues	Elsenhans *et al.* (1991)

4.1 High Dose to Low Dose Extrapolation

High dose to low dose extrapolations of pharmacokinetic interactions are critical to the better characterization of the dose-response relationships for chemical mixtures. A scientifically sound approach for high dose to low dose extrapolation requires that the nonlinear kinetic behaviour of the interacting chemicals be taken into account along with the dose-dependent impact of the interaction mechanism (e.g. enzyme induction, competitive inhibition). In this regard, the PBPK models have been demonstrated to facilitate the conduct of high dose to low dose extrapolations of the consequence of pharmacokinetic interactions. **Figures 4** and **5** illustrate the dose-dependent change in internal dose metrics of toluene (TOL) (blood concentration and amount metabolized) in rats with PBPK models (or equivalent steady-state algorithms) (Isukapalli *et al.*, 2008). Accordingly, the impact of increasing concentration of *m*-xylene (*m*-XYL) on the internal dose of inhaled TOL varies as a function of both their exposure concentrations. These differences in internal dose are adequately simulated with the PBPK models and such algorithms because they not only take into account the nonlinearity due to saturable metabolism, but also the additional impact of metabolic inhibition occurring during mixed exposures. This simulation approach has successfully been used for the determination of the interaction threshold, that is, the exposure concentration of mixture components at which their internal dose does not deviate significantly compared to individual chemical exposures (Tardif *et al.*, 1995; El-Masri *et al.*, 1996a). Several studies have investigated the interaction threshold as a function of mechanism and exposure concentrations of interacting chemicals (Tardif *et al.*, 1993; 1995; 1997; El-Masri *et al.*, 1996a; 1996b; Simmons, 1996; Haddad *et al.*, 2001; Dobrev *et al.*, 2002). More recently, Yang and Dennison (2007), based on PBPK modelling of gasoline and six of its components, concluded that the interaction thresholds during mixed exposures would appear to stay within the bounds of the thresholds of its respective component chemicals.

4.2 Route to Route Extrapolation

The temporal change in the effective concentration of an inhibitor at the site of interaction and the tissue dose of the toxic moiety of mixture components might vary depending upon the route of exposure (e.g. intravenous, oral, inhalation, dermal). For systemic toxicants in mixtures, the resulting toxicity is likely to depend upon the route of exposure since it is the key determinant of the bioavailability, rate of absorption and extent of first pass clearance (IGHRC, 2005). These route-dependent factors, influencing the magnitude and profile of the effective concentration of a chemical at the interaction site, may lead to differences in the magnitude of the interaction during mixed exposures. Krishnan and Pelekis (1995), as well as Nong and Krishnan (2005) demonstrated the route-to-route extrapolation (oral to inhalation) of the occurrence of metabolic interactions for human exposure to a mixture of TOL and dichloromethane (DCM) as well as mixtures of TOL, *m*-XYL and ethylbenzene (EBZ). These limited studies have shown that the PBPK models, by facilitating the consideration of route-specific phenomena, permit the simulation of the internal dose for combinations of systemically acting chemicals for each exposure route (or for aggregate exposures), to facilitate the extrapolation of the threshold of interactions from one exposure route to another.

4.3 Interspecies Extrapolation

When the dose-response information for individual chemicals and mixtures is initially obtained in laboratory animals, the determination of the toxicologically equivalent dose in humans becomes necessary. The PBPK models have been shown to be powerful in the conduct of interspecies extrapolations of single chemicals (Reddy *et al.*, 2005; Krishnan and Andersen, 2007). The determination of toxicologically equivalent doses of chemicals in mixtures not only requires adjustment for species differences in physiology, metabolism and volume of

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Table 4 List of PBPK models developed for mixtures of environmental contaminants

Mixture components	Species	Exposure pattern	References
Binary mixtures			
Isofluorane/dibromomethane	R	Simultaneous	Clewell and Andersen (1985)
Trichloroethylene/dichloroethylene	R	Simultaneous	Andersen *et al.* (1987), El-Masri *et al.* (1996a), El-Masri *et al.* (1996b)
Benzene/toluene	R	Simultaneous	Purcell *et al.* (1990)
Bromotrichloromethane/mirex	R	Simultaneous	Thakore *et al.* (1991)
Bromotrichloromethane/phenobarbital	R	Simultaneous	Thakore *et al.* (1991)
Bromotrichloromethane/chlordecone	R	Simultaneous	Thakore *et al.* (1991)
Ethanol/trichloroethylene	H	Sequential	Sato *et al.* (1991)
Toluene/*m*-xylene	R, H	Simultaneous	Tardif *et al.* (1993), Tardif *et al.* (1995)
1,3-Butadiene/styrene	R, M	Simultaneous	Filser *et al.* (1993), Medinsky *et al.* (1994), Leavens and Bond (1996)
1,3-Butadiene/benzene	M, R	Simultaneous	Bond *et al.* (1994)
1,3-Butadiene/ethanol	M, R	Simultaneous	Bond *et al.* (1994)
Trichloroethylene/vinyl chloride	R	Simultaneous	Barton *et al.* (1995)
Carbon tetrachloride/kepone	R	Sequential	El-Masri *et al.* (1996c)
Carbon tetrachloride/ methanol	R	Sequential	Evans and Simmons (1996)
Toluene/dichloromethane	R	Simultaneous	Pelekis and Krishnan (1997)
n-Hexane/toluene	R, H	Simultaneous	Yu *et al.* (1998), Ali and Tardif (1999)
Methyl chloroform/*m*-xylene	R	Simultaneous	Tardif and Charest-Tardif (1999)
Dibromomethane/bromochloromethane	R	Simultaneous	Jepson and McDougal (1999)
Toluene/trichloroethylene	R	Simultaneous	Thrall and Poet (2000)
Ethylbenzene/*m*-xylene	H	Simultaneous	Jang *et al.* (2001)
Trichloroethylene/perchloroethylene/ methylchloroform	R	Simultaneous	Dobrev *et al.* (2001)
Polychlorinated biphenyls (PCBs)	M	Simultaneous	Lee *et al.* (2002)
Trihalomethanes and haloacetic acids	R	Simultaneous	St-Pierre *et al.* (2005)
Chlorpyriphos and diazinon	R	Simultaneous	Timchalk and Poet (2008)
Ternary mixtures			
Toluene/ethylbenzene/*m*-xylene	R, H	Simultaneous	Tardif *et al.* (1997), Dennison *et al.* (2005)
Trichloroethylene/perchloroethylene/ methyl chloroform	R, H	Simultaneous	Dobrev *et al.* (2001), Dobrev *et al.* (2002)
Mixtures of more than three components			
Benzene/toluene/ethylbenzene/*m*-xylene/dichloromethane/ tetrachloroethylene/ styrene	R	Simultaneous	Haddad *et al.* (2000a)
Dichloromethane/ benzene/ toluene/ethylbenzene/*m*-xylene	R	Simultaneous	Haddad *et al.* (1999), Haddad *et al.* (2000b)
Benzene/toluene/ethylbenzene/*m*-xylene	H, R	Simultaneous	Haddad *et al.* (2001)
Gasoline components	R	Simultaneous	Dennison *et al.* (2003), Dennison *et al.* (2004)
PCBs	R	Simultaneous	Emond *et al.* (2005)

R = Rat, M = mice, H = humans

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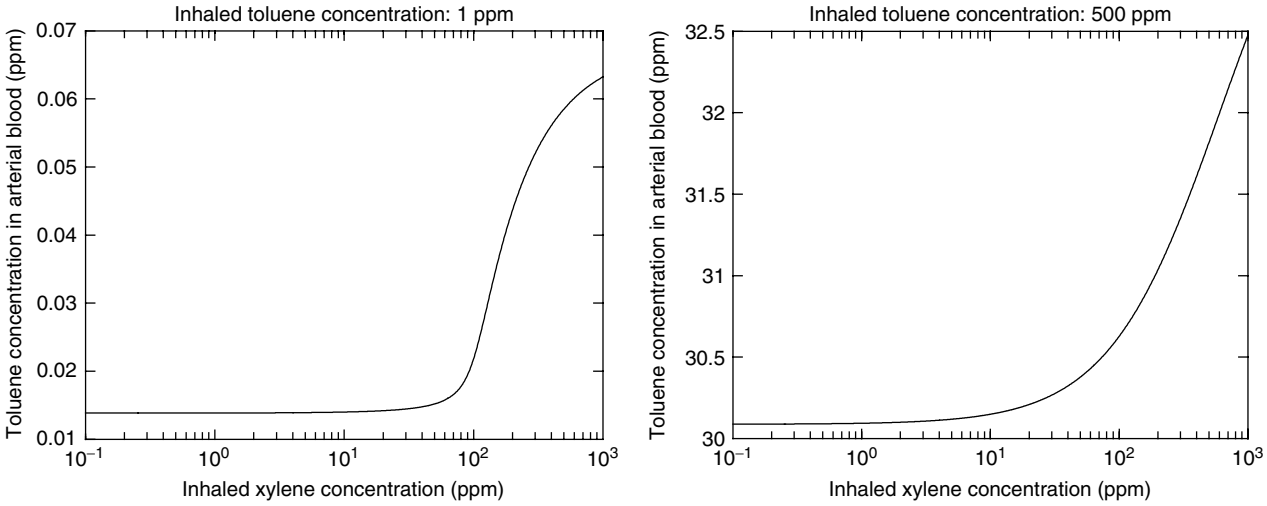

Figure 4 Impact of competitive inhibition of *m*-xylene on the steady-state arterial concentration levels of toluene in rat.

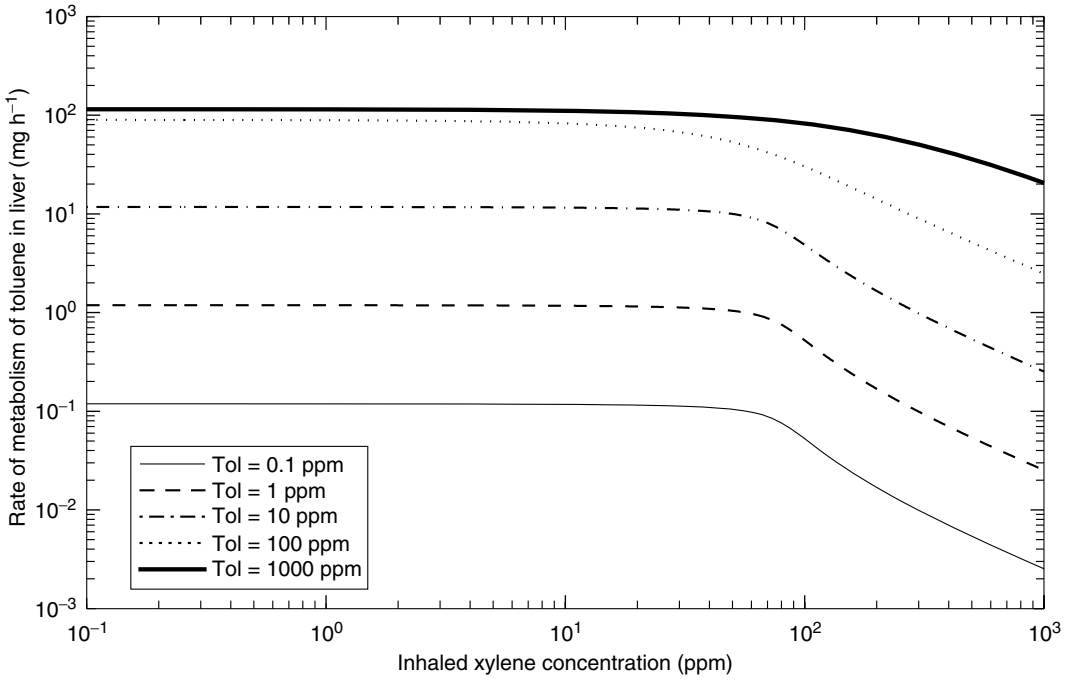

Figure 5 Impact of competitive inhibition of *m*-xylene on the rate of metabolism of toluene in humans.

distribution, but also necessitates the consideration of the possible interspecies differences in the extent of interactions. There are only a few examples demonstrating the validity of the use of rodent mixture PBPK models for predicting the pharmacokinetics of chemicals in humans during mixed exposures. The rat-to-human extrapolation of pharmacokinetic interactions using the PBPK modelling approach has been demonstrated for a binary mixture (TOL and *m*-XYL) and a ternary mixture (TOL-XYL-EBZ) (Tardif *et al.*, 1995; 1997). In these studies, the physiological parameters (i.e. alveolar ventilation rate, cardiac output, tissue volumes, tissue blood flow rates) of the rat PBPK models were replaced with those of humans, the maximal velocity for metabolism was scaled on the basis of body surface, whereas the affinity constants and inhibition constants representing interactive effects were considered to be species-invariant, based on the observation that the same isozyme (CYP2E1) is involved in the metabolism of these substances in both the test species and humans.

4.4 Mixture Complexity

The toxicity and dose-response relationships of mixture components may not only be influenced by the outcome

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of interactions at the binary level, but also by interactions at higher levels (e.g. ternary, quaternary). Historically, there has been a lack of predictive tools essential for simulating higher-order interactions within complex mixtures. In this regard, considerable body of work by Krishnan and colleagues (Tardif *et al.*, 1995; 1997; Haddad *et al.*, 1999; 2000a; 2000b; 2001; Krishnan *et al.*, 2002) has demonstrated the unique usefulness of PBPK models in conducting extrapolations of interactions from binary to more complex mixtures. The approach involves identifying and linking the relevant individual chemical PBPK models via interaction terms [e.g. metabolic inhibition **(Table 5)**]. PBPK models for mixtures of any level of complexity can then be created as long as the quantitative information on the mechanism for each interacting chemical pair is available or can be hypothesized. According to this methodology then, for modelling the kinetics of the components of complex mixtures, plausible binary interactions need only be characterized. In a mixture of three chemicals, for example, there are three two-way interactions. The first step here is to develop PBPK models for each component of the mixture. Then the single chemical models are interconnected at the binary level by modifying the appropriate equations (e.g. metabolism terms). If we consider competitive metabolic inhibition as the mechanism of interaction, then the equation for calculating the rate of the amount metabolized (RAM) of each component should be modified appropriately **(Table 5)**. If we consider interactions at the binary level alone, then how is it possible to simulate the consequence of a higher-order interaction (e.g. involving three chemicals)? This is where the unique usefulness of PBPK modelling becomes evident. Let us consider a mixture of three chemicals (c1, c2, c3), competing for metabolism by a P450 isoenzyme. The initial step is to model the binary chemical interaction between c1 and c2. Then, following the addition of another chemical, c3, the binary combinations c3–c1 and c3–c2 should be characterized. Subsequently, the PBPK model not only simulates the binary interactions involving c3, but also the modulatory effect of c3 on the interaction between c1 and c2 (**Figure 6**). Because, once the inhibitory effect of c3 on c2 is accounted for, this automatically impacts the inhibitory effect of c2 on c1 (i.e. $1 + C_{c2}/Km_{c2}$) (**Figure 6**). Since the exposure to chemical c3 increases C_{c2}, this then translates into a modification of the magnitude of the interactive effect of c2 on c1. Similarly, c3 may also affect the concentration of c1, which would then result in a change in the magnitude of the interactive effect of c1 on c2. Based on this analogy, it is possible to predict the influence of the addition of another chemical c4, c5, and so on. As a new chemical becomes an integral part of the network of the components of the mixture, any modulation of a binary interaction involving that new chemical has repercussions on all the others. The novel thing about this PBPK modelling approach is that it only requires data on binary interaction mechanisms for predicting the magnitude and consequence of multiple interactions within complex mixtures. This approach was initially validated for predicting the kinetics of components of a ternary mixture of alkyl benzenes (Tardif *et al.*, 1997). Further validation of the conceptual and mechanistic basis of this approach has been performed using other experimental data on interactions collected during mixed chemical exposures (Haddad *et al.*, 1999; 2000a; 2000b; Dobrev *et al.*, 2001; 2002; Dennison *et al.*, 2005).

5 PBPK MODELS AND DOSE-RESPONSE ASSESSMENT OF CHEMICAL MIXTURES

The tissue dose simulated using the interaction-based PBPK models can in turn be used to conduct dose-response analysis for mixtures. In this regard, the PBPK models are useful in simulating the change in the tissue dose of a toxic moiety (often referred to as the 'dose

Table 5 Description of metabolic interactions (inhibition) among chemicals during mixed exposures used in PBPK models

Type of inhibition	Rate of metabolism of chemical 1	Rate of metabolism of chemical 2
No interaction	$\frac{V_{max_1} \cdot C_{1,v,liv}}{Km_1 + C_{1,v,liv}}$	$\frac{V_{max_2} \cdot C_{2,v,liv}}{Km_2 + C_{2,v,liv}}$
Competitive inhibition	$\frac{V_{max_1} \cdot C_{1,v,liv}}{Km_1 \left(1 + \frac{C_{2,v,liv}}{K_{I,2-1}}\right) + C_{1,v,liv}}$	$\frac{V_{max_2} \cdot C_{2,v,liv}}{Km_2 \left(1 + \frac{C_{1,v,liv}}{K_{I,1-2}}\right) + C_{2,v,liv}}$
Noncompetitive inhibition	$\frac{V_{max_1} \cdot C_{1,v,liv}}{(Km_1 + C_{1,v,liv}) \cdot \left(1 + \frac{C_{2,v,liv}}{K_{I,2-1}}\right)}$	$\frac{V_{max_2} \cdot C_{2,v,liv}}{(Km_2 + C_{2,v,liv}) \cdot \left(1 + \frac{C_{1,v,liv}}{K_{I,1-2}}\right)}$
Uncompetitive inhibition	$\frac{V_{max_1} \cdot C_{1,v,liv}}{Km_1 + C_{1,v,liv} \cdot \left(1 + \frac{C_{2,v,liv}}{K_{I,2-1}}\right)}$	$\frac{V_{max_1} \cdot C_{1,v,liv}}{Km_2 + C_{1,v,liv} \cdot \left(1 + \frac{C_{2,v,liv}}{K_{I,1-2}}\right)}$

Based on Dobrev *et al.*, 2001.

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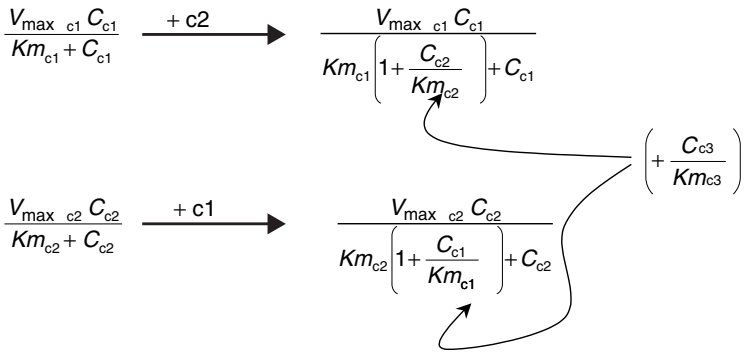

Figure 6 Predictability of the effect of chemical c3 on the interacting binary pair of c1 and c2. These chemicals are substrates of the same enzyme and are assumed to compete for metabolism. V_{max} refers to the maximal velocity for biotransformation; Km refers to the Michaelis-Menten affinity constant; C terms are concentrations of unchanged chemicals in the venous blood leaving the liver. The equations on the left-hand side of the thick arrows represent metabolism of chemicals c1 and c2 when they are present alone, and on the right-hand side represent the modification of the rate of their metabolism in the presence of an inhibitor (chemical c2 or c1). With the addition of chemical c3 to the binary mixture of chemicals c1 and c2, all one has to do is to include the binary-level interaction constants representing metabolic inhibition between chemical c3 and chemical c1, and chemical c3 and chemical c2 in the PBPK model. In this specific example, for the sake of simplicity, the inhibition constants are set equal to the Km of the inhibitor.

metric') following mixed chemical exposures. A change in the level of the tissue dose in certain cases leads to a corresponding change in toxicity. For example, as shown in the dose response curve in **Figure 7A**, an increase in tissue dose of a chemical during mixed exposures leads to an increase in the toxicity. Here, the tissue dose per unit exposure concentration is altered and the resulting increase in tissue dose is significant enough to enhance the toxicity of the chemical during mixed exposures. On the other hand, if the change in tissue dose occurs only to a smaller extent and within the threshold region of the chemical(s), then a change in tissue dose does not translate to a change in toxicity (**Figure 7B**).

Based on the tissue dose vs response relationship shown in **Figure 7A**, for genotoxic carcinogens, the probability of excess cancer in a population [P(d)] exposed to a mixture can be calculated as:

$$P(d) = q^*_{\text{tissue}} d_{\text{tissue}} \tag{4}$$

where q^*_{tissue} = the tissue dose-based cancer slope factor for each of the mixture components (1,2, . . . ,n), and d_{tissue} refers to the tissue dose of each mixture component simulated using an interaction-based PBPK model.

The application of the above approach to the dose-response assessment of chemical mixtures is illustrated in **Table 6**. In this case, using the mixture PBPK model that takes into account the interactions among the components, the change in tissue dose of the toxic moiety of DCM [i.e. the glutathione (GSH) conjugate] was simulated for various mixed exposure scenarios. The tissue dose, as well as the cancer risk attributed to DCM during mixed exposure (in comparison with single chemical exposures) increase as the interactions cause a decrease in the flux of this chemical through the CYP450 pathway (due to competitive inhibition of metabolism) combined with a proportional increase in the flux through the GSH pathway. The latter metabolic pathway is thought to be responsible for the carcinogenicity of DCM (Andersen *et al.*, 1991).

For systemic toxicants affecting the same target organ, the tissue dose-based dose-response analysis is conducted as follows:

$$\text{HI} = \sum_{i=1}^{n} \frac{C_{\text{tissue,exp},i}}{C_{\text{tissue,ref},i}} \tag{5}$$

where $C_{\text{tissue,exp},i}$ refers to the tissue concentration of the

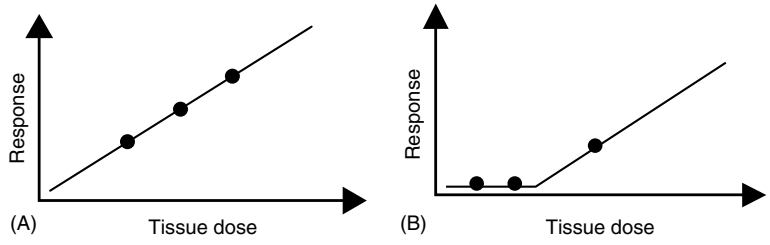

Figure 7 Dose-response relationships between tissue dose and tissue response, without a threshold (A) and with a threshold (B). (Based on Krishnan *et al.*, 2002.)

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Table 6 PBPK model simulations of tissue dose and risk level of inhaled dichloromethane (DCM) in humans during coexposure to benzene (BEN), toluene (TOL), ethylbenzene (EBZ) and *m*-xylene (*m*-XYL)

Exposure concentration (ppm)					Change in risk level
DCM	BEN	TOL	EBZ	m-XYL	for DCM
50	0.5	50	100	100	4.04
25	0.5	25	50	50	3.59
12.5	0.5	12.5	25	25	2.57
20	0.5	10	10	10	1.74
10	0.5	10	10	10	1.72

Based on Haddad *et al.*, 2001.

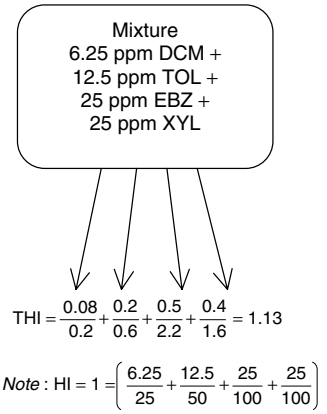

Figure 8 Comparison of the tissue dose-based hazard index (THI) with the conventional hazard index (HI) for a chemical mixture. The data for THI were obtained using the simulations of an interaction-PBPK model for mixtures. (Based on Haddad *et al.*, 2001.)

dose surrogate of component i predicted using a PBPK model that accounts for multiple interactions occurring in the mixture, and $C_{\text{tissue,ref},i}$ is the tissue concentration of the dose surrogate of component i corresponding to its maximum allowable concentration.

The PBPK-based mixture dose-response assessment for systemic effects [e.g. central nervous system (CNS) effects] is illustrated in **Figure 8**. As seen in this figure, for the mixture of DCM, benzene (BEN), TOL, EBZ and *m*-XYL, the tissue dose-based hazard index (THI) is greater than the HI, which does not take into account the metabolic interaction and its impact on the tissue dose of the components.

6 CONCLUDING REMARKS

The dose-response assessment for chemical mixtures and their components can be improved by developing integrated pharmacokinetic/PD models, even though such models are only beginning to be developed. Recently, Timchalk and Poet (2008) developed a PBPK/PD model for the mixture of chlorpyriphos and diazinon, by taking into account the mechanisms of toxicokinetics, toxicodynamics (cholinesterase inhibition), as well as the metabolic interaction between these chemicals. The PBPK/PD model was then able to simulate the cholinesterase inhibition as a function of the dose of the mixture components, thus facilitating the construction of a dose-response relationship for this mixture. Mechanistic models of receptor interactions at various levels—binding, receptor activation, as well as signal transduction—have been developed and applied for the analysis of combined drug responses (Jonker *et al.*, 2005), but not for the dose-response assessment of mixtures of environmental contaminants as yet. With the on-going research in the areas of omics, molecular toxicology and computational toxicology, it will increasingly become feasible to construct/simulate dose-response relationships for chemical mixtures as a function of the pharmacokinetic and PD interactions, as well as the concentrations of the mixture components in the human microenvironment.

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Toxicology of Chemical Mixtures

Kannan Krishnan and Jonathan Boyd

CONTENTS

1 INTRODUCTION

Multichemical exposure is the rule rather than the exception in both the general and occupational environments. Even though toxicity testing protocols and health risk assessment methods applicable to single chemicals are fairly well developed, it is not the case for chemical mixtures. The fundamental additional considerations in mixture toxicology relate to the concept of additivity and the possible occurrence of interactions. The toxicity of chemicals in mixtures might be altered due to interactions occurring in the exposure, toxicokinetic and/or toxicodynamic phases (see **Evaluation of Toxicological Interactions for the Dose-Response Assessment of Chemical Mixtures**). The exposure-phase interactions may be dealt with by quantifying the parent chemicals and the reaction products in a mixture (Krishnan and Brodeur, 1991).

Toxicity assessment may be more complicated if interactions occur among the components of the mixture at the toxicokinetic or toxicodynamic level (Calabrese, 1991; Krishnan and Brodeur, 1991). Even though data on the occurrence of toxicokinetic and toxicodynamic interactions are scanty, some evidence of their occurrence in humans is available (Krishnan and Brodeur, 1994). For example, joint toxicity of sulfur dioxide and ammoniacal compounds is thought to have been encountered during the London fog disaster of 1952 (Waldbott, 1973); the increased joint toxicity of sulfur dioxide and ozone was a likely causative factor of the high mortality of Japanese children observed in the early 1970 s (Bates and Hazucha, 1974); isomalathion contributed to enhance the cholinergic effects observed in 1976 in Pakistani spraymen handling malathion (Baker *et al*., 1978); and the attenuation of the toxic effects of mercury in a Japanese population was attributed to the simultaneous exposure to elevated levels of selenium (Suzuki *et al*., 1980). Other examples of interactions among essential and nonessential elements characterized in humans include: zinc and lead, as well as iron and mercury, cadmium or lead (reviewed in Krishnan and Brodeur, 1991; Calabrese, 1991). In most cases, however, there is a lack of toxicity data (i.e. the target organ as well as the dose–response information) for the mixture of concern or there is insufficient information on the components and interactions among them.

The purpose of this chapter is to describe the concepts and methods for evaluating the toxicity of chemical mixtures. This chapter is organized in terms of the assessment of whole mixtures, similar mixtures or mixture components.

2 TOXICITY ASSESSMENT OF CHEMICAL MIXTURES

The toxicity of chemical mixtures is evaluated using integrative, dissective or synthetic strategies (Mauderly, 1993; Eide, 1996). The integrative studies focus on evaluating the mixture as a whole, whereas the synthetic approach evaluates the toxicity and interactions among components. The integrative and synthetic approaches are also referred to as 'top-down' and 'bottom-up', respectively, in mixture toxicology. The dissective

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approach, on the other hand, involves the reconstruction of the toxicity of the whole mixture on the basis of toxicological evaluation of selected submixtures. In this regard, various fractionation approaches are useful for regrouping mixture constituents for further characterization of their biological activity (Mauderly, 1993; Eide, 1996).

Frequently, the purpose of mixture toxicity studies is to address one or more of the following questions:

- What are the critical effects and toxicity benchmarks (no-observed-adverse-effect level (NOAEL), lowest-observed-adverse-effect level (LOAEL)) associated with a given chemical mixture?
- How does the toxicity of one mixture (e.g. cancer slope factor) compare with another similar mixture?
- How does the whole mixture toxicity compare with the toxicity of individual chemicals or submixtures?

These concerns can be addressed using whole mixture studies, comparative potency studies and component or fraction-based studies, as discussed in the following sections.

3 TOXICITY ASSESSMENT OF WHOLE MIXTURES

The toxicity evaluations of whole mixtures focus on identifying the target organ(s) and characterizing the dose–response relationship in the target or test species. Here, the mixture is considered as a single entity; thus preparations are made taking into account the absolute and relative concentrations of the various components, their miscibility and volatility, among other factors (Yang and Rauckman, 1987). The challenges, study design and approaches for identifying critical effects and toxicity benchmarks (NOAEL, LOAEL) using whole mixtures are essentially the same as for any single chemical. Since the 'whole mixture approach' allows qualitative and quantitative evaluations of the toxicity of the mixture in consideration, there is no need to guess or judge the pertinence of data on interaction between components. The major advantage is that the dose–response data generated using the whole mixture are directly relevant for risk assessment purposes even if the composition of the mixture is not known completely, as long as it is the same for the mixture of concern and the mixture used in toxicity tests (US EPA (Environmental Protection Agency), 1997). For instance, an assessment of the toxicity of commercial hexane, which contains 52% *n*-hexane and 48% of other materials (3-methylpentane, methylcyclopentane, 2-methylpentane, cyclohexane, 2,3-dimethylbutane, 2,2-dimethylpentane, 2,4-dimethylpentane, 2,2-dimethylbutane, 2,2,2-trimethylbutane and 2-methylhexane) was conducted by Daughtrey *et al.* (1999). For this purpose, groups of animals (50 per sex) were exposed to 0, 900, 3000 and 9000 ppm of the mixture, to identify the chronic effects. Clearly, in this case the assessment of the whole mixture (commercial hexane) provided the most direct information on the toxicity of this mixture and it did not require assumptions regarding interactions among components. Another example of such data collection relates to observations made in a cohort of workers exposed to various risk factors simultaneously (e.g. Rabinowitz *et al.*, 2008). The extent to which these data can be extrapolated to other situations (i.e. high dose to low dose, interspecies and interindividual) is not known. This source of uncertainty is of concern because the extent and magnitude of interactions might vary between the doses of mixtures evaluated experimentally and the dose levels of relevance to the end user (e.g. occupational exposures vs. environmental exposures).

Even though the whole mixture approach is applicable for uncharacterized or partially characterized mixtures whose composition is stable and does not vary with time and space, the dose–response data required even for a single whole mixture can be time and resource consuming. The number of end points to be investigated in such studies can be unrealistically large (e.g. haematotoxicity, hepatotoxicity, developmental toxicity) and would be difficult to narrow down without some relevant knowledge of mixture components.

Cancer slope factors for whole mixtures may also be determined using this approach. When such data are available for mixtures of interest, they can be of use only if the identity of mixture components, their proportions and concentrations do not change with time and space. This limitation is highlighted with the cancer slope factors for polychlorinated biphenyl (PCB) mixtures. The cancer slope factors for three PCB mixtures were reported to range from 0.07 to 2.0 mg/kg(day)$^{-1}$ (Warren *et al.*, 2004). Therefore, it would appear that the cancer slope factor for Aroclor-1268 could not be assumed to be the same as that of another PCB mixture, namely Aroclor-1254 (Warren *et al.*, 2004). In such cases, an analysis of the similarity of mixtures should be undertaken.

4 TOXICITY ASSESSMENT BASED ON SIMILAR MIXTURES

A relative potency determination or comparative assessment of responses obtained in laboratory assays may be conducted for mixtures that are considered to be similar based on chemical composition, mode of action or toxicity end point (US EPA (Environmental Protection Agency), 2000). Let's assume that A is the mixture of concern, whereas the toxicity benchmark

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for another similar mixture B has already been characterized. The human dose–response or toxicity information for mixture A can then be evaluated by making use of the dose–response information for mixture B in a parallelogram approach, in which the human dose–responses are assumed to be related in the same way as the responses in *in vitro* or short-term assays for both mixtures (Lewtas, 1985; Schoeny and Margosches, 1989). Accordingly, the relative potency of the two mixtures in a particular assay (e.g. mouse skin tumour initiation assay) is used in conjunction with the human risk estimates for the 'similar' mixture, to solve for the unknown (i.e. the human risk estimate for the mixture in hand), as follows:

$$\frac{R_A}{P_A} = \frac{R_B}{P_B} \quad (1)$$

where R_A and R_B refer to human risk estimates for mixtures A and B, and P_A and P_B are the potencies of mixtures A and B in a particular bioassay.

Additionally,

$$RP_1 = k(RP_2) \quad (2)$$

where RP_1 and RP_2 are the relative potencies of a mixture in assays 1 and 2, and k is a constant (Lewtas, 1983).

The above approach has been applied for deriving cancer risk estimates for humans based on short-term studies with roofing tar and diesel exhaust mixtures in comparison with the coke oven emissions (Lewtas, 1983). Since this approach treats the complex mixture as a single entity, potential toxicological interactions occurring among components are accounted for. Conceptually then, the comparative potency approach assumes that for similar, but not necessarily defined, complex mixtures, a measure of comparative potency based on results of *in vitro* or short-term bioassays can be correlated in a constant manner with human data. The comparative potency approach requires that human data be available for a similar mixture and this requirement seriously limits the applicability of this methodology. The assumption that the human responses for an uncharacterized mixture and a similar, characterized mixture are related in the same manner as they would be in a rodent/*in vitro* bioassay may not be true in all instances. In this regard, the *in vitro/in vivo* comparisons of dose–response behaviour are empirical, since the tissue dose of the toxic moiety and the extent of interactions might not be identical in both systems. Additionally, the comparative evaluation procedure applied for mixtures is end-point driven (e.g. genetic toxicity), and as such it only provides a biased and not a complete appraisal of the potential spectrum of critical toxic effects associated with a given mixture. This problem can be resolved with knowledge of the composition of mixtures and the target organ toxicity of components.

5 COMPONENT-BASED ASSESSMENT OF MIXTURE TOXICITY

5.1 Evaluation of the Joint Toxicity of Similarly-Acting Mixture Components

The component-based approaches focus on evaluating the relative or absolute toxicity of mixture constituents, to eventually predict the toxicity of the whole mixture. In the case of similarly acting systemic toxicants, the reconstruction of the toxicity of mixtures is frequently based on (i) dose addition, or (ii) toxic equivalency factor (TEF) approaches. Where the components of a mixture are isomers or congeners of the same chemical, or the components act by a common mechanism, the TEF approach has been used for evaluating the mixture toxicity (US EPA (Environmental Protection Agency), 2000). Dose addition assumes that the constituents of a chemical mixture behave as if they are dilutions of each other (Guidelines for the Health Risk Assessment of Chemical Mixtures, 1986; Calabrese, 1991; Mumtaz *et al*., 1993; Seed *et al*., 1995; Teuschler and Hertzberg, 1995; Hertzberg *et al*., 1998). The basis here, then, is that the true dose–response curve of each of the mixture constituents for a common end point may be different, but the slopes of these curves would be identical if their dose levels were adjusted for potency differences (**Figure 1**). Even though the dose-addition approach is applied for assessing the toxicity of mixtures of systemic toxicants (e.g. organophosphorus pesticides) that exhibit common mechanism of action, recent observations question such a narrow definition of the application domain. In fact, the study by Rider *et al*. (2008) suggests that chemicals acting on the same tissue during a critical period induce

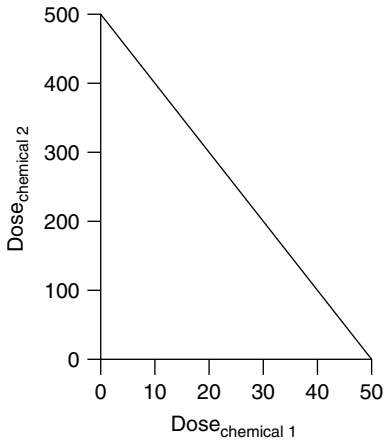

Figure 1 Illustration of the combinations of two chemicals (along the solid line) yielding the same level of response. Notice the difference in potency of the chemicals (Y axis vs. X axis; dose in arbitrary units).

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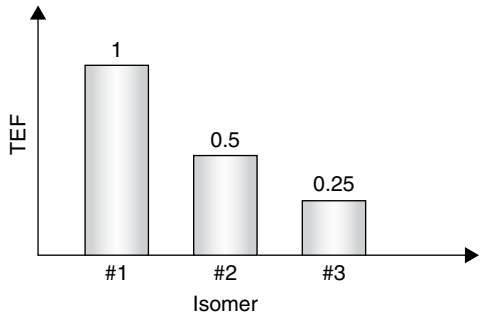

Figure 2 The toxic equivalency factor (TEF) for a reference isomer (#1) as well as for other less potent chemicals in a mixture (#2, #3).

dose additivity even though they act by different molecular mechanisms.

The dose addition approach is based on the premise that the slopes of the dose–response curves for mixture components would be identical if their dose levels are adjusted for potency differences. The potency difference correction for the dose addition approach might be improved if this was done on the basis of the target tissue dose of individual chemicals (Haddad *et al*., 2001) but this has not been done routinely.

When the components of a mixture are congeners or isomers of a chemical, the dose of (and thereby the toxicity associated with) the whole mixture has been computed on the basis of the product of isomer-specific environmental concentration and the TEF for each isomer (Safe, 1994; 1997). The TEF is the ratio of the potency of a given isomer to the 'signature or reference' isomer in a relevant toxicological assay (Barnes *et al*., 1991; Ahlborg *et al*., 1994; Petry *et al*., 1996) (**Figure 2**). The reference isomer is normally the most potent, and as such the concentrations of other isomers in the mixture are adjusted according to potency differences. This procedure then converts all isomers into the equivalents of the most potent isomer (Eadon *et al*., 1986; Barnes *et al*., 1991; Ahlborg *et al*., 1994; Petry *et al*., 1996), and sums them to get an equivalent total dose in terms of the most potent isomer, as follows:

$$TEQ = \sum_{i=1}^{n} C_i TEF_i \qquad (3)$$

where

TEQ = toxic equivalency of a mixture in terms of the most potent isomer,
TEF_i = toxic equivalency factor of isomer i with reference to the most potent isomer, and
C_i = concentration of isomer i in the mixture.

This TEQ is then used along with the dose–response data for the reference isomer to derive estimates of risk associated with the mixture (Guidelines for the Health Risk Assessment of Chemical Mixtures, 1986; US EPA (Environmental Protection Agency), 2000). A key assumption of this approach is that the toxicity induced by each of the various isomers in the mixture is qualitatively similar to that of the 'reference' isomer, even though the potency may vary. The equivalents of each isomer estimated in terms of the 'reference' isomer may be considered as being toxicologically equipotent to the 'reference' isomer. Thus, product of TEFs and exposure concentrations of all isomers/congeners in a mixture are then summed up, assuming dose additivity. TEFs for dioxins and dioxin-like PCBs have been derived (e.g. van den Berg *et al*., 1998). Accordingly, the TEF for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is 1, whereas that for 2,3,7,8-tetrachlorodibenzofuran (TCDF) and 1,2,3,4,6,7,8-heptachlorodibenzofuran (HCDF) respectively it is 0.1 and 0.01. In other words, exposure to TCDF is similar to being exposed to a tenth of the dose of TCDD, when the exposure doses are equal; this is due to the difference in potency; that is, the chemicals are not equipotent regarding their ability to induce a particular toxic effect or perturbation of a biological process, but rather proportionally potent.

The TEF method does not take into account the possible interactions between the isomers/congeners constituting a mixture. There are a number of examples demonstrating or suggesting the occurrence of competitive interactions between structurally similar chemicals both at the toxicokinetic and toxicodynamic levels (Chen *et al*., 1991; Rao and Unger, 1995a; 1995b; Wölfle, 1997).

The TEF approach also assumes that the isomers act by a similar mechanism contributing to the induction of the same critical effect. This is not necessarily true; and as an extension of this argument it may be suggested that the most potent isomer in one bioassay may not be so with respect to another end point/assay. Therefore, the conceptual basis of this method to enable the consideration of multiple end points in deriving the TEFs should be carefully evaluated (Ahlborg *et al*., 1994). A detailed treatment of the theoretical basis of the TEF approach and limitations can be found in Neumann (1996), Putzrath (1997) and Toyoshiba *et al*. (2004).

5.2 Evaluation of the Joint Toxicity of Dissimilarly-Acting Chemicals

The component-based approach for mixtures of dissimilarly acting chemicals (i.e. independent mode of action) involves the summation of the responses due to each one of them, accounting for the level of independence (Cassee *et al*., 1998). The most conservative form of

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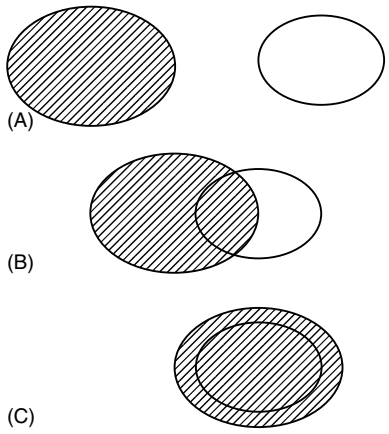

Figure 3 Illustration of the statistical concept of varying levels of independence between two events (or two chemicals in a mixture).

independent action assumes mutual exclusiveness for the events associated with chemical A and chemical B; that is, that the individuals most susceptible to chemical A will not be susceptible to chemical B and vice versa. Therefore, the response from mixed exposure to A and B will be equal to the sum of the probability of the individual events, that is, addition of the response due to A and B determined individually assuming no overlap in terms of susceptibility or occurrence of the events (**Figure 3A**). When the population susceptible to chemical A is also susceptible to chemical B, the susceptibilities are 'correlated positively'. In such cases, the proportion of individuals responding to the mixture AB will be equal to the response of the most toxic agent (**Figure 3C**) (Cassee *et al*., 1998). The intermediary scenario is one where the adverse events associated with the mixture components are not mutually exclusive (**Figure 3B**). Here, in statistical terms, one event has no effect on the occurrence or not of the other event except that both are independent. Thus, the mixture toxicity is calculated as the sum of the responses due to component 1 and component 2 minus the area of overlap (i.e. P(A) × P(B)) (**Figure 3B**).

Based on this theory of response addition and assumption of complete independence, the probability of excess cancer incidence in a given population is estimated by summing up the product of actual or projected environmental concentration (E) and the cancer slope (q*) for each of the mixture components (Guidelines for the Health Risk Assessment of Chemical Mixtures, 1986). The response addition approach so far has only been applied for the assessment of the potential health risk associated with mixtures of chemical carcinogens. Assuming additivity of responses associated with multiple chemical carcinogens is reasonable, since some (genotoxic) carcinogens are believed to act by nonthreshold mechanisms. Further, the assumption of the independence of mechanisms may be reasonable based on the premise that the greater the number of carcinogens in a mixture, the greater the probability that the mechanisms involved are dissimilar (Calabrese, 1991). However, summing the individual estimates of upper bound risks for components has been questioned because it overestimates the true upper bound. More realistic approaches that account for the possibility that all components are not likely to be present at their maximal risk level simultaneously have been described (Chen *et al*., 1991; Kodell and Chen, 1994).

As with the dose addition approach, there is a concern related to the assumption of absence of supra- and infra-additive interactions among mixture components. This assumption may not always be justified, as suggested by the available experimental data on interactions among chemical carcinogens (Rao *et al*., 1989).

Further, the response addition methodology is currently used for mixtures of substances inducing cancer in different tissues and/or via different routes of exposure. This practice involves assignment of equal 'biological' weight regardless of the site-specificity of the tumour induced by mixture components. It is well known that the 'adverse' nature of tumours is also related to the anatomical site of their location. An improved method would be to use tissue dose measures specific for each exposure route (obtained with mechanistic pharmacokinetic models) rather than exposure concentrations in the mixture risk calculation (Haddad *et al*., 2001). This methodology can also facilitate the consideration of change in tissue dose due to interactions among mixture components (see **Evaluation of Toxicological Interactions for the Dose-Response Assessment of Chemical Mixtures**).

5.3 Evaluation of Departure from Additivity

The assessment of the direction and magnitude of interactions among mixture components for various exposure conditions is a challenge. Interactions at the metabolism level have been observed in human volunteer or occupational exposure studies for the following solvent combinations: trichloroethylene and 1,1,1-trichloroethane (Pfaffli and Backman, 1972), benzene and toluene (Inoue *et al*., 1988a), ethylbenzene and *m*-xylene (Angerer and Lehnert, 1979), xylene and toluene (Tardif *et al*., 1991), trichloroethylene and tetrachloroethylene (Inoue *et al*., 1988b), as well as *m*-xylene and methyl ethyl ketone (Liira *et al*., 1988). These metabolic interactions, essentially competitive in nature, lead to reduced/delayed production and excretion of metabolites, as well as, in some cases, increased concentrations of unchanged parent chemical(s) in the blood and alveolar air. Such interactions are expected to result in supra-additive toxicity if the parent chemical is the toxic moiety. Accordingly, supra-additive toxicity during exposure

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to binary mixtures of solvents has been observed in animal studies, whenever one of the two solvents was a potent inducer of activating enzymes, or an inhibitor of detoxication enzyme system (Pass *et al*., 1985; Brady *et al*., 1989). Thus, solvents inducing hepatic CYP2E1 (cytochrome P450 2E1) upon prior administration (e.g. ethanol, isopropanol, ketones) have been shown to increase the toxicity of other solvents bioactivated by the same isoenzyme. In fact, the toxic interaction between isopropanol and carbon tetrachloride (mediated by CYP2E1) is thought to be responsible for the hepatorenal toxicity associated with two separate industrial accidents (Folland *et al*., 1976; Deng *et al*., 1987). Similarly, the outbreak of an occupational neuropathy among the textile workers in Ohio (Billmaier *et al*., 1974; Allen *et al*., 1975) and among glue sniffers in West Berlin in the 1970s (Altenkirch, 1979) were attributed to the interactive neurotoxicity involving methyl ethyl ketone and *n*-hexane/methyl *n*-butyl ketone (MnBK). However, the toxicological consequences of all metabolic interactions have not been confirmed in occupational monitoring studies (Olson *et al*., 1985; Yin *et al*., 1987).

Some of the confusion in mixture toxicology literature relates to what constitutes interaction. Generally, the term 'interaction' in mixture toxicology is used to refer to departure from additivity; however, investigators often fail to provide the definition of additivity. As discussed in the preceding section, additivity is based either on the dose or response—depending upon whether the mixture components act by similar action or dissimilar (independent) action. Comparing the toxicity of a mixture with that of its components requires an appropriate statistical model for additivity (Bliss, 1939; Finney, 1952). In the scientific literature, the generic terms such as 'infra-additive' and 'supra-additive' are increasingly being used to describe toxicological interactions. The infra-additive interactions (commonly referred to as antagonism) are said to result when the observed toxicity is less than that expected based on the addition of the dose or response of the components. Supra-additive interactions (commonly referred to as potentiation or synergism) are said to occur when the toxicity of the mixture is greater than that of its components evaluated individually (**Figure 4**).

In the case of similarly acting chemicals then, the departure from additivity can be identified as follows (Finney, 1952; Smyth *et al*., 1969):

$$\frac{1}{LD_{50_{\text{mixture}}}} = \frac{P_A}{LD_{50_{\text{component A}}}} + \frac{P_B}{LD_{50_{\text{component B}}}} \qquad (4)$$

where P_A and P_B represent the proportion of A and B in the mixture.

Here the left side of the equation represents the outcome associated with the mixture, whereas the right-hand side relates to the components taken individually. When both sides are equal (allowing for the variability and uncertainty associated with the measurements), it is considered that the mixture toxicity is additive and that interactions would not appear to contribute to change the potency of the components or the mixture as a whole. On the other hand, when the outcome for the tested mixture is greater or less than that of the sum of the contributions of individual components, then infra-additive or supra-additive interactions are said to occur.

The above concept can be understood by comparing the experimental data on the median lethal dose (LD_{50}) of mixtures and components of some organic solvents evaluated by Smyth *et al*. (1969; 1970). In the case of acetonitrile and butylether, the mixture LD_{50} is 10.43 ml kg^{-1}, whereas the LD_{50}s of the individual chemicals are 8.27 and 14.1 ml kg^{-1} for acetonitrile and butylether, respectively. Here 1/10.43 would equal the sum of 0.5/8.27 and 0.5/14.1, indicating that the 50 : 50 mixture of acetonitrile and butylether is additive. Alternately, the mixture of acetonitrile and 1,4-dioxane is supra-additive because the LD_{50} of the mixture is lower than that predicted on the basis of additivity. Thus, the LD_{50} for the mixture was 2.42 ml kg^{-1}, whereas the LD_{50}s for the individual

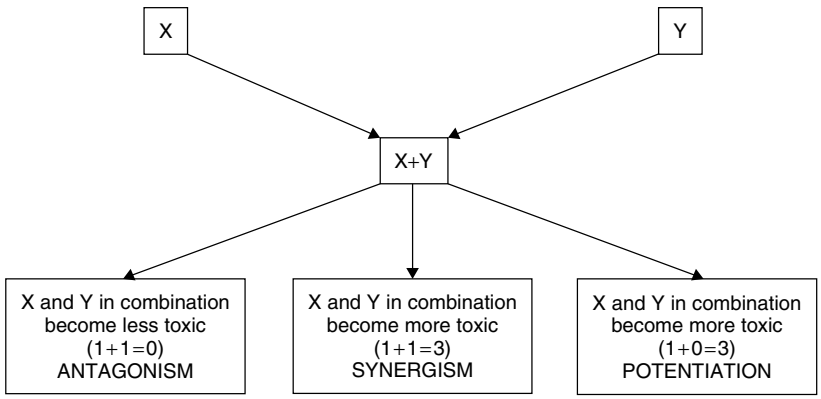

Figure 4 Illustration of the types of interactions (i.e. departure from additivity) occurring during combined exposure to chemicals X and Y.

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Table 1 Toxic interactions among environmental pollutants

End point	Supra-additivity	Infra-additivity
Hepatotoxicity	Carbon tetrachloride + trichloroethylene	Carbon disulfide + chloroform
Haematotoxicity	Carbon monoxide + nitric oxide	Lead + iron
Immunotoxicity	PCBs/dioxins	Benzene + toluene
Irritation	Sulfur dioxide + manganese dioxide	Acrolein + sulfur dioxide
Neurotoxicity	Methyl ethyl ketone + methyl *n*-butyl ketone	Toluene + *n*-hexane
Nephrotoxicity	Dimethyl aniline + methyl *iso*-butyl ketone	Cadmium + mercury
Pneumotoxicity	Sulfuric acid + nitrogen dioxide	Formaldehyde + ozone
Reproductive toxicity	Lead + cadmium	Cadmium + selenium
Teratogenicity	Lead + cadmium	Carbon monoxide + sulfur dioxide

Based on Krishnan and Brodeur (1991).

chemicals were 8.27 and 7.07 ml kg^{-1}, respectively, for acetonitrile and dioxane. Here, the experimental LD_{50} for the mixture is about one-third of the expected value based on additivity, indicating supra-additive joint toxicity of acetonitrile and dioxane in the rat (Smyth *et al*., 1970).

In the case of dissimilarly acting chemicals, the outcome (response) associated with the mixture exposure is compared with the sum of the responses due to individual components, assessed separately. When the mixture response is greater (lower) than the sum of the responses of the components, then supra-additivity (infra-additivity) has resulted. For example, if the individual responses due to components A and B are 5 units each and the mixture (A + B) response is 15 units, then this is evidence of synergism. Note that both mixture components (A, B) are toxicologically active in this case. On the contrary, if one of the components is inactive (i.e. does not induce measurable responses) and the combined response is greater than the sum of the individual responses, then such a departure from additivity is referred to as potentiation. For example, if chemical A induces 5 units of response whereas chemical B causes no measurable change in response but the combined exposure to A and B yields 20 units of response, then *potentiation* is said to have occurred. Here chemical B potentiates the toxicity of chemical A during mixed exposures. **Table 1** lists a number of interactions identified to occur among environmental pollutants.

In order to facilitate the identification of the occurrence of interactions, or verify whether the components of a mixture in fact follow additivity, a number of experimental designs are used (factorial design, ray design, central composite design and simplex design) and the results analysed using a statistical method (e.g. isobole method, response surface analysis), which essentially facilitate the definition of the boundary of additivity (Carter and Carchman 1988; Greco *et al*., 1995; Henchler *et al*., 1996; Cassee *et al*., 1998; McConkey *et al*., 2000). The full factorial design, even though representing the most comprehensive way of evaluating chemicals individually as well as in all possible combinations (Narotsky *et al*., 1995), does become cumbersome and unmanageable with increasing numbers of mixture components (**Figure 5**).

Despite the importance of the *in vivo* studies in characterizing the toxicity of mixtures and interactions, there is greater potential for *in vitro* studies to contribute to an understanding of not only the mode of action of chemical mixtures but also the occurrence of interactions (i.e. deviation from additivity) at the mechanistic level. As emphasized in the recent National Academy of Sciences (NAS) report on toxicity testing in the twenty-first century, the focus of the toxicity studies with mixtures, as with single chemicals, would need to shift from adverse effects seen at high doses to the characterization of perturbations of biological pathways (National Academy of Sciences (NAS), 2007). In this regard, *in vitro* studies have a critical role of advancing the future of dose–response evaluation of mixtures. The following section illustrates the evaluation of biological perturbations by a mixture of three chemicals using an *in vitro* system.

5.4 *In Vitro* Assessment of Mixture Toxicity: a Case Study

Rotenone, deguelin and pyridaben are natural and synthetic inhibitors of complex I of the electron transport chain (ETC), a well-studied pathway critical for maintaining cellular health. These inhibitors interfere with ubiquinone reduction and not only act upon the same enzymatic subunit within the ETC pathway, but have also been shown to interact at the same binding site (Schuler and Casida, 2001; Ino *et al*., 2003), suggesting that the mixture might follow dose addition. Beyond their central role in cellular respiration, mitochondria are key players in apoptosis, generation of reactive nitrogen and oxygen species, transduction of electrical signals, and calcium homeostasis (Skulachev, 2001; Droge, 2002; Balaban *et al*., 2005; Rizzuto and Pozzan, 2006). Thus, it is no surprise that, while complex I inhibitors are known to interrupt proton translocation at the inner mitochondrial membrane, they have also been shown

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Number of mixture components (*n*)	Complexity of the combinations (*c*)										Total
	1	2	3	4	5	6	7	8	9	10	
1	1										1
2	2	1									3
3	3	3	1								7
4	4	6	4	1							15
5	5	10	10	5	1						31
6	6	15	20	15	6	1					63
7	7	21	35	35	21	7	1				127
8	8	28	56	70	56	28	8	1			255
9	9	36	84	126	126	84	36	9	1		511
10	10	45	120	210	252	210	120	45	10	1	1023

Figure 5 The number of mixture components (*n*) vs. number of submixtures (*c*) as a function of complexity.

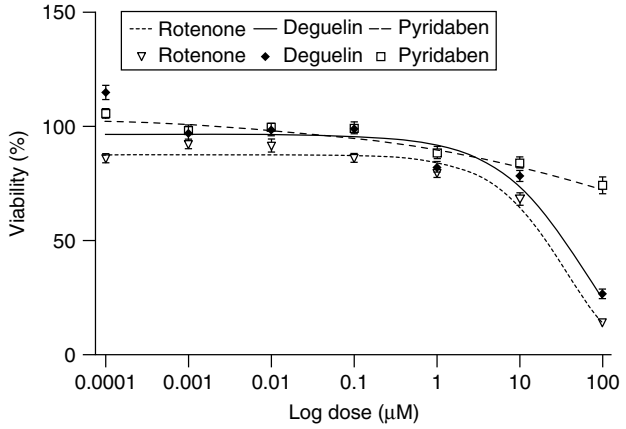

Figure 6 Dose–response of the individual complex I inhibitors: rotenone, deguelin and pyridaben. HepG2 cells were treated with individual inhibitors across a dose range and MTT analysis was performed. (Reproduced from Boyd *et al.*, 2008. © Oxford University Press.)

to lead to alterations in general cellular processes. For evaluation of the toxicity of mixtures of complex I inhibitors (rotenone, deguelin and pyridaben), HepG2 cells were exposed to them singly (0.001–100 μM) or as mixtures (total dose, 0.001–100 μM) for 24 hours, and cell viability was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Boyd *et al.*, 2008). Mixtures dosing included all binary and tertiary combinations of rotenone, deguelin and pyridaben. All mixtures dosing employed a fixed-ray design (Svendsgaar and Hertzberg, 1994) based upon the calculated LD_{50} values of the individual inhibitors. Briefly, the calculated LD_{50} for each inhibitor is used to determine the relative fraction of that component within a mixture that is expected to yield an equipotent response. The mixture fraction of each individual component is then multiplied by the total dose to yield the concentration of the component at that dose. Preliminary LD_{50} values for the individual inhibitors were determined to be 67.19, 596.1 and 179.4 μM, for rotenone, deguelin and pyridaben, respectively. As an example of the dosing regime, the mixture fraction of rotenone and deguelin was calculated with each total dose (0.001–100 μM) being composed of 67.19/663.3 or 10.13% rotenone and 596.1/663.3 or 89.87% deguelin. This experimental design has been shown to be particularly well suited for side-by-side comparisons when analyzing multiple mixtures by both dose and response addition models (Payne *et al.*, 2000).

As shown in **Figure 6**, cytotoxicity, as deduced from MTT reduction, increased in a dose-dependent manner for each of the three inhibitors tested. Following 24 hour exposures, rotenone, deguelin and pyridaben reduced HepG2 viability to 14, 27 and 74%, respectively at the highest concentration examined (100 μM). At the lowest dose tested (0.0001 μM), deguelin and pyridaben both resulted in greater than 100% viability, indicating a possible hormetic response (**Figure 6**). Overall LD_{50} values for the individual inhibitors were calculated to be 20.76, 34.16 and 4610 μM for rotenone, deguelin and pyridaben, respectively. The best-fit slopes for the entire sample set were found to be -2.034 ± 0.1117 for rotenone, -1.739 ± 0.1492 for deguelin and -0.4394 ± 0.1435 for pyridaben (**Table 2**). Pairwise comparison with an F-test showed rotenone and deguelin to have similar slopes within error ($p = 0.1605$), but both were found to be significantly different from pyridaben ($p < 0.0001$).

To assess the overall predictive capability of the additivity models in these experiments, the dose–response curves were compared by F-test analysis of best-fit parameters for observed versus predictive values for mixtures (**Table 3**). The corresponding graphs of observations and their respective dose addition and response addition predictive curves are

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Table 2 Slope comparison of individual dose–response curves

Comparison	Slope ± SEM	Fit
Rotenone Deguelin	−2.034 ± 0.1117 −1.739 ± 0.1492	$p = 0.1605$
Rotenone Pyridaben	−2.034 ± 0.1117 −0.4394 ± 0.1435	$p < 0.0001$
Deguelin Pyridaben	−1.739 ± 0.1492 −0.4394 ± 0.1435	$p < 0.0001$
Rotenone Deguelin Pyridaben	−2.034 ± 0.1117 −1.739 ± 0.1492 −0.4394 ± 0.1435	$p < 0.0001$

Table 3 Comparison of best-fit parameters between observed and predicted dose-response curves

Mixture components	LD_{50} mixture fraction	LD_{50} F-value (p)	
		observed vs. dose-additive predicted	observed vs. response-additive predicted
Rotenone	0.1013	1.899 (0.2308)	1.629 (0.2794)
Deguelin	0.8987		
Rotenone	0.2725	11.51 (0.0067)	1.283 (0.3625)
Pyridaben	0.7275		
Deguelin	0.7687	12.00 (0.0060)	7.797 (0.0171)
Pyridaben	0.2313		
Rotenone	0.07973	21.27 (0.0013)	127.1 (<0.0001)
Deguelin	0.7074		
Pyridaben	0.2129		

shown in **Figures 7A–D** for the mixtures tested. Examination of the observed versus predicted values from the dose addition model resulted in significant differences ($p < 0.05$) between all combinations, except rotenone and deguelin (**Table 3**). The observed versus predicted values from the response addition model resulted in a significant difference ($p < 0.05$) only for the tertiary mixture (**Table 3**). Since the individual dose–response curves of rotenone and deguelin were found to be similar within error, it was expected that both dose addition and response addition would be predictive based upon relative similarity of all parameters. Overall, the analysis of the dose–response curves resulting from challenge with binary and tertiary mixtures revealed that the appropriate model may be inhibitor-specific: rotenone and deguelin may be dose additive, whereas mixtures with pyridaben appear to fit the response addition model (Boyd *et al*., 2008).

6 CONCLUDING REMARKS

The fundamental concern with both the component-based and mixture-based toxicity assessment relates to the lack of knowledge regarding the role and importance of toxic interactions among components. There are several examples of supra-additive and infra-additive interactions among environmental pollutants (Krishnan and Brodeur, 1991; Krishnan and Brodeur, 1994). When one component interferes with the toxicokinetics or toxicodynamics of another component in the mixture, the net result is a change in the magnitude of the toxicity of the mixture, that is, deviation from the summation of the toxicity of mixture components. Despite reports of this phenomenon in several situations where humans were exposed occupationally or accidentally (Krishnan and Brodeur, 1994), there has not been any progress until recently in developing methods that permit the consideration of animal or human data on interactions in the risk assessment of mixtures. Given the enormous resources essential to characterize interactions occurring at various doses, routes and exposure scenarios, pragmatic and predictive approaches should be explored.

A pragmatic experimental approach deals with the fractionation of the complex mixture with reference to the biological activity. Alternatively, testing of the top 10 chemicals of the mixture of concern may be undertaken to guide the process of toxicity evaluation (Cassee *et al*., 1998). The relevance of such observations for

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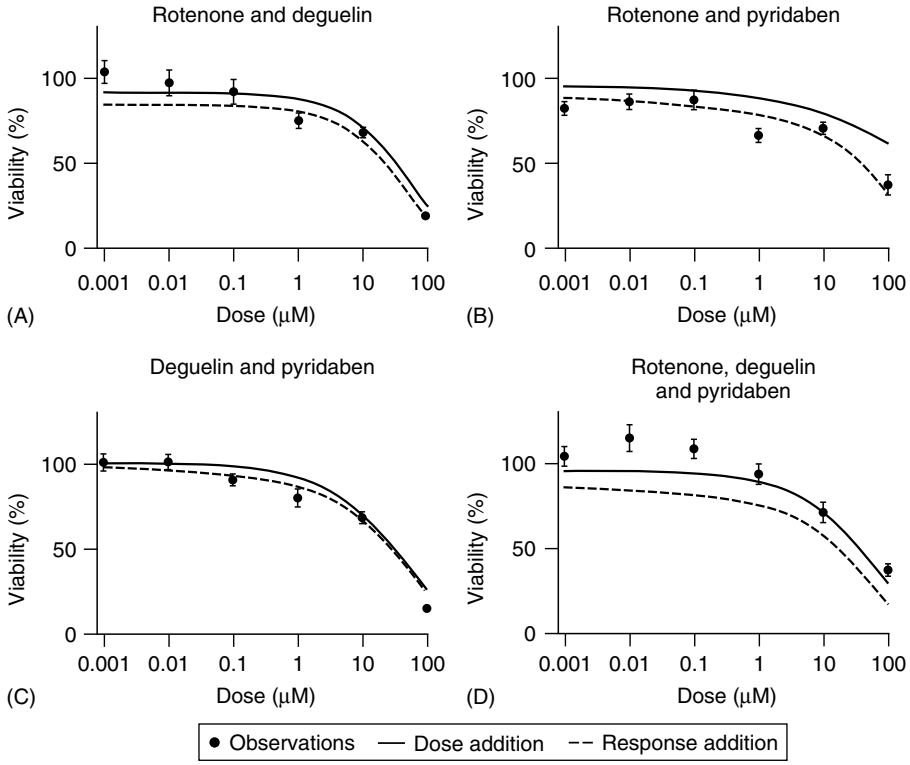

Figure 7 Dose response of a mixture of complex I inhibitors. HepG2 cells were dosed with mixtures of the complex I inhibitors rotenone, deguelin and pyridaben based upon calculated equipotent LD_{50} fractions. Filled circles indicate average observed data points and error bars represent the standard error of the mean (SEM). Solid lines indicate the predicted curve as calculated from the dose additivity model and dashed lines indicate the predicted curve as calculated from the response additivity model. (Reproduced from Boyd *et al.*, 2008. © Oxford University Press.)

humans exposed to low doses should be evaluated with physiologically based pharmacokinetic (PBPK) models (e.g. Haddad and Krishnan, 1998; Haddad *et al.*, 1998; Haddad *et al.*, 2001; Yang and Dennison, 2007). These models can help identify the threshold of interactions for mixtures of varying complexity, composition and dose levels (see **Evaluation of Toxicological Interactions for the Dose-Response Assessment of Chemical Mixtures**).

The wealth of information on joint action of chemicals has already been used to develop a web-based tool, for evaluating the potential additivity or interactions among components of mixtures (Vyskocil *et al.*, 2007). This web tool in English and French can be found at *www.irsst.qc.ca/en/_outil_100037.html*. This tool has primarily been developed for facilitating the assessment of joint toxicity of chemicals in the occupational environment but should be useful as a starting point to identify environmental mixtures with potential concerns of supra-additivity.

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Molecular and Cellular Concepts in Toxicology

Michael A. Lynes

CONTENTS

1 INTRODUCTION

Toxicant-induced changes at the molecular and cellular scale represent a microcosm of the events that occur at the tissue, organism and population levels of organization. These changes in cellular and molecular events propagate through the different levels of organization to disturb normal biological activities, and can initiate disease as a consequence. Not only does the understanding of these events enhance our ability to manage toxicant-induced diseases, but they can also be used to illuminate the normal functions of the affected biological systems. Toxic responses that occur as a consequence of exposures to environmental toxicants are largely viewed as undesirable, as are 'off-target' toxic effects of pharmaceuticals designed to modify some specific molecular target. On the other hand, there are contexts in which the toxic effect is the desired goal of the pharmaceutical, and selective toxic effects in those instances can have beneficial or therapeutic consequences. A thorough understanding of the changes that occur at the molecular and cellular levels enable both the management of biological consequences of environmental toxicant exposures, the therapeutic benefits of pharmacological toxicants and the prediction of toxic effects induced by new drugs and contaminants.

2 'THE DOSE MAKES THE POISON'

Paracelsus' (1493–1541) writings include the widely quoted, 'All things are poison and nothing is without poison, only the dose permits something not to be poisonous.' We can extend that idea by noting that toxicants are often reactive compounds that will interact with a wide array of targets in the body, and that the set of targets will be in part determined by the local concentration of the toxicant, the local concentration of relevant targets, and the delivery and processing of each of these components by the body. Often, toxicants will accumulate in specific organelles, cells, tissues and organs of the body, and thus will have their greatest effect at those locations. For example, lipid-soluble toxicants can accumulate in the liver, brain and adipose tissues.

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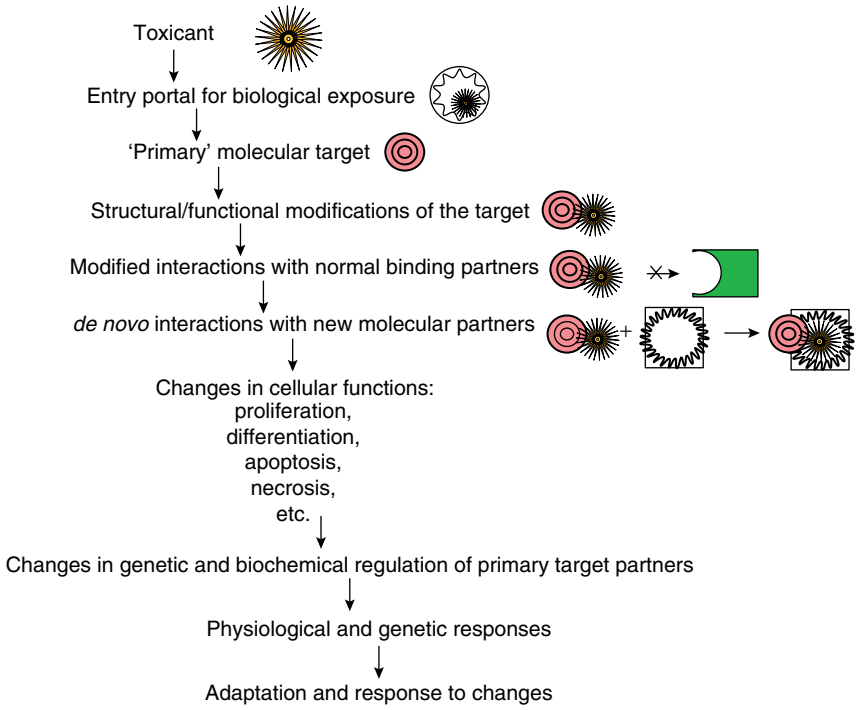

Figure 1 Initial molecular interactions with a primary biological target, and the cascade of molecular and cellular events that can ensue.

It is also true that the greatest effect may be observed where the most sensitive molecular target is at highest concentrations. Finally, it may be that the greatest cellular effects are observed in cells that are given the least time to effect repairs before autodeletion mechanisms are activated (e.g. apoptosis) to eliminate the danger posed by damaged cells. Thus, cells that have sustained toxicant-induced genetic damage and which replicate with high frequency (e.g. lymphocytes) are particularly prone to toxicant-induced death.

At the same time, the processing and bio-activation or detoxification may influence target tissues and the resulting biological effects of toxicants. For example, cadmium (Cd) initially accumulates in liver tissue (Habeebu *et al*., 1998) and can initiate the synthesis of the metal-binding protein, metallothionein (MT), while effecting hepatic cell survival and functioning (Klaassen and Liu, 1997). Subsequently, cadmium-metallothionein (Cd-MT) has been found in the circulation and appears to accumulate in the kidney. Recent reports suggest that megalin may be the membrane receptor that facilitates Cd-MT endocytosis at the apical side of kidney proximal tubule cells (Wolff *et al*., 2006; 2008). Once in the kidney, metal and metalloprotein can each have toxic effects on renal cells, though distinctions regarding the mechanisms of these effects have not yet been elucidated (Liu *et al*., 1999).

The consequences of toxicant exposure can thus be quite disseminated in the body, and overlapping timing in the occurrence of these events may make it difficult to identify a 'primary' molecular target of the toxicant (see **Figure 1**). Not only is the 'primary' molecular target altered by interactions with the toxicant, but toxicant exposure often initiates a cascade of parallel molecular events, and each of these changes may further influence the overall effect of the toxicant on cellular structure and function. Historically, the goal of many scientific investigations has been to identify the initial molecular change that initiates biological change, but recent technological advances may ultimately facilitate a systems-based approach to the understanding of these molecular changes.

3 BIOMOLECULAR SIGNATURES OF TOXICANT EXPOSURE AND THEIR DETECTION

Traditional histological and biochemical assays have long been used to identify the primary targets of toxicant exposure. These experimental approaches can yield considerable information, but are limited in the number of potential targets that can be examined in each experiment. Early large-scale investigations of the molecular changes associated with toxicant exposure often employed two-dimensional (2D) gel electrophoresis. For the most part, 2D gel electrophoresis simultaneously separates proteins in a heterogeneous mixture according to the isoelectric point and molecular weight of each

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protein in a 2D array. The separated proteins can be nonspecifically stained, and changes to specific protein spot location and size can be compared between samples that have been simultaneously separated in parallel gels. Because the electrophoretic separation of individual protein spots is very susceptible to subtle changes in the conditions of the experimental protocol, identification of toxicant effects by traditional 2D gel electrophoresis can be difficult to exactly replicate in gels run simultaneously for comparison. Thus, this technique has been modified to label the proteins in each of a pair of samples with a different fluorescent label (differential in-gel electrophoresis, DIGE). The two samples can thus be mixed and run simultaneously on the same gel, enabling the separation of the individual proteins from each sample under identical 2D electrophoresis conditions. Each set of labelled proteins is then imaged separately, and the relative proportions of each label in the mixture at individual spots in the gel can be compared, to characterize the consequences of toxicant exposure on the entire population of separated proteins. For example, the prototype nephrotoxicant 1,2-(dichlorovinyl)-L-cysteine (DCVC) changes the expression of 23 proteins in exposed renal epithelial cells (de Graauw *et al*., 2005) as measured by DIGE. Fourteen proteins increased in concentration and nine decreased as a consequence of exposure to DCVC. The most prominent of the changes was found to involve altered Hsp27 (heat-shock protein 27) phosphorylation that appeared to be dependent on p38 kinase activity. Information about a set of proteins that is altered as a consequence of toxicant exposure can be regarded as representing a biomarker signature of such exposure. The identification of changes to a single molecular species will probably not be exclusively linked to a single toxicant because similar changes may be associated with other toxicants or disease processes. However, there may be specific patterns of molecular changes at the protein level that are able to suggest the toxicant involved, and indicate the sequence of changes that result from such exposure (Merrick and Bruno, 2004). Unique molecular signatures have been linked to the occurrence of breast cancer (Vazquez-Martin *et al*., 2007; 2008) and to other forms of cancer (Villanueva *et al*., 2006), but the identification of such signatures for specific toxicant exposures is still in its infancy (Zieziulewicz *et al*., 2003).

Assessment of the spectrum of molecular changes that result from toxicant exposure can also be done using DNA microarray technology. For the most part, DNA microarrays are filter-based or use DNA oligonucleotide targets that have been immobilized on other solid substrates as short (about 25 nucleotides) sequences arranged in a densely packed set of spots. Messenger RNA (mRNA) harvested from cells is usually converted to a complementary DNA (cDNA) coupled to a fluorescent tag, and hybridized to the immobilized oligonucleotides. A high-resolution image of the substrate is collected, and the fluorescent intensity of each individual spot on the chip is measured, as a way of determining how much of the cognate mRNA was present in the sample. Adjacent regions often are used as controls in which a single nucleotide has been substituted in the immobilized oligonucleotide in order to assess nonspecific binding. This type of analysis has been used to analyse mRNA samples harvested following toxicant exposure *in vivo* (Jeong *et al*., 2006), *in vitro* (de Longueville *et al*., 2003) and *ex vivo* (Elferink *et al*., 2008). *Ex vivo* analysis of toxicant effects has not been widely used because there are technical challenges to the maintenance of tissue explants, but this approach does offer some advantages, in that it maintains the organization and potential interactions of cells that are exposed to toxicant (Catania *et al*., 2007), without adding the complications associated with exposures in intact animals.

As with the analysis of the proteome, studies of the transcriptome may identify how the cell changes as a consequence of toxicant exposure, but characterizations of the sequence of molecular events that leads to a particular signature may be a complex challenge. This is in part due to the large number of changes that can result (both in mRNA levels and in protein concentration) following toxicant exposure. Liver samples harvested from zebrafish exposed to arsenic in the aquarium water for up to 96 hours showed an increasing number of gene functions that had been altered over the course of the experiment. By eight hours, about 500 gene functions were altered in comparison to tissues from untreated fish, and this number tripled in the ensuing 40 hours (Lam *et al*., 2006).

Recent advances in microarray technology now enable massively parallel studies of genome structure and activity using instruments such as the 454 Pyrosequencer. This instrument can use fragmented DNA coupled to beads for sequencing in picolitre volumes at rates of up to 10^9 bases per day. The system can evaluate epigenetic influences (Korshunova *et al*., 2008), and can assess the influences of cellular changes on gene expression (Sugarbaker *et al*., 2008). These types of analysis can identify structural changes to the genome that occur as a consequence of toxicant exposure, and can also identify specific changes to gene activity patterns.

The comparison of a 2D gel pattern produced with cellular extracts of control cells with a different pattern produced with separated proteins from toxicant-exposed cells can result in the identification of specific protein spots that are up- or down-regulated as a consequence of toxicant exposure. Specific identification of the protein spots from these gels often depends upon recovering the proteins from the gel, and sequencing them by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF) or another type of mass spectrometry.

New technologies are emerging to further these microarray-based protein analyses. One of these, grating-

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coupled surface-plasmon resonance imaging (GCSPRI) is based on measurements that determine changes to the refractive index at the interface between a metal (e.g. gold) sensor chip and a dielectric (e.g. water). This technology is based on the use of a diffraction grating to couple incident light energy to metal electrons (Brockman and Fernandez, 2001). Under the appropriate conditions, light can initiate a density oscillation in the electrons at the gold surface (termed a plasmon). The coupling of light to the metal electrons is sensitive to the incident angle of the illuminating light. Thus, by sweeping through a small range of incident light angles, the optimum angle for coupling can be identified. Proteins adsorbed to the surface of the sensor chip will increase the refractive index of the dielectric at the gold/dielectric interface, and will thus increase the critical angle at which maximum coupling occurs. Since the sensor chip can be simultaneously imaged and analysed for changes to the critical angle at multiple regions on the sensor chip, the assay can be used to assess multiple events of protein capture at different regions that represent different analytes measured from the same sample. Propagation of plasmons within the gold sensor chip limits the density of sensor spots on a sensor chip to approximately $10^3\,cm^{-2}$. In one configuration, individual antibodies spotted to the sensor chip at regions of interest (ROIs) have been simultaneously interrogated by GCSPRI for the capture of cognate antigens. GCSPRI has been used to assess protein levels in biological samples (Unfricht *et al*., 2005) as well as to capture cells (Jin *et al*., 2006), and can be multiplexed to make both of these measurements at once from complex biological samples. It is possible to capture and enumerate cells on the sensor chip, and to culture these cells for extended periods of time, while secreted or released analytes are detected by nearby sensor spots on the chip. In this way, microarray analysis of cellular functioning can be done in real time. Thus, the composition of a toxicant-induced biomarker signature may ultimately include individual proteins and other captured macromolecules from the biological sample, the phenotype of cells found in target tissues and the functional characterization of those cells. A second generation of the GCSPRI technology relies on coupling a fluorescent emission to plasmon generation (Reilly *et al*., 2006). Preliminary studies have shown this variation of the basic technology to be significantly more sensitive without sacrificing microarray capacity (manuscript in preparation).

4 THE INTERACTOME

As the data set that represents the biomarker signature of a cellular response to toxicant grows, it will eventually become possible to match this data with data sets that attempt to describe the molecular interactions of cellular proteins. These data sets are collected as the 'interactome' and represents data collected for a whole organism: each protein–protein or protein–DNA interaction within the network of interactions is represented by a series of directed graphs that link individual molecular components to those other components with which they interact. Many of the data sets used to create these graphic representations come from various kinds of affinity studies such as yeast two-hybrid experiments and phage display studies of molecules and molecular motifs that can bind to one another (Barrios-Rodiles *et al*., 2005). There are now a number of interactomes being developed, representing a variety of different species such as human (Brown and Jurisica, 2005), *Drosophila* (Sanchez *et al*., 1999), yeast (Shi *et al*., 2008) and *C. elegans* (Simonis *et al*., 2009).

5 SUBCELLULAR TARGETS OF TOXICANTS

5.1 The Plasma Membrane

Just as toxicants can have effects on primary and secondary molecular targets, and just as interactions between these components can influence the spectrum of affected molecular species, a variety of subcellular compartments and organelles can be altered in both structure and function as a consequence of toxicant exposure. To a certain degree, these effects depend upon the fundamental chemical characteristics of the toxicant, and on the access that they have to specific targets. **Figure 2** depicts representative cellular targets of toxic agents, ranging from those targets at the plasma membrane all the way to the genetic material of the nucleus and mitochondria. One of the initial cellular targets of toxicants can be the plasma membrane itself, where they can have their effects both at the external surface of the plasma membrane, or can have indirect effects on membrane structure by initiating apoptotic or necrotic death processes in the cell. Direct chemical effects on the membrane include changes to the lipids comprising the membrane bilayer, and proteins associated with the membrane. For example, oxidants are known to initiate lipid peroxidation, a self-sustaining cascade of events that can alter membrane integrity (Young and McEneny, 2001). **Figure 3** illustrates the consequences of lipid interactions with oxidant, and the proliferation of free radicals as a consequence of these interactions. The accumulation of these lipid peroxides can produce changes in the structure of proteins associated with the plasma membrane, the interactions of proteins/lipids in the lipid bilayer and can ultimately change the both the physical nature and the functional capacities of the membrane

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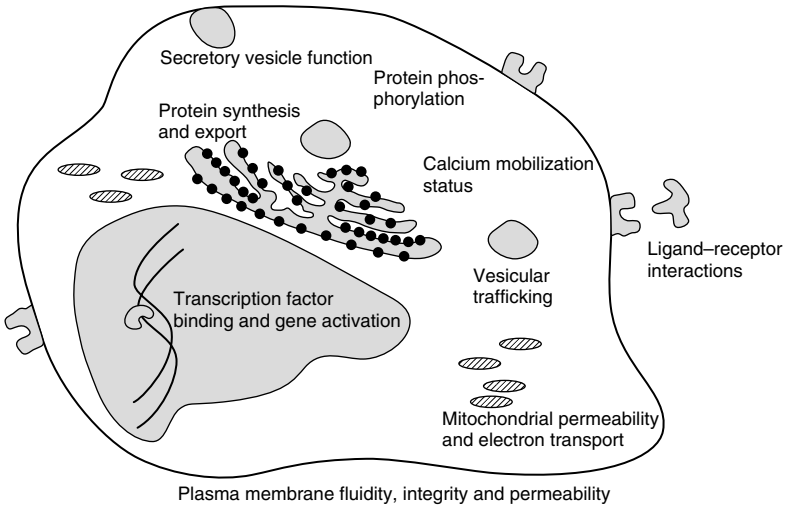

Figure 2 Various cellular targets of toxicant-mediated damage.

itself. An illustration of physical changes in membrane integrity that can result from lipid peroxidation is shown in **Figure 4**. **Figure 4A** shows a scanning electron micrograph of normal discoid erythrocytes, while the **Figure 4B** is an image of the same erythrocytes after exposure to hydrogen peroxide. The oxidation of the lipids in the bilayer prevents the interactions between the leaflets that sustain normal erythrocyte discoid shape and in some cells this produces a change in shape called an echinocyte. In some cases, the appearance of these echinocytes is indicative of systemic oxidative damage, and can be used as an indicator of *in vivo* exposure to toxicants that elicit oxidative damage (Chukhlovin, 1996).

Direct access of the toxicant to the exterior leaflet of the plasma membrane may also result in chemical modifications of proteins found on the plasma membrane. For example, toxicants may oxidize thiols associated with surface proteins to dithiols and may inactivate these proteins as a result. This change has been suggested to render the lipid bilayer less mobile and more fragile as a result, but can also influence ion channels and other membrane functions (Padmini and Sundari, 2008; Zavodnik *et al*., 2001; Dumaswala *et al*., 2001). One of the primary defences against this oxidative stress is glutathione, which plays an important role as an antioxidant (Dumaswala *et al*., 2001; Fidelus *et al*., 1987). Other cellular antioxidant defences include other thiol-rich proteins such as MT, and the antioxidant enzymes such as catalase and superoxide dismutase. Intriguingly, there are marked differences in antioxidant capacity in different cells and that distinguishs individual species of animal. This results in differential sensitivity to oxidative stresses that influences the survival of cells under stress and can reflect the differential toxicity of some agents in certain species. For example, mouse

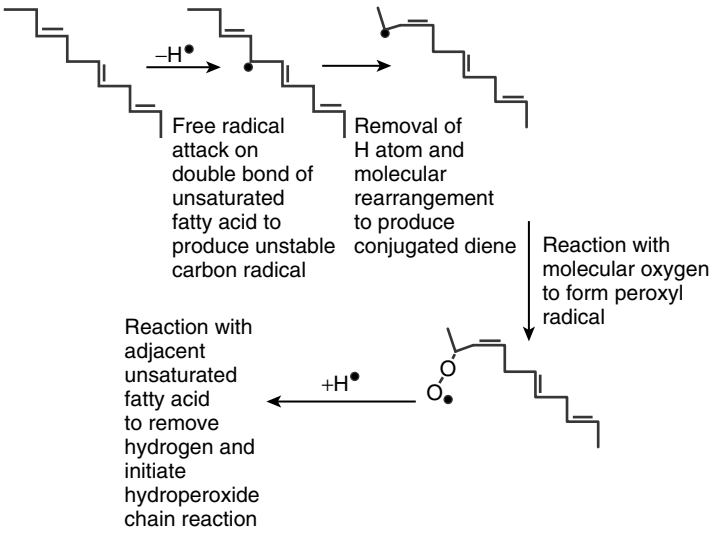

Figure 3 Lipid peroxide cascade. (Adapted from Young and McEneny, 2001.)

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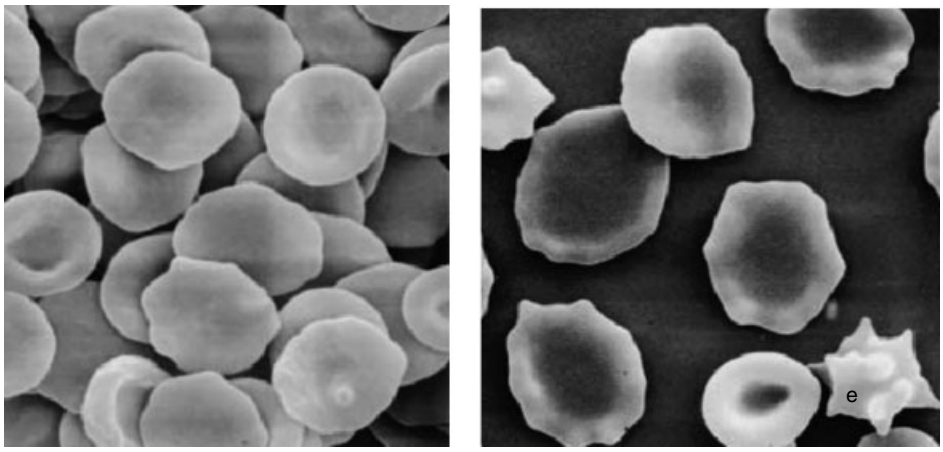

Figure 4 Effects of toxicant exposure on erythrocyte membrane structure as seen in scanning electron micrographs. (A) Normal discoid erythrocytes. (B) H_2O_2-damaged erythrocytes; '**e**' indicates echinocyte configuration.

lymphocytes have been shown to possess lower concentrations of glutathione than human lymphocytes, making the mouse cells less resistant to dissolved oxygen in tissue culture media (Messina and Lawrence, 1992). As a consequence, to facilitate the normal function of murine lymphocytes, tissue culture media is often supplemented with β-mercaptoethanol (Chen and Hirsch, 1972) as a reducing agent.

The interactions of toxicants with the bilayer may also alter membrane and cytoskeletal dynamics in ways that influence membrane processes. Thus, the processes of phagocytosis, endocytosis and pinocytosis can all be directly disturbed by the influences of toxicants (Hodge *et al*., 2007). Oxidants can initiate actin polymerization (Hinshaw *et al*., 1991) and alter a range of cellular activities that rely on the cytoskeleton and result in cellular shape changes. Similarly, damage to the membrane can also influence protein secretion and the expression of newly synthesized membrane proteins.

Passive transport requires that the toxicant be capable of crossing the lipid bilayer and can be driven by the characteristics of the transported molecule or by membrane transporters that facilitate the transport. Active transport requires the expenditure of energy from adenosine triphosphate (ATP) hydrolysis. Membrane-permeable oxidants can cross this barrier, so agents that can generate oxidants near the plasma membrane surface can produce indirect effects without themselves crossing into the cytosol, but there is also evidence that the oxidative changes that occur at the plasma membrane itself can also change intracellular signal-transduction cascades (Urano *et al*., 1997; Waghray *et al*., 2005; Pelle *et al*., 2005). One way to experimentally separate the two phenomena (oxidation at the plasma membrane vs. the effects of oxidants within the cell) is to use membrane-impermeant oxidants such as copper phenanthroline (Duncan and Lawrence, 1989) in comparison to agents that can oxidize intracellular targets. One way in which these changes can occur is by the influences that toxicants can have on ion channels within the plasma membrane. In some cases, toxicants can traverse the membrane via ion channels (Friedman and Gesek, 1994). Noncharged molecules such as hydrogen peroxide (a nonpolar molecule that is approximately the same size as water) have recently been shown to move across plasma membranes via the aquaporin water channel (Bienert *et al*., 2007), a membrane channel that is impermeable to charged molecules. In some cases there are receptors that will be used as transition points for the movement of toxicants into the cell. For example, Cd complexed with MT has been shown to be taken up by renal proximal tubule cells after binding to megalin (Wolff *et al*., 2006). Finally, there are a range of membrane-associated uptake mechanisms (e.g. pinocytosis, endocytosis, phagocytosis) that may facilitate the uptake of toxicants in a nonspecific fashion (Kaminski *et al*., 1986).

In some instances, the act of internalizing particulate material from dying cells (which might be induced to enter a programmed death cascade by toxicant exposure) can initiate a propagating damage cascade. For example, phagocytic Kupffer cells of the liver that engulf apoptotic hepatocytes are stimulated to produce inflammatory cytokines and to induce fibrotic lesions that can perpetuate liver damage (Canbay *et al*., 2003). Internalization of quartz microparticles by alveolar macrophages has been associated with reactive oxygen species (ROS) generation, tumour necrosis factor α (TNF-α) generation and cellular toxicity, and depends upon the reorganization of the cytoskeleton to permit this internalization (Haberzettl *et al*., 2007). In instances of silica-mediated death of alveolar macrophages that follows internalization of a toxicant, ROS do not appear to be the principal

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mechanism, but rather lysosomal damage may act to initiate apoptosis (Thibodeau *et al*., 2004).

5.2 Other Cellular Membrane Targets

As noted above, silica internalization can induce lysosomal leakage that may precede apoptotic cell death, demonstrating that not all toxicant effects on lipid bilayers operate at the external surface of the cell. The endoplasmic reticulum (ER) is a central target of many forms of toxicant-induced damage, and 'ER stress' is a term applied to any condition that disturbs the normal functioning of this important organelle in protein synthesis, protein folding and secretion. The consequences of toxicant-induced damage to this cascade of protein synthesis and folding has been called proteotoxicity (Hightower, 1991), and is accompanied by the cellular stress response in the form of synthesis of a range of molecular chaperones localized in the ER (e.g. the Grp78 and Grp94 glucose-regulated proteins (Lass *et al*., 2008; Liu *et al*., 1997)).

5.3 Toxicant-Induced Damage to Signalling Cascades

The consequences of damage to plasma membranes and the receptors expressed on the surface of the target cell can be changes to the downstream signalling mechanisms and ultimately to changed gene function and cellular activity. Similar consequences can also arise when toxicants directly or indirectly alter protein phosphorylation via influences on both kinases and phosphatases and/or second messengers in these signalling cascades. Inorganic mercury has been shown to alter CD95-dependent apoptosis-signalling cascades (Laiosa *et al*., 2007) in ways that may both illuminate this toxicant's ability to initiate autoimmune disease in humans, as well as to depress adaptive immunity (McCabe *et al*., 2007; Silbergeld *et al*., 2005). Conversely, manipulation of these targeted signalling cascades may offer therapeutic opportunities to ameliorate cellular damage by toxicants. Renal cells injured with DCVC can be protected by activation of protein kinase B (Akt) which improves mitochondrial respiratory function and stabilizes these cells (Shaik *et al*., 2008). Cd toxicity has been linked to influences on about 290 different genes in *C. elegans*, and, using interactome analysis tools, the signalling protein KEL-8 was shown to protect *C. elegans* from Cd exposure in a MAPKK (mitogen-activated protein kinase kinase)-dependent manner (Cui *et al*., 2007).

There are three different types of second messengers: the hydrophilic molecules, such as calcium ions, inositol triphosphate (IP_3) and cyclic adenosine monophosphate (cAMP) found in the cytosol; the hydrophobic molecules, such as membrane-associated diacylglycerol and the phophatidylinositols; and the small molecule gases, such as carbon monoxide (CO) and nitric oxide (NO) that can act both across membranes and within the cytosol. Calcium is a particularly sensitive indicator of cellular activation, and is quite susceptible to some forms of toxicant-mediated cellular activation or inactivation. Methylmercury, a ubiquitous environmental toxicant, is associated with elevations in free cytosolic calcium (Ca^{2+}) (Kang *et al*., 2006). Cd-induced apoptosis has also been linked to increases in free cytosolic calcium and this cell death can be attenuated by increasing the intracellular levels of the calcium-binding protein calbindin-D (Jeon *et al*., 2004).

5.4 Toxicant-induced Damage to Genetic Machinery

As receptor proteins and signalling proteins can be targets of toxicant-induced damage, so too can the enzymes and transcription factors that are responsible for regulating gene activity and for DNA and RNA synthesis. Changes to proteins that sort the chromosomes during cell division can also have toxic effects on the cell. Acrylamide has many industrial uses and is also found in some cooked foods. This neurotoxicant has been found to affect the spindle fibre proteins that are used to sort chromosomes during cell division, which could explain some of its clastogenic effects (Exon, 2006). Toxicants can also interact directly with the genetic material, forming adducts that interfere both with replication and with function of specific genes. Polycyclic aromatic hydrocarbons (PAHs) are a widespread example of an environmental toxicant that can alter the physical nature of DNA (Hu *et al*., 2008), but a broad range of agents can alkylate DNA, generate thymidine dimers, cause frameshifts in codon structure or otherwise cause chromosomal breakage and mutation. In each of these instances, the response of the cell to this genetic damage can be drawn from a range of repair mechanisms (e.g. nucleotide excision repair, photoreactivation, recombinational repair). Alternatively, the cell can sense the mutation at cell-cycle checkpoints and respond by entering the apoptotic pathway to self-deletion. A recent recognition is that early changes to the genetic structure of the individual may also have consequences to the onset of disease later in life, or the susceptibility to disease in offspring of the toxicant-exposed individual. Some of these epigenetic changes may be the result of changes to imprinted genes, or to other patterns of DNA methylation that regulate gene function (Dolinoy *et al*., 2007). The undesirable consequences of these genetic changes can include the release of the cells from normal patterns of cell

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division. If the cell evades cell-cycle checkpoints, these cells may ultimately produce neoplasias that progress to cancer.

6 FUNCTIONAL CHANGES IN CELL BEHAVIOUR FOLLOWING TOXICANT EXPOSURE

A host of cellular functions fall prey to toxicant exposure. The molecular structures that are altered as a consequence of exposure may be primary changes that directly result from the exposure, they may be downstream consequences of such primary molecular changes or they may be cellular attempts to re-establish molecular or cellular homoeostasis. There are a broad range of molecular changes that together transfer information regarding changes in the extracellular environment to the cytoplasm and finally to the nucleus. These changes are often initiated by ligand interactions with receptors expressed at the plasma membrane, and precipitate a signalling cascade that includes changes in the patterns of protein tyrosine phosphorylation, phospholipid metabolism, calcium mobilization from intracellular and extracellular stores and transcription factor activation. Because these different molecular changes are tightly intertwined, it is often difficult to assess the direct effect of the toxicant when many different changes to the cascade may be altered (Pounds, 1990). However, there are many instances of change that ultimately influence cellular behaviours and result in the initiation of dramatically different outcomes including cell division, differentiation and apoptosis.

6.1 Protein Synthesis as a Consequence of Cellular Stress

The original identification of cellular protein stress responses that occur as a consequence of physical insult was done using cultures of fruit fly (*Drosophila melanogaster*) salivary gland cells (reviewed in Haak and Kregel, 2008). In these cells, polytene chromosomes are produced by multiple rounds of DNA replication without centromere replication and without cell division. As a consequence, the giant chromosomes have banding patterns that correspond to the genetic organization of the chromosome.

These bands expand, or 'puff', in a microscopically visible structure when a gene within the region becomes activated to transcribe new mRNA. When *D. melanogaster* salivary cells were cultured at an elevated temperature, many active genes were turned off, while a distinct set of new genes, the heat-shock proteins (Hsps), were activated. We now know that this set of proteins, that are turned on when cells encounter stress, are activated upon exposure to a host of different stressors, including a wide array of chemical toxins and toxicants. The initiation of synthesis is apparently induced by mechanisms within the cell that sense protein misfolding, and some of the heat-shock proteins serve as molecular machinery charged with either disassembling the misfolded proteins, or refolding them to a normal configuration. The mechanisms for the recognition of misfolded proteins are still unclear, but it appears that Bip/Grp78 recognizes the unveiling of hydrophobic regions (more often at the core of the protein in β-strands) that often accompanies misfolding (Schroder and Kaufman, 2005). An interesting hypothesis suggests that the misfolded proteins and protein aggregates are damaging to cellular processes, and that the molecular chaperones are responsible for the maintenance of normal protein configurations as a way of reducing the damage caused by toxicants that initiate these processes (Hightower, 1991).

Inhibition of the Hsps can influence a cell's progression through the cell cycle, as well as its propensity to undergo apoptosis (Okamoto *et al*., 2008). These proteins serve as a hallmark of toxicant exposure, and can also influence the functional capacities of the cell, the survival of the exposed cell and the death pathway taken by cells too damaged to recover from injury. There is a growing body of literature that shows that stress proteins of a variety of types can be released from the cell (see **Table 1** for examples of various forms of stress proteins). The original observation of the selective release of Hsps (hsp110, hsp71, hsp73) (Hightower and Guidon, 1989) has been supported with numerous subsequent observations of extracellular stress proteins (Galloway *et al*., 2008; Luo *et al*., 2008a; 2008b; Calderwood *et al*., 2007). While the actual mechanisms of release of these proteins is not well understood, there are a number of different pathways that stress-response proteins can take to exit the cell (Lynes *et al*., 2006). Since many of these stress-response proteins are synthesized on free polysomes, the mechanism of secretion must differ from the classical secretory pathway. While these alternative, nonclassical pathways are poorly defined in mammalian cells, there are software approaches to the identification of these proteins that rely on the assumption that the molecular motifs of extracellular proteins should be similar in some ways that are independent of the mechanisms of secretion or release. SecretomeP is available as online software that can be used to interrogate proteins sequences for these motifs (Bendtsen *et al*., 2004). Once released from cells, these stress-response proteins have been shown to influence cellular behaviors, as what has been termed a 'danger signal' (Matzinger, 2002). Heat-shock proteins and glucose-regulated proteins can influence immune capacity by some potentiating some responses (Galloway *et al*., 2008) and by suppressing others (Luo *et al*., 2008b).

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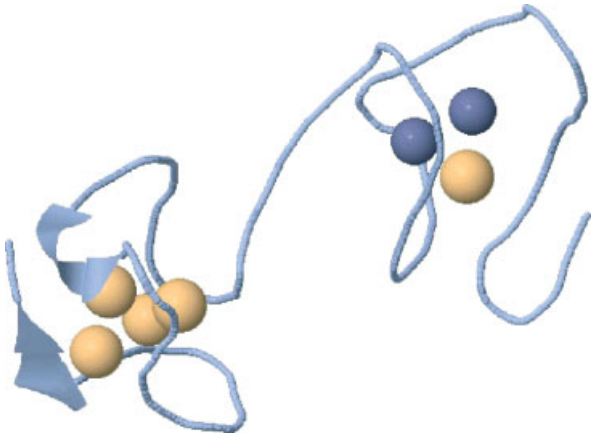
Table 1 Toxicant-induced stress-response proteins

Stress protein	Specific examples	Example of inducing conditions	Normal cellular role
Heat-shock proteins	Hsp70, Hsp27	Heat, denaturing agents	Protein folding
Metallothionein	MT-I, MT-II	Metals, inflammatory cytokines, reactive oxygen	Redox management, essential metal reservoir
Acute phase proteins	Serum amyloid A	Inflammation	Cholesterol transport, induction of extracellular matrix digestion
Stress hormones	Glucocorticoids	Psychological and physical stress	Control of endocrine and exocrine cell development
Proinflammatory cytokines	IL-1, IL-6, TNF-α	Infection, toxicant-induced tissue damage	Cell regulation
Others	Ubiquitin	Protein damage	Protein metabolism

6.2 Metallothionein as a Central Stress-Response Protein

MT is an unusual kind of stress-response protein in a biochemical, regulatory and functional sense (Borghesi and Lynes, 1996; Lynes *et al*., 2006). The protein is very small (about 7 kDa for the most common isoforms), and is both rich in cysteine residues (about 33 mol%) and lacks histidines and aromatic amino acids (**Figure 5**). The apoprotein can be associated with Zn and Cu in nonintoxicated organisms, and can capture other divalent heavy metals such as Hg and Cd when they are present. Metal binding occurs as a consequence of hydrogen bonding and metal–thiolate bonds between the many cysteines and the metals. While MT expression can serve to protect against the damage induced by heavy metals, both by sequestering the metal and by scavenging the reactive oxygen species that can be generated by redox-active metals, there appears to be no effective mechanism for MT secretion. This may be part of the reason for the long biological half-life of metals such as Cd, which is thought to have a biological half life in humans of 20 to 30 years. Genes for MT or MT-like proteins and polypeptides are found in both prokaryotes and eukaryotes; in the mouse the genes are located on chromosome 8, and in humans they are found on chromosome 16q13. The structural genes are composed of three exons interrupted by two introns and are expressed upon exposure to a host of different inducers (see **Figure 6**). Intriguingly, MT synthesis can be induced both by toxic and nontoxic events within and surrounding the cell. Cytokines and stress hormones that influence the immune system can often produce MT biosynthesis activity, suggesting that MT may act in a variety of ways to regulate cellular biochemistry. For example, MT has been shown to suppress some



MDPNCSCSTGGSCTCSSSCGCKNCKCTSCKKSCCSCCPVGCSKCAQGCVCKGASDKCTCCA

Figure 5 Metallothionein structure. The amino terminus of this rat Cd_5, Zn_2-MT is located at the right of the image. The two quasi-domains each surround a group of heavy-metal diavalent cations: the smaller blue ones are Zn and the slightly larger tan ones are Cd. The structure was produced by Robbins *et al*. (1991), and was rendered with Jmol. The amino-acid sequence of rat MT1 is shown below and was obtained from *Uniprot.org*. (Reproduced from The UniProt Consortium, The Universal Protein Resource (UniProt).)

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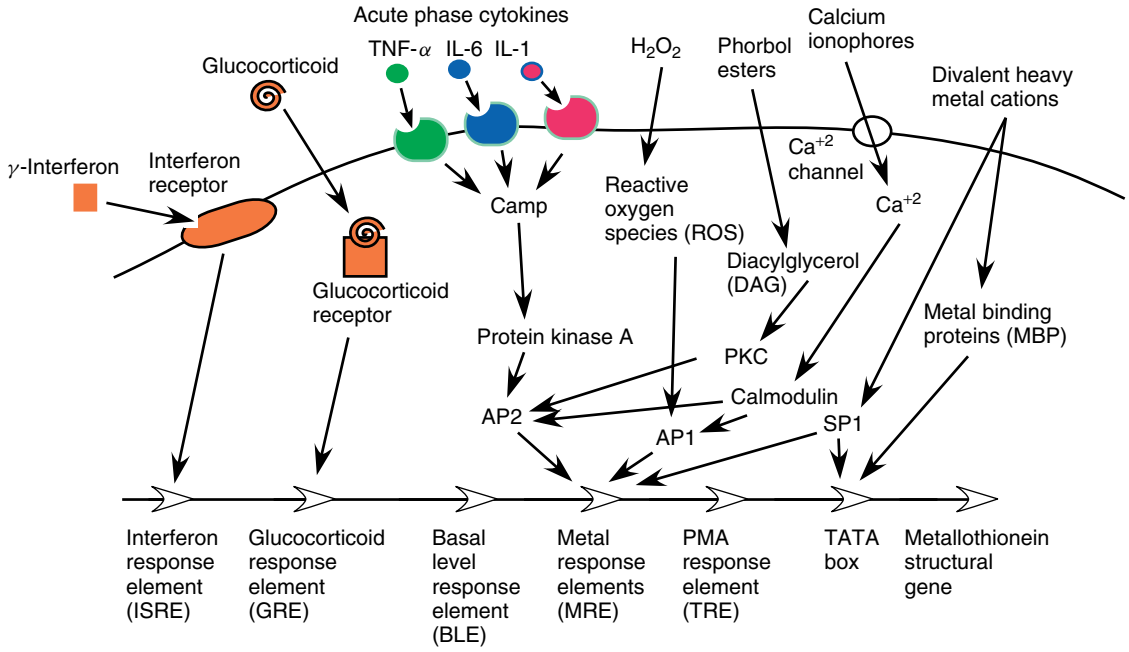

Figure 6 Metallothionein gene regulatory cascades. A variety of agents and cytokines can cross the plasma membrane or bind to receptors, activating of signalling cascades that result in transcription factor binding to the upstream regulatory regions controlling metallothionein mRNA synthesis.

immune capacities (Lynes *et al*., 2006; Youn and Lynes, 1999), while it activates lymphocyte proliferation and chemotactic responses (Lynes *et al*., 1990; Yin *et al*., 2005). MT has been shown to regulate NF-$\kappa\beta$ and Sp1, two transcription factors with important roles to play in inflammation.

Cellular interactions with stress-response proteins can take several different forms. These proteins can serve simply as delivery agents that move the associated toxicant to a new target cell or tissue. For example, MT has been shown to transport toxic heavy metals from the liver to the kidney (Klaassen and Liu, 1997). Uptake of MT at the kidney may rely on kidney-associated surface proteins; there are reports that Cd-MT can bind to megalin on kidney cells (Wolff *et al*., 2006; 2008). Other groups have reported that MT can bind other cells, suggesting that the protein may disseminate toxicants to multiple cell types (Chung *et al*., 2008; El Refaey *et al*., 1997). In addition to the simple distribution of toxicants that are associated with stress-response proteins, the production of these proteins may serve to activate cellular processes via interactions with cell surface receptors that initiate signalling cascades in the target cells. For example, some Hsps are known to bind to pattern recognition receptors (PRRs) such as Toll-like receptor (TLR)-2 and -4 (Binder *et al*., 2004). Hsp22, Hsp70 and Hsp90 all have been found in association with TLR4 (Roelofs *et al*., 2006; Triantafilou and Triantafilou, 2004), and each has been suggested to modify the immune response in the context of stress. One of the intriguing therapeutic opportunities that this raises is the possibility of using these stress proteins to intentionally manipulate the immune response in beneficial ways (Wang *et al*., 2008; Barginear *et al*., 2008; Lynes *et al*., 2006).

7 CELLULAR DEATH CASCADES

One of the consequences of cellular damage that follows toxicant exposure can be the entry into the apoptotic cascade. While this process is extensively covered in anther chapter of this work (**Cell Death and Apoptosis**), it is appropriate to briefly consider it's implications here. In this response, a series of linked enzymatic events can produce changes to the total cellular volume, resulting in shrinkage of both organelles and the entire cell body. One of the earliest events is the activation of a family of proteins known as caspases (cysteinyl aspartate-specific proteinases). Additionally, the cell will experience mitochondrial depolarization and cytochrome C release, and a redistribution of phosphatidyl serine from an asymmetric presence on the inner leaflet of the plasma membrane to an equilibrated presence on both the inner and outer leaflet. DNA fragmentation is often associated with the activation of an endonuclease that cleaves the DNA randomly between nucleosomes, producing a DNA ladder comprised of fragments in multiples of the 200 bp that encircle each nucleosome. As apoptosis progresses, there is often an endonuclease-mediated fragmentation of the DNA and a process of partitioning of the cell into smaller apoptotic bodies that appear

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to arise from surface 'blebs' (Lane *et al*., 2005). One hypothesis is that these blebs are more readily processed by phagocytic cells without the release of intracellular structures that might otherwise activate inflammatory processes and inappropriate immune responses (O'Reilly and Strasser, 1999). However, apoptosis that occurs in excess of these processing mechanisms has been associated with the onset of autoimmune disease in experimental models and human disease (Schulze *et al*., 2008), suggesting that toxicants that initiate apoptosis may (in some instances) be responsible for inappropriate autoimmune activities via some similar mechanisms. There are some instances where toxicants have been shown to inhibit apoptosis mechanisms, thereby enabling the survival of cells that can be responsible for subsequent autoimmune disease. This disease could result from the survival of undesirable autoreactive cells, or it could result from the secondary necrotic cell death and initiation of inflammation by those cells that did not successfully undergo apoptosis. For example, mercury interferes with apoptosis by blockade of the assembling death-inducing signal complex (DISC) that is activated by Fas/FasL interactions (McCabe *et al*., 2005; 2003). Trichloroethylene (TCE) has recently been reported to interfere with apoptosis by activating a metalloproteinase to cleave FasL from CD4+ T cells (Blossom and Gilbert, 2006). These cells are thus potentially able to initiate some forms of T-cell-mediated autoimmune disease. A parallel mechanism may relate to toxicants that interfere with phagocytic processing of apoptotic cells, resulting in a similar autoimmune propensity (Russell and Cameron, 1986).

Another cellular death cascade is termed autophagy. This process results in the packaging of cytosol and organelles into large vesicles within a double membrane (Klionsky, 2004; Klionsky *et al*., 2008). When these autophagosomes fuse with a lysosome, the contents of the vesicle are degraded by the lysosomal enzymes. Under most circumstances, this process enables the cell to recycle cellular materials, but this process can be inappropriately initiated by the exposure to toxicants such as arsenic, hexavalent chromium (Cr[VI]) and Cd (Cheng *et al*., 2007; Di Gioacchino *et al*., 2008).

Necrosis is distinguished from these orderly processes of programmed cell death by several different characteristics. Cells that are necrotic swell as the total cell volume increases; organelles will also swell during this process. Membrane integrity is damaged, and intracellular macromolecules will be released from the cell. Unlike the orderly catabolism of the DNA that occurs during apoptosis, endonucleases fragment the DNA without regard to sequence or structure, producing a wide range of DNA fragment sizes that electrophorese as a 'smear' of DNA due to the wide range of overlapping sizes that are produced.

8 PROLIFERATION AND DIFFERENTIATION

Cells under stress can also respond by proliferation. Lymphocytes cultured in the presence of Hg, Cd and other divalent heavy-metal cations at certain concentrations, for example, will proliferate (Warner and Lawrence, 1988; 1986; Lawrence, 1981). This proliferation can result in inappropriate immune activities, as well as diminish the capacity to mount an effective immune response against antigenic challenges. Other toxicants, such as asbestos, can also initiate proliferation, in ways that implicate oxidative stress via the actions of myeloperoxidase and signal transduction cascades that involve protein kinase C (Haegens *et al*., 2005; Shukla *et al*., 2007). Neurons will make the choice to proliferate or differentiate depending on the local redox environment (Noble, 2006; Noble *et al*., 2005) which can be influenced by a range of different toxicants. In this case as well, the choice of proliferative activity by a neuronal cell can disturb normal neural circuitry and have deleterious effects on normal tissue activity.

8.1 Molecular Toxins and Toxicants as Probes and Modifiers of Biological Function

In a research context, toxicants are often useful as molecular tools with which to manipulate the biochemistry of cells. Agents that we think of as toxic in the clinical context are often employed to inhibit or modify specific aspects of cell function. For example, liposomal clodronate (dichloromethylenebisphosphonic acid) can delete macrophages after *in vivo* exposure in many tissues, including the eye (Biewenga *et al*., 1995; Slegers *et al*., 2000). This experimental deletion of this cell population enables the evaluation of macrophage contributions to a variety of disease processes. Biological mechanisms can also be studied by comparing the effects of a specific toxicant on the proteome to those induced by a different toxicant. Two types of information can come from these studies. The first is an appreciation of the similarities or distinctions in the molecular events altered by the two toxicants. In some cases, a novel toxicant mechanism may best be understood by analogy to a similar, previously studied agent. The second is a better understanding of the array of proteins that are functionally linked in normal cells, and the ways they respond to toxic insult. This approach was used to explore the effect of the toxicants 1,1-dichloroethylene (DCE) and diclofenac, which each covalently adduct hepatic proteins. In this study, bile was collected and compared after treatment with each of these agents, and was shown to differentiate the kinds of

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protein changes that are unique to these different agents (Jones *et al*., 2003).

In similar fashion, toxicants have a variety of uses as therapeutic agents where the consequence of exposure is to change molecular or cellular activities within cells. The challenge is to balance the desirable toxic effects that diminish cancer cell survival or metastasis, while minimizing the unintentional toxic effects on other cell types. Cisplatin has a number of uses in cancer therapy; it is useful for the treatment of osteosarcoma, early stage non-small-cell lung cancer (NSCLC), and other malignancies, but can also effect hearing and nervous system tissues (Lewis *et al*., 2009; Sioka and Kyritsis, 2009), and can be immunotoxic as well (Kouchi *et al*., 1996).

Another new area of both experimental and therapeutic manipulation of molecular and cellular systems is also challenged by unanticipated toxic consequences. Small interfering ribonucleic acids (siRNAs) have been explored as potential immunotherapeutics for a wide range of diseases such as AIDS (Kumar *et al*., 2008) and diabetes (Marzban *et al*., 2008). These molecules belong to a class of short (20–25 nucleotides long) double-stranded RNA molecules that operate in the RNA interference pathway to block or interfere with the stability of a targeted messenger RNA. As a consequence, the role played by that mRNA is suppressed, and changes to biological activities can be elicited. While the goal is to specifically and selectively interfere with a single target's function, there have been a number of reported instances of unanticipated off-target effects of siRNAs that result from unfortunate homologies between the targeted mRNA and others (Tschuch *et al*., 2008; Colombo and Moll, 2008). Moreover, *in vivo* delivery of siRNAs can activate cellular sensors for viral nucleic acids, resulting in kinase activation and the synthesis of Type I interferon, TNF-α and other proinflammatory cytokines.

9 CONCLUSIONS

The molecular and cellular responses to toxins and toxicants underlie all other aspects of the effects of toxins, the diagnosis and management of toxicant exposure and (perhaps most importantly) the inferences we can draw about the basic biological mechanisms of the exposed organism. The study of these mechanisms has a broad range of implications for our understanding of biological systems and offers a rich opportunity for the use of this information in the therapeutic management of disease. At the same time, we have only begun to understand the influences of an exposed individual's genetic background to disease susceptibility, both as a component of the development of personalized drugs for specific patient populations and in the context of individual susceptibilities to specific toxic environments.

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Cell Death and Apoptosis

Sidhartha D. Ray and George B. Corcoran

CONTENTS

1 INTRODUCTION

The study of the cellular reaction to injury is fundamental to the study of disease in all living systems. There is a basic similarity in such reactions whether the cell is that of an invertebrate, vertebrate or plant. Through this conceptual oversimplification, the science of toxicology has contributed significantly to advancing our understanding of human disease. Among the many known cellular alterations in disease, cell death, the irreversible loss of vital cellular functions and structures, is certainly the most drastic and one of the most frequently encountered and easily recognized. Cell death, a near equilibrium terminal end-stage, induced by severe physiological homeostatic perturbation, is a phenomenon inherent to all biological organisms. This endpoint can be induced by ischaemia or hypoxia, drugs or chemicals, immune reactions (complement attack), microorganisms, high temperature or radiation and a variety of naturally occurring and anthropogenic toxins. Historically, the first recognized mode of cell death was necrosis, a general term referring to the morphological stigmata of a cell that has committed to die. During this process, a cell passes through a reversible phase, often followed, with

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explosive rapidity, by irreversible changes resulting from adverse stimuli.

Mechanisms suggested to lead to the irreversibility of necrotic changes involve the release of lysosomal enzymes, activation of membrane-active proteases/phospholipases, global impairment of ion homeostasis, damage by dangerous oxy-radicals or biological reactive intermediates (BRIs), a precipitous drop in cellular energy status, with depletion of intracellular cytoprotective agents like antioxidants, adenosine triphophate (ATP), glycogen and glutathione, malfunctioning of cytoprotective enzymes, like superoxide dismutase, catalase, peroxidase, glutathione peroxidase, poly-adenosine diphosphate (ADP)-ribose polymerase (PARP) and substantive insult to the integrity of genomic DNA beyond a critical point, culminating in failure of the plasma membrane. A comparatively recent advance in our understanding of lethal injury, however, establishes that cell death occurs via another major and distinct dynamic process termed apoptosis. Whereas necrosis is considered as unprogrammed and typically accidental or unplanned cell death resulting from adverse stimuli, apoptosis, on the other hand, is regarded as a programmed, physiological and beneficial form of cell death which can be elicited by both normal and adverse stimuli. Because the details of necrotic cell death are thoroughly discussed in other chapters of this book, this chapter will focus on drug- and chemically induced apoptotic processes and their mechanisms of action.

When apoptosis (pronounced Ap oh ′tow sis or A ′poop tow sis derived from the Greek *apo*, meaning leaf, and *ptosis*, meaning to fall off) was first described over a third of a century ago (Kerr *et al.*, 1972a), different existing terminologies were used to describe some of the seemingly unrelated changes seen in different tissues (councilman or acidophilic bodies in liver diseases (Klion and Schaffner, 1966 and see Child and Ruiz, 1968); civatte bodies in lichen planus (Montgomery, 1967); tingible bodies in lymphoid germinal centres (Nossal and Ada, 1971); basophilic or Benirschke granules characteristic of premenstrual endometrial glands (Ehrmann, 1969, etc.). The newly coined term defined and differentiated apoptosis from necrosis based solely on changes in cellular ultrastructure (Kerr, 1969; 1971; Kerr *et al.*, 1972b). The consolidation and renaming the processes of shrinkage necrosis and others noted above to apoptosis evolved exclusively on morphological grounds, when it was found to play a global role in regulating tissue morphogenesis and tissue size (see Majno and Joris, 1995). The relevance of this unique cell death process was widely underappreciated until an upsurge in interest during the 1990s focussed efforts on unraveling the mechanisms underlying this process. After two decades of intensive research and thorough examination, it is now accepted that apoptosis is a tightly regulated, energy-dependent, genetically programmed and evolutionarily conserved self-destruction process through which cells undergo organized suicide, typically for beneficial purposes.

In multicellular organisms, such beneficial functions include maintenance of optimal tissue growth and development and physiological activity involving a balance between proliferation, growth arrest and programmed cell death (PCD). Apoptotic cells, upon completion of the death mission, conclude the suicide programme by forming apoptotic bodies which are rapidly removed by the reticuloendothelial system, including macrophages and phagocytes, and to some extent neighbouring cells. Massive cell suicide by apoptosis usually does not lead to organ dysfunction, yet this can be one of the particularly cataclysmic outcomes of necrotic cell death. Another noteworthy attribute is the lack of involvement of inflammation during the progression of events. Although apoptosis and necrosis are often described as diametrically opposed modes of cell death, some ask whether they are parts of a genetic and biochemical continuum (Corcoran and Ray, 1992). Since PCD is often used synonymously with apoptosis, it is important to clarify the use of the terms programmed cell death and apoptosis. It has been appreciated for many years that not all PCD occurs by apoptosis (Clarke, 1990; Abraham *et al.*, 2007), and that apoptosis is not always programmed or scheduled to occur, as observed during development and metamorphosis (**Scheme 1**). For these reasons and for the purpose of this chapter, apoptosis and PCD are not used interchangeably. The term apoptosis is used strictly to designate cells that die with the hallmark morphology associated with this process, whereas PCD is reserved for cases in which cell death is scheduled and is not necessarily apoptotic.

Apoptosis is characterized by a series of well-defined morphological changes (see **Figures 1–4**). Initially, there is a decrease or shrinkage in cell volume (cytoplasmic condensation), the generation of a pyknotic nucleus, and condensation of chromatin along the nuclear periphery. This is followed by loss of the nuclear membrane, fragmentation of nuclear chromatin and subsequent formation of multiple fragments of condensed nuclear material, often with cytoplasm, called apoptotic bodies. These may later appear on the cell surface and be shed as vesicles, blebs and apoptotic bodies. The next stage is the phagocytosis of apoptotic bodies by phagocytes or neighbouring cells, preventing the release of cellular contents into the surrounding areas and avoiding an inflammatory response. The morphological changes associated with apoptosis contrast with those of necrosis, another primary form of cell death typically associated with pathological processes. Necrosis has been viewed as an unprogrammed and chaotic sequence characterized by cellular swelling, in which swollen intracellular structures disintegrate rather than shrinking, and by the release of cellular contents that induce an inflammatory responses in the surrounding tissue (**Figures 1–4**).

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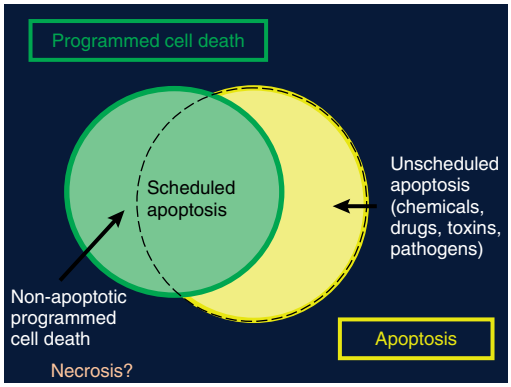

Scheme 1 Programmed cell death, apoptosis and necrosis. Apoptosis and programmed cell death (PCD) are often used interchangeably in the literature. Apoptosis involves a genetic programme that is responsible for a cell death process that is defined strictly on morphological criteria. In metazoans, there are developmental, hormonal and other programmes that schedule the triggering of cell death. Here, the term programmed cell death has been used to denote the scheduling of cell death, independent of the mode and morphology of cell death. There are many examples of cell death that are programmed, but do not involve the morphology or the genetic steps that are hallmarks of apoptosis. Similarly, there are many instances of apoptotic cell death which are unscheduled. To minimize confusion based on ambiguous terminology, it would be logical to reserve use of the term programmed to indicate that cell death is scheduled, and that programmed cell death not be equated with the genetic steps, biochemistry, morphology or occurrence of apoptosis. Thus, programmed cell death (PCD) and apoptosis should not be used interchangeably, and the former should not be used to imply the scheduling of cell death. Finally, growing evidence suggests that necrosis may be programmed in part. If this is borne out, the far left portion of the left circle that is labelled nonapoptotic programmed cell death may include a large component of cells dying by necrosis.

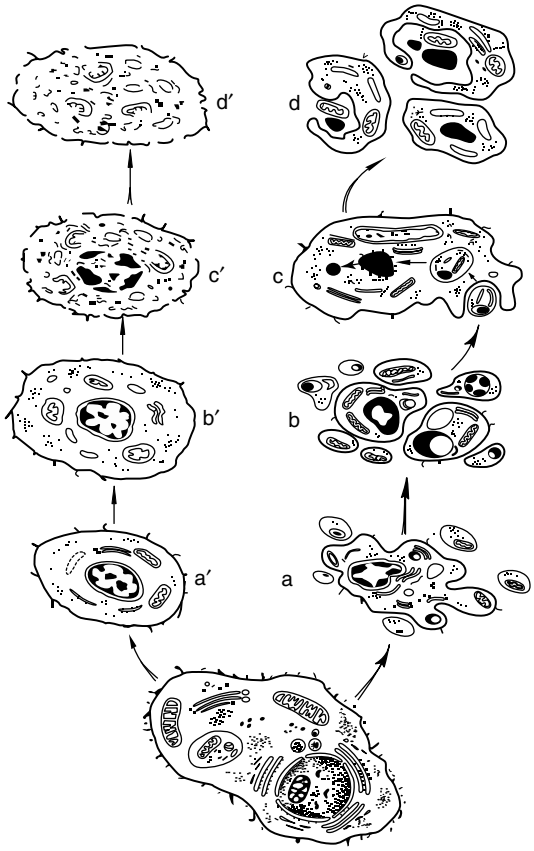

Figure 1 Morphological changes characteristic of necrosis and apoptosis. In apoptosis (right pathway, a–d), the cell condenses and shrinks (a and b) rather than swells (a′ and b′). In necrosis (left pathway, a′–d′), cell lysis follows generalized swelling and is preceded by disintegration of organelles (mitochondria, nucleus and the plasma membrane breakage (c′–d′). In contrast, cell–cell contacts which are initially retained in necrosis, are broken down early in apoptosis. This is followed with explosive rapidity by stereotypical changes that include loss of plasma membrane surface structures and compaction of the cytoplasm, coupled with the condensation of heterochromatin and its fragmentation. This phase is followed by migration of masses of condensed chromatin to the periphery of the nuclear membrane, and chromatolysis usually involving caspase-activated DNAse (Ca^{2+} and Mg^{2+}-dependent endonuclease) activation. Condensed, membrane-bound cell fragments or apoptotic bodies containing cytoplasm and intact organelles, including mitochondria, are phagocytosed by macrophages/phagocytes or other neighbouring cells (c–d). See details of phagocytosis under higher magnification in the electron micrograph in Figure 5A. Engulfed materials are internally digested to avert inflammation. Stages depicted in this figure are as defined by Searle *et al.* (1982). (Reproduced with permission from SD Ray, Long Island University.)

In recent years, two dominant dogmas regarding necrosis have come under significant challenge and this has implications for our understanding of apoptosis. First, necrosis is increasingly becoming viewed as a process that may be partially or substantially programmed (Edinger and Thompson, 2004; Bizik *et al.*, 2004; Chan *et al.*, 2003; Henriquez *et al.*, 2008). This confronts the notion that necrosis is wholly unregulated, and questions a cardinal mechanistic demarcation between necrosis and apoptosis. Second, some instances of necrosis appear to represent an aborted attempt to execute apoptosis, or perhaps more importantly, a fundamental integration of the processes underlying necrosis and apoptosis, sometimes termed aponecrosis or necroptosis (Degterev and Yuan, 2008; Formigli *et al.*, 2004; Cheng *et al.*, 2003). The latter concept may include instances of the well-recognized phenomenon of secondary necrosis that has been observed following the initiation of apoptosis. Secondary necrosis was first reported largely for *in vitro* models, but now is also well documented *in vivo* including in mammals (Silva *et al.*, 2008). Apoptotic bodies that are not phagocytosed and fully digested, and that progress to secondary necrosis,

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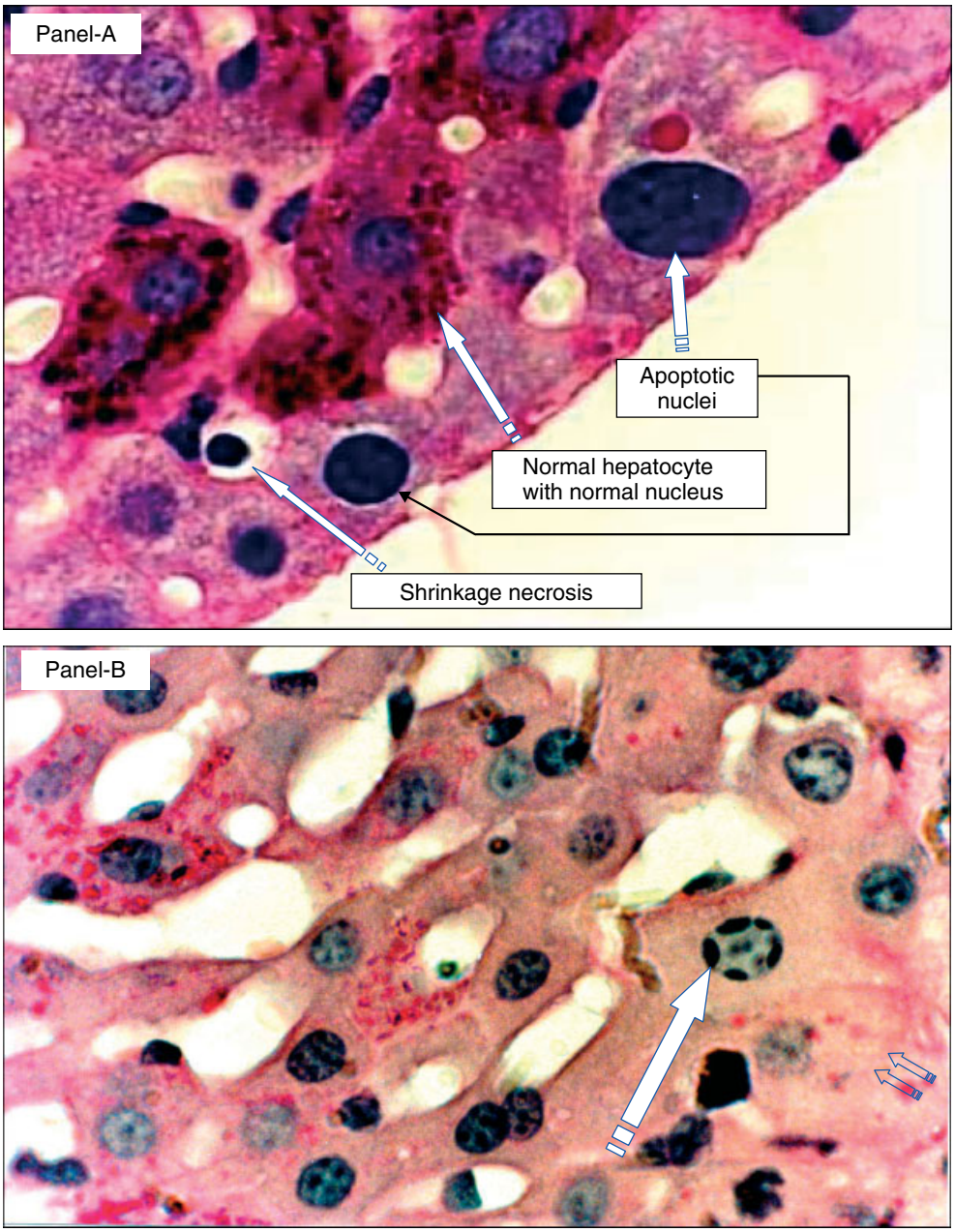

Figure 2 Paracetamol-(acetaminophen)-induced hepatocellular apoptosis and necrosis (low magnification). Light photomicrographs of paraffin-embedded mouse liver sections show early morphological changes typical of apoptosis induced by acetaminophen (500 mg/kg, ip at six hours, periodic acid-Schiff (PAS) staining, ×1000). (A) presents examples of normal hepatocytes with normal nuclei and apoptotic hepatocytes with dense apoptotic nuclei at various stages, including shrinkage necrosis. The section exhibits the beginning of apoptotic changes including condensation of chromatin, as well as abnormal enlargement of the nucleus characterized by very dense staining. Nuclear alterations are evident in the near total loss of volume regulation. Necrotic changes include nuclear and cytoplasmic damage with weak staining. A strongly PAS-positive glycogen-loaded normal hepatocyte shows normal nuclear morphology. (B) shows nuclear condensation, chromatin fragmentation and margination of the condensed chromatin to the nuclear membrane periphery, a trait typical of apoptosis. The short double arrows point to a possible necrotic cell very close to nuclear dissolution, characterized by poor nuclear staining. The long single arrow shows a hepatocyte with an apoptotic nucleus where chromatin has condensed, fragmented and migrated to the nuclear membrane. (Reproduced from Ray and Jena, 2000. © Springer.)

are the proposed basis for various serious pathologies including some autoimmune diseases (Rovere-Querini *et al.*, 2008; Zhang and Xu, 2002; Rovere *et al.*, 2000), chronic inflammation (Henson, 2005) and septicaemia (Wesche-Soldato *et al.*, 2007). Further discussion of these potentially important developments in the understanding

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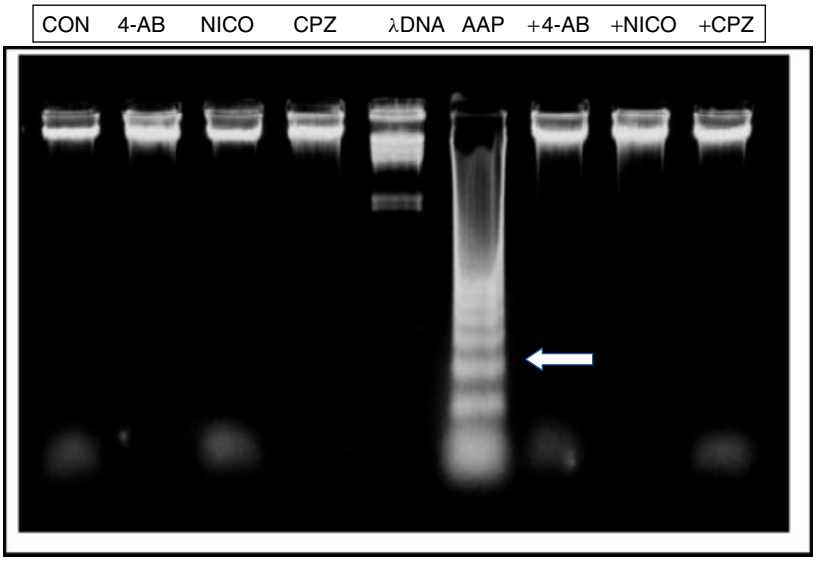

Figure 3 Paracetamol (acetaminophen)-induction of a DNA fragmentation ladder. This ethidium bromide-stained agarose gel shows DNA damage in mouse liver 24 hours after acetaminophen overdose. Mice were treated with a PARP inhibitor (4-aminobenzamide (4-AB) or nicotinamide NICO) or the Ca^{2+}-calmodulin antagonist chlorpromazine (CPZ) to assess their effects on acetaminophen-induced DNA fragmentation (15 μg per lane of DNA extracted from three animals and pooled). The loss of large genomic DNA with concomitant appearance of a classic DNA ladder typical of apoptosis was observed following acetaminophen treatment (lane 6). The two PARP inhibitors and CPZ prevented acetaminophen-induced DNA fragmentation and DNA-laddering (lanes 7–9), whereas these antagonists alone failed to alter the integrity of the genomic DNA. Legend for lanes: 1—vehicle control (CON); 2—4-AB, 3—NICO; 4—CPZ; 5—λDNA BstEII-digest; 6—acetaminophen (APP); 7—acetaminophen + 4-AB; 8—acetaminophen + NICO; 9—acetaminophen + CPZ. The appearance of a DNA ladder (lane 6) is characteristic of dissociation of the CAD-ICAD complex and subsequent activation of DFF. Severe loss of large genomic DNA is evident in the acetaminophen-exposed animals (lane 6). Note the gradual loss of fluorescent intensity close to the well, reflecting total disappearance of large molecular weight DNA. (Reproduced from Ray *et al.*, 2001. © Elsevier.)

of cell death is beyond the scope of this chapter. Interested readers are referred to other chapters in this book and to the cited publications for more information on these rapidly evolving topics.

Key characteristics of apoptosis and necrosis are described in **Table 1** and **Figures 1–4**. In apoptosis, it has been shown that cells activate an intrinsic death programme and thus actively contribute to their own demise. Apoptosis is gene-directed and influenced by extrinsic and intrinsic signals (**Table 2**). The initiating signals are fundamentally similar. For example, cells exposed to ionizing radiation may undergo apoptosis, which is highly predictable in terms of the proportion of cells affected, but they are not programmed to die unless they are exposed to radiation. Another example of apoptosis involves the ischemic death of the endometrial lining of the uterus at the end of menstrual cycle (**Table 2**). In addition, apoptosis can be affirmatively predicted in specific individual cells of the well-studied nematode, *Caenorhabditis elegans*. *Caenorhabditis elegans* matures as an adult hermaphrodite with 1090 cells, of which 131 then undergo PCD (Ellis *et al.*, 1991). Nearly all of these are apoptosis-designated cells and found at various locations. They automatically trigger suicidal death pathways at different, but precisely defined times.

This finding led to the proposed scenario in which cells receiving death signals or loss of survival signals may turn on a dormant genetic death sequence regardless of the cause. It is now clear that the regulatory mechanisms controlling apoptosis are as fundamental to life, and are fully as complex as those regulating cell proliferation. Based on the observations of the past three decades, the apoptotic response of a cell to a drug or toxin is determined at five distinct levels: (i) stimulus (e.g. drug, chemical or toxin) and its bioavailability and metabolism; (ii) cellular defense mechanisms; (iii) signal transduction pathways; (iv) status of relevant genes, particularly oncogenes and (v) intrinsic cellular susceptibility to apoptosis (**Table 2**).

2 APOPTOSIS: OCCURRENCE

Apoptosis is an operationally, morphologically and, for the most part, biochemically distinct form of cell death from necrosis (Kerr *et al.*, 1972b; Wyllie, 1987). Owing to the multitude of signals and requisite cellular metabolic events which can lead to apoptosis, a surprisingly wide range of physiological and toxicological stimuli effectively initiate this death pathway in an array

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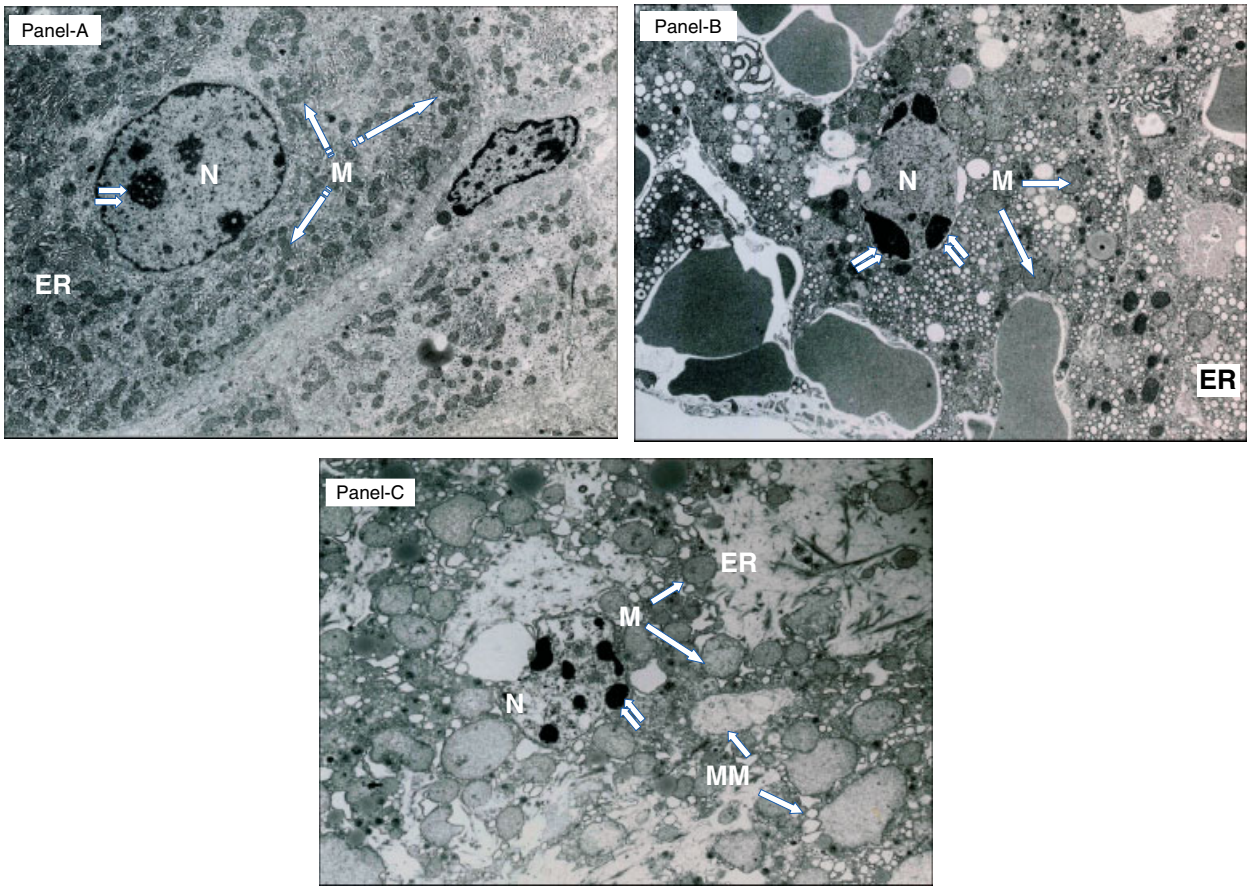

Figure 4 Paracetamol- (acetaminophen)-induced hepatocellular apoptosis and necrosis (high magnification). Electron photomicrographs of mouse liver show ultrastructural details of a normal healthy hepatocyte from a vehicle control animal (A), and an apoptotic (B) and a necrotic (C) hepatocyte from animals treated with 500 mg kg^{-1} acetaminophen i.p. at 24 hours. The nucleus of the apoptotic hepatocyte (B, ×8000) presents with characteristic morphology of apoptosis, including hypertrophic cytoplasmic changes. Note the condensation, fragmentation and margination of heterochromatin in the absence of a significant loss of mitochondrial volume. Frank degradative changes are evident in the cytoplasm and nucleus of the necrotic hepatocyte (C, ×8000). Compare volumes of cells and organelles in (A), (B) and (C). (C) shows severe mitochondrial swelling (MM) and collapse of the mitochondrial boundary, extensive nuclear damage with fragmented chromatin and collapse of the nuclear membrane, and other morphological aberrations in the cytoplasm of a necrotic cell. The double arrows signify fragmented chromatin. The single arrows identify mitochondria. Legend: ER—endoplasmic reticulum; M—mitochondria; MM—mega mitochondria; N—nucleus. Scale: 1 μm = 8 mm at ×8000. (Reproduced with permission from Ray *et al.*, 1996. © American Society of Pharmacology and Experimental Therapeutics.)

of different cell types. It is clearly evident, however, that the induction of apoptosis in any given tissue is tightly regulated, such that suicide can often only be induced by a particular tissue-specific signal. Consequently, a signal that turns on execution in one tissue may have very little or no apoptotic effect, or may even cause an opposing outcome such as proliferation, in another tissue. The wide range of cells that can undergo apoptosis *in vitro* and *in vivo*, including invertebrates, vertebrates and plants, confirms that practically each and every type of cell has a latent or active ability to execute the terms and conditions of apoptosis.

The tissue specificity of the induction of apoptosis and the molecular mechanisms by which the apoptotic process occurs have been intensively studied but remain incompletely understood. Cell death by apoptosis has been reported in plants (Basile *et al.*, 1973; von Wangenheim, 1987), nematodes (Yuan and Horvitz, 1990), insects (Lockshin and Williams, 1965), fish (Daoust *et al.*, 1984), birds (Glucksmann, 1951; Hurle *et al.*, 1977; Oppenheim, 1985), amphibians (Searle *et al.*, 1982), vertebrates (Glucksmann, 1951) and mammals (Baruch *et al.*, 1963; Compton and Cidlowski, 1992). Regression of the tadpole tail during amphibian metamorphosis serves as one of the prime examples of ontogenic apoptosis, perhaps only rivaled by the cataclysmic metamorphosis of some insects (Lockshin, 1981; Bowen and Bowen, 1990). Collapse of the tadpole's tail followed by degradation of the tail muscle is a captivating event that has been captured by classical histopathologists. Biochemists and cell and molecular biologists have

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Table 1 Distinguishing features of apoptosis and necrosis

Charcteristic	Apoptosis	Necrosis
Distribution	Affects individual cells scattered throughout the tissue	Affects massive and contiguous cells
Adhesion between cells and to basement membrane	Lost early	Lost but late
Cellular morphology	a. Chromatin condensation (karyorrhexis) followed by margination as large crescents to the periphery of the nuclear membrane; fragmentation in large masses (convolution). See Figure 1B	a. Irregular clumping of chromatin, pyknosis or karyolysis, nucleolysis occasionally preceeds collapse of nuclear membrane; cells occasionally maintain their boundaries with some or no organelles. See Figure 1C
	b. Loss of cell volume (cytoplasmic compaction)	b. Very early swelling of cell, ballooning occurs frequently
Damage to organelles, for example, mitochondrion	Late (organelles mostly retain integrity), occasionally organellar swelling and bleb formation on cell surface appear very late (organelles found in blebs)	Very early swelling of organelles; cells disintegrate and lyse, appear chaotic, form blebs early (organelles are not found in blebs)
DNA breakdown pattern	Internucleosomal cleavage (Ladder-like pattern on agarose gel)	Random or irregular damage (appears as a smear on gel)
Release of lysosomal enzymes	Absent	Present
Duration of biochemical and morphological changes	Minutes to hours; propelled by macromolecular synthesis; mitochondrial permeability transition pore formation	Hours to days; lack of macromolecular synthesis; involvement of mitochondrial permeability transition pore formation unknown
Ultimate outcome	Forms apoptotic bodies, occasionally containing intact organelles	Swelling, disintegration, dissolution
Cell removal	Usually via phagocytosis by all types of resident and nonresident cells	Usually cells are not removed
Inflammation	Absent	Present
Adhesion between cells and to basement membrane	Lost very early	Lost very late
Energy requirement and signaling dependency	Strictly energy-dependent, very tightly regulated, can easily be delayed but can be inhibited only with difficulty, signaling-dependent (well-defined extrinsic & intrinsic pathways)	Energy and signaling-independent, occasionally energy-dependent, can be blocked prior to irreversible changes (e.g. plasma membrane leakage)
Genomic control	Strictly dependent	Usually independent
Scar formation	Absent (organ dysfunction reported)	Present (organ dysfunction reported)
Reversibility	Onset can be delayed but cannot be reversed after morphological changes develop	Can be blocked if detected early prior to point of no return in the absence of significant membrane damage
Overall regulation	Very tightly regulated; mostly dependent on energy status, protein synthesis, gene transcription	Independent of energy status or protein synthesis or gene transcription, may be partially regulated

also done much to unravel the mechanisms underlying this event (Weber, 1969; 1977; Kerr *et al.*, 1987).

Another classic example of apoptosis is the loss of cells from the interdigital zones during development in chickens, mice and humans (Bowen and Bowen, 1990). During embryological and foetal development of amphibians, chickens and mammals, experiments have confirmed that peripheral innervation regulates the level of cell death (Levi-Montalcini and Brooker, 1960). Later, Levi-Montalcini and Aloe (1981) demonstrated that nerve growth factor (NGF) channelled via peripheral innervation plays a significant role in controlling the number of cells in developing neuronal ganglia, and in determining the final size of ganglia. Large numbers of

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Table 2 Apoptosis associated with physiological and pathological states

State or condition	Cells/organs involved
1. Normal cell turnover (in adult tissues)	Hepatocytes, adrenal cortex, intestinal crypt epithelium, spermatogonia, megakaryocytes, neutrophils
2. Embryogenesis/ metamorphosis	Epithelial cell of small intestines; deletion of interdigital webs; regression of Müllerian ducts in males and Wolffian ducts in females; palate midline epithelium fusion; most neurons; pronephros, mesonephros and metanephros regression to form nephron and tubules; human tail regression; auditory vesicle invagiation and detachment from ectoderm during the ear development; foetal penderm, intermediate epidermal cells and appendages during development and remodelling of skin
3. Thymocyte deletion	T-cell deletion during negative selection
4. Normal involutional processes	Uterine endometrium after menstrual cycle; mammary epithelium regression after lactation; hair follicles, ovarian follicular atresia, vaginal epithelial cells (stratum spinosum, stratum basalis) during late metoestrus and early dioestrus
5. Atrophic processes	Prostate changes after castration; adrenal cortex after ACTH depletion; kidney during hydronephrosis; thymus during glucocortocoid imbalance; pancreatic ductular obstruction
6. Immune-mediated cell killing	T-cells, killer cells and natural killer cells, allograft rejection, graft vs host disease, tumour necrosis factor-induced (TNF)
7. Cellular injury	Radiation, ischaemia, hyperthermia, calcium ionophores, many drugs and chemicals, toxins of plant and bacterial toxins, steroids, oxidants, carcinogens, anti-cancer agents
8. Viral diseases	Promoters: HIV (human immunodeficiency virus), human T-cell leukaemia virus, human papilloma virus, parvo virus. Inhibitors: herpes simplex virus, human cytomegalovirus, Epstein-Barr virus, vaccinia virus, cowpox virus, baculovirus, hepatitis B virus
9. Neurological diseases	Parkinson's disease, Alzheimer's disease, stroke, spinal muscular atrophy, HIV, encephalopathy, prion disease, Huntington's disease, amyotropic lateral sclerosis
10. Trophic factor withdrawal	Epidermal growth factor, insulin-like growth factor-I, estrogen, IL-2, thrombin, nerve growth factor (NGF) and transforming growth factor dysregulation (TGF-β)
11. Others	Aging, autoimmune disease, neoplasms, thermal stimuli (heat shock), drugs, chemicals

cells are deleted by apoptosis, especially in the nervous system (Oppenheim, 1985; Clarke, 1990). In mammals, the secondary palate that separates the oral and nasal cavities develops by growth, rotation and fusion of the left and right palatal shelves. The fusion of the shelves is mediated by decreased proliferation, increased adhesiveness and apoptosis of medial edge epithelial cells (Greene and Pratt, 1976; Pratt and Greene, 1976).

During vertebrate sexual differentiation and maturation, the reproductive organs show spectacular changes that involve massive apoptosis. Despite the fact that both male and female embryos begin with the same reproductive rudiments, the Wolffian duct differentiates into epididymis and vas deferens whilst the Müllerian duct regresses in the male. The opposite occurs in the female. The Müllerian duct differentiates into the uterus in mammals or oviduct and shell gland in avian species. These events are directed by hormonal scheduling programmes, and cell removal occurs by apoptosis.

All of these observations indicate that apoptosis is an equal and opposite force to mitosis (Kerr *et al.*, 1987). In certain tissues, some cells survive until the organism dies, while other cells are continually produced in self-renewing tissues. These cells undergo differentiation to perform specific functions, and later die. Noted examples of these events are the life cycles of keratinocytes and haematopoietic cells (Lord, 1979; 1986; Muel *et al.*, 1986; McCall and Cohen, 1991; von Wangenheim, 1987). Prior to their destruction by apoptosis, these cells pass through a transit compartment and a maturation compartment. Many hormone- and growth factor-sensitive tissues operate in a similar fashion and mimic the progression of events in order to maintain tissue homeostasis. Cells belonging to tissues and organs such as the liver (Benedetti *et al.*, 1988a; 1988b; Terada and Nakanuma, 1995), kidney (Glucksmann, 1951; Koseki *et al.*, 1992; Coles *et al.*, 1993), thymus (Compton and Cidlowski, 1996; Duke *et al.*, 1996), prostate (Kyprianou and Isaacs, 1988), ovary (Hopwood and Levison, 1976; Sandow *et al.*, 1979), uterus (Sandow *et al.*, 1979; Hopwood and Levison, 1976), adrenal cortex (Wyllie *et al.*, 1973), lymphoid cells (Koury and Bondurant, 1990; Williams *et al.*, 1990), brain (Mukherjee *et al.*,

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1997), nervous system (Glucksmann, 1951; Oppenheim, 1991) and intestinal crypt cells (Milligan and Schwartz, 1996; see also Duke *et al.*, 1996) are vulnerable to apoptosis naturally or otherwise. The death of immature thymocytes in our body is a classic example of apoptosis (Duke *et al.*, 1996).

3 MORPHOLOGY OF APOPTOSIS

Since emerging from a relatively brief period of skeptical acknowledgement, apoptosis is now widely accepted, based in part on confirmation of its highly characteristic and remarkably stereotyped morphological changes observed under light and electron microscopy. Although the most practicable and reliable way of unequivocally distinguishing between apoptosis and necrosis is by transmission electron microscopy, high-power ($\times$1000) brightfield microscopy has generated valuable and informative results in many studies (Ray *et al.*, 1996; 2006; Ray and Jena, 2000). Pioneering pathologists and microscopists agree that nuclear morphology is the gold standard for distinguishing apoptotic from necrotic cell death.

Apoptosis is an ongoing natural process in living systems and can progress very quickly, on a scale of minutes to hours (Sanderson, 1976; Matter, 1979). Cells and apoptotic bodies are removed quite rapidly after the onset of apoptosis (Green, 2005). This has made it a cumbersome and challenging task to capture cells in various stages of this process for complete morphological analysis (Wyllie, 1987). Apoptotic cells die a clean death. The dead cells are normally engulfed and digested by phagocytes and by neighbouring cells with such speed and efficiency that their passing is histologically inconspicuous. This contrasts with necrotic cell death which is untidy and conspicuous (Savill, 1994). Under the influence of an inducing agent *in vitro* or in disease states *in vivo*, abundant apoptotic cells can be found for discrete observations. A variety of representative stimuli and inducers that can activate this process are listed in **Tables 3** and **4**. The time to onset of apoptosis after a lethal stimulus is variable, but the changes are rapid. In apoptosis, coordinated changes occur in the nucleus, in the cytoplasm, and on the cell surface. *In vivo*, apoptosis usually affects single cells or small groups of cells in an asynchronous fashion. The entire apoptotic process can be divided into four steps.

Step I. Cell shrinkage: The earliest changes observed include the loss of cell junctions and other specialized plasma membrane structures such as microvilli. Simultaneously the cytoplasm becomes hypertrophied (**Figure 4B**) and condenses, and the entire cell shrinks. Unlike necrosis, an apoptotic cell lacks swelling, but shrinks and pulls away from its neighbours without causing disturbance to tissue architecture or function.

Step II. Nuclear or chromatin condensation: This is one of the most widely observed and distinguishing features of apoptosis. At this stage, condensing fragments of chromatin coalesce into one or more large masses or several smaller masses and migrate towards the nuclear periphery and the nuclear membrane. As the process continues, the nucleus breaks down into several fragments. Under an electron microscope, these fragments appear dense and dark with the near total maintenance of volume regulation of organelles. The contraction of cytoplasmic volume is associated with loss of intracellular fluid and ions (see **Figures 2** and **4**, which depict stereotypic changes of Step II).

Step III. Formation of apoptotic bodies: The cell membrane transiently adopts a deeply convoluted outline and shows extensive surface blebbing. Subsequently, the cell breaks up into several membrane-bound smooth-surfaced apoptotic bodies that contain a variety of tightly compacted organelles and some nuclear fragments. These are designated apoptotic bodies (**Figure 5A**).

Step IV. Phagocytosis of apoptotic cells or bodies: Apoptotic bodies vary greatly in size and shape. There is no limit to the number of apoptotic bodies that are formed from a single cell, although the number can vary with the size of the cell. Apoptotic bodies are typically phagocytosed by professional phagocytes (macrophages) and to some extent by neighbouring cells serving as amateur phagocytes (glomerular mesangial cells (Savill *et al.*, 1993), renal tubular cells (Savill, 1994) and hepatocytes (Ray *et al.*, 1996)). Primary engulfing cells are typically members of the reticuloendothelial system (mononuclear phagocytes, such as macrophages and phagocytes), but also can be any other normal or abnormal cell capable of phagocytosis (Wyllie *et al.*, 1980). In fact, the largest number of *C. elegans* cell death genes identified in the seminal work of Horvitz and colleagues participate in, or are required for, efficient phagocytosis of the remnants of cells undergoing apoptosis (Ellis *et al.*, 1991; Horvitz *et al.*, 1994). Genes required for phagocytosis during apoptosis (Mangahas and Zhou, 2005) and necrosis (Chung *et al.*, 2000) differ somewhat and are now well delineated. The endocytosed apoptotic masses are rapidly degraded within lysosomes. Adjacent cells migrate or proliferate to replace the space occupied by the recently deleted apoptotic cell, unless loss of the cell is part of an ontogenic or other beneficial process. The roles of genes and signalling pathways in apoptosis are more fully discussed elsewhere in this chapter.

Although apoptotic bodies provide a potent stimulus for phagocytosis, the mechanism of recognition of apoptotic bodies by the engulfing cell is only partially understood. Apoptotic bodies are rarely found in the extracellular space without approaching or adhering

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Table 3 Agents that induce apoptosis *in vivo*

Agent	Target organ(s)	References
Acetaminophen (paracetamol)	Mouse liver	Ray *et al.* (1996; 2000a; 2006), Nagy *et al.* (2007)
Actinomycin D	Heart cells (*Tfam* knockout)	Wang *et al.* (2001)
Adriamycin (Doxorubicin)	Rat heart; Rat kidney	Nakamura *et al.* (2000), Kairaitis (2005)
Aflatoxin	Rat liver	Meki *et al.* (2001)
Amiodarone	Rat lung	Uhal *et al.* (2003)
Amphotericin-B	Rat kidney	Varlam *et al.* (2001)
Asbestos (Chrysotile)	Rat alveolar epithelial cells	Kido *et al.* (2008)
Benzene	Mouse lung	Weaver *et al.* (2007)
Bleomycin	Mouse lung	Li *et al.* (2003), Genovese *et al.* (2005)
Cadmium chloride	Rat kidney; Rat liver	Kukner *et al.* (2007)
Cisplatin	Rat kidney	Luo *et al.* (2008)
Clenbuterol (β_2-adrenergic receptor agonist)	Rat cardiac myocyte	Burniston *et al.* (2005)
Colchicine	Duodenal crypts	Gonzalez *et al.* (2005)
Copper (Trientine)	Mouse fibrosarcoma cells	Hayashi *et al.* (2007)
Cycloheximide	Rat liver	Faa *et al.* (1994)
Cyclophosphamide	Rat germ cells/mouse tumours	Cai *et al.* (1997), Meyn *et al.* (1994)
Cyclosporine	Rat kidney	Bobadilla and Gamba (2007)
Cytosine arabinoside (Ara-C)	Mouse brain	Yamauchi *et al.* (2004)
Diclofenac	Mouse kidney	Hickey *et al.* (2001), Bhatt *et al.* (2008)
Diethylstilbestrol	Rat testis (sperm cell)	Nair and Shaha (2003)
7, 12-Dimethylbenz[a]anthracene	Mouse thymus; Mammary glands	Burchiel *et al.* (1992), Ray *et al.* (2007)
D-Galactosamine	Mouse	Tsutsui *et al.* (2003)
1,2-Dimethylhydrazine	Mouse colon	Oumouna-Benachour *et al.* (2007)
Dimethylnitrosamine	Mouse liver	Ray *et al.* (1992; 2000a, 2000b, 2003, 2005)
Doxorubicin	Rat heart; Mouse heart	Childs *et al.* (2002), Chatterjee *et al.* (2008)
Enalapril (losartan and nifedipine)	SHR smooth muscle cell	deBlois *et al.* (1997)
Ethanol	Rat liver/SHR liver	Baroni *et al.* (1994), Manolas *et al.* (1997)
Ethanol	Mouse neuron	Nowoslawski *et al.* (2005)
5-Fluorouracil	Rat thymus, spleen and ileum	Sakaguchi *et al.* (1994)
Furosemide	Mouse liver	Ray *et al.* (2004)
D-Galactosamine	Mouse liver	Imao *et al.* (2006)
4-Hydroxynonenal	Mouse muscle cell	Braga *et al.* (2008)
Indomethacin	Mouse lung	Murdoch (1996)
Lead nitrate	Rat liver	Pagliara *et al.* (2003)
Lipopolysaccharide	Rat liver	Zhang *et al.* (1993), Norimatsu *et al.* (1995)
Menadione	Mouse liver, thymus	Chiou *et al.* (1997)

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Table 3 (*continued*)

Agent	Target organ(s)	References
Mercury ($HgCl_2$)	Rat kidney	Kanda *et al.* (2008)
Methylazoxymethanol	Rat brain	Ciani *et al.* (2003)
Methamphetamine	Mouse brain	Jayanthi *et al.* (2004)
Microcystin	Rat thymus and spleen	Milutinović *et al.* (2003)
Methylmercury	Rat kidney	Inouye *et al.* (1991)
Methotrexate	Rat liver (after partial hepatectomy)	Kobayashi *et al.* (2002)
Morphine	Mouse macrophage	Bhat *et al.* (2004)
MPTP	Mouse brain	Karunakaran *et al.* (2007)
Mitomycin C	Rat/Mouse (retinal photoreceptor cell)	Nakagawa *et al.* (1997)
N-Methyl-*N*-nitrosourea (MNU)	Mouse retina	Miki *et al.* (2007)
Nicotine	Mouse lung (macrophages)	Demiralay *et al.* (2006)
Ricin	Mouse macrophage	Korcheva *et al.* (2007)
Silica	Rat lung cells	Wang *et al.* (2005)
Thermal burns	Rat and mouse—many organs	Gravante *et al.* (2007)
Thioacetamide	Mouse thymus	Ledda-Columbano *et al.* (1991), Faa *et al.* (1992)
T-2 toxin	Rat ovaries	Murshedul *et al.* (2000)
4-Vinylcyclohexene diepoxide	Rat ovarian follicle	Thompson *et al.* (2005)
Valproic acid	Mouse heart	Li *et al.* (2005)
Zidovudine (AZT)	Monocytes, Cardiac myocyte	Miró *et al.* (2005), Purevjav *et al.* (2007)
Zoledronate	Human kidney	Markowitz *et al.* (2003)

phagocytes. The recognition of an apoptotic body by the phagocytic cells appears to involve a receptor-mediated process. Three different receptor-mediated phagocytosis processes have been implicated, including macrophage lectins and carbohydrate receptors, vitronectin (thrombospondin) receptors and phosphatidylserine receptors (Savill *et al.*, 1993). Lectin-mediated binding involves an integrin-polypeptide type of molecular bridge, complemented with sugar–lectin interaction either directly or indirectly (Morris *et al.*, 1984; Duvall *et al.*, 1985; Arends and Wyllie, 1991). This notion is further supported by the observations that simple sugars (e.g. *N*-acetylglucosamine, *N*,*N*′-diacetyl-chitobiose and mannosamine) will block the recognition of apoptotic cells by macrophages (Duvall *et al.*, 1985; Savill *et al.*, 1989). The loss of sialic acid from membrane glycoproteins would expose normally masked residues such as *N*-acetylglucosamine, *N*-acetylgalactosamine and galactose, rendering these available for interaction with macrophage lectins (Dini *et al.*, 1992).

Another type of recognition of apoptotic thymocytes by macrophages involves movement of phosphatidylserine from the inner to the outer surface of the apoptotic cell. Although this phospholipid is normally located on the inner surface of the plasma membrane, its movement to the outer surface of apoptotic cells and bodies is facilitated by a decrease in the activity of aminophospholipid translocase, an enzyme which normally transports phosphatidylserine from the outside of the plasma membrane to the inside, and/or by an increase in the activity of scramblase, an enzyme that moves phospholipids in both directions (Fadok *et al.*, 1992; 1993). Yet another possibility involves exposure of the phosphatidylserine moiety on the apoptotic cell surface due to a loss of membrane symmetry. The third type of receptor, identified on the macrophage surface, is known to recognize apoptotic neutrophils by a mechanism involving vitronectin, one of the heterodimeric integrin molecules, $a_v\beta_3$, CD_{36} and thrombospondin (Savill *et al.*, 1990; 1991) receptor complexes. The Horvitz group has suggested the involvement of various genes (*ced-1*, *-2, -5, -6, -7, -8, -10*) in the corpse removal process. This type of cell removal observed during apoptosis generally benefits the organism and does not threaten neighbouring cells or structures, because toxic contents of dying cells are normally digested internally, avoiding an inflammatory outcome. Apoptotic bodies are distinct membrane-encapsulated entities that can exclude vital dyes until they are degraded. Occasionally, apop-

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Table 4 Agents that induce apoptosis *in vitro*

Agent	Cell type/model system(s) *in vitro*	References
Acetaminophen (paracetamol)	Mouse hepatocytes	Shen *et al.* (1991), Shen *et al.* (1992)
Adriamycin (Doxorubicin)	CML-lymphocytes, Tumor cells	Cheng *et al.* (2006), Anand *et al.*, (1995); Skladanowski and Konopa (1993)
d-Allose	Human postate cancer cells	Naha *et al.* (2008)
Aphidicolin	CHO cells	Barry *et al.* (1990)
Arachidonic acid	Hep-G_2 cells	Chen *et al.* (1997)
Ara-C	Human myeloid leukaemia cells	Grant *et al.* (1994)
Ardipusilloside III	Human U251MG glioblastoma cells	Lin *et al.* (2008)
Asbestos—crocidolite	Human A549 lung epithelial cells	Baldys *et al.* (2007)
ATP	Thymocytes	Zheng *et al.* (1991)
Buthionine sulfoximine (BSO)	GT1-7 neural cells	Kane *et al.* (1993)
Bleomycin	Human hepatocytes/Hep-G_2	Galle *et al.* (1995)
Butyrate	CRC cells	Bordonaro *et al.* (2008)
Calphostin C	HL-60 promyelocytic leukaemia cells	Jarvis *et al.* (1994b)
Camptothecin	MIL cells	Kaufmann (1989)
Capsaicin	Human PC-3 androgen-independent prostate cells	Sánchez *et al.* (2007)
Celecoxib	Adult T-cell leukaemia/lymphoma	Sinha-Datta *et al.* (2008)
C2-ceramide	U937 cells	Jarvis *et al.* (1994c), Verheij *et al.* (1996)
Chromium	CHO AA8 cells/macrophage J-774A.1 cells	Blankenship *et al.* (1994), Bagchi *et al.* (1998a)
Cisplatin	CHO cells	Barry *et al.* (1990)
Colchicine	Rat hepatocytes	Tsukidate *et al.* (1993)
Etoposide	Mouse L929 fibroblast cells	Mizumoto *et al.* (1994)
Erythropoietin	Erythroid progenitor cells	Koury and Bondurant (1990)
5-Fluorodeoxyuridine	CHO cells	Barry *et al.* (1990)
Gossypol	HL-60 promyelocytic leukaemia cells	Jarvis *et al.* (1994b)
Hoechst 33258	HT-29-II Colon adenocarcinoma cells	Oberhammer *et al.* (1993)
Hydrogen peroxide	U 937 cells	Coppola *et al.* (1995)
Hydroxyurea	CML-lymphocytes	Anand *et al.* (1995)
Interleukin-lrf	Pancreatic Rlm5F cells	Ankarcrona *et al.* (1994)
Methotrexate	CHO cells	Barry *et al.* (1990)
Melphalan	HL60/U937 cells	Fernandes and Cotter (1994)
Mimosine	Human U-937 monocytic leukemia cells	Hallak *et al.* (2008)
Mitomycin C	B-lineage lymphocytes	Potchinsky *et al.* (1995)
Okadaic acid	Rat NRK-52 normal kidney cells	Davis *et al.* (1994)
	Rat hepatocyte, GH3 pituitary cell	Boe *et al.* (1991)
Sodium nitroprusside	RAW 264.7 macrophage cells	Messmer *et al.* (1995)
TCDD	Immature thymocytes	McConkey *et al.* (1988b)
Tributyltin	Thymocytes	Raffray *et al.* (1993)
Trifolin	HL-60 promyelocytic leukaemia cells	Torres *et al.* (2008)
Vinblastine	Rat hepatocytes	Tsukidate *et al.* (1993)
VP-16 (podophyllotoxin)	MOLT-4 cells	Catchpoole and Stewart (1995)

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totic bodies bypass phagocytosis and are released into the adjacent lumen. In such instances, apoptotic bodies demonstrate progressive dilation and degradation of the engulfed contents, particularly mitochondria, in a process resulting in secondary necrosis (Searle *et al.*, 1975). There has been an expansive growth in our understanding of secondary necrosis and its role in health and disease (see Silva *et al.*, 2008).

4 BIOCHEMICAL CHANGES INVOLVED IN APOPTOSIS

Following the morphological classification of apoptosis, many isolated molecular and biochemical events were characterized, often from studies on immature thymocytes *in vitro*. Because this is a tightly regulated energy-dependent active process, cells succumbing to this phenomenon show orderly changes in a highly synchronous fashion. Some of the key elements that orchestrate this beneficial form of death are Ca^{2+} and Mg^{2+} ions, DNA and the nuclear enzyme endonuclease. Wyllie (1980) and Cohen and Duke (1984) showed convincing evidence that chromatin undergoes cleavage early during the killing of immature thymoctyes by glucocorticoids. Activation of a Ca^{2+}-sensitive endonuclease appears to be an obligatory step in the appearance of small DNA cleavage products (**Figure 5**). These investigators highlighted the role that Ca^{2+} plays in the process and proposed that faltering Ca^{2+} regulation within the cell is needed for glucocorticoids to propel cell killing in this model. In a study that examined the killing of thymocytes by the immunotoxin 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), Orrenius and coworkers concluded that this environmental chemical also promotes endonuclease-mediated DNA damage in a Ca^{2+}-dependent manner (McConkey *et al.*, 1988b).

These discoveries raise a number of important questions. How do the mechanisms of acute cell killing gleaned from these *in vitro* models relate to cell death: (i) from other forms of cell insult, (ii) in other types of cells such as the hepatocytes and neurons and (iii) under more relevant conditions such as in living animals. Although most of these questions are not yet fully answered, leading laboratories throughout the world moved forward with apoptosis research employing the DNA ladder as a biomarker to confirm drug- and chemical-induced apoptosis in a wide range of model systems. Over time, the DNA ladder became so popular as a biochemical hallmark that its use resulted in a record number of publications for a decade (**Figure 3**). In 1980, Wyllie showed that the morphological changes of apoptosis are associated with distinctive, endonuclease-dependent double-strand cleavage of nuclear DNA at the linker regions between nucleosomes. The resulting oligonucleosomal fragments are readily demonstrated using agarose gel electrophoresis (**Figure 3**). Necrosis, in contrast, is generally accompanied by random DNA degradation and histone digestion, with diffuse smearing of DNA on agarose gels.

Identification of the DNA ladder as a simple biochemical hallmark led to an explosion of investigations attempting to identify agents capable of inducing apoptosis based on their ability to produce a ladder. Several prominent exceptions to this rule were noted during this period, leading to an appropriate revision that internucleosomal DNA cleavage cannot be used as a sole criterion for diagnosing apoptosis (Collins *et al.*, 1992) in the absence of other dependable markers. The demonstration of internucleosomal chromatin degradation continues to be a valuable diagnostic aid, particularly when supplemented with morphological results and biochemical data, such as caspase activation and M30 neoantigen release (Schwartzman and Cidlowski, 1993; Wieckowska *et al.*, 2006; Hetz *et al.*, 2007). These characteristics are reproducible in both *in vivo* and *in vitro* systems comprising normal or abnormal cells. Apoptotic and necrotic cells share DNA as a common target. Most necrotic cells also show considerable DNA degradation, appearing in a smear-like pattern with haphazard, random or nonspecifically degraded fragments migrating as a continuous spectrum of sizes on agarose gels. Apoptotic cells, on the other hand, most often show a characteristic ladder-like pattern (Huang *et al.*, 1997) except when DNA cleavage ends early resulting in the formation of higher-molecular-weight fragments ranging from 50 to 700 kb (Bicknell and Cohen, 1995).

A rise in intracellular and/or intranuclear Ca^{2+} is a prerequisite for chromatin fragmentation, since the primary enzymes responsible for cleaving DNA appear to be Ca^{2+}- and Mg^{2+}-dependent. Calcium homeostasis must be under as careful regulation in the nucleus as elsewhere throughout the cell (Ray *et al.*, 1990; 1991; 1993; Ray and Corcoran, 1991). The Ca^{2+} concentration gradient present between the nucleus and the cytoplasm (Williams *et al.*, 1988) implies the existence of structures that transport Ca^{2+} across the nuclear membrane. Skeletal muscle nuclei demonstrate Ca^{2+}-stimulated ATPase activity (Kulikova *et al.*, 1982). Nuclei isolated from rat liver accumulate Ca^{2+} *in vitro* via an ATP- and calmodulin-dependent process and contain a constitutive endonuclease that responds to pathophysiological Ca^{2+} concentrations in the submicromolar range by cleaving DNA into periodic intranucleosomal fragments (Jones *et al.*, 1989).

Considerable evidence now establish that the role of Ca^{2+} is pivotal in the early stages of toxic injury leading to cell death (reviewed by Reed, 1990; Barry and Eastman, 1992; Corcoran and Ray, 1992; Trump and Berezesky, 1995; Jeong and Seol, 2008; Vanlangenakker *et al.*, 2008; Dong *et al.*, 2006). Ca^{2+} ion appears to be acting in a well-known capacity as a principal intracellular messenger to convey the consequences of initial damage from diverse sites to one or more specific

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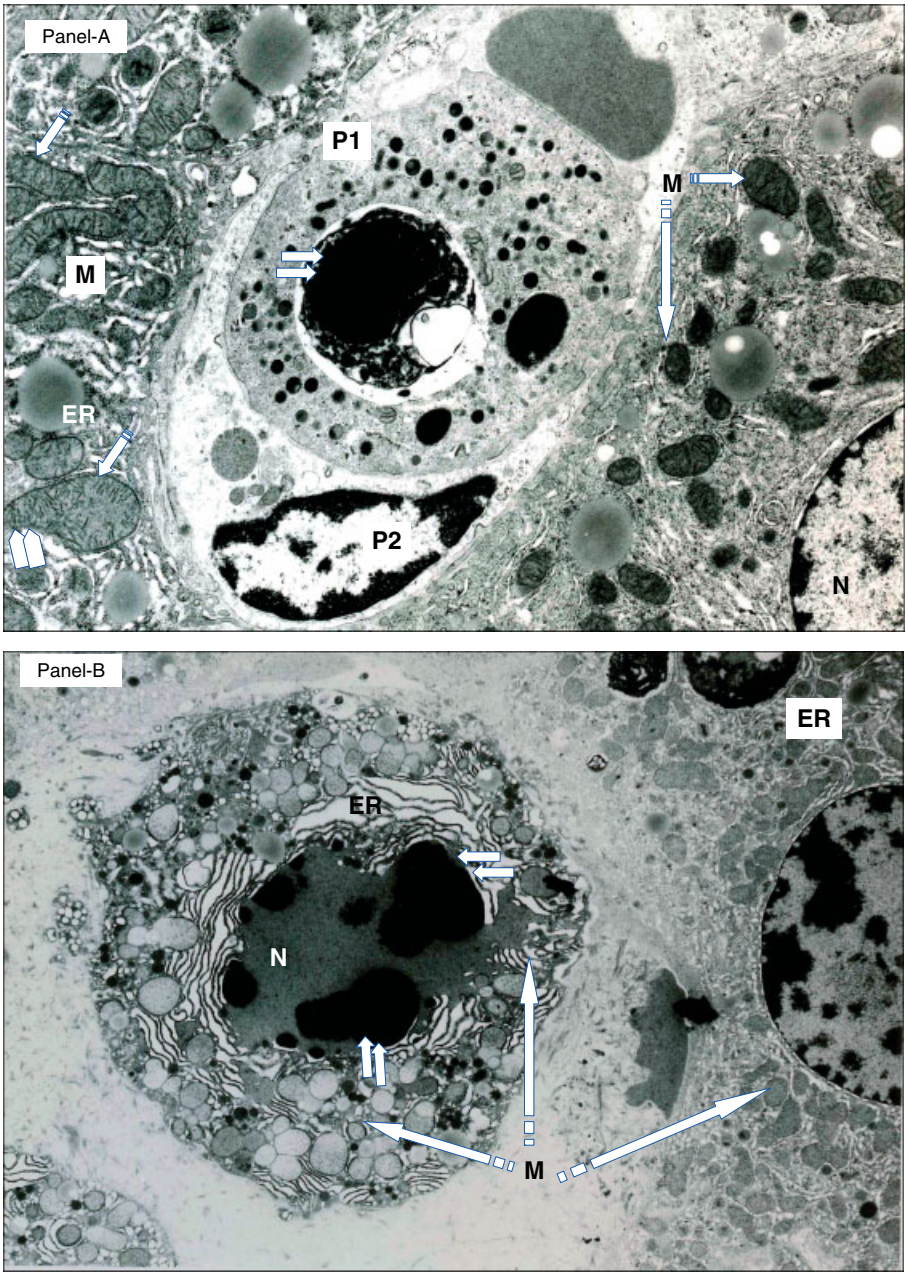

Figure 5 Dimethylnitrosamine-induced hepatocellular apoptosis and necrosis involving phagocytosis. Electron photomicrographs of mouse liver show ultrastructural details over time of apoptotic and necrotic hepatocytes from animals treated with 100 mg kg^{-1} dimethylnitrosamine i.p. In (A), a portion of a nucleus is visible at the bottom right, and the endoplasmic reticulum and numerous mitochondria appear normal. Mitochondrial inner folds are clear in several mitochondria as shown by arrows. This panel presents the phagocytosis (P1) of apoptotic bodies and remnants by a macrophage at 6 hours (×8000). Note that the macrophage-apoptotic body complex is being approached by a second cell for scavenging. Double blunt overlapping arrows (bottom left) point to disruption of the mitochondrial membrane. (B) shows both apoptotic and necrotic changes 18 hours after dimethylnitrosamine exposure (×12 000). Necrotic changes in the cytoplasm and mitochondria, and some apoptotic morphology (condensed and fragmented chromatin) are evident. In comparing the changes in volume regulation in Figure 3A and B with those in this figure, note advanced plasma membrane, mitochondrial, cytoplasmic and ER damage (distortion), including other unidentifiable morphological aberrations of the cytoplasm of a hepatocyte possibly exhibiting some features of apoptosis and others of necrosis (termed apocrosis or necraptosis) in the vicinity of a normal hepatocyte (right half of the section). Scale: 10 μm = 10 mm at ×10 000; 12 μm = 12 mm at ×12 000. Legend: M—mitochondria; N—nucleus; ER—endoplasmic reticulum; P1—phagocytosis; P2—secondary phagocytosis. (Reproduced with permission from Ray *et al.*, 1996. © American Society of Pharmacology and Experimental Therapeutics.)

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secondary loci that are critical for cell viability. In addition to Wyllie's (1980) experiments involving immature thymocytes, many laboratories reported that, in apoptosis, an elevated level of Ca^{2+} after a toxic insult was the cause, while endonuclease-mediated DNA fragmentation leading to cell death was the effect (Cohen and Duke, 1984; McConkey *et al.*, 1989; 1990a; Ray *et al.*, 1990). This cause-and-effect relationship was further strengthened by results of studies exposing thymocytes to agents that elevate intracellular Ca^{2+} levels (e.g. glucocorticoids, calcium ionophores, radiation, TCDD, tributyltin, natural killer cells or cytotoxic T-lymphocytes and anti-CD_3 antibodies) or agents known to prevent intracellular Ca^{2+} accumulation [e.g. ethylene glycol tetraacetic acid (EGTA)], verapamil, chlorpromazine, nifedipine, promethazine).

Because protein synthesis is also important for mediating the influx of calcium under many circumstances, it is assumed, in the case of thymocytes, that the interaction of a glucocorticoid with its plasma membrane receptor rapidly induces the synthesis of a protein that acts as a calcium pore (McConkey *et al.*, 1996). It has been confirmed that glucocorticoid-induced thymocyte apoptosis is sensitive to treatment with actinomycin D (an inhibitor of RNA synthesis) and cycloheximide (an inhibitor of protein synthesis), whereas apoptosis induced by tributyltin is not. Further, the endonuclease inhibitor, aurintricarboxylic acid, and Zn^{2+} ion are known to inhibit fragmentation of DNA in some model systems (Benchokroun *et al.*, 1995; Zalewski and Forbes, 1995). Together, these results suggest that, although not universal, apoptosis is an active process requiring RNA and protein synthesis, especially for cell death to become irreversible. Studies demonstrating the necessity for *de novo* macromolecular synthesis for the execution of this cell death pathway make the important distinction that toxic cell death (necrosis) does not require such macromolecule synthesis.

Tributyltin-induced apoptosis provides a useful and convincing example refuting the need for new macromolecular synthesis in apoptosis. Nickel, an agent that blocks non-voltage-dependent Ca^{2+} channels, blocks tributyltin-induced apoptosis. This suggests that tributyltin interacts directly with the plasma membrane of thymocytes to open Ca^{2+} channels, facilitating Ca^{2+} entry into the cell and bypassing the need for intracellular signalling and new protein synthesis. Pathways in which new protein synthesis or new gene expression is required for apoptosis after exposure to the stimulus may be referred to as *induction* pathways. Alternatively, apoptosis can result from inhibition of protein or messenger RNA synthesis, suggesting that a cell death pathway is constitutively expressed, yet kept in abeyance by short-lived inhibitors. Pathways that induce apoptosis by inhibiting protein synthesis are termed *release* pathways, and pathways that are not influenced by inhibition of protein synthesis, such as cytotoxic T-cell mediated apoptosis, have been termed *transduction* pathways (Patel and Gores, 1995). This body of thinking stresses the global role that ion regulation plays in continued cell vitality, and emphasizes, in particular, the large number of regulatory systems that rely upon the actions of calcium ions.

The integrity of no single molecule appears to be as vital to the cell as that of DNA. Its safeguarding from any entity and its perpetuation are among the most intricate and aggressively pursued goals of homeostatic regulatory controls. In the entire apoptosis literature, three distinct patterns of DNA fragmentation have so far been reported (Huang *et al.*, 1997): (i) internucleosomal cleavage of genomic DNA (Wyllie *et al.*, 1984); (ii) 30–50 kb and larger fragment cleavage (Roy *et al.*, 1992) and (iii) single-stranded nicking (Ucker *et al.*, 1992). The classic ladder-like pattern that stems from DNA breakdown at the internucleosomal nodes generates fragments in 180–200 bp increments, bearing 3′-OH DNA termini. This type of fragmentation appears long before the onset of many of the stereotypic morphological changes and continues even after the removal of the death signal. The second type of DNA degradation appears after cleavage and involves fragmentation of a higher order loop structure of chromatin (700 and 300 kb), into about 50 kb products that anchor to the nuclear matrix in the interloop or matrix attachment regions (MARS). Release of these 50 kb fragments is thought to result from cleavage in the MARS. The larger fragments appear to be intermediates in the process of DNA fragmentation and ultimately give rise to DNA ladders. However, it is not clear whether the large fragments are produced by the activity of the enzymes responsible for forming the oligonucleosomal fragments, since concentrations of divalent cations required for their formation are different from those required by latter enzymes. It is hoped that future studies will determine whether this type of chromatin cleavage is universal, and is an irreversible commitment in all apoptotic cells, since a similar pattern of DNA fragmentation has also been found in necrotic cells (Sun *et al.*, 1994; Bicknell and Cohen, 1995; Kataoka *et al.*, 1995).

The third type of fragmentation is single-stranded breaks in core-histone-associated DNA and internucleosomal areas by the Ca^{2+}-dependent endonuclease DNase-I (Peitsch *et al.*, 1993). Addition of the enzyme to isolated nuclei, and other reconstituted systems promotes the formation of DNA strand breaks which possess the same 5′-PO_4 and 3′-OH end groups as those found in DNA fragments isolated from apoptotic cells. Two closely spaced single-stranded breaks on opposing strands of DNA will result in double-stranded DNA cleavage, providing a strong signal for the apoptotic cascade, but this does not offer a mechanism to explain overall chromatin cleavage. Parrish and Xue (2006), Widlak and Garrard (2005), Huang *et al.* (1997), and others have reviewed the characteristics of candidate

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nucleases isolated from a variety of model systems. To date, DNA fragmentation factor (DFF)40/caspase-activated DNase (CAD) and EndoG (CSP-6 in *C. elegans*) have emerged as leading candidates for nuclease activity in apoptosis in mammals (see Genetic Regulation of Apoptosis). Other nucleases may also be important in various instances of apoptotic and necrotic cell death, including apoptosis-inducing factor (AIF) and its *C. elegans* homologue WAH-1, CRN1-CRN6, CYP-13, DNase-I, DNase-II, NUC1, NUC18, NUC40, NUC58, a 27 kDa nuclease and a 37 kDa nuclease. It appears that in *C. elegans*, CSP-6, WAH-1 and several CRN nucleases interact together to form a multinuclease complex called a degradasome. Endonuclease activity in cells undergoing apoptosis can be regulated directly or indirectly by protooncogenes and tumour suppressor genes, such as *c-myc*, *Ha-ras*, *bcl-2* and *p-53* (Vanderbilt *et al.*, 1982; Milligan and Schwartz, 1996; Huang *et al.*, 1997), which are discussed elsewhere in this chapter.

Transglutaminases, a family of Ca^{2+}-dependent enzymes, catalyse the formation of protein crosslinks that are biologically irreversible. No enzymes capable of hydrolysing the crosslinks formed by transglutaminases in substrate proteins have been found in vertebrates (Fesus *et al.*, 1991). These cytosolic enzymes accumulate to achieve high levels in some terminally differentiated cells and also in a number of cell types undergoing apoptosis both *in vivo* and *in vitro.* Transglutaminase is both induced and is activated during apoptosis. Although the role of transglutaminase in apoptosis is poorly understood, it is considered likely that the cross-linking of proteins stabilizes apoptotic bodies and prevents the leakage of intracellular constituents into the extracellular space, which would trigger unwanted inflammation. Intracellular activation of this enzyme can change adhesive properties (Byrd and Lichti, 1987; Nara *et al.*, 1989) and may contribute to the altered cell surface properties of the apoptotic bodies (Fesus *et al.*, 1991). Intriguingly, overexpression of the enzyme has been reported to activate apoptosis (Fesus *et al.*, 1987; Knight *et al.*, 1991).

Another group of thiol-containing and Ca^{2+}-dependent proteases, linked directly or indirectly to apoptosis, is the calpains (Martin *et al.*, 1995). Calpains, known as calcium-dependent and papain-like proteases, occur in two forms, μ-calpain and m-calpain. μ-Calpain and m-calpain have identical regulatory subunits with distinct catalytic subunits, but the Ca^{2+} requirement for activation is higher for m-Calpain. Although their distribution is ubiquitous, calpains are nonlysosomal cytosolic modulatory proteases, not digestive proteases, and are occasionally localized at the plasma membrane. Calpain substrates include cytoskeletal proteins (actin-binding proteins, e.g. fodrin/spectrin, talin and filamin and microtubule-associated proteins), membrane proteins [growth factor receptors, e.g. epidermal growth factor (receptor); adhesion molecules, e.g.integrin and cadherin; ion transporters, e.g. Ca^{2+}-ATPase; ion channels, e.g. Ca^{2+}-channel] and enzymes [kinases including protein kinase C (PKC)], calmodulin-dependent kinase; phosphatases such as calcineurin and phospholipases]. Either directly or indirectly, calpains may influence cell shape, inactivate transcription factors (*c-fos* and *c-jun*) and jeopardize signal transduction pathways by attenuating PKC activity.

Numerous Ca^{2+}-linked processes, such as activation of the interleukin-1β-converting enzyme (ICE) family of proteases, PKC, growth factor-dependent events and various signalling processes may be under the regulation of calpains (see Genetic Regulation of Apoptosis). Expression of calpains in apoptotic cells is an early event occurring long before the onset of morphological changes. The activation of calpains is thought to play a role in glucocorticoid-induced thymocyte apoptosis and FAS/APO-1-mediated apoptosis of T-cell hybridomas (Squier *et al.*, 1994). Engagement of FAS/APO-1, a tumour necrosis factor (TNF)-receptor superfamily member (CD95 in mammalian cells) with specific antibodies, or upon exposure to its natural ligand, transduces an extremely rapid, synchronous and potent apoptotic signal to a variety of cell types (McConkey *et al.*, 1996). Since calpains are Ca^{2+}-binding thiol (-SH)-containing proteins, it has been suggested that their cleavage products may be involved in promoting some of the morphological features of apoptosis. Interestingly, in *C. elegans*, the products of the cell death genes *ced-3* and *ced-4* are a sulfydryl-containing protease and a Ca^{2+}-binding protein, respectively. Other Ca^{2+}-linked processes are discussed elsewhere in this chapter.

Many different types of proteins that are either cleaved or selectively expressed during apoptosis include histone HI, topoisomerases I and II, lamins, Ul-70 kDa and the nuclear enzyme PARP (Tanaka *et al.*, 1984; Kaufmann, 1989; Ray *et al.*, 1992; McConkey *et al.*, 1995). Among these proteins, PARP has gained considerable attention. Activation of the DNA repair enzyme PARP (EC 2.4.2.30), considered another prominent biochemical event associated with apoptotic death, involves ribosylation of nuclear proteins (Marks and Fox, 1991). This process is mediated by the enzymes mono(ADP-ribosyl)transferase and poly(ADP-ribose) synthetase and involves the transfer of ADP-ribose groups from nicotinamide adenine dinucleotide (NAD) to protein acceptor sites and to existing ADP-ribose polymers. The ribosylation process facilitates DNA-excision repair and this antagonizes apoptotic processes. Massive activation of this enzyme leads to lethal depletion of NAD, and indirectly ATP. Conversely, mild activation, often seen with submaximal injury, is associated with increased repair and decreased cytotoxicity or cytolethality. Therefore, some investigators have argued that depletion of NAD and ATP induced by DNA strand breakage is a critical event in apoptotic death (Degterev and Yuan, 2008;

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Carson *et al.*, 1986; Wielckens and Delfs, 1986; see review by Ratnam and Low, 2007).

This ostensible antiapoptotic action of PARP is substantiated by observations that poly(ADP-ribose) synthetase inhibitors such as nicotinamide and 3-aminobenzamide may either increase or decrease the sensitivity of apoptosis-inducing agents, depending upon the concentration employed (Ray *et al.*, 1992; Yahya *et al.*, 1995). NAD, the PARP substrate, is also known to maintain cellular redox balance. Any gross shift in the physiological level of this component perturbs the tightly regulated myriad of metabolic functions coupled with cellular homeostasis, ultimately resulting in irreversible cytotoxicity. Likewise, ATP depletion leads to failure of ATP-dependent membrane pumps and results in the disturbance of the cellular ionic equilibrium, particularly Ca^{2+}, eventually causing activation of cytotoxic mechanisms involving proteases, phospholipases and endonucleases. In contrast to all these observations, H_2O_2, a potent necrogen which induces DNA strand breaks, is a powerful inducer of PARP in *in vitro* model systems. Hence both inhibition and activation of poly(ADP-ribose) synthetase may set the stage for endonucleases to proceed with their goals, either to complete or to oppose the final mission of cell, death by apoptosis (Nelipovich *et al.*, 1988; Ray *et al.*, 1992).

5 PROTEIN KINASES, SIGNALLING MECHANISMS AND APOPTOSIS

The past two decades have witnessed tremendous progress in understanding how extracellular signals are transduced across the plasma membrane and then transmitted within the cell to elicit specific physiological responses. Recently, intense interest has focussed upon the potential involvement of diverse signalling processes in the regulation of apoptosis (McConkey *et al.*, 1990b). However, many elements of the signalling system(s) involved in the induction of apoptosis remain unknown. The signal generated by a stimulus must be transmitted to effector molecules that often lie in different subcellular compartments. Transduction pathways vary according to trigger stimulus, but two major areas of focus are cytosolic calcium and protein kinases. Participation of Ca^{2+}-/phospholipid-dependent protein kinase (PKC) in intracellular signalling processes has been demonstrated in many cell types. Physiologically, the activity of PKC is regulated upon its translocation from cytosol to the membrane, and it is at the plasma membrane that the enzyme is activated by its endogenous activator diacylglycerol. The breakdown of phosphatidylinositol (PI) triphosphate by phospholipase C results in the production of diacylglycerol, a second messenger cofactor that mediates activation of the PKC. Besides the reversible activation of PKC by Ca^{2+}, phospholipids, diacylglycerol, unsaturated fatty acids and phorbol esters, this enzyme is also irreversibly activated by limited proteolysis involving the intracellular protease calpain (see discussion elsewhere in this chapter). One of the pathways of the transmembrane signalling system operates through the activation of PKC, whereas the other signal system involves receptor-mediated activation of cyclic adenosine monophosphate (cAMP)-dependent protein kinase. Information concerning a role for PKC in apoptosis mostly comes from studies with phorbol esters [12-0-tetradecanoylphorbol-13-acetate (TPA)]. These tumour promoters act by binding to the diacylglycerol-binding site on the enzyme and promoting its activation. Ca^{2+} mobilization and the calmodulin-dependent regulatory system appear to work in concert with TPA (Kizaki *et al.*, 1989) involving the pathway of interleukin-2 (IL-2) gene expression.

PKC-mediated phosphorylation of numerous protein substrates is associated with a wide range of biological effects, including stimulus secretion coupling, induction of cellular proliferation and differentiation, activation of nuclear transcription factors and cell surface receptors and tumour promotion. PKC is expressed in mammalian systems as a family of diverse serine-threonine kinases, consisting of at least nine isoforms differing in both substrate specificity and dependence upon Ca^{2+}. Mechanisms that explain the divergent actions of PKC are speculative at present. In haematopoietic cells, pharmacological agents that inhibit PKC activity, such as the isoquinolines, staurosporine and H7, inhibit the growth of both normal and leukaemic progenitors, suggesting the involvement of basal PKC activity in cell proliferation. In addition, PKC inhibitors block leukaemic cell differentiation in response to tumour-promoting phorboides. Given that cell differentiation is associated with a loss of proliferative potential, these observations indicate that PKC inhibitors may exert pleiotropic effects on haematopoietic cell behaviour. Efforts to define the role of PKC in the induction of apoptosis have been complicated by conflicting reports. Take, for example, the observations that activation of PKC by exposure to phorbol-12-myristate-13-acetate (PMA), either alone or in conjunction with Ca^{2+} ionophores, induces apoptosis in cells of lymphoid origin and that inhibition of PKC by exposure to H7 prevents glucocorticoid-induced apoptosis in murine thymocytes (McConkey *et al.*, 1995). These findings suggest that PKC activation promotes this process. On the other hand, the ability of PMA to oppose steroid-induced apoptosis in thymic lymphocytes and to prevent growth-factor-deprived haematopoietic cells from undergoing apoptotic cell death is instead consistent with an antagonistic influence (Jarvis *et al.*, 1994a; 1994b). Although most studies argue in favour of a role for PKC in apoptosis, it is important to appreciate that a specific contributory role in apoptosis is highly dependent on the context of events and cell types.

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Besides PKC, other kinases have been reported to be associated with apoptosis. PI3 kinase is activated by different growth factors. Activation of PI3 kinase leads to phosphorylation of AKT (protein kinase-B). Phosphorylated AKT induces phosphorylation of Bcl-xL/Bcl-2-associated death promoter (Bad) through other kinases. Phosphorylated Bad remains inactive because it is squelched by 14-3-3 proteins, resulting in cell survival. However, dephosphorylation of Bad in the absence of phosphorylated AKT results in Bad being dissociated from 14-3-3. The dissociated Bad binds to Bcl-xL releasing Bax. This leads to apoptosis. AKT also phosphorylates procaspase-9, thereby inactivating caspase-9-mediated activation of the caspase cascade (Rohn *et al.*, 1998; Cardone *et al.*, 1998; see Section 6).

Other important biochemical determinants of apoptosis include cAMP, ceramide and the redox status of a cell. The sphingomyelin pathway, initiated by hydrolysis of the phospholipid sphingomyelin in the cell membrane to generate the second messenger ceramide, is thought to mediate apoptosis in response to TNFα, to FAS ligand (Fas-L), to photodynamic therapy, and to X-rays. Generation of ceramide, a hydrolysis product of sphingomyelin, through the sphingomyelin pathway results in the induction of apoptosis in several cell lines. At least two specific intracellular targets for ceramide have been identified: (i) a membrane ceramide-activated protein kinase and (ii) a cytoplasmic ceramide-activated protein phosphatase. Ceramide promotes the formation and release of oligonucleosomal DNA fragments, produces corresponding loss of integrity of DNA, and elicits the expression of the classical morphology of apoptosis. Interestingly, ceramide-related DNA damage consists exclusively of double-stranded breaks introduced into mature DNA, but not nascent DNA. Surprisingly, apoptosis is not initiated by diglyceride or other glycerophospholipid-derived messengers (e.g. lysophosphatidylcholine, arachidonic acid or phosphatidic acid) in U937/HL60 cells. However, a report claims that Hep-G2 cells overexpressing P4502E1 undergo apoptosis upon exposure to arachidonic acid (Chen *et al.*, 1997).

As with ceramide, cAMP promotes palatal fusion which involves apoptosis of medial edge epithelial cells (Pratt and Martin, 1975). McConkey *et al.*, (1990a) have reported that agents that elevate cAMP stimulate DNA fragmentation and produce apoptosis in thymocytes act via activation of cAMP-dependent protein kinase A. In contrast, Edwards *et al.*, (1991) have observed that analogues of cAMP inhibit apoptosis. This disparity may be due to the model system tested. Similar to cAMP and ceramide, Ca^{2+} and other agents with a role in maintaining cellular redox status including antioxidants (e.g. vitamin C, vitamin E), ADP/ATP, NAD/NADH, NADP/NADPH and GSH/GSSG ratios may influence apoptotic death by varied mechanisms. Fernandez *et al.* (1994) reported that marked GSH depletion enhances the cytotoxicity of alkylating agents (e.g. melphalan, chlorambucil), switching from the pathway of apoptosis to necrosis, whereas Cotter and coworkers (Verhaegen *et al.*, 1995) claim that antioxidants including butylated hydroxyanisole (BHA) inhibit apoptosis. Similarly, agents that deplete intracellular glutathione typically render cells more vulnerable to oxidative stress-induced apoptosis (Zhong *et al.*, 1993). The redox potential of a cell turns out to be as critical to cell survival as maintaining intracellular ion homeostasis or genomic integrity. An oxidative shift by reactive oxygen species (ROS) may modify the nature of the stimulatory signal, resulting in alteration of the direction of the response (apoptosis/necrosis/proliferation). Efficient activation of NFκB-dependent genes by TNF requires that a cell be in an oxidized state, suggesting that stimuli such as TNF and PMA may exert only a limited response if the cell is not in appropriate redox equilibrium (Israel *et al.*, 1992).

In this context, growing attention is now being devoted to mitochondrial functions during apoptosis. ROS, Ca^{2+}, the mitochondrial permeability transition (MPT) and cytochrome c (cyt-c) are presumably all important for apoptosis. The first two entities, ROS and Ca^{2+}, are directly related under a number of conditions. For example, ROS stimulate Ca^{2+} release from mitochondria, and when mitochondria cycles Ca^{2+} excessively their ROS production increases (Richter *et al.*, 1996). Mitochondria can initially protect the cell against toxic increases in Ca^{2+}. However, excess Ca^{2+} cycling can culminate in vigorous perturbation of the $\Delta\psi$ (mitochondrial membrane potential [MMP]), resulting in a variety of lethal molecular lesions. Similarly, TNFα stimulates ROS production by mitochondria, and the TNFα-induced apoptotic killing of some cell types is due to ROS-induced mitochondrial Ca^{2+} cycling since apoptosis is blocked by mitochondrial Ca^{2+} uptake inhibitors (Hennet *et al.*, 1993a; 1993b). An important observation, which provides a specific clue as to which mitochondrial parameter(s) may be important for the control of apoptosis, comes from the observation that the stabilization of $\Delta\psi$, either by overexpression of a death inhibitory gene (*bcl-2*) or by the ionophore nigericin, prevents apoptosis induced by TNFα. Many laboratories have reported that $\Delta\psi$, which is the driving force for mitochondrial ATP synthesis, decreases prior to DNA damage, and that maintenance of ATP prevents apoptosis (Shimizu *et al.*, 1996).

Ca^{2+}, oxidant chemicals and a range of other inducers (e.g. oxidative stress and alteration in cellular phosphorylation state) promote the onset of the MPT in mitochondria. The MPT reflects the opening of a highly regulated pore within the inner mitochondrial membrane. Opening of high-conductance permeability transition pores causes the onset of the transition, leading to membrane depolarization, release of ions, uncoupling of oxidative phosphorylation, permeation of sucrose and other solutes

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of molecular weight less than 1500 and large amplitude mitochondrial swelling. Ca^{2+}, oxidant chemicals, Pi (inorganic phosphate) and membrane depolarization promote pore opening, whereas, Mg^{2+}, ADP, low pH and immunosuppressant ciclosporin (cyclosporine-A) favour the closed state. Oxidants, including the drug doxorubicin, are the most thoroughly studied class of inducing agents. Doxorubicin interferes with the capacity of isolated cardiac mitochondria to accumulate and retain calcium, and it does this by selectively inducing the MPT, presumably via the free-radical-mediated oxidation of critical protein thiols associated with the voltage-sensing element of the pore. Associated with this is an increased sensitivity to calcium-induced membrane depolarization and osmotic swelling. Mitochondrial osmotic swelling in response to pore formation may be responsible for the release of mitochondrial factors that are crucial in triggering the late stages of the apoptotic pathway. One such factor is cyt-c, the release of which occurs in the absence of significant changes in *Aip*, suggesting a novel mechanism for cell death commitment. cyt-c molecules from a variety of metazoan species, including the insect *Manduca sexta*, all function to induce apoptosis in *Xenopus* egg extracts. Collectively, these studies indicate that loss of mitochondrial function is a pivotal event in modulating cellular energy status and influencing cell-death processes including apoptosis (Lemasters and Nieminen, 1998; Qian *et al.*, 1999; Theruvath *et al.*, 2008).

6 GENETIC REGULATION OF APOPTOSIS

The concept of active cell death being a genetically encoded process stimulated an aggresive search for the genes involved in the regulation of cell-death. The number of ground-breaking discoveries and seminal articles published in this arena in the last decade alone exceeds the very impressive body of information accumulated in the preceding several decades. As more genetic engineering protocols and transgenic models became available, these tools produced a rapid advance of progress in this field and resulted in the discovery of many genes and vital regulatory elements across a range of model systems. In addition to the caspase-regulated pathways, **Table 5** lists of proapoptotic and antiapoptotic genes and their relationships. Active involvement of a number of these proteins has been demonstrated in either the causation or prevention of apoptotic death. Although the entire field has only grown in complexity, understanding and designing access to predictable cell death pathways may have become easier. The cornerstone for many of these findings has been the genetic blueprint of the cell death pathway described in *C. elegans*. The central components of this pathway were found to have mammalian counterparts that exhibit considerable homology and redundancy, often through multigene families. Molecular biological experiments in mammalian cells and in a wide variety of transformed cells have provided a rich and varied foundation of information and understanding.

6.1 TNFR Superfamily

The TNF family of ligands and receptors is a well characterized group of cell surface and soluble molecules capable of initiating apoptosis *in vivo* and *in vitro*. Prodeath members of this family contain a cytoplasmic death domain (DD), a protein–protein interaction motif critical for engaging downstream components of the signal transduction machinery. Following the activation of these receptors by their cognate ligands, a death-inducing signalling complex (DISC) is formed by recruitment of cytoplasmic DD-containing proteins to the receptor DD motif. Among the most carefully studied death-inducing ligand-receptor pairs is TNF/TNF receptor-associated factor type 1 (TNFR1) and CD95L (Fas)/CD95 (Apo-1). The variety of downstream effector proteins regulating and controlling apoptotic signalling cascades has resulted in links between extrinsic and intrinsic signals in order to execute a death command or a survival command. Understanding how such signals are produced and propagated is a key goal for the many laboratories studying drug and chemical-induced cell injury and cell death (**Figure 6**).

Members of the TNFR superfamily, TNFR1 (which is expressed ubiquitously), TNF receptor-associated factor type 2 (TNFR2, which is expressed primarily by the cells of the immune system) and Fas (Apo-1/CD95) primarily trigger the *extrinsic pathway* of apoptosis. TNFR1 and TNFR2, which both bind to the cytokine TNF, were the first members of the TNFR family to be identified. This superfamily includes many death receptors (DR) besides Fas and TNFR (**Figure 6**). Members of this family exhibit pleiotropic action. These receptors can trigger proliferation, survival, differentiation or death depending on the cell type, the signal type that is received by the cell, and the cellular context. Members of this superfamily play important roles in immunity mechanisms and cellular remodelling, in addition to apoptosis and cell survival. These DD proteins, TNFs and Fas-L, function as rapid inducers of cell death through the cytoplasmic tails of the receptors harbouring DDs. The amino acid sequences of these DDs may slightly vary across receptors but all share a very high degree of sequence homology. Most importantly, they provide the foundation for protein–protein interaction. The DD in a receptor tail also recruits DD-containing intracellular adaptor proteins. Upon interaction with a death ligand, the DR rapidly launch apoptosis. This scenario is well exemplified in the case of immunocompetent cells where most signals (survival and death) are

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Table 5 Genes and gene products involved in regulating apoptosis

Gene or gene product	Influence on apoptosis/putative mechanism(s)	References
Bcl-2	Inhibits/delays apoptosis, binds to *Bax and Bak*	Vaux *et al.* (1992), Hockenbery *et al.* (1993a,b), Hengartner and Horvitz (1994)
Bcl-xL	Inhibits apoptosis; binds *Bax and Bak*	Boise *et al.* (1993)
A1	Inhibits apoptosis *Bcl-2* family member, early response gene, transcriptionally induced by GM-CSF	Lin *et al.* (1993)
Mcl-1	Inhibits apoptosis; *Bcl-2* family member	Kozopas *et al.* (1993), Reynolds *et al.* (1994)
Ced-9	Apoptosis inhibitor, *C. elegans* homolog of *Bcl-2*	Hengartner *et al.* (1992), Hengartner and Horvitz (1994)
BHRF-1	Bcl-2 homolog that inhibits apoptosis, Epstein Barr Virus gene	Marchini *et al.* (1991)
ASFV HMWF5-HL	African Swine Fever Virus gene homologous to *Bcl- 2*	Neilan *et al.* (1993)
Bad	Accelerates apoptosis, interacts with *Bcl-2* and *Bcl-xL; can displace Bax from Bcl-xL*	Yang *et al.* (1995)
Bak	Promotes or inhibits apoptosis; binds to *Bcl-2, Bcl-xL,* and E1B 19K; sequence homology with *Bcl-2* within BH1 and BH2 regions	Chittenden *et al.* (1995), Farrow *et al.* (1995), Karbowski *et al.* (2006)
Bax	Promotes apoptosis, binds to *Bcl-2, Bcl-xL,* E1B 19K	Oltvai *et al.* (1993)
Bcl-Xs	Promotes apoptosis, binds *Bcl-2*	Boise *et al.* (1993)
Nbk, Bik 1	Apoptosis promoter, binds to *Bcl-2,* E1B 19K, *Bcl-xL,* and BHRF1, has only BH3 domain	Boyd *et al.* (1994)
Ced-3	*C. elegans gene,* apoptosis promoter	Yuan *et al.* (1993), Kumar *et al.* (1994)
Ced4	*C. elegans gene,* apoptosis promoter homologous to caspases and Apaf-1 in mammals	Ellis and Horvitz (1986), Yuan and Horvitz (1990)
P^{35}	Baculovirus gene, apoptosis inhibitor, binds to and inhibits caspases	Bump *et al.* (1995)
CrmA	Apoptosis inhibitor, cowpox viral protein, inhibits apoptosis induced by caspases	Gagliardini *et al.* (1994), Tewari *et al.* (1995)
MORT-1/FADD	Apoptosis promoter, binds Fas; same as FADD	Boldin *et al.* (1995), Chinnaiyan *et al.* (1995)
TRADD	Apoptosis promoter, binds TNFR-1	Hsu *et al.* (1995)
Reaper	Activator of programmed cell death in Drosophila, similarity to death domain of TNFR-1	White *et al.* (1994)
P^{53}	Apoptosis promoter, tumour suppressor gene, transactivates *Bax* expression	Yonish-Rouach *et al.* (1991), Miyashita *et al.* (1994)
C-myc	Apoptosis promoter, protooncogene, transcription factor	Fanidi *et al.* (1992), Evan *et al.* (1992)
R-ras	Apoptosis promoter, binds *Bcl-2*	Fernandez-Sarabia and Bischoff (1993), Wang *et al.* (1995)

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Table 5 (*continued*)

Gene or gene product	Influence on apoptosis/putative mechanisms	References
Rb	Involved during apoptosis, tumour suppressor	Freeman *et al.* (1994), de Jong *et al.* (1998)
C-fos	Upregulated during apoptosis, transcription factor, IER (immediate early response) genes	Buttyan *et al.* (1988), Janssen *et al.* (1997)
C-Jun	Upregulated during apoptosis, IER gene, AP-1 group of transcription factors	Estus *et al.* (1994), Pandey and Wang (1995), Walton *et al.* (1998)
Hsp70	Involved during apoptosis, heat shock protein	Buttyan *et al.* (1988)
Sgp-2/TRPM-2	Involved during apoptosis, transfer lipids (?), complement inhibitor (?), include *clusterin* in this family	Buttyan *et al.* (1989)
cdk-4/cdk-5	Involved during apoptosis, cyclin dependent protein kinases	Freeman *et al.* (1994)
cyclin DI/cyclin D3	Involved during apoptosis, cell cycle regulator	Freeman *et al.* (1994), de Jong *et al.* (1998)
egr-1	Apoptosis promoter, transcription factor	Liu *et al.* (1998)
Rp-2/Rp-S	Apoptosis promoters, integral membrane protein (?) and DNA binding protein (?)	Owens *et al.* (1991)
Fas/AP01/CD95	Apoptosis promoter, members of TNF receptor superfamily	Nagata and Goldstein (1995)
TNF-R1	Apoptosis promoter, cell surface receptor	Cleveland and Ihle (1995)
Dad-1	Apoptosis inhibitor	Nakashima *et al.* (1993)
Calpain	Accelerates apoptosis, induces proteolysis	Squier and Cohen (1997), Spinedi *et al.* (1998)
Granzyme B	Apoptosis promoter, serine protease, activates caspases, cytolytic to T cells	Shi *et al.* (1996a), Trapani *et al.* (1998)
Survival factors	Growth factors and cytokines such as IGF-1 and various interleukins that act to promote cell survival	Raff (1992), Harrington *et al.* (1994), O'Connor (1998)
TGF-β	Apoptosis promoter, growth factor, may influence Bcl-2 expression but not dependent upon Bax or p53	Oberhammer *et al.* (1992), Selvakumaran *et al.* (1994), Buske *et al.* (1997), Roberson *et al.* (1997)

prompted via these receptors located on the membrane surfaces. Prime examples of Fas-mediated apoptosis are: (i) cell removal under normal physiological conditions, (ii) elimination of activated mature T cells from peripheral blood at the end of an immune response, (iii) deletion of self-reacting B cells, (iv) killing of virus-infected cells by cytotoxic T lymphocytes and (v) killing of inflammatory cells at the site of immune privileged organs. Since TNFRs are associated with both inflammatory and apoptotic responses, interaction of trimeric TNF with TNFR leads to the trimerization of TNFR molecules. This leads to binding of the DD of the TNFR-associated death domain protein (TRADD) to the DD of TNFR. TNF-signalling is linked to the Fas signalling pathway through interaction of TRADD with the Fas-associated death domain protein (FADD), and binding of FADD to TRADD leads to the propagation of apoptotic death through activation of caspase-8, as in the case of the Fas and Fas-L interaction. However, interaction of TRADD with TRAF2 and receptor-interacting protein (RIP) leads to the activation of NFκB or c-Jun NH_2-terminal kinase (JNK)/activator protein (AP)-1, which in turn leads to the induction of proinflammatory and immunomodulatory genes (**Figure 11**). NFκB activation to its full capacity usually stalls apoptosis, since the second complex recruits caspase-8 inhibitor FADD-like IL-1β-converting enzyme (FLICE) inhibitory protein (FLIP). The activation of caspase-8 via proximal

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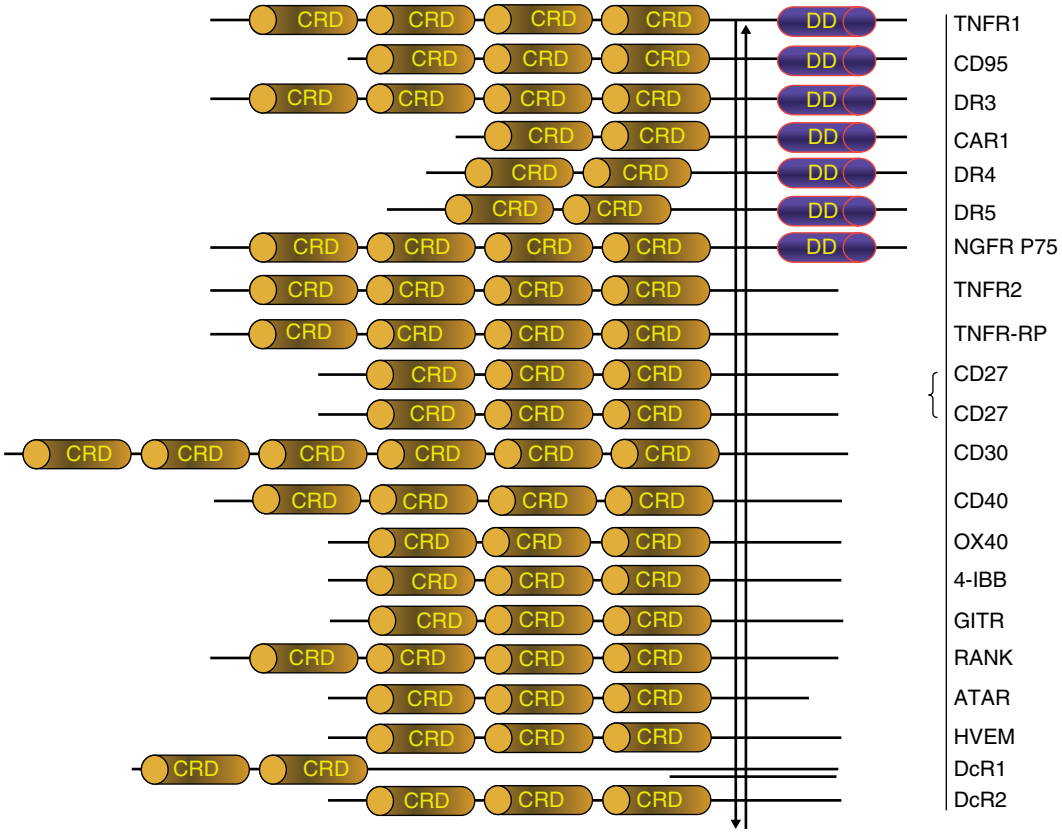

Figure 6 These TNF death receptor superfamily proteins have extracellular ligand-binding regions characterized by variable numbers of cysteine-rich (CRD) repeats. Death receptors contain death domains (DD) in the intracellular regions, which are essential for apoptosis signalling and cell death. Decoy receptors DcR1 and DcR2 compete with DR4 and DR5 for binding to TRAIL. DcR3 is a soluble receptor for FasL. The members of the TNF receptor superfamily function structurally as trimers and multimers of trimers. Single polypeptide chains are shown for simplicity. Proteins shown are mammalian and CAR1 from chicken. The TRAF family consists of six distinct proteins. Each except TRAF1 contains a ring finger domain, a zinc-finger motif, an amino-terminal TRAF domain and a carboxy-terminal TRAF domain. TRAF binds indirectly to the death domain of the receptor. TRAF2, TRAF5 and TRAF6 function as adaptor proteins that link the cell-surface receptors to downstream kinase cascades, resulting in the activation of NFκB and AP1. Neither TRAF1 nor TRAF3 activates kinase cascades. (With permission, from the Annual Review of Biochemistry, Volume 69 © 2000 by Annual Reviews.)

death signals can further activate caspase-3, cleave BH3-interacting domain death agonist (Bid) forming truncated Bid (tBid) and turn on mitochondria-centric death-promoting events. The Bid cleavage product tBid migrates to mitochondrial membrane prior to its action. Another well-known pathway involves the following: (i) caspase-8 cleaves procaspase-3 directly and activates it, (ii) the active caspase 3 cleaves DFF-45 into two heterodimeric factors DFF40 and DFF45, (iii) cleaved DFF45 dissociates from DFF40, inducing oligomerization of DFF40 that shows very high DNase activity and ultimately causes orderly internucleosomal genomic cleavage, considered a prime hallmark of apoptosis (**Figures 6** and **10**).

Another TNF family member, designated TNF-related apoptosis-inducing ligand (TRAIL), or alternatively APO-2, is also known as a rapid inducer of apoptosis in a broad range of tissues and a wide variety of transformed cell lines of diverse origin. Interest in TRAIL has risen because of its reported ability to induce apoptosis in tumour cells while bypassing normal cells. The presence of cysteine-rich domains (CRDs) characterizes the TNFR superfamily that mediates binding between ligands and these types of transmembrane domain receptors. Among these DR, the potential to induce apoptosis by TNFR1, Fas and the TRAIL receptors, DR4, DR5 and DR6 have been the centre of several investigations. To date, decoys identified for TRAIL are DcR1, DcR2 and osteoprotegerin (OPG), and a soluble Fas decoy receptor, DcR3. Interestingly, however, only DR4 and DR5 can trigger apoptosis and decoy receptors fail to elicit the activation of the downstream apoptotic signalling pathway since they lack a functional DD. This raises the issue as to how the signalling initiated by engagement of TNFR superfamily members is transduced within the cell. In response to ligand binding,

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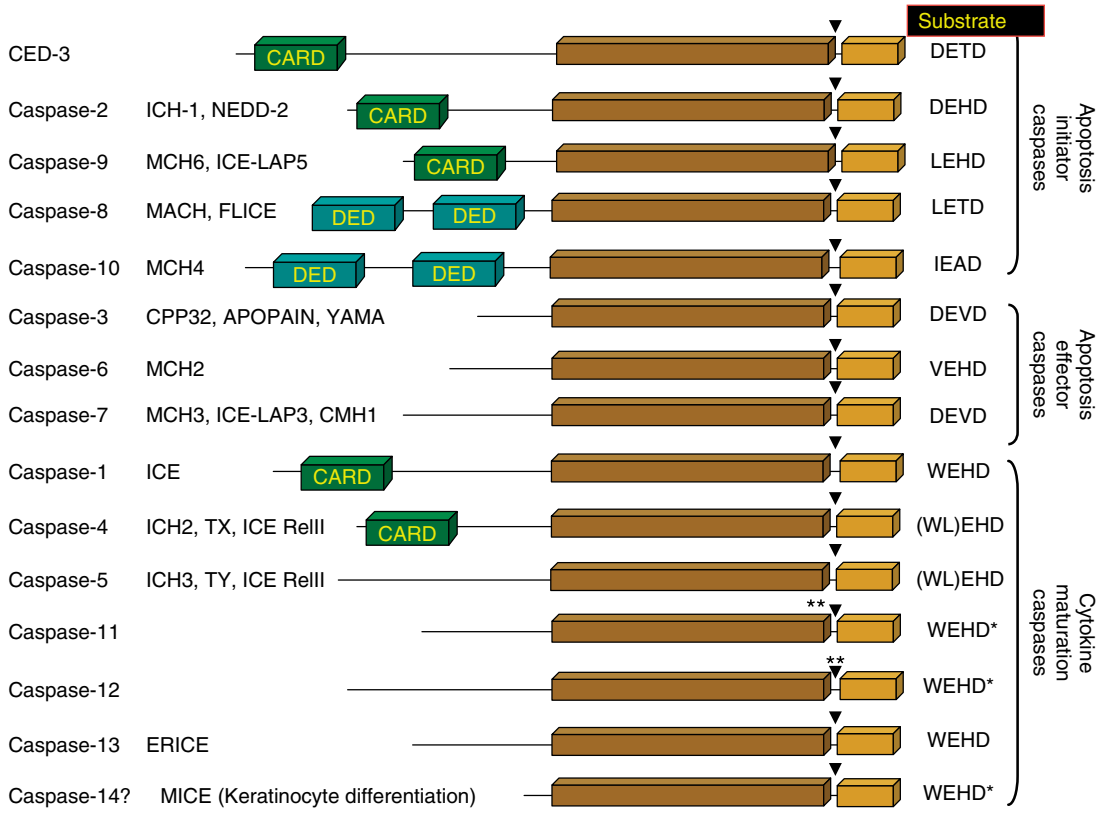

Figure 7 Classification and specificity of caspase enzymes. Caspases are cysteine aspartic acid-specific proteases because they cleave their substrates after specific tetrapeptide motifs (P4-P3-P2-P1), where P1 is an Asp residue. Members of this family are subdivided into initiators that are able to autoactivate and initiate the proteolytic processing of other caspases, and effectors that are activated by other caspase molecules and execute the final stages of apoptosis. Human caspases 1–10, mouse caspases 11–14 and *C. elegans* Ced-3 target endogenous substrates that are cleaved by effector caspases during the onset of apoptosis. All caspases have a similar structure comprised of a propeptide domain followed by a large and a small subunit. Large brown and small tan bars represent large and small subunit cleavage products, and arrow heads show cleavage sites. The propeptide can be of variable length and, in the case of initiator caspases, can be used to recruit the enzyme to activation scaffolds such as the APAF1 apoptosome. Two distinct, but structurally related propeptide motifs have been identified, the caspase recruitment domain (CARD) and the death effector domain (DED). These domains facilitate interaction with proteins that contain the same or similar motifs. Caspase activation is initiated through proteolytic processing of the caspase to generate large and small subunits that assemble into heterodimers. This rearranges the caspase active site into the active conformation. Caspases function as heterotetramers formed through dimerization of two caspase heterodimers. Initiator caspases exist as monomers in healthy cells, and effector caspases are present as preformed dimers. Not all mammalian caspases participate in apoptosis. Caspase-1, -4, -5 and -12 are activated during innate immune responses and in the regulation of inflammatory cytokine processing (such as IL1β and IL18). Caspase-12 is expressed as a truncated, catalytically inactive protein in most humans. A subset of individuals of African descent express full-length caspase-12 and appear to be more susceptible to inflammatory diseases. 400 substrates for the mammalian caspases have been identified, but the significance of many of these cleavage events remains unknown. The caspase proteases have overlapping substrate specificities that suggest partially overlapping functions. For example, despite evidence for a central role for caspase-3 in apoptosis, mice deficient in this enzyme display a defect in apoptosis only in the brain, suggesting that in most other tissues enzymes such as caspase-1 and -7 function redundantly with caspase-3. Known caspase inhibitors include Caspase-1: YVAD, DEVD, WEHD, IAP, p35, crmA; Caspase-2: WEHD, YVAD, DEVD, IETD, AEVD, IAP, p35; Caspase-3: WEHD, YVAD, IETD, DEVD, p35; Caspase-4: IETD, AEVD, YVAD, DEVD; Caspase-5: AVED, IATD; Caspase-6: VEID; Caspase-7: WEHD, YVAD, DEVD, p35; CMH-1; Caspase-8: YVAD, crmA; Caspase-9: YVAD, WEHD, AEVD; and Caspase-10: YVAD, WEHD, AEVD, DEVD. ** = aspartate cleavage site is not known;* caspase 11–14 = mouse caspases. (With permission, from the Annual Review of Biochemistry, Volume 69 © 2000 by Annual Reviews.)

various proteins are recruited to specific domains within the cytoplasmic tails of these receptors. It is these downstream effectors, which vary in both structure and function, that determine signalling outcomes. Some effectors are enzymes, such as kinases, phosphatases and proteases, some are adaptor proteins that serve to recruit still more signalling intermediaries and some are regulatory proteins. Understanding the nature and

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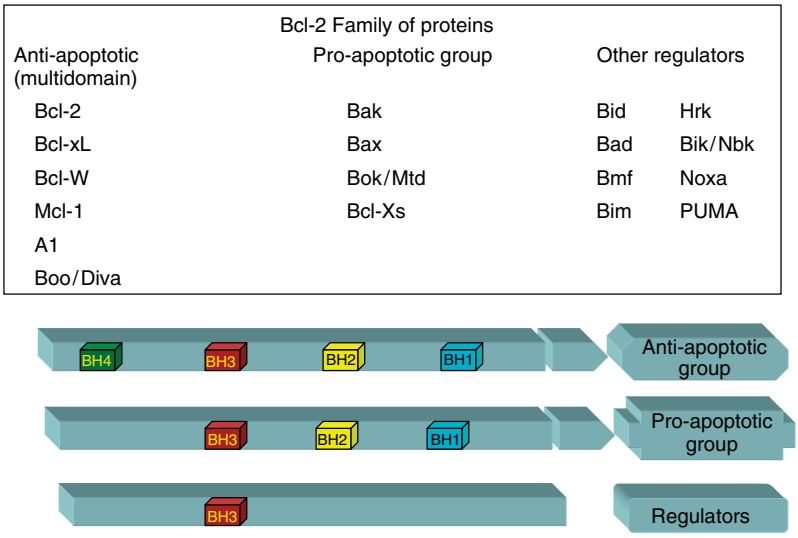

Figure 8 The Bcl-2 family of anti- and pro-apoptotic proteins. This group of proteins is comprised of three subfamilies that contain between one and four Bcl-2 homology (BH) domains. Members of the anti-apoptotic group contain all four BH domains. Most members of this subfamily also contain transmembrane domains (TM) and are therefore typically associated with membranes. The pro-apoptotic Bax-like subfamily members lack BH4 domains and promote apoptosis by forming pores in mitochondrial outer membranes. Members of the BH3-only subfamily form a structurally diverse group of proteins that only display homology within the small BH3 motif. The mammalian BH3-only protein family currently includes eight members (Bid, Bad, BmF, Bim, Hrk, Bik/Nbk, Noxa, PUMA), all of which promote apoptosis when over expressed. These proteins share little sequence homology apart from the BH3 motif, and are regulated in distinct ways. Noxa, PUMA and, to a lesser extent, Bid are transcriptionally upregulated by p53. DNA damage therefore results in the increased synthesis of these BH3-only proteins in a p53-dependent manner. Bid is activated through proteolysis by caspase-8. Bad is inactivated through growth-factor-receptor signals that result in phosphorylation of this BH3-only protein at several sites, leading to sequestration by 14-3-3 proteins. Bim is tethered to microtubules and Bmf is tethered to actin microfilaments, possibly through interactions with dynein light chain-1 and dynein light chain-2, respectively, although this remains to be conclusively demonstrated. Disruption of the cytoskeleton can liberate these proteins to promote apoptosis. Growth factors stimulate the ERK-dependent phosphorylation of Bim, and this results in proteasome-mediated degradation of this BH3-only protein. Conversely, growth factor withdrawal results in a decline in ERK signalling and elevated levels of Bim. Bcl-2 and its close relatives (Bcl-xL, MCL1, BCL2A1, Bcl-W and Bcl-B) have four Bcl-2 homology (BH) domains and all block apoptosis. Members of the anti-apoptotic group of Bcl-2 proteins inhibit apoptosis by preventing BH3-only protein-induced oligomerization of the proapoptotic Bcl-2-family members Bax and/or Bak in mitochondrial outer membranes, which would otherwise lead to the efflux of cytochrome c and other mitochondrial intermembrane space proteins. The antiapoptotic Bcl-2 proteins differentially bind to BH3-only proteins. Some BH3-only proteins, like Bid and Bim interact with essentially all anti-apoptotic Bcl-2 proteins, whereas others, like Noxa, interact only with certain Bcl-2-family members. (With permission, from the Annual Review of Biochemistry, Volume 69 © 2000 by Annual Reviews.)

functions of these downstream effectors will allow us to explain how particular cellular outcomes result from signalling through a limited number of receptors.

Experiments employing embryonic fibroblasts from caspase-deficient mice or using specific caspase inhibitors demonstrate that caspase-8 is essential for DR4-, DR5- and DR3-transduced apoptosis. However, it remains to be determined whether these receptors activate caspase-8 through FADD and/or TRADD or whether they utilize adaptors that are unknown. These receptors also have the ability to compete for the binding of death ligands to block apoptosis triggered by DR. Preassembly or self-association of DR through a distinct functional domain in the extracellular domain of the DR, termed the preligand assembly domain (PLAD), is critical for ligand binding. The activation of a conformational change or the formation of higher-order receptor complexes to acquire signal competence is achieved upon ligand binding to the preformed TNFR complex. Formation of a DISC that recruits the corresponding adaptors through the DD results from ligation of DR with ligands. Once the DISC is fully assembled and activated (death receptors and adaptors), effector molecules such as procaspase-8 bind to it and induce the activation of the downstream apoptotic signalling pathway. The aggregation of the receptors is also followed by internalization or endocytosis into an endosomal pathway. Inhibition of this internalization either chemically or by mutation may abrogate the biological response.

TNFR2, CD30, CD40, lymphotoxin receptor, the osteoprotegerin ligand (OPGL), receptor activator of

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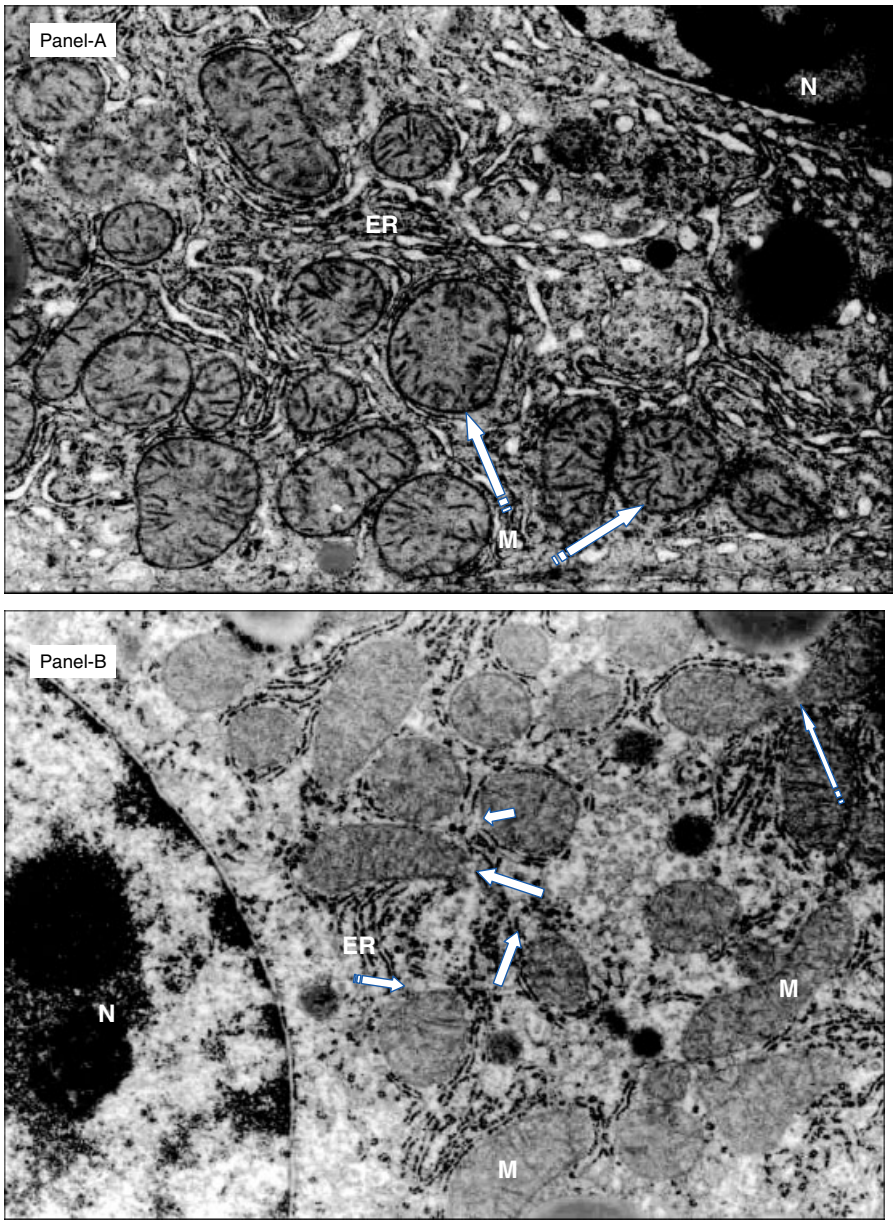

Figure 9 Dimethylnitrosamine-induced mitochondrial changes. Electron photomicrographs of mouse liver show ultrastructural details of mitochondria over time from animals treated with 100 mg kg^{-1} dimethylnitrosamine i.p. (A) shows the presence of abundant mitochondria of different shapes and sizes in a normal control animal. See intact outer peripheries of mitochondrial membranes (arrows) and prominent inner folds. (B) shows mitochondrial membrane alterations in liver after dimethylnitrosamine. This section shows the presence of abundant mitochondria of different shapes and sizes. However, the outer mitochondrial membranes are no longer intact (arrows) in several mitochondria while inner folds remain prominent. These changes in outer mitochondrial membranes accompany cytochrome c release via mitochondrial transition pores (arrows). Scale: 1 μm = 28 mm at ×28 000. Legend: M—mitochondria; N—nucleus; ER—endoplasmic reticulum. (Reproduced with permission from SD Ray, Long Island University.)

NFκB (RANK) and B-lymphocyte activating factor (BAFF) receptors constitute a second class of receptors in the TNFR superfamily. Interestingly, DDs are missing from the cytoplasmic tails of these receptors. These receptors have sequences which allow them to interact with a special set of intracellular adaptors called TNF receptor-associated factors. Cytoplasmic tails of the second class of receptors bind to only five members (TRAF1, TRAF2, TRAF3, TRAF5 and TRAF6) out of the six TRAF proteins (TRAF1–TRAF6) identified to date. Transgenic mouse models that lack expression of TNFR1 demonstrate that this receptor is involved in signalling leading to inflammation and cell survival. Ironically, however, TNFR1$^{-/-}$ mutants display impaired apoptosis. In response to a single ligand, TNF, TNFR1 mediates not only survival and inflammatory

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signalling, but also apoptotic signalling, whereas, in response to TNF binding, survival and inflammation signals are primarily transduced by TNFR2. The Fas (CD95) receptor, which was originally discovered as the product of the mutated gene in MRL (Murphy Roths large)/lpr (lymphoproliferation) mice, mediates mainly signals for apoptosis in response to the binding of its ligand FasL. Fas- or FasL-deficient mice show massive lymphoadenopathy and deregulated lymphocyte homeostasis due to a lack of apoptotic death (Strasser *et al.*, 2000; Mak and Yeh, 2002; Jin and El-Diery, 2005; Elmore, 2007; Schütze *et al.*, 2008).

A relatively new TNF family member, BAFF (also called BlyS, TALL-1 or zTNF4), has recently drawn some attention due to its ability to facilitate B-cell survival. Mature B-cell hyperplasia coupled with systemic lupus erythematosus-like symptoms are associated with mice overexpressing BAFF. In contrast, a complete loss of follicular and marginal-zone B lymphocytes is observed in BAFF-deficient mice. Moreover, mice lacking BAFF exhibit deregulated T-cell-dependent and T-cell-independent antibody response. B-cell maturation antigen (BCMA), transmembrane activator and calcium modulator, cyclophilin ligand interactor (TACI) and BAFF-R are accessible to BAFF for binding. Binding to BAFF can instigate diverse signalling pathways, including the activation of caspases, the translocation of NFκB, or the activation of mitogen-activated kinases such as JNK or extracellular signal regulatory kinase (ERK). BAFF and APRIL [a proliferation-inducing ligand or TRDL 1α (TNF-related death ligand 1α)] were recently found to be trophic factors in lymphocyte malignancies and immune-related disorders. They have been identified in bronchial tissue, and a number of immune-related and immune-independent tissues (spleen, liver, lung, heart, intestine, kidney and thymus) which makes them toxicologically important.

Moment-to-moment differences in the balance between cytotoxic and cytoprotective factors and a gross imbalance between oxidants and antioxidants can dictate the severity of toxicant-induced organ injury. This general framework has been used to develop investigational models that can be studied to characterize molecular targets for future therapeutic interventions. At least in this field, several investigators have identified TNF as a critical mediator of toxicant-mediated organ injury, particularly in the liver. Hepatotoxins including tetrachloromethane (CCl_4), dimethylnitrosamine (DMN), D-galactosamine, ethanol, fumonisin B1, lipopolysaccharide, methotrexate, several peroxisome proliferators (clofibric acid, bezafibrate, ciprofibrate and nafenopin) and the plasticizer di-(2-ethylhexyl)phthalate influence liver cell death via modulation of TNFα. Similarly, TNF has also been shown to play an important role in apoptosis of both chronic and acute human neurodegenerative diseases, antineoplastic drug doxorubicin-induced cardiomyopathy, arsenic-induced immunotoxicity and oxygen-induced pulmonary toxicity. The apoptosis-enhancing effect of Fas-FasL has been reported in liver injury caused by acetaminophen (paracetamol) (Tagami *et al.*, 2003a), CCl_4 (Jameel *et al.*, 2009), ethanol (Tagami *et al.*, 2003b), bleomycin-induced dermal sclerosis (Yammamoto *et al.*, 2007), 5-fluorouracil-induced breast cancer cell death (Chhipa and Bhat, 2007), cadmium-induced podocyte apoptosis (Eichler *et al.*, 2006), valproic acid-induced prostate cancer cell death (Angelucci *et al.*, 2006) and nephrotoxicity induced by cyclosporine A, cisplatin and gentamicin (see reviews by Rezzani *et al.*, 2008; Servais *et al.*, 2008).

6.2 DISC (Death Inducing Signalling Complex)

Initiation of apoptotic death can be either receptor-mediated (e.g. Fas/CD95) or nonreceptor chemically mediated (stress-induced). Usually when Fas mediates cell death, a multiprotein complex DISC is assembled internal to the plasma membrane, which activates downstream caspases, setting the stage for the cell suicide process. The DISC was first described in the FasL-Fas apoptotic signalling pathway. Binding to Fas-L promotes receptor trimerization that in turn results in intracellular clustering of DDs followed by complex internalization via an endosomal pathway. This allows an adaptor protein called FADD to associate with the receptor through an interaction between homologous DDs on both molecules. FADD contains a death effector domain (DED) that allows binding of procaspase 8 to the CD95–FADD complex. Procaspase 8 (also known as FLICE) associates with FADD through its own DED. Caspase-8, the main initiator caspase in CD95 signalling, is expressed as two isoforms, caspase-8/a and -8/b. Both are recruited to the activated CD95 receptor. These molecules, FADD and caspase-8 are the key components of the CD95 DISC (**Figures 10** and **11**). FasL-induced clustering of Fas, FADD and caspase-8 within the DISC leads to autoproteolytic processing of caspase-8 by induced proximity and dimerization, followed by the release of the processed active proteases. Following the autoproteolytic cleavage of the enzyme, caspase-8 is released from the DISC as an active heterotetramer containing two p18 and two p10 subunits. Recently one study showed that all cleavage products of procaspase-8 actually remain bound to the DISC, including the p10 and p18 subunits of caspase-8. Studies in FADD and caspase-8 deficient mice indicated that both are required for FAS-mediated apoptosis.

Cells can be divided into two types according to the requirements of their mitochondrial pathway in FAS-induced apoptosis. In *type I* cells, processed caspase-8 is sufficient to activate directly other members of the caspase family, whose action on defined substrates leads

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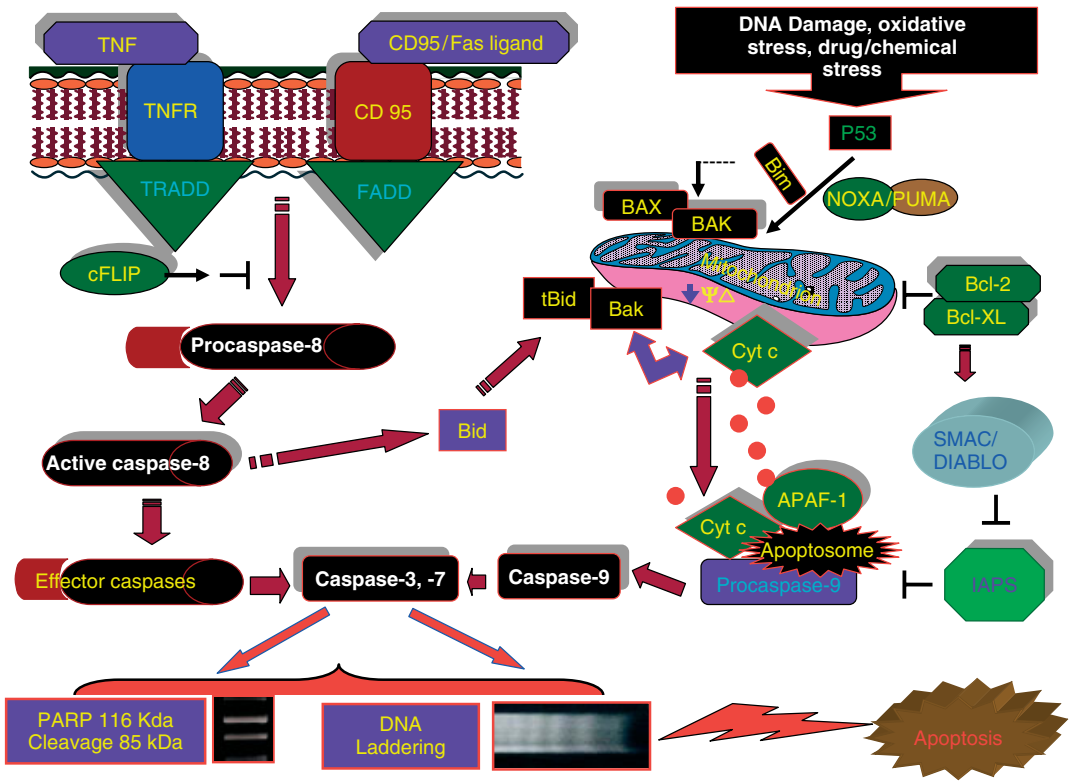

Figure 10 Pathways of apoptosis. The extracellular binding of ligands such as TNF-α or Fas-L to their respective death receptors initiates the extrinsic signalling cascades of apoptosis. Ligands bind to activate their receptors by inducing receptor trimerization. Once stimulated, the intracellular binding domains of these receptors containing death effector domain (DED) motifs initiate the assembly of the death-inducing signalling complex (DISC) and the recruitment and assembly of initiator caspase-8 and -10. Once activated DED-containing proteins TRADD and FADD have recruited procaspase-8 and -10 to the receptor complex, the latter undergo autocatalytic activation involving cleavage at specific sites. Activated caspase-8 activates caspase-3 via two mechanisms. In the first mechanism, caspases-8 cleaves Bid to tBid which translocates to mitochondria where it triggers cytochrome c release. t-Bid mediates the assembly of proapoptotic Bcl-2 family members like Bax and Bak into hetero-oligomeric complexes that form pores in the outer membrane of the mitochondria, resulting in the release of apoptosis-regulating factors such as cytochrome c. Bax and Bim can translocate to mitochondria in response to death stimuli, including survival factor withdrawal. These events are triggered following DNA damage, where p53 induces the transcription of Bax, Noxa and PUMA. Pro-caspase-9, APAF1 and cytochrome c form the apoptosome complex, which induces activation of caspase-9 and -3, and begins the cell demise process. The release of cytochrome c can be inhibited by Bcl-2 and related antiapoptotic proteins. Bcl-2 is regulated by binding to Bad, and for Bcl-2 to exert its antiapoptotic activity, the Bcl-2/Bad complex must be broken down by phosphorylation of Bad by Akt. The intrinsic (mitochondrial) and extrinsic (death-receptor) pathways converge at the level of caspase-3 activation. Its activation and activity are antagonized by IAP proteins, which themselves are antagonized by Smac/DIABLO protein released from mitochondria. In the second mechanism, activated caspase-8 directly cleaves and activates procaspase-3. Active caspase-3 cleaves DFF-45 in the heterodimer DFF40/DFF45. Cleaved DFF45 dissociates from DFF40, inducing oligomerization of DFF40 that has high DNase activity. The active DFF40 oligomer causes internucleosomal DNA fragmentation and the DNA ladder, considered hallmarks of apoptosis. This sequence is the same as proposed for CAD-ICAD cleavage, dissociation and orderly DNA fragmentation. Caspases cleave nuclear lamins, causing the nucleus to break down and lose normal structure. Fas-induced apoptosis can be effectively blocked at several stages by FLICE-inhibitory protein (FLIP), by Bcl-2, or by the cytokine response modifier A (CrmA). (Reproduced with permission from Jin and El-Diery, 2005. © Landes Bioscience.)

to the execution phase of apoptosis. In *type II* cells, efficient activation of effector caspases by Fas depends on an amplification loop that relies on caspase-8-mediated cleavage of Bid and subsequent release of mitochondrial proapoptotic factors such as second mitochondria-derived activator of caspase (SMAC)/direct inhibitors of apoptosis protein binding protein with low pI (Diablo) or cyt-c to drive the formation of the caspase-9-activating apoptosome. Active caspase-9 activates the executioner caspase-3, which in turn activates caspase-8, thereby completing a positive feedback loop. Another DED-containing protein caspase-10 has also been shown to be recruited to the DISC (**Figures 6** and **11**). Although *in vitro* studies show that caspase-10 shares similar

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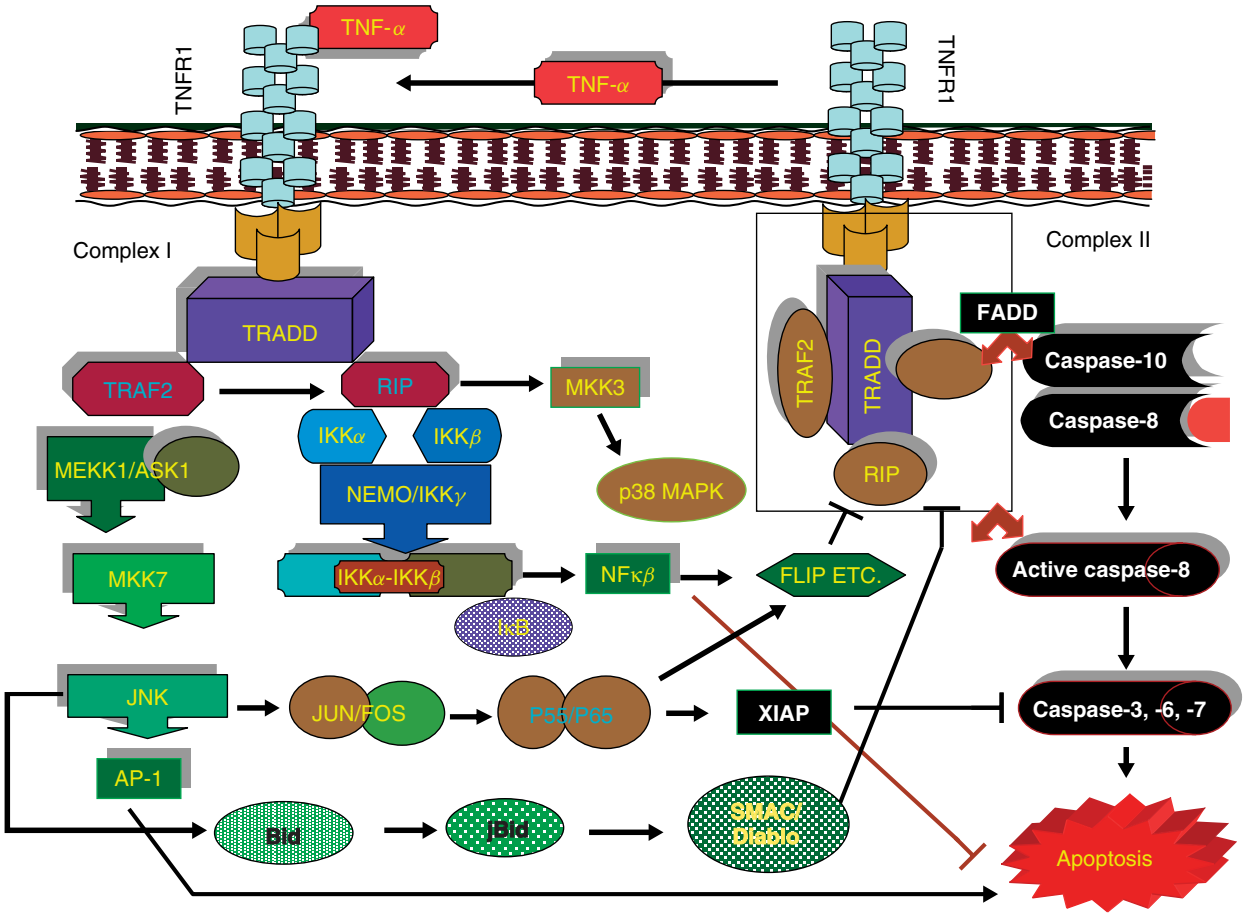

Figure 11 Cross-talk between multiple pro-survival and pro-death signaling pathways. Recent studies have demonstrated that TNFR1-induced apoptosis involves 2 sequential signaling complexes. Complex I, the initial plasma membrane-bound complex, consist of TNFR1, the adaptor TRADD, the kinase RIP1, and TRAF2 and rapidly signals activation of NFκB or JNK. In a second step, TRADD and RIP1 associate with FADD along with caspase-8, forming a cytoplasmic complex, complex II. When NFκB is activated by complex I, complex II harbors the caspase-8 inhibitor FLIP-L and the cell survives. Otherwise, cells undergo apoptosis through a complex II-mediated signaling pathway. Thus, TNFR1-mediated signal transduction includes a checkpoint, resulting in cell death (via complex II) in instances where the initial signal (via complex I and NFκB) fails to be activated. A large intracellular signaling complex forms after binding of TNF-α to its receptor and trimerization. The intracellular domain associates with the IKK complex, of which IKKα and IKKβ are essential components. NIK phosphorylates and activates these kinases, allowing them to dissociate and cause phosphorylation of IκB, the inhibitor of NFκB. The phosphorylated inhibitor can be ubiquinated, and can undergo proteolysis. Liberated NFκB translocates to the nucleus and activates gene transcription. NFκB plays an important role in TNF-α mediated expression of adhesion molecules, enzymes, cytokines and chemokines in endothelial cells. When sufficient copies of the inhibitory factor IκB are made, NFκB is sequestered in the cytoplasm using p55/p65 proteins. This process regulates inflammation. This figure depicts additional pathways such as: (i) MEKK1/ASK1, JNK, AP1 route, (ii) TRADD, RIP, IKK?/IKK?, NFκB and FLIP route, (iii) TRAF2, RIP, MKK3, p38/MAPK route, (iv) TRAF2, FADD, caspase-10, -8, and caspase-3, -6, -7 route, and (v) TRAF2, MKK7-JNK, JUN/FOS, p55/p65, XIAP, either caspase 3, -6, -7 and/or FLIP route and their impact on cell surviva/death. For example, TNFα-mediated caspase-8 cleavage and apoptosis require the sequential activation of a cascade involving JNK, Bid, and Smac/DIABLO. Typically JNK activation, which induces caspase 8-independent cleavage of Bid at a distinct site to generate the jBid, can favor apoptosis by triggering the release of Smac/DIABLO into the cytosol, inhibiting the TRAF2-IAP1 complex and consequently activating caspase-8. Translocation of jBid to mitochondria leads to preferential release of Smac/DIABLO, but not cyt-c. Released Smac/DIABLO disrupts the TRAF2-cIAP1 complex. It has been proposed that inhibition imposed by TRAF2-cIAP1 on caspase-8 activation must be relieved for the JNK pathway to induce apoptosis. Additionally, RIP (receptor-interacting protein) can also cause receptor stimulation of the inhibitor of the IKK cascade, NFκB. A third complex involves TNFR-associated factor 2 (TRAF2), which couples receptor engagement to the JNK cascade, stimulating transcription factor AP-1. Death receptor DR3, which was identified by virtue of its homology to TNFR1, assembles signaling complexes that are similar to those of TNFR1. See genetic regulation of apoptosis section for further details. (Adapted with permission from Jin and El-Deiry, Cancer Biol. Int., 2005, © Landes BioScience.)

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features with caspase-8 in many aspects, findings *in vivo* demonstrated that it cannot substitute for caspase-8 in the DISC to trigger apoptosis. The reason why caspase-10 is associated with the DISC while not being essential for cell death signalling remains unclear.

6.3 Apaf-1 and the Apoptosome

Apaf-1, the central component of the apoptosome, consists of three different domains: (i) the N-terminal protein interaction domain known as the caspase activation and recruitment domain (CARD), which can bind via a homophilic interaction to the CARD of procaspase-9; (ii) a nucleotide-binding domain that shares homology with the *C. elegans* homologue CED-4 and (iii) a C-terminal regulatory domain containing multiple repeats of tryptophan and aspartate residues. The CARD is responsible for interactions with the prodomain of caspase-9. This interaction is essential to the recruitment and activation of procaspase-9. The CARD and the nucleotide-binding domains are responsible for oligomerization of Apaf-1 in the presence of cyt-c and deoxyadenosine triphosphate (dATP). WD40 repeats are thought to interact with cyt-c, as removal of this domain in Apaf-1 results in constitutive binding and activation of caspase-9. Apaf-1 exists in an inactive conformation in cells and is activated through binding to cyt-c and dATP. Structural analysis suggests that binding of nucleotides may induce significant conformational changes in Apaf-1 and that these conformational changes may drive the formation of the caspase-9-activating apoptosome.

Assembly of activated Apaf-1 into an apoptosome is complex. The structure of the apoptosome, determined by cryoelectron microscopy, reveals a wheel-shaped complex with sevenfold symmetry. The CARD and the expanded nucleotide-binding domain of Apaf-1 are located at the central hub, whereas the WD40 repeats constitute the extended spokes. Importantly, the form of this complex confirms the structural involvement of cyt-c in the formation of the apoptosome. Docking of caspase-9 to the apoptosome results in a dome-shaped structure. However, the structure of the bulk of caspase-9 remains only partly identified. The complex structure of the apoptosome is a limitation for accurate identification of domain boundaries and interfaces. Therefore, the molecular underpinnings of caspase-9 activation remain unclear. Caspase-9 plays a role in brain development and its deficiency results in embryonic lethality coupled with brain malformation. The rate of apoptosis declines in neuronal tissues derived from such mutant embryos. Caspase-$9^{-/-}$ cell types show that, like caspase-3, caspase-9 is not required in all cell types for all apoptotic events. Caspase-9 is necessary for the apoptotic death of embryonic stem cells and of thymocytes exposed to dexamethasone or γ-irradiation. However, caspase-$9^{-/-}$ thymocytes readily undergo apoptosis in response to UV irradiation or engagement of FAS (**Figures 6–11**).

6.4 *Bcl*-2: The Monarch of Cell Survival Genes

The three phases of cell death—induction, initiation and execution—characterize the progression of apoptosis. The induction phase corresponds to the initiation of the apoptotic signal, which has a specific goal. The stimuli may be composed of combinations of altered physiological signals, chemical or physical damage, or lack of survival signals. Cell death signals are predominantly transmitted via two major biochemical routes in mammalian cells: the *intrinsic* pathway and the *extrinsic* pathway (**Figures 5** and **11**; see review by Orrenius *et al.*, 2007).

The intrinsic pathway normally responds to proapoptotic signals, such as the interplay between *Bcl*-2 family members that share the BH3 domain (primarily *Bcl*-2 and *Bax*) and relies heavily on mitochondrial events. *Bcl*-2 and its family members have emerged as central regulators of the mitochondrial pathway of apoptosis (**Figure 8**). These proteins manipulate mitochondrial functions and carefully orchestrate events either to initiate the death pathway and release proapoptotic factors such as cyt-c, endonuclease-G and AIF, or to forestall the onset of the death pathway. The release of cyt-c leads to Apaf-1-mediated activation of initiator caspase-9, which in turn activates effector caspases. The *extrinsic* pathway, in contrast, is triggered by the ligation of DR belonging to the TNF-receptor superfamily. Stimulation of the Fas/TNFR receptor family by a toxicant can trigger a canonical extrinsic apoptosis pathway. Several genes and gene products participating in this process are listed in **Table 5**, **Figures 5** and **8**.

The maintenance of normal cell number in tissues (homeostasis) requires a delicate balance between the rate of cell division and the rate of cell death. When this balance favours the direction of increase in the rate of proliferation, a decrease in the rate of cell death, or both, it leads to benign or malignant tumours. Oncogenes positively influence cell proliferation and growth, whereas tumour suppressor genes (antioncogenes) act as negative regulators of growth. Since apoptosis essentially counterbalances cell proliferation, genes involved in apoptosis are essential to regulating growth. Based on this assumption, cell death research has primarily focussed upon death regulation and survival genes. Death genes maneuver the intracellular microenvironment via one-way traffic to set up execution by transducing signals, triggering other molecules and/or driving the cell cycle to a required point for exit, following the death pathway. Once apoptosis is initiated in a productive context, it

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proceeds via the action of gene products that are common to all types of cell death. This notion is further substantiated by the fact that once apoptosis is turned on, it typically can be delayed, but cannot be stopped.

In this arena, the first anti-apoptotic gene to be clearly identified in humans was Bcl-2 (B-cell lymphoma-leukaemia-2 gene), cloned from the breakpoint of the t(14 : 18) translocation found in the majority of follicular lymphomas. The discovery of the Bcl-2 oncogene helped to stimulate recognition of the concept that gene products that modulate the susceptibility of certain cell types to apoptosis may play an important role in the process leading to malignant transformation. This is primarily due to the survival of cells in inappropriate physiological situations. In both pathological and physiological conditions, the *Bcl*-2 gene product has emerged as a critical regulator of apoptosis. Although *Bcl*-2 is a proto-oncogene, the mechanism by which the Bcl-2 protein promotes tumour growth is very different to other oncogenes. Since *Bcl*-2 has the unique ability to rescue cells that are destined to die without affecting the rate of proliferation, it is possible that its location may be a key feature of its function. *Bcl*-2 is involved in maintaining homeostasis, including the MMP and balance of the interactions among the members of the Bcl-2 protein family [Bad, Bax, Bcl-2-interacting mediator of cell death (Bim)]. In addition, Bcl-2 has a role in the regulation of calcium homeostasis in the cell. The *intrinsic* pathway is highly sensitive to the ratio of anti- to proapoptotic factors and it serves as a rheostat that sets the threshold of susceptibility to apoptotic death. Bcl-2-deficient transgenic mice are normal at birth, but later develop to polycystic kidney disease and catastrophic postnatal immune function failure. *In vivo*, Bcl-2 prevents many forms of apoptotic death. Cumulatively, all these observations suggest an important role for Bcl-2 *in vivo*. Expression of the Bcl-2 protein has been shown to prevent apoptosis by directly enhancing cellular antioxidant capacity, possibly through directly scavenging reactive oxygen radicals, or indirectly by counteracting oxidative stress. The other potential mechanisms of action of this gene product include a role in intracellular calcium regulation, and nuclear transport and control of signal transduction pathways.

Numerous proteins with structural similarity to Bcl-2, the prototypical inhibitor of apoptosis, have been discovered in the past decade (**Figure 8**). Included in this family are Bcl-xL (long form of spliced product of *Bcl*-x), Bcl-w, A1/Bfl-1, Mcl-1, Boo/Diva, *C. elegans* Ced-9 and a number of viral homologues. These members have three or four regions with wide-ranging sequence homology (Bcl-2 homology regions BH1–BH4). Mcl-1 was cloned during a screen for genes with increased expression when a human myeloblastic leukaemia cell line was induced to differentiate by phorbol ester. It is considerably larger than Bcl-2, it is primarily membrane bound and its expression delays apoptosis induced by deregulated c-myc. Another relative of Bcl-2, A1, which was isolated from mouse bone marrow induced to proliferate with granulocyte macrophage colony-stimulating factor (GM-CSF), resembles an early response gene and is transcriptionally induced by GM-CSF. Distant relatives of the same family which favour apoptotic death are mammalian Bax, Bcl-xS (short form of spliced product of it Bcl-x), Bak and Bok/Mtd (contain 2 or 3 BH regions). A most compelling proapoptotic group of proteins, which share only a BH3 region, consists of mammalian Bad, Bik/Nbk, Bid, Hrk/DP5, Bim/Bod and Blk and *C. elegans* Egl-1.

Interestingly, proapoptotic and antiapoptotic members of the Bcl-2 protein family exhibit physical interaction, and several members of the Bcl-2 family have a conserved C-terminal transmembrane region. This region localizes proteins to the outer leaflet of the nuclear envelope, the outer mitochondrial membrane and the endoplasmic reticulum (ER). Mounting evidence suggests that the bulk of the Bcl-2 protein is on the cytosolic face of these membranes. Similar subcellular localization has been reported for Bcl-xL, A1, Mcl-1, Bax and Bak. Subcellular localization for some family members appears to be permanent, but others can change their subcellular localization. It has been reported that Bax is a cytosolic protein until the cell is directed to undergo apoptosis, when it becomes redistributed to mitochondrial membranes. It is possible that such subcellular redistribution affords a mechanism of regulating the activity of Bcl-2 family members. The limited common features of the proapoptotic members of the Bcl-2 family (all they share is a 16-amino-acid BH3 domain) may mean that each has some specific activity, such as interaction with specific upstream signalling molecules, and that their common function is to bind and antagonize the anti-apoptotic effects of their ligands. The current consensus view is that the biochemical effects of the Bcl-2 family on apoptosis are mediated through the anti-apoptotic members. Studies with transgenic mice have shown that Bcl-2-inhibitable pathways can also be blocked by Bcl-xL, Bcl-w, A1 and Mcl-1, and Bcl-2-insensitive death inducers fail to activate death by its homologues. Bcl-2/Bcl-xL expression can be regulated by cytokines, colony-stimulating factors and mitogens, whereas the activities of different proapoptotic Bcl-2 family members are regulated by distinct posttranslational modifications.

Although the precise mechanisms of how the Bcl-2 family proteins govern apoptosis remains to be fully elucidated, at least three different models of regulation have been proposed. In the first model, the proapoptotic Bax and Bak are maintained in an inactive conformation through direct interactions with one or two different antiapoptotic Bcl-2 proteins. In response to an apoptotic stimulus, BH3-only proteins bind to and neutralize the antiapoptotic Bcl-2 proteins, thereby releasing Bax and Bak. Overexpression of Bcl-2 or Bcl-xL has been reported to prevent Bax translocation and activation, and

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Bak has been demonstrated to be sequestered by Bcl-xL and Mcl-1 in the mitochondria under normal conditions. In addition, certain BH3-only proteins display selective binding to specific antiapoptotic Bcl-2 family members. For instance, Bad interacts with Bcl-2 and Bcl-xL, but not with Mcl-1, whereas Noxa binds to Mcl-1, but not to Bcl-2 or Bcl-xL. Together, these observations may suggest that death-stimulus-dependent activation of more than one BH3-only protein may be required to release a proapoptotic protein to initiate apoptosis, allowing for fine regulation of apoptosis. Similarly, Bid is a proapoptotic Bcl-2 family member that is cleaved and activated by caspase-8 in response to DR signalling in a seemingly unique manner. One mechanism proposes that granzyme B enters the cytosolic compartment of the target cell by a poorly understood mechanism involving perforin, a pore-forming protein. Two early targets of granzyme B are Bid and procaspase-3, forming active caspase-3 and tBid. tBid modulates the organization of the outer mitochondrial membrane inducing formation of MPT pores and releasing the proapoptotic factors cyt-c, HtrA2/OMI (which blocks inhibitors of apoptosis) and endonuclease-G (a putative enzyme producing DNA damage) which together lead to apoptotic changes. Although the role of cyt-c was once considered limited to the electron transport chain and ATP production in mitochondria, its powerful role in apoptosis has been recognized in the last decade of the twentieth century.

A second model may involve tBid-mediated insertion and oligomerization of proapoptotic Bax into the outer mitochondrial membrane. Together, these events create a positive environment for pore formation that allows the release of cyt-c into the cytosol. Further, tBid can directly induce oligomerization of membrane-associated Bak leading to pore formation, and it has the ability to interact with cardiolipin to influence its distribution between the mitochondrial membranes or induce changes in the mitochondrial ultrastructure. cyt-c is normally bound to the inner mitochondrial membrane by cardiolipin and facilitates electron transport from Complex III to Complex IV. During apoptosis, cyt-c detaches from cardiolipin and appears as a soluble protein in the intermembrane space. Detachment of cyt-c from cardiolipin can be mediated either by direct formation of oxidized cardiolipin (CL-OOH) induced by the cyt-c-cardiolipin peroxidase, or through oxidative modification of cardiolipin by ROS originating from the respiratory chain or by phospholipase-A2-mediated formation of lysocardiolipin. Bim is another proapoptotic Bcl-2 family member that is not involved in the DR pathway, but is critical for thymocyte negative selection. Bim is also known to mediate mitochondria-regulated particulate matter-induced apoptosis in alveolar epithelial cells. Bim was isolated independently by two groups that exploited its ability to bind Bcl-2 or Mcl1 (myeloid cell leukemia-1). Apoptotic-signal-initiated induction of Bim can cause mitochondriacentric apoptosis. Alternative splicing of *Bim* yields three BH3-containing prodeath variants including BimEL and BimL. Bim-deficient lymphocytes accumulate in peripheral blood and in the spleen, and these cells are resistant to apoptotic stimuli, such as cytokine deprivation and microtubule perturbation.

Bad is a member of the BH3-only subfamily of Bcl-2 apoptosis-regulating proteins, regulated extensively by phosphorylation of serine residues and sequestration by 14-3-3 proteins. Growth factor or cytokine deprivation results in the inactivation of AKT, which induces the dephosphorylation and activation of Bad. The 14-3-3 proteins constitute a family of chaperones that are particularly abundant in the brain, like -synuclein. The 14-3-3 protein family consists of seven known mammalian isoforms. It exhibits a remarkable degree of sequence conservation, among both species and isoforms. 14-3-3 binds to many different proteins, most of which contain phosphorylated serine residues. An emerging role for 14-3-3 proteins as effectors in prosurvival signalling has captured the attention of several investigators. Binding of 14-3-3 to phosphorylated Bad (S112 and S136) appears to stabilize Bad maintenance in the cytoplasm. At least three sites on Bad can be phosphorylated *in vivo*, including S112, S136 and S155 of the murine protein. Dephosphorylated Bad is localized to the mitochondria along with Bcl-2 and Bcl-xL, where it can induce apoptosis. Mutation of any of the phosphorylation sites enhances the cell-killing ability of Bad, suggesting that Bad phosphorylation is a critical mechanism for inhibiting its activity (Hengartner, 2000; Strasser *et al.*, 2000; Zhang *et al.*, 2004–2005; Jin and El-Diery, 2005; Chen *et al.*, 2006; Youle and Strasser, 2008).

6.5 ER Stress

Because the ER is a primary site for the metabolism of drugs and toxicants, this compartment plays a major role in regulating mechanisms of cell injury and cell death. From a toxicology perspective, sizeable amounts of free radicals and biological intermediates are produced in the ER during xenobiotic biotransformation, making this site a decisive factor in the *intrinsic* pathway of apoptotic cell death. Crosstalk between ER and mitochondria catalyse numerous prodeath and antideath signals (**Figure 11**). A primary responsibility of the ER is to authenticate and allow only properly folded proteins to pass along the secretory pathway. Major forms of stress to the ER, including oxidative and reductive stress, chemical toxicity, exposure to agents regulating intracellular/extracellular Ca^{2+}-concentration, such as Ca^{2+} channel blockers and Ca^{2+} ionophores, and exposure to inhibitors of glycosylation can result in deregulated calcium homeostasis and lead to the unfolded protein response. This protein-related event is further promoted

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when stress impairs the synthesis of proteins that specifically prevent further aggregation and accumulation of unfolded proteins, that induce ER-resident chaperones coupled with folding catalysts and that activate ER-associated protein degradation to remove aggregates. The continuation of such responses in the face of the ongoing stress load leads to necrosis, apoptosis, or both kinds of cell death.

Many forms of apoptosis, including those involving the ER rely on Ca^{2+}. The futile cycling of Ca^{2+} across the ER, cytosol and mitochondria during toxic redox reactions is not uncommon and creates ER stress that is unfavourable to the cell. For example, staurosporine- and ceramide-induced apoptosis employ calcium as a messenger that coordinates the amplification loop between the mitochondria and the ER. Minute amounts of released cyt-c diffuse to the adjacent ER and bind to InsP3 receptors, thereby enhancing Ca^{2+} release from the ER. Elevated levels of intracellular Ca^{2+} go on to evoke mass release of cyt-c from neighbouring mitochondria. This amplifying interplay between Ca^{2+} and cyt-c results in a dramatic activation of caspases and, in many instances, involves Bcl-2 family members including Bax or Bak. To date, only caspase-12 activation has been directly linked to Bax- or Bak-induced ER stress. The translocation of activated caspase-12 from the ER to the cytosol leads to cleavage of procaspase-9, followed by caspase-3 ultimately driving events to apoptotic death. Interestingly, caspase-12 has only been cloned from two rodent species, (mouse and rat) and several investigators continue to search for a human homologue of caspase-12. Although mice that are deficient in caspase-12 are resistant to ER stress-induced apoptosis, their cells undergo apoptosis in response to other death stimuli. Furthermore, caspase-12-deficient cortical neurons are defective in apoptosis induced by β-amyloid protein, but not by staurosporine exposure or trophic factor deprivation. Caspase-12 mediates an ER-specific apoptosis pathway and may contribute to β-amyloid neurotoxicity. Other signalling molecules, such as GADD153/CHOP, ALG-2, VCP and BAP31, may also be involved during ER stress-induced apoptosis (Rizzuto *et al.*, 2003; Liu and Baliga, 2005; Jin and El-Diery, 2005; Elmore, 2007; Schütze *et al.*, 2008).

Among various caspase-independent pathways of cell death, the granzyme A (GrA)-mediated pathway has captured considerable attention. Granzymes A, B, and 3 are members of distinct groups of serine proteases that work synergistically with perforin to induce apoptosis in target cells recognized by cytotoxic T lymphocytes and natural killer cells. Upon entry into the cell via a Ca^{2+}-dependent perforin-mediated pore, GrA triggers a caspase-independent pathway that forms single-stranded DNA nicks coupled with the appearance of apoptotic morphology. GrA-activated DNase (GAAD), which is also known as NM23-H1, is considered the prime candidate in the formation of these DNA strand-breaks. GrA triggers cell death with apoptotic features by targeting ER-associated SET complex containing the GAAD, NM23-H1, its inhibitor, the nucleosome assembly protein SET and Ape1 (apurinic endonuclease-1, also known as redox factor-1). This complex contains an inhibitor of protein phosphatase 2A (pp32), SET, HMG2, and Ape1. The SET complex presumably translocates to the nucleus in response to oxidative stress and GAAD to induce DNA strand breaks. GAAD activity is inhibited by its specific inhibitor IGAAD. In this pathway, GrA cleaves SET, HMG2, and Ape1, but not pp32, to release and activate GAAD (MacDonald *et al.*, 1999; Liu and Baliga, 2005; Li *et al.*, 2006; Elmore, 2007).

6.6 FADD, TRAF2 and RIP

It is now clear that different adaptor proteins appropriate for specific paths, upon receptor aggregation, proscribe whether TNFR1 mediates signals for apoptosis or cell survival. In fact, when TRADD binds to TNFR1, TRADD can then associate with either FADD or TRAF2 and with RIP. Therefore, recruitment of FADD to the TNFR1 complex would lead to apoptosis, whereas recruitment of TRAF2 and RIP would signal cell survival (**Figure 11**). This hypothesis was tested in genetically altered mice deficient in FADD or TRAF2 expression. FADD-deficient mice tend to die at day 11.5 of embryogenesis, indicating that FADD is essential for embryonic development. Subsequent studies in this direction revealed that FADD is expressed widely during embryogenesis, consistent with its role as being fundamental to the developmental processes. However, the precise role of FADD during embryonic development remains incompletely understood (Wang *et al.*, 1999; Strasser *et al.*, 2000; Zhang *et al.*, 2004–2005; Jin and El-Diery, 2005).

6.7 Mitogen-activated Protein 3 Kinases

During the induction of cell survival by TNFR1 engagement, a number of mitogen-activated protein 3 kinase (MAP3K) family members have been shown to associate with TRAF2 or RIP (**Figure 11**). NFκB inducing kinase (NIK) was initially proposed to be the downstream target of TRAF2 in mediating TNF-induced NFκB activation leading to survival. However, NIK-deficient mice show a specific defect in lymph node development and in lymphotoxin-β-receptor signalling, and cells lacking NIK respond normally to TNF in activating NFκB. Instead, the mitogen-activated protein extracellular signal regulated kinase kinase (MEKK-1) has been implicated in this NFκB activation pathway. In addition, MEKK-1 and apoptosis signal-regulating kinase 1 (ASK-1) are reported to mediate TRAF2-triggered JNK activation. From studies of knockout mice, however, it seems

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that MEKK-1 is required for TNF-induced JNK activation only in embryonic stem cells and not in fibroblasts or T cells. Similarly, ASK-1 is not required for early phase TNF-induced JNK activation, and ASK-$1^{-/-}$ cells exhibit only a partial defect in sustained stress kinase activation. A new member of the MEKK family (MEKK-3) has recently been found to associate with RIP, meaning that it could play a role in downstream survival signalling (**Figure 11**). Indeed, disruption of MEKK-3 severely impairs the activation of NFκB induced by TNF, and MEKK-$3^{-/-}$ cells are highly sensitive to TNF-induced apoptosis (Jin and El-Diery, 2005; Takeda *et al.*, 2007).

6.8 NFκB and IKK

NFκB was discovered as a factor in the nucleus of B cells that binds to the enhancer of the κ light chain of immunoglobulin. Seven members have been reported in the IκB family—IκBα, IκBβ, Bcl-3, IκBε, IκBγ, and the precursor proteins p100 and p105—that bind the dimerization domain of NFκB dimers. Transcription factor NFκB is involved in cellular responses to endotoxin, inflammation and various environmental stresses. Among these, the IκB kinase (IKK) complex composed of the IKKα and IKKβ catalytic subunits and the IKKγ/NEMO (NFκB essential modulator) regulatory subunit is essential for activation of NFκB transcription factors. In the absence of environmental stress, resting NFκB is maintained in the cytoplasm through the interaction with inhibitory-κB (IκB) protein. TNFα is normally used to stimulate NFκB in various cells where NFκB is a key transcription factor whose activation promotes cell survival. The biological system in which NFκB plays the most important role is the immune system. In order to achieve NFκB activation, IκB must be removed via phosphorylation followed by ubiquitination and proteasomal degradation. Phosphorylation of IκB is mediated primarily by the IKK complex containing the proteins IKKα, IKKβ and NEMO (also known as IKKγ). IKKα and IKKβ are active kinases, while NEMO is a regulatory protein that binds tightly to both kinases. Upon optimal activation, IKK phosphorylates the IκB protein, leading to dissociation and subsequent translocation of NFκB into the nuclear compartment. Translocated NFκB binds to a κB binding site on the promoter regions and stimulates the transcription of genes responsible for expressing a variety of cytokines, chemokines, transcriptional factors and enzymes that produce secondary inflammatory mediators and adhesion molecules. The release of these cytoactive factors is cell and cellular-demand specific. In addition, NFκB activation is required for protection of cells from apoptosis, especially from that caused by members of the TNF family of death-inducing cytokines. The cytotoxic effect of TNF is only manifested when NFκB activation is blocked, either by inhibition of protein synthesis or overexpression of dominant negative mutant IκB. TNF-dependent liver failure is observed in mice deficient in either RelA (p65) or TNF-induced NFκB activation elements. The death-inducing potential of TNF is blocked or substantially limited with concomitant activation of NFκB.

Ironically, some proapoptotic DR (TRAIL-R, FAS) also have the ability to turn on the NFκB pathway. TRAF2 can recruit the IKK complex to the TNFR1 signalling complex, whereas RIP seems to stabilize the IKK complex. Mice lacking RelA (p65), a principal subunit of NFκB, die during embryogenesis due to massive hepatocellular apoptosis. In the classical NFκB signalling pathway, IKKβ is both necessary and sufficient for phosphorylation of IκB. The role of IKKα in the classical pathway is unclear, although recent studies suggest it may regulate gene expression in the nucleus by modifying the phosphorylation status of histones. The alternative pathway, however, depends only on the IKKα subunit, which functions by phosphorylating, and is activated in response to a subset of NFκB inducers including LTβ and BAFF.

Deficiency of IKKβ is embryonic lethal, with mice displaying a liver cell apoptosis phenotype similar to that of RelA knockout mice. In addition, the activation of NFκB by TNF or IL-1 is defective in IKK$\beta^{-/-}$ cells. Lethal irradiation of hosts with IKKβ-deficient foetal liver cells reveals a defect in T-lymphocyte survival. In contrast, IKKα-deficient mice do not exhibit a foetal liver defect like IKK$\beta^{-/-}$ mice. Instead, IKK$\alpha^{-/-}$ mice display abnormal limb and skeletal patterns and are defective in epidermal differentiation. Defective skin formation seems to be independent of NFκB activity and is due instead to failed secretion of a keratinocyte-differentiation-inducing factor. IKKα is not required for IKK or NFκB activation in response to inflammatory cytokines. Further studies have revealed that IKKα is required for B-cell maturation and secondary lymphoid organogenesis. NEMO (IKKβ and IKKγ/NFκB essential modulator)-deficient mice also display a phenotype of foetal liver cell apoptosis and embryolethality, consistent with an essential role for NEMO in the central pathway mediating NFκB activation. Like RelA$^{-/-}$ cells and IKK$\beta^{-/-}$ cells, NEMO$^{-/-}$ cells show an increased susceptibility to TNF-induced apoptosis. NEMO is an X-linked gene, and female NEMO$^{+/-}$ mice develop a self-limiting inflammatory skin disorder characterized by hyperkeratosis and increased apoptosis (**Figures 10** and **11**; Mak and Yeh, 2002; Hayden and Ghosh, 2004).

A typical PKC, ζPKC, has been implicated in TNF-induced NFκB activation via RIP and the adapter protein p62. ζPKC-depleted cells exhibit impaired cellular response to TNF that depends on NFκB transcriptional activity. However, ζPKC-deficient mouse embryonic fibroblasts show normal IKK activation and lower levels of TNF-dependent DNA binding to NFκB. Interestingly,

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RelA (a p65 subunit of NFκB) phosphorylation is defective in ζPKC$^{-/-}$ cells, suggesting that an alternative pathway may orchestrate NFκB activation (**Figure 11**). While ζPKC$^{-/-}$ cells are hypersensitive to TNF-induced apoptosis, ζPKC$^{-/-}$ mice do not exhibit foetal liver apoptosis or embryonic lethality. In addition to ζPKC, a new family of proteins known as Pyrin proteins has been shown to associate with the IKK complex, affecting activation of NFκB. Pyrin proteins are derived from a new family of genes that accounts for several hereditary periodic fever syndromes (PFSs).

NFκB signalling is directly dependent upon the intracellular redox environment. The thiol reactive agents *N*-ethylmaleimide (NEM) and iodoacetic acid (IAA) inhibit NFκB binding to DNA, and this DNA binding can also be prevented by reducing agents, such as β-mercaptoethanol. The exact DNA binding locus of the p50 subunit includes cysteine residue Cys62, which is particularly redox sensitive. While ROS production appears to be necessary to initiate the events leading to the dissociation of the NFκB/I-κB complex and subsequent NFκB activity, excessive ROS production can oxidize Cys62 and inhibit NFκB binding to DNA. In some studies, it has been observed that translocation of constitutive NFκB into nuclei is considerably reduced, thereby interrupting the role of NFκB as an antiapoptotic factor when a reactive thiol, such as Cys179 of IKK undergoes modification by ROS, electrophiles or BRIs. For example, arsenite-mediated suppression of TNFα-induced NFκB activation is through its inhibitory action on IKK activity, and due to its covalent binding to Cys179 in the activation loop of IKK. Suppression of constitutive activation of NFκB during exposure of Hodgkin/Reed-Sternberg (HRS) cells to arsenite (iAsIII) contributes to the induction of apoptosis because constitutive NFκB activation is instrumental for cell proliferation, cell division and cell survival. This observation points to a therapeutic possibility that iAsIII-induced apoptosis in HRS cells via the inhibition of NFκB might be an effective means of treating Hodgkin's lymphoma. Likewise, substantial induction of apoptotic cell death can be achieved by inhibiting PI3K/Akt signalling by iAsIII. Some observations claim that iAsIII acts as an activator of NFκB through oxygen free radicals. This could be an early antiapoptotic response and this action of NFκB should not be ignored (Rahman and MacNee, 2000; Mak and Yeh, 2002).

TNF also has the ability to induce activation of the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) pathway (**Figure 11**). TNF-induced activation of the JNK pathway occurs through a TRADD-TRAF2 axis. TNF exposure to TRAF2 knockout mice impairs JNK activation. Analyses of MKK7- and MKK4-deficient mouse embryonic fibroblasts suggest that MKK7 is essentially involved in TNF-induced JNK-activation (**Figure 11**). JNK kinases, upon activation, translocate into the nucleus and enhance the activity of transcription factors including c-Jun and ATF2 by phosphorylating their amino-terminal activation domains. c-Jun belongs to a group of basic region-leucine zipper proteins (ZIPs) that dimerize to form transcription factors commonly designated as AP-1. The AP-1 proteins have an important role in a variety of cellular processes, including proliferation, differentiation and induction, as well as prevention of apoptosis. The AP-1 proteins very effectively interact with antioxidant response elements (ARE) (see more about ARE in Section 7.5). Unlike Fas and TRAIL signalling, TNF does not induce cell death spontaneously. Activation of NFκB by TNF mediates a strong prosurvival signalling pathway. The NFκB pathway targets several antiapoptotic factors, including c-FLICE (caspase-8) inhibitory protein (cFLIP), IEX-1L, Bfl-1/A1, X-linked inhibitors of apoptosis (XIAP), cellular inhibitors of apoptosis protein (cIAP)1, cIAP2 and Bcl-xL. Toxic effects of TNF are only manifested when NFκB activation is blocked, either by inhibition of protein synthesis or by overexpression of dominant negative mutant IkB (Nguyen *et al.*, 2003; see **Figures 10** and **11**).

Besides the above interactions, several studies now strongly attest to the fact that the prodeath potential of TNF is blunted if NFκB undergoes concomitant activation *in vivo*. Interestingly, receptors that show notable proapoptotic inclination (TRAIL and Fas) can also activate the NFκB pathway. Genetic evidence from knockout mice and mutated cell lines support the notion that all DR investigated to date require caspase-8 and the DD-containing adaptor protein FADD to induce cell death. Nevertheless, in contrast to Fas and TRAIL DR, TNFR1 is indirectly linked to FADD by TRADD, which is also responsible for bridging TNFR1 to TRAF2 and the IKK complex. These disparate TNFR1-mediated apoptotic responses have given rise to the proposal of two signalling complex cascades: (i) Complex I, the initial plasma membrane-bound complex consisting of TNFR1, the adaptor TRADD, the kinase RIP1 and TRAF2, has the ability to coordinate rapid signalling and activation of NFκB or JNK; (ii) Complex II, which assembles in the cytoplasm, involves coherent association of TRADD and RIP1 with FADD and caspase-8. In circumstances where NFκB is activated by Complex I, Complex II docks the caspase-8 inhibitor FLIP-L and the cell survives. Otherwise, the Complex II-mediated signalling pathway predominates and drives cells to undergo apoptosis. In this way, TNFR1-mediated signal transduction introduces a checkpoint, resulting in Complex II-mediated cell death in situations where the initial signal via Complex I and NFκB remains dormant or inactive for one reason or the other (**Figure 11**).

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6.9 Apoptosis Execution Machinery: The Complex Interplay of Caspases

The dismantling of cellular structures during apoptosis requires extensive cooperation of proteases. An involvement of proteases in apoptosis was implicated with the discovery of Ced-3. ICE became the first homologue of the cysteine protease Ced-3 to be discovered in mammals. To date, at least 14 distinct mammalian caspases have been identified, with 11 coming from the human genome. Seven mammalian caspases, including four initiator and three effector caspases, are known to play important roles in apoptosis. All caspases are produced in cells as catalytically inactive zymogens. As a consequence of intrachain cleavage, the catalytic activity of effector caspases is enhanced by several orders of magnitude. In their zymogen forms, caspases typically undergo proteolytic cleavage at two Asp-X sites, producing an autocatalytic activation cycle. Activation of the caspase cascade is the commonest mode of initiating cell death involving the downstream effector arm (caspase-3, -6 and -7) and/or the upstream initiator arm (caspase-8 or caspase-9). Downstream partners are usually more abundant and active than members of the upstream group. These observations are overwhelmingly supported by the results of *in vitro* studies demonstrating that procaspase-3 and -7 can be activated by caspases-6, -8, -9 and -10.

Caspases are the central players in apoptosis. Two classes of caspases exist, including the **initiator** caspases, which begin the apoptotic cascade in response to extracellular signals transduced by cell surface receptors such as TNFR1, and the **effector** caspases, which serve as the executioner caspases. Functionally, however, caspases can be classified into a third group. Caspase-1, -4 and -5 and mouse caspase-11 and -12 are designated **inflammatory** caspases because they primarily function in the regulation of inflammatory processes. Caspases are directly implicated in several mechanisms that trigger inflammation, including caspase-1 during exposure to bacterial components and subsequent changes in the intracellular ionic environment, and activation of the inflammasome by ASC, Ipaf and the Nacht leucine-rich-repeat protein (NALP)s group (members of two subgroups of the NOD-LRR family). The inflammasome, which has been termed the linebacker of innate defense (Drenth and van der Meer, 2006) and bears strong similarity to the DISC and Apaf-1, has been considered vital for the onset of activation of inflammatory caspases. Caspase-2, -8, -9, and -10 belong to a functional group of long prodomain **initiator** caspases, whereas, caspase-3, -6, and -7, belong to a functional group of short prodomain **effector** enzymes. Various apoptotic signalling pathways activate initiator caspases when assisted by a series of polyprotein complexes, whereas the receptor-mediated extrinsic pathway is usually launched after ligation of receptors and followed by formation of the DISC before subsequent activation of procaspase-8. Cleavage of caspases is a common denominator of the two pathways of apoptosis operating in mammalian cells. The **intrinsic** pathway depends on mitochondrial involvement, whereas the **extrinsic** pathway is generally independent of mitochondria involvement. Mitochondria play a vital role at this crucial juncture to decide the fate of the cell. This organelle evaluates the intensity of the extrinsic signals and the intrinsic signals, but finally takes a stand to dispense what is necessary or beneficial for the organism. If the net result commands the triggering of apoptosis, cyt-c is released from mitochondria at a level sufficient to initiate the activation of **effector** caspases. If the level of apoptotic signalling is not sufficient, then survival signals are typically propagated to neutralize or delay death signals. Genetic regulation of the apoptotic pathways varies considerably across cell types and in response to toxicological reactions. The evidence now shows, perhaps not surprisingly, that not all elements are required in all cell types or in all response to apoptotic agents Shi *et al.*, (1996b).

As noted, **inflammatory** caspases (1, 4, 5, 11, 12, 13 and 14) are involved in inflammation instead of apoptosis. Initiator caspases have long prodomains and contain either a DED (caspase-8 and -10) or a CARD (caspase-2, -9), which mediates the interaction with upstream adaptor molecules. Effector or executioner caspases (3, 6, and 7) contain short prodomains and are typically processed and activated by upstream caspases and perform the downstream execution steps of apoptosis by cleaving multiple cellular substrates. Initiator and effector caspases are activated by different mechanisms. Typically, signalling pathways that lead to caspase zymogen processing can be subdivided into two major categories: DR-mediated and mitochondria-mediated pathways. Some apoptotic signals trigger oligomerization of death adaptor proteins like Apaf-1 and FADD. Death adaptor oligomers, in turn, induce the aggregation of procaspases. Based on insightful studies, it was presumed that initiator caspases are autoproteolytically activated when brought into close proximity to each other, giving rise to the concept of the induced proximity model. This model was later redefined by the proximity-induced dimerization model. The apoptosome-mediated facilitation of homodimerization of caspase-9 is derived from these concepts. Increased local concentrations of caspase-9 may be an operational consequence of this proximity-induced dimerization. Similarly, the DISC induces dimerization and subsequent autoactivation of caspase-8. The productive conformation of the active site in initiator caspases may be stabilized in these complexes. The adaptor-driven activation of caspases depends on conserved motifs within their long prodomain. Two such motifs, the CARD of caspase-9 and -2 and the DED of caspase-8 and -10 mediate their homophilic interactions with CARD- or DED-containing adaptor proteins Apaf-1 or FADD. Evidence from knockout mice indicates

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that caspase-8 is required for all known DR-mediated apoptotic pathways whereas caspase-9 is mostly involved in mitochondria-mediated apoptotic pathways.

To date, nearly 400 substrates for the caspases have been discovered. Caspase targets can be subdivided into major categories that include (i) apoptosis mediators, (ii) apoptosis regulators, (iii) structural proteins, (iv) DNA repair proteins and (v) proteins critical to cell-cycle regulation. PARP and other DNA repair enzymes, lamin, gelsolin, PKC, and the p53 inhibitor MDM2 often become the targets of effector caspases. Some caspases, like caspase-3, are capable of inducing morphological changes typically associated with apoptosis, like plasma membrane blebbing by removing a negative regulatory domain from the kinase p21-activated protein kinase 2. Among the most well-known caspase effects observed during apoptosis is cleavage of the CAD–iCAD complex. CAD is normally held dormant because of its binding to an inhibitor, iCAD (also called DNA fragmentation factor). During apoptosis, iCAD is cleaved by caspases from the CAD–iCAD complex, leading to the release of an active endonuclease and producing characteristic internucleosomal DNA cleavage. This DNA cleavage ladder became a parameter considered to be the hallmark for apoptosis for over two decades. Internucleosomal DNA cleavage is now known to occur in forms of nonapoptotic dell death. Today, the DNA ladder remains a useful diagnostic tool in assessing cell death, but is no longer accepted as an absolute biomarker of apoptosis.

It is worth noting that within the caspase cleavage cascade, both proapoptotic and antiapoptotic protein kinases can also become targets of proteolysis. Some of the antiapoptotic kinase targets of caspases are AKT, focal adhesion kinase (FAK) and RIP. The activation of transcription factor NFκB is an important prosurvival mechanism. Apoptosis can become favoured via its signalling pathway when the p65RelA subunit of NFκB and upstream activating kinase IKKβ are inhibited through caspase-dependent cleavage. Proapoptotic kinases, such as ROCK1 kinase, PAK2 (p21-activated kinase 2 and MEKK1) are known to be cleaved and activated by caspases in various tissues. TNF-dependent ROCK1 kinase was reported to be modulated by caspase-3 and was considered necessary and sufficient for membrane blebbing during apoptosis. Likewise caspase-3-mediated cleavage of an active form of PAK2 has also been implicated during the formation of apoptotic bodies. Some form of stress during genotoxicity and Fas-induced apoptosis activates MEKK1 and apoptotic MEKK1 signalling involving JNK activation. These are some of the unique properties of caspases.

This arm of apoptosis research has not only generated considerable interest, but also produced a foundation of high quality research publications. Most mechanisms of activation of initiator caspases were explored using isolated proteins, and in the absence of involvement of adaptor protein complexes that are responsible for their intracellular activation. Adapters belong to continuously evolving classes of proteins that are major contributors to apoptosis signalling pathways. These proteins contain a variety of protein-binding modules that link protein partners together and facilitate the creation of larger signalling complexes. By linking specific proteins together, cellular signals are propagated to elicit appropriate responses back from the cell to the environment. In this arena, the induced proximity model, which stresses the importance of active site conformation and interaction within the adapter–protein complex, may best describe the initiator caspase activation mode, whereas the proximity-driven dimerization model, which emphasizes the dimerization process, may best describe how initiator caspases respond to induced proximity. The former offers a specific explanation as to how the initiator caspases are activated. Irrespective of how these caspases launch their activation scheme, their enhanced catalytic activity stems from their altered active site conformation. The particular role of adapter proteins is to bring the caspase zymogens together in a precise conformation, enabling them to undergo autocatalytic activation.

6.10 Caspase-1

Caspase-1 is the prototypical member of the **inflammatory** subclass of caspases involved in cytokine maturation. This class also includes caspases 4, 5, 11, 12, 13 and 14. Caspase-1 is synthesized as a 45 kDa proenzyme that undergoes proteolytic cleavage at Asp residues to produce the active enzyme. The active caspase-1 enzyme is a hetrotetramer comprised of two P20 and two P10 subunits, with the catalytic site being formed by amino acids from both these subunits. The active cysteine is located within the P20 subunit. Caspase-1 is considered a proapoptotic caspase containing an N-terminal CARD domain. It undergoes activation through interactions with other CARD-containing proteins such as ASC, RIP2 and NLRC4 via homotypic CARD–CARD interactions. Because of its long N-terminal prodomain, caspase-1 can be considered to belong to the initiator group of caspases, and therefore could potentially act proximally in a cascade leading to apoptosis.

A breakthrough in the identification of the mechanisms controlling activation of inflammatory caspases came from the identification and characterization of the 'inflammasomes'. Inflammasomes are large, multimeric protein complexes (700 kDa) that recruit inflammatory caspases and trigger their activation after sensing various antigenic and metabolic stresses. It has now become evident that several NLR family members fulfill important roles in the assembly of inflammasome complexes to induce caspase-1 activation. NALP1 and NALP2 are founding members of the NOD-like receptor family, and these inflammasomes are often defined by the distinguishing NLR family member that

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links recognition of specific pathogens to the proximity-induced autoactivation of caspase-1. Currently, four distinct inflammasomes have been identified: the NALP1 inflammasome (NACHT domain-, leucine-rich repeat-, and PYD-containing protein 1b), the NALP2 inflammasome (NACHT-LRR- and pyrin domain-containing protein 2), the NALP3 inflammasome (NACHT-LRR-PYD-containing protein-3) or cryopyrin, and the IPAF (ICE protease-activating factor) inflammasome. Other NOD-like receptors (NLRs), including NALP3 and NOD2, which are associated with inflammatory disorders, have also been described. The NALP1 and NALP3 inflammasomes are located in the cytoplasm and can, therefore, detect intracellular infection through recognition of microbial pathogen-associated molecular patterns. The inflammasome pathways cooperate with Toll-like receptor pathways to mediate a rapid and appropriate response to pathogens and genotoxic stress (Lamkanfi *et al.*, 2007). Future studies will unravel the exact role of this complex during drug or chemical-induced toxicity.

6.11 Caspase-2

Caspase-2 (CASP-2, ICH-1L, ICH-1L/1S, ICH1, NEDD2), a member of the ICE family, is activated during apoptosis by another ICE member, a caspase-3 (CPP32)-like protease(s). When cells are induced to undergo apoptosis, endogenous caspase-2 is first cleaved into three fragments of 32–33 and 14 kDa, which are then further processed into 18 and 12 kDa active subunits. The caspase-3-selective peptide inhibitor *N*-acetyl-Asp-Glu-Val-Asp-aldehyde (DEVD-CHO) inhibits caspase-2 activation, DNA fragmentation and mitochondrial dysfunction in a concentration-dependent manner in *in vivo* models. In addition to being localized in the cell nucleus, caspase-2 is found in the Golgi complex where it cleaves golgin-160 at a unique site insensitive to cleavage by other caspases. Because caspase-2 is located at the Golgi complex, and it cleaves a Golgi substrate early during apoptosis, it has received attention as a potentially important site for generating and transducing unique proapoptotic signals. The contributions of golgin family members are also being studied carefully for their role in Golgi structure and function, particularly in vesicular traffic. The fact that golgin-160 is a substrate for three different caspases suggests that it may be involved in apoptosis. By virtue of its physical location and its role in membrane traffic, the Golgi complex is gaining acceptance as an important new regulator for sensing and integrating information about the intracellular environment. The significance of this organelle is further highlighted by the fact that proteins involved in apoptotic signalling—TNFR1, Fas, an isoform of PKC, PI(3) kinase and ceramide—are situated at Golgi membranes. Caspase-2 is unique among the caspases in that it has features of both upstream caspases, a long prodomain, and downstream caspases, DEXD substrate specificity. The prodomain of caspase-2 has been shown to be essential for the dimerization and autoprocessing of precursor caspase-2 molecules. This caspase-2 prodomain also interacts with a homologous domain in the death adaptor protein RAIDD, implicating caspase-2 as an upstream activator of a distinct caspase pathway.

6.12 Caspase-3

Most caspase $3^{-/-}$ mice die at or before birth. The few survivors are smaller than their wild-type littermates and have severe abnormalities limited to the brain, expressed as enlarged brains, skull defects, excess accumulation of neurons and visible head masses. These masses are ectopic protrusions of supernumerary cells that accumulate in place of the pyknotic clusters usually derived from apoptosis during normal brain development. Caspase-3 is thus crucial for normal embryonic development. Caspase-3 deficiency also dramatically reduces the ability of many cell types to undergo apoptosis in different settings. For example, apoptosis of oncogenically transformed mouse embryonic fibroblasts induced by a chemotherapeutic agent is greatly compromised in the absence of caspase-3. Activation-induced cell death of peripheral T cells is also greatly reduced. However, while caspase-3 is necessary for efficient apoptosis of embryonic stem cells following UV irradiation, it is not required for apoptosis induced by γ-irradiation.

The requirement for caspase-3 in apoptosis induced by a given stimulus can be tissue specific. For example, while TNF induces the apoptosis of caspase-$3^{-/-}$ thymocytes, transformed caspase-$3^{-/-}$ mouse embryo fibroblasts (MEFs) are resistant to this stimulus. Furthermore, caspase-3 knockout mice display an apoptotic defect in response to both intrinsic and extrinsic pathway stimuli. It appears likely that caspase-3 is required for certain critical events during apoptosis, but is not required for all the events. In response to certain stimuli, caspase-$3^{-/-}$ cells fail to develop oligonucleosomal DNA degradation or chromatin condensation yet exhibit other physical signs characteristic of apoptosis. Thus, while caspase-3 is an important or required player in many instances of apoptosis, it is dispensable for apoptosis in other settings. Almost all caspases, including caspase-3, have the ability to cleave PARP, and the 85 kDa PARP cleavage product has become regarded as a hallmark of apoptosis. In addition, the 45 kDa subunit of DFF undergoes cleavage into two intermediate fragments of 30 and 11 kDa under the influence of caspase-3.

Numerous drugs and chemicals directly or indirectly impair Ca^{2+} homeostatsis to induce cytotoxic reactions. The restoration of tight regulation of intracellular Ca^{2+} concentration in various locations, such as the mitochondrion, the ER, the cytosol and the nucleus, can rescue

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a cell from otherwise lethal consequences of lost regulation. Protracted impairment of regulation triggers an irreversible cascade to death. One means that cells use to maintain close control of internal Ca^{2+} is via channels at the cell membrane which change in response to calcium levels in the ER. These are called Ca^{2+}-release activated Ca^{2+} channels (CRAC; Orai proteins) channels. CRACs operate in conjunction with regulator proteins called stromal interacting molecules (STIMs), which act as the ER calcium sensor that activates the Ca^{2+} channels when Ca^{2+} levels drop. Interestingly, caspases do not spare this regulatory system.

Various components of the Ca^{2+} signalling machinery are cleaved by caspases, with potentially different cellular consequences. The IP3 receptor type 1 (IP3R1) has been identified as a caspase-3 substrate, and IP3R1 cleavage results in the inhibition of IP3-induced Ca^{2+} release and activity. Since Ca^{2+} release may act as a potentiating loop of apoptosis, such an effect could represent a negative feedback mechanism, reducing apoptosis. Besides IP3R1, caspase-3 can also target Ca^{2+}-permeable glutamate receptors of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subtype and contribute to neuronal apoptosis associated with Alzheimer's disease. Inactivation of this target can abrogate excitotoxicity and Ca^{2+} overload in neurons destined for apoptosis. Another vital target that undergoes cleavage under the influence of this caspase is plasma membrane Ca^{2+}-ATPase (PMCA), the most effective channel contributing to the rapid return of $[Ca^{2+}]$ to basal levels. Isoforms of this channel (neuron-specific PMCA2 and the ubiquitous PMCA4) are substrates of caspases. While PMCA2 is cleaved in neurons in the *in vivo* setting following excitotoxic stimulation and ischaemia stress in the brain, PMCA4 is cleaved in non-neuronal cells during staurosporine-induced apoptosis. Cleavage of PMCA results in loss of function and aberrant intracellular Ca^{2+} transients. Other important targets of caspase-3 are (i) Naρ/Ca2ρ transporter (NCX) type 1 in cerebellar granule neurons undergoing apoptosis, and (ii) hepatitis B virus X protein-expressing liver cells. Taken together, these alterations of Ca^{2+} concentrations and signalling capability constitute a powerful potentiation loop, facilitating the rapid commitment of cells to caspase-3-dependent apoptotic death. Future studies may reveal cooperative roles for other organelles during Ca^{2+} signalling and caspase-dependent apoptosis.

6.13 Caspase-8/FLICE Inhibitory Protein

Caspase-8 (Mach, Mch5) is the key initiator caspase downstream of the apoptosis pathway induced by TNFR1 and other DR. The binding of TNFα to its receptor TNFR makes the receptor intracellular DD available for binding to TRADD. TRADD is an adaptor protein that in turn elicits the binding of FADD, which in turn mediates the binding of procaspase-8 to this multiprotein complex. Caspase-8 also interacts with FADD through homologous DED regions, which result in the proteolytic processing of the inactive procaspase-8 into the active caspase-8 enzyme. Usually, aggregation and close proximity result in the activation of caspase-8 such that its caspase domain is processed into two active subunits: (i) c-FLICE (caspase-8) inhibitory protein (c-FLIP: FLICE inhibitory protein), and (ii) a cellular homologue of viral FLICE (caspase-8) inhibitory protein. However, c-FLIP lacks the enzymatic activity of a caspase, and it is thought that c-FLIP recruited by FADD to the receptor complex in the place of caspase-8 hinders the progression of apoptotic signals (**Figure 11**). FLIP, acting as an important regulator of DR-mediated apoptosis, has also been identified in the DISC. So far, two forms of FLIP have been cloned, termed c-FLIPS (short) and c-FLIPL (long). c-FLIPS contains tandem DEDs and is highly homologous to the N-terminus of caspase-8. c-FLIPL contains, not only the tandem DEDs, but also a protease-like domain, homologous to caspase-8, but without enzymatic activity. The role of FLIP in DR-mediated apoptosis is still controversial, and recruitment of FLIP to the DR of DISC can disable DISC-mediated processing and release active caspase-8. Typically, FADD-deficient embryos and embryos lacking c-FLIP do not show signs of imbalanced apoptotic cell death. Therefore, the developmental defects observed in these mutant mice are probably apoptosis-independent. In contrast, cells lacking c-FLIP are highly sensitive to DR signalling and DR activation. Caspase-8 and c-FLIP thus appear to play critical, but opposite, roles in regulating DR signalling. With caspase-8 being an initiator caspase, it can proteolytically activate several other effector caspases, including caspases-3, -6 and -7. Activated effector caspases proteolytically digest a number of target proteins and set the apoptotic cell death into motion (Shi, 2005; Kuribayashi and El-Diery, 2008).

6.14 Caspase-11

Caspase-11 is present only in vertebrates and commonly known as an inflammatory caspase (also known as a group I caspase). These are encoded by three main genes in humans, caspase-1, caspase-4 and caspase-5, and three main genes in mouse, caspase-1, caspase-11 and caspase-12. In mammals, all these caspases are characterized by the presence of a CARD domain at the N-terminus. Human, chimpanzee and mouse inflammatory caspases share significant sequence homology and are organized in a single locus. Caspase-11 can physically interact with caspase-1 to promote its activation. Elevated expression of caspase-11 under pathological conditions is directly responsible for activation of caspase-1, making caspase-11 an upstream regulator of caspase-1. The expression of caspase-11 is undetectable in healthy mice

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and highly induced upon injection of lipopolysaccharide. These mice fail to produce mature IL-1β and are resistant to shock induced by bacterial endotoxins. Moreover, caspase-11-deficient embryonic fibroblasts are resistant to apoptosis induced by ectopic expression of caspase-1, further supporting caspase-11 as an upstream activator of caspase-1. Based on expression profiles, human functional orthologues to caspase-11 have been proposed to be caspase-5 and caspase-4. Caspase-5 together with caspase-1 are found to be components of the NALP1 inflammasome, a complex involved in the activation of caspase-1. The inflammasome is a unique intracellular complex which is assembled upon activation of inflammatory caspases and has the ability to cleave and activate IL-1 and IL-18. The role of the inflammasome is becoming increasingly studied in the field of caspase activation and caspase–caspase cooperation. It appears that different inflammatory caspases may cooperate to exhibit their maximum activity. Sequence comparisons of the caspase domain and prodomains of the inflammatory caspases suggest that both caspase-4 and caspase-5 probably arose following the duplication of a caspase-11 ancestor gene. Emerging evidence with regards to the capase-11 orthologue caspase-4 suggests that caspase-4 may play a role in ER stress-induced apoptosis, an interpretation that awaits further confirmatory evidence.

Caspase induction during apoptosis has been studied using intracellular zinc-depleted cells. The depletion of intracellular zinc is achieved with the chemical N,N,N',N'-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN). Among the caspases examined, only caspase-11 was increased by TPEN. Caspase-11 activity also increased, which results in caspase-3 activation. Cycloheximide or actinomycin D blocks caspase-11 induction, reduces caspase-11 and caspase-3 activation, and attenuates TPEN-induced neuronal apoptosis. Blockade of caspase-11 by a chemical inhibitor or its genetic deletion attenuates TPEN-induced apoptosis, indicating a critical role of caspase-11 in TPEN-induced cell death. Although mitochondria-mediated caspase-9/-3 activation also contributes to TPEN-induced apoptosis, caspase-11 is likely a key inducible apoptosis-inducing protein.

6.15 ASK

ASK-1 is a MAP3K that is involved in the cellular apoptotic response resulting from oxidative stress, ER stress, calcium overload, lipopolysaccharide and receptor-mediated inflammatory signals such as TNF. ASK-1 overexpression induces mitochondrial-dependent caspase activation leading to apoptosis. ASK-1-deficient mice have revealed that ASK-1 is required for apoptosis. However, the functions of ASK-1 are not limited to the apoptotic pathway, because this protein is also involved in the control of cellular proliferation, differentiation and survival. The mitogen-activated protein kinase (MAPK) cascades are multifunctional intracellular signalling pathways that are evolutionarily conserved in all eukaryotic cells. Three main MAPK cascades include the ERKs, JNKs and p38 MAPKs pathways which have been well characterized (**Figure 11**). Each MAPK cascade consists of three classes of serine/threonine kinases, MAPK, MAPK kinase (MAP2K) and MAP2K kinase (MAP3K). MAP3K phosphorylates and thereby activates MAP2K, and activated MAP2K in turn phosphorylates and activates MAPK. A growing number of MAP3Ks has been identified, each of which activates single or multiple downstream MAPK cascades. Extensive characterization of several MAP3Ks has demonstrated that MAP3Ks are crucial to sensing extracellular and intracellular stimuli and regulating cellular responses through the activation of downstream MAPK pathways (Takeda *et al.*, 2007).

6.16 Signalosome and Apoptosis

The COP9 signalosome (known as CSN) is an evolutionary conserved large multiprotein complex that consists of eight polypeptide subunits termed CSN1-CSN8, found in all eukaryotes. In order to stabilize cellular proteomics, ubiquitination and proteasome-mediated degradation of diverse proteins are crucial for eukaryotic physiology and development. The diverse functions of the COP9 complex include regulation of several important intracellular pathways, including the ubiquitin/proteasome system, DNA repair, angiogenesis, cell cycle, developmental changes, and some aspects of immune responses. The largest class of E3 ubiquitin ligases is the cullin-RING ligases (CRLs) which are themselves positively regulated through conjugation of the ubiquitin-like peptide RUB/NEDD8 to cullins. The CSN interacts with components of the ubiquitin(Ub)-proteasome system (UPS) known as cullin-RING Ub ligases. The CSN behaves genetically as an activator of CRLs, although it neutralizes CRL activity *in vitro*. This apparent paradox was recently reconciled in different organisms, as the CSN was shown to prevent autocatalytic degradation of several CRL substrate adaptors. RUB modification is antagonized by the CSN and it dissociates covalent bonding of RUB from cullins. Although the UPS and the CSN determine the stability of regulatory molecules involved in apoptosis such as p53, Bax, Bak and Smac, the exact function of the system in apoptosis is still not known. Inhibitors of CSN-associated kinases induce accumulation of p53 and apoptosis in tumor cells, and the UPS in concert with the CSN controls the degradation of IκBα, a major regulator of the key survival factor NFκB. A recent study has demonstrated that CSN-mediated deneddylation can be regulated by active caspase 3 and that the CSN executes a specific function during the apoptotic process (Hetfeld *et al.* 2008).

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Another group of investigators has shown that endogenous ASK1 constitutively forms a high molecular mass complex, which they designate as ASK1 signalosome, and ASK2 is a presumed novel component of the ASK1-signalosome in which ASK2 may modulate the capacity and sensitivity of the signalosome for various stresses and elicit appropriate cellular responses. The basal activity and stimulation-dependent activation of ASK1 are highly regulated by ASK1-signalosome. Various ASK1-interacting proteins that have been shown to regulate ASK1 activity are candidates for components of the ASK1-signalosome. Among them, thioredoxin (Trx) was the first identified ASK1-interacting protein and plays a critical role in the regulation of ASK1 activity. Trx inhibits ASK1 kinase activity by direct binding to the N-terminal region of ASK1. Members of ASK family serve as a MAP3K of the JNK and p38 MAP kinase pathways and play critical roles in response to cellular stress and the innate immune response. ROS-dependent activation of ASK1 is accomplished by the dissociation of Trx from ASK1 followed by inclusion of TRAF2 and TRAF6 in the ASK1-signalosome (Fujino *et al.*, 2007). These stresses may include diabetes, various cardiovascular diseases, oxidative stress, UV, anticancer agents and metal-induced toxicity (Noguchi *et al.*, 2005; Fujino *et al.*, 2007; Takeda *et al.*, 2007; 2008).

7 INTRINSIC APOPTOTIC PATHWAYS

7.1 Mitochondria: Sensor and Master Operator

This vital intracellular energy-producing organelle governs most intrinsic apoptotic pathways. The same characteristics that make mitochondrial uniquely important for cell health, its dependency on oxygen, its ATP synthase for ATP synthesis, its anatomy and its genetic autonomy, also make it a sensitive and powerful control point for adverse events affecting the cell. One critical characteristic of mitochondrial function is the importance of its outer membrane and the vulnerability of this membrane to toxicants and free radicals which can induce the MPT. MPT is primarily governed by Bcl-2 family members. The MPT results in pores that comprise the voltage-dependent anion channels (VDAC) in the outer membrane, the adenine nucleotide translocator (ANT) in the inner membrane, and several auxiliary Bcl-2 family apoptosis-regulating proteins. The basic unit of the MPT pore is the voltage-dependent anion channel-adenine nucleotide translocase-cyclophilin D (VDAC-ANT-CyP-D) complex located at contact sites between the mitochondrial inner and outer membranes. Pro-apoptotic signals open the ANT in the inner mitochondrial membrane and the VDAC in the outer mitochondrial membrane. Pore opening causes water and ions to invade into the matrix, leading to an equilibration and transient dissipation of the MMP. This phenomenon leads to matrix swelling and eventually to the rupture of the outer membrane, releasing proapoptotic proteins into the mitochondrial intermembrane space. In cases where toxic effects are independent of the MPT, other proteins like VDAC may promote formation of pores to advance the death process. Mild MPT perturbations slightly and reversibly compromise ATP production, whereas gross MPT perturbations set the stage and switch the death programme via release of proapoptotic molecules. Both Bcl-2/Bcl-xL and Bax/Bak interact with VDAC to modulate MPT pores. The antiapoptotic Bcl-2 and Bcl-xL close the VDAC channel, whereas the proapoptotic Bax and Bak open the VDAC channel to release cyt-c. A delicate balance between antiapoptotic members (Bcl-2 and its relatives Bcl-xL, Bcl-w, A1 and Mcl-1) and multi-BH domain pro-apoptotic members (Bax, Bak and Bok) maintain cell viability, whereas an imbalance between these two groups favouring proapoptotic members leads to cell death.

In addition, up to eight short BH3-only protein members, such as Bid, Bad and Bim also show proapoptotic activity. Prior to launching of the death programme, the damage-sensing BH3-only proteins clearly lie upstream of Bax/Bak, because they cannot kill cells that lack both Bax and Bak. Prominent investigators in the field suggest that a transcriptionally independent pathway leads to loss of MPT at the outer mitochondrial membrane, permitting efflux of pro-apoptotic proteins in a Bax/Bak-dependent manner. Although cytosolic Bax exists as a monomer in healthy cells, it undergoes conformational changes at both polar ends, migrates to the outer membrane, and oligomerizes during onset of apoptosis. Likewise, Bak a monomer, localized to mitochondrial membrane in normal cells, also undergoes conformational changes and forms larger aggregates during apoptosis. Small numbers of Bak molecules are usually found to be bound with VDAC2 in healthy cells. In the absence of inhibition by VDAC2, Bak demonstrates an enhanced allosteric conformational activation resulting in the release of pro-apoptotic factors conducive to increased apoptotic death. For example, ethanol intoxication involves oxidative stress accompanied by mitochondrial membrane permeabilization and cyt-c release. Bax translocation to mitochondria is oxidative stress-dependent, and the translocated Bax interacts with VDAC. Free radicals such as superoxide can also open the VDAC channel, resulting in the release of cyt-c and cell death. The VDAC–ANT complex is also chemotactic for other proteins, particularly kinases such as hexokinase and glycerol kinase. ANT has been identified as an important target for ROS during apoptosis induced by doxorubicin and arsenic trioxide (**Figures 5–11**).

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A significant change in the mitochondrial outer membrane potential is known to release an important group of proteins known as cellular inhibitors of apoptosis proteins, including Smac/DIABLO, HtrA2 (Htr2/Omi: high temperature requirement protein A2) and GSPT1/eRF3. The cIAPs are emerging as key proteins in the control of cell death. cIAPs have the ability to halt the progression of apoptosis by inhibiting caspase activity. The first four amino acids of mature Smac/DIABLO, AVPI, interact tightly with the BIR3 domain of XIAP. Peptides with identical sequence homology have proven to be strong inhibitors of cIAPs and greatly sensitize cells to TNFα- and TRAIL-induced apoptosis. Omi/HtrA2 can also proteolytically cleave and inactivate cIAPs and is presumably a more efficient cIAP suppressor. Smac/DIABLO, HtrA2/Omi and GSPT1/eRF3 bind cIAPs in a competitive manner similar to caspases. For example, Smac/DIABLO and cyt-c are released simultaneously during apoptosis to neutralize the inhibitory influence of cIAPs and promote cyt-c -dependent caspase activation. Smac/DIABLO seems likely to play an important protective role in DR-mediated apoptosis. Some studies suggest that caspase-3 and caspase-7, but not caspase-1, can be ubiquitinated by cIAP2 and that caspase-7 and caspase-3 are specific targets for cIAP2-mediated ubiquitination. cIAPs can act via inhibition and/or through ubiquitination. This later interaction may be another novel way of controlling the induction of apoptosis through the negative regulation of caspases. Additional evidence suggests that cIAPs prevent apoptosis initiated by Fas signalling and etoposide, whereas in nonapoptotic cells, inhibition of unwarranted caspase activation by cIAPs can be deemed a blessing in disguise to prevent the catastrophic consequences of caspase activation. Nevertheless, most members of this evolutionarily conserved family clearly maintain an antiapoptotic function.

In addition to their release of caspase activating proteins, mitochondria are the prime sources of several other pro-apoptotic proteins that are independent of caspase activity. In recent years, much effort has been invested in identifying caspase-independent apoptotic cell death pathways during toxic reactions, degenerative diseases and tumour growth. It is now known that in addition to their critical role in the production of cellular energy, mitochondria are also the source of key proapoptotic molecules that are distinct from caspase activation. For example, AIF, once released into the cytosol, translocates to the nucleus in response to death stimuli provoked by p53, UVB, *N*-methyl-*N*-nitro-*N*-nitrosoguanidine, H_2O_2 and *N*-methyl-aspartate. Once in the nucleus, AIF specifically induces chromatin condensation at the peripheral nuclear membrane, and stimulates extensive genomic DNA fragmentation. Under some circumstances, AIF cooperates with cytosolic factors to permeabilize mitochondrial membranes and accelerate apoptosis. Classic experiments have demonstrated the inability of zVADfmk to forestall AIF-induced apoptosis, implying that its actions are caspase-independent despite cells demonstrating the classic morphologic features of apoptosis. Definitive genetic evidence establishes an essential role for AIF during early mammalian development. AIF is instrumental for the first wave of apoptotic death during embryonic development, tissue morphogenesis and cavitation. Another vital caspase-independent factor that is released by mitochondria into the cytosolic compartment is endonuclease-G. This genome-sensitive enzyme translocates to the nucleus and produces oligonucleosomal DNA fragmentation in an orderly fashion. It is believed that endonuclease-G is indispensable for DNA fragmentation, particularly during caspase-independent apoptosis. Interestingly, however, adequate expression of Bcl-2 in a timely manner effectively protects cells from both caspase-dependent and caspase-independent apoptosis. This unique property of Bcl-2 may be attributable to its ability to prevent mitochondrial membrane perturbations and release of endonuclease-G.

It is established that cells of diverse origin utilize several mitochondrial proteins to orchestrate apoptotic cell death. cyt-c, IAP and endonuclease-G are located inside the mitochondria, whereas their targets are in cytoplasm (Apaf-1 and Smac/DIABLO) or the nucleus (genomic DNA). The purpose of this spatial segregation insures that these target proteins execute their lethal actions only when needed during apoptosis, and that cells have adequate opportunity to counteract them if and when necessary. It is also worth noting that these proteins are only transiently released into the cytoplasm, yet their lethal actions require processing and endorsement by the mitochondria. For example, Apaf-1 activation will not occur unless cyt-c is in its appropriate configuration (folded into its mature haeme-bound form), and IAP antagonism may falter if the target sequence of Smac/DIABLO in mitochondria is not removed. In metabolically active healthy cells, spatial partition of these proteins is advantageous for mitochondria because: (i) cyt-c is a requisite element of the mitochondrial respiratory chain, (ii) AIF safeguards the intracellular microenvironment from oxidative stress through its pyridine nucleotide-disulfide oxidoreductase domain and (iii) HtrA2/Omi degrades denatured proteins once serving as molecular chaperones. Several lines of evidence suggest that HtrA2/Omi has a duality of function, promoting DNA fragmentation in a pro-apoptotic environment and maintaining mitochondrial functions in normal cells in a fashion analogous to cyt-c. It will be interesting to see whether future studies demonstrate other functions for Smac/DIABLO and endonuclease-G that may even be anti-apoptotic in nature.

Ca^{2+}-mediated mitochondrial regulation of apoptotic cell death has been a focus of research for decades. Ca^{2+} signals directly or indirectly activate various kinases,

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including the PKC family. At present, 12 PKC isozymes have been identified based on cofactors required for maximal activation. These isozymes include the classical isozymes α, $\beta 1$, $\beta 2$ and γ, which require calcium and diacylglycerol for activation, the novel isozymes δ, ε, η, θ and μ, which require only diacylglycerol for activation, and the atypical isozymes ζ, ι and λ, which require phosphatidylserine, but not calcium or diacylglycerol for activation. Thus, pro-apoptotic Ca^{2+} signals are well known, but with considerable variation in experimental models, results regarding protein kinases have been often contradictory. In most experimental systems, the classical isoforms, PKCα, PKC$\beta 2$ and PKCε, as well as the atypical isoforms PKCζ, PKCι and PKCλ, appear to be antiapoptotic, whereas overexpression of PKCδ and PKCθ show proapoptotic activities. In fact, under special circumstances, PKCα plays the role of a survival factor. PKCα is often inhibited during apoptosis, as seen in its dephosphorylation after treatment with ceramide. The fact that both calpains and caspases result in PKCα degradation lends credence to this view. Although the atypical PKCs ζ and λ have been proposed to antagonize apoptosis, their mode of interaction remains poorly understood.

The novel PKCδ has been suggested to participate in the mitochondria-dependent apoptotic pathway triggered by different drug and chemical stimuli. A catalytically active fragment of PKC is generated by proteolysis in cells undergoing apoptosis in response to ionizing radiation, DNA-damaging drugs, and antiFas antibody. As for the site of action of PKC, it is known to translocate during apoptosis to mitochondria, as well as to the Golgi complex (Emoto *et al.*, 1995; Konishi *et al.*, 1999), where it is activated through a tyrosine-phosphorylation step. Among the multiple subtypes of PKC, PKCδ has been implicated as a proapoptotic kinase, mostly by acting as a target of caspase-3 (Li *et al.*, 1999; Roderick and Cook, 2008).

7.2 Apoptosome and Caspase-9

Caspase-9 is considered to be an initiator caspase. The apoptosome, a multimeric complex involving Apaf-1, cyt-c and the cofactor dATP/ATP, is instrumental for its activation. It has been more than a decade since the mitochondrial respiratory chain pigment cyt-c was found to be a classic activator of caspase-3, and its receptor was affirmatively identified as Apaf-1. Later, investigators working on the oncoprotein Bcl-2 demonstrated that cyt-c leakage from mitochondria can be blocked by this anti-apoptotic gene. Multiple mechanisms of cyt-c release can occur cuncurrently within one model of cell death. This crucial event is demonstrated by the formation of a cyt-c and dATP-dependent Apaf-1/caspase-9 complex, and its ability to trigger some of the effector caspases, such as the caspase-3 and -7. Further biochemical characterizations have disclosed that a coherent assembly of dATP/ATP, cyt-c and Apaf-1 forms the apoptosome. The convergence mechanism involves interaction between the apoptosomal caspase recruitment domain of Apaf-1 with the prodomain of procaspase-9, resulting in zymogen procaspase-9 recruitment and its activation. Activated caspase-9 binding to the apoptosome increases catalytic activity severalfold through apoptosomal allosteric regulation of caspase-9 activity.

Apoptosis execution requires the closely coordinated activation of initiator caspases. p53-induced protein with death domain (PIDD) appears to be a bystander during the complex interplay of caspases. Prototypically, caspase activation is triggered upon complex-mediated clustering of inactive zymogen molecules, such as in the caspase-9-activating apoptosome complex. Likewise, caspase-2, which is involved in stress-induced apoptosis, is recruited into a large protein complex that contains PIDD. Increased amounts of PIDD expression result in spontaneous activation of caspase-2 and sensitization to apoptosis by genotoxic stimuli. PIDD has also been shown to play a critical role in DNA damage-induced NFκB activation. Upon genotoxic stress, a complex involving PIDD, RIP1 and NEMO is formed. Cells stably expressing PIDD show enhanced genotoxic stress-induced NFκB activation, through augmented sumoylation (a small ubiquitin-like modifier) and ubiquitination of NEMO. Knockdown of PIDD and RIP1 expression abrogates DNA damage-induced NEMO-modification and NFκB activation (Shi, 2005).

7.3 p53

Considerable focus has been directed to the role of another gene product in apoptosis, p53, also termed the guardian of the genome. It has earned a prominent position in the periodic table of the genes, and has been implicated in such fundamental cellular functions as growth inhibition, redox regulation, coactivation of numerous pathways, apoptosis regulation via extrinsic and intrinsic pathways, apoptosome activation, abrogation of survival signals via the AKT pathway and cancer therapy. This suppressor gene codes for a 53 kDa protein that orchestrates how organisms cope with DNA damage by either stalling cell division or inducing cell death. Transcriptional activation of the $p21^{WAF1}$ cyclin-dependent kinase inhibitor plays a key role in the induction of cell cycle arrest by p53. p53 contains three functional domains: an amino-terminal transcriptional activation domain, a central sequence-specific DNA binding domain and a carboxy-terminal oligomerization domain. The majority of p53 mutations sequenced from human tumours map to the sequence-specific DNA-binding domain and impair the ability of p53 to bind DNA. This nuclear p53 tumour suppressor gene is the most widely mutated gene

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in human cancers. p53 encodes a transcriptional activator whose targets include genes that regulate genomic stability, cellular response to DNA damage and cell cycle progression. It was appealing to think that p53-induced tumour suppression arises through the activation of cell suicide that is irreversible, rather than through imposition of an inherently reversible arrest of cell growth, as previously thought. The consensus now is that p53 can induce growth arrest or apoptosis depending on the physiological or pathophysiological circumstances or cell type, and that both activities are potentially involved in tumour suppression.

While the p53-mediated pathway is the most common mechanism of radiation-induced apoptosis, a large body of evidence suggests ceramide may be a second messenger in mediating radiation-induced apoptosis in specific systems *in vitro* and *in vivo*. Emerging data suggest that radiation acts directly on the plasma membrane of several cell types, activating acid sphingomyelinase, which generates ceramide by enzymatic hydrolysis of sphingomyelin. Ceramide then acts as a second messenger in initiating an apoptotic response via the mitochondrial system. Radiation-induced DNA damage can also initiate ceramide generation by activation of mitochondrial ceramide synthase and *de novo* synthesis of ceramide. In some cells and tissues, Bax is activated downstream of ceramide, regulating commitment to the apoptotic process via release of mitochondrial cyt-c. p53 also accumulates and directs apoptosis in response to DNA damage in skin, thymocytes and intestinal epithelium. High levels of constitutive c-myc expression or exposure to chemotherapeutic agents elicits a p53-dependent apoptotic response.

The past three decades have witnessed intense scrutiny of the activities of p53 and how at the molecular level it oversees a kind of cellular damage-control system to limit aberrant cell growth in response to DNA damage, oncogene activation, hypoxia and the loss of normal cell–cell contact. p53 implements cellular growth restriction by inducing senescence, cell cycle arrest (G_1-S and G_2-M checkpoints) or apoptosis. The molecular rationale for inducing cell cycle arrest vs apoptosis continues to be investigated and remains incompletely understood. Normally, p53 expression level, the type of stress signal, the cell type and the cellular context at the time of stress are principal factors that guide how p53 will maximize the benefit to the host. The binding of p53 to its canonical binding sequence in target genes has been proposed as a possible mode to deal with cells going through chaotic consequences. Surprisingly, however, the ability of p53 to activate target genes through a noncanonical sequence has also been reported. At the genetic level, the sequence of events is envisioned as follows. The binding affinity of p53 for the promoter of the p21WAF/CIP1 (p21: a cell-cycle-regulating gene) remains more-or-less unaffected in response to DNA damage, whereas binding to the promoter of the DNA-repair-associated gene GADD45 is reduced. UV and alkylating agents can induce two specific genes in human and hamster cells, known as growth arrest and DNA damage-inducible proteins 45 and 153 (GADD45, GADD153). This is due primarily to oxidation of Cys277 residing within human p53. Although the mechanism of Cys277 oxidation remains to be defined, it may be associated with oxyradicals that are produced in response to exposure to high-dose UV light. Under reducing conditions, the affinity of p53 for the GADD45 promoter is increased, which suggests that the reduction of Cys277 is necessary to permit binding of p53 to C-rich binding sequences, such as that of GADD45. It is interesting that selenomethionine-mediated reduction of Cys275 and Cys277 residues can signal p53 to recruit the p53-binding redox factor Ref1 and stimulate DNA-repair instruments through GADD45 induction, without affecting cell growth. Thus, the redox state of Cys277 on p53 appears to serve as a switch for activating the DNA repair machinery, and this event may have implications in cancer chemoprevention (Goodrich and Lee, 1992; Miyashita and Reed, 1995; Haupt *et al.*, 2003; Li *et al.*, 2006; Kuribayashi and El-Diery, 2008).

p53 is important but is not the lone player in this arena. Its new family members p63 and p73 have been implicated in assisting p53 in inducing apoptosis. The high level of sequence homology between p63, p73 and p53 proteins, particularly in the DNA binding domain, allows p63 and p73 to transactivate p53-responsive genes causing cell cycle arrest and apoptosis. Therefore, p63, p73 and p53 genes form a family of transcription factor. Investigations of the effect of p63 and p73 on p53 transcriptional activity with knockout MEFs has disclosed two discrete groups of target genes. For one group, p53 alone is sufficient to induce p21 and mdm2 genes, whereas the induction of apoptotic genes PERP (p53 apoptosis effector related to PMP-22), Bax and Noxa requires a co-operative effort of p63 and p73 together with p53. This would suggest an essential role for both p63 and p73 in p53-induced apoptosis. Cooperative roles for these genes have been described during normal mouse development and DNA damage with participation of Abl tyrosine kinase and p73.

Keeping all of these actions in focus, p53 has been shown to be able to activate the extrinsic apoptotic pathway through the induction of genes encoding three transmembrane proteins: Fas, DR5 and PERP. More specifically, p53 induces *Fas* mRNA expression by binding to elements found in the promoter and first intron of the *Fas* gene. This induction is tissue specific and γ-irradiation-dependent. P53 can enhance *Fas* transcription, causing overexpression of Fas at the cell surface via increased trafficking of the Fas receptor from the Golgi. The other pro-apoptotic member that is induced by p53 is DR5/KILLER, the death-domain-containing TRAIL. p53-induced DR5 in response to DNA damage can propagate cytolethality via caspase-8. P73 or p53 expression

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transactivates endogenous p53 expression through direct binding of p73 or p53 protein to the *p53* promoter, thereby forming a previously unknown positive feedback loop regulating human p53 expression. Importantly, interference with p53-mediated autoregulation of *p53* transcription results in aberrant cell cycle regulation and abolishes a p53-mediated apoptotic response to DNA damage. The other target involved during genomic damage is PERP, which has a p53 response element. Significantly higher levels of PERP mRNA in apoptotic cells suggest a role for this gene, which is directly targeted by p53 (Goodrich and Lee, 1992; Miyashita and Reed, 1995; Haupt *et al.* 2003; Li *et al.*, 2006; Wang and El-Diery, 2006; Kuribayashi and El-Diery, 2008).

p53 can function as a transcription factor and activate the transcription of death genes like Bax or repress the transcription of survival genes like Bcl-2. Compared with p53-expressing wild-type controls, tissues from p53-null mice show reduced Bax expression and elevated Bcl-2 expression. Several Bcl-2 family members are p53 targets, including Bax, Noxa, PUMA and the previously discussed Bid. Bax is the first member of this group shown to be induced by p53, and p53-responsive elements have also been identified in the *Bax* gene. During activation-induced stress, Bax forms a homodimer and releases cyt-c from mitochondria, which results in caspase-9 activation. The requirement for Bax in p53-mediated apoptosis appears to be cell-type dependent, however, in humans, PUMA encodes PUMA-α and PUMA-ß, both BH3-domain-containing proteins. The onset of growth arrest or death in response to exogenous p53 expression requires a specific balance between PUMA and p21. If p21 is disrupted via gene targeting, the cells die through apoptosis. If the PUMA gene is also disrupted in such cells, apoptosis is prevented. These observations suggest that the balance between PUMA and p21 is pivotal in determining the response to p53 activation and provide a model for understanding p53 mutation in cancer. Moreover, it is well established that PUMA expression generates a prodeath environment via mitochondrial translocation and mulitmerization of Bax. Thus, Bax is an absolute requirement for PUMA-dependent apoptosis. Unlike PUMA, Noxa contains a single p53-responsive element in its promoter and is induced in response to X-ray irradiation. Noxa encodes a BH3-only protein and hence it is speculated that it assists p53-mediated apoptosis in a manner consistent with *PUMA* and *Bax*. Besides *Bax*, *PUMA* and *Noxa*, *Bid* mRNA is also known to increase considerably in a p53-dependent manner. Thus, it appears that p53 expression is mitochondria-sensitive and some intrinsic pathways are intimately linked to p53, which can tilt the balance towards proapoptotic events.

Another p53-dependent direct pathway involves the formation of the apoptosome. p53 is known to facilitate cyt-c release through the induction of target genes encoding BH3-only proteins, and it also induces *Apaf*-1 expression through a response element within the *Apaf*-1 promoter. p53 also has a direct impact on the caspase cascade in both transcription-dependent and -independent mechanisms. Caspase-6 activation has been achieved under the influence of p53. In response to DNA damage, p53 directly induces caspase-6 expression through a response element within the third intron of the gene. There are several other ways that p53 participates in the induction of apoptosis. Some apoptotic signals accentuate localization of p53 to the mitochondria preceding cyt-c release and procaspase-3 activation. p53-deficient Saos-2 cells undergo apoptosis when exposed to mitochondrial-import leader peptides. Permeabilization of the outer mitochondrial membrane by formation complexes with antiapoptotic Bcl-xL and Bcl-2 proteins is another event linked to p53 expression. Therefore, it is reasonable to speculate that the mitochondrial localization of p53 is not the only transcription-independent mechanism by which p53 promotes apoptosis (Haupt *et al.*, 2003).

p85 is another gene that appears to be sensitive to oxidative stress, although its precise role in cell death is unclear. PI3K activates AKT, a serine/threonine kinase, through phosphorylation of Ser473 by the 3′-phosphoinositide-dependent kinase PDK1. This results in phosphorylation of a range of targets by AKT, with the most prominent being Mdm2, a major inhibitor of p53. The final outcome of this event is that the Mdm2 accumulates in the nucleus, boosts Mdm2 interactions with p300, and reduces the affinity of Mdm2 for p19ARF. Consequently, AKT augments the inhibition and destabilization of p53 by Mdm2. Interestingly, stress-induced activation of p53 counteracts the inhibitory effects of this survival pathway by multiple mechanisms. The well-characterized pathways are: (i) promotion of caspase-mediated cleavage by p53, and subsequent degradation of the AKT protein itself, (ii) induction of p53-mediated expression of the PTEN tumour suppressor gene, and subsequent coding of a phosphatase that dephosphorylates PI3K, thereby impairing AKT activation and (iii) p53-induced expression of cyclin G, which recruits the phosphatase PP2AB′ to the Mdm2-p53 complex where it dephosphorylates Mdm2 at the AKT phosphorylation sites. These feedback loops determine survival vs apoptotic outcome in the interplay between p53 and the AKT survival pathway. This balance is often interrupted in cancer, either by mutations in PTEN or the amplification of Mdm2.

Lastly, the *ATM*/p53 (ataxia telangiectasia-mutated gene) signalling pathway is another route that involves p53 and tumorigenesis. The *ATM* gene encodes a protein kinase that acts as a tumour suppressor. Ionizing radiation-induced DNA damage activates ATM, stimulates DNA repair and blocks cell cycle progression. ATM-dependent phosphorylation of p53 is considered the prime interaction that makes this route relevant.

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7.4 c-Myc

c-Myc is a nuclear transcription factor that functions as a master regulator of the cell cycle and thus proliferation, differentiation, neoplasia and cell death. The *c-Myc* gene is the cellular homologue of the viral oncogene *v-myc*, and is known to play a prominent role in carcinogenesis. To date, three Myc proteins have been reported in mammals, c-Myc, N-Myc and L-Myc. The *c-Myc* mRNA encodes two short-lived phosphoproteins initiated at independent CUG and AUG translational start sites. c-Myc is the prototypical member of a family of DNA binding proteins that contain carboxy-terminal basic helix-loop-helix leucine zipper (bHLH-LZ) domains. The transcriptional activity of c-Myc requires dimerization with Max, another member of the family. These heterodimers bind specifically to CACGTG sites in DNA. c-Myc harbours N-terminal transcriptional activation domains, and c-Myc–Max heterodimers preferentially activate transcription. Myc–Max heterodimers regulate the transcription of many target genes, and the *c-Myc* gene encodes a nuclear phosphoprotein of approximately 60 kDa. *c-Myc* was identified as a protooncogene and an immediate early response gene and, thus, attention initially focussed on its role in the cell cycle. Enforced activation or cells that are deprived of survival factors readily induce expression of c-Myc and undergo apoptosis. The expression of c-Myc and cofactor Max appears transiently during castration-induced and post-lactation breast regression-induced apoptotic death of prostate and mammary cells, respectively.

In apoptotic cell death, the apparent role for c-Myc was unexpected and paradoxical because of its already recognized role in cell cycle progression through G_1–S and in cell transformation. Extensive experimental evidence shows that cdc25A, a phosphatase, activates cyclin-dependent kinases (Cdks) essential for a G_1-to-S transition and its expression is induced by c-Myc. c-Myc levels are higher in growing cells than in quiescent cells, indicating that expression of c-Myc is necessary for cell proliferation. This is supported by the fact that its downregulation triggers growth arrest and differentiation. c-Myc targets may include the ornithine decarboxylase (ODC), p53 and cdc25A genes. Increased ODC activity leads to increased superoxide levels as a consequence of polyamine metabolism, suggesting that oxidative stress may be involved in c-Myc-induced apoptosis. Nonetheless, ODC inhibition attenuates, but does not prevent apoptosis in c-Myc-overexpressing myeloid cells. Several recent publications have focussed on the connection of apoptosis, induction of p53 and c-Myc. It is clear that the roles of p53 and c-Myc in apoptosis are multidimensional.

In mammalian cells, another pathway by which c-Myc and apoptosis are linked is via $p19^{ARF}$ localization to nucleus. ARF responds to proliferative signals generated by p53 activation using c-Myc because the ARF promoter responds to c-Myc. The ARF tumour suppressor protein acts at a checkpoint that protects against unscheduled cell proliferation with tumorigenic signalling. c-Myc activation is known to upregulate ARF transcription, and in turn, ARF promotes the p53 response by inhibiting Mdm2. This ARF-Mdm2-p53 axis sets a threshold for cellular proliferative signalling. Receipt of excessive proliferative signals can be pro-apoptotic due to the induction of ARF-mediated p53-dependent apoptotic signalling. Alternatively, ARF can interact and bind with c-Myc and substantially reduce the ability of Myc to activate transcription, and push the balance towards inducing hyperproliferation coupled with transformation. Thus excessive Myc expression provides a strong selection pressure for events that dismantle apoptotic signalling pathways. Because ARF is a target of Myc activation, the loss of ARF, like the loss of p53, can attenuate Myc-induced cell death. Overall, it seems like the normal role ARF is to respond to hyperproliferative signals, thereby facilitating p53 activation through a signalling mechanism that differs from those induced by DNA damage. c-Myc-induced apoptosis has also been shown to cooperate with extrinsic death-receptor signalling, such as Fas-L and Fas receptor expression. This is achieved by c-Myc-mediated down-regulation of FLIP expression. Transcriptional stimulation of c-Myc-dependent ODC activity is another way that proapoptotic and/or proliferative responses are regulated.

The role of c-Myc in cellular metabolism is an emerging field of research. Besides ARF, another factor is becoming increasingly prominent in the arena of c-Myc-regulated apoptosis. This factor, called nuclear respiratory factor 1 (NRF-1), is a member of a unique family of evolutionarily conserved transcription factors that are critical for eukaryotic development, mitochondrial respiration and biogenesis in particular. Human NRF-1 has been shown to transactivate the promoters of a number of mitochondrial genes. Well-designed studies have concluded that c-Myc is capable of up-regulating the expression of a number of NRF-1 target gene products, including cyt-c, COX5B, and COX6A, and possibly mitochondrial oxidative phosphorylation. NRF-1 is particularly important in cell growth and development, as homozygous knockouts of this gene are embryonic lethal. Functional NRF-1 sites have been found in genes encoding at least one subunit of each of the respiratory complexes III, IV and V. One of the well-studied NRF-1 target genes is cyt-c, a gene shown to play a critical role in both the electron transport chain and in apoptosis. cyt-c-null mice do not live beyond day 10 owing to respiratory insufficiency, and cells derived from these embryos are apoptosis resistant. The addition of serum restores induction of synthesis of cyt-c coupled with enhanced respiration. Ultrastructural studies with electron microscopy have revealed intramitochondrial electron-dense zones, formation of mega mitochondria, disorganized mitochondrial membranes, fragmented nuclei and

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membrane blebbing. The distinct electron-dense mitochondrial phenotype and increase in mitochondrial size seen in NRF-1-overexpressing cells suggest extensive import of mitochondrial proteins, which may be responsible for disruption of the mitochondrial membrane. These events clearly establish a link between apoptosis and the selective activation of NRF-1 genes. Further, the ability of Bcl-2 to inhibit both NRF-1-and c-Myc-induced apoptosis is consistent with the induction of shared apoptotic signalling pathways, resulting in mitochondrial dysfunction and cell death.

Several toxicological studies have shown that a variety of stimuli including cytokines, ionizing or UV irradiation, heavy metals and oxidative stress can elevate c-Myc mRNA levels in a range of tissues *in vivo* and *in vitro*. Environmental and industrial toxins trichloroethylene and dichloroacetylene produce a genotoxic metabolite *S*-(1, 2-dichlorovinyl)-L-cysteine (DCVC) that is highly nephrotoxic. DCVC-exposed cells die either via apoptosis or necrosis as a result of multiple mechanisms, such as covalent binding of a reactive metabolite, loss of glutathione, increased levels of cellular Ca^{2+} and ROS, disruption of the actin cytoskeleton and membrane lipid peroxidation. Interestingly, DCVC induces c-Myc mRNA in a manner that is linked, at least in part, to an increase in cellular Ca^{2+} content and oxidative stress. Cells exhibiting dysregulated c-Myc expression in the absence of adequate levels of growth factors tend to undergo apoptosis, whereas in lymphocytes, antisense oligonucleotides to c-Myc block activation-induced apoptosis but not steroid-induced apoptosis. Together, these events may be interpreted the following relationships. Growth arrest, c-Myc off and growth factors absent; population expansion, c-Myc on and growth factors present; and apoptosis, c-Myc on and growth factors absent. In summary, the effects of c-Myc depend on cell type and stimulus and are not needed for all forms of apoptosis. Inappropriate expression of c-Myc may have evolved as a mechanism to prevent the development of neoplasia. This is particularly true for cells with dysregulated c-Myc expression in situations where complementary growth-factor-mediated signals are absent. The molecular mechanisms mediating these opposing functions of the c-Myc protein will require additional investigation (Morrish *et al.*, 2003; Vafa *et al.*, 2002).

7.5 ARE

Oxidative stress is now a phenomenon being investigated by many disciplines, including toxicology because of its involvement in many drug- and chemical-induced toxic reactions. Impairment of the respiratory chain can cause oxidative stress through superoxide production. Oxidative stress, once substantial, can overwhelm the cell when the available antioxidants fail to oppose the oxidative burden. Therefore, antioxidant research in toxicology has gained considerable momentum in the twenty-first century. Oxidants, antioxidants, and inflammatory and antiinflammatory agents modulate the activation of redox-sensitive AP-1 and NFκB, which were discussed earlier in this chapter. AP-1 and AP-1-like AREs have also been reported to modulate the expression of γ-glutamylcysteine synthetase (γ-GCS), the rate-limiting enzyme in *de novo* GSH biosynthesis. A catalytic heavy subunit, c-GCS-HS, and a regulatory light subunit, c-GCS-LS, constitute γ-GCS. It has also been reported that the 5′-flanking promoter region of both the human catalytic γ-GCS-HS and regulatory γ-GCS-LS genes contain putative AP-1 and AREs (Antioxidant Response Elements). These are necessary for γ-GCS expression in response to diverse toxic stimuli. Various inflammatory lung diseases exhibit elevated lung fluid glutathione levels and this increase in GSH may be due to up-regulation of GSH synthesis by AP-1 and ARE activation. The cytoprotective role of GSH during toxic reactions remains paramount, and contributes to the substantial interest in AREs (Rahman and MacNee, 2000; Shih *et al.*, 2003; Lee and Johnson, 2004).

Promoter regions of genes encoding conjugating (phase II) detoxification enzymes and of antioxidant proteins contain *cis*-acting regulatory elements or enhancer sequences known as ARE. ARE were first described in 1989 as enhancer elements, similar to a TPA-responsive element (TRE) or an AP-1 site in the rat glutathione S-transferase (GST)-P gene. Soon, similar *cis*-acting regulatory elements or enhancer sequences were found in rat GST Ya, mouse GST Ya, and human NAD(P)H: quinone oxidoreductase-1 (NQO1). Collectively, these studies provide a common ground for exploring related mechanisms of detoxification gene induction. It is now established that ARE sequences are found in many genes, and that they play an important role in downstream gene expression. Some prominent ARE-driven genes that encode rat GST A1, mouse GST A1, rat GST P1, rat NQO1, human NQO1, human glutamate-cysteine ligase (GCL), mouse ferritin-L, mouse metallothionein-1, and mouse uridine diphosphate (UDP)-glucuronosyl transferase (UGT). Interestingly, the aryl hydrocarbon (Ah) receptor mediates expression of functionalization (phase I) drug metabolizing enzymes through XRE regulatory elements. However, little was known about transacting factors or binding proteins for ARE sequences until the discovery of Nrf2 (NF-E2 related factor 2).

Nrf2, a close relative of Nrf1, is a basic leucine zipper protein (bZIP) in the Cap'n'Collar (CNC) transcription factor family characterized by the presence of a 45 amino acid homology region referred to as the CNC domain. It has been proposed that CNC family member, Maf transcription factor, can also bind to the ARE sequence based on the striking resemblance of the ARE consensus sequence to the Maf recognition element

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(MARE). The primary structures of members of the CNC-bZIP subfamily are also well conserved in the basic DNA-binding and protein dimerization domains. This family includes p45NFE2, Nrf1, Nrf2, Nrf3, Bach1 and Bach2. The Nrf2-dependent ARE-driven gene NQO1 is important because of its ability to combat oxidative stress. In the meantime, the role of the AP-1 transcription factor in ARE activation has been explored because of its similarity in binding sequence to the ARE sequence. It has been shown that PMA and *tert*-butylhydroquinone (tBHQ) increase GST Ya gene expression through AP-1, whereas AP-1-mediated-ARE activation is PKC-independent. Many proteins were suggested to be regulated by ARE, but the mechanisms underlying ARE activation did not begin to unfold until the identification of Nrf2. It was proposed that the transcription factor binding to ARE might be an AP-1- related protein because both human NQO1-ARE and rat NQO1-ARE contain AP-1 and AP-1-like elements. Tissue-specific expression of the Nrf2 transcription factor is identical to that of NQO1, and the DNA binding sequence for Nrf2 is identical to the ARE sequence. Together these findings indicate that Nrf1 and Nrf2 interact with ARE and drive cellular defense pathways on demand (Rahman and MacNee, 2000; Shih *et al.*, 2003; Lee and Johnson, 2004).

Studies using Nrf2 knockout mice show decreased expression levels of GSTs and NQO1 compared to wild-type. Neurons from $Nrf2^{-/-}$ mice are more sensitive to oxidative stress compared with those from $Nrf2^{+/+}$ mice and overexpression of Nrf2 dramatically increases resistance of neurons to oxidative cell death. Microarray analysis of oligonucleotides has revealed that Nrf2 regulates the orchestrated gene expression of detoxification enzymes, antioxidant proteins, anti-inflammatory proteins, calcium homeostasis proteins and signalling molecules. An oxidative stimulus with simultaneous up-regulation of ARE-driven genes by Nrf2 appears to be efficient in increasing cellular detoxification and antioxidant capacity, implying a role for the Nrf2-ARE pathway as a cellular antioxidant defense mechanism. Nrf2 is sequestered in the cytoplasm by Keap1, whereas ARE activation signals from protein kinase pathways and electrophiles disrupt the Nrf2–Keap1 complex leading to nuclear translocation of Nrf2 and transcriptional activation of ARE-driven genes. This relationship is supported by evidence that Nrf2 knockout mice are more vulnerable to 1-methyl-4-phenylpyridinium (MPP^+) and rotenone-induced oxidative stress. Biochemical analysis of brain tissues from post-mortem Parkinson's disease patients have disclosed elevated ARE-regulated enzymes such as haeme oxygenase-1 (HO-1) and NQO1, suggesting the possibility of common transcriptional regulation (Rahman and MacNee, 2000; Shih *et al.*, 2003).

Additional evidence comes from studies with the most commonly used analgesic in the world, acetaminophen (paracetamol). Acetaminophen overdose leads to an immediate adaptive defense response in the liver involving various mechanisms, including the nuclear translocation of redox-sensitive transcription factors such as Nrf-2, which sense chemical danger and set cell defenses into motion. Thus, with respect to acetaminophen, Nrf-2 genes of immediate significance are those involved in glutathione synthesis such as γ-GCS, GSTs, UGTs and haem oxygenase. Importantly, it has been observed that nuclear translocation occurs at nontoxic doses of acetaminophen and at time-points before overt toxicity is observed. However, with increasing doses of acetaminophen, there is a progressive decline in nuclear translocation, transcription, translation and protein activity as the rate of drug bioactivation and the adverse consequences of oxidative stress overwhelm cell defenses. There have been several reports describing the modulation of Fas- or TNF-RI-mediated apoptosis by GSH. According to these reports, acute GSH depletion induced insensitivity to DR stimulation, while during prolonged GSH depletion increased DR-mediated apoptosis occurred. Several reports conclude that Nrf2 activity influences the sensitivity of death signalling, and overexpression of Nrf2 protects cells from Fas-induced apoptosis. In addition, *N*-acetyl-L-cysteine (NAC), a precursor to GSH, protects cells from Fas-mediated killing in HeLa cells. All of these results advance our understanding of the genetic link between xenobiotic-induced oxidative stress, ARE and apoptotic death (Morito *et al.*, 2003; Xu *et al.*, 2005).

In conclusion, genetic analysis of cell death has identified key genes, proteins and interlinked pathways that regulate and execute cell death. Structurally related vertebrate genes that appear to play similar roles have been identified and characterized, indicating that the general framework of cell death mechanisms has been conserved over the course of evolution. Depending upon the circumstances, cells may either oppose or follow the instructions of a single gene or of several genes in a concerted manner to orchestrate or bypass the apoptotic pathway, whichever is sensed to be more beneficial for the organism. Since the discipline of toxicology primarily deals with unraveling mechanisms of cell injury and cell death, considerable interest in apoptosis research will continue well into the foreseeable future. Additionally, efforts are underway to manipulate or connect key regulatory points on apoptotic pathways in order to develop novel strategies to delay, reverse or prevent the contributions of this mode of cell death to major diseases such as cancer and neurodegeneration. It is clearly established that protection from apoptosis can be achieved at four different levels: (i) interception of an apoptosis-inducing stimulus, (ii) antagonism of an apoptosis-inducing trigger; (iii) interference with signal transduction cascades and (iv) blockade of catabolic enzymes participating in cell death. The race to develop pharmaceutical agents based on our understanding of apoptosis began at the end of the last century and will continue on into the twenty-first

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century, with the hope of developing break-through therapeutic classes with new abilities to intervene or prevent diseases for which the current prognosis is poor.

The advantages of a self-orchestrated cell death process in the removal of unnecessary, damaged or dangerous cells are abundant. However, this strategy can and often does fail during toxic injury to cells. A fundamental flaw of apoptosis is that its emergence, development and maintenance as a system are driven by the evolution and effectiveness of self-preservation and survival genes. Apoptosis-deficient cells, as seen in many cancers, refuse to die and require new strategies to be defeated. The pathways that lead to activation of apoptosis vary among different cells despite similar stimuli, and different cells respond differently to varied stimuli. Yet the final pathway leading to cell death remains nearly the same. Emerging trends in this field show that gene and antisense agents directed at *Bcl*-2 have promise in some models and may evolve into acceptable therapies in the treatment of some human cancers. Intracellular and extracellular interference with specific protein–protein interactions by select inhibitors may offer another viable approach. Progress achieved in this direction appears rather impressive when viewed in the context of the age of this field, which spans only a couple of decads. Several approaches now show some promise in new apoptosis-based therapies.

8 DRUG- AND CHEMICAL-INDUCED APOPTOSIS

It seems at first paradoxical that cell life is dependent on cell death. Perturbations of normal life processes by toxic chemicals enable us to learn about the life processes themselves and represent an important dimension of the inherent value of the science of toxicology in achieving health and safety. Life processes are highly dynamic, involving the continuous turnover of cells, subcellular structures and biological molecules that often produce opposing effects exemplified by death and regeneration, damage and repair, injury and compensation, and degradation and resynthesis. Therefore, in order to understand the science of toxicity more precisely and within its broadest contexts, the result of exposures to drugs and chemicals might be regarded as the product of divergent biological processes. This includes homeostasis on the one hand and injury followed by compensation and/or repair on the other (see Lotti, 1995). Advances in our journey to understand lethal injury at the molecular level have built on discrete, novel observations. Successive observations have stimulated activity related to that biochemical or regulatory change and have often resulted in heightened interest in one or another implicated subcellular organelle. Over time, attention has focussed on a specific favoured organelle, including the plasma membrane, the cytoplasm, mitochondrion, the lyososome and other subcellular organelles, only to wane and give way to the next target. In considering this history of events, a most important stimulus for increased interest in the role of the nucleus in lethal injury has come from the studies of apoptosis. With the advent of the millennium, research emphasis continues to be placed upon cytoprotection and disease prevention strategies based on the knowledge gained from these mechanistic studies.

Among an ever-increasing number of agents that trigger or cause apoptosis, cancer chemotherapeutic agents, glucocorticoids and retinoids are among the most extensively characterized. Environmental contaminants such as dioxin, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and tributyltin are also capable of triggering apoptosis in target tissues under environmentally relevant exposure scenarios. Natural and anthropogenic agents capable of provoking apoptosis include sanguinarine (Ahmad *et al.*, 2000; Mackraj *et al.*, 2008), the cannabinol anadamid (Pushkarev *et al.*, 2008) ochratoxin-A, gliotoxin, diphtheria toxin, thapsigargin, staurosporine and genistin (McConkey *et al.*, 1995). From the abundance of accumulating reports it appears that many drugs and chemicals capable of causing necrosis are potential apoptogens and vice versa. Likewise, most antinecrogens can be effective inhibitors of apoptosis and vice versa (Ray *et al.*, 1996; 2006).

Studies on acetaminophen offer some insights into these important but unresolved relationships. It is known that acetaminophen directly alkylates genomic DNA both *in vivo* and *in vitro,* and that this widely consumed analgesic produces genotoxic effects in a variety of test systems (Dybing *et al.*, 1984). These findings have now been extended to show that acetaminophen inhibits DNA synthesis and increases single-strand breaks and sister chromatic exchanges of V79 Chinese hamster cells (Hongslo *et al.*, 1988). Although some of these events appear relatively late and may relate more to the carcinogenic potential rather than the hepatotoxic potential of acetaminophen, they are important in that they demonstrate the ability of acetaminophen to damage DNA directly or to inhibit systems that maintain DNA integrity.

A series of publications by Ray, Corcoran and collaborators established that acetaminophen-induced cell death *in vivo* (Ray *et al.*, 1990; 1991; 1993; 1996; Ray and Corcoran, 1991) closely resembles aspects of apoptosis *in vitro* and *in vivo*, particularly as apoptosis is described for the lymphoid cell model using immature thymocytes (Wyllie, 1980; Cohen and Duke, 1984). Classic apoptotic damage including Ca^{2+} accumulation, DNA fragmentation in the form of a ladder, and decreased nuclear DNA recovery all appear prior to major hallmarks of necrosis, that is, extensive leakage of alanine aminotransferase (ALT) into plasma and quantifiable liver histopathology in mice. The timing of these events suggests that faltering Ca^{2+} regulation throughout the cell, but particularly in the nucleus, leads to the same kind of endonuclease

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activation seen relatively early during apoptosis, and thoroughly documented during thymocyte killing *in vitro* (Wyllie, 1980; Cohen and Duke, 1984). Shen *et al.* (1991; 1992) recapitulated and further explored these acetaminophen-induced *in vivo* effects in cultured hepatocytes *in vitro.* It is now well established that hepatotoxic doses of acetaminophen *in vivo* induce death of hepatocytes by apoptosis as well as necrosis (Alison and Sarraf, 1994; Ray *et al.*, 1996; 2006; Liu *et al.*, 2003).

Numerous cells undergoing apoptosis are found in the liver long after the progression of toxicity due to acetaminophen, even 24 hours after drug exposure. Given that apoptotic cells are very rapidly removed from *in vivo* systems, the presence of a large number of this type of cell very late in the toxicosis raises two possible explanations: (i) A rapid onset of apoptosis in a large population of cells that cannot be quickly removed or (ii) A decreased efficiency of the removal process as it is overwhelmed during advancing toxicosis and declining homeostasis (Ray *et al.*, 1996). Similarly, Pritchard and Butler (1989), using the powerful carcinogen and potent hepatotoxin, immunotoxin and nephrotoxin, DMN, demonstrated its ability to induce apoptosis in the liver *in vivo.* Later, Ray *et al.* (1992) discovered some of the biochemical steps, demonstrating the involvement of Ca^{2+} dysregulation in this DMN-induced death cascade. Changes leading to cell death were consistent with histochemical and ultrastructural changes of apoptosis observed by Pritchard and Butler (1989).

The biochemical changes involved in both DMN- and acetaminophen-induced apoptosis (Ray *et al.*, 1992; 1993) were similar and primarily involved intranuclear Ca^{2+}-mediated endonuclease-dependent fragmentation of genomic DNA. This observation was further substantiated by the effects of Ca^{2+}-channel blockers verapamil and chlorpromazine, which prevent Ca^{2+} entry into the hepatocellular nucleus and protect DNA from the endonucleolytic attack, ultimately resulting in the prevention of hepatotoxicity and lethality (Ray *et al.*, 1993). In some of the *in vitro* experiments, aurintricarboxylic acid, an inhibitor of endonuclease and protein synthesis, successfully blocked DNA fragmentation and cell death (McConkey *et al.*, 1989; Shen *et al.*, 1992). Strika *et al.* (1998), using the loop diuretic furosemide, showed that it can induce apoptosis in addition to necrosis in the mouse liver and kidney. Faa *et al.* (1994) using a translational inhibitor, cycloheximide, demonstrated its potential to induce apoptosis in rat liver by electron microscopy. The ultrastructural changes observed in this study are classically apoptotic. Later, using transgenic L_{37}SV40 liver cells, the apoptosis-inducing potential of cycloheximide was shown to be due to growth factor deprivation (Bulera *et al.*, 1996).

The interaction of toxicants and apoptosis is complex and the mechanisms of interaction differ across toxicants. The apoptosis-inducing potential of a variety of carcinogens has been addressed at great length. For many years, research with PAHs has focussed on the ability of these common environmental chemicals to induce cell transformation. Many investigators share the notion that carcinogens antagonize or alter the apoptotic pathway, improving the chances that a cell with aberrant DNA or oncogenic mutations will survive. In contrast, carcinogens can also be powerful inducers of apoptosis. PAHs offer a classical example. Within the PAH chemical group, 7,12-dimethylbenzaanthracene (DMBA) is among the most cytotoxic and immunosuppressive agents that has been studied. DMBA causes apoptotic death of cells in a variety of tissues *in vivo* and in A20.1 murine B-cell lymphoma *in vitro* (Burchiel *et al.*, 1992; 1993; Yamaguchi *et al.*, 1997). Aryl hydrocarbon receptor (AhR)-mediated effects on a Ca^{2+}-endonuclease may be responsible for triggering the apoptotic events observed in these studies. The involvement of a Ca^{2+}-endonuclease has been unequivocally demonstrated in TCDD -induced thymic cell apoptosis (McConkey *et al.*, 1988a). Fuchs and Pruett (1993) have shown that morphine induces mouse thymocyte apoptosis *in vivo* but not *in vitro,* and suggest that both glucocorticoid and opiate receptors are involved in the process. The antitumour antibiotic mitomycin C, a DNA synthesis inhibitor, induces aspermatogenic cell apoptosis in mouse testes (Nakagawa *et al.*, 1997). Representative agents that induce apoptosis *in vivo* and *in vitro* are listed in **Tables 3** and **4**.

Numerous studies indicate that chemotherapeutic agents are capable of triggering apoptosis of both malignant and normal cells. Drugs as diverse as alkylating agents [*N*-(5,5-diacetoxypentyl)doxorubicin], DNA topoisomerase II inhibitors (fostriecin, etoposide, dactinomycin), DNA-intercalating compounds [adriamycin 1-β-D-arabinofuranosylcytosine; Ara-C], DNA-binding compounds (actinomycin-D), DNA crosslinking agents (cisplatin; nitrogen mustard), protein phosphatase (1A, 2A) inhibitor (okadaic acid), PKC inhibitors (Ara-C, staurosporine, H7) and microtubule-disrupting agents (vinblastine, vincristine, colchicine, nocodazole) have all been demonstrated to be potent inducers of apoptosis although by different mechanisms (**Tables 3** and **4**).

Another highly relevant and widely studied hepatotoxin, ethanol, has received heightened attention for many decades. Accumulating data indicate that apoptotic death occurs in addition to necrotic death in several hepatobiliary diseases, including ethanol-induced liver injury *in vivo* (Benedetti *et al.*, 1988a; 1988b; Goldin *et al.*, 1993; Baroni *et al.*, 1994), cholestasis, viral hepatitis, alcoholic and nonalcoholic steatohepatitis and fibrosis (Malhi and Gores, 2008), as well as in *in vitro* model systems (Chen *et al.*, 1997). Unlike acetaminophen and some higher alcohols [C_3 (propan-2-ol) to C_8 (octanol)], however, one bolus LD_{50} dose of ethanol fails to induce apoptosis in the rat liver. Higher alcohols such as butanol, pentanol, hexanol and octanol are potent inducers of

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apoptosis in the normal rat liver. Ray and coworkers also found that spontaneously hypertensive rats are more sensitive to the apoptosis-inducing potential of alcohols (Manolas *et al.*, 1997). The same group found that moderate ethanol exposure ramping from 3 to 5% over three weeks followed by low-dose acetaminophen (300 or 400 mg kg^{-1}, i.p.) dramatically potentiate the acetaminophen-induced apoptotic death rate in mouse liver cells *in vivo* (Ray, 1997).

Although oxidative stress has been proposed to be a principal stimulus leading to apoptosis in this case (Kurose *et al.*, 1997), it is difficult to pinpoint a definitive mechanism for ethanol-induced apoptosis, since the dose, duration, route of exposure and models are diverse. Increased expression of Bcl-2 protein has been demonstrated in ethanol-exposed rat livers (Yacoub *et al.*, 1995). However, this increase in Bcl-2 expression was found to corretate with the presence of an excess number of inflammatory cells and may not have involved an increase in expression of the gene in the liver cells. Similar observations were reported by Dhruva and Ray (1996), Dhruva *et al.* (1996), and Ray (1998) in acetaminophen-treated mouse livers, where Bcl-2 protein was found in mouse liver. Regulation of hepatocellular apoptosis by Bcl-2-dependent and Bcl-2-independent mechanisms may exist (Oberhammer *et al.*, 1992). Future studies with ethanol are needed to elucidate its interaction at the organelle level (e.g. MPT or cyt-c release) and/or at the genetic level (e.g. Bcl-2 regulation).

Oxidative damage induced by ROS is a normal aspect of aerobic life. Damage occurs at the structural level to cellular and subcellular organelles, and at the functional level to macromolecules including enzymes, DNA, proteins and carbohydrates. Damage inflicted by ROS species including $O^{2-\cdot}$, H_2O_2, $HO\cdot$ and others has been termed oxidative stress. The role of oxidative stress in apoptosis has been extensively reviewed (Buttke and Sandstrom, 1994; Ray *et al.*, 2001; 2004; Hickey *et al.*, 2001; Surh, 2003). Many agents, whether physical (ionizing/UV radiation) or chemical (redox cyclers including antineoplastic agents) are capable of provoking oxidative stress in addition to inducing apoptosis mediated by a variety of ROS (Buttke and Sandstrom, 1994; Pallardy *et al.*, 1997; Bagchi *et al.*, 1998b). Evidence that oxidative stress can induce apoptosis comes from studies in which apoptosis induced by ROS can be inhibited by antioxidants.

One such example is TNFα. Stimulation of the TNFα receptor promotes intracellular production of ROS through increased mitochondrial superoxide anion formation. Inhibition of ROS production by *N*-acetylcysteine or the free radical scavenger thioredoxin prevents apoptotic cell death (Buttke and Sandstrom, 1994; Pallardy *et al.*, 1997). The Fas antigen, which is structurally related to the TNF receptor, also induces apoptosis when crosslinked with antiFas antibodies. Its ability to induce apoptosis in cells has been shown to be blocked by thioredoxin. Exogenous sources of ROS such as H_2O_2, and exogenous *tert*-butyl hydroperoxide induce apoptosis at doses well below the necrogenic doses. Quinone compounds that undergo redox cycling and cause superoxide radical formation, such as menadione and 2,3-dimethoxy-1,4-naphthoquinone also induce apoptosis (McConkey *et al.*, 1988b). Chronic inhibition of superoxide dismutase transcription or activity causes apoptotic death. Intracellular ROS appear to activate genes responsible for apoptosis, conceivably through an oxidative-stress-responsive nuclear transcription factor such as NFκB (Sen and Packer, 1996).

Over the past two decades, free radicals related to ROS and nitric oxide (NO) have been the subject of intense study and considerable debate. NO orchestrates a range of useful biological activities as well as deleterious pathophysiological activities. It is synthesized from molecular oxygen and the guanidino group of L-arginine by a family of closely related enzyme isoforms of nitric oxide synthase (NOS: EC 1.14.13.39). NO is a messenger molecule that participates in drug-induced hepatotoxic reactions, relaxation of smooth muscle, neurotransmission and killing of tumour cells and bacteria. Evidence suggests that biochemical and toxicological effects of NO are mediated, in part, by $ONOO^-$ (peroxynitrite: a reaction product of NO with superoxide anion, $O^{2-\cdot}$). NO and $ONOO^-$ have each been shown to induce necrosis in model systems (Ignarro, 1990; Nathan, 1992), and their apoptotic potential in murine macrophages, human epithelial cells and HL-60 cells is well established (Messmer *et al.*, 1995; Sandoval *et al.*, 1997 Sen *et al.*, 2008).

Mechanisms proposed for NO toxicity include its interaction with protein thiol groups or iron–sulphur proteins or by direct DNA damage. The genomic damage induced by radiation or by drugs such as etoposide can result in apoptosis. NO may signal different forms of cell death depending on the type and/or the steady-state concentration of the NO redox species involved. A link between p53 expression and NO synthesis dependence has been established (Messmer *et al.*, 1994), although the connection between antideath gene *bch*, NO and apoptosis remains open for investigation (Dhruva and Ray, 1996; Dhruva *et al.*, 1996). Similarly, the precise role of Kupffer cells, macrophages, neutrophils and phagocytes requires further scrutiny, because these cells play an important role during NO-induced apoptosis *in vivo.*

Many agents are genotoxic while others lack genotoxicity. Among the latter, TCDD, trichloroethylene (TCE), dichloroethylene (DCE), CCl_4, trichloromethane ($CHCl_3$), hexavalent chromium compounds, cadmium compounds, nickel compounds and tributyltins have been thoroughly investigated. Surprisingly, some of the historically known compounds like ethyl-methane sulfonate, CCl_4 and $CHCl_3$, lack the ability to induce apoptosis despite the observations that their toxicities

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are Ca^{2+}-dependent (Long *et al.*, 1989; Ray and Fariss, 1994). In contrast, other agents including DCE, TCE, and TCDD are efficient inducers of apoptosis (Blankenship *et al.*, 1994; Costa, 1998). In addition to TCDD (McConkey *et al.*, 1988b), other mechanistically well-defined examples from this category are tributyltin oxide (bis(tri-*n*-butyltin) oxide) and tributyltin-induced apoptotic death of immature thymocytes in culture (Raffray *et al.*, 1993). Investigators have demonstrated that tributyltin oxide, a potent but relatively nonspecific agent, activates thymocyte apoptosis independent of protein synthesis and under conditions of severely compromised cellular energetics. This argues against the well-accepted notion that apoptosis is a highly regulated intracellular event which is energy dependent and requires transcriptional and translational control. Unlike the oxide form of tributyltin, tributyltin chloride is a potent and rapid inducer of apoptosis *in vitro*. *In vivo*, dibutyltin chloride seems to be much more potent in inducing thymic atrophy (Dobbelsteen *et al.*, 1997).

Accumulating evidence indicates that metals may either positively or negatively influence apoptotic processes. Some forms of metals, such as calcium, magnesium, cadmium and chromium, favour apoptosis, whereas Zn^{2+} may either favour or antagonize apoptosis (Sunderman, 1995; Wolf *et al.*, 1997). Furthermore, some metals exert an antagonistic influence on the action of other metals. Cadmium suppresses chromium-induced apoptosis (Shimada *et al.*, 1998). Metal-induced apoptosis is a leading issue for toxicology and remains at the centre of public health concerns over environmental exposures.

Although the mechanisms of many antiapoptotic agents such as cholesteryl hemisuccinate are unknown, the fact that dimethyl sulfoxide, a membrane-permeable solvent hydroxyl radical scavenger, thioredoxin, an intracellular thiol reducant, *o*-phenanthroline, a hydroxyl radical formation blocker, *N*-(2-mercaptoethyl)propane-1,3-diamine, an inhibitor of membrane peroxidation and the antioxidant vitamins C and E, as well as exogenous catalase, prevent apoptotic death in a variety of model systems (Buttke and Sandstrom, 1994) substantiate the role of ROS in apoptosis. Cholesteryl hemisuccinate prevents both of the apoptotic and necrotic forms of cell death induced by acetaminophen *in vivo* (Ray *et al.*, 1996).

Varied studies offer evidence that agents that influence PARP activity, for example, 3- and 4-aminobenzamide (**Figure 3**), NAD, nicotinamide, caffeine and theophylline, depending upon the model system and concentration used, either abrogate or escalate drug- or chemically induced cell death. However, the same agents interfere with transmembrane signalling mechanisms in different model systems, such as thymocytes (Ray *et al.*, 1992; McConkey *et al.*, 1995; Yahya *et al.*, 1995). The nitroso-containing free radical scavenger 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) blocks methylprednisolone-induced apoptosis, and the Ca^{2+}-ATPase inhibitor thapsigargin, the Ca^{2+} chelator EGTA, the Zn^{2+} chelator *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine, Zn^{2+} salts (Bagchi *et al.*, 1997) and the topoisomerase II inhibitor etoposide block apoptosis in a variety of model systems under diverse conditions. The commonly used antioxidant BHA (frequently added as a preservative in foods), dithiocarbamates (DTCs), diethyl dithiocarbamate (DETDTC) and dimethyl dithiocarbamate (DMDTC) serve as anti-apoptotic agents in radiation-induced leukaemic cells (Verhaegen *et al.*, 1995).

9 SUMMARY AND CONCLUSIONS

Apoptosis is a process of single-cell deletion requiring active participation of the cell in its own demise. It occurs during both physiological conditions, such as neural development, and pathological conditions such as acute myocardial infarction, ischaemia and stroke. It is morphologically distinct from necrosis and characteristically presents with cell shrinkage, dense fragmented chromatin and cellular budding with fragmentation. The process results in the formation of apoptotic bodies that are quickly phagocytosed by a variety of cells, thereby keeping the integrity of the tissue intact. In contrast to necrosis, apoptosis typically does not induce an inflammatory response. Apoptosis can result from an imbalance between the activation of effector genes which promote cell death and repressor genes which enhance cell survival. Of these, Ca^{2+} regulation and several Ca^{2+}-dependent events have been scrutinized. Much of the evidence remains indirect and some even speculative. It is also not clear once a particular cell turns on the death programme *in vivo* or *in vitro*, how the neighbouring cells react. Although in *in vivo* model systems many of the biochemical pathways for apoptosis are well characterized yet not fully understood, the TNFR superfamily, the caspases and the *bcl-2* gene products are clearly implicated in effector roles, and ceramide, ROS, MPT and cellular energy status have been implicated as mediators. Cell cycle regulatory elements have gained considerable attention, and it is established that apoptosis may require the action of several molecules that are normally involved in regulating cellular proliferation. It is also evident that the mechanisms necessary for completing apoptosis exist in every cell.

Much has occurred to propel apoptosis into the forefront of basic and clinical research. This outcome was founded upon identification of genes that control cell death and has resulted in an appreciation of the roles of apoptosis in development, health and disease. Apoptosis is often a beneficial form of cell death in development and therapeutics, but is increasingly recognized as the basis of many drug- and chemical-induced toxicities. The induction of apoptosis after a toxic assault

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can also be seen as beneficial, since loss of aberrant cells by apoptosis can impose a minimal negative impact on the host. Instances have been reported where apoptosis has been observed either in the near total absence of genomic DNA digestion (Ucker *et al.*, 1992) and in the absence of a nucleus (Jacobson *et al.*, 1994). It now appears that apoptotic and necrotic processes paradoxically share many similar intracellular mechanisms, including oxidative and reductive stress, Ca^{2+} dysregulation, DNA fragmentation, MPT, cellular energy status and membrane potential fluctuations. Raffray and Cohen (1997) elegantly addresses these concepts, and offer their opinion that apoptosis and necrosis are essentially distinct modes with limited molecular and cell biology overlaps. Yet increasing evidence indicates that apoptosis and necrosis are parts of a cell death continuum (Wyllie, 1987; Corcoran and Ray, 1992; Ray and Raje, 1994; Tomei *et al.*, 1994; Ray *et al.*, 1996).

The search for commonalities that unify drug- and chemical-induced cytotoxic mechanisms has met with limited success. From an operational standpoint, the killing of some cells can be viewed as occurring via direct means, whereas the killing of others takes place via indirect means. In toxicant-induced cell injury which ultimately results in cell death, changes that are responsible for cell death are joined and effectively concealed by a host of powerful but futile stimuli from a network of control systems that attempt to return the cell to homeostasis. While the control network strives to return to homeostasis, systems that are devoted to cellular repair and regeneration also become engaged through a number of common mechanisms. It is apparent why most cell responses during lethal injury are not directly related to the ultimate cause of cell death. Only a select response or subset of responses is considered to be capable of producing permanent irreversible damage and the ultimate loss of viability.

The cell is considered the smallest autonomous unit of living systems that has the capability to receive information from its environment, to transduce it according to the genetic information encoded in DNA and finally to put out appropriately processed signals into its environment in order to realize teleonomically designed organized functions (Ji, 1997). The widespread involvement of apoptosis in diverse conditions gives rise to the notion that targeting this response will lead to the development of novel therapeutic regimens. The presence of apoptotic machinery in tumours suggests that its induction could be used as a therapy. The ability to modify sensitivity to apoptosis through the regulatory pathways has clear implications for the treatment of malignancy. Potential strategies fall into three categories: directly inducing apoptosis by cytotoxic agents, enhancing vulnerability to apoptosis to increase the efficacy of other therapies and boosting the resistance of normal cells to apoptosis (Bellamy *et al.*, 1995). Among these, direct antitumour therapy targeting apoptotic modulation may prove to be much less systemically toxic than standard chemotherapy and could also be used in an adjuvant manner, to increase the susceptibility of tumours at the time they are exposed to chemotherapy (Rudin and Thompson, 1997). The question of cause–effect in drug-induced apoptosis seems to be most crucial in order to appreciate the impact of apoptosis on chemotherapy and to position the protective action of death-inhibitory genes. The successful developments of agonists and antagonists of this process can provide important new access points of various regulatory pathways. Already, newly acquired knowledge concerning the regulation of apoptotic cell death is being applied clinically in the development of prognostic indicators to predict disease progression and for the treatment of cancer. Apoptosis-targeting strategies currently being tested in clinical trials involve the use of antisense oligonucleotides (*Bcl*-2, XIAP, ML-IAP), recombinant biologicals (TRAIL receptor, DR5, CD95-Fc, Survivin-2B, TNFα, caspases) and classical organic pharmaceuticals (caspases, IAP, p53, cyt-c, JNK). It is rather early to tell whether the overall strategy of modulating cell death signalling pathways will prove to be clinically useful, but treatment successes, should they be realized would represent important victories for the strategy of rationale drug development.

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A Metabolomic Perspective of Small Molecule Toxicity

Andrew D. Patterson and Jeffrey R. Idle

CONTENTS

1 INTRODUCTION

Recently, the Society of Toxicology redefined toxicology for the National Library of Medicine as follows: 'Toxicology is the study of the adverse effects of chemical, physical or biological agents on living organisms and the ecosystem, including the prevention and amelioration of such adverse effects' (http://www.toxicology.org/AI/PUB/SI05/SI05_Define.asp). The key word in this catch-all definition, that separates toxicology from other disciplines such as pharmacology, therapeutics or ecology, is 'adverse'. Immediately, we notice that a value judgement is required. After all, Lucretius wrote in 50 BC 'What is food to one man is bitter poison to others' (*De Rerum Natura*, book IV, line 637) and therefore the adverse effects of chemicals have been long recognized as nonuniversal. Adrien Albert appreciated that toxicity is often species-selective (Albert, 1951) and, moreover, Charles Darwin understood that 'No-one supposes that all the individuals of the same species are cast in the same actual mould'. This has become an axiom of the field of pharmacogenetics (Idle, 1988). Thus, 'adverse' is, at worst, a subjective, and, at best, a relative, concept. This is one reason why it is desirable to have objective biological indicators of toxic effects, biomarkers that do not depend on a qualification by species, individual or phenotype, but that reflect the interaction between an exogenous chemical and an enzyme, receptor or other biological mediator of toxicity. Such biomarkers may be a sign of changes in gene expression, that is, transcriptomic biomarkers, they may indicate changes in protein expression and/or stability, that is, proteomic biomarkers, or they may reflect fluxes of small cellular metabolites, that is, metabolomic biomarkers. In this chapter we will discuss metabolomic biomarkers of small molecule toxicity and provide an introduction to methods of metabolomic analysis, discuss the short history of metabolomics in toxicology, and present a future perspective of metabolomics in toxicological science.

2 WHAT IS METABOLOMICS?

There are many descriptions of metabolomics and a few definitions to be found in the scientific literature. The situation is complicated by the existence of an apparent rival name, 'metabonomics'. This term was introduced by Jeremy Nicholson and his colleagues at Imperial College London and defined as follows: 'the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification' (Nicholson *et al.*, 1999). However, the term has been used almost

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exclusively to describe nuclear magnetic resonance spectroscopy (NMR)-based studies, many of which have been carried out by the Imperial College group itself. A more detailed discussion of the two apparently rival terms has been made (Robertson, 2005). In PubMed, citations for 'metabonomics' are only a third of those for 'metabolomics'. Although interchangeable, the two terms appear to mean different things to different investigators, so it is worth clarifying here that the definition of Nicholson for metabonomics equally applies to metabolomics. The attraction of the latter is that the complete set of small molecules in a cell, tissue or organism is usually referred to as the metabolome, and therefore metabolomics would seem to follow logically, as genomics follows the genome or proteomics follows the proteome. Some have proposed the compromise term 'metabolic profiling' (Clarke and Haselden, 2008). Nevertheless, it is not the definition of metabolomics that is important, but its characteristics. A further remark must be made about some of the published studies on NMR-based metabonomics and that is that they do not, in fact, adhere to the definition of metabonomics. Specifically, metabonomics is said to be 'the quantitative measurement of...' and yet, almost invariably, no attempt at quantitation of analytes is made. There is little doubt that the considerable body of NMR-based literature has contributed significantly to the noninvasive and semiquantitative investigation of small molecule toxicity. The message here is that metabolomics should be employed to identify candidate elevated or attenuated biomarkers of chemical toxicity and then these should be evaluated quantitatively outside of the metabolomic platform.

3 LCMS-BASED METABOLOMIC METHODS

Many of the problems inherent in NMR-based metabolomics studies can be overcome through the use of liquid chromatography-mass spectrometry (LCMS). In this case, instead of each high concentration constituent of the biological matrix giving rise to multiple, sometimes overlapping, signals in a highly complex NMR spectrum, each constituent is usually resolved in time as a single peak in a chromatogram that yields an accurate mass and can be subjected to tandem mass spectrometry (MS/MS) that yields unequivocal identities of large numbers of analytes. Such peaks are readily amenable to quantitation. When combined with ultraperformance liquid chromatography (UPLC), MS instrumentation, such as quadrupole–orthogonal time-of-flight mass spectrometry (QTOFMS), can typically yield ~5000 ions in both positive and negative electrospray ionization (ESI) mode. Each of these ions will have a retention time (RT) value (on the UPLC column), a mass-to-charge ratio (m/z value) and an intensity value. Each biological sample analysed may therefore generate 30 000 data points. A typical metabolomic experiment that employs UPLC-ESI-QTOFMS methodologies can easily generate a million data points. Yet, hidden in these data may be a single elevated biomarker. Thus, the need for multivariate data analysis (MDA) methods to reduce the data set to a manageable form.

Figure 1 shows a typical metabolomics protocol in which either humans or laboratory animals are subjected to a '±' sample collection protocol. In one arm of this study blank samples, that is, devoid of experimental chemical exposure, are collected from each individual subject or animal. These might be spot urines, timed urines, blood samples or even saliva, sweat or sebum samples. In the second arm of the study the individual subjects or animals are exposed to a chemical or mixture of chemicals. This might entail administration by mouth of a drug or other xenobiotic, or could involve exposure by inhalation or in the diet. Multiple samples at different time points after either an acute or chronic exposure can be taken. The next phase of the study involves analysis of the samples. Ideally, samples should be labelled and then randomized. For LCMS-based metabolomics, sample preparation requires minimum effort. For urine, addition of acetonitrile, chilling and centrifugation is sufficient to remove any particles and also precipitate and remove proteins. Samples can then be injected into the analytical instrument.

Figure 2A shows a working Waters Q-Tof Premier™ (right) coupled to a Waters Acquity UPLC® (left). The UPLC employs small-particle-size columns, typically 1.7 μm. This permits higher chromatographic resolution, shorter chromatography times and the use of less solvent per sample analysis. A typical UPLC chromatogram will be of 10 minutes duration, with a total of 12 minutes for equilibration, run time and analysis. An equivalent high-performance liquid chromatography (HPLC) run would take of the order of 50 minutes. This improved throughput is essential for metabolomic studies, which can generate very large numbers of samples that may need to be injected both in positive ion (ESI+) and negative ion (ESI−) modes. Exiting from the UPLC column, the eluate is electrosprayed into the mass spectrometer. Many biological molecules form positive and negative ions that are inherently unstable and fragment in the front end of the instrument. Accordingly, attention must be given to voltage settings in this part of the instrument to minimize in-source fragmentation, while permitting a usable ion yield. **Figure 2B** depicts the workings of a QTOFMS instrument. Ions pass through an ion guide immediately preceding initial resolution by a quadrupole. Ions traverse the collision cell, which is used to fragment the ions in MS/MS experiments. Finally, ions enter the time-of-flight chamber, where they are resolved by mass, with a mass accuracy of typically 1–5 ppm. The instrument is capable of acquiring spectra, say from 50–850 Da, at a rate up to 20 per second. Thus, in a

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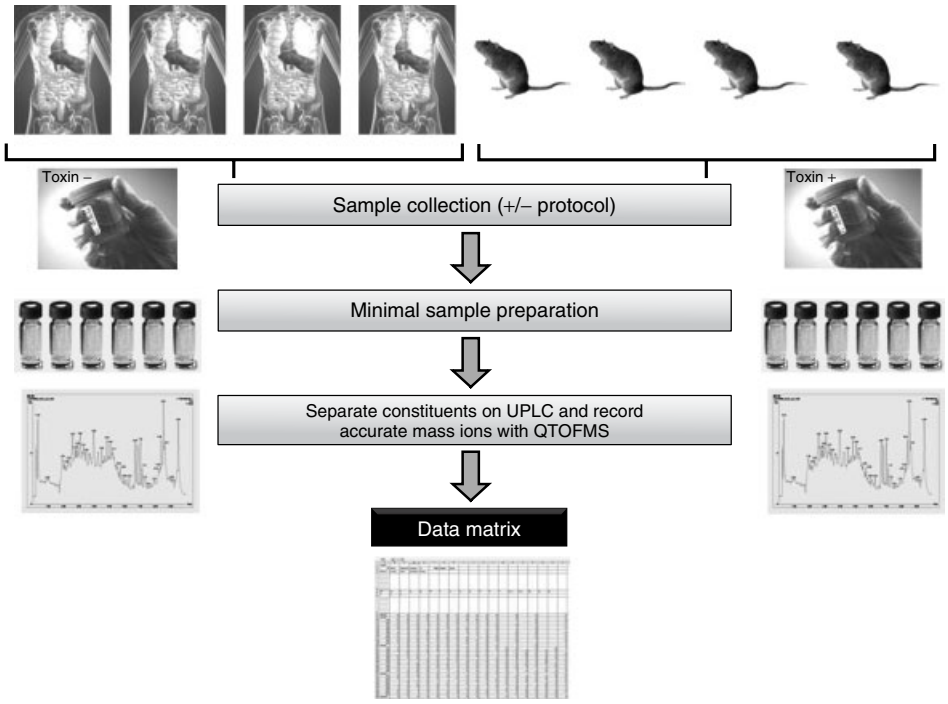

Figure 1 Metabolomics workflow. Biological samples, such as urine, serum, sweat, saliva or sebum, are collected from human subjects or laboratory animals that have been exposed (TOXIN+) or not exposed (TOXIN−) to a small molecular toxicant. Minimal sample preparation and analysis by UPLC-ESI-QTOFMS leads to production of a data matrix of retention time, m/z and ion abundance. The primary objective of a metabolomics experiment is production of this data matrix.

typical 10 minute chromatogram, over 10 000 full spectra can be collected. It is not unusual in a metabolomics experiment with only 20 samples to acquire 5000 negative and 5000 positive ions per sample, thus a total of 200 000 ions. These data are stored in a data table with values of RT, m/z and relative abundance for each ion. The total number of data for this small experiment is thus 600 000. The primary objective of a metabolomics experiment is to produce this data spreadsheet. Since a variable in the data table should correspond to the same ion in all of the samples, the first manipulation of the data is to align the peaks, that is, to ensure as best as possible that the positive ion of, say, $m/z = 116.0711$ (corresponding to proline at a mass error of 0.9 ppm) has an RT = 0.31 min for all samples. This peak alignment procedure, carried out by the instrument software MarkerLynx™, requires the judicious setting of peak width parameters so that the software does not record multiple proline peaks at slightly different RT values. Noise in the data set can also be reduced by means of data scaling, commonly using the Pareto scaling method, whereby the value of each variable is divided by the square root of its standard deviation. Once the aligned and scaled data table is in hand, the data must be analysed using chemometric methods. Therefore, metabolomics first involves analytical chemistry followed by a mathematical solution.

There has been a proposed initiative in the metabolomics field to report, for each experiment, the 'metadata' or information about the experiment as well as to standardize terminology and construct databases for disseminating information to the public (Castle *et al.*, 2006; Fiehn *et al.*, 2006; 2008; Jenkins *et al.*, 2004; Sansone *et al.*, 2007; Scholz and Fiehn, 2007). The metadata includes such details as sample preparation, chromatography and mass spectrometry (MS) conditions, as well as descriptions of data preprocessing and analysis. Based on standards set forth by these groups, the metabolomics standard initiative (MSI) was established such that independent validation of experiments could be conducted and to facilitate the exchange of data (Metabolomics Standards Initiative, 2008; Sansone *et al.*, 2007). With this in mind, each metabolomics experiment should be carefully documented and annotated in as much detail such that the experiment could be reproduced from scratch.

3.1 Quality Control

Before proceeding with the data analysis it highly recommended to inspect visually the chromatograms to ensure that the downstream statistical analyses are robust and reliable. For this purpose a set of standard operating procedures must be established and followed well before entering the data analysis stage. The following are few important suggestions:

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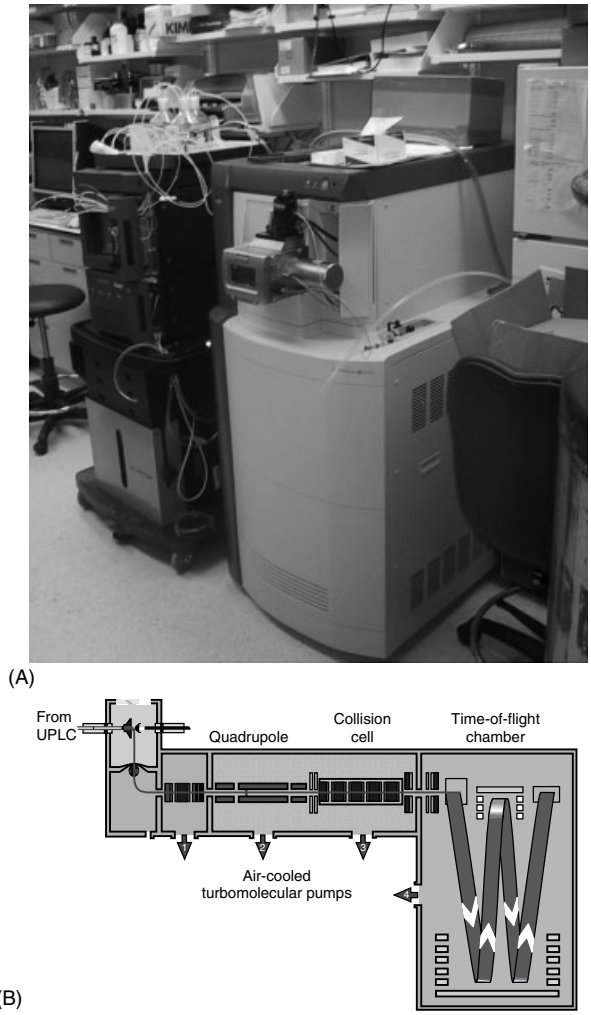

Figure 2 (A) A working UPLC-ESI-QTOFMS. Samples are injected into a Waters Acquity™ UPLC® and the eluant electrosprayed into a Waters Q-Tof Premier™ QTOFMS. (B) Production of ions by electrospray and their resolution by QTOFMS. Ions are first partially resolved by a quadrupole and pass through a collision cell which is used to fragment ions in MS/MS experiments. Ions are resolved by accurate mass and detected with a typical mass error of 1–5 ppm.

1. Inspect the internal standard peak shape and require that its RT is relatively stable across all samples. Other known compounds, such as creatinine in urine, can be monitored similarly. Deconvoluting software will find it difficult to align peak RTs if the chromatography is not consistent and may report missing values when they are indeed present. If these conditions are not met, it may be possible to use relative RT to correct for any chromatography problems.
2. Inspect the intensity of the internal standard and other known compounds. Ensure they are within historical ranges. Reduced sensitivity will hinder investigations looking for low abundance biomarkers and may indicate a need for maintenance of the instrumentation.
3. Investigate the influence of injection order. Ensure samples were properly randomized and that the variability associated with the injection order does not influence the results (Enot *et al.*, 2008).
4. If using instrumentation capable of accurate mass measurements, be sure the reported m/z values have been acquired with acceptable mass error (<10 ppm, preferably <5 ppm). In addition to checking the accurate mass of the internal standard or from a cocktail of compounds, it is wise to also check the accurate mass measurement of compounds known to be contained within a sample. Otherwise it will be exceedingly difficult to identify unknowns based on inaccurate mass measurements. For example, in metabolomic analysis of urine, it is possible to determine the accurate mass of creatinine (Formula = $C_4H_7N_3O$, MW = 113.0589) and calculate the mass error in ppm. Error in ppm can be calculated as follows, where mass_e is the experimental mass and mass_c is the calculated mass:

$$\text{Error in ppm} = \frac{\text{mass}_e - \text{mass}_c}{\text{mass}_e} \times 10^6$$

4 CHEMOMETRICS

If your experiment needs statistics, you ought to have done a better experiment.

Lord Rutherford

The analysis of such huge data sets cannot be accomplished manually. Chemometrics, the application of mathematical or statistical methods to chemical data (Lavine and Workman, 2008), provides a means of extracting useful information from data-rich experiments (Trygg *et al.*, 2007; Wiklund *et al.*, 2008). Chemometric methods of data analysis include the MDA methods of principal components analysis (PCA) and projection to latent structures (PLS). In addition, a new method of increasing popularity is the machine learning algorithm 'random forests'. This has found utility in analysis of DNA microarray data and in proteomic analysis (Barrett and Cairns, 2008; Datta, 2008; Diaz-Uriarte and Alvarez de Andres, 2006; Shi *et al.*, 2005) but shows great potential as a tool for metabolomics.

4.1 Data Matrix and Pre-Processing

In the case of hyphenated techniques such as liquid chromatography coupled with mass spectrometry (LC-MS), it is necessary to generate a data matrix that is suitable for MDA. This process includes deconvoluting and aligning the peaks across all the samples and recording

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the measured values such as peak area. Both freely available (e.g. XCMS) and commercial (e.g. MarkerLynx™, Waters Corporation) software are useful for data extraction (Smith *et al.*, 2006). Once the data matrix has been populated it is wise to normalize (e.g. divide by an internal standard or cocktail of internal standards, or sum the total ion chromatogram to some arbitrary value) to reduce the influence of sample preparation and instrument variability on the outcome and to improve the overall quality of the data for downstream statistical analysis and modelling (Enot *et al.*, 2008; Sysi-Aho *et al.*, 2007). Additionally, the normality of the data should be inspected given that many statistical methods assume an approximate Gaussian distribution (Steuer *et al.*, 2007). Normalization should be done with great care as it may introduce unwanted artefacts (Sysi-Aho *et al.*, 2007). Additionally it is recommended that the data matrix be inspected for very low or zero values, and while these may be removed using some predetermined criteria, it should be investigated if these values are truly not important. Once the data matrix has been generated and the data is determined to be of sufficient quality, then the next phase of data analysis can be undertaken. This complete data handling process is shown in **Figure 3**.

4.2 Statistical and Data Modelling Approaches

4.2.1 Principal Components Analysis (PCA)

PCA is a so-called unsupervised method of analysis, meaning that manual labels of inputs are not used. Put more simply, if the analysis is one of urine samples from chemically exposed and chemically unexposed animals, classification by exposure is not used in the PCA analysis. PCA is a common and highly popular unsupervised method for analysing metabolomics data, particularly as a 'first pass' analysis to discover important latent variables as well as potential outliers. PCA is a dimension reduction tool permitting the visualization of high-dimension data in, typically, two- or three-dimensional space. PCA works by first finding the maximum variance in the dataset (i.e. the first principal component) and then describes the remaining variance in subsequent, orthogonal components (Eriksson *et al.*, 2006). Information can be obtained by viewing the scores plot (i.e. the samples projected onto principal components) and loadings plot (i.e. the variables influencing the location of the samples in the scores plot) to understand which variables are contributing to the observed group separation

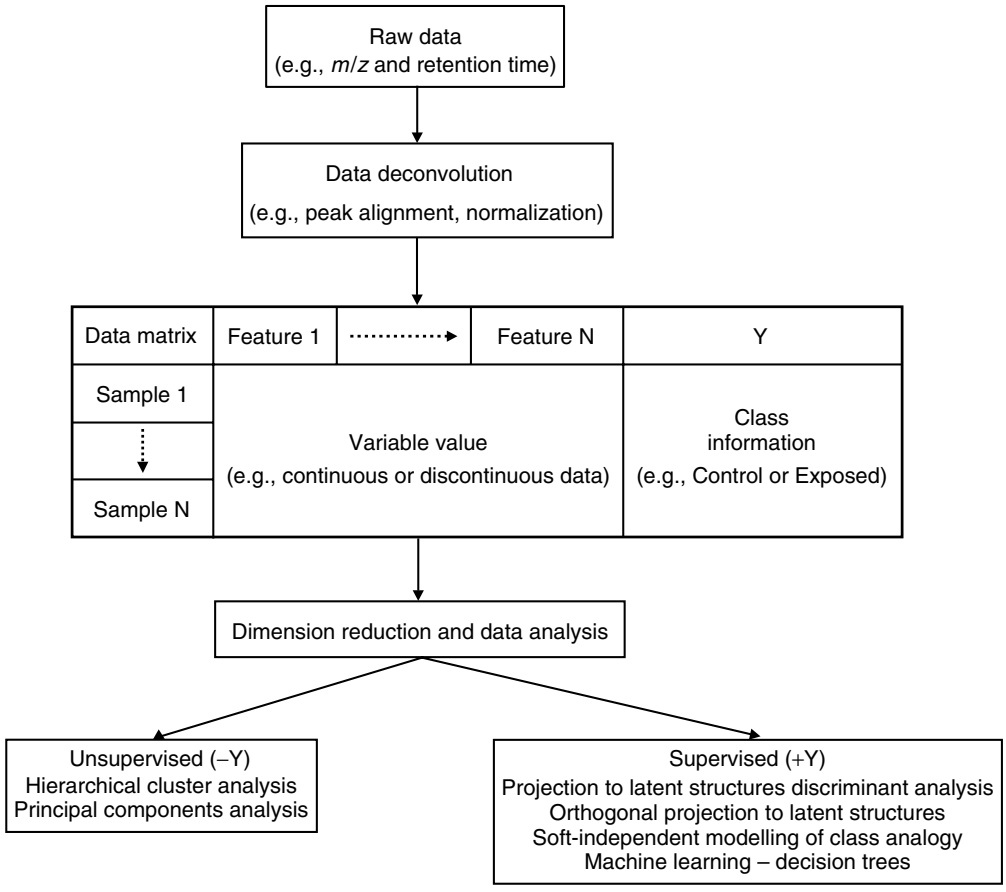

Figure 3 Mathematical and statistical manipulation of the raw data matrix to yield metabolomic solutions. Single ion chromatographic peaks are typically aligned and normalized using the instrument software MarkerLynx™.

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(Eriksson *et al.*, 2006). Data modelling and visualization by this method, particularly for MS, requires that the data be scaled in order to cope with the different ranges of input data. Scaling methods such as unit variance (UV), dividing each variable by its standard deviation across the samples, is useful to eliminate the influence of highly abundant variables, and hence those with greater variance, on the resulting model. Other popular scaling methods include Pareto scaling (see above) (van den Berg *et al.*, 2006). It is important to note that scaling is likely to be experiment dependent and that various scaling methods should be investigated. A more detailed description of PCA can be found elsewhere (Trygg *et al.*, 2007)

We will illustrate PCA using data from a very simple experiment whereby four male C57BL/6 mice were allowed access to a normal diet (NIH31 chow) and their urine collected for 24 hours. The mice were then switched to a synthetic diet (AIN-93G) and, after seven days, a second 24 hour urine sample was collected. The samples were analysed according to the protocols in **Figures 1** and **3**. Based upon positive ions, the PCA score scatter plot (or scores plot) for these eight mouse urine samples is shown in **Figure 4A**. It is immediately apparent that the four samples from mice on the normal chow and the four samples from mice on the synthetic chow form two distinct clusters. They have separated

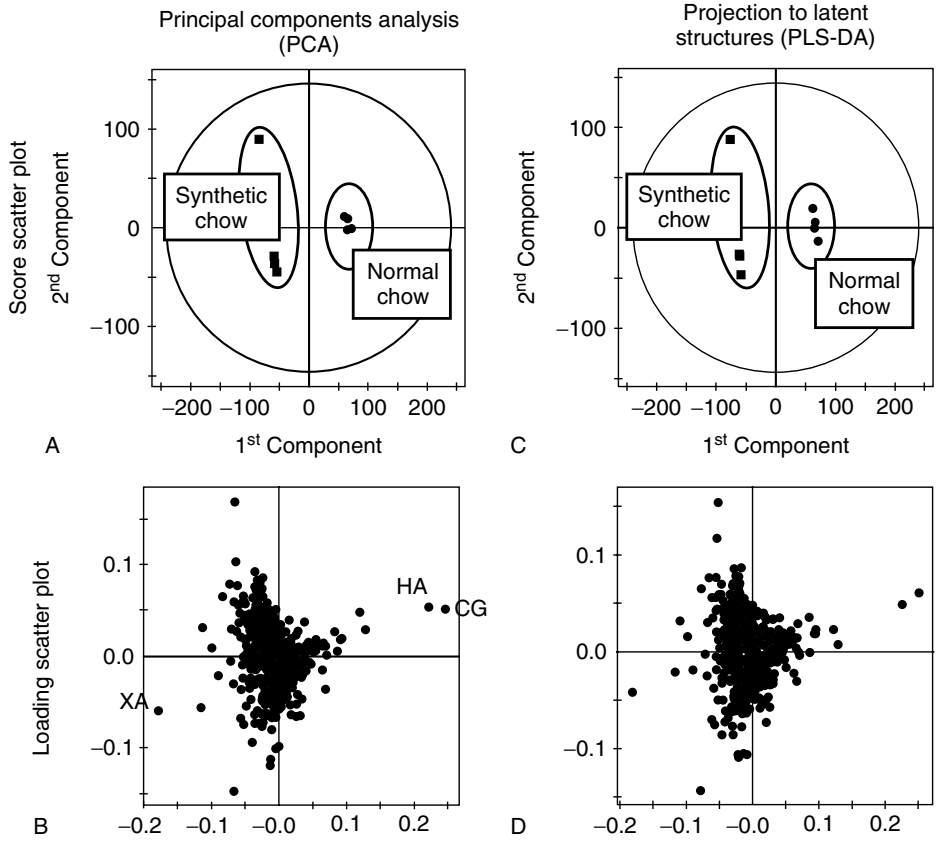

Figure 4 (A) A score scatter plot (scores plot) for a principal components analysis (PCA) of urine from mice fed on a normal chow and then switched to a synthetic chow. Note that the principal variation is encompassed in principal component 1 (*x*-axis). The mean value of the second component is approximately the same for both groups. PCA is an unsupervised analysis, meaning that class information, that is, knowledge of the diet for each sample, is not used in the analysis. (B) A loading scatter plot (loadings plot) for a principal components analysis (PCA) of urine from mice fed on a normal chow and then switched to a synthetic chow. Two principal outliers are associated with the normal chow (CG, cinnamoylglycine; HA, hippuric acid) and one with the synthetic chow (XA, xanthurenic acid). (C) A score scatter plot (scores plot) for a projection to latent structures (PLS-DA) of urine from mice fed on a normal chow and then switched to a synthetic chow. Note that the principal variation is encompassed in principal component 1 (*x*-axis). The mean value of the second component is approximately the same for both groups. PLS-DA is a supervised analysis, meaning that class information, that is, knowledge of the diet for each sample, is used in the analysis to help build a model to separate the classes. Note that this scores plot is virtually identical to the PCA scores plot in Figure 4A because the PCA separation is so good that little supervision using class information (normal vs synthetic chow) is required to give a separation. (D) A loading scatter plot (loadings plot) for a partial least squares discriminant analysis (PLS-DA) of urine from mice fed on a normal chow and then switched to a synthetic chow. Note the same outliers are present as in the PCA loadings plot in Figure 4B.

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in principal component 1 (PC1) but not in principal component 2 (PC2), mean values of PC2 being similar for the two clusters. From this, we can immediately deduce that there are urinary constituents detectable by UPLC-ESI-QTOFMS that are quantitatively, or even qualitatively, different between the two sets of urines. These inbred mice of the same sex and age differed only in their diet, and this was sufficient to yield separation in the PCA scores plot. Exposure to a toxic chemical or mixture of chemicals would surely, therefore, give a similar result. The ions that gave rise to the clustering into distinct metabolomic phenotypes can be seen in the loading scatter plot (loadings plot) in **Figure 4B**. The black data cloud represents the urinary metabolome of male C57BL/6 mice. However, there are some ions that have moved out from this cloud and a shift to the right is associated with the normal chow and a shift to the left with the synthetic chow. The two right-side outliers have masses of 131.049^{+} and 105.034^{+}, corresponding to fragment carbonyl ions from cinnamoylglycine (CG) and hippuric acid (HA; benzoylglycine), respectively. The single left-side outlier has a mass of 206.044^{+}, corresponding to the protonated molecular ion $[M-H^{+}]$ of xanthurenic acid (XA). These three biomarker assignments were confirmed by cochromatography with authentic standards and by tandem MS (data not shown). According to this preliminary metabolomic analysis, switching male C57BL/6 mice from a normal to a synthetic diet causes a decrease in urinary output of CG and HA, and an increased urinary excretion of XA. At this point, it is possible to return to the original UPLC-ESI-QTOFMS chromatograms and verify this finding. **Figure 5** shows an extracted ion chromatogram for the three masses referred to above, both for urine from a mouse that had been fed the synthetic chow for nine days and urine from the same mouse that had previously been fed the normal chow for nine days. These raw data clearly substantiate the metabolomic findings that, out of ~5000 positive ions, three dietary markers emerged. Normal chow is associated with the excretion of these two glycine conjugates of aromatic acids that are derived from gut floral metabolism of the plant metabolite chlorogenic acid (CGA), an ester of caffeic acid (CfA) and quinic acid (QA) (see Section 18.5). The synthetic chow does not contain these precursors of cinnamic and benzoic acids (BAs). XA, in contrast, is not made by the colonic microbiota, but rather is a degradation product of tryptophan (Zhen *et al.*, 2007). Its urinary excretion from the normal chow can be seen from **Figure 5** to be rather meagre. However, the switch to the synthetic diet provokes an approximately 50-fold increase in its urinary excretion, which may represent an increased synthesis of XA, presumably because the synthetic diet is rich in tryptophan. Nevertheless, this simple metabolomics experiment, with PCA analysis, has revealed two diet-dependent distinct urinary metabolomic phenotypes, a 'HA/CG' and a 'XA' phenotype.

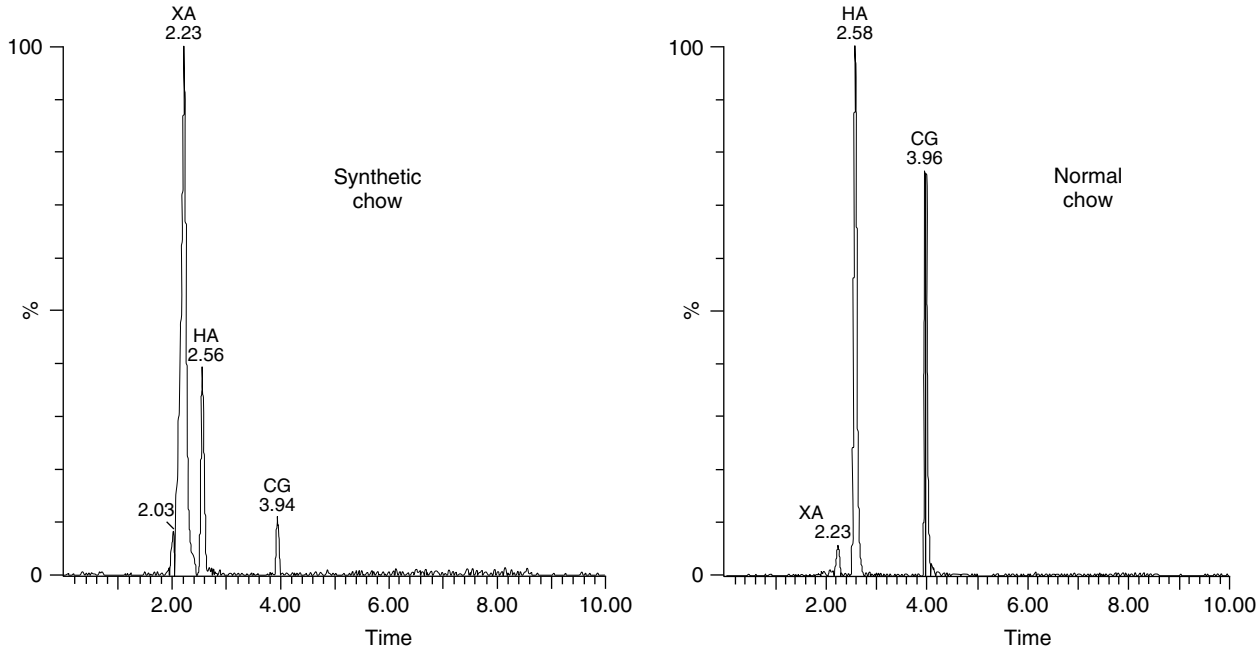

Figure 5 Extracted ion chromatograms for positive ions corresponding to xanthurenic acid (XA), hippuric acid (HA) and cinnamoylglycine (CG). These mass chromatograms confirm the findings of the loadings plots (Figure 4B and D), that XA urinary excretion is low on normal chow, but is highly elevated after switching to the synthetic chow. Additionally, relative urinary excretions of HA and CG are decreased after switching to the synthetic chow, consistent with the metabolomic findings.

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4.2.2 Projection to Latent Structures (Partial Least Squares)—Discriminant Analysis

PCA is a simplistic view of the data matrix and, as might be expected, complex datasets may not be well resolved by PCA. Projection to latent structures-discriminant analysis (PLS-DA) is a supervised method where user-provided class information is employed to maximize the class separation in the model. It can be a useful approach to uncover latent variables (from the loadings) and can be used to classify new input samples. As in PCA, information can be obtained by inspecting the scores and loadings plot. However, using the scores plot may be misleading and, instead, class predictions may be a worthwhile substitute (Westerhuis *et al.*, 2008). Like PLS-DA, orthogonal PLS-DA (orthogonal projection to latent structures-discriminant analysis (OPLS-DA)) is a supervised method, but unlike PLS-DA, OPLS-DA concentrates the intended class variation into the first component and places the unwanted variation, or noncorrelated variation, into the subsequent components (Johan Trygg, 2002). However, these approaches are powerful and are likely to model noise, making the interpretation of these models difficult (Westerhuis *et al.*, 2008). It is therefore essential that the models are properly validated by permutation tests or other cross-validation methods. Ideally, the data should be split into training and test sets, but, as is common with most metabolomics studies, sample number is often small making this validation method difficult.

The utility of PLS-DA supervised analysis can be demonstrated using the same data set as was used for the PCA analysis above. **Figure 4C** shows that the PLS-DA scores plot is virtually identical to the PCA scores plot in **Figure 4A**. This is because the separation by PCA is so good that little model building by PLS-DA is required. In addition, the PLS-DA loadings plot in **Figure 4D** is virtually identical to the PCA loadings plot in **Figure 4B**, with the same three biomarkers being identified. However, there is one last data manipulation that is possible and that is production of a loadings S-plot from the PLS-DA model, shown in **Figure 6**. The S-plot has a unique feature not found in the regular loadings plots (**Figure 4B** and **D**) and that is the depiction of relative abundance of the ion on the *x*-axis and the fit to the PLS-DA model on the *y*-axis. Careful inspection of this S-plot reveals that there are a number of lower abundance ions with almost a perfect fit to the model. These ions will be tightly associated with either the normal chow (top right) or the synthetic chow (bottom left) and, once identified, they are likely to reveal the effects of switching the diet on the metabolism of the mouse. In stark contrast, the two ions marked with arrows, while appearing to be outliers and therefore of interest, are actually poorly correlated to the PLS-DA model and are therefore not worthy of further investigation.

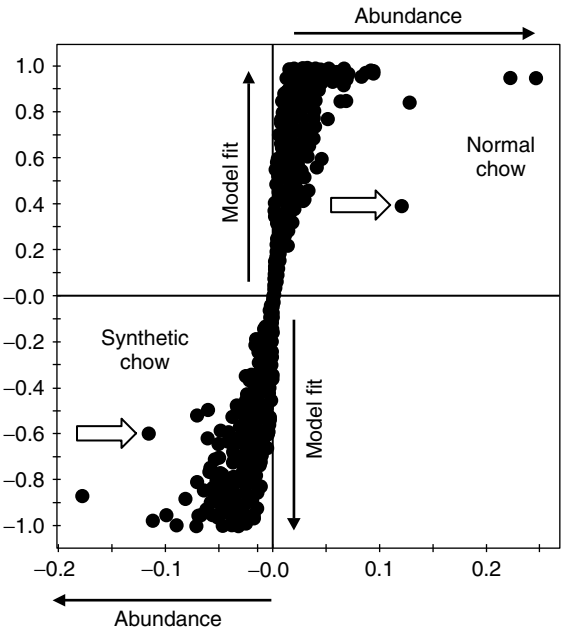

Figure 6 A loadings S-plot from a PLS-DA model of the urinary data from mice maintained on normal chow and then switched to a synthetic chow. This type of data presentation shows ion abundance (*x*-axis) vs goodness of fit to the PLS-DA model (*y*-axis). The same outliers are present as in PCA and PLS-DA loadings plots (Figure 4B and D) in the normal chow quadrant (upper right; CG and HA) and the synthetic chow quadrant (lower left; XA). Note that the arrowed markers, while clearly outliers, are poorly correlated to the PLS-DA model, that is, the difference between normal and synthetic diets.

4.2.3 Random Forests Machine Learning Algorithm

In order to expand and improve the sensitivity of discovery efforts, analytical tools that are less prone to over-fitting are positive additions to any metabolomics programme. Random forests, a machine learning algorithm, is useful in this regard and is freely available for use in the R software environment (http://cran.r-project.org). The random forests algorithm builds an ensemble of decision trees using the classification and regression tree method (Breiman, 1984). The algorithm is simple to deploy, and, unlike PLS-DA and OPLS-DA, overparameterization is relatively rare (Breiman, 2001; Enot *et al.*, 2006; 2008). Additionally, random forests easily handles high-dimensional data and can incorporate additional data points (e.g. clinical data, gene expression) into the analysis. The output is easily interpreted as it gives ranked estimates of the most important variables that can be displayed as box plots. However, the rankings of the top variables can vary slightly, so it is

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advisable to run multiple ($n = 25$) random forests iterations and keep track of those consistently top-ranked variables. Furthermore, features of the random forests, such as the proximity matrix, can be used to display graphically the similarities and dissimilarities existing in the data set (e.g. multidimensional scaling (MDS) plots).

The utility of random forests can be demonstrated with a data set of the same four male C57BL/6 mice maintained on normal chow and four male DBA mice housed and fed similarly. **Figure 7** shows the MDS plot after the eight urine samples were analysed in ESI+ mode by UPLC-ESI-QTOFMS and the data table subjected to random forests analysis. A clear segregation of the two strains of mice was obtained. The difference between these samples is due to genetic and not environmental factors. This analysis also produces a list of ions that most varied between the two groups (data not shown) and this can be employed in the normal manner for biomarker discovery (see Section 18.4.3).

4.2.4 Self-organizing Maps

The metabolome, unlike the genome, transcriptome or proteome, is poorly annotated, making final structural determination a difficult and time-consuming task. While methods such as PCA, PLS-DA, OPLS-DA and random forests are useful for data modelling and for defining the most important variables associated with each class, they are not useful for providing a global view of the metabolome. The self-organizing map (SOM) algorithm is useful in this regard because it can present the metabolomic data in a manner that is visually appealing and intuitive (Kohonen, 2001). Moreover, simple comparisons can be made among samples without the need for any metabolite identification. SOMs are not widely used in metabolomics analysis but they have been applied to metabolomic studies of *Arabidopsis*, diabetes and ionizing radiation (Hirai *et al.*, 2005; 2004; Makinen *et al.*, 2008; Patterson *et al.*, 2008).

Gene expression dynamics inspector (GEDI) is one example of a software package that can be used for analysis and visualization of patterns in metabolomic data matrices (Eichler *et al.*, 2003). This software was developed originally for the interpretation and visualization of gene expression data. Its utility as a tool in metabolomics investigation has recently been realized (Patterson *et al.*, 2008; Tyburski *et al.*, 2008). GEDI creates a two-dimensional heatmap-like mosaic of coloured tiles for each sample based on the SOM algorithm. Additionally, GEDI can compute 'average' mosaics in order to identify common patterns within each group. The complete GEDI program package can be found at http://www.childrenshospital.org/research/ingber/GEDI/gedihome.htm.

The utility of GEDI SOMs can be seen in **Figure 8**. This represents in each window a holistic view of the male C57BL/6 mouse urinary metabolome and the effect of diet on it. What is clear is that the representations illustrated by PCA and PLS-DA loadings plots (**Figure 4**), and even in the PLS-DA loadings S-plot (**Figure 6**), do not give the fullest picture of the effect of diet change on the urinary metabolome of these mice. On average, each of the 247 tiles in this 19×13 mosaic contains 20 ions. Deep red indicates high abundance and deep blue low abundance, with shades of red, orange, yellow, green and blue in between. A large

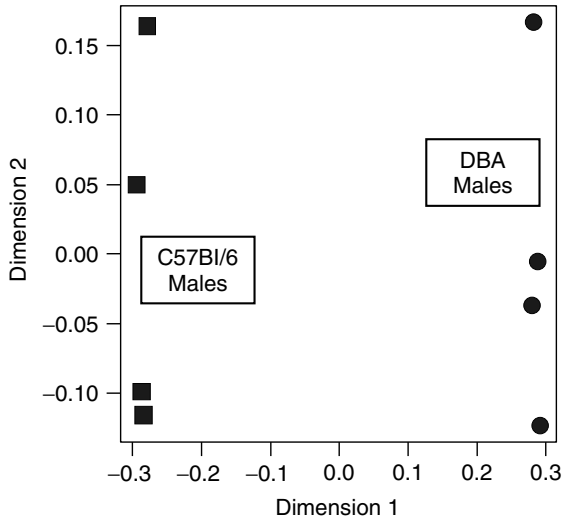

Figure 7 Multidimensional scaling (MDS) plot derived from a random forests analysis of mouse urine from four male C57BL/6 mice and four male DBA mice, all maintained on normal chow. This clear separation is due to genetic and not environmental differences.

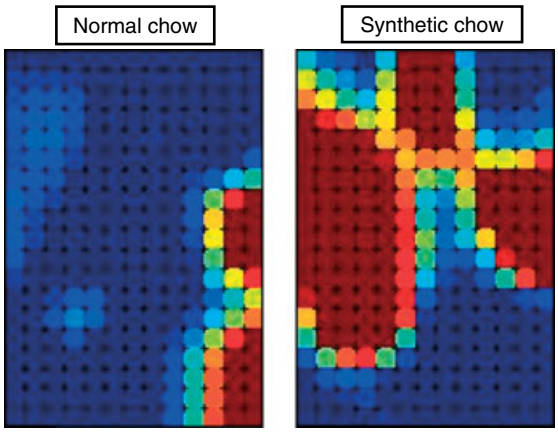

Figure 8 GEDI self-organizing maps (SOMs) giving a holistic view of the mouse urinary metabolome and how it changes in response to diet. Each tile in the mosaic contains approximately 20 ions. Colour indicates abundance of ions—deep blue for low abundance to deep red for high abundance. These heat maps have shades of red, orange, yellow, green and blue in between. Note that a significant proportion of the urinary metabolome 'heats up' after changing to the synthetic chow, indicating an increased urinary excretion of analytes.

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proportion of the urinary metabolome 'heats up' on changing from a normal to a synthetic diet, with a small area (bottom right) 'cooling down'. Returning to the PCA and PLS-DA loadings plots in **Figure 4B** and **D**, the same phenomenon is now apparent, with the lopsided shift of the urinary metabolome towards the synthetic chow side (left). Understanding which ions covary under different conditions will be useful for metabolic pathway construction.

4.3 Biomarker Solution

Anonymous ions are not biomarkers, they are the footprints of biomarkers. Each m/z value still needs to be translated into a physical chemical entity. Unlike the preceding parts of the metabolomic process, there is relatively little computational or bioinformatics assistance to be had with this process. However, there are some useful databases, but in the processing of ions into chemicals, this is where the bottleneck lies. In addition, provisional biomarkers require confirmation with authentic standards, which may or may not require chemical synthesis. Two principal factors determine the degree of difficulty of biomarker solution: (i) the m/z value and (ii) the mass error in the estimation of m/z. The larger the value of m/z and the bigger the error, the greater number of empirical formulae are possible. Discussed below are a number of tools that aid conversion of an accurate mass (m/z value) into candidate empirical formulae and then candidate biomarkers.

4.3.1 Seven Golden Rules

Even the use of instrumentation such as a Fourier-transform ion cyclotron mass spectrometer (FT-MS) or an Orbitrap mass spectrometer, that can routinely give ultrahigh mass accuracies of <1 ppm, is insufficient for calculating unique empirical formulae without resort to the use of isotope ratio data (Kind and Fiehn, 2006). To assist in this process, the Metabolomics Fiehn Laboratory at the University of California (Davis) has a description of a useful piece of downloadable software called Seven Golden Rules on its web site (http://fiehnlab.ucdavis.edu/projects/Seven_Golden_Rules). Examples of its use and a full account of its functionality have been published (Kind and Fiehn, 2007). Unlike many other tools, Seven Golden Rules employs heuristic filtering of molecular formulae, eliminating those that match the accurate mass, but that (i) contain elements other than C, H, N, O, S and P, (ii) do not obey certain chemical rules regarding orbitals and valency, (iii) have incorrect M, M + 1, M + 2 isotope patterns, (iv) have C to H ratios outside of the range 0.125–3, (v) have inappropriate ratios of C to N, O, P and S, that (vi) contain multiple high-element counts, such as $C_{26}H_{28}N_{17}O_1P_3S_8$ (which would pass the other rules) and (vii) yield improbable formula once trimethylsilyl groups have been subtracted. This last rule applies only to metabolomic studies performed by GC-MS (gas chromatography coupled with mass spectometry), where polar protons are frequently replaced by trimethylsilyl groups [$(CH_3)_3$-Si-] by chemical derivatization. These are the Seven Golden Rules. Isotope ratios are particularly important and these same authors have reported that even a mass accuracy of 0.1 ppm is insufficient to derive an empirical formula for masses >185.9760 Da when the elements C, H, O, N, S and P are included in the search (Kind and Fiehn, 2006). A surprising and important finding during the development of this software was that correct isotope ratio measurement was more important than mass accuracy (Kind and Fiehn, 2007).

4.3.2 Madison Metabolomics Consortium Database (MMCD)

Madison Metabolomics Consortium Database (MMCD), which is maintained by the National Magnetic Resonance Facility at Madison, is a resource for metabolomics research based on NMR spectroscopy and MS. The goal of the MMCD has been to support high-throughput NMR and MS approaches to the identification and quantitation of metabolites present in biological samples (Cui *et al.*, 2008). This web site (http://mmcd.nmrfam.wisc.edu) permits an accurate mass to be entered, together with a mass error value and will seek to solve the formula of protonated ($[M+H]^+$) and deprotonated ($[M-H]^-$) molecular ions, together with Na^+, K^+ or NH_4^+ adducts. Solutions are presented as hits in databases such as PubChem (http://pubchem.ncbi.nlm.nih.gov), Chemical Entities of Biological Interest (ChEBI) (http://www.ebi.ac.uk/chebi), Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp) and the Human Metabolome Database (HMDB; http://www.hmdb.ca). This is a very useful means of identifying endogenous molecules based upon accurate masses.

4.3.3 ChemSpider

Providing access to millions of chemical structures and integration into a multitude of other online services, ChemSpider is the richest single source of structure-based chemistry information, with almost 20 million chemical structures (http://www.chemspider.com). Using the tool 'properties search' in ChemSpider it is possible to input an accurate mass, set a mass error limit and adjust the mass by the addition or subtraction of hydrogen and electrons, to allow for protonated and deprotonated molecular ions. Unlike Seven Golden Rules and MMCD, it is currently not possible to allow for adducts with sodium and so on. However, the databases used by ChemSpider contain over 60% of all

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chemical entities ever synthesized, so this is nevertheless an extremely valuable resource for solving protonated and deprotonated molecular ions. That notwithstanding, many biological molecules may evade solution even with ChemSpider. For example, the vitamin E component α-tocopherol is excreted in urine as the β-D-glucuronide of α-carboxyethyl hydroxychroman (α-CEHC) (Pope *et al.*, 2002), which has an empirical formula of $C_{22}H_{33}NO_{10}$ and a protonated molecular ion of $m/z = 472.2183$. Searching this mass in ChemSpider at mass tolerances of 10, 5, 1 and 0.1 ppm yielded 1134, 282, 128 and 7 hits, respectively, none of which was α-CEHC glucuronide.

4.3.4 Lipid Mass Spectrometry Prediction Program

This is a small program that can be downloaded from LIPID MAPS (http://www.lipidmaps.org). This software permits input of accurate masses with mass tolerances of $\pm$0.1, 0.2, 0.5 and 1.0 amu (Da) for the following lipid classes, glycerophospholipids, glycerolipids, cardiolipins, sphingolipids, fatty acids and cholesterol esters. In addition, a variety of modified molecular ions can be specified, for example, $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, $[M+NH_4]^+$, $[M-H]^-$ and $[M.Cl]^-$. This is a very useful tool for analysing metabolomic data from serum or plasma. Target lipid biomarkers can then usually be purchased from Avanti Polar Lipids (http://www.avantilipids.com), the supplier of authentic standards to LIPID MAPS.

4.3.5 MassTRIX

Apparently, MassTRIX, an online tool for annotating metabolites, stands for mass translator into pathways and is housed on servers at the Helmholtz Zentrum, München (http://mips.gsf.de/proj/metabolomics/index.html). It is possible to paste in a list of ions, perhaps the top 100 hits from random forests or OPLS-DA, nominate a mass error of 0.1, 0.2, 0.5, 1.0, 2.0, 3.0 (default), 4.0 or 5.0 ppm, choose a KEGG database to search, choose one of a huge number of species from which your experimental data were derived and, if necessary, restrict the search to a particular list of KEGG metabolic maps or KEGG enzymes. The job runs in the background on your browser and will usually take over one hour to complete. The output should be a list of positive hits for endogenous compounds, which can be viewed in their respective KEGG metabolic maps. This tool is useful for starting to build a systems biology view of the problem under investigation. A fuller description has been published (Suhre and Schmitt-Kopplin, 2008).

4.3.6 Common Sense, the Human Expert System

It is a regular experience that the above tools can yield biomarker solutions to exact masses that, when passed through a human expert system, are clearly nonsense. Such examples include simple esters of carboxylic acids, halogenated compounds, cyano compounds, *N*- and *O*-ethyl, isopropyl, butyl, isobutyl, and so on, compounds. Many other types of chemicals are biologically implausible. A human expert system that applies a biochemical common sense to the solutions obtained is an absolutely essential component of the biomarker solution process. Finally, when all these hoops have been passed, the final question is availability of the compound. A search of the Sigma-Aldrich catalogue (http://www.sigmaaldrich.com) is a first step, then other suppliers, as found in ChemExper (http://www.chemexper.com). Failure to find a supplier of the authentic compound naturally leads to chemical synthesis.

4.3.7 Quantitative Studies

As mentioned in Section 18.2, historical metabolomic studies have very often failed to quantitate biomarkers that are reported as being 'elevated', 'raised' or 'decreased' in certain biological circumstances. We believe that, wherever possible, it is important that metabolomics yields quantitative data as well as a qualitative solution to biological problems such as in toxicology, for example. Metabolomics is therefore useful for identifying candidate biomarkers, which then need to be confirmed by tandem MS methods against authentic standards. Then the job is done and investigators may leave the metabolomics platform and carry out standard analytical chemical determinations of concentrations of the confirmed biomarkers in the appropriate biological samples, for example, urine or tissue extracts of animals exposed to toxicants vs unexposed, or from exposed transgenic mice and control wild-type mice. Knowing the quantitative, as well as the qualitative, biochemical changes that are provoked by the toxicant at the organ level provides a more solid foundation for discerning mechanisms of toxicity. Where urine has been the biological matrix of choice, it is prudent to normalize excretions as μmol mmol^{-1} creatinine, unless the study involves a change in protein intake, alteration of renal function, or some other variable that would *per se* alter the creatinine output, in which case a mole quantity should be quantitated from a 24 hour timed urine collection.

5 PITFALLS IN METABOLOMIC STUDIES

There are many pitfalls to be aware of when conducting a metabolomics study. Even when using a single inbred strain of mouse, it is possible to introduce experimental

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Table 1 Confounding variables in metabolomic studies

Confounding factor	Rationale
Diet	The influence of diet on the metabolome is well documented and has been reported in both animal (Fardet *et al.*, 2007; Fearnside *et al.*, 2008; Gu *et al.*, 2007; Phipps *et al.*, 1998) and human (Assfalg *et al.*, 2008; Gu *et al.*, 2007; Lenz *et al.*, 2004; Rezzi *et al.*, 2007; Stella *et al.*, 2006; Walsh *et al.*, 2006; Zeisel, 2007) studies. While not always possible and/or feasible with human studies, metabolomic investigations with animals should be strictly controlled for diet such that control and disease/treated animals are receiving matched diets. Purified diets, or in the case of humans, precollection diets, may be useful for eliminating or reducing metabolomic 'noise' associated with plant-based diets (Walsh *et al.*, 2007).
Gut microbiota	An often overlooked and underappreciated contributor to the host metabolome is the gut microbiota (Martin, 2007; Martin *et al.*, 2007; Robosky *et al.*, 2005; Robosky *et al.*, 2006; Turnbaugh *et al.*, 2006; Wang *et al.*, 2004). The gut flora population numbers near 10^{13} bacteria and estimates suggest that there may be anywhere from 500 to 1000 different species of bacteria present in the human gut (Backhed *et al.*, 2005). There is nearly 10 times the number of bacteria compared to all the somatic and germ cells in humans, and remarkably, the gut flora contains nearly 100 times the number of genes present in the human genome (Xu and Gordon, 2003). Particular care should be taken to ensure that animal models have similar complements of gut microbiota.
Age Gender Genetic background Environment	Metabolomic profiles are strongly influenced by diurnal variation, age, gender, genetic background and other environmental stimuli (Bollard *et al.*, 2001; Fearnside *et al.*, 2008; Goodacre, 2007; Lutz *et al.*, 2006; Plumb *et al.*, 2003; Plumb *et al.*, 2005; Rock *et al.*, 2007; Slupsky *et al.*, 2007; Williams *et al.*, 2005). Urine collection from animals in metabolic cages may induce unwanted stress associated with isolation and handling (Bollard *et al.*, 2001). These factors must be extremely well-controlled and their potential influence on the metabolomic outcome understood before conducting any metabolomic investigation.

confounders. A list of potential confounders is given in **Table 1**. A major consideration is diet and its impact on the gut microbiota. It has already been ably demonstrated in **Figures 4–6** and **8** that the composition of the diet can radically affect the urinary metabolome, for example. In major part this is due to the cooperation of the gut microbiota, which contributes significantly to the host metabolome. This process is worthy of consideration in further detail because of its potential impact on the raw data of experiments and the interpretations made from them. **Figure 9** shows the relationship between just one small aspect of plant metabolism, the catabolism of phenylalanine and both host and gut microbiota metabolism. Plants synthesize *trans*-cinnamic acid (CnA) from phenylalanine (Phe). This is converted through *p*-coumaric acid (CmA) and *p*-coumaroyl-CoA (Cm-CoA) to CGA or alternatively through CfA and caffeoyl-CoA to CGA. Dietary CGA passes through the gut where it is metabolized by colonic microbiota to CfA and QA. The QA is aromatized through cyclohexane carboxylic acid (CHCA) to BA. The CfA is metabolized by gut microbiota to CnA and phenylpropionic acid (PPA). These end-products of bacterial metabolism are absorbed and are all are reconciled in the liver as BA, conjugated with glycine and excreted in urine as HA, giving rise to the 'HA phenotype'. However, in the small intestine, CGA may be absorbed and converted in the liver and kidneys to CfA and QA, and further to ferulic acid (FA), protocatechuic acid (PcA), then to vanillic acid (VA). These catechol acids (CfA, FA, PcA and VA) are excreted in urine as sulfate and glucuronic acid conjugates and this comprises the 'chlorogenic acid metabolite phenotype' ('CGAM phenotype'). Thus, the HA phenotype is indicative of gut microbiota metabolism and the CGAM phenotype of host hepatic and renal metabolism.

When conducting metabolomics studies in humans there is much less control over the potential confounding variables listed in **Table 1**. However, for metabolomics to be a useful tool it needs to work in human populations, not just for inbred strains of laboratory animals. Targeted determination of metabolites in relation to human health and disease has been the highly successful activity of clinical chemistry departments in hospitals the world over. The practice of clinical chemistry dates back

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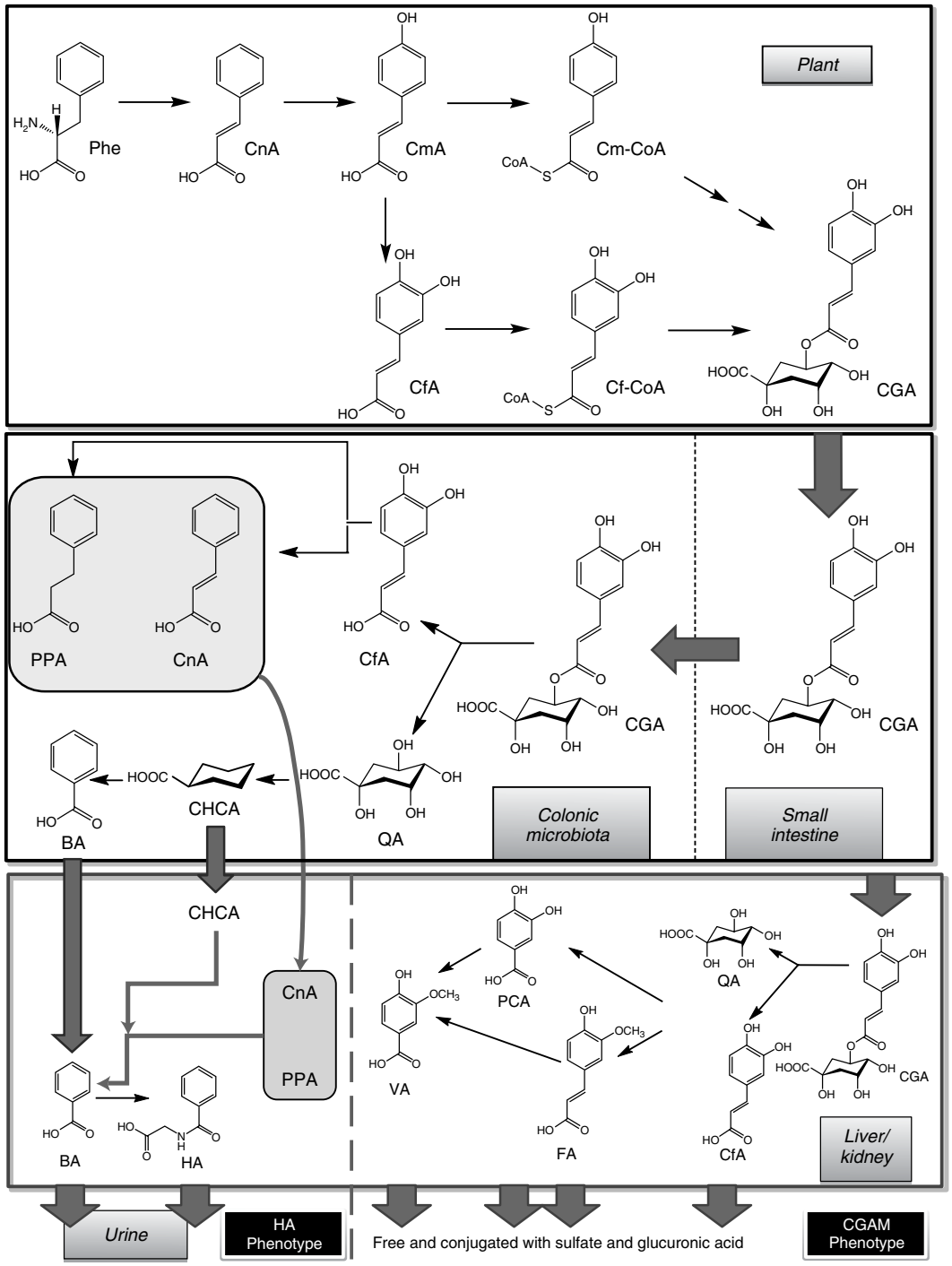

Figure 9 Origin of differential urinary metabolomic 'HA' and 'CGAM' phenotypes. CnA = *trans*-cinnamic acid, Phe = phenylalanine, CmA = *p*-coumaric acid, Cm-CoA = *p*-coumaroyl-CoA, CGA = chlorogenic acid, CfA = caffeic acid, CfA-CoA = caffeoyl-CoA, QA = quinic acid, CHCA = cyclohexane carboxylic acid, BA = benzoic acid, PPA = phenylpropionic acid, HA = hippuric acid, FA = ferulic acid, PcA = protocatechuic acid, VA = vanillic acid.

to nineteenth-century Germany, with the evolution of simple tests for constituents of blood and urine. Today, there are of the order of 500+ discrete chemical tests that can be performed on human biological matrices for the purpose of diagnosis. However, as a research tool, small molecule biochemistry has been eclipsed for some decades by the pursuit of macromolecules, in particular DNA and proteins. This was not always the case and the contribution of small metabolite profiling to human health has been discussed elsewhere (Idle and Gonzalez, 2007). In 1971, Evan and Marjorie Horning, from Baylor College of Medicine in Houston, published an account of

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metabolic profiling of urinary organic acids and steroids, which stated, 'In essence, "metabolic profiles" are multicomponent GC analyses that define or describe metabolic patterns for a group of metabolically or analytically related metabolites" (Horning and Horning, 1971). In the same year, a second paper on urinary profiles of organic acids was published (Mamer *et al.*, 1971). Metabolic profiling, in particular, for urinary organic acids and amino acids, has been common practice in the diagnosis of inborn errors of metabolism, since these early pioneering studies. Metabolomics, on the other hand, is not biased towards one or more chemically related groups of analytes, such as organic acids. There is no reason, however, why metabolomics should not provide at least the same quality of insight as targeted metabolic profiling that has been used for decades by clinical chemistry laboratories. Obviously, interindividual differences in diet and the gut microbiota will play a major part in determining the metabolome of different human subjects, but it is anticipated that this higher level of background metabolic noise should not disguise the potent metabolic signals that derive from pathological states.

6 METABOLOMICS AND SMALL MOLECULE TOXICITY

Chemical toxicity frequently has a metabolic component. The classical chemicals in this regard are the so-called genotoxic carcinogens. Recognition that polycyclic aromatic hydrocarbon, nitrosamine and heterocyclic amine carcinogens required metabolic activation by host enzymes in order to react chemically with nucleic acids and proteins has been one of the great milestones in cancer research. Perhaps the central metabolic contribution to toxicology is detoxication, the rendering of molecules less toxic, more water soluble and readily excretable by metabolic processes commonly referred to as 'drug metabolism' (Williams, 1963). So, drug metabolism plays a major role in determining plasma, urine and target organ concentrations of chemical toxicants and their metabolites, which may or may not harbour biological activity. Metabolomics is able to offer a means to uncover metabolites of chemical toxicants in various biological matrices and also biomarkers of the biological responses of tissues to such toxicants.

6.1 Metabolomic Biomarkers of Tissue Response to Small Molecule Toxicants

6.1.1 Studies Using NMR

Virtually all the original studies were performed using NMR under the rubric of metabonomics. Studies up to 2002 have been comprehensively reviewed in the context of the 'development of high throughput toxicity screening systems for lead candidate selection' by the pharmaceutical industry (Shockcor and Holmes, 2002). A huge amount of compendia data has been catalogued. A summary of the data up until 2002 is given in **Table 2**. This clearly shows that there are no more than about 30 specific metabolomic biomarkers that were detected by NMR and that these were described as being elevated, decreased or unchanged, in relation to various toxic stimuli. The commonest findings reported in these studies were: (i) decreased levels of citric acid, creatinine, 2-oxoglutaric acid, HA and succinic acid or (ii) increased levels of creatine, taurine, acetic acid and glucose. Robertson has reported a list of 11 'usual suspects', comprising the aforementioned list plus lactic acid and trimethylamine/trimethylamine *N*-oxide (TMAO) (Robertson, 2005). It would seem that NMR-based metabolomic studies of liver and kidney toxicity (primarily) have a rather limited perspective on the metabolome. It is worth noting that the 11 'usual suspects' are among the highest abundance analytes in urine and could probably be detected by techniques much less sophisticated than high-field NMR. The purpose of using a $500 000 MS platform in metabolomic investigations is precisely to get as broad a coverage of the metabolome as possible. There have been reports, however, that contradict the foregoing reports of studies with a high yield of unusual biomarkers that are revealing of mechanisms of toxicity. A good example is the hepatotoxicity of hydrazine in the rat (Nicholls *et al.*, 2001). Analysis of urine from hydrazine-dosed rats revealed decreases in hippuric, citric, succinic, 2-oxoglutaric and fumaric acids, together with creatinine and TMAO (except for fumaric acid, all from the 'usual suspects' (Robertson, 2005)). In addition, urinary taurine, creatine, threonine, *N*-methylnicotinic acid, β-alanine, lactic acid, 2-aminoadipic acid, citrulline, $N\alpha$-acetylcitrulline and arginosuccinate (only 3/10 were 'usual suspects') were all elevated. In plasma, glycine, alanine, isoleucine, valine, lysine, arginine, tyrosine, citrulline, 3-D-hydroxybutyric acid, creatine, histidine and threonine were all elevated. Presumably, this increased flux of amino acids reflects proteolysis in the liver arising from necrosis and apoptosis. The most interesting finding in this report was the elevated levels of 2-aminoadipic acid, a lysine degradation product (http://www.genome.ad.jp/dbget-bin/show_pathway?map00310+C00956). Moreover, citrulline is a degradation product of the amino acids arginine and proline (http://www.genome.ad.jp/dbget-bin/show_pathway?map00330+C00327) and also an intermediate in the urea cycle, which produces arginine and fumaric acid by arginosuccinate lyase (EC 4.3.2.1; http://www.genome.ad.jp/dbget-bin/show_pathway?map00220+4.3.2.1). Collectively, these findings are consistent with an increased protein and nitrogen turnover due to hepatic necrosis and apoptosis. It was proposed by

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Table 2 Metabolomic biomarkers in relation to organ toxicity of small molecule toxicants

NMR biomarker	Up or down	Toxicity (see footnotes)
Citric acid	↓	A, AA, ANIT, CC, CT, DCVHC, HCB, LA, MC, PAP, P, PA, SC, SF, TCTFP, TA, UN
Citric acid	↑	CP, DCVC
Creatine	↑	A, AA, CC, CT, G, H, LA, PA, TA
Creatinine	↓	DCVHC, HCB, H, MC, PAP, P, SF, TCTFP, UN
Taurine	↑	A, AA, BHT, CT, E, G, LA, PA, TA
2-Oxoglutaric acid	↑↓	A, PI
2-Oxoglutaric acid	↓	AA, ANIT, CT, DCVHC, E, G, HCB, LA, MC, PAP, PA, SC, SF, TCTFP, UN
Lactic acid	↑	AA, CP, IFO, LA, P
Phenylacetylglycine	↑	AA, AM, CQ
N-Methylnicotinamide	↑	AA
N-Acetylglycine	↑	BE, CE, PI
Acetic acid	↑	ANIT, DCVHC, G, HCB, LA, MC, PAP, PA, SF, TCTFP, TA, UN
'Bile acids'	↑	ANIT, G
Dimethylglycine	↑	AM, BE, CE, CQ
Glucose	↑	ANIT, BHT, CC, CF, CP, CPT, DCVHC, E, HCB, IFO, LA, MC, PAP, P, PA, SC, SF, TA, TCTFP, UN
Hippuric acid	↓	ANIT, CF, CP, CPT, DCVHC, G, HCB, H, IFO, LA, MC, PAP, P, SC, SF, TA, UN
Succinic acid	↑↓	BE, CE
Succinic acid	↑	DCVC
Succinic acid	↓	ANIT, CT, DCVHC, G, HCB, LA, MC, PAP, SF, TCTFP, TA, UN
Adipic acid	↑	BE, CE
Glutaric acid	↑	BE, CE
Alanine	↑	CP, PA
Trimethylamine *N*-oxide	↑↓	BE, CE, PI
Trimethylamine *N*-oxide	↑	IFO, PA, TA
Trimethylamine *N*-oxide	↓	H
Glutamine	↑	CP
Glycine	↑	CP, IFO
'Amino acids'	↑	DCVHC, HCB, MC, PAP, P, SF, TCTFP, TA, UN
'Organic acids'	↑	DCVHC, G, HCB, MC, PAP, P, SF, TCTFP, TA, UN
Betaine	↑	G
Urocanic acid	↑	G
2-Aminoadipic acid	↑	H
β-Alanine	↑	H
Fumaric acid	↓	H
Histidine	↑	IFO
N-Acetylcitrulline	↑	H
'β-Hydroxybutyric acid and other ketone bodies'	↑	IMI
'*N*-Acetyls'	↑	LA
Valine	↓	P
Formic acid	↑	PA
'Macromolecules'	↑	PA
Threonine	↑	SF
Urea	↑	UN

Abbreviations of toxicant (tissue or process): A = adriamycin (heart, kidney), AA = allyl alcohol (liver), AM = amiodarone (lung), ANIT = α-naphthylisothiocyanate (liver), BE = 2-bromoethanamine (kidney, mitochondrial dysfunction), BHT = butylated hydroxytoluene (liver), CC = cadmium chloride (testis), CT = carbon tetrachloride (liver), CF = cefoperazone (unspecified), CP = cephaloridine (unspecified), CPT = cephalothin (unspecified), CE = 2-chloroethanamine (kidney, mitochondrial dysfunction), CQ = chloroquine (liver), DCVC = *S*-(1,2-dichlorovinyl)-L-cysteine (kidney), DCVHC = *S*-(1,2-dichlorovinyl)-L-homocysteine (kidney), E = ethionine (liver), G = galactosamine (liver), HCB = hexachlorobutadiene (kidney), H = hydrazine (liver), IFO = ifosfamide (unspecified), IMI = imipenem (kidney), LA = lead acetate (liver, lung, kidney), MC = mercuric chloride (kidney), PAP = *p*-aminophenol (kidney), P = paraquat (kidney, lung), PI = propyleneimine (kidney), PA = puromycin aminonucleoside (kidney), SC = sodium chromate (kidney), SF = sodium fluoride (kidney), TCTFP = 1,1,2-trichloro-3,3,3-trifluoro-1-propene (kidney), TA = thioacetamide (liver, kidney), UN = uranyl nitrate (kidney). Adapted from published data (Shockcor and Holmes, 2002).

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the authors (Nicholls *et al.*, 2001) that this elevated 2-aminoadipic acid results in a reduction of kynurenic acid in the brain and thus acts as a glial toxin, perhaps explaining the neurotoxic effects of hydrazine. These authors revisited this problem using ^{1}H magic-angle spinning (MAS) NMR on various tissues (Garrod *et al.*, 2005). They were unable to detect 2-aminoadipic acid in the hippocampus, but did find it in millimolar quantities in the kidney cortex, where it was proposed to be the result of inhibition of 2-aminoadipate aminotransferase, an enzyme that, in common with other aminotransferases, uses pyridoxal 5′-phosphate as a cofactor, which is sequestered by hydrazine. The authors also state that this sequestration is what leads to the elevation of many amino acids in plasma and urine. They note that the role of 2-aminoadipic acid in hydrazine toxicity was still elusive, but that the kidney may play a role. This report, like so many other in this field, contains no quantitation beyond an 'increase (↑) or decrease (↓)' in the biomarkers discovered. Nevertheless, these reports (Garrod *et al.*, 2005; Nicholls *et al.*, 2001) contribute an important advance in the understanding of the toxicology of hydrazine.

6.1.2 Studies Using MS

MS has a number of distinct advantages in metabolomic studies. In one structured comparison of NMR and MS methods in metabolomics (Robertson, 2005), MS is given a 'big advantage' over NMR in two areas, sensitivity and resolution of metabolites and an 'advantage' over NMR in selectivity. It would appear that MS fails on 'reproducibility (across labs)', 'potential for sample bias' and in 'quantitation'. The major criticism of LC-MS, specifically ESI methods, has always been ion suppression (Annesley, 2003). Many studies have shown that constituents in the sample matrix suppress the formation of ions unevenly across the chromatogram. In a metabolomics study, this could have the effect of suppression of ionization, and therefore detection, of certain analytes and thus reduce the scope of the data matrix generated. However, the yield of biomarkers using, for example, UPLC-ESI-QTOFMS is so high that ion-suppression effects can be tolerated, but not ignored. These are, in general, specific to a particular segment of the chromatogram. Providing that off-platform calibration and quantitation is performed to determine actual concentrations of biomarkers, for example, in $\mu mol\,mmol^{-1}$ creatinine in urine, then ion suppression becomes less of a problem. If LC-MS-based metabolomic studies adopted the same standards as typical NMR-based studies, that is, reporting merely increases and decreases of analytes, then ion suppression could have a much greater impact.

The power of UPLC-ESI-QTOFMS applied to metabolomics is ably demonstrated by a study of the activation of the nuclear receptor PPARα in mice. PPARα ligands are hepatocarcinogens in rodents (Gonzalez and Shah, 2008) and robust biomarkers for their nuclear receptor-mediated effects would be of great value. A comparison of PPARα-null and wild-type mice that were fed for 14 days a diet containing the potent PPARα ligand Wy-14,643 (0.1%) revealed three urinary metabolomic phenotypes associated with the mouse *Ppara* $+/+$ and $-/-$ genotypes (Zhen *et al.*, 2007). **Figure 10A** shows a PCA scores plot with both treated and untreated $-/-$ mice clustered into one phenotype. This firstly demonstrates the absence of any major 'off-receptor' effects, that is, in the absence of the PPARα receptor, no detectable effect on the urinary metabolome was observed. It should be noted that all potential metabolites of Wy-14,643 had first been purged from the data matrix, which is relatively straightforward since they can be identified from the chlorine isotope ratio, since Wy-14,643 contains a single chlorine atom. If this procedure had not been carried out prior to data deconvolution (**Figure 3**), the treated and untreated $-/-$ mice would have separated on the basis of urinary metabolites of Wy-14,643. **Figure 10A** also shows that the wild-type ($+/+$) mice cluster and separate, both on the basis of treatment (first component) and genotype ($+/+$ vs $-/-$; second component). The net effect of treating $+/+$ mice and $-/-$ mice is a shift in both first and second components of variance, in this case, from upper left to bottom right. The intriguing separation of the untreated $+/+$ and $-/-$ mice into two distinct urinary metabolomic phenotypes, is indicative of the presence of endogenous activators of the PPARα receptor. **Figure 10B** shows the presence of two major biomarkers for PPARα receptor activation in the mouse, labelled HDOPA and DHOPA. These correspond to 11β-hydroxy-3,20-dioxopregn-4-en-21-oic acid (**Figure 10C**) and 11β,20-dihydroxy-3-oxopregn-4-en-21-oic acid (**Figure 10D**), respectively, two corticosteroid acids formed from corticosterone. The identity of the PPARα target genes responsible for the formation of these two compounds, as well as their interconversion, is the subject of current investigation. This study (Zhen *et al.*, 2007) underscores the power of MS-based methodologies in metabolomics.

6.2 From Drug Metabolism to Drug Metabolomics

As was stated above, small molecule toxicity comprises a discernible metabolic component, involving the metabolic detoxication and also metabolic activation of chemical toxicants. Knowledge of the metabolic disposition of drugs, environmental chemicals and other xenobiotics to which humans may be exposed is essential. The field of drug metabolism has its origins in the mid-nineteenth century. Early studies invariably involved the

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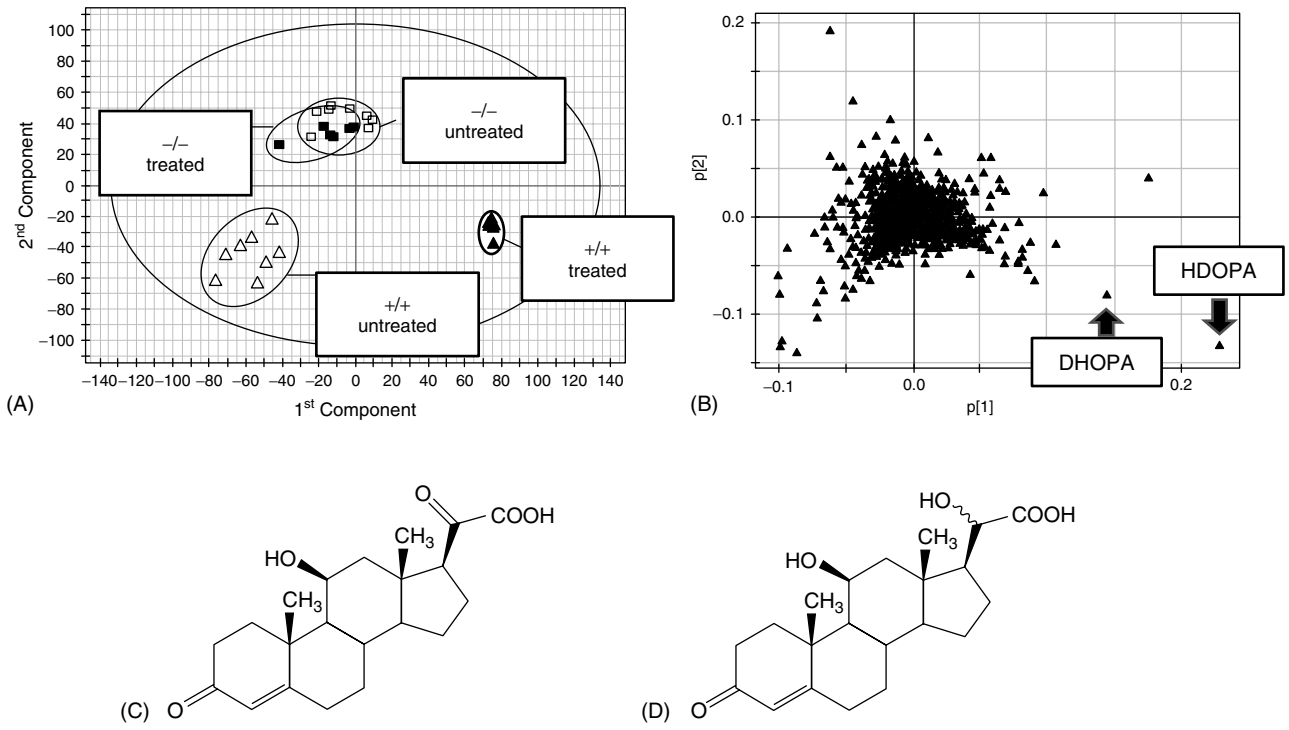

Figure 10 Discovery of urinary biomarkers for PPARα activation in the mouse by UPLC-ESI-QTOFMS based metabolomics. (Adapted from published data (Zhen *et al.*, 2007).) (A) PCA scores plot showing clustering of treated (0.1% Wy-14,643 diet for two weeks) and untreated (normal chow) *Ppara*-null $(-/-)$ mice and clear clustering and separation of treated and untreated *Ppara*-wild-type $(+/+)$ mice. (B) PCA loadings plot showing two principal ions (361.201^+ = HDOPA and 363.216^+ = DHOPA; see below) that contributed to the separation of treated $+/+$ and $-/-$ mice in the scores plot. (C) Structure of HDOPA (11β-hydroxy-3,20-dioxopregn-4-en-21-oic acid). (D) Structure of DHOPA (11β,20-dihydroxy-3-oxopregn-4-en-21-oic acid).

isolation, purification and often crystallization of metabolites from urine. Degradative chemical reactions and derivative formation were the underpinnings of the field, based almost exclusively on a knowledge of organic chemistry on the part of investigators. Little was known about the biology of drug metabolism in these early days. Technology has always spurred the quantum leaps in drug metabolism, as in other areas of life sciences. The introduction of visible and ultraviolet spectrophotometry in the 1930 s (Beinert, 1994), radiolabelled compounds (Dziewiatkowski, 1949; Dziewiatkowski and Wingo, 1949), spectrofluorimetry (Klatzkin *et al.*, 1949) and partition chromatography in the 1940s (Martin and Synge, 1941a; Martin and Synge, 1941b) catalysed the expansion of the field of drug metabolism. However, the subsequent introduction of GC, GC-MS, HPLC and LC-MS into drug metabolism has provided the underpinning to the explosion of studies in this field. Today, the phrases 'drug metabolism' and 'xenobiotic metabolism' appear in the abstracts of over 10 000 papers listed in PubMed.

A relatively short time ago, drug metabolism studies were laborious, time-consuming and often incomplete. **Figure 11** contrasts an experiment conducted in 1974 with one in 2008 that reflects a major revolution in technology. In **Figure 11A**, the fate of BA is being investigated in a particular species of African fruit bat, Franquet's epauletted bat (*Epomops franqueti*) (Collins *et al.*, 1977; Idle, 1976). A related bat, the Indian fruit bat or flying fox (*Pteropus giganteus*) had been reported to be the only species of 21 studied not to convert BA to HA (Bridges *et al.*, 1970). Subsequently, it was reported that this species conjugated BA by a novel alternative route, with glutamic acid (Idle *et al.*, 1975). **Figure 11** shows the relatively poor chromatographic resolution of thin-layer chromatography (TLC) and that the use of radiolabelled substrates and cochromatography with authentic metabolites could still leave the job unfinished. There are at least two unidentified metabolites, A and B. **Figure 11B** shows the extreme resolving power of UPLC, in this case with a longer column eluted over 20 minutes. All available acylcarnitine derivatives are resolved by this method. Moreover, accurate mass spectra at a rate of 20 per second are collected across the whole chromatogram, ensuring identity of analytes. It is the ultrahigh resolution of UPLC, combined with the accurate mass determination of QTOFMS and MDA that furnishes a unique toolbox in drug metabolism studies.

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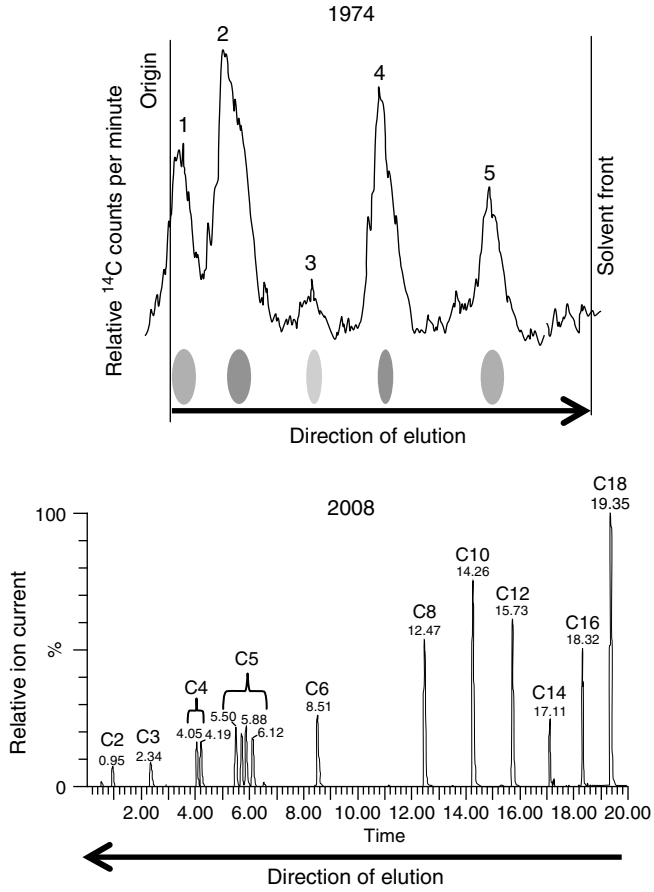

Figure 11 Advances in drug metabolism since 1974. (A) A radiochromatogram of urine from a Fraquet's epauletted fruit bat (*Epomops franqueti*) that had been administered [^{14}C]benzoic acid. This experiment was conducted in 1974 by elution of urine on silica gel thin-layer chromatography (TLC) plates with benzene: acetone: glacial acetic acid (6 : 2 : 1 v/v). 1 = unidentified metabolite A, 2 = benzoyl-β-D-glucuronide, 3 = unidentified metabolite B, 4 = benzoylglutamic acid + benzoylglycine (hippuric acid), 5 = benzoic acid. (B) Separation by UPLC and detection by ESI-QTOFMS of 15 acylcarnitines, from C2 (acetylcarnitine) up to C18 (stearoylcarnitine). This experiment was conducted in 2008.

6.2.1 A Re-examination of the Metabolism of Aminoflavone

Aminoflavone (NSC686288) is a candidate antitumour agent active against a broad range of tumour cell lines whose selectivity was believed to be due to metabolism by CYP1A1 to DNA-reactive metabolites. The sulfotransferase SULT1A1 had also been demonstrated to play role in aminoflavone cytotoxicity *in vitro*. However, the *in vivo* metabolism of aminoflavone had not been characterized. Using a metabolomic approach in the mouse, aminoflavone was shown to be biotransformed into 13 metabolites, 12 of which were novel (Chen *et al.*, 2006). **Figure 12** shows the chemical structures of aminoflavone and 13 metabolites. Only $N^{4'}$-hydroxy-aminoflavone was known prior to this study. This study demonstrated that aminoflavone undergoes hydroxylation in positions 3-, $N^{4'}$- and N^5, together with certain dihydroxylations. Conjugations include *O*-glucuronides, *N*-*O*-glucuronides, *N*-glucuronides, *O*-sulfates and *N*-*O*-sulfates. The *N*-*O*-glucuronides and sulfates probably represent chemically reactive and cytotoxic metabolites. This study demonstrates the power of the UPLC-ESI-QTOFMS metabolomics approach in understanding the toxicology of small molecules. Moreover, metabolomics in combination with genetically modified mice considerably improves our understanding of the chemical–biological interactions *in vivo* of aminoflavone. Using *Cyp1a2*-null mice and *CYP1A2*-humanized mice, the role of murine Cyp1a2 and human CYP1A2 in the metabolism of aminoflavone was greatly advanced (Chen *et al.*, 2006).

6.2.2 A Re-examination of the Metabolism of Arecoline, Arecaidine and Arecoline 1-oxide

Arecoline and arecaidine are the principal areca nut alkaloids to which some 600 million people are exposed through the habit of areca nut chewing. Despite areca alkaloids being the fourth most prevalent exposure from habitual use, after alcohol, caffeine and nicotine, little

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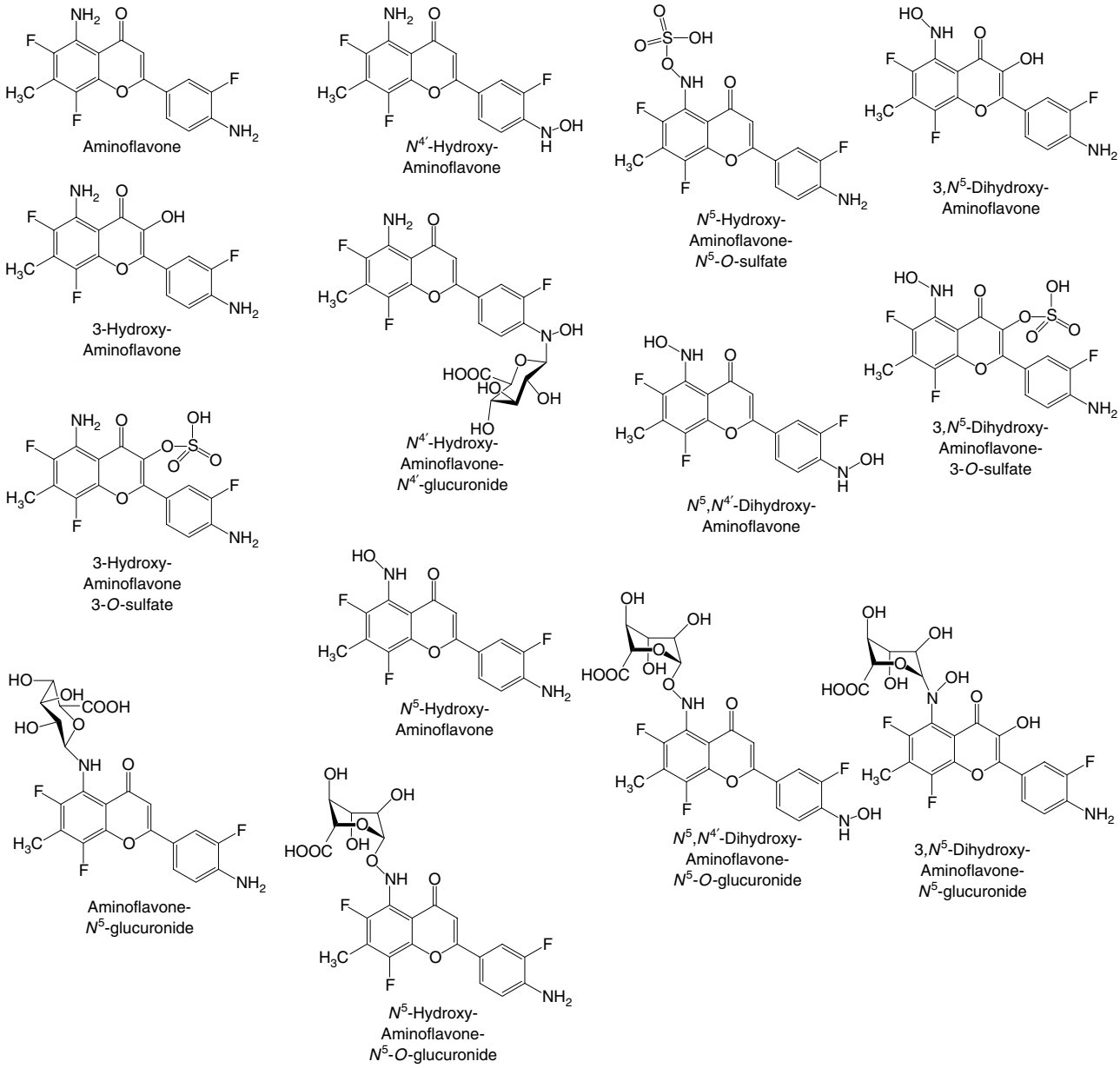

Figure 12 Aminoflavone and 13 urinary metabolites in the mouse determined by UPLC-ESI-QTOFMS metabolomics. Metabolite identities appear under each structure.

was known about their metabolism *in vivo* (Giri *et al.*, 2006). Arecoline had been previously reported to be metabolized to arecaidine by demethylation and to an *N*-oxide metabolite (Nery, 1971). In addition, an *N*-acetylcysteine conjugate of arecaidine had been reported (Boyland and Nery, 1969). A metabolomic study was undertaken after administration of arecoline and arecaidine to mice (Giri *et al.*, 2006). Additionally, the further metabolism of arecoline 1-oxide, the principal metabolite of arecoline, was investigated using a metabolomic protocol, also employing UPLC-ESI-QTOFMS (Giri *et al.*, 2007). These studies led to the expansion of known arecoline metabolites from 3 to 14. In addition to three *N*-oxides, three mercapturic acid metabolites and their mercaptan degradation products were reported (**Figure 13**). These novel metabolic insights should lead to further studies on the toxicity and potential carcinogenicity of areca alkaloids.

6.2.3 A Re-examination of the Metabolism of 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is ubiquitous in the human diet and is a potent rodent carcinogen, relying on metabolism to exert its mutagenic and carcinogenic potential (Turesky, 2002). In common

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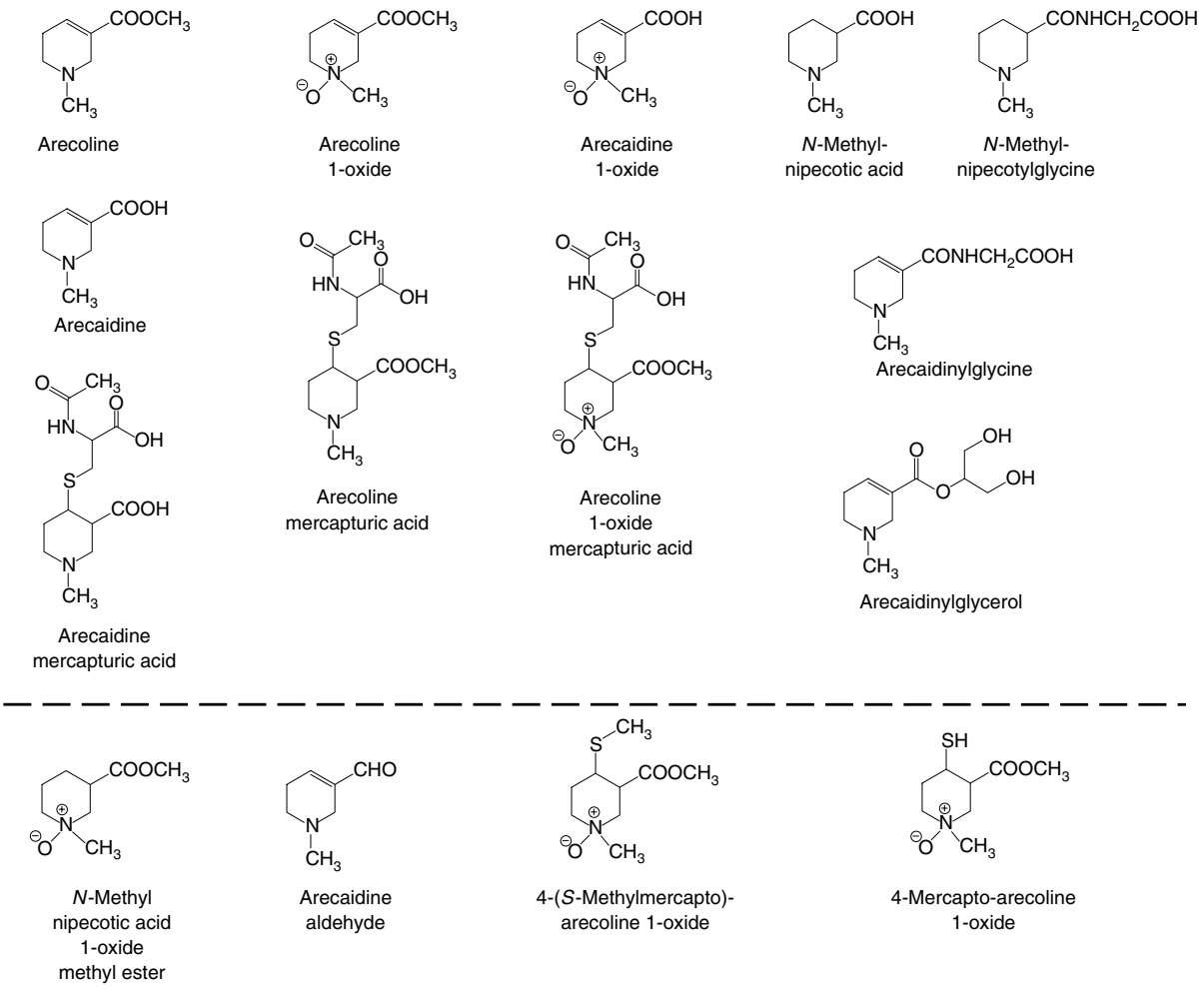

Figure 13 Arecoline and 14 urinary metabolites in the mouse determined by UPLC-ESI-QTOFMS metabolomics. Above the dotted line are metabolites determined after administration of arecoline to mice. Below the dotted line are additional metabolites determined after administration of the principal arecoline metabolite arecoline 1-oxide. Metabolite identities appear under each structure.

with other dietary heterocyclic amines, PhIP undergoes *N*-oxidation by cytochromes P450 followed by acetylation or sulfation to yield DNA-reactive metabolites (Turesky, 2002). The metabolism of PhIP in the mouse was re-examined using a metabolomic protocol after analysis of treated and untreated mouse urine by UPLC-ESI-QTOFMS (Chen *et al.*, 2007). **Figure 14** shows that this methodology identified 17 urinary metabolites of PhIP in the mouse from which the formation of two others, 4′,5-dihydroxy-PhIP and 4′,N^2-dihydroxy-PhIP, can be inferred by the presence of their conjugates in urine. Identities of metabolites were confirmed by mass fragmentography. Of the PhIP metabolites detected, eight were novel. These insights into the metabolism of PhIP, by hydroxylation and sulfate and glucuronic acid conjugation, aid our understanding of both the metabolic activation and detoxication of this important potential human carcinogen.

6.2.4 A Re-examination of the Metabolism of Melatonin

Melatonin is a pineal hormone that contributes to biological rhythmicity in a wide number of species. Oral melatonin preparations are sold without prescription in many countries. Consequently, millions of people are exposed to pharmacological doses, orders of magnitude above amounts released daily by the human pineal gland. Melatonin is claimed to possess antioxidant properties, but doubts exist regarding the extent to which these antioxidant properties are influenced by the metabolism of melatonin (Ma *et al.*, 2006). In addition, there has been a debate regarding the nature of the principal urinary metabolite of melatonin in the mouse, whether it is the 6-*O*-sulfate (Skene *et al.*, 2006) or the 6-*O*-glucuronide (Kennaway *et al.*, 2002). UPLC-ESI-QTOFMS-based metabolomics is particularly well

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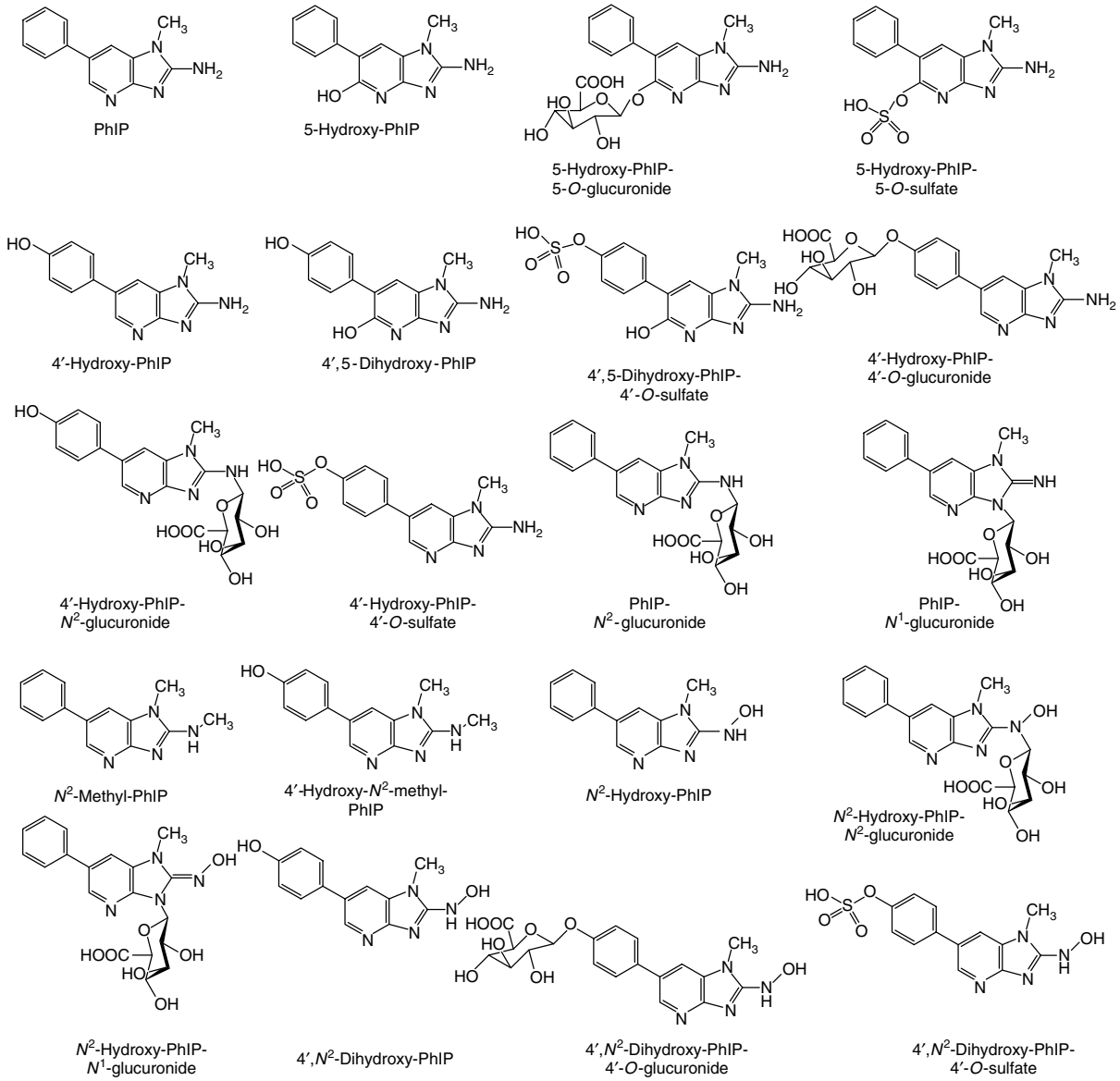

Figure 14 PhIP and 19 urinary metabolites in the mouse determined by UPLC-ESI-QTOFMS metabolomics. Metabolite identities appear under each structure. PhIP = 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine.

placed to contribute to these issues. A study has been reported in which melatonin was administered to mice and the MS data matrix analysed by OPLS to determine the presence of melatonin metabolites in mouse urine (Ma *et al.*, 2008). **Figure 15** shows the OPLS loadings S-plot from this study. Fourteen metabolites of melatonin in the mouse were characterized, seven of which were novel. This study also reported that N^1-acetyl-N^2-formyl-5-methoxykynuramine and N^1-acetyl-5-methoxykynuramine, known melatonin antioxidant products, were not detected in mouse urine. Finally, the debate over sulfation vs glucuronidation of 6-hydroxymelatonin was settled using the same strains of mice as the previous investigators (Kennaway *et al.*, 2002; Skene *et al.*, 2006). The metabolomic study, combined with ESI− and ESI+ mass fragmentography and specific enzymic deconjugation reactions established that the principal excretory product of melatonin in the mouse is 6-hydroxymelatonin 6-*O*-glucuronide, with the 6-O-sulfate virtually undetectable (Ma *et al.*, 2008).

6.2.5 A Re-examination of the Metabolism and Toxicity of Paracetamol

Paracetamol (*N*-acetyl-*p*-aminophenol; APAP; acetaminophen in the USA) is the biggest single cause of avoidable and fatal hepatotoxicity in developed countries. Its metabolism has been studied for over 50 years and provides an important case example of the essential role of metabolism in drug toxicity (Gonzalez, 2007). A metabolomics study has been reported in which three novel metabolites of APAP were uncovered in

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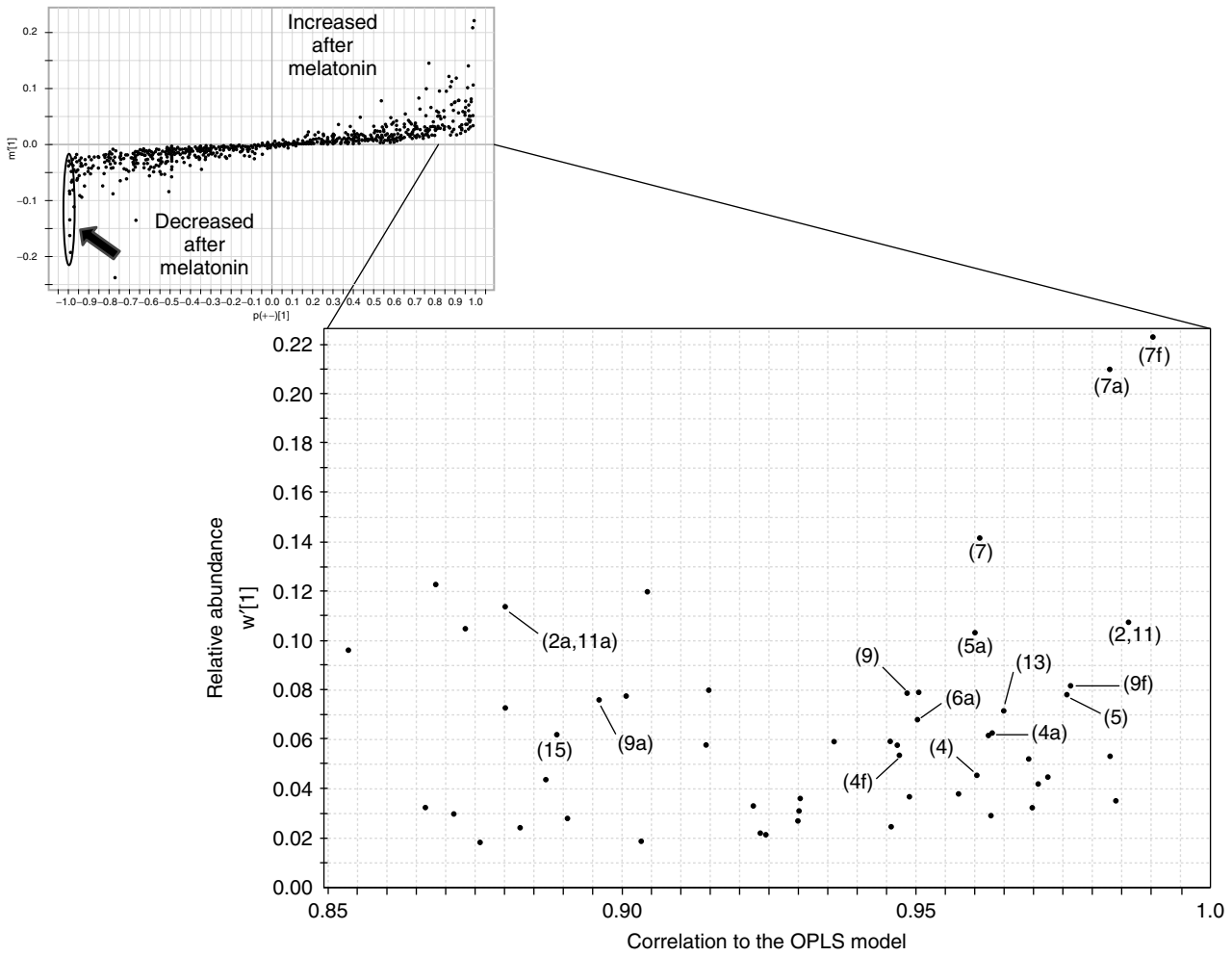

Figure 15 OPLS loadings S-plot for determination of the urinary metabolites of melatonin in the mouse. (Adapted from published data (Ma *et al.*, 2008).) The top panel shows quadrants containing ions that are increased and decreased after melatonin administration. The arrowed group of ions represents urinary constituents that were suppressed, and with perfect correlation to the OPLS model, after melatonin administration. The expanded quadrant shows melatonin metabolites that were identified from authentic standards, by specific enzymic deconjugation reactions, and by mass fragmentography. Metabolites are: 1 = unchanged melatonin (ion not shown), 2 = 6-hydroxymelatonin, 3 = *N*-acetylserotonin (ion not shown), 4 = melatonin *N*-glucuronide, 5 = 2-oxomelatonin, 6 = dihydroxymelatonin, 7 = 6-hydroxymelatonin 6 − *O*-glucuronide, 8 = 6-hydroxymelatonin 6-*O*-sulfate (ion not shown), 9 = *N*-acetylserotonin 5-*O*-glucuronide, 10 = *N*-acetylserotonin 5-*O*-sulfate (ion not shown), 11 = *cyclic*-3-hydroxymelatonin, 12 = 6-hydroxy-*cyclic*-3-hydroxymelatonin (ion not shown), 13 = *cyclic*-*N*-acetylserotonin 5-*O*-glucuronide, 14 = *cyclic*-melatonin (ion not shown), 15 = 5-hydroxyindolylacetaldehyde. Subscripts a and f refer to Na^+ adducts and in-source fragments, respectively.

the mouse (Chen *et al.*, 2008a). The study detected the usual APAP metabolites, specifically, unchanged APAP, APAP cysteine conjugate, *N*-acetylcysteine conjugate, glucuronic acid conjugate, sulfate conjugate and 3-methoxy-APAP glucuronide. However, an APAP dimer was detected (**Figure 16**) that was shown in additional experiments to derive from the action of reactive oxygen species (ROS) on APAP. Moreover, a benzothiazine derivative was deduced by mass fragmentography of an ion of $m/z = 281.0054^+$, corresponding to the empirical formula $C_{11}H_9N_2O_3S_2^+$. The incomplete structure is given in **Figure 16**. In common with model benzothiazine derivatives, this metabolite also arises from the action of ROS. Finally, it was reported that the cysteine conjugate of APAP underwent transamination to the corresponding keto acid, and that this reaction occurred almost exclusively in the kidney (Chen *et al.*, 2008a). Through the use of *Cyp2e1* +/+ and −/− mice, it was possible to conclude that CYP2E1 contributes to the toxicity of paracetamol by the generation of ROS. This study combined genetically modified mice with

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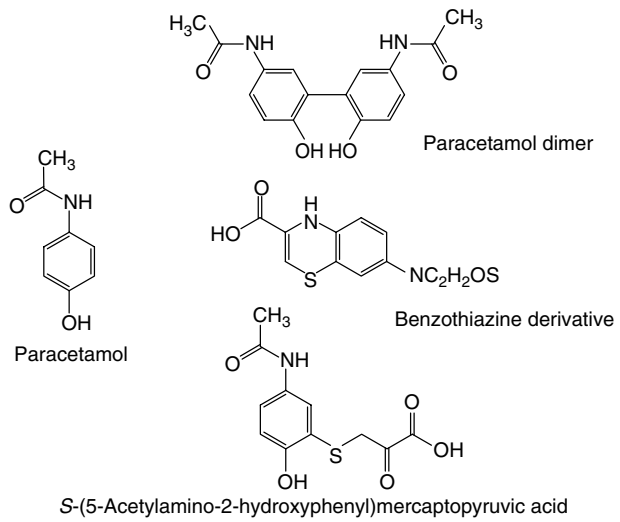

Figure 16 The structure of paracetamol and three novel metabolites in the mouse determined by UPLC-ESI-QTOFMS.

high-resolution UPLC, accurate mass ESI-QTOFMS, unsupervised PCA and mass isotopomer analysis, and demonstrated that this unique platform is able to derive novel metabolites of a highly investigated drug like paracetamol.

7 FUTURE PERSPECTIVES FOR METABOLOMICS IN TOXICOLOGY

The literature ably demonstrates that rapid screening for biomarkers of organ toxicity can be performed using either NMR- or MS- based metabolomic methodologies. In the same way that there is unlikely to be a consensus on the use of the terms metabolomics, metabonomics and metabolic profiling, there is unlikely to be agreement on the relative advantages and disadvantages of the NMR and MS platforms. This is a young science, still yet to establish its relevance and value to a host of more recognized disciplines. The pharmaceutical industry has adopted the subject very rapidly and it is becoming clearer that the potential of metabolomics as a high-throughput, relatively low-cost, technology is considerable.

While the high abundance so-called 'usual suspects' (see Section 18.6.1.1) may be sufficient for high-throughput screening protocols, they do not have sufficient information content to inform about mechanisms of toxicity. It is possible to generate NMR data beyond this dataset that is more informative, as was the case with hydrazine toxicity (Garrod *et al.*, 2005; Nicholls *et al.*, 2001). However, it is our experience and contention that the best starting point in a metabolomics study of a small toxic molecule is to deploy MS-based protocols, especially UPLC-ESI-QTOFMS, with its high resolution of metabolites and accurate mass determination of resultant ions. The potential of ion suppression must be worked around and this becomes less of a concern when preliminary biomarkers of toxicity are further investigated on a second platform, for example, triple quadrupole MS or GC-MS, not just for the purposes of qualitative confirmation, but also for quantitation. Examples of this protocol have been published (Tyburski *et al.*, 2008; Zhen *et al.*, 2007). Regarding the mechanisms of toxic responses, UPLC-ESI-QTOFMS-based metabolomics has also proved useful in delineating the inflammatory response of the mouse gut to administered sodium dextran sulfate (Chen *et al.*, 2008b) and in improving the understanding of biological responses to ionizing radiation, both *in vitro* (Patterson *et al.*, 2008) and *in vivo* (Tyburski *et al.*, 2008). Further exploration of such questions by the addition of GC-MS and GC-time-of-flight (TOF) platforms is likely to be very productive. One of the major limitations of standard rapid-throughput UPLC methods is that most of the highly polar urinary metabolites elute in under one minute. However, solid-phase extraction, followed by derivatization and GC-MS analysis produces excellent resolution of polar metabolites. GC-MS has provided the underpinning to plant metabolomics since its inception (Dixon *et al.*, 2006; Fiehn, 2002; Fiehn *et al.*, 2008; Taylor *et al.*, 2002) and, together with UPLC-ESI-QTOFMS, will surely become a major force in 'toxicometabolomics' in the future.

Finally, it must not be forgotten that a significant proportion of human and animal toxicology is dependent upon metabolism of small molecules, by the host and microbiota. Understanding detoxication and metabolic activation pathways for small molecules is essential to a fuller recognition of their potential as toxicants. It has been demonstrated ably here (see Section 18.6.2) that MS-based metabolomics produces unrivalled insights into the metabolic disposition of small chemical entities. Given the technologies available and likely to evolve in the short term, one may anticipate an exciting future as metabolomics combines with functional genomics and proteomics to deliver systems toxicology. This threshold is akin to that in molecular biology in the early 1980s, just prior to the invention of the polymerase chain reaction (PCR) and the subsequent dissemination of PCR thermocyclers throughout the scientific community. A subject with an enormous perceived potential went, almost overnight, from an esoteric and exclusive domain to a widespread and highly democratic set of simple techniques that, through automation due to commercial interests, permitted the sequencing of the human genome, and over 180 others. Functional genomics is also in the process of democratization, with the spread of technology for DNA microarrays and single nucleotide polymorphism (SNP) mapping. Metabolomics waits in the wings for its turn. It is not helpful to the average investigator if metabolomics can only be carried out in labs with high-field NMR spectrometers or time-of-flight, Fourier-transform ion cyclotron resonance, or Orbitrap

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mass spectrometers that are expensive to purchase and maintain and require great expertise for the acquisition and interpretation of data. What is needed is the PCR thermocycler equivalent for metabolomics that can bring metabolomics into any laboratory. As has been stated elsewhere (Idle and Gonzalez, 2007), this will require a commitment of the best minds in academic research, as well as a willingness of industrial manufacturers to fulfil the collective vision of a world with simple, cheap and reproducible metabolomic profiling, a true electronic dog's nose.

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Endocrine Disruption in Toxic Responses

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CONTENTS

1 INTRODUCTION

Many endocrine-disrupting agents, including industrial materials, pesticides, pharmaceuticals and phytochemicals, have been identified by the use of *in vitro* assay systems and *in vivo* studies in laboratory animals. These chemicals are widely distributed in the environment, and are able to mimic or antagonize the biological functions of natural hormones. Indeed, abnormalities thought to be due to such agents have been found in animals throughout the world. There is also thought to be a risk to humans, for example, diethylstilboestrol (DES) syndrome. Xenoestrogens can accumulate in our environment, and may play a role in the increasing incidences of breast cancer, testicular cancer and other problems of the reproductive system in humans. Risks due to endocrine-disruptors in the environment are discussed in this chapter.

2 ENDOCRINE DISRUPTING ACTIVITY *IN VITRO* AND *IN VIVO* IN ANIMALS

Many environmental xenobiotics have been shown to exert hormonal effects at the cellular and organism levels. These compounds are able to mimic the biological activity of sex hormones and thyroid hormones (TH), and are called endocrine-disrupting chemicals (EDCs). They include insecticides, such as kepone, o,p'-dichlorodiphenyltrichloroethane (o,p'-DDT), dieldrin, methoxychlor, and industrial chemicals used in the plastics and detergent industries, such as alkylphenols and bisphenol A (BPA), and polychlorinated biphenyl (PCB) congeners (Soto *et al.*, 1994; Gaido *et al.*, 1997; Andersen *et al.*, 1999). Quantitative structure–activity relationship (QSAR) work on the structural features of oestrogen receptor (ER) ligands shows that an unhindered hydroxyl group on an aryl ring and a hydrophobic group attached *para* to the hydroxyl group are essential (Anstead *et al.*, 1997; Fang *et al.*, 2000; Shi *et al.*, 2001; Hong *et al.*, 2002). Ligand binding assays and studies in a reporter/transcriptional system for the ER support the requirement for these structural features (Nishihara *et al.*, 2000; Blair *et al.*, 2000). Among these compounds, those which are lipophilic and persistent may be accumulated through the food web, posing a health threat to humans and animals. Xenoestrogens can accumulate in our environment, and may play a role in the increasing incidence of breast cancer, testicular cancer and other problems of the reproductive system in humans. Interactions of oestrogenic and antiandrogenic compounds with the respective hormone receptors have been demonstrated to account for most of the endocrine-disrupting actions, and these chemicals

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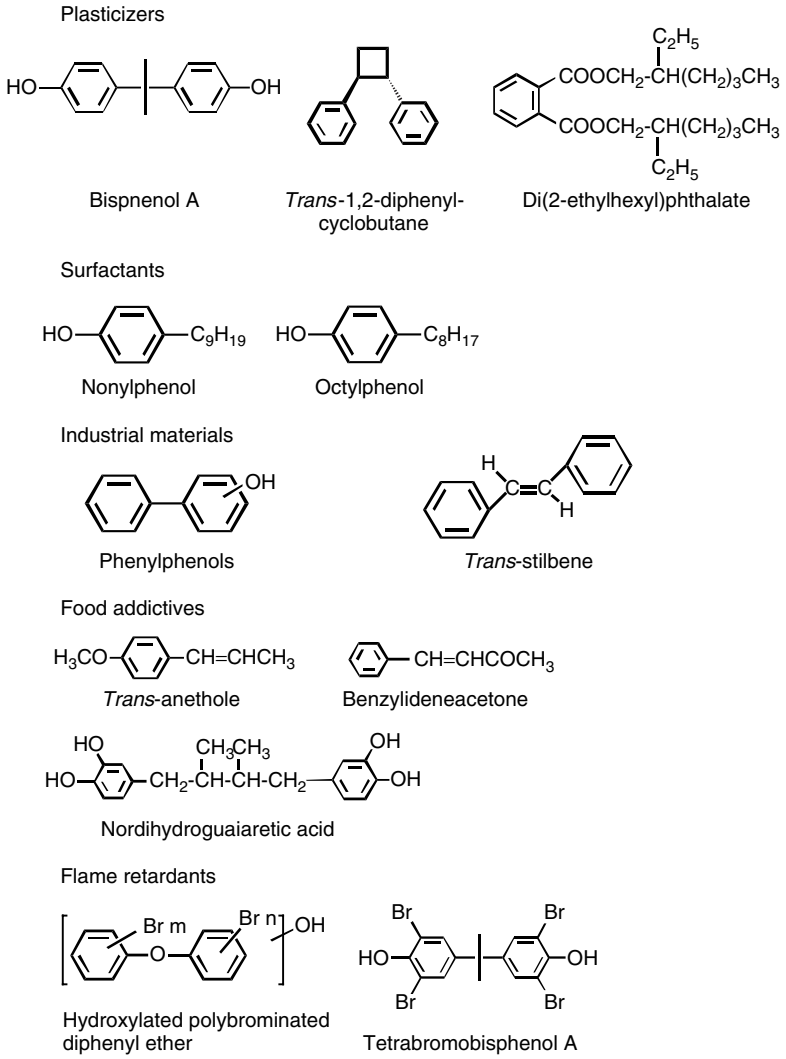

Figure 1 Endocrine-disrupting chemicals on environments.

can alter reproductive development in mammals. The effects of proestrogens, which are activated to oestrogens by metabolic systems, must also be taken into account. EDCs including proestrogens shown in this chapter are listed in **Figure 1**.

2.1 Oestrogenic, Antiandrogenic and Thyroid Hormone Disrupting Activities of Endocrine Disrupters

2.1.1 Plasticizers

BPA (2,2-bis-(4-hydroxyphenyl)propane) is an industrial raw material for manufacture of polycarbonate and epoxy resins, and contaminates the end products. It can be detected in liquid from canned vegetables and in the saliva of patients treated with dental sealants (Brotons *et al.*, 1995; Olea *et al.*, 1996; Hashimoto *et al.*, 2001). Bisphenol B (2,2-bis-(4-hydroxyphenyl)butane), bisphenol F (4,4′-dihydroxydiphenylmethane), bisphenol AD (ethylidenebisphenol), bisphenol AF (1,3-trifluoro-2, 2-bis-(4-hydroxyphenyl)propane), tetramethylbisphenol A (TMBPA), 3,3′-dimethylbisphenol A and bisphenol S (bis-(4-hydroxyphenyl)sulphone) are also used as materials for polycarbonate resin. Tetrachlorobisphenol A (TCBPA) has been found in the effluent from waste-paper recycling plants (Fukazawa *et al.*, 2001). Kuruto-Niwa *et al.* (2002) reported that oestrogenic polychlorinated BPAs were not easily biodegraded.

BPA and some related compounds exhibit oestrogenic activity towards cell lines, such as oestrogen-responsive breast cancer cell line MCF-7 cells, and endocrine-disrupting effects *in vivo* (Krishnan *et al.*, 1993; Gaido *et al.*, 1997; Ashby and Tinwell, 1998; Ashby *et al.*, 2000; Tinwell *et al.*, 2000; Kim *et al.*, 2001; Matthews *et al.*, 2001). But there were remarkable differences in activity. TCBPA showed the highest activity, followed by bisphenol B, BPA and TMBPA. Antioestrogenic activity against 17β-oestradiol (E2) was

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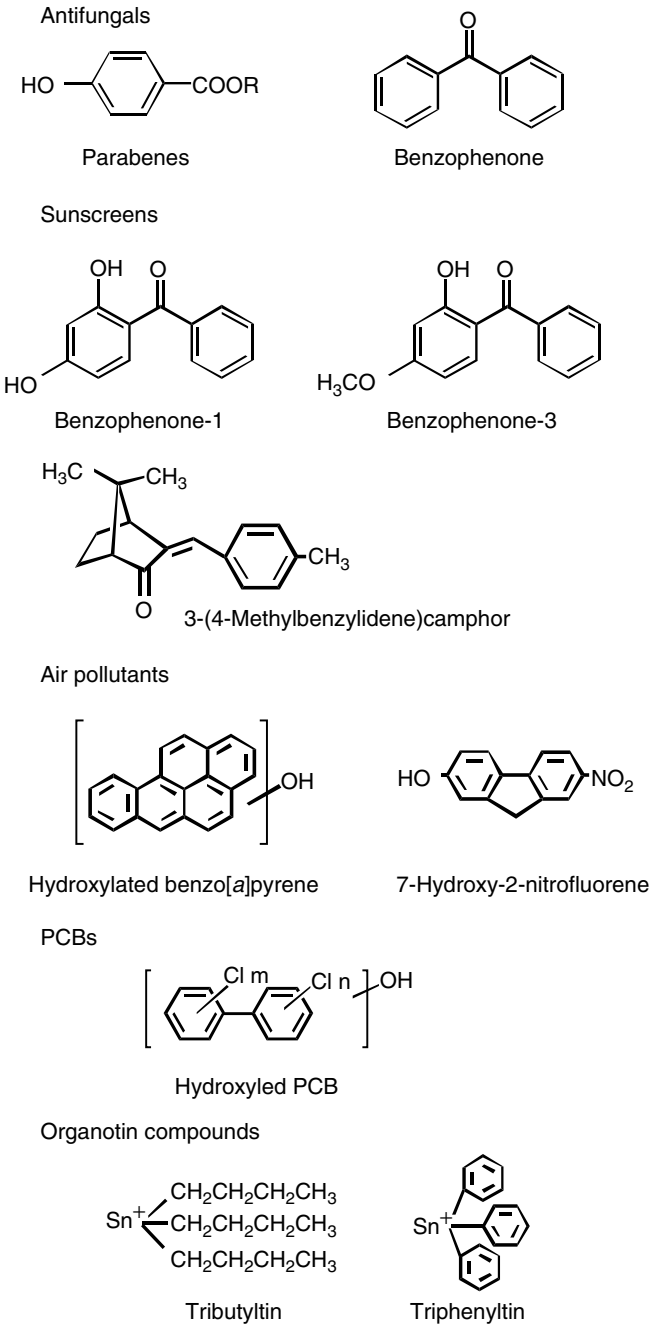

Figure 1 *continued*.

observed with TMBPA. BPA gave positive responses in *in vivo* uterotrophic assays using ovariectomized mice. In contrast, BPA and some related compounds showed significant inhibitory effects on the androgenic activity of 5α-dihydrotestosterone (DHT) in mouse fibroblast cell line NIH3T3. TMBPA showed the highest antagonistic activity, followed by bisphenol AF, bisphenol AD, bisphenol B and BPA. In contrast, TMBPA and 3,3′-dimethylbisphenol A exhibited significant thyroid hormonal activity towards rat pituitary cell line GH3, which releases growth hormone (GH) in a TH-dependent manner. These results suggest that the 4-hydroxyl group of the A-phenyl ring and the B-phenyl ring of BPA derivatives are required for these hormonal activities, and substituents at the 3,5-positions of the phenyl rings and the bridging alkyl moiety markedly influence the activities (Kitamura *et al.*, 2005a). In the metabolism of BPA, the 3-hydroxyl metabolite (catechol-type metabolite) was formed by human and rat liver microsomes and exhibited oestrogenic activity (Elsby *et al.*, 2001a). The glucuronide metabolite proved to have no oestrogenic activity (Pottenger *et al.*, 2000). BPA is further activated when it is incubated with rat S-9 mix (Yoshihara *et al.*, 2001), affording dimer-type metabolites which

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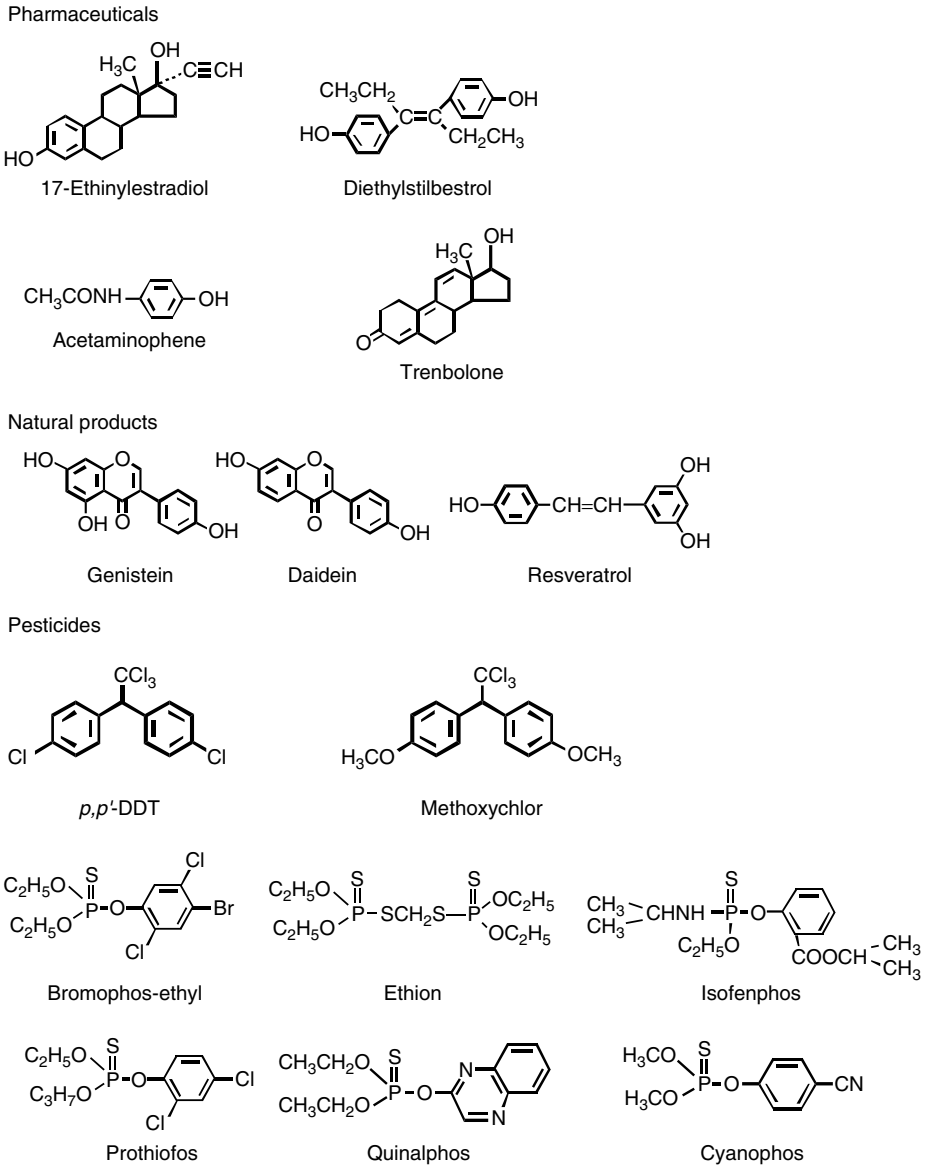

Figure 1 *continued*.

show higher activity than BPA. Such further activation of xenoestrogens must also be considered in the risk assessment of xenoestrogens.

Styrene oligomers, such as *trans*-1,2-diphenylcyclobutane, *cis*-1,2-diphenylcyclobutane, 1,3-diphenylpropane, 2,4-diphenyl-1-butene, 2,4,6-triphenyl-1-hexene and 1α-phenyl-4β-(1′-phenylethyl)tetralin are incorporated into polystyrene resin as impurities in the course of manufacture, and may have a variety of biological actions, including hormonal activity (Kawamura *et al.*, 1998). Polystyrene has been used to manufacture food containers for take-away, such as coffee cups, meat trays, salad boxes and soup bowls, as well as instant food containers, in which instant foods such as Japanese noodles, buckwheat noodles, Chinese noodles, chow mein, spaghetti and rice are cooked by adding hot water. There are reports indicating that styrene oligomers migrate from these containers into the food contents (Sakamoto *et al.*, 2000).

Ohyama *et al.* (2001) reported that some styrene dimers and trimers were oestrogenic in a cell proliferation assays with oestrogen-responsive MCF-7 cells. However, Nobuhara *et al.* (1999) reported that styrene oligomers did not induce the proliferation of MCF-7 cells. Kitamura *et al.* (2003a) also reported that these styrene oligomers were negative in the oestrogen screening assay. However, some styrene oligomers, such as *trans*-1,2-diphenylcyclobutane, *cis*-1,2-diphenylcyclobutane, 1,3-diphenylpropane and 2,4-diphenyl-1-butene, exhibited oestrogenic activity after metabolic activation with liver microsomes of phenobarbital-treated rats. *Trans*-1-(4-Hydroxyphenyl)-2-phenylcyclobutane, which was produced by liver microsomes, exhibited a significant oestrogenic activity. Recombinant human cytochrome

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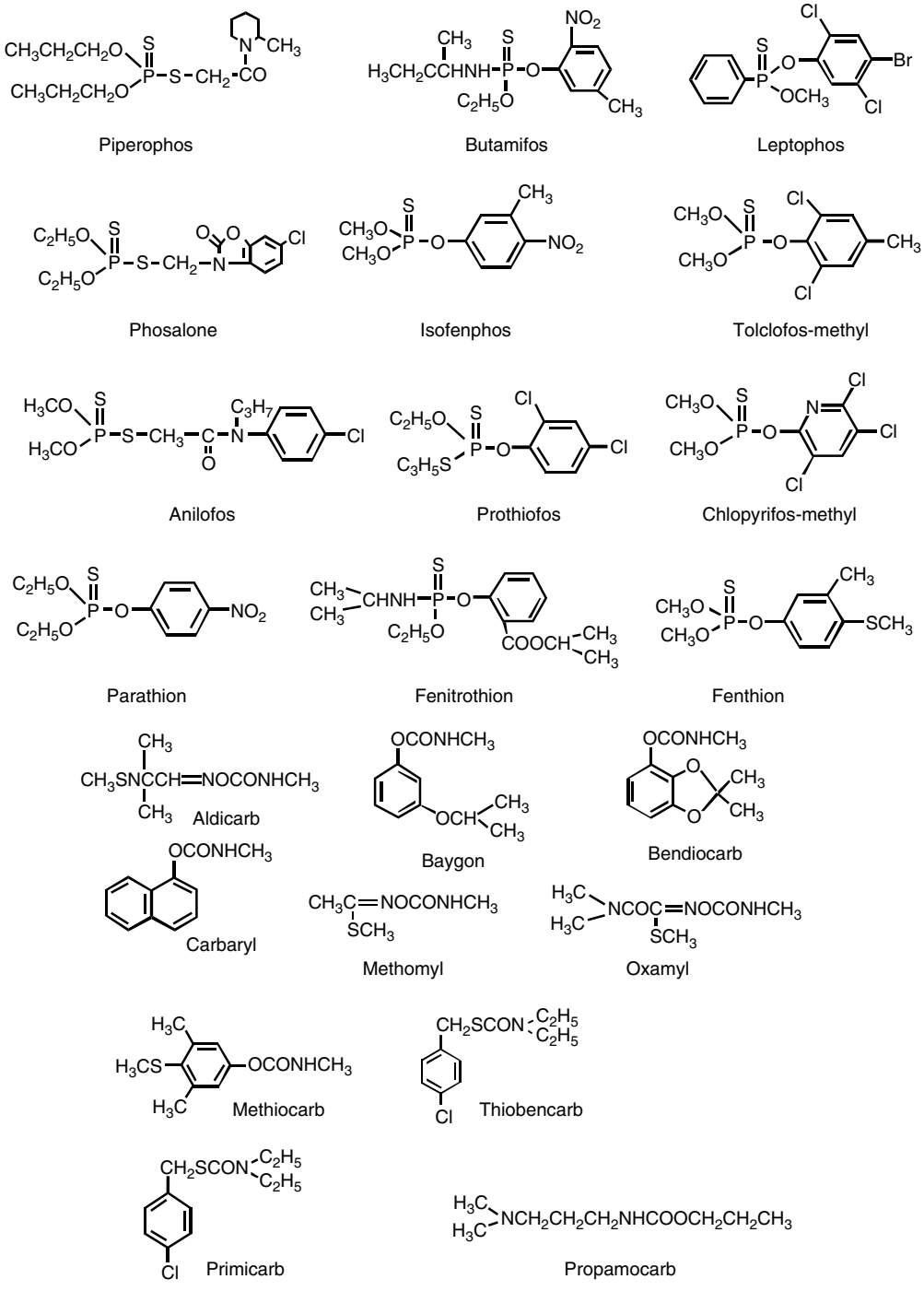

Figure 1 *continued*.

P450 (P450) 2B6 and rat P450 2B1 were responsible for the activation. In contrast, P450 1A may be mainly responsible for the activation of 2,4-diphenyl-1-butene. Thus, some styrene oligomers exhibit oestrogenic activity after metabolic activation to form the hydroxylated metabolite.

Phthalate esters are the most abundant man-made chemical contaminant in our environment. They are mainly used as plasticizers to impart flexibility to nitrocellulose, polyvinyl resins for vinyl floors, food wraps, cosmetics, medical products and toys, and are produced industrially in large quantities. They can leach out of these materials into water, soil or food (Murature *et al.*, 1987). Thousands of tons of plastics are disposed of annually in landfill sites, thus enabling phthalate esters to migrate into groundwater via the soil. The ubiquity of these compounds in the aqueous environment is well known, and their presence has been reported in river water and drinking water, as well as in fish and sediments. High levels of di(2-ethylhexyl) phthalate (DEHP)

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was detected in diet samples obtained from hospitals (Tsumura, 2002). Commonly detected species include di(*n*-butyl) phthalate (DBP), dimethyl phthalate, diethyl phthalate, DEHP, di(*n*-octyl) phthalate and butyl benzyl phthalate. The general population may be exposed to these chemicals via their diet, either from food contamination, or from food or drinks directly contaminated by plastic wraps containing phthalates. In most cases, the greatest exposure is from food. Levels of DBP in foods range from 50 to 500 mg kg^{-1} in the USA. Recently, phthalates have been attracting attention because of their disrupting action on the endocrine system. Some phthalate plasticizers are reported to show oestrogenic activity due to their affinity for ER (Jobling *et al.*, 1995; Nakai *et al.*, 1999). Disruption of androgen-regulated male reproductive development has also been reported (Mylchreest *et al.*, 1999; Hoyer, 2001). Mylchreest *et al.* (1999) reported that the reproductive toxicity of DBP is not due to affinity for androgen receptors (ARs). Phthalate is metabolized via multiple pathways, resulting in the production of deacylated derivatives and their glucuronide conjugates, and hydroxylated derivatives (Saillenfait *et al.*, 1998). The monoester metabolites are believed to be the toxic species, including reproductive toxicity (Foster *et al.*, 2000, 2001; Fukuoka *et al.*, 1995; Oishi and Hiraga, 1980). In contrast, Picard *et al.* (2001) reported that the metabolites of butyl benzyl phthalate are easily produced in MCF-7 cells used for the test, but do not show an oestrogenic effect on MCF-7 proliferation.

Jobling *et al.* (1995) reported that some phthalate esters are weak oestrogens in a yeast reporter assay. Picard *et al.* (2001) demonstrated that butyl benzyl phthalate shows oestrogenic activity in E-screen, and the phthalate is deactivated by dealkylation. Nakai *et al.* (1999) also reported that dialkyl phthalates have binding affinity for ER. We have shown that the 4-hydroxyl and 3-hydroxyl metabolites are major metabolites of dimethyl phthalate and DBP in a rat liver microsomal system. The *in vivo* oestrogenic activity of phthalate esters had previously been believed to be due to monoalkyl metabolites. It is necessary to reconsider the mechanism of reproductive toxicity of phthalate esters. It is also important to consider the activity of metabolites produced from the parent compounds in the assessment of the toxicity of chemicals. Butyl benzyl phthalate is antiandrogenic in *in vitro* yeast-based assays (Sohoni and Sumpter, 1998). Di-*n*-amyl phthalate was positive in the Hershberger test (Yamasaki *et al.*, 2004). Hydroxylated derivatives of phthalate esters were positive in the oestrogen test (Toda *et al.*, 2004).

Recently, nitrile-butadiene rubber has been used instead of polyvinyl chloride to make disposable gloves for use in the food industry. It is reported that 4,4′-butylidenebis(6-*t*-butyl-*m*-cresol) and 2,4-di-*t*-butylphenol, which were eluted from the gloves, are antiandrogenic in *in vitro* reporter gene assays (Satoh *et al.*, 2008). The activity of 4,4′-butylidenebis(6-*t*-butyl-*m*-cresol) was higher than that of *p*,*p*′-DDE (dichlorodiphenyldichloroethene) and similar to that of BPA. 4,4′-Butylidenebis(6-*t*-butyl-*m*-cresol) also showed antioestrogenic activity in the assay system.

2.1.2 Surfactants

Nonylphenol is a degradation product of nonylphenol ethoxylate, which is used as a nonionic surfactant in detergents, paints, herbicides and cosmetics. Nonylphenol is also used as a stabilizer and antioxidant in plastics. Nonylphenol is a mixture of various isomers that have alkyl side chains represented by C_6H_{19}. It is commonly found in rivers, estuaries and other aquatic environments. Nonylphenol is an endocrine-disruptor, having oestrogenic and antiandrogenic activities (White *et al.*, 1994). Watanabe *et al.* (2004) demonstrated that 4-nonylphenol binds both ER α and β. Branched 4-nonylphenol isomers exhibited especially high oestrogenic activity (Shioji *et al.*, 2006). 4-Nonylphenol also binds to and activates pregnane X-receptor (PXR), and induces P450 3A (Masuyama *et al.*, 2000). *In vivo* studies also demonstrated that nonylphenol is uterotrophic in immature rats (Lee and Lee, 1996). Nonylphenol has been shown to weakly bind androgen and progesterone receptors in mammalian *in vitro* assays and yeast-based assays (Sohoni and Sumpter, 1998; Laws *et al.*, 2000). Paris *et al.* (2002) demonstrated that 4-*tert*-octylphenol exhibits antiandrogenic activity with α and β oestrogen activities in reporter cell lines. AR was down-regulated with ER α and β in gonadal tissue of hermaphroditic fish after exposure to nonylphenol (Seo *et al.*, 2006). Nonylphenol in wastewater in India exhibited antiandrogenic activity in the Hershberger assay (Kumar *et al.*, 2008). Octylphenol, a degradation product of alkyl ethoxylates, is also oestrogenic in *in vitro* oestrogen assays (White *et al.*, 1994).

2.1.3 Industrial Materials

Phenylphenols are used as household insecticides, especially for indoor applications (Grossman, 1995; Paris *et al.*, 2002), and as intermediates in the manufacture of rubbers and resins. These hydroxylated derivatives all showed oestrogenic activity. However, 2-hydroxydiphenyl and 3-hydroxydiphenyl showed lower activities than 4-hydroxydiphenyl and 4,4′-dihydroxydiphenyl. Soto *et al.* (1997) reported that 2-hydroxydiphenyl, which is used as an antifungal, is a weak oestrogen, and the related compounds 4-hydroxydiphenyl and 3-hydroxydiphenyl are oestrogenic.

Trans-4-Hydroxystilbene and *trans*-4,4′-dihydroxystilbene both show oestrogenic activity, being about as potent as DES in oestrogen screening tests. *trans*-Stilbene was converted to the active metabolites, *trans*-4-hydroxystilbene and *trans*-4,4′-dihydroxystilbene, by rat

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liver microsomes, and the oestrogenic activity of *trans*-stilbene might be due mainly to *trans*-4-hydroxystilbene and slightly to *trans*-4,4′-dihydroxystilbene. In contrast, *cis*-stilbene was not metabolized to the corresponding hydroxylated metabolites in a liver microsome system (Sugihara *et al.*, 2000). In an *in vivo* oestrogen test using ovariectomized (OVX) rats, *trans*-stilbene, as well as 4-hydroxylated stilbene, was positive (Sanoh *et al.*, 2006). This indicates that *trans*-stilbene exhibits oestrogenic activity after metabolic activation *in vivo*.

2.1.4 Food Additives

It is also reported that *trans*-anethole, a flavour agent, is not oestrogenic, but 4-hydroxy-1-propenylbenzene, the demethylated metabolite of *trans*-anethole, exhibits oestrogenic activity (Nakagawa and Tayama, 2003). Butylated hydroxyanisole, a food additive used as an antioxidant and food preservative in food, food packing, feed, cosmetics and pharmaceuticals, has antioestrogenic activity, but not oestrogenic or antiandrogenic activity *in vivo* in rats (Kang *et al.*, 2005).

Benzylideneacetone also has a use as a flavouring additive for cosmetics, detergents, cigarettes and foods (Opdyke, 1973). This food additive is metabolically activated to an oestrogen by a microsomal enzyme system, and the 4-hydroxy derivative, which is a major metabolite in the microsomal system, has oestrogenic activity (Kohno *et al.*, 2005).

Nordihydroguaiaretic acid (NDGA) is an antioxidant which has been used to preserve oils and food. It also has anticancer activity. NDGA is a member of the lignans, which are generally regarded as phytoestrogens. NDGA has oestrogenic activity *in vitro* with the estrogen-responsive element (ERE)-luc reporter system using NIH3T3 cells and the oestrogen-responsive cell growth assay in a pituitary cell line MtT/E-2. Uterotropic assays in rats indicated oestrogenic activity *in vivo*, but only at a dose as high as $100\,mg\,kg^{-1}$ body weight/two days for 10 days. NDGA is able to bind both ERα and ERβ. Interestingly it preferentially induced ERα-mediated ERE-luc activity, although the binding affinity of NDGA was about the same with both receptors. However, with ERβ, NDGA showed only weak agonistic action, and it antagonized the oestrogenic action when administered with E2 (Fujimoto *et al.*, 2004).

2.1.5 Flame Retardants

Brominated flame retardants are widely used throughout the world in building materials, paints, synthetic textiles and plastic products, including epoxy resin electronic circuit boards and other electronic equipment (Alaee and Wenning, 2002; Darnerud *et al.*, 2001; Darnerud, 2003; De Wit, 2002; Sjödin *et al.*, 2003). Polybrominated diphenyl ethers (PBDEs) and tetrabromobisphenol A (TBBPA) have been detected in environmental samples, such as sediments, fish and birds, and also in human breast milk and blood (Sjödin *et al.*, 2001; Thomsen *et al.*, 2002; Oberg *et al.*, 2002; Fischer *et al.*, 2006; Harrard and Porter, 2007; Inoue *et al.*, 2006; Schecter *et al.*, 2006). The halogenated compound was also found in air samples at an electronics recycling plant (Sjödin *et al.*, 2001). Thomsen *et al.* (2001) detected TBBPA and TCBPA in human plasma lipids at levels of $4–200\,pg\,g^{-1}$ plasma. Some PBDEs may influence the endocrine system in wildlife. It has become evident that exposure to PBDEs can lead to TH disturbances and neurotoxicity in experimental animals, wildlife and humans (Hallgren and Darnerud, 2002; Mariussen and Fonnum, 2003; Viberg *et al.*, 2003; Fukuda *et al.*, 2004; Hamers *et al.*, 2006; Sakai *et al.*, 2003; Zhou *et al.,* 2001a; 2002). It has been suggested that hydroxylated PBDEs may disrupt TH status, because of their structural similarity to TH. Hydroxylated PBDEs may have high binding affinities for the serum TH binding protein transthyretin (TTR) and thyroid hormone receptor (TR), thereby displacing the natural ligand. TBBPA and TCBPA exhibited significant thyroid hormonal activity towards rat pituitary cell line GH3, which releases GH in a TH-dependent manner (Kitamura *et al.*, 2002a). 1,2,5,6,9,10-Hexabromocyclododecane also exhibited TR-mediated gene expression (Yamada-Okabe *et al.*, 2005).

Affinity for TR of some hydroxylated PBDEs was comparatively examined. 4-Hydroxy-2,2′,3,4′,5-pentabromodiphenyl ether and 3-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether showed binding affinity for TR. However, 2,2′,3,4,4′,5′-hexabromodiphenyl ether, decabromodiphenyl ether, 4-methoxy-2,2′,3,4′,5-pentabromodiphenyl ether, 4-hydroxy-2,2′,3,4′-tetrabromodiphenyl ether and 4′-hydroxy-2,2′,4-tribromodiphenyl ether did not show affinity for TR. In contrast, 4′-hydroxy-2,2′,4-tribromodiphenyl ether exhibited significant oestrogenic activity in an oestrogen-responsive reporter assay in MCF-7 cells. However, the adjacent bromo-substitution of 3- or 4-hydroxylated PBDEs markedly decreased the oestrogenic activity. These findings suggest that hydroxylated PBDEs act as TH-like agents, as well as oestrogens. A 4- or 3-hydroxyl group in PBDEs is essential for thyroid hormonal and oestrogenic activities, while adjacent dibromo substitution favours thyroid hormonal activity, but not oestrogenic activity.

It has been reported that some PBDEs and TBBPA have oestrogenic, antiandrogenic and antiprogesteronic activities *in vitro* (Kitamura *et al.,* 2002a; Olsen *et al.*, 2003; Stoker *et al.*, 2005; Hamers *et al.*, 2006). Ceccatelli *et al.* (2006) reported that a PBDE, PBDE 99, may cause ER disruption *in vivo* in rats. Richardson *et al.* (2008) reported that PBDE 47 disturbed TH in mice *in vivo*. Lilienthal *et al.* (2006) also reported that PBDE 99 influences sexual development and causes sexually dimorphic behaviour in rats. Antiestrogenic activity against E2 was observed with TBBPA. TBBPA and TCBPA gave positive responses in an *in vivo* uterotrophic assay using

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ovariectomized mice. In contrast, BPA and some related compounds showed significant inhibitory effects on the androgenic activity of DHT in mouse fibroblast cell line NIH3T3.

Recently, organophosphorus esters such as tris-(2-chloroethyl) phosphate and tris-(2-chloropropyl) phosphate have been widely used as flame retardants, especially as fire-preventing agents in polyurethane foams. These flame retardants exhibited cytotoxicity in the presence of S9 mix, but did not show oestrogenic or antioestrogenic activity in *in vitro* assay systems (Follmann and Wober, 2006).

2.1.6 Antifungals

Parabens are in widespread use as preservatives in foods, pharmaceuticals and cosmetics. Since the human population is widely exposed to parabens, their endocrine-disrupting effects may be a serious problem for humans. The oestrogenic activities of parabens such as methylparaben, ethylparaben, propylparaben and butylparaben have been reported in *in vitro* screening systems (Byford *et al.*, 2002; Terasaka *et al.*, 2006).

Antiandrogenic activities of some parabens were examined using reporter gene assays with Chinese hamster ovary (CHO)K1 cells. Isobutylparaben, *n*-butylparaben, isopropylparaben and *n*-propylparaben exhibited antiandrogenic activity. However, relatively short side chain parabens, ethylparaben and methylparaben, had no effect (Satoh *et al.*, 2005). Prusakiewicz *et al.* (2007) also reported that parabens exerted their oestrogenic effects by elevating levels of oestrogens through inhibition of oestrogen sulfotransferasesin skin. The inhibitory activity increased with increasing paraben ester chain length, similarly to paraben sulfotransferase oestrogenicity. In contrast, some parabens were active in the yeast two-hybrid system, but their activities were eliminated by S9 treatment (Morohoshi *et al.*, 2005).

Propylparaben shows an oestrogenic effect in rainbow trout. When sexually immature, rainbow trout were kept in water containing propylparaben at 225 $\mu g\,l^{-1}$, plasma vitellogenin was markedly increased after 12 days (Bjerregaard *et al.*, 2003). *p*-Hydroxybenzophenone, which is formed from benzophenone, an antifungal agent, in rat hepatocytes, is also oestrogenic (Nakagawa and Tayama, 2001; Nakagawa and Suzuki, 2002).

2.1.7 Sunscreens

Hydroxybenzophenones, such as 2,4-dihydroxybenzophenone (benzophenone-1) and 2,2′,4,4′-tetrahydroxybenzophenone (benzophenone-2), have the ability to absorb and to dissipate ultraviolet (UV) light A (400–315 nm) (Klein, 1992). Consequently, they are used as UV stabilizers in plastic surface coatings on food packaging to prevent polymer degradation and loss of quality of the packed food owing to UV light irradiation. These compounds may be transferred from the packaging to the contents, and subsequently ingested by humans. However, they are natural components of plants such as mango and muscat grape, and are also used as flavourings. It has been reported that some derivatives have cytotoxic effects (Matsumoto *et al.*, 2003). Hydroxybenzophenones and their derivatives are also used as sunscreens for humans. These compounds are reported to be absorbed through human skin, and bioaccumulation may occur in wildlife and humans (Felix *et al.*, 1998; Hagedorn-Leweke and Lippold, 1995; Hayden *et al.*, 1997; Jiang *et al.*, 1999). In spite of some toxic effects of benzophenone, such as hepatotoxicity, hydroxylated benzophenones are safe for topical application to humans. Acute and subchronic systemic toxicity of these compounds after dermal application is considered to be rather low (Burdock *et al.*, 1991; Dutta *et al.*, 1993; Okereke *et al.*, 1995). However, some problems have arisen with photoallergic reactions in patients with suspected clinical photosensitivity (Schauder and Ippen, 1997).

Some hydroxylated benzophenones also show oestrogenic activity in the yeast two-hybrid system with ER and coactivators (Kawamura *et al.*, 2003). It was also reported that 2-hydroxy-4-methoxybenzophenone (benzophenone-3), 2,4-dihydroxybenzophenone, 2,2′,4,4′-tetrahydroxybenzophenone and 4-hydroxybenzophenone are weakly positive in the uterotrophic assay using immature rats (Schlumpf *et al.*, 2001; Nakagawa and Tayama, 2001; Koda *et al.*, 2005). Jarry *et al.* (2004) reported that 2,2′,4,4′-tetrahydroxybenzophenone is oestrogenic in the uterotrophic assay using immature rats, and also decreased l-3,3′,5-triiodothyronine (T3) and thyroxin (T4) levels in the rats. Androgenic and antiandrogenic properties of hydroxylated benzophenones are also an important problem. Yamasaki *et al.* (2003) reported that some hydroxybenzophenones do not act as antiandrogens in the Hershberger assay. Satoh *et al.* (2001) also found that benzophenone has no affinity for AR. Ma *et al.* (2003) reported that benzophenone-3 is a weak antagonist for AR. Suzuki *et al.* (2005) comparatively examined the oestrogenic and antiandrogenic activities of benzophenone and 16 of its derivatives, which are used as UV stabilizers, by means of hormone-responsive reporter assays in various cell lines. Hydroxylated benzophenones exhibited oestrogenic activity in human breast cancer cell line MCF-7, but their activities varied markedly. The highest activity was observed with 2,4,4′-trihydroxybenzophenone (2,4,4′-triOH-BP), followed by 2,3′,4,4′-tetrahydroxybenzophenone, 4,4′-dihydroxybenzophenone, 2,2′,4,4′-tetrahydroxybenzophenone, 4-hydroxybenzophenone and 2,4-dihydroxybenzophenone. Benzophenone itself showed little activity in the assay. In contrast, benzophenone and some related compounds showed significant inhibitory effects on the androgenic activity of DHT in rat fibroblast cell line

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NIH3T3. The highest activity was observed with 2,4,4′-triOH-BP, followed by 2,3′,4,4′-tetrahydroxybenzophenone, 2,2′,4,4′-tetrahydroxybenzophenone, 3-hydroxybenzophenone and 2,2′-dihydroxybenzophenone. 2,4-Dihydroxybenzophenone, 2,4,4′-triOH-BP and benzophenone gave positive responses in uterotrophic assays using ovariectomized rats, and 2,4,4′-triOH-BP was positive in the Hershberger assay using castrated rats. They suggested that a 4-hydroxyl group on the phenyl ring of benzophenone derivatives is essential for high hormonal activities, and the presence of other hydroxyl groups markedly alters these activities.

4-Methylbenzylidene camphor (4-MBC) and 3-benzylidene camphor are also UV absorbers used in sunscreens. They stimulated MCF-7 cell proliferation. 4-MBC and 3-benzylidene camphor bound to ERβ (Mueller *et al.*, 2003; Schlumpf *et al.*, 2004; Klann *et al.*, 2005). In uterotrophic assays in immature rats, 3-benzylidene camphor showed high potency, being almost as active as genistein. 4-MBC and 4-hydroxybenzoic acid isobutyl ester also had a stimulating effect on uterine weight (Tinwell *et al.*, 2002; Schlumpf *et al.*, 2004; Koda *et al.*, 2005). They suggested that active metabolites with different receptor binding characteristics are formed, as the activity on immature rat uterus could not be explained in terms of binding to ERβ. 4-MBC also interferes with the thyroid axis after the developmental exposure of rats (Maerkel *et al.*, 2007). UV filters also enter aquatic ecosystems, and 4-MBC was most frequently found, followed by benzophenone-3 (Balmer *et al.*, 2005). In contrast, 4-octylphenylsalicylate and 2,2′-dihydroxy-4,4′-dimethoxybenzophenone acquired oestrogenic activity after metabolic activation by S9-mix in the yeast two-hybrid assay system (Morohoshi *et al.*, 2005).

2.1.8 Air Pollutants

Polycyclic aromatic hydrocarbons (PAHs) are environmental pollutants formed during the incomplete combustion of organic materials such as fossil fuel, wood and others, including automobile exhaust, domestic heating and industrial processes. Humans are exposed to PAHs from air, water, diet and cigarette smoke. PAHs are carcinogenic in humans and animals. Many PAHs are negative in oestrogen screening tests. However, some reports suggest that hydroxylated PAHs act as xenoestrogens. It was demonstrated that hydroxylated metabolites of benzo[*a*]pyrene exhibit oestrogenic activity. The 1-,3-,7- and 9-hydroxylated metabolites had binding affinity for ER, but the 7,8- and 9,10-dihydroxy metabolites did not (Charles *et al.*, 2000; Fertuck *et al.*, 2001; Bader, 2005). De Wiele *et al.* (2005) also reported that polycyclic aromatic hydrocarbons were transformed to oestrogenic hydroxylated metabolites by human colon microbiota. As oestrogenic compounds in cigarette smoke condensate, 2-hydroxyfluorene, 2- and 3-hydroxyphenanthrenes, and 1-hydroxypyrene were identified (Kamiya *et al.*, 2005). Some hydroxylated PAHs were also reported to be antiestrogenic in a yeast assay system (Tran *et al.*, 1996; Hirose *et al.*, 2001). 1-Hydroxypyrene was identified as an oestrogenic compound in diesel exhaust particulates (Noguchi *et al.*, 2007). 4-Nitrophenol in diesel exhaust particles is both oestrogenic and antiandrogenic (Li *et al.*, 2006). Hayakawa *et al.* (2007) compared the oestrogenic/antioestrogenic activities of 63 monohydroxylated PAHs using the yeast two-hybrid assay system, and found that hydroxylated PAHs having three to five rings show these activities. Hydroxylated PAHs having four rings, such as 3-, 4- and 10-hydroxybenzo[*a*]anthracenes, and 2-hydroxychrysene showed especially high oestrogenic activity, and hydroxylated PAHs having four rings such as 2- and 3-hydroxybenzo[*c*]phenanthrenes, 2-hydroxybenz[*a*]anthracene and 3-hydroxychrysene showed high antioestrogenic activity. Antiandrogenic activity of some PAHs was reported in a reporter gene assay system (Vinggaard *et al.*, 2000; Kizu *et al.*, 2003). Both Vinggaard and Kizu's studies demonstrated that aryl hydrocarbon receptor (AhR) was required for the antiandrogenic effect of PAHs. Lin *et al.* (2004a) also demonstrated that the AhR signalling pathway is involved in the effect of benzo[*a*]pyrene on AR expression.

Nitropolycyclic aromatic hydrocarbons, which are carcinogenic and mutagenic, enter the environment from diesel engine exhaust, urban pollution sources, cigarette smoking and so on (Wang *et al.*, 1980; Rosenkranz and Mermelstein, 1983). Nitropolycyclic aromatic hydrocarbons should also be examined to see whether nitro reduction or hydroxylation of the aromatic rings activates these compounds to xenobiotic oestrogens, as is the case for their carcinogenicity. 2-Nitrofluorene is a typical carcinogenic nitropolycyclic aromatic hydrocarbon (Möller *et al.*, 1989). 2-Nitrofluorene was detected in diesel exhaust particles as a major component, together with nitropyrenes, and was also detected as a major pollutant in the atmosphere (Campbell and Milton, 1984). It was shown that 2-nitrofluorene exhibits a significant oestrogenic activity after activation by rat liver microsomal mixed function oxidase of 3-methylcholanthrene-treated rats. In this case, 7-hydroxy-2-nitrofluorene was formed as a major metabolite and exhibited a significant oestrogenic activity (Fujimoto *et al.*, 2003).

2.1.9 Polychlorinated Biphenyls

PCBs have been widely used throughout the world as industrial chemicals for heat transfer and electrical insulation. However, they have contaminated almost every component of the global ecosystem, including wildlife and human adipose tissue, breast milk and serum, due to their lipophilic character. They have been reported

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to have a variety of toxic effects, including immunotoxicity, neurotoxicity, developmental toxicity, hepatotoxic effects, reproductive toxicity and carcinogenesis (Safe, 1994). In recent years it has become evident that exposure to PCBs can also lead to TH disturbances in wildlife and humans (Brouwer *et al.*, 1998; Porterfield and Hendry, 1998). Decreased levels of circulating plasma T4 following PCB exposure have been found in laboratory animals (Brouwer *et al.*, 1998; Barter and Klaassen, 1994). Disturbance of TH metabolism, such as glucuronidation, sulfation or deiodination, with PCBs was also reported (van Birgelen *et al.*, 1995; Brouwer *et al.*, 1998).

Some hydroxy-PCBs, which are metabolites of PCBs, are reported to show antithyroid hormonal activity in addition to oestrogenic activity (Lans *et al.*, 1994; Connor *et al.*, 1997; Korach *et al.*, 1988; Cheek *et al.*, 1999; Kitamura *et al.*, 2005b). The potency of hydroxylated PCBs to compete for binding with TR was tested, in view of their structural resemblance to the THs. Korach *et al.* (1988) reported that some hydroxy-PCBs, such as 4,4′-dihydroxy-3,3′,5,5′-tetrachlorobiphenyl, competitively bind to ER and exhibit oestrogenic activity in the mouse uterus. Other reports also show that hydroxylated PCBs are oestrogenic or antioestrogenic with respect to binding to ER (Moore *et al.*, 1997; Ramamoorthy *et al.*, 1997). Connor *et al.* (1997) demonstrated complex structure–oestrogenicity/antioestrogenicity relationships for hydroxylated PCBs. In the case of hydroxy-PCB, adjacent chloride substituents decreased the oestrogenic activity (Kitamura *et al.*, 2005c). The catechol-type metabolite of PCBs was also shown to induce oestrogenic activity (Garner *et al.*, 1999).

It has also been reported that hydroxylated PCBs can disrupt TH status by high binding affinity for the serum TH binding protein TTR (Lans *et al.*, 1993; 1994; Cheek *et al.*, 1999). Kitamura *et al.* (2005c) comparatively examined the TH-disrupting and oestrogenic activities of 14 hydroxylated PCBs by means of binding assays with TR, as well as TH-dependent growth assays and assays of production of GH in pituitary cell line GH3 cells, and also evaluated the oestrogenic activity observed in ERE-luciferase reporter assays. 4-Hydroxy-2,2′,3,4′,5,5′-hexachlorobiphenyl, 4-hydroxy-3,3′, 4′,5-tetrachlorobiphenyl and 4,4′-dihydroxy-3,3′,5,5′-tetrachlorobiphenyl, which have been detected as metabolites of PCBs in animals and humans, and six other 4-hydroxylated PCBs markedly inhibited the binding of triiodothyronine to TR. These hydroxy-PCBs also enhanced the proliferation of rat pituitary cell line GH3 cells and stimulated their production of GH. However, 4-hydroxy-2′, 4′, 6′-trichlorobiphenyl, 4-hydroxy-2,2′,5,5′-tetrachlorobiphenyl, 4-hydroxy-2,3,3′, 4′-tetrachlorobiphenyl, 2,3′,5,5′-tetrachlorobiphenyl and 2,3′, 4′,5,5′-pentachlorodiphenyl did not show affinity for TR. Only 4-hydroxy-2′, 4′, 6′-tetrachlorobiphenyl exhibited a significant oestrogenic activity in oestrogen-responsive reporter assays in MCF-7 cells. However, 3,5-dichloro substitution of 4-hydroxylated PCBs markedly decreased the oestrogenic activity. Thus, it was suggested that a 4-hydroxyl group in PCBs is essential for thyroid hormonal and oestrogenic activities, and that 3,5-dichloro substitution favours thyroid hormonal activity, but not oestrogenic activity.

The levels of these active metabolites in the body would be critical in determining whether these compounds actually show activity *in vivo*. Sandau *et al.* (2000, 2002) reported that the amount of total hydroxylated PCBs in normal human populations is of the order of $0.15-0.46\,\mathrm{ng\,g^{-1}}$ wet weight of blood, but in highly exposed populations, such as the Inuits, the concentration can reach as much as $11.6\,\mathrm{ng\,g^{-1}}$ wet weight of blood. The average values for Inuit men and women are 1.73 and $1.01\,\mathrm{ng\,g^{-1}}$ blood, respectively. The concentration of hydroxylated PCBs in humans is thus two or three orders of magnitude less than the concentration shown to cause thyroid hormonal activity in this *in vitro* study. Therefore, these effects may not occur in the real world. High levels of hydroxylated PCBs in marine mammals may be expected (Hoekstra *et al.*, 2003). These compounds may be accumulated in the animal body, especially marine mammals, through the generations and by bioconcentration in the food chain.

Serum TH levels are tightly regulated through the hypothalamus–pituitary–thyroid axis *in vivo*, but this may not be the case for these chemicals, which may act directly on the endocrine organs. In contrast, it was reported that hydroxylated PCBs disrupt the central nervous system through antagonistic action against THs (Iwasaki *et al.*, 2002; Miyazaki *et al.*, 2004). If hydroxylated PCBs are taken up into foetuses, these compounds may induce TH-disrupting effects through this action in combination with the agonistic mechanism. Darnerud *et al.* (1996) also reported that hydroxylated PCBs bind with foetal TTR, and this is accompanied with a decrease in foetal plasma thyroxin levels. It is necessary to assess the *in vivo* endocrine-disrupting action of hydroxylated PCBs, taking into account the other TH-disrupting actions of these compounds.

2.1.10 Organotin Compounds

Organotin compounds have been used as heat stabilizers in vinyl chloride polymers, industrial catalysts for polyurethane foam and silicones, and biocides for bacteria and fungi. In particular, tributyltin and triphenyltin have been widely used as biocides, agricultural fungicides, wood preservatives and disinfecting agents in circulating industrial cooling waters, as well as in antifouling paint for ships and aquaculture nets. Organotin compounds are toxic to sexual development of

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marine species at low concentrations (Beaumont and Badd, 1984). Molluscs are the most sensitive species, and shell malformations have been observed in oysters, as well as reduced growth and population decline (Alzieu, 1991; Widows and Page, 1993). Imposex, imposition of male characters in female organisms of some gastropod species, has been widely observed, and leads to reproductive failure and consequent population decline (Gibbs and Bryan, 1994; Horiguchi *et al.*, 1997). Organotin compounds are also neurotoxic and immunotoxic in mammals (Cooke *et al.*, 2004). Contamination with organotin compounds has been reported in wild animals, especially in marine organisms (Snoeij *et al.*, 1987). Organotin compounds also contaminate human food, especially shellfish, and water, mainly through contributions from industrial effluents and from leaching of polyvinyl chloride water pipes. Humans exposed accidentally or occupationally to organotin compounds develop seizures, episodes of severe pain and psychic disturbances (Fortemps *et al.*, 1978; Ross *et al.*, 1981). Kannan *et al.* (1999) reported that butyltin compounds were detected in the order of monobutyltin > dibutyltin > tributyltin in human blood, and the average level of total butyltin compounds in blood collected from subjects in Michigan was 21 ng ml^{-1}, with a maximum of 101 ng ml^{-1}, which corresponds to the lower levels in sea mammalians.

Imposex can be caused by increased androgen levels through inhibition of aromatase (CYP19) activity by tributyltin (Betin *et al.*, 1996). However, the concentration required for the inhibition is very high compared to environmental levels. Recently, it was demonstrated that organotin compounds function as retinoid X receptor (RXR) agonists, and aromatase is inhibited as a result of the high affinity of organotin compounds for RXR (Nakanishi *et al.*, 2005; Nakanishi *et al.,* 2006a). Yonezawa *et al.* (2007) also reported that tributyltin and triphenyltin inhibited osteoclast differentiation in mouse monocytic RAW264.7 cells through RXR, but monobutyltin and dibutyltin did not show such activity. In contrast, organotin compounds also are reported to inhibit the activity of steroidogenic enzymes such as 3β-hydroxysteroid dehydrogenase type II, 5β-reductase type II and 17β-hydroxysteroid dehydrogenase types I and III with aromatase in animals *in vitro* (Doering *et al.*, 2002; Lo *et al.*, 2003). However, Nakanishi *et al.* (2006b) found that tripropyltin, tributyltin and triphenyltin enhanced the activity of 17β-hydroxysteroid dehydrogenase type I in human placenta. Thus, these organotins stimulated 17β-oestradiol biosynthesis, and are expected to induce higher levels of E2 in human placenta. Haemoproteins such as P450 isozymes and neuronal nitric oxide synthase (nNOS) were also inhibited by organotin compounds, like aromatase (Kim *et al.*, 1998; Ohashi *et al.*, 2004).

2.1.11 Pesticides

2.1.11.1 Action of Pesticides In Vitro

Chlorinated insecticides, such as kepone, *o*, *p*′-DDT, dieldrin and methoxychlor, are known to have oestrogenic activity (Andersen *et al.*, 1999). *p*, *p*′-DDE, vinclozolin and ketoconazole (antifungal agents), and linuron, iprodione, chlozolinate, procymidone, chlornitrofen, fenitrothion and fenthion (insecticides) have antiandrogenic activity (Kelce *et al.*, 1995; Gray *et al.,* 1999a; Tamura *et al.* 2001; Kojima *et al.*, 2003; Kitamura *et al.*, 2003b; Wong *et al.*, 1995; Ostby *et al.*, 1999; Lambright *et al.*, 2000; McIntyre *et al.*, 2000). *p*, *p*′-DDT (1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane) is metabolized to *p*, *p*′-DDD (1,1-dichloro-2,2-bis(4-chlorophenyl)ethane) and *p*, *p*′-DDE (1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene) by reductive dechlorination and dehydrochlorination, respectively, and *p*, *p*′-DDD is further oxidized to *p*, *p*′-DDA (2,2-bis(4-chlorophenyl)acetic acid) in animals and fish (Esaac and Matsumura, 1980; Kitamura *et al.*, 2002b). *p*, *p*′-DDD shows oestrogenic activity and *p*, *p*′-DDE shows antiandrogenic activity (Kelce *et al.*, 1995). The metabolites of *p*, *p*′-DDT and *o*, *p*′-DDT described above exhibit oestrogenic activity, like the early established environmental oestrogen *o*, *p*′-DDT (Forster *et al.,* 1975; Chen *et al.*, 1997; Nelson *et al.*, 1978). Nelson (1974) showed that some of these DDT metabolites have uterotropic effects *in vivo*. Gray *et al.* (1999) reported that *p*, *p*′-DDT was antiandrogenic, as well as *p*, *p*′-DDE. Methoxychlor requires demethylation by liver microsomal mixed function oxidases, including CYP 1A2 and 2C19, to elicit oestrogenic activity (Stresser and Kupfer, 1998; Elsby *et al.*, 2001b). Schlenk *et al.* (1997) also reported that methoxychlor was activated to oestrogen in fish. The widespread use of these pesticides and fungicides in the environment may alter male sexual development and reproductive processes (Kelce and Wilson, 1997).

Andersen *et al.* (2002) tested 22 pesticides for effects on proliferation of MCF-7 cells, and interference with the activation of ER and AR. They reported that chlorpyrifos, prochloraz, fenarimol, endosulfan, dieldrin, pirimicarb, propamocarb, methiocarb and tolclofos-methyl exhibited oestrogenic activity in cell proliferation assays and transactivation assays using MCF-7 cells. Kojima *et al.* (2004) examined the oestrogenic activity of 56 pesticides in *in vitro* reporter gene assays using CHO cells. They found that prothiofos, bromophos-methyl, tolclofos-methyl, quinalphos, leptophos, cyanofenphos, dichlofenthion, EPN (ethyl *p*-nitrophenyl phenylphosphonothionate), ethion and bromophos-ethyl showed affinity for ERα and ERβ. Butamifos showed the highest activity among positive compounds for ERα. No antioestrogenic activity was observed with any of pesticides tested.

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Kojima *et al.* (2004) also reported that fenitrothion, anilofos, EPN, prothiofos, parathion, methyl parathion, tolclofos-methyl, piperophos, ethion, butamifos, phosalone, dichlofenthion, fenthion, cyanophos, leptophos, bromopho-ethyl, quinalphos, isofenphos and fenitrothion (MEP) oxon showed inhibitory effects on the androgenic activity of DHT in a reporter gene assay using CHO cells transfected with human AR (hAR). Fenthion, an organophosphorus pesticide, which is widely used throughout the world as a broad-spectrum insecticide for numerous crops, showed antiandrogenic activity against the androgenic activity of DHT in a reporter assay using NIH3T3 cells (Kitamura *et al.*, 2003b). Tamura *et al.* (2001) reported that fenitrothion competitively inhibited DHT-dependent human AR activation in HepG2 cells. The potency of fenitrothion was about 8- to 35-fold higher than those of *p*, *p*′-DDE and linuron, well-known environmental antiandrogens (Maness *et al.*, 1998; McIntyre *et al.*, 2001). Sohoni *et al.* (2001) observed activity of fenitrothion in recombinant yeast expressing the human AR. In this literature, fenitrothion showed the highest activity among the pesticides tested. Fang *et al.* (2003) reported that methyl parathion, parathion and triphenyl phosphate were positive in a comparative binding assay to AR.

Chlorpyrifos induced gene expression of gonadotropin-releasing hormone (GnRH) in a hypothalamic cell line, GT1-7, suggesting that it may have an impact on the hypothalamic–pituitary–gonad axis (Gore, 2002; Kojima *et al.*, 2004). It is also reported that chlorpyrifos may interfere with testosterone metabolism, potentially leading to hormonal imbalance (Usmani *et al.*, 2003). Bisson and Hontela (2002) reported that diazinon inhibited adrenocorticotropic hormone (ACTH)- and dibutyryl-cAMP-stimulated cortisol secretion in adrenocortical cells of rainbow trout. Takeuchi *et al.* (2006) examined agonistic activities of 200 pesticides for peroxisome proliferator-activated receptor α (PPARα), which regulates fatty acid metabolism. They showed that diclofop-methyl, pyrethrins and imazalil act as agonists for PPARα, and further, PPARα-inducible P450 4A was induced in mice treated with diclofos-methyl and pyrethrins.

Pyrethroid insecticides are widely used throughout the world as a wide-spectrum insecticide for numerous crops and also for indoor pest control in the public health sector and housing (Casida *et al.*, 1983; Roberts and Hutson, 1999). Pyrethroids act on the axons in the peripheral and central nervous systems of insects, causing prolonged opening of sodium channels (Aldridge, 1990; Miyamoto *et al.*, 1995). Some authors have indicated that hydrolysis products of permethrin have endocrine-disrupting potential (Stratton and Corke, 1982; Tyler *et al.*, 2000; McCarthy *et al.*, 2006). Several endocrin-disrupting actions, such as oestrogenic, antioestrogenic, antiandrogenic and antiprogestagenic activities, have been reported (Eil and Nisula, 1990; Garey and Wolff, 1998; Go *et al.*, 1999; Kim *et al.*, 2004). Chen *et al.* (2002) reported that some pyrethroid pesticides were positive in oestrogen screening assays, E-screen assays, ER competitive binding assays or pS2 expression assays. Go *et al.* (1999) reported significant oestrogenicity of sumithrin and fenvalerate in an assay using MCF-7 cells. Antioestrogenic activity of pyrethroids was also reported (Kim *et al.*, 2004). Several synthetic pyrethroids, such as sumithrin, fenvalerate and permethrin, as well as naturally occurring pyrethrins, interact with AR (Parker *et al.*, 1984; Eil and Nisula, 1990). Tyler *et al.* (2000) showed that metabolic and environmental degradation products of pyrethroids have oestrogenic, antioestrogenic and/or antiandrogenic activity.

Urea-type pesticides, propanil, linuron and diuron, which are herbicides widely used around the world, have the ability to bind to AR (Cook *et al.*, 1993; Bauer *et al.*, 1998). Indeed, linuron is structurally related to the nonsteroidal antiandrogen, flutamide, and its IC50 value for competition at the AR is approximately three and half times that of flutamide. In contrast, prochloraz, inhibited the aromatase activity of human placental microsomes and human JEG-3 choriocarcinoma cells (Mason *et al.*, 1987; Vinggaard *et al.*, 2000; Andersen *et al.*, 2002). Linuron decreased accessory sex organ weights in sexually immature and mature rats treated with linuron. In linuron-treated mature rats, serum E2 and LH levels were increased (Cook *et al.*, 1993). Imidazole-like fungicides such as imazalil and prochloraz were potent inhibitors of aromatase (Sanderson *et al.*, 2002). Atrazine, which is widely used as a selective pre-emergence herbicide, has a low affinity for ER and AR. However, atrazine influenced these hormone levels through inhibition or induction of hormonal-regulating enzymes. Atrazine and vinclozolin increase aromatase levels by binding to and inhibiting phosphodiesterase, resulting in increased transcription of the aromatase gene through elevated cyclic adenosine monophosphate (cAMP) levels (Sanderson *et al.*, 2002; Roberge *et al.*, 2004). The pesticide vinclozolin is also known to exhibit antiandrogenic activity after metabolic activation (Kelce *et al.*, 1994; Gray *et al.*, 1994). A diphenyl ether-type herbicide, chlornitrofen, exhibited oestrogenic and antiandrogenic activities in *in vitro* reporter gene assay system using CHO cells. The antiandrogenic activity was higher than that of *o*, *p*′-DDT (Kojima *et al.*, 2003). Nitrofen also caused TR-mediated gene expression (Yamada-Okabe *et al.*, 2005).

Some carbamate pesticides such as aldicarb, Baygon (propoxur), bendiocarb, carbaryl, methomyl and oxamyl weakly activated oestrogen- or progesterone-responsive reporter genes in breast MCF-7 and endometrial (Ishikawa) cancer cells (Klotz *et al.*, 1997). Kojima *et al.* (2004) examined the oestrogenic activity of 22 carbamate pesticides and reported that methiocarb is agonistic against human ER (hER)α and ERβ. Further, they found that the activity against ERβ was higher than that against ERα. In contrast, Andersen *et al.* (2002)

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reported that methiocarb exhibits oestrogenic activity in cell proliferation assays and transactivation assays using MCF-7 human breast cancer cells. Methiocarb acted as an AR antagonist, as well as an ER agonist. It inhibited the androgenic response to R1881 at the concentration of 20 μM in transactivation assays using CHO cells (Andersen *et al.*, 2002). Kojima *et al.* (2004) reported that methiocarb and thiobencarb inhibited the androgenic activity of DHT in CHO cells transfected with hAR at 2.8×10^{-6} M and 9.4×10^{-6} M (IC20), respectively. Activation or inhibition of enzyme activities involved in steroid hormone synthesis might also alter endogenous hormone levels. Pirimicarb and propamocarb enhanced the activity of CYP 19 (aromatase), which catalyzes the conversion of C19 steroid to oestrogens (Andersen *et al.*, 2002).

2.1.11.2 Action of Pesticides In Vivo in Animals

Quinalphos, an organophosphate insecticide, exhibited oestrogen-like action in vaginal cornification and increased the uterine wet weight (uterotrophic assay; Clark *et al.*, 1980) in immature and ovariectomized mature rats at 1 mg kg day^{-1}, subcutaneous (SC), for three days, but the activity was weaker than that of aldrin (Chatterjee *et al.*, 1992). Regarding antiandrogenic activity, fenthion was positive *in vivo* in the Hershberger assay (Hershberger *et al.*, 1953) using castrated male rats (Kitamura *et al.*, 2003b). When fenthion (25–50 mg kg day^{-1}) was subcutaneously dosed together with testosterone propionate (0.5 mg kg day^{-1}) for seven days, the effect of testosterone on the weights of the prostate and seminal vesicles was significantly suppressed. Antiandrogenic activity of fenitrothion has also been reported in the Hershberger assay using castrated male rats (Tamura *et al.*, 2001). Chlorpyrifos-methyl showed antiandrogenic activity after metabolic activation in the Hershberger test using rats (Kang *et al.*, 2004). To investigate whether fenitrothion has an impact on androgen-dependent sexual differentiation, the effects *in utero* exposure were further examined in rats (Turner *et al.*, 2002). Pregnant Sprague–Dawley (SD) rats were administered fenitrothion by gavage from gestation day 12 to 21. In male offspring maternally exposed at 25 mg kg day^{-1}, a reduction in anogenital distance (AGD) was evident on postnatal day 1, although the effect was transient, and no abnormalities in male organ development were noted at postnatal day 100. Administration of fenitrothion at high dosages (55 mg kg^{-1}, three days) has been reported to reduce testicular cytochrome P450 (Clos *et al.*, 1994). A decrease in serum testosterone levels also was detected at this dosage.

The ability of parathion to interfere with the metabolism of androgens, as well as with androgen uptake, in male accessory organs in rats was described back in the 1960s (Kupfer, 1967; Schein and Thomas, 1976; Thomas and Schein, 1974). The oral administration of parathion (1.3–5.2 mg kg day^{-1}) caused significant alterations in the metabolism of testosterone in mice. An early study suggested that this compound competitively inhibits androgen binding to its receptor (Schein *et al.*, 1979). Parathion was reported to interfere with mouse spermatogenesis, and this may be related to its prenatal toxicity in mammals. An organ culture study with mouse testis revealed that parathion directly suppresses normal testicular differentiation (Rojas *et al.*, 1998). An increase of apoptosis in spermatogonia by this pesticide was also reported (Bustos-Obregon *et al.*, 2001). Quinalphos is also known to have adverse effects on the testis and male accessory glands in rats (Ray *et al.*, 1991; Ray *et al.*, 1992). When Wistar rats were given this compound at 0.25 mg kg day^{-1} (interperitoneal; ip) for 26 days, massive degeneration of germ cells in the testis, with a reduction in the sperm count, was evident (Ray *et al.*, 1992).

Malathion can disrupt thyroidal activity. In young adult rats, administration of malathion at 60 μg rat day^{-1} for 21 days resulted in decreased serum T3 and T4 and increased TSH levels (Akhtar *et al.*, 1996). A significant delay of growth was observed in tadpoles in water containing malathion at 1 mg l^{-1}, suggesting a decrease in thyroid function (Fordham *et al.*, 2001). In the freshwater catfish, *Clarias batrachus*, malathion decreased serum T3 level, but accelerated T4 synthesis in the pharyngeal thyroid. Extrathyroidal conversion of T4 to T3 was also inhibited (Sinha *et al.*, 1991b; 1992). Rawlings *et al.* (1998) reported that chlorpyrfos and dimethoate decrease serum T4 levels in ewes treated with these chemicals at 12.5 and 0.2 mg kg^{-1}, respectively, three times a week, for 43 days. The teleost fish, *Channa punctatus* (Bloch), exposed to cythion at 2–4 ppm in water developed hypertrophy and hyperplasia in the follicular epithelium and a reduction of colloid content in the thyroid (Ram *et al.*, 1989). Treatment of bullfrog tadpoles (*Rana catesbeiana*) with malathion significantly delayed developmental progression (Fordham *et al.*, 2001). Malathion inhibited T3 binding to TTR, but did not bind to the ligand-binding domain of TRβ in a study using recombinant Japanese quail TTR and TR (Ishihara *et al.*, 2003). This affinity for TTR may account for the effect of malathion on serum TH levels *in vivo*.

When a high dose of carbaryl was orally administered to male rats (100 mg kg^{-1} body weight, five days a week), marked histopathological changes in the testes were seen, with degeneration of spermatogenic cells (Pant *et al.*, 1995). These effects were associated with declines in epididymal sperm count and % sperm motility and increased abnormal sperm morphology. Adult male Wistar rats fed with laboratory chow containing 1.0, 6.3 or 203 ppm benomyl exhibited decreased ejaculate sperm counts, decreased testicular weight and a lowered male fertility index (Barnes *et al.*, 1983). In catfish, carbaryl exposure produced a reduction in the number of oocytes,

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deformity in oocytes at different stages, and a reduction in the gonadosomatic index (Kulshrestha and Arora, 1984). When female Swiss mice were orally administered with this chemical at 1 mg kg^{-1} body weight per day, there was a significant decrease in the number of oestrous cycles and in the duration of the phases of each cycle (Baligar and Kaliwal, 2002). Rats dosed with carbofuran (1.5 mg kg^{-1}, sc) showed transient endocrine disruption, that is, the levels of progesterone, cortisol and E2 were significantly increased, while the levels of testosterone were decreased (Goad *et al.*, 2004). In fish, treatment with 0.5–1 mg l^{-1} of carbofuran inhibits oocyte maturational processes in females (Chatterjee *et al.*, 1997) and causes deleterious testicular changes, included necrosis of Leydig cells (Ram *et al.*, 2001). In contrast, an impairment of the duration of oestrous cycles in rats treated with mancozeb was observed (Mahadevaswami *et al.*, 2000).

Carbamate insecticides disrupt serum TH levels in rodents and fishes. In a study with SD rats fed with aldicarb at 10 ppb, metribuzin at 10 ppm or methomyl at 1 ppm in drinking water (6–16 weeks), the serum thyroxin levels increased significantly (Porter *et al.*, 1999). In catfish, carbaryl exposure (12 mg l^{-1}) for 96 hours suppressed serum T4, but elevated serum T3 levels, while 16 days' exposure (5 mg l^{-1}) decreased both T3 and T4 (Sinha *et al.*, 1991a). Rawlings *et al.* (1998) reported that carbofuran caused a significant increase in serum thyroxin level, and triallate increased the basal LH level compared with that of control ewes. In the teleost fish, *Channa punctatus*, treatment with carbaryl at 1.7 ppm for 30 days also caused a decline in the level of T4 and an elevation in T3 (Ghosh *et al.*, 1989). In the same species, long-term exposure (six months) to carbofuran at 4.5 ppm led to histological abnormalities of the thyroid gland, including hypertrophy, hyperplasia and degeneration of follicular epithelial cells, and a reduction in colloid content, along with retardation of thyroid function. Another study also found a greater susceptibility of the younger group to chronic toxicity of carbofuran as compared with adults in this species (Ram, 1988). Mancozeb can disrupt thyroid function, and its degradation product, ethylene thiourea, is causally related to thyroid cancer in animals (Steenland *et al.*, 1997; Chhabra *et al.*, 1992).

2.1.12 Pharmaceuticals

Some pharmaceuticals have high endocrine-disrupting activity in wildlife. Their residues in aquatic environments may present a serious problem, because these medicines have high biological activities. For example, acetaminophen (paracetamol), clofibrate, ibuprofen, cimetidine, 17α-ethinylestradiol, antibiotics and so on are detected in aquatic environments (Boxall, 2004). 17α-ethinylestradiol at aquatic environmental concentrations causes reproductive failure in zebrafish (Nash *et al.*, 2004). However, Folmar *et al.* (2000) reported that 17α-ethinylestradiol did not enhance vitellogenin (a biological marker for oestrogens in fish) levels in the blood of male sheepshead minnow at environmental concentrations of this pharmaceutical. DES, a potential oestrogen, has been used medically as a substitute for endogenous oestrogen and as hormonal therapy for prostate or breast cancer, and also to prevent threatened abortions (Metzler, 1984; Smith and Brookline, 1948). However, it may induce vaginal adenocarcinoma (Herbst *et al.*, 1971). Its oestrogenic activity is about the same as that of E2 in *in vitro* oestrogen screening tests.

Acetaminophen exhibited weak antioestrogenic activity in Ishikawa cells (Dowdy *et al.*, 2003). Trenbolone acetate, which is used to promote growth in cattle, exhibited androgenic activity *in vivo* in the Hershberger assay and *in vitro* androgen-dependent gene expression assays. The activity is exhibited by the active metabolite, 17β-trenbolone, and is as potent as that of testosterone propionate (Hotchkiss *et al.*, 2007).

Natural hormone steroids and synthetic steroids, such as 17α-ethinylestradiol and mestranol, are also a group of endocrine disruptors, which are excreted by humans and animals. Although these steroids are found in various environments, the risk arising from them is not adequately understood (Ying *et al.*, 2002). Most natural steroids may be short-lived and do not accumulate in tissues. In contrast, synthetic steroids may be more stable and may remain in the body longer than natural steroids (Tapiero *et al.*, 2002).

2.1.13 Natural Products

Phytoestrogens are plant chemicals, which have various actions, including fungicidal, plant-hormonal and plant-protective activities. Some phytoestrogens structurally resemble endogenous oestrogens. These oestrogens have higher potential to disrupt oestrogen signalling than industrial xenoestrogens, and exposure is generally repeated or prolonged. However, dietary phytoestrogens show some beneficial effects in patients with oestrogen-dependent diseases. Asian populations consume a diet that is rich in phytoestrogens, and suffer lower rates of hormone-dependent cancers compared with Western populations. Thus, are phytoestrogens friends or foes? This has been discussed for a long time (Barrett, 1996). Unlike industrial xenoestrogens, phytoestrogens are readily metabolized and rapidly excreted from the body. Phytoestrogens exert pleitotropic effects involving kinase inhibition, cell cycle regulation and antioxidative properties. These actions are are likely to contribute to the beneficial effects of phytoestrogens.

Phytoestrogens are usually termed isoflavones. They tend to bind preferentially to ERβ, and act as agonists and antagonists (Tikkanen and Adlercreutz, 2000; Ikeda *et al.*, 2002). Aglycones of soy isoflavones such as genistein, dihydrogenistein and equol bind to ER receptors.

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Their binding ability to ERβ, which is comparable to that of E2, is higher than that to ERα (Morito *et al.*, 2001). Breinholt and Larsen (1998) investigated the oestrogenic activity of 23 flavonoids in assays based on the transcriptional activity of ER in yeast and proliferation of MCF7 cells, and showed that eight flavonoids, including naringenin, apigenin and kaempferol, were active. Daidzin and genistin prevent bone loss in ovariectomized rats (Ishida *et al.*, 1998). In contrast, oestrogens regulate lipid metabolism. Phytoestrogens also reduce obesity and decrease adiposity (Bhathena and Velasquez, 2002; Cederroth *et al.*, 2007).

It was demonstrated that resveratrol, which is a derivative of stilbene found in grapes and wine, is an agonist for ER (Gehm *et al.*, 1997). A comparison of the oestrogenic activities of some stilbene derivatives indicated that the 4-hydroxyl group of the A-ring plays a most important role and the vinyl linkage is necessary for high activity. Hydrophobicity of the B-ring also plays an important role, because the oestrogenic activity of hydroxystilbene is higher than that of hydroxystyrene. A *p*-hydroxy group in the A-phenyl ring, a vinyl linkage, a B-phenyl ring and a hydrophobic linkage are necessary for maximal activity of stilbene derivatives (Sanoh *et al.*, 2003).

Koda *et al.* (2007) demonstrated that all-*trans*-retinoic acid has antioestrogenic activity in an *in vivo* uterotrophic assay. They suggested that up-regulation of retinoic acid receptor and RXR mRNA expression levels was responsible for the activity.

2.2 Developmental Toxicity of Endocrine-Disrupting Chemicals

Exposure to EDCs during the development period, pre- or postnatal, can cause irreversible effects in humans and animals. In this period, nuclear receptors have different roles from those in adults, and the adverse effects of EDCs appear after development.

DES and BPA disrupt normal development of the reproductive tract (vom Saal *et al.*, 1998; Laws *et al.*, 2000; Gupta, 2000) and prolonged administration is a risk factor for carcinogenesis. Exposure to $10\,\mu g\,kg^{-1}$ body weight BPA during gestation days 14–18 altered maternal behaviours in mice (Palanza *et al.*, 2002). In another study, the UV filters 4-MBC and 3-benzylidene camphor were administered to the parent generation before mating, during pregnancy and lactation, and to the offspring until adulthood. Neonates exhibited enhanced prostate growth after 4-MBC and altered uterine gene expression after both chemicals. 4-MBC and 3-benzylidene camphor delayed male puberty and affected reproductive organ weights of adult offspring (Schlumpf *et al.*, 2008). Mice prenatally exposed to methoxychlor showed changes in reflex development and periadolescent mice showed decreased reaction times in exploring both a novel environment and a novel object (Palanza *et al.*, 1999).

Administration of antiandrogenic chemicals may alter sexual differentiation. *In utero* treatment with AR antagonist, flutamide, vinclozolin and p,p'-DDE, results in ventral prostate agenesis and testicular nondescent, and male offspring display increased incidences of epididymal and testicular lesions (Gray, 1999b).

Concern about thyroid-disrupting chemicals has increased because of the critical role that TH plays in brain development. Thyroid disrupting chemicals alter the structure or function of the thyroid gland, alter regulatory enzymes associated with TH homeostasis, or change circulating or tissue concentrations of THs. More important effects of EDCs are disruption of TH in the prenatal and postnatal stages, resulting in brain developmental dysfunction, especially in the nervous system (Crofton, 2008). Organochlorines such as dioxins, PCBs, and PBDEs could all lead to TH disruption, and affect serum total T4 concentration (Crofton *et al.*, 2005).

2.3 Disruption of Thyroid Hormone Homeostasis in Wildlife

Global climate change is receiving particular attention because it has the potential to cause habitat destruction, desertification and biodiversity loss, and also may have a serious impact on human health and society. The problem of man-made chemicals released into the environment has also been of great concern in recent years.

One example of the importance of TH to vertebrate growth and development is amphibian metamorphosis (Kollros, 1961; Kaltenbach, 1966; Dodd and Dodd, 1976). Frog THs are structurally identical to mammalian THs (Dodd and Dodd, 1976), and frog TRs are highly homologous to other vertebrate counterparts. Type II deiodinase (DI-2) converts T4 into the much more biologically active T3 through the removal of an outer ring iodine molecule, whereas type III deiodinase (DI-3) inactivates T4 and T3 by removal of an inner ring iodine molecule. The biological functions of T3 are mediated by interaction with TR, which belongs to the nuclear hormone receptor family (Zhang and Lazar, 2000). TR forms a heterodimer with a RXR, thereby acquiring binding ability to thyroid hormone response elements (TREs) located in the promoter regions of target genes, followed by binding of T3 to enhance gene transcription (Shi, 2000). Amphibian metamorphosis is triggered and controlled by THs, and thus it seems highly probable that TH synthesis, TH transport and TH metabolism are all potential target areas for the disrupting effects of environmental chemicals.

Tadpole tail resorption during anuran metamorphosis is a dramatic change resulting from apoptosis, or programmed cell death, and is controlled by TH (Shi,

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2000). Addition of THs to the water or medium used in rearing premetamorphic stage tadpoles results in an enhanced metamorphic process, and such TH-induced enhancement has proven to be a useful tool for investigating the effects of environmental chemicals. The expression of key genes important for the TH signalling pathway can be easily monitored in real-time throughout development in transgenic tadpoles and frogs carrying enhanced green fluorescent protein (EGFP) transgenes relevant to the thyroid axis and to metamorphosis. TH function is regulated by a complex interplay between the hypothalamus, pituitary and thyroid glands (HPT) (Lechan and Fekete, 2006). For mammals the hypothalamic thyrotropin-releasing hormone (TRH) stimulates the biosynthesis and release of thyroid stimulating hormone (TSH) in the pituitary gland, followed by release of T4 and T3 from the thyroid gland into the circulation. For developing tadpoles the hypothalamus controls TSH release via corticotrophin-releasing factor (CRF), rather than TRH (Shirey *et al.*, 2006).

Several investigations have shown that PCBs adversely affect amphibian metamorphosis. Jelaso *et al.* (2005) reported that long-term dietary exposure to Aroclor 1254 (A1254) delayed progression through metamorphosis in developing *Xenopus (X.) laevis*. Shirey *et al.* (2006) found that exposure of *X. laevis* tadpoles to an environmentally relevant concentration (50 ppb) of A1254 resulted in delayed metamorphosis and altered gene expression of TTR and DI-2 and DI-3. This change in DI-2 expression is consistent with the findings of Morse *et al.* (1996), who reported that conversion of T4 to T3 is significantly decreased in the developing brains of rats after exposure to A1254 *in utero*. Long-term exposure (up to completion of metamorphosis) of *X. laevis* tadpoles to the technical PCB mixture Clophen A50 reduced plasma T4 levels and significantly delayed metamorphosis (Gutleb *et al.*, 2000; 2007).

Goto *et al.* (2006) reported that T3-treated *R. ruosa* tadpole tails displayed marked apoptotic features, including DNA fragmentation and ladder-like DNA profiles, as opposed to little or no fragmentation or ladder formation for T3 + BPA-treated tails. They also found that BPA suppressed spontaneous *Silurana tropicalis* metamorphosis controlled by endogenous circulating TH. These results indicate that BPA acts as a TH antagonist. BPA was also found to block spontaneous and T3-induced metamorphosis in *X. laevis* tadpoles (Iwamuro *et al.*, 2003). Iwamuro *et al.* (2006) reported that BPA acts as a T3 antagonist by suppressing TRα and TRβ gene expression in *X. laevis* tail tissue. Goto *et al.* (2006) reported that in transgenic *X. laevis* tadpoles carrying plasmid DNA containing TRE and 5′-upstream promoter regions of the TRβA1 gene linked to an EGFP gene, T3 induced strong EGFP expression in the hind limbs. This expression was suppressed by BPA, indicating that BPA may act as an antagonist to prevent the binding of T3 to TR, resulting in suppression of TR-mediated gene expression.

TBBPA also suppresses T3-enhanced tadpole tail absorption, and suppresses spontaneous and T3-induced amphibian metamorphosis, including tadpole tail apoptosis. It also inhibited strong T3-induced EGFP expression in transgenic tadpole hind limbs. These findings indicate that TBBPA acts as a T3 antagonist (Kitamura *et al.*, 2005b; Goto *et al.* 2006). Exposure to TBBPA, however, results in accelerated metamorphosis in Pacific tree frog *Pseudacris regilla* tadpoles, suggesting that this chemical substance acts as a TH agonist (Veldhoen *et al.*, 2006). The effect of TBBPA on TH action may show a substantial species-dependence.

3 TOXICOLOGICAL EFFECTS ON THE REPRODUCTIVE SYSTEM

EDCs originally called environmental oestrogens, bind to steroid hormone receptors and act as agonists (‘mimicking oestrogens’) or antagonists. They are naturally occurring chemicals or environmental contaminants, and could affect the reproductive system physiologically regulated by sex steroid hormones. A primary concern therefore is whether these chemicals alter the reproductive system and reduce the number of offspring without displaying significant acute toxicity (Hollander, 1997; Hotckiss *et al.*, 2008; Markey *et al.*, 2002; Waring and Harris, 2005).

3.1 Invertebrates

Well-documented imposex in molluscs caused by tributyltin exemplifies the reproductive effects of environmental chemicals in invertebrates (Birchenough *et al.*, 2002). Ttributyltin was widely used in antifouling paint on ships, and although its use was restricted during the 1990s, high levels of contamination are still found in some areas. Ttributyltin causes the imposition of male sex organs (penis and vas deferens) and leads to reproductive failure. Early studies demonstrated that imposex in these organisms was related to an increased level of testosterone, which may not have comparable functions to those in vertebrates (LeBlanc and McLachlan, 2000). The investigation of the effects of EDCs on invertebrates, which forms a part of ecotoxicology, is a huge challenge, considering that they comprise 95% of all terrestrial and aquatic animal species, with huge diversity (Hutchinson, 2002).

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3.2 Fish, Amphibians, Reptiles and Birds

In vertebrates, oestrogens play a fundamental role in sex-differentiation, development of sex characteristics and reproduction. Environmental oestrogens could disrupt the reproductive function, although the dose ranges may be very diverse and mechanisms differ. In fishes, exposure to sewage effluent induced abnormal synthesis of vitellogenin (the precursor of egg yolk protein) in male liver. It is associated also with an increase in 'intersex' population, which reduces fertility (Jobling *et al.*, 2002; Jobling and Tyler, 2003). High levels of contaminations of nonylphenol, octylphenol and BPA, as well as synthetic pharmaceutical oestrogens, have been found in rivers (Jobling *et al.*, 2006). In frogs, a herbicide, atrazine, disrupts male gonadal development at the concentration of 0.1 ppb, well below the level of environmental contamination. This malformation is associated with the depletion of androgens and production of oestrogens, probably due to the activation of aromatase (Hayes *et al.*, 2006; Tavera-Mendoza *et al.*, 2002). The effect was well documented in Florida alligators (Gallagher *et al.*, 2001; Lind *et al.*, 2004; Semenza *et al.*, 1997). At Lake Apopka, Florida, which was polluted with ficofol, DDT and its metabolites, male alligators were feminized (smaller penises and testis) and the females had ovarian abnormalities, resulting in low hatching rates of eggs. In birds, an early study showed that injection of DDT into gull eggs led to feminization of males, with development of ovarian tissue and oviducts (Fry and Toone, 1981). In Japanese quail, DDT exposure altered the morphology of the oviduct and the shell gland, impairing egg laying, although male reproductive glands were not significantly affected (Halldin, 2005).

3.3 Mammals (Laboratory Rodents)

In mammals, the reproductive system is orchestrated by oestrogen, androgens and pituitary hormones, comprising the hypothalamic–pituitary–gonad axis. Moreover, oestrogens and androgens play a key role in foetal and neonatal development of the reproduction system. Although endocrine disruptors could affect the function of the adult reproductive system, this requires high exposure and the effects are generally reversible. One of the rare cases of EDCs affecting reproductive functions in adults has been recognized in sheep since the 1940s. Feeding clover containing phytoestrogens, quol and coumestrol causes infertility in these animals (Rossiter, 1970). Nevertheless, toxicological effects by EDCs at environmentally relevant levels could occur in the developmental stage of the reproductive system (Maffini *et al.*, 2006; Markey *et al.*, 2003). There was much argument about the existence of so-called 'low-dose effects' before the recognition of this problem (vom Saal and Hughes, 2005). In any event, we should keep in mind that, as with other toxicological studies, our present knowledge about the effects of EDCs in mammals largely comes out of investigations with laboratory rodents (**Table 1**).

3.3.1 Male Reproductive Glands

Since the development of male reproductive glands is regulated by oestrogen as well as androgens, both oestrogenic and androgenic EDCs may cause adverse effects. In addition, AhR agonists such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and PCBs also cause problems, probably through disruption of the androgenic and oestrogenic pathways.

Foetal exposure to antiandrogens may cause malformations in androgen-dependent tissues. Male SD rats treated

Table 1 Effects on reproductive glands with perinatally administered antiandrogenic and estrogenic chemicals

Representative chemicals	Male	Female	
Flutamide DDE, vinclozolin prochloraz, linuron	Cryptorchidism, hypospadias, prostate agenesis, decrease in weight of testis, SV and epididymides	—	Gray *et al.* (1999), McIntyre *et al.* (2001)
DES (high dose)	Cryptorchidism, hypospadias, decreased sperm output and decrease in prostate size	Cystic ovaries, virginal adenocarcinoma, uterus hyperplasia	Cheek and McLachlan (1998), Newbold (2004)
DES (low dose)	Increase in prostate weight, decrease in weight of epididymides	Smaller vagina, smaller endometrial lamina propria	Gupta (2000), Markey *et al.* (2005), Walker *et al.* (1990)
BPA (low doses or environmentally relevant doses)	Increase in prostate weight, decrease in epididymides, decreased ERb in POA	Smaller vagina, smaller endometrial lamina propria, increased ducts and end buds in mammary gland, altered pattern of oestrous cycle	Markey *et al.* (2001), Markey *et al.* (2003), Ramos *et al.* (2003), Rubin *et al.* (2001)

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with an antiandrogen, flutamide, during gestation days 12 to 21, showed prostate agenesis and decrease in weight of the seminal vesicles, testes and epididymides, along with a reduction of AGD and retained nipples (McIntyre *et al.*, 2001). Perinatal administration of vinclozolin, a fungicide whose metabolites are androgen antagonists, reduced prostate, seminal vesicle and epididymal weights (Gray *et al.,*1999; Kelce *et al.*, 1994). Another antiandrogen, DDE, was reported to exhibit similar effects (Kelce *et al.*, 1995; 1997; You *et al.*, 1998). In the category of antiandrogenic chemicals, there are inhibitors of testosterone synthesis, such as prochloraz (fungicide) and linuron (herbicide), which have similar prenatal effects (Blystone *et al.*, 2007; Hotchkiss *et al.*, 2004; Lambright *et al.*, 2000; McIntyre *et al.*, 2000; Vinggaard *et al.*, 2005; Wilson *et al.*, 2004).

The effects of high doses of synthetic oestrogens, DES and ethinyl oestradiol, on male reproduction have been well documented (Cheek and McLachlan, 1998; Newbold, 2004). When pregnant mice were treated with DES, the incidence of cryptorchidism and hypospadias increased and sperm concentration decreased in the male offspring (McLachlan *et al.*, 1982; Yoshida *et al.*, 1999; 2000). Ethinyl oestradiol also causes disruptions, including poorly formed testis, cryptorchidism and reduced number of Sertoli cells. Similar reproductive dysfunctions have been reported in humans, that is, sons of mothers treated with DES during pregnancy, although the data are not consistent (Atanassova *et al.*, 2005; Joffe, 2003; Steinberger *et al.*, 1977; Steinberger and Chowdhury, 1977; Swan, 2000). Neonatal exposure to high doses of oestradiol permanently altered prostatic growth. Prins has shown that rats treated with oestrogen at postnatal day (PND) 3 showed a decrease in prostate size, along with reduced prostatic responsiveness to testosterone in adulthood (Prins, 1992; Prins *et al.*, 1993). Changes in expression of oestrogen and ARs were reported be involved (Prins *et al.*, 1998; Prins and Birch, 1997).

The development of the prostate gland seems to be very sensitive to oestrogenic chemicals, including BPA. vam Saal and colleagues have demonstrated that foetal treatment with BPA (2–20 $\mu g\,kg^{-1}$ body weight) affected the prostate development and adult prostate weight was increased by 30% (Welshons *et al.*, 1999). They also showed that foetal low-dose exposure to other xenoestrogens, methoxychlor and DES, had the same effect (Palanza *et al.*, 2002; vom Saal *et al.*, 1997). AR mRNA in mesenchyme cells isolated from foetal mouse prostate is up-regulated by oestradiol as well as BPA, which may account for the facilitation of androgen responses (Richter *et al.*, 2007). Neonatal low-dose exposure to oestrogen increases prostate weight, though the effect may not be permanent through adulthood (Putz *et al.*, 2001a; 2001b). Increase in size of the preputial gland and decrease in epididymis size by BPA have been reported (Cagen *et al.*, 1999a; 1999b).

Prenatal exposure to TCDD, a strong AhR agonist, also has an impact on reproductive parameters. Pregnant mice were treated with TCDD on gestation day 14 and the male offspring exhibited decreases in ventral prostate, coagulating gland and thymus weights (Ko *et al.*, 2002; Lin *et al.*, 2002; 2004b; Mably *et al.,* 1992a, 1992b; Theobald and Peterson, 1997). Decreases in pituitary gland weight and epididymal sperm numbers were also found. Disruption in oestrogenic signals may be involved, since TCDD could interfere with the oestrogen pathways (Biegel and Safe, 1990; Ohtake *et al.*, 2003).

3.3.2 Female Reproductive Glands

Since the development of female reproductive glands is regulated primarily by oestrogen, EDCs with oestrogenic activity could cause adverse effects. AhR agonists are also able to alter the oestrogen signalling. Effects of *in utero* exposure to high doses of DES on female reproductive glands have been well described, including structural malformations in oviduct, uterus and vagina. High doses of oestrogenic EDCs, such as *p-tert*-octylphenol, induce abnormal differentiation in developing rat uteri via abnormal ER expression and subsequent alteration of cell proliferating activity (Yoshida *et al.*, 2002).

Prenatal exposure to BPA induced anatomical changes in mouse ovaries, an increase in the ovarian tissue occupied by antral follicles, and a decrease in the corpora lutea. Environmentally relevant doses of BPA cause changes in the reproductive tract including decrease in weight of the vagina and endometrial lamina propria and increased proliferative activity of epithelium in the endometrial glands (Markey *et al.*, 2003; Newbold *et al.*, 2007; Schonfelder *et al.*, 2002; Suzuki *et al.*, 2002). Prenatal administration of TCDD also alters female reproductive development. *In utero* exposure to TCDD induces cleft phallus, vaginal thread formation, and reduced ovarian weight (Gray *et al.*, 1997; Gray and Ostby, 1995; Wolf *et al.*, 1999).

3.3.3 Hypothalamic–Pituitary–Gonad Axis

Toxic effects of EDCs on the reproductive system occur indirectly through the hypothalamus–pituitary axis that regulates gonadal function via pituitary hormones, such as luteinizing hormone (LH) and follicle stimulating hormone (FSH). It is well known that high dose administration of oestrogen or DES suppresses GnRH release from the hypothalamus, reducing LH/FSH secretion from the pituitary gland, which results in reduction of testosterone production in males (feminization) and halting of the oestrus cycle in females. Prenatal exposure to a low level of BPA (0.1 $\mu g\,kg^{-1}$ body weight per day) altered patterns of oestrous cyclicity and decreased levels of plasma LH in adulthood in SD rats (Rubin *et al.*, 2001). In male Long–Evans rats,

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perinatal exposure to BPA ($2.4\,\mu g\,kg^{-1}$ body weight per day) resulted in a decrease in plasma LH level (Akingbemi *et al.*, 2004). In male Wistar rats, lower levels of administration of BPA (25 and $250\,ng\,kg^{-1}$ body weight per day) caused changes in expression of ERα in the preoptic area of the hypothalamus (Ramos *et al.*, 2003). TCDD administered perinatally also may alter the hypothalamic–pituitary–gonad axis (Theobald and Peterson, 1997).

4 EFFECTS OF ENDOCRINE DISRUPTERS ON THE CENTRAL NERVOUS SYSTEM

Another important toxicity that should be considered is neurotoxicity. Most toxic actions against neurons are irreversible and can lead to serious disorders, because neurons are not readily replaceable and neuronal circuits are vulnerable. Environmental chemicals penetrate especially easily into the brain during the foetal and neonatal periods, because the blood–brain barrier is not completely formed.

The gonads are key organs in sexual differentiation, but sexual differentiation of the brain is also important, because the hypothalamic–pituitary axis controls spermatogenesis or oogenesis via gonadotropin secretion. There are differences in the brain, as well as other organs, between males and females, and the difference in the hypothalamus is especially striking. Several lines of evidence suggest that the number of apoptotic cells in these nuclei is sex-dependent in postnatal developing rats. Sex differences in the number of apoptotic cells have been found in the central division of the medial preoptic nucleus (MPNc) and the anteroventral periventricular nucleus (AVPV) in developing rats. The sexually dimorphic nucleus of the preoptic area (SDN/POA), which involves the MPNc, is the pivotal region in sexual differentiation. In rats, the SDN/POA in male hypothalamus is two to five times larger than that in female hypothalamus (Gorski *et al.*, 1978; 1980), and this brain region regulates sexual behaviour and gonadotrophin levels (Arnold and Gorski, 1984; De Jonge *et al.*, 1987; Lephart *et al.*, 2001; Preslock and McCann, 1987).

SDN/POA is affected by perinatal exposure to hormones. When female rats were given testosterone in the perinatal period, the SDN/POA in adults became as large as in males. In contrast, when rats were castrated in the neonatal period, the SDN/POA in adults became as small as in females. Therefore, differentiation of the SDN/POA depends on systemic testosterone exposure at the early stage, as well as in the genital tract. However, this masculinization is due to oestradiol formed from testosterone by aromatase.

In contrast, the AVPV in female rats is larger than that in males. Sexual differentiation of the AVPV results from exposure of the male nervous system to oestrogen formed by aromatization of testicular testosterone secreted in the first few days after birth. Thus, it has been hypothesized that exposure to EDCs during this critical period could alter the sexually dimorphic expression of TH and the overall expression of ERα in the AVPV.

Chemicals which mimic or inhibit these hormone actions are therefore possible sexual differentiation-disrupting compounds. When female rats were treated pre- and postnatally with testosterone or DES they became anovulatory and their SDN/POA developed to be equivalent in size to that of normal males. Identical treatment of male rats resulted in deficient testicular development, but had no influence on SDN/POA volume (Döhler *et al.*, 1984). In female rats, treatment with DES either perinatally (from day 16 of gestation to PND 10) or postnatally (birth to day 10) was equally effective in increasing the volume of the SDN/POA compared to that in controls. Prenatal treatment (day 16 of gestation to birth) with DES also increased the volume of the SDN/POA, but this increase was significantly smaller than that achieved with the other treatments (Tarttelin and Gorski, 1988). In another study, neonatal rats received injections of either corn oil, DES, genistein or zearalenone on Days 1–10 of life and were castrated on Day 21. The animals were killed by decapitation on Day 49. Volumes of the SDN/POA of the exposed groups were compared. In females, 0.1 mg DES, 1000 mg genistein and 1000 mg zearalenone increased SDN volume; 100 mg genistein and 100 mg zearalenone had no effect. There was no difference in SDN size among the male groups (Faber and Hughes, 1991).

The volume of the SDN/POA in Long–Evans rats was significantly altered by a change in diet at 80 days of age, at which point one-half of the males or females fed phytoestrogen-rich diet (containing 600 mg of phytoestrogens per gram of diet) from birth were switched to phytoestrogen-free diet until 120 days of age (Lund *et al.*, 2001). Males initially fed a phytoestrogen-rich diet but subsequently changed to a phytoestrogen-free diet had significantly smaller SDN/POA volumes than males fed a phytoestrogen-rich diet for a long time. These data suggest that consumption of phytoestrogens via a soy-rich diet significantly influences SDN/POA volume during adulthood.

A subsequent report showed that males fed a phytoestrogen-free diet displayed decreased SDN/POA volumes compared to males on a phytoestrogen-rich diet (Lephart *et al.*, 2003). However, in females, the SDN/POA volumes were not affected by the diets. Females fed a phytoestrogen-rich diet displayed larger AVPV volumes than males on the same diet, or females on a phytoestrogen-free diet. Males fed the phytoestrogen-free diet had larger AVPV values than did phytoestrogen-rich diet-fed males. When the SDN/POA region was examined in lifelong phytoestrogen-free diet-fed males, more apoptotic cells were present than in

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males fed the phytoestrogen-rich diet, while in the AVPV region, the opposite results were obtained. In summary, dietary consumption of phytoestrogens can alter hormone-sensitive hypothalamic brain volumes in rodents during adulthood.

Nevertheless, many other reports have found little effect of EDCs on SDN/POA. To evaluate the impact of dietary exposure to EDCs during the sensitive period of brain sexual differentiation, maternal SD rats were fed three representative chemicals, methoxychlor (24, 240 and 1200 ppm), genistein (20, 200 and 1000 ppm) or diisononyl phthalate (DINP; 400, 4000 and 20 000 ppm), from gestational day 15 to PND 10. These three chemicals caused signs of maternal toxicity at high doses; females showed endocrine disrupting effects, such as irregular oestrous cyclicity and histopathological alterations in the reproductive tract and anterior pituitary. But, the SDN/POA volume remained unchanged in all cases (Masutomi *et al.*, 2003).

The reproductive function, as well as pathohistological change in reproductive organs, in postpuberty rats treated subcutaneously with 300 mg g^{-1} BPA or 2 mg g^{-1} oestradiol benzoate from PND 1 to 5 was examined. Oestradiol benzoate markedly reduced the volume of the SDN/POA in males. On the other hand, all male and female rats treated postnatally with BPA showed normal reproductive function and no histopathologic abnormalities of reproductive organs. BPA did not affect the volume of the SDN/POA (Nagao *et al.*, 1999).

BPA was administered to pregnant Crl:CD BR SD rats by gavage at 0, 3.2, 32 or 320 mg kg day^{-1} from gestation day 11 to PND 20. DES at 15 mg kg day^{-1} was also used, as a chemical with known oestrogenic effects. Treatment with BPA also had no detectable effect on the volume of the SDN/POA. However, DES at 15 mg kg day^{-1} increased the volume of the SDN/POA of female offspring, as well as influencing their normal oestrous cyclicity following puberty (Kwon *et al.*, 2000).

It is also reported that oestrogenic compounds affect the AVPV. Animals were given four subcutaneous injections of 50 mg E2, 250 mg genistein, or 250 mg BPA at 12 hour intervals over PND 1 and 2 and sacrificed on PND 19. E2 treatment masculinized TH immunoreactivity in the female AVPV, while exposure to genistein or BPA demasculinized TH immunoreactivity in the male AVPV. In addition, they identified a population of neurons coexpressing TH and ERα, located primarily in the medial region of the AVPV. Normally, females have nearly three times as many double-labelled cells as males, but these cell numbers were defeminized by E2, genistein or BPA treatment (Patisaul *et al.*, 2006).

Bax and Badare proapoptotic proteins, while Bcl-2 and Bcl-xL are antiapoptotic proteins. The ratio of these proteins is important for apoptosis. The expression levels of Bcl-2 family members and active caspase-3 were compared in postnatal male and female rats (Tsukahara *et al.*, 2006). In the AVPV-containing tissues of PND 1 rats, there were significant sex differences in the levels of Bcl-2 (female > male) and Bax (female < male) proteins, but not of Bcl-xL or Bad proteins. In the MPNc-containing tissues of PND 8 rats, there were significant sex differences in the protein levels of Bcl-2 (female < male), Bax (female > male) and Bad (female < male), but not Bcl-xL. Immunohistochemical analyses showed significant sex differences in the number of active caspase-3-immunoreactive cells in the AVPV on PND 1 (female < male) and in the MPNc on PND 8 (female > male) (Tsukahara *et al.*, 2006). Oestrogen up-regulated Bcl-2 expression and down-regulated Bax expression in the MPNc of postnatal rats (Tsukahara *et al.*, 2008).

The female AVPV contains a higher number of cells expressing tyrosine hydroxylase. DES (1 mg) injected daily during the first week after birth, like oestradiol or testosterone, significantly reduced later tyrosine hydroxylase expression in the AVPV of females by approximately 40–65% compared to oil-treated controls (Lansing and Lonstein, 2006).

Chemicals which modify AhR-mediated neuroendocrine functions are candidate neurotoxic endocrine disrupters. AhR is an important transcription factor which mediates transcription of various molecules, such as P450 1A, but AhR ligands also affect many other functions. Many environmental chemicals can change endocrine physiology and neuroendocrine functions, but AhR-mediated effects have been well studied. The AhR pathway also crosstalks with oestrogen (Hombach-Klonisch *et al.*, 2005; Ohtake *et al.*, 2003), androgen (Jana *et al.*, 1999; Morrow *et al.*, 2004) and thyroid (Yamada-Okabe *et al.*, 2004) hormone receptor pathways.

Sex-specific physiological changes are recognized with perinatal treatment of TCDD or other AhR ligands in animal models. These studies started about 15 years ago: Peterson *et al.* and Gray *et al.* demonstrated that perinatal exposure toTCDD of male offspring of rats resulted in less masculine sexual behaviours and an increased propensity to show female mating behaviours (Mably *et al.,* 1992; Bjerke *et al.*, 1994; Gray *et al.*, 1995). TCDD changes hormone action by binding to AhR. Therefore, the distribution of AhR and its coactivators, such as aryl hydrocarbon receptor nuclear translocator (ARNT), is important for TCDD action. AhR is expressed in the SDN/POA, in which ER is coexpressed, and which is sensitive to perinatal steroid hormone actions. AVPV also has abundant expression of AhR (Petersen *et al.*, 1989a; 1989b), and this raises the possibility that TCDD may affect sexual differentiation in these regions.

The hippocampus is a part of the limbic system and plays a part in long-term memory and spatial navigation. It has an essential role in the formation of new memories about experienced events. Some researchers prefer to consider the hippocampus as part

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of a larger medial temporal lobe memory system responsible for general declarative memory. The hippocampus mainly consists of the CA1, CA2 and CA3 regions, and the dentate gyrus is also conventionally included in it.

Synaptic plasticity is the ability of the connection between two neurons to change. There are several underlying mechanisms that cooperate to achieve synaptic plasticity, including changes in the quantity of neurotransmitter released into a synapse and changes in how effectively cells respond to those neurotransmitters. Since memories are postulated to be represented by enormously interconnected networks of synapses in the brain, synaptic plasticity is one of the important neurochemical foundations of learning and memory. Long-term potentiation (LTP) and long-term depression (LTD) are two basic processes of long-term plasticity. LTP is the long-lasting enhancement in communication between two neurons that results from stimulating them simultaneously. Since neurons communicate via chemical synapses, and because memories are believed to be stored within these synapses, LTP and its opposing process, LTD, are widely considered to be the major cellular mechanisms that underlie learning and memory. LTP and LTD are observed in glutamatergic excitatory neurons, mediated by Ca^{2+} via the *N*-methyl-D-aspartic acid (NMDA) receptor.

Oestradiol has a rapid influence on the synaptic plasticity of rat hippocampal glutamatergic neurons in slices. Many electrophysiological studies showed the effects of oestradiol on LTP and LTD in rats and mice (Foy *et al.*, 1999; Vouimba *et al.,* 2000). Moreover, the role of oestradiol in slowly modulating hippocampal plasticity has been researched, because the hippocampus is known to be a target for the actions of gonadal oestrogens reaching the brain via the circulation. For example, the density of dendritic spines in CA1 pyramidal neurons is modulated *in vivo* by oestrogen replacement in ovariectomized animals (Gould *et al.*, 1990; Woolley and McEwen, 1992) and by androgens in castrated animals (MacLusky *et al.*, 2005), leading to the increase of spines.

Since the synaptic plasticity is modulated by oestradiol, environmental endocrine disruptors might affect synaptic plasticity (Kawato, 2004). When BPA was injected to pregnant mice or Japanese monkeys, it was detected in maternal and foetal brains (Uchida *et al.*, 2002). BPA can reach the brain and disturb oestrogen functions in the brain. Though endocrine disrupters are efficiently metabolized in the liver, only low levels of drug-metabolizing enzymes were expressed in the brain (Kishimoto *et al.*, 2004; Miksys and Tyndale, 2002). Therefore, endocrine disrupters are expected to stay in the brain for a long time. These findings suggest that endocrine disrupters actually reach mammalian brains and may disrupt their functions. It has been demonstrated that 10–100 nM BPA, DES and 4-nonylphenol have effects on LTD and spinogenesis (Ogiue-Ikeda *et al.*, 2008).

5 EFFECTS OF ENDOCRINE DISRUPTERS ON THE IMMUNE SYSTEM

BPA can modulate both the endocrine and immune systems, resulting in alterations of homeostasis, reproduction, development and behaviour (Al-Hiyasat *et al.*, 2002; Tyl *et al.*, 2002; Sawai *et al.*, 2003; Funabashi *et al.*, 2004). Furthermore, halogenated derivatives of BPA and BPA-dimethacrylate, as well as BPA, are widely used as plastic products, dental sealants, and flame-retardants for building materials, paints, polystyrene resins and electronic circuit boards. Depolymerization of these products allows BPA and its derivatives to leach into foods, into infant formulas from plastic bottles, into saliva of patients treated with dental sealants, and into packed fresh foods (Brotons *et al.*, 1995; Biles *et al.*, 1999; Olea *et al.*, 1996; Vivacqua *et al.*, 2003). Alkylphenol ethoxylates are widely used as surfactants and detergents in domestic and industrial products and are commonly found in wastewater. These compounds are degraded to more resistant alkylphenols, such as 4-*n*-nonylphenol and 4-*n*-octylphenol, and have been detected in fish muscle tissue and foods. These compounds were also reported to exhibit oestrogenic activity (Andersen *et al.*, 1999).

Alizadeh *et al.* (2006) studied whether or not administration of BPA modulates T helper (Th) cell immune responses in mice challenged with ovalbumin (OVA), and found that BPA treatment of naïve animals resulted in lower titres of total IgE and higher levels of IgG2a, followed by higher levels of IFN-γ, interleukin (IL)-12 and intact IL-4 in response to OVA. In contrast, in OVA-pretreated groups, BPA did not affect production of total or OVA-specific IgE, or OVA-specific IgG2a, and resulted in lower production of IFN-γ. Furthermore, BPA resulted in impaired lymphocyte proliferation in response to concanavalin A in naïve animals, but not in tolerant animals. They concluded that BPA results in augmentation of Th1 immune responses, but has no significant effect on an established tolerance to OVA.

It has been reported that both BPA and 4-*n*-nonylphenol significantly enhance IL-4 production in keyhole limpet haemocyanin (KLH)-primed $CD4^+$ T cells in a concentration-dependent manner, and treatment with BPA or nonylphenol (NP) *in vivo* resulted in a significant increase of IL-4 production in $CD4^+$ T cells and of antigen-specific IgE levels in the sera of KLH-primed mice. The enhancing effect mapped to a region in the IL-4 promoter containing binding sites for nuclear factor (NF)-AT. Furthermore, it appeared that the transcription factor NF-AT was involved in

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the enhancing effects of BPA and 4-nonylphenol on IL-4 production (Lee *et al.*, 2003). Occupational health hazards that may be associated with BPA include allergic contact dermatitis (ACD) secondary to BPA exposure. Most ACD occurs in workers handling BPA-containing products, such as plastic products, and in workers exposed to epoxy adhesive tapes, foams and dental products. The cutaneous penetration of BPA through pig skin was examined *in vitro* using a Franz cell. After 2, 5 and 10 hours of exposure, total BPA content in skin was 3, 6.9 and 11.4% of the applied dose, respectively. BPA remained essentially on the skin surface and accumulated mainly in the dermis. As the pig skin model is a reliable predictor of percutaneous penetration in humans, these findings may be reassuring for workers in contact with BPA-containing products.

EDCs may cause immune disturbance, because disruption of endocrine function seriously affects the immune system. A number of biological mediators, including tumour necrosis factor (TNF)-α and nitric oxide (NO), participate in the defence mechanisms, and they are produced by macrophages in response to microbial toxins, such as bacterial endotoxin (Hong *et al.*, 2004; Araya *et al.*, 2002; Goering *et al.*, 2000). Hong *et al.* (2004) reported that carbaryl, alachlor, nonylphenol, octylphenol, tributyltin and triphenyltin inhibited lipopolysaccharide (LPS)-induced NO production *in vitro*, whereas 2,4-dichlorophenoxyacetic acid and BPA enhanced its production. On the other hand, zineb and alachlor enhanced LPS-induced TNF-α production by mouse peritoneal macrophages *ex vivo*, while alachlor inhibited LPS/IFN-γ, (interferon-γ)-induced NO production *ex vivo*. Therefore, they considered that some EDCs exert modulatory activity on endotoxin-induced macrophage activation, either positively or negatively, suggesting that these compounds may affect the development of infectious diseases.

Yoshino *et al.* (2004) studied the effect of prenatal exposure to BPA on the immune system in mice, and demonstrated that prenatal exposure to BPA may result in the up-regulation of immune responses, especially Th1 responses, in adulthood. It is widely accepted that females have superior immune responses to males, but the mechanisms through which sex hormones may enhance T cell responses are still poorly understood. Some researchers have suggested that oestrogen may enhance humoral immune responses and may be involved in the pathogenesis of autoimmune diseases, and also that it may be able to alter cytokine production and T-cell subset distribution (Ito *et al.*, 2001; Correale *et al.*, 1998). Several reports have indicated that oestrogen significantly increased IFN-γ and IL-2 mRNA in concanavalin A-activated thymocytes, splenic lymphocytes and enriched splenic T cells, though it had no marked effect on IL-4 mRNA. Therefore, it has been suggested that oestrogens may modulate the immune system by regulating cytokines (Fox *et al.*, 1991; Karpuzoglu-Sahin *et al.*, 2001a; Calemine *et al.*, 2003). DES is a synthetic oestrogen which was given to millions of women. Karpuzoglu-Sahin *et al.* (2001a) found that the DES-induced increase in IFN-γ secretion is due to hormonal effects on T cells, but not on antigen-presenting cells (APC), suggesting that: (i) oestrogens upregulate secretion of IFN-γ, a vital immunoregulatory cytokine and (ii) inappropriate exposure of developing foetuses to DES may permanently alter the cytokine programming of lymphocytes (Karpuzoglu-Sahin *et al.*, 2001b).

Maret *et al.* (2003) analysed the effect of oestrogens on CD4 T cell activation and differentiation after immunization with exogenous antigens, and they found that administration of low doses of E2 to castrated female mice produced a striking increase of antigen-specific CD4 T cell responses, as well as selective development of IFN-γ-producing cells. Quantitative assessment of the frequency of T cells bearing a public TCR β chain CDR3 motif demonstrated that the clonal size of primary antigen-specific CD4 T cells was dramatically increased in immune lymph nodes from E2-treated mice, and ERα, but not ERβ, was necessary for the enhanced E2-driven Th1 cell responsiveness. Furthermore, ERα expression in haematopoietic cells was essential, since E2 effects on Th1 responses were only observed in mice reconstituted with bone marrow cells from ERα+/+, but not ERα-deficient mice. Therefore, they considered that oestrogen administration promotes strong antigen-specific Th1 cell responses through a mechanism that requires functional expression of ERα in haematopoietic cells. In the mouse, OVX leads to significant reductions in cancerous bone volume, while oestrogen (E2) replacement not only prevents bone loss, but also can increase bone formation. Zhou *et al.* (2001b) reported that mesenchymal stem cells (MSCs) treated *in vitro* with E2 displayed a significant increase in ERα mRNA and protein expression, as well as alkaline phosphatase (ALP) activity and proliferation rate. In contrast, E2 treatment resulted in a decrease in ERβ mRNA and protein expression, as well as apoptosis, in both OVX and sham-operated mice. Therefore, they considered that oestrogen *in vitro* directly augments proliferation and differentiation, ERα expression and osteogenic gene expression, and inhibits apoptosis and ERβ expression in MSCs obtained from OVX and sham-operated mice. Coexpression of ERα, but not ERβ, and osteogenic differentiation markers might indicate that ERα functions as an activator and ERβ functions as a repressor in osteogenic differentiation in MSCs.

It has been reported that E2 attenuates responses to endoluminal injury of the rat carotid artery, at least in part, by decreasing inflammatory mediator expression and neutrophil infiltration into the injured vessel, with a major effect on the neutrophil-specific chemokine cytokine-induced neutrophil chemoattractant (CINC)-2 β (Xing *et al.*, 2007). Miller *et al.* (2004) investigated

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whether or not E2 inhibits expression of adhesion molecules, chemokines and proinflammatory cytokines in rat carotid arteries in the early hours after balloon injury, and found that expression of mRNAs for adhesion molecules (P-selectin, vascular cell adhesion molecule-1 and intercellular adhesion molecule-1), chemoattractants CINC-2beta and monocyte chemoattractant protein (MCP)-1, and proinflammatory cytokines (IL-1β and IL-6) was markedly increased in injured arteries of ovariectomized + vehicle rats at two hours and was reduced at 24 hours, thus attenuating the stimulus for leukocyte entry and negatively modulating the injury response. In contrast, E2 significantly attenuated expression of the proinflammatory mediators at two hours and E2 significantly inhibited neutrophil chemotactic activity of arterial homogenates. From these results, they concluded that E2 attenuates the early vascular injury response, at least in part, by negatively modulating proinflammatory mediator expression and the resultant chemotactic activity of injured vessels for neutrophils. Segura *et al.* (1999) investigated the effects of BPA on the viability of macrophages obtained from Wistar rats and reported that BPA did not significantly alter macrophage viability at high concentrations, but it did dose-dependently decrease the adherence index of rat peritoneal macrophages. A high concentration of BPA also caused significant inhibition of the adherence index. They therefore considered that BPA can alter macrophage adhesion. Because adhesion is the first step in the phagocytic process of macrophages and in antigen presentation, BPA could inhibit macrophage function and modulate immune and inflammatory responses in dental pulp and periapical tissues.

The effects of BPA on prolactin (PRL) secretion and the expression of a PRL-regulating factor from the posterior pituitary in the oestrogen-sensitiveFischer 344 (F344) rats were investigated, and it was reported that BPA increased PRL gene expression, PRL release and proliferation of anterior pituitary cells, albeit with 1000- to 5000-fold lower potency than oestradiol. In contrast, BPA had similar efficacy to oestradiol in inducing hyperprolactinemia in oestrogen-sensitive F344 rats, though SD rats did not respond to BPA. Posterior pituitary cells from E2- or BPA-treated F344 rats showed strongly increased PRL gene expression upon coculture with GH3 cells stably transfected with a reporter gene. Like E2, BPA induced ERE activation in transiently transfected anterior and posterior pituitary cells. These results indicated that: (i) BPA mimics E2 in inducing hyperprolactinemia in genetically predisposed rats; (ii) the *in vivo* action of E2 and BPA in F344 rats is mediated, at least in part, by increasing PRL-regulating factor activity in the posterior pituitary; (iii) BPA appears to regulate transcription through an ERE, suggesting that it binds to ERs in both the anterior and posterior pituitaries (Steinmetz *et al.*, 1997). This brings into question the safety of sealants and other resin-based dental materials, due to the reported presence in them of BPA and its dimethacrylate ester. Schafer *et al.* (1999) noted that both BPA and BPA-dimethacrylate at concentrations at or above 10^{-6} M significantly increased cell proliferation, and the effect was similar to that seen with 10^{9} M oestrogen. Furthermore, flow cytometric studies showed that these mitogenic effects occurred within 24 hours of exposure to oestrogen, BPA, or BPA-dimethacrylate. Therefore, BPA and BPA-dimethacrylate cause cell proliferation at micromolar concentrations that exceed the effective concentrations of oestrogen by 1 to 10 000-fold.

Oestrogen also plays a role in the immune system. For instance, previous studies have shown that oestrogen has stimulatory effects on humoral immune responses, but suppressive effects on cellular responses. More recent studies demonstrate that oestrogen increases the secretion of IFN-γ from splenic lymphocytes, which play a major role in regulating the function of all key immune cells. It has been reported that BPA decreases the substrate adherence capacity of antigen-presenting cells, including macrophages, *in vitro*. BPA also increases the nonspecific proliferation of spleen cells in response to the mitogen concanavalin A *in vitro.* Yoshino *et al.* (2004) investigated the effect of exposure to BPA on antigen-specific antibody production and proliferative responses of lymphoid cells in mice. They also examined whether exposure to BPA-modulated Th1 immune responses, which play a role in cellular responses, as well as Th2 immune responses, which are involved in humoral responses.

In vitro BPA exposure stimulates breast cancer cell lines to proliferate, increases progesterone receptor expression in a uterine cell line, and induces c-*fos* gene expression (Steinmetz *et al.*, 1998; Bergeron *et al.*, 1999; Schafer *et al.*, 1999). Several *in vivo* studies document reproductive effects of low-dose BPA exposure. Microgram quantities of BPA were shown to be oestrogenic in F344 rats, as judged from increased prolactin expression (Steinmetz *et al.*, 1997). Male offspring of BPA-treated pregnant mice showed increased prostate size and decreased epididymal weight (Gupta, 2000; Nagel *et al.*, 1997). A review of these studies led the Endocrine Disruptors Low-Dose Peer Review Panel to conclude with caution that exposure to very low doses of BPA can cause biological effects (Kaiser, 2000). Few reports have appeared concerning BPA and immune function. *In vitro*, BPA has been shown to inhibit lymphocyte mitogenesis (Sakazaki *et al.,* 2002), MCP-1 production (Inadera *et al.*, 2000) and macrophage adhesion (Segura *et al.*, 1999). The inhibition of INF-γ by BPA appears to be long-lasting and may be due to remodelling of the INF-γ promoter. Recruitment of the histone acetylase CREB-binding protein (CBP) to a promoter contributes to increased transcription (Valapour *et al.*, 2002). Both the nuclear factor-κB (NF-κB) and ERα pathways use CBP (Harnish *et al.*, 2000; Kushner *et al.*, 2000). NF-κB is a critical transcription factor for several cytokine

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genes, including INF-γ (Sica *et al.*, 1997). BPA bound to the ER may sequester proteins, such as CBP, required for NF-κB-induced cytokine gene expression, thereby reducing cytokine production. On the other hand, a recent report indicates that BPA activates the transcription factor cAMP-responsive element binding protein (CREB) (Quesada *et al.*, 2002). Expression of the INF-γ promoter is down-regulated by CREB-ATF1 binding to both proximal and distal elements and by their competition for binding with Jun/ATF2 proteins (Penix *et al.*, 1996; Zhang *et al.*, 1998). Activation of CREB due to BPA exposure may explain the diminished production of INF-γ (Sawai *et al.*, 2003).

6 INFLUENCE OF ENDOCRINE-DISRUPTING CHEMICALS ON HUMANS

Human exposure to individual EDCs is generally considered to be low, and in addition, the compounds are much less potent than natural hormones. However, humans are expected to be exposed to mixtures of potential EDCs. Hence, potential additive or synergistic effects have to be considered to assess the human risk. Furthermore, *in vivo* potency is often different from *in vitro* potency, since the mechanisms of hormonal action of chemicals may not be adequately reflected by the *in vitro* assays.

Human exposure to BPA has increased due to an increase in the use of epoxy resins and polycarbonate plastics. The primary route of human exposure is foods, via migration from containers. As BPA has been detected in river water samples, intake of fish or seafood may be a route for human exposure (Belfroid *et al.*, 2002). The daily intake is thought to be below $1\,\mu g\,kg^{-1}$ body weight per day (Kang *et al.*, 2006). Careful study is needed to see whether these levels of BPA may have an endocrine disruptive effect on humans. Foetuses are especially susceptible, because the level of uridine 5' -diphosphate (UDP)-glucuronosyl transferase, which is a major detoxification enzyme for BPA, is extremely low (Cappiello *et al.*, 2000). Several reports indicate that BPA can pass through the placenta into infants (Miyakoda *et al.*, 2000; Takahashi and Oishi, 2000; Schonfelder *et al.*, 2002).

Pesticides such as DDT and methoxychlor are well known to act as endocrine disruptors (Guillette *et al.*, 1994). The putative endocrine-disrupting effect of pesticides in wildlife is largely a prediction based on data obtained in laboratory animals. However, investigations of incidents involving misuse of pesticides have revealed virtually no endocrine-disrupting activity of these chemicals (Flickinger *et al.*, 1984).

Permethrin causes some toxic symptoms at high oral doses (Bradbury and Coats, 1989). Accidental and occupational human exposures involving dermal contact have caused acute poisoning (Altenkirch *et al.*, 1996; Chen *et al.*, 1991; Laskowski, 2002). Developmental neurotoxicity of pyrethroids has also been suggested. Neonatal rats are more sensitive than adults to pyrethroids (Shafer *et al.*, 2005). A neurotoxic effect of pyrethroids on mammalian cells was observed (Soderlund *et al.*, 2002; Kakko *et al.*, 2004). A variety of reversible symptoms, such as headache, dizziness, nausea, irritation of skin and nose and paresthesia, following exposure have been reported (He *et al.*, 1989). Exposure to the pesticide could be cumulative as regards neurotoxicity. There is potential human exposure to pyrethroids. Attempts have been reported to detect urinary metabolites of permethrin, such as phenoxybenzoic acid, in humans, as an indicator of exposure (Angerer and Ritter, 1997; Leng *et al.*, 1997; Shan *et al.*, 2004). Phenoxybenzoic acid in the urine of pest control operators was in the range $0.5–277\,\mu g\,l^{-1}$, whereas it was undetectable in the urine of nonexposed subjects (Leng *et al.*, 1997). Asakawa *et al.* (1996) also reported that phenoxybenzoic acid was detected in the urine of agricultural workers who applied permethrin to cabbages grown in open fields in Japan. Pyrethroid exposure of the general population in Germany was suggested by the fact that its metabolites were detected in the urine of volunteers (Schettgen *et al.*, 2002). It was demonstrated that metabolites of permethrin are formed by carboxylesterase and the P450 system (Nakamura *et al.*, 2007). The activities of these enzymes could be important determinants of pyrethroid exposure in humans. Fan *et al.* (2007) suggested that atrazine-induced aromatase expression was correlated with reproductive cancers in humans.

Organophosphate and carbamate insecticides are widely used in agriculture and for pest control. Chlorpyrifos, an organophosphate insecticide, has been found in human blood in Spain (Pitarch *et al.*, 2003), in human urine in the USA (Olsson *et al.*, 2003) and in human breast milk in India (Sanghi *et al.*, 2003), together with organochlorine pesticides. There are many opportunities for human exposure. As the pesticides are thought to be readily absorbed through skin, monitoring is an essential component of exposure assessment. In a study of exposure to chlorpyrifos, diazinon, propetamphos and diazinon in volunteers, almost all of these pesticides were found to be excreted after an oral dose, but large amounts were recovered in the skin surface after a dermal dose (Cocker *et al.*, 2002; Garfitt *et al.*, 2002). Tuomainen *et al.* (2002) reported that after application of malathion in greenhouses, the level of the pesticide in urine of workers reached a maximum at 6–7 hours and then rapidly decreased. Colosio *et al.* (2002) measured the ethylenethiourea levels in urine of workers in vineyards as an indicator of mancozeb, and reported that the levels in the workers were much higher than in controls, being especially high in operators of open tractors. In contrast, infants and children may have other opportunities to be exposed to

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organophosphate and carbamate insecticides. Gurunathan *et al.* (1998) reported that chlorpyrifos persistently accumulates on residential surfaces and toys accessible to children after household application. Exposure of children to organophosphate insecticides can be evaluated by measuring urinary biomarkers and their metabolites, and may be related to potential adverse health effects (Eskenazi *et al.*, 1999).

Environmental antiandrogens may affect the development of the male reproductive system (Kelce and Wilson, 1997). As androgens initiate and maintain spermatogenesis, environmental antiandrogens may contribute to the decline in sperm content that is suspected to be occurring in humans and other animals. It is reported that organohalogen compounds, such as PCBs, TCDD and DDE, may contribute to the decreased sperm counts and male reproductive capacity (Sharpe, 1995). Furthermore, inhibition of AR-mediated actions during the embryonic stage may lead to developmental alteration of male external genitalia (Foster, 1997). In humans, although a causal relationship between exposure to endocrine-disrupting pesticides and reproductive disorders has not been established, some recent reports predict an effect on humans. Sherman (1996) reported birth defects of the central nervous system in children exposed *in utero* to chlorpyrifos. Weidner *et al.* (1998) indicated increased occurrence of cryptorchidism in sons of female gardeners. Abell *et al.* (2000) also indicated reduced fecundity in female greenhouse workers. There are conflicting results concerning the association of exposure of pregnant women to pesticides with changes of foetal growth or length of gestation (Grether *et al.*, 1987; Savitz *et al.*, 1989; Fenster and Coye, 1990; Restrepo *et al.*, 1990; Thomas *et al.*, 1992; Willis *et al.*, 1993; Kristensen *et al.*, 1997; Xiang *et al.*, 2000; Perera *et al.*, 2003; Eskenazi *et al.*, 2004). Further examination is needed to clarify the influence of insecticides on humans and wildlife.

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Nitric Oxide Pathways in Toxic Responses

Jeffrey D. Laskin, Diane E. Heck and Debra L. Laskin

CONTENTS

1 INTRODUCTION

Originally described as a by-product in the chemical manufacturing industry and as an urban air pollutant, the discovery that nitric oxide gas is produced biologically and functions as an important physiological mediator was quite unexpected. Prior to the 1980s, crystallographers capitalized on the free-radical nature of nitric oxide for protein analysis, using its ability to bind to iron as a method to identify precise structural features of haem-containing proteins (Chien and Dickinson, 1977). The finding that nitric oxide could bind to, and activate, guanylyl cyclase, an important class of haem-containing enzymes, provided a novel tool for investigating enzyme activation and suggested a potential mechanism underlying the actions of vasodilators that release nitric oxide (Arnold *et al*., 1977; Katsuki and Murad, 1977; Katsuki *et al*., 1977). Further work showing that nitric oxide is produced endogenously and induces relaxation of smooth muscles led to studies of other physiological actions of nitric oxide, including its ability to regulate vascular tone, neuronal activity and macrophage-mediated cytotoxicity, and the enzymes that mediate nitric oxide biosynthesis (Furchgott, 1995). It is now well established that at low concentrations, nitric oxide modulates many biological processes in cells and tissues. However, when produced in excessive amounts or at inappropriate times and/or places, nitric oxide contributes to pathophysiological responses. Whether or not this causes tissue injury and/or induces significant pathology depends on local concentrations of enzymes that generate nitric oxide, its metabolism to toxic intermediates, and its degradation and detoxification in target organs. As a highly diffusible gas that can readily cross cell membranes, nitric oxide acts both as an autocrine and paracrine mediator of toxicity (Wood and Garthwaite, 1994; Porterfield *et al*., 2001). Thus, xenobiotics can induce nitric oxide production in target cells, causing oxidative and nitrosative stress and cytotoxicity. Nitric oxide can also diffuse out of the cells into surrounding cells and tissues, amplifying tissue injury.

2 NITRIC OXIDE SYNTHASES

Nitric oxide is derived from L-arginine in a sequential two-step reaction mediated by the enzyme, nitric oxide synthase (Stuehr, 2004). This enzyme catalyzes a five-electron oxidation of L-arginine to nitric oxide and L-citrulline, NADPH (nicotinamide adenine dinucleotide phosphate) and oxygen serving as cosubstrates. Three isoforms of nitric oxide synthase have been identified. These include two relatively low-output forms of the enzyme, endothelial nitric oxide synthase (eNOS or NOS-3) and neuronal nitric oxide synthase (nNOS or NOS-1), and a high-output inducible or macrophage form of the enzyme (iNOS or NOS-2). Whereas eNOS plays an important role in regulating vascular tone, signalling and remodelling, as well as leucocyte–endothelial interactions, platelet aggregation and angiogenesis, nNOS mediates neurotransmission (Stuehr, 2004; Sessa, 2004; Chatterjee and Catravas, 2008). In contrast, iNOS is important in cytotoxicity and inflammatory tissue injury, and is expressed in large amounts in macrophages (Lowenstein and Padalko, 2004). eNOS is localized in peripheral vascular endothelial cells, as well as in brain

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vasculature and in motor neurons, while nNOS is found in astrocytes, myocytes, the prostate and the adrenal medulla. An additional form of nitric oxide synthase has been identified in the inner membranes of mitochondria, which may be derived from one of the three isoforms of the enzyme (Carreras *et al*., 2007). Although the specific gene product for this enzyme is unknown, it is thought to function in the regulation of mitochondrial electron transport (Haynes *et al*., 2004; Carreras *et al*., 2007).

The different isoforms of nitric oxide synthase are homodimers. Each of the enzymes contains flavin cofactors which mediate electron transfer from NADPH in one subunit of the enzyme to haem iron in an oxygenase domain of the other subunit. The oxygenase reaction of nitric oxide synthase requires tetrahydrobiopterin; eNOS and nNOS are also calcium/calmodulin-dependent (Stuehr *et al*., 2004). Enzyme activity and nitric oxide biosynthesis is regulated by the availability of L-arginine, as well as essential cofactors, including oxygen, NADPH, flavin adenine dinucleotide, flavin mononucleotide and tetrahydrobiopterin, and for eNOS and nNOS, calcium. Enzyme activity can also be regulated by post-translational modification and protein interactions (Roman *et al*., 2002). For example, nitric oxide biosynthesis via eNOS is regulated by S-nitrosylation, fatty-acid acylation and phosphorylation, as well as interaction with hsp90 and β-actin (Sessa, 2004; 2005; Gratton *et al*., 2004; Tummala *et al*., 2008). The activity of eNOS is also controlled by the membrane scaffolding protein caveolin-1, which sequesters eNOS in caveolae and inhibits its activity (Minshall *et al*., 2003; Su *et al*., 2007). Similarly, nNOS is negatively regulated by phosphorylation (Bredt *et al*., 1992). In post-synaptic terminals, this form of nNOS is activated by calcium following glutamate-induced activation of *N*-methyl-D-aspartate (NMDA) receptors (Zhou *et al*., 2008).

Nitric oxide production is also regulated by expression of nitric oxide synthase proteins. For example, iNOS is controlled transcriptionally by the stability of the transcripts and their translation (Kleinert *et al*., 2003). Initially thought to be expressed only in macrophages and to participate in cytotoxicity, it is now known that many different cell types can be induced to express iNOS and to generate nitric oxide, an activity that may be important in chemical toxicity (see further below). A number of diverse stimuli, including endotoxin, inflammatory cytokines and growth factors, are known to regulate expression of iNOS (Heck *et al*., 1992; Pacher *et al*., 2007). The endothelial and neuronal forms of nitric oxide synthase are also regulated by changes in protein expression. For example, eNOS protein is up-regulated in endothelial cells in diabetes (Hink *et al*., 2001) and nNOS protein in spinal-cord injury (Conti *et al*., 2007).

3 NITRIC OXIDE SIGNALLING AND TOXICITY

Containing a single unpaired electron, nitric oxide is a highly reactive free radical, a property that contributes to its cell signalling and cytotoxic activity (Nathan, 2003; 2004). The most well-characterized target for nitric oxide is ferrous iron in haem (Angelo *et al*., 2008), an interaction that is key to the ability of nitric oxide to regulate guanylyl cyclase (Arnold *et al*., 1977; Katsuki and Murad, 1977; Katsuki *et al*., 1977; Nakane, 2003). By binding to haem, nitric oxide allosterically activates its catalytic site, leading to increased intracellular cGMP (cyclic guanosine monophosphate) and the activity of cGMP-dependent kinases, enzymes that control numerous physiological activities including vascular tone, neuronal activity, cell growth and differentiation (Angelo *et al*., 2008). A critical role for the nitric-oxide–cGMP pathway in pathology has been identified in cardiovascular diseases, where diminished activity can lead to increases in vessel tone and an increased risk of thrombosis (Nakane, 2003; Hare and Stamler, 2005; Marin and Sessa, 2007).

Other important targets for nitric oxide include enzymes that contain transition metals and those that generate free radicals in their catalytic cycle (Pacher *et al*., 2007). For example, nitric oxide readily binds tyrosyl radicals in ribonucleotide reductase, resulting in inhibition of enzyme activity and DNA synthesis (Kwon *et al*., 1991). Nitric oxide also reacts rapidly with the superoxide anion, forming peroxynitrite (Pacher *et al*., 2007; Szabó *et al*., 2007). In fact, there is much evidence to support the idea that cytotoxicity caused by excessive production of nitric oxide is mediated in large part by peroxynitrite. Superoxide anion is formed in cells from many sources, including mitochondrial respiration, redox cycling, NADPH oxidases and xanthine oxidase (McCord, 1998). In either direct one- or two-electron oxidation reactions, or via indirect radical-mediated reactions, peroxynitrite can react with biological molecules, including lipids, proteins and nucleic acids, processes that trigger oxidative injury and perturbations in cell signalling (Alvarez *et al*., 1999; Radi *et al*., 2000; Alvarez and Radi, 2003; Rubbo *et al*., 2008). Peroxynitrite-induced tissue injury has been linked to many diseases, including neurodegeneration, diabetes, stroke, heart disease and cancer. Additional reactive nitrogen species derived from nitric oxide that can contribute to toxicity include nitrite, nitrogen dioxide, nitronium and nitrosonium cations, nitroxyl, nitrosoperoxycarbonate anion and nitryl chloride (Nathan, 2003). Each of these intermediates possesses distinct chemical and biological reactivities that may be important in the cytotoxic actions of nitric oxide in cells and tissues.

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Modification of proteins and subsequent alterations in their structure and function is an important mechanism by which reactive nitrogen species initiate biological activity and/or cause cellular damage. The best characterized modifications are nitration, glutathionylation and S-nitrosylation (Alvarez and Radi, 2003; Shelton and Mieyal, 2008; Townsend, 2007). Both nitric oxide and peroxynitrite induce protein nitrotyrosine formation; peroxynitrite is significantly more effective than nitric oxide in generating nitrotyrosine modifications and this is often used as a marker of peroxynitrite production in cells and tissues (Pacher *et al*., 2007). Nitric oxide can also form nitrogen dioxide; further reactions with nitric oxide generate N_2O_3, a strong nitrosating agent that is highly reactive with protein thiols, as well as with primary and secondary amines (Pacher *et al*., 2007). S-Nitrosylation of critical sulfhydryls groups in proteins is known to regulate the activity of such groups; examples of modified proteins include cytoskeletal proteins, membrane receptors and channels, and protein kinases and phosphatases (Foster *et al*., 2003; Hess *et al*., 2005). The enzyme-independent and reversible attachment of glutathione or other low-molecular-weight thiols to cysteine sulfdryls in proteins is referred to as glutathionylation (Shelton and Mieyal, 2008). Formed via the S-nitrosylation of glutathione and protein thiols, S-nitrosylglutathione may serve as a store and/or transporter for nitric oxide (Foster *et al*., 2003; Hess *et al*., 2005). Nitric oxide can be removed from S-nitrosylglutathione enzymatically by S-nitrosylglutathione reductase, an intracellular regulator of S-nitrosothiols (Liu *et al*., 2004; Hedberg *et al*., 2003).

Reactive nitrogen species can also react with nucleic acids and lipids leading to toxicity. Damage to DNA can cause mutations, leading to necrosis and/or apoptosis (Szabó and Ohshima, 1997; Ohshima *et al*., 2006; Sawa and Ohshima, 2006), as well as altered cell growth and differentiation, potentially resulting in the development of cancer (Sawa and Ohshima, 2006). Peroxynitrite is also known to form 8-nitroguanine adducts in RNA and DNA (Szabo *et al*., 1996; Szabó and Ohshima, 1997; Ohshima *et al*., 2006). In DNA, this can lead to G:C to T:A transversions, which are mutagenic (Yermilov *et al*., 1995). The reaction of reactive nitrogen species with lipids can either directly or indirectly generate a variety of biologically active products, including electrophilic lipids such as 4-hydroxynonenal and 15-deoxy-Δ12,14-prostaglandin J2 (Catalá, 2009; Forman *et al*., 2008; Uchida and Shibata, 2008), lipid peroxides (Kim *et al*., 2008) and receptor agonists, such as nitrolinoleic acid and lysophosphatidylcholine (Villacorta *et al*., 2007; Manini *et al*., 2008). Electrophilic lipids can directly modify proteins and DNA (Zmijewski *et al*., 2005), while nitrolinoleic acid and other nitrated unsaturated fatty acids can inhibit smooth muscle cell proliferation. Reactive nitrogen species can also regulate metabolism of sphingolipids, which are important in cell growth, differentiation and apoptosis (Perrotta *et al*., 2008).

Detoxification of reactive nitrogen species is important in limiting tissue injury and a number of pathways have been identified that are involved in this process. However, some of these products also possess cytotoxic activity. For example, the reaction of nitric oxide with oxyhaemoglobin leads to its conversion to inactive nitrate (Angelo *et al*., 2008). In contrast, the reaction of peroxynitrite with carbon dioxide generates nitrogen dioxide and carbonate radicals; both of these products are reactive and can modify critical carbohydrates, proteins and nonprotein thiols and intracellular thiols (Denicola and Radi, 2005; Pacher *et al*., 2007). Cellular antioxidants, including glutathione, uric acid and selenium compounds also react with and protect cells against reactive nitrogen species (Pacher *et al*., 2007). It should be noted that nitric oxide itself can also function as an antioxidant. By eliminating superoxide anion, an important intermediate in the generation of highly reactive hydroxyl radicals, nitric oxide can limit cellular damage induced by reactive oxygen species (Pacher *et al*., 2007; Coulter *et al*., 2008). Nitric oxide has also been reported to act as a chain-terminating agent when it reacts with thiyl radicals, forming nitrosothiols (Benhar *et al*., 2006; Gaston *et al*., 2006).

4 NITRIC OXIDE AS A MEDIATOR OF CHEMICAL TOXICITY

It is now well established that nitric oxide plays an important role in the toxicity of a variety of xenobiotics in many different tissues, including the liver, lung, skin, brain and cardiovascular system (**Table 1**). That nitric oxide contributes to toxicity is based on findings that cell damage is associated with excessive production of nitric oxide and/or by increased expression of NOS protein and activity and consequent expression of biomarkers of nitric oxide production (e.g. nitrosyl-modified proteins, lipids and DNA; the appearance of oxidation products of nitric oxide in body fluids such as blood and urine). Indirect markers of nitric oxide production, including increases in cGMP, as well as downstream effectors of the nitric-oxide–cGMP pathway are also detectable in target tissues (Arnold *et al*., 1977; Katsuki and Murad, 1977; Katsuki *et al*., 1977; Fesenko *et al*., 1985; Fiscus and Murad, 1988; Boswell-Smith *et al*., 2006). Evidence to support a role for nitric oxide in chemical toxicity is also based on the findings that selective NOS inhibitors effectively prevent tissue damage. Abrogation of chemically induced tissue injury is also observed in genetically engineered mice lacking the different isoforms of NOS. It should be pointed out, however, that in some cases, xenobiotic-induced nitric oxide production is associated with protection against tissue injury. This is thought to be

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due to the antioxidant properties of nitric oxide. Whether or not nitric oxide plays a pathological or protective role in toxicity is dependent on the toxicant, the target tissue and the extent to which toxicity is mediated by reactive oxygen species.

Numerous studies have demonstrated that nitric oxide participates in chemically-induced hepatotoxicity (Nussler *et al*., 1995; Milbourne and Bygrave, 1995; Losser and Payen, 1996; Gardner *et al*., 1998; 2002; Jaeschke *et al*., 2002). iNOS has been identified in both resident and inflammatory liver macrophages, as well as in hepatocytes, sinusoidal endothelial cells and stellate cells (Helyar *et al*., 1994; Laskin *et al*., 1995; Gross and Wolin, 1995; Gardner *et al*., 1998). By binding to haem-containing proteins in hepatic drug-metabolizing enzymes, nitric oxide can either inhibit or activate drug metabolism. Exposure to hepatotoxicants such as paracetamol (acetaminophen), ethanol, carbon tetrachloride, and endotoxin has been reported to activate Kupffer cells, as well as inflammatory macrophages, sinusoidal endothelial cells, stellate cells and/or hepatocytes, to express iNOS and produce excessive quantities of nitric oxide (Helyar *et al*., 1994; Laskin *et al*., 1995; Laskin and Pendino, 1995; Chamulitrat and Spitzer, 1996; Rockey, 1997; Rockey and Chung, 1997; Matsumoto *et al*., 2000; Spitzer and Spitzer, 2000; Zhu and Fung, 2000; Gardner *et al*., 1998; 2002; 2003; Rockey, 2003). Although this has been correlated with nitrotyrosine staining of the liver (Hinson *et al*., 1998; Gardner *et al*., 2002; 2003), the precise role of nitric oxide and peroxynitrite in hepatotoxicity appears to be dependent on the toxicant. Thus, while some studies have suggested that reactive nitrogen species are toxic, in other models, they appear to play a protective role. For example, in animals pretreated with inhibitors of iNOS, such as aminoguanidine, or in transgenic mice with a targeted disruption of iNOS, hepatotoxicity induced by acetaminophen or endotoxin is significantly reduced (Ialenti *et al*., 1992; Wright *et al*., 1992; Losser and Payen, 1996; Gardner *et al*., 1998; 2002; Laskin *et al*., 2001c). In contrast, hepatotoxicity is augmented in iNOS knockout mice treated with carbon tetrachloride (Morio *et al*., 2001). Similar increases in carbon tetrachloride-or endotoxin/*C. parvum*-induced hepatotoxicity have been described in animals pretreated with iNOS inhibitors (Harbrecht *et al*., 1992; Muriel, 1998; Taylor *et al*., 1998; Zhu and Fung, 2000), which is thought to be due to a loss of the ability of nitric oxide to reduce levels of cytotoxic oxidants (Pacher *et al*., 2007).

In the lung, the toxicity of a number of toxicants, including ozone, silica, particulate matter and paraquat is reported to be mediated by nitric oxide (Zhu *et al*., 1998; Porter *et al*., 2006; Fakhrzadeh *et al*., 2002). Several prominent cell types in the lung, including alveolar and interstitial macrophages and bronchiolar and alveolar Type II epithelial cells have the capacity to produce nitric oxide via iNOS after exposure to proinflammatory cytokines or bacterial-derived endotoxin (Pendino *et al*., 1993; Punjabi *et al*., 1994; Shan *et al*., 2007). Nitric oxide is known to play a key role in regulating airway responsiveness and bronchiole relaxation (Bhalla, 1999; Castranova *et al*., 2002), as well as macrophage-mediated cytotoxicity and cell proliferation (Laskin *et al*., 2001a; Laskin and Laskin, 2001). These latter activities are likely to be significant in tissue damage and in the regulation of alveolar epithelial regeneration following injury (Laskin *et al*., 2001c; Laskin and Laskin, 2001). Acute exposure of rodents to ozone or silica results in increased production of nitric oxide via iNOS by alveolar macrophages and Type II cells (Zeidler *et al*., 2004; Fakhrzadeh *et al*., 2008). Lung macrophages, as well as Type II cells isolated from animals exposed to ozone and silica produce increased amounts of nitric oxide in response to inflammatory mediators, indicating that the toxicants sensitize these cells to produce cytotoxic mediators (Laskin *et al*., 1995; 2001a; 2001b). Tumour necrosis factor-α (TNFα) is a critical inflammatory mediator stimulating nitric oxide production in lung macrophages following ozone inhalation in mice; animals deficient in TNFα are protected from ozone toxicity and fail to produce nitric oxide in response to ozone (Fakhrzadeh *et al*., 2008). Both ozone-and silica-induced toxicities are ameliorated by inhibitors of nitric oxide biosynthesis, as well as in iNOS knockout mice (Fakhrzadeh *et al*., 2002; Zeidler *et al*., 2004), strongly supporting a cytotoxic role for nitric oxide in the pathogenesis of lung injury caused by these agents.

Nitric oxide-mediated toxicity in the lung and brain is also associated with exposure to paraquat (Day *et al*., 1999; Denicola and Radi, 2005; Djukic *et al*., 2007). Paraquat is known to induce toxicity via redox cycling, a process mediated by a number of different enzymes, including NADPH cytochrome P450 reductase, thioredoxin reductase and nNOS (Smith and Brian, 1991; Day *et al*., 1999; Gray *et al*., 2007). Paraquat toxicity in the lung is associated with increased nitric oxide production and inhibitors of NOS have been reported to mitigate tissue injury (Mustafa *et al*., 2002). In the brain, dopaminergic neurons are susceptible to paraquat toxicity and this may be important in the development of Parkinson's disease. Toxicity is attributed to both oxidative and nitrosative stress (Hara *et al*., 1991; Djukic *et al*., 2007). Nitric oxide has been linked to excitotoxicity, a process of neuronal cell death caused by activation of excitatory amino-acid receptors, in particular, NMDA/glutamate receptors (Knott and Bossy-Wetzel, 2008). NMDA is coupled to nitric oxide production, presumably through the activation of nNOS via receptor-mediated calcium mobilization (Dawson and Dawson, 1998). NOS inhibitors, as well as haemoglobin, are effective in blocking glutamate-mediated neurotoxicity *in vitro* (Parathath *et al*., 2007). It has been suggested that

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Table 1 Examples where nitric oxide contributes to xenobiotic toxicity

Tissue	Xenobiotic	References
Liver	Endotoxin	Wong and Billiar (1995)
	Paracetamol (acetaminophen)	Gardner *et al*. (1998)
	Carbon tetrachloride	Zhu and Fung (2000), Morio *et al*. (2001)
	Ethanol	Dey and Cederbaum (2006)
Lung	Ozone	Laskin *et al*. (2001a)
	Silica	Porter *et al*. (2006)
	Paraquat	Berisha *et al*. (1994)
	Particulates	Zhu *et al*. (1998)
Skin	UVB light	Romero-Graillet *et al*. (1997)
		Gonzalez Maglio *et al*. (2005)
Kidney	Cisplatin	Jung *et al*. (2009)
Brain	Paraquat	Djukic *et al*. (2007)
	MPTP[a]	Denicola and Radi (2005), Schulz *et al*. (1995)
Cardiovascular	Arsenic	Bunderson *et al*. (2004), Chen and Chen (2008)
	Doxorubicin	Vasquez-Vivar *et al*. (1997)

[a](*N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine).

paraquat initiates excitotoxicity by stimulating glutamate efflux via a nitric-oxide-dependent mechanism (Shimizu *et al*., 2003a; 2003b; Dinis-Oliveira *et al*., 2006). Nitric oxide has also been reported to mediate the toxicity of *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in the brain. MPTP is structurally similar to paraquat and may induce nitric oxide production via oxidative stress (Yokoyama *et al*., 2008). NOS inhibitors have also been shown to inhibit MPTP toxicity in mice (Watanabe *et al*., 2008) and in astrocytes. Anti-inflammatory agents suppress MPTP-induced NOS, suggesting that they may be effective in treating Parkinson's disease (Carbone *et al*., 2009).

Nitric oxide is also known to contribute to the toxicity of doxorubicin and arsenic in the cardiovascular system. Doxorubicin is one of the more prominent clinically utilized antitumour agents (Wadler *et al*., 1986). However, toxicity, which includes chronic heart failure and degenerative cardiomyopathy, has limited its use (Ferreira *et al*., 2008). Like paraquat, doxorubicin is known to redox cycle and its toxicity is thought to be due, at least in part, to the generation of cytotoxic reactive oxygen intermediates (Doroshow, 1983; Davies and Doroshow 1986; Doroshow and Davies, 1986). Redox cycling can occur via eNOS, where electrons are diverted from nitric oxide biosynthesis (Day *et al*., 1999). Nitric oxide is crucial for cardiovascular function and inhibiting its activity may be an important mechanism of toxicity (Schulz *et al*., 2004). Doxorubicin redox cycling also leads to the production of the superoxide anion. Thus, localized production of low amounts of nitric oxide may result in the generation of cytotoxic concentrations of peroxynitrite. Indeed, production of peroxynitrite has been described in mouse models of doxorubicin-induced heart failure (Weinstein *et al*., 2000; Mihm and Bauer, 2002; Mihm *et al*., 2001; 2002). Moreover, cardiac dysfunction following doxorubicin treatment is associated with nitration of cardiac myofibrils (Weinstein *et al*., 2000). Cardiomyocyte mitochondria are also targeted by peroxynitrite generated following doxorubicin exposure (Mihm *et al*., 2002). That peroxynitrite is important in doxorubicin-induced cardiomyopathy is evident from the findings that metalloporphyrin peroxynitrite decomposition catalysts protect against the development of doxorubicin-induced cardiac dysfunction (Pacher *et al*., 2003). Recent studies have also shown that disruption of eNOS protects against cardiac injury, dysfunction and mortality induced by doxorubicin (Neilan *et al*., 2007). Interference with doxorubicin-induced cardiotoxicity by a competitive inhibitor of FasL, a mediator of apoptosis and cellular inflammatory responses, was also reported to be associated with reduced expression of iNOS, as well as nitric oxide and peroxynitrite production (Niu *et al*., 2009).

Epidemiological studies have demonstrated a strong association between arsenic exposure and cardiovascular disease (Engel and Smith, 1994; Wang *et al*., 2002). Arsenic is thought to function by activating a membrane-bound NAD(P)H oxidase in vascular endothelial cells (Smith *et al*., 2001) and vascular smooth muscle cells (Lynn *et al*., 2000), a process that generates superoxide anion. Localized reaction of superoxide anion with nitric oxide can lead to cytotoxic concentrations of peroxynitrite in the cardiovascular tissue. In a mouse model, arsenic exacerbates artherosclerotic plaque formation and increases nitrotyrosine formation within the plaque (Bunderson *et al*., 2004). In rats, arsenic increases vascular leakage, a process abrogated by NOS inhibitors (Chen and Chen, 2008). Arsenic has also been found to induce peroxynitrite formation in bovine aortic endothelial cells (Bunderson *et al*., 2002). Taken together, these data suggest an important contribution of nitric oxide in arsenic-induced cardiovascular toxicity.

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It is well recognized that the clinical use of cisplatin is limited by nephrotoxicity (Hensley *et al*., 2009). Cisplatin is known to induce oxidative damage in the kidney, including lipid peroxidation and to reduce intracellular antioxidants (Weijl *et al*., 1997; Yoshida *et al*., 2000; Antunes *et al*., 2001; Mora *et al*., 2003). Nitric oxide is an important mediator of kidney function including the control of renal microcirculation and in tubule activity. Recent studies have shown that cisplatin-induced renal injury is associated with up-regulation of mitochondrial NOS and nNOS, and an accumulation of nitrites in mitochondria (Jung *et al*., 2009). Cisplatin-induced increases in iNOS have also been described (Adams *et al*., 2009). In a rat model, an inhibitor of iNOS has been shown to reduce cisplatin-induced histological damage and to cause increases in proteinuria, decreases in creatinine clearance, tubulointerstitial infiltration and staining for 3-nitrotyrosine (Chirino *et al*., 2008). These data indicate that nitric oxide is involved in cisplatin toxicity, and suggest that reducing or preventing renal oxidative/nitrosative stress may improve therapeutic outcomes with this antitumour agent.

Nitric oxide has also been reported to mediate ultraviolet light B (UVB)-induced skin toxicity (Romero-Graillet *et al*., 1997; Gonzalez Maglio *et al*., 2005). Early studies showed that nitric oxide is produced by mouse and human keratinocytes (Heck *et al*., 1992). Subsequently, Romero-Graillet *et al*. (1997) reported that UVB irradiation induces the release of nitric oxide and the generation of cGMP in cultures of human keratinocytes. Nitric oxide also mediates a delayed vasodilator response to ultraviolet light (Warren *et al*., 1993; Warren, 1994). It has been demonstrated that all isoforms of NOS are present in the skin and that substantial levels of nitric oxide are generated during the wound-healing response (Stallmeyer *et al*., 1999). Nitric oxide production during wounding has been identified as a critical regulator of vascular endothelial growth factor expression, an important angiogenic factor in the proliferative phase of skin repair (Frank *et al*., 1999). UVB light is known to induce keratinocyte apoptosis. An antiapoptotic role for nitric oxide in the skin has been proposed, since UVB-induced apoptosis of dermal and epidermal cells is enhanced in iNOS- and eNOS-knockout mice, respectively (Weller *et al*., 2003). UVB light has also been reported to inhibit γ-interferon-stimulated nitric oxide production in macrophages and keratinocytes (Sur *et al*., 2002).

5 CONCLUSIONS

Nitric oxide is well recognized as a key soluble mediator in the pathogenesis of tissue injury induced by many toxicants. Xenobiotics or their metabolites can directly injure tissue, generating reactive oxygen and nitrogen species. Excessive production of these mediators leads to oxidative stress and may result in production of additional proinflammatory and cytotoxic mediators by target cells and/or infiltrating leucocytes. Resulting nitric oxide and its oxidation products can activate many different biochemical and molecular signalling cascades that contribute to toxicity. Nitric oxide can also participate in inflammatory processes that promote tissue damage. Further studies on the pathways by which nitric oxide induces pathology will be crucial for a better understanding of the mechanisms leading to xenobiotic-induced cell damage and tissue injury, and for the development of therapeutic interventions aimed at mitigating toxicity resulting from chemical exposures.

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Introduction to Mitochondrial Toxicity

Yvonne Will and James A. Dykens

CONTENTS

1 INTRODUCTION

Mitochondria have been the subject of study since their independent discovery in the mid-to-late 1800s by cytologists, including Kölliker and Flemming, who described them as 'granules' or 'filaments', respectively. In 1890 Altmann used fuchsin staining to demonstrate that mitochondria, which he presciently characterized as 'elementary living particles' and called 'bioblasts', are present in almost all cells. In 1898, using alizarin and crystal violet stains, Benda coined the name mitochondrion to reflect their plastic morphology *in situ*: mitos (Greek: a thread), khondrion (Greek: a grain). The gradual illumination of the central role mitochondria play in bioenergetics has led to several Nobel prizes: Otto Warburg in 1931 for the discovery that oxygen consumption resides within the mitochondria; Hans Krebs in 1953 for the elucidation of the citric-acid cycle; Peter Mitchell in 1978 for his insights and description of the chemiosmotic coupling of electron transfer reactions and phosphorylation and John E. Walker and Paul D. Boyer in 1997 for their work revealing the molecular structure of adenosine triphosphatase (ATPase) (ATP synthase, Complex V) that allows its function as a rotating motor complete with stators.

Mitochondriology has enjoyed a recent renaissance, sparked by the observations that mitochondrial dysfunction caused disease (Luft *et al*., 1962), that mutations in mitochondrial DNA (mtDNA) are linked to diseases, that mitochondria are key players in apoptosis, and that mitochondria have signalling function. Most recently, study of mitochondria has accelerated in response to findings that many xenobiotics and pharmaceuticals with unexplained organ toxicity and adverse clinical events impair mitochondrial function as an 'off-target' effect.

Mitochondria generate 95% of the cell's energy, and also most of the reactive oxygen- and nitrogen-centred

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free radicals that undermine cell integrity. In this light, it should not be surprising that loss of mitochondrial capacity or functional impairment due to xenobiotic exposure could contribute to untoward drug toxicity. Nevertheless, that this is indeed the case has only recently gained appreciation, in large measure due to inadequacies in the techniques typically used to assess potential toxicity during preclinical drug development.

In this chapter we will provide a brief overview of mitochondrial morphology and function, followed by a discussion of drugs that undermine function and organ toxicities related to mitochondrial toxicity. We will then provide selected examples of recently developed methodologies to study mitochondrial toxicities *in vitro* and *in vivo*. We will close with consideration of how such mitochondrial impairment translates into cytotoxicity and organ toxicity via threshold effects, how bioaccumulation of mitotoxicants confounds classical pharmacokinetics, and how these provide a new model of previously unexplained idiosyncratic drug toxicity. Throughout, in the citations we have emphasized comprehensive literature reviews that will introduce the interested reader to the more detailed primary publications, and we apologize to our many colleagues whose work has been slighted as a result.

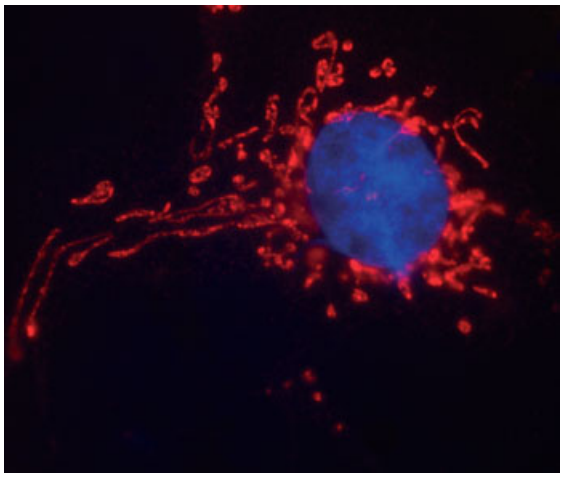

Figure 1 COS cells stained with Mitotracker Red displaying the mitochondrial reticulum. (Courtesy of Sandra Wiley, UCSD, San Diego.)

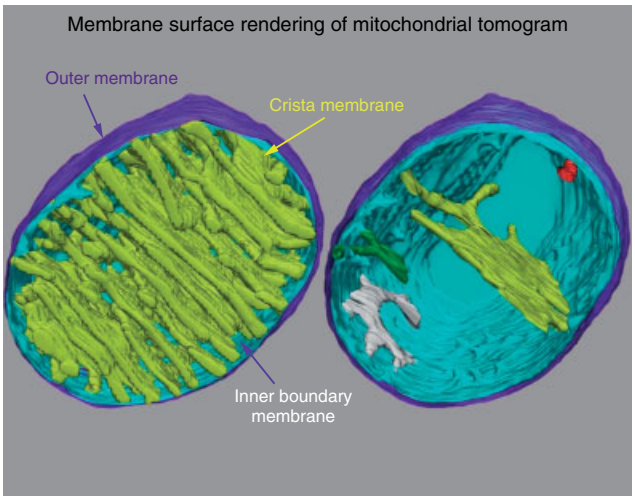

Figure 2 Mitochondrial compartmentalization. Computer tomography. (Courtesy of Dr. T. Frey, SDSU, San Diego.)

2 OVERVIEW OF MITOCHONDRIAL BIOLOGY

2.1 Morphology

Most of our knowledge of mitochondrial structure has been founded upon electron-microscopic examination. Today, three-dimensional reconstruction using computer tomography and confocal and electron microscopy have broadened our understanding of mitochondrial morphology. Whereas electron microscopy projected mitochondria as individual round or threadlike organelles (depending on the tissue), we now know that mitochondria form a dynamic reticulum that is constantly undergoing fission and fusion, depending on the metabolic or pathological state of the cell, to form individual organelles or merge into back into the network (**Figure 1**).

From the first ultrastructural studies it was apparent that mitochondria are composed of four compartments: an outer membrane, an inner membrane, an intermembrane space and an interior chamber called the matrix. Both membranes are a mixture of phospholipids and integral and peripheral proteins. In the outer membrane the proportion of proteins and lipids is approximately 1:1, which is typical of most cellular membranes. However, the inner membrane contains a much higher proportion of proteins (80%) and only 20% phospholipids, of which 10% is cardiolipin, an atypical phospholipid with four acyl tails that is only found in the inner mitochondrial membrane. The inner membrane is impermeable to protons, which is crucial for mitochondrial ATP production, and so has many transmembrane transporters that regulate import and export of metabolites, including the adenine nucleotide translocator (ANT) that exchanges adenosine diphosphate (ADP) for ATP.

The outer membrane is freely permeable to solutes up to 5000 Da and contains ion channels, such as voltage dependent anion channels (VDACs) that work in concert with transporters in the inner membrane. For larger molecules, shuttles and carriers are in place (**Figure 2**).

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The impermeable inner mitochondrial membrane can be passively crossed only by weak acids, lipophilic cations, depending on chemical characteristics, oxygen and ammonia. Electrically charged and hydrophilic compounds, such as carboxylic anions (including respiratory substrates) and inorganic ions, are unable to pass the inner membrane without participation of specialized transport-protein carriers. This impermeability is required to maintain the coupling of the proton gradient generated by the redox reactions of the electron transport system (ETS) to phosphorylation. ETS and its reduction of oxygen is separate from, but coupled with, phosphorylation, and when taken together are called oxidative phosphorylation (OXPHOS); they are mediated by a series of respiratory complexes numbered I–V, all embedded in (Complexes, I, III–V) or adjacent to (Complex II), the inner membrane.

2.2 Oxidative Phosphorylation

The complete oxidation of glucose yields 38 ATPs:

$$C_6H_{12}O_6 + 6O_2 + 38ADP \longrightarrow 38ATP + 6CO_2 + 6H_2O \quad (1)$$

Of the 38 ATPs, two are from glycolysis, so that mitochondria generate 95% of the cells energy ((2/38)*100 = 5.3). Under physiological conditions (as opposed to standard) the overall efficiency is approximately 50%, with the remaining energy lost as heat. A simple calculation demonstrates the importance of mitochondrial functional integrity to organismal energetic budgets. Human resting metabolism, for example, while sitting quietly in a chair or lying at ease on a cot, is a function of gender, but averages 6127 and 7983 kJ day^{-1} in women and men, respectively (De Lorenzo *et al*., 2001). Under physiological conditions, ATP hydrolysis yields 42–50 kJ mol^{-1} (Campbell, 1993), with the result that women turn over ~133 mol ATP day^{-1} and men ~173 mol ATP day^{-1}. The molecular weight of ATP is 507 g mol^{-1}, so that women turn over 67 431 g day^{-1} (=148 lbs), and men turn over 87 711 g day^{-1} (=193 lbs). We essentially turn over our body weight or more in ATP every day! Moreover, humans have aerobic scope (i.e. can increase activity) of between 10- and 20-fold over rest, so a well-trained marathon runner could turn over >1 kg of ATP per minute.

This ATP production is due to the mitochondrial ETS that contains four respiratory complexes plus ATP synthase. Details are beyond the scope of this review, and can be found in several reviews and biochemistry books (Lehninger *et al*., 2008; Scheffler, 2007; Berg *et al*., 2006). Nevertheless, the process bears some attention in the context of drug-induced impairment.

Pyruvate from glycolysis is transported across the inner mitochondrial membrane by a specific transporter (Halestrap, 1978) that is a member of the mitochondrial carrier family with homologues in all other species examined thus far (Hildyard and Halestrap, 2003). Once inside the matrix, pyruvate dehydrogenase (PDH) catalyses the highly exergonic (irreversible) decarboxylation reaction between pyruvate and coenzyme A to yield the key metabolic intermediate acetyl CoA, plus CO_2 and NADH (nicotinamide adenine dinucleotide). PDH is an interesting enzyme because it is part of a huge (4600 kDa) PDH complex made up of some 60 subunits that constitute the three enzymes, cofactors and regulatory components necessary for the complete reaction (Lodish *et al*., 1995). Acetyl CoA is also a key intermediary in the β-oxidation of fatty acids within the mitochondria, and from amino-acid catabolism of the ketoacid following deamination. Regardless of the source, acetyl CoA enters the Krebs cycle (citric acid cycle) by condensing with oxaloacetate to yield citrate, which, in a series of eight reactions, yields reducing equivalents in the form of six NADH and two FADH (flavin adenine dinucleotide), plus four CO_2 molecules and one substrate-level phosphorylation.

The potential energy inherent in reducing equivalents of NADH and FADH is gradually released during mitochondrial OXPHOS by passing the electrons down a redox gradient from Complex I (NADH-ubiquinone-oxidoreductase) to oxygen as the terminal acceptor in a process called respiration (see **Figure 3**, which is the electron transport chain, ETC). Electrons also enter via Complex II (succinate dehydrogenase), and are passed from both Complexes I and II via a membrane-bound carrier, ubiquinone, to Complex III (ubiquinol-cytochrome-c-oxidoreductase). From Complex III, the electrons are carried to Complex IV (cytochrome c oxidase) via cytochrome c, where four electrons are collected, and used to tetravalently reduce oxygen to water.

At Complexes I, II and IV, the potential energy of the redox reaction is sufficient to translocate a proton from the matrix, against a gradient, into the inner membrane space. This yields a membrane potential of some 180 mV (matrix negative) due to the charge of the electric potential ($\Delta\Psi$), plus an additional 40–60 mV from the proton ion pH gradient (ΔpH) of about 1 pH unit, so that the average mitochondrial proton motive force (PMF) is about 220 mV (matrix negative). Clearly, if the inner membrane were permeable to protons, they would diffuse back down the gradient as fast as they are pumped out, with no energy captured (i.e. lost as heat and doing no work). But as long as the inner membrane is impermeable, this gradient persists, and it can be thought of resistance that the ETS must work against. In response to decreases in PMF, ETS can accelerate to a maximum flux.

As an aside, generations of students learned this process as the 'electron transport chain', which connotes a one-to-one linkage between the various components.

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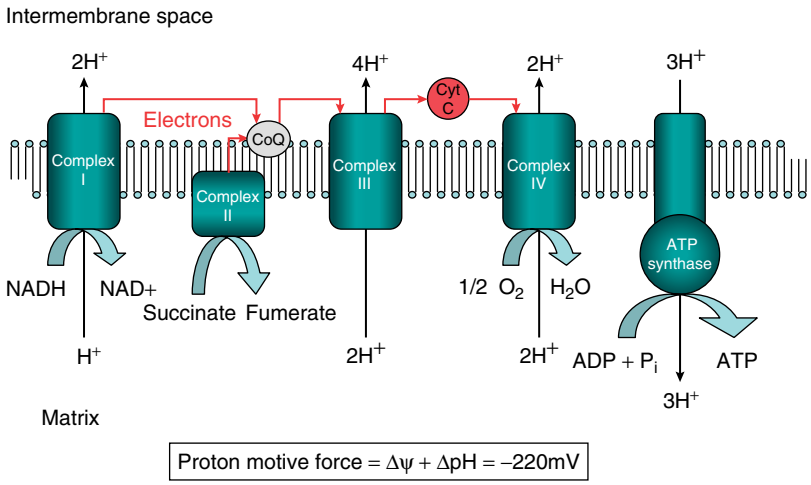

Figure 3 The mitochondrial electron transport chain (ETC) consists of Complex I (NADH-oxidoreductase), Complex II (succinate-dehydrogenase), Complex III (ubiquinol-cytochrome-c-oxidoreductase), Complex IV (cytochrome oxidase) and Complex V (ATPase). Electrons are passed from either Complex I or II to Complex III via the mobile carrier, ubiquinone. Electrons are passed to Complex IV, the final electron acceptor at which oxygen undergoes the four-electron reduction to combine with hydrogen to yield water. The energy released in this process is used to drive ATP synthesis.

However, the molar ratios between the various components are not equal; for every mole of Complex I, there are 3 moles of Complex III, 7 of Complex IV, 9 of cytochrome c and 50 of ubiquinone. Only when the lateral diffusion coefficients are included in the analysis does the equation approach unity for rates of electrons transport (Chazotte and Hackenbrock, 1988). In this light, this process is more akin to an ETS rather than a serial chain. Moreover, recent evidence indicates that the complexes aggregate into 'super complexes' that facilitate the requisite redox reactions (Genova *et al.*, 2003).

Regardless of the molecular architecture of the ETS, the potential energy inherent in the PMF is captured by Complex V (F_0F_1-ATPase) as the flow of protons down the ion and pH gradient into the matrix is transformed into phosphorylation of ADP. The molecular mechanism of Complex V is wonderfully elegant; it is a complex protein motor where protons pass through a channel, and the catalytic domains rotate on an axle held in place by an arching external stator.

Note that ETS and flow of electrons to oxygen is coupled to phosphorylation of ADP by Complex V (OXPHOS), and that such coupling is dependent on the integrity of the inner membrane. If this membrane becomes permeable to protons, ETS is no longer coupled to phosphorylation and ATP production declines to the extent of this uncoupling, reaching zero if ETS can not accelerate and re-establish the PMF. Under these uncoupled conditions, respiration is unopposed by PMF, and oxygen reduction reaches maximum rates. In addition, respiration does not respond to ADP availability. Conversely, under coupled conditions and in the presence of ample substrate and cofactors, respiration accelerates to close to maximum upon exposure to ADP, and then it returns to basal rates after all ADP has been phosphorylated.

2.3 Free-Radical Production and Antioxidants

Molecular oxygen has a high affinity for electrons ($E = +0.82$ V), larger than all the other components of the ETS. As such, any of the 'upstream' components are capable of passing the transiting electron to molecular oxygen. Because of orbital spin restrictions, molecular oxygen preferentially undergoes univalent reductions, the first product of which is the superoxide anion, a free radical (contains an unpaired electron in the outermost orbital). This is thought to underlie the selection pressures resulting in the reaction dynamics at Complex IV, where four electrons accumulate to completely reduce oxygen to water avoiding serial reduction through several reactive products. Superoxide spontaneously dismutates (dismutation is a reaction in which two identical reactants yield two different products, most commonly via simultaneous reduction and oxidation) at fairly fast rates ($\sim 10^5\ M^{-1}\ s^{-1}$ at pH 7) to yield hydrogen peroxide (H_2O_2) and O_2 (ground state). However, this reaction is catalysed by the enzyme superoxide dismutase more than 70 000 times faster ($7 \times 10^9\ M^{-1}\ s^{-1}$), with essentially diffusion-limited kinetics. All aero-tolerant organisms contain at least one form of this metalloenzyme, and mammals have three: a dimeric Cu-Zn superoxide dismutase (SOD) 1 in the cytoplasm and mitochondrial intermembrane space, a Mn-containing tetramer SOD

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2 in the mitochondrial matrix and a Cu-Zn tetrameric isozyme excreted into the intercellular space.

Superoxide is reactive; it can depolymerize hyaluronate, but the Fe–S centres found in several of the electron transport complexes are especially susceptible, resulting in impairment of respiration. Although superoxide is a reactive radical, in the presence of reduced transition metals plentiful in biology, such as Cu and Fe, the H_2O_2 that results from superoxide undergoes Fenton chemistry via the Haber–Weiss reaction to yield the hydroxyl radical (OH·), the most reactive radical known from biological systems:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH\cdot + OH^- \qquad (2)$$

Hydroxyl reacts extraordinarily rapidly with a half-life of 10^{-9} s and so rarely diffuses further than five atomic radii, which essentially precludes enzymic mechanisms to remove it. The radical damages all types of macromolecules, including nucleic acids, yielding mutations, and lipids, initiating peroxidation chain reactions that destabilize membranes, among many other injurious reactions. Importantly, superoxide also efficiently reacts with nitric oxide radicals to yield peroxynitrite ($ONOO^-$), a strong oxidizing agent, that in turn can react with CO_2 to form nitrosoperoxycarbonate ($ONOOCO_2^-$) that homolyses to form the carbonate radical and nitrogen dioxide. The conjugate acid of $ONOO^-$ is peroxynitrous acid (ONOOH), and with a pK_a of ~6, it can also homolyse to yield the hydroxyl radical. It bears reiteration that although in the context here, free-radical production is deleterious, these same reactive molecules are essential for killing pathogens during the inflammatory response, among other signalling and physiological processes.

Regardless of the complexity of the ensuing biological chemistry, production of superoxide is the initial domino in this deleterious cascade, and all organisms maintain antioxidant defences against radicals and the damage they cause that can lead to cell death via either necrotic or apoptotic pathways. The protective systems include a number of low-molecular-weight antioxidants and enzymatic systems. The former category includes vitamin C and vitamin E, as well as reduced glutathione (GSH) and the reduced pyridine nucleotides NADH and NADPH (nicotinamide adenosine dinucleotide phosphate). The first step in the detoxification of the superoxide anion is catalysed by superoxide dismutase (Mn-SOD), which converts two superoxide anions into H_2O_2. The subsequent detoxification of H_2O_2 is catalysed by catalase, which is believed to be intrinsic to heart mitochondria. Another enzyme detoxifying H_2O_2 is glutathione peroxidase, which reacts with reduced GSH as an electron (or oxygen) acceptor, oxidizing it to glutathione disulfide (GSSG).

GSSG resulting from the reactions catalysed by glutathione peroxidases must be reduced back to GSH to enable the process to continue. This is catalysed by glutathione reductase and specifically utilizes NADPH as the electron donor. NADPH can be generated from isocitrate or malate by the action of the respective dehydrogenases, $NADP^+$-dependent mitochondrial isocitrate dehydrogenase or decarboxylating malate dehydrogenase. Thus, regeneration of reduced GSH is an energy-consuming process and can compete with ATP synthesis for the PMF or respiratory substrates, which means that protection against oxidative stress is energetically costly.

There are at least two sites that autoxidize to yield superoxide; Complexes I and III are the primary ones, although under pathogenic conditions other components, such as partially reduced ubiquinone (ubisemiquinone) can also. It stands to reason that when ETS is impaired, particularly if blockade is 'downstream' of these two components, they remain in the fully reduced state, which increases the probability of autoxidation to superoxide, and this can be demonstrated pharmacologically with inhibitors such as antimycin. Under normal conditions, however, when mitochondria are exposed to conditions that induce the irreversible permeability transition (PT; see next section) that uncouples OXPHOS, superoxide and hydroxyl radicals are directly detected using spin trapping and electron paramagnetic resonance technologies. See **Figure 4**, which is the ETC with superoxide formation at Complexes I and III).

2.4 Mitochondria and Ca^{2+} Homoeostasis

In most mammalian cells, the concentration gradient of Ca^{2+} across the plasma membrane is around 10 000-fold, in contrast with that for Na^+ (12-fold), K^+ (35-fold) or Cl^- (29-fold) (Star *et al*., 1987). These gradients are actively maintained via plasma-membrane ion-dependent ATPase that translocates ions against the concentration gradients, often in exchange for a cotransported ion, for example, Na^+-K^+ ATPase. The steep gradient for Ca^{2+} reflects its utility as a cell signal in a wide variety of physiological processes. For example, excitation contraction coupling of muscle cells and neurotransmitter release at neuronal synapses are mediated by transient increases in cytosolic Ca^{2+}. In muscle cells, relaxation is mediated by removal of Ca^{2+} by ATP-dependent sequestration into the sarcoplasmic reticulum and in most other cells into the endoplasmic reticulum and also back out via the plasma membrane. Mitochondria figure prominently in Ca^{2+} homoeostasis because it is rapidly accumulated into the matrix via a trans-inner-membrane transporter, the Ca^{2+} uniporter, driven by the net negative mitochondrial membrane potential. The uniporter has an extraordinarily high V_{max} and is more reasonably considered a channel rather than an classical ion transporter

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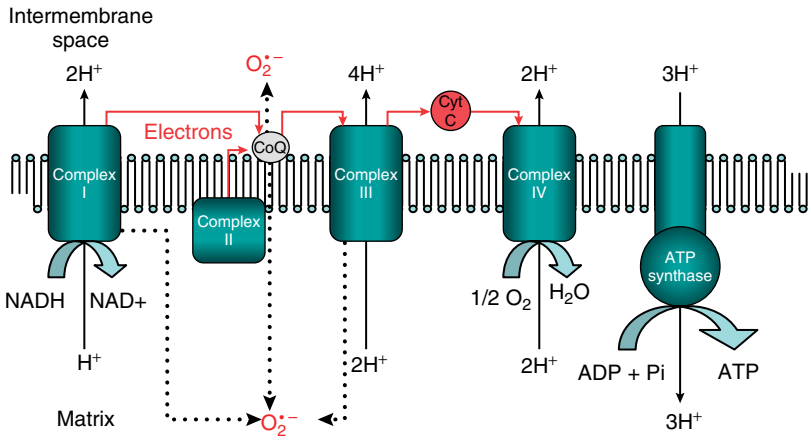

Figure 4 Superoxide production in the ETC by Complexes I and III.

or exchanger such as Na+/K^+-ATPase (Kirichok *et al.*, 2004).

Under physiological conditions, such Ca^{2+} uptake is benign, and even beneficial in that modest Ca^{2+} increases accelerate Krebs cycle fluxes (Szabadkai and Duchen, 2008; Rimessi *et al.*, 2008). Similarly, under physiological conditions, the accumulated Ca^{2+} is pumped out of the mitochondria and reticulia, and hence out of the cell, via ion-dependent exchangers, some of which require ATP directly, while others dissipate membrane potential to fuel ion exchange, and so secondarily require cell energy. However, when cytosolic Ca^{2+} increases beyond a finite level, and/or when such exposures persist, Ca^{2+} accumulates in the mitochondria and gradually destabilizes them and imperils the cell. This is a continuum, ranging from modest to lethal Ca^{2+} exposures, that is influenced by timing issues and whether coincident pathology is afoot, such as hypoxia (Dong *et al.*, 2006). For example, if such Ca^{2+} exposure is rapid, excessive and especially when accompanied by hypoxia, which further reduces ATP production, mitochondria undergo irreversible collapse by a process called the permeability transition (see next section). In the absence of ATP to fuel transmembrane ion-dependent ATPases, the cell undergoes an acute osmotic crisis, and dies by necrosis, eliciting an inflammatory response. When such Ca^{2+} exposure is more gradual, the cell will continue to respond until to a threshold is reached where the Ca^{2+} buffering capacity of the reticulum and mitochondrial compartments is exceeded, and mitochondrial PT will induce apoptosis rather than necrosis.

2.5 Mitochondrial Permeability Transition

The mitochondrial permeability transition (MPT) refers to the opening of a nonselective pore in the inner mitochondrial membrane, which results in permeability to solutes up to 1500 Da. When MPT occurs, mitochondrial membrane potential will collapse and mitochondria will swell (Hunter *et al.*, 1976). The molecular composition of the pore has been subject to many studies and several models have been postulated. In one model, the pore consists of the adenine nucleotide translocator (ANT), the VDAC and cyclophilin D (CypD), which is capable of binding cyclosporin A (CyA), a potent inhibitor of the PT (Halestrap and Brennerb, 2003; Crompton *et al.*, 1999). Recently, it has been reported that MPT can occur in ANT-, VDAC- and CypD-deficient mitochondria, which challenges the proposed model (Kokoszka *et al.*, 2004; Basso *et al.*, 2005; Baines *et al.*, 2007). Another model describes the MPT as being formed from misfolded membrane proteins, which aggregate. In this model, CypD and other chaperones are thought to protect mitochondria from depolarization and swelling. When the formation of pores exceeds the amount of chaperones, unregulated MPT will occur, which in this case cannot be protected by CyA (He and Lemasters, 2002).

2.6 mtDNA Transcription/Translation

Mammalian mitochondria contain a circular genome (mtDNA) with ~16 500 bp, which is well preserved between species. The mtDNA encodes two ribosomal RNAs (rRNAs) for the large and small ribosomal subunits, 22 tRNAs and 13 proteins that are subunits of four of the five OXPHOS complexes of the electron transport chain. From proteomic studies it is estimated that mitochondria contain >1000 distinct proteins, of which ~600–800 have been definitely identified by mass spectroscopy. Thus, the vast majority of proteins in mitochondria are imported.

Mitochondria also contain their own transcriptional and translational machinery necessary to synthesize the 13 essential proteins. The mechanism of mtDNA replication is relatively well understood. There is a major

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and specific mtDNA polymerase belonging to the polymerase gamma family. This enzyme functions not only in DNA replication, but also in mtDNA repair, and in recombination. The mitochondrial translation system in the mammalian organelle is responsible for the synthesis of 13 proteins that constitute subunits in the complexes (I–IV) of the electron transport chain and ATP synthase (V), but not Complex II. Seven subunits are found in Complex I (NADH-ubiquinone-oxidoreductase), one subunit is found in Complex III (the bc1 complex, or ubiquinone-cytochrome-c-oxidoreductase), three subunits form the core of Complex IV (cytochrome c oxidase) and two subunits are part of the ATP synthase (Complex V).

3 OVERVIEW ON DRUG-INDUCED MITOCHONDRIAL TOXICITY

Figure 5 shows the many ways drugs can cause mitochondrial toxicity. Drugs can inhibit the individual OXPHOS complexes, including ATPase, and many drug classes have been shown to do so. Many studies have been performed using classical inhibitors. For example, rotenone, a plant poison still used for fish killing in the Amazon, inhibits Complex I of the ETC. Rotenone is also used to model Parkinson-like syndromes in rats, as it selectively targets the x neurons in the brain. 3-Propionic acid, an inhibitor of OXPHOS Complex II, succinate dehydrogenase, is a model compound to study Huntington's disease, a condition characterized by progressive motor impersistence, chorea and physical instability, accompanied by cognitive decline leading eventually to frank dementia. Antimycin A, an antibiotic, is an inhibitor of OXPHOS Complex III. Incubation of mitochondria with either rotenone or antimycin will generate superoxide, which can lead to oxidative stress, damaging proteins, lipids and DNA within the mitochondria, leading to dysfunction.

The classical compound to inhibit OXPHOS Complex IV, cytochrome oxidase, is KCN. Oligomycin, another antibiotic inhibits ATPase. More than 50 drug classes have been shown to inhibit OXPHOS Complex I, which might not be surprising considering the complexity of Complex I, with more than 50 polypeptides. Another way to cause mitochondrial dysfunction is through uncoupling of the OXPHOS from ATP synthesis, through uncontrolled hydrogen shuttling (uncoupling) across the inner mitochondrial membrane, resulting in dissipation of the mitochondrial transmembrane potential and subsequent energy depletion and injury to the organelle. Compounds can achieve this either through hydrogen shuttling across the membrane, though channel formation or simply through disturbance of the bilayer. Examples are nonsteroidal anti-inflammatory drugs (NSAIDs).

Another mechanism leading to mitochondrial toxicity is through inhibition of mitochondrial carrier proteins such as ANT, and anion and cation carriers. This will lead to substrate depletion, resulting in decreased ATP production. Another way to lead to mitochondrial toxicity is through the inhibition of mtDNA replication and mitochondrial protein synthesis, as described for antivirals (nucleoside reverse transcriptase inhibitors; NRTIs) and antibiotics (tetracyclines, oxazolidinediones), respectively.

Oxidative stress can also lead to mitochondrial dysfunction and can be produced through redox cycling or depletion of antioxidants such as GSH. Drugs can also induce apoptosis through induction of the MPT, which leads to loss of important mitochondrial components (<1200K) such as ATP, NAD, GSH. The result is intrusion of water into the mitochondria leading to swelling, and mitochondrial and cell death. Many cancer drugs will induce the MPT. Drugs can also inhibit enzymes within the glycolytic and FA (Fanconi anaemia) pathways and also in the citric-acid cycle, leading to depletion of substrates.

The following section will provide examples for these different mechanisms in more depth. **Table 1** summarizes the text below and provides relevant references.

4 EXAMPLES OF DRUGS CAUSING MITOCHONDRIAL TOXICITY

4.1 Drugs Effecting Complexes I–V

4.1.1 Complex I

Complex I inhibitors are found throughout many different drug classes. The most studied xenobiotic is rotenone, which is found in plants and is still used as an insecticide. Rotenone is an irreversible inhibitor of Complex I, inhibiting it at two sites, one buried in the hydrophobic part of the inner membrane and the other at an external site on the matrix face. This binding blocks electron transport from the dehydrogenase iron–sulphur cluster to coenzyme Q and results in reactive oxygen species (ROS) formation. Rotenone acts as a semiquinone antagonist and Complex I ROS formation has been attributed to autoxidation of the flavin semiquinone of NADH dehydrogenase. Rotenone treatment of rats leads to selective Complex I inhibition in the brain and can cause Parkinson-like symptoms, which makes it a useful model in Parkinson's research (Horgan *et al.*, 1968).

Another model compound for Parkinson's is MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), identified as a by-product of the 'new heroin' (meperin) synthesis in 1983, when illicit drug users were diagnosed

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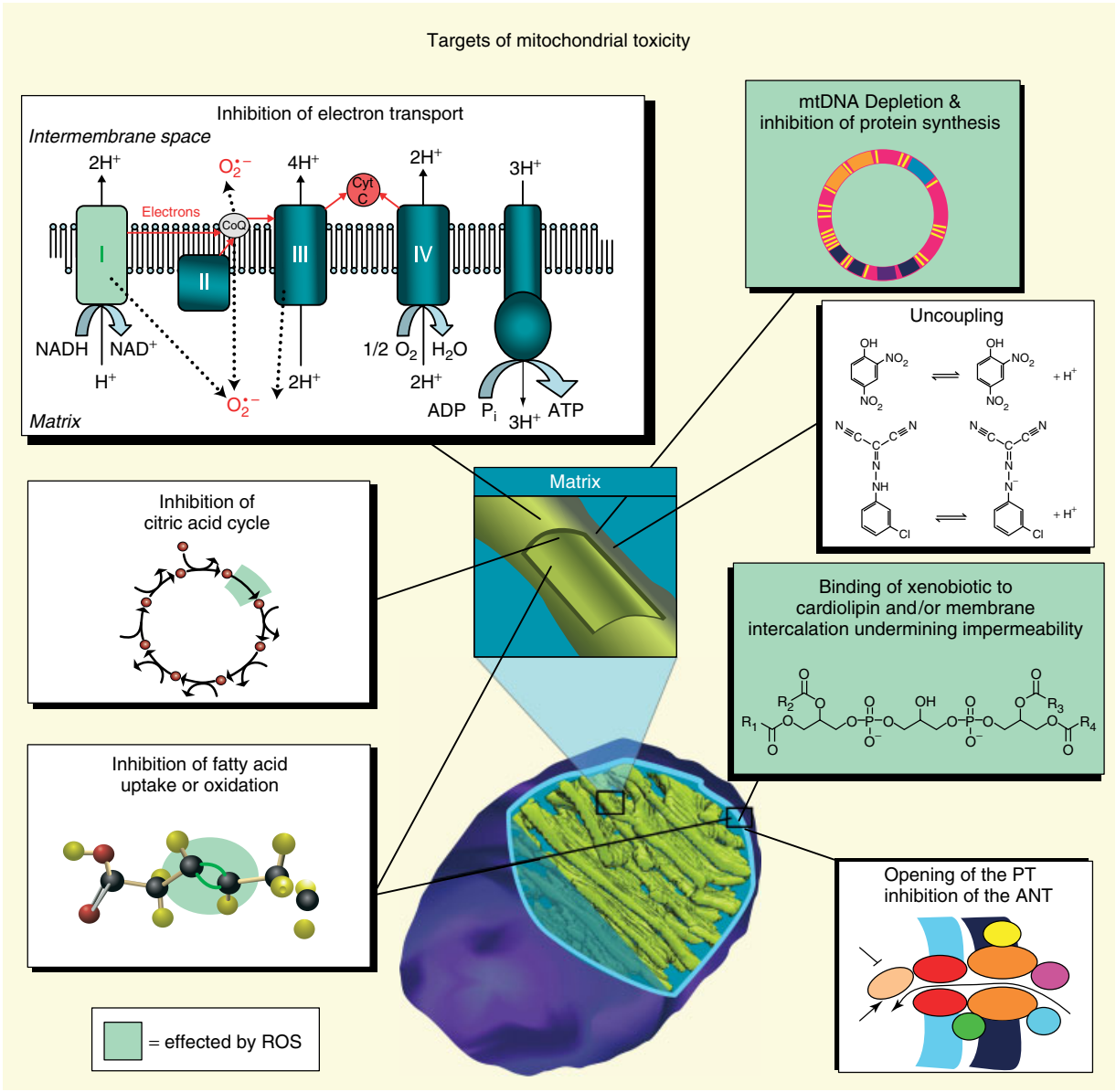

Figure 5 Mitochondrial function can be impaired in many different ways, such as inhibition of ETC proteins, carriers, uncoupling, metabolic-pathway inhibition, mtDNA depletion, inhibition of protein synthesis.

with Parkinson's. MPTP is very lipophilic and therefore can cross the blood–brain barrier. In the brain it is taken up by glial cells. In the astrocytes it is then converted by monoamine oxidase B (MAO-B), which is a mitochondrial enzyme, to MPDP+ (l-methyl-4-phenyl-2,3-dihydropyridinium). MPDP+ autoxidizes to MPP+ (1-methyl-4-phenylpyridinium). MPP+ is then taken up through the dominergic receptor and accumulates in the mitochondria where it inhibits Complex I, causes ROS formation and leads to mitochondrial damage and an energy crisis for the neuron (Ramsay *et al.*, 1987).

There are a variety of other antipsychotic neuroleptic drugs that also inhibit Complex I such as haloperidol, chlorpromazine, fluphenazine and risperidone. Like rotenone and MPTP, these drugs also cause Parkinson-like syndromes, as well as tardive dyskinesia. The rank order of potency for inhibiting Complex I activity in rat brain cortex was haloperidol > chlorpromazine > fluphenazine > risperidone. Similar results were found in human brain cortex (Balijepalli *et al.*, 1999; Maurer and Möller, 1997). Fibrates, such as clofibrate, ciprofibrate and fenofibrate are used therapeutically to decrease hyperlipidaemia. They are agonists of PPARα (peroxisome proliferator-activated receptor α), whose activation induces peroxisomes containing high palmitoyl–CoA ligase activity, which metabolizes fatty acids and lowers blood triglycerides, low-density lipoprotein (LDL) cholesterol and increases high-density

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Table 1 Examples of drugs that cause mitochondrial impairment and their proposed mechanisms

Effect on mitochondria	Compound	Reference
Complex I	Neuroleptics (haloperidol, chlorpromazine, fluphenazine, risperidone, zotepine)	Balijepalli *et al*. (1999) Maurer and Möller (1997)
	Classical inhibitors (rotenone)	Horgan *et al*. (1968)
	Model compound (MPP+)	Ramsay *et al*. (1987)
	Antidiabetics and antihyperlipaedimics: fibrates (clofibrate, ciprofibrate, fenofibrate), thiazolidinediones (rosiglitazone, troglitazone) and biguanides (phenformin, buformin, metformin)	Dykens *et al*. (2008a) Nadanaciva *et al*. (2007) Brunmair *et al*. (2004a) Brunmair *et al*. (2004b) El-Mir *et al*. (2000)
	Antiandrogen (flutamide)	Coe *et al*. (2007)
	Antidepressant (nefazodone)	Dykens *et al*. (2008b)
	Anticancer (sorafenib)	Will *et al*. (2008)
Complex II/III	Cancer and autoimmune (cyclophosphamide) and antifungal (ketoconazole)	Souid *et al*. (2003)
	Classical inhibitors (antimycin)	Schagger *et al*. (1995)
	Anticancer (tamoxifen, sorafenib)	Will *et al*. (2008) Tuquet *et al*. (2000)
Complex IV	Thiazolidinediones (darglitazone, troglitazone)	Nadanaciva *et al*. (2007)
	Anticancer (tamoxifen, sorafenib)	Will *et al*. (2008) Tuquet *et al*. (2000)
Complex V	Classical inhibitor (oligomycin)	Walker *et al*. (1995)
	Dietary supplement (resveratrol)	Gledhill *et al*. (2007)
Uncouplers	Classical uncouplers (FCCP, DNP)	Cunarro and Weiner (1975)
	NSAIDs (flufenamic acid, diflunisal, tolfenamic acid, mefenamic acid, indomethacin, benoxaprofen, naproxen, fenoprofen, nimesulide, diclofenac)	Ong *et al*. (2006) Boelsterli (2003) Masubuchi *et al*. (1999)
	Anaesthetics (bupivacaine, etidocaine, lidocaine)	Sztark *et al*. (1998)
	Parkinsons (tolcapone)	Haasio *et al*. (2002)
	Anticancer (tamoxifen)	Tuquet *et al*. (2000)
	Antidiabetic (WY-14643, fenofibrate)	Zungu *et al*. (2006)
	Antidepressant (fluoxetine)	Souza *et al*. (1994)
Inhibitors of mtDNA replication	Antivirals (ddC > ddI > d4T > AZT)	Zungu *et al*. (2006)
Inhibitors of mt protein synthesis	Antibiotics (chloramphenicol, tetracyclines, aminoglycosides, macrolides, lincosamides, oxazolidinones (linezolid)).	Duewelhenke *et al*. (2007) McKee *et al*. (2006)
Inhibitors of Krebs cycle enzymes and fatty acid metabolism	Anticonvulsant (valproic acid)	Nulton-Persson *et al*. (2004)
	Antidepressant (amineptine, tianeptine)	Pessayre *et al*. (1999)
	Antibiotics (tetracycline derivatives)	Fromenty and Pessayre (1997)
	Salicylates (aspirin, ibuprofen)	
Oxidative stress	Analgesic (acetaminophen)	Jaeschke *et al*. (2003)
	Anticancer (doxorubicin, danunorubicin)	Berthiaume and Wallace (2007)
	Antidiabetic (chlofibrate, ciprofibrate)	Qu *et al*. (2001)
	Antianginal (amiodarone, perhexillin)	Ribeiro *et al*. (1997)
Induction of the mitochondrial permeability transition	Anticancer (lamellarin D)	
	NSAIDs (ibuprofen, nimesulide)	Berson *et al*. (2006)
	Analgesic (acetaminophen)	Kon *et al*. (2004)
	Antidiabetic (gemfibrozyl, WY-14643, troglitazone)	Zhou and Wallace (1999)

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lipoprotein (HDL). Fibrates can cause liver toxicity, hepatomegaly and muscle toxicity in some patients. All fibrates inhibit Complex I (Brunmair *et al*., 2004b; Nadanaciva *et al*., 2007).

The biguanide agents, phenformin, buformin and metformin act as antidiabetic drugs by inhibiting hepatic glucose release, gluconeogenesis and β-oxidation of fatty acids. Phenformin and buformin both were discontinued in 1977 and 1978, whereas metformin is still available due to its 20-fold less potency for lactic acidosis. All three compounds inhibit Complex I of the OXPHOS. Using immunocapture, the IC50 (half maximal inhibitory concentration) for Complex I inhibition was 1.2, 11.3 and 66 mM for phenformin, buformin and metformin, respectively (Dykens *et al*., 2008b). Phenformin was shown to have an IC50 of 4.97 $\pm$ 0.87 μM for lactic acidosis and also was shown to significantly inhibit mitochondrial respiration after 40 minutes preincubation (Dykens *et al*., 2008b). In contrast, buformin was much less potent in inhibiting the oxygen consumption and its reported IC50 for lactic acidosis is 119 $\pm$ 18 μM. The least toxic compound, which is the only one of the three still on the market is metformin, where the IC50 for lactic acidosis is close to mM concentrations (734 $\pm$ 168 μM). It is thought that these compounds bioaccumulate in the mitochondria, a fact that is supported by our findings that 30 minutes preincubation was required to achieve inhibition of glutamate/malate-driven respiration (Dykens *et al*., 2008b).

Thiazolidinediones are antihyperglycemic drugs used for Type 2 diabetes. The effectiveness of thiazolidinediones for inhibiting Complex I or causing lactate release in skeletal muscle or rat liver homogenates was found to be in the order: rosiglitazone > troglitazone $\gg$ metformin (Brunmair *et al*., 2004b). In addition, a recent study reported that the major targets for thiazolidinediones are Complexes IV and V of the OXPHOS (Nadanaciva *et al*., 2007). Results from this study showed the rank order of potency on mitochondrial respiration to be: rosiglitazone, pioglitazone > darglitazone, muraglitazar > troglitazone. Troglitazone was withdrawn form the market in 1997, due to idiosyncratic hepatotoxicity. Darglitazone and muraglitazar were discontinued in development. Rosiglitazone and pioglitazone recently received a black box label[a] for potential cardiotoxicity. The link between mitochondrial toxicity and the potential contribution to cardiotoxicity remains to be established.

Flutamide also inhibits Complex I (Coe *et al*., 2007) and causes hepatotoxicity. It is thought to mainly inhibit Complex I even though inhibition of succinate-driven respiration has also been shown to occur. Flutamide decreases ATP in hepatocytes and leads to GSH depletion. This might result in inhibition of succinate dehydrogenase, an enzyme containing SH groups in its catalytic site.

Recently, nefazodone was also found to inhibit Complex I, which potentially could contribute to the idiosyncratic hepatotoxicity that led to its withdrawal in 2004 (Dykens *et al*., 2008a).

4.1.2 Complex II/III Inhibitors

Only very few drugs are known to inhibit Complexes II and III of the OXPHOS. Oxaloacetate and malonate are competitive inhibitors of Complex II. Cyclophosphamide or ketoconazole, and hydrazine also inhibit succinate dehydrogenase. 3-Nitropropionic acid inhibits Complex II and is used to mimic Huntington's disease (Souid *et al*., 2003).

The classical inhibitor for Complex III is antimycin. Its binding will cause ROS formation in the OXPHOS chain (Schagger *et al*., 1995). Another Complex III inhibitor is stigmatellin.

Tamoxifen has also been shown to inhibit Complex III (Tuquet *et al*., 2000).

4.1.3 Complex IV Inhibitors

As mentioned above, it has been shown that the main targets of OXPHOS inhibition for the thiazolidinediones are Complexes IV and V (Nadanaciva *et al*., 2007).

Many local anaesthetics inhibit cytochrome c oxidase following order of potency: quinisocaine > butacaine > pramocaine > bupivacaine > carticaine > lidocaine, procaine > prilocaine. This inhibition is in accord with their potency as anaesthetics, and correlates with their lipophilicity (log P) (Casanovas *et al*., 1983; Stringer and Harmon, 1990).

4.1.4 Complex V Inhibitors

The classical Complex V inhibitor is oligomycin (Walker *et al*., 1995). Resveratrol also inhibits Complex V (Gledhill *et al*., 2007), but *in vivo* translation has not been established.

4.2 Drugs Uncoupling OXPHOS

The most well-known mitochondrial uncoupler is dinitrophenol (DNP), which was widely used as a wood preserver. In the 1930s it was used as a diet pill. It caused dramatic weight loss. However, no feedback mechanism exists *in vivo* to regulate the upper limit of body temperature and so people died with overdose. DNP is still illegally used by body builders (Cunarro and Weiner, 1975).

Many members of the NSAIDs are uncouplers. They contain a carboxyl (weak) acid group which can bind to the arachinonate binding sites for COXI and COXII (cyclo-oxygenases I and II). The lipid-soluble nature of the NSAIDs and also the acid group allows the NSAIDs

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to interact with the inner mitochondrial membrane of enterocytes, binding to phospholipids. They act as protonophores, leading to an increase in oxygen consumption causing ATP depletion and subsequent cell injury and cell death. There are different classes of NSAIDs, such as salicylic acids (aspirin), phenylacetic acids (diclofenac), carbo and heterocyclic acids (indomethacin, sulindac), profionic acids (ibuprofen, fenoprofen, ketoprofen) and fenamic acids (flufenamic acid). NSAIDs potency for increasing basal respiration and inhibiting ADP-stimulated respiration was found to be in the order: flufenamic acid, diflunisal > tolfenamic acid > mefenamic acid > diclofenac > indomethacin > benoxaprofen, naproxen, fenoprofen (Masubuchi *et al*., 1999).

The nitroaromatic compound, nimesulide, also uncouples mitochondrial respiration. Unlike the NSAIDs described above, nimesulide causes less gastroinsestinal toxicity, but rare and serious hepatic dysfunction (Ong *et al*., 2006).

Diclofenac also can cause rare, but serious hepatotoxicity. Diclofenac is taken up by the mitochondrial anion carrier. It uncouples and also induces the MPT (Boelsterli, 2003).

Several long-acting anaesthetics (bupivacaine and etidocaine) have caused severe cardio and myotoxicity. These compounds are lipophilic amphiphilic amines, which can act as true protonophores. Lidocaine also uncouples mitochondria, but to much less extent (Sztark *et al*., 1998).

Tolcapone was withdrawn after only being on the market for six months due to several deaths through hepatic failures. Tolcapone uncouples rat liver mitochondria at 10 μm whereas 50 μM of DNP or 200 μM of entacapone is needed for the same amount of uncoupling (Haasio *et al*., 2002).

The last compound to be discussed is tamoxifen, one of the most widely used agents to treat breast cancer. Tamoxifen may induce fatty liver and nonalcoholic steatohepatitis (NASH) in patients. Tamoxifen has been shown to uncouple mitochondria at 10 nmol mg^{-1} protein, whereas at a dose of 40 nmol mg^{-1} protein, ATP-dependent respiration is inhibited (Tuquet *et al*., 2000).

4.3 Drugs Causing Oxidative Stress

Doxorubicin, an anthracycline, is used for the treatment of various solid tumours and also lymphomas. Its use is somewhat compromised by the fact that it causes cumulative and dose-dependent nonreversible damage to the heart. An increased incidence of arrhythmias has been reported, as well as degeneration of the cardiac muscle. Much work has been done in trying to understand the mechanism leading to the cardiac-specific injury. It is now understood that doxorubicin selectively redox cycles on Complex I of the OXPHOS (see **Figure 4**). The consequences of this are GSH depletion and induction of the MPTP, as well as oxidation of mtDNA. Progress also has been made in trying to understand the cardio-specificity of doxorubicin. Several hypotheses have been postulated: (i) the heart has a high energy demand and therefore relies on continuous ATP production; (ii) the heart has low antioxidant capacity and therefore is prone to oxidative stress and its consequences; (iii) doxorubicin alters cardiac-specific gene transcription; (iv) doxorubicin causes alterations in sarcomeric protein organization and potentially targets nuclear lamina proteins (Berthiaume and Wallace, 2007).

4.4 Drugs Effecting Mitochondrial DNA Synthesis

The adverse effects that are predominantly attributed to NRTIs include peripheral neuropathy, cardiomyopathy, hepatic steatosis and hepatotoxicity, hyperlactataemia and lactic acidosis, Type 2 diabetes or insulin resistance, and lipodystrophy. *In vitro* inhibition of mtDNA transcription shows the following rank order of toxicity: ddC > ddI > d4T > AZT > ABC = 3TC = TDF. The *in vitro* findings correlate well with the observed clinical adverse events related to mitochondrial toxicity (Zungu *et al*., 2006); specifically, ddC and d4T are known to be highly toxic. The European Medicines Evaluation Agency actually issued a guidance document that requires testing of all newly submitted antivirals for mitochondrial DNA depletion (EMEA, 2005). In the USA, there is also guidance for industry (CDER, 2006).

4.5 Drugs Effecting Mitochondrial Protein Synthesis

Classes of antibiotics that bind to the bacterial ribosome and inhibit bacterial protein synthesis include: chloramphenicol, tetracyclines, aminoglycosides, macrolides, lincosamides and most recently the oxazolidinones. IC50 values obtained *in vitro* for inhibition of mitochondrial protein synthesis are well in accord with therapeutic doses. Toxicities observed are different for different antibiotics. For example, chloramphenicol causes a dose-dependent and reversible bone-marrow depression and fatal aplastic anaemia in certain genetically sensitive individuals. Tetracyclines can cause anaemia, thrombocytopenia, central nervous system effects, endocrine and metabolic effects, tooth discolouration, skin photosensitivity, and renal and liver toxicity, especially liver steatosis and myopathy. Members of the aminoglycosides are known to concentrate and persist in the endolymph

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and perilymph of the inner ear, causing ototoxicity when given at high concentrations.

Linezolid, which belongs to the newer class of the oxazolidinones, causes dose-dependent and reversible bone-marrow suppression (McKee *et al*., 2006; Duewelhenke *et al*., 2007) (**Table 1**).

4.6 Methods to Investigate Mitochondrial Dysfunction

4.6.1 Oxygen Consumption/pH

The traditional way to measure mitochondrial function/dysfunction is by measuring oxygen consumption (oxygraph). Most used for this purpose is still the Clark electrode which is connected to a small glass chamber holding 0.5–3 ml buffer, substrates and mitochondria (Examples of suppliers are *www.oroboros.at* and *www.hansatech-instruments.com*; Hansatech and Orosboros). The chamber is sealed with a plunger to avoid exchange of oxygen with the environment. A chart recorder or computer programmes can be used to monitor oxygen consumption over time. By comparing basal respiration (state 2/4) with ADP-stimulated respiration, one gains understanding on how functional/dysfunctional the mitochondria are.

Figure 6 shows a typical respiration measurement. First mitochondria (usually at 0.5–1 mg ml^{-1}) are added to the chamber. Respiration should be zero if mitochondria have been isolated intact. Upon addition of the substrate, basal respiration is measurable as State 2, which should be low, as no phosphorylation occurs. Upon addition of a small amount of ADP, respiration rapidly increases (State 3). Upon complete phosphorylation of ADP, mitochondria will return to basal respiration (State 4). Often respiratory ratios are calculated by dividing the rate at State 3 by the rate at State 4. In isolated rat-liver mitochondria, this rate would be approximately 6–8 if glutamate/malate is used to fuel OXPHOS Complex I and approximately 4–5 when succinate is used as a substrate.

Naturally, respiratory control ratios (RCRs) are higher for heart and muscle mitochondria with glutamate/malate and can easily reach values of 8–10. An excellent method for oxygraph measurements is described by Heisler (1991).

As mentioned above, drugs can have different effects on respiration, which is illustrated in the figure. For example, if a drug is added to the mitochondria and, after addition of glutamate/malate, no change in oxygen consumption is observed, this compound is a Complex I inhibitor. The block could be bypassed by addition of succinate. If a drug increases basal respiration, it is an uncoupler. Several different degrees of inhibition and uncoupling can be observed, depending on the potency of the compound/drug.

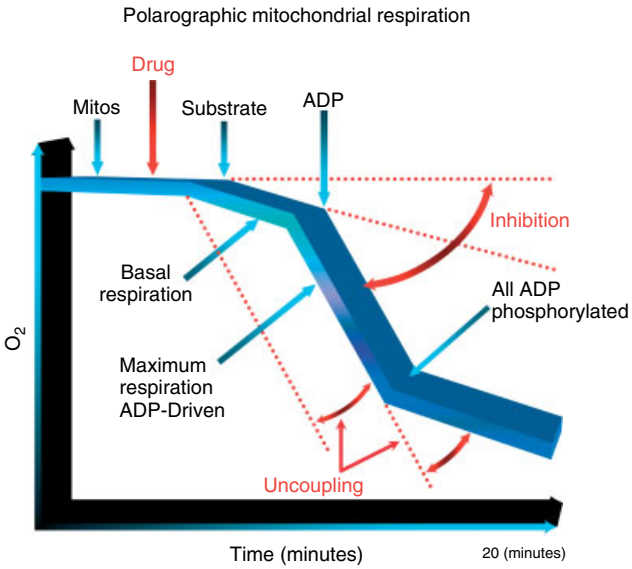

Figure 6 Polarographic measurement of oxygen consumption.

Whereas the oxygraph is the main work horse for students and academics, the method is not applicable to the industrial setting. Here, many compounds will have to be screened to avoid mitochondrial liabilities. Static and soluble sensor technology has allowed much higher throughput (Seahorse and Luxcel). Oxygen consumption can be measured in isolated mitochondria after *in vivo* treatment with drugs or *in vitro* by adding drugs of interest to isolated mitochondria.

Oxygen consumption can also be measured in cells. Whereas the oxygraph will require a larger amount of cells and low throughput, the sensors allow measurement at higher throughput. Oxygen-consumption measurement in cells becomes even more meaningful when paired with pH measurement. A true mitochondrial toxicant would cause an increase in oxygen consumption with a subsequent increase in acidification as compensation through glycolysis, whereas drugs not acting through the mitochondria will lead to a decline in oxygen consumption without a concomitant increase in acidification. pH measurements can also be made using solid and soluble sensor technologies.

4.7 OXPHOS Activities

Often it is also important to understand the possible mechanisms of mitochondrial toxicity as described in **Figure 5**. Activities of the OXPHOS complexes can be measured by spectrophotometry, either in isolated mitochondria, cells or tissues. After careful solubilization with an appropriate detergent, substrates are added, the reaction is monitored over time and rate constants are calculated (Birch-Machin *et al*., 1993). By combining the measurements of amount and activity

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through immunocapture, sensitivity can be increased even more (Nadanaciva *et al*., 2007).

5 MITOCHONDRIAL PERMEABILITY TRANSITION

Induction of MPT can be monitored in isolated mitochondria and cells, where it can be measured as swelling, which manifests itself in a decrease in absorbance, monitored at 680 nm spectrophotometrically. Compounds are usually tested in the presence of calcium, which in itself can induce MPT at high concentrations. The classical inhibitors of MPT are cyclosporin A, bongrecic acid and ATP. In cells, MPT is much more difficult to demonstrate. It is measured using calcium and membrane-potential dyes in combination using confocal imaging or other live cell imaging. (Nieminen *et al*., 1995; Qian *et al*., 1997; Trollinger *et al*., 1997; Kim *et al*., 2006).

5.1 Oxidative Stress (ROS)

Oxidative stress (ROS) generation can also be measured in both isolated mitochondria and cells, using specific dyes for superoxide formation such as dihydroethidium (Tay *et al*., 2005), mitosox (Fauconnier *et al*., 2007) and amplex red (Starkov and Fiskum, 2003).

5.2 Cell-Based Assays

Modern drug-discovery and development programmes rely heavily on cell-culture models to assess drug activity and toxicity. The organ toxicities of the drugs discussed in this chapter were not predicted by such culture models, and so drug-induced mitochondrial dysfunction is only now becoming more widely recognized as an important aspect of drug toxicity. Even potent mitotoxicants failed to kill cells in culture, or cause organ toxicity in animal models designed to detect it. Now that we know more about mitochondrial failure, we understand why the standard cell-based assays did not reveal off-target mitochondrial impairment. Indeed, many cells in culture are resistant to canonical mitotoxins such as cyanide, antimycin, rotenone and oligomycin, let alone to less potent xenobiotics being developed as therapeutics. This is because cells grown in culture are almost always provided with abundant glucose as oxidizable substrate, typically 25 mM, which is fivefold higher than physiological. Because of this excess glucose, the medium does not have to be changed as frequently. However, under these high-glucose conditions, the cells are almost completely glycolytically poised, that is, relying on glycolysis for all their ATP needs.

This condition was noted as early as 1929 when Crabtree and Warburg independently found that under high glucose conditions respiration mitochondrial OXPHOS is repressed (Crabtree effect), and that lactate is produced despite the presence of competent mitochondria (Warburg effect). Although there are differences between these two effects, they are essentially variations on a metabolic theme that has generally been attributed to the fundamental glycolytic nature of cancer cells. However, the notion that cancer cells are glycolytic by disposition does not necessarily hold. In a metanalysis of studies where both oxygen consumption and lactate efflux were concomitantly monitored, Zu and Guppy (2004) concluded from 16 studies of normal, freshly isolated cells, that glycolysis contributes anywhere from less than 1% to almost 60% of the total ATP, with an average of 20%, but with a standard deviation that exceeds the mean (SD = 21). A similar analysis of more than 30 cancer cell lines revealed that, on average, these transformed cells derive 18% of their ATP from glycolysis, with an SD that also exceeds the mean (SD = 19). Zu and Guppy (2004) concluded that solid tumours are glycolytically poised because they are hypoxic *in vivo*, not because of fundamental reorganization of metabolic poise. Interestingly, glycolysis increases 34-fold when rabbit thymocytes shift from a resting to a proliferating state, which increases the percentage of glycolytic ATP by an order of magnitude. In this way, the susceptibility of cells to metabolic impairment also depends on what growth phase or state of stimulation the cells are in. Debate continues, no doubt confounded by the nature of these moving targets, but it is clear that we still do not fully understand regulation of metabolic poise.

To render glycolytic cells susceptible to mitotoxicants and hence useful for prediction *in vivo*, glucose in the medium can be replaced by galactose, a sugar that, together with glucose, forms the disaccharide lactose. The net ATP yield from glycolysis with glucose as substrate is 2 mol per mol of fuel. However, for galactose to enter glycolysis, two ATP equivalents have to be invested, so that the net yield from glycolysis to lactate is zero ATP. Under these conditions, the cells are compelled to generate ATP via OXPHOS, and as such become susceptible to mitotoxins (Marroquin *et al*., 2007). Indeed, 100% of HepG2 cells grown in galactose are killed by concentrations of oligomycin in which more than 80% of the glucose-grown cells remain viable (Marroquin *et al*., 2007). The facility with which various cell lines can accomplish this switch is a characteristic of the line. In our hands, hepatoma cells (e.g. HepG2) show immediate and spontaneous increases in respiration and dimunition of extracellular acidification rates, indicating that fully functional mitochondria are present, but not being used much for ATP production. However, other cell lines, such as neuroblastoma (SH SY-5Y) die when acutely transferred into galactose media, so that these

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cells require gradual replacement of glucose by galactose over numerous passages. No doubt clonal selection results form the imposed metabolic bottleneck in addition to upregulation of organelle biosynthesis. The use of galactose-grown cells for drug-toxicity screening is just now becoming more widely appreciated, but we suspect the model will gain wide acceptance once its utility for predicting drug safety in *in vivo* models is confirmed. This raises the question of why the standard preclinical *in vivo* models used to evaluate drug safety also fail to predict organ toxicity of many mitotoxins, not to mention many drugs without mitochondrial liabilities. It should be noted in this context that almost all such *in vivo* evaluations are conducted in young, perfectly healthy and drug-naïve animals, precisely the circumstances where drug-induced mitochondrial impairment (DIMI) is least likely to be detected.

The model whereby DIMI yields cell and organ pathology is based on threshold effects and physiological scope, which is the spare capacity inherent in any metabolic pathway. This model also incorporates nonclassical pharmacokinetics, where selective tissue bioaccumulation of the drug can yield cytosolic and mitochondrial concentrations that far exceed plasma levels, which are the primary measures of drug exposure in most current drug-development programmes. In this way, a drug at plasma concentrations insufficient to yield mitochondrial impairment in one cell type can selectively accumulate to injurious levels in another, resulting in toxicity to that organ.

The threshold at which DIMI emerges is likely to be relatively fixed and varies among different cell types. For example, an unstressed hepatocyte needs some finite amount of ATP to conduct normal business, which includes albumin secretion, xenobiotic detoxification, glucose homoeostasis and a host of other processes. This is also the case for more aerobically demanding cells, such as myocardiocytes and neurons that have higher ATP turnover, but also a minimum requirement for homoeostasis. So, although the basal energetic demands varies in different cell types, in a given cell type it is likely to be relatively constant across the population, and therefore unlikely to underlie the idiosyncratic nature of many toxic drug responses. Rather, it is more likely that the aerobic reserve capacity above the threshold needed to maintain basal function varies more widely among individuals. For example, the mitochondrial capacity in a hepatocyte from an alcoholic is substantially less than that found in a drug-naive person in liver disease, so that the impaired hepatocyte will tolerate loss of mitochondrial capacity via drug exposure less well than a cell with robust reserve capacity, and so will show organ toxicity sooner, that is, idiosyncratically (Sastre *et al*., 2007).

6 MITOCHONDRIAL IMPAIRMENT IN IDIOSYNCRATIC DRUG TOXICITY

In an elegant review, Roger Ulrich (2007) points out those idiosyncratic drug reactions, that is, rare toxicity not predicted by preclinical data and unrelated to dose, result from the convergence of several variables that are characteristic for individuals, but that vary across the population. As several of these risk factors converge, the probability of drug-induced organ toxicity increases. The most important factor, that is, the one which accounts for the largest fraction of variance, is increasing age. But Ulrich also identified inhibition of key cellular function, physical activity, genetics and inherited metabolic defects, concurrent exposures, heterogeneity in drug metabolism and bioactivation, underlying disease, nutrition and innate immune response, as other risk factors.

It is telling that all of these risk factors are also unambiguously implicated in idiosyncratic toxicity caused by mitochondrial impairment. In light of the preceding discussion of threshold effects, the mitochondrial capacity declines as we age, reducing the physiological scope and lowering the bioenergetic threshold for cell death. Going through the list of risk factors, almost all have direct mitochondrial counterparts. For example, with inhibition of key cellular function, under normoxic physiological circumstances mitochondrial OXPHOS generates >90% of cellular ATP and, depending on the tissue, energy demand and physiological scope, even modest impairment could prove injurious. Mitochondrial biomass is directly related to physical activity; depending on the timing and type of exercise, the more we exercise, the greater the mitochondrial capacity. As such, a well-trained athlete has greater mitochondrial reserves, and hence can tolerate more DIMI before pathology emerges. This is particularly true for tissues where the bioenergetic capacity is subject to conditioning, such as skeletal muscle and heart, but less so for an organ such as the liver, where bioenergetic capacity reflects organ history more than condition.

Earlier we mentioned that mutations or deletions in mtDNA cause a host of pathologies, and exposure to drugs with mitochondrial toxicity is reported to reveal a previously silent mtDNA disorder, or to exacerbate already frank pathology (Schon *et al*., 2008). In addition, polypharmacy with several mitochondrial toxicants is at least additive (Dykens *et al*., 2007). Heterogeneity in drug metabolism is also an important factor; for example, there are at least six metabolites of troglitazone that have been identified, and the complement of the various cytochrome P450s and other drug-metabolizing systems is thought to contribute to idiosyncratic responses in the population. Not every individual will metabolize the drug in the same way. However, the parent molecule without any such metabolism is a potent mitotoxin, so

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that we need not invoke a reactive metabolite to explain its toxicity (Dykens *et al*., 2007). However, we may find that such metabolism reduces its toxicity, and in this way heterogeneity in the population would still serve to render some individuals more susceptible to the drug than others. Also, mitochondria may well be capable of catalysing some drug-metabolizing reactions, particularly hydroxylations.

In this light, idiosyncratic drug reactions, and hence organ toxicity due to idiosyncratic mitochondrial reactions, depend on age, organ history and genetics, among others, as noted. However, the animals used in almost all preclinical studies for drug toxicity are young, with abundant mitochondrial capacity not yet diminished by age or drug exposure, and hence possessing maximum bioenergetic scope. As noted above, these are exactly the wrong circumstances to detect drug-induced mitochondrial dysfunction. For example, hepatotoxicity by troglitazone is not detected preclinically in such healthy animals until the activity of the mitochondrial form of the antioxidant enzyme superoxide dismutase is reduced by 50% (Ong *et al*., 2007). In these animals, with artificially repressed reserve antioxidant capacity, troglitazone yields frank hepatic lesions. It remains to be determined whether such pathology is precipitated by the increased oxidative stress resulting from loss of antioxidant capacity *per se,* or to secondary mitochondrial impairment resulting from oxidative inactivation of OXPHOS Fe–S centres. Given that both pathological processes are so functionally intertwined, it seems likely that both contribute to the aetiology of the lesion.

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NOTES

[a] A black box warning in the USA is information on the package insert for prescription drugs warning that they may cause serious adverse effects.

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Toxicity of Peroxisome Proliferators

John P. Vanden Heuvel

CONTENTS

1 PEROXISOMES: HISTORY AND FUNCTION

Peroxisomes were first described in mouse kidney cells as 'microbodies' characterized as subcellular organelles with single lipid membranes surrounding a granular matrix of soluble proteins (de Duve, 1969). Subsequently, peroxisomes were shown to be present in many cell types in numerous organisms including plants and mammalian cells. The distribution and relative size of peroxisomes in mammals is variable. Cells containing considerable amounts of peroxisomes include liver, kidney, adrenal cortex and sebaceous cells, while peroxisomes are rarely found in smooth muscle or fibroblasts. In rat liver, peroxisomes account for approximately 2.5% of total protein and are approximately 1.5% of the cell volume (Weibel *et al.*, 1969).

Urate oxidase, D-amino acid oxidase and catalase were some of the first enzymes identified that were localized in peroxisomes (de Duve, 1969). The term 'peroxisome' was first used to describe these organelles, based on the biochemical property of peroxide formation by the enzymes associated with peroxisomes. Since this time, numerous enzymes have been localized within peroxisomes and include many that catalyze reactions central to lipid metabolism. Among the general groups of enzymes are those involved in fatty acid activation (acyl-CoA synthases), β-oxidation, carnitine acyltransferase, bile acid synthesis, glycerolipid synthesis, cholesterol synthesis, fatty acid elongation, metabolism of oxygen or reactive oxygen species and nucleotide binding proteins (see Osmundsen *et al.* (1991) for review).

Biological roles of peroxisomes in respiration, gluconeogenesis, thermogenesis, purine catabolism and lipid metabolism have all been described. There are similar enzymes to those found in peroxisomes in other cellular compartments, including the cytoplasm and mitochondria. This is particularly true for the fatty acid metabolizing enzymes, where similar steps in β-oxidation occur in both peroxisomes and mitochondria (see **Figure 1**). Peroxisomes preferentially oxidize long-chain fatty acids prior to further mitochondrial oxidation to modulate energy production, since peroxisomal oxidation yields H_2O_2 and heat, rather than mitochondrial oxidation which produces energy in the form of NADH or $FADH_2$. A key component of the peroxisome is the enzyme catalase. This enzyme detoxifies hydrogen peroxide generated from the first and rate-limiting enzyme in the peroxisomal β-oxidation pathway, acyl-CoA oxidase (ACO). The relationship between catalase, ACO and cancer will be discussed below under the auspices of oxidative stress.

2 PEROXISOME PROLIFERATION

The phenomenon of peroxisome proliferation was first reported by several groups in the mid-1960s (Hess *et al.*, 1965). These investigators demonstrated significant increases of hepatic peroxisomes in both size and

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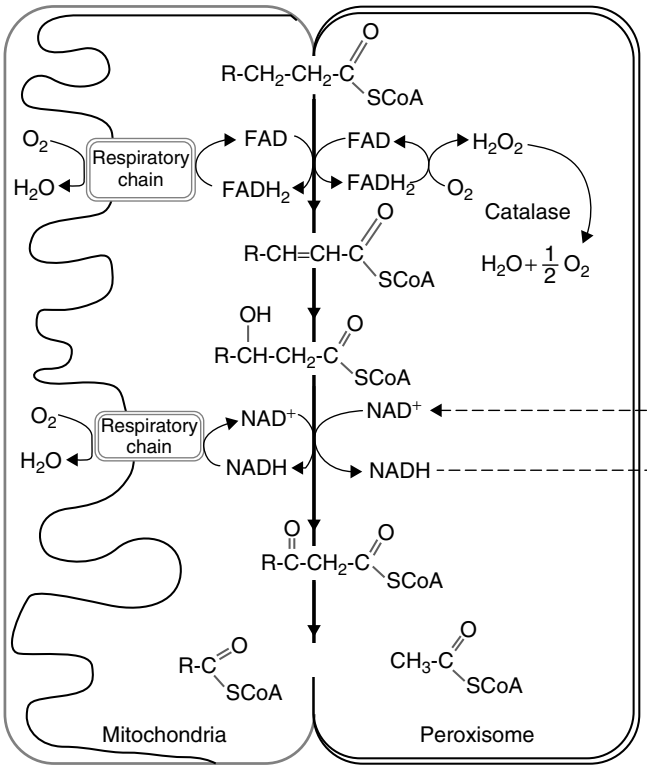

Figure 1 Comparison of the fatty acid β-oxidation pathway in the mitochondria versus the peroxisome.

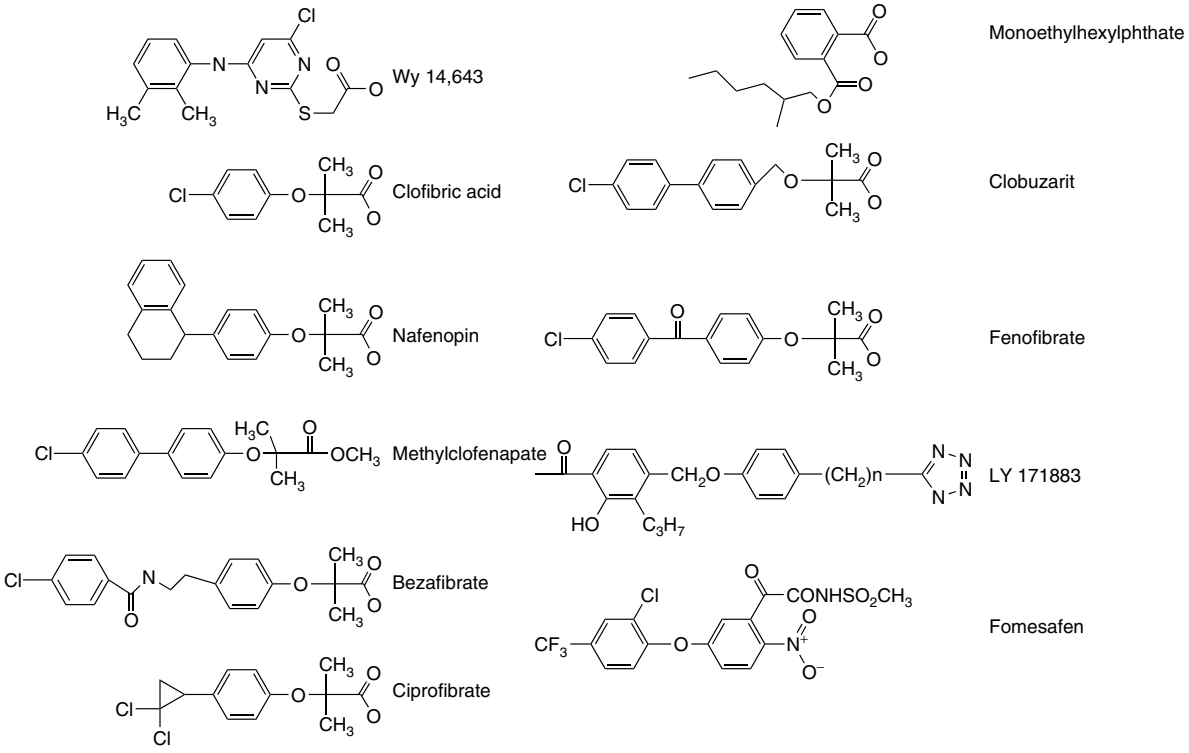

Figure 2 Structure of representative peroxisome proliferators.

number in response to administration of the hypolipidaemic drug clofibrate in rats. This peroxisome proliferation accompanied hepatomegaly in these animals. Peroxisomes typically occupy less than 2% of the cytoplasmic volume, while treatment with fibrate drugs increases this volume to as much as 25%. In addition to

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peroxisome proliferation and hepatomegaly, peroxisomal fatty acid oxidation is induced, and long-term administration of hypolipidaemic drugs causes hepatocarcinogenesis (Reddy *et al.*, 1976), as will be discussed in more detail below. A number of structurally diverse compounds were later identified that share the morphological and biochemical response of clofibrate and deemed 'peroxisome proliferators'. Peroxisome proliferators (PPs) are a diverse class of chemicals and include the fibrate class of hypolipidaemic drugs (clofibrate, ciprofibrate), Wy-14,643 (often used as the prototypical PP), commercially used plasticizers (phthalates), steroids (dehydroepiandrosterone (DHEA)), and dietary fatty acids or derivatives (**Figure 2**). Further, starvation or feeding high levels of fatty acid can induce peroxisome proliferation, although the magnitude of the effect is substantially lower than that found with treatment with xenobiotics.

During the mid-1980s, it was suggested that the biological effects induced by PPs were mediated by an unidentified cytosolic receptor that modulated gene expression to cause the pleiotropic effect of these chemicals (Reddy and Lalwai, 1983). There were several reasons for this hypothesis. First, the peroxisome proliferation response was tissue-, species- and sex-dependent. Second, although PPs are structurally diverse, structure–activity relationships were identified, and most chemicals able to cause peroxisome proliferation were found to share a fatty acid-like structure. Last, PPs regulate a battery of genes involved in fatty acid metabolism with a similar dose–response relationship. Based on the hydrophobic nature of PPs, it was hypothesized that the heretofore-unidentified receptor would be a nuclear hormone receptor. In 1990, a receptor termed peroxisome proliferator-activated receptor (PPAR) was cloned from mouse liver using the oestrogen receptor DNA binding domain as a probe (Issemann and Green, 1990). Discovery of PPAR has proven to be pivotal to the understanding of how PPs regulate gene expression and ultimately result in toxicity.

3 PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS (PPARs)

3.1 Introduction

PPARs are members of the nuclear receptor (NR) superfamily of transcription factors, similar in both structure and function to the steroid, thyroid and retinoid receptors. The NR superfamily of proteins is subdivided into six sub-families, based on their amino acid sequence similarities (Nuclear Receptors Nomenclature Committee, 1999). In the systematic nomenclature for NRs, PPARs comprise the group C of subfamily 1 (Nuclear Receptors Nomenclature Committee, 1999). The multifaceted responses of PPARs are mediated by three subtypes expressed in different tissues and at different times in development. The subfamily has been defined as PPARα (NR1C1), PPARβ (also called PPARδ and NUC1, NR1C2) and PPARγ (NR1C3), each with a possibility of different ligands, target genes and biological role (**Figure 3**).

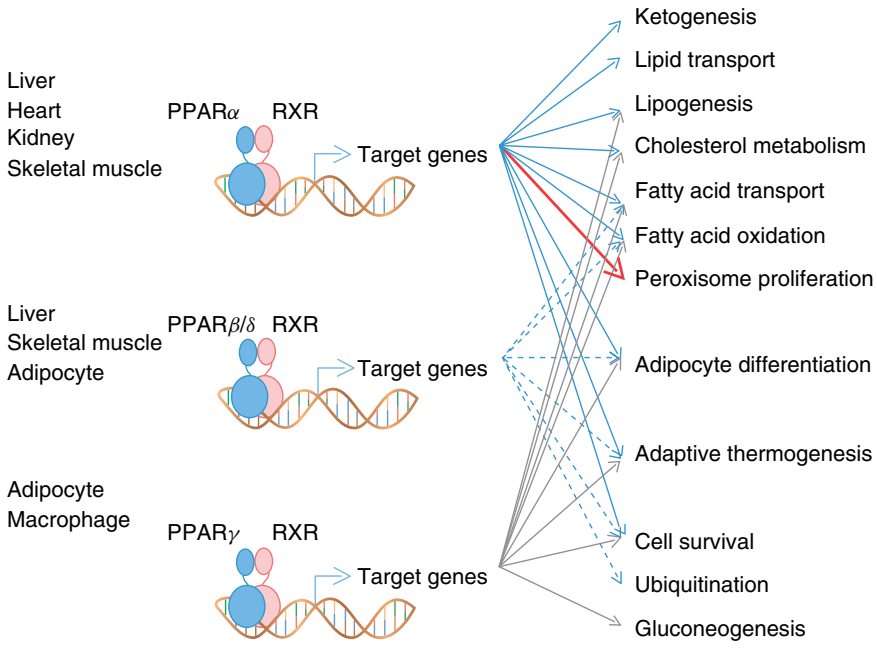

Figure 3 Basic mechanism of action of peroxisome proliferator-activated receptors. (Summarized from KEGG Pathways, http://www.genome.ad.jp/kegg/pathway/hsa/hsa03320.html).

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3.2 Mechanism of Action

The structure and mechanism of action of all NRs is very similar. In general, there are three functional domains: (i) a ligand-binding/dimerization domain, (ii) a DNA-binding/weak dimerization domain, and (iii) transactivation domains activation function 1 (AF-1) and activation function 2 (AF-2). The ligand-binding domain at the carboxy terminus of the receptor is where endogenous or xenobiotic ligands bind, resulting in a conformational change of the receptor that alters its biophysical properties. Many receptors, including PPARs, require heterodimerization with retinoid X receptor (RXR) (Mangelsdorf and Evans, 1995) for DNA binding. The DNA binding domain of the receptor is responsible for recognition of a receptor-specific response element consisting of a sequence of nucleotides in the promoter region of the target gene. The transactivation domains consist of a ligand-independent AF-1 domain in the amino terminus and a ligand-dependent transcription AF-2 found in the ligand-binding domain. These domains are protein–protein interface domains that direct recruitment of transcriptional coactivators to the target gene (Glass and Rosenfeld, 2000). In short, NRs are ligand-activated transcription factors that result in their pleiotropic responses by directing target gene expression.

3.3 Target Gene Expression

The PPAR/RXR complex controls gene expression by interacting with specific DNA response elements (peroxisome proliferator response elements, PPREs) located upstream of target genes (Tugwood *et al.*, 1992). Genes containing PPRE motifs include ACO (Tugwood *et al.*, 1992), peroxisomal bifunctional enzyme (PBE or BIF (Zhang *et al.*, 1992)), liver fatty acid-binding protein (L-FABP (Issemann *et al.*, 1992)) and microsomal CYP4A (Muerhoff *et al.*, 1992), although many more have been described (Juge-Aubry *et al.*, 1997). Members of the RXR-interacting subgroup of NRs typically bind to DNA elements containing two copies of direct repeat arrays spaced by 1–6 nucleotides (DR1–DR6). The idealized consensus binding site (AGGTCA) is similar for most members of this class of NR, with the specificity dictated by the number of nucleotides between half-sites as well as the 5′ flanking elements (Juge-Aubry *et al.*, 1997). In the case of PPAR, a DR1 motif is preferred, with PPAR interacting with the 5′ repeat and RXR (α, β or γ) binding to the 3′ motif (Ijpenberg *et al.*, 1997). PPREs are similar for all three PPAR subtypes (Ijpenberg *et al.*, 1997; Juge-Aubry *et al.*, 1997).

In addition to regulating PPRE-target genes, PPARα can affect gene expression in a non-DNA binding mechanism via protein–protein interactions. Including its heterodimerization partner RXRα, PPARα associates with heat shock protein 70 (hsp70 (Huang *et al.*, 1994)), hsp90 (Sumanasekera *et al.*, 2003a; 2003b), hepatitis X-associated protein (XAP2) (Sumanasekera *et al.*, 2003b), liver X receptor (LXR) (Miyata *et al.*, 1996), C/EBPα (Hollenberg *et al.*, 1997) and NF-κB subunits (Fruchart *et al.*, 1999). These interactions may influence the activity of numerous transcription factors and hence regulate genes that do not contain PPREs. A good example of such a gene is prolactin, which is regulated by PPs independent of PPARα's DNA binding (Tolón *et al.*, 1998).

3.4 PPARα and Gene Expression

The PP response (hepatomegaly, increased peroxisome enzymes) is due almost exclusively to the PPARα subtype and will be the focus of the subsequent discussion. **Figure 4** provides a summary of mRNAs that are altered as a result of treatment with PPs. As noted, administration of PPs causes changes in numerous mRNAs, and many of these genes are central to lipid metabolism. For example, mRNAs encoding peroxisomal, mitochondrial and microsomal fatty acid metabolizing enzymes are all increased by PPs. Further, liver mRNAs encoding proteins involved in lipid transport including fatty acid binding protein, fatty acid transporters, lipoprotein lipase and apolipoproteins are also regulated by these chemicals. Expression of mRNAs encoding other lipid-related proteins that are altered as a result of PPs includes HMG CoA synthase, lecithin:cholesterol acyl transferase, stearoyl-CoA desaturase 1, fatty acid synthase, S14 and malic enzyme, which are involved in cholesterol metabolism and lipogenesis. In addition to modifying gene expression essential to lipid metabolism, PPs can also influence mRNAs encoding proteins that regulate cell proliferation or the acute phase response. Lastly, PPARα can mediate alterations in mRNAs associated with steroid metabolism (17β-HSD), prostaglandin synthesis (COX-2), iron metabolism (transferrin), nitrogen metabolism (GOT, GPT) and hepatitis B viral replication. While it is clear that PPARα is critical in the regulation of lipid metabolism, and that this receptor likely regulates cell proliferation/apoptosis underlying PP-induced hepatocarcinogenesis, the precise role for some of these alterations in gene expression is not understood. For many of the target genes that regulate lipid metabolism, functional PPREs have been identified in their respective promoter region, including peroxisomal fatty acid metabolizing enzymes, fatty acid transporter, apolipoproteins and lipoprotein lipase.

Gene expression profiling has been performed on a wide variety of PPs including monoethyhexylphthalate (MEHP), clofibrate, Wy-14,643 and perfluorooctanoic acid (PFOA), to name a few (reviewed in Woods *et al.*, 2007). Toxicogenomic analysis has been used to discriminate between transcriptional responses to

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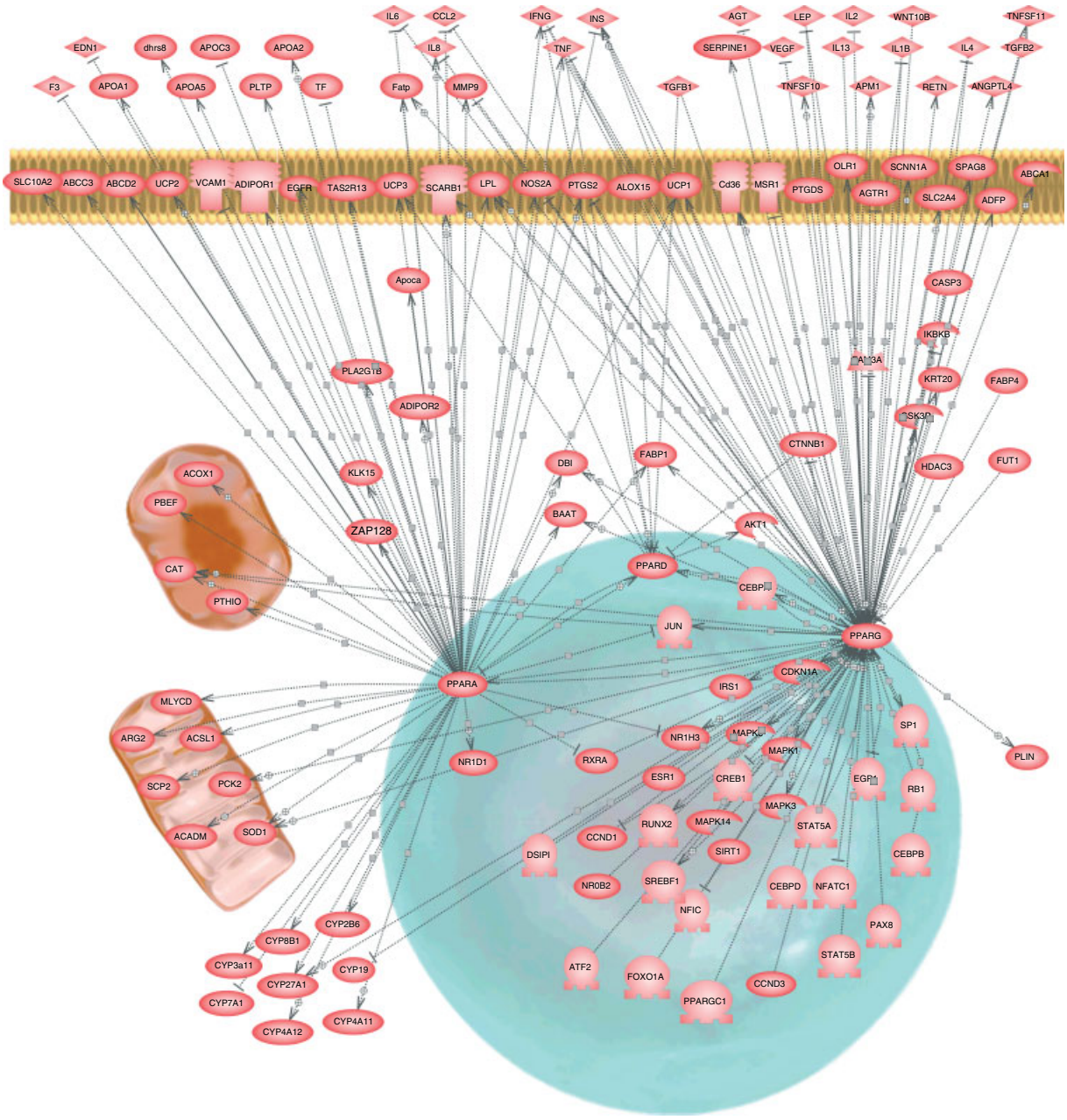

Figure 4 Regulatory network of genes regulated by peroxisome proliferators via PPARα. (Prepared using PathwayStudio® 4.0 software (Ariadne Genomics, Rockville, MD). Using Medscan natural language processing, information from all abstracts on PubMed and other public data sources was extracted to assemble molecular networks.)

chemicals of different classes. For example, in mouse liver the transcript profiles of activators of PPARα (Wy-14,643, clofibrate or gemfibrozil) were much more similar to each other than to that of a constitutive androstane receptor (CAR) agonist phenobarbital (PB) (Hamadeh *et al.*, 2002). All compounds caused an induction of genes involved in fatty acid metabolism, cell proliferation and acute phase proteins and a suppression of genes associated with gluconeogenesis. However, PPARα agonists produced a significantly greater increase in β-oxidation pathways, whereas PB caused a large response of genes encoding for detoxification and microsomal enzymes. It is generally believed that the similar responses of the PPs are those that are regulated via PPARα, although each chemical may have specific, off-target effects.

4 PPARα-DEPENDENT TOXICITY

PPARα agonists are collectively referred to as PPs because of the hyperplastic effects on liver peroxisomes following exposure in rodents (Reddy and Krishnakantha, 1975). In addition to peroxisomal proliferation, the

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size and number of hepatocytes also increases, causing significant liver enlargement. Chronic administration of PPs in rodents leads to liver tumourigenesis by a nongenotoxic mechanism (Marsman and Popp, 1994). These two phenomena (hepatic peroxisome proliferation/hepatomegaly and hepatocarcinogenesis in rodents) are seen with this class of compound and are dependent on the presence of PPARα (Klaunig *et al.*, 2003). Other toxic responses have been reported for PPs (see **Table 1**). For example, male reproductive and developmental toxicity and carcinogenic effects in testis, pancreas and kidney have also been associated with chronic administration of several PPARα agonists (Biegel *et al.*, 2001; Kurokawa *et al.*, 1988). However, this is not seen with all PPs and there is either lacking or conflicting evidence of whether PPARα is the key mediator (reviewed in Peraza *et al.*, 2006). In the subsequent discussion we will focus on the accepted PPARα-dependent responses, how they have been definitively studied and what this may mean to human health risk.

4.1 PPARα-Null Mouse

In 1995, the PPARα-null mouse was first described (Lee *et al.*, 1995). Targeted disruption of the murine PPARα gene was not developmentally lethal, thus providing an excellent model to examine the role of PPARs in homeostasis, toxicity and cancer. In the initial report, PPARα-null mice were described as refractory to peroxisome proliferation, hepatomegaly, and increased expression of mRNAs encoding peroxisomal and microsomal fatty acid metabolizing enzymes. Subsequently, this mouse model has been used extensively to examine the role of the PPARα in PP hepatocarcinogenesis, lipid homeostasis and alterations in gene expression elicited from a variety of stimuli, such as feeding experimental diets, fasting and cold acclimation (Klaunig *et al.*, 2003; Gonzalez and Shah, 2008). Combined, these data have provided definitive *in vivo* evidence that the PPARα has a critical role in modulating gene expression to maintain lipid homeostasis. Further, these reports conclusively demonstrate that the PPARα is the only member of this receptor subfamily capable of mediating peroxisome proliferation and, most importantly, hepatocarcinogenesis induced by PPs. Hence, the term peroxisome proliferator-activated receptor best describes the PPARα, yet will continue to be used for the other two PPAR isoforms due to significant sequence homology.

Table 1 Toxicity of peroxisome proliferators in laboratory animals

Toxic response	PPARα dependence
Hepatocarcinogenesis	Yes
Other tumours (Leydig, pancreatic acinar, kidney)	Questionable
Hepatotoxicity	Questionable
Cardiotoxicity/lipotoxicity	Yes
Reproductive/developmental	Questionable
Muscle toxicity	Questionable

4.2 Peroxisome Proliferator Hepatocarcinogenesis

Long-term administration of PPs causes hepatocarcinogenesis in rodent models (Klaunig *et al.*, 2003). The carcinogenic effect of these chemicals is only seen when there is significant peroxisome proliferation (i.e. greater than four-fold increase in peroxisomal ACO activity) (Ashby *et al.*, 1994). Due to limited peroxisome proliferation, hepatocarcinogenesis is not likely to occur in response to physiological activation of PPARα-dependent transcription such as that observed with fasting or feeding a diet rich in fatty acids. PPs that cause hepatocarcinogenesis include the fibrate class of hypolipidaemic drugs, Wy-14,643, DHEA, di(2-ethylhexyl) phthalate (DEHP) and trichloroethylene (reviewed in Gonzalez and Shah, 2008). The incidence of hepatocarcinogenesis in wild-type mice fed the potent PP Wy-14,643 for 11 months is 100%, while PPARα-null mice are refractory to this effect (Peters *et al.*, 1997). Microscopic examination of livers from PPARα-null mice fed Wy-14,643 also failed to identify any preneoplastic lesions. Thus, it is clear that the carcinogenic effect of these chemicals is mediated by PPARα; however the specific mechanisms underlying this effect have not been fully elucidated. Further, it is likely that a species difference in sensitivity to PPs exists, and this will be discussed later. A number of possible mechanisms for PP-induced hepatocarcinogenesis have been hypothesized, including: (**i**) increased oxidative damage to intracellular macromolecules (DNA, protein, lipids) that contribute to gene mutations indirectly; (**ii**) increased cell replication in the presence of damaged cells; and (**iii**) inhibition of apoptosis, resulting in the persistence of damaged cells.

4.2.1 Oxidative Stress

One of the first hypotheses to explain the carcinogenic effect of these chemicals is that peroxisome proliferation causes oxidative damage, resulting from increased intracellular concentration of H_2O_2 produced by peroxisomal ACO (Reddy and Rao, 1989). Hydrogen peroxide in the presence of metals facilitates free radical formation, which may in turn damage macromolecules. Oxidative stress may occur as a result of the large induction of ACO caused by PPs in the absence of significant increases of catalase (Nemali *et al.*, 1988). Evidence demonstrating intracellular oxidative damage resulting from PPs is inconsistent. Peroxide-modified lipids have been detected in hepatocytes from rats treated with either Wy-14,643, clofibrate or DEHP (Conway *et al.*,

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1989; Marsman *et al.*, 1992). However, more sensitive measures of oxidative damage, including ethane exhalation and hepatic levels of esterified F2-isoprostanes, are not markedly influenced by administration of Wy-14,643 (Conway and Popp, 1995; Soliman *et al.*, 1997). Evidence showing oxidative damage to DNA resulting from PPs is also inconsistent. DNA damage in the form of 8-hydroxydeoxyguanosine (8-OH-dG) has been reported in liver from rats after long-term treatment with PPs, including DEHP (Takagi *et al.*, 1990). However, the increase in 8-OH-dG residues is small (less than two-fold), not sustained with chronic DEHP treatment, and the correlation between 8-OH-dG levels and tumour multiplicity is weak (Marsman *et al.*, 1988). In contrast to the studies demonstrating oxidative damage to DNA, no measurable increase in 8-OH-dG or thymidine glycol was reported in rat liver after administration of nafenopin (Hegi *et al.*, 1990).

Despite evidence that PPs cause intracellular oxidative damage to lipids or DNA, most investigations support the idea that PPs are nongenotoxic carcinogens (Klaunig *et al.*, 2003). Numerous assays designed to determine the genotoxicity of chemicals have been utilized to assess the effect of PPs, including mutation, genotoxicity, DNA damage, chromosomal damage and cell transformation assays. Nonetheless, there is one report suggesting that H_2O_2 produced from overexpression of ACO in NIH3T3 cells causes cell transformation (Chu *et al.*, 1995). While this suggests that oxidative damage can lead to cell transformation *in vitro*, this has not been clearly demonstrated *in vivo*.

Interestingly, spontaneous peroxisome proliferation, induction of many PPARα target genes and hepatocarcinogenesis are all found in ACO-null mice after one year of age (Fan *et al.*, 1996). This phenotype is remarkably similar to that of rodents fed PPs, suggesting that in the absence of fatty acid oxidation by peroxisomal acyl-CoA oxidase, a fatty acid metabolite accumulates and increases PPARα-dependent transcription. Thus, ACO may function to metabolize this unidentified fatty acid metabolite to prevent accumulation that ultimately would result in peroxisome proliferation and hepatocarcinogenesis.

4.2.2 Cell Cycle Control

Alterations in cell cycle control constitute another postulated mechanism of PP-induced carcinogenesis. A significant increase in liver weight is always found in rodents fed PPs, and this is due in large part to increased cell proliferation as measured by ^{3}H-thymidine or bromodeoxyuridine incorporation in hepatocytes (Plant *et al.*, 1998; Anderson *et al.*, 2001). Since hepatomegaly and increased expression of cell cycle control proteins are not found in PPARα-null mice treated with PPs (Peters *et al.*, 1997; 1998), the mitogenic effect of these chemicals clearly requires this receptor. Perhaps PPs, acting through PPARα, alter the expression of a particular subset of genes that in turn affects the rate of proliferation of cells. Several early studies indicated that the degree of peroxisome proliferation correlated poorly with the relative hepatocarcinogenicity of PPs. However, a strong correlation was observed between the relative tumour promoting activity of the PPs DEHP and Wy-14,643, with the ability to induce a persistent increase in replicative DNA synthesis (Marsman *et al.*, 1988).

Although most genes known to be regulated by these chemicals are involved in fatty acid metabolism (reviewed in Desvergne *et al.*, 1998; Desvergne and Wahli, 1999), there are several PP-responsive genes with a link to cell cycle control. Induction of the oncogenes c-Ha-ras, jun and c-myc by PPs has been reported, and the ability to induce these genes correlates well with tumour promoting potential (Vanden Heuvel, 1999). For example, the tumour promoters Wy-14,643, clofibrate, ciprofibrate and DEHP were inducers of c-fos, c-jun, junB egr-1 and NUP475, whereas the noncarcinogenic peroxisome proliferator DHEA was ineffective (Ledwith *et al.*, 1996). In addition, an immediate early gene (IEG) critically involved in lipid metabolism, tumour promotion and inflammation, COX-2, is also regulated by PPs (Ledwith *et al.*, 1997). A novel IEG involved in neuronal differentiation, rZFP-37, is a PP-regulated gene in rodent liver (Vanden Heuvel *et al.*, 1998). Both rZFP-37 and c-myc have been demonstrated to be controlled by PPARα (Vanden Heuvel *et al.*, 1998; Belury *et al.*, 1998), using the null mouse model. Many of the genes described above are critical in the progression of the cell cycle, in particular the G_1 to S transition. PP-induced expression of growth regulatory genes precedes entry of the cell into S phase (Ledwith *et al.*, 1997). Also, expression of cell cycle control proteins associated with cell replication (cyclins, cyclin-dependent kinases) are increased as a result of treatment with PPs (Rininger *et al.*, 1996; Ma *et al.*, 1997).

A genomics study in immortalized hepatocytes from PPARα wild-type and knockout mice treated with Wy-14,643 demonstrated a PPARα-dependent induction of ACO and genes regulating cell cycle, along with changes in other genes implicated in cancer, such as JunB, and retinoblastoma protein 1 (Rb1) (Tien *et al.*, 2003). Also, the Wy-14,643-induced increase in growth rate was PPARα dependent, but this response has not been observed in other pure cell cultures. Previous studies conducted *in vitro* using primary cells have demonstrated that hepatocytes, in absence of Kupffer cells or Tumor necrosis factor (TNF)α, do not exhibit Wy-14,643-induced cell proliferation (Parzefall *et al.*, 2001). One major difference between primary and permanent cultures is the capacity for proliferation, with permanent cultures exhibiting higher cell turnover, which likely explains the difference between the observations in this study and historical data.

Hepatocyte growth factor (HGF) is altered as a result of PP treatment and thus could influence hepatocyte cell

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proliferation. Administration of PPs to rats decreases hepatic HGF mRNA after short-term feeding with Wy-14,643 (Motoki *et al.*, 1997; 1999), with a continuing decline to less than 50% of control levels after 40 weeks of feeding (Motoki *et al.*, 1997). The reduction in liver HGF mRNA levels is found in nontumour portions of cancerous livers in PP-treated rats, although a more striking decrease is found in the tumour regions of the same livers (Motoki *et al.*, 1997; 1999). Since HGF can also inhibit colony formation in soft agar of neoplastic or preneoplastic liver cells from Wy-14,643-fed rats, this suggests that decreased HGF expression resulting from PP treatment could contribute to the mechanisms underlying PP-induced hepatocarcinogenesis. Importantly, PP-induced reduction of HGF requires a functional PPARα (Kiss *et al.*, 2001). However, these studies also showed that hepatomegaly and expression of mRNAs encoding cell cycle control proteins resulting from PP feeding are not prevented in two different lines of transgenic mice overexpressing liver HGF at substantially high levels. Thus, the precise role of reduced HGF expression in the pleiotropic response resulting from PP treatment is unclear.

Consistent with peroxisome proliferators' role as hepatic tumour promoters, these chemicals decrease the rate of programmed cell death, thereby altering the balance between mitosis/apoptosis, a key mechanism in carcinogenesis (Bayly *et al.*, 1993; James and Roberts, 1994; Roberts *et al.*, 1995). A proteomics study was conducted with clofibrate-treated rats (Léonard *et al.*, 2006). As expected, the altered proteins were involved in lipid metabolism, fatty acid metabolism, amino acid metabolism, protein metabolism, citric acid cycle, xenobiotic detoxification and oxidative stress. However, several proteins were implicated in apoptosis, such as prohibitin, 10-formyl tetrahydrofolate dehydrogenase, senescence marker protein-30, pyridoxine 5′-phosphate oxidase and vimentin. PPARα is an essential component of PP repression of cell death as determined with the null mouse model (Hasmall *et al.*, 2000). In addition, overexpression of a dominant negative PPARα, (thereby abolishing PPARα activity) abrogates the suppression of apoptosis by PPs (Roberts *et al.*, 1998). Additionally, hepatocytes from PPARα-null mice are refractory to suppression of apoptosis caused by PPs (Hasmall *et al.*, 2000). Combined, there is evidence that PPARα can regulate the response to PPs by preventing programmed cell death that potentially results in cell proliferation of 'damaged' cells which would normally undergo apoptosis.

Microarray studies in mouse liver following two weeks of dietary treatment of potent PP, 4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid (Wy-14,643) revealed similar results with altered expression of genes involved in lipid and glucose metabolism, transcription, apoptosis and cell cycle (Cherkaoui-Malki *et al.*, 2001). A novel set of 27 PP-regulated genes were identified and included cell death inducing DNA fragmentation factor α, retinoic acid early transcript γ, cell surface receptors Cd39, Cd24, pyruvate dehydrogenase-kinase-4 (Pdk-4), Cyp2b9 and Cyp2b10. Furthermore, PPARα dependency was demonstrated for many of these genes, as no increase in expression was observed in PPARα knockout mice.

Another possible mechanism by which PPs could effect gene expression and hepatocarcinogenesis is through influencing the expression of microRNAs (miRNAs) that in turn could alter gene expression or levels of specfic mRNAs. Indeed, miRNA arrays revealed a marked change in the expression of several miRNAs after a four-hour short-term Wy-14,643 treatment (Gonzalez and Shah, 2008). The mechanism of hepatocellular proliferation involves downregulation of the miRNA let-7c gene by PPARα. Let-7c controls levels of c-myc by destabilizing its mRNA. Thus, upon suppression of let-7c, c-myc mRNA and protein are elevated, resulting in enhanced hepatocellular proliferation.

4.2.3 Species Differences

There is considerable debate concerning the mechanisms by which PPs cause liver tumours in rodent models and whether these chemicals represent a human cancer risk. It is well established that long-term administration of PPs results in hepatocarcinogenesis in rats and mice (reviewed in Klaunig *et al.*, 2003). In contrast, liver tumours are not found in Syrian hamsters after long-term administration of PPs. Similar investigations in nonhuman primates indicate that these species are also refractory to the PP-induced liver cancer, although these experiments were not carried out over the lifespan of the animals. Consistent with this idea, hepatic peroxisome proliferation is not found in nonhuman primates treated with these chemicals. Liver biopsies from humans treated with gemfibrozil, clofibrate or fenofibrate for periods ranging from 27 to 94 months did not reveal convincing evidence of peroxisome proliferation, supporting the hypothesis that humans are less responsive to PPs. Further support of this idea is provided by two limited epidemiological studies that showed no evidence of increased cancer risk as a result of fibrate therapy. While these observations suggest that a significant species difference in the response to PPs exists, it is critical to delineate the mechanisms of this putative difference in order to increase the confidence of risk assessments made for this class of chemicals.

An important advance in understanding the human relevance of PP hepatocarcinogenesis came with the development of the 'humanized' PPARα mouse (Cheung *et al.*, 2004). This line expresses the human receptor in liver in a PPARα null background by placing the human peroxisome proliferator-activated receptor alpha (hPPARα) cDNA under control of the Tet-Off system of doxycycline control with the liver-specific LAP1 (C/EBPβ) promoter (reviewed in Gonzalez and Shah,

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2008). In the absence of doxycycline, hPPARα is constitutively expressed and after administration of a PP, target genes are affected specifically in the liver. For example, treatment of humanized mice with Wy-14,643 resulted in increased expression of ACO, bifunctional enzyme (BIEN) and thiolase (THIOL) and the fatty acid transporter CD36. Upon treatment with Wy-14,643, hPPARα mice had lower levels of fasting serum total triglycerides similar to wild-type mice expressing native mPPARα. Importantly for this discussion, hPPARα mice do not show any significant hepatocellular proliferation, nor did they have an induction of cell cycle control genes (PCNA, c-myc, c-jun, Cdk1, Cdk4 and several cyclins) upon treatment with Wy-14,643, unlike the wild-type mice. Humanized PPARα mice were resistant to Wy-14,643-induced hepatocarcinogenesis after 11 months of Wy-14,643 feeding in contrast to a 100% incidence in the wild-type mouse group (Morimura *et al.*, 2006) (**Table 2**). These findings suggest that the species-specific effects of fibrates are likely due to differences in the profile of genes activated by mPPARα versus hPPARα following fibrate treatment (Gonzalez and Shah, 2008).

4.3 Cardiotoxicity/Lipotoxicity

Activation of PPARα and to some extent PPARβ/δ plays a major role in heart metabolism. The postnatal mammalian heart uses mitochondrial fatty acid oxidation as the chief source of energy, and PPARα regulates the expression of several key enzymes involved in cardiac mitochondrial fatty acid oxidation. In cardiac hypertrophy and heart failure, myocardial energy utilization reverts to the foetal pattern, and metabolic substrate switches from fatty acid to glucose with a decrease of PPARα expression. Overexpression of PPARα in heart in mice increases myocardial fatty acid oxidation and decreases myocardial glucose uptake and oxidation (Hopkins *et al.*, 2003), a metabolic phenotype strikingly similar to that of the diabetic heart (Snyder, 1991). The hearts of these mice exhibited signs of diabetic cardiomyopathy including ventricular hypertrophy, activation of gene markers of pathologic hypertrophic growth, and transgene expression-dependent alteration in systolic ventricular dysfunction. In addition, the recovery from ischaemic injury is affected in the mice with overexpressed PPARα (Sambandam *et al.*, 2006). Insulin-resistant mice crossed into a PPARα-null background did not exhibit evidence of mitochondrial biogenesis or induction of mitochondrial gene expression indicative of the insulin resistant heart (Duncan *et al.*, 2007). Conversely, transgenic mice with cardiac-specific overexpression of PPARα exhibited signatures of cardiac mitochondrial biogenesis. Uptake of fatty acids by the heart is crucial in the development of lipotoxicity in the transgenic PPARα animals (Yang *et al.*, 2007). There is no report indicating that available PPARα activators (fibrates) induce either deleterious or beneficial effects on cardiac function in patients with heart failure. Nevertheless, it has been reported that PPARα and PPARγ agonists prevent left ventricular diastolic dysfunction in diabetic rats (Kim *et al.*, 2003), possibly through an improvement of the hyperglycaemia and/or hyperlipidaemia. Thus, the physiological importance of PPARα in cardiac tissue is evident and there is evidence of unfavourable effects of PPARα activation in lipid metabolism (lipotoxicity) in cardiomyocytes. Whether exogenous PPARα ligands can exacerbate pathologic cardiac hypertrophy in individuals with diabetes type 2, metabolic syndrome or obesity needs to be further studied.

Table 2 Incidences of liver tumours in hPPARα and wild-type (mPPARα) mice treated with Wy-14,643[(a)]

Genotype	Treatment	Tumours (%)
hPPARα	Control	0
hPPARα	Wy-14,643	5
mPPARα	Control	0
mPPARα	Wy-14,643	71

[(a)] Mice were fed with 0.1% Wy-14,643 for up to 44 weeks. Adapted from (Gonzalez and Shah, 2008).

5 SUMMARY AND CONCLUSION

In summary, PPs are a diverse class of chemicals that are capable of producing a consistent pleiotropic response. The effects observed in rodent liver include peroxisome proliferation, hepatomegaly, regulation of gene expression, cell cycle control and, ultimately, carcinogenesis. The majority of biological effects induced by PPs are mediated by the PPARα, and include physiological, toxicological and carcinogenic pathways. Acting as transcription factors, PPARα functions in classic NR fashion by ligand binding, heterodimerization, recruitment/dissociation of coactivators/co-repressors, and modulation of transcription after binding to specific responsive elements of target genes. Definitive evidence of the biological and toxicological role of this receptor has been provided by extensive analysis of PPARα-null mice. The mechanisms underlying PP-induced hepatocarcinogenesis are not clear, although oxidative stress, increased cell proliferation and/or repressed apoptosis are likely to have roles in this process. Whether or not the hepatocarcinogenic effect of PPs routinely observed in rodent models is relevant to humans is a controversial issue and requires ongoing molecular and cellular research.

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Reactive Oxygen Species in the Induction of Toxicity

Giorgio Lenaz and Paola Strocchi

CONTENTS

1 INTRODUCTION

Reactive oxygen species (ROS), although recognized to be physiologically involved in cell signalling by affecting the oxidoreductive (redox) state of signalling proteins, are among the major determinants of toxicity in cells and organisms. These aggressive derivatives of partial oxygen reduction exert their damaging effects in mainly two ways: (i) by direct modification of cellular or extracellular macromolecules that are in this way altered in their function, leading to pathological effects; (ii) by alterations of their physiological action on the redox state of factors involved in signal transduction, thus leading to hyper- or hypo-functionality of the signalling pathways.

ROS is a collective term including oxygen derivatives, either radical or nonradical, that are oxidizing agents and/or are easily converted into radicals (Halliwell, 2006). A free radical is a species containing one or more unpaired electrons. Diatomic oxygen O_2 is a radical because it has two unpaired electrons each located in a different π^* antibonding orbital, but both with the same spin quantum number: this parallel spin is the reason for its low reactivity with nonradical molecules. However, inverting the spin of one of the unpaired electrons by an energy input converts O_2 into the much more reactive singlet oxygen O_2 in which both unpaired electrons have been moved to the same π^* orbital. Transition metals such as iron and copper, when in a free state, have a strong capacity to reduce O_2, thus generating radical species.

If a single electron is supplied to O_2, it enters one of the π^* orbitals to form an electron pair there, thus leaving only one unpaired electron in the superoxide radical anion $O_2^{\bullet -}$; addition of another electron gives the peroxide ion, which is a weaker acid and is protonated to hydrogen peroxide H_2O_2; addition of two more electrons breaks the molecule producing water H_2O. If one single electron is added to H_2O_2 by a reduced metal ion (e.g. Fe^{2+}), the hydroxyl radical $OH^{\bullet}$ is produced by the Fenton reaction. The hydroxyl radical is extremely reactive with a half-life of less than 1 ns, thus it reacts

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close to its site of formation.

$$O_2 + 1e^- \longrightarrow O_2^{\bullet -} \quad (1)$$

$$O_2^{\bullet -} + 1e^- + 2H^+ \longrightarrow H_2O_2 \quad (2)$$

$$H_2O_2 + 2e^- + 2H^+ \longrightarrow 2H_2O \quad (3)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\bullet} + OH^- \quad \text{(Fenton reaction)} \quad (4)$$

Reaction 1 is catalysed in cells by several systems described in Section 15.2. Hydrogen peroxide can also be formed *in vivo* by two-electron reduction of O_2 catalysed by a number of oxidases. Reaction 2 is catalysed by superoxide dismutases (SODs) and Reaction 3 by catalase, glutathione peroxidases (GPxs) and other peroxidases (Section 15.5).

Since there are other reactive species besides those of oxygen, the term reactive species (RS) has been expanded to include reactive nitrogen species (RNS), reactive chlorine species (RCS), and so on. Among RNS is nitric oxide $NO^{\bullet}$, a not very reactive radical well known as a signalling molecule (Rubio and Morales-Segura, 2004; Schlossmann and Hofmann, 2005). If $NO^{\bullet}$ reacts with $O_2^{\bullet -}$ the nonradical product peroxynitrite is formed and rapidly protonated at neutral pH to the aggressive peroxynitrous acid, which can further undergo homolytic fission to hydroxyl radical and nitrogen dioxide radical.

$$NO^{\bullet} + O_2^{\bullet -} + H^+ \longrightarrow ONOOH \quad (5)$$

$$ONOOH \longrightarrow NO_2^{\bullet -} + OH^{\bullet} \quad (6)$$

Hypochlorous acid HOCl is a RCS that can also generate the hydroxyl radical by reacting with Fe^{2+}

$$Fe^{2+} + HOCl \longrightarrow Fe^{3+} + OH^{\bullet} + Cl^- \quad (7)$$

Several other radicals are produced by reaction of RS with organic compounds and macromolecules, as we shall see in Section 15.3.

2 SOURCES OF ROS IN THE CELL

ROS arise in cells from exogenous and endogenous sources. Exogenous sources of ROS include ultraviolet (UV) and visible light, ionizing radiation, drugs and environmental toxins. Among endogenous sources there are xanthine oxidase (XO), cytochrome P-450 enzymes in the endoplasmic reticulum, peroxisomal flavin oxidases, plasma membrane nicotinamide adenine dinucleotide phosphate (NADPH) oxidases; nevertheless, the major source of ROS is usually considered to be the mitochondrial respiratory chain in the inner mitochondrial membrane, although other enzyme systems in mitochondria can be important contributors to ROS generation.

In healthy cells, the continuous production of ROS is balanced by various detoxification reactions by a number of antioxidant systems. Only excessive ROS production or defects in protective systems lead to oxidative stress and pathological conditions. Under normal conditions, ROS are maintained within narrow boundaries and contribute to the redox balance in the cell (Linnane and Eastwood, 2006): redox balance, the ratio between oxidizing and reducing species, is involved in regulation of signalling pathways, including protein kinase and phosphatase activity and gene expression through modulation of transcription factor function (Fruehauf and Meyskens, 2007) (Section 15.4); glutathione redox state plays a central role in maintaining such redox homeostasis and the GSH/GSSG ratio is an estimate of cellular redox buffering capacity (Schafer and Buettner, 2001).

2.1 Mitochondria as Sources of ROS

Within a cell, mitochondria largely contribute to the production of ROS via the respiratory chain (Lenaz, 1998; 2001). The relevance of mitochondrial production of ROS within a cell is indirectly revealed by the results of deficiency of mitochondrial antioxidant enzymes. Mitochondria contain an isozyme of SOD (SOD-2) and GPx (see Section 15.5). The lack of SOD-2 (Melov *et al.*, 1999) and of mitochondrial GPx (Esposito *et al.*, 2000) is deleterious to cells.

2.1.1 Overview of the Mitochondrial Respiratory Chain

The electron transfer chain or respiratory chain consists of four major multisubunit complexes designated as NADH-coenzyme Q (CoQ) reductase (Complex I), succinate-CoQ reductase (SQR) (Complex II), ubiquinol-cytochrome c reductase (Complex III) and cytochrome c oxidase (Complex IV). The best fit unit stoichiometry between complexes in beef heart mitochondria is 1 Complex I : 1.3 Complex II : 3 Complex III : 6.7 Complex IV (Schägger and Pfeiffer, 2001). In addition there are 0.5 adenosine triphosphate (ATP) synthase (also called Complex V) and three to five units of the ADP/ATP translocase (catalysing the equimolar exchange of adenosine diphosphate (ADP) and ATP across the inner membrane) for each cytochrome oxidase, and there is one NADH/$NADP^+$ transhydrogenase per Complex I.

The respiratory chain collects reducing equivalents (hydrogen atoms) from mitochondrial oxidations of the tricarboxylic acid cycle, from pyruvate oxidation, fatty acid and amino acid catabolism and other oxidative reactions, and conveys them to molecular oxygen that is reduced to water (four electron reduction). The free energy decrease accompanying electron transfer is exploited to create an electrochemical proton gradient ($\Delta\mu_{H^+}$) by proton translocation from the mitochondrial inner space, the matrix, to the space between

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the inner and outer mitochondrial membranes (Nicholls and Ferguson, 2002). The proton gradient is then used as a source of energy to synthesize ATP from ADP and inorganic phosphate (Pi) by the ATP synthase complex (oxidative phosphorylation (OXPHOS)), or alternatively to drive other energy-linked reactions such as NADH/$NADP^+$ transhydrogenation and up-hill ion movements across the inner membrane. The ATP synthesized is moved to the cytoplasm in exchange with ADP by the ATP/ADP translocase.

The inner membrane contains several other proteins having electron transfer activity in smaller amounts (Ernster and Schatz, 1981); among these there are electron transfer flavoproteins (ETFs) capable of feeding electrons to the respiratory chain by pathways not involving Complex I and/or NAD, that is, glycerol-3-phosphate dehydrogenase, ETF-ubiquinone oxidoreductase, dihydroorotate dehydrogenase, choline dehydrogenase, besides alternative NADH dehydrogenases in mitochondria from several organisms, especially plants and fungi.

2.1.2 The Respiratory Chain as Source of ROS

The major sites of superoxide formation in the respiratory chain are within respiratory Complexes I and III. Further sites, however, may have importance and physiological relevance. It is worth noting that mitochondria from different tissues may vary conspicuously in their capacity to produce ROS using different substrates (Kwong and Sohal, 1998), and this capacity is also related to animal species and age.

2.1.2.1 Complex III

Complex III (ubiquinoe-cytochrome c reductase) represents a confluence point for reducing equivalents from various dehydrogenases: it catalyses the transfer of electrons from ubiquinol ($CoQH_2$) to water-soluble cytochrome c and concomitantly links this redox reaction to translocation of protons across the membrane, converting the energy associated with electron flow into an electrochemical proton gradient.

$$CoQH_2 + 2\ \text{cyt.c}\ Fe^{3+} + 2H^+\ (\text{in}) \longrightarrow CoQ + 2\ \text{cyt.c}\ Fe^{2+} + 4H^+\ (\text{out}) \quad (8)$$

The formation of superoxide in Complex III depends on the peculiar mechanism of electron transfer, the so-called Q-cycle (Crofts, 2004) (**Figure 1**).

Since the electron transfer from cytochrome b_L to b_H occurs against the electrical gradient (from the positive to the negative side), it is strongly retarded when the electrochemical potential is high, as in the controlled state (State 4); this retardation prolongs the lifetime of Qo and allows reaction of the semiquinone with O_2 forming superoxide (Jezek and Hlavata, 2005).

Antimycin A (AA) is known not to completely inhibit electron flow from ubiquinol to cytochrome c: the AA-insensitive reduction of cytochrome c is mediated by superoxide radicals. According to the Q-cycle, AA blocks ubiquinone reduction by cytochrome b_H at centre i, at the inner or negative side of the membrane. The antimycin-stimulated production of ROS is inhibited by the inhibitors acting at centre o (at the outer or positive side), where ubiquinol reduces both the Rieske iron–sulphur cluster and cytochrome b_L. Thus, we may locate the site of one-electron reduction of oxygen in the presence of antimycin at a component located at centre o, presumably ubisemiquinone (Casteilla *et al.*, 2001).

Myxothiazol was found to enhance ROS production using different respiratory substrates in the absence of antimycin, and the effect was reversed by stigmatellin (Starkov and Fiskum, 2001). Muller *et al.* (2003) found that proximal Qo site inhibitors, acting close to the b_L niche induce superoxide formation in yeast submitochondrial particles (SMPs) supplemented with decylubiquinol; the effect is not modified by antimycin, showing that the source of radicals is not at the Qi site. Muller *et al.* (2003) suggested that oxidation of ubiquinol at centre o is biphasic, with delivery of the first electron to the Rieske iron–sulphur cluster, leaving a semiquinone that in absence of further oxidation by cytochrome b_L would interact with oxygen, forming superoxide.

Ubisemiquinone is relatively stable only when protein-bound, therefore the CoQ pool in the lipid bilayer should not be a source of ROS. Exogenously administered CoQ has not been found to exert pro-oxidant effects *in vivo*: thus, the pro-oxidant species deriving from its antioxidant action (Nohl *et al.*, 1998) would not seem to be operative in *in vivo* supplementation.

2.1.2.2 Complex I

Complex I (NADH CoQ reductase) oxidizes NADH in the mitochondrial matrix and reduces CoQ in the lipid bilayer of the inner mitochondrial membrane.

$$NADH + H^+ + CoQ + 4H^+\ (\text{in}) \longrightarrow NAD^+ + CoQH_2 + 4H^+\ (\text{out}) \quad (9)$$

Several prosthetic groups contribute to electron transfer within the enzyme: flavin mononucleotide (FMN) is the entry point for electrons, that are then transferred to a series of iron–sulphur clusters (Ohnishi *et al.*, 1998). Enzymes from different sources have different numbers of iron–sulphur clusters, most of which share the same midpoint potential. The N2 iron–sulphur cluster, which is of the kind Fe_4S_4, has the highest midpoint potential (E_m between -150 and -50 mV) and is considered to be the direct electron donor to ubiquinone. N2 is most likely located in the connection between the peripheral and the membrane arms. The magnetic interaction with the semiquinone radical, corresponding to a distance of about 10 Å (Ohnishi and Salerno, 2005), suggests that

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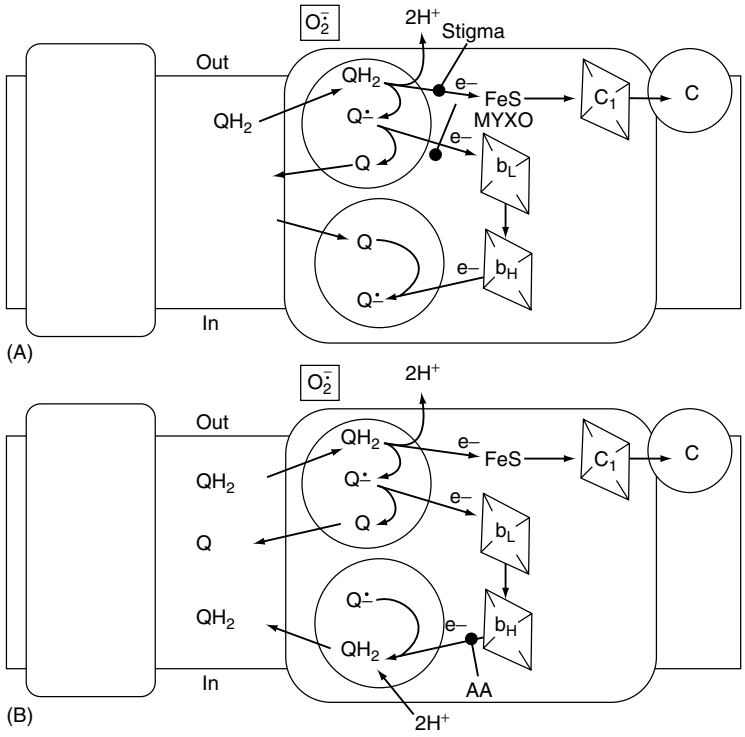

Figure 1 The Q-cycle in Complex III as a source of superoxide. In the Q-cycle, QH_2 delivers the first electron at the outer positive site (thus called site o or P) of the inner membrane to the Rieske iron–sulphur protein and hence to cytochromes c_1 and c; the result is release of two H^+ in the intermembrane space and formation of an unstable semiquinone anion $Q^{\bullet-}$ at the Qo site, which is immediately oxidized to Q by the low-potential cytochrome b_{566} (or b_L) at the cytosolic side; the electron is then delivered to the high-potential cytochrome b_{562} (or b_H) at the internal negative side (thus called site i or N); b_H is then reoxidized by CoQ at site i (Q_i) forming another semiquinone. The cycle is completed by oxidation of a second molecule of QH_2. Superoxide is thought to arise mainly from the CoQ semiquinone at centre o under conditions stabilizing this intermediate, and is released in the intermembrane space. STIGMA, stigmatellin; MYXO, myxothiazol; AA, antimycin A.

the ubiquinone headgroup could somehow reach up into the peripheral arm, as recently assumed by Brandt *et al.* (2003), who have hypothesized an amphipathic 'ramp' guiding ubiquinone into the catalytic site. Recently, the arrangement of iron–sulphur clusters in the hydrophilic domain of Complex I from *T. thermophilus* has been determined by X-ray crystallography, showing a linear chain of all clusters except N1a and N7 (Hinchliffe and Sazanov, 2005).

Early experiments proved the involvement of Complex I in ROS production (Takeshige and Minakami, 1979); addition of NADH at low concentration led to copious ROS production detected by lipid peroxidation; addition of NADH at high concentration, but in presence of rotenone, also induced peroxidation. Water-soluble CoQ homologues used as electron acceptors from isolated Complex I stimulated H_2O_2 production, whereas CoQ_6 and CoQ_{10} were inactive (Cadenas *et al.*, 1977). More recent studies confirmed that Complex I is a major source of superoxide production in several types of mitochondria. The superoxide production by Complex I is higher during the reverse electron transport from succinate to NAD^+ (Korshunov *et al.*, 1997; Turrens, 2003; Jezek and Hlavata, 2005), whereas during the forward electron transport it is much lower. Reverse electron transfer-supported ROS production requires high membrane potential and is inhibited by uncouplers and by processes dissipating membrane potential (Kushnareva *et al.*, 2002; Starkov and Fiskum, 2003). Rotenone enhances ROS formation during forward electron transfer (Herrero and Barja, 2000; Genova *et al.*, 2001) and inhibits it during reverse electron transfer (Lambert and Brand, 2004; Ohnishi *et al.*, 2005; Vinogradov and Grivennikova, 2005).

The identification of the oxygen reducing site has been the subject of extensive investigation, and several prosthetic groups in the enzyme have been suggested to be the direct reductants of oxygen. These include FMN, ubisemiquinone and iron–sulphur cluster N2 (Lenaz *et al.*, 2006a; 2007a). In isolated Complex I, FMN is considered the major electron donor to oxygen to form the superoxide anion. In mitochondrial membranes, however, the identification of flavin as the site of oxygen reduction is incompatible with the finding that two classes of inhibitors both acting downstream of the iron–sulphur clusters in the enzyme have opposite

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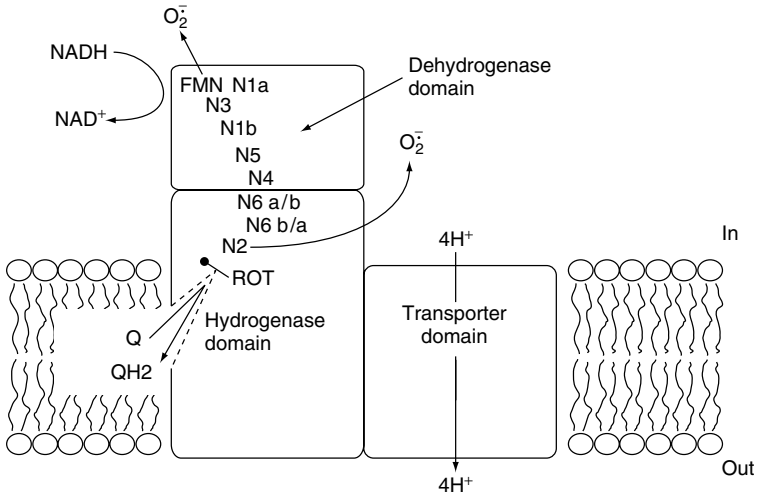

Figure 2 Schematic representation of Complex I formed by the apposition of three different modules: a dehydrogenase domain where NADH is oxidized, containing FMN and iron–sulphur clusters N1a, N1b, N3, N4, and N5; a hydrogenase domain where CoQ is reduced, containing FeS clusters N6a, N6b and N2; and a transporter domain containing no prosthetic groups and involved in proton translocation. Superoxide formation presumably occurs at both FMN and N2 (see text) and is released in the mitochondrial matrix. ROT, rotenone.

effects, in that rotenone enhances superoxide production, whereas stigmatellin inhibits it (Lenaz *et al.*, 2006a). A possible explanation is that two sites for oxygen reduction exist in the complex, represented by flavin and an iron–sulphur cluster; the latter site would be predominant in membrane particles, whereas the former might be available after Complex I isolation.

The electron donor to the first molecule of bound ubiquinone in the complex is most probably iron–sulphur cluster N2 (Ohnishi *et al.*, 1998). It is likely that this centre is also the electron donor to oxygen both directly and via one-electron reduction of several exogenous quinones (**Figure 2**). Studies in CoQ-depleted and reconstituted mitochondria indicated that endogenous CoQ is not required for superoxide generation (Genova *et al.*, 2001). It is worth noting that reconstituted mitochondria, containing a large excess of CoQ_{10}, produce the same amount of superoxide as CoQ-depleted mitochondria, indicating that endogenous CoQ_{10} is not a source of ROS.

2.1.2.3 Complex II

The enzyme is also a component of the Krebs cycle (succinate dehydrogenase) and catalyses the reduction of CoQ by succinate, without formation of a proton gradient.

$$\text{succinate} + \text{CoQ} \longrightarrow \text{fumarate} + \text{CoQH}_2 \qquad (10)$$

The indirect evidence that often ROS production is higher when electrons are channelled through Complex II than through Complex I, while in both cases reaching Complex III, would be in line with the idea that Complex II may be a source of ROS (McLennan and Degli Esposti, 2000).

Ishii *et al.* (1998), in a study on *C. elegans*, produced evidence that a mutation in succinate dehydrogenase cytochrome b induces oxidative stress and ageing. In *E. coli*, fumarate reductase (QFR), active under anaerobic conditions, is structurally analogous to Complex II (SQR), active under aerobic conditions; in contrast with Complex II, however, *E. coli* QFR has no *b* haeme. Significantly, *E. coli* QFR is a potent source of H_2O_2, whereas SQR is not (Messner and Imlay, 2002). The source of electrons to oxygen is fully reduced flavin adenine dinucleotide (FAD). The difference between SQR and QFR has been ascribed to the electron attracting capacity of cytochrome *b*, due to its high redox potential (Yankovskaya *et al.*, 2003): thus, in the absence of cytochrome *b*, the electrons would be held preferentially on the flavin, favouring leak to oxygen.

Direct demonstration of ROS production by Complex II was obtained by Zhang *et al.* (1998) in purified SQR and succinate dehydrogenase; auto-oxidation of flavin was the source of the superoxide; reconstitution with the bc1 complex, to yield an active succinate cytochrome c reductase, inhibited superoxide formation.

2.1.2.4 Glycerol-3-Phosphate Dehydrogenase

This enzyme catalyses the oxidation of glycerol-3-phosphate by CoQ:

$$\text{glycerol-3-P} + \text{CoQ} \longrightarrow \text{dioxyacetone-P} + \text{CoQH}_2 \qquad (11)$$

A high rate of ROS production was detected in insect flight muscle mitochondria (Bolter and Chefurka, 1990) and in brown adipose tissue mitochondria (Sekhar

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et al., 1987) when glycerol-3-phosphate was used as the respiratory substrate. This suggested that mitochondrial glycerophosphate dehydrogenase (mGPDH) could be the source of ROS. Drahota *et al.* (2002) demonstrated that mGPDH in the presence of antimycin is a powerful source of hydrogen peroxide, that was strongly stimulated by addition of the one-electron acceptor ferricyanide. The ferricyanide-induced H_2O_2 production is a specific feature of mGPDH: it is completely inhibited by mGPDH inhibitors, and is negligible using NADH or succinate as substrates. It is reasonable that ferricyanide takes up one electron and the other is used to reduce oxygen.

In a study on *Drosophila* mitochondria (Miwa *et al.*, 2003) ROS production by mGPDH and the relative contributions of Complex I by reverse electron transfer of centre o of Complex III and of mGPDH were assessed by use of specific inhibitors, demonstrating that mGPDH was the major source of superoxide.

2.1.2.5 Dihydroorotate Dehydrogenase

This essential enzyme of pyrimidine biosynthesis catalyses the oxidation of dihydroorotate to orotate:

$$\text{dihydroorotate} + \text{CoQ} \longrightarrow \text{orotate} + \text{CoQH}_2 \qquad (12)$$

The enzyme was found to be involved in the production of superoxide in liver mitochondria (Forman and Kennedy, 1975) and in malarial parasite cells (Krungkrai, 1991). It is worth noting that the major domain of the enzyme, carrying the carboxyl terminal, protrudes in the intermembrane space, so that it is likely that superoxide is released in this space similarly to glycerol-3-phosphate dehydrogenase.

2.1.2.6 ETF and ETF Dehydrogenase

ETF is a small flavoprotein that accepts electrons from fatty acyl coenzyme As (CoAs) and other lipid compounds and is then reoxidized by CoQ in the respiratory chain:

$$\text{ETF}(\text{FADH}_2) + \text{CoQ} \longrightarrow \text{ETF}(\text{FAD}) + \text{CoQH}_2 \qquad (13)$$

It was observed that the oxidation of palmitoyl carnitine by mitochondria leads to production of H_2O_2 (Boveris *et al.*, 1972). More recently, in a study on the topology of ROS production by the respiratory chain, St-Pierre *et al.* (2002) observed copious production of H_2O_2 by rat muscle and heart, but not liver, mitochondria when oxidizing palmitoyl carnitine; since the rate was only slightly enhanced by exogenous SOD, it was suggested that superoxide production occurred at the matrix side, therefore it was generated at a site different from centre o of Complex III. The authors considered it likely that the flavoproteins ETF and ETF dehydrogenase, involved in fatty acid oxidation, were the sites for generation of superoxide.

(**Figure 3**) summarizes the superoxide production sites of the respiratory chain.

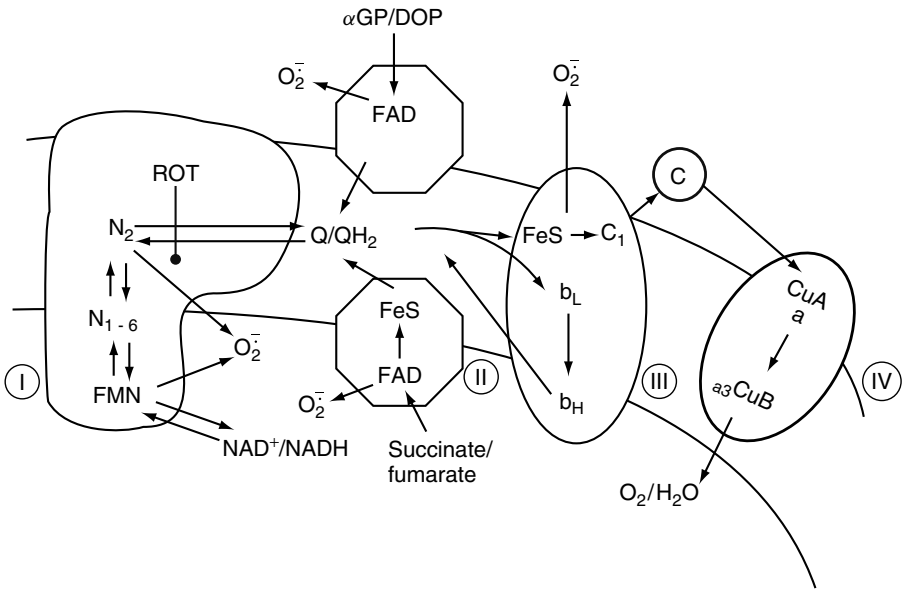

Figure 3 Overall cartoon showing the main sites of generation of superoxide in the mitochondrial respiratory chain. The scheme shows $O_2^{\bullet-}$ formation by Complex I, with release in the matrix during forward and reverse electron transfer, by Complex III during the Q-cycle, with release in the intermembrane space, and by Complex II in the matrix and glycerol-3-phosphate dehydrogenase in the intermembrane space. ETF dehydrogenase behaves as Complex II, while dihydroorotate dehydrogenase behaves as glycerol-phosphate dehydrogenase. ROT, rotenone; α-GP, glycerol-3-phosphate; DOP, dioxyacetone phosphate.

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2.1.3 Modulation of Mitochondrial ROS Production

Are free radicals produced by mitochondria physiologically released to the cytosol? Staniek and Nohl (2000) applied a noninvasive detecting system for hydrogen peroxide and found that isolated intact rat heart mitochondria do not produce detectable H_2O_2, unless using succinate in the presence of antimycin. Korshunov *et al.* (1997) also found no hydrogen peroxide formation by intact rat heart mitochondria, unless pretreated in such a way as to deplete them of endogenous antioxidants. It may be inferred that under normal conditions ROS are not exported out of mitochondria. There is, however, overwhelming evidence that ROS production detected in different cells under pathological conditions has a mitochondrial origin.

Although the first product of oxygen reduction by the respiratory chain is superoxide, this radical has a short life and is rapidly converted into hydrogen peroxide by mitochondrial SOD or can attack other molecules, such as lipids, before being able to escape the mitochondrion. Although hydrogen peroxide is also removed by GPx, it is much more stable than superoxide, so that some molecules can escape the organelle and be detected outside. Most of superoxide is generated at the matrix side of the inner membrane, as appears from the observation that superoxide is detected in SMPs which are inside-out with respect to mitochondria. A study with suitable spin traps, however, demonstrated the formation of superoxide radical in mitoplasts (Han *et al.*, 2001) indicating that a significant aliquot of this species is released at the outer face of the inner membrane (St-Pierre *et al.*, 2002). It is likely that Complex I releases ROS in the matrix while Complex III releases them in the intermembrane space. The superoxide anion released in the intermembrane space may be exported to the cytoplasm through an anion channel related to a voltage-dependent anion channel (VDAC) (Han *et al.*, 2003).

It is not easy to demonstrate that ROS detected in cells are produced by mitochondria; the effect of respiratory inhibitors appears to be the best way to discriminate between mitochondrial and nonmitochondrial ROS. However, the effect of inhibitors is ambiguous. Although antimycin is usually found to stimulate ROS production (Boveris *et al.*, 1972) in intact cells, as it does in mitochondria, the effect of rotenone is contradictory (Lenaz, 2001). Some studies showed that rotenone enhances ROS production in intact cells, whereas others showed inhibition of cellular ROS production by the same inhibitor. Since rotenone decreases ROS production by Complex III while enhancing ROS production by Complex I, the relative contribution of the two complexes to ROS production may vary in different cells. Since ROS production by reverse flux of electrons is decreased by rotenone (Kushnareva *et al.*, 2002), another critical point is represented by membrane potential and the contribution of reverse electron transfer in Complex I.

Mitochondrial ROS production is enhanced in State 4 and when the rate of electron transfer is lowered (Skulachev, 1996). The rationale is in a more reduced state of the respiratory carriers capable of donating electrons to oxygen. To this purpose, uncoupling and release of excessive membrane proton potential may protect mitochondria from damage due to excessive free-radical production. In rat hepatocytes the futile cycle of proton pumping and proton leak may be responsible for 20–25% of respiration (Brand, 2000); in perfused rat muscle the value is even greater. Uncoupling may be obtained by activating proton leak through uncoupling proteins (Casteilla *et al.*, 2001). In this way a tissue may dissipate a conspicuous part of the energy conserved by its mitochondria, however it keeps the mitochondrial respiratory chain under more oxidized conditions preventing the formation of damaging free radicals (**Figure 4**).

The ROS production by the respiratory chain complexes may be under physiological control; this is particularly evident for Complex I. Events leading to decrease of the rate of electron flow in the complex also lead to overproduction of ROS; physiological states, such as subunit phosphorylation, which inhibits Complex I activity, may modify its ROS-generating capacity (Raha *et al.*, 2002; Maj *et al.*, 2004; Scacco *et al.*, 2006). It is therefore tempting to speculate that endocrine alterations may affect the capacity of ROS formation by hyper- or hypophosphorylation of the complex.

There is increasing evidence that pathological states in which Complex I activity is impaired also lead to ROS overproduction (Fato *et al.*, 2008); in cell lines from patients with Complex I deficiency, an inverse relationship was found between superoxide production and residual enzyme activity (Verkaart *et al.*, 2007).

2.1.4 Other Mitochondrial Enzymes

2.1.4.1 $p66^{Shc}$

Recently, an additional source of ROS in mitochondria (directly in the form of hydrogen peroxide) was demonstrated in the $p66^{Shc}$ protein (Migliaccio *et al.*, 2006); $p66^{Shc}$ is a splice variant of $p46^{Shc}$/$p52^{Shc}$, two cytoplasmic proteins involved in signal transduction from tyrosine kinases to Ras. $p66^{Shc}$ has the same modular structure of the former proteins, but contains a unique N-terminal region and is not involved in Ras regulation. Its function has been recently discovered to be in the regulation of ROS metabolism and apoptosis (Migliaccio *et al.*, 1999). $p66^{Shc-/-}$ cells are resistant to apoptosis induced by a variety of different signals (Migliaccio *et al.*, 1999); expression of the protein is required for mitochondrial depolarization and release of cytochrome c after a variety of proapoptotic signals (Trinei *et al.*, 2002). $P66^{Shc}$ deletion in mice decreases the incidence of ageing-associated diseases (Napoli *et al.*, 2003; Menini *et al.*, 2006; Rota

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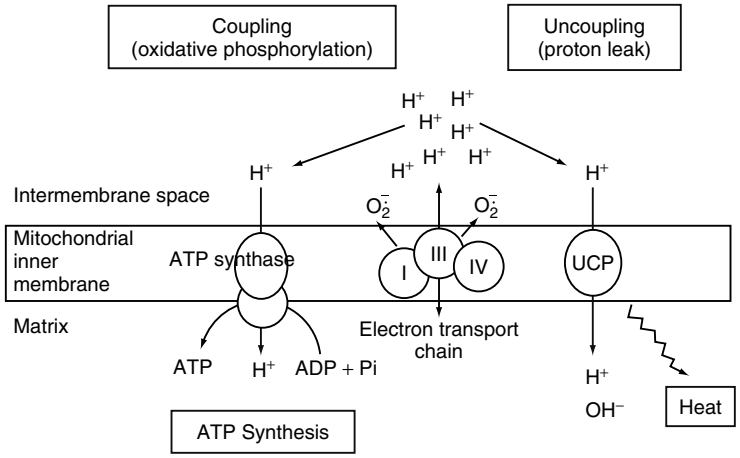

Figure 4 Scheme showing the competition of ATP synthase and uncoupling proteins (UCPs) for the proton gradient. Both systems utilize protons from the intermembrane space and release them in the matrix, determining an increase of the rate of electron transfer in the respiratory chain. Under both these conditions (State 3 and uncoupled state respectively) the membrane potential is decreased and ROS production is low. When ATP is not synthesized and UCPs are not expressed, the membrane potential is high, electron transfer is slow and the chain is more reduced, allowing abundant ROS formation.

et al., 2006) and prolongs lifespan of animals (Migliaccio *et al.*, 1999).

Pathways leading to increase of $p66^{Shc}$ expression in oxidative stress involve p53 (Trinei *et al.*, 2002) and protein kinase C (PKC)-β isoform (Pinton *et al.*, 2007); moreover $p66^{Shc}$ expression and phosphorylation is involved in stabilization of the hypoxia-inducible factor (HIF)1α (Bianchi *et al.*, 2006; Carraro *et al.*, 2007) (cf. Section 15.5).

A fraction of $p66^{Shc}$ has a mitochondrial localization in the intermembrane space, where it is bound in an inactive form in a high-molecular-weight complex including the inner membrane transporter (TIM)/outer membrane transporter (TOM) protein import system (Giorgio *et al.*, 2005); proapoptotic signals dissociate the protein from the complex and activate it to a form inducing a permeability transition by opening a high-conductance channel in the inner membrane, the permeability transition pore (Bernardi *et al.*, 2001) involved in the events leading to apoptosis. This effect is due to the intrinsic property of $p66^{Shc}$ to act as a redox protein accepting electrons from cytochrome c and directly producing hydrogen peroxide (Giorgio *et al.*, 2005) (**Figure 5**). Since the reaction equilibrium of cytochrome c oxidation by $p66^{Shc}$ is low ($K_{eq} = 0.1$), the reaction is thermodynamically favoured when the level of cytochrome c reduction is high (Giorgio *et al.*, 2005).

This means that H_2O_2 production by this mechanism should be enhanced when cytochrome c oxidase (the enzyme catalysing cytochrome c reoxidation by oxygen)

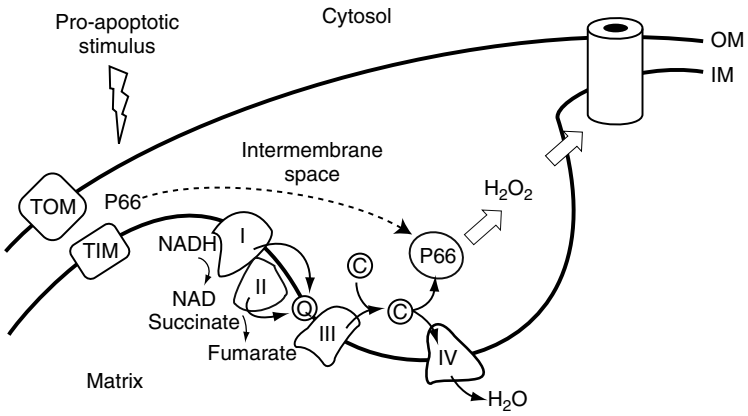

Figure 5 Role of $p66^{Shc}$ in H_2O_2 production in mitochondria. In the presence of proapoptotic stimuli, p66 is released from the protein transport complex (TOM, outer membrane transporter, TIM, inner membrane transporter) and becomes free in the intermembrane space, where it may receive electrons from cytochrome c, with production of H_2O_2. The generation of H_2O_2 would be favoured by conditions where cytochrome oxidase (IV) is inhibited. OM, outer membrane; IM, inner membrane. (Reproduced by Giorgio *et al.*, 2005. © Elsevier.)

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is inhibited. The K_m of cytochrome oxidase for oxygen is very low (<1 μM), thus allowing its activity, even at low oxygen tensions; however, low oxygen tensions promote the activation of HIF by a still controversial mechanism (Taylor, 2008) that triggers a series of metabolic changes, among which is the alteration of cytochrome oxidase subunits and activity. The activation of the $p66^{Shc}$ pathway may in part explain the paradoxical enhancement of ROS production during hypoxia (Guzy and Schumacker, 2006).

Another factor leading to decrease of cytochrome oxidase activity is nitric oxide $NO^{\bullet}$, which inhibits the enzyme competitively with oxygen (Sarti *et al.*, 2000), thus elevating the K_m. The $NO^{\bullet}$ inhibition may not be relevant at normal oxygen tensions (about 30 μM), but may become important when the oxygen tension is lowered, for example during ischaemia.

2.1.4.2 Dihydrolipoamide Dehydrogenase

Dihydrolipoamide dehydrogenase (DLD) is a flavoprotein subunit of the α-ketoglutarate dehydrogenase and pyruvate dehydrogenase complexes situated in the mitochondrial matrix, catalysing the reoxidation of protein-bound lipoate (lipoamide) by NAD^+.

$$\text{lipoamide (SH)}_2 + \text{NAD}^+ \longrightarrow \text{lipoamide (S-S)} + \text{NADH} + \text{H}^+ \quad (14)$$

This subunit of α-ketoglutarate dehydrogenase has been shown to be a source of superoxide by electron leak from the flavin (Starkov *et al.*, 2004; Tretter and Adam-Vizi, 2004) under State 4 conditions, when the enzyme is more reduced; the contribution of the pyruvate dehydrogenase analogous subunit to superoxide formation was found to be 50% of that of α-ketoglutarate dehydrogenase. In *S. cerevisiae* under conditions of NAD depletion or at high $NADH/NAD^+$ ratios, the contribution of α-ketoglutarate dehydrogenase to ROS formation was higher than that of the respiratory chain (Tahara *et al.*, 2007) as demonstrated by strong ROS formation by adding α-ketoglutarate or pyruvate, but not malate, to wild-type permeabilized mitochondria, and by prevention of oxidative stress in deletion mutants of the DLD gene; the oxidative stress was also prevented by calorie restriction, which is known to keep the $NADH/NAD^+$ ratio low (Lin *et al.*, 2004).

2.1.4.3 Monoamine Oxidase

Amine oxidases are a class of enzymes catalysing the oxidation of amines by O_2 forming the respective aldehydes and H_2O_2; they are subdivided into FAD-dependent oxidases (monoamine oxidases (MAOs), polyamine oxidases) and copper and topaquinone-dependent oxidases (Strolin Benedetti *et al.*, 2007). Amine oxidases are involved in the oxidation of endogenous amines, such as dopamine (DA), serotonin and so on, but also of xenobiotic amines, such as tyramine and several drugs (Strolin Benedetti *et al.*, 2006).

$$\text{R-CH}_2\text{NH}_2 + \text{O}_2 \longrightarrow \text{R-CH=NH} + \text{H}_2\text{O}_2 \quad (15a)$$

$$\text{R-CH=NH} + \text{H}_2\text{O} \longrightarrow \text{R-CHO} + \text{NH}_3 \quad (15b)$$

MAO exists in two isoforms bound to the outer mitochondrial membrane, MAO-A and MAO-B; it is a flavoprotein (Cadenas and Davies, 2000) (for a review on the molecular mechanism see Edmondson *et al.*, 2007). MAO-A has been shown to represent an important source of ROS in rat heart (Maurel *et al.*, 2003); oxidative stress mediated by MAO-A results in cardiomyocyte apoptosis and MAO-A inhibition reduces myocardial damage induced by ischaemia-reperfusion (Bianchi *et al.*, 2005). The increase of MAO-A activity in a heart subjected to ischaemia-reperfusion has been attributed to increased availability of its substrate serotonin. Use of specific MAO inhibitors may represent a tool for cardioprotection (Di Lisa *et al.*, 2007).

Table 1 summarizes the sites of ROS formation in mitochondria.

2.2 Other Cellular Sources of ROS

Besides mitochondria, other systems in the cell may significantly contribute to ROS generation and sometimes

Table 1 Summary of the known sites for ROS production in mitochondria

Respiratory chain	Comments
Complex I	Forward and reverse e-transfer; $O_2^{\bullet -}$ in the matrix
Complex III	High membrane potential, $O_2^{\bullet -}$ in the IMS
Complex II	$O_2^{\bullet -}$ in the matrix
α-GP-DH	$O_2^{\bullet -}$ in the IMS
ETF-DH	$O_2^{\bullet -}$ in the matrix
Dihydroorotate DH	$O_2^{\bullet -}$ in the IMS
P66	H_2O_2 from cyt c, in the IMS
α- ketoglutarate DH complex	From the E_3, DLD subunit- $O_2^{\bullet -}$ in matrix
Pyruvate DH complex	From the E_3, DLD subunit- $O_2^{\bullet -}$ in matrix
Monoaminooxidase (MAO)	Outer membrane

α-GP-DH = α-glycerophosphate dehydrogenase; ETF-DH = electron transfer flavoprotein dehydrogenase; DLD = dihydrolipoate dehydrogenase; IMS = intermembrane space.

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become the overwhelming source of such species. This is particularly true in cells where mitochondria are not a substantial mass percentage or in specialized systems such as the phagocytic cells. Nevertheless some authors consider extramitochondrial sources of ROS as prevalent and endowed of higher damaging potential for the cell.

2.2.1 Xanthine Oxidase

This enzyme catalyses the oxidation of hypoxanthine to xanthine and in turn the oxidation of xanthine to urate and is involved in the degradation of purine nucleotides. The enzyme in healthy tissues exists mainly in the form of NAD-dependent xanthine dehydrogenase (XDH), but under ischaemic conditions is converted by a protease into XO, which reduces oxygen to both superoxide and hydrogen peroxide.

$$\text{hypoxanthine} + \text{NAD}^+ \longrightarrow \text{xanthine} + \text{NADH} + \text{H}^+ \text{ (xanthine dehydrogenase)} \quad (16)$$

$$\text{hypoxanthine} + \text{O}_2 \longrightarrow \text{xanthine} + \text{H}_2\text{O}_2 \text{ (xanthine oxidase)} \quad (17)$$

$$\text{hypoxanthine} + 2\text{O}_2 \longrightarrow \text{xanthine} + 2\text{O}_2^{\bullet -} \text{ (xanthine oxidase)} \quad (18)$$

The enzyme from animal sources is a homodimer with a molecular weight of 300 kDa; each subunit binds a molybdopterin cofactor, two nonidentical iron–sulphur centres and one FAD (Nishino *et al.*, 2008). The enzyme is largely localized in peroxisomes, but is also found in the cytoplasm of hepatic endothelial cells.

Stirpe and Della Corte (1969) first reported that the enzyme originally exists in the XDH form, but is converted to XO by proteolysis or by oxidation of cysteine residues to form S-S bridges. The difference between XDH and XO is the changed reactivity of reduced FAD towards NAD and O_2. Indeed FAD is the only redox centre in the enzyme undergoing significant changes in redox properties when XDH is converted into XO, so that the flavin semiquinone $FADH^{\bullet}$ is thermodynamically stable in XDH, but not in XO (Nishino *et al.*, 2008).

Under ischaemic conditions XDH rapidly converts to XO generating both $O_2^{\bullet -}$ and H_2O_2: ATP depletion results in loss of ATP-dependent Ca^{2+} homeostasis increasing its concentration in the cell; one important consequence of Ca^{2+} increase is the activation of a Ca-dependent protease that cleaves XDH to XO, thus inducing massive production of ROS by oxidation of hypoxanthine generated by cleavage of AMP, the major nucleotide present in the cell under ischaemic conditions. The protective effect of the XO inhibitors oxypurinol or allopurinol against ischaemia-reperfusion injury in several tissues (e.g. Viňa *et al.*, 2000; Rieger *et al.*, 2002) is an indirect demonstration of the important role of XO in ROS generation under ischaemic conditions.

2.2.2 Cytochrome P450

Cytochrome P450 (CYP) constitutes a superfamily of enzymes acting as mono-oxygenases inserting one oxygen atom from O_2 into a variety of endogenous and exogenous compounds (Zangar *et al.*, 2004).

$$\text{RH} + \text{NADPH} + \text{H}^+ + \text{O}_2 \longrightarrow \text{R-OH} + \text{NADP}^+ + \text{H}_2\text{O} \quad (19)$$

These include physiological molecules such as fatty acids, sterols, eicosanoids, amino acids and so on, or xenobiotic molecules such as drugs, procarcinogens, antioxidants, organic solvents, plant products and so on. CYPs are haeme proteins defined by an absorption spectrum, where the $Fe^{2+}(CO)$ complex has a characteristic Soret band near 450 nm. The CYP enzymes are membrane-bound (mostly to the endoplasmic reticulum) existing in a multienzyme system including a flavin-containing NADPH-CYP reductase. The microsomal complex also contains an alternative system of cytochrome b_5 and the flavoprotein NADH-cytochrome b_5 reductase; the role of cytochrome b_5 in P450 function is unclear.

There are many different CYP isoforms, and there can be over 30 CYP genes expressing their products in the same organism, many of them in the same tissue (Nebert *et al.*, 1991). Some CYP isoforms are specific in their choice of substrates, but many, particularly those in the endoplasmic reticulum, can react with a wide number of endogenous and exogenous compounds. The vast majority of CYPs are expressed in the liver, but some are specifically detected in other tissues, including heart (Gottlieb, 2003).

The efficiency of electron transfer from NADPH through the reductase to P450 for oxygenation reactions is called the degree of coupling (Yasui *et al.*, 2005); in microsomal P450 the degree of coupling is poor, less than 50% and often much lower. For this reason the rate of NADPH and oxygen consumption by P450 is weakly dependent on the presence of substrates, and then the microsomal P450 generates ROS, including superoxide, hydrogen peroxide, hydroxyl radical and singlet oxygen (Yasui *et al.*, 2005).

The mechanism of mono-oxygenation involves several steps (Sono *et al.*, 1996) (**Figure 6**).

Besides producing radicals, P450 may also inactivate fatty acid hydroperoxides (Zangar *et al.*, 2004). Fatty acid hydroperoxides (cf. Section 15.3.1) may act as direct oxidizing agents to P450 leading to substrate oxidation and monohydroxylated fatty acids; in the absence of substrate co-metabolism, P450 can catalyse the isomerization of fatty acid hydroperoxides to the dihydroxylated fatty acids.

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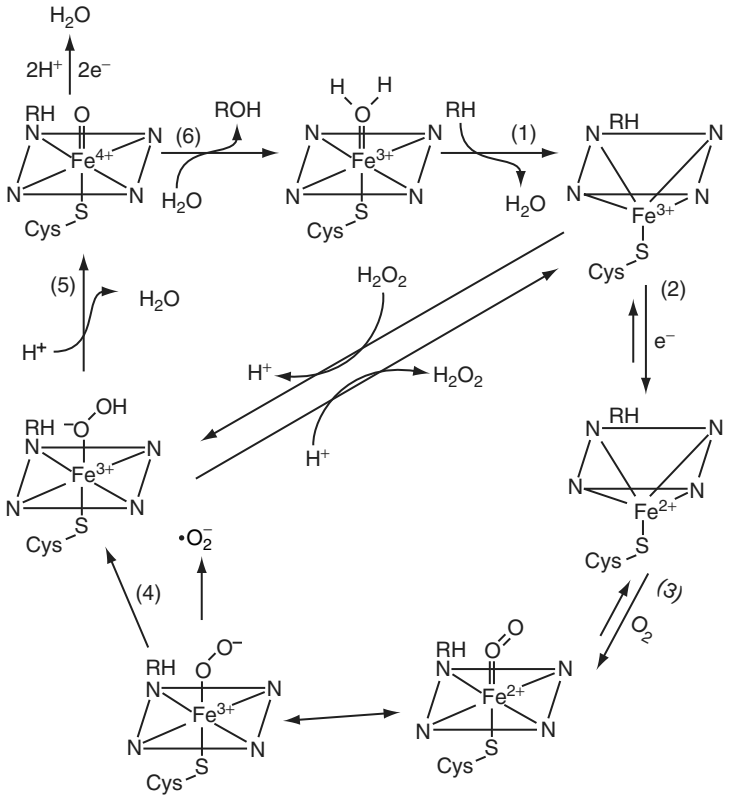

Figure 6 The catalytic cycle of cytochrome P450 and the sites of ROS production. The release of superoxide would occur after transfer of one electron to the haeme iron and binding of oxygen, producing a six-coordinate haeme–Fe^{3+}–$O_2^{\bullet -}$ intermediate (step 3) that can release $O_2^{\bullet -}$; after addition of a second electron and protonation (step 4), the hydroperoxo-Fe^{3+} intermediate may dissociate H_2O_2, regenerating the free Fe^{3+}-haeme (peroxide shunt). (Reproduced by Sono *et al.*, 1996. © American Chemical Society.)

P450 appears to contribute significantly to the overall ROS formation in the cell, even in the absence of xenobiotics induction, in some tissues, such as the liver.

2.2.3 Nitric Oxide Synthase

Nitric oxide synthases (NOS) are a family of enzymes including endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS); NOS is a CYP reductase-like enzyme that catalyses flavin-mediated electron transfer from NADPH to a prosthetic haeme group; the enzyme also requires tetrahydrobiopterin (BH_4) to transfer electrons to arginine to release $NO^{\bullet}$ (Bredt, 1999). Although NOS is mostly a cytoplasmic enzyme, a mitochondrial isoform has been described (Ghafourifar and Sen, 2007), but its existence is still debated (Burwell and Brookes, 2008).

The reaction catalysed by NOS is still little understood; the reaction occurs in two steps with formation of *N*-hydroxyarginine, but the detailed mechanism is uncertain:

$$\text{arginine} + 2O_2 + \text{NADPH} + H^+ \longrightarrow \text{citrulline} + NO^{\bullet} + \text{NADP}^+ \quad (20)$$

$NO^{\bullet}$ is a relatively stable radical with a half-life of 1–10 s (Brown and Borutaite, 2004).

In the absence of either BH_4 or arginine, NOS uncouples, that is, it reacts with oxygen, generating $O_2^{\bullet -}$ instead of $NO^{\bullet}$ (Pou *et al.*, 1999); ROS generated by other sources are involved in a feed-forward mechanism inactivating BH_4 and leading to NOS uncoupling (Alp and Channon, 2004). Simultaneous formation of $NO^{\bullet}$ and $O_2^{\bullet -}$ would favour the production of peroxynitrite.

2.2.4 Peroxisomal Enzymes

Peroxisomes are cellular organelles which oxidize a number of substrates, consuming oxygen, the latter being reduced to H_2O_2. In rat liver, peroxisomal oxidations account for about 20% of total O_2 consumption (Reddy and Mannaerts, 1994). Peroxisomes play a key role in both the production and scavenging of ROS in the cell (Schrader and Fahimi, 2006). Besides H_2O_2, peroxisomal enzymes also generate $O_2^{\bullet -}$ and $OH^{\bullet}$ radicals; it was also found that the inducible isoform of NOS is localized in the peroxisomes in its monomeric inactive state, and in the cytoplasm in its dimeric active state (Loughran *et al.*, 2005).

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The main pathway of peroxisomes involved in H_2O_2 production is fatty acid β-oxidation (Hiltunen and Poirier, 2006) via three different acyl-CoA oxidases specific for fatty acyl chains of different lengths and bile acid intermediates. Other enzymes contained in peroxisomes and contributing to hydrogen peroxide generation are D-amino acid oxidases, sarcosine oxidase, L-α-hydroxy acid oxidase, polyamine oxidase (Schrader and Fahimi, 2006), and, most important, XO described in Section 15.2.2.1.

Peroxisomes also contain enzymes that scavenge these reactive species (Schrader and Fahimi, 2006): catalase is the typical marker enzyme of peroxisomes that removes with high efficiency peroxides formed by peroxisomal enzymes; in addition these organelles also contain GPx, Mn-superoxide dismutase (SOD-2) (more typically present in mitochondria), Cu, Zn-superoxide dismutase (SOD-1), peroxiredoxin (Prdx) I, and others (cf. Section 15.5). **Table 2** shows the major ROS-generating and ROS-scavenging enzymes of peroxisomes.

An elevation of oxygen concentration induces a moderate proliferation of peroxisomes, but a more pronounced proliferation, with a many-fold induction of H_2O_2-generating enzymes, occurs after treatment with peroxisome-proliferating chemicals such as hypolipidaemic drugs, industrial compounds and agrochemicals (Beier and Fahimi, 1991); this effect is due to the nuclear transcription factor peroxisome proliferator-activated receptor (PPAR) α and its coactivators (Reddy, 2004). On then other hand, the regulation of peroxisomal proliferation and enzyme induction by oxygen and ROS is not yet well understood (Schrader and Fahimi, 2006). The exposure of cultured cells to UV light and oxidizing agents induces peroxisomal proliferation; the massive proliferation induced by a variety of xenobiotics and the subsequent tumour formation in rats is due to an imbalance in the formation and scavenging of ROS and is mediated by PPARα (Yu *et al.*, 2003).

Studies of knockout (KO) mice in which the PEX genes required for peroxisomal biogenesis and protein import have been deleted (Baes and Veldhoven, 2006) showed absence of functional peroxisomes, mitochondrial alterations, but no evidence of oxidative damage nor increased peroxide production.

Table 2 Peroxisomal enzymes involved in ROS formation and detoxification in mammals

ROS generation	ROS degradation
Acyl-CoA oxidases	Catalase
Palmitoyl-CoA oxidase	Glutathione peroxidase
Pristanoyl-CoA oxidase	Mn-SOD
Trihydroxycoprostanoyl-CoA oxidase	Cu, Zn-SOD
Urate oxidase	PMP20
Xanthine oxidase	Epoxide hydrolase
D-Amino acid oxidase	Peroxiredoxin-1
Pipecolic acid oxidase	—
D-Aspartate oxidase	—
Sarcosine oxidase	—
L-α-Hydroxy acid oxidase	—
Polyamine oxidase	—
NO synthase	

SOD, superoxide dismutase; PMP, peroxisomal membrane protein.

2.2.5 Plasma Membrane NADPH Oxidase

A phagocyte NADPH oxidase, later called NOX-2, was found to be responsible for the respiratory burst accompanying phagocytosis (Rossi and Zatti, 1964) and producing superoxide. Much later it was found that NOX isozymes are present in many other cell types (Bedard *et al.*, 2007; Lambeth, 2007; Sumimoto, 2008). All NOX family members are transmembrane proteins that transport electrons across biological membranes to reduce O_2 to superoxide; all have a strong preference for NADPH over NADH as the donor substrate.

$$\mathrm{NADPH} + 2\mathrm{O}_2 \longrightarrow \mathrm{NADP}^+ + 2\mathrm{O}_2^{\bullet -} + \mathrm{H}^+ \qquad (21)$$

Much of what is known about the topography and structure of NOX isoforms is derived from studies of NOX-2 (**Figure 7**); the protein has six transmembrane domains and contains FAD in the cytoplasmic domain and two asymmetrical haeme groups embedded in the membrane domain, so that electrons are transferred from

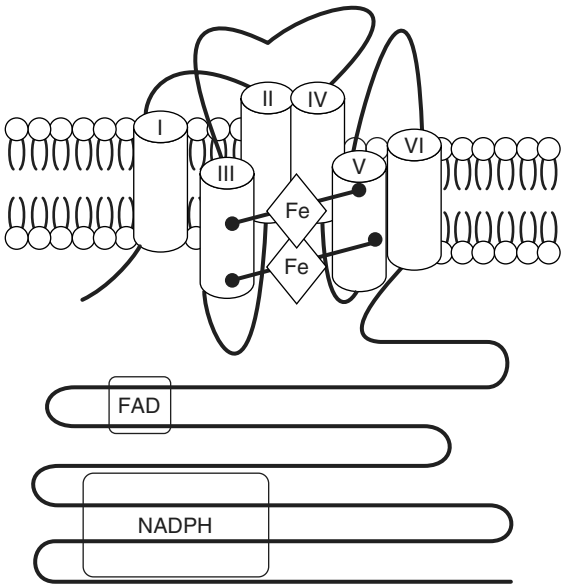

Figure 7 Structure of NOX-2 (gp91^{phox}). The six cylinders I-VI are transmembrane α-helices; the two rhombs connecting helices III and V are the haeme groups, while FAD and the NADPH binding region are in the cytoplasmic domain. NOX-2 is constitutively linked to membrane-bound p22^{phox} (not shown).

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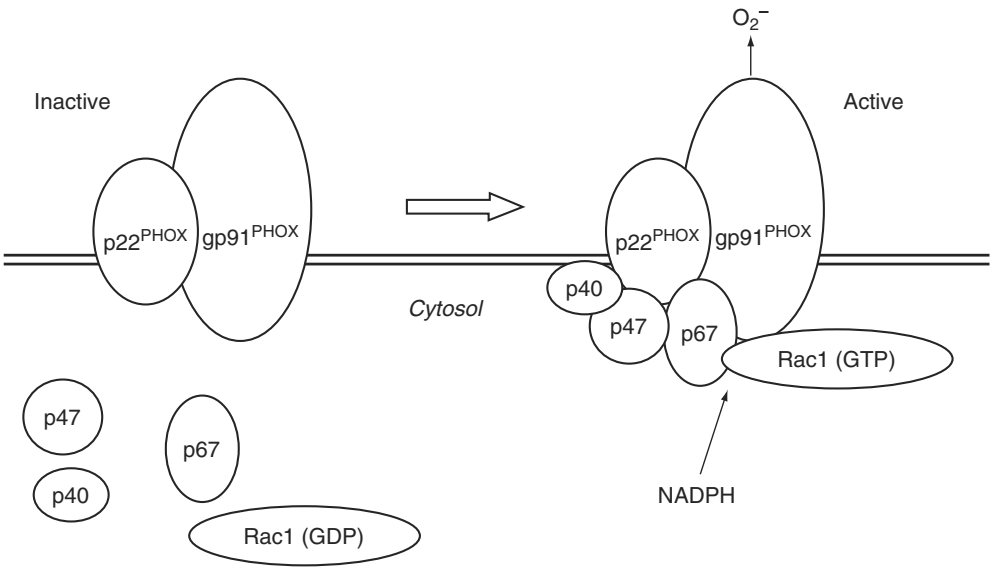

Figure 8 Activation of NOX-2 by cytoplasmic factors. Upon cellular stimulation, the p40, p47, p67 and the GTPase Rac1 associate from forms free in the cytoplasm to a supramolecular complex with the gp91-p22^{phox} complex. This activated complex is able to transfer electrons from cytoplasmic NADPH to extracellular oxygen to form superoxide.

NADPH to FAD in the cytoplasm and translocated to oxygen on the exterior of the cell.

The activation of NOX-2 occurs through a complex series of protein–protein interactions; NOX-2 (gp91^{phox}) forms a constitutive complex with p22^{phox}; phosphorylation of p47^{phox} allows its interaction with the NOX2-p22^{phox}; p47^{phox} is an organizer subunit allowing the translocation of other cytosolic subunits (the activator subunit p67^{phox}, the smaller p40^{phox} and then finally the guanosine triphosphate (GTP)ase Rac) (**Figure 8**); once assembled, the complex is active in catalysing the reaction of NADPH with oxygen.

Although NOX-2 is regarded as the typical phagocyte-specific NADPH oxidase, widely expressed in neutrophils and macrophages, nevertheless it is found in a number of tissues. In phagocytes, NOX-2 localizes in both plasma membranes and in intracellular granules; upon phagocyte stimulation, NOX-2 translocates to the surface by fusion of the granules with the plasma membrane.

Once superoxide is formed (Reaction 21), this is converted to H_2O_2 by SOD and then to hypochlorous acid by myeloperoxidase:

$$2O_2^{\bullet -} + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad (22)$$

$$H_2O_2 + Cl^- + H^+ \longrightarrow HOCl + H_2O \qquad (23)$$

It is not clear how much the bacterial killing activity is due to superoxide, to H_2O_2 or to hypochlorous acid, or even to hydroxyl radical, singlet oxygen or peroxynitrite (Bedard and Krause, 2007); probably the combination of all of these factors is active *in vivo*.

More recently, several homologues of NOX-2 were discovered, each present in major amounts in a specific tissue, but all expressed in a wide variety of tissues (Bedard and Krause, 2007) (cf. **Table 3**). All the NOX homologues, except NOX-5, require p22^{phox} for activity, while the requirement for cytosolic subunits is clear only for NOX-1.

Two additional enzymes resembling NOX, called dual oxidase (DUOX)-1 and -2, were identified in the thyroid gland (Dupuy *et al.*, 1999). The first product of these enzymes is likely to be superoxide, which is, however, very rapidly dismutated to hydrogen peroxide (Ameziane-el-Hassani *et al.*, 2005); DUOX enzymes do not appear to require cytosolic factors.

Besides the well-understood role of NOX-2 in bacterial killing, the function of NOX from other sources is essentially cell regulation; this function occurs through oxidation of redox-sensitive cysteine residues in signal transduction proteins (Hunter, 2000). Nevertheless, the

Table 3 Human NOX isoforms

Enzyme	Location	Regulatory factors
NOX-1	Inducible (colon and smooth muscle)	NOX01, NOXA1, P22^{phox}
NOX-2	Phagocytes	P22^{phox}, P47^{phox}, P67^{phox}, P40^{phox}, Rac1
NOX-3	Foetal kidney	P22^{phox}
NOX-4	Widespread	—
NOX-5	Spleen, sperm, brain	Ca^{2+}
DUOX-1	Thyroid and others	Ca^{2+}
DUOX-2	Thyroid and others	Ca^{2+}

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biochemical and physiological properties of NOX enzymes *in situ* are not yet fully clarified (Nauseef, 2008).

NOX-derived ROS have been implicated in a wide variety of pathological dysfunctions, including cardiovascular diseases, neurodegenerative diseases, hyperinflammatory response to *Helicobacter* infection, inflammatory colon disease and colon cancer, liver cirrhosis, diabetic nephropathy, asthma, chronic obstructive pulmonary disease and others (Bedard and Krause, 2007; Lambeth, 2007).

In addition to the NADPH oxidases described above, Morré's group has described a family of cell surface hydroquinone and NADH oxidases with protein disulfide thiol exchange activity, referred to as ECTO-NOX (Morré and Morré, 2003); one member of this family, designated tNOX, is specific for cancer cells and for cells in early developmental stages (Cho and Morré, 2008). These enzymes react with O_2, generating superoxide, and are characterized by the requirement for ubiquinone in the membrane as a redox mediator between quinone reductase and NOX proper.

2.2.6 Lipoxygenases

Lipoxygenases catalyse the conversion of arachidonic acid into a broad class of signalling molecules, such as leukotrienes, hydroxyeicosatetraenoic acid and lipoxins (Schewe, 2002). The enzymatic cycle involves formation of enzyme-bound radicals and lipid hydroperoxides that, under certain conditions, escape the active site and attack surrounding molecules. Lipid hydroperoxides can also be formed by nonenzymatic oxidation of polyunsaturated fatty acids (PUFAs) and further modified by lipoxygenases.

2.2.7 Metal-induced Oxidative Stress

The generation of ROS is closely linked to the participation of redox-active metals (Valko *et al.*, 2005; 2006). The redox state of the cell is largely linked to an iron redox couple; it has been shown that an excess of superoxide releases free iron from iron proteins (Liochev and Fridovitch, 2002); the released Fe^{2+} can participate in the Fenton reaction, generating the highly reactive hydroxyl radical (Reaction 4). Since superoxide is able to reduce Fe^{3+} to Fe^{2+}, it participates in the Haber–Weiss reaction, which combines the Fenton reaction and the reduction of Fe^{3+} by superoxide (Reaction 24 + 4):

$$Fe^{3+} + O_2^{\bullet -} \longrightarrow Fe^{2+} + O_2 \quad (24)$$

$$O_2^{\bullet -} + H_2O_2 \longrightarrow O_2 + OH^{\bullet} + OH^{-}$$

(Haber–Weiss reaction) (25)

The most realistic *in vivo* production of $OH^{\bullet}$, according to the Fenton reaction, occurs when the reduced metal is iron, copper, chromium or cobalt; the pathophysiological significance of Fenton chemistry is however still debated, due to the effective sequestration of metals, namely iron, by metal-binding proteins (Kakhlon and Cabantchik, 2002); however, organisms overloaded with iron contain large amounts of free iron; free iron is transported into an intermediate pool of exchangeable and easily chelated iron.

The role of metal ions in enhancing ROS formation is considered the main basis of their toxicity and carcinogenicity (Valko *et al.*, 2006). Besides the well-characterized carcinogenic action of arsenic, cadmium, chromium and nickel (Durham and Snow, 2006), iron in excess may also be carcinogenic due to direct action on DNA (Goetz and Luch, 2008).

ROS are continuously produced in erythrocytes due to the high O_2 tension and their high iron content (Cimen, 2008); the source of ROS in erythrocytes is haemoglobin (Hb), which undergoes auto-oxidation to produce $O_2^{\bullet -}$ and meta-Hb having a ferric iron. Oxidative stress may damage the red cell itself, but ROS may also leave the cell and damage other components of the circulation (Johnson *et al.*, 2005).

2.2.8 Role of Electron Mediators and Xenobiotics: Redox Cycling

Many physiologically active substances and xenobiotics have electron transfer functionalities, either per se, or more usually in their metabolites. These main groups include quinones (or phenolic precursors), metal complexes (or complexors), aromatic nitro compounds (or reduced derivatives), and conjugated imines or iminium species (Kovacic and Cooksy, 2005). *In vivo* redox cycling with oxygen can occur in a catalytic fashion giving rise to a futile cycle that regenerates the parent compound and releases ROS. Redox cycling has been suggested for several drugs, like cocaine, other abused drugs, catecholamines and several other compounds (Halliwell and Gutteridge, 1999). Electron transfer with redox cycling occurs through interference with physiological electron transfer reactions such as microsomal CYP, XO and the mitochondrial respiratory chain.

A typical example is the anticancer agent, adriamycin, which exhibits severe cardiotoxicity; the toxic effect is distinct from the anticancer mechanism, and involves ROS formation, as also suggested by the protective effect of overexpressing antioxidant enzymes in transgenic animals (Sun *et al.*, 2001).

Studies in perfused rat hearts showed that adriamycin largely localizes to mitochondria. Thus, although the drug can be reduced univalently by a variety of systems, such as CYP, XO and mitochondrial Complex I, the oxidative activation in the heart appears to involve mainly mitochondria (Berthiaume and Wallace, 2007). Conversely, in the liver CYP appears to account for a substantial portion of activation (Cribb *et al.*, 2005). The mechanism of redox cycling is shown in (**Figure 9**): the

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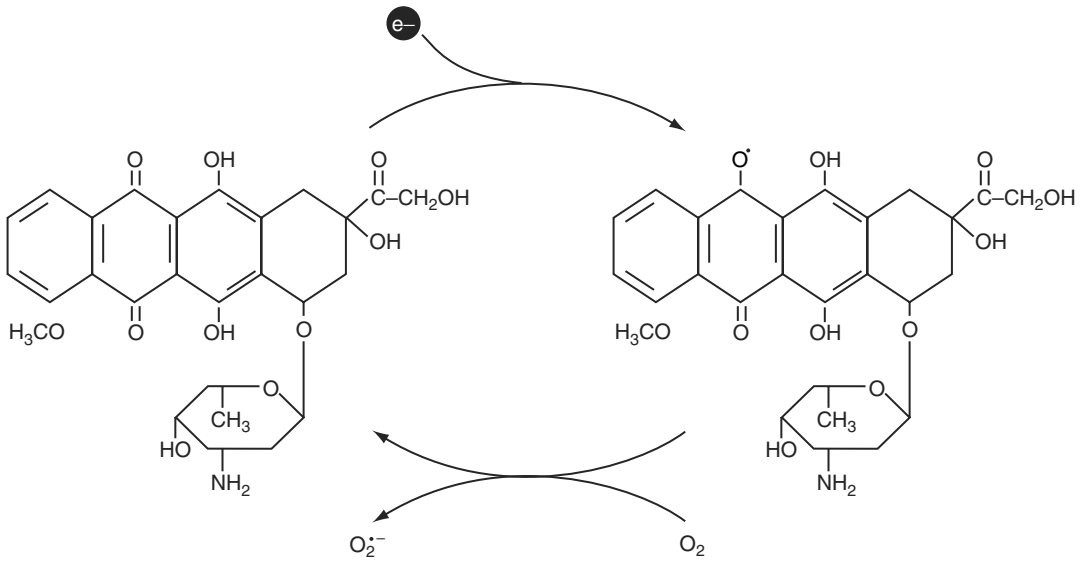

Figure 9 Redox cycle of adriamycin. The drug is reduced by one electron (from CYP or mitochondrial Complex I) to its semiquinone radical, which reacts with oxygen, producing superoxide.

drug is first reduced to a semiquinone radical by a one-electron transfer, and then reacts with oxygen, releasing superoxide and regenerating adriamycin.

Recent studies with physiological doses confirm that adriamycin induces acute ROS generation that occurs mainly at the level of Complex I (Salvatorelli *et al.*, 2006). The mechanism of cardiotoxicity, however, implies long-term exposure, indicating that acute oxidative stress per se is not sufficient to explain cardiac failure. The major mechanisms involved in chronic toxicity also appear to involve mitochondria and are ascribed to secondary damage induced by ROS to Complex I and other mitochondrial complexes, with loss of OXPHOS efficiency, and to oxidative damage to mitochondrial DNA leading to permanent loss of the OXPHOS machinery (Berthiaume and Wallace, 2007).

The activation pathway followed by carcinogenic polycyclic aromatic hydrocarbons is more complex (Luch, 2005) (**Figure 10**); mono-oxygenation by CYP generates *trans*-dihydrodiols with the subsequent formation of dihydrodiol epoxides, which form adducts with DNA, thus initiating carcinogenesis. Alternatively, *trans*-dihydrodiols may be converted by enzymatic oxidation into cathecols, which undergo spontaneous auto-oxidation with O_2 to the corresponding *o*-quinones, by two one-electron steps, with intermediate formation of the semiquinone anion radicals and reduction of O_2 to either $O_2^{\bullet -}$ or H_2O_2; both the semiquinone and the quinone forms can undergo catalytic one-electron redox cycling, being reduced by either microsomal CYP and cytochrome b_5 reductase or mitochondrial Complex I (Luch, 2005). One-electron reduction and auto-oxidation steps establish futile redox cycles in which ROS generation is amplified multiple times (Goetz and Luch, 2008). Biotransformation of polycyclic hydrocarbons also leads to formation of polynuclear quinones, which may also undergo redox cycling with the corresponding quinols.

A similar mechanism has been suggested for benzene: initial metabolism occurs in the liver by CYP, with resulting formation of, among other compounds, benzene dihydrodiol, which is oxidized to cathecol and then

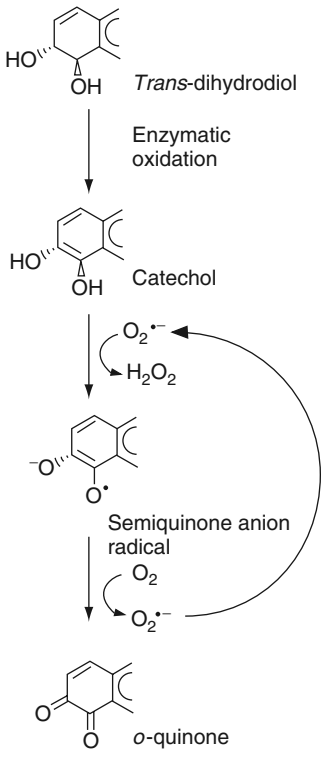

Figure 10 Redox cycling of aromatic polycyclic hydrocarbons. The *trans*-dihydrodiols are oxidized to cathecols that undergo redox cycling as described in the text.

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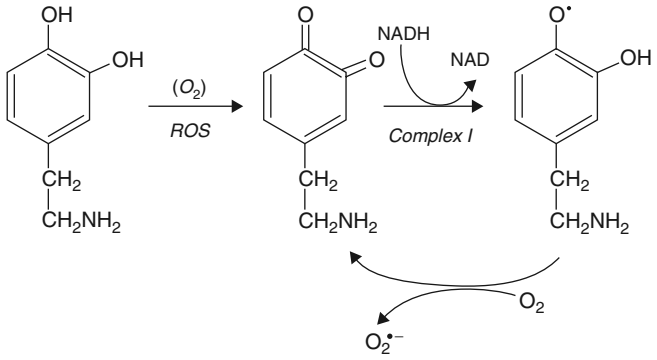

Figure 11 Redox cycling of cathecolamines: the example of dopamine is shown in the figure. Dopamine is first oxidized to dopaminochrome, which is reduced by Complex I by one-electron transfer to the semiquinone, which then auto-oxidizes generating superoxide and perpetuating the cycle.

to benzoquinone (Shen *et al.*, 1996), which undergoes redox cycling with ROS production. To this purpose, *p*-benzoquinone derivatives, such as short-chain CoQ homologues and analogues undergo redox cycling with oxygen at the level of mitochondrial Complex I (Genova *et al.*, 2001); the mechanism of activation involves a site close to the CoQ physiological reduction site, but situated upstream of the rotenone block, presumably the iron–sulfur centre N2 (Lenaz *et al.*, 2006a). When, however, the quinones are added in the absence of a Complex I inhibitor, but in presence of a Complex III inhibitor, thus allowing their reduction, they behave as potent antioxidants preventing ROS formation (Fato *et al.*, 2008).

Adrenaline may undergo oxidation and cyclization to adrenochrome in a multistep process in which the main oxidant under physiological conditions is the superoxide anion (Bindoli *et al.*, 1992). On the other hand adrenochrome can be reduced to the corresponding semiquinone by NADPH in liver microsomes and by mitochondrial Complex I in bovine heart (Bindoli *et al.*, 1990). A redox cycle is then established in which the semiquinone reacts with O_2, producing superoxide and regenerating adrenochrome (Genova *et al.*, 2006).

Since adrenochrome reduction to the semiquinone is totally insensitive to both rotenone (which acts at the level of iron–sulphur centre N2) and *p*-hydroxymercuribenzoate (which inhibits at the start of the iron–sulfur chain), the site of electron delivery to adrenochrome is presumably FMN. Similar events may also occur with other catecholamines such as DA and noradrenaline (**Figure 11**).

2.2.9 UV and Ionizing Radiation

The UV light region occurs between 100 and 400 nm and is subdivided into thee main categories: UVA (315–400 nm), UVB (280–315 nm) and UVC (100–280 nm). The ozone layer absorbs all UVC and most UVB, but the damage to the protective ozone layer now allows an increased amount of UVB to reach the ground (De Gruijl and van der Leun, 2000). UVA is totally absorbed by the skin and penetrates deep into the epidermis and dermis, whereas UVB is less penetrating, but much more active. The UVC light is the most energetic, but is completely absorbed by the Earth's atmosphere.

To exert its biological effects UV light must be transmitted across skin layers and be absorbed by cellular molecules. Damage is induced via two different mechanisms (Pattison and Davies, 2006): one is direct absorption of photons by cellular chromophores leading to photoinduced reactions; the second is indirect and involves photosensitization processes where endogenous and exogenous photosensitizers absorb UV light and induce damage, either by direct interaction of the excited photosensitizer with other molecules, with free radical formation, or by energy transfer to molecular oxygen, with production of ROS, mostly singlet oxygen, but also superoxide (**Figure 12**).

The formation of ROS as mediators of photooxidative stress in UV-irradiated skin depends on a number

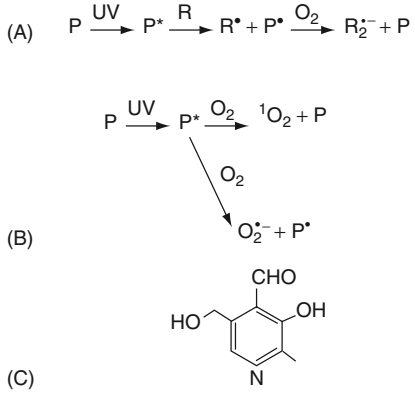

Figure 12 Mechanism of photosensitizers in ROS formation: (A) direct interaction of the excited photosensitizer (P*) with another compound R, with radical formation and secondary autooxidation; (B) interaction of P* with oxygen, with formation of either singlet oxygen or superoxide; (C) an example of endogenous photosensitizer: pyridoxal.

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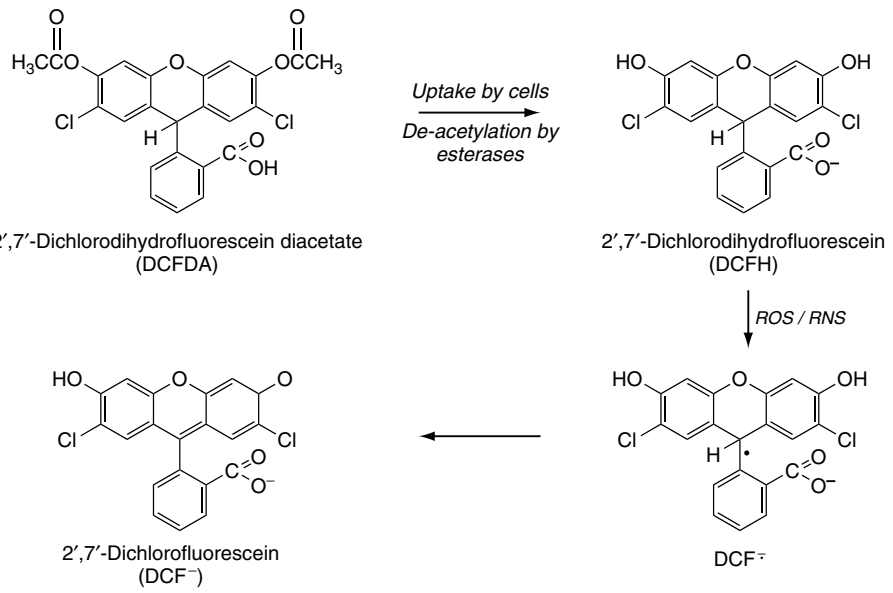

Figure 13 Mechanism of dichlorofluorescein diacetate (DCFDA) as a probe for ROS. DCFDA is taken up by cells, deacetylated to DCFH and oxidized by ROS to DCF via an intermediate radical DCF•.

of photosensitizers present in the skin, such as urocanic acid, riboflavin, melanin precursors, advanced glycation end products (AGE, see Section 15.3.3) and also endogenous 3-hydroxypyridine derivatives (Wondrack *et al.*, 2004); the latter derivatives may arise from various sources, such as B_6 vitamins, the extracellular matrix-associated crosslinks pyridinoline and deoxypyridinoline, and various AGE that accumulate during aging.

The cellular damage induced by ionizing radiation initiates with radiolysis of water, producing radicals, mostly OH•. The direct effect of radiation on ROS production is rather modest and much inferior to the amount of radicals produced by normal metabolic processes ($1\,\mu M\ Gray^{-1}$ compared with hundreds of $mmol\ day^{-1}$ produced by mitochondria). Thus the few primary ionization events must be amplified to account for the rapid and robust activation of signal transduction pathways (Mikkelsen and Wardman, 2003); amplification mechanisms may involve stimulation and uncoupling of NOS with formation of peroxynitrite, release of metal ions and ROS-induced opening of the mitochondrial permeability transition pore (MPTP). This latter mechanism is of major importance since rho^0 cells deficient in mitochondrial DNA (mtDNA) and mitochondrial electron transfer do not show amplification. It has been proposed that radiation damage in a few mitochondria is transmitted via a wave of Ca^{2+}-dependent permeability transitions to adjacent mitochondria, with enhanced ROS/RNS formation.

2.3 Measuring ROS in Cells

Most ROS persist for very short times *in vivo* and cannot be measured directly; there are two approaches to detect transient ROS (Halliwell and Whiteman, 2004): (i) attempt to trap these species and measure the levels of the trapped molecules; (ii) measure the amount of oxidative damage done by ROS. In addition, sometimes the antioxidant activity of body fluids or cells is taken as an index of oxidative stress.

The only technique that can detect free radicals directly is electron spin resonance (ESR). Since reactive radicals are transient, one solution is to add probes that act as 'traps' that intercept them, forming stable radicals detectable by ESR; a wide range of traps has been used in animals and cells in culture, mostly nitroxides. Measuring the amount of oxidative damage (see Section 15.3) by selecting appropriate biomarkers is widely used, but there is often little correlation between the extent of oxidative stress and the enhancement of a biomarker, and often different biomarkers behave differently.

The most widely used methods to detect ROS in cells are based on the use of probes, mainly fluorescence probes. The most popular is 2′-7′-dichlorofluorescein diacetate (DCFDA) (**Figure 13**); DCFDA enters cells and accumulates, then is deacetylated by endogenous esterases to yield the non-fluorescent 2′-7′-dichlorohydrofluoroscein (DCFH) which is oxidized by endogenous ROS to the strongly fluorescent 2′-7′-dichlorofluoroscein DCF: although DCF is usually considered a probe for peroxides, it reacts slowly with H_2O_2, and also with superoxide, while reacting mostly with OH• and peroxynitrite. Other widely used probes are listed in **Table 4**.

The fluorescence can be measured by putting the cells in a plate reader or better by flow cytometry, which measures only intracellular fluorescence. It has to be borne in mind that all studies with probes are amenable

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Table 4 Detection of ROS

ESR spin traps
Biomarkers of ROS damage
Probes:
Dichlorofluorescein diacetate (DCFDA)
Dihydrorhodamine-123
Dihydroethidine
Luminol and lucigenine
Parinaric acid

to artefacts and should be interpreted with much caution (Halliwell and Whiteman, 2004).

3 EFFECTS OF ROS ON CELLULAR MOLECULES AND CELLULAR STRUCTURES

All biomolecules in the cell are targets of ROS and undergo chemical modifications that accumulate with age (Diplock, 1994; Lenaz, 1998): protein carbonylation and methionine oxidation, AGE, lipid peroxidation and nucleotide modifications. However, it is not completely understood which species are responsible for the damage *in vivo* and how the increased availability of ROS translates into the accumulation of specific oxidative damage (Schoneich, 1999); for instance, some proteins are better targets than others for oxidative damage.

The damage induced by ROS depends on the extent and specificity of their reactivity (Halliwell, 2006). If two free radicals meet, they can join their unpaired electrons to form nonradical products; this may lead to loss of reactivity, but in some cases the products may be even more dangerous than the reactants, for example, the reaction of $O_2^{\bullet -}$ and $NO^{\bullet}$ generates the toxic peroxynitrite (Reaction 5).

When a radical meets a nonradical species, several options are possible (Halliwell, 2006). If the radical is reactive enough it attacks the nonradical, generating a new radical and initiating a chain reaction. There are several types of reaction: (i) the radical can add to the nonradical, forming an adduct still having an unpaired electron; (ii) the radical may behave as a reductant, donating a single electron to the nonradical; (iii) the radical may be an oxidant, taking a single electron from the nonradical; (iv) a radical may abstract a hydrogen atom from a –CH bond, for example, $OH^{\bullet}$ can abstract $H^{\bullet}$ from a hydrocarbon, forming water and a carbon-centred radical.

3.1 Lipid Peroxidation

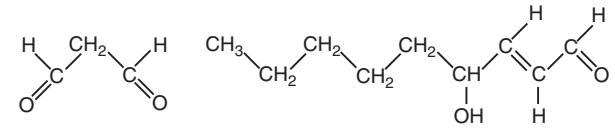
The polyunsaturated fatty acyl chains of phospholipids are very sensitive to oxidation by ROS (Balazy and Nigam, 2003). Since subcellular membranes contain high amounts of polyunsaturated fatty acids (PUFAs), formation of carbon-centred radicals within membranes results in peroxidation of fatty acids.

The overall process of lipid peroxidation can be divided into three stages:

(i) Initiation, that is, extraction of hydrogen from allylic or bis-allylic positions of PUFAs: carbon radicals in the presence of O_2 generate peroxyl radicals ($LOO^{\bullet}$) and random mixtures of cyclic peroxides and hydroperoxides. The initiating species may be the $OH^{\bullet}$ radical, but also a $Fe^{2+}–O_2–Fe^{3+}$ complex or a $ADP–Fe^{2+}$ complex (Valko *et al.*, 2006).

(ii) Radical chain propagation (**Figure 14**): peroxyl radicals may react with side chains of PUFA, yielding hydroperoxides ROOH and other carbon radicals, thus initiating a chain reaction. Phospholipid hydroperoxides may be cleaved by phospholipase A_2 to yield the free fatty acid hydroperoxides (Williams and Gottlieb, 2002). In addition, hydroperoxides can further degrade to alkoxyl ($LO^{\bullet}$), alkyl ($L^{\bullet}$) or $OH^{\bullet}$ radicals, thereby initiating another cycle of chain reactions.

(iii) Termination: when a radical meets another radical or an antioxidant, the chain reaction is terminated. However, peroxyl radicals can react with O_2 by cyclization to produce a cyclic peroxide, which can undergo a second cyclization to form a bicyclic peroxide and then an endoperoxide; the latter is cleaved to produce aldehyde products, the major being malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE), but including also alkadienals, crotonaldehyde, acrolein and so on, all cumulatively designated as advanced lipid peroxidation end products (ALE) (Negre-Salvayre *et al.*, 2008). Oxidation of ω-6 PUFA yields 4-HNE, while oxidation of ω-3 PUFA yields 4-hydroxy-2-hexanal.



MDA is a volatile molecule that reacts via Schiff base formation with amino groups of protein, lipid and DNA. In addition MDA also reacts with DNA bases forming adducts that are mutagenic (Marnett, 1999). 4-HNE is more reactive than MDA with proteins; in addition the modified proteins are not degraded efficiently by the proteasome (Friguet and Szweda, 1997).

Other nonenzymatic products of arachidonic acid oxidation are isoprostanes, which are formed *in situ* within phospholipids, and are then removed by action of phospholipase A_2; isoprostanes are similar to prostaglandins, exert numerous biological functions, but also may

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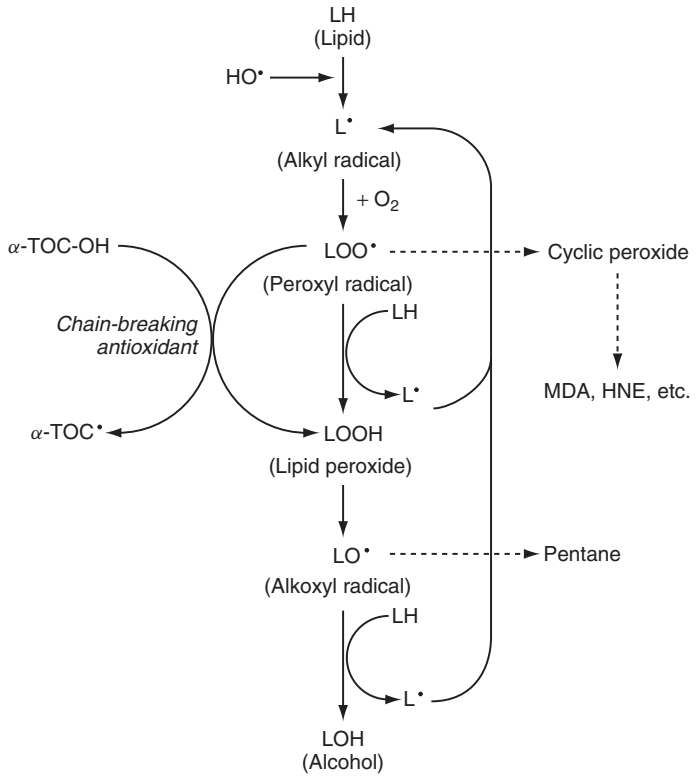
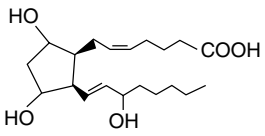
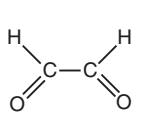


Figure 14 Mechanism of lipid peroxidation. After initiation by attack with an OH•, polyunsaturated fatty acids (LH) form an alkyl radical L• that reacts with oxygen, forming a hydroperoxide radical LOO•. By reacting with other fatty acids LOO• starts a chain reaction with formation of lipid peroxides LOOH; the chain reaction may be interrupted by an antioxidant, a molecule generating more stable radicals, such as α-tocopherol (α-Toc-OH). Alternatively LOOH may react with Fe^{2+}, generating the alkoxyl radical LO•, which may establish a further chain reaction or be degraded to hydrocarbons (pentane). Usually LOOH are degraded to aldehydes such as MDA, 4-HNE, and so on (see text).

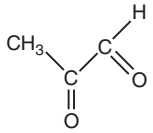
form protein adducts with lysine side chains. Isoprostanes are used as indicators of oxidative stress (Morrow, 2005; Dalle Donne *et al.*, 2006).



Isoprostane



Glyoxal



Methylglyoxal

Another class of reactive ALE precursors is represented by α-oxoaldehydes (methylglyoxal and glyoxal); they are basically derived from sugars in hyperglycaemic conditions, however they are also generated from lipid peroxidation (glyoxal) or the catabolism of glycerol and ketone bodies (methylglyoxal) (Negre-Salvayre *et al.*, 2008). Methylglyoxal metabolism is also linked to ROS production both in the generation and in the degradation of this compound (Kalapos, 2008).

The initial H• abstraction from PUFAs can occur at different points in the carbon chain; thus the peroxidation of arachidonic acid (20 : 4) gives six lipid hydroperoxides, eicosapentaenoic acid (20 : 5) can give eight, and docosahexaenoic acid (22 : 6) can give 10, plus high numbers of cyclic peroxides and isoprostanes (Halliwell, 2006).

Cholesterol can be peroxidized at different positions (5α, 6α, 6β, 7α, 7β) by free radical or singlet oxygen attack. Subsequent breakage of the B ring will produce aldehydes (secosterols), that induce protein misfolding (Bieschke *et al.*, 2006). A common product in atherosclerotic plaques is 27-hydroxy-cholesterol.

Metal ions may split lipid hydroperoxides to produce alkoxyl (LO•) and more peroxyl (LOO•) radicals, that will continue the chain reaction.

$$LOOH + Fe^{2+} \longrightarrow Fe^{3+} + LO^{\bullet} + OH^{-} \quad (26)$$

$$LOOH + Fe^{3+} \longrightarrow Fe^{2+} + LOO^{\bullet} + H^{+} \quad (27)$$

Lipid hydroperoxides have relatively long lifetimes, compared with lipid radicals; for this reason they

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can easily translocate relatively long distances (Girotti, 2008). A well-known example is the transbilayer movement of peroxidized phosphatidyl serine in cells destined for apoptosis. In addition ROOH can move from one to another membrane within a single cell, or from the plasma membrane of a cell to that of another cell or to a lipoprotein (Girotti, 2008). Movement of peroxides from one membrane to another may greatly expand their range of toxic damage, particularly if they move to a compartment rich in iron and/or poor in antioxidants (Vila *et al.*, 2000).

A distinct lipid modification is the reaction of the *cis* double bonds of arachidonic acid with the nitrogen dioxide radical ($NO_2^{\bullet}$) forming nitroeicosanoid derivatives that can then split to four different *trans* isomers; these compounds have a number of biological effects, including induction of apoptosis, and may be regarded as selective biomarkers of nitro-oxidative stress (Balazy and Chemtob, 2008).

At the cellular level, lipid peroxidation induces changes in cell membranes with modification of their permeability and alterations of lipid-dependent enzymes. This latter effect is well studied in mitochondria where oxidation of cardiolipin induces a decrease in activity of respiratory complexes (Paradies *et al.*, 2000; 2002) and loss of supercomplex organization (Lenaz *et al.*, 2007b).

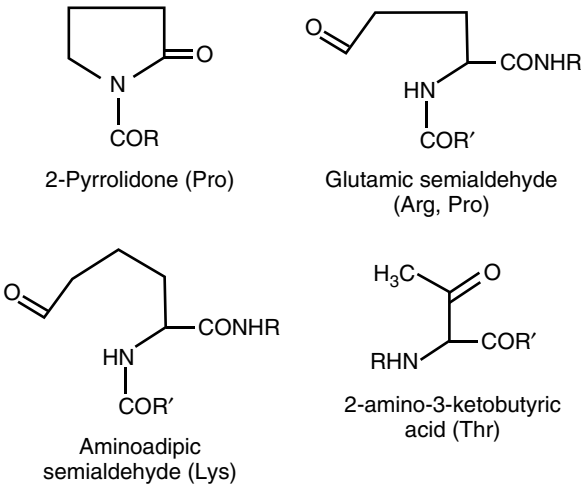

Figure 15 Structures of protein carbonyls arising from oxidation of amino acid side chains. The parent amino acids are indicated in parentheses. R and R′ indicate the remainder of the polypeptide chains.

3.2 Protein Oxidation

Hydroxyl radicals produced by ionizing radiation or by the Fenton reaction are able to attack the polypeptide backbone of proteins to form a carbon-centred radical, which may react with O_2 to form a peroxyl radical (Stadtman, 1992; Stadtman, 2004). The peroxyl radical is then converted to alkyl peroxide by reaction with protonated superoxide $HO_2^{\bullet}$. The alkoxyl derivatives of proteins may then undergo peptide bond cleavage. In the absence of radiation or metal ions, superoxide and H_2O_2 would be inactive.

The side chains of all amino acids in proteins are susceptible to oxidation; however some residues like proline, histidine, arginine, lysine and cysteine are highly sensitive to oxidation by metal ions. Thus it is believed that Fe^{2+} binding to the above residues undergoes the Fenton reaction with H_2O_2 to yield $OH^{\bullet}$ at those specific sites.

The amino acid residues that are most vulnerable to attack by ROS are arginine, glutamate, histidine, tyrosine, valine, cysteine, proline, threonine and methionine (Stadtman, 2004). Cysteine and methionine residues are particularly susceptible to oxidation by ROS. The oxidation of methionine residues by ROS forms methionine sulfoxide (Met-SO). The oxidation of the amino acid residues, as well as the glycation of lysine groups (cf. Section 15.3.3) generates carbonyl groups: protein carbonylation is the most widely used marker of protein oxidation both *in vitro* and in pathological conditions (Dalle Donne *et al.*, 2006) (**Figure 15**).

Peroxynitrite formed by the reaction of $NO^{\bullet}$ with $O_2^{\bullet -}$ may react with cysteine SH groups, oxidizes methionine to methionine sulfoxide and reacts with tyrosine forming 3-nitrotyrosine (another important marker of oxidative protein damage).

Protein oxidation by ROS is associated with the formation of many different kinds of inter- and intraprotein crosslinkages, such as by addition of a lysine amino group to a carbonyl group of the oxidized protein, by interaction of two carbon-centred radicals of polypeptide backbones, by oxidation of cysteine residues to form S-S crosslinks, or by oxidation of tyrosine residues to form tyr-tyr crosslinks. The cysteine S-S crosslinks are reversible, since they can be repaired by disulfide exchange reactions. Oxidation of cysteine residues can also lead to reversible formation of mixed disulfides, particularly with glutathione (GSH) (S-glutathionylation); S-glutathionylated proteins accumulate under conditions of oxidative stress (Dalle Donne *et al.*, 2005).

Besides being altered by direct oxidation by ROS, proteins may be covalently modified by products of lipid peroxidation, such as the reactive aldehydes described in Section 15.3.2.1 (Bieschke *et al.*, 2006).

3.3 Advanced Glycation End Products (AGE)

AGE are a group of heterogeneous compounds that form constantly in the body, but are markedly increased in conditions of hyperglycaemia and oxidative stress (Vlassara, 2005). AGE are the end-products of the Maillard

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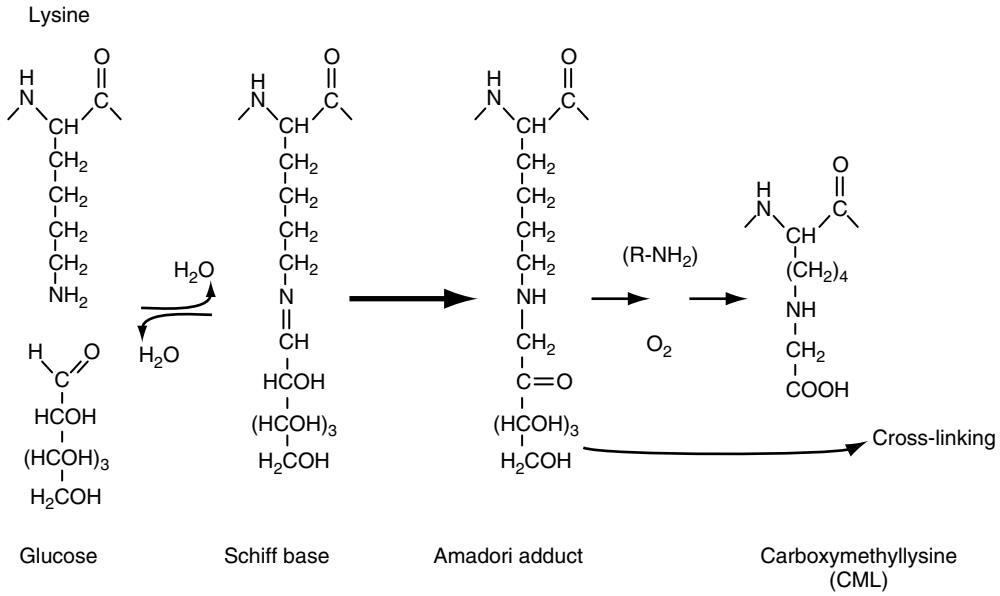

Figure 16 Mechanism of formation of AGE. The example concerns glucose and protein-bound lysine residues. The formation of the Schiff base has a timescale of hours, whereas Amadori products are formed within days. The formation of stable crosslinks takes weeks or months.

reaction between sugars and proteins: a sugar reacts with a protein amino group to form a Schiff base, is then stabilized in the form of an Amadori product and its subsequent rearrangement leads to formation of stable and irreversible AGE compounds (**Figure 16**).

The final step of the Maillard reaction is driven by oxidative stress, and AGE accelerate oxidation, thus favouring their own production. Protein adducts also arise from lipid oxidation and are often included among AGE, although they should be more properly called ALE (Section 15.3.1).

For the process of AGE formation, glucose is the predominant substrate, but other sugars, such as fructose, glucose-6-phosphate, glyceraldehyde-3-phosphate, ascorbate and methylglyoxal, may react with proteins and produce chemically distinct AGE (Bohlender *et al.*, 2005; Soskic *et al.*, 2008). AGE-modified proteins are recognized by specific receptors (RAGE) whose activation is responsible for the cellular effects of AGE and their pathological consequences (Wendt *et al.*, 2003). Binding and activation of RAGE can promote further oxidative stress and AGE formation via the NADPH oxidase pathway (Peppa *et al.*, 2008).

The diet is a major source of AGE and dietary AGE are called glycotoxins; exaggerated intake of thermally treated food may exert oxidative stress and pathological consequences (Vlassara and Striker, 2007).

3.4 DNA Damage

The hydroxyl radical reacts with all components of the DNA molecule damaging the purine and pyrimidine bases and the deoxyribose backbone (Dizdaroglu *et al.*, 2002; Cooke *et al.*, 2003). Permanent modifications of genetic material are the first step involved in mutagenesis, cancerogenesis and ageing. To date more than 100 products have been identified from the oxidation of DNA, including single- or double-stranded DNA breaks, purine, pyrimidine and deoxyribose modifications, and DNA crosslinks (Valko *et al.*, 2006). DNA damage can result in either arrest or induction of transcription, induction of signal transduction pathways, replication errors and genome instability.

The $OH^{\bullet}$ radical is able to add to double bonds of DNA bases and to abstract an H atom from the methyl group of thymine and each of the C atoms of deoxyribose; the formation of 8-hydroxyguanine (8-OH-G) (**Figure 17**) is the most prominent example among over 20 base modifications.

8-OH-G is the most easily formed base alteration, is mutagenic and carcinogenic, and is a good biomarker of oxidative DNA damage. This modification occurs in one in 10^5 G residues in normal cells, but increases in oxidative stress, ageing and a number of diseases (Halliwell and Gutteridge, 1999). mtDNA is more susceptible than nuclear DNA (nDNA) to oxidative damage (Ott *et al.*, 2007) due to the close proximity of the respiratory chain and the lack of protective histones; the incidence of 8-OH-G is 10- to 20-fold higher in mtDNA than in nDNA. The oxidative damage to mtDNA can lead to deletions or other rearrangements (Meissner, 2007). In particular the 4977 base pair deletion ('common deletion') has been studied in many tissues and found to increase with ageing (Lenaz *et al.*, 2006b).

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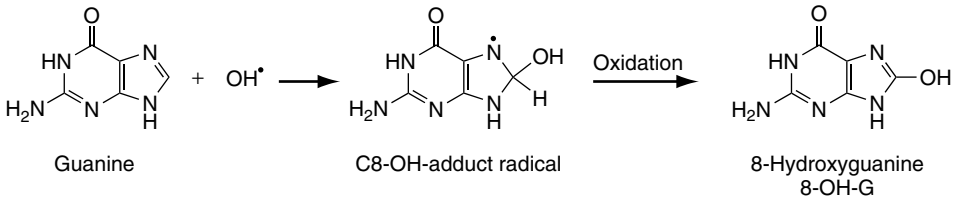
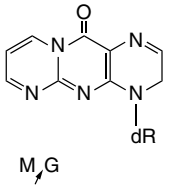


Figure 17 Formation of 8-hydroxy-guanine (8-OH-G) by reaction of $OH^{\bullet}$ with guanine. The reaction occurs via the intermediate formation of an 8-hydroxy adduct radical.

Also, products of lipid peroxidation, such as MDA, can react with DNA bases forming adducts (Marnett, 1999); for example, the M_1G adduct can rise to 1.2 per 10^6 nucleotides and is mutagenic.



4 ROS AND CELL SIGNALLING

It is clear nowadays that ROS (and RNS) act as second messengers (Forman *et al.*, 2008) by interfering with the expression of a number of signal transduction pathways and genes. Because ROS are oxidants, they influence the redox state of signalling proteins. To produce a redox-dependent modification of an enzyme, enzymatic catalysis is usually required to allow the modification under physiological conditions. For the same reason not all ROS are equally suitable for signal transduction; for instance the $OH^{\bullet}$ radical is too unspecific to undergo catalysed reactions, while $O_2^{\bullet -}$, H_2O_2 and lipid peroxidation derivatives like HNE are widely used. Depending on their concentration, ROS can have positive responses (cell proliferation) or negative responses (cell death).

The ROS concentration within cells is largely dependent on the redox state of glutathione (GSH/GSSG) and thioredoxin (Trx), which has two critical cysteine residues (see also Section 15.5). GSH reduces H_2O_2 enzymatically (GPx) generating the oxidized form GSSG, but the GSH/GSSG ratio also depends on disulfide exchange with proteins catalysed by protein disulfide isomerase (PDI).

$$2GSH + H_2O_2 \longrightarrow GSSG + 2H_2O \text{ (GPx)} \quad (28)$$

$$\text{Protein-SH} + GSSG \longrightarrow \text{Protein-SSG} + GSH \text{ (PDI)} \quad (29)$$

Also Trx is involved in disposing of H_2O_2 by Trx peroxidases or Prdxs:

$$\text{Trx (SH)}_2 + H_2O_2 \longrightarrow \text{Trx(S-S)} + 2H_2O \text{ (Prdx)} \quad (30)$$

Proteins may be glutathionylated by ROS by a two-step mechanism:

$$\text{Protein-S}^- + H_2O_2 \longrightarrow \text{Protein-SO}^- + H_2O \quad (31a)$$

$$\text{Protein-SO}^- + GSH \longrightarrow \text{Protein-SSG} + OH^- \quad (31b)$$

Glutathionylation has been proposed to act as a protective mechanism to prevent overoxidation of cysteines in signal transducing proteins.

The protein thiol oxidation by ROS also occurs in the thiolate form ($Cys\text{-}S^-$); signalling proteins thus modified include phosphoprotein phosphatases (PTPs), Ras, large G-proteins, serine/threonine kinases of the mitogen-activated protein kinase (MAPK) families, transcription factors, such as AP-1, NFκB, p53 and others. The effect of oxidants is different with different proteins: PTPs are inhibited, nuclear transcription factors are activated (Valko *et al.*, 2006). Thus for example, activation of ERK1/2, Akt and NFκB promotes cell survival, whereas activation of c-jun N-terminal kinases (JNKs), p38 kinase and p53 would lead to cell cycle arrest and apoptosis. In addition, ROS appear to activate the HIF-1α (Kietzmann and Gorlach, 2005) by inhibiting prolyl-4-hydroxylase (which hydroxylates a critical proline directing the factor to proteolytic digestion), similarly to what happens in anoxia, for lack of O_2 required for HIF hydroxylation. During neoplastic transformation certain signalling pathways bypass the programmes leading to apoptosis and senescence.

Numerous studies have shown that HNE at physiological concentrations stimulates cell proliferation, differentiation and cytoprotective response through affecting multiple signalling pathways: activation of PKC, of the family of MAPK, and of tyrosine kinase receptors, inhibition of Ca-ATPases with consequent accumulation of Ca^{2+} in the cytoplasm, and others (Forman *et al.*, 2008). It is beyond the scope of this chapter to provide a detailed description of the signalling pathways modified by ROS.

A special mention is needed for the role of mitochondria in relation to ROS signalling (Ott *et al.*, 2007). Apoptotic stimuli induce release of protein-bound $p66^{Shc}$ (Section 15.2.1.4) that interacts with cytochrome c,

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generating H_2O_2. A key player of ROS signalling is p53, which is stabilized by DNA damage from ROS and activates the apoptotic machinery in mitochondria. Direct action of mitochondrial ROS within mitochondria on the apoptotic cascade can be effected through opening of the permeability transition pore, a large channel of the inner membrane activated by various stimuli and involved in outer membrane permeabilization and release of apoptotic factors, such as cytochrome c, from the intermembrane space (Rasola and Bernardi, 2007). The pore is activated by ROS, presumably via redox-sensitive cysteines (Fruehauf and Meyskens, 2007). The role of oxidative stress in apoptosis is beyond the scope of this chapter; an important reference is Ryter *et al.* (2007). Additional information is provided in **Cell Death and Apoptosis** of this book.

Finally, mitochondrial damage induced by ROS or other agents and resulting in decreased membrane potential may induce a retrograde response in the nucleus to activate nuclear genes involved in compensatory mitochondrial biogenesis via decreased mitochondrial Ca^{2+} uptake and concomitant rise in the cytosol (Butow and Avadhani, 2004).

5 ANTIOXIDANT DEFENCES

ROS production and ROS-mediated damage are counteracted in cells by different mechanisms (Zimniak, 2008) that will be described here only in general terms.

First, cells have devised mechanisms capable of repairing damage once occurred. The best known example is represented by DNA repair, which removes oxidative and other lesions from chromosomal and mitochondrial DNA and restores the original strands (Hazra *et al.*, 2007); the mtDNA-repairing systems are usually considered less efficient than those operating in the nucleus (De Souza-Pinto *et al.*, 2008).

Damaged molecules, in particular proteins, are recognized by degrading systems; oxidatively modified proteins are extensively ubiquitinated and directed to the proteasome where their complete digestion takes place (Grune *et al.*, 1997; Ghazi *et al.*, 2007). Incapability of the proteasome to digest some proteins or accumulation of insoluble protein aggregates may lead to profound alterations in cells. Also, damaged mitochondrial proteins are degraded, presumably by the mitochondrial Lon protease (Bulteau *et al.*, 2006).

Cellular structures and organelles undergo turnover and after a suitable lifetime are directed to autophagy by lysosomal digestion (Bergamini, 2006; Chen and Gibson, 2008). Autophagy systems recognize damaged organelles, such as mitochondria, and preferentially address them to digestion. The term mitoptosis has been suggested (Skulachev, 2006) to define the removal of oxidatively damaged mitochondria.

In a way, apoptosis (see Section 15.4 and also **Cell Death and Apoptosis** of this book) is an extreme device to eliminate cells where extensive damage has taken place, rather than allowing them to propagate the oxidative stress to other cells, for example, by plasma membrane NOX (Section 15.2.2.5). The notion that ROS may activate apoptosis through p53 and the mitochondrial permeability transition (Section 15.4) is in line with this assumption.

Other defence mechanisms operate to reduce ROS production. In mitochondria, uncoupling proteins UCPs have been suggested to fulfil this function, producing mild uncoupling and limiting ROS generation by the respiratory chain (Section 15.2.1.3). Also, substrate supply down-regulation and decrease of pO_2 would limit ROS production (Barja, 2007).

An opposite strategy of defence is biosynthesis of macromolecules resistant to oxidative stress (Pamplona and Barja, 2007). For instance, the presence of less unsaturated fatty acyl chains in membrane lipids represents a powerful defence against lipid peroxidation. Likewise, lowering the amount of components highly susceptible to oxidation, such as methionine in proteins and guanine in nucleic acids, renders them more resistant to alteration. On the other hand, the presence of a nonessential methionine in proteins may represent a sink against ROS damage (Levine *et al.*, 1996). It was shown that a mtDNA polymorphism (mt5178C→A) substituting Leu with Met at position 237 in Complex I subunit ND2 confers a longevity trait (Tanaka *et al.*, 1998) possibly by reducing oxidative damage to other more functional amino acids.

Finally, cells and body fluids contain molecules that neutralize or scavenge ROS, collectively termed antioxidants. Antioxidants may be divided into antioxidant enzymes that neutralize ROS by forming less toxic species, and antioxidant small molecules; the latter may either be endogenous metabolites and biosynthesized cofactors, or exogenous molecules taken up from the diet. Certain antioxidants are able to regenerate other antioxidants establishing an antioxidant network (Sies *et al.*, 2005).

5.1 Enzymatic Antioxidants

SOD catalyses the dismutation of superoxide to H_2O_2 and oxygen (Reaction 2); it exists in three isoforms in humans: cytosolic Cu, Zn-SOD, mitochondrial Mn-SOD and extracellular SOD (Landis and Tower, 2005). SOD must work in concomitance with enzymes removing H_2O_2, otherwise accumulation of the latter in the presence of reduced metal ions would activate the Fenton reaction (Reaction 4).

The systems capable of removing H_2O_2 are represented by catalase and peroxidases. Catalase (Mates *et al.*, 1999) is localized in peroxisomes and is very efficient, having one of the highest turnover numbers known

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for an enzyme.

$$2H_2O_2 \longrightarrow 2H_2O + O_2 \qquad (32)$$

GPx exists in two main forms, one selenium-dependent and the other not (Mates *et al.*, 1999); humans have four different Se-GPx. The enzyme catalyses Reaction 28 using H_2O_2 as an oxidant for GSH, but can also use organic peroxides:

$$2GSH + ROOH \longrightarrow GSSG + ROH + H_2O \qquad (33)$$

GSH must be regenerated by glutathione reductase (GR), using NADPH as the reductant; the major source of NADPH for cytosolic GR is the pentose phosphate pathway, while in mitochondria it is energy linked NADH/$NADP^+$ transhydrogenation.

$$GSSG + NADPH + H^+ \longrightarrow 2\ GSH + NADP^+ \qquad (34)$$

Perhaps Prdxs, catalysing Reaction 30, are the most important peroxide-removing systems (Rhee *et al.*, 2005). Trx reductase is an Se-enzyme that regenerates reduced Trx.

5.2 Nonenzymatic Antioxidants

Some of these are water-soluble and act in the cell's soluble fraction or in plasma, others, being lipid-soluble, act within membranes, while amphipathic molecules can act in both environments.

Vitamin C (ascorbic acid) is water-soluble and cooperates with lipid-soluble vitamin E to regenerate α-tocopherol from the tocopheroxyl radical produced during the vitamin E radical scavenging activity; the product of the reaction is a very stable ascorbate radical ($Asc^{\bullet -}$) (**Figure 18**).

Although excessive doses of ascorbate may be pro-oxidant, physiological amounts have always been proven to be antioxidant even in the presence of metal ions (Valko *et al.*, 2006).

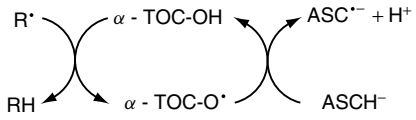

Figure 18 Cooperation between vitamin E (α-tocopherol, α-TOC) and ascorbate ($ASCH^-$ is the partially ionized antioxidant form) in scavenging radical species $R^{\bullet}$.

Vitamin E includes eight different derivatives, of which α-tocopherol is the most potent in humans. It is the most important lipid-soluble antioxidant and mainly prevents lipid peroxidation; its dietary supplementation is beneficial, however high doses may be pro-oxidant (Valko *et al.*, 2006). Its reaction with lipid radicals (mainly peroxyl radicals) generates the α-tocopheroxyl radical, which can be re-reduced to α-tocopherol by ascorbate in the water phase or by reduced Coenzyme Q ($CoQH_2$) in the lipid phase. $CoQH_2$ can also directly scavenge radicals like $LOO^{\bullet}$, generating its semiquinone form (Ernster and Dallner, 1995).

$$QH_2 + LOO^{\bullet} \longrightarrow Q^{\bullet -} + LOOH \qquad (35)$$

Coenzyme Q (ubiquinone, CoQ) is an essential electron carrier in the mitochondrial respiratory chain, but is also present in serum lipoproteins, endomembranes and the plasma membrane (Turunen *et al.*, 2004). Chemically, CoQ is constituted by a benzoquinone ring and a lipid side chain with several isoprenoid units, its number being species specific. Its redox reactions can be driven either by the simultaneous transfer of two electrons in a single step, or by two sequential steps of one-electron transfer through a partially reduced semiquinone intermediate (**Figure 19**). CoQ is the only lipidic antioxidant that is synthesized in mammals, and its biosynthesis is a complex process that involves the participation of at least eight gene products.

Besides the mitochondrial respiratory chain, several enzymes catalyse CoQ reduction to achieve its antioxidant

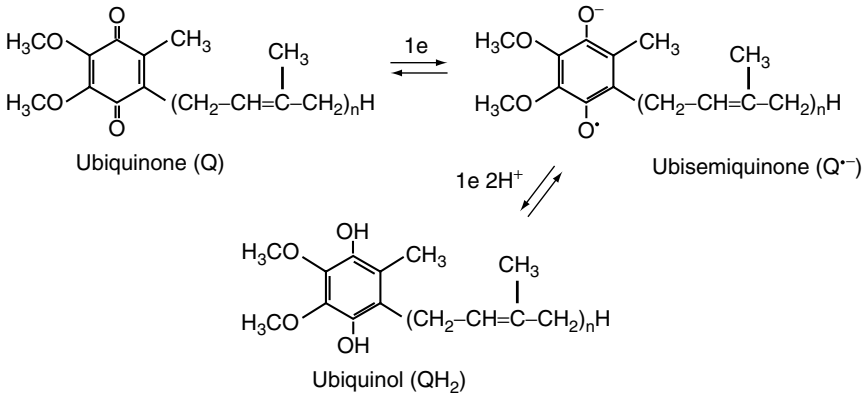

Figure 19 The redox states of Coenzyme Q; n is the number of isoprenoid units.

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reduced state in eukaryotic cells. NADH-cytochrome b_5 reductase can reduce CoQ through a one-electron reaction mechanism (Arroyo *et al.*, 1998). The soluble enzyme NAD(P)H:quinone oxidoreductase 1 (NQO1, DT-diaphorase) can reduce quinones by a two-electron reaction and maintains the reduced state of CoQ_{10} *in vitro* (Beyer *et al.*, 1996). A distinct cytosolic NADPH-CoQ reductase different from NQO1 has been also described (Takahashi *et al.*, 1995).

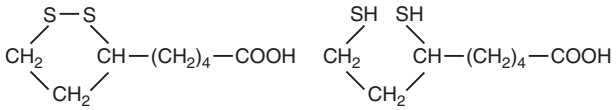
Glutathione GSH, besides scavenging H_2O_2 enzymatically can also scavenge radicals directly, forming thiyl radicals that can dimerize, generating oxidized glutathione GSSG:

$$GSH + R^{\bullet} \longrightarrow GS^{\bullet} + RH \quad (36)$$

$$GS^{\bullet} + GS^{\bullet} \longrightarrow GSSG \quad (37)$$

GSH is also able to regenerate vitamins C and E to their active forms.

Another dithiol antioxidant is lipoic acid in its reduced form dihydrolipoic acid; it is able to quench ROS and to regenerate other antioxidants as described above.



Lipoate Dihydrolipoate

Finally there is a great number of dietary antioxidants contained in fruit and vegetables that are not vitamins or part of coenzymes, such as carotenoids and flavonoids. They may act as quenchers of singlet oxygen and peroxyl radicals forming more stable radicals that do not propagate and may act as chain terminators by reacting with other radicals (Valko *et al.*, 2006).

6 INVOLVEMENT OF ROS IN PATHOLOGY

6.1 ROS and Ageing

The 'mitochondrial theory of ageing' (Linnane *et al.*, 1989) is based on the hypothesis that mitochondrial DNA somatic mutations, caused by accumulation of oxygen radical damage, induce alterations of the OXPHOS machinery culminating in an energetic failure that is at the basis of cellular senescence. Moreover a vicious circle (Ozawa, 1997) can be established since the accumulated damage to the respiratory chain would enhance ROS generation (**Figure 20**). Many reports (reviewed in Lenaz *et al.*, 2006b) demonstrate that the rate of production of ROS from mitochondria increases with age in mammalian tissues and in fibroblasts during replicative

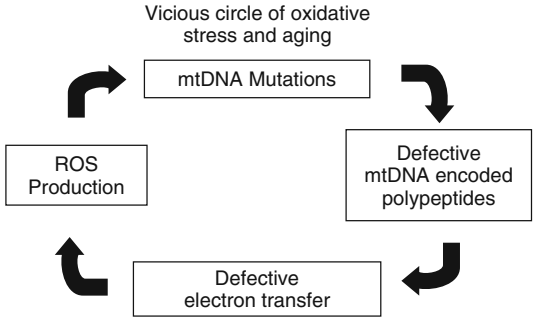


Figure 20 The proposed vicious circle of mtDNA mutations, ROS generation and oxidative phosphorylation decay in ageing.

cell senescence, considered to represent a plausible model of *in vivo* ageing (Hayflick, 2003).

Compelling evidence for the mitochondrial theory of ageing derives from several observations: the strong negative correlation existing between expected lifespan and rate of ROS production of different species (Sohal *et al.*, 1995; Barja and Herrero, 2000), within the same species under different activity conditions (Yan and Sohal, 2000) and between lifespan and membrane lipid unsaturation (Pamplona *et al.*, 2002). Furthermore the demonstration that caloric restriction, while prolonging lifespan in mammals, reduces ROS production (Barja, 2004) provides additional points in favour of the role of ROS in ageing.

A large number of large-scale deletions, point mutations and tandem duplications have been observed in tissues of aged individuals (Lee and Wei, 1997; Ozawa, 1997; Wei and Lee, 2002). Michikawa *et al.* (1999) reported that a T414G transversion in the D-loop of mtDNA is accumulated in skin fibroblasts from old subjects, while two different point mutations (A189G and T408A) accumulate in the D-loop in muscle of old individuals (Wang *et al.*, 2001). These mutations are located in the control region of mtDNA and may impair its replication and transcription.

A minimal threshold level of 50–95% mutated mtDNA is usually necessary to impair respiratory chain function, depending on the type of mutation and the tissue affected (Rossignol *et al.*, 2003). Since the proportions of mutated mtDNA within ageing human tissues rarely exceed 1%, it has been questioned how these levels may cause significant bioenergetic effects. Hayakawa *et al.* (1996), using 180 kinds of polymerase chain reaction (PCR) primers, found that mtDNA in elderly subjects is extensively fragmented into minicircles of different sizes. As a result, the amount of mtDNA mutations may reach such a high level as to cause significant impairment of OXPHOS. Furthermore, mutated mtDNA molecules may be distributed unevenly among the cells of affected tissues in a mosaic pattern of mtDNA segregation (Linnane *et al.*, 1995). Exponential accumulation of mutated mtDNA during life suggests that there is a preferential replication of the mutant genomes.

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Are mtDNA mutations the cause of ageing, or do they just accompany the ageing process as interesting biomarkers of senescence? Trifunovic *et al.* (2004) showed that expression of a proof-reading deficient mtDNA polymerase in a homozygous knock-in mouse strain leads to increased levels of somatic mtDNA mutations causing progressive respiratory chain deficiency; the mice develop symptoms strikingly reminiscent of ageing. Even if these mice do not have an enhanced ROS production (Trifunovic, 2006; Trifunovic and Larsson, 2008), it is likely that this effect results from the severe extent of mutation in the mutator mice, while the 'natural' way to induce mutations is ROS attack.

There is overwhelming evidence that the bioenergetic function of mitochondria declines with ageing, specially in postmitotic tissues (Lenaz *et al.*, 2006b). If mtDNA mutations/deletions are at the basis of human ageing, the existence of a biochemical threshold complementing mtDNA mutations is critical, as discussed above. The notion that the respiratory chain is mainly controlled at the level of Complex I suggests that the main alterations due to ageing must be found at the level of this enzyme (Barrientos and Moraes, 1999; Lenaz *et al.*, 2000). Flux control analysis in aerobic respiration in coupled liver mitochondria (Ventura *et al.*, 2002) showed that Complex I has little control in young rats, but very high control in the old animals, meaning that ageing induces a profound alteration of Complex I that is reflected in the entire OXPHOS system.

Recent investigations attempt to relate the mitochondrial changes with the cellular environment. Changes in the redox status of cellular components by oxidative stress during ageing are considered the cause of the increased contents or DNA-binding activities of transcription factors NF-kB, AP-1, HIF-1 and of heat-shock proteins (Calabrese *et al.*, 2001; 2004); their increased activity is considered a compensatory mechanism for cellular protection; caloric restriction, the main known factor recognized to delay ageing, was found to prevent their activation. Available data on redox-responsive transcription factors suggest that their uncontrolled activation in ageing could lead to serious chronic pathogenic conditions characterized by what has been called 'molecular inflammation' (Chung *et al.*, 2002).

The role of oxidative stress in ageing has also been extended to age-related degenerative diseases, such as neurodegenerative diseases (Section 15.6.3) and ocular diseases (Brennan and Kantorow, 2008).

6.2 ROS and Ischaemic Diseases

Atherosclerosis is the major cause of cardiovascular diseases; it is characterized by accumulation of cholesterol deposits in macrophages in arteries with progressive formation of plaques that can rupture and induce thrombi. The hypothesis receiving highest consensus on atherogenesis is that of the oxidative modification of low-density lipoprotein (LDL) (Stocker and Keaney, 2004); oxidized LDL, but not native LDL, is readily internalized by macrophages through the 'scavenger receptors' so that cholesterol esters are accumulated, generating the so-called foam cells and initiating the atherosclerotic process. LDL oxidation is effected by ROS generated by the endothelial cells and affects both lipids and proteins; it is the oxidation of lysine groups in apo-B100 that allows LDL uptake by macrophages.

A major consequence of arterial thrombosis is vessel obstruction with consequent ischaemia. Ischaemia, especially if followed by a reperfusion phase, is involved in most cardiovascular diseases. ROS are involved in tissue lesions due to ischaemia-reperfusion (I/R) in heart, brain and other tissues. A number of mechanisms have been invoked to explain this phenomenon, all involving participation of ROS and oxidative stress (Blomgren *et al.*, 2003; Wang and Lo, 2003; Paradis *et al.*, 2004).

The mitochondria are the primary intracellular source of ROS: when anoxia is followed promptly by reperfusion, the resulting increase in oxygen supply leads to overproduction of ROS. This increase in ROS formation could arise from different patterns of mitochondrial respiration provoked by the ischaemic insult and the reperfusion phase. Mitochondrial respiration is significantly diminished at the level of Complex IV at the early reperfusion phase (Perez-Pinzon *et al.*, 2002), but is exacerbated in Complexes I and II after 24 hours (Piantadosi and Zhang, 1996).

Moreover, during ischaemia, when ATP is progressively depleted, ion pumps cannot function, resulting in a rise in calcium (Ca^{2+}), which further accelerates ATP depletion. The rise in Ca^{2+} during I/R leads to mitochondrial Ca^{2+} accumulation, particularly during reperfusion when oxygen is reintroduced. Mitochondrial Ca^{2+} overload and increased ROS can result in opening of the MPTP, which further compromises cellular energetics. The resulting enhanced permeability of the outer mitochondrial membrane allows the release of proapoptotic proteins such as cytochrome c from the intermembrane space to the cytosol, with activation of caspases and cell death (Szeto, 2008) (**Figure 21**). Another effect of the high cytosolic Ca^{2+} levels is activation of phospholipase A_2, with consequent liberation of arachidonic acid that also would activate the MPTP (Penzo *et al.*, 2004).

Numerous other sources of induced cellular ROS following I/R have been implicated in cell signalling. Among them, XO and myeloperoxidase produce extracellular ROS, as well as associated endothelial cell damage, during the reperfusion injury phase that follows ischaemia (Bulkley, 1993). Finally, NOXs appear also to serve as a source for ROS following I/R (Liu *et al.*, 2004).

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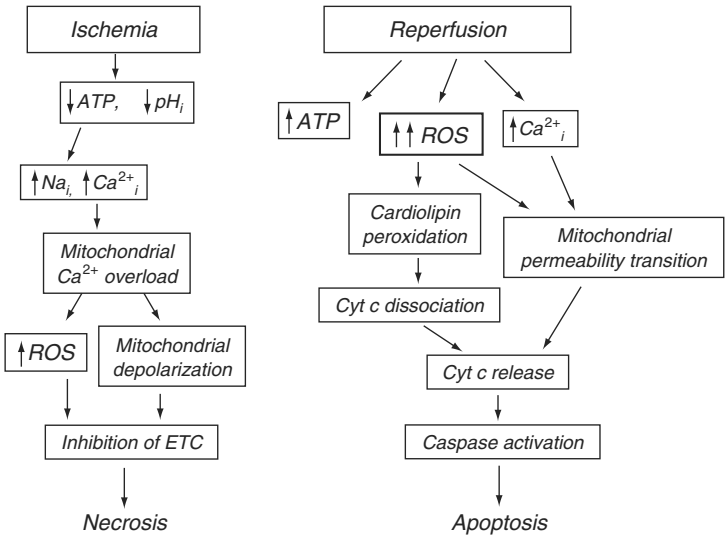

Figure 21 Pathways leading to cell death by necrosis and apoptosis in ischaemia reperfusion injury.

6.3 ROS and Neurodegenerative Diseases

Age-related neurodegenerative diseases are characterized by pathological changes in disease-specific areas of the brain; in each disease, these pathological processes lead to degeneration of distinct subsets of neurons. However, despite the differences in the clinical manifestations and selective neuronal vulnerability, the underlying pathological processes on cellular and molecular levels appear similar across different diseases, suggesting common pathways of neurodegeneration. Increasing lines of evidence suggest a key role of oxidative stress, which can modify cellular bioenergetic capacity, further increasing ROS generation resulting in cellular damage and programmed cell death.

6.3.1 Parkinson's Disease

Parkinson's disease (PD) affects approximately 1% of the population at the age of 55 and increases in prevalence to roughly 5% by the age of 85. PD is characterized clinically by bradykinesia, resting tremor and rigidity. The symptoms of PD are predominantly due to a profound reduction in striatal DA content caused by the loss of dopaminergic neurons in the substantial nigra (SN) pars compacta and its projections to the striatum. The pathological lesions of PD are neuronal inclusions known as Lewy bodies (LBs) (Forno, 1996) and dystrophic neurites termed Lewy neurites (Braak *et al.*, 1994); the protein α-synuclein (syn) is the major component of Lewy pathology (Spillantini *et al.*, 1997).

The role of oxidative stress in neuronal degeneration in PD is substantiated by pathological findings and animal models that have provided experimental paradigms to delineate the possible mechanisms (Chinta and Andersen, 2008). Markers of oxidative stress, such as products of lipid peroxidation and oxidation of mtDNA and cytoplasmic RNA, are increased in dopaminergic neurons of PD brains. Increased oxidation in the SN of PD patients also may be partially due to the reported accumulation of iron, which in the form of Fe^{2+} can catalyse the formation of strong oxidants. The presence of AGEs in Lewy pathology and the demonstration that α-syn is a specific target of nitration suggests that oxidative damage may be involved in the formation of these inclusions.

The selective loss of DA neurons in the SN might be due to DA neuron glutathione deficiency (Loeffler *et al.*, 1994) (resulting in a reduced antioxidant capacity), high content of DA, a redox active molecule (Section 15.2.2.8) (Zigmond *et al.*, 2002) or elevated iron concentrations (Section 15.2.2.7) (Zecca *et al.*, 2004). The normal enzymatic metabolism of DA results in the generation of hydrogen peroxide, and the nonenzymatic auto-oxidation of DA results in the formation of reactive quinones and semiquinones that react to generate hydrogen peroxide, superoxide anions and hydroxyl radicals (cf. Section 15.2.2.8) (Fato *et al.*, 2008). These findings have provided further credence to the proposal that DA metabolism results in oxidative stress (Baumgarten and Grozganovic, 2000).

The possible involvement of oxidative stress as an aetiological factor of PD is further supported by studies with specific neurotoxins that are extremely potent inducers of parkinsonism in humans and animals. The best studied of these toxins are 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenylpyridinium (MPP^+), the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which selectively destroys catecholaminergic neurons. Both toxins have been shown to generate hydroxyl radicals in the caudate of treated animals (Chiueh *et al.*, 1993). Moreover, administration of rotenone, an inhibitor of mitochondrial Complex I also inducing ROS generation (15.2.1.2.2), recapitulated the

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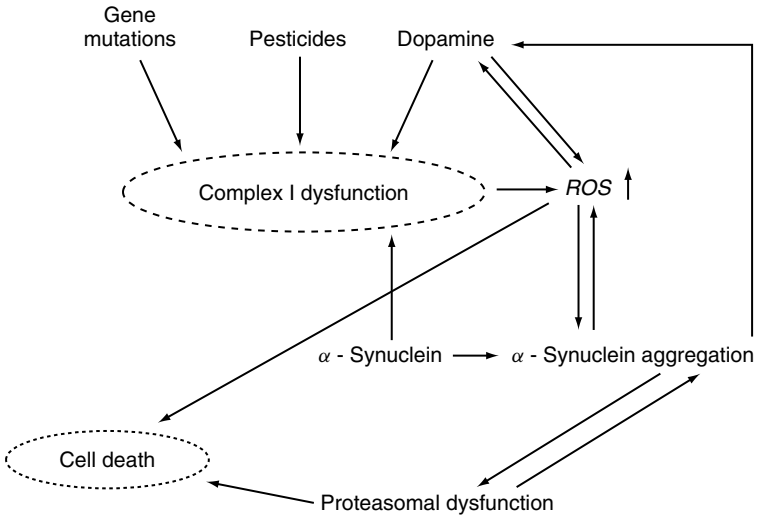

Figure 22 Postulated role of Complex I in Parkinson's disease.

major pathological features of PD, including selective loss of dopaminergic neurons and α-syn-positive LB-like inclusions, indicating that the inhibition of Complex I might be sufficient to trigger PD pathogenesis (Betarbet *et al.*, 2000).

Multiple lines of evidence suggest a pathogenic role of oxidative damage and mitochondrial dysfunction in causing PD (Schapira, 2008). The direct relation between mitochondrial dysfunction and PD came from the post-mortem description of Complex I deficiency in the SN of patients with PD. Subsequently, the deficiency was also seen in the skeletal muscle and platelets. Consistent deficits in the subunits and activity of mitochondrial Complex I of the electron transport chain in blood platelets and SN of PD patients is a prominent phenomenon (Keeney *et al.*, 2006). Reduced Complex I activity is also seen in cytoplasmic hybrid (cybrid) cell lines containing mtDNA from PD patients (Swerdlow *et al.*, 1996). The Complex I deficiency in the substantia nigra and platelets implies that it is a systemic defect in a proportion of cases ($\sim$25% on the basis of platelet activities) and this might be due to genetic or environmental causes. The specificity of the Complex I defect and its relation to oxidative stress have been supported by the recent finding that this is the only respiratory chain protein complex that is affected by endogenous oxidative damage and reduced structural stability (Keeney *et al.*, 2006).

Mutations or polymorphisms in both mtDNA and nDNA were implicated in causing PD or in affecting PD risk (Bogaerts *et al.*, 2008). To this purpose, mtDNA mutations may be involved in the aetiology and predisposition to the idiopathic disease, since cybrids containing mitochondria from patients exhibit a reduced activity of Complex I (Swerdlow *et al.*, 2001) and generate Lewy bodies (Trimmer *et al.*, 2004). However, exhaustive sequencing of mtDNA has not revealed mutations that consistently associate with PD (Pankratz and Foroud, 2007); nevertheless, somatic deletions of mtDNA are found more frequently in SN from PD patients. The possible role of Complex I in PD is shown in (**Figure 22**).

Besides mitochondria, plasma membrane NOX have also been implicated in ROS generation in PD. Activated glial cells are observed at sites of neurodegeneration in PD (Lawson *et al.*, 1990; Kim *et al.*, 2000; Miller *et al.*, 2008). By altering concentrations of intracellular ROS, NADPH oxidase is also reported to prime the microglial response to additional stimuli. For example, neuronal toxins such as MPTP and rotenone are also shown to prime microglia, resulting in synergistic microglial activation and associated neurotoxicity on additional insult with LPS.

6.3.2 Alzheimer's Disease

Alzheimer's disease (AD) is the most common cause of dementia in developed countries. Senile plaques and neurofibrillary tangles (NFTs) are, together with neuronal loss and cortical atrophy, characteristic neuropathological features of the disease. Senile plaques contain β-amyloid (Aβ) peptide, produced by cleavage of the amyloid precursor protein (APP) by β- and γ-secretases. NFTs are twisted helicoidal strands of hyperphosphorylated τ protein, a microtubule-associated protein (Selkoe, 2001; Reynolds *et al.*, 2007).

Oxidative stress is one of the earliest events of AD, with implications as an important mediator in the onset, progression and pathogenesis of the disease. The sources of ROS-mediated damage appear to be multifaceted in AD, with interactions between redox transition metals, mitochondria and other factors. Over the past decade, modification to virtually all classes of biomacromolecules

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indicative of oxidative stress has been described in association with the susceptible neurons of AD (Smith, 1998; Zhu *et al.*, 2007).

Mitochondrial dysfunction is a consistent hallmark of AD (Moreira *et al.*, 2007): a deficient activity of cytochrome oxidase is a constant feature in AD and has been associated with the presence of pathogenic mtDNA mutations (Cardoso *et al.*, 2004). According to Swerdlow and Khan (2004) mitochondrial dysfunction is the primary event leading to the development of AD. Elson *et al.* (2008), however, failed to find consistent mtDNA differences in 270 AD patients with respect to controls. Recent studies have demonstrated that Aβ progressively accumulates within the mitochondrial matrix, providing a direct link to mitochondrial toxicity. β-amyloid-binding alcohol dehydrogenase (ABAD) is localized to the mitochondrial matrix and binds to mitochondrial Aβ exaggerating Aβ-mediated mitochondrial and neuronal perturbation (Takuma *et al.*, 2005; Devi *et al.*, 2006; Chen and Yan, 2007). It is therefore likely that the initial stress is provided by Aβ deposition and is further enhanced by mitochondrial dysfunction.

In AD, overaccumulation of iron in the hippocampus, cerebral cortex and basal nucleus of Meynert colocalizes with AD lesions. Iron is an important cause of oxidative stress in AD because it accumulates in the brain and is involved in the formation of $OH^{\bullet}$ via the Fenton reaction (Castellani *et al.*, 2007). As with iron, copper concentrations are also highly INCREASED within Aβ plaques, setting up conditions for Fenton-type chemistry through the reduction of Cu(II) by Aβ–H_2O_2 reactions. Recently, it has been shown that RNA-bound iron plays a pivotal role for RNA oxidation in vulnerable neurons in AD (Nunomura *et al.*, 2007; Shan *et al.*, 2007; Moreira *et al.*, 2008).

Growing evidence supports the role of oxidative stress in AD, such as increased levels of lipid peroxidation, glycation and protein oxidation products in AD brains. Modification to sugars is marked by increased glycation and glycoxidation. Accumulated AGE were detected in NFTs, and the glycation of tau has been proposed to play a role in stabilizing paired helical filament aggregation leading to neurofibrillary tangle formation (Takeuchi and Yamagishi, 2008). Furthermore, AGE, by activating RAGE receptors, increase intracellular ROS production.

Oxidative modification of proteins is marked by significantly elevated levels of protein carbonyls and widespread nitration of tyrosine residues (Malinski, 2007; Polidori *et al.*, 2007). Moreover, crosslinking of proteins, by oxidative processes, may lead to the resistance of the lesions to intracellular and extracellular removal, even though they are extensively ubiquitinated, and this resistance of NFTs to proteolysis might play an important role in the progression of AD, as revealed by immunostaining of NFTs for 3-NT (Smith, 1998). Some specifically oxidized proteins have recently been identified by proteomics and it is notable that many are either enzymes related to ATP generation or involved in glycolysis, for example, creatine kinase BB, involved in production of high-energy phosphate used for ATP synthesis. Therefore, oxidative modification may lead to metabolic impairment in AD (Castegna *et al.*, 2003).

Both microglia and astrocytes cluster at sites of Aβ deposition. Several lines of evidence suggest that one of the sources of ROS is the microglial NADPH oxidase. Aβ peptide can directly activate the NADPH oxidase of microglia, which results in a burst of superoxide radicals and increased production of hydrogen peroxide; not surprisingly, microglial expression of NADPH oxidase subunit $p22^{phox}$ is enhanced in AD brain, implicating increased microglial respiratory burst activity (Wilkinson and Landreth, 2006).

6.3.3 Huntington's Disease

Huntington's disease (HD) is an autosomal dominant neurodegenerative disease caused by a CAG trinucleotide repeat expansion encoding an abnormally long polyglutamine tract in the huntingtin protein (Htt). HD is characterized by extensive loss of striatal neurones. There is evidence that oxidative damage occurs in HD brain and in models of the disorder (Browne *et al.*, 1997; Polidori *et al.*, 1999; Browne and Beal, 2004), as also shown by enhanced lipid peroxidation, protein carbonylation and DNA damage. Products of the lipid peroxidation process, such as 4-HNE, facilitate the induction of mitochondrial permeability transition, which could lead to cell death by release of apoptogenic factors (Stavrovskaya and Kristal, 2005). Oxidative stress may be a direct mechanism of huntingtin-linked cellular damage; however, findings that increased oxidative damage to DNA and lipids occurs after symptom onset in the R6/2 transgenic mouse model of HD suggest that it is a downstream event in neuronal dysfunction (Bogdanov *et al.*, 2001). It is therefore possible that oxidative damage is induced by energetic defects (Greenamyre, 2007).

More recent findings provided evidence that mitochondrial dysfunction is central to the pathogenesis of HD. Human 143B cells expressing mutant Htt exhibited a reduced activity of Complex III (Fukui and Moraes, 2007), possibly by interference of Htt aggregates with mitochondrial trafficking and distribution in the cell (Solans *et al.*, 2006). Conversely, treatments that inhibited Complex III activity significantly promoted the formation of Htt aggregates. This effect of Complex III inhibition on the Htt aggregates appeared to be mediated by the inhibition of proteasome activity, but not by ATP depletion or production of ROS. Accordingly, Complex III mutant cells also showed decreased proteasome activity. These results suggest the presence of a feedback system connecting the mitochondrial respiratory Complex III and the production of Htt aggregates. This observation indicates that therapeutic interventions

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targeting Complex III and/or proteasome could ameliorate the progress of HD (Fukui and Moraes, 2007).

6.3.4 Friedreich's Ataxia

Friedreich's ataxia (FRDA) is a neurodegenerative disorder arising from a deficit of the mitochondrial iron chaperone, frataxin. In typical FRDA patients the gene has undergone a triplet (GAA) expansion within the first intron (Grabczyk and Usdin, 2000). Reduction of frataxin results in the accumulation of iron and ROS, which leads to oxidative damage, neurodegeneration and a diminished lifespan. FRDA patients display signs of increased oxidative stress, including elevated markers of free radical damage to DNA, lipid peroxidation, reduced plasma levels of glutathione, reduced aconitase activity and elevated iron regulatory protein 1 (Karthikeyan *et al.*, 2003; Lobmayr *et al.*, 2005).

Mouse models expressing low levels of human frataxin show decreased aconitase activity and mild oxidative stress with iron accumulation as a late event (Al Mahdawi *et al.*, 2006). Yeast, *Drosophila*, mouse and cell culture models of frataxin deficiency have revealed important roles for frataxin and its homologues in mitochondrial iron storage, regulation of intracellular iron trafficking, iron–sulphur cluster and haeme biogenesis and reactivation of the labile iron–sulphur cluster of mitochondrial aconitase. A role for frataxin in preventing formation of ROS has been well established (O'Neill *et al.*, 2005), invoking a paradigm of FRDA pathology in which ROS toxicity leads to mitochondrial dysfunction with subsequent cell death (Puccio and Koenig, 2002). The loss of intracellular iron chaperone capacity imposed by frataxin deficiency could elicit a Fenton chemistry-based mechanism of ROS toxicity. In the *Drosophila* model of FRDA, *Dfh* suppression recapitulates the principal biochemical hallmarks of FRDA, including diminished activity of iron–sulphur-containing enzymes, susceptibility to iron toxicity, loss of intracellular iron homeostasis and early-onset adult mortality (Anderson *et al.*, 2005). Overexpression of frataxin in the mitochondria increases resistance to oxidative stress and extends lifespan in *Drosophila*.

6.3.5 Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that primarily affects motor neurons in brain cortex, brainstem and spinal cord. ALS occurs in both sporadic (~80–90% cases) and familial (5–10% cases) forms, which are clinically and pathologically indistinguishable. Among the familial cases, 10–20% can be attributed to point mutations in the gene coding for the antioxidant enzyme Cu/Zn SOD (SOD1) (Rosen *et al.*, 1993).

In human sporadic and familial ALS, markers of oxidative damage of proteins, lipids and DNA are elevated in the brain and spinal cord (Beal *et al.*, 1997). Recently, it has been observed that lipid peroxidation is significantly increased in the erythrocytes of sporadic ALS patients. On the other hand, catalase activity was found to be significantly lower. The activities of glucose-6-phosphate dehydrogenase, glutathione reductase and glutathione levels were also significantly reduced in ALS patients (Babu *et al.*, 2008).

Several potential mechanisms of motor neuron degeneration in ALS have been proposed based on animal model and cell culture system analyses. Markers of oxidative damage have been detected in animal models (Aguirre *et al.*, 2005; Perluigi *et al.*, 2005; Liu *et al.*, 2007). SOD1 mutants expressed in human cell lines directly stimulate NOX by binding to Rac1, with overproduction of damaging ROS. Diminishing ROS by treatment with the NOX inhibitor apocynin or by elimination of NOX extends survival in ALS mice, reviving the proposal that ROS mediate ALS pathogenesis (Boillée and Cleveland, 2008; Harraz *et al.*, 2008).

6.4 ROS and Diabetes

Mitochondrial dysfunction has been considered as a pathogenic factor for both Type 1 and Type 2 diabetes (Stark and Roden, 2007). The development of insulin resistance in obese individuals has been linked to alterations of lipid metabolism in skeletal muscle leading to increase of diacylglycerols, which interfere with the insulin receptor-mediated pathway via PKC stimulation (Petersen and Shulman, 2006). A primary mitochondrial defect with alteration of fatty acid β-oxidation or a secondary deficiency due to elevated malonyl-CoA levels inhibiting carnitine acyl transferase I (CAT I) (Bandyopadhyay *et al.*, 2006) have been involved in the pathogenesis of lipid metabolic dysregulation leading to insulin resistance. Even though a primary mitochondrial defect in skeletal muscle as the cause of insulin resistance has been recently challenged (Asmann *et al.*, 2006), mitochondrial dysfunction in muscle and other tissues has been frequently observed in diabetic individuals (McCarty, 2005).

A substantial body of evidence implicates oxidative stress as an important pathogenic factor in diabetic complications in both Type I and Type II diabetes mellitus; decreased uptake of glucose into muscle and adipose tissue leads to chronic hyperglycaemia, which results in tissue damage and pathophysiological complications. The drivers of this oxidative stress include hyperglycaemia, hyperinsulinaemia and the elevated free fatty acids and lipids that are usually associated with diabetes. ROS such as superoxide, hydroxyl radical and hydrogen peroxide arise from many cellular sources in response to hyperglycaemia. These sources include OXPHOS (Brownlee, 2005), glucose auto-oxidation (Wolff and Dean, 1987; Robertson *et al.*, 2003), activation of NADPH

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oxidase (Li and Shah, 2003; Cave *et al.*, 2006) and other enzymes, such as XO, lipoxygenase, CYP monooxygenases and nitric oxide synthase. ROS also arise from the production of AGE and activation of RAGE (Wautier *et al.*, 2001). In addition, shifts in redox balances, decreased tissue concentrations of low molecular weight antioxidants such as reduced glutathione (GSH) and vitamin E, and impaired activities of antioxidant defense enzymes such as SOD and catalase (Baynes and Thorpe, 1999), contribute to oxidative stress in diabetes.

Increased oxidative stress in diabetes is postulated to promote the development of neuropathy (Feldman, 2003), nephropathy (Ha and Kim, 1999; Hinokoi *et al.*, 2002), myocardial injury (Cai and Kang, 2001) and retinopathy (Kowluru, 2006).

A unifying mechanism in the development of diabetic complications has been suggested by Brownlee in a central role of ROS production by mitochondria (**Figure 23**). The increase of NADH together with the increased flux of glucose through glycolysis and of free fatty acids through β-oxidation have been suggested to enhance the pressure of electron donors in the respiratory chain with a rise in the transmembrane electrochemical potential and consequent ROS production. Furthermore, the glucose-induced increase in superoxide induces mutations in mitochondrial DNA resulting in defective subunits of the electron transport complexes eventually causing increased superoxide production at physiological concentrations of glucose (Brownlee, 2001; 2005). After the initial enhancement, a secondary decrease in glycolytic flux is thought to occur through inhibition of glyceraldehyde phosphate dehydrogenase; this key glycolytic enzyme is inhibited by poly-ADP-ribose synthesized by poly-ADP-ribose polymerase (PARP), which is activated by the increase of ROS (Du *et al.*, 2003). The accumulation of glycolytic intermediates upstream of the inhibited step activates major pathways of hyperglycaemic damage: (i) the hexosamine pathway from fructose-6-phosphate, leading to increased glycosylation and activation of proinflammatory factors by uridine diphosphate (UDP)-*N*-acetyl-glucosamine; (ii) PKC from dihydroxyacetone phosphate via diacylglycerol, leading to further activation of NFκB and proinflammatory genes and of plasma membrane NADPH oxidase; (iii) the AGE pathway from glyceraldehyde phosphate via methylglyoxal; (iv) in addition, the polyol pathway from glucose is enhanced with consequent decrease of NADPH (**Figure 23**).

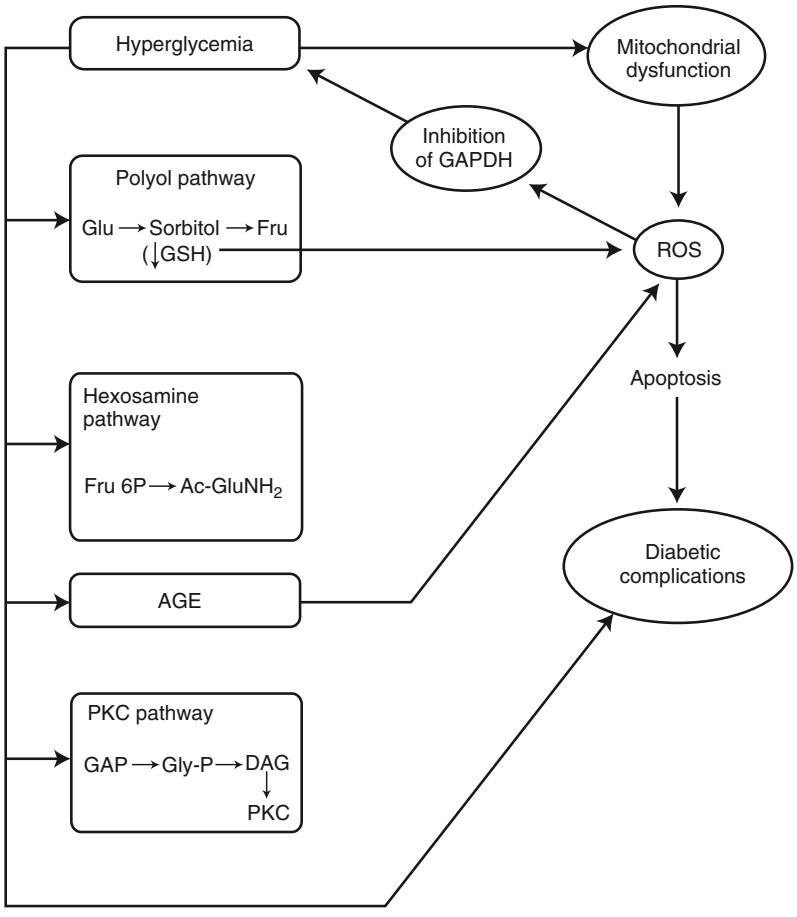

Figure 23 Central role of mitochondrial dysfunction and ROS production in the generation of diabetic complications. (Glu, glucose; Fru, fructose; AC-GluNH$_2$, N-acetylglucosamine; GAP, glyceraldehyde P; Gly-P, glycerol-P).

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The modulation of the permeability transition of mitochondrial membrane represents another dysfunction caused by ROS. The inner mitochondrial membrane space contains several soluble proteins including cytochrome c; the release of cytochrome c from mitochondria into the cytoplasm and Bax translocation from the cytosol to mitochondria, which could drive cell apoptosis, are increased in the retina and its capillary cells in diabetes (Kowluru and Abbas, 2003).

6.5 ROS and Cancer

The development of cancer in mammals is a multistep process, the aetiology of which is still largely unknown. Mitochondrial dysfunction and subsequent oxidative stress are suspected to contribute to cancer.

6.5.1 The Warburg Effect

A shift in cellular energy production from aerobic oxidation in mitochondria to anaerobic glycolysis is a fundamental property of cancer cells, also called the Warburg effect (Warburg, 1930; 1956; Garber, 2004). Otto Warburg postulated that damage of the aerobic energy metabolism is a primary and irreversible event in tumour formation (Warburg, 1956).

The early characterization of cancer metabolism by Warburg demonstrated that tumour cells exhibit a two- to threefold increased rate of glycolysis when compared to the tissue from which they have been derived (Andjelkovic *et al.*, 1996). The reasons for this apparent switch away from mitochondrial respiration are still contentious. It has been suggested that mitochondrial inactivation is beneficial for the tumour cell, as it removes the mitochondrial apoptosis pathway; in addition, blockade of the complete oxidation of pyruvate saves precursors for biosynthetic pathways that are required for rapid growth of the tumour (Deberardinis *et al.*, 2007). It has been proposed that the Warburg effect is a consequence of the tumour's hypoxic environment, making glycolysis the more viable metabolic pathway for energy generation (Zu and Guppy, 2004). Hypoxia due to rapid tumour growth stimulates the expression of the transcriptional factor HIF-1α, which induces transcription of glycolytic enzymes; the concomitant decrease in mitochondrial respiration has been thought to be a direct consequence of the HIF-1α increase, since the enhanced expression of pyruvate dehydrogenase kinase inhibits pyruvate dehydrogenase and decreases carbon input into the Krebs cycle (Kim *et al.*, 2006). At the same time HIF-1 causes a switch in the nuclear-encoded subunit 4 of cytochrome oxidase from the isoform COX4-1 to COX4-2 resulting in more efficient electron delivery to oxygen (Fukuda *et al.*, 2007; Semenza, 2007). On the other hand, a decrease of activity of the respiratory chain has been observed in several tumours (Wallace, 2005). This latter observation has brought the postulate of an opposite sequence of events, with reduction of respiration preceding a compensatory increase of glycolysis. In favour of the second interpretation is the observation of a primary mitochondrial dysfunction due to transcriptional deregulation of several mitochondrial proteins (Chiaradonna *et al.*, 2006). In this scenario, p53 mutations, frequently observed in cancer, have been proposed to represent the primary cause of respiration decrease by preventing the correct assembly of cytochrome oxidase (Matoba *et al.*, 2006). Recent studies indicate that mitochondrial dysfunction is one of the more recurrent features of cancer cells (Modica-Napolitano and Singh, 2004; Wallace, 2005), given that substantial differences between mitochondria of normal and cancer cells have been reported at microscopic, molecular, biochemical, metabolic and genetic levels. Tumour cells have long been known to possess an abnormal redox state, characterized by aberrant energy metabolism, down-regulation of antioxidant enzymes and increased production of ROS (Szatrowski and Nathan, 1991). This is advantageous for cancer progression as elevated ROS production increases the basal rate of mutation of the genome (Jackson and Loeb, 2001) and so accelerates the progression of the cancerous phenotype by the acquisition of mutations.

6.5.2 The Mitochondrial Energetics and ROS Biology of Cancer

Mitochondrial DNA mutations, extremely frequent in cancer cells, have also been involved in the generation of mitochondrial abnormalities (Carew and Huang, 2002; Petros *et al.*, 2005).

Only a few reports have been able to identify a strict association between metabolic changes in cancer cells and mitochondrial complex composition and activity; for example, in a cell line derived from a thyroid oncocytoma, Bonora *et al.* (2006) found a decrease of Complex I activity that was ascribed to a specific mutation in the ND1 gene of mitochondrial DNA. Evidence that dysfunction of the respiratory chain, resulting from mutations in mtDNA, plays a role in tumour genesis is based on data of cultured cells, but has not been shown directly in tumour tissues (Petros *et al.*, 2005; Modica-Napolitano *et al.*, 2007). Recently, it has been demonstrated that mutations in the gene encoding subunit ND6 of Complex I produced a deficiency in Complex I activity and were associated with ROS overproduction. Pretreatment of the highly metastatic tumour cells with ROS scavengers suppressed their metastatic potential in mice. These results indicate that mtDNA mutations can contribute to tumour progression by enhancing the metastatic potential of tumour cells (Ishikawa *et al.*, 2008).

Mutations in nDNA or mtDNA OXPHOS genes that impede the flow of electrons through the ETC increase mitochondrial ROS production. The resulting H_2O_2, which is relatively stable, diffuses out of the mitochondrion, through the cytosol, and into the nucleus.

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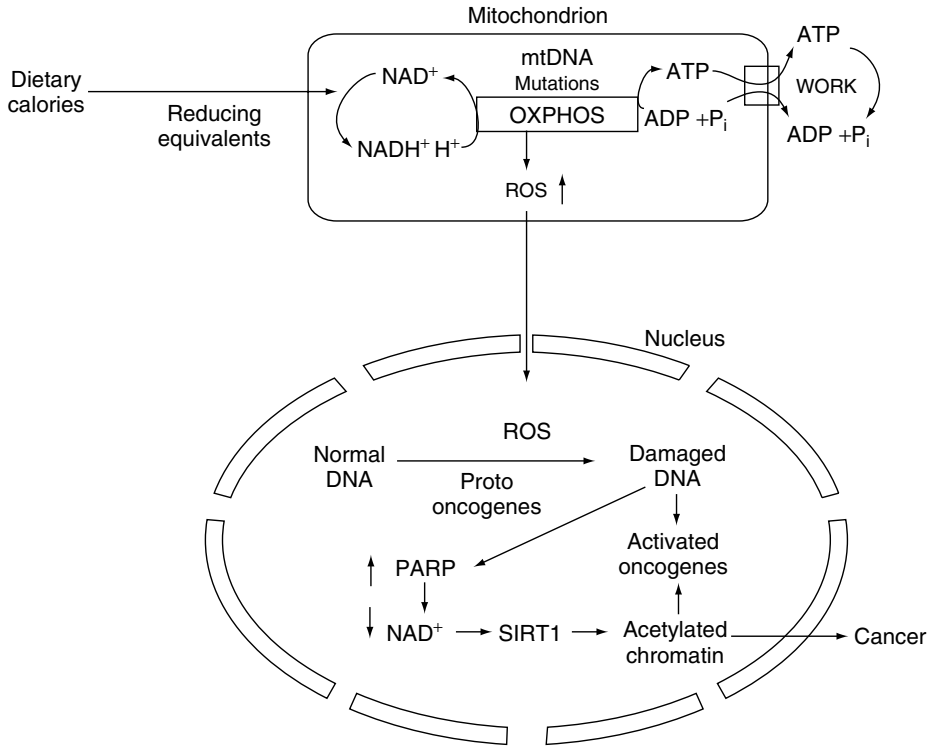

Figure 24 Model for the proposed role for mitochondrial dysfunction in cancer (Wallace, 2005). Caloric overload or inhibition of OXPHOS (mtDNA somatic mutations) perturbs the cellular mitochondrial energetic balance, resulting in increased ROS. The increased ROS also diffuse into the nucleus where they can mutate and activate protooncogenes (initiation) and can interact with NFκB, APE-1 and various kinases to initiate cell division (promotion) leading to neoplastic transformation (cancer). Damaged DNA activates PARP resulting in NAD^+ depletion and consequent inhibition of SIRT-1, a deacetylase of histones and other proteins; the resulting prevalent activity of histone acetylases allows further activation of proto-oncogenes. Figure abbreviations are PARP, poly ADP-ribose polymerase; SIRT1, the mammalian homologue to Sir2.

In the nucleus, it interacts with transition metals and is converted to $OH^\bullet$, which mutagenizes the nDNA. The resulting nDNA damage activates the nDNA repair systems, including the PARP. The activated PARP degrades the nuclear NAD^+ in the process of adding poly ADP-ribose chains to histones and other nuclear proteins (Wallace, 2005) (**Figure 24**). The degradation of the nuclear NAD^+, together with the high $NADH/NAD^+$ ratio, inactivates nuclear SIRT1. SIRT1 is a histone deacetylase, and nuclear transcription is repressed by the deacetylation of histones and activated by histone acetylation. Therefore, inhibition of SIRT1 permits histone acetylation to predominate, turning on the transcription of normally inactive genes. In postmitotic tissues, histone acetylation permits the activation of the genes that regulate cell replication and differentiation, the protooncogenes. The diffusion of H_2O_2 into the nucleus and its conversion to $OH^\bullet$ can then mutate the protooncogenes, converting them into functional oncogenes. Moreover, increased cytosolic and nuclear H_2O_2 activates a variety of cellular signal transduction factors including NFκB, APE-1, *Fos*, *Jun* and tyrosine kinases. This drives the cell into replication. Consequently, mutations in mitochondrial genes that inhibit electron flow through the ETC result in chronically increased mitochondrial ROS production, which can act as both a tumour initiator (mutation of protooncogenes) and tumour promoter (activation of transcription and replication).

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Toxicogenomics and the Evolution of Systems Toxicology

Michael D. Waters and B. Alex Merrick

CONTENTS

1 INTRODUCTION

Toxicology, the study of poisons, is focussed on substances and exposures that cause adverse effects in living organisms. Studies in toxicology measure the effects of an agent on an organism's food consumption and digestion, its body and organ weights, microscopic histopathology and cell viability, immortalization, necrosis and apoptosis (Mattes *et al*., 2004). Toxicogenomics combines toxicology with genetics and genomics to provide a comprehensive view of the function of the genetic and biochemical machinery (genes, proteins and metabolites) of the living cell. A critical part of the study of toxicology, and by extension, toxicogenomics, is the empirical and contextual characterization of adverse effects at the various levels of organization of the organism—ranging from animal health and function to organs, tissues, cells and intracellular and intercellular molecular systems. Thus, toxicology and toxicogenomics are rapidly evolving into systems toxicology (Waters and Fostel, 2004).

Toxicology and toxicogenomics are multidisciplinary in nature and both have evolved significantly as relevant technologies have advanced. By analogy, the invention of the microscope in the seventeenth century was the beginning of a long road of technological development to modern histology and pathology, which is an inherent component of toxicology. The intricacies of tissue organization and the complex architecture of organs at a cellular level were made accessible by light microscopy, which then gave way to higher resolution with the advent of electron microscopy and more recently atomic force microscopy (Maunsbach and Afzelium, 1999; Braga and Ricci, 2004). The twentieth century saw the maturing of histology as a refined tool for pathology and in a like manner presages the potential of toxicogenomics for the field of toxicology.

Similarly, the modern achievements of sequencing whole genomes have been quickly followed by

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Table 1 Some relevant terms defined[a]

Functional genomics: The development and application of global (genome-wide or system-wide) experimental approaches to assess gene function by making use of information and reagents provided by physical mapping and sequencing of genomes.
Knowledgebase: An archival and computational system that uses data, information and knowledge captured from experts to carry out tasks that create new information and new understanding.
Metabolomics or metabolite profiling: The directed use of quantitative analytical methods for analysing the entire metabolic content of a cell or organism at a given time (the metabolome).
Metabonomics: Techniques that detect changes in the concentration of low molecular weight metabolites present in a cell or organism at a given time (the metabonome) by using nuclear magnetic resonance or mass spectrometry coupled to gas or liquid chromatography.
Proteomics: A collection of techniques used to measure the structural and functional properties of proteins through the use of 2D gel electrophoresis or liquid chromatography, typically followed by protein identification using some form of mass spectrometry.
Systems biology: The integrated study of biological systems (cells, tissues, organs or entire organisms) at the molecular level. It involves perturbing systems, monitoring molecular expression, integrating response data and modelling the molecular structure and network function of the system. See GENE REGULATORY NETWORK.
Systems toxicology: The study of the perturbation of biological systems by chemicals and stressors, monitoring changes in molecular expression and conventional toxicological parameters, and iteratively integrating response data to describe the functioning organism.
Toxicoinformatics: The description of a toxicological stress and the annotation of the dose-dependent molecular responses that are elicited over time.
Transcriptomics: Techniques that measure the full complement of activated genes, mRNAs or transcripts in a particular tissue at a particular time, typically through the use of cDNA or oligonucleotide microarrays.

[a]Waters and Fostel, 2004.

gene expression profiling technologies that allow comprehensive queries of the transcriptome, to the refinement of traditional proteomics, and to the creation of other -omic technologies (see **Table 1** for definitions). Toxicogenomics (Nuwaysir *et al*., 1999) evolved from the desire to characterize how genomes respond to environmental stressors or toxicants by combining genome-wide mRNA expression profiling (transcriptomics) with global protein expression patterns (proteomics) that are interpreted by the use of bioinformatics to understand the role of gene–environment interactions in disease and dysfunction.

The increasing resolution of toxicogenomic analysis with oligonucleotide probe sets is becoming sufficiently refined for the production of single nucleotide polymorphism (SNP) genotyping arrays that can be used for cytochrome P450 (CYP450) genotyping and SNP mapping for identifying relevant gene loci for sensitivities or resistant responses (Mandal *et al*., 2005; Shi, 2002; Wen *et al*., 2003). The inherently reductive nature of toxicogenomic analysis down to the level of DNA, mRNA and protein sequences is also being counterbalanced by a concerted attempt to reassemble these molecular pieces of information into pathways and networks (Jiang *et al*., 2008; Hellerstein, 2008; Grzegorczyk, 2008; Gohlke *et al*., 2008; Ganter *et al*., 2008) that form the new field of systems toxicology (Ideker *et al*., 2001a; 2001b; Ekins *et al*., 2005; Heijne *et al*., 2005; Wetmore and Merrick, 2004; Waters and Fostel, 2004; Waters *et al*., 2003b; Waters and Yauk, 2007; Gant, 2007).

The result of these concurrent reductive and assembly activities in gene expression information is a much greater depth of field now possible for examining toxicant responses. Toxicogenomics is leading the next revolution towards a better understanding of molecular pathology. Expectations are that toxicology and pathology of the future will merge three areas: traditional pathology and toxicology, differential protein and gene expression analysis and systems biology (Lobenhofer *et al*., 2006; Ruepp *et al*., 2005; Boorman *et al*., 2002). The teaming of these technologies will extend the sensitivity of toxicity detection beyond what is currently achievable and may even uncover the earliest beginnings of acute toxicity onset or the molecular signatures of long-term toxicant exposure and disease (Storck *et al*., 2002; Suter *et al*., 2004; Jiang *et al*., 2008).

The ability to discern mechanisms of toxicity as related to health issues is an important challenge facing scientists, public health decision-makers and regulatory authorities, whose aim is to protect humans and the environment from exposures to hazardous drugs, chemicals and environmental stressors (e.g. nonionizing radiation). The problems of performing safety and risk assessments for drugs and chemicals and of identifying environmental factors involved in the aetiology of human disease have long been formidable issues. Genomic technologies offer the potential to change the way in which toxicity and human health risk are assessed.

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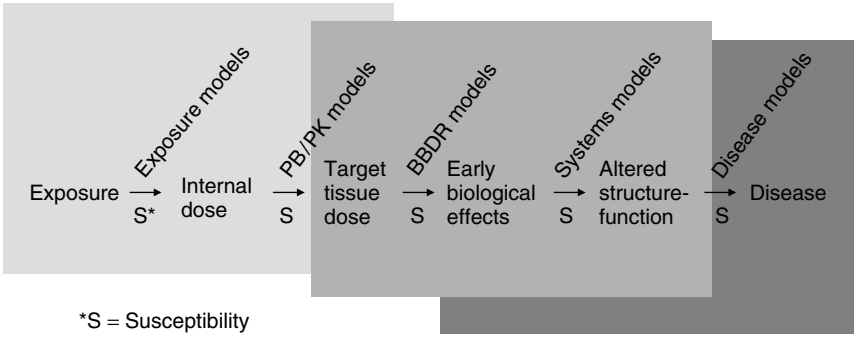

Figure 1 The role of genetic susceptibility and computational modelling on the continuum from exposure to disease outcome. The sequence of events between initial exposure and final disease outcome are shown from left to right. Following exposure, the body's 'ADME' (absorption, distribution, metabolism, excretion) systems control local concentrations of a chemical stressor in various body compartments. The impact of genetics is felt in specific alleles encoding various transporters, xenobiotic metabolizing enzymes, etc. Mathematical models such as exposure models, PB/PK and BBDR models can be used to approximate these processes. PB/PK models are a set of differential equations structured to provide a time course of a chemical's mass-balance disposition (wherein all inputs, outputs and changes in total mass of the chemical are accounted for) in preselected anatomical compartments. BBDR models are dose–response models based on underlying biological processes. Once the target tissue is exposed to a local stressor the cells respond and adapt or undergo a toxic response; this process can be modelled with systems toxicology approaches. Finally, the disease outcome itself can be mimicked by genetic or chemically induced models of particular diseases.

The rapid accumulation of genomic sequence data and associated gene and protein annotation has catalysed the application of gene expression analysis to understand the modes of action of chemicals and other environmental stressors on biological systems (see **Figure 1**). These developments have facilitated the emergence of the field of toxicogenomics, which aims to study the response of a whole genome to toxicants or environmental stressors (Aardema and MacGregor, 2002; Afshari, 2002; Ulrich and Friend, 2002; Fielden and Zacharewski, 2001; Hamadeh *et al*., 2002a; Tennant, 2002; Thomas *et al*., 2001; Olden and Guthrie, 2001; Olden, 2006; Waters *et al*., 2003b; 2003c; Lobenhofer *et al*., 2001; 2006; Burchiel *et al*., 2001). The related field of toxicoproteomics (Merrick and Tomer, 2003; Merrick and Madenspacher, 2005; Petricoin *et al*., 2004; Wilkins *et al*., 1996) is similarly defined with respect to the proteome, the expression of gene products of the genome. The emerging field of toxicometabonomics combines high-throughput intermediary metabolite or pharmaceutical metabolite profiling with computer-assisted pattern recognition approaches (Lindon *et al*., 2006; 2007; Kasper *et al*., 2005; Griffin, 2006). Global technologies, such as oligonucleotide microarrays, protein chips, mass spectrometry (MS) and nuclear magnetic resonance (NMR)-based molecular profiling, for example, can simultaneously measure the expression of numerous genes, proteins and metabolites, respectively, thus providing the potential to accelerate the discovery of toxicant pathways, modes-of-action and specific chemical and drug targets. Toxicogenomics therefore combines toxicology with genetics, global -omics technologies (see Appendix A) and appropriate pharmacological and toxicological models (**Figure 1**) to provide a comprehensive view of the function of the genetic and biochemical machinery of the cell.

This review explores the new field of toxicogenomics, delineates some of its research approaches and success stories, and describes the challenges it faces. It discusses how integrating data derived from transcriptomics, proteomics and metabonomics studies can contribute to the development of a toxicogenomics knowledgebase (**Figure 2**) and to the evolution of systems toxicology as it relates to molecular expression profiling. In many ways, current gene, protein and metabolite expression profiles are simple 'snapshots'; by contrast, systems toxicology, like systems biology (Ideker *et al*., 2001a; Nurse, 2003), attempts to define the interactions of all of the elements in a given biological system, under stress or toxicant perturbation, to achieve a mechanistic understanding of the toxicological response.

2 TOXICOGENOMICS: AIMS AND METHODS

Toxicogenomics has three principal goals: to understand the relationship between environmental stress and human disease susceptibility (**Figure 1**), to identify useful biomarkers of exposure to toxic substances and biomarkers of disease and to elucidate the molecular mechanisms of toxicity. A typical toxicogenomics study

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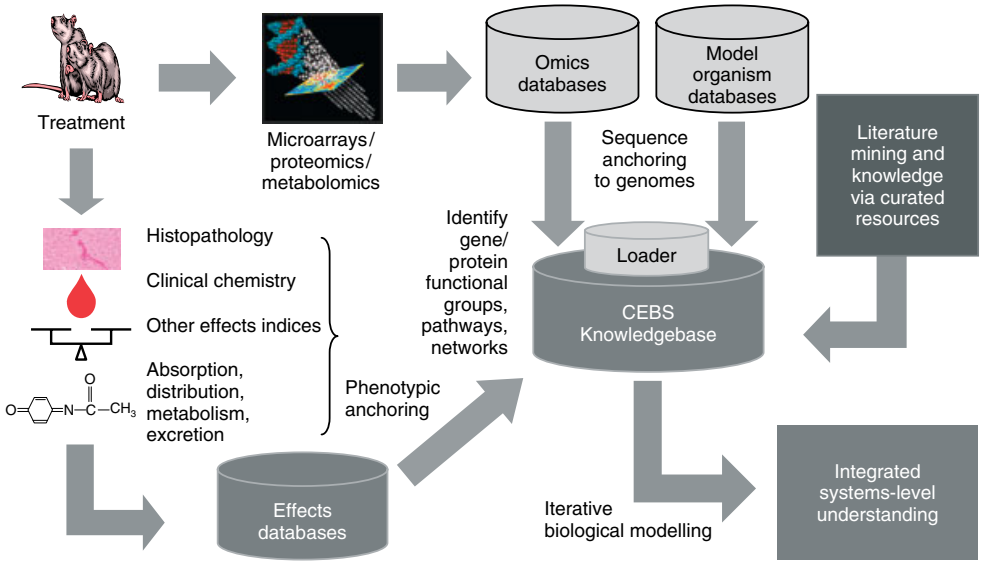

Figure 2 A framework for systems toxicology. The figure indicates the paths from the initial observation (rat in upper left) to an integrated toxicogenomics knowledgebase and thence to systems toxicology. The '-omics datastream' is shown by the clockwise path from rat to knowledgebase, and the 'traditional' toxicology approach is shown in the counterclockwise path. The knowledgebase will integrate both data streams, along with literature knowledge, and by virtue of iterative modelling lead to a systems toxicology understanding. The framework involves 'phenotypic anchoring' (to toxicological endpoints and study design information) and 'sequence anchoring' (to genomes) of multidomain molecular expression datasets in the context of conventional indices of toxicology, and the iterative biological modelling of the resulting data.

might involve an animal experiment with three treatment groups: high- and low-dose treatment groups and a vehicle control group that has received only the solvent used with the test agent. These groups will be observed at two to three points in time, with three to five animal subjects per group. In this respect a toxicogenomics investigation resembles a simple, acute toxicity study. Where the two approaches differ is in the scope of the response they each aim to detect, and in the methods used. The highest dose-regimen is intended to produce an overtly toxic response, which in a toxicogenomics study can be detected using the global measurement techniques described below (see also Appendix A).

In a typical toxicogenomics experiment, lists of significantly differentially expressed genes are created for each biological sample (Hamadeh *et al*., 2002e). Alternatively, profile analysis methods can be applied to dose and time-course studies (Hamadeh *et al*., 2002e) to identify genes and gene profiles of interest. Then, with the aid of the relevant knowledge that is systematically extracted and assembled (Zweiger, 1999) through literature mining, comparative analysis and iterative biological modelling of molecular expression datasets, it is possible to differentiate adaptive responses of biological systems from those changes (or biomarkers) associated with or precedent to clinical or visible adverse effects. Over the past 5–10 years, the field of toxicogenomics has validated the concept of gene expression profiles as 'signatures' of toxicant classes, disease subtypes or other biological end points. These signatures have effectively directed the analytical search for predictive biomarkers of toxicant effects and contributed to the understanding of the dynamic alterations in molecular mechanisms associated with toxic and adaptive responses.

The experimental work involved in a toxicogenomics study and the amount of gene expression data generated are vast. To examine even only one tissue per animal in the study design described above requires 18–45 microarrays (more if technical replicates are used) and the attendant measurement of as many as 30 000 or more transcripts per array. In addition, each animal will typically have treatment-associated data on total body and organ weight measurements, clinical chemistry measurements (often up to 25 parameters) and microscopic histopathology findings for several tissues. The careful collection, management and integration of these data, in the context of the experimental protocol, are essential for interpreting toxicological outcomes. Thus all data must be recorded in terms of dose, time and severity of the toxicological and/or histopathological phenotype(s). The compilation of such experimental data, together with toxicoinformatics tools and computational modelling are important in deriving a new understanding of toxicant-related disease (Tennant, 2002).

Toxicogenomics integrates the multiple data streams derived from transcriptomics, proteomics and metabonomics with traditional toxicological and histopathological end-point evaluation (**Figure 2**). This integration has the potential to synergize our

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understanding of the relationship between toxicological outcomes and molecular genetics. Furthermore, toxicology and toxicogenomics are progressively developing from studies done predominantly on individual chemicals and stressors into a knowledge-based science (Waters *et al*., 2003a). However, the evolution of a truly 'predictive toxicology'—wherein knowledge of toxicogenomic responses of a prototypic agent in one species and strain is used to predict the mode-of-action of a similar agent in a related strain or another species—will require that the results of numerous toxicogenomics investigations across genotypes and species be assimilated into a multidomain, multigenome, knowledgebase (**Figure 2**). This knowledgebase must be searchable by chemical formula/stressor-type, by gene/protein/metabolite molecular signature, or by phenotypic outcome, among other entities, to find results analogous to those observed with a newly tested agent. It must capture current knowledge of chemical mode-of-action and facilitate the discovery of new modes. Toxicology will then have become an information science, and public health and risk assessment will be the beneficiaries.

3 TOXICOGENOMICS: EVOLUTION OF THE FIELD

Toxicogenomics has evolved from early gene expression studies, which described the response of a biological system to a particular toxicant or panel of reference agents, towards more mature investigations that integrate several -omics domains with toxicology and pathology data (see **Table 2**). Exposure- and outcome-specific patterns of gene, protein and metabolite profiles have been used to identify molecular changes that serve as biomarkers of toxicity (Thomas *et al*., 2001; 2007; 2002; Waring *et al*., 2001b; Mortuza *et al*., 2003; Kramer *et al*., 2004; Heinloth *et al*., 2004; Hamadeh *et al*., 2002c; Bulera *et al*., 2001; Bartosiewicz *et al*., 2001) and provide insights into mechanisms of toxicity (Cutler *et al*., 1999; Coen *et al*., 2004; Donald *et al*., 2002; Fertuck *et al*., 2003; Fountoulakis *et al*., 2002; Hamadeh *et al*., 2002d; Huang *et al*., 2001; Kramer *et al*., 2003; Peterson *et al*., 2004; Ruepp *et al*., 2002; Waring *et al*., 2002) and disease causation (Wagenaar *et al*., 2004; Lu *et al*., 2001; Hamadeh *et al*., 2004; Iida *et al*., 2003; Ellinger-Ziegelbauer *et al*., 2004). Critical to this evolution were extensive and ongoing genome sequencing and annotation efforts (Twigger *et al*., 2002; Higgins *et al*., 2003) and the ability to describe response profiles in genetically and toxicologically important species such as mouse, rat, dog and human. Another important contribution to toxicogenomics has been the formation of collaborative research consortia (Ulrich *et al*., 2004; Pennie *et al*., 2004; Lindon *et al*., 2003), which bring together scientists from regulatory agencies, industrial laboratories and academic and government scientists to identify and address important issues for the field.

3.1 Profiles of Response to Toxicants

Nuwaysir *et al*. popularized the term 'toxicogenomics' to first describe the use of microarrays to measure the responses of toxicologically relevant genes, and to identify selective, sensitive biomarkers of toxicity (Nuwaysir *et al*., 1999). The initial published toxicogenomics study compared the gene expression profiles of human cells responding to the inflammatory agent lipopolysaccharide (LPS) or to mitogenic activation by phorbol myristate acetate (PMA) (Heller *et al*., 1997). RNA samples isolated at various times following exposure showed the expected increases in cytokine, chemokine and matrix metalloproteinase transcripts. Similar expression profiles were seen in synoviocytes and chondrocytes from a patient with rheumatoid arthritis, confirming the ability of the system to mimic the biological changes that occur during inflammatory disease. Subsequent studies extended this type of observation in other tissues and for a wide variety of toxicants, enabling the association of specific molecular profiles with specific toxicities (Merrick and Bruno, 2004).

4 PHENOTYPIC ANCHORING

Conventional toxicology has employed surrogate markers correlated with toxic responses to monitor adverse outcomes in inaccessible tissues. For example, liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are released following hepatic damage, and levels of these enzymes in serum correlate with histopathological changes in the liver (Travlos *et al*., 1996). These serum enzyme markers, in conjunction with histopathology, facilitate the 'phenotypic anchoring' of molecular expression data (Tennant, 2002; Waters *et al*., 2003c; Paules, 2003). 'Phenotypic anchoring' is the process of determining the relationship between a particular expression profile and the pharmacological or toxicological phenotype of the organism for a particular exposure or dose and at a particular time (Tennant, 2002). The dose and time alone are often insufficient to define the toxicity experienced by an individual animal; thus another measure of toxicity is needed for full interpretation of the data obtained during a toxicogenomics study. Conversely, the phenotype alone may be insufficient to anchor the molecular profile, since, for example, an elevated value for serum ALT can be observed both

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Table 2 The scope and evolution of toxicogenomics

Study aim	Key references
Toxicogenomics tools and model systems	Toxicogenomics began with 'toxicology-specific' cDNA microarrays designed to measure the levels of acute phase and xenobiotic-metabolizing enzymes such as cytochrome P450s (Nuwaysir *et al*., 1999; Bartosiewicz *et al*., 2000). These were superseded as commercial platforms were developed for toxicologically important species such as rat. The armamentarium of preclinical gene expression platforms was completed with the canine microarray (Higgins *et al*., 2003). It is now possible to use commercial oligonucleotide microarrays to measure expression responses in species ranging from nematode (*Caenorhabditis elegans*), to Daphnia (*Daphnia magna*) (Poynton *et al*., 2007), to frog (*Xenopus laevis*), and zebrafish (*Danio rerio*) (Langheinrich, 2003; Alestrom *et al*., 2006; Hoyt *et al*., 2003; Scholz *et al*., 2008), to rodents (rat and mouse), to nonhuman primates and man. Toxicogenomics tools for sentinel aquatic species have been developed as well (Hogstrand *et al*., 2002). Later experiments began to focus on more challenging subjects such as subcellular organelles (Jiang *et al*., 2004), nonstandard tissue such as saliva (Vitorino *et al*., 2004), less well-characterized species, genetic models of diseases (Talamo *et al*., 2003), and integration of data from different -omics disciplines (Coen *et al*., 2004; Ruepp *et al*., 2002; Iida *et al*., 2003; Hogstrand *et al*., 2002; Juan *et al*., 2002). Additionally, comprehensive studies of yeast have become increasingly important.
Some tissues used in toxicogenomics studies	Many toxicogenomics studies have involved hepatotoxicants (Thomas *et al*., 2002; Hamadeh *et al*., 2002e; Waring *et al*., 2001a; Mortuza *et al*., 2003; Kramer *et al*., 2004; Heinloth *et al*., 2004; Hamadeh *et al*., 2002c; Bulera *et al*., 2001; Bartosiewicz *et al*., 2001; Dong *et al*., 2002; Fountoulakis *et al*., 2002; Hamadeh *et al*., 2002d; Kramer *et al*., 2003; Peterson *et al*., 2004; Ruepp *et al*., 2002; Waring *et al*., 2002; Lu *et al*., 2001; Iida *et al*., 2005; Iida *et al*., 2003; Weiss *et al*., 2004), as the liver is the primary source of xenobiotic metabolism and detoxification, and because liver injury is the principal reason for withdrawal of new drugs from the market (Lee, 2003). However, toxicogenomics studies have also addressed, nephrotoxicity (Cutler *et al*., 1999; Bartosiewicz *et al*., 2001; Huang *et al*., 2001), neurotoxicity (Xie *et al*., 2002; Dam *et al*., 2003) and reproductive toxicity (Fertuck *et al*., 2003), for example, as well as lung toxicity (Mortuza *et al*., 2003; Wagenaar *et al*., 2004), skin toxicity (Hamadeh *et al*., 2002f) and cardiotoxicity (Hu *et al*., 2002).
Phenotypic anchoring	Phenotypic anchoring relates expression profiles to specific adverse effects defined by conventional measures of toxicity such as histopathology or clinical chemistry (Beyer *et al*., 2007; Daston, 2008; Moggs *et al*., 2004; Moggs, 2005; Paules, 2003; Powell *et al*., 2006). Experiments have been designed to correlate expression patterns with disease pathologies such as necrosis, apoptosis, fibrosis or inflammation (Hamadeh *et al*., 2002e; Waring *et al*., 2001b; Wagenaar *et al*., 2004; Higgins *et al*., 2003; Waring *et al*., 2001a). Additionally, phenotypic anchoring can be used to provide biological context for toxicogenomics observations made at subtoxic doses (Heinloth *et al*., 2004; Peterson *et al*., 2004).
Some classes of toxicants characterized	Studies have examined responses to toxicants with established mechanisms of toxicity (Waring *et al*., 2001b; Bulera *et al*., 2001; Bartosiewicz *et al*., 2001; Fountoulakis *et al*., 2002; Hamadeh *et al*., 2002d; Kramer *et al*., 2003; Ellinger-Ziegelbauer *et al*., 2004; Hamadeh *et al*., 2001), environmental toxicants (Lu *et al*., 2001; Hogstrand *et al*., 2002; Hamadeh *et al*., 2002f; Witzmann *et al*., 2003), or exposures to suprapharmacological levels of drugs (Mortuza *et al*., 2003; Heinloth *et al*., 2004; Coen *et al*., 2004; Donald *et al*., 2002; Peterson *et al*., 2004; Ruepp *et al*., 2002; Iida *et al*., 2003; Fountoulakis *et al*., 2000; Hu *et al*., 2002).
Examples of toxicant or stressor mechanisms	Acetaminophen or paracetamol (Heinloth *et al*., 2004; Coen *et al*., 2004; Fountoulakis *et al*., 2000; Ruepp *et al*., 2002; Huang *et al*., 2004; Beyer *et al*., 2007) Arsenic (Ghosh *et al*., 2008; Liu *et al*., 2006; Liu *et al*., 2004), arsenite (Nohara *et al*., 2008) Endocrine disruptors (Phillips and Foster, 2008; Phillips *et al*., 2008; Iguchi *et al*., 2007; Villeneuve *et al*., 2007) Oestrogenic agents (Fertuck *et al*., 2003; Adachi *et al*., 2004) Oxidant stress (Nadadur *et al*., 2000; Weiss *et al*., 2004)

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Table 2 (*continued*)

Study aim	Key references
	Peroxisome proliferators[a] (Thomas *et al*., 2001; Hamadeh *et al*., 2002c; Bartosiewicz *et al*., 2001; Hamadeh *et al*., 2002d; Kramer *et al*., 2003)
Importance of reporting husbandry and other technical details	Expression profiles are altered by experimental conditions including harvest method, *in vitro* culture method, vehicle used to deliver an agent, time of day of sacrifice and diet. Up to 9% of the transcripts in mouse liver fluctuated with circadian cycling (Akhtar *et al*., 2002). These included genes controlling glucose metabolism and vesicle trafficking or cytoskeleton, as might be anticipated from changes in the diet of animals during the day and night. In addition, however, transcript levels of Cyp17 and Cyp2a4, which are important for steroid synthesis, and Cyp2e1, which is important for detoxification of xenobiotics, also fluctuated. These changes might be expected to impact the response to test agents, and reflect a requirement to report the time of day of dosing and sacrifice, along with the diet, vehicle and harvest and culture methods when summarizing or publishing results of toxicogenomics studies.
Commercial database resource for toxicogenomics	Toxicogenomics studies for the purpose of developing a commercial database have been performed by GeneLogic (*http://www.genelogic.com/*). This company has gathered data from several hundreds of samples produced from short-term exposures of agents at pharmacological and toxicological dose levels. Customers of the company can access the database to classify the mode-of-action of novel agents of interest.
Integration	*Of toxicogenomics efforts:* ILSI Committee on the Application of Toxicogenomics to Risk Assessment (Ulrich *et al*., 2004; Pennie *et al*., 2004), Toxicogenomics Research Consortium (TRC), Consortium on Metabonomics and Toxicology (COMET) (Lindon *et al*., 2003). Through such Consortia the technical factors affecting data can be identified and overcome, approaches to data analysis and interpretation can be agreed upon, and high-quality public datasets prepared. The field of toxicoproteomics is currently not represented by a consortium, while the ILSI Genomics Committee and the TRC are working toxicogenomics consortia in transcriptomics, and COMET is a working toxicogenomics consortium in metabonomics.
	Of data domains: (Coen *et al*., 2004; Ruepp *et al*., 2002; Hogstrand *et al*., 2002; Juan, 2002; Heijne *et al*., 2004). Integration of data can provide a more complete picture of the expression profiles associated with a particular treatment, shedding light not only on what the cell is planning (transcriptomics), but also what occurred in the proteome and metabonome.

[a]Peroxisome proliferators are compounds that induce increased numbers of peroxisomes—single-membrane cytoplasmic organelles that metabolize long-chain fatty acids.

before peak toxicity (as it rises) and after peak toxicity (as it returns to baseline). Thus, anchoring the molecular expression profile in phenotype, dose and time helps to define the sequence of key molecular events in the mode-of-action of a toxicant.

Phenotypic anchoring can also be used in conjunction with lower doses to classify agents and to explore the mechanisms of toxicity that occur before histopathological changes are seen. For example, transcriptional changes that occur following both low- and high-dose exposures of acetaminophen (paracetamol) were identified, indicating that biological responses can be detected using transcriptome measurements before histopathological changes are easily detected (Heinloth *et al*., 2004; Beyer *et al*., 2007). Follow-up work shows the accumulation of nitrotyrosine and 8-hydroxy-deoxyguanosine adducts phenotypically anchors an oxidative stress gene expression signature observed with a subtoxic dose of acetaminophen (paracetamol), lending support to the validity of gene expression studies as a sensitive and biologically meaningful end point in toxicology (Powell *et al*., 2006). Additionally, phenotypic anchoring can help to elucidate a toxicant's mechanism of action. For example, the transcriptional responses in a rat model to superpharmaceutical doses of WAY-144122 (a negative regulator of insulin) were observed before histopathological changes were seen in either liver or ovary, and reflected different mechanisms of toxicity in the two organs (Peterson *et al*., 2004).

5 BIOMARKERS AND SIGNATURES

A biomarker is an objective measure that can indicate health, disease, pharmacologic response to therapy, or adverse response to toxicants (Guerreiro *et al*., 2003; Frank and Hargreaves, 2003). Several reviews have been written about expression profiling and biomarker discovery (Frank and Hargreaves, 2003; Kennedy, 2002; Gunn and Smith, 2004; Hanash, 2003; Walgren and Thompson, 2004; Wallace *et al*., 2004; Roberts *et al*.,

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2003). Many classic biomarkers, such as serum ALT, sorbitol dehydrogenase, ornithine carbamyltransferase and others, can almost function as singular indicators of specific types of liver pathology and dysfunction (Amacher, 2002). But at the current level of understanding for complex diseases and toxicities, it is unlikely that any single readout may be a sufficient indicator. Instead, multiple markers that function as a 'molecular signature' or 'metabolic fingerprint' appear needed for classifying and better describing toxicant mechanisms of action (Guerreiro *et al*., 2003; Lindon, 2003; Bailey and Ulrich, 2004).

To some degree, the wide use of cluster analysis in classifying toxicity and disease (Bergmann *et al*., 2003; Abe *et al*., 2003) favours toxicity or disease characterization by transcript signature profile over a single biomarker. However, one appraisal of toxicogenomic studies outlined the following challenges that exist in developing such a profile: (i) it is difficult to generate a transcriptional profile that is truly predictive rather than diagnostic of an expected outcome; (ii) few of the studied toxicants have a specific molecular target and unique mechanism of action that could provide a well-defined response and (iii) it remains a challenge to distinguish between primary and secondary (cause or consequence) transcriptomic changes in toxicity vs adaptive responses unrelated to toxicity (Bailey and Ulrich, 2004). In addition, some of the other major factors that detract from robust DNA microarray toxicity signatures are variation in signal generation, use of different transcriptomic platforms, variation in the number of arrayed genes and sequences and interindividual animal differences due to genetic or microenvironmental factors (Bailey and Ulrich, 2004). Such difficulties encountered in transcript profiling of toxicants in both experimental and preclinical studies will undoubtedly be a shared experience for proteomic analysis in similar settings. Alternatively, part of the challenge in defining predictive signatures are frequent internal redundancies in biochemical pathways and dilution effects by nonresponding cells in complex multicellular tissues that might combine to either mask or dampen the expression of key, singular gene transcripts and proteins in target cells that are the rate-limiting step for toxicity during profiling studies.

Biomarker discovery studies using proteomic profiling methods in toxicological settings are reflected in the fewer numbers of citations in proteomics and toxicology compared to toxicogenomic studies to date. However, a survey of the toxicoproteomic literature reveals keen research and commercial interests for how to best use proteomic technologies for biomarker discovery. The terminology used in biomarker research as a result of toxicogenomic and toxicoproteomic studies is also likely to continue expanding from the conventional terms, 'biomarkers', 'signatures' and 'profiles' to other terms like 'barcode' (Choe *et al*., 2002) for simultaneous detection of multiple bioanalytes for a specific disease or 'footprint' (Bratton and Cohen, 2003) from the signal transduction community to indicate proteins held in a partially activated state. Once a more sophisticated integration of proteomics and toxicogenomic technologies with toxicology occurs and biological responses brought on by subtle changes in activation status or posttranslational modifications are more readily determined, the use of these terms will likely become more widespread. The use of transcriptomics in combination with proteomics for discovering individual biomarkers can accelerate the discovery process, particularly when conventional biomarkers are lacking. As an example, a class of lead compounds identified in a discovery programme based on gamma secretase inhibition as therapy for Alzheimer's disease also had an undesirable effect of inhibiting cleavage by Notch1 of the Hes1 gene product, a process important for differentiation of intestinal epithelial cells. Through the use of gene expression profiling and subsequent protein analysis, Searfoss *et al*., 2003 identified adipsin as a novel biomarker for this toxicity.

Carcinogenic potential is conventionally measured using a two-year study, incurring significant expense in both animals and human resources. It is therefore of great interest to identify biomarkers of carcinogenicity that can be detected in acute, short-term studies and efforts towards this have been reported (Hamadeh *et al*., 2002e; 2004; Kramer *et al*., 2004; Iida *et al*., 2003; Ellinger-Ziegelbauer *et al*., 2004; Newton *et al*., 2004). Biomarkers with clinical relevance have also been found using toxicogenomics approaches. For example, (Petricoin *et al*., 2002) found a set of protein markers that distinguished patients with high levels of prostate specific antigen (PSA), a clinical marker correlated with prostate cancer, from those with low PSA levels and thus presumed to be healthy. In addition, the marker set also correctly predicted 71% of patients with intermediate PSA levels. In a second example (Zhang and Gant, 2004), three plasma biomarkers were discovered and then independently validated to detect early stage invasive epithelial ovarian cancer from healthy controls with high sensitivity and specificity compared to the traditional marker CA125 alone. These biomarkers demonstrate the potential to improve the detection of early stage ovarian cancer using toxicoproteomics technologies.

Doubtless, the sequencing of the human genome has brought about a systematic means of viewing the molecular basis of disease through genes and their many levels of regulation. Genomics-based tools will be developed for diagnosis and prediction of disease onset or recurrence, personalized medicine and assess treatment response (Ginsburg and Haga, 2006). Although in its beginnings, genomic biomarker research has the capability of providing high definition viewing into pathophysiological processes and providing more precise predictors of outcome not previously possible with traditional biomarkers. Before genomic biomarkers can be regularly integrated into clinical practice, however, the

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necessary levels of evidence must be brought to bear to demonstrate analytical and clinical validity and ultimately their utility in improving patient care. Similar caveats apply prior to being embraced by the pharmaceutical and regulatory sectors for preclinical safety assessment.

6 PROTEOMIC ANALYSIS OF TOXIC SUBSTANCES

Toxicoproteomics has been considered by Merrick and colleagues (Wetmore and Merrick, 2004; Merrick and Madenspacher, 2005) as a new field involving a distinct relationship of toxicologic pathology with toxicogenomic disciplines. Other reviews explore its importance in serum protein pattern diagnostics (Petricoin and Liotta, 2004), delineate new biomarkers of toxicity and toxicity signatures (Merrick and Bruno, 2004), highlight the field's achievements and limitations in the development of biomarkers (Sinha *et al*., 2007), and trace discovery of early markers of drug toxicity (Collins *et al*., 2007). Data in these reviews resulting from toxicoproteomic studies were categorized by: (i) pharmaceutical or chemical toxicant under study; (ii) the model organism or clinical subjects involved; (iii) target organs analysed, such as liver, kidney, brain and heart; (iv) biofluids analysed, such as serum, plasma, urine or cerebrospinal fluid; (v) proteomic method of analysis that was conducted, such as 2D gel, differential gel electrophoresis (DIGE), isotope coded affinity tags (ICATs), antibody array; (vi) the use of *in vivo* or *in vitro* model systems and (vii) the number of differentially expressed and identified proteins. While specific proteins were identified as differentially expressed in each of the cited investigations, these reviews noted that validation and follow-up studies to confirm either individual proteins or sets of proteins as 'biomarkers' were extremely limited. There was a general consensus among reviews of toxicoproteomics studies (Collins *et al*., 2007; Merrick and Bruno, 2004; Merrick and Madenspacher, 2005; Petricoin *et al*., 2004; Wetmore and Merrick, 2004) about common aims of the field. First, the discovery potential of proteomics technologies can be exploited to find new biomarkers or toxicity signatures during preclinical safety assessment or hazard evaluation and in diagnosing and treating human disease. Second, toxicoproteomics can be used to achieve a better understanding of molecular mechanisms underlying chemically induced toxicity in preclinical and experimental settings. Third, toxicoproteomics can integrate with data from other -omics technologies, bioinformatics, imaging and computation tools and toxicogenomics databases for a systems biology approach to predictive mechanistic toxicology. These collective aims represent a practical stratification of the discovery to knowledge process that often begins with biomarker(s) development that leads to an improved understanding of toxicity mechanisms.

As multiple consequences of mechanistic research mature and expand into a larger context of systems biology, initial molecular toxic insults will eventually be translated into a predictable series of downstream events that form a visible phenotype of toxicity. The range of toxicant effects analysed by proteomics from tissues, biofluids, subcellular fractions and modified proteins by adduction or other posttranslational effects is impressively broad and promising for toxicology and pathology evaluations. The application of new proteomic platforms and an integration of proteomics with transcriptomic and metabolomic analysis in databases promises to greatly enhance the biomarker discovery capability for toxicoproteomics (Patterson, 2003).

7 SERUM AND PLASMA PROTEOMES AND ACCESSIBLE BIOFLUIDS

Complete proteomic mapping of the human serum and plasma and specialized blood components is being undertaken in healthy individuals and in patients with disease. Serum and plasma are arguably the most informative, accessible biofluids of the body for biomarker development. The reason is that almost all cells of the body are in contact with blood and, as such, the cells and the tissues they make up very often communicate through active endocrine secretion or passive release of peptides and proteins into the circulation (**Figure 3**). Further, analysis of low concentration, bioinformative proteins can be greatly improved after immunoaffinity removal of abundant (but less informative proteins) that make up 85–90% of plasma. Such abundant plasma proteins include albumin, IgG, IgA, transferrin, haptoglobin, α-1-antitrypsin, hemopexin, transthyretin, α-2-HS glycoprotein, α-1-acid glycoprotein, α-2-macroglobulin and fibrinogen from human plasma (Pieper *et al*., 2003a; 2003b). Although any process of abundant protein depletion by affinity chromatography may inadvertently remove some desirable proteins, the gain for detecting low-level protein markers during subsequent proteomic analysis is greatly worthwhile and is in part due to a 10–20-fold enrichment over untreated plasma or serum.

Why has there been such interest in biomarker development from clinical blood samples? One reason is that despite the enormous utility of clinical chemistry in diagnostics and medicine, remarkably few novel biomarkers have been discovered over the last decade (Anderson *et al*., 2002; Anderson and Anderson, 2002). Newer mass spectrometry-based methodologies in the last 8–10 years have transformed proteomics. While this has led to a dramatic increase in biomarker research, the discovery of new biomarkers has been slow to realize. Current

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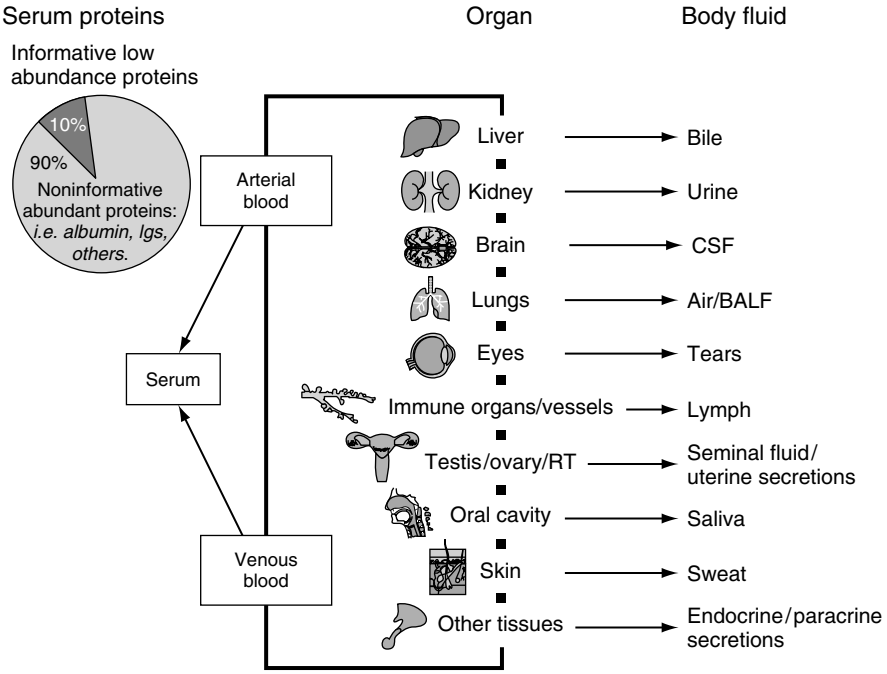

Figure 3 Protein profiling of serum and target organs: systems toxicology and pathology. Blood circulation in organs and tissues makes it an ideal body fluid to assess early, overt injury and recovery periods of acute toxicity. More comprehensive profiling of the serum proteome could lead to improved markers of toxicity and disease, a better understanding of recovery and repair processes, interorgan and intertissue communication, and a more unified, systems toxicology and pathology approach to temporal changes in the serum proteome and its relationship to human health in development, maturity, reproduction and ageing. Similar advances should occur in biomarker development after mapping the proteomes of tissue-specific biofluids and monitoring changes during toxicity and disease. (CSF = cerebrospinal fluid; BALF = bronchoalveolar fluid; RT = reproductive tract).

proteomic technologies can analyse extremely small amounts of proteins. However, a consistent problem has been in detecting and quantifying proteins that are present at 2–3 orders of magnitude lower than the more abundant proteins. The commercial availability of 'immunosubtraction' materials available for human sera and soon for rodent serum, and recent studies that survey the human serum and plasma proteomes (described below) will have major impacts on biomarker research. Three studies published over the past few years have attempted to comprehensively map the human plasma and serum proteomes defining them to 490 (Adkins *et al*., 2002), 325 (Pieper *et al*., 2003a) and 341 (Tirumalai *et al*., 2003) unique proteins with almost 3700 electrophoretically separable features (Pieper *et al*., 2003a). However, the number of serum and plasma proteins is thought to be much higher than 1000 unique proteins, but an exact number is unknown. The detection of nuclear, DNA binding, cytoplasmic and kinesin complex proteins in serum poses exciting possibilities about how the serum proteome reacts and adjusts during the course of disease and toxicity (Merrick and Tomer, 2003). The complete mapping of the human proteome has taken on international importance since this is one of the prime goals of an international consortium called the Human Proteome Organization (HUPO) (Merrick, 2003). Cataloguing of the many other biofluids from various organs (**Figure 3**), such as saliva (Vitorino *et al*., 2004), urine (Pieper *et al*., 2004), cerebrospinal fluid (Puchades *et al*., 2003; Jiang *et al*., 2003) and others (Kennedy, 2001) should lead to important advances in biomarker development. While there are no corresponding organizations dedicated to the proteomes of primates, mice, rats and other experimental animals, such interests will evolve in time either as an outgrowth of HUPO or as separate entities.

One other area in plasma proteome research deserves mention. While most proteins isolated from blood are generally soluble, there are solid protein substances or 'microparticles' in blood that are found at low levels. Microparticles are intact vesicles derived from cell membranes of 0.2–2 μM in size that are shed or secreted from tissues into blood (Piccin *et al*., 2007). Microparticles constitute a 'subproteome' of plasma which has a high likelihood of containing specialized proteins associated with injury and disease (Smalley and Ley, 2008; Merrick, 2008). While this subproteome makes up less than 0.01% of the total plasma proteome, it is rich in proteins altered under a variety of pathological conditions. Membrane activation processes and apoptosis can create microparticles from cell types as diverse

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as platelets, endothelial cells, monocytes and granulocytes. Many normal cell types, including tumour cells (Ginestra *et al*., 1997), release vesicles by ectocytosis either spontaneously or in response to various stimuli (MacKenzie *et al*., 2001; Gasser and Schifferli, 2004). Review of various methods for microparticle isolation shows that flow cytometry has been widely adopted, while ultracentrifugation in less than 2 hours provides sufficient amounts for proteomic analysis (Piccin *et al*., 2007). Detection of these emitted membrane vesicles have wide application in immune and cancer diseases and have physiologic roles in coagulation, vascular function, angiogenesis, wound healing, inflammation and development (Pilzer *et al*., 2005; Ardoin *et al*., 2007). The proteome of human platelet microparticles has recently been mapped (Garcia *et al*., 2005) and the likelihood seems high that microparticle changes in response to chemical exposure and injury represent a prime opportunity for facile enrichment and discovery of new biomarkers during toxicoproteomic studies.

8 TRANSCRIPTOMICS AND PROTEOMICS: DISAGREEMENT OR COMPLEMENTARITY?

An important issue that continues to arise in gene expression studies is the reportedly poor correlation between mRNA and protein levels reported for some studies that compared transcriptomic and proteomic datasets from the same experiment (Ideker *et al*., 2001a; Gygi *et al*., 1999). The correlation issue factors into how -omic integration efforts proceed at an experimental design level, at a database level and eventually would affect choices of candidate protein biomarkers predicted from transcript changes in DNA microarrays and vice versa.

Several studies have reported variable correlations between protein and transcript expression in different studies that range from poor to very positive. For example, earlier studies reported relatively poor correlation between changes in transcript and protein levels in yeast, *Saccharomyces cerevisiae* (Gygi *et al*., 1999; Duan *et al*., 2002; Griffin *et al*., 2002) and also in lung adenocarcinoma (Duan *et al*., 2002), while yet another study in yeast reported a weakly positive correlation (Washburn *et al*., 2003). In the latter study (Washburn *et al*., 2003), a clustering approach took into account transcription and posttranscriptional control of expression for several proteins and arrived at a more positive correlation than previous reports. A positive correlation was noted for a limited set of proteins and transcripts in neuroblastoma cells during apoptosis (Weinreb *et al*., 2003) and a high correlation was noted for mRNAs and the corresponding 39 leukaemia-relevant (AML and ALL) proteins in 113 patients (Kern *et al*., 2003). In a recent study, expression of 82 proteins identified from platelets were closely mirrored in the transcriptome (McRedmond *et al*., 2004). In a study from chromium (VI)-treated rats, results from a 380-antibody array probing lung tissue suggested an uncoupling of transcriptome and proteome data under normal homeostatic conditions, but an establishment of parallel mRNA and protein expression when lung cells responded to acute genotoxic damage (Izzotti *et al*., 2004). An interesting computational approach compared published literature knowledge with DNA microarray data of normal and malignant breast tissue and found correlation only among gene expression changes with a >10-fold difference (Hu *et al*., 2003). A recent high impact study with the NCI-60 (60 human cancer cell lines) and controls demonstrated a high correlation of cell-structure-related proteins between mRNA and protein levels across the NCI-60 cell line set, whereas non-cell-structure-related proteins were poorly correlated. The latter two studies suggest that highly expressed, abundant structural proteins appear to be most highly correlated at the transcript and protein levels. One other important study for consideration reports that proteomic mapping of mitochondria in the mouse produced a list of 591 mitochondrial proteins, including 163 proteins not previously associated with this organelle (Mootha *et al*., 2003). These researchers found protein expression data were largely concordant with large-scale surveys of RNA abundance and both measures indicated tissue-specific (brain, heart, kidney and liver) differences in organelle composition. These researchers concluded that the combined proteomic and transcript analysis identified specific genes of biological interest, such as candidates for mtDNA repair enzymes, offered new insight into the biogenesis and ancestry of mammalian mitochondria, and provided a framework for understanding the organelle's contribution to human disease (Mootha *et al*., 2003).

The preceding studies suggest that some of the variability in correlating protein and transcript levels involves: (i) sampling time after treatments (mRNA changes precede protein expression), (ii) the high information density of DNA arrays and proteomic platform capacity for identified proteins (many transcripts compared to few proteins), (iii) very different bioinformatic methods among studies for comparing correlated expression of protein and transcripts and (iv) differences among these studies in the particular cell or tissue sample, time of specific toxicant exposure and experimental design. It is expected that levels of low concentration, short half-life, cell-specific regulatory proteins, such as transcription factors or phosphorylated signalling intermediates, would be highly variable in their transcript and protein isoform levels at any one point in time and cellular spatial compartment. The difficulty in correlating protein and transcript levels appears to escalate when considering entire transcriptomes and proteomes as encountered in reviewing

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published studies. Since each -omic discipline has developed with distinct technical platforms and bioinformatic approaches, the challenge of integration should not be unexpected. However, the complementarity and sizeable insight gained by performing proteomic and transcriptomic analysis as illustrated by gene expression studies of mouse mitochondria (Mootha *et al*., 2003) seem highly justified by new knowledge gained. Further, it is essential to eventually acquire transcriptomics, proteomics and metabolomics data to construct a systems biology framework for toxicology and pathology. The variable nature of each experiment emphasizes the need to capture all pertinent experimental details in expression databases designed to accommodate and inter-relate proteomics, transcriptomic and metabolomic data.

9 INTEGRATION OF DATA

A key objective in toxicogenomics is to integrate data from different studies and analytical platforms to produce a richer and biologically more refined understanding of the toxicological response of a cell, organ or organism. For example, one would like to describe the interplay between protein function and gene expression, or between the activity of certain metabolizing enzymes and the excretion into serum or urine of populations of small metabolites. The integration of data from different domains such as proteomics and transcriptomics (Ruepp *et al*., 2002; Hogstrand *et al*., 2002; Juan *et al*., 2002), or transcriptomics and metabonomics (Coen *et al*., 2004) has been reported. In these experiments, tissue samples using different technologies derived from the same individual animals or from comparably treated animals were analysed in parallel using different technologies. However, the data from different studies were integrated only after a short list of differentially responsive transcripts or protein spots had been derived.

The experience gained from integrating global proteomics or metabonomics data, such as spot intensities from 2D gels or metabonomics fingerprint data from NMR, tells us that cluster or principle component analysis may be performed to derive global signatures of molecular expression in much the same way as in transcriptomics analyses. If biological samples segregate into unique clusters that show similar expression characteristics, additional efforts can be undertaken to discern the novel proteins or metabolites that are expressed in these samples. Further steps can also be taken to evaluate these proteins or metabolites as potential biomarkers and as a means to determine the underlying toxicological response.

Although software is plentiful for managing expression profiling data at the laboratory level, there is a compelling need for public databases that combine profile data with associated biological, chemical and toxicological end points (Mattes *et al*., 2004). Comparisons of gene, protein and metabolite data within public databases will be valuable for promoting a global understanding of how biological systems function and respond to environmental stressors (Lindon *et al*., 2003; Amin *et al*., 2002). As these repositories are developed, experiments will be deposited from disparate sources, using different experimental designs, yet targeting the same toxicity end point or a similar class of toxicant. In these cases, it will be important that the databases integrate data from related studies before performing data mining. To maximize the value of deposited datasets, the repositories must also be able to integrate data from different technological domains (see Appendix A). Members of regulatory bodies are working with scientists from industry, academic and government laboratories participating in the International Life Sciences Institute (ILSI) Genomics Committee and Clinical Data Interchange Standards Consortium/Standards for Exchange of Nonclinical Data (CDISC/SEND) consortia to develop standards for the exchange, analysis and interpretation of transcriptomics data.

A proposal has been made to extend toxicogenomics and combine it with computational approaches such as physiologically based pharmacokinetic (PBPK) and pharmacodynamic (PD) modelling (Waters *et al*., 2003b). PBPK modelling can be used to derive quantitative estimates of the dose of the test agent or its metabolites that are present in the target tissue at any time after treatment, thereby allowing molecular expression profiles to be anchored to internal dose, as well as to the time of exposure and to the toxicant-induced phenotype. Relationships among gene, protein and metabolite expression can then be described both as a function of the applied dose of an agent, and the ensuing kinetic and dynamic dose–response behaviours that occur in various tissue compartments. Such models also must take into account the fact that the transcriptome, proteome and metabolome are themselves dynamic systems, and are therefore subject to significant environmental influences, such as time of day and diet (Kita *et al*., 2002).

Despite the numerous successes of toxicogenomics in the context of toxicology, a poorly addressed, but confounding issue pertinent to drug safety and human risk assessment is the impact of the individual genetic background on the response of the individual animal or human patient. The PharmGKB pharmacogenetics knowledgebase (Klein and Altman, 2004) catalogues the relationship between different human genetic backgrounds and susceptibility to drug therapy. In addition, the National Institute of Environmental Health Sciences (NIEHS) Environmental Genome Project (EGP) (Olden and Guthrie, 2001) is identifying SNPs in genes that are important in environmental disease, detoxification and repair. Linking toxicogenomics knowledgebases with those containing information about SNPs and human susceptibility will gradually lead to a more complete

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picture of the relevance of the responses and genotypes of surrogate animal species to human risk assessment.

10 CHALLENGES AND TECHNICAL CONSIDERATIONS

Predicting potential human health risks from chemical stressors raises three general challenges. These are: the diverse properties of thousands of chemicals and other stressors that are present in the environment; the time and dose parameters that define the relationship between exposure to a chemical and disease; and the genetic diversity of human populations and of organisms used as surrogates to determine the adverse effects of a toxicant. **Figure 1** illustrates the effect of genetic susceptibility on the continuum from toxic exposure to disease outcome. Knowledge of this continuum, and the role that genetics has in it, can help us to understand environmentally induced diseases, assess risk and make public health decisions. Associated with these challenges are others of a more technical nature; these pertain specifically to toxicogenomics studies and are described below.

Although genome-wide alterations in mRNA, protein or metabolite levels in tissue extracts clearly are useful in identifying 'signature' gene changes, verifying that one or more gene products are involved in a toxic process depends on knowing the specific cell types in which the target-gene transcripts and products are located. Experimental methods, such as Northern or Western blotting, or real-time polymerase chain reaction (PCR), are typically used to verify the expression profile of a gene or to selectively analyse its expression as a function of toxicant dose or time of exposure. *In situ* hybridization, immunohistochemistry and other techniques can be used to identify the specific cell types that express the gene(s).

The ability to focus molecular expression analysis on only a limited number of cell types depends upon cell separation methods that minimize the opportunity for other cell types to contribute to gene expression *in situ*. Even the most carefully gathered biological samples contain many cell types, especially if the sample is from inflamed or necrotic tissue. More homogeneous samples are provided by laser capture microdissection (LCM), a method that isolates individual cells or sections of tissue from a fixed sample (Kabanov, 2006; Karsten *et al*., 2002; Bonner *et al*., 1997; Emmert-Buck *et al*., 1996; Jain, 2002; Wittliff and Erlander, 2002). The use of LCM minimizes contributions by nontarget cell populations in comparisons of diseased and normal tissues, but also introduces handling and preparation steps that can affect detection accuracy.

Simultaneously with new technology that selectively samples cell populations must come the ability to reliably detect signals from increasingly smaller samples. For example, it will frequently be necessary to amplify mRNA from the same biological sample that was used for transcriptomics analysis. The need to detect weak signals or small, but biologically important, changes in expression levels remains, as toxicologists explore the initial steps in biological signalling cascades and compensatory processes. At present, complimentary DNA (cDNA) microarray hybridization can detect strong signals within a mixed cell population in samples that are diluted by up to 20-fold (Hamadeh *et al*., 2002b). Thus, this technology is likely able to detect a strong signal from a population comprising 5–10% of the total tissue, but might miss more subtle changes associated with signalling or other initial responses to a stressor. With LCM a relatively pure cell population could be sampled, thus the technology would be expected to detect much more subtle changes, for instance responses seen only in a subpopulation, or asynchronous responses occurring in 10% of the cells at the time of sampling. The ultimate goal would be the ability to quantify genomic changes occurring in a single cell.

Although mRNA analysis is a powerful tool for recognizing toxicant induced effects, analysis of protein structure and modification and, more importantly, of global protein expression provides distinct advantages for understanding the functional state of the cell or tissue. Promising new methods are emerging, including the capacity to profile proteins with antibody arrays (Huang, 2001) and surface-enhanced laser desorption/ionization MS (SELDI; see Appendix A) (Merchant and Weinberger, 2000; Liotta and Petricoin, 2000). Alterations in patterns of mRNA and protein expression in accessible tissues such as serum (Petricoin *et al*., 2004) may offer new insights into the function of genes in the context of toxicity and guide the search for protein biomarkers of toxicant exposure or predictive toxicity.

Whole genome queries by DNA array platforms are starting to address the complexity of gene expression in mammalian systems by incorporating experiments that include RNA splicing, inhibition and editing and transcriptional silencing and modulation (Herbert, 2004). The perspective for quantifying and cataloguing the human genome has changed markedly since the first maps were published (Venter *et al*., 2001; McPherson *et al*., 2001). Estimates have been placed for those portions of the human genome derived from alternative splicing of multiexon genes at 41–60%, for retrotransposition at 45%, for antisense transcription at 10–20% of genes, and for non-protein-coding RNA at approximately 7% of full-length cDNAs (Herbert, 2004).

Complete transcriptomic analysis with existing DNA array technology, although not routine, is becoming more practical with the use of tiled oligonucleotide microarrays (Mandal *et al*., 2005). However, generation of tissue-specific, normalized and subtracted cDNA libraries has the potential to characterize the expression of rare transcriptional units not represented on available oligonucleotide DNA microarray gene chips. A

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detailed study that compared expressed sequence tag (EST) sequences of a subtracted library generated from mouse retina to those MG-430 consensus sequences was undertaken, using UniGene build 124 as the common reference. A set of 1111 nonredundant transcript regions, not represented on the commercial array, was identified from murine cDNA clone collections and subtracted cDNA libraries selected for expression of rare transcriptal units, including seven transcripts and splice variants with retina-specific expression (Shearstone *et al*., 2005).

The challenge can also be exemplified by the p53 gene family. The p53 tumour suppressor protein generally fits a linear expression pattern from gene, to transcript, to single protein; however, its two homologous family members, p63 and p73, have the peculiarity of sharing many splice variants that give rise to at least six major transcripts and subsequent proteins including TAp63/p73 α, β and γ isoforms and ΔNTAp63/p73 α, β and γ isoforms (Benard *et al*., 2003; Courtois *et al*., 2004; Demonacos and La Thangue, 2003). Even p53 splice variants and proteolytic cleavage products have been frequently observed in many different malignancies as well as developmental processes (Courtois *et al*., 2004). Despite the complex expression pattern of some genes and difficulties in distinguishing their variants with current microarray technologies, the current DNA microarray platforms for transcriptome assessments now provide an ever-improving coverage of gene expression.

Proteomic platforms do not yet deliver the same quantity or type of information as the transcriptome. Indeed, for the human genome, the relationship between gene number and proteome size is far from simple when taking into account splice variants and pseudogenes (Harrison *et al*., 2003). One estimate has the number of protein-coding transcripts at about 100 000, but that number might be higher (Harrison *et al*., 2003). Also, accounting for posttranslational modifications of each protein would create many more structurally unique variants and hints at the enormous complexity of the human protein expression. Despite the vastness of the proteome, proteomics is creating revolutionary insights into protein expression of unicellular and higher-order species that will reshape pathology, toxicology and many other disciplines.

First, the composition of subcellular structures such as the nucleolus (Andersen *et al*., 2002), mitochondria (Mootha *et al*., 2003), Golgi (Wu *et al*., 2004) and others are being comprehensively catalogued by new methods in proteomic analysis at a rapid rate and in a manner that will eventually apply to protein dysfunction underlying disease. Second, proteomes of important biological fluids such as serum and plasma, are planned for complete mapping, which will be crucial for biomarker development (Anderson and Anderson, 2002). Third, development of global analysis methods for posttranslational modifications, such as phosphorylation, glycosylation, ubiquitination and others, will help elucidate the active forms of protein during homeostasis and disease. Fourth, identification of specific xenobiotic–protein adducts by proteomics will provide insights, not only into acute cellular injury and necrosis (Liu and Kaplowitz, 2002), but also into immune system activation (Ju and Pohl, 2001) and idiosyncratic responses to therapeutics and environmental chemicals (Ju and Uetrecht, 2002). Fifth, rapid, parallel and nanovolume protein analysis of biofluids will be entering research and clinical realms ranging from therapeutic target discovery to diagnosis and to epidemiological investigations. These contributions of proteomics to toxicology and pathology represent a technological exploitation of the complex properties of proteins, such as posttranslational modifications (i.e. signal transduction) spatial location and structure (i.e. subcellular organelles), and functions (enzyme activities).

One of the great challenges in metabolomics, whether one is using NMR spectroscopy, MS, electrochemical detection or other methods, is poor annotation (metabolite identification) of the observed signals. In a typical NMR study, fewer than 30 metabolites of the estimated few hundred compounds within a spectrum will be unambiguously identified. This limits the metabolic information that can be extracted from the spectral data and restricts the mechanistic insight that could potentially be gained of the biological system under study. Furthermore, peak annotation is a prerequisite for instrument-independent metabolic data, which is both the desired format for the construction of metabolic databases and facilitates the comparison of datasets regardless of the instrument on which they were measured. This problem must be addressed urgently if metabolomics is to fulfill its potential.

11 BIOINFORMATICS CHALLENGES

Full realization of the potential of molecular profiling in toxicogenomics requires a very substantial investment in bioinformatics in order to extract biological sense from the myriad of interrelated numerical molecular identifiers and their associated annotations. Advances in bioinformatics and mathematical modelling provide powerful approaches for identifying the patterns of biological response that are imbedded in genomic datasets (**Figure 4**). However, facile interpretation of global molecular datasets derived from -omics technologies is currently constrained by the 'bioinformatics bottleneck'. Bioinformatics and -omics technologies themselves must improve in gene, protein and metabolite identification and annotation to open the field of toxicogenomics to high-throughput applications in drug development and toxicant evaluation. Several useful resources address the annotation problem by linking identifiers used in genomic databases at the National Center for Biotechnology Information (NCBI), European

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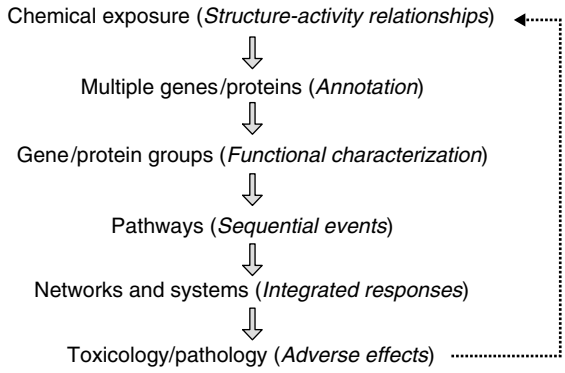

Figure 4 Bioinformatics challenges and biological complexity. The focus of bioinformatics in interpreting molecular expression data depends on the level of biological complexity—here shown progressing from genes/proteins/metabolites to networks and systems. For toxicology/pathology the focus is on phenotypic anchors—observed biological responses that can be related to the chemical structure of the test agent or exposure.

Molecular Biology Laboratory (EMBL) and DNA Data Bank of Japan (DDBJ) to other annotation resources. Critical to resolving annotation inconsistencies is the knowledge of the sequence of the actual nucleotide or protein that is used to query the genome.

The use of advanced bioinformatics tools to extract information from microarray results (Quackenbush, 2001) is valuable only if the data employed by these tools have a high degree of internal specificity and accuracy (Mattes *et al*., 2004). Additionally, the interpretation of molecular expression profiles must emphasize biological coherence as well as statistical validity when deriving knowledge from toxicogenomics experiments. This means that once a set of genes with altered expression is identified, their biological functions must be ascertained. Mechanistic interpretation of transcript changes may be impeded by the nonstandard or imprecise annotation of a sequence element (i.e. gene). Without appropriate synonyms for gene names, the effectiveness of a literature search may be limited. Differences in annotation within and among different microarray platforms may hamper the comparison of results. Such inconsistency frequently arises from annotation resources using different lexicons, or from annotation information being compiled at different times.

Additional bioinformatics and interpretive challenges arise at many levels of biological organization (**Figure 5**). Our current focus and level of understanding of the global molecular landscape encompasses only the lower levels of complexity (genes/proteins, gene/protein groups, functional pathways). The resolution of this knowledge might be termed linear toxicoinformatics, that is, the description of environmental stimuli and responses over dose and time following a toxicological stress. Toxicologists and risk assessors typically define a sequence of key events and linear modes-of-action for environmental chemicals and drugs (Farland, 1992; 1996; Larsen *et al*., 2000). By contrast, the networks and systems level of biological organization may demonstrate highly nonlinear cellular expression state changes in response to environmental stimuli (Hughes *et al*., 2000; Begley *et al*., 2002). Thus, the statistical and bioinformatics-based separation of the complex adaptive, pharmacological and toxicological responses of drugs, chemicals and even dietary constituents will probably very much be a matter of degree. This reflects the kinetic and dynamic responses of specific tissues to toxicants as directed by the genome, the genetic heritage of the individual, and that individual's current and prior exposures.

12 SYSTEMS TOXICOLOGY

Ideker *et al*. (2001a) used the phrase 'systems biology' to describe the integrated study of biological systems at the molecular level—involving perturbation of systems, monitoring molecular expression, integrating response data and modelling the systems molecular structure and network function. Here we similarly use the phrase 'systems toxicology' to describe the toxicogenomic evaluation of biological systems, involving perturbation by toxicants and stressors, monitoring molecular expression and conventional toxicological parameters, and iteratively integrating response data to model the toxicological system (Waters *et al*., 2003a).

A number of approaches are being developed to model network behaviour, with different assumptions, data requirements and goals. However, it is not likely that toxicogenomics and systems toxicology models will be assembled exclusively from knowledge of cellular components, without equivalent knowledge of the response of these components to toxicants (Begley *et al*., 2002). Thus the 'stress testing' of the structural biology of the system and the capture of that data in the context of the functioning organism adapting, surviving or succumbing to the stress will be required.

Development of a knowledgebase to accurately reflect network-level molecular expression and to facilitate a systems-level biological interpretation requires a new paradigm of data management, data integration and computational modelling. A knowledgebase that fully embraces systems toxicology (Waters *et al*., 2003a; 2008) will use precise sequence data to define macromolecules, interaction data based experimentally on colocalization, coexpression and analyses of protein–protein interactions; and functional and phenotypic data based on gene knockouts, knockins and RNA-interference studies, in addition to studies of responses to chemical, physical and biological stressors. These data will allow specific molecules to be accurately

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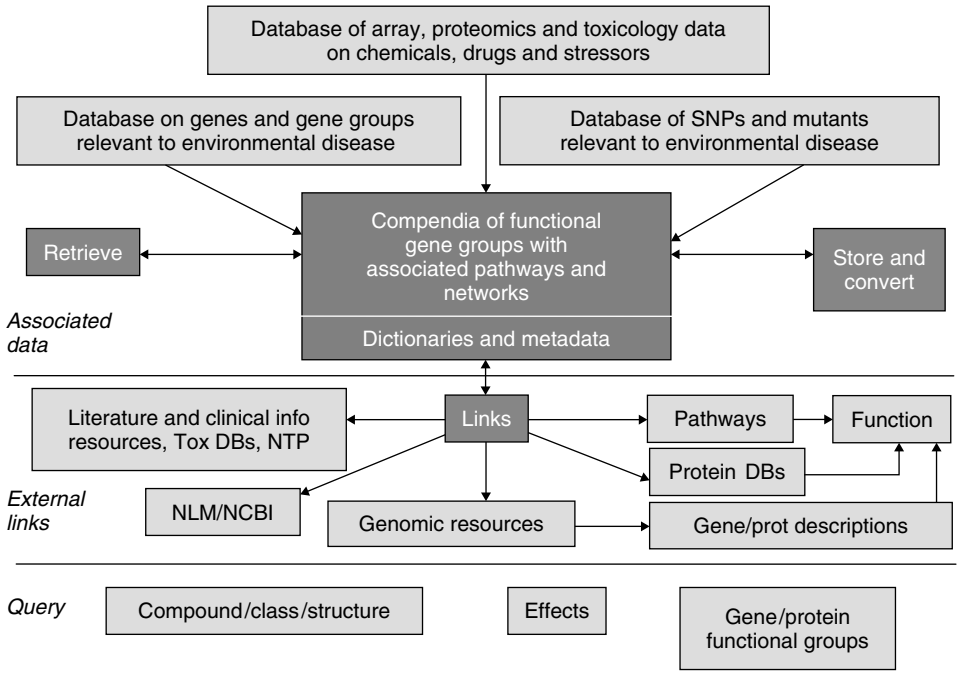

Figure 5 Conceptual framework for the development of the chemical effects in biological systems (CEBS) knowledgebase. CEBS knowledgebase is a cross-species reference toxicogenomics information system on chemicals/stressors and their effects. The figure has three sections—the upper section, indicating data associated in CEBS, the centre section, indicating external links from CEBS, and the lower section, indicating example query types that CEBS will support. The boxes in the upper section include primary data, important genetic loci and genetic markers such as SNPs. In the central section, links to databases and unstructured data are shown. SNPs, Single Nucleotide Polymorphisms; Tox, toxicology; DBs, databases; NTP, National Toxicology Program; NTP, National Library of Medicine; NCBI, National Center for Biotechnology Information; Prot, protein.

related to biological phenomena that reflect the normal as well as the stressed cell, tissue, organ and organism. In the best of circumstances, a systems toxicology approach will build a toxicogenomic understanding from global molecular expression changes that are informed by PBPK/PD modelling and biologically based dose–response (BBDR) modelling. The challenge in constructing a robust systems toxicology knowledgebase is formidable.

13 THE FUTURE OF TOXICOGENOMICS

New toxicogenomics methods have the power and potential to revolutionize toxicology. Technological innovations that are already in use permit RNA profiling of formalin-fixed tissues (Lewis *et al*., 2001), potentially making archived tissues from generations of toxicological studies accessible to gene expression analysis. Methods to array hundreds of toxicologically relevant protein antibodies, and to profile hundreds of small molecules in high-throughput mode using gas chromatography (GC)/liquid chromatography (LC)/MS are in development.

Toxicoproteomics research is anticipated to lead to the identification, measurement and evaluation of proteins and other biomarkers that are more accurate, sensitive and specific than those available now, and which might be targeted to particular human genetic subpopulations. Metabonomics research will help to identify small endogenous molecules as important in a sequence of key metabolic events; such 'metabolite fingerprints' might then help to diagnose and define the ways in which specific chemicals, environmental exposures or stressors cause disease. This, coupled with the ability to detect damage to particular organs by observing alterations in serum and urine components, is expected to lead to the more sensitive detection of exposure or risk factors (Wetmore and Merrick, 2004). Additional considerations in assessing the toxicogenomic response to environmental exposures are the individual genotype, lifestyle, age and exposure history (Kaput and Rodriguez, 2004). Toxicogenomics will help to ascertain the degree to which these factors influence the balance between healthy and disease states.

Toxicogenomics will increase the relevance of toxicology through the global observation of genomic responses with therapeutically and environmentally realistic dose regimens. It will help to delineate

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the mode-of-action of various classes of agents and the unique genetic attributes of certain species and population subgroups that render them susceptible to toxicants (Farland, 1996; Waters *et al*., 2003b). Studies on strains within a species that are sensitive or resistant to the chemical induction of specific disease phenotypes will be particularly valuable. Extending this thinking to the phylogenetic analysis of both core conserved biological processes (Stuart and Berry, 2003) and to the toxicological responses seen in different species will provide additional comparative insight on genetic susceptibility and on probable disease outcomes.

The combined application of the -omics technologies will improve our overall understanding of mechanisms of toxicity and disease aetiology as integrated toxicogenomics databases are developed more fully (Waters *et al*., 2003b). Data on gene/protein/metabolite changes collected in the context of dose, time, target tissue and phenotypic severity across species from yeast to nematode to man will provide the comparative information needed to assess the genetic and molecular basis of gene–environment interactions. Toxicology will emerge as an information science that will facilitate scientific discovery across biological species, chemical classes and disease outcomes (Tennant, 2002). Although there are large challenges in developing public toxicogenomic data repositories, the nucleotide sequence databases—GenBank, EMBL and DDBJ—provide an excellent example of the benefit to the larger scientific and medical community of sharing data.

Concomitant with development of toxicogenomics databases must be the evolution of bioinformatics methods and data-mining tools, and individuals trained to apply them (Quackenbush, 2001). We believe that a predictive systems toxicology will gradually evolve, aided by knowledge that is systematically generated (Zweiger, 1999) through literature mining (Chaussabel and Sher, 2002; Sluka, 2002), comparative analysis and iterative biological modelling of molecular expression datasets over time. Given the vast numbers and diversity of drugs, chemicals and environmental agents, and the diversity of species in which they act, we believe, however, that it is only through the development of a comprehensive and public knowledgebase that toxicology and environmental health can rapidly advance. The ultimate goal of the National Center for Toxicogenomics (NCT) is to create the Chemical Effects in Biological Systems (CEBS) knowledgebase (Waters *et al*., 2003a; Waters *et al*., 2008), a public resource that will enable health scientists and practitioners to understand and mitigate or prevent adverse environmental exposures and related diseases.

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APPENDIX A: DESCRIPTIONS OF SELECTED -OMICS TECHNOLOGIES

The terms transcriptomics, proteomics and metabonomics or metabolomics refer to highly parallel analytical technologies wherein simultaneous measurements are made of expressed genes, proteins or metabolites, respectively. These technologies are used to ascertain the function of the genome. Toxicogenomics makes use of all of these functional genomics technologies in the study of toxicology. The terms toxicoproteomics and toxicometabolomics are sometimes used in a technology-centric sense to discuss the response of the proteome or metabolome to toxicants. Toxicoproteomics, as part of the larger field of toxicogenomics, seeks to identify critical proteins and pathways in biological systems that are affected by and respond to adverse chemical and environmental exposures using global protein expression technologies.

A.1 Transcriptomics—DNA Microarray Hybridization and Analysis

Early gene-expression profiling experiments that were carried out for toxicogenomics studies employed cDNA microarrays (Nuwaysir *et al*., 1999). Although this cDNA technology has been supplanted by synthetic short and long oligonucleotide microarrays, the technological concepts underlying the two approaches are largely analogous: cDNAs are derived from sequence-verified clones representing the 3′ ends of the genes, which are either spotted onto glass slides using a robotic arrayer or synthesized *in situ*. Each RNA sample is labelled with dye-conjugated 2′-deoxyuridine 5′-triphosphate (dUTP) by reverse transcription from an oligo deoxythymine (dT) primer. The fluorescently labelled cDNAs are then hybridized to the microarray and the microarray is scanned using laser excitation of the fluorophores (Hamadeh *et al*., 2002e). Raw pixel intensity images derived from the scanner are analysed to locate targets

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on the array, measure local background for each target, and subtract it from the target intensity value. Prime discovery features of cDNA and oligonucleotide microarrays are the sequences representing ESTs, hypothetical proteins, homologues, orthologues or genes of unknown function. ESTs or similar features are not currently represented on antibody arrays. When examining differential transcript profiles, however, many researchers focus more upon annotated genes of known or homologous function that currently account for less than half the genes predicted for the human genome. Changes in expression of ESTs might be tracked in transcriptomic studies, but they are often not followed up in many gene expression experiments (except when used as part of discriminating signatures to classify disease of toxicity).

A.2 Proteomics

Global measurement of proteins and their many attributes in tissues and biofluids defines the field of proteomics. An established proteomics strategy (Patterson and Aebersold, 2003) uses global protein stratification systems, such as polyacrylamide gel electrophoresis (PAGE), followed by protein identification by MS. 2D PAGE separation, by charge and by mass, can resolve thousands of proteins to near homogeneity. This separation is a necessary prerequisite to enzymatic digestion and MS identification, which requires unique peptide fingerprint masses or amino-acid sequence tags. Where proteins are separated by LC instead of PAGE, a new and promising platform involving multidimensional LC can be used to fractionate and reduce the complexity of the protein mixture before peptide sequencing by MS or tandem MS (LC/MS/MS). This approach is being augmented by SELDI-TOF MS (surface-enhanced laser desorption/ionization time-of-flight mass spectrometry), a method that results in the isolation of tens- to hundreds-of-thousands of low molecular weight fragments representing a proteome.

The focus of proteomics can range from global protein analysis (Davis, 2004; Zhu *et al*., 2003) via approaches such as 'shotgun proteomics' (Wolters *et al*., 2001; MacCoss *et al*., 2002) where researchers strive for the maximum number of protein identifications, to a more discrete level of protein analysis often termed 'targeted proteomics' (Dongre *et al*., 2001). In targeted proteomics, protein groupings or subproteomes (Cordwell *et al*., 2000) may be organelles (Dreger, 2003), portions of structures like nuclear membrane proteins (Schirmer *et al*., 2003), signalling pathways (Ping, 2003) and protein complexes or protein families (Lee *et al*., 2003). Inherent in this range of different biochemical perspectives is the assumption that protein structure and spatial location within cell(s) or tissue remain critical features in proteomic analysis. Other characteristics of proteins, such as function, protein–protein interactions, 3D structure or specific posttranslational modifications, have also helped define various subdisciplines of proteomics.

A.3 Metabolomics and Metabonomics

Quantitative analytical methods have been developed to identify metabolites in pathways or classes of compounds. This collective directed approach has been called metabolite profiling or metabolomics. Semiquantitative, NMR-based metabolic fingerprinting has also been applied to high abundance metabolites and has been termed 'metabonomics' (Nicholson *et al*., 2002). Peaks detected in NMR spectra carry information regarding the structure of the metabolites, whereas peaks detected by MS have associated molecular weights. In addition, specific MS methods can be established to fragment the parent molecule, allowing metabolites to be identified through investigation of fragmentation patterns.

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Environmental and Endogenous Factors Determining the Expression of Toxicity

Karen E. Stine

CONTENTS

1 INTRODUCTION

One of the basic principles of toxicology is the concept of the molecular site of action, or molecular lesion. This interaction of a toxicant with a target, typically a cellular macromolecule or macromolecules, leads to cellular dysfunction with consequences that are often expressed at the physiological or organismal level. The focus of this chapter, however, is not on the nature of the molecular lesion itself, but rather on the environmental and endogenous factors that influence its expression. Relevant factors that will be discussed are illustrated in **Figure 1**, and include exposure to environmental stressors (including other chemical exposures), as well as intrinsic characteristics of the organism (such as species, sex, age, physiologic parameters or genetic variation), which may either influence delivery and binding of the toxicant to the target molecule or alter the ultimate physiological consequences of that binding.

2 ENVIRONMENTAL FACTORS

2.1 Interactions between Chemical Toxicants

There are a number of environmental factors that may have a significant influence on expression of toxicity relating to a given chemical exposure. Perhaps chief among these, however, would be coexposures to other chemicals in the environment. Interaction between chemical toxicants can be complex, and may be mediated through a number of different mechanisms, including impacts on toxicokinetics, metabolism or binding at the target site. Identifying and assessing these interactions between biologically active compounds, however complex, is critical to the accurate prediction of both human health and environmental risks (Robinson and MacDonell, 2004; McCarty and Borgert, 2006; Pomati *et al.*, 2008).

2.1.1 Impact of Coexposures on Toxicokinetic Parameters

There are several mechanisms by which one toxicant has been reported to impact the absorption and distribution of another in an organism. These would include modification of properties of the epidermis, including changes which may enhance or reduce the ability of other compounds to undergo absorption through the skin. For example, in one study, parathion absorption was significantly altered by coadministration of sodium lauryl sulfate (which enhanced absorption) and stannous chloride (which reduced absorption) (Qiao and Riviere, 1995).

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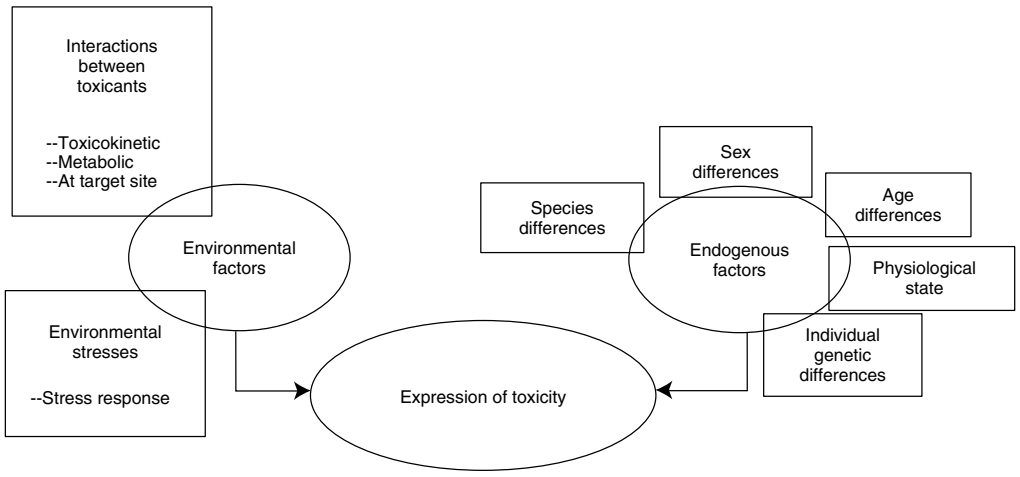

Figure 1 A schematic showing the types of environmental and endogenous factors that may influence expression of toxicity.

Likewise, compounds that affect conditions in the gastrointestinal tract may alter absorption of other compounds. For toxicants absorbed by simple diffusion, an increase in epithelial permeability such as that produced by polyamines, for example (Gao *et al.*, 2008), may enhance absorption of other compounds. On the other hand, competition for carrier-dependent transport (such as occurs between cadmium, calcium, zinc and other ions) can potentially decrease toxicant absorption (Glover and Hogstrand, 2003). Toxicant absorption can also be decreased by direct modulation of the activity of a transporter molecule, as in the case of the effects of acetaldehyde, a metabolite of ethanol, on the PEPT1 drug transporter (Fisher *et al.*, 2008). Of course, gastrointestinal coexposures may certainly include compounds found in foods, making diet an important source of variation in absorption. For example, plant flavonoids, found both in food and in dietary supplements, have been shown to interact with and alter transport of drugs such as vinblastine, doxorubicin and digoxin (Cermak and Wolffram, 2006). These effects are mediated through action on intestinal drug transporters including MRP2 and a P-glycoprotein transporter. The P-glycoproteins (two of which are found in humans) belong to the family of adenosine triphosphate (ATP)-binding cassette (ABC) transporters (Hennessy and Spiers, 2007). The genes coding for these proteins are expressed in many tissues, and play a critical role in blocking uptake of toxicants into those tissues. P-glycoproteins have been shown to be inducible and while the mechanism has yet to be completely elucidated, it appears to involve multiple signalling pathways and may possibly involve nuclear receptors such as pregnane X (PXR) (Callaghan *et al.*, 2008). Unfortunately, however, P-glycoprotein is frequently expressed in cells with a neoplastic phenotype, where its protective effects provide a daunting barrier to the success of chemotherapy (Cordon-Cardo *et al.*, 1990).

Compounds that affect general gastrointestinal motility can also affect absorption of toxicants through altering the time available for absorption to take place (DeSesso and Jacobson, 2001). Similarly, respiratory absorption of toxicants can be affected by a number of factors that affect rate and depth of respiration, including both exercise and chemical exposures (Mautz, 2003).

Once absorbed into the blood stream, toxicants are then delivered to the various body tissues. The rate of this delivery is dependent on a number of factors, including the binding affinity of the toxicant for binding to plasma proteins such as albumin. Competition for binding to albumin and other proteins can lead to displacement of toxicants from binding, which may lead to increased tissue toxicant levels, and, thus, increased potential toxicity. For example, acetylsalicylic acid has been shown to reduce binding of both propranolol (Rezaei *et al.*, 2007) and imipramine (Juarez-Olguin *et al.*, 2002) to human serum albumin, an action which led to a significant increase in the incidence of side effects in patients receiving the imipramine.

Finally, a special factor influencing distribution of toxicants is the blood–brain barrier. The blood–brain barrier is a set of anatomical and physiological modifications that result in a reduced passage of toxicants from the bloodstream into central nervous system tissues. This complex combination of tight junctions between capillary endothelial cells, transporters (such as the P-glycoprotein pump, Cordon-Cardo *et al.*, 1989), and additional layers of glial cell membrane and cytoplasm to cross renders many toxicants incapable of entry into the central nervous system (Zlokovic, 2008). However, chemical compounds that reduce the effectiveness of the barrier may lead to enhanced central nervous system toxicity of other compounds. This would be a concern, for example, with the usage of potential P-glycoprotein inhibitors such as verapamil for cancer chemotherapy (Kars *et al.*, 2008).

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2.1.2 Impact of Coexposures on Metabolism of Toxicants

One of the most well-characterized ways in which multiple toxicants interact is through impact on xenobiotic metabolism. Xenobiotic metabolism has been generally subdivided into two phases: Phase I, involving oxidation, hydrolysis and occasionally reduction; and Phase II, involving conjugation of either the parent compound or a Phase I metabolite with glucuronic acid, glutathione or another compound, which typically confers increased size and hydrophilicity to the substrate. Many of the enzymes involved in these processes are inducible, and the process is thus subject to alteration based on the chemical environment within the cell. For example, one of the major enzyme families involved in Phase I metabolism, the cytochrome P450 family, contains a number of isozymes that can be both induced and inhibited by substrates. This, of course, would affect not only the metabolism of those specific substrates, but also any other toxicants which are substrates for metabolism by that isozyme.

Specific examples of P450 inducers include polycyclic aromatic hydrocarbons (PAHs) such as 3-methylcholanthrene, which are inducers of the cytochrome P450 CYP1A1 and CYP1A2 isozymes. This induction is mediated through PAH binding to and activation of a cytosolic receptor known as the aryl hydrocarbon (Ah) receptor. This receptor, when activated, stimulates gene transcription through its interaction with DNA regulatory sequences such as the dioxin-responsive elements (DRE) or xenobiotic responsive elements (XRE) (Beischlag *et al.*, 2008). Also among the inducible members of the cytochrome P450 family is the most abundant human cytochrome, CYP3A4. This isozyme can be induced by rifampin and phenytoin, through a mechanism involving the PXR nuclear receptor (Zhou, 2008). Inducers of other P450 isozymes include barbiturates, pregnenolone-16α-carbonitrile (PCN), clofibrate and isoniazid (Runge *et al.*, 2000). Again, induction of a P450 isozyme through exposure to an inducing agent is significant not only because of effects on the metabolism of the inducer, but also because of effects on the metabolism of any other compounds metabolized by that isozyme. Interestingly, many of the same compounds that induce P450 isozymes are also inducers of P-glycoprotein (Callaghan *et al.*, 2008).

Inhibition of cytochrome P450 isozymes can also play a significant role in impacting toxicity. For example, members of a class of compounds known as mechanism-based inactivators are activated by P450, only to then bind to and irreversibly inhibit the enzyme itself. These compounds tend to be specific for particular P450 isozymes, and include compounds with terminal acetylenes that alkylate the P450 haem group, alkyl and aromatic amines, furans, and sulphur-containing compounds (Masubuchi and Horie, 2007) For example, 17α-ethinylestradiol and mifepristone (also known as RU-486), as well as erythromycin and the calcium-channel blocker verapamil are all inhibitors of CYP3A4 (Guengerich, 1990; He *et al.*, 1999; Ma *et al.*, 2000), leading to significant potential impact on other drugs being administered to a patient simultaneously.

One additional group of compounds that can impact expression of toxicity are compounds that lead to depletion of glutathione. Glutathione, a small peptide consisting of a glycine, a cysteine and a glutamic acid residue, undergoes conjugation with hydrophobic, electrophilic xenobiotics through the action of a family of enzymes known as glutathione S-transferases (GSTs). Because of the reactive nature of the substrates for this reaction, glutathione conjugation is one of the body's major detoxification reactions. Exposure to compounds, such as ethanol, that deplete glutathione, has been shown to potentiate toxicity of other compounds, such as acetaminophen (paracetamol), which depend on conjugation to glutathione for effective detoxification (Zhao and Slattery, 2002). Levels of glutathione also vary significantly with levels of food consumption, making diet yet another factor that can impact toxicity (Qin *et al.*, 2007).

2.1.3 Impact of Coexposures on Physiological Effects of Toxicants

Another level at which one compound may affect the toxicity of another is through interactions mediated at or near the site of the molecular lesion. Sites where these effects may be seen would include cellular targets such as enzymes and receptors. Interactions between chemicals have often been described as additive, synergistic (when the combined effect is greater than that predicted on the basis of either alone) or antagonistic. Another category, potentiation, is used to refer to a chemical that may be inactive at a receptor itself, but which acts to enhance the activity of a second chemical at that site (Eaton and Klaassen, 2001). However, the situation is complicated by the existence of partial agonists, which produce effects upon binding that are nonetheless reduced from those produced by a full agonist, as well as inverse agonists, which actually reverse receptor activity. For example, naloxone, long categorized as an opioid antagonist, has actually proven to be an inverse agonist at the mu receptor (Sadee *et al.*, 2005). Antagonistic effects have also been reported in binding of halogenated aromatic compounds to the Ah receptor, leading to varying responses in CYP1A1 induction (Petrulis and Bunce, 2000).

It should be noted, however, that physiological antagonism between two drugs or toxicants may exist without necessarily implying that the two compounds share a single molecular site of action. Atropine, for example, is an antidote to all organophosphates (OPs) such as sarin exposure: sarin targets acetylcholinesterase, leading to an increase of acetylcholine in the synapse; atropine

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counters that effect through blocking the muscarinic acetylcholine receptor (Cannard, 2006) (see **Toxicology of Chemical Warfare Agents**).

The mechanism of action of some toxicants relates to the ability of the toxicant, or often a metabolite, to initiate oxidative damage through the mechanism of lipid peroxidation. This is particularly true of toxicants such as chlorinated hydrocarbons, which are metabolized to form free radicals, species whose unpaired electrons render them highly reactive. These reactive molecules can, and will, bind to cellular macromolecules such as lipids, initiating degradation of the fatty acid components and resulting in both membrane damage and production of additional reactive molecules that can damage other cellular components. Interaction of free radicals with cellular macromolecules can, however, be reduced or blocked by the presence of other molecules that act as antioxidants. For example, in one study, the antioxidants α-tocopherol and selenium were shown to reduce hepatic damage by malathion in chicks, through inhibition of lipid peroxidation (Sodhi *et al.*, 2008).

2.2 Effects of Environmental Stressors on Toxicity

2.2.1 Basics of the Heat Shock or Stress Response

The response of organisms to environmental stressors has been documented in multiple biological systems (Moseley, 1997). A common feature of this response is the induction of a group of proteins which were first termed heat shock proteins (hsps) due to their initial discovery in cells exposed to hyperthermia (Tissieres *et al.*, 1974). An entire family of these proteins, now also more generally known as stress proteins, has since been identified (Morimoto *et al.*, 1997; Horvath *et al.*, 2008). Ranging in size from approximately 15 to 110 kDa in molecular weight, some of these proteins are constitutive, while others have been found to be induced in response to a variety of cellular stresses, including heavy metals (Bauman *et al.*, 1993), oxidative stress (Drummond and Steinhardt, 1987) and ischaemia (Myrmel *et al.*, 1994). This seems to be an adaptive response, as the presence of stress proteins was shown early on to confer resistance to further stresses, such as additional hyperthermia (Li and Werb, 1982). In fact, the existence of stress proteins in so many diverse species certainly argues strongly for a central role for these proteins in basic cellular processes.

Stress proteins play a variety of roles in the cell. Many stress proteins seem to function as molecular chaperones by regulating protein folding (Hendrick and Hartl, 1993; Roth *et al.*, 2008). This seems to be the major function of many hsps including the hsp60, hsp70 and hsp100 families (Saibil, 2008). Other hsps play a variety of roles, including regulation of the function of receptors such as the glucocorticoid receptor (Pratt *et al.*, 1992), regulation of membrane structure and function (Horvath *et al.*, 2008) and control of apoptosis (Lanneau *et al.*, 2008). Hsp90, for example, is a eukaryotic cytosolic protein that occurs in two isoforms (α and β). In humans, it is primarily the hsp90-α gene that is inducible. One of the best known interactions of hsp90 is with glucocorticoid, progesterone and oestrogen receptors. The protein binds to the hormone-binding domain of these receptors, keeping them partially unfolded in the configuration that binds the hormonal ligand with the highest affinity (Pratt, 1997). The presence of hsp90 on the cell surface may indicate a role in presenting proteins to immune cells. In fact, geldanamycin (a specific inhibitor of hsp90 activity which binds to the N-terminal chaperone site) blocks the CD28 costimulation signal for T lymphocytes (Schnaider *et al.*, 1998). Intriguing evidence also implicates hsp90 in the stabilization of mutant proteins in *Drosophila*, allowing mutations to accumulate in the genome (Tomala and Korona, 2008). A final group, the so-called small hsps with molecular weights ranging from around 15 to 30 kDa, have divergent structures, but with a common α-crystalline domain with several highly conserved segments. Most are strongly inducible, some are also constitutively expressed (Arrigo and Landry, 1994; Shemetov *et al.*, 2008).

The mechanism of how induction of the stress response occurs in eukaryotes is relatively well understood and involves binding of an activated heat shock factor (HSF) protein to a responsive heat shock element (HSE) in the genome, which initiates the processes of transcription and translation for these proteins (Shamovsky and Nudler, 2008). There is some evidence, also, that protein kinase A (PKA) and protein kinase C (PKC) may play a role in regulation of promoter activity, as the binding of HSF to DNA in a glioblastoma cell line was suppressed by PKC and PKA inhibitors and the accumulation of hsp72 was induced by PKC and PKA activators (Ohnishi *et al.*, 1998).

The stress response can be triggered by a number of factors besides the classic exposure to elevated temperatures. Numerous chemicals have been reported to induce various hsps, including heavy metals (Ivanina *et al.*, 2008), pesticides (Sharma *et al.*, 2008) and other environmental toxicants (Ait-Aissa *et al.*, 2000).

2.2.2 The Relevance of the Stress Response to Toxicology

Obviously, induction of stress proteins in response to one chemical exposure has the potential to alter the impact of a second exposure to the same or different chemical. For example, exposure to oxalate has been shown not only to lead to elevated levels of hsp70 in LLC-PK1 (renal epithelial) cells, but also to protect against subsequent exposure to oxalate (Koul *et al.*, 2008). And

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not only can prior exposure to heat protect against later chemical exposure (Venkatakrishnan *et al.*, 2006), but chemical exposure (in this case, to sodium azide) can also induce thermotolerance (Massie *et al.*, 2003). However, some toxicants may actually reduce stress protein expression (either specifically or through general inhibition of protein synthesis) and might thus contribute to an increase in vulnerability of the cells (Skandrani *et al.*, 2006).

There are other reasons, as well, to be interested in the impact of stress protein induction on cell survival. Stress proteins are not only certain to play a role in cell death, but also possibly in oncogenic transformation and expression of neoplastic phenotypes (Romanucci *et al.*, 2008). For example, hsp70 has been reported to confer some degree of resistance to apoptosis in lymphoid and myeloid cell lines (Lasunskaia *et al.*, 1997) and expression of hsp27 in tumorigenic mouse fibroblasts reduced the cytotoxicity of TNF-α to these cells (Mehlen *et al.*, 1995). In addition, hsp90 has been shown to form complexes with members of the cellular src family of tyrosine kinases, and may be a participant in src-mediated oncogenic transformation (Whitesell *et al.*, 1994). A member of the hsp90 family has also been shown to be constitutively expressed in drug-resistant cancer cells, and may be involved in stabilization of the function of the P-glycoprotein molecule involved in multidrug resistance (Bertram *et al.*, 1996). The relationship between expression of small hsps in cancer cells and prognosis has been studied, but is not entirely clear. Hsp27 has been detected in several, but not all types of brain tumours, with expression roughly correlating with degree of malignancy (Khalid *et al.*, 1995), and overexpression of hsp27 in breast cancers has been associated with increased risk of relapse, although it may, in fact, render those cells more susceptible to being targeted by some immune system cells (Mahvi *et al.*, 1993). Stress proteins may also play a role in neurodegenerative diseases (Morimoto, 2008).

There are, of course, other cytoprotective molecules besides the stress proteins which may also be induced and which may then protect against subsequent toxic exposures. These would include the P-glycoprotein pump (the ABC transporter mentioned earlier), which, again, is often found overexpressed in neoplastic cells. The mechanism for this overexpression is not clear, but may well result at least in part from response to cell stress (Callaghan *et al.*, 2008). Also in this grouping would be the metallothioneins, low molecular weight metal-binding proteins rich in cysteine residues. Ubiquitous in all kingdoms of life, in mammals these inducible proteins are found primarily in liver and kidney, where they seem to serve primarily as protection against heavy metal toxicity (Coyle *et al.*, 2002). Metallothioneins also appear to play a role in apoptosis (Dutsch-Wicherek *et al.*, 2008).

2.3 New Techniques for Sorting Out Complex Mechanistic Questions

Whether one is discussing sorting out complex effects of multiple toxicants, or a complex physiological reaction to environmental stressors, the number of variables involved in both cause and effect can be daunting. A few new molecular techniques, however, have been developed that show promise in helping sort out these complex relationships. One of these is the microarray technique, which can be used to compare the effects of chemical toxicants individually and in mixtures as well as to identify the complex responses of inducible genes to environmental stressors. Briefly, a microarray consists of a glass chip covered by a chemical matrix to which pieces of DNA (generally a short segment of a gene of interest) are attached. A typical microarray may contain tens of thousands of these DNA segments. Typically, fluorescently labelled mRNA is prepared from the tissues of interest, and allowed to react with the chip, where binding of the labelled mRNA to complementary DNA on the chip can be quantified by measuring fluorescence at each location. A virtual snapshot of gene activity at that point in time is thus produced (Jaluria *et al.*, 2007).

This technique and others have led to the development of the new field of toxicogenomics, a field which focuses on the response of the genome to toxicants and stressors (Thomas *et al.*, 2002; Gomase and Tagore, 2008). In one example of a toxicological application of this technology, Sen, *et al.* employed DNA microarrays to examine the effects of cigarette smoke (a complex mixture) on mouse fibroblast cells (Sen *et al.*, 2007), demonstrating upregulation of genes coding for metallothioneins, heat shock proteins and other stress and growth-related proteins. They were also able to demonstrate upregulation of a number of CYP isozymes in human bronchial cells, including CYP1A1 and CYP1B1. Microarrays have also been utilized in ecotoxicological studies (Nesatyy and Suter, 2007). Hook, *et al.*, examined the gene expression profile in rainbow trout liver following exposure to three individual chemicals and then a mixture of the three. Underscoring the complexity involved in mixture toxicology, they found that although the patterns of the individual exposures could be seen in the mixture results, they were not simply additive (Hook *et al.*, 2008).

3 ENDOGENOUS FACTORS

3.1 Species Differences

3.1.1 Species Differences in Toxicokinetics and Metabolism

It is well known that there are significant species differences in terms of response to toxicants, particularly as concerns xenobiotic metabolism. Differences

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in metabolism between species can be characterized as either qualitative (different enzymes and pathways) or quantitative (same pathways, but different relative activity levels). In Phase I metabolism, differences in structure and function have been identified between mouse, rat, dog, monkey and human P450 isozymes, with the fewest differences between species seen for CYP2E1, and greater differences seen between species for CYP1A, CYP2C, CYP2D and CYP3A (Martignoni *et al.*, 2006). These differences may prove significant in choosing animal models for assessment of first-pass metabolism, as CYP3A is a major factor in bioavailability of the many drugs that are substrates of this isozyme (Komura and Iwaki, 2008). One focussed comparison between CYP3A gene structure and expression in human and chimpanzee tissues has indicated that CYP3A metabolism in adult humans and chimpanzees most likely does not differ significantly (Williams *et al.*, 2007). Other studies have also examined CYP3A activities, indicating that mouse, monkey, minipig and dog CYP3A activities are all more similar to human activities than is rat CYP3A (Turpeinen *et al.*, 2007).

Enzymes involved in Phase II metabolism also show interspecies differences. For example, differences have been identified between humans, monkey, dogs and rats in glucuronidation and sulfation reactions (Wang *et al.*, 2005; 2006) as well as between dogs and humans in *N*-acetylation reactions (Gao *et al.*, 2006).

3.2 Sex Differences

3.2.1 Sex Differences in Toxicokinetics and Metabolism

Sex differences with the potential to impact toxicology have been characterized in humans as well as in other organisms (Gochfeld, 2007). In looking at humans, for example, differences in gender roles may lead to differences in exposure levels to toxicants. This observation holds true both on and off the job, but may be particularly significant in the work environment (Kennedy and Koehoorn, 2003). Physiological factors such as differences in respiratory rates between the sexes may also affect the toxicokinetics of absorption, although the actual impact of this in humans is not well established (Ernstgard *et al.*, 2003a; 2003b). Few, if any, studies have identified sex differences in dermal absorption. And whereas some studies (Schwartz, 2003) have indicated differences in gastrointestinal absorption, this observation may, in fact, be complicated by differences in metabolism. Differences between sexes may also exist in toxicokinetics of elimination. For example, sex differences have been reported in the handling of organic anions in rat liver (Wang *et al.*, 2008), and multiple studies have addressed the impact of possible sex differences in transporter proteins such P-glycoprotein and breast cancer resistance protein (BCRP) in humans (Modjtahedi *et al.*, 2006).

While many gender-related studies have focussed on pharmaceuticals, some have also examined the role of sex in toxicant handling. Twenty years after the industrial accident at Seveso in 1976 (which led to the release of 2,3,7,8-tetrachlorodibenzodioxin (TCDD) over a wide area), women in the area were found to have higher plasma levels of TCDD than men, a difference potentially due to differences in metabolism or elimination (Landi *et al.*, 1998). Cadmium, also, has been found to accumulate to significantly higher levels in retinal tissues in older women as opposed to older men (Wills *et al.*, 2008).

Differences between sexes are almost certainly present in the metabolism of many compounds in several species. Not only have differences in cytochrome P450 metabolism of xenobiotics been detected in rodents and other laboratory animals (Ramesh *et al.*, 2000; Visalli *et al.*, 2005); but studies in humans have also indicated the existence of sex-related differences (Tanaka, 1999). While there may be at least approximate equivalence between many human male and female P450 activities for a variety of substrates (Schwartz, 2003), differences in P450 induction and activity in humans have been reported in the cases of CYP3A which may show higher activity in women (Schwartz, 2003; Wolbold *et al.*, 2003; Gorski *et al.*, 2004; Wenk *et al.*, 2004) and some of the CYP2 subfamilies, which may show higher activities in men (Tanaka, 1999). Some differences have also been seen in Phase II metabolism in humans, although the significance of these are not yet clear (Tanaka, 1999; Schwartz, 2003).

In wildlife toxicology, as well, increasing attention is being given to the potential impact of sex differences on exposure, metabolism and elimination of toxicants (Burger *et al.*, 2007). Exposure may vary with sex, for example, due to different feeding patterns, leading to differences in tissue levels of environmental contaminants.

3.2.2 Sex-related Differences in Molecular Targets

Of course, the physiological differences between males and females also present the possibility for differential toxicity through interaction of a toxicant with different molecular targets. While the most obvious example of this would involve toxicants that exclusively target male or female reproductive structures and functions (the impact of dibromochloropropane (DBCP) on spermatogenesis, for example) a more subtle variation would involve toxicants which may impact either reproductive system, but in different ways. A candidate for this category would be the environmental oestrogens, or endocrine disrupting chemicals (EDCs). These compounds interact with steroid receptors and have been postulated to be linked to a variety of human health problems, including breast cancer in women, reproductive

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tract problems in men and fertility in both sexes (Caserta *et al.*, 2008). They have also been linked to changes in sexual structure and function in wildlife (Goksoyr, 2006; Sonne *et al.*, 2006; Fossi *et al.*, 2007). There are conflicting viewpoints, however, and the issue of links between EDC exposures and human diseases, in particular, remains controversial (Safe, 2005).

Endocrine disrupters almost certainly exert their effects through interaction with receptors, including not only oestrogen receptors, but also receptors such as the PXR and constitutive androstane receptor (CAR) (Kretschmer and Baldwin, 2005). In fact, the mechanism through which toxicants display gender differences in effects may well involve variation in expression of receptors. Differences in kidney toxicity between male and female rats has been linked to differences in expression levels of genes coding for the peroxisome proliferator-activated receptor α (PPAR-α), Ah receptor and oestrogen receptor α, among others (Lu *et al.*, 2006). Another possible mechanism is disruption of steroid hormone biosynthesis (Sanderson, 2006).

3.3 Age Differences

Obviously, the fundamental effects of toxicants on developing organisms may be entirely different than the effects on adults due to the complex developmental processes taking place at that stage in the life cycle. Addressing these differences is a task that is clearly beyond the scope of this review. However, the impact of age on the other factors that influence toxicity, such as toxicokinetics, can be examined. There are many toxicokinetic differences between adults and children including increased gastrointestinal absorption rates and reduced gastrointestinal motility in children, as well as increased deposition of particles in the lungs (Oskarsson *et al.*, 1998; Alcorn and McNamara, 2003; Ginsberg *et al.*, 2004). On the other side of the coin, alterations in gastrointestinal function such as decreased motility with ageing (Salles, 2007) may also impact absorption of toxicants in older adults.

In terms of distributional factors, incomplete development of the blood brain barrier in infants (Saunders *et al.*, 2000) as well as differences in relative lipid content in younger children (Clewell *et al.*, 2002) may lead to age-related differences in distribution of toxicants. There is also evidence that plasma protein levels are lower in neonates than in adults (McNamara and Alcorn, 2002), a factor that also has the potential to affect distribution of toxicants to tissues.

Biotransformation is also significantly affected by age. Levels of many Phase I enzymes including CYP1A2 and CYP3A4 are markedly reduced in neonates while other activities such as CYP3A7 are at high levels at birth and decline with age (Alcorn and McNamara, 2003; Ginsberg *et al.*, 2004; Hines, 2008). The substrate profile of CYP3A7 has not been completely determined, but evidence indicates that its metabolic capacity is most likely reduced when compared to CYP3A4 (Williams *et al.*, 2002). For Phase II reactions, glucuronidation capacity is reduced at least through two months of age, (Ginsberg *et al.*, 2002), and neonates appear to be slow acetylators (Parientekhayat *et al.*, 1991). On the other hand, sulphation in neonates seems to approach adult levels (Richard *et al.*, 2001). There is a neonatal GST isozyme, GST-pi, although it may not be as effective at detoxification as adult GST for at least some substrates (Pacifici *et al.*, 1988). In the elderly, on the other hand, evidence indicates that reduced drug and toxicant clearance may be primarily attributed to reduced blood flow and liver volume, rather than decline in P450 activities (Schmucker, 2001).

Any discussion of the impact of age on toxicokinetics must also address kidney function. Glomerular filtration rate (GFR) and renal blood flow are reduced in neonates, and the processes of secretion and reabsorption are significantly reduced as well (Alcorn and McNamara, 2003). The functional capabilities of the kidneys are reduced with age, as well, making the elderly also at increased risk for drug and toxicant-related toxic effects (Zhou *et al.*, 2008).

3.4 Physiological States

Another factor that must be considered in expression of toxicity is the physiological state of the organism in question. Variations here can range from physiological changes imposed by exercise (alterations in cardiovascular or respiratory parameters, for example) to physiological changes accompanying disease states. One area of interest has been in the effects of exercise on absorption of air pollutants. Lower amounts of benzene, for example, have been shown to be absorbed by individuals breathing at a higher rate over a shorter duration of time than by individuals breathing at a slower rate over a longer period of time (McNabola *et al.*, 2007). For smokers, as well, lung retention of particles depends on depth of inhalation and other respiratory factors; interestingly, rates of nicotine absorption are much less dependent on these parameters (Baker and Dixon, 2006). Physical activity has also been shown to impact absorption of organic solvents (Lof and Johanson, 1998).

More attention is now being shown to potential interactions between microbes and toxicants, both as regards normal gut flora and disease-causing pathogens (Ilback and Friman, 2007). Evidence indicates that the composition of gastrointestinal flora can affect xenobiotic absorption either through microbial production of metabolites that are absorbed to a greater or lesser extent than the parent compound, or through reversal of detoxification processes such as conjugations (Humblot *et al.*, 2007). In general, impact of nutritional state on effect of toxicants

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must also be considered. This is of particular concern, since in developing countries undernutrition and toxicant exposure may go hand in hand (Ahamed *et al.*, 2007; Wasserman *et al.*, 2008).

3.5 Individual Differences: Another Role for Genomics in Toxicology

Along with their usefulness in sorting out complex environmental interactions, genomic techniques can also help elucidate the elusive biochemical differences between individuals of the same species. The question of individual susceptibility to toxicants can then be addressed through examination of genetic variation within key enzymes involved in the toxicologic process. One major area of research has been in characterization of individual variations in the enzymes involved in xenobiotic metabolism.

There are many well-characterized genetic polymorphisms in human cytochrome P450 genes (Thier *et al.*, 2003). One recent review (Ingelman-Sundberg *et al.*, 2007) estimates over 350 different P450 alleles, with the highest number of variations described for CYP2D6, CYP2B6, CYP1B1 and CYP2A6. Some of these variations show ethnic differences. For CYP2A6, for example, one common allele which results in inactivity occurs at much higher frequencies in Asian than in Caucasian populations (Ingelman-Sundberg *et al.*, 2007). On the other hand, CYP2C9*2, a variant with reduced activity is present mainly in Caucasians (Schwarz, 2003). Many of these polymorphisms have been shown to modify metabolism of several drugs and toxicants, and several have been associated with increased risk for diseases such as cancer (Kamataki *et al.*, 2005; Shimada, 2006), as well as reduced responsiveness to drugs due to increased metabolism (Kawanishi *et al.*, 2004). Molecular modifications underlying these P450 polymorphisms include deletions, duplications and copy number variations (Ingelman-Sundberg *et al.*, 2007). There are also genetic polymorphisms in the gene for cytochrome P450 oxidoreductase, the electron donor for CYP-catalysed oxidations (Hart and Zhong, 2008).

GSTs are the enzymes responsible for glutathione conjugation, an important Phase II reaction. Two isozymes, GSTT1-1 and GSTM1-1 (which participates in metabolism of PAHs) are actually deleted in a segment of the human population (Bolt and Thier, 2006). Affected individuals are homozygous for the *GSTM1*0* or *GSTT1*0* deletion polymorphism, and the frequency of the deletion varies between different ethnic groups (Bolt and Thier, 2006). The functional significance of this deletion in terms of susceptibility to toxicants and/or disease is not entirely clear; however, one study has found the *GSTM1*0* and *GSTT1*0* polymorphisms to be positively associated with an increased risk for heavy metal (mercury) accumulation (Gundacker *et al.*, 2007). *GSTM1*0* has also been found to be associated with increased risk of cirrhosis (Ghobadloo *et al.*, 2004), increased cataract risk in the indoor workplace (Saadat and Farvardin-Jahromi, 2006), increased risk for tobacco-induced DNA damage (Palma *et al.*, 2007) and increased risk of smoking-related coronary artery disease (Manfredi *et al.*, 2007). Individual differences in expression of these and other GST isozymes may also play a role in individual susceptibility to a number of cancers (Hayes and Pulford, 1995). Also involved in Phase II, the *N*-acetyltransferases NAT1 and NAT2 show polymorphisms, as well, with a percentage of the population characterized as 'slow acetylators' (Thier *et al.*, 2003).

Genetic variability is best characterized in mammals, but it is certainly recognized that genetic polymorphisms in other species may be of critical importance in ecotoxicology. Reductions in genetic variation due, for example, to population bottlenecks may affect the ability of populations to tolerate exposure to environmental chemicals. One organism in which this has been demonstrated is the midge *Chironomus riparius* (Nowak *et al.*, 2007). In addition, genetic variations between strains chosen for laboratory testing may significantly impact results of ecotoxicological testing (Picado *et al.*, 2007).

3.6 Epigenomics

Perhaps the final word in assessing the factors that influence expression of toxicity is epigenomics. Epigenomics focuses on regulation of gene expression rather than the structure of the genes themselves, and involves mechanisms such as modification of chromatin structure through histone modification and methylation of DNA, as well as control of gene expression through microRNAs (miRNAs) (Ingelman-Sundberg *et al.*, 2007; Szyf, 2007). While the specific impact of these mechanisms on expression of toxicity is not yet well understood, preliminary indications are that they do play an important role. One study, for example, has implicated alterations in the methylation of promoter and enhancer regions of the CYP1B1 gene in prostate cancer (Tokizane *et al.*, 2005). MicroRNAs also appear to be involved in regulation of this gene (Tsuchiya *et al.*, 2006).

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Chronotoxicology

Karam F. Soliman and Elizabeth A. Mazzio

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1 INTRODUCTION

The study of kinetics, dynamics, toxicological responses and side effects of drugs, poisons or toxic substances relative to temporal rhythms occurring in living organisms is referred to as 'chronotoxicology'. Temporal rhythms are dynamic changes in the body guided by environmental time cycles with variation in frequency (i.e. day: circadian, week: circaseptan, month: circalunar, annual: circannual or seasonal) which are governed by the momentum of the earth's rotation about its central axis (rotation speed: 467 m s^{-1}) and its elliptical orbit around the sun (a 365.25 day cycle). Each day the earth's single rotation about its central axis completes a full day/night 24 hour cycle, where the earth is further split into day and night by a dividing edge called the 'circle of illumination'. Temporal rhythms are also contingent upon the earthly position of an organism in terms of its location–degrees latitude/longitude.

Temporal rhythmic patterns in living organisms have been documented throughout history often describing observable photoperiodicities in almost all terrestrial life forms, including plants, single-celled organisms, insects, cells, yeasts and mammals (Pierre *et al*., 1985; Lloyd and Stupfel, 1991; Egan *et al*., 1999; Herzog *et al*., 2004; Chen and McKnight, 2007; Michael *et al*., 2008). In mammals, the main metronomes to which temporal biorhythms align themselves are photoperiodic stimuli derived from natural sunlight (photophase) or lack of illumination (scotophase) throughout the 24 hour period. An endogenous rhythmic flow is further influenced by local external time cues in the environment such as the daily sound of an alarm clock, a daily routine, social schedules, exercise, feeding patterns or routine noises which in the absence of photoperiodic stimuli create '*zeitgebers*' (German for 'time giver', synchronizer) (Lax *et al*., 1999; Carney *et al*., 2006; Kosobud *et al*., 2007; Korczak *et al*., 2008; Ni *et al*., 2008).

There is a vast complexity in understanding biological rhythms because of the number of variables that alter timing systems such as disease states, drugs, lifestyles or the very process of ageing (Van Someren and Riemersma-Van Der Lek, 2007). While biological circadian rhythms are in essence endogenous free-running systems, they are also entrained (synchronized) to the world around them and adapt to ensure survival of life, acclimatization to environmental or seasonal conditions, and to ensure productivity and species reproduction. Units of measurement most often used to quantify biological temporal rhythms are 'periods' (length of time to complete a single cycle), 'oscillations' (the repetitive

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motion of a cycle), 'amplitude' (magnitude of a biological response) and 'phase' (a representative peak or valley of an amplitude).

1.1 Entrainment of SCN by Photoreceptive Retinal Ganglion Cells

In humans, the anatomical 'biological clock' is the suprachiasmatic nucleus (SCN), which is housed within the anterior hypothalamus proximal to the optic chiasm. The SCN receives information from intrinsically photosensitive retinal ganglion cells (ipRGCs) through a monosynaptic retinohypothalamic tract (RHT), which detects illumination through a blue-light sensitive photopigment called melanopsin (Turner and Mainster, 2008; Ruggiero *et al*., 2009). The blue spectral absorbance of melanopsin in ipRGCs peaks between 460 and 480 nm making it excitable by direct sunlight, which has a wavelength of approximately 477 nm (Stevens *et al*., 2007; Turner and Mainster, 2008). The photic receptivity of ipRGCs differs vastly from parts of the eye required for vision such as the basic rod-mediated scotopic response to light (night vision) and cone-mediated photopic response to light (day vision). Melanopsin in ipRGCs is responsible for the non-image-forming photosensitive system that regulates circadian timing, where rods and cones relay visual information such as colours, images, shapes and patterns to the brain (Ruggiero *et al*., 2009). For this reason, therapeutic use of short wavelength blue monochromatic light is more effective in evoking circadian phase shifts, suppressing melatonin, enhancing alertness and treating disorders associated with disruption of circadian rhythms (Stevens *et al*., 2007) (**Figure 1**). Any degenerative loss of ipRGCs as in the case with glaucoma can adversely affect entrainment and circadian rhythmicity (Drouyer *et al*., 2008), whereas visually impaired/blind animals continue to be entrained by the light/dark cycle, provided ipRGCs are still functional (Silva *et al*., 2005).

SCN entrainment is necessary to establish physiological temporal rhythms that promote the optimization of health. In brief, darkness to the back of the retina signals adrenergic innervation to the pineal gland through the superior cervical ganglia functioning to regulate the production of melatonin and its release into the bloodstream to regulate sleep (Cardinali and Vacas, 1987). Exposure to natural or artificial daylight entrains the SCN, initiates suppression of melatonin during the day and improves quality of sleep (Adler *et al*., 1992; Murphy *et al*., 1993; Park *et al*., 2007). However, for daylight exposure to properly synchronize the clock, it must be applied during the actual solar day because exposure to artificial daylight during dark phase (night) can counter proper phase adjustment leading to disturbed sleep patterns and impaired cognitive function.

The amount of daylight required to entrain the SCN should reach a certain light intensity and duration each day with varying requirements set forth for varying species. However, in mammals, SCN entrainment generally requires light intensities of >1000 lux for several hours (Sack *et al*., 2007a; 2007b) consisting of monochromatic light at 460 nm rather than photopic

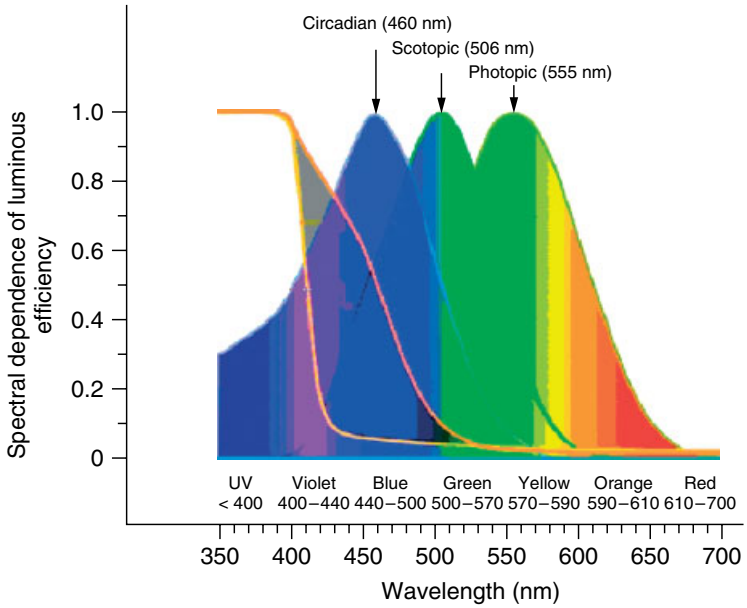

Figure 1 Spectral sensitivity peaks of photopic, scotopic and circadian (melatonin suppression) photoreception. Peak sensitivities of circadian, scotopic and photopic photoreception are 460 nm (blue), 506 nm (green) and 555 nm (green-yellow). (Reproduced from Turner and Mainster, 2008. © BMJ Group.)

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light at 555 nm (Lockley *et al*., 2003). And light exposure must be directed and received by the eye, because sunlight directed at the skin does not entrain human circadian rhythms (Lindblom *et al*., 2000).

1.2 Entrainment of Nonphotoreceptive Peripheral Oscillators

The SCN sets its tempo to environmental light/dark cycles as a self-sustained clock in response to light. It also serves to orchestrate rhythms in thousands, if not millions, of concurrent biological clocks located within cells, tissues and organs that are not responsive to light but respond through neural and humoral SCN outputs, which then regulate peripheral oscillatory rhythmicity (Yamazaki *et al*., 2000) (**Figure 2**).

Secondary peripheral oscillators entrain to various frequency rhythms that regulate life by maintaining a plethora of basic physiological functions occurring in major organs such as the heart, liver kidney and so on, that serve to regulate body temperature, blood pressure, appetite and health of the cardiovascular, nervous, digestive, immune, reproductive, endocrine and neuromuscular systems. These peripheral clocks respond both to the SCN and zeitgebers, allowing for coordination of precisely timed events to sustain biological life (Prasai *et al*., 2008).

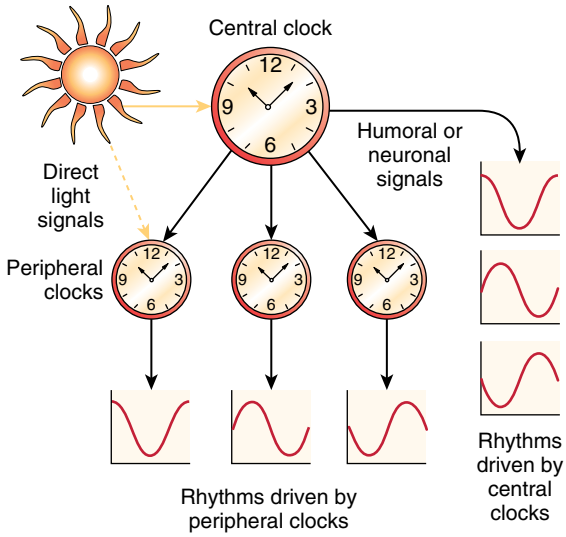

Figure 2 Circadian rhythms are generated by the central clock, which is under the influence of light. Peripheral clocks are under the direct control of the central clock, although they may also be regulated by other stimuli. Peripheral clocks receive input from the central clock, which can be neural, diffusible or hormonal. So the SCN directly induces a variety of physiological rhythms, while synchronizing or directing peripheral clocks, which may have distinctive characteristics. (Reproduced from Cermakian and Sassone-Corsi, 2000. © Nature Publishing Group.)

1.3 Desynchronization of the SCN from Peripheral Oscillators

A dissociation of the SCN main clock from peripheral oscillators is thought to lead to disorders of circadian rhythm and disease. Experimentally, lesions to the SCN in rats can lead to a functional loss of peripheral neuroendocrine cycles and desynchronized circadian rhythms (Eastman *et al*., 1984; Nagai *et al*., 1994; Filipski *et al*., 2004), which can occur even when peripheral oscillators continue to display rhythms in a self-sustaining manner (Yoo *et al*., 2004). In other words, peripheral oscillators not communicating properly with the SCN in essence have lost sight of the 'orchestra conductor', but continue to play their instruments without working in concert to perform a symphony. The result is an undesirable disjointed and nonharmonious sound.

In humans, a disruption of peripheral oscillators to the SCN leads to desynchronization or internal rhythms being jolted out of alignment. This is often the case in instances such as abnormal eating patterns which alter food-entrainable oscillators (Bechtold, 2008), jet lag where the body is rapidly transported through different time zones given no chance to acclimatize (Reilly *et al*., 2008; Srinivasan *et al*., 2008; Locke *et al*., 2008), or night-shift work which can lead to chronically impaired sleep, cardiovascular disease, peptic ulcers, glucose intolerance, mood/cognitive deficits, reproductive problems, accidents and increased risk for breast cancer (Yoon and Song, 2002; Van Dongen, 2006; Biggi *et al*., 2008; Mosendane *et al*., 2008; Kolstad, 2008). A lack of sunlight can also adversely affect circadian rhythms leading to winter depression (seasonal affective disorder) (Monteleone and Maj, 2008), major depressive disorder or exacerbate conditions such as Alzheimer's disease, schizophrenia and bipolar disorder (Lamont *et al*., 2007; Kaladchibachi *et al*., 2007). Other common conditions of desynchronization are classified as circadian-rhythm sleep disorders and include advanced sleep-phase disorder, delayed sleep-phase disorder, free-running disorder, irregular sleep–wake rhythm and premenstrual dysphoric disorder (Sack *et al*., 2007b).

When peripheral oscillators dissociate from the main SCN clock, phase adaptation and time would be required before entrainment to the central clock could be re-established. This process can be expedited by various therapeutic regimes such as bright light therapy, hypnosis, oral administration of melatonin, vitamin B_{12}, stimulants, melatonin MT_1 and MT_2 receptor agonists or implementing structured sleeping patterns (Brunner and Wirz-Justice, 1993; Morgenthaler *et al*., 2007;

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Pandi-Perumal *et al*., 2008; Santhi *et al*., 2008; Lieverse *et al*., 2008). Physical activity or exercise can also assist in phase shift of the circadian clock (Sack *et al*., 2007a; 2007b) in addition to manipulating the timing of nonphotic stimuli such as establishing routine eating patterns (Mendoza *et al*., 2005; Bechtold, 2008).

2 THE ANATOMY OF THE SCN

Understanding the anatomy of the SCN is a critical foundation for the study of any physiological process as it is affected by universal planetary time cycles. A closer look at the SCN main clock reveals it to be a small population of approximately 10 000 γ-aminobutyric acid (GABA)-ergic neurons further comprised of an inner core (ventrolateral) containing vasoactive intestinal polypeptide (VIP), somatostatin, gastrin-releasing peptide (GRP) and androgen receptors (ARs) where the outer 'shell' (dorsomedial) contains arginine vasopressin (AVP)-positive neurons (Moore *et al*., 2002; Karatsoreos and Silver, 2007). The SCN shell displays rhythmic oscillation patterns associated with neuronal firing, whereas the core displays relatively lower rhythmic amplitude. Destruction of the SNC core results in loss of all circadian controls indictating that the unit functions as a whole self-sustaining rhythmic entity. Incoming information travels through afferent pathways which intervene on various parts of the SCN resulting in neuronal firing and circadian biological timekeeping (Karatsoreos and Silver, 2007; Locke *et al*., 2008; Kent and Meredith, 2008). **Figure 3** shows the core and shell through caudal to rostral sectioning, as determined by immunocytochemistry.

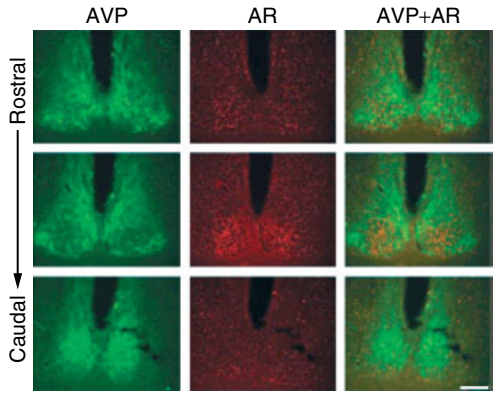

Figure 3 Photomicrographs depict double-label immunocytochemistry for arginine vasopressin (AVP) (green) and androgen receptor (AR) (red) from the rostral to caudal SCN in a male mouse. AVP delineates the shell region of the SCN, where as AR is largely restricted to the core region. Scale bar 150 μm. The core marker ARs are involved with circadian rhythms of the neuroendocrine system that regulate male and female reproductive physiology. (Reproduced with permission from Karatsoreos *et al*., 2007. © The Endocrine Society.)

2.1 SCN: Major Afferent Pathways

The SCN receives major afferent input via three main pathways. The main zeitgeber (light intensity) establishes the foremost afferent pathway, which receives photic information about the position of the sun and its rotation around the earth.

Retinal ganglion cells containing melanopsin connect through the RHT, which is a glutaminergic innervation also containing pituitary adenylate cyclase activating polypeptide (PACAP), which junctions at a VPAC 2 receptor/VIP-positive SCN core (Hannibal *et al*., 1997; 2000; Fukuhara *et al*., 1997; Itri and Colwell, 2003). Animals that lack VIP or VIP-PACAP Type 2 (VPAC2) receptors show reduced amplitude of circadian gene expression with loss of rhythmicity or electrical firing within the SCN (Maywood *et al*., 2007). This is because SCN firing is synchronized by VIP acting on VPAC2, which is required for circadian rhythmicity and coupling of the SCN core to the shell (Kalló *et al*., 2004), which then regulates genes in peripheral tissue through neural or hormonal pathways (Toh, 2008). Both light and glutamate are capable of stimulating the SCN core via glutamatergic *N*-methyl-D-aspartate (NMDA)/non-NMDA retinal terminals which then can trigger oscillatory changes in $[Ca^{2+}]_I$, via calcium-dependent cascades involving mitogen-activated protein kinases (MAPKs)/extracellular signal-regulated kinase (ERK), protein kinase G, phosphorylation, nitric oxide, c-fos and calcium/cyclic adenosine monophosphate (AMP) response elementcyclic AMP (cAMP) response-element-binding protein (CREB) control of SCN circadian clock gene expression and thus circadian rhythms (Hastings *et al*., 2007; Pulivarthy *et al*., 2007; Irwin and Allen, 2007; Akashi *et al*., 2008) (*see* Section 3).

Both photic (light) and nonphotic stimuli (i.e. physical activity, brain stimulation reward and foot shock) can act on a second branch of the RHT, directing information to the intergeniculate leaflet (IGL), enkephalin and neuropeptide Y (NPY)-positive cells, which via the geniculohypothalamic tract (GHT) terminate in areas that overlap the direct RHT–SCN projections (Pickard *et al*., 1987; Kumar and Rani, 1999; Cain *et al*., 2007; Kim and Harrington, 2008; Toh, 2008). Lesions to the IGL impair light-induced periodicities (Harrington and Rusak, 1986) and block phase-shifting responses to triazolam, indicating that both light and benzodiazepine GABA receptors relay information to establish rhythm and entrainment through the IGL (Johnson *et al*., 1988).

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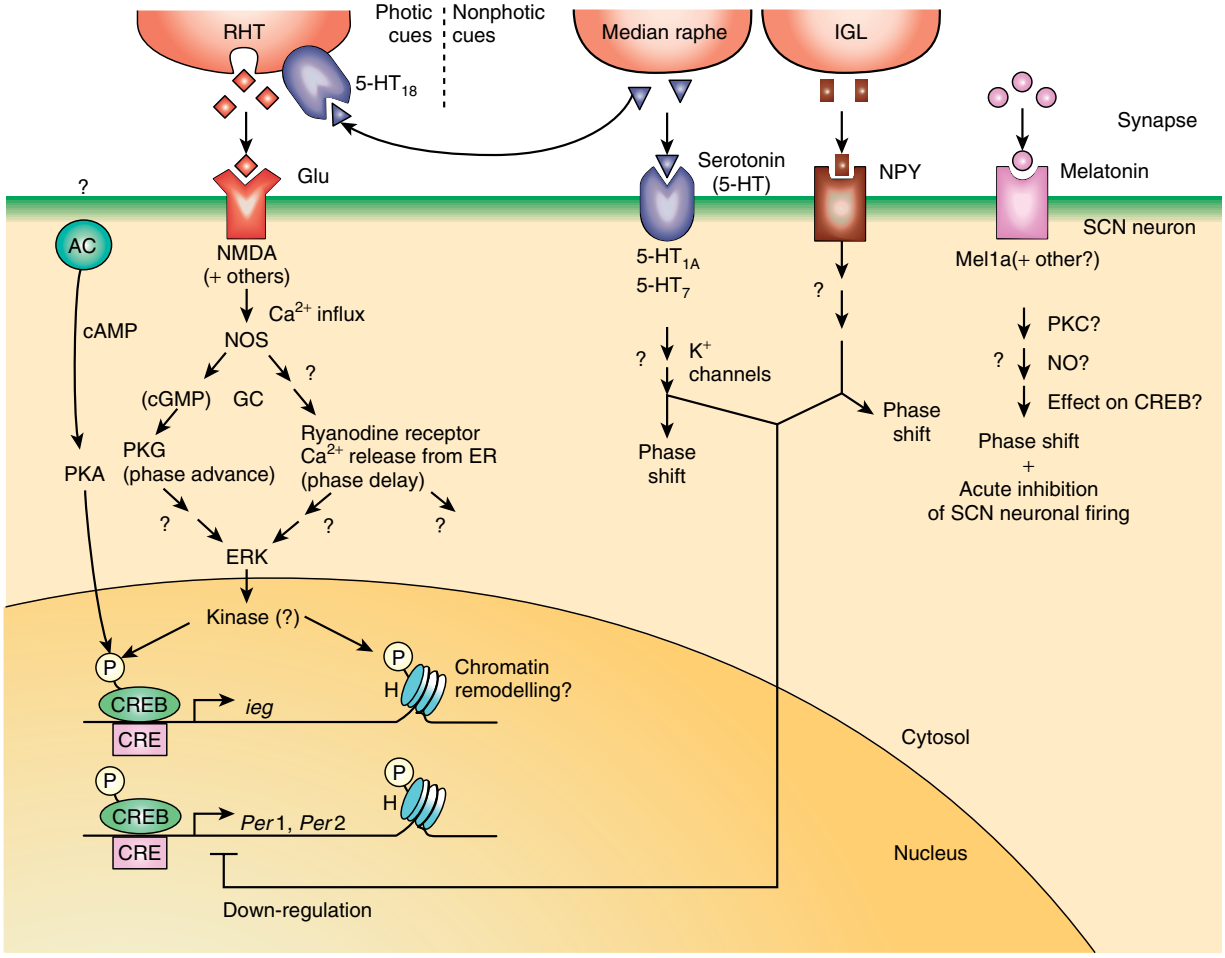

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Figure 4 The SCN circadian clock is affected by light (photic cues) through the RHT leading to use of glutamate (Glu) as a neurotransmitter. Glutamate receptor triggering induces various intracellular responses, leading ultimately to gene expression and phase shifts. Nonphotic cues involve a variety of other neurotransmitter and signalling pathways (three of which are shown here). The photic and nonphotic pathways can crossregulate each other pre- and postsynaptically. AC, adenylate cyclase; CRE, cAMP response element; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; GC, guanylyl cyclase; H, histone; for example immediate early genes (e.g. c-*fos*); IGL, nerve terminus from the intergeniculate leaflet (thalamus); NMDA, *N*-methyl-D-aspartate; NOS, nitric oxide synthase; NPY, neuropeptide Y; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PKG, cyclic guanosine monophosphate (cGMP)-dependent protein kinase. This diagram shows the multilevel regulation of the circadian clock. (Reproduced from Cermakian and Sassone-Corsi, 2000. © Nature Publishing Group.)

The third major afferent to the SCN comprises serotonergic neurons, which stem from the median raphe nucleus (Meyer-Bernstein and Morin, 1996). Light or serotonin-altering drugs may affect circadian rhythms due to both the SCN and the IGL receiving serotonergic input from the midbrain raphe nucleus (Ying *et al*., 1993). Lesions to the median raphe will render animals more sensitive to light at high intensity, indicating serotonin to be an inhibitory modulator of circadian rhythmicity (Muscat *et al*., 2005). IGL innervation from the dorsal raphe is also modulated by inhibitory effects of serotonin, which regulate circadian and sleep/arousal systems (Blasiak *et al*., 2006). **Figure 4** is an illustrative showing the three primary afferent projections extending to the SCN, which ultimately control transcription of clock genes.

2.2 SCN: Major Efferent Pathways

SCN efferent pathways are quite numerous and include those that extend to many areas of the brain, including the caudal projections to the subparaventricular zone and hypothalamic arousal systems in the posterior/lateral hypothalamic areas (regulate rest activity) (De la Iglesia

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and Schwartz, 2002; Moore and Danchenko, 2002), the ventrolateral preoptic nucleus, via GABAergic neuron projections targeting the medial preoptic area and the dorsomedial hypothalamic nucleus (regulate sleep wake cycle) (Deurveilher *et al*., 2002), ipsilateral neural efferents from the SCN to leutinizing hormone (LH)-containing neurons in the preoptic area (regulating LH cycles) (De la Iglesia and Schwartz, 2002), and efferents from the SCN to the paraventricular nucleus (PVN), the preoptic area and the mediobasal nuclei to (regulate daily rhythms of adrenocorticotrophins, gonadotrophins and metabolic hormones, respectively) (De la Iglesia and Schwartz, 2002; Kalsbeek *et al*., 2007). The hypothalamic PVN is an important target area of biological clock output and harbours the preautonomic neurons that control peripheral sympathetic and parasympathetic activity which in turn regulate plasma glucose and insulin concentrations (Kalsbeek *et al*., 2008).

Secondary projections from the SCN involve those that project to the neocortex, limbic system, hippocampus, anterior pituitary and reticular activating system to provide temporal information required for thermoregulation, feeding, memory and learning, mental performance and endocrine function. And, one of the main SCN efferents is to the upper thoracic intermediolateral cell column which extends to the superior cervical ganglion sympathetic neurons, which in turn innervate the pineal gland. Via this pathway, the SCN regulates the circadian rhythm by pineal secretion of melatonin, the compound responsible for sleep onset (Toh, 2008).

Secretion of melatonin is mediated by sympathetic efferents to the pineal gland and is the primary regulator of circadian sleep patterns. Melatonin released from the pineal gland is highest in the evening and suppressed by sunlight, therefore the term dim-light melatonin onset 'DLMO' is used as a diagnostic position marker to assess physiological parameters of circadian rhythm that occur under dim light, which include plasma or saliva melatonin, as well as cortisol, sleep onset, core body temperature or urinary 6-sulfatoximelatonin concentrations (Sarabia *et al*., 2008; Hofstra and de Weerd, 2008). In the absence of light, pineal melatonin secretion is at it highest (3–10×) (Touitou, 1998; Thapan *et al*., 2001), whereas SCN clock genes up-regulate the transcription of N-acetyl transferase (NAT) and hydroxy indole-O-methyl transferase to maximize melatonin synthesized from tryptophan (Touitou, 2005; Ravindra *et al*., 2006). Nocturnal melatonin synthesis is critical in regulating sleep, and individuals with polymorphisms in the promoter region of the NAT gene are at greater risk for developing late-sleep-onset/short-duration circadian-rhythm disorder (Wang *et al*., 2004). In contrast, sunlight suppresses melatonin synthesis via photic non-vision-related retinal ganglion cells, the SCN, the PVN and the superior cervical ganglion through down-regulating NAT (Thapan *et al*., 2001; Stevens *et al*., 2007). Exposure to bright light marks a new day corresponding to elevation of heart rate, circulating cortisol, core body temperature, mental alertness (Rüger *et al*., 2006) and a rise in dopamine (DA) which inhibits melatonin synthesis through acting on D_2-like DA receptors (Tosini *et al*., 2008).

The circadian cyclic amplitude of melatonin is often dampened during the process of ageing, limited exposure to daylight, seasonal variation or night-shift work—factors which correlate to greater incidence of coronary heart disease, orthostatic hypotension, Alzheimer's disease and a diverse range of human cancers (Ravindra *et al*., 2006). For this reason, oral or sublingual melatonin supplementation holds considerable promise in the amelioration of altered sleep/wake cycles in the elderly, patients with circadian-rhythm sleep disorders and for the treatment of cancer (Ravindra *et al*., 2006). In addition, melatonin also provides additional health benefits such as its inherent immunostimulant, antioxidant, oncostatic, thermoregulatory, antidepressive, anti-inflammatory and cytoprotective properties (Karasek, 2004; 2007; Touitou, 2005; Ravindra *et al*., 2006).

3 REGULATION OF CLOCK GENES

Biological clocks are regulated by genetic transcriptional-translation feedback loops that respond to the day/night cycle and information received by the SCN. In brief, retinal stimulation via glutaminergic activation of VIP in the SCN core then extends through GABA/GRP signalling to the shell which then influences clock genes through signalling systems involving calcium calmodulin kinase, the p42/44 MAPK pathway, nitric oxide, protein kinase G and ERK, which activates CREB, thereby controlling rapid or slow genetic transcription of SCN clock-related proteins. The major SCN elements controlling circadian oscillation transcriptional feedback loops are period (Per 1–3), cryptochrome (Cry 1, 2), the nuclear orphan receptors: reverse erythroblastic leukaemia viral oncogene homologs (Rev-erbα, Rev-erbß), retinoic acid-related orphan receptors (RORs) (Rorα, Rorß) and the CLOCK (circadian locomotor output cycles kaput)/BMAL1 (brain and muscle ARNT-like protein 1) heterodimers (Challet, 2007).

CLOCK and BMAL1 contain basic helix–loop–helix motifs for DNA binding at the NPAS2 domain which heterodimerize and translocate from the cytosol to the nucleus, where the positive (+) molecular feedback loop consists of CLOCK-BMAL1 binding to the E-box promoter element sequence upstream of *Per* (1,2,3 genes)/*Cry* (1,2 genes) and nuclear receptors Ror and Rev-erbβ. CLOCK also acts as a histone acetyltransferase involved with the activation of *Per* and *Cry*

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genes (Doi *et al*., 2006; Zhu *et al*., 2008). The negative (−) molecular feedback loop occurs when attainment of critical concentrations of Per accumulate in the cytoplasm, which then complex with Cry, enter the nucleus and down-regulate the nuclear transcriptional activator CLOCK-BMAL1 and thus downregulate further transcription of the *Cry* and *Per* genes and other clock-related genes. This negative feedback cycle largely regulates the oscillation of the circadian clock.

More specifically, the cycle starts with heteromeric proteins CLOCK and BMAL1 activating clock genes by acting on the m*Per*1 promoter. Per then enters the cytoplasm where it is phosphorylated by casein kinase I ε (CKI-ε, CKI-δ), protein kinase A, protein kinase C (PKC) and other kinases that regulate its stability, where phosphorylation delays nuclear entry and that of Cry. Post-translational modification of Per by CKI is also genetically regulated, where a peak in CKI in the SCN is observed at the start of a subjective night (Agostino *et al*., 2008). When Per and Cry proteins attain critical mass, they dimerize and translocate to the nucleus where they suppresses CLOCK-BMAL1 transcription of *Per, Cry, Ror and Rev-erb*β in a negative feedback loop. The existing Per and Cry complexes are degraded by the end of circadian night, where a surge of BMAL1 expression emerges initiating a new cycle of *Per* and *Cry* gene expression to start a new day (Preitner *et al*., 2002; Sato *et al*., 2004; Hastings *et al*., 2007).

As demonstrated in **Figure 5**, the core loop is stabilized by feedback pathways involving the transcription of *Rev-erb* (α,β) and *ROR* (α,β), which translocate to the nucleus and compete for binding to a nuclear target sequence called retinoic acid-related orphan receptor response elements (RORE) in the BMAL1 promoter.

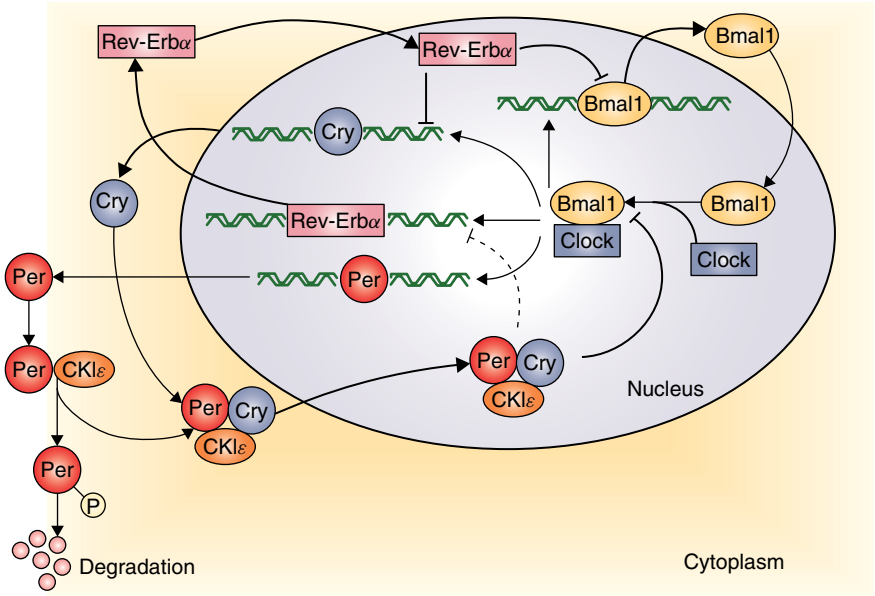

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Figure 5 Circadian rhythms are generated by the feedback loops of the core circadian genes. In the SCN neurons, the intracellular levels of CLOCK remain steady throughout the 24 hour period, whereas BMAL1 expression levels are high at the beginning of a subjective day and low at the beginning of a subjective night. The high level of BMAL1 promotes the formation of BMAL1–CLOCK heterodimers. These bind to E-box sequences in the promoters of the *Cry, Per* and *Rev-erb* genes to activate transcription at the beginning of a circadian day. The BMAL1–CLOCK heterodimer can also inhibit *Bmal1* transcription. After transcription and translation, the Rev-erb protein enters the nucleus to suppress the transcription of *Bmal1* and *Cry* genes. As the Per proteins, such as Per2, accumulate in the cytoplasm, they become phosphorylated (P) by CKI. The phosphorylated forms of Per are unstable and are degraded by ubiquitylation. Late in the subjective day, however, Cry accumulates in the cytoplasm, promoting the formation of stable CKI/Per/Cry complexes, which enter the nucleus at the beginning of a subjective night. Once in the nucleus, Cry1 disrupts the CLOCK/BMAL1-associated transcriptional complex, resulting in the inhibition of *Cry, Per* and *Rev-erb* transcription, and depression of *Bmal1* transcription. It is not clear whether Per and Cry must dissociate from the CKI/Per/Cry complex to inhibit the activity of the CLOCK/BMAL1 heterodimer and to stimulate *Bmal1* transcription in the nucleus. The interacting positive and negative feedback loops of circadian genes ensure low levels of Per and Cry, and a high level of BMAL1 at the beginning of a new circadian day. Solid lines indicate direct regulation, and dashed lines indicate indirect regulation (Fu and Lee, 2003). (Reproduced from Loning and Cheng, 2003. © Nature Publishing Group.)

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The *Rev-erbα* promoter contains E boxes and its transcription is activated by CLOCK-BMAL1 heterodimer and repressed by Per/Cry1 resulting in the oscillations of Rev-erbα. In turn Rev-erbα can repress *BMAL1* and *CLOCK* transcription through RORE, which occurs during the day cycle, ending early in the evening (Preitner *et al*., 2002; Sato *et al*., 2004). Their antagonistic effects generate a rhythmic level of BMAL1 and thus CLOCK-BMAL1. Additionally, Per2 activates *BMAL1* transcription in a positive feedback loop. ROR-*α* competes with Rev-erbα for the binding of shared DNA response elements, and genes containing RORE in their promoter are repressed by Rev-erbα, whereas RORα activates their transcription (Duez and Staels, 2008) and RORs tend to activate transcription of *BMAL1* mRNA (Hasting *et al*., 2007; Maywood *et al*., 2007). The amplitude of Rev-erb dwindles by early morning and it acts as a repressor of BMAL1 in tandem with the actions of Per and Cry.

There are many other SCN transcription factors capable of binding to E-Box cis-elements in response to photic light stimuli that contribute to temporal circadian oscillations, the scope of which exceeds this review (Honma *et al*., 2002; Porterfield *et al*., 2007; Rossner *et al*., 2008). In brief, SCN clock genes ultimately regulate humoral or neuronal output signalling that subsequently drive peripheral oscillations (i.e. liver, adipose, skeletal, muscular, vascular, etc.) in cells containing genes that have E boxes in their promoter and/or RORE sites in their regulatory regions (Duez and Staels, 2008).

4 CHRONOTOXICOLOGY

Biological rhythms established by the SCN/peripheral oscillators influence most all biological processes ('chronobiology'), where anomalies can predicate disease onset ('chronopathology'), which can influence time-dependent exacerbation/reduction of toxic effects of drugs and poisons ('chronotoxicology') and alter the responsiveness to drugs ('chronopharmacology'), which then may require time-wise delivery of medications ('chronotherapeutics'). These terminologies basically describe the means by which SCN/peripheral rhythmic oscillation patterns regulate physiological function, eating patterns, drug delivery, metabolism, detoxification, nutrient drug/utilization or excretion.

The term 'chronotoxicology' in some cases is not independent of chronotherapeutics, which is dependent upon the study of chronopathology. For example, rhythmic patterns of disease onset or symptoms can peak and trough at certain hours or seasons, thereby opening an avenue by which chronotherapeutic application can be applied to optimize drug efficacy and reduce unwanted toxic side effects (Halberg *et al*., 2006). In discussing the term chronotoxicology, we will use as an example a disease most impacted by circadian rhythms: cancer. The study approach to chronotoxicology in general involves various separate segments: (i) chronopathology of cancer—the study of how various clock genes regulate circadian rhythms that create risk factors for developing cancer, (ii) the extent to which clock genes control cancer specific genes: cell proliferation, DNA repair and angiogenesis and (iii) assessing the chronotoxicology of various chemotherapy agents to assess optimal drug-delivery times and reduce unwanted side effects.

4.1 SCN Clock Desynchronization and Cancer Risk

A higher incidence of cancer is associated with aberrant circadian rhythms brought about by night-shift work, jet lag or inherent mutations in clock genes (Hansen, 2001; Stevens, 2005; Zhu *et al*., 2006; 2008; Wood *et al*., 2008; Tokunaga *et al*., 2008). It has been hypothesized that cancer may develop from desynchronization as a result of night-time light exposure during the normal nocturnal sleep phase, creating hormonal fluctuations and suppressing melatonin production (Reiter *et al*., 2007; Kloog *et al*., 2009). Once a malignant tumour is established, circadian patterns then govern tumour growth, as described by Filipski *et al*. (2002). In the following study, desynchronization established by SCN bilateral electrolytic lesions in animals with Glasgow osteosarcoma or pancreatic adenocarcinoma resulted in a loss of circadian rhythmic patterns (**Figure 6A**) parallel to acceleration of tumour growth (**Figure 6B**).

4.2 Clock-Gene Coupling to Cell Division/Proliferation

The undisputable link between the cancer and circadian rhythms may be deep-rooted in evolution, where the process of cell division, proliferation, DNA repair and cell cycling was timed in order to prevent DNA damage from the mutagenic effects of ultraviolet light during the day (Chen and McKnight, 2007). As mentioned previously, the circadian oscillation transcriptional feedback loop is under the control of Per 1–3, Cry 1,2 (stabilized by the casein kinases), Rev-erbα, Rev-erbß, Rorα, Rorß, CREB and the CLOCK/BMAL1 heterodimers (Challet, 2007) many of which regulate cell division, cell-cycle gene transcription or DNA repair (Arjona and Sarkar, 2005; Chen and Mcknight, 2007) such as Wee1, the cyclins and c-Myc that in turn modulate the expression of active cyclin B1-Cdc2 kinase, a regulator of mitosis (Matsuo *et al*., 2003; Chen-Goodspeed and Lee, 2007).

Cell-cycle and circadian rhythms, both controlled by clock genes, can lead to varying therapeutic efficacy

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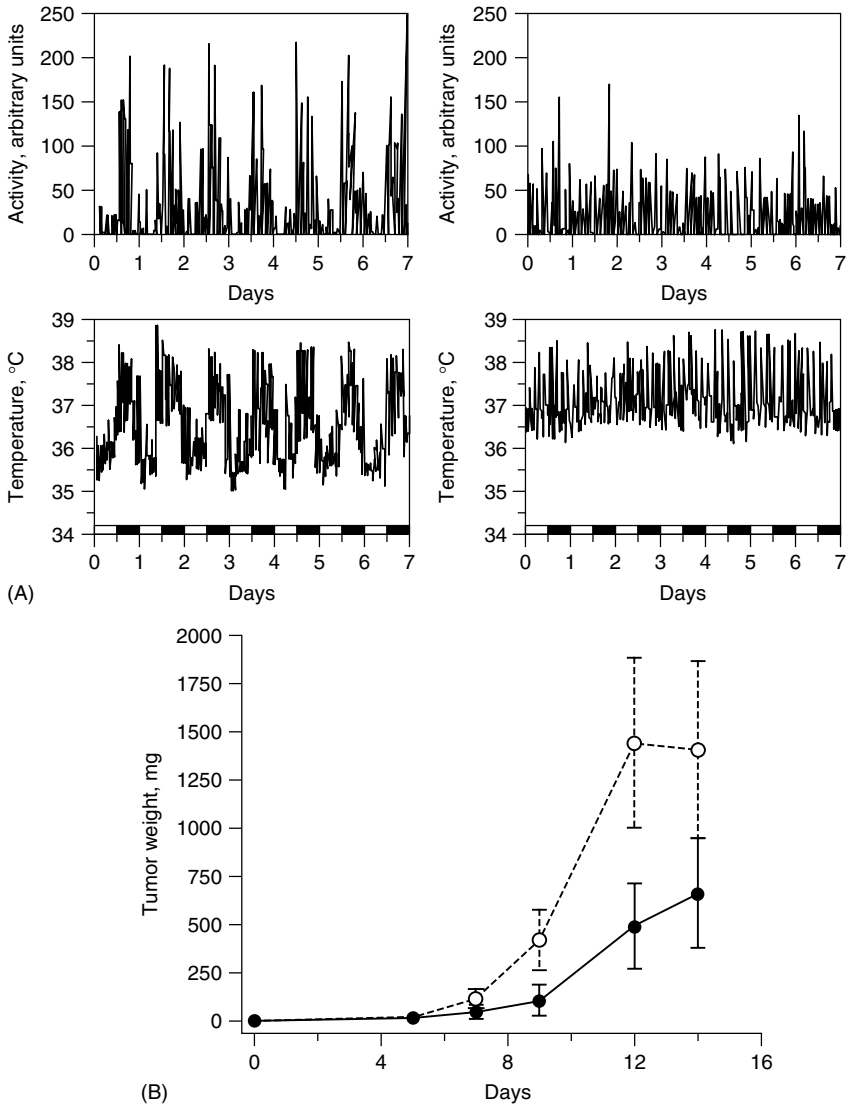

Figure 6 (A) Representative sample: loss of circadian rhythms in SCN lesioned tumour bearing animals evidenced by quantification of changes in core body temperature or rest activity. Pattern of rest activity (top) and temperature (bottom) in a sham-operated mouse (left) and in a mouse with lesions in SCN (right). A rhythmic pattern was clearly recognizable in the sham-operated mouse, with no rhythm obvious in the mouse with SCN lesions, and no 24 hour period stood out in Fourier analysis. The rest-activity cycle was suppressed in 79 of 110 mice with SCN lesions and body-temperature rhythm was suppressed in 60 of the 75 mice with SCN lesions (Filipski *et al*., 2002). (B) The effect of SCN lesion on Glasgow osteosarcoma tumour growth in sham-operated (o) and mice with lesions in the SCN (•). Mean tumour weights are shown with their respective 95% confidence intervals. Accelerated growth of tumours in mice with SCN lesions was statistically validated with ANOVA (analysis of variance), $p < 0.001$. Tumour size was measured three times a week using a calliper. Tumour weight (mg) was estimated from two perpendicular measurements (mm): tumour weight = (length × width)/2. (Reproduced from Filipski *et al*., 2002. © Oxford University Press.)

of chemotherapy drugs (as measured by suppressed proliferation of tumour cells) with drug dosing at various time points throughout the day (**Figure 7**). Iurisci *et al*. (2006) investigated osteosarcoma tumour growth in mice with administration of Seliciclib (which down-regulates casein kinases, ERK2 and cyclin-dependent kinase) administered at three time points ZT (zeitgeber time = lights on) 3, 11, 19 for five days using a 12/12 light/dark cycle.

The results from this study show that administration of the drug during the early morning phase or ZT3, 11 resulted in greater tumour suppression, which also corresponded to changes in tumour mRNA for the clock genes (*Per2, Rev-erbα* and *BMAL1*) and clock-controlled cell-cycle genes as well (*c-Myc, Wee1, cyclin B1* and *Cdk1*) (Iurisci *et al*., 2006).

Many studies have attempted to define the nature of the relationship between clock genes and cell-cycle genes,

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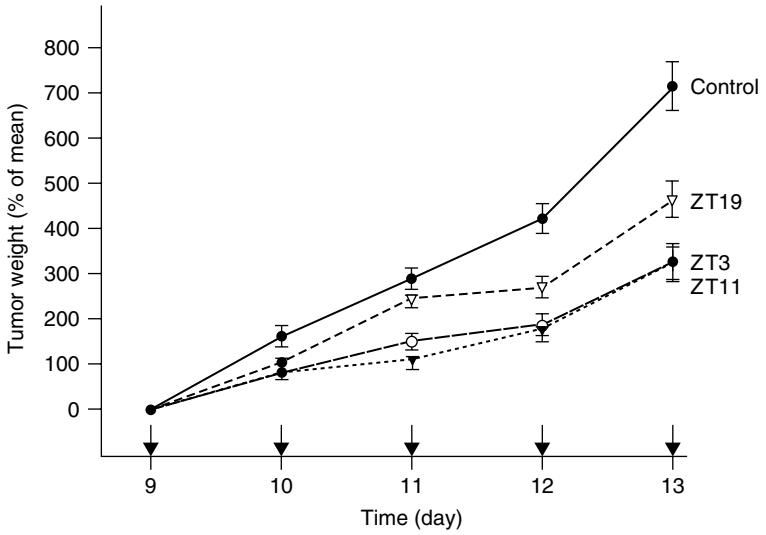

Figure 7 Effects of time-dependent dosing of Seliciclib on tumour growth. Tumour weight change as a function of Seliciclib administration time. •, controls; o, treated at ZT3; ▲, treated at ZT11; △, treated at ZT19. Treatment started on day 9 after tumour inoculation. Arrows, days of drug injection. Thirty-six controls and 32 mice were allocated to each treatment group. Points, mean; bars, SE. Differences between controls and animals treated at ZT3, ZT11 or ZT19 were validated with ANOVA ($p < 0.001$). (Reproduced with permission from Iurisci *et al*., 2006. © American Association for Cancer Research.)

with the most consistently reported clock gene to control cell growth being Per 1 ± 2 proteins. High levels of Per 1,2 are potent tumour suppressors that regulate ataxia telangiectasia mutated (ATM)/ATM and Rad3 related (ATR) protein kinase phosphorylation of Chk1/Chk2 which halt the cell cycle through G1-S/G2-M (Yu, 2007) and facilitate the DNA repair pathway (Chen-Goodspeed and Lee, 2007; Chen and Mcknight, 2007). The phosphorylation/stabilization of Per2 by CK1-ε) and both Per 1,2 potentiates the expression of several tumour suppressor proteins such as Cyclin D1, Cyclin A, Mdm-2 and Gadd45α (Fu *et al*., 2002).

The overexpression of Per 1,2 leads to greater vulnerability for cancer cells to undergo apoptosis due to suppressed expression of anti-apoptotic c-Myc, Bcl-X and Bcl-2 and elevating expression of proapoptotic p53 and Bax (Hua *et al*., 2006). In contrast, a deficiency or inhibition of Per 1,2 led to aggressive tumour proliferation, a stronger cell survival response, resistance to apoptosis and a reduction in tumour suppressor proteins, including the cyclins (Fu *et al*., 2002; Gery *et al*., 2006; Chen-Goodspeed and Lee, 2007). Low levels of Per 1, 2 are found in samples taken from patients with non-small-cell lung cancer (Gery *et al*., 2007), sporatic and familial primary breast cancer (Winter *et al*., 2007) and aggressive colorectal tumours (Krugluger *et al*., 2007). **Figure 8** shows the results of realtime reverse-transcription polymerase chain reaction (RT-PCR) for quantification of *Per 1,2, CLOCK* and dihydropyrimidine dehydrogenase (5-fluorouracil-metabolizing enzyme) in Grade 2 or Grade 3 primary colorectal tumours vs. normal colon mucosa from the same patients.

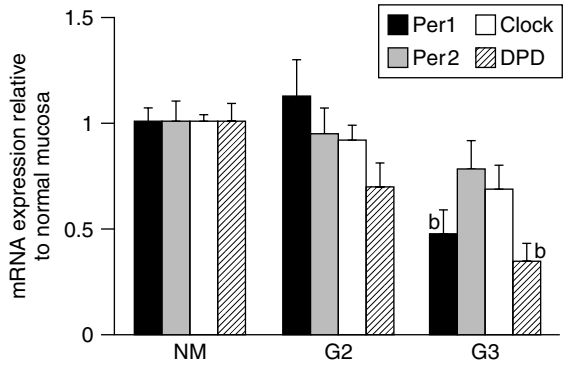

Figure 8 Relative change of Per1, Per2 and DPD mRNA levels in call patients with colorectal tumours grade 2 (G2) or grade 3 (G3) compared with normal mucosa (NM). Data represent fold change ±SD of specific mRNA levels in tumour specimen as compared with specimen of NM. a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.001$ (Reproduced with permission from Krugluger *et al*., 2007. © American Association for Cancer Research.)

The results show attenuation of clock genes in the order of *Per 1 > CLOCK > Per 2* in Grade 3 tumours derived from 30 cancer patients.

While Per 1 appears to be a primary regulator of tumour growth, a less pronounced association has been reported for other clock genes including Cry (Gauger and Sancar, 2005; Chen and McKnight, 2007) or Clock

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(Antoch *et al*., 2008). However, mutations in genes that encode factors which regulate clock genes *Per* and *Cry* contribute to a predisposition or risk for developing cancer. This is the case for mutations in the *CSNK1E* gene which encodes for CK1-ε which can adversely affect phosphorylation of clock-gene products and p53 tumour suppressor proteins thereby altering tumour growth and circadian rhythmic patterns (Knippschild *et al*., 1997; 2005) or mutations in NPAS2 which forms heterodimers with BMAL1 to regulate the clock genes *Per* and *Cry* (Zhu *et al*., 2008). Lastly, the direct administration of melatonin (as a chronobiotic agent to induce phase shift or establish synchronization) inhibits tumour growth, angiogenesis, proliferation and metastasis (Ravindra *et al*., 2006) possibly through attenuating fatty-acid growth-factor-uptake, telomerase, endothelin-1 synthesis (angiogenic factor) and activating the tumour suppressor p53 gene (Blask *et al*., 2005; Stevens *et al*., 2007).

4.3 Clock-Gene Coupling to DNA Repair

DNA repair enzymes are closely linked to the circadian clock and regulated by daily variations in exposure to UV radiation or genotoxins (Shadan, 2007). It is important to note that SCN clock genes are not necessarily aligned to the timing of peripheral clock genes, where the fluctuation of Cyr/Per that drive the motor could be different for diverse tissues based on need. However, as an example, the following study (Kang *et al*., 2009) assesses the activity of DNA repair enzymes monitored in reference to changes in clock genes: *Clock, Bmal1, Per* and *Cry* in cerebrum tissue derived from mice which were exposed to a 12 hour light/dark cycle: ZT0 = (lights on) and a 24 hour dark cycle where CT0 = (subjective time point zero, corresponding to ZT0 for the 12 hour light/dark cycle). **Figure 9A** represents the excision rate as assessed by protein A/+ substrate of 140 bp duplex with a 6–4 photoproduct in the centre and a ^{32}P label at the fifth phosphodiester bond 5′ to the damage; **Figure 9B** represents quantified excision activity.

These findings show that peak hours of DNA repair (**Figure 9A,B**) correspond to a reduction in Per2/Cry1 (**Figure 9C**) which occurs simultaneously at approximately 10–14 hours CT/ZT.

4.4 Clock-Gene Controls and Angiogenesis

Cyclical oscillation of Per 2/Cry 1 also regulate diurnal fluctuations in vascular endothelial growth factor (VEGF) promoter activity, where the efficacy of antiangiogenic agents can be optimized by administering drugs when VEGF production increases (Ohdo, 2007). In the following study (Shimizu *et al*., 2008) the effect of circadian rhythms on VEGF/tumour growth using antineovascular therapy (ANET), an Ala-Pro-Arg-Pro-Glyc peptide in a liposome were assessed in Colon 26 NL-17 tumour-bearing mice. The results demonstrate variable effects of tumour suppression at various hours after circadian light onset expressed as 'ZT = 0' on a 12 hour light/dark cycle, where tumour suppression peaked at ZT 14 (♦) (**Figure 10A**), also corresponding to lower plasma VEGF concentrations and greater tumour suppression (**Figure 10B**) (Shimizu *et al*., 2008).

Similar results were reported by Koyanagi *et al*. (2003), where a drop in VEGF gene transcription in hypoxic tumour cells implanted in mice occurred between ZT 10 and 14, near the early dark phase correlating to/or subsequent to a rise in Cry 2 or Per 1,2 which were in antiphase to BMAL1 and CLOCK (**Figure 11**).

Here, circadian fluctuation in VEGF protein levels in tumour-bearing mice (tumour mass and liver) showed similar temporal profiles as that reported by Shimizu *et al*. (2008) also providing information that fluctuating levels appear to be linked to the time clock genes *Per 1,2* .

4.5 Chronotherapeutic Applications and Chronotoxicology

In establishing effective time-wise delivery of chemotherapy drugs, it is important to ascertain how each drug works and to determine the circadian rhythms of the particular drug targets (Altinok *et al*., 2009), drug detoxification systems (Lévi, 2008) and fluctuations in hormonal or immunological variables (Lissoni *et al*., 2008). Applied chronotoxicology can render fewer side effects and greater efficacy, which is often obtained following treatment delivery at which side effects are least observed (Lévi, 1999; 2001; 2008), and achieved with drugs which are formulated to release drug concentrations to accommodate these patterns (Ohdo, 2007). Some applications include peak infusions of 5-fluorouracil at 04:00, folinic acid at 04:00, doxorubicin at 06:00, oxaliplatin at 16:00 and cisplatin at 16:00–20:00 hours (Ohdo, 2007). **Figure 12** reflects circadian rhythms in anticancer drug tolerability in laboratory mice or rats, as presented by Mormont and Levi (2003).

While time-guided administration of drugs continues to be a valuable tool in reducing side effects and improving therapeutic response rates, future research will be required to assess the efficacy of chronotoxicology

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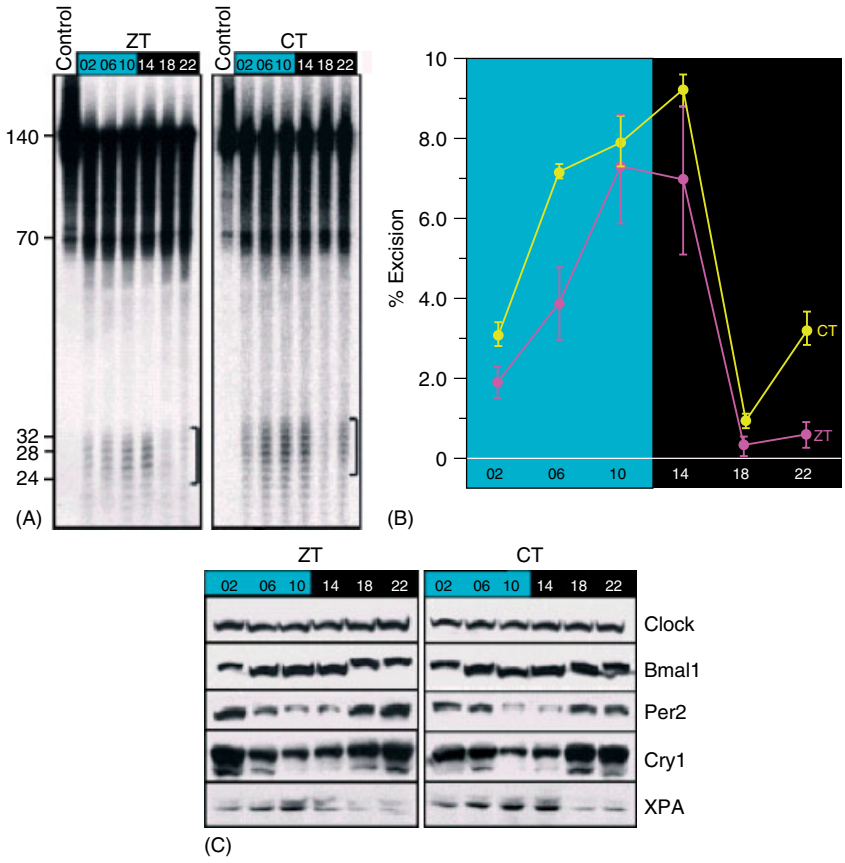

Figure 9 Circadian oscillations of nucleotide excision repair activity in the mouse brain. (A) Excision assays with mice cerebrum extracts prepared from brains harvested at the indicated ZT and CT times. Ten femtomoles of substrates were loaded as a control. (B) Quantitative analysis of the excision activity as a function of ZT or CT. For ZT, each data point represents the average of excision activity from the cerebrums of three mice, and the bars represent the SE. For CT, the data points represent the averages of duplicate experiments conducted with extracts from a single mouse for each data point. Bars indicate SD. (C) The effect of circadian clock on 'day/night' expression patterns of the clock genes and excision repair. Extracts from brains were harvested at ZT18 (night) and ZT06 (day) and were analysed by immunoblotting. Circadian oscillations of xeroderma pigmentosum A (XPA) determined by immunoblotting with the four core clock proteins. XPA peaks in the afternoon/evening hours and is in phase with BMAL1 and antiphase with *Cry* 1 and *Per* 2 indicating they are controlled by clock genes. (Reproduced with permission from Kang *et al*., 2009. © National Academy of Sciences.)

on long-term survival rates, in order to establish the relative impact of this science on tactical advantage in the treatment of cancer.

4.6 Chronokinetics and Chronotoxicology

While chronotherapeutics is synonymous to chronotoxicology in the study of cancer treatment or chemotherapy drugs to selectively kill tumour cells, the term chronotoxicology also describes the toxic effects of drugs or poisons relative to circadian rhythmic patterns. This requires an in-depth review of how each individual toxin is processed, metabolized and excreted in the human body ('chronokinetics'), the directed target or means by which it exerts its lethal effects ('chronodynamics') and the extent of toxicological damage (chronotoxicity) relative to 24 hour time points. Chronokinetics is further defined as a dosing time-dependent and predictable (rhythmic) change used to characterize the pharmacokinetics (or the bioavailability) of a drug, chemical or toxic substance which is often dependent on the solubility/physical properties (i.e. lipophilicity, hydrophilicity) of a compound (Reinberg, 1992). The distribution of poisonous substances throughout the body is governed by circadian rhythms inherent to peripheral tissues and organs that regulate absorption (dermal, digestive or pulmonary), distribution (delivery from the blood or lymph to tissue dependent upon circulation/binding capacity of plasma proteins), metabolic rate,

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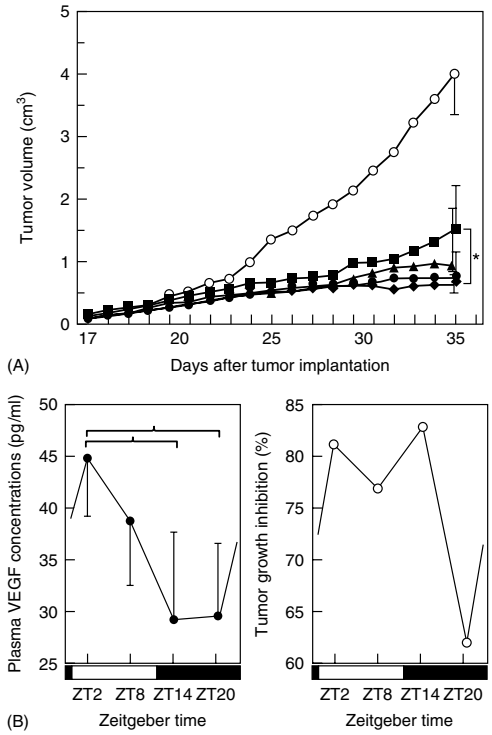

Figure 10 (A) Chronotherapy approach using APRPG-LipADM against C26 NL-17 carcinoma-bearing mice. C26 NL-17 carcinoma cells were subcutaneously implanted into Balb/c mice. The mice were intravenously administered glucose 0.3 M (O) or APRPG-LipADM (10 mg kg^{-1} as ADM) through a tail vein at ZT 2 (•), ZT 8 (▲), ZT 14 (♦) or ZT 20 (+) on days 18, 21 and 24 after tumour implantation. Tumour volume in each mouse was measured to evaluate therapeutic effects. Data are presented as a mean tumour volume and SD where the SD bars are shown only for the last points for sake of graphic clarity. Significant differences between APRG-LipADM treated groups are indicated; $^{*}p < 0.05$ (Reproduced with permission from Shimizu *et al.*, 2008. © The Pharmaceutical Society of Japan.). (B) Chronopharmacological relationship between VEGF concentration in the plasma and therapeutic effect. C26 NL-17 carcinoma cells were subcutaneously implanted into Balb/c mice and the mice were killed on day 18 at ZT 2 (•), ZT 8 (▲), ZT 14 (♦) or ZT 20 (+). Plasma VEGF concentration was determined (left panel) and tumour growth inhibition on day 35 as in the preceding figure are shown (right panel). Significant differences are indicated; $^{*}p < 0.05$. (Reproduced with permission from Shimizu *et al.*, 2008. © The Pharmaceutical Society of Japan.)

tissue storage capacity, liver function and excretion (i.e. glomerular filtration, tubular reabsorption and urinary pH), all of which establish a unique chronokinetic profile that varies from substance to substance (Reinberg, 1992).

The diurnal fluctuation in observed adverse effects of toxic substances is highly reliant on circadian rhythms that occur in liver detoxification processes. The liver is the major processing centre for drug metabolism, biotransformation and detoxification of lipid and soluble drugs/poisons involving oxidative Phase I (NADPH-cytochrome P450 reductase/cytochrome P450 electron transfer systems) or conjugative Phase II (uridine-5′-diphospho-glucuronosyltransferase) reactions, which regulate the rate and capability to eliminate toxic substances from the body through excretion. The cytochrome P450s (CYPs) involved with Phase I make the field of chronotoxicology extremely broad because there are >2500 cytochrome P450 sequences known and, in humans alone, there are 18 families of genes and 43 subfamilies which in large part control many aspects of steroid, drug and fatty-acid metabolism (Nelson, 1999; Nelson, 2003). The CYP (1–3) families and their 17 subfamilies play the most critical role in the metabolism of drugs and, just for one example alone, the CYP 2D6 family processes a large classification and number of drugs, including several of the antiarrhythmics (flecainide, mexiletine, propafenone); antidepressants (amitriptyline, paroxetine, venlafaxine, fluoxetine (Prozac), trazadone); antipsychotics (chlorpromazine, haloperidol, thoridazine); β-blockers (labetalol, timolol, propanolol, pindolol, metoprolol) and analgesics (codeine, fentanyl, meperidine, oxycodone, propoxyphene) (Nelson, 1999; Nelson, 2003). Other individual CYPs such as CYP 2E or CYP 1A2 are responsible for the metabolism of ethanol (Diaz Gómez *et al.*, 2006) and caffeine (Kot and Daniel, 2008), respectively.

Further, some drugs/substances can alter their own rate of detoxification by acting on hepatic tissue, as in the case with CYP inhibitors (blocking the detoxification of a drugs), that is, (−) CYP 3A4, CYP 2D6, CYP 2 C9 (grapefruit juice) (Girennavar *et al.*, 2007), CYP potentiators (enhancing detoxification of the drug), agents that alter the catalytic biotransformation of drugs, that is CYP 2 C8, CYP 3A4 (taxol) (Spratlin and Sawyer, 2007), induce drug interactions (Darwish *et al.*, 2008) or even gene mutations linked to disease predisposition, that is CYP 1B1 (congenital glaucoma) each which contribute confounding variables to a chronotoxicological profile (Firasat *et al.*, 2008).

Circadian rhythms in the liver are governed by clock genes which regulate the diurnal transcription of Phase I/Phase II enzymes, detoxification and transport of xenobiotics (Akhtar *et al.*, 2002; Storch *et al.*, 2002; Lim *et al.*, 2006). For example, the clock nuclear orphan receptors: RORα and RORγ are highly expressed in liver tissue and, in mice, a concentration peak is observed at approximately 16–24 HALO 'hours after light onset', also governing the transcription of genes encoding Phase I/Phase II metabolic enzymes (Kang *et al.*, 2007). Kang *et al.*, examined gene alterations in RORα and RORγ double knockout mice (DKO), where results show induction of genes (corroborating

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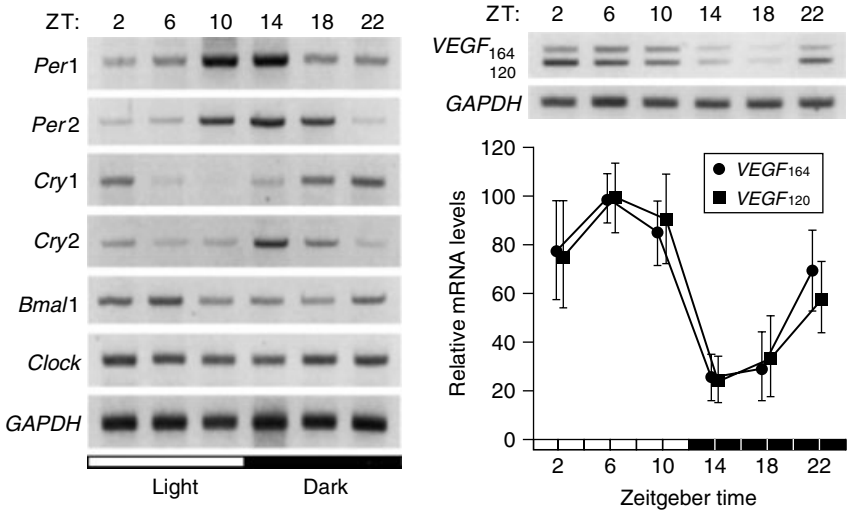

Figure 11 Circadian expression of clock genes and *VEGF* in implanted tumour cells. (A) Representative electrophoretic image of RT-PCR products of clock genes in tumour masses. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control for transcripts whose expression was constant throughout the day. The horizontal bar at the bottom indicates light and dark cycles. (B) Temporal profiles of mRNA expression of $VEGF_{164}$ and $VEGF_{120}$ in tumour masses. For plots of RNA, the mean peak values for $VEGF_{164}$ and $VEGF_{120}$ are set at 100. Each point represents the mean ± SE (bars; $n = 4–6$). The mRNA levels for both $VEGF_{164}$ and $VEGF_{120}$ exhibit significant circadian variations ($p < 0.01$, respectively; ANOVA). The upper panel shows a representative electrophoretic image of RT-PCR products of the *VEGF* gene. The horizontal bar at the bottom indicates light and dark cycles. (Reproduced with permission from Koyanagi *et al*., 2003. © American Association for Cancer Research.)

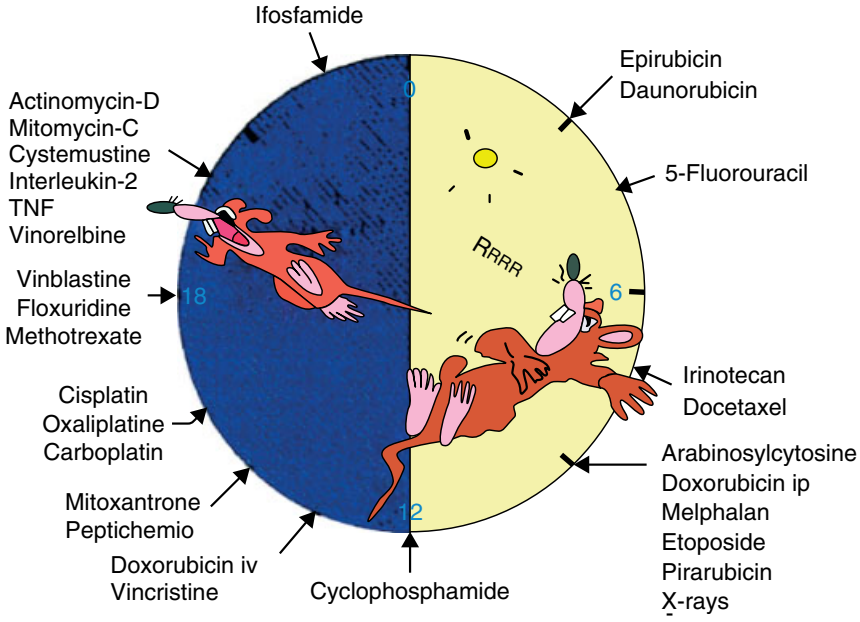

Figure 12 Circadian rhythms in anticancer drug tolerability in laboratory mice or rats. The least toxic dosing time is indicated for each cytostatic or immunologic agent as a function of the rest-activity cycle. iv: intravenous; TNF: tumor necrosis factor. (Reproduced from Mormont & Levi, 2003 © American Cancer Society.)

repressor effects in DKO mice) occur for a large number of P450s including (i.e. *Cyp2b9, Cyp2b13, Cyp4a, Cyp2b, Cyp39a1, Cyp4a10, Cyp3a25, Cyp3a41, Cyp2c38, Cyp2a4, Cyp3a16, Cyp4a1, Cyp3a11, Cyp2c37, Cyp2e1*), sulfotransferases (*Sult2a1, Sult1e1, Sult1d1, Sult1a1, Sult1c1, Sult5a1, Sult3a1*) and several glutathione S-transferase genes (*Mgst3, Gstm4, Gsta1*) to name a few (Kang *et al*., 2007).

Clock genes are also governed by other retinoid-related nuclear receptors, such as the peroxisome

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proliferator-activated receptor α (PPARα), which serves as a transcription factor regulating the expression of genes (Lemberger *et al*., 1996; Oishi *et al*., 2005). PPARα is highly expressed in liver tissue (Desvergne *et al*., 2006) and regulates circadian output expression of Phase I/Phase II proteins, such as Cyp4a-UGT (Johnson *et al*., 2002; Barbier *et al*., 2003; Teboul *et al*., 2008). Briefly, PPARs heterodimerize with the retinoid X receptor (RXR) and bind to DNA sequences (peroxisome proliferator hormone response elements) that turn on/off gene transcription. However, similar to ROR/Rev-erb, the PPARα/RXRα can also modulate CLOCK/BMAL1-mediated transcription of *Per* and *Cyr* (Nakamura *et al*., 2008) and for this reason, PPARα agonists are capable of entraining both liver and systemic circadian rhythms (Canaple *et al*., 2006; Shirai *et al*., 2007). These findings suggest that circadian expression of proteins involved in xenobiotic detoxification are largely regulated by ligand-dependent transcription factor nuclear receptors, which appear to be responsible for the daily fluctuation in drug metabolism and changes of drug effectiveness and toxicity.

Experimental analysis of daily fluctuations in toxicity requires routine chronotoxicological assessment, often carried out by determination of an LD_{50} (median lethal dose) in mice synchronized to a 12 hour light/dark cycle, with varying administration times measured by 'HALO'. Many studies show that the time of administration plays an enormous role in the variability of the toxicity/lethal effects of drugs. Examples of this would include that observed for ondansetron (a chemotherapy adjunct antinausea agent) in mice; where dosing at 1 HALO corresponded to an absence of mortality vs. dosing at 19 HALO, which dropped the survival rate by approximately 50% (Khedhaier *et al*., 2003). Similarly, florfenicol shows 2.5 times greater toxicity at 16 HALO compared to 4 HALO in mice (Picco *et al*., 2001). Opposite diurnal patterns are also observed for drugs such as cetirizine (histamine H1 receptor antagonist), where drug dosing at 17 HALO was associated with greater survival rate than at 5 HALO (Dridi *et al*., 2005). The chrontoxicological profile obtained for a chemical/drug or foreign substance has in its foundation a rationale for the observed effects, which are dependent upon how clock genes affect the specific drug target, such as receptors, ion channels, signalling systems and so on (chronodynamics) or specific enzymes in the liver responsible for its metabolism or detoxification (chronokinetics).

In summary, the chronotoxicological effects of poisons or toxic substances are highly dependent upon chronopharmacokinetics, which render a variation in biodistributions or absorption patterns, or chronodynamics, rhythmic variation in toxin target proteins or processes. The study of time and life processes are complex due to the many multifactorial interfering variables comprising oscillations, patterns governed by the reception and transmittance of all types of information occurring simultaneously in many tissues, organs and cells. Future investigations should aim to compile data between inter- and intraspecies variability for all types of chronological studies in tissues, organs and cells, to determine if there is a potential means by which to assess the probability of an outcome.

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Dedicated to the memory of Dr. Charles A. Walker who pioneered Chronotoxicology.

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Effects of Chirality on Toxicity

Mae Grace Nilos, Jay Gan and Daniel Schlenk

CONTENTS

1 INTRODUCTION

We live in an asymmetric world where life is neither fair nor even-handed—sociologically and physically.
David Triggle (2002)

Louis Pasteur was the first to describe 'chirality' as an inherent property of compounds which are products of biological processes (Tombo and Bellus, 1991; Kurihara and Miyamoto, 1998; Ali *et al*., 2003). His pioneering work on the resolution of tartaric acid in the middle of the nineteenth century allowed chemists to realize that optical activity results from molecular dissymmetry of the individual molecules of (+)- and (−)-stereoisomers, related as nonsuperimposable mirror-image forms (Mason, 1986). Following Pasteur's resolution of the tartaric acid racemate, J. H. van't Hoff and J. A. Le Bel, developed the concept of molecular asymmetry (Kurihara and Miyamoto, 1998). Pasteur's term, 'dissymétrie', for enantiomorphism (from the Greek enantios morphe which means opposite shape), was later replaced by 'chirality', from the familiar correlation to the mirror-image relationship between the left and the right hands (Mason, 1986). The significance of stereoisomerism, not only in relation to physicochemical, but also to biological properties, has since been recognized by scientists (Mason, 1986; Tombo and Bellus, 1991).

The first definitive example of differential bioactivity of a pair of drug enantiomers was that of British pharmacologist Arthur Cushny almost 100 years ago (Waldeck, 2003; Patel and Hutt, 2004). He demonstrated the higher potency of the natural, levorotatory alkaloid (−)-hyoscyamine over its racemate, atropine ((±)-hyoscyamine), in antagonizing cholinergic stimuli. He showed that endogenous adrenaline, which is also levorotatory, is twofold more potent than synthetic adrenaline, which is racemic (Waldeck, 2003). This was followed in 1933 by Easson and Steadman's postulate of a three-point interaction between a drug and its receptor, explaining stereoselectivity in the action of chiral drugs (Waldeck, 2003; Patel and Hutt, 2004).

However, apart from relatively few instances, stereoselectivity was essentially ignored after it was first described in the early years of the last century (Maher and Johnson, 1991; Patel and Hutt, 2004; Garrison, 2006). Following decades of essentially 'limited dimensionality' in pharmacology and therapeutics, in which racemates dominated the pharmacopoeia, the significance of stereochemistry is only beginning to be rediscovered, first in pharmaceuticals, and more recently in agrochemicals (Tombo and Bellus, 1991; Crossley, 1992; Shah *et al*., 1998; Agranat *et al*., 2002; Brocks *et al*., 2004; Patel and Hutt, 2004; Garrison, 2006). Several factors need to be considered when addressing the need for enantiomer-specific toxicological data for bioactive agents, including availability of suitable analytical techniques and appropriate assay methods (Waldeck, 2003). It is therefore not so much the lack of interest, but rather the challenges and the lack of necessary technology that kept scientists from looking into the importance of chirality in chemical toxicity.

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The separation and quantification of enantiomers is probably the greatest challenge in chiral chemical analysis (Gorog, 2007). Separation of enantiomers can only be achieved in chiral environments, mostly on chiral chromatographic columns containing chiral derivatives (e.g. β-cyclodextrin and α_1-acid glycoprotein (AGP)). In addition, enantiomeric separation of chemicals with two or three stereogenic centres can be challenging, since they consist of up to four to eight enantiomers. The difficulty in chiral analysis was further complicated by the lack of enantiomer standards, which are hard to synthesize and purify. Also, for compounds with labile chiral centres, enantiomers may undergo isomer inversion or racemization, which further complicates quantitative analysis of enantiomers (Testa *et al*., 1993; Lamzin *et al*., 1995; Reist *et al*., 1998; Wsol *et al*., 2004). The past 30 years saw significant advances in chiral separation and synthesis techniques, which helped facilitate the routine separation of enantiomers of a significant number of chiral compounds for stereoselective toxicity assessment (Tombo and Bellus, 1991; Wong, 2006; Agranat *et al*., 2002).

This chapter is not intended as an exhaustive compilation of enantioselective properties of chiral bioactive agents, but rather to provide an overview of the complexities that may result from chirality in order to underscore the importance of stereochemical considerations in both human and ecotoxicology. Discussions will focus on toxicological processes that might be subject to stereoselectivity. Because the nomenclature for stereoisomers in the literature is not uniform, optical activity, whenever possible, will be noted as (−)- and (+)- for the levo- and dextrorotatory enantiomers, respectively. When the absolute configuration is known, the designations (*R*) and (*S*) will be used.

2 DEFINITIONS AND NOMENCLATURE

A considerable number of organic chemicals exist in a number of isomeric forms, which can be classified as either structural isomers or stereoisomers (**Figure 1**). Stereoisomers have the same chemical groups connected in the same sequence of bonds and only differ in their 3D structures. Stereoisomers are comprised of nonsuperimposable mirror-image enantiomers and the non-mirror-image stereoisomers (comprising multiple chiral centres) diastereomers (McMurry, 2004; Brocks, 2006; Ulrich, 2007). Geometric isomers, which have as their basis for asymmetry restricted bond movement about a plane, are also possible, but are not common as pharmaceutical or agrochemical agents (Brocks, 2006).

The term 'chiral', as used to describe the molecular asymmetry of carbon-containing compounds was first coined by Lord Kelvin in his Baltimore Lectures on molecular dynamics (Lamzin *et al*., 1995; Ali *et al*., 2003). The term is derived from the Greek word 'cheir', which means 'hand'. Thus, chirality, in very simple terms is 'handedness' (i.e. the existence of left/right opposition) (Lamzin *et al*., 1995).

Chirality results from the asymmetric positions in the structure of a molecule (Davies and Teng, 2003). The occurrence of asymmetry in organic compounds results from a tetrahedral sp^3 hybridized atom (the 'chiral centre', which is usually carbon, but also phosphorus, sulphur and other tetrahedral-substituted atoms) on which are attached four different constituent atoms or groups (**Figure 2**) (Lamzin *et al*., 1995; Davies and Teng, 2003; Brocks, 2006; Sasaki, 2008). The presence of a carbon–carbon double bond that renders the molecule rigid may also result in a chiral structure. Steric hindrance arising from ring structures that prevents free rotation about a bond allows enantiomeric atropisomers to exist. For many of the polychlorinated biphenyl (PCB) congeners with three or four *ortho* chlorine substituents and PCB metabolites (e.g. PCB methyl sulphones), the restricted rotation around the biphenyl bond provides molecular stability, allowing for the formation of two stable rotational isomers that are nonsuperimposable mirror images of each other (Kania-Korwell *et al*., 2006; Wong, 2006).

The presence of a chiral centre in a molecule is associated with its ability to rotate plane-polarized light. Optical activity by a chiral compound is typically described by the direction of optical rotation. A clockwise rotation is generated by the dextrorotatory (*d*) or (+)-enantiomer, while a levorotatory (*l*) or (−)-enantiomer will rotate the plane-polarized light counterclockwise. It should be noted that this property has no influence on biological activity, but serves as a useful marker in the analysis of enantiomers (Maher and Johnson, 1991; Karim, 1996; Davies and Teng, 2003; Patocka *et al*., 2004; Brocks, 2006).

Another method of describing chiral compounds, as proposed by the International Union of Pure and Applied Chemistry (IUPAC), is the 'Cahn–Ingold–Prelog (CIP) (sequence) rule' or the '*R*–*S* convention' which indicates the absolute configuration and stereochemical description of a chiral entity (Sewell *et al*., 1998; Tucker, 2000; Agranat *et al*., 2002; Davies and Teng, 2003). It defines a chiral centre as being *R* (derived from the Latin rectus or clockwise) or *S* (sinister or counterclockwise), in reference to the spatial arrangement of the constituents around the chiral centre (van der Goot and Timmerman, 1988; Davies and Teng, 2003). If a chemical has more than one chiral centre, it is necessary to assign absolute configuration to each chiral centre.

The spatial orientation of the constituents surrounding the chiral carbon atom in amino acids and sugars is often identified by the prefix D or L (in small uppercase letters), referring to the absolute configuration of the compound's α-carbon (the chiral carbon of D- and

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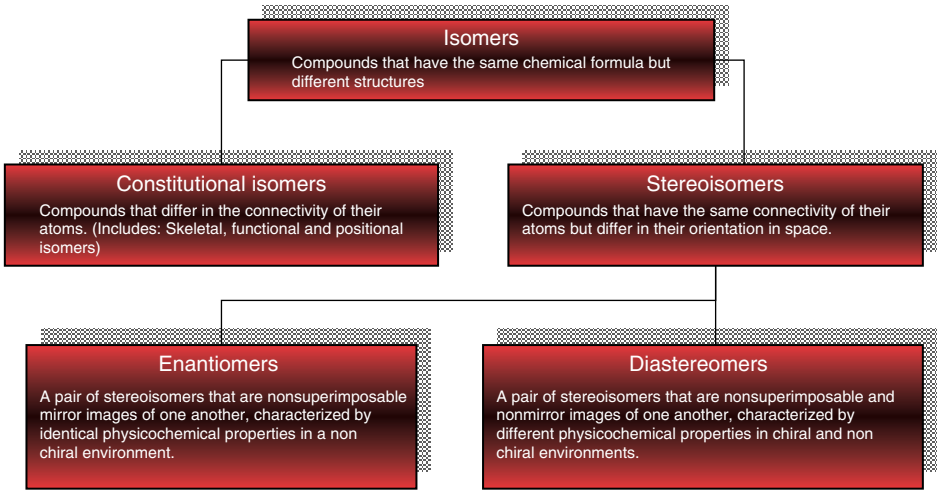

Figure 1 Basic terms in stereochemistry.

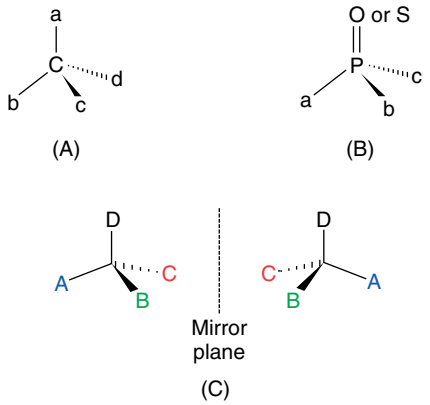

Figure 2 Structural representation of a chiral carbon (A); a chiral phosphorus (B) and pair of mirror image enantiomers (C) where A, B, C and D are different chemical groups attached to the chiral (asymmetric or stereogenic) centre.

L-glyceraldehyde) (van der Goot and Timmerman, 1988; Maher and Johnson, 1991; Lamzin *et al*., 1995).

The number of possible enantiomers increases with the number of asymmetric centres in a chiral molecule and so does the complexity of the required nomenclature. The number of enantiomers in a chiral compound is determined by the asymmetric (chiral) centres, which is given by the relationship 2^n, where n is the number of asymmetric positions (Maher and Johnson, 1991; Karim, 1996; Daniels *et al*., 1997). The term racemate generally denotes an optically-inactive equimolar (50 : 50) mixture of optical enantiomers (Mason, 1986). With the exception of optical activity, individual enantiomers possess the same physical and chemical properties when placed in an achiral environment. Biologically, however, enantiomers can be regarded as different compounds (Ariens, 1986; Agranat *et al*., 2002).

For a particular action (e.g. therapeutic or pesticidal), enantiomers are sometimes described based on their relative activity. The terms 'eutomer' and 'distomer' refer to the more active and less active enantiomer, respectively (Lehmann *et al*., 1976; Ariens, 1986; Maher and Johnson, 1991; Karim, 1996). The degree of selectivity for any given activity, is given by the 'eudismic ratio', or the potency ratio between the eutomer and distomer (Waldeck, 2003; Patel and Hutt, 2004). These terminologies and designations refer to a particular single activity (desired or adverse) of a chiral agent, so that the eutomer for one activity may be the distomer for another.

Table 1 provides a summary of the nomenclatures and their definitions commonly encountered in chiral chemistry.

3 CHIRAL RECOGNITION IN BIOLOGICAL SYSTEMS

Having observed that the mold, *Penicillium glaucum*, metabolized (+)-tartrate more rapidly than (−)-tartrate, Pasteur in 1858 commented, 'There cannot be the slightest doubt that the only and exclusive cause of this difference in the fermentation of the two tartaric acids is caused by the opposite molecular arrangements of the tartaric acids. In this way, the idea of the influence of molecular asymmetry of natural organic products is introduced into physiological studies, this important characteristic being perhaps the only distinct line of demarcation between dead and living matter.' (Booth *et al*., 1997; Triggle, 2002; Patel and Hutt, 2004).

The significance of chirality has long been recognized in relation to the biological activity of natural compounds and synthetic drugs (Caldwell, 1995; Karim, 1996; Kurihara *et al*., 1997; Ladik and Szekeres, 2006). At the molecular level, chirality is ubiquitous in proteins, carbohydrates, nucleic acids, lipids and steroids (Lamzin *et al*., 1995; Greer and Wainer, 2002;

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Table 1 Commonly used nomenclature in chiral chemistry and their definitions

Notation	Identification	Comments
	Optical activity	
(−) or *l*	Levorotatory—rotates plane-polarized light in a counterclockwise direction.	Optical activity is a property of an enantiomer that has no influence on its biological activity. However, it serves as a useful analytical marker in the identification of enantiomers in the laboratory.
(+) or *d*	Dextrorotatory—rotates plane-polarized light in a clockwise direction.	
	Absolute configuration	
R	Rectus (right)—if a curve arrow is drawn from the highest to the lowest priority substituent, the direction of rotation is clockwise.	Based on the Cahn–Ingold–Prelog (CIP) sequence rule, the *RS* convention indicates the absolute configuration of the molecule based on priorities assigned to atoms directly bonded to the chiral centre. Priorities are assigned in order of decreasing atomic numbers. *R* and *S* are italicized and connected with a hyphen only when used as a prefix. If a chemical has more than one chiral centre, it is necessary to assign absolute configuration to each chiral centre.
S	Sinister (left)—if a curve arrow is drawn from the highest to the lowest priority substituent, the direction of rotation is counterclockwise.	
	Amino acids and sugars	
D	In α-amino acids, the configuration is D if the amino group is on the right of the Fisher projection formula with the carboxylate written on top. In sugars, the configuration is D when the −OH is on the right of the Fisher projection formula with the C=O written on top and CH_2OH on the bottom.	The DL system is currently used only in amino acids and carbohydrates. Configuration at the chiral centre is assigned based on the planar (Fischer) projection rather than 3D formulas. The descriptors used are small capital D and L.
L	In α-amino acids, the configuration is L if the amino group is on the left of the Fisher projection formula with the carboxylate written on top. In sugars, the configuration is L when the −OH is on the left of the Fisher projection formula with the C=O written on top and CH_2OH on the bottom.	

McConathy and Owens, 2003; Inoue, 2005). These biomolecules are made up of units that have the same sense of chirality, so that any polymer chain folded into a three-dimensional structure generally adopts one of the two stereochemical configuration. This determines the fit of the enantiomer with biomolecular targets (Simonyi *et al*., 1986; Garrison, 2006).

Most carbohydrates have the D-configuration (Agranat *et al*., 2002). The 21 essential amino acids are L-enantiomers (with very few exceptions) (Lamzin *et al*., 1995). Moreover, many of the natural substrates and ligands for these systems are single stereoisomer chiral molecules (e.g. neurotransmitters, endogenous opioids and hormones) (Lamzin *et al*., 1995; Patel and Hutt, 2004). Therefore, when an exogenous chiral compound is introduced into the body, it is not surprising to see markedly different effects of enantiomers as a consequence of their differential interaction with receptors, enzymes and ion channels (Crossley, 1992; Triggle, 1994; Agranat *et al*., 2002; Hegeman and Laane, 2002; Brocks, 2006; Ladik and Szekeres, 2006). The interaction between chiral chemicals and receptor molecules may result in a number of possibilities, including: (i) equal activities of enantiomers, (ii) one enantiomer possessing all the expected activity, (iii) quantitatively different activities between enantiomers and (iv) qualitatively different activities of enantiomers (Triggle, 1994). However, it should be noted that chirality is certainly not a requirement for biological activity. However, in situations where the bioactive agent contains one or more chiral centres, the desired biological property is often determined by a specific

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absolute configuration (Tombo and Bellus, 1991; Brocks, 2006; Garrison, 2006).

The cornerstone in the understanding of chiral discrimination in biological systems is the multipoint attachment theory, postulated by Easson and Stedman in 1933, which states that the difference in activity results from the differential binding of a pair of enantiomers at a common site on an enzyme or receptor surface (Waldeck, 1993; Booth *et al*., 1997). According to this theory, at a minimum three of the four possible interactions occur for the 'more' active enantiomer as opposed to only two interactions with the 'less' active enantiomer (Lamzin *et al*., 1995; Greer and Wainer, 2002). In the experimental comparison of activities of (+)- and (−)-adrenaline, three functional groups, NH_2^+, phenylcatechol and alcoholic OH, attached to the asymmetric carbon of (−)-adrenaline were involved in the interaction with the receptor. In the mirror image (+)-adrenaline, the alcoholic OH group is oriented away from the site of the receptor, resulting in only two functional groups interacting between the molecule with the receptor, which according to Easson and Stedman explained the lower potency of the (+)-form (Patil and Miller, 2002).

Since the mid 1950s the three-point model has essentially become a key element in structure–activity relationship studies in medicinal chemistry and served as the basis for the design of a number of chiral stationary phases developed for chromatography (Booth, 1997; Mesecar and Koshland, 2000; Patel and Hutt, 2004). However, later studies showed that the simplified model does not universally hold.

For instance, Ogston's 1948 findings on the enzymatic decarboxylation of L-serine to glycine were also initially explained by the three-point interaction model. However, a re-examination of the process suggested that stereoselectivity was largely influenced by the 'correct' conformation at the binding site, which does not necessarily lie at the catalytic site. The critical step in the process was the stereoselective decarboxylation of a prochiral (nonchiral molecules that can be converted to a chiral molecule in one step) intermediate metabolite, aminomalonic acid (Booth *et al*., 1997). In which case, there were actually two interaction sites that produced binding interactions between the substrate and the enzyme. But because of the chirality of the enzyme, the catalytic site was positioned in such a way that only one of the conformations could be decarboxylated (Crossley, 1992; Booth *et al*., 1997). The same 'conformationally driven' chiral recognition mechanism was also used in describing the enantioselective discrimination by α-alkyl arylacetic acids on amylose tris(3,5-dimethylphenylcarbamate) chiral stationary phase (Booth *et al*., 1997).

More recently, by using the enzyme isocitrate dehydrogenase (IDH), Mesecar and Koshland (2000) proposed that a fourth 'location', which is not necessarily an 'interaction site', is essential in distinguishing between enantiomers in a protein structure. In their study, when a racemic mixture of isocitrate was introduced to Mg^{2+}-free crystals of IDH, only the L-isomer (2*S*,3*R*) bound to the enzyme. But when a racemic mixture of isocitrate was introduced to the enzyme crystals in the presence of Mg^{2+}, only the D-isomer (2*R*,3*S*) bound the enzyme. Further investigation revealed that three of the four groups attached to the tetrahedral C2 atom of D- and L-isocitrate bind to the same three locations in the IDH active site, the only difference being the fourth group (the hydroxyl of the C2 carbon). The –OH group of the L-isocitrate associates with an arginine residue at position 119 in the metal-free enzyme, while the –OH group of D-isocitrate associates with the metal and with two aspartate residues at positions 283′ and 307 in the enzyme. The authors concluded that it is the position of the fourth group that makes it possible for IDH to distinguish between the two enantiomers, further noting that the three-point attachment theory only worked if it is assumed that the ligand can approach a flat protein surface in only one direction (Mesecar and Koshland, 2000). If the binding sites on the protein are in a cleft or on protruding residues, a three-point attachment will not be sufficient to discriminate between isomers (Booth *et al*., 1997; Mesecar and Koshland, 2000). The 'four-location' model appears to be the current consensus among chiral-recognition mechanism studies (Patel and Hutt, 2004).

4 IMPORTANCE OF CHIRALITY IN XENOBIOTIC TOXICITY

The past 25 years have seen significant improvements in the asymmetric synthesis and chiral separation technologies that have allowed for the isolation of an increasing number of enantiomers on a laboratory scale and consequently, the evaluation of pharmacological properties of individual enantiomers (Agranat *et al*., 2002; Gorog, 2007; Shimazawa *et al*., 2008). An off-shoot of this is the rapid growth in the proportion of enantiomerically pure drugs entering the new drugs market.

From the mid to late 1980s, racemates accounted for 25% of the drug market (Simonyi *et al*., 1986; Patel and Hutt, 2004). Recent reports indicate that approximately 50% of commercially available drugs are chiral (Rentsch, 2002; McConathy and Owens, 2003). The proportion of single-enantiomer drugs that are being introduced in the market has grown steadily, rising from approximately 20% of new drugs 10 years ago to almost 75% in 2002 (Agranat *et al*., 2002). The world market share of single-enantiomer drugs increased from 27% in 1996 to 39% in 2002 (Shimazawa *et al*., 2008). The development of single enantiomers from existing racemates, known as 'chiral switches', has also become an important component of drug development portfolios

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(Agranat *et al*., 2002). Beginning in the early 1990s, a number of regulatory authorities worldwide have asked for pharmacological, pharmacokinetic and toxicological profiles for individual enantiomers prior to the registration of a new chiral drug (Rentsch, 2002).

4.1 Toxicodynamic Complexities

The most significant factor responsible for differential pharmacological activities between enantiomers is in drug–receptor interactions, which are essentially 3D events (Maher and Johnson, 1991; Patel and Hutt, 2004). If the drug molecule is chiral this interaction can be expected to be stereoselective (Crossley, 1992; Triggle, 1994; Waldeck, 2003; Brocks, 2006). Moreover, stereoselectivity usually occurs at specific sites in the receptor (Ariens, 1986; Kwon and Triggle, 1991; Triggle, 1994). This is because interaction is primarily based on structural 'complementarity' of the drug molecule and its receptor site (Ariens, 1986).

A chiral molecule may interact with a number of receptor systems, including metabolic enzyme systems, neurotransmitter receptors linked to a second messenger system, or ligand/voltage-gated ion channels. These systems usually occur in structurally related families so that it is possible for enantiomeric pairs to have different pharmacodynamic actions as a result of activation of entirely different receptor populations, thereby contributing to side effects sometimes found in the racemates (Crossley, 1992; Maher and Johnson, 1991). For instance, differentiation between muscarinic acetylcholine receptor subtypes has been demonstrated by stereoisomers of atropine-like compounds. In the case of atropine itself, all the presynaptic muscarinic autoreceptor activity is due to the *R*-(+) enantiomer, but the postsynaptic activity is mainly due to the *S*-(−) enantiomer (Crossley, 1992).

A study of the enantiomers of hexahydrodifenidol and its chiral acetylenic analogues showed that the antimuscarinic potencies and receptor subtype selectivity of the compound depend on various structural parameters, including absolute configuration. The Ml (neuroblastoma), M2 (cardiac), M3 (pancreatic) and M4 (striatum) muscarinic receptors make qualitatively and quantitatively different stereochemical demands for the (*R*)- and (*S*)-enantiomers, resulting in different receptor selectivity profiles (Feifel *et al*., 1990). In addition, *S*-hexahydrodifenidol was found to be nonselective between muscarinic receptor subtypes, whereas the *R*-enantiomer was selective for the Ml receptor (Waelbroeck *et al*., 1991).

A number of psychiatric drugs currently in the market are chiral compounds whose individual enantiomers show marked differences with regard to their interaction with receptors and metabolizing enzymes. These include antidepressants (tricyclics, selective serotonin reuptake inhibitors, monoamine oxidase inhibitors, viloxazine, buprion, trazodone, mianserine, venlafaxine), benzodiazepines, zopiclone and several antipsychotics (Rentsch, 2002). *In vitro* studies have indicated (*S*)-fluoxetine, (*R*)-fluoxetine and (*S*)-norfluoxetine have similar potencies as 5-hydroxytryptamine (5-HT) reuptake inhibitors, but (*R*)-norfluoxetine is approximately 20 times less potent (Rentsch, 2002; Patel and Hutt, 2004). Similarly, *in vitro* studies have also shown that the *S*-(+)-enantiomers of another antidepressant drug, citalopram and its metabolites are more potent serotonin reuptake inhibitors over the corresponding *R*-(−)-enantiomers (Rentsch, 2002).

It is not uncommon that therapeutic effects are predominantly associated with one enantiomer, but adverse effects may be derived from the other enantiomer. For example, the potencies of the individual enantiomers of the anaesthetic and analgesic, ketamine, indicate a much higher potency of *S*-(+)-ketamine as compared with the *R*-(−)-enantiomer. However, *R*-(−)-ketamine, but not *S*-(+)-ketamine, was found to be responsible for the side effects associated with the racemate (e.g. hallucinations, restlessness and agitation during operative recovery in surgical patients) (Lien, 1995; Rentsch, 2002; Patel and Hutt, 2004).

Ligand- and voltage-gated ion channels represent major classes of pharmacologic receptors that show stereoselectivity in drug interactions (Triggle, 1994; 1996). The general characteristic of ion channels is that each possesses multiple drug binding sites. For instance, the voltage-gated Na^+ and Ca^{2+} channels, as well as the γ-amino butyric acid $(GABA)_A$-gated Cl^- channel each has at least five separate classes of drug binding sites (Triggle, 1994). Stereoselectivity of drug action at these binding sites is quite common (Clarkson, 1989; Triggle, 1994; Patel and Hutt, 2004).

The interpretation of stereoselectivity at these channels is complicated by differences in affinity and access to different channel states (Kwon and Triggle, 1991). In addition, membrane potential can also influence the stereoselectivity of drug interactions. For example, RAC-109 and its quaternary *N*-methyl derivative RAC-421 are local anaesthetics that act at the Na^+ channels without stereoselectivity in the resting state. However, stereoselectivity is observed with increased depolarization, as determined in the increased activity of the (−)-enantiomer, possibly resulting from differences in the binding site conformation between the resting, open and inactivated states of the Na^+ channel (Triggle, 1994). Similarly, the (*S*)-enantiomers of 1,4-dihydropyridines are activators, while the (*R*)-enantiomers are antagonists at the L-type Ca^{2+} channel. The activator properties of the (*S*)-enantiomer are membrane-potential-dependent, and at a sufficiently depolarized state it becomes antagonistic (Triggle, 1994).

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Enantiomers may also have opposing activities at the same receptor (Patel and Hutt, 2004). For example, the (+)-enantiomer of picenadol, a phenyl piperidine opioid analgesic, was shown to be two to three times as potent as morphine, while its antipode has about 1/10 of the activity of the morphine antagonist, naloxone. The (3*R*,4*R*)-(+)-picenadol is a strong μ-opioid receptor agonist while the (−)-antipode (3*S*,4*S*) is a weak antagonist at the same receptor (Leander and Zimmerman, 1983; Froimowitz and Coy, 1995; Shah *et al*., 1998).

There are a number of excellent reviews that detail both pharmacokinetic and pharmacodynamic similarities and differences between enantiomeric pairs of current pharmacologically important chiral drugs (Shah *et al*., 1998; Rentsch, 2002; Brocks *et al*., 2004; Mehvar and Brocks, 2004).

4.2 Toxicokinetic Complexities

The interaction of compounds with molecular targets is one of the fundamental rules of pharmacodynamics (Mayer and Testa, 1994). This rule also applies to pharmacokinetic and toxicokinetic processes. In addition to the so-called principal activity of stereoisomers, individual enantiomers may behave as distinctly different chemicals with respect to their absorption, distribution, metabolic transformation, excretion and toxicological properties in the body (Walle and Walle, 1986; Tocco *et al*., 1990; Tombo and Bellus, 1991; Mayer and Testa, 1994; Karim, 1996; Patel and Hutt, 2004). Therefore, stereoselectivity not only in toxicodynamic (pharmacological or toxicological), but also toxicokinetic (dispositional) processes may be responsible for the differential bioactivity of enantiomers.

4.2.1 Uptake

Stereoselectivity in a biological process often starts with the stereospecific recognition of a substrate by a biochemical entity, most often a protein, such as receptor or enzyme, responsible in facilitating that process. With respect to absorption, there are only a limited number of examples in the literature. In general, these studies indicate no significant stereoselectivity for chiral drugs, except in cases involving active transport (Walle and Walle, 1986; Karim, 1996; Waldeck, 2003; Brocks *et al*., 2004; Brocks, 2006).

The absorption and transport of a xenobiotic compound occur through a number of mechanisms. Passive diffusion depends on physicochemical properties of the agent, such as lipophilicity, polarity and molecular size. Carrier-mediated mechanisms such as facilitated diffusion occur via transmembrane carriers along concentration gradients. Active transport is mediated through carrier proteins, like P-glycoprotein, independent of the concentration gradient (Tsuji and Tamai, 1996; Zschiesche *et al*., 2002). For most drugs, the primary absorption and transport process across a biological membrane is through passive diffusion, suggesting that enantioselectivity may not play a prominent role in the absorption of most drugs (Walle and Walle, 1986; Mayer and Testa, 1994; Tsuji and Tamai, 1996; Patel and Hutt, 2004; Brocks, 2006). Enantioselectivity is not expected for drugs transported via passive diffusion, because enantiomers have identical physicochemical properties, such as lipid–water partitioning coefficients (Maher and Johnson, 1991; Patel and Hutt, 2004). In contrast, enantiomers of chiral drugs can be stereoselectively absorbed through carrier-mediated (active) membrane transport mechanisms such as those involved in intestinal absorption (Tsuji and Tamai, 1996; Itoh *et al*., 2001; Brocks, 2006).

Chiral drugs such as methotrexate, leucovorin, L-3,4–dihydroxyphenylalanine (L-DOPA), cephalexin and terbutaline have been shown to be stereoselectively absorbed (Wade *et al*., 1973; Tamai *et al*., 1988; Karim, 1996; Itoh *et al*., 2001; Patel and Hutt, 2004). L-DOPA, used in brain neurotransmitter replacement therapy for the treatment of Parkinson's disease, has been shown to be selectively absorbed in the intestine over the D-enantiomer (Wade *et al*., 1973). In fact, researchers have used these findings as evidence for the existence of an active transport mechanism for the intestinal absorption of L-DOPA.

Stereoselectivity was also reported in the absorption of methotrexate (L-(+)-enantiomer), a folic acid analogue, clinically used as an antineoplastic and antirheumatic agent (Hendel and Brodthagen, 1984; Itoh *et al*., 2001). Both folic acid and methotrexate were shown to be actively taken up into the brush-border membrane vesicles through the folate transporter of rabbit small intestines. In contrast, the affinity of the antipode D-amethopterin (D-(−)-enantiomer) to the folate transporter was approximately 60-fold lower than that of methotrexate (Itoh *et al*., 2001).

For most transporters, however, the magnitude of stereoselectivity is much smaller or almost negligible (Itoh *et al*., 1998; 2001). Only a 3.5-fold difference in affinity to the canalicular multispecific organic anion transporter was observed between amethopterin enantiomers (Itoh *et al*., 2001). Also, while significant diastereoselective renal secretion of carbenicillin stereoisomers was observed *in vivo*, *in vitro* studies using basolateral membrane vesicles of rabbit kidneys showed that transport by the organic anion transporter was not stereoselective (Itoh *et al*., 1998).

No stereoselectivity was observed for the mammalian P-glycoprotein-mediated intestinal secretion of the chiral β-adrenoreceptor-blocking drug, talinolol (Gramatte and Oertel, 1999; Zschiesche *et al*., 2002). But because most P-glycoprotein substrates are chiral (e.g. chloroquine, primaquine, itraconazole, cisapride, fexofenadine and

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talinolol), there is currently great interest in the potential stereoselectivity in P-glycoprotein-mediated efflux mechanisms (Patel and Hutt, 2004; Brocks, 2006).

4.2.2 Distribution

The most studied components of the human serum that play major roles in drug binding are human serum albumin and AGP (Fernandez *et al*., 1999; Patel and Hutt, 2004). Human serum albumin, the most abundant protein in the blood, has long been regarded as a general binder and carrier component (Simonyi *et al*., 1986). However, the AGP molar concentration in blood is approximately 30 times lower than that of human serum albumin, and therefore the binding capacity is expected to be much smaller. Nevertheless, its contribution to overall plasma protein binding has been observed (Albani *et al*., 1984), and in some cases, dominates certain slightly basic drugs (e.g. β-adrenergic blockers and psychotropic agents) (Simonyi *et al*., 1986; Patel and Hutt, 2004).

For instance, stereoselective binding of propranolol has been observed in human plasma, where *S*-(−)-propranolol showed higher mean binding over the *R*-(+)-enantiomer (Albani *et al*., 1984; Simonyi *et al*., 1986). The observed stereoselectivity has been attributed to the predominance of the enantioselective binding of the (−)-enantiomer to the AGP, rather than the human serum albumin, to which the (+)-propranolol enantiomer showed a higher (although not statistically significant) mean binding (Albani *et al*., 1984). Stereoselectivity in drug binding to human serum albumin and AGP has also been described for disopyramide (Lima *et al*., 1984), hydroxychloroquine (McLachlan *et al*., 1993) and the nonsteroidal anti-inflammatory drug (NSAID) 2-arylphenylpropionic acid (Jones *et al*., 1986). From these observations, the highly enantioselective nature of AGP has been exploited in the development of chiral stationary phase columns for the chromatographic resolution of a wide range of chiral compounds (Simonyi *et al*., 1986; Hermansson, 1989; Li and Lloyd, 1993).

Plasma protein binding studies of zopiclone (a short-acting hypnotic agent, available commercially as a racemate), showed (+)-zoplicone to have higher affinity for human serum albumin and AGP, although overall, the (−)-enantiomer was preferentially bound to plasma proteins, possibly due to the higher affinity of (−)-zoplicone to other plasma proteins (e.g. globulins and lipoproteins) (Fernandez *et al*., 1999).

Some drugs display interspecies differences in the direction of stereoselectivity in pharmacokinetic processes, including plasma protein binding (Fernandez *et al*., 1999; Brocks, 2006). For example, in the differential binding of disopyramide enantiomers, (*S*)-(+)-disopyramide was bound in higher concentrations than its antipode to serum proteins in humans, gorillas and pigs, while the reverse was true in serum and albumin from cows and sheep (Lima, 1988). Similarly, stereoselectivity in plasma protein binding of MK-571 (a potent leukotriene D_4 receptor antagonist developed for asthma treatment), enantiomers was found to be species dependent. Lin *et al*. (1990) were able to classify 12 mammalian species into three groups: (i) those that bind the *S*-(+)-enantiomer to a greater extent than the *R*-(−)-enantiomer (human, baboon, monkey, cow, dog and cat); (ii) those that bind the *R*-(−)-enantiomer more extensively (rat, guinea pig and sheep) and (iii) those that show no stereoselectivity (rabbit, hamster and mouse). Serum albumin was also found to be the major binding component for MK-571 enantiomers, and that the species differences in stereoselective binding were largely influenced by structural differences in the albumin molecule (Lin *et al*., 1990; Tocco *et al*., 1990). (−)-Propranolol is also more strongly bound in human plasma than (+)-propranolol, but the opposite is true in rats (Fernandez *et al*., 1999).

Specific and/or nonspecific binding to tissue proteins is another factor that may impart enantioselectivity in drug distribution (Simonyi *et al*., 1986; Patel and Hutt, 2004; Brocks, 2006). However, the molecular events involved are more complex than in plasma protein binding, and therefore not as well studied. Overall, the stereoselectivity in plasma protein binding of chiral drugs is well documented (Brocks, 2006). Summaries of some of these studies are provided by Brocks (2006), Mehvar and Brocks (2004) and Patel and Hutt (2004).

4.2.3 Metabolic Transformation

The wanted (therapeutic) and unwanted (toxicity) effects of drugs can be due to the parent compound and/or its metabolites. Biotransformation of a drug can lead to the formation of inactive metabolites that are eventually eliminated, or to therapeutically and/or toxicologically active metabolites responsible for part or all of the therapeutic and/or toxic effects (Testa, 1986) (*see* **Biotransformation of Xenobiotics**). As discussed previously, the interaction of enantiomeric substrates with macromolecules such as enzymes results in specific orientations to the catalytic site and the formation of energetically different pairs of complexes (Mayer and Testa, 1994; Caldwell, 1995). Thus, enantiomeric pairs are frequently metabolized at different rates and/or via different routes to yield products that vary in nature and/or extent (Caldwell, 1995; Patel and Hutt, 2004).

The largest enantiomeric differences in the pharmacokinetic properties of a chiral chemical are primarily derived from hepatic metabolizing activity (Walle and Walle, 1986). However stereochemical differences in presystemic drug metabolism is worth mentioning as well (Echeilbaum, 1988; Brocks, 2006). A few of the examples include the stereoselective first-pass metabolism of the more active *S*-(−)-verapamil enantiomer following oral dosing (Vogelgesang *et al*., 1984; Brocks, 2006; Hanada *et al*., 2008), as well as

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S-carvedilol, which undergoes more extensive first-pass metabolism than *R*-carvedilol (Ishida *et al*., 2007), and the observed higher first-pass metabolism of the (*R*)-enantiomer of the anti-inflammatory compound tazofelone in humans (Clay *et al*., 1999).

Stereoselectivity in metabolic transformation has been classified into two basic types, substrate stereoselectivity and product stereoselectivity (Caldwell, 1995). Substrate stereoselectivity is observed when, under similar conditions, the biotransformation of enantiomers of a chiral drug is quantitatively and/or qualitatively different (Mayer and Testa, 1994; Patel and Hutt, 2004). On the other hand, product stereoselectivity occurs when quantitatively or qualitatively different stereoisomeric metabolites are generated from a single chiral, prochiral or proachiral substrate (Mayer and Testa, 1994; Caldwell, 1995). Additionally, in asymmetric inductions involving enzymatic reactions where there is a preferential formation of a particular diastereomeric product from one enantiomer, the term 'substrate–product stereoselectivity' was proposed (Mayer and Testa, 1994; Patel and Hutt, 2004).

Alternatively, stereoselective metabolic events can be classified according to the outcome of the transformation events, which include: (i) prochiral to chiral; (ii) chiral to chiral; (iii) chiral to diastereomeric; (iv) chiral to nonchiral transformations and (v) chiral inversion (Caldwell, 1995; Patel and Hutt, 2004).

The S- and N-oxidation of xenobiotic compounds is primarily mediated by the microsomal flavin-containing mono-oxygenase (FMO), in addition to the cytochrome P450 systems (Beedham, 1997; Cashman, 1998; Cashman, 2000). Prochiral to chiral transformation occurs readily with each system (Cashman *et al*., 1993; Moroni *et al*., 1995; Rodrigues *et al*., 1995; Cashman, 1998; Cashman, 2000). For example, for the ulcer treatment drug, cimetidine, S-oxidation is the preferred metabolic route leading to inactivation *in vivo*. Cashman *et al*. (1993) have shown that this process is primarily catalysed by FMO3 in human liver, leading to the formation of a chiral sulfoxide. Moreover, FMO showed stereopreference for the formation of (−)-cimetidine-S-oxide *in vivo*, as indicated in the urinary ratio of (+)25.5 : (−)74.5% (Cashman *et al*., 1993). Similarly, the broad-spectrum antihelmintic human and veterinary drug, albendazole is another example of a prochiral drug that is stereoselectively oxidized to S-oxide metabolites (Moroni *et al*., 1995; Velik *et al*., 2003). A high level of stereoselectivity was also observed in the extrahepatic sulfoxidation of the organophosphate and carbamate insecticides fenthion and methiocarb by FMO1 (Furnes and Schlenk, 2005). In fact, stereochemistry can be used to differentiate the contributions of FMO isoforms, as FMO1 and FMO2 are more stereoselective with regard to tolyl-sulfide sulfoxidation, whereas FMO3 provides a 50 : 50 mixture of enantiomers (Rettie *et al*., 1995).

In the racemic mixture of warfarin however, where the (*S*)-enantiomer is three to five times more potent than *R*-warfarin, both enantiomers are extensively metabolized in the liver. The biotransformation of the more potent *S*-enantiomer is stereoselective to (*S*)-7-hydroxywarfarin by CYP2C9, whereas the *R*-enantiomer is metabolized to (*R*)-6, (*R*)-7, (*R*)-8 and (*R*)-10-hydroxywarfarin by several cytochrome P450 isozymes, including CYP1A2, CYP3A4 and CYP2C19 (Uno *et al*., 2008). The enantiomers of warfarin in this case are transformed by different routes and/or at different rates producing metabolites that are not stereochemically different from the parent compound (Caldwell, 1995).

Other studies on the stereochemistry of warfarin metabolism in man have shown that ketone reduction in the side chain following the aromatic hydroxylation of mainly the *R*-enantiomer, produces a new chiral centre and diastereoisomers, (*R*,*S*)-warfarin alcohol or (*S*,*S*)-warfarin alcohol (Lewis *et al*., 1975; Hermans and Thijssen, 1992). This is an example of a stereoselective metabolic transformation of a prochiral group in chiral drugs yielding a diastereoisomer (Caldwell, 1995). Another example is hexobarbital. In addition to the relatively more rapid rate of metabolism of (+)-hexobarbital by rat liver microsomes, (+)-hexobarbital forms β-3′-hydroxyhexobarbital, whereas the (−)- enantiomer is preferentially metabolized to α-3′-hydroxyhexobarbital (Caldwell, 1995). Conjugation reactions, in some cases, may also result in enantiomers of chiral xenobiotics being conjugated at different rates with an achiral or chiral endogenous conjugating substrate to give pairs of diastereoisomeric conjugates. Some well-known examples include the glucuronide conjugates of 2-phenylpropionic acid and oxazepam, and the glutamine and glutathione conjugates of 4-chlorophenoxypropionic acid and bromovaleric acid, respectively (Caldwell, 1995; Hayball, 1996; Patel and Hutt, 2004).

Although it does not commonly occur, when chiral compounds undergo biotransformations at the chiral centre, chirality may be lost (Caldwell, 1995). An example is the oxidative aromatization of the dihydropyridine calcium-channel-blocking drug, nilvadipine at the N-chiral centre to produce an achiral pyridine analogue (Niwa *et al*., 1989). The stereoselective oxidation of omeprazole at the chiral sulfoxide centre to sulfone is another example (Abelo *et al*., 2000; Patel and Hutt, 2004; Brocks, 2006). The metabolic deamination of amphetamines to nonchiral phenylacetone and ammonia in rabbit liver microsomes was also stereoselective to (−)-amphetamine (Patil and LaPidus, 1972).

It has also been observed that certain chiral drugs may undergo interconversion wherein an enantiomer converts to another configuration, via enzymatic or nonenzymatic processes (Testa *et al*., 1993; Wsol *et al*., 2004). Thalidomide is perhaps the most well-known example. Banned for use as a sedative in the 1960s due to

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its propensity to cause serious birth defects, thalidomide recently attracted interest for its anti-inflammatory properties first demonstrated in the treatment of leprosy (Caldwell, 1995; Reist *et al*., 1998; Waldeck, 2003; Brocks, 2006). Initial studies using oral administration to rabbits and mice showed that both thalidomide enantiomers were equally teratogenic and had the same sedative effect. However, (*S*)-(−)thalidomide, rather than (*R*)-(+)-thalidomide, was shown to cause dose-dependent teratogenicity in both rats and mice when given intraperetoneally, leading to the premature conclusion that the adverse effect of thalidomide might have been avoided if only the pure (*R*)-(+)-enantiomer had been used (Vermeulen, 2003).

Further studies later confirmed that the sedative effect by thalidomide in man is related to the (*R*)-thalidomide (Shah *et al*., 1998). However, the teratogenic effect of thalidomide may still not have been avoided by using enantiopure (*R*)-thalidomide because thalidomide enantiomers undergo rapid bidirectional (racemization) interconversion in biological media, as shown in *in vitro* as well as *in vivo* studies (Waldeck, 2003; Brocks, 2006). This makes the determination of enantioselectivity in therapeutic and toxic action complicated for chiral drugs like thalidomide. Albumin, hydroxyl ions, phosphates and amino acids (in particular, basic amino acids like arginine and lysine), were found to influence the base-catalysed thalidomide enantiomer inversion *in vivo* (Testa *et al*., 1993; Reist *et al*., 1998). The rapid interconversion of thalidomide following oral administration explains the apparent lack of selectivity in the orally administered drug.

A few other examples of enzymatic reactions that lead to chiral interconversion are those of chiral NSAIDs, classified as arylpropionic acid derivatives (also known as the 'profens'), which undergo substrate- and species-dependent metabolic stereoisomer conversion (Testa *et al*., 1993; Caldwell, 1995; Hayball, 1996; Wsol *et al*., 2004; Hao *et al*., 2005; Brocks, 2006).

Chiral profens possess a chiral centre α- to the carbonyl group and their therapeutic activity is mainly concentrated in the *S*-(+)-enantiomer, with the *R*-(−)-enantiomers being weakly active or inactive (Caldwell, 1995). Most arylpropionates undergo unidirectional inversion from the *R*-(−)- to *S*-(+)-configuration, so that the reaction is essentially a bioactivation of the *R*-enantiomers, which in this case may be regarded as prodrugs for the pharmacologically active *S*-enantiomer (Hao *et al*., 2005). The molecular mechanism of the inversion reaction of profens involves a stereospecific formation of a coenzyme-A thioester from the *R*-enantiomer, which undergoes a series of steps (Caldwell, 1995; Wsol *et al*., 2004). The first step is the stereoselective activation of the *R*-(−)-enantiomer via the formation of acyl-CoA thioester in the presence of coenzymeA (CoA), adenosine-5′-triphosphate (ATP) and Mg^{2+}. This is followed by the enzymatic epimerization of the *R*-thioester to the *S*-(+)-thioester. The final step is the release of the active *S*-(+)-enantiomer by hydrolysis of the thioester (Williams *et al*., 1986; Hayball, 1996; Wsol *et al*., 2004). The toxicological importance of such chiral inversion processes has been attributed to the acyl-CoA thioester intermediates, which can modulate lipid metabolism by inhibiting mitochondrial β-oxidation of fatty acids and forming lipid acylglycerols that can be incorporated into membranes and could potentially alter membrane structure, function or second messenger signalling (Caldwell, 1995; Wsol *et al*., 2004).

4.2.3.1 Toxicological Consequences of Stereoselective Metabolism

The occurrence of stereoselectivity in metabolism can have serious consequences on the beneficial activity of the enantiomers of a racemic compound. For example, toxicity was shown to result from differential metabolism of the pyrethroid insecticide fenvalerate to a variety of species. Fenvalerate exists as a mixture of four enantiomers due to the presence of two chiral centres in its structure. Studies have shown that administration of the mixture of fenvalerate enantiomers resulted in granulomatous changes in mouse liver, spleen and mesenteric lymph node (Kaneko *et al*., 1986; Miyamoto *et al*., 1986; Okuno *et al*., 1986; Kaneko *et al*., 1988). Later toxicity evaluations of individual enantiomers indicated that the stereoselective metabolism of the 2*R*,α*S* enantiomer to a lipophilic conjugate, cholesteryl 2*R*-2-(4-chlorophenyl) isovalerate causes the granulomatous changes observed in the various tissues (Takamatsu *et al*., 1987). *In vitro* and *in vivo* biotransformation studies in mice, rats, dogs and monkeys, showed that all fenvalerate enantiomers were hydrolysed to 2-(4-chlorophenyl) isovalerate. However, only the 2*R*,α*S*-fenvalerate formed the cholesterol conjugate (Miyamoto *et al*., 1986; Takamatsu *et al*., 1987). As a result, the enantiopure fenvalerate that contains only the active isomer (2*S*,α*S*-fanvelerate), esfenvalerate, which does not produce the toxicity associated with fenvalerate, was developed and marketed.

The toxicological consequence of stereoselectivity in biotransformation is also depicted in chemotherapeutic agents (Vermeulen and Koppele, 1993). These compounds are usually prodrugs, characterized as having both desired (cytostatic) and undesired (cytotoxic) effects, the induction of which is largely influenced by the configuration of the parent compound (Vermeulen and Koppele, 1993). For example, animal model studies and clinical data of the anticancer prodrug, ifosfamide, showed enantioselective differences in cytochrome P450 metabolism between its two enantiomers (Roy *et al*., 1999). (*R*)-Ifosfamide contains the unique antitumour activity of this agent, and the ifosfamide-related toxicity is primarily related to the (*S*)-enantiomer (Vermeulen, 2003). Urotoxicity has been observed as a result of the production of

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acrolein during the N-dechlorethylation of the drug. The CYP2B6 and CYP3A4 mediated N-dechlorethylation (inactivation, urotoxic), is a competing pathway for the 4-hydroxylation reaction (activation to desired effect) of the drug by the same enzyme (Roy *et al*., 1999; Brocks, 2006). 4-Hydroxylation was shown to be more rapid for the *R*-enantiomer, which was N-dechloroethylated to a significantly lesser extent than the corresponding *S*-enantiomer (Roy *et al*., 1999).

4.2.3.2 Interspecies Differences in Stereoselective Metabolism

Significant interspecies variation also exists in the activity of biotransformation enzymes, which could have serious consequences, especially since biotransformation studies on drugs and other chemicals are mostly based on experimental work in a restricted number of species, and findings extrapolated from animal studies to humans. These differences could be due to differences in enzyme structures or expression levels of enzymes between species (Walle and Walle, 1986; Cashman *et al*., 1993; Beedham, 1997; Wsol *et al*., 2004; Brocks, 2006).

For example, in the S-oxidation of the ulcer treatment drug, cimetidine, pig liver FMO1 showed a stereopreference for the formation of (+)-cimetidine-S-oxide (Cashman *et al*., 1993). This is in contrast to the human liver FMO (FMO3), which has stereopreference for the formation of the (−)-S-oxide. In the previous example, for the pyrethroid fenvalerate, interspecies differences were also observed in 2*R*-2-(4-chlorophenyl) isovalerate cholesterol ester formation, which could be due to differences in carboxylesterase isoforms (Miyamoto *et al*., 1986).

Another example is propranalol, which is extensively metabolized by several oxidative routes, which is then followed by glucuronidation. The *S*-enantiomer, which contains most of the racemic propranolol's activity is selectively glucuronidated in dog and human liver microsomes. In rats and rabbits however, UDP-glucuronosyltransferase (UGT) is more selective to the *R*-enantiomer. Moreover, *S*-propranolol was found to be a noncompetitive inhibitor of *R*-propranolol glucuronidation in dog liver microsomes, while *R*-propranolol noncompetitively inhibits the glucuronidation of its enantiomer in rabbit liver microsomes (Testa, 1986; Walle and Walle, 1986). Examples of interspecies differences in chiral inversion have also been reported, including in microorganisms (Wsol *et al*., 2004).

Carboxylesterases are involved in detoxification or metabolic activation of important drugs and agrochemicals (Godin *et al*., 2006; Anand *et al*., 2006; Nishi *et al*., 2006; Ross *et al*., 2006; Satoh and Hosokawa, 2006). Species differences and substrate specificities for stereoselective hydrolysis have been reported for various esterase substrates (Glickman *et al*., 1979; Yoshigae *et al*., 1997; Stok *et al*., 2004; Nishi *et al*., 2006). Carboxylesterases are also important enzymes involved in prodrug activation (Satoh and Hosokawa, 2006). For example, propranolol shows poor oral bioavailability, due to extensive first-pass metabolism, therefore ester prodrugs of propranolol have been designed to avoid presystemic metabolism via masking of glucuronidation site of propranolol (Yoshigae *et al*., 1997; Satoh and Hosokawa, 2006). The *R*-enantiomer of propranolol prodrugs were preferentially hydrolysed, however, rat liver cytosol showed extremely low hydrolytic activities, broad substrate specificities and poor stereoselectivities compared to the microsomes. In contrast, dog liver cytosol showed similar enantioselectivity and substrate specificity to microsomes (Yoshigae *et al*., 1997).

In conclusion, it is important to consider species differences every time extrapolation is to be made between species. It should be noted that differential metabolism could alter the enantiomer ratio significantly and, sometimes, different metabolites are formed in different species. Interspecies differences in the stereoselective metabolism of chiral compounds are also the subject of recent reviews (Wsol *et al*., 2004; Brocks, 2006).

4.2.3.3 Genetic Polymorphism

Another factor to consider in stereoselective metabolism is that of genetic polymorphism (a characteristic of some of the most important enzymes that mediate xenobiotic transformation) (Shah *et al*., 1998). The biotransformation of a number of drugs is under genetic control (Caldwell, 1995; Cashman, 2004; Cashman, 2005). When genetic polymorphism is a factor in stereoselective metabolism, the likely existence of sensitive subpopulations further complicates risk assessment of the chiral chemicals. The single nucleotide polymorphisms in CYP2C9 and CYP2C19 are among the most common examples (Uno *et al*., 2008).

Because of their importance in the metabolism of a wide variety of xenobiotics, the cytochrome P450 superfamily of metabolic enzymes is a major determinant of interindividual differences in the rate of drug elimination and pharmacological or toxicological response (Vermeulen and Koppele, 1993). Both enantiomers of warfarin, which exhibit huge quantitative differences in their activity, are eliminated via hepatic metabolism (Rettie *et al*., 1992; Rentsch, 2002; Veenstra *et al*., 2005). The polymorphic CYP2C9 is responsible for the metabolism of the pharmacologically active *S*-enantiomer (Rettie *et al*., 1992). Seven alleles of CYP2C9 that could be responsible for reduced or null enzyme activity have been identified (Lee *et al*., 2002; Veenstra *et al*., 2005). The CYP2C9*3 allele has been implicated with the retarded elimination of (*S*)-warfarin in polymorphic individuals (Rentsch, 2002; Veenstra *et al*., 2005; Gage *et al*., 2008). The CYP2C9*3 and the CYP2C9*2 variants are present in approximately 35% of Caucasian individuals, but significantly less prevalent in African-American and Asian populations (Lee

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et al., 2002). CYP2C9 is also the main enzyme in the 7-hydroxylation of the (*R*)-enantiomer of another anticoagulant acenocoumarol, but CYP1A2 and CYP2C19 are also involved (Suszuki *et al*., 2004). CYP1A2 and CYP2C19 are also polymorphic, so that individuals with low enzyme activity could be at higher risk for bleeding episodes after a normal dosage (Rentsch, 2002).

CYP2D6 is an important oxidative enzyme responsible for metabolism of more than 30% of frequently prescribed drugs (Masuda *et al*., 2006). The lack of functional CYP2D6 has been reported in approximately 7.7% of Caucasian and 1.9% of Asian populations (Shimizu *et al*., 2003). Bufuralol, a nonselective β-adrenoceptor antagonist, consisting of two enantiomers, 1′*R*-bufuralol and the more active 1′*S*-bufuralol, is a well-known substrate of CYP2D6 (Caldwell, 1995; Narimatsu *et al*., 2003). Studies have shown bufuralol undergoes enantioselective and regioselective oxidation by CYP2D6 in extensive metabolizers, but not in poor metabolizers (Testa, 1986; Caldwell, 1995; Narimatsu *et al*., 2003; Masuda *et al*., 2006). Studies on substitution of phenylalanine by alanine at position 120 resulted in the elimination of substrate enantioselectivity of CYP2D6 (Masuda *et al*., 2006).

Oxazepam, a benzodiazepine receptor agonist, is metabolized primarily by hepatic glucuronidation. Oxazepam is formulated as a racemic preparation of *S*- and *R*-stereoisomers, although the *S*-enantiomer is thought to be much more active than its antipode. *S*-oxazepam has been shown to be stereoselectively glucuronidated by UGT2B15, whereas *R*-oxazepam is glucuronidated by multiple UGT isoforms. Studies have shown that *S*-oxazepam (but not *R*-oxazepam) is polymorphically glucuronidated in humans. A common genetic variation is seen in aspartate (UGT2B15*1) or tyrosine (UGT2B15*2) at position 85 of the UGT2B15 protein. Microsomes from human embryonic kidney (HEK)-293 cells overexpressing UGT2B15*1 showed five times higher *S*-oxazepam glucuronidation activity than did UGT2B15*2 microsomes (Court *et al*., 2002).

Human paraoxonase-1 (PON1), which belongs to the family of serum paraoxonases consisting of PON1, PON2 and PON3, has been extensively studied for protection from organophosphorus compound toxicity (Amitai *et al*., 2006; Yeung *et al*., 2008). Nerve agents such as cyclosarin and soman are racemic mixtures of two and four enantiomers, respectively. The stereoisomers of cyclosarin and soman differ significantly in their acetylcholinesterase (AChE)-inhibiting potency. This is mainly due to the preferential hydrolysis of wild-type PON1 of the less toxic stereoisomers P(+)-cyclosarin, and C(+)P(+)- and C(−)P(+)-soman enantiomers (Amitai *et al*., 2008). In humans, several polymorphisms of human PON1 are known to exist, which can have a substantial impact on PON1 activities (Yeung *et al*., 2008). In addition, variants of PON1 have been developed in recent years to increase catalytic efficiency of hydrolysis of the toxic stereoisomers of soman and cyclosarin, and other chemical warfare agents (Amitai *et al*., 2006; Amitai *et al*., 2008; Yeung *et al*., 2008).

The various stereochemical aspects of xenobiotic transformation of chiral drugs are the subject of extensive reviews by Vermeulen and Koppele (1993), Mayer and Testa (1994), Caldwell (1995), Cashman (1998; 2000), Mehvar *et al*. (2002), Vermeulen (2003), Wsol *et al*. (2004), Patel and Hutt (2004) and Brocks (2006).

4.2.4 Excretion

It should be noted that stereoselective appearance of enantiomers in the excreta may be reflective of stereoselectivity in the other dispositional pathways and may not necessarily be reflective of stereoselectivity of the excretion process itself. For the unbound drug, glomerular filtration across the kidney seldom shows enantioselectivity. However, transport proteins, such as P-glycoprotein, have been reported to be responsible for transporting drug molecules across canalicular cells and renal tubular cells, which could lead to enantioselective excretion (Lemma *et al*., 2006; Crettol *et al*., 2007). If any, in the majority of instances the selectivity is relatively modest, with enantiomeric ratios between 1.0 and 3.0. Often, it is only when carrier proteins are involved that enantioselectivity in the rates of filtration is observed (Patel and Hutt, 2004; Brocks, 2006).

5 ECOTOXICOLOGICAL CONSEQUENCES OF CHIRAL CONTAMINANTS

5.1 Chiral Agrochemicals

Generally, chiral agrochemicals are synthesized and supplied as racemic mixtures (**Table 2**). However, as with chiral drugs, the desired biological activity may be limited to individual enantiomers (Kurihara *et al*., 1997; Liu *et al*., 2005; Garrison, 2006). The percentage of chiral pesticides has increased from 19% in the 1980s to approximately 30.6% currently (Williams, 1996; Ulrich, 2007). Among these, pyrethroids and organophosphorus compounds are two of the most widely used pesticides worldwide (Amweg *et al*., 2006; Garrison, 2006; McCarthy *et al*., 2006; Scholz and Hopkins, 2006; Singh and Walker, 2006) (*see* **Toxicology of Pesticides**).

Physical and chemical processes in the environment (e.g. leaching, air–water exchange, sorption and other nonselective chemical reactions) do not usually differentiate between the enantiomers of the same compound. However, in biologically mediated processes enantioselective environmental attenuation has been observed in chiral legacy insecticides, chiral phenoxy

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Table 2 Some well-known examples of chiral pesticides

	Number of enantiomers	Category
Pyrethroids		
cis-Bifenthrin	2	Insecticide
Permethrin	4	Insecticide
Fenvalerate	4	Insecticide
Cypermethrin	8	Insecticide
Cyfluthrin	8	Insecticide
Cyhalothrin	8	Insecticide
Organophosphorus		
Fonofos	2	Insecticide
Trichloronate	2	Insecticide
Leptophos	2	Insecticide
Profenofos	2	Insecticide
Methamidophos	2	Insecticide
Fenamiphos	2	Insecticide
Malathion	2	Insecticide
Acetamides		
Acetochlor		Herbicide
Metolachlor	4	Herbicide
Dimethenamid	4	Herbicide
Metalaxyl	2	Fungicide
Phenoxyalakanoic acids		
Dichlorprop	2	Herbicide
Mecoprop	2	Herbicide
Phenylpyrazole		
Fipronil	2	Insecticide
Organochlorine		
cis-Chlordane	2	Insecticide
trans-Chlordane	2	Insecticide
2,4′-DDT	2	Insecticide
2,4′-DDD	2	Insecticide
Hexachlorocyclohexane	2	Insecticide

herbicides and fungicides (Moller *et al*., 1994; Tanabe *et al*., 1996; Muller and Buser, 1997; Buser and Muller, 1998; Bidleman and Falconer, 1999; Lewis *et al*., 1999; Jarman *et al*., 2005; Garrison, 2006). Of the modern use insecticides, enantioselective degradation has been reported for a number chiral organophosphorus and pyrethroid insecticides (Garrison, 2004; Liu *et al*., 2005; Qin *et al*., 2006). In addition, isomer conversion or isomerization may also occur during enantioselective biodegradation, potentially influencing the activity and contributing to side effects of chiral pesticides in the environment (Muller and Buser, 1997; Jarman *et al*., 2005). Enantiomers of the same compound may also differ in their toxicity to target and nontarget species (Garrison, 2006). In most chiral pesticides only one of the two (or more) enantiomers is usually responsible for most, if not all, of its pesticidal activity. The other enantiomer(s), although inert to the target pest, end up as an undesirable chemical load to the environment and could be toxic to nontarget organisms.

Until recently, enantiomer-specific ecotoxicology studies have been lacking for most of the current-use chiral pesticides (*see* **Aquatic Toxicology: Concepts, Practice, New Directions**). Early experiments with rats have demonstrated that the (−)-*o*,*p*′-dichlorodiphenyltrichloroethane (DDT) enantiomer is a more active oestrogen mimic of the human oestrogen receptor in a yeast-based assay than the (+)-enantiomer (McBlain, 1987; Hoekstra *et al*., 2001). In the fish, Japanese medaka (*Oryzias latipes*), significant differences in the induction of the egg yolk precursor protein, vitellogenin, which is under oestrogen-receptor control, in adult male fish was reported for *cis*-bifenthrin enantiomers (Wang *et al*., 2007) (*see* **Endocrine Disruption in Toxic Responses**).

Enantioselectivity in acute toxicity to the aquatic invertebrate *Ceriodaphnia dubia* was also observed in fipronil, a phenyl-pyrazole broad-spectrum insecticide (Garrison, 2006). The (+)-enantiomer was found to be the more toxic component. Additionally, *in vitro* binding and functional assays with human oestrogen, androgen

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or progesterone nuclear receptors, (−)-fipronil showed significantly higher androgen and progesterone activities than the corresponding (+) form (Konwick *et al*., 2005; Garrison, 2006).

Pyrethroids are highly toxic to fish and aquatic invertebrates due to the high affinity of pyrethroids to the molecular target in these species and/or significant differences in the pathways and rates of metabolism, from that in mammals (Bradbury and Coats, 1989; Chamberlain *et al*., 1998). Among the currently used pesticides, pyrethroids can have up to four to eight enantiomers resulting from multiple chiral centres, but only one or two enantiomers are responsible for the acute insecticidal activity (Kurihara and Miyamoto, 1998). For example, of the eight enantiomers in cypermethrin or cyfluthrin, only two (1*R*-3*R*-αS and 1*R*-3*S*-αS) have insecticidal activity, and the rest are mostly inert to the target organism (Naumann, 1990; Leicht *et al*., 1996). In *cis*-bifenthrin, only the 1*R*-*cis* enantiomer is biologically active, while the 1*S*-*cis* enantiomer is inactive (Naumann, 1990). Liu *et al*. (2005) reported large differences of up to 40-fold for acute toxicities of pyrethroid enantiomers to aquatic invertebrates, *Daphnia magna* and *Ceriodpahnia dubia*, indicating that aquatic toxicity is due to a specific enantiomer.

Similarly, chiral organophosphorus insecticides have been shown to stereoselectively inhibit cholinesterase enzymes. For example, acetylcholinesterase inhibition by the enantiomers and racemates of profenofos, fonofos and crotoxyphos was determined *in vivo* in aquatic invertebrates and fish, and also *in vitro*, with electric eel (*Electrophorus electricus*) (EE-AChE) and human recombinant (HR-AChE) acetylcholinesterases (Nillos *et al*., 2007). Variable sensitivity between acetylcholinesterase enzymes from different species, as well as variable magnitude of enantioselectivity in enzyme inhibition was observed. Similarly, the enantiomers of malathion were also enantioselective in their toxicity to anthropods (Garrison, 2004).

The stereoselective sulfoxidation of the organophosphate insecticide fenthion to (*R*)-(+)-fenthion sulfoxide by FMO represents a detoxification pathway. However, studies have also shown that subsequent oxidative desulfuration *in vivo*, could result in the bioactivation of (*R*)-(+)-fenthion sulfoxide to the significantly more potent acetylcholinesterase inhibitor, (*R*)-(+)-fenoxon sulfoxide (Gadepalli *et al*., 2007).

Enantioselective bioaccumulation is possible in a range of organisms in the environment (Buser and Muller, 1998; Bidleman and Falconer, 1999). For example, in almost all studies on α-hexachlorocyclohexane (α-HCH), the (+)-enantiomer was found to be more abundant in tissues of most aquatic organisms and mammals (Moller *et al*., 1994; Tanabe *et al*., 1996; Wiberg *et al*., 2000). This enantioselective bioaccumulation may have been a result of either greater accumulation rate of the (+) enantiomer, or faster metabolism of (−)-α-HCH, or both. In current-use pesticides, enantioselective bioaccumulation of fipronil has been observed in tissues of rainbow trout (*Oncorhynchus mykiss*) exposed to fipronil (Konwick *et al*., 2006). Significant selectivity was observed in the uptake of *cis*-bifenthrin enantiomers in the liver of Japanese medaka (Wang *et al*., 2007).

5.2 Chiral Pharmaceuticals in the Environment

Driven by advances in analytical techniques, the presence of pharmaceuticals in the environment is an issue that has received increasing attention from environmental scientists in recent years (Dietrich *et al*., 2002; Fent *et al*., 2006; Ankley *et al*., 2007; Daughton, 2007; Khetan and Collins, 2007; Stanley *et al*., 2007; Cooper *et al*., 2008). The occurrence of various classes of drugs has been reported in terrestrial and aquatic environments, particularly in Europe and North America (Bound *et al*., 2006; Ankley *et al*., 2007). While wastewater treatment could substantially reduce sewage concentration over time, it is not designed to completely remove these chemicals from the waste streams (Wong, 2006). Because these compounds have been designed to be biologically active, scientists began to question the consequences of exposure, particularly in nontarget organisms (Ankley *et al*., 2007; Stanley *et al*., 2007) (*see* **Toxicological Considerations for Pharmaceutical Products**).

Currently, knowledge of the ecotoxicological significance of pharmaceuticals in the environment is insufficient, even more so, the importance of stereochemistry in the environmental fate and effects of chiral drugs. As noted in this chapter, many of these pharmaceuticals are used as racemates. Studies on the occurrence of the chiral NSAID, ibuprofen, in surface waters and wastewater from water-treatment plants have shown high enantiomeric excess of the *S*-enantiomer (the pharmacologically active enantiomer) in wastewater influents (Buser *et al*., 1999). The same study showed similar enantiomeric excess in surface waters, although laboratory incubation of racemic ibuprofen with lake water indicated a more rapid dissipation, thus resulting in *R*-enantiomer excess after several days of exposure. Similarly, enantiomeric fractions (ratio of a single enantiomer concentration over the total concentration) of propranolol showed a nonracemic mixture following secondary wastewater treatment, indicating a biotransformation process occurring during treatment (Fono and Sedlak, 2005). More recently, Stanley *et al*. (2007) investigated the enantiomer-specific sublethal effects of the antidepressant, fluoxetine, in aquatic invertebrates and fish. Their findings indicated *S*-fluoxetine was more toxic to juvenile teleost fish (*Pimephales promelas*) in a short-term (seven days) survival and growth study. In contrast, in the same study, no significant difference was

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observed between fluoxetine enantiomers to *D. magna* following a 21-day chronic exposure.

These studies suggest that enantiomers may have vastly different toxicological characteristics and may degrade at significantly different rates in the environment. Consequently, enantioselectivity in these processes may result in enhanced or reduced ecotoxicological risks that are not yet fully understood. The significance of enantioselective attenuation of chiral contaminants both in laboratory and field samples is of limited consequence if enantiomers of the same compound have comparable toxicity to nontarget organisms. The combined toxicity of the enantiomers in such case is expected to remain unchanged, and therefore changes in entiomeric ratios alone will not result in differential effects. Accordingly, if enantioselectivity only occurs in toxicity, but not in degradation, enantiomeric ratios would not be expected to change over time, so that environmental effects can be predicted from the racemate. It is therefore essential that enantioselective degradation studies are complemented with toxicity investigations in order to reduce uncertainty in the risk assessments of chiral contaminants (*see* **Risk Assessment of Chemicals**).

6 CONCLUSION

From molecules to humans, 'chirality' is a prominent feature of the living world, and also occurs in man-made chemicals, especially pharmaceuticals and agrochemicals. Thus, stereoselectivity can be expected in the interactions of chiral chemicals with biological macromolecules. In most chiral bioactive agents only one of the two (or more) enantiomers that comprise the chiral molecule is responsible for most or all the desired activity. The other enantiomer(s) is often assumed to be inactive 'isomeric ballast' of little or no activity. However, a significant number of enantiomer-specific toxicokinetic and toxicodynamic studies have shown that the 'inactive' enantiomer could be the stereoisomer that carries the toxic (side) effect.

The stereochemistry of a chemical should not be ignored when biomolecules (enzymes, carrier molecules, specific receptors and ion channels) are involved. As illustrated in the studies reviewed in this chapter, there are a number of possibilities that can occur when a racemic mixture interacts with a biological system, including: (i) only one of the enantiomers possesses all the desired activity, with the other enantiomer(s) biologically inert; (ii) both enantiomers have similar activities or (iii) the enantiomers have significantly different activities or different potencies. In addition, interindividual and interspecies sensitivity to the activity, as a result of difference in biotransformation enzymes, should also be considered when dealing with chiral compounds.

The last 30 years has seen a significant increase in published literature highlighting the important relationship between molecular geometry and bioactivity, particularly for chiral pharmaceuticals and agrochemicals. Nevertheless, a recent rough estimate of studies on popular chiral pharmaceuticals revealed that less than 100 publications on racemic drugs contain information on the chiral state of the drug or discussions on the consequences thereof (Waldeck, 2003). On average, that number accounts for $<3.0\%$ of the total number of published studies for any given drug. Although a similar evaluation is yet to be done, it is probably safe to assume a far more inferior publication record exists for pesticides and other chiral pollutants. However, as chiral agrochemicals become more prevalent in the market, the interest in these areas has exponentially increased in the last five years. However, there continues to be a significant lack of judiciousness when it comes to the need for properly identified stereochemical properties of these bioactive agents. The persistent reluctance to acknowledge the risks associated with the chirality of a chemical is no longer justified. Chiral technology has significantly developed to a point where we are allowed ample opportunities in enantiomer resolution and preparation techniques, which in turn offer new avenues for the pharmacologists and environmental toxicologists to explore the stereochemical properties of these ubiquitous agents.

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The Influence of Temperature on Toxicity

Christopher J. Gordon and Pamela J. Rowsey

CONTENTS

1 INTRODUCTION

Why should temperature be considered as a significant factor in most toxicological studies? First, while body temperature of most mammals, including rodents, is normally stable over a wide range of ambient temperatures, altered regulation is seen upon exposure to toxicants, with the effects usually being more pronounced in rodents and other small mammals. Second, large mammals, such as humans, are able to regulate a stable core temperature following toxicant exposure, but environmental heat and cold stress will nonetheless exacerbate the physiological and behavioural responses to a toxicant. Third, by virtue of the stable nature of body temperature, any environmental perturbation or insult that changes temperature should be considered as a biologically significant event. Thus, one should look at temperature regulation as a hallmark of homoeostasis and consider the significance of temperature regulation as affected by a toxicant. To this end, one can use body temperature as a benchmark of toxicological exposure because a temperature change suggests a significant change in physiological homoeostasis. This chapter endeavours to explain the general effects of environmental toxicants on the thermoregulatory response of rodents and humans.

1.1 Thermoregulatory Profile

The toxicity of drugs, environmental contaminants and other chemicals is commonly evaluated in rats and mice. Their thermoregulatory response to toxic insults is pronounced, compared to that of humans and other large mammals. As will be discussed later, the increased thermal sensitivity of rodents is primarily attributed to their small size. Understanding the reasons behind their increased sensitivity requires an explanation of the rodent thermoregulatory profile (**Figure 1**). A thermoregulatory profile is an assessment of the effects of environmental temperature on the core and skin temperature, and activity of autonomic thermoeffectors. Measuring the activity of thermoeffectors; that is, metabolic rate (MR), evaporative water loss and skin blood flow (SkBF), over a range of ambient temperatures reveals a pattern of thermoeffector activity that is typical for rodents and other mammals (**Figure 1**). There is a range of ambient temperatures termed the *thermoneutral zone*, where MR is at or near basal levels. In this zone of ambient temperatures, temperature regulation is achieved by control of sensible heat loss without regulatory changes in MR or evaporative water loss. The control of body temperature in the thermoneutral zone is achieved with modulations in SkBF which controls the rate of heat loss with no additional metabolic requirements. As ambient temperature decreases below the thermoneutral zone, the

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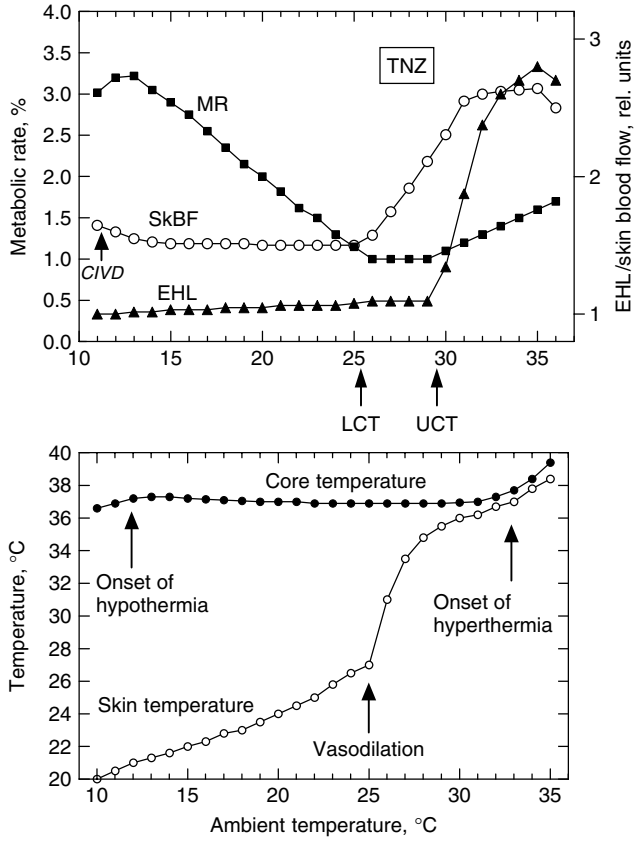

Figure 1 General pattern of core and skin temperature and activity of autonomic thermoeffectors as a function of ambient temperature in a homoeotherm. SkBF—skin blood flow, EHL—evaporative heat loss; MR—metabolic rate; LCT—lower critical temperature; UCT—upper critical temperature; TNZ—thermoneutral zone.

blood flow to the skin is minimized as a result of peripheral vasoconstriction. As ambient temperature decreases further, metabolism must increase above basal levels by shivering and nonshivering thermogenesis in order for heat production to match heat loss to the environment. The ambient temperature where MR increases is termed the *lower critical temperature* (LCT). As ambient temperature decreases below the LCT, skin temperature falls passively, but may increase with extreme cold exposure as a result of cold-induced vasodilation (CIVD) of the peripheral blood vessels. This is a protective response to keep exposed tissues from freezing. Eventually, a temperature is reached where MR cannot compensate for the heat loss and the core temperature decreases, leading eventually to hypothermic death.

As ambient temperature increases above the thermoneutral zone, SkBF increases and there is a disproportionate rise in skin temperature. However, if SkBF is maximal, skin temperature cannot increase above the internal core temperature. This means that as ambient temperature increases, the gradient between the skin and ambient temperature becomes smaller, thus limiting the rate of heat loss by convection, conduction and radiation. Hence, at temperatures above the thermoneutral zone, evaporative heat loss (EHL) mechanisms (i.e. panting, sweating, saliva grooming) must be activated to maintain thermal balance. This ambient temperature is termed the *upper critical temperature* (UCT). It is also identified with the point where core temperature and metabolism begin to rise (IUPS Thermal Commission, 2001). At around the point of the UCT, skin temperature has increased to a level that is just below core temperature, reflecting maximal redistribution of warm blood from the core to the periphery. With further increase in ambient temperature, skin and core temperature parallel each other until the point of thermoregulatory failure. At this point, evaporation is ineffective to maintain sufficient heat loss and core temperature spirals upward leading eventually to hyperthermic death.

1.2 Pharmacokinetic Modelling and Temperature

When studying the role of temperature on the efficacy of a drug or toxicant using *in vitro* or *in vivo* systems, thermal sensitivity is commonly expressed in terms of either the Q_{10} or the *temperature coefficient*. Q_{10} is defined as 'the ratio of the rate of a physiological process at a particular temperature to the rate at a temperature 10 °C lower, when the logarithm of the rate is an approximately linear function of temperature' and is calculated by the following equation (IUPS Thermal Commission, 2001):

$$Q_{10} = (R_2/R_1)^{10/(T_2-T_1)} \quad (1)$$

where R_1 = rate of activity at one temperature, T_1; and R_2 = rate of activity at another temperature, T_2.

Thus, a Q_{10} of two means that the activity doubles with each 10 °C elevation in temperature, a Q_{10} of three is tripling of activity, and so on. A Q_{10} of one means there is no change in activity with temperature and a Q_{10} of less than one means that activity increases with a reduction in temperature. The Q_{10} normally ranges between two and three for most biological processes. Comparative physiologists and toxicologists studying the effects of toxicants and drugs on the lower vertebrates and invertebrates often rely on Q_{10} to express the effects of temperature on physiological processes. Q_{10} varies with temperature, usually decreasing in magnitude with a progressive rise in temperature. Hence, it is essential to report the temperature range of its determination when reporting Q_{10} values (Cossins and Bowler, 1987).

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The temperature coefficient is also a commonly used parameter to describe thermal sensitivity and interaction with drugs and toxicants and is defined as 'the ratio between the change in any temperature-dependant activity and the defined temperature range within which this change occurs' (IUPS Thermal Commission, 2001). In other words, the temperature coefficient is an expression of the change in activity of a process for a 1.0 °C change in temperature. Unlike the Q_{10} parameter, the temperature coefficient is either positive or negative, depending on how temperature affects the physiological process. Temperature coefficients are probably used more often than the Q_{10} value in studies on the effect of temperature on toxicological and pharmacological processes in birds and mammals. Like Q_{10}, temperature coefficient will also be dependent on the temperature range used in its calculation.

Temperature coefficients and/or Q_{10} values are critical in the development of physiologically based pharmacokinetic (PBPK) models. PBPK models are essential in toxicology and are used to predict the disposition and fate of a chemical and its physiological effects. PBPK modellers must factor temperature into their models because the temperature coefficient for all metabolic processes involved in the metabolic activation, deactivation and clearance of a toxicant will be a factor if the toxic agent affects body temperature. However, in addition to core temperature, there are other aspects of the thermoregulatory system that must be factored into PBPK models, including temperature acclimation and exercise. This was a popular area of research and is thoroughly reviewed in a paper by Ballard (1974).

We incorporate a basic PBPK model with a simple thermoregulatory model to show the potential interactions between core, skin and ambient temperature, and the efficacy of a toxicant (**Figure 2**). Homoeothermic organisms maintain a stable core temperature over a wide range of ambient thermal conditions. A stable core temperature is achieved with relatively large fluctuations in temperature of the peripheral tissues (skin and

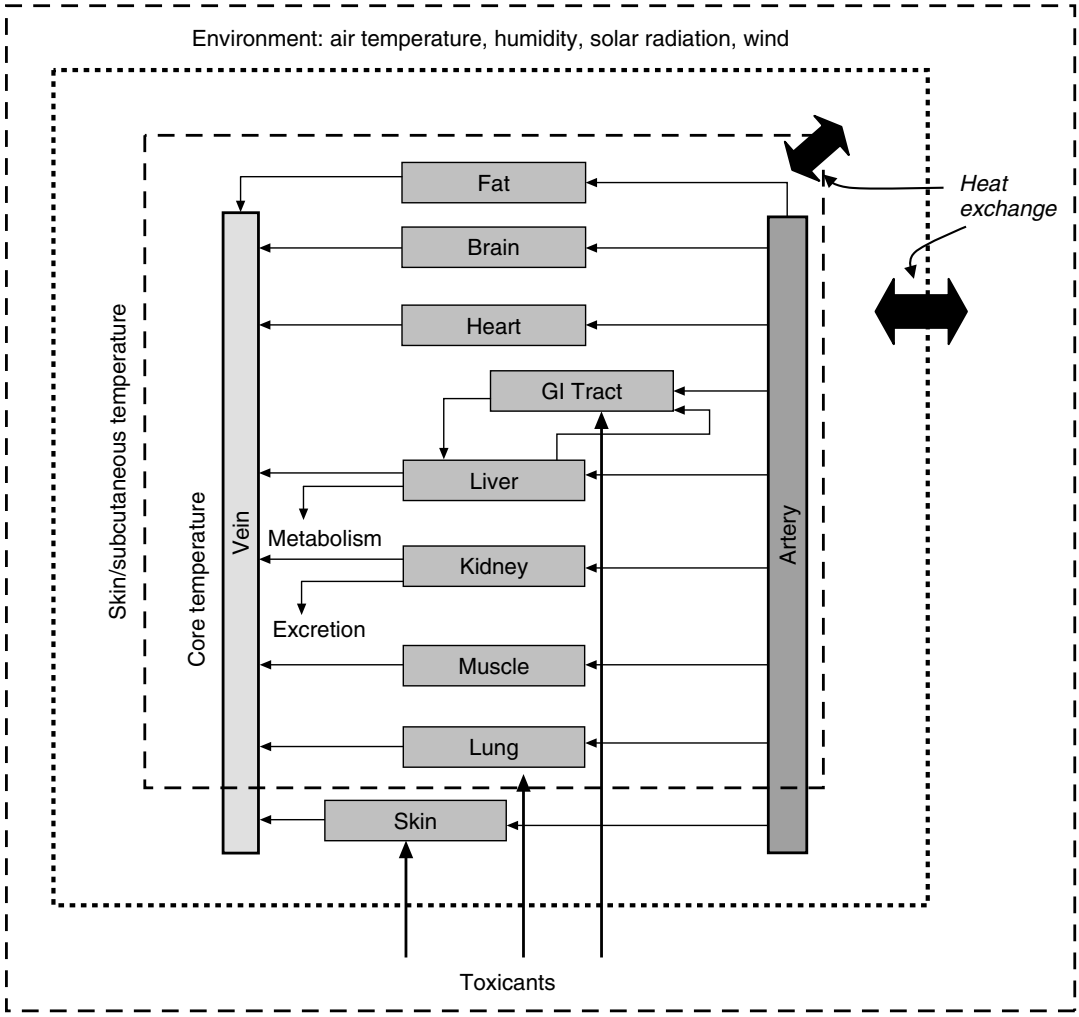

Figure 2 Combination of a simple pharmacodynamic/pharmacokinetic model and thermoregulatory model to demonstrate how core, peripheral and ambient thermal environments influence the kinetics and dynamics of toxicants (see Gordon, 2005).

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subcutaneous temperature) that are influenced by changes in ambient temperature, relative humidity, solar radiation and wind speed. Most components of a PBPK model are influenced by the core temperature, including brain, heart, gastrointestinal (GI) tract, liver, kidney, lung and skeletal muscle. If exposure to a toxicant changes core temperature, then the pharmacokinetics in these organ systems will be affected, depending on the temperature coefficients of each system. The temperature of the peripheral tissues is also critical because it may influence the uptake of certain toxicants. Warm temperatures raise skin temperature leading to an increase in SkBF and sweating, both of which encourage the transcutaneous absorption of organophosphate insecticides and nicotine from drug patches, and others (Gordon and Leon, 2005). For example, parathion absorption from the skin of humans increased by 180% when the ambient temperature of exposure was raised from 28 to 40.5 °C (Funckes *et al*., 1963). Although the lungs and GI tract are essentially surrounded by the *milieu* of the thermal core, their role in the intake of toxicant is also influenced by the ambient environment. The intake of an airborne toxicant will be dependent on minute ventilation which, in turn, is dependent on ambient temperature. Heat stress increases ventilation, resulting in greater intake of airborne toxicants, such as ozone (Mautz, 2003). Animals that pant to thermoregulate in the heat, such as dogs, sheep and other species, would be especially susceptible to this mode of action. Finally, the intake of toxicants in the GI tract can also be influenced by the thermoregulatory system. This can be pertinent in the study of wildlife species subjected to toxicant or natural toxins in their food supply. The increased energy demands in the winter mean greater intake of food that is tainted with toxic agents.

1.3 Forced and Regulated Changes in Temperature

It is important to understand whether a drug- or chemical-induced change in body temperature is forced or regulated (**Figure 3**). Normothermic body temperature can increase or decrease in a forced or regulated fashion. If the thermal response can be identified as being regulated, then one can be assured that the toxicant is affecting central nervous system (CNS) thermoregulatory mechanisms. A forced response could be mediated with or without activation of CNS pathways. An increase in the set-point temperature (T_{set}), as occurs with a fever, means there is a transient period where T_{set} is suddenly elevated above the core temperature (**Figure 3A**). That is, with an infection there is an inflammatory response and release of a variety of cytokines and other factors into the circulation. Cytokines such as IL-1 and IL-6 directly affect the CNS thermoregulatory centres in the hypothalamus resulting in an upward shift in the set-point. The animal responds as if it was cold and selects warmer ambient temperatures and activates thermoeffectors to increase heat production (shivering and nonshivering thermogenesis) and reduce heat loss (peripheral vasoconstriction).

Thermal physiologists view infectious fever as the cornerstone of a set-point elevation. Noninfectious agents can also increase the set-point and this phenomenon is also termed *regulated hyperthermia*. As the hyperthermic response progresses, there is eventually an equalling of T_{set} and core temperature and the animal reaches a steady state with an elevated body temperature. During *forced hyperthermia*, core temperature increases above T_{set}, as

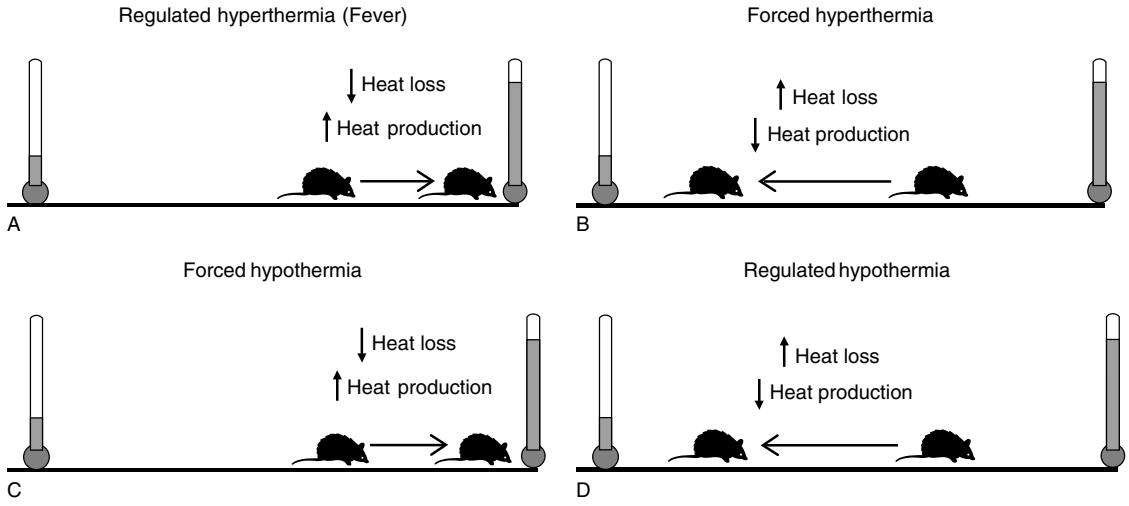

Figure 3 Summary of the possible thermoregulatory response to a toxicant or drug. A rodent under normothermic conditions prefers an ambient temperature in its thermoneutral zone (~30 °C). A chemical, pyrogen or other agent may affect the central control of thermoregulation or the ability for thermoeffectors to control body temperature leading to forced or regulated changes in core temperature. (Reproduced from Gordon, 1983. © Elsevier.)

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would occur by exposure to high ambient temperatures or by administering toxicants or drugs that stimulate metabolic thermogenesis, but without affecting the CNS control mechanisms (**Figure 3B**). During forced hyperthermia, thermoeffectors are activated to reduce heat gain and increase heat loss to lower its core temperature. The animal seeks a colder environment to facilitate heat loss and lower body temperature to normal. *Forced hypothermia* refers to the state where core temperature is forced below T_{set}, as would occur during acute cold exposure or treatment with toxicants or drugs that impair metabolic thermogenesis, without affecting CNS control mechanisms (**Figure 3C**). The organism responds with an activation of thermoeffectors to minimize heat loss and increase heat production. A warmer environment is sought to reduce heat loss. *Regulated hypothermia* occurs when a chemical or drug causes a sudden lowering of T_{set} (**Figure 3D**). This is essentially opposite to that of a fever because the organism feels hot and responds by seeking cooler temperatures and activating thermoeffectors to increase heat loss and reduce heat production. These thermoeffector responses persist until T_c is equal to T_{set}, but at a lower body temperature. One also finds the term *anapyrexia* used to describe a pathological condition in which there is a regulated decrease in body temperature (IUPS Thermal Commission, 2001). Anapyrexia and regulated hypothermia are essentially the same, but we prefer the latter, especially in describing the responses to toxic agents.

1.4 Impact of Environmental Temperature on Toxic Response

Whether a toxicant will cause an increase, decrease or have no effect on body temperature will depend largely on the combined thermal environment of exposure (i.e. air temperature, relative humidity, wind speed and insulative quality of the cage and bedding material) and the animal's thermoregulatory response to the toxic agent (**Table 1**). The thermal environment of the test species should always be evaluated in any drug/toxicity study; however, this is often overlooked. Researchers not cognizant of thermoregulation often consider rats and mice to be poor thermoregulators. This is a misconception because untreated mice and rats are quite capable of defending a relatively constant body temperature over a wide range of ambient temperatures (for review, see Gordon, 1993b). This is done through the appropriate activation of thermoeffectors to increase heat production in the cold or activate heat loss mechanisms upon exposure to warm temperatures. When these effector responses are hampered by experimental manipulation (e.g. restraint) or by treatment with a drug or chemical that specifically targets one or more of the effector systems, then one will see marked changes in the rodent's body temperature.

The potential changes in temperature, indicated by the number of arrows in each block in **Table 1**, is dependent on whether the animal is housed in a cool, warm or thermoneutral environment. For example, a drug that blocks metabolic thermogenesis will manifest the most effective change in body temperature when exposure occurs at a relatively cool ambient temperature. A toxicant that induces peripheral vasoconstriction, thus restricting blood flow and heat loss from the skin will have relatively minor effects in a cold environment because the animal is in a state of peripheral vasoconstriction, but would lead to hyperthermia in a thermoneutral and warm environment. On the other hand, an agent that causes peripheral vasodilation would be mostly ineffective in a warm environment because SkBF is already elevated and an additional vasodilatory action should have little effect on total heat loss. Blocking salivation in rodents or sweating in humans would have little effect in the cold, but would lead to dramatic hyperthermia if the blocking agents are administered in a warm environment.

If the toxicant impairs one thermoeffector without affecting CNS thermoregulatory control, then one would expect the animal to utilize other thermoeffectors to maintain thermal homoeostasis. For example, if SkBF was elevated in a cold environment, then metabolic thermogenesis could increase to counter for the increased heat loss. Stimulation of metabolism in a warm environment, such as occurs by exposure to chemical agents that uncouple oxidative phosphorylation, is accompanied by a marked increase in evaporation (Wood *et al*., 1983). Overall, these are idealized situations and drugs and toxicants generally affect the function of more than one thermoeffector system.

Table 1 Relative hyperthermic (↑) and hypothermic effects (↓) of a toxicant or drug that stimulates or blocks thermoeffectors at cool, thermoneutral and warm ambient temperature. Number of arrows indicates relative magnitude of change in core temperature[a]

	Thermogenesis increased	Skin blood flow increased	Evaporation increased	Thermogenesis blocked	Skin blood flow blocked	Evaporation blocked
Cool temperature	↑	↓↓	↓	↓↓↓↓	—	—
Thermoneutral temperature	↑↑↑	↓	—	↓↓	↑↑↑	↑
Warm temperature	↑↑↑↑	—	—	↓	↑↑↑↑	↑↑↑↑

[a]Reproduced from Gordon, 2005. © Taylor and Francis Group.

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1.5 Acute Thermoregulatory Responses

There is an extensive database on the acute effects of drugs and chemicals on the body temperature of rodents and other species (Clark and Lipton, 1985a; 1985b). In test batteries that screen for toxicants, body temperature is frequently used as a benchmark of overt toxicity (Moser, 1995; Tamborini *et al*., 1990). A decrease in body temperature in a test species is considered a significant sign of acute toxicity, placed in the same class of sequelae as body weight loss, decreased appetite and reduced motor activity. There is often a problem with the manner in which body temperature is measured in rodents. Most studies rely on hand-held probes to measure the colonic or rectal temperature of animals that are extensively handled and manipulated. In many of these studies, one finds the so-called baseline core temperature of the control rats to be at least 38 °C, a value which is at least 1 °C above the rat's normal daytime temperature. This hyperthermic response obviates any subtle effects that a toxicant at low doses would have on body temperature and limits the usefulness of body temperature as a sensitive indicator of toxicity. Radiotelemetry provides the best means of monitoring the core temperature of undisturbed animals (Gordon *et al*., 2008). Recently, a set of recommendations was developed for researchers studying the effects of drugs and toxicants on the thermoregulatory system (**Table 2**). Overall, the best assessment of the effects of a toxicant on thermoregulation is made in undisturbed animals monitored remotely with telemetry. Restraint or repeated insertion of a temperature probe are the least desired methods because of the stress-induced changes in body temperature.

In the study of the acute effects of drugs and chemicals on thermoregulation, one needs to first understand

Table 2 Recommendations for rodent thermometry in toxicology studies, starting with the most ideal and ending with least ideal method[a]

1. Radiotelemetry. Monitoring of a temperature probe surgically implanted in a thermal core of the animal (abdomen, brain). Remote monitoring of animals housed in a vivarium isolated from disturbances from personnel. Maintenance of animals in a thermoneutral environment and/or with bedding that allows animals to adjust insulative qualities of microenvironment.
Disadvantages: Expensive start up costs; surgical implantation with anaesthesia required.
Advantages: provides best measure of true thermoregulatory patterns of mice and rats; continuous, automated monitoring of core temperature 24 hours/day. Allows for observations of acute, subchronic and chronic effects of drugs and toxicants.

2. Subcutaneous transponder ID chip. ID chips implanted under skin with trocar.
Advantages: less costly than telemetry; accurate measure of the subcutaneous temperature; no surgery or anaesthesia required.
Disadvantages: subcutaneous temperature is not a measure of the core temperature; personnel must be close proximity to animal to position receiver to collect temperature data. Data collection not automated; no 24 hour monitoring.

3. Infrared thermography
Advantages: provides noncontact measurement of skin temperature which can be valuable for interpreting effects of toxicants and drugs. Hand held devices relatively inexpensive.
Disadvantages: skin temperature does not provide any indication of core temperature; continuous, automated monitoring is costly. Personnel must be in close proximity to animal.

4. Colonic/rectal temperature probes (unrestrained rodents). Used on single housed animals undisturbed immediately prior to measurement.
Advantages: inexpensive and easy to perform; provides an accurate, single time-point measurement of the core temperature provided animal is undisturbed prior to probe insertion.
Disadvantages: Technique evokes stress resulting in change in body temperature; repeated use of device leads to inaccurate estimates of the core temperature due to stress. Difficult to use with multiple housed animals because disturbance causes stress and elevation in temperature as multiple animals are being measured.

5. Colonic/rectal temperature probes in semi-restrained animals (e.g. probe inserted and taped to tail)
Advantages: inexpensive, accurate measure of core temperature
Disadvantages: continuous stress of probe insertion leads to abnormally high body temperatures in rats. Animals must be restrained or carefully observed to assure probe remains in place.

6. Restraint with colonic probe
Advantages: Restrained animal easy to manipulate (i.e. nose only inhalation).
Disadvantages: Marked effects on thermoregulatory system. Rodents are much more sensitive to ambient temperature when restrained. Unable to use behaviour and thermoeffectors as effectively when restrained.

[a] Gordon *et al*., 2007

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if the effect is forced or regulated, then consider how ambient temperature will impact the response (Gordon, 1983). The effects of changes in ambient temperature on the toxicity of a chemical could be diametrically opposed depending on whether the body temperature change is forced or regulated. That is, many toxicologists and pharmacologists are cognizant of the temperature-dependency and attempt to apply corrective measures to maintain a consistent thermal environment within the test subject. In many cases, the hypothermic effect of an agent is blocked by raising ambient temperature or by placing the animal on a heating pad. However, if the chemical is eliciting a regulated hypothermia, then raising ambient temperature and preventing the development of hypothermia is, in fact, more stressful. If the chemical simply impairs thermoeffectors that increase heat production, then raising ambient temperature will be beneficial.

Measuring core temperature alone is not sufficient to decide if the chemical is eliciting a forced or regulated change in temperature. Performing a thermoregulatory profile in a rodent exposed to a toxicant (e.g. **Figure 1**), can help elucidate if the change in body temperature is forced or regulated. For example, an increase in tail SkBF and a reduction in MR in a cold environment would suggest a CNS drive to lower core temperature. In fact, behavioural thermoregulation measured concomitant with core temperature is usually sufficient information to distinguish between forced and regulated changes in core temperature. Behavioural thermoregulation in rodents is measured using a temperature gradient or operant system. Temperature gradients mimic a natural type of option for the animal to seek out its optimum thermal environment with little energy expenditure. This can be important if the toxicant impairs motor activity. Moreover, thermoregulatory behaviour in a gradient can be learned quickly. Operant systems are also useful in toxicology studies because the animals can be instrumented easily to monitor physiological processes while quantifying its behaviour. However, considerable training is needed for animals to learn to use thermoregulatory behaviour in an operant system and the animal must continually work to maintain thermal reinforcements.

Our laboratory and others have used the temperature gradient to monitor telemetered rats and mice while dosed with toxic chemicals. The time-course of selected ambient temperature and core temperature in the rat monitored by telemetry exemplifies the regulated hypothermic response induced by administration of chlorpyrifos, an anti-choline esterase (ChE)-based insecticide (**Figure 4A**). When dosed with a control vehicle (corn oil), there was a transient decrease in selected temperature that reflects a heat dissipatory response from the stress of handling and injection. When dosed with chlorpyrifos, selected ambient temperature decreased from 30 to 25 °C and the behavioural response preceded a 2.5 °C decrease in core temperature (**Figure 4B**). At the nadir of the decrease in core temperature, selected temperature increased rapidly, a response that facilitated the recovery of core temperature. It is important to note that the rat has the option of selecting ambient temperatures as warm as 36 °C in the gradient. If the rat simply moved to a temperature range that was slightly above the thermoneutral zone, the hypothermic effects of chlorpyrifos would have been blocked. Administration of the organophosphate diisopropyl fluorophosphate (DFP) also induced an abrupt selection for cooler temperatures that occurred concomitantly with a decrease in core temperature (Gordon, 1994; 1997).

There are innumerable studies showing that acute exposure to toxic chemicals elicits hypothermia in mice and rats. A small number of studies have used techniques to measure autonomic and/or behavioural thermoeffectors to determine the nature of the hypothermic response and have shown that the hypothermia is regulated. Mice or rats placed in a temperature gradient and given toxic doses of heavy metals (nickel, cadmium, lead), solvents (ethanol, sulfolane) and antiChEs (chlorpyrifos, DFP) will consistently prefer cooler ambient temperatures as they become hypothermic (for review, see Gordon, 2005; Gordon *et al*., 1988b). So far, there is no report that an animal will select a warm temperature to block the acute hypothermic effects of the toxicant. It follows that the majority of toxic agents studied in mouse and rat that elicit hypothermia are likely evoking a regulated hypothermia. The hypothermic response is generally an adaptive response that improves survival with the toxicant (see section 1.8)

The best understood mechanism of action of regulated hypothermia has been documented with antiChE-based agents. The stimulation of CNS muscarinic pathways appears to be a primary cause of the acute hypothermic response elicited by antiChE insecticides. At high levels of stimulation of the muscarinic pathways in the thermoregulatory centres, the heat loss pathway is stimulated, meaning that the animal reacts as if it is hot and attempts to lower core temperature by increasing SkBF and selecting cooler ambient temperatures. Coadministration of muscarinic antagonists such as scopolamine or atropine blocks most of the hypothermic response elicited by antiChE agents (Gordon and Grantham, 1999; Maickel *et al*., 1991). The mechanism of action for other toxicants is not understood. How toxicants with a diversity of structures elicit a similar hypothermia response is not known. There may be common modes of action derived in peripheral tissues that drive the CNS thermoregulatory responses.

1.6 Thermoregulatory Effects of Alcohol

Special attention is given to the thermoregulatory effects of alcohol (ethanol). The toxicity of alcohol on the

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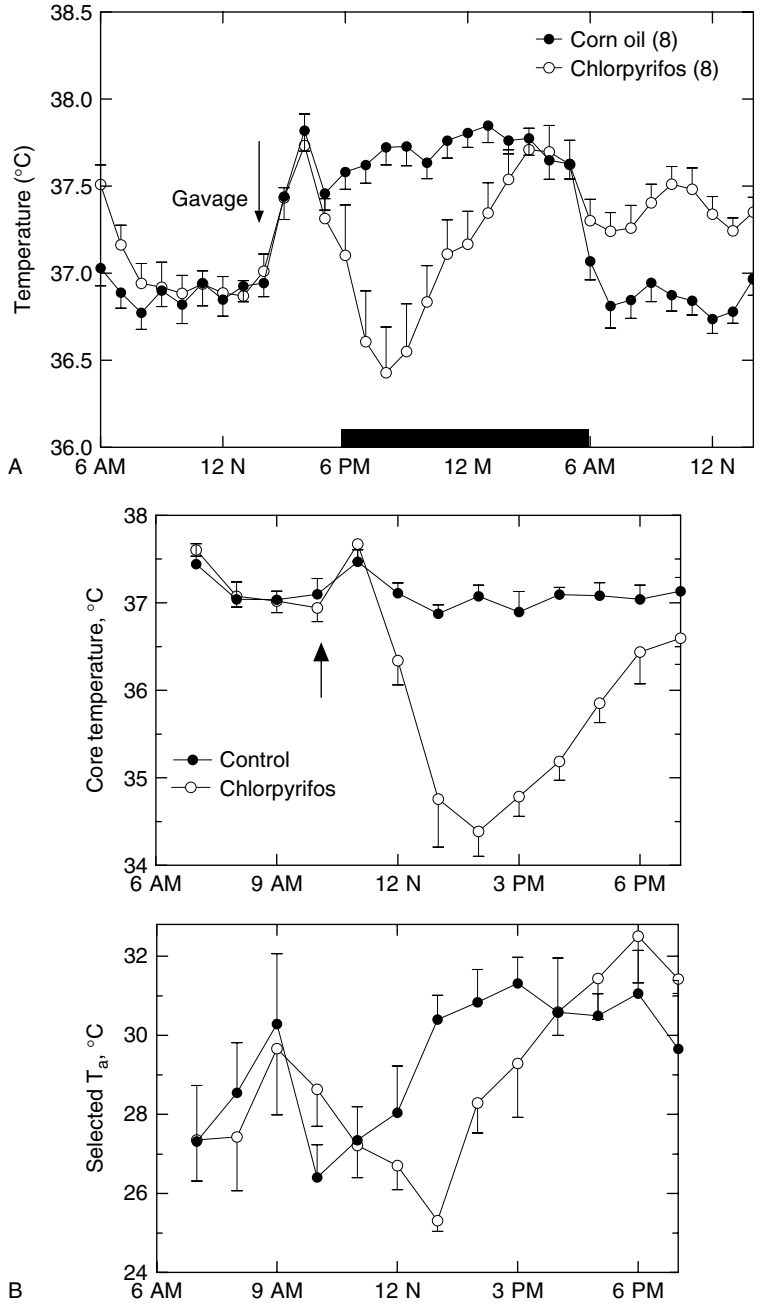

Figure 4 (A) Time-course of core temperature in female Sprague–Dawley rats given chlorpyrifos (from Rowsey *et al*., 2003). (B) Behavioural thermoregulatory response of female Long–Evans rats dosed with chlorpyrifos and housed in a temperature gradient to monitor selected ambient temperature and core temperature. (Reproduced from Gordon, 1997. © Elsevier.)

thermoregulatory system is well understood compared to any other toxicant. Hence, alcohol is an ideal agent to study in the extrapolation of thermoregulatory responses of toxicants from small to large species. It was well known that acute exposure to alcohol via an oral, inhalation or parenteral route elicits hypothermia in rats and mice housed at standard laboratory temperatures (~22 °C) (for a review, see Kalent and Le, 1984; Gordon, 2005). The hypothermic effects of alcohol are ambient-temperature-dependent and hyperthermia is elicited in rats and mice exposed to alcohol at ambient temperatures above their thermoneutral zone. In view of this response, Myers (1981) termed the thermoregulatory response of alcohol in the rat a 'poikilothermic' response, meaning that with acute intoxication, the rat's thermoregulatory system becomes dependent on the prevailing ambient temperature. In other words, alcohol intoxication reduces thermal homoeostasis to a condition normally

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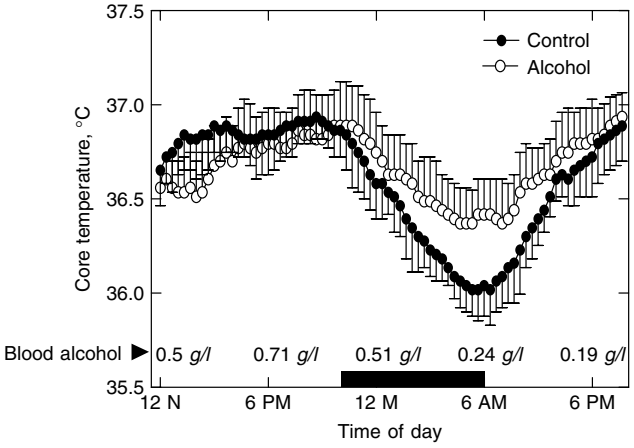

Figure 5 Circadian profile of core temperature of nine healthy men given a control fluid or 256 g of alcohol administered regularly over a 26 hour period. Note significant hyperthermic effect of ethanol only during the night. Also note transient hypothermia at start of alcohol exposure. Blood alcohol levels measured at selected hours is given in italics. (Data modified from Danel *et al*., 2001.)

observed in most of the so-called lower vertebrate (reptiles, amphibians, fish) that have little autonomic control of thermoregulation.

Poikilothermic species do have behavioural control of their body temperature and can regulate a relatively stable core temperature when housed in an environment that allows for behavioural thermoregulation. The same is true for rats and mice exposed to alcohol and given a choice of selecting from a range of ambient temperatures. Studies in our laboratory and others showed that when rats and mice were dosed with alcohol and allowed to behaviourally thermoregulate, they selected cooler ambient temperatures concomitant with a reduction in body temperature (Gordon and Stead, 1986; Gordon *et al*., 1988a; O'Connor *et al*., 1989; Briese and Hernandez, 1996). Thus, alcohol, like many other toxicants, evokes an apparent regulated hypothermia in rodents. Lomax (1980) postulated that alcohol elicited a reduction in T_{set} since they noted that rats dosed with alcohol became hypothermic and avoided a heat source.

Under standard laboratory conditions, a small hypothermic response can be elicited in humans who consume moderate doses of alcohol (**Figure 5**). As discussed later, a large body mass is a key factor in limiting the hypothermic response to toxicants and drugs. Thus, the marked hypothermic response seen in small laboratory rodents will be relatively meagre in healthy adult humans. On the other hand, there are emergency room reports showing marked hypothermia in some instances. These cases arise from acute intoxication of individuals in poor health combined with exposure to cold temperatures.

Measuring autonomic and behavioural thermoeffectors in humans dosed with alcohol under controlled conditions provides evidence of a regulated hypothermic response. Yoda *et al*. (2005) maintained human subjects at a warm ambient temperature of 33 °C (i.e. just above their thermoneutral temperature) while measuring SkBF, sweating and core temperature. Following a moderate amount of alcohol consumption (0.36 g kg^{-1}), the subjects had a feeling of warmth and this was accompanied by an increase in sweating and SkBF. With the onset of thermoeffectors to increase heat loss, core temperature fell by 0.3 °C. Following the principles outlined in **Table 1**, by maintaining the subjects at a warm ambient temperature, the effects of alcohol on sweating and SkBF should be easier to activate. That is, if alcohol activates a regulated hypothermic response then this response should be more profound if the subjects are housed in a warm environment. Overall, humans respond to alcohol with autonomic responses to increase heat loss. Moreover, the sensation of a feeling of warmth following moderate alcohol consumption should lead to a selection of a cooler ambient temperature. These responses are homologous to that of rodents with the exception that the hypothermia is miniscule in human subjects.

1.7 Toxic Chemicals Eliciting Hyperthermia

There are three classes of toxic chemicals that induce marked hyperthermia in laboratory rodents: dichlorodiphenyltrichloroethane (DDT), 2,4-dinitrophenol (DNP) and certain pyrethroids. DDT is an organochlorine that was well studied for its thermoregulatory and other effects prior to being banned. It is tremorigenic and a convulsant and is one of the few pesticides that can elicit an acute and apparently lethal increase in the core temperature (Hudson *et al*., 1985). The thermoregulatory effects of DDT are dependent on ambient temperature. When given a lethal dose (LD) of DDT (1000 mg kg^{-1}) and housed at 22 °C, tail skin temperature of the adult rat begins to increase prior to any change in core temperature by 3–5 hours after injection (Woolley, 1973). There is then a precipitous decrease in tail skin temperature that is concomitant with a marked increase in core temperature. At the point of death, around 6 hours after dosing, core temperature is 40–42 °C while tail skin temperature is nearly below pretreatment levels. It is interesting to note that if the DDT-treated rat is placed in a cold room maintained at 3–4 °C, core temperature will fall dramatically, reaching near hypothermic lethal levels within a few hours (Woolley, 1973). Hence, in spite of the intense tremorigenic activity of this agent, DDT-treated rats in the cold are unable to thermoregulate against a cold stress that is well tolerated by untreated rats. As with

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other tremorigenic compounds, such as kepone, the heat from tremor in rats dosed with DDT appears to contribute little to the overall heat balance of the rat.

DNP is a classic uncoupling agent of oxidative phosphorylation and elicits a marked elevation in whole-body oxygen consumption. It is interesting to find that there is so little known about the thermoregulatory mechanisms of DNP in spite of the long history of its use as a herbicide (Takehiro *et al*., 1979). Intravenous infusion of DNP in the awake rat caused a near instantaneous elevation in MR, ventilatory frequency and minute ventilation concomitant with a steady rise in body temperature. The MR of the rat was increased from 34 to 51 ml kg^{-1} min^{-1} following repeated intravenous injections of DNP, with a total dose of 24 mg kg^{-1} in rats maintained at a cold ambient temperature of 15 °C. The increase in MR was associated with a 1.1 °C increase in core temperature. Pentachlorophenols (PCPs) have antifungal properties and have been used for many years as a wood preservative, and also uncouple oxidative phosphorylation. Human exposures in the timber industry are common with cutaneous and inhalation routes. Dangerous, prolonged elevations in core temperature, accompanied with profuse sweating have been reported in workers subjected to acute PCP exposure. For example, a 22-year-old male exposed to PCP on the work site was admitted to the emergency room with a core temperature of 41.8 °C; within 22 hours core temperature had increased to 42.2 °C and the subject died (Wood *et al*., 1983). One finds that the hyperthermia in these studies is termed 'fever' or 'hyperpyrexia'. However, assuming that the CNS control of body temperature is unaffected by PCP, then it can be assumed that the rise in core temperature is essentially a forced hyperthermia and not a true fever (see **Figure 3**).

The pyrethroid-based insecticides are widely used in domestic and agricultural applications, but there is surprisingly little information on their effects on thermoregulation. Pyrethoids are generally classified as Type I or Type II, depending on their neurophysiological and behaviour effects (see Ray and Fry, 2006 for summary). Type I pyrethroids keep sodium channels open momentarily (i.e. milliseconds), whereas Type II agents keep channels in an open state for minutes. In general, Type I pyrethroids elicit hyperthermic responses whereas Type II elicit hypothermia. However, the hypothermia elicited by Type II pyrethroids occurs at relatively high doses. For example, in rats monitored by radiotelemetry, the thermoregulatory response to deltamethrin, a commonly studied Type II agent, shows a biphasic response of hyperthermia at low doses and hypothermia at high doses (**Figure 6**). In spite of all that is known on

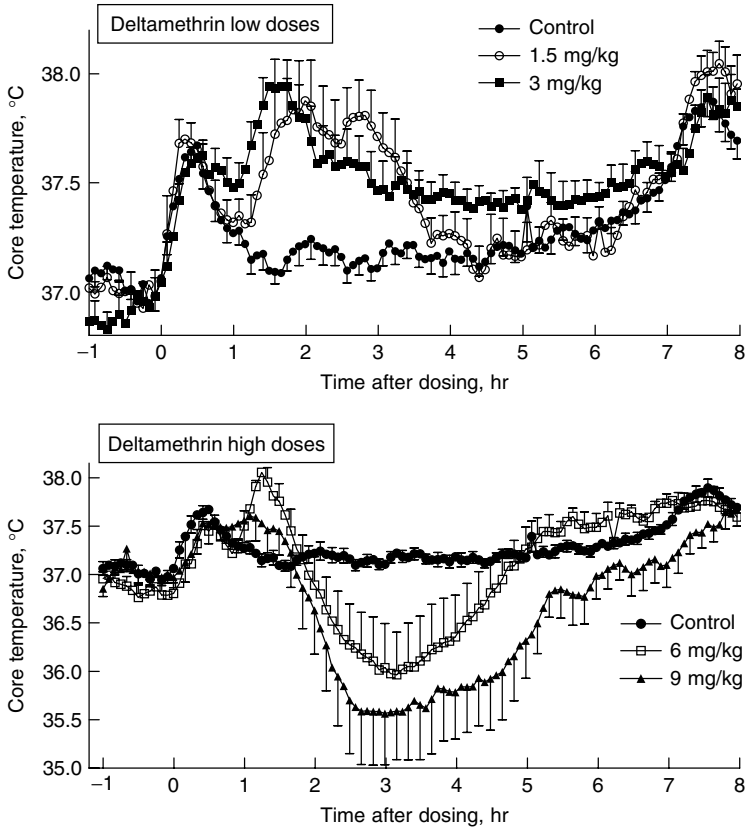

Figure 6 Time course of core temperature of rats monitored by radiotelemetry and administered deltamethrin by oral gavage. (Data from Wolansky *et al*., 2007.)

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the neural mechanisms of pyrethroid toxicity, there is no understanding of how certain classes and doses of pyrethroids elicit hypothermic or hyperthermic responses in the rat.

1.8 Advantages of a Hypothermic Response

The direct effects of body temperature on the toxicity of drugs and toxic chemicals were recognized well over 100 years ago (for review, see Fuhrman, 1946). Prior to the development of modern analytical methods, frogs were often used as a species for bioassays of drugs and other chemicals. Because body temperature of frogs and other poikilotherms is directly dependent on ambient temperature, it was quickly determined in these early studies that body temperature had a marked effect on pharmacokinetics of drugs and toxicants. For example, studies in the 1890s on the toxicity of colchicines showed that the frog was 400–500 times as sensitive when dosed at an ambient temperature of 32 °C as compared to 20 °C. Toxicity studies in amphibians maintained at different temperatures led to a general conclusion for cholchicine that '. . . the temperature of the body is the chief factor influencing toxicity' (Fuhrman, 1946). The LD (e.g. LD_{50}) has conventionally been used in rodent studies as a benchmark of toxicity. While tests using death as an end point are rarely used in current research studies, these older studies are very useful for illustrating how overt toxicity is affected by body and ambient temperature. Keplinger *et al*. (1959) was one of the first to perform a systematic analysis of the LD of a variety of drugs and chemicals in the rat. The approximate LD of 58 compounds was determined in rats housed for 45 minutes before and 72 hours after dosing at environmental temperatures of 8, 26 and 36 °C. When compared to a temperature of 26 °C (i.e. slightly below the rat's thermoneutral zone) the LD was consistently lowered for all compounds administered to rats at an ambient temperature of 36 °C. In the selected examples in **Figure 7**, the percent lethality in mice administered given doses of toxicants and maintained at different ambient temperatures is plotted. In all cases, the incidence of lethality increases when the mice are maintained at warm temperatures that block their ability to become hypothermic. It is interesting to note the consistent effect of higher environmental temperatures on chemical lethality in spite of marked differences in the chemical's mechanism of action. For example, in rats, DDT causes tremor and hyperthermia and would be expected to be more toxic at higher temperatures by inducing hyperthermic death. On the other hand, many other agents that normally induce hypothermia such as ethanol, toluene and chlorpromazine at 26 °C are nonetheless more toxic in the heat (Keplinger *et al*., 1959).

1.9 Magnitude and Duration of a Toxicological Response

The physiological response or sensitivity to a drug would be expected to depend on both the tissue concentration (or magnitude) and length of time that the toxicant

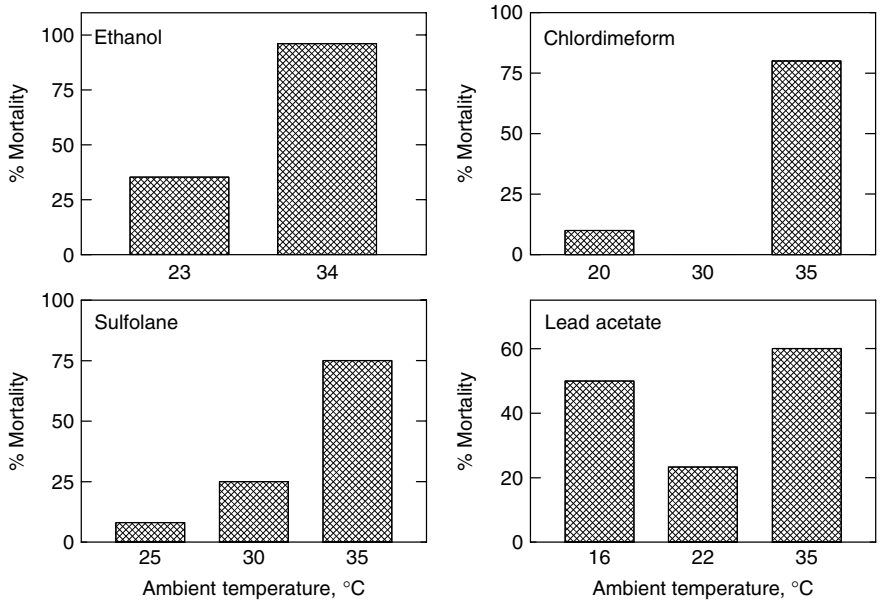

Figure 7 Effects of ambient temperature on lethality of various toxicants administered to mice. (Details from studies reviewed by Gordon, 2005.)

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persists in the body (Doull, 1972). In most cases, hypothermia is going to prolong the duration, but reduce the magnitude of toxicity. That is, when body temperature is lowered, the mechanism(s) responsible for metabolizing and excreting the toxicants are going to be depressed because the temperature coefficients for these processes are positively affected by temperature. Doull (1972) developed a general prediction of the response of biological systems to toxic levels of drugs that can also be applied to xenobiotic agents, 'Temperature is directly correlated with the magnitude and inversely correlated with duration of drug response in biological systems'. In other words, while the concentration of a toxicant will persist longer during hypothermia, the toxicity of the agent is reduced.

This tenet of Doull's was stated over 30 years ago and remains a critical issue in toxicology and pharmacology. The thermoregulatory system of mice and rats can respond rapidly with a hypothermic or hyperthermic response. These integrated thermoregulatory responses will have a marked impact on the pharmacokinetics and overall toxicity of the chemical or drug. Larger mammals, such as humans, do not sustain the changes in body temperature, as is seen with rodents. Thus, PBPK modellers who develop predictions based on data collected in laboratory rodents should consider how the potential thermoregulatory responses in the smaller species should be extrapolated to larger species.

The clearance of ethanol in mice maintained at different ambient temperatures illustrates the magnitude vs. duration principle of Doull (1972) (**Figure 8**). Ethanol elicits a marked hypothermic response in mice, but the magnitude of hypothermia can be modulated by altering ambient temperature. After an acute dose of ethanol and housing the mice at ambient temperatures of 4–35 °C, it was shown that the clearance of ethanol was directly affected by the core and ambient temperature. Mice housed at 22 °C became hypothermic with a core temperature of 35 °C and their ethanol elimination rate was 0.012 mg ml min^{-1}. When ambient temperature was raised to 35 °C, the hypothermic effect of ethanol was essentially blocked and the clearance of ethanol increased by 30%. Body temperature influences the rate of ethanol elimination, presumably through the thermal modulation of alcohol dehydrogenase activity in the liver (Romm and Collins, 1987; Bejanian *et al*., 1990). Hence, a warmer core temperature allows for a rapid clearance of the drug, but this is also associated with increased mortality (see **Figure 7**). Overall, hypothermia from alcohol exposure delays the clearance of the drug, but reduces its overall toxicity.

1.10 Delayed and Chronic Effects

Radiotelemetric monitoring of undisturbed rodents has provided valuable information on the delayed and chronic effects of toxicants on the thermoregulatory system. The stress of repeated handling and insertion of colonic probes in rats and mice tends to obviate any subtle effects of a toxicant on thermoregulation. Gallaher and Egner (1987) first used radiotelemetry to document a delayed hyperthermic response to acute ethanol exposure in rats. That is, following recovery of the hypothermic effects of ethanol at doses of 4–6 g kg^{-1}, there was a prolonged elevation in core temperature the day after dosing. In the time course of core temperature of humans dosed for several hours with alcohol (**Figure 5**), a delayed rise in core temperature is observed, similar to that observed in rodents exposed acutely to alcohol.

Our laboratory first showed a similar response to an organophosphate, DFP (Gordon, 1993a). The acute hypothermic effects of DFP in rats and mice were well characterized (Gordon, 1994). We found that the DFP-exposed rat maintained an elevated core temperature during the day for at least 48 hours after exposure. This delayed elevation in body temperature has been

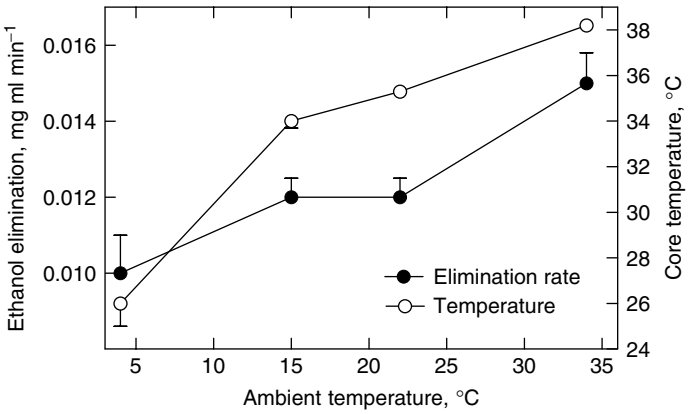

Figure 8 Effect of body temperature on the clearance of ethanol in mice. Ethanol-induced hypothermia was modulated by maintaining mice at different ambient temperatures. (Reproduced from Bejanian *et al*., 1990. © Elsevier.)

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shown in rats dosed with organophosphate-based insecticides chlorpyrifos and diazinon, and carbaryl, a carbamate-based insecticide (Gordon and Mack, 2003). The elevation in core temperature is manifested during the daytime of the rat's circadian rhythm, corresponding with the time when core temperature is normally at its lowest point (see **Figure 4A**). This is a typical pattern in rodents subjected to infectious fevers (for discussion, see Gordon, 2005). In addition, the delayed rise in core temperature following exposure to an organophosphate is probably not a rebound from the acute period of hypothermia. Blocking the hypothermic response by maintaining the rats at thermoneutral ambient temperature has no effect on the development of the hyperthermic response to chlorpyrifos (Gordon, 1997). Moreover, the rise in temperature is blocked by administration of an antipyretic, suggesting that the temperature rise is akin to a fever and is mediated by activation of the cyclooxygenase pathway (Gordon *et al*., 1997; Gordon and Mack, 2003).

1.11 Human Responses

It is important to consider the differences in body size, surface area : mass ratio and MR when extrapolating toxicological effects from rodent to human. As discussed earlier for alcohol, the magnitude of the acute hypothermic response to toxicants and drugs that is observed in laboratory rodents is generally not seen in adult humans and other large mammals. In fact, a hyperthermic response is commonly seen in humans subjected to antiChE insecticides, metal fumes and a variety of other toxicants (Gordon, 2005; Saadeh *et al*., 1996; Namba *et al*., 1971). The hyperthermic response may be regulated, being similar to a fever, or it may be a forced elevation in core temperature. Humans exposed acutely to organophosphate insecticides can exhibit an elevated temperature that persists for days, suggesting that it is regulated.

As body size increases, the surface area : body mass ratio decreases, thus limiting the ability to dissipate heat. For example, a 30 g mouse has approximately 17 times the surface area : body mass ratio as compared to an adult 80 kg human. Hence, there are physical limitations to dissipate heat normalized per unit body weight with increasing body size. Rodents dissipate heat faster and have the ability to quickly lower core temperature in response to toxicant exposure. The thermal inertia of a large mammal, such as a human impedes the ability to lower core temperature in a rapid manner.

It appears that rodent models of thermoregulation are reasonable predictors of human responses provided that the aforementioned issues are taken into consideration. In view of the temperature dependency of biochemical and physiological systems (see Section 2), scaling the thermoregulatory responses from rodent to human can be important in the extrapolation of a variety of toxicological responses that are not associated with thermoregulation. Adult humans and other relatively large species are essentially trapped in their stable thermal milieu and cannot respond with a natural hypothermic response when faced with a toxic insult. The fact that hypothermia is protective and rodents are able to use this as an adaptive mechanism to a toxic insult, one may under estimate the risk of a toxicant when extrapolating from a small rodent to an adult human.

2 SUMMARY

Our understanding of the effects of temperature on toxic response has seen resurgence in the past several decades with the development of better methods to study the integration of autonomic and behavioural thermoregulatory responses in rodents and other species. Radiotelemetry provides researchers with the best tool to study the thermoregulatory responses in undisturbed rodents exposed chronically or acutely to toxic chemicals. The thermoregulatory response to acute exposure to many toxic chemicals involves a regulated hypothermic response, characterized by an increase in autonomic thermoeffectors to increase heat loss and a behavioural preference for cooler temperatures. This thermoeffector response is quickly manifested by a marked drop in the core temperature in rodents. However, in humans and other large mammals, the hypothermic response is meagre due to their large thermal inertia. A combination of exposure to cool temperatures and a moderate hypothermic response has been found to benefit survival to a variety of toxic agents. Thus, the integrated thermoregulatory response of rodents to lower their core temperature seems to be an adaptive response. On the other hand, fever or hyperthermia is often seen in humans and other large mammals exposed to various toxicants. A fever is also seen in rodents provided that core temperature is monitored without disturbing the animal (e.g. telemetry). The universal effects of temperature on chemical toxicity calls for researchers to have a better understanding of the thermoregulatory effects of environmental toxicants.

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Development of Tolerance to the Toxicity of Xenobiotics

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CONTENTS

1 INTRODUCTION

Tolerance due to exposure to xenobiotics is a well-known phenomenon and is of great importance in our daily life. It is known to occur in animals, plants and microorganisms exposed to all types of chemicals that surround us. Tolerance, due to the chronic presence of a compound, is believed to result from a homeostatic process, in which a newly established effect is counteracted, thus transferring the situation back to normal. This can, in principle, be achieved either by reducing the response at the target or by preventing access of the active compound to the target.

Tolerance can be developed in several ways. It is often obtained by receptor changes, either the number of receptors or by decreased affinity for the receptor molecule. This is particularly noted in the superfamily of seven transmembrane G protein-coupled receptors (GPCRs). This represents the largest family of plasma membrane receptors. Examples of such receptors are the β-adrenergic receptor, the cholinergic muscarinic receptor and the opiate receptor. These GPCRs can be desensitized to activation by agonists and thereby develop tolerance. Desensitization requires phosphorylation of the receptor by G protein-coupled receptor kinases (GRKs). The phosphorylated GPCRs will then bind strongly to a group of proteins called arrestins which thereby uncouples them from G proteins. This prevents further stimulation and downstream signalling. Arrestins bind with much higher affinity (<1 nM) to GRK-phosphorylated GPCRs than to unphosphorylated receptors, and the presence of multiple phosphate groups on the receptor (i.e. more than three per receptor) is thought to be the primary trigger for high-affinity arrestin binding (Gurevich and Gurevich, 2004). The arrestin-bound receptor is then internalized to endocytes and removed.

Tolerance can also be developed by the presence of proteins that can bind or inactivate the agonist, thereby making it less readily available. An example of such a mechanism is the action of sublethal doses of cadmium on most organisms. Cadmium is toxic to several organs, but in particular the liver. Pretreatment with low concentrations of cadmium leads to tolerance to a large number of metals. Cadmium induces a protein with several SH groups called metallothionein, which has the ability to bind large amounts of cadmium and thereby prevent any toxic effects (Klaassen and Liu, 1998). Metallothionein plays an important part in the development of tolerance towards several metals, such

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as cadmium, zinc, copper and mercury. Metallothionein, due to its high number of SH groups also shows tolerance to free-radical damage (Thornalley and Vasák, 1985). Animals with little or no metallothionein, such as sheep, are very sensitive to grazing on copper-containing soil (Søli, 1980). Cross-tolerance occurs when an organism is submitted to small doses of one compound leading to the induction of a protein that will also protect against other compounds simultaneously. Metallothionein, as shown above, can protect against several metals, although it is induced by only one of them. Another example of cross-tolerance is the induction of cytochrome P450 (CYP) enzymes, by administration of small doses of phenobarbital. This is accompanied by in increase in the enzyme CYP2E1 in the liver and some other organs. This enzyme has the ability to metabolize and detoxify several other substances, including ethanol. Therefore tolerance to alcohol is increased by exposure to phenobarbital.

Tolerance is a term used to describe the adaption of a single individual rather than the population. Tolerance usually occurs rapidly if it only requires an induction of a protein or takes more time when it requires more subtle changes in a signal system adhered to a receptor. When the stress is removed the tolerance is usually lost within a few weeks.

In general we separate the term tolerance into resistance and tolerance. The term 'resistance' suggests that the genetic constitution of a population allows it to resist a physiological effect. This means that the action is reduced or abolished by a few genes in the population, allowing some of the organisms to survive. An example is the discovery that dichlorodiphenyltrichloroethane (DDT) lost its toxic effects on several insects. The insects that survived the action of DDT contained an enzyme, DDT hydrochlorinase, that was able to metabolize DDT to the inactive dichlorodiphenyltrichloroethylene (DDE) (Sternburg *et al*., 1954). This enzyme was later identified as a glutathione transferase. Also, DDT attaches itself to one of the sodium channels of insects. A few individuals had two amino acid mutations which did not have any physiological effect on the channel, but prevented DDT acting on the channel. Again, these insects survived and were allowed to multiply and caused DDT to lose its insecticidal property. This simply means that organisms which do not have these genes do not survive, and thus allow those that do to survive and multiply. If these survival genes were present before the action we call this natural selection and this property will remain in the generations to follow.

2 TOLERANCE TO INHIBITORS OF ACETYLCHOLINESTERASE (AChE-I)

In the following we will discuss tolerance developed to acetylcholinesterase (AChE)-I because this involves changes to several different receptors and to different enzymes, which either bind or hydrolyse the inhibitors. AChE-I consists of two large groups of compounds known as organophosphates (OPs) and carbamates. They are important mainly as insecticides, but also as chemical warfare agents. The use of these compounds for peaceful purposes causes several accidental deaths every year. The compounds inhibit the enzyme AChE and thereby increase the level of acetylcholine (ACh) in the synaptic gap. The acute toxic effects of AChE-I are due to stimulation of the muscarinic and nicotinic receptors by ACh. Most emphasis will be given to OPs because they are partly irreversible inhibitors and their effects are more chronic.

The recovery of AChE in vertebrates is slow and the half-time for resynthesis of the enzyme is several days. There are several examples that prolonged exposure to toxic concentrations of OP give physiological effects that are smaller than expected from the degree of AChE inhibition (Bushnell *et al*., 1991). Barnes and Denz (1954) gave rats 50 ppm of the OP parathion for several months and observed that after three months several of the effects of AChE inhibition, such as fasciculation, lacrimation and salivation disappeared, although the animals still occasionally showed tremors. Normally animals die after having been exposed to 1 mg kg^{-1} day octamethylpyrophosphoramide (OMPA) for five days. But Rider *et al*. (1952) showed that after pretreatment of animals with 0.5 mg OMPA kg^{-1} day^{-1} for five days, the animals did not show any toxic effects after a dose of 1 mg kg^{-1} day for 41 days. Another example is that rats survived daily administration of 0.5 LD_{50} of the irreversible inhibitor soman for 11 days (Sterri, 1981). In fact, often the animals were symptom-free. There are several observations of physiological effects of sublethal doses of OP that cannot be explained by the recovery of AChE. Thus, when treating the pupil with the irreversible inhibitor soman, miosis, as a sign of AChE inhibition of the iris, was noted. After a single topical soman instillation into the conjunctival sac there was an almost linear relationship between the reduction in AChE activity and the reduction in pupillary diameter. Repeated topical administration of soman at 24 hour intervals, in doses capable of almost complete inhibition of AChE in the iris, was accompanied, however, by a reduced miotic effect. There were a reduced rate of soman-induced pupillary constriction, a less pronounced reduction in pupillary diameter and a more rapid return of the pupillary diameter to normal size on repeated administration. The decrease in response to repeated administration could not be explained by a reduced inhibitory effect of soman on AChE, nor by *de novo* synthesis of AChE, but could be a sign of developed tolerance (Soli *et al*., 1980). Also AChE inhibition led to hypothermia in animals and the rapid return from hypothermia seen after treatment could not be explained by recovery of AChE activity. It must have been due to some form of developed tolerance

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(Gordon, 1997). More recently, animals with knockout (KO) AChE both in the peripheral and central nervous system have been produced and they survive up to one year (Xie *et al*., 2000). This can only occur because the animal develops tolerance to the absence of AChE.

Tolerance to AChE inhibitors has been noted after different forms of administration and in several species, such as mouse, rat, guinea pig and man. Tolerance can be developed in several ways. It can be due to changes in the number of or by decreased affinities of the cholinergic muscarinic or nicotinic receptor molecules. It can, however, also be obtained by proteins that can bind or inactivate the inhibitor. Examples are binding of the inhibitor to carboxylesterases (CarbEs), butyryl-cholinesterases (BuChEs) or albumin. In addition, tolerance can, in principle, be achieved through more rapid detoxification of OP compounds by OP-hydrolysing enzymes, such as paraoxonases (PONs) and somanases.

2.1 The Importance of Muscarinic Receptors

Muscarinic acetylcholine receptors (mAChRs) are heavily involved in the development of tolerance to AChE-I. The hypothesis that tolerance to chronic treatment with AChE-I could be due to inactivation of the cholinergic receptor was first stated by Brodeur and Dubois (1964). Changes in the muscarinic receptor have, in fact, been shown to be one of the most important ways for a cell or an individual to obtain tolerance towards AChE inhibitors. The presynaptic muscarinic autoreceptors respond rapidly to a high concentration of ACh in the synaptic gap by reducing release. The postsynaptic receptor responds, usually more slowly, by reduction in the number of mAChRs after AChE inhibition in the brain or peripheral nervous system (Ehlert *et al*., 1980; Schiller, 1979; Gazit *et al*., 1979) Some authors reported both a decrease in the number of mAChRs and in the affinity to the ligand in the ileum and striatum (Costa *et al*., 1981), but most authors have reported only a decrease in the number of mAChRs in the brain.

There are five subtypes of mAChRs, named m1, m2, m3, m4 and m5. Subtypes m1, m3 and m5 are coupled to the α-subunit of the Gq/11 class of G-proteins. The receptor–agonist interaction activates phosphatidylinositol-specific phospholipase C, resulting in the production of diacylglycerol and phosphoinositol. On the other hand, subtypes m2 and m4 inhibit adenyl cyclase through α-subunits of G-protein Gi/0 (Bonner, 1989). The liberated complex of β- and γ-subunits regulate the function of potassium and calcium channels (Herlitze *et al*., 1996). There is now good evidence that internalization of muscarinic receptors can take place via phosphorylation by GRKs and further binding to arrestin (Gainetdinov *et al*., 2004).

Assay of the muscarinic receptor is generally performed using binding of labelled 3-quinuclidinyl benzylate (QNB) which attaches all subtypes. QNB can be used to assay both mAChRs at the plasma membrane and in the cytoplasm, when they are internalized. In contrast *N*-methylscopolamine (NMS) is water soluble and does not penetrate cell membranes. NMS can only measure mAChRs localized at the plasma membrane and not when they are internalized. NMS binding has therefore been found to be more sensitive than binding with QNB. In two cell lines, NG108-15 and SK-N-Sh, carbachol caused a loss in NMS binding, but the binding of QNB was unchanged, thus indicating internalization of the receptor (Baumgold *et al*., 1989).

2.2 Postsynaptic Receptors

Prolonged treatment with a cholinergic agonist leads to a decrease in mAChRs. This is common for G-protein-linked receptors. Down-regulation follows receptor internalization and degradation, a process which takes several hours. For example, Chinese hamster ovary (CHO) cells stably transfected with the m1 subtype were exposed to the cholinergic agonist carbachol for 24 hours. This resulted in a decrease of 66% in muscarinic receptor binding and a 73% decrease in the m1 messenger RNA (mRNA) level (Wang *et al*., 1990). Likewise, in cultured granule cells, both subtypes m2 and m3 were reduced after carbachol treatment. This also resulted in a decrease in cyclic adenosine monophosphate (cAMP) production (50%) and a reduction in the corresponding mRNA by 30–50% (Fukamauchi *et al*., 1993). In contrast the m4 subtype receptors of neuroblastoma cells were reduced by 30% by 24 hour carbachol treatment, but in this case there was no effect on the corresponding m4 mRNA.

The decrease in mAChRs depends both on the duration of AChE inhibition and the degree of inhibition in experimental animals. Schwab *et al*. (1981) and Costa *et al*. (1981) showed that there was no immediate effect after acute inhibition of AChE in rats with OP. Jett *et al*. (1994) injected parathion over a 21 day period resulting in 84–90% AChE inhibition in the brain without overt signs of toxicity. To their surprise they found a reduction in m1 subtype receptors and m1 mRNA in the frontal cortex and m4 subtype receptors and m4 mRNA in the striatum, but no change in hippocampus mAChRs, although AChE inhibition was similar in all three regions. They concluded that hippocampus had different feed-back mechanism for regulation of the mAChR.

Yagle and Costa (1996) studied the effect of disulfoton (2 mg kg^{-1} for a fortnight) on proteins and mRNA levels of mAChR subtypes in brain tissue (m1 and m2) and

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peripheral mononuclear cells (m3). The AChE activity in the cerebral cortex was reduced to 19% of original level after 14 days and QNB binding was reduced to 72% during this period. The m1 subtype dominated in the cortex, hippocampus and striatum, whereas m2 dominated in the medulla and cerebellum. After the treatment m1 and m2 mRNA was reduced by about 25% in the hippocampus, whereas in the medulla m2 mRNA was reduced by 19%. Subtype m3 was reduced by 10% in the cortex, but not in lymphocytes. In contrast to Jett *et al*. (1994), there was no difference between the observed effects in the different regions.

In mice exposed to parathion in the diet there was a large variation in AChE inhibition due to intake of food (Jett *et al*., 1993). In these animals it was therefore possible to show a correlation between AChE inhibition and reduction in both QNB binding and 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) binding. There was no effect on the affinities for these ligands. The maximal reduction in binding for these was 58%. In contrast, NMS binding was also reduced to 58%, but this occurred already at only 10% AChE inhibition.

A correlation between ChE inhibition and QNB and AF-DX 384 binding in the cortex and striatum of young and adult rats was also found by Li *et al*. (2005). They gave young rats (age seven days) and adult rats (age 90 days) chlorpyrifos and methyl parathion for seven days and found a correlation between ChE inhibition and muscarinic binding at one day and seven days postexposure.

The tolerance observed to hypothermia after treatment with OPs has also been suggested to be due to a decrease of mAChRs in the temperature-regulated region of the brain (Overstreet and Yammamura, 1979; Russel *et al*., 1975).

In conclusion, studies from AChE inhibition in the brain show that there is generally a correlation between AChE inhibition and reduction in mAChR binding. The degree of AChE inhibition is higher than the reduction in receptor binding. NMS binding, which can only measure the extracellular muscarinic receptor, is more sensitive than the other antagonists, which measure both extracellular and internalized receptor molecules. In general, the reductions in mAChRs are similar in different brain regions. In almost all cases examined there was a reduction in the number of muscarinic binding sites and not an effect on affinity to the binding site after exposure to AChE-I.

A complicating factor in the discussion of the effect of OP inhibition and mAChRs is the direct action between several OPs and mAChRs (Ward *et al*., 1993; Huff *et al*., 1994). Diisopropyl fluorophosphate (DFP), paraoxon, malaoxon and chlorpyrifos-oxon inhibit 1,3-dioxalan binding, which is a high-affinity ligand to a subset of the m2 subtype. QNB binding was only affected by echothiophate. The effect of OPs on dioxalan binding occurs in the same concentration range as for AChE inhibition. The m2 receptor is coupled to cAMP production and paraoxon, malaoxon and chlorpyrifosoxon all inhibit forskolin-stimulated c-AMP formation (Ward and Mundy, 1996).

2.3 Muscarinic Autoreceptor

Autoregulation of ACh release seems to play an important role in presynaptic mAChRs (Molenaar and Polak, 1980; Nordstrom and Bartfai, 1981; Dolezal and Wecker, 1991; Kirkpatrick and Richardson, 1993). This is a common feature of G-protein-linked receptors. The importance of the autoreceptor is demonstrated by the fact that the addition of atropin, the well-known cholinergic antagonist, to various brain slices or peripheral tissues increased the release of ACh. In contrast, the cholinergic agonist carbachol inhibited the release of ACh (Nordstrom and Bartfai, 1980; Aas and Fonnum, 1986; Dolezal and Wecker, 1990). Using antagonists specific for subtype m2 and m4 receptors, it was suggested that the regulation of ACh release from brain tissue was controlled by these two subtypes (Quirion *et al*., 1995). This was further investigated by the use of an antisense to m2 receptor and by using a very specific m4 antagonist during microdialysis of the hippocampus. In this way it was shown that the responsible subunit in the hippocampus was m2 (Kitaichi *et al*., 1999). The use of brain slices from an m2/m4 receptor single KO mice confirmed that autoinhibition is mediated primarily by the m2 receptor in the hippocampus and cerebral cortex, but predominantly by the m4 receptor in the striatum (Zhang *et al*., 2002).

The presynaptic autoreceptors in the brain reduce the release of ACh and thereby reduce the cholinergic effects on the postsynaptic receptors. The mechanism of inhibition of ACh release involves the stimulation of presynaptic GPCRs and is due to a restriction of the activity of the high-voltage-activated N or P/Q calcium channels (Krejcí *et al*., 2004). This restriction takes place by liberated β/γ subunits of the receptor, which interact with the activated calcium channels and inhibit calcium influx. The effect of autoreceptors on ACh release may have therapeutic consequences in, for example, Alzheimer's disease. AChE inhibitors used in therapy can inhibit the release of ACh. A consequence of this could be that the use of high-affinity M2 muscarinic antagonists may prevent inhibition of ACh release and facilitate learning and memory in experimental animals (Quirion *et al*., 1995).

In adult muscle, the m1-receptor-selective antagonist pirenzepine (10 μM) reduced evoked neurotransmission (approximately 47%), whereas the m2-receptor-selective antagonist methoctramine (1 μM) increased the evoked release (approximately 67%). Both the m1- and

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m2-mediated mechanisms depend on calcium influx via P/Q-type synaptic channels (Santafé *et al*., 2006).

2.4 Nicotinic Autoreceptors

The nicotinic receptor dominates at the neuromuscular junction, but it is also widely distributed in the mammalian brain. The brain receptors are different from those in the ganglion and motor endplates. Nicotinic receptors are localized on both presynaptic axon terminals and postsynaptic somatodendritic sites (Sargent, 1993). The concept of ionotropic autoreceptors has been established primarily by indirect evidence measuring the overflow of radioisotope-labelled transmitters or whole-cell recording of excitatory postsynaptic current (EPSC) amplitude at postsynaptic cells (Bowman *et al*., 1990). In the brain the nicotinic receptor has been implicated in various physiological and pathological conditions, including cognition, Alzheimer's disease, Parkinson's disease, anxiety and addiction to tobacco products (Nordberg *et al*., 1989; Arneric *et al*., 1995).

The positive modulation of neurotransmitter release appears to be a widespread and potentially important role of presynaptic nicotinic ACh receptor channels (McGehee *et al*., 1995). Nicotinic autoreceptors have also been proposed to regulate ACh release at motor nerve terminals (Bowman *et al*., 1990) and in central cortical and hippocampal synaptosomes (Rowell and Winkler, 1984; Wilkie *et al*., 1996).

The increased release of ACh from brain synaptosomes caused by the nicotinic autoreceptor can only be detected in the presence of atropine, which inhibits the muscarinic autoreceptor (Wu *et al*., 2003). The nicotinic antagonist mecamylamine, but not nicotine, inhibits the release of ACh, indicating the specificity of the receptor. Sublethal DFP treatment ($2\,mg\,kg^{-1}$) over several days leads to a decrease in AChE activity, muscarinic receptor and nicotine binding in rat brain (van de Kamp and Collins, 1992). The mechanism behind the increased release may be due to an increased intracellular calcium level (Wilkie *et al*., 1996). Present studies indicate that the most important function of presynaptic neuronal nicotinic receptors (nAChR)s in either synaptic or nonsynaptic localization is to directly effect intracellular Na^+ and Ca^{2+} levels, followed by a depolarization sufficient to activate local voltage-sensitive Ca^{2+} channels, resulting in transmitter release (Szabo *et al*., 2008).

Several antiChEs have a direct effect on the nAChRs. The most potent OPs to inhibit the $\alpha4\beta2$ nAChRs are the oxidized metabolites of disulfoton, parathion-ethyl, parathion-methyl and fenthion (Smulders *et al*., 2004). The same oxidized OPs are effective AChE inhibitors and also the best for interaction with the muscarinic receptor. Both paraoxon and chlorpyrifos-oxon inhibit the nAChR and desensitize the receptor by binding to a different site from the ACh binding site (Katz *et al*., 1997). These OPs inhibit the release of ACh and will delay the symptoms of AChE poisoning. Even very toxic AChE-Is such as VX and sarin have effects on ion channels or receptors at levels close to their inhibitory potency of AChE. OPs like ecothiopate, DFP and VX block the open ion channel of the nicotinic receptor and desensitize the receptor of the electric organ of the torpedo and muscle endplate (Bakry *et al*., 1988; Eldefrawi *et al*., 1988).

A special case is that galantanine and physiostigmine, both AChE-Is, increase ACh release (Coyle *et al*., 2007). This is unexpected, but is due to their effect as allosteric ligands of the nicotinic receptors in the brain. It has been suggested that they act through the phosphatidyl-3-kinase and AKTpathway (Takada-Takatori *et al*., 2006). Nicotinic agonists have therefore been suggested to be used in the treatment of Alzheimer's disease, since they increase ACh release.

2.5 Adenosine Receptors

Adenosine has been shown to have powerful inhibitory action on neuronal activity and on the release of excitatory transmitters (Kirkpatrick and Richardson, 1993; Broad and Fredholm, 1996; Jin and Fredholm, 1997). In both the central nervous system (CNS) and the vertebrate neuromuscular junction, adenosine both inhibits and stimulates ACh release, depending on the type of adenosine receptor. In general, inhibition of release is mediated via A1 receptors and excitation through A2 receptors. The final outcome depends on the degree of the activation of the two receptors. Adenosine triphosphate (ATP) is known to be coreleased with ACh from the synaptic vesicles, and can be hydrolysed extracellularly by ecto-5-nuclidases to adenosine. Adenosine is also released from purinergic vesicles, motor endplates and postjunctional tissue. Adenosine is the active component, but ATP can substitute for adenosine if hydrolytic enzymes are not available.

Since adenosine and ATP are present in cholinergic structures and interfer with ACh release, the adenosine receptor can therefore be expected to be involved in tolerance to AChE-I poisoning. In agreement, in perfused rat cortical slices, the A1-selective agonist, N^6-cyclopentyladenosine (CPA), caused a dose-dependent inhibition of ACh release, which was attenuated in the added presence of the A1-selective antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX; $1\,\mu M$). In the neostriatum, the evoked release of [^{3}H]-ACh was significantly enhanced by the A2a agonist CGS 21680 but decreased by the A1 agonist, R-PIA. The effects of NECA (5′-*N*-ethylcarboxamidoadenosine), which is equipotent at both receptor subtypes, were dependent on the concentration used. With high concentrations NECA inhibited, and with low concentrations

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enhanced, the evoked release of [^{3}H]-ACh. As an example of the importance of the adenosine receptors in modifying ACh release, the selective adenosine A1 receptor agonist CPA (N^6-cyclopentylxanthine) effectively attenuated the cholinergic symptoms and prevented mortality in lethally tabun- or sarin-intoxicated rats (van Helden *et al*., 1998). During AChE-I poisoning, the CPA-treated animals showed only a small elevation of extracellular ACh concentrations in the brain relative to the baseline value, whereas an 11-fold increase in transmitter levels was observed in animals not treated with CPA (Bueters *et al*., 2003).

In the motor endplate, the excitatory A2a receptors facilitate ACh release by interaction with the presynaptic nicotinic AChRs. This probably occurs through a cAMP-dependent mechanism (Correia-de-Sa and Ribeiro, 1994). Blockade of A2a receptors enhances desensitization, and blockade of A1 receptors enhances sensitization of the presynaptic nAChRs.

2.6 Tolerance in AChE Knockout Mice

The maximal tolerance to a high concentration of ACh must be found in the knockout animal for AChE (AChE (−/−)). Inhibitors of AChE like OPs are fast acting and lethal; it is therefore with some surprise that such a knockout animal can survive up to almost one year (Xie *et al*., 2000). Investigation of the peripheral nervous system using the phrenic nerve-hemidiaphragm showed that the knockout animal had developed markedly reduced and fragmented nicotinic receptors. To compensate for the absence of AChE activity, the size of the endplate was reduced. The junctional folds of the endplate and the number of nAChRs were heavily reduced. This allows smaller responses to ACh and also increases ACh diffusion out of the synaptic gap. The decay time of the miniature endplate potential was increased from 1 ms in the wild-type to 5 ms in the AChE-KO. BuChE activity was not increased, nor had it changed its general localization. Twitch tensions elicited in diaphragms of AChE (−/−) mice by single supramaximal stimuli had larger amplitudes and slower rise and decay times than did those in wild-type animals. In AChE (−/−) preparations, repetitive stimulation at frequencies of 20 and 50 Hz, and at 200 and 400 Hz produced decremental muscle tensions. Muscles from control mice maintained tension at all frequencies examined. Inhibition of BuChE activity led to larger responses of evoked released ACh, suggesting that the enzyme hydrolysed the extrasynaptic ACh (Adler *et al*., 2004; Girard *et al*., 2007).

In the central nervous system AChE was also eliminated, but there was no effect on the level and localization of choline acetyltransferase or on the ACh vesicle transporter. Extracellular ACh levels in the hippocampus were 60-fold elevated in AChE (−/−) mice compared with wild-type AChE (+/+) animals. In AChE (+/+) animals inhibition of BuChE did not affect extracellular ACh level, but in AChE (−/−) inhibition of BuChE increased extracellular ACh (Hartmann *et al*., 2007). There was a large increase in the choline plasma membrane transporter, probably to compensate for there being less choline available through reduced hydrolysis of ACh in the synaptic gap. There was a major reduction in mAChR numbers, including subtypes m1, m2 and m4, and there wase also a high degree of internalization of the mAChR subtypes. There were, however, no changes in the mRNA of the muscarinic receptor. Many of the symptoms usually seen in wild-type animals following the action of muscarinic agonists, such as induced hypothermia, tremor, salivation and analgaesia, and pilocarpine-induced seizures, could not be found in AChE (−/−) mice. It seems, therefore, that the most important way of obtaining tolerance in AChE (−/−) animals is a reduction in receptor numbers and not in regulation of ACh synthesis (Mesulam *et al*., 2002; Volpicelli-Daley *et al*., 2003).

A correct balance between muscarinic and adrenergic receptors was important to maintain the proper function of the lung in AChE (−/−). Muscarinic receptors were decreased to 50%, α1-adrenoceptors to 23% and β-adrenoceptors to about 50% of control. Changes were subtype specific, as α1A-, α1B- and β2-adrenoceptors were decreased while α1D-adrenoceptors remained unchanged. In contrast, receptor signalling into the cell, as measured by coupling to G-proteins, cAMP content and PI-phospholipase C activity was the same as control (Myslivecek *et al*., 2007).

3 ENZYMES

3.1 Carboxylesterase

Irreversible AChE-I, such as chemical warfare agents like sarin or soman (Sterri *et al*., 1980; Fonnum and Sterri, 1981) have a very slow or nonexistent recovery (Coult *et al*., 1966). Still, when 0.5 LD_{50} of these compounds are administrated daily for 11 days to rats, they will survive. This is caused by the presence of a protein which inactivates and binds OPs and thereby prevents them from reaching their target. In rodents this is due to an enzyme called carboxylesterase, which has a high affinity for OPs and is present in high concentrations in the plasma (Myers, 1952; Goutier, 1956). Unlike choline esterase (ChE), CarbE spontaneously recovers from inhibition with nerve agents and in that way may act as a scavenger.

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There are three criteria which define such a scavenger:

1. The binding between plasma CarbE and soman or sarin is rapid enough to pick up the compound before it leaves the blood.
2. The plasma CarbE is present in sufficient amounts to be able to inactivate each dose of soman or sarin.
3. The plasma CarbE–OP complex is spontaneously and sufficiently recovered before the next dose of soman or sarin is administered.

The quantitative contribution by plasma CarbE for repetitive exposure to the most toxic ChE inhibitors could be confirmed by pretreatment of the animals with specific inhibitors of CarbE. The estimated rate of detoxification of repetitively administered soman was reduced to 15% of the rate in the absence of triorthocresyl phosphate (TOCP), a CarbE inhibitor (Fonnum and Sterri, 1981; Sterri and Fonnum, 1984). This means that 80–90% of the repetitively administered soman must be inactivated through binding to the TOCP-sensitive active site of CarbE. In mice the LD_{50} of soman was reduced to about 6% by pretreatment with 35–50 mg kg^{-1} of the CarbE inhibitor CBDP (2-(*o*-cresyl)-4H-1,2,3-benzodioxa-phosphorin-2-oxide) (Casida *et al*., 1961; McKay *et al*., 1971; Boskovic, 1979). The acute LD_{50} of soman in rats and guinea pigs was reduced to one third by pretreatment with 100 mg kg^{-1} TOCP (Fonnum and Sterri, 1981; Sterri *et al*., 1981). The LD_{50} of sarin was reduced to about 20% by pretreatment with 40–50 mg kg^{-1} TOCP in rats (Myers, 1959; Polak and Cohen, 1969). The effects of CBDP on the LD_{50}s for several OP compounds confirm the importance of CarbE against the acute toxicity of highly toxic soman, sarin, tabun and paraoxon, but not against less toxic compounds such as dichlorvos and DFP (Maxwell, 1992). The less toxic ChE inhibitors have toxic concentrations in the plasma that are far beyond the plasma concentration of CarbE. Treatment with CarbE inhibitors showed that paraoxon toxicity depended on CarbE, but that DFP did not (Dettbarn *et al*., 1999).

As the name indicates, CarbE hydrolyses ester bonds and can inactivate insecticides with an active ester bond. The most prominent of such insecticides is malathion. Inhibition of CarbE with TOCP leads to a 100-fold increase in toxicity for malathion (Murphy *et al*., 1976).

Young rats have a low content of endogenous CarbE in the plasma (14 days old), and were protected from soman exposure by intravenous (iv) pretreatment with exogenous CarbE protein, partly purified from liver (Fonnum *et al*., 1985). This is the first example of use of a scavenger to reduce toxicity towards nerve agents. This has later inspired a large number of investigators to use other scavengers, like BuChE (Wolfe *et al*., 1987; Raveh *et al*., 1989; 1997; Broomfield *et al*., 1991; Maxwell *et al*., 1991; 1992; Doctor *et al*., 1993).

The recovery of CarbE after nerve-agent inhibition is due to the spontaneous reactivation of the inhibited enzyme (Polak and Cohen, 1969). Maxwell and Brecht (2001) suggested that the spontaneous reactivation may be due to a highly conserved histidine located in the active site of CarbE, but not present in AChE and BuChE. The position of histidine is similar to the histidine site introduced by Millard *et al*. (1995) to produce an OP-hydrolysing mutant of human BuChE.

The scavenger function of plasma CarbE is obviously of great importance for the understanding of soman detoxification in rodents. Rats, guinea pigs and primates, including man, are species with respectively high, medium and minor (zero) concentrations of plasma CarbE (Myers, 1952; Aldridge, 1953; Cohen *et al*., 1971; Li *et al*., 2005). It is therefore possible to explain the differences between species with respect to acute toxicity and to the effect of pyridostigmine prophylaxis on soman poisoning (Sterri and Fonnum, 1989). Thus, from insight into the mechanism of interference by CarbE, both the theoretical LD_{50} of soman and the theoretical protection factor of carbamate prophylaxis could be predicted for rat, guinea pig, marmoset and rhesus monkey (or man). The LD_{50} and protection factor found correspond well with the experimental value for subcutaneous (sc) LD_{50} of soman in rat (Sterri *et al*., 1980; 1985), guinea pig (Sterri *et al*., 1981) marmoset and rhesus monkey (Dirnhuber *et al*., 1979). The CarbE data also agreed with the experimental protection factor obtained for pyridostigmine prophylaxis against soman in rodents (Gordon *et al*., 1978) and primate species (Dirnhuber *et al*., 1979). From the experimental LD_{50}s of soman, it is suggested that rodent plasma CarbE may be a main scavenger for soman in both inhaled, iv and sc administration, whereas with percutaneous (pc) and interperitoneal (ip) administration there may be additional dermal and hepatic parameters for the threshold of soman (Sterri, 1989).

3.2 Acetylcholinesterase and Butyrylcholinesterase

The spontaneous reactivation of dimethoxy and diethoxy phosphorylated ChEs occurs within a few hours (Burgen and Hobbiger, 1951). Animals exposed to insecticides such as schradan (Rider *et al*., 1952), demeton (Barnes and Denz, 1954), disulfoton (Brodeur and Dubois, 1964), paraoxon (Wecker *et al*., 1977) and dichlorvos (Sterri, 1981) tolerate repeated exposure to sublethal doses for a long time. The symptoms and recovery from symptoms of dichlorvos poisoning correlated well with the timeframe of inhibition and spontaneous reactivation of AChE in different tissues (Sterri, 1981). The concentrations of plasma BuChE is 30-fold less that that of CarbE

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(Li *et al*., 2005). Both the low concentration and insufficient recovery of plasma BuChE observed after each dose of soman or sarin in repetitive-exposure experiments exclude any tolerance by binding to BuChE (Fonnum and Sterri, 1981; Sterri *et al*., 1981). In accordance, Grubic *et al*. (1988) excluded any contribution of plasma BuChE to the tolerance developed to acute soman toxicity by using iso-OMPA to differentiate between inhibition of plasma BuChE and CarbE.

Recovery of plasma BuChE, AChE activity and muscle protein by *de novo* synthesis has been reported to be vital for development of tolerance to repetitive exposure to DFP in rats, especially restoration of AChE activity in skeletal muscle (Gupta and Dettbarn, 1986). Also, modifications in affinity of AChE for ACh in paraoxon-tolerant rats have been reported by Milatovic and Dettbarn (1996). They observed 20–25% increase in affinity (decrease in K_m) for ACh by both the brain and diaphragm AChE. This may be the result of structural changes in AChE or the result of altered levels of pre-existing isozymes of AChE.

3.3 Phosphoric Triester Hydrolases

Aryldialkylphosphatase/PON (EC 3.1.8.1) and DFPase/somanase (EC 3.1.8.2) are enzymes hydrolysing OP compounds with generally low affinity (K_m = 0.1–10 mM) (Aldridge and Reiner, 1972). The plasma enzymes therefore prefer to hydrolyse relatively high concentrations of OPs present in the blood. They are mainly important for detoxification of the less toxic OP compounds. As an example, PON1-knockout mice were extremely sensitive to the less toxic diazoxon, but did not show increased sensitivity to paraoxon (Li *et al*., 2000). In agreement, following injection of exogenous PON1 to the knock-out mice, the same authors observed protection against the less toxic diazoxon and chlorpyrifos-oxon, but not against paraoxon. PON1 is polymorphically distributed in human populations with an amino acid substitution (Gln/Arg) at position 192, which determines the catalytic efficiency of this 354 amino-acid protein. In addition to the variable catalytic efficiency determined by the position 192 amino acid, protein levels of PON1 vary by as much as 15-fold among individuals with the same genotype. Sensitivity to diazoxon exposure is primarily determined by the plasma level of PON1, whereas for chlorpyrifos-oxon exposure, both the plasma PON1 level and the position 192 amino acid are important. Mouse PON1 has good catalytic efficiency for the hydrolysis of diazoxon and chlorpyrifos-oxon, but a poor efficiency for paraoxon hydrolysis relative to rabbit PON1 (Li *et al*., 2000).

Somanase in blood has little effect on soman detoxification, but in liver may be important. Liver perfusion experiments with soman demonstrated 80–90% detoxification of soman during a single pass through the liver, and this was not due to inhibition of ChE or CarbE (Sterri *et al*., 1983). The high capacity of soman detoxification in liver might therefore mainly be due to somanase activity, and might explain the tolerance to acute toxicity of soman by ip administration, compared to sc administration (Fonnum and Sterri, 1981).

The organophosphorus nerve agent soman contains two chiral centres (a carbon and a phosphorus atom), resulting in four stereoisomers (C+P+, C−P+, C+P− and C−P−). The P-isomers exhibit a mammalian toxicity that is ~1000-fold greater than that of the P+ isomers. The different capacity for the binding or hydrolysis of the four stereoisomers is important to evaluate scavengers that have the potential to protect against soman intoxication. Using a gas chromatography-mass spectrometry-based approach, it was found that BuChE were found to bind soman with a relative preference for the more toxic stereoisomers (C−P− > C+P− > C−P+ > C+P+), while human serum albumin and PON1 interacted with the less toxic isomers (C−P+/C+P+ > C+P−/C−P−).

4 TOLERANCE TO MORPHINE

Tolerance to morphine is an important topic, both because of its use in medicine and because of its abuse. There are three opioid receptors, namely μ-, κ- and δ-opioid receptors, which are all of the GPCR type. There are large differences in development of tolerance among the opioid agonists. Many of them, particularly the peptides, follow the normal pattern of receptor internalization, whereas tolerance to morphine seems to follow another mechanism (Keith *et al*., 1996). It is therefore interesting to show this tolerance mechanism as an example. Mechanistic studies suggest that morphine does not promote significant internalization of μ-opioid receptors because it fails to stimulate adequate receptor phosphorylation by GRKs. This makes the subsequent attachment to arrestin difficult and receptor recruitment to the clathrin-dependent endocytosis pathway becomes limited (Zhang *et al*., 1998). There is significant circumstantial evidence in support of this supposition, because only overexpression of GRKs promotes morphine phosphorylation, and overexpression of arrestins also promotes morphine-induced μ-opioid receptor internalization (Whistler and von Zastrow, 1998). This suggests that morphine is not an optimal substrate for phosphorylation and receptor interactions with arrestins. Subsequent work has shown, however, that morphine may induce rapid internalization of opioid receptors in neuronal dendrites in the nucleus accumbence (Haberstock-Debic *et al*., 2005). This property may only be present in limited cell types.

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The lack of receptor internalization stimulated scientists to look at processes beyond the receptor action and rather to study the pathway from the receptor. It was established many years ago that both acute and chronic exposure of morphine to cells *in vitro* produced effects on cAMP. Acute opiate exposure lead to inhibition of the cAMP pathway, whereas chronic stimulation led to the opposite effect, namely the return of cAMP to normal levels (Sharma *et al*., 1977). Opiates act on several parts of the brain, but two regions are particularly important, namely the locus coeruleus and the nucleus accumbence (MacDonald *et al*., 1992).

Aghajanian *et al*. (1983), using electrophysiological techniques were able to repeat the observations from *in vitro* experiment on cells in the locus coeruleus. They found that cells stopped firing after acute addition of morphine, and the cells returned to normal after chronic exposure to morphine. Nestler and Tallman (1988) showed that chronic stimulation of morphine activated c-AMP-dependent protein kinase activity in locus coreleus cells. Later they identified a series of phosphoproteins stimulated by morphine treatment (Guitart and Nestler, 1989).

Subsequent studies pointed to cAMP-response-element-binding protein transcription factor (CREB) as an important target. Phosphorylation of CREB is inhibited acutely by opiate and recovers during chronic treatment. These changes could in part be mediated by the induction of CREB expression by morphine in the locus coeruleus (Guitart *et al*., 1992).

Lane-Ladd *et al*. (1997) first demonstrated that the morphine-induced increases in adenylyl cyclase and protein kinase A (PKA) activity in the locus coeruleus are associated with selective increases in levels of immunoreactivity of types I and VIII adenylyl cyclase and of the catalytic and type II regulatory subunits of PKA. By using antisense oligonucleotides directed against CREB they found that this transcription factor mediated some of these effects.

5 CONCLUSION

Tolerance to a xenobiotic can be developed either by reduction if the response to a receptor or by proteins that are capable of binding or inactivating the compound. For many receptors the response is reduced by internalization and removal of the receptor. The large group of G-protein-binding receptors display a similar mechanism. An exception is morphine where the signal system attached to the receptor changes.

Examples of proteins binding xenobiotics are metalothionein, albumin and CarbE. A problem with albumin binding is that the binding is reversible and the xenobiotics may be released by other compounds.

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Statistics for Toxicology

Peter N. Lee and David Lovell

CONTENTS

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1 INTRODUCTION

This chapter concerns statistical aspects of the design, conduct, analysis and interpretation of toxicological data. Intended mainly for the reader not qualified in statistics, it is more concerned with principles than with techniques. As such it avoids, where possible, the use of technical statistical terms, though Section 10 contains a glossary of terms which may be of interest. A major concern is with the typical long-term rodent carcinogenicity study, particular attention being given to the appropriate treatment of pathological data on the incidence of tumours and non-neoplastic findings. However, many of the principles and methods described apply much more widely in toxicology. The chapter ends with a section referring to additional reading which may be helpful when analysing, not only pathology, but also a range of other types of toxicological data.

2 ROLE OF THE STATISTICIAN

Having read this chapter and the references, the pathologist or toxicologist may then be in a good position to be able to carry out appropriate analyses in a number of standard situations. However, it is still important that an expert statistician be available for advice and assistance, even if only to confirm quickly that what has been done is sensible. All too frequently, where a statistician is not involved at any stage, reports and papers end up with conclusions that are invalid because the statistics have been carried out wrongly.

Although a statistician may carry out the statistical analysis, it is vital that the pathologist or toxicologist and the statistician have regular discussions. The pathologist or toxicologist should make it clear what questions are to be answered and, where relevant, describe how different variables inter-relate biologically. The statistician should ensure the pathologist or toxicologist fully understands why the selected methods of analysis have been chosen and what any output means. The pathologist or toxicologist should be reluctant to accept reports from the statistician consisting of hundreds of pages of computer-produced statistical tables where the vast majority of the output is irrelevant to the questions of interest.

3 SOME GENERAL PRINCIPLES

3.1 Bias and Chance

Any toxicological study aims to determine whether a treatment elicits a response. An observed difference in response between a treated and control group need not necessarily be a result of treatment. There are, in principle, two other possible explanations—*bias*, or systematic differences other than treatment between the groups, and *chance*, or random differences.

A major objective of both experimental design and analysis is to try to avoid bias. Wherever possible, treated and control groups to be compared should be alike in respect of all other factors. Where differences remain, these should be corrected for in the statistical analysis.

Chance cannot be wholly excluded, since identically treated animals will not respond identically. While even the most extreme difference might in theory be due to chance, a proper statistical analysis will allow the experimenter to assess this possibility. The smaller the probability of a 'false positive' the more confident the experimenter can be that the effect is real. Good experimental design improves the chance of picking up a true effect with confidence by maximizing the ratio between 'signal' and 'noise'.

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3.2 Hypothesis Testing and Probability (p) Values

A relationship of treatment to some toxicological end point is often stated to be 'statistically significant ($p < 0.05$)'. What does this really mean? A number of points have to be made.

First, statistical significance need not necessarily imply biological importance, if the end point under study is not relevant to the animal's well-being.

Second, the statement will usually be based only on the data from the study in question and will not take into account prior knowledge. In some situations—for example, when one or two of a very rare tumour type are seen in treated animals—statistical significance may not be achieved, but the finding may be biologically extremely important, especially if a similar treatment was previously found to elicit a similar response.

Third, the p value does not describe the probability that a true effect of treatment exists. Rather it describes the probability of the observed response, or one more extreme, occurring, on the assumption that treatment actually had no effect whatsoever. A p value that is not significant is consistent with a treatment having no effect, but is also consistent with a treatment having a small effect, not detected with sufficient certainty in this study.

Fourth, there are two types of p value. A 'one-tailed (or one-sided) p value' is the probability of getting by chance a treatment effect in a specified direction as great as or greater than that observed. A 'two-tailed p value' is the probability of getting, by chance alone, a treatment difference in either direction which is as great as or greater than that observed. By convention p values are assumed two-tailed unless the contrary is stated. Where, unusually, one can rule out in advance the possibility of a treatment effect except in one direction, a one-tailed p value can be used. Often, however, two-tailed tests are to be preferred, and it is certainly not recommended to use one-tailed tests and *not* report big differences in the other direction. In any event it is important to make it absolutely clear whether one-tailed or two-tailed tests have been used.

It is a great mistake, when presenting results of statistical analyses, to mark, as do some laboratories, results simply as significant or not significant at one defined probability level (usually $p < 0.05$). This is a poor practice, as it does not allow the reader any real chance to judge whether or not the effect is a true one. Increasingly statisticians present the actual p value for every comparison made. While this gives precise information, it can make it difficult to assimilate results from many variables at the same time. One practice that can be used is to mark p values routinely using plus signs to indicate positive differences (and minus signs to indicate negative differences) as follows: +++ $p < 0.001$, ++ $0.001 \leq p < 0.01$, + $0.01 \leq p < 0.05$, (+) $0.05 \leq p < 0.1$. This highlights significant results more clearly and also allows the reader to judge the whole range from 'virtually certain treatment effect' to 'some suspicion'. Note that using two-tailed tests, bracketed plus signs indicate findings that would be significant at the conventional $p < 0.05$ level using one-tailed tests, but are not significant at this level using two-tailed tests. In interpreting p values it is important to realize they are only an aid to judgement to be used in conjunction with other available information. One might validly consider a $p < 0.01$ increase as chance when it was unexpected, occurred only at a low dose level, with no such effect seen at higher doses, and was evident in only one subset of the data. On the contrary, a $p < 0.05$ increase might be quite convincing if it occurred in the top dose and was for an end point one might have expected to be increased from known properties of the chemical or closely related chemicals.

3.3 Multiple Comparisons

When a p value is stated to be <0.05 this implies that, for that particular test, the difference could have occurred by chance less than one time in 20. Toxicological studies frequently involve making treatment–control comparisons for large numbers of variables and, in some situations, also for various subsets of animals. Some statisticians worry that the larger the number of tests, the greater the chance of picking up statistically significant findings that do not represent true treatment effects. For this reason, an alternative 'multiple comparisons' procedure has been proposed in which, if the treatment was totally without effect, then 19 times out of 20 *all* the tests should show nonsignificance when testing at the 95% confidence level. Automatic use of this approach cannot be recommended. Not only does it make it much more difficult to pick up any real effects, but also there is something inherently unsatisfactory about a situation where the relationship between a treatment and a particular response depends arbitrarily on which other responses happened to be investigated at the same time. It is accepted that in any study involving multiple end points there will inevitably be a grey area between those showing highly significant effects and those showing no significant effects, where there is a problem distinguishing chance and true effects. However, changing the methodology so that the grey areas all come up as nonsignificant can hardly be the answer.

3.4 Estimating the Size of the Effect

It should be clearly understood that a p value does not give direct information about the size of any effect that

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has occurred. A compound may elicit an increase in response by a given amount, but whether a study finds this increase to be statistically significant will depend on the size of the study and the variability of the data. In a small study, a large and important effect may be missed, especially if the end point is imprecisely measured. In a large study, on the other hand, a small and unimportant effect may emerge as statistically significant.

Hypothesis testing tells us whether an observed increase can or cannot be reasonably attributed to chance, but not how large it is. Though much statistical theory relates to hypothesis testing, medical statisticians increasingly prefer confidence interval (CI) estimation with differences between test and control groups expressed in the form of a best estimate, coupled with the 95% CI. Thus, if one states that treatment increases response by an estimated 10 units (95% CI 3–17 units), this would imply that there is a 95% chance that the indicated interval includes the true difference. If the lower 95% confidence limit exceeds zero, this implies the increase is statistically significant at $p < 0.05$ using a two-tailed test. One can also calculate, for example, 99% or 99.9% confidence limits, corresponding to testing for significance at $p < 0.01$ or $p < 0.001$.

In screening studies of standard design, the tendency has been to concentrate mainly on hypothesis testing. However, presentation of the results in the form of estimates with CIs can be a useful adjunct for some analyses, and is very important in studies aimed specifically at quantifying the size of an effect.

4 GOOD LABORATORY PRACTICE AND QUALITY ASSURANCE

Before going on to discuss specific considerations relating to the design, conduct and analysis of toxicological studies, it is important to refer to the concept of studies being performed to Good Laboratory Practice (GLP) with the inclusion of a formal quality assurance (QA) component. This aspect has been a response to examples of poor, and in some cases fraudulent, practice in studies in the past. GLP compliance requires written description of the experimental protocol and the development of standard operating procedures (SOPs) which govern how procedures are carried out. Specific attention is given to the collection and maintenance of the integrity of the raw data collected in a study. QA personnel responsible for monitoring studies for GLP compliance are likely to require evidence that the study was conducted according to the defined protocol, and that any statistical analyses and reporting of the data were carried out as described in the study protocol. Evidence will be needed that any statistical package used had previously been validated to ensure that it was providing appropriate results. GLP is now a central feature of standard toxicological tests carried out for regulatory purposes. Its influence and importance is likely to increase and the development of the related concept good scientific practice (GSP) for use in more basic experimental studies is now in progress.

5 EXPERIMENTAL DESIGN AND CONDUCT

5.1 Introduction

Issues relating to experimental design and conduct are considered in detail in Chapter 3 of Gart *et al*. (1986) and in Lee (1993), and will only be discussed briefly here. In many cases, the experimental design is governed in part by regulatory requirement and little further input of a statistical nature is either feasible or needed.

There are 10 facets of any study which may affect its ability to detect an effect of a treatment. The first six facets are concerned with minimizing the role of chance, while the last four relate to avoidance of bias. These are discussed in the following sections.

5.2 Choice of Species and Strain

Ideally, the responses of interest should be rare in untreated control animals, but should be reasonably readily evoked by appropriate treatments. Some species or specific strains, perhaps because of inappropriate diets (Roe, 1989) have high background tumour incidences, which make increases both difficult to detect and difficult to interpret when detected.

5.3 Dose Levels

This is a very important and controversial area. In screening studies aimed at hazard identification, it is normal, to avoid requiring huge numbers of animals, to test at dose levels higher than those to which man will be exposed, but not so high that marked toxicity occurs. A range of doses is usually tested to guard against the possibility of a misjudgement of an appropriate high dose and that the metabolic pathways at the high doses differ markedly from those at lower doses, and, perhaps, to ensure no large effects occur at dose levels in the range to be used by man. In studies aimed more at risk estimation, more and lower doses may be tested to obtain fuller information on the shape of the dose–response curve.

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5.4 Sample Size

This is obviously an important determinant of the precision of the findings. The calculation of the appropriate number depends on: (i) the critical difference, that is, the size of the effect it is desired to detect; (ii) the false positive rate—that is, the probability of an effect being detected when none exists (equivalent to the 'α level' or 'Type I error'); (iii) the false negative rate, that is, the probability of no effect being detected when one of exactly the critical size exists (equivalent to the 'β level' or 'Type II error') and (iv) some measure of the variability in the material.

Lee (1993) gives some tables showing the number of animals required to detect a critical difference for various combinations of α and β for normally and binomially distributed variables. These values can also be calculated more generally by software such as STUDYSIZE or nQUERY ADVISOR. As a rule of thumb, to reduce the critical difference by a factor n for a given α and β, the number of animals required will have to be increased by a factor n^2.

5.5 Duration of the Experiment

It is obviously important not to terminate the study too early for those fatal conditions which are normally strongly age-related. Less obviously, going on for too long in a study could be a mistake, partly because the last few weeks or months may produce relatively little extra data at a disproportionate cost, and partly because diseases of extreme old age may obscure the detection of tumours and other conditions of more interest. For nonfatal conditions, the ideal is to carry out a post-mortem examination of the animals when the average prevalence of the condition is around 50%.

5.6 Accuracy of Determinations

This is of obvious importance. Though GLP and improving technology have improved the situation here, there is an ever-present need for those taking part in the study to be diligent.

5.7 Stratification

To detect a treatment difference with accuracy, it is important that the groups being compared are as homogeneous as possible with respect to other known causes of the response. In particular, suppose that there is another known important cause of the response for which the animals vary, so that the animals are a mixture of hyper- and hyporesponders from this cause. If the treated group has a higher proportion of hyper-responders it will tend to have a higher response even if treatment has no effect. Even if the proportion of hyper-responders is the same as in the controls, it will be more difficult to detect an effect of treatment because of the increased between-animal variability.

Given that this other factor is known, it will be sensible to take it into account in both the design and analysis of the study. In the design, it can be used as a 'blocking factor' so that animals at each level are allocated equally (or in the correct proportion) to control and treated groups. In the analysis, the factor should be treated as a stratifying variable, with separate treatment–control comparisons made at each level, and the comparisons combined for an overall test of difference. This is discussed further in section 6.7 where we refer to factorial design as one example of the more complex designs that can be used to investigate the separate effect of multiple treatments.

5.8 Randomization

Random allocation of animals to treatment groups is a prerequisite of good experimental design. If not carried out, one can never be sure whether treatment–control differences are due to treatment or to 'confounding' by other relevant factors. The ability to randomize easily is a major advantage animal experiments have over epidemiology.

While simple randomization of all animals eliminates bias (at least in expectation), it may not be the optimal technique for producing a sensitive test. If there is another major source of variation (e.g. sex, or batch of animals), it will be better to carry out stratified randomization, (i.e. carry out separate randomizations within each level of the stratifying variable).

The need for randomization applies, not only to the allocation of the animals to the treatment, but also to anything that can materially affect the recorded response. The same random number that is used to apply animals to treatment groups can be used to determine cage position, order of weighing, order of bleeding for clinical chemistry, order of sacrifice at termination, and so on.

5.9 Adequacy of Control Group

While historical control data can, on occasion, be useful, a properly designed study demands that a relevant concurrent control group be included with which results for the test group can be compared. The principle that like should be compared with like, apart from treatment,

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demands that control animals should be randomized from the same source as treatment animals. Careful consideration should also be given to the appropriateness of the control group. Thus, in an experiment involving treatment of a compound in a solvent, it would often be inappropriate to include only an untreated control group, as any differences observed could only be attributed to the treatment/solvent combination. To determine the specific effects of the compound, a comparison group given the solvent only by the same route of administration would be required.

5.10 Animal Placement

It is not always generally realized that position of the animal in the room in which it is kept may affect the animal's response. An example is the strong relationship between incidence of retinal atrophy in albino rats and closeness to the lighting source. Systematic differences in cage position should be avoided, preferably via randomization.

5.11 Data Recording

Two distinct sources of systematic bias may occur in data recording. One is that awareness of treatment may, consciously or subconsciously, affect the values recorded by the measurer. This can be avoided by organizing data-recording so that observations are made blind of treatment. The second is that there is a systematic shift in the standard of measurement with time, coupled with a tendency for different groups to be measured at different time points. This is particularly important when a pathologist grades a lesion for severity and when the control and high-dose animals are read before the intermediate-dose animals. In some situations it may be necessary to reread all the slides blind and in random order to be sure that 'diagnostic drift' is avoided.

Valid analysis cannot be conducted unless one can distinguish animals which were examined and did not have the relevant response and animals which were not examined. It can also be important to identify why data are missing.

6 STATISTICAL ANALYSIS: GENERAL CONSIDERATIONS

6.1 Introduction

In the next section different techniques that might generally be applied to different types of toxicological data are summarized. Before doing so, a number of points are worthy of discussion. Some are quite general, while others relate more specifically to problems in dealing with data from pathological investigations.

6.2 Variables to be Analysed

Although some pathologists still regard their discipline as providing qualitative rather than quantitative data, it is abundantly clear that pathology, when applied to routine screening of animal toxicity and carcinogenicity studies, has to be quantitative to at least some degree so that statistical statements can be made about possible treatment effects. Inevitably, there will be some descriptive text which will not be appropriate for statistical analysis. However, the main objective of the pathologist should be to provide information on the presence or absence (with severity grade or size where appropriate) of a list of conditions, consistently recorded from animal to animal by well-defined criteria, which can be validly used in a statistical assessment.

Given statistical analysis is worth doing and data are available that could be analysed, should one then analyse all the end points recorded? Some arguments have been put forward against analysing all the end points studied, but none are convincing.

One argument is that some end points are not of interest. Perhaps the study is essentially a carcinogenicity study, so that non-neoplastic end points are considered to be 'background pathology' and almost *per se* unrelated to treatment. In our view, this is illogical. If the pathologist has gone to the trouble of recording the data, then surely, in general, they ought to be analysed; otherwise, why record them in the first place? After all, the costs of the statistical analysis are much less than those of doing the study and the pathology. While one might justify failure to analyse non-neoplastic data where tumour analysis has already shown the compound is clearly carcinogenic and no longer of market potential, the general rule ought to be to analyse everything that has been specifically investigated.

Another argument put forward against doing multiple analyses is that it may yield many chance significant p values that have to be 'explained away'. This seems to us a poor reason for not exploring the data fully. A detailed look at the data can only aid interpretation, provided one is not hide-bound by the false argument that statistical significance necessarily equates with biological importance and definitely indicates a true effect of treatment.

Another reason not to analyse might be that visual inspection of summary tables reveals no suspicion of an effect for some end point. This seems to be, in this age of rapid and efficient computer programs, totally the wrong way to organize things. Given the data are on

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computer, it is much better and quicker to do the actual analysis than to do the inevitably subjective, unreliable and slow prescreening process. In any case, where there are substantial differences in survival between groups, it is very difficult to form a reliable view by inspection of non-age-adjusted frequencies on whether an effect might or might not have occurred.

A final, more valid, reason is that some end points occur only very rarely. One should, however, be clear what 'very rarely' is. For a typical study with a control and three dose groups of equal size, one would get a significant trend statistic if all three cases occurred at the top dose level or in the control group (two-tailed $p = 0.03$), so a total of three cases will normally be enough for statistical analysis. End points occurring once or twice only are not worth analysing formally, though, if only seen in the top-dose group, they may be worth noting in the report. This is especially true if they are lesions that are rarely reported.

6.3 Combination of Pathological Conditions

There are four main situations where one might consider combining pathological conditions in a statistical analysis.

The first is when essentially the same pathological condition has been recorded under two or more different names or even under the same name in different places. Here failure to combine these conditions in the analysis may severely limit the chances of detecting a true treatment effect. It should be noted, however, that grouping together conditions which are actually different may also result in the masking of a true treatment effect, particularly if the treatment has a very specific effect.

The second is when separately recorded pathological conditions form successive steps on the pathway of the same process. The most important example of this is for the incidence of related types of malignant tumour, benign tumour and focal hyperplasia. It will normally be appropriate to carry out analyses of: (i) incidence of malignant tumour, (ii) incidence of benign or malignant tumour, and, where appropriate, (iii) incidence of focal hyperplasia, benign or malignant tumour. It will not normally be appropriate to carry out analyses of benign tumour incidence only or of the incidence of hyperplasia only.

The third situation for combining is when the same pathological condition appears in different organs as a result of the same underlying process. Examples of this are the multicentric tumours (such as myeloid leukaemia, histiocytic sarcoma or malignant lymphoma) or certain non-neoplastic conditions (such as arteritis/periarteritis or amyloid deposition). Here analysis will normally be carried out only of incidence at any site, although in some situations site-specific analyses might be worth carrying out.

The final situation where an analysis of combined pathological conditions is normal is for analyses of overall incidence of malignant tumour at any site, of benign or malignant tumour at any site, or of multiple tumour incidence. While analyses of tumour incidence at specific sites are normally more meaningful, since treatments often affect only a few specific sites, these additional analyses are usually required to guard against the possibility that treatment had some weak, but general, tumour-enhancing effect not otherwise evident.

In some situations, one might also envisage analyses of other combinations of specific tumours, such as tumours at related sites (e.g. endocrine organs if the compound had a hormonal effect) or of similar histological type.

6.4 Taking Severity into Account

The same line of argument that suggests that if the pathologist records data it should be analysed, also suggests that if the pathologist chooses to grade a condition for severity, the grade should be taken into account in the analysis. There are two ways to carry out analysis when the grade has to be taken into account. In one, analyses are carried out, not only of whether or not the animal has a condition, but also of whether or not the condition is at least grade 2, at least grade 3, and so on. In the other approach, nonparametric (rank) methods are used. The latter approach is more powerful, as it uses all the information in one analysis, although the output may not be so easily understood by those without some statistical training.

Note that the analyses based on grade can be carried out only if grading has been consistently applied throughout. If a condition has been scored only as present/absent for some animals, but has been graded for others, it is not possible to carry out graded analyses unless the pathologist is willing to go back and grade the specific animals showing the condition.

6.5 Using Simple Methods Which Avoid Complex Assumptions

Methods for statistical analysis can vary considerably in their complexity and in the number of assumptions they make. Although use of statistical models has its place, more so for effect estimation that for hypothesis testing, and more so in studies of complex design than in those of simple design, there are advantages in using, wherever possible, statistical methods that are simple, robust and make as few assumptions as possible. There are three reasons for this. First, such methods are more

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generally understandable to the toxicologist. Second, there are hardly ever extensive enough data in practice to validate any given formal model fully. Third, even if a particular model were known to be appropriate, the loss of efficiency in using appropriate simpler methods is often only very small.

The methods we advocate for routine use for the analysis of tumour incidence tend, therefore, not to be based on the use of formal parametric statistical models. For example, when studying the relationship of treatment to incidence of a pathological condition and wishing to adjust for other factors (in particular, age at death) that might otherwise bias the comparison, methods involving 'stratification' are recommended, rather than a multiple regression approach or time-to-tumour models. Analysis of variance (ANOVA) methods can be useful in the case of continuously distributed data for estimating treatment effects. However, they involve underlying assumptions (normally distributed variables, variability equal in each group). If the assumption of normality is violated, nonparametric methods based on the rank of observations, rather than their actual value, may be preferable for hypothesis testing.

6.6 Using All the Data

Often information is available about the relationship between treatment and a condition of interest for groups of animals differing systematically in respect of some other factor. Obvious examples are males and females, differing times of sacrifice, and differing secondary treatments. While it will be necessary, in general, to look at the relationship within levels of this other factor, it will also generally be advisable to try to come to some assessment of the relationship over all levels of the other factors combined. There are some situations where the effect of treatment varies markedly by level of the other factor, and where a combined inference is not sensible, but in far more situations this is not the case, and using all the data in one analysis allows a more powerful test of the relationship under study. Some scientists consider that conclusions for males and females should always be separately drawn, but there are strong statistical arguments for a joint analysis.

6.7 Combining, Pooling and Stratification

Suppose, in a hypothetical study of a toxic agent which induces tumours that do not shorten the lives of tumour-bearing animals, the data are as shown in **Table 1**.

Table 1 Tumour rates[a] by period and treatment in a hypothetical study

Period of study	Control	Exposed	Combined
Early deaths	1/20 (5%)	18/90 (20%)	19/110 (17%)
Late deaths	24/80 (30%)	7/10 (70%)	31/90 (34%)
Total	25/100 (25%)	25/100 (25%)	50/200 (25%)

[a]Data shown are number with tumour out of number examined (percentage with tumour).

It can be seen that if the time of death is ignored and the *pooled* data are studied, the incidence of tumours is the same in each group, resulting in the *false* conclusion that treatment had no effect. Looking within each time of death, however, an increased incidence in the exposed group can be seen. An appropriate statistical method would *combine* a measure of difference between the groups based on the early deaths and a measure of difference based on the late deaths, and conclude *correctly* that incidence, after adjustment for time of death, is greater in the exposed groups.

In this example, time of death is the 'stratifying variable', with two strata—early deaths and late deaths. The essence of the methodology is to make comparisons only within strata (so that one is always comparing like with like except in respect of treatment) and then to combine the differences over strata. Stratification can be used to adjust for any variable, or indeed combinations of variables.

Some studies are of factorial design, in which combinations of treatments are tested. The simplest such design is one in which four equal-sized groups of animals receive: (i) no treatment, (ii) treatment A only, (iii) treatment B only and (iv) treatments A and B. If one is prepared to assume that any effects of the two treatments are independent, one can use stratification to enable more powerful tests to be conducted of the possible individual treatment effects. Thus, to test for effects of treatment A for example, one conducts comparisons in two strata, the first consisting of groups 1 and 2 not given treatment B and the second consisting of groups 3 and 4 given treatment B. Combination of results from the two strata are based on twice as many animals, and are therefore markedly more likely to detect possible effects of treatment A than is a simple comparison of groups 1 and 2. There is also the possibility of identifying interactions, such as synergism and antagonism, between the two treatments.

6.8 Multiple Control Groups

In some routine long-term screening studies, the study design involves five groups of (usually) 50 animals of

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each sex, three of which are treated with successive doses of a compound and two of which are untreated controls. Assuming that there is no systematic difference between the control groups (e.g. the second control group in a different room or from a different batch of animals), it will be normal to carry out the main analyses with the control groups treated as a single group of 100 animals. It will usually be a sensible preliminary precaution to carry out additional analyses comparing incidences in the two control groups.

Table 2 Tumour rates[a] by period and treatment in a further hypothetical study

Period of study	Control	Exposed
Early deaths	0/20	0/20
Middle deaths	1/10	9/10
Late deaths	20/20	20/20
Total	21/50	29/50

[a]Data shown are number with tumour out of number examined.

6.9 Trend Analysis, Low-Dose Extrapolation and NOEL Estimation

While comparisons of individual treated groups with the control group are important, a more powerful test of a possible effect of treatment will be to carry out a test for a dose-related trend. This is because most true effects of treatment tend to result in a response which increases (or decreases) with increasing dose and because trend tests take into account all the data in a single analysis. In interpreting the results of trend tests, it should be noted that a significant trend does not necessarily imply an increased risk at lower doses. Nor, conversely, does a lack of increase at lower doses necessarily indicate evidence of a threshold (i.e. a dose below which no increase occurs).

Note that testing for trend is seen as a more sensitive way of picking up a possible treatment effect than simple pairwise comparisons of treated and control groups. Attempting to estimate the magnitude of effects at low doses, typically below the lowest positive dose tested in the study, is a much more complex procedure, and is heavily dependent on the assumed functional form of the dose–response relationship.

Such low-dose extrapolation is typically only conducted for tumours believed to be caused by a genotoxic effect which some, but by no means all, scientists believe have no threshold. For other types of tumours and for many non-neoplastic end points, a threshold is generally considered likely to exist. While the precise threshold cannot be estimated directly from data at a limited number of dose levels, a no observed effect level (NOEL) can be estimated by finding the highest dose level at which there is no significant increase in effects.

6.10 Need for Age Adjustment

Where there are marked differences in survival between treated groups, it is widely recognized that there is a need for an age adjustment (i.e. an adjustment for age at death or onset). This is illustrated in the example above, where, because of the greater number of deaths occurring early in the treated group, the true effect of treatment disappears if no adjustment is made. Thus, a major purpose of age adjustment is to avoid bias.

It is not so generally recognized, however, that, even where there are no survival differences, age adjustment can increase the power to detect between-group differences. This is illustrated in **Table 2**.

Here treatment results in a somewhat earlier onset of a condition which occurs eventually in all animals. Failure to age-adjust will result in a comparison of 29/50 with 21/50, which is not statistically significant. Age adjustment will essentially ignore the early and late deaths, which contribute no comparative statistical information, and be based on the comparison of 9/10 with 1/10, which is statistically significant. Here age adjustment sharpens the contrast, rather than avoiding bias, by avoiding diluting data capable of detecting treatment effects with data that are of little or no value for this purpose.

6.11 Need to Take Context of Observation into Account

It is by now widely recognized that age adjustment cannot properly be carried out unless the context of observation is taken into account. There are three relevant contexts, the first two relating to the situation where the condition is only observed at death (e.g. an internal tumour), the third where it can be observed in life (e.g. a skin tumour).

In the first context the condition is assumed to have caused the death of the animal—that is, to be *fatal*. Here the incidence rate for a time interval and a group is calculated by:

$$\frac{\text{(number of animals dying in the interval because of the lesion)}}{\text{(number of animals alive at the start of the interval)}} \quad (1)$$

In the second context, the animal is assumed to have died of another cause—that is, the condition is

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incidental. Here the rate is calculated by:

$$\frac{\text{(number of animals dying in the interval with the lesion)}}{\text{(total number of animals dying in the interval)}} \quad (2)$$

In the third context, where the condition is *visible*, the rate is calculated by:

$$\frac{\text{(number of animals getting the condition in the interval)}}{\text{(number of animals without the condition at the start of the interval)}} \quad (3)$$

A problem with the method of Peto *et al*. (1980), which takes context of observation into account, is that some pathologists are unwilling or feel unable to decide whether, in any given case, a condition is fatal or incidental. A number of points should be made here.

First, where there are marked survival differences, it may not be possible to conclude reliably whether a treatment is beneficial or harmful unless such a decision is made. This is well illustrated by the example in Peto *et al*. (1980), where assuming all pituitary tumours were fatal resulted in the (false) conclusion that *N*-nitrosodimethylamine (NDMA) was carcinogenic, while assuming they were all incidental resulted in the (false) conclusion that NDMA was protective. Using, correctly, the pathologist's best opinion as to which were, and which were not, likely to be fatal, resulted in an analysis which (correctly) concluded NDMA had no effect. If the pathologist, in this case, had been unwilling to make a judgement as to fatality, believing it to be unreliable, no conclusion could have been reached. This state of affairs would, however, be a fact of life, and *not* a position reached because an inappropriate statistical method was being used. Alternative techniques, such as the PolyK test (Bailer and Portier, 1988), which do not take context of observation into account, cannot provide a valid analysis of data such as these (Lee and Fry, 2002a; 2002b).

Although it will normally be a good routine for the pathologist to ascribe 'factors contributory to death' for each animal that was not part of a scheduled sacrifice, it is in fact not strictly necessary to determine the context of observation for all conditions at the outset. An alternative strategy is to analyse under differing assumptions: (i) no cases fatal, (ii) all cases occurring in decedents fatal, (iii) all cases of same defined severity occurring in decedents fatal, with, under each assumption, other cases incidental.

If the conclusion turns out the same under each assumption, or if the pathologist can say, on general grounds, that one assumption is likely to be a close approximation to the truth, it may not be necessary to know the context of observation for the condition in question for each individual animal. Using the alternative strategy might result in a saving of the pathologist's time by only having to make a judgement for a limited number of conditions where the conclusion seems to hang on correct knowledge of the context of observation.

Finally, it should be noted that, although many non-neoplastic conditions observed at death are never causes of death, it is, in principle, as necessary to know the context of observation for non-neoplastic conditions as it is for tumours.

6.12 Experimental and Observational Units

In many situations, the animal is both the 'experimental unit' and the 'observational unit', but this is not always so. For determining treatment effects by the methods of the next section, it is important that each experimental unit provides only one item of data for analysis, as the methods all assume that individual data items are statistically independent. In many feeding studies, where the cage is assigned to a treatment, it is the cage, rather than the animal, that is the experimental unit. In histopathology, observations for a tissue are often based on multiple sections per animal, so that the section is the observational unit. Multiple observations per experimental unit should be combined in some suitable way into an overall average for that unit before analysis.

6.13 Missing Data

In many types of analysis, animals with missing data are simply removed from the analysis. There are, however, some situations where this can be an inappropriate thing to do. One situation is when carrying out an analysis of a condition that is assumed to have caused the death of the animal. Although an animal dying at week 83 for which the section was unavailable for microscopic examination cannot contribute to the group comparison at week 83, one knows that it did not die because of any condition in previous weeks, so it should contribute to the denominator of the calculations in all previous weeks.

Another situation is when histopathological examination of a tissue is not carried out unless an abnormality is seen at post mortem. In such an experiment one might have the data shown in **Table 3**.

Ignoring animals with no microscopic sections, one would compare $2/2 = 100\%$ with $14/15 = 93\%$ and conclude that treatment nonsignificantly decreased incidence. This is likely to be a false conclusion, and it would probably be better here to compare the percentages of the total animals in the group which had a post mortem abnormality which turned out to be a tumour, that is, $2/50 = 4\%$ with $15/50 = 30\%$. Unless some aspect of treatment made tumours much

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Table 3 Hypothetical data for a tissue not examined routinely

Treatment group	Number of animals			
	Total	Abnormal at post mortem	Examined microscopically	With specific tumour
Control	50	2	2	2
Treated	50	15	15	14

easier to detect at post mortem, one could then conclude treatment did have an effect on tumour incidence.

Particular care has to be taken in studies where the procedures for histopathological examination vary by group. In a number of studies conducted in recent years, the protocol demands full microscopic examination of a given tissue list in decedents in all groups, in terminally killed controls and high-dose animals. In other animals, terminally killed low- and mid-dose animals, microscopic examination of a tissue is only conducted if the tissue is found to be abnormal at post mortem. Such a protocol is designed to save money, but leads to difficulty in comparing the treatment groups validly. Suppose, for example, responses in terminally killed animals are 8/20 in the controls, 3/3 (with 17 unexamined) in the low dose and 5/6 (with 14 unexamined) in the mid dose. Is one supposed to conclude treatment at the low and mid doses increased response, based on a comparison of the proportions examined microscopically (40, 100 and 83%), or decreased response, based on the proportion of animals in the group (40, 15 and 25%)? It could well be that treatment had no effect, but some small tumours were missed at post mortem. In this situation, a valid comparison can only be achieved by ignoring the low- and mid-dose groups when carrying out the comparison for the age stratum 'terminal kill'. This, of course, seems wasteful of data, but these are data that cannot be usefully used due to the inappropriate protocol.

6.14 Use of Historical Control Data

In some situations, particularly where incidences are low, the results from a single study may suggest an effect of treatment on tumour incidence, but be unable to demonstrate it conclusively. The possibility of comparing results in the treated groups with those of control groups from other studies is then often raised. Thus, a nonsignificant incidence of two cases out of 50 in a treated group may seem much more significant, if no cases have been seen in, say, 1000 animals representing controls from 20 similar studies. Conversely, a significant incidence of five cases out of 50 in a treated group as compared with none out of 50 in the study controls may seem far less convincing if many other control groups had incidences around five out of 50.

While not understating the importance of looking at historical control data, it must be emphasized that there are a number of reasons why variation between studies may be greater than variation within studies. Differences in diet, in duration of the study, in intercurrent mortality and in who the study pathologist is, may all contribute. Statistical techniques that ignore this and carry out simple statistical tests of treatment incidence against a pooled control incidence may well give results that are seriously in error, and are likely to overstate statistical significance considerably. Appropriate tests allow for the possibility of these additional sources of variation (Gart *et al*., 1986; Tarone, 1982).

7 STATISTICAL ANALYSIS: SUMMARY OF APPROPRIATE METHODS

7.1 Types of Response Variable

Responses measured in toxicological studies can normally be classified into one of the following three types:

1. Presence/absence: a condition either occurs or it does not.
2. Ranked: a condition may be present at various discrete levels.
3. Continuous: a condition may take any value, at least within a given range.

Strictly, each type of response requires a different sort of statistical technique. Methods for presence/absence data can be used for ranked or continuous data by defining a cut-off point above which is deemed present, but this is rather wasteful of data. Methods for ranked data can also be used for analysing continuous data, and probably should be if one is not confident about the assumptions of normality and homogeneity of variance underlying the standard parametric methods used, such as ANOVA.

7.2 Types of Comparison

There are three principal different types of comparison between groups. The first is the pairwise comparison, in which the responses in two different groups, usually a treated group and the control group, are compared. The second, only applicable to studies with more than two treatment groups, is the test for heterogeneity, which tests whether, taken as a whole, there is significant evidence

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of departure from the (null) hypothesis that the groups do not differ in their effect. The third, only applicable to studies with more than two treatment groups in which the different groups represent different doses of the same compound, is the test for a dose-related trend.

7.3 Recommended Methods for Between-Animal Comparisons

7.3.1 Presence/Absence Data

For pairwise comparisons, Fisher's exact test is recommended for unstratified data, though a 2×2 corrected chi-squared test will yield a sufficient approximation unless incidences are low. For stratified data, a corrected chi-squared test is usually used, although exact versions of this are available in some programs, and may be preferable where data are sparse.

For dose-related trend, the Cochran–Armitage test is often appropriate for stratified or unstratified data. Like the chi-squared test, it is only approximate, and again exact tests may be preferable.

To test for overall heterogeneity over k groups, the $2 \times k$ chi-squared test should be used for unstratified and stratified data. Exact versions of this are now available in many software packages.

The tests described above are illustrated in Lee (1993) with fuller details given in many textbooks.

Where context of observation has to be taken into account, the Peto/IARC (International Agency for Research on Cancer) method (Peto *et al.*, 1980) is appropriate for pairwise comparisons and tests for dose-related trend and heterogeneity, though exact versions of this method are available in some programs (see Section 8).

7.3.2 Ranked Data

It is clearly possible to use a cut-point, defining values above it as 'present' and below it as 'absent', with the methods described in Section 7.3.1 then being used. However, this is wasteful of information.

For unstratified data, pairwise comparisons should be made using the Wilcoxon (Mann–Whitney U) test, with a heterogeneity test based on Kruskal–Wallis one-way ANOVA. Both tests are described in Conover (2003). For trend tests, the nonparametric analogue of the Cochran–Armitage test, derived by Marascuilo and McSweeney (1967) and described by Lee (1993), is recommended, although alternatives are available (Jonckheere, 1954; Shirley, 1977).

The method of Fry and Lee (1988) (see also Lee, 1993) can be used to provide stratified rank tests of pairwise comparison, heterogeneity and dose-related trend.

7.3.3 Continuous Data

Bartlett's test (see Lee, 1993) should be used to test for homogeneity of variance. If the data show appreciable heterogeneity, transformation of the individual data items by logarithms and/or square roots can be considered, but analysis of the data using the ranked methods described in Section 7.3.2 is a simple and sound alternative.

Assuming the data are reasonably homogeneous, they should be analysed using standard methods for normally distributed data, that is, the Student's t-test for pairwise comparisons, the one-way ANOVA F test for heterogeneity and linear regression for the trend test (Guenther, 1964; Johnson and Leone, 1977). The Williams test (Williams, 1971; 1972) is an alternative trend test which, like the Shirley test (Shirley, 1977), attempts to estimate the lowest dose level showing a significant relationship with treatment.

Analysis of continuous data to correct for sources of potential bias is not normally carried out by methods involving stratification, but by the methods of analysis of covariance, multiple regression and linear models. These, and the more complex ANOVA methods required for factorial designs, probably need discussion with a statistician.

7.3.4 Multiple Comparison Tests

Although multiple comparison tests should be used with caution (see Section 3.3), it is worth pointing out that there are various standard procedures available. For parametric (continuous) data, there are the Dunnett test (Dunnett, 1955; 1964) and the Stepwise Dunnett test (Dunnett and Tamhane, 1991), while for nonparametric data there are the Bonferroni and Stepwise Bonferroni tests (Hochberg and Benjamini, 1990), the Steel test (Steel, 1959) and the Dunn test (Dunn, 1964). A detailed discussion of these is beyond the scope of this chapter, though it should be noted that Bonferroni correction is often used with quantitative data, such as toxicogenomic data.

7.4 Other Statistical Methods

The methods described in the previous subsection have concentrated on the common experimental situation where interest is centred on evidence of variation between groups in respect of a single variable, with the possible confounding effect of other variables taken account of by stratification. There are a number of different situations, considered briefly below, where alternative statistical techniques are required.

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7.5 Relationships between Two Variables

For presence/absence data, relationships between two variables can be studied by Fisher's exact test or a corrected chi-squared test. For ranked and continuous data, the correlation coefficients of, respectively, Spearman and Pearson can be used. Techniques are also available for determining whether these relationships differ for different treatment groups.

7.6 Multivariate Methods

Situations involving the inter-relationship of a large number of variables normally require the advice of a professional statistician. Multivariate statistical methods are now widely used in the developing discipline of toxicogenomics and its related field of proteomics and metabolomics. They are aimed at producing lists of genes with significantly different expression between treated groups, and profiles of genes with different expression which distinguish between samples and have potential predictive ability. A wide range of multivariate statistical methods are involved, such as cluster analysis, principal component analysis, discriminant functions and partial least squares. Specialized software is available for these approaches in both proprietary packages and through the use of the Bioconductor suites of programs developed using the statistical programming language R (Gentleman *et al*., 2005). The results of these analyses are linked with the appreciable Bioinformatic resources on the genes, proteins and metabolic pathways maintained by organizations such as the NCBI (National Center for Biotechnology Information) and the EBI (European Bioinformatics Institute). The statistical methods used are beyond the scope of this chapter, but the large numbers of statistical tests carried out raise issues with respect to multiple comparisons and the interpretation of statistical significant results. Other multivariate methods commonly used in other applications are multiple regression analysis and factor analysis.

7.7 Multiple Observations on the Same Animal

In long-term studies, it is common to make similar measurements—for example, of body weight—on the same animal at different stages of the study. To look for evidence of a between-group difference in changes in responses over a given period, the preferred technique is analysis of covariance, where differences in mean values at the second time-point are studied after adjustment for values observed at the first time-point. Alternatively, parametric growth curves may be fitted and the groups compared in respect of these parameters, but such techniques tend to be complex. Diggle *et al*. (2002) provide a comprehensive review of methods for the longitudinal analysis of data. Methods for the analysis of repeated measures data can be found in Crowder and Hand (1990).

7.8 Paired Data

Sometimes studies consist of pairs of animals, each pair of animals being similar in respect of important potential confounding variables, for example, litter, and differing only in respect of treatment. It is important to take the pairing into account for analysis. Appropriate techniques to use are the sign test or McNemar's test for presence/absence data, the Wilcoxon matched-pair signed-ranks test for ranked data and the matched-pair *t*-test for continuous data. Considerable care is needed to ensure that the appropriate paired or unpaired test is used. A common error in analysis is to use a paired test on unpaired data or vice versa.

7.9 LD_{50}

Some toxicological studies calculate the dose of a substance at which 50% of the animals respond. This is known as the LD_{50} when the response is death [lethal dose (LD)] and more generally as the median-effective dose. The classical technique to calculate the LD_{50} from data consisting of the percentage responding at each of a range of dose levels is known as probit analysis. Sequential methods which require fewer animals are increasingly being applied to reduce the number of animals used in this controversial and unpopular toxicological test.

8 SOFTWARE

The recommended methods for between-animal comparisons are all available in the comprehensive computer program for data entry, reporting and statistical analysis of pathological data, ROELEE. Various standard statistical packages, such as GENSTAT, MINITAB, SAS, S-PLUS, SPSS and SYSTAT, include many of the techniques described here. With the advent of much more powerful computers, there has been a tendency in many situations to move from the formerly used approximate asymptotic statistical tests to exact randomization tests. Most packages, including SAS, SPSS and STATXACT have facilities for exact tests, while ROELEE includes an exact version of the Peto *et al*. (1980) test, including an exact trend test. STUDYSIZE and nQUERY ADVISOR

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are helpful for determining appropriate group sizes when designing a study. See Section 11 for software addresses.

9 RECOMMENDED READING

Lee (1993) provides a simple text, aimed at toxicologists and including worked examples, for many of the methods we recommend. Peto *et al*. (1980) describes and justifies the most commonly used test for comparison of age-adjusted tumour incidences taking context of observation into account. Gart *et al*. (1986) is a more detailed text, aimed more at statisticians, describing methods of analysis of carcinogenicity data, while Krewski and Franklin (1991) is an extensive textbook covering a wide range of issues relating to statistics in toxicology. Two good general biostatistical textbooks are Altman (1991) and Armitage *et al*. (2001). For those concerned with estimation of effect rather than with determining statistical significance, Altman *et al*. (2000) gives details of how to calculate confidence limits for many tests. Gad (2005) also contains useful material on other types of data, such as body and organ weights, clinical chemistry, haematology, reproduction, teratology, dominant lethal assay and mutagenesis. Kirkland (1989) provides an overview of the statistical issues and methods used in genetic toxicology and mutagenicity studies.

10 GLOSSARY OF STATISTICAL TERMS

10.1 Types of Data

Dichotomous data: Data that can take one of two possible values, such as dead/alive, smoker/nonsmoker, present/absent. Also called binary data.
Ordinal data: Data that are classified into more than two categories which have a natural order, for example, nonsmokers, ex-smokers, light smokers and heavy smokers. Also called ranked data. Ordinal data are often reduced to two categories to simplify analysis and presentation, which may result in a considerable loss of information.
Categorical data: Data that are classified into two or more nonoverlapping categories. Race and type of drug (aspirin, paracetamol, etc.) are examples of categorical variables.
Continuous data: Data with a potentially infinite number of possible values within a given range. Height, weight and blood pressure are examples of continuous variables.

10.2 Measures of Central Tendency

Mean: An average value, calculated by adding all the observations and dividing by the number of observations. Also called the arithmetic mean.
Median: The value for which one-half of the observations (when ranked) will lie above that value and one-half will lie below that value. When the number of values in the sample is even, the median is computed as the average of the two middle values.
Mode: The value which occurs most frequently in the sample.

10.3 Measures of Variability

Variance: The average of the squared differences between data values and the mean.
Standard variation: A measure of the spread of values, calculated as the square root of the variance.
Standard error of the mean: The theoretical standard deviation of all sample means of size n drawn from a population. It depends on both the variance and the sample size (n), decreasing as n increases.

10.4 Distributions and Related Terms

Normal distribution: A bell-shaped statistical distribution with known properties commonly used as the basis of models to analyse continuous data. Key assumptions in such analyses are that the data are symmetrically distributed about a mean value, and the shape of the distribution can be described using the mean and standard deviation.
Skewness: This measures the deviation of the distribution from symmetry. If the skewness is clearly different from zero, then that distribution is asymmetrical, while Normal distributions are perfectly symmetrical. Often in toxicology, the data values themselves have a skew distribution, with many low values and a few high values, but the logarithms of the data values are symmetrically distributed and approximately satisfy a Normal distribution.
Other distributions: Other distributions commonly used in statistics are the t-distribution (or Student t-distribution), used to compare means of samples where the variance is estimated from the data, the F-distribution, used to compare two variances, the chi-squared distribution, used to compare frequencies in

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categorical data, and the Poisson distribution, used to describe the distribution of rare events.

Degrees of freedom: This is the number of categories or classes being tested minus 1. Thus, for example, the ratio of two variances, both calculated from a normally distributed sample, one of size n_1 and the other of size n_2, has an F distribution with degrees of freedom $n_1 - 1$ and $n_2 - 1$.

Quartile: Technically, this is any of the three values which divide ranked data into four equal parts, so that each part represents a quarter of the sampled population. In practice, the term quartile is often used to describe the parts themselves, for example, 'the data were divided into four quartiles', rather than 'the data were divided by three quartiles'.

***n*-tiles:** This is any of the $n - 1$ values which divide ranked data into n equal parts, so that each part represents an nth of the sampled population. The term is often used to describe the parts themselves. The median ($n = 2$), tertiles ($n = 3$), quartiles ($n = 4$), quintiles ($n = 5$), deciles ($n = 10$) and centiles ($n = 100$) are examples of n-tiles which are often used.

10.5 Some Other Terms

CI: For a specified statistic, such as the mean, the CI gives us a range of values around the statistic where the 'true' (population) statistic can be expected to be located with a given level of certainty. 95% CIs are very commonly used.

Correlation: The linear association between two variables, measured by a correlation coefficient, with values ranging from -1 to $+1$. A value of $+1$ implies perfect positive correlation, so that the values when plotted on a graph would fall perfectly on an increasing straight line.

Regression analysis: A statistical modelling technique used to estimate or predict the influence of one or more independent variables on a dependent variable, for example, the effect of age, sex and educational level on the prevalence of a disease. Logistic regression and meta-regression are types of regression analysis.

***p* value:** the probability (ranging from zero to one) that the results observed in a study (or results more extreme) could have occurred by chance if in reality the null hypothesis was true.

Power: The probability of rejecting the null hypothesis when a specific alternative hypothesis is true.

Nonparametric tests: Nonparametric tests are often used in place of their parametric counterparts when certain assumptions about the underlying population are questionable. Nonparametric tests may be, and often are, more powerful in detecting population differences when certain assumptions are not satisfied. All tests involving ranked data, that is, data that can be put in order, are nonparametric.

11 SOFTWARE ADDRESSES

GENSTAT 10—VSN International Ltd., 5 The Waterhouse, Waterhouse Street, Hemel Hempstead, HP1 1ES, UK. *www.vsn-intl.com*

MINITAB—Minitab Inc., Quality Plaza, 1829 Pine Hall Road, State College, PA 16801-3008, USA. *www.minitab.com*

nQUERY ADVISOR—Statistical Solutions, Stonehill Corporate Center, Suite 104, 999 Broadway, Saugus 01906, USA. *www.statsol.ie*

R (The R Project for Statistical Computing)—*www.r-project.org*

ROELEE—P. N. Lee Statistics & Computing Ltd., 17 Cedar Road, Sutton, Surrey, SM2 5DA, UK. *www.pnlee.co.uk*

S-PLUS®—Insightful Corporation, North Carolina Office, 5317 Highgate Drive, Suite 213, Durham, NC 27713, USA. *www.statsci.com*

SAS—SAS Institute Inc., SAS Worldwide Headquarters, 100 SAS Campus Drive, Cary, NC 27513-2414, USA. *www.sas.com*

SPSS—SPSS Inc., 233S. Wacker Drive, 11th Floor, Chicago, IL 60606-6307, USA. *www.spss.com*

STATXACT—Cytel Statistical Software, 675 Massachusetts Avenue, Cambridge, MA 02139-3309, USA. *www.cytel.com*

SYSTAT—Systat Software Inc., 1735 Technology Drive, Suite 430, San Jose, CA 95110, California, USA. *www.systat.com*

STUDYSIZE—Creostat HB, Enbarsvagen 11, 42655 V. Frolunda, Sweden. *www.creostat.com*

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Pathological Techniques in Toxicology

Peter Greaves

CONTENTS

1 INTRODUCTION

For over 100 years microscopic evaluation of pathological alterations in human tissues has made major contributions to our understanding of diseases and it remains a significant diagnostic tool in human medicine. Rather than diminish in importance in the face of novel imaging techniques, recent advances in biotechnology have served to reaffirm the central role of histopathology in the diagnoses of human diseases, as well as in the study of adverse effects of xenobiotics on people. This has occurred partly because the transfer of new technologies into the pathology laboratory and the development of new histological visualization procedures. These new methods represent a logical extension to the use of special stains on tissue sections and have provided the pathologist with additional objective techniques to allow more precise morphological and functional correlation of changes in tissues.

Histopathological evaluation of tissue sections also represents a major technique in experimental studies of human disease. It has a central role in conventional toxicology studies which are performed to assess the toxicity of xenobiotics. The particular contribution of histopathology is its ability to assess the interaction of xenobiotics on complete body and organ systems rather than single cells or organs. The important place of histological assessment of tissues in regulatory toxicology has given rise to the development of the discipline of toxicological pathology. It needs to be underlined that this discipline is not simply the diagnosis of pathological lesions, but also the translation and integration of pathological diagnoses into meaningful summaries that can be understood by a diverse audience. A thorough knowledge of pathological techniques applicable to experimental pathology and problem solving in toxicology is an important and integral part of the skills needed by the toxicological pathologist.

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1.1 Basic Techniques in Autopsy and Histology Practice

Fundamental to good histopathological evaluation of tissue sections is a detailed autopsy or necropsy. This requires careful observation of the organs for any abnormalities and appropriate selection of tissues. Whereas in human autopsy practice and in experimental pathology studies, tissue sampling is usually directed towards particular lesions or organs of direct relevance to the problem under study, a more systematic approach is usually adopted in conventional animal toxicity studies. Most international regulatory guidelines require 30 or 40 tissues to be taken from all animals in most conventional toxicity studies. These are defined by the standard protocols of laboratories performing work for government regulatory authorities, and recommended lists are available (Jacobs *et al*., 2003). However, it is important to remain acutely aware that even the most complete tissue list remains a selective process and a careful inspection of the tissues for abnormalities at autopsy remains mandatory.

The precise selection of blocks, orientation of tissues and number of slides examined varies between laboratories. However, these procedures have become more standardized over recent years and recommended tissue blocking and orientation procedures have been published (Ruehl-Fehlert *et al*., 2003; Kittel *et al*., 2004; Morawietz *et al*., 2004).

For routine purposes, most tissues are fixed in a formalin solution by most laboratories, followed by embedding in paraffin wax and cutting of histological sections. Exceptions are eyes and testes, for which formalin is a poor fixative. Here, Bouin's, Zenker's or Davidson's fixatives are used. Bouin's fluid is generally considered the best fixative for the testis although there has been a move away from this because of the safety concerns of its picric acid content (Latendresse *et al*., 2002). Formalin, when used under stringent conditions, is usually good as a fixative for many immunocytochemical and molecular biological techniques on tissue sections. Frozen sections are, however, usually needed for studies of enzyme activity or requiring intact RNA.

Haematoxylin and eosin remains the most widely used stain, supplemented where appropriate by a Romanovsky stain for haemopoietic cells, periodic acid Schiff (PAS) stain for hepatic glycogen, glomerular basement membrane and the acrosome on testicular germ cells, trichrome and elastic stains for the myocardium and blood vessels and oil red O for neutral lipids. Immunocytochemical techniques are now extensively used (see below).

The use of larger, semithin (1–3 μm thick) plastic- or resin-embedded sections is a cost-effective compromise between electron microscopy and conventional light microscopy. Sometimes termed 'high-resolution light microscopy', light microscopic evaluation of semithin sections provides a means of avoiding extensive use of the electron microscope because it can locate cytoplasmic organelles in a way sometimes not possible in paraffin wax-embedded material.

1.2 Organ Weights

The extent to which organs are weighed varies between laboratories, and government guidelines are not always consistent (Michael *et al*., 2007). Heart weight is a guide to potential cardiac alterations and especially important in the assessment of cardiovascular drugs. Likewise, the lungs are weighed in inhalation studies for this can provide a useful indication of the extent of oedema or accumulation of exudate. Brain weight is employed as stable reference point in adult animals for it is fairly independent of body weight changes and can be useful in the evaluation of weight change in some organs (Bailey *et al*., 2004). The weights of endocrine organs are useful guides to alterations in the endocrine status of laboratory animals.

Testicular weights correlate with testicular toxicity and weights can be compared with in-life measurement of testicular size (Heywood and James, 1978). Weighing the testis is a useful precaution at the early phase of development of a novel drug prior to any assessment of male fertility and is generally recommended (Sellers *et al*., 2007). By contrast, ovarian weight is highly variable, as a consequence of cyclical ovarian development and is therefore a less sensitive indicator of treatment-induced changes in the female reproductive system, so that choosing to weigh ovaries needs to be considered on a case-by-case basis.

1.3 Histopathological Evaluation of Toxicological Studies

Histopathological assessment requires, above all, a systematic and meticulous approach, correlating in-life observations, autopsy findings, organ weight and clinical pathology changes with histopathological alterations. Careful selection of diagnostic terminology is essential because many of the terms utilized in experimental pathology have been borrowed from human pathology, where they may by applied to quite specific clinical conditions not always relevant to laboratory animal species. Lucid summary reports are essential. Good practices for pathology reports in toxicology studies have been recently summarized (Morton *et al*., 2006).

In conventional subacute and chronic toxicity studies, as well as in carcinogenicity bioassays, a complete set of tissues from the top-dose and control animals is

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routinely examined. In addition, any target organs or tissues showing tumours or other macroscopic alterations at autopsy in animals from intermediate-dose groups are also assessed. This baseline approach is outlined in some of the regulatory guidelines. However, some pathologists avoid this selective approach and examine all tissues sampled from all animals. This approach is perhaps more costly from a histological processing point of view, but permits rapid reporting of all the salient histopathological findings in every dose group. Cause-of-death assignment is another component of the assessment of carcinogenicity studies in rodents which is widely practised to aid statistical evaluation (Kodell *et al*., 1995).

The error which develops from the increased awareness of a lesion by the pathologist only after examining a considerable number of animals has been aptly termed 'diagnostic drift' (Roe, 1977). Another point of debate is the appropriateness of blind slide reading as a means to avoid bias, and opinion is much divided (Holland, 2001). Blind slide reading as a routine process conflicts with the need to have relevant clinical, clinical pathology and macroscopic data to make an appropriate tissue diagnosis. For this reason, only doubtful lesions which persist after a careful and integrated nonblind review of tissue sections are those which should be evaluated in a properly designed blind review (Newberne and de la Iglesia, 1985).

Peer review of pathological findings and quality assurance audit of the processes in histopathological assessment have also become more important components of regulatory toxicology studies in recent years. Peer review is believed to ensure integrity of the pathological diagnoses, encourage consistency and facilitate learning (Ward *et al*., 1995). Moreover, it is generally accepted that a peer review or check of a percentage of the diagnoses in a study by a second pathologist provides a level of security for regulatory authorities. Reviews by groups of pathologists (pathology working groups) have also been proposed as a means of resolving difficult diagnostic issues in toxicological pathology (Ward *et al*., 1995).

For over 30 years the pathologist has been assisted in the evaluation of toxicity and carcinogenicity studies by computer recording, storage and collation of macroscopic findings and their corresponding histopathological diagnoses. This has been an important contribution to the timely and accurate reporting of carcinogenicity studies, where the number of observations in older animals renders hand recording and tabulation a time-consuming process, prone to transcription error.

Finally, it is worth noting that in the use of new genetically engineered animal models in carcinogenicity testing, the same basic principles of evaluation of tumours are still valid. Indeed, the range of pathological lesions in these genetically engineered animals demands a particularly good knowledge of both human and animal pathology.

1.4 Histochemistry and Cytochemistry

Histochemistry is a tool that is a system of chemical morphology which adds another dimension to the characterization of alterations in tissues. Changes to structures, cells or organelles can be assessed by the changes in their chemical structure or enzymatic activity. For instance, the well-studied methods for alkaline phosphatase demonstrate activity at the cell membrane where active transport occurs. This can be used to localize and detect changes in structures, such as the surface of the intestinal epithelium and the brush border of proximal renal tubules. The histochemical method for 5′-nucleotidase is also an excellent marker for plasma membrane. Measures of peroxidase activity can be used to localize peroxisomes. Succinate dehydrogenase is used as an indicator for the Krebs' cycle and mitochondrial enzyme activity (Chayen *et al*., 1973). The enzyme, γ-glutamyl transpeptidase has long been regarded as the classical marker for neoplastic foci in the rodent liver. Characterization of pH-sensitive adenosine triphosphatase (ATPase) activity is also the conventional method for identification of different skeletal muscle-fibre types, which is still employed (Westwood *et al*., 2005). It is important to underline that the use of histochemical methods for the simple localization of tissue constituents or pathological alterations is conceptually different to using histochemistry to measure enzyme activity at the tissue level. This approach has been referred to as quantitative cytochemistry. This form of cytochemistry combines procedures for the production of insoluble chromophores by rigorous, controlled biochemical reactions with optical methods for their measurement (Chayen, 1984).

1.5 Immunocytochemistry

The use of classical histochemical methods for the identification of tissue constituents has largely been superseded by immunocytochemistry, which can usually be performed on conventional sections rather than the fresh frozen sections required by many histochemical methods.

Immunocytochemistry of routinely fixed and processed tissues has been one of the major technological advances in the histopathological evaluation and diagnosis of human diseases, because it represents an independent, objective method of cell identification against which traditional subjective morphological criteria can be compared. Immunocytochemistry has been particularly useful in the characterization of human neoplasms. However, unlike enzyme cytochemistry, it can usually only demonstrate the presence of antigenic determinants and is less able to demonstrate activity of cellular systems. Nevertheless, it represents a powerful

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tool for solving of certain problems in toxicology. Although many available antibodies do not work well on tissue sections from laboratory animals, a number of monoclonal and polyclonal antisera crossreact well with the tissues of laboratory animals and can be used in the histopathological evaluation of toxicity studies, provided appropriate controls are applied. Examples include polypeptide and protein hormones, metabolizing enzymes, structural proteins and cell markers, cell cycle proteins and activated caspases for apoptosis (Jones *et al*., 1996; Eckle *et al*., 2004; Mikaelian *et al*., 2004; Kunder *et al*., 2007). Although haemopoietic cells retain species-specific surface markers, monoclonal antibodies to lymphocytic and monocytic surface-membrane markers are available for rodents and dogs, and can be used in the characterization of xenobiotic-induced alterations in the haemopoietic and lymphoid systems (Ward *et al*., 2006).

A number of web sites are available to help in the selection and use of antibodies for use in immunocytochemistry in animal toxicity studies (Erexson and Faucette, 2006).

1.6 Lectin Histochemistry

A technique closely allied to immunocytochemistry is that of lectin histochemistry. The term 'lectin' is applied to proteins and glycoproteins extracted from invertebrates and lower vertebrates that have the capacity to bind sugar groups and glycoproteins in specific ways. Cell surface glycoconjugates play a key role in cellular recognition, cell-to-cell adhesion and act as receptor molecules, so they can be important in the reaction of cells to xenobiotics and pathogens. For many years studies on red blood cells have been performed with the lectin, Concanavalin A, which possesses haemagglutination properties. Lectins labelled with peroxidase or fluorescein have been used to demonstrate specific sugar groups and glycoproteins histochemically in tissue sections (Nicholson, 1974; Spicer and Schulte, 1982). Lectin histochemistry has been used for the characterization of mucins, the demonstration of cell structures in normal tissues and changes in cell surface expression in malignant cells. In toxicology, their primary use is in the characterization of changes induced by xenobiotics in structures which are well delineated by labelled lectins such as the biliary canaliculus, testicular germ cell acrosome, renal tubule, bronchial and gastrointestinal epithelium, haemopoietic cells and microglia (Geleff *et al*., 1986; Masson *et al*., 1986; Fix *et al*., 1996; Dullmann *et al*., 2002; Gheri *et al*., 2002). Conventional formalin fixation and paraffin-wax embedding is frequently adequate for the application of labelled lectin histochemistry, although alcoholic fixation or unfixed frozen sections may provide superior results in some cases.

1.7 Electron Microscopy

Although electron microscopy is widely used as a basic research tool, transmission electron microscopy also has a well established role in the characterization of structural alterations in cells and tissues which have been modified by the effects of xenobiotics. Despite it providing only a static morphological assessment of cells, its ability to characterize changes in subcellular organelles provides valuable information about functional deficits. A conventional application of electron microscopy in regulatory toxicology studies is the characterization of cytoplasmic alterations associated with liver weight changes and hepatocellular hypertrophy. Electron microscopy allows the definition of changes such as proliferation of the smooth endoplasmic reticulum, peroxisomal or mitochondrial proliferation, apoptosis and phospholipidosis. It is also important in the exclusion of subcellular degeneration in vital organs such as the heart when unexplained macroscopic or weight changes are seen without light microscopic alterations.

A variety of methods for the selection, perfusion and immersion fixation of tissues have been proposed for the application of electron microscopy in toxicity studies. Optimum fixation is obtained by whole-body perfusion with an aldehyde fixative, but this may conflict with procedures necessary for other components of a toxicity study such as routine histopathological examination, biochemical, metabolism and kinetic studies. One good compromise is perfusion fixation of freshly isolated samples of organs such as lung or liver. This provides superior tissue preparation to that obtained by immersion fixation (Roberts *et al*., 1990).

Despite the technological advances in transmission electron microscopy, especially semi-automated tissue processing and staining, it remains a demanding and labour-intensive process. Furthermore, it is highly selective and only small samples of tissues can be examined. Therefore, any electron microscopic work performed within the context of a toxicity study should have precisely defined objectives so that appropriate samples are selected and examined. Electron microscopy is not a method for speculative study of tissues in toxicity studies and defining no-effect dose levels. Standard operating procedures for application to toxicity studies designed for government regulatory authorities have been proposed (Dykstra *et al*., 2002).

Scanning electron microscopy has some applications in the study of early chemical-induced changes on epithelial surfaces such as the gastrointestinal mucosa and the bladder epithelium. It also forms the basis for a robust technique for the examination of the middle ear in laboratory animals treated with ototoxic agents (Astbury and Read, 1982).

Immunocytochemistry can also be applied to electron microscopic study in order to define the subcellular distribution of antigenic sites (Dinsdale *et al*., 2004).

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Ultrastructural autoradiography using tritium-labelled xenobiotics, especially when performed using quantitative methods such as the so-called hypothetical grain analysis, is valuable for the identification of sites of accumulation of drugs and chemicals within cells and may provide information on mode of cellular action (Read *et al*., 1985).

1.8 Microscopic and Imaging Technologies

Increasingly sophisticated imaging technologies are available for the analysis of pathological changes in tissues (Ying and Monticello, 2006). The use of the mainstay conventional microscope is now usually aided by the use of modern digital camera technology. Automated or semi-automated morphometric systems are also widely available. These represent potentially powerful tools applicable to the light and electron microscopic evaluation of xenobiotic-induced cellular and tissue changes. Whatever system used, morphometric analysis requires good statistical methods in the choice of numbers of animals, number of samples per animal, the quantity of data points per sample and in the mode of data analysis. In the measurement of basic parameters such as volume density, surface density and numerical density, account should be taken of potential artefacts due to shrinkage of tissues following fixation and processing, as well as the problems posed by magnification factors.

Another computer-based technological advance also being evaluated for use in toxicological pathology is so-called 'virtual microscopy'. This represents creating a digital replica of the content of a whole glass microscope slide and manipulating it on the computer to closely simulate looking at a slide with a traditional microscope (Dee, 2006). Virtual slides can be moved in two dimensions and through multiple magnifications, be annotated, compared side by side with slides stained by immunohistochemistry and used for image analysis.

Confocal imaging systems are based on the principle that both the illumination and detection systems are focussed on the same single volume element of the specimen. This enables examination of optical sections through intact tissue without the need for fixation, embedding, sectioning and staining. This has been applied to the study of a number of tissues, but notably to the translucent tissues of the eye in ocular toxicity studies (Jester *et al*., 1996).

Laser capture microscopy represents another innovation by which cells and tissues can be dissected from a histological section using a microscope combined with a laser system to remove and harvest for molecular biological analysis. In this way DNA can be extracted from specific tissues or cells in formalin-fixed slides or RNA from frozen sections (Green *et al*., 2003).

1.9 Molecular Biology Technology

Recombinant DNA technology represents the most recent transfer of basic research technology into the pathology laboratory. Molecular probes are being used to examine the structure and expression of specific genes in both normal and neoplastic human tissues. Over the last two decades these techniques have been widely applied in experimental pathology, where they have provided pathologists with new opportunities for the study of mechanisms in pathological processes. They have been used in the study of experimental tumourigenesis, examination of cell proliferation and gene rearrangements in various rodent organs (Malarkey and Maronpot, 1996). Although technically demanding, *in situ* hybridization enables demonstration of xenobiotic-induced alterations to the expression of specific messenger RNA (mRNA) in cells in histological sections (Doughty *et al*., 1995). Methods for the assessment of apoptosis in tissue sections are based on DNA polymerase-mediated or terminal deoxynucleotidyl transferase-mediated incorporation of biotinylated nucleotides to DNA strand breaks, so-called *in situ* end-labelling methods (Wheeldon *et al*., 1995).

Particular leading edge technology is so-called 'toxicogenomics' based on transcriptional profiling of tissues using one of a number of microarray platforms to assess induced alterations to gene expression (see **Toxicogenomics and the Evolution of Systems Toxicology**). In some laboratories this is being applied in an almost semiroutine basis in toxicological assessment (Foster *et al*., 2007). A well-designed genomic experiment undoubtedly provides a molecular signature of transcriptional events in response to xenobiotic exposure. It can aid in the characterization of toxic effects and provide the basis for mechanistic analysis. However, it should be underlined that gene changes do not necessarily indicate functional alteration and for this reason these technologies are best when they can be correlated with phenotypical alterations that can be seen using conventional evaluation, notably histopathology (Gant *et al*., 2005).

2 METHODS APPLICABLE TO SPECIFIC ORGAN SYSTEMS

2.1 Skin and Subcutaneous Tissue

Careful visual inspection remains the principle component of the assessment of skin alterations or changes in the pelage of laboratory animals in conventional toxicity

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studies. Drugs and chemicals given systematically may affect the skin in a number of ways. Pigmented skin or hair may lose its normal colour and albino skin may become coloured by administration of pigmented compounds or chemicals with pigmented metabolites. Agents with effects on sebaceous glands may alter the normal glossy fur of laboratory animals. Ulceration and inflammation may be the result of diverse factors, both spontaneous and as a result of toxicity. Therefore, skin abnormalities detected during life or at autopsy in conventional toxicity studies should be examined histologically. In addition, sections taken from carefully selected standard sites should also be sampled for routine histopathological examination. Standard sites are important because skin thickness and structure varies between different anatomical regions. Haematoxylin and eosin staining is sufficient for most purposes.

Studies conducted to study topical irritancy, contact sensitization or phototoxic activity usually employ rabbits or guinea pigs (*see* **Cutaneous Toxicology**). Here, reliance is commonly placed on visual inspection and a semiquantitative assessment of the degree of erythema, swelling, erosion and ulceration of the treated skin. However, histopathological examination can be useful in assessing the nature of any changes and immunohistochemical study of inflammatory and immune cells, blood vessels, collagen and other structural elements can be helpful in the characterization of skin toxicity.

Histological examination of injection site injury and the local damage around subcutaneously implanted biomaterials and implanted controlled-release treatment systems is an essential part of the assessment of their irritancy potential. It is important to not only characterize the nature and severity of the surrounding cellular reaction to these materials, but also the time course of the inflammatory and healing process, in comparison with known negative and positive control substances (Autian, 1972; Andersen, 2001).

In carcinogenicity studies performed in rodents, tumours of the skin and subcutaneous tissues are usually diagnosed by conventional microscopic techniques and this is usually adequate. In certain instances, particularly when tumours are induced by treatment, immunocytochemistry for antigens such as myoglobin, lysozyme, α-1 antichymotrypsin, smooth muscle actin, intermediate filaments, the mitochondrial uncoupling protein and S100 is performed, although it often adds little to overall interpretation of the relevance of the findings (Hardisty *et al*., 2007).

2.2 Mammary Gland

In laboratory animals, the mammary gland represents a sensitive indicator of the pituitary–gonadal axis. In rodents it is liable to develop neoplasms either spontaneously with advancing age or following prolonged hormonal derangement induced by administration of xenobiotics. For these reasons mammary glands are examined carefully in conventional toxicity studies both during life and at autopsy. This is followed by histological examination. Very occasionally the mammary glands of experimental animals have been used to test the tissue compatibility of mammary implants using histological assessment (Devor *et al*., 1993).

As laboratory rodents and beagle dogs possess several pairs of mammary glands, routine histological examination is usually conducted on a selected number of sites, frequently on one gland from each side plus any other showing macroscopic abnormality. Conventional haematoxylin and eosin stained sections are usually sufficient for mammary gland assessment. Special staining techniques include immunocytochemical staining for cytokeratins and myosin for myoepithelial cells, collagen Type IV and laminin for basement membrane and Type I collagen and fibronectin as connective tissue markers as well as sex hormone receptors (Greaves, 2007).

2.3 Haemopoietic and Lymphatic Systems

In conventional toxicity studies, blood smears, haemopoietic organs and lymphoid tissues are routinely examined by light microscopy in conjunction with automated analysis of peripheral red and white blood cells (*see* **Haematology and Toxicology**). Weighing and careful histological examination of thymus, spleen and lymph nodes are widely considered to be important components in the examination of effects of xenobiotics on the immune system. They form part of the first tier of procedures adopted in immunotoxicity testing batteries in the National Toxicology Program and at the Chemical Industry Institute of Toxicology, as well as in government testing guidelines (Luster *et al*., 1988; Ruehl-Fehlert *et al*., 2005).

Blood smears are usually examined using classical Romanowsky stains and conventional histological sections are taken from spleen, selected lymph nodes, thymus and decalcified bone marrow sections. The cellularity of bone marrow varies between different sites in laboratory animals. In rodents, bone marrow from the femur, sternum and vertebral bodies are considered to be the most representative (Cline and Maronpot, 1985; Wright, 1989). The cytology of bone marrow cells in decalcified, paraffin-embedded sections is not ideal and Romanowsky-stained bone marrow smears provide better cytological detail. The technical problems posed by the need to decalcify bone have been surmounted in some toxicology laboratories by the use of 3 µm thick methyl methacrylate-embedded sections stained with modified Giemsa supplemented by Gomori's stain for

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reticulin, a technique originally developed for human bone marrow biopsies (Burkhardt *et al*., 1982).

Critical histological examination of lymph nodes requires good orientation of the tissue to provide the basis for a clear assessment of the relative sizes of cortex, paracortex and medulla and their three-dimensional orientation. A semiquantitative assessment based on a standard approach defined by the World Health Organization, represents one good method for characterization of functional alterations in the B and T cell zones of the lymphoid system (Cottier *et al*., 1972). A descriptive approach to each lymph node compartment has been designed for toxicity studies (Haley *et al*., 2005; Ruehl-Fehlert *et al*., 2005). A similar approach has been recommended for the examination of the mucosal-associated lymphoid tissue (MALT) (Elmore, 2006). A detailed illustrated monograph on histopathological evaluation of lymphoid organs in toxicity studies has been published (Maronpot, 2006).

Whereas the spleen functions as a blood storage organ and a site of extramedullary haematopoiesis, its discrete nature in rodents makes is a useful organ with which to undertake morphometric analysis of the periarteriolar lymphoid sheaths (T cell zones) and germinal follicles (B cell areas). Spleen weight does not appear to be a reliable indicator of haemopoietic cellularity in the dog.

Conventional histological stains are readily supplemented by a variety of monoclonal antibodies for the immunohistochemical localization of surface markers of monocytic and lymphoid subsets. A large number of antibodies to rodent haemopoietic cell antigens are available and some have been reported to work on conventional paraffin-wax-embedded sections and occasionally antibodies to human T cell antigens react across different species (Ward *et al*., 2006; Kunder *et al*., 2007). However, more generally, antibodies to human antigens can be used on histological sections from monkey tissues (Li *et al*., 1993; Neubert *et al*., 1996). Antibodies to immunoglobulins or immunoglobulin light chains are usually sufficiently robust to be used in the characterization of B-lymphocytes in paraffin-wax-embedded sections. Lectin histochemistry may also help visualization of endothelial cells lining blood vessels and sinuses, dendritic cells and macrophages in lymphoid tissue in rodents (Dullmann *et al*., 2002).

2.4 Musculoskeletal System

An assessment of long bone growth is conveniently made by measurement of long bone length. The femur is frequently used for this purpose in laboratory animals. Appropriate laboratory processing of bone is a prerequisite of its good histological assessment. Formalin is adequate for most purposes, followed by decalcification using acidic fluids and embedding in paraffin wax. Use of polarized light microscopy is helpful in the identification of bone types in decalcified sections, for it can demonstrate the patterns of collagen orientation. Undecalcified sections using methacrylate or similar hard embedding media, supplemented by stains such as von Kossa, Goldner trichrome or solochrome cyanine are essential for the assessment of bone mineralization.

Choice of sampling site is particularly important for assessment of bone in toxicity studies in laboratory animals (*see* **Cartilage and Bone as Target Tissues for Toxic Materials**). It should be an identical site in all animals within a particular study, for it has been shown that even modest differences in sampling site can influence histomorphological variables of bone (Anderson and Danylchuck, 1978). In long bones, histological examination is usually directed to the metaphysis. Chondrocytes dividing in the metaphysis as columns of proliferating cells and maturing into hypertrophic cells are responsible for bone growth. Therefore, examination of this zone, particularly if morphometric analysis is also carried out, is an ideal place to evaluate bone formation and resorption in studies designed to evaluate xenobiotics which affect this process (Schenk *et al*., 1986). Vital fluorescent labels such as tetracycline, alizarin red S, calcein, procion or haematoporphyrin can be used to visualize sites of bone mineralization in tissue sections of bone from laboratory animals (Solheim, 1974).

Articular cartilage reacts to chemical insult only in a limited way. Like bone, it represents a stratified structure growing from the chondrosseous junction of the epiphysis which can be assessed histologically using conventional processing and staining procedures. Special stains such as toluidine blue, safranine O, lectin histochemistry and immunocytochemistry for collagens, fibronectin, cell cycle proteins and activated caspases, as well as electron microscopy represent further tools for the study of cartilage (Farnum and Wilsman, 1988; Burkhardt *et al*., 1997; Wancket *et al*., 2005).

Whether skeletal muscle is examined as part of a systemic toxicity study or for the study of the local irritancy of implanted or injected substances, sections are usually processed and stained conventionally. In the examination of implantation or injection sites, it is important to assess the extent and nature of local damage and the time course of the repair process.

The use of cryostat and histochemical reactions for ATPase activity has been accepted as the basis for identification of the slow twitch or Type I fibres and the fast twitch or Type II fibres for many years (Brooke and Kaiser, 1970). However, immunocytochemical staining for fast and slow isoforms of myosin correlate reasonably well with fibre analysis using the traditional method, but are applicable to formalin-fixed material in the study of the adverse effects of xenobiotics on muscle fibres (Behan *et al*., 2002; Westwood *et al*., 2005). Immunohistochemical stains for structural components such as collagen, fibronectin, myoglobin, laminin and desmin

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have also be used as adjuncts to assess alterations induced by xenobiotics in skeletal muscle (Helliwell, 1988).

2.5 Pulmonary System

Nasal chambers are the structures which are first subjected to the effects of inhaled substances. Xenobiotic-induced damage to the lining of the nasal passages of both humans and laboratory animals is often site specific and dependent on the sensitivity of the nasal epithelial tissue to the specific chemical. Knowledge of the comparative anatomy of the nasal chambers is required (Harkema *et al*., 2006). Although nasal passages are not studied in great detail in conventional toxicity studies, they are examined histologically when drugs and other chemicals are administered by inhalation. In rodents, the small size of the bones of the nose and nasal sinuses make a simple transverse-blocking procedure following decalcification a cost-effective way to provide standardized histological sections. A series of standard diagrams for the examination and recording of lesions in the nasal passages of rats and mice have been devised (Mery *et al*., 1994). For large animals, a complex dissection is required for histological assessment of the nasal mucosa.

Careful inspection of the lungs in a good light after opening the thoracic cavity at autopsy provides important information about pathological alterations. Changes may be manifest by uneven collapse, enlargement or over-inflation, patchy discolouration or stiffness of the lung parenchyma, as well as the presence of oedema or pleural effusions. Total weight or dry/wet weight ratios can be sensitive indicators of lung damage and oedema (Parker and Townsley, 2004).

Various methods of lung fixation are available. Immersion fixation in neutral buffered formalin is widely employed for most routine purposes and has the advantage of retaining exudates and fluids within the air spaces and airways. Instillation of fixative via the trachea under constant pressure (25–30 cm water) shows structural alterations well, although it has a tendency to dislodge exudates. Use of formalin vapour, perfusion fixation via the vasculature and freezing in liquid nitrogen are other methods which are employed. Fixation via the trachea is generally the preferred method because its advantages seem to outweigh its disadvantages.

A standardized approach to selection of pulmonary tissue for histological examination in rodent toxicity studies has been proposed (Kittel *et al*., 2004). Specialist stereological methods have a place in the detailed investigation of pulmonary toxicity, although a particularly rigorous approach is needed to avoid sampling bias (Hyde *et al*., 2007). Ultrastructural study is used in the elucidation of selective structural damage to the pulmonary parenchyma, but the size of the lungs and diversity of its cellular components creates problems of fixation and sampling (Tyler *et al*., 1985).

Haematoxylin and eosin remains the most important stain supplemented by stains for reticulin, collagen and elastic fibres as well as mucins. As in other tissues, a variety of immunocytochemical, cytochemical and autoradiological techniques are used in the study of the effects of xenobiotics on lung tissue. These include immunocytochemical demonstration of lymphoid cells, xenobiotic metabolizing enzymes and endocrine cells, as well asstructural components such as the collagen and laminin (Greaves, 2007).

2.6 Cardiovascular System

Key to the pathological evaluation of the heart in toxicity studies is the careful visual inspection of the pericardium, myocardium, endocardium and valve cusps of each cardiac chamber. Cardiac weight in laboratory animals varies with body weight, body length, age and sex, and circulatory demand. For this reason, weighing the heart is not only a useful guide to the toxic effects of xenobiotics on the myocardium, but also a measure of functional adaptation to cardiac work load.

Careful sampling of representative sections of each cardiac chamber and large blood vessels is also important for adequate histological assessment. These should include segments of the coronary arteries, free walls of the atria and ventricles, valves and zones sensitive to ischaemia, such as the endocardium and papillary muscles of the left ventricle. This is relatively easily achieved in rodents, where a single, well-orientated section of the heart can reveal most of these areas (Morawietz *et al*., 2004). However, for study of the heart in larger animals and humans a careful sampling procedure is needed, which requires several sections for each heart (Keenan and Vidal, 2006). Histological examination of the cardiac conducting tissue is not conducted routinely in regulatory toxicity studies. for it requires a large number of tissue sections for thorough study. However, a simplified method for examination of the sinuatrial and atrioventricular nodes in the dog has been proposed which can be more routinely applied (Palate *et al*., 1995).

Conventional fixation, paraffin-wax embedding, haematoxylin and eosin with connective tissue, fibrin and elastic stains are the cornerstones for detection of myocardial and vascular alterations.

Other histological techniques include the detection of early myocardial damage in formalin-fixed tissue using demonstration of the loss of myosin, tropomyosin ATPase, creatine kinase or lactate dehydrogenase from muscle fibres (Doran *et al*., 1996; Zhang *et al*., 2006). Immunohistochemical markers for neurofilaments and glutamate receptors have also been used to study neural

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structures and the conducting system (Mueller *et al*., 2003). Electron microscopy remains important for the characterization of damage to cytoplasmic organelles, particularly mitochondria (Hirano *et al*., 2001).

2.7 Digestive System

The mouth is subjected to visual inspection in conventional toxicity studies and the tongue is usually sectioned for histopathological examination of the oral mucosa. Major salivary glands are examined using conventional processing and staining techniques, although mucin histochemical stains are useful in the study of alterations in mucin secretion in these glands.

Whilst teeth are usually only inspected by the naked eye, it may be necessary to examine the dentition histologically in animals treated with xenobiotics. This is performed using the techniques employed for bone. Standardized sections for examination of rodent incisors have been proposed (Kuijpers *et al*., 1996).

Histological examination of the gastrointestinal tract is complicated by its great length and the fragility of the mucosa before fixation. Careful inspection without vigorous washing is essential to both locate lesions and to avoid artefacts which can mimic inflammation and ulceration in histological sections. Although selective blocking is usually appropriate if preceded by careful macroscopic inspection, the so-called 'Swiss roll' techniques are valuable for a detailed histological survey of the gastrointestinal epithelium. These methods can be adjusted for use in laboratory rodents, larger animal species and humans. They may be performed on fresh tissue or after fixation.

Mucin stains are particularly useful tools in the study of induced changes in the stomach and intestinal mucosa. This can be approached by either conventional mucin histochemistry or lectin histochemistry on formalin-fixed and paraffin-wax-embedded material. The techniques are applicable to study of the gastrointestinal mucosa from laboratory animals and humans, although there are considerable interspecies differences in the distribution of glycoconjugates demonstrated by these methods (Sheahan and Jarvis, 1976; Ishihara *et al*., 1984). The use of argyrophil staining is the traditional approach the characterization of enterochromaffin cells of the stomach and intestine because of their endogenous reducing activity. Immunohistochemistry is now more widely used for the characterization of enterochromaffin cells in the mucosa, notably using antisera to histamine, histidine decarboxylase, neurone-specific enolase and chromogranin A (Betton *et al*., 1988; Norlen *et al*., 2001). The study of cell proliferation using immunocytochemistry and antibodies to proliferating cell nuclear antigen (PCNA), Ki67 or bromodeoxyuridine is also useful in the assessment of the effects of xenobiotics on the gastrointestinal mucosa. The various techniques used for the characterization of apoptosis complete the repertoire in the study of the effects of drugs on cell turnover in the gastrointestinal tract (Pritchard and Watson, 1996).

Monoclonal antibodies against lymphoid surface markers are used for the study of the mucosa-associated lymphoid tissue or MALT in laboratory animals (Bland and Warren, 1985; Martin *et al*., 1986). Mucosal mast cells in rats have been shown to be demonstrable in conventionally processed tissues by prolonged toluidine blue staining (Wingren and Enerbäck, 1983).

As the principle site of metabolism and detoxification of drugs and other xenobiotics, the liver is a frequent site of alterations in toxicity studies, and is therefore the focus of careful histopathological assessment. In conventional studies, the liver is weighed prior to fixation and selection of blocks for processing for light and electron microscopy, as well as other techniques. The relatively large size of the liver presents problems in selection of tissue blocks and considerable variation exists between laboratories in the hepatic blocking schedule. In the National Toxicology Program rat carcinogenicity studies, the procedure involves the selection of two blocks taken from the widest parts of the left lobe and the right median lobe (Maronpot *et al*., 1989). Selection of two blocks appears to be the generally accepted practice for routine rodent liver histopathological examination in many laboratories.

As the liver is one of the most important sites for the adverse effects of xenobiotics, numerous special techniques have been employed in the microscopic evaluation of liver sections (*see* **Hepatotoxicity**). Not to be neglected are the conventional special stains such as oil red O for lipid, stains for collagen, reticulin and pigments. Electron microscopy, enzyme cytochemistry, immunocytochemistry and molecular biological methods all have well-accepted roles in the characterization of changes induced by xenobiotics in the liver of laboratory animals. It is frequently necessary to characterize proliferation of smooth endoplasmic reticulum, peroxisomes or mitochondria and lysosomal alterations such as phospholipidosis when treatment-induced changes in liver weight are recorded, or there is light microscopic evidence of hepatocyte hypertrophy. Quantification of both hepatic cell proliferation using PCNA, Ki67 or bromodeoxyuridine, and apoptosis with immunocytochemical detection of activated caspase and other methods such as *in situ* end labelling, are also useful (Wheeldon *et al*., 1995; Eckle *et al*., 2004).

As the liver possesses considerable functional heterogeneity within the lobule, cytochemical methods for reactions such as catalase, uricase, various dehydrogenases, alkaline phosphatase, 5′-nucleotidase and $NADH_2$ diaphorases, as well as glutathione, form a bridge between morphology and activity measured by classical biochemical techniques (Chayen, 1984).

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A number of histochemical methods have been applied to the study of foci of cellular alteration in the rodent liver. These include the classical marker γ-glutamyl transpeptidase, glucose-6-phosphate dehydrogenase and immunohistochemical demonstration of placental glutathione S-transferase (Tatematsu *et al*., 1987; Tsuda *et al*., 2003).

Gene expression analysis of liver tissue is also believed to provide additional insights to the mechanisms involved in xenobiotic-induced hepatic pathological alterations (Heinloth *et al*., 2007). It has also been recently argued that conducting multiple hepatic microarray analyses can provide a general view of potential metabolic changes that occur (Mori *et al*., 2007).

The pancreas is usually examined using conventional stains, although the different populations of endocrine cells of the islets of Langerhans can be readily stained using immunocytochemical staining of insulin, glucagon, somatostatin and pancreatic polypeptide, as well as other peptides (Wieczorek *et al*., 1998).

2.8 Urinary Tract

The kidney is particularly important in toxicology because many xenobiotics or their metabolites are eliminated primarily through the urinary tract (*see* **Responses of the Kidney to Toxic Compounds**). In addition, the kidney is liable to be exposed quickly to peak concentrations of circulating xenobiotics by virtue of its high blood flow. Its ability to concentrate toxic solutes in tubular cells or in the tubular lumen is an additional risk factor.

Renal weight changes are a useful guide to renal toxicity, although renal weight may also alter as a physiological response to changes in renal demand. Hence, it is essential that assessment of renal toxicity includes careful visual inspection of the renal parenchyma at autopsy for appearances of toxicity such as swelling, pallor, congestion and haemorrhage followed by histopathological examination. Inspection of the contents of the renal pelvis for crystals, mineral and cellular debris is also important, for these substances may be lost in subsequent tissue handling and processing.

Conventional fixation and processing, followed by haematoxylin and eosin staining supplemented by PAS, notably to stain basement membrane is usually appropriate for most conventional toxicity studies. It is important that all parts of the nephron are examined microscopically, so histological sections should comprise both cortex and medulla and include the tip of the papilla.

A number of techniques are available for more detailed study of induced alterations in the nephron. Electron microscopy has a time-honoured place in the study of pathological alterations in both the glomerulus and renal tubule, although perfusion fixation may be needed for the appropriate degree of preservation of tubular cells. Light microscopy of plastic embedded sections 1–3 μm thick is a useful compromise which provides excellent resolution of renal structures.

Various parts of the renal tubule show different enzyme activities which relate to the function of different segments. Histochemical demonstration of enzyme activities can therefore be used as markers for components of the nephron and for structural–functional studies. For instance, lysosomal enzyme activity is highest in the proximal tubule, reflecting the role of this segment in degradation of reabsorbed macromolecules. Therefore, enzyme cytochemical demonstration of acid phosphatase or other lysosomal enzymes outlines the proximal tubule. Of interest to the pathologist as cytochemical markers are brush border enzymes, alkaline phosphatase, 5′-nucleotidase and γ-glutamyl transpeptidase which are useful in the study of tubular damage. Measurement of enzyme activity in the urine is also a useful complementary, noninvasive technique for the assessment of tubular toxicity in humans and laboratory animals, because cellular enzymes from damaged tubular cells spill into the urine in increased amounts. However, immunocytochemistry is now more often used to demonstrate the presence of brush border enzymes such as γ-glutamyl transpeptidase (Yasuda *et al*., 1986). Immunocytochemical demonstration of Tamm–Horsfall proteins, localized at the surface membrane of the thick ascending loop of Henle, has also been used for the study of renal tubular changes induced by xenobiotics. In the study of drug-induced alterations to the juxtaglomerular apparatus, immunocytochemical staining using antisera to renin or labelling of renin mRNA expression by *in situ* hybridization are superior to the classical, nonspecific techniques for renin-containing granules, such as the Bowie and Hartroft stains. Immunocytochemical staining for α-smooth muscle actin not only reveals smooth muscle in vascular smooth muscle, but also activated mesangial cells (Kaneko *et al*., 2001).

Labelled lectins can be used to localize different segments of the nephron histochemically and this can often be performed on formalin-fixed, paraffin-wax-embedded material. For instance, peanut lectin (*Phaseolus vulgaris*) and *Ricinus communis* stain the S1 and S2 segments of the rat proximal tubule whereas the S2 remains poorly stained. The mouse proximal tubule brush border stains with *Lotus tetragonolobus* (Schulte and Spicer, 1983).

Histological study of the bladder mucosa requires special care particularly in the study of proliferative lesions of the urothelium (Cohen *et al*., 2007). The best orientation of the epithelium is obtained following inflation of the bladder with fixative at autopsy, for this removes folds which can give a misleading impression of the thickness of the epithelium. However, this procedure may dislodge exudates and other material from the bladder lumen, so care should be exercised in its application. Assessment of cell proliferation can be important

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in the assessment of hyperplasia and neoplasia of the bladder epithelium. Scanning electron microscopy is a useful special technique for the examination of chemically induced alterations to the superficial transitional epithelium (Cohen *et al*., 2007).

2.9 Reproductive System

The effects of xenobiotics on reproduction are usually examined in special reproductive studies, in which histopathological examination plays relatively little part (*see* **Reproductive Toxicology**). However, histopathological examination of the male and female reproductive tract is an important component of conventional toxicity studies. At an early stage of the development of a new medicine this histopathological examination may represent the only assessment of treatment-related effects on the reproductive organs and germ cells and needs to be performed with care.

In males the testes and accessory sex organs are examined routinely. Special fixatives are employed for light microscopic examination of the testes because formalin is a poor method of preservation of the germinal epithelium prior to embedding in paraffin wax. Immersion in Bouin's, Zenker's or Helly's fluids is commonly used, followed by paraffin wax embedding, sectioning and staining with haematoxylin and eosin (Latendresse *et al*., 2002). PAS is often used for the demonstration of the germ cell acrosome, for this enables the most precise staging of the germ cell cycle in laboratory animals and in humans. The approach to microscopic assessment of the testis in toxicity studies has been the subject of discussion for many years and international guidelines now exist. The recommended approach is a qualitative assessment of testicular histology followed by quantitative assessment or evaluation of seminiferous stages in special time-course studies, only when lesions are detected in conventional studies (Lanning *et al*., 2002; Creasy, 2003).

The female reproductive system is examined histologically using conventional histological techniques, paying close attention to the cyclical alteration in the endometrium, vaginal mucosa and ovarian tissue. The ovary requires careful orientation if sections are to include cortex, medulla and hilar tissue. As ovarian function is closely linked to the endocrine system, it is important that in any assessment of the reproductive tract, account is taken of endocrine organs.

2.10 Endocrine System

Histological examination of endocrine tissues is important in toxicity studies. When it is technically possible, histological examination is preceded by weighing of the organs. However, this may not be advisable for firmly bound organs such as the thyroid gland, for danger of disruption of histological sections.

Although haematoxylin and eosin staining is appropriate in most circumstances, immunocytochemistry is of importance. Antisera for many peptide hormones are widely available and, provided appropriate dilution and absorption controls are employed, they can be used in the examination of the endocrine tissues of most conventional laboratory animal species. C-cells in the thyroid gland can be particularly difficult to delineate in haematoxylin and eosin stained sections, but they can be clearly seen by immunocytochemistry for calcitonin. Immunohistochemical demonstration of the acidic protein, chromogranin A, is also a good marker in different species for many endocrine cells containing secretory granules.

The chromaffin reaction, which represents darkening on exposure to aqueous solutions of potassium dichromate as a result of oxidation and polymerization of catecholamines, remains a useful stain in the study of catecholamine activity in the medulla (Tischler *et al*., 1997). Neurone-specific enolase is consistently present in the rat and mouse adrenal medulla and can be used as a histochemical marker for proliferative and neoplastic alteration in this tissue (Wright *et al*., 1990). Others have used immunohistochemical staining for phenylethanolamine-*N*-methyltransferase and chromogranin A (Tischler *et al*., 1996). The examination of electron dense granules in the medulla is also useful for the detailed characterization of medullary cells.

2.11 Nervous System and Special Sense Organs

Histopathological assessment of the nervous system requires careful selection of nervous tissues, appropriate fixation, processing and staining in ways that are often different from the techniques employed for other tissues.

Perfusion fixation of the brain is the method of choice for the avoidance of artefacts, although immersion fixation followed by conventional processing is adequate for most routine purposes, but this must also be done with care (Fix and Garman, 2000). It is essential to be aware of the artefacts which can develop in the immersion fixation of brain so that they are not confounded with treatment-induced alterations. These artefacts have been described in detail (Garman, 1990).

Blocks for histological examination should be selected to include appropriate cerebral nuclei and fibre tracts. Sectioning should be sufficient to include grey and white matter from the cerebrum, cerebellum, brain stem, spinal cord as well as spinal ganglia and peripheral nerves. Key areas include basal ganglia, substantia nigra, dentate nuclei, Purkinji cells and optic tracts (Garman,

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2003; Morawietz *et al*., 2004). Standard sections can be obtained by the use of a metal mould with cross channels for accurate slicing of the fixed brain and appropriate zones assessed by use of one of the stereotaxic atlas of neuroanatomy (Sidman *et al*., 1971; Paxinos and Watson, 1997; Bolon, 2000).

In addition to haematoxylin and eosin, special stains for myelin, neuronal bodies, axons and glial cells are helpful. Traditional silver methods still have a place in the evaluation of neuronal degeneration (Grant *et al*., 2004). Two novel anionic fluorescent compounds, Fluoro-Jade and Fluoro-Jade B have also been proposed as tracers for neuronal degeneration in toxicity studies (Schmued and Hopkins, 2000). Changes in peripheral nerve fibres are particularly well characterized in semithin plastic or epoxy resin embedded, toluidine-blue-stained sections.

Immunocytochemistry using a wide range of antisera also has a place in the assessment of toxicity of the nervous system. Immunocytochemistry using antisera to glial fibrillary acidic protein (GFAP). S100 protein, neurone-specific enolase and cytoskeletal components may provide valuable information for the identification of cell types and characterization of changes within them. Immunocytochemical localization of GFA protein is now considered a useful addition to the assessment of the effects of toxins on the central nervous system, particularly when used together with more conventional stains (Fix *et al*., 1996). The lectin *Griffonia simplicifolia* isolectin B_4 has also been used to stain in the study of the toxic effects of xenobiotics on the rat cerebral cortex (Fix *et al*., 1996). Electron microscopy also remains a valuable tool for problem solving in neuropathology although it is technically demanding and labour-intensive (Jones, 1988).

Eyes are examined by use of conventional histological techniques after use of special fixatives such as Davidson's fluid to avoid as much artefact as possible to the cornea, lens and retina. The lens poses particular problems in histological processing, but excellent results can be achieved using plastic-embedding procedures. Confocal microscopy has been shown to be a potentially powerful technique in the histological study of corneal irritation because it can be applied in a noninvasive manner (Jester *et al*., 1996).

The cochlea is not usually examined histologically in routine toxicity studies and reliance is placed on clinical assessment or specialist tests (*see* **Ototoxicity**). However, the middle and inner ear can be examined histologically after decalcification of the petrous bone and preparation of conventional sections. However, sectioning the cochlea to achieve the correct orientation requires considerable skill and this method can only demonstrate small numbers of sensory cells in the organ. One technique is a surface preparation technique which involves dissection of the cochlea and subdivision of the organ of Corti into short segments for examination by phase contrast microscopy (Engström *et al*., 1964). A modification of this technique using scanning electron microscopy is relatively simple to perform and can be used in the rat and other experimental animals (Astbury and Read, 1982; Liberman, 1990).

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Clinical Chemistry in Toxicity Testing: Scope and Methods

Sylvie Gosselin, Lila Ramaiah and Lesley Earl

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1 INTRODUCTION

Clinical pathology, consisting of routine haematology, coagulation, clinical chemistry and urinalysis, is an important part of safety assessment for food ingredients, drugs and chemicals, and is useful in predicting potentially important toxicity endpoints, detecting potential human responses to hazardous substances (chemicals) and selecting parameters for clinical monitoring (drugs) (OECD, 2001; Weingand *et al*., 1996; Redbook, 2003). Serial sampling with time allows the evaluation of the development, progression and recovery of changes to the test system. Some government and regional regulatory agencies and international organizations provide guidelines for clinical pathology testing in animal toxicity and safety studies. These testing guidelines vary between agencies and the scientists should be aware of these differences (see Regulatory Agency Web Sites).

1.1 Core Clinical Chemistry Testing

The core clinical chemistry tests appropriate for all test materials include evaluation of protein, lipid and carbohydrate metabolisms, liver and kidney functions and electrolyte balance (see **Table 1** for industry consensus). Additional clinical chemistry tests may be necessary to extend the search for toxic effects or elucidate toxic effects attributable to a test material (Redbook, 2003; OECD, 2001).

1.2 Blood Sampling and Intervals

The number of occasions of blood sampling for clinical pathology testing is dependent on study objectives, study duration, test material, animal species and regulatory requirements. Blood volume and techniques for each animal species should comply with all appropriate laws and regulations concerning animal welfare and husbandry (Weingand *et al*., 1996). Blood sample volume and frequency should not compromise the physiological status of the animal and the route and method should be chosen to maximize the possibility of an efficient and low-stress bleed (for both the technician and the animal). Guidance is provided by a joint European Centre for the Validation of Alternative Methods (ECVAM)/European Federation of Pharmaceutical Industries and Associations (EFPIA) publication (Diehl *et al*., 2001) and the principles of this guidance have general industry-wide agreement. Technical expertise in handling and sampling the chosen species is paramount to obtaining good samples, minimizing the variability between samples and reducing the likelihood of a failed sample.

There are no clinical pathology requirements in single-dose toxicity studies for test material from the nonpharmaceutical industry. In the pharmaceutical industry, clinical pathology evaluation is usually not required, except for acute animal studies conducted to provide the primary safety data supporting single-dose safety/kinetic studies in human trials. Clinical pathology should be performed ideally for maximum effect (24–48 hours post-dose) and recovery (14 days post-dose) (FDA, 1996). Where nonrodents are appropriate for investigation, the studies should be designed so that a maximum amount of information is obtained from the smallest number of animals (escalating dose or dose-range-finding studies are acceptable); in these studies, clinical pathology testing may or may not be extensive depending on the class of compounds to be tested, the objectives of the study and the company strategy (FDA, 1996; Smith *et al*., 2005).

The minimum requirements for blood sampling in repeated-dose studies in rodents and nonrodents are based on a document published in 1996 representing the consensus of a joint international scientific committee (Weingand *et al*., 1996). With respect to the rodents, the blood sampling should at least be done at termination. Interim testing may not be necessary for long-duration studies if testing was done in short-duration studies that used dose levels not substantially lower than those of the long-duration studies. For repeated-dose studies in nonrodents, testing should be done at least once before initiation (to allow some measure of intra- and interanimal variability), at least once during the study and at termination. The small number of animals generally used in nonrodent toxicity studies is balanced by the fact that pretreatment and serial samples can be taken, making interpretation of individual changes with time quite practical.

While clinical pathology testing is not recommended (Weingand *et al*., 1996) for rodents in studies longer than 52 weeks, because naturally occurring geriatric conditions obscure meaningful interpretation of laboratory data, some regulatory agencies suggest or require the testing to be done to maximize the information obtained from those animals (Redbook, 2006; EMEA, 2002).

Routine clinical chemistry analysis is carried out either in serum or plasma. For serum collection, no anticoagulant is required, whereas for plasma collection tubes coated with either heparin or lithium heparinate are required. Ethylenediaminetetraacetic acid (EDTA) is not suitable, since it will affect some electrolyte and enzyme measurements as it acts as an ion chelator. For most routine parameters both serum and plasma are suitable, but it is important to be consistent and build experience and background data in at least one matrix. In the USA serum is usually collected. In the UK and mainland Europe, plasma is usually collected. There may be differences in the clinical chemistry values between these matrices (Young *et al*., 2006).

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Table 1 Core clinical chemistry tests in standard regulatory toxicity studies[a]

Total protein	Alanine aminotransferase	Sodium	Urea nitrogen
Albumin	Aspartate aminotransferase	Chloride	Creatinine
Cholesterol	Alkaline phosphatase	Potassium	—
Triglyceride	Gamma-glutamyltransferase	Calcium	—
Glucose	Total bilirubin	Phosphorus	—

[a]The laboratories, surveyed in 2007 and 2008, consisted of 54 clinical pathology laboratories in the pharmaceutical industry and contract research organizations (Parzych and Kimball, 2008).

Rat urine sampling in rodents is usually carried out by collection from specially designed collection cages, where animals can be deprived of food and water for several hours. Analysable samples from mice are difficult to obtain and not recommended because of the low volumes. In some nonrodent species, such as dogs and macaques, it may be practically possible to obtain bladder samples by catheterization or cystocentesis, but it can be stressful to animals (and it does not allow a timed sample to be collected to estimate volume). Of course, this has to be balanced against the stress associated with overnight collection and the scientific objectives of the study. In addition, overnight urine collection systems need to be scrupulously cleaned to avoid contamination. Several nonpharmaceutical regulatory guidelines require the collection of a timed urine sample, which mitigates towards the former method (Redbook, 2003; OECD, 1995b).

2 DATA INTERPRETATION AND REPORTING

Assuming that the current choice of animal models and the design of the studies are predictive of human hazard (Olson *et al*., 2000; Greaves *et al*., 2004), toxicity study interpretation and reporting assure that drugs in development or on the market will not cause harm to the patient, or that the use of chemicals will not be detrimental to human welfare/health. Therefore, the quality, integrity and completeness of the reporting of experimental data, including clinical pathology, from toxicity studies are essential. To avoid losing important aspects in an unnecessarily long document, reports should be as concise and precise as possible (Wells and Gosselin, 1996; OECD, 2001; Black, 2002).

In regulatory toxicity studies, clinical pathology data from animals treated with increasing doses of a test material are compared with data from concurrent control animals for rodent and nonrodent studies and pretest data for nonrodent studies (OECD, 2001). Clinical pathology data can be among the most difficult data within a given study to evaluate, because interpretations of many clinical pathology findings are interdependent and can be influenced by several factors, such as limitations of the assays, preanalytical variations, route of administration, environment, social housing and naturally occurring diseases. Clinical pathology results should also be correlated with in-life observations, necropsy findings, organ weights and histopathological changes, in order to make plausible and informative interpretations (Wells and Gosselin, 1996; Boone *et al*., 2005; Hall and Everds, 2008).

2.1 Preanalytical Variability

Reviews of factors (physiological, procedural or artefactual) which can complicate the interpretation of clinical chemistry findings may be found in the following references (Robinson and Evans, 1996; Loeb and Quimby, 1999; Young and Bermes, 2006; Hall and Everds, 2008). In a regulated environment, the protocol should be able to control several of the variables by specifying the species, strain, geographic region (monkeys), supplier, age of animals at the start of study, the use of certified diet and by fasting the animals prior to bleeding. The in-depth knowledge of standard operating procedures (SOPs) and appropriate technical training should control variables associated with blood collection site, order of sample collection, anaesthesia and poor blood samples (clotting, haemolysis, dilution due to excess of anticoagulant, evaporation, delay in serum/plasma separation). Fine-tuned scheduling of blood sample collection will take into consideration variations related to circadian rhythm (e.g. hormones), the most appropriate time after dosing for specific assays (e.g. troponins) and may help reduce fear/excitement/stress in the pretest period, by giving a chance for the animals to acclimatize to their new environment and activities.

2.2 Analytical Variability

Interpreting clinical chemistry results in regulatory toxicity studies requires the ability to discern toxicity from physiologic variability and laboratory error. Often, subtle trends in data that have a wide physiologic range may be masked by analytical variability. Minimizing variability is central to generating quality results from which

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meaningful conclusions can be drawn, having a direct impact on the outcome of the test material under investigation. The quality of laboratory results is determined by the quality of the sample (preanalytical variation), the quality of the analysis (analytical variation) and the quality of laboratory records (postanalytical variation) (Stockham and Scott, 2002). Some degree of random analytical variation is unavoidable due to the physicochemical nature of analytical methodologies. In a regulatory environment, the acceptable degree of variability must be defined, monitored and controlled. A quality assurance (QA) programme must be implemented which specifies the procedures used to produce data of known variability (precision) and bias (accuracy). This set of operating principles serves as a means of monitoring analytical variability to ensure validity of results.

In a laboratory's QA programme, analytical variability must first be defined by means of equipment qualification and method validation. A review of policies and procedures that can be implemented in order to develop a validation process may be found in the following references (Bansal *et al*., 2004; CLSI, 2007; Garber and Carey, 2003). Thereafter, variability and bias are monitored and managed by way of specified equipment maintenance and analytical quality control (QC) SOPs. QC consists of a systematic process within a sample analysis methodology to assure that the process is in control. In QC, assay variability and bias are measured over time and compared against set criteria (Blick and Passey, 2003; CLSI, 2006). This is typically accomplished using commercially available QC (control) material that is analysed at the same time as the test sample. The recovered concentration of the control material is evaluated against specified acceptance criteria. In addition, external QC or proficiency programmes determine the quality of data produced by the laboratory by comparing laboratory results for an unknown (laboratory intercomparison samples and performance evaluation samples) with those of other laboratories using the same methods and equipment (Veterinary Laboratory Association, College of American Pathologists). Proper implementation of these robust internal and external QA programmes provides reasonable confidence regarding suitable instrument performance and accurate, precise and reproducible data generation, as prescribed within regulatory environments.

2.3 Are the Changes Related to the Test Material?

Safety evaluation of new chemical entities, whether they are industrial chemicals or potential new drugs, is not simple. It requires objectivity, careful attention to detail, knowledge of experimental models, historical perspective, knowledge of government regulatory requirements and experience in data interpretation. Safety evaluation of potential new drugs differs in approach to that used with industrial chemicals. The risk/benefit analysis in drug safety assessment must take into account the disease for which the drug is intended, the natural history of the disease without therapy and the availability of other treatments. With potential new drugs, not every effect seen in a preclinical study can be considered as unwanted or harmful. For example, drugs designed to treat diabetes would be expected to show significant to life-threatening hypoglycaemia in preclinical studies using euglycaemic animals (Dorato and Engelhardt, 2005).

The main goals for conducting most regulatory toxicity studies are (Wells and Gosselin, 1996; ECETOC, 2002; Lewis *et al*., 2002; Boone *et al*., 2005):

1. To define if the effects are due to the test material
2. If they are judged to be due to test material, it is to discriminate between those that are adverse and nonadverse
3. To identify the no observed adverse effect level (NOAEL)/no observed effect level (NOEL).

The difference between controls and animals treated with the test material is likely an effect of the test material if:

1. There is a dose response
2. It is observed in several animals (not an outlier) and in both sexes
3. The changes are consistent between individual animals (same direction either increase or decrease)
4. It is noted over more than one interval or the effect progressed over time
5. It was reported in previous studies with this test material (same animal species or other species)
6. It is a precursor to a known effect of the test material
7. The effect is expected with this class of test material.

Toxicokinetic results should be evaluated for isolated test material effect that may be due to erratic bioavailability of the test material. If the clinical pathology measurements are physiologically not credible, the measurements should obviously be excluded from consideration. The effect should also be differentiated from changes due to the vehicle, procedure (repeat bleedings), background findings associated with route of administration (continuous intravenous infusion, intramuscular or subcutaneous injection) and from naturally occurring diseases/disorders.

2.3.1 Statistics

Appropriate statistical methods should be used to analyse clinical pathology data. Regardless of the outcome of

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statistical analysis, scientific interpretation is necessary for the ultimate determination of test material effects. Statistical significance alone should not be used to infer toxicological or biological relevance of clinical pathology findings. Additionally, the absence of statistical significance should not preclude the possibility that test material effects exist (Weingand *et al*., 1996; OECD, 2001). Some statistical tools used in toxicology studies differ between laboratories and comparison of strategies for application have been evaluated recently (Kobayashi *et al*., 2008); however, each laboratory has a preferred approach.

In nonrodents, statistical analysis of changes in clinical pathology data is further complicated by interindividual and intraindividual variability and by the low number of animals per dose group. For these reasons, the use of statistical analysis in the evaluation of clinical pathology data augments, but does not replace, comparisons of individual data with concurrent controls in all species, nor does it replace comparisons of individual data with pretest values in nonrodents (Boone *et al*., 2005). For example, hepatotoxic signals such as elevations in alanine aminotransferase (ALT) in nonrodent preclinical studies may be found in one or two animals and not achieve statistical significance. The biological significance of relevant hepatotoxic signals should be clarified (EMEA, 2008).

2.3.2 Historical Control Data

The concurrent control data are more appropriate than historical reference ranges for comparison with test material treated groups. Ideally, historical control data (HCD) must come from the same laboratory, utilizing the same strain, age and sex of animals obtained from the same supplier, with identification of study procedures (route of administration, vehicle, clinical pathology methods/instrumentations, serum/plasma, site of collection, etc.) which could affect the values (OECD, 2001). HCD used to clarify a potential effect in a study should be reasonably contemporary to the study under evaluation (ECETOC, 2002; Lewis *et al*., 2002). In some studies, particularly when the rabbit is used as the animal model, the reproductive status (pregnant vs. nonpregnant) of the animals should be identified (Wells *et al*., 1999; de Rijk *et al*., 2002).

HCD are useful in evaluating the acceptability of the 'normal' data obtained from nonrodents in the pretest period or from controls during the treatment period, and may have some utility in placing test material changes into the context of severity (changes that remain within the limit of HCD have a lower risk than those that do not) and should not be seen as a convenient device for discounting test material findings (ECETOC, 2002; Clemo, 1997).

2.4 Are the Test Material Changes Adverse or Nonadverse?

An adverse effect may be considered to be a change (biochemical, functional or morphological) that may impair performance and generally have a detrimental effect on the general well-being, growth, development or life span of animals in toxicity studies. More specifically, an adverse effect in toxicity studies conducted for drug approval should be an effect that would be unacceptable if it occurred in a human clinical trial (Wells and Gosselin, 1996; OECD, 2001; Lewis *et al*., 2002; ECETOC, 2002; FDA, 2005; Boone *et al*., 2005; Dorato and Engelhardt, 2005).

In order to identify if a clinical pathology effect is adverse, it should be quantified (fold or % change vs. controls or in some cases pretest values), qualified (minimal to severe) and corroborated with clinical observations, morphologic pathology findings and/or any other clinical pathology changes. The change is unlikely adverse if it is transient and if it is considered an adaptive response or secondary to severe reduction in food consumption/anorexia, the moribund condition of the animal (liver/kidney failure, electrolyte imbalance) or excitement/stress (increased ALT/AST in monkeys). A change that is readily and completely reversible on cessation of treatment would suggest a lower level of concern. The change is more likely considered to be adverse if the effect is inconsistent with class effects, mode of action or what is otherwise known or expected of the test material. Before concluding that an effect is adverse, the intended clinical indication should be taken into consideration (hypoglycaemia with antidiabetic drugs; hypocholesterolaemia with lipid-lowering drugs), as well as if the finding has any human relevance. Microsomal enzyme inducers in rodents resulting in increased thyroid hormone turnover, thyroid stimulating hormone (TSH) stimulation, follicular hypertrophy/hyperplasia and the eventual progression to thyroid tumour is a well-known example which is irrelevant to humans. Other points to consider would be if the animal model is relevant (e.g. biologically responsive animal species are recommended for biopharmaceuticals), if there is tolerance over time (particularly with central nervous system drugs), and if the toxicity is due to a toxic metabolite not identified in humans.

The identification of the NOAEL can be a challenging process and should be determined for each toxicity study as a whole, rather than for individual findings (Lewis *et al*., 2002; Dorato and Engelhardt, 2005). In some cases, however, there may be more than one NOAEL (e.g. neoplastic vs. non-neoplastic findings, local vs. systemic toxicities) in a toxicity study. The initial opinions on where harmful and nonharmful effects occur may change as additional information related to the molecule is generated over the course of developing a

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test material. Data from longer-term studies may give the toxicologist new, or additional, perspective on whether or not an observation thought to be subtle or minor in a short-term study is an early indicator of a harmful effect. For example, a liver enzyme elevation in a short-term preclinical study, without a histopathology correlate, will be noted, but may not be considered to be harmful. The outcome of longer-term studies, indicating a progression of effect and the occurrence of histological alteration in the liver, provides a new interpretation of the importance and/or relevance of the response and the duration of exposure associated with the first indications of an unwanted response (Dorato and Engelhardt, 2005).

3 PROTEINS

Plasma proteins perform a nutritive function, exert colloidal osmotic pressure and aid in the maintenance of acid–base balance. Individual proteins serve as enzymes, antibodies, coagulation factors, hormones and transport substances. The major site of plasma protein synthesis is the liver (albumin (ALB), most α and β globulins (GLOBs)) and the second major contributor is the immune system (γ GLOBs). Serum contains all of the plasma proteins except fibrinogen, factor V and factor VIII. Age is an important consideration in the interpretation of protein variations and it must be kept in mind that few protein changes are pathognomonic (Evans and Duncan, 2003; Kaneko, 1997b; Stonard and Evans, 1999).

ALB represents ~50–70% of the serum/plasma total protein (TPROT) concentration in laboratory animals (rats, dogs and monkeys); in humans, it ranges from ~64–69%. Because of its abundance and small size, ALB accounts for 75% of the colloidal osmotic activity of plasma. TPROT and ALB concentrations are directly measured while GLOB concentrations usually are calculated (total protein—albumin = globulin). GLOB and ALB can be separated and directly quantified by electrophoresis, however it is not recommended by the Committee for International Harmonization of Clinical Pathology Testing and is now rarely performed on routine toxicity studies (Evans and Duncan, 2003; Kaneko, 1997; Weingand *et al*., 1996).

In toxicity studies, the most frequent reason for increased TPROT concentration is dehydration. Possible correlative clinical observations include gastrointestinal (GI) fluid losses (e.g. vomiting, diarrhoea, excessive salivation), polyuria and reduced water consumption. Since water consumption in rodents is closely associated with food consumption, any cause of decreased food consumption in rodents has the potential to cause relative dehydration (Hall and Everds, 2008). Increased TPROT also has been associated with inflammatory conditions that stimulate the production of acute phase proteins (APPs) and immunoglobulins. Neither of these two parameters is routinely evaluated in regulatory toxicity studies. Immunoglobulins are discussed in Chapter **Immunotoxicology** of this book.

Decreased TPROT concentrations result from either decreased protein synthesis or increased protein loss. Concomitant decreases in ALB and GLOB occur with malnutrition, haemorrhage, intestinal malabsorption, protein-losing enteropathy, severe exudative skin lesions/burns and overzealous administration of fluids. Hypoalbuminaemia can be the result of hepatic insufficiency, anorexia/cachexia, glomerulopathy with proteinuria and high protein effusion (Evans and Duncan, 2003; Kaneko, 1997). In toxicity studies, small decreases in ALB are frequently observed in animals administered high doses of a test material. This may correlate with effects on other parameters (e.g. haematocrit, glucose, cholesterol (CHOL) and body weight) and is usually considered an indication of the overall poor condition of the animals rather that evidence of a specific toxic mechanism (Hall and Everds, 2008).

3.1 Acute Phase Proteins (APPs)

Most APPs migrate to the α- and β-globulin areas on routine electrophoresis. This method is much less sensitive than the individual APP assays. The current interest in APPs is generated by their potential use in providing an early and reliable signal for the presence of inflammatory processes and to monitor animals in toxicity studies and human patients (Epstein, 1999; Shao, 2006; Ceron *et al*., 2005; Eckersall, 2006). The usefulness of APPs has been shown with inflammatory lesions caused by phosphodiesterase inhibitors (Dietsch *et al*., 2006; Zhang *et al*., 2006). The APPs can be broadly classified into two categories depending on their blood circulating levels: ALB and transferrin are identified as negative APPs, while C-reactive protein, serum amyloid A, haptoglobin, α-1-acid glycoprotein (AGP) and ceruloplasmin are positive APPs. The magnitude of the increases varies from about 50% to as much as 1000-fold depending on the APPs and animal species (Epstein, 1999; Gentry, 1999; Ceron *et al*., 2005). The major APPs for humans, dogs and monkeys are C-reactive protein and serum amyloid A and for rats, α-2 macroglobulin and α-1 AGP (Kaneko, 1997; Gentry, 1999; Ceron *et al*., 2005; Car *et al*., 2006; Shao, 2006). Fibrinogen, previously the most widely used APP, is not a major responder in any species, including humans. Some proteins, such as AGP and ALB, function to bind drugs and they can have important pharmacokinetic implications because variations in the plasma levels of APPs during inflammation can alter the free plasma concentrations of drugs (Stonard and Evans, 1999; Ceron *et al*., 2005).

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4 LIPIDS

CHOL is an essential lipid found in all mammalian cells, and is a major component of the membrane. In addition to its structural role in cellular membranes, CHOL is a precursor molecule for sterol-based compounds, including bile acids, oxysterols, neurosteroids, glucocorticoids, mineralocorticoids and sex hormones. In toxicity studies, CHOL and triglyceride (TRIG) concentrations are the most frequently measured lipid compounds in the blood. Insoluble TRIG and CHOL are transported through the blood by lipoproteins. Lipoproteins consisting of variable quantities of TRIG, CHOL, CHOL esters and phospholipids are synthesized by the liver and small intestine and secreted into plasma. Lipoprotein lipase produced by several tissues, mainly gastric mucosal cells, pancreatic acinar cells, adipocytes, myocytes and hepatocytes, and the presence of appropriate cell receptors are required for clearance of lipids from blood. Chylomicron, very low-density lipoproteins (VLDLs), intermediate-density lipoproteins (IDLs), low-density lipoproteins (LDLs) and high-density lipoproteins (HDLs) are the main lipoprotein fractions. The HDL and LDL fractions are also classified as the good and bad CHOL carriers, respectively (Rifai and Warnick, 2006; Stockham and Scott, 2002; Evans and Duncan, 2003).

There are large species differences in lipoprotein profiles and the percentage of total CHOL carried by each lipoprotein class. Dog, rat and mouse are HDL-predominant species and are considered relatively resistant to atherosclerosis compared to LDL-predominant species, such as humans and nonhuman primates. Atherogenesis occurs in dogs only if CHOL is above 750 mg dl^{-1}. It is believed that HDLs exert a protective effect for the development of coronary artery disease because of their role in reverse cholesterol transport (RTC). RTC refers to the efflux of CHOL from peripheral tissues (including arteries) to HDLs for esterification and subsequent hepatic clearance. CHOL esters are retained by HDLs or transferred to VLDLs or LDLs for transport to the liver for disposal. This process is believed to be an efficient way of redistributing CHOL among lipoproteins for reuse by peripheral tissues or removal from the circulation through delivery to the liver and excretion in bile. In humans, two enzymes participate in the process (lecithin cholesterol acyltransferase (LCAT) and cholesteryl ester transfer protein (CETP)). It is of interest that CETP activity has been documented in nonhuman primates, but not in dogs or rats (Wagner *et al.*, 1999; Bauer, 2004; Movva and Rader, 2008).

In toxicity studies, increases or decreases in CHOL and TRIG concentrations are relatively frequent findings. The changes are usually small and are generally believed to represent minor alterations in lipid metabolism that do not adversely affect the health of the animals. Factors to consider include food consumption and assimilation, body weight and composition, liver function and hormone balance. TRIG concentration is elevated postprandially, while CHOL concentration is relatively stable. When fat is mobilized to meet energy requirements because of significant anorexia, starvation, malabsorption or maldigestion, TRIGs are usually increased, sometimes markedly; CHOL levels, however, are variable (Hall and Everds, 2008). Lipaemia, a term used for the milky appearance of serum/plasma caused by increased concentrations of TRIG-carrying lipoproteins, may interfere with laboratory assays (glucose, several electrolytes, haemoglobin, total bilirubin (TBILI)).

Conditions in veterinary medicine causing secondary hyperlipidaemia are relatively common. Increased CHOL concentrations reflect increased concentrations of the cholesterol-rich lipoproteins (e.g. LDL, HDL) and TRIG increases usually indicate chylomicronaemia and/or increased VLDL. In hypothyroidism, CHOL use decreases and CHOL synthesis increases. Hyperlipidaemia is variable, ranging from mildly increased CHOL and HDL concentrations to marked lipaemia with CHOL greater that 1000 mg dl^{-1}, hypertriglyceridaemia, and panhyperlipoproteinaemia. In diabetes mellitus, lack of insulin results in reduced lipoprotein lipase activity (insulin enhances lipoprotein lipase activity). Lipolysis is increased as lipids are used as an alternative source of energy. Marked hypertriglyceridaemia is associated with increased VLDL concentration; CHOL concentrations are mildly increased. Hyperchylomicronaemia may also be present. Liver disorders, including cholestasis, are frequently accompanied by alterations of serum and tissue lipids. In nephrotic syndrome, hypercholesterolaemia is secondary to enhanced synthesis of CHOL-containing lipoproteins. Lipoprotein synthesis presumably is stimulated by hypoalbuminaemia, and decreased plasma oncotic pressure. Sometimes TRIG concentration also is increased. Excess corticosteroid administration may result in hypertriglyceridaemia from increased lipolysis, insulin resistance and decreased lipoprotein lipase activity. Moderate hypercholesterolaemia, predominantly due to increased LDLs, may occur (Evans and Duncan, 2003). In the dog, TRIG concentration greater than 500 mg dl^{-1} usually mandates treatment, while TRIG concentration greater than 1000 mg dl^{-1} has been associated with pancreatitis, lipaemia retinalis, seizures, cutaneous xanthomas, peripheral nerve paralysis and behavioural changes (Elliott, 2005). In toxicity studies, intravenous infusion of lipid emulsion has been associated with significant neurologic toxicities in dogs; emesis/retching, somnolence and loss of consciousness (Miles *et al.*, 1991). In our experience, the severity of the clinical observations is dependent on the rate of the infusion and the composition of the lipid formulation.

CHOL, HDL, LDL and TRIG may be the pharmacologic targets of hypolipidaemic agents, leading to

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low blood concentrations at dose levels used in toxicity studies (Walsh, 1996; Von Keutz and Schluter, 1998). Some other compounds have been unexpectedly associated with markedly low CHOL in rats, with individual values less than 10 mg dl^{-1} to below the level of quantification (Everds, 2005; Wason *et al*., 2003; Abell and Mosbach, 1962; personal communication). The consequence of these low to very low CHOL concentrations is unclear in those instances, but teratogenicity, characteristically holoprosencephaly, was reported with compounds interfering with the final step of CHOL synthesis with secondary accumulation of 7-dehydrocholesterol (Roux *et al*., 1979; Kolf-Clauw *et al*., 1997; Dietschy and Turley, 2004). Drugs inhibiting 3-hydroxy-3-methylglutarate-coenzyme A (HMG-CoA) reductase (statins) in the early steps of the CHOL metabolic pathway have produced teratogenic effects, predominantly skeletal defects, while others with the same mechanism of action and at high maternal toxic exposures, did not produce teratogenicity (Dostal *et al*., 1994; Physicians' Desk Reference, 2008). Because animal studies have generated conflicting results and human data in pregnant women are limited, HMG-CoA reductase inhibitors currently on the market are contraindicated for pregnant women (Kazmin *et al*., 2007; Physicians' Desk Reference, 2008).

5 CARBOHYDRATES

The balance of glucose production and its uptake and utilization in peripheral tissues are regulated by a network of hormones, neural pathways and metabolic signals. Insulin plays a dominant and pivotal role in glucose production and utilization. In the fasting state, insulin is suppressed, allowing increased glyconeogenesis in the liver and the kidneys and enhancing glucose generation by the breakdown of liver glycogen. Low insulin levels also reduce glucose uptake and utilization in peripheral tissues and allow lipolysis and proteolysis to occur, which leads to the release of precursors for gluconeogenesis and provides alternative energy sources. Other hormones, such as glucagons, adrenaline (epinephrine), growth hormone and cortisol play less important roles in the control of glucose flux during normal physiologic circumstances. However, these hormones are critically important in response to hypoglycaemia where glucagon increases glucose concentration by glycogenolysis and gluconeogenesis while corticosteroids and catecholamines increase glucose concentrations via gluconeogenesis and glycogenolysis, respectively (Kaneko, 1997; Sacks, 2006).

In toxicity studies, the most frequent causes of increased glucose concentrations in the blood are failure to fast an animal and catecholamine release secondary to excitement, pain or fear. The fasting glucose of wild-caught or purpose-bred monkeys can be confused with diabetes mellitus (e.g. >150 mg dl^{-1}) when the animals have not yet been accustomed to their new environment. Moribund animals occasionally exhibit marked hyperglycaemia, probably as a result of both corticosteroid and catecholamine release (Hall and Everds, 2008). Animals infused intravenously with a glucose-containing formulation will have higher glucose concentration than pretest animals infused with saline; in this instance, the effect would be considered an artefact (Evans and Duncan, 2003). A large number of drugs may cause hyperglycaemia in normal individuals or impair metabolic control in diabetic patients. Potential hyperglycaemic drugs may have a direct effect on peripheral tissues that counter the actions of insulin or may inhibit insulin secretion directly or indirectly via depletion of K^+ (diuretics) (Davis, 2006). Corticoids, thiazide diuretics, megestrol acetate, ketamine and morphine are some of the drugs used in veterinary medicine that can be associated with increased glucose levels (Evans and Duncan, 2003).

In toxicity studies, decreases in glucose concentration are most commonly observed in animals that fail to thrive and gain body weight, with or without a concurrent decrease in food consumption. The differences between controls and test material-treated animals are considered secondary effects and likely not to affect the animals adversely (Hall and Everds, 2008). Hypoglycaemia has been associated with an excess of insulin or insulin-like factors, with decreased glucose production (hypoadrenocorticism, hypopituitarism, growth hormone deficiency, severe liver diseases, fasting, malnutrition, pregnancy) or with excess glucose consumption by tissues (sepsis, extreme exercise) (Hess, 2005).

Because severe hypoglycaemia can ultimately result in coma and death, animals must be carefully monitored when they are tested with rapid-acting insulin secretagogues and other hypoglycaemic drugs. Most clinical signs arise from the effect of hypoglycaemia on the CNS or from hypoglycaemia-induced release of catecholamines. The severity of the clinical signs (weakness, ataxia, disorientation, tremors, seizures, collapse) is proportional to the severity of the hypoglycaemia, its duration and the rate at which it develops (Hess, 2005). Clinical suspicion of hypoglycaemia should be associated with glucose levels of less than 60 mg dl^{-1} in the dog. Clinical signs of neuroglycopenia have been reported in monkeys at blood glucose concentrations of $\sim$40 mg dl^{-1} (2.2 mmol l^{-1}) or less (Chen *et al*., 1998).

6 ELECTROLYTES, FLUID BALANCE AND ACID–BASE

Electrolytes play integral roles in pH and fluid balance, electrical membrane potentials, muscular function, nerve

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conduction and enzymatic reactions. The efficacy of many drugs relies on their desired target effects on electrolytes, acid–base and fluid balance. However, the same electrolyte changes can be manifested as serious adverse effects in the case of toxicity. Endocrine and transmembrane regulatory mechanisms work to maintain serum electrolyte concentrations tightly controlled within a narrow range. The resultant narrow standard deviations may yield changes with statistical significance that are not dose-related and do not correlate with histopathologic findings (Evans, 1996). However, even minor changes in electrolyte concentration can have a devastating impact on cellular function, with nervous, muscular and renal tissue being most sensitive. Sodium, potassium and calcium abnormalities are commonly associated with cardiotoxicity and nephrotoxicity (DiBartola, 2006b). Detection and monitoring of electrolyte abnormalities is essential in preclinical safety assessment programmes.

The interpretation of electrolyte abnormalities requires an understanding of the distribution of body water and of homoeostatic mechanisms for osmotic and electro-neutrality. Osmotically active solutes (sodium (Na^+), chloride (Cl^-), potassium (K^+), glucose, bicarbonate (HCO_3^-) and urea) determine water distribution in body compartments. Of lean body mass, 60% is composed of water located in the intracellular fluid (ICF) or extracellular fluid (ECF). Regulated ECF–ICF shifts maintain electroneutrality and tonicity. Potassium, magnesium (Mg^{2+}), phosphate and proteins predominate in the ICF, while Na^+, calcium (Ca^{2+}), Cl^- and HCO_3^- are the major ECF solutes. These molecules carry a positive (cation) or negative (anion) charge, and though ECF and ICF solute composition is different, electroneutrality and osmotic equilibrium are maintained. Changes in electrolyte concentrations result from altered hydration and electrolyte intake, transmembrane shifts, altered renal handling or loss via alimentary tract, skin or airways. Massive cellular lysis, loss of plasma membrane integrity, severe acid–base disturbances and multiorgan malfunction, as seen in moribund animals or in shock, are the most common nonspecific causes of mixed electrolyte abnormalities (DiBartola, 2006b).

Sodium must be interpreted with consideration to hydration status, ECF volume and measured osmolality. The most common causes of abnormalities in Na^+ are dehydration (hypernatraemia) and dietary insufficiency (hyponatraemia). The adverse clinical consequences of hyper- and hyponatraemia depend largely on the rapidity of onset and associated osmolality. In dogs, neurologic signs (disorientation, ataxia, seizures and coma) manifest themselves when Na^+ values are <120 mEq l^{-1} or >170 mEq l^{-1}. Other clinical signs are associated with hypovolaemia (tachycardia, decreased capillary refill time) or hypervolaemia (pulmonary oedema) (DiBartola, 2006a). Though many drugs target renal handling of Na^+, alterations in serum Na^+ concentrations and adverse effects may not be observed until high doses are attained due to compensatory homoeostatic mechanisms. While Na^+ is the major extracellular cation, Cl^- is the major extracellular anion, and the two ions tend to follow one another. As such, any alteration in Cl^- must be interpreted in the context of sodium. Dehydration and water overload both result in proportional changes in Cl^- and Na^+. Nonproportional changes between Na^+ and Cl^- occur secondary to losses or gains of one ion but not the other, or reflect the role of Cl^-, but not Na^+, in renal acid–base homoeostasis (de Morais and Biondo, 2006). Clinical signs of hyperchloraemia are associated with hypernatraemia and hyperosmolality, while signs of hypochloraemia are associated with metabolic alkalosis (Willard *et al*., 1999; Galla *et al*., 1984).

Plasma K^+ concentration is primarily determined by dietary Na and K intake, renal excretion and acid–base status (Church, 2005). Any cause of cellular lysis may result in release of potassium from the ICF and result in hyperkalaemia. In dogs, hyperkalaemia becomes adverse at values ≥ 7.5 mEq l^{-1} due to altered cardiac muscle conductance. Hypokalaemia may occur secondary to decreased intake, gastrointestinal loss or renal loss due to tubular injury and tubular acidosis (DiBartola and de Morais, 2006). Pharmaceutical compounds such as aldosterone, aminoglycosides, diuretics, antineoplastic agents and β-adrenergic agonists cause hypokalaemia (Evans, 1996). Hypokalaemia becomes clinically adverse when concentrations fall below 3.0 mEq l^{-1}, due to severe muscle weakness and cardiac contraction abnormalities (Willard *et al*., 1999).

Calcium (Ca^{2+}), magnesium (Mg^{2+}) and inorganic phosphate (Pi) are ions with many important structural and functional roles whose plasma concentrations are regulated in concert by way of dietary intake, GI absorption, renal function, parathyroid hormone (PTH), parathyroid hormone-related protein (PTHrP), calcitriol (1,25-dihydroxyvitamin D_3) and calcitonin.

Ca^{2+} exists in three fractions: ionized (iCa^{2+}), complexed and protein bound. Ionized calcium is the physiologically active fraction and its concentration is tightly regulated within a narrow range by PTH and calcitriol. Total calcium (tCa) as measured by most automated methods does not always reflect iCa^{2+} due to variability of protein binding that changes with pH and protein concentration. Increases in TPROT cause increases in tCa (pseudohypercalcaemia) while decreases result in pseudohypocalcaemia. Even after correction for TPROT, tCa may not reflect abnormalities in iCa^{2+}. Therefore, tCa is used only as a screening tool. A test panel including calcitriol, PTH and iCa^{2+} is recommended for compounds that are suspected to interfere with calcium homoeostasis. True hypercalcaemia is an uncommon but important finding. Though hypercalcaemia is normal in growing animals, in adults excessive iCa^{2+} is toxic to cells and results in nervous, gastrointestinal, cardiac and renal dysfunction. Most

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often, adverse effects of hypercalcaemia (polydipsia/polyuria, anorexia, dehydration, lethargy, weakness and vomiting) are noted when tCa $\geq$15 mg dl^{-1}. However, tCa values of 12–14 mg dl^{-1} may also be adverse, depending on rapidity of onset and presence of acidosis or Na^+ and K^+ abnormalities. Dogs with tCa >19 mg dl^{-1} are severely ill and likely to develop soft tissue mineralization and concentrations >20 mg dl^{-1} are considered life threatening. Histopathologic changes correlating with clinical signs are more likely to be identified with persistent hypercalcaemia (Schenk *et al*., 2006). Causes of hypercalcaemia include hyperparathyroidism, hyperthyroidism, hypoadrenalism, poor venipuncture technique and exposure to drugs or toxins such as thiazides, lithium and calciferol (Evans, 1996).

Hypocalcaemia may occur with renal failure, fasting or inadequate intake of calcium or vitamin D, hypoparathyroidism, acute pancreatitis, hypercalcitoninism or drug/toxin exposure (diuretics, anticonvulsivants, fluoride, ethylene glycol) (Evans, 1996). The duration, magnitude and rate of decline determine severity of clinical signs. Hypokalaemia and alkalosis precipitate the onset of and magnify clinical signs. In dogs, concentrations <6.5 mg dl^{-1} cause hyperexcitability, disorientation, muscle tremors, pyrexia, cramping, stiff gait and seizures. Other signs include polyuria, polydipsia, hypercalciuria, anterior and posterior cataracts, tachycardia and increased QT intervals. Severe hypocalcaemia (<5.0 mg dl^{-1}) may cause death from cardiovascular failure or respiratory failure. A rapid decline to <4.0 mg/dl can cause left-sided myocardial failure and death (Schenk *et al*., 2006).

Phosphate is the major intracellular anion. Serum phosphate is primarily inorganic orthophosphate (Pi) and is protein bound, free anion, or complexed to Na^+, Mg^{2+} or Ca^{2+}. Concentrations are affected by diurnal cycle and pH. Hyperphosphataemia is normal in growing animals and may occur after a protein meal. Hyperphosphataemia results from increased GI uptake, cell lysis, tissue necrosis, acidosis, altered bone metabolism, decreased glomerular filtration rate (GFR) and hypoparathyroidism. In dogs, clinical signs of hyperphosphataemia (soft tissue mineralization) occur when the calcium phosphate solubility product ([Ca] × [Pi]) is greater than 60 (DiBartola and Willard, 2006). In rats, hyperphosphataemia ($\geq$13 mg dl^{-1}) causes myocardial hypertrophy, kidney dysfunction and osteoporosis-like lesions without soft tissue calcification (Neves *et al*., 2004). Hypophosphataemia results from diminished intestinal absorption, vomiting, transmembrane shifts due to dietary carbohydrates, insulin and iv dextrose, hyperventilation, hypothermia, tubular injury and hyperparathyroidism. Hypophosphataemia <1.5 mg dl^{-1} causes haemolysis, impaired leucocyte and platelet function, metabolic encephalopathy, decreased cardiac contractility and bone demineralization.

Magnesium is an important structural component of bone and muscle that predominates in the ICF and is found free, complexed or protein bound in blood. However, it is not well characterized or routinely assessed in preclinical studies. Hypomagnesaemia (<1 mg dl^{-1}) is more clinically significant than hypermagnesaemia, with important effects on cardiac and smooth muscle function. Studies in rats and dogs given magnesium-deficient diets showed poor growth rates, poor skin and hair, peripheral vasodilation, seizures and myocardial necrosis. Magnesium's function as a cofactor for adenosine triphosphatase (ATPase) pumps also results in potassium depletion accompanying magnesium deficiency (Bateman, 2006). In dogs, clinical signs associated with hypermagnesaemia (>10 mg dl^{-1}) include respiratory depression, apnoea, coma and cardiac arrest (Willard *et al*., 1999).

Blood gas analysis is required to evaluate acid–base balance, gas exchange and respiratory function. An evaluation of blood gas may be useful in some cases because many drugs, such as acetazolamide, ammonium chloride (NH_4Cl), calcium chloride ($CaCl_2$), antacids, gluconate and loop diuretics, affect acid–base balance and gas exchange. However, blood gases are not routinely measured in preclinical safety assessment because of extensive blood sampling and handling requirements. Instead, total carbon dioxide (tCO_2) is measured on automated chemistry analysers as an approximation for bicarbonate (HCO_3^-). Underfilled or uncapped tubes may have decreased tCO_2. Values <12 mEq l^{-1} indicate severe metabolic acidosis while values >30 mEq l indicate metabolic alkalosis. Adverse effects due to metabolic alkalosis are uncommon, and are generally a consequence of hypocalcaemia (decreased iCa^{2+}) (Fraser *et al*., 1991).

7 ENZYMOLOGY

Enzymes are powerful early indicators of drug-induced tissue damage and metabolic effects, including damage to the liver and bile duct (AST; ALT; ALP, alkaline phosphatase; LDH, lactate dehydrogenase; ID, iditol dehydrogenase; SDH, sorbitol dehydrogenase; GGT, γ-glutamyl transferase), cardiac and skeletal muscle (CK, creatine kinase; LDH; AST; ALT), bone (B-ALP, bone alkaline phosphatase), pancreas (amylase and lipase), intestine (amylase) and kidney (amylase; lipase; GGT; NAG, *N*-acetyl glucosaminidase; GST, glutathione-(S)-transferase), to name a few. As such, enzymology is indispensable to any safety assessment programme. Enzymes that are useful biomarkers of toxic injury originate from specific tissues and cells and their expression is usually confined to a specific cellular compartment (cytosolic, organelle, plasma membrane). Their activity in blood reflects changes in membrane integrity (leakage), metabolism or synthesis (induction), excretion/half-life, and/or inactivation (Stockham and Scott, 2002). The type and spectrum of change may

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reflect the mechanism of toxicity while its magnitude often correlates with severity (Thrall, 2006).

Enzymes are proteins that catalyse chemical reactions. Those that catalyse the same reaction but arise from different genes are termed isoenzymes, whereas those that differ due to post-translational modification of the same gene product are termed isoforms (Stockham and Scott, 2002). Isoenzymes and isoforms tend to be highly tissue-specific and have unique half-lives. Most automated clinical chemistry analysers quantify the enzymatic catalysis of a specific chemical reaction, but cannot distinguish isoenzymes and isoforms. Therefore, increases in blood enzyme activity as determined by automated methods is variably tissue-specific depending on the enzyme activity in question, species and isoenzyme/isoform half-life. Though not routinely assessed in preclinical safety assessments, isoenzyme/isoform activity should be pursued when thorough evaluation of effects on a specific target organ is required.

8 LIVER

Hepatotoxicity has been the most frequent single cause of safety-related drug marketing withdrawals for the past 50 years and is not uncommonly observed in experimental studies with other test materials, such as pesticides. Hepatotoxicity discovered after approval for marketing has also limited the use of many drugs (FDA, 2007). Liver toxicities appear to be the consequence of the unique blood vessel, secretory, synthetic and metabolic features of this organ. About 75% of hepatic blood comes directly from the GI tract and spleen via the portal vein bringing drugs and xenobiotics absorbed by the gut directly to the liver in concentrated form (Jaeschke *et al*., 2002). There are six basic mechanisms or sites of action for chemically induced liver injury (Lee, 2003). These include:

1. Disruption of calcium homoeostasis leading to cell surface blebbing and lysis
2. Cholestasis and biliary canalicular injury
3. Stimulation of autoimmunity
4. Stimulation of apoptosis
5. Mitochondrial injury
6. Metabolic bioactivation of chemicals via cytochrome P450 to reactive species.

It is important to keep in mind that many of these mechanisms can be in play at the same time in an affected liver. Furthermore, the active proliferative responses of hepatocytes to cellular damage and injury makes the liver an important target for carcinogens (Jaeschke *et al*., 2002).

Hepatotoxicity in standard preclinical studies may be identified from clinical chemistry, histopathology and ultrastructural pathology, bioaccumulation, expression of xenobiotic metabolizing enzymes, generation of reactive metabolites and immune-related hepatotoxicity (EMEA, 2008). ALT, AST and ALP activities and TBILI, direct (conjugated) bilirubin, TPROT and ALB concentrations are considered suitable and applicable parameters for the evaluation of potential hepatic injury in preclinical studies because they can be correlated with parameters recommended for identification of hepatic injury in humans (Boone *et al*., 2005). This list can be modified to include other parameters that are optimized to the species of interest or to specific patterns of hepatic injury (EMEA, 2008).

8.1 Hepatocellular Toxicity

ALT and AST are recommended parameters for the assessment of hepatocellular injury in rats, dogs and nonhuman primates. Of these, ALT activities is considered the more specific and sensitive test. When activity of both enzymes are increased by hepatic injury, the magnitude of the ALT increase is usually greater than AST, in part due to the longer half-life of ALT, but also because a substantial amount of AST is present in mitochondria, ranging approximately 30–40% in dogs and 81–85% in rats (Boone *et al*., 2005; Solter, 2005). Assessment of AST release from rat liver following ischaemia has shown that hepatocytes do not lose mitochondrial AST until almost all cytosolic AST has leaked and not until there is appreciable loss of cell integrity, such as necrosis. This implies that more severe forms of hepatic injury are necessary for the release of mitochondrial enzymes than cytosolic enzymes (Solter, 2005).

Hepatic causes of increased serum ALT, with or without increased AST, include hepatocellular necrosis, injury or regenerative/reparative activity (Boone *et al*., 2005). The mechanism proposed to explain the appearance of cytosolic enzymes in blood with reversible damage involves the formation of membrane blebs that detach and allow the cell membrane to reseal without cell death. Greater increases of serum enzyme activity likely reflect principally irreversible cell damage and necrosis, while mild increases may indicate mostly membrane blebbing and therefore, reversible cell damage (Solter, 2005; Stockham and Scott, 2002).

Increases (rat and dog) in ALT may occur in conjunction with hepatic microsomal enzyme induction. In general, they are not associated with evidence of histopathological hepatic injury and therefore are considered adaptive in nature (Amacher *et al*., 1998; 2001). Occasional decreases in ALT have been associated with hepatic microsomal enzyme induction and assay interference by the test material (ECETOC, 2002; Boone *et al*., 1998; Young, 2000). Interestingly, hepatocytes and biliary epithelium

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proliferation following the administration of epithelial growth factors have resulted in dose-related decreases in liver enzymes (ALT, AST and ALP) in the rat, but not in the monkey (Maraschin *et al*., 1995; Reindel *et al*., 2001). Reduced ALT/AST activities have a tendency to be dismissed even if they are identified as test material-related effects. The determination of whether a reduction in ALT/AST activities is adverse may become of greater importance when it is seen in the absence (at the same dose level) of any clear adverse findings when an overall NOAEL is being set for a study (PSD, 2007). Test material interference with the liver enzyme assays may reduce the sensitivity of ALT/AST activity to detect hepatocellular damage. Other potential causes of decreased ALT/AST activity may include decreased hepatocellular synthesis or release of the enzymes and inhibition or reduction of the enzyme activity (PSD, 2007; Hall and Everds, 2008).

ALT can also be affected by extrahepatic factors. Muscle injury can cause increases in the transaminases, but AST is generally higher than ALT when both are concurrently increased. In such cases, measurement of creatinine kinase activity, a specific indicator of muscle injury, may distinguish muscle injury from hepatic disease as the cause of increased enzyme activity (Bain, 2003). Procedure-related handling and type of restraint can cause increases in AST with or without increases in ALT in mice and nonhuman primates (Boone *et al*., 2005). Compounds resulting in hypotension or hypoxia (severe anaemia, congestive heart failure) with secondary hepatocellular damage may also be accompanied by liver enzyme increases (Webster, 2005). All data generated during toxicity studies (clinical signs, cardiovascular assessment, clinical pathology, histopathology) should be evaluated thoroughly to identify if the effect on liver enzymes is of hepatic or nonhepatic origin (skeletal muscle, heart, red blood cells (RBCs)).

Supplementary parameters of hepatocellular injury, such as activity of SDH, a cytosolic enzyme, and glutamate dehydrogenase (GLDH), mainly a mitochondrial enzyme, are useful when additional indicators of hepatic pathology are desired. Both parameters have been shown to be specific for liver injury (Boone *et al*., 2005). SDH has been considered the most sensitive and specific indicator of hepatocellular toxicity in rats and its interpretation is not confounded by compound interference with the assays as occurs with ALT/AST (Car *et al*., 2006). SDH is cleared fairly rapidly, yet remains in blood long enough to be of diagnostic value in acute hepatocellular injury (Hoffman *et al*., 1999). One disadvantage about the determination of SDH activity is that it requires rapid processing of samples from blood collection to chemistry analyser, unlike the more stable analytes, AST and ALT (Car *et al*., 2006). GLDH may become a more effective biomarker of acute hepatic injury than ALT, AST, SDH or ALP in the rat, based primarily on the large increase following hepatocellular injury, prolonged persistence in the blood following injury, high sensitivity for detection of injury, high tissue specificity and lower susceptibility to inhibition or induction. GLDH activity is higher in the centrilobular region of the liver than in the periportal zone. In contrast, AST is approximately homogeneously distributed in the liver lobules and ALT is mainly periportal (O'Brien *et al*., 2002). Recently, GLDH has been studied as a potential biomarker of liver injury in the dog (Jordan, 2007). Of note, neither GLDH or SDH are routinely utilized by physicians to evaluate human hepatic damage (Boone *et al*., 2005).

The magnitude of the increase in enzyme activities (ALT, AST, SDH, GLDH) depends on the number of hepatocytes affected, the severity of the injury, intracellular enzyme concentration, intracellular enzyme location and half-life of the enzyme (Bain, 2003; Stockham and Scott, 2002). If increases in ALT correlate with detrimental histologic changes it should be considered adverse. Additionally, concurrent increases in the activity of two or more enzymatic indicators of hepatic injury may potentially be considered adverse (Davies, 1992). Increase of ALT activity in the range of two- to fourfold and higher, compared to concurrent control mean or individual pretest values, without any other hepatic clinical chemistry abnormalities or in the absence of hepatic histological correlates, should raise concern as an indication of potential hepatic injury, unless a clear alternative explanation is present (Boone *et al*., 2005).

8.2 Hepatobiliary Toxicity

ALP and TBILI are recommended for identification of hepatobiliary injury, with GGT as a supplementary indicator (Boone *et al*., 2005). ALP and GGT are inducible enzymes, typically membrane-bound, and are not released into the blood with increased membrane permeability, while TBILI concentration is a measure of liver function (Bain, 2003). The mechanisms of drug-induced cholestasis are not clearly understood. A key component of bile secretion involves a series of ATP-dependent export pumps, such as the canalicular bile salt transport that moves bile salts and other bile constituents from the hepatocyte cytoplasm to the lumen of the canaliculus. Some drugs may bind to these canalicular transporter molecules and lead to the arrest of bile formation or movement within the lumen of the canalicular system. Secondary injury can result as bile salts have a detergent action that can damage cell membranes and injure biliary epithelium or hepatocytes in areas of cholestasis. Another possible mechanism leading to cholestasis involves disruption of actin filaments situated around the bile canaliculi preventing the normal pulsatile contractions that move bile through the canalicular system to the bile duct (Cullen, 2005).

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In laboratory animals, hepatobiliary pathology and bone growth/disease are the two most common causes of increased ALP activity. Increases in ALP due to hepatobiliary pathology generally precede increases in bilirubin. Increased (rat and dog) or decreased (rat) ALP activity from the liver in the absence of cholestasis has been reported with concurrent microsomal enzyme inductions (Boone *et al*., 2005). In rats, a transient postprandial increase in circulating ALP may occur due to presence of the intestinal ALP isoenzyme, while fasting results in a decrease in circulating ALP (Loeb, 1999b).

Although ALP elevation is a sensitive indicator of hepatobiliary disease, its low specificity for liver disease confuses interpretation. The low specificity of ALP activity stems from the fact that increased ALP activity may reflect physiologic or pathologic changes other than those from hepatic origin (Fernandez and Kidney, 2007). For example, nonhepatic increases in ALP activity are found in young animals, in pregnant and lactating females and in association with high fat diets. Bone disease, endocrine disease, neoplasia, illness or stress, drugs (glucocorticoids and anticonvulsants) and other disorders can result in increased ALP activity. In a group of dogs with histologically confirmed hepatic disease (Center *et al*., 1992), the highest ALP activity (~10-fold increase) was reported in animals with cholestasis, followed by chronic hepatitis, corticosteroid-induced hepatopathy and hepatic necrosis (approximately sixfold increase).

Bilirubin is produced as a breakdown product of haemoglobin. The unconjugated bilirubin is transported in the blood (ionically bound to ALB) to the liver. Unconjugated bilirubin is taken up by hepatocytes, conjugated and excreted into bile (Bain, 2003). Secretion of conjugated bilirubin into bile canaliculi is an energy-dependent process and is the rate-limiting step in bilirubin excretion in most species (Cullen, 2007). Increases in circulating bilirubin level are generally due to retention subsequent to an impairment of intra- or extrahepatic bile flow (cholestasis), overproduction associated with accelerated RBC destruction/haemolysis or altered bilirubin metabolism. The unconjugated fraction (indirect-reacting) generated from the degradation of RBC and conjugated fraction (direct-reacting) comprise the TBILI. In toxicity studies, bilirubin fractionation is generally only of value for exploring drug-related inhibition of the bilirubin conjugation enzyme, uridine diphosphate glucuronosyltransferase (UGT1A1). Concomitant bilirubinuria is supportive of an increase in the circulating conjugated bilirubin level, which is often due to cholestasis (Boone *et al*., 2005).

In the absence of haemolysis or changes in other hepatic parameters, increases in TBILI alone are unlikely to be adverse to the animal or to indicate potential hepatic injury in humans (Boone *et al*., 2005). Dogs with TBILI concentrations $\geq 0.5\,\text{mg dl}^{-1}$ would be considered hyperbilirubinemic; icterus may not be detected clinically until TBILI exceeds $2\,\text{mg dl}^{-1}$ (Cullen, 2007). Additionally, concurrent increases in ALT and TBILI should be critically evaluated given the potential for serious outcomes with this pattern of changes in humans (Boone *et al*., 2005; FDA, 2007).

GGT in all species is found in highest concentration in the kidney, pancreas and liver. GGT increases in serum/plasma as a result of hepatobiliary disease and in urine during early renal tubular toxicity (Hoffman *et al*., 1999). GGT, a canalicular enzyme, is increased in cholestatic liver disease secondary to impaired bile flow and/or biliary epithelial necrosis. An increase in circulating GGT without evidence of biliary pathology is associated with the use of some anticonvulsants in humans and dogs and an increase in circulating glucocorticoids in dogs and rats. In both dogs and rats, GGT is less sensitive, but more specific for cholestasis, compared to ALP (Boone *et al*., 2005). In healthy rats, GGT is often undetectable; however, cholestasis results in marked GGT elevation (Loeb, 1999). Concurrent increases in GGT and ALP are considered more indicative of a hepatic enzyme source, but do not exclude concurrent bone disease (Boone *et al*., 2005).

8.3 Supplementary Tests for Hepatic Synthetic Function

The liver is responsible for the synthesis and homoeostasis of a wide variety of blood constituents. Approximately 70% or more of the functional hepatocytes must be lost before alterations of hepatic function are detectable by biochemical testing. Therefore it should not be unexpected to detect changes in enzyme activity before hepatic failure is identified (Bain, 2003). Total protein, ALB, TRIG, CHOL, glucose, blood urea nitrogen (BUN), activated partial thromboplastin time and prothrombin time may be instrumental in the identification of deleterious effects on glucose metabolism and hepatic synthesis of proteins, lipids and coagulation factors (Boone *et al*., 2005).

Additional parameters such as ALP isoenzymes, 5′-nucleotidase, α-glutathione-(S)-transferase (α-GST), lactate dehydrogenase (LDH), total bile acids and several others are not routinely assessed in preclinical studies for a number of reasons, such as no added value, not widely used by physicians, less sensitivity and/or lacks hepatic specificity (Boone *et al*., 2005).

Overt hepatotoxicity due to drug administration is a real and current issue in drug development and regulatory circles (Senior, 2007; Hunt *et al*., 2007; FDA, 2007; EMEA, 2008). The standard preclinical experimental designs conducted in at least two species, one rodent and one nonrodent for at least two weeks of repeated

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dosing and using various multiples of the intended human therapeutic dose can predict subsequent adverse hepatic effects in the clinic over 50% of the time (Olson *et al*., 2000; Greaves *et al*., 2004). However, preclinical models are not infallible and are not always predictive. Whether the lack of predictivity is due to individual human genetic sensitivities, immunologically mediated phenomena, disease mediation or idiosyncratic reactions, the animal models are limited in detecting these characteristics and other low-incidence phenomena (Peters, 2005; Boelsterli and Lim, 2007; Kaplowitz, 2005). The draft EMEA (2008) guideline introduces a stepwise approach in an effort to identify hepatotoxicity signals. The first step is to optimize the interpretation of data generated during standard preclinical studies, followed by mechanistic studies to assess the clinical relevance of preclinical hepatotoxicity signals in terms of clinical settings.

9 KIDNEY

The kidney is a vital organ responsible for plasma filtration and maintenance of the body's internal biochemical environment. The kidney performs a wide variety of homoeostatic functions, including regulation of acid–base, electrolytes and water, metabolism and excretion of waste products and toxins, and endocrine regulation of minerals, blood pressure and tissue oxygenation capacity (Sands and Werander, 2005). The kidney's triple roles in plasma filtration, ultrafiltrate concentration and metabolism render it exceptionally sensitive to the toxic effects of xenobiotics. The kidney filters more than one quarter of cardiac output every day, exposing the glomerulus to high levels of drugs with even very short half-lives. Tubular epithelial cells metabolize drugs to generate products often with greater toxicity. In addition, tubular reabsorption concentrates toxins and metabolites in the ultrafiltrate. The 100- to 1000-fold increase in local drug exposure renders even low toxicity compounds quite damaging to the tubular epithelium (Evans, 1996; Sands and Werander, 2005).

Drug-induced nephrotoxicity accounts for approximately 35% of cases of renal failure in humans, and results in a high mortality rate (Zhang *et al*., 2005; Trof *et al*., 2006). In clinical drug development, the rate of project termination is highest for renal toxicity. Approximately 70% of urinary system toxicities result in compound termination, in contrast to 55% for hepatobiliary injury and function test abnormalities and 47% for cardiovascular (Olson *et al*., 2000). The pathogenesis of kidney injury involves a variety of mechanisms including vasoconstriction, altered glomerular haemodynamics, blood vessel injury, thrombotic microangiopathy, glomerular membrane damage, direct tubular toxicity, ischaemic tubular nephrosis, immune-mediated inflammation, drug crystallization/precipitation, osmotic nephrosis, retroperitoneal fibrosis and mineral deposition (Lash, 2005; Markowitz and Perazella, 2005; Evenepoel, 2004; Choudhury and Ahmed, 2006). The effects can be acute, beginning two weeks after drug exposure or sooner, or they may be chronic (Markowitz and Perazella, 2005). Though acute effects tend to be reversible, with function returning to baseline after the compound is withdrawn, chronic exposures can lead to irreversible damage (Zimmerman *et al*., 2007). Severe or prolonged damage leads to impaired renal function and eventual uraemia.

9.1 Serum Biochemistry/Renal Function

In preclinical toxicity studies, nephrotoxicity is routinely assessed by serum biochemistry, urinalysis and histopathology (Weingand, 1996; Greaves *et al*., 2004). Histopathology correlates best with human toxicity and is currently the gold standard for detection and monitoring of nephrotoxicity in animals (Olson *et al*., 2000). However, serum biochemistry and urinalysis provide *in vivo* assessments of kidney function and injury that can be correlated with clinical endpoints (Olson *et al*., 2000; Emeigh Hart, 2005). Traditional *in vivo* indicators of nephrotoxicity are of limited utility because they have low specificity and sensitivity for kidney injury and function. In contrast, the newer, nontraditional serum and urinary biomarkers offer promising alternatives for early detection of nephrotoxicity that correlates well with human toxicity and does not require invasive biopsies.

Changes in functional nephron (glomerulotubular unit) mass are most readily identified by estimating changes in GFR. BUN and creatinine are traditional indices of GFR in both humans and animals because they are freely filtered across the glomerulus and excreted in the urine with minimal secretion or reabsorption. Increases in BUN and creatinine above reference ranges (azotaemia) indicate that GFR is decreased, and form the basis for an investigation into renal drug effects.

Azotaemia can be classified as prerenal, renal or postrenal. Renal azotaemia occurs when loss of urine concentrating ability renders the urine isosthenuric (same concentration as glomerular filtrate: specific gravity (sp gr) = 1.008–1.012) (DiBartola, 2005). However, there can be significant renal disease without azotaemia. Because of compensatory hypertrophy and hyperfiltration mechanisms, damage to 90% of the original parenchyma has probably occurred before azotaemia is detected (Thrall, 2006). In rats, renal azotaemia correlates with microscopic lesions (vacuolar degeneration, acute tubular necrosis and inflammation) when BUN is $>20\,\text{mg dl}^{-1}$ and creatinine is $>1\,\text{mg dl}^{-1}$ (Sajedianfard *et al*., 2007). Increased inorganic phosphorus and potassium, decreased

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TPROT, ALB, sodium and chloride, and acidosis or alkalosis provide support for a diagnosis of renal azotaemia (DiBartola, 2005). In chronic studies, nonregenerative anaemia may occur, due to diminished renal production of erythropoietin or anaemia of chronic disease (DiBartola, 2005). Clinical signs of renal azotaemia (uraemia) are nonspecific (lethargy, dehydration, depression, reduced food consumption, vomiting, ulcerative stomatitis and weight loss) (DiBartola, 2005).

Postrenal azotaemia results from urinary tract obstruction. Although drugs that cause retriperitoneal fibrosis (e.g. methysergide, ergotamine, atenolol, hydralazine) or crystalluria/lithiasis (acylclovir, methotrexate, sulfanilamide) may result in obstructive nephropathy, postrenal azotaemia is relatively uncommon in toxicologic studies (Choudhury and Ahmed, 2006). Postrenal azotaemia is associated with oliguria or anuria, severe hyperkalaemia (increased potassium) and metabolic acidosis. Prolonged postrenal urinary tract obstructions (>48–72 hours) can result in acute intrinsic renal failure, cardiac conduction abnormalities and coma.

Prerenal azotaemia occurs secondary to increased production of BUN/creatinine or from decreased renal blood flow. Since renal function is intact, urine is concentrated (sp gr >1.030). Causes of prerenal azotaemia that are not drug-related include age-related decreases in renal blood flow, blood loss and dehydration. Sex, age, strain, body weight and group housing are additional prerenal variables that impact upon GFR (Vadiei *et al*., 1990; Hackbarth and Hackbarth, 1981; Hackbarth *et al*., 1981; Henegar *et al*., 2001). BUN increases with hepatic ureagenesis secondary to high-protein diets, GI bleeding, starvation, infection and fever. Conversely, decreased ureagenesis due to diminished hepatocellular function or anorexia may decrease BUN. In contrast to BUN, creatinine synthesis is relatively constant (proportional to muscle mass) and concentrations are not as dependent on extrarenal factors. Age, gender, diet and endogenous muscle catabolism during sepsis or cachexia may affect serum creatinine concentrations. Because meals cause a transient increase in GFR (Thrall, 2006), fasting status must be consistent across groups and collection intervals. Drug-related prerenal azotaemia is common and must be distinguished from drug-induced renal azotaemia (nephrotoxicity). Volume depletion secondary to diuresis is frequently observed with antihypertensives due to their diuretic or vasodilator effects. Vasoconstrictors (ciclosporin, osmotic contrast dye) also reduce GFR in a prerenal manner. Though these effects are reversible and not indicative of renal toxicity per se, prolonged exposure can lead to loss of renal concentrating ability and tubular necrosis, resulting in renal azotaemia (Choudhury and Ahmed, 2006). In chronic studies, normal age-related deterioration of kidney function magnifies drug-induced nephrotoxicity (Espandiari *et al*., 2007).

BUN and creatinine are reabsorbed and secreted, respectively, by renal tubules resulting in under- and overestimation of GFR (Thrall, 2006; Darling and Morris, 1991; Emeigh Hart, 2005; Dieterle *et al*., 2008). Alternative blood markers that do not undergo tubular handling are currently actively sought and developed. Endogenous small molecules and low molecular weight (MW) proteins such as cystatin C and 2-(α-mannopyranosyl)-L-tryptophan (2-MPT) are emerging as sensitive indices of GFR (Emeigh Hart, 2005). Cystatin C is not affected by diet, age, muscle mass or hydration status. In rats, it is freely filtered in the glomerulus and is 99.5% reabsorbed and degraded by the tubules (Tenstad *et al*., 1996).

9.2 Urinalysis

Urine is an easily obtainable and valuable analytical substrate for the evaluation of nephrotoxicity. Urinalysis must take into consideration the urine collection method, hydration status, water intake, diet and presence of nonrenal disorders that alter renal fluid or electrolyte handling (Emeigh Hart, 2005; DiBartola, 2005). In large studies, overnight (16 hours) collections of freely voided urine are routine. Pans or trays placed beneath cages may be contaminated with drinking water, food and faeces. Though metabolic cages can be carefully designed to prevent contamination, in many cases, contamination is inevitable. This may significantly complicate interpretation of parameters, including water intake, urinary volume, concentration, pH, urobilinogen, bilirubin, protein and sediment examination. Timed urine sampling (24 hour) provides a more quantitative assessment of glomerulotubular function that is not as influenced by diurnal fluctuations in urine volume (common in rodents) and is more likely to detect early release markers. Bladders must be completely emptied at the beginning of a specified period after dosing and prior to urine collection. Water evaporation, solute decomposition and bacterial growth can be minimized by collecting samples into cooled containers (Emeigh Hart, 2005). Cystocentesis is a time-consuming urine collection method that is useful when contamination must be avoided, but is only amenable to small studies and does not provide timed urine volume measures.

Routine urinalysis for toxicologic studies in laboratory animals includes visual assessment, volume, concentration, semiquantitative biochemical analysis with reagent strips and microscopic sediment examination. Interpreted together, azotaemia, urine volume and urine concentration are important indicators of kidney function. Loss of urine concentration capacity is an earlier indicator of renal injury than azotaemia, occurring after loss of 66% of the functional renal parenchyma, in contrast to 75–90% for azotaemia (Thrall, 2006). Azotaemia

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with dilute urine (sp gr <1.012), and decreased urine volume (oliguria or anuria) indicates decreased functional nephron mass. Dehydration with dilute urine strongly suggests tubular dysfunction (DiBartola, 2005). Azotaemia with large volumes of dilute urine indicates loss of urine concentrating capacity. Interpretation of urine volume and concentration must take into account other factors that impact free water balance. Drugs that undergo renal excretion may cause osmotic diuresis. Psychogenic polydipsia (central diabetes insipidus) is to be suspected in nonhuman primates with abundant, dilute urine (Rosenberg and Loomis, 1980; Fanton *et al*., 1987). In rodents, water consumption varies with strain, body weight and kidney weight (Hackbarth and Hackbarth, 1982) and diminishes with decreased food consumption. Decreases in water consumption may cause dehydration, prerenal azotaemia and decreased urine volume, but urine will still be concentrated (sp gr >1.035) (Griffiths *et al*., 2007).

Reagent test strips designed for human use are commonly used to semiquantitatively screen for pH, protein, glucose, ketones, bilirubin and occult blood in animals. Biochemical quantification of protein and glucose confirms reagent strip findings and detects more subtle changes (Espandiari *et al*., 2007). Other tests available on reagent strips, including specific gravity, leucocyte esterase and nitrites, are not useful in animals (Paquignon *et al*., 1993). Urinary pH is highly variable and dependent on dietary factors (Bertani *et al*., 1989), bacterial metabolism and evaporative loss of CO_2. Urinary pH does not substitute for blood pH determination because of renal handling of bicarbonate and hydrogen ions. Proteinuria is observed with haemorrhage, inflammation, renal tubular degeneration or glomerulopathy. If renal tubular degeneration is suspected as the cause of proteinuria, quantitative biochemical assessments of urinary proteins is indicated (see below). Alkaline urine may cause false positives for protein (Reagan *et al*., 2007). Proteinuria is normal in male rats due to androgen-dependent rat urinary protein α-2 μ-globulin. Furthermore, ageing male rats with chronic renal disease often have marked proteinuria (Bertani *et al*., 1989; Quimby, 1999). Proteinuria is also a normal finding in mice due to the presence of uromucoid, GLOBs, and major urinary protein (MUP) (Quimby, 1999). Spontaneous glomerular amyloidosis should be suspected in proteinuric rabbits (Hinton, 1981). Detection of urinary glucose by the reagent strip method is an abnormal finding that should prompt further investigation into hyperglycaemia or loss of tubular function. In most species, the proximal tubules reabsorb over 99% of filtered glucose (except in the gerbil in which glucosuria is normal) (McClure, 1999). Unless glucose reabsorption mechanisms are saturated (hyperglycaemia), glucosuria indicates proximal tubular dysfunction and occurs even prior to histological evidence of injury (Finco, 1997). Bilirubinuria occurs with disorders that cause hyperbilirubinaemia, while urobilinogenuria is not informative in animals. Many drugs cause false ketonuria (Willard *et al*., 1999). Other causes of ketonuria include excessive lipid catabolism due to anorexia and diabetes mellitus. Occult blood in urine may result from haematuria or haemoglobinuria, or may represent blood contamination due to external trauma.

Identification of particulate urine components such as drug crystals, erythrocytes, leucocytes, renal epithelial cells, casts or lipid droplets requires microscopic sediment examination. Normal urinary crystals must be morphologically differentiated from those associated with liver disease or with drug toxicity. The presence of intact RBCs distinguishes haematuria from haemoglobinuria. Large numbers of hyaline casts suggest proteinuria and glomerular damage. Renal tubular epithelial cells and granular or waxy casts composed of these cells are strong indicators of tubular degeneration (da Silva Melo *et al*., 2006). High numbers of leucocytes are indicators of inflammation in the urinary tract, while casts containing leucocytes confirm their renal origin. Finally, large quantities of lipid droplets can be seen in urine sediments after chronic glomerular damage in humans and rodents (Emeigh Hart, 2005).

9.3 Nonroutine Tests of Renal Injury and Function

Serum (BUN/creatinine) and urine analysis are the current standards for the identification and monitoring of nephrotoxicity. Though they are useful screening tools in both humans and animals, the many challenges associated with their interpretation limit their utility for early, sensitive and predictive assessments of drug-related kidney toxicity. Attention has focussed on quantitative biochemistry and molecular approaches that may better detect early kidney toxicity. Though not routinely performed, quantitative biochemical urinalyses can be useful for focussed evaluations to detect subtle renal injuries or functional deficits. This approach is particularly amenable to well-controlled safety pharmacology or investigative toxicity studies for which compounds are suspected to influence renal function. Quantitative urine biochemistry measurements can be used to specifically evaluate the functional nephron mass (clearance tests), glomerular injury/function (high MW proteins), tubular function (glucose, low MW proteins, pH, concentration, fractional excretion (FE) of electrolytes) and tubular injury (tubular enzymes and proteins) (Emeigh Hart, 2005).

Timed renal clearance studies are used to quantify GFR. Freely filtered endogenous molecules such as creatinine or cystatin C are most commonly measured to evaluate functional nephron mass by clearance tests. Exogenous molecules, including radiolabelled compounds,

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inulin and sulfanilate are less practical, but more accurate alternatives. However, even clearance tests lack the sensitivity to adequately monitor renal function because of abundant functional reserve. In dogs, a GFR <3.5 ml min kg^{-1} is associated with nephrotoxicity (Narita *et al*., 2007). In humans, significant renal haemodynamic toxicity has been defined as a $>20\%$ fall in GFR (Edwards *et al*., 1994).

The renal tubules are responsible for electrolyte homoeostasis as well as maintaining the renal medullary concentration gradient that determines urine concentration capacity. Alterations in urinary electrolytes relative to plasma reflect changes in tubular reabsorption and excretion. FE should be calculated when there are unexplained blood electrolyte abnormalities (Emeigh Hart, 2005). Accurate calculation of FE requires timed urine collection, though it can also be estimated by normalizing for creatinine (Stockham and Scott, 2002). Values depend on requirements for homoeostasis, and vary according to species, strain, nutrition, circadian and seasonal rhythm and body weight (Finco, 1997; Lefebvre *et al*., 2008; Hackbarth and Hackbarth, 1982). When dietary factors are controlled and there is no nonrenal electrolyte loss, increased electrolyte FEs are early indicators of tubular dysfunction and reduction in GFR (Emeigh Hart, 2005). In dogs, FE of sodium increases rapidly (hours) after an acute renal toxic insult, long before the onset of azotaemia (days) (Thrall, 2006). In rats, metabolic acidosis complicates interpretation of FE because it has multiple opposing effects on renal handling of sodium (Faroqui *et al*., 2006). Extrarenal causes of NaCl depletion result in loss of renal medullary concentration gradient, while potassium depletion causes collecting tubules to be refractory to antidiuretic hormone (ADH), both diminishing renal concentrating ability.

The glomerulus is a filtration barrier that limits the size and charge of ultrafiltrate solutes, with ALB and larger molecules being mostly restricted from passage (Lash, 2005). The little ALB that passes is reabsorbed by the tubules such that urinary ALB is negligible. Mild glomerular or tubular damage results in low-level albuminuria (microalbuminuria). Glomerular damage can be distinguished from tubular damage by the presence of protein markers of low (tubular injury) or high (glomerular injury) MW (Emeigh Hart, 2005). Low MW rat protein markers that are translatable to humans include cystatin C and retinol binding protein (RBP) (Emeigh Hart, 2005). Substantial glomerular damage is associated with high-level proteinuria of large proteins such as ALB. Timed urinary protein excretion measures are best and should be normalized to body weight. Normal values in dogs range from 5.1 to 22.4 mg kg^{-1}. When timed sampling is not possible, protein can be normalized for creatinine excretion by calculating the urine protein to creatinine ratio (U(P:C)). In dogs, U(P:C) values less than 1.0 are normal, while values greater than 2.0 are abnormal (Thrall, 2006). Additional potentially useful urinary protein biomarkers of tubular injury include fibronectin, collagen IV, α-GST, pap 5C10 antigen (Pap A1), clusterin, kidney injury molecule-1 (KIM-1), liver fatty acid binding protein (L-FABP) and cysteine-rich protein 61 (CYR 61) (Emeigh Hart, 2005). At present, α-GST, clusterin and KIM-1 demonstrate the most promise as early markers of tubular injury for preclinical drug-safety studies (Zhou *et al*., 2008; Yang *et al*., 2007; Espandiari *et al*., 2007; Vaidya *et al*., 2006). Clusterin is a proximal tubular glycoprotein whose secretion is induced rapidly after tubular injury and sustained during chronic injury. Rat urinary clusterin levels correlate with severity of damage (Hidaka *et al*., 2002). Antibodies for α-GST and clusterin are commercially available and these cross-react with both human and rat clusterin (Emeigh Hart, 2005).

Enzymes or protein biomarkers that are selective for tubule segment and subcellular location can be used to localize and estimate the severity of the tubular injury. These biomarkers are not present in blood or filtered by the glomerulus. As such, their presence in urine is highly specific and sensitive for renal tubular injury. The proximal convoluted tubule (PCT) is highly susceptible to toxic injury because it is the first site of ultrafiltrate concentration. It also metabolizes relatively harmless compounds into more reactive metabolites. Enzymes produced by the PCT include GGT, NAG, lysozyme, β-galactosidase, β-glucuronidase, β-glucosidase, alanine aminopeptidase (AAP) and intestinal alkaline phosphatase (IALP). The enzyme μ-GST is considered to be specific for distal tubules while renal papillary antigen (RPA) has been shown to be specific for papillary collecting ducts.

The most common enzyme measurements used in animals are GGT and NAG (Yang *et al*., 2007; Narita *et al*., 2007). Urinary GGT and NAG levels increase prior to azotaemia (Thrall, 2006). They are markedly increased in dogs with acute renal disease, whereas only slight increases are detected with chronic disease (Loeb, 1999a). Urinary NAG activity in male dogs is double that of female dogs (Nakamura *et al*., 1983). In the rat, urinary enzyme excretion followed a weekly biorhythm, emphasizing the importance of contemporaneous controls (Loeb, 1999b). After an acute toxic insult, rat urinary markers peak at 6 hours and return to normal levels within 48 hours (da Silva Melo *et al*., 2006; Yang *et al*., 2007). In one rat study, GGT, α-GST and μ-GST were more sensitive for low-level toxic insults than NAG (Yang *et al*., 2007). Enzymes used in humans, but with variable utility in animals, include lysozyme, β-galactosidase, β-glucuronidase, β-glucosidase, AAP and IALP (da Silva Melo *et al*., 2006; Emeigh Hart, 2005).

Traditional indices of renal injury and function such as BUN and creatinine are influenced by numerous nonrenal confounding factors and are late indicators of

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nephrotoxicity. Quantitative urinalyses and functional tests are not amenable to large screens and are fraught with sources of variability. These factors make interpretation of drug-related changes difficult and complicate decision-making in drug development. In preclinical safety assessment, there is a dire need for early, sensitive and predictive *in vivo* biomarkers of nephrotoxicity that are translatable to humans (Greaves *et al*., 2004). Molecules such as cystatin C, clusterin and KIM-1 are highly promising and are currently being developed and validated for use in animals. The utility of urinary markers as indicators of nephrotoxicity is greatly dependent on the agent, dose, species, sex and study design. Improvements in diagnostic sensitivity and specificity are likely to be based on panels comprised of multiple markers rather that single tests (Nahas *et al*., 1997). The growing genomics and proteomics arenas are becoming powerful tools for identification of new markers and promising platforms for drug-induced nephrotoxicity screening panels (Dieterle *et al*., 2008; Bandara *et al*., 2003; Schnackenberg *et al*., 2007).

10 CARDIAC MUSCLE

Cardiotoxicity is a significant cause of failure of compounds in clinical and preclinical development. Of the market withdrawals, 90% were caused by toxicity and of those, two out of three were either associated with hepatotoxicity or cardiotoxicity (Schuster *et al*., 2005). From 1960 to 1999, cardiovascular toxicity accounted for 9% of withdrawals of prescription drugs from worldwide markets (Fung *et al*., 2001) The predictivity of animal models for cardiotoxicity in humans is quite high (approximately 80%) particularly in nonrodents (Olson *et al*., 2000). Other xenobiotics, such as solvents, halogenated hydrocarbons and heavy metals are known to be cardiotoxic. There are several basic mechanisms of toxicity: (i) disturbance of ion homoeostasis (ii) inhibition of Na/K ATPase (iii) altered coronary blood flow (iv) oxidative stress (v) organellar dysfunction and (iv) apoptosis or oncosis. It is likely that no one mechanism of toxicity operates alone and more than one mechanism combines to elicit toxicity. Functional effects on the heart, for example arrhythmia, are often completely dissociated from degenerative structural effects, for example, myocardial necrosis. Purely functional effects do not result in the release of cellular constituents into plasma and are therefore not detected by clinical chemistry. The mainstay of detecting and evaluating cardiotoxicity remain the electrocardiogram and histopathology. However, clinical chemistry parameters have been of value in detecting and evaluating degenerative structural cardiotoxicity. Very few parameters are specific for cardiotoxicity and care is required in interpreting data. In addition, toxicity to the heart may be secondary to toxicity in other organs, for example kidney, erythrocytes.

CK and LDH were the standard traditional parameters used to identify cardiotoxicity. Increases in these enzymes, however, do not specify cardiac damage since their distribution is widespread in other tissues. CK has three isoenzymes which differ in their distribution CK-MM (muscle), CK-BB (brain) and CK-MB (cardiac muscle). Generally total CK is measured in toxicology studies and so interpretation of changes in this enzyme alone should be made with caution. In addition, the half-life of the CK enzyme is quite short and certainly less than one day (Lindena *et al*., 1986); therefore acute damage is not likely to be detected by CK at the end of a 28 day toxicity study, but may be of value where active damage is occurring. CK values are notably variable between individuals and are sometimes elevated as a result of the dose route (e.g. intramuscular), particularly since most CK is muscular in origin. Therefore the value of CK as an indicator of cardiotoxicity is limited. Similarly LDH distribution is widespread and there are multiple isoforms of LDH which differ with species. Handling of animals may also result in mild muscle trauma, which can result in release into plasma (O'Brien, 2008).

Troponins (Tns) are the three protein filament components of cardiac and skeletal muscles. TnI in the absence of Ca^{2+} binds to actin and inhibits actinomysin ATPase-induced contraction. TnT links the troponin complex to tropomyosin. TnI and TnT each have two different isoforms, each specific for cardiac and skeletal muscle. Thus the tissue origin of each can be identified. The two isoforms of TnC, which bind calcium do not discriminate between cardiac and skeletal muscle. Therefore only TnI or TnT are specific to the heart. Cardiac troponins have been the preferred biomarker for clinical myocardial infarction because of their high specificity and sensitivity (Babuin and Jaffe, 2005) and practical detection period postdamage (O'Brien *et al*., 2006). Its use as a biomarker of cardiotoxicity in laboratory animal models particularly for pharmaceuticals is growing and now becoming routine in pharmaceutical development for the major nonclinical species, although interlaboratory validation of the assays in order to ensure sensitivity, precision and accuracy is still active since there are a variety of commercially available assays and variable results have been obtained (ILSI HESI Technical Committee, 2008). O'Brien *et al*., (2006) note that there are several caveats to the effective use of cardiac troponins as markers of damage, including a background of high TnI as a result of stress, restraint, exertion, spontaneous cardiomyopathy in Sprague–Dawley rats, blood collection by cardiac puncture, and so on. However, relative to other biomarkers currently available it is considered as the 'gold standard' for myocardial injury associated with cardiotoxicity (O'Brien, 2008). For reviews of preclinical applications see Wallace *et al*. (2004),

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Adamcova *et al*. (2005) and Walker *et al*. (2006). The question whether to use TnT or TnI is best answered by the fact that there are currently multiple commercial suppliers of assay kits for TnI and only one for TnT.

Another potential marker of degenerative structural myocardial damage is fatty acid binding protein 3 (FABP3). This cytosolic protein involved in intracellular transport of long chain fatty acids is readily released from damaged cells and is thought to be highly sensitive as a marker of myocardial injury, perhaps more sensitive than troponins; however, its ready release means that FABP3 may be elevated in serum when physiological stress, for example, exercise or handling restraint trauma occurs. Somewhat like LDH and CK, FABP3 is also distributed in other tissues (e.g. muscle, brain and kidney). Therefore the cardiac origin of elevated serum FABP3 cannot be absolutely identified where FABP3 alone is the biomarker (Zhen *et al*., 2007). The analytical techniques used to detect FABP3 are not yet open to routine analysis in a clinical pathology laboratory.

11 SKELETAL MUSCLE

Increases in serum LDH and CK occur with degenerative or necrotic muscle injury. Most serum CK is muscular in origin. It is thought that CK is the more sensitive indicator of muscle injury, with peak activity reached in approximately 16–24 hours. The main isoenzyme found in skeletal muscle is MM. AST is often used alongside LDH and CK to investigate muscular injury. However, AST is fairly ubiquitous and without differential isoenzyme investigation of LDH and CK the origin of the increased serum enzymes is often uncertain. Variability associated with physiological and mechanical stress due to handling or exercise adds another layer of difficulty in interpretation. In addition, animals which are given intramuscular or other parenteral injections may have skeletal muscle damage resulting in elevated serum CK and LDH. Therefore good dosing and sampling techniques are vital to obtaining interpretable data. Monitoring of serum electrolytes would be useful in helping to identify myotoxicity since intracellular calcium, potassium and sodium concentrations are all vital in the functioning muscle fibre.

12 BONE

Many of today's pharmaceuticals directly target bone diseases (e.g. hormone replacement drugs, oestrogen receptor modulators, bisphosphonates and etidronate to treat osteoporosis, hyperparathyroidism, rickets/osteomalacia, Paget's disease of bone) or diseases characterized by secondary bone resorption (rheumatoid arthritis, diabetes mellitus, nephrotic syndrome, ethanol toxicity, oestrogen deficiency, acromegaly, hyperthyroidism). Conversely, many drugs induce toxicities that directly or indirectly affect bone metabolism (e.g. corticosteroids, anticonvulsivants, thiazide diuretics). Most common are indirect effects resulting from a wide variety of disorders including renal disease, disruption of hormonal or metabolic states, altered calcium/phosphorus/zinc homoeostasis, oxidative stress, inflammation, immobility and microvascular disease. Drug toxicity to bone is not routinely assessed in preclinical studies, though nonspecific indicators such as ALP, calcium and phosphorus, may raise suspicion for drug effects on bone (Woodard *et al*., 2002). In these cases, or when the drug has expected direct or indirect effects on bone, monitoring of bone toxicity is indicated.

Bone toxicity is most often assessed by quantifying changes in bone turnover rates (formation and resorption). Histopathology and histomorphometry are the gold standard to assess bone toxicity in terminal studies, while bone mineral density (BMD) is the gold standard *in vivo*. The drawback of BMD is that it can only detect significant changes after chronic exposures (more than six months) (Kleerekoper, 2001). Newer biochemical markers of bone turnover have the potential to detect *in vivo* changes earlier (within a few weeks, to three to six months) and with greater sensitivity. A detailed explanation of each marker is beyond the scope of this chapter (See reviews: Kleerekoper, 2001; Pagani *et al*., 2005; Delmas *et al*., 2000; Delmas, 2001). In general, markers should be selected such that results reflect both formation and resorption. Animal bone biomarkers are usually quantified by immunoassay using commercially available kits (RatLaps ELISA (Nordic Bioscience Diagnostics A/S, Denmark), Rat-MID osteocalcin ELISA (Nordic Bioscience Diagnostics A/S, Denmark), BTI Rat Osteocalcin EIA kit (Biomedical Technologies, MA), antirat TRACP-5b ELISA (IDS, AZ) and antimouse RANKL (R&D Systems) (Rachon *et al*., 2007; Brzoska *et al*., 2007; Stolina *et al*., 2005).

12.1 Sources of Variation

Because many markers are present in tissues other than bone, and most are influenced by nonskeletal processes, it should be borne in mind that changes are not tissue specific. In addition, changes are not disease specific, but reflect alterations in skeletal metabolism independent of cause. Renal clearance of biomarkers increases with decreasing renal function. Bone formation decreases with age, and is higher in males. Factors such as fracture repair, pregnancy/lactation and immobility all significantly impact results. Finally, blood levels vary on a circadian rhythm that can be modified by drugs (Shao *et al*., 2003; To *et al*., 2003).

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12.2 Measures of Bone Formation

In new bone formation, osteoblasts develop new bone matrix, including osteocalcin (OCN) and osteonectin (OST), and secrete bone-specific alkaline phosphatase (BALP) to stimulate bone mineralization (Lee *et al*., 2008). Commonly used biomarkers of osteoblastic synthetic activity include OCN (bone gla-protein (BGP), OST), type I collagen propeptides and bone ALP (Pagani *et al*., 2005). OCN is the most abundant noncollagenous protein of bone (Lee *et al*., 2008). It is a small calcium-binding protein that leaks into blood during bone formation by osteoblasts, odontoblasts and hypertrophic chondrocytes. It is specific for bone formation because it does not enter the blood when released from bone matrix during resorption (Woodard *et al*., 2002; Delmas *et al*., 2000). OCN is rapidly degraded in serum to release intact peptide and various fragments that can all be quantified: undercarboxylated osteocalcin (ucOC), total OC (intact + N-mid fragment), intact OC (OC [1–49]) and N-mid fragment of OC (OC[1–43]). Measuring both intact and OCN fragments increases sensitivity. Urinary OCN quantification has also been described in humans (Ivaska *et al*., 2005). Increases in OCN may be seen in growing animals (Lee *et al*., 2008) or with conditions that cause increased bone turnover.

New bone formation leads to the production of collagen cleavage products and other bone matrix proteins that are elaborated during osteoblast differentiation, such as type I procollagen propeptides (C- and N-terminal procollagen I carboxyterminal propeptide (PICP) and procollagen type I N propeptide (PINP)) (Woodard *et al*., 2002; Delmas, 2001). Intact trimer of PINP is the most sensitive marker of bone formation in humans (Delmas *et al*., 2000).

In domestic animals, ALP is comprised of two isoenzymes: (i) I-ALP (intestinal) and (ii) tissue nonspecific ALP. Isoforms of tissue nonspecific ALP include L-ALP (hepatocytes and biliary epithelium) and B-ALP (osteoblasts). In addition, canine hepatocytes exposed to glucocorticoids express C-ALP (Stockham and Scott, 2002). Increased ALP activity identified in routine biochemistry panels represents total ALP activity, with variable relative contributions from the different isoforms. B-ALP may be responsible for increases in total ALP during new bone formation. Specialized assays such as heat denaturation, electrophoresis, precipitation, selective inhibition and immunoassays are required to distinguish B-ALP from other ALP isoforms, but are not well suited to regulatory environments.

12.3 Measures of Bone Resorption

During bone resorption, activated osteoclasts mediate the hydrolytic degradation of bone, releasing its structural constituents (calcium, phosphorus and collagen and noncollagen proteins) into blood and urine. (Brzoska *et al*., 2007; Lee *et al*., 2008). Simultaneous increases in both calcium and phosphorus are strongly suggestive of bone resorption, though other causes, such as renal failure and hypervitaminosis D must be ruled out. Over 90% of organic bone is type I collagen, which is released as a series of peptides that can be measured in serum and urine (Rachon *et al*., 2007). Collagen proteins that can be used as markers of bone resorption include hydroxyproline (HYP), hydroxypyridium crosslinks of collagen (pyridinolone (PYD) and deoxypyridinolone (DPD)) and type I collagen telopeptide crosslaps (N-terminal crosslinking telopeptides of type I (NTX-I) collagen and C-terminal crosslinking telopeptides of type I (CTX-I) collagen, C-terminal telopeptide of Type I Collagen (ICTP) (Delmas *et al*., 2000; Do *et al*., 2006). The majority of these are measured in urine. HYP is not recommended because it is nonspecific and affected by diet (Woodard *et al*., 2002; Delmas *et al*., 2000). DPD is formed during extracellular maturation of fibrillar collagens and released upon the degradation of mature collagens. It is more specific for bone collagen that PYD (Woodard *et al*., 2002).

Noncollagen proteins released from bone during resorption include bone sialoprotein (BSP) and tartrate-resistant acid phosphatase (TRAP isoform 5b). BSP accounts for 5–10% of noncollagenous matrix of bone and is the major synthetic protein product of osteoblasts and odontoblasts. TRAP-5b reflects osteoclastic activity as well as activity from prostate, blood cells and pancreas (Woodard *et al*., 2002). A newer biomarker of bone resorption is RANKL (receptor activator for nuclear factor κ B ligand). RANKL mediates osteoclast activation and is a useful indicator of osteoclastic activity. It is one of the earliest indicators of bone erosion and systemic bone loss (Stolina *et al*., 2005).

13 BLOOD VESSEL

Blood Vessel injury induced by drugs in preclinical studies is of concern. Dogs appear to be particularly sensitive to arterial injury and have a pattern of spontaneous arteriopathy (Stephan-Gueldner and Inomata, 2000), and as such are thought to be problematic models of human susceptibility. There are no established specific clinical pathology markers for blood vessel disease. Blood Vessel changes were traditionally predicted by blood pressure collapse associated with reflex tachycardia. Candidate molecules which have a mechanistic relevance to blood vessel damage originating from endothelial cells and/or smooth muscle cells are being evaluated and include von Willebrand factor, von Willebrand factor propeptide and caveolin-1 (Brott *et al*., 2007).

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14 ENDOCRINE SYSTEM

The endocrine system is instrumental in regulating metabolism, growth, development and puberty, and tissue function. This is achieved by releasing hormones into blood and ECF which act as chemical messengers and modulate the function of organs and tissues. The endocrine glands include the hypothalamus, pituitary (anterior and posterior), thyroid, parathyroid, pancreas, adrenals, ovaries, testes and pineal gland. Other organs, such as the kidney, GI tract, liver and heart, also have endocrine functions. Toxicity associated with the endocrine glands is usually confined to the adrenals, testes, ovaries and thyroid (Ribelin, 1984). These tissues are the focus of this section; however, the measurement of hormones is generally not undertaken in toxicity screening studies unless there is cause for concern related to a known or suspected pharmacological or toxicological action. Measurement of the circulating levels of hormones can be a powerful technique to identify functional impairment of the endocrine system. The serum concentrations of many hormones are dependent on several factors, including time of day, age, sexual maturity, gender, nutritional status and stress, to name but a few. The effects of these variables can often diminish the diagnostic value of hormone assays. Therefore the standardization of the procedures and timing for blood collection is crucial to obtaining interpretable data. Hormones are generally one of four chemical types. Some examples of hormones from each of the chemical types secreted into the blood and their glandular origin are provided below (**Table 2**).

In order to have a robust biomarker for the detection of functional change, the hormone must be released in sufficient quantities to be detected in biological fluids (serum, plasma or urine), the origin of the hormone must be identifiable and it must be sufficiently stable to allow for practical application of the assay after the toxicological event.

14.1 Adrenals

The adrenal is the most common toxicological target in the endocrine system (Ribelin, 1984; Colby, 1996). The need for assessment of adrenal function is clear, yet in regulatory screening toxicity tests it is uncommon for adrenal function to be specifically assessed. There are no specific biomarkers of adrenal toxicity in the battery of clinical chemistry parameters routinely used.

Table 2 Chemical classes of major hormones and their glandular origins

Chemical type	Hormone	Tissue origin
Peptides and proteins	Follicle stimulating hormone (FSH)	Anterior pituitary
	Luteinizing hormone (LH)	Anterior pituitary
	Prolactin (PRL)	Anterior pituitary
	Thyroid stimulating hormone (TSH)	Anterior pituitary
	Adrenocorticotrophic hormone (ACTH)	Anterior pituitary
	Thyrotropin releasing hormone (TRH)	Hypothalamus
	Antidiuretic hormone (ADH)	Posterior pituitary
Steroids	Aldosterone	Adrenal cortex
	Cortisol/corticosterone	Adrenal cortex
	Oestradiol	Ovary/placenta
	Progesterone	Ovary/placenta
	Testosterone	Testis/ovaries/adrenal cortex
	Calcitriol	Kidney
Amino acid derivatives	Adrenaline (epinephrine)	Adrenal medulla
	Noradrenaline (norepinephrine)	Adrenal medulla
	Dopamine	Adrenal medulla
	Tri-iodothyronine (T_3)	Thyroid
	Thyroxine (T_4)	Thyroid
Fatty acid derivatives	Prostaglandin E_2	Most cells
	Thromboxane A2	Platelets
	Prostacyclin (PGI_2)	Endothelial cells

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The adrenal is divided into two distinct regions, the adrenal medulla (inner) and the adrenal cortex (outer). The cortex is further differentiated into the zona reticularis adjoining the adrenal medulla, and moving outwards the zona fasciculata followed by the outermost zona glomerulosa. The hormones of the cortex are steroid hormones formed by the further metabolism of CHOL, whereas the hormones of the medulla are amino acid derivatives. The high cytochrome P450 enzyme activities of the cortex attest to its biosynthetic function and are targets for drugs which inhibit steroidogenesis. The pituitary hormone adrenocorticotrophic hormone (ACTH) regulates the biosythesis of the mineralocorticoids (e.g. aldosterone), synthesized in the zona glomerulosa, and the glucocorticoids of the zona (e.g. corticosterone/cortisol). Evaluation of toxicity to the adrenals relies on histopathology, at least in screening studies. Further evaluation of suspected adrenal toxicity and functional change can be carried out by measurement of the three main hormones, corticosterone/cortisol, aldosterone and adrenaline. Adrenal toxicity may also result in perturbations to sodium, potassium, chloride and hydrogen in both serum and urine (which can be measured without resorting to hormone assays), since the mineralocorticoids regulate renal reabsorption of these ions. Similarly, since glucocoticoids stimulate gluconeogenesis, and fat and protein catabolism, secondary to disturbance in cortisol or corticosterone can be a reduction in serum glucose, CHOL and/or protein. Aminoglutethimide, etomidate, metyrapone, ketoconazole and suramin are associated with impaired steroidogenesis and in humans cortisol synthesis is inhibited by these drugs. Fungicides of the conazole group, used as pesticides on plants, inhibit sterol synthesis in fungi and have some activity on steroid metabolism in animals (see **Toxicology of Pesticides**).

A clinical chemistry approach to evaluating adrenal impairment can be direct measurement of adrenal hormones or evaluation of the response of the hormones to adrenal challenge by ACTH (Harvey *et al*., 2007; Harvey and Everett, 2003). The circulating background serum concentrations of adrenal hormones vary markedly, particularly associated with stress, and so blood-sampling techniques which minimize stress are crucial. Indwelling catheters may be recommended for investigative studies, but this approach is uncommon. Other factors affecting serum steroid concentrations include age, season, strain, sex, and light, handling and environment, circadian rhythm and episodic secretion (Evans, 1996). There are also species differences in the synthesis of corticosteroid (see **Table 3**). Rats and mice synthesize corticosterone as the predominant form of the hormone, whereas all other species, including humans, sythesize cortisol. Therefore one of the most valuable models of adrenal function and toxicity is the guinea pig, because of its small size and relatively large adrenals.

Table 3 Species differences in corticosteroid form

Species	Predominant form of corticosteroid	
	Cortisol	Corticosterone
Human, dog, primate, minipig, rabbit, hamster, guinea pig, sheep, cattle	Yes	No
Rat and mouse	No	Yes

In cases of adrenal insufficiency caused by inhibition of steroidogenesis, often the testis is affected too.

14.2 Gonads

The gonads comprise the testes in males and the ovaries in females. Both have dual functions in gametogenesis and as endocrine glands secreting the sex steroids. In males, testosterone, dihydrotestosterone and even small quantities of oestrogens are secreted. In females the sex steroids are oestrogens (principally oestradiol) and progesterone. Overall hormonal control of gonads is maintained by the hypothalamic–pituitary–gonadal axis. The gonadotropins of the anterior pituitary regulate the gametogenic and secretory functions of both male and female gonads. They are luteinizing hormone (LH) and follicle stimulating hormone (FSH). The major stimulatory effects of these two hormones are described in **Table 4** below.

The functional control of the gonads is complex and requires complex feedback mechanisms to work in concert. For a further review of the function of the reproductive system see **Hepatotoxicity**.

14.2.1 Testis

Histopathology is the traditional method to assess toxic insult to the testis, but is sometimes accompanied by seminology or flow cytometry. Several efforts have been made to identify noninvasive clinical chemistry markers of testicular toxicity in the battery of routine laboratory animal clinical chemistry tests, however, none

Table 4 Major stimulatory roles of FSH and LH

Hormone	Testis (male)	Ovary (female)
FSH	↑Spermatogenesis	↑Follicular development and maturation in the ovary
LH	↑Testosterone secretion from Leydig cells	↑Ovulation

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are commonly used in screening tests. The isoenzyme LDH-C4 (LDH-X) is testis-specific and is found in several stages of germ cells. It has been reported as being of value in evaluating testicular toxicity (Reader *et al*., 1991; Shen and Lee, 1984) and was seen to be elevated for up to 14 days in plasma after an acute toxic insult by ethylene glycol monomethyl ether. Androgen binding protein (ABP) is a marker of Sertoli cell function since it is secreted by the cell and has affinity for testosterone and dihydrotestosterone. ABP was reported to be a valuable marker of 1,3-dinitrobenzene toxicity when measured in plasma and was elevated both in testicular tissue and in plasma (Suter *et al*., 1998). However, this is not commonly used as a marker and the variability of ABP testicular values was very high, reducing the sensitivity of the marker. Urinary creatine has also been reported to be of value as a noninvasive marker in identifying testis-specific toxicity. Draper *et al*. (1996) showed that 2-methoxyethanol caused dose-dependent pathological damage to the testes which was detectable at the lowest dose in rats. Urinary creatine excretion was significantly raised at all doses, but testis weight was only significantly decreased at the highest two doses; serum testosterone was relatively insensitive and LDH-C4 was not significantly increased at any dose. In cadmium chloride-induced testicular toxicity, similarly creatine was a sensitive marker of testicular damage even in vasectomized rats demonstrating that the creatine reaches the urine via the blood stream (Draper and Timbrell, 1996). Creatine was also found to be of value as a murine marker of testicular toxicity caused by 2-methoxy acetic acid (Traina *et al*., 1997).

Measurement of the hormones and controlling gonadotropins associated with testicular cell function is the primary method for determining alterations in androgen status. Methods for steroid hormones, such as testosterone by radioimmunoassay are relatively easily transferable between species since the structure is the same, but for peptide or protein hormones, for example LH, FSH, the crossreactivity between species may be weak, leading to the need for robust validation of methods in the laboratory. The pulsatile and/or circadian rhythms of the sex hormones in different species add a layer of variability which make the control of timing and sampling procedure absolutely vital. Where possible, multiple samples should be taken from baseline and maximally stimulated animals during the days of evaluation (Creasy and Foster, 2002). Stimulation of LH/FSH release can be achieved by administering luteinizing hormone releasing hormone (LHRH) (gonadotropin releasing hormone (GnRH)), and testosterone release may be stimulated by administration of human chorionic gonadotropin (hCG). These functional tests may not be desirable or practical on screening studies, but are an effective method to further evaluate suspected testicular toxicant modes of action.

Age has an influence circulating levels of sex hormones. In ageing male rats, decreases in serum testosterone (Karpas *et al*., 1983; Wang *et al*., 1993; Zirkin *et al*., 1993; Chen *et al*., 1994) and gonadotropins (Zirkin *et al*., 1993) have been reported. In male dogs, conflicting reports show no decrease (Taha and Noakes, 1982; Lowseth *et al*., 1990) or a decrease (Bondarenko *et al*., 1979; Gunzel Apel *et al*., 1990). Similarly dog serum gonadotropin concentrations have been reported to decrease with age by Gunzel Apel *et al*. (1990).

14.2.2 Ovary

The ovary, like the testis, has the dual functions of gametogenesis and endocrine production of hormones. The female reproductive system has a regular cycle of oestrus, metestrus, diestrus and proestrus, which is of different lengths for different mammalian species. This cycle is regulated by hormones of the hypothalamic–pituitary–gonadal axis, largely LH and FSH. The structure and secretory activity of the ovary at any one time will reflect the stage of oestrus. Regulation of the female reproductive system is complex and the reader is referred to standard reference texts for further discussion. Therefore there is an inherent need to understand the stage of oestrus that each animal (both control and treated animals) is undergoing in order to interpret hormonal or structural differences. Ideally, baseline sampling should be carried out at multiple timepoints in order to provide an intra-individual control. For rodents this is generally not practical, but is possible for large animals, such as macaques, dogs and minipigs, providing sample volumes are minimized. The investigation of female gonadal function is rarely carried out on screening studies, since the assays often require serial sampling and circulating hormone concentrations are influenced by many variables including age, stress, nutritional status and so on.

Methods for measurement of female reproductive hormones in laboratory animals do exist, but they are often developed from the human clinical counterparts and therefore the volumes of plasma or serum required can be too high to be practical in rodent and primate assays. Hawkins *et al*. (1975) investigated the use of radioimmunoassay for measuring 17β-oestradiol and prolactin (PRL) levels in rats, and demonstrated significant fluctuations related to the oestrus cycle. In addition to the stage of the oestrous cycle, the PRL concentration was influenced by mode of blood collection, degree of haemolysis and choice of serum or plasma.

Measurement of PRL, FSH and LH, as well as circulating oestrogens, are the primary methods for clinical evaluation of female reproductive status. Assays for these are commercially available as immunoassays for the rat, dog and primate, but kits for other species may be available too. Validation of the assays with regard to sensitivity and range, accuracy and precision prior to use

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in other species is important to ensure the quality of the data.

Further variability in gonadotropins and steroid hormones is related to age and reaching towards the end of the reproductive life span in female humans and in animals (Nass *et al*., 1984; LaPolt and Lu, 2001).

14.3 Thyroid

The thyroid and its secretions have been the focus of many a risk assessment. In laboratory animal toxicity and carcinogenicity studies the thyroid is a relatively common target organ, but in humans it is not often associated with drug treatment. In 1394 rodent carcinogenicity studies conducted by the National Toxicology Program the thyroid was the sixth most common site for tumours (Huff *et al*., 1991). The rodent is particularly susceptible and highly sensitive to chemically induced thyroid toxicity and carcinogenesis because of the species differences between humans and rodents.

The mammalian thyroid gland produces the major thyroid hormones thyroxine (T4), tri-iodothyronine (T3) and calcitonin. Calcitonin is produced by the C-cells or parafollicular cells, which are either located within the thyroid follicles, between the basal region of the follicular cells and the basement membrane, or in an interfollicular location (Capen *et al*., 2002). Calcitonin and PTH act in concert to control calcium in ECFs. PTH is thought to be responsible for the prevention of hypocalcaemia, particularly in calcium deprivation, and acts synergistically with vitamin D3 (calcitriol), whereas calcitonin is thought to prevent the development of hypercalcaemia due to postprandial absorption and protects against excessive calcium and phosphorus loss from maternal bone during pregnancy (Capen *et al*., 2002). Disturbances in calcium homoeostasis can be picked up routinely in screening studies by measuring serum calcium. However, the interpretation of fluctuations of serum calcium alone should be undertaken with care, since calcium homoeostasis is achieved by the interplay of many factors, including diet, intracellular storage, bone storage and tubular resorption/excretion.

The follicular thyroid consists of follicles of varying size (20–250 μm) that contain colloid in the lumen produced by cuboidal or columnar follicular epithelial cells. Thyroid hormone synthesis begins with the very efficient energy-dependent active transport of iodide into the follicular lumen where it is oxidized by thyroid peroxidase to iodine. Tyrosine is subsequently iodinated and the building blocks 3-monoiodotyrosine and 3,5-diiodotyrosine are formed. Thyroid hormones stored in the colloid are released as T3 and T4 bound to carrier proteins. Synthesis and release of thyroid hormones is under the control of the hypothalamic–pituitary–thyroid axis. Hypothalamic thyrotrophin releasing hormone (TRH) is released in response to low circulating T3/T4. This then, in concert with the low T3/T4 levels, stimulates the thyrotrophic cells of the anterior pituitary to secrete TSH (thyrotrophin). TSH acts directly on the thyroid to stimulate T3/T4 release. Therefore there are several points at which thyroid function can be disrupted. Serum T3, T4 and TSH are not commonly measured on routine screening studies for toxicity, but if there is histopathological evidence of functional change in the thyroid or a previous concern about thyroid function, then investigative studies can be useful to identify a mode of action. The measurement of thyroid parameters and functional challenge is a key part of the tool set available. Measurement of T3 and T4, which are amino acid derivatives, is straightforward and is quite transferable between species. TSH is, however, a glycoprotein and therefore the immunoassay method transferred from one species to another requires careful evaluation for sensitivity, accuracy and precision. There are marked species differences in the transport of thyroid hormones as shown in **Tables 5** and **6** below. Generally, the low-affinity, high-capacity binding to ALB, prealbumin and postalbumin of rodents is quite different from the high-affinity binding to thyroglobulin in other species. Consequently, the kinetics of T3/T4 clearance in serum differs between species, for example, serum T4 half-life in rats is approximately 12–24 hours, whereas in humans it is five to nine days. Dogs, monkeys and minpigs are more similar to humans in the way that they handle thyroid hormones. The differences in binding and kinetics of thyroid hormones probably accounts for much of the unique sensitivity to thyroid carcinogenesis induced by TSH stimulation in rodents.

Investigation of mode of action of thyroid functional disturbance starts with the measurement of circulating TSH, T3 and T4. There are a number of environmental and physiological factors which can have an impact on thyroid hormones, including food deprivation, handling, temperature and diurnal variation (Dohler *et al*., 1979), mandating extreme care in constructing the protocol, sampling and handling methods. Generally the volumes required for T3/T4 only allow for terminal sampling in the mouse. Further investigation of a potential disturbance in thyroid function may require investigation of iodide accumulation and organification by the perchlorate discharge test, usually carried out in rats, in which sodium perchlorate is administered and the ratio of thyroid to blood ^{125}I is measured. Propylthiouracil, an inhibitor of iodide organification reduced the thyroid:blood ratio, whereas SK&F 93479 which increases thyroid hormone clearance and thereby increases TSH drive, did not. This test helps to distinguish between direct and indirect effects (e.g. effects on metabolism and clearance of thyroid hormones) (Atterwill *et al*., 1987; Coelho-Palermo Cunha and van Ravenzwaay, 2007). Methods to investigate mode of action on the thyroid include investigation of the metabolism of T3

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Table 5 Thyroxine (T4) binding to serum proteins in selected vertebrate species (Dohler *et al*., 1979)[a]

Species	T4-binding globulin	Postalbumin	Albumin	Prealbumin
Human	++	−	++	+
Monkey	++	−	++	+
Dog	+	−	++	−
Mouse	−	++	++	+
Rat	−	+	++	−

[a]Degree of T4 binding to serum proteins: + or + + ; Absence of binding: −

Table 6 Triiodothyronine (T3) binding to serum proteins in selected vertebrate species (Dohler *et al*., 1979)[a]

Species	T3-binding globulin	Postalbumin	Albumin	Prealbumin
Human	+	−	+	−
Monkey	+	−	+	−
Dog	+	−	+	−
Mouse	−	+	+	−
Rat	−	−	+	−

[a]Degree of T3 binding to serum proteins: + or ++; Absence of binding: −

and T4, TSH response to TRH stimulation and investigation of the T3/T4 response to TSH stimulation.

An important group of thyroid toxicants is the alkylenebisdithiocarbamate fungicides. At least part of this toxicity is mediated by plant metabolites, ethylene thiourea in the case of the ethylenebisdithiocarbamates and propylene thiourea in the case of propineb. The Joint Expert Meeting on Pesticides Residues (FAO/WHO, 2000) cited a study by Freyberger, 1996, who concluded that depression of thyroid hormone synthesis and consequent stimulation of the hypothalamic–pituitary–thyroid axis caused the thyroid lesions induced by these compounds.

14.4 GI Tract and Pancreas

14.4.1 GI Tract

In humans, chemicals and drugs which are either intentionally or accidentally ingested through contaminated food or drink have the greatest potential for exposure in the highest concentration to the GI tract because this is the portal of entry. Once absorbed systemically, chemicals or metabolites that are secreted in the bile as a route of excretion are again potentially in contact with the gut at high concentrations. Finally enterohepatic circulation tends to prolong residence in the gut, again resulting in significant exposure. For these reasons and others related to specific tissue susceptibility metabolism or physicochemistry, the GI tract may have a unique susceptibility to toxicants. Therefore it is not surprising that the GI tract is a particular target and in toxicology studies, compounds which are given either in the diet or drinking water or by oral gavage, especially at high doses, are those where the potential for GI toxicity is highest.

Treatment-related effects on GI function of laboratory animals are often accompanied by disturbances in electrolyte and fluid balance, diarrhoea or constipation, vomiting (except in rodents, which cannot vomit) and, if prolonged, poor bodyweight gain and other conditions related to poor nutrition.

ALP is a marker enzyme of microvillius membranes and exists as soluble and membrane-bound forms. The tissue distribution of ALP is wide, but there are several tissue-specific isoenzymes found in serum, which include hepatic, bone, placental and intestinal forms. Generally found in the brush border of secretory/absorptive cells, in the rat, the bone and intestinal forms dominate, but in other species, the hepatic form generally dominates. In order to investigate the origin of increases in serum ALP, electrophoresis is used to separate the isoforms. Changes in intestinal ALP in response to intestinal toxicants have been documented (e.g. cysteamine, Japundzic *et al*., 1991; 1990; mepirizole and propionitrile), but there are few reports of the value of this enzyme in chemical-induced GI tract toxicity. Diamine oxidase, which metabolizes polyamines has been shown to be a useful serum biomarker of toxicity to the upper villus cells of the rat intestinal mucosa, for example, anticancer drugs (Moriyama *et al*., 2005), but use is not widespread. The GI damage induced by xenobiotics can also result in increased permeability to markers such as sucrose (gastroduodenal) and ^{51}Cr-EDTA (intestinal). When the urinary excretion of these orally administered substances was measured, the degree of excretion correlated with gross toxicological and pathological changes to the GI tract, including ulceration and bleeding due

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to nonsteroidal anti-inflammatory drugs (NSAIDs) or cisplatin (Yanez *et al*., 2003).

Haemorrhage into the GI tract is a consequence of loss of integrity of the mucosal lining. This is typical of NSAID toxicity and cytotoxic anticancer agents. Measurement of occult (hidden) blood in faecal samples can be carried out in laboratory animals to determine if loss of integrity resulting in haemorrhage has occurred. This procedure is generally carried out if an effect is suspected and is not used as a screening test. The methods used are based on the pseudoperoxidase activity of haemoglobin, however they are not specific to haemoglobin and there are high levels of false positives reported, at least in the clinical setting. This is also a concern in toxicology, particularly if the test compound has peroxidase activity, or if foods high in peroxidase activity are ingested (red meat, broccoli, parsnips and cantaloupe melon (by primates, dogs or minipigs). In contrast, foods high in vitamin C can produce false negative results (since vitamin C is an effective reducing agent).

14.4.2 Pancreas

The pancreas is a dual-functioning gland having both endocrine and exocrine secretions. The endocrine pancreas is comprised of clusters of cells called islets of Langerhans which are α cells secreting glucagon, β cells secreting insulin, δ cells secreting somatostatin or PP cells secreting pancreatic polypeptide. The exocrine pancreas secretes digestive enzymes into the small intestine (see **Toxicology of the Exocrine Pancreas**). Digestive secretions include trypsin, chymotrypsin, pancreatic lipase and pancreatic amylase produced by the acinar cells. In addition, centroacinar cells which line the pancreatic ducts secrete biocarbonate and salt into the small intestine.

Serum and/or urinary amylase activity has been used as a marker for toxicity to the exocrine pancreas. Isoenzymes are used to identify the tissue origin, since amylase may also arise from the salivary gland and intestine. Lipase is more specific for pancreatic injury. Recent work has shown that two peptide products of proteolytic cleavage (RA1609 and RT2864) are sensitive markers of exocrine pancreatic toxicity (Walgren *et al*., 2007a; Walgren *et al*., 2007b) in animals treated with caerulein but the surface-enhanced laser desorption/ionization (SELDI)-mass spectrometry analytical technique required to measure the peptides is outside the current scope of the average clinical chemistry laboratory. Whilst not ready now for use as a preferred marker in investigating pancreatic toxicity, these markers may be potentially useful in the future, once further evaluation and validation has taken place.

Testing β-cell function can be carried out by determining serum glucose or insulin after a single administration of glucose by oral gavage or by a parenteral route to bypass the insulin-stimulating gut hormones.

15 NERVOUS SYSTEM

There are very few biomarkers of damage to the central or peripheral nervous systems. Routine serum and urine samples have been of limited value in routine screens to identify neurotoxicity. Sampling of CNS is not a routine procedure and in laboratory animals (rats, mice, marmosets) it is a technical challenge requiring anaesthesia or an indwelling canula. The volume of CSF that can be obtained is very small and there is potential for contamination from surrounding tissues. There are essentially only two established specific markers of neurotoxicity, as described below.

15.1 Neural Esterases

Inhibition of acetylcholinesterase activity is a known effect of organophosphate and carbamate pesticides (Costa, 1988; Padilla, 1995; *see also* **Toxicology of Pesticides**). Acetylcholinesterase hydrolyses and therefore degrades and controls the concentration of the neurotransmitter acetylcholine. Inhibition of the enzyme in either the central or peripheral nervous system prolongs the action of the neurotransmitter and is thought to be responsible for the effects that these chemicals produce. However, reduction in cholinesterase activity does not necessarily indicate clinical symptoms of neurotoxicity, but it may impair the organism's ability to respond to additional exposures to anticholinesterases (US EPA, 1998a). In mammals and birds, signs of anticholinesterase poisoning include miosis, excessive salivation, tremors, respiratory difficulty and tetany. The cholinesterases that have to be considered are RBC or 'true' acetylcholinesterase, butyrylcholinesterase (plasma or pseudocholinesterase) and brain cholinesterase. Brain cholinesterase is predominantly acetylcholinesterase as is found in the RBC, indeed they are the same gene product, although butyrylcholinesterase activity is also present in the brain. Butyrylcholinesterase has a different substrate specificity from acetylcholinesterase and is a different gene product: plasma butyrylcholinesterase is synthesized in the liver. Inhibition of nonspecific butyrylcholinesterase has no known toxicological consequence but it is a potential indicator of exposure and acetylcholinesterase inhibition and can be measured in life, whereas brain acetylcholinesterase can only be measured by taking samples at necropsy. RBC and brain data are preferred for risk assessment. When assessing the

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safety of new organophosphates or carbamates, measurements of brain, RBC and plasma cholinesterase are required in rat and dog. Studies in the dog use fewer animals per group, but they have the advantage that pretreatment values can be obtained, thus each animal will have its own control data. The method normally used to measure cholinesterase activity is based on a colorimetric reaction Ellman (1961), however it has the potential to underestimate reversible cholinesterase inhibition (Padilla *et al*., 2007). The method is liable to underestimation if pH changes or if the incubation time variable is not controlled, particularly for reversible inhibition (carbamates) in which reactivation can occur soon after sampling, or if diluted. An alternative method is a radiometric method. For further information on the measurement of cholinesterases and their use in risk assessment see US EPA (2000). A small number of organophosphates, for example phosporamidates and phosphonates have the potential to produce human delayed or late-onset manifestation of neurotoxicity or organophosphate-induced delayed polyneuropathy (OPIDP) also known as 'dying back polyneuropathy'. The clinical signs of neurotoxicity do not develop until at least six days after exposure in humans, but the rat and dog are not good models for this clinical syndrome. The adult chicken is a sensitive model for OPIDP and is the preferred species for the measurement of neuropathy target esterase (NTE) by hydrolysis of phenyl valerate (US EPA, 1998a; OECD, 1995b).

15.2 Glial Fibrillary Acid Protein

Astrocytes are the major glial cells in the brain with important physiological and supportive activities. In response to neurotoxic injury, they may undergo hypertrophy and produce more glial fibrillary acid protein (GFAP) (O'Callaghan, 1988; Garman *et al*., 2001). When increases in GFAP are corroborated by neuropathological change then GFAP can be used as a biochemical marker of neurotoxicity in tissue sections. In contrast, lack of response does not always mean that a neurotoxic effect has not occurred, since other neural targets will not involve an astrocyte response. Trimethyl tin, methylmercury, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and cadmium have all been shown to increase brain GFAP (Dorman *et al*., 2002).

16 SUMMARY

This chapter describes the routine measurement of the noncellular blood compartment (plasma or serum) and urine for biochemical composition in toxicology studies conducted in commonly used laboratory species. The measurement of a range of clinical chemistry parameters provides an indication of the overall health of the animal and is used in conjunction with other parameters to investigate the toxicity of drugs and chemicals. The range of parameters commonly used in toxicology studies has been carefully selected to include those considered by experts to be the most useful in determining health status and identifying change as a result of toxicity. The methods used are well defined and must be conducted according to SOPs, and the data generated are subject to strict QC procedures in order to minimize variability unrelated to the effect of the test article. The advantage of clinical chemistry is that provided the volume of blood taken or the method of urine collection is strictly controlled, generally the procedures are only minimally invasive and have a negligible or transient impact on the physiological status of the animal. Therefore serial samples can be taken, which is useful for investigating progression and recovery of changes in live animals within several organs often associated with toxicity. There are few routine clinical chemical parameters which are specific indicators of a single target organ toxicity; the interpretation of changes in the blood and urine composition requires integration of information from a variety of measurements, such as clinical signs, food consumption and bodyweight, haematology, electrocardiography, blood pressure and histopathology, to understand the pattern of change and put it into the context of identifying toxicity. Changes in clinical chemistry can help to identify toxicological end points such as the NOAEL and the maximum tolerated dose.

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Haematology and Toxicology

Timothy C. Marrs and Simon Warren

CONTENTS

1 INTRODUCTION

Haematological investigations are of importance in toxicology in two different sets of circumstances. Firstly, toxicological effects on the blood and blood-forming organs may occur in humans; these effects may cause similar changes in animal studies. Secondly, toxicological parameters are routinely measured during toxicological studies, regardless of whether the test compound is thought to be specifically haematotoxic, and guidelines are available about how these investigations should be carried out (Weingand *et al*., 1996). Both in humans and in experimental animals, abnormalities observed may be due to toxicity to the haemopoietic organs such as the bone marrow, or be produced indirectly through effects on animal nutrition, or on organs such as the kidney, which affect haemopoiesis. Moreover, some xenobiotics can affect the peripheral blood directly, an example being the production of haemolysis by certain drugs. One of the most interesting aspects of haematology in humans is the frequent interaction of inherited traits, for example, porphyria and glucose-6-phosphate dehydrogenase deficiency, with xenobiotics, including pharmaceuticals.

2 STRUCTURE AND FUNCTIONS OF THE BLOOD AND ASSOCIATED ORGANS

Blood is a fluid containing a number of cellular elements, including the red cells, which contain the oxygen-transporting pigment, the white cells and the platelets. The white cells are frequently categorized into neutrophils, eosinophils, basophils, monocytes and lymphocytes.

2.1 Haematopoiesis

Haematopoiesis occurs from stem cells. In studies with irradiated mice it was noted that certain bone marrow cells had the ability to form haematopoietic colonies in irradiated mice (Till and McCulloch, 1961). It appears that the stem cells in the marrow can give rise to all types of blood cells and these stem cells are thus known as pluripotent stem cells (Barton *et al*., 1980). The pluripotent stem cells are also known as colony-forming

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units spleen (CFU-S) from their properties in studies *in vitro* and they have a marked ability for self-renewal. The CFU-S assay can be used to quantitate pluripotent stem cells. Factors that control haematopoiesis have been studied (Opferman, 2007; Metcalf, 2008).

The pluripotent stem cell can differentiate into stem cells that are capable of giving rise to myeloid cell types, the colony-forming unit granulocyte/erythroid/monocyte/megakaryocyte (CFU-GEMM), which in turn give rise to progenitor cells, whose potential is more restricted, such as the colony-forming unit granulocyte/macrophage (CFU-GM), the colony-forming unit erythroid (CFU-E), and so on (Chanarin, 1985; Irons, 1991; Young and Weiss, 1997; Means, 1997; Dzierzak and Speck, 2008). There is some evidence that there may be more than one pathway to the various lineage fates (see review by Buza-Vidas *et al*., 2007).

In most mammals, including humans, haemopoiesis is first seen in the yolk sac and later in foetal life, in the liver and spleen. After birth, the bone marrow is, in humans, the major site of haemopoiesis. In rats and mice virtually all the bone medullary space is occupied by haematopoietic tissue, whereas in adult humans, dogs and rabbits such tissue becomes limited to the proximal epiphyses of the long bones and skull, the remainder of the marrow being occupied by fat. Increased haematopoietic activity is, in man, usually confined to the medullary space, except under conditions of extreme demand. In the mouse and the rat, this is not the case and splenic (and hepatic) haematopoiesis is much more common in conditions of very high demand. The bone marrow contains a network of thin-walled branching vessels called the venous sinuses. The wall of the sinuses consists of the endothelium, basement membrane and the adventitia, the last of which is associated with adventitial reticular cells. The endothelium is complete, that is, not fenestrated, but the basement membrane is discontinuous. Mature blood cells cross the wall of the sinuses to enter the circulation (Chanarin, 1985). In normal circumstances, the cellular component of the marrow remains fairly constant, unless disturbed by factors such as loss of blood; in such conditions the marrow can respond rapidly to bring the number of cells in the peripheral circulation back to normal; this is controlled by a number of factors that stimulate haemopoiesis, such as erythropoietin (see below).

2.1.1 The Red Cell and Haemoglobin: Erythropoiesis

The main role of the red blood cell is the reversible combination of the haemoglobin therein and oxygen. The erythroid series of cells is derived from the stem cell and thence the CFU-GEMM. The earliest progenitor restricted to the red cell lineage is the burst-forming unit-erythroid (BFU-E), from which is derived the CFU-E. This gives rise to the precursor cells, the various normoblasts (Irons, 1991; Means, 1997). The last stage in maturation to the non-nucleated biconcave erythrocyte is the reticulocyte, so-called because of its fine basophilic reticular network (see Sieff and Nathan, 1996). In situations in which erythrocyte production has been stimulated, reticulocytes may be seen in the peripheral blood. The regulation of production of red cells is dependent upon the oxygen requirement of the tissues, being controlled by humoral factors, including erythropoietin, whose main source is the kidney (Spivak and Graber, 1980; Koury *et al*., 1988), where the rate of production is dependent on tissue oxygenation, with a contribution from the liver (see reviews by Chanarin, 1985; Koury, 2005).

Haemoglobin is one of a number of oxygen-binding proteins that have evolved in the animal and plant kingdoms, and contains a tetrapyrole ring system, with ferrous iron at the centre. Chlorophyll, in the plant kingdom, resembles haemoglobin in so far as it has a similar ring, though magnesium takes the place of the iron of haemoglobin. Copper, found in haemocyanin, replaces the iron atom in many invertebrates and, in some sea squirts, vanadium is present. The structure of haemoglobin can be summarized: each molecule of haemoglobin consists of four amino acid chains: a pair of alpha chains and a pair of beta chains (Perutz, 1967–1968). The chains are folded and each carries a haem group, attached to a histidine residue at position 87 in the alpha chains and 92 in the beta chains. The haem group comprises a tetrapyrole ring with a central ferrous iron atom. The iron remains in the ferrous state during binding to both oxygen and carbon monoxide. If oxidized to the ferric state methaemoglobin (MetHb) is produced: this is discussed below.

The iron atoms of haemoglobin can each accept electrons from a maximum of six oppositely charged ions or neutral substances (Strang, 1977). Each iron atom forms six bonds: four with the nitrogen atoms of the tetrapyrole ring; one with the histidine residue of the amino acid chain and one with oxygen or, in cases of poisoning, with carbon monoxide. As the haemoglobin molecule takes up oxygen, its quaternary structure changes due to changes in the extent of loose bonding, or attraction, of the iron atom to other amino acid residues in the amino acid chains. These conformational changes lead to the haemoglobin molecule passing from a ‘tense’ configuration when in the deoxygenated state into a ‘relaxed state’ as oxygen atoms are taken up. In the relaxed state the affinity for oxygen is high: saturation occurs when the haemoglobin molecule has bound four molecules of oxygen. This sequential change in affinity for oxygen defines the sigmoid shape of the oxyhaemoglobin dissociation curve. It is interesting to note that if haemoglobin bound only one oxygen atom, that is, consisted of only one amino acid chain with a single haem group, the dissociation curve could be represented by a hyperbola: this is the case with myoglobin, a one-chain molecule. The

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structure of haemoglobin differs in different species and that in the human foetus is different from that in the adult human: foetal haemoglobin (haemoglobin F) gives up its oxygen less readily than normal adult haemoglobin (Hoffbrand and Pettit, 1984). Abnormal haemoglobins in humans can produce diseases such as sickle cell anaemia and some types of hereditary methaemoglobinaemia.

In addition to haemoglobin, the red cell contains enzymes, such as glucose-6-phosphate dehydrogenase (G6PD). G6PD is of great importance in maintaining NADPH levels. NADPH is essential for reducing oxidized glutathione, reduced glutathione being required for the protection of intraerythrocytic sulfhydryl groups and the structural integrity of the cell. Maintenance of the reducing potential of the red cells is also necessary to prevent methaemoglobinaemia. Oxidant stress of the red cells will produce haemolytic anaemia, methaemoglobinaemia and/or Heinz bodies (see below).

2.1.2 White Cells

2.1.2.1 Granulocytes

The granulocytic series are derived from the CFU-GEMM, which gives rise to the CFU-GM. Three lineages give rise to mature granulocytes, which are known as polymorphonuclear leucocytes or polymorphs. They have segmented nuclei and cytoplasmic granules and in humans are divided into neutrophils, eosinophils and basophils according to the staining characteristics of their cytoplasmic granules (Irons, 1991; Liesveld and Lichtman, 1997). The granules contain a variety of enzymes and other substances characteristic of the inflammatory response and phagocytosis and the cells have a turnover rate of a few hours (Marsh, 1985). The primary role of polymorphs is phagocytosis and they perform the major part of this role in the tissues, into which they pass between the capillary endothelial cells. The granules of neutrophils are notable for their high alkaline phosphatase activity and polymorph leucocytosis is characteristic of bacterial infection, but also may occur in other conditions, for example, myeloid leukaemias. Eosinophils have a distinct progenitor cell, the colony-forming unit eosinophil (CFU-EO) (Young and Weiss, 1997) and are frequently present during allergic phenomena and in parasitic infestations. The granules of eosinophils have high peroxidase activity and contain a variety of hydrolytic enzymes. Basophils are the circulating equivalent of mast cells and the granules contain heparin and histamine: the function of basophils has long been obscure, but evidence is accumulating for a role in allergic disease. In humans, basophils are the prime early producers of cytokines crucial for initiating and maintaining allergic responses. Basophils are the main cellular source for early production of interleukins IL-4 and IL-13 (see reviews by Gibbs, 2005; Falcone *et al*., 2006). Granulocytopoiesis is under the control of factors such as granulocyte colony-stimulating factor (Shochat *et al*., 2007).

2.1.2.2 Monocytes

Monocytes are derived from the CFU-GM, in common with neutrophils. Once they reach the tissues they transform to tissue macrophages or more specialized forms such as the Kupffer cells of the liver. There is some evidence in mouse and human embryos that embryonic macrophages do not follow the monocyte pathway, that is, that the ontogeny of macrophages in early development is different from that occurring during adult development (Shepard and Zon, 2000). Monocytes are large cells, whose function is phagocytosis (see Takahashi *et al*., 1996).

2.1.2.3 Lymphocytes

Lymphocytes can be divided into T-lymphocytes and B-lymphocytes. These are so-called because of the involvement of the thymus in T-lymphocyte production, whereas, in birds, the site of B-lymphocyte development is the Bursa of Fabricius. In mammals the primary lymphoid organs are the thymus and bone marrow.

2.1.3 Platelets and Coagulation

Platelets are formed in the bone marrow by fragmentation of megakaryocytes, large cells (diameter 50–200 μm), which are derived from megakaryoblasts (Tarallo, 1985; Irons, 1991). Factors involved in the commitment and differentiation of these cells have been reviewed (Long, 1998). The function of platelets is to stop bleeding: thus platelet aggregation can produce primary haemostasis, while platelets are also concerned in the initiation of coagulation, by liberating various molecules. In addition, thrombocytopenia results in increased capillary fragility. If the platelet count falls below about $50\,000\,\mu l^{-3}$, spontaneous haemorrhage and haemorrhage after minor trauma are likely to occur (Chanarin, 1985). The process of coagulation involves both plasma factors and platelets to produce the so-called coagulation cascade. There is an opposing system, fibrinolysis, which removes unwanted fibrin.

2.1.4 Interspecies Differences

There are notable differences in the ratios of the various cell types of the blood in different laboratory animals. Thus in the rat, guinea pig and certain types of primate (e.g. lemurs), lymphocytes predominate over polymorphs in the blood, whereas in other animals, including humans, polymorphs predominate. Thus haematological data should be compared with control data from the same species. Historical control data, where used, should be from the same species, and, as strain differences can occur, also from the same strain. As well as quantitative differences between blood cell numbers, there are

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some notable morphological differences between species. There are major differences in red cell morphology between some species not commonly used in the laboratory, but the red cell of nearly all laboratory species resembles that in humans in being a biconcave disc; however central pallor is not seen in cats' red cells. Reticulocytosis is frequently observed in rats and a severalfold change may be taken as adverse, whereas in other species a more modest change is an indicator of increased red cell production. The size of erythrocytes varies considerably between species, being about 8 μm in humans and as small as 3 μm in the goat (Irons, 1991). The life span of the human red cell is approximately 120 days (Ashby, 1919), whereas in many other species, it is considerably shorter, for example, 30 days in the mouse (Irons, 1991). Amongst the white cells, amphophils, pseudoeosinophils or heterophils are the rabbit equivalent of the neutrophils of other species, as are the pseudoeosinophils of guinea pigs (Loeb *et al*., 1978); although these cells carry out approximately the same functions as do neutrophils in other species, the specific granules stain orange-red with Romanowsky stains. The size of the granules in basophils varies considerably depending upon the species. The bone marrow of mice and rats is notable for the paucity of fat by comparison with human marrow, while rat haematopoietic tissue is remarkable for its large number of mast cells.

3 PRACTICAL CONSIDERATIONS IN TOXICOLOGY STUDY TECHNIQUES

Given the high degree of automation available in a modern haematology laboratory, practical techniques used to get blood samples from the animal to the laboratory constitute one of the greatest sources of avoidable variability in a toxicology study. There are important considerations in training and adequacy of staff, sample volume, sampling technique, selection of anticoagulant and anaesthesia and sequencing of the procedure, each of which has the potential to influence the outcome of a study.

The benefit to the study and to the animals themselves of properly trained and proficient sampling personnel cannot be over-emphasized. Quite correctly, adequate training and maintenance of training is a Good Laboratory Practice (GLP) requirement; but even under non-GLP conditions animal use ethics require the attention to animal well-being and integrity of the sampling process that only full competence satisfies. Staff who are well-trained and adept are less likely to cause excessive stress to the animals and to sampling personnel themselves, and will tend to reduce the chance of injury to the animals, perform with appropriate rapidity (reducing time-to-sample), perform with greater consistency and have an established routine for organization of samples and equipment. Lower levels of stress should result in fewer mistakes; a shortened procedure time results in less duration of stressful stimuli in the animal room; appropriate attention may be given to documentation of the procedure, mixing of samples with anticoagulant and prompt despatch of samples for analysis. In some species, stress may cause the spleen to contract (Morton *et al*., 1993) releasing erythrocytes to the circulation, with the consequence that red blood cell count (RBC), packed cell volume (PCV) and haemoglobin concentration become temporarily increased. Avoidable errors might include use of too small a syringe needle (forcing blood through the needle resulting in a degree of haemolysis), or inadequate mixing in the sample tube (with potential for clotting). A toxicologist should be aware of these factors; they can vary with practice during the sampling procedure, and an appropriate sequence of test and control animals helps mitigate the consequences of procedural variability.

The volume of blood available for sampling is rarely a limitation in haematology studies with large species. However, in smaller animals (e.g. rodents) study design requires a balance between the volume of sample required for analysis (each parameter on the wish list adding to the volume required) and the volume of blood which might reasonably be drawn. Hence, useful considerations include choice of analytical instrument (which volume is required for each parameter) and potential selection of methodology for which a smaller sample is required. The sample size needed for a complete haematology blood count, complete blood count (CBC) is generally relatively small and manageable; but haematology samples are frequently drawn along with clinical chemistry samples, hence volume for haematology may not be the only limitation. Under such circumstances, selection of the size of the sample tube may be a relevant factor. Haematological CBC can currently (2008) be performed on samples of 100–200 μl; these volumes are readily obtainable from rats and mice, and sample tubes of appropriate volume are commercially available. However, most test guidelines in which haematology is required also include coagulation, and a substantial international consensus (Weingand *et al*., 1996) suggests prothrombin time and activated partial thromboplastin time (APTT) as a minimum. Sample volumes for coagulation (requiring citrate as anticoagulant) are larger than those for CBC, and therefore generally available from rodents mainly as a terminal procedure, although venepuncture sampling of appropriate volume is feasible from rats at 13 weeks or beyond in a toxicity study.

The site from which blood is sampled will to some extent be dictated by the sample size required; and may in turn dictate necessity for anaesthesia. Blood sampling methodology and conditions are extensively addressed by various rules, guidelines and national legislation, and are requently guided by expertise within each facility. For insightful guidance on blood sampling, including detailed

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Table 1 Recommended sampling sites for blood

Species	Site
Mouse:	amputation of the tail tip, or venepuncture of the tail vein.
Rat:	venepuncture of the tail vein, or of the jugular vein
Rabbit:	venepuncture of the ear vein
Dogs:	venepuncture of the cephalic vein (forelimb) or jugular vein.

instructions for the various procedures, influence on the wellbeing of the animals, and limitations on volumes which might be drawn, the reader is referred to the excellent web site of the National Centre for the three Rs, *http://www.nc3rs.org.uk/bloodsamplingmicrosite/page.asp?id=313* which, although largely specific for the UK, has a European perspective and is a useful resource for toxicologists world-wide (see also **table 1**). The following paragraphs should be taken as guidance for consideration.

Small samples (e.g. the single drop required for a blood smear) may be taken by simple piercing of the tail vein in rodents. The tail vein of rats may also be used for slightly larger samples, of perhaps up to 500 µl. For such sampling it is helpful to warm the animal in a thermostatically controlled warming chamber so that the tail veins become dilated; warming of the tail vein alone does not reliably increase flow of blood sufficient for a sample as great as 500 µl. The tail tip of mice can be carefully amputated, but with care not to damage bone, thus limiting this procedure to no more than one or two occasions.

If a greater volume of blood is required (up to 0.3 ml in mice, or 1–2 ml in rats), blood can be drawn from the jugular (rats and mice) or from the sublingual vein (rats). These volumes can repeatedly be taken so long as intervals are no less than approximately 28 days. Advice on sample volumes are based on the basis that circulating blood volume is approximately 50–70 ml kg^{-1} bodyweight, and that up to 10% of the circulating blood volume may be drawn at this interval without undue consequence. Clearly, as animals grow larger greater volumes (perhaps up to 3.5 ml from a 500 g rat) might be drawn. Review of modern toxicology reports shows that sampling from the orbital sinus remains a very common technique, and provides excellent blood samples in well-practiced hands; however, it carries the possibility of severe consequences to the animal—including infection behind, and loss of function of, the eye—and for this reason use is discouraged by many experts. It must be recognized that apparatus such as the butterfly needle has been available for many years, making sampling from veins of small animals a more manageable technique than it once was. With all techniques, precautions to restrict 'dead volume' in the sampling apparatus are prudent; blood discarded in the sampling apparatus is wasted, and lengthy cannulae are unhelpful in this respect.

Far greater quantities of blood (5–15 ml in rats) can be drawn at sacrifice. This is generally well in excess of requirement for routine CBC haematology, but may be helpful if coagulation or extensive clinical chemistry investigation is required at the same time. Blood should be drawn as soon as possible after death, with the abdominal vena cava being an easily accessible site at necropsy. Temptation for a preterminal sample can be resisted. However, modern automation permits that the majority of results from a preterminal investigation can be available within hours of the samples being taken, giving opportunity to plan confirmatory or alternative assays in samples taken at termination. The toxicologist should be aware that sampling a few days before termination may result in changes to certain clinical chemistry and haematology values in terminal samples; hence this option is unlikely to be feasible following preterminal sampling restricted to control and top-dose animals. Further, samples drawn from different locations may differ slightly in cell content or biochemical properties; hence, samples taken at necropsy from the abdominal vena cava may not be directly comparable with samples drawn at an earlier timepoint from the tail vein or jugular vein. While this in no way invalidates the comparison of control and treated groups, longitudinal comparisons (changes over the duration of the study) should be done only with care, as should use of historical data, to account for possible differences in sampling methods.

Haematology sampling requires anticoagulant, typically ethylenediaminetetraacetic acid (EDTA) (for cell counts) or citrate (for coagulation tests). A specific quantity of anticoagulant is present in the sample tube, appropriate to the required volume of blood, so it is prudent for the volume of blood in the tube to be as close as reasonable to the specification. In toxicity studies, animals of the top dose are generally expected to show modest toxicity, which occasionally results in technical difficulty obtaining the same volume of blood as from controls. Should these cause the volume of blood sampled from any group of treated animals to be systematically smaller than that of the corresponding controls, there is the potential to create as a sampling artefact, an apparently treatment-related variation in anticoagulant concentration. This might be reflected as a degree of cell shrinkage. To minimize such artefacts, good practice suggests anticoagulant samples are drawn before the clotted sample used for clinical chemistry.

In considering use of any anaesthetic, the toxicologist must bear in mind the potential for unwanted toxicological consequences (e.g. hepatic enzyme induction). Dogs are normally sufficiently docile and compliant that blood samples may be drawn without anaesthesia, particularly if animals have even slight training. Use of general anaesthesia will aid restraint of small animals during blood sampling, thus reducing risk of injury, as well as

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reducing the level of discomfort suffered by the animal, and for particularly invasive sampling methods such as cardiac puncture or orbital sinus sampling, general anaesthesia should be regarded as mandatory. There are several inhalation anaesthetic agents that are suitable for small animal use. All volatile anaesthetic agents may impair well-being of the operator unless used in a closed system with good ambient ventilation. With appropriate apparatus (e.g. a metered inhalation chamber) these agents have the advantage of being convenient, with rapid recovery. Use of ether as an anaesthetic is undesirable; not only is the vapour potentially flammable and explosive, but is irritant to nasal mucosae hence causing discomfort. More modern volatile agents such as methoxyfluorane are a preferred alternative. Use of carbon dioxide (e.g. a 70% CO_2/30% oxygen mix) may be permitted, although it must be questioned if this method is anaesthetic (prevents pain); further, risk of mortality is somewhat greater than with a well-metred volatile anaesthetic. The risk is yet greater if 100% CO_2 is used and, given the investment in time and expense required (i.e. to bring an experimental animal in a toxicology study to the point of blood sampling with recovery being required) use of CO_2 may easily be false economy. Use of CO_2 is a common method of rodent sacrifice and terminal blood samples will frequently be obtained via use of this agent; but it causes alkalosis of the blood, so is unsuitable if simultaneous blood gas measurements are attempted. Injectable anaesthetics may be used, particularly for large species, but do not have the popularity of inhalation agents.

3.1 Useful Parameters

Parameters recommended in the great majority of regulatory guidelines (e.g. those of the Organisation for Economic Cooperation and Development (OECD)) provide a screening procedure to identify if the blood is a target organ system. These parameters are discussed by Weingand *et al*. (1996). Further parameters may be helpful to help elucidate a process by which changes occurs.

3.1.1 Complete Blood Count (CBC)

The key parameters are red blood cell count, haematocrit, haemoglobin concentration, white blood cell count and platelet (thromobocyte) count. These values are easily determined using modern automated haematology analysers from a small blood sample, easily obtained from the mammalian species used in toxicity testing. It is important to be aware that haematology instrumentation is usually designed for analysis of human blood samples. While it is usual for a toxicology laboratory to have access to some of the many instruments with software that permits appropriate analysis of samples from most animal species, different instruments may, however, vary slightly in how 'abnormalities' in cell size or morphology are determined or detected.

Examination of blood smears (for reticulocytes, erythrocyte appearance or white cell differential counts) is frequently performed manually, so is labour intensive. For this reason, most guidelines permit examination of control and high-dose groups in the first instance. This limited procedure is not necessary if automated examination is available.

A key principle in haematology autoanalysers is that the blood sample is aspirated through a small aperture allowing few cells at a time, permitting cell number and size to be measured by light scattering or changes in electrical resistivity. The primary data obtained is thus cell count and cell volume, with haematocrit calculated from this information; cell indices of mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) are generally provided. Mean cell volume (MCV) is a key parameter in determination of whether an observed anaemia is macrocytic, microcytic or normocytic, an essential first step in understanding an anaemic process. White blood cells are frequently distinguished by presence of a nucleus, and differentiated either by biochemical and size characteristics, or by nuclear size and shape. Platelets are distinguishable by size.

Should results indicate a change in blood parameters—particularly anaemia—examination of a blood film may be helpful; this process is frequently manual, although some features may be determined by instrumentation. A blood smear requires particularly little blood, and it may be good practice to ensure a film is fixed and preserved (even if not necessarily examined) from each blood sample. From a blood film variation in cell shape, degree of staining, cellular inclusions and other features can be determined, and can be highly informative as to the cause of toxicological change. Reticulocytes are often part of the automated count and should be reported if available; reliable estimation of reticulocytosis in a blood smear, however, requires use of a vital stain additional to other procedures.

3.1.1.1 White Blood Cell Parameters

Total white blood cell counts, part of the automated CBC, can occasionally be informative, but the differential count is usually more helpful. A typical differential count will give the proportion of lymphocytes, neutrophils (banded and segmented), monocytes, basophils and eosinophils. Automated instrumentation is variable in methodology and ability to perform these counts, and while automated counts are sometimes presented, the count is often performed manually. A typical differential count will present results for each cell count as a percentage of total white cells; correction for total white cell count to give each cell type per unit volume (e.g. thousands per μl^3) will give a more accurate picture of how the differential

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count may be changing. A typical differential count will, however, not differentiate T and B lymphocytes, for which more specialist techniques are possible should immunotoxicity be suspected.

It is of interest to note that a guideline requirement for 'blood smears in all animals' to be examined for differential count at 52 weeks and termination of carcinogenicity studies, in the absence of CBC measurement in most of the individuals concerned, is for the purpose of detecting haematologic neoplasia. Presenting and interpreting these data as group mean values is seldom useful.

3.1.2 Clotting Function

Most testing guidelines require some measure of clotting function; Weingand *et al*. (1994) indeed recommend both prothrombin time and APTT. Although platelet count may give indication of changes in clotting ability, this is, in isolation, not a sufficient measure. Since tests of clotting function require a larger sample of blood than the CBC, these are perhaps conducted less often than guidelines recommend, particularly in rodent studies, although means to obtain sufficient sample are discussed in an earlier paragraph. Prothrombin time (the Quick test) indicates effectiveness of the intrinsic clotting system, while APTT measures effectiveness of the extrinsic system. An effect on either parameter can usefully be followed up by more detailed examination to determine which elements of the clotting cascade might be specifically affected, although the volumes of blood required are unlikely to be available with ease in rodents.

3.1.3 Bone Marrow Smears

Toxicological effects on red or white cell production can be inferred from CBC, but confirmation of a process affecting cell production requires a bone marrow smear. Histology of 'bone and bone marrow' does not permit the differential count required, since cells are too tightly packed for a differential count. Marrow smears require a separate procedure usually conducted at termination; marrow should be sampled as soon as possible after sacrifice. From large animals it may be possible to take a known volume of marrow and obtain absolute counts (thousands per μl^3). From smaller animals, it is usually necessary to either flush marrow from bones with a small volume of saline, or to smear marrow directly onto a slide using a paintbrush. Either method yields differential counts, of white and red cell (myeloid and erythroid) precursor cell types. Appropriate cell density in the marrow smear facilitates the counting procedure (cells must be spread thinly enough for cytoplasm to be easily visible, but closely enough to be easily counted within the microscope field of view). 'Painting' a long smear up and down the slide with a paintbrush can be a helpful technique. A key piece of information obtained from marrow smears is the 'myeloid/erythroid ratio', showing a balance of white cell to red cell production. Interpretation of this ratio indicates if either myeloid or erythroid cell production shows impairment.

3.1.4 Methaemoglobin

MetHb measurement is not commonly done, but may be a key mechanistic parameter in studies where indications of haemolytic anaemia are seen, for example, decreases in numbers of erythrocytes, presence of Heinz bodies or increases in spleen size. MetHb should also be investigated if the chemical structure gives rise to suspicion that the test compound or metabolites may give rise to MetHb, for example, aminophenols and aniline derivatives. Sampling and measurement requires careful preparation; the assay needs be conducted within a very short time of the sample being drawn and typically, the analysis occurs in or very close to the animal room. MetHb levels in normal samples are very low (0–4% varying with species and more so with analytical methodology), but since haemoglobin in a sample tube oxidizes slowly to MetHb, time from sampling to analysis is critical. Since the assay is not commonly performed, historical data in a relevant time frame is usually lacking. The author has personal experience of one case in which the tempo of sampling was disturbed when samples for one group of animals needed to be reanalysed; reanalysis complied with the standard operating procedure (SOP) specification (within 10 minutes of the sample being drawn), but the additional delay—no more than 3–5 minutes—resulted in increases in MetHb, to a measurable 0.2–0.4%. While the degree of increase was biologically insignificant (levels in excess of 25% being thought necessary for notable impairment of oxygen transport), the position of some regulatory authorities is that 'any increase in MetHb is adverse' (Luttik and van Raaij, 2001)—as indeed was confirmed in this case. Hence, care with methodology can be of great importance, study designs capable of confirming experimental repeatability are advisable (adequate sequencing would have avoided the example cited above), and expert interpretation of results may be critical. MetHb can be rapidly reversible *in vivo*, so timing of sample relative to administration of the test material must be considered; rats are good at recovering from MetHb formation due to high innate MetHb reductive ability, while dogs are considered more closely representative of man in this respect (see also Section 4.3.2). MetHb levels may be measured by spectrophotometric means either directly (e.g. the IL 682 Co-Oximeter System[a]®) or before and after the addition of cyanide (the latter is the principle of the method of Evelyn and Malloy, 1938). Spectrophotometric measurement of MetHb is subject to a number of interferences; these are rarely a problem in animal toxicological studies, but may be important in the treatment of human cyanide

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poisoning by MetHb formation. This is further discussed below.

4 ABNORMALITIES IN HAEMATOLOGICAL TESTS

As has been discussed at the beginning of this chapter, xenobiotics can affect the blood in numerous different ways. Experimental animals are, of course, generally used as models for human toxicological disease, but it must be remembered experimental animals are not perfect models for humans. As a generalization, laboratory animals are better predictors of dose-related haematological phenomena than of idiosyncratic human responses.

In both experimental animals and humans, increases and decreases in the numbers of particular blood cell types may be observed: a decrease in the number of red cells is characteristic of anaemia, while a diminution in the number of white cells is called leucopenia. A low platelet count is called thrombocytopenia. Leucopenia can affect all white cell types, or be more specific (e.g. granulocytopenia or lymphopenia). Cytopenias can be due to decreased production of the affected cell type, or to increased destruction. Increases in circulating cell types can also be seen: an increase in circulating red cells is called polycythaemia, of white cells, leucocytosis and of platelets, thrombocytosis. Again, specific white cell types may be affected, giving rise for example, to lymphocytosis. An increase in circulating eosinophils is often called eosinophilia. A feature of haematotoxicity is that changes in cell types rarely occur alone. Thus aplastic anaemia brings about a reduction in all formed elements of the blood and polycythaemia rubra vera is often characterized by increased circulating granulocytes and platelets, as well as red cells.

4.1 Changes in Red Blood Cells

4.1.1 Anaemia

Anaemia is a decrease in the number of red cells in the circulation, in the MCV or the MCH or any combination of these three (Irons, 1991). There are a number of mechanisms whereby xenobiotics may cause anaemia (**Table 2**). In some instances this may be a result of an interaction between an inherited predisposition; this is clearly the case in the haemolytic anaemia associated with G6PD deficiency and certain xenobiotics, where anaemia will only occur in the minority of the population carrying the deficiency. Toxic anaemias can be normocytic (e.g. aplastic anaemia), megaloblastic or microcytic. Toxic microcytic anaemias are often sideroblastic (see below).

4.1.1.1 Aplastic Anaemia

Aplastic anaemia is but one example of a group of conditions, the cytopenias, which result from bone marrow failure. Bone marrow failure may have various effects, granulocytopenia, thrombocytopenia, and so on, depending on the species, individual, xenobiotic involved or precise experimental conditions. These differences may be related to factors such as the rate of production and turnover of the various cell populations; a good example is sulphur mustard, where in experimental animals studies, all types of bone marrow failure can be produced depending on the type of study and the period that has elapsed after dosing (see **Toxicology of Chemical Warfare Agents**). Conditions where leucopenia or thrombocytopenia predominate are discussed below; those including anaemia are discussed in this section.

Aplastic anaemia is a severe form of pancytopenia. A number of xenobiotics, both drugs and other chemicals, give rise to bone marrow depression and, ultimately, aplastic anaemia (**Table 2**). There is a marked decrease in the cellularity of the bone marrow associated with a decrease in the number of circulating red cells, white cells and platelets. Immunosuppression may occur. In some cases, for example, with cytotoxic drugs, the condition is inevitable if the dose is sufficient, and the effect is dose-related, whereas in other cases the reaction is an idiosyncratic one, and is not dose-related. A large number of drugs have been reported on occasion to give rise to idiosyncratic aplastic anaemia. There is often difficulty with demonstrating a cause and effect relationship and this has also been a difficulty with some nondrug xenobiotics, for example, lindane (IPCS (International Program on Chemical Safety), 1991; Irons, 1992).

In the case of benzene, excessive exposure results in pancytopenia, and in severe cases aplastic anaemia (USDHHS, 1997), while low-dose exposure may cause falls in specific cell counts (i.e. anaemia, leucopenia or thrombocytopenia may occur separately). Lymphocytopenia seems to be a particularly reliable indicator of repeated benzene exposure (Irons, 1991). In animal studies, abnormalities of cellular and humoral immunity have been noted (Rozen and Snyder, 1985), while benzene and its metabolites may interfere with uptake of iron into bone marrow precursors, and it seems likely that reactive metabolites may play a part in benzene toxicity (USDHHS, 1997; Khan, 2007). Exposure to benzene is associated with leukaemia (see below).

Chloramphenicol has been known for many years to be haematotoxic. This antibiotic produces a dose-related, reversible anaemia with reticulocytopenia, sometimes with leucopenia and thrombocytopenia; this form of toxicity develops during drug treatment. Chloramphenicol, albeit rarely, can also produce aplastic anaemia, evident in the blood as a severe pancytopenia. The precise mechanisms whereby the toxicity arises is still unknown, but has been ascribed to single-stranded DNA breaks

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Table 2 Xenobiotic-induced anaemia

Type of anaemia		Examples[a]
Aplastic		Benzene Trichothecene toxins Drugs: Chloramphenicol Phenylbutazone Gold salts
Megaloblastic	Interference with vitamin B_{12} absorption or action	Nitrous oxide Drugs: PAS
	Dihydrofolate reductase inhibitors	Drugs: Methotrexate Trimethoprim
	Interference with other aspects of folate utilization	Drugs: Phenitoin Primidone Barbiturates
	Antimetabolites	Drugs: 6-Mercaptopurine
Microcytic, including sideroblastic		Alcohol Lead Drugs: Isoniazid
Haemolytic	Oxidant materials	Aminophenols Anilines Nitrites Drugs: Dapsone Salazopyrin Phenacetin Pesticides: Propanil Sodium chlorate
	Immune mechanisms	Various drugs including: α-Methyldopa PAS Sulfonamides Sulfonylureas Penicillin Cephalosporins
	Glucose-6-phosphate dehydrogenase deficiency[b]	Various xenobiotics including fava (broad) beans[c] Drugs: Primaquine Tafenoquine
	Others	Copper Arsine

[a]Ueno (1983), Hoffbrand (1996), Weatherall (1996), Amess (1993), Fisher (1992), Shanks *et al*. (2001), Beutler *et al*. (2007).

[b]This deficiency is relatively common in populations exposed to malaria against which the deficiency appears to provide some protection (Beutler *et al*., 2007).

[c]The toxic component of fava beans responsible is not entirely clear, but such a component may be divicine, an unstable aglycon metabolite of the fava bean pyrimidine β-glucoside, vicine (McMillan *et al*., 1993).

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produced by the chloramphenicol metabolite nitrosochloramphenicol, to the production of reactive oxygen species and to actions on mitochondria (Holt *et al*., 1993; 1997). There is disagreement as to whether the topical use of chloramphenicol represents a hazard (Rayner and Buckley, 1996; Smith *et al*., 1997; Walker *et al*., 1998; Doona and Walsh, 1998). Chloramphenicol is commonly used for eye infections in the UK, but rarely in the USA.

A number of other compounds can cause anaemia as part of pancytopenia, but the leucopenia is usually more prominent than the anaemia, for example, trichothecene mycotoxins (see below).

Pure selective red cell aplasia is unusual as a toxic effect, but can occur as an immune reaction to phenytoin and sulfathiazides (Krantz, 1983).

4.1.1.2 Megaloblastic Anaemia

Megaloblastic anaemia is characterized by large erythrocytes (macrocytosis), so that the haemoglobin is reduced and the MCV increased; additionally there is usually anisocytosis and poikilocytosis. The morphology of red cell precursors in the bone marrow is abnormal: changes are seen in the erythroid, granulocytic and megakaryocytic precursor cells, including large nuclei and Howell–Jolly bodies. The underlying abnormality is often disturbance of DNA synthesis, most commonly in human medicine as a result of an effect on vitamin B_{12} or folate. Megaloblastic anaemia as a toxic end point may result from interference with the absorption or metabolism of vitamin B_{12} or folate, or by a direct action on DNA metabolism. In fact, interference with the absorption of vitamin B_{12} by xenobiotics rarely causes severe deficiency, although a number of substances, such as ethanol, *p*-aminosalicylic acid (PAS) and colchicine are known to cause malabsorption of vitamin B_{12} (Hoffbrand, 1996). A number of drugs can interfere with folate metabolism: dihydrofolate reductase inhibitors interfere with the conversion of dihydrofolates to tetrahydrofolates, and include methotrexate and trimethoprim. Anticonvulsants, such as phenytoin, primidone and barbiturates can cause a deficiency of folate, possible due to poor absorption, enzyme induction, excess utilization or a combination of these three.

4.1.1.3 Sideroblastic and Other Microcytic Anaemias

In microcytic anaemia the haemoglobin is reduced, as is the MCV. Sideroblastic anaemia is one type of microcytic anaemia, in which there is excess iron in the bone marrow (see review by Alcindor and Bridges, 2002). The erythroblasts in the haemopoeitic organs contain granules of iron arranged in a ring around the nucleus ('ring sideroblasts'), these granules staining very easily with Prussian Blue (Weatherall, 1996). Alcohol intoxication is said to be the most common cause of toxic sideroblastic anaemia (Hines, 1969). Sideroblastic anaemia is characteristic of toxicants which interfere with the biosynthesis of haem and occurs in lead poisoning (Ramsden and Pawade, 2007), and after exposure to antituberculous drugs (isoniazid) and chloramphenicol. The microcytic anaemia of lead poisoning is only sometimes characterized by sideroblastic change in the bone marrow, but this anaemia is characterized in the periphery by prominent basophil stippling of the erythrocyte (Fried, 1997). Increased δ-aminolaevulinic acid (ALA) appears in the urine. Lead interferes with haem biosynthesis (see below) and also increases the fragility of the erythrocyte by binding to its membrane (Irons, 1992).

4.1.1.4 Haemolytic Anaemia

Haemolytic anaemias are characterized by reduced red cell survival, often accompanied by a raised bilirubin, reticulocytosis and, if severe, the presence of nucleated red cells in the peripheral circulation. Hyperplasia of erythropoeitic tissue occurs and extramedullary haematopoiesis can cause splenomegaly, which may be particularly prominent in rodents (Irons, 1991). There are two main mechanisms for xenobiotic-induced haemolytic anaemia: (i) oxidative stress and (ii) immune mechanisms. A combination of inherited G6PD deficiency and certain foreign compounds can give rise to a severe type of oxidative haemolysis, sometimes accompanied by intravascular haemolysis. This can be a serious problem with malaria therapy as this deficiency is relatively common in populations exposed to malaria, against which the deficiency appears to provide some protection (Beutler *et al*., 2007), precisely where antimalarials such as primaquine and tafenoquine are needed. With some drugs, for example, primaquine, the dose required to produce haemolysis of normal human erythrocytes is very much greater than that required in cells that are enzyme-deficient (Dern *et al*., 1955), but this is not the case with all drugs.

At least three mechanisms have been identified for immune haemolytic anaemia. Methyldopa is associated with an autoimmune reaction, while immune complexes can be responsible for PAS, sulfonamide and sulfonylurea-induced haemolytic anaemia. Penicillin and cephalosporins can act as membrane associated haptenes. The Coombs test is usually positive in drug-induced immune haemolytic anaemia (Hoffbrand and Pettit, 1984; Jacobs *et al*., 1990). Haemolytic anaemia due to oxidative stress may be accompanied by methaemoglobinaemia, sulfaemoglobinaemia and/or the appearance of Heinz bodies (see below). There are some compounds that can cause haemolytic anaemia, where the cause remains unclear, for example, copper (Fisher, 1992).

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4.1.2 Polycythaemia

Polycythaemia is the opposite of anaemia and is characterized by an increase in the haemoglobin and red blood cell count. An apparent polycythaemia will be observed in dehydration. Polycythaemia is seen in both experimental animals and humans as a compensatory phenomenon, where potential for tissue hypoxia exists. In human populations, polycythaemia is seen in those who dwell at high altitudes and in smokers, because of the intake of carbon monoxide by the latter. Cobalt is another cause of polycythaemia, apparently due to stimulation of renal erythropoietin release (Templeton, 1992). Polycythaemia rubra vera is an uncommon myeloproliferative disease seen in humans in which the polycythaemia is often accompanied by an increase in granulocytes and/or platelets. In the vast majority of cases the aetiology is not known, but cases have been described in personnel who took part in a test of nuclear weapons (Caldwell *et al*., 1984).

4.1.3 Toxicants Affecting Haemoglobin

The physiology of haemoglobin is considered at the beginning of this chapter. Toxicants can affect haemoglobin by: (i) influencing the synthetic pathway for haem (e.g. lead), (ii) by altering the final haemoglobin (e.g. various oxidant materials to produce MetHb) or (iii) by reacting with haemoglobin (e.g. carbon monoxide). The first process may cause anaemia, whereas methaemoglobinaemia and carbon monoxide poisoning both effectively decrease the oxygen carrying power of the blood, without necessarily causing anaemia.

4.1.3.1 Porphyria

The synthesis of haem involves the synthesis of porphyrins. This process involves the condensation of glycine and succinyl coenzyme A to form ALA and this is followed by a number of steps to protoporphyrin into which iron is inserted to form haem. Porphyrias are mostly hereditary diseases, but their importance to the toxicologist is twofold: (i) certain toxicants, for example lead, can cause acquired porphyria in normal human subjects and in experimental animals (ii) the (hereditary) acute porphyrias are often precipitated by various drugs in those predisposed to these diseases. These conditions are characterized by increased excretion of porphyrin precursors. Acute intermittent porphyria[b] is the most severe and most common of these conditions and is inherited as an autosomal dominant. The disease is particularly common in northern Sweden and is characterized by gastrointestinal, neurological and psychiatric abnormalities; it is caused by deficient activity of porphobilinogen deaminase (Deybach and Puy, 1995) and a notable diagnostic feature is that the urine turns dark (red-brown) on standing. Attacks may be precipitated by drugs such as phenobarbital, sulfonamides, oestrogens and alcohol. A full list of drugs that may precipitate attacks is available at *http://www.uq.edu.au/porphyria/*. In variegate porphyria, which is also inherited as an autosomal dominant, there is notable photosensitivity, systemic signs occur less frequently than in acute intermittent porphyria, but, as in the latter, coloration of the urine occurs (see McColl *et al*., 1996; Hift *et al*., 1997; Bari, 2007). This condition is particularly common in white South Africans and is caused by deficient activity of the protoporphyrinogen oxidase gene (Frank *et al*., 1998). Porphyria cutanea tarda is a chronic condition, only a proportion of cases being genetic: these are due to a reduction in activity of the enzyme, uroporphyrinogen III decarboxylase and the condition is inherited as an autosomal dominant. The main symptoms and clinical signs are photosensitivity, which leads to skin damage and scarring (Sarkany, 2001), and discoloured urine may also be reported (Rich, 1999). Lead produces an acquired porphyria by inhibiting the haem biosynthetic enzyme ALA dehydratase, while the activity of ferrochelatase is also reduced. By contrast, the activity of ALA synthetase is increased. As a result, levels of ALA in the blood and urine are increased, as are coproporphyrin III and zinc protoporphyrin (see Feldman, 1999). A number of other chemicals are porphyrogenic, for example, dichlorobenzene (NTP, 1987), the organochlorine pesticides lindane and heptachlor in sufficient dose (Simon and Siklósi, 1974; Mylchreest and Charbonneau, 1997; Taira and San Martin de Viale, 1998), hexachlorobenzene (WHO, 1997) and certain herbicides (Jinno *et al*., 1999). Indeed, hexachlorobenzene has been used to produce a model of porphyria cutanea tarda in the rat (Llambías *et al*., 2003). Disorders of haem biosynthesis have been described in animals, including cattle, pigs, sheep and cats. Porphyria cutanea tarda has been reported in pigs (Nezamzadeh *et al*., 2005). Where porphyria has been seen in experimental animals, discoloured urine is the usual clinical sign. The porphyrins causing discolouration are not specifically detected by the usual urine test strips; more detailed studies of pigments in the urine, liver and blood are then indicated.

4.1.3.2 Oxidant and Other Changes in Haemoglobin

Methaemoglobinaemia and carboxyhaemoglobinaemia, the latter resulting from the binding of carbon monoxide to haemoglobin, cause changes in the colour of the blood; carboxyhaemoglobin is a cherry-red colour and MetHb brown. In addition, a number of other derivatives of haemoglobin have been described (**Table 3**).

4.1.3.2.1 Methaemoglobin (MetHb)

MetHb is the pigment that results when the iron of haemoglobin, normally in the ferrous (Fe^{2+}) state, is oxidized to the ferric state (Fe^{3+}). The toxicological importance of MetHb is twofold:

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Table 3 Oxidant and other derivatives of haemoglobin

Pigment	How formed
Methaemoglobin (MetHb)	Oxidation of Fe^{2+} to Fe^{3+} in haemoglobin
Sulfhaemoglobin	Ill-characterized pigment associated with oxidant changes in the erythrocyte
Cyanmethaemoglobin	Reaction of cyanide with MetHb
Sulfmethaemoglobin	Reaction of sulfide with MetHb
Carboxyhaemoglobin	Reaction of carbon monoxide with haemoglobin

1. MetHb is incapable of carrying oxygen reversibly in the way that haemoglobin does, with the result that methaemoglobinaemia represents a loss of oxygen carrying power of the blood and very high levels of MetHb (>50%) are thus potentially lethal.
2. MetHb can combine with cyanide, sulfide and azides and thereby ameliorate their toxic effects (see **Antidotal Studies**).

A large number of compounds that produce other oxidant changes in the erythrocytes (**Table 2**) can also produce methaemoglobinaemia. However, there are also other causes of methaemoglobinaemia, some of which are nontoxicological. Two MetHb reductase enzymes serve to keep MetHb levels in the red cells low; deficiency, which may be inherited, causes raised level of MetHb. In human populations, certain unusual haemoglobins are more susceptible to oxidation that normal haemoglobin. This can result in high levels of MetHb, as can inherited low MetHb reductase activity. MetHb levels after exposure to MetHb-generating xenobiotics are determined by levels of the xenobiotic or its active metabolite, the intrinsic sensitivity of the haemoglobin to oxidation and the activity of MetHb reductase (Marrs and Bright, 1986; Bright and Marrs, 1986). But the marked interspecies differences that have been observed appear mainly to result from differences in the ability of the erythrocyte to reduce red cell MetHb levels, laboratory rodents having a high capacity and dogs, cats and humans and (probably) nonhuman primates having a much lower capacity (Kiese and Weis, 1943; Malz, 1962; Robin and Harley, 1966; Stolk and Smith, 1966; Smith and Beutler, 1966; Smith *et al*., 1967; Bolyai *et al*., 1972; Agar and Harley, 1972; Hawkins *et al*., 1981; Marrs *et al*., 1987; Purnell and Singh, 2005). In toxicological studies, where only rodents are used, the low propensity of rodents for demonstrating methaemoglobinaemia may cause the potential of a xenobiotic to cause toxic methaemoglobinaemia to be missed, a factor which is often not adequately appreciated by regulatory bodies. Suspicion of toxic methaemoglobinaemia in rodent studies should be triggered by anaemia with enlargement and discolouration of the spleen, and/or presence of Heinz bodies in erythrocytes. In practice, of species used in toxicological studies, dogs are probably the best models for humans.

Table 4 Some compounds producing methaemoglobinaemia[a]

Chemical or drug group	Examples
Aminophenols and derivatives	2-Aminophenol 4-Aminophenol 4-Dimethylaminophenol
Anilines	Aniline 4-Aminopropiophenone Propanil
Antimalarial drugs	Chloroquine Primaquine
Antileprotic drugs	Dapsone
Chlorates	Sodium chlorate
Nitrites	Amyl nitrite Sodium nitrite
Hydroxylamines	Hydroxylamine Phenylhydroxylamine
Sulphonamides	Sulfamethoxazole

[a]Kiese *et al*. (1950), Beutler (1985), Hall *et al*. (1986), Watt *et al*. (2005).

Chemically induced methaemoglobinaemia can result from exposure to a very large number of toxicants, many of which are aniline derivatives, aminophenols or nitrites (**Table 4**).

Aminophenol and its derivatives, including 4-dimethylaminophenol, appear to form MetHb by establishing an intraerythrocytic cycle in which a quinoneimine is formed by oxidation of the aminophenol by the oxygen from oxyhaemoglobin. On the reduction of the quinoneimine to the aminophenol, the Fe^{2+} iron of haemoglobin is oxidized to its trivalent state. With the aniline derivatives, including 4-aminopropiophenone (*p*-aminopropiophenone, PAPP), a nitroso intermediate is involved in the formation of MetHb. The methaemglobin formation observed with the antileprotic drug, dapsone, appears to be mediated by hydroxylamine metabolites (Vage *et al*., 1994). MetHb formation is strongly influenced by pO_2 (Kiese, 1974; Mansouri, 1985; Marrs *et al*., 1987).

Spectrophotometric measurement of MetHb is subject to a number of interferences, such as hydroxocobalamin (Lee *et al*., 2007) and methylene blue (Dötsch *et al*., 1999). It should be noted that the spectrophotometric characteristics of cyanmethaemoglobin and MetHb are different, so that in cyanide poisoning, incorrect dosages of MetHb-forming antidotes may be calculated, and misinterpretation of MetHb measurements can result from methods that fail to discriminate between the various pigments (Shih and Korte, 1996).

Methaemoglobinaemia may be treated with methylene blue or toluidine blue (Kiese *et al*., 1972; IPCS/CEC (International Program on Chemical Safety/Commission of the European Communities), 1993).

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4.1.3.2.2 Heinz Bodies

Heinz bodies are inclusions in red blood cells that consist of denatured globin (White *et al*., 1951). Heinz bodies can be seen in wet preparations of blood, using phase contrast microscopy and they can also be stained with methyl violet (Bushby, 1970). Their appearance may be an accompaniment to haemolytic anaemia, produced by oxidant xenobiotics ('Heinz body anaemia'), including those substances that produce MetHb. However, there does not appear to be close correlation between ability to produce MetHb and Heinz bodies, or of the latter with erythrocytic depletion of reduced glutathione (Miller and Smith, 1970). Moreover, in toxicological studies, there can be marked species differences in the production of Heinz bodies with the same substance (Marrs *et al*., 1984).

4.1.3.2.3 Sulfhaemoglobin

Sulfhaemoglobin is a rather ill-characterized pigment that differs from MetHb by resistance to reducing agents. It should be distinguished form sulfmethaemoglobin (**Table 3**).

4.1.3.2.4 Carboxyhaemoglobin

Carbon monoxide binds to haemoglobin in precisely the same way as does oxygen, though the affinity of carbon monoxide for haemoglobin is about 245 times that of oxygen. This means that if the carbon monoxide saturation of haemoglobin is plotted against the partial pressure of carbon monoxide, a curve of precisely the same shape as the standard oxyhaemoglobin dissociation curve would be obtained. Indeed, if one adjusted the scales on the partial pressure axes, the curves could be superimposed. It is also worth recalling that, at saturation, 1.34 ml of either oxygen or carbon monoxide is carried per gram of haemoglobin. If haemoglobin is exposed to a mixture of oxygen and carbon monoxide, the gases will compete for the binding sites. At equilibrium the, relative concentrations of oxyhaemoglobin and carboxyhaemoglobin are given by the Haldane Equation:

$$[\mathrm{COHb}]/[\mathrm{O_2Hb}] = \mathrm{M} \cdot [\mathrm{PCO}]/[\mathrm{PO_2}] \qquad (1)$$

M is referred to as the Haldane coefficient.

The effect of the great affinity of carbon monoxide on the uptake of the gas relative to that of oxygen is discussed in **Air Pollution**.

In addition to reducing the capacity of haemoglobin to carry oxygen, carbon monoxide inhibits the release of oxygen by haemoglobin. Under normal circumstances, as oxygen atoms dissociate from haemoglobin, the affinity of the molecule for oxygen drops rapidly. This produces the sudden change in gradient at the shoulder of the oxyhaemoglobin dissociation curve. To move from the relaxed, high-oxygen-affinity state to the tense, low-affinity state, oxyhaemoglobin must lose oxygen atoms. In carbon monoxide poisoning, haemoglobin molecules do not carry either oxygen alone or carbon monoxide alone; on the contrary, haemoglobin molecules will likely carry a mix of oxygen and carbon monoxide molecules. As the partial pressure of oxygen falls, at the tissues, for example, oxygen molecules would normally be released and haemoglobin would move from the relaxed to the tense state. However, if the haemoglobin molecule is carrying carbon monoxide molecules in addition to oxygen, the carbon monoxide molecules will not be released and the relaxed, high-oxygen-affinity state will be maintained. This effect can be summed up as: the binding of carbon monoxide molecules to haemoglobin inhibits the release of oxygen. This is seen in carbon monoxide poisoning as a shift of the oxyhaemoglobin dissociation curve to the left. This shift is sometimes referred to as the Haldane shift, though the term is confusing, as it is also used to describe the shift in affinity of haemoglobin for carbon dioxide that occurs when haemoglobin loses oxygen molecules. The above details were worked out by J. S. Haldane and his son J. B. S. Haldane in the early years of the century (Haldane, 1912; Douglas *et al*., 1912).

The shift in the dissociation curve causes a major problem for the release of oxygen at the tissues. An often quoted example is the difference between the effects of anaemia, with the haemoglobin concentration reduced to 50% of normal, and carbon monoxide poisoning, with a carboxyhaemoglobin concentration of 50%. In the case of the anaemia the venous pO_2 would be about 3.6 kPa (27 mmHg); in the case of carbon monoxide poisoning the venous pO_2 would be about 1.9 kPa (14 mmHg): very significantly lower (Nunn, 1993).

Exposure to carbon monoxide results in equilibration of the gas across the alveolar/capillary barrier. As has been already stated, the equilibrium conditions are given by the Haldane Equation. The rate of increase of carboxyhaemoglobin conforms to a 'wash in' exponential function. More detailed modelling of the kinetics of carbon monoxide uptake has been undertaken (Coburn *et al*., 1965; Coburn, 1979) and complex equations have been produced.

Carbon monoxide poisoning is relatively common because the gas, which is odourless, can be formed by partial combustion of natural gas, a fuel commonly used for heating in many countries. At high concentrations of carboxyhaemoglobin (>50%), carbon monoxide causes death, while concentrations as low as 3–4% may cause angina, presumably through impaired oxygen transport (DoE, 1994). Additionally, delayed effects can occur in the central nervous system after apparent recovery.

The affinity of foetal haemoglobin for oxygen is greater than that of adult haemoglobin (Longo, 1976; Garvey and Longo, 1978; Longo *et al*., 1967; Power and Longo, 1975). In addition, the foetal oxyhaemoglobin dissociation curve is displaced to the left of the equivalent adult curve. Foetal haemoglobin also has a greater affinity for carbon monoxide than adult haemoglobin, though the

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foetal/adult difference in affinity is not as great as that for oxygen. Engel gave the CO/O_2 affinity ratio as 175 for foetal haemoglobin, as compared with 245 for adult haemoglobin (Engel *et al*., 1969).

As in the adult, carbon monoxide shifts the foetal oxyhaemoglobin dissociation curve to the left. This is particularly dangerous, as the foetus is always at a significantly lower partial pressure of oxygen than the adult. A 10% level of carboxyhaemoglobin will move the maternal venous pO_2 to 26 mmHg, but the foetal venous pO_2 to 11 mmHg. Uptake into and release of carbon monoxide from the foetus are delayed compared with the mother. This is a reason for considering treatment with hyperbaric oxygen whenever carbon monoxide poisoning occurs during pregnancy.

It should be noted that carboxyhaemoglobinaemia can occur due to metabolism of methylene chloride, commonly used as a paint remover (Hughes and Tracy, 1993).

4.2 Changes in White Cells

Reductions in the number of white cells (neutropenia) may affect all, a few, or just one type of white cell. It may be part of a pancytopenia or, even if all blood cell types are affected, the leucopenia may predominate; this is common with T-2 mycotoxin, poisoning with which is often characterized by some degree of anaemia, but marked leucopenia and thrombocytopenia are usually the most prominent features (Smalley, 1973; Hayes *et al*., 1980; Ueno, 1984). Trichothecene toxins produced by fungal species such as *Fusarium* are responsible for a number of syndromes, including alimentary toxic aleukia (ATA) in Russia and red mould disease in Japan. ATA occurs after the consumption of over-wintered mouldy grain and includes leucopenia, haemorrhages, sepsis and bone marrow failure (Lutsky *et al*., 1981; Lautraite *et al*., 1995; Ueno, 1983; 1984; Wang *et al*., 1998).

4.2.1 Neutropenia/Granulocytopenia/Agranulocytosis

Severe neutropenia or granulocytopenia is often called agranulocytosis. Neutropenia may result from inadequate production or increased destruction, while an apparent neutropenia can result from a shift into the blood marginal pool (Marsh, 1985). The former may occur as part of aplastic anaemia, but many compounds capable of producing aplastic anaemia, sometimes affect granulocytes and their precursors more severely than cells of the erythrocyte series, perhaps because of the erythrocyte's longer life span. Granulocytopenia can be a dose-related predictable effect as with radiation (Cronkite, 1949), various anticancer drugs, chloramphenicol and the mustards (including the chemical warfare agent, sulphur mustard (Marrs *et al*., 1996; see also **Toxicology of Chemical Warfare Agents**) or an idiosyncratic response. Idiosyncratic neutropenias fall into two groups: the slowly-developing dose-dependant type which has been reported with chloramphenicol, phenothiazines, sulfonamides and numerous other substances. The rapidly developing type of idiosyncratic neutropenia is rare, often accompanied by eosinophilia and is sometimes catastrophic. There is often a history of prior exposure (Marsh, 1985) and the effect is notoriously difficult to predict from animal studies. Amongst compounds producing this effect are chloramphenicol, gold salts and nonsteroidal anti-inflammatory drugs. It will have been noted above that chloramphenicol can produce several types of neutropenia.

Neutropenia, analogous to haemolytic anaemia, can occur because of increased cell destruction that may result from immunological mechanisms, for example, with pyrazolone analgesics, including aminopyrine (Brogden, 1986; see review by Uetrecht, 1996).

Increases in the polymorph count are found in acute infections, in leukaemias of the myeloid series and also with lithium carbonate. A notable toxicological cause of eosinophilia was the eosinophilia-myalgia syndrome seen in the early 1990s, after contamination of certain preparations of the food supplement, and essential amino acid, L-tryptophan, with a contaminant (Harati, 1994).

4.2.2 Leukaemias

Leukaemias are a heterogeneous group of malignant diseases involving the precursors of peripheral blood cells. Most arise in the bone marrow and exhibit high levels of circulating white cells. They are classified by the cell type involved and also into acute and chronic (see Tsongas, 1985). Leukaemias are seen in many species, including man and experimental animals such as mice and rats (for review of appearances and diagnostic criteria in experimental animals see Della Porta *et al*., 1979). There are notable strain differences in susceptibility of mice to leukaemia and also in rats (F-344 having a notably high incidence) (see Haseman *et al*., 1998).

Exposure to a number of drugs and other chemicals can be associated with leukaemias, as can radiation. A notable example is benzene (see also above), where exposure of human populations seems causally related to acute nonlymphocytic leukaemia and chronic myelogenous leukaemia (Paxton *et al*., 1994; Jex and Wyman, 1996; Mehlman, 2006). Benzene causes chromosomal abnormalities in a number of cell types, including blood. The precise mechanisms of benzene leukaemogenesis have not been elucidated: benzene is metabolized to benzene oxide and thence to phenol in the liver (Tunek *et al*., 1978; Sawahata *et al*., 1985) and chronic exposure to benzene results in the presence of metabolites in the bone marrow, including hydroquinone and, additionally benzene itself. These compounds produce a reduction

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in circulating lymphocytes and are immunotoxic (Pyatt *et al*., 1998) and they also bring about an increase in cells of the granulocyte series. Further, benzene and its metabolites have been shown to induce DNA damage, which may contribute to leukaemogenesis, plausibly perhaps in combination with the increased replication of primitive progenitor cells, as has been observed in laboratory animals (Farris *et al*., 1997). Another possibility is that activation of protein kinase C may alter activation and expression of cellular proto-oncogenes (see review by Snyder and Kalb, 1994). There is some evidence that benzene may be associated with other malignant conditions, including Hodgkin's disease (see Jex and Wyman, 1996).

Drugs where there is an association with leukaemias in laboratory rodents include anticancer drugs (Schmahl and Habs, 1978). Hydrocarbons such as methylcholanthrene, benzpyrene and dimethylbenzanthacene can also produce leukaemias in rodents (Rappaport and Baroni, 1962). Exposure to a number of chemicals in humans is associated with increased prevalence of leukaemia; furthermore there is an association of increased risk of leukaemia and certain occupations, for example, the rubber industry (Alder *et al*., 2006), where, however, the precise causative agent is not known (Irons, 1992).

4.3 The Clotting System

4.3.1 Trombocytopenia

Thrombocytopenia is amongst the commonest blood dyscrasias reported as due to xenobiotics, especially drugs (Irons, 1991). The results are purpura and spontaneous haemorrhage, or bleeding in response to minor trauma. As with red cells and granulocytes, a reduction in circulating platelets can result from decreased production or increased destruction. Decreased production of platelets may be seen with thiazide diuretics, which are toxic to megakaryocyte precursors (Levin, 1974). Other drugs which may cause thrombocytopenia by toxicity to the bone marrow include chloramphenicol (Yunis, 1973), sulfonamides (Pena *et al*., 1985) and phenytoin (Brown and Chun, 1986), however, many drugs that cause thrombocytopenia by an effect on the bone marrow can also produce other signs of bone marrow failure, depending on the circumstances, and isolated thrombocytopenia with some drugs, for example chloramphenicol, may proceed to aplastic anaemia. Increased destruction of platelets is generally of immunological origin, although not in the case of ristocetin, an antibiotic which has a dose-related effect on platelets, by a nonimmunological mechanism. Drugs that produce thombocytopenia by immune mechanisms include quinine and quinidine (Christie *et al*., 1985), and gold salts (Stavem *et al*., 1988).

4.3.2 Drugs Affecting the Coagulation System

Anticoagulants of the coumarin group, some of which are used as rodenticides, interfere with vitamin-K dependent blood-clotting factors (see Proudfoot, 1996; **Toxicology of Pesticides**. Coumarol, in mouldy sweet clover, is an important cause of haemorrhagic disease in cattle (Blood *et al*., 1983).

5 CONCLUSIONS

The components of the blood can be affected by many xenobiotics, most particularly therapeutic drugs. Many of the techniques used in human and veterinary medicine have not been widely applied to laboratory animal toxicology studies. This limits the use to which information from routine laboratory animal toxicology studies may be put. Part of the reason for this is that haematology is often the Cinderella of disciplines in the toxicology laboratory by comparison with clinical chemistry and particularly histopathology; there is also the tendency for guidelines rapidly to become a recipe, which has to be followed, but whose scope should not be exceeded unless specifically required by regulators. This is a great pity, because the haematological data in toxicology studies represent a great deal of effort and investment and, insofar as satellite groups of animals are employed, a cause of usage of additional experimental animals. It is therefore only right that such data should be exploited to the hilt and used to their best possible advantage.

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NOTES

a. GMI, Inc. Ramsey, MN, USA.

b. This is the form of porphyria from which it is alleged King George III of Great Britain suffered.

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Imaging Techniques in Neurotoxicology Research and Development

Philippe Hantson and Thierry Duprez

CONTENTS

1 INTRODUCTION

The brain as a major target organ in toxicology can be injured by numerous substances ranging from pharmaceuticals to illicit drugs and environmental agents. Dramatic improvements have been obtained in the last two decades in the knowledge of toxic damage to the brain regarding both morphological changes and functional disturbances of the neural circuitry (Lingford-Hughes, 2005). This review focusses on recent advancements in neuroimaging techniques and their implementation in clinical practice and on fundamental aspects of research and development (Laureys *et al*., 2002). The most commonly involved toxic substances are reviewed, together with the morphological and functional changes they are responsible for. A key feature of neuroimaging after toxic exposure seems to be the selective susceptibility of some brain subareas to toxins (Hantson and Duprez, 2006; Dietemann *et al*., 2004). The grey matter seems more vulnerable to anoxic or ischaemic insults because of higher demands in oxygen. The corpus striatum (putamina and caudate nuclei) is the most vulnerable grey area (**Figure 1A–E**). It is also affected by toxic-related metabolic disorders (metabolic acidosis, hypoglycaemia) and contains many sensitive dopaminergic and serotoninergic pathways which have major functional roles. Lesions to the white matter seem less frequent, but have been up to now less

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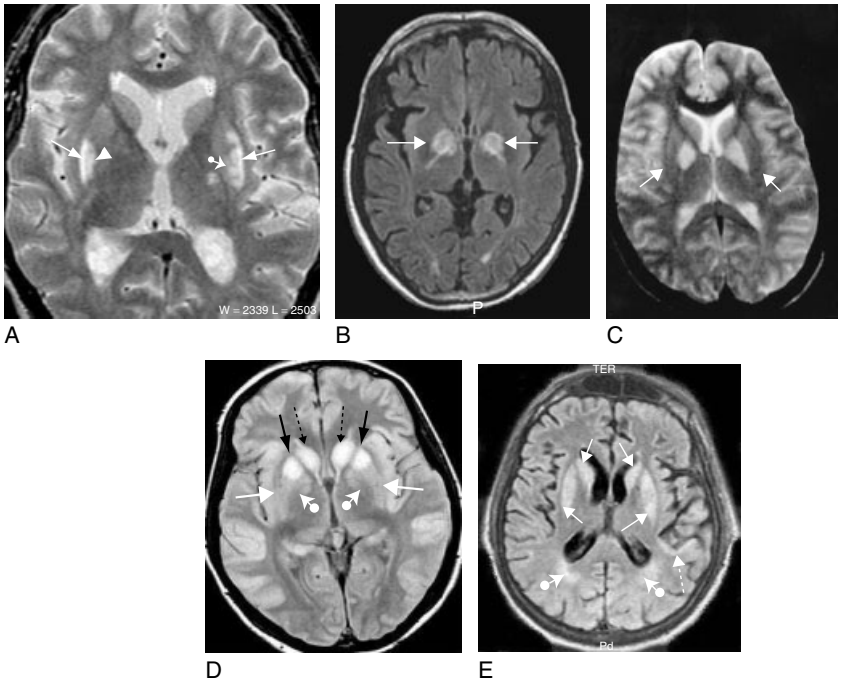

Figure 1 Illustration of the lenticulate involvement in different toxic or metabolic conditions. (A) Methanol-induced putaminal changes predominating in the posterior areas on T2-weighted sequences. (B) Relative preservation of the putamina, contrasting with the bilateral and symmetric involvement of the medial part of the globi pallidi in a patient exposed to cocaine and ecstasy. (C) Carbon monoxide poisoning with preferential injury of the whole globi pallidi also with sparing of the putamina. (D) Insulin overdose, with involvement of the anterior areas of the putamina. (E) Putamina and caudate nuclei appearing hyperintense after hypoxic injury due to cardiac arrest. (Reproduced with permission from Hantson, 1997. © Informa Healthcare.)

extensively investigated than grey matter damage. Besides morphological changes, recent neuroimaging techniques allow the additional investigation of ultrastructural parameters, metabolism, regional cerebral blood flow (CBF) and neuronal activation/reactivity. A deeper insight into the pathophysiology of drug abuse is expected from isotopic techniques using radiolabelled tracers acting as ligands to specific membrane receptors.

2 PRINCIPLES

2.1 Computed Tomography

The introduction of computed axial tomography in clinical practice in the early 1970s dramatically improved the diagnostic performance of neuroimaging. The technique, which used X-rays, was immediately considered to be best suited for the detection of cerebral abnormalities until the introduction of magnetic resonance imaging (MRI) one decade later, which demonstrated unsurpassed soft tissue contrast (see below). The technique, which is routinely called a CT scan uses an X-ray generating tube placed in opposition to X-ray detectors on a rotating frame. During rotation around the patient, the detectors collect continuous data about the attenuation of the X-rays when passing through the body at many different incidences. A backspace reconstruction algorithm of attenuation data allows the generation of a matrix image in which the attenuation coefficient of each voxel is visualized by a calculated value on a greyscale. Technological improvements, such as reduction in the size of the detectors, the helical acquisition technique, increases in the rotation speed of the tube-detector frame and the multirow detector configuration have led to higher temporal and spatial resolution, but without true impact on brain tissue contrast. Intravenous perfusion of an iodinated contrast agent allows enhancement of vascularized cerebral areas in which the breakdown of the blood–brain barrier (BBB) results in interstitial leakage of contrast molecules. Up-to-date systems enable reconstruction of 'isotropic' voxels of similar size in the three orthogonal dimensions, thereby resulting in a similar image quality for reformats in all planes.

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2.2 Magnetic Resonance Imaging

MRI is derived from the NMR phenomenon described in 1948 by Bloch and Purcell. The initial technical application of the technique was the elucidation of the atomic structure of complex organic macromolecules such as the double helix of DNA. The MRI technique uses the magnetic properties of the hydrogen nuclei—the 'protons'—which are the main atomic component of water and fat. Protons behave like spinning charges and generate a very small dipolar magnetic field. In standard conditions, the proton dipoles have completely random orientation and statistically cancel each other so that there is no net magnetization of the tissues. When the patient's body is immerged into strong external 'basic' or 'static' magnetic field, the protons are compelled to align either in a 'parallel' low-energy state, or in an 'antiparallel' high-energy state. The excess in low-energy parallel spins constitutes the 'net magnetization' of the tissues. When an excitatory radiofrequency pulse is applied at a similar frequency to the spinning of the proton dipoles, the resonance phenomenon occurs and energy is transferred to the parallel spins, which are tipped in the antiparallel direction, thereby creating the disappearance of the longitudinal 'T1' magnetization. Moreover, the application of a resonant pulse generates a phase coherence of the processing spins, which creates the appearance of a transverse 'T2' magnetization. When the external pulse is stopped, the system comes back to equilibrium. The time needed to recover 66% of the value of the longitudinal magnetization is called the 'T1 relaxation time' and the time needed to destroy 66% of the transverse magnetization is called the 'T2 relaxation time'. T1 and T2 values, as well as the spin density, are intrinsic properties of the tissues. They differ for water and fat, and for free water when compared to bound water adsorbed to macromolecules. By appropriate manipulation of the pulse sequence parameters, one may create tissue contrast mostly depending on the T1 time (the so-called 'T1-weighting'), or on the T2 time ('T2-weighting'). Since grey and white matter have different proportions of water (e.g. cytoplasm) and fat (e.g. myelin), high-quality tissue contrast will be obtained for brain tissue. Anatomy is best depicted on T1-weighted images, but increases in free water content, such as inflammation, oedema or demyelination, is best detected on T2-weighted images. Intravenous paramagnetic contrast agents are magnetic polluters strongly decreasing the T1 relaxation time of adjacent protons, thereby leading to a dramatic increase in signal intensity on T1-weighted images. Additional contrast manipulations are available. In fluid-attenuated inversion recovery (FLAIR) images, the signal of free water is nulled, resulting in darkening of cerebrospinal fluid (CSF), but with preservation of the T2 contrast of the brain tissue. In diffusion-weighted (DW) images, the signal intensity is reduced according to the degree of restriction of free-water motion, that is, by microanatomical barriers, such as membranes, thereby giving ultrastructural information on tissues. A quantitative apparent diffusion coefficient (ADC) can be calculated to generate functional mapping of the restriction of water diffusion. In processes increasing the extracellular compartment, the ADC is increased. In turn, in processes in which diffusion is restricted, such as ischaemic cytotoxic oedema, or proliferative disorders involving dense cellularity, the ADC is lowered.

2.3 Functional Magnetic Resonance Imaging

Functional MRI of neuronal activation relies on the blood oxygen level dependant (BOLD) contrast principle (Ogawa *et al.*, 1990). Oxygenated haemoglobin (Hb) has a low magnetic susceptibility effect. In turn deoxyhaemoglobin has a higher susceptibility effect, resulting in a marked decrease in signal intensity. The overall drop in signal intensity is a function of the ratio between oxy- and deoxyHb within blood in capillaries. At baseline, a certain proportion of deoxyHb is present within a voxel. When neurons of the voxel are activated, a local increase in blood flow suddenly occurs, which exceeds the true increase in oxygen needs by neuronal and synaptic activity, resulting in a relative decrease in the ratio between deoxy- and oxyHb. The signal attenuation is diminished and the comparison of signal at rest and during the task allows the delineation of the brain voxels being activated by the task.

2.4 Magnetic Resonance Spectroscopy

Magnetic resonance spectroscopy (MRS) was the original technique designed in the late 1940s and early 1950s to investigate the atomic structure of organic macromolecules. It relies on the principle of chemical shift, which is the small change in precession frequency of a defined atom according to its electronic, atomic and molecular environments. When combined with MRI, the technique allows the detection and quantification of defined metabolites. Proton magnetic resonance spectroscopy (^{1}H-MRS) is the most commonly used in the brain, allowing, for example, the study of choline (Ch), creatinine (Cr), *N*-acetyl aspartate (NAA), myoinositol (MI), alanine and lactate. Spectroscopic analysis of other nuclei (phosphorus, carbon, sodium, lithium) can be performed. Quantitative analysis of adenosine triphosphate (ATP) concentration is enabled by ^{33}P spectroscopy.

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2.5 Positron Emission Tomography

The initial step leading to the development of positron emission tomography (PET) was tissue autoradiography which investigated *in vitro* the accumulation of organic molecules labelled with radioactive elements, thereby acting as tracers. Because glucose is the unique source of energy for neurons, a radioactively labelled form of glucose can be used to measure cerebral metabolism. Positron-emitting tracers are detected and located by multiple sensors positioned around the head. Functional PET imaging can be combined with high-anatomic-resolution CT or MRI (Otte and Halsband, 2006). The kind of information obtainable depends upon the nature of the radioisotopes. The most commonly used radioisotopes are oxygen-15, fluorine-18, carbon-11 and nitrogen-13. They can be combined with different molecules like water, glucose, benzodiazepine-receptor ligands etc. [^{18}F]-fluorodeoxyglucose (FDG) is captured by neurons. Since it is not an appropriate metabolite for the glucose kinase, it can not be further metabolized and remains trapped within the intracellular compartment. An FDG-PET scan thereby allows the mapping of regional variations in cerebral glucose uptake. PET using water labelled with oxygen-15 ($H_2{}^{15}O$) allows precise measurement of regional cerebral blood flow (rCBF). The very short half-life of the tracer (2 minutes) allows repeated experiments in the same session and prevents potentially harmful effects of longer exposure to radioactivity. Specially tailored PET tracers allow receptor imaging in clinical pharmacology and toxicology.

2.6 Single Photon Emission Computed Tomography

In contrast to PET, single photon emission computed tomography (SPECT) is based on the detection of individual photons (low-energy gamma rays) rather than positrons, which are paired. The radioisotopes used for SPECT have longer half-lives than those used for PET, thereby avoiding the need for the availability of an on-site cyclotron. However, the spatial resolution is lower with SPECT as is the range of the cerebral activities that can be investigated. Absolute quantification is not possible. The standard radioisotopes for SPECT are technetium-99m and iodine-123. Tc-99m-hexamethyl propylamine oxime (Tc-99m-HMPAO) is widely used for the determination of CBF. As the tracer remains within the brain for a longer period than PET isotopes, the capability for analysing long-lasting processes is an advantage of SPECT over PET (Marshall *et al*., 1997). Iodine-123-labelled ligands have been specifically developed to investigate interactions between brain receptors and neurotransmitters or drugs.

2.7 Electroencephalography

Electroencephalography (EEG) is the oldest technique for detecting electrical activity in the brain from the scalp. EEG is a routine validated procedure for the diagnosis of numerous brain disorders in neurology, neurosurgery and psychiatry. The EEG recording is the sum of activities of a large number of neurons, thereby resulting in low spatial resolution. In turn, excellent temporal resolution with a range of a few milliseconds can be obtained. Nonspecific EEG changes (diffuse slowing) are frequently observed in subjects with impaired consciousness due to toxic exposure. High sensitivity in detecting infraclinical seizure activity is the main advantage of the technique in day-to-day practice.

2.8 Evoked Potentials

Evoked potentials (EPs) are a recording of the electroencephalographic activities under different stimuli: visual, auditory or somatosensory. They can be used to test the integrity of the corresponding neural pathways. They are mainly used in patients presenting acute neurological disorders (brain anoxia, brain trauma).

Brainstem auditory evoked potentials (BAEPs) and median nerve somatosensory evoked potentials (SEPs) investigate the brainstem, while the cerebral cortical level is assessed by median nerve SEPs and flash visual evoked potentials (fVEPs). At the same time, the peripheral structures are also assessed.

BAEPs bring information about the functioning of cochlea (wave I), VIIIth nerve (waves I and II), cochlear nuclei (wave II), trapezoidal bodies (wave III) and lateral lemniscus (waves IV and V). Median nerve SEPs allow, thanks to a multilevel recording, checking of the somatosensory lemniscal pathways from peripheral nerve (N9 wave at the Erb's point) to associative cortex (parietal P45 and frontal P30 waves) going through the spinal structures (cervical N13 wave), the medial lemniscus (P14 wave) and the primary cortex (parietal N20 wave). fVEPs give information on retinal functioning (wave I), on primary occipital cortex functioning (waves III and IV) but also on associative cortex activation (wave VII and rhythmical after-discharge). A bilateral occipital recording allows the detection of a retrochiasmatic lateralization between left and right occipital areas.

The latency between peaks gives an idea of the conduction time between these different structures (interpeak latencies between the BAEPs wave, central

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conduction time between cervical N13 and parietal N20 of the median nerve SEPs) and allows, among others, the assessment of subcortical conduction. In the case of some specific question, other modalities can be used. If a prechiasmatic visual assessment is necessary, a monocular stimulation can be used; if the patient is conscious, checkerboard reversal stimulation can be performed in order to evaluate more precisely the macular function and the optic nerve, since the flash assesses more specifically the peripheral retina and is less sensitive to an optic nerve lesion.

Like EEGs, EPs are potentially influenced by some drugs or toxic substances. They will affect one or several modalities, according to the properties of the substance, and will influence the latency, amplitude, duration or topography of the waves. Some EP components could be related to the mental attention of the subject when the stimulus occurs (the so-called 'cognitive' or 'endogenous' EPs).

3 APPLICATIONS TO CLINICAL PRACTICE AND RESEARCH IN TOXICOLOGY

3.1 Carbon Monoxide

The radiological features of carbon monoxide (CO) intoxication have been extensively documented. While brain CT examination is often unremarkable in the condition, the exquisite tissue contrast yielded by MRI allows the technique to depict diseased brain as early as 1 hour after exposure in experimental models. Distinction between early changes of the acute phase and delayed changes due to subacute encephalopathy has to be made (Lo *et al*., 2007). At the acute phase, the globus pallidus is the most commonly involved brain area in CO poisoning (O'Donnel *et al*., 2000; Chu *et al*., 2004) (**Figure 1C**). As expected, damage is usually symmetrical. Extension to other areas (putamina, caudate nuclei, thalami and substantia nigra) are frequently seen. Tissue characterization of CO-related brain damage can be further done using different kinds of MRI sequences: cystic necrosis appears very bright on T2-weighted images and very dark on T1-weighted ones. Cytotoxic oedema—which is often present within striatum—is detected by reduced ADC values due to cell swelling on DW parametric maps and high signal intensity on DW images (Kinoshita *et al*., 2005). Haemorrhagic transformation commonly observed in pallidi (Bianco and Floris, 1996) is best depicted by magnetic susceptibility artefacts due to the presence of haemoglobin degradation products on gradient-echo T2-weighted sequences. However, necrosis of the globi pallidi does not necessarily mean parkinsonism, and vice versa, probably because the damage to the nigrostriatal pathway is incomplete (Prockop, 2005). The prevalence of white matter hyperintensities on T2-weighted images appears highly variable (Parkinson *et al*., 2002). They seem preferentially located deep at the border of the lateral ventricles and centrum semiovale. White matter changes are usually permanent, even though some reversibility, which could be due to remyelination of the injured areas, has been demonstrated (Choi *et al*., 1993; Yoshii *et al*., 1998). The relationship between white matter hyperintensities and neuropsychological outcome is still debated, as MRI changes are not prominent in a majority of patients with cognitive impairment. Delayed encephalopathy following CO poisoning is characterized by the recurrence of neurological or psychiatric symptoms after complete clinical recovery of variable duration (usually between two and three weeks) from acute phase symptoms. White matter demyelination may be responsible for delayed neuropsychiatric syndrome (Chang *et al*., 1992; Murata *et al*., 2001; Kim *et al*., 2003) DW images highlight confluent areas of high signal intensity in the periventricular and centrum semiovale white matter corresponding to cytotoxic oedema, as demonstrated by ADC parametric maps. (Kim *et al*., 2002).

Regional CBF disturbances in the acute phase of CO poisoning have been described in the frontal cortex, basal ganglia and parietal cortex (Pach *et al*., 2004; 2005). A significant degree of asymmetry between the two hemispheres has been detected in basal ganglia and the prefrontal cortex. Delayed follow-up ^{99m}Tc-HmPAO SPECT studies have demonstrated the best reversibility of the anomalies within the parietal cortex. No unequivocal (precise) correlation between imaging features and neuropsychological testing at six months has been demonstrated.

3.2 Cyanide

Cyanide is toxic either by inhalation or by ingestion of cyanide salts. Cyanide has a similarly high affinity to CO for anatomical structures with very high oxygen demands. Again, lesions are prominent within the putamina and globi pallidi. They can be associated with the onset of extrapyramidal signs. Extension of tissue damage to the cortical grey matter may occur. Cortical laminar necrosis, previously described in neuropathological studies, may also be seen on MRI. (Kasamo *et al*., 1993; Riudavets *et al*., 2005). In a patient who survived a suicide attempt after ingestion of a high amount of potassium cyanide, biphasic changes were observed in CBF measurement by 99m-Tc-HMPAO SPECT (Zaknun *et al*., 2005). CBF values at the upper normal range were noted at the early stages, at a time the patient had choreoathetotic movements. Later on, after akinetic mutism had clinically developed, the CBF

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dropped to abnormally low values. [^{18}F]-FDG-PET revealed a global decreased tracer uptake within the cortical grey matter. The reduction was marked in the frontal lobes and striatum bilaterally, and to a lesser extent in the anterior part of the temporal lobes. A SPECT investigation was also done using [123-I]-2β-carbomethoxy-3-β-(4-iodophenyl)-tropan (β-CIT) as tracer, reflecting the density of striatal presynaptic dopamine transporters (DATs). A progressive loss in nigrostriatal dopaminergic neurons was suspected on two serial examinations.

3.3 Insulin

Insulin overdose is responsible for cerebral hypoglycaemic insult which prominently involves the grey matter. Within basal ganglia, hyperintensities on T2-weighted images are bilaterally seen within caudate and lenticular nuclei, but thalami are usually spared for unknown reasons (Jung *et al*., 2005) (**Figure 1D**).

3.4 Methanol

Methanol poisoning results in severe metabolic acidosis due to formic acid accumulation that usually becomes critical after a delay of several hours. Ancient neuropathological descriptions suggested that methanol-related brain injury involved basal ganglia, mainly the putamina. The first CT investigations reported that putaminal necrosis and haemorrhages were frequent. There was, however, no clear relationship between CT findings and clinical outcome. The preferential involvement of the putamina was further highlighted by MRI (Sefidbakht *et al*., 2007) (**Figure 1A**). DW sequences demonstrate cytotoxic oedema within the putamina, appearing after several hours. This delay may correspond to the time needed for the biotransformation of methanol into formic acid. Involvement of the subcortical white matter within frontal and/or occipital lobes has also be reported (Hantson *et al*., 1997). Patients with extensive putaminal necrosis more frequently develop permanent visual impairment. But, the origin of this deficit is an optic nerve injury that cannot be demonstrated by neuroimaging techniques, but well by visual evoked potential (VEP) examination. Blindness or severely decreased visual acuity may be observed in patients who survive methanol poisoning. There is strong scientific evidence that formic acid is the metabolite responsible for this specific toxicity. Experiments in nonhuman primates have shown that the ocular toxicity is usually biphasic. Early retinal dysfunction can be diagnosed by electroretinography (ERG). Retinal dysfunction is potentially reversible and a relationship can be found between ERG changes and blood formate concentrations, with a threshold value for retinal dysfunction. With large excesses of formate, retinal dysfunction will be followed within a few hours or days by a toxic optic neuropathy. This injury could still progress weeks after exposure, but a partial recovery is possible. Investigations of visual impairment in human methanol poisoning cannot be easily achieved, particularly in comatose patients. Hantson *et al*. (1999) were able to investigate 17 methanol-poisoned patients at the acute stage. They confirmed the biphasic pattern of visual injury using fVEPs. The risk for developing permanent visual injury was well correlated with the severity of metabolic acidosis and with the peak value of blood formate concentration (Hantson *et al*., 1999).

3.5 Ethylene Glycol

In contrast to methanol poisoning, ethylene glycol intoxication is usually not followed by permanent neurological impairment. Brain death is, however, possible in hyperacute forms complicated by severe metabolic acidosis and brain oedema. On CT images, the early appearance of diffuse cerebral oedema may appear, but usual hypointense areas within the central white matter, basal ganglia, thalami, midbrain and upper pons are seen only after a delay of 24–48 hours. (Morgan *et al*., 2000). Nonspecific cerebellar white matter abnormalities have also be described on brain MRI (Freilich *et al*., 2007).

3.6 Ethanol

Neuroimaging techniques have demonstrated structural and functional abnormalities in uncomplicated alcoholics who are cognitively impaired (Pfefferbaum *et al*., 1997; Lyons *et al*., 2001). Alcoholics have a reduction in brain volume and weight that is largely accounted for by a reduction in white matter volume. The reduction of the prefrontal white matter is particularly important in patients with Wernicke–Korsakoff syndrome. The mechanism underlying the white matter changes can be investigated by ^{1}H-MRS, which shows a decrease in NAA signal in the frontal white matter, suggestive of demyelination and loss of axonal integrity (Videen *et al*., 2001).

At low doses, ethanol can act as a stimulant (increased cerebral activity, behavioural disinhibition, etc.), while at higher doses, ethanol can result in motor and sensory impairments. Imaging studies in humans have shown that with low doses of ethanol intake, there was an increase in CBF in a region-specific manner. The frontal cortex seems sensitive to the lowest doses, while higher doses are required to elicit effects in the temporal cortex

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(Tiihonen *et al*., 1994). High doses of ethanol decrease CBF and local cerebral glucose utilization globally in humans. The cerebellum is the site where functional activity is frequently reduced at moderate doses. It has still to be determined whether the regions in which blood flow is elevated (prefrontal regions and temporal cortex) are those involved in the cerebral reward system, as it has been also suggested for cocaine craving.

Alcoholics usually exhibit poor task performance. This could be associated, not only with structural changes (brain atrophy), but also with functional disorders in some specific areas. Several studies have focussed on the prefrontal cortex. One of the limitations of scintigraphic studies is that an apparent decrease in functional activity in the frontal cortex could be only the result of a structural decrease in brain tissue with otherwise normal metabolic activity. Nevertheless, consistent results were obtained by SPECT studies. They showed repeatedly a reduction of rCBF in the prefrontal cortex that was greater than in any other region. The changes in rCBF appear to be independent of the magnitude of brain atrophy, which is usually more pronounced in the periventricular regions (Melgaard *et al*., 1990). Alcoholics with poor task performance have frontal hypoperfusion, irrespective of brain atrophy. Interestingly, the subjects without significant brain atrophy have a better recovery of frontal perfusion after abstinence. The delay for perfusion recovery may be as long as several years.

Ethanol abuse may be complicated by different types of acute or subacute encephalopathy: central pontine myelinolysis, Marchiafava–Bignami encephalopathy or Wernicke–Korsakoff encephalopathy). These entities are now easily identifiable by MRI. Marchiafava–Bignami encephalopathy is a rare disease and is seen in chronic ethanol abusers. The main pathological feature is acute demyelination or necrosis of the corpus callosum (mainly the splenium). Several MRI reports have shown an extension of the lesions to the hemispheric white matter. This is particularly well explored by the FLAIR or DW imaging sequences (Johkura *et al*., 2005). Central pontine myelinolysis was first described in alcoholic and malnourished patients. It can also be observed following metabolic disorders, and particularly after rapid correction of hyponatraemia. Pathologically, it is defined by a symmetric area of myelin disruption in the centre of the basis pons, but extrapontine myelinolysis can be documented in other brain areas (cerebellar and neocortical white/grey matter junctional areas, thalamus and striatum). Typically, central pontine myelinolysis is confirmed with T2-weighted MRI showing a typical trident-like hyperintensity on the centre of the pons. DW imaging findings in the acute phase are consistent with cytotoxic oedema, which is potentially reversible. MRS data are scarce, but suggest a decreased NAA/Cr ratio and an increased Ch/Cr ratio during the acute phase (Cramer *et al*., 2001; Guo *et al*., 2006).

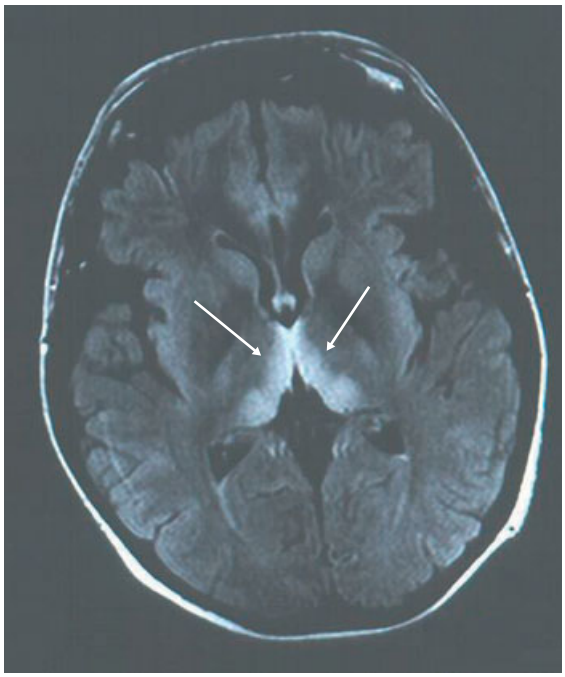

Figure 2 Brain MRI performed in a 33-year-old woman admitted with altered consciousness and ophthalmoplegia. Thiamine deficiency due to chronic ethanol abuse. Hyperintense lesions were seen on T2-weighted sequences in the thalamic area, around the third (see arrows) and fourth ventricle and the periaqueductal midbrain. Further development of Korsakoff syndrome.

Wernicke–Korsakoff encephalopathy is also not restricted to alcoholics, but is reported in other diseases, particularly after starvation. The causative mechanism is a thiamine deficiency. Typically, the T2-weighted and FLAIR sequences demonstrate hyperintense lesions in the medial thalami and the periventricular region of the third ventricle, in the periaqueductal area, in the mamillary bodies, in the tectal plate or in the dorsal medulla (Zuccoli *et al*., 2007) (**Figure 2**). The value of DW imaging as a prognostic tool should be further investigated (Lapergue *et al*., 2006).

Global decreases or no change in functional activity were found in patients presenting with Wernicke's encephalopathy. In the acute phase, decreased rCBF can be observed in the frontal cortex (Hunter *et al*., 1999). As the Papez circuit is involved in the generation of memories, it appears logical to observe a reduction of local cerebral glucose utilization in the cingulate gyrus, basal forebrain, hippocampus and thalamus (Fazio *et al*., 1992; Crews *et al*., 2005).

Spectroscopic alterations of NAA- and Ch-containing compounds have been found in the frontal cortex and in the cerebellum of alcoholic-dependent patients. In particular, the decreased Ch signal observed in frontal white matter and cerebellar regions could be at least partially reversible with abstinence. There is also a

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correlation between the increase of Ch signals and recovery from grey and white matter atrophy (Ende *et al*., 2006).

Ethanol withdrawal syndrome is characterized by various clinical signs, the most severe being cardiovascular or neurological disturbances. The direct consequences of ethanol withdrawal on long-term brain function are not precisely known, but it can be at least suspected that withdrawal could play a role in the cognitive deficits known to occur in alcoholics. Increase or decrease in global CBF was reported during withdrawal. In the context, agitation and auditory or visual hallucinations are common experiences. This may be associated to an increased functional activity in the frontal lobes (Berglund and Risberg, 1981). Experimental animal models confirmed the possibility of localized changes in brain function. The reactivities of some brain structures are clearly different during acute ethanol intoxication or withdrawal. The sensorimotor areas, the sensory systems and the cingulate cortex exhibit increased glucose utilization during withdrawal, without significant changes during intoxication. In turn, the cerebellum and the limbic regions are highly sensitive to the acute effects of ethanol, but do not show increased functional activity during withdrawal.

3.7 Cannabis

Cannabis is the most commonly used illicit substance. There is minimal evidence that chronic cannabis use could induce structural brain abnormalities, and in particular a reduction of white or grey matter volumes (Tzilos *et al*., 2005).

The principal psychoactive compound of cannabis, delta-9-tetrahydrocannabinol (THC), acts centrally via the cannabinoid 1 (CB1) receptor. Thalamic nuclei and limbic regions have a higher receptor density, as do the globi pallidi and ventral pallidum within the basal ganglia.

Numerous studies have looked at the effects of cannabis on brain function (Quickfall and Crockford, 2006). However, their study designs differed in regard to several aspects: definition of 'chronic' or 'occasional' cannabis use, association or not with other substances, period of prestudy abstinence and so on. Nevertheless, the results of the reported studies are reasonably consistent (Pearlson, 2001). Quantification of exposure remains a great concern, as the THC content of smoked cannabis has substantially increased over the past 20 years. Concerning the methodology, SPECT also appears less sensitive than PET or functional magnetic resonance imaging (fMRI) to detect changes in some specific brain regions. During exposure, there is an overall increase in brain activity, and more specifically in frontal, limbic and cerebellar regions. In turn, there is a reduction of activity in the same areas during abstinence. There is an overlap of the regions involved with those usually implicated in other substance use disorders (e.g. ethanol). The current neuroimaging data are insufficient to clarify whether residual cognitive disorders either resolve or not with abstinence alone.

3.8 Ecstasy

3,4-Methylene dioxymetamphetamine (MDMA, 'ecstasy') is a synthetic amphetamine with stimulant and hallucinogenic effects. MDMA influences both serotoninergic and dopaminergic transmission. The primary effects of MDMA are believed to be indirect, but it also binds to a number of postsynaptic receptor sites, with a great affinity for the serotonin transporter (SERT) and for the serotonin (5-hydroxytryptamine (5-HT)) receptor, particularly the 5-HT_2A type. There is now considerable evidence that MDMA has highly selective neurotoxic effects on the 5-HT brain neurons, but in some species the neurotoxic effects could extend to the dopaminergic neurons. Experiments with MDMA in nonhuman primates seem to indicate that the loss of brain serotonin markers following repeated MDMA exposure may persist for several years. Some recovery is still possible, but probably with an aberrant neural organization. The early studies that attempted to determine the neurotoxic effects of MDMA in humans used indirect markers, like the measurement of 5-hydroxyindolacetic acid (5-HIAA) in the cerebrospinal fluid. These indirect measures probably underestimated the potential of MDMA-induced neurotoxicity.

The possibility of long-term neurotoxicity is still a matter of debate in humans. There is a limited comparability of MDMA doses in animal models or human studies. The lowest dose to induce neurotoxic changes in nonhuman primates was found to be 40 mg kg^{-1} body weight. The usual 'ecstasy' doses used by humans per weekend are in the range 75–250 mg.

Several studies have addressed either the morphological or functional changes induced by MDMA in humans (Cowan, 2007).

Structural changes to brain grey matter seem limited after MDMA exposure, in humans as well as in animal models. Cowan *et al*. (2003) used voxel-based morphometry with MRI to compare regional brain grey matter concentration in MDMA users and controls. These authors found that MDMA users had multiple areas of reduced grey matter concentration in the brainstem, cerebellum and mostly the left-sided neocortex. However, these results could not be replicated by the subsequent studies; there was no consistent overlap with brain regions showing altered serotoninergic function or altered neurochemistry or metabolism in other neuroimaging studies.

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Heavy ecstasy use has been associated with neurocognitive deficits in various behavioural and brain imaging studies. The few available fMRI studies have primarily focussed on working memory and generated inconclusive results concerning the effects of ecstasy on brain activity patterns and the specific brain areas that are affected (Daumann *et al*., 2004). In a recent study, Jager *et al*. (2008) tried to clarify the specific effects of ecstasy on neurocognitive brain function by separating heavy ecstasy users from polydrug users. They concluded that ecstasy alone had no effect on working memory and attention, while polysubstance ecstasy users had reduced associative memory performance. Incidental use of ecstasy seems devoid of significant effects on cognitive brain function (Jager *et al*., 2007).

Contrasting results have been published concerning the patterns of rCBF measured by SPECT. Reneman *et al*. (2001) investigated the changes in rCBF in a group of MDMA users abstinent for a period of at least three weeks. Compared with controls, the MDMA users had a relative increase of rCBF values in the globi pallidi. This could be the result of a loss of serotoninergic vasoconstrictive effects to globus pallidus arterial vessels. The same authors found, with MRI, an increased ADC in this specific area in the MDMA users cohort. In an other rCBF study, Chang *et al*. (2000) explored the effects of prior MDMA exposure on rCBF in drug-abstinent MDMA users at baseline and again after administration of MDMA. At baseline, there was no difference in rCBF between MDMA users and controls. After the readministration of MDMA, a significant reduction of rCBF was noted in multiple brain regions (bilateral caudate, bilateral superior cortex, right dorsolateral prefrontal cortex).

The investigation of MDMA-induced neurotoxicity progressed after the development of radioligands capable of binding selectively to brain serotonin terminals (McCann *et al*., 2001). The technique was first developed for SPECT ligands and thereafter for PET. The first SPECT studies in MDMA users were performed using a cocaine analogue, [123I]β-CIT, that binds to both SERT and DAT. A reduction in cortical [123I]β-CIT binding was found primarily in the primary sensorimotor cortex. This reduction was more evident in cases of recent MDMA use (Semple *et al*., 1999). The density of SERT can be investigated in humans by PET studies using different specific ligands. The first documentation by PET of MDMA-induced 5-HT neurotoxicity was obtained in baboons. With the same radioligand, (+) [11C]McN5652, it was possible to demonstrate global and regional reductions of binding sites in MDMA users. A direct correlation was found with the extent of previous MDMA use (McCann *et al*., 2005). In abstinent MDMA users, compared to a nonMDMA control group, a global reduction in brain SERT density was demonstrated, with a variability within the different brain areas. Some degree of regeneration of serotonin axons and axon terminals was possible. It appears also that the reduction in the PET-binding parameters could underestimate the true extent of SERT loss. Analyses suggest a positive relationship between SERT binding and duration of abstinence, and a negative relationship between SERT binding parameters and MDMA use intensity. The interpretation of these PET findings remains challenging, as the potential relationship between brain measures of SERT and functional consequences of MDMA has still to be determined. The effect of lower exposure is also not precisely known. (McCann *et al*., 1998; Buchert *et al*., 2004). An other PET human study was conducted in MDMA users with 2–18[F]- FDG as tracer. The subjects were chronic MDMA users (cumulative dosages ranging from 12 to 840 tablets), who were observed after an abstinence period of at least two months. A reduced glucose metabolic uptake was noted in the amygdala, hippocampus and frontal cortex. This is more indirect evidence for persistent effects on neural activity resulting from brain 5-HT neurotoxicity (Obrocki *et al*., 1999).

Two brain metabolites, NAA and MI, have been extensively investigated by MRS in MDMA users. To date, no consistent results have been obtained. In particular, a reduction of NAA in cortical frontal areas was only observed in the population with the greatest MDMA exposure (Daumann *et al*., 2004).

3.9 Cocaine

With 3,4-MDMA ('ecstasy') and cocaine, there is a well-documented risk of cerebrovascular accidents. As expected, the globi pallidi seem highly vulnerable. (De Roock *et al*., 2007; Reneman *et al*., 2000) (**Figure 1B**).

The primary neurochemical action of cocaine in the brain is the blockade of the reuptake of dopamine, norepinephrine and serotonin. The reinforcing effects of cocaine are thought to be mediated by the dopaminergic pathway extending from the ventral tegmental area to the nucleus accumbens. PET studies in humans were helpful to demonstrate that the higher concentrations of synaptic dopamine corresponded to the peak physiological effects of cocaine (Schlaepfer *et al*., 1997). Additional data were procured by fMRI. The fMRI signal changes are influenced by alterations both in perfusion and function. Following acute cocaine administration, an increased signal intensity is usually observed in the ventral striatum/subcallosal cingulate, ventral tegmentum and several other cortical regions. It is still debated whether chronic cocaine use can produce long-term changes in brain structure and function. It seems likely, according to *in vivo* experiments in nonhuman primates and also in humans, that repeated exposure to cocaine

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could increase the density of DAT in some brain subregions (Little *et al*., 1999). However, it is exceedingly difficult to examine the temporal development of changes in DAT in binding sites in human cocaine abusers. Data obtained from cocaine self-administering monkeys seem to indicate that the increases associated with prolonged cocaine exposure begin to occur on the order of months rather than years (Letchworth *et al*., 2000). Cocaine users have significantly lower levels of dopamine D2 receptors when compared to drug-naive subjects. This down-regulation could persist for a long period following cocaine abstinence (Volkow *et al*., 1993). In parallel, changes in both resting levels of cerebral metabolism and blood flow, as well as altered responses to pharmacological challenge, have been demonstrated in cocaine abusers. Biphasic changes in cerebral metabolism can be found according to the duration of abstinence. Within the first few weeks, increases in cerebral metabolism have been reported within the basal ganglia and orbitofrontal cortex, while, at a later stage, lower rates of cerebral metabolism is correlated to decreased D2 receptor availability.

As craving is a hallmark feature of cocaine addiction, special attention has to be given to the neuroimaging techniques in an effort to describe the neural substrates involved in craving (Porrino *et al*., 2001). PET scans are able to demonstrate increases either in CBF or glucose utilization in some specific brain areas (frontal cortex, amygdala, cerebellum).

3.10 Opioids

With regard to illicit substances, chronic exposure to heroin has been particularly well investigated by MRI. The typical pattern is called 'spongiform encephalopathy'. It corresponds to spongiform degeneration of the white matter associated with multivacuolar degeneration of the oligodendrocytes. The lesions usually appear after chronic inhalation of heated heroin ('chasing the dragon'). On T2-weighted MRI, hyperintense areas within the white matter of the cerebellar hemispheres and brainstem are described, while the subcortical white matter and the anterior limbs of the internal capsules are usually spared. (Gacouin *et al*., 2003; Keogh *et al*., 2003).

The opiod-receptor system is characterized by three basic forms of receptors, mu (μ), delta (δ) and kappa (κ). In particular, the μ receptor is thought to mediate the analgesic and euphoric effects of opioids, as well as the addictive properties. PET studies using ligands for the opioid system can be performed either in normal volunteers or in opioid-dependent subjects (Nutt and Daglish, 2001). In healthy volunteers, the main areas of concentration of opioid receptors are found in the thalamus, caudate nucleus, and the temporal, frontal and parietal cortices (Jones *et al*., 1988). More specifically, the μ-opioid receptor is mainly represented in the thalamus, while the δ-opioid receptor is chiefly present in the putamen and caudate nucleus. It is essential to know that the number and distribution of opioid-binding sites vary with age and gender. The usual finding in the opioid system is that the density of binding sites may increase with age, but with significant differences throughout the brain, and also according to gender (Zubieta *et al*., 1999). PET studies in monkeys have shown that the elimination half-life of the opioids from the brain will depend upon their lipophilic characteristics. For ethical reasons, studies on the acute effects of opioids in healthy volunteers are rather limited. Fentanyl can cause an increase of rCBF in the anterior cingulate cortex and the prefrontal and orbitofrontal cortices, but a decrease in other areas (frontal, temporal, cerebellar) (Firestone *et al*., 1996). The increase of rCBF is already observed at rest but is still augmented by pain. The study by neuroimaging of the effects of acute doses in addicts is complicated by many practical problems. For example, in animal models, there are differences in effect, dependent on whether the drug is self-administered or not. If the subjects are opioid-free as a result of a recent detoxification, the ethics of a new administration of opioid have to be considered carefully. Even in chronic opioid addicts, the washout period of opioids from the brain can be extremely variable. Finally, the circumstances of drugs use can also influence the subjective effects and the neuroimaging results. When rCBF is measured by HMPAO SPECT in subjects who are currently abstinent or actively dependent, according to the nature of the opioid-receptor that is stimulated, a correlation between changes in rCBF and subjective effects can be demonstrated. 'Bad effects' could be associated with an increase in rCBF in the anterior temporal lobes, whereas euphoria is accompanied by increased rCBF in the anterior cingulate, thalamus and amygdala regions (Schlaepfer *et al*., 1998). By contrast, the measurements of regional cerebral metabolic rate of glucose are consistent with a widespread decease of cerebral metabolic activity throughout the majority of brain regions. The correlation between the subjective responses and the reduced cerebral activity is usually poor (London *et al*., 1990).

The next issue to be discussed is the chronic effects of these substances. It has been shown that hypoperfusion defects can be found following chronic use of cocaine or heroin. Ideally, the studies should be performed using high doses of opioids because opioid-dependent subjects develop tolerance. Due to practical and ethical problems, most of the paradigms are analysing the effects of methadone or buprenorphine substitution in a group of heroin-dependent individuals. Contrasting with the acute studies, a reduction in rCBF is observed in a population receiving chronic methadone treatment. The subjects who had higher methadone maintenance doses had smaller

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decreases in perfusion (Danos *et al*., 1998). Interestingly, a different pattern could be observed with buprenorphine. In comparison with the placebo group that had worsening perfusion deficits following detoxification, there was a dose-dependent improvement in hypoperfusion for the subjects treated by buprenorphine (Holman *et al*., 1993). Methadone treatment is also able to influence the opioid-receptor availability. PET studies have illustrated that methadone could produce a decrease in the index of free-receptor density; no correlation was obtained with plasma methadone levels (Melichar *et al*., 1999).

Opioid withdrawal is also investigated by different neuroimaging techniques. In heroin-dependent subjects undergoing detoxification, HMPAO SPECT at one week revealed focal perfusion deficits, with a delayed improvement at three weeks (Rose *et al*., 1996). The interpretation of such results remains difficult, as the scans are usually obtained after the resolution of the withdrawal signs. In an other experimental model using the administration of an opioid antagonist (e.g. naloxone or naltrexone) to subjects maintained on methadone or buprenorphine, conflicting results regarding CBF changes (increase or decrease) have been published (van Dyck *et al*., 1994; Krystal *et al*., 1995). Finally, the involvement of the mesocortical-limbic system in the opioid reward system can be confirmed by both animal and human studies. There is increasing evidence that the nucleus accumbens is the main site of dopamine release (Wise *et al*., 1995).

3.11 Nicotine

There are a limited number of morphological date available regarding the structural brain damage induced by nicotine. In comparison with never-smokers, it was possible to demonstrate, in a study performed on 22 smokers, that there was a significantly smaller grey matter volume and lower grey matter density in the frontal regions (anterior cingulate, prefrontal and orbitofrontal cortices), the occipital lobe and the temporal lobe, including the parahippocampal gyrus (Gallinat *et al*., 2006).

Nicotine affects cognitive strategies on working memory tasks and improves attention and performance on working memory tasks. There are conflicting data on whether administration of nicotine increases or decreases CBF. When fMRI is used to investigate possible alterations in task-related activation as a result as nicotine use, it is not clearly demonstrated that the changes in CBF could be coupled to changes in neuronal activity (Murphy *et al*., 2006).

Nicotine may share with other substances neural circuits implicated in the processes of reinforcement. Nicotine also illustrates the importance of the manner in which the drug is delivered. Nicotine given intravenously or from cigarette smoking may result in a somewhat different pattern of increased rCBF. The increased rCBF that is observed in the frontal cortex and in the nicotine-receptor-rich reticular system of the pons, midbrain and thalamus is greater after inhalation rather than after intravenous administration. It appears also that dopamine release within the ventral striatum is observed in nicotine-dependent individuals when smoking a cigarette. By contrast, intravenous nicotine administration to nonhuman nondependent primates should only produce minor changes (Brody *et al*., 2004; Rose *et al*., 2003).

3.12 Metals

3.12.1 Manganese

Manganese is a well-identified neurotoxicant. Exposure to excessive amounts of manganese is associated with a variety of psychiatric or motor disturbances (parkinsonian symptoms). An increased signal in T1-weighted images was observed in experimental manganese poisoning in nonhuman primates. A similar MRI pattern has also been reported in workers exposed to manganese. There is a bilateral symmetrical increase in signal intensities that is confined to the globus pallidus and midbrain. However, it is essential to understand that the increased signal intensities reflect a target site, but not necessarily manganism. A reversibility is possible after cessation of professional exposure (Kim, 2006).

3.12.2 Methylmercury

Methylmercury poisoning may cause visual impairment and deafness, especially if exposure occurs prenatally. Children could also be particularly vulnerable after exposure to contaminated seafood. Electrophysiological investigations have revealed delays in EP latencies, and particularly of the peak III in BAEPs and of the N145 in pattern-reversal VEPs (Murata *et al*., 1999; 2007). EPs could therefore be considered as an objective method for assessing methylmercury toxicity. However, the clinical relevance of minimal changes following low-level methylmercury exposure is still debated.

3.12.3 Lead

As lead exposure impairs hearing and nerve conduction in the auditory system, there is also evidence that BAEPs could be affected in both children and adults. Some publications suggest that lead exposure could interfere with BAEPs in a dose-dependent manner (Bleecker *et al*., 2003).

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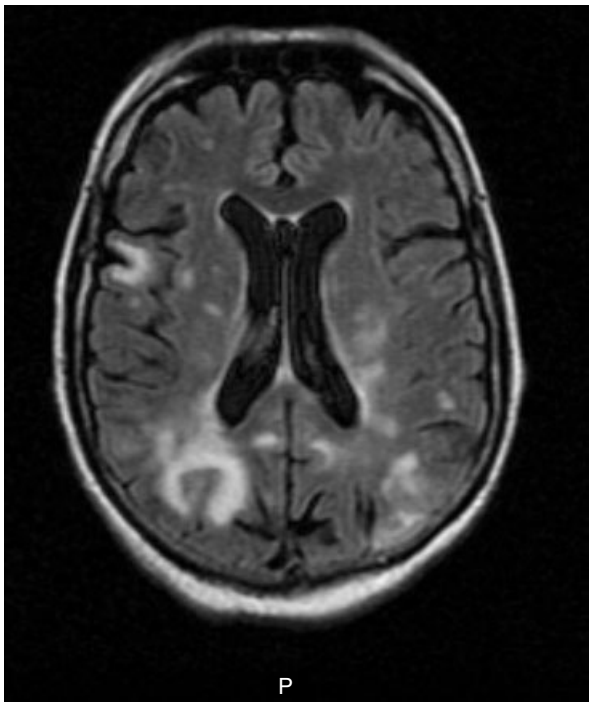

Figure 3 Brain MRI obtained in a 54-year-old patient admitted with coma. Recent medical history of heart transplantation. Immunosuppressive treatment with tacrolimus leading to excessive blood concentration after the progression of renal failure. FLAIR and T2-weighted sequences demonstrated hyperintense lesions bilaterally within the white matter in the frontal, parietal, temporal and occipital regions. The apparent diffusion coefficient (ADC) is increased in the corresponding areas. This is suggestive of vasogenic oedema.

3.13 Solvents

Organic solvents are responsible for chronic demyelinating lesions within the white matter. Hyperintensities on T1- and T2-weighted images are seen with trichloroethane in the lenticular nuclei, in the putamina and in the internal occipital region. Chronic exposure to toluene determines global corticosubcortical atrophy with changes also in the white matter and grey nuclei.

VEPs have also been used to investigate solvent-related encephalopathy (Verberk *et al*., 2004). A decrease of visual contrast sensitivity was confirmed by VEPs performed under specific experimental conditions ('low contrast').

3.14 Posterior Reversible Encephalopathy

Posterior reversible encephalopathy syndrome (PRES) was first identified in patients with eclampsia and in those who had undergone transplantation. The typical syndrome is combining headache, altered mentation, visual disturbance, severe hypertension and generalized seizures. The role of immunosuppressive drugs (cyclosporine, tacrolimus) or of some chemotherapy agents (methotrexate) has been discussed. The mechanisms behind this neurotoxic syndrome remain controversial. Disruption of the blood–brain barrier, with subsequent vasogenic oedema is one hypothesis (Lamy *et al*., 2004). The MRI pattern consists of hyperintensities in the T2-weighted and FLAIR sequences present in the subcortical white matter, and particularly in the parietal and occipital lobes (**Figure 3**). Involvement of the basal ganglia, brainstem or deep white matter is less common (Bartynski and Boardman, 2007).

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Alternatives to *In vivo* Studies in Toxicology

Shayne C. Gad

CONTENTS

1 INTRODUCTION

The key assumptions underlying modern toxicology are: (i) that other organisms can serve as accurate predictive models of toxicity in man; (ii) that selection of an appropriate model to use is the key to accurate prediction of potential hazard in man and (iii) that understanding the strengths and weaknesses of any particular model is essential to translating potential hazards identified in these models to assess relevant hazard in man, and in the subsequent management of actual risks. The nature of models and their selection in toxicological research became the subject of critical scientific review starting in the 1980s. Usually in toxicology, when we refer to 'models', we really have meant test organisms or systems, although, in fact, the manners in which parameters are measured (and which parameters are measured to characterize an end point of interest) are also critical parts of the model (or, indeed, may actually constitute the 'model').

Although there have been accepted principles for test organism selection, these have not generally been the actual final basis for such selection. It is a fundamental hypothesis of both historical and modern toxicology that adverse effects caused by chemical entities in higher animals are generally the same as those induced by those entities in man. There are many who point to individual exceptions to this and conclude that the general principle is false. Yet, as our understanding of molecular biology advances and we learn more about the similarities of structure and function of higher organisms at the molecular level, the more it becomes clear that the mechanisms of chemical toxicity are largely identical in all higher life forms, including humans. The target sites are molecular, and differences in responses are all about similarities in receptor populations, receptor population distribution in organ systems, and in the manner and means of getting toxicophores to these sites or preventing them from reaching these sites. In this sense, it is now the age of translational toxicology. This increased understanding has caused some of the same people who question the general principle of predictive value to in turn suggest that our state of knowledge is such that mathematical models or simple cell culture systems could be used just as well as intact higher animals to predict toxicities in man. This last suggestion has unfortunately missed the point that the final expressions of toxicity in man or animals are frequently the summations of extensive and complex interactions on cellular and biochemical levels. Zbinden (1987) and Gad (1996) published extensively in this area, including a very advanced defence of the

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value of animal models. Lijinsky (1988) has reviewed the specific issues about the predictive value and importance of animals in carcinogenicity testing and research. Although it was once widely believed, and may still be believed by many animal rights activists, that *in vitro* mutagenicity tests would entirely replace animal bioassays for carcinogenicity, this is clearly not the case on either scientific or regulatory grounds (despite the limitations of the current bioassay models). Although there are differences in the responses of various species (including man) to carcinogens, the overall predictive value of such results, when tempered by judgement, is clear. At the same time, a well-reasoned use of *in vitro* or other alternative test model systems is essential to the continued development of a product safety assessment programme that is effective, efficient and relevant to human safety (Gad, 1990a; 1996; 2002; 2007).

The subject of intact animal models and their proper selection and use has been addressed elsewhere (Gad, 2007) and will not be further addressed here. However, alternative models which use other than intact higher organisms are seeing increasing use in toxicology for a number of reasons.

The first and most significant factor behind the interest in so-called *in vitro* systems has clearly been philosophical and political—an unremitting campaign by a wide spectrum of individuals concerned with the welfare and humane treatment of laboratory animals (Singer, 1975), though some are also clearly simply antiscience and antitechnology. Russell and Burch (1959) first proposed what have come to be called the three Rs of humane animal use in research—replacement, reduction and refinement. These have served as the conceptual basis for reconsideration of animal use in research.

Replacement means utilizing methods that do not use intact animals in place of those that do. For example: veterinary students may use a canine cardiopulmonary-resuscitation simulator, Resusci-Dog, instead of living dogs; cell cultures may replace mice and rats that are fed new products to discover substances poisonous to humans. In addition, using the preceding definition of animal, an invertebrate (e.g. a horseshoe crab) could replace a vertebrate (e.g. a rabbit) in a testing protocol.

Reduction refers to the use of fewer animals. For instance, changing practices allow toxicologists to estimate the lethal dose of a chemical with as few as 1/10 the number of animals used in traditional tests. In biomedical research, long-lived animals, such as primates, may be used in multiple sequential protocols, assuming that they are not deemed inhumane or scientifically conflicting. Designing experimental protocols with appropriate attention to statistical inference can lead to decreases or to increases in the numbers of animals used. Through coordination of efforts among investigators, several tissues may be simultaneously taken from a single animal. Reduction can also refer to the minimization of any unintentionally duplicative experiments, perhaps through improvements in information resources.

Refinement entails the modification of existing procedures so that animals are subjected to less pain and distress. Refinements may include administration of anaesthetics to animals undergoing otherwise painful procedures; administration of tranquilizers for distress; humane destruction prior to recovery from surgical anaesthesia and careful scrutiny of behavioural indices of pain or distress, followed by cessation of the procedure or the use of appropriate analgesics. Refinements also include the enhanced use of noninvasive imaging technologies that allow earlier detection of tumours, organ deterioration or metabolic changes, and the subsequent early euthanasia of test animals.

Progress towards these first three Rs has been previously reviewed (Gad, 1990b; 1994; Salem, 1995; Salem, 1998; Gribaldo, 2007). However, there is a fourth R—responsibility, which was not in Russell and Burch's initial proposal. To toxicologists this is the cardinal R. They may be personally committed to minimizing animal use and suffering, and to doing the best possible science of which they are capable, but at the end of it all, toxicologists must stand by their responsibility to be conservative in ensuring the safety of the people using or exposed to the drugs and chemicals produced by our society.

Since 1980, issues of animal use and care in toxicological research and testing have become one of the fundamental concerns of both science and the public. Are our results predictive of what may or may not be seen in man? Are we using too many animals, and are we using them in a manner that gets the answer we need with as little discomfort to the animals as possible? How do we balance the needs of man against the welfare of animals?

During the same timeframe, interest and progress in the development of *in vitro* test systems for toxicity evaluations have also progressed. Early reviews by Hooisma (1982), Neubert (1982) and Williams *et al.* (1983) record the proceedings of conferences on the subject, but Rofe's (1971) review was the first found by this author. Although it is hoped that in the long term some of these (or other) *in vitro* methods will serve as definitive tests in place of those that use intact animals, at present it appears more likely that their use in most cases will be as screens. Frazier (1992), 1994 and Gad and Chengelis (1997) give recent overviews of the general concepts and status of *in vitro* alternatives.

The entire product-safety assessment process, in the broadest sense, is a multistage process in which none of the individual steps is overwhelmingly complex, but the integration of the whole process involves fitting together a large complex pattern of pieces. The single most important part of this product-safety evaluation

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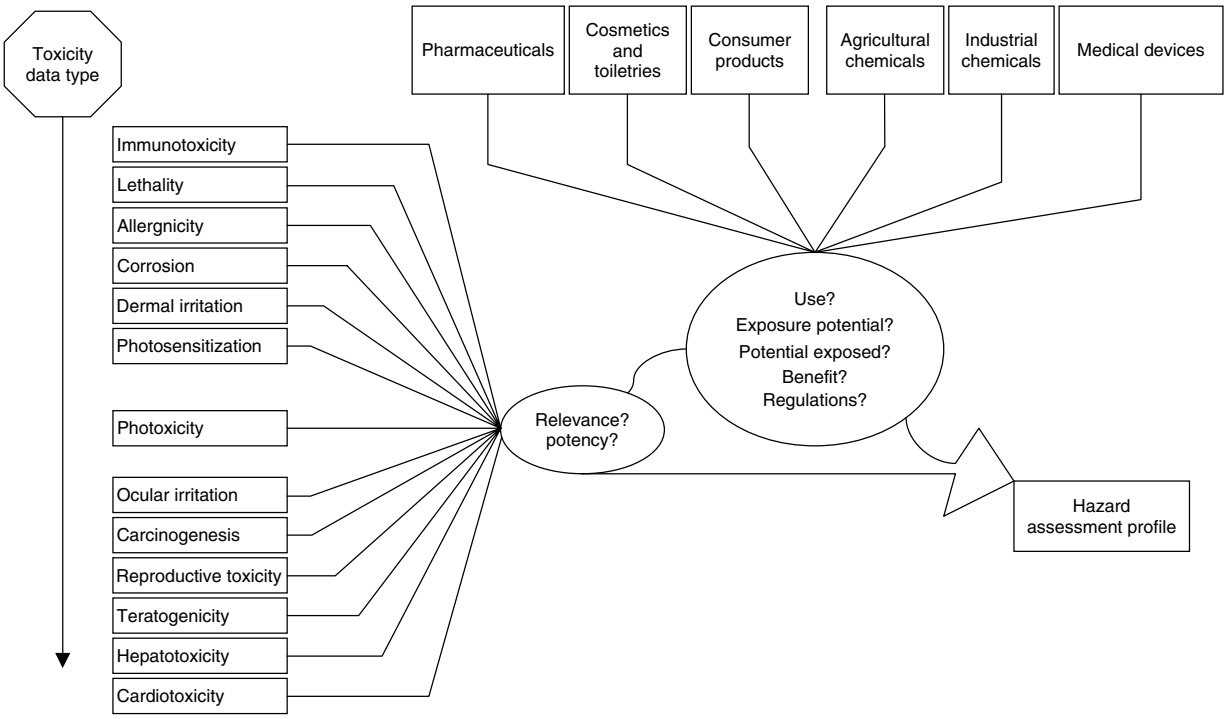

Figure 1 The hazards associated with a new product are a multidimensional problem depending on the product's intended use, its innate toxicity, its physiochemical properties, and the potential human and environmental exposure. This matrix diagrammatically illustrates the key questions involved in developing the final hazard assessment profile. There is also the special case of pharmaceutical and pesticide products, where there are regulatory mandated minimum test batteries.

programme is, in fact, the initial overall process of defining and developing an adequate data package on the potential hazards associated with the product life-cycle (the manufacture, sale, use and disposal of a product and associated process materials). To do this, one must ask a series of questions in a highly interactive process, with many of the questions designed to identify and/or modify their successors. The first is—what information is needed?

Required here is an understanding of the way in which a product is to be made and used, and the potential health and safety risks associated with exposure of humans who will be associated with these processes. Such an understanding is the basis of a hazard and toxicity profile. Once such a profile has been established (as illustrated in **Figure 1**), the available literature is searched to determine what is already known.

Taking into consideration this literature information and the previously defined exposure profile, a tier approach (**Table 1**) has traditionally been used to generate a list of tests or studies to be performed. What goes into a tier system is determined by regulatory requirements imposed by government agencies, as well as the philosophy of the parent organization, economics and available technology. How such tests are actually performed is determined on one of two bases. The first (and most common) is the menu approach: selecting a series of standard design tests as 'modules' of data. The second is an interactive/iterative approach, where strategies are developed and studies are designed, based on both needs and what has been learned to date about the product. This process has been previously examined in some detail. Our interest here, however, is in the specific portion of the process involved in generating data—the test systems.

2 TEST SYSTEMS: CHARACTERISTICS, DEVELOPMENT AND SELECTION

Any useful test system must be sufficiently sensitive to ensure that the incidence of false negatives is low. Clearly a high incidence of false negatives is intolerable. In such a situation, large numbers of dangerous chemical agents would be carried through extensive additional testing only for it to be found that they possess undesirable toxicological properties after the expenditure of significant time and money. One the other hand, a test system that is overly sensitive will give rise to a high incidence of false positives, which will have the deleterious consequence of rejecting potentially beneficial chemicals. The 'ideal' test will fall somewhere between

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Table 1 The usual way of characterizing the toxicity of a compound or product is to develop information in a tier approach manner. More information is required (a higher tier level is attained) as the volume of production and potential for exposure increase. A common scheme is shown

Tier Testing			
Testing tier	Mammalian Toxicology	Genetic Toxicology	Remarks
0	Literature review	Literature review	Upon initial identification of a problem, database of existing information and particulars of use of materials are established.
1	Cytotoxicity screens GPMT or LLNA Acute systemic toxicity Receptor binding i.e., hERG	Ames test *In vitro* SCE *In vitro* cytogenetics Comet assay	R&D material and low-volume chemicals with severely limited exposure.
2	Subacute studies ADME Primary dermal irritation Safety pharmacology Eye irritation	*In vivo* SCE *In vivo* cytogenetics	Medium-volume materials and/or those with a significant chance of human exposure.
3	Subchronic studies Reproduction Developmental toxicity Chronic studies Mechanistic studies	K	Any materials with a high volume or a potential for widespread or long-term human exposure or one that gives indications of specific long-term effects.

these two extremes and thus provide adequate protection without unnecessarily stifling development.

The 'ideal' test should have an end-point measurement that provides data such that dose–response relationships can be obtained. Furthermore, any criterion of effect must be sufficiently accurate in the sense that it can be used reliably to resolve the relative toxicity of two test chemicals that produce distinct (in terms of hazard to humans), yet similar responses. In general, it may not be sufficient to classify test chemicals into generic toxicity categories. For instance, if a test chemical falls into an 'intermediate' toxicity category, yet is borderline to the next more severe toxicity category, it should be treated with more concern than a second test chemical that falls at the less toxic extreme of the same category. Therefore, it is essential for a test system to be able to both place test chemicals in an established toxicity category and rank materials relative to others in the category.

The end-point measurement of the 'ideal' test system must be objective. This is important, to ensure that a given test chemical will give similar results when tested using the standard test protocol in different laboratories. If it is not possible to obtain reproducible results in a given laboratory over time or between various laboratories, then the historical database against which new test chemicals are evaluated will be time/laboratory-dependent. If this condition is the case, then there will be significant limitations on the application of the test system since it could potentially produce conflicting results. From a regulatory point of view this possibility would be highly undesirable. Along these lines, it is important for the test protocol to incorporate internal standards to serve as quality controls (QCs). Thus, test data could be represented utilizing a reference scale based on the test system response to the internal controls. Such normalization, if properly documented, could reduce interest variability.

From a practical point of view, there are several additional features of the 'ideal' test which should be satisfied. Alternatives to current *in vivo* test systems basically should be designed to evaluate the observed toxic response in a manner as closely predictive of the outcome of interest in man as possible. In addition, the test should be fast enough to ensure that the turnaround time for a given test chemical is reasonable for the intended purpose, very rapid for a screen, timely for a definitive test. Obviously the speed of the test and the ability to conduct tests on several chemicals simultaneously will determine the overall productivity. The test should be inexpensive, so that it is economically competitive with current testing practices. And finally, the technology should be easily transferred from one laboratory to another without excessive capital investment (relative to the value of the test performed) for test implementation.

It should be kept in mind that although some of these practical considerations may appear to present formidable limitations for any given test system at the present time, the possibility of future developments in testing technology could overcome these obstacles. In reality, these practical considerations are grounds for consideration of multiple new candidate tests on the basis

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Table 2 Rationale for using *in vivo* test systems

1. Provides evaluation of actions/effects on intact animal organ–tissue interactions.
2. Either neat chemicals or complete formulated products (complex mixtures) can be evaluated.
3. Either concentrated or diluted products can be tested.
4. Yields data on the recovery and healing processes.
5. Required statutory tests for agencies under such laws as the Federal Hazardous Substances Act (unless data are already available), Toxic Substances Control Act, Federal Insecticides, Fungicides and Rodenticides Act (FIFRA), Organization for Economic Cooperation (OECD) and Food and Drug Administration Laws.
6. Quantitative and qualitative tests with scoring systems generally capable of ranking materials as to relative hazards.
7. Amenable to modifications to meet the requirements of special situations (such as multiple dosing or exposure schedules).
8. Extensive available database and cross-reference capability for evaluation of relevance to human situation.
9. The ease of performance and relative low capital costs in many cases.
10. Test are generally both conservative and broad in scope, providing for maximum protection by erring on the side of over prediction of hazard to man.
11. Tests can be either single end point (such as lethality, corrosion, etc.) or shot-gun (also called multiple end point, including such test systems as a 13 week oral toxicity study).

of competitive performance. The most predictive test system in the universe of possibilities will never gain wide acceptance if it takes years to produce an answer or costs substantially more than other test systems that are only marginally less predictive.

The point is that these characteristics of the 'ideal' test system provide a general framework for evaluation of alternative test systems in general. No test system is likely to be 'ideal'. Therefore, it will be necessary to weigh the strengths and weaknesses of each proposed test system in order to reach a conclusion on how 'good' a particular test is.

In both theory and practice, *in vivo* and *in vitro* tests have potential advantages. **Tables 2** and **3** summarize these advantages. How, then, might the proper tests be selected, especially in the case of the choice of staying with an existing test system or adopting a new one? The next section will present the basis for selection of specific tests.

3 CONSIDERATIONS IN ADOPTING NEW TEST SYSTEMS

Any new test system starts out promising advantages in every way over previous systems. Over time, however, the scientific community comes to recognize the strengths and weaknesses of what may turn out to be a valuable tool in the overall field of risk 'identification, assessment and management'. Conducting toxicological investigations in two or more species of laboratory animals

Table 3 Limitations of *in vivo* testing systems which serve as a basis for seeking *in vivo* alternatives for toxicity tests

1. Complications and potential confounding or masking findings of *in vivo* systems.
2. *In vivo* systems may only assess short-term site of application or immediate structural alterations produced by agents. Specific *in vivo* tests may only be intended to evaluate acute local effects (i.e. this may be a purposeful test system limitation).
3. Technician training and monitoring are critical (particularly in view of the subjective nature of evaluation).
4. *In vivo* tests in animals do not perfectly predict results in humans if the objective is to exclude or identify severe-acting agents.
5. Structural and biochemical differences between test animals and humans make extrapolation from one to the other difficult.
6. Lack of standardization of *in vivo* systems.
7. Variable correlation with human results.
8. Large biological variability between experimental units (i.e. individual animals).
9. Large, diverse and fragmented databases which are not readily comparable.

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remains the generally accepted standard as the prudent and responsible practice in evaluating the systemic toxicity of a new chemical, entity, especially one that is expected to receive widespread use and to have exposure potential over human lifetimes.

But this paradigm—and that one of the species needs to be (or even should) be a mouse is now widely open to question. Adding a second or third species to the testing regimen offers an extra measure of confidence to the toxicologist and the other professionals who will be responsible for evaluating the associated risks, benefits and exposure limitations or protective measures. Although undoubtedly broadening and deepening a compound's profile of toxicity, the practice of enlarging on the number of test species is an indiscriminate scientific generalization, as has been demonstrated in multiple points in the literature (Gad and Chengelis, 1997). Moreover, such a tactic is certain to generate the problem of species-specific toxicosis. This is defined as a toxic response or an inordinately low biological threshold for toxicity that is evident in one species or strain, while all other species examined are either unresponsive or strikingly less sensitive. Species-specific toxicosis usually implies that either different metabolic pathways for converting or excreting xenobiotics or anatomical differences are involved. The investigator confronting such findings must be prepared to address the all-important question, 'Are humans likely to react positively or negatively to the test agent under similar circumstances?' Assuming that numerical odds prevail and that humans automatically fit into the predominant category would be scientifically irresponsible, whether on the side of being safe or at risk. Such a confounded situation can be an opportunity to advance more quickly into the heart of the search for predictive information. A species-specific toxicosis can frequently contribute towards a better understanding of the general case if the underlying biological mechanism either causing or enhancing toxicity is defined, and especially if it is discovered to uniquely reside in the sensitive species.

The design of our current tests appears to serve society reasonably well (i.e. significantly more times than not) in identifying hazards that would be unacceptable. However, the process can just as clearly be improved from the standpoint of both improving our protection of society and doing necessary testing in a manner that uses fewer animals in a more humane manner.

4 *IN VITRO* MODELS

In vitro models, at least as screening tests, have been in use in toxicology since the early 1980s. The last 20+ years have brought a great upsurge in interest in such models. This increased interest is due to economic and animal welfare pressures and technological improvements.

Criteria against which an *in vitro* model should be evaluated for its suitability in replacing (partially or entirely) an accepted *in vivo* model are incorporated in the process detailed in **Table 4**, which presents the proposed steps for taking a new *in vitro* testing technology from being a research construct to a validated and accepted test system.

There are substantial potential advantages in using an *in vitro* system in toxicological testing, which include isolation of test cells or organ fragments from homeostatic and hormonal control, accurate dosing and quantification of results. It should be noted that, in addition to the potential advantages, *in vitro* systems per se also have a number of limitations, which can contribute to there not being acceptable models. Findings from an *in vitro* system that either limit their use in predicting *in vivo* events or make them totally unsuitable for the task include wide differences in the doses needed to produce effects, or differences in the effects elicited. Some reasons for such findings are detailed in **Table 5**.

Tissue culture has the immediate potential to be used in two very different ways by industry. First, it has been used to examine a particular aspect of the toxicity of a compound in relation to its toxicity *in vivo* (i.e. mechanistic or explanatory studies). Second, it has been used as a form of rapid screening to compare the toxicity of a group of compounds for a particular form of response. Indeed, the pharmaceutical industry has used *in vitro* test systems in these two ways for years in the search for new potential drug entities.

The theory and use of screens in toxicology have previously been reviewed (Gad, 1988a; 1988b; 1989a; 1989b). Mechanistic and explanatory studies are generally called for when a traditional test system gives a result that is either unclear or is one for which the relevance to real-life human exposure is doubted. *In vitro* systems are particularly attractive for such cases because they can focus on very defined single aspects of a problem or pathogenic response, free of the confounding influence of the multiple responses of an intact higher-level organism. Note, however, that first one must know the nature (indeed the existence) of the questions to be addressed. It is then important to devise a suitable model system which is related to the mode of toxicity of the compound.

There is currently much controversy over the use of *in vitro* test systems—will they find acceptance as 'definitive tests systems' or only be used as preliminary screens for such final tests? Or, in the end, not be used at all? Almost certainly, all three of these cases will be true to some extent. Depending on how the data generated are to be used, the division between the first two is ill-defined at best.

Before trying to definitely answer these questions in a global sense, each of the end points for which *in vitro*

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Table 4 Multistage scheme for the development, validation and transfer of *in vitro* test system technology in toxicology

STAGE I: STATEMENT OF TEST OBJECTIVE
(A) Identify existing test system and its strengths and weaknesses.
(B) Clearly state objectives for alternative test system.
(C) Identify potential alternative test system.

STAGE II: DEFINE DEVELOPMENTAL TEST DESIGN
(A) Identify relevant variables.
(B) Optimize test performance.
(C) Understand what the test does in a functional sense.
1. Is it a simulation of an *in vivo* event?
2. Is this simply a response to the presence of the agent?
3. Is the measured response a functional step or link in the *in vivo* event of interest?
4. Is the measured response a functional step or link in the *in vivo* event of interest or some intermediate stage?
5. Is this an effect on some structure or function analogous to the *in vivo* structure or function?

STAGE III: EVALUATE PERFORMANCE OF OPTIMUM TEST
(A) Develop library of known positive- and negative-response materials of diverse structure and a range of response potencies (i.e. if the end point is irritation, then materials should range from nonirritating to severely irritating).
(B) Use optimum test design to evaluate the library of 'knowns' under 'blind' conditions.
(C) Compare correlation of test results with those of other test systems and with real cases of interest—results in humans.

STAGE IV: TECHNOLOGY TRANSFER
(A) Present and publish results through professional media (at society meetings, in peer-reviewed journals).
(B) Provide hands-on training to personnel from other facilities and facilitate internal evaluations of test methods.

STAGE V: VALIDATION
(A) Arrange for test of coded samples in multiple laboratories (i.e. interlaboratory validation).
(B) Compare, present and publish results.
(C) Gain regulatory acceptance.

STAGE VI: CONTINUE TO REFINE AND EVALUATE TEST SYSTEM PERFORMANCE AND UTILIZATION
(A) Continually strive for an understanding of why the test 'works' and its relevance to effects in man.
(B) Remain skeptical. Why should any one of us be the one to make the big breakthrough? Clearly, there is some basic flaw in the design or conduct of the study which has given rise to these promising results. Doubt, check and question; let your most severe critic review the data; go to a national meeting and give a presentation; then go back home and doubt, check and question some more!

STAGE VII: REGULATORY ACCEPTANCE AND ADAPTATION
(A) Accepted use will first be side by side with existing system, which it will come to supplant.
(B) It will be modified in use as practical factors reveal means of optimization.

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Table 5 Possible interpretations when *in vitro* data do not predict results of *in vivo* studies

1. Chemical is not absorbed at all or is poorly absorbed in *in vivo* studies.
2. Chemical is well absorbed, but is subject to 'first-pass effect' in the liver.
3. Chemical is distributed so that less (or more) reaches the receptors than would be predicted on the basis to its absorption.
4. Chemical is rapidly metabolized to an active or inactive metabolite that has a different profile of activity and/or different duration of action from that of the parent drug.
5. Chemical is rapidly eliminated (e.g. through secretory mechanisms).
6. Species of the two test systems used are different.
7. Experimental conditions of the *in vitro* and *in vivo* experiments differ and may have led to different effects from those expected. These conditions include factors such as temperature or age, sex and strain of animal.
8. Effects elicited *in vitro* and *in vivo* by the particular test substance in question differ in their characteristics.
9. Tests used to measure responses may differ greatly for *in vitro* and *in vivo* studies, and the types of data obtained may not be comparable.
10. The *in vitro* study did not use adequate controls (e.g. pH, vehicle used, volume of test agent given, samples taken from sham-operated animals), resulting in 'artefacts' of method rather than results.
11. *In vitro* data cannot predict the volume of distribution in central or in peripheral compartments.
12. *In vitro* data cannot predict the rate constants for chemical movement between compartments.
13. *In vitro* data cannot predict the rate constants of chemical elimination.
14. *In vitro* data cannot predict whether linear or nonlinear kinetics will occur with specific dose of a chemical *in vivo*.
15. Pharmacokinetic parameters (e.g. bioavailability, peak plasma concentration, half-life) cannot be predicted solely on the basis of *in vitro* studies.
16. *In vivo* effects of chemical are due to an alteration in the higher-order integration of an intact animal system, which cannot be reflected in a less complex system.

systems are being considered should be overviewed and considered against the factors outlined to this point.

5 LETHALITY

Many of the end points of interest in toxicology present a fundamental limitation to the development and use of an *in vitro* or nonmammalian system in place of established *in vivo* methods. While cytotoxicity is a component mechanism in many of these toxic responses, disruption or diminution of the integrated function of multiple cells and systems is just as important.

The evaluation of lethality (symbolized in the public mind by the LD_{50} test) would seem to offer a unique opportunity for the development and use of alternatives. Approaches to alternatives for lethality testing include no living materials at all (the structure–activity relationship (SAR) or computer model approaches), those that use no intact higher organisms (but rather cultured cells or bacteria) and those that use lower forms of animal life (invertebrates and fish, for example). Each of these approaches presents a different approach to the objective of predicting acute lethality in humans or, rarely, economic animals, and will be examined in turn.

There are systems that do not directly use any living organisms but, rather, seek to predict the lethality (in particular, the LD_{50}) of a chemical on the basis of what is known about structurally related chemicals. Such SAR systems have improved markedly over the last 10 years (Enslein *et al*., 1983a; Lander *et al*., 1984), but are still limited. Accurate predictions are usually possible only for those classes of structures where data have previously been generated on several members of the classes. For new structural classes, the value of such predictions in minimal. Accordingly, this approach is valuable when working with analogues in a series, but not for novel structures. It is also a strong argument for getting as many data as possible into the published literature.

A more extensive and once promising approach has been the use of various cultured cell systems. Kurack *et al*. (1986), for example, have developed and suggested a system based on cultured mammalian hepatocytes. The system does metabolize materials in a manner like mammalian target species, and has shown promise in a limited battery of chemicals. Such mammalian cell culture and bacterial screening systems have significant weaknesses for assessing the lethality of many classes of chemicals, since they lack any of the integrative functions of a larger organism. Thus, they would miss all agents that act by disrupting functions, such as the organophosphate pesticides, most other neurologically mediated lethal agents and agents that act by modifying hormonal or immune systems.

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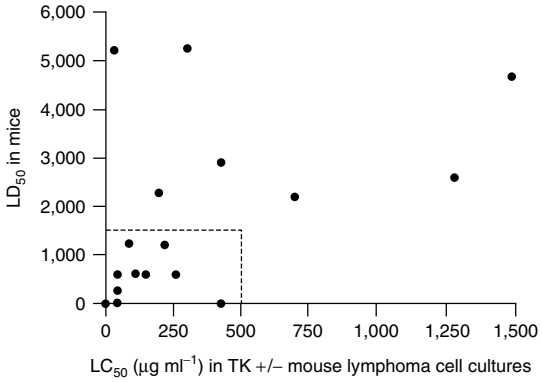

Figure 2 Graph showing a comparison of the lethalities of a group of 18 drugs of diverse structure in *in vivo* (mouse) and *in vitro* (cultured mouse lymphoma cells) test systems. Correlation of these LD_{50}/LC_{50} values is very poor, though extreme high- and low-scale values seem to be more closely associated in the two systems.

Clive *et al*. (1979) have reported on the correlation of the LC_{50}s of a variety of chemicals in mouse lymphoma cell cultures with their oral LD_{50}s in mice, as shown in **Figure 2**. No linear correlation is present, but highly cytotoxic substances (in this group) are significantly more toxic orally. Given the impression of some LD_{50} values, due to such factors as steepness of slope of the lethality curve, the lack of linear correlation should be no surprise. Most recently, Ekwall *et al*. (1989) have reported on the Multicenter Evaluation of *In Vitro* Cytotoxicity (MEIC) programme system, which utilizes a battery of five cellular systems. For a group of 10 chemicals, the system provided good correlation with, or predictive power of, rat LD_{50}s.

Recently Parce *et al*. (1989) reported on a biosensor technique in which cultured cells are confined to a flow chamber through which a sensor measures the rate of production of acidic metabolites. It is proposed to use this as a functional measure of cytotoxicity and as a screening technique for a number of uses, including *in vivo* lethality.

Three lower species of intact animals have been proposed for use in screening or testing of the lethal effects of chemicals. First, some researchers have shown a good correlation between the LD_{50} of the same chemicals in rats. This correlation is nonlinear, but still suggests that more toxic materials could be at least initially identified and classified in some form of screening system based on *Daphnia*. A broader range of chemical structures will need to be evaluated, however, and some additional laboratories will need to confirm the findings. It must also be kept in mind that the metabolic systems and many of the other factors involved in species differences (as presented in Gad and Chengelis, 1988; 1992; Gad, 2006) contribute to a nonlinear correlation and may also make the confidence in prediction of human effects in cases somewhat limited.

Earthworms have been one of the more common species used to test chemicals for potential hazardous impact on the environment. The 48 hour contrast test has proved to be a fast and resource-effective way of assessing acute toxicity of chemicals in earthworms and is outlined in **Table 6**. The standardized method, approved by the EEC, is discussed by Neuhauser *et al*. (1986). This test is for environmental impact assessment where crosslaboratory comparisons are important. If, however, one wishes to adopt this technology for the purpose of screening new chemicals or releasing batches of antibiotics, then variants of this method may be acceptable, as internal consistency is more important than interlaboratory comparisons. There are two important considerations. First, because of seasonal variations in the quality of earthworms obtained from suppliers, positive controls or comparator chemicals should be

Table 6 Earthworm 48 hour contact test—acute lethality

1. Place filter paper of known size (9 cm or 12 × 6.7 cm) in a Petri dish or standard scintillation vial.
2. Dilute test article in acetone or some other volatile solvent.
3. Slowly and evenly deposit known amounts of test article solution onto filter paper.
4. Dry thoroughly with air or nitrogen gentle stream.
5. Add 1.0 ml of distilled water to filter paper.
6. Add worm (*L. rubellus*). Use 400–500 mg body weight range.
7. Ten replicate vials per concentration.
8. Store/incubate in the absence of light at 15–20 °C for 48 h.
9. Examine for lethality (swollen, lack of movement upon warming up to room temperature, lack of response to tactile stimulation).
10. Express dose as μg cm^{-2} and mortality as usual. Calculate LD_{50} using standard techniques.
11. Always include negative and positive (benchmark) controls.

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Table 7 Earthworm toxicity—toxicity rating

Rating	Designation	Rat LD_{50} (mg kg^{-1})	*Eisenia foetida* LC_{50} (μg cm^{-2})
1	Supertoxic	<5	<1.0
2	Extremely toxic	5–50	1.0–10
3	Very toxic	50–500	10–100
4	Moderately toxic	500–5000	100–1000
5	Relatively nontoxic	>5000	>1000

From Neuhauser *et al*. (1985a, 1985b).

included on every assay run. Second, distilled water must be used, as worms are quite sensitive to contaminants that may occur in chlorinated water. The filter paper should completely cover the sides of the vessel; otherwise the worms will simply crawl up the sides to escape the adverse stimulus the chemical contact may provide.

Using these techniques, Roberts and Dorough (1985) and Neuhauser *et al*. (1986) have compared acute toxicity in a variety of organic chemicals in several earthworm species. While there are some obvious differences between worm species, in general the rank order of toxicity is about the same. *Lumbricus rubellus* tends to be the most sensitive species. All earthworms are very sensitive to carbofuran under the conditions of this test. Neuhauser *et al*. (1985a, 1985b) have proposed a toxicity-rating scheme based on acute lethality in the earthworms which is similar to the more familiar scheme based on acute lethality in rodents (**Table 7**). Roberts and Dorough (1985) and Neuhauser *et al*. (1986) have published extensive compilations of acute lethality in worms and compared these with acute lethality in rats and mice. A selection of these is shown in **Table 8**. Applying the rating scheme of Neuhauser, most chemicals receive about the same toxicity rating based on results in *Eisenia foetida* and mice. This may suggest that replacing the LD_{50} with the LC_{50} for rating toxicity (for transportation permits, for example) deserves serious consideration.

The main advantages of the 48 hour contact test are the savings of time and money. The cost savings fall into three categories. First, earthworms are cheap. One hundred *L. rubellus* will cost about US$2.00. The 100 mice they could replace in screens and QC testing, for example, would cost $300–400 (€150–200 at an exchange rate of $2.00 = €1.00). Second, earthworms require no vivarium space, and their use could reduce the number of rodents used, resulting in a net decrease in vivarium use. Third, adapting the 48 hour contact test would require little capital investment, other than a dedicated under-the-counter refrigerator set as 15–20 °C. Otherwise, the assay can be easily performed in a standard biochemistry laboratory. With regard to time savings, the standard lethality test with rodents requires 7–14 days of postdosing observations. The 48 hour contact test is completed in 48 hours. Not only is the turnaround time faster, but also the amount of time that technical personnel will have to spend observing animals and recording observations will be reduced. An incidental advantage of earthworms is that they are cold-blooded invertebrates, and thus as exempt from current animal welfare laws.

Table 8 Earthworm acute lethality—comparative values

Chemical	*Eisenia foetida* (LC_{50})	Mouse (LD_{50})
2,4-Dinitrophenol	0.6 (1)	45 (2)
Carbaryl	14 (3)	438 (3)
Benzene	75 (3)	4 700 (4)
1,1,1-Trichloroethane	83 (3)	11 240 (5)
Dimethylphthalate	550 (4)	7 200 (5)

There are two main disadvantages to the use of earthworms in acute toxicity testing. First, there are a limited number of end points. Other than death and a few behavioural abnormalities (Stenersen, 1979; Drewes *et al*., 1984), the test does not yield much qualitative information. Second, there probably is some institutional bias. Because the test is basically low-technology (no tissue culture) and uses a nonmammalian model, it may be easy to dismiss the utility of the test.

Finally, the use of smaller species of fish as a surrogate for man has gained some supporters. Currently, the zebra fish has shown significant promise as a surrogate model for mammalian species toxicity (Hill *et al*., 2005; Chiu *et al*., 2008; Tanguay and Reimers, 2008) and as a screen for carcinogens (Stern and Zon, 2003; Berghmans *et al*., 2005). There is certainly no reason why they could not be used for screening water-soluble compounds for extreme acute toxicity.

Although the intact organisms would seem to be the most utilitarian on the face of it, they still will not totally replace mammalian systems, owing to the need to be concerned about those systems that are significantly different in higher organisms. Still, it would appear that for those compounds for which human exposure is not intentional, testing in an intact lower organism systems (or perhaps

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Table 9 Rationales for seeking *in vitro* alternatives for eye irritancy tests

1.	Avoid whole animal and organ *in vivo* evaluation.
2.	Strict Draize scale testing in the rabbit assesses only three eye structures (conjuctiva, cornea, iris) and traditional rabbit eye irritancy tests do not assess cataracts, pain, discomfort or clouding of the lens.
3.	*In vivo* tests assess only inflammation and immediate structural alterations produced by irritants (not sensitizers, photoirritants or photoallergens). Note, however, that the test was (and generally is) intended to evaluate any pain or discomfort.
4.	Technician training and monitoring are critical (particularly in view of the subjective nature of evaluation).
5.	Rabbit eye tests do not perfectly predict results in humans, if the objective is either the total exclusion of irritants or the identification of truly severe irritants on an absolute basis (that is without false positives or negatives). Some (such as Reinhardt *et al*., 1985) have claimed that these tests are too sensitive for such uses.
6.	There are structural and biochemical differences between rabbit and human eyes which make extrapolation from one to the other difficult. For example, Bowman's membrane is present and well developed in man (8–12 μm thick) but not in the rabbit, possibly giving the cornea greater protection.
7.	Lack of standardization.
8.	Variable correlation with human results.
9.	Large biological variability between experimental units.
10.	Large, diverse and fragmented databases which are not readily comparable.

even in a cell culture system) should be sufficient to identify agents of significant concern. In these cases, lethality testing in intact mammals is probably unwarranted.

5.1 Ocular Irritation

Testing for potential to cause irritation or damage to the eyes remains the most active area for the development (and validation) of alternatives, and the most sensitive area of animal testing in biomedical research. This has been true since the beginning of the 1980s. **Table 9** presents an overview of the reasons for pursuing such alternatives. The major reason, of course, has been the pressure from public opinion.

Indeed, many of the *in vitro* tests now being evaluated for other end points (such as skin irritation and lethality) are adaptations of test systems first developed for eye irritation uses. A detailed review of the underlying theory of each test system is beyond the scope of this chapter. Frazier *et al*. (1987) and Gad (2007) performed such reviews, and **Table 10** presents an updated version of the list of test systems overviewed in those volumes.

There are six major categories of approach to *in vitro* eye irritation tests. Because of the complex nature of the eye, the different cell types involved and interactions between them, it is likely that a successful replacement for existing *in vivo* systems (such as the rabbit) would require some form of battery of such test systems. Many individual systems, however, might constitute effective screens in defined situations. The first five of these aim at assessing portions of the irritation response, including alterations in tissue morphology, toxicity to individual component cells or tissue physiology, inflammation or immune modulation, and alterations in repair and/or recovery processes. These methods have the limitation that they assume that one of the component parts can or will predict effects in the complete organ system. While each component may serve well to predict the effects of a set of chemical structures which determine part of the ocular irritation response, a valid assessment across a broad range of structures will require the use of a collection or battery of such tests.

The sixth category contains tests that have little or no empirical basis, such as computer-assisted SAR models. These approaches can only be assessed in terms of how well or poorly they perform. **Table 11** presents an overview of all six categories and some of the component tests within them, updated from the assessment by Frazier *et al*. (1987) along with references for each test.

Given that there are now some 80 or more potential *in vitro* alternatives, the key points along the route to the eventual objective of replacing the *in vivo* test systems are thus: (i) how do we select the best candidates from this pool?; (ii) how do we want to use the resulting system (as a screen or test)?; (iii) how do we gain regulatory and user acceptance of the appropriate test systems?

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Table 10 *In vitro* alternatives for eye irritation tests

MORPHOLOGY
Enucleated superfused rabbit eye system (Burton *et al*., 1981).
Balb/c 3T3 cells/morphological assays (HTD) (Borenfreund and Puerner, 1984).
CELL TOXICITY
Adhesion/cell proliferation
BHK cells/growth inhibition (Reinhardt *et al*., 1985).
BHK cells/colony formation efficiency (Reinhardt *et al*., 1985).
BHK cells/cell detachment (Reinhardt *et al*., 1985).
SIRC cells/colony forming assay (North-Root *et al*., 1982).
Balb/c 3T3 cells/total protein (Shopsis and Eng, 1985).
BCL/D1 cells/total protein (Balls and Horner, 1985).
Primary rabbit corneal cells/colony forming assay (Watanabe *et al*., 1988).
Membrane integrity
LS cells/dual dye staining (Scaife, 1982).
Thymocytes/dual fluorescent dye staining (Aeschbacher *et al*., 1986).
LS cells/dual dye staining (Kemp *et al*., 1983).
RCE-SIRC-P815-YAC-1/Cr release (Shadduck *et al*., 1985).
L929 cells/cell viability (Simons, 1981).
Bovine red blood cell/haemolysis (Shadduck *et al*., 1987).
Mouse L929 fibroblasts-erythrocin C staining (Frazier, 1988).
Rabbit corneal epithelial and endothelial cells/membrane leakage (Meyer and McCulley, 1988).
Agarose diffusion (Barnard, 1989).
Corneal protein profiles (Eurell and Meachum, 1994).
Cell metabolism
Rabbit corneal cell cultures/plasminogen activator (Chan, 1985).
LS cells/ATP assay (Kemp *et al*., 1985).
Balb/c 3T3 cells/neutral red uptake (Borenfreund and Puerner, 1984).
Balb/c 3T3 cells/uridine uptake inhibition assay (Shopsis and Sathe, 1984).
HeLa cells/metabolic inhibition test (MIT-24) (Selling and Ekwall, 1985).
MDCK cells/dye diffusion (Tchao, 1988).
CELL AND TISSUE PHYSIOLOGY
Epidermal slice/electrical conductivity (Oliver and Pemberton, 1985).
Rabbit ileum/contraction inhibition (Muir *et al*., 1983).
Bovine cornea/corneal opacity (Muir, 1984).
Proposed mouse eye/permeability test (Maurice and Singh, 1986).
INFLAMMATION/IMMUNITY
Chlorioallantonic membrane (CAM)
CAM (Leighton *et al*., 1983).
HET-CAM (Luepke, 1985).
Bovine corneal cup model/leucocyte chemotactic factors (Elgebaly *et al*., 1985).
Rat peritoneal cells/histamine release (Jacaruso *et al*., 1985).
Rat peritoneal mast cells/serotonin release (Dubin *et al*., 1984).
Rat vaginal explant/prostaglandin release (Dubin *et al*., 1984).
Bovine eye cup/histamine (Hm) and leukotriene C4 (Lt/C4) release (Benassi *et al*., 1986).
RECOVERY/REPAIR
Rabbit corneal epithelial cells-wound healing (Jumblatt and Neufeld, 1985).
OTHER
EYTEX assay (Gordon and Bergman, 1986; Soto *et al*., 1988).
Computer-based structure-activity (SAR) (Enslein, 1984; Enslein *et al*., 1988).
Tetrahymena/motility (Silverman, 1983).

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Table 11 *In vitro* dermal irritation test systems

System	End point	Validation data?[a]	References
I.			
Excised patch of perfused skin	Swelling	No	Dannenberg (1987)
Mouse skin organ culture	Inhibition of incorporation of [^{3}H]-thymidine and [^{14}C]-leucine labels	No	Kao *et al*. (1982)
Mouse skin organ culture	Leakage of LDH and GOT	Yes	Bartnik *et al*. (1989)
II.			
Test skin—cultured surrogate skin patch	Morphological evaluation (?)	No	Bell *et al*. (1988)
Cultured surrogate skin patch	Cytotoxicity	No	Naughton *et al*. (1989)
III.			
Human epidermal keratinocytes (HEKs)	Release of labelled arachidonic acid	Yes	DeLeo *et al*. (1988)
Human polymorphonuclear cells	Migration and histamine release	Yes (surfactants)	Frosch and Czarnetzki (1987)
Fibroblasts	Acid	—	Lamont *et al*. (1989)
HEKs	Cytotoxicity	Yes	Gales *et al*. (1989)
HEKs	Cytotoxicity (MTT)	Yes	Swisher *et al*. (1988)
HEKs, dermal fibroblasts	Cytotoxicity	Yes	Babich *et al*. (1989)
HEKs	Inflammation mediator release	No	Boyce *et al*. (1988)
Cultured Chinese hamster ovary (CHO) cells	Increases in $\gamma\vartheta$-hexosaminidase levels in media	No	Lei *et al*. (1986)
Cultured $C_3H10T_{1/2}$ and HEK cells	Lipid metabolism inhibition	No	DeLeo *et al*. (1987)
Cultured cells—BHK21/C13	Cell detachment	Yes	Reinhardt *et al*. (1987)
BHK21/C13	Growth inhibition		
primary rat thymocytes	Increased membrane permeability		
Rat peritoneal mast cells	Inflammation mediator release	Yes (surfactants)	Prottey and Ferguson (1976)
IV.			
Hen's egg	Morphological evaluation		Reinhardt *et al*. (1987)
SKINTEX—protein mixture	Protein coagulation	Yes	Gordon *et al*. (1989)
V.			
Structure-activity relationship (SAR) model	NA	Yes	Enslein *et al*. (1987)
SAR model	NA	No	Firestone and Guy (1986)

[a]Evaluated by comparison of predictive accuracy for a range of compounds compared with animal test results. Not validated in the sense used in this chapter.
NA = not available.

There have been some large-scale validations of some of these tests (IRAG, 1993). Most of the individual investigators have performed smaller 'validations' as part of their development of the test system, and in a number of cases trade associations have sponsored comparative and/or multilaboratory validations. At least for screening, several systems should be appropriate for use and, in fact, are used now by several commercial organizations. But the Interagency Regulatory Alternatives Group (IRAG, 1993) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM, 1997) have coordinated and reported on large-scale evaluations. In terms of use within defined chemical structural classes, use of *in vitro* systems for testing of chemicals for nonhuman exposure should supplant traditional *in vivo* systems once validated on a broad scale by multiple laboratories. Broad use of single tests based on single end points

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(such as cytotoxicity) is not likely to be successful, as demonstrated by such efforts as those of Kennah *et al*. (1989).

5.2 Dermal Irritation

Extensive progress has been made in devising alternative (*in vitro*) systems for evaluating the dermal irritation potential of chemicals since this author first reviewed the field (Gad and Chengelis, 1988). **Table 11** overviews 20 proposed systems which now constitute five very different approaches.

The first approach (I) uses patches of excised human or animal skin maintained in some modification of a glass diffusion cell, which maintains the moisture, temperature, oxygenation and electrolyte balance of the skin section. In this approach, after the skin section has been allowed to equilibrate for some time, the material of concern is placed on the exterior surface and wetted (if not a liquid). Irritation is evaluated either by swelling of the skin (a crude and relatively insensitive method for mild and moderate irritants), by evaluation of inhibition of uptake of radiolabelled nutrients or by measurement of leakage of enzymes through damaged membranes.

The second set of approaches (II) utilizes a form of surrogate skin culture comprising a mix of skin cells which closely mirror key aspects of the architecture and function of the intact organ. These systems seemingly offer a real potential advantage but, to date, the 'damage markers' employed (or proposed) as predictors of dermal irritation have been limited to cytotoxicity.

The third set of approaches (III) is to use some form of cultured cell (either primary or transformed), with primary human epidermal keratinocytes (HEKs) preferred. The cell cultures are exposed to the material of interest, then either cytotoxicity, release of inflammation markers or decrease of some indicator of functionality (lipid metabolism, membrane permeability or cell detachment) is measured.

The fourth group (IV) contains two miscellaneous approaches—the use of a membrane from the hen's egg with a morphological evaluation of damage being the predictor end point (Reinhardt *et al*., 1987), and the SKINTEX system, which utilizes the coagulation of a mixture of soluble proteins to predict dermal response.

Finally, in group V there are two SAR models which use mathematical extensions of past animal results correlated with structure to predict the effects of new structures.

Many of these test systems are in the process of evaluation of their performance against various small groups of compounds for which the dermal irritation potential is known. Evaluation by multiple laboratories of a wider range of structures will be essential before any of these systems can be generally utilized.

5.3 Irritation of Parenterally Administered Pharmaceuticals

Intramuscular (im) and intravenous (iv) injection of parenteral formulations of pharmaceuticals can produce a range of discomfort resulting in pain, irritation and/or damage to the muscular or vascular tissue. These are normally evaluated for prospective formulations before use in humans by evaluation in intact animal models—usually the rabbit (Gad and Chengelis, 1988).

Currently, a protocol utilizing a cultured rat skeletal muscle cell line (the L6) as a model is in an inter-laboratory validation programme among more than 10 pharmaceutical company laboratories. This methodology (Young *et al*., 1986) measures creatine kinase levels in media after exposure of the cells to the formulation of interest, and predicts *in vivo* im damage based on this end point. It is reported to give excellent rank-correlated results across a range of antibiotics (Williams *et al*., 1987), and in a recent multilaboratory evaluation a broader structural range of compounds (PMA/Drusafe In Vitro Task Force, 1994).

Another proposed *in vitro* assay for muscle irritancy for injectable formulations is the red blood cell haemolysis assay (Brown *et al*., 1989). Water-soluble formulations are gently mixed at a 1:2 ratio with freshly collected human blood for 5 s, then mixed with a 5% w/v dextrose solution and centrifuged for 5 min. The percentage red blood cell survival is then determined by measuring differential absorbance at 540 nm, and this is compared with values for known irritants and nonirritants. Against a very small group of compounds (four), this is reported to be an accurate predictor of muscle irritation.

There is no current candidate alternative for the venous irritation test, but the *in vitro* alternative for pyrogenicity testing—the *Limulus* test—is one of the success stories for the alternatives movement. It has totally replaced the classical intact rabbit test in both research and product release testing. The test is based on the jellying or colour development of a pyrogenic preparation in the presence of the lysate of the amoebocytes of the horseshoe crab (*Limulus polyphemus*). It is simpler, more rapid and of greater sensitivity than the rabbit test it replaced (Cooper, 1975).

5.4 Sensitization and Photosensitization

There are actually several approaches available for the *in vitro* evaluation of materials for sensitizing potential.

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These use cultured cells from various sources and, as end points, look at either biochemical factors (such as production of migration inhibition factor (MIF)) or cellular events (such as cell migration or cell 'transformation').

Milner (1970) reported that lymphocytes from guinea-pigs sensitized to dinitrofluorobenzene (DNFB) would transform in culture, as measured by the incorporation of tritiated thymidine, when exposed to epidermal proteins conjugated with DNFB. This work was later extended to guinea-pigs sensitized to phenylenediamine. He also reported (Milner, 1971) that his method was capable of detecting allergic contact hypersensitivity to DNFB in humans, using human lymphocytes from sensitized donors and human epidermal extracts conjugated with DNFB.

Miller and Levis (1973) reported the *in vitro* detection of allergic contact hypersensitivity to dinitrochlorobenzene (DNCB) conjugated to leucocyte and erythrocyte cellular membranes. This indicated that the reaction was not specifically directed towards epidermal cell conjugates. Thulin and Zacharian (1972) extended others' earlier work on MIF-induced migration of human peripheral blood lymphocytes to a test for delayed contact hypersensitivity. Burka *et al*. (1981) reported on an assay system based on isolated guinea-pig trachea. No further mention of this has been found in the literature. None of these approaches has yet been developed as an *in vitro* predictive test, but work is progressing. Milner (1983) published a review of the history and state of this field which still provides and accurate and timely overview.

Any alternative (*in vitro* or *in vivo*) test for sensitization will need to be evaluated against a battery of 'known' sensitizing compounds. The Consumer Product Safety Commission in 1977 proposed such a battery, which is shown in **Table 12**. This has not yet been done for any of the proposed systems. Owing to the complexity of the system involved, it is unlikely that a suitable *in vitro* replacement system will be available soon.

Gad *et al*. (1986) have published comparative data on multiple animal and human test system data for some 72 materials. Such a database should be considered for the development and evaluation of new test systems.

Table 12 Requested reference compounds for skin sensitization studies (US Consumer Product Safety Commission)

Hydroxylamine sulfate	Penicillin G
Ethyl amino benzoate	*p*-Phenylenediamine
Iodochlorohydroxy quinoline (Clioquinol, Chinoform)	Epoxy systems (ethylenediamine, diethylenetriamine, diglycidyl ethers)
Nickel sulfate	Toluene-2, 4-diisocyanate
Monomethyl methacrylate	Oil of Bergamot
Mercaptobenzothiazole	

5.5 Phototoxicity and Photosensitization

The Daniel test for phototoxicity (also called photoirritant contact dermatitis) utilizes the yeast *Candida albicans* as a test species and has been in use for more that 20 years (Daniel, 1965). The measured end point is simply cell death. The test is simple to perform and cheap, but does not reliably predict the phototoxicity of all classes of compounds (for example sulfanilamide). Test systems utilizing bacteria have been suggested as alternatives over the last 10 years (Harter *et al*., 1976; Ashwood-Smith *et al*., 1980) for use in predicting the same end point.

Most recently, ICI has conducted studies on an *in vitro* phototoxicity assay which involves using three cultured cell lines: the A431 human epidermal cell line (a derived epidermal carcinoma), normal HEKs (a primary cell line derived from cosmetic surgery) and the 3T3 Swiss mouse fibroblast cell line. The protocol for this assay involves subculturing the particular cell type into microtitre tissue-culture-grade plates and incubating them over a period of 24 hours. Following incubation, the cultures are exposed to the test compound at a concentration predetermined as nontoxic. After a 4 hour exposure to the compound, the cell cultures are exposed to either UV A (320–400 nm) or UV A/B (280–400 nm) radiation for varying lengths of time. The degree of enhanced toxicity effected by either UV A or UV A/B radiation in the presence of the test compound relative to the control is assessed, using the MTT assay. MTT, abbreviated from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, undergoes a reduction reaction which is specific to mitochondrial dehydrogenases in viable cells. Work on validation of this test using 30 compounds of know phototoxic potential has shown a high degree of correlation between *in vitro* and *in vivo* results. Jackson and Goldner (1989) have described several other *in vitro* assay systems for this end point.

The area of development of *in vitro* photosensitization assays has been a very active one, as the review of McAuliffe *et al*. (1986) illustrates. Such tests have focussed on being able to predict the photosensitizing potential of a compound and variously employed cultured mammalian cell lines, red blood cells, micro-organisms and biochemical reactions. McAuliffe's group has developed and proposed a test that measures the incorporation of tritiated thymidine into human peripheral blood mononuclear cells as a predictive test (Morison *et al*., 1982). They claim to have internally validated the test, using a battery of known photosensitizers.

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Bockstahler *et al*. (1982) have developed and proposed another *in vitro* test system which uses the responses of two *in vitro* mammalian viruses—host cell systems to the photosensitizing chemicals proflavine sulfate and 8-methoxypsoralen (8-MOP) in the presence of light as a predictive system. They found that infectious simian virus 40 (SV40) could be induced from SV40-transformed hamster cells by treatment with proflavine plus visible light or 8-MOP plus near-UV radiation. The same photosensitizing treatments inactivated the capacity of monkey cells to support the growth of herpes simplex virus. SV40 induction and inactivation of host cell capacity for herpes virus growth might be useful as screening systems for testing the photosensitizing potential of chemicals. Advantages (ease and speed of conduct) and disadvantages (use of potentially infective agent and the limited range of compounds evaluated to date) were found to be associated with both of these test systems.

5.6 Development Toxicity

The area of developmental toxicology actually is one of the earliest to have alternative models suggested for it, and has one of the most extensive and oldest literatures. This is, of course, partly owing to such models originally being used to elucidate the essential mechanisms and process of embryogenesis.

Because of the complicated and multiphasic nature of the developmental process, it has not been proposed that any of these systems be definitive tests, but rather that they serve as one form or another of a screen. As such, these test systems would either preclude or facilitate more effective full-scale evaluation in one or more of the traditional whole-animal test protocols.

The literature and field are much too extensive to review comprehensively here. There are a number of extensive review articles and books on the subject (Wilson, 1978; Clayton, 1981; Kochhar, 1981; Saxen, 1984; Homburger and Goldberg, 1985; Faustman, 1988; Daston and D'Amato, 1989), which should be consulted by those with an in-depth interest.

The existing alternative test systems fall into six broad classes: (i) lower organisms; (ii) cell culture systems; (iii) organ culture systems; (iv) submammalian embryos; (v) mammalian embryos and (vi) others.

Table 13 provides an overview of the major representatives of these six groups, along with at least one basic reference to the actual techniques involved and the system components for each.

The comparative characteristics of these different classes of test systems are presented in **Table 14**. The key point is that these systems can be used for a wide range of purposes, only one of which is to screen compounds to determine the degree of concern for developmental toxicity.

The utility of these systems for screening is limited by the degree of dependability in predicting effects primarily in people and secondarily in the traditional whole-animal test systems. Determining the predictive performance of alternative test systems requires the evaluation of a number of compounds for which the 'true' (human) effect is known. In 1983 a consensus workshop generated a so-called 'gold standard' set of compounds of known activity (Smith *et al*., 1983). The composition of this list has been open to a fair degree of controversy over the years (Flint, 1989; Johnson, 1989; Johnson *et al*., 1989). However, an agreed-upon 'gold standard' set of compounds of known activity is an essential starting point for the validation of any single test system or battery of test systems because of the multitude of mechanisms for developmental toxicity. It is unlikely that any one system will be able to stand in place of segment II studies in two species, much less to accurately predict activity in humans. Their use as general screens or as test systems for compounds with little potential for extensive or intended human exposure will, however, probably be appropriate.

5.7 Target Organ Toxicity Models

This last model review section addresses perhaps the most exciting potential area for the use of *in vitro* models—as specific tools to evaluate and understand discrete target organ toxicities. Here the presumption is that there is reason to believe (or at least suspect) that some specific target organ (nervous system, lungs, kidney, liver, heart, etc.) is or may be the most sensitive site of adverse action of a systemically absorbed agent. From this starting point, a system that is representative of the target organ's *in vivo* response would be useful in at least two contexts.

First, as with all the other end points addressed in this chapter, a target organ predictive system could serve as a predictive system (in general, a screen) for effects in intact organisms, particularly man. As such, the ability to identify those agents with a high potential to cause damage in a specific target organ at physiological concentrations would be extremely valuable.

The second use is largely specific to this set of *in vitro* models. This is to serve as tools to investigate, identify and/or verify the mechanisms of action for selective target organ toxicities. Such mechanistic understandings then allow for one to know whether such toxicities are relevant to man (or to condition of exposure to man), to develop means either to predict such responses while they are still reversible or to develop the means to intervene in such toxicosis (i.e. first aid or therapy), and finally to potentially modify molecules of interest to avoid

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Table 13 Alternative developmental toxicity test systems

Category	Test system	Model	References
I: Lower organisms	Sea urchins	Organism	Kotzin and Baker (1972)
	Drosophila	Intact and embryonic cells	Abrahamson and Lewis (1971)
	Trout	(Fish species)	MacCrimmon and Kwain (1969)
	Planaria	Regeneration	Best *et al*. (1981)
	Brine shrimp	Disruption of elongation; DNA and protein levels in *Artemia nauplii*	Kerster and Schaeffer (1983), Sleet and Brendel (1985)
	Animal virus	Growth of poxvirons in culture	Keller and Smith (1982)
	Slime mold	Dictyostelium discoidem	Durston *et al*. (1985)
	Medaka	(Fish species)	Cameron *et al*. (1985)
	'Artificial embryo'	*Hydra attenuata*	Johnson *et al*. (1982)
II: Cell culture	Protein synthesis of cultured cells	Pregnant mouse and chick lens epithelial cells	Clayton (1979)
	Avian neural crest	Differentiation of cells	Sieber-Blum (1985)
	Neuroblastoma	Differentiation of cells	Mummery *et al*. (1984)
	Lectin-mediated attachment	Tumour cells	Braun and Horowicz (1983)
III: Organ culture	Frog limb	Regeneration	Bazzoli *et al*. (1977)
	Mouse embryo limb bud	Inhibition of incorporation of precursor and of DNA synthesis	Kochhar and Aydelotte (1974)
	Metanephric kidney organ cultures	From 11 day mouse embryos	Saxen and Saksela (1971)
IV: Submammalian embryo	Chick embryo	—	Gebhardt (1972)
	Frog embryo	*Xenopus laevis*	Davis *et al*. (1981)
V: Mammalian embryo	Rat embryo culture	Whole postimplantation embryos	Brown and Fabro (1981), Cockroft and Steele (1989)
	Chernoff	Mouse embryo short test	Chernoff and Kavlock (1980)
	'Micromass cultures'	Rat embryo midbrain and limb	Flint and Orton (1984)
VI: Other	Structure–activity relationships (SAR)	Mathematical correlations of activity with structural features	Enslein *et al*. (1983b) Gombar *et al*. (1990)

unwanted effects while maintaining desired properties (particularly important in drug design).

In the context of these two uses, the concept of a library of *in vitro* models (Gad, 1989c; 1996) becomes particularly attractive. If one could accumulate a collection of 'validated', operative methodologies that could be brought into use as needed (and put away, as it were, while not being used), this would represent an extremely valuable competitive tool. The question becomes one of selecting which systems/tools to put into the library, an how to develop them to the point of common utility.

Additionally, one must consider what forms of markers are to be used to evaluate the effect of interest. Initially, such markers have been exclusively either morphological (in that there is a change in microscopic structure), observational (is the cell/preparation dead or alive or has some gross characteristic changed?) or functional (does the model still operate as it did before?). Recently it has become clear that more sensitive models do not just generate a single-end-point type of data, but rather a multiple set of measures which in aggregate provide a much more powerful set of answers.

There are several approaches to *in vitro* target organ models.

The first and oldest is that of the isolated organ preparation. Perfused and superfused tissues and organs have been used in physiology and pharmacology since the late nineteenth century. There is a vast range of these available, and a number of them have been widely used in toxicology (Mehendale, 1989 presents an excellent overview). Almost any end point can be evaluated in most target organs (the central nervous system being a notable exception), and these are closest to the *in vivo* situation and therefore generally the easiest to extrapolate or conceptualize from. Those things that can be measured or evaluated in the intact organism can largely also

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Table 14 Developmental toxicity test system considerations

Possibility	*In vivo*	Organ culture	Cell culture	Lower organisms	Mammalian embryo culture	Submammalian embryos	Other
To study maternal and organ factors	Yes	No	No	No	No/Yes	No/Yes	NA
To study embryogenesis as a whole	Yes	No	No	No	Yes	Somewhat	NA
To eliminate maternal confounding factors (nutrition, etc.)	No	Yes	Yes	No	Yes	Yes	NA
To eliminate placental factors (barrier differences)	No	Yes	Yes	No	Yes	No	NA
To study single morphogenetic events	Difficult	Yes	No	Maybe	Yes	Yes	NA
To create controllable, reproducible conditions	Difficult	Yes	Yes	Yes	Yes	Yes	NA
For exact exposure and timing	Difficult	Yes	Yes	Yes	Yes	Yes	NA
For microsurgical manipulations	Difficult	Yes	No	Maybe	Yes	Yes	NA
For continuous registration of the effects	Difficult	Yes	Yes	No	Yes	Yes	NA
To collect large amounts of tissue for analysis	Yes	Difficult	Yes	No	Yes	No	NA
To use human embryonic tissue for testing	No	Yes	Yes	No	No	No	NA
Screening	Expensive	Yes	Yes	Yes	Yes	Yes	Yes

NA = not available.

be evaluated in an isolated tissue or organ preparation. However the drawbacks or limitations of this approach are also compelling.

An intact animal generally produces one tissue preparation. Such a preparation is viable generally for a day or less before it degrades to the point of losing utility. As a result, such preparations are useful as screens only for agents that have rapidly reversible (generally pharmacological or biomechanical) mechanisms of action. They are superb for evaluating mechanisms of action at the organ level for agents that act rapidly, but not generally for cellular effects of for agents that act over a course of more than a day.

The second approach is to use tissue or organ culture. Such cultures are attractive, owing to maintaining the ability for multiple cell types to interact in at least a near-physiological manner. They are generally not as complex as perfused organs, but are stable over a longer period of time, increasing their utility as screens somewhat. They are truly a middle ground between perfused organs and cultured cells. Only for relatively simple organs (such as the skin and bone marrow) are

Table 15 Representative *in vitro* test systems for respiratory system toxicity

System	End point	Evaluation	References
Isolated perfused rat and rabbit lungs (S)	Damage markers: exudate of hormones	Correlation with results *in vivo*	Anderson and Eling (1976), Roth (1980), Mehendale (1989)
Alveolar macrophages (S)	Cytotoxicity: as a predictor of fibrogenicity	Correlation with *in vivo* fibrogenicity across a broad range of compounds	Reiser and Last (1979)
Lung organ culture (M,S)	Morphological: structure and macromolecular composition	Proposed from prior experience in pharmacology	Placke and Fisher (1987)
Hamster lung culture (M)	Morphological: structure and cell death	Correlation of *in vivo* effects of cigarette smoke	Stammati *et al*. (1981)

Letters in parentheses indicate primary employment of system: S = screening system; M = mechanistic tool.

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Table 16 Representative *in vitro* test systems for neurotoxicity

System	End point	Evaluation	References
Perfused rat phrenic nerve—hemi-diaphragm (M)	Functional: release of acetylcholine, conduction velocities, muscle response	Correlates with *in vivo* effects of trialkyltins	Bierkamper (1982)
Primary rat cerebral cells (S)	Observational: cell growth and differentiation	Cell diameter and outgrowth	Hooisma (1982)
Primary rat tissue culture (S)	Functional: receptor-ligand binding	Binding rates	Bondy (1982), Volpe *et al*. (1985)
Organotypic neural cultures (S)	Functional: electrophysiological and pharmacological properties	Correlation with *in vivo* results for a range of known active agents	Spencer *et al*. (1986), Kontur *et al*. (1987)
Isolated perfused brain (M)	Functional: biochemical and electrophysiological	Unknown	Mehendale (1989)
Cultured mouse otocyst (M)	Morphological	Unknown—a tool for potentially evaluating ototoxins	Harpur (1988)

Letters in parentheses indicate primary employment of system: S = screening system; M = mechanistic tool.

good models which perform in a manner representative of the *in vitro* organ available.

The third and most common approach is that of cultured cell models. These can be either primary or transformed (immortalized) cells, but the former have significant advantages in use as predictive target organ models. Such cell culture systems can be utilized to identify and evaluate interactions at the cellular, subcellular and molecular level on an organ- and species-specific basis (Acosta *et al*., 1985). The advantages of cell culture are that single organisms can generate multiple cultures for use, that these cultures are stable and useful for protracted periods of time and that effects can be studied very precisely at the cellular and molecular levels. The disadvantages are that isolated cells cannot mimic the interactive architecture of the intact organ, and will respond over time in a manner that becomes decreasingly representative of what happens *in vivo*. An additional concern is that, with the exception of hepatocyte cultures, the influence of systemic metabolism is not factored in unless extra steps are taken. Stammati *et al*. (1981) and Tyson and Stacey (1989) present some excellent reviews of the use of cell culture in toxicology. Any such cellular systems would

Table 17 Representative *in vitro* test systems for renal toxicity

System	End point	Evaluation	References
Rat proximal tubular cells (S)	Functional: methylglucose uptake or organic ion transport	Correlation with effects of known nephrotoxin	Boogaard *et al*. (1989)
Rat cortical epithelial cells (S)	Functional: biochemical	Good correlation with *in vivo* for nephrotoxic metals and acetaminophen	Smith *et al*. (1986, 1987) , Rylander *et al*. (1987)
Isolated perfused kidney (M)	Functional: biochemical and metabolic morphological	Correlation with *in vivo* findings for some nephrotoxins	Mehendale (1989)
Renal slices (S,M)	Full range of functional (biochemical and metabolic)	Correlation with *in vivo* findings for a range of nephrotoxins. Still allows evaluation of a degree of cell-to-cell and nephron-to-nephron interactions	Smith *et al*. (1988)

Letters in parentheses indicate primary employment of system: S = screening system; M = mechanistic tool.

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Table 18 Representative *in vitro* test systems for cardiovascular toxicity

System	End point	Evaluation	References
Coronary artery smooth muscle cells (S)	Morphological evaluation—vacuole formation	Correlates with *in vivo* results	Ruben *et al*. (1984)
Isolated perfused rabbit or rat heart (M,S)	Functional: operational, electrophysiological, biochemical and metabolism	Long history of use in physiology and pharmacology	Mehendale (1989)
Isolated superfused atrial and heart preparations (S,M)	Functional: operational and biochemical	Correlation with *in vivo* findings for antioxidants	Gad *et al*. (1977, 1979)

Letters in parentheses indicate primary employment of system: S = screening system; M = mechanistic tool.

be more likely to be accurate and sensitive predictors of adverse effects if their function and integrity were evaluated while they were operational. For example, cultured nerve cells should be excited while being exposed and evaluated.

A wide range of target-organ-specific models have already been developed and used. Their incorporation into a library-type approach requires that they be evaluated for reproducibility of response, ease of use and predictive characteristics under the intended conditions of use. These evaluations are probably at least somewhat specific to any individual situation. **Tables 15–20** present overviews of representative systems for a range of target organs: respiratory, nervous system, renal,

Table 19 Representative *in vitro* test systems for hepatic toxicity

System	End point	Evaluation	References
Primary hepatocytes (S,M)	Multiple: • Biotransformation • Genotoxicity • Peroxisome proliferation • Biliary dysfunction • Membrane damage • Ion regulation • Energy regulation • Protein synthesis	NA	See Tyson and Stacey (1989)[a], Stammati *et al*. (1981)
Hamster hepatocytes (S)	Functional: biochemical	Correlates with *in vivo* effects of acetaminophen	Harman and Fischer (1983)
Rat liver slices (S)	Functional: alterations in ion content, leakage of damage markers, changes in biosynthetic capability Morphological: histopathological evaluation	Rank correlation with *in vivo* findings for a wide range of chemicals	Gandolfi *et al*. (1989), Adams (1995), Fisher *et al*. (1995)
Isolated perfused liver (M)	Functional: biochemical and metabolic	Correlation with *in vivo* findings for a wide range of chemicals	Mehendale (1989), Wyman *et al*. (1995)

[a]Tyson and Stacey estimated in 1989 that there were 800 unpublished studies of a toxicological nature on cultured hepatocytes.
Letters in parentheses indicate primary employment of system: S = screening system; M = mechanistic tool. NA = not available.

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Table 20 Representative *in vitro* test systems for other target organ studies

Organ	System	End point	Evaluation	References
Pancreas	Isolated perfused intestines (M)	Functional: biochemical and metabolic	Correlation with *in vivo* findings for methylprednisolone	Mehendale (1989)
GI tract	Isolated perfused intestines (M)	Functional: biochemical and metabolic	Limited	Mehendale (1989)
Reticuloendothelial	Erythrocytes (S)	Observational: cytotoxicity	Correlation with haemolytic effects	Stammati *et al*. (1981)
		Functional: inhibition of colony formation		
Testicular	Sertoli and germ cell cultures (S)	Observational: cytotoxicity	Correlation with *in vivo* effects for phytholate esters and glycol ethers	Garside (1988)
		Functional: steroid and hormone production		
Adrenal gland	Primary adrenocortical ADC cell cultures (S,M)	Functional: cortisol production	Correlation with *in vivo* effects for three known ADC toxicants	Wolfgang *et al*. (1994)
Thyroid	Cultured thyroid cells (S,M)	Functional: biochemical and metabolic	Correlation with *in vivo* findings for a wide range of agents with thyroid- specific toxicity; evaluation against 'negative' compounds not significant	Brown (1988)

Letters in parentheses indicate primary employment of system: S = screening system; M = mechanistic tool.

cardiovascular, hepatic, pancreatic, gastrointestinal and reticuloendothelial. These tables do not mention any of the new coculture systems in which hepatocytes are 'joined up' in culture with a target cell type to produce a metabolically competent cellular system.

While in Europe there are numerous validated (by European Centre for the Validation of Alternative Methods (ECVAM) Standards) *in vitro* alternatives for personal care products, industrial and agricultural chemicals (though only two—theLAL assay for pyrogenicity and the 3T3 cell assay for phototoxicity—have acceptance for use with pharmaceutical regulators) with regulatory acceptance (as summarized in **Table 21**), this is not the case in the US. Indeed, only four alternatives have been approved by the ICCVAM process to date (Gaul, 2008). Though there is considerable research continuing on the development (and putatively, on the validation) of many more methods (Kuehn, 2008), progress in obtaining regulatory acceptance of such methods to take the place of animal test methods is very slow, and those methods that are accepted are limited in scope screens for eye and skin irritation pyrogenicity, phototoxicity and genotoxicity.

This regulatory acceptance is, indeed, as it has been for 20 years, the final frontier and true obstacle to any further significant reduction in animal use.

6 SUMMARY

The tools are currently at hand (or soon will be) to provide the practising toxicologist with unique opportunities both for identifying potentially toxic compounds in a much more rapid and efficient manner than before and for teasing apart the mechanisms underlying such toxicities on an integrated basis (from the level of the molecule to that of the intact organism). The *in vitro* systems overviewed here, once understood (by investigators and

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Table 21 Status of nonanimal methods that have regulatory standing

Test method	Test system	End point	OECD/TG or other regulatory comments
In vitro test methods for which there are OECD health effects test guidelines (including draft guidelines under review for acceptance) can be found at http://www.oecd.org/home/			
Transcutaneous electrical resistance test (TER)	Monitors changes in the electrical resistance as a measure of loss of corneum integrity and barrier function; involves skin disks from euthanized rats	Skin corrosion (topical agents)	TG 430
Human skin models (EpiDerm™, EPISKIN™)	Reconstructed human epidermal equivalent (commercial system) used to assess cell viability, involving the MTT reduction test	Skin corrosion (topical agents)	TG 431
3T3 NRU phototoxicity test	BALB/c 3T3 (murine) cell line cytotoxicity based on Neutral Red uptake to measure cell viability; not direct replacement alternative, as there is no *in vivo* equivalent test	Phototoxicity	TG 432/FDA guidance
Corrositex™ membrane barrier test	An artificial barrier system coupled to a pH-based chemical detection system	Skin corrosion (topical agents)	Draft TG 435
Bacterial reverse mutation test (Ames)	Revertant bacteria detected by their ability to grow in the absence of the amino acid	Genotoxicity	TG471/ICH,ISO, FDA guidance
In vitro mammalian chromosome aberration test	Microscopic detection of chromosomal damage to cells in culture	Genotoxicity	TG473/ICH, ISO, FDA guidance
In vitro mammalian cell gene mutation test	Functional bioassays to monitor mutations in enzyme encoding genes	Genotoxicity	TG476
Sister chromatid exchange assay	Cells in culture are examined after two rounds of division by metaphase arrest and chromosomal preparation; chromatid exchange is monitored by microscopy	Genotoxicity	TG479/ICH, ISO, FDA guidance
Gene mutation assay in yeast	*Saccharomyces cerivisiae* exposed to the test substance are grown under different culture conditions used to monitor mutagenic potential (cf. Ames test)	Genotoxicity	TG 480
Mitotic recombination assay in yeast	Crossover or gene conversion following exposure of yeast to the test substance; relies on different growth requirements of mutated and wild-type yeast strains	Genotoxicity	TG 481
Unscheduled DNA synthesis in mammalian cells	Measures the DNA repair synthesis after deletions caused by the test substance based on the incorporation of radioactive nucleotides into the newly synthesized DNA	Genotoxicity	TG 482/ICH, ISO and FDA guidance
In vitro micronucleus test	Cell-based assay; supplement to TG 474 (*in vivo* micronuleus test); detection of chromosome damage and formation of micronuclei in interphase	Genotoxicity	Draft TG 487/ICH guidance
Sex-linked recessive lethal test	*Drosophila* are exposed to the test substance. Germline transmission of mutations is monitored through two successive generations	Reproductive toxicity	TG 477

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Table 21 (*continued*)

Test method	Test system	End point	OECD/TG or other regulatory comments
Validated methods that are yet to be introduced into regulatory use			
EpiOcular™	Human keratinocyte derived model of the corneal epithelium barrier function	Eye irritation (topical application)	Retrospective (weight-of-evidence) validation (ECVAM)
In vitro micronucleus test	CHL/IU, CHO, SHE or V79 cell lines are commonly used, with or without metabolic activation, to monitor damage and formation of micronuclei in interphase	Mutagenicity	Retrospective (weight-of-evidence) validation (ECVAM)
Embryonic stem cell test	3T3 cell cytotoxicity and differentiation of embryonic stem murine cell lines used to examine teratogenic potential	Developmental toxicity	Endorsed as screening test (EU)
Postimplantation rat whole embryo test	Morphological assessment of rat embryos	Developmental toxicity	Endorsed as screening test (EU)
Micromass test	Micromass cultures of rat limb are bud monitored for inhibition of cell proliferation and differentiation	Developmental toxicity	Endorsed as screening test (EU)
Methods undergoing validation			
EPISKIN™	Reconstructed human skin system used with MTT assay to monitor barrier function	Skin irritation	Report stage in EU
EpiDerm™	Similar to EPISKIN™	Skin irritation	Report stage in EU
Prevalidated methods			
SkinEthic eye model	Epithelial corneal cell line used for cytotoxicity testing based on the MTT reduction assay	Eye irritation	Appraisal stage in EU
Methods undergoing development, prevalidation or evaluation			
Tissue culture models	Neutral Red release and silicon microphysiometry or fluorescein leakage bioassays with human keratinocytes and MDCK cells, respectively; red blood cell (RBC) haemolysis test	Eye irritation	Being reviewed by ICCVAM for possible retrospective (weight-of-evidence) validation
Organotypic models	Bovine corneal opacity and permeability (BCOP) assay, with postmortem corneas; hen's egg test on the chorioallantoic membrane (HET-CAM assay); isolated rabbit and chicken eye tests (IRE and ICE)	Eye irritation	Being reviewed by ICCVAM for possible retrospective (weight-of-evidence) validation
Modified Leydig cell line	Analysis of progesterone production as a measure of the test substance effects on steroid hormone production	One/two generation study	For use as part of test battery
Testis slices	Assessment of steroid production capacity of the Leydig cells upon exposure of *ex vivo* rat tissue to toxicants	One/two generation study	For use as part of test battery
Human carcinoma cell line	Assay to allow entire steroid pathway effects to be mapped	One/two generation study	For use as part of test battery
Placental microsomal aromatase assay	Monitors the ability of substances to affect steroid production; a subcellular microsomal assay is used industrially	One/two generation study	For use as part of test battery

This is a comprehensive list of methods that have been validated or that are at various stages of development for toxicity testing. More information about these methods and how they can be applied is available from: *http://www2.defra.gov.uk/research/project_data/more?!=CB01067&M=KWS&V=reach&scope=0* and OECD, 2005.

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regulators) in how they function and fail (just as *in vivo* systems have come to be understood), will allow this to happen while reducing the need to have recourse to intact mammalian test systems. However, the intact animal models—and, indeed, human for pharmaceuticals—will still be an essential element in the safety assessment armamentarium for the foreseeable future.

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Nanotoxicology—The Toxicology of Nanomaterials

Kristin Gellein and Tore Syversen

CONTENTS

1 INTRODUCTION

Since ancient times, humans have been exposed to nanosized materials originating from combustion processes, such as forest fires and volcanoes, as well as natural sources, such as sea spray. This exposure drastically increased with the industrial revolution, when anthropogenic sources of nanosized particles were introduced. Now, with the rapid development of nanotechnology, we have a new source of exposure to nanosized materials.

Nanotoxicology is a new field of study that emerged because nanometre-scale particles may behave different from their physically larger chemical counterparts (Donaldson *et al*., 2004). The term nanotoxicology refers to the study of interactions of nanostructures with biological systems, with an emphasis on the relationship between the physical and chemical properties of nanostructures and induction of toxic responses (Fischer and Chan, 2007).

Nanomaterials (NMs) are defined as engineered structures with at least one dimension of 100 nm or less. They include nanoparticles, nanofibres and nanotubes, composite materials and nanostructured surfaces. Engineered NMs have unusual physicochemical properties because of their small size, chemical composition, surface structure, solubility, shape and aggregation (Nel *et al*., 2006). NMs usually differ substantially from bulk materials of the same chemical composition.

NMs are expected to have a substantial impact on many industrial products and areas of society because of their unique and novel properties (Hardman, 2006). However, such widespread use will inevitably also raise concern about possible adverse biological effects (Nel *et al*., 2006). Our understanding of the interactions of nanostructures with biological systems is still very limited, and it is still unclear to what extent exposure to humans, animals, insects and plants may produce harmful biological responses (Fischer and Chan, 2007). In particular, our understanding of the ecotoxicology of NMs is very limited compared to other chemicals with a similar distribution in the environment.

The interaction of NMs with biological systems is affected by several factors such as: size, surface area, shape, chemical composition, lattice structure, surface chemistry, surface charge and aggregation state. These are all physicochemical properties that can affect the toxicity of NMs (Zuin *et al*., 2007). Because of their special physical and chemical features, the study of NMs as potential toxic agents requires an interdisciplinary approach, including disciplines ranging from physics and chemistry to biology and medicine (Fubini *et al*., 2007).

Nanotoxicology is a fast-growing discipline. The concept and background for this new discipline has been thoroughly reviewed by Oberdörster *et al*. (2005a), Donaldson *et al*. (2004) and Nel *et al*. (2006).

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2 NANOMATERIALS

2.1 Properties and Use

Size is the major characteristic which differentiates these materials from other chemically similar materials. However, size has an important impact on a range of other important features that may influence toxicity. Such properties are summarized below (Borm and Kreyling, 2004; Nel *et al*., 2006):

- Nanosize
- Extremely large surface
- High surface-to-mass ratio
- High surface reactivity
- High strength, conductivity and reactivity
- Catalytic promotion of reactions
- Ability to absorb and carry other compounds
- Ability to escape defence systems *in vivo*
- Ability to cross cellular and subcellular membranes
- Specific reactivity arising at the nanolevel
- Strong interparticle forces.

Some of these properties make NMs very attractive for a broad range of applications. Engineered NMs may increase the performance of existing products as well as induce the development new products (Oberdörster *et al*., 2005a). NMs are increasingly being used for commercial purposes such as cosmetics, sunscreens (e.g. TiO_2, ZnO), car tyres, electronics, microelectronics and semiconductors. Other applications which are rapidly developing are, for example, fuel cells, coatings and catalysts. There is also a potential for the use of NMs in applications such as high-performance materials, energy storage and conversion, self-cleaning surface coatings and stain-resistant textiles (Oberdörster *et al*., 2005a). Perhaps one of the most exciting and promising areas of nanotechnology is within medical diagnosis and therapy. In this area NMs are increasingly being utilized in medicine for the purposes of diagnosis, imaging and drug delivery (Nel *et al*., 2006).

2.2 Different Types of Engineered NMs

There is a broad range of engineered NMs, for example nanoparticles, carbon-based NMs, metal particles and oxides, and quantum dots (QDs). Within these groups there are numerous subgroups.

2.2.1 Nanoparticles

Nanoparticles are defined as single spherical particles with a diameter less than 100 nm (Oberdörster *et al*., 2005a). Nanoparticles can be divided into combustion-derived nanoparticles, such as diesel soot, engineered nanoparticles, and naturally occurring nanoparticles from volcanic eruptions, atmospheric chemistry and so on. Examples of engineered nanoparticles include titanium dioxide, zinc oxide, alumina, carbon black and buckminster fullerenes (C_{60}) (Borm *et al*., 2006).

2.2.2 Carbon-Based NMs

Carbon-based NMs are composed of carbon, often with some residues from the production process (Hurt *et al*., 2006). This group of products include, but are not limited to, carbon dots, nanodiamonds and fullerenes such as buckyballs (e.g. C_{60}), nanotubes and nanofibres. CNTs are considered among the most promising materials in nanotechnology because of their extraordinary electronic and mechanical properties (Helland *et al*., 2007). They are tubelike structures of graphene sheets, with diameters as small as 1 nm and potential lengths of several hundred nanometres. CNTs include single-walled carbon nanotubes (SWCNTs) and multiwalled carbon nanotubes (MWCNTs).

2.2.3 Quantum Dots

QDs are semiconductor nanocrystals (2–100 nm) with unique optical and electrical properties currently applied in biomedical imaging and electronic industries (Hardman, 2006). QDs have a fluorescence spectrum that renders them optimal fluorophores for biomedical imaging (Hardman, 2006). They can also target specific biological events and cellular structures and are therefore attractive in site-specific gene and drug delivery. For biological applications QDs typically have a core/shell conjugate structure where the core typically is composed of metals from Groups 3–5 (e.g. Cd/Se, Cd/Te and Zn/Se). Many of these core metals are known to be toxic at low concentrations, and toxicity related to degradation of the core is therefore expected (Lewinski *et al*., 2008).

3 TOXICOLOGY OF NANOMATERIALS

3.1 Background

Our understanding of NMs toxicity is limited as they are novel materials and methods for toxicity testing are not readily available (Yang *et al*., 2008). NMs are currently available in a wide variety of compositions, from very simple structures, such as titanium oxide, to very complex structures with surface modifications or coatings. As a result, the traditional approaches of toxicity testing are not directly applicable (Donaldson *et al*., 2004). NMs

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are not toxic entities *per se* just because of their size. However, it is clear that these materials hold unusual properties due to their nanosize, which may affect their toxicity. A current paradigm to nanotoxicology is the adverse health effects of ultrafine particles (UFPs) found in air and working environments, which indicates that exposure to nanosized materials can cause significant health problems, such as pulmonary and cardiovascular diseases (Donaldson *et al*., 2004; Oberdörster *et al*., 2005a).

Particle size and surface area are important characteristics from a toxicological viewpoint. In a study of carbon black, it was found that biological effects such as inflammation, genotoxicity and histology were related to surface area and not particle mass (Yang *et al*., 2008). Additionally, the extremely small size of NMs means that they more readily gain entry into the human body compared to larger-sized particles. The behaviour of these nanoparticles inside the body is a major question that needs to be resolved. There is also concern because of their potential for interaction with biological processes inside the body. Because of their large surface area, nanoparticles will adsorb on to their surface some of the macromolecules present in their environment. This may, for instance, affect the regulatory mechanisms of enzymes and other proteins. There is also the question about what happens when nondegradable or slowly degradable NMs accumulate in body organs.

As CNTs are considered to be perhaps the most promising materials in nanotechnology, they are among the most studied NMs. *In vitro* studies in rats have shown that they have the potential to cause pulmonary toxicity (Lam *et al*., 2004; Warheit *et al*., 2004). Due to their needle-like fibre shape, these nanotubes have a structural resemblance to asbestos, which is known to induce asbestosis, lung cancer and malignant mesothelioma of the pleura (LaDou, 2004). In a recent study that introduced CNTs into the abdominal cavity of mice, CNTs showed the same effects as long thin asbestos fibres, raising concerns that exposure to CNTs may lead to mesothelioma (Poland *et al*., 2008).

3.2 Human Exposure to Nanomaterials

People become exposed to NMs in a variety of ways, such as at the workplace (e.g. engineers, researchers and clinicians), as patients during therapeutic or diagnostic administration or as consumers through the use of products containing nanostructures (e.g. cosmetics, sunscreens). At this point, not much is known about levels of exposure via the different routes for NMs and the relative importance of direct exposure or indirect exposure through environmental contamination (Oberdörster *et al*., 2005a).

Potential routes of NMs exposure include the gastrointestinal tract, skin, lung and systemic administration for diagnostic and therapeutic purposes. In all cases, there will be unique routes of exposure that will dictate the specific fate of the nanostructures. Inhalation is the most significant exposure route for airborne NMs, and inhaled nanoparticles are efficiently deposited by diffusion mechanisms in all regions of the lung (Oberdörster *et al*., 2005a). Consumer exposure to nanoparticles presents another exposure route for these materials. Engineered nanoparticles are used in personal-care products, for example, cosmetics and sunscreens. It is impossible to assess the quantities and types of NMs in these products as such information is often protected from public disclosure by trade secrets (Colvin, 2003). Even though dermal exposure to NMs occurs regularly through the use of cosmetics and sunscreens, the epidermis in healthy skin provides excellent protection against particles spread to the dermis (Nel *et al*., 2006).

Once the NMs has entered the body they will distributed through the bloodstream to various tissues by processes of partitioning and diffusion (Garnett, 2007). At the target site, NMs may trigger tissue injury. The nanostructures may also enter cells of target organs and remain in the cells for some time before moving to other organs or being excreted (Fischer and Chan, 2007). The interactions of NMs with cells, body fluids and proteins play a important role in both their biological effects and their ability to distribute throughout the body (Nel *et al*., 2006). NMs binding to proteins may generate complexes that are more mobile and can enter tissue sites that would normally be inaccessible. Depending on the chemical composition, the NMs may remain unchanged, become modified, or even metabolized. Known sites of localization of nanoparticles are bone marrow, spleen, brain and lymph nodes (Garnett, 2007). All of these organs contain macrophages, and thus it is probable that nanostructures are taken up by phagocytic cells. Opsonization is the interaction of nanostructures with proteins and this process contributes to the uptake of NMs by the liver and several other tissues (Garnett, 2007). Little is known about what happens to the nanostructures after they have been distributed and sequestered into cells. The breakdown of the nanostructures could elicit unique molecular responses that are difficult to predict.

It is important to recognize, evaluate and control the exposure of NMs to protect the health of researchers, production workers, users and members of the public. The exposure of workers and consumers to nanotechnology products is of immediate concern. Many of these products will eventually enter the environment and in the longer term, there will be a much wider exposure of the entire ecosystem to engineered NMs through water and soil (Nel *et al*., 2006).

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3.2.1 Ultrafine Particles

Increases in urban air pollution have been found to be associated with a range of adverse health effects. Because few of the components in this type of pollution are intrinsically very toxic, the focus has been towards the ultrafine components. UFPs (<100 nm) in urban environmental air pollution are most often produced by combustion processes. Contrary to engineered NMs, these particles have a wide size range and represent a heterogeneous chemical composition (Colvin, 2003). Many methods exist to quantify and characterize UFPs. The number of particles can be measured by a condensation particle counter (CPC), a scanning mobility particle sizer (SMPS) or an electrical low-pressure impactor (ELPI) (Brouwer *et al*., 2004). Particle characterization is also done by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (Brouwer *et al*., 2004).

UFPs may exist as singlet particles or they may exist as aggregates and the latter will change their deposition characteristics due to a greater diameter than single particles (Donaldson *et al*., 2001). Studies have shown that UFPs can contribute to adverse health effects in the respiratory tract as well as in extrapulmonary organs (Oberdörster *et al*., 2005a). Such health effects are primarily found in susceptible subgroups and not in healthy people unless they are exposed at very high concentrations (Donaldson *et al*., 2001). *In vivo* and *in vitro* studies have shown that these UFPs can cause both oxidative stress and proinflammatory effects (Donaldson and Stone, 2003). The mechanisms are still unclear, but it seems that the large surface area of UFPs play an important role (Donaldson and Stone, 2003).

3.2.2 Nanomaterials in the Workplace

As the production demand for NMs develops there will be an increase in occupational exposure, both during primary manufacture of the production and use of NMs in consumer products. Animal studies suggest that engineered NMs may pose a health hazard to employees, which underscores the need for preventive measures against both adverse affects and exposure (Schulte *et al*., 2008; Seaton and Donaldson, 2005). Even though NMs differ from their bulk counterparts, many of the workers using these substances have no formal safety training beyond those adapted for bulk solids (Colvin, 2003). At present, there is considerable uncertainty as to how we should assess the potential work-related health risks and how we should manage such risks.

In the absence of appropriate techniques we must consider occupational exposure to NMs as a logical subset of traditional exposure assessment for chemical hazards in the workplace. As we develop improved techniques for the relevant toxicology and health assays we will be able to more accurately characterize hazardous materials or procedures (Schulte *et al*., 2008). The large and diverse group of manufactured NMs arising from nanotechnology represents inhalation, skin and oral hazards of unknown potential. The actual body burden of NMs will be a mixture of these exposure possibilities. Workers can be exposed through the transfer of nanoparticles directly to skin, clothing or other objects. This can again generate airborne nanoparticles, some of which may be removed by ventilation systems, settle on surfaces or be inhaled. Contaminated surfaces can include equipment, tools, floors and stairs, clothing and areas of exposed skin. Settled particles may also be transferred to clothing or hands through direct contact with contaminated surfaces (Hoover *et al*., 2007). The proper way to handle such exposure situations is through preventive measures such as gloves, appropriate clothing and respirators, as well as procedures to keep the workplace clean and tidy.

Measuring airborne NMs in the workplace is an important element of the evaluation of possible health hazards to employees. Particle (dust) measurements are usually based on mass only, but for NMs such characterization should include surface area and number of particles as well (Brouwer *et al*., 2004; Han *et al*., 2008). Also, chemical composition, surface reactivity, aggregation or agglomeration and other physicochemical factors can influence the toxicity and should be considered, depending on the type of NMs being encountered (Schulte *et al*., 2008).

Epidemiological data specifically from nanoparticle-exposed humans are so far not available (NIOSH, 2007). The very few studies with experimental short-term human exposure to NMs have failed to demonstrate reproducible health outcomes (Beckett *et al*., 2005; Scharrer *et al*., 2007). In the absence of human data, extensive experience with welders and flame-based metal cutters may be a model (although imperfect) for estimating the potential health effects of occupational exposure to NMs (Nasterlack *et al*., 2008). Decrements in lung function have been observed in a small number of heavily exposed welders, infrequent cases of fibrosis have been attributed to improper workplace ventilation or mixed dust exposure, while central nervous system (CNS) and reproductive effects have been absent or inconsistent (Nasterlack *et al*., 2008). A relationship between lung cancer and welding has never been established, except for stainless-steel welding. This experience derived from welding cannot, of course, dismiss health concerns related to engineered NMs, but may help to set some of the concerns in perspective (Nasterlack *et al*., 2008).

From *in vivo* and *in vitro* studies we know that engineered NMs have the potential to cause mild inflammatory effects, generate reactive oxygen species (ROS) and oxidative stress, increase macrophage sensitivity to complementary components and inhibit phagocytosis (Oberdörster *et al*., 2004). The ability of NMs to redistribute from their original site of deposition is of special

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concern. Also, NMs have the ability to pass the normal phagocytosis defence without being detected, and thereby get access to, for example, blood and the nervous system (Oberdörster *et al*., 2004). It has also been reported that NMs have the potential to translocate to the brain via the olfactory nerve endings in the nose (Oberdörster *et al*., 2004).

3.2.3 Pulmonary Effects

Pulmonary toxicology is a well-established area of medical research, and appropriate techniques and procedures have been established for toxicity testing, as well as characterization of occupational diseases. Although the danger of inhaling fumes or very small smoke particles has been recognized since ancient times, systematic research into the effects of nanometre-sized particles on health did not start until the 1980s (Donaldson *et al*., 2005). Most of the toxicity research on NMs *in vivo* has been carried out in mammalian systems, with a focus on respiratory exposure (Oberdörster *et al*., 2005a).

The main mechanism for deposition of inhaled nanoparticles in the respiratory tract is diffusion due to displacement when they collide with air molecules (Oberdörster *et al*., 2005a). Inhaled nanoparticles of different sizes can target all three regions of the respiratory tract, the nasopharyngeal, tracheobronchial and alveolar region. The larger surface area of the alveoli and the intimate air–blood contact in this region make the alveoli less well protected against inhaled substances, such as nanoparticles, compared to the trachea, bronchi and bronchioles (Yang *et al*., 2008). Concern that inhalation of engineered nanoparticles may have adverse effects arises from the fact that, compared to coarse or fine particles, nanoparticles exhibit a high degree of deposition in the conducting and respiratory zones of the lung (Shvedova *et al*., 2005).

Clearance of inhaled nanoparticles from the lung depends mainly on particle size and on particle surface characteristics. In addition, alveolar macrophages appear to be unable to recognize particles less than 70 nm as being foreign, thus allowing such particles to gain access to the pulmonary interstitium and thence the capillary blood flow. Particle size may also affect the clearance mechanism. Nanofibres measuring more than 20 μm in one axis are too long to be phagocytosed and will be cleared very slowly, staying in the lungs for months or possibly years. This can, again, induce a general nonspecific pulmonary inflammatory response.

Once the nanoparticles have escaped clearance by alveolar macrophages and entered the alveolar interstitium, they can migrate to the pulmonary capillary blood and translocate to systemic sites by different transfer routes and mechanisms. One of the mechanisms is trancytosis across epithelia and access to the blood circulation directly or via lymphatics, from where the particles are then distributed throughout the body. Another mechanism is uptake by sensory nerve endings embedded in airway epithelia, followed by axonal translocation to ganglionic and CNS structures (Oberdörster *et al*., 2004). This transport mechanism is specific for NPs. It should be noted, however, that no definite conclusion about the systemic translocation of inhaled nanoparticles can be drawn to date, based on the conflicting results of animal and human studies.

3.2.4 Cardiovascular Effects

The relationship between air pollution and adverse cardiovascular effects is well documented in epidemiological studies (Donaldson *et al*., 2005). In both epidemiological and experimental studies, a positive association between particulate matter in air pollution and adverse cardiovascular outcomes has been found (Peters *et al*., 2004; Pope *et al*., 2004). One of the key processes that could mediate adverse effects on the cardiovascular system is inflammation of the lung caused by deposited particles. So far, little is known about the potential of NMs to affect the cardiovascular system, however it is plausible that exposure to NMs might trigger similar effects as have been observed to result from particulate air pollution.

Inhaled NPs may influence the vasculature through indirect effects mediated by pulmonary inflammation or through the direct action of particles that have become blood-borne (Duffin *et al*., 2007). Inflammatory effects and blood translocation could explain adverse cardiovascular effects observed in epidemiology studies with particulate air pollution (Duffin *et al*., 2007). Support for this contention comes from studies using model NPs and combustion-derived NPs, where adverse effects such as clotting, plaque development and endothelial dysfunction were enhanced after NP exposures using several different models (Mills *et al*., 2005; Radomski *et al*., 2005; Sun *et al*., 2005). Atherosclerosis is an inflammatory process, initiated via endothelial injury. Exposure of NMs may exacerbate the vascular inflammation of atherosclerosis and promote plaque development or rupture (Duffin *et al*., 2007; Sun *et al*., 2005).

Several studies have demonstrated that SWCNTs induce pulmonary toxicity (Lam *et al*., 2004; Shvedova *et al*., 2005; Warheit *et al*., 2004). The unique physical characteristics of NTs, as well as the pulmonary toxicity, are predictors for potential cardiovascular injury (Simeonova *et al*., 2007). Initial studies have demonstrated that respiratory exposure to high concentrations of SWCNTs provokes, not only pulmonary toxicity, but vascular effects related to mitochondrial oxidative modifications and accelerated atheroma formation (Simeonova *et al*., 2007). SWCNTs may induce cardiovascular effects either directly or indirectly through mitochondrial oxidative perturbations, which can result in altered vessel homeostasis (Simeonova *et al*., 2007).

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There is some evidence that carbon microscale particles can affect vascular haemostasis and precipitate thrombosis. Radomski and colleagues (Radomski *et al*., 2005) have studied the *in vitro* effects of engineered NMs on human platelet aggregation and rat vascular thrombosis. They found that nanotubes and carbon black particles, but not C_{60} bucky balls, stimulated platelet aggregation, and the same ranking was observed in their ability to induce vascular thrombosis in rat carotid arteries.

3.2.5 Neurotoxicology

The brain and other parts of the nervous system are protected by well-developed barriers. However, small and hydrophobic molecules can penetrate these barriers and nanoparticles in the size range 20–50 nm may enter the central nervous system. Acute toxicity would seem to be unlikely except in extreme circumstances, but chronic toxicity might occur (Gibson, 2007).

Nanoparticles may enter the brain where the blood–brain barrier (BBB) is absent or limited, such as the circumventricular structures, or after pathophysiological changes to the BBB induced by injury, disease or ageing (Gibson, 2007). Alternatively, following inhalation, nanoparticles may gain access to the brain via the olfactory nerve pathway (Oberdörster *et al*., 2004). The olfactory system represents a unique interface between the nervous system and the external environment, since there is an intimate association between the nasal epithelium and olfactory neurons (Gibson, 2007). This route has been investigated thoroughly for metals such as manganese (Aschner *et al*., 2005), and has also been demonstrated for CNTs and manganese oxide (Oberdörster *et al*., 2004).

Today, little is known about the consequences of manufactured nanoparticles reaching the CNS. It has been proposed that the large surface area and high surface reactivity of nanoparticles are likely to induce oxidative stress, ROS and inflammation (Nel *et al*., 2006). The nervous system is very sensitive to oxidative stress. There is evidence that ambient UFPs are linked to brain inflammation and neurological effects (Campbell *et al*., 2005), but a direct contribution from UFPs has been difficult to prove (Gibson, 2007). Recent studies do, however, suggest that pure nanoparticles can induce inflammation in the brain (Elder *et al*., 2006). Clearly, there is a need to study the neurotoxicology of nanoparticles further.

3.2.6 Dermal Effects of NMs

Skin is a complex and dynamic organ that has several functions, the primary one being to act as a barrier to the external environment. The skin is the largest organ of the body and serves as a primary route of environmental and/or occupational exposure. It consists of three layers, the epidermis, dermis and hypodermis. The epidermis consists of five layers and the outermost layer, the stratum corneum, serves as an effective barrier (Madison, 2003). However, the epidermis also provides an environment where particles could potentially lodge and not be susceptible to removal by phagocytosis (Monteiro-Riviere *et al*., 2007). Even though the skin is a primary route of potential exposure to toxicants, there is little information on whether NMs are absorbed across the stratum corneum barrier or whether systemically administered particles can accumulate in dermal tissue (Monteiro-Riviere *et al*., 2007). The ability of NMs to traverse the skin is a primary determinant of their dermatotoxic potential.

Dermal toxicology studies have shown that NMs can interact with human epidermal keratinocytes (HEK), and the effects depend on the composition, size and surface properties of the NMs (Monteiro-Riviere *et al*., 2005; Sayes *et al*., 2006). Titanium dioxide (TiO_2) is a naturally occurring mineral that is widely used in sunscreens and cosmetics due to its ability to filter ultraviolet (UV) radiation. The ability of TiO_2 in sunscreen preparations to penetrate the protective stratum corneum barrier is controversial. Larger particles of zinc and titanium oxide used in topical skincare products have been shown to penetrate the stratum corneum barrier of rabbit skin (Lansdown and Taylor, 1997). Nanoscale TiO_2 is now being proposed for use in sunscreens due to greater UV filtering ability and greater transparency. These potential benefits are countered with concerns of increased depth of skin penetration, increased reactivity and increased toxicity (Monteiro-Riviere *et al*., 2007). Studies thus far, however, indicate that nanosized TiO_2 does not penetrate the viable layers of skin in healthy adults (Kiss *et al*., 2008; Sayes *et al*., 2006). Even though, more studies on exposure to TiO_2 via the dermal route is needed.

Data is limited on the dermal effects of manufactured carbon-based NMs. HEK has showed significant decrease in viability after being exposed to SWCNTs ($0.00005–0.05\ mg\ ml^{-1}$) for 24 hours (Zhang *et al*., 2007). HEK exposed to MWCNTs has shown a concentration-dependent decrease in viability and also increased interleukin-8 (IL-8) (Monteiro-Riviere *et al*., 2005). These studies suggest a significant dermal hazard after topical exposure to SWCNTs and MWCNTs, provided that they penetrate the stratum corneum barrier. Topical administration of 200 µg of fullerenes to mouse skin over 72 hours, however, did not show any effect on either DNA synthesis or ornithine decarboxylase activity (Nelson *et al*., 1993).

QDs can be obtained commercially in liquid and powder forms, to which both producers and consumers may be exposed. It has been shown that QDs are able to penetrate porcine skin after exposure for 8–24 hours (Ryman-Rasmussen *et al*., 2006). Accumulating *in vitro* evidence indicates that exposure to QDs during manufacture and handling may be hazardous, depending upon the physicochemical composition of the QDs, the cell types

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involved and environmental factors (Monteiro-Riviere *et al*., 2007).

3.3 Ecotoxicology

The environment does contain many natural sources of particles in the nanometre scale, such as volcanic dust, seaspray and particles from soil erosion. Since these materials have been in the environment since ancient times, organisms have adapted to the presence of many of these materials. The presence of manufactured NMs in the environment now seems inevitable, given the rapidly growing number of products containing these materials, and the possible environmental impacts of these materials remain largely unknown. Engineered NMs are designed to have particular surface properties and chemistries that are less likely to be found in natural particles. They might therefore present enhanced or novel physicochemical or toxicological properties in comparison to natural NMs (Handy *et al*., 2008c). As a result of their remarkably high surface-area-to-volume ratio and complexing capability, engineered NMs may adsorb pollutants, which may change the transport and bioavailability of both the NMs and the pollutants in natural systems (Navarro *et al*., 2008).

Although environmental concentrations of manufactured NMs have not yet been routinely measured, there are concerns that NMs will be released from products over their life cycle. These novel materials will be released from products that are in current use and we are only just starting to explore their ecotoxicology and environmental chemistry. Data on biological effects show that NMs can be toxic to bacterial algae, invertebrates and fish species, as well as mammals, often in concentrations as low as $1\,\mathrm{mg\,l^{-1}}$ (Handy *et al*., 2008b). However, much of the ecotoxicological data is limited to species used in regulatory testing and freshwater organisms. Data on bacterial, terrestrial species, marine species and higher plants is particularly lacking. There are recent reviews on NM effects on algae (Navarro *et al*., 2008), bacteria and other microbes (Neal, 2008), invertebrates (Baun *et al*., 2008), and fish and lower vertebrates (Handy *et al*., 2008a). It is clear that more studies are needed on marine and terrestrial invertebrate species, as well as other vertebrates, including amphibians, reptiles and birds. Data on terrestrial plants and other photosynthetic organisms are also lacking.

The detection of NMs in the environment is complicated because of low concentrations and relatively high background levels of natural nanoparticles. Methods must be developed for samples collected in the field in order to characterize physical and chemical properties. Such methods must be validated before they can be used in environmental monitoring programmes. Most likely these methods will involve size fractionation, and total element or isotope analysis, along with suitable labelling and ancillary measurements (Handy *et al*., 2008c). Handy *et al*. (2008c) has summarized methods that may be useful in characterizing and measuring NMs in the environment.

3.4 Nanomedicine

Nanomedicine uses nanotechnology to maintain and improve human health. Nanomedicine includes the treatment, monitoring, repair, construction and control of human biological systems at the molecular level, using engineered nanodevices and nanostructures. Current and potential applications of nanotechnology in medicine are in diagnostic devices, drug delivery, nutraceuticals and the production of improved biocompatible materials. The development of a wide spectrum of nanoscale technologies is beginning to change the foundations of disease diagnosis, treatment and prevention. These technological innovations have the potential to turn molecular discoveries arising from genomics and proteomics into widespread benefit for patients (Moghimi *et al*., 2005).

The advantage of nanomedicine over conventional medicine lies in the size. Nanotechnology contributes to the development of novel drugs, especially in the area of cancer therapy and treatment to neurological disorders. It produces nanosized drugs that are only slightly larger in size than proteins and are therefore small enough to move across barriers, escape the vasculature and enter cells directly (Chan, 2006). The potential to cross the BBB, especially, may open new ways for drug delivery to the brain. Drugs of nanosize also have an earlier onset of therapeutic action and can be used in lower concentrations, thus reducing the risk of adverse effects (Chan, 2006).

Nanoparticles are attractive for medical purposes because of their unique features, such as their high surface-to-mass ratio, their quantum properties and their ability to adsorb and carry other compounds. The surface is able to bind, adsorb and carry other compounds, such as drugs, probes and proteins. Thus, nanoparticles can act as carriers of therapeutic agents which can be encapsulated, covalently attached or adsorbed onto the surface. This opens the possibility of controlled drug delivery by directing these carriers to a specific location (Moghimi *et al*., 2005). The carrier can then be activated on demand in a limited region. This is a great advantage and provides new opportunities, especially in cancer therapy. Also, by nanosizing a formulation, the drug dissolution rate can be increased, leading to enhanced drug adsorption and bioavailability. Improved tissue selectivity can also be achieved due to the selective uptake of nanoparticles in certain tissues.

Nanoparticles for medical use are based on material of both biological and chemical origins. Source materials

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of biological origin are, for example, phospholipids, lipids, lactic acid, dextran and chitosan, while chemical sources include various polymers, carbon, silica and metals (De Jong and Borm, 2008). Colloidal gold, iron oxide nanocrystals, nanoshells and QDs are examples of nanoparticles that have diagnostic applications in biology and medicine (Moghimi *et al*., 2005). Incorporation of cancer-killer genes into nanocapsules is also being investigated (Chan, 2006).

While nanomedicine provides great promise, it also implies substantial challenges to public health. Nanoparticles for imaging and drug delivery are often purposely coated with bioconjugates such as DNA, proteins and monoclonal antibodies to target specific cells. As these nanoparticles are intentionally engineered to interact with cells, it is important to ensure that these enhancements do not cause any adverse effects (Lewinski *et al*., 2008). Not all NMs are toxic, and it is important to identify those with undesirable properties in order to control risk towards patients (Chan, 2006). It is obvious that the potential interaction with tissues and cells, and thus toxicity, greatly depends on the actual composition of the nanoparticle formulation. For biomedical purposes, especially *in vivo* applications, toxicity is a critical factor to consider when evaluating potential use in patient care.

3.5 Toxicity Testing

3.5.1 Sample Preparation

The collection and preparation of relevant samples of nanostructures for toxicity testing is a critical step in obtaining results that are comparable between studies/laboratories and provides reliable information about the toxicity. It is important that all physicochemical characteristics that are potentially significant are measured in the samples (Warheit, 2008). The following physicochemical properties should be characterized in the context of toxicity screening tests: particle size distribution, agglomeration state, particle shape, crystalline structure, chemical composition, surface area, surface charge and porosity (Tsuji *et al*., 2006). The relevant inhalation dosimetry in risk assessment of NMs may be surface area rather than mass per air volume or mass per body weight. The characterization of the test material should therefore as a minimum also include surface area per mass and particle number per mass (Oberdörster *et al*., 2005b). As mass may not be the proper dose metric for comparing the toxicity of nanoparticles, the dose should be monitored as mass delivered or inhaled. The dose can then be converted to a surface area or particle-number dose as appropriate.

It is recommended that NMs preparation methods are fully documented, including the selection of dispersion media, methods of dispersion in the media and agglomeration state in the medium (Oberdörster *et al*., 2005b). The specific preparation techniques will depend on the material and test protocols being used. However, caution is advised when using ultrasonic agitation to disperse materials, as at high energies the method may be sufficiently aggressive to alter the characteristics of the material.

3.5.2 Testing in Animals

In vivo studies of inhalable particles are complicated, but provide important information about the toxicity of NMs. Nanostructures may interact with biological components, such as proteins and cells, possibly leading to unique biodistribution, clearance, immune response and metabolism. An understanding of the relationship between the physical and chemical properties of the nanostructures and their *in vivo* behaviour would provide a basis for assessing toxic response and more importantly could lead to predictive models for toxicity (Fischer and Chan, 2007).

Inhalation is the physiologically relevant and therefore preferred method of pulmonary exposure. Physicochemical characterization of the generated aerosol is essential in these studies. Inhalation studies can be performed as whole-body exposure or nose-only exposure (**Mixed Routes of Exposure**, Section 2.2.1). Nose-only exposure is more stressful on the animal. However, dermal and oral exposure is minimized in nose-only studies. With whole-body exposure, grooming will inevitably result in some oral exposure. A critical barrier to conducting inhalation studies with NMs is that the amount of material is often limited. Intracavity instillation, where the NMs are administered in a liquid medium, uses less material than inhalation exposure. Intracavity instillations can be performed as intratracheal, pharyngeal or laryngeal instillations.

In vitro experiments may be helpful in determining the dose range to be used for *in vivo* studies. *In vitro* cell cultures, flow-through diffusion cells and perfused skin models can be used (Holsapple *et al*., 2005). Although standard OECD protocols for dermal testing are well established, such protocols are not directly applicable to NMs. However, the procedures for occluded testing should be used, and exposure time and so on must be determined by the aim of the study (e.g. systemic absorption, irritation). The quantitative prediction of the rate and extent of percutaneous penetration (into skin) and absorption (through skin) of topically applied NMs is complicated because the processes driving nanoparticles into skin may be different from those governing other materials (Holsapple *et al*., 2005). It is important to have analytical methods that are able to discriminate between epidermis, dermis and hair follicle.

It is possible that NMs may appear in the water supply or be inadvertently ingested and the effects of oral exposure to NMs should also be investigated. Peroral

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exposure could be done by a single gavage at a dose which would represent the worse-case human exposure, or by continuous administration (**Peroral Toxicity**). Rats or mice are the recommended model system (Oberdörster *et al*., 2005b). The administration of the test materials could be by capsules or tablets, where a stainless steel or flexible tube is used to deliver the substance to the stomach, or it can be incorporated into the diet or drinking water.

3.5.3 In Vitro

In vitro studies are an important tool towards understanding how an agent will react in the body. Compared to animal experimentation, cellular testing is less ethically ambiguous, easier to reproduce and control and less expensive. *In vitro* techniques are, in general, seen as important adjuncts to *in vivo* studies. Studies in animal models will identify the organs of interest, in turn leading to identification of the best cell types for *in vitro* studies and the focus of studies on how these cells respond to the nanostructures. *In vitro* studies allow specific biological pathways to be tested under controlled conditions that are not available in studies *in vivo*. There are of course, well-documented problems with *in vitro* approaches, such as lack of validation against *in vivo* adverse effects, dosimetry mismatch, oversimplicity and noninvolvement of the complete inflammatory response (Oberdörster *et al*., 2005b).

An increasing number of *in vitro* studies of NMs have been published. These studies include a wide range of nanoparticle concentrations and exposure times, which makes it difficult to compare studies and determine whether the observed cytotoxicity is physiologically relevant. A wide range of different cell types, culturing conditions and end points can and have be used for *in vitro* studies. This adds to the problem of comparison between different studies. *In vitro* tests can be divided into portal-of-entry toxicity and target-organ toxicity. Cell lines represent the most amenable source of human cells for analysis of the potential toxicity of NMs. These cells are largely, but not exclusively derived from tumours, and a number of cell lines are available, derived from a wide variety of organs. For studies on the nervous system, primary cells are widely used. These cells are derived from dissected embryonic or early postnatal tissue, which are dissociated either mechanically or enzymatically.

The majority of cytotoxicity assays measure cell death and viability by colorimetric methods. There is, however, a technical problem in using such cytotoxicity tests for carbon-based NMs. These materials are black and absorb light in the tests, which are based on colorimetric measurement. The carbon-based NMs interact with the light absorption of a number of the indicator dyes commonly used. MTT (2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Pulskamp *et al*., 2007; Worle-Knirsch *et al*., 2006), Alamar Blue (Casey *et al*., 2007), WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5 (2,4-disulfophenyl)-2H-tetrazolium) (Casey *et al*., 2007) and Neutral Red (Casey *et al*., 2007) all interact with these carbon-based materials, leading to false readings and misinterpretations. For carbon-based NMs, other end points need to be developed and validated. The clonogenic assay has been introduced by Herzog *et al*. (2007) as an alternative to colorimetric methods. This method was first developed by Puck and Marcus (1956) and measures a single cell's ability to form a colony. The method has been further developed and validated for carbon nanotubes (Gellein *et al*., 2009).

4 FUTURE CHALLENGES

The revolution in nanotechnology is bringing advantages to almost every area of our lives. Nanotechnology itself has the potential to exceed the impact of the industrial revolution and it has been projected to become a $1 trillion market by 2015. As the quantities and types of NMs used in society increase, so does the potential for unintended consequences on human health and the environment. A proactive research is critical to ensure a sustainable and safe nanotechnology industry.

The diversity of engineered NMs and potential effects represent major challenges and research needs in nanotoxicology. A multidisciplinary effort is needed, involving researchers in toxicology, materials science, medicine, molecular biology, bioinformatics, chemistry and their subspecialties. The establishment and standardization of test procedures to ensure safe manufacture and use of NMs in the marketplace is urgently required and achievable.

NMs cannot simply be considered as a single homogeneous class. The toxicity of NMs depends on specific physiochemical factors, and the toxic potential of each type of material needs to be evaluated individually. In the testing of NMs, there needs to be an emphasis on the characterization of the materials themselves. The appropriate route of exposure and the appropriate end points also need to be considered in the design of toxicity studies. Another very important issue is determining the appropriate dose. Exposure levels relevant to human and environmental exposure should be utilized.

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Antidotal Studies

D. Nicholas Bateman and Timothy C. Marrs

CONTENTS

1 INTRODUCTION

An antidote is defined in Webster's New Collegiate Dictionary (Webster, 1986) as a remedy to counteract the effects of a poison. Antidotes in this sense are usually considered to be specific chemical entities, but sometimes the definition is broadened to include nonspecific measures, such as administration of oral activated charcoal, or treatments such as haemoperfusion, dialysis and so on (Bateman and Chaplin, 1989). This chapter deals solely with chemical antidotes.

Experimental studies on antidotes are carried out for the same reasons as on other drugs, to demonstrate efficacy and to assess safety in use. However, antidotes differ from many other drugs both in the way they are used and in the manner in which their effectiveness and toxicity need to be assessed. Antidotes are usually only used in life-threatening situations and are often administered as a single dose or, at most, as a short treatment course. They are sometimes used, principally under conditions of war, is a prospective manner, but more often after exposure to the toxin. This means that many of the toxicological data required for drugs used over longer periods in less serious diseases may well be considered unnecessary by many people. Indeed many antidotes, particularly older ones, have been introduced after only minimal animal toxicity studies and in some cases the impetus to the introduction of antidotes has been military. A further difference from many other drugs is that randomized clinical trials, including placebo groups, are rarely possible because of ethical considerations. Although trials on efficacy in humans can be designed, these often use a retrospective control group or a parallel group treated with an established antidote and may be less than totally satisfactory from a scientific point of view. This means that one is more than usually reliant on animal studies for the evaluation of efficacy. Assessment of safety may also be more difficult as the clinical action of the antidote in a nonpoisoned animal or patient may be different to that when the toxin is present. This is perhaps best exemplified by the cyanide antidote dicobalt edetate, which is more likely to result in acute adverse effects in the absence of cyanide.

2 THE POISON

A programme of antidote development requires some knowledge of the toxicology of the poison. There are two purposes in this: firstly, to identify an antidotal approach that is likely to be successful and secondly, to enable the design and interpretation of an antidote efficacy study.

The acute toxicity of a poison compound has traditionally been quantified by the LD_{50}. The use of the LD_{50} test has been criticized on both humane and scientific grounds (British Toxicology Society, Working Party on Toxicity, 1984; Society of Toxicology, 1989; DePass, 1989), but many antidotal efficacy studies use experimental designs that require the use of this test. When the LD_{50} is carried out, the slope of the log dose–probit mortality line should be recorded because the slope is

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often changed by antidotal treatment (Natoff and Reiff, 1970); furthermore, the slope will be useful in calculating the dosing schedules of the poison in studies in which any antidote is to be administered. Ideally acute toxicity studies should be carried out in several species of animal. Animals may show marked differences between one another and from humans in the quantitative or qualitative toxicity of the poison; such species differences will clearly influence the choice of a suitable animal model for experimental work. It is also relevant to consider the dosage of antidote and the dosage of poison in any model. Doses of a poison close to the LD_{50} are more likely to benefit from an antidote. Scaling the appropriate dosages to man also requires consideration. Species differences in paracetamol (acetaminophen) toxicity are a good practical example of these issues.

Where the objective of the treatment is the amelioration of a nonlethal, but crippling, effect, for example, blindness in methanol poisoning, measurement of the ED_{50} (lowest dose giving the effect of interest in 50% of the animals) for the effect will be needed. In such cases, species sensitivity to the appropriate toxic effect will be an important consideration in the choice of species in efficacy studies.

The subacute or chronic toxicity of a poison is not usually of interest specifically in the design of experiments to measure the effects of antidotes. However, delayed effects from acute toxicity may be important, for example, the delayed exposure toxicity of carbon monoxide (Garland and Pearce, 1967; Werner *et al*., 1985; Lee and Marsden, 1994; Lo *et al*., 2007) and the 'intermediate syndrome' associated with acute organophosphorus toxicity (Senanayake and Karalliedde, 1992; Karalliedde *et al*., 2006). The ED_{50} may be a suitable measure of toxicity where the delayed effects are nonlethal.

It is reassuring for the toxicologist to know that the target organ is the same in an animal species as in man. Sometimes an indication of this may be gleaned from acute lethality studies, but on most occasions the effect of sublethal doses of the poison will have to be studied. This is usually necessary since death of some of the animals during acute lethal toxicity studies makes interpretation of the histopathology, a useful indicator of organ specific effects, difficult; moreover, other investigations, for example, haematological and clinical chemistry parameters, helpful in demonstrating organ-specific toxicity, are typically not measured during such studies. Mechanistic studies on particular cell types within the target organ, while desirable and possibly helpful in the initial design of an antidotal approach, are in practice rarely necessary in the assessment of antidote efficacy. Newer imaging techniques, including magnetic resonance imaging (MRI) and positron emission tomography (PET) scanning are another way in which organ toxicity can be followed in real time.

Elucidation of the metabolic pathways of a poison is most useful where conversion to a toxic metabolite is a prerequisite for toxicity. In such instances it is essential that the species used in experimental studies handle the poison in a similar manner to man. Data on this aspect are often weak, but new metabomic and genomic techniques should offer better insights in future. In the situation where the antidote and poison directly react together, for example chelating agents and metals, differences in tissue distribution of the toxin or antidote are likely to be less important. Species differences in rates of metabolism may suggest a difference in the time relationship of antidote dosage and efficacy. Thus knowledge of the pharmacokinetics of the poison in animals and man, while less useful in identifying an antidotal mechanism, may be useful in designing animal efficacy studies, particularly with respect to the choice of an animal model and the choice of appropriate dosing regimes.

3 INTRODUCTION OF NEW ANTIDOTES

The introduction of new antidotal approaches have only rarely occurred by the chance finding of antidotal action, for example, as part of a screening programme. It has usually been based on an extensive substructure of the knowledge of the mechanism of action of the poison, or, in the case of pharmacological antagonists, study of structure–activity relationships. With application of pharmacological, biochemical or chemical expertise it has then been possible to find a way in which the poison could be detoxified or its toxic effects reversed. If one considers the introduction of antidotes at present available, it is clear that most antidotes evolved from the study of an antidote which was less than optimal, a 'lead compound' (Burger, 1982). Analogous compounds were then studied and usually the one with the best therapeutic index was that eventually adopted as the standard treatment. Examples of this approach include the introduction of sodium nitrite, a component of the classic therapy for cyanide which arose from the discovery by Pedigo (1988) of the usefulness of amyl nitrite in cyanide poisoning. Another example is the use of acetylcysteine for the treatment of paracetamol poisoning, which followed the earlier introduction of cysteamine (see Prescott *et al*., 1976). The discovery of the oxime organophosphorus antidotes, pralidoxime chloride (2-PAM), obidoxime and HI6, followed from the discovery of the antidotal effect of hydroxylamine (Bismuth *et al*., 1992). The introduction of dicobalt acetate as a cyanide antidote by Paulet (1960) was an attempt to improve upon the experimentally effective, but toxic, inorganic cobalt salts. More recent examples include 4-methyl pyrazole (fomepizole) as an antidote to toxic alcohols and hydroxocobalamin as a chelating agent in cyanide poisoning. Thus probably the single most challenging part of the process

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of bringing new antidotes into clinical practice is the discovery of 'lead antidotes' and this is usually dependent on a hypothesis for the mechanism of toxicity of a particular poison.

4 MECHANISM OF ANTIDOTAL ACTION

It is difficult to produce a classification of antidotes that is satisfactory in all respects, although a number of workers have attempted to do so (Marrs, 1987; 1992; Bismuth, 1987; Bateman and Chaplin, 1989). To some extent this is because the mode of action of certain well-known antidotes is controversial, while others appear to act in more than one way.

It is possible to envisage a number of ways in which the toxicity of poisons might be opposed (**Table 1**). Broadly they can be divided into three main classes: (i) antidotes that remove the active poison from its site of action, usually by bringing about chemical detoxification of the poison; (ii) antidotes that act specifically at pharmacological receptors or other macromolecules and (iii) antidotes that act in a functional manner.

4.1 Antidotes that Act Chemically

4.1.1 Antidotes that Act Directly on the Poison

The most simple and most easily understood mode of antidotal action is direct chemical reaction between an antidote and a poison to form a product which is less toxic, and may be more rapidly excreted. There are numerous examples of such antidotes, including the chelating agents used in poisoning with a variety of toxic metals, as well as the cobalt-containing cyanide antidotes and Fab fragments, used in digoxin poisoning. Studies *in vitro*, often unhelpful in antibody assessment, have been used in some of these examples to elucidate the chemistry of the reactions.

4.1.1.1 Chelating Agents

The term 'chelation' is often used with a lack of precision. The word comes from the Greek for a claw and it has been argued that monothiol compounds should not be described as chelating agents since they only have one reactive group. Nevertheless, the term is often used as a general term to describe those drugs whose action is to complex metals. The beneficial effect is the result of a number of processes: the complex may be less toxic than the free metal, mobilization from critical sites of toxic actions may occur or else elimination from the body. In many instances all three processes contribute to an antidotal efficacy.

One of the earliest chelating agents was dimercaprol (British anti-Lewisite (BAL)). Like many advances in toxicology the introduction of this substance was stimulated by military considerations. Dimercaprol was intended for use in treatment of poisoning by the chemical warfare agent lewisite, an extremely toxic organic arsenical compound. The use of dimercaprol had its origin in the suspicion, subsequently confirmed, that the toxicity of arsenic was due to the ability to combine with sulfydryl groups in biological molecules, in particular in lipoic acid, part of the pyruvate decarboxylase complex. Dimercaprol and some similar compounds were extensively studied by Stocken and Thompson (1946), and dimercaprol was the first dithiol chelating agents to be used clinically. More recently, two more related drugs have been introduced, dimercaptosuccinic acid (DMSA) and dimercaptopropane sulfonic acid (DMPS). These possess the same dithiol chelating grouping as dimercaprol, but the molecules as a whole are more hydrophilic. Unlike dimercaprol, which has to be injected, DMSA and DMPS can be used orally and both have better therapeutic indices than the older drug (Aposhian *et al.*, 1984).

The toxicity of many metals other than arsenic is due, at least in part, to reaction with sulfydryl groups. It is, therefore, not surprising that the active part of some other chelators also contain sulfydryl groups. Penicillamine, which is a monothiol chelator has been used for some years for the treatment of Wilson's disease, a condition in which copper overload is responsible for hepatic and central nervous system damage. Therapy with penicillamine will chelate a variety of other metals, including lead. Other chelating agents contain active groups other than sulfydryl groups. Disodium calcium ethylene diamine tetra-acetate and its analogues chelate lead and zinc and can be used in acute cadmium poisoning. Desferrioxamine is a compound of natural origin that binds iron and aluminium. A considerable amount of work has been carried out on reactions of the chelating agents (ligands) with metals, and it is possible to predict the efficacy of particular chelating agents, in individual metal poisonings on the basis of the affinity constant of the metal and chelator (Ringbom, 1963; Pearson, 1968; Ayers, 2007). However, despite their logical derivation, net affinity (conditional stability) constants of metal complexes can be misleading. As discussed above the beneficial action of chelating agents is probably the result of a combination of effects, including detoxication by complexation (i.e. the complexed metal is less toxic than the uncomplexed metal), mobilization and elimination. In order to detoxify effectively, chelating agents must gain access to the tissue were the metal is exerting its action. In the case of mobilization, the process must occur in a toxicologically desirable direction; that is away from the critical site of toxic action of the metal. Unfortunately, net affinity constants cannot predict the extent to which

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Table 1 Mechanism of action of antidotes

Class	Subclass			Poison
Antidotes that act chemically	Direct chemical detoxicants	Chelating agents		Metals
		Cobalt compounds		Cyanide
		Antibodies and derivatives	Monoclonal antibodies	Soman
			Fab fragments	Digoxin
Enzymatic detoxicants	Co-substrates	Sodium thiosulfate		Cyanide
	Enzymes	Rhodanese		Cyanide
		Acetylcholinesterase		OPs
	Prevention of formation of toxic metabolite	Ethanol		Methanol, ethylene glycol
Antidote gives rise to a detoxifying substance	Methaemoglobin-formers			Cyanide and sulfide
Antidote reacts with enzyme-poison complex	Oximes			OPs
Antidote reacts with toxic metabolite	*N*-acetylcysteine and methionine			Paracetamol
Antidote that act pharmacologically	At characterized pharmacological receptors	Naloxone		Opiates
		Flumazanil		Benzodiazepines
		Prenalterol		β-Blockers
	At other macromolecules	Oxygen		Carbon monoxide
Functional antidotes	Diazepam			OPs

chelation occurs *in vivo*, or whether mobilization of the metal occurs in a beneficial direction. Thus, Catsch and Harmuth-Hoehne (1975) found that penicillamine was a more effective mobilizing agent in mercury poisoning than diethylene triamine pentacetate, whereas the corresponding affinity constants would suggest otherwise. In an attempt to refine studies *in vitro* Yokel and Kostenbauder (1987) hypothesized that hydrophobicity of the chelated complex was important in successful chelation therapy. They therefore studied chelating agents for use in aluminium poisoning *in vitro* in an octanol–aqueous system and *in vivo* in rabbits poisoned with this metal. They concluded that the ideal chelator should have sufficient affinity for the metal of interest, be sufficiently water soluble to take by mouth and be sufficiently lipid soluble to distribute to sites of accumulation of the metal. If this theoretical model were to be directly applicable to experimental investigations of antidotal efficacy *in vivo*, it would be a great advantage. Unfortunately the *in vivo* situation cannot be adequately approximated *in vitro*, even for chelating agents, a group of antidotes where *in vitro* studies would seem the most promising.

4.1.1.2 Cobalt-Containing Cyanide Activities

It has been known from many years that transition metals can form stable and often relatively nontoxic complexes with cyanide. Clinically, this property of the transition metals has only been exploited in the case of iron (see methaemoglobin below) and cobalt. Cobalt is a metal whose toxicity is well recognized and it has therefore generally been considered that toxicity of inorganic cobalt salts preclude their clinical use. Muschett *et al.* (1952) showed that hydroxocobalamin (Vitamin $B12_a$) was an effective antidote in experimental cyanide poisoning in mice. In clinical practice, hydroxocobalamin presents a number of problems. The molecular weight

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is high and the compound binds cyanide in a ratio of 1 mole of hydroxocobalamin:1 mole of cyanide. This means that very large quantities of hydroxocobalamin are necessary for meaningful cyanide antidotal action. Paulet (1960) studied a number of cobalt derivatives in order to find a compound of lower mass which had the antidotal effectiveness of hydroxocobalamin, but which lacked the toxicity of inorganic cobalt salts. The cobalt compounds studied were the chloride, acetate, gluconate and glutamate salts and cobalt histidine and dicobalt edetate. The last two were effective and less toxic than the other compounds. On the basis of efficacy studies in dogs and acute lethality studies in mice, dicobalt edetate, in the formulation known as Kelocyanor, was widely adopted in Europe. Kelocyanor has given rise to adverse reactions, especially when given in the absence of substantial cyanide poisoning. More recently, studies have been undertaken into other organic derivatives of cobalt, notably porphyrins (McGuinn *et al*., 1994). There has been a recrudescence of interest in hydroxocobalamin. A retrospective review of cases of acute cyanide poisoning (mostly ingested potassium cyanide) treated with hydroxocobalamin was undertaken by Borron *et al*. (2007); this suggested that hydroxocobalamin was an effective antidote. Erdman (2008) suggests that there is, as yet, insufficient evidence to demonstrate either the safety or clinical efficacy of hydroxocobalamin when administered after exposure to hydrogen cyanide, which is rapidly absorbed and distributed, and essentially acts immediately. Hydroxocobalamin also has logistical drawbacks for emergency use, in that it would have to be given intravenously over at least 15 minutes in a large volume of fluid to counteract the effects of a poison with rapid effects.

4.1.1.3 Fab Fragments and Monoclonal Antibodies

Antisera have long been used to treat poisoning with toxins of biological origin, such as botulinus toxin and toxins in snake venom: this approach can theoretically be adopted from other poisons. It is an attractive option for many poisons where no chemically detoxifying antidote of sufficient efficacy and an adequate lack of toxicity is available. Thus, monoclonal antibodies have reportedly been successful in experimental poisoning by the organophosphorus nerve agent soman (Lenz *et al*., 1984), while monoclonal antibodies against Paraquat have been produced (Johnston *et al*., 1988). In the best known example of immunotherapy for poisoning with a drug, poisoning with digoxin, whole antibodies are not used. Instead poisoning with this cardiac glycoside is treated with Fab antibody fragments (Stolshek *et al*., 1988). Fab fragments have the advantages that they can be eliminated by glomerular filtration through the kidney (Cole and Smith, 1986) and are less immunogenic than whole antibodies (see review by Bateman, 2004). Immunotherapy can, in theory, be used to treat any poisoning where detoxicating antibodies can be made against a toxicant. However, in practice the size of the dose of poison makes the approach of limited practical value for many clinical poisonings. Biotechnology, making, as it does, large-scale manufacture of monoclonal antibodies easier, adds to the attractiveness of immunotherapy, making wider use more likely in the future.

4.1.2 Antidotes that Act on the Poison via an Enzyme-Catalysed Reaction

The existence of enzymatic pathways of detoxication can be exploited in two main ways. Detoxifying cosubstrates can be used, but will usually only be effective if the rate of reaction of that particular metabolic pathway is cosubstrate limited. Alternatively, the amount of enzyme present can be increased by injecting enzyme derived from an exogenous source. A further way of influencing an enzymatic process is the exogenous supply of an alternative substrate, or the use of an enzyme inhibitor. This may be helpful where poisoning results from metabolism of an indirectly acting poison to a toxic metabolite.

4.1.2.1 Cosubstrates

Sodium thiosulfate is probably not the physiological sulphur donor of the enzyme rhodanese. Nevertheless, this cyanide antidote appears to act by increasing the supply of sulphur for the enzyme, which is normally rate-limited by sulfane sulphur availability. The rhodanese reaction accelerates the rate of cyanide transulfuration to thiocyanate, an ion which is considerably less toxic than cyanide. Sodium thiosulfate, when used alone, is not particularly effective because, although it can increase the rate of cyanide transulfuration very considerably (Cristel *et al*., 1977), the blood level of cyanide does not fall fast enough, in the context of acute cyanide poisoning, to counteract the poisoning effectively. The possible reason for this is that rhodanese is a mitochondrial enzyme, while sodium thiosulfate administered intravenously remains largely extracellular. Because of the slow nature of the fall in cyanide blood levels that are observed after the use of sodium thiosulfate, the main use of this cyanide antidote has been as a second-line antidote to one of the methaemoglobin formers (see below), such as sodium nitrate or 4-dimethylaminophenol.

Other sulphur compounds, including sodium ethane thiosulfonate and propane thiosulfonate have been studied in the expectation that they would enter the mitochondria. In some animal studies, these sulfonic acid derivatives were superior to sodium thiosulfate, but they have not been used clinically. Cyanide detoxication by transulfuration has also been carried out using another endogenous enzyme: β-mercaptopyruvate sulphur transferase. In this case sodium β-mercaptopyruvate was the experimental antidote (Way *et al*., 1985).

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4.1.2.2 Exogenous Enzymes

Exogenous enzymes suffer from the disadvantage of being potentially foreign proteins, but have nevertheless been used experimentally as antidotes. Thus, in an attempt to place the rhodanese in the extracellular space, the use of intravenous bovine heart rhodanese accompanied by sulphur-containing cyanide antidotes has been studied in animals (Frankenberg, 1980). Another antidote that has been studied is acetylcholinesterase in the treatment of anticholinesterase poisoning. Thus Wolfe *et al*. (1992) found that foetal bovine serum acetylcholinesterase and horse serum butyrylcholinesterase were effective pretreatments in experimental soman toxicity in rhesus monkeys.

4.1.2.3 Alterations of Toxic Metabolite Formation

An enzymatic method of detoxication which is only applicable to indirectly acting poisons is the inhibition of the formation of a toxic metabolite. Clinically, such an approach is adopted when ethanol is used to compete at the active site of the enzyme alcohol dehydrogenase and thus to inhibit the formation of formic acid and formaldehyde in methanol poisoning (Cooper and Kini, 1962). Ethylene glycol poisoning can be treated in the same way (see review by Kraut and Kurtz, 2008). Additionally, in experimental studies, pyrazole or 4-methylpyrazole have been used to inhibit alcohol dehydrogenase in these poisonings (Clay *et al*., 1975).

4.1.3 Antidotes Giving Rise to Detoxifying Substances

4.1.3.1 Methaemoglobin-Forming Antidotes

A group of antidotes that do not themselves act by chemically binding a poison, but produce a substance that does, are the cyanide antidotes that induce a therapeutic methaemoglobinaemia. Methaemoglobin is a form of haemoglobin in which the iron has been oxidized from Fe^{2+} to $Fe3^{+}$. Methaemoglobin is unable to carry oxygen reversibly in the way that haemoglobin does, but it has a high affinity for cyanide and sulfide. The first of this group of antidotes introduced for the use of cyanide poisoning was amyl nitrite. Although this is still sometimes used, the most widely available methaemoglobin producer is sodium nitrite and this is still used in the USA and elsewhere as a primary cyanide antidote. The more recently-introduced 4-dimethylaminophenol (DMAP) is used in Germany while 4-aminopropiophenone (PAPP) is primarily of military interest (Bright, 1987). Sodium nitrite and DMAP produce methaemoglobin, but in different ways, the former somewhat more slowly. The aim in both instances is to produce methaemoglobinaemia of sufficient degree to bind substantial quantities of cyanide, without producing such a high level of methaemoglobin as to produce an appreciable danger of tissue anoxia. In fact, there appears to be a number of instances where dangerously high levels of methaemoglobin have been produced, perhaps because of individual susceptibility, or overenthusiastic use of the antidotes. It is unfortunate that the therapeutic monitoring of methaemoglobin during the treatment of cyanide poisoning is usually not possible, since common methods for measuring methaemoglobin do not separately measure haemoglobin, cyanmethaemoglobin and methaemoglobin (see **Haematology and Toxicology**). Cyanmethaemoglobin is the pigment produced by the reaction of cyanide with methaemoglobin. Adverse outcomes seem to be more common with DMAP than sodium nitrite, a fact which is surprising in view of the much longer time during which the latter has been used (Van Heijst *et al*., 1987; Marrs, 1989). It is also worth noting that some authorities doubt whether the main action of sodium nitrite is for it to form methaemoglobin, and suggest a vasoactive action (Way *et al*., 1987).

4.1.3.2 Antidotes that React with an Enzyme–Poison Complex

4.1.3.2.1 Oximes

Hydroxylamine was studied as an antidote to poisoning with organophosphate anticholinesterase because it was, in certain respects, similar to the substrate of the enzyme, namely acetylcholine. It was superseded by the pyridinium oximes and organophosphate poisoning is now often treated by an oxime together with atropine, an anticholinergic drug. The oxime that is used in many countries is the monopyridinium oxime, 2-PAM. Additionally one or two countries use other salts of pralidoxime; the methanesulfonate is used in the United Kingdom and the methylsulfate in France and some other countries; the methiodide is also available in certain pharmacopeias. The bis-pyridinium oxime, obidoxime has certain advantages in the therapy of organophosphate chemical warfare agents, particularly in that there is evidence that it may be effective in tabun poisoning. Even here, Marrs *et al*. (2006) suggested that there are no clinically important differences between pralidoxime, obidoxime and HI-6 (another bis-pyridinium oxime) in the treatment of nerve-agent poisoning, if studies employing pretreatment with pyridostigmine are excluded, except possibly with cyclosarin. There appear to be no clear differences between 2-PAM and obidoxime in organophosphorus pesticide poisoning (see review by Marrs and Vale, 2005). The principle action of oximes is the dephosphorylation and consequent reactivation of acetylcholinesterase, and, in this respect, the main weakness in their activity is against acetylcholinesterase which has undergone ageing. The ageing process, which is monodealkylation of the dialkyl phosphorylated enzyme, renders the enzyme refractory to both oxime-induced and spontaneous reactivation (Bismuth *et al*., 1992). It is probable that ageing occurs to some extent with all

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organophosphate anticholinesterases and it is possible that it may cause clinical problems in organophosphate pesticide poisoning, where treatment is initiated very late. It is, however, a very serious problem with the nerve agent soman, whose complex with acetylcholinesterases ages with a half life of a few minutes (see Wilson *et al*., 1992). Of the numerous oximes that have been studied, the only ones where much activity is exerted on aged enzyme complexes are the Hagedorn oximes such as HI-6. However, there is controversy whether the activity of this oxime in the situation where appreciable ageing has occurred is attributable to acetylcholinesterase reactivation or to other direct effects of the oxime (see Marrs *et al*., 1996a).

4.1.3.3 *Antidotes that Act on a Toxic Metabolite of the Poison*

In the case of poisons requiring metabolism before they become toxic, any of the above antidotal methods could, in principle, be applied. In practice, however, poisoning with such materials is treated by bringing about direct reaction with a toxic metabolite. An example is paracetamol poisoning. Paracetamol (acetaminophen) is toxic by virtue of its metabolic transformation to a reactive metabolic *N*-acetyl-*p*-benzoquinoneimine (NABQI). Under normal conditions of use, paracetamol is harmless, but in an overdosage causes cell damage and this leads to, among other things, hepatic necrosis. The lead antidote cysteamine, although effective in animal models, caused adverse reactions in man, especially nausea. Acetylcysteine probably acts by conjugating with NABQI; methionine, an alternative antidote may also do so, but only after conversion in the liver to homocysteine (Prescott, 1983; Seddon *et al*., 1987). Recent data suggests that the adverse effect profile of acetylcysteine in man is worse in the absence of paracetamol, raising interesting questions on the relationships between the antidote and the mechanisms of its adverse effects (Pakravan *et al*., 2008).

4.2 Antidotes that Act Pharmacologically

It is convenient to divide antidotes that act pharmacologically into those antidotes where antagonism occurs at characterized pharmacological receptors and those were antagonism occurs at other macromolecules: this division, though somewhat artificial, is nevertheless useful.

4.2.1 *Antidotes that Act as Characterized Pharmacological Receptors*

Such antidotes include naloxone, an antidote for opiates (Evans *et al*., 1973; IPCS, 1993) and flumazanil, which is effective in reversing the effects of benzodiazepines (Scollo-Lavgizzari, 1983), although not licensed for the management of poisoning in the UK. In severe poisoning with β-blockers, adrenergic agonists, such as isoprenaline or the more specific and cardioselective prenalterol, provide examples of receptor antagonism (Wallin and Hulting, 1983). In some cases the receptor can be bypassed and second messenger systems stimulated directly, as with the action of glucagon in β-blocker poisoning. Development of compounds within the class requires a knowledge of the pharmacological profile of a drug and the particular pharmacological property responsible for toxicity.

4.2.2 *Antidotes that Antagonize Other Macromolecules*

Carbon monoxide is poisonous by virtue of its tight binding to haemoglobin and other cellular components. Carbon monoxide can be displaced competitively from such sites by oxygen.

4.2.3 *Related Antidotal Mechanisms*

Antagonism of clinical poisoning does not necessarily take place at the same receptor as that at which the poison acts. Atropine is an anticholinergic drug and acts upon muscarinic cholinergic receptors. However, this drug is used as an antidote in poisoning with organophosphate and carbamate anticholinesterases, substances whose major action is not directly on the cholinergic receptor. The macromolecule to which the anticholinesterases binds is the enzyme acetylcholinesterase, and the poisoning is a consequence of accumulation of acetylcholine, the normal substrate of that enzyme. It is this effect which is antagonized by atropine (see review by McDonough and Shih, 2007). Analogous to this is the use of physostigmine in atropine poisoning: this anticholinesterase promotes acetylcholine accumulation, which overcomes the effect of atropine.

4.3 Functional Antagonism

There are a number of antidotes which are used symptomatically in poisoning and as such are difficult to classify. In some cases, further study may show these to have antagonistic actions that belong to one of the above groups. An example is the wide use of diazepam to combat the convulsions in a wide range of poisoning, and, in the case of organophosphate poisoning, an additional benefit is reduction in muscle fasciculations (Sellström, 1992; Marrs and Sellström, 2007). Diazepam acts allosterically on the γ-aminobutyric acid A ($GABA_A$) receptor. The GABA system is an inhibitory neurotransmission system and the agonistic effect of diazepam is to ameliorate or prevent the convulsions

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which may occur in organophosphate poisoning (see review by Marrs, 2004).

5 ASSESSMENT OF ANTIDOTAL EFFICACY

Antidotal efficacy can be assessed *in vitro*, in experimental animals and, to some extent, in human poisonings. All these approaches have limitations: studies *in vitro* cannot adequately simulate the situation *in vivo*, even in the case of the most straightforward antidotes which react with and detoxify the poison. Studies *in vitro*, however, are useful preliminaries to animal studies, particularly in narrowing down the choice amongst a series of related antidotes (Marrs, 1992). Moreover, in mechanistic studies of the action of antidotes, an *in vitro* approach is often extremely useful. Studies in experimental animals, possibly the most useful of the three approaches, are limited by the problems of extrapolation for two different substances, the toxin and the antidote, from animals to man, and by the need for very careful attention to experimental design. The limitations inherent in data on human poisonings are quite different: for example, Poison Centre data normally relate to suicidal and accidental exposure and so the dose of poison is uncontrolled and often unknown. While large multicentre trials would be very useful, these are extremely difficult to conduct, as ethical committees often require consent in advance of intervention, which is difficult to obtain in distressed patients. Such studies are extremely rare, and may be negative (Eddleston *et al*., 2008). Thus these do not usually provide information which can properly replace animal studies.

5.1 Assessment of Antidotes in Experimental Animals

There are many ways in which studies of antidotal efficiency can be performed and some of the factors to be considered in experimental design are listed in **Table 2**.

Table 2 Variables

Animal model	a. Species, strain and sex b. Numbers and controls
Poison	a. Dose b. Route of administration c. Solvent and excipients
Antidote	a. Dose b. Route of administration c. Solvent and excipients d. Time between administration of poison and antidote
End point	a. Lethality b. Other i. Clinical ii. Biochemical iii. Haematological iv. Electrophysiological v. Histopathological vi. Behaviour

5.1.1 Animal Models

One of the main features requiring attention is the choice of species of experimental animal. However well constructed the study is in other respects, unless the behaviour of the poison and antidote are similar in the chosen species and in humans, the results will be valueless (see Calabrese, 1982; Marrs, 1987). Species' suitability will be determined by similarity between the chosen species and humans in absorption, distribution, metabolism, excretion and response to the poison. The relative importance of these considerations depends on the mode of action of the poison: if it is a directly active poison, for example, cyanide, similarity in rate of endogenous detoxification would appear to be the most important consideration, while, for those substances which are toxic in humans only after a metabolic activation step, the occurrence of this conversion at a similar rate in the chosen species to that in man is probably the most important factor.

Major quantitative differences in lethality of chemicals between a given species and humans indicate that the species is probably a poor choice. Such differences exist with respect to methanol (Clay *et al*., 1975), ethylene glycol (Gessner *et al*., 1961), certain organophosphates (Crawford *et al*., 1976) and paracetamol (Prescott, 1996). It must be further borne in mind that the choice of scarce, large or exotic animals will increase the cost of the experiment and may thereby tend to reduce the numbers that can be used. This will clearly decrease the power of the study. Moreover normative data on such animals is usually scanty or nonexistent. All the forgoing considerations apply, of course, to the evaluation of any xenobiotic in animals; however, with antidotal studies the same considerations also apply with respect to the antidote, so that the species chosen must be similar to the human in its handling of both the poison and the antidote. This fact may greatly complicate the choice of animal model and indeed there may be no perfect solution.

A particular difficulty arises with the organophosphate nerve agent, soman. This is because this substance forms a complex with acetylcholinesterase which ages (loses an alkyl group) rapidly, rendering the complex refractory to reactivation by most pyridinium oximes. Ageing rates

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are species-dependant, so that realistic antidotal studies undertaken in species other than primates must be interpreted with great care (Eyer and Worek, 2007).

The species having been decided upon, the numbers of animals and controls must be determined. Two main types of experimental design have been adopted for the evaluation of antidotes. In the first, 'LD_{50} ratio', the LD_{50} of the poison is measured with and without the administration of the antidote. This procedure has the advantage that the result produced is a single figure, the protection ratio, but is has the disadvantage that it is necessary to use a relatively large number of animals. Moreover, the slope of the treated and untreated log dose–probit mortality curves may not be the same (Natoff and Reiff, 1970), and this will impair the value of the single figure. The protection ratio design is usually employed when small laboratory animals are used, although there are instances of the use of this type of design with larger animals, such as sheep by Burrows and Way (1979), studying cyanide antidotes, and monkeys by Dirnhuber *et al.* (1979), studying pyridostigmine prophylaxis in soman poisoning. Although performance of LD_{50} ratio-type studies sounds easy, multiple pilot experiments are often needed to bracket the median lethal dose. This difficulty is aggravated by the fact that LD_{50}s are often not measured nowadays for regulated chemicals such as pesticides, other measures of acute toxicity being used for classification purposes (see **Acute Toxicity**).

The other principle design is the comparison of survival in groups of animals given the same supralethal dose of poison, one group being treated with the antidote and the other being left untreated. The number of animals used is much less than with protection-ratio design, but the information supplied is also less. This approach has been criticized on the grounds that antidotes capable of increasing the LD_{50} of the poison by comparatively small amounts can produce dramatic increases in the proportion of animals surviving (Way *et al*., 1987); nevertheless, such experimental designs are frequently being used with large laboratory animals such as dogs. An example of the use of this design with laboratory rodents is a study by Jokanović and Maksimović (1995), who compared the four pyridinium oximes, trimedoxime, obidoxime, pralidoxime and HI-6 in the treatment of oral poisoning with a number of organophosphorus pesticides in the male Wistar rat.

If this approach is adopted, it is essential to have an untreated, but poisoned, control group within the study. The reliance on literature LD_{50}s, where the animals, normally of the same strain, may have been studied under different conditions and with different formulations of poisons, is reprehensible and renders the study valueless.

Whichever type of design is adopted, consideration should be given to the inclusion of a treatment-only group.

5.1.2 Poison

The dose of poison used depends on the overall design of the study. Where a design of protection-ratio type is used, dosing is, as with any LD_{50} estimate, designed to bracket the lethal dose. Adjustment of the dose range upwards may be required in the antidote-treated group. Often siting shots will be necessary preliminaries to a substantive experiment in both antidote-treated and untreated groups. Where survival is compared in two groups of animals, one poisoned and untreated and the other poisoned and treated with the antidote, the dose of poison chosen is usually a dose which will be supralethal in animals not treated with antidote. It must also be a dose against which there is a reasonable chance of survival with antidote. To obtain a reasonable idea of the maximum dose of toxicant against which an antidote will protect, it may be necessary to use several different doses of poison.

The poison should normally be given by the route by which it commonly gains access to man. In the case of a large number of poisons, this will be by mouth, so that gavage is appropriate in animal studies. Percutaneous and inhalation poisoning present problems, the former because the reproducibility of lethality figures tends to be poor and human skin is different in structure to animals, the latter because of the relative scarcity of inhalation facilities in laboratories. Intratracheal installation has been used as a substitute for inhalation studies, but it does not appear to be satisfactory, particularly with toxicants acting locally on the lungs (Richards *et al*., 1989).

Where poisoning by a pharmaceutical preparation is being studied, formulations are readily available for experimental purposes and are often used. The use of the formulation, rather than the active ingredient by itself, should be considered with other formulated materials, such as pesticides.

5.1.3 Antidote

In the case of an antidote already in use in man, it may be appropriate to adjust the dose to the size of the animal in experimental studies. In certain instances, however the choice of dose is more complicated. For example, it would be unwise to ignore species differences in methaemoglobin generation when studying methaemoglobin-producing cyanide antidotes.

The antidote is usually assessed after administration by the route by which it will be used. Furthermore, while in the early stages of development, the antidote will be frequently studied in its pure state, at some point it should be evaluated in the formulation in which it will be used clinically.

One of the most difficult points to resolve is the time relationship between administration of the poison and the antidote. This is a problem which is particularly important with rapidly acting poisons, and careful

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attention must be applied to this aspect of the design of the study if data are to be produced which can be extrapolated to clinical human poisonings. The use of prophylactic administration of antidotes is not valid, except where mechanistic studies are being carried out or where the antidote is intended for prophylactic use (Way *et al*., 1987). Even when the antidote is given after poisoning, there is still the problem of the precise time interval between the challenge and the administration of the antidote. Marrs *et al*. (2006) cited unpublished data from Porton Down which showed a drastic decrease in the effect of atropine, pralidoxime and diazepam on protection ratios in guinea pigs given pyridostigmine 30 minutes before nerve agent, depending on the interval between poisoning and the administration of therapy. With the three nerve agents, sarin, soman and VX, delay from one to four minutes approximately halved the efficacy of the atropine/pralidoxime/diazepam therapy. Thus the interval between poisoning and treatment may be critical. Usually the antidote is given at a fixed time after poisoning or at the onset of a well-defined clinical sign. Unfortunately the time interval has often been chosen so as to be unrealistic compared with the time between clinical poisoning and when clinical therapy is likely to be available. On the other hand, the use of clinical signs as a cue for antidote administration invites the possibility of observer bias. A possible solution is to use a fixed time interval, but one somewhat longer than those customarily employed. In addition, it is of value to consider a study design in which the antidote is administered at intervals after the toxin, a situation that more closely resembles clinical poisoning. Temporal considerations (discussed above) tend to be much less important with poisons whose clinical effects have slow onset, or in the treatment of chronic or delayed poisoning, than in acute poisonings.

5.1.4 End Point

Although the vast majority of antidote efficacy studies employ death or survival as the end point, a possible alternative is change in time of survival. Clinical end points other than lethality may be used, but observer bias should be carefully avoided. The efficacy of the antidote against sublethal effects, often behavioural ones, has also been used; this approach is attractive in military contexts, where knowledge of a poisoned individual's ability to keep fighting is desired. Biochemical end points, such as liver function and clotting changes in paracetamol overdose, are well established. Others, such as reactivation of cholinesterase in organophosphate poisoning, or mobilization of a metal in heavy-metal poisoning may be used (e.g. Kreppel *et al*., 1995; Tandon *et al*., 1996), but can have a potential problem, in that biochemical improvement does not always correlate with clinical efficacy of the antidote.

5.1.5 More Complex Studies

5.1.5.1 Comparison of Antidotes

It is frequently necessary to evaluate a new antidote against an existing one, or to survey a series of structurally related antidotes. The aim should be study the antidotes under reasonably realistic conditions. In this case the animal model chosen must be suitable for the evaluation of the toxic effects of the poison and of more than one antidote, as well as be able clearly to show comparative efficacy. It may be appropriate to use control groups, these animals receiving each antidote alone, another group receiving the poison alone and further groups receiving the poison together with each treatment. Alternatively, a full dose–response evaluation, which will yield a protection ratio for each antidote, can be carried out and in fact has frequently been done (see, for example, Schwartz *et al*., 1979). Although most of the variables discussed earlier will also apply to an evaluation of two or more antidotes in the same study, it is usually more difficult to standardize experimental conditions in a study employing more than one antidote.

5.1.5.2 Use of More than One Antidote in the Same Animal

More than one antidote, used together, may be used to treat a poisoning. Thus, organophosphates are usually treated with atropine and an oxime, and cyanides frequently with sodium nitrite and sodium thiosulfate (see above). The combined effects of such treatments may be additive, synergistic or less than additive. There is no particular problem with assessing treatment regimes of this type, the usual procedure being to appraise each antidote separately in experimental animals and then together: an example is the study of the efficacy of dimercaptosuccinic acid and calcium disodium edetate in lead-intoxicated rats by Flora *et al*. (1995). It will frequently be necessary to use multiple dosing combinations and it should not be forgotten that antidotes may interact chemically if mixed together before administration.

5.1.6 Antidotal Studies for Regulatory Purposes

Antidotal studies are sometimes done for regulatory purposes, notably on anticholinesterase organophosphate and carbamate insecticides. Many studies are in the open scientific literature, while others are in regulatory submissions and can be obtained from national pesticide registration authorities. Many have been reviewed in the toxicological monographs prepared by the Joint Expert Meeting on Pesticide Residues and published by the Food and Agricultural Organization in Rome (*http://www.inchem.org/pages/jmpr.html*). These studies are not always well-designed, insufficient attention often being given to statistical power.

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5.2 Studies of Antidotes *In Vitro*

In Section (4.1.1) it was stated that studies *in vitro* were often unhelpful in antibody assessment. Nevertheless they can be useful in investigating the mechanism of antidotal action. Another role of studies *in vitro* is where it is desired to compare similar antidotes, whose mechanism of action is known. Thus, Worek *et al*. (1997; 1998a; 1998b) compared the reactivating capability of various pyridinium oximes against nerve agent-inhibited acetylcholinesterase. The problem with using studies *in vitro* to assess the likely efficacy of antidotes in humans is that such studies cannot allow for the pharmacokinetics of the poisons and antidotes.

5.3 The Assessment of Antidotes in Human Beings

Provided that certain ethical guidelines are followed (Royal College of Physicians of London, 1986) and given appropriate ethical committee approval, studies of poisons or antidotes may be carried out on human volunteers. Such studies would be performed for a number of different purposes.

It may be necessary to establish the pharmacokinetics or pharmacodynamics of a poison or antidote in man; further, the observer may wish to study the distribution and metabolism of both in humans. Thus a number of pesticides have been studied in human volunteers (Wilks and Woolen, 1994), as have chemical warfare agents (Marrs *et al*., 1996b; Sidell, 2007). Human studies may be carried out during clinical use, either as part of a controlled clinical trial or by observation during routine clinical use; antidotes can also be studied in human healthy volunteers. Some compounds that are used as antidotes have already passed through such studies, since they are already used as therapeutic substances in other clinical situations. An example might be the use of β-adrenergic antagonists in the management of theophylline poisoning.

The second area where it may be necessary to study effects of antidotes in volunteers is in the evaluation of adverse effects. Sometimes, this may occur after the antidote has undergone clinical use, as was the case in studies that were carried out on acetylcysteine. In these experiments, intradermal acetylcysteine was given to volunteers, and to patients who had undergone treatment for paracetamol poisoning and had suffered adverse reactions (Bateman *et al*., 1984). More recent work has been carried out in patients to further explore the nature and mechanisms of adverse effects from this antidote (Pakravan *et al*., 2008).

For antidotes that act as agonists and antagonists at pharmacological receptor sites, studies of the pharmacodynamic reaction in volunteers may be carried out prior to the administration of the drugs to patients. Thus, the opiate antagonist naloxone and the benzodiazepine antagonist flumazanil were studied in volunteers prior to being given to patients.

Clinical studies in patients may involve the use of single doses in intoxicated patients, and comparison with control groups to assess response. This technique was used when naloxone was introduced, and had been shown to be efficacious in opiate poisoning, but without affecting benzodiazepine and barbiturate poisoning. In addition, clinical studies need to be done in patients to establish the appropriate dosing regime, and this is particularly the case for pharmacological antagonists such as naloxone, for which studies were carried out to clarify the most appropriate dosing format (Goldfrank *et al*., 1986).

6 CONCLUSION

Poisons and their antidotes may be studied in experimental animals, *in vitro* or in humans. The design of studies in all three situations presents major challenges. In the case of animal studies, great rigour is needed to ensure that studies are designed to simulate realistic treatment scenarios.

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Toxicity Data Obtained From Human Studies

Martin F. Wilks and Hugo Kupferschmidt

CONTENTS

1 INTRODUCTION

Animal or *in vitro* models for toxicity testing are assumed to offer a reasonable prediction of what may happen in humans as the ultimate species of interest in most cases. The main tasks of experimental toxicology have been defined as relating to the spectrum of toxicity (detection of adverse effect and description of dose–response relationship), extrapolation to other species, particularly to humans, and prediction of safe levels of exposure (Zbinden, 1991). In contrast, most toxicity data from humans are observational and subject to much larger uncertainties and variations than animal toxicology studies (**Table 1**). Although regulators as well as the concerned public are looking for actual human data to confirm or refute perceived health risks from chemical exposure, they appear increasingly unwilling to accept experimental data from human studies, even though they may have been obtained in accordance with strict ethical and scientific guidance.

The purpose of this chapter is to explore the ethics, benefits and limitations of various types of human studies which are of use in toxicity evaluations. This will be made in the context of specific subsets of human exposure to xenobiotics, for example exposure to industrial chemicals, pharmaceuticals or agricultural chemicals, in which certain types of studies have a particular relevance.

2 ETHICAL ISSUES

All research involving human subjects should only be conducted once ethical issues have been formally considered and documented. The principle of an ethical 'Code of Conduct' for such research was first enshrined following the trial of Nazi physicians who had conducted atrocious experiments on prisoners and detainees during the Second World War (US Government Printing Office, 1949). This so-called 'Nuremberg Code' was designed to protect the integrity of the individual, setting out conditions for the ethical conduct of research involving human subjects, in particular the principle of 'voluntary consent' to research. In 1966, the General Assembly of the United Nations adopted the International Covenant on Civil and Political Rights, of which Article 7 states: 'No one shall be subjected to torture or to cruel, inhuman or degrading treatment or punishment. In particular, no one shall be subjected without his free consent to medical or scientific experimentation.'

The Declaration of Helsinki, adopted in 1964 by the World Medical Association, has become the fundamental document in the area of biomedical research, and has formed the basis for international and national legislation and codes of conduct. The Declaration has been revised and updated several times, most recently at the Seoul Meeting of the Association in 2008. The Declaration sets out ethical guidelines for physicians engaged in clinical (patient) and nonclinical

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Table 1 Some characteristics of animal experiments and human studies in toxicology

	Animal experiment	Human study
Genetic variability	Homogenous	Heterogenous
Age distribution	Uniform	Variable
Pre-existing morbidity	None	Frequent
Diet	Controlled	Uncontrolled
Environment	Controlled	Uncontrolled
Exposure	Onset and duration known	Onset and duration usually not known, or variable
	Single-agent exposure	Multiple-agent exposure
	Exposure route defined	Multiple exposure routes
	Multiple dose levels	Dose unknown
Effect	Prospectively defined by observation, laboratory analysis and histopathology	Frequently established by retrospective analysis of morbidity and mortality data

(nonpatient volunteer) biomedical research (WMA, 2008).

In view of the special circumstances of developing countries, the Council for International Organizations of Medical Sciences (CIOMS) and the World Health Organization (WHO) issued in 1982 the 'Proposed International Guidelines for Biomedical Research Involving Human Subjects'. The purpose was to indicate how the ethical principles enshrined in the Declaration of Helsinki could be effectively applied, particularly in developing countries. The 'Proposed Guidelines' were adopted by the XXVIth CIOMS Conference in 1992, and, in revised form, in 2002 (CIOMS—Council for International Organizations of Medical Sciences, 2002).

In the revision of the 'Proposed Guidelines' it became clear that special attention needed to be paid to epidemiological studies because of their growing importance, particularly in the public health sector, and the relative paucity of guidance available. The result was the issuing of the 'International Guidelines for the Ethical Review of Epidemiological Studies' (CIOMS—Council for International Organizations of Medical Sciences, 1991). These focus particularly on the safeguarding of confidentiality in the absence of informed consent.

Numerous pieces of legislation and other guidelines are available at national and international level (for details see CIOMS—Council for International Organizations of Medical Sciences, 2002). The controversy that has surrounded human volunteer studies with pesticides in recent years is discussed in detail later in this chapter. In general, a central question in ethical considerations regarding human volunteer studies is concerned with the risk of harm. In the case of nonpatient human volunteer studies there is no direct benefit to the individual participating in the investigation. It follows that, where there is no direct benefit, the risk to the volunteer of coming to harm as a result of participation in the study should be no greater than minimal. In the third edition of their guidelines on the practice of ethics committees the Royal College of Physicians of London (1996) has elaborated on the term 'minimal risk': this could include everyday risks such as travelling on public transport or in a private car (the latter having considerably higher risk), but would not include travel by pedal or motorcycle—in other words, minimal risk is seen at a lower level than many people are prepared to take in many areas of life. This notion has been challenged as overly restrictive and it has been argued that research participants might be exposed to more than minimal risk of harm if, first, they give valid informed consent; second, the potential benefit of the research is large and, third, there is unlikely to be a public reaction against research as a result of harm to participants (Hope and McMillan, 2004).

However, such an approach is not without problems. Where there is no direct benefit to the individual, the knowledge gained is expected to somehow benefit the community. This carries the burden of demonstrating the societal value of the research, something which is very dependent on the position of the enquirer (Dayan, 1997). For example, improving the therapy of a rare but particularly aggressive form of cancer might be regarded as a worthier cause than demonstrating that a particular deodorant does or does not cause itching. Yet, we are all likely to use the latter, whereas the majority of us fortunately do not suffer the former.

The issue of risk of harm is also directly linked to the question of what happens when harm occurs. The CIOMS Guideline 19 deals with this in two distinct but closely related ways (CIOMS—Council for International Organizations of Medical Sciences, 2002). The first is the uncontroversial entitlement to free medical treatment and compensation for accidental injury which may have been caused by procedures performed exclusively to accomplish the purposes of research (nontherapeutic procedures). The second is the entitlement to material compensation for death or disability occurring as a direct result of study participation. In nontherapeutic research, it is generally unreasonable to assume that an investigational drug or other substance studied holds out the prospect of direct benefit for the individual subject; accordingly,

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compensation is usually owed to individuals who become disabled as a result of participating as subjects in such studies. Subjects must not be asked to waive their rights to compensation or required to show negligence or lack of a reasonable degree of skill on the part of the investigator in order to claim free medical treatment or compensation. The informed consent process or form should contain no words that would absolve an investigator from responsibility in the case of accidental injury, or that would imply that subjects would waive their right to seek compensation for impairment, disability or handicap. Prospective subjects should be informed that they will not need to take legal action to secure the free medical treatment or compensation for injury to which they may be entitled. They should also be told what medical service or organization or individual will provide the medical treatment and what organization will be responsible for providing compensation. Before the research begins, the sponsor should agree to provide compensation for any physical injury for which subjects are entitled to compensation, or come to an agreement with the investigator concerning the circumstances in which the investigator must rely on his or her own insurance coverage (for example for negligence or failure of the investigator to follow the protocol, or where government insurance coverage is limited to negligence). Sponsors should seek adequate insurance against risks to cover compensation, independent of proof of fault.

All research involving volunteers must be approved by an appropriately constituted, independent ethics committee (in the USA the term 'institutional review board' (IRB) is frequently used). This requires a balance of medical and nonmedical, scientific and lay men and women who act independently of the study sponsor. It is the ethics committee's duty to ensure that there is adequate justification for the proposal, that the previously mentioned acceptability criteria are being followed, and that every effort is being made to safeguard the health and welfare of the volunteers. The ethics committee must be prepared to co-opt experts to advise on specific scientific aspects of the study or the risks involved. The Committee will have to pay particular attention to adequate volunteer information and informed consent, and it approves payment of the volunteers. Ethics committees should be informed without delay of any significant untoward event occurring during the course of the study, and should be consulted about significant changes in a project. For some types of study a class approval may be given to avoid repetitive submissions of projects differing only in detail. This is particularly appropriate for projects that pose no risk of distress or injury to subjects (Royal College of Physicians of London, 1996).

Ethical considerations should also include the timely publication of study results, irrespective of whether they are positive or negative, and public access to such information. In recent years, the registration of clinical trials with independent agencies has become commonplace. Following the establishment of the first clinical trials registry by the National Library of Medicine in the USA (*www.clinicaltrials.gov*), many registries have been set up in other countries. The International Committee of Medical Journal Editors (ICMJE) announced in 2004 that it would in future only consider manuscripts dealing with clinical trials if they had been registered with a public trials registry meeting certain criteria (DeAngelis *et al.*, 2004). The definition of a clinical trial was subsequently expanded to include any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes (Laine *et al.*, 2007). The World Health Organization maintains a clinical trials registry platform which allows a single point of access to different registries and the unambiguous identification of clinical trials (WHO, 2008). While the above-mentioned registries are primarily concerned with providing information about the existence of ongoing trials, the latest developments concern the provision of results from such studies. For example, pharmaceutical companies have set up a repository for providing results of all hypothesis-testing studies of marketed drugs (*www.clinicalstudyresults.org*).

Most of the available legislation and guidance have stemmed from the need to regulate the development of medicines, with particular attention being paid to human volunteer studies. The basic principles, however, are equally applicable to other areas of human studies. Thus, a framework has been established with criteria which any human study needs to fulfil (Wilks, 2001):

1. There must be a detailed experimental protocol including provisions for appropriate ethical review.
2. The objectives of the research must be scientifically sound and directed to an advancement of biomedical knowledge. The desired information must not be obtainable otherwise, for example through animal or *in vitro* studies. The study should use as few subjects as possible, but as many as necessary to ensure scientific validity.
3. The procedures must be justifiable in terms of the objectives. This requires respect for the individual and the avoidance of unnecessary, trivial or degrading procedures.
4. A formal risk—benefit analysis must have been carried out. As a general rule, the smaller the direct benefit to the individual involved, the lower the risk must be.
5. The responsible investigators must be appropriately qualified to carry out the research. The welfare of the individual participating in the study must be paramount and all data generated must be confidential.
6. Informed consent has to be obtained from all subjects participating in a study, except in clearly defined

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circumstances. For example, a retrospective analysis of hospital records may not require informed consent, provided that anonymity is guaranteed. Informed consent requires a full explanation of the study and the risks involved in language which the subject can understand. Subjects must be free to withdraw from the study at any time without the need for explanation and without penalty. Special consideration needs to be given to informed consent when studying population groups which are not considered to be independent, self-controlling individuals, such as children, those with defects of cognition and people who do not enjoy a free life (e.g. prisoners, members of the armed forces).

7. Study subjects should be compensated for the inconvenience of participating in a study, but the level of compensation should not act as an inducement to participate against their better judgement and should never be for undergoing risk.

3 EVALUATION OF DATA FROM HUMAN STUDIES

All human studies should be designed and conducted according to accepted scientific principles. This is nowhere more important than in the evaluation of data derived from the study. It is therefore paramount to establish *a priori* what the purpose of the study is, and whether the data generated are likely to fulfil the objective. Human studies fall broadly into two categories: those which are used to generate a hypothesis, and those which test a hypothesis (Cohrssen and Covello, 1989).

Hypothesis-generating studies are usually of a descriptive nature. They include single or multiple case reports, reports of adverse reactions and studies which demonstrate a correlation between the occurrence of illness and the existence of occupational or environmental hazards.

In contrast, hypothesis-testing studies are observational, looking at individuals or small groups of people. These studies are analysed using established statistical methods to determine if an association exists between presence of an exposure hazard and the occurrence of illness. Often, the hypothesis examined has been previously generated by a descriptive study. Examples include two widely used types of epidemiological study: case—control and cohort studies (see **Essentials of Epidemiology for Toxicologists**), as well as most human volunteer studies.

3.1 Qualitative Evaluation of Human Data

The process of human-data evaluation has traditionally been one of inductive reasoning, beginning with fragments of data from which to derive a hypothesis which aims to explain the development of illness by attributing its origin to one or more causes or risk factors (Hoel and Landrigan, 1987). The observational nature of many of the studies has led to the establishment of principles to examine cause—effect relationships (Hill, 1962). While the focus has largely been on the traditional epidemiology studies, the criteria proposed by Hill (1965) can and should be applied to any human data generated in the process of establishing whether an observed association represents causality:

1. **The strength of the association:** In epidemiological studies this is usually expressed in terms of relative risk, that is, the stronger the relative risk, the greater the likelihood that the association is of a causal nature. However, confounding factors need to be carefully taken into account, for example smoking when studying the chemical origins of respiratory disease or lung cancer.
2. **The consistency of the association:** The reproducibility of a finding 'by different persons, in different places, circumstances and times' (Hill, 1965) is one of the strongest arguments for the existence of causality.
3. **The specificity of the association:** If exposure to a specific hazard is associated with one type of illness and vice versa, there is strong evidence for causality. While this is not all that common in xenobiotic-induced illness, there are examples such as asbestos and mesothelioma, aromatic amines and bladder cancer, or vinyl chloride and haemangiosarcoma of the liver. However, absence of specificity does not exclude causality.
4. **The temporal relationship between cause and effect:** For causality to exist exposure must have preceded illness. In the case of latency periods it is important to conduct the study at a time when the exposure is likely to have produced an effect.
5. **The biological gradient of the association:** The establishment of a dose—response relationship is a powerful argument for causation. Likewise, any alteration in exposure leading to a change in disease frequency supports a causal relationship.
6. **The plausibility of the association:** It is helpful if the suspected causation is biologically plausible. This means that it should not seriously conflict with known biological and pathophysiological disease mechanisms. However, this is by no means an absolute criterion since it very much depends on the biological knowledge of the day.
7. **The coherence of the association:** The cause and effect interpretation of the data should not seriously conflict with the known facts about the natural history and biology of the disease.
8. **Experimental evidence for the association:** It is sometimes possible to test the presumed association

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in intervention studies, for example reduction in occurrence of lung cancer in people who have stopped smoking.

9. **Analogy with other known associations:** If the situation under review is similar to situations where causality has already been accepted, for example by similarity of agents or exposure circumstances, this may increase confidence in causal association by analogy.

3.2 Quantitative Evaluation of Human Data

Analysis of human data is considered a critical component of quantitative risk assessment. This is a stepwise process in which the occurrence of disease in an exposed population is related quantitatively to the intensity and duration of exposure (for a definition of the terminology used see IPCS—International Programme on Chemical Safety, 2004). The individual steps in the process are:

1. **Hazard identification:** This answers the question of whether a particular agent can cause a specific biological effect or illness. Although hazard assessment is the domain of experimental studies, human data sometimes provide the initial information.
2. **Dose—response relationship:** Is there a linear, nonlinear or threshold pattern in the relationship between exposure and effect?
3. **Exposure assessment:** What is the intensity, frequency and duration of human exposure to the agent in question? This is often the most difficult part of the assessment, particularly when direct exposure measurements are not available, as is the case for most retrospective studies.

The latter two steps combine to form the *risk characterization* part of the process. This relies heavily on the use of models for the calculation of risk. However, any such calculations have to be treated with great caution because of the uncertainties, in particular when combining data from animal studies, usually carried out using high doses, and estimates of human exposure which themselves are rarely precise. It is customary to include uncertainty or assessment factors when trying to quantify risk to account for some of these variables. A default value of 100 is frequently used, which consists of two 10-fold factors: one to account for interspecies difference and one to reflect human intra-individual variability. Refinements to this general approach using human data have been suggested (Dorne and Renwick, 2005). Details of the methodologies involved and their applications can be found elsewhere (Richmond *et al*., 1981; Cohrssen and Covello, 1989; Hallenbeck, 1993).

Since there is little formal guidance on how human data can be interpreted and used for risk assessment it is not surprising that such data are not consistently used and evaluated in chemical risk assessments. The European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), in collaboration with the International Programme on Chemical Safety (IPCS), the Organisation for Economic Co-operation and Development (OECD) and the EU Joint Research Centre, therefore held a workshop with the aim of discussing and bringing consensus to the topic (ECETOC, 2004). The workshop concluded that all forms of human data can be useful when making risk assessment decisions, but that good-quality human data are often not readily accessible and/or their usefulness is frequently constrained by a lack of information on exposure. However, this situation could be accounted for if a framework could be developed that enabled different sources of human data to be collected, evaluated and applied as part of the risk assessment process. Subsequent to the 2004 workshop, the IPCS held a follow-up activity that examined how the information held by poisons centres could be developed and assembled to be more useful for risk assessors (IPCS—International Programme on Chemical Safety, 2005). The IPCS activity served to bring clarity to how poisons centre data might be collected and archived in order to increase the likelihood of it being useful for subsequent risk assessments. As a further step, a framework for the integration of human and animal data in chemical risk assessment has now been proposed (ECETOC, 2009). This report is particularly concerned with quality aspects of human as well as animal data, since they play an important role in the choice of key data sources regarding the lead health effect(s) in the risk assessment process.

4 SOURCES OF HUMAN DATA

There are various ways of looking at sources of human data, depending on their ultimate use. One which has already been mentioned is the classification into hypothesis-generating and hypothesis-testing studies. Another way would be to focus on the study subjects or populations in terms of whether an intervention is carried out, such as withdrawing a subject from exposure, or whether observations are being made without interventions. If the study focuses on the individual, it is often referred to as a clinical study, whereas epidemiological studies look at population groups or subsets. Finally, the nature of the study can be either retrospective, that is, taking an individual's or population group's health status or disease incidence and trying to correlate it with past exposure (e.g. case—control studies), or prospective, where the development of the disease is followed in exposed and nonexposed subjects over time, possibly over many years (cohort studies).

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Some principles of epidemiology have already been referred to above; further details can be found elsewhere in this book (see **Essentials of Epidemiology for Toxicologists**). For the purpose of this chapter, those sources of human data are selected which allow a direct, though not always easily quantifiable, assessment of individual chemical exposure and health effects. Thus, the principle sources of information are case reports and surveillance schemes, use of chemicals as therapeutic agents, exposure and health surveys, and human volunteer studies.

4.1 Case Reports and Surveillance Schemes

Case reports identify one or more cases of disease which have been detected, usually by clinicians, as a result of the treatment of a patient, whereas case series often stem from active surveillance (e.g. in occupational medicine) or passive reporting (e.g. adverse drug reaction reporting). In many instances, case reports have served to alert physicians to the existence of occupational or environmental illness. The earliest recorded examples of case series of occupational or environmental illness are reports of lung disease among potters and weavers (Ramazzini, 1713), and observations of scrotal cancer among chimney sweeps (Pott, 1775). Many of the known human carcinogens were identified through case reports (Miller, 1978). For example, the first three cases of haemangiosarcoma of the liver in vinyl chloride-exposed workers were reported by a company physician, John Creech, in a PVC (poly(vinyl chloride)) resin manufacturing plant in Louisville, Kentucky, USA (Creech and Johnson, 1974). Frequently, such case reports are then followed by further epidemiological studies and/or animal studies to confirm or refute the validity of the original observation. Thus, in most instances, case reports serve to generate a hypothesis.

A distinct advantage of case reports over most other types of studies is their low cost. Most of the work necessary to allow reporting of a case or case series is performed by healthcare workers in the course of their normal duties, thus the cost is largely absorbed by the medical care system. In addition, the time interval between identification of cases and publication is often short, indeed, many medical journals allow for the rapid publication of case reports by speeding up editorial review. Case reports are often highly specific: in many instances they contain extensive descriptions of symptoms and clinical signs. Frequently coupled with good exposure information (e.g. analytical verification in the case of drug overdose), they are uniquely able to define a dose—response relationship of chemical exposure.

Limitations of case reports are publication bias (i.e. preferential reporting of positive associations between an exposure and a health outcome) and the fact that they usually deal with single incidents or relatively small numbers. Extrapolation or generalization may therefore be difficult, especially since there is no information on the size or definition of the population at risk. They also rely on the individual effort of usually very busy healthcare workers who need to find the time for writing and literature searches. The role of case reports as 'early warning systems' is less useful when cases are sporadic, the relative risk is low, the outcome is a common disease, the aetiology is multifactorial, or there is a long latency period between exposure and effect (Cone *et al*., 1987).

Case reports which are generated through a formal reporting system are of enormous use in disease surveillance, either as part of drug monitoring or toxicovigilance.

4.1.1 Drug Surveillance Schemes

These are used extensively to evaluate toxicity resulting from the use of pharmaceuticals, and may include chemicals which form part of prescription pharmaceuticals. Serious, but rare toxicity, occurring with a frequency of 0.1% or less, may not be manifest until after widespread general usage, and general patterns of toxicity may not be recognized unless systematic drug surveillance schemes are employed. The statistical background to adverse drug reactions is well defined (Talbot and Waller, 2004) and gives an indication of the size of the exposed patient population required to manifest adverse drug reactions which occur infrequently.

Efforts have been undertaken to develop a new framework of risk management, particularly in the last 10 years, by the International conference on Harmonization (ICH) and the CIOMS (Tsintis *et al*., 2004), together with formal regulatory guidelines from the Committee for Medicinal Products for Human Use (CHMP) of the European Medicines Agency (EMEA) (Moseley, 2004) and the US Food and Drug Administration (FDA) (FDA, 2005a; 2005b; 2005c). The documents describe methods and provide clarification and harmonization of practices for overall safety surveillance, early risk detection and risk-management processes. The ICH E2E (ICH, 2004), CIOMS VI (CIOMS—Council for International Organizations of Medical Sciences, 2005), US FDA and EMEA guidelines on safety risk management are all complementary. The new framework makes safety risk management more continuous throughout the life cycle of a product. Thus pharmacovigilance, traditionally associated more with postmarketing activities, is now increasingly applied throughout the life cycle of a drug, making premarketing and postmarketing activities a continuum (Hartford *et al*., 2006).

The ICH E2E guideline recommends that company pharmacovigilance experts get involved early in product development. Planning consists of the 'safety specification', which reflects the status of safety issues being followed during development and includes review of

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nonclinical as well as clinical data, and the 'pharmacovigilance plan', which is based on the safety specification and describes actions for safety concerns identified.

The most common methods of postmarketing drug surveillance are described below.

4.1.1.1 Spontaneous Reporting

Much valuable safety information may be gleaned by the reporting of 'spontaneous' adverse events from routine clinical usage of a drug. Reports may be either from the consumer directly to the pharmaceutical company (commonly in the USA) or from a healthcare professional. Pharmaceutical companies have an obligation to submit an initial report on life-threatening and unexpected serious adverse events, received in this manner, to regulatory authorities within a given time frame (usually within 15 days of receipt). Clarification, obtained from a request for further information regarding concomitant disease, medication and outcome, may be submitted later in order to complete the overall assessment. In the UK, the additional mechanism of the 'Yellow Card' scheme (administered by the Medicines and Healthcare Products Regulatory Agency (MHRA) encourages voluntary reporting by healthcare professionals and patients of all suspected reactions to new drugs (defined as the first year of a drug's licence; 'black triangle' designation), and serious or unusual reactions to established drugs. Reports number over 20 000 per year (MHRA, 2007). However, it is estimated that only 10% of serious reactions and 2–4% of nonserious reactions are reported in this way. In contrast, in France and other countries physicians have a legal obligation to report to pharmacovigilance centres. The WHO also administers a scheme (Programme for International Drug Monitoring) encompassing 86 countries (WHO, 2009). Reporting from these schemes may be influenced by many factors including drug promotion, time on the market and adverse publicity. A balanced picture of a drug's safety may not necessarily be obtained, but this method is particularly useful in detecting signals worthy of further study.

4.1.1.2 Clinical Trials

Development trials may be regarded as potentially artificial situations because of limitations on the types of patients entered and concurrent medications, defined dosage, duration of treatment and the use of comparators, including placebo. Nevertheless, clinical trials may provide useful information. During the course of clinical trials (whether premarketing or postmarketing), pharmaceutical companies have an obligation to report all serious adverse events (fatal, life-threatening, disabling/incapacitating and those causing or prolonging hospitalization or congenital abnormalities or birth defects) to regulatory authorities (European Commission, 2001; ICH, 2002; 2008). In general marketed usage, a wider spectrum of patients may receive a drug, occasionally even for an unlicensed (i.e. unproven/untested, so-called 'off-label') indication and at higher doses and for longer periods. Previously unrecognized or unpredictable drug toxicity may also occur from such usage.

In many countries, there are legal requirements for pharmaceutical companies to submit periodic safety update reports (PSURs) to regulatory authorities on any new safety information which comes to light after a drug is marketed; correspondingly CIOMS introduced a development safety update report (DSUR) for the premarketing phase (EMEA, 2005; Klepper, 2004). However, there are rarely any formal requests for large-scale pharmacovigilance studies. Countries such as the UK and USA may request postmarketing surveillance studies (PMS), particularly for drugs of a new class and for those where potentially significant but relatively infrequent adverse events have been seen (e.g. hepatic disturbance) in the development phase. Such large-scale, company-sponsored studies, involving many thousands of patients, may report a multitude of adverse events which are often difficult to interpret even when the studies are well designed. Indeed, it has often been considered that PMS can only give bad news about a new drug. However, with careful interpretation, such studies can yield useful safety information which will assist the passage of the drug into more widespread clinical usage.

Phase 4 clinical trials which are well controlled may yield useful safety information, in the same manner as development trials.

4.1.1.3 Safety Assessment of Marketed Medicines (SAMM)

This is distinct from Phase 4 studies as it involves primarily noninterventional studies in which a drug is prescribed in the usual way by the patient's physician. Their aim is to collect safety data from routine clinical use. Safety assessment of marketed medicines (SAMM) studies involving any form of intervention (e.g. randomization and nonroutine investigations) are subject to normal clinical trials ethics and regulatory requirements. They should have a protocol or study plan including precise objectives, stating whether the study is hypothesis testing (investigating known or putative safety issues) or generating (identifying previously unrecognized safety issues). SAMM studies are conducted by pharmaceutical companies, are approved, and are sometimes required by regulatory authorities (e.g. MHRA), as a condition of granting a product licence. Such studies should not be conducted for promotional purposes. Different types of study may be conducted. They include observational cohort studies, case—control studies and case surveillance. Results of these studies are presented to regulatory bodies and are usually published. Reported adverse events from these are included in the PSUR. The various different types of study are described below.

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4.1.1.3.1 Observational Cohort Studies

Patients who are about to receive a prescribed drug are identified and followed prospectively, whereby certain conditions apply:

1. The study population should reflect the population expected to receive the drug in normal clinical practice and is subject to the stipulated contraindications in the prescribing information.
2. The decision to prescribe the drug should have been made on clinical grounds before the patient is entered into the study.
3. The product is supplied by the family physician and not by the pharmaceutical company.
4. A control group of patients with the same condition should generally be prescribed an alternative therapy.
5. The physician reports adverse events during routine, rather than specially arranged consultations.

A special type of cohort study, commonly used in the UK since 1982 by the Drug Safety Research Trust, is where the patient is identified by the prescription (prescription event monitoring (PEM)) (Drug Safety Research Unit, 2009). General Practitioners are, after an interval of six months, sent a questionnaire requesting information on 'events', that is, new diagnoses, deterioration or improvement in a condition, any suspected adverse drug reaction or any other significant finding, including cessation of treatment and subsequent follow-up details. The results for one drug can be compared with those for other drugs subjected to this scrutiny.

Many general practices in the UK have computerized databases and there are many computerized healthcare systems (e.g. Kaiser-Permanente and Medicaid in the USA). These databases can be used either to investigate in greater detail previously identified clinical conditions that are suspected of being drug related (e.g. human insulin is associated with hypoglycaemia without warning) or to identify all patients on a specific drug or with a condition and follow them up to determine what clinical events subsequently occur. The latter is effectively an 'electronic' cohort study.

4.1.1.3.2 Case—Control Studies

These are hypothesis-testing, retrospective studies comparing the history of drug exposure of patients experiencing a disease or event with control patients without the disease or event. These studies have been employed to study associations between thromboembolism and oral contraceptives, endometrial cancer and hormone replacement therapy, and upper gastrointestinal haemorrhage and nonsteroidal anti-inflammatory drugs.

4.1.1.3.3 Case Surveillance

These are again retrospective studies looking at patients with diseases or events that are likely to be related, and ascertaining drug exposure.

In conclusion, each method has advantages and disadvantages both in terms of ease of use, reliability and cost. The principal benefit is that otherwise unidentified associations can be detected by virtue of the population approach that these techniques employ.

4.1.2 Toxicovigilance

While drug surveillance schemes provide useful information on pharmaceuticals in 'normal use', they cover by their very nature only a very limited number of chemicals. There is a need to collect and review data related to drugs in overdose situations as well as to the vast range of nonpharmaceutical chemicals which people are exposed to at work or in their everyday lives. Such information can be collected from various sources, such as statistics on mortality and morbidity, and accident surveillance schemes.

Official statistics can provide overall numbers for deaths and hospital admissions and are useful for trend analysis, but they do not provide detailed clinical and toxicological information. Furthermore, the nature and extent of data collection varies enormously between countries, and international comparisons are therefore extremely difficult. Accident surveillance schemes have been developed to facilitate health education and to monitor product safety. In the UK, for example, the Home Accident Surveillance Scheme administered by the Royal Society for the Prevention of Accidents (ROSPA, 2008) monitors home and leisure accidents. However, experience has shown that suspected poisoning accounts for only 3–4% of such accidents, and that, while overtly dangerous products can be identified, little information is collected from which to assess toxic effects and dose response (Volans *et al.*, 1991). A special situation exists in the case of pesticide exposure. The UK Health and Safety Executive runs a scheme which allows pesticide operators, as well as members of the general public, to report incidents relating to pesticide use. The data thus collected are evaluated by a panel of experts who decide, on the basis of the available information, whether the alleged health effect is likely to have been caused by the exposure (Health and Safety Executive, 2007). In addition, companies in the UK are required to submit immediately any new information on potentially dangerous effects of a product on human or animal health, ground water or the environment. The Pesticide Safety Directorate (PSD) publishes an annual survey of human health incidents report to companies each year (PSD—Pesticide Safety Directorate, 2008). This lists the active ingredient, symptoms, description of the incidents and the actions taken by the company.

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The most important source of data on toxicovigilance comes from poison control centres (PCCs). Such centres exist now in many countries, and their primary role is the provision of advice on potential risks and management of cases of suspected poisoning and toxicological emergencies. This advice is usually given by telephone, in many cases on a 24-hour basis, either to healthcare professionals or members of the general public. In order to be able to give accurate information, the PCC must receive details of the incident, patient information, the time course of symptoms and any treatment already given. This information has enormous potential as a source of human toxicology information, and, increasingly, PCCs see it as their role to collect and evaluate such data for the purpose of health surveillance and promotion. As with other surveillance schemes, there are limitations in the way the data are collected and analysed, principally because of the lack of standardization of systems used to obtain and record information from the caller. In the recent past, some of the PCCs have developed Internet-based information systems (NPIS, 2008; RIVM, 2008; TOXINZ, 2008). However, while these systems are of proven efficacy with regard to delivering information, they are less suitable to collect data on human poisoning due to their lack of personal interaction.

However, efforts are being made at a national and supranational level to improve the systems of toxicovigilance. In the USA all PCCs accredited by the American Association of Poison Control Centers provide annually an agreed set of data on their incident-related activities that is reported in the National Poison Data System (NPDS) (Bronstein *et al*., 2008). Data from 60 PCCs are automatically uploaded via the Internet to a central server at least once per hour, thus enabling 'real-time' toxicosurveillance. This database can be searched according to product, circumstances of exposure, therapy, type and severity of effect and medical outcome. The data thus obtained can be used for further analysis. For example, in 2007 there were approximately 2.4 million exposure cases of which more than 1.2 million occurred in children younger than six years. Of the total number of exposures 0.7% experienced a major outcome, and there were 1415 (0.1%) fatalities. The most commonly implicated substance categories were analgesics, cosmetics (including personal care products) and household cleaning substances. The low hazard factors (sum of major and fatal outcomes per number of exposures) indicate that frequent exposure does not necessarily imply toxicity. Pharmaceutical preparations accounted for most of the fatalities, with sedatives/hypnotics/antipsychotics being the most frequent cause.

Many European countries require their PCCs to provide annual reports. Under the auspices of the European Association of Poison Control Centres and Clinical Toxicologists efforts are being made towards a better harmonization of data collection. In conjunction with the European Commission and the IPCS, a standardized scheme (poisoning severity score) for grading the severity of poisoning has been developed and has been in use for 10 years (Persson *et al*., 1998). Initial application of the scoring system has shown that it is helpful in accurately assessing the initial clinical severity and the likelihood of further deterioration. Furthermore, it helps in deciding which enquiries to refer to a clinical toxicologist (Casey *et al*., 1998).

One of the aims of the IPCS is to encourage countries to establish PCCs, and it has published a policy overview and technical guidance on PCC organization and management (WHO, 1997). These guidelines recommend the establishment of internationally agreed mechanisms for the collection, validation and analysis of data relating to poisonings. The IPCS has for a number of years developed a poison centre information system (Meredith and Haines, 1996). This 'INTOX' system is particularly aimed at helping developing countries to establish their own poison information services, and contains a large volume of product and treatment information (*www.intox.org*). At the same time the comprehensive incident recording facilities of the system help to foster standardization and integration of data collection and reporting not only across centres but also across countries.

4.2 Use of Chemicals as Therapeutic Agents

Chemicals have been used as therapeutics throughout history. Arsenic in its naturally occurring disulphide form was recommended by Hippocrates and Galen for treating ulcers, and cautery of tumours by arsenic salts was practised by Avicenna in the tenth century. Potassium arsenite solution (Fowler's solution) was used extensively for treating numerous ailments, including until recently in some treatments of leukaemia (Sollmann, 1957). Similarly, mercury ointments were used by Arab physicians from the tenth century. From around 1500 onwards, the use of mercury salts or vapour was standard treatment for syphilis (though it is a matter of debate whether more patients died from the disease or the treatment). The diuretic potential of mercury has been known at least since the seventeenth century, and mercurial diuretics were used as late as the mid 1970s.

The reason why therapeutic use of chemicals is of interest for toxicologists is because of the first-hand, often extensive human information which becomes available when a chemical is taken through the stages of pharmaceutical development. There are three principal reasons why a chemical may become of interest for such development.

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4.2.1 Incidental Discovery of Therapeutic Potential

Nitroglycerin (glycerol trinitrate) was discovered in 1847 and incorporated by Alfred Nobel into a major component of dynamite in 1867. Effects from occupational exposure were first described by Darlington (1890) as throbbing headaches, breathing difficulties, drowsiness, weakness and vomiting. These effects are due to the pharmacological action of nitroglycerin on the smooth muscle, where it produces relaxation.

The ability of organic nitrates to relieve anginal pain was discovered in 1867 by Lauder Brunton, who noticed that amyl nitrite caused marked flushing, tachycardia and a fall in blood pressure when its vapour was inhaled (cited in Rang and Dale, 1987). It was soon realized that the symptoms caused by nitroglycerin were due to the same action as that of amyl nitrite, and it was found to be just as effective in angina. It is still widely used but because of its short action other organic nitrates have been developed, the most commonly available being isosorbide dinitrate or mononitrate and pentaerythritol tetranitrate.

4.2.2 Therapeutic Use as a Direct Consequence of the Known Properties of the Chemical

Treatment of human vector-borne parasitic disease relies heavily on the use of insecticides. DDT (dichlorodiphenyl-trichloroethane) was the first major chemical to be used with spectacular success by American troops during the Second World War in malaria vector control and against other insect pests (Smith, 2001). During the 1940s and 1950s it became an important element of public health campaigns, until concerns over its persistence in the human body and the environment, as well as the development of insect resistance led to restriction of usage and an eventual ban in many countries. However, because of its cost-effectiveness and relatively low acute toxicity, DDT remains an important vector control agent in many parts of the world (IPCS—International Programme on Chemical Safety, 1979). The widespread use of DDT prompted many investigations into possible health effects, including oral, dermal, inhalation and bioaccumulation volunteer studies at doses up to 1500 mg (for a summary see Smith, 2001). Apart from its antiparasitic use, DDT was also studied for a possible bilirubin-reducing effect in selected patients with jaundice (Thompson *et al*., 1969) at doses up to 3 mg kg^{-1} day^{-1} for six months. There were no side effects in this study, nor were there any when DDT was used as an antidote in phenobarbital intoxication as a single dose of 5000 mg (Rappolt, 1970).

The products nowadays used in vector parasitic control fall into different chemical categories, mainly organochlorines, organophosphates and pyrethroids. Lindane, malathion and permethrin are effective against scabies while malathion and permethrin are recommended for head lice.

4.2.3 Use as a Therapeutic Adjuvant

Many chemicals are used as coformulants in medicinal preparations. For example, propylene glycol is used as a solvent in parenteral preparations, either on its own or in combination with ethanol. The chemically related diethylene glycol, an industrial solvent and antifreeze, was used in 1937 to prepare a liquid formulation of the newly introduced sulfanilamide antibiotic. The resulting death of over 100 people had profound consequences for the licensing of drugs in the USA, leading to new legislation which stipulated the requirements for data submission to the Food and Drug Administration (Wax, 1995). Unfortunately, there have been at least six other mass poisonings with analgesic elixirs containing diethylene glycol in South Africa, India, Nigeria, Bangladesh, Haiti and Panama, resulting in the death of several hundred patients, the majority of them children (Wax, 1996; Barr *et al*., 2007; Schier *et al*., 2008). In each case, the cheaper diethylene glycol appears to have been substituted for the more expensive propylene glycol or glycerol.

Chlorofluorocarbons (CFCs) are still widely used as refrigerants, propellants, degreasers, fire extinguishers and industrial cleaning agents. Human health effects such as cardiac arrhythmias and even fatalities from asphyxiation have been described at high concentrations (McGee *et al*., 1990). On the other hand, CFCs are used as propellants in metered-dose inhalers prescribed for the treatment of asthma. Doses dispensed from these devices contain typically between 50 and 100 mg of CFCs, the most common ones being trichlorofluoromethane (FC-11), dichlorodifluoromethane (FC-12) and dichlorotetrafluoroethane (FC-14) (Pierce *et al*., 1991).

4.3 Exposure and Health Surveys

Human studies of chemical exposure are at their most powerful if they include adequate information on both exposure and health effects. Such an ideal is rarely achieved; most studies concerned with exposure measurement look at relatively low levels of exposure over short periods, which are unlikely to result in short-term health effects. Conversely, most epidemiological studies looking at health effects from chemical exposure suffer from a lack of quantifiable exposure information, especially where there is a long latency period between exposure and onset of illness (see **Essentials of Epidemiology for Toxicologists**).

Exposure and health surveys can in theory be carried out wherever there is a likelihood of exposure occurring,

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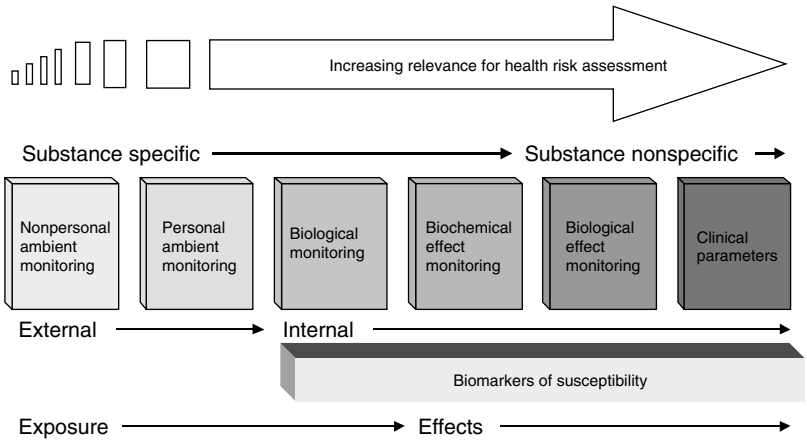

Figure 1 Monitoring techniques as part of the exposure-disease continuum. (Reproduced with permission from ECETOC. © 2005.)

including environmental exposure of the general public. In practice, it is mainly the surveillance of occupationally exposed workers which is likely to yield substantive information on the link between chemical exposure and health effects (see **Occupational Toxicology**). Exposure and health surveys are useful either as components of risk assessment or risk management. The former takes place ideally before people are exposed to the chemical in question, the latter is part of an ongoing process to safeguard the health of the individual who may be exposed.

4.3.1 Methods for Exposure Assessment

Exposure can be determined by means of nonpersonal ambient monitoring or personal monitoring, the latter being further categorized into personal external monitoring and variants of biomonitoring (**Figure 1**). The choice of a method depends principally on the purpose of the investigation, the chemical involved, the conditions at the workplace and the resources available.

Nonpersonal ambient monitoring is designed to assess the concentration of chemicals either as particulate, gas or vapour in the workplace air. It is achieved by installing collection devices containing filters or other absorptive material (e.g. activated charcoal, silica gel) which can subsequently be analysed. The high sampling volume means that very low levels of detection can be achieved (Schlatter, 1992). This method is particularly suitable for process checks and to monitor engineering controls designed to minimize exposure.

Personal ambient monitoring is usually carried out to assess inhalation exposure. The distribution of a nongaseous chemical depends largely on the physicochemical properties of the particle or droplet. In terms of inhalation risk assessment, two categories of airborne particulates are usually considered; the inhalable fraction (the mass fraction which is inhaled through the nose and mouth) can for sampling purposes be considered to be of a size below 100 µm. The respirable fraction (the mass fraction which penetrates into the unciliated airways) is considered to be below 15 µm (International Organization for Standardization, 1995). The collection devices are attached to work clothing in the vicinity of the breathing zone and are connected to personal sampling pumps.

The contamination of workers' skin and clothing can be measured using a variable number of multilayered gauze patches attached to defined areas of the body. However, because of the nonuniformity of dermal exposure, whole body monitoring using clothing which acts as sampling medium has been proposed as an alternative (Chester, 2001). This has the advantage that the capture and retention properties of normal work clothing are mimicked as closely as possible.

Biomonitoring is a general term comprising a number of subcategories (ECETOC, 2005 and **Figure 1**):

1. **Biological monitoring:** This method analyses for biomarkers of exposure designed to provide a measure of internal dose or body burden. It can be defined as measurement of a chemical or its metabolites in the body fluids of exposed persons, and conversion to an equivalent absorbed dose of the chemical based on a knowledge of its human metabolism and pharmacokinetics (Woollen, 1993). Examples include the determination of metals (e.g. mercury, arsenic or lead) in blood or urine, the determination of unchanged substances (e.g. PCBs (polychlorinated biphenyls), dioxins or benzene) in adipose tissue, milk, urine or blood, the determination of specific metabolites of a chemical (e.g. s-phenylmercapturic acid as a metabolite of benzene) in urine, or volatile compounds (unchanged substances or metabolites) in exhaled breath.
2. **Biochemical effect monitoring:** While also being a measure of exposure, the biomarker reflects the effective dose of a chemical, usually a potentially genotoxic substance, by measuring its reaction

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product with an endogenous macromolecule indicating that the chemical was not only bioactive but also was absorbed into the body and reached the tissues (hence: tissue dose). Examples are the determination of adducts of a specific chemical (e.g. ethylene oxide or polycyclic aromatic hydrocarbons) to DNA or a protein, such as albumin or haemoglobin.

3. **Biological effect monitoring:** This measures early and often reversible biological effects, which do not necessarily lead to specific health effects. Because the effect may be influenced by various physiological and environmental factors unrelated to chemical exposure, most methods of biological effect monitoring are intrinsically nonspecific. Examples include measurements of acetyl cholinesterase activity in blood, zinc protoporphyrin, sister chromatid exchanges and other chromosomal aberrations, and microproteinuria.
4. **Clinical parameters (biomarkers of disease):** These are an expression of (beginning) disease. Some parameters assessed in biological effect monitoring may also be used in clinical practice and form a continuum from a reversible biological to a clinically relevant effect. For example albuminuria may be used in biological effect monitoring (microalbuminuria) as a biomarker of early renal function effects but it is also used as a clinical parameter, for instance to assess renal function impairment in diabetics.

Biomarkers are influenced by factors such as lifestyle and physiology, including genotype and phenotype. The term 'biomarker of susceptibility' refers to indicators of an inherent or acquired ability of an organism to respond to exposure to a specific substance, for instance through the expression of certain isoforms of cytochrome P450, glutathione transferases or *N*-acetyltransferases. In addition, factors such as iron status, nutritional status, and so on, may also be regarded as biomarkers of susceptibility. In contrast to the other biomarkers they reflect potential differences in uptake and metabolism of chemicals between individuals and, consequently, potential differences in health risks. Biomarkers of susceptibility may, like lifestyle factors, such as smoking and drinking behaviour, explain differences in biomarker results between individuals with identical exposure profiles (Angerer *et al*., 2004).

Recent developments in molecular biology have raised the possibility of using 'omics' technologies in the study of biomarkers, for example:

- In proteomics, new mass spectrometric (MS) techniques such as analysis of the single combined spectra extracted from full-scan MS data may allow the detection and structural characterization of chemical adducts on proteins (Brock *et al*., 2007).
- Differences in biological responses to exposure to hazardous airborne substances between children and adults have been studied using genome-wide gene expression profiles in peripheral blood of children in regions with different levels of air pollution (Van Leeuwen *et al*., 2008).
- The use of molecular spectroscopic approaches that capture environmental and selected genomic influences is now being used to investigate the connections between phenotype variation and disease risk factors (Nicholson *et al*., 2008).

4.3.2 Worker Exposure and Health Surveys

With hundreds of thousands of chemicals in production and use, it is clearly neither possible nor desirable to establish a human database for all of them, since the vast majority pose no appreciable risk to humans. Human data should therefore be obtained in a systematic, priority-based way with an emphasis on those chemicals which are most likely to lead to adverse health effects as a result of their intrinsic hazard *and* the likely human exposure.

Surveys of occupationally exposed populations can be of considerable benefit in the risk assessment process, particularly when a considerable amount of human use experience data has accumulated (see **Occupational Toxicology**). In addition, health surveys may help to refine assessment factors for the calculation of acceptable risk, particularly where there are questions about the applicability of animal data at high doses to the human use conditions which may involve exposure at doses several orders of magnitude lower than encountered in animal studies.

The registration and regulation of pesticides probably involves the most sophisticated risk assessment process outside the pharmaceutical area. This is not surprising given that pesticides are designed to have an effect on biological systems leading to the control or destruction of rodents, weeds, insects or fungi. Most countries operate regulatory systems, with the best known existing in the USA (EPA, 2002) and the European Union (SANCO European Commission Health and Consumer Protection Directorate General, 2008). Internationally, the Food and Agriculture Organization (FAO) and the WHO carry out toxicological evaluations of pesticides (Joint Meeting on Pesticide Residues (JMPR)) for the purpose of setting acceptable daily intakes (ADIs) (IPCS—International Programme on Chemical Safety, 2008).

It has been proposed to take a tiered approach to the generation and use of human data for the purpose of pesticide operator risk assessment (Henderson *et al*., 1993; OECD, 1997). This is shown schematically in **Figure 2**. Each tier involves the comparison of an exposure data set with the appropriate NOAEL (no observed

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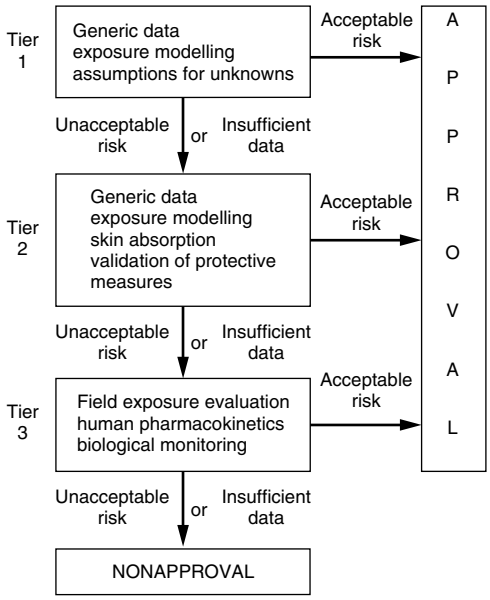

Figure 2 Three-tiered approach for the assessment of worker exposure in a risk assessment procedure for registration of a pesticide. Requirement of actual human data (field exposure evaluation, biological monitoring) depends on whether the calculated exposure shows an acceptable risk when considering the toxicology profile of the compound. (OECD, 1997, modified and reproduced by permission of OECD.)

adverse effect level—usually obtained from animal toxicology studies) and applying an assessment or safety factor to account for uncertainties when extrapolating from animal data to human exposure, as well as interindividual differences in human response.

The first tier in the risk assessment process would typically involve the use of generic databases which allow model calculations for the likely exposure level (Van Hemmen, 1993). The source and type of data used should be clearly identified and the database or model used must be validated and applied correctly. At this stage, very conservative assumptions are used (e.g. no protective equipment, 100% skin absorption). In the second tier the data set is refined, for example using actual skin absorption data (animal or human *in vitro*) and building in factors to account for personal protection. The third tier involves the generation of actual human data. These could be exposure measurements (Chester, 1993) or, ideally, biological monitoring backed by human pharmacokinetic information (Woollen, 1993). If the risk assessment still indicates excessive exposure, further risk mitigation factors may be considered to reduce the absorbed dose to an acceptable level.

Chemically-induced occupational illnesses are prevented by the control of exposure to chemical hazards in the workplace. Sometimes this needs to be supplemented by surveillance of the people potentially exposed to chemicals at work. It is important that such surveillance is fully integrated in the risk management process (Tordoir and Maroni, 1994). If this is not the case the surveillance will be out of focus and neither effective nor efficient. Some of the basic principles which should be followed are:

1. The surveillance should give information over and above that already available.
2. It should provide information which will assist in evaluating control measures.
3. If individual (biological) monitoring is involved, there must be an appropriate sampling strategy and analytical technique, preferably involving noninvasive sampling.
4. There must be clear criteria for interpreting the results.
5. There must be adequate consultation with employees, including feedback of the results.
6. Appropriate action is taken as a result of the surveillance, and its effectiveness is evaluated.

Medical examinations form an important part of occupational health programmes (see **Occupational Toxicology**). The content and frequency must depend on the results of the risk assessment process. In particular, the content of medical examinations should be related to the identified health hazards and to the characteristics of chemical exposure, while the frequency of periodical examinations should be determined on the basis of the magnitude of risk to human health and the natural course of the relevant adverse health effects (Tordoir and Maroni, 1994). Any surveillance programme involving clinical care must contain appropriate safeguards for the participating individuals. Employees should be aware of the possible consequences of surveillance, and individual medical records should be kept confidential. Before disclosing such information the individual's consent needs to be obtained. However, as a result of surveillance employees may be transferred to a job that entails less exposure to a particular hazard, and this should be explained clearly to the participants in a programme at the outset. It is also important that surveillance programmes are not used to discriminate against specific employee groups (e.g. women or older workers).

Any occupational surveillance programme is critically dependent on the quality of data recording and retention. The records should document data relevant to individual workers, they should be able to identify health trends and priority areas for action, fulfil legal requirements, and enable future epidemiological studies to be carried out. It is important to keep records for long enough periods, for example, in the case of exposure to ionizing radiation 60 years is recommended.

The main items of information that are useful for future studies may be considered under a prioritization system

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of data recorded along the following lines (Cooke *et al*., 1999):

1. A basic data set aimed primarily at identification of subjects, employment and exposure to potentially hazardous substances. The latter should include as much information as possible about job profile, employment dates and sites, as well as all available information on chemical exposure, in particular any available monitoring data.
2. Data which frequently prove desirable in epidemiological studies and which could be available or recorded at medical interviews and examinations, such as information about previous employments, ethnicity, residence abroad, recreational activities and hobbies.
3. Medical (clinical) data where it is considered useful or advisable to carry out a full clinical examination. These may include detailed histories, medical examinations and laboratory tests.

It is recognized that ideal data records are rarely, if ever, available. Furthermore, data records may not follow the individual if they change their employer. Nevertheless, analysis of data records may be the only way to identify health effects from chemicals with long latency periods between exposure and the occurrence of illness.

4.3.3 Surveys in the General Population

Historically, (bio)monitoring has mainly been used in occupational settings, for example for surveys, and also as a research tool to study uptake and metabolism of chemicals to help with the assessment of health risks. However, due to rapid technical developments, biomonitoring is increasingly being used to study environmental exposure. In recent years many countries around the world have established environmental biomonitoring programmes that report representative values of selected xenobiotics in nonoccupationally exposed populations. These programmes complement a range of other biomonitoring programmes that study levels of chemicals in the environment, such as in drinking water, food, fish and other biota and air. One population group of particular interest in many programmes is children.

A coordinated approach to human biomonitoring in Europe has been initiated under the European Environment and Health Strategy. In a pilot project a number of work packages were completed, such as establishment of an electronic inventory of human biomonitoring activities, drafting of various guidelines, consideration of ethical issues and work on the utility and sensitivity of biomarkers (ESBIO—Expert Team to Support Biomonitoring in Europe, 2007). Projects at member state level have included biomonitoring studies, for example in Germany (Pesch *et al*., 2002), Poland (Jakubowski and Trzcinka-Ochocka, 2005) and France (Huel *et al*., 2002). A number of related research activities and projects have also been set up at European level, in particular through the EU Framework Programmes, for example ChildrenGenoNetwork (2008), AIRNET (2008), Plutocracy (2008), MENDOS (2008) and BIOMONECS (2008).

In the USA, the third National Report on Human Exposure to Environmental Chemicals has recently been published (CDC, 2005), providing part of an ongoing assessment of the exposure of the US population to selected environmental chemicals using biomonitoring. The report provides data for 148 chemicals, including metals, polycyclic aromatic hydrocarbons, dioxins and dioxin-like substances, PCBs, phthalates, phyto-oestrogens, organochlorine and organophosphate pesticides and pyrethroids. This report was based on measurements taken from selected participants in the National Health and Nutrition Examination Survey (NHANES), which is undertaken serially to collect data on the health and nutrition status of the US population. Trends in the levels of chemicals are reported where these chemicals were also measured in earlier reports. Results are interpreted with reference to the ranges of concentrations found in the population studied. In some instances, results are also interpreted against reference values.

With regard to existing information it is useful and important to differentiate between *research projects*, which aim at the generation or the improvement of knowledge on the causal links between environmental factors and health by hypothesis generation and testing, and *survey projects*, which aim at periodic measurements to produce information on the prevalence of exposure to environmental agents. Survey projects may be used in the public health arena with a view to developing and evaluating policies designed to protect the health of the general population.

4.4 Human Volunteer Studies

Human studies are commonplace in drug development. New chemical entities are taken through the stages of safety and efficacy testing, the first one usually involving the administration of a single dose to volunteers (Phase I testing). In contrast, there is very little information on human pharmacokinetics and toxicity for the majority of nonpharmaceutical chemicals. However, such information can be very useful, in some cases even essential, for the risk assessment process. While the previously mentioned types of studies can all make a very valuable contribution to the understanding of the effects of chemicals in humans, they almost invariably suffer from a lack of control regarding exposure. In contrast, human volunteer studies give information on health effects in relation to well-defined exposures.

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4.4.1 The Controversy over Human Volunteer Studies

The underlying principles and international guidelines on ethical aspects of research involving human subjects have already been extensively discussed (see above). Historically, safe exposure levels for chemicals were established through experience of safety in use. For less overtly toxic compounds it may have taken a long time to recognize the link between exposure and harmful effects. Even when this was achieved, certain effects may have been tolerated as an occupational hazard if the benefit from use was seen to be substantial. Increasing awareness of the effects of chemical exposure has gradually led to the introduction of regulations covering the control of chemical exposure in the workplace. Biological monitoring has been established as a cornerstone of exposure surveillance (see above), but it requires an understanding of human metabolism and pharmacokinetics (Wilks *et al*., 1993). A second line of argument concerns the use of human volunteer studies for the purpose of reduction, refinement or replacement of animal studies (Volans *et al*., 1991). This is of particular importance in areas where public opinion is increasingly reluctant to accept animal testing for basic safety assessments. A typical example would be testing for topical effects of cosmetics, personal care and household products.

Some types of human volunteer studies, in particular those with pesticides, have attracted considerable controversy in recent years. In the USA, the Food Quality Protection Act of 1996 introduced the concept of an additional uncertainty factor of up to 10 into the risk assessment process for pesticides, to account for the possible increased sensitivity of infants and children (EPA, 2009). A number of dosing studies with volunteers were subsequently submitted to the agency with the intent of providing human data which would allow reduction of the existing intraspecies uncertainty factor. The resulting protracted and intensely polarized debate has been summarized in detail elsewhere (NRC Committee on the Use of Third Party Toxicity Research, 2004). Some scientists, environmental and other activist groups challenged the ethical and scientific validity of the studies, contending among other things that people should not be put at risk for the purpose of reducing the stringency of regulatory standards (Lockwood, 2004; Oleskey *et al*., 2004; Sass and Needleman, 2004; Needleman *et al*., 2005; Krimsky and Simoncelli, 2007). Other scientists and industry groups argued that the human dosing studies were needed to ensure the scientific quality and accuracy of the EPA's safety evaluations and that they had been, and could be, conducted ethically (Charnley and Patterson, 2003; Chart *et al*., 2004; McAllister, 2004; Tobia *et al*., 2004; Resnik and Portier, 2005). In response to the controversy, the EPA commissioned the National Academy of Sciences to provide advice on whether, and under what circumstances, the agency should accept and consider intentional human dosing studies. The National Research Council committee which considered this question recommended that such studies should be conducted and considered for regulatory purposes only if all of the following conditions were met (NRC Committee on the Use of Third Party Toxicity Research, 2004):

- The study is necessary and scientifically valid—that is, it addresses an important regulatory question that cannot be answered with animal studies or nondosing human studies, and has been designed, conducted and reported in a manner that ensures the study will be adequate scientifically to answer the question.
- The societal benefits of the study outweigh any anticipated risks to participants.
- Intentional human dosing studies that are to be used only to improve the accuracy of a reference dose (RfD), and that otherwise provide no health or environmental benefit, can be justified only when there is reasonable certainty that participants will experience no adverse effects.
- All of the recognized ethical standards and procedures for protecting the interests of study participants are observed, including equitable selection and recruitment of participants, informed consent and independent review of the scientific and ethical merits of the study by an IRB or its foreign equivalent.

On the basis of this advice the EPA issued a final rule in 2006 which established the framework under which data from intentional human dosing studies would be considered in future (EPA, 2006). The rule gives details of the many difficult questions the agency had to struggle with and the reasons for its decision. The final rule categorically prohibits any EPA-funded research, or the consideration of other ('third party') research involving intentional exposure of pregnant women and children. It also extends the federal policy which governs protection of human subjects in research ('Common Rule') to other intentional exposure studies involving nonpregnant adults intended to be submitted to EPA under the pesticide laws. Furthermore, it requires protocols for such studies to be submitted to the EPA before the research is started, and it establishes an independent Human Studies Review Board to review new research proposals and research reports intended to contribute to decision making.

In Europe, the regulatory framework concerning authorization and use of pesticides is governed by European Union legislation. In Annex II, Part A, Point 5.9 of Council Directive 91/414/EEC of 15 July 1991 on the placing of plant protection products (PPPs) on the market, it is stated that '... Data and information relevant to the effects of human exposure, where available, are of particular value, in confirming the validity of extrapolations made and conclusions reached with respect to target organs, dose—response relationships

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and the reversibility of toxic effects. Such data can be generated following accidental or occupational exposure' (Commission of the European Communities, 1991). Furthermore, in an opinion expressed by the Scientific Committee on Plants, it was stated that 'Human data are most useful because they provide reassurance on the extrapolation process' (SCP—Scientific Committee on Plants, 2002). However, the Scientific Committee went on to note that 'apart from ethical issues, studies conducted in humans may have limitations (e.g. reduced number of subjects, the use of only one sex, the possibility of studying only selected end points). The Committee stresses that human data should be used in the context of the entire toxicological profile of the PPP under consideration.' The latest draft guidance document on the setting and application of acceptable operator exposure levels (AOELs) describes the intended restrictions concerning the use of human data as follows: 'Toxicological studies conducted in humans with the purpose of determining a human No Observed Effect Level of an active substance have not been and will not be used *per se* to derive regulatory limit values (such as an ADI, an AOEL or an Acute Reference Dose) for the substance. Rather, such studies if they are scientifically and ethically valid, will be evaluated and used as supplementary information to confirm the validity of regulatory limit values which will continue to be derived from extrapolations from appropriate studies in laboratory model species' (SANCO European Commission Health and Consumer Protection Directorate General, 2006). The document goes on to say that if appropriate scientifically valid and ethically generated human data are available which show that humans are more sensitive and which would lead to a lower AOEL value, these data should take precedence over animal data. Significantly, no such provision has been made for the reverse case, that is, human data showing decreased sensitivity. This position has been criticized by the European Food Standard Authority's Panel on Plant Protection Products and their Residues (PPR), who stated in an opinion on an earlier draft of the guidance document that 'the results of tests involving humans when ethically and scientifically acceptable should be used to derive reference values, including AOELs, and not be considered simply supportive of reference values derived from animal data. However, this position should not be interpreted as condoning the injudicious study of human subjects involving deliberate or intentional exposure' (PPR, 2006). It appears, nevertheless, that the opinion of this scientific advisory body may not find its way into legislation, in particular the revision of the PPP directive 91/414/EEC. The latest proposal by the European Commission repeats on a number of occasions the point made in the draft AOEL guidance document, that deliberate human administration studies should not be used to determine a human No Observed Effect Level of an active substance, and explicitly states in Article 4: 'In relation to human health, no data collected on humans shall be used to lower the safety margins resulting from tests or studies on animals' (Commission of the European Communities, 2006).

In contrast, the World Health Organization has considered human data in the context of chemical risk assessment for many years through the IPCS. Regarding human volunteer studies, the IPCS concluded that such studies have contributed particularly to our understanding of kinetics and to the development of air quality guidelines and standards for traditional pollutants (IPCS—International Programme on Chemical Safety, 1999). The 1998 Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment, and the WHO Core Assessment Group discussed the use of data from biomedical testing involving human subjects in hazard evaluation (JMPR, 1998). The meeting concluded that in order for human studies to be considered in the hazard evaluation of a pesticide, it must be shown that they were conducted in accordance with principles such as those expressed in the Declaration of Helsinki or equivalent statements. Studies should be designed with sufficient rigour and robustness to ensure their applicability to diverse populations. Therefore, the study design should include a sufficient number of subjects of both sexes (to be determined, in part, on the basis of the effects to be evaluated, to account for inherent normal variability and to ensure adequate statistical rigour). The subjects should be adults, and any females should not be pregnant. Provided that studies could be shown to be scientifically sound and to adhere to appropriate ethical guidelines, the meeting agreed to make continued use of them in future evaluations.

4.4.2 Preliminary Safety Data

An assessment of the risk to volunteers participating in any study involving the administration of drugs or chemicals can only be made on the basis of available hazard information on the compound. There is a trend within the pharmaceutical industry to study new chemical entities in humans increasingly early in the development. However, a recent life-threatening adverse event in a Phase I clinical trial has demonstrated that unforeseen human reactions to novel products can occur even in the presence of drug safety information which does not indicate any specific risk (Suntharalingam *et al*., 2006). In this case, six healthy male volunteers were given a recombinant anti-CD28 monoclonal antibody in a clinical trials facility. Within 90 minutes of receiving a single intravenous dose of the drug, all volunteers experienced inflammatory reactions of an increasing severity and became critically ill over the following 12–16 hours with pulmonary infiltrates and lung injury, renal failure and disseminated intravascular coagulation. They were transferred to an intensive care unit where they received intensive cardiopulmonary support including dialysis,

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high-dose methylprednisolone and an anti-interleukin-2 receptor antagonist antibody. Despite the severity of the cytokine release syndrome, all six patients survived although two required intensive organ support for 8 and 16 days, respectively. A subsequent investigation by the MHRA concluded that the adverse incidents did not involve errors in the manufacture of the antibody or in its formulation, dilution or administration to trial participants (MHRA, 2006). In the MHRA's opinion an 'unpredicted biological action of the drug in humans' was the most likely cause of the severe adverse reactions in the trial participants, despite apparently adequate preclinical testing. The report highlights the important scientific and medical issues raised by the incident, in particular those concerning the potential risks associated with this type of drug and how to make the transition from preclinical testing to trials in humans. However, an editorial accompanying the publication of the event concluded that while it is important to do whatever is necessary to minimize risk, it is not possible to prevent all such events in future, and rare and idiosyncratic adverse events should not put an end to medical progress (Drazen, 2006).

Human dosing studies with a nonpharmaceutical are almost invariably carried out comparatively late, for example in the case of pesticides when data on operator exposure are required. This means that there will be substantially more toxicity information available than that which is required for a Phase I single oral dosing study in drug development. The WHO/FAO Expert Panel of the Joint Meeting on Pesticide Residues has suggested that the entire standard toxicological database in the appropriate animal species to establish the ADI should be available (JMPR, 1998). The benefit of this situation in the case of the pesticide is that a risk assessment for humans will be improved as more toxicity information becomes available. On the other hand, there may be a tendency to overemphasize adverse effects occurring in chronic and lifetime studies in animals at high dose levels, which may have little or no relevance for possible effects expected from a single low dose administered to volunteers. A minimum toxicity dataset has therefore been suggested which should be available before considering a human volunteer study with nonpharmaceutical chemicals (Wilks and Woollen, 1994). This would, of course, need to be adjusted depending on the type of study and the chemical in question, but for a single oral dosing study it should contain at least the following, carried out according to OECD guidelines:

1. *In vitro* and *in vivo* tests of genotoxicity.
2. Acute oral toxicity tests in one rodent and one nonrodent species.
3. Subchronic (90 days) toxicity in one rodent and one nonrodent species.
4. Balance and biotransformation studies in one species.
5. Assessment of fertility in a single generation study.
6. Teratology in one species (in case of participation of female volunteers).

Particular attention should be given to the results of short-term tests which are most relevant to a single exposure of humans. For a single dose dermal study additional information would be required on:

1. Acute dermal toxicity in one rodent species.
2. Percutaneous absorption, either using animal *in vivo* or human skin *in vitro* data.

In addition to any toxicological information, human experience in use should be considered whenever possible.

4.4.3 Types of Human Volunteer Studies

Two principal types of human volunteer studies with nonpharmaceutical chemicals need to be distinguished. There are studies which look at an adverse or toxic effect as the outcome measurement. The second type involves the administration of chemicals to study their metabolic fate and pharmacokinetic behaviour.

4.4.3.1 Effect Studies

The term 'minimal risk' in human volunteer studies has already been discussed, and this is of paramount importance when considering effect studies. It therefore follows that the study of adverse or toxic effects in volunteers can only be justified on the assumption that no lasting damage will occur. In practice, this restricts such studies to acute and inherently reversible changes at dose levels which people are likely to encounter at their workplace or in the general environment. For example, the acute neurobehavioural effects of solvents have been studied in volunteers at exposures around permitted occupational exposure limits (Iregren and Gamberale, 1990). Such studies include the effects of exercise and alcohol as modifiers of the CNS effects of solvents. Another example is the study of sensory effects of pyrethroid insecticides. Synthetic pyrethroids have the potential to induce transient, fully reversible paraesthesia (variously described as tingling, burning or numbness) in exposed skin of susceptible individuals (LeQuesne *et al*., 1980). The underlying mechanisms of this effect, as well as the potential for therapy, have been studied using human volunteers (Flannigan and Tucker, 1985; Flannigan *et al*., 1985).

Organophosphorus insecticides have been tested in volunteer studies for their potential to inhibit cholinesterase (ChE) activity as a biochemical marker of exposure long before the present controversy regarding such studies (see above). Oral administration of malathion at doses up to 16 mg person^{-1} day^{-1} for up to 47 days had no significant effect on either

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plasma or red cell ChE activity, and the volunteers remained asymptomatic (Moeller and Rider, 1962). Parathion-methyl has been studied in a whole series of volunteer studies, with oral doses between 1 and 22 mg person^{-1} day^{-1} producing no significant effect on plasma or red cell ChE (for a summary see Rider *et al*., 1969). A dose of 30 mg person^{-1} day^{-1}, or approximately 0.4–0.5 mg kg^{-1} day^{-1} showed a maximal mean red cell ChE depression of 37%, thus being defined as the level of minimal incipient toxicity for parathion-methyl (Rider *et al*., 1971). In studies designed to test the safety of dichlorvos as a disinsectant of aircraft, 15 volunteers were intermittently exposed to a concentration of 0.5 mg m^{-3} for five hours per night, four nights per week over two weeks (Rasmussen *et al*., 1963). There was no effect on red cell ChE activity, but a gradual reduction of plasma ChE with the lowest single plasma value observed being 34% of normal. No clinical signs were seen, and detailed studies of visual performance, complex reaction time, neurological function and airways resistance showed no abnormalities.

The largest number of effect studies is carried out in the testing of consumer products for topical irritancy and sensitization. It is now recognized that the standard animal tests, such as the Draize skin and eye irritation tests (Draize *et al*., 1944; Draize, 1959) frequently overpredict the severity of effects occurring in humans (Freeberg *et al*., 1984; Nixon *et al*., 1975). With regard to eye effects, volunteer studies have been employed to establish the threshold of effects seen with mild to moderate irritants (Freeberg *et al*., 1986). This has shown that a modification of the Draize test using a lower instillation volume is more representative of human effects. For the predictive testing of skin irritancy a human four-hour patch test has been developed (Basketter *et al*., 1994; Dykes *et al*., 1995; York *et al*., 1995). It has been proposed to use this test as an alternative to the Draize test for the classification and labelling of substances and preparations (York *et al*., 1996).

4.4.3.2 Metabolism and Pharmacokinetic Studies

These types of studies have two main purposes. The first is the identification of metabolic pathways and target metabolites which can subsequently be used in biological monitoring studies at the workplace or in the general population. Second, in order to interpret the results of biological monitoring studies and extrapolate back from the excretion of a metabolite to exposure to the parent compound, a detailed knowledge about the human pharmacokinetics of the compound is required. When a compound is administered to humans for the first time it is prudent to carry out a pilot study with a single volunteer. This serves several purposes. It provides assurance that adverse reactions are unlikely (particularly relevant since the events surrounding the Phase I trial of a novel monoclonal antibody in 2006, see above), confirms whether the dose is adequate in terms of analytical sensitivity, provides material for the improvement of analytical methods, and allows the sample collection times to be optimized. For the main study, it may be desirable to study more than one dose level, and, where dermal or inhalation uptake is important, more than one route of administration.

For example, the pyrethroid insecticide cypermethrin was administered to volunteers as a single oral and dermal dose (Woollen *et al*., 1992). Urine was collected for 120 hours after dosing and analysed for the metabolites *cis*- and *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (DCVA), 3-phenoxybenzoic acid (3PBA) and 3-(4-hydroxyphenoxy)benzoic acid (4OH3PBA). Following oral dosing, the ratio of *trans*- to *cis*-DCVA metabolites was approximately 2 : 1, and similar amounts of DCVA and PBA (3PBA + 4OH3PBA) metabolites were excreted. In contrast, in the dermal study the ratio of *trans*- to *cis*-DCVA metabolites excreted in urine was approximately 1 : 1, and the total amount of PBA metabolites recovered was four times higher than the total amount of DCVA metabolites. In comparison, the metabolite excretion profiles in a field worker exposure study involving hand-held spraying were consistent with a predominance of absorption by the oral rather than the dermal route, since the ratios of *trans*- to *cis*-DCVA were usually close to 2 : 1 and similar amounts of DCVA and PBA metabolites were excreted in urine (Wilks *et al*., 1993). These data illustrate route-specific metabolism of cypermethrin that may not have been predicted from animal studies, as well as the importance of both oral and dermal volunteer studies and the usefulness of measuring multiple metabolites. The results could be used to provide indicators of the main route of absorption during occupational exposure, thus allowing better targeting of safety advice and protective measures.

Di-2-ethylhexyladipate (DEHA) is a plasticizer which is added to PVC to produce 'cling film' type food wrappings. DEHA can migrate into foods, particularly those with a high fat content, and the maximum likely daily intake has been estimated to be around 8 mg day^{-1} (MAFF, 1990). An oral human volunteer study was carried out using deuterium-labelled compound to distinguish between metabolites derived from the test substance and background levels (Loftus *et al*., 1993). The most abundant metabolite found was 2-ethyl hexanoic acid (EHA), whereas mono-2-ethylhexyl adipate, the major metabolite found in a primate study at a 100-fold higher dose level, was not found. This example illustrates a potential problem in risk assessment. Certain metabolic pathways may become overloaded at higher doses and bring other pathways into operation. Consequently there will be some uncertainty about comparisons between animal toxicology studies at

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high dose levels and human volunteer studies at much lower doses. Follow-up biological studies were carried out using EHA as a biological marker in 24 hour urine samples from 112 individuals from five geographic locations in the UK. Estimates of daily intake of DEHA showed a skewed distribution with a median value of 2.7 mg (Loftus *et al*., 1994).

5 CONCLUDING REMARKS

The use of toxicity data from human studies is still very much the domain of pharmaceutical safety assessment, but the benefits of the collection of human data are now recognized in other areas, most notably those involving exposure to industrial chemicals and pesticides, as well as the safety assessment of consumer products. This chapter has looked at the main sources of human data in toxicity assessment, in particular for nonpharmaceutical chemicals. They all have their unique advantages and limitations, and no single type of human study is able to answer all questions.

Case reports often contain a high level of detailed and verified exposure and give a rare insight into dose–response relationships. In the case of adverse reaction reporting they also allow an estimate of the likelihood of adverse effects occurring. Their main limitation is that they are reactive, often retrospective and rely on the individual effort of clinicians or relatively few data collection systems, for example those of PCCs and the pharmaceutical industry.

In the case of human therapeutic use of chemicals, the systematic use of human data normally seen with pharmaceuticals applies through a combination of volunteer studies and adverse data reporting. However, this is only applicable to a small number of chemicals.

Exposure studies and health surveillance provide invaluable information on presence or absence of effects from long-term exposure to chemicals in the workplace or environment. However, they can be time consuming and expensive, and the link between exposure and effect may be difficult to verify, particularly for retrospective studies.

Human volunteer studies with nonpharmaceutical chemicals have the advantage of well-characterized exposure combined with information on the pharmacokinetic behaviour and/or effects of the compound. Nevertheless, outside the area of consumer products their usefulness is limited by ethical concerns and scientific challenges such as study design and small study populations. It is hoped that the present controversy surrounding some aspects of volunteer studies can be resolved through rational, scientific debate involving all stakeholders.

In the end, it is not so much the type of human study which will determine the ultimate usefulness of the data generated, but the rigorous application of established scientific principles. For the reasons discussed in this chapter, it is neither feasible nor desirable that human studies will replace experimental toxicology, but the two should be complementary. The danger is that toxicologists will continue to do experiments and epidemiologists will continue to collect data, but they fail to constructively interact with each other (Wilks *et al*., 1996). As observed by Sir Richard Doll, most occupational hazards of cancer have been discovered as a result of clinical intuition or epidemiological observation. However, most could have been avoided if modern laboratory techniques had been employed to test the substances used before men and women were exposed to them in an industrial environment (Doll, 1984).

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Quality Assurance in Toxicology Studies

J. Rodney Pateman

CONTENTS

1 INTRODUCTION

The concept of any quality-related controls in respect of scientific research can at first seem nonsensical. The very nature of research, including toxicology, is to discover hitherto unknown and sometimes unexpected information: if a study generates the first information in the field, against what measures can it be assessed for quality, and what would be the purpose of such assessment?

Some of these issues become clear when a distinction is made between regulatory and nonregulatory toxicology. The purpose of regulatory toxicology is to provide information to government agencies, such that they can make sound decisions as to the precautions necessary to protect against dangerous properties of a chemical substance. The dangers may relate to human or animal health, or to the environment, and may be mitigated by limitations on the amount of material permitted on the market, limiting the scope or manner of the use of the material, or advice and warnings as to precautions during use (such as protective clothing, hazard labelling, etc.). The reports of the studies on which these regulatory decisions are based must be reliable, or else dangerous material may be inadequately controlled with potentially disastrous consequences. For these reasons, such work must be done within a statutory legal framework.

However, historically (and in some ways paradoxically), it was fundamental research that was first subject to quality control. Such work is generally published in the scientific press, and the process of publication almost invariably includes a process of peer review. Then, when published, other workers in the field attempt to verify and extend the original observations. This process, although retrospective, serves to control overenthusiasm in the original authors, enhance the authenticity of the observation, if it can be replicated by others, and allow the work to fade away if it cannot. These processes continue today, but their retrospective nature and protracted timescale can allow considerable waste of resource and exposure to risk, until the feedback mechanisms take effect.

More immediate assurance of the integrity of toxicological work would clearly be of advantage, and this need was vividly illustrated by the nature of some regulatory studies submitted to the US Food and Drug Administration (FDA), during the mid-1960s. The story is an interesting one, but the full telling is for another place. In essence, a chance observation of an anomaly between submitted dossiers by one of the FDA data integrity assessors led to a major investigation of the whole of the American regulatory testing business (US Food and Drug Administration, 1976). The original anomaly arose from error, oversight and endemic sloppiness in the testing laboratory, but the wider investigation revealed spectacular scientific fraud in some other organizations. Studies had not been conducted properly, or, in some cases, the work had not been done at all and 'results' were simply made up. There was a general lack of proper management and supervisory control, including poor data review, inadequate training and inappropriate

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equipment; combined with a tendency to under- or over-report to satisfy the perceived preference of the sponsor company. This all culminated in hundreds of instances in which it was considered that false statements had been made to the US Government agencies. Custodial sentences were imposed on the main players in the fraud, but a significant improvement in general control and quality was needed if the FDA were to reach proper decisions in future, and provide adequate protection for the populace. There was no choice: regulatory toxicology, subject to inherent commercial pressures as it inevitably will be, had to be brought to heel. Following a several-million-dollar investigation lasting over two years, the FDA published legislation for regulatory toxicology (US Food and Drug Administration, 1978) that demanded specific standards and controls. This was 'Good Laboratory Practice' (GLP). Although this US Federal Regulation directly affected US laboratories and did not extend beyond US territory, any foreign company submitting data to the FDA was also required to meet the same standards and attest to so doing, in writing; otherwise the FDA would not consider the submission.

2 GOOD LABORATORY PRACTICE

The FDA GLP regulations were designed to ensure that data presented to the Administration was sound and reliable, and so prevent a recurrence of the earlier problems. Given the nature, impact and extent of those problems (people were in jail and test laboratories had ceased business), the requirements were accepted by the US pharmaceutical industry relatively easily. Once this ground had been broken, other authorities followed. In 1983 the US Environmental Protection Agency published almost identical legislation, as did authorities around the world, notably in Japan, the Netherlands and the UK. Aware that a plethora of slightly different national GLP regulations might constitute a multifaceted barrier to trade, the Organisation for Economic Cooperation and Development (OECD) developed generic *Principles on Good Laboratory Practice* (OECD, 1998), which the agencies of all OECD member countries agreed to accept. The list of countries agreeing to accept the common standard is shown in **Table 1**, and this gives an indication of the power of this MAD (Mutual Acceptance of Data) agreement. This is essentially the situation today: although laboratories undertaking regulatory studies must comply with their domestic GLP legislation, the domestic law is invariably based on the OECD principles and is accepted, not only by OECD member countries, but by many others as well. As the number of OECD member countries grows and the new members are seen to meet the necessary standards, the extent of mutual acceptance of toxicology data also expands. Within each country, government-appointed inspectors visit laboratory premises and observe work in progress, examine records and reports, interview staff and monitor compliance with GLP laws. The outcomes of such inspections are widely available, so that the authorities can reject work from unsatisfactory laboratories, wherever in the world they may be based.

Table 1 Countries Mutually Accepting GLP Data Under the OECD Scheme (January 2008)

Argentina[a]	Australia	Austria
Belgium	Brazil[a]	Canada
China[b]	Chinese Taipei[b]	Czech Republic
Denmark	Finland	France
Germany	Greece	Hungary
Iceland	India[a]	Ireland
Israel[c]	Italy	Japan
Korea	Luxembourg	Malaysia[b]
Mexico	Netherlands	New Zealand
Norway	Poland	Portugal
Singapore[a]	Slovakia	Slovenia[c]
South Africa[c]	Spain	Sweden
Switzerland	Thailand[b]	Turkey
United Kingdom	United States	

[a]Provisional adherents.
[b]Observer status.
[c]Non-OECD member countries mutually accepted as adherent to OECD GLP.

What do the GLP Principles or Regulations demand? The requirements cover the way in which regulatory studies are planned, performed, monitored, recorded, reported and archived. For a full and authoritative account of these six elements the reader is referred to the text of the *OECD Principles* (or national GLP statute), but the official wording is sometimes vague, thereby permitting a variety of interpretations of detail by nations, even by individual inspectors. The basis of each of these six elements will be addressed here, but this account should not be the sole source of information for those intending to implement a GLP system. Read the rules, and speak to the relevant inspectorate. In the experience of the author, a constructive and helpful dialogue can often be had.

3 GLP REQUIREMENTS

3.1 Study Planning

Each individual study must be the subject of a written plan, agreed between those sponsoring the work and the individual responsible for coordinating the study events. In GLP terms, the latter person is styled 'study director'. Although a laboratory may employ several individuals in this role, at any one time there must be only one study director per study, acting as the single point of study coordination and control. The purpose of the plan is to ensure that no one involved is in any

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doubt as to the nature and extent of the study: one of the contributory causes of the error and oversight in the 1960s. This plan (or protocol) was initially perceived as one of the most difficult aspects to achieve in a scientific environment, because the future course of a study may depend on results obtained to date. However, regulatory studies are usually well defined, with clear end points and internationally agreed methodology. Plans for such studies can be prepared, sometimes by pasting the variables, such as the identity of the item under test, study schedule, and so on into the text of a standard method. Component technical procedures need not be specified in detail if they can be standardized in other documents held by the laboratory. These are defined as standard operating procedures (SOPs), and cover routine operations, such as use and calibration of equipment, preparation of reagents, animal husbandry, laboratory cleaning and so on. The regulation requires that the following points are addressed in each study plan—although addressing the issue might simply amount to stating that the item is not relevant to the particular study.

- Descriptive title
- Statement of purpose
- Identity of test item and any reference item
- Identification of the sponsor
- Location of test laboratory(ies)
- Identification of the study director
- Identification of any principal investigator(s) and their areas of responsibility
- Dated approval of the plan by the study director
- Proposed dates for starting and completing the study
- Reference to any relevant published test guideline
- Justification for the choice of test system
- Relevant characterization of the test system
- Method/route of administration of the test item to the test system, and justification for the choice
- Dose or concentration to be used, frequency or duration of application
- Experimental design, chronology, materials, methods and conditions
- Type and frequency of analysis, observations, measurements and examination.

Once agreed between the study sponsor and the laboratory, the plan is fixed and authorized by the dated signature of the study director. For such a document to be effective as an instrument of control on the scope and conduct of the study, the plan must be made available to all technical staff.

The plan may take on a significant level of complexity when several disciplines are required to work in harmony on a study, such as veterinarians, analytical and clinical chemists, pathologists and statisticians, and if some aspect of the study is located in another company or facility, especially if that location is in another country speaking a different language, the communication and drafting skills of the study director are tested indeed. In such cases, he/she may call on the services of one or more local GLP-compliant peers, styled 'principal investigators'. The extent of any such assistance is of course defined in advance in the study plan, and it is self-evident that principal investigators must maintain excellent contact with the study director, if the latter is to retain his/her position as a single point of study coordination.

The proposed study must be entered on the laboratory's 'master schedule' of studies. Historically a paper ledger, but nowadays usually a computerized database, this schedule displays the study identifier, study director, material under test, test type and key dates, often with other information useful to the laboratory in the management of work. The schedule must be kept current as the work proceeds, so providing a clear picture of the extent of work done, planned or in progress. The GLP purpose, however, relates to the quality monitoring of the test facility—as described later.

3.2 Study Performance

Each study must follow its study plan and the relevant SOPs, but the legislators recognized that toxicology studies do not always run on fixed tracks, and that changes of direction may be necessary as a result of emerging information. Such changes might include changes to dose levels, the addition (or removal) of observation time-points or appointment of a replacement study director. In some cases, it may be necessary to terminate a study early. Whatever the change from the originally approved plan, it must be formalized in a written amendment to the study plan, approved by the dated signature of the study director and distributed to all holders of the original document. The study may then proceed according to the plan as amended. In the course of a study there may be several such amendments, and to ensure that there is no doubt about the sequence of events, all must be sequentially numbered.

Life in the toxicology laboratory can suffer the same vicissitudes as anywhere else. The study that follows the plan (as amended) exactly, from start to finish, is relatively rare. A timed observation may be a few minutes late, controlled temperature may temporarily exceed defined limits, a proportion of samples or specimens may be lost in processing, specified equipment may cease functioning and be replaced. The list of potential deviations from plan or from SOP is endless, and few can be anticipated in an amendment. In this situation, GLP requires that the deviation is acknowledged in writing by the study director, and an assessment made of its impact. Clearly, the impact may range from negligible, to invalidation of the study; the purpose of formal documentation of deviations is to ensure that the individual best placed

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to perceive the widest consequences to the study (the study director), makes the most appropriate decision and is seen so to do.

Regulatory toxicology studies often involve the use of living test systems: animals, plants, cell cultures, bacteria. The reliability of the study may be compromised if the test population is diseased or not homogenous, so GLP requires that the source, strain and health of the test species are known. However impeccable the source, a period of quarantined acclimatization to laboratory conditions should be allowed, during which any latent disease may be manifest—rather than appear as a complicating factor or possible effect of treatment during the study. In the case of microorganisms or cell lines, specific tests may be necessary to verify the expected characteristics and lack of contamination. In each case, the choice of the test system must be justified in writing to minimize the risk of deliberate or accidental selection of an insensitive species, potentially resulting in serious underestimation of toxicity.

The study environment must be appropriate to the health of the species and assure a stable and protected platform for the study. The health of rodents supplied with adequate diet and water can be indicated by behavioural observation and weight gain: unicellular plants in log phase of growth are generally considered healthy. As test systems, both must be protected from external interference; allegedly, the ability of field mice to come and go in the rodent house did not help the integrity of one early study! Granada Television's 'World in Action' called 'Tried Untested'(Granada Television, 1981).

GLP requires equal attention to the integrity of the test item (substance or material under test). The study director must be sure that the test item is stable for the duration of the study, and will not degrade as the work proceeds—otherwise the test results may be misleading as to the toxicity of the item provided for test. Similarly, if it is necessary to administer the substance as a preparation in vehicle (water, oil, feed, etc.), tests must be done to assure the stability, homogeneity and achieved concentration of the preparation.

Substances are often much less stable in vehicle than as the supplied chemical, especially in liquid formulation, so the same concerns about the identity of the chemical causing any effects on the study apply as for the stability of the test item itself. Homogeneity is a concern in dietary mixtures and liquid suspensions, since it is necessary to ensure that all test subjects receive the specified dose (another early problem related to inadequate mixing of a test item in feed. Animals could have eaten the feed, but ignored the lumps of chemical).

An assessment of achieved concentration is required to verify the accuracy of the preparation process. Should any one of these aspects not be assured, it may be difficult for the study director to state, with confidence, that the effects observed related to the specified dose of the specified substance.

Weak, contaminated, inappropriate or expired reagents may also be a source of misleading results. The GLP regulations require that all reagent containers show the identity of the contents, storage conditions and expiry date, and, not unreasonably, state that expired reagents should not be used.

Equipment used in studies must be reliable and fit for purpose. Proper cleaning and maintenance to minimize the risk of breakdown, and calibration of measuring equipment against traceable standards may seem self-evident, but GLP requires, not only that this is done, but that it is seen to be done. Inspection, cleaning and calibration logs for many instruments are therefore mandatory, and the scheme for scheduling and recording these assessments must be described in SOPs. All this information relating to the status of equipment must be readily available to users, for as we shall see later, the users of measuring equipment are required to put their names to the results. The increasing use of computerized systems in regulatory studies has presented new facets to the equipment aspects of GLP compliance, in which extensive validation work may be required to demonstrate that systems perform as required—and continue to do so, following any modification or upgrade. (Here I should emphasize the word 'required'—it is not necessary to demonstrate that systems perform at their peak, or to manufacturer's specification; the needs of the individual laboratory must be determined, then the system must be assessed against those needs). Clear documentation of the laboratory's needs, the actions required to test system performance against those needs, the execution of those actions, the outcome of those actions and final management approval of the system as fit for use will all be required for a claim of GLP compliance.

Possibly the most significant contributors to the reliability of a study and its report are the personnel involved in the work—not just technical staff in the laboratory, but managers, data processors, maintenance engineers, clerical workers, animal husbandry staff, quality monitors and so on. GLP requires that all these staff are aware of their responsibilities and are appropriately qualified, trained, experienced and competent—although the determination of 'appropriately' and assessment of competence are left to facility management. Nevertheless, documentation such as job descriptions, curricula vitae (CVs—résumés) and records of training must be maintained and kept current.

All samples or specimens arising from the study and data records, whether paper-based or digital, must carry a unique study identifier. All raw data (records of original observations and activities, i.e. primary data) must be made promptly, legibly, accurately and directly, and be dated and initialled by the staff making the entry (see later). Electronic signatures and dates are acceptable, so long as they are as secure, and as legally valid as their

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handwritten equivalents. Changes to data are acceptable, so long as any change is itself signed, dated and carries a written explanation. These requirements arose from work in which data appeared to have been provided retrospectively by persons unknown, or in which records had been changed for no apparent good reason but, suspiciously, resulted in a better outcome for the study. Nowadays, the dates and signatures allow cross-reference to the competence records, so that it can be demonstrated, at any time in the future, that the persons making the observations and records were deemed competent by management, at the time that the work was done.

3.3 Study Monitoring

For toxicologists, perhaps one of the most bizarre concepts was that their work should be monitored, not for technical excellence, but for compliance with GLP; and not by their peers, but by an independent quality assurance (QA) function, reporting to management. Often seen initially as a group of interfering (and ignorant) nit-pickers, asking naïve questions and easily misdirected by scientific argument, this group of people not only picked over the data records and reports arising from studies, but they also intruded into laboratories to observe the toxicologist at work.

The background to this requirement justified the intrusion. The FDA investigation had revealed that some work reported to government agencies had been misreported, fraudulently manipulated or manufactured. Although the FDA had considered placing an Agency investigator in each facility to ensure integrity, this was over-burdensome (financially, at least) and the requirement was dropped in favour of an internal quality assurance unit (QAU). This unit monitors and evaluates all regulatory studies, but its purpose is to report outcomes to management. Management is ultimately responsible for the standards within a facility, including GLP compliance; the QAU acts as a management agent providing the information upon which management may take appropriate corrective and preventive action. For this reason, the members of the QAU report directly to management, and cannot be placed organizationally within any technical department working on regulatory studies. (There are exceptions for very small organizations, but these are uncommon and require very careful justification).

This principle of independence extends to the QA tasks themselves. Self-inspection is neither ideal nor particularly effective, and QA must take care not to become embroiled in technical procedure. In making their observations on quality and compliance, QA should not become part of the processes they are monitoring; they should be the metaphorical 'fly on the wall'. Although the subject of QA monitoring of study reports will be addressed later, one of the most likely ways for this principle to fail is when QA of study reports drifts into quality control (QC), so it is worth expanding somewhat on this danger now.

In principle, the study director's team should produce a draft study report that is perfect in all respects; then QA confirm to management that the system has worked well. In the real world of course, even the best draft will doubtless contain some transcription errors, typographical errors, ambiguity or obscurity—even though it may meet the requirements for a generally full and accurate account. The training and psychology of most QA auditors will not allow them to ignore presentational glitches—and why should it? The auditor, as much as the scientist, has an interest in the general quality of the report, the only part of the study that most people will see. So the auditor will bring the study director's attention to ambiguous phraseology, incorrect numerology and typographical fumbles. All well and good, but as this process is repeated in report after report, the study director may begin to rely on the QA process to pick up any errors, to the extent that the draft is no longer proofed before submitting it to QA scrutiny. Presto! Quality control, part of management's role of *ensuring* quality, is transferred to the *assurance* function; QA has become an integral part of the process and is on the brink of disqualifying itself as an independent GLP QA unit.

Over almost three decades, the scope of QAU activity has expanded into advisory roles, and the skills of the individuals within the QAU are often of value in customer contact and in assessing matters other than GLP compliance. When planning their work, many QAUs now use fairly sophisticated risk-analysis techniques, but the fundamental activity, required by legislation, is unchanged. They are required to inspect regulatory studies in progress, and to audit the reports emanating from those studies.

3.3.1 Study Inspection

All study QA inspection work is designed to assess the level of compliance: with the GLP regulations, with the plan for the study (protocol) and with the company's written procedures. Aspects of compliance that cannot easily be associated with a specific study may well be inspected separately on a facility basis; such aspects might include the operation of storage areas, maintenance of the SOP system, staff competence review procedures and cleaning or husbandry.

To ensure that all regulatory work in the facility is covered by the internal monitoring scheme, the QA inspectors will refer to the laboratory's master schedule of studies. From this, the inspectors are able to determine the type of each study, identify the study director and see when each study starts and ends. In this way, QA can ensure that an appropriate level of monitoring is applied to every piece of work intended for regulatory submission.

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During some early inspections, it was not uncommon for technicians to attempt to demonstrate their skill by performing tasks as speedily as possible. Unfortunately for them, QA were less concerned about the rate of pipetting, instead concentrating on whether the volume and identity of the material being dispensed agreed with the study plan, whether the containers were properly labelled, and the extent to which cross-contamination was controlled. Of course, excess speed can be associated with lack of care so technical enthusiasm often backfired, resulting in even closer scrutiny!

Further, there may have been a tendency for scientific managers to wheel out their best technicians for inspection, or QA inspectors could have been excused for thinking they had wandered onto the *Marie Celeste* when entering a laboratory for an arranged inspection. Such displays will be rare in an organization with well-established GLP and QA systems, and the QA inspector will be recognized as an essential and normal element of the study infrastructure. Study staff will recognize that the QA inspections lend credence to their work through independent observation of normal practice, and they act no differently whether QA are present or not.

The QA role is to assess compliance with written standards, not to make judgements on scientific merit. It is for management to decide, on receipt of the report from QA, whether (for example) an SOP should be updated to reflect better practice, or whether technical staff should be disciplined for failing to follow the official procedure—or both.

Quality control traps, similar to those mentioned previously for the audit of study reports, may be found in relation to the QA role in study inspection. The QA inspector that checks that all the sample labels are correct before the technical staff proceed does *their* job for them. QA should be concerned with assessing systems and processes, not directly checking every last item; QA should ask themselves whether there is evidence that someone else has checked every last item. Of course, there are circumstances in which a QA inspector must interfere: if a technician has picked up the wrong syringe and is just about to dose an animal with an incorrect material, it would be inappropriate for the inspector to silently observe the error, then write a report to management about it. And there is no harm in pleasantries such as holding a door or passing a towel—so long as the inspector does not lose sight of the potential danger of involvement.

3.3.2 Study Report Audit

In most QA units, the majority of effort goes into report audit, and the master schedule of studies is often used to identify study reports that are imminently available for attention, and so manage workloads. In essence, the audit procedure is intended to provide assurance to management that reports of toxicology studies are complete (all the study events are addressed; no actions or outcomes are suppressed), true (all the described events and outcomes are supported by study records), the study followed the plan (no planned activities omitted and no extra activities included) and that the report addresses all the required elements listed in the GLP Regulations. Usually, the review also includes an assessment of data quality, and an assessment of 'internal consistency'—that is, tables of individual data, group means or summary tables, graphical representations, textual accounts, summaries and conclusions are all in logical agreement. It is not difficult to see how such minute scrutiny can drift into quality control or issues of presentation!

To perform such an audit, QA must have access, not only to the draft report, but also to all the study records and supporting information. This will include the study protocol, records of study activities and results, receipt, characterization, dispensing, formulation and analysis of the substance under test; receipt, characterization and health records of the test species; environmental control, data processing; relevant correspondence, and so on, in whatever form (handwritten or machine output paper documents, computer databases, electronic files, photographs, chart output, specialist reports, dictation tapes, microscope slides, etc.). Records of reagent preparation, instrument calibration and staff competence may also be called for. The access to this material is direct and by right, not by gift of the study management. For example, QA staff will have personal, (normally read-only) access to computer data, rather than relying on the study staff to supply a printout.

The QA auditor's role is not executive. Although some detected errors may appear to be typographical in origin or easily and plainly corrected ('three samples' should be 'four samples'), the auditor should at most propose a change to the report, for ratification or rejection by the study director. For anomalies that are less clear-cut ('the reported result does not seem to be obtained using the equation specified in the SOP'), the auditor should neither recalculate the result according to the equation, nor assume that an alternative equation is relevant on this occasion. In all cases, the auditor should aim to display the anomaly and the study director should assess the issue, then make any appropriate correction to the report or explanation in the data, simply indicating to QA that the audit observation is accepted and resolved, or rejected.

From time to time, but hopefully rarely, the QA audit will reveal such a fundamental error that the study is invalid (say, an incorrect material had been tested or a key reagent was past its expiry date). In such a case the auditor will notify the study director immediately and not persist with the audit of that study. Much more commonly, subject to slight correction, study reports will be approved for issue and a Statement of Quality Assurance, listing the occasions on which the study

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was monitored and audited, will be signed by a QA representative.

3.3.3 External Monitoring

The company QA unit serving a toxicology laboratory will be very familiar with the processes and procedures in that laboratory, and will therefore be well placed to detect anomalies (and, with experience of individual technicians, even predict them!). However, despite the best intentions, over-familiarity with a single environment can restrict the auditor's ability to perceive; and in any case, the opinion of an internal auditor employed by the toxicology facility (directly or indirectly) will have limited influence on government regulatory bodies. Greater assurance is obtained by occasional monitoring at a higher (and even more independent) level, by government-appointed national GLP monitoring authorities.

Administratively, these government inspectorates occupy various positions in the national framework. In some cases, inspectors are associated with the regulatory bodies themselves, for example in the USA, Japan and Switzerland, in which each ministry has its own inspectors. In the Netherlands and the UK, a single inspectorate within the Department of Health monitors GLP compliance on behalf of all agencies. Elsewhere, autonomous or quasi-autonomous organizations may be accredited to inspect on behalf of government.

Whatever the legal framework, the GLP monitoring authorities of OECD countries make inspection visits to facilities wishing to enter the GLP programme; then annual, biennial or three-yearly short-notice routine surveillance inspections of GLP-registered test facilities, as well as extra inspections in relation to specific issues (for-cause). These inspections assess all aspects of facility operation in respect of GLP compliance, often using high-level documents such as site plans and master schedules to determine whether the facility has the capability to perform all the reported work. Inspection observations are reported to the laboratory management, who must indicate that appropriate action has been taken or is intended in respect of each reported deficiency. The inspectors generally reserve the right to verify such statements, but the final outcome results in the laboratory continuing on the list of compliant facilities, or removal. The current list of UK compliant facilities is maintained on the Medicines and Healthcare Producers Regulatory Agency (MHRA) web site (MHRA, 2008). The national GLP authority is also required to report the overall outcome to the OECD (and within the European Union (EU), to the European Commission). There is therefore considerable pressure on the test facilities to maintain their compliance status, since the report of the national authority to OECD is the basis of Mutual Acceptance of Data between OECD countries.

3.4 Study Recording

GLP addresses some features of the way in which study events and outcomes are recorded. Data should be recorded directly, promptly and legibly, entries should be signed and dated and any changes to the record must leave the original clear, be explained and themselves be signed and dated. Why?

The problems discovered in the 1970s included many data entries that had been obliterated and rewritten, without apparent justification, and which aroused the suspicion of the FDA review team. The laboratory managers were, in the main, unable to account for the changes. On judicial enquiry, it further transpired that other data had been entered after the study had finished, not by the study technicians, and clearly not at a time when the recorded observations could have been made.

Signing and dating by the technicians responsible for recording the data allows verification that these personnel were competent to make the observations (by reference to training and competence records) and indicates that the records were made at a time that the observations could have been made. To a large extent, these signatures and dates also limit the possibility of fraudulent generation of data purporting to reflect contemporaneous observations, since any such entries will be relatively easy to detect. Similarly, signatures and dates associated with changes to the records provide clarity, and associated with the explanation or justification and the ability to see the original entry, assurance that the change was made for good reason. This was not a new concept, but a new application; consider the process for correcting a written error on a bank cheque.

Prompt and direct recording is required to reduce transcription errors and enhance credibility. Observations committed to memory for later documentation must carry a degree of suspicion: 'It must have been number 21 that was immobile yesterday, because it's immobile today—or was it number 23 yesterday?'. The requirement for direct recording relates to the GLP definition of 'raw data'. These are not just unprocessed, unadjusted or uncorrected individual records, but the earliest records of events, observations or decisions. An interim note on a scrap of paper, which is destroyed after the information is later rewritten into the formal record, is not acceptable. The scrap of paper was the raw data: the only record that was not potentially open to mistranscription. It should have been retained—and signed and dated!

Identical requirements apply when data is captured and retained by a computer system. The person responsible must be identified (usually carried into the record from the user log-on information along with the date—and often the time), and changes must be written to the database in such a way that the new entry (with its new user id and date) logically overrides the original, but does not physically overwrite it. The ability of such a system to maintain a clear, complete audit trail covering possibly

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multiple levels of correction to a variety of types of data will be an essential element of system validation.

Original data may therefore take many forms, and the same data type may not be in the same form between laboratories. Paper records are used by almost all laboratories, even if only as backup in case of computer failure. Other forms might include photographs, sound recordings, machine printout as charts or tally-rolls, specimens, microscope slides, optical or magnetic discs, determined solely by data capture mechanisms. The list of possibilities is endless, the key issue is whether is this is the original, retainable record.

3.5 Study Reporting

The GLP Regulations require a full, written account of every study. The study director is responsible for the report and for signing a statement to indicate the extent to which the study complies with the Regulations. A properly designed and executed study in a well-managed facility will have few if any deviations from GLP, but the intent of the statement is to highlight any significant or systematic failures to comply. The occasional administrative error—such as a single missing signature in the data—would not constitute non-ompliance, but the routine omission of signatures by an individual, or throughout one section of the data, would do so. This is an area requiring judgement by the study director, often following advice from the QA department, but noncompliance does not necessarily imply that the study is not reliable. It is the responsibility of the study director to declare such shortcomings, and for the receiving authorities to make judgements on their impact.

The study director must consider his/her statement of compliance very carefully. In the UK, there are specific legal penalties (financial and custodial) for making, copying, holding or supplying a false 'Good Laboratory Practice Instrument' with the purpose of inducing a regulatory authority to accept it as genuine. The definition of such instruments includes the study director's statement, required in every report.

The previously mentioned signed statement of quality assurance monitoring for the study is also a required element. Other elements specified in the Regulation are:

- A descriptive title
- Identification of the test item by code or name (IUPAC, CAS number, biological parameters, etc.)
- Identification of any reference item
- Characterization of the test item including purity, stability, and homogeneity
- Name and address of the study sponsor
- Name and address of any test facilities and test sites involved
- Name and address of the study director
- Name and address of any principal investigators and the phase of the study delegated, if applicable
- Name and address of scientists having contributed reports to the final report
- Experimental starting and completion dates
- Description of methods and materials used
- Reference to OECD test guidelines or other test guidelines or methods
- A summary of results
- All information and data required in the study plan
- A presentation of the results, including calculations and determinations of statistical significance
- An evaluation and discussion of the results and, where appropriate, conclusions
- The location where the study plan, samples of test and reference items, specimens, raw data and the final report are to be stored.

In the event that a published report requires amendment for any reason, the amendment must be done in the form of an addendum, justifying the correction and clearly identifying the area affected. This parallels the principles of correction to data entries and amendment to study plans, that is, formal, justified, signed and dated documentation that does not obscure the original.

3.6 Study Archiving

A major difficulty encountered in the 1970 investigations was the absence of complete records. Whether the records had never been generated, or had been destroyed during normal housekeeping, simply mislaid or were deliberately hidden will never be known. Whatever the reason, significant parts of the data for many studies were not available, and therefore it was not possible for the FDA investigators to fully reconstruct the study events. As the events could not be reconstructed, there was no way of assessing whether or not the events were well controlled and accurately represented in the study reports. Therefore, the GLPs specify that all records supporting the study should be retained in secure archives.

The archive need not resemble a bank vault. GLP does not describe the physical nature of the archive, it simply specifies what is to be achieved. The size of an archive facility is wholly at management discretion, and will depend on the amount and type of material to be retained. The archive may be a building, a series of rooms, a single dedicated room, one or more filing cabinets or a desk drawer. It is the way that such facilities are operated and the assurance they provide that meets GLP standards, not the physical scale.

The archive must be 'secure'. Risks to security range from the accidental, through negligence to malice, and may originate with persons or nature. GLP defines some

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minimum features for security arrangements, but every company must assess the potential risks to its archive, and then incorporate policies, controls, protection and detection to the extent necessary in each individual case.

Company management is responsible for establishing GLP archive arrangements, for appointing a person in charge and for the safekeeping of the materials. This responsibility is critical. The archive must not be open to those that manage the studies or generate the data; this is a necessary security feature since it separates the keeping of the material from those who might be considered to have most interest in interfering with it.

Access to the archive area must be limited to the management-appointed archive staff, and the area must be secured whenever these are not present. Other persons may have legitimate reasons for access, such as maintenance engineers, government inspectors, potential sponsors or QA personnel, but archive staff should control all such visits by remaining present and keeping a record of the event.

The design and positioning of the archive should protect against leakage or other water damage, fire, seismic disturbance, malicious incursion and, in the case of magnetic records, dust and electromagnetic flux. Although many risks may be minimized by design, it will not be possible to eliminate them completely, so it will be necessary to monitor the storage areas. In any case, monitoring can demonstrate the effectiveness of the precautions.

If special storage temperatures are necessary (say for some types of specimens), the operating temperatures of fridges and freezers should be monitored and recorded, just as for any other such equipment in the laboratory areas. Infestation control may also be appropriate.

Although it is not a strict GLP requirement, many GLP-compliant facilities generate backup copies of archived material, and retain them in a separate location. In the case of paper or electronic media, this is particularly easy (in some respects too easy, since a plethora of copies may confuse the identity of the original or erode concern for its integrity). However, such measures can significantly enhance the security of the archived materials.

The named individual in charge of the archive (the 'archivist'), should have at least one named assistant so that the archive is routinely accessible for reference. Archive staff may have other responsibilities, but crucially, must not be involved in any way with the generation of GLP material to be archived.

Just like any other GLP area, the archive must be operated according to SOPs. These SOPs should address all archive procedures, including receipt, storage and eventual disposal of materials, as well as environmental control, staff training and emergency measures.

The whole point of a GLP archive is that the material must be readily available for authorized inspection. This has implications for the organization and indexing of materials whilst in storage. It also requires a formal system, whereby material can be brought out of storage for review, without compromising its integrity. Management is responsible for authorizing any access, which should be seen as a normal aspect of a working archive; technical staff, managers, quality assurance and clerks compiling registration dossiers will all have genuine need to refer to previous records.

GLP does not require that materials be retained forever, so the collection may be reviewed at intervals to allow the removal of unwanted material. Most national GLP regulations state that study material should be retained for a period relating to the notification, submission or registration of the material under test. Some national authorities define exact retention times, such as US EPA and Switzerland (10 years) or Germany (15 years). It can be difficult to establish the point at which material that is not specific to a particular study is no longer required, such as equipment calibration records or staff training records. Many companies retain such items indefinitely.

Even prior to their temporal retention limit, GLP allows disposal of specimens that cannot sustain evaluation, so specimens that are likely to have a limited life in storage may be assessed from time to time and any that have deteriorated beyond use may be removed.

In common with all other areas of a GLP facility, records should be retained for all archive activities, such as movement of records, training and competence reviews of staff and the dates and circumstances of visits by anyone else and the whole operation should be subject to regular review by QA.

3.6.1 Archive Contents

Any item that relates to the conduct of GLP work should be retained. This simple statement belies the variety of material that will encompass not only the study plan, amendments, data (in all its forms), specimens, correspondence and final report of every study, but all supporting material. This will include items such as personnel records, historical SOPs, equipment calibration and maintenance records, environmental controls, performance or validity checks on test systems, test item and test system receipt and distribution information, animal health and husbandry records, reagent preparation logs, quality monitoring records, master schedule of studies, the list is almost endless.

At management discretion, the archive may also retain other records, such as those from non-GLP areas, financial data, business contracts, submission dossiers and so on. However, to avoid compromising the integrity of GLP materials, identical standards of control and archive management must apply to this material as to GLP data.

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3.7 GLP Today

The basic requirements of GLP have not changed for decades. However, the adverbial nature of the requirements has allowed their application to study designs not originally envisaged. These encompass the increasing variety of nonanimal, *in vitro* or *ex vivo* alternative toxicology testing strategies without recourse to separate legislation or extended facility accreditation; although OECD has produced a guidance document (OECD, 2004) on the application of GLP principles to *in vitro* studies, the guidance can all be derived from the basic GLP principles. In the same way, GLP has extended successfully into areas such as agricultural field work, determination of physicochemical properties, subatomic spectroscopy analysis, direct capture and reporting with computerized systems and studies with biological test items.

Currently, several government agencies are beginning to take account of risk in their programmes of GLP monitoring, such that facilities with a long history of satisfactory inspection, performing standard, noncritical work may be inspected with less frequency or rigour than those with large volumes of immediately preclinical work. There is a growing body of interpretative advice published by national monitoring authorities (a reflection of the imprecise language of the OECD principles, and unfortunately not always enjoying international harmony in either scope or content). The frequency with which GLP deviations are found remains apparently high, even in facilities categorized as low risk. However, the high incidence of shortcomings generally relates to administrative matters, which in all conscience do not impact directly on the integrity of studies. This is often true even in the case of shortcomings categorized as 'major' deviations by the inspectorate, since even a major deviation (such as a lack of characterization information on the item under test) does not result in a misleading outcome so long as the circumstances are clearly reported.

4 NONREGULATORY TOXICOLOGY

Although there is no legal obligation for quality systems in nonregulatory toxicology, the inclusion of quality-based controls can be of significant benefit. In the early days of GLP, much was made of the cost to industry of implementation, but the passage of time has shown that early detection and resolution of issues can avoid very costly cumulative error. The same is likely to be true in nonregulatory work, whether in research or judicial process. The financial cost of pursuing a line of research arising from a miscalculation in an earlier result, or of a team of coworkers attempting to develop a discovery whilst misunderstanding the original procedure, could be great indeed. The cost of miscarriage of justice arising from erroneous conclusions presented in court could be devastating, not only to those wrongly convicted and their families/employees, but to the reputation of the legal system and the scientific community as a whole, not to mention the effect on society when the guilty are not brought to book. Sadly, science often has a poor reputation with the general population nowadays, possibly because scientific opinion has too small a voice when competing with politicians and the mass media.

For all the above reasons, any aspect of toxicology would be well advised to instigate some form of quality control. If further encouragement is needed, ask any investigator whether they would be confident that they could repeat an observation they made five years ago, and obtain the same result (within experimental error, or course!). Would they stake their career on it? Aren't they, in effect, doing just that anyway? What is it about the human condition, that allows us to repeat a rushed piece of work 'properly' but resists doing it 'properly' in the first place?

The value of quality systems in nonregulatory toxicology is recognized by organizations such as the International Association of Forensic Toxicologists, the British Association of Research Quality Assurance, the American Academy of Forensic Sciences and the European Society for Toxicological and Forensic Chemistry. All of these organizations have produced guidelines or standards to help enhance the status of nonregulatory toxicology—and minimize the occurrence of unreliable data and the bad publicity that inevitably follows. Poor practice will out, eventually, as an American State Patrol toxicology laboratory discovered. A panel of District Court judges ruled in 2008 that the laboratory's alcohol analyses should not be admitted in trials, because they were so unreliable. The problems included selective reporting and rejection of results, mix-ups in sample identity and malfunctioning software. As a result, the judges wrote, there was the possibility that 'the innocent may be wrongly convicted and the guilty may go free'. This related to the determination of a common, small hydrocarbon in body fluids. If this cannot be done reliably, what of more complex techniques such as DNA analysis or screening of athletes for performance-enhancing substances? Errors in forensic toxicology may not directly affect such a potentially worldwide population as inappropriate marketing permits for chemicals, but they do have significant influence on the outcome of judicial process. Any defined quality system will enhance the authority and credibility of results, assisting juries and disciplinary panels with their conclusions and helping assure the population at large that judgements are soundly based.

Much common ground exists between the various statements, policies and codes with respect to scientific quality. For example, the contractual agreement required between funding bodies such as the Biotechnology and Biological Sciences Research Council, Department for

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Environment, Rood and Rural Affairs (DEFRA), Food Standards Agency (FSA) and the Natural Environment Research Council and their contractors, (Joint Code of Practice for Research), covers the same major elements as ISO 9000, national accreditation schemes such as United Kingdom Accreditation Service (UKAS) and GLP. Such commonality surely indicates the fundamental nature of the benefit of the component requirements. Detailed mechanisms differ: some schemes accredit specific techniques, others relate to management systems under which a laboratory operates. Some schemes involve regular interlaboratory comparisons of standard samples to assure that test sensitivity is similar (and therefore more likely to be fundamentally accurate); most involve external and internal audit. Documented calibration of measuring instrumentation traceable to national standards is routine, as are documented validation exercises to demonstrate that computerized systems do, in fact, do what the toxicologists require and expect of them.

Take the inevitable bureaucracy out of these systems, and you are left with common-sense, fundamental precautions. Where there is no statutory requirement to implement a formal system, toxicological investigations of any type carry increased credence if they can be associated with a demonstrable quality scheme, however basic. This can give comfort and confidence to governments, the judiciary, toxicological researchers and the public in general.

Quality research—done by knowledgeable people, thought through, with all circumstances clearly documented, all observations accurately recorded and reported in an unbiased manner and double-checked—isn't that a concise summary of the requirements for regulatory toxicology? Toxicologists owe it to themselves, their profession, society and science to do no less.

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Animal Welfare in the Toxicology Laboratory

Roy C. Myers and Bennett J. Varsho

CONTENTS

1 INTRODUCTION

Justification for proper care and humane treatment of animal models should be obvious. From a moral standpoint, it contradicts our sense of decency to cause pain and suffering in another species for no real benefit (see **Ethical, Legal, Social and Professional Issues in Toxicology**). Researchers must develop a measure of respect for laboratory animals and fully realize they feel pain and have basic needs for sustaining good health. Beyond moral issues, it must be remembered that animal welfare is crucial for good science. Animals experiencing pain, stress or poor health do not make good test subjects. One of the basic precepts of science is that a test group must contain a minimal number of variables other than those purposely introduced (such as a chemical dose for assessing biologic response). Subjects must be uniformly healthy and stress-free; otherwise, results are suspect and interpretations are difficult, if not impossible.

Animal researchers feel the mounting public scrutiny of their work from the media, the government and various animal advocacy groups. Laboratories are frequently targets of protests and other forms of negative publicity, with accusations of mistreatment or even torture. In some cases, animal rights activists break into laboratories to cause damage and/or to 'liberate' the research animals (see **Ethical Issues Related to Animal Welfare Considerations in the Toxicology Laboratory**). Obviously, all researchers must strive to avoid even a hint of animal abuse. A final reason to be concerned about animal welfare is direct and critical—it is the law. Most nations and international governing bodies clearly prohibit animal misuse. As will be seen in subsequent sections, there are specific requirements regarding the care, treatment, and disposition of laboratory animals. Failure to obey the laws can result in loss of licenses, rejection of studies and even fines or imprisonment. These all are powerful inducements for the proper use of animals.

2 BRIEF HISTORY OF ANIMAL WELFARE

Animal welfare and antivivisection activities were relatively common during the nineteenth century in the UK (Johns Hopkins Center for Alternatives to Animal Testing, 2008a). By 1876, the British government was pressured to pass the Animal Cruelty Act. In more recent times (1979), the Farm Animal Welfare Council

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developed guidelines for animal use that became the 'Five Freedoms' (Wikipedia, 2008):

1. Freedom from thirst, hunger, and malnutrition
2. Freedom from discomfort due to environment
3. Freedom from pain, injury and disease
4. Freedom to express normal behaviour for the species
5. Freedom from fear and distress.

Political and social pressure to prohibit animal research also developed in the nineteenth century in the USA (Johns Hopkins Center for Alternatives to Animal Testing, 2008b). In 1866, the American Society for the Prevention of Cruelty to Animals was established. Numerous bills prohibiting animal research were considered by various legislatures between 1896 and 1900, but were all defeated. The advent of World War I and the growing enthusiasm for medical research tempered the opposition to animal use somewhat over the next several decades. However, a magazine story (Stan Wayman, Life Magazine, 1966) documented the poor living conditions for some dogs used in research. The US Congress, flooded with mail demanding controls on animal research, responded by passing new legislation (Laboratory Animal Welfare Act, 1966) which required that research animals receive humane care and treatment. Under this act, standards for care, treatment and transportation of animals became the responsibility of the US Department of Agriculture (USDA).

3 ANIMAL WELFARE LEGISLATION AND POLICY

Beginning with the Animal Welfare Act of 1966, several laws and regulations have been enacted in the USA to protect animals. Policies in other nations and international agreements are considered to be similar in content to the US policies. Examples are found in the *Guide to the Care and Use of Experimental Animals* published by the Canadian Council on Animal Care (CCAC; Canadian Council on Animal Care, 1993), guidelines produced by the Australian and New Zealand Council for the Care of Animals in Research and Teaching (ANZCCART; Australia and New Zealand Council for the Care of Animals in Research and Teaching, 2008), *Guidelines for Proper Conduct of Animal Experiments* published by the Science Council of Japan (2006), and so on. An effort to develop worldwide principles for laboratory animal use began in 1983, resulting in the *International Guiding Principles for Biomedical Research Involving Animals*, published in 1985 by the Council for International Organizations of Medical Sciences (CIOMS) (Association for Assessment and Accreditation of Laboratory Animal Care International, 1997a). The system in the UK is defined by the Animals (Scientific Procedures) Act of 1986. This law requires researchers to obtain a personal licence, a project licence and a certificate for the research facility. Whenever new research techniques are proposed, amended licences must be obtained (Association for Assessment and Accreditation of Laboratory Animal Care International, 1997b). Especially important in Europe is the European Directive 86/609 adopted by the Council of the European Communities (now the European Union) in 1986. This directive is fairly comparable to the US Animal Welfare Act (Association for Assessment and Accreditation of Laboratory Animal Care International, 1997c). More recently, the Council of Europe (separate from the European Union) published new guidelines for animal welfare, *Guidelines for Accommodation and Care of Animals* (Council of Europe, 2006), which are similar to those in the guide issued by the US National Research Council (discussed later in this chapter).

3.1 Animal Welfare Act

The Animal Welfare Act of 1966 (Public Law 89–544) has been amended several times and is administered by the US Department of Agriculture (1995). Within the USDA, the Animal and Plant Inspection Service (APHIS) enforces the Act (US Department of Agriculture, 1996). Recent amendments to the Act generally deal with issues not related directly to toxicology or they provide better definitions of animals to be excluded (US Department of Agriculture, 2008). The Act provides standards for animal husbandry, veterinary care, exercise (dogs) and psychological health (primates). Specifically excluded are rats and mice (by far the most commonly used research models), farm animals not specifically used for research and birds. To the extent possible, however, the conscientious investigator attempts to apply the principles of the Act to rodents during toxicological research. Researchers are required, under specified circumstances, to administer anaesthesia or analgesics to minimize distress. Unnecessary research or duplication of animal testing is prohibited. A very important aspect of the Act is the formation of an Institutional Animal Care and Use Committee (IACUC) at each facility to oversee animal use. The IACUC must include three or more members, one being a veterinarian, another being an individual not affiliated with the facility and others being from a variety of backgrounds, scientific and nonscientific (US Department of Agriculture, 1995). Study management personnel, such as study coordinators or study directors should be encouraged to serve on the IACUC to gain insight into animal welfare issues. It is often recommended that the 'outside' person be a nonscientist, possibly an ethicist or educator. The IACUC makes sure that the facility complies with the Act through

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inspections (every six months), reviews of test protocols and formal reporting. One often-overlooked mandate of the Act is that researchers and facilities 'endeavour to build public confidence in animal research'. In other words, they should be open and truthful about the importance and practice of animal research.

In considerable detail, the Act specifies animal care conditions, promoting a healthy and stress-free atmosphere. These cover housing, ventilation, lighting, temperature, interior surface composition, caging, sanitation, pest control, feed and water, record keeping, veterinary care, training, handling and security. Specially trained APHIS inspectors (veterinarians or animal health technicians) are given full access to all areas where regulated animals are kept. Any deficiencies discovered by the inspectors are made known to laboratory management along with deadlines for correction. Failure to address the deficiencies adequately could result in suspension of the facility's animal licence or in legal action.

3.2 Health Research Extension Act

Passed in 1985 (P.L. 99–158) and implemented in 1986, the Health Research Extension Act has resulted in publication of the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals (Office for Protection from Research Risks, 1996). All facilities involved in PHS-sponsored projects must comply with this policy. Administration of the policy is the responsibility of the Office for Protection from Research Risks (OPRR) at the National Institutes of Health (NIH). Failure to comply results in a loss of NIH funding. In 2000, the OPRR Division of Animal Welfare was moved from OPRR, becoming the Office of Laboratory Animal Welfare (OLAW) within NIH.

Like the Animal Welfare Act, the Health Research Extension Act details the requirements for facility maintenance, employee training, handling techniques, veterinary care and record keeping associated with animal research. Highlighted are the IACUC, certification of the facility and compliance with the PHS *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996). The Guide, considered by most researchers as the definitive source for animal welfare instruction, will be described more fully later. Also emphasized is the appropriate use of analgesia and anaesthesia, together with methods of euthanasia consistent with those originally recommended by the American Veterinary Medical Association (AVMA) Panel on Euthanasia, 1993) and by the more recent AVMA Guidelines on Euthanasia (American Veterinary Medical Association, 2007). The PHS Policy is summarized by nine US Government Principles (driven by the Guide) which must be agreed to through submission of an 'assurance' of animal welfare prior to initiation of the study. The testing facility must be fully evaluated by an appropriate IACUC and/or accredited by the AAALAC International.

3.3 Good Laboratory Practices

With the adoption of the US Food and Drug Administration (FDA) Good Laboratory Practices (GLPs) regulations (US Food and Drug Administration, 1978; 1999), animal care and use considerations have become part of an overall plan for the conduct of scientifically sound studies. Basically, research under FDA standards must comply with the Animal Welfare Act and with animal treatment conditions recommended by the Guide. GLP requirements for detailed standard operating procedures (SOPs) and a quality assurance unit review also help ensure appropriate attention to animal welfare. Other agencies in the USA followed suite, with the passing of GLP regulations under the US EPA (Environmental Protection Agency) Toxic Substance Control Act (US Environmental Protection Agency, 1989a) and the US EPA Federal Insecticide, Fungicide and Rodenticide Act (US Environmental Protection Agency, 1989b). The US EPA revised animal-related GLPs in 1997 (US Environmental Protection Agency, 1997). Also providing guidance on basic animal welfare are several national or international GLPs such as those from the Organization for Economic Cooperation and Development (OECD; Organization for Economic Cooperation and Development, 1981; 1998).

3.4 Specific Testing Guidance

Study guidelines by the US EPA, FDA and OECD and many other agencies contain recommendations on the conduct of tests in a manner reflective of animal welfare. They especially promote euthanasia of distressed or moribund animals, limits of numbers of animals used, nonanimal screening and employment of available published data. As testing guidelines are updated, the emphasis on animal welfare issues typically increases.

4 ANIMAL WELFARE IN PRACTICE

It should be clear at this point that it is ethically and legally important to conduct animal research with as much attention to animal welfare as is practically possible. There are some very specific practices to achieve this, both from a standpoint of basic animal care and from the standpoint of humane research with animals. Responsibilities fall upon all personnel involved: institutional management, the investigator, technical staff and support staff (veterinary

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to maintenance). All must be committed to the proper treatment of animals and to maintaining a suitable environment.

4.1 Animal Care

It would be impossible to discuss all principles of animal care in detail within this chapter. Instead, some basic concepts, as presented in the 'Guide' (National Research Council, 1996) and the Animal Welfare Act (US Department of Agriculture, 1995), are summarized below. An outline of the essential animal care considerations is given in **Figure 1**. Further information may be found in the *Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research* (Institute for Laboratory Animal Research, 2003). It should be emphasized that all phases of animal care must be clearly detailed in facility SOPs with appropriate management review and approval.

4.1.1 General Animal Environment

In general, facilities used for animal research should provide for separation of animal rooms from those used for administrative purposes (offices, conference rooms, supply rooms, etc.). Moreover, when possible, animals should be housed in areas isolated from laboratories where tests are conducted. Such isolation permits

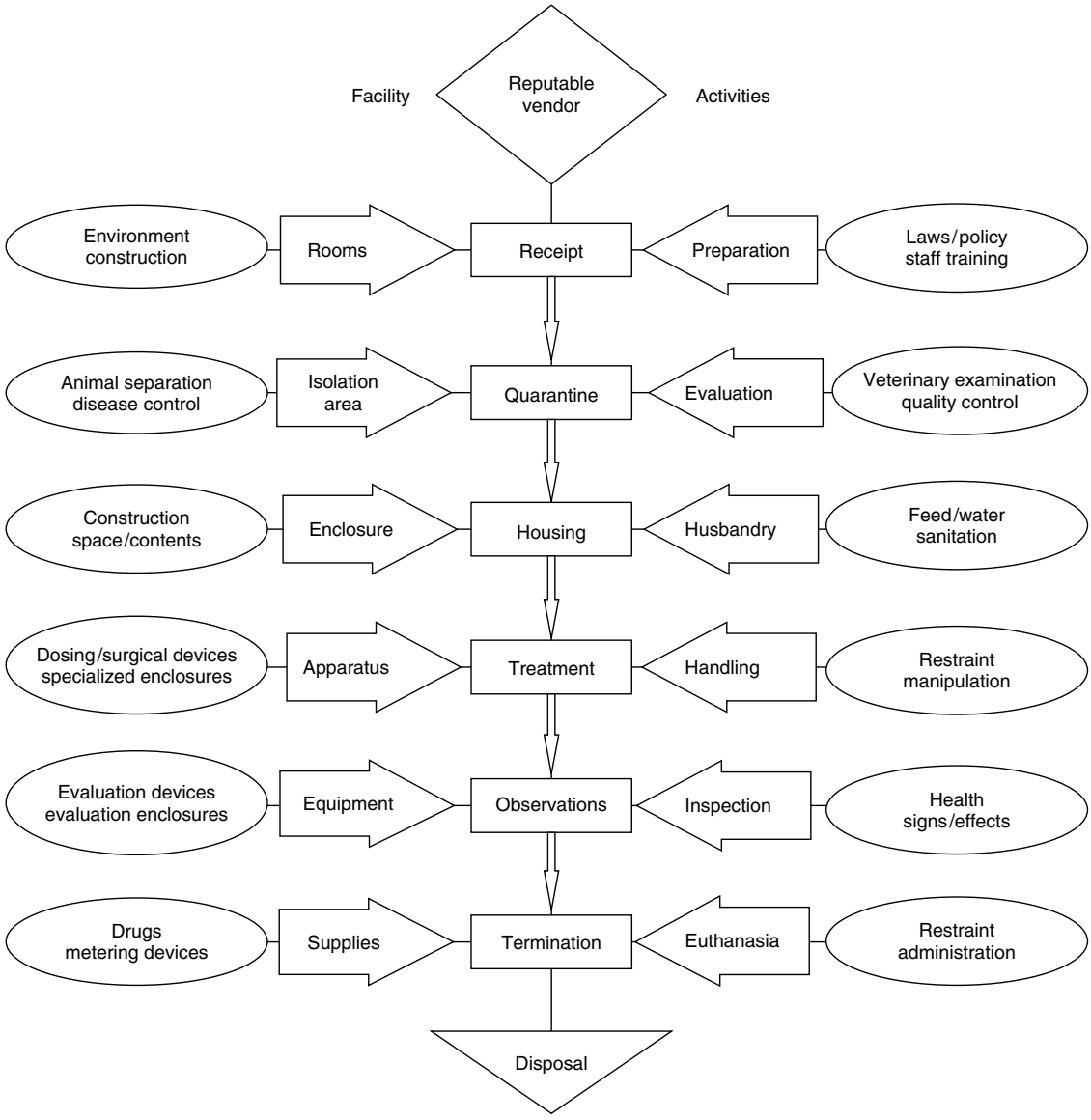

Figure 1 Essential considerations for animal care in the toxicology laboratory are shown diagrammatically. The central core includes the basic activities involving animals during their presence at the laboratory. Facility considerations and influencing factors are given on the left; animal care processes and influencing factors appear on the right.

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minimal stress and confusion for the test animal. Surfaces within animal rooms should be resistant to moisture, vermin, cleansers and deterioration from frequent scrubbing. Porous surfaces and wooden surfaces, which allow collection of dirt and microorganisms, must be avoided. Exposed plumbing, ductwork and lighting fixtures must be minimized. Animals must be obtained from only reputable vendors or bred under carefully controlled conditions within the facility. Newly received animals should be quarantined until their health status can be fully determined.

There should be space dedicated to sanitation of caging and supplies, storage of feed and bedding, performance of specialized animal treatment (dosing, surgery, necropsy, etc.) and storage of scientific apparatus and supplies. Humidity in animal rooms should be relatively consistent and controllable within a range of 30–70%, while temperatures should be appropriate for the species housed and controllable within $\pm 1\,^{\circ}C$. Room lighting should be regulated by a timing device to provide a uniform diurnal cycle (typically 12 hours of light and 12 hours of darkness). Appropriate monitoring systems (recording devices and possibly alarms) should be used to ensure proper temperature, humidity and light cycles are maintained. There must be adequate air filtration to control the spread of dust and pathogens, and provisions must be made for temporary ventilation in the event of a mechanical failure. To avoid excess noise, rooms should be constructed of sound-limiting materials. Moreover, sounds generated by personnel and equipment should be kept to a minimum.

If animal surgeries are to be performed, there should be adequate isolation from sources of contamination. It is suggested that separate areas be provided for support materials and equipment, prepping, surgery and recovery. The recovery area should permit adequate observation of treated animals, as well as provide for the comfort of the animal (low lighting, reduced noise, special bedding, etc.).

4.1.2 Immediate Animal Environment

Adequate control of the immediate surroundings is crucial to animal research projects. Primary enclosures (cages, boxes, pens) must provide for basic needs such as feeding, maintenance of body temperature, moving, urination/defaecation, access to adequate airflow and resting. They must be constructed of materials with a smooth, impervious surface that resists rust and damage from frequent handling. Their design must permit animal observation, efficient changing of feed and water, and ease of cleaning and sanitation. Standard caging, especially for rodents and rabbits, typically is constructed of stainless steel with a mesh floor (although some researchers feel that a solid bottom with bedding is preferable). The wire mesh bottom permits animal waste to fall onto a collection tray underneath (**Figure 2**). A solid bottom (using plastics or vinyl-coated metal) may be more appropriate for larger animals. Some housing systems, for special types of research, involve closed boxes or cages with independent ventilation and air filtration systems. They also may have integral urine and faeces collection systems. Very large research models, such as sheep, goats, horses or cattle, may require barns, corrals or pastures. The basic needs for these animals will be similar to those of smaller research subjects, but with appropriate adjustments specific to each species.

Figure 2 A typical animal housing area is shown here. Note that the animal carriers are clean, well organized and well labelled. The cages are made of stainless steel with a mesh floor to allow waste to fall onto the collection trays (with special absorbent paper) underneath. (Photograph courtesy of WIL Research Laboratories, LLC.)

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Table 1 Recommended housing conditions for selected laboratory animal species[a]

Species	Housing type[b]	Size	Minimum cage/pen floor area (per animal)	Minimum cage/pen height (cm)	Room temperature (°C)
Mouse	Group	10–15 g	39–52 cm^2	13	18–26
		15–25 g	52–77 cm^2	13	18–26
		>25 g	>97 cm^2	13	18–26
Rat	Group	<100 g	110 cm^2	18	18–26
		100–200 g	110–148 cm^2	18	18–26
		200–400 g	148–258 cm^2	18	18–26
		400–500 g	258–387 cm^2	18	18–26
		>500 g	>452 cm^2	18	18–26
Hamster	Group	60–80 g	65–84 cm^2	15	18–26
		80–100 g	84–103 cm^2	15	18–26
		>100 g	>123 cm^2	15	18–26
Guinea pig	Group	<350 g	387 cm^2	18	18–26
		>350 g	651 cm^2	18	18–26
Rabbit	Single	<2 kg	0.14 m^2	36	16–22
		2–4 kg	0.14–0.27 m^2	36	16–22
		4–5.4 kg	0.27–0.36 m^2	36	16–22
		>5.4 kg	>0.45 m^2	36	16–22
Rabbit (nursing)[c]	Single (plus litter)	2–4 kg	0.46 m^2	36	16–22
		4–5.4 kg	0.56 m^2	36	16–22
Cat	Single	<4 kg	0.27 m^2	61	18–29
		>4 kg	>0.36 m^2	61	18–29
Dog	Single	<15 kg	0.72 m^2	Species–specific[d]	18–29
		15–30 kg	1.1 m^2	Species–specific[d]	18–29
		>30 kg	>2.2 m^2	Species–specific[d]	18–29
Monkey	Single	<1 kg	0.14 m^2	51	18–29
		1–10 kg	0.14–0.39 m^2	51–76	18–29
		10–25 kg	0.39–0.72 m^2	76–91	18–29
		25–30 kg	0.72–0.90 m^2	91–117	18–29
		>30 kg	>1.4 m^2	117	18–29
Ape	Single	<20 kg	0.90 m^2	140	18–29
		20–35 kg	0.90–1.4 m^2	140–152	18–29
		>35 kg	>2.3 m^2	213	18–29
Poultry[e]	Single	<0.25 kg	0.02 m^2	—[f]	16–27
		0.25–1.5 kg	0.02–0.09 m^2	—	16–27
		1.5–3.0 kg	0.09–0.18 m^2	—	16–27
		>3.0 kg	>0.27 m^2	—	16–27
Sheep/goat	Single	<25 kg	0.9 m^2	—	16–27
		25 to >50 kg	0.9–1.8 m^2	—	16–27

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Table 1 (*continued*)

Species	Housing type[b]	Size	Minimum cage/pen floor area (per animal)	Minimum cage/pen height (cm)	Room temperature (°C)
Swine	Single	<15 kg	0.7 m^2	—	16–27
		15–50 kg	0.7–1.4 m^2	—	16–27
		50–200 kg	1.4–4.3 m^2	—	16–27
		>200 kg	>5.4 m^2	—	16–27
Cattle	Single	<75 kg	2.2 m^2	—	16–27
		75–350 kg	2.2–6.5 m^2	—	16–27
		350–650 kg	6.5–11.2 m^2	—	16–27
		>650 kg	>13.0 m^2	—	16–27
Horse	Single	Adult	13.0 m^2	—	16–27

[a]Recommendations for primary enclosure from the 'Guide' (National Research Council, 1996); larger animals may also require secondary enclosures for exercise, mating, or other activities.
[b]Typical preference for housing; group-housed animals are frequently single-housed according to study protocol.
[c]Space recommendation from the Animal Welfare Act (US Department of Agriculture, 1995).
[d]Recommended cage height for dogs is 6 inches above the head during normal standing position.
[e]Based primarily on the chicken.
[f]Height recommendation not specified; allowance should be made for typical postures.

Space recommendations are proportional to size, but are also based on expert judgement. These are detailed in the 'Guide' and summarized in **Table 1**. Attention must be given not only to floor area, but also to enclosure height. Volumes should be adjusted to account for special needs (mating animals, pregnant animals, nursing animals, etc.) and for addition of feed/water containers or other objects. Moreover, required volumes for singly housed animals may differ from each animal housed in a group. It is essential that temperature be maintained within species-specific limits (**Table 1**). Extreme temperatures (above 29 °C or below 4 °C) may cause clinical effects, including death. Relative humidity is not as critical, but for the sake of animal comfort and wellbeing, should be controlled to a range of 30–70%.

Animal enclosure ventilation provides a supply of breathing air, temperature control and removal of odours and contaminants. Under normal conditions, 10–15 air changes per hour are considered to be adequate. Special conditions, such as a large population density, presence of noxious gases, excessive heat generation by equipment or lighting, or potential air contamination with toxic materials, would require upward adjustment of airflow. If the primary enclosure system contains its own ventilation and filtration system, the room airflow could be adjusted downward if so desired.

Lighting in an animal area must be carefully controlled, because factors such as intensity, duration, wavelength and schedule can affect the physical and behavioural wellbeing of research subjects. Lighting should be diffuse throughout the housing area. The photoperiod should reflect each species' natural diurnal rhythm. For example, rodents are nocturnal and will feed mostly during darkness. They will maintain regular feeding patterns when lights are properly controlled. Moreover, rats are susceptible to phototoxic retinopathy; therefore, intensity should be maintained at 130–325 lux, according to the 'Guide'.

Many research animals are easily stressed by excess noise. They should be isolated from loud equipment, other animals (especially dogs, swine or nonhuman primates), or human activity that produces intense, persistent, frequent or high-frequency sounds. Technical staff should be discouraged from loud talk, excessive banging of equipment, playing of radios and excessive use of cell phones or pagers. Animals that typically rest during daylight hours may be severely disturbed by such activities.

Some species may require special features in their primary enclosures. For example, cats prefer a raised resting area within their cage. Other species (notably higher species such as nonhuman primates) require perches, tunnels, swings, toys or foraging devices. These permit natural behaviours that, in turn, enhance the animal's physical and psychological health. Animals such as dogs should have frequent access to runs or large pens where they can exercise. Many species (dogs, cats, primates) may benefit from frequent contact with humans. Consideration should also be given to an animal's natural social environment. For many species, individuals may communicate with other individuals through tactile, visual, auditory and olfactory contact. Therefore, whenever not otherwise required by the testing protocol, such animals should be housed in groups

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or within a short distance of fellow members of the species. This may best be exemplified by the rat, which typically huddles with other rats during rest periods and tussles with its cage mates during active periods.

4.1.3 Animal Husbandry

All research subjects must be provided nutritious, uncontaminated feed specific for the test species. Supplies of feed should be chosen carefully with special attention to feed content, methods of storage, vermin control and notations of manufacture date and shelf life. Analytical reports should be available to the researcher, who should review them for nutritional content, as well as the presence of contaminants that may cause unwanted physiological responses. Feed must be stored in a dry, cool, clean area. It should be elevated from the floor using pallets, shelves or carts. Most feeds can be used for approximately six months after manufacture, although some special diets, such as those containing vitamin C supplements, may expire after just three months. Out-of-date feed should always be promptly discarded.

Feed containers should permit unimpeded access to their contents and should discourage contamination with animal waste. They must not be transferred between animals without being sanitized. In many instances feed should be restricted. For example, young rabbits received from a feed-restricted environment and placed into an *ad libitum* environment may initially overeat, develop diarrhoea and then stop feed intake. These animals become severely anorexic if not treated and may have to be euthanized. A useful practice is to restrict feed intake at first, gradually permitting *ad libitum* feeding. In some instances, intubation with fruit juice will stimulate normal feed patterns. Provision of a leafy vegetable (such as kale) may also help the rabbit regain its appetite. There have been several studies (quoted in the 'Guide') indicating that unrestricted feed consumption will lead to obesity, shortened life spans and increased disease (including cancer). These are undesirable conditions in long-term research projects. Some species thrive on a variety of diets and on special additions like fresh vegetables or grains.

Like feed, water supplied to animals must be palatable and uncontaminated. Water should be analysed by the facility or the supplier for contaminants, microbes and pH. Investigators should review reports from these analyses to ensure that the water is of sufficient quality. Water can be provided by individual bottles, pans or tubes, although automated systems may be preferable. For any system, the apparatus should be examined daily for proper operation and cleanliness. Automated systems should be flushed frequently, perhaps daily, and subjected to appropriate filtration and frequent sanitation. Some species may require training before they can learn to use the watering systems, usually by pressing the tips or levers to permit water flow and then holding the animal's mouth to the fluid. Care must be taken to avoid excess pressure in water systems for small animals such as mice. They may be unable to press the release devices and will quickly dehydrate as a result.

Choice of animal bedding can have a considerable effect on animal wellbeing. It should not have strong odours (for example, cedar chips can induce enzymatic and cytotologic changes) and should not produce excessive particulates. Bedding can consist of wood chips or other granular materials. Frequently, it is appropriate to place specially developed paperboard in trays under caging to collect waste (**Figure 2**). Such paperboard often minimizes odours and contains components that reduce microorganism growth. Like feed, bedding must be stored in a dry, clean area with elevation from the floor. The requirement for bedding change depends on the species, but typically varies from once a week to once a day. For rats and mice, at least, three changes a week is recommended.

Cleaning and sanitation of cages, racks and feeders should occur at least every two weeks for typical research animals. Devices such as solid-bottom cages and water containers should be sanitized every week. Primary enclosures should be treated with hot water (typically at 80 °C) and/or chemical disinfectants. Water and feed containers must be treated with hot water and detergents that kill microorganisms. All room surfaces must be cleaned and disinfected to prohibit disease. Special commercial solutions can be used to scrub floors and walls; they can also be used in room foggers. The effectiveness of sanitation should be monitored through analysis of wipe samples for the presence of bacteria.

Another method of controlling disease includes incineration of animal carcasses and waste. Recently, other methods for waste disposal have been developed, including use of a tissue digestor (containing potassium hydroxide, heat and pressure). Pesticides may be used to control vermin present outside the facility and in some areas inside. If chemical pest control is not practical or desirable, traps may be more appropriate for eliminating rodents.

4.1.4 Animal Identification

A discussion about animal identification might seem to be beyond the scope of this chapter, but there are considerations that have animal welfare implications. All research animals must be carefully tracked throughout the course of their presence at the research facility, from arrival to euthanasia and disposal. Failure to do this effectively could result in confusion in study data and possibly the need to repeat a portion (or all) of an animal research project (and excessive use of animals). Part of the tracking process involves the appropriate labelling of animal cages, pens, stalls or rooms with specific animal data (usually a unique assigned animal number or code, sex, possibly the source and strain of animal,

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study identification, treatment group, etc.). Labelling can be accomplished through use of printed cards, bar-coded stickers or some sort of electronic transponder.

According to the 'Guide', unique direct animal identification may include collars, bands, plates, tabs, coloured stains, ear notches or tags, tattoos, subcutaneous transponders and freeze brands. Toe-clipping (removal of certain toes according to a designated numbering code) for identification of small rodents is discouraged and only to be used whenever other methods are not feasible (and with approval of the facility IACUC). This method, if absolutely required, should be limited to altricial neonates and, along with other invasive procedures (ear notching, tattooing, subcutaneous implantations), must be done as painlessly and aseptically as possible. Veterinary guidance about proper technique and possible use of analgesics, anaesthetics or antiseptics should be considered.

4.1.5 Veterinary Care

A qualified veterinarian (certified and experienced in laboratory animal medicine) must be available on site or on call to provide oversight of animal care and handling. This includes direct or indirect responsibility for administration of veterinary drugs, euthanasia and surgical procedures. The veterinarian should ensure that laboratory animals are healthy as received (free of injury or disease) and that proper care is provided while the animal remains in the research facility. Any health status reports provided by the vendor or generated by the facility should be reviewed by the designated veterinarian to ensure that only healthy animals are used and to prevent unhealthy new arrivals from infecting the rest of the colony. This means that each new animal shipment must be separated from other animals on site.

The facility should have a programme by which a schedule of animal examinations is followed and periodic quality control is conducted. Typically, several animals from large shipments or a few animals chosen periodically from smaller shipments are subjected to necropsy, evaluation for clinical pathology, haematology, serology, clinical chemistry and possibly histopathology. These evaluations, to be completed under veterinary control, will help ensure that only disease-free animals are used in research projects. Otherwise, results could be confounded by the presence of disease or other anomalies. For example, certain rodent viruses can alter toxicological response from test substances, even though the animals may show no outward signs of disease. Any animals displaying infectious disease must be isolated from others, and preferably destroyed, before disease can spread. Any room contaminated by an infectious agent must be emptied and sanitized before reuse.

4.1.6 Technical Training

In typical research situations, substantial responsibility for the care of animals must belong to the animal technicians. As they tend to feeding, watering, cleaning and observation duties, these persons will normally have daily contact with the animals. In a sense, they comprise the first line of defence against illness, injury or excess distress from treatment. No research facility can afford to leave such efforts to poorly trained or otherwise ill-equipped individuals.

Ideally, animal technicians will have received some training in biology or animal science prior to employment. A number of colleges or community colleges offer degrees in these fields. Whether such formal training is received or not, they must be given on-the-job training with experienced technical staff, including direct involvement with staff veterinarians, before being permitted to work alone. Research facilities should have a programme in place to provide theoretical and practical instruction in animal care. There should be SOPs in place for reference, and progress in the training programme should be documented.

Probably the best insurance for adequate training of personnel is by formal certification. In Europe, guidelines for training courses in laboratory animal science have been developed by the Federation of European Laboratory Animal Science Associations (FELASA; Federation of European Laboratory Animal Science Associations, 2008), covering four categories of personnel—animal technicians, research technicians, scientists and specialists. Through a programme offered by the American Association for Laboratory Animal Science (AALAS), a nonprofit organization begun in 1950, technicians have the opportunity to become certified at three levels of achievement (American Association for Laboratory Animal Science, 2008). The beginning level of certification is for Assistant Laboratory Animal Technician (ALAT), followed by Laboratory Animal Technician (LAT) and Laboratory Animal Technologist (LATG). To determine qualification for certification at one of these levels, the candidate's education and experience are reviewed (up to five years of experience is required for a nondegreed LATG certification, for example). AALAS provides a number of training materials for each level. In some instances, formal training programmes are conducted (by local AALAS chapters, facility management, etc.) to help candidates through a preparatory course. The candidate must submit a formal application and fee to take the required certification examination. The examination can be taken throughout the year on a designated personal computer workstation or once a year at the AALAS National Meeting.

Difficulty and subject emphasis in the examination (and the training materials) reflect the level of certification involved. As the technician advances, less emphasis is placed on the basics of animal husbandry, health and

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welfare, while increasing attention to facility administration and management. Consistent emphasis is placed on general knowledge. The goal is to encourage technicians to become proficient in many phases of animal care, with achievement of appropriately higher certification levels as experience and education progress. AALAS members are also able to advance in their careers through attendance of local or national meetings, and through numerous educational resources that the association provides. AALAS also provides a programme for animal care management certification. A candidate must have 5–10 years of total work experience (depending on level of formal education), with at least three years of managerial experience. To achieve the Certified Manager Animal Resources (CMAR) status, he or she must complete three examinations by the Institute for Certified Professional Managers (ICPM) and one examination by AALAS. Recipients of CMAR designation must pay an annual recertification fee and must earn a specified number of continuing education credits.

As a final note about animal care certification, it is often desirable for nontechnical staff, such as study coordinators or study directors to complete one or more level of AALAS certification. It is a useful means of fully appreciating the needs of the animals and the function of technical staff. A bonus is that scientific decisions by researcher will more likely be reflective of humane animal use during study planning and conduct.

4.1.7 Laboratory Accreditation

Just as personnel should be certified for the proper use and care of animals, the research facility itself should be accredited. The organization that provides the standards of animal care excellence against which the facility can be measured is the Association for Assessment and Acreditation of Laboratory Animal Care (AAALAC) International (Association for Assessment and Accreditation of Laboratory Animal Care International, 1997d; 2008). The core of this organization was formed in 1950 by a group of Chicago veterinarians who established the Animal Care Panel. Over time, one of the key functions of this panel, through a number of publications and specialized committees, became the establishment of professional standards for laboratory animal care. These standards were used to develop an accreditation programme. In 1965, AAALAC was incorporated as a voluntary accreditation body with 14 medical and veterinary charter member organizations.

Today, over 750 institutions (companies, universities, hospitals, government agencies, research laboratories) have been accredited by AAALAC International including facilities in 30 countries. Over 180 consultants assist AAALAC International in evaluating animal care and use programmes, several of whom are international specialists. AAALAC International is administered by a board of trustees representing 68 member societies, several relating directly to medical or toxicology research (Society of Toxicology, American College of Toxicology and others). Interaction with the member societies ensures that AAALAC International remains responsive to the research community. Actual accreditation is conducted by a council of accreditation consisting of 45 animal medical experts, research scientists and other specialists.

Any private or public organization using animals for research, teaching or testing may apply to AAALAC International for accreditation. The application must describe the organization's animal care and use policies and responsibilities, animal environment, housing, management, veterinary medical care and the physical plant. AAALAC International assesses the institution's animal care and use programme by conducting a site visit. If the institution demonstrates excellence in animal welfare according to AAALAC International 'rules of accreditation' (including the principles presented in the 'Guide', EEC Directive 86/609 and individual national standards), full accreditation may be granted. Conditional or provisional accreditation may be awarded if correctable deficiencies are noted. Otherwise, accreditation is withheld. For accredited facilities, an annual report must be submitted detailing current animal facility status and any changes in animal care or use. Renewal visits are made every three years, after which continued full accreditation, conditional or deferred accreditation (short-term deficiencies noted, requiring correction) or probationary accreditation (serious deficiencies seen) is awarded. Serious and uncorrected flaws will result in revoked accreditation.

4.2 Humane Animal Research

Certainly, the proper care of animals is a crucial aspect of animal welfare in the toxicology laboratory. However, the other major aspect, the humane use of animals in research, is also crucial although somewhat more subject to interpretation. The actual research techniques, including restraint, treatment, manipulation, observation and euthanasia must be conducted with minimal discomfort. Yet the goals of the research project are to be accomplished as fully as possible. A number of organizations have published their policies on animal research, including the Society of Toxicology, the American College of Toxicology and many others. Basically, these policies state that there should not be unnecessary research, excessive numbers of animals in animal testing, or excessive pain or discomfort in animal research. Furthermore, all applicable regulations and policies regarding animal welfare must be followed. The conscientious researcher will make a serious commitment to these ends.

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4.2.1 Restraint of Animals

Many types of toxicological research require some sort of animal restraint, such as the use of straps, harnesses, boxes, boards, collars, wraps, leashes and others. Even manually holding an animal during transportation, treatment or observation could be considered a form of restraint. Ideally, any restraint would be for a short duration, but this is not always the case. Any type of mechanical or manual restraint must be conducted with the animal's comfort and wellbeing in mind. Any apparatus should be of appropriate design, size and materials to minimize discomfort or injury. The 'Guide' offers some basic guidelines, quoted below:

- Restraint devices are not to be considered normal methods of housing.
- Restraint devices should not be used simply as a convenience in handling or managing animals.
- The period of restraint should be the minimum required to accomplish the research objectives.
- Animals to be placed in restraint devices should be given training to adapt to the equipment and personnel.
- Provision should be made for observation of the animal at appropriate intervals, as determined by the IACUC.
- Veterinary care should be provided if lesions or illnesses associated with restraint are observed. The presence of lesions, illness, or severe behavioural change often necessitates temporary or permanent removal of the animal from restraint.

Large animals may be lead by a leash and lifted by the trunk. Handling of small animals requires a firm but careful grip (**Figure 3**). The specific technique depends on the species, and proper training is essential. Some animals may require grasping around the entire trunk; some may be grasped temporarily by a foot or limb; others may be picked up by the scruff of the neck; a few (especially the mouse) may be held briefly by the tail. The goal is to cause the least stress, while minimizing chance of injury (through biting or scratching) to the handler.

4.2.2 Animal Treatment/Dosing

It would be quite difficult to discuss, in detail, all the possible types of treatment employed during a toxicology project. Test substances may be administered by gavage (peroral intubation), feed inclusion, intravenous injection, dermal application, ocular application, inhalation, parenteral injection, intramuscular injection, subcutaneous injection, endotracheal intubation and others. Some projects require special techniques, such as surgery, implantation, introduction of probes or other invasive procedures. Each treatment presents a unique set of concerns for animal welfare. As discussed in other sections of the chapter, only adequately trained personnel should be permitted to perform the various treatments and subsequent observations. Moreover, all the concepts of proper animal use (minimum pain or distress, consultation with or conduct by veterinary staff, approval by the IACUC, appropriate use of anaesthetics or analgesics, minimal numbers of test subjects, observation for unacceptable distress, possible early termination of treatment) must be adhered to.

4.2.3 Animal Observation

Prior to and following treatment, all animals in a study must be carefully examined for any signs of illness and/or

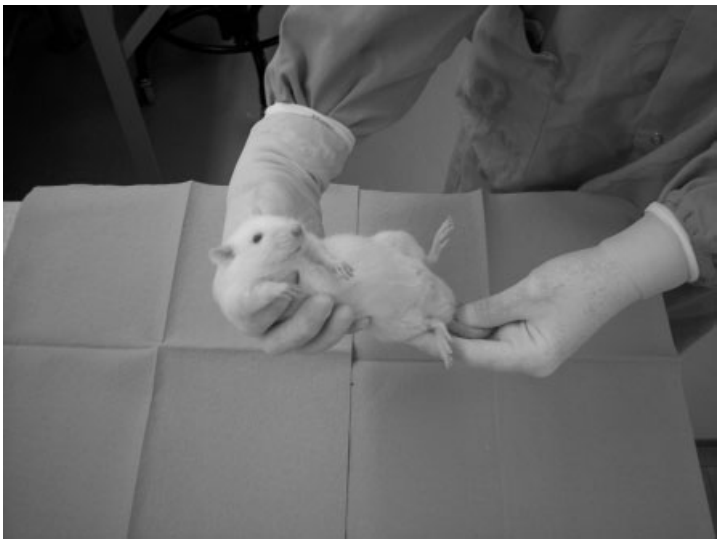

Figure 3 Physical examination of study animals is a crucial aspect of animal welfare and good science. Technical staff must be well trained in the handling and observation of test animals. (Photograph courtesy of Hilltop Lab Animals, Inc.)

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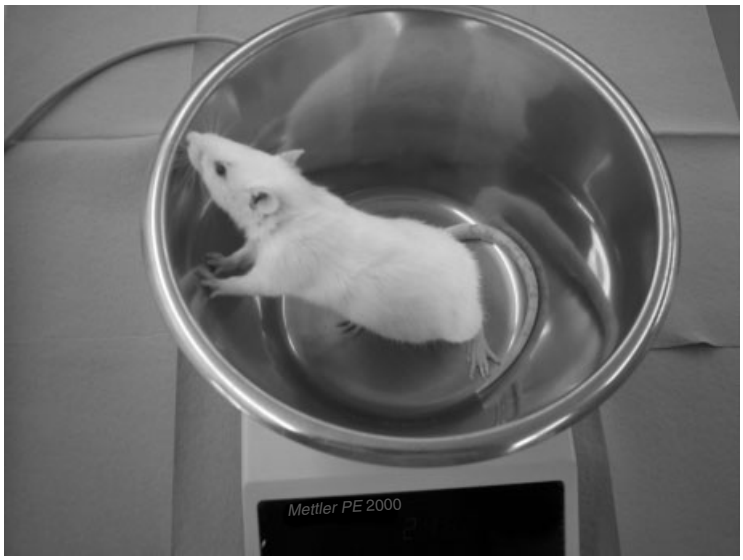

Figure 4 Weight is one of the basic measurements for monitoring the health of test animals. Those affected by illness, injury, or treatment/dosing may lose weight or fail to gain weight normally. The rat shown here appears to be healthy and curious about it surroundings as it is weighed. (Photograph courtesy of Hilltop Lab Animals, Inc.)

toxic effect. Typically, trained technical staff perform observations on a regular basis, but other study personnel (study coordinator, study director, veterinarian, other specialists) should also be involved in the examination process. Depending on the type of study and specific protocol, timing of observations will vary. The protocol and SOPs will detail the process of animal observation, which should include visual assessment, handling and perhaps a battery of simple determinations (such as righting reflex, blink response, startle response, toe pinch response and others). Animals should be examined for local irritation or lesions, changes to skin or fur, breathing difficulty, loss of locomotor ability, nervous system effect (tremors, convulsions, paralysis, coma), behavioural change, salivation, diarrhoea and so on (Myers and DePass, 1993).

For most studies, a critical assessment is body weight measurement (**Figure 4**). Weight loss is often the first indication that an animal is experiencing adverse effects from treatment. All observations must be carefully recorded and if effects are believed to be significant, supervisory or veterinary staff should be consulted. Many study protocols require additional assessment beyond simple physical examination, such as clinical chemistry (with urinalysis), haematology and (upon scheduled or unscheduled death) gross pathology and histopathology.

4.2.4 Surgery in Animals

Toxicologic studies requiring surgery present some unique concerns about animal welfare. According to the 'Guide' there are several categories of surgery. They can be considered to be major or minor, with further divisions of survival and nonsurvival surgeries. Major surgery penetrates and exposes a body cavity or results in significant impairment of physical or physiologic function. This type of procedure should only be performed once on any single animal, unless there is scientific justification (and IACUC approval). Cost savings alone do not justify multiple surgeries in one animal. In minor surgery, there is no exposure of a body cavity and little or no physical impairment is caused. In any case, the fate of the animal could be survival or euthanasia (or unplanned mortality). Basic requirements for any surgery are presurgical planning, involvement of qualified and well-trained personnel, suitable anaesthesia, good aseptic technique (sterile equipment, supplies, gloves, surgical site, etc.), use of proper facilities and equipment, and proper postoperative care. Of course, some of these factors may not be critical if the animal is scheduled for euthanasia immediately after the procedure. Surgery should be performed in an area separated from other animals to avoid excessive stress in them. While minor surgeries, or any surgeries on rodents, may not require a dedicated facility, major surgery on nonrodents should occur only in facilities (ideally, operating rooms) intended for that purpose.

After surgery, the animal should be placed in a clean, dry area where it can be observed for recovery from anaesthesia and for any signs of excessive distress. The investigator and veterinarian are responsible for determining whether unacceptable pain, unusual behaviour, respiratory distress or cardiovascular changes are present. Intervention, in the form of analgesics or even euthanasia, might be indicated. It should be noted that drugs such as sedatives, anxiolytics (drugs to reduce anxiety) and neuromuscular blocking agents are not analgesic or

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anaesthetic and so are not appropriate for use on their own. The staff veterinarian should provide guidance as to the timing of the animal's return to regular housing.

4.2.5 Euthanasia of Animals

The 'Guide' defines euthanasia as 'the act of killing animals by methods that induce rapid unconsciousness and death without pain or distress'. At some point, every test animal will either die on study or will be put to death. Euthanasia may be scheduled as part of the study protocol or may be the ultimate means to relieve an animal of pain or distress. Two major factors must be considered here. The first is the recognition of distress that is sufficiently severe to warrant euthanasia. The second is the determination of the most appropriate method of euthanasia. Neither of these considerations is trivial for the investigator.

Nearly all toxicology protocols recommend euthanasia of animals experiencing pain or suffering. Difficulty arises, however, in defining when distress is sufficiently severe to justify early termination. It might be worth noting that distress is not the same as stress (Institute for Laboratory Animal Research, 2008). Stress is a real or perceived disturbance of an animal's physiological or psychological wellbeing (such as relatively short-term pain, discomfort, physical injury, fear or anger from a dosing procedure or from a toxic effect). Distress is a negative state in which the animal is unable to adapt to one or more stressors, and thus cannot return to a normal state of wellbeing. Since the research animal cannot tell us how it feels, we must pay close attention to a number of visible signs. Recognition of pain and suffering is extensively discussed in the literature (Mroczek, 1992; Carstens and Moberg, 2000; Institute for Laboratory Animal Research, 2008). Broadly speaking, it can be manifested by any abnormal behaviour, unusual physical appearance or alteration of bodily function. More practically, only those signs indicative of prolonged, at least moderately severe, and possibly life-threatening abnormalities should trigger consideration of euthanasia. A number of these are listed in **Table 2**. By this reasoning, salivation or tremors may not indicate need for euthanasia; laboured breathing or convulsions may indicate that euthanasia is advisable.

In acute studies, one must be very careful in determining the need for early sacrifice. By their nature, signs up to and including death are frequently observed. Premature euthanasia will skew LD_{50} calculations, eliminate the evaluation of reversal of signs and confound determination of survivability. This is where technical experience becomes crucial. Technical and veterinary personnel familiar with the various signs and probable outcomes should be consulted for advice. Again, a balance between science and animal welfare is needed. Clearly, moribund (close to death) animals, those with lingering distress, or severe local effects (such as ocular damage) should always be euthanized.

In longer-term projects, the guidelines for euthanasia may be more flexible. Typically, the objectives are geared more towards subtle changes (such as altered tissue structure or function, weight effects, clinical chemistry or haematologic alterations, etc.). Animals showing substantial clinical signs or severe lesions, if suffering is clearly present, should be seriously considered for euthanasia, especially if the study is relatively long and sufficient numbers of animals remain available for evaluation. Animals with well-developed tumours should be euthanized, especially if they are part of a cancer study and they obviously represent a positive response. Any animal that is judged to be moribund, should be euthanized to end its distress and to make sure that appropriate necropsy and tissue study can be performed before autolysis occurs. Of course, animals showing signs of disease (particularly contagious disease) should be euthanized quickly to avoid distortion of study data and possible infection of other animals.

The methodology for euthanasia, carried out for either the relief of distress or for scheduled study termination, should be considered thoughtfully. It is not automatically a matter of injecting the animal with a lethal dose of sodium pentobarbital (although it may often be). In fact, depending on the species and circumstances of the study, some methods may seem rather exotic (such as microwave irradiation or decapitation). One of the best-known guidances for euthanasia is the *Report of the American Veterinary Medical Association (AVMA) Guidelines on Euthanasia* (American Veterinary Medical Association, 2007). This document details the elements of humane euthanasia, common (and uncommon) agents, species-specific methods and AVMA recommendations. According to the AVMA, euthanasia should be without pain or stress, preferably producing rapid unconsciousness, followed by cardiac or respiratory arrest and loss of brain function. Moreover, it is helpful (more for the researcher than the animal perhaps) if the method produces little animal movement immediately following the procedure. Personnel performing euthanasia must be appropriately trained and experienced so that the process itself (handling, restraint, euthanasia technique) is relatively painless and stress-free. The 'Guide' notes that euthanasia should be performed in a professional and compassionate manner and personnel should be able to clearly recognize the cessation of vital signs. It is recommended that euthanasia not take place in the presence of other animals, as possible, because they may become stressed by vocal or nonvocal (behaviour, odours) reactions of the euthanized animal.

Some examples of euthanasia procedures, and their advantages, are listed in **Table 3**. The AVMA has classified euthanasia agents into those producing hypoxia (usually due to depression of vital organs or processes), depression of vital nerve function and physical disruption

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Table 2 Observable signs of severe pain or distress

Local effects[a]	
Skin corrosion	Severe erosion or ulceration penetrating most or all of dermis, large areas of severe necrosis
Ocular injury	Severe corneal opacity, corneal ulceration, purulent or bloody discharge, severe periocular necrosis
Extremity injury	Severe swelling, ulcerative lesions, apparent fractures, severe cutaneous erosion/sloughing, gangrenous appearance
Systemic effects[a]	
Nervous signs	Severe or persistent tremors, convulsions, narcosis, catalepsy
Locomotor/muscular signs	Hunched posture, ataxia, paralysis, prostration
Respiratory signs	Gasping or laboured breathing, very slow or rapid breathing, audible breathing (rales, wheezing)
Cardiovascular signs	Very slow or rapid heart rate, severe pallor, redness or cyanosis of extremities
Gastrointestinal signs	Persistent vomiting, severe diarrhoea, anorexia
Behavioural effects[b,c]	
Excessive vocalization[b]	Squealing, grunting, growling, whimpering, howling (especially during movement or handling)
Atypical actions[b]	Self-mutilation, stereotypic activity, restlessness, head shaking, apparent apathy to stimulants
Direct response to pain[b]	Licking, biting or scratching of affected area; unusual posture to relieve pressure on affected area (hunched or stretched appearance); excessive struggling or biting during handling; grimacing or baring teeth
Absence of actions[b]	Failure to groom, decreased socialization, poor reflexes, marked decrease in feed/water intake
Aggression[c]	Resistance to handling, biting, scratching, kicking, baring teeth, vocalization, attacking run, wrestling

[a]Myers and DePass (1993).
[b]Mroczek (1992), US Department of Agriculture (1995).
[c]Institute for Laboratory Animal Research (2008).

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Table 3 Methods of euthanasia in the toxicology laboratory[a]

Method	Species	Major advantages	Major limitations	AVMA recommendation
Barbiturate injection	Most	Rapid, safe, inexpensive	Requires training, restraint, drug control	Acceptable (preferred) method
Anaesthetic inhalation	Most	Rapid, multiple animals exposed	Initial irritation, hazardous to staff	Acceptable method for small species
Penetrating captive bolt	Large farm species	Rapid	Requires training, equipment, restraint	Acceptable method for large species
Carbon dioxide inhalation	Several small lab animals	Rapid, safe, inexpensive, multiple animals	Some species stressed or very tolerant, ineffective in immature rodents	Acceptable at high concentrations
Carbon monoxide inhalation	Most small	Rapid loss of consciousness	Hazardous to staff, difficult to detect	Acceptable method with appropriate generation (bottled gas only)
Microwave irradiation	Mice, rats	Rapid, safe, brain enzymes fixed	Specialized training, equipment, expensive	Acceptable method with appropriate equipment
Tricaine/benzocaine injection	Fish, amphibians	Rapid, safe	Expensive	Acceptable method
Cervical dislocation	Birds, small rodents/rabbits	Rapid, safe, inexpensive, no drug residue	Requires training, unpleasant for staff	Conditionally acceptable method when justified, some size limitations
Decapitation	Most small	Rapid, no drug residue	Requires training, some hazard, unpleasant	Conditionally acceptable method when justified
Gunshot	Large farm or wildlife species	Rapid, ease for certain species	Requires training, dangerous, unpleasant	Conditionally acceptable method when necessary
Electrocution	Foxes, sheep, swine, mink	Rapid, inexpensive, no drug residue	Requires special equipment, hazardous, unpleasant, severe contractions	Conditionally acceptable method in specialized instances
Pithing	Small amphibians	Rapid, no drug residue	Requires training, unpleasant, tissue damage	Conditionally acceptable method in specialized instances
Nitrogen/argon inhalation	Most small	Rapid, safe, readily available	Stressful in some species, must limit O_2, ineffective in immature rodents	Conditionally acceptable method in specialized instances
Exsanguination	Several	Safe, inexpensive	Very stressful	Unacceptable method without anaesthesia
Rapid freezing	Several small	Safe, inexpensive	Very stressful	Unacceptable method without anaesthesia
Air embolism injection	Several	Safe, inexpensive	Causes convulsions, other sign of distress	Unacceptable method without anaesthesia

(*continued overleaf*)

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Table 3 (*continued*)

Method	Species	Major advantages	Major limitations	AVMA recommendation
Drowning	Several	Safe, inexpensive	Very stressful, slow	Unacceptable method
Hypothermia	Rodent neonates	Possibly convenient	Stressful, slow	Unacceptable method
Strychnine dosing	Several	Possibly convenient	Causes convulsions, painful contractions	Unacceptable method
Chloroform injection	Several	Possibly convenient	Very hazardous to staff	Unacceptable method
Cyanide dosing	Several	Possibly convenient	Unpleasant, hazardous	Unacceptable method
Stunning (blow to head)	Several	Rapid, no drug residue	Unpleasant, sufficient force required	Unacceptable method without other lethal procedure (conditionally acceptable for pigs less than three weeks old)

[a]Summarized from the American Veterinary Medical Association (AVMA) Guidelines on Euthanasia (American Veterinary Medical Association, 2007).

of brain or vital nerve function (such as by cervical dislocation). In some instances, inhalant anaesthetics (such as ether, halothane, methoxyflurane) are preferred because of the rapid unconsciousness produced. These may be easily placed into a closed chamber to build up high vapour concentrations followed by introduction of the animal. Some species are more susceptible than others, and factors such as age or health status may affect reactions. A high concentration of carbon dioxide is useful for anaesthesia/euthanasia, especially for groups of small laboratory animals, except that times to death appear to be extended for neonatal rodents. Some inhalants may produce lung discolouration or other lesions and these agents should be avoided when pulmonary assessment is critical to a study.

Another major class of euthanasia agents consists of those typically injected intravenously (ear, tail, neck or limb veins) or, less commonly, intraperitoneally. Probably the most prevalent of these are the barbiturates, such as sodium pentobarbital. It produces rapid anaesthesia with minimal apparent distress when delivered in sufficient doses. For larger animals, chloral hydrate may be the preferable agent. Some available euthanasia solutions contain a barbiturate combined with local anaesthetics to make injection relatively painless.

The third method of euthanasia is by physical means such as cervical dislocation (by stretching and twisting the base of the skull from the first cervical vertebra), decapitation (by commercial guillotine), microwave irradiation (by equipment designed specifically for mice and rats) and exsanguination (by draining the blood from already unconscious animals). These procedures should be performed quickly and smoothly by experienced personnel. They typically are only used for specialized studies in which certain tissues or fluids must be preserved without drug contamination or degradation.

4.3 Improvements in Animal Research

The basis for making animal research more humane is found in the three Rs (Russell and Burch, 1959). These represent reduction, refinement and replacement—all important considerations while conducting animal testing. As the term suggests, reduction means limiting the numbers of animals to levels essential to meet the goals of the study. Refinement is improvement in test methods to reduce potential for pain and distress caused the test subjects. Replacement involves the use of alternative tests to substitute for whole-animal procedures. Salem and Katz (1998) have proposed a fourth R, responsibility. This introduces the commitment factor required for meeting the challenges of the first three Rs.

4.3.1 Reduction in Animal Numbers

Prior to modern concerns about animal use, many researchers paid little attention to the numbers of animals used in their research. The more animals tested, the more statistically meaningful the results became. Moreover, results could often be obtained more quickly if larger numbers of animals were tested at the start rather than awaiting preliminary findings and then planning studies accordingly. Today, the approach has become very different. Through improved planning, more patient study conduct and better data analysis, fewer animals

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are needed for most research projects. Additionally (and very significantly), regulators around the world have decreased the numbers of animals required for several testing protocols.

Possibly one of the fields most significantly changed over the last two decades has been that of acute toxicity testing (see **Acute Toxicity**). Acute tests, by their nature, are more likely to result in distress and death as relatively high doses are administered by various routes (oral, cutaneous, intraperitoneal, intravenous, inhalation). In fact, for acute LD_{50} studies, the goal is determination of the dose that would be expected to kill half of the subject population. Animal rights activists find this test particularly objectionable. The classical method has been to dose several animals of each sex on each of several dose levels to give what was considered to be sufficient numbers for the LD_{50} calculation, such as was required by the probit analysis method (Bliss, 1935; Finney, 1971). The end result was typically a total of 50–100 animals for a single LD_{50} test to fulfil the requirement of obtaining several treatment levels with fractional mortality. Numerous evaluations have shown that acceptable LD_{50} values can be obtained with far fewer animals. For example, Weil *et al*. (1953) showed that for 24 compounds studied, the mean peroral LD_{50} in rats varied only from 4.76 to 4.64 $g\,kg^{-1}$ when the number of animals per dose level was decreased from 10 to 5. DePass *et al*. (1984) obtained comparable results with 11 compounds when numbers per level and numbers of dose levels were reduced.

The statistical method used to calculate the LD_{50} affects the number of animals required. Procedures like the moving average method (Thompson, 1947), approximate lethal dose method (Deichmann and LeBlanc, 1943) and up-and-down method (Bruce, 1985) require minimal numbers of test subjects. These methods have been summarized by DePass (1989), who reported that the conventional method (probit analysis) typically required roughly 50 animals (rats) per study; the moving average method required 10–20 animals; the approximate lethal dose method used about 5–10 animals and the up-and-down test required 6–8 rats. In the up-and-down method, a single rat is dosed at a time. Doses are increased or decreased to provide multiple lethal and nonlethal levels and permit calculation of an approximate LD_{50}. This method has been fully discussed and compared to other methodologies by Lipnick *et al*. (1995). According to the European Centre for the Validation of Alternative Methods (ECVAM; European Centre for the Validation of Alternative Methods, 2008a), the up-and-down method was adopted by the OECD as an alternative to the traditional LD_{50} test in 1998. The US EPA also listed this test as an acceptable substitute for the LD_{50} test in 1998 (US Environmental Protection Agency, 1998a). After further review by ECVAM in 2001, a revised up-and-down protocol was validated. The OECD now has adopted it as a replacement (rather than an alternative) for the LD_{50} test and discourages the use of the traditional LD_{50} test procedure (deleted from OECD guidelines in 2002).

Another significant issue in the LD_{50} test is whether both sexes require investigation. In the work of DePass *et al*. (1984), it was found that mean LD_{50}s (from 91 rat oral tests) were 2.47 $g\,kg^{-1}$ for males and 2.13 $g\,kg^{-1}$ for females. It has been the experience of several other researchers that females are somewhat more sensitive than males (although some have also disputed this), but that, overall, good correlation exists between the two sexes. Thus, several guidelines (US EPA, OECD) recommend using just one sex (the potentially more sensitive one) of animal for the LD_{50} determination (or estimate) and a few confirmatory animals of the opposite sex at critical dose levels.

Whichever method is used for an LD_{50} determination, it is most useful to do a preliminary test with one or two animals at different dose levels suspected to be near the LD_{50} (based on chemical structure and/or literature data). After a day or two, additional animals may be treated to begin to fill in levels. It may take a few more days of 'wait-and-see' before all required animals are dosed. This approach has been detailed by Myers and DePass (1993). If conducting the up-and-down test, one animal per level may be sufficient.

In cutaneous irritation tests, the classic protocol (Draize *et al*., 1944) requires that at least six rabbits receive a small dose (0.5 ml or 0.5 g) of sample on the clipped skin. A number of investigators have indicated that fewer animals are sufficient. Derelanko *et al*. (1993) analysed data from 224 studies, which provided skin irritation classifications based on six rabbits. The data were randomly reduced to include three, four and five rabbits per study and skin irritation was reclassified for each chemical tested. Agreements between classification with fewer animals and those with six animals ranged from 69 to 95% for negligible irritants, 76 to 85% for mild irritants and 90 to 100% for severe irritants. Absolute differences in dermal irritation indices (the numerical score to quantify degree of reaction, ranging from 0.0 to 8.0) among groups of three, four, five and six animals were small, with an average of no more than 0.3. The authors concluded that as few as three rabbits would normally be adequate. In fact, most recent guidelines (OECD, US EPA and others) have recommended no more than three in the skin test in recent years (Organization for Economic Cooperation and Development, 2002; US Environmental Protection Agency, 1998b). The recommended approach to skin irritation evaluation is to start with a single rabbit. If the irritation is severe (necrosis or ulceration noted) additional testing is unnecessary. More moderate reaction indicates the need for the full set of three animals. If results are equivocal, a few more rabbits may be dosed (up to six) to aid in the final interpretation.

Ocular irritation testing may be approached in a manner similar to that of the skin irritation procedures.

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The researcher should remember, however, that the 'Draize Test' is probably one of the most controversial (to the public) of the short-term toxicity procedures. Every effort should be made to reduce animal use, while not compromising the data required. Previously, six eyes were usually required. However, three eyes are probably sufficient, although some protocols may require more, especially if the benefit of washing out the eye is a desired end point. A reasonable way to approach the eye test is first to review results of cutaneous testing if available. Severe skin irritants would be expected to cause substantial eye injury (of course, there are exceptions). This could be confirmed in a single eye test with a single animal if providing some ocular data is crucial. In any event, a single eye dose should be used as a preliminary guide to the need for further testing. Just as in the skin irritation test, if one eye is severely affected, dosing should be terminated. Otherwise, a total of three eyes should be dosed, or up to six eyes for equivocal findings. Both the OECD and the US EPA have adopted this approach.

The above approaches or variations of these may also be applied to other types of toxicological evaluations, including longer-term projects, such as chronic oral or inhalation studies, cancer studies or reproductive studies. The USDA has recommended a few general methods for animal reduction (Bennett, 1994), including animal sharing, improved statistical design, phylogenetic reduction and better quality animals. In animal sharing, certain study animals may be reused for procedural development, training, alternative route testing (e.g. screening for oral toxicity following cutaneous irritancy testing), tissue harvesting or routine physiologic/pathologic measurements. This should only take place when animals are not stressed from the original study and there is little chance that the goals of the secondary procedure will be compromised. Another method for sharing animals involves the use of common controls. A negative control, vehicle control or positive group used for one study may sometimes be shared with other studies if test parameters are equivalent. An example would be a series of dermal sensitization studies conducted in tandem, using a single set of naive, vehicle and positive control groups.

Statistical design for a toxicity study may be improved by maintaining state-of-the-art knowledge of applied statistics and by using the latest computer statistical packages (see **Statistics for Toxicology**). Inappropriate statistical design can result in failure to reach the study goals and, therefore, a necessary repetition of the project. Moreover, proper statistical analysis can permit the use of minimal numbers of animals (possibly fewer treatment groups or fewer animals per group). There must be sufficient numbers of animals to make a meaningful comparison among the treatment and control groups. No more subjects than statistically required should be used, as discussed by Erb (1990) and McCance (1989), among others. Phylogenetic reduction can be considered a type of reduction or replacement of animal use. It involves the use of the least advanced species that will permit attainment of the study objectives. An example might be increased use of rodents and fewer dogs or nonhuman primates in a dietary inclusion project. The end result could be fewer animals used overall and/or fewer animals from a phylogenetic higher order used.

The use of better quality animals is easily overlooked as a means of reducing animal numbers. However, there is considerable logic in the practice of purchasing the most healthy and genetically consistent animals possible. The loss of animals to disease or abnormality can necessitate repetition of toxicity studies. Otherwise, study findings may become indecipherable from inherent flaws in inferior animal populations.

It is also important that animal quality be confirmed as soon as shipments arrive, preferably by veterinary personnel. In conjunction with the use of better animals should be the practice of ordering fewer animals. Typically, a few extra animals are ordered to compensate for losses prior to or during the study. The investigator should always make a habit of ordering only enough to meet study needs, and insist that the vendor only deliver the quantity ordered.

4.3.2 Refinement of Animal Procedures

Methodologies in toxicology should continually be improved to provide for better science. Additionally, this process of *refinement* may frequently promote decreased pain and distress in the animal model. Some improvements may also decrease the numbers of test subjects required. There are several examples of refinement that apply to the acute toxicity procedures discussed previously.

In the acute toxicity tests, there should be a practical dose limit beyond which further treatment is unnecessary. Most agencies, such as the OECD and the US EPA have recommended this 'limit test' for a number of years. For example, acute peroral dosing with 2.0 or $5.0\,g\,kg^{-1}$ of test substance (depending on the agency involved) will generally be sufficient if all or most animals survive and no LD_{50} determination will be required. The premise is that larger doses (up to $16.0\,g\,kg^{-1}$ or even higher in some older studies) would be unlikely to be encountered by humans. There are exceptions, such as the need to evaluate potential cumulative doses or clearance of very large doses, but reasonable limits should be established just the same. The 'limit test' effectively reduces total animal numbers and decreases the numbers of animals experiencing distress, since sublethal doses are more frequently involved.

Many researchers have questioned the utility of using death as an end point in acute toxicology. It may be sufficient to determine only levels producing observable clinical signs. This is the approach of the 'fixed-dose

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procedure' (van den Heuvel *et al*., 1990). Using a series of standardized dose levels, effect and no-effect levels are established. Materials are assigned toxicity classifications according to a specified scheme. In an international validation study, these classifications were found to correlate well to those assigned on the basis of full LD_{50} studies. Like the limit test, the fixed-dose procedure limits the degree of distress in animals, since lethality is avoided as much as possible. A variation of this method, which does include death as an end point, is the 'acute-toxic class method' (Schlede *et al*., 1994). It also uses a series of established dose levels to be given in a relatively complex pattern designed to efficiently determine lethal/nonlethal levels with few animals (usually three per group). After considerable review, the OECD and then the US EPA adopted both the fixed-dose procedure and the acute-toxic class method as acceptable alternatives to the traditional LD_{50} test. As of December 2001, the OECD has labelled these procedures as replacements for the LD_{50} test (European Centre for the Validation of Alternative Methods, 2008a).

As fewer animals are employed in a toxicology study, it becomes more desirable to gather all the data reasonably possible from those animals studied. Rather than simply observe for lethality and gross signs on an acute study, one could consider evaluation for subtle behavioural effects, tissue changes, haematological alterations and so on. This type of approach was recommended by Gad *et al*. (1984), and by Gad and Chengelis (1998; 1988). These authors proposed inclusion of interval necropsies to help identify target organs before tissues have time to recover from treatment. A few animals euthanized at three or four days (instead of the typical 14 days) could have selected tissues (kidneys, liver, spleen, lungs, brain, reproductive organs, stomach, intestine) removed, weighed and processed. Comparisons are made with tissues of animals terminated at 14 days. Additionally, Gad *et al*. recommended employment of a neurobehavioural screen designed to assess the peripheral and central nervous system. Benefits from such approaches, from an animal welfare standpoint, are decreased likelihood of additional testing to gather these data, better preparation for longer-term studies, and (perhaps) identification of subtle end points for other tests on similar materials.

A few relatively simple practices can substantially enhance the value of acute tests. One is routinely to test animal urine for occult blood (by Ames Hemastix® Reagent Strips), a potential early indication of kidney damage. Reagent strips are also available for measuring protein, glucose, nitrites, leucocytes and other urine parameters. These could prove useful in screening for acute toxic effects. Basic tests for righting reflex, grip strength, startle response and pupillary contraction give clues about neurologic damage. Suspected target tissues can be saved from selected animals at death or euthanasia for possible subsequent evaluation. Whenever study results indicate a potential organ effect, appropriate tissues are available for histologic assessment.

Skin and ocular irritancy tests can also be 'refined' to diminish stress in animals. Most regulatory agencies have already refined these procedures by eliminating the need for them when the pH is less than 2.0 or greater than 11.5, based on the assumption that strongly acidic or basic materials are severely irritating. However, this is not always a valid assumption, because irritation also depends on the characteristics (such as potential for buffering) of the chemical in question. In the skin test, lower dose amounts (0.05–0.1 ml instead of the usual 0.5 ml) might be administered for suspected severe irritants. Alternatively, diluted test substances may be applied. Of course, care must be taken to ensure the goals of the study and applicable regulations are fulfiled. Various transportation regulations, such as those of the US Department of Transportation (DOT; US Department of Transportation, 1991; 2000) or several international transportation agencies, have required evaluation for several contact periods, typically four hours (the standard contact time in most protocols), one hour and three minutes. Obviously, materials not severely irritating after four hours would not require the shorter contact periods. Materials suspected to be severely irritating should first be applied for one of the shorter time periods (preferably on a single animal) and results evaluated to determine whether longer contact periods are needed. Thus, a kind of range-finding study can be first conducted to establish irritant doses or contact times. This approach is also recommended by the US Environmental Protection Agency (1998b) and other agencies. If the specific test protocol permits, several doses may be applied simultaneously to a single animal set, with variations in dose amounts, contact times or even test substances. This process might be more appropriate for initial screens rather than definitive testing. A further refinement of the cutaneous irritation test could be the microscopic evaluation of dermal tissue. This will permit lesions not apparent grossly to be discovered, especially for low-dose or reduced-contact-period studies.

For ocular testing, one of the most effective methods to reduce stress is through low-volume doses. Instead of the typical dose of 0.1 ml, doses of 0.01 or 0.005 ml may be applied for potentially severe irritants (possibly in one or two animals). Additionally, treated eyes may be washed with lukewarm water at various times to limit the severity of response, as long as this does not interfere with the study goals (such as observation of healing time). Some of the most promising refinements in ocular testing have stemmed from improved techniques for assessing eye injury. These permit an increased likelihood of predicting ocular injury following doses that do not cause severe gross effects. Examples are measurements of intraocular pressure, examination by a slit-lamp biomicroscope, and corneal thickness measurements (Ballantyne, 1986; Myers *et al*., 1998). These and

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other methods have been well correlated with traditional Draize scoring results and could become standard in the toxicology laboratory. A number of guidelines recommend use of a topical anaesthetic in eyes exhibiting severe injury, or even in conjunction with the initial treatment procedure. This may be acceptable wherever only the most basic ocular effects are to be evaluated (perhaps in comparative studies). However, topical analgesics or anaesthetics must be used with caution, as they may substantially affect corneal permeability, with ocular injury underestimated (Myers *et al*., 1998) or possibly overestimated. Thus, the investigator will be compelled to find a balance between scientific objectives and animal welfare. There are a number of reviews of eye testing available in the literature and on the Internet, including a fairly comprehensive one by Scheiwiller (2005).

The preceding considerations, while specific for acute toxicology, should also be applicable to toxicology in general. The USDA (Bennett, 1994) has generalized some of these approaches, and others, into four categories: decreased invasiveness, improved instrumentation, improved control of pain and improved control of techniques. The first two (decreased invasiveness and improved instrumentation) are somewhat related. Technologies such as magnetic resonance imaging, improved clinical chemistry/haematology analysers, video systems, computer-driven activity detectors and modern monitoring devices (using microelectronics, fibre optics and laser instrumentation) now may be used to collect data that formerly required considerable animal surgery, manipulation or restraint. The end result is that more data may be obtained with fewer animals and less invasiveness. Even simple procedures, such as the use of Hemastix®, may be considered a means of decreased invasiveness.

As for the improved control of pain, the use of tranquilizers, analgesics and anaesthetics should be considered when test animals are experiencing discomfort. Certainly, these are imperative during surgery, invasive blood collection (for example from the retro-orbital sinus) or invasive dose administration such as cannulation. Pain resulting after treatment, either the result of the procedure (such as surgery) or the result of a toxic response, should be relieved with the appropriate drug(s) if this does not conflict with the study objectives. An example might be application of an analgesic salve on sites of severe irritation following a repeated skin application study. In most instances, a qualified veterinarian should directly oversee administration of drugs controlling pain. The fourth USDA category for refinement, improved control of techniques, entails proficiency in the handling and restraint of animals. Animals must be handled in a gentle, consistent fashion. Both the handler and the animal should be properly trained. The test animal can be preconditioned to accept conventional or unconventional handling methods required by the study protocol. In this way, there will be minimal stress during the study.

4.3.3 Replacement of Animals

Much attention has been directed towards alternatives to animal research. While detailed discussion of these is beyond the scope of this chapter (see **Alternatives to In vivo Studies in Toxicology**), they will be considered in general terms as means to reduce animal numbers and distress. Alternative test systems may be employed as screens, supplements or even substitutes for whole-animal tests. They may permit rapid evaluation of multiple chemical substances for relative potential for toxicity or irritancy. They may also be used to predict reasonable starting doses for more definitive animal studies. Sometimes, alternative methods provide early indications of severe toxicity or irritancy so that methodologies can be appropriately adjusted (fewer animals, shorter exposure times, smaller doses, less invasive techniques, etc.) to minimize animal discomfort. Additionally, the alternative techniques may provide insight into the most appropriate species, route and other test conditions for a toxicity study.

Replacement methodologies may be divided into three general classifications: living systems, nonliving systems and computer simulations (Bennett, 1994). Living alternatives usually refer to *in vitro* models such as organ, tissue or cell cultures, invertebrate animals such as insects or simple marine species, and microorganisms such as bacteria or yeasts. In nonliving systems, chemicals or chemical matrices may be used to evaluate certain biologic reactions, such as enzymatic or immunological action. For example, there are commercially available artificial skin systems, which may or may not contain human or animal cellular components. These are used to evaluate potential for cutaneous penetration and/or irritation. Recent advances in computer and mathematic modelling have resulted in some promising methods of predicting acute toxicity, chronic toxicity, carcinogenicity, genotoxicity, reproductive toxicity and primary irritancy of chemical substances. Essentially, the computer models are based on structure–activity relationships (SARs), the assumption that similar chemical structures should produce similar biological effects. A few examples of the various types of alternatives are presented in **Table 4**. Extensive descriptions and reviews of alternatives to animal testing can be found in the literature, such as the book by Salem and Katz (1998), or online at the web sites of ECVAM, ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods), CAAT and others.

It may be useful to look at some specific examples of alternative methodologies to see how they relate to animal welfare. A number of years ago, Kurt Enslein published his work on predictability of oral LD_{50}s using a computer-assisted SAR approach (Enslein, 1988). He compared computer-estimated LD_{50}s to those derived

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Table 4 Examples of alternative methods used for replacement of animals in toxicological research

Test system	Alternative classification	Potential toxicity end point	Basis for procedure and measurement
Daphnia magna[a]	Living system	Lethality, endocrine disruption	Correlation of LC_{50}s to LC_{50}s of other species; evaluation of offspring sex ratio
Cultured mammalian hepatocytes[b]	Living system	Metabolic changes	Prediction of and disturbance in metabolic pathways from chemical doses
Neutral red dye uptake[c]	Living system	Metabolism, ocular injury, cytotoxicity, phototoxicity, lethality, others	Indication of cell injury by reduced dye uptake
Enucleated rabbit or chicken eye[d]	Living system	Corneal opacity	Evaluation of opacity, other morphologic changes in isolated eyes
Computerized structure/activity[e]	Computer simulation	Developmental toxicity, carcinogenicity	Prediction of effects based on computer input of chemical structure
Frog embryo teratogenesis assay[f]	Living system	Teratogenicity	Evaluation of microscopic morphology changes in frog embryos
Embryonic stem cell test[g]	Living system	Embryotoxicity, teratogenicity	Evaluation of inhibition of differentiation or growth of embryonic stem cells (mouse)
Rat skin transcutaneous electrical resistance assay[d]	Living system	Skin corrosion	Evaluation of electrical resistance change in explanted rat skin
EPISKIN™ and EpiDerm™ *in vitro* systems[d]	Living system	Skin corrosion	Evaluation of cell viability by mitochondrial activity
Spermatozoa swimming analysis[h]	Living system/computer simulation	Cellular motion effects	Mathematical analysis of changes in cellular movement or structure
Mutatox® bioluminescent bacterial test[i]	Living system	Genotoxicity	Assessment of light produced in presence of genotoxic agents
Cultured human or mouse neurons[j]	Living system	Neurotoxicity	Measurement of anticholinesterase activity
Corrositex® *in vitro* system[k]	Nonliving	Dermal corrosion	Assessment of lysis in biobarrier through determination of colour change

[a]Guilhermino *et al*. (2000), Tatarazako (2007).
[b]Gad and Chengelis (1988), Sacco *et al*. (2004).
[c]Chu and Toft (1993), Zurlo *et al*. (1993), Interagency Coordinating Committee on the Validation of Alternative Methods (2008a).
[d]Interagency Coordinating Committee on the Validation of Alternative Methods (2008a).
[e]Enslein (1988), Richardt and Benigni (2002).
[f]Sklarew (1993), Interagency Coordinating Committee on the Validation of Alternative Methods (2008a).
[g]European Centre for the Validation of Alternative Methods (2008b).
[h]Cohoon *et al*. (1998).
[i]Wang *et al*. (1998).
[j]Ehrich (1998).
[k]Gordon (1998), Interagency Coordinating Committee on the Validation of Alternative Methods (2008a).

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from animal studies for over 2000 chemicals and found that 50% of the LD_{50}s were equivalent within a factor of two and 95% of the LD_{50}s were within a factor of eight. With improvements in his programmes over the years and through the work of many others, this approach should be helpful in predicting relative toxicity for a number of actual or developmental molecules. At least in theory, numerous time-consuming and animal-intensive assays could be avoided and those chemicals with the best toxicity profiles would be pursued.

Ocular irritancy testing has been an area of intense alternative research, with considerable progress noted (Chu and Toft, 1993; Interagency Coordinating Committee on the Validation of Alternative Methods, 2008a). One promising commercial alternative (EpiOcular™, MatTek Corporation) contains normal, human-derived epidermal keratinocytes which have been cultured on a special medium to form a multilayered structure similar to the corneal epithelium. Following administration of the test substance, a series of biochemical procedures is conducted to measure relative potential for irritancy, cytotoxicity and permeability. EpiOcular™ or other alternatives could give useful preliminary indications of the intensity of ocular response. The investigator could then adjust the conditions for *in vivo* testing (sample volume, number of animals, timing of washing) to provide the maximum benefit with minimam animal distress. While these procedures continue to be evaluated, others have been validated as acceptable alternatives to animal testing (European Centre for the Validation of Alternative Methods, 2008a). One of the validated methods is another commercial product, Corrositex (In Vitro International)—an *in vitro* system which can be used to predict severity of dermal corrosion. It has been shown to correlate well with animal and human test results and has been accepted by several agencies, including the DOT, US EPA, OECD and others, as a replacement for animal testing.

One of the most animal-intensive studies is the chronic carcinogenesis assay. This costly, time-consuming work can be conducted on only selected test substances. An *in vitro* screen has been developed by Ames *et al.* (1975) to assess the genotoxicity/carcinogenicity potential of chemicals. The Ames Test utilizes mutated bacterial strains (*Salmonella typhimurium*) which are dependent on histidine for survival. These strains are sensitive to chemical carcinogens and react by reverting to histidine independence. Thus, colony growth in a histidine-free media indicates genetic mutation and the potential of carcinogenicity. Frequently, this assay is included in a battery of acute tests as a relatively fast and inexpensive cancer screen. Such a battery may be the only testing done for many of the new materials being developed. No animals are used in the Ames Test and subsequent long-term animal tests may or may not be indicated.

4.3.4 Responsibility for Animal Welfare

The 'fourth R', responsibility, applies to all who have an influence on how animals are treated. Each person, organization and agency must make a commitment to conduct or promote the most stress-free animal research possible. The technical animal staff must be fully trained and observant in animal care and handling. SOPs must be understood and followed. Communications must be open between technical and supervisory personnel regarding animal health and wellbeing. The facility management must be fully supportive of animal welfare and humane research. Even though pressures are high to keep costs low, finish projects rapidly, and maintain a high volume of data output, these goals should not overshadow good science and animal welfare issues. Efforts should be made to track any trends in poor treatment technique, dosing errors, accidental animal deaths or any hints of abuse. Senior staff should make sure that the facility, personnel, policy and work environment are sufficiently directed towards animal welfare. In the long term, this approach will project a respectable reputation for animal research. Along with this approach should be a commitment by those companies or organizations that sponsor animal research. They may have very special goals to reach from the research, but they must also be sensitive and supportive towards humane treatment of animals.

Since much research is guided by governmental or international agencies, it is most important that they keep current with validated animal research techniques. However, they should not yield to political or social pressures to eliminate needed animal testing. Instead, refinements or replacements of animal testing should be carefully integrated into research guidelines. Importantly, agencies should formalize accepted improvements in animal testing quickly to avoid delays or confusion in procedural development in the toxicology laboratory. There must be continual efforts for harmonization among agencies of one nation and among the nations of the world. Unfortunately, tests have been sometimes repeated because evaluation under the guidance of one agency may not fulfil the requirements of another agency or another country. This situation has now been undergoing rapid improvement through the efforts of the United Nations (UN) and the OECD (United Nations Economic Commission for Europe, 2008). Their efforts have resulted in the 'Globally Harmonized System of Classification and Labelling of Chemicals' (GHS). The first edition of GHS was approved in 2002, followed by revisions in 2004 and 2006 (published in 2007). Agencies, industries and countries around the world are reviewing the GHS requirements and deciding how to implement them. GHS is not a regulation and compliance is voluntary, but there will be considerable pressure for companies doing global business to adopt it.

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The most significant and most direct responsibility for animal welfare falls on the investigators (study directors, primary researchers, project managers). They must be thoughtful and knowledgeable in their approach to animal research issues. The welfare of animal subjects should become as much a part of study planning as are the scientific aspects. The investigator should first review the study goals and decide if sufficient work has been done previously. To do this, a review of available literature and historic data from the facility is helpful (see **Information Resources for Toxicology**). There are many sources of related literature on the Internet, including the AGRICultural OnLine Access of the USDA (AGRICOLA; AGRICultural OnLine Access, 2008) database containing millions of citations. If there is a precedence of similar testing, the new project should be aborted or at least altered to gain only the data still required. Some thought should be given to alternative methods, if they could provide adequate data. Throughout the process of protocol development, animal receipt, study conduct and study observations, animal welfare must be considered, like the approach shown in **Figure 5**.

If excessive suffering or pain is anticipated or apparent during a research project, procedures must be revised and/or animals euthanized. Sometimes, this decision is made at the expense of study data and, therefore, options must be considered carefully, with input from veterinary staff and the IACUC. Sponsoring organizations must be notified and the rationale provided. Moreover, animal welfare does not end with the termination of a toxicology project. Studies should be reviewed afterwards in light of potential improvement for animal welfare. Whenever possible, serious consideration should be given to publication of study findings so that other investigators will have access to them and avoid duplication of the work involved.

4.4 The Institutional Animal Care and Use Committee

As described briefly above, The Animal Welfare Act (US Department of Agriculture, 1995) requires the animal research facility to form an IACUC for oversight of animal-related issues, following the standards set forth by the 'Guide'. Additional information can be found in the *Institutional Animal Care and Use Committee Guidebook* published by the Office of Laboratory Welfare/Applied Research Ethics National Association (Office of Laboratory Animal Welfare/Applied Research Ethics National Association, 2002). Also, there is a web site dedicated to IACUC information at iacuc.org. An active, well-informed IACUC is one of the best guarantees that animal welfare policies are supported and followed. Although research facilities are sometimes criticized for self-policing (IACUC members are chosen by the management of the facility), there are numerous checks and outside (USDA, AAALAC) inspections that, along with practical considerations already noted, make most IACUCs effective. The required membership of an IACUC has been noted previously, and the total number of members depends on the size of the facility and the extent of its research. Beyond the mandated composition of the groups, it is desirable to include members from diverse backgrounds among scientific and nonscientific disciplines. Outside experts may also serve as consultants. There may be need for subcommittees to carry out the many functions of the IACUC.

As outlined in the 'Guide', the IACUC oversees and assesses the animal care programme and the use of animals on research. At least every six months, it inspects the facility (with emphasis in all animal housing, testing, sanitation and supply areas), reviews protocols (study and animal care) and carefully records all findings. A written report (containing meeting minutes, documents reviewed, areas inspected and items discussed) signed by most or all members, is issued to principal investigators and facility management detailing any deficiencies. Specifically, study protocols, and subsequent animal use and euthanasia, are evaluated on the following bases (the 'Guide'):

- Rationale of proposed animal use.
- Justification of the species used.
- Justification of the number of animals used.
- Availability of alternative methods.
- Adequacy of staff training and experience.
- Atypical housing or husbandry requirements.
- Consideration of sedation, analgesia and anaesthesia.
- Unnecessary duplication of testing.
- Use of repeated and/or major surgical techniques.
- Criteria for excessive pain or distress.
- Criteria and procedures for intervention (removal from study or euthanasia).
- Methods of euthanasia.
- Safety of working environment for personnel.

Standardized procedures (commonly used techniques), in addition to specialized research projects, must be considered by the IACUC. Standardized protocols should not require full review each time they are employed, but any revisions affecting animal use must be considered by the IACUC. All specialized protocols should each be reviewed by the IACUC. There should be a review/approval form for each project, which should be maintained as part of study records. These records should also be maintained in facility files, available for independent review, such as a USDA inspection. The IACUC report must reflect adherence to the Animal Welfare Act, with minor and major deviances noted. A major deficiency is one that threatens the health, wellbeing or safety of the animals.

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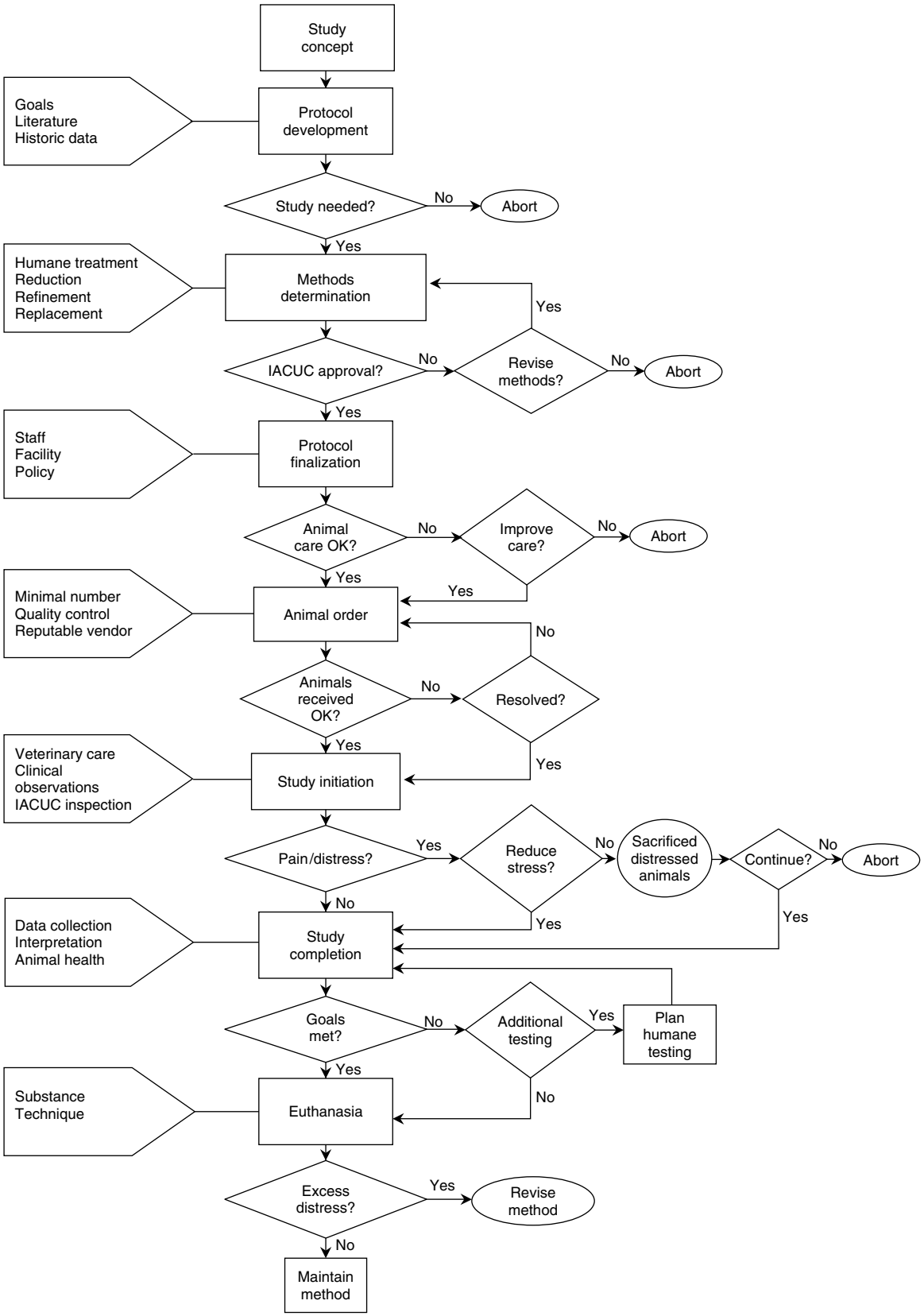

Figure 5 The schematic for humane treatment of animals in the toxicology laboratory shows the complexities of the decision processes. The central portion indicates the basic steps and decisions regarding animal use to be considered during a research project. Influencing factors appear on the left; options and secondary decisions are given on the right.

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Identification of animal welfare deficiencies need not only originate from formal inspections, however. Any staff member should be encouraged to present concerns to the IACUC. Technical or nontechnical personnel, for example, should be free (without any negative consequences) to inform a direct supervisor or an IACUC member of any excessive pain/distress witnessed during the course of a toxicology project. Such information may be kept confidential, but careful documentation must be made. It then becomes the responsibility of site management and/or the IACUC to investigate and take appropriate corrective action as warranted. This process is discussed by Silverman (1994).

There must be a reasonable and specific plan, with an outlined schedule, for correction of significant deficiencies. Resolution of any major deviation should occur through a joint effort of the researcher, facility management officials of the area of concern (animal care supervisor, environmental or maintenance staff, purchasing agent, etc.), and veterinary staff, along with the IACUC. If major deficiencies are not resolved according to the plan and schedule, the remaining deficiencies 'shall be reported in writing by the IACUC, through the Institutional official, to APHIS and any Federal agency funding that activity' (The 'Act'). The IACUC will also withhold approval for the specific studies affected until concerns are resolved. Of course, conflicts may occur among staff, researchers, management and study sponsors. These will have to be resolved in light of the principles presented in the introduction—ethics, science, social pressures and legal requirements.

Secondary responsibilities of the IACUC are training staff members, providing information on animal use, interacting with other personnel (health and safety officers, managers, legal staff, etc.), developing a liaison with outside organizations promoting humane research and engaging in public education (Dell, 1994). Any complaints or charges of animal misuse made by the public or the media should be investigated by the IACUC. This should aid facility management in arriving at an appropriate response.

5 FUTURE DIRECTIONS

It is apparent that the issue of animal welfare has not lessened in importance over the last several years and will continue to be a major concern. Animal rights groups continually question the value and humaneness of animal research. They are well funded, highly visible, and often associated with the 'politically correct' among celebrities and government officials. The public and the media will certainly continue to question the need for animal research. In turn, government legislation will continue the attempts to ban or, at least, severely limit certain types of research. In 1992 the European Community's Council of Ministers voted to prohibit sales of cosmetics containing ingredients tested on animals after 1 January 1998 (Zurlo *et al*., 1993). This was done with the assumption that suitable alternatives would be available. After several delays in implementation, the current rule (European Directive 2003/15/EC) is that finished cosmetic products may not be tested in animals (American Veterinary Medical Association, 2003). Ingredients may not be tested in animals after March, 2009. Over the last several years, groups in the USA, such as the Soap and Detergent Association and the Cosmetic, Toiletry, and Fragrance Association have substantially reduced animal use in product safety testing, and have begun increased reliance on alternative methodologies.

Several US agencies such as the DOT, EPA and FDA have increasingly incorporated animal welfare concerns and alternative testing procedures into their guidelines. Recently, the US EPA announced that a 'Memorandum of Understanding' has been developed in collaboration with the NIH and the National Toxicology Programme (NTP) to use high-speed automated robots to screen chemicals for toxicity (US Environmental Protection Agency, 2008). The screening will use cells and isolated molecular targets instead of laboratory animals. Validation and implementation will take place over many years.

A number of research facilities continue to be proactive in developing alternatives to animal testing. One well-known facility in the USA is the Johns Hopkins Center for Alternatives to Animal Testing. It is funded by several major chemical and health product companies, food and cosmetic companies, animal welfare groups and Federal agencies. For the most part, such organizations recognize that whole-animal research will continue to be needed. Their goals are to more fully develop and validate *in vitro* tests, computer modelling, reduction in animal use and other alternative methods of toxicologic research (Johns Hopkins Center for Alternatives to Animal Testing, 2008c). They serve as information sources for alternative methods, with data on advantages and disadvantages. In 1997, the National Institute of Environmental Health Sciences (NIEHS) established the Interagency Coordinating Committee on the Validation of Alternative Methods (2008b). This group is composed of representatives from 15 Federal regulatory and research agencies involved with toxicologic research and information. ICCVAM, administered by the National Toxicology Program Interagency for Evaluation of Alternative Methods (NICEATM), promotes validation and acceptance of methods that reduce, refine or replace animal tests.

In Europe, the ECVAM spends millions of dollars on alternative research (Zurlo *et al*., 1993; European Centre for the Validation of Alternative Methods, 2008b). Numerous alternative methods have been validated by ECVAM and many more are under consideration. In the UK, FRAME (Fund for the Replacement of Animals in Medical Experiments) has been active for many years in

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the development/validation of alternative methods. These and comparable organizations worldwide are expected to provide new directions and new options in toxicologic research over the years to come.

All individuals and organizations involved with animal research will be compelled to be increasingly vigilant in their approach to animal research, staying current with new legislation, new methodologies and changing public perceptions as they develop in the future. The basic need for animal research is not likely to disappear for many years. The approaches and attitudes towards animal welfare will need to evolve as new standards and technologies become available.

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Chemical and Biological Occupational Hazards in the Toxicology Laboratory

Bryan Ballantyne

CONTENTS

1 GENERAL CONSIDERATIONS

Many of the occupational hazards in the toxicology laboratory are common to those of laboratories specializing in other branches of science and technology. These cover a kaleidoscope of situations, including the handling of flammable and explosive materials, heating equipment, electrical devices, sharp instruments, glassware, lasers and radioactive isotopes. These general aspects of laboratory safety have been discussed in detail in various monographs (National Research Council, 1981; Fawcett, 1983; Armour, 1991).

Toxicology laboratories have specific biological and chemical hazards, and special precautionary measures, protective procedures and management protocols are required. There is exposure to a very wide range of substances, many at the research and development stage, whose human health hazards are unknown. Indeed the reason for conducting investigations on such chemicals is to determine various aspects of their toxicity and potential health hazards. Knowledge of the physicochemical properties of materials to be investigated, and of any relevant structure–biological activity relationships, should be considered in order to allow the most appropriate guidance on safe-handling practices. However, considerable caution is required, since many aspects of toxicity are not predictable. Some materials may have a relatively well-defined acute toxicological profile, but other special aspects of toxicity are being investigated in the laboratory, for example, long-term toxicity, genotoxicity, reproductive toxicity or metabolism and toxicokinetic studies. Because of the potential for repeated exposure to these substances in the laboratory, specific protective and precautionary measures may be necessary. Also, certain materials of high biological activity are used as positive controls; for example, in genotoxicity, developmental toxicity and sensitization studies. Clearly, for such materials the most stringent of storage and handling procedures are required. Additionally, there is a potential for exposure to chemicals used routinely in laboratory procedures, for example, in the histology laboratory. Since the toxicology and potential human health hazards of these latter materials are usually well documented, any necessary protective and precautionary procedures should be known.

Physical injury can occur during the handling of animals and from instruments such as scalpels, scissors

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and needles. Exposure to, and handling of, laboratory animals may cause physical injury, infections and irritant or allergic reactions. These aspects of toxicology laboratory practice, considered later (Sections 4 and 5), can be a significant cause of morbidity. Risk factors may be somewhat greater for certain groups of workers than for others; for example, animal cage cleaners are exposed to chemicals, mechanical equipment, high-pressure water and high temperatures.

2 ROUTES AND NATURE OF HAZARDOUS EXPOSURES

Exposure may occur by various routes and involve different toxicopathological processes. In general, containment of chemicals for acute studies can be accomplished more easily than for chronic studies because of the smaller number of animals used in acute studies, thus allowing the work to be conducted in exhaust hoods, which is not feasible with the larger number of animals used in repeated-exposure studies (Snellings, 1992).

During the dosing of animals there may be exposure to the test chemical. For example, whilst incorporating test material into feeds for peroral studies, a dust may be created during milling and mixing operations, and also during transfer of test diets. This could result in respiratory tract and cutaneous exposure. Test material applied to the skin of experimental animals may be disseminated by handling of animals, reclipping of hair, changing of bedding and sweeping animal room floors (Darlow *et al*., 1969). Exposure to vapour may occur during application of volatile test material to the skin. With exposure to airborne materials there may be a potential for fire or explosion. Some chemicals, such as pyrophorics, must be diluted with nitrogen before mixing with air. Exposure to test material may occur from leaking chambers or from the handling of animals just removed from a chamber, particularly if exposed to dust or aerosol. Chemical exposure may also occur from contaminated protective clothing. Although gloves may give protection against test chemicals, they may be a source of allergic reactions (see Section 3).

Some health hazards in the toxicology laboratory are known and well appreciated. These include irritant and allergic reactions to chemicals, animals and gloves. For certain potential adverse toxic effects the evidence may be conclusive, but in many other cases may be suggestive, but unproven. Thus, benzene is classified as an IARC (International Agency for Research in Cancer) Group 1 carcinogen with human evidence for leukaemia induction (Glass *et al*., 2003); however, other animal carcinogens are suspect (but unproven) as human carcinogens. There is limited evidence for an increased cancer incidence in laboratory workers (Nenzan, 1990; Summary, 1991) and for adverse developmental and reproductive effects (Heidam, 1984; Legge, 1986). Possible influences on reproductive performance and effects on the developing conceptus have been considered, but with variable findings. Thus, in some studies the risk of abortion or congenital malformation was not increased in laboratory workers (Baltzar *et al*., 1979; Olsen, 1983; Ayelsson *et al*., 1984; Heidam, 1984), but in others there have been indications of increased abortions (Strandberg *et al*., 1978; Lindbohm *et al*., 1984), or an increased risk of malformations (Meirik *et al*., 1979; Ericson *et al*., 1982; 1984) with specific or classes of substances. In a retrospective case-report study, Taskinen *et al*. (1994) found a possible association between exposure to toluene, xylene and formalin/formaldehyde during early pregnancy and an increased risk of abortion, but cautioned that the findings need careful interpretation because of frequent simultaneous chemical exposures. Wennborg *et al*. (2005) investigated major congenital and neural crest malformations in a cohort of Swedish university-employed women working in scientific laboratories; the entire cohort consisted of 2676 women from biomedical laboratories (exposed) and 1147 from other departments (unexposed). They found no significantly increased risk of congenital malformations in relation to laboratory work in general. However, an excess risk was found for organic solvents, especially benzene, for major congenital malformations and for neural crest malformations in offspring of women who had worked with benzene before the end of the second trimester of pregnancy. Thus, odds ratios (with confidence intervals, CI) for various groups were: laboratory work in general, 1.2 (CI = 0.7–2.0); solvent exposure before the third trimester, 1.8 (CI = 1.0–2.9) and neural crest malformations with benzene use, 5.3 (CI = 1.4–21.1). Benzene has previously been associated with reduced birth weight (Chen *et al*., 2000) and increased spontaneous abortion (Xu *et al*., 1998).

A relatively new area of investigation is that of nanotoxicology in support of health hazard evaluations of the technological uses of nanoparticles, in the length range of approximately 1–100 nm. Nanoparticles currently in use have been made from transition metals, silicon, carbon and metal oxides (zinc dioxide and titanium dioxide) (Dreher, 2004), and in may cases engineered nanoparticles exist as nanocrystals (Murray *et al*., 2000). A substantial proportion of inhaled nanoparticles are likely to deposit in the respiratory tract; ~30 to >90%, depending on breathing rate and particle size (Schulte, 2008). They readily reach the deep lung, and the alveolar region is the primary site of deposition for nanoparticles in the range 10–100 nm. There are indications that these materials may pose a hazard locally to the lung through oxidative stress, inflammation and tumourigenesis, and also cause systemic effects by redistribution to other organs following pulmonary deposition

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(Donaldson *et al*., 2005; Donaldson *et al*., 2006). Translocation from lungs has been shown for brain liver, kidney and spleen (Elder *et al*., 2006; Geiser *et al*., 2005), and the rate depends on physicochemical properties of the nanoparticles, including size, chemical nature, shape and charge (Oberdörster *et al*., 2002; Kreyling *et al*., 2006). Occupational health precautions in the toxicology laboratory should be primarily preventive in nature, with appropriate health monitoring (Schulte *et al*., 2008). Currently many resources, particularly in the USA, Europe and Japan, are being devoted to the development of risk and safety evaluations of nanomaterials, environmental fate, human health effects, workplace and environmental monitoring and precautionary measures (Thomas *et al*., 2006a). A detailed review of nanotoxicology is given in **Nanotoxicology-The Toxicology of Nanomaterials**.

Whilst all chemicals should be characterized for potential adverse human health effects, and appropriate protective and precautionary measures be developed for their use and handling in the laboratory, certain groups of substances are clear candidates for this exercise. This includes volatile organic solvents because of the likelihood for exposure and their known local systemic toxicity.

3 LATEX ALLERGY

3.1 General Considerations

The rubber accelerator 2-mercaptobenzothiazole (MBT) has been reported to cause allergic contact dermatitis from gloves. It has been suggested that the thiol group is essential to MBT's covalent binding and/or haptenation to nucleophilic protein residues. Oxidation of MBT and its possible haptenation pathways in the pathogenesis of allergenicity were investigated by Chipinda *et al*. (2007). Oxidative transformation of MBT to the disulfide 2,2′-dithiobis(benzothiazole) (MBTS) was detected in glove matrix when hypochlorous acid, iodine and hydrogen peroxide were used as oxidants. Cysteine reduced MBTS to MBT with subsequent formation of the mixed disulfide 2-amino-3-(benzothiazol-2-yl disulfanyl) propionate. Spectrophotometry and mass spectrometry studies demonstrated the simultaneous reduction of MBTS and disulfide formation with Cys34 on bovine serum albumin, suggesting a potential route of protein haptenation through covalent binding between protein cysteinyl residues and the MBT/MBTS thiol moiety. The investigators concluded that the studies suggest that the critical functional group on MBT is thiol, and haptenation is via the formation of mixed disulfides between the thiol group MBT and a protein sulfydryl group. Allergens present in latex (Turjanmaa, 1987; Alenius *et al*., 1991), may result in the production of IgE antibodies and/or initiate T cell-mediated reactions following exposure (Frosch *et al*., 1986). As a consequence, reactions to latex glove materials that have been documented include urticaria, contact dermatitis, angio-oedema, conjunctivitis, rhinitis, bronchospasm, asthma and anaphylaxis (Seaton *et al*., 1986; Turjanmaa, 1987; Shama, 1991; Zotti *et al*., 1992; Warshaw, 1998; Toraason *et al*., 2000; Taylor and Erkek, 2004). Thus, the clinical syndromes that result from latex hypersensitivity may be variable, and include both type I- (IgE) and type IV-mediated responses. Overall, the prevalence of latex allergy may differ considerably for various exposure populations. For example, cited prevalences of latex allergy for healthcare workers in general in reliable series are 7.0% (Turjanmaa, 1987) and 12.1% (Liss *et al*., 1997). In one large detailed study of 741 nurses, the overall prevalence of latex sensitization, based on serum positive reactions for antilatex IgE, was 8.9% (Grzybowski *et al*., 1996). In another study, 21 out of 384 hospital nurses (5.5%) were positive for the presence of latex-specific IgE antibodies (Kaczmarck *et al*., 1996). It is recognized that atopy is a high-risk factor for the development of latex allergy (Kam *et al*., 1997; Liss *et al*., 1997).

3.2 Allergic Contact Dermatitis

Allergic contact dermatitis is a well documented type IV hypersensitivity reaction associated with latex exposure. Typical incidences are 7% in health care workers (Fisher, 1992) and 22% in dental workers (Katelaris *et al*., 1996). Contact urticaria, a type I reaction, may occur within 5–60 minutes after cutaneous exposure, and be clinically present as erythema, hives, pruritis and eczema. Rhinitis, conjunctivitis and periorbital oedema may also be present (Kam *et al*., 1997). For laboratory workers with established dermatitis, suspected of being caused by the gloves, patch testing with the glove rubber may be useful for diagnostic purposes. The possible contribution of glove powder to delayed onset cutaneous hypersensitivity may be assessed by patch testing with epichlorhydrin and preservatives, including isothiazolin-3-one (Heese *et al*., 1991). Localized urticaria may also be investigated by skin-prick tests. Radioallergosorbent tests (RASTs), enzyme-linked immunosorbent assays (ELISAs) and Western blot tests may be useful, but apparently have a low sensitivity in healthcare workers (Holzman, 1993; Slater, 1994). Filon and Radman (2006) found a significant relationship between latex glove allergy and positive skin test for latex with an odds ratio of 9.70 (95% CI 5.5–17.0). Suli *et al*. (2006) reported that skin-prick tests displayed the highest diagnostic efficiency, having higher sensitivity and specificity than specific IgE determination and use tests. They also noted that patch testing with rubber chemicals had a low sensitivity, but a good specificity; however, basophil histamine release and immunoblot showed low sensitivity and low specificity.

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The investigators concluded that a combination of clinical history and skin-prick test should be used in order to diagnose latex allergy.

3.3 Respiratory Sensitization

Asthma occurs in a small population of those exposed to latex. Respiratory exposure to latex allergen is usually related to the use of powdered gloves, with allergens being transferred from gloves to powder, which may then become airborne as gloves are handled (Swanson *et al*., 1992). In one series in which airborne latex was measured in a healthcare institution, the latex aeroallergen concentrations ranged from 5–618 mg m^{-3} (Liss *et al*., 1997). Bronchial provocation testing has shown that airborne latex allergens produced a late asthmatic response. In one series, latency was 4–16 hour postexposure, and with a duration of 3–48 hours (Brugnami *et al*., 1994). The possible contribution of latex to occupational asthma requires, initially, confirmation of the presence of asthma by appropriate respiratory function tests, followed by challenge tests with monitoring of peak expiratory flow rate (PEFR) and forced expiratory volume in 1 s (FEV_1) (Marcos *et al*., 1991; Vandenplas *et al*., 1996). However, it should be borne in mind that provocation tests must be undertaken with considerable care and under skilled medical supervision, since severe systemic reactions, including anaphylaxis, may be induced (Pisati *et al*., 1994). Clearly, laboratory workers presenting with respiratory tract hypersensitivity reactions should also be investigated for the possible contribution of laboratory animal allergy (LAA). Hamilton and Adkinson (1998) in a multicenter study found that the Grear NAL (nonammoniated latex allergen preparation) skin test reagent at 100 μg ml^{-1} is a safe and effective method for confirmation of latex allergy.

With respect to pre-employment screening of laboratory animal workers, a complete history should be obtained, which should include factors that may indicate atopy. Special investigations should include respiratory function tests and, possibly, skin patch tests. For those with established allergic contact dermatitis, cotton liners and barrier creams may be effective, but the use of alternative forms of gloves is probably the best corrective measure. For those with established respiratory sensitization, a change in work conditions may be necessary.

4 LABORATORY ANIMAL ALLERGY

4.1 General and Background Considerations

LAA has been recognized for several decades as a health-related problem in those handling animals in various types of biological laboratories. Its high incidence and severity are such that a change of employment may be necessary. In the UK, LAA is a prescribed industrial disease under the Social Security Act of 1975. The prevalence of LAA in several surveys is shown in **Table 1**, where the range is 19.2–56.0%. It is therefore a factor of considerable occupational health concern in toxicology laboratories, particularly with respect to precautionary and protective measures. Lutsky (1987) surveyed 252 laboratory animal facilities in 21 nations, and concluded that LAA is a world-wide occupational disease. Other estimates of the range in incidence include 5–40% of research workers exposed to animals within the first year (Tfelt-Hansen *et al*., 2007). A high incidence, 59.5%, for LAA on skin-prick testing was recorded for veterinarians (Krakowiak *et al*., 2007). In addition to direct health consequences, those with LAA may be more susceptible to life-threatening anaphylaxis secondary to rodent bites (Teasdale and Davies, 1983; Hesford *et al*., 1995) or to minor injuries from needles used in animal procedures (Watt and McSharry, 1996).

The rat and mouse are most frequently cited as causes of LAA, but hamsters, guinea pigs, rabbits and cats

Table 1 Prevalence of laboratory animal allergy in different geographical locations, based on clinical effects, according to various surveys

Country	Group size	Incidence (%)	Reference
Japan	5641	23.1	Aoyama *et al*. (1992)
UK	138	44.0	Venables *et al*. (1988a)
UK	146	30.0	Slovak and Hill (1981)
UK	9	22.9	Beeson *et al*. (1983)
Sweden	101	32.0	Willers *et al*. (1992)
Sweden	146	41.0	Agrup *et al*. (1986)
Sweden	110	20.9	Weissenbach *et al*. (1988)
The Netherlands	99	19.2	Kruize *et al*. (1997)
Australia	228	56.0	Bryant *et al*. (1995)
Australia	121	32.0	Schumacher *et al*. (1981)

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have also been implicated (Sjostedt *et al*., 1995). Antigens responsible for LAA are usually associated with animal hair, dander and urinary protein. Two allergens have been identified in rat urine, saliva and pelt. Originally described were Rat n 1A, believed to be a prealbumin and Rd Rat n IB, an α_{2U}-globulin (Newman Taylor *et al*., 1977; Eggleston *et al*., 1989). Studies, confirming nucleotide and amino acid sequences, have shown that both allergens are variants of α_{2U}-globulin (Bush *et al*., 1998). In addition to the physiological proteinuria in rats, a raised excretion of urinary protein occurs in sexually mature male rats due to nephritis (Longbottom, 1984; Schumacher, 1987). Three mouse allergens have been identified (Sirayanian and Sandberg, 1979; Schumacher, 1980; Price and Longbottom, 1987). The major allergen, Mus m 1, previously referred to as Agl, is a prealbumin found in urine, hair follicles and dander. As a result of testosterone-dependent gene expression, the concentrations in urine and serum are four times greater in male than female rats. The second mouse allergen, Mus m 2, is a glycoprotein in hair follicles and dander, but not urine. The third mouse allergen is albumin (Price and Longbottom, 1987). Airborne mouse allergens appear to be present in direct relationship to the number of mice present, and the degree of work activity in animal rooms or laboratories (Twiggs *et al*., 1982). In guinea pigs, two allergens (Cau p I and Cau p II) have been proposed (Schou, 1993), which are probably concentrated in the fur, possibly explaining the higher allergenic potency in dander from guinea pigs compared with urine and saliva (Sjostedt *et al*., 1995). In the cat, the major allergen (Fed d 1) occurs principally in the pelt and sebaceous and mucous salivary glands, but not serum and urine (Anderson and Baer, 1981; Brown *et al*., 1984). Fed d 1 is produced in sebaceous glands and transferred to fur by licking. At least 12 cat proteins have been determined to be allergenic, but Fed d 1 is the major one (Charpin *et al*., 1991; Warner and Longbottom, 1991; Kleine-Tebbe *et al*., 1993; Bush *et al*., 1998). In the dog, the most important antigenic protein is Can f 1, a polypeptide present in hair, dander and saliva (Schou *et al*., 1991; Bush *et al*., 1998). In addition to the common causes of LLA, there are cases of allergy related to geographically specific causes; one potentially fatal example is occupational laboratory allergy to the African migratory grasshopper, *Locusta migratoria*. Lopata *et al*. (2005) evaluated scientists and technicians exposed these locusts in the laboratory. Six of 10 individuals experienced symptoms ranging from urticaria and rhinoconjunctivitis to asthma. Seven individuals demonstrated sensitivity on skin-prick testing, and five had specific IgE anybodies to *L. migratoria*. Novel antigens with molecular weights of ~700 kDa were identified in locust wings.

In general, animal aeroallergens are associated with relatively small particles, which can remain suspended in the air for long periods and have a respirable fraction. Airborne mouse allergen is in particles in the size range 3–18 μm (Price and Longbottom, 1990; Ohman *et al*., 1994). Rat allergens are in particles in the size range from <1 to >20 μm, with the majority being <7 μm in diameter (Platts-Mills *et al*., 1986; Corn *et al*., 1988).

4.2 Clinical Presentation

Commonly affected sites are the respiratory tract, eye and skin. Typical combinations of signs and symptoms for these sites are shown in **Figure 1** (Aoyama *et al*., 1992). Rhinitis and conjunctivitis are common (Agrup *et al*., 1986; Venables *et al*., 1988a; Aoyama *et al*., 1992; Seward, 2001; Ruoppi *et al*., 2004), as are cough, dyspnoea and wheezing. Rhinoconjunctivitis is usually the presenting symptom complex, with nasal congestion, rhinorrhoea, sneezing and itching eyes with excess lacrimation. In one study investigating the incidence of allergy in workers exposed to laboratory animals over a 12-year period in a pharmaceutical manufacturing facility, the most common symptoms were related to rhinitis rather than to asthma (Elliot *et al*., 2005a). It is in the more serious cases that asthma occurs, with reported incidences in the range 17–71% (Kruize *et al*., 1997; Hunskaar and Fosse, 1990). Urticaria and angio-oedema may be present. Slovak and Hill (1981) have described two distinguishable forms: regional LAA with classical rhinitis and negative skin-prick tests, and progressive LAA with rhinitis leading to asthma and positive skin-prick tests. It has been noted that the presence of symptoms of LAA are a major risk factor for the development of asthma. For example, Elliot *et al*. (2005b) studied 603 workers with 2527.4 person years over a 12.3 year period, and determined that the probabilities of experiencing asthma symptoms by the 11th year of follow-up were 0.367 for workers with allergy

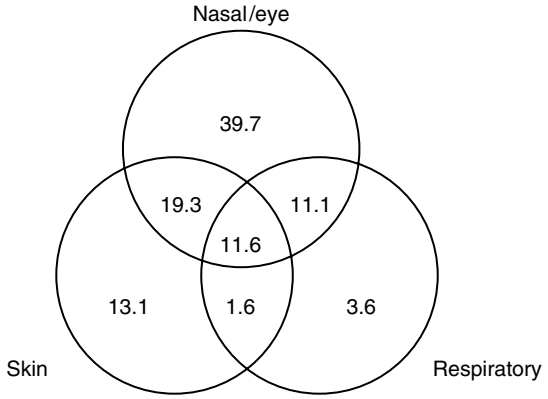

Figure 1 Combinations of symptoms in subjects with subjects with laboratory animal allergy in a survey by Aoyama *et al*. (1992). (Reproduced from Aoyama *et al*., 1992. © BMJ Group.)

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symptoms compared with 0.052 for those without allergy symptoms. The hazard ratio for asthma symptoms when comparing workers with and without allergy symptoms was 7.39 (95% CI, 3.29–16.60) after adjustment for sex and familial history of allergy. Female subjects developed asthma at a rate 3.4 times that for males.

Willers *et al*. (1992) found that those with LAA have a significant increase of trapped gas after pulmonary provocation with methacholine, and hence they associated LAA with small airway hyper-reactivity. However, pulmonary function testing does not reveal a decrement in forced vital capacity (FVC) or FEV_1 (Agrup *et al*., 1986; Willers *et al*., 1992). In a prospective study, Sjostedt *et al*. (1998) found increased methacholine responsiveness in FEV_1 at follow-up in skin-prick positive subjects. They suggest that airway responsiveness may start in the smaller airways and subsequently affect the larger airways. In the Aoyama *et al*. (1992) series, 70% of LAA subjects developed signs and symptoms within three years of exposure and 33% of these occurred within one year, particularly in those with respiratory symptoms. Agrup *et al*. (1986) found that first symptoms appeared within one year of the start of animal handling in 7 of 41 subjects: for the whole group the mean time to symptoms was 2.3 years (range one month to seven years). Kruize *et al*. (1997) reported a mean time of development of LAA symptoms of 109 months in nonatopics and 45 months in atopics. A longer latency of 0.5–12.0 years was described by Davies *et al*. (1983).

Family members of laboratory animal workers may be at risk of developing LAA due to spread of allergens outside the work facility. Thus, Krop *et al*. (2007) measured mouse and rat urinary proteins in samples of mattress dust and in extracts of hair-covering caps worn during work, and found that the levels of these allergens were significantly higher in mattress samples of laboratory animal workers than in controls. Hair-covering caps used in animal facilities had large amounts of rat and mouse urinary proteins, a finding that stresses the need for these to be used as part of industrial hygiene programmes to prevent spread of occupational allergens.

4.3 Predisposing Factors

4.3.1 Atopy

Predisposition of subjects (atopy) in most studies has been defined by a clinical history of allergy, positive skin-prick tests against nonlaboratory environmental allergens, and increased concentrations of serum IgE. Atopy has been frequently correlated with the development of LAA (Agrup *et al*., 1986; Kibby *et al*., 1989; Aoyama *et al*., 1992; Willers *et al*., 1992; Bryant *et al*., 1995; Fuortes *et al*., 1996; Ruoppi *et al*., 2004; Filon and Radman, 2006). However, not all cases are associated with atopy. For example, Gross (1980) found that prior allergies were more common in subjects with LAA (58%) in comparison with the general population (15%). Beeson *et al*. (1983) found the overall prevalence of atopy was 67% in LAA. The latency to onset of signs of LAA may be shorter in atopic than nonatopic subjects (Weissenbach *et al*., 1988; Kruize *et al*., 1997), and atopics may progress to a more severe form of the disease (Davies *et al*., 1983). Also, atopy may be strongly associated with the more severe manifestations of LAA, notably asthma (Cockroft *et al*., 1981; Slovak and Hill, 1987). In a study by Kruize *et al*. (1997), 13% of atopics with LAA had asthmatic symptoms compared with 6% nonatopics that had LAA.

4.3.2 Intensity of Exposure

The degree of exposure to allergens varies considerably and depends on, amongst other factors, stock density, tasks conducted, ventilation rates, cage design, bedding, air filtration and humidity. Although several studies have shown an association with the intensity of exposure expressed as concentration and/or duration of exposure (Cullinan *et al*., 1994; Elliot *et al*., 2005a; Kruize *et al*., 1997; Krakowiak *et al*., 2007), others suggest that intensity of exposure may not be a significant factor in LAA. However, analysis is complicated by the possibility of a self-selection bias. Thus, Venables *et al*. (1988b) found an inverse relationship between the prevalence of LAA, determined by symptoms and the duration of employment; they suggested that workers with LAA avoid exposure to animals. The available evidence indicates that environmental exposure is a strong determinant for the development of LAA, and that appropriate control measures may reduce morbidity (Bush *et al*., 1998). In the study by Kruize *et al*. (1997), intensity as expressed by time of exposure was a clear determinant; thus, an increased relative risk for developing LAA was found for nonatopics exposed for more than two hours per week, and an even higher relative risk for atopics with more than two hours exposure per week.

4.3.3 Cigarette Smoking

Several studies have not shown an association between cigarette smoking and susceptibility to LAA (Agrup *et al*., 1986; Willers *et al*., 1992; Fuortes *et al*., 1996; Kruize *et al*., 1997). However, Venables *et al*. (1988a) examined data from three cross-sectional surveys of 296 laboratory workers exposed to small animals and found evidence for an association between smoking and occupational asthma and skin weals to animal urine extracts. They suggested that smoking might be a risk factor for LAA.

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4.4 Clinical Confirmation

Skin-prick tests are usually conducted with specific animal allergen extracts, but may not always be diagnostic. For example, Willers *et al*. (1992) noted a false positive incidence of 24% and a false negative incidence of 28%. Slovak and Hill (1987) found that 48% of those with LAA showed a positive skin reaction to allergens from the relevant species. They suggested that only those with a positive prick test develop progressive LAA (i.e. a strong correlation with asthma and a poor correlation with rhinitis only).

The RAST is used to measure specific IgE antibodies to preparations from urine protein extracts or superficial skin scrapes. Beeson *et al*. (1983) found that in subjects with LAA, 53% had positive skin-prick tests, and the RAST was also positive in 53%. Several groups (Vanto *et al*., 1982; Davies *et al*., 1983; Agrup *et al*., 1986; Venables *et al*., 1988a) have found that in those showing positive skin-prick tests, there was a good correlation with the results from RAST. Weissenbach *et al*. (1988) and Willers *et al*. (1992) consider that skin tests are more sensitive than RAST. Crossreactions between various species may occur (Agrup *et al*., 1986). Portengen *et al*. (2004) studied IgG4 in 529 laboratory animal workers, and found IgG4 titres were higher in atopic than nonatopic subjects, and increased with higher allergen exposures. Titres were highest in subjects who were sensitized and reported respiratory symptoms to rats compared with those who were not; 202 ± 5.7 GSD (geometric standard deviation) vs. 8.4 ± 18.3, respectively. IgG4 was a strong predictor of newly occurring sensitization and symptomatic rat allergy during follow-up in atopic and rat-sensitized subjects. The investigators consider that high anti-rat urinary allergen IgG4 is a strong predictor of prevalent and incident sensitization and symptomatic rat allergy in atopic and rat-sensitized subjects.

Studies by Adisesh *et al*. (1998) showed raised nitric oxide (NO) in exhaled air, increasing with symptom severity, suggesting this measurement might be a useful monitor in LAA. Hewitt *et al*. (2008) undertook serial measurements of NO in exhaled air in order to assess if the approach could detect exposure-related airway inflammation in 50 laboratory animal workers, of whom 11 had work-related symptoms and two of these has positive serology for LAA. Baseline NO was high (>150 ppb) in the two seropositive workers and increased progressively during a working week in one subject, confirming exposure-related airway inflammation. In seronegative subjects, mean NO concentrations were 19.8 ± 20.1 SD (standard deviation) ppb and 21.7 ± 20.8 ppb in the symptomatic and nonsymptomatic groups respectively, with no significant changes in expired air NO over time. The authors concluded that serial measurements of exhaled NO might provide complementary information in the assessment of possible occupational sensitization. Krakowiak *et al*. (2003) investigated the biochemical and nasal characteristics of nasal lavage fluid (NLF) and nasal resistance changes due to challenge with laboratory animal allergens in 25 patients with occupational asthma and rhinitis, in 22 patients with atopic asthma and rhinitis sensitized to house dust mite and in 15 healthy subjects. In patients with occupational allergy, nasal symptoms of varying severity developed directly after specific nasal challenge, and total symptom score immediately and 24 hours after challenge was significantly correlated with expiratory nasal resistance (ENR). The parentage of eosinophils and basophils in NLF increased significantly 5 and 24 hours after specific challenge and was correlated with ENR. The investigators did not find any significant increase in eosinophils, basophils and albumin in NLF, or changes in ENR, with nonoccupational asthma patients or in healthy subjects. The authors concluded that the changes in eosinophils, basophils and albumin in NLF reflect allergic inflammation, and are related to symptomatology and ENR in occupational allergy.

4.5 Workplace Management and Protective Measures

Lutsky *et al*. (1983) reviewed employer practices and concluded, at that time, there were no consistent or formal policies for addressing LAA. However, as discussed below, there have been marked improvements since then:

1. Pre-employment medical screens: these should include a history with particular, although not exclusive, reference to prior sensitization problems, personal habits, other occupational diseases and pulmonary and skin problems; respiratory function tests (including FVC, FEV_1 and PEFR); skin-prick testing; and measurement of serum IgE. However, pre-employment medical screens need to be interpreted with caution. Some atopics will not develop LAA, and a history of atopy may not be sufficient to exclude from employment. Thus, Botham *et al*. (1995) found that if atopy or pre-existing sensitization had been used as an exclusion criterion, this would have resulted in incorrect decisions for about one-third of their population. They believe that skin-prick tests and possibly RAST analysis should contribute to the pre-employment medical assessment. Also, they consider that atopics, and those already sensitized to laboratory animals, can be advised of their increased chances of developing LAA, but should not necessarily be excluded from employment. Greater consideration should be given to those

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with a history of LAA, chronic skin disease, asthma or cardiovascular diseases, since these appear to make individuals more susceptible to induction and consequences of LAA than either a history of atopy or the presence of IgE antibodies alone. Important factors are a history of allergy to environmental antigens (especially domestic animals), strong reactions to skin-prick testing and high specific IgE to animal antigens. Fuortes *et al*. (1996) recommend that workers who are atopic or have respiratory symptoms should be placed in low-exposure situations.

2. Postplacement periodic medical evaluations: these are essential, and should include history, physical examination, respiratory function tests and possibly measurement of IgE as a potential indicator of the development of sensitization. In those developing LAA, the cause and possible progression of the disease should be carefully explained. With subjects who express a desire to continue working despite the development of symptoms, the prophylactic use of sodium cromoglycolate may provide some protection (Gross, 1980), but this should be regarded only as a temporary measure. In those with LAA, and particularly if asthma has developed, the only safe measure is one of job relocation where exposure to animal allergens does not occur.
3. Workplace management: this is the major way to control and/or prevent the development of LAA. The most important element is to reduce airborne allergen particles in workplaces and laboratories. For example, Gordon *et al*. (1992) demonstrated that rat urine aeroallergens can be significantly reduced by decreasing the stock density of rat housing areas and the use of filter cages or, when these cannot be employed, by replacing contact litter with noncontact absorbent litter. As noted previously, exposure concentrations of allergen vary considerably within the same facility, and between different facilities. Major determinants are stock density, tasks conducted, ventilation rates, cage design, bedding, air filtration and humidity. Clearly, attention should be directed at efficient extraction ventilation, handling procedures, personal protective equipment (including respiratory protective equipment), cage design and placement, the development of safe handling practices and worker education.

In the context of the number of laboratories using animals, there have been relatively few detailed measurements of aeroallergen exposure, but methods for measuring workplace aeroallergens have been described. These can be used, not only to determine the degree of exposure, but also to assess the effectiveness of engineering and other controls to reduce exposure. Gordon *et al*. (1997a) have described a method for measuring urinary protein in personal air samples for assessment of occupational exposure. They found high exposures in laboratory technicians having direct handling contact with animals. Hollander *et al*. (1997) described the measurement of rat and mouse urinary aeroallergens in airborne dust samples by the use of a sandwich enzyme immunoassay, having a detection limit of $0.075\,\mathrm{mg\ ml^{-1}}$. The highest levels of exposure were found during removal of contaminated bedding from cages. Nieuwenhuijsen *et al*. (1994), using a RAST procedure on specimens from personal air samples, found exposure concentrations to animal technicians over a work shift to be $32.4\,\mu\mathrm{g\ m^{-3}}$ (geometric mean). While handling rats the value was $68.0\,\mu\mathrm{g\ m^{-3}}$ and during cleaning out, $53.6\,\mu\mathrm{g\ m^{-3}}$. They also detected low concentrations of rat urinary aeroallergen close to, but outside, animal facilities.

Gordon *et al*. (1997b), who measured air samples for rat and mouse urinary aeroallergens by RAST inhibition, found that there was a sevenfold reduction in concentrations when mice were housed in ventilated cages operated at positive pressure, compared with conventional caging. There was a twofold reduction in personal exposures when soiled litter was removed by vacuum. Rat urinary aeroallergens associated with handling rats were reduced 25-fold when performed in a ventilated cabinet, compared with handling on an open bench. They concluded that a significant reduction in exposure to aeroallergens can be obtained by the use of ventilation systems for housing and handling, but the removal of soiled litter was less effectively reduced by vacuum procedures, and thus respiratory protective equipment may be desirable for this operation. A one-way airflow ventilation system drawing airborne allergens from the working area has been reported to reduce airborne particulates significantly (Yamauchi *et al*., 1989).

Fisher *et al*. (1998) described a comprehensive programme to reduce exposure to environmental allergens that resulted in the incidence of LAA being reduced from 10 to 0%. The programme included education and training: modification of work practices; engineering controls; the use of personal protective equipment including respiratory protection (generally dust-mist respirators); a medical surveillance programme for symptom assessment and RAST testing for animal allergies. The medical surveillance (including RAST), education and personal protective equipment instruction are performed annually and are mandatory for those working with laboratory animals. Engineering controls included filter-typed cages, high-efficiency particulate air-filtered room ventilation, increased room air exchanges and dust-free bedding. Reeb-Whitaker *et al*. (1999) found that for control of laboratory aeroallergen exposure, three strategies are the use of filter cage tops, operation of negatively pressurized

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cages and the use of ventilated changing tables. Schweitzer *et al*. (2003) studied the influence of housing conditions on animal allergen exposure. They found that housing mice in ventilated cages operated under negative pressure and using ventilated change tables reduced ambient mouse allergen (Mus M 1) concentrations 10-fold compared with values when mice were housed in conventional caging and using conventional (nonventilated) changing tables. Housing mice in positively pressurized cages vs. conventional cages did not reduce ambient allergen values. They also noted that the percentage of animal workers reporting allergy symptoms correlated significantly with ambient allergen concentration. Thus, in rooms with the lowest allergen concentration (0.14 ± 0.30 ng m^{-3}) 12.9% workers reported allergy symptoms, but in rooms with the higher allergen concentrations (2.3 ± 00.4 ng m^{-3}) 45.9% of workers reported symptoms. These findings indicate that reduced exposure to laboratory animal allergens can improve the health status of animal care workers. The value of protective and precautionary measures is also demonstrated by the study of Botham *et al*. (1987) who found that the first-year incidence of LAA fell from 37% in subjects who started work with animals in 1980–1981 to 12% in those who started in 1984. This lower level of LAA was maintained in a subsequent study. The reduction coincided with the introduction of improved engineering controls and personal protective measures, including the use of educational programmes.

5 ZOONOSES

A concern about handling laboratory animals is the transmission from animals to the handler of viruses, bacteria or parasites through contact, a bite, or inhalation. Examples for large animals include Simean monkey virus B (Hull, 1973), tuberculosis from primates (Lennette, 1973) and Q fever from sheep (Lennette, 1973). Cat scratch disease is of presumed viral aetiology with the cat acting as a vector. There is regional lymphadenitis secondary to a primary cutaneous lesions; the disease is usually benign, with a good prognosis (Petersdorf, 1976). Rat bite fever may follow a bite from a rat, mouse or other rodent. The most common form, usually seen in laboratory workers, is caused by *Streptohaeillus meliniformis*, a Gram-negative bacillus found in the oropharynx of more than 50% of healthy rats. It presents as a febrile illness with myalgia, weakness and vomiting, followed by rashes and arthralgias (Sandle, 1983).

Eradication programmes have reduced the incidence of zoonotic disease. Most toxicology laboratories use only specific pathogen-free animals from suppliers with quality control programme. Details on the diagnosis, prevention and control of zoonoses are given by Acha and Szyfrei (1980).

6 SPECIFIC CONSIDERATIONS

In addition to the general physical and chemical hazards common to most laboratories of a toxicology facility, certain specialized areas may present particularly high-level concerns. In the pathology laboratory, personnel may be exposed to fixatives and solvents used in tissue preparation. For example, Kilburn *et al*. (1985) reported results of area sampling for formaldehyde, xylene and toluene in rooms where tissues and slides are processed, and determined the ranges to be: formaldehyde 0.2–1.9 ppm, xylene 3.2–102 ppm and toluene 8.9–12.6 ppm. They found that disturbances of memory, mood, equilibrium and sleep, which occurred simultaneously with headache and indigestion, were experienced more frequently in female histology technicians than in unexposed female clerical workers. Over a four year period, there was no evidence for cumulative effects (Kilburn and Warshaw, 1992). Formaldehyde has been shown to be carcinogenic in laboratory animals, but the currently available epidemiological information does not give convincing evidence that it is oncogenic in humans. Contact dermatitis from epoxy resin in immersion oil for microscopy has been described (Le Coz *et al*., 1999).

Several studies require positive control materials which are of high cytotoxicity, for example, in genotoxicity, carcinogenesis and reproductive toxicity investigations. Although most studies on exposure and effects of cytotoxic materials have been conducted on healthcare workers (see **Laboratory Recognition of Potential Xenobiotic Respiratory Sensitizers**), the results are relevant to toxicology laboratory situations. Some studies on the effects of occupational exposure to cytostatic drugs have shown increases in urine mutagenicity, peripheral blood lymphocyte cytogenetics and sister chromatid exchanges (Falck *et al*., 1979; Waksvik *et al*., 1981; Chrysostomou *et al*., 1986; Pohlova *et al*., 1986), but others have failed to demonstrate such effects (Gibson *et al*., 1984; Venitt *et al*., 1984). Differences are probably related to the degree of exposure and the protective measures used. In an animal toxicology study with cyclophosphamide, it was demonstrated that animal technicians could be exposed to the material (Sessink *et al*., 1993). Air filter samples showed <0.1–1.0 µg day^{-1}; surface wipes showed cyclophosphamide in adjacent rooms (<0.02–44 mg cm^{-2}); gloves were contaminated (2–99 µg per pair) and one of 87 urine samples from four animal technicians showed cyclophosphamide (0–7 µg). Several facilities have designed limited access facilities and specific safety programmes for genetic toxicology laboratories. Facilities include special considerations on room air flow and filtration, cupboard hoods, countertop

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designs, sink placement, type of flooring, wall sealing, storage facilities and traffic flow (Inmon *et al*., 1985). Associated with these design features are considerations on protective clothing, safety responses, monitoring and training. The US Occupational Safety and Health Administration (OSHA) has published guidelines for handling antineoplastic drugs (OSHA, 1986). Many of the recommendations are applicable to the toxicology laboratory. Design features for high toxicity laboratories have been published by DiBerardinis *et al*. (1993).

7 PROTECTION, PRECAUTION AND MANAGEMENT

Several texts on the design of toxicology laboratories, safety measures and routine safe practices have been written (Fawcett, 1983; Armour, 1991). Only representative examples of some of the more important features will be considered here.

The facets of particular relevance to laboratory design include the provision of adequate general ventilation and of specific ventilation for particular operations (e.g. laminar flow cabinets for high-toxicity materials) and the use of extractor lines or scavenger systems to prevent localized exposure of laboratory personnel to vapours, gases or dusts. The design of ventilation, scavenger systems and fume cupboards should be such that air from the work area is not carried up in front of the facial area of personnel. Systems should be designed with sufficient flexibility to allow for future laboratory modifications. The design of inhalation units for safe working conditions has been considered elsewhere (McClellan *et al*., 1984). There should be sufficient storage space and shelving to avoid possible hazards from overcrowding of the bench. Separate appropriately illuminated, ventilated and temperature-regulated accessible storage is required in order that the quantities of test and working chemicals may be kept to a minimum in open laboratory conditions.

Protective clothing requires detailed consideration on an individual basis. However, because of the universal potential for breakage of glassware or splashing of chemicals, safety glasses should be worn routinely. Appropriate protective clothing is required when handling chemicals or animals, which may include laboratory coats, gowns and gloves. Contaminated laboratory coats should be discarded. Front-buttoned laboratory coats may not give the same degree of protection as the wraparound variety. Gloves should be chosen on the basis of known permeability characteristics. Although for widely used laboratory chemicals it is frequently known which is the most appropriate type of glove material, with unknown materials it may be appropriate to undertake permeability tests to ensure adequate protection. In some cases it may be necessary to wear two pairs of gloves to give extra protection. Smith and Grant (1988) found a six times lower puncture rate for the inner glove when two pairs of gloves were worn during surgery. In some situations, differing glove requirements may be necessary. For example, during animal restraining procedures one technician may require to wear more physically protective gloves (e.g. leather) whilst restraining an animal, whilst the experimenter may require thinner gloves for palpation or procedures such as intravenous injections. It should be remembered that latex gloves themselves might be a source of adverse effects as discussed above (Section 3). Also, severe urticaria has been documented following the use of glove powder (van der Meeren and van Erp, 1986). Respiratory protective devices should be made available for use when working with chemicals having an offensive smell, potent peripheral chemosensory irritant effect or high vapour toxicity, or they should be available ready for use in strategic positions in case of spills or vapour leakage. In most cases cartridge respirators are used, although full-face air-supplied devices are preferable. Respirators should not be used at the expense of carefully controlled ventilation, since they restrict vision and may have medical problems associated with their use (Ballantyne, 1981). The choice of respirator is critical; for example, Sakaguchi *et al*. (1989) studied the use of respiratory protective equipment in animal facilities to protect against animal allergens. They found that most respirators designed for removal of particulates did eliminate a significant proportion of room air allergens; however, one had an efficacy of only 65%. The use of respirators requires a management programme to cover the following (Ballantyne, 1981):

- Medical examination to eliminate those with medical conditions prohibiting or restricting their use
- Training programme for use and maintenance
- Routine inspection programmes
- Random audit programme.

Management, safety and training programmes should be in the form of carefully developed, readable and readily referenced protocols. For example, they should include factors such as the following. For all chemicals used, and all test materials, there should be a review of the known toxicity and occupational health problems in order that potential handling hazards can be determined and appropriate protective and precautionary measures undertaken. The Material Safety Data Sheet, if available, should help in this respect. Technical staff in training should handle materials and contaminated glassware, equipment and clothing under supervision. Safe methods should be developed for the legal disposal of hazardous materials without causing contamination of the environment (Pitt and Pitt, 1985). There should be strict laboratory regulations to prevent secondary routes of exposure, for example, smoking, eating and drinking should be prohibited in working laboratories.

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Before being assigned to a laboratory position, persons should have a full pre-employment medical examination to assess their physical, haematological, biochemical and physiological functions as a basis for future reference. Also, this examination should be sufficiently detailed to allow the exclusion or restriction of those with predisposing factors to the development of laboratory-associated adverse health effects, for example, LAA, or those with existing medical conditions that could be exacerbated by exposure to chemical agents. Provision should be made for future periodic medical examinations with appropriate special investigations. Ideally these medical examinations should be conducted by a physician with experience in occupational medicine.

There should be adequate planning and facilities for the first-aid management of chemical and/or physical accidents. This should include the immediate access to rapid-release, high-volume showers and facilities for copiously washing the eye. A periodically maintained standard first-aid kit should be available and medical grade oxygen accessible; these should be placed in a readily available and conspicuously marked area. If specific antidotes are available for chemicals used in laboratory, these should also be readily available; for example, hydroxocobalamin for cyanides and atropine/oximes for anticholinesterases. A number of laboratory personnel, available on an on-call basis, should be specially trained in first-aid procedures. Additionally, the designated safety officer should be sufficiently familiar with chemicals in the laboratory and able to offer relevant information to a treating physician. A permanent and active safety committee is essential for the development of policies, their oversight, random audits and the success of safety programmes.

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Information Resources for Toxicology

Philip Thomas Copestake

CONTENTS

1 INTRODUCTION

Our ability to access information, of all kinds, is now staggering. The Internet provides a window to an almost bewildering level of knowledge and detail on any chosen subject—much of it at little, or essentially no, cost—and available at our desks with just a few clicks at a computer. We have had to develop new skills in order to keep pace with these rapid advances in technology, not only in how we search for information, but also in assessing what we are looking at. We are often making judgements on the selection and value of information almost subconsciously, and at great speed, perhaps also under the pressure of work commitments and deadlines. Toxicology—like most scientific disciplines—has grown in parallel with the monumental changes that have taken place in recent decades in the ability to store, manage and disseminate information. It is essential that we have not only an appreciation of what information resources are available, but also an understanding of the processes by which they have been produced.

Information on toxicology is, of course, sought by a diverse array of people. They may be research scientists in industry or academia, seeking to understand the mechanisms by which materials are causing adverse effects, or occupational safety consultants advising on the risks to employees of a particular set of exposure conditions. They may be regulators, trying to establish guidance or legislation at a wider level to protect the general public from adverse health effects from the chemicals to which they may be exposed in their daily life. They even might be lawyers, entering the fray when something has gone seriously wrong. Poisons information centres will assist the medical profession with responding to acute emergencies. There is even the desire amongst the general public to seek information on the chemicals that have become an everyday part of their modern lives. It may be a highly specific question that is being addressed, or a more general requirement for data. The ultimate aim usually is to ascertain and understand the hazards that are inherent to a particular chemical or product, that can then be put alongside an associated level of exposure to determine the risks that those circumstances may present. If necessary, appropriate communication or management of those risks can then be attempted, whether it is on a local, national or international scale. Such an integral part of the world in which we live is toxicology, that it is inevitable that it can become subject to commercial and political influence. Toxicological information needs to be clear and widely available if we are to ensure chemical safety, minimize environmental damage and, on animal welfare grounds, reduce the level of unnecessary toxicity testing.

The aim of this chapter is not to attempt to provide a comprehensive list of resources that are available currently in the area of toxicology. Such compilations leave their author(s) open to criticism from those whose favoured tomes or preferred databases have been omitted, and can also quickly go out of date. Rather, an attempt is made to point readers in a valuable direction, provide some insight into the relative usefulness of the various forms in which information is made available, and offer advice on the factors that need to be acknowledged in order that they may be used effectively. Hopefully, it will provide a useful basis on which the various

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available toxicological resources can be selected and used appropriately. Although key examples are given, these are by no means necessarily endorsements of these particular sources over others.

2 INFORMATION RESOURCES IN TOXICOLOGY—A BRIEF HISTORY

A particular problem with the science of toxicology is that information can be found amongst the resources of a diverse array of disciplines. The reasons for this are essentially to do with the way toxicology has evolved as a subject, and it is useful in this context to look first at how many of the information resources in toxicology have come to exist.

An independent scientific discipline is recognized by virtue of it having its own professional societies and specialist journals, textbooks and undergraduate courses. By these criteria, toxicology can be regarded as being a relatively young science in comparison with other medical disciplines. Although toxicology could perhaps be said to have matured late on in the twentieth century, its roots go back much further, intertwined with those of pharmacology and general medicine. In many respects, toxicology may even predate these older sciences, as man first became aware of the toxic effects of animal venoms and poisonous plants and utilized this knowledge for hunting and warfare. A historical overview of information resources in toxicology reflects, to a large degree, the growth of the science of toxicology itself; the development of many systems for information dissemination, from early texts to online databases and databanks, has been marked by landmarks in its history.

Early resources demonstrate the intimate association with medicine. The 'birth' of toxicological literature could probably be placed with the Ebers Papyrus of around 1500 BC. This ancient Egyptian text includes descriptions of the effects of a number of 'poisons', and outlines remedies for various diseases. Later, around 900 BC, Indian herbal medicine, in the form of Ayurvedism, provided the Vedic texts. Hippocrates, at around 400 BC, introduced rational medicine and described methods for controlling the absorption of toxic metals. Theophrastus (370–286 BC) described numerous poisonous plants, whilst Dioscorides (around AD 50) made an attempt to classify poisons. In 1198, the physician Maimonides wrote a volume entitled *Poisons and Their Antidotes.*

Most graduate toxicologists will know the name Paracelsus. It was in the sixteenth century that he wrote his treatise *Bergsucht,* which included, for essentially the first time, descriptions of the health effects associated with exposure to chemicals found in mining, including arsenic and mercury poisonings, and the asthmatic and gastrointestinal symptoms common to miners' disease. Paracelsus had a profound influence at that time, presenting many ideas which remain important concepts of toxicology today.

Developments in the Industrial Revolution gave rise to further information on occupational diseases associated with chemical exposure. Percival Pott recognized the role of xenobiotics in human disease and linked the occupation of chimney sweep with cancer of the scrotum in a publication of 1775. The Spanish physician Orfelia, often cited as the founder of modern toxicology, produced his *A General System of Toxicology* in 1817, perhaps the first comprehensive textbook in the field.

Although many topics of a toxicological nature would have appeared in the medical journals of the nineteenth and early twentieth centuries, it was not until 1930 that the first scientific journal devoted to toxicology emerged. Published in Berlin, this was *Sammlung von Vergiftungsfällen,* the precursor of the publication *Archives of Toxicology,* which continues today on behalf of the German Society for Experimental and Clinical Pharmacology and Toxicology. This journal was followed in 1938 by *Farmakologiia i Toksikologiia,* which was published in Moscow, and by *Acta Pharmacologica et Toxicologica* (now *Pharmacology and Toxicology*) in Copenhagen, in 1945. The growth of industry, particularly in the chemical and pharmaceutical sectors, following the Second World War was a major driving force for the increase in toxicological literature, and many organizations devoted to the science of toxicology have their origins in this period. The journal *Toxicology and Applied Pharmacology* began publication in 1959 and became the official organ of the Society of Toxicology, which was founded in 1961. It was joined by the journal *Fundamental and Applied Toxicology,* first published in 1981. The 1960s and 1970s saw the birth of what became known as the environmental movement—the push for more detailed information was coupled to this growing public awareness of, and concern about, the effects of chemicals on human health and the environment. The publication in 1962 of Rachel Carson's book *Silent Spring,* which highlighted the potential damage caused by the indiscriminate use of pesticides, was a jolt to public consciousness, and a major turning point in this aspect of the history of toxicology. There was greater regulatory control in response to such demands—companies increasingly became required to test their products and provide regulatory authorities with documented data to assure safety. Such requirements for information were paralleled through the latter half of the twentieth century by a steep growth in the number of journal titles available in toxicology. There was increased specialization in the toxicological sciences as they diversified, and this was reflected by the ever-growing number of specialist journals covering areas such as genetic toxicology and cancer, epidemiology, reproductive toxicology, contact dermatitis and ecotoxicology. We see such trends continue with more recent developments in areas such as nanotechnology

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and the consequent concern about the toxicology of nanoparticles, and genomics—the study of the effects of chemicals at the molecular level in the cell.

The enormous proliferation in information brings with it the problems of how to manage and track down relevant data. 'Secondary' indexes to the literature were developed to allow the search, identification and retrieval of information by specific subject headings. Major publications in the field included *Index Medicus* and *Excerpta Medica,* both of which covered the medical literature, *Biological Abstracts,* which took in the whole of the life sciences, and *Chemical Abstracts,* covering the entire field of chemistry. With the advent of computer technology in the latter half of the twentieth century, many of these resources became available in electronic form. Coupled with developments in telecommunications, large resources of information became readily accessible to researchers. Some regulations themselves involved the compilation of datasets in electronic form, establishing databanks of toxicological data made available in a standardized layout. Many of these resources continue to be important today. The fundamental collation and cataloguing of information, however, remains the same. What have changed are the availability and cost of information, and the speed at which it can be disseminated.

3 INITIAL GENERATION OF DATA—THE SCIENTIFIC COMMUNITY

Like most scientific information, toxicological data will begin life as the results from experimental research and observation. Early dissemination will take place on an informal level with immediate colleagues and peers. More formal presentation of results is likely to occur at professional seminars and meetings, and such gatherings frequently represent the first outside disclosure of new data, albeit usually to a closed audience. The early communication and discussion of data amongst scientists is an important aspect of the so-called 'scientific process' and the formation and possible rejection of ideas and hypotheses. Participation in such a process can be vital as a means of becoming aware of emerging toxicological knowledge, particularly to those working in the research environment or needing to respond to new detail that could impinge on how a particular chemical is viewed and controlled. The interdisciplinary nature of toxicology makes this human network and its organization critical to effective coordination and the spread of information. Often, toxicological topics also find their way into meetings covering broader subject areas such as food technology, cosmetology or other medical and life sciences.

The more tangible forms of information that result from such early processes may be the minutes or proceedings of meetings, conferences or seminars, news articles or company reports. Although much of this information can be highly valuable, by its very nature there is generally no systematic answer to tracking it down or being alerted to its existence. A weather eye on a multitude of relevant web sites is possibly one of the best strategies, together with participation in appropriate trade and research associations or scientific societies. Most will have web sites and perhaps e-mail or web-based alerting services to indicate when news or new content is available.

4 PRIMARY SOURCES—THE SCIENTIFIC LITERATURE

As is common with all science, the validity of toxicological findings is enhanced by formal publication. The vehicle for the primary publication of results of toxicological endeavour remains the scientific journal. It is generally through this respected part of the scientific process that experimental results are reported, then scrutinized, and perhaps re-evaluated by the wider scientific community.

Many scientific journals are, of course, commercial ventures by publishers seeking to fill a niche in the market of scientific literature, and they jostle for supremacy in their particular field through impact factors or citation statistics. The scientific reputation of a journal, however, can be a rather nebulous concept, and may have been built up over many years, perhaps as a result of it being the official publication of a distinguished association, society, or other scientific organization, or maybe through hard-won respect in the quality of the science being published. Every journal will have its own particular aims and scope. The editorial policy and list of those that sit on the editorial board may give some indication of its prestige.

Whilst many items, such as short communications or letters, may take the form of a narrative, most scientific papers appear in the well-established standard format, usually consisting of a Summary or Abstract, an Introduction—putting the study into context and providing an indication of why the investigation was carried out, the Methods—containing enough detail to allow the investigation, if necessary, to be repeated independently, Results—indicating in quantitative and/or qualitative terms what was actually recorded and observed, and a Conclusion—presenting a discussion of how these results could be interpreted, and lastly the appropriate bibliographic References to any documents cited in the paper.

Once a paper has been submitted to a journal, an initial decision will be made as to its suitability for

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publication—whether, for example, the paper fits in with the aims and scope of the journal, and represents a novel advancement in knowledge. An aspect of particular importance, and one on which the value of any journal is usually judged, is the so-called peer-review process. This mechanism involves the scrutiny of submitted papers by a number of independent referees who are chosen as experts in the particular field of interest. They will use their judgement as to whether the paper should be accepted for publication or make recommendations for amendment. The peer-review process is often regarded as the basis of scientific credibility and ensures that much science of poor quality never appears in the good scientific literature. The mechanism has worked well over the years, although it is by no means infallible. It is a system which is dependent to a large degree on the goodwill and professionalism of the referees, who generally give their time and skill free of charge.

One of the frequent problems with toxicological information is that much can languish unpublished as commercially sensitive reports. Companies are often understandably interested in protecting the huge investment that they may have made in terms of conducting laboratory studies. Although there will, of course, be obligations to ensure the safety of their products, and perhaps to inform relevant regulatory authorities if studies provide new insight into toxicology, there may not be tremendous desire to commit resources to putting the information into the open scientific literature.

For those that rely on ready access to the published literature, the cost of subscribing to scientific journals can be substantial. Specialist libraries have, over many years, faced stark choices and often difficult deliberations with the scientists that they serve over the merits or otherwise of subscribing to a particular journal. Many of the key publishers have responded to these difficulties, often for their own commercial survival as journal sales decrease, and the growing norm now is for individual articles to be bought. Tables of Contents that are made available as new journal issues are published can be scanned easily on the web, the abstracts perused, and those items of interest purchased as a download.

Another trend in scientific publication, and one being pushed hard in some quarters, is the development of the 'open access' journal. A significant number of journals, including several in the field of toxicology, are freely accessible as full text; the publishers rely on page or 'administration' charges to those submitting papers for publication, advertising, or charging for add-on alerting services, to cover their costs and make the publications viable on a profitable basis.

Currently, there are perhaps well over 100 journals devoted primarily to toxicology. As the science has developed, emerging subdisciplines have given rise to new titles. A very restricted core list for those interested primarily in hazard and risk assessment gives some indication of the range of titles now available:

American Journal of Epidemiology and Epidemiologic Reviews
Archives of Environmental Contamination and Toxicology
Archives of Toxicology
Basic and Clinical Pharmacology and Toxicology
Birth Defects Research Part B: Developmental and Reproductive Toxicology
Bulletin of Environmental Contamination and Toxicology
Cancer Causes and Control
Carcinogenesis
Chemical Research in Toxicology
Comparative Biochemistry and Physiology C—Toxicology and Pharmacology
Contact Dermatitis
Critical Reviews in Toxicology
Drug and Chemical Toxicology
Ecotoxicology and Environmental Safety
Environmental Health Perspectives
Environmental Toxicology and Pharmacology
Experimental and Toxicologic Pathology
Food and Chemical Toxicology
Fundamental and Applied Toxicology
Human and Experimental Toxicology
Inhalation Toxicology
International Journal of Toxicology
Journal of the American College of Toxicology
Journal of Applied Toxicology
Journal of Environmental Pathology, Toxicology and Oncology
Journal of Occupational and Environmental Medicine
Journal of Occupational Medicine and Toxicology
Journal of Pharmacological and Toxicological Methods
Journal of Toxicological Sciences
Journal of Toxicology and Environmental Health
Mutagenesis
Mutation Research
Neurotoxicology
Neurotoxicology and Teratology
Particle and Fibre Toxicology
Pharmacology and Toxicology
Regulatory Toxicology and Pharmacology
Reproductive Toxicology
Toxicological Sciences
Toxicology
Toxicology and Applied Pharmacology
Toxicology and Industrial Health
Toxicology In Vitro
Toxicology Letters
Veterinary and Human Toxicology

Other scientific journals also impinge vastly on the area of toxicology, most notably those dealing in medical, pharmacological or epidemiological research, and, with varying degrees of frequency, will publish useful data. More general, but highly prestigious, scientific periodicals such as *Nature* or *Science* also sometimes include reports which are of toxicological significance.

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Keeping up to date with the scientific journal literature can be a daunting task. Current-awareness publications appear frequently, often weekly, or even daily. *Current Contents,* from Thomson Reuters (*http://thomsonreuters.com/products_services/scientific/Current_Contents*), is perhaps one of the better known of these services in the biomedical field, but other, often more specialized services, are available in both printed and electronic format, or as part of a service offered by the information unit of a trade association or scientific organization. Such 'selective dissemination of information' (SDI) can provide, on a regular, specified frequency, a focussed set of documents tailored to the specific requirements as outlined by the user.

Most scientific journals are now available in electronic format on the Internet, usually ahead of their printed version, and the web has also allowed for additional content such as data tables and perhaps lengthy comment or discussion to be included. Most of the key scientific journal publishers also have facilities on their web sites to search their full text journal content by keyword, date or author. The search capabilities vary in sophistication, with some only allowing fairly simple single keyword searching, whilst others have far more advanced search interfaces. These can be another useful way of keeping alerted to new publications of interest, as tailored searches can be saved, and any new content matching these search criteria e-mailed at specified intervals, perhaps daily, weekly or monthly. The searches can be across all of the journals published by the particular provider, or to a selected group of chosen subject journals. Such searches would, of course, need to be set up for each of the relevant publishers, a task that initially can be somewhat time-consuming.

5 STANDARD TEXTS, MONOGRAPHS AND EXPERT REVIEWS

Standard reference works compile and condense information into a form that can be seen to represent a culmination of the scientific process; they usually present a considered and generally widely accepted view in a particular area. They are often written, compiled or edited by distinguished experts in the field of interest. Many regularly used texts provide concise overviews or summary data on the toxicology of chemicals or chemical groups and can be useful for background or general material.

It is not within the scope of this chapter to commit to print a reading list of particular books recommended over others. New texts are constantly emerging and old favourites updated and reprinted; the usefulness of each will depend very much on the environment in which it is being used—the particular subject area of interest and the type and depth of information required. Some books have gained enviable reputations as essential texts to grace the shelves of any self-respecting toxicologist. What is important, however, is to acknowledge the limitations of some such material. One of the primary considerations is that of topicality. The gestation period and publication timetable of a large textbook are inevitably protracted. In a specialist area that is rapidly changing, the information may be somewhat out of date, perhaps by as much as two to three years, even as the text first appears on the bookshop shelves. Well-loved textbooks may remain on a library shelf for many years, and that same book, consulted 10 years after its initial publication, may present a very dated view from that currently prevailing. Although generally making use of experts, the task of writing or contributing to a book is usually essentially unpaid and thus the authors may not have been able to afford the time and resources to conduct a comprehensive and in-depth evaluation of all the information that might be available on a particular topic. Typographical errors are, unfortunately, not uncommon within published scientific works and the misplacement of a decimal point or the misprinting of a unit of measure, for instance, can have profound consequences to any dose–response analysis. Errors are sometimes even found to have been repeated in citations by other works. Consequently, it is always advisable, if the reference is available and resources permit, to check original data. This is not always an easy task, as, unfortunately, referencing within textbooks can be poor, and sometimes perhaps even nonexistent, making it at best difficult to trace and verify the cited data. Many texts are written with a very specific readership in mind and it is necessary to appreciate the context in which the book has been written, along with its published aims. Frequently, there is large overlap in the information provided by different texts so it is worth considering carefully the merits of purchasing any particular book.

Current details of textbooks that are available in toxicology are best sought from the catalogues, brochures and web sites of the major scientific publishers. Announcements of new books frequently appear in scientific journals and magazines and will inevitably be posted or e-mailed directly to those whose contact details lie within the publisher's marketing department. It is also worth taking note of published book reviews which may appear in trade-association journals, society information bulletins and in the more mainstream primary biomedical journals. Clearly, it is important to evaluate the suitability of any particular text to personal requirements. Cost will certainly be a consideration as many large textbooks (or even those smaller ones with a more specialist, and thus limited, audience) can be expensive. Many standard works are also now available electronically, making searching for particular topics easier.

Of particular use in the toxicological arena are monographs or reviews of data compiled and evaluated by

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expert groups. Many sources of toxicological information emanate from national and international organizations. The nature of toxicology and its application are such that it has an extensive network of bodies that have some role in the generation, dissemination or use of toxicological information. Many of these will be governmental or quasigovernmental agencies, such as health, food or agriculture departments, or established research organizations. The industrial and consumer sectors will have their own interests represented by trade associations and consumer groups. On an international scale, organizations such as the World Health Organization (WHO), International Labour Organisation (ILO), the Organisation for Economic Co-operation and Development (OECD) and the European institutions of regulation will all have an input. Many well-established publications of this type can be of great value in providing a perhaps authoritative compilation of the data and particular view of toxicological significance:

WHO: The WHO and its various agencies publish a number of key reviews, some under joint sponsorship with the United Nations Environment Programme (UNEP) and the ILO in its collaborative International Programme on Chemical Safety (IPCS) (*www.who.int/ipcs/en/*). These include:

- *Environmental Health Criteria* (EHC) monographs (*www.inchem.org/pages/ehc.html*), and the more recent *Concise International Chemical Safety Assessment Documents* (CICADs) (*www.inchem.org/pages/cicads.html*), both of which provide comprehensive summaries of toxicological data on particular chemicals or groups of chemicals, together with details on the derivation of appropriate human and environmental guidance values for risk assessment.
- Evaluations published by the Joint FAO (Food and Agriculture Organization)/WHO Expert Committee on Food Additives (JECFA) (*www.inchem.org/pages/jecfa.html*) and those of the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) (*www.inchem.org/pages/jmpr.html*), which assess data relating to food additives and contaminants.
- *Monographs on the Evaluation of the Carcinogenic Risk (of Chemicals) to Humans,* published by the International Agency for Research on Cancer (IARC) (*www.inchem.org/pages/iarc.html*), which provide summaries of the data pertinent to an assessment of carcinogenicity of chemicals to man, and present a consensus classification as to overall risk.
- *WHO Drinking-Water Guidelines* (*www.who.int/water_sanitation_health/dwq/guidelines/en/index.html*), *WHO Air Quality Guidelines* (*www.euro.who.int/air/activities/20050222_2*) and the *WHO Recommended Classification of Pesticides by Hazard and Guidelines to Classification* (*www.inchem.org/documents/pds/pdsother/class.pdf*).
- *Health and Safety Guides* (HSGs) (*www.inchem.org/pages/hsg.html*), which provide concise information in nontechnical language for use by decision-makers on risks from exposure to chemicals, with practical advice on medical and administrative issues.
- *Pesticide Data Sheets* (PDSs) (*www.inchem.org/pages/pds.html*), containing basic information for safe use of pesticides. Prepared in collaboration with the Food and Agriculture Organization (FAO), they give toxicological information on individual pesticides, with priority to those with wide use in public-health programmes and/or in agriculture.
- *Poisons Information Monographs* (PIMs) (*www.inchem.org/pages/pims.html*), which evaluate information on substances (chemicals, pharmaceuticals, poisonous plants, and poisonous and venomous animals) commonly involved in cases of poisoning. PIMs are designed as a concise, practical document to facilitate the work of poisons information specialists.

US Agency for Toxic Substances and Disease Registry (ATSDR): The ATSDR is an agency of the US Department of Health and Human Services and publishes a series of Toxicological Profiles (*www.atsdr.cdc.gov/toxpro2.html*), in depth reviews of substances found at hazardous waste sites.

US Environmental Protection Agency (EPA): The US EPA's Integrated Risk Information System (IRIS) (*http://cfpub.epa.gov/ncea/iris/index.cfm*) provides consensus information on human health effects from exposure to various chemicals found in the environment, and background to the development of oral and inhalation human health guidance values.

European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC): ECETOC (*www.ecetoc.org/*) Technical and Special Reports focus on the manufacture, processing, handling and use of chemicals.

OECD: The OECD-coordinated high production volume (HPV) programme (*http://cs3-hq.oecd.org/scripts/hpv/*) develops Screening Information Data Sets (SIDSs) and SIDS Initial Assessment Reports (SIARs).

National Industrial Chemical Notification and Assessment Scheme (NICNAS): The Australian NICNAS produces a number of Priority Existing Chemicals (PECs) Assessments (*www.nicnas.gov.au/Publications/CAR.asp*) on industrial chemicals.

By no means an exhaustive list, the above provides just a small selection of what is available. Many can be found for free over the Internet. The difficulty can be in determining quickly whether, for a particular chemical of interest, such an expert review is available. A trawl through the various expert group web sites is one—albeit

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rather laborious—option. The date that any particular review was carried out is crucial, and it is important to appreciate that the data collation (search date) itself may have actually taken place some time before this. Unless it is clear what date the data search was undertaken (and the extent of that search), it is always a good idea to then search the literature from one or two years prior to the date of the review so ensuring that no additional key data have become available. Sadly, it is still rare for reviews to detail the search strategy that has been employed to identify relevant data—what databases were interrogated and when, and what keyword terms were used.

6 DATABASES

The meanings of many of the terms associated with the practice of information retrieval have been somewhat blurred as a consequence of modern technology; whilst the word 'database' today relates almost exclusively to any structured computer-held information, it was traditionally used as a term to describe any set of 'secondary' (or bibliographic) data compiled as an index to the primary scientific literature. Secondary publications such as *Index Medicus, Chemical Abstracts*, and *Biological Abstracts,* which were developed, as has been discussed, as tools to search the ever-expanding scientific literature, would therefore fall within this definition; they were essentially pointers to where the actual data—the primary journal articles—could be found.

Computerized, or 'online', databases were developed in the mid 1960s as a spin-off from the computer phototypesetting processes that were used in the production of these printed indexes; the bibliographic details to scientific papers were captured in machine-readable form which then allowed reformatting into suitable hard copy format, together with the creation of subject, author and cumulative indexes. With developments in computer and telecommunications technology during the 1960s and early 1970s it became possible for these machine-readable master tapes to be used directly for information retrieval. Large quantities of data could be searched and matched against any specified search word. Although at first the technology was cumbersome and slow by modern standards, and it was impossible to change the search strategy during the search, with improvements in the speed of electronic communications, *interactive* online searching quickly became a reality; the results of any search could be viewed as the search progressed and the strategy that had initially been employed to identify relevant information could be altered to provide, as necessary, a more focussed or expanded set of data. It was a revolution in the way information was held and disseminated and a huge advance in the mechanism by which literature searching was carried out.

We now take very much for granted the speed and ease of searching for information electronically, but it is worth pondering on what a tremendous improvement such systems made in locating relevant information. A search that would have taken many hours using traditional library resources could now be accomplished in a matter of minutes, and consequently at a fraction of the cost of a professional librarian's or information searcher's time.

Two large US corporations, Lockheed DIALOG and Systems Development Corporation (SDC), were the first to exploit the developments in computer technology for information retrieval. They were followed, in the mid 1970s, by European services such as ESA-IRS (European Space Agency—Information Retrieval Service), operated by the European Space Agency, and BLAISE (British Library Automated Information Service), an online system developed by the British Library in the UK.

Medicine was one of the first sciences to benefit from this new information technology. Medical Literature Analysis and Retrieval System (MEDLARS) became operational at the US National Library of Medicine (NLM) in 1964, followed by MEDLINE, the interactive online version of *Index Medicus,* in 1971.

MEDLINE continues to be one of the key biomedical bibliographic databases available, currently holding in excess of 15 million references to journal articles. Its stated subject scope is 'biomedicine and health', defined to encompass those areas of the life sciences, behavioural sciences, chemical sciences and bioengineering needed by health professionals and others engaged in research and clinical care. Increased coverage of the life sciences more generally began in 2000. Coverage is mostly from 1950 to the present, with some older material also now included. Approximately 5000 worldwide journal titles, published in around 40 different languages, are cited. MEDLINE is the primary component of PubMed (*www.ncbi.nlm.nih.gov/sites/entrez*), a service provided by the NLM's National Center for Biotechnology Information (NCBI) (*www.ncbi.nlm.nih.gov/*), and is accessible through their web site or via a number of commercial online database providers as discussed below.

As has been discussed, the period of computerization during the second half of the twentieth century was one in which toxicology as a science was coming into its own, with the consequent huge expansion in its information base. There was developing concern amongst researchers in the field about the dispersion of toxicological information over a diverse array of published scientific literature. The Toxicology Information Program was established at the NLM in 1967 in an attempt to address this, and one of its early achievements was the development in 1972 of TOXLINE. TOXLINE was set up in the hope of providing a 'one-stop shop' for searching the toxicological literature. At the time there was no unified bibliography of toxicological information available, as there was, for example, with medicine

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in the form of *Index Medicus.* The solution was to combine various subsets specifically relating to toxicology from a number of different electronic secondary data sources. Thus, sections from *Index Medicus* and *Chemical Abstracts* that were relevant to toxicology were included, together with others such as from *Pesticides Abstracts,* and the *International Pharmaceutical Abstracts* (IPA). More specialist collections of bibliographic information were also included, such as the files of the Environmental Mutagen Information Center (EMIC) and the Environmental Teratology Information Center (ETIC).

Currently, TOXLINE (*http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?TOXLINE*) comprises the following:

Standard biomedical/toxicology journal literature: *PubMed/MEDLINE*
Special journal and other research literature: *Developmental and Reproductive Toxicology (DART)*
International Labour Office (CIS — Centre international d'information de sécurité et de santé au travail (International Occupational Safety and Health Information Centre))
Technical reports and research projects: *Federal Research in Progress (FEDRIP)*
Toxic Substances Control Act Test Submissions (TSCATS)
Toxicology Document and Data Depository (NTIS — National Technical Information Service (US Department of Commerce))
Toxicology Research Projects (CRISP— Computer Retrieval of Information on Scientific Projects (US National Institutes of Health))
Archival collection (no longer being updated): *Aneuploidy (ANEUPL)*
Environmental Mutagen Information Center File (EMIC)
Environmental Teratology Information Center File (ETIC)
Epidemiology Information System (EPIDEM)
Hazardous Materials Technical Center (HMTC)
Health Aspects of Pesticides Abstract Bulletin (HAPAB)
International Pharmaceutical Abstracts (IPA)
NIOSHTIC ((US) National Institute for Occupational Safety and Health (NIOSH) Technical Information Center)
Pesticides Abstracts (PESTABs)
Poisonous Plants Bibliography (PPBIB)
Swedish National Chemicals Inspectorate (RISKLINE)
Toxicological Aspects of Environmental Health (BIOSIS)

Despite this heterogeneous array of subsets which go to make up the full files, the user of TOXLINE is presented with an interface which suggests a single, apparently homogeneous, system covering toxicology.

In terms of breadth of scope and size, TOXLINE remains the premier online bibliographic database in the field of toxicology. The number of citations it holds now runs into several millions. Thousands of journal titles are cited at least once, although a more limited core set of journals, running into the hundreds, provides the bulk of the records.

Toxicology and medicine are, of course, subjects where other general or more specialized online computer databases may provide useful information, and a search in TOXLINE alone may not be considered adequate. The wider scope of MEDLINE may result in additional hits, and databases such as EMBASE (*www.embase.com/*), the online version of *Excerpta Medica*, or BIOSIS Previews (*www.thomsonreuters.com/products_services/scientific/BIOSIS_Previews*), which includes the indexing service *Biological Abstracts*, are certainly worth considering for a general and comprehensive trawl for toxicological information. EMBASE, from the publisher Elsevier BV, is a comprehensive pharmacological and biomedical database renowned for extensive indexing of drug information. Currently, it contains more than 18 million records primarily dating from 1974 to present, including a MEDLINE subset, and covering the literature from more than 7000 journal titles. BIOSIS Previews is produced by Thomson Reuters and includes *Biological Abstracts* records, now from 1926, together with additional material from the review literature, books, meeting abstracts and patents. Again, more than 18 million records are included from 5000 source titles. Coverage is to virtually every life sciences discipline, including traditional biology (botany, ecology, zoology), together with the interdisciplinary subjects of biomedicine (including toxicology), biochemistry and biotechnology.

There is much overlap between databases in the information they select and index and it is certainly worth considering carefully whether a particular database will cover the area of interest comprehensively, but not be so broad as to generate a large amount of unwanted information. Specialized and more selective databases may be more appropriate for a search in a particular subject category. The scope of literature selection may also be an important factor in database choice. Whilst many bibliographic databases are fairly strong on conventionally published scientific information, they do not cope as well with the less structured literature such as meeting or conference reports, pronouncements from expert groups, dissertations and theses, or even press releases from government departments or agencies, all of which may be useful to the toxicologist. The TRACE database (*www.bibra-information.co.uk/trace.html*), developed by **bibra**—toxicology advice & consulting, is an important example of a more specialized bibliographic database, designed primarily to identify key data for hazard and risk assessment, and covering the scientific journal literature as well as much of this other valuable material.

Web-based interfaces provide easy access to most of the databases discussed here. TOXLINE and MEDLINE are available free via the NLM's TOXNET Toxicology

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Data Network (*http://toxnet.nlm.nih.gov/*) or the NLM home page (*www.nlm.nih.gov/*). They also form part of the services available through commercial database providers such as STN (*www.stn-international.de/*), ProQuest (Dialog, DataStar) (*www.dialog.com/sources/subject/*), and Wolters Kluwer (Ovid) (*www.ovid.com/*). These host services can provide vastly more-complex search capabilities and customized interfaces, and also allow tailored alerting services to be set up using specified delivery formats such as text, HTML, XML and RSS (Really Simple Syndication) web feed. Whilst such services come at a cost—generally with up-front registration fees and charges for content—they can clearly be of value in terms of time saved.

Searches for information can be constructed and refined in most online databases by combining search terms using the Boolean operators AND, OR and NOT. The inclusion of the operator AND will retrieve articles which are common to both sets, whilst OR will add the two sets together. For example, a search can be narrowed by indicating that documents that contain the search word CANCER must also contain the word MAN (i.e. CANCER AND MAN), whilst it could be broadened by specifying that the document should contain any of the words CANCER OR CARCINOGENICITY OR TUMOUR, as well as (AND) MAN OR HUMAN OR WORKER. The logical operator NOT will eliminate documents that contain the specified search term, for instance CANCER NOT STOMACH would retrieve any records that contain the search word CANCER, but not any that also contain the word STOMACH. It should be used with care, since documents that do contain useful information may be unintentionally eliminated; if the topic of interest is cancer of the colon, a paper which discusses both colon and stomach cancer would have been eliminated using the above strategy.

In addition to the use of Boolean logic, other search techniques involve the use of relational operators allowing a user to specify, for example, that two search words must appear adjacent to each other (STOMACH next to CANCER, for instance), or within a specified number of words from one another (say CANCER within four words of STOMACH, to retrieve both STOMACH CANCER and CANCER OF THE STOMACH). It may be possible also to require that words appear in a specified order, or within the same sentence or paragraph. The search may be limited to words appearing in the title only, or to just the keywords that have been added to the record by the databases indexers. Truncation of terms is another possibility, such that MUTA-, for instance, could be used as a search term to include papers containing any of the words MUTATION, MUTANT, MUTAGEN, MUTAGENIC, MUTAGENICITY, and so on. Search statements can often be complex and may need careful and expert construction, but once developed can often be saved for future use, with perhaps a different chemical or date range.

The key to effective searching and identification of relevant information lies not only with a suitable search strategy, but also with how the documents within any database are indexed. Each record in a bibliographic database represents one scientific paper, journal article or document. The record generally has standard elements or fields which may include the Authors, Title, Publication Type and Reference, Language of the Paper, Year of Publication, Country of Publication, Organization(s) for which the authors work, Keywords or Descriptors, and perhaps the Abstract. Each of these fields may be used as an entry point for searching or to limit the search using such methods as mentioned above. The Title, Keywords and Abstracts are most frequently used as the basis for searching, although full text searching is also now possible.

TOXLINE, as is common with many systems, is essentially 'free text'; that is, there is no fully controlled vocabulary applied across all of the subfiles for the purposes of indexing and searching. In order to search comprehensively on a particular subject, it is therefore necessary to consider including as many synonyms for each search term as possible. The computer will then look for any documents in the database where this word appears, in the title, abstract or keyword fields.

More valuable for information retrieval is the use of a restricted and structured thesaurus of index terms such, for example, that the word MAN would be used as a keyword to the exclusion of any others, even if these others, such as HUMAN, WORKER or CHILD, and so on, were the only ones to appear in the text in relation to a paper that includes human data. In this instance, a search restricted to the keywords field on 'MAN' would retrieve *all* relevant papers; searching with a list of synonyms is not needed. A controlled vocabulary such as this is well developed, for instance, with the MEDLINE, EMBASE and BIOSIS Previews databases. The MEDLINE indexers have at their disposal a large hierarchical tree structure of terms, known as Medical Subject Heading (MeSH) terms. The indexers select the most appropriate of these descriptors to represent the subjects covered by a particular paper. The tree structure allows for broad and narrow concepts to be described. Whilst the most specific term available is applied in each case, 'explosion' of a broader term on searching would retrieve this and related documents. The search word ASTHMA would miss any articles indexed under ASTHMA IN CHILDREN, unless the term ASTHMA was exploded in the search. The MeSH thesaurus is continually evolving, with new terms added frequently, as the sciences that it describes grow and develop. Often the terms applied can be given added weight by the indexers as major or minor concepts in the article. This again allows refinement of searches if large sets are initially being retrieved.

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The selection of keywords and, in particular, the use of structured vocabularies requires particular knowledge and experience by both the database indexers themselves and by those wishing to search. Expert indexing is crucial to the effectiveness of a database and, unfortunately, on occasion is clearly lacking. Inappropriate or inadequate indexing of a particular paper will severely decrease the likelihood of it being retrieved during a specific search, or may lead to it appearing as a false drop. Similarly, errors during the entering of a paper's details, perhaps of a simple typographical nature, can result in an item failing to be retrieved.

Chemical names can present particular difficulties when searching for data, and thus are a major problem for those seeking information pertinent to toxicological hazard and risk assessment. Chemical nomenclature can be complex and all synonyms and permutations may have to be included in a comprehensive free text search, necessitating perhaps a high degree of chemical knowledge. Searching precisely on a parent compound without retrieving large numbers of papers on derivatives which include the parent name can also be a huge problem, as can searching for information on a generic class of substances, which would depend on a high degree of detailed chemical indexing, including generic details. Such comprehensive indexing is, unfortunately, lacking in many databases. The inclusion of Chemical Abstracts Service (CAS) Registry numbers in database records can eliminate many of the problems associated with chemical indexing. CAS Registry numbers are unique numbers for specific chemicals, assigned by the CAS. Unfortunately, comprehensive and accurate inclusion of CAS Registry numbers across the biomedical databases is, again, rather limited.

Although such considerations are, sadly, often given little thought today by those who use databases, the indexing policies and the expertise of the database indexing, together with the initial selection criteria for documents which should appear as records, clearly are vitally important aspects of database construction and the consequent use to which they can be put. The value of any bibliographic database must be judged by its ability to identify all relevant data sources.

Two criteria are important in assessing performance. The first of these is the ability of any search to 'recall' information, which is defined as the number of relevant citations retrieved out of the total number of relevant citations in the database being searched, and can also be viewed as the 'sensitivity' of the search. The second criterion is 'specificity' (or 'precision'), which measures the ability of the search to discriminate between relevant and nonrelevant citations and is the proportion of those records identified which prove, on final analysis, to be of relevance. Large, broad-scope databases can perform very poorly on the basis of these criteria. The varied requirements of the different types of user—the basic research community, applied researchers, those charged with evaluating hazard and risk, and so on—are accommodated at the expense of specificity, while the use of nonspecialist selection and indexing reduces both specificity and recall. It is not unusual when searching for toxicological data on a particular well-researched chemical to retrieve a hit list running into several hundreds or even thousands. The ideal is for a database to be subject specific, retrieving only information of relevance, which is easy to search, and comprehensive for that subject. In toxicological hazard and risk assessment, the loss of information may undermine an opinion on safety in use.

7 DATABANKS

Under its precise definition, a 'databank' provides factual information in the form of numeric data or textual statements. In contrast, as described above, a *database* acts as an index—providing the bibliographic location of where that factual information can be found. The electronic availability of many of the compilations of data we have discussed from, for example, expert groups, could be said to place them firmly into this category. A number of additional key compilations of structured toxicological data are useful—and frequently used—and therefore mentioned here.

Some of the issues in relation to the indexing and searching of databases discussed above would also apply to computer-based databanks. What makes the latter different is that the factual nature of their content presupposes some degree of value addition. The criteria for the selection and evaluation of the data become more crucial, and the nature and purpose of the databank should be considered carefully. An example of a commonly used databank in toxicology is the Registry of Toxic Effects of Chemical Substances (RTECS) (*www.cdc.gov/niosh/rtecs/*), produced by the US National Institute for Occupational Safety and Health (NIOSH) in response to the US occupational safety and health regulations. It contains useful information on an enormous number of chemicals, although the data are limited in nature, and certainly are not intended to provide a complete assessment of hazard or risk.

The Hazardous Substances Data Bank (HSDB) (*http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB*) is another favoured and well resourced US databank, supported by the NLM. It contains extensive data including toxicity, safety and handling, emergency response, environmental fate, and exposure potential information on a large number of potentially hazardous chemicals. Information is selected for inclusion by a scientific review panel, the initial aim of which was to establish a databank of 'evaluated' data. The result has been a predilection for qualitative statements on hazard rather than quantitative insights on dose–response, essential to any evaluation of hazard and risk.

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Additional useful resources include others produced by the NLM:

Chemical Carcinogenesis Research Information System (CCRIS) (*http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CCRIS*): This is a scientifically evaluated and fully referenced databank developed and maintained by the US National Cancer Institute (NCI). It contains over 9000 chemical records with carcinogenicity, mutagenicity, tumour promotion and tumour inhibition test results. Data are derived from studies cited in primary journals, current awareness tools, NCI reports and other special sources, with the test results reviewed by experts in carcinogenesis and mutagenesis.

GENE-TOX (*http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?GENETOX*): Created by the US EPA, GENE-TOX contains genetic toxicology (mutagenicity) test data resulting from expert review of the open scientific literature. Data on more than 3000 chemicals are included.

International Toxicity Estimates for Risk (ITER) (*http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?iter*): Compiled by Toxicology Excellence for Risk Assessment (TERA), ITER contains over 650 chemical records with key data in support of human health risk assessments. ITER provides a comparison of international risk assessment information from the ATSDR, Health Canada, the Netherlands National Institute of Public Health and the Environment (RIVM), US EPA, IARC and other independent parties whose risk values have undergone peer review.

Regulatory drives also result in important compilations of toxicological data. Renewed efforts within Europe under the Registration, Evaluation, Authorisation and restriction of CHemicals (REACH) regulation (*http://echa.europa.eu/*), for example, aim to make publicly available large amounts of toxicological data on the thousands of chemicals on the market within the European Union (EU). The level of detail that will have to be supplied by manufacturers or importers will be determined by the overall tonnage and will use the structured International Uniform Chemicals Information Database (IUCLID) format. It will be a huge and costly undertaking for industry, but the resulting databank should prove a valuable resource of up-to-date toxicological information.

8 THE INTERNET—WORLD WIDE WEB

We now take the Internet, and particularly the World Wide Web, for granted; it has become for most of us a key daily interface with the outside world. It acts as the portal to many of the resources that have been described in this chapter—the primary scientific journals, many of the expert review publications, access to the various databases and databanks. It also provides access via general search engines such as Google (*www.google.com/*), Yahoo! (*http://yahoo.com/*) or AltaVista (*www.altavista.com/*) to an unimaginably huge repository of information from all manner of organizations, companies, individuals, pressure groups and others from around the world. A general search for toxicological data can be useful, using chemical names, CAS Registry number or other chemical identifier. The crucial thing here is to validate what is being viewed—essentially anybody can post information on the Internet and it is easy to disguise spurious information as being legitimate.

9 ACCESSING THE RESOURCES

As we have seen, there are now many entry points to the various toxicological resources available—be it direct to a particular provider's web site, via the compilation of several sources into a search portal, or through a commercial host provider; the bewilderment from choice seems to be a modern phenomenon. There is always a continuing desire to collate information into user-friendly interfaces, and several are worthy of note in the field of toxicology.

The NLM is, as is clear, a big player in toxicological resources. Its freely available TOXNET Toxicology Data Network (*http://toxnet.nlm.nih.gov/*) brings together the key resources it provides in this area, including the TOXLINE, HSDB, CCRIS, GENE-TOX, IRIS and ITER databases/banks, amongst others. Even more advanced is its ChemID*plus* web-based search system (*http://chem.sis.nlm.nih.gov/chemidplus/chemidheavy.jsp*) that provides access to structure and nomenclature files used for the identification of chemical substances cited in the NLM databases, including those from TOXNET. ChemID*plus* provides structure searching and direct links to many biomedical resources, both within NLM and on the Internet. The system contains over 380 000 chemical records, of which around 270 000 include chemical structures. It is searchable by name, synonyms, CAS Registry numbers, molecular formula, chemical structure and/or biological or chemical properties.

The INCHEM interface (*www.inchem.org/*) is the IPCS portal to the various documents and expert reviews with which it is involved, including IARC Monographs, EHC documents, CICADs, JECFA and JMPR monographs and evaluations, and SIDS/SIARs for HPV chemicals.

Other players have also done their utmost to make our lives easier—the OECD's eChemPortal (*http://webnet3.oecd.org/echemportal/*) provides access to a range of databanks, including those of the IPCS (INCHEM), SIDS, and the European Commission (the European Chemical Substances Information

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System—ESIS (*http://ecb.jrc.ec.europa.eu/esis/*)). It is this clever packaging and repackaging of resources that is likely to remain a feature of our lives as toxicologists for many years to come.

10 CONCLUSION—QUALITY AND QUANTITY

With the huge growth in information and the diversification of media and speed by which it is distributed, it is easy to lose sight of issues of quality. The proliferation of new sources not only brings tremendous advantages in terms of accessibility and speed, but also introduces new problems, in particular those of accuracy and verification. More is not necessarily better, and it is particularly important these days to consider how the information is being produced and compiled, and by whom. Unfortunately, the tremendous increase in toxicological data in recent times has not been matched by better quality control or analysis. Information is frequently misapplied or misunderstood. Expert selection and evaluation are something that comes at a price, and the adage 'you get what you pay for' is an important one in the area of toxicological information. It is no easy task to assimilate a focussed set of quality information from a diverse set of scientific disciplines, and particular expertise is required to add significant value to that information such that it becomes meaningful within the context in which it is being sought.

Another difficulty is presented by the nature of the science. Toxicology is often described as an 'art' as well as a science. The large subjective component in the interpretation of toxicological data can lead to conflicting views among experts in the field. Many of these areas are controversial and are thus open to political factors. Toxicology impinges on so much of our daily lives that it perhaps suffers from this more than many other sciences. The issue of independence can thus be an important factor.

It is difficult to predict what future moves will be made in information resources for toxicology. What is certain, however, is that the electronic revolution will continue to make a big impact. Predictions of the imminent demise of traditional methods such as the hard copy publication of primary journals have not yet been realized, however. The printed textbook and journal article are still popular media. The most important changes in information resources for toxicology are likely to be those prompted by the increasing specialization within the science and, of growing necessity, those of information quality control and analysis.

There has always been a move towards trying to provide data sources that can be readily accessible to the end (perhaps nonexpert) user. The danger is that the layman may not appreciate the issues of selectivity, validation and evaluation, and the intricacies of chemical searching that are crucial to the effective and appropriate use of toxicological information. The hope is that the future will see a growing understanding of the benefit of quality, value-added resources over poor-value quantity.

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Toxicities Associated with Parenteral Nutrition

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CONTENTS

1 INTRODUCTION

Parenteral drug and nutritional therapies are mainstays of modern medicine. Parenteral nutrition contains both the macronutrient building blocks and the micronutrients required for optimal human nutrition. Prior to the development of modern parenteral nutrition formulations in the late 1960s, patients with short-bowel syndrome and functional gastrointestinal diseases died of malnutrition. Today, more than 40 000 patients in the United States are permanently dependent on parenteral nutrition to survive (Mullady and O'Keefe, 2006). Many tens of thousands more are sustained for finite periods of time with complex nutritional therapy via the intravenous route. Despite the unquestionable value of parenteral nutrition for patients with structural or functional intestinal diseases, life-threatening complications related to the presence of unintended chemical and microbial contaminants are well recognized. In addition to these iatrogenic and often preventable toxicities, insufficient knowledge of the optimal dosing and ratios of parenteral nutrition additives may pose risks to human health. In the case of both contaminants and supplements added in potentially toxic quantities, infants and children represent a particularly vulnerable population. This chapter will review the potential toxicities associated with both unintended contaminants in parenteral nutrition, including microbes, phthalates, bisphenol-A (BPA), particulates, lipid-peroxidation products and aluminium, as well as intentional supplementation with potentially toxic substances, including trace metals, amino-acid mixtures and lipid emulsions (**Table 1**). Special attention will be paid to parenteral nutrition-associated liver disease (PNALD), which remains an unpredictable, poorly understood and potentially life-threatening complication of prolonged exposure to parenteral nutrition.

2 CONTAMINANTS IN PARENTERAL SOLUTIONS

Parenteral nutrition, while life-saving, carries the potential for serious risks to human health related to the presence of unintended contaminants. Parenteral contaminants that can affect patient safety include microbes, phthalates, BPA, particulates, lipid peroxidation products and aluminium (**Table 1**). The toxicities associated with these contaminants are discussed in detail below.

2.1 Microbial Contamination

Blood-stream infections are associated with significant morbidities and mortality. Microbial contamination of intravenous solutions has been uncovered during investigation of some hospital outbreaks of septicaemia. The

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Table 1 Parenteral toxicities

Contaminants in parenteral solutions
Microbes
Phthalates
Bisphenol-A
Particulates
Lipid peroxidation products
Aluminum
Potentially toxic components in parenteral nutrition
Amino acids
Lipid emulsions
Trace metals
Iron
Chromium
Copper
Manganese

products most likely to be the source of parenteral microbial contamination are blood products, including red blood cells and clotting-factor concentrates, heparin–sodium chloride solutions and components of parenteral nutrition, particularly lipids (Vonberg and Gastmeier, 2007). The most common patient-care settings are haematological–oncological departments and intensive care units. The neonatal intensive care unit is a unique high-risk environment, where prolonged use of parenteral nutrition and high utilization of blood products are coupled with a relatively immunocompromised patient population.

In a review by Vonberg and Gastmeier (2007), accidental microbial contamination of parenteral nutrition was associated with a high mortality. The ability of parenteral nutrition to support the growth of organisms is dependent on the nutritional composition of the solution. Increased parenteral caloric intake, particularly the administration of large concentrations of intravenous lipids, increases the risk for bloodstream infections in patients receiving parenteral nutrition (Vonberg and Gastmeier, 2007). Mortality rates as high as 22–50% have been reported in patients receiving contaminated lipid-containing intravenous products, such as total parenteral nutrition (TPN) or propofol. This is significantly higher than the associated mortality rate of under 10% in patients who received contaminated parenteral solutions that did not contain lipids (Vonberg and Gastmeier, 2007). Lipid emulsion has been shown to support pathogens at 25 °C (Jarvis and Highsmith, 1984).

The spectrum of pathogens involved in hospital-acquired outbreaks depends on the type of contaminated product administered. The pathogens most commonly associated with contaminated blood products include bacteria, such as *Yersinia enterocolitica* and *Serratia* spp., and viruses, such as hepatitis A, hepatitis C and parvovirus B19. Contamination of blood products with human immunodeficiency virus (HIV) is rare in the current era of universal screening. The most likely microbial contaminants in parenteral nutrition are *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Staphylococcus saprophyticus*, *Enterobacter cloacae*, *Klebsiella oxytoa*, *Serratia marcescens*, *Acinetobacter calcoaceticus*, *Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Flavobacterium* spp. and *Candida albicans*. These organisms have been implicated in >95% of cases of blood-stream infections associated with contaminated parenteral nutrition (Habsah *et al*., 2005). *Candida parapsilosis* has also been detected in batches of parenteral nutrition products after routine monitoring using randomly amplified polymorphic DNA (RAPD) (Marais *et al*., 2004). Outbreaks of *Enterobacter* spp. septicaemias have been described in neonatal intensive care units in Brazil caused by microbial contamination of TPN solutions (Campos *et al*., 2007; Tresoldi *et al*., 2000). In Malaysia, a neonatal intensive care unit outbreak of *Pantoea* spp. sepsis in eight patients was attributed to the administration of contaminated parenteral nutrition. Seven of the eight infected neonates died (Habsah *et al*., 2005). Systemic infections and central venous catheter colonization with the lipophilic yeast, *Malassezia furfur*, has also been described in neonatal intensive care unit patients receiving lipid emulsions as part of their TPN (Aschner *et al*., 1987).

Guidelines have been established by the American Society of Health-System Pharmacists (2000) for the preparation of parenteral nutrition admixtures. Sterility and quality-assurance measures to avoid extrinsic contamination and to ensure accurate delivery of parenteral nutrition additives must be in place if an automated compounding device is used for preparation of parenteral nutrition (Kochevar *et al*., 2007). Contamination can occur through a variety of mechanisms including: (i) the use of contaminated components during manufacturing, (ii) inadequate or inappropriate aseptic technique for solution preparation, (iii) failure to sterilize the solution, (iv) contamination of the lipid emulsion or the dextrose solution, (v) contamination of the solution during storage, (vi) administration problems and (vii) an ascending method of infection (Tresoldi *et al*., 2000). The mortality and morbidities associated with microbial contamination of parenteral nutrition solutions are preventable. Standardized procedures, continuous quality-improvement initiatives and on-going surveillance should eliminate this source of iatrogenic morbidity.

The use of multidose drug vials deserves special mention. Numerous preventable infections and unnecessary deaths have been traced to the practice in hospitals of using drug vials intended for single use for multiple patients in an attempt at cost containment (Vonberg and Gastmeier, 2007). The Centers for Disease Control and Prevention in the United States and most manufacturers

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warn against this practice. Furthermore, the cost effectiveness of using manufacturer-intended multidose vials has been called into question (Sheth *et al*., 1983).

2.2 Phthalates

Polyvinyl chloride (PVC)-based medical devices contain 20–40% (by weight) di-(2-ethylhexyl) phthalate (DEHP) (Kavlock *et al*., 2006). DEHP is the only plasticizer or phthalate approved by the United States Food and Drug Administration (FDA) for medical devices (Calafat *et al*., 2004). DEHP provides PVC with its desired properties of flexibility, strength, usability at a range of temperatures, ability to withstand sterilization, resistance to kinking, optical clarity, weldablity, barrier capability and centrifugability (Kavlock *et al*., 2006). PVC-containing devices include blood bags, tubing for intravenous (iv) therapy, ventilator tubing, extra corporeal membrane oxygenation (ECMO) circuits, enteral and parenteral nutrition containers, and oxygen and suction equipment (Pak *et al*., 2007). DEHP is not bound to PVC and is released from these devices. The rate at which it is released is dependent on the temperature at which the device is stored and used, the storage time, the flow rate through the tubing, the percentage of DEHP in the PVC and the presence of lipids in the solution stored in the device (Calafat *et al*., 2004). DEHP is lipophilic in nature and will dissolve in solutions containing lipids such as parenteral nutrition, lipid emulsions, plasma, platelets and blood. Extraction of DEHP from PVC devices is increased when comparing parenteral nutrition containing lipids to formulations of parenteral nutrition with no added lipids (Pak *et al*., 2007).

In experimental animals, DEHP has been shown to produce a wide range of adverse effects, including liver toxicity and testicular atrophy. The ability of DEHP to produce adverse effects in humans is controversial and has been an active topic of debate in scientific and regulatory circles. The FDA Center for Devices and Radiological Health (CDRH) performed a safety assessment to determine the tolerable intake (TI) values for DEHP and develop an index of safety or risk in patients exposed to DEHP (*http://www.fda.gov/cdrh/ost/dehp-pvc.pdf* , accessed 8 April 2009). A TI value is defined as the dose of a compound that is not expected to result in adverse effects following exposure for a defined period. Based on the results of the safety assessment, CDRH concluded that the amount of DEHP released from PVC bags used to store and administer crystalloid fluids or intravenous drugs poses little to no risk to patients. The possibility of risk of potentially toxic DEHP exposure in patients receiving TPN with lipids is age-dependent. While the dose of DEHP received by adult patients receiving TPN admixtures is estimated to be less than the TI, the dose of DEHP received by neonates undergoing TPN supplementation is uncertain, with at least some, but not all, studies suggesting that neonates can receive very high doses of DEHP. Calafat *et al*. (2004) reported premature infants who received iv therapy for more than two weeks had urinary levels of mono-(2-ethylhexyl) phthalate (MEHP) that were approximately 26 times higher than that compared to the median for children aged 6–11 years who experienced only common environmental exposures to DEHP (Calafat *et al*., 2004). Exposure to DEHP from sources besides for TPN commonly utilized in neonatal patients requiring intensive care raises the overall level of concern about increased risk of DEHP-mediated adverse effects in critically ill neonates. One such source is transfusion of blood products. Relatively high doses of DEHP can be received by patients who are transfused with large volumes of blood and blood products over a short period. Thus, neonates undergoing exchange transfusion or ECMO, as well as trauma or surgical patients of any age requiring massive blood transfusions, might be at risk.

DEHP is hydrolysed rapidly to MEHP, which is then oxidized through a multistep pathway to other metabolites including mono-(2-ethyl-5-hydroxyhexyl) phthalate (mEHHP) and mono-(2-ethyl-5-oxohexyl) phthalate (mEOHP). mEHHP and mEOHP are excreted in the urine and faeces either as the free form or as conjugates. Exposure to DEHP can be assessed by monitoring urinary levels of MEHP, mEHHP and mEOHP. MEHP can also be formed exogenously by lipase enzymes in stored plasma or blood or by hydrolysis in stored and heated iv fluid, resulting in conversion of released DEHP in stored blood, plasma or iv fluids to MEHP before reaching the patient. Exposure to MEHP is important since this compound is thought to be more toxic than DEHP.

2.2.1 Special Considerations in Children

Children may be more susceptible than adults to the toxic effects of DEHP. Several studies show that testicular toxicity occurs in prepubertal rats at lower oral doses than is required to produce this effect in sexually mature rats (Gray *et al*., 1977; Sjoberg *et al*., 1985b; Dostal *et al*., 1988). However, this age-related effect in testicular toxicity was not seen following intravenous administration of DEHP to rats (Sjoberg *et al*., 1985a). Various physiological factors and metabolic differences between children and adults may result in increased sensitivity of children to DEHP. Compared to adults, children (especially neonates) have a reduced glucuronidation capacity. Approximately 60% of DEHP is excreted in humans as the glucuronide conjugate (Albro *et al*., 1982). Reduced glucuronidation capacity could result in delayed excretion of DEHP. Bilirubin is excreted as a glucuronide conjugate. The DEHP metabolite, MEHP, also undergoes glucuronidation and has been shown to interfere with bilirubin conjugation (Sjoberg *et al*., 1991), posing

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a particular hazard to neonates who are at risk of kernicterus.

Lipase enzymes in the gastrointestinal tract convert DEHP to the toxic metabolite, MEHP. Infants have high gastric lipase activity to aid in the digestion of fats in milk (Hamosh, 1996; Lee *et al*., 1993), raising the possibility that infants convert DEHP to MEHP more efficiently than do older children or adults. An increased permeability of the blood–testis barrier in children as compared to adults could result in increased exposure of the testes to DEHP or MEHP (Furuya *et al*., 1978). DEHP may exert toxic effects on the testes through depletion of zinc or Vitamin E. Both zinc and Vitamin E deficiencies are common in preterm infants and DEHP could exacerbate zinc and Vitamin E deficiency from other causes in preterm infants (Obladen *et al*., 1998; Chan *et al*., 1999).

Children typically receive a larger dose of DEHP than adults do when the dose is expressed on a $mg\,kg^{-1}$ basis. Further, medical procedures that are almost exclusively performed in children, such as ECMO and double-volume exchange transfusion, can result in potentially large DEHP exposures. Critically ill infants spend weeks to months in the neonatal intensive care unit exposed to multiple medical devices, many of which are made of plastic and contain PVC (Calafat *et al*., 2004). It has been speculated that the well-known effects of DEHP on hepatic enzyme activity contribute to the unexplained hepatobiliary dysfunction in neonates requiring TPN (Loff *et al*., 2000).

In 2000, the first expert panel of the National Toxicology Program's (NTP) Center for the Evaluation of the Risks to Human Reproduction (CERHR) suggested that the DEHP intake for infants who are critically ill may be 2–3 orders of magnitude higher than the general population. The benefits of the medical interventions were recognized to outweigh the risks, but the panel expressed concerns that DEHP in male infants and toddlers may adversely affect the development of their reproductive tract (Calafat *et al*., 2004; Kavlock *et al*., 2006). In 2003, concerns were also raised by the American Academy of Pediatrics and the FDA, based on animal data documenting DEHP to be toxic to the male reproductive tract at doses nearing those resulting from intensive medical procedures in humans (Calafat *et al*., 2004; Shea, 2003). Further research on the effects of DEHP and safe alternatives to DEHP are needed.

2.3 Bisphenol-A

BPA is used in the production of polycarbonate plastics and epoxy resins, which have many applications, including as components of certain medical products and their packaging. On 14 April 2008, the NTP released a draft brief that raised concerns that exposure to BPA could be linked to developmental problems of the endocrine system in infants and young children. NTP released a final report on BPA on 3 September 2008. Following the release of the NTP report, public concern focussed on exposure to BPA from plastic baby bottles and the linings of baby-food containers. However, exposure from parenteral sources represents an unknown risk. There are many FDA-regulated products that are known to contain BPA including medical devices and biological products such as blood products, vaccines and drugs. Unfortunately, information on the presence and levels of BPA for products with direct patient contact is largely unknown. Important areas for future study include the rate and extent of BPA release from devices and biologics, the conditions that affect the release and leaching of BPA, estimates of patient exposure to BPA from parenteral exposures, possible alternatives to BPA in medical devices, and studies that evaluate the toxicity or justify the safety of BPA in these products Department of Health and Human Services, Food and Drug Administration (2008).

2.4 Particulates and Precipitates

Safe administration of parenteral nutrition depends upon knowledge of the stability and compatibility of its various components. Both the stability and compatibility of parenteral nutrition solutions are affected by factors such as the infusion container, administration set, filter and reactive, but necessary, additives (Driscoll, 2003). The simple act of opening multiple commercial injectables that compose a parenteral admixture begins the degradation of these individual components. Complex reactions begin to occur when they are combined into a single container. The responsibility rests on the pharmacist to assign an expiration date that is based on the best available information to ensure degradation does not proceed to a point where infusion is clinically dangerous (Driscoll, 2003).

2.4.1 Problems with Instability

Instability of parenteral nutrition admixtures and breakdown of nutritional components over time may lead to formulation failure or disruption (Mirtallo *et al*., 2004; Driscoll, 2005). Certain amino acids, intravenous fat emulsions (IVFE) and vitamins are subject to instability (Mirtallo *et al*., 2004).

Vitamins are susceptible to physicochemical instability in parenteral nutrition formulations. Several vitamins are known to degrade over time and in the presence of oxygen. Very few have a clinically significant impact in the acute-care setting. However, when the patient is dependent on long-term TPN and body reserves are low, the impact can be significant. The best example of this is the degradation and absorption of vitamin

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A into the plastic matrix of the infusion container, following preparation of TPN in weekly batches. In a case report, a home TPN patient developed night blindness within six months. The patient was treated with a large intramuscular dose of vitamin A and symptoms resolved. Following a relapse, daily dosing of vitamins was provided and the problem resolved (Mirtallo *et al*., 2004).

Ascorbic acid, or vitamin C, when added in batch fashion, is degraded and results in the formation of large particulates. Degradation is avoided by adding such vitamins just prior to infusion (Mirtallo *et al*., 2004). Protection from light may also reduce degradation; it has been shown that vitamin C degrades with phototherapy (Smith *et al*., 1988).

Another potential toxicity associated with vitamin C administration is the formation of oxalate particulates. Oxalate ($C_2H_2O_4$) is a relatively strong dicarboxylic acid with a molecular weight of 90.04 Da. Oxalate is derived directly from ascorbic acid, glyoxalate, glycolate and glycoaldehyde. Glycine, hydroxyproline, ethanolamine, hydroxypyruvate and xylulose can also serve as indirect precursors of oxalate formation (Rockwell *et al*., 1998). Oxalate particulates have been described in parenteral nutrition solutions and have been associated with catheter occlusions (Das, 1986), and nephocalcinosis (Rockwell *et al*., 1998) in neonatal patients receiving parenteral nutrition. TPN solutions contain the oxalate precursors ascorbate and glycine. Glycine is present in all commercial amino-acid solutions. Ascorbic acid is a component of the intravenous multivitamin formula for parenteral nutrition (M.V.I. Pediatric; Armour Pharmaceutical, Kanakee, IL, USA). Increased urinary oxalate excretion has been reported in infants receiving TPN (Campfield and Braden, 1989). Degradation of parenteral ascorbate has been reported to cause elevated serum oxalate concentration and systemic oxalosis in adults with renal insufficiency (Balcke *et al*., 1984; Swartz *et al*., 1984) and in patients receiving long-term TPN (Buchman *et al*., 1995).

Trace metals, particularly copper, iron and zinc, potentiate ascorbate degradation to oxalate (Kassem *et al*., 1969). In contrast, ascorbate is protected by the presence of amino acids, especially cysteine. Unfortunately, routine amino-acid solutions typically used in TPN contain relatively little cysteine. Thus, in part to protect neonates from the metabolic load associated with oxalogenesis, supplemental cysteine beyond that provided by amino-acid solutions is recommended for infants receiving TPN.

2.4.2 Problems with Incompatibility

Incompatibility generally involves the coexistence of two or more electrolytes in solution that leads to precipitation of an insoluble product (Driscoll, 2005). The most dangerous risk of incompatibility in parenteral nutrition formulation is the development of macroprecipitates exceeding 5 μm. These macroprecipitates may subsequently pass into the central circulation causing vascular accidents. Two forms of precipitates can appear in a prepared formulation—solid and liquid (Mirtallo *et al*., 2004).

2.4.2.1 Solid Precipitates

Solid precipitates develop when incompatible combinations of salts are added to a parenteral nutrition formulation (Mirtallo *et al*., 2004). Perhaps one of the most lethal consequences of mishandling TPN is the formation of rigid, crystalline precipitates. This can be the result of: (i) poor compounding techniques, (ii) inadequate knowledge of the admixture components and (iii) assignment of inappropriately long expiration dates (Driscoll, 2003).

Calcium salts are very reactive and form insoluble products with numerous additives (Mirtallo *et al*., 2004). Most notorious is the precipitation of insoluble dibasic calcium phosphate formed as a result of either poor compounding technique or inadequate knowledge of admixture components or patient condition (Driscoll, 2003). Dibasic calcium phosphate precipitates have resulted in embolic deaths. This can be avoided through a variety of measures, which include the choice of calcium gluconate as the preferred form of calcium in multicomponent parenteral nutrition formulations. Order of compounding is important to avoid formation of insoluble precipitates—generally phosphate is added first and calcium is added closer to the end to benefit from the larger volume of solution (Mirtallo *et al*., 2004). The methods for determining calcium-phosphate precipitation vary widely and rely on unaided and aided visual detection using high-intensity lamps against a contrasting background or Tyndall beam, turbidimetric analysis, polarized light microscopy and, to a limited extent, the actual counting and sizing of particulate growth over time using light obscuration. The United States Pharmacopeia, the British Pharmacopoeia and the European Pharmacopoeia state that for the determination of particulate matter in aqueous injectables, the measurement can be made to meet pharmacopeia specifications using light obscuration (the reference method) or a microscopic particle count test (Driscoll, 2005).

There are alternative parenteral salts that may reduce the risk of calcium-phosphate precipitates. Organic phosphates such as glucose-1-phosphate, fructose-1,6-diphosphate and glycerol phosphate have been shown to be compatible with organic calcium. Glycerol phosphate is the most commonly used; the organic phosphates are not readily available or approved worldwide (Driscoll, 2005).

Bicarbonate salts present risks for precipitation as they react with calcium to form an insoluble precipitate, calcium carbonate. Therefore, bicarbonate salts should not be added to TPN solutions. If an alkalinizing salt is needed, sodium acetate or potassium acetate should be

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used. Acetate salts are frequent components in neonatal TPN.

Ascorbic acid in supraphysiologic doses to achieve an antioxidant effect is highly unstable. It readily degrades in the presence of oxygen to form oxalic acid. Oxalic acid is highly reactive with calcium and will form an insoluble precipitate, calcium oxalate. For this reason, ascorbic acid should be administered as a separate infusion (Mirtallo *et al*., 2004).

2.4.2.2 Liquid Precipitates

Phase separation with the liberation of free oil will result in liquid precipitation (Mirtallo *et al*., 2004). All IVFE are thermodynamically unstable and have short shelf lives (24 months). Instability of IVFE is manifested by an increasing population of large fat globules greater than 5 μm, a process known as coalescence (Driscoll, 2003). The major concern about destabilization of IVFE and formation of large fat globules is that lipid droplets greater than 5 μm can occlude the microvasculature (pulmonary capillaries) and cause embolic syndrome and death (Driscoll, 2005). Evidence in rats and guinea pigs has demonstrated tissue damage associated with unstable IVFE compared to stable IVFE (Driscoll, 2003). Egg lecithin is the primary emulsifier used in IVFE as a stabilizer and imparts a negative charge to the lipid droplets. The addition of cations (sodium, potassium, calcium, magnesium) impedes stabilization by neutralizing the droplet surface charge (Driscoll, 2005). The physicochemical limitation of the IVFE is clearly outlined by the manufacturer. The pharmacist should use this brand-specific information and not extrapolate to other products (Mirtallo *et al*., 2004). Appropriate methods for analysis of the stability of IVFE have been debated. The United States Pharmacopeia has proposed the light obscuration method with a pharmacopeia limit defined as the volume-weighted percentage of fat greater than 5 μm, or less than 0.05%, irrespective of the final lipid concentration (Driscoll, 2005).

Total nutrient admixtures containing fat emulsion, as well as other components of parenteral nutrition, such as carbohydrates, amino acids, vitamins, minerals and trace elements in a single container have been formulated for neonates and infants. However, given the limited amount of published stability information available, the practice guidelines from the American Society for Parenteral and Enteral Nutrition (ASPEN) recommend the use of 2-in-1 formulations with separate administration of IVFE for neonates and children (Mirtallo *et al*., 2004).

2.5 Lipid Peroxidation Products

Lipids are the primary source of energy for infants and young children and thus are an essential component of paediatric parenteral nutrition. Prior to the introduction of parenteral lipid emulsions in TPN in the 1960s, essential-fatty-acid deficiency was commonplace. Current lipid emulsions provide an important energy source, essential fatty acids and, in some countries, required lipid-soluble vitamins. However, concern has been raised about the potential toxic effects of lipid-peroxidation products, which can pose a particular hazard to premature infants with low antioxidant defences. Lipid emulsions for parenteral use can be peroxidized during storage; these peroxidation products can induce cell damage, at least *in vitro* (Pitkanen, 1992). Lipid peroxidation can also occur *in vivo* in adult and neonatal patients receiving parenteral lipid emulsions. Fat emulsions have been linked to increased oxygen-derived free-radical activity in infants (Hasanoglu *et al*., 2005). Lipid emulsions contain various amounts of polyunsaturated fatty acids (PUFAs), which can serve as substrates for the formation of lipid hydroperoxides by free radicals. Factors that increase lipid peroxidation include ambient light, phototherapy for hyperbilirubinaemia, contamination by air and presence of trace elements (Laborie *et al*., 2000).

As PUFA are essential components of biological membranes, their peroxidation *in vivo* may cause cellular damage. Lipid peroxidation has been implicated in the development of common complications of prematurity, such as bronchopulmonary dysplasia, necrotizing enterocolitis and retinopathy of prematurity, although a causal relationship between these neonatal morbidities and peroxidation of parenteral lipid emulsions has not been proven (Krohn and Koletzko, 2006). Studies showing that *in vitro* lipid peroxidation is inversely related to the α-tocopherol/PUFA ratio of the fat emulsion suggest that additional vitamin E may be beneficial (Pitkanen, 1992; Pironi *et al*., 2003). L-Carnitine was also shown to be effective in inhibiting lipid peroxidation of a linoleic acid emulsion (Gulcin, 2006). However, L-carnitine has not been shown to improve lipid tolerance, ketogenesis or weight gain in neonates receiving TPN (Cairns and Stalker, 2000). This study did not address the impact of carnitine supplementation on disease parameters related to oxidative stress in premature infants.

As noted above, exposure of TPN to ambient light generates organic peroxides (Neuzil *et al*., 1995; Silvers *et al*., 2001) and hydrogen peroxide (Laborie *et al*., 1998; Khashu *et al*., 2008). Furthermore, photosensitized riboflavin present in parenteral multivitamin preparations catalyses electron transfer between electron donors such as vitamin C, amino acids or lipids and dissolved oxygen, producing hydrogen peroxide (Laborie *et al*., 1998; Khashu *et al*., 2008). Light exposure also induces a loss of antioxidant vitamins in TPN (Chessex *et al*., 2007). Shielding TPN from light represents an approach to protect the solution from the generation of peroxides and by-products of lipid peroxidation (Laborie *et al*., 1999; 2000). It has been hypothesized that these reactive species may play an important role

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in the pathogenesis of various neonatal conditions, including bronchopulmonary dysplasia. Khashu *et al*. (2008) examined whether generation of oxidants in light-exposed TPN impacts lipid metabolism and impairs glucose uptake in preterm neonates. They reported slightly higher blood glucose and significantly higher triglyceride levels in preterm infants receiving light-exposed TPN, compared to a comparable group receiving light-protected TPN (Khashu *et al*., 2008). These same investigators reported that photoprotection of TPN solutions facilitated advancement of minimal enteral nutrition (Khashu *et al*., 2006) and, most provocatively, was associated with a 30% relative reduction in the incidence of death or bronchopulmonary dysplasia (Chessex *et al*., 2007). These results should be examined in a large, randomized, controlled trial. If replicated, the simple and inexpensive intervention of shielding TPN from light could have a major impact on the medical outcomes and healthcare costs associated with neonatal intensive care.

2.6 Aluminium

Aluminium is the third most abundant component of the earth's crust. It is a nonessential toxic metal with no known physiologic role. The gastrointestinal tract provides a protective barrier against the absorption of aluminium when consumed orally, with only 1% absorbed systemically. However, when delivered parenterally the gastrointestinal barrier is bypassed and aluminium is deposited in bone, liver, spleen, kidney, brain and other tissues. Aluminium contamination of parenteral nutrition and associated aluminium toxicity has been recognized since the 1980s (Gura and Puder, 2006; Advenier *et al*., 2003). Aluminium toxicity has been implicated in vitamin D-resistant osteomalacia, dementia and fracturing bone disease in dialysis patients and TPN-associated cholestasis in neonates. With a half-life of up to seven years, aluminium will remain in the body for an extended period of time (Gura and Puder, 2006).

Much of the raw material used to manufacture solutions used for intravenous nutritional support of hospitalized patients is contaminated with aluminium (Klein, 2005). Pharmaceutical manufacturers have only recently obtained the technology necessary to detect aluminium contamination of their products. Aluminium contamination in parenteral nutrition can be found in a variety of parenteral nutrition components (Gura and Puder, 2006). Casein hydrolysate, a common protein source in parenteral nutrition in the 1980s, was highly contaminated with aluminium. Since then, there has been a substitution of crystalline amino acids and a reduction of the aluminium burden (Klein *et al*., 1991; Vargas *et al*., 1988). Other additives, such as multivitamins, trace elements, calcium and phosphate salts, contain measurable amounts of aluminium (Gura and Puder, 2006) ranging from a concentration of $3–3439\,\mu g\,l^{-1}$ (Klein *et al*., 1991).

In July 2004, the United States FDA mandated that manufacturers measure aluminium content of parenteral nutrition products and disclose this information on the label (Klein, 2005). This regulation regarding aluminium content labelling applies only to products that are routinely used in the preparation of parenteral nutrition. Large-volume parenterals (dextrose solutions, sterile water for infusion, fat emulsions and crystalline amino acids) are required to limit their aluminium content to less than $25\,\mu g\,l^{-1}$ (Gura and Puder, 2006). The rationale for this recommendation originates from a 1986 report that demonstrated that teens and adults on long-term TPN receiving $25–30\,\mu g\,l^{-1}$ aluminium had no significant aluminium accumulation in bone, serum or urine (Heyman *et al*., 1986). This does not, however, take into consideration the aluminium contribution from small-volume parenterals, which can be significant (Klein *et al*., 1991). No upper limit for aluminium has been established for small-volume parenterals, such as trace elements, salts and multivitamins. These products are among those most highly contaminated with aluminium. Regulation simply requires that the aluminium content be labelled at the time of expiration. When the product contains less than $25\,\mu g\,l^{-1}$, it is sufficient to state 'contains no more than $25\,\mu g\,l^{-1}$ aluminium'. Those small-volume parenterals that exceed $25\,\mu g\,l^{-1}$ must list the maximum amount at expiration for each batch product (Gura and Puder, 2006). Although these regulations have been instituted with the goal of limiting exposure to less than $4–5\,\mu g\,kg^{-1}\,day^{-1}$, which is the maximum amount deemed 'safe' by the FDA, pharmacists are not required to notate the total aluminium content on the labels of patient parenteral nutrition containers (Gura and Puder, 2006).

Manufacturers of these solutions are required to provide the FDA with the validated method they are using to measure aluminium content, samples of their results and assurance that the method conforms to 'Good Manufacturing Practices' specified by the FDA (Klein, 2005). A statement must be added in the warning section of the package insert that states 'levels of aluminium greater than $4–5\,\mu g\,kg^{-1}\,day^{-1}$ accumulate at levels associated with central nervous system (CNS) and bone toxicity' (Driscoll and Driscoll, 2005). In other countries, limitations are different; the British Pharmacopoeia requires only that calcium chloride salts meet specific aluminium content limits (Gura and Puder, 2006).

Many factors affect the extent of aluminium contamination in the parenteral admixture (Gura and Puder, 2006; Klein *et al*., 1991). The source of raw materials may account for batch-to-batch variability of the same product manufactured by the same company. Glass and rubber

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contain more aluminium than plastic and play an important role with regards to container design. As aluminium may leach from glass containers, the quality of the glass is an important variable. The type of product contained is important, as calcium and phosphate salts have high affinity for aluminium and increase leaching from glass containers. Aluminium also leaches from glass according to the stability constant of the amino acids for aluminium with cysteine, cystine, aspartic acid and glutamic acid having the highest affinity. Heat sterilization enhances the leaching of aluminium from glass and rubber stoppers. The longer a product is on the shelf, the more potential there is for leaching of aluminium into the solution (Gura and Puder, 2006). Thus, aluminium contamination is unpredictable and highly variable (Klein *et al*., 1991).

Options for decreasing aluminium contamination include substituting potassium phosphate with sodium phosphate. More importantly, a change to sodium phosphate in plastic vials would decrease the leaching of aluminium from the glass containers. Another alternative is the use of calcium chloride in place of calcium gluconate, which is highly contaminated. However, this would predispose premature infants to hyperchloraemia and the associated metabolic acidosis. Koo *et al*. (1986b) have recommended using a 1:1 ratio of calcium gluconate and calcium chloride with sodium and potassium acetate instead of sodium and potassium chloride (Koo *et al*., 1986b). Finally calcium acetate is a consideration as it has minimal contamination, but it is less soluble and there are minimal data regarding compatibility (Gura and Puder, 2006).

2.6.1 Special Considerations in Infants and Children

There have been concerns about aluminium toxicity in infants and children receiving parenteral nutrition since the 1980s. Parenteral nutrition solutions are the main source of aluminium exposure in neonates, contributing as much as 88.7% of total aluminium intake among neonates in intensive care nurseries (Moreno *et al*., 1994). The major route of aluminium elimination is renal. Preterm kidneys have limited ability to excrete aluminium (Koo *et al*., 1986a). Compared with adults, preterm infants excrete only 23% of an intravenous aluminium load. Marked aluminium accumulation in bone, urine and plasma has been demonstrated in infants after only three weeks of TPN (Sedman *et al*., 1985). Long-term moderate aluminium exposure causes osteomalacia with alterations in bone formation and mineralization, parathyroid hormone secretion and urinary calcium excretion. Acute intoxication causes an encephalopathy. The risk of aluminium toxicity is dependent on the length of exposure and on renal function, although toxic levels have been observed in patients with normal renal function on long-term TPN.

Aluminium toxicity has been implicated in the pathogenesis of PNALD and cholestasis. Hepatic aluminium concentrations in children on TPN were noted to be 5–17 times normal (Klein *et al*., 1984). Furthermore, aluminium loading in piglets and rats causes hepatic accumulation and damage (Klein *et al*., 1984; 1987; 1988; 1989).

There are strong data supporting the adverse effects of aluminium on bone mineral content, metabolic bone disease and bone growth. TPN containing high concentrations of aluminium is associated with reduced serum 1,25 hydroxy-vitamin D (1,25(OH)2D) levels in serum in children (Klein *et al*., 1985). In adults on long-term TPN containing high aluminium concentrations, bone disease is manifest by long-bone pain and pain in weight-bearing joints, decreased bone-formation rate and patchy osteomalacia. Children develop evidence of radiographic rickets and bone fractures (Klein *et al*., 1982). In one study, bone formation varied inversely with both plasma aluminium and bone-surface aluminium, suggesting that aluminium acquired during TPN can reduce bone formation and lead to patchy osteomalacia (Vargas *et al*., 1988).

Aluminium can be toxic to the CNS of both preterm and term newborns. High brain-tissue levels of Al have been identified at autopsy (Popinska *et al*., 1999). In one highly concerning randomized, controlled trial comparing infants fed aluminium-depleted TPN to those on standard TPN, infants receiving standard TPN had significantly lower mental developmental indices, with an adjusted loss of one point per day of TPN (Bishop *et al*., 1997). This finding, reported in 1997, requires replication. If reproducible, stricter limitations on aluminium content in parenteral formulations for neonates should be mandated or techniques for aluminium depletion should be incorporated into routine parenteral nutrition administration practices.

Questions remain as to 'safe' levels of aluminium in intravenous solutions for various patient populations. The FDA defined the maximum safely tolerated amount as $5\,\mu g\,kg^{-1}\,day^{-1}$, noting that amounts in excess may cause CNS or bone toxicity. It is concerning that aluminium intake from parenteral nutrition solutions in neonates has been reported to be as high as $6.6–16.7\,\mu g\,kg^{-1}\,day^{-1}$ (Moreno *et al*., 1994; Popinska *et al*., 1999). ASPEN defined aluminium intakes as safe when they are $<2\,\mu g\,kg^{-1}\,day^{-1}$; a dose that does not result in accumulation in tissues and fluids. ASPEN defined unsafe aluminium content as $2–15\,\mu g\,kg^{-1}\,day^{-1}$, as this is an amount that results in tissue loading, but no documented toxicity. Toxic levels are defined as $60\,\mu g\,kg^{-1}\,day^{-1}$, which is a dose that results in tissue accumulation and symptoms of toxicity (Klein *et al*., 1991). For term and preterm infants, a 'safe' dose has not been established. However, based on the literature, it appears that neonates are being exposed to greater aluminium loads than are known to be safe for adolescents and adults. In the absence of

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reliable label information and to prevent iatrogenic toxicity, the aluminium content of parenteral nutrition should be assessed regularly. Aluminium contamination of TPN additives is an under-recognized danger to TPN patients, with infants on prolonged TPN at greatest risk.

3 POTENTIALLY TOXIC COMPONENTS IN PARENTERAL NUTRITION

In addition to toxicity associated with the presence of inadvertent contaminants in parenteral nutrition, risks may be associated with intended components, such as amino-acid mixtures and lipid emulsions. Deliberate supplementation of parenteral nutrition with essential trace metals may also pose risks when present in high concentrations or when the underlying health status of the patient alters the metabolism or requirements of these trace elements.

3.1 Amino Acids

The goals of parenteral nutrition amino-acid preparations are to provide essential amino acids of sufficient quantity and appropriate composition to support normal growth and a normal plasma amino-acid profile. The earliest parenteral amino-acid preparations were protein hydrolysates, with fixed amino-acid compositions. Complications from infusions of these early protein hydrolysates included hyperammonaemia, acid–base disorders and allergic reactions, particularly in paediatric and neonatal patients (Storm and Helms, 2007).

The 1970s ushered in the pharmaceutical-grade crystalline amino-acid formulations which were based on adult amino-acid requirements. When these adult parenteral crystalline amino-acid formulations were administered to infants, new complications related to the amino-acid composition of these adult formulations occurred. Manufacturers had ignored the growing body of literature suggesting that paediatric parenteral amino-acid requirements were different to adult amino-acid requirements. For example, it was already known that phenylalanine hydroxylase activity was absent or low in infants and young children, making tyrosine a conditionally essential amino-acid in paediatric populations (Raiha, 1973). Likewise, cystathionase is not fully functional in neonates and infants, making cysteine and taurine essential amino-acids (Sturman *et al*., 1970). Winters *et al*. (1977) documented the abnormal plasma amino-acid profile of infants receiving any parenteral amino-acid formulation that had been developed based on the amino-acid requirements of adults. The abnormal profiles included low levels of branched-chain amino acids, abnormally low plasma levels of cystine and high normal to elevated levels of methionine. Plasma levels of phenylalanine were in the low normal range, but plasma tyrosine levels were consistently below normal. The total glutamine and glutamic acid composition was also found to be below the requirements needed in childhood (Winters *et al*., 1977). The conclusion was that creation of paediatric-specific amino-acid formulations was needed.

The ultimate result was the introduction of F-14 or TrophAmine in July 1984. TrophAmine was the culmination of many phases of formulation development, normal volunteer testing, plasma amino-acid profile determination and reformulation to engineer the first successful paediatric amino-acid formulation (Storm and Helms, 2007). Compared with adult formulations, TrophAmine contained increased essential amino-acid content, increased branched-chain amino-acid content, a broader nonessential amino-acid content which included aspartic acid, glutamic acid and taurine, with modifications in the level of almost every amino acid compared with that in adult amino-acid formulations. Also, the pH of the amino-acid formulation was lowered to 5.5 to enhance calcium and phosphate solubility (Storm and Helms, 2007). Further enhancements included the addition of *N*-acetyl-L-tyrosine (NAT) and L-cysteine-HCl. NAT, a soluble and stable source of tyrosine, resulted in normal or near-normal plasma tyrosine levels in paediatric patients. L-Cysteine-HCl both enhanced plasma cystine levels and further lowered the pH, thus improving calcium and phosphate solubility, which is important in achieving the increased calcium and phosphate requirements of infants and children receiving parenteral nutrition. Use of this paediatric formulation in term infants achieved normalization of all plasma amino acids at an intake of $2.4\,g\,kg^{-1}\,day^{-1}$, with the exception of tyrosine, and a positive nitrogen balance. The new paediatric amino-acid formulations also improved growth and nitrogen balance and resulted in more normal amino-acid profiles in premature infants compared with the traditional adult formulations (Helms *et al*., 1987).

The newly approved paediatric amino-acid formulations, TrophAmine (McGraw, Bethlehem, PA) in 1984 and Aminosyn PF (Abbott, Abbott Park, IL) in 1985 represented a significant advancement in the delivery of paediatric parenteral nutrition. However, as is often the case in paediatric medicine, a one-size-fits-all approach is rarely optimal. Scrutiny of these formulations in low-birth-weight premature infants revealed plasma phenylalanine levels that were more than two standard deviations above normal and poorer utilization of cysteine and NAT as tyrosine (Heird *et al*., 1988; Adamkin *et al*., 1991). Future improvements are needed in formulations for preterm infants to address their higher tyrosine requirements and poor retention of NAT.

The problem of PNALD and the identification of the inciting ingredient(s) contributing to this persistent problem in neonatal medicine have yet to be resolved. In

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its most severe form, it can lead to hepatic failure and death or the need for liver transplant. The incidence of TPN-induced hepatic steatosis and cholestasis is clearly higher in the paediatric than in the adult population. It is correlated directly with the degree of prematurity and duration of parenteral nutrition, and inversely with the amount of enteral feedings tolerated. Compared with adults, young children conjugate more of their bile-acid pool with taurine instead of glycine. Therefore, higher concentrations of taurine have been incorporated into paediatric amino-acid formulations (Storm and Helms, 2007). A number of observational and retrospective studies suggested that use of the newer paediatric amino-acid formulations resulted in a relatively low rate of TPN-associated cholestasis that was most dependent on the length of TPN exposure, rather than the commercial product used (Beck, 1990; Storm and Helms, 2007; Forchielli *et al*., 1995), although at least one retrospective comparison study favoured TrophAmine over Aminosyn PF (Wright *et al*., 2003). An examination of the aetiology of hepatic failure among young children on liver transplant lists suggests that despite the introduction of paediatric-specific amino-acid formulations, PNALD remains a significant problem. Unfortunately, the role of amino-acid formulations vs. other TPN components, such as lipids and aluminium contamination, remains unresolved.

3.2 Lipid Emulsions

Most commercial fat emulsions used in TPN are composed of soybean oil or a combination of soybean oil and safflower oil in which long-chain triacylglycerols have been partially replaced with medium-chain triacylglycerols (MCTs) (Chen and Yeh, 2003). Lipids are necessary in parenteral nutrition-dependent patients due to their high caloric value and essential fatty-acid content. However, they too have been implicated in PNALD (Alwayn *et al*., 2005; Gura *et al*., 2008; Diamond *et al*., 2008). Soybean oils contain phytosterols, which are thought to have a deleterious effect on biliary secretion. Soybean oil-based ω-6-fatty-acid emulsions are not cleared in the same manner as enteral chylomicrons, which may lead to accumulation of lipids in the hepatic Kupffer cells, impairment of liver function and steatotic liver injury. Thus, the development of hepatic steatosis results from an imbalance in the rates of entry, synthesis or clearance of fat from the liver (Day and James, 1998). Standard lipid emulsions may also enhance the production of proinflammatory cytokines, because they are a relatively rich source of ω-6-fatty acids, which are the precursors to arachidonic acid (Mayer *et al*., 2003c; Fernandes, 1994).

The metabolism of lipids depends on their route of administration. Enteral lipid micelles are absorbed by the enterocyte, packaged into chylomicrons and released into the portal venous system for uptake in the liver. The emulsified particles of commercially available lipid emulsions mimic the size and structure of chylomicrons, but differ in their content. Artificial lipid particles, which primarily contain essential fatty acids and ω-6-triglycerides, are devoid of cholesterol or protein. These ω-6-fatty-acid-containing emulsions are dependent on lipoprotein lipase, apolipoprotein E and low-density lipoprotein receptors for clearance, and they release less essential fatty acids than do chylomicrons. These factors are thought by some to contribute to the steatohepatitis associated with the intravenous administration of the lipid emulsions most commonly used today (Allardyce, 1982; Colomb *et al*., 2000; Alwayn *et al*., 2005).

Until very recently, fish-oil emulsions for use in parenteral nutrition were not commercially available. In the United States, in 2008, they are considered experimental and require human-subjects review by institutional review boards. Fish oils are rich sources of ω-3-fatty acids, especially eicosapentaenoic acid (EPA; C20:5) and docosahexaenoic acid (DHA; C22:6). Numerous studies have demonstrated the hypotriglyceridemic, antiatherogenic and antihypertensive effects of oral dietary fish oils (Harris, 1989; Nestel, 1990; Knapp and FitzGerald, 1989; von Schacky, 1987). In contrast, the use of fish oil in human parenteral nutrition has not been well studied.

The mechanism of clearance of ω-3-fatty-acid-containing lipid emulsions is incompletely understood. In humans, the ω-3-fatty acids have been shown to accelerate the clearance of chylomicron triglycerides (Park and Harris, 2003). In animal models, ω-3-fatty-acid solutions have been shown to decrease lipogenesis and prevent or attenuate parenteral nutrition-induced hepatosteatosis in rats and guinea pigs (Van Aerde *et al*., 1999; Yeh *et al*., 1997). They also decrease interleukin 6 and other proinflammatory cytokine levels *in vitro* and *in vivo* (Mayer *et al*., 2002; 2003c; Rallidis *et al*., 2003) and displace arachidonic acid from tissue fatty-acid pools and inhibit the arachidonic-acid pathway of inflammation (Chen *et al*., 1992; Simopoulos, 2002). In 2005, Alwayn *et al*. investigated the role of ω-6-and ω-3-fatty acid emulsions in a murine model of nutritionally induced liver damage. They showed that mice exclusively fed a fat-free, high-dextrose liquid diet developed hepatic steatosis and essential-fatty-acid deficiency. Supplementation with ω-3-fatty acids by either the intravenous or enteral routes prevented dietary-induced hepatic steatosis. In contrast, supplementation with a conventional ω-6-lipid emulsion worsened hepatic steatosis (Alwayn *et al*., 2005). These data suggest that the content or the ratio of ω-6-:ω-3-fatty acids delivered intravenously is aetiologic in hepatic steatosis. It has been shown that a high polyunsaturated ω-6-:ω-3-long-chain fatty acid ratio is associated with nonalcoholic fatty-liver

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disease and may favour lipid synthesis over oxidation and secretion (Araya *et al*., 2004). The standard soybean-based lipid emulsion contains 15 times higher polyunsaturated ω-6-:ω-3-fatty-acid ratio compared with the ω-3-fish-oil-based fatty-acid emulsion and 11 times higher than the normal diet (Alwayn *et al*., 2005).

Despite experimental evidence that ω-3-fatty acids may be beneficial in preventing or reversing hepatic injury, the mechanisms for the salutary effects are poorly understood. Chen *et al*. (1996) described the effects that various fatty acid compositions had on rats that received TPN. They demonstrated a lower hepatic fat content in rats that received TPN containing lipid emulsions that were high in ω-3-fatty acids compared with those given TPN with fat emulsions derived from olive or safflower oils (Chen *et al*., 1996). Proposed mechanisms include stimulation of both peroxisomal and mitochondrial β-oxidation of fatty acids, thereby decreasing the availability of nonesterified fatty acids as substrates for triacylglycerol synthesis and secretion (Chen and Yeh, 2003; Burdge *et al*., 2003; Neschen *et al*., 2002; Willumsen *et al*., 1993). ω-3-fatty acids have also been shown to inhibit *de novo* lipogenesis in rat livers by reducing the activity of acetyl CoA carboxylase (Neschen *et al*., 2002). Other potentially protective properties of ω-3-fatty acids include their propensity to lower the activity of superoxide dismutase and glutathione peroxidase (Yeh *et al*., 1997), their limited ability to be esterified to glycerol (Rustan *et al*., 1988; Nossen *et al*., 1986) and their ability to reduce the synthesis of arachidonic acid (Chen *et al*., 1992; Alwayn *et al*., 2005).

Despite these promising human and animal studies, fish-oil-based lipid emulsions are not commonly used in medicine and have only recently become commercially available. Omegaven™ is a fat emulsion comprised of ω-3-fatty acids from fish oil produced by Fresenius Kabi AG (Bad Homburg, Germany). It is not approved for use in the United States. This or a similar ω-3-based lipid product has been used in Europe and Asia in adult trauma and surgical patients (Morlion *et al*., 1996; Grimm *et al*., 2006; Wichmann *et al*., 2007), adults with sepsis and systemic inflammatory response syndrome (SIRS) (Mayer *et al*., 2003a; 2003b), patients with inflammatory bowel disease (Grimminger *et al*., 1993a) and adults with psoriasis (Grimminger *et al*., 1993b; Mayser *et al*., 1998), atopic dematitis (Mayser *et al*., 2002) and rheumatoid arthritis (Proudman *et al*., 2008).

Paediatric experience with Omegaven™ is limited. It has been hypothesized that use of Omegaven™ in infants and young children with TPN-induced liver disease could potentially reverse or prevent the cholestasis and development of hepatic failure (Diamond *et al*., 2008; Gura *et al*., 2008). The published evidence is limited to a case report of two patients from Boston, both with severe end-stage liver disease who demonstrated reversal of PNALD, and in one case removal from the transplant list, following a switch from ω-6-fatty-acid-based lipid solution to Omegaven® (Gura *et al*., 2006). This was followed by a recently published case series from Boston of 18 patients treated with Omegaven™, all of whom demonstrated resolution of their PNALD (Gura *et al*., 2008). Clearly there is a need for well-designed prospective studies to determine the optimal approach to parenteral nutrition comparing ω-3-fatty-acid lipid emulsions, alone or in combination with ω-6-fatty-acid emulsions, to the standard ω-6 solution. Such a trial will not only answers questions as to the efficacy of ω-3-fatty-acid lipids, but also affords an opportunity for optimal safety assessment (Diamond *et al*., 2008).

3.3 Trace Metals

Until 30 years ago, trace elements were thought to be of little significance in human nutrition. It is now recognized that many factors can render individuals and population groups susceptible to disease resulting from trace element deficiencies or excess. However, there is insufficient knowledge about the nutritional requirements, causes and effects of micronutrient deficiencies and toxicities.

All nutrients have an optimal range of intake, with either deficiency or excess being potentially harmful. Clinical deficiencies have been described for six of the elements: iron, iodine, zinc, copper, chromium and selenium. The trace metals that are typically added to PN solutions include zinc, copper, chromium, manganese and selenium. Most often these trace metals are added as a cocktail containing fixed ratios of zinc, copper, chromium and manganese, with selenium often added separately. The goals for trace metal supplementation in parenteral nutrition should include prevention of trace-metal deficiencies and avoidance of toxicity from excess intake. Although trace-metal deficiencies have been commonly reported in patients dependent on prolonged parenteral nutrition, this chapter will focus on trace-metal toxicities associated with inadvertent excess concentrations of, or adverse reactions to, parenterally delivered trace metals.

3.3.1 Iron

Anaphylactic reactions have been reported following administration of iv iron dextran (Kumpf, 2003). Although the occurrence risk is low, the reaction can be severe. Therefore, a test dose is recommended prior to infusion of therapeutic iron dextran. Routine addition of iron to parenteral nutrition solutions is not recommended. Intravenous iron may worsen infection risk. Moreover, iron should not to be used in lipid-containing solutions because trivalent cations (Fe^{3+}) can destabilize lipid emulsions. Iron dextran has, on occasion, been added to lipid-free parenteral nutrition solutions, but iron overload is a risk, requiring periodic

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monitoring. An alternative to iron dextran is iron citrate, which does not carry the same risk of adverse reactions.

3.3.2 Chromium

Chromium is an essential trace metal important in glucose tolerance and lipid metabolism (Leung and Galbraith, 1995). Chromium deficiency during prolonged TPN is well documented. It causes hyperglycaemia and glycosuria, impaired growth and negative nitrogen balance. The current recommendation for chromium supplementation to TPN is $0.2\,\mu g\,kg^{-1}\,day^{-1}$ for paediatrics patients and $10–15\,\mu g\,day^{-1}$ for adults. This amount may exceed that required for sufficiency in neonates. Oral chromium is poorly absorbed; breast fed infants receive only $0.05\,\mu g\,kg^{-1}\,day^{-1}$. Chromium is excreted primarily via the urine. Therefore, reduction in dosage may be required in the face of renal dysfunction.

Chromium is a known contaminant of crystalline amino-acid solutions and chromium as a contaminant may provide amounts in excess of recommendations. Elevated chromium is associated with mutagenesis and carcinogenesis. The long-term use of multiple trace-element products at recommended doses has also been associated with excessive serum concentrations of chromium. Chromium was found in high concentrations in up to 94% of 382 patients on short-term TPN (Leung and Galbraith, 1995) with approximately 50% having serum levels greater than 10-fold of normal. The source of excess chromium was found to be the amino-acid solution. Elevated chromium levels have been reported in TPN patients in Germany, the United Kingdom and the United States (Leung, 1995). Chromium excess has also been reported in children receiving TPN contaminated with chromium from amino acid and phosphate additives (Moukarzel *et al*., 1992; Bougle *et al*., 1993). It has been suggested that chromium supplements may not be needed in routine TPN administration. Further study is required to determine the effects of high concentrations of chromium during prolonged parenteral nutrition.

3.3.3 Copper

Copper is an essential constituent of many enzymes, including the important antioxidant enzyme, Cu-Zn superoxide dismutase and cytochrome oxidase, the terminal oxidase in the electron transport chain. Normal copper levels and homeostasis are essential for normal growth and development, as demonstrated by the inborn errors of copper metabolism: Wilson's disease and Menkes disease. In Menkes disease, mutations in the ATP_7A gene results in generalized copper deficiency, neurological dysfunction and connective tissue disease. In Wilson's disease, mutations in the ATP_7B gene cause copper accumulation in the liver and copper overload. Wilson's disease has facilitated the recognition of copper as a highly toxic trace metal with significant oxidizing potential (Houwen, 2008).

Copper deficiency in patients receiving unsupplemented TPN is well documented and can cause neutropenia and anaemia unresponsive to iron therapy (Leung and Galbraith, 1995). In infants, copper deficiency also causes osteoporosis and delayed bone growth. Copper toxicity is rare, but can be seen in infants on parenteral nutrition who also have liver disease, as 80% of copper is eliminated via biliary excretion. Excess copper intake is thought to contribute to liver cirrhosis. Current TPN recommendations call for $20\,\mu g\,kg^{-1}\,day^{-1}$ for preterm and term infants with normal biliary excretion and 0.5–1.5 mg daily in adults. Higher amounts may be needed in patients with excess gastrointestinal losses. Copper should be withheld when hepatic cholestasis is present.

3.3.4 Manganese

Manganese is an essential metal required for normal growth, development and cellular homeostasis. However, exposure to high manganese concentrations can result in manganese deposition in the brain and an irreversible psychological and neurological syndrome known as manganism. Manganism, which clinically resembles Parkinson's disease, is associated with destructive symmetric lesions in the globus pallidus, substantia nigra, subthalamic nucleus and, to a lesser extent, in the caudate and putamen.

In adults, the concentration of manganese in the diet is known to influence the amount of manganese absorbed from the gastrointestinal tract and the amount eliminated in the bile. When dietary manganese levels are high, adaptive changes reduce gastrointestinal absorption of manganese, enhance manganese liver metabolism and increase biliary excretion of manganese (Britton and Cotzias, 1966; Davis *et al*., 1993; Dorman *et al*., 2001; Finley and Davis, 1999; Mahoney and Small, 1968; Malecki *et al*., 1996). Thus, despite large fluctuations in oral manganese intake, brain and other tissue manganese levels remain relatively constant. No formal recommended dietary allowance for manganese has been established, but the US National Research Council has established an estimated safe and adequate dietary intake (ESADDI) of $2–5\,mg\,day^{-1}$ for adults (Greger, 1998).

The earliest introduction of long-term parenteral nutrition for patients with intestinal failure was accompanied by morbidities due to inadequate replacement of micronutrients. Although evidence for the essentiality of manganese in humans is limited, guidelines for its inclusion in parenteral nutrition supplements were developed by the American Medical Association (1979) and the American Academy of Pediatrics, Committee on Nutrition (1985). However, it is important to note that there have been no reported cases of manganese deficiency in long-term parenteral-nutrition patients (Dickerson, 2001). Rather, recognition has been growing of hypermanganesemia and its associated complications in

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patients receiving long-term parenteral nutrition. Patients with cholestatic liver disease are at particularly high risk for hypermanganesaemia because more than 90% of manganese is excreted through the bile (Malecki *et al*., 1996; Agency for Toxic Substances and Disease Registry (ATSDR), 2000).

The published literature cites a broad, 200-fold range for the recommended adult daily parenteral dose of manganese, from a low of 0.18–0.91 μmol (10–50 μg) to a high of 40 μmol (2.2 mg) (Aschner and Aschner, 2005). The ASPEN Task Force for the Revision of Safe Practices for Parenteral Nutrition recommends the addition of 60–100 μg daily manganese supplements to adult parenteral nutrition formulations (Mirtallo *et al*., 2004). In 1985, The American Academy of Paediatrics Committee on Nutrition recommended a manganese intake of 2–10 $\mu g\,kg^{-1}\,day^{-1}$ for exclusively parenterally fed preterm infants (1985). This became the standard of care throughout the USA and Europe, and has influenced the composition of currently available commercial multiple trace-element products. In 2003, the recommendations for manganese supplementation in parenteral nutrition for paediatric and neonatal patients were revised. The Pediatric Nutrition Handbook—5th edition recommends 1 $\mu g\,kg^{-1}\,day^{-1}$ American Academy of Pediatrics (2003). The 2004 ASPEN Special Report also recommends 1 $\mu g\,kg^{-1}\,day^{-1}$ for preterm neonates <3 kg and for term neonates 3–10 kg. However, this recommendation, like the 1985 recommendation, is not evidence-based.

It is believed that parenteral exposure poses a risk for manganese toxicity, since the bioavailability of manganese in parenteral fluids is near 100% (Dobson *et al*., 2004) and parenteral administration bypasses the normal regulatory mechanisms of the gastrointestinal track. Furthermore, many components of parenteral nutrition are contaminated with manganese, particularly KPO_4 (280 $\mu g\,l^{-1}$) and $MgSO_4$ (225 $\mu g\,l^{-1}$) (Kurkus *et al*., 1984; Hambidge *et al*., 1989; Wilson *et al*., 1992). Wilson *et al*. (1992) reported that the manganese content of parenteral nutrition solutions in the absence of trace element supplementation was 7.3 $\mu g\,l^{-1}$ (range 5.6–8.9 $\mu g\,l^{-1}$). Hambidge *et al*. (1989) recommended that manganese supplements be omitted from the parenteral nutrition solutions when there is evidence of cholestasis, concluding that the amount of manganese present as contaminant in intravenous solutions appears to provide adequate manganese in these patients.

Manganese intoxication has been reported in association with parenteral nutrition solutions providing ≥0.1 mg manganese per day, ~1.5–2.0 $\mu g\,kg^{-1}\,day^{-1}$ for an average adult (Ono *et al*., 1995; Nagatomo *et al*., 1999; Bertinet *et al*., 2000; Takagi *et al*., 2002). As with other forms of manganese poisoning, these patients developed elevated serum manganese levels and had symmetrical high-intensity T1-weighted magnetic resonance (MR) signals in the globus pallidus consistent with manganese accumulation at this site. Some patients demonstrated characteristic psychiatric symptoms and clinical signs of manganese-induced parkinsonism-like syndrome. Withdrawal of the manganese supplement from the parenteral nutrition significantly decreased manganese levels in both the blood and brain (Bertinet *et al*., 2000).

Data in children are more limited than in adults. Fell *et al*. (1996) reported 11 children on prolonged parenteral nutrition with hypermanganesaemia and cholestasis. In a child with a movement disorder, T1-weighted MR showed bilateral symmetrically increased signal intensity in the globus pallidus and subthalamic nuclei. Similar changes were seen in five other children, one with hypermanganesaemia and cholestasis, and four on long-term parenteral nutrition without liver disease, but with elevated manganese blood levels (Fell *et al*., 1996).

As a paramagnetic trace element, manganese can be detected by magnetic resonance imaging (MRI). Manganese deposition appears as a hyperintense signal (i.e. a shortened T1) in the basal ganglia, specifically the globus pallidus on T1- (short TE/short TR) but not T2- (long TE/long TR) weighted MRI. Other metals, such as copper and iron, do not manifest this appearance. There are numerous case reports in the literature of increased T1-weighted signal intensities in older children and adults exposed to prolonged parenteral nutrition (Mirowitz *et al*., 1991; Mirowitz and Westrich, 1992; Ejima *et al*., 1992; Dietemann *et al*., 1998; Mizoguchi *et al*., 2001). When blood manganese levels were measured in these patients, hyperintense T1-weighted signals in the basal ganglia were associated with elevated manganese levels. Discontinuation of manganese supplementation was associated with improvement in clinical symptoms and MRI findings.

It is standard clinical practice to supplement adult and pediatric patients receiving parenteral nutrition with a multiple trace-element solution containing a fixed ratio of zinc, copper, chromium and manganese. Sometimes selenium is added to this trace-element cocktail. The use of trace-element solutions with fixed ratios of multiple components limits flexibility that may be needed to regulate manganese intake (or that of other trace elements) under various clinical conditions, such as cholestatic liver disease.

There are growing concerns about potentially toxic manganese levels in neonates who require prolonged parenteral nutrition. Standard clinical practice in the United States includes the daily addition of the trace-metal supplement, Multitrace®-4 Neonatal (American Regent, Inc.), which contains 25 $\mu g\,ml^{-1}$ of manganese. This supplement is most often added at a dose of 0.2 $ml\,kg^{-1}$ or 0.2 $ml\ dl^{-1}$ of parenteral nutrition solution. For the infant receiving 150 $\mu g\,kg^{-1}\,day^{-1}$ of PN, this will provide an additional 5.0–7.5 $\mu g\,kg^{-1}\,day^{-1}$ of manganese. This amount is far in excess of the amount of manganese absorbed

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by healthy infants on a diet of human milk. There are additional considerations that raise concern about manganese toxicity in the parenterally fed neonate. Not only is the absorptive control of the intestine bypassed in infants receiving manganese-supplemented parenteral nutrition, but the excretion of manganese is likely to be poor, as parenterally fed infants pass little or no stool and frequently develop overt evidence of hepatic dysfunction and cholestasis. The immature and more permeable blood–brain barrier of neonates confers a further risk of manganese-induced CNS toxicity during a critical stage of brain development. There have been sporadic cases in which children with excessive exposure to manganese have developed overt signs of neurotoxicity (Cawte, 1985; Fell *et al*., 1996; Woolf *et al*., 2002). Rodent studies suggest that neonatal animals, when compared to adult animals, are at an increased risk for manganese-induced neurotoxicity, based on their propensity to achieve higher brain manganese levels and altered brain dopamine concentrations following similar oral exposures (Chandra and Shukla, 1978; Kontur and Fechter, 1988; Pappas *et al*., 1997; Dorman *et al*., 2000). In monkeys and rats, a correlation exists between the severity of CNS symptoms and manganese brain concentrations, with both the rate and extent of manganese transport into the CNS influencing the clinical outcome (Suzuki *et al*., 1975; Roels *et al*., 1997). Tran *et al*. (2002) demonstrated that high dietary manganese intake in neonatal rats resulted in developmental deficits (Tran *et al*., 2002).

A reciprocal relationship between iron status and manganese uptake has been established. In the plasma, both metals are bound to albumin and transferrin, and both compete for the same carrier transport systems on endothelial cells (Mena *et al*., 1974; Aschner and Aschner, 1990; Aschner and Aschner, 1991; Erikson *et al*., 2002). Transport of manganese and iron to extrahepatic tissues, including the brain, occurs via transferrin-mediated endocytosis and via the divalent metal transporter 1 (DMT-1) (Chua and Morgan, 1997; Malecki *et al*., 1999; Erikson *et al*., 2002; Garcia *et al*., 2006). In the presence of low iron, both DMT-1 and transferrin receptors (TfRs) are up-regulated to maximize the body's ability to sequester iron; this also results in increased manganese transport. The converse is also true; in the presence of high levels of iron, DMT-1 and TfR levels are decreased, resulting in decreased manganese transport (Erikson *et al*., 1997; 2002; Andrews, 1999; Roth and Garrick, 2003). *In vivo, iv* ferric-hydroxide–dextran complex inhibits the net uptake of manganese across the blood–brain barrier and high iron-containing diets likewise reduce the concentration of manganese in the CNS (Diez-Ewald *et al*., 1968; Aschner and Aschner, 1990). Conversely, iron deficiency results in more manganese uptake into the brain, both in adults (Mena *et al*., 1974; Aschner and Aschner, 1990; Erikson *et al*., 2002; 2004) and in neonates (Garcia *et al*., 2006).

Worldwide, iron deficiency is the most prevalent nutritional deficiency, affecting over two billion people; infants are one of the most vulnerable populations. Iron stores in sick newborns are low. Iron-deficiency anaemia in this population is exacerbated by the lack of a safe preparation for parenteral iron supplementation, and by frequent phlebotomy losses. This problem is particularly acute in the premature infant as iron stores mainly accumulate in the foetus in the third trimester (Rao and Georgieff, 2002). Since iron and manganese use similar transport systems, it has been hypothesized that anaemic populations are more susceptible to manganese intoxication, even when exposed to low or normal levels of manganese. A significant gap in our knowledge exists with respect to the interaction between iron deficiency and manganese neurotoxicity. Specifically, the role of iron status in the distribution patterns and accumulation of manganese in the CNS in infants is unknown.

Unfortunately, published prospective studies or randomized, controlled trials of manganese supplementation in parenterally fed adults and children are entirely lacking. Given the anecdotal evidence of excessive blood and tissue levels of manganese in patients requiring prolonged TPN, such prospective studies are sorely needed. Target populations for such investigations include adults with hepatic disease, patients of any age with iron deficiency and neonates on prolonged TPN. Based on current knowledge of trace element homeostasis in health and disease, it seems likely that an individualized approach to trace-metal supplementation in parenteral nutrition patients is required. As noted by the 2004 ASPEN Special Report, 'The ratios of trace elements in commercially available paediatric multiple trace element products result in excessive intake of manganese if recommended doses of zinc are given. It is clear that micronutrient requirements for children receiving parenteral is a fertile area for research and an area in which further commercial product development is required' (Mirtallo *et al*., 2004). There is a need to better define the safe and effective dose of parenteral manganese and to identify practical methods for assessing tissue stores in patients requiring long-term parenteral nutrition (Dickerson, 2001; Erikson *et al*., 2007).

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Peroral Toxicity

William J. Brock

CONTENTS

1 INTRODUCTION

Every day humans are exposed to a multitude of chemicals. Humans are impacted by those exposures, either adversely or beneficially or even without a health outcome. Oral exposure represents the principle route of exposure to substances for humans. Each day we consume foods that contain a plethora of chemicals deemed to be safe. However, there are many reports in the scientific literature and in the lay press that suggest that exposure to some substances, including microorganisms, represents a potential for adverse health outcomes. In 2007, there were reports in the lay press about an increase in lead exposure to children from toys manufactured in China, and even our pets were at risk for kidney toxicity and subsequent lethality from a contaminant in pet foods being imported into the United States. Exposure to pharmaceuticals has led to an increased risk of cardiovascular effects, liver toxicity and other toxicities that had not been revealed in either preclinical studies or controlled clinical studies. The occurrence of toxicity has led to product withdrawals and in some cases litigation. Hence, assessing the potential for adverse health effects in humans, an understanding of the similarities in absorption and the physiology of systemic distribution and metabolism of these agents in animals is of prime importance. Changes in regional blood flow to the gastrointestinal tract, the presence of food and nutrients, as well as disease states will all have some influence on the degree of absorption and the biological fate of the agent. Therefore, a great deal of time and energy has been devoted to furthering our knowledge relative to *in vitro* and *in vivo* models used in toxicological assessment of environmental and therapeutic agents.

In this chapter, we will examine the role of oral exposures in the potential for systemic toxicity, mechanisms involved in absorption from the gastrointestinal tract and local toxicity associated with oral administration.

2 CELLULAR ENVIRONMENT OF THE GASTROINTESTINAL TRACT

The gastrointestinal tract is a tube open to the environment with absorption occurring along the entire length.

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The extent of absorption is dependent on a variety of factors, as described below, but the epithelial lining, regional blood flow along the 'tube' and the local environment within the lumen will influence absorption. Epithelium lines the entire gastrointestinal tract, although specialized cells will be present in certain regions of the gastrointestinal tract. The structure of the epithelial cell with its highly developed involutions leads to a greater surface area for absorption.

The cellular lining of the buccal cavity and oesophagus provides opportunities for the absorption of potentially toxic compounds. The circulatory drainage of the buccal cavity, the mouth and the tongue is to the jugular vein and hence xenobiotics directly enter the general systemic circulation thereby bypassing the hepatic-portal loop. Indeed, absorption from the buccal cavity has long been recognized as a way to achieve high therapeutic levels quickly, and has been an advantageous route for administration of nitrites for treatment of cardiovascular disorders, for example, angina. In addition, a number of investigators have examined buccal absorption of insulin as a means of treatment for diabetes (Oh and Ritschel, 1990; Hoffman and Ziv, 1997; Chetty and Chien, 1998). However, this route has not yet proven to be completely efficacious, but continues to be the subject of research for a variety of drugs and over-the-counter (OTC) preparations, for example, steroids, anti-inflammatory agents, and so on.

The oesophagus serves as the pipeline for delivery of substances to the stomach. The cellular structure of the oesophagus is composed of a stratified squamous epithelial lining with varying degrees of keratinization dependent on the diet of the animal species. Rodents and primates (omnivores) will have a greater degree of cellular keratin compared with rabbits that are principally herbivores. The muscular layer of the oesophagus is composed of striated muscle or a combination of striated and smooth muscle. Animals with more striated muscle partially explains why certain species do not vomit, for example, the rat, in contrast to animals that regularly vomit (dog and guinea pig) because of the greater amount of smooth muscle.

The stomach of an animal represents arguably the most important structure when considering oral administration of substances. The cellular structures and functions of the stomach, local pH and bacterial environments, as well the presence or absence of food will have dramatic effects on local toxicity, as well absorption of nutrients and a wide variety of xenobiotics. In the rodent species, for example, the nonglandular stratified squamous epithelium is adjacent to a cardiac (fundic) portion of the stomach. The two areas of the stomach are separated by a limiting ridge. The nonglandular portion serves for food storage. The cardiac portion of the stomach is proximal to the oesophagus. Acid secretion is stimulated by histamine (H_2 receptors) and by acetylcholine (M_3 muscarinic receptors). The H_2 receptors increase intracellular cyclic adenosine monophosphate (cAMP), and the muscarinic receptors and the gastrin receptors exert their effects by increasing intracellular free calcium. Gastrin also acts by stimulating the secretion of histamine from enterochromaffin-like cells (ECL cells). Vesicle- and granule-containing cells are the predominant endocrine cell types in the acid-secreting portion of the stomach.

The cellular make-up of the fundic portion of the stomach is comprised of numerous specialized cells. Parietal and chief cells function to secrete pepsinogen and hydrochloric acid and serves to create the acidic environment of the stomach. Mucoid cells, as the name implies, secrete mucous into the lumen of the stomach and help preserves the cellular integrity of the stomach from the corrosive action of hydrochloric acid. Also present in the stomach are enteroendocrine cells that serve to secrete various endocrine substances including histamine, serotonin, gastrin and other inflammatory mediators. In general, these cellular structures also appear within the cardiac portion of the stomach. Sato (2007) recently described tuft cells, also known as brush cells, as a widespread cell type found in the digestive tract. The most characteristic morphological features of these cells are their long and blunt microvilli. Although the function of tuft cells is not yet completely known, these cells seem to possess the cellular and molecular basis for chemoreception. Interestingly, these cells also have been characterized in the gallbladder and respiratory tract of various species.

The intestinal tract makes up the longest structure of the gastrointestinal tract and serves primarily for the absorption and secretion of nutrients. A comparison of the length of the intestine of rat and human certainly shows the human intestine to be longer. However, the length of the intestine relative to the entire length of the gastrointestinal tract reveals that the rat and human are similar, that is, the intestine makes up about 81–83% of the gastrointestinal tract (**Table 1**). Furthermore, it is this section of the gastrointestinal tract that functions in the metabolism of endogenous and exogenous compounds, either from cellular enzyme systems of the intestinal tract or from bacterial action on compounds. Morphological aspects of the intestinal tract result in an increase in the functional capacity of the small intestine, including its length, villi and microvilli. These latter characteristics will influence the mucosal surface area, thereby resulting in changes in transit time of a compound through the gastrointestinal tract. Transit time through the intestinal tract usually occurs within a few hours compared to a much longer duration within the stomach and colon.

An osmotic gradient of ion and water transport occurs, with differences in functional capacity occurring between the duodenum and the jejunum. In the duodenum passive movement of sodium and water is from the lumen into systemic circulation. In contrast, fluid and sodium movement of fluid and ions occurs from systemic circulation

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Table 1 Absolute and relative length of small intestine in humans and animals[a]

Species	Region	Absolute length (cm)	Relative length (%)
Human	Small intestine	680	81[b]
	Duodenum	25	4[c]
	Jejunum	260	38
	Ileum	395	58
Rat	Small intestine	125	83
	Duodenum	9.5–10	8
	Jejunum	90–135	90
	Ileum	2.5–35	2
Pig	Small intestine	182	78
Dog	Small intestine	414	85
Rabbit	Small intestine	356	61

[a]Modified from DeSesso and Jacobson (2001), Kararli (1995).
[b]Value is relative to the entire length of gastrointestinal tract.
[c]Value is relative to region of the gastrointestinal tract, for example, small intestine.

into the lumen of the jejunum. Net secretion occurs in the ileum and jejunum of guinea pigs, the ileum of rabbits and the proximal portion of the jejunum in neonatal swine. The jejunum absorbs sodium, chloride and bicarbonate against an electrochemical gradient, but this decreases with animal age. Bile salts are primarily absorbed from the ileum.

The cells lining the mucosa of the small intestine are composed of simple columnar epithelium on the villi and cuboidal epithelium in the crypts. Intestinal cells secrete fluids and electrolytes, but these secretions are then reabsorbed by intestinal villus cells. Enterocytes absorb simple carbohydrates and amino acids and then actively transport them into systemic circulation. Transport of electrolytes across epithelial cells occurs by multiple mechanisms, including passive ionic diffusion and energy-dependent active transport.

Goblet cells are present throughout the intestine, with the numbers of cells increasing through the distal end of the intestine. Paneth cells containing lysozymes also are present in the intestinal tract and are found in monkeys, mice, rats, hamsters and guinea pigs, but not in dogs or swine. Paneth cells secrete mercury and other heavy metals into the intestinal lumen and become necrotic in chronic, for example, methyl mercury, intoxication of primates. Phagocytic M cells are located on the epithelium overlying the lymphoid tissues and serve to transfer antigen material from the intestinal lumen to the immune system. In addition, M cells engulf pathogenic microbes and foreign particulates.

The circulatory pattern of the gastrointestinal tract is an important consideration when extrapolating toxicological data obtained from oral toxicity studies to other routes of exposure, as well as assessing the risks associated with oral administration of xenobiotics. Arterial blood is supplied by a number of arteries, but it is the venous circulation that becomes important for toxicology, particularly for 'first-pass' hepatic metabolism. All portal vein blood empties into the sinusoids of the liver. Hence, all material absorbed from the lower portion of the oesophagus to the proximal end of the rectum are cleared to the liver prior to entering the general systemic circulation. Substances metabolized by the liver can potentially reappear in the gastrointestinal tract (enteroheptic circulation) leading again to absorption, further metabolism and even bioactivation by metabolic enzymes present in the gastrointestinal tract (see **Toxicokinetics of Xenobiotic and Metabolite Distribution and Excretion**).

3 MECHANISMS OF ABSORPTION OF XENOBIOTICS

The gastrointestinal system should be viewed as a long tube that is open to the outside environment, with absorption occurring across the entire length of the gastrointestinal tract. The cellular make-up of this system has been described above, but because of this cellular environment the surface area available for absorption of xenobiotics is quite large. Absorption can occur by a several processes, and these are the same processes as for absorption of nutrients. Furthermore, absorption of xenobiotics is dependent on the molecular size of the compound and the microenvironment of the gastrointestinal lumen, as well as the lipophilicity of the agent, that is, the greater the lipid solubility of the agent the greater potential for absorption. Absorption of xenobiotics and nutrients from the gastrointestinal tract occurs primarily by passive diffusion or active transport. Other means of absorption, however, include filtration, facilitated transport, pinocytosis, and so on.

Although the cellular structures are important in absorption of substances, for example, nutrients, these

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Table 2 pH of contents in different regions of the gastrointestinal tract[a]

Species	Stomach		Small intestine			Colon
	Anterior	Posterior	Duodenum	Ileum	Jejunum	
Human	1.5–3.5		5–7	6–7	7	5.5–7
Monkey	4.8	2.8	5.6	5.8	6.0	5.1
Dog	5.5	3.4	6.2	6.2	6.6	6.5
Pig	4.3	2.2	6.0	6.2	6.9	6.8
Rabbit	1.9	1.9	6.0	6.8	7.5	7.2
Rat	5.0	3.3	6.5	6.7	6.8	6.6
Guinea pig	4.5	4.1	7.6	7.7	8.1	6.7
Mouse	4.5	3.1	—	—	—	—

[a]Modified from Kararli (1995).

structures also serve as barriers to impede the potential absorption of molecules, thereby serving to protect the host. In simple diffusion, molecules cross the membranes of the epithelial cells, but first must partition from the gastrointestinal fluid adjacent to the membrane, termed the unstirred water environment, and then cross the mucus layer coating the membrane before crossing the lipid membrane into the cell. The molecule must pass through the cytoplasm and then through the basement membrane and the capillary or lymphatic wall membranes. The lipid membrane favours absorption of hydrophobic species, thereby accounting for the greater absorption rate of un-ionized species, although absorption of ionized forms of a molecule may be absorbed to a limited degree most likely due to the microenvironment at the cellular interface. The process of diffusion is related to the concentration gradient across the membrane. The fluid content of the gastrointestinal tract, innervations of the tissues and blood flow to the tissues will influence the rate of absorption.

The intestine can absorb vast quantities of fluid from the intestinal lumen to maintain normal homeostasis. Greater than 98% of consumed fluid is absorbed. The balance between absorptive and secretory processes is a highly regulated process. Disruption of these integrated processes can result in disease states or conditions that can result in excessive fluid secretion. Under certain conditions, the secretory function can exceed the absorptive function leading to diarrhoea and loss of fluid. An extreme example has been noted in cholera patients where fluid loss can be up to 20 l per day (Montrose *et al*., 2003). In the stomach, about 2500 ml of gastric juice is secreted daily, and contains a variety of substances, for example, ions, gastric enzymes, and so on. The hydrochloric acid secreted by the gastric tissue kills ingested bacteria, provides the necessary pH for pepsin to start protein digestion and stimulates bile flow. The mucosal cells of the stomach secrete HCO_3^- to maintain a pH gradient of pH 1–2 at the luminal side to pH 6–7 at the surface of the epithelial cells. The pH within the gastrointestinal tract will also depend on the type of food consumed, and there will be a difference in the pH of the lumen depending on whether the measurements were made on an empty stomach, for example, or the measurement is taken of the contents (**Table 2**).

Implicit in the foregoing discussion is the absorption of substances in the 'normal' homeostatic state. Disruption of normal gastrointestinal homeostasis potentially will affect the absorptive environment of the gastrointestinal tract for drugs and nutrients. Indeed, under some circumstances, disease states of the gastrointestinal tract can affect viability and survival. Anand *et al*. (2007) described a disease state of preterm infants, necrotizing enterocolitis, which results in multiple organ failure and death principally related to the degeneration of the gastrointestinal tract thereby leading to gastrointestinal pathogens invading the body, termed bacterial translocation. Although somewhat of an extreme example, a disease state, whether chronic (Crohn's disease) or acute (the 24-hour gastrointestinal 'bug') will have an effect on absorption. Indeed, in the latter case antiemetics are given, for example as a suppository to help relieve symptoms.

3.1 Passive Diffusion

Permeation of xenobiotics across biological membranes occurs primarily by diffusion with absorption into systemic circulation dependent on the concentration gradients across the gastrointestinal epithelium. Small molecules of molecular weight less than 600 are readily absorbed across the epithelia through aqueous pores within the membrane, whereas larger molecules are absorbed across the gastrointestinal epithelium based primarily on the physiochemical properties of the agent, for example, hydrophobicity. The degree of gastrointestinal absorption tends to increase with an increase in hydrophobicity.

Estimation of absorption has historically been associated with an assessment of the octanol–water partition coefficient, log P, and is the log of the equilibrium ratio,

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Table 3 Octanol: water partition coefficients of selected agents

Compound	log *P*
Glucose	−3.24
Glutathione	−3.05
Citric acid	−1.72
p-Aminohippuric acid	−0.25
2-Butanone	0.30
Aniline	0.90
Methylene chloride	1.25
Benzoic acid	1.90
Trichloroethylene	2.61
1,4-Dichlorobenzene	3.40
Phenanthrene	4.50
Vitamin A	5.68
Dichlorodiphenyltrichloroethane (DDT)	6.20

K_{ow}, of the solute concentrations in the two phases:

$$K_{ow} = \frac{[\text{solute}]_{oct}}{[\text{solute}]_{w}} \quad (1)$$

High positive values of log *P* indicate very lipid soluble compounds that can readily traverse biological membranes (**Table 3**).

Absorption across biological membranes is also based on the ionization of the molecule as many compounds are organic acids or bases. The ionized form of the molecule will be more water soluble and less likely to be absorbed, although some absorption will occur through aqueous pores. In contrast, the unionized form of the molecule will have greater absorption with the limit of that absorption, being based on molecular size and lipid solubility, as noted above. The degree of ionization is dependent on the pK_a and pH of the solution according to the Henderson–Hasselbalch equation. This basic principle of ionization that we learned in introductory chemistry was that the pK_a is the ratio of the concentration of the un-ionized form of the molecule to the concentration of the ionized form of the molecule:

$$\text{For acids} : pK_a - \text{pH} = \log\frac{[\text{unionized}]}{[\text{ionized}]} \quad (2)$$

$$\text{For bases} : pK_a - \text{pH} = \log\frac{[\text{ionized}]}{[\text{unionized}]} \quad (3)$$

As the acidic pH of the stomach is encountered, organic acids would be largely unionized and readily available for absorption. For example, benzoic acid (pK_a 4) is about 50% ionized at stomach pH. As the compound moves into the small intestines, the local pH increases to about 7, so that the molecule becomes largely ionized. For organic bases, the opposite occurs. At low pH, an organic base is largely ionized, with very little of the compound available for absorption, whereas within the intestines, the degree of ionization declines and absorption potential increases.

3.2 Active Transport

Although a majority of xenobiotics will be absorbed across the gastrointestinal epithelium by simple diffusion, a number of compounds, including many nutrients, are absorbed via active transport mechanisms. Active transport is the movement of a molecule across a membrane that is driven by energy in the form of expenditure of adenosine triphosphate (ATP) and against a concentration gradient. In addition, specific transport proteins are often found that facilitate transport so that even large molecules can be moved across the membrane. Therefore, active transport results in greater concentrations of compounds within the cell. Indeed, active transport of compounds into the cell becomes particularly important when structurally related toxicants, such as 5-fluorouracil, compete for the transporter proteins. This competition can be used for therapeutic benefits.

In the last decade or more, a great deal of research has been reported on various protein transporters. Intestinal transporters can be classified as either influx or efflux (Hurst *et al*., 2007; Kunta and Sinko, 2004). The influx transporters are expressed throughout the intestine, although there are regional differences in levels of expression. Examples of different transporters with some examples of substrates are shown in **Table 4**. The reader is referred to the excellent review of transporters by Bleasby *et al*. (2006) who demonstrated, using *in silico* preparations, variations in species of the transporters as well as tissue distribution. In addition, the publication by Wagner *et al*. (2001) provides a thorough review of transporters, substrates and effects of foods and other factors on drug absorption and elimination. Although a complete description of all intestinal transporters is beyond the scope of this chapter, numerous intestinal transporters have been described in the literature each with its own specificity for nutrients and xenobiotics. Clearly, inhibition of these transport mechanisms or competitive inhibition with xenobiotics can potentially lead to toxicity that will be expressed in various tissues. In some pharmaceutical applications, knowledge of these transport mechanisms may lead to therapies, including treatment for obesity.

The multidrug resistant protein (MDR) was one of the first family of protein efflux transporters identified. This transporter was found to transport chemotherapeutic agents out of gastrointestinal cells, as well as cells of other organs. Other gastrointestinal transporters that have been identified include nucleotide transporter (NT), divalent-metal ion transporter (DMT) and peptide transporter (PEPT). Absorption of xenobiotics may be

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Table 4 Examples of gastrointestinal influx and efflux transporters

Transporter	Regional expression	Substrates	References
Influx Transporters			
Di/tripeptides (PEPT1)	Small intestine	Angiotensin converting enzyme inhibitors; β-lactam; penicillins	Friend (2004), Katsura and Inui (2003), Oh *et al*. (2002)
Organic anion-transporting polypeptides (OATP1A2; OATP1B1; OATP1B3)	Small intestine (apical membrane of villi enterocytes)	Fexofenadine; taurocholic acid; oestradiol 17-β-glucuronide; pravastatin	Glaeser *et al*. (2007), Katsura and Inui (2003)
Fatty acid translocase (FAT; CD36)	Proximal small intestine (apical membrane of villi enterocytes)	Long-chain fatty acids; cholesterol	Nassir *et al*. (2007), Baillie *et al*. (1996), Poirier *et al*. (1996)
Concentrative nucleoside transporters (N1, N2, N3)	Intestine with maximal transport in jejunum	Pyrimidine and purines	Pastor-Anglada *et al*. (2007)
Sodium-dependent bile salt transporter, ASBT (SLC10A2)	Ileum	Bile acids	Groen *et al*. (2007)
Efflux Transporters			
P-glycoprotein (P-gp)	Stomach, jejunum, ileum, colon	Anticancer drugs; digoxin; glucuronide conjugated drugs; ketoconazole	Yokooji *et al*. (2007), Thiebaut *et al*. (1987), Bleasby *et al*. (2006), Canaparo *et al*. (2007)
Breast cancer resistance protein (BCRP)	Intestine	Topotecan; glucuronide conjugated drugs	Wagner *et al*. (2001), Suzuki and Sugiyama (2000)
Multidrug resistance-associated protein (MRP)	Duodenum, colon	Anticancer drugs; digoxin; phase II conjugated drugs	Cao *et al*. (2006), Doherty and Charman (2002), Wagner *et al*. (2001)

increased from what would be predicted based solely on physiochemical properties, for example, K_{ow}, if the substance is a potential substrate for the influx transporters. Efflux transporters, in contrast, are able to function as an absorption barrier.

The jejunum of the human is a region of the intestinal tract with the highest levels of protein carriers and surface area (Lennernas, 2007). Human jejunal permeability (P_{eff}) is a useful determinant for approximating the fraction of oral dose absorption. As reviewed by Lennernas (2007), intestinal P_{eff} are often based on multiple parallel transport processes. Site-specific jejunal P_{eff}, however, do not reflect the permeability along the entire intestinal tract, but are useful for approximating the fraction oral dose absorbed. A jejunal P_{eff} value of $>1.5 \times 10^{-4}$ cm s^{-1} suggests that a substance will be completely absorbed no matter which transport mechanism is utilized. Moreover, jejunal P_{eff} determined for drugs transported mainly by active carriers may be predictive of the fraction absorbed.

Nassir *et al*. (2007) characterized the regional absorption of fatty acids and cholesterol in enterocytes isolated from transgenic and wild-type species. They reported on the transporter CD36, a membrane protein that is expressed in the intestine on the luminal surface of enterocytes. CD36 null ($CD36^{-/-}$) mice exhibit impaired chylomicron secretion, but no significant changes in lipid absorption. In addition these authors reported CD36 levels decreased from the proximal (duodenal) to distal (ileum) regions of the intestine. Moreover, enterocytes isolated from the small intestines of CD36 knockout mice, when compared with wild-type counterparts, exhibited reduced uptake of fatty acid and cholesterol in the proximal end of the intestine. These authors concluded that proximal intestinal absorption of dietary fatty acid and cholesterol is based on CD36 expression, whereas absorption of these nutrients is independent of CD36 expression.

Like that of fatty acids, an ileal transport protein has been described by Groen *et al*. (2007) and others (Kanda *et al*., 1996; Grober *et al*., 1999), and these authors have suggested that inhibition of this transport protein may contribute to reduced bile acid absorption potentially leading to reduced bile flow. The intestinal bile

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acid-binding protein (I-BABP) is a cytosolic protein that binds bile acids with a high affinity with its expression restricted to the ileum where it is involved in the enteroheptic circulation of bile acids. Groen *et al*. (2007) further showed that in wild-type and Atp8b1$^{G308V/G308V}$ mice (mouse model of cholestasis), intestinal taurocholate absorption was mediated by the ileal bile salt transporter ASBT (sodium-dependent bile acid salt transporter). However, no difference in the ileal protein expression of ASBT was noted between these two strains, suggesting that increased plasma taurocholate is not related to increased intestinal bile salt absorption.

Cellular efflux pumps such as P-glycoprotein (P-gp) serve as possible defence mechanisms and also influence the bioavailability and disposition of xenobiotics (Zhang and Benet, 2001). P-gp is a plasma membrane-bound drug efflux protein found primarily in drug eliminating organs and presumably functions as a detoxifying transporter because it actively extrudes xenobiotics from the body (Ambudkar *et al*., 1999). In the small intestine, P-gp has been localized to the apical membrane of the intestinal epithelial cells (Thiebaut *et al*., 1987), consistent with a role in efflux of compounds into the intestinal lumen. Pharmacokinetic studies of paclitaxel, digoxin and cyclosporine A in knockout mice have revealed the importance of intestinal P-gp in limiting the oral bioavailability of these drugs (Schinkel *et al*., 1995; Sparreboom *et al*., 1997).

In humans, intestinal P-gp contributes to the variability in the pharmacokinetic properties of cyclosporine A (Lown *et al*., 1997) and tacrolimus (Hashida *et al*., 2001; Fukatsu *et al*., 2001) of organ transplant patients. There also is evidence that these systems can be up-regulated by environmental xenobiotics. Lecoeur *et al*. (2006) analysed the ability of diazinon to act as an efflux modulator in human Caco-2 cells and concluded that following repeated oral exposure, diazinon increased P-gp expression and activity. This suggests the involvement of P-gp in the transfer of diazinon that may have potential consequences for xenobiotic interactions.

Absorption from the colon has a clear benefit in certain disease cases, and has long been recognized as means to avoid first-pass liver metabolism. The degree of absorption depends on many factors, and this route has proven useful for many therapeutic agents. Calcagno *et al*. (2006) compared the gene expression of the Caco-2 cell line to normal colon to determine if this cell line would be predictive of colonic absorption. These investigators found that the gene expression pattern of the cell line closely patterned the transporter profile of normal colon, but was distinctly different from tumour samples. Hence, colonic drug absorption will parallel normal colon. However, as noted previously, caution needs to be exercised when attempting to predict absorption in various disease states, for example, colon cancer.

3.3 Filtration

Passage of small solutes can occur with movement of water through pores and tight epithelial junctions within the gastrointestinal tract. Transport of molecules with molecular weights less than 200 would be carried through by the hydrostatic forces of water—'solvent drag' (Blanchard, 1975). Although filtration is more common in the kidney, it can occur to a limited extent in the gastrointestinal tract, for example, calcium absorption.

3.3.1 Facilitated Diffusion

Facilitated diffusion is a carrier-mediated process of absorption similar to active transport. However, absorption does not occur against a concentration gradient, thereby distinguishing this process from active transport. Furthermore, facilitated diffusion does not require the expenditure of cellular energy and is not subject to inhibition by cell poisons.

The transport of glucose into the cell occurs with the Na^+-K^+ pump, which generates an Na^+ gradient across the cell membrane. The glucose-Na^+ symport protein uses this Na^+ gradient to transport glucose into the cell. In the gastrointestinal tract, cells take in glucose and Na^+ from the intestine and transport them through the cell into the blood stream by the Na^+-glucose transporter, glucose permease (a glucose-facilitated diffusion protein) and Na^+-K^+ pumps. A similar mechanism for glucose transport occurs in the central nervous system.

Morgan *et al*. (2007) proposed a jejunal facilitated transport mechanism of glucose absorption. In this model, the authors proposed that intestinal absorption occurs by the sodium-dependent glucose cotransporter, SGLT1, which causes insertion and activation of a high-affinity facilitated-transport glucose transporter (GLUT2). These authors showed that perfusion of calcium-depleted rat jejunum with glucose and the calcium-channel blockers nifedipine and verapamil, reduced GLUT2, but not SGLT1, suggesting that dietary sufficiency may mediate a significant pathway of glucose-stimulated calcium uptake and that luminal supply of calcium is necessary for GLUT2-mediated glucose absorption.

3.4 Pinocytosis

Cells have the ability to transport macromolecules (proteins, polysaccharides, polynucleotides) to their interior through endocytosis, a broad term that includes phagocytosis and pinocytosis. Phagocytosis is the process of enveloping a particle by modifying its membrane to form a phagosome around the particle. Phagosomes are then pulled by cytoskeleton motion of the cytosol into one or more lysozymes for digestion of the particles.

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In contrast, pinocytosis transports liquid substances into the cell. Pockets occur in a given area of the cell membrane, capturing the liquid and forming vesicles that are then pulled by cytoskeleton motion into the cytoplasm. Pinocytosis may be selective or nonselective. Selective pinocytosis occurs in two stages. The liquid substance adheres initially to membrane receptors and then the substance is transferred to vesicles that leave the membrane surface and transport their contents into the cytoplasm. In nonselective pinocytosis, the vesicles envelop all the solutes present in the extracellular fluid.

Absorption of nutrient biomolecules occurs via this route and the disposition of particles, for example cadmium and ferretin are absorbed via this mechanism. Uptake of proteins via pinocytosis has been observed in neonatal rats, guinea pigs and rabbits (Leece and Broughton, 1973). Interestingly, immune complexes responsible for food allergies may be absorbed into systemic circulation by pinocytosis (Walker and Bloch, 1983; Sasser and Jarboe, 1980; LeFevre and Joel, 1977).

4 FACTORS AFFECTING GASTROINTESTINAL ABSORPTION

Normal physiological functioning of the gastrointestinal tract, for example food intake and bodily responses to food intake, can affect the systemic absorption of xenobiotics as well as nutrients. In addition, absorption into the systemic circulation from the gastrointestinal tract is affected by metabolism of the agent by intestinal microflora or metabolic enzymes present in the gastrointestinal epithelium. In addition to physiological effects, the chemical nature of substances administered will affect intestinal transport into systemic circulation, but also may affect the normal functioning of the gastrointestinal tract, for example, transit time.

Alternative routes of drug administration continue to be an area of intense research. However, the oral route remains the preferred route of drug administration. During the process of drug discovery, oral bioavailability is often a prerequisite when considering a candidate drug for further development with poor bioavailability often resulting in the rejection of a potential drug candidate and leading to higher product costs for drug development of alternative drugs, formulations and salt forms. Since each new candidate drug is expected to offer unique physicochemical characteristics, the study of its absorption and bioavailability marks a critical step in the development of a new drug.

4.1 Chemical Factors

Most pharmaceutical preparations are marketed as racemic mixtures, although the stereoselective preparations have become important in recent years as drug manufacturers have explored the use of stereochemistry in extending patent protection, for example with fluoroquinolone antibiotics and omeprazole, as well as examining differences in pharmaceutical activity. This 'chiral switching' has resulted in a number of drugs becoming available as both a single enantiomer and a racemic mixture. There are two principles in chiral drug development—*de novo* development of a single enantiomer or a switch from an existing racemic drug to one of its isomers in a pure form. Single enantiomer drugs are a rapidly growing proportion of new drugs, with about 20% of new drugs coming to the pharmaceutical market about 10 years ago, compared to almost 75% of the pharmaceuticals being single enantiomer drugs within the last few years (Agranat *et al*., 2002). 'Chiral switches' exploit single enantiomers of racemic mixtures and have become important in drug development portfolios. In recent years, comparability of biologics has emerged as an important area of development, with small differences in structure resulting in similar, or at times, significant differences in pharmaceutical activity and bioavailability.

Stereoisomers often have very similar physicochemical properties, so it is difficult to distinguish between them in achiral environments. As noted, however, in the body these stereoisomers can have very different effects. Drug absorption processes are likely to be stereospecific when mediated by carrier molecules. Passive diffusion is not, however, governed by such a specific mechanism. The importance of stereochemical aspects in considering the bioavailability of drug molecules has been well emphasized by Jamali *et al*. (1989) and Jamali (1992). Brocks *et al*. (1991) showed that following an oral dose of the racemic anti-inflammatory drug etodolac, the blood vs. time area under the curve (AUC) for the active *S*-etodolac was about 10-fold lower in plasma and synovial fluid than the inactive *R*-enantiomer. Although it is unclear why the differences existed, the authors postulated it may be due to stereoselective metabolism. In a subsequent study, these authors showed that the lower AUC in rat plasma of *S*-etodolac, compared to the *R* enantiomer, was related to a greater distribution to tissues, owing to a lesser degree of binding to plasma proteins and to a higher rate of glucuronidation (Brocks and Jamali, 1991).

In a similar example, propranolol enantiomers are stereoselective when high doses of the racemic drug are given by oral administration (Marier *et al*., 1998). At low oral doses, the kinetics of the propranolol enantiomers are identical, but at much higher doses the kinetics of the enantiomers are stereoselective, that is, the AUC for *S*-propranolol is greater than the AUC for *R*-propranolol. Propranolol enantiomer plasma-protein binding is not stereoselective. *In vitro* incubation of the individual enantiomers showed that the intestinal intrinsic clearance of *R*-propranolol is greater than that of *S*-propranolol. Tracy

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(1995) has reviewed a number of drugs that show stereoselectivity on pharmacokinetic and pharmacodynamic properties.

As described by Sabnis (1999), chemical complexes of interest in the pharmaceutical industry can be divided into three categories based on the energy of attraction between the components of the complexes: (i) covalently linked complexes, (ii) ionic complexes and (iii) inclusion complexes. Within the gastrointestinal tract, there will be minimal changes to a molecular complex held together by covalent bonds, although the metabolic capability of microorganisms within the cellular constituents of the gastrointestinal tract can readily metabolize substances (see below). Prodrugs can take advantage of the metabolic competency of the gastrointestinal tract as these xenobiotics are prepared by chemical modification of the drug by the addition of a labile moiety to improve the solubility, absorption or uptake by the target organ (Hussain *et al*., 1987; Lai *et al*., 1987; Hansen *et al*., 1992; Lokind and Lorenzen, 1996). Ionic complexes are held together by weak forces such that these substances will be generally labile and, therefore, susceptible to configurational changes in the structure. These interactions, referred to as 'soft' or 'dynamic', occur rapidly and can readily be reversed (Tsuchida, 1991).

Inclusion compounds, which form the third category of complexes, result more from the general architecture of the compound. In these cases, the constituents of the complex are 'trapped' in the open structure of another molecule to yield a stable arrangement. Examples of this would include cyclodextrins, as these compounds trap lipophilic drugs to form a complex having a comparatively more hydrophilic character (Albers and Muller, 1995). Cholestyramine also serves an example, as this compound can trap bile acids (Zhu *et al*., 1992).

As described above, pH and partition coefficients will have a great influence on systemic absorption of substances from the gastrointestinal tract. The importance of ionization in drug absorption is based on the observation that the nonionized form of the drug has a greater K_{ow} than the ionized form, which becomes of prime importance in determining the degree of membrane penetration (Navia and Chaturvedi, 1996; Sabnis, 1999). Therefore, the partition coefficient and its bioavailability are influenced by the structural changes brought about by the substitution of different chemical groups. Since an alteration in the chemical structure may affect the pharmacological activity, formation changes or examination of a prodrug are means to improve the bioavailability.

Lui *et al*. (1986) examined differences in stomach pH between humans and dogs, as the dog is often used to examine absorption pharmaceutics. In this investigation, pH was continuously recorded through the use of Heidelberg capsules that transmit data through a radio frequency transmitter. Gastric pH was significantly higher in dogs, compared to humans (**Table 5**). The authors suggested that the differences between dogs and humans were related to differences in gastric secretion and pancreatic bicarbonate secretion, with gastric secretion being lower in dogs and pancreatic secretion being higher in humans. Hence, it would be expected that dogs would have a greater gastric and intestinal pH. The implications of this difference could be that xenobiotic absorption may differ. However, the time–pH profiles for dogs and humans tend to be similar, such that absorption of compounds would not significantly differ. Clearly, where the pK_a of poorly soluble drugs falls within the range pH 5–8, there may be discrepancies between absorption in dogs and humans. The purest specimens of parietal cell secretion that have been obtained contain approximately 0.17 N HCl, with a pH as low as 0.87 (Montrose *et al*., 2003). The pH of the cytoplasm of the parietal cells, like that of other cells, is 7.0–7.2.

Table 5 Regional gastrointestinal pH values[a]

Region	Human	Dog	Rat
Stomach (lumen)	2.5	2.8	3.2–3.8
Duodenum	5–7.8	5–7.6	7.5
Jejunum	5–7	6.2–7.3	6.5–6.8
Ileum	7	6.6–7.9	7.1
Colon	7.5	6.5	6.5–6.6

[a]Adapted from Sutton (2004), Martinez *et al*. (2002), Davies and Morris (1993).

As previously discussed, the stomach contains cells responsible for acid secretion. The acidic stomach aids in the digestion of foods, as well as the absorption of xenobiotics and nutrients. Gastrin secretion is activated by the presence of food, whereas gastrin secretion is reduced during fasting because of the low pH of the stomach environment. Species differences in stomach pH exist, although the differences are not widely variable. As seen in **Table 5**, the difference in stomach pH between dogs and humans is not significant. Sutton (2004) showed some differences between dogs, rats and humans with respect to regional gastrointestinal pH.

4.2 Physiological Factors

A major factor that can affect absorption of xenobiotics and, hence, influence toxicity is transit time of substances through the gastrointestinal tract. When foods and other substances enter the intestinal tract, peristaltic action is triggered by distension of the wall in the intestine. The movement of substances from the stomach through the intestine to the rectum for elimination can take several days, although in normal circumstances this occurs within about 24 hours. Although the origin of these coordinated contractions is not well understood, they appear to be dependent on a combination of

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Table 6 Mean retention time (h) of solid materials[a]

Species	Mean retention time (h) of particles
Human	46
Pig	48
Rabbit	15
Rat	29
Dog	23

[a]Adapted from Martinez *et al*. (2002).

internal rhythmic contractions and neural, paracrine and humoral factors. The presence of food increases motility through the gastrointestinal tract due to signals propagated throughout the enteric nervous system from the walls of the stomach. In addition, the presence of fats in the duodenum appears to trigger another mass peristaltic reflex. Protein-rich food leaves more slowly, and emptying is slowest after a meal containing fat. The rate of emptying also depends on the osmotic pressure of the material entering the duodenum. Since fats are particularly effective in inhibiting gastric emptying, some people drink milk, cream, or even olive oil before a cocktail party. The fat delays intestinal transport from the stomach where its absorption is slower. Hence alcohol enters the small intestine more slowly so that a sudden rise of the blood alcohol to a high level and consequent embarrassing intoxication are avoided. Fraser (1997) suggested that alcohol interacts with drugs and foods delaying their absorption and gastric emptying time. Therefore, it remains somewhat uncertain whether foods affect alcohol transit time or alcohol affects food transit time. Interspecies differences in fluid retention and solid material as a measure of transit time are shown in **Table 6**.

Measuring transit time has become, in certain cases, part of the safety pharmacology study programme for new pharmaceutical compounds. The description of the methods is very limited in the International Conference on Harmonisation (ICH) Guidance (ICH, 2000). However, in this study design, a 15% activated charcoal suspension is administered orally to fasted rats about 30 minutes after the oral dose of test article. The rats are subsequently euthanized, about 60 minutes after receiving the charcoal administration, and the intestines removed. The distance travelled by the charcoal as a fraction of the length of the intestine is measured, providing an indication of whether the test article had an effect on transit time relative to controls. Atropine and morphine, substances known to affect transit time, are usually included as a positive control substance. Harrison *et al*. (2004) suggested that this study design, which is most common, should be supplemented with the use of isolated cell systems, ligated segments of the intestine, and so on.

Another factor related to intestinal transit time is the relative length of the intestinal tract (see **Table 1**). Clearly, as the length increases, so too will the residence time. The intestinal tract of the dog is relatively short and simple compared to, for example, ruminant animals. Intestinal transit appears to be quite different, in a variety of ways, from movement through the stomach. The time taken by the material to reach the ileocaecal valve, once emptied from the stomach, is relatively consistent. Apparently, transit through the small intestine is less dependent on the size or state of the matter, and the presence of food does not influence the intestinal transit (Davis *et al*., 1986). Therefore, it is during intestinal transit that the maximum opportunity exists for drug absorption for most dosage forms.

The residence time for a dose taken on an empty stomach depends on the time of dosing relative to the occurrence of the 'housekeeper wave', the peristaltic action that moves contents from the stomach into the pylorus (Gleysteen *et al*., 1985). Langguth *et al*. (1994) studied the influence of various fasting-state gastrointestinal parameters on the variability in absorption of cimetidine in dogs. These authors found that gastric emptying increased the variability of cimetidine concentration in blood, affecting the pharmacokinetic profile of the drug. Lipka *et al*. (1995) reported similar results for celiprolol. Sababi and Bengtsson (2001) used an *in situ* model of intestinal motility using two animal anaesthetics that result in different intestinal motility patterns, that is, Rapinovet (propofol) displayed a migrating motor complex with Inactin (thiobutabarbital) showing more of a fed-like motility response. These authors found an increase in plasma of 3-methyl-glucose when the animals were anaesthetized with Inactin compared to the other anaesthetic.

Gastauer *et al*. (1979) reported on a sex-related difference in the bioavailability in dogs of different oral dosage forms of digoxin, with the drug more rapidly eliminated in female dogs. Gender-related differences in metabolism are well known. It is not clear from this publication what role metabolism within the gastrointestinal tract played in this difference.

Adusumalli *et al*. (1992; 1993) evaluated age and sex of the animal as possible factors that affect the bioavailability of dosage forms. In their studies, no sex-related differences in the pharmacokinetics of azelastine were observed. When comparing age-related differences, however, they observed a higher maximum systemic concentration of the drug (C_{max}) and a lower volume of distribution at steady state in young dogs (four to six weeks old), although the bioavailability was not affected. In contrast, when these investigators examined the pharmacokinetics of felbamate, they did see age-related differences in the bioavailability (Adusumalli *et al*., 1992). Gender- and age-related differences in intestinal metabolism and absorption of substances have been reported for ethanol (Thomasson, 1995). Jones (2003), however, suggested that there are insufficient data to conclude a gender difference, but that the lower volume of distribution in women explains sex-related

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differences. In women, nickel absorption has been shown to be less than men (Nielsen *et al*., 1999).

More recently, Duan *et al*. (2006) reported that ageing enhanced cholesterol absorption by suppressing expression of the jejunal and ileal sterol ATP-binding cassette efflux transporters and up-regulating expression of the duodenal and jejunal sterol influx transporter, Npc1l1. In addition, these authors found that oestradiol treatment increased cholesterol absorption through intestinal up-regulation of these transporters. Doyle-McCullough *et al*. (2007) examined the intestinal uptake *in situ* of latex microparticles as a function of species, age and gender. These authors found that some parameters, species and gender, did not affect the extent of microparticle uptake, but that uptake varied with age, with uptake being significantly greater in young adult males (seven weeks) than in younger (three weeks) and older (17 and 52 weeks) age groups of animals. A lack of a gender effect on intestinal uptake also has been shown for naproxen and pilocarpine (Fagerholm *et al*., 2005; Omori *et al*., 2004). However, Bai *et al*. (2005) reported that men have a greater malabsorption of nutrients in coeliac disease, despite this disease being more common in women. Overall, therefore, gender differences have been observed with certain xenobiotics, but not with others, suggesting that generalities in gender differences related to intestinal transport or metabolism cannot be conclusively identified.

A number of investigators have examined jejunal and ileal absorption of compounds, as these segments of the gastrointestinal tract represent those areas where the greatest absorption occurs for pharmaceutical agents. Studies with Caco-2 cells, everted intestinal sacs and other membrane systems have been used to examine the active transport of xenobiotics (Berggren *et al*., 2004). In these studies by these investigators and others, the *in vitro* models were predictive of jejunal and ileal absorption of compounds such as digoxin, verapamil, bile acids, and so on. (Amelsberg *et al*., 1996; Johnson *et al*., 2003; Berggren *et al*., 2004). In a review by Bohlen (1998), the role of intestinal blood flow and other physiological factors, and cellular mechanism had an influence on intestinal absorption. Such a conclusion was similarly reached by Winne (1980) nearly 30 years ago.

4.3 Food Interactions

Food has long been recognized as a factor that influences the absorption of drugs, chemicals and other nutrients. Indeed, in routine acute oral toxicity testing of substances, fasting of animals is often utilized, as suggested by regulatory guidance. As alluded to throughout this chapter, foods can modify the absorption of substances, including other food substances, by changing dissolution, solubility and enhancing binding to food-containing materials, as well as changing gastric residence time, altering blood flow, and so on. In an extensive review of the subject, Singh (1999) classified drugs into categories of 'Decreased', 'Delayed', 'Accelerated', 'Increased' and 'Unaffected' in terms of the effects of foods on gastrointestinal absorption (**Table 7**).

The classification was based on an alteration of the maximum blood concentration ($C_{\max}$) and the area under the blood concentration and time curve (AUC) of the pharmaceutical. For 'Decreased' and 'Delayed' absorption, the author indicates that the extent of absorption is not affected, but the time course of absorption is changed. Whether such a change is due to a direct interaction of the food with the pharmaceutical or whether there is a delay in gastric emptying time from the stomach to the intestine appears to largely depend on the pharmaceutical. In contrast, drugs that show 'Accelerated' or 'Increased' absorption appear to be those with poor solubility. Furthermore, the altered absorption results from a greater gastric residence time allowing for an increased dissolution in the presence of, for example, bile acids. Overall, however, a majority of the drugs that Singh (1999) examined were 'Unaffected' by the coadministration of foods, with only a few drugs showing 'Accelerated' absorption in the presence of foods. Weber and Ehrlein (1998) also showed that gastric emptying time affects intestinal absorption. In their studies, carbohydrates were absorbed at a higher rate compared to fats and proteins, although there continued to be a large absorptive reserve within the intestine. Numerous authors have examined the role of foods and beverages on the absorption of drugs and nutrients, with the outcome of the research showing a great deal of disparity between the drugs and the food substances (Lee *et al*., 1997; Fraser, 1997; Kinoshita *et al*., 1996; Watkins *et al*., 1992).

The influence of foods on absorption has been recognized for many years. Dahan and Altman (2004), Palumbo *et al*. (2005) and numerous other authors (Lilja *et al*., 2004; Paine *et al*., 2004; 2005) have shown that grapefruit juice coadministered with drugs will alter the pharmacokinetics of some drugs due to an inhibition of cytochrome P450 3A4 in the small intestine. Wagner *et al*. (2001) showed that grapefruit juice resulted in about a twofold increase in the $C_{\max}$ of talinolol. The active ingredients in grapefruit juice, flavanols, appear to affect various gastric transporters for drugs and other substances with the multidrug transporter P-gp being the more characterized transporter (Wagner *et al*., 2001; Glaeser *et al*., 2007; de Castro *et al*., 2007). Edwards *et al*. (1996) also identified 6′,7′-dihydroxybergamottin, a furancoumarin in grapefruit juice, as a potent inhibitor of cytochrome P450 3A4 that also may be responsible for the altered pharmacokinetics of certain drugs.

Probably the most common example of food influencing the toxicity of a substance is with ethanol. Ethanol is completely absorbed from the gastrointestinal tract, with about 20% of an ingested dose being absorbed from

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Table 7 Examples of substances classified based on effects of food

Therapeutic class	Decreased	Delayed	Accelerated	Increased	Unaffected
CVS	Desmopressin Furosemide Hydralazine	Isosorbide Verapamil Isradipine	Diltiazem	Diprafennone Lovastatin Quinidine gluconate	Atorvastatin Digoxin Pravastatin
Anti-infective	Ciprofloxacin Ethambutol Rifampicin	Fluconazole Ketoconazole Levofloxacin	Temafloxacin	Albendazole Atovaquone Clofazimine	Adefovir Artemisinin Azithromycin
CNS	Levodopa Carbidopa Avitripan	Pyridoxal Rizatriptan Dolasetron	Oxybutynin	Morphine Ziprasidone Dixyrazine	Oxycodone Valproic acid Bromocriptine
Respiratory	Theophylline	Domitroban Salbutamol Procaterol	Theophylline[a]	Montelukast	Loratadine Phenylpropanolamine Pseudoephedrine
Antineoplastic	Estramustine Mercaptopurine Methotrexate	Letrozole[a]		Cyclosporin	Bicalutamide Finasteride

[a]Modified release formulation. Adapted from Singh (1999).

the stomach and the balance in the duodenum (Jones, 2003). Absorption of ethanol is significantly delayed when taken concurrently with a diet high in lipid content. Moreover, when the lipid diet is administered directly into the duodenum, ethanol absorption is delayed for several hours. The absorption of ethanol in the presence of food may occur because of binding to food particles, increase in local metabolism of ethanol or increased presystemic metabolism through the hepatic first-pass effect. What has clearly been demonstrated, however, is that ethanol ingestion on an empty stomach leads to a greater blood-alcohol concentration.

There is an abundance of literature on the effects of foods and dietary supplements on the bioavailability of drugs and chemicals. An online search using PubMed (*www.ncbi.nlm.nih.gov*) with the topic of foods and food-drug interactions returned well over 1000 references. In the previous edition of this book, Tyler (2000) described how the acute oral toxicity of various glycol ethers was modified in the fasted and fed state of rats and mice. In general, the toxicity (as measured by the LD_{50}) was greater in the fasted animal, a finding that is consistent with much of the literature for a lengthy list of substances. Hence, the impact of foods on the absorption of xenobiotics is clearly established. However, there are circumstances where the presence of food can have a negative or opposite effect, that is, decrease the absorption of substances. A classic example would be the binding of some antibiotics and other drugs, for example, tetracycline, mercaptopurine, levofloxacin, to the components of milk (Matsumoto *et al*., 2001; Jung *et al*., 1997; Amsden *et al*., 2003; de Lemos *et al*., 2007). The negative effect of food on absorption can be related to a number of mechanisms including chelation, adsorption of the drug to food, acid instability within the gastrointestinal tract, enzymatic hydrolysis of peptides, increased viscosity and, as described above, altered motility.

4.4 Xenobiotic Interactions

In addition to the influence of foods on gastrointestinal absorption of substances, a large number of publications are available on the effects of interactions between xenobiotics on absorption. Xenobiotic interactions may affect absorption by virtue of one substance altering the local environment within the gastrointestinal tract, affecting the metabolic competency of cellular enzymes, binding the other, altering motility, and so on.

Larsson *et al*. (1983) showed that omeprazole, picoprazole and cimetidine possess gastric antisecretory properties that may alter the bioavailability of a concurrently administered drug. Choi *et al*. (2004) demonstrated that coadministration of flavone enhanced the pharmacokinetics of paclitaxel, prolonging the half-life and increasing the AUC. The authors suggested that the alteration in the pharmacokinetics of paclitaxel was related to the inhibition of cytochrome P450 and the P-gp efflux pump. Similarly, Zhang *et al*. (2003) had previously suggested that Tween 80, a commonly used solvent for drug delivery, inhibits P-gp, resulting in an increased absorption of digoxin administered to rats.

Alterations in gastrointestinal absorption by inhibition of transporters or alteration of the metabolism of substances are clearly recognized as one means of enhancing or inhibiting absorption. However, other factors will be of importance, and include binding the substances to gastrointestinal contents, for example, as

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described above for foods, or through chelation. *In vitro* studies by Tanaka *et al*. (1993), demonstrated that levofloxacin and other quinolones will bind to different aluminium salts, resulting in lower bioavailability. Similarly, Deppermann and Lode (1993) showed a significant decrease in bioavailability by aluminium-based antacids on ciprofloxacin, ofloxacin, norfloxacin and lomefloxacin. Mechanistically, the authors suggested that the absorption of quinolones by aluminium hydroxide reprecipitation may be responsible for the reduced bioavailability. Similarly, absorption of inorganic ions, for example iron, is disrupted by the presence of other cations, for example copper. Iturri and Nuñez (1998) found that cadmium and copper, manganese or lead, but not mercury, inhibited the uptake of iron in perfused mouse duodenal preparations. Furthermore, the inhibition by copper and cadmium was dependent on the iron redox state, since neither cation inhibited Fe^{3+} uptake. The authors proposed that Fe^{2+} and Fe^{3+} are transported from the gastrointestinal tract by different mechanisms, or that Fe^{3+} is reduced in enterocytes to Fe^{2+} and the subsequent absorption is not inhibited by either cadmium or copper.

The mucosal layer of the gastrointestinal tract serves to protect the intestinal epithelium from the acidic environment of the stomach, as well as protecting the cellular environment from mechanical and other chemical forces of digestion. Nonsteroidal anti-inflammatory drugs (NSAIDs), for example aspirin, have long been recognized as causing an ulcerogenic effect in the stomach. Indeed, the search for new NSAIDs has focussed not only on the potential for these compounds to affect the inflammatory process, but also on being less ulcerogenic to the stomach. The newer generation COX2 inhibitors have shown some success. Regardless, ulcer formation of the gastrointestinal tract will certainly change the bioavailability of drugs much in the same way as disease states alter absorption.

4.5 Microbial Influences

There has been long recognition that gastrointestinal microflora play a major role in the metabolism of xenobiotics in the gastrointestinal tract (Goel *et al*., 2005; Smith *et al*., 2005). Turner *et al*. (2003) demonstrated that human gut microflora influence the metabolism of isoflavones, thereby altering the bioavailability of this class of compounds. Clearly, local interactions at the cellular level between bacteria and food constituents may have dramatic influence on the metabolism of xenobiotics, thereby influencing the absorption and hence toxicity.

Bacteria are present throughout the length of the gastrointestinal tract although there is a significant bacterial concentration in the large intestine. Few bacteria are present in the gut at birth, but a rapid colonization occurs shortly after. It has been estimated that the average adult has 1014 different intestinal flora. The stomach has the least amount of microflora ($<10^3$CFU ml^{-1}), primarily because the low pH destroys many organisms. In contrast, almost the entire digestion process can be attributed to the large number ($>10^{14}$ CFU ml^{-1}) of anaerobic bacteria present in the large intestine (Patel, 2005). Normal gastrointestinal bacteria produce antibacterial chemicals (called bacteriocins) as a result of their metabolism. This by-product can generate a local antibiotic effect that hinders the reproduction of invading flora. Secretions in the large intestine consist primarily of alkaline mucus that protects the epithelium and neutralizes acids produced by bacterial metabolism.

5 GASTROINTESTINAL METABOLISM

Historically, the metabolism of xenobiotics has been classified as either a Phase 1 or a Phase 2 reaction. Although such terminology is somewhat outdated, this classification remains convenient for the purposes of description of the mechanisms of metabolism. Phase 1 reactions result primarily in the oxidation of parent compounds, but also involve reduction reactions. Cytochrome P450 reactions often result in an increased polarity of the parent molecule and generally precede conjugation reactions (Phase 2 metabolism). These enzyme systems are responsible for the metabolism of xenobiotics, but are also responsible for the metabolism of nutrients and endogenous compounds. Over the last 20 years, different isoforms of cytochrome P450 have been characterized for their metabolic specificity. For example, cytochrome P450 3A4 is the major isoform that metabolizes most drugs, whereas halogenated hydrocarbons are generally substrates for cytochrome P450 2E1. Parkinson (2001) has described the different isoforms of cytochrome P450 with regard to substrate specificity, and inhibitors and inducers of these enzyme systems. The liver contains the highest concentration of metabolic enzymes, and the enzymes are located within the endoplasmic reticulum. In the gastrointestinal tract, heterogeneity of the enzyme concentrations can be found, with a decrease in levels of activity from the upper to the lower intestinal tract (Martinez *et al*., 2002).

Distribution studies by Chhabra and Fouts (1976) revealed that enzyme activity declines from the proximal end of the intestine to the distal-colonic end of the intestine. These authors examined aniline hydroxylase, aminopyrine *N*-demethylase and aryl hydrocarbon hydroxylase in intestinal microsomes and found these enzymes along the entire length of small intestine. Maximal activity was present in the proximal intestine. Mizuma *et al*. (1997) examined the intestinal transport and metabolism of kyotorphin (KTP) in rat everted

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Table 8 Intestinal cytochrome P450 activity in animals treated with phenobarbital (PB) or 3-methylcholanthrene (3-MC)[a]

Species	Control	PB	Control	3-MC
Guinea pig	0.22 ± 0.02	0.231 ± 0.03	0.26 ± 0.014	0.26 ± 0.004
Rabbit	0.41 ± 0.06	0.40 ± 0.04	0.54 ± 0.12	0.34 ± 0.03

[a]Values are the mean (±SE) expressed as nmol/mg protein; from Chhabra and Eastin (1984).

small intestine. These authors found that KTP on the mucosal side was metabolized within 60 minutes. No metabolism was detected on the serosal side of the intestine. In intestinal homogenates, KTP was metabolized and was decreased by peptidase inhibitors, bestatin, *o*-phenanthrolin and tryptophan hydroxamate.

In perfusion studies of the rat small intestine, regional differences in intestinal metabolism and elimination of cimetidine were observed (Piyapolrungroj *et al*., 2000). The metabolite cimetidine *S*-oxide was formed to a greater extent in the jejunum compared with the ileum, and the occurrence of the metabolite was a function of the pH-dependent intracellular uptake. Perfusion studies with inhibitors of cimetidine mucosal transport and inhibitors of microsomal *S*-oxidation provide an inhibition profile suggesting that jejunal cimetidine permeability decreases with increasing intracellular cimetidine concentration.

Inhibitors and inducers of cytochrome P450 enzymes have been extensively examined. Chhabra and Eastin (1984) presented data from their laboratory demonstrating induction of intestinal metabolism in various species. In their study (**Table 8**), the authors found that intestinal cytochrome P450 activity was refractory to induction by phenobarbital or 3-methylcholantrene, although it is well known that hepatic cytochrome P450 isozymes are well induced by either substance (Parkinson, 2001).

As discussed above, dietary components have an influence on the absorption of xenobiotics. Similarly, diet will influence the metabolism of xenobiotics, and the literature is full of examples (see Domeneghini *et al*., 2006; Ramesh *et al*., 2004).

6 BIOPHARMACEUTIC CLASSIFICATION SYSTEM AND HUMAN DRUG ABSORPTION

In 1995 Amidon *et al*. (1995) devised a biopharmaceutics classification system (BCS) to classify drugs based on their aqueous solubility and intestinal permeability. The authors suggested that dissolution rate had a minimal impact on bioavailability of highly soluble and highly permeable (BCS Class 1) drugs when the dissolution of a drug is sufficiently rapid (Kaus *et al*., 1999). As a result, various regulatory agencies, including the Food and Drug Administration (FDA) now allow bioequivalence waivers of formulations of BCS Class 1 drugs to be demonstrated by *in vitro* dissolution (often called a biowaiver). Definitive BCS classification is done when a potential Class 1 candidate enters human testing, with classification according to methods outlined in the FDA guidance (Food and Drug Administration (FDA), 2000; **Table 9**). For instance, solubility is determined at pH 1.2, 7.5 and at a pH approximating the pK_a (pK_a − 1, pK_a and pK_a + 1) of the agent.

Kasim *et al*. (2004) undertook an analysis of approximately 260 oral immediate-release drugs, and compared the BCS classification of these drugs based on the partition coefficient (log *P* or *C*log*P*) or dose number.[a] For a majority of the drugs examined, BCS classification was the same. About 67% of the drugs were classified as 'high solubility' drugs.

For classification of drugs, preference is often given to data developed from clinical or preclinical studies with a preference given to data developed from renal excretion data in human studies, or a human mass balance study with radiolabelled material. Human absolute bioavailability studies and preclinical permeability studies using rat intestinal perfusion or Caco-2 cells also are considered for classification. For immediate-release dosage forms, a product is considered to be a rapidly dissolved substance when not less than 85% of the labelled amount of the substance dissolves within 30 minutes in acidic media (0.1 N HCl or simulated gastric media), pH 4.5 media and pH 6.8 media, or simulated intestinal fluid without enzymes. Recently, Benet *et al*. (2008) recommended the extent of drug metabolism (≥90%) as an alternate method in defining Class 1 marketed drugs suitable for a waiver of *in vivo* studies of bioequivalence.

Class 1 substances are those that are well absorbed and where the rate-limiting step for absorption is dissolution of the agent. For immediate-release dosage forms, that is, those formulations that dissolve very quickly, gastric emptying time becomes rate limiting for absorption. Amidon *et al*. (1995) proposed that the dissolution profile should be determined over time and for at least 85% dissolution at several different pH values. This proposal has been incorporated into the FDA guidance FDA 2000. For Class 3 drugs, absorption is limiting, and drugs of this class show a large variation in permeability. Monographs for several pharmaceuticals have been published in the open literature with an eye towards obtaining a biowaiver from conducting *in vitro* or *in vivo* bioequivalence and bioavailability studies (Yu *et al*., 2002; Blume and Schug, 1999; Kortejarvi *et al*., 2005). Without the waiver, pharmaceutical companies would be mandated

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Table 9 Biopharmaceutical classification system[a]

Biopharmaceutical classification	Description	Examples
Class 1	High solubility—high permeability	Chlorpheniramine, cloxacillin
Class 2	Low solubility—high permeability	Clofazimine, gibenclamide
Class 3	High solubility—low permeability	Hydralazine, methotrexate
Class 4	Low solubility—low permeability	Theophylline, trimethoprim

[a]Adopted from Food and Drug Administration (FDA) (2000) and Amidon *et al*. (1995).

to undertake *in vivo* bioavailability and bioequivalence testing for newly formulated generic drugs or reformulation of existing patented drugs. Such testing could have considerable cost ramifications. Class 4 drugs represent those drugs that would be difficult to formulate for oral dosage forms.

In vivo differences in the rate and extent of absorption of a drug of two equivalent solid oral products may be due to differences in drug dissolution. When the *in vivo* dissolution of a solid oral dosage form is rapid in relation to gastric emptying time, the drug has high permeability and the rate and extent of drug absorption is unlikely to be dependent on dissolution and/or gastrointestinal transit time. Under such circumstances, the FDA suggests that a demonstration of *in vivo* bioavailability or bioequivalence may not be necessary for drug products containing Class 1 drug substances. The BCS approach outlined by the FDA may be used to justify a biowaiver for highly soluble and highly permeable drug substances, Class 1 agents, for an immediate-release, solid oral dosage form.

A drug substance is considered highly soluble when the highest dose strength is soluble in 250 ml or less of aqueous media over the pH range 1–7.5. The permeability class boundary is based on the fraction of dose absorbed in humans and on the rate of mass transfer across human intestinal membrane. A drug substance is considered to be highly permeable when the extent of absorption in humans is determined to be 90% or more of an administered dose based on a mass balance determination or in comparison to an intravenous reference dose. A drug substance is considered rapidly dissolving if 85% or greater of the labelled amount of the drug substance dissolves within 30 minutes. Lennernas (1998) examined several methods used to predict human intestinal permeability. The author suggested a good correlation exists between the measured human effective permeability values and the extent of absorption of drugs in clinical pharmacokinetic studies. Estimations of the absorption half-lives from the measured effective intestinal permeability (P_{eff}) agree very well with the time to maximal amount of the dose absorbed. Human *in vivo* permeability can be predicted using preclinical permeability models, such as *in situ* perfusion of rat jejunum, the Caco-2 model and excised intestinal segments.

Prediction of passively transported compounds can be accurately predicted, although those agents that are absorbed by carrier-mediated transport mechanisms need to be evaluated with a special degree of caution (Lennernas, 1997; 1998). Clearly, added research is needed to further characterize the influence of active transport mechanisms, for example, multidrug-resistance transporters and intestinal metabolism, for example, Cytochrome P450 3A4, on drug bioavailability that is observed with anticancer agents (Schellens *et al*., 2000).

7 ORAL ADMINISTRATION OF XENOBIOTICS: PRACTICAL CONSIDERATIONS

Guidance standards for toxicological protocols have been published by the OECD and the EPA. For oral toxicity assessments, the guidance protocols are available for acute oral toxicity, 28- and 90-day toxicity and long-term chronic and oncogenicity studies. In the guidances, the rat and mouse are indicated as preferred rodent species with the dog specified as the preferred nonrodent species.

Generally, the oral administration of xenobiotics to experimental animals will be by bolus gavage or continuous administration. The first case, the most common means of administration, most often utilizes rodents, although dogs, rabbits, minipigs, nonhuman primates and variety of other animals are used, with each presenting their own advantages and disadvantages. By gavage, a stainless steel or flexible tube is used to deliver the xenobiotic. For rodents, the tube usually deposits the substance within the oesophagus, but with the force of depression of the syringe, the material is forced into the stomach. For larger species, a flexible tube is used that can deliver the material directly into the stomach with a wash of the tube included, using water or the vehicle. In addition, capsules or tablets can be delivered into the stomach for larger species, for example dogs, rabbits, monkeys, although with skilled technical ability tablets can be administered to rats. The dog is very prone to emesis following administration of substances in liquid formulations. Therefore, the use of capsules or tablets may potentially overcome this problem. Recently, the minipig has gained favour as an alternative nonrodent

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species in safety assessments, as it is less prone to emesis (Svendsen, 2006; Dincer *et al*., 2006; Kano *et al*., 2005).

Alternatively, studies are carried out in which the test material is incorporated into the diet or drinking water. When included with the feed or water, measurements of food and water consumption becomes necessary in order to calculate a dose rate. In addition, a homogeneous distribution of the test material within the feed is necessary and could include suspending the test material in corn oil or other vehicles to ensure distribution. Including the test article in drinking water poses additional issues in study conduct. Spillage of diet and water can potentially lead to inaccurate determination of dosage. Furthermore, for water administration, ensuring homogenous distribution of substances can be problematic if the compound is not completely soluble. Incorporating test chemicals into diet or water also may result in an unacceptable taste for the animal such that there is reduced consumption. In these cases, it is often advisable to undertake a short-term study to test for the palatability of the compound prior to undertaking longer-term studies.

Although dispersion of substances in water is not usually a problem with soluble test materials, stability of a compound in water can be problematic. Hence, determining the stability of the waster solution prior to study initiation becomes mandatory. The instability of a water solution of a compound was evident in a chronic drinking water study with vinyl acetate, in which the compound was formulated on a weekly basis (Lijensky and Reuber, 1983). In a subsequent chronic study, the water solutions were prepared daily to compensate for the rate of hydrolysis of the compound (Bogdanffy *et al*., 1994). In addition to instability, volatile materials may evaporate from the water. Often, however, volatile liquids are formulated in another solvent, for example corn oil, and then suspended in water. Although not an ideal means of compound delivery, fluorocarbons were incorporated into vehicles, for example, corn oil, for administration many years ago (Longstaff *et al*., 1984).

With gavage administration, animals can be treated fairly rapidly and automated systems have been adapted from equipment designed for cutaneous application. Administration of dosing solution or suspensions can be labour intensive and represents a major cost factor in toxicology studies. In a typical subchronic study with a control and three dose groups, the number of rats often included is about 80–100 animals that may need to be treated for as long as 90 days. As the duration of the study increases, the numbers of rats included in these studies increases such that for a two year carcinogenicity study, upwards of 400–600 animals may be used. Well-trained technicians, however, can gavage a specific animal quickly, and the skilled technician often can gavage a few animals within a minute or so. This can also be a disadvantage. With the administration of compounds that are somewhat viscous, for example corn oil solutions and suspensions, there is a greater chance of gavage errors, whether through perforation of the oesophagus, directly discharging the compound into the trachea or through aspiration. In a recent experience by this author, a rather viscous dosing solution appeared to adhere to the gavage needle and as the needle was withdrawn the compound was aspirated into the lung by the rat. This led to an increase in mortality across the dosing groups. Having the technicians delay for a few seconds before withdrawing the dosing needle prevented further mortalities.

When conducting studies by gavage, the dosing vehicle selected to solubilize or suspend the test article is critical to ensure absorption of the compound without the vehicle having an effect on absorption or, most importantly, that the vehicle does not exert toxicity (Gad *et al*., 2006). Generally, aqueous solutions of the test article are preferred. However, other solvents are used in preclinical studies and include a rather lengthy list of neat materials and combinations of materials. The formulation must be suitable for the intended route of administration, maintain the stability of the active ingredient and preferably maximize the systemic bioavailability of the drug. Occasionally, the vehicle is specified by the sponsor of a study, but more frequently the laboratory conducting the studies has experience with different vehicles and can provide advice. Because the process of vehicle selection has been mostly one of custom or personal choice, there are many vehicles which have seen use in preclinical formulation. Gad *et al*. (2006) provided various characteristics that make vehicle selection preferable. Although the intent of their paper was to review vehicles for different routes of administration, it is important to note that many of the cited characteristics are useful for oral administration, for example maintain stability, minimize dose volumes, and so on.

As alluded to previously in this chapter, a dosing vehicle can alter the bioavailability of a compound. A classic example of this was reported many years ago by Kim *et al*. (1990). These authors found that a single dose of carbon tetrachloride at l0 or 25 mg kg^{-1} in corn oil caused less severe hepatic injury than when given at the same dosages undiluted or as an aqueous emulsion to rats. In contrast to this report, however, the hepatotoxicity of chloroform was enhanced when administered in corn oil (Bull *et al*., 1986). Koporec *et al*. (1995) subsequently showed that carbon tetrachloride hepatotoxicity was not influenced by the vehicle. In their study, rats orally administered carbon tetrachloride at doses of 0, 25 or 100 mg kg^{-1} by gavage in either corn oil or a 1% Emulphor aqueous emulsion for 13 weeks. These authors found that corn oil did not significantly alter the subchronic hepatotoxicity of carbon tetrachloride compared to the aqueous emulsion. Clearly, discrepancies in experimental outcomes for similar compounds further complicate the choice of a vehicle for compound administration.

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Other vehicles have become more often used, and include carboxymethyl cellulose (CMC), polyethylene glycols (PEGs), dextrans, and so on (see Gad *et al*., 2006). In selecting these other vehicles, caution needs to be exercised to ensure toxicity to the animal does not occur or have an affect on intestinal metabolism. Mountfield *et al*. (2000) reported the effects of various commonly used formulation ingredients on cytochrome P450 3A inhibition using dog, monkey and human microsomes. Although these authors did not observe changes in cytochrome P450 3A with most formulation ingredients, greater inhibition of cytochrome P450 3A activity was observed with amphiphilic ingredients, for example, Tween 80 and oleic acid. Yoo *et al*. (1999) showed that bioavailability following sublingual administration of clomipramine was enhanced hydroxypropyl-β-cyclodextrin compared with saline administration.

Toxicity produced by the vehicle also needs to be considered. For example, it is well known that Tween 80 may cause anaphylactic reactions in dogs, with intravenous administration (Varma *et al*., 1985; Eschalier *et al*., 1988). In addition, Tween 80 has been shown to have an electrophysiological effect in anaesthetized dogs (Torres-Arraut *et al*., 1984), as does Cremophor EL (Lorenz *et al*., 1977). Dogs are susceptible to emesis such that oral administration of substances that cause gastrointestinal irritation may evoke an emetic response. Hence, the use of vehicles in the dog that may be mucosal irritants, for example, Tween 80, might result in emesis, thereby affecting the bioavailability of the drug. Dose selection of the vehicle, therefore, becomes an important consideration in the design of the study. The maximum dose volume used in toxicity studies should not exceed 20 ml kg^{-1} as a single dose. Indeed, it is often best to minimize the dose volume to 10 ml kg^{-1} or less unless divided doses are considered. Many of the vehicles used in toxicology studies have been used for many years and have been found to be acceptable to ensure limited effects on experimental animals and have limited effects on the stability of the test material. Clearly, many of these factors should be determined prior to undertaking a study.

8 SUMMARY

From the above discussion, it is clear that numerous factors will affect the passage of drugs and chemicals through the gastrointestinal tract into systemic circulation. These factors involve anatomical features, species differences, physiological features and biochemical features, including intestinal metabolism and effects on transporters. With regard to assessing the potential risk of chemicals, these features will need to be considered, as there will be differences between experimental animals and humans.

The cellular environment of the gastrointestinal tract and the molecular mechanisms of absorption will have a major influence on the bioavailability of xenobiotics. As noted, the pH of the local cell environment will affect the ionization status of the compound, whereas a drug may inhibit cellular transporters of other substances, for example, nutrients, thereby having an affect on the nutritional status of the animal. Moreover, there has been demonstrated evidence that some substances will affect the metabolic status of the gastrointestinal tract. Cytochrome P450 3A is the more common form of cytochrome isoforms found in the intestine, but there are numerous examples beyond those described in this chapter that can inhibit, or induce, metabolism, much in the same way that hepatic metabolism is affected by chemicals. In addition, the bacterial content of the gastrointestinal tract can affect the absorption of substances, primarily by bacterial metabolism. The fasting status of the animal also will affect absorption, and is probably one of the more important considerations in the design of a toxicology study. It has been clearly shown that absorption occurs more readily in the fasted animal, and that toxicity may be enhanced, since there is a greater surface area of the gastrointestinal tract available for absorption. Finally, chemical interactions will potentially modify absorption through binding of the compound or changing the local cellular environment.

From a practical perspective, oral administration of compounds is the most common method for compound administration in toxicology studies. In oral toxicity studies, one will need to consider the drug formulation and the potential effects of the vehicle on the stability, as well as the potential toxicity, of the vehicle. For the most part, a majority of the vehicles being used currently in laboratories have a long history of use, such that toxicity of the vehicle does not become too much of a concern, but the principle of toxicology, the dose makes the poison, is always a consideration.

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NOTES

[a.] Dose number (Do) was calculated as highest dose strength divided by a predetermined volume (250 ml) and the result divided by the solubility. *C* log*P* is an estimate of gastrointestinal permeability (see Kasim *et al*., 2004).

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Cutaneous Toxicology

Jim E. Riviere and Nancy A. Monteiro-Riviere

CONTENTS

1 INTRODUCTION

Skin is one of the largest organs of the body and therefore dictates that particular attention should be paid to it when considering systemic toxicoses. However, the skin is also a primary interface between the body and the environment, making it particularly relevant to assess dermal toxicity after occupational or environmental exposure to chemicals. The skin is often a primary exposure route for cosmetics and is the preferred route of administration for topically applied drugs targeted either locally for dermatological applications or transdermally for systemic therapy. Toxicity to this organ is manifested by alteration to one of its biological functions including loss of barrier function, damage to dermal collagen or vascular structures, hair loss, damage to adnexial structures such as sweat and sebaceous glands, carcinogenic transformation of epidermal cells or activation of the resident immune system. The unique exposure of skin to the environment also makes this organ susceptible to direct interaction with ultraviolet (UV) radiation that may result in sunburn, phototoxicity or cancer.

Absorption and toxicity are inextricably linked in evaluating the potential for a chemical or drug to cause dermal toxicity. A *barrier* is defined as something that obstructs or impedes. For most applications in dermatotoxicology, this obstruction refers to the impediment of chemical movement across or into the skin. This barrier can be viewed as restricting diffusion, but also could be viewed as impeding absorption of intact drug through metabolism in the skin. Topically applied chemicals may either *penetrate* the skin to have a local effect and/or be *absorbed* across the skin and result in systemic absorption and distribution throughout the body. It is important to keep these terms separate since both are important parameters with different experimental end points.

Any review of skin toxicology must divide the discussion into three sections; percutaneous absorption or penetration, toxicological effects and the *in vivo* or *in vitro* model system used to evaluate these. This chapter will focus on introducing the reader to the succinct elements of the structure and function of skin that are relevant to understanding dermal toxicity. More advanced texts should be consulted for further discussion in this area (Riviere, 2006; Wilhelm *et al*., 2007; Roberts and Walters, 2008).

2 STRUCTURE AND FUNCTION OF SKIN

The skin is the largest organ of the body, with the integument accounting for some 20% of body weight in most species. Skin has the function of protecting the internal organs of the body from extremes in temperature fluctuations, allergens, pollutants, toxic chemicals and organisms such as bacteria, fungi, parasites and viruses found ubiquitously in the environment. The skin of humans and most animals is actually quite similar morphologically across species. Perforating the skin are several types of appendages (depending on the species), including hair follicles, sebaceous and sweat glands, spines, quills, scales, spurs, horns, claws, nails and hooves. The specific and comparative anatomy of

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skin and hair has been reviewed extensively elsewhere (Montagna, 1967; Monteiro-Riviere, 2006).

On the histological level, the skin can be divided into two very distinct regions: the epidermis and the dermis. The epidermis consists of stratified squamous keratinized epithelium that undergoes a programmed proliferation and differentiation that will eventually result in the formation of the major barrier to chemical penetration: the stratum corneum. Two primary cell types exist in the epidermis: keratinocytes and nonkeratinocytes (melanocytes, Langerhans cells and Merkel cells).

Five distinct layers of keratinocytes can be present in the epidermis, as shown in **Figure 1**. Starting from the deepest layer, they are the stratum basale (basal layer), stratum spinosum, stratum granulosum, stratum lucidum and the stratum corneum. Each cell layer originates from the stratum basale layer, a single layer of cuboidal or columnar cells that rest on the basement membrane. These cells are attached to the basement membrane by hemidesmosomes, and to each other and to the cells of the stratum spinosum by desmosomes. The stratum basale cells continuously divide, with some remaining as basal cells and others moving upward and maturing by changing their intracellular content and biochemical pathways through the process called keratinization. It is this viable and dividing epidermal cell layer that is the target for chemical transformation into cancerous cells.

The next more superficial layer, the stratum spinosum, is composed of irregularly shaped polyhedral cells that make up much of the bulk thickness of the epidermis. The next layer composes the stratum granulosum consisting of several layers of cells that begin to flatten horizontally. Of primary interest within these cells are the membrane-bound organelles, the lamellar granules, which contain polar phospholipids including glycosphingolipids and free steroids, as well as numerous hydrolytic enzymes. Nonmembrane-bound electron-dense keratohyalin granules are also present. These consist of profilaggrin, a precursor protein of filaggrin and play a role in keratinization and barrier function. As lamellar granules accumulate within these cells they will release their products via exocytosis into the intercellular spaces between the stratum granulosum and stratum corneum layers of the epidermis, eventually forming the extracellular lipid matrix of the stratum corneum that participates in the penetration barrier of the stratum corneum. As these epidermal cells continue their migration upwards, they may form the stratum lucidum, a translucent line of cells found only in areas of very exceptionally thick skin such as the plantar and palmar surfaces (foot pads) (Monteiro-Riviere, 1991; 2006).

The stratum corneum is the outermost superficial epidermal layer and is the most important when considering drug or chemical absorption after topical exposure, since it is the primary barrier to percutaneous absorption. In addition to the barrier function for xenobiotics trying to enter the body from the environment, the stratum corneum also provides a barrier to insensible water loss, an evolutionary adaptation that allows terrestrial animals to exist in a nonaquatic environment. Most cosmetics are targeted to improve this function. It is this function that is assessed when transepidermal water loss (TEWL) is

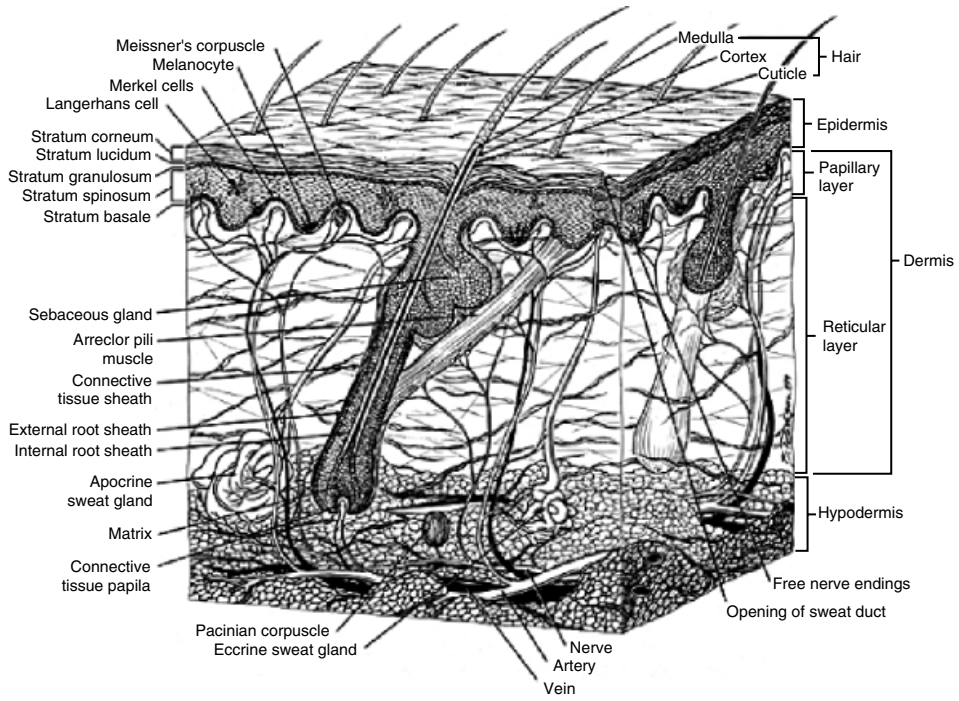

Figure 1 Structure of skin.

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measured as a biomarker of barrier function in cutaneous toxicology studies.

The stratum corneum consists of several dead layers of cells, organized into vertical columns in a tightly packed geometrical configuration, the thickness of which varies depending on location (Monteiro-Riviere, 1991). Each corneocyte is embedded in the lipid matrix produced by the lamellar granules. These cells are bathed in the lipid matrix, forming the so-called 'brick and mortar structure' (Elias, 1983) where the dead corneocytes represent the 'bricks' and the intercellular lipids represent the 'mortar'. There is a consensus that chemicals are absorbed through skin via this intercellular pathway, with partitioning into and diffusion through the lipid predictive of compound absorption. These intercellular lipids consist of a mix primarily of sterols, other neutral lipids and ceramides. These dead cells are also surrounded by a thick plasma membrane with a submembranous layer of involucrin, also produced earlier in development. Although highly water retarding, the dead, keratinized cells are highly water absorbent (hydrophilic), a property that keeps the skin supple and soft as they absorb water on its way to being evaporated from the surface. With intracellular and intercellular barriers firmly in place, the stratum corneum has the ability to constrain the passage of unwanted chemicals and toxins from the environment. A natural oil covering the skin, the sebum, appears to maintain the water-holding capacity of the epidermis, but has no appreciable role in retarding the penetration of xenobiotics. This is especially prevalent in some species including sheep.

In addition to the keratinocytes, epidermal nonkeratinocytes are present throughout the epidermis and do not participate in the formation of keratin. They include the melanocytes, Merkel cells (tactile epithelioid cells) and the Langerhans cells (intradermal macrophages).

Melanocytes are dendritic cells located in the basal layer of the epidermis and hold pigment-containing ovoid cytoplasmic granules referred to as melanosomes. These cells impart colour to the skin by transferring melanosomes to neighbouring keratinocytes. Merkel cells are located in the basal region of the epidermis and are thought to function as slow-adapting mechanoreceptors for touch. Langerhans cells are dendritic cells and are located in the stratum spinosum but may also be present in dermal lymph vessels, lymph nodes and dermis. The Langerhans cells' primary function is to detect antigens penetrating the stratum corneum layer and then migrate out of the epidermis once activated and present the antigens to lymphocytes in regional lymph nodes. They are often considered the initial receptors for cutaneous immune responses. It should be stressed that an antigen need only penetrate the skin to elicit a dermatological immune response. Systemic absorption is not necessary. The epidermis is not perfused by vascular capillaries and thus obtains nutrients by diffusion from the underlying dermis. The basal cells of the epidermis sit on the epidermal–dermal junction (EDJ) which may be a target for vesicating agents such as sulphur mustard or lewisite.

The dermis is composed of connective tissue consisting of collagen, elastic and reticular fibres dispersed in an amorphous ground substance and can be divided into two rather poorly demarcated areas. The superficial papillary layer consists of loose connective tissue that connects the epidermis (stratum basale/basal lamina) to the deeper reticular layer of the dermis. The reticular layer consists of dense irregular connective tissue connected to the hypodermis, which is composed mostly of superficial fascia or fat. Dispersed throughout both layers of the dermis is a network of arterial and venous blood vessels and connecting capillaries needed to nourish the cells of the dermis and epidermis via diffusion, as well as take part in the final stages of the percutaneous absorption of compounds. Lymph vessels, nerves, apocrine and eccrine sweat glands, sebaceous glands, Pacinian (pressure receptor), Meissner's (touch receptor), and Ruffini (mechanical receptor) corpuscles, hair follicles and smooth muscles (arrector pili) are some of the other major structures found in the dermis. Cutaneous blood flow rates may be one of the factors affecting the passive percutaneous absorption of chemicals.

Figure 2 shows photomicrographs illustrating the appearance of skin in humans and pigs.

There are interesting aspects of skin biochemistry that may impact dermal toxicity. The skin, specifically the

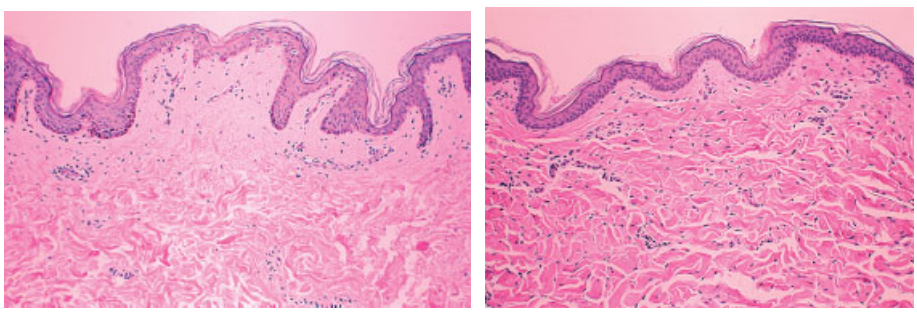

Figure 2 Photomicrograph of human (A) and porcine (B) skin.

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epidermis, is mostly an anaerobic organ. The absence of capillaries directly feeding oxygen to the epidermal cells makes the epidermal cells relatively oxygen poor in the normal state when compared to other tissues with a more direct blood supply. Due to this low oxygen tension, the epidermis produces 70–80% of its total energy requirements (adenosine triphosphate) through the anaerobic metabolic pathway (glycolysis), with lactic acid being the end product of glucose utilization. The epidermal cells, the most active of which are those in the single bottom layer of the stratum basale, are the primary cells involved in this energy production, with the lactic acid end product passively diffusing into the dermis and then into the blood vasculature, eventually being recycled by the liver back into glucose. Although the glycolytic pathway produces most of the energy requirements of the epidermis, other energy pathways (tricarboxylic acid cycle and pentose phosphate shunt) are also utilized to lesser degrees in some phases of epidermal growth (Freinkel, 1983).

The stratum corneum is the primary line of defence to prevent percutaneous absorption of drugs. However, any drug passing through the stratum corneum may face a metabolic, rather than a physical, diffusional barrier. The skin has a remarkable ability to metabolize xenobiotics. These metabolic reactions consist mainly of oxidation, reduction, hydrolysis, and the phase I and II conjugation reactions. Some of these reactions are major pathways for the metabolism of topical steroids as well as other drugs. Numerous studies have demonstrated the metabolic capabilities of skin when topically exposed to caffeine, testosterone, butylated hydroxytoluene, salicylic acid, norepinephrine, benzo(a)pyrene and benzoyl peroxide—to name only a few—in many species of laboratory animals, as well as in human skin. The cytochrome P450 enzyme system, most commonly associated with the liver, is present and is inducible in skin (depending on the compound that was applied topically) (Riviere and Chang, 1992; Mukhtar, 1992; Bashir and Maibach, 2005). These studies all suggest that the skin has formidable metabolic functions in addition to the traditional barrier functions. For many compounds, such as benzo(a)pyrene, this could result in metabolic activation to toxic moieties or active molecules after topical prodrug administration.

In addition to these metabolic activities, skin also has a marked ability to synthesize lipid, which is used to construct the extracellular epidermal barrier. Epidermal cells manufacture a variety of neutral lipids, ceramides, glycosylceramides, gangliosides, sterol esters, fatty acids, alkanes and phospholipids, which are largely found in the extracellular barrier of the stratum corneum. Some of these lipids tend to be site-specific, with phospholipids and sterols residing mostly in the basal cell layers and some sterols and neutral lipids occupying the upper regions of the epidermis, mainly the stratum corneum. It is now accepted that this complex intercellular lipid matrix provides the primary barrier to drug penetration. The effects on percutaneous absorption by the intercellular lipid structure has been discussed in greater detail elsewhere (Wertz, 1992; Potts and Francoeur, 1992; Elias and Feingold, 1992; Monteiro-Riviere *et al*., 2001b). The primary protein product of the skin is keratin, which is the main intracellular component of the stratum corneum layer. In tandem with extracellular lipid, keratin forms the major component of the epidermal barrier. Keratin primarily consists of cysteine, serine, glutamic acid, arginine, aspartic acid and glycine amino acid residues. Of significance within the keratin molecule structure is the intrachain cysteine disulfide bridging that further strengthens the overall keratin macrostructure. Keratin is the major protein foundation of hair (Monteiro-Riviere, 1991; 2006).

3 PERCUTANEOUS ABSORPTION

For a topically applied chemical that is not a direct corrosive agent (see discussion below), penetration and/or absorption through the epidermal barrier are required for a dermatotoxic response to be expressed. The major end points for assessing a chemical's ability to be absorbed through skin are either to determine its permeability constant or estimate the rate and extent of absorption after topical dosing from a specific concentration and formulation, including the chemical of interest. In this latter vein, dosing conditions can also be designed to mimic occupational or environmental exposure scenarios in response to the needs of specific regulatory issues. These approaches are fully developed and discussed elsewhere (Cleek and Bunge, 1993; Bunge and Cleek, 1995; Riviere, 2006).

The movement of chemicals across the stratum corneum barrier into the epidermis occurs primarily by passive diffusion driven by the thermodynamic force arising from the greater applied concentration of the drug on the surface of the skin compared to that in the skin and/or blood. This is best quantitated using Fick's law of diffusion that states that the steady state of drug flux across a membrane can be expressed as:

$$Flux(J) = \frac{DP}{h}(Concentration\ Gradient \times (Applied\ Surface\ Area \quad (1)$$

D is the diffusion coefficient or diffusivity of the drug in the intercellular lipids of the stratum corneum, P is the partition coefficient for the drug between the stratum corneum and the dosing medium on the skin surface and h is the skin thickness or actual path-length through which the drug diffuses across the diffusion barrier.

Because of the aforementioned 'brick and mortar' structure of the stratum corneum, the diffusion path length is tortuous and much greater than the actual

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thickness. The driving force for this thermodynamic process is the concentration gradient that exists between the applied dose and the dermal perfusate or blood.

The compound's permeability coefficient Kp is defined as DP/h. Kinetically, this is a first-order rate constant that forms the basis for the absorption rate constant (Ka) used in pharmacokinetic models describing chemical percutaneous absorption and transdermal drug delivery. Dermal absorptive flux should always be expressed in terms of the surface area of the dosing site, making the two important properties of dosage after topical exposure the concentration of drug applied, as well as the surface area of application. Finally, Fick's law expresses the steady-state flux of drug that occurs when this rate becomes constant. In skin diffusion studies, this occurs after the passage of a *lag time* that is a function of the drug 'loading' the stratum corneum and dermis, diffusivity and thickness of the skin. Lag time can be calculated as $(h^2)/6D$. For molecules with small diffusivities, initial absorption rates may be very low. If steady state is not achieved, Fick's second law of diffusion may be employed, although data requirements and experimental design are much more complex.

The diffusivity of a chemical is a function of the molecular weight, molecular size, molecular interactions with skin constituents (e.g. hydrogen bonding, hydrophobic interactions, etc.), the drug's solubility in the membrane milieu and the degree of ionization. These properties form the basis of the quantitative structure activity relationship (QSAR) equations presented below. Large molecular weight drugs (approximately greater than 500 Da, e.g. proteins) have extremely low diffusivities, thus effectively preventing them from being absorbed across the skin barrier and result in significant systemic fluxes. When diffusivity is very low, an exceedingly long lag-time could occur, due to its increased dependency on this parameter. Note that this rule of thumb applies relative to the ability to generate significant transdermal fluxes for pharmacological activities, and not necessarily absorption of small quantities of chemicals that could exert a local effect. However, from the perspective of cutaneous toxicology, very small quantities of chemical may still penetrate into the skin and elicit a toxicologic response, especially when the mechanism of such toxicity is not dose related (e.g. allergic response, carcinogenicity). For compounds that are partially ionized, diffusivity is significantly decreased, suggesting that only the nonionized fraction of a weak acid or base is available for diffusion across the stratum corneum. This is a function of the pH of the dosing medium as well as the pH of the skin.

The penetrating drug must have some propensity to partition into the intercellular lipids of the stratum corneum for diffusion to occur. The partition coefficient determines the ability of the drug to gain access to the diffusion pathway and is usually determined in experimental systems by measuring octanol/water or lipid/water partitioning. The higher the ratio, the greater is the lipophilicity. It is generally accepted that the optimal log octanol/water partition coefficient for a drug to penetrate the stratum corneum is approximately two. In other words, the drug is partitioned in the lipid phase approximately 100-fold. For hydrophilic drugs with low partition coefficients, prodrugs could be formulated (e.g. by esterification) which increases the drug's permeability across the stratum corneum. The lipophilic moiety (e.g. ester) is then cleaved in the epidermis, dermis or even plasma and the active parent drug is then distributed throughout the systemic circulation. If the partition coefficient is too great, the drug may have a tendency to sequester in the stratum corneum and not enter the more aqueous dermis, thereby decreasing systemic delivery. If it does penetrate into the dermis, the high lipid partition coefficient may favour formation of a dermal depot.

It must be noted, that the drug must also have partitioning properties that are favourable for entering into solution in the aqueous plasma, or be able to bind to plasma proteins for systemic absorption to occur. Finally and most importantly for many pharmaceutical and risk assessment issues, the tendency to partition is counterbalanced by solubility in the dosing vehicle. For a compound to be absorbed, it must have sufficient solubility to be in solution while still possessing sufficient lipid solubility to partition into the stratum corneum lipids. Optimization of these two independent properties may be required to obtain significant dermal absorption.

There are other factors that also are important in assessing absorption. After the lag time has passed, chemical flux across skin is at steady state. It is this value that should be used to calculate Kp. In an infinite-dose experiment where the amount absorbed is minimal compared to that applied, a plot of cumulative flux will easily allow precise measurement of flux. However, in a finite-dose experiment, where a significant fraction of the dose actually gets absorbed, the flux after the lag time has passed will ultimately decrease as the chemical is depleted from the dosing solution. The slope of the initial portion of this flux profile is thus usually termed *pseudo-steady state*. At steady state, the flux (J) can be presented as $J = Kp \cdot$ concentration.

Experimental data can also be reported as maximum flux ($J_{\max}$) and the time to this flux ($T_{\max}$). This term is often used as the parameter to quantify the absorption properties for toxicological end points since there are no assumptions inherent to its determination. $J_{\max}$ for a specific chemical can be determined from a saturated solution of the compound and will give an estimate of the worst-case dermal exposure for a chemical. However, if a supersaturated solution is applied, $J_{\max}$ may be even greater, its value being a function of thermodynamic activity in the dosing vehicle countered by the extent of interaction between the vehicle and the skin. Depending on the circumstances and the nature of the chemical

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and vehicle involved, $K\mathrm{p}$ or $J_{\max}$ may be the optimal parameters to characterize exposure.

A great deal of research has been focussed on relating the physical chemical properties of the penetrant to its permeability through skin (Moss *et al*., 2002); an area coined quantitative structure permeability relationships (QSPeR). The first such relationship widely used to assess chemical absorption was that of Potts and Guy (1992):

$$\mathrm{Log}K\mathrm{p} = 0.71\log PC_{\mathrm{octanol/water}} - 0.0061MW - 6.3(R^2 = 0.67) \quad (2)$$

where MW is the molecular weight.

This equation was subsequently modified (Potts and Guy, 1995) to relate $K\mathrm{p}$ to molecular properties of the penetrants as:

$$\mathrm{Log}K\mathrm{p} = 0.0256MV - 1.72\sum\alpha - 3.93\sum\beta - 4.85(R^2 = 0.94) \quad (3)$$

where MV is molecular volume, $\sum\alpha$ is the hydrogen-bond donor acidity and $\sum\beta$ is the hydrogen-bond acceptor basicity.

The most promising approach is to generalize this approach using linear free-energy relationships (LFERs) to relate permeability to the physical properties of the penetrant under defined experimental conditions (dose, membrane selection, vehicle). Geinoz *et al*., (2004) should be consulted for a critical review of most such QSPeR models applied to dermal absorption. Abraham's LFER model is representative of the dermal QSPeR approaches presently available (Abraham *et al*., 1999). This model was selected since it is broadly accepted by the scientific community as being descriptive of the key molecular/physiochemical parameters relevant to solute absorption across skin. This basic model can be written as:

$$\log K\mathrm{p} = c + a\sum\alpha + b\sum\beta + s\pi + rR_2 + vV_x \quad (4)$$

where π is the dipolarity/polarizability, R_2 represents the excess molar refractivity, V_x is the McGowan volume and the other parameters are as described earlier. The variables c, a, b, s, r and v are strength coefficients coupling the molecular descriptors to skin permeability in the specific experimental system studied.

All of these approaches have modelled permeability in single aqueous vehicle systems. Formulations and solvents have long been know to alter a chemical penetrant's solubility and partition coefficient into the stratum corneum, the two factors which determine dermal absorption. For a lipid-soluble drug, a lipid base formulation would tend to decrease absorption by retaining the applied drug at the skin surface. In contrast, an aqueous base would promote absorption solely by this partitioning phenomenon, which would favour drug movement out of the formulation into the more favourable lipid environment of the stratum corneum. The actual solubility of the compound in the vehicle further modifies this relationship. The reverse scenario would be operative for a hydrophilic drug.

Our laboratory has focussed significant research on the effects of chemical mixtures on dermal absorption of penetrant compounds (Riviere and Brooks, 2005; 2007). In order to quantitate mixture effects, we have been exploring using an additional term, referred to as the mixture factor (MF), to modify a QSPeR equation to better predict absorption from mixtures:

$$\log K\mathrm{p} = c + m\mathrm{MF} + a\sum\alpha + b\sum\beta + s\pi + rR_2 + vV_x \quad (5)$$

The nature of the MF is determined by examining the residual plot (actual—predicted log $K\mathrm{p}$) generated from the base LFER equation based on molecular descriptors of the permeants, against a function of the physical chemical properties of the mixture/solvents in which they were dosed. The selection of an appropriate MF is based on its correlation to the residuals of the normal QSPeR model. Importantly, the MF seems to improve prediction of permeability and absorption across different LFER models (e.g. Potts and Guy as well as Abraham approaches).

4 DERMAL TOXICITY

If a cytotoxic chemical is capable of dermal penetration, it may have a high probability of causing toxicity to the skin. The clearest approach to assessing chemical-induced damage to skin is to assess what abnormalities occur when the specific anatomical structures discussed above are perturbed after exposure to topical compounds.

4.1 Direct and Contact Irritants

A large number of cutaneous irritants specifically damage the barrier properties of skin, which results in an irritation response. These include organic solvents, which extract the intercellular lipid and perturb the skin's barrier, as assessed by increasing TEWL. Some chemicals destroy or digest the stratum corneum and underlying epidermis with a mode of action classified as corrosive. Such compounds cause chemical burns and include strong acids, alkalis and phenolics. They directly attack the epidermal barrier and chemically destroy the underlying viable cell layers. The best treatment in these cases is to dilute and remove the offending agents by flushing

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with water. Time is of importance since even with immediate treatment with specific decontaminants after experimental phenol exposure, phenol was observed to still penetrate skin (Monteiro-Riviere *et al*., 2001a). The exception is CaO (quicklime), which violently reacts with water and generates heat, resulting in further thermal damage, and metallic (e.g. tin, titanium) tetrachloride compounds, which hydrolyse to hydrochloric acid on exposure to water causing further skin damage. These types of reactions are easy to assess using *in vitro* models such as the Corrositex® system, which detects macromolecular damage by acids and bases to a collagen matrix resulting in a chemical colour change in an associated detector system.

Direct irritation may be defined as an adverse effect of compounds directly applied topically to the skin not involving prior sensitization and thus initiation by an immune mechanism. Epidermal cells may be directly affected resulting in the initiation of a series of sequelae leading to irritation. Several types of adverse effects and chemical interactions may be manifested when a compound is placed upon the skin surface. Irritation is usually assessed by a local inflammatory response characterized by erythema (redness) and/or oedema (swelling). Other responses may be present that do not elicit inflammation, such as an increase in thickness. Irritant reactions may be classified by many characteristics, including acute, cumulative, traumatic and pustular, however two classifications are generally studied by toxicologists. Acute irritation is a local response of the skin usually caused by a single agent that induces a reversible inflammatory response. Cumulative irritation occurs after repeated exposures to the same compound and is the most common type of irritant dermatitis seen. The mechanisms of irritation are thus very complex and are still being characterized, since they involve interaction of inflammatory cytokines, with subsequent involvement of the immune system.

If a penetrating compound is capable of interacting with the immune system, the manifestations seen will be dependent upon the type of immunologic response elicited (e.g. cellular versus humoral, acute hypersensitivity, etc.). This response is termed contact dermatitis. Immune cells localized within skin (e.g. Langerhans cells, lymphocytes, mast cells) may modulate the reaction or the keratinocytes themselves may initiate the response. In fact, keratinocytes were once thought to produce only keratin and mucopolysaccharides, but recent studies have shown that they can produce growth factors, chemotactic factors and adhesion molecules. Keratinocytes may act as the key immunocyte in the pathophysiology of allergic contact and irritant contact dermatitis. Direct irritation of keratinocytes by toxic chemicals may also initiate this cytokine cascade without involvement of the immune system, blurring the distinction between direct and indirect cutaneous irritants (Nickoloff, 1993; Nettis *et al*., 2002). Damage in irritant contact dermatitis is a function of the duration of contact, strength of the irritant (penetrability and inherent potency) and frequency of contact.

It must be stressed that the primary mechanism by which most chemicals cause dermal irritation (e.g. organic solvents, corrosives) is through impairment of the stratum corneum barrier properties discussed earlier, reflected by an increase in TEWL. If the stratum corneum barrier is perturbed, a feedback response may be initiated, whereby regeneration of the barrier occurs. This reaction is mediated by cytokines (especially tumour necrosis factor (TNF)-α) originating locally within the epidermis. However, additional responses to these inflammatory mediators may in themselves launch an irritation response mediated by the keratinocytes. Some workers also believe that this disruption of barrier function is the initiating event resulting in atopic dermatitis, suggesting that therapies aimed at restoring barrier function are more important than those targeted at down-regulating the immune response.

Regardless of the initiating mechanism (direct irritation, immunologically mediated contact dermatitis), the sequelae to many irritants are the same, being primarily erythema and oedema. Testing strategies used to assess both types of dermatotoxicologic insults will be presented below.

4.1.1 Phototoxicity

Unlike other internal organs, the skin has the potential to be constantly exposed to sunlight. This often results in adverse effects. UV radiation encompasses short-wave UVC (200–290 nm), mid-wave UVB (290–320 nm) and long-wave UVA (320–400 nm). With the recently identified increase in UVB exposure in certain geographical regions apparently secondary to ozone depletion, this topic is becoming increasingly important. Although the ozone layer surrounding the earth filters out all UVC radiation, enough UVB and UVA reach the earth's surface to pose chronic and acute health hazards. The most common adverse effects from this exposure include sunburn and skin cancer, mostly be attributed to UVB exposure. Sunburn, the excessive cutaneous exposure to UVB radiation, is the most frequent and familiar phototoxic reaction. Phototoxicity is a nonimmunological UV-induced response. The effects on skin are similar to a typical sunburn characterized by erythema, oedema, vesication, increased skin temperature and pruritis followed by hyperpigmentation. Irradiated skin injury is histologically characterized by the presence of sunburn cells in the stratum basale layer of the epidermis. These cells are dyskeratotic with a bright eosinophilic cytoplasm and a pyknotic nucleus in H&E stained tissue. Ultrastructurally, they possess cytoplasmic vacuoles, and condensed filament masses mixed with remnants of other cytoplasmic organelles (Monteiro-Riviere *et al*., 1994). Exposure to UV light may also induce carcinogenicity in all skin

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cell types, including basal and squamous cell carcinomas as well as the more invasive and lethal malignant melanoma.

In addition to the direct effects of UV light on skin described above, some chemicals may be photoactivated to toxic intermediates that then cause skin toxicity. Exogenous phototoxic chemicals can be found in therapeutic, cosmetic, industrial or agricultural formulations, with tetracycline antibiotics being the classic example. Phototoxicity may also be secondary to endogenous substances resulting from abnormal products of intermediates of haeme biosynthesis such as porphyrins, caused by an inherited or acquired enzymatic defect. Several types of phototoxic events may occur, classified based on whether they are oxygen dependent or not. When a reaction involves oxygen, the molecules can absorb photons and transfer the energy to oxygen molecules, thereby generating singlet oxygen, superoxide anions and hydroxyl radicals that cause damage to the skin. This occurs when protoprophyrins are UV irradiated. A photochemical reaction involves absorbance of photons by the chemical resulting in an excited state that will react with the target molecules to form photoproducts. A good example of this type of reaction is 8-methoxypsoralen (xanthotoxin), which reacts with specific sites on DNA by forming covalent bonds between the pyrimidine base and the furocoumarin. This phototoxic reaction may be restricted to areas of skin exposed to UV light, an attribute that has been employed with therapeutic advantage to treat accessible skin tumours and psoriasis. With other compounds like chlorpromazine and protriptyline, the molecules absorb photons to form stable photoproducts which then induce cutaneous toxicity (Marzulli and Maibach, 1996; Moulton-Levy and Maibach, 2008).

4.1.2 Vesication

Another mode of chemical-induced damage to the skin is vesication where agents cause blisters, which are separations of the epidermal and dermal layers of the skin. There are two histological classes of blisters that reflect different mechanisms of vesication. Intraepidermal blisters are formed due to the loss of intercellular attachment caused by cytotoxicity or cell death, while defects in the basement membrane components contribute to the blisters that occur within the EDJ. The chemical warfare agent sulphur mustard (bis-2-chloroethyl sulphide; HD) is the prototypical chemical that has been used to define this type of toxicity. HD is a bifunctional alkylating agent that is highly reactive with many biological macromolecules, especially those containing nucleophilic groups such as DNA and proteins. HD is known for its severe cutaneous damage and systemic toxicity.

The HD-induced dermal lesion is characterized by vesication and slow wound healing (Papirmeister *et al*., 1991). Our laboratory has shown that the epidermal–dermal separation associated with vesication occurs in the upper lamina lucida of the basement membrane after HD-induced dermal injury in the isolated perfused porcine skin flap (IPPSF) (Monteiro-Riviere and Inman, 1995). It was once thought that alkylation of DNA with subsequent DNA crosslinks or breaks were the primary and initial event responsible for HD cutaneous toxicity (Papirmeister *et al*., 1991). This lead to the hypothesis that DNA alkylation, metabolic disruption and proteolytic activity had occurred. Studies in our laboratory have shown that gross blisters and microvesicles, with separation occurring at the upper lamina lucida of the basement membrane, were present at 5 hours after HD exposure (Zhang *et al*., 1995a; Monteiro-Riviere and Inman, 1995). This suggests that a basement membrane component is the target of HD alkylation. Vesication is also accompanied by inflammation, necrosis and apoptosis (Zhang *et al*., 1995b; Monteiro-Riviere and Inman, 1997).

5 MODELS FOR EVALUATION OF ABSORPTION AND TOXICITY

The focus of dermal absorption studies is to predict chemical behaviour in humans. Studies are often conducted directly on humans for many drugs and cosmetics, however, for screening of unknown molecules or severe toxicants, or to serve the needs of traditional toxicology testing for chemicals with suspected toxicity, surrogate animal species must be used. The selection of an appropriate species to study dermal absorption is largely based on the use of the data that will be generated. These objectives include assessing penetration or absorption and systemic exposure after topical application, dermal sensitivity testing, carcinogenesis and potential for direct toxicity to skin. Anatomical factors that play a role in selection of a model species include diseases, skin thickness, hair follicle density, blood flow and regional species differences (Bronaugh *et al*., 1982; Monteiro-Riviere *et al*., 1990; Monteiro-Riviere, 1991; 2006; 2008).

5.1 Absorption and Penetration

A large amount of literature compares dermal absorption of a wide variety of chemicals in humans and multiple animal species (Feldmann and Maibach, 1974; Maibach and Feldmann, 1974; Bronaugh *et al*., 1982; Scott *et al*., 1991; Wester *et al*., 1998; Riviere, 2006). To compile such data, factors such as applied dose, surface area, use of occlusive dressings, and vehicle or formulation should

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be controlled. Since rodents are the primary laboratory animal used to assess safety of numerous drugs and chemicals, their use in dermal absorption studies is required for making route-to-route extrapolations. Mice and rats are also employed for carcinogenicity 'skin-painting' studies. In general, rodents (mice, rats) are more permeable to chemicals than humans, making them suitable for defining worst-case absorption scenarios for toxicological end points. Rabbits and guinea pigs have been utilized in dermal immunology protocols assessing issues such as contact dermatitis and skin sensitization. Rabbits have also been used to assess dermal absorption, but in many studies they have showed an increase in absorption with many compounds compared to humans.

Animal species with minimal hair or fur should be used when the goal of the study is to predict the rate and extent of chemical absorption in humans. As hair follicle density increases to the level seen in furred rats ($\approx 300\,cm^{-2}$) or mice ($\approx 650\,cm^{-2}$), the interfollicular skin becomes thinner, thereby increasing absorption. In contrast, hair follicle density in humans is only $11\,cm^{-12}$, which is the same as pigs. Hairless rodents usually lack hairs, but actually have a lower hair follicle density ($\approx 75\,cm^{-2}$), the term hairless being applied to their lack of hair shafts and not follicles (Bronaugh *et al*., 1982). Apes are often selected due to their evolutionary closeness to humans, however regions of the body with minimal hair (ventral abdomen) must be used.

The domestic pig is widely touted as an appropriate animal model (Feldmann and Maibach, 1974; Maibach and Feldmann, 1974; Scott *et al*., 1991; Wester *et al*., 1998). In addition to similarities in hair follicle density, number of epidermal cell layers, skin thickness and cutaneous blood flow, the biochemistry and biophysics of the stratum corneum lipids are comparable to humans. Since body mass/surface area ratios are also like humans, extrapolations of systemic exposure are facilitated. In whatever species employed, one should be cognizant of the fact that regional differences in skin anatomy exist making the crucial animal descriptor both species and body site. In addition, when pig skin obtained from an abattoir is employed, one must be sure to harvest skin before scalding occurs in the carcass decontamination process.

Once an appropriate species is selected, the decision must be made between using intact animals *in vivo* versus numerous *in vitro* approaches. The 'gold-standard' for absorption studies are *in vivo*, however the extent of variability inherent to this work often precludes intact animals being used for detailed probing of the mechanisms involved. Additionally, *in vivo* approaches are expensive, require special facilities, and are not accepted by some regulatory authorities for cosmetic screening. These approaches are extensively reviewed elsewhere (Riviere, 2006).

5.1.1 In Vivo Approaches

The classic technique used to assess absorption of any compound exposed to the body is by measuring the amount excreted into urine and faeces compared to that excreted after intravenous administration of an equivalent dose. The ratio of the total amount excreted after dermal to intravenous dosing is termed the *bioavailability*, referred to as F. This parameter can be calculated by collecting the plasma concentrations and comparing the ratio of their areas under the concentration-time profile (AUC). The reason F has to be determined relative to a parenteral route such as intravenous is that differences in metabolism between chemicals may change the rate and or extent of excretion or the percentage of a drug present in the central plasma compartment. Once the fraction of the dose eliminated in urine or faeces is known, then only the excreta needs to be collected to get future estimates of absorption. An extension of this is used when urine is monitored for assessing systemic exposure. If creatinine concentrations are also collected (marker of urine production), and drug mass normalized by it, then monitoring of only chemical concentrations and creatinine is sufficient to make estimates of relative systemic exposure or absorption after different topical chemical treatments.

All of these approaches require timed samples and careful analysis. Experiments should only be ended when the majority of the compound has been eliminated (e.g. approximately 80%). For dermal applications with very slow rates of absorption, the length of the study required to insure complete systemic distribution and elimination can be lengthy. Truncating studies earlier may lead to erroneous conclusions. When conducting such studies, care must be taken to insure the dose completely covers the marked exposure area and that this surface area is measured.

Additional approaches have recently been developed which attempt to assess *in vivo* absorption by monitoring the drug absorption process from the perspective of concentrations in stratum corneum tape strips (Rougier *et al*., 1985; Nylander-French, 2000). This approach, termed *dermatopharmacokinetics*, analyzes the amount of drug in skin at the application site by sequentially removing and analyzing the drug on the surface (nonabsorbed drug) by gentle washing and swabbing. Various approaches are then employed to model the diffusion gradient reflected in the stratum corneum depth profiles. These techniques must first be calibrated to normal human absorption end points before reliable predictions can be made.

5.1.2 In Vitro Approaches

A primary technique used to study dermal absorption in both humans and animals is to mount skin samples into a diffusion cell and measure chemical flux into a perfusate bathing the dermal side of the skin sample. Specific

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protocols have been established relative to membrane preparation and perfusate composition (Bronaugh and Stewart, 1984; Bronaugh and Stewart, 1985). Skin may be either full-thickness, heat-separated epidermal membranes or dermatomed skin (skin sliced into discs of predetermined thickness). If haired animal skin is employed, the skin section must be sufficiently thick to avoid cutting off the base of the hair follicles in the dermis. Skin is then clamped between two chambers, one of which contains a vehicle from which the absorbed chemical will be sampled. *Static* diffusion cells sample this chamber and replace with new perfusate at each time point. *Flow-through* cells use a pump to pass perfusate through the receptor chamber and collect flux by repeatedly collecting the perfusate.

In some systems, the skin disc is first fully hydrated and allowed to equilibrate with media before dosing. Receptor fluid is usually saline for pharmaceutical drug studies or a media containing albumin or a surfactant/solvent for studies of organic chemicals where some degree of lipid solubility in the receptor fluid is required. Perfusate should be heated to 32 or 37 °C according to experimental guidelines. If the chemical is dissolved in water and dosed in the donor chamber at a dose that far exceeds the amount absorbed, this experimental condition is termed an *infinite dose* experiment. In contrast, if the dose is applied at typical exposure situations and exposed to ambient air or covered (occluded), the experiment is termed *finite dose*. In this case, relative humidity of the dosing environment may be important.

The skin source is an important consideration. In human studies, skin may either be obtained fresh from reconstructive surgical procedures, or dead from cadaver sources. In the latter case, skin is often frozen and then thawed before use. In these cases, a membrane integrity test is conducted using ^{3}H water absorption to screen skin discs for lack of barrier integrity. Fresh skin should be used to insure an intact barrier and healthy tissue, a factor extremely important for metabolism studies and to minimize hydration from the ^{3}H integrity studies. These skin samples are often perfused with oxygenated media to maintain metabolic functions. Artificial skin grown using air–liquid interface cultures are also available, although the permeability through such systems is presently much greater than that of normal human skin. Many artificial 3D model systems only contain keratinocytes and do not contain all of the other cell types or appendages that are present in skin. When only the stratum corneum is to be studied, some workers have used shed snake skin as a model to assess the absorption properties and have reported results comparable to humans.

In vitro models are the predominant approach used to assess dermal absorption today. They are the sole method used to calculate absorption parameters, such as permeability discussed above. Numerous protocols have been proposed for specific purposes, such as assessing absorption of lipophilic pesticides versus transdermal delivery of more hydrophilic drugs. Differences between such protocols often relate to surface decontamination methods (swabs, wash, etc.), perfusate composition (addition of vehicle to enhance solubility of penetrant) and length of experiment. If penetration is to be assessed, chemical concentrations in the remaining skin disc after surface drug removal must be measured. Finally, specific types of experimental designs must be used to provide data for specific mathematical modelling approaches, considerations that often dictate specific approaches.

5.2 Toxicity

There are several types of irritancy testing protocols that are performed in order to comply with federal and international safety regulations. The most common test, developed in 1944, is the Draize test to measure acute primary irritation (Draize *et al*., 1944). The test compound is applied in an occluded fashion to a clipped area of abraded and intact skin of at least six albino rabbits, and evaluated at 24 hours, when the patches are removed. The exposed areas are re-evaluated at 24 and 72 hours and scored according to **Table 1**, which depicts scores for erythema and oedema ranging from one to four depending on the severity of the irritation. Many modifications have been made to this scoring system over the years and most cosmetic and pharmaceutical companies have their own modified Draize test. Since these tests are conducted in an occluded fashion, irritancy is potentiated due to hydration reducing the skin barrier and promoting penetration. The Draize test may be modified to assess sensitization and contact dermatitis by pre-exposing animals to a sensitizing dose of the study chemical or a general sensitizer such as dinitrophenol (DNP) and then rechallenging the animals at a later date to illicit the immune-mediated response.

More sensitive markers of *in vivo* irritancy than gross examination have been developed. Many studies have correlated Draize scores with noninvasive biophysical estimates of erythema, the hallmark of skin irritation. Erythema may be assessed using a myriad of different colour-measuring instruments that attempt to objectively quantitate, on a continuous scale, the subjective categorical assessment inherent to human scoring systems. These systems are based on reflectance principles and operate by irradiating skin with specific light wavelengths and measuring the colour of the reflected light. These systems can also detect toxin reactions that alter the melanization process and produce altered skin pigmentation. Erythema, which results from increased blood flow, may be directly assessed using noninvasive laser Doppler velocimetry, which measures dermal blood flow. As mentioned in the absorption section above, a sensitive indicator of loss of barrier function associated with dermal irritation is TEWL.

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Table 1 Draize skin reaction assessment scores

Erythema	Score	Skin reaction
	0	No significant change
	1	Very slight (barely perceptible)
	2	Well defined; pale red
	3	Moderate to severe; definite red; area well defined
	4	Severe; beet or crimson red
Oedema	**Score**	**Skin reaction**
	0	No significant change
	1	Very slight (barely perceptible)
	2	Slight; edges show definite raising
	3	Moderate; edges raised 1 mm
	4	Severe; raised more than 1 mm and extending beyond area of exposure

Table 2 *In vitro* skin irritation test systems adopted by OECD

Corrosivity testing: Corrositex®
- Assess chemical reactivity of acids and bases to collagen matrix
- OECD 404 and OECD 431 adopted April, 2004

Transepidermal electrical resistance system
- OECD 430 adopted April, 2004

Sensitization: Local lymph node assay
- OECD 429 adopted April 2002

Phototoxicity: In vitro 3T3 neutral red uptake
- OECD 432 adopted April 2004

A great deal of research has been conducted to develop humane alternatives to the Draize test using principles of modern *in vitro* science. A number of test systems have been approved by the Organization for Economic Cooperation and Development (OECD) and are listed in **Table 2**. Additional systems employ a skin organ culture approach that attempts to provide the stratum corneum barrier and viable epidermal cells, which can react to penetrated compounds. Since these are essentially organ culture systems, the cell viability may be assessed by sampling the bathing culture medium. Neutral red (3-amino-7-dimethyl amino-2-methylphenazine hydrochloride) can be used to probe lysosome integrity while thiazol blue (3-4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide; MTT) is a mitochondrial enzyme substrate that can assess if these organelles are metabolically viable. Leakage enzymes such as lactate dehydrogenase (LDH) can be assayed to detect cell membrane damage. Reactive products (cytokines, prostaglandins) produced by keratinocytes may be detected in the culture medium. Recent work in our laboratory assessing the potential cutaneous toxicity of manufactured nanomaterials has demonstrated that these systems, fully validated for chemical toxicity assessment, may not be optimal for nanomaterials due to their interaction with dye production (binding, catalysis) or cell imaging (Monteiro-Riviere and Inman, 2006; Monteiro-Riviere *et al*., 2009).

5.3 Absorption/Toxicity Models

The optimal *in vitro* cutaneous model should possess viable cells and structures similar to intact skin, as well as a functional vasculature. Such a model would allow topical chemical absorption to be assessed simultaneously with direct toxicity. Our laboratory has developed the IPPSF, an *ex vivo* perfused skin preparation precisely for this purpose (Bowman *et al*., 1991; Riviere *et al*., 1986; Monteiro-Riviere, 1990; Riviere and Monteiro-Riviere, 1991). An anatomically intact alternative animal model, the IPPSF possesses a viable epidermis and an accessible and functional microcirculation. It is experimentally much closer to *in vivo* than excised skin and is thus a more appropriate *in vitro* model for assessing percutaneous absorption. The IPPSF model has been shown to be predictive of *in vivo* human absorption (Wester *et al*., 1998). This model was also used to study UV phototoxicity (Monteiro-Riviere *et al*., 1994). In addition to morphologically assessing the dose-dependent formation of sunburn cells, decreased

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glucose utilization was observed, accompanied by increased vascular resistance and cell proliferation decreased by assaying proliferating cell nuclear antigen (PCNA). Levels of prostaglandin E_2 in the perfusate from UVB exposed skin flaps also increased in a dose-dependent manner, reflecting the cutaneous irritation response. This study demonstrated the utility of this model, since the morphological, biochemical and physiological changes could be experimentally monitored and evaluated in the same preparation.

6 CONCLUSION

There is continued and renewed interest in assessing dermal absorption and toxicity of topically applied drugs and chemicals, since skin is a common route of exposure for occupational and cosmetic entities, as well as dermatological and transdermal drug preparations. Penetration is inextricably linked to dermal toxicity, since most toxicants, no matter how potent, must first penetrate the protective stratum corneum barrier to reach cellular target sites (e.g. Langerhans cells or other epidermal cells). Testing approaches that ignore this barrier function may overestimate the potential for dermal toxicity. Another unique aspect of dermal toxicology is that for most types of topical irritants, once initiated, the cutaneous response characterized by erythema and oedema are similar and fairly independent of initiating mechanism. A major challenge to most research protocols is to move towards *in vitro* platforms that are predictive of *in vivo* responses. This challenge will continue to drive dermatotoxicology for the foreseeable future.

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Inhalation Toxicity

Geoff H. Pigott

CONTENTS

1 INTRODUCTION

Of the three main portals of entry into the body, inhalation, ingestion and dermal penetration, the first two are quantitatively dominant. The main portal of entry for bulk materials is via ingestion and the digestive tract is adapted to facilitate this, in particular, for the entry of nutrients. To achieve sufficiently rapid exchange, the epithelium lining the gut is folded with an extensive network of microvilli so the total area available for absorption is very large. In the case of inhalation, the main function of the lung is to facilitate the entry of oxygen and dispose of surplus carbon dioxide. This also requires a large surface area for efficient function and the total gas exchange area of the lung is about half that available for absorption via the gastrointestinal tract. By comparison with these structures the skin area exposed to external influences is small and better adapted to protect the organism from the environment.

Extraneous materials, both gaseous and particulate, may be entrained in the inspired air and thus have the capacity to enter the circulation by inhalation exposure. The physiological adaptations that facilitate gas exchange will clearly also maximize absorption of other external agents which might penetrate to the alveolar spaces. Chemicals absorbed in this way are readily dispersed in the blood. Unlike ingested materials, these do not automatically pass through the liver before entering into the general circulation. Thus, the potential for metabolic detoxification may be significantly reduced for chemicals absorbed by inhalation, and this can lead to different dose–response relationships for inhaled compounds when compared with oral exposures, although there are fewer differences in the character of response expressed.

Oral exposure is relatively simple to execute, and dosimetry easy to measure with acceptable accuracy, and this is often the primary route by which the toxicity of xenobiotics is assessed. Thus in many cases, a primary aim of the inhalation toxicologist may be to define whether inhaled material will significantly influence the character of the response and/or lead to very different dose–response relationships compared with oral exposures. In some instances, for example, gaseous pollutants, the lung may be the primary route of entry, but for others, such as liquid or solid aerosols, oral exposure may be quantitatively significant even when the exposure is via the air.

Nevertheless, inhalation exposures are significant, both for their potential systemic effects and, often more importantly, for the local response in the lung itself. Assessment of these responses, especially establishing quantitative dose relationships is challenging, both technically

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and intellectually. Much human disease is transmitted via the air and the mechanisms by which the various responses are induced are at best only poorly understood and often not at all at the molecular level. Elucidation of the mechanisms and kinetics involved is a major challenge.

This text outlines the major features of lung structure and physiology as a backdrop to the range of potential effects that can be induced. The various responses to toxicants, both gaseous and particulate are described, together with the major factors that determine tissue dose. Finally the model systems developed to assess inhalation toxicity are outlined.

2 BASIC STRUCTURE AND FUNCTION OF THE LUNG

2.1 Lung Anatomy

The primary purpose of the lung is gas exchange of oxygen from ambient air with carbon dioxide produced by respiration. The physical exchange between air and circulating blood is achieved by diffusion and thus may be approximated by applying the equations of Fick's laws. These determine that the main requirements for rapid equilibration will be a short path for gas exchange, that is, the capillaries carrying the blood must be only a short distance from the external air, and a large surface area to accommodate the necessary volume exchange for the organism. The total surface area required is a function of body size, although not directly proportional. The structures evolved to achieve this are more complex than this limited function might suggest. Respiratory exchange is vulnerable to many factors. Water loss is perhaps the most difficult to control. Incoming air may be dry, the diffusive surface needs to be moist to facilitate both solution of the gas and its rapid diffusion. Unless this process is controlled, the resultant loss of water would be rapidly fatal to most land-dwelling organisms. A second feature is the potential for infection. A moist warm area, plentifully supplied with nutrients via blood, provides perfect conditions for culturing microorganisms, including pathogens. In higher organisms these problems have been resolved by two different structures: those present in mammals and those in birds. Most important, for the purposes of toxicology, is the mammalian system (**Figure 1**). All such structures consist of a system of nasal turbinates which warm and condition incoming air and remove any larger particles that may be suspended in it. Air is then conducted via the trachea into a series of branching tubes. The branches may be symmetric or asymmetric, but their purpose is to distribute air flow into a series of balloon-like sacs, the alveoli, in which the process of gas exchange takes place. From the trachea down, the organ is encased in a sealed cavity in the chest which may be expanded and contracted by muscular activity, thereby pumping air in or out. For many of the smaller mammalian species, nose breathing is obligatory, although in others, for example primates, dogs, there is also the option of inhaling and exhaling via the mouth (oronasal breathers)

This basic structure consists of several different types of cell. The greater part of the internal nasal passage is covered by respiratory epithelium. Embedded within this are mucus-producing goblet cells, ciliated cells, cuboidal cells and basal cells, together with other specialized structures, in particular the olfactory epithelium occupies part of this structure. This latter epithelium consists of sensory cells with densely packed nerve endings, together with the sustentacular (supporting) cells. Responses of this structure are beyond the remit of this chapter. The larger conducting airways are lined with a pseudo-stratified epithelium containing ciliated cells and secretory cells. The most important of the latter are the mucus cells that produce a high molecular weight glycoprotein, the basic component of respiratory tract mucus, and the serous cells, which produce a more

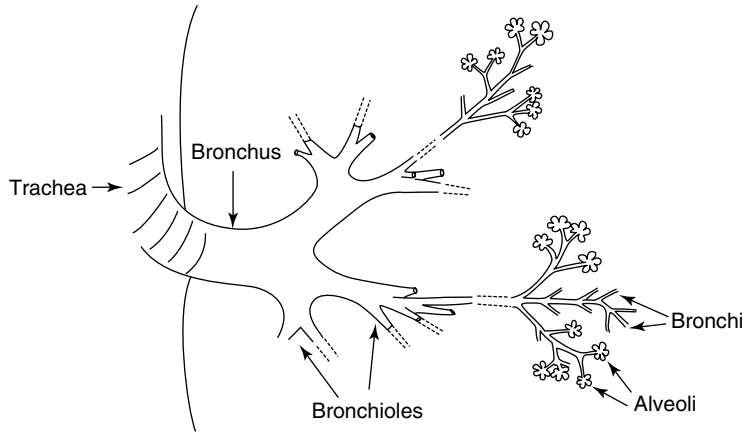

Figure 1 Basic structure of the mammalian lung.

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fluid matrix in which the mucus partially dissolves. This system provides a sticky surface which can trap particles, including bacteria and other microorganisms present in the incoming air, in the surface layer, with a more mobile layer beneath. Flagellae on the surface of the ciliated cells beat in a regular fashion such that the mucus is cleared towards the larynx, where it is eventually either swallowed or expectorated. This is an important primary defence mechanism for lungs; material deposited on the 'mucociliary escalator' may be cleared from the lung within an hour or so. Inhibition of the system may therefore have significant influence on lung dosimetry and factors that cause such inhibition can enhance the toxicity of other pollutants by effectively increasing the received dose. The conducting airways also contain connective tissue within the walls, which imparts stiffness so that they remain patent under reduced pressure, and smooth muscle, which can contract and constrict the airways in response to various adverse stimuli.

Respiratory bronchioles (about 2 mm diameter in man) are the transition zone between the purely conducting airways and the area of gas exchange. These are lined with a cuboidal ciliated epithelium and also the nonciliated Clara cells. The Clara cells have several roles including secretion of components of pulmonary surfactant. They also have metabolic capability for xenobiotics via a P450-type enzyme system, are considered the major sites for metabolism of mucus that has strayed beyond the ciliated airways and are the progenitor cells for the ciliated bronchiolar epithelium.

Beyond the respiratory bronchioles are alveolar ducts and finally alveoli. This region is marked by a transition to a different epithelial type, consisting of Type I and Type II epithelial cells. Type I cells are flattened and have a very large surface area. These are the locus for gas exchange. Type II epithelial cells produce lung surfactant and may also divide to replace damaged Type I cells. In most species they are slightly more numerous than the Type I cells, although they occupy much less of the lung surface area, being more cuboidal. The alveolar spaces contain specialized macrophages (alveolar macrophages). These are derived from similar stem cells to macrophages found elsewhere in the lymphatic systems and reside in the interstitium before entering the alveolar spaces. However, in their terminally differentiated form they are distinct from the circulating macrophages.

The above describes primarily the 'air side' of the lung. On the 'body side' there is a branching structure of blood vessels ending in a capillary bed which closely lines the interior of the alveolar spaces. The whole is supported by a structure consisting primarily of connective tissue and cartilage and contained within an airtight sac (the visceral pleura) consisting primarily of mesothelial cells. The lung is contained within the thoracic cavity, a bony cage formed by the ribs, which also contains an airtight lining (the parietal pleura) consisting of mesothelial cells. The base of the cavity is sealed by a muscular membrane (the diaphragm) which can expand and contract, together with the ribcage, to vary the air space within the chest cavity. This creates the bellows effect which is responsible for the process of breathing.

The lung lymphatics are mainly situated around the bronchial vascular channels that drain to the hilar lymph nodes. In addition, there is a fine system of lymphatic vessels on the pleural surface, which drain into the pleural space and ultimately through the diaphragm and into the peritoneal cavity.

This picture is simplified in this text in order to emphasize the main structures involved and to direct the reader towards the functional areas that may be affected by various toxicants. More details on the anatomical structure of the lung and of the cellular types present may be found in standard anatomical texts. A detailed understanding of the cell types involved may be important in the assessment of mode of action of individual toxicants, but, as with many aspects of toxicology, it is more important in the first instance to consider the basic principles.

The second anatomical structure which has evolved for gas exchange is exemplified by the lung in birds. This is sometimes described as a 'bottle brush' lung and consists of a series of air sacs leading into branching airways, which then recombine to give a 'flow through' structure rather than the 'in and out' structure seen in most mammalian systems. In some respects this provides more efficient gas exchange, but at the expense of being more prone to external influences and infection. This is arguably the price which much be paid for the greater efficiency per unit weight and hence facilitation of flight. More information on these structures can be found, for example Duncker, 1971; Duncker, 2004; Powell, 2000, but they will not be considered further in this text.

2.2 The Physiology of Gas Exchange

The movement of air into and out of the lungs follows a bellows-type action. The chest is expanded so that the rib cage moves upwards and outwards while the diaphragm is moved downwards. This increase in volume of the thoracic cavity allows the lung to expand passively in response to the partial vacuum created. Air flows through the nasal passages and/or the oral cavity via the trachea and the branching tube systems towards the alveoli. After the process of gas exchange this process is reversed, with the rib cage contracting, the diaphragm moving upwards and the consequent reduction in volume of the thoracic cavity driving the air from the lung back into the atmosphere. The process typically involves expansion of the lung from a minimum of approximately 1.2 l to a value of approximately 5.5 l in the typical 60 kg adult. These values are known as the residual volume (RV) and total lung capacity (TLC) respectively and show a relationship to body size in

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all mammalian species. The maximum difference which can be achieved between these values is termed the forced vital capacity (FVC). Normal respiration involves a lesser change in lung capacity, which may be as small as 500 ml for a person at rest. This change is termed the tidal volume (TV), which increases markedly in response to stress and/or exercise. In oronasal breathers increases in TV are likely to coincide with an increase in the frequency of mouth breathing, rather then nose breathing. This has significance, especially for the intake of particulates and aerosols; these are discussed later. Measurements of the various capacities of the lung can provide important indices of injury. These measurements are well developed in humans, where a series of normal physiological values has been established over a long period of time. These depend mainly on age and sex, but have also been developed for specific populations.

The volume change associated with a unit of pressure change across the lung is defined as compliance. Measurements of compliance show that the values obtained during expiration (lung deflation) differ from those achieved during inspiration (lung inflation). This hysteresis illustrates the contribution of pulmonary surfactant in maintaining the structure and integrity of the lungs. A brief consideration of the nature of the alveoli, that is, small air-filled sacs bound with an aqueous fluid, leads to the conclusion that other things being equal, once deflated they should be extremely difficult to reinflate, due to the effects of surface tension. The gas pressure (P) needed to keep equilibrium between the collapsing force of surface tension (T) and the expanding force of gas pressure in an alveolus of radius (r) is expressed in the equation:

$$P = \frac{2T}{r} \text{(Laplace's rule)} \qquad (1)$$

indicating that once collapse commences, this is an accelerating force. The surfactant produced by Type II pneumocytes is highly surface active and consists predominantly of phospholipids, mainly phosphatidyl choline. Other phospholipid components include phosphatidyl glycerol and phosphatidyl inositol. Surfactant also contains about 10% protein and 10% neutral lipids, including cholesterol. All components have been shown to be important for the overall function (Goerke, 1998). This surfactant not only reduces the overall surface tension within the lung, but has the unusual property of further decreasing the surface tension as it is compressed. Thus, as alveoli deflate, the surface tension decreases, so that at the end of expiration the value approaches zero. This makes the lungs relatively easy to reinflate. Conversely, as inflation continues the surface tension increases, imposing an effective limit on the size to which individual alveoli can expand. Surfactant also plays an important role in entrapment of particles that may be deposited in the alveolar spaces. Abnormalities in pulmonary surfactant, whether induced or congenital, may be important contributors to pulmonary disease (Devendra and Spragg, 2002).

Gas exchange across the alveolar membrane is primarily passive. Oxygen diffuses inwards and combines with the iron contained in haemoglobin in the red corpuscles. The diffusion path is sufficiently short for this process to be completely saturated well within the half second or so that the red corpuscles spend adjacent to the alveoli. At the same time carbon dioxide is released from the blood and diffuses across the membrane into the alveoli. This is enhanced by the presence of the enzyme carbonic anhydrase in the red blood cells, which dissociates the bicarbonate formed when carbon dioxide is collected from the tissues. Each inspiration does not result in a complete change of air in the alveolar spaces; although there is mixing of the inspired air, the flow rate becomes very low beyond the terminal bronchiole. As a result alveolar air contains about 5.5% carbon dioxide and diffusion from the blood results in equilibrium with this value, (a feature that is well recognized in tissue culture).

2.3 Defence Mechanisms in the Lung

These can be divided into two categories, mechanical and biological. Together they are effective in reducing exposure of the more sensitive areas of the lung to many toxicants, especially those in particulate form.

2.3.1 Mechanical Defence Mechanisms

The mechanical defence mechanisms reflect the structure of the respiratory tract. These are effective against particulate (liquid or solid) contaminants in air and provide a defence against invading microorganisms. The primary air intake through the nares will include entrained particles. However, in general, the initial airflow is upwards and this creates a vertical elutriation effect which eliminates the largest particles. It is generally agreed that particles much larger than about 50 μm will not enter the respiratory tract, at least during nose breathing. In obligate nose breathers, such as small rodents (and other underground dwelling animals), the nasal turbinates are convoluted. Thus, in addition to warming and humidifying incoming air, they create a turbulent flow, which causes the impaction of much of the particulate burden onto the mucus-lined cellular surface. Air flow over the larynx creates further turbulence and further deposition so that as air enters the trachea only relatively small particles (less than about 10 μm) remain entrained in the air flow.

For oronasal breathers, the nasal turbulence may be bypassed and larger particulates remain entrained in the airflow at the larynx. Nevertheless, the turbulence created

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at this point is sufficient to remove most of the larger particles such that air entering the trachea is substantially depleted of particles $>15\,\mu m$. Thus, although the pattern of deposition in the oronasal region will vary between obligate nose and mouth breathers, air entering the trachea is depleted of large particles in both groups.

In the trachea and bronchi further deposition mechanisms operate. As outlined above, the major deposition route for larger particles is inertial impaction, which is enhanced by turbulent airflow. This mechanism continues to influence deposition within the ciliated airways although it becomes progressively less important as the larger particles are deposited. In a system of branching tubes, with a consequent division of the airflow into substreams, inertial deposition of particles on the carina is inevitable, and there is substantial evidence for such deposition in both humans and experimental animals. The mucus coating of the conducting airways ensures that, once deposited, by whatever mechanism, particles remain trapped in this surface and are subsequently cleared and not resuspended. However, some exposures lead to inhibition of the clearance mechanism and in these cases carinal deposits may be significant in the development of lung disease, including the development of bronchial carcinoma in cigarette smokers.

Gravitational settlement becomes increasingly significant as a route for particle deposition as the diameter of the bronchioles decreases. This is readily modelled in principle as the particles essentially follow Stoke's Law. (Quantitative aspects of deposition are addressed in Section (4.3.3).) The tortuous route for airflow means that the absolute importance of this deposition mechanism varies within lung lobes and possibly between them, but settlement becomes the dominant process for particle removal in all the bronchiolar regions. At this point, diffusion is relatively unimportant, although it becomes increasingly so as airway size diminishes further. By the time the incoming air has penetrated to the respiratory bronchiole, where the mucus coating of the conducting airways is no longer present, most particles $>2.5\,\mu m$ have been removed. Particle deposition in the respiratory bronchioles and alveolar areas depends primarily on diffusion. Although gravitational settlement still plays a part, the remaining particles are sufficiently small for Brownian motion to be a significant factor in maintaining their suspension. Particles above about $0.5\,\mu m$ diameter may be substantially deposited in the alveolar region, although the very smallest particles (less than $0.1\,\mu m$) may remain in suspension for sufficient time to allow a proportion to be exhaled.

All of the above assumes that the particles involved are insoluble. Particle solubility or even wettability will influence behaviour within the respiratory tract and hence the ability of the organism to remove them from the air. At one extreme, hygroscopic particles will increase in diameter as they penetrate the respiratory tract. This leads to the possibility of devising particles with properties that maximize their deposition in specific regions of the tract. This property is exploited for therapeutic purposes in a limited number of cases.

2.3.2 Physiological Defence Mechanisms

A primary defence mechanism is the ability of the major airways to constrict. Smooth muscle in the bronchial walls contracts in response to various stimuli, effectively restricting airway size and thus the TV. In the short term this reduces exposure to contaminated atmospheres, but may rapidly become an impediment when the restricted airflow becomes physiologically limiting. Chronic contraction of this musculature is associated with asthma.

The contribution of the mucus layer in the branching airways has already been mentioned. This is an important feature for the removal of deposited materials within the conducting airways. Pulmonary surfactant plays a similar role in the nonciliated airways, but in this instance excess surfactant is removed by alveolar macrophages. In addition to clearing excess or damaged surfactant, these mobile phagocytes will engulf any particulate material found within the alveoli/terminal bronchioles. In common with other macrophages, phagocytosis results in particles being confined in secondary lysosomes, where they may be digested or dissolved by a combination of enzyme attack and low pH. The latter may be especially important for mineral particles. The pH range of the secondary lysosome is variously quoted in the range 4–5.5, but these values have limited applicability. In effect, hydrogen ions may be secreted directly through the lysosome membrane onto the contained particle, with limited water present. This is a probable mechanism for the aggressive dissolution of particles seen in the secondary lysosome, a process that is more effective than the nominal pH value would indicate.

Particles refractory to digestion may suffer various fates. Some particles, especially crystalline silica may disrupt the membrane in the secondary lysosome. This may be important in various disease processes. Particles nontoxic to the lysosome membrane may be maintained for longer periods. The fate of particle-laden macrophages depends to a degree on the materials ingested, but many may re-enter the interstitial space and the lymphatic circulation and significant burdens of particulate contaminants may subsequently be found in local drainage lymph nodes. Alternatively, in cases of heavy contamination, some macrophages accumulate in groups of adjacent alveoli. This concentration may be sufficient to fill the relevant alveolar spaces to the point where gas exchange is significantly inhibited. At this point, various pathological reactions may occur, including the formation of giant cells and the induction of a 'foreign body' reaction. This latter usually results in the collected macrophages, with their burdens of contaminant particles, being sequestered within a fibrous

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capsule, where they may remain for very long periods. Finally, some alveolar macrophages migrate to the ciliated airways, where they are entrained in the mucociliary escalator and eventually find their way to the pharynx, where they are subsequently ingested, or occasionally expectorated.

3 RESPONSES OF THE LUNG TO TOXIC INSULT

In addition to systemic effects following absorption into the lung, there are several local reactions that can be induced and which may have more or less of an effect on lung function. These range from primary irritation, which can result in a temporary and reversible, but occasionally very severe, reduction in the exchange capacity of the lung, to longer-term effects involving permanent damage, reduction of exchange capacity and in some cases to lung cancer. Further responses may be mediated by the immune system and can lead to permanent disability via airway constriction in an asthmatic-type response. These latter reactions, which can occur in response to gases, liquids or particulates are addressed in **Risk Assessment of Chemicals** (respiratory sensitization). The local reactions have a relatively limited range of outcomes, primarily manifest as reduced lung function, but there is a range of underlying mechanisms. These mechanisms relate to the intrinsic properties of the toxicants concerned, in particular their physical state (gases, liquid aerosols and particles), as well as their mode of action. Thus it is convenient to consider each of these physical states separately when attempting to model and predict the effect on the respiratory system. However, in adopting this broad classification for pollutants it is necessary to remember that in a significant number of cases the states will overlap, for example, liquid aerosols will be in equilibrium with vapour,[a] low melting point solids may change from dusts to liquid aerosols as they warm in the lung.

3.1 Toxicity of Gases

These are the simplest form to consider, at least in terms of entry into the lung, as they will mix completely with incoming air. Concentration is usually expressed in terms of partial volume (v/v) usually as parts per million (ppm), but for some of the more common and nontoxic gases this can be expressed as a percentage. Defence against such toxicants is limited to restriction of air intake overall.

For gases, the lung is the most important and usually the only portal of entry. Their subsequent fate depends on the nature of the gas. Small and relatively inert molecules may have no specific effect and enter and leave with the air stream, though their presence in sufficient quantities may dilute the oxygen content of the air to the extent where asphyxiation occurs. More commonly, hydrocarbon and other lipid-soluble gases may dissolve in the alveolar membranes and reduce gas exchange by that route. Once in the alveolar spaces, gases will be absorbed by diffusion and can exert systemic affects. This property is widely exploited in the use of anaesthetic gases, (for example see Franks, 2006). As mentioned previously, this portal of entry bypasses the metabolizing systems present in the liver and thus may modify dose–response relationships in comparison with oral exposures. Though there is metabolic capacity in the lung (mainly in the Clara cells found in the terminal bronchioles), this is less extensive than the liver and it is possible for gaseous pollutants, in particular, to bypass these specialized cells. Once absorbed, gaseous pollutants can, in theory, exert effects throughout the body, although in practice these are most commonly seen in the blood. Specific examples include carbon monoxide poisoning and hydrogen sulfide poisoning. Both of these are attributed to the combination of the inhaled gas with haemoglobin, with consequential effects on oxidative processes for energy production.

A common feature of gases that are known to provoke systemic effects is their lack of water solubility. The more soluble gases are generally associated with a localized response. These include the acid halides, sulphur oxides and nitrogen dioxide, all of which will dissolve in the fluids lining the lung surface. The most common response is irritation of the lung. The form which this takes depends on how far the gas has penetrated into the lung before dissolution and hence on its solubility. The most soluble gases induce an acute sensory irritation, characterized by an irritant or burning sensation in the upper respiratory tract throat and chest; the eyes may also be irritated by similar mechanisms. There is a reflex decrease in breathing rate and there may be bronchial constriction. The onset is very rapid and severity tends to be concentration dependant. The immediate effect is to reduce the intake of contaminated air and thus limit exposure. The effects are usually sufficiently severe to induce exposed individuals to escape from the situation in order to alleviate them. This is the principle of the irritant gases used in riot control. Generally, minor exposures of this nature have no long-term consequences. However, prolonged exposures and/or higher concentrations may result in severe respiratory distress caused by lung irritation and inflammation. Chronic exposures result in a degree of resistance to initial irritant effects, but may also lead to a range of longer-term deleterious consequences, including emphysema, bronchitis and in some cases squamous metaplasia of epithelia. All of these are a consequence of cellular damage and in the case of 'pure' acid gases, are likely to be consequent on the concentration dissolving in the surface liquid being sufficient to overcome the buffering capacity of

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natural fluids, with consequent loss of homeostasis for the underlying epithelia. There may be a similar response to alkaline gases (of which ammonia is the only common example), although high pH tends to be more directly damaging to tissue than low pH, so that effects are more severe.

For gases that can penetrate deeper into the lung, either by dint of concentration or lower rate of dissolution, the effects can be more severe. Where dissolution occurs in alveolar spaces, a severe lung oedema may result, which in extreme cases can be fatal. This is an acute phase of lung injury where fluid can seep into the alveolar spaces, presumably as a consequence of damage to the Type I epithelial cells and/or a loss of integrity in the junctions between them. The result is an accumulation of a serous fluid in the alveolar space, which severely limits the diffusive exchange of oxygen and carbon dioxide, even in the absence of frank structural damage. With continued exposure, this condition may become rapidly progressive and ultimately fatal. However, if exposure is discontinued, in many cases the condition will resolve completely, with no obvious residual injury. Chronic lower-level exposures to such agents can result in lasting tissue damage, with various consequences which will be discussed later. Tissue damage in the alveolar area may be more extensive and more difficult to repair than that in the upper airways. Thus, the longer-term consequences of exposure to these gases, which include sulphur trioxide and nitrogen dioxide, tend to be much more severe.

In the case of several pollutant gases, additional mechanisms contribute to the overall effect. Examples include chlorine and bromine. Although both of these gases are sensory irritants, this is not a reflection of their intrinsic properties, but rather of their ability to react with water to produce both the acid halide and the relevant hypohalide. This latter is sufficiently reactive to produce oxidative damage in the underlying tissue. It is probable that the oxidative damage caused by exposure to agents such as hypochlorites (and other oxidizing agents, such as ozone), is mediated through superoxide/free radicals and is evident when these are present at sufficient concentrations to overwhelm local defence mechanisms. It is likely that the lung is particularly susceptible. Most tissues are exposed to oxygen only in combined form. Even under these conditions, formation of superoxides and free radicals can be a mechanism of cellular injury, although most cells are equipped with adequate mechanisms to detoxify these radicals as they are formed. Uniquely, the lung is exposed to molecular oxygen and its primary function is to capture this and convert it to its combined form with haemoglobin. Thus, within the pulmonary tissues, cellular defence systems must be sufficiently sensitive to capture and detoxify free radicals and superoxides, but not sufficiently active to react directly with molecular oxygen. This is a difficult equilibrium to maintain and prolonged exposure of the normal lung to enhanced oxygen levels will result in long-term damage.

As with the sensory irritants, the less soluble or less reactive gases may penetrate deeper into the lung where there is a larger surface area exposed and where the consequences of such exposures may be more severe, as the surface cells are not protected by a mucus layer.

3.2 Toxicity of Liquid Aerosols

There are two main features that distinguish exposures to liquid aerosols from those to gases. The first is the relative dose that may be achieved. Particles have an inherently higher molecular density than gases, so that the rate of transfer to the lung from a liquid (or solid) aerosol can be much higher than that from gases. Secondly, they behave as particulates and thus aerosol size is a major determinant of the site of action (see below). In the case of liquid aerosols, the range of response is not dissimilar to that from gases. Sensory irritation is an important response and because of the higher quantities of material delivered to the epithelium by particle deposition this can be especially severe. In most circumstances this will limit exposure. The nature of the aerosol will also determine its ability to penetrate into the alveolar spaces. Those aerosols that are hygroscopic are less likely to penetrate more deeply, as their particle size will increase due to the humidity in the lung. However, nonhygroscopic and particularly hydrophobic liquid aerosols of sufficiently small size can be deposited efficiently in the alveolar spaces and the dose delivered may be substantial. This high dose to the lung can result in significant potential for local injury. In particular, aerosols which penetrate to the alveolar spaces may produce severe pulmonary oedema.

3.3 Particulate Aerosols

There is an especially wide range of potential responses to these exposures. There are several reasons for this. As with liquid aerosols, regional deposition is strongly dependant on particle size. However, once deposited, especially in the alveolar region, where residence times are long, the range of response observed depends strongly on particle properties. Those particles which are soluble can exert either a local or systemic effect as a result of the properties of the inhaled material. As with liquid aerosols, the relative quantity delivered can be substantially in excess of that achieved by gaseous inhalation and the effects more severe. This is well exemplified by the response known as metal fume fever. This acute response to inhalation of very finely divided aerosols (individual particle sizes of the order of 1 μm or less)

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has historically been encountered in metal-working and welding. Although it is most commonly associated with copper, magnesium and more especially zinc, inhaled as the oxides, it has also been less frequently encountered following exposure to fumes from a range of metals. Symptoms include chills and fever, nausea and occasionally vomiting, fatigue, weakness and aching of the head and body. Lung capacity may also be reduced. The symptoms have been likened to malaria or influenza leading to misdiagnosis (Kurzbaum *et al*., 2007). However, the condition is transient and recovery is complete after a day or so. No fatalities have been recorded. This disease is now extremely rare, as modern industrial hygiene practices preclude exposures of sufficient intensity to induce the response. The mechanism is believed to be a result of metal–protein complexes being released into the bloodstream, inducing a response akin to a foreign protein, although this is not true immunity.

Larger and less readily soluble particles may induce a different spectrum of response. This may include fibrosis, emphysema and lung cancer.

A fibrotic response is most commonly seen after exposure to siliceous minerals (especially crystalline silica). It is probably associated with toxicity to alveolar macrophages, which attempt to clear the particles deposited in the alveolar space. As previously indicated, even benign dusts, when present in sufficient quantity, can induce limited local collagen production, to sequester the material from the surrounding tissue. This 'foreign body response' can be regarded as a minimal reaction to what is effectively an overloading of the clearance mechanism. In fibrosis this effect is amplified and may progress for a long period, even in the absence of continued exposure. It is most likely that the relevant particles prove toxic to macrophages following phagocytosis. The macrophages then release cytokines which cause the ingress of fibroblasts and the induction of collagen synthesis in order to repair localized damage and to sequester the toxic particles. However, the particles remain active and further macrophages may attempt clearance and thus amplify the process. The exact mechanism(s) involved remain to be elucidated, but cytokine production and fibroblast recruitment is an important contributor and suggest possibilities for treatment (Thannickal *et al*., 2004; Kuwano *et al*., 2001). In the case of crystalline silica, the process produces a nodular fibrosis, which can be scattered through the lung. In extreme cases individual nodules can become the size of a hen's egg. The presence of these nodules reduces lung compliance and may eventually result in respiratory insufficiency. The associated inflammatory response may also be important. Silica and similar minerals are regarded as lung carcinogens, although they are not mutagenic, at least in the classical sense. Fenton-type[b] actions at the particle surface can give rise to peroxides at least *in vitro* (Castranova *et al*., 2004), but these reactions seem to be of lesser importance *in vivo* and are considered unlikely candidates for mutagenic activity. The tumours may be secondary to the fibrosis seen: inflammatory reactions at the site of fibrosis lead to cellular proliferation that could act as a carcinogenic promoter.

Particles may also induce lung tumours by other means. Examples include various metals, especially the transition metals nickel and chromium. The exact mechanism for these tumours has not been elucidated, but plausible theories include the catalysis of oxidative damage or direct interaction with the DNA–histone complex resulting in changed gene expression.

Although many of the occupational diseases of mining and quarrying, and associated industries can be traced to the presence of quantities of crystalline silica in the materials handled, this is not always the case. It is evident that the inhalation of even inert particles in sufficient quantity can result in respiratory insufficiency. It is well established in experimental animals that there comes a point where the clearance mechanisms in the lung are overwhelmed. In these circumstances, there is a continued and progressive build-up of inhaled material within alveolar spaces which eventually, by dint of the quantity involved, results in reduced efficiency of gas exchange within the lung. This process is known as lung overload and is generally associated with exposure to very high dust concentrations. Consequences in experimental animals include the induction of a chronic inflammatory response, which leads to epithelial hypertrophy, metaplasia and ultimately to lung tumours (Hext, 1994). There is limited evidence for similar responses in humans in the past, mainly in coal miners, but the reaction does not include the tumours seen in rats (Levy, 1995). The condition is rare in modern working conditions.

The other major change seen in response to alveolar damage is emphysema. In this condition the alveolar walls are destroyed, leading to large air spaces within the lung parenchyma. This apparently increases lung compliance, but reduces the efficiency of gas exchange by effectively decreasing the area available. The main cause of emphysema is considered to be cigarette smoking, but exposure to other pollutants can produce a similar response. As with fibrosis, it is believed that this process is mediated by macrophages, but in this instance liberation of digestive enzymes, in particular, elastase is considered the proximal cause. Emphysema is associated with severe or recurrent inflammation and it is believed that the participating leukocytes are responsible for the release of proteolytic enzymes. One of the main defences against uncontrolled digestion by proteolytic enzymes, including elastase, is the protein α-1 antiprotease. It was once believed that a concomitant deficiency of this protein in the circulation was necessary for the induction of emphysema. However, it is now evident that proteases other than elastase are important in the aetiology and that the previous theory was an oversimplification. Current theories on the molecular mechanisms

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of emphysema have been reviewed by Morris and Sheppard (2006) and Wright and Churg (2007).

The inhalation of particulates may also induce disparate responses dependant on particle size. At one extreme, irritant particles such as caustic soda may cause localized damage to the upper respiratory tract and larynx as a consequence of their alkalinity. This may be particularly important in the case of larger particles that are entrained during mouth breathing. The size of the particles can give rise to a sufficient local concentration of alkali to be damaging. This is speculative, but may be a rare example of reversal of the convention that larger particles are of lesser importance in the induction of responses in the respiratory tract. At the other extreme, nanoparticles may be especially efficient at delivering systemic doses of chemical via the inhalation route. These particles are sufficiently small to penetrate the alveolar epithelium, presumably mainly through gap junctions. This aspect of toxicology is addressed in **Nanotoxicology-The Toxicology of Nanomaterials**.

In the case of poorly soluble particles, material entrained on the mucociliary escalator may contribute to systemic exposure by the oral route. These particles are cleared relatively rapidly (within an hour or so in man) to the larynx where they are then swallowed. In some circumstances this may be a significant route of exposure although this is mainly at the high concentrations used with exposures to experimental animals, rather than for human exposures, which are generally at very much lower levels.

3.3.1 Fibres

These represent a special case of particulates. Readily soluble fibrous minerals and other materials behave in most respects in a manner identical to more compact particles. However, durable, and perhaps semidurable, fibres have specific effects which are not seen following oral exposure, even at very high levels.

The internationally accepted definition of a fibre is a particle with a length/diameter ratio $>3:1$. To further differentiate these from acicular particles, which occur in many minerals, a minimum length of 5 µm is generally specified. The aerodynamic behaviour of fibres is dominated by their diameter (d). Falling speed is proportional to d^2 and directly proportional to the density, with the length making only a minor contribution. This may be rationalized by the observation that fibres will align in any air flow so that they are parallel to the direction of travel, as this represents the minimum air resistance. The physiological consequence is that relatively long fibres penetrate deeply into the lung. Fibres over 100 µm length have been reported in alveolar spaces although these are relatively rare. Nevertheless, fibres 20–40 µm long penetrate with reasonable frequency.

The literature on the effects of fibres is dominated by the observed responses to asbestos. This is understandable given the history of this group of substances, but not always illuminating when attempting to quantify dose–response relationships. There are more than 140 silicate minerals that qualify for the term asbestos, but only five of these have been studied in any detail or have any commercial importance. The most commonly mined and used asbestos was chrysotile (white asbestos), which is a serpentine mineral of similar chemical composition to talc. This is chemically and biologically distinct from the amphibole asbestos minerals, of which crocidolite (blue asbestos), amosite (brown asbestos), anthophyllite and actinolite have been exploited commercially, especially the first two. Another amphibole (tremolite) is frequently encountered as a contaminant of both chrysotile asbestos and talc, especially in the large North American ore body, which has been a prominent source of both these minerals. Although there are significant differences in both chemistry and in physical properties between these minerals (and especially between chrysotile and the amphiboles), they have been processed in the same facilities and by similar techniques, such that, aside from the initial mining and milling, pure exposures are rare. Thus, although there is ample experimental evidence for differentiating between the effects of these minerals, the practical response has been to classify and control them as a group. Although this solution has resulted in effective control of exposures, it can be argued that it has hindered understanding of fibre toxicology, (especially carcinogenesis) and has possibly resulted in over-regulation of some uses of these minerals.

During the early phase of asbestos exploitation, from approximately 1900 to 1930, little attention was paid to workplace hygiene and workers were often exposed to massive levels of airborne fibres. Exposure to all types of asbestos can cause a progressive fibrosis of the lung. This is differentiated from that caused by silica in that, probably due to penetration of fibre into the interstitium, the accumulation of fibrous tissue is reticulated rather than nodular. Asbestosis can thus be differentiated from silicosis by X-ray and fibrosis produced in response to asbestos is often described as 'honeycomb lung'. *In extremis*, this fibrosis can be rapidly fatal and the incidence of death in asbestos-processing operations provoked regulations (in 1930 in the UK) to control the dust levels in the workplace. At about the same time, the link between lung cancer and asbestos exposure was first made, although this was only firmly established much later, when the synergy with cigarette smoking was also discovered. These discoveries led to further tightening of the rules relating to exposure levels in the workplace. The occurrence of mesothelioma (a rare tumour of the pleura or peritoneum) in populations exposed to amphibole asbestos, was first reported in Wagner *et al*. (1960); this is now seen as a major problem from exposure to asbestos (and by extension possibly to

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fibrous minerals in general). The tumour has a very long latent period (maybe more than 40 years). This latter feature contributes significantly to problems of control, since there is a very long interval before the efficacy (or otherwise) of exposure reduction is established.

Once the range of effects of asbestos was understood, the extent (if any) to which these extended to other fibrous particulates, whether natural or man-made, was an obvious concern. The discovery of high local incidence of mesothelioma in villages in Turkey, where there was significant exposure to the natural zeolite mineral erionite (Baris *et al*., 1978), reinforced this concern. However, the extent to which this property extends to other fibres remains controversial. The relevant evidence is summarized below.

Asbestosis is established as being caused by even short asbestos fibres, indeed these may be more potent. It is almost certainly related to the surface chemistry of the fibres concerned and does not differ in mechanistic terms from fibrosis produced by particulates. In this respect, control presents no special challenge and there are short-term assays (including inhalation assays) that are good predictors of fibrosis, at least in the progressive form.

The above is true for reactions in the lung parenchyma. Asbestos has also been observed to cause a fibrotic thickening of the pleura, both parietal and visceral. This reflects the ability of very fine fibres to penetrate the junctions between Type 1 cells and enter the lymphatic circulation. Where, as with asbestos, this effect is progressive and pleural thickening severe, the response may be significant, although this generally is seen only at levels of exposure where pulmonary fibrosis is also evident. More problematic are the relatively fine pleural plaques which are seen after some low exposures to asbestos and may also be seen after exposure to some other mineral fibres, notably refractory ceramic fibres (Lockey *et al*., 1996). In the latter case the plaques may be considered to be no more severe than a foreign body response. The location of these lesions suggests that they are precursors to, or a site of initiation of mesothelioma, especially when they are severely fibrotic, as seen following heavy asbestos exposure. Epidemiological evidence does not show an association with refractory ceramic fibre exposure and mesothelioma, so that the plaques seen in these workers may serve mainly as an index of exposure rather than a potential focus for disease. Nevertheless, given the particularly long latent period of this tumour and the relatively recent development of the man-made mineral fibres (MMMF) industry, at least in respect of the tonnages currently handled, this interpretation cannot be considered conclusive.

In the case of lung cancer, epidemiology suggests that fibre length is an important contributory factor. No mechanism has been directly established and it should be noted that incidence in experimental animals, although high, tends to be at different locations from that seen in man. Human lung cancer following asbestos exposure is frequently bronchogenic. In animals it is almost exclusively peripheral. The additional association with cigarette smoking in man may give a clue to the reasons for this difference. Deposition of dusts and fibres on the bronchial carina is well known. At this point, interaction with inhaled cigarette smoke, a mixture of fine particulate and gaseous contaminants, is both possible and likely. Absorption of some of this mixture by deposited asbestos fibres may serve as a delivery mechanism. The well-established inhibition of mucociliary clearance in heavy smokers may further enhance the received dose. Alternatively the fibre itself, due to its irritant properties, may promote clonal expansion of cells previously initiated by the carcinogens in cigarette smoke.

Despite many attempts, there is currently no model of the induction of lung cancer in rodents by cigarette smoke and hence no rodent model of tobacco-related lung cancer. Thus, it is possible that the aetiology of tumours in animals could differ from that in humans. There is definitive epidemiological evidence that, at least in man, exposure to asbestos and cigarette smoking interact in the induction of lung cancers (though not mesothelioma), such that this can approach a multiplicative relationship. Evidence from animal studies must be viewed with some caution in view of the inability to duplicate the effect. In this context, it is not possible to determine whether the effect might extend to other fibres. However, there is now a consensus that this response does not occur without at least minimal lung fibrosis, so that control of this response will also control lung cancer incidence.

Mesothelioma is perhaps the most interesting of the responses. This tumour arises from the epithelial cells lining the pleural and peritoneal cavities. It is thus evident that either fibres exert their effect remotely or that they must penetrate through at least one layer of interstitial cells before reaching their target. The latter is the most probable route and the consequent incidence of pleural plaques has already been mentioned. Asbestos fibres may be found throughout the lymphatic circulation and in particular in the fluid draining from the pleural space. This fluid passes into the peritoneal cavity, which is the second (after the pleurae) most common primary site for mesothelioma.

It is generally agreed that in order to induce mesothelioma, fibres must be long, thin and durable. However, all three properties have been challenged and a closer definition of these terms continues to prove difficult. The long and thin components are relatively easy to explain qualitatively. In order to reach the target tissues, the fibres must be respirable. This, for mineral fibres, puts an effective diameter limit at 3 μm, although significant deposition in numerical terms is not seen until about 2 μm diameter, with peak deposition being in the region of 0.5–1 μm diameter. This is reflected in the epidemiology where there are well-documented differences in human disease associated with exposures from different sources

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of nominally the same mineral. These were related to fibre diameter (Timbrell, 1972) based on a study of the relative sizes of asbestos fibres from various sources.

Animal models confirm the importance of dimension. Wagner *et al*. (1973) demonstrated that a high incidence of mesothelioma could be induced in animals by intrapleural injection and that this could be related loosely to fibre size, with submicron diameter fibres being the most active. Similar experiments in the USA by Stanton's group (Stanton and Wrench, 1972; Stanton *et al*., 1977; 1981) examined mineral fibres with a wide range of compositions and sizes, and also concluded that there was a clear correlation between diameter and proportion of dosed animals with mesothelioma. The Stanton group classified fibres only into broad categories, but were able to conclude that the probability of mesothelioma was greatest for fibres of less than 1.5 μm diameter and greater than 8 μm in length. The findings have been widely accepted, in particular as showing that 8 μm is the critical length above which fibres could cause mesothelioma, although this is a simplistic overinterpretation of Stanton's results. More recent experiments have shown that 8 μm is an underestimate and 20 μm is now considered the most likely discriminating length. This has at least a potential mechanistic justification, as alveolar macrophages can fully incorporate fibres up to approximately 16 μm length into a secondary lysosome. Fibres longer than 16 μm may thus have at least a proportion of their length 'free' in the alveolar space, at least until additional macrophage(s) engulf the remainder and form a giant cell. It is also clear that penetration into the interstitium, or the pleural space, is an important step in tumour induction. Partially for this reason, but also because of experimental determinations with a range of fibres, it has been suggested that for the development of mesothelioma, fibre diameter must be less than 0.5 μm. These thin fibres may be translocated readily from their site of deposition via the lymphatic system to the pleural surfaces and elsewhere.

The definition of durability is also unresolved. As mesothelioma is induced in relatively low yield in inhalation studies, even at enhanced exposure rates, most evidence on durability has been derived from injection experiments, which are used as a surrogate for inhalation, although these generally measure mainly dissolution and are thus of less value for fibres that fragment. Durability can be considered to act by means of dose limitation. Continuing exposure to fibres that dissolve or fragment to the point where they are more readily cleared, will result in a lesser cumulative dose than similar exposures to more durable fibres. There is now significant evidence (Hodgson and Darnton, 2000) that there is a level of exposure to asbestos that will not result in disease and this has been equated with a total lung burden of about 10^6 fibres g^{-1}. Thus fibres that are sufficiently fragile to keep the lung burden below this value would not be expected to be toxic. Various values for dissolution/clearance half-life have been proposed in order to exonerate specific fibre types, but none has been rigorously calibrated by reference to primary inhalation exposures. Thus, although the importance of durability is recognized, the property remains difficult to quantify.

Chrysotile asbestos falls into the category considered less durable, since this mineral consists effectively of bundles of fibrils which can fragment over a relatively short period. The fibrils are readily dispersed and can be detected in the circulation and in urine. These fibrils are approximately 20 nm diameter and <1 μm long. They are not associated with any of the manifestations of asbestos-related disease. This observation is in agreement with the epidemiological observations of a relatively low incidence of mesothelioma following exposures to 'pure' chrysotile. Thus, Churg *et al*. (1993) argued that no more than 50 mesotheliomata worldwide could have been caused by exposure to chrysotile and it was possible that these cases were not asbestos related, but were the result of a 'spontaneous' mesothelioma rate of 1 per million deaths. However, all conclusions on the carcinogenicity of chrysotile are complicated by the frequent contamination of this mineral with fibres of the amphibole tremolite. There is evidence that mesothelioma cases in chrysotile workers are associated with an increased burden of long tremolite fibres compared to exposed, but tumour-free controls. However, experimental studies in animals can induce mesothelioma with apparently pure chrysotile, provided the fibres are sufficiently long. This distinction remains controversial, but is unlikely to be resolved, since all forms of asbestos are now subject to significant restrictions worldwide and most investigative work is now directed elsewhere.

The nonasbestos fibre that has been shown unequivocally to induce mesothelioma in man is the durable zeolite mineral, erionite (Wagner *et al*., 1985). This is at least as potent as asbestos in animal experiments and in many cases more so. Whether this tumour might be induced by other fibres, in particular MMMF, remains controversial. Some fibres, particularly the crystalline whiskers (mainly produced in relatively small quantities, often for research) can induce mesothelioma in high yield in animals. There has been insufficient exposure to constitute a human risk and as a matter of prudence exposures to these fibres are carefully controlled. In the case of the fibres produced in greater bulk, mainly for insulation, such as glass wools, rock wool and slag wools, there is no epidemiological association with mesothelioma. This statement must be tempered by the knowledge that 'pure' exposures to these fibres are relatively rare. Those whose exposures are sufficiently historic to establish a true estimate of risk are likely also to have been exposed to asbestos, since the fibre was used in similar circumstances. More recent exposures have been controlled to levels where even if there were a potential risk, significant incidence of the tumour would be unlikely.

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Another property of fibres, especially man-made fibres, is their potential to cause irritation. This is seen mainly as irritation of the skin and mucus membranes, but can also affect the upper respiratory tract, larynx and eyes. The properties which influence this property are stiffness and diameter. It has long been established that fibre diameters $>5\,\mu m$ are generally necessary to induce significant skin irritation (Heisel and Hunt, 1968), probably reflecting the fact that this is principally a mechanical effect (inducing limited localized histamine release). Fibres of this size may be impacted on the larynx during mouth breathing and this can lead to a dry cough and discomfort. It is believed not to have long-term consequences, but in practical terms, as the discomfort is immediate and directly attributable to exposure, it may provoke more adverse reaction from workers than the longer-term health risks associated with finer fibres. It is possibly this immediate reaction to certain MMMF, notably the glass wool extensively used in domestic insulation, that raises public concern over the possible long-term effects of these materials.

4 ASSESSMENT OF INHALATION RESPONSES

One of the most useful sources of information for inhalation toxicity is studies with human volunteers. The advantages are that the target species is directly assessed and the use of carefully controlled atmospheres gives reliable information on dosimetry. The disadvantages include: for ethical reasons only healthy volunteers are used, (generally males); exposure concentrations are relatively low and for practical purposes, the studies are of relatively short duration. Examples include the assessment of sensory irritation responses, especially in the upper respiratory tract, to gases which have been previously characterized in other systems; this has been reviewed by Doty *et al*. (2004).

Although human exposures give the most useful information they are clearly unsuitable as a primary screen. Other models have therefore been devised for this purpose.

4.1 Properties Required of a Model for Inhalation Exposures

An acceptable model for the assessment of inhalation toxicity will:

- Show an identical response to humans for a full range of toxicants to be tested.
- Show a similar dose relationship to humans for the lesions observed.
- Have identical size-selective properties to the human respiratory tract.
- Allow accurate quantification of both the atmosphere and the dose received.
- Induce long-term responses within a considerably shorter timescale than that required for humans.

There are no model systems that fully meet all of these requirements. As with many aspects of toxicology, mammalian systems tend to react in a similar manner and this is as true for the lung as it is for other organs. The use of a short-lived species allows even complex responses, such as lung fibrosis and lung cancer, to be modelled within a relatively short time compared to the human lifespan, although these experiments may take one to two years. The size-selective properties of the human lung are difficult to model exactly: small animal species have smaller respiratory tracts and the limit values for respirable and inhalable fractions are lower than those for humans. This difference is enhanced further when the model species is an obligate nose-breather. Dose quantification is possible in most of the model systems employed; establishing the received dose in humans is more problematic.

Inhalation exposures are the most technically challenging of the models used in toxicology, at least in terms of equipment required and range of expertise needed to conduct a satisfactory experiment.

The most common animal model for the inhalation toxicologist to study is the rat, although the dog, mouse, hamster and guinea pig have also found significant use. Studies with primates are rare, but not unknown.

Historically, rats were often exposed in whole-body chambers, with the atmosphere distributed throughout. This system has now been almost totally superseded by a head-only or, more commonly, snout-only exposure system. In such systems, the rats (or other small rodents) are confined in tubes, usually of transparent plastic, from which their snout protrudes at a tapered end. Some form of restraint is applied at the rear, with the tail usually free (**Figure 2**) to facilitate thermoregulation in the test animal. Rodents in particular readily acclimatize to such devices, especially if exposures are performed in full illumination, when they will normally be at rest. Animals to be exposed are placed, via suitable ports, such that their noses protrude into the test atmosphere, which in some cases is delivered specifically to the breathing zone of each animal. The test atmosphere is maintained at a slight positive pressure compared to the restraining tubes so that there is no dilution from air flow past the test animal. The whole apparatus is generally enclosed in a ventilated space so that the test atmosphere, including any leaks, may be disposed of in a safe manner. Duration of exposure varies, but is usually in the region of 4–6 hours for acute and subacute exposures and either five or seven days per week. This system offers several advantages over whole-body

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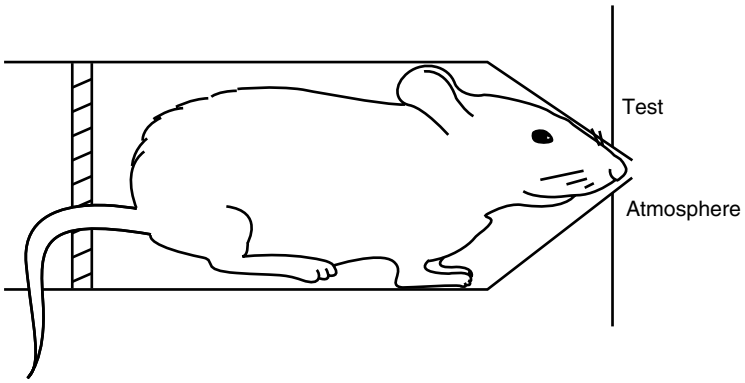

Figure 2 Restraining tube for nose-only exposure of small rodents.

exposures. First, the contamination of fur is minimal. This is especially important with liquid and solid aerosols where, with a whole body chamber, oral exposure (via deposition on the fur with subsequent grooming), may significantly exceed exposure to the lung. This may make interpretation of response especially difficult. Secondly, the system is easy to control. The smaller volume in which the test atmosphere must be generated leads to shorter path length between the generator and the final exhaust, thus minimizing the possibility of atmosphere deterioration in the process. The system is also economical with test compound. The restraining tubes may be adapted to act as a whole-body plethysmograph so that the respiration rate, TV and lung compliance of the individual animals can be measured in real time. For all these reasons whole-body chambers are generally used only for chronic exposures, especially when these are designed to be continuous; such experiments are rarely conducted.

Selection of equipment for atmosphere generation must take account of the properties of the compound. The simplest system is for gases, where adjustment of the relative flow rates of test gas and dilution air will give the desired atmospheric concentration. However, it is important to ensure that within the system, there is a chamber that achieves turbulent mixing to avoid stratification of the test atmosphere. This is especially important where the test involves heavy vapours. In circumstances where very high concentrations of low reactive gases are to be tested, it may be necessary to supplement the test atmosphere with oxygen, in order to maintain more or less normal levels. In general, it is advisable to restrict dilution to maintain minimum oxygen content of 19% of the total atmosphere, in order to avoid changes which may be spuriously attributed to the compound. In the case of volatile liquids, generation is normally achieved by bubbling air through a reservoir of the material. The reservoir may or may not be heated, dependant on the volatility of the compound concerned. The saturated vapour may then be diluted in air to give suitable concentration(s). Where heat is applied, the possibility of condensation aerosols cannot be ruled out and, if a pure gaseous exposure is desired, then the atmosphere may need to be passed through a suitable cyclone to remove particulates prior to entering the test chamber.

Liquids of low volatility are generally tested as aerosols. These are almost universally generated with a nebulizer, using the Venturi effect to produce sufficiently small droplets to be respirable for the species concerned. For more difficult liquids, simple nebulizers may be replaced by more complex versions, such as the ring nebulizer. Nevertheless, for very high-viscosity liquids, it may be necessary to dilute the test article with a suitable inert solvent, in order to generate a respirable atmosphere. Where this is done it is important to consider the nature of the aerosol to which the test animal may be exposed. Exposure to an aqueous solution is generally as acceptable as exposure to the neat material. However, if an organic solvent is used (acetone and alcohol are the most common, but others might be considered) then it is necessary either to ensure a sufficient path length for the solvent to substantially evaporate from the aerosol droplets prior to exposure, or to make an assessment of the potential effects of such solvent on the likely reaction in the test animal. The latter may be difficult. Typically particle size for aerosols is measured in the breathing zone. If the particle consists of a significant proportion of solvent then this may continue to evaporate after the aerosol is inhaled, resulting in a progressive size reduction as the particle penetrates the respiratory tract. Thus, respirability may be significantly under-estimated by the measurement in the breathing zone. In addition, if the particle penetrates to the alveolus, the presence of residual solvent may influence the behaviour once deposited. For example, it may accelerate the dispersal of the aerosol into lung surfactant.

Fortunately most of the changes induced are likely to enhance rather than reduce the apparent toxicity of the test aerosol, such that this system can be regarded as failsafe. However, where it is adopted it is important that the experiment include a solvent control, where test animals are exposed to the maximum concentration of solvent that could be achieved by any of the test groups. This provides some indication of the potential for solvent

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effects, but, as the pure solvent will evaporate more rapidly than the test solution, does not remove the need for expert judgement of the contribution of the solvent to any reaction seen.

In the case of particulates, there is a wide range of generation systems available. Most depend on similar principles. Possibly the simplest system is the air ejector, although its use is restricted to free-flowing powders. This makes use of the Venturi effect in a similar manner to the generation of liquid aerosols, except that in this case powder is entrained into the system rather than a liquid. Typically, the test powder will be delivered from a reservoir into a groove in a rotating table, with the intake for the air ejector positioned above the groove. Adjustment of air flow and rotation speed can be used to regulate the ultimate atmospheric concentration. Many test substances are not sufficiently free flowing to be generated in this simple system: even a few aggregates may block the system. Other devices that overcome this sensitivity rely on mechanical lofting of material, usually from a compacted plug of dust. One of the most frequently used is the Wright's dust feed, where a rotating blade scrapes dust continuously from a plug of compacted material in a reservoir. Air blown over the blade entrains the dust into the test atmosphere. A common variant replaces the blade with a rotating brush. In each case the dust plug is advanced by a suitable mechanism and adjustment of the rate of advance and the airflow determines atmospheric concentration.

Fibre generation uses similar equipment, although compromises are necessary. Severe mechanical attrition may fragment test fibres so that the ultimate atmosphere is not representative of the material selected for test. A number of designs for generators aim to reduce this mechanical attrition. These include the Timbrell generator (converted from a coffee grinder) where a rapidly rotating paddle scrapes fibres from the end of a plug (which is advanced in a similar manner to that in the Wrights dust feed) and thus entrains them into the airflow. A variant involves a rotating brush, where the stiffness of the bristles can be adjusted such that fibre comminution is avoided. As with all such systems it is important to establish the extent (if any) to which the generation system influences fibre size, but in any case characterization of test atmospheres is of paramount importance in these experiments, if the observed responses are to be correctly interpreted.

4.2 Measurement of Test Atmospheres

It is important to establish both the characteristics and stability of test atmospheres in order to achieve an accurate estimate of dose. This involves measurement at frequent intervals. For achieved concentration, this measurement should be made at least once per hour during exposure, preferably more frequently for shorter-term experiments, so that adjustments can be made to maintain the specified concentration.

This is readily achieved for gaseous mixtures, where direct measurements from a side stream or directly from the breathing zone via a gas-tight syringe are a matter of routine.

4.2.1 Assessment of Aerosols

While the defence mechanisms leading to the removal of particulates from the air stream before it reaches the alveolar region is relatively simple to describe in qualitative terms, measuring and modelling deposition is more challenging. This is nevertheless important because exposures to particulate atmospheres of pollutants of synthetic or natural origin involve a range of particle sizes and the size spectrum can have substantial influence on the reaction induced. Definition and measurement of the size range that will enter the respiratory tract (the inhalable fraction) is an important aspect of atmosphere characterization. Dust or aerosol particles larger than this, although presenting visual hazards and possibly an ingestion hazard to oronasal breathers, will have no effect on the lung itself. Similarly, particles which penetrate beyond the conducting airways to the alveolar regions are less readily cleared and have the potential for longer-term residence which may result in substantial effects. In addition, the thin alveolar epithelium (to maximize gas exchange) is less well protected than the mucus covered epithelia of the upper respiratory tract. This combination of factors means that material which can penetrate to the alveolar area (the respirable fraction) receives most attention from the toxicologist. Thus, definition of these fractions and their measurement is of major significance in the assessment of occupational and environmental exposures and for characterizing test atmospheres.

Total atmospheric concentration is readily measured by gravimetric sampling of nonvolatile aerosols and suitable capture and assay methods, such as absorption tubes, are available for other substances. However, particle size distribution is of special importance and this must be established (and confirmed periodically) for each test atmosphere. For any given generation system, particle size distribution tends to be more stable than total concentration, so measurement once or twice during each exposure period is usually sufficient to establish the characteristics. Once stability has been established (usually in proving trials before the test species is introduced) measurements should be made at least once per exposure period and preferably more frequently.

Various mechanical devices can be used to assess particle size distribution in aerosols. All depend on impaction or elutriation.[c] The horizontal elutriator is the simplest in principle. In this device size-selective properties are conferred by parallel plates or a cyclone. The

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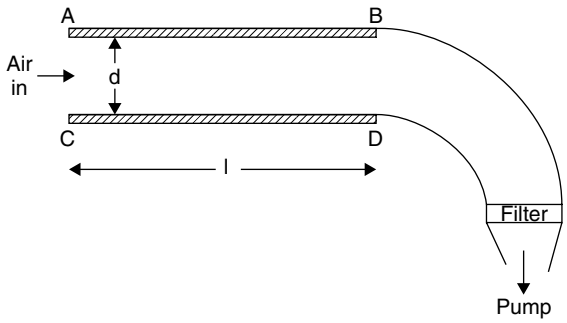

Figure 3 The principle of the horizontal elutriator.

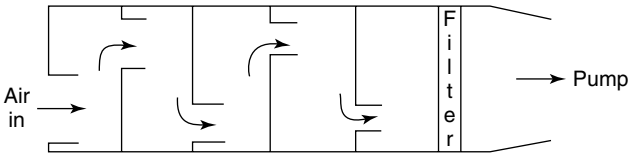

Figure 4 The principle of the cascade impactor. Air is drawn through at a constant rate so that velocity increases at each stage as aperture size decreases.

parallel-plate device illustrates the principles involved. Parallel plates AB and CD with length *L* are a fixed distance *d* apart. Airflow is introduced into one end of the device and a collector is connected to the other end (**Figure 3**). In such devices, airflow is essentially laminar so that ignoring the (relatively small) effect of the reduced airflow at the surface of the plate, the cut-off size of the elutriator is defined by the maximum size of a particle that entering at point 'A' in the airflow falls at such a speed that it just emerges at point 'D' into the collector. Particles larger than this will deposit within the elutriator, particles smaller will penetrate to a greater or lesser extent. This device illustrates the contribution of the probability function in the assessment of deposition potential, and in actual deposition in the lung. Essentially, all particles entering at point 'C' will deposit rather than emerge. Particles entering at positions intermediate between 'A' and 'C' have a probability of collection that is proportional to the square of their aerodynamic equivalent diameter (AED) and the exact point at which they enter. Such devices can be calibrated with aerosols of known size and density and can produce reliable and reproducible results from very diverse atmospheres. Although simple in concept, commercial devices may be complex. Replacing gravimetric assessment with laser systems can give instant readouts rather than time-weighted averages.

It is obvious that this device models only gravitational settlement and diffusion. These are the most important deposition mechanisms in the lower respiratory tract and therefore the device will give a reasonable (upper) estimate of the fraction which might penetrate, for example, to the alveolar spaces. Definition of the respirable fraction is the most common use for these devices. It is equally obvious that the fixed cut-off is determined by the construction of the device and therefore is not readily varied, except to the degree that airflow can be manipulated across the device without introducing turbulence. Thus, although the device will give a reasonably accurate assessment of the respirable fraction, or some other selected cut-off, it gives no further information on particle size distribution. Thus definition of exposure to different species (each having specific lung aerodynamics) is difficult if these are the only measurements available.

The cascade impactor makes use of the inertial properties of particles and can give significant information on particle size distribution within a given atmosphere. The principle is also simple to describe. The system maintains a fixed airflow into the device which consists of a series of plates or stages (**Figure 4**). At each stage, airflow is further restricted so that velocity increases. Each stage is also arranged so that airflow is off-set from the previous stage. Particles above a specified size, defined by the velocity of the airflow, have sufficient inertia to impact on the relevant plate, so that consecutive stages collect ever smaller particles. The devices have four or more stages usually followed by collection of the very finest material on a filter. Devices of this type are relatively versatile. The size of particle collected on each plate can be varied, dependent on the overall airflow, although the proportional size relationship between the plates is fixed by the design of the device. At the end of the sampling period the plates are weighed (or the test substance washed off and analysed) to determine the relative fraction deposited in each plate. These impactors may also be calibrated by standard reference materials.

These systems still find wide use and are reliable, but have the disadvantage of giving only retrospective results. Devices using lasers and applying the Tyndall effect can give real-time measurement of particle size distributions. Such devices are currently beyond the resources of many laboratories, although they offer clear benefits for dose assessment.

4.3 Other Models

4.3.1 Animal Models

Currently, there is no reliable alternative to an inhalation study in the whole animal to assess the full range of potential responses to unknown materials. Various alternatives may allow assessment of specified responses if suitably calibrated. For example, sensory irritation has been successfully modelled in mice as a predictive screen (Alarie, 1981) and results from this have been used extensively for setting air quality standards. This

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system has been refined over time, but the basic principles remain. The main strength of such systems is the focussed response, but this is also the main disadvantage when a wider assessment is required.

Various attempts have been made to develop systems that overcome the complexities of generating reliable and reproducible test atmospheres. These include administration to experimental animals by nonphysiological routes. Two routes predominate, intratracheal injection and intracavity injections.

Intratracheal injections are used to assess the primary response of the lung to a bolus of material, usually in saline suspension. It is typically used to investigate the toxicology of particulates, although it is probably more useful for investigation of liquids. Problems arise when either the amount injected is excessive and/or particle sizes are not representative of material that would naturally deposit in the alveolar spaces. Injection volumes are relatively large (typically 0.1–0.5 ml for a rat) to ensure that the material is washed down to the lower respiratory tract, rather than lodging in the trachea, where it could be readily cleared. In practice, much of the material reaches the alveolar spaces, but if the quantity injected exceeds about 0.1 mg (in the rat) it may accumulate in localized foci which are refractory to the normal clearance mechanisms. While this technique may give basic information about the fundamental response to injected particles, the reactions seen may not be a true representation of that which would occur during inhalation exposure. In particular, accumulations of a dust in the alveolar spaces tend to dissolve much more slowly than the same amount of material deposited by inhalation. This may result in a more prolonged and/or qualitatively different response to that normally seen. Nevertheless, when correctly calibrated and used comparatively this system can provide useful information. Use in isolation, especially when larger quantities are injected, gives results that are frequently misleading.

Intracavity injections include injections into the pleural space and into the peritoneal cavity. Both primarily expose mesothelial cells and therefore potentially assess the capacity for mesothelioma induction. Intrapleural injection and implantation has been outlined in Section (3.3.1) and was used calibrate the carcinogenic potential of a range of fibres. Based on these results, deductions concerning the importance of different size ranges were derived. Both techniques have been criticized, in that they used a constant mass of fibre. Thus, the numbers of fibres applied depend on particle size.

The technique of intraperitoneal injection was first developed in the 1930s in the USA, where injection of a fixed mass into the peritoneal cavity of guinea pigs was used to assess the fibrogenic potential of dusts. These studies were of three to six months duration. As the response is not markedly quicker than that seen in the lung following inhalation, the more physiological route is generally preferred. More recently Pott (1992) used intraperitoneal injection in rats to assess the carcinogenic potential of mineral fibres; these injections used constant fibre number. This technique appeared to maximize the potential for fibres to produce tumours. The technique is open to several criticisms. The numbers of fibres injected are beyond the range which might physiologically penetrate to the peritoneal space. The exact dimensions of the latter have not been determined, but clearly since 'natural' deposition is via the lung, nonrespirable fibres would not be present, although they are not excluded from injected material. The effects of the inclusion of these fibres, which may provoke an irritant response in the cavity, have not been established. Administration of a constant number of fibres may involve injection of very large masses of sample, if the fibres involved are mainly coarse. In the extreme, the quantities involved may be lethal and the injected mass may be controlled for this purpose. The combination of these factors is likely to lead to 'false positive' results.

Injection techniques may overcome one problem associated with inhalation exposures. When the rat is used as a model the respirable limit for fibres is below than that in humans. The respirable limit for mineral fibres in rats is normally considered to be about 1.5–2 μm diameter (equivalent to about 5 μm AED) with peak deposition below 1 μm physical diameter (2.5 μm AED). Corresponding values for humans are approximately 3 μm physical diameter as the respirable limit, with maximal deposition at about 1 μm. Thus, the rat model does not fully assess the potential effects of those fibres that lie between 2 and 3 μm diameter. Injection overcomes this restriction as the fibres injected may be of any selected size. Nevertheless, dosimetry considerations indicate that the fraction within the 2–3 μm size range that is deposited in the human alveoli will be small and injection may overestimate responses. One difficulty with these models, as with other surrogates, is well illustrated by reference to estimation of the potential for mesothelioma induction. The range of fibres known to induce mesothelioma in humans, and thus calibrate the system, is limited. Thus the test system is optimized to give a strong response to these materials. However a 'positive' result in the test is usually defined by a lesser response. Thus, the true significance of 'positive' results in such assays is debatable, although these systems are almost certainly oversensitive by comparison with the epidemiological results.

4.3.2 In Vitro Tests

There are various *in vitro* tests, which allow assessment of particular responses and/or the elucidation of mechanisms. These systems, which include lung slices and cultured cell lines, can be very sensitive and the tissue dose received accurately quantified. The main disadvantage is the narrow focus of response. A further problem with such systems is that the properties assessed are

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seldom the subject of routine evaluations of significant numbers of 'new' materials. In these cases the system may require recalibration on each occasion that it is used and confidence in the result may be limited. In practice, such techniques are valuable when developed to support specific research projects, especially those elucidating mechanism, but at present have limited application as a more routine screen.

4.3.3 In Silico Assessments

Current use of computer simulations is confined mainly to the assessment of deposition of aerosols in the respiratory tract.

As stated earlier, calculation of the proportional deposition of particles of different sizes in various areas of the lung is difficult because of the complex nature of airflows within it. Nevertheless, deposition curves for the human lung were developed many years ago, and find extensive use in the interpretation of measurements in the workplace air and for environmental air pollution. These curves are based on measurements of deposited particles *post mortem* and are refined from time to time. Perhaps the best known is that developed by the American Conference of Government Industrial Hygienists (ACGIH, 2001).

Although it is possible (at least in theory) to define a level in the respiratory tract below which a particle of a specific size will not penetrate, due to its physical properties, it must be recognized that the forces of impaction, gravitational deposition and diffusion act on particles at all levels of the respiratory tract, although their importance varies with region and development of models to assess this is complex.

One of the most important concepts for such modelling is the recognition that particles in air behave in accordance with Stoke's Law, that is, falling rate in still air may be described by the equation:

$$V = \frac{g \cdot d^2(\rho_{\text{particle}} - \rho_{\text{air}})}{18\mu} \qquad (2)$$

where V = falling speed of particle; g = acceleration due to gravity; d = particle diameter; ρ_{particle} = density of particle; ρ_{air} = density of air and μ = viscosity of air.

This equation is valid for spherical particles. It may be seen that there are two components of importance, the first being the particle diameter, the second the particle density. This means that the equation is generally directly applicable to liquid aerosols where the particles are more or less spherical and the density reflects that of the bulk liquid. For particulate aerosols, this equation is less directly useful, in that the particles may have a variety of shapes and densities. The use of the AED concept to overcome this problem has already been described and it is extensively applied in model development.

Diffusion may be modelled using Fick's law, and extension of the models to include interception, though more difficult, can be encompassed by the equations developed to describe the cascade impactor. More difficulty is encountered when attempting to fit these equations with airflows within the lung. Nevertheless these may be approximated by the use of physical models including whole lung casts prepared from cadavers (Martonen *et al*., 2000). Thus all the basic elements to construct models are available.

As computing power has become more accessible, sophisticated models have been developed and there are several algorithms for deposition that are reasonably successful at predicting the behaviour of specific particles, for example Heyder *et al*. (1986). Although all are to some extent empirical, the algorithms have been developed to the point where models of aerosol deposition in the human lung that allow for the effects of nose or mouth breathing are available (Esmen *et al*., 2002). Results from these simulations emphasize the importance of aerosol size distribution in determining received dose to the deep lung, and thus demonstrate the limitations of relatively simplistic measurements of respirable fraction for exposure assessment. Similar calculations have been made for deposition of fibres ranging from relatively simple algorithms (Burke and Esmen, 1978) to more advanced models (Dai and Yu, 1998). Nevertheless all of the above are to a greater or lesser extent empirical in that they rely on deposition measurements in animals and humans for initial calibration and algorithm development.

The problem of calibration of these models by comparison with the living lung is not trivial. This has been attempted in a number of ways. For human deposition arguably the most accurate is the measurement of particles within the alveolar spaces *post mortem*. This should give a reasonable estimate of the maximum size particle that can penetrate to this region. It can be argued that diseased states will increase or decrease this size by virtue of dilating or narrowing the airways, but the effect is likely to be small. The probability function illustrated with reference to the horizontal elutriator above (and elaborated by Esmen *et al*., 2002) shows that in practice a precise definition of the upper limit is not critical as the deposition curve is asymptotic. This is especially true when particle number is the determinant factor in any response. Nevertheless reasonable accuracy is needed when computing received mass dose since the contribution of these larger particles will be disproportionate to their number.

These direct measurements have been supplemented by other assays such as spheres of known size tagged with radiolabel, but for ethical reasons these studies have been relatively less numerous and generally confined to healthy volunteers. Similar measurements in experimental animals are probably more representative and these have also extended to deposition modelling of sized fibres (Morgan *et al*., 1980), but their relationship to the deposition in the human lung is uncertain. This is well illustrated by the size-selective properties of the rat

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lung. For many years this was assumed to be similar to that for humans, but the (substantial) difference is now well established. This may also extend to clearance rates (Snipes *et al*., 1989), another factor of critical importance for assessment of long-term dose to the lung. Further data development for calibration remains a major constraint on model development.

Accurate predictions of deposition are an important component for risk assessment, but may also help to resolve questions of mechanism. For soluble particulates, particle size will determine both deposition site and dissolution rate, as the latter must depend on exposed surface area. Thus the difference in respirable size between rat and human lungs may have profound effects on the ability to model response, especially for soluble materials, as the weight of material in the 4–7 μm AED fraction (approximately the difference between human respirable and rat respirable aerosols) can be substantial and perhaps lead to an underestimation of response if uncorrected. Conversely, if physical size is important (as may be the case with fibres), then these differences can *in extremis* invalidate model systems completely. Attempts to enhance exposures in animal models may increase or even introduce the property to be assessed, while size-dependent toxicity of fibres in the 4–7 μm AED range cannot be assessed fully in rodent models. Other observations also emphasize the need for careful size measurement of airborne particulates. Epidemiological studies have shown that response to airborne pollution can be correlated with the PM_{10} and the $PM_{2.5}$ fractions (see **Air Pollution**) and these correspond with peak human deposition values for inhalable and respirable dust respectively. The relative difficulty in recreating responses in animal models may be at least in part, a reflection of the relative dose received for a given atmospheric concentration. Accurate predictions of deposition rates in model species compared to man will allow this aspect of uncertainty to be reduced or eliminated.

5 DOSIMETRY

Historically, a primary aim of toxicology, including inhalation toxicology, has been hazard identification. Measurements of received dose and the kinetic aspects of distribution and elimination have largely concentrated on pharmacological agents, although basic information on absorption, distribution, metabolism and excretion (ADME) may be collected for some chemicals (see **Toxicokinetics of Xenobiotic and Metabolite Distribution and Excretion**; **Toxicokinetics**). It is less common to collect ADME data for other than oral exposures except by indirect means. As a consequence, assumptions are often made concerning received dose from inhalation exposures in order to conduct risk assessments. These tend to be oversimplified, but fail-safe. As a result, many risk assessments may be unduly cautious. While this ensures safety, it may be wasteful of resource if controls are more stringent than necessary to achieve the desired level of protection. Improved understanding of dosimetry, especially by the inhalation route, is a primary means by which risk assessments can be improved.

For inhalation exposures an initial assumption is that dosimetry follows Haber's law. This can be expressed in the simple form (Witschi, 1999):

$$C \times t = K \tag{3}$$

where C is the concentration of a toxicant required to produce a specified response; t is a measure of time and K is a constant representing a particular effect induced.

This postulates equivalence of short intense exposures with lower, but more prolonged, administration (the basis of most toxicology models). Thus, experimentally a one hour acute exposure can be roughly related to a four hour exposure by simple proportionality. Although this law has been extensively employed in risk assessments (Gaylor, 2000), it has long been recognized as oversimplification except under limited conditions. This is most evident at the extremes. There must always be a finite time before any effect is expressed, irrespective of the intensity of the dose. Similarly, at very low doses the effect will either never be expressed or perhaps (theoretically) expressed only in a period that exceeds the lifetime of the exposed species. Nevertheless, the rule generally holds within limits, especially for gases. The interpretive problem is the definition of the limits of applicability. Extension to longer-term exposures is much less certain, but it has frequently been adopted, even for aerosols. In particular, for comparative assessment of responses, in both experimental exposures and epidemiological studies, the 'cumulative dose' is often used. This may conventionally be expressed in units of exposure and time, for example, as mg m^{-3} h, and is a simple product of the average concentration and the duration of exposure. While the relationship is reasonably secure within relatively narrow exposure bands (e.g. to allow for small fluctuations in daily exposure rates in a long-term experiment), as exposures become more diverse so the uncertainty must increase. There are multiple reasons for this uncertainty, but the major influence is that of the various clearance mechanisms operating within the lung. Clearance is seldom negligible, especially at low concentrations, although higher concentrations of pollutants may well inhibit the process. Thus diversity of a computed cumulative dose and the real lung burden increases with time, often to the extent where true comparison becomes impossible. This is seen experimentally, where the lung burden will generally reach an equilibrium level after a relatively short period (<100 days), at least in rats. At very high concentrations, deposition rate exceeds the clearance capacity, leading to a continuing increase in lung burden. This 'lung overload' is well documented

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and known to produce responses that are atypical of less intense exposure (see Section 3.3).

Miller *et al*. (2000) attempted to allow for at least some of the above deficiencies by proposing a power law formula that recognizes Haber's law as being a special case in the more general relationship:

$$(C - C_0)^{\alpha} \times t^{\beta} = K \quad (4)$$

where C = concentration in air; C_0 = a threshold concentration below which no effect is expected; K = constant representing effect and α and β are constants that reflect clearance.

The utility of the equation in specific circumstances depends on the ability to determine α and β with appropriate precision. Currently this is not possible without extensive experimental data. Even if these data were to be available, extrapolation to man may alter the values of one or both constants. Nevertheless, this equation suggests that cumulative doses might be estimated by relatively simple relationships; the challenge is to determine how these can be employed generically to avoid the need for extensive experimentation on individual chemicals.

Even less well understood are the relative effects of lower cumulative doses, in particular the response to a short, but intense exposure, as opposed to low-level, but prolonged intake. For the purpose of setting hygiene and environmental quality standards, these are often equated, with epidemiological results from industrial exposures extrapolated to environmental exposures at much lower, but more constant levels. It should be noted that even when occupational exposures are reasonably well documented, these consist of relatively few measurements by comparison to the rigour with which atmospheres are assessed experimentally, and can seldom be related to 'true' exposure of specified individuals. Several simple observations can show how individual exposures can vary quite widely, so categorizing exposure into, for example, low, medium and high bands and assigning nominal exposure limits to these may provide a helpful indication of likely exposures but is subject to high uncertainty. Subsequent extrapolation of these bands to environmental exposure leads to even greater uncertainty, which is reflected in the wide confidence limits that are often calculated for such exposures. It is common practice to adopt, for example, a 95% lower confidence limit for an acceptable exposure. This is then often further reduced by the application of a 'correction' or 'assessment' factor to reflect the fact that the measured exposures spanned only a fraction of the working day and involved healthy adults only. Resultant environmental quality standards can be very low. These values can be difficult to achieve and significant resource may be expended in order to reduce exposures to levels that are deemed acceptable. One of the major challenges for the inhalation toxicologist is therefore to refine these indices of exposure to give a more accurate assessment of dosimetry. The aim of this exercise will be to improve ability to compare dissimilar exposures and thus to ensure that, where necessary, resources are applied cost effectively to ensure public safety.

6 FURTHER DEVELOPMENTS

Current systems for experimental inhalation exposure have been refined to the point where major developments are unlikely. Although the rat has limitations as a model species for man, the large database that has been developed over many years in this species mitigates against the adoption of alternatives and those which have been proposed, such as the hamster, have different limitations. Thus it is unlikely that there will be any major change in the assessment screens used for new (or existing) chemicals, although limited experimentation with larger species may be needed to model deposition kinetics for particles that are respirable in man, but not in rats.

A major concern with the current database is the lack of quantitative information, especially for human exposures, that can be used in risk assessments. Development of more usable exposure data will depend on experimental results. Only in carefully controlled settings is it possible to elucidate the fine details of all aspects of human exposures. However, basic research in this area should allow the development of more accurate predictive models that can then be more widely used. Such models would have profound effects on the risk assessment process, as it should be possible to estimate the range of exposures in the population rather than relying on additional (arbitrary) factors to allow for the variations between individuals and diurnal fluctuations.

Specific mechanisms for lung injury have proved difficult to elucidate. Emphysema and fibrosis are major causes of disability worldwide. Despite many years of experimentation, the molecular basis of the development of these conditions is still not fully understood. Recent developments in cellular signalling and gene expression may help to establish the precise pathways involved and this in turn may provide a more secure basis for intervention.

Cancers of the lung remain a major problem. It is likely that current developments will increase understanding of the aetiology of cancer in general and that these developments will be as relevant to the lung as they are to other sites. However, the specific issue with mesothelioma may provide a further challenge and also an opportunity to elucidate at least part of the mechanism for cancer induction. Efforts to explain the genesis of this tumour by surface properties of 'active' fibres have shown that this is not the answer—or at best only part of the answer—and there remains a strong association

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with fibre size. The combination of physical attributes and association with a single cell class suggests that a specific mechanism may be involved. This may be more accessible to the investigator than the more widespread effects of less specific carcinogens.

Overall it is expected that inhalation toxicology will continue to develop from the model development and descriptive phase into a fuller understanding of mechanism. This is likely to be facilitated by the development of specific model systems, probably *in vitro,* to allow cellular responses to be isolated and investigated.

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NOTES

[a.] The term vapour is used in various contexts and may thus be regarded as inexact. In this text it is used to mean the gaseous phase of a substance that is liquid at the temperature in question.

[b.] In Fenton's reaction iron cycles between the Fe(II) and Fe(III) oxidation states. The oxidation step produces a highly reactive hydroxyl radical (·OH) that attacks organic molecules. A silica surface potentiates the reaction.

[c.] The dictionary definition of this term is separation of particles by an upward air flow. In practice many such devices, including that described here, use a horizontal air flow.

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Mixed Routes of Exposure

John J. Clary

CONTENTS

1 INTRODUCTION

Mixed routes of exposure to chemicals are very common in environmental settings (air, water, soil, food) and occupational settings that may also include environmental exposure to the same chemicals. While many human exposures are by multiple exposure routes, one route usually predominates. There may be a tendency to think of the predominant route as the only route of major concern. For example, in an occupational setting inhalation exposure may be the primary concern. This leads to the establishment of acceptable workplace, exposure levels, that is, threshold limit values (TLVs) and permitted exposure levels (PELs) by the American Conference of Governmental Industrial Hygienists (ACGIH) and Occupational Safety and Health Administration (OSHA), respectively. Airborne material, especially vapours, can result in pulmonary, as well as oral and dermal exposure in the workplace. In some cases a skin notation is added to a TLV or PEL due to a concern for potential irritation to skin, mucous membranes and eye from the chemical in question. Concern about assessing total exposure is addressed by the ACGIH TLV Committee, in some cases, where adequate data exist by the use of biological monitoring and the establishment of a biological exposure index (BEI). This involves measuring a chemical or its metabolite in biological specimens (blood, urine, hair, etc). Biological monitoring may not always feasible due to problems in sampling, etc.). Adequate data must exist to use this approach. Analytical data may be more difficult to collect, but it is clear that this is the best method to assess total exposure in the workplace.

Environmental exposures are often from several routes of exposure. A toxic material may be in the air, on food and/or in drinking water. Dermal exposure is possible if water is used for bathing. If the toxic material is volatile and found in the water, the heating of the water could also result in pulmonary exposure. Although one route of exposure is the major one, the other routes may be a factor in determining both total exposure and metabolite production.

Many regulatory agencies and industries rely on risk assessment in making risk management decisions. Risk assessments usually examine several routes of exposure, independently for each route of potential exposure, and then the different routes of exposures are added together to define the total exposure and risk. While this approach has limitations, total exposure data usually do not exist in human or animal experiments used in the risk assessment. Many times very conservative default assumptions are used.

Differences in absorption rate and metabolic breakdown by different routes of exposure may result in changes in the metabolite blood level in key tissue affecting toxicity. The ultimate toxic agent may be formed as a result of metabolism by the liver. Portal

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circulation following oral exposure will result in the production of metabolites more rapidly than systemic circulation (pulmonary). Distribution to the target organ is also a factor to consider. In addition, species difference in absorption and metabolism may further complicate the issue.

The route of exposure(s) is a factor both in the design of toxicity studies and in the evaluation of a chemical's effect in humans. When testing a chemical for toxicity in animals, the route of primary concern should be the route of exposure(s) during human use. Various factors have to be incorporated into experimental design. If pulmonary exposure is the primary concern, then an inhalation study should be conducted. A whole-body exposure study has been used in evaluating many chemicals, but a nose-only exposure might give a better indication of just the effect of pulmonary exposure. Aerosol, vapours or dust exposure in a whole-body inhalation will result in test material being deposited on the fur of experimental animals. Dermal and oral exposure (as a result of grooming) may result in a significant exposure under these conditions.

If the oral route is the primary route of concern, then the questions of incorporating the test material in drinking water or feed (for foods) or using a gavage technique (drugs) should depend on the most likely human exposure. If the material is a pesticide, in addition to inhalation and dermal workplace exposure, oral exposure, on or in food, is also possible.

The length of exposure period, short time, such as intravenous (instantaneous) or oral (bolus), compared with longer exposures such as in drinking water, inhalation exposure over a six hour period per day or continuous dermal exposure, may also be important for the response if multiple routes of exposure are of concern. A workplace exposure could result in both inhalation and dermal exposure. Dermal exposure would most likely continue until the exposed individual changed clothes and washed the affected areas. Absorption and metabolism most likely will proceed at different rates, and this possibly could affect the course and nature of the toxic response.

This chapter will discuss the question of multiple exposure routes in both experimental animals and humans. Multiple routes of exposure could have a significant effect on the toxic response in animal experimentation and in human experience.

2 MIXED EXPOSURE IN ANIMALS

2.1 Inhalation

2.1.1 Nose Only vs. Whole Body

Nose-only inhalation exposure is selected over whole-body exposure many times because of cost or availability of test material, or other concerns such as contamination of the chamber by radio-labelled material. Whole-body exposure is usually less labour intensive and therefore may be less costly to conduct. Handling of the animals in a nose-only study requires more labour and the exposure is more likely to be stressful than a whole-body exposure. In a nose-only exposure, the test agent is much less likely to be found on the fur or skin. Dermal and oral exposures are minimized under these conditions. Grooming in whole-body exposure studies could result in significant ingestion of test materials, especially dusts, mist or aerosol by experimental animals or their offspring in multigeneration studies. This is demonstrated in an inhalation study of rats exposed for six hours by the whole-body route to radioactive 4,4-methylene diphenyl diisocyanate (MDI). Radioactivity was found in all tissues. The highest dose of radioactivity was found in the respiratory and gastrointestinal tract suggesting that grooming after exposure was a major source of exposure (Gadhill *et al*., 2005).

Oral exposure also results from vapour or particle exposure as a result of being trapped or dissolved in the mucus fluid in the respiratory tract and being removed by the mucociliary escalator and then swallowed.

There have been a few studies that have compared nose-only with whole-body exposure in terms of response. Several studies are listed below that illustrate the difference in response.

In an inhalation study in mice comparing water aerosol exposure with nose-only or whole-body inhalation, a small increase in maternal toxicity, foetal malformations and variations were observed in the nose-only exposed mice, suggesting that stress of the exposure method was a factor (Tyl *et al*., 1994).

In a rat inhalation study of respirable chromium, rats were housed in conventional cages (whole-body exposure) or fibre-glass tubes that allowed exposure to the nose-only (Langard and Nordhagen, 1980). The whole-body exposed rats excreted 8.4 times more faecal and 5.5 times more urinary chromium than the rats in the nose-only exposure. This suggests that significant additional exposure is primarily by the oral route (grooming).

In an acute inhalation study in rats exposed to chloropicrin, responses to nose-only and whole-body exposure were compared. The LC_{50} values were 14.4 ppm whole-body vs 6.6 ppm nose-only. The higher LC_{50} in the whole-body animal could be a result of the rats trying to avoid the vapour by huddling together and placing their noses in their fur or their cage-mates' fur as a filter. In the whole-body exposure the response (irritation and/or damage to respiratory tract) was biphasic. The first phase lasted about three days and the second phase was observed 6–14 days after exposure. In the nose-only animals, effects were observed in the first three days only. The response seen on days 6–14 only in the whole-body

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exposure could be a result on grooming of material from fur (Yoshida *et al*., 1991).

In a mouse study of aerosol exposure to two viruses, *Klebsiella pneumoniae* (KB) and Venezuelan equine encephalitis (VEE) virus, the viral concentration reached a maximum tissue level 23–36 hours earlier in the nose-only vs. the whole-body exposure. Higher levels of KB were found in tissues with nose-only exposure. KB was found in the cerebellum at 73 hours only in the nose-only exposed mice, but the resulting toxic response was similar by either route of exposure (Stephenson *et al*., 1988).

An argument for whole-body inhalation exposure in animal experiments, in addition to cost, may be that this type of inhalation exposure results in some dermal exposure, more like the normal exposure scenario in humans for workplace chemicals. It is common for occupationally exposed workers to receive both inhalation and dermal exposure to airborne chemicals during the normal workday, if adequate protective measures are lacking.

2.2 Oral

In early nutrition experiments to establish nutrient requirements for trace elements and vitamins, the problem of ingestion of the faeces by the rat was an experimental detail that had to be considered to prevent underestimating the requirement. Ingestion of faeces could still be a source of continuous oral exposure if the test agent or its metabolites (given by inhalation, or the dermal or oral routes) is excreted in the faeces. The resulting total exposure might be higher and the duration of exposure might be a factor in the production of toxic effects, no matter what the route of exposure used.

The effect of the duration of the exposure can be a factor in the response. This was demonstrated when a one week gavage dosing was compared with administration of the same concentration of test chemical in the drinking water for one week (La *et al*., 1996). Formation of DNA adducts and cell proliferation was measured. The gavage dosing resulted in two to four times the adduct formation and up to three times the cell proliferation, demonstrating the effect of a bolus dose vs. continuous dosing also by the oral route.

2.3 Dermal

In dermal studies, exposure by the oral route is possible through grooming. This may be prevented by the use of a collar, the placement of the test material on the animal and individual housing. If the test agent is volatile and care is not taken to prevent volatilization, inhalation exposure is possible.

2.4 Group vs. Individual Housing

Group housing vs. individual housing may be a factor in multiple routes of exposure. Inhalation exposure to irritating material may cause rats to huddle together and use the fur of other rats in the group as a filter and thereby reduce inhalation exposure. Individual housing may be useful in preventing or reducing exposure from coprophagy, grooming or off-gassings from fur of test materials.

2.5 Multiple Routes of Exposure

Toxicity studies, in general, normally use a single route of exposure. This makes the calculation of dose and the interpretation of results easier. Very few studies make an attempt to determine, calculate or estimate the effect of exposure from all routes simultaneously on the toxic response. This is understandable, as the contribution by exposure from other routes is most likely very small in comparison with the main route of exposure.

In a 1983 study, an attempt was made to study the effects of total exposure to shale (inhalation, ingestion and dermal) in hairless mice (Bernfeld and Hornberger, 1983). The exposures were carried out by mixing the test material (shale) with the bedding for 68 weeks of the study. Five groups of 200 hairless female mice were used: one group on conventional bedding material (dried and ground corncob as a negative control group), one group on processed shale (termed 100% processed shale), one group on a 1:1 mixture of processed shale and conventional bedding material (termed 50% processed shale), one group on a 1:9 mixture of processed shale and conventional bedding material (termed 10% processed shale) and one group on natural shale (termed 100% natural shale). Half of the animals in each group (100 mice) received about 25 µg of lanolin per mouse per week, over approximately 1 in^2 area of the lower back to simulate possible synergistic effects of ointments or skin lotions applied simultaneously with the shale. The other half received five weekly portions of 0.1 ml of acetone, as a negative control group to the lanolin treatment. The particle size distribution of processed shale ranged from particles of 0.1 µm diameter to a mesh size of 60 or more. About 2% of the weight of the shale consisted of particles of 1 µm or less, about 23% of 10 µm or less and about 36% of 44 µm or less. This suggests that up to approximately 20% of the shale was respirable.

A dose-related coat of black powder was observed during the experimental period covering the surface area of the skin of the hairless mice. In addition, visual evidence at necropsy supported an observation that dose-related amounts of shale had been ingested and inhaled as estimated by lung, trachea and larynx colour.

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Histopathological evaluation of all sites of abnormal gross appearance and selected sections of skin, large and small intestines, caecum, lungs, trachea and larynx of all animals revealed no deleterious effects due to the exposure of the mice to processed or natural shale. The only major pathological findings were a significant number of lymphomas in all groups, including the controls. The prevalence of lymphoma was unrelated to the bedding materials and a high incidence was found in historical controls.

This study is an example of an investigation of potentially chronic and carcinogenic effects evaluated simultaneously as a result of dermal, ingestion and dust inhalation exposure in a single study.

This study would be criticized today because of Good Laboratory Practice issues and the use of an unacceptable protocol from a regulatory point of view, but it is still a rational attempt to evaluate the combined toxic effects of three routes of exposure simultaneously. It might be considered today as an interesting screening study. The same approach could be used for screening pigments or other coloured nonvolatile materials.

In another rat study with a combined chronic exposure to chloroform, exposures were by inhalation only (six hours per day five day per week for 104 weeks), oral only (1000 ppm for 104 weeks) and both routes at the same time (104 weeks). It was concluded that the combined exposure enhanced carcinogenicity and chronic toxicity in the kidneys was greater than the additive rate (Nagano *et al*., 2006).

3 HUMANS

Exposure of humans (occupationally or from environmental sources) is usually mixed, from both the route point of view and perhaps the agents involved. In a single day we are all exposed to environmental airborne chemicals from many ambient sources, low-level contaminants in food and dermal exposure to other chemicals in material we handle. Occupational exposure can also be significant in chemical production and use.

3.1 Occupational Exposure

Occupationally exposed workers can have dermal, inhalation and possible oral exposure when working with many chemicals. The evaluation of agricultural workers exposed to pesticides is a good example of mixed exposure from the inhalation, dermal and oral routes. Inhalation and dermal exposure would be expected during normal use. Interestingly, it appears at least in some cases dermal exposure appears to be the major route of exposure in occupationally exposed pesticide workers. This suggests biological monitoring may be a more accurate predictor of exposure than airborne levels (He, 1993). Oral exposure could result from vapour or particles being trapped or dissolved in the mucus fluid in the respiratory tract and being removed by the mucociliary escalator and then swallowed. Ingestion of food from pesticide-treated crops may contain a pesticide residue, or a low level of pesticide may be found in the drinking water.

Dermal exposure to chemicals can result in significant inputs to the total dose in many workplace situations. Dermal exposure may increase in relative importance when airborne occupational exposures are reduced. Dermal exposure normally occurs by one of three pathways: (i) immersion (direct contact with a liquid or solid chemical substance); (ii) deposition of aerosol or vapour and uptake through the skin or (iii) surface contact (transfer from contaminated surfaces such as clothes).

Even the eye may be an important route of exposure for some chemicals such as nerve agents. Airborne nerve agents, in addition to pulmonary exposure, have been reported to penetrate the conjunctiva rapidly and stimulate muscarine receptors. Individuals exposed to nerve gas (such as the sarin released in the Tokyo subway) experience lacrimation and rapid miosis. Miosis is not normally seen or is delayed following dermal exposure to sarin (Holstege *et al*., 1997).

The most common method for measuring exposure in occupationally exposed workers is the air level of the chemical in question. The occupational exposure limits most commonly used are the TLV and PEL, which are normally based on air levels. Biological monitoring, where possible, is recommended as the most precise means of estimating the total absorbed dose of a chemical or pesticide by multiple routes. Dermal exposure is difficult to measure routinely, but estimates can be derived for the measurement of dermal exposure. Personal air sampling is the preferred method for the measurement of inhalation exposure, including measurement of the respirable fraction and/or any vapour component of a chemical.

The potential for significant dermal exposure should not be ignored. In an exposure assessment in pharmaceutical workers exposed to fentanyl during production, air and hand wipe samples were collected and all showed exposure. A significant correlation between hand exposure and urinary excretion was observed. Inhalation exposure results appeared to correlate with the dermal values, suggesting that dermal exposure is the primary route of exposure to fentanyl in production workers (Van Nimmen *et al*., 2006). This suggests that routine air monitoring would be a less accurate measure of exposure than dermal exposure. The best measure of total exposure appears to be urinary levels (biological monitoring)

Airborne exposure monitoring conducted simultaneously with biological monitoring is useful in determining

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the sources of exposure. If biological monitoring indicates a much greater exposure than predicted by just airborne exposure, then dermal exposure or even dietary exposure may be a significant source of exposure, as demonstrated above.

3.2 Environmental Exposure

Inhalation, dermal and oral exposure are all potential routes of exposure in the environment. Environmental exposures are often mixed. The sources of the exposure are more varied than those found in the workplace. Inhalation exposure can come from factory emissions, incineration, spills of volatile chemicals, volatilization from bodies of water and automobile exhaust, to name only a few. Dermal exposure can come from the air, water, soil and direct contact. Oral exposure can come from food, soil and water. All these exposures may be occurring at the same time and contributing to the total exposure of the individual.

An example of these multiple routes of exposure is a study concerned with the risks to humans and wildlife posed by dioxin-contaminated soil (Paustenbach, 1989). Exposure was by dermal contact (soil), inhalation (dust) and ingestion (soil by children and food and water by adults). Environmental exposure to plants, fish, birds, wildlife and grazing livestock can result in contamination of food, in addition to potential toxic effects to these environmental species. Other factors that influenced the exposure were absorption by the various routes, bioaccumulation (soil-wildlife, fish food chain, soil sampling, residential vs. industrial sites), uptake by plants, run-off, biological half-life, leaching into groundwater, weather and others.

3.3 Other Exposures

Multiple routes of exposure may occur from other sources, such as cosmetics, food and drugs. The use of cosmetics results in primary dermal exposure, but oral exposure may also take place from lipstick. Other cosmetic ingredients may be added to food or be present as a natural component (e.g. lactic acid). Both dermal and oral exposure will occur. Several routes may be used to administer a drug. The rate of absorption of drugs may be a key factor in determining the route(s) selected by a physician. Ideally, the correct concentration of a drug or active ingredient at the target site is desired. Differences in absorption, distribution and metabolism considerations could lead to multiple routes of administration (exposure) in some cases.

3.4 Biological Markers of Exposure

Biological monitoring can use human data and animal experimentation data. It gives the best picture of total exposure from all routes. Biological monitoring may be used as an identification of total exposure, a marker of a toxic effect, or a marker of susceptibility to a toxic agent. The biological marker can be the chemical itself or a metabolite. It can be measured in human blood, urine, hair, fingernails or other biological fluids or samples. Depending on the nature of the biological marker, either short-or long-term exposure may be estimated. Currently the ACGIH TLV Committee has set acceptable BEI values based on biological monitoring for approximately 50 chemicals found in the workplace (American Conference Governmental Industrial Hygienists (ACGIH), 2007). These values are established by determining the level of the biological marker in individuals exposed just to inhalation exposure of the chemical of concern. The use of biological markers allows total exposure from all routes to be compared with inhalation-only exposure.

Blood lead is a good example of biological monitoring, measuring total exposure from inhalation and ingestion in a workplace situation. Inorganic blood lead has been used as background for occupational exposure limits for lead (Skerfving, 1993). A two-compartment model is used to describe lead metabolism. There is a rapid compartment (reflecting soft tissues), with a lead half-time of about one month, and a slow lead half-time (reflecting the bone lead pool), with a half-time of approximately a decade. There are significant interindividual variations in lead metabolism. The whole-blood lead level is useful for biological monitoring for recent absorption. The relationship between exposure and blood lead concentration is curvilinear, with a decreasing impact of rising exposure. Blood lead level may be affected by the slow release of lead from the bone lead pool. The average blood lead level in individuals exposed only to 'background' lead exposure varies considerably. Blood lead levels in workers are roughly related to air lead levels in the workplace. These air levels may underestimate exposure, as they do not take into account additional exposure from other routes such as food, drink and tobacco. Total exposure can be estimated by biological monitoring.

Biomarkers based on metabolites have also been used. For example, benzene exposure results in muconic acid in urine, resulting from the ring opening of a benzene metabolite. 5-Phenylcysteine resulting from the addition of benzene oxide to a cysteine sulfydryl group in albumin and in haemoglobin is another biomarker for benzene exposure (Bechtold and Henderson, 1993).

Biological monitoring can be used as an estimate of the absorbed dose of a chemical, especially if animal and human metabolism and pharmacokinetic data are available. An example of this approach is a study of atrazine applicators (Lucas *et al*., 1993). Enzyme-linked

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immunosorbent assays (ELISAs) were used to detect atrazine and its metabolites in the urine. The primary urinary metabolite was the mercapturic acid conjugate of atrazine. This study demonstrated a correlation between cumulative dermal and inhalation exposure and total atrazine equivalents excreted over a 10 day period.

Most biomarkers are correlated with air concentrations. Once this correlation has been established it can be used to determine the different sources of exposure and the amount that each route of exposure contributes to the total exposure. Air concentrations and biological monitoring have been used to evaluate the exposure of occupationally exposed factory workers to the solvent methylene chloride (Ghittori *et al*., 1993). The air concentrations were determined by personal passive dosimeters. The biological monitoring of workers was performed by determining the concentration of carbon monoxide in alveolar air and methylene chloride in urine. A correlation between the methylene chloride concentration in air and the carbon monoxide concentration in alveolar air was found when workers who smoked were removed from the analysis. Smokers who worked with methylene chloride had elevated carbon monoxide levels compared with nonsmoking workers, thus demonstrating the impact of smoking as another source of exposure that elevates carbon monoxide levels. In this case, a significant linear correlation was found between the air concentration of methylene chloride in the breathing zone and the methylene chloride concentration in urine.

4 RISK ASSESSMENT

Risk assessment is widely used to make risk management decisions. All potential sources of exposure are considered in risk assessment. Exposure results in potential inhalation, dermal and oral exposure if the chemical of concern is airborne and respirable. In addition to direct inhalation and dermal exposure, airborne fallout can enter the water supply and be deposited on growing food crops, resulting in additional dermal and oral exposure. Leachate from landfill or contamination of surface water from surface run-off or airborne deposition can result in contamination of the water used for drinking and personal hygiene.

USEPA has developed a relative source contribution (RSC) policy for chemical exposure from drinking water comparing oral with the contribution from other sources. However RSC is based more on professional judgement that actual data (Howd *et al*., 2004).

In most cases, risk assessment is based on data from each source that come from different experiments and possibly different species. Physiologically based pharmacokinetic (PBPK) modelling is used to integrate all exposures and other factors such as absorption, distribution, metabolism and elimination by the different routes and also species differences (Medinsky and Klaassen, 1996). PBPK modelling can be used to examine toxicity resulting from separate exposure routes and the influence of the time separating two routes of chemical exposure. PBPK modelling provides a basis for extrapolation across species, routes and doses and is a useful tool for risk assessment.

In an assessment of bis-(pentabromophenyl) ether in a worker exposed population, the major routes of exposure were inhalation of dust and skin exposure. Consumer exposure was considered negligible by all routes. Oral was considered the main environmental exposure. In an occupationally exposed worker the contribution of environmental oral exposure was considered insignificant to the total exposure in workers (Anonymous, 2004). This assessment is a good example of demonstrating that the major route of exposure varies with the population at risk.

Risk assessment as practiced has built-in conservative default assumptions, such as water consumption, absorption by various routes and animal to human extrapolation. The resulting risk assessment is most likely predicting a risk greater than the real risk because of the conservative approach used. This in itself is acceptable, but determining the actual risk would require that a realistic exposure scenario is established and that the effect on the total toxic response is determined. Any synergistic or inhibiting effects of one route vs. another should also be determined.

Many of these exposure estimates are derived from modelling. For example, a modelling approach has been used to demonstrate the effects of gasoline-contaminated drinking water (Shehata, 1985). The model estimated ambient and indoor air quality from volatilization from drinking water. Oral and dermal burdens were estimated using benzene, toluene and xylene as surrogates for gasoline. Exposure to vapours during showering in the confined area of a bathroom was estimated to be possibly high enough to cause acute mucous irritation. While this type of information is useful for risk assessment, it is based on exposure modelling of several routes of exposure and not actual data. The use of risk assessment is widespread and these types of exposure estimates from multiple-route exposure can be a source of concern. Exposure assessments can have a large impact on the risk assessment process. More sound experimental data in both animal and humans are needed to evaluate how good these estimates really are.

A simple two-compartment PK model has been the standard for studies describing the kinetics of radiolabelled tracers, such as manganese. In an attempt to describe exposure by both inhalation and oral routes of exposure at the same time, a unified model from the simple two-compartment PK model failed to work. The model was revised for route of exposure differences in availability, and proved to describe distinguishable pools of manganese, one in the gastrointestinal (GI)

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tract-derived portal blood and the other in venous/arterial blood. The authors concluded that route-dependent differences in handling of absorbed manganese are a necessary component in PBPK modelling (Clewell *et al*., 2004).

Another example of modelling is illustrated by an assessment of total exposure from trichloroethane (TCA)-contaminated water during showering (Byard, 1989). Exposure from three sources and two routes was estimated (inhalation dermal water and dermal vapour). It is interesting that in this assessment, the predicted exposures during showering by the inhalation and dermal water routes were similar. The exposure to the skin by dermal vapour exposure was estimated to be 1% of the inhalation or dermal water dose.

Biological monitoring is a good way to validate PBPK modelling. Biological monitoring should be encouraged if practical, especially when the experimental data used for PBPK modelling are limited (absorption, distribution, metabolism and elimination) and from different species.

5 SUMMARY

While risk management decisions are based on risk assessments that estimate total exposure from all sources, the design of toxicological experiments in animals is usually route specific. A comparison of acute lethal doses by different routes points out how the difference in the route of exposure can affect the toxic response. There are very few cases where experiments in animals were designed to assess total exposure from several routes. Multiple routes of exposure are found, however, in many of the human responses to toxic agents. Thought should be given to conducting mixed-exposure studies in animals to evaluate how predictive the PBPK and risk-assessment modelling are in estimating the actual response.

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Neurotoxicology

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CONTENTS

1 CELL TYPES IN THE NERVOUS SYSTEM

The brain contains several different types of neurons, glial cells and epithelial cells. A substance is considered neurotoxic when it causes a pathological effect on the function of the nervous system. A neuron consists of cell body with a nucleus, dendrites, axons and nerve terminals. **Figure 1** shows a schematic drawing of a nerve cell and illustrates how different toxic substances may interact with different parts of the neuron. Dendrites, cell bodies, axons and terminals of the neuron may be targeted by different toxic substances. All protein synthesis in the neurons takes place in the cell body. The dendrites and the cell bodies are covered with receptors and receive incoming signals in the form of chemical transmitters from other neurons. Excessive excitation of

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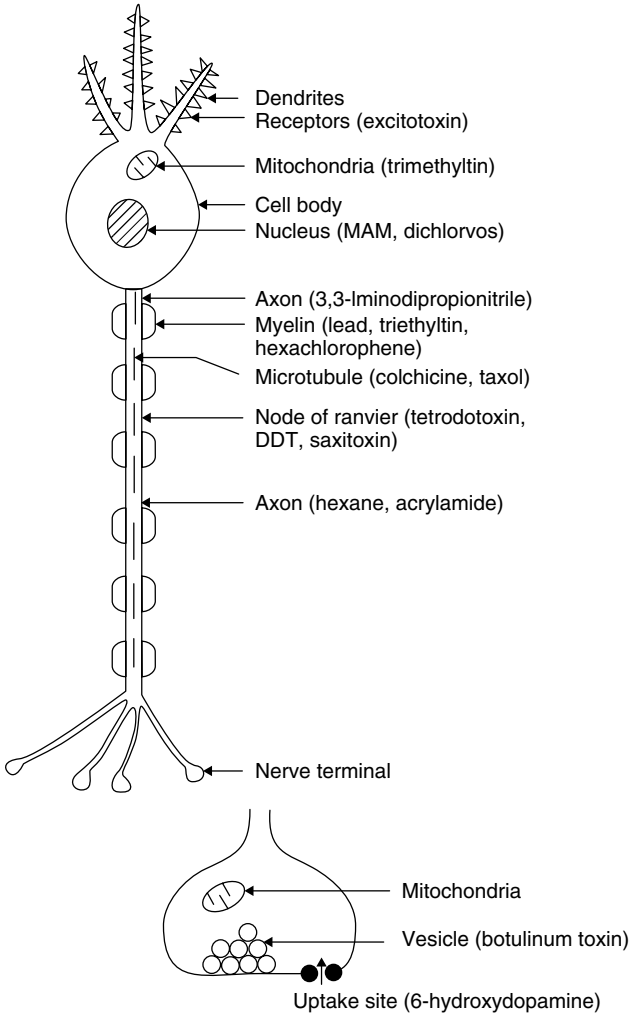

Figure 1 Schematic drawing of a nerve cell with dendrites, cell body, nucleus, axon and terminals, giving examples of toxic compounds which target different parts of the cell. Excitotoxins target receptors on dendrites and cell bodies. Methylazoxymethanol (MAM) and dichlorvos react with DNA and its repair system. 3,3-Dipropionitrile causes proximal axonopathy, whereas hexane and acrylamide cause distal axonopathy. Tellurium and lead cause myelinopathy. 6-Hydroxydopamine is an example of a compound which targets the terminal through its plasma membrane uptake, whereas botulinum toxin targets proteins in the synaptic vesicles.

receptors may cause neuron degeneration by a phenomenon called excitotoxicity. Degeneration of neurons is accompanied by gliosis, that is, the proliferation of astroglial cells. The transmitter signal received by the cell is then transferred electrically down the axon. The axon is usually thinner than the dendrites and may extend from several micrometres up to a metre. The axons contain cytoskeletal proteins, such as neurofilaments and microtubules that are involved in the transport of proteins from the cell body to the nerve terminals. Proteins are also transported retrogradely, that is, from the terminal to the cell body. The transport involves the proteins kinesin and dynein which act as a microtubule-associated adenosine triphosphatase (ATPase). Membrane-bound proteins are transported at a rate of 400 mm per day in the anterograde direction by kinesin, whereas similar proteins are carried in the retrograde direction by means of dynein. The transport of mitochondria occurs with an intermediate rate of 50 mm per day. Soluble proteins and the cytoskeletal proteins move with a slow rate of 1–4 mm per day (Barry *et al*., 2007). The axons are usually surrounded by an insulating layer consisting of myelin cells that are named oligodendroglia in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS). Several toxic compounds are specifically directed to the myelin cells. Myelin has a complex multilamellar structure. Compounds affecting energy metabolism and membrane stabilization may cause intramyelinic oedema, a pathological condition often seen in myelinopathy. Other compounds may lead to a selective loss of myelin called demyelination. In the peripheral nervous system remyelination may occur after a toxic insult, whereas in the central nervous system this is rare. The insulation of axons by myelin increases the impulse transport down the axon (Blakemore, 1984). The surface of the nerve cells contains ion channels, and they are highly localized in the nodes of Ranvier. Several toxins have been found to affect ion channels.

The axon branches out and ends in small enlargements called the nerve terminals. A simple neuron usually has several thousand terminals. The terminals contain synaptic vesicles filled with neurotransmitters and the vesicles release the transmitter by exocytosis. The neurotransmitters are amino acids (glutamate, γ-aminobutyric acid (GABA) and glycine), amines (catecholamines and serotonin), acetylcholine, adenosine derivatives or different peptides. The terminals have high affinity uptake mechanisms for many transmitters. Uptake of false transmitters or inhibition of the transport mechanisms, which will prolong transmitter action, may have fatal consequences.

The glial cells are the myelin-forming cells, astrocytes and microglia. The astrocytes have a large cell body from which a number of processes radiate. The processes sometimes end in thickenings called end feet which surround the capillaries in the CNS. The astroglial cells contain active uptake mechanisms for many neurotransmitters and interact strongly with the neurons metabolically. The astrocytes take up GABA and glutamate from the extracellular space after their release from the nerve terminals and transport glutamine, lactate and some other amino acids back to the neuron. The astrocytes play an important part in the replenishment of the tricarboxylic acid (TCA) cycle of the neurons (Fonnum and Hassel, 1996).

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Microglia cells are widely distributed and are extremely sensitive to neuronal damage. It was established early on that they proliferate after axotomy. Now it is recognized that they become activated and undergo a series of morphological and phenotypic changes within minutes after a toxic insult. The phenotypic changes involve new types of antigen such as major histocompatibility complexes not normally encountered on resting cells. These antigens are normally expressed in the immune system (Streit *et al*., 1989). Activation of microglia may result in transformation into macrophages that phagocytize necrotic neuronal debris (Streit and Kreutzberg, 1988).

2 THE BLOOD–BRAIN BARRIER (BBB)

The blood–brain barrier (BBB) establishes an essential barrier between the brain and the blood circulation, and protects the brain from many polar toxic substances. The BBB consists of nonfenestrated endothelial cells surrounding the brain's microvessels (Rees and Karnovsky, 1967). The nonfenestrated cells are linked by tight junctions, whereas in the rest of the body the endothelial cells are spaced apart and allow substances, including proteins, to penetrate (**Figure 2**). The endothelial cells in the BBB also have low pinocytotic activity. The endothelial cells around the capillaries are surrounded by the end feet of the astrocytes, but it is uncertain to what extent the astrocytes contribute to the BBB. Transport through the BBB is limited to nonpolar substances and to several nutrients for which there are a specific uptake systems. There are uptake systems for hexoses, monocarboxylic acids, neutral, acidic and basic amino acids, nucleosides and purine bases (Miller *et al*., 1985). Many toxic substances can enter the brain with the aid of these uptake systems.

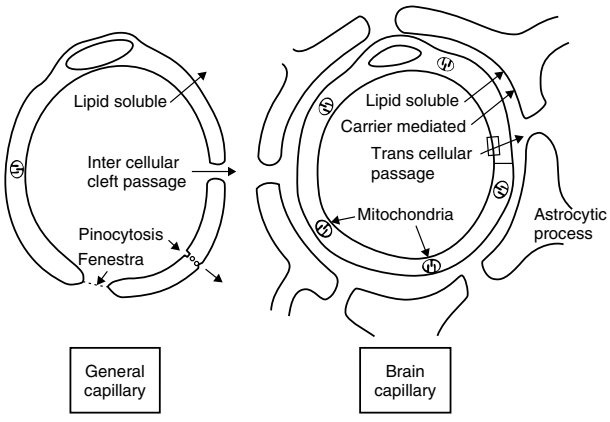

Figure 2 A capillary vessel normally found in the body and a capillary vessel in the BBB. In the brain capillary, the epithelial cell is nonfenestrated and is linked by a tight junction. Outside the epithelial cells are the end feet of astrocytes. Toxic compounds can only enter the brain if they are lipophilic or if a transport system is available.

In humans, the BBB is established at birth, whereas in rats it is on postnatal day 10. There are some areas in the brain that have a weaker BBB, namely the choroid plexus and the circum ventricular organs (median eminence, subfornical organ, area postrema and neurohypophysis). These regions have endothelial cells of the fenestrated type, but they maintain the tight junctions between the cells.

The peripheral nerves are also protected from many toxic substances. The peripheral nerves are protected from the extracellular fluid by the epineurum which consists of connective tissue. The endoneural capillary system also has some similarities with the endothelial cells of the BBB, but the barrier is less efficient. In the brain the cerebrospinal fluid (CSF) acts as a sink for toxic agents, but in the peripheral nervous system this sink is lacking. Some parts of the peripheral nervous system are less well protected. The perineurium is neither present in the nerve terminal branches nor in the myoneural junctions. These are therefore easily attacked by tetanus and botulinum toxins. The peripheral ganglia are covered by an efficient perineurium barrier, but the ganglionic cells do not have an efficient barrier. The sensory and autonomous ganglia are therefore readily exposed to neurotoxins such as lead and mercury (Olsson, 1990).

3 TOXIC AGENTS ACTING ON NEURON CELL BODIES AND DENDRITES

The toxic mechanism leading to degeneration of neurons is complex and can be regarded as an interplay between several mechanisms. This chapter shows that there is a close connection between excitotoxicity, oxidative stress formation, mitochondrial damage, removal of damaged protein and DNA repair. It is often not possible to say which is the most important factor.

3.1 Excitotoxins

Excitotoxins are a group of neurotoxic substances that excite somatic and dendritic receptors in such a way that the neurons may die (**Table 1**). All excitotoxins are, in principle, agonists of the glutamate receptors in the brain and are structurally related to glutamate. There is a good correlation between their excitatory potency and neurotoxicity (Olney, 1969). There are three classes of glutamate receptors linked to an ion channel, namely the α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), *N*-methyl-D-aspartic acid (NMDA) and kainate receptors. The receptor most often

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Table 1 Excitotoxins

Endogenous toxins	Exogenous toxins
L-Glutamate	Kainate
L-Aspartate	*N*-Methylamino-L-alanine
L-Homocysteate	Ibotenate
L-Cysteine sulfinate	Domoate
L-Cysteine sulfonate	*β*-*N*-Oxatylamino-L-alanine
Glutamate	L-Alosine

linked to neurotoxicity is the NMDA receptor, which has an ion channel for calcium and sodium (Hollmann and Heineman, 1994).

Kainate has been used as an antihelminic agent and is extracted from seaweed. It has been used as a medicine in low doses for children and this explains why they did not observe a toxic effect (Olney, 1981). Kainate does not cross the BBB and has to be injected to enter the brain. Kainate injected into the neostriatum was used to produce the first model for Huntington's disease (Coyle and Schwarcz, 1976). Ibotenate is found in *Amariata* mushrooms. Domoate is produced by algae (*Nitzchia pungens*) and was believed to be responsible for cognitive deficits and death after mussel poisoning in Canada (Stewart *et al*., 1990). *β*-*N*-oxalylamino-L-alanine (L-BOAA) and *β*-*N*-methylamino-L-alanine (BMAA) are two plant-derived excitatory amino acids. Zeevalk and Nicklas (1989) found that L-BOAA and L-BMAA cause exitotoxic damage in chick retina, BOAA through a non-NMDA receptor and BMAA through the NMDA receptor. L-BOAA has been found in the bean *Lathyrus sativas* and is believed to be the toxic agent causing lathyrism, a well-known disease in certain parts of India and also in the Mediterranean basin. Lathyrism is characterized by specific paraparesis which can occur after consumption of the bean. The toxic agent should normally be removed from the bean by a special treatment before the bean is consumed (Ravindranath, 2002). BMAA is another excitatory amino acid present in cycad flour derived from sago palm seeds (*cycas cincinalis*). It has been suggested that it is responsible for 'Guam disease' where the patients obtain Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (ALS) together (Spencer *et al*., 1987). Today this is considered less likely, since BMAA is not active enough. Wilson *et al*. (2002) showed that neurological lesions were induced in mice fed cycad flour even when the amounts of methylazoxymethanol (MAM), BOAA and BMAA had been reduced to very low or undetectable levels. They concluded that an as yet undiscovered toxin must be responsible for the neurodegenerative disease in Guam. Khabazian *et al*. (2002) have isolated and characterized other putative neurotoxins in cycad flour. The most toxic fractions of the washed cycad flour contained several sterol glucosides, of which *β*-sitosterol glucoside (BSSG) occurred in the largest amounts, between 21 and 155 mg kg^{-1} washed cycad flour. BSSG was shown to be neutrally active, evoking a rapid depolarizing field potential and causing cell loss. BSSG also evoked a significant release of labelled glutamate, an effect that could be attenuated by AP5 (2-amino-5-phosphonopentanoate, an NMDA antagonist). As no or only low amounts of cycasin or MAM were present in the cycad flour, the authors concluded that the molecule responsible for the cycad-induced neurodegeneration was BSSG, which activates neuronal cells via glutamate release leading to NMDA receptor activation, culminating in cell death.

The mitochondria are also involved in excitotoxicity. Novelli *et al*. (1988) showed that reduction of ATP synthesis, by KCN or the omission of glucose, increased the toxicity of glutamate. Several other mitochondrial inhibitors, such as 3-nitropropionate or malonate, inhibitors of succinic dehydrogenase, or aminooxyacetate, an inhibitor of aspartate aminotransferase were linked to excitotoxicity. Glutamate antagonists ameliorated the effect of the mitochondrial inhibitors and showed that there was a link between mitochondrial enzyme inhibitors and excitotoxicity (McDonald and Schoepp, 1993; Greene and Greenamyre, 1995).

Excitotoxicity is not a single event, but is a result of a cascade of events (Fonnum and Lock, 2004). This cascade, which involves receptor activation, an increase in intracellular calcium and a generation of reactive oxygen species (ROS), is illustrated in **Figure 3**. It may be initiated by a high concentration of extracellular glutamate caused by inhibition of glutamate uptake, or by excessive release of glutamate. Alternatively, it starts with exposure to an extracellular excitatory compound. In all cases this leads to the activation of one of the glutamate receptors, either the AMPA or the kainate receptor. Activation of the AMPA receptor is accompanied by the entry of sodium ions and depolarization of the membrane. The NMDA receptor is normally blocked by Mg^{2+} in the ion channel. On depolarization of the membrane, Mg^{2+} is released from the NMDA ion channel and this allows the permeation of calcium through the ion channel into the neuron (Choi, 1988). A high intracellular concentration of calcium has severe consequences. It may be sequestered into the mitochondria and induce depolarization of the mitochondrial inner membrane. Such depolarization may increase the release of ROS, which are produced by the electron-transport chain (Schinder *et al*., 1996). Such reactive oxygen radicals can also be formed by calcium activation from xanthine oxidase, nitric oxide synthetase or arachidonate in the nerve terminal (Fonnum and Lock, 2004). The oxygen radicals may attack proteins, lipids and DNA, and be the final step in the excitotoxic events. The high intracellular calcium can also activate membrane-destructive enzymes such as calpain, endonucleases and phospholipase A. In some cases the excitotoxic effect occurs via

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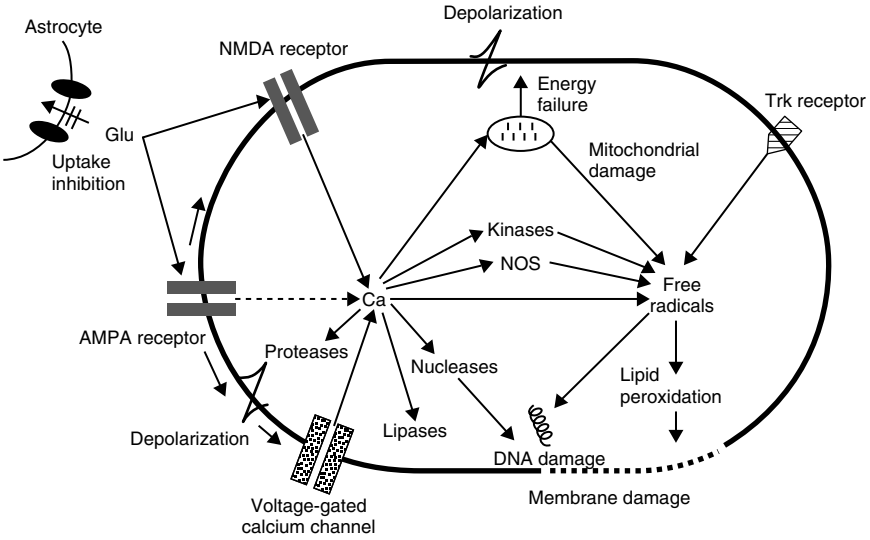

Figure 3 The cascade of reactions during excitotoxicity. Excitotoxins activate the glutamate receptors and depolarize the neurons. Calcium enters the neuron through the ion channel of the NMDA receptor and through voltage-activated calcium channels. The high intracellular calcium leads to the formation of reactive oxygen species either through the electron-transport chain of the mitochondria, by xanthine oxidase or nitric oxide synthetase. Free radicals lead to protein damage, DNA damage and peroxidation of membranes. Calcium also activates protease, lipase and DNAase. (Figure is taken from (Fonnum and Lock, 2004) by permission of Journal of Neurochemistry.)

the kainate receptor and in this case ROS also play an important role.

Some of the excitotoxins are also believed to play an important role in the development of several neurodegenerative disorders. One of the difficulties in explaining the action of several excitotoxins, such as domoate and L-BOAA, is the fact that they pass poorly through the BBB. The BBB has certain weak sites and it can also be weakened by different mechanisms, including infections. In experimental studies on young animals before the establishment of the BBB, glutamate, aspartate, homocysteate and other excitatory amino acids may produce brain damage through an excitotoxic mechanism (Olney, 1969; Karlsen and Fonnum, 1976). Olney *et al*. (1990) claimed that the toxic effect of cysteine was substantially increased in the presence of bicarbonate. Alternatively, the toxic mechanism of cysteine could be a special case of excitotoxicity, where cysteine will exchange with intracellular glutamate or lower the redox potential of the NMDA receptor to a more sensitive mode (Mathisen *et al*., 1996).

When evaluating the importance of excitotoxicity in neurodegenerative disorders, one should bear in mind that many of these disorders probably originate through different mechanistic steps. There is strong evidence from the high extracellular level of excitatory amino acids that excitotoxicity plays a role in the development of brain damage due to both focal and global ischaemia, and hypoglycaemia (Ikonomidou and Turski, 1995). Head trauma like concussion (Pugilist brain), high cerebral pressure and acute subdural haematoma also cause high extracellular levels of glutamate in human brain (Engelsen *et al*., 1985) and the primary damage is ameliorated by glutamate antagonists (Lipton, 1993). The neurological effects of HIV also involve the glutamate receptor (Raber *et al*., 1996). The toxic mechanism in development of amyotrophic lateral sclerosis may include both a high extracellular level of glutamate due to a loss of glutamate transport (Rothstein *et al*., 1996) and increased ROS due to mutations in Cu/Zn superoxide dismutase (Bowling *et al*., 1993). Excitotoxicity is also involved in the development of neurological degenerative diseases such as Huntington's and Parkinson's diseases.

4 BRAIN DAMAGE BY OXIDATIVE STRESS

The three most important free radicals in the brain are superoxide ($\bullet O_2^-$), nitric oxide ($\bullet$NO) and the hydroxyl ($\bullet$OH). Together with peroxynitrite and hydrogen peroxide they are called reactive oxygen species and play an important role in oxidative stress in the brain. The brain is regarded as particularly sensitive to oxidative stress for several reasons (Halliwell, 2007). Firstly, the brain, which is 1–2% of the body weight, utilizes about 20% of the oxygen being taken up in the body. About 1–5% of this oxygen is lost from the electrochemical chain in the mitochondria as superoxide (Fridovich, 1978). Several brain regions such as the basal ganglia (substantia nigra, globus pallidus, putamen

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and nucleus caudatus), red nucleus and cerebellar nuclei have a high content of protein-bound iron, for example in transferrin and haemoglobin (Beard *et al*., 1993). A small amunt of iron can be released by the action of superoxide on iron-binding proteins and participate in the formation of the active hydroxyl radical. Also, injured neurons can release their intracellular iron into the surrounding tissue. The brain contains enzymes such as monoamine oxidase (MAO), producing high concentrations of H_2O_2, which, in the presence of metal ions, can produce the active OH radical. The brain also contains neurotransmitters, such as dopamine, which are auto-oxidative. The brain is also sensitive to ROS because of its high content of unsaturated fatty acids in membranes. These acids are rapidly peroxidated (Halliwell, 2007).

The brain also has only a modest antioxidant defence. Enzymes such as catalase are hardly present in the brain and superoxide dismutases are only present at 10–20% of levels in the liver. The most important antioxidant in the brain seems to be glutathione (GSH) peroxidase, which is a selenium-dependent enzyme (LeBel and Bondy, 1991). Selenium deficiency leads to toxic effects in the brain. Further, the peroxiredoxin system, with a low K_m, is important, since it may reduce oxidative stress at a low concentration (Rhee *et al*., 2005). The low-molecular-weight antioxidants, ascorbate, GSH and vitamin E, are naturally occurring in the brain. Ascorbate is present in a high concentration (mM) and there is a specific uptake system for ascorbate in the brain and in the neuron (Qiu *et al*., 2007). The highest level of GSH (mM concentration) is found in the astrocytes and they transport cysteine into the brain for GSH synthesis (Dringen and Hirrlinger, 2003). Vitamin E is found in micromolar concentrations and has several different functions in the brain. The level of vitamin E in brain is strongly regulated and it takes at least six months to modify the level. Vitamin E deficiencies give neurological symptoms (Muller and Goss-Sampson, 1990).

Superoxide radicals are obtained from the electron chain or are formed from hydrogen peroxide by superoxide dismutase. The superoxide radical has only limited reactivity. Its main importance is to participate in the formation of the very active hydroxyl radical and peroxynitrite. Hydrogen peroxide is also a poor oxidizing agent, but it penetrates cell membranes easily. Superoxide and hydrogen peroxide can react in the presence of iron or copper ions to give the hydroxyl radical via the Haber–Weiss reaction:

$$\bullet O_2^- + H_2O_2 \rightarrow \bullet OH + OH^- + O_2 \qquad (1)$$

In the cells hydrogen peroxide yields hydroxyl radicals by reaction with metal ions through the Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^- \qquad (2)$$

In fact, the damage to cells does not occur from the reactivity of superoxide or hydrogen peroxide, but rather from the hydroxyl radical. In this context it is obviously important that iron or copper ions are available.

•NO is synthesized by various forms of nitric oxide synthetase that catalyse the conversion of arginine to citrulline and •NO (Bredt and Snyder, 1994). Neurons produce •NO by means of a calcium-activated enzyme, often linked to the NMDA receptor, whereas glial cells synthesize •NO in a calcium-independent manner. The latter enzyme is induced by pathological conditions (Bolaños and Almeida, 2006). •NO itself is not a very strong oxidizing agent, but together with superoxide radicals it will produce the toxic peroxynitrite (Beckman *et al*., 1990). The mechanism of •NO neurotoxicity is not yet fully understood. Peroxynitrite will interfere with a key enzyme in the TCA cycle and cause energy deficiency. Mitochondrial complexes II–III and IV seem at present to be the main target for peroxynitrite (Almeida *et al*., 1998).

The hydroxyl radical is very reactive and will react with DNA, membrane lipids and carbohydrates. In DNA it will react with deoxyribose, oxidize purine and pyrimidine bases and cause strand breakage. In membranes it will react particularly with the polyunsaturated fatty acids. It will form lipid radicals, which will react with oxygen to form peroxyl radicals and this can start a cascade of reactions (Halliwell, 2007).

Oxidative stress is involved in brain damage often caused by excitotoxic mechanisms. Examples are the toxic mechanism of methylmercury (Sarafian and Verity, 1991), 3-nitropropionate (Dawson *et al*., 1995), 2-chloropropionate (Sturgess *et al*., 2000), polychlorinated biphenyls (PCBs) and brominated flame retardants (BFRs) (Mariussen and Fonnum, 2006), 1-methyl-4-phenyl-1,2,3,5-tetrahydropyridine (MPTP), X-rays, toluene and glucocorticoid treatment (LeBel and Bondy, 1991). Oxidative stress is probably also involved in Alzheimer's, Parkinson's and Huntington's diseases, Friedrich's ataxia and amyotrophic lateral sclerosis (Butterfield, 2006; Kwong *et al*., 2007). Oxidative stress can be attenuated by enzymes and antioxidants. Spin traps such as α-phenyl-*N*-*t*-butylnitrone is a widely used agent to trap radicals *in vitro* (Carney *et al*., 1991). In cell-culture experiments and in animal models, the oxidative stress can be modified by antioxidants like *Ginkgo biloba*, but it is rare to find good evidence of antioxidants in human brain disease (Halliwell, 2007). In principle, iron chelating agents should be very effective in preventing oxidative damage. So far, desferol has shown great potency in cardiac disease, but it passes the BBB only with difficulty (Gassen and Youdim, 1997).

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4.1 Effects on DNA

Neurons, which are postmitotic cells, depend on an active DNA repair mechanisms in order to survive. This is consistent with the fact that extensive neurodegeneration is found in people with the rare autosomal recessive photosensitive disorders xeroderma pigmentosum and Cockayne's syndrome, which are associated with a defect in nucleotide excision repair (Kohji *et al*., 1998; Rapin *et al*., 2000). It is also interesting that permeabilized neurons exposed to a methylating agent (*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; MNNG) and glutamate, increase the number of single-strand breaks by two- to fivefold and double-strand breaks of DNA by two- to sixfold (Rao, 2007). DNA repair mechanisms play an important role in preventing oxidative stress damage. 8-Oxo-7,8-dihydro-2′-deoxyguanosine (oxo8dG) is one of the most common adducts formed as a result of the action of ROS on DNA. This adduct is repaired by an active mechanism of base excision repair (BER) also involving specific *N*-glycosylases, DNA polymerases and DNA ligases. Oxo8dG is removed from DNA by a bifunctional glycolyase/AP lyase enzyme, termed 8-oxoguanine glycosylase 1 (OGG1) (Liu *et al*., 1996). Oxo8dG is the result of several toxic compounds and found in a series of neurological diseases. A less successful repair of this and other oxidative lesions in DNA will have severe consequences.

DNA damage to mammalian cells, including cerebellar granule cells, initiates transient phosphorylation cascades, which modify the activities of proteins regulating both apoptosis and repair. In these cases the production of DNA damage or inhibition of repair commits the damaged neurons to apoptosis (Sastry and Rao, 2000). In the following, the role of DNA damage in the selective toxicity of foreign compounds to granule cells in the cerebellum is shown as an example.

4.1.1 Methylazoxymethanol (MAM)

MAM is an alkylating agent which may be metabolized to diazomethane and subsequently methylates nucleotide bases. It has been extensively been used as a scientific tool and is found as one of the constituents of cycasine from Guam. A single administration of MAM to a pregnant rat during foetal development induces a permanent atrophy of the brain structures formed at that time. Treatment of rats at gestational day 15 has the most dramatic effect since this is associated with the most rapid growth in the brain (Johnston *et al*., 1981). Postnatal treatment with MAM affects the cerebellar granular cells. The repair enzyme for methylated bases is O^6-methylguanine-DNA methyltransferase, which is less active in the brain than in other organs.

4.1.2 Trichlorfon and Dichlorvos

Trichlorfon is an organophosphonate widely used in the treatment of ecto- and endoparasites, and in the aquaculture industry to control salmon lice. In human medicine it is used against the trematode *Schistosoma haematobium*. Trichlorfon is spontaneously converted to dichlorvos, which is responsible for its anticholinesterase action. It was early discovered that when sows were treated late in gestation with trichlorfon, the piglets showed severe locomotion disturbances, including ataxia (Berge *et al*., 1987). The toxic effect has been reproduced in guinea pigs, but not in rats. When pregnant sows or guinea pigs were exposed to trichlorfon or dichlorvos, the cerebellum, medulla and hypothalamus were the brain structures most heavily affected. Histological examination showed degeneration of dividing cerebellar granular cells (Mehl *et al*., 2007). The lesion was neither due to anticholinesterase activity nor to an effect on neuropathic esterase, since other inhibitors of these enzymes do not produce such effects. It was suggested that dichlorvos and probably trichlorphon alkylates DNA and inhibits the DNA repair enzyme O^6-methylguanine-DNA methyltransferase (Mehl *et al*., 1994).

5 AXONOPATHY

Axonopathy is used to describe a lesion in which the primary site is the axon itself. During axonopathy the axon and the nerve terminals degenerate, often together with the myelin surrounding the axon, whereas the cell body is left intact. This is caused by an insufficient amount of proteins transported to the axons and the terminals, and therefore impaired function and eventually neuropathy will follow. Also the retrograde transport, which sends information from the terminals to the cell body, is probably essential for the cell function. The different forms of transport seem to be dependent on the proper functioning of microtubules and neurofilaments. There is a large number of compounds which can cause axonopathy, including acrylamide, carbon disulfide, chlordecane, colchicine, disulfiram, hexane, 3,3-iminodipropionitrile, organophosphate insecticides, taxol and vincristine (Spencer and Schaumberg, 1980).

6 DISTAL AXONOPATHY

6.1 Hexane Metabolites

Hexane has been used as a solvent in the leather and graphic industries. It was recognized early that daily exposure to this solvent could be accompanied

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by sensorimotor distal axonopathy. Histologically, the distal part of the axon shows massive swellings of the neurofilament, often proximal to the nodes of Ranvier. This results in marked distortions of the axons including retraction of the myelin.

The toxic mechanism of hexane has been investigated using a large number of hexane metabolites. The mechanism involves the oxidation of hexane to 2-hexanol, 2-hexanone and finally to 2,5-hexandione, which is the toxic agent (Krasavage *et al*., 1980). Particularly important was the finding that 2,5-hexanedione forms crosslinks with amino groups of the amino acid lysine and thereby forms a pyrrole (Graham *et al*., 1991). 3,3-Dimethyl-2,5-hexanedione, 2,3-hexandione and 2,4-hexandione cannot form pyrroles with lysine for steric reasons and are consequently not toxic. In contrast, 3,4-dimethyl-2,5-hexanedione, which forms a pyrrole rapidly, is toxic. 3-Acetyl-2,5-hexanedione, which also forms a pyrrole readily, but does not allow the pyrrole group to oxidize, is not neurotoxic. Thus pyrrole formation is not enough to produce the neurofilamentous swelling observed during hexane exposure. Pyrrole oxidation, followed by nucleophilic attack and neurofilament crosslinkage, seems to be necessary for the development of axonopathy (St. Clair *et al*., 1988). In both humans and experimental animals, the loss of sensory impulses begins distally and then, on continuous exposure, progresses proximally.

6.2 Carbon Disulfide

Carbon disulfide has been used in the rubber and viscose rayon industry. Exposure to high concentrations of carbon disulfide causes effects both on the nervous system and on the cardiovascular system. The pathology of the axon caused by carbon disulfide exposure is identical to that caused by hexane. The sequence of events begins with the initial reaction between carbon disulfide and lysine residues on proteins to form a dithiocarbamate (Lam and DeStefano, 1986). The dithiocarbamate protein adduct subsequently undergoes decomposition to isothiocyanate adducts. The isothiocyanate–protein adduct can crosslink to other proteins (Amarnath *et al*., 1991).

6.3 Acrylamide

Acrylamide is used as a precursor for polyacrylamide, which is found in building material as well as laboratory equipment. The toxic agent is acrylamide itself, whereas polyacrylamide is apparently nontoxic. The axonopathy induced by acrylamide begins with distal degeneration of the axon and then progresses proximally if the exposure continues. The axonopathy is characterized by multifocal paranodal swellings of distal parts of the axons, both in the peripheral and the central nervous system. The swellings contain neurofilaments, fragments of smooth endoplasmatic reticulum and degenerating mitochondria. In the peripheral nervous system, the tibial, plantar and sural nerves are most often affected. Acrylamide axonopathy is reversible with time, but full recovery depends on the severity of the intoxication (Smith and Oehme, 1991). In the CNS, acrylamide causes the loss of Purkinje cells and of nerve cell volume in the cerebral cortex (Cavanagh, 1982). The potential of acrylamide to cause developmental neurotoxicity has been tested in rats and the conclusion is that the offspring are not affected at lower doses than the mother. The mechanism of action is controversial and at least three different explanations have been offered. LoPachin (2005) and coworkers suggest that the lesions arise in the nerve terminal. Based on longitudinal silver stain they have shown progressive degeneration of nerve terminals in all CNS regions and in the Purkinje cells after acrylamide exposure. They therefore conclude that the axon degeneration is secondary to the degeneration of the nerve terminal. On the other hand, Sickles *et al*. (2002) suggest that the toxic effect is linked to fast anterograde and retrograde transport. They found that vesicle transport, as a measure of fast transport, is inhibited in animals exposed to acrylamide. They therefore proposed that acrylamide reacts with the motor of the fast axonal flow, namely the protein kinesin. Due to the wide reactivity of acrylamide, they did not exclude that other proteins involved in axonal transport are also affected. Sabri and Spencer (2002) agreed with the suggestion that fast axonal flow is affected, but maintain that energy perturbation is involved. This is based on their early finding that glyceraldehyde dehydrogenase is inhibited by acrylamide.

6.4 Organophosphorus Compounds

The toxic organophosphorus compounds which cause axonopathy after exposure are triorthocresylphosphate (TOCP), which is used in machine oil, and a series of earlier used insecticides. The degeneration of axons does not occur immediately after exposure, but is usually delayed by 7–10 days. The effect can only readily be seen in hens and humans. Major outbreaks of axonopathy occurred during prohibition in the USA when a popular drink (Ginger Jake) was contaminated with TOCP. Another outbreak was in Morocco when olive oil for consumption was contaminated with TOCP. Altogether 40 000 human cases of paralysis have occurred after exposure to organophosphorus compounds such as TOCP and several different herbicides or cotton defoliants (Johnson, 1982). TOCP is metabolized to 2-(*o*-cresyl)-4,4-1,2,3-benzodioxaphosphorin-2-oxide, which will phosphorylate an esterase called neuropathy

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target esterase (NTE). It was earlier established that the enzyme possessed phenyl valerate esterase activity in the presence of paraoxon and was inhibited by mipafox (Johnson, 1982). For a toxic effect to appear *in vivo*, more than 70% of NTE must be inhibited and the inhibited NTE must further be dealkylated (i.e. loss of an alkyl group). The enzyme has now been purified and the mechanism revealed (Johnson and Glynn, 1995). NTE reacts with those organophosphates that cause paralysis with swelling and degeneration of distal parts of long nerves in the legs and spinal cord. Cloning of NTE complementary DNA allowed the generation of constitutive and brain-specific NTE-null mice; the former die by midgestation, whereas the latter may display age-dependent neurodegeneration. NTE is not required by dividing cells, but is needed for survival of postmitotic cells such as placental secondary giant cells and brain neurons. NTE is localized to the cytoplasmic face of the endoplasmic reticulum (ER) and catalyses the deacylation of ER-membrane phosphatidylcholine to soluble products: glycerophosphocholine and fatty acids. Phosphatidylcholine is the major phospholipid of eukaryotic cell membranes. When NTE is inhibited by organophosphorus agents it probably disrupts membrane phospholipid homeostasis and ER functions. This will affect axonal transport and glial–axonal interaction. The distal parts of long axons will be particularly vulnerable to loss of these support functions (Glynn, 2006).

The identification of NTE has allowed the relationship between the enzyme and the delayed neuropathy to be studied with series of organophosphorus compounds (Johnson, 1982). The best inhibitors of NTE are alkyloctylphosphorofluoridates and dialkylphosphorofluoridates. The best inhibitors are alkyl phosphates with alkyl groups 12–14 carbon atoms (Wu and Casida, 1995). When one of these compounds, ethyl octylphosphorofluoridate, was administered to mice, which are insensitive to delayed neuropathy, the animals developed ataxia, paralysis and died within one to three days.

7 PROXIMAL AXONOPATHY

3,3′-Iminodipropionitrile is a neurotoxic nitrile affecting both the peripheral and central nervous system and produces a proximal axonopathy (Griffin and Price, 1980; Llorens *et al*., 1993). This axonopathy is characterized by neurofilament-filled swellings of the proximal part of the axon. This damages the transport of neurofilaments, but not the axonal transport of actin and tubulin (Griffin *et al*., 1983). 3,3-Iminodipropionitrile is also responsible for the 'waltzing syndrome' which manifests itself as choreiform and circling movements. This has been assumed to be due to impaired function of and damage to the vestibular hair cells (Llorens *et al*., 1993). Another compound, which in high concentrations also leads to a block of neurofilament transport proximally, is 3,4-dimethyl-2,5-hexanedione. As already discussed, this hexanedione rapidly forms a pyrrole and also allows oxidation of the pyrrole. It has been suggested that the position of the filamentous swelling along the axon reflects the rate of the reaction (Anthony *et al*., 1983). Hexanedione will therefore block filaments at the proximal part of the axon. At lower concentrations it leads to more distal axonopathy.

7.1 Cytoskeletal-Active Compounds

The microtubules that are present in the axons play an important part in axonal transport. Both vincristine and colchicine, which bind to tubulin and inhibit the formation of microtubules, have been found to cause peripheral neuropathy. Taxol, which also leads to axonopathy, stabilizes the polymerized form of tubules and prevents the dissociation of microtubules into subunits. It is interesting that both the inhibition of formation and the dissociation of microtubules cause axonopathy (Hanson and Edström, 1978). All three compounds are in use clinically and have caused neuropathy in humans.

When colchicine and later other microtubule poisons, such as the *Vinca* alkaloids were given intracerebroventricular, they specifically caused degeneration of the hippocampal granule cells (Goldschmidt and Steward, 1980; 1989). Such administration of colchicine produces cognitive impairment due to the degeneration of hippocampal neurons. Intracerebroventricular colchicine injection resulted in free-radical generation characterized by alterations in oxidative stress markers with a significant increase in malondialdehyde (MDA) and nitrite levels and depletion of reduced GSH activity in the rat brains (Veerendra Kumar and Gupta, 2002). When colchicine was stereotactically injected into the brain, it destroyed several different cholinergic neurons (Fonnum and Contestabile, 1984).

8 MYELINOPATHIES

Loss of myelin is accompanied by slower conduction in the axons and also in aberrant conduction of impulses between adjacent processes (Rasminsky, 1980). The effect of toxic compounds either result in segmental demyelination or in intramyelinic oedema (**Table 2**). Demyelination is often accompanied by axonopathy.

8.1 Hexachlorophene

Hexachlorophene was used to treat newborn infants to avoid staphylococcal infections (Mulick, 1973). The compound penetrated the skin and resulted in

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Table 2 Compounds causing myelinopathy

Agent	Effects
Diphtheria toxin	Segmented demyelination
Hexachlorophene	Intramyelinic oedema CNS and PNS
Tellurium	Demyelination
Triethyltin	Intramyelinic oedema CNS
Lead	Intramyelinic oedema PNS

intramyelinic oedema. Hexachlorophene has experimentally been shown to bind tightly to cell membranes and destroy ion gradients across membranes. It also uncouples mitochondrial activity and reduces the production of ATP. Both effects could be responsible for development of the oedema (Towfighi, 1980). The high pressure from the intramyelinic oedema may injure the axon and lead to axonal degeneration.

8.2 Lead

Man has been exposed to lead, through lead moulding, soldering and through lead pipes, for centuries. In some areas the consumption of lead pigments in paint caused lead poisoning in children. In this connection there have been many reports showing a correlation between increased brain lead level and decreased school performance (Needleman and Gatsonis, 1990). In experimental animals, lead exposure leads to demyelination. In humans the picture is less clear. Children seem to be more susceptible to lead encephalopathy than adults. In children lead exposure can result in severe brain oedema, probably due to damage to the endothelial cells (Johnston and Goldstein, 1998). Children exposed to lead are, however, often exposed to other environmental toxins.

In humans demyelination is less well developed, but axonopathy is often found. One often finds, however, decreased conductance in peripheral axons, which indicate demyelination. The effect of lead exposure is predominantly localized to the motor axons (Chang, 1990).

8.3 Triethyltin

In 1950s a number of severe cases of triethyltin poisoning occurred after exposure to Stalinon, a preparation used for treatment of acne. Triethyltin is readily absorbed through the skin, and the compound caused intramyelinic oedema in the CNS and consequently a rapid increased intracranial pressure (Krigman and Silverman, 1984; Chang, 1990). In experimental animals, there was first weakness of the hind legs followed by paralysis and later tremor and convulsions. Of the alkyltins, only triethyltin produces an effect on myelin.

8.4 Diphtheria Toxin

Diptheria toxin comes from the bacteria *Corynebacterium diphtheriae*, which is transported via the blood to the PNS where it passes into the parenchyma. It inhibits the synthesis of the proteolipid of myelin and the synthesis of the basic protein of the Schwann cell, and thus causes segmented demyelination both in man and in experimental animals. The lesion appears to be most extensive in spinal nerves and dorsal root ganglia (McDonald and Kocen, 1984).

9 ION-CHANNEL NEUROTOXINS

Ion-channel neurotoxins are widely distributed in nature and offer important protection for prey. The ion-channel toxins generally target voltage-activated channels. In this review we will focus on a few important toxins acting on the voltage-activated sodium channel, although toxins acting on potassium channels and calcium channels are also found. The sodium channel is highly selective for small univalent cations ($Na^+ = Li^+ > K^+ > Rb^+ > Cs^+$) and has relatively small functional diversification, compared to other ion channels. Nevertheless, small differences have been noted. The voltage-activated sodium channel is a tetrameric protein (230 kDa) with an ion pore, a selectivity filter and two gates. One of the gates is closed at the resting membrane potential and opens rapidly during depolarization (m-gate). The other is open at the resting potential and closes slowly during depolarization (h-gate). The combined action of these gates allow for the generation of rapid 'spikes', the so-called action potentials.

There are five different binding sites for sodium-channel toxins (**Figure 4**). Tetrodotoxin, which is isolated from the ovaries and liver of the puffer fish (*Tetraodan*), is one of the most toxic low-molecular-weight compounds known. Saxitoxin is produced by several dinoflagellates, and may also be found in shellfish. Tetrodotoxin and saxitoxin block the ion transport by direct blockage of the ion port outside the so-called selectivity filter. Another class of toxins includes batrachotoxin, dichlorodiphenyltrichloroethane (DDT), pyrethroids, veratridine, aconitine and grayanotoxin. They are hydrophobic and facilitate opening of the m-gate and stabilize the h-gate in the open position thereby preventing or slowing down repolarization of excitable cells. Batrachotoxin is derived from the skin of a south or central American frog and is the most selective and potent toxin of sodium channels. Aconitine

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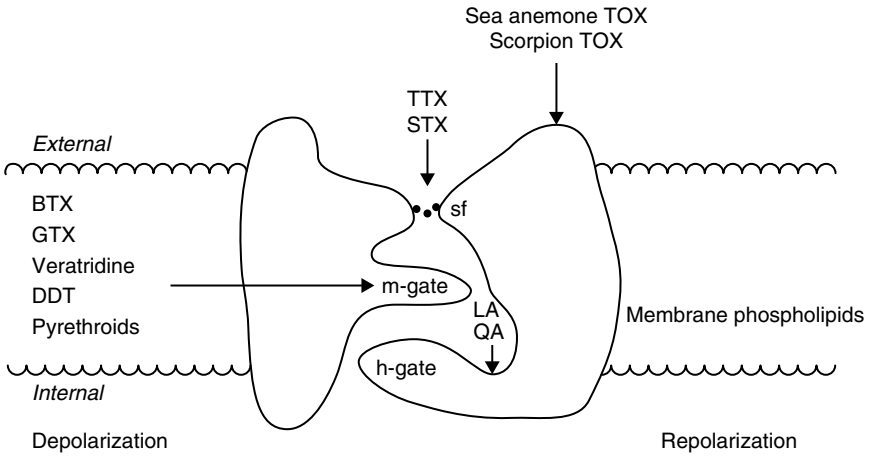

Figure 4 The sodium ion channel with its selectively filter and h- and m-gates. The binding sites for tetrodotoxin (TTX), saxitoxin (STX), scorpion and sea anemone toxins (TOX), batrachotoxin (BTX) and LA (local anaesthetic) are shown. GTX: grayanotoxin; DDT: dichlorodiphenyltrichloroethane; QA: quaternary ammonium salts; sf: selectivity filter.

is a plant alkaloid and grayanotoxin, which is composed of a group of three diterpenoids, is also obtained from plants.

DDT and pyrethroids are important pesticides which act on the sodium channel. Pyrethroids were originally extracted from the flowers of *Chrysanthemum*, but a large number are now synthesized. The pyrethroids are usually separated in two groups based on their chemical structure. Group 1 pyrethroids include the conventional compounds without a cyanogroup, and Group 2 include several compounds which contain a cyanogroup. DDT and pyrethroids of Group 1 will cause repetitive firing of the neuron, whereas pyrethroids of Group 2 do not cause repetitive firing. Instead, Group 2 pyrethroids cause gradual depolarization of the membrane and thereby block impulse conduction (Narahashi *et al*., 1994; 2007).

The third group of toxins is derived from some African scorpions and sea anemones. Their lethal action derives mainly from secretion of neurotransmitters which cause arrhythmia in the heart and repetitive firing and depolarization in nerves. The toxins from scorpions and sea anemones have different chemical structures, but seem to bind to the same site outside the sodium channel. The binding of these toxins is voltage dependent (Catterall, 1995).

10 TOXINS ACTING ON THE NERVE TERMINAL

10.1 Clostridium Neurotoxins

Tetanus toxin and botulinum toxins A, B, C, D, E, F and G are proteins produced by the bacterium *Clostridium basili*. All the toxins recognize a receptor on the motor endplate or the preterminal axon, and are taken up by receptor-mediated endocytosis. The tetanus toxin acts on the inhibitory neurons in the spinal cord, whereas the botulinum toxins target the cholinergic endplates. They all have a similar chemical structure and all block the release of neurotransmitters. The toxins consist of two peptides which are linked together by an S–S bridge. The heavy peptide chain recognizes the cell to be attacked. The botulinum toxins are split after being taken up into the terminal (Simpson, 1990). The the isoforms A, B and C enter neurons by binding to the synaptic vesicle protein SV2 (Dong *et al*., 2006). Membrane translocation of the toxin triggered by acidification of the vesicular lumen is followed by structural rearrangement of the toxin. The light peptide chain has a zinc-dependent endoprotease activity and is released into the cytoplasm. The proteolytic activity is directed against specific proteins involved in transmitter release. Botulinum toxins A and E cleave SNAP-25 (soluble NSF attachment protein), B, D, F and G cleave VAMP (vesicle-associated membrane protein) and C cleaves syntaxin. These peptides are part of the SNARE complex, which is essential for vesicle fusion to presynaptic membranes and transmitter release. The effect of botulinum toxins can last for a long period—up to 30 days. Botulinum toxins do not lead to neuronal cell death. When the toxins are removed and *de novo* synthesis has occurred the cells regenerate their function (Rossetto and Montecucco, 2008).

The tetanus toxin is transported retrogradely to the cell bodies in the spinal cord, where it crosses the synaptic cleft and can be taken up into inhibitory nerves by a similar mechanism as described for the botulinum toxin. In the inhibitory terminals it inhibits transmitter release by cleavage of VAMP. As a result, inhibitory feedback regulation is lost, leading to overexcitation of the neuron and muscle cramps (Turton *et al*., 2002).

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10.2 α-Latrotoxin

Black widow spider venom potentiates release of neurotransmitters from nerve terminals. The active component is a protein called α-latrotoxin. It depletes the terminal of small, clear synaptic vesicles, but does not alter the content of dense core vesicles. α-Latrotoxin probably binds with high affinity to neurexin 1-α, a specific protein in the nerve terminals (Ushkaryov *et al*., 2008).

10.3 Convulsants

Several convulsants in the CNS act on the GABA synapse, either as inhibitors of GABA synthesis and release, or by blocking the chloride channel of the GABA receptor. GABA is synthesized by the pyridoxal phosphate-dependent enzyme glutamate decarboxylase. Compounds which bind to pyridoxal phosphate such as isoniazid, semicarbazide and thiosemicarbazide, or compounds which inhibit glutamate decarboxylase directly, such as allylglycine and 3-mercaptopropionate, decrease GABA and cause convulsions (Abe and Matsuda, 1979).

Several other convulsants interact with the $GABA_A$ receptor. This receptor complex has three distinct binding sites: the GABA binding site, the benzodiazepine binding site and the picrotoxin binding site (**Figure 5**). Compounds which react with the GABA receptor are anxiolytic and convulsant β-carbolines, barbiturates, some anaesthetics, such as alfoxalone, and some anthelmintics and insecticides, such as avermectin and lindane. The binding of GABA to the receptor opens the ion channel and thereby increases the chloride conductance; the binding of benzodiazepine increases the probability of the opening of the chloride channel, whereas the binding of picrotoxin blocks the opening of the channel. The sedative barbiturates work by prolonging the opening time of the chloride channel. Other convulsants, such as the bicyclophosphate esters, penicillin G, and various important insecticides like aldrin, lindane, endosulfan, dieldrin and avermectin, bind at or close to the picrotoxin site and modify the opening of the chloride channel (Sieghart, 1992; Vergnes *et al*., 2000). Bicuculline, a GABA antagonist, is also a convulsant. β-Carbolines are a group of compounds which bind at or close to the benzodiazepine site. Unlike the benzodiazepines they are, however, convulsants and are inverse agonists of benzodiazepines (Lumis and Martin, 1992).

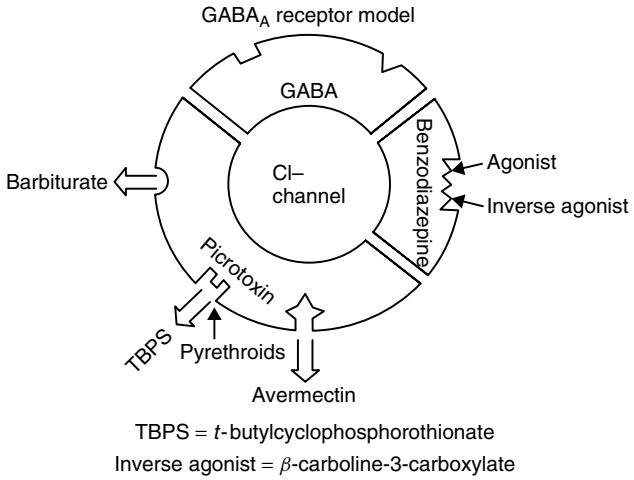

Figure 5 $GABA_a$ receptors have three main sites that interact with each other. They are the GABA site, the bezoxydiazepine site and the picrotoxin site. Substances modify the chloride channel of the GABA receptor by interacting with these three main sites.

11 CHEMICAL LESIONS OF SPECIFIC NEURONS

A series of specific compounds which can selectively destroy groups of neurons, have been developed. Several of these are used mainly for research purposes and they do not pass the BBB, but must be injected intracerebrally. Stereotactic injections of ibotenate, quinolinate and kainate have been used to obtain destruction of cell bodies, while sparing axons passing the sites. Subcutaneous administration of glutamate has been used to destroy specific cell groups before the BBB is fully established (Karlsen and Fonnum, 1976).

During ageing, there is a loss of catecholaminergic neurons resulting from their auto-oxidation. In extreme cases this results in Parkinson's disease. The metabolism of catecholamine involves monoamine oxidase, yielding H_2O_2 as a by-product. H_2O_2 will either give a superoxide radical from a dismutase reaction or a hydroxyl radical by the Fenton reaction. Both radicals are important for cell destruction, as discussed previously.

6-Hydroxydopamine is an analogue of dopamine which is readily taken up into catecholaminergic terminals. When administrated systemically it will be taken up and subsequently undergoes auto-oxidation in sympathetic neurons. The result is therefore a selective destruction of the sympathetic innervation (Malmfors and Sachs, 1968). 6-Hydroxydopamine does not penetrate the BBB, but when administrated stereotactically, it will destroy catecholaminergic cells in the substantia nigra.

Another compound which selectively destroys catecholaminergic structures is MPTP. MPTP was synthesized as a by-product from the production of the illegal opiate analogue meperidine. Within hours to days after consumption it results in parkinsonian-like symptoms (Davis *et al*., 1979). MPTP is uncharged and therefore passes readily through the BBB and is taken up into

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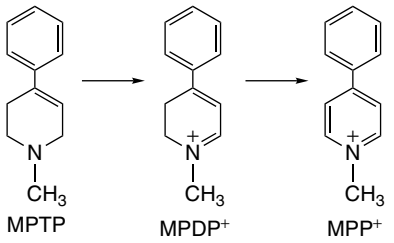

Figure 6 MPTP is taken up across the BBB and then diffuses into astrocytes. In astrocytes it is oxidized by monoamine oxidase B to the methyl phenyldihydropyridinium ion ($MPDP^+$) and finally to the methyl phenylpyridinium ion (MPP^+). This is taken up into dopaminergic cells and inhibits the mitochondrial activity.

astrocytes, where it is converted by monoamine oxidase B to the methyl phenyldihydropyridinium ion ($MPDP^+$) and further to the methyl phenylpyridinium ion (MPP^+) (**Figure 6**). The toxic effect of MPTP is to a large extent blocked by the monoamine oxidase B inhibitor deprenyl. MPP^+ is released from the astrocytes into the extracellular space before a toxic concentration is reached within the glial cells (Wu *et al*., 1992). It is then taken up into the dopaminergic cells by the same uptake mechanism as for dopamine (Javitch *et al*., 1995). The toxic MPP^+ is therefore accumulated and trapped within these cells, and makes them a target for its toxic action. It has also been shown that MPP^+ can be formed from MPTP, catalysed by iron. MPP^+ inhibits oxidative phosphorylation and kills the cells. MPTP is an example of a compound that is much more toxic to monkeys and man than to rodents (Nagatsu and Sawada, 2006). The findings of the toxic effects of MPTP and 6-hydroxydopamine have opened up the possibility that environmental toxins may play a role in development of Parkinson's disease.

5,6-Dihydroxytryptamine and its 5,7-isomer are taken up into serotonergic cells by the transport system for serotonin. Since there are few serotonergic structures peripherally, they are usually administrated stereotactically into the brain, and act in a manner similar to that of 6-hydroxydopamine (Bjørklund *et al*., 1974).

Reports from northern China indicate that 3-nitropropionic acid (3-NPA) is also likely to be responsible for the development of putaminal necrosis, with delayed dystonia in children, after ingestion of mildewed sugar cane. The compound inhibits succinic dehydrogenase, activates palpain and causes massive oxidative stress. The effect is degeneration in the striatum and the compound is suggested as a model for Huntington's chorea. The effect in the striatum cannot fully be explained by inhibition of succinic dehydrogenase because the enzyme is equally inhibited in other brain regions. It is therefore believed that the neurotransmitter activities in the striatum make this a more vulnerable part of the brain (Brouillet *et al*., 2005).

AF64A (ethylcholine mustard aziridinium ion) is a choline analogue, and this has been used specifically to destroy cholinergic cells. This selectivity of this compound is a result of selective uptake into cholinergic cells and its subsequent reaction with the protein synthesis system (Fisher and Hanin, 1986). The specificity of the compound, however, has been criticized (Villani *et al*., 1986).

Intraperitonal injection of 3-acetylpyridine has been found to cause selective degeneration in the inferior olive nucleus and hypoglossus nucleus (Desclin and Escubi, 1974). The specificity is believed to depend on the specific uptake into certain cell types of acetylpyridine, which acts as an antimetabolite. More detailed studies have shown loss of cells in other brain structures, particularly the hippocampal region (Balaban, 1985).

Trimethyltin (TMT) has been used as a stabilizer of plastic and as a pesticide. After systemic administration it gives relatively specific lesions to the pyramidal cells in the hippocampal region (Naalsund *et al*., 1985). TMT has been suggested to act as a mitochondrial uncoupler blocking mitochondrial ATPase. This will lead to oxidative stress. Recently, a peptide, termed stannin, has been specifically localized to TMT-sensitive cells, and blocking stanin synthesis in primary neuronal cultures with an antisense directed against stannin messenger RNA has been reported to protect neurons from TMT toxicity (Toggas *et al*., 1993). It therefore seems possible that the binding of TMT to stannin initiates the toxic reaction. In addition, tumour necrosis factor and other compounds seem to be involved in the toxic mechanism (Reese *et al*., 2005).

12 ENVIRONMENTAL TOXINS

12.1 Polychlorinated Biphenyls

PCBs are a group containing 209 different congeners. The compounds were, until 1977, used in electrical insulation, fire prevention and as additives to paints and building material, but have since been forbidden in most countries. PCBs are stable and lipophilic compounds, and therefore they are biomagnified in the food chain. Top predators such as polar bears, seals and seagulls contain high concentration of PCBs. The highest level of PCBs in brain was found in dead polar seagulls from Svalbard and ranged from 0.9–29.5 mg kg^{-1} brain wet weight (Gabrielsen *et al*., 1995). The neurotoxicological significance of PCBs is well documented after several incidents of accidental exposure. In 1968, more than 1000 people in the Kyushu province in Japan were exposed to cooking oil containing several different chlorinated aromatic chemicals, including PCBs. Symptoms were neurological abnormalities. About 10 years later, a similar incident took place in Taiwan. Some

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of the children in the exposed groups had problems in neurological development and speech (Mariussen and Fonnum, 2006). In the United States there have been two studies of children in Michigan and in North Carolina, respectively, where they found a correlation between the PCB level and poor performance in behavioural tests (Jacobson and Jacobson, 1996). This indicated that exposure to PCB mixtures during development affects neurological development and impairs cognitive function. Developmental neurotoxicity of PCBs has been confirmed in several laboratory animal species, including the nonhuman primates. In young animals there is an effect on learning and memory, in adult animals there is an effect on motor behaviour (for a review see Kodavanti and Tilson, 1997; Mariussen and Fonnum, 2006).

During the last 20 years there has been an attempt to understand the cellular bases of PCB-induced behavioural and neurological effects in animal models. PCBs are divided into two groups based on their ortho substitution. PCBs that have no ortho substitution will have a planar configuration and a high affinity for an aryl hydrocarbon (Ah) receptor, like other planar polychlorinated compounds, including 2, 3, 7, 8-tetrachlorodibenzodioxin (TCDD). Other PCB congeners with ortho-substituted congeners are nonplanar and have no access to the Ah receptor. These congeners, which dominate in PCB mixtures, are regarded as neurotoxic. The mechanisms behind the neurotoxic effects are not well understood, but ortho-substituted PCB congeners have been reported to act on different parameters of importance for brain function (Mariussen and Fonnum, 2006).

Repeated exposures to PCB doses appear to affect brain dopamine levels, both during development and in adults (Seegal, 1996). Low concentrations of ortho-substituted congeners have been reported to inhibit uptake of neurotransmitters, particularly dopamine, into synaptosomes and synaptic vesicles (Mariussen *et al*., 1999; Mariussen and Fonnum, 2001). Bemis and Seegal (2004) exposed rat-brain synaptomes to PCBs and conclude that the reduction of dopamine in synaptosomes was primarily due to inhibition of the vesicular uptake. This has been confirmed by *in vivo* studies. Seegal *et al*. (2002) showed by microdialysis that extracellular PCBs increased dopamine for the first three days after giving PCBs, as an indication of dopamine synaptosome-transporter inhibition. It decreased thereafter, as evidence of vesicular-transport inhibition. Caudle *et al*. (2006) found a reduction in both the dopamine synaptosome transporter and vesicular transporter after giving rats 7.5 and 15 $mg\,kg^{-1}\,day^{-1}$ of PCBs for 30 days. PCBs are weaker inhibitors of glutamate and GABA transport into synaptosomes (Mariussen and Fonnum, 2001), although an effect of higher molecular weight PCBs cannot be excluded. It has been suggested that the cholinergic receptors could be involved to explain the cognitive and hypoactive behaviour (Eriksson *et al*., 2001). The effects found were small and it should be kept in mind that in the striatum there is an interplay between dopaminergic and cholinergic structures.

Several studies have shown that the ortho-PCBs can induce both apoptotic and necrotic cell death of several neuronal populations, such as cerebellar granule cells, hippocampal cells and mesencephalic cells *in vitro* (Kodavanti *et al*., 1994; Mariussen *et al*., 2002; Lee and Opanashuk, 2004). Mariussen *et al*. (2002) further showed that the cell death could be related to the formation of oxidative stress. Both parameters were reduced by Vitamin E, MK801 (an NMDA receptor blocker), the AMPA receptor agonist NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione), and NAME (*N*-ω-nitro-L-arginine methyl ester, a nitric oxide synthase inhibitor). Lee and Opanashuk (2004) also showed that dopaminergic cells were more susceptible to PCBs than nondopaminergic cells and that protection were offered by antioxidants. The PCB congeners also interfere with the signal transduction system in neuronal cells, namely the production of 1,4,5-inositol trisphosphate and the subsequent mobilization of intracellular calcium. Increased activity of protein kinase C is also reported (Kodavanti *et al*., 1994).

Thyroid hormones and retinol are important regulators during the development of the nervous system. PCBs have been reported to bind competitively to the transport protein for these compounds, and PCBs consequently lower the plasma level of thyroid hormone and retinol. PCBs have also been reported to block the thyroid hormone receptor. Such effects may have severe consequences for the development of the foetus (Ulbrich and Stahlmann, 2004).

12.2 Brominated Flame Retardants (BFRs)

There are several groups of flame retardants (FBRs), but three groups dominate with regard to production volume, namely tetrabromo bisphenyl A (TBBA), polybrominated diphenyl ether (PBDE) and hexabromocyclododecane (HBCD). The total annual production of the three reaches about 200 000 tonnes per year. BFRs are found in textiles, furniture, clothing and electronic equipment. Humans are mostly exposed to BFRs through house dust and food consumption. TBBA is bound chemically to the material, whereas PBDE and HBCD are added to the material. This means that, in principle, TBBA is less readily released into the environment. TBBA is also less lipophilic and probably less important as a neurotoxic agent. From a toxicological point of view PBDE is the most important. PBDEs are structurally similar to PCBs and they consist of 209 congeners. They

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are provided as mixtures named tetraBDE, pentaBDE, hexaBDE and decaBDE after the number of bromo constituents. When males rats were given tetraBDE ($10.5\,mg\,kg^{-1}$), pentaPBE (0.8 and $12.5\,mg\,kg^{-1}$) and hexaPBE (0.45 and $9\,mg\,kg^{-1}$) on days 3, 10 and 19, and were tested after two and four months, they showed permanent aberrations in spontaneous behaviour (Viberg *et al*., 2003; 2004). Rats receiving pentaBDE and hexaBDE also showed affected learning and memory functions when tested in the Morris water maze (Eriksson *et al*., 2001; Viberg *et al*., 2003). They registered a 20% decrease in nicotinic binding sites in the hippocampus in their experiments.

There are several similarities displayed by the FBRs and the PCBs. Mariussen and Fonnum (2003) showed that pentaBDE, HBCD and TBBA inhibited the uptake of dopamine into both synaptic vesicles and into the synaptosomes at low (μM) concentrations similar to findings for PCBs. TBBA also inhibited the transport of other neurotransmitters, such as glutamate and GABA. The three groups, TBBA, HBCD and pentaBDE also induced cell death in cerebellar granule cells at similar concentrations to PCBs. Again, as shown for PCBs, vitamin E and MK 801 protected against cell death indicating intrasynaptosomal increase of calcium and oxidative stress (Reistad and Mariussen, 2005; Reistad *et al*., 2006; 2007). Others also showed that pentaBDE was cytotoxic (Madia *et al*., 2004). Further, pentaBDE and the PCB mixture Arochlor 1254 released arachidonic acid and thereby activated phospholipase A2 (PLA2) from cerebellar granule cells. They also induced protein kinase C (PKC) translocation and disrupted calcium homeostasis in the microsomes (Kodavanti and Ward, 2005; Kodavanti and Derr-Yellin, 2002).

13 MERCURY

Mercury exists in several different forms, such as metallic mercury, HgCl, $HgCl_2$ and methylmercury. The lipophilic methylmercury has been the toxic agent in poisonings in Japan and Iraq. In 1971, in Iraq, 500 people died and 5000 were severely injured after eating seed grain dusted with methylmercury fungicide. The wheat was not intended for eating, but for planting. In some areas the wheat arrived too late and during a famine the seed grain was therefore used for human consumption. Several weeks after consumption, the first symptoms, which were loss of sensation in the skin and blurred vision, appeared. Later severe symptoms such as blindness, deafness, loss of coordination and death occurred. The use of this fungicide is now forbidden. In Minamata in Japan industrial effluent containing mercury was released into the bay and converted by micro-organisms to methylmercury. The methylmercury in the sediment was transferred, via smaller organisms, to fish, which were consumed by the inhabitants (Cranmer *et al*., 1996).

In daily life, humans are most commonly exposed to methylmercury by consumption of contaminated fish and other foodstuffs. There have been three large investigations, in the Seychelles (van Wijngarden *et al*., 2006), New Zealand (Crump *et al*., 1998) and the Faroe islands (Debes *et al*., 2006), concerning the possible side effects to children in populations having a high proportion of fish in their diet. It was found that the benchmark value of methylmercury during pregnancy was 10–12 ppm in maternal hair in these investigations of children (van Wijngarden *et al*., 2006). But other forms of exposure, such as occupational and dental material, should also be kept in mind. Mercury vapour taken up through the lung can combine with cysteine in the blood and be transported over the BBB. The intraoral concentration of mercury is correlated to the number of tooth fillings; mercury is continuously released from tooth fillings.

In general, it can be said that mercury compounds have a wide range of effects, both in the nervous system and in other organs, particularly the kidney. In the brain, methylmercury particularly injures the neurons of the visual cortex and the small granular cells of the cerebellar cortex (Shiraki, 1979). In the cerebellum, larger neurons, such as the Purkinje cells, seem to tolerate methylmercury better than the smaller neurons. In the methylmercury particularly affects the dorsal root ganglion cells (Schiønning *et al*., 1998).

The molecular mechanism of the toxicity of methylmercury is not known. It reacts with protein SH groups and leads to a production of ROS in nervous tissue (Sarafian and Verity, 1991). At an early stage, it has an effect on protein synthesis, either by inhibiting the transport of amino acids across the BBB or by a direct effect on the ribosomes.

14 LESIONS IN ASTROCYTES

The astrocytes play an important role in regulating extracellular fluid, and in the synthesis and metabolism of neurotransmitters. These cells have the ability to take up potassium ions and also buffer the pH of the extracellular fluid. Further, the cells have the ability to take up, glutamate and to a lesser extent GABA, from the extracellular space and convert these compounds to glutamine. The astrocytes, in contrast to neurons, contain pyruvate carboxylase, which is important for the replenishment of the TCA cycle, and glutamine synthetase which converts glutamate to glutamine. Glutamine can be transported back to the neuron and used for replenishment of the TCA cycle (Fonnum and Hassel, 1996).

Fluoroacetate and its toxic metabolite fluorocitrate have received considerable attention as specific inhibitors of glial metabolism and they have been used in several studies to characterize the importance of glial cells (Fonnum *et al*., 1997). Fluoroacetate is the toxic ingredient of the poisonous plant *Dichapetalum* (Peters,

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1963). Fluoroacetate is an example of a nontoxic substance which is converted to a toxic metabolite, fluorocitrate, *in vivo*. The selective gliotoxic action of these two substances is due to the fact that they are preferentially taken up into glial cells only. Further, in the case of fluoroacetate, it is converted by acetyl thiokinase to fluoroacetyl CoA and further to fluorocitrate. Currently, sodium fluoroacetate is licensed in the USA for use against coyotes, which prey on sheep and goats, and in Australia and New Zealand to kill unwanted introduced species.

Fluoroacetate crosses the BBB. It is, however, not brain specific, since it is also toxic to the heart and kidney. Fluorocitrate does not cross the BBB, and has to be injected intracerebrally. The toxic action of fluorocitrate is due to its reversible inhibition of the TCA enzyme aconitase. Ultrastructural investigation shows that four hours after intracerebral injection of fluorocitrate, the astrocytic cytoplasm looks empty, and there is a loss of glycogen. The endoplasmatic reticulum is swollen and fragmented, and the mitochondria look swollen. Biochemically, there is a large loss of glutamine and GSH, and smaller changes in glutamate, aspartate and GABA. The synthesis of glutamine from acetate is completely inhibited (Paulsen *et al*., 1987).

The accumulation of citrate during inhibition of aconitase is believed to be an important part of the toxic mechanism *in vivo*. Citrate will chelate calcium and Bosakowski and Levin (1986) showed a strong negative correlation between the calcium concentration in serum and the toxic effect of fluorotoxins. Calcium administration increased the survival time of rats poisoned with fluoroacetate.

Since fluoroacetate is only useful close to lethal doses, intracerebral injection of fluorocitrate is mainly used in inhibition of glial metabolism. Fluorocitrate and fluoroacetate have both been used in *in vitro* studies of cell culture or brain slices, but fluoroacetate has to be used in 100-fold higher concentrations. Both compounds have also been used in microdialyses (Fonnum *et al*., 1997). As a method for evaluating the glia specificity of the inhibitors, the glial metabolism can be restored with glial-specific substrates, such as isocitrate or 2-oxoglutarate (Fonnum *et al*., 1997).

Methionine sulfoximine is an excellent inhibitor of glutamine synthetase and treatment with this compound is followed first by a decrease in the level of glutamine and later by a decrease in glutamate in the brain. There will also be an increase of ammonia in the brain that will produce pathology in the astrocytes. Animals exposed to high concentrations of methionine sulfoximine develop seizures after a few hours, whereas low doses give pathological-looking astrocytes (van den Berg and van den Velden, 1970). The related buthionine-l-sulfoximine more specifically inhibits GSH formation.

Another compound with a potential as a gliatoxin is D,L-aminoadapic acid. Pedersen and Karlsen (1979) showed that intravitreal injection was accompanied by specific degeneration of Müller cells in the retina. Subsequent work has shown that L-aminoadipic acid is a selective gliatoxin in the brain. The primary mechanism seems to be that aminoadipic acid is a selective substrate of the cystine-glutamate antiporter in glia cells (Pow, 2001). Whether the subsequent effect is due to an effect on protein synthesis (Nishimura *et al*., 2000) or whether it is due to a reduction in GSH (Reichelt *et al*., 1997) is an open question.

Whereas there are several neurotoxic substances, there are only few gliotoxic substances. To some extent it seems that astrocytes can survive for a long time with no or minimal metabolism. Astrocytes also have a more powerful glycolytic activity and a high amount of ATP can be supplied in the absence of the TCA cycle. Further, astrocytes have a higher level of the antioxidant GSH and can therefore resist attack by free radicals to a much larger extent than neurons.

15 CONCLUSION

The effect of toxic compounds on the CNS is often difficult to evaluate due to the complex function of the brain and the specificity of different brain areas. We can therefore expect to find evidence of CNS effects from several toxic compounds in the future. The development stage seems particularly vulnerable.

Both excitotoxicity and free-radical formation may be common toxic mechanisms for a wide variety of toxic substances. Also, an evaluation of the importance of neurotoxic substances and genes in the development of neurological degenerative diseases should be an important research topic for the future.

A group of toxic compounds not dealt with in this review are the acetylcholinesterase inhibitors. They are dealt with under the chapters on pesticides (**Toxicology of Pesticides**) and warfare gases (**Toxicology of Chemical Warfare Agents**).

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The Role of Behavioural Toxicity in Risk Assessment

Bernard Weiss

CONTENTS

1 INTRODUCTION

Over 30 years ago, the United States Congress set into motion the Toxic Substances Control Act (TSCA). It denoted the beginning of a new era in environmental regulation. It called for evaluating the safety of chemicals before commercial production. To the astonishment of many, it acknowledged behaviour as a valid index of environmental health on the same scale as cancer and reproductive disorders. It was included in a workshop held in 1974 to prepare for TSCA by illustrating the variety of methods to be used for such evaluations (Weiss *et al*., 1975).

Behavioural toxicology at that time represented a concept and perspective just emerging in toxicology. Pharmacologists had begun to recognize it in the late 1960s, due to its coupling with behavioural pharmacology, as an independent subject matter (Weiss and Laties, 1969). The first meeting entirely devoted to the discipline had taken place in Rochester in 1972 (Weiss and Laties, 1975). Following TSCA, signed into law in 1976, the discipline, which had hardly passed through infancy at the time, took an accelerated path to maturity.

One source to which we must grant credit is the USSR, whose scientists insisted that functional measures, particularly of central nervous system (CNS) status, provided a much more sensitive and valid criterion of toxicity than pathology, the dominant criterion in the West. Although Soviet dogma and perceived political advantage granted functional measures superior status in the hierarchy of standards, veneration of Pavlovian doctrine also exerted a marked influence. US scientists, comparing generally stricter Soviet with more lenient US exposure standards for many substances, were compelled to ask whether the differences arose from the measures used to determine toxicity.

Behaviour as a criterion of toxicity is now firmly embedded in the toxicological sciences and in regulatory

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directives as well. Most large manufacturers of chemicals and pharmaceuticals now include some form of functional neurotoxicity assessment, largely behavioural, in screening new compounds. The basis for what is considered a 'level of concern' for blood lead, in the US Environmental Protection Agency's (USEPA) phrasing, is how children perform on psychological tests. Debates over 'safe' levels of methylmercury in fish are also based on psychological test performance.

Occupational settings provided another ecological niche in which behavioural toxicology could thrive. The committees that stipulate threshold limit values (TLVs) for workers had long relied on behavioural criteria of excessive exposure, as in their definition of the short-term exposure limit (STEL): 'The maximum concentration to which workers can be exposed for a period of up to 15 minutes continuously without suffering from . . . narcosis of sufficient degree to increase accident proneness, impair self-rescue or materially reduce work efficiency . . . '.

Therapeutic drugs provide still another terrain open to exploration by behavioural questions. One compelling example is cancer chemotherapy. Although oncologists have always been aware of the neurotoxic potential of chemotherapeutic agents, they confronted them in the form of severe reactions such as seizures, disorientation and overt peripheral neuropathies. During the past decade, however, they have learned that patients may suffer much more subtle forms of toxicity, such as cognitive impairment, which patients describe as 'chemobrain', and which have been documented by neuropsychological tests (Weiss, 2008).

The content of this chapter is guided by three aims. First, it seeks to familiarize readers from other disciplines with the terminology and tenets of behavioural toxicology. These tenets channel the way methods are developed and applied; methods cannot be applied in isolation without appreciating the principles supporting their use. A second aim is to discuss the methods themselves, the kinds of information they are designed to yield and the objectives that govern their application. Automated techniques will be featured, whenever possible, because of the unique demands of behavioural testing in the regulatory and risk assessment arenas. Observational techniques are valuable as pointers to functional deficits, but are rarely definitive and are much more difficult to reproduce from one laboratory to another. The third aim is to provide examples from current research in behavioural toxicology to amplify the way in which methods are applied. Laboratory animal testing will be the primary context for these descriptions, although some of the methods, especially those designed for sensory and motor assays, are applicable to or have been derived from human testing.

1.1 Principles and Definitions

Behavioural toxicology rests on a simple but significant premise: in essence, to fully determine how a chemical affects an organism, it is crucial to evaluate how the whole organism functions when exposed to that chemical. Many other approaches occupy special niches and yield important information, of course. *In vitro* systems, such as cell cultures, may provide helpful mechanistic data. Neurochemical research yields information about changes in the chemical milieu of the brain, such as neurotransmitter activity. Morphological assays convey critical information about the structural integrity of the brain. Neurophysiological observations offer insights about the electrical activity that reflects the locus and timing of nervous system events. Advances in imaging technology, such as magnetic resonance imaging (MRI) and positron emission tomography (PET) scans, furnish an even more unique window on the brain's universe. All of these sources of information, however, are designed ultimately to extract answers to one fundamental question: what are the processes and structures that govern the organism's behaviour? For toxicology, the question is transformed: is the organism's ability to function at risk?

The ancillary premise of behavioural toxicology is that the myriad functions that collectively define behaviour can be isolated for study. This principle does not assert that one particular function can be severed from all other functions. It simply means that it can be observed selectively by appropriate investigative techniques, much like a muscle group selectively subjected to stress by someone lifting weights; the muscle group is not detached from the rest of the body. Memory, for example, cannot be isolated from the ability to perform a motor response when reacting to a stimulus, but the form of the response can be specified so that it is minimally confounded with memory function. The degree of isolation depends on the experimenter's aims. Some tests, often designated as apical, call upon many functions at once. Others, such as some sensory tests, are designed as narrow probes of a single function. Narrow or broad, however, behavioural tests are aimed at the total organism and its governing systems. Tests of colour vision span a complex pathway from the eye to the brain and involve multiple sites in the brain itself.

The chapter is organized around different classes of techniques. Although the sequence proceeds approximately from simpler to more complex methods, the progression is not wholly monotonic. Sensory function testing, for example, may embody both relatively simple and relatively complex techniques. The sequence of topics is guided mainly by how an investigator might proceed in asking increasingly deeper and more detailed questions of a chemical. Rarely, however, will one measure or end-point suffice to illuminate fully the nature of a toxic effect on behaviour. Behaviour is

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simply too abundant a source of functions, questions and methods. This chapter provides a survey of the leading methods, some of their findings and descriptions of others that seem to offer noteworthy potential for toxicological questions.

2 REFLEXIVE AND NATURALISTIC TECHNIQUES

Questions about a chemical's neurobehavioural toxicity may be provoked for many reasons. Chemicals already established in use sometimes spur questions because of new regulations, new exposure information, accidents and other situations calling for additional information. In these circumstances, the questions tend to be directed towards specific effects. For new chemicals, information has to be gathered more broadly because of the diverse functions that might be affected. Simple screening tests are typically applied in the early phases of an assessment, and tend to rely on easily observable or measured end points. Many of these fall into the category of reflexive and naturalistic behaviours not requiring extended training or advanced instrumentation. Naturalistic is often used as a synonym for spontaneous, or to denote behaviours not explicitly defined by an experimental procedure. All behaviours are naturalistic, however, to the extent that they fall within the capabilities of the organism under study.

2.1 Functional Observation Batteries

A common element in many arrays of preliminary screening tests is a functional observation battery (FOB), now a standard procedure in most testing protocols, and based primarily on reflexive behaviours. Their role is not new; pharmacologists, testing a new chemical, would typically administer it to rats or mice to observe whatever overt effects it would produce. Such observations would also parallel determination of the LD_{50}, currently out of fashion. FOBs in essence represent a more systematic and more focused scheme for conducting a clinical examination and have been adopted internationally. **Table 1** lists some of the measures typically included in current rodent test batteries.

Table 1 Typical measures examined in functional observation batteries

- Home-cage and handling
- Posture
- Ease of handling
- Piloerection
- Vocalizations
- Open field
- Time to first step
- Urination, defaecation
- Gait
- Bizarre behaviour
- Rearing behaviour
- Reflex and physiological
- Approach response
- Touch response
- Finger snap response
- Righting reflex
- Grip strength
- Catalepsy
- Forelimb grip strength

The limitations of FOBs are not always appreciated. The prime developer of the FOB battery at the USEPA (Moser *et al*., 1995) cautions users against inflated expectations from such procedures. First, their role in the assessment process is to gauge the potential of a chemical to produce neurotoxicity and to suggest which more specific tests, such as those aimed at cognitive function, might be undertaken. They are not designed to identify underlying mechanisms. Second, they are not intended to provide the basis for setting exposure standards, even though, for some chemicals, their sensitivity may achieve parity with more advanced procedures. FOB data can also be used to model dose–response functions (Zhu *et al*., 2005), but like other methods that depend upon observer judgements, FOB observers need to be trained to employ scoring criteria consistently because observational methods are notoriously subject to observer drift in how the nominal criteria are interpreted. In addition, at sites where FOBs are conducted, concordance between observers must be monitored to ensure that all base their scoring on the same criteria. Furthermore, observers should be kept unaware of the identity of the chemical under test; covert bias of which the observer is unaware can easily contaminate a study. Because of the training required, and because the observers themselves need to be observed, FOBs tend to be labour intensive.

A comprehensive neurological examination is even more daunting, and is not suitable for screening or simple hazard identification. The numerous facets of such an examination are described by O'Donoghue (1996) and include many more specific assessments than included in the typical FOB. Such an expanded FOB is designed for other purposes such as securing information needed to identify the locus of damage.

2.2 Naturalistic Behaviours

Animal behaviour observed in a natural or quasinatural setting is the domain of the discipline of ethology. Instead of attempting to isolate specific forms or expressions of behaviour in the laboratory, ethologists undertake to study behaviour in the context of an animal's total

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environment as it appears in nature. Ethology has played a minor role in behavioural toxicology because it requires major investments of observer time and often presents interpretive puzzles in the form of what specific circumstances and events control specific behaviours. Laboratory investigators can select aspects of the behaviours that animals show in natural settings and transfer them to a laboratory setting.

Psychobiologists have studied laboratory analogues of naturalistic behaviours from many vantage points. Such behaviours have undergone an expansion of interest with the emergence of endocrine disruption as an element of neurobehavioural risk assessment. It has promoted the contrivance and use of techniques that might reflect disorders of endocrine function attributable to environmental chemicals. Sexually dimorphic end points are especially responsive to interference with gonadal hormone dysfunction, particularly when exposure occurs during early development, a stage at which the brain undergoes sexual differentiation (Weiss, 2002). Virtually all drugs and chemicals produce sexually dimorphic effects, so that even preliminary screening should be alert to sex differences in response.

2.2.1 Reproductive Behaviours

The major role accorded gonadal hormones in discussions of endocrine disruptors highlights sexual behaviour as a prime end point. Male rat sexual behaviour is of particular interest because of the striking array of chemicals labelled as 'environmental oestrogens'. In the standard assessment of copulatory function, observers record measures such as intromission latency following the introduction of a receptive female into the male's space. Although this is the standard technique, and useful, it suffers from its lack of concordance with the natural ethological setting in which the female has a wide range of opportunities to set the pace of copulation. It measures only male copulatory behaviour in a situation with a restrained female.

Abnormalities such as undescended testes, which can occur following gestational exposure to phthalates and other antiandrogenic chemicals, can cloud the relation between the motivational states underlying reproductive behaviours and endocrine function. To preclude such confounding, investigators can assay sexual motivation rather than the mechanics of copulation. The partner preference paradigm offers such an assay. The partner preference test is based on studies demonstrating that olfactory stimuli govern the appropriate selection of sexual partners in rats, mice, ferrets and other mammalian species. Normal males spend more time near oestrous females, and normal females spend more time near sexually active males. Furthermore, hormonal status plays a critical role in partner preference in both male and female rats (Harding and McGinnis, 2003; Clark *et al*., 2004).

Preference testing can be conducted with a set of three attached enclosures. The test animal is placed in the central enclosure, giving it the opportunity to see and smell the two stimulus animals without contacting them directly. For testing males, one side enclosure will contain a male. The other will contain an ovariectomized stimulus female brought into oestrus by injecting 10 µg per rat of oestradiol benzoate 48 hours and 1.0 µg per rat progesterone 1 hour before testing. Choice is measured by proximity to one or the other side of the central enclosure over a period such as 10 minutes. Gonadectomized males prefer the odour of sexually active males rather than that of receptive females (Xiao *et al*., 2004).

2.2.2 Play Behaviours

'Rough-and-tumble' play, and play fighting in particular, is sexually differentiated. In juveniles from many mammalian species, including humans, males engage in it more frequently than females (e.g. Hines, 2003 and Vreugdenhil *et al*., 2002a,b, in humans; Pellis, 2002 in rats) and the sex differences are determined by perinatal exposure to gonadal hormones (Hotchkiss *et al*., 2002). Males castrated at birth exhibit frequencies of play fighting similar to those seen in their female littermates. Females treated perinatally with androgens exhibit frequencies similar to those of males. In rats, play fighting begins to appear at about 18 days of age and peaks between 30 and 40 days of age. Its frequency wanes subsequently, but does not disappear entirely, and also undergoes changes in form.

Smith *et al*. (1997) describe an established protocol. After weaning at postnatal day (PND) 21, same-sex paired littermates are maintained in regular housing. Before the assigned day, for example, PND 34, each pair is habituated to the test enclosure (50×50 cm in area) for 15 minutes a day for three successive days (e.g. PND 30–33). Each rat is then socially isolated for 24 hours in a standard cage; social isolation increases the frequency of play fighting substantially. On the test day, the two members of each pair are placed in the test chamber and video recorded for 10 minutes with illumination provided by red LED lighting. Play fighting is scored in accordance with the categories below (Pellis and Pellis, 1997; Smith *et al*., 1997):

Nape contact: One animal brings the tip of its snout into contact with or within 1 cm of its partner's body.
Pouncing: One animal lunges towards its partner with forepaws extended.
Pinning: One animal is astride its partner, who is supine, with its forepaws on the partner's ventral surface.
Wrestling: The two animals rolling and tumbling over one another.

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Chasing: One animal rapidly pursues the other.
Boxing: The two animals stand erect on their hind legs with forelegs raised.

Hotchkiss *et al*. (2003) and Colbert *et al*. (2005) found that the antiandrogenic fungicide, vinclozolin, altered play behaviour in male rats exposed during early development.

2.2.3 Aggression

Aggressive behaviours are displayed by most animals, including common laboratory species (Scott, 1972). A standard method for eliciting attack behaviour in rodents is to introduce an intruder male into another male's home cage, and record the following behaviours on the part of the resident (Miczek *et al*., 1984): (i) attack: leaping towards the intruder; (ii) threat posture: arched back posture; (iii) tooth chattering and (iv) boxing posture: standing on hind legs with forelegs extended; and, on the part of the intruder, escape and defensive behaviours, primarily adoption of a supine posture.

Kawai *et al*. (2003) measured aggressive behaviour in male mice exposed prenatally to the plasticizer bisphenol A, which is an oestrogenic compound currently found in most humans because of its widespread use in many products. They used the resident intruder protocol as their criterion. At eight weeks of age, the treated mice exhibited considerably more aggressive behaviours than their controls. In interpreting such results, it should be recalled that masculinization of the brain results from a process by which testosterone, released by male foetus testicular tissue, is converted by brain aromatase (CYP 19) to oestradiol (Weiss, 2002).

2.2.4 Maternal Behaviour

The early environment of both rodents and humans is governed by the mother's behaviour. In rodents, subtleties in maternal behaviour, such as the amount of licking and grooming behaviours engaged in by rodent dams largely determine how robustly offspring respond to stress when they mature (Cameron *et al*., 2005). Such outcomes are expressed through the hypothalamic–pituitary–adrenal axis. Moreover, licking and grooming behaviours are transmitted to the female offspring, apparently epigenetically, so that daughters behave like their mothers. Maternal stress alters dam behaviour, which may account for how it changes the response of offspring to lead exposure (Cory-Slechta *et al*., 2008).

For those reasons, chemical exposures *in utero* that modify maternal behaviour in the female offspring are important end points to measure in assessing neurobehavioural toxicity. Palanza *et al*. (2002) provide one example. They administered bisphenol A (BPA) to pregnant rats, then studied the maternal behaviour of the female offspring. Dams exposed to BPA prenatally spent less time nursing their pups and more time out of the nest compared with control females. The F2 females were not tested for their own maternal proclivities, but the report by Cameron *et al*. (2005) suggests that they, in turn, would also show reductions in duration of maternal behaviours.

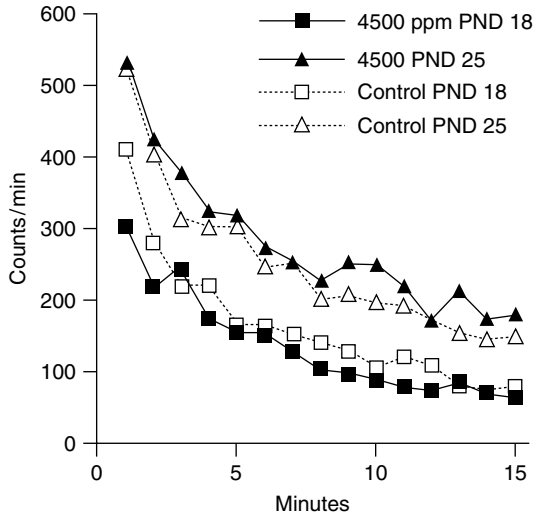

Figure 1 Time course of habituation in motor activity, in control rats and in rats exposed to methanol vapour (4500 ppm) or air from gestational day (GD) 6 to PND 21. The rats were placed in a circular runway where movements were recorded by a ring of photocells. Statistical analysis established a significant interaction between methanol exposure and postnatal day ($p < 0.01$). (Reproduced from Stern *et al*., 1997. © Elsevier.)

2.3 Motor Activity

Motor activity is used extensively in evaluating neurotoxicity. It is considered 'apical' (Geyer and Reiter, 1985) in that it is the joint outcome of several functional capacities. The overall level of motor activity shows a biphasic pattern over time in the normally developing rat, with increases up to about four weeks of age, followed by a decline to a more steady-state level. Typically, female rodents are more active than males before attaining sexual maturity (e.g. Elliott and Grunberg, 2005).

Exemplifying the usual developmental pattern, prenatal and neonatal exposure to methanol vapour depressed activity on PND 18 and elevated it, compared to controls, on PND 25 (Stern *et al*., 1997). Like other investigators, Stern *et al*. (1997) recorded a gradual decline in activity over the 15 minute test period used in their experiment (**Figure 1**). This habituation phenomenon, which represents a form of learning, is a

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useful and easy measure to implement, that ought not to be neglected. Rate of adaptation to the environment provides information independent of absolute number of counts.

As with FOBs, activity measures are designed only to reveal potential neurotoxicity, not to indicate possible mechanisms. Devices in common use include photocell arrays that can measure both horizontal and vertical (rearing) movements, and motion detectors based on disturbed ultrasonic fields or capacitance changes (see **Figure 2**). Typical enclosures consist of running wheels and unencumbered spaces (open field) equipped with photocells to identify exploratory activity. Some laboratories have devised means for measuring activity in the home cage, which allow them to collect data over the full 24 hour period. Despite differences in design, responses to chemicals in different devices are relatively congruent and replicable across laboratories (Crofton *et al*., 1991).

A newly developed device expands the possibilities afforded by recordings of locomotor activity (McKerchar *et al*., 2006). The instrument, a force-plate actometer, uses a stiff, low-mass horizontal plate coupled to four supporting force transducers positioned at the corners of the plate. Movements of the subject are sensed by the transducers, whose signals are processed by computer to yield measurements of locomotor activity in different areas of the field, rotation around the centre and whole-body tremor. Spatial resolution is less than 1 mm, and temporal resolution is 0.02 seconds. A similar device is described by Zumwalt *et al*. (2006). One advantage of such a device is that it can also measure features such as whole-body tremor, as shown in **Figure 3**.

The predominant questions aroused by activity data arise from their lack of specificity. Especially, what does it mean to measure a decrease in activity? Couldn't any chemical, at a sufficient dose, produce enough debility to curtail spontaneous activity? The answer is obvious, but hardly precludes the usefulness of motor activity as one component of an ensemble of tests. A single measure, in isolation, is not sufficient to render a decision. Increases in spontaneous activity are usually more informative than decreases because they indicate a nervous system site of action.

Activity measures need not be confined to presumed neurotoxicants. One of the flaws attributed to such measures is that they may be responsive to other kinds of variables, such as illness lacking a neurogenic aetiology. This feature may prove an advantage for certain questions. Activity measures proved edifying in demonstrating some of the indirect effects of ozone exposure. Ozone is primarily a deep-lung irritant, not a neurotoxicant, but it can evoke subjective complaints at low levels, especially in conjunction with exercise, and depress athletic performance. In controlled experiments with humans, some subjects cease exercising, due to symptoms such as painful breathing. Most animal experiments with ozone, however, feature lung pathology or pathophysiology rather than discomfort. Tepper *et al*. (1982) adopted, instead, a behavioural perspective framed by the human studies. They measured wheel running in rats exposed concurrently to different concentrations of ozone ranging from 0.03 ppm (control) to 1.0 ppm. Running wheels are standard tools for measuring locomotor activity and are used widely in psychobiology for tracking circadian and ultradian rhythms. Even at the lowest tested concentration of ozone, 0.125 ppm, the 1 hour standard specified by the USEPA, activity fell significantly. These findings are consistent with observations that indicate correlations of reduced respiratory function (forced expiratory volume in 1 second; FEV_1) in children with ambient oxidant concentrations. In a subsequent study, Tepper and Weiss (1986) attached a brake to the running wheel that the subjects could release for 10 second periods by pressing a lever on a fixed-ratio schedule. In this situation, ambient ozone at 0.08 ppm reduced the reward potency

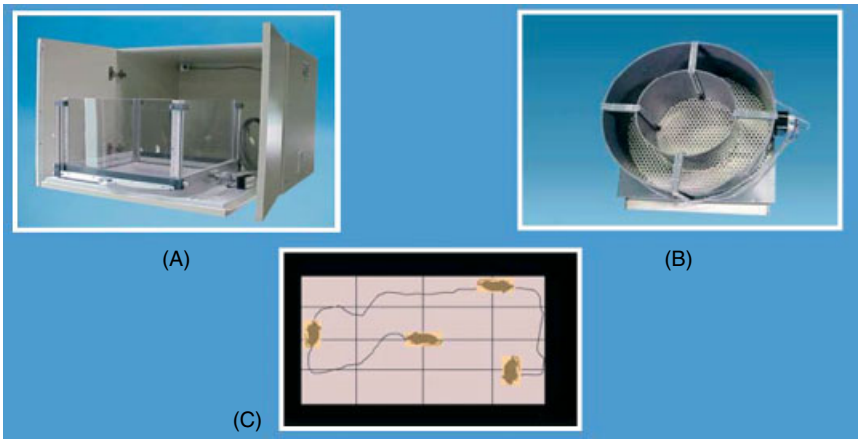

Figure 2 Examples of systems used for the study of locomotor activity. (A) Chamber with a photocell array above the floor; activity is measured as number of photocell beam interruptions. (B) Photocells arranged in a ring configuration. (C) Schematic of an open field; an observer records every crossing of a line.

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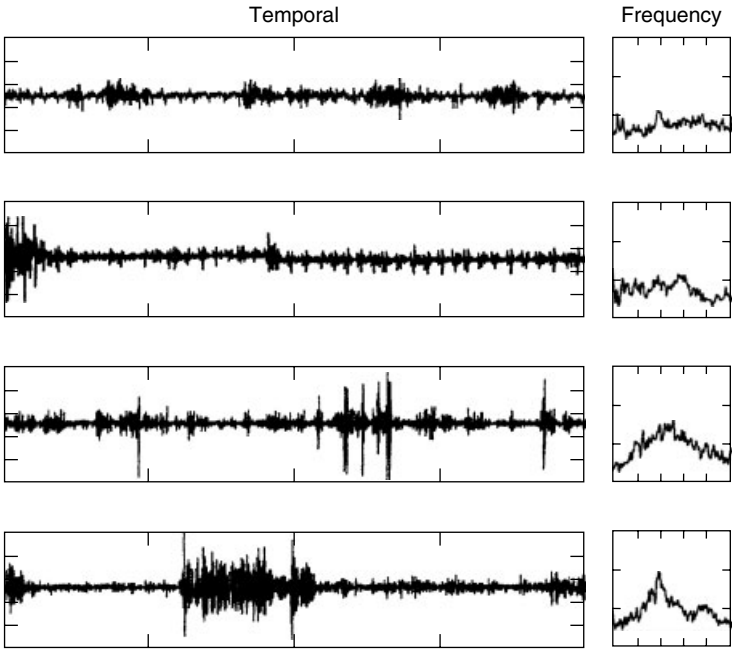

Figure 3 Tracings of a rat's response to the tremorgenic drug harmaline in the force-plate actimeter. From USA Patent No. 6,601,010, by Steven Fowler and Troy J. Zarcone. Also see McKerchar *et al*. (2006).

of wheel running as reflected by lower lever-pressing rates, indicating that ozone reduced the motivation to run.

Cyclical variations in several different behaviours can prove more informative. One form of cyclical motor activity pertinent to neurotoxicology is that coordinated with the oestrus cycle. Normal female rats housed in running wheels show peaks in activity during proestrus. Disturbances of normal oestrus cyclicity by chemicals may provide clues both to toxicity and its mechanisms. Weekly administration of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) to female Sprague–Dawley rats beginning prenatally led to a loss of normal oestrus cyclicity and hastened reproductive senescence (Franczak *et al*., 2006), a result attributed to its endocrine disruptor properties.

The reward properties of wheel running were studied by Markowski *et al*. (2001), who adopted another approach. They studied the reward properties of wheel running, much like Tepper and Weiss (1986), in female rats exposed prenatally to low doses of TCDD on gestational day 18. Because TCDD disturbs oestrus cyclicity, it was hypothesized that the motivation of females to run would change. In this arrangement, shown in **Figure 4**, the subjects had to press a lever on a fixed-ratio schedule to release the brake for 15 second periods. **Figure 5** reveals that TCDD reduced the motivation to run, as measured by lever-pressing to release the break. As plotted by a benchmark dose model, the largest effect occurred at 60 ng kg^{-1}, yielding the kind of U-shaped dose–response function common in endocrine disruption research (**Figure 6**).

3 SIMPLE, SPECIFIC, FUNCTIONAL TESTS

3.1 Motor Function and Coordination

At some phase of the evaluation process, investigators may need to probe further into possible functional deficits than permitted by the methods described above. For explicit assessments of motor function, such as might be required for evaluating potential organophosphorous-induced delayed neuropathy, FOBs may not be adequate for quantitative grading. Two useful techniques for such a situation are foot splay measurements and rotarod performance.

Foot splay is the simpler of the two and requires no instrumentation. The hind feet of a rat or mouse are inked with a food dye. It is then dropped from a specified height (such as 30 cm for rats and 15 cm for mice) after the experimenter has gripped it firmly by the loose skin at the shoulders. The distance between the marks made by the feet is a measure of hindlimb splay resulting from neuropathy induced by the compound under study. Several consecutive trials are typically used to calculate a mean.

Weiss *et al*. (2005) administered methylmercury to pregnant mice and, in some groups, continued to administer methylmercury to the offspring until 26 months of age. Hind-limb splay proved a sensitive measure of methylmercury neurotoxicity, even in those offspring exposed only prenatally, but also showed interactions with ageing. Norreel *et al*. (2001) found it useful in

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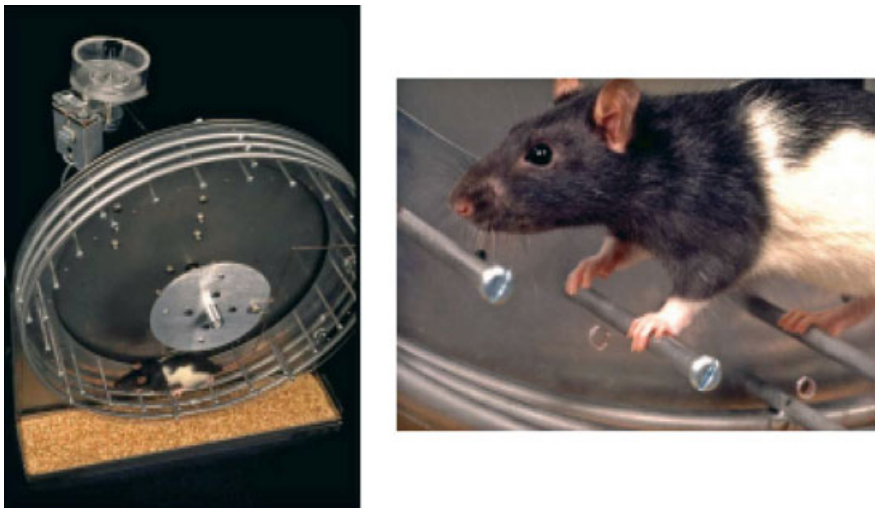

Figure 4 Apparatus used to study reward value of wheel-running. Pressing a lever on the wall a required number of times (a fixed ratio) releases a brake, and allows the rat to run for a specified number of seconds. (Reproduced with permission from Markowski *et al*., 2002. © National Institute of Environmental Health Sciences.)

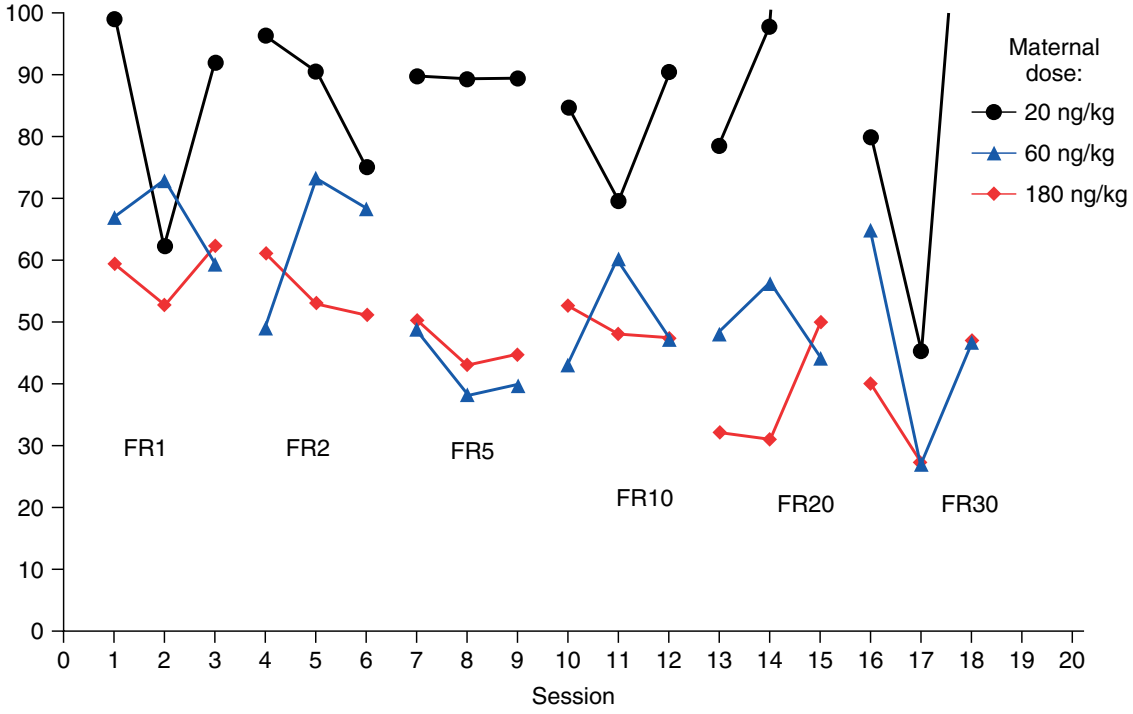

Figure 5 Plot showing effects of prenatal TCDD exposure on fixed ratio (FR) responding for access to running wheels by female offspring. (Reproduced with permission from Markowski *et al*., 2002. © National Institute of Environmental Health Sciences.)

surveying motor deficits in a mouse model of a neurological disease. In rats, LoPachin *et al*. (2002) found splay measures sensitive to agents producing peripheral axonopathies.

Upper body strength typically diminishes with age, and is also sensitive to a variety of neurotoxicants. One method used to assess it is to determine the subject's ability to grasp a horizontal wire with its forepaws and to remain suspended. In their study of rat ageing, Shukitt-Hale *et al*. (1998) observed a steady decline in the latency to fall (i.e. hang time). At six months of age, the rats could hang on for a mean of 20 seconds. At 22 months of age, that duration decreased to about seven seconds.

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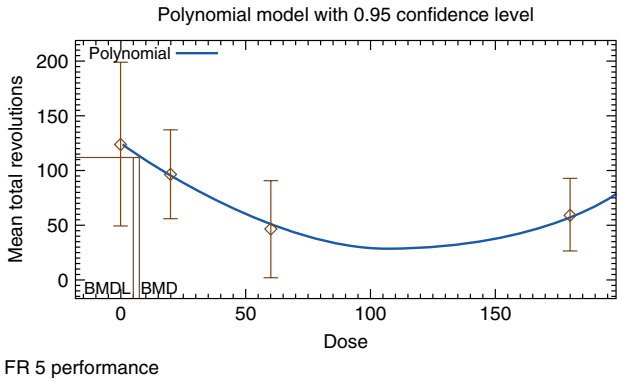

Figure 6 Benchmark dose plot showing quadratic relationship between prenatal TCDD exposure and amount of wheel running in female offspring. (Reproduced with permission from Markowski *et al.*, 2002. © National Institute of Environmental Health Sciences.)

Rotarod techniques place the animal on a grooved, rotating rod to which it clings to avoid a fall. At low rates of revolution, unimpaired animals have no difficulty remaining on the rod. As rates increase, animals can no longer cling for extended periods of time. The rod is typically divided into sections so that several animals can be tested at once and number of revolutions is counted automatically. By using individual counters beneath each animal's position that cease incrementing when it falls, a score can be assigned to each subject. One common effect of methylmercury exposure is impaired coordination. Sakamoto *et al*. (2004) administered methylmercury to neonatal rats, presumably a vulnerable period, and observed dose-related deficits in rotarod performance that were correlated with the severity of neural damage.

3.2 Sensory Function

Reflex, that is, involuntary behaviours such as those included in FOBs, predominate in the assessment of sensory function because they are relatively simple and inexpensive. They do not require extended training, as do the operant procedures discussed later. Neither do they provide the vital quantitative information required for extrapolation to the covert varieties of dysfunction that threaten humans. The acoustic startle response (ASR), and its transformation into reflex modification procedures, demonstrates how it is possible to convert a reflex into a readily quantifiable functional criterion.

The ASR is frequently used as a measure of sensory-motor reactivity. It describes the neuromuscular reaction to a brief loud sound. The central nervous system mechanisms underlying the ASR have been delineated by investigations extending over more than two decades. In the typical procedure used with rodents, a subject is restrained in an enclosure in which the response to the evoking stimulus is measured by an accelerometer or strain gauge attached to the enclosure platform. The output of the transducer, following stimulus delivery, is processed to yield measures of integrated response amplitude and latency and amplitude of the multiple peaks and troughs characteristic of this kind of signal.

Localized brainstem and midbrain circuitry mediate the response although the behavioural output can be conditioned, probably via processing by higher-level brain regions such as the amygdala and/or cortex (Davis *et al*., 1997). If the startle-eliciting stimulus is preceded by a weaker 'prepulse' acoustic stimulus, the response amplitude will be reduced. By administering a range of prepulse intensities across different tone frequencies, and measuring the amplitude of the response correlated with each prepulse frequency and intensity, an auditory sensory threshold can be established. Threshold differences observed at specific frequencies are frequently the result of neurotoxicant-induced damage to sensory processing mechanisms. In contrast, a reduced response amplitude noted across the entire prepulse range is often the outcome of motor system impairment.

Goldey and Crofton (1998) used this procedure to examine damage to the auditory system inflicted by developmental exposure to polychlorinated biphenyls (PCBs) in the form of Aroclor A1254. They found that the resulting hypothyroxinemia and hearing loss induced by exposure could be attenuated by the administration of thyroxin.

Reflex procedures are less adaptable for visual function, somatosensory function and even advanced auditory function because the complex nature of the relevant stimuli, such as visual patterns or speech, precludes their use as modulators for reflex modification. The complexity of the stimuli engenders a need for correspondingly complex behavioural assessments, especially when the pivotal questions about toxicity hinge on emerging, covert effects.

3.2.1 Visual Function

Vision is the sensory system of greatest concern in human toxicology because of its dominant role in our behaviour. With improved understanding of how the system works, scientists whose interests span both toxicology and visual science have been able to ask questions directly applicable to humans. Among the environmental neurotoxicants associated with damage to the visual system, methylmercury engenders many concerns. At high exposure levels, due to the damage it inflicts on areas in the brain subserving visual function, it severely impairs vision. Human poisonings in adults, as in the catastrophe in Minamata, Japan, and primate experiments (e.g. Evans *et al*., 1977) reveal damage in the visual cortex and associated functional impairment. Because of the extreme

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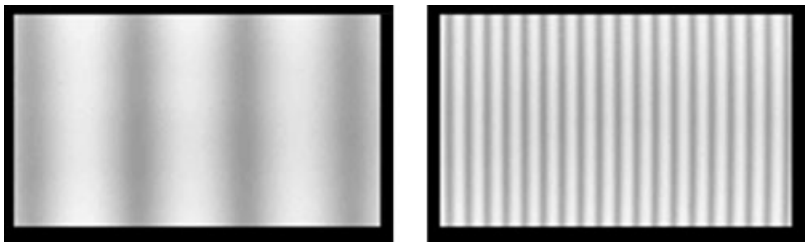

Figure 7 Oscilloscope display showing two spatial frequencies used in contrast sensitivity testing. (A) Low spatial frequency. (B) High spatial frequency.

sensitivity of the foetal brain to methylmercury, which underlies current exposure guidelines in fish, Burbacher *et al*. (2005) undertook to study nonhuman primates exposed only through maternal ingestion during gestation.

They presented stimuli consisting of alternating black and white sinusoidal gratings that varied in spatial frequency and contrast. The subjects, restrained in primate chairs, faced two oscilloscopes on which the stimuli were presented. One oscilloscope was blank; the other presented a sinusoidal grating of specified spatial frequency and contrast (see **Figure 7** for examples). The subject had access to two response buttons. If it pressed the button corresponding to the grating, it received an apple juice reward. By varying spatial frequency and contrast, the experiments were able to construct contrast sensitivity functions. The exposed monkeys exhibited a significant loss of contrast sensitivity, particularly for the higher spatial frequencies. Because exposure had occurred 11–14 years earlier, these results indicated that permanent damage to the visual system had occurred early in development.

Rats and mice are not visual animals. Despite their more primitive visual capacities, however, they are capable of complex visual discriminations and even of rudimentary colour discrimination. The mouse retina contains both rods and cones, although the latter account for only 3% of the total receptor population. Jacobs *et al*. (2004) designed an arrangement for assessing colour vision in mice that can serve as a model for testing rodent visual capacity. They presented subjects with a three-alternative forced-choice discrimination task. Test stimuli were projected on to three circular test panels. If the mouse chose the correct panel, it received a liquid reward from feeder tubes mounted above each panel. With this system, the experimenters could measure a variety of functions, including spectral discriminations. By this means, they determined that these discriminations were connected to two classes of cones with different peak sensitivities at two different wavelengths. A similar procedure has been used with rats (Jacobs *et al*., 2001).

3.2.2 Somatosensory Function

Cutaneous or somatosensory discriminations are important for neurotoxicology because, for mechanoreceptors especially, they require intact peripheral nerves and receptors, while peripheral neuropathies are induced by members of many classes of chemicals: organophosphorus pesticides, heavy metals, solvents and cancer chemotherapy drugs. Sensitivity to vibration is a recognized measure of somatosensory function. Maurissen *et al*. (1983) administered acrylamide to six monkeys trained to press a key when they detected vibration applied to a fingertip (**Figure 8**). Correct detections were reinforced with fruit juice delivered through a tube. On catch trials, when no stimulus was applied, juice was delivered if the monkey refrained from responding. Over a period of many weeks the monkeys were given 10 mg kg^{-1} each weekday until signs of overt toxicity, such as body-weight loss, began to appear. Vibratory thresholds rose during this time and remained elevated for many weeks after the other signs of toxicity had faded.

This study exemplifies several important features of sensory testing. First, the magnitude of the stimulus, produced by an electronically controlled and monitored rod, was specified as displacement amplitude, the appropriate measure. Many publications on altered vibration sensitivity induced by chemicals use a tuning fork, which offers no prospect of measuring amplitude, or simply report the voltage setting of one of the popular electronic instruments, which conveys no useful information. Second, the psychophysical procedure itself was constructed to yield unbiased threshold measures because of the introduction of catch trials. Third, the vibrator itself had to be calibrated to a resolution of fractions of a micrometer because of the high sensitivity of cutaneous sensation. Simple devices are useless for setting human exposure standards because they cannot detect incipient dysfunction.

Precise instrumentation is clearly crucial in securing precise estimates of somatosensory function. Additional examples of the level of instrumentation required can be found in Bensmaia *et al*. (2005) and Tommerdahl *et al*. (2005). Equally important, but frequently slighted, is the psychophysical procedure that determines the pattern and

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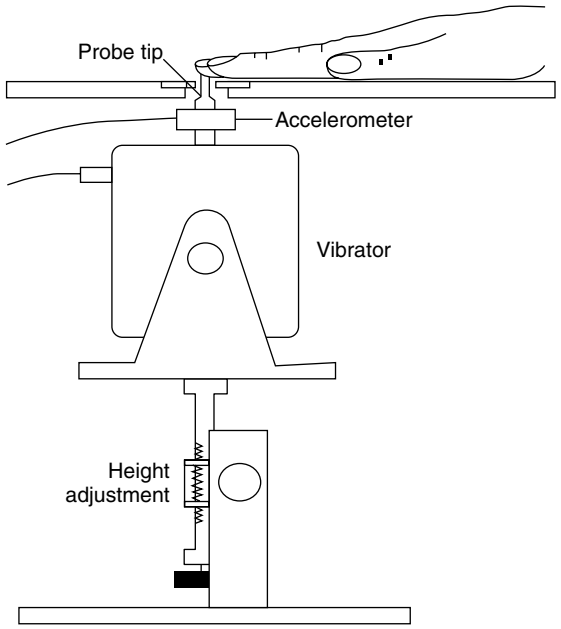

Figure 8 Apparatus used for testing sensitivity to vibration in humans and nonhuman primates. An electronically-driven vibrator under computer control determines both amplitude and frequency.

sequence of stimulus presentation, the appropriate physical dimensions of the stimulus and the form of the subject response. Commercial producers of equipment habitually seem oblivious to these requirements, but even laboratory investigators, much too often, choose unsuitable experimental models. Maurissen (1985) noted that many reports purporting to measure vibration sensitivity provided flawed descriptions of the stimuli. Rather than measuring or noting the amplitude of stimulus displacement, the appropriate measure, they report irrelevant, meaningless or improper values such as a voltage setting. In addition, many alleged measures of sensory function fail to use the appropriate psychophysical testing procedures. Maurissen (1985) divides psychophysical methods into four categories:

- Method of limits
  - Ascending and descending series of stimulus intensities from above and below thresholds, respectively
- Method of constant stimuli
  - Equally spaced stimulus values presented in random sequence
- Method of adjustment
  - Observer varies stimulus intensity to exceed and dip below detection limits
- Adaptive methods
  - Stimulus intensity rises with failure of detection and falls with correct detection.

He divides response requirements into three categories:

- Forced-choice procedures
  - Subject must choose among alternatives
- Yes–no procedures
  - Subject responds if stimulus is detected, refrains if it is not
- Rating procedures
  - Subject reports likelihood of presence or absence using rating scale.

Because rodents are the species of choice in most laboratory experiments, methods suitable for their evaluation warrant consideration. Xerri *et al*. (2005) designed a situation requiring rats to discriminate a pattern of roughness on the floor of an operant chamber. Under one condition, the chamber contained a heterogeneous floorboard covered with a combination of squares of high or low roughness arranged in a checkerboard pattern. Under the other condition, it contained a homogeneous floorboard covered with low roughness squares. Each condition was associated with either the left or right lever mounted on the chamber wall, and the rat received a food pellet reward for a correct choice.

Many experiments have assessed tactile discriminations in rodents by relying on their exquisitely sensitive vibrissae, an advantageous sensing device for nocturnal species. Mehta *et al*. (2007) designed an apparatus consisting of a tube into which the rat inserted part of its head. Its task was to discriminate the position of a rod lowered into either the rostral or caudal vibrissae field, and to make the appropriate lever choice. Other experimenters have used mazes whose walls are coded by roughness, or have applied vibratory stimuli to the whiskers.

4 COGNITIVE FUNCTION

4.1 Simple Cognitive Tests

Perhaps the most vexing questions about neurotoxicity are directed towards functions such as learning and complex performance. Human behaviour is largely acquired behaviour and often emitted in response to circumstances that require discriminating judgements and decisions. The data that come from FOBs, motor activity and reflex measures offer little guidance about the possibility of impaired cognitive function. Learned behaviours are usually included in screening batteries for that reason. There are many varieties, but the one used most often today is the water maze, followed by other maze techniques.

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Mazes were a staple of experimental psychology for many years, and some psychologists and other neuroscientists still rely upon them as behavioural assays. For toxicity testing, they represent a considerable investment that is often unacknowledged: the cost of technician time. When a full-time technician is assigned to test animals, usually one at a time, the cumulative cost far exceeds outlays for the kind of automated equipment devoted to schedule-controlled operant behaviour, discussed later. Repeated handling of the animals is a confounding factor often ignored as well, although handling acts in complex ways on arousal and stress mechanisms.

One of the most widely used techniques to assay cognitive function in rodents is known as the Morris water maze. It was devised to study spatial learning. The apparatus is simplicity itself: a tub of water into which the animal is placed. The animal can escape from this aversive situation by climbing onto a submerged platform. Because the water is rendered opaque by dissolving dried milk powder in it, the subject must learn to rely on the surrounding visual cues to learn the location of the platform. Most investigators now use automated visual tracking to record the patterns of movement in the tub during the acquisition process. It is especially attractive to investigators with limited research budgets because of its simplicity, so that human labour can be substituted for automated equipment, although technology is now available to record the subject's path as it searches for and learns the location of the platform.

Many neuroscientists find the apparent simplicity of the technique appealing, but only if they have failed to consider its defects. One glaring problem is its sensitivity to water temperature, especially in the form of temperature–chemical interactions (Rauch *et al*., 1989; Sandi *et al*., 1997). It is a particularly questionable technique for questions that may involve stress. Engelmann *et al*. (2006) conducted an extensive study in rats of the response of various stress indicators to water maze testing. Microdialysis measures taken with an indwelling catheter were seen to reflect a significantly increased release of arginine vasopressin within the periventricular nucleus and to elevate plasma adrenocorticotropic hormone (ACTH) and corticosterone. These findings duplicate those observed when rats are subjected to a forced swimming procedure, which is explicitly designed to stress the animals and to study the phenomenon of learned helplessness (Bowers *et al*., 2008).

The radial arm maze was designed to provide another measure of spatial learning and discrimination. It takes the form of a central zone from which multiple runways, typically eight in number, extend in a radial pattern like the spokes of a wheel (**Figure 9**). Usually, the end of each runway is baited with a food pellet, but experimenters may choose to bait less than the maximum number. The subjects learn to return to the central zone and then proceed to investigate other spokes. Eventually, they develop the strategy of following a reliable sequence of spokes so that they do not miss a baited arm. The number of trials required to learn to retrieve all the pellets without error is the most frequent measure of performance. Radial arm mazes are used in many areas of neuroscience; one notable application is in studies of ageing, where they have provided criteria for investigating the source of performance deficits associated with advanced age.

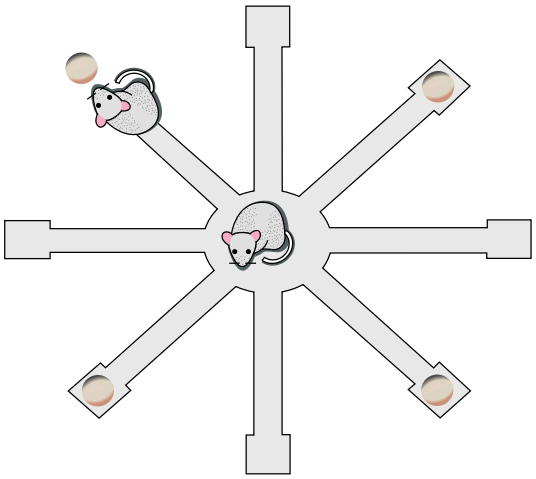

Figure 9 Schematic of a radial maze. A trial begins with the subject placed on the central platform. The arms are baited with food, and performance measures are based on the sequences of arm choices employed by the subject to secure the food.

Radial maze performance proved sensitive to developmental exposure to A1254 in a study by Roegge *et al*. (2000). They administered it to pregnant and lactating female rats from GD 6 to weaning at PND 21. Both male and female offspring were tested on a 12 arm radial maze when they reached adulthood, but only eight of the 12 arms were baited with food pellets to enhance task difficulty. The PCB-exposed males made significantly more working and reference memory errors than control males, but the exposed females performed as well as control females. Such sexually dimorphic effects are common.

5 ADVANCED BEHAVIOURAL ASSESSMENTS

5.1 Operant Techniques

The test procedures described above would generally be applied during the earlier phases of a programme to evaluate the neurotoxicity of a chemical. They serve more to identify its potential for neurotoxicity, rather than to yield information about the more advanced functions critical for human performance. In our accustomed environments, we make decisions based on analyses of

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intricate data, we make subtle distinctions among stimuli, we combine motor and cognitive functions in acts such as typing manuscripts and driving, and we alter our behaviour in accord with a long history of experience. The likelihood of a neurotoxicant contaminating our environment at a level high enough to produce an overt, simple defect is far less than the chance of such a chemical insinuating itself at low levels into that environment and inducing undetected and covert functional disorders. Identifying an agent as potentially neurotoxic is only the first, and largely minor, step in the risk-assessment process. Determining the full consequences of environmental levels to which the population might be or is exposed is a far more abstruse and demanding art. Simply buffering no-observed adverse effect levels (NOAELs) derived from simpler assays with large uncertainty factors is not always a feasible solution. Even now, with lead no longer contaminating gasoline, many individuals continue to be exposed from dust deposited in earlier years, and from residential dust from paint. The question now posed is how much to invest in cleaning up the remaining lead contamination, which invokes the question of how much it needs to be reduced to eliminate, in particular, its undesirable effects on brain development. Those questions continue to make lead's neurotoxicity an important research question whose central theme is derived from its effects on complex performances such as those assayed by psychological tests (Gilbert and Weiss, 2006). The clearest effects in animal models are those documented by schedule-controlled operant behaviour (Cory-Slechta, 2001).

Organisms interacting with their environment, the crux of behaviour, exemplify a dynamic system. Those incapable of adjusting to the demands of this continuous transaction fail to survive. Many techniques attempt to capture a slice in time of this capacity, like the water maze, or claim one specific functional aspect as their territory. Schedule-controlled operant behaviour targets the fluid rather than static relationship between an organism's behaviour and its environmental consequences.

Operant behaviour refers to learned or acquired behaviour that is controlled by its consequences. It describes most of the repertoire of human behaviour. Some of the other techniques, such as maze performance, also study learned behaviour and have long histories in behaviour science. These techniques primarily offer an index of the acquisition phase of learned responses, which can also be conceived of as a transition behaviour, but they provide snapshots of learning. In many experiments, this transition phase confounds the specific response to be learned, such as locating the hidden platform in the water maze, with the subject's adjustment to a novel environment. Schedule-controlled operant behaviour can provide a clearer and more detailed view of the acquisition process by building on what experimenters call steady-state performance

Figure 10 A standard chamber for the study of schedule-controlled operant behaviour. In this chamber, provision has been made for three response levers, three banks of lights and a pellet feeder; pellets are visible in the food magazine under the centre lever. Auditory signals can also be presented.

(Weiss, 1970). The latter term, in fact, describes quasi-stable performance because absolute stability is never attained; the inherent variability of our responses means that our transactions with the environment are continuously in flux. A simple example: the skill of automobile driving is tested constantly as the conditions of traffic and weather change. The fixation of many investigators on a single score, such as that derived from a maze, neglects this essential property of behaviour. Schedule-controlled operant behaviour captures its fluctuating essence.

The term, schedule-controlled, refers to the way in which experimenters define the relationship between a specified response by the organism and the effects of that response. A typical operant chamber is shown in **Figure 10**. A prototypical situation is one in which a rat, by depressing one of the levers mounted on the front panel, can trigger the release of a small food pellet. The food pellet is termed a reinforcer and the process is termed reinforcement. The rat's responses produce food delivery according to the contingencies, or schedule, programmed by the experimenter.

The USEPA regards behavioural assessments based on operant schedules as both definitive and sensitive measures of complex cognitive function. In listing the criteria by which solvent neurotoxicity was to be determined, it noted that, 'Solvents may have neurotoxic effects on memory, learning and performance which can be permanent. These effects are less well understood . . . The schedule-controlled operant behaviour test has . . . typically been required as a second tier test . . . it

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is proposed as a first-tier test . . . because of EPA's desire to obtain data on the effects of solvents on learning, memory, and performance'.

Schedule contingencies come in many varieties. Some are based primarily on time. Interval schedules specify relationships between elapsed time and the availability of reinforcement. A fixed-interval schedule might specify that the first response five minutes since the last reinforcement will produce the next reinforcement (FI 5). Another way to construct a schedule based on elapsed time is to specify the interval between successive responses; a differential reinforcement of low rate schedule might require a minimum of 20 seconds between responses (DRL 20) for reinforcement. Response number, in the form of ratio schedules, is another widely used performance criterion. A fixed-ratio schedule might require 100 responses (FR 100) for reinforcement delivery.

The primary virtue of schedule-controlled operant behaviour is its flexibility. It can be used to study rate of responding during steady-state behaviour, or the acquisition of new behaviour against a background of stable behaviour, or the ability to distinguish related visual stimuli, or the speed of responding to a stimulus, or the accuracy and other characteristics of motor control.

One of the most useful features of operant behaviour is that animal subjects can train themselves. Experimenters typically begin with a rat, say, which is maintained at 85% of its free-feeding body weight. At this weight, rats will respond consistently for food pellets provided that they do not earn enough food during a session to become sated. One advantage of this regimen is that leaner rats remain healthier than rats allowed to feed freely, making it more feasible to conduct extended chronic studies. Once a rat has reached the targeted body weight, it can be trained in an experimental chamber where food pellets are occasionally released into a receptacle mounted on the wall. Rats quickly learn to associate food pellet delivery with the receptacle and the sounds accompanying pellet release by the food magazine. Because rats tend assiduously to explore their surroundings, a lever mounted on a wall near the pellet receptacle will almost inevitably be depressed. If a press is programmed to deliver a pellet, it is almost certain that it will soon be pressed again. Those animals who are not adventurous enough to explore the lever can be encouraged to do so by placing some food on it or (as we have done) smearing it with peanut butter. In a short time, the rat is pressing the lever repeatedly. We take advantage of overnight sessions, when the chambers usually lie unused, for this kind of training because it requires no experimenter intervention. After one or several sessions on this continuous reinforcement schedule (FR 1), the rat is ready to be introduced to more complex contingencies.

Different experimental approaches have been employed to address questions about how to use operant procedures as indices of behavioural toxicity. One approach focusses on the initial, transition-state performances that occur in response to changes in experimental conditions (Weiss, 1970). Transition-state performances are of particular interest because they demonstrate the capability of the subject to learn, or adapt or adjust to a changing environment. Those behavioural adjustments may indicate an adverse effect not seen under final steady-state conditions when compensatory factors have had an opportunity to emerge. A transition-state procedure can also be viewed as a dynamic 'challenge', which imposes burdens on the subject and thereby reveals deficits or vulnerabilities not detected under steady-state conditions. Other investigators have demonstrated the sensitivity of transition states in studies of prenatal or early development effects of ethanol (Gentry and Middaugh, 1988), methylmercury (Newland *et al*., 1994; 2004), mercury vapour (Newland *et al*., 1996) and lead (Newland *et al*., 1994). In addition to our interest in transition-state performance as an index of learning, and its demonstrated sensitivity to environmental changes, including chemical exposures, we find it appealing for the practical reason that, under many conditions, less time may be required to examine transition-state than steady-state performances.

In a study of the effects of prenatal TCDD (Hojo *et al*., 2002) on operant performance, food-reinforced, fixed-ratio lever pressing was used under a procedure in which the fixed-ratio criterion is maintained for only a few sessions before being shifted to a new value. The procedure is similar to one used by Gentry and Middaugh (1988) to study the effects of prenatal ethanol exposure and by Paletz *et al*. (2006) to study the effects of prenatal methylmercury exposure and diet. As shown in **Figure 11**, transitional performances showed marked sex differences; male offspring showed reduced responding while female offspring exhibited elevated response rates. In general, as the ratio requirement increased, the dose differences followed suit.

These results illustrate experimental approaches that are especially useful for neurotoxicology. They demonstrate that transitions from one schedule value to another can be a potent index of functional capacity. These data testify to the practicality of operant performance in developmental studies. The discipline of behavioural pharmacology, whose methods correspond closely with those of behavioural toxicology, most typically relies upon the stability of schedule performance to study several doses in the same organism to plot dose–effect functions. Behavioural teratology is unable to take advantage of repeated-measures designs and so finds it burdensome to train animals to asymptotic performance. Focussing on early transition phases requires no prolonged training. At the same time, if investigators so choose, the animals have already been equipped with a foundation of training enabling them to be tested with more complex and demanding schedules.

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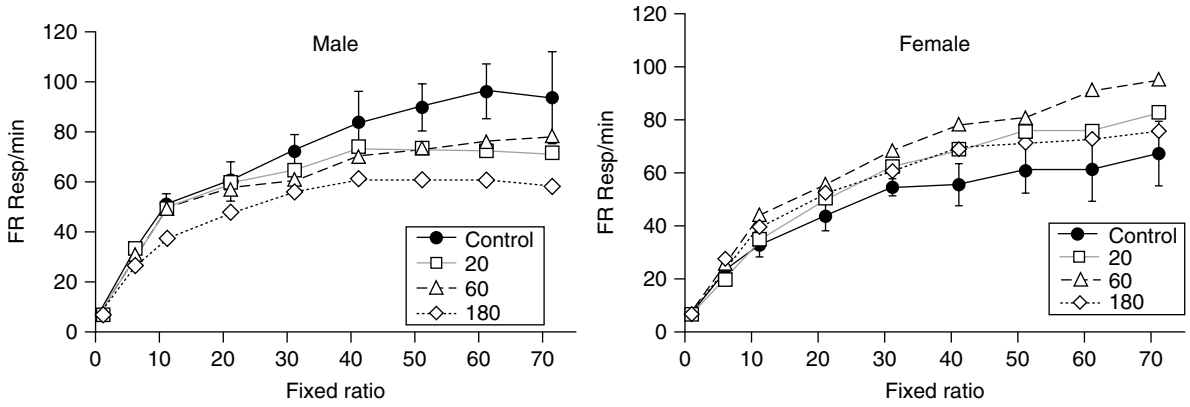

Figure 11 Incremental fixed-ratio performance by male and female offspring exposed prenatally to different doses of TCDD. Control male response rates (responses per minute) exceeded control female response rates. TCDD exposure decreased male rates and increased female rates. (Reproduced with permission from Hojo *et al*., 2002. © National Institute of Environmental Health Sciences.)

5.1.1 Complex Schedules

All schedules depend upon intact short-term memory for optimal performance, but the relation can be made more explicit. Rice and Karpinski (1988) measured the ability of lead-treated and control monkeys to remember the location of a previous response. The monkeys faced a panel with two levers. On such a delayed spatial alternation task, they are required to alternate responses between the two levers to secure reinforcement. Delays of varying durations between successive response opportunities can be programmed to measure short-term spatial memory function. Monkeys dosed with lead during infancy, after infancy, or continuously from birth, with doses that generated steady-state blood lead levels of 11 or 13 $\mu g\ dl^{-1}$, showed impaired performance compared to controls. Both initial learning, and subsequent performance at the longer delay values, faltered because of indiscriminate and preservative responding, a consistent finding in the lead literature.

A delayed spatial orientation arrangement for mice and rats is shown in **Figure 12**. Markowski *et al*. (2002) used such an arrangement to study the effects of TCDD on working memory. Offspring were exposed to a single maternal dose of 0.0, 0.06, 0.18 or 0.54 $\mu g\ kg^{-1}$ by mouth (po) of TCDD on gestation day 15. A random series of delay intervals (0.01, 3, 6, 9, 12 or 24 s) was tested during each session, with the expected outcome that more incorrect choices were made at the longer delays. For both male and female offspring, the dose of 0.18 $\mu g\ kg^{-1}$ produced the worst performance.

5.1.2 Assessment of Complex Motor Function

The elementary procedures used to screen for neurotoxic potential rely heavily on motor deficits as indices of

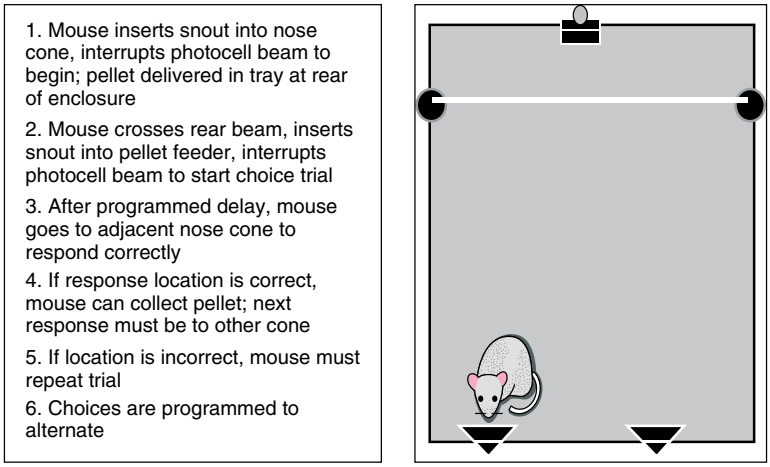

Figure 12 Schematic showing protocol for testing effects of delays on spatial alternation performance.

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dysfunction. Coarse, blatant disturbances of motor function are only the first phase of an adequate assessment. On many occasions, the central question is not the potential for neurotoxicity, which typically has been identified much earlier, but whether prevailing environmental exposure levels may be responsible for covert impairment. Furthermore, such questions frequently apply to fine motor control as a component of more complex tasks, such as driving. Responding to such questions in the animal laboratory requires extensive training of subjects and the proper selection of criteria. For human testing, although extensive training may not be necessary, precision instrumentation is still as vital.

The basic physical properties that define movement comprise time or duration, displacement (either linear or angular) and mass. From these basic units, the following combinations, among others, can be derived (Newland, 1988): linear velocity, angular velocity, acceleration and force. The simple measures of motor function included in screening batteries are not conveyed in terms of such properties, but, instead, report data such as number of photocell interruptions (locomotor activity) or the length of time a rat can remain on a rotating rod. Most operant performance measures are given as response rates derived from switch closures. A rich variety of information about motor function, however, can be secured from situations ordinarily yielding relatively gross data. All that is required is a small investment in proper instrumentation.

Nonhuman primates are especially suited for questions about complex motor function. Newland and Weiss (1991) trained squirrel monkeys (*S. sciurea*) to grasp a rod attached to a sensitive angular position transducer and to maintain it in a prescribed angular band for eight seconds. During that time, the position of the rod was sampled at a high rate and stored to allow for spectral analysis. Ethanol at doses ranging from 0.25 to 1.0 g kg^{-1} po reduced tremor power significantly, most markedly at the dominant frequency band of about 7–10 Hz. These data support the claims by elderly individuals with essential tremor that modest consumption of alcohol reduces their tremor.

Many environmental neurotoxicants induce such effects. Among them are virtually all classes of insecticides, several heavy metals and volatile organic solvents. Newland (1988) noted that abnormal tremor could serve as an apical test for motor system dysfunction because it can be induced by impairment in many parts of the central nervous system devoted to movement. An investigator pursuing such measures should be aware of the precautions required to secure and process such data. Physiological tremor is composed of many contributing frequencies of varying amplitudes, so that the analysis of its electrical analogue is aimed at dissecting the signal into its components. The distribution of variance over frequency, the power spectrum, defines the composition of the tremor. Such process is radically different from observing tremor clinically; neurologists are capable of detecting grossly deviant tremor, but such observations are incapable of providing the information needed to set exposure standards or to detect incipient dysfunction.

One of the earliest signs of manganese intoxication is abnormal fatigue. Miners who have suffered excessive exposure to the ore complain of extreme tiredness. Because primates are the only nonhuman species in which the signs of manganese neurotoxicity correspond to those seen in humans, Newland and Weiss (1992) constructed a device requiring monkey subjects to respond, for fruit juice reinforcement, with a response similar to rowing. The monkeys pushed with their feet and pulled with their arms against a spring whose resistance approximated their body weight. On the FR component of a multiple fixed-ratio fixed-interval schedule, manganese chloride administered intravenously (iv) on several occasions over a period of several months reduced response rates, shifted the distribution of inter-response times to longer intervals, and increased the number of incomplete responses.

Effort is a variable too often neglected, rather than exploited, in behavioural testing. Zarcone *et al*. (2007) trained mice to press on a disk behind a wall of an operant chamber, as shown in **Figure 13**. If the force exerted exceeded a prescribed criterion, such as 8 g, for a prescribed duration, the mouse earned 0.05 ml of sweetened condensed milk delivered via an electromechanical dipper. The mice displayed remarkable precision in performance, exerting just enough force to exceed the criterion.

The experiments described above comprise no more than a minute sample of possibilities. The technology afforded by the experimental analysis of behaviour permits behaviour to be moulded to answer whatever questions experimenters devise. Complex motor responses, amenable to detailed, precise analyses such

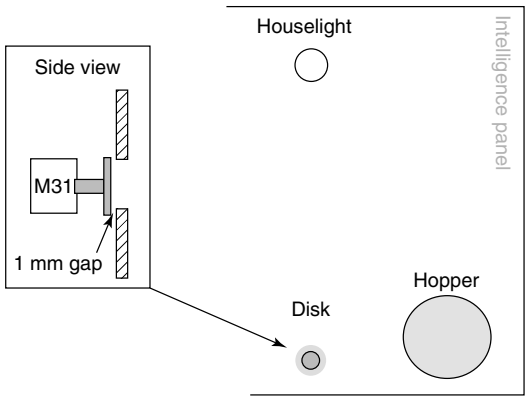

Figure 13 System for assessing response force requirements on responding for food rewards (Zarcone *et al*., 2007). The rat inserts its paw into the opening to press the disk. (Reproduced with permission from Zarcone *et al*., 2007. © Society for the Experimental Analysis of Behavior.)

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as those exemplified by the mathematical tools applied to tremor, can be elicited even in the rat. The correlation may not be perfect, but it generally holds that the order of complexity in response topography and its analysis is correlated with its usefulness for extrapolation to humans.

6 HUMAN TESTING

6.1 Adults

Most of what is known about neurotoxic chemicals was driven by observations in humans. Almost all of the literature in neurotoxicology, in fact, remains based on substances identified through their adverse effects on humans. Few, if any of the screening procedures described earlier in the chapter have successfully prevented the marketing of agents or classes of agents that had been planned for introduction into environments presenting the possibility for human exposure. The marked success stories of neurotoxicology, exemplified by lead, are successes because they revealed neurobehavioural effects at exposure levels previously believed to be safe, and because they unearthed unanticipated facets of neurotoxicity. Lead's neurotoxicity, recognized for over two millennia, had not been questioned, only its potency. Similarly, organophosphorus insecticides are universally recognized as powerful nerve poisons; neurobehavioural testing, however, disclosed their ability to impair function in the absence of overt clinical toxicity or long after the disappearance of overt signs. Much of neurotoxicological research is still aimed at the identification of potential neurotoxicants, but the primary issues confronting risk assessment derive from another question: how much is safe? Human behavioural testing is still the ultimate arbiter.

Many of the earliest attempts to assess neurotoxicity in humans adopted procedures that had been developed for clinical neuropsychological testing. Such procedures often proved poorly designed for research in neurotoxicology because they evolved as diagnostic instruments, not as tools with which to screen populations or for experimental investigations. They typically were used to provide a functional profile of a patient, often one who had suffered brain damage. They had not been contrived to determine, for example, whether workers exposed to pesticides differed from controls on various psychological dimensions, or to yield a dose–response function for acute exposures relating concentration to performance. Nevertheless, lacking more appropriate tools, they were invoked to respond to some pressing questions about exposed populations. The pioneering reports from the Finnish Institute of Occupational Health (Hanninen, 1988) relied heavily on clinical instruments. Neurotoxicology also borrowed techniques from the experimental psychology laboratory. Such techniques lacked the standardization and norms provided by most clinical tests, but offered the virtue of greater specificity, flexibility and a scientific basis.

Moreover, the time taken for evaluation may be limited, especially in the workplace, so that a compact but comprehensive test battery is more suitable than the typical, largely paper and pencil tests administered by clinical neuropsychologists. Responses on paper and pencil tasks also have to be scored and transcribed, leading to transcription errors and rescoring.

Faced with the need to assess specified populations exposed to defined hazards, or to evaluate particular stressors experimentally, neurotoxicology turned to the development and adoption of computerized testing. It made the mechanics of testing more efficient; it offered considerably more uniformity in how test stimuli were presented; it made it possible to test several subjects simultaneously; it could use testers who did not require advanced clinical training; it could automate scoring and analysis; it allowed remote testing (as in an exposure chamber) and it proved adaptable for translation of procedures used in the animal laboratory. Perhaps most important of all, it moved human testing from clinical diagnosis to the realm of performance.

Performance tests differ from conventional clinical tests in several respects (Wetherell, 1996). Tests devised for clinical applications aim to differentiate between individuals. Performance tests are designed to differentiate among stressors such as drugs, toxic chemicals and conditions such as sleep deprivation. Clinical tests should be relatively insensitive to environmental perturbations because they should serve to identify stable traits in the individual, but performance tests are expressly designed to reflect such perturbations. Finally, clinical tests generally are meant to be given only once; in contrast, performance tests should be capable of repeated administration. Practice effects can be minimized with alternate forms.

Slikker *et al*. (2000) offer a comprehensive discussion of the properties and usefulness of computerized test batteries and how they reflect and extend traditional approaches. Several current batteries have been used widely enough, and are well-enough established, to be considered as appropriate instruments for neurotoxicology. The Cambridge Neuropsychological Test Automated Battery (CANTAB) (Fray and Robbins, 1996) consists of a suite of computerized tests, now numbering 22, that embrace a variety of cognitive functions: visual memory, executive function, working memory, semantic and verbal memory, attention, decision-making and response control (designed to assess behaviours such as impulsivity). Most of the tests are explicitly designed to be independent of language and culture. Alternate forms are available for repeated testing. The CANTAB has been used extensively in patients with Alzheimer's and Parkinson's diseases (Blackwell *et al*., 2004). The

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BARS (Behavioural Assessment and Research System) battery is specifically designed for the detection of neurotoxicity in populations with limited education or literacy (Rohlman *et al*., 2003). It too can be used for repeated assessments.

One of the newer features of computer-based testing is the incorporation of instructional materials. Particularly because of the variety of populations that undergo assessment for neurobehavioural function, including those unfamiliar with testing procedures and that are often illiterate, more effective means for communicating test instructions have been sought by investigators. The computer itself is a tool that can be adapted for such a purpose. Anger *et al*. (1996), in response to such a need, use computer graphics for the BARS battery to teach subjects how to perform the tests before the test items themselves are presented. The technique relies on a sequence of approximations to the final performance, much like the technique, called shaping, used to train animals on schedule-controlled operant behaviour.

6.2 Children

New initiatives, such as the formation of an office devoted to child health at the USEPA, and heightened public and legislative awareness of the special vulnerabilities of children to toxic agents, have promoted the design of test batteries specifically targeted to developmental neurotoxicity. These differ from the more established, traditional tests that have helped propel behavioural teratology into its current prominence. Intelligence tests such as the Stanford–Binet and the Wechsler Intelligence Scale for Children (WISC) occupied critical roles in demonstrating the threats posed to brain development by lead and PCBs. The Bayley Scales of Infant Development, suitable for earlier ages, also enjoyed wide use in such contexts. The virtue of such instruments lies in the detailed amount of information available about their reliability, about the population used for standardization and in the extensive literature describing their application to an enormous diversity of problems.

These and other traditional tests, by themselves, cannot serve all the purposes of investigations undertaken to discriminate between populations that differ in exposure sources and levels. Test scores are meant primarily to yield information about the child and its unique traits rather than about the population to which the child belongs. They also provide information mostly about cognitive function rather than a more complete assessment of all functions.

In an attempt to construct a more comprehensive battery, Davidson *et al*. (2006) recruited graduates of the University of Rochester Neonatal Intensive Care Unit (NICU) and compared their performance to controls on a broad array of measures. The NICU population is known to be at risk for both major and mild anomalies in perception, motor functioning, learning, memory and cognition. They were chosen for study as a surrogate population for those at risk due to exposure to elevated levels of neurotoxic chemicals. The study aimed to evaluate the capacity of these tests and tasks to predict such potential deficits.

The test battery included the following measures:

- Audiometry and tympanometry
- Auditory processing
  - Pitch pattern sequence test (auditory information processing)
  - Auditory continuous performance test (cognitive)
  - Dichotic digits test-double pairs (auditory information processing)
- Electrophysiological tests
  - Cognitive event related potentials (cognitive)
  - Auditory evoked potentials (auditory information processing)
  - Otoacoustic emissions (auditory information processing)
- Experimental tasks (neuropsychological)
  - CANTAB (cognitive, auditory and visual information processing)
  - Multiple fixed-interval schedule self-control (cognitive)
- Experimental tasks (sensory and motor)
  - Fine motor control
  - Scotopic visual form discrimination (somatosensory)
  - Visual spatial contrast sensitivity (somatosensory)
  - Monitoring and vigilance (complex perceptual motor).

In total, the battery yielded 63 measures. A battery this comprehensive would not necessarily be suitable for every investigation. It was devised to include most of the end points from which investigators would choose in studies of specific exposures, thereby providing guidance for such choices. A special aim of the work was to provide data on sensitivity and specificity.

Over one-third of the 63 measures proved capable of detecting prediagnosed lower IQ, the presence of a learning disability (LD) or a neonatal risk profile with at least 70% sensitivity and specificity. Sensitivity and specificity were obtained from curves estimating receiver operating characteristics (ROC). A variety of covariates were included in the statistical analysis, such as gender, age, hearing status or familiarity with computers. Tests were also eliminated from the battery if they were affected by too many covariates. Further studies are planned to confirm the capability of the battery to detect subtle changes associated with neurotoxic exposures.

The CANTAB has also been used successfully in children with various behavioural disorders (Rhodes *et al*., 2006; Goldberg *et al*., 2005). It was also used

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successfully in defining specific behavioural and cognitive deficits produced by lead exposure in four- to five-year-old children in Rochester (Canfield *et al*., 2004) and to study the effects of lead on executive function in children with a particular dopamine receptor polymorphism (Froehlich *et al*., 2007).

Testing of infants, both human and animal, for cognitive function presents unique problems simply because of their limited physical repertoire. A useful guide to the kinds of tests that offer cross-species comparisons was provided by Sharbaugh *et al*. (2003). It is a useful demonstration of the possibilities offered by approaches that rely on fundamental behavioural principles for experimental design.

7 RISK ASSESSMENT ISSUES

Toxicology is an applied science. It is supported as a research enterprise because it yields information about threats to human health. Its contributions to basic science, which are substantial, are gratifying bonuses. Behavioural testing is only one of the biological measures, however, relied on to establish health risks. From that vantage point, it can be appreciated that research should seek the most direct extrapolation to humans. The methods described in this chapter can be divided roughly into two categories. One set of methods is most suitable for determining if an agent is a potential neurobehavioural toxicant. Screening tools, such as FOBs, enjoy widespread adoption because they serve this role in helping to determine if a new chemical might pose unjustified health hazards. They are primarily instruments for hazard identification.

The other set of methods is used in a different way. If screening is classified as a first-tier effort, methods such as schedule-controlled operant behaviour are classified as second-tier approaches. That is, they amplify and extend the first-tier information and may provide more specific indices of toxicity. Such a classification system copes only obliquely with the situation that still prevails: first, almost all the neurotoxicants we know about have been identified as a result of human exposures rather than from simple preliminary screens; second, rigorous probing with behavioural measures almost invariably discloses impaired function at exposure levels lower than previously believed to present health risks. The main lesson from such a history might be that preliminary screening is most efficiently carried out by employing complex, advanced behavioural assays at the beginning of an assessment programme rather than assigning them to a secondary role. Whatever the strategy chosen, however, as this chapter demonstrates, the supply of behavioural test procedures is virtually limitless and can be moulded to virtually any need.

Behavioural toxicology in the year 2009 can boast of impressive progress during the past four decades. Clever and productive investigators transformed it from a science originally at the margins of traditional toxicology to a science that now exercises leadership in policy disciplines such as risk assessment. An inevitable by-product of success, however, is the enlargement of expectations. Some of it we compulsive malcontents have imposed on ourselves. Some of it is due to an expansion of opportunities. And some is due to newer kinds of questions asked of us. The past is an imperfect, but illuminating guide to the next stages of this evolution.

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Peripheral Chemosensory Irritation: Fundamentals, Investigation and Applied Considerations

Bryan Ballantyne

CONTENTS

1 INTRODUCTION AND BACKGROUND

The major causes of adverse effects from occupational and general environmental exposure to xenobiotic chemicals, and the investigation of such effects, relate to toxicological processes. However, certain induced, but reversible, pharmacological and physiological effects may result in discomfort, distraction or inattention in an affected person(s). Included in such transient effects are, as examples, nauseating odours and the induction of narcosis, either of which may lead to uncomfortable working conditions, a feeling of ill health and be distracting, and hence a factors contributing to physical accidents. For these reasons both effects have been used as a basis to quantitatively assign exposure limits for certain chemicals in the occupational environment (Amoore and Hautala, 1983; ACGIH, 2007). A further common source of discomfort and distraction in the workplace and general environment is that of peripheral chemosensory irritation (PCSI), in which there is an interaction between a substance having PCSI properties and sensory receptors in exposed body surfaces (skin and mucosae), resulting in local discomfort or pain at the site of contact, together with certain related local and systemic reflexes. This chapter reviews the nature, causation and clinical manifestations of the PCSI effect, factors influencing the response, approaches to animal and human investigations, quantitation, physiological significance and practical implications and applications.

2 THE NATURE AND GENERAL FEATURES OF PERIPHERAL CHEMOSENSORY IRRITATION

The words irritant and irritancy are often used somewhat loosely in many toxicological texts, and may not be specified or defined with respect to whether the words are being applied to pathological or pharmacological processes. With acute pathological processes irritancy should be used to imply a local vascular and cellular inflammatory reaction (primary irritation), and in pharmacological processes it should indicate a generally reversible functional interaction between the causative molecule and peripheral nerve sensory receptors (Ballantyne, 1983).

PCSI is a pharmacological process in which causative molecules interact with sensory nerve receptors in skin or mucosae and produce a local sensation (discomfort, itching, burning or pain) together with induced related local and some systemic (autonomic) reflexes. PCSI effects subside after removal of the stimulus and do not result in any long-term adverse sequelae. Thus, the major characteristics associated with a PCSI event are that the causative material acts locally (in exposed surfaces) by stimulation of peripheral nerve sensory receptors, which results in transient local sensations with locally mediated and some systemic (autonomic) reflexes, and not per se in any longer-term sequelae (Ballantyne, 2006). Although the pharmacological and pathological processes of irritancy should be clearly mechanistically and functionally differentiated, it should be noted that many substances capable of causing a pharmacological PCSI effect may, but usually at higher applied concentrations, also result in an inflammatory response. In this respect a PCSI effect may give a biological warning of exposure to a potentially harmful substance. Early in the history of investigations into the mechanistic causation of PCSI effects, it was considered that they were attributable to a 'common chemical sense' that was independent of touch, temperature and pain (Parker, 1912). However, it was subsequently shown that PCSI effects are mediated by several types of receptors, most of which also respond to noxious, thermal and/or mechanical stimuli (Green, 2000). This concept that PCSI effects are principally mediated by chemically sensitive neural elements of pain and temperature, that is, a process of PCSI, replaced the idea of a common chemical sense. The word 'chemesthesis' was introduced to stress that chemosensory irritation is a multimodal sense (Green *et al*., 1990). Sites generating a PCSI effect are discussed in Section 3.

A characteristic feature of the PCSI response from many PCSI molecules is the development of a progressive decrease in the sensory irritant response with sequential applications of the causative material, a phenomenon referred to as tachyphylaxis (or tolerance). Also, crosstachyphylaxis between chemically different PCSI materials may occur with some, but not all, materials. When crosstachyphylaxis occurs it is possible that this may be the result of a similar mode of action of the responsible materials and stimulation of the same peripheral sensory nerve receptor (Foster and Ramage, 1981). For example, Chang and Barrow (1986) used a depression of respiratory rate (DRR) model (see Section 8.2.5) in the rat to investigate crosstolerance between formaldehyde and chlorine. They demonstrated formaldehyde pretreatment induced a significant crosstolerance to the PCSI effects of chlorine, as shown by a shift-to-the-right of the concentration–response curves. Also, Babiuk *et al*. (1985) demonstrated that pre-exposure of rats to formaldehyde vapour caused crosstolerance with acetaldehyde, as evidenced by the concentration causing a DRR by 50% (RD_{50}) being increased three to five times, and similarly with acrolein with which the RD_{50} increased five times by formaldehyde pre-exposure. However, PCSI crosstolerance was not seen with propionaldehyde, buytyraldehyde, crotonaldehyde, cyclohexanecarboxal-

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dehyde, cyclohex-3-ene-1-carboxaldehyde and benzaldehyde, confirming that crosstolerance is not a generalized phenomenon. Tolerance and crosstolerance are clearly of practical relevance because repeated exposure to a specific PCSI material, or pre-exposure to another PCSI, may result in a reduced sensory warning on subsequent exposures, and hence a correspondingly lesser degree of protection. Although the mechanism of production of tolerance is not totally understood, it has been proposed that in some cases a chemically induced histopathological change in the nasal epithelium may result in tolerance and crosstolerance. Tolerance has been reviewed in detail by Bos *et al*. (1992).

3 SITES GENERATING A PERIPHERAL CHEMOSENSORY IRRITANT RESPONSE

From a practical viewpoint, the principal surface sites at which a PCSI effect can be experienced are the mucosal surfaces of the eye and respiratory tract, and the skin. At all these sites local sensations and related local reflexes are produced. Additionally, systemic reflexes develop, which include transient increased systolic blood pressure (SBP) and diastolic blood pressure (DBP) and bradycardia; these are common to all sites of contact, but often of variable severity, depending on the potency of the local effects.

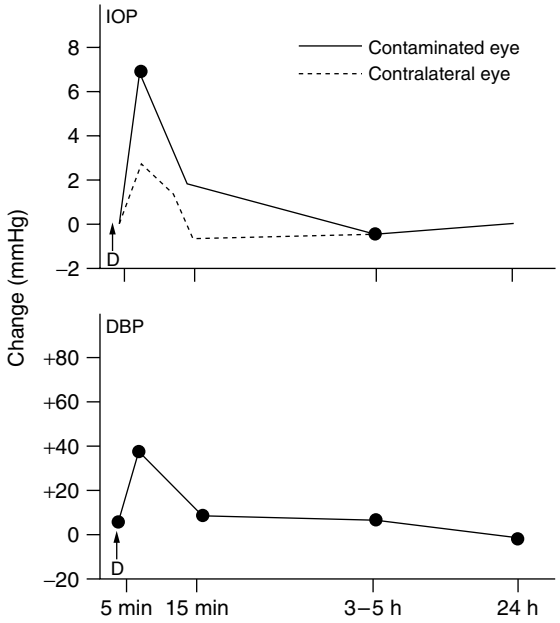

Figure 1 Effects of potent peripheral chemosensory irritation of the human eye on intraocular pressure (IOP) and diastolic blood pressure (DBP). At D, one eye of the volunteer had 0.05% dibenz[b.f]-1,4-oxazepene (CR) dissolved in polyethylene glycol 300 applied to the cornea of one eye. There was a transient increase of IOP in the contaminated eye and, to a lesser extent, in the contralateral eye (A). Also, (B) there was a transient increase in DBP. (Reproduced with permission from Ballantyne *et al*., 1977. © Elsevier.)

3.1 Eye

Following local contamination of the eye with a PCSI material there develops (depending on the concentration of PCSI applied) local itching, discomfort or pain, accompanied by excess lacrimation and blepharospasm as local reflexes. These effects produce transient variable degrees of visual impairment, and result in a warning of exposure to an irritant substance. Also, systemic autonomic reflexes may occur, including increased SBP and DBP with bradycardia. Also, there may be a transient increase in intraocular pressure (IOP). Following cessation of PCSI stimulation, the sensory, local and autonomic reflexes diminish; for example, effects on IOP and DBP are shown in **Figure 1**.

The transient increase in IOP (**Figure 1**) is most likely to occur following splash contamination of the eye with a PCSI material in solution (Ballantyne *et al*., 1976; 1977a; Ballantyne, 1983), but has also been demonstrated following exposure of the eye to a PCSI in the vapour phase (Ballantyne *et al*., 1976; 1977a). There appears to be an association between the magnitude and the duration of the increase in IOP and the inflammatory potential of the causative PCSI material, as evidenced by conjunctival hyperaemia. Changes in local blood flow, mostly congestion in the episcleral venous plexus, may be causal factors. However, following contamination of an eye with a PCSI material, there is an increase in IOP both in the contaminated eye and, to a lesser degree, in the contralateral eye (**Figure 1**). This indicates the possible existence of a systemic, as well as a local, cause of the increase in IOP (Ballantyne *et al*., 1977a). Thus, in addition to local changes in blood flow, the following may be factors in the production of PCSI-induced transient ocular hypertension; (i) squeezing of the eyeballs during blepharospasm; (ii) reflex contraction of the extraocular muscles and (iii) increase in central venous pressure resulting from the pain and stress of potent sensory irritation (PSI) (Collins *et al*., 1967; Miller, 1967; Rengstorff, 1975; Ballantyne, 1977a). The transient increase in IOP that occurs with a noninjurious concentration of PCSI substance is unlikely to be detrimental to ocular function, except possibly for susceptible individuals in whom there may be precipitation or exacerbation of glaucoma (Ballantyne *et al*., 1973a; 1973b; Rengstorff, 1975).

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3.2 Respiratory Tract

Effects are experienced in human subjects inhaling PCSI materials as discomfort or pain in the nasal mucosa, oronasal pharynx, larynx, trachea and bronchi, with associated local reflexes of coughing, sneezing, rhinorrhoea, increased respiratory tract secretions, changes in breathing rate and decreased tidal volume (TV). The clinical presentation varies somewhat based on the chemistry of the PCSI material, its solubility and the principal sites of receptor activation (**Table 1**). A characteristic of respiratory tract chemosensory responses to PCSI substances is that they stimulate afferent cholinergic nerves in the nasal mucosa leading to reductions in breathing rate and TV (Cauna *et al*., 1969; James and Daly, 1969; Ulrich *et al*., 1972). Various sensory receptors are present in the lower respiratory tract (Crofton and Douglas, 1981; Widdicombe, 1981). With respect to sensitivity to inhaled chemosensory irritant materials, the most important of these appear to be J receptors and airway irritant receptors (Kristiansen *et al*., 1986; 1988; Nielsen and Vinggaard, 1988). Stimulation of J receptors results in apnoea, rapid shallow breathing and systemic hypertension with bradycardia. Stimulation of airways irritant receptors causes hyperpnoea and bronchoconstriction (Douglas, 1981). The overall effect of stimulation of lower respiratory tract receptors is, in most species, an increase in breathing rate and TV (**Table 1**). However, with most PCSI substances effects are produced at lower concentrations by trigeminal nerve stimulation than occurs with lower respiratory tract receptor stimulation. Therefore, with a biologically effective PCSI challenge by respiratory route exposure, the trigeminal reflex will usually predominate. These respiratory regional differences in chemoreceptor sensitivity form the basis for the tRD_{50}, concerned with assessing the margin of warning provided by the PCSI response for respiratory tract injury (see Section 8.2.5).

3.3 Skin

An itching or burning sensation follows contamination of the skin with a PCSI material, and is often accompanied by a local erythema.

Table 1 Effects produced by peripheral chemosensory irritant materials on respiratory tract chemoirritant receptors

Location	Effect
Nasal mucosa	Decreased breathing rate Decreased tidal volume
Lung	Increased breathing rate[a] Decreased tidal volume Bronchospasm

[a]A few species have decreased breathing rate (e.g. mouse).

3.4 Other Sites

Contamination of the oral cavity with a PCSI material can result in a stinging or burning sensation on the tongue or palate, accompanied by increased salivation.

4 MOLECULE–SENSORY-RECEPTOR INTERACTIONS AS THE MECHANISTIC BASIS FOR PERIPHERAL CHEMOSENSORY IRRITATION

Several investigators believe that molecules capable of producing a PCSI response interact nonspecifically with sensory nerve receptors and, as noted in Section 2, the expression 'common chemical sense' was originally used to differentiate PCSI function from other chemically induced sensations, such as taste and smell (Keele, 1962). Molecules producing PCSI effects have widely varying chemical structures and, in general, there are no morphologically identifiable specific receptors for the PCSI response. In skin, however, PCSI molecules demonstrate some selectivity in that they excite polymodal nociceptive receptors and warm thermoreceptors (Foster and Ramage, 1981), but the sensitivity of these receptors is divorced from their mechano- and thermoreceptive functions (Green and Tregear, 1964). The most distinct and marked PCSI effects result from stimulation of corneal and nasal mucosal sensory receptors, and in these tissues it is considered that a direct interaction occurs between PCSI molecules and trigeminal nerve chemoreceptors that are on C-fibres and possibly Aδ-fibres (Nielsen, 1991; Nielsen *et al*., 1996). The potent PCSI material capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide) is capable of binding with receptors on both C- and Aδ-fibres and desensitizing them to other PCSI molecules; this desensitization may be sustained (Bevan and Szolesanyi, 1990; Holzer, 1991; Thurauf *et al*., 1991). Hence, pretreatment with capsaicin can be used as a method to recognize PCSI effects generated from capsaicin-sensitive nerves and it is used as a pharmacological tool to investigate sensory irritant effects (Ballantyne, 2006). For example, Hansen and Nielsen (1994a) studied the sensory irritant effects of *n*-propanol and ethylbenzene in mice after pretreatment with capsaicin and found that intranasal preapplication with capsaicin decreased the sensory irritant response to these materials. They also showed that pretreatment with indomethacin had no effect on the PCSI response, indicating that there was a direct receptor interaction rather than an indirect effect from tissue damage. Other aspects of desensitization have led to suggestions concerning differential receptor binding. Thus, Hansen *et al*. (1992), using a reflexly induced decrease in breathing rate in mice as an index of PCSI, found that methyl ethyl

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ketone at low vapour concentrations caused a desensitization of the response, but little desensitization was seen at higher concentrations. In contrast, *n*-propanol desensitized the receptor at all concentrations. However, pre-exposure of mice to *n*-propanol did not influence the response to methyl ethyl ketone. The authors concluded that the results indicated that the two ketones bind to different receptor sites, which have different properties.

Molecules with a variety of differing chemical structures are capable of causing PCSI effects (Nielsen, 1991); for example, molecules having a C=C and carbonyl grouping, in particular in the presence of a halogen, have sensory irritant properties (Dixon and Needham, 1946). With a material having known PCSI effects, the introduction of certain groupings into the molecule may alter its sensory irritant potential, the magnitude of which depends on the specific grouping introduced. For example, based on RD_{50} measurements, compared with the sensory irritant styrene, the chloro-derivative, 3-chlorostyrene, is 2.6 times more potent and β-nitrostyrene is 364 times more potent (Alarie, 1973). Some homologous series of chemicals may show a clear relationship between molecular weight (MW) and PCSI potential. Thus, for a series of C_3–C_7 *n*-alkylamines Nielsen and Vinggaard (1988) noted that PCSI potential increased, as reflected by decreasing values for the nasal exposure, as the chain length increased. Also, **Table 2** indicates that for a series of *N*-(3′-hydroxy-4-methoxyphenyl)-2-chloroamides (**Figure 2**), the PCSI potential, as determined by a guinea pig blepharospasm (GPB) test increased (became more potent) as the C chain length increased (**Table 2**, **Figure 3**). Less clear relationships have been have been shown for other series; for example, differences exist within a monoalkylamine series by RD_{50} measurements (**Table 3**). The primary saturated aliphatic monoamines in this series show a decrease in RD_{50} value (i.e. increased potency) by nasal exposure (nRD_{50}) as the MW increases (**Figure 4**). Thus, for this series, whose nRD_{50} values ranged from 17 to 157 ppm, analysis by linear regression showed a significant ($p = 0.01$) correlation between nRD_{50} and MW (**Figure 4A** $r = -0.79$). If *t*-octylamine is omitted from the analysis, because this compound does not fall into the generally decreasing nRD_{50} series (**Table 3**), then the association between MW is stronger (**Figure 4B**; $r = -0.9874$, $p = 0.0006$). In contrast, the secondary saturated aliphatic monoamines do not show a statistical relationship between nRD_{50} and MW ($r = 0.4262$, $p = 0.47$). The unsaturated aliphatic amines, allylamine and diallylamine, were the most potent PCSI materials in the series, with respective nRD_{50} values of 9 and 4 ppm. This agrees with measured nRD_{50} values for other allyl compounds, including allyl acetate (2.9 ppm), allyl glycidyl ether (5.7 ppm), allyl alcohol (3.9 ppm) and allyl ether (5 ppm) (Gagnaire *et al*., 1987; Nielsen *et al*., 1984). However, with many homologous series, MW has little influence on PCSI potential, as shown in **Table 4** for GPB studies with a series of *N*-phenyl-2-chloroamides. Over the range of MWs tested, the PCSI effects were similar.

Experimental studies have suggested that PCSI molecules interact with membrane-associated binding sites, and that binding affinity is a major factor in determining the potency of the PSI response (Green *et al*., 1979). In certain cases these effects may be associated with –SH or $–NH_2$ groups of membrane proteins or enzymes, and/or the amide nitrogen of a peptide bond (Alarie, 1973; Dixon and Needham, 1946; Douglas, 1981; Schauenstein *et al*., 1977; Silver *et al*., 1967). However, different mechanisms probably operate for different chemical groupings. For example, the PCSI potential of saturated aliphatic aldehydes decreases with their hydration constant, which may determine their degree of crosslinking with receptor proteins. In contrast, unsaturated aliphatic aldehydes such as acrolein and crotonaldehyde do not hydrate to any degree, but they undergo addition reactions with –SH, $–NH_2$ and other groups (Schauenstein *et al*., 1977; Steinhagen and Barrow, 1984). For higher MW, less reactive aldehydes, the sensory irritant effects may be due to a physiological mechanism involving thermodynamic and solubility properties of the molecule in a lipid bilayer containing receptor proteins (Luo *et al*., 1983; Nielsen and Alarie, 1982). It was shown by Gagnaire *et al*. (1993) that

Table 2 The effect of molecular weight on the sensory irritant potential of a homologous series of *N*-(3′-hydroxy-4′-methoxyphenyl)-2-chloroamides as shown by the guinea pig blepharospasm test[a]

Chloroalkylamide[b]	Number of side chain C atoms	EC_{50} (95% CL)[c] (molar)
2-Chlorobutyramide	4	$5.37\ (4.27–6.75) \times 10^{-5}$
2-Chlorovaleramide	5	$1.20\ (1.05–1.66) \times 10^{-5}$
2-Chlorohexamide	6	$7.08\ (5.45–9.20) \times 10^{-6}$
2-Chloroheptamide	7	$2.83\ (2.18–3.68) \times 10^{-6}$
2-Chlorooctamide	8	$2.03\ (1.57–2.63) \times 10^{-6}$
2-Chlorononamide	9	$9.50\ (7.15–12.6) \times 10^{-6}$

[a]Data from Ballantyne (1999).
[b]Attached to 3′-hydroxy-4′-methoxyphenyl nucleus.
[c]EC_{50} = effective concentration 50% to cause blepharospasm (with 95% confidence limits).

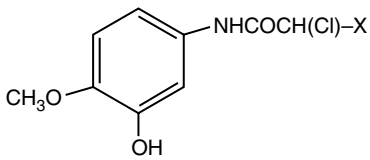

Figure 2 Nucleus for a series of *N*-(3′-hydroxy-4′-methoxyphenyl)-2-chloroamides tested for determination of the PCSI RD_{50} by the guinea pig blepharospasm procedure.

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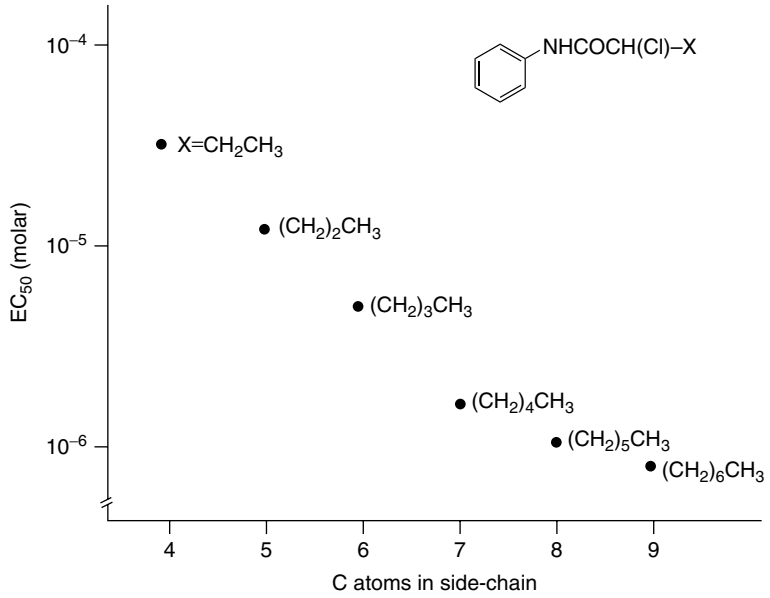

Figure 3 The influence of molecular weight (MW) on peripheral sensory irritant response for a homologous series of *N*-(3′-hydroxy-4′-methoxyphenyl)-2-chloroamides in a guinea pig blepharospasm test. (Source Ballantyne, 1999.)

Table 3 Depression of respiratory rate by 50% produced by vapour of several monoamines in male Ssc:CF-1 or Swiss OF_1 mice by nasal breathing (nRD_{50}) and breathing through a tracheal cannula (tRD_{50})[a]

Amine	Formula	Molecular weight	nRD_{50} (ppm)	tRD_{50} (ppm)	t/n[b]	logP[c]
Primary saturate aliphatic monoamines						
n-Propyl	$CH_2CH_2CH_2NH_2$	59.12	115	500	4.35	0.58
i-Propyl	$(CH_2)_3CHNH_2$	59.12	157	489	3.11	0.41
n-Butyl	$CH_2CH_2CH_2CH_2NH_2$	73.13	84	226	2.69	1.13
i-Butyl	$(CH_3)_2CHCH_2HNH_2$	73.13	91	406	4.46	0.92
n-Pentyl	$C_5H_{11}NH_2$	87.23	64	119	1.86	1.68
n-Hexyl	$CH_3(CH_2)_5NH_2$	101.25	42	93	2.21	2.11
n-Heptyl	$CH_3(CH_2)_6NH_2$	115.28	25	62	2.48	3.00
n-Octyl	$CH_3(CH_2)_7NH_2$	129.31	17	35	2.06	3.10
t-Octyl	$(CH_3)_3CCH_2C(CH_3)_2NH_2$	129.31	80	96	1.20	2.19
Secondary saturated aliphatic monoamines						
Diethyl-	$(C_2H_5)_2NH$	73.13	184	549	2.98	0.58
Di-*n*-propyl	$(CH_3CH_2CH_2)_2NH$	101.19	92	222	2.41	1.69
Di-*i*-propyl	$[(CH_3)_2CH]NH$	101.69	161	102	0.63	2.91
Di-*n*-butyl	$(CH_3CH_2CH_2CH_2)_2NH$	129.31	173	106	0.61	2.81
Di-*i*-butyl	$[(CH_3)_2CHCH_2]_2NH$	129.31	300	289	0.96	2.67
Tertiary saturated aliphatic monoamine						
Tri-ethyl	$(C_2H_5)_2N$	101.19	186	691	3.72	1.45
Primary unsaturated aliphatic monoamine						
Allyl-	$CH_2{=}CHCH_2NH_2$	57.10	9	56	6.27	0.15
Secondary unsaturated aliphatic monoamine						
Diallyl	$(CH_2{=}CHCH_2)_2NH$	97.16	4	157	39.25	1.98
Primary alicyclic monoamine						
Cyclohexyl-	$C_6H_{11}NH_2$	99.18	27	78	2.87	1.54

[a]Data from Gagnaire *et al*. (1989), Gagnaire *et al*. (1993) and Nielsen and Yamagiwa (1989).
[b]Ratio of tRD_{50}/nRD_{50}.
[c]LogP_{ow} = *n*-octanol–water partition coefficient.

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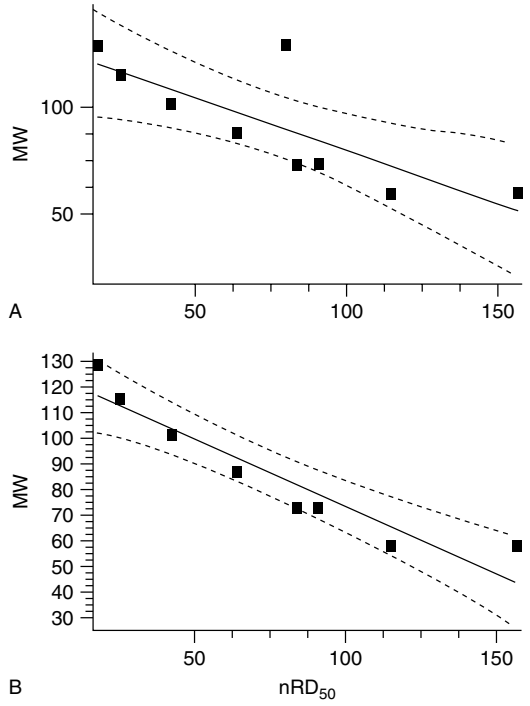

Figure 4 Linear regression analysis for assessment of the correlation between nRD_{50} in mice and molecular weight (MW) for the primary aliphatic monoamines listed in **Table 2**. (A) Analysis for all nine primary amines shown in **Table 2**; $r = -0.79$, $p = 0.01$. (B) Analysis for primary amines excluding *t*-octylamine; $r = -0.9874$, $p = 0.0006$.

RD_{50} values for aliphatic amines were related to their octanol–water partition coefficients (P_{ow}), with a linear relationship between lipophilicity and sensory irritation. For the series of monoamines shown in **Table 3**, the primary saturated aliphatic compounds show a strong association between PCSI potential (as RD_{50}) and log P_{ow} (**Figure 5A**; $r = -0.9524$, $p = 0.0003$), indicating a linear relationship between lipophilicity and PCSI potency. However, this does not extend to the secondary saturated aliphatic monoamines (**Figure 5B**; $r = 0.3945$, $p = 0.51$). Cometto-Muñiz and Cain (1995) studied a homologous series of aliphatic alcohols, ketones and alkylbenzenes, and found that thresholds for eye irritant decreased with increasing C chain length, implicating lipophilicity in the irritancy potential. Dudek *et al.* (1992) compared the Swiss–Webster mouse RD_{50} value for toluene (4900 ppm) with those for various halogenated derivatives of toluene, which were found to be significantly more irritant (range 4.3–27 ppm). They believed that their findings were compatible with the interaction of these PCSI molecules with a receptor protein in a lipid bilayer. The trends in RD_{50} values were related to the development of a partial positive charge on the toluene α-carbon–halogen bond by the positioning of a ring chlorine, and the bond dissociation energies of the α-carbon–halogen bond for the halogenated isomers of benzyl halide. Quantitative structure–activity relationship (QSAR) considerations have suggested the importance of hydrogen bonding in relation to the activation of PCSI receptors by nonreactive volatile organic molecules (Abraham *et al.*, 1990; Nielsen, 1991). The effect of hydrogen bonding on upper respiratory tract irritation, assessed in detail by QSAR, demonstrated that the process involved nonreactive irritants as hydrogen bond acids; that is, the receptor site is a hydrogen bond base. However, the range of nonreactive irritants was not large, with most hydrogen bond acid compounds being simple alcohols. To further investigate this area, Nielsen *et al.* (1996) studied the following three compounds with differing hydrogen bond acidities; methyl hexafluoroisopropyl ether, trifluoroethanol and hexafloropropan-2-ol. The respective RD_{50} values determined in CF-1 mice were $\geq$160 000, 110 400–23 300 and 165 ppm. QSAR showed that methyl hexafluoroisopropyl ether and trifluoroethanol behaved as predicted nonreactive sensory irritants, whereas hexafluropropan-2-ol was

Table 4 Guinea pig blepharospasm studies on a series of *N*-phenyl-2-chloroamides. Over the range of molecular weights tested the PCSI effects were similar[a]

N-Phenyl derivative	Chloroalkyl group (X) attached to *N*-phenylamide[b]	EC_{50} (95% CL) ($\times 10^{-5}$ molar)[c]
2-Chloro-acetamide	$-CH_2Cl$	40.6 (30.5–54.5)
2-Chloro-propionamide	$-CH(Cl)CH_3$	8.70 (6.61–11.43)
2-Choro-butyramide	$-CH(Cl)CH_2CH_3$	4.30 (3.52–5.37)
2-Chloro-valeramide	$-CH(Cl)(CH_2)_2CH_3$	3.54 (2.72–4.60)
2-Chloro-hexanamide	$-CH(Cl)(CH_2)_3CH_3$	2.87 (2.18–3.77)
2-Chloro-heptanamide	$-CH(Cl)(CH_2)_4CH_3$	2.68 (2.05–3.50)
2-Chloro-octanamide	$-CH(Cl)(CH_2)_5CH_3$	1.15 (0.87–1.50)
2-Chloro-nonamide	$-CH(Cl)(CH_2)_6CH_3$	1.07 (0.82–1.40)
2-Chloro-decanamide	$-CH(Cl)(CH_2)_7CH_3$	1.23 (0.92–1.65)

[a]Data after Ballantyne (1999).
[b]Phe-NHCO-X.
[c]Effective concentration 50% (95% confidence limits).

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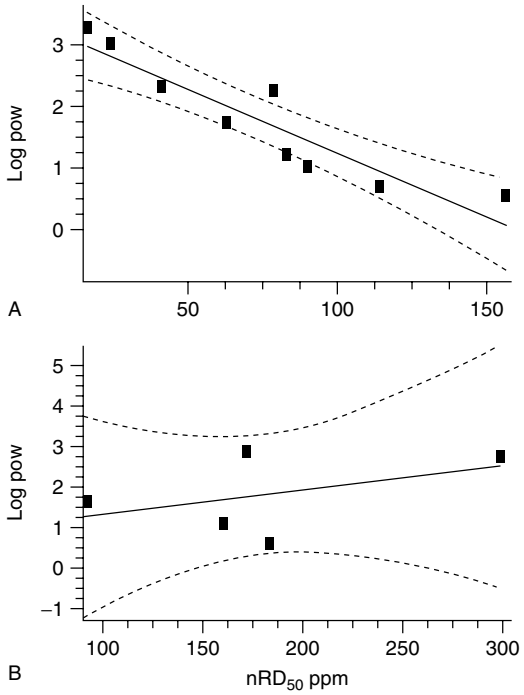

Figure 5 Linear regression analysis for assessment of the correlation between nRD_{50} in mice and P_{ow} (octanol–water partition coefficient) for the saturated aliphatic monoamines shown in **Table 2**. (A) Analysis for all nine primary saturated aliphatic monoamines listed in **Table 2** shows a strong correlation; $r = -0.9524$, $p = 0.0003$. (B) Analysis for the five secondary saturated aliphatic monoamines listed in **Table 2** shows a poor correlation; $r = 0.3945$, $p = 0.51$.

much more potent than predicted, which could result from a coupled reaction involving both strong hydrogen bonding and weak Brønsted activity. Kasanen *et al.* (1998) have studied stereospecificity in detail.

5 THE FUNCTIONAL BIOLOGICAL AND PHYSIOLOGICAL SIGNIFICANCE OF PERIPHERAL CHEMOSENSORY IRRITANCY

There are several inter-related aspects of the functional biological aspects of the PCSI response in normal biological circumstances, as discussed below.

5.1 Biological Warning and Protection Functions

The local sensations produced in the PSI response give a warning that such materials, which may produce tissue injury at higher concentrations, are present in the immediate environment. The sensations produced cause the affected individual to seek an uncontaminated area. The local reflexes resulting from sensory irritation are important from the viewpoint of protective mechanisms that limit further exposure. For example, with ocular sensory irritation the resultant excess lacrimation removes material from the corneal and conjunctival surfaces, and blepharospasm restricts further access of material to the eye. Sensory irritation of the respiratory tract results in decreased breathing rate, decreased TV and cough, all of which limit inhalation of the irritant material. However, certain potent PSI materials may inhibit the phagocytic capacity of pulmonary macrophages (Hogg *et al.*, 1983). Because many materials causing a PCSI effect may also produce tissue injury (inflammation) at higher exposure concentrations, a determination of the exposure conditions leading to a PCSI response can be an important factor in determining, or quantifying, what are suitable safe occupational or environmental exposure limits (Ballantyne, 1983). For materials that produce a PCSI response, the eye irritation threshold is usually above odour thresholds. For example, Cometto-Muñiz and Cain (1995) studied a homologous series of aliphatic alcohols, ketones and alkylbenzenes, and found that the ratio of eye irritation thresholds to odour thresholds varied from 15 with 2-propanone to 2 695 for propyl benzene, This indicates that with a known PCSI material, the odour may increase the warning margin of exposure to the material.

Whilst the biological importance of a PSI response is clear, it is also obvious that the absence of such a response with a material capable of causing tissue injury can be a potential hazardous situation. Such an absence of a warning on contact can be conducive to local injury. Examples of materials that may injure due to the absence of sensory warning on contact include dimethyl sulfate and methyl bromide (Grant and Schuman, 1995). In these circumstances it may be required to add a material with PSI properties; for example, addition of chloropicrin to ethyl bromide used for fumigation purposes.

5.2 Harassment and Incapacitation

Both the sensory effects (discomfort and pain) and the local reflex effects may be distracting and harassing, and thus produce variable degrees of incapacitation. For example, blepharospasm and excess lacrimation result in an impairment of vision. This may be detrimental to efficient working, and also may predispose the affected individual to accidents. Therefore, PCSI is an important consideration in relation to safe working conditions.

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5.3 Effect on Pulmonary Defence Mechanisms

The effect of PCSI materials on the pulmonary defence mechanisms is variable. For example, sulphur dioxide and formaldehyde at low concentrations may stimulate phagocytosis (Katz and Laskin, 1976), whereas other PCSI materials may inhibit the phagocytic capacity of pulmonary macrophages. A dose-related inhibition of the normal phagocytic response of the pulmonary alveolar macrophage has been shown with cigarette smoke (Green and Carolin, 1967) and marijuana smoke (Cutting *et al*., 1974). In a study of the effect of dibenz[b.f]-1,4-oxazepine (CR) and 10 of its derivatives on the phagocytosis of rabbit pulmonary alveolar macrophages towards *Staphylococcus epidermis*, it was found that CR was the most potent inhibitor, and the 1- and 2-chloro-derivatives were the least potent (Hogg *et al*., 1983). Whereas inhibition of phagocytosis was dose dependent, the degree of inhibition was not correlated with either the nature or the position of the substituent groups on the CR molecule. Also, no discernable relationship existed between the inhibition of phagocytosis and the sensory irritancy of the compounds tested.

6 FACTORS INFLUENCING THE PERIPHERAL CHEMOSENSORY IRRITANCY RESPONSE

Numerous endogenous and exogenous factors may influence the concentration or dosage required to induce a PCSI response, its subjective severity, and its latency and duration. Some of the more important of these factors are discussed below.

6.1 Concentration

PCSI effects are not produced until a certain concentration of the causative substance [the threshold concentration (TC)] is attained. At suprathreshold concentrations, the exposure concentration is a determinant of the following.

- The proportion of the exposed population that respond; the higher the concentration, the larger the proportion of the population that are affected. On a linear basis, in general there is a typical biological sigmoid curve (with proportionate values) or Gaussian curve (with absolute data). This is illustrated in **Figure 6**.

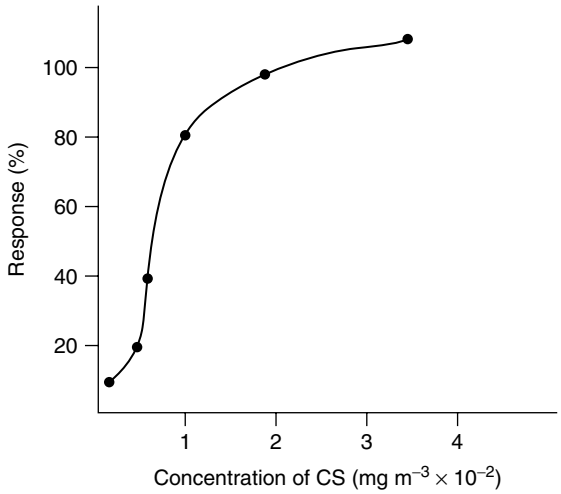

Figure 6 Sigmoid concentration–response curve of the threshold PCSI response for sensation induced in the human eye by an aerosol of 2-chlorobenzylidene malononitrile (CS). The % response is the proportion of the population responding for each concentration tested. By probit analysis, the concentration causing a 50% response in the population studied (TC_{50} with 95 confidence limits) is 4.0 (2.3–6.6) × 10^{-3} mg m^{-3}. (Ballantyne, 1999).

- The latency to onset of PCSI effects; the higher the concentration, the shorter the time to onset of effects (**Figure 7**).
- The duration of the sensory irritant effect, particularly after a brief (pulsed) exposure. The higher the concentration, in general the longer is the persistence of the PCSI effect (**Figure 7**).
- The subjective experience of the response. For example, the higher the concentration of PCSI material applied to the eye, the more marked is the induced blepharospasm (e.g. intermittent to sustained) and the more severe is the induced pain.

Although concentration is an overall determinant of sensory irritant response, molecular size and shape is most likely an integral component of this determinant. For example, Cometto-Muñiz *et al*. (2006; 2007) studied 'cut-off' effects for the detection of ocular irritation for vapours of homologous *n*-alkylbenzenes, 2-ketones, aldehydes and carboxylic acids, the results of which indicated that cutoffs rest on limitations related to the dimensions of the molecules rather than limitations related to their vapour concentration. For example, the stimulus molecule could exceed the size that permits it to fit into the receptor pocket of a receptive protein.

6.2 Particle Size

For PSI materials in particulate form, size may be an important determinant of both the severity and the site of

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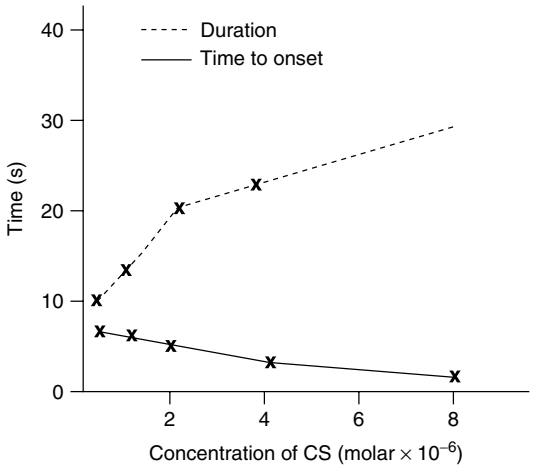

Figure 7 Effect of the concentration of 2-chlorobenzylidene malononitrile (CS) on the latency to onset (solid line) and duration (interrupted line) of discomfort in the human eye. As the concentration of CS in solution applied to the eye increases, latency decreases and the duration of the induced effect increases (Data after Ballantyne and Swanston, 1973a.)

the response. For example, small particles of respiratory dimensions rapidly produce both ocular and respiratory PSI effects, whereas larger particles produce predominantly ocular irritation with prolonged recovery. This was shown by Owens and Punte (1963) using particles of *o*-chlorobenzylidene malononitrile having respective sizes of 1 and 60 μm mass median aerodynamic diameter (MMAD).

6.3 Motivation and Distraction

Both increased motivation and distractions can raise the threshold for the induction of PCSI effects, and enhance tolerance for suprathreshold sensory irritancy. For example, in the workplace the concentration at which sensory discomfort becomes apparent with a particular material may be significantly higher than that causing threshold effects with the same material tested under carefully controlled laboratory conditions. It thus follows that the quantitative values obtained for sensory irritant effects in carefully controlled experimental situations probably represent the most sensitive index for those effects, and thus represent the most reliable and safest estimates on which to base occupational exposure guidelines (OEGs).

6.4 Tolerance

Subjects may develop tolerance to a PSI material if exposure is gradual and at low concentration; the individual adapts by a decrease in the apparent feeling of discomfort and harassing effects (Beswick *et al*., 1972). The development of tolerance is clearly of importance in the workplace, since an adaptation to a warning of potentially harmful chemicals may lead to a decrease in safe working conditions, particularly if there is not strict adherence to carefully derived occupational exposure limits (OELs).

6.5 Personality

There are significant personal and psychological factors in tolerance to pain, and a similar variability exists between individuals with respect to the discomfort produced by PCSI materials. Thus, under a given set of circumstances some will tolerate the discomfort more readily than others. This also is a factor to be considered when assessing the variability to sensory irritants in the workplace.

6.6 Personal Habits and State of Health

Smoking, medication and certain diseases may affect susceptibility to PCSI materials. For example, in a study of worker sensitivity to sodium borate dust, Woskie *et al*. (1998) found that those who smoked, used nasal sprays, or who had allergies or colds were more sensitive to sensory irritant effects, and Dunn *et al*. (1982) showed that smokers were less sensitive to nasal CO_2 irritation.

6.7 Temperature and Humidity

It has been demonstrated that increased environmental temperature and humidity may decrease endurance to peripheral sensory irritation (Punte *et al*., 1963).

6.8 Vehicle

With sensory irritants in solution the vehicle used to dissolve or suspend the active PCSI substance may modify its irritant potential. Surface-active vehicles, for example, can facilitate penetration and enhance the PCSI response, as shown by a lowering of the concentration necessary to induce both threshold and incapacitating effects.

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7 QUANTITATION AND INTERPRETATION OF PERIPHERAL SENSORY IRRITANT OBSERVATIONS

As noted above (Section 6.1), there is usually a clear positive relationship between exposure concentration for a PCSI material and the proportion of the exposed population responding, and this is sigmoid or Gaussian in form (**Figure 6**). This indicates that whilst the majority of the exposed population respond over a well-defined region about the median, a small proportion (at the left-hand side of the curve) is hyper-reactive to peripheral sensory irritant stimulation and a small proportion (to the right) is hyporeactive. For ease of presentation and calculations the concentration–response data are usually converted to a linear configuration by a log-probit plot. It is conventional to calculate a 50% response level, with 95% confidence limits (CLs), in order to allow statistical comparison of the relative potency of the PCSI response under defined conditions. This comparison should also include a determination of the slope on the concentration–response regression line, in order to obtain the best possible estimate of comparative sensory irritant potency. A variety of values, discussed below, are calculated to allow the sensory irritant response to be quantified.

For effects that can be objectively evaluated it is common to refer to the effective concentration (EC); that is, the exposure concentration that produces the specific effect under consideration. When sufficient data exists it is common to calculate the sensory irritant potency as the EC_{50}; that is, the concentration, calculated from the exposure concentration–response data, which causes a specific irritant response to occur in 50% of the exposed population under the particular conditions of the observations. Examples of effects that may be quantified and expressed as an EC_{50} include depression of breathing rate, blepharospasm and subjectively assessed discomfort (e.g. pain). Certain defined effects may be referred to by specific expressions; for example, a 50% depression in breathing rate is usually referred to as the RD_{50}. For the subjective evaluations in humans, such as the degree of discomfort, it is also possible to determine and cite 50% response levels; for example, thethreshold concentration 50% (TC_{50}) and, above TCs, if the degree of discomfort is severe, the incapacitating (intolerable) concentration 50% (IC_{50}) can be obtained. In some situations it may be useful to calculate different levels of incapacitation; for example, IC_{10} or IC_{90}.

As with proportion of the population responding to various exposure concentrations of a PCSI challenge, there is also variability between individuals in the latency to effect and duration of effect. Therefore a complete description of a PCSI response requires information on the exposure concentration, proportionate response, latency and duration of response. **Figure 8A** shows the relationship between the exposure concentration

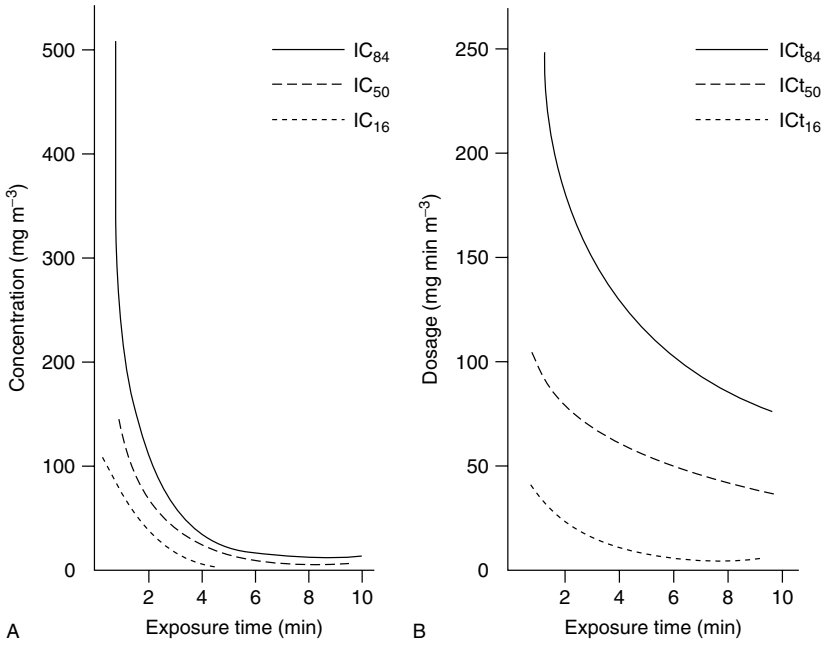

Figure 8 Graphical representation showing the relationship between the atmospheric concentration of 1-chloroactephenone (CN) and the exposure time (A) and the exposure dosage (Ct) of CN and time (B) required to produce defined levels of incapacitation. The incapacitating concentrations (IC) and incapacitating doses (ICt) are plotted as a function of time required to cause intolerable effects in 16, 50 and 84% of the population studied. (Data after McNamara *et al.*, 1968.)

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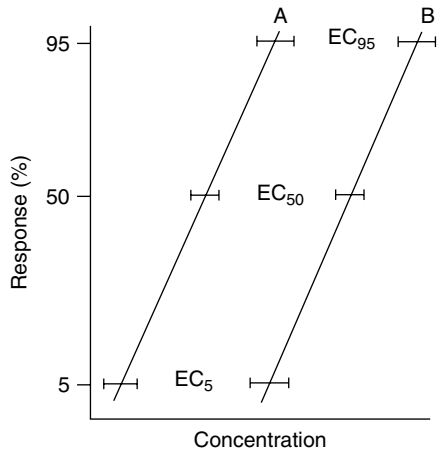

Figure 9 Comparison of two PCSI materials (A and B) having significantly different EC_{50} values, but similar slopes of the concentration–response regression lines. In view of the parallel nature of the two regression lines, the EC_5 and EC_{95} values are also significantly different for materials A and B. Thus, A and B are biologically significantly different from each other, with respect to PCSI potency, over a wide concentration range.

and exposure time to produce different degrees of incapacitation in a particular group of human subjects. It can be seen that for any given exposure concentration, the proportion of the population responding increases with exposure time, reflecting variable latency within the group. Also, and a further indication of variability, within limits a given degree of incapacitation can be produced by various reciprocally related exposure times and exposure concentrations. The reciprocal relationship between exposure time and concentration to produce a given degree of incapacitation also has implications for the exposure dosage (Ct) received. **Figure 8B** shows that for any given level of incapacitation the exposure dosages are greater for the high concentration–short exposure duration conditions than for the lower concentration–more prolonged conditions. Thus, whilst increasing the exposure concentration shortens latency to response, it also results in a greater exposure dosage to produce a given equipotent incapacitating effect.

Ideally, data derived from the use of animal models should indicate the range of concentrations over which PCSI effects may be expected to occur in humans. However, as discussed later, most animal models are less sensitive than the human to PSI materials. In general, animal models will detect those materials having a potential to produce PCSI effects in humans, but normally do not rank different materials in their order of potency for the human. Also, they do not accurately predict the concentration range over which effects may be anticipated in humans.

In comparing the relative potency of different PCSI materials, it is important not only to compare the EC_{50} and 95% CL, but also to examine the slope of the regression line. For example, if two different PCSI materials have significant EC_{50} values and the slopes of the regression lines on the dose–response lines are similar, then it may be concluded that the materials are not significant, with respect to PCSI potency, over a wide exposure concentration range (**Figure 9**). In contrast, even with materials of closely comparable EC_{50} values, if the slopes are different this may have important practical relevance with respect to proportionate population response. This is illustrated in **Figure 10** for two materials having the same EC_{50} values, but differing slopes. Due to the differing slopes, the values of the EC for the two materials differ significantly from each other at both high response (e.g. EC_{95}) and low response (e.g. EC_5) levels. It follows that for the material having the steeper slope, once the TC is exceeded then only a small incremental increase in exposure concentration is required to cause an effect in the majority of the population. Thus, in such situations in the workplace it may be necessary to add a wider concentration margin below the relatively narrow concentration range required to convert a threshold to an incapacitating effect. In contrast, with the material having the shallower slope the concentration range necessary to convert a small response rate to a large response rate will be much greater. However, in these circumstances it may be

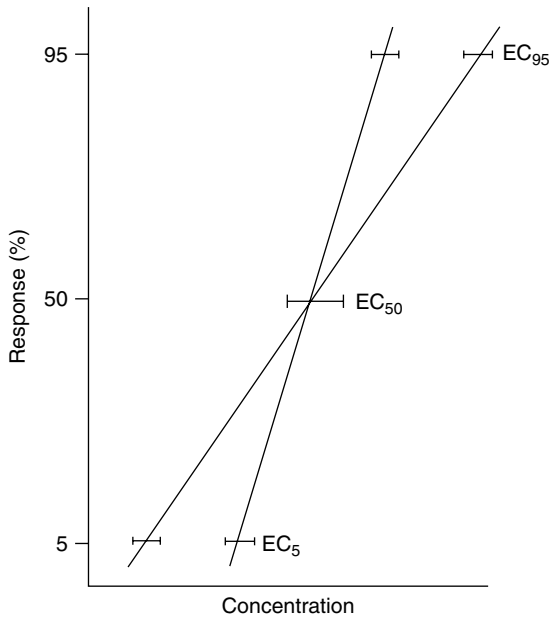

Figure 10 Comparison of two PCSI materials having the same EC_{50} value, but differing slopes on the concentration–response regression lines. This results in the respective EC_5 and EC_{95} values being significantly different from each other. This has implications with respect to hyper-reactive individuals in the group with the shallow slope, and safety margins for the group with the steeper slope (see text; Section 7).

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necessary to consider the measures necessary to protect the small proportion (EC_5) that is hypersensitive.

When differences in slopes are present in a comparative evaluation, there are two major implications. First, the relative potency of two materials may not be the same at different levels of response. Second, it may not be statistically valid to calculate a potency ratio. However, in such circumstances it may be useful to calculate the comparative potencies (CPs) at different levels of irritancy. This is defined as follows (Ballantyne, 1977):

$$CP_x = [A] \text{ causing } (x) \text{ response}/[B] \text{ causing } (x) \text{ response}$$

where [A] and [B] are concentrations of PCSI materials A and B, and (x) is a specific response rate.

For individual specific materials, it is often useful to compare their threshold and ICs; for example, IC_{50}/TC_{50}. This effectiveness ratio (ER) can be used to indicate if the threshold value gives an adequate warning of potentially incapacitating exposure; the greater the value of the ratio, the more effective is the warning of the threshold effect. The value can also be used to show how many times the TC has to be increased to result in an incapacitating effect. **Table 5** gives an example of values for the CP of *o*-chlorobenzylidene malononitrile (CS) and dibenz(b.f)-1,4-oxazepine (CR), and the ERs for the individual materials. It can be seen that as the concentration increases, the comparative potency of CR is greater than that for CS; thus the aerosol concentration of CS required to attain a TC_{50} is twice that for CR, for an IC_{50} is 5.1 times and for an IC_{75} response is 9.1 times. Also, there is a wide margin between the TC_{50} and IC_{50} for CS and CR, indicating a significant PCSI warning at the TC_{50}. However, the increase in concentration required to convert threshold to incapacitating effects is greater with CS (900×) than with CR (350×), indicating the latter to have a greater PCSI potency. A similar situation applies with the IC_{75}, but the proportionate increases required are even greater with CS.

8 METHODOLOGIES FOR ASSESSING SENSORY IRRITANT POTENTIAL

Nonanimal methods for determining the potential of substances to produce PCSI effects have included QSAR considerations and chemical models. Biological approaches include the use of *in vitro* and *in vivo* models, and the controlled exposure of human volunteer subjects. Some of these approaches with their relative advantages and disadvantages are considered below.

8.1 Chemical Models

Some correlations have been made between PCSI potential and certain physicochemical characteristics, although this has been only for a limited series of materials, and comparisons between irritant and nonirritant materials are sparse. In an effort to predict what materials are likely to have PCSI properties, various artificial membranes have been devised to simulate biological membranes. These have included monolayers and bilayers of lecithin, with monitoring of phase transitions to measure interaction with a PCSI. Approaches used to investigate transitions have included differential scanning calorimetry, electron spin resonance and X-ray diffraction. In general, the use of such chemical membrane simulants has not been a reliable predictor for PCSI effects.

8.2 Animal Biological Models

There are several biological models, both *in vivo* and *in vitro* and of varying degrees of sophistication and sensitivity, which have been used to qualitatively or quantitatively assess the PSI potential of various classes of chemicals. These include the following.

Table 5 Comparative potencies (CP) and effectiveness ratios (ERs) for aerosols of 2-chlorobenzylidene malononitrile (CS) and dibenz(b.f)-1,4-oxazepine (CR) by respiratory exposure of male human volunteer subjects[a]

Material	Degree of irritancy (mg/m^3)[b]			ER	
	TC_{50}	IC_{50}	IC_{75}	IC_{50}/TC_{50}	IC_{75}/TC_{50}
CS	4.0×10^{-3}	3.6	10.0	900	2500
CR	2.0×10^{-3}	0.7	1.1	350	550
CP	2.0	5.1	9.1	—	—

[a] Data after Ballantyne (1977).
[b] TC_{50} = threshold concentration 50% (1-minutes exposure).
IC_{50} = incapacitating concentration 50% (1-minutes exposure).

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8.2.1 Isolated Intestinal Segment

This is a nonspecific method based on the use of segments of small intestine, which are suspended in an incubating bath in such a manner that permits contraction of the segment to be mechanically measured. The PSI is added to the bath at varying concentrations, and a record kept of those concentrations causing contraction of the intestinal segment.

8.2.2 Frog Flexor Reflex (FFR)

This approach, also nonspecific, involves a determination of the minimum concentration of irritant that causes withdrawal of the hind limb of decerebrate frogs from test solutions of PCSI materials added to an incubating bath. The method involves the sequential immersion of the hind-limb preparation in increasing concentrations of PCSI substance in solution. After each immersion, the limb is washed with saline and after a period of 5 minutes the limb is immersed in the next solution. The time (T) between immersion of the hind limb and its reflex withdrawal from the solution is noted. The log-molar concentration of PCSI is plotted against T and the curve extrapolated to infinite time by the use of a template of a standard curve, in order to permit the minimum irritant concentration (T_m) to be determined. The mean minimum irritant concentration from six frog preparations ($\check{T}_m$) is used for comparison between different materials. The method is essentially *in vitro*, simple, economic, easy to conduct and has been used by some as a reliable screening test for detection of materials having a PCSI potential (Ballantyne, 1999; Feniak, 1966). However, with some materials the frog flexor reflex (FFR) test will show poor reproducibility and tachyphylaxis may develop. Although the FFR test is relatively insensitive compared with other methods, such as the *in vivo* GPB and mouse depression of respiration (RD_{50}) tests (**Table 6**), the procedure correlates well ($r = 0.99$) with the GPB test (**Table 7**), but is less well correlated with the mouse RD_{50} ($r = 0.76$) (**Table 8**).

Table 6 Relative sensitivity of the frog flexor reflex (FFR), guinea pig blepharospasm (GPB) and mouse respiratory rate depression (RRD) tests[a]

Compound[b]	FFR $\check{T}_m$ (molar)[c]	GPB EC_{50} (molar)[d]	RRD RD_{50} (molar)[e]
CS	9.78×10^{-6}	2.16×10^{-5}	6.00×10^{-8}
CR	1.10×10^{-4}	9.00×10^{-5}	3.40×10^{-7}
HMPC	2.14×10^{-6}	7.70×10^{-7}	3.82×10^{-8}

[a] Data after Ballantyne (1999).
[b] CS = 2-chlorobenzylidene malononitrile.
CR = dibenz(b.f)-1,4-oxazepine.
HMPC = *N*-(4′-hydroxy-3-methoxyphenyl)-2-chloro-octanamide.
[c] $\check{T}_m$ = mean minimum irritant concentration.
[d] EC_{50} = effective concentration 50%.
[e] RD_{50} = depression of respiration 50%.

8.2.3 Neurophysiological Preparations

Several peripheral nerves known to contain afferent nerves carrying PCSI-induced action potentials from skin or mucosal surfaces have been used to detect materials having sensory irritant properties. Measurement

Table 7 Comparative peripheral chemosensory irritant potential for various chemicals measured by the frog flexor reflex (FFR) and guinea pig blepharospasm (GPB) tests[a]

Compound	FFR $\check{T}_m$ (molar)[b]	GPB EC_{50} (molar)[c]
N-(4′-Hydroxy-3′-methoxyphenyl) 2-chlorodecanamide	1.64×10^{-6}	3.58×10^{-7}
N-(3′-Hydroxy-3′-methoxyphenyl) 2-chloroheptanamide	1.89×10^{-6}	2.83×10^{-6}
N-(3′-Hydroxy-4′-methoxyphenyl) 2-chloronanamide	1.97×10^{-6}	9.50×10^{-7}
N-Nonanoylvanillylamide	2.00×10^{-6}	3.30×10^{-7}
N-(4′-Hydroxy-3′-methoxyphenyl) 2-chlorononamide	2.10×10^{-6}	3.17×10^{-7}
N-(4′-Hydroxy-3′-methoxyphenyl) 2-chloro-octanamide	2.14×10^{-6}	7.69×10^{-7}
N-(3′-Hydroxy-4′-methoxyphenyl) 2-chloro-octanamide	3.32×10^{-6}	2.03×10^{-6}
N-(4′-Hydroxy-3′-methoxyphenyl) 2-chloroheptanamide	5.23×10^{-6}	1.90×10^{-6}
2-Chlorobenzylidene malononitrile	9.78×10^{-6}	2.16×10^{-5}
Dibenz(b.f)-1,4-oxazepine	2.09×10^{-5}	3.48×10^{-5}
4-Methyldibenzoxazepine	2.20×10^{-6}	9.30×10^{-5}
3-Methyldibenzoxaxepine	4.16×10^{-5}	5.40×10^{-5}
β,β-Diacetyl-3-chlorostyrene	9.43×10^{-5}	1.30×10^{-4}
1-Chloroacetophenone	1.0×10^{-4}	9.00×10^{-5}
Phenanthrine	1.77×10^{-3}	4.11×10^{-3}

[a] Data after Ballantyne (1999).
[b] $\check{T}_m$ = mean minimum irritant concentration.
[c] EC_{50} = effective concentration 50%.
Correlation coefficient (r) = 0.99 ($p < 0.001$).

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Table 8 Comparative peripheral chemosensory irritant potential for various chemical classes measured by the frog flexor reflex (FFR) and mouse respiratory rate depression (RRD) tests[a]

Compound	FFR $\check{T}_m$ (molar)[b]	RRD RD_{50} (molar)[c]
o-Chlorobenzylidene malononitrile	9.78×10^{-6}	6.00×10^{-8}
Dibenz(b.f)-1,4-oxazepine	2.09×10^{-6}	2.30×10^{-7}
ω-Chloroacetophenone	1.10×10^{-4}	3.40×10^{-7}
N-(4′-hydroxy-3′-methoxyphenyl) 2-chloro-octanamide	2.14×10^{-6}	3.82×10^{-8}
N-(4′-hydroxy-3′-methoxyphenyl) 2-chlorononamide	2.10×10^{-4}	1.43×10^{-7}
N-(4′-Hydroxy-3-methoxyphenyl) 2-chlorodecanamide	1.64×10^{-6}	2.16×10^{-7}

[a]Data after Ballantyne (1999).
[b]$\check{T}_m$ = mean minimum irritant concentration.
[c]RD_{50} = respiratory rate depression 50%.

of afferent nerve activity has been used with the following neurophysiological preparations.

8.2.3.1 Ciliary Nerve

Ciliary nerve recording following corneal stimulation is frequently undertaken using an excised cat eye mounted on a warm chamber, with the cornea exposed and the attached long ciliary nerve laid over recording electrodes (Green and Tregear, 1964). Test sensory discharge can be induced by light tactile stimuli or cooling the surface. The respective thresholds are 0.1–1.0 g and 5–20 °C (Green and Tregear, 1964).

8.2.3.2 Nasopalatine, Ethmoidal and Sphenoidal Nerves

Nasopalatine, ethmoidal and sphenoidal recording following nasal mucosal stimulation has been carried out with success in the rat (Cooper, 1970; Kulle and Cooper, 1975). For example, Tsubone and Kawahi (1991) used the ethmoidal nerve to record afferent impulses during exposure of the rat to various irritant gases. They found that formaldehyde and acrolein stimulate nasal mucosal sensory endings at about 1.0 ppm, with acetaldehyde producing a significantly lesser degree of stimulation. The concentrations producing a 50% increase in nerve activity were 1.8 ppm for formaldehyde, 1.2 ppm for acrolein and 908 ppm for acetaldehyde.

8.2.3.3 Laryngeal Nerve

Laryngeal nerve recording has been conducted following PCSI stimulation of the laryngeal mucosa. It is usual to conduct the study in anaesthetized and tracheotomized rats, with action potentials recorded from the recurrent laryngeal nerve. Unlike the corneal (ciliary nerve) preparation, the laryngeal model is more complex and slowly adapting (Dirnhuber *et al*., 1965).

8.2.3.4 Cutaneous Nerves

For cutaneous application of a PCSI material, a convenient model is by topical application to the hind-limb, with recording of afferent action potentials from the saphenous nerve (Foster and Ramage, 1981).

8.2.4 Blepharospasm Test

The induction of blepharospasm in conscious animals in response to topical application of a PCSI substance to the cornea, or from exposure to atmospherically dispersed material, is a frequently used approach to assess the PCSI potential on the cornea. It is a simple and reliable *in vivo* method, which can also be performed in most species, including human subjects. For solutions, the test involves sequentially applying increasing concentrations to the surface of the cornea and noting the proportion of the test population that develop blepharospasm following the initial blink reflex. In this way, the response rate can be calculated and an EC_{50} for blepharospasm calculated. If the test substance is in solution, or has to be diluted, it is important that an inert be used. For materials dispersed in the atmosphere a similar observation is employed to sequential increasing concentrations of the test substance.

There is species variation in the sensitivity of the blepharospasm test for the same substances applied to the eye (**Table 9**). In general the guinea pig is the most suitable test species because of cost, size and sensitivity to PSI materials. It is reproducible, free from tachyphylaxis, and differentiates PCSI molecules having close similar structures. However, it does not correlate well with the depression of breathing rate test in the mouse ($r = 0.68$; **Table 10**).

In addition to detecting chemicals having PCSI potential, the GPB test has found use in various drug applications. For example, it has been successfully used as a screen for the development of comfortable ophthalmic drug preparations. In studies by Bar-Ilan (1997), results from a GPB test were compared with the number of human subjects who noted pain or discomfort with various concentrations (2.5–17.5%) of sulfacetamide. The dose–response curves for the guinea pigs and humans were essentially identical, with a threshold at 5% sulfacetamide and a linear increase up to a maximum at 12.5–15.0%. The good relationship between the GPB test and the human nociceptive response with sulfacetamide indicated the usefulness of the procedure for predicting the degree of ocular discomfort in humans.

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Table 9 Comparison of the blepharospasm-inducing effects in various species of solutions (in polyethylene glycol 300) of 2-chlorobenzylidene malononitrile (CS) and dibenz(b.f)-1,4-oxazepine (CR) applied topically to the cornea[a]

Material	Blepharospasm as EC_{50} with 95% CL (molar)[b]		
	Guinea pig	Rabbit	Human
CS	2.2 (1.9–2.4) $\times 10^{-5}$	5.9 (3.8–10.0) $\times 10^{-5}$	3.2 (2.1–6.1) $\times 10^{-6}$
CR	3.5 (2.8–4.3) $\times 10^{-5}$	7.9 (5.1–12.5) $\times 10^{-5}$	8.6 (6.8–12.5) $\times 10^{-7}$

[a]Data after Ballantyne and Swanston (1973a, 1973b).
[b]Results as effective concentration 50% (with 95% confidence limits).

Table 10 Comparison of the peripheral chemosensory irritant potential for various classes of compounds as assessed by the guinea pig blepharospasm (GPB) and mouse depression of respiratory rate (DRR) tests[a]

Compound	DRR RD_{50} (molar)[b]	GPB EC_{50} (molar)[c]
N-Undec-10-enoyl-4-hydroxy-3-methoxy benzylamine	2.04×10^{-8}	1.55×10^{-7}
cis-N-(4-cyclohexylmethyl)-cyclohexylacetamide	2.95×10^{-8}	8.83×10^{-7}
N-(4′-Hydroxy-3-methoxyphenyl) 2-chloroheptanamide	3.82×10^{-8}	7.69×10^{-7}
2-Chloro-3,4-dimethoxy-Ω-nitrostyrene	7.10×10^{-8}	9.26×10^{-6}
N-(4′-hydroxy-3′-methoxyphenyl) 2-chlorononamide	1.43×10^{-7}	3.17×10^{-7}
N-(4′-hydroxy-3-methoxyphenyl) chlorodecanamide	2.16×10^{-7}	3.58×10^{-7}
Dibenz(b.f)-1,4-oxazepine	2.30×10^{-7}	3.48×10^{-5}
N-Phenyl-2-chloro-actanamide	3.08×10^{-7}	1.15×10^{-5}
1-Chloroacetophenone	3.40×10^{-7}	9.00×10^{-5}

[a]Data after Ballantyne (1999).
[b]Depression of respiratory rate 50%.
[c]Effective concentration 50%.
Correlation coefficient (r) = 0.60 (p = 0.06).

8.2.5 Depression of Breathing Rate

Inhalation of PSI materials by laboratory animals results in a decrease in breathing rate, which is reflexly induced by stimulation of trigeminal sensory receptors in the nasal mucosa. The decrease in breathing rate is due to a pause in the expiratory phase of the breathing cycle, resulting in a characteristic notching pattern (**Figure 11**). The approach basically involves head-only exposure of animals to various concentrations of test material, and recording the breathing rate by means of a plethysmograph. Using a minimum of four animals, the mean proportionate decrease in breathing rate for a given concentration is calculated. Up to a limiting value, there is usually a relation between the increase in concentration (C) of the test substance inhaled and the resultant decrease (D) in breathing rate (**Figure 12**). Since C and C/R are linearly related, a least squares regression between the two can be undertaken and the concentration causing a 50% depression in the breathing rate (RD_{50}) calculated. To ensure that the most sensitive response is obtained, preliminary studies are necessary to determine if the exposure time is long enough to obtain the maximum decrease in breathing rate at the exposure concentrations. This can vary with different chemicals. For example, with glutaraldehyde vapour

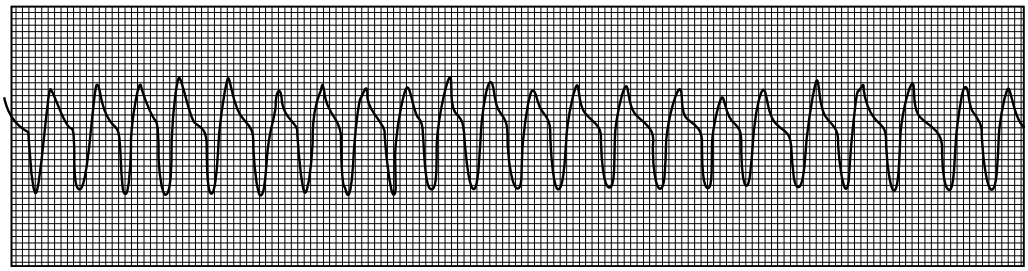

Figure 11 Effect of exposure to glutaraldehyde vapour on the breathing cycle of the mouse. The respiratory cycle shows a prolongation of the expiratory phase that produces a characteristic notching.

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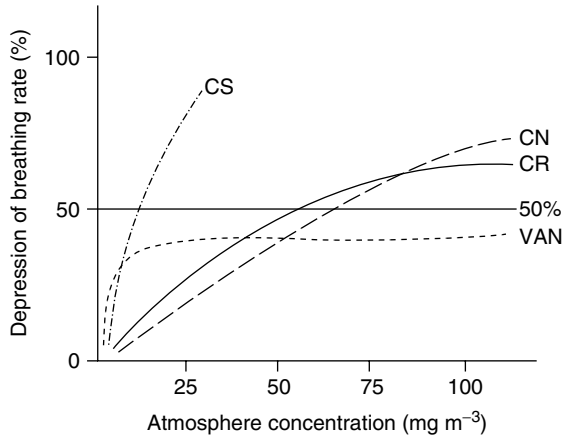

Figure 12 Relationship between depression of breathing rate in the conscious mouse and the airborne concentration of several PCSI substances: CS = 2-chlororbenzylidene malononitrile; CN = 1-chloroacetophenone: CR = diben[b.f]-1,4-oxazepine: VAN = *N*-nonanoylvanillylamide. (Data after Ballantyne *et al*., 1977.)

a plateau for decrease in breathing rate was obtained within 5–10 minutes (**Figure 13**), and with methyl isocyanate within 10–15 minutes (Ferguson *et al*., 1986). If the exposure time is not sufficiently long (i.e. a plateau is not reached), then a misleadingly high value may be obtained; for example, with 1,6-hexamethylene di-isocyanate the 10 minute RD_{50} was 0.96 ppm and the 120 minute RD_{50} was 0.22 ppm; for toluene the 10 minute RD_{50} was 0.81 ppm and the 120 minute value was 0.25 ppm (Sangha *et al*., 1981).

Species variations in sensitivity occur, with the mouse being the usual animal used for testing. It has also been shown that strain variations also occur. A factor of 10 has been demonstrated between the least sensitive and the most sensitive strains of mice (Alarie *et al*., 1980). Swiss–Webster mice are most frequently used species, although no significant differences in sensitivity have been shown between Swiss–Webster and B6C3F_1 mice (Steinhagen and Barrow, 1984). The rat is not an appropriate species (Babiuk *et al*., 1985). In order to permit head-only exposure of the test species, and to simultaneously record the breathing movements, it is necessary to use a whole body plethysmograph with a neck restraint made of rubber. Studies involving multiple exposures have suggested that this method could result in pituitary gland lesions (Kaempfe and Dudek, 1994). Subsequent detailed studies with ND-4 Swiss–Webster mice given single sham and test material (dust) exposures did show pituitary lesions with both the sham (7/7) and test material (52/80) groups (Werley *et al*., 1996). Histology revealed pituitary gland haemorrhages. The authors concluded that the lesion is produced by increased pressure in the blood supply to the pituitary gland, but believe that the lesion (as an artefact of methodology) should have little effect on the usefulness of the procedure as a screening test. Details of the RD_{50} investigation have been published elsewhere (Ballantyne *et al*., 1977b), and comprehensive lists of measured RD_{50} values have been published (Alarie, 1981a; 1981b; Bos *et al*., 1992; Schaper, 1993).

For any substance there is a difference in the concentration causing a typical PCSI response by stimulation

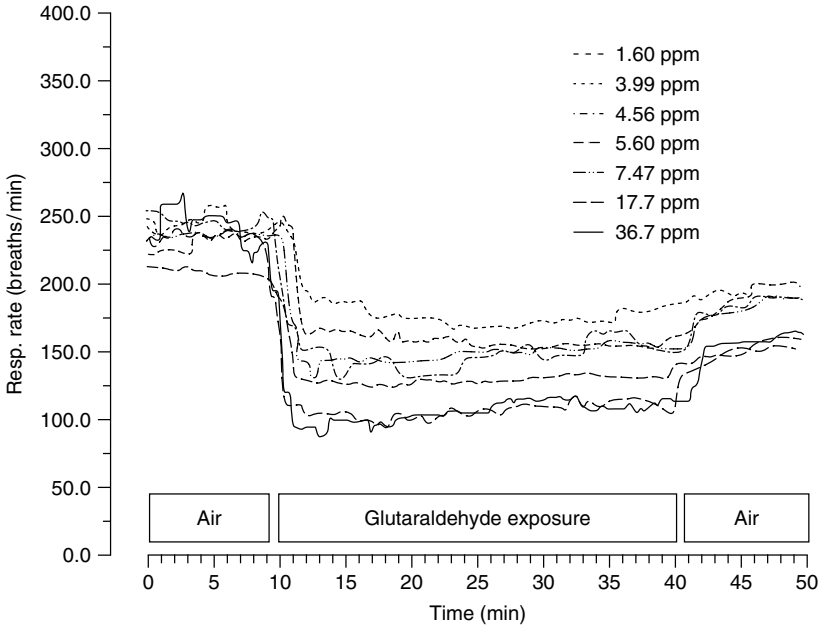

Figure 13 Average time–response curves for changes in breathing rate as a function of exposure time for male ND-4 Swiss–Webster mice exposed to air for 10 minutes, followed by a 30 minutes exposure to various concentrations of glutaraldehyde vapour, and then exposed postglutaraldehyde to air. (Data after Werley *et al*., 1995.)

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of receptors in the nasal mucosa and the concentration that is required to stimulate pulmonary receptors. In most cases, and in the intact conscious animal, the nasal trigeminal afferent response will predominate. It follows that a determination of the difference between the concentration causing a nasal response and that causing pulmonary irritation could be used as an index of the margin of warning for potential lung injury. This has been studied by using intact mice to determine the PCSI response from trigeminal stimulation and comparing the result with that resulting from exposure of tracheal-cannulated mice to determine the pulmonary response (Alarie, 1981c). The mouse, unlike other species, develops a decrease in breathing rate in response to pulmonary receptor stimulation due the development of a pause between the end of expiration and the start of the subsequent inspiration. The duration of this pause increases with exposure concentration. The decrease in breathing rate in the tracheal-cannulated mice and calculated RD_{50} (tRD_{50}) are performed as for the nasal exposure route (nRD_{50}). The ratio of RD_{50} values in the tracheal-cannulated and intact mice (tRD_{50}/nRD_{50}) can be used as the warning margin produced by the intact PCSI response. The closer this ratio is to unity, the closer is the concentration to cause pulmonary irritation to that causing nasal trigeminal stimulation. Examples are shown in **Table 3** for several monoamines. It can be seen that only one primary saturated aliphatic monoamine, *t*-octylamine, has close nRD_{50} and tRD_{50} values, with a tRD_{50}/nRD_{50} (t/n) ratio of 1.2. However, three secondary saturated aliphatic amines have tRD_{50}/nRD_{50} ratios <1.0; namely, di-isopropylamine (0.63), di-*n*-butylamine (0.61) and di-isobutylamine (0.96), indicating that OEG for these chemicals need to be strictly controlled. Methods that maybe used for a determination of concentrations of irritant-induced structural and/or functional pulmonary injury for a direct comparison with nRD_{50} values include ultrastructural changes, alterations in lung function (including resistance; Matijak-Schaper *et al*., 1983), detection of pulmonary oedema by a sensitive method based on the intravenous injection of ^{51}Cr-ethylenediamone tetra-acetate following lung lavage (Valentini *et al*., 1983), and measurement of protein and lactate dehyrogenase in lung lavage fluid.

8.3 Studies in Human Volunteer Subjects

8.3.1 General Comments

The animal studies discussed above (Section 8.2) in general allow the detection of substances that have a potential to cause objective PCSI effects. They do not, however, permit for the assessment of subjective effects such as discomfort or pain that are experienced in human subjects. In view of this, and because the human may be more sensitive to some PCSI substances than is estimated in animal models, it may be desirable to conduct PCSI tests in human volunteer subjects. These studies allow, not only an assessment of subjective responses, but, when appropriately planned and conducted, permit a quantitation of the PCSI response at threshold and suprathreshold levels and allow a determination of group variability and of the influence of environmental conditions. Additionally, the physiological and potential medical effects of exposure can be monitored simultaneously; for example, systemic reflexes (such as blood pressure and heart rate). These tests should, of course, only be conducted in volunteer subjects who have been fully informed of the nature and conduct of the tests, are aware of any possible discomfort and risks, have been fully informed that they may withdraw from the study at any time and have not been attracted by possible overcompensation if the volunteers are to be paid. The toxicology and pharmacology of the test substance should have been fully investigated, the potential for adverse health effects should be fully understood, and any possible side effects should carry the minimum of risk for the subjects. The protocol should be reviewed and approved by an appropriate independent institutional review board (Ballantyne, 2005).

8.3.2 Cutaneous Exposure

Measurement of PCSI of the skin has been discussed in detail by Green (2000) and Green and Bluth (1995). Various concentrations of test material are applied to the skin and the subjective degree of discomfort assessed. It should be remembered that there are anatomical regional variations in the sensitivity of the skin to PCSI substances (Ballantyne *et al*., 1973a). Because the epidermis acts as a barrier to the access of PCSI materials to the subepidermal sensory nerve endings, a method was devised to overcome this and obtain comparative evaluation of different substances. This is the blister base technique and involves the creation of a blister on the forearm by covering the area of skin with tape having perforations at regular intervals, and to which is applied a paste of 0.2% cantharidin in kaolin. The tape and paste are removed after a 6 hour contact period and the blisters allowed to develop overnight. Human facial sensory discomfort (sting) testing has been used as an approach for product assessment of cosmetic formulations (Dang *et al*., 2007).

8.3.3 Eye Irritation

Human ocular chemoreceptors are located mainly in the central area of the cornea (Coe and Douglas, 1984). As with animal *in vivo* models, in the human it is possible to determine the concentration of PCSI material that causes blepharospasm. However, in addition, in the human it is possible to assess the degree of discomfort or

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pain produced at various concentrations and to calculate concentrations that are thresholds for sensation and also incapacitating (e.g. TC_{50} and IC_{50}). PCSI effects of solutions on the eye are relatively easy to determine by careful sequential applications of small measured volumes of solutions to the surface of the cornea (Ballantyne and Swanston, 1973a; 1973b). For vapour and gases, tests can be conducted in a chamber with the subject wearing oronasal respiratory protective equipment or in a chamber with specially constructed eye ports (Bender *et al*., 1983). Also, specially designed goggles are available for localized exposure of the eye and prevention of simultaneous exposure of the respiratory tact (Kjaergaard, 1992; Hempel-Jørgensen *et al*., 1996; 1999). Some examples of EC_{50} values for blepharospasm are shown for humans and compared with animal findings in **Table 9**; values for subjectively assessed discomfort are shown in **Table 11**. These values indicate that, for the species studied, the blepharospasm-inducing effects of the test materials were greater in the human; that is, the human is more sensitive. Also, sensory discomfort was a more sensitive indicator of exposure to a PCSI material than was blepharospasm.

Kjaergaard *et al*. (1990) described a potentially valuable technique for measurement of what they described as sensory irritation of the eye, in which they undertook photographic measurement of changes in conjunctiva redness before and after exposure to an airborne irritant. It is a sensitive and reproducible approach. Using a series of volatile organic compounds, Hempel-Jørgensen *et al*. (1998) found that the degree of conjunctival hyperaemia in human volunteer subjects was positively related to the exposure concentration. This noninvasive method may be a valuable approach for studies on the irritating potential of environmental pollutants. It has been used, for example, to study exposure to formaldehyde, *n*-decane, tobacco dust and birch pollen (Bach *et al*., 1988; Kjaergaard and Pederson, 1989; Kjaergaard *et al*., 1989; 1990).

8.3.4 Respiratory Tract Exposure

For exposure to airborne PCSI materials it is usual to determine the concentration causing discomfort within the respiratory tract. Depending on the reason for conducting the study this may be nasal irritation, cough or chest discomfort. Nasal irritation may be experienced principally as an uncomfortable sensation and stinging (pungency). There are a variety of approaches for exposures, the most frequently used being whole body chambers of sufficient size to house the volunteers comfortably with any required monitoring equipment. In some cases, and when available, the use of a wind tunnel may offer a more convenient approach to exposure control and to alter the concentration of airborne test material. To avoid complications of interpretation due to ocular irritation either comfortable airtight goggles may be worn, or the test material can be delivered by mask. The latter approach, however, may have psychological disadvantages with some subjects. Comparative measurements of nasal pungency in those with normal olfaction vs. anosmics suggest that the presence of normal olfactory function may decrease the threshold for nasal pungency (Cometto-Muñiz and Cain, 1990). They also found that eye irritation thresholds were very close to nasal pungency thresholds in anosmics (Cometto-Muñiz and Cain, 1995), and suggested that eye irritation data may serve as a basis for assessing potency for the induction of nasal pungency; that is, nasal trigeminal threshold. Eye irritation thresholds generally do not differ significantly between those with normal olfaction and anosmics of similar age, gender and smoking status (Cometto-Muñiz and Cain, 1998). In this respect it is of interest to note that human pungency thresholds correlate with some RD_{50} values in animal studies (Cometto-Muñiz and Cain, 1994).

Techniques to evaluate and differentially compare both olfactory and chemosensory irritant thresholds have been described. One of these depends on the observation that if a volatile compound is sniffed into one nostril, and simultaneously air is sniffed into the contralateral nostril, then only if the material is a PCSI (stimulating the nasal trigeminal nerve receptors) can it be readily identified which nostril is being stimulated; olfaction by itself does not permit this spatial discrimination (Dalton *et al*., 2000; Roscher *et al*., 1996; Wysocki *et al*., 1992). Nasal localization thresholds in those with normal olfactory function, in general, are approximately equivalent to nasal pungency thresholds in anosmics, and the threshold for nasal localization thus offers a suitable way to measure nasal irritation in those with normal olfaction (Cometto-Muñiz and Cain, 1998). The lateralization, or monorhinal stimulation, technique was used by Wysocki *et al*. (1997) to study the odour and sensory irritant thresholds for acetone. In acetone-exposed workers (36 699 ppm) they found that lateralization thresholds were elevated compared with a control group not occupationally exposed (15 758 ppm), indicating that acetone is a weak sensory irritant and that adaptation is an important factor in the response. Olfactory adaptation was also noted (855 vs. 41 ppm).

Table 11 Concentrations for solutions of 2-chlorobenzylidene malononitrile (CS) and dibenz[b.f]-1,4-oxazepine (CR) to produce threshold sensations in the human eye[a]

Material	TC_{50} (M)[b]
CS	$7.3\ (4.2–11.2) \times 10^{-7}$
CR	$4.9\ (3.8–6.5) \times 10^{-7}$

[a] Data after Ballantyne and Swanston (1973a, 1973b).
[b] Threshold concentration 50% (with 95% confidence limits).

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8.3.5 Total Body Exposure

With unprotected workers in the occupational environment, PCSI materials in the atmosphere may come into contact with the skin, eye and respiratory tract and thus produce PCSI responses in one or all of these sites. Hence, for such situations it is meaningful for studies to be conducted by whole body exposure, simulating the workplace. Such studies can indicate if any particular tissue is more susceptible to the PCSI potential of an airborne material and would thus be a determinant for sensitivity as a warning effect. Appropriately planned, such studies can yield significant amounts of information. For example, Lundquist *et al*. (1992) investigated the effects on human volunteer subjects of diethylamine vapour that was increased in concentration in the atmosphere from 0 to 12 ppm over a 1 hour period (average concentration over this period was 10 ppm). A moderate to strong olfactory response with distinct subjective nasal and ocular irritation was noted. Some, but not all, subjects showed an adaptation. Nasal irritation appeared to be a slightly more sensitive index, and a significant correlation existed between nasal and ocular irritation ($r = 0.87$; $p < 0.001$). To study acute physiological changes in the nasal mucosa, they exposed adult male volunteers to 25 ppm diethylamine vapour in a chamber for 15 minutes. Changes in nasal volume and nasal resistance were measured by acoustic rhinometry and rhinomanometry. No reaction occurred for either nasal volume or resistance, indicating that these procedures are not useful measures for the detection of PCSI effects at lower (near threshold) concentrations.

9 APPLIED RELEVANCE OF PERIPHERAL CHEMOSENSORY IRRITANT EFFECTS

The biological effects of PCSI are of relevance in the following practical situations.

9.1 Occupational Exposure and Protection

As noted above, both the local sensory and reflex effects resulting from the PCSI response may be detrimental to safe and efficient working conditions, and in many cases measured values for PCSI effects may be a basis for assigning airborne OELs (discussed in detail below (Section 10.2).

9.2 Harassing Agents

The use of PCSI materials for warning purposes by mixing with other materials not giving a warning on contact has been mentioned above (Section 5.1). More frequent is the use of certain PSI materials as riot-control agents. By the very nature of their intended usage, riot-control agents produce marked PCSI effects, which result in harassment, hindering the conduct of unlawful activities, and causing malefactors to leave the contaminated area. These aspects of PSI have been discussed in detail elsewhere (Ballantyne, 1977; Ballantyne and Salem, 2005; Salem *et al*., 2005).

9.3 Combustion Products

Many chemically differing materials are produced during the processes of combustion, the nature and amounts of which depend on the circumstances of the fire. Some of these materials may produce PCSI effects; for example, hydrogen chloride, sulphur dioxide, isocyanates. The resulting harassing or incapacitating effects may impede escape from a fire. This may be additive to other factors hindering escape from a fire; for example, obstacles, physical injury, hypoxia and absorbed products which produce a disturbance of consciousness, such as carbon monoxide, hydrogen cyanide and volatile organic solvents (Ballantyne, 1981; Norris and Ballantyne, 1999).

9.4 Environmental Considerations

Many airborne chemicals in the environment can evoke chemosensory irritant effects. Perceived irritation, particularly in the indoor environment, is a common symptom and resembles the effects experienced in 'sick building syndrome (SBS) or in multiple chemical sensitivity (MCS) (Koren *et al*., 1992; Otto *et al*., 1992; Dalton *et al*., 1997; Anderson and Anderson, 1999; Sparks, 1999). Trigeminally medited ocular, nasal and pharyngeal irritation is generally regarded as an important component of symptom reporting with indoor 'problem buildings' (Shusterman, 2006). Imputed agents include extremes of temperature and humidity, the presence of volatile organic compounds, combustion products (including cigarette smoke) and ozone from electronic equipment. In addition to the primary irritation, mucosal irritants trigger various secondary reflexes, such as nasal congestion, rhinorrhoea and cough.

There are several syndromes, including SBS and MCS, that are classified together by some authorities as idiopathic environmental intolerance (IEI) (Sparks, 1999) and that are characterized by irritation of the face (burning sensation), sore eyes and throat, difficulty with breathing

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(including asthma), often (particularly with MCS) neurological problems including confusion, fatigue, difficulty with concentration and poor memory. The considerable overlap of symptoms related to PCSI, pulmonary irritation and airflow limitation with the various types of IEI has led to proposals that common air pollutant mixtures (occupational, domestic and environmental) can cause these various syndromes. The term IEI has been preferred by organizations such as the World Health Organization (WHO) and International Labour Organization (ILO) because 'sensitivity' is a description that implies an immunological basis; the relation between environmental exposure and symptomatology is, as yet, not totally proven, and the underlying pathophysiological mechanisms and criteria for diagnosis are yet to be fully defined. However, it is established that air samples taken from domestic sites associated with repeated human complaints of poor air quality have caused sensory irritation, pulmonary irritation and airflow limitation in mice (Anderson and Anderson, 1997a; 1997b; 1998; 1999). Also, behavioural changes have been detected by functional observation battery studies in mice after exposure to product emissions or air from sites where individuals have made complaints. Findings of these types suggest that many symptoms of SBS and MCS could be the result of exposure to airborne irritant chemicals, such as those liberated by common commercial products and found in polluted air, For example, Kjaergaard *et al*. (1989) found that *n*-decane caused a concentration-dependent irritation of mucosae and increased the sensation of odour intensity. They showed that even at low concentrations, *n*-decane caused symptoms similar to those of SBS. Many individuals with MCS have been reported to have a heightened sense of smell or lower odour threshold (Fielder *et al*., 1996). However, Caccappolo *et al*. (2000) were not able to demonstrate lower olfactory threshold sensitivity or enhanced ability to identify odour accurately compared with age and gender-matched healthy controls. Where many relatively nonreactive chemicals are present in the atmosphere at concentrations below their respective PCSI thresholds, their combined effect could induce irritation of the mucosae (Cometto-Muñiz *et al*., 1997). Anderson and Anderson (1999) have noted that repeated exposures (two or three 1 hour exposures over 24 hours) of mice to certain airborne mixtures of chemicals (e.g. emissions of a solid air freshener) yielded the same degree of irritation, whereas repeated exposures to other mixtures (e.g. emissions from some fabric softeners, disposable nappies and vinyl mattress covers) resulted in increased sensory irritant responses (two- to fourfold). Thus, in the latter cases the intensity of each exposure was constant in magnitude, but the magnitude of the sensory irritant response was increased, leading the authors to conclude that the sensitivity of the mice to chemical mixtures had changed. The response is specific to certain mixtures of chemicals.

Individuals who complain of symptoms of IEI are a heterogeneous group, and more than one mechanism may operate in different cases (NIEHS, 1997). The suggested pathogenesis has included exposure to an airborne mixture of materials having PCSI properties, as discussed above, and to preceding or concurrent psychiatric illness, sensitivity to odours or combinations of these factors. However, the role of exposure to airborne materials having PCSI properties, notably in mixtures, offers a major aetiologic avenue, for which there is some laboratory support. IEI has increasingly impacted the overall burden of chronic disability, and deserves further medical and scientific investigation and evaluation.

10 PERIPHERAL CHEMOSENSORY IRRITATION AS A FACTOR IN DETERMINING OCCUPATIONAL EXPOSURE GUIDELINES

10.1 General Considerations on Occupational Exposure Guidelines

OEGs are usually recommended on the basis of providing protection from adverse health effects of overexposure for a working lifetime of exposure. Depending on the major adverse health effects determining the quantitative value of the guideline, they may be set as permitted average exposure values or may be further qualified for additional restriction of exposure potential. Thus, the threshold limit values (TLVs) recommended by the American Conference of Governmental Industrial Hygienists (ACGIH) (ACGIH, 2007) may be set as time-weighted average (TWA) exposure values to be permitted over an 8 hour work day (TWA over 8 hours for a 40 hour workweek; TWA_8). Further considerations may lead to additional qualifications as follows:

1. The recommended guideline is a set (fixed) value that may not be exceeded during any part of the working period, a ceiling (C) value.
2. The TWA is qualified (supplemented) by an additional concentration, to which it is believed that workers can be exposed for a short period without developing irritation, narcotic effects or chronic or irreversible tissue damage, providing that the daily TWA_8 is not exceeded. This qualification is the short-term exposure limit (STEL) and is usually set for a period of 15 minutes that should not be occur for more than four times a working day, with a

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period of at least 60 minutes between successive STEL exposures.

3. A written 'notation' of certain effects may be given in order to draw attention to these particular potential adverse effects and that specific precautionary measures may be required; for example, these have been cited for irritation, narcosis, sensitization. Also, carcinogenic effects are indicated by the notations give in **Table 12**.

TLVs are based on credible information derived from *in vivo* and *in vitro* toxicology laboratory investigations, controlled human volunteer studies, in-use occupational medical studies and experience, epidemiology studies and available industrial hygiene observations. The values are usually recommended on the basis of the most sensitive index of potential adverse effects that may occur in exposed workers. These may sometimes be toxic effects causing an impairment of health of variable concerns and the result of either acute and/or repeated exposure; examples include carcinogenic effects, nononcogenic chronic health effects, neurological effects, reproductive effects, developmental toxicity, immune-mediated effects (including asthma) and other organ-specific or enzyme-specific pathology. In other cases the effects may have a physiological or pharmacological basis, such as PCSI or narcosis, both of which are detrimental to efficient working conditions and may result in accidents due to impairment of vision or locomotion, or from distraction. In yet other cases, a qualified TLV may be assigned on the potential for differing adverse effects from the same material. In reaching conclusions concerning the most appropriate value for a TLV-TWA, in addition to the particular substance-related adverse effect(s), it is necessary to take into account how other workplace or personal factors may influence the expression of adverse effects, and how a hypersusceptible proportion of the exposed worker population would react; from these considerations the TLV is appropriately modified. These additional considerations will include, for example, the possible influence of age, gender, smoking habits, genetic factors and general state of health. For many diverse effects resulting from repeated exposure, the TLV-TWA provides the most satisfactory and convenient method for controlling workplace exposure. These values should take account of both the inhaled dose of material and, where relevant and data is available, the possible percutaneous absorption of the material, which in some cases may make a significant contribution to the systemic toxicity from airborne gases, vapours or mists. Information on the possible relevance and quantitative contribution from percutaneous absorption can be derived from repeated cutaneous and percutaneous toxicokinetic studies in laboratory animals, or from exposed human volunteer subjects by wipe sampling and measurement of parent material or metabolite in sequential blood samples (Leung and Paustenbach, 1994; McDougal *et al*., 1990). Excursions permitted on the TWA_8 depend on a number of factors, including the nature of the workplace, the duration of the excursion periods and whether higher concentrations produce acute toxicity. TWA excursions are permitted above the TLV if they are compensated for by equivalent excursions below the TLV-TWA during the workday. The magnitude of the excursions is not usually definable with any degree of precision based on toxicological data. However, Leidel *et al*. (1975) reviewed numerous industrial hygiene surveys conducted by the National Institute of Occupational Safety and Health (NIOSH) and found that short-term exposure measurements were, in

Table 12 ACGIH classification categories for notations of grades for carcinogenic potential

Grade	Notation	Definition
A1	Confirmed human carcinogen	Epidemiological evidence for carcinogenicity in humans.
A2	Suspect human carcinogen	Human data are adequate, but conflicting or insufficient to confirm human carcinogen. Carcinogenic in experimental animals at doses, by exposure routes, at sites, of histopathological or mechanisms relevant to worker exposure. Limited human data but sufficient animal data.
A3	Confirmed animal carcinogen	The substance is carcinogenic in experimental with unknown relevance to animals at high doses by route(s), at sites, of humans histopathological types, or by mechanism(s) that may be relevant to human exposure. Available epidemiology does not confirm an increased risk of cancer in exposed humans.
A4	Not classifiable as a human	Lack of information excludes a definitive carcinogen assessment of potential carcinogenicity in humans. *In vitro* or animal studies do not provide positive Indications of a carcinogenic potential.
A5	Not suspected as a human	Based on credible epidemiology studies, or when carcinogen, the evidence suggesting a lack of carcinogenicity in experimental animals is supported by mechanistic studies.

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general, log normally distributed with geometric standard deviations generally in the range 1.5–2.0. If short-term exposure values in a given situation have a geometric standard deviation of 2.0, then 5% of all values will exceed 3.13 times the geometric mean. This forms the basis for excursions permissible on TLV-TWA values that do not have a qualifying STEL; that is, excursions may exceed three times the TLV-TWA for no more than a total of 30 minutes during a workday and under no circumstances should they exceed five times the TWA, provided the TLV-TWA is not exceeded. The STEL supplements the TWA where acute effects are recognized from a material whose more serious effects are primarily of long-term or chronic nature; a STEL is usually only assigned where toxic effects have been established from high concentration–short duration exposures. The ceiling (C) value is appropriate for acute effects (principally PCSI), usually occurring at low concentrations, which have a demonstrable threshold that is below the exposure concentration [no observed adverse effect level (NOAEL)], which may result in cumulative or long-term toxicity. A variety of other, mainly environmental, exposure guidelines exist, which are designed to protect individuals who may be incidentally exposed from workplace or transport releases. These include acute exposure guideline levels (AEGLs) which are derived to protect the population from adverse effects in the case of a single exposure due to an accidental release of chemicals into the environment. They are intended to provide estimates of exposure concentrations for a range of exposure durations (10 and 30 minutes, 1, 4 and 8 hours) that are predicted to result in various categories of severities of adverse effects; that is, mild (AEGL-1); severe, irreversible, potentially disabling adverse health effects (AEGL-2) or life-threatening (AEGL-3) (Krewski *et al*., 2004; Bos *et al*., 2006). PCSI effects may play a determinant role in AEFL-1 assessments (Alexeeff *et al*., 2008).

Countries other than the US have different approaches and terminologies for their own national exposure limits for the workplace. In the UK, for example, under the Control of Substances Harmful to Health (COSHH) Regulations, the Health and Safety Executive (on advice from an independent Advisory Committee on Toxic Substances, ACTSs) set OELs. COSHH defines two types of OELs—the occupational exposure standard (OES) and the maximum exposure level (MEL). OESs are set for materials for which it is possible to identify a concentration at which there are no significant risks to health, and MELs are set for materials that have serious implications for health (notably carcinogens and asthmagens), and for which an OES cannot be set (Topping, 2001).

10.2 Peripheral Chemosensory Irritation in the Assignment of Occupational Exposure Guidelines

Because PCSI materials induce distracting, harassing and/or incapacitating effects that are not conducive to efficient working conditions and may predispose to accidents, this is the basis for assigning OEGs to many substances causing PCSI. This is particularly valuable when PCSI effects occur at concentrations lower than those resulting in toxicity by acute or repeated exposure, because sensory irritants then give a warning of potential overexposure. About 40% of ACGIH TLVs are set on the basis of PCSI as the most sensitive index. Since a threshold exists for a PCSI effect and the slope on the exposure concentration–response data varies between different substances, a TWA_8 by itself is not appropriate for workplace protection. Therefore, either a C-value is recommended for a substance having a potent PCSI effect as the main biological factor, or if a TWA_8 is recommended based on other toxicity that can be qualified by a STEL. For a limited number of substances, quantitative information is available on the peripheral chemosensory irritant potential in humans, which permits a reasonable estimate of threshold and incapacitating effects, and allows a reliable OEG to be assigned. Where data are available, attention should be paid to the threshold and incapacitating concentrations, slope on the exposure concentration–response relationship and ERs. Also, with both animal and human-derived data, consideration should be given to the following and how they may influence the value of the OEG: development of tolerance, environmental conditions, the nature of the workplace, type of occupation and the physical characteristics and pattern of the workplace exposure.

In the absence of reliable human data, or else to support and confirm human data, PCSI studies in animals have been widely used. Most frequently employed have been nRD_{50} studies in mice. Those who recommend the value of nRD_{50} studies have supported their case by comparing these values with TLVs for known PCSI substances that have been assigned by ACGIH or other recognized agencies (Barrow *et al*., 1977; Alarie *et al*., 1980; Kane *et al*., 1980; de Ceaurriz *et al*., 1981; Alarie, 1984; Steinhagen and Barrow, 1984; Nielsen and Yamagiwa, 1989; Schaper, 1993). Predictive conversion methods that have been proposed include the following:

1. The TLV should lie between 0.01 nRD_{50}, where it is predicted that there will be either no sensory irritation or it will be at threshold levels, and 0.1 nRD_{50}, which is likely to be an uncomfortable, but tolerable concentration (Barrow *et al*., 1977; de Ceaurriz *et al*., 1981). At the nRD_{50} the predicted response in humans is intolerable or incapacitating.

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2. Since there is a good correlation between the then available TLVs, 0.03 nRD_{50} was recommended for establishing a TLV based on chemosensory irritation (Alarie, 1981b; Schaper, 1993).
3. Because at an airborne concentration of 0.1 nRD_{50} it is anticipated that humans will experience slight discomfort, this should be the highest exposure concentration, and form the basis for a STEL to qualify the TLV-TWA_8 defined on the basis of 0.03 nRD_{50} (Gagnaire *et al*., 1994).
4. Where data are available from tracheal-cannulated animals, and the tRD_{50} is close to the nRD_{50}, because of the prediction of pulmonary irritation, somewhat grater caution is recommended, with the TLV set at 0.01 tRD_{50} (Weyel *et al*., 1982; Weyel and Schaffer, 1985; Nielsen, 1991).

The need for a safety margin between the measured nRD_{50} and the recommended OEG is supported by the observation that respiratory histopathological inflammatory lesions may be induced at the nRD_{50} concentration. For example, Buckley *et al*. (1984) found that with 10 sensory irritant substances of widely differing mouse nRD_{50} values, short-term repeated exposures at the RD_{50} caused inflammatory lesions in the reparatory tract. The materials studied (with nRD_{50} values) were 2,4-toluene di-isocyanate (0.4 ppm), acrolein (1.7 ppm), formaldehyde (3.1 ppm), chloropicrin (8.0 ppm), sulphur dioxide (117 ppm), ammonia (303 ppm), hydrogen chloride (309 ppm), dimethylamine (5111 ppm) and epichlorohydrin (687 ppm). After exposure of the mice for six hours per day for five days, all irritants produced lesions in the nasal mucosa with an anterior–posterior severity gradient. The lesions ranged from slight epithelial hypertrophy or hyperplasia to epithelial erosions and ulceration with variable subepithelial inflammatory cell infiltration. Only chlorine, chloropicrin and epichlorohydrin caused lesions in the lower respiratory tract.

Some illustrative examples of the use of measured RD_{50} values in mice to derive OEGs are as follows:

1. Rydzynski and Jedrychowski (1994) established an nRD_{50} of 5.9 mg m^{-3} (95% confidence limts1.3–13.0; slope 1.37) for cyanuric chloride, indicating the material to be a potent PCSI substance and suggested an OEG of 0.17 mg m^{-3} for the substance (0.03 nRD_{50}).
2. The respiratory PCSI potential of chlorine and of nitrogen trichloride were studied by mouse plethysmography by Gagnaire *et al*. (1994), who found respective nRD_{50} values of 3.5 and 2.5 ppm. Based on 0.03 and 0.1 of the nRD_{50} they proposed TWA_8 (and STEL) values of 0.1 (0.5 ppm) for chlorine and 0.1 (0.3 ppm) for nitrogen trichloride.
3. Werley *et al*. (1995) determined the nRD_{50} for glutaraldehyde vapour in ND-4 Swiss–Webster mice as 13.86 ppm (95% CL 9.86–23.58 ppm), which is in the range of nRD_{50} values measured for other aliphatic aldehydes (Bos *et al*., 1992). Using a correlation factor of 0.03 nRD_{50}, this suggests an OEG of 0.42 ppm, which is close to the threshold irritancy concentration of 0.3 ppm found for exposed human volunteer subjects (Ballantyne and Jordan, 2001). The current ACGIH TLV for glutaraldehyde is 0.05 ppm as a C value; although this is in part based on PCSI effects, the value is recommended mainly because of a suggested potential for respiratory sensitization by glutaraldehyde (ACGIH, 2007).
4. Steinhagen *et al*. (1982) found the nRD_{50} value for dimethylamine vapour to be 573 ppm in male Fischer 344 rats and 511 ppm in male Swiss–Webster mice. Use of the RD_{50} value from the mouse, and the range of 0.01–0.1 RD_{50} as a guideline interval in which the OEG should be set, suggested an OEG between 5 and 51 ppm; the current ACGIH TLV for dimethylamine is 5 ppm with a STEL of 15 ppm (ACGIH, 2007). The next higher alkylamine, diethylamine, has the following mouse RD_{50} values (Nielsen and Yamagiwa, 1989); an nRD_{50} value of 550 mg m^{-3} (184 ppm; i.e. approximately one-third that of dimethylamine) and a tRD_{50} of 1650 mg m^{-3}. These would suggest TLV values of 16.5 mg m^{-3} (0.03 RD_{50}) and 5.5 mg m^{-3} (0.01 RD_{50}), respectively. It is relevant to note that a 1 hour exposure of human volunteers to an average concentration of 30 mg m^{-3} diethylamine vapour caused a distinct nasal and eye sensory irritation. The current ACGIH TWA_8 for diethylamine is 5 ppm (15 mg m^{-3}) and a STEL of 15 ppm (45 mg m^{-3}) (ACGIH, 2007). Di-isopropylamine is a secondary monoamine whose tRD_{50} (102 ppm) is lower than the nRD_{50} (161 ppm) with a t/n ratio of 0.63, indicating a potential for lung injury at a concentration below that giving a typical nasal PCSI warning (**Table 3**). Therefore an OEG of 0.01 tRD_{50} would be most appropriate; that is, 1.0 ppm. However, the current ACGIH TLV is set at a TWA_8 of 5 ppm (ACGIH, 2007), a value that is more appropriate for one derived from the nRD_{50} ($\times 0.03 = 4.83$ ppm).

RD_{50} values for aerosols can be measured readily in mice. For example, Ballantyne *et al*. (1994) exposed mice, nose-only, for 30 minutes to triethylene glycol aerosols of MMAD of 2.45–3.10 µm. Maximum decrease in breathing rate occurred at 15–25 minutes of the start of exposure and were sustained. The nRD_{50} was calculated to be 5.14 mg l^{-1}, from which it would be calculated (0.03 nRD_{50}) that the OEG for irritation would be 150 mg m^{-3}.

A comparison of isocyanates provides an instructive example of the use of PCSI data for establishing

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OEGs and the additional influence of pulmonary irritation on establishing the values. James *et al*. (1987) determined the nRD_{50} for methyl isocyanate was 2.9 ppm in mice, and Ferguson *et al*. (1986) established a value for the nRD_{50} of 1.3 ppm and for the tRD_{50} of 1.9 ppm. These RD_{50} values indicate that methyl isocyanate is a potent PCSI substance. With respect to nasal irritancy, methyl isocyanate is not as potent as (60 minutes values) the aliphatic hexyl di-isocyanate and the following aromatic mono- and di-isocyanates: phenyl isocyanate (0.9 ppm), *p*-toluene isocyanate (0.84 ppm) and toluene di-isocyanate (0.39 ppm). It is, however, more potent than hexyl isocyanate (9.2 ppm; Sangha *et al*., 1981). Although the nRD_{50} for methyl isocyanate (1.3 ppm) is numerically larger than that for toluene di-isocyanate (0.39 ppm), the main difference between these two isocyanates is that there was no evidence for pulmonary irritation with the toluene compound (even in cannulated mice inhaling $5 \times RD_{50}$) (Sangha and Alarie, 1979), but the methyl compound had a tRD_{50} of 1.9, and thus the ratio of tRD_{50}/nRD_{50} was 1.46, indicating that methyl isocyanate is a potent pulmonary as well as sensory irritant. In comparison with other potent PCSI substances, such as acrolein (nRD_{50} 1.7 ppm) and formaldehyde (nRD_{50} 3.7 ppm), methyl isocyanate is much more hazardous by inhalation, as indicated by the respective tRD_{50}/nRD_{50} ratios of 142, 31 and 1.46. The current ACGIH (2007) TLV values for isocyanates agree with measured nRD_{50} values as follows:

1. Methyl isocyanate; 0.03 $nRD_{50} = 0.04$ ppm; 0.01 $tRD_{50} = 0.0$ ppm; TLV = 0.02 ppm
2. 1,6-Hexamethylene di-isocyanate: 0.03 $nRD_{50} = 0.0066$ ppm; TLV = 0.005 ppm (also a respiratory sensitizer)
3. Toluene-1.4-di-isocyanate: 003 $nRD_{50} = 0.00$ 75 ppm; TLV = 0.005 ppm (also a respiratory sensitizer).

Several investigators have expressed reservations about the suitability of the RD_{50} test as a basis for assigning OEGs. For example, Bos *et al*. (1992) have questioned the suitability of this approach based on considerations of interlaboratory variations, the finding that toxicity (inflammation) may be seen at or below RD_{50} concentrations, and the rationale for deriving an OEG from RD_{50} data is based on empirical findings. Nikula and Lewis (1994), for example, demonstrated that exposure to pyridine, six hours per day for four days, at 5 ppm (ACGIH TLV) produced lesions in the olfactory mucosa which were only slightly less than those produced by comparable exposures to pyridine vapour at 444 ppm. Histology demonstrated vacuolar degeneration of sustentacular cells; focal and marked attenuation of the epithelium; loss of neurons; presence of intraepithelial luminal structures. As noted above, although effects may be observed at nRD_{50} concentrations, using a correction factor up to 0.1 nRD_{50} allows, at least in part, for slight effects at the RD_{50}. Bos *et al*. (1992) recommended the following procedures with respect to the use of the sensory irritation procedure:

1. In view of interspecies variations, two species (rat and mouse) should be used, with the results from the most sensitive species being chosen.
2. Time–response and log concentration–response curves should be obtained.
3. Exposure should be continued until a plateau is obtained.
4. There should be verification that no pulmonary irritation is occurring.
5. For every material tested, it should be verified whether the observed response is due to irritation or toxicity, and that toxicity is not occurring below the concentration causing sensory irritation.

It has been stressed that many nonsensory factors, such as exposure history, attitudes and expectations, and personality variables can significantly alter the perception of odour and irritation following exposure to volatile chemicals (Arts *et al*., 2006; Dalton, 2002). Objective measures of irritation obtained in conjunction with subjective responses can lend valuable input to determinations on OELs, but should take into account other factors, such as cognitive or emotional, that may modulate the subjective response (Dalton, 2002). It is suggested that studies evaluating chemosensory irritation should take the following into account: (i) distinguish between annoyance elicited by odour sensation and that elicited by true sensory irritation; (ii) evaluate exposure-related factors that affect odour and irritancy responses and (iii) separate true adverse health effects from those mediated by psychosocial factors (Dalton, 2003).

The latter point is important, and it cannot always be assumed that by preventing irritation the occurrence of other manifestations of systemic irritancy can be prevented. For example, the mouse nRD_{50} values for 1,2-dichlorobenzene and hexachloro-1,3-butadiene were found to be 181 and 211 ppm, respectively. The median active level of exposure (MAL) for 1,2-dichlororbenzene to produce a 50% decrease in hepatic glucose-6-phosphate dehydrogenase (an index of hepatotoxicity) was 598 ppm, and the MAL for hexachloro-1,3-butadiene to cause a 50% renal tubular injury (based on alkaline phosphatase histochemistry) was 7.2 ppm. Thus the MAL for 1,2-dichlorobenzene hepatotoxicity is about 3.3 times that of the nRD_{50}, and hence this material can be treated as a PCSI substance from the OEG point of view. However, the nRD_{50} for hexachloro-1,3-butadiene vapour is 39.3 times higher than the MAL for nephrotoxicity, and thus the latter

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should receive greater consideration in the assignment of an OEG (de Ceaurriz *et al*., 1988).

OEGs are generally based on irritancy and toxicology information collected at ambient temperature. However, in some workplace situations there may be an elevated environmental temperature; for example, tropical conditions. Also, certain industrial processes may not have been totally investigated. For example, thermoplastic resins are heated to liquefy them for processes such as blowing, extrusion or injection molding. This can result in the liberation of residual monomers, additives or thermal decomposition products, to which workers may be exposed. Schaper *et al*. (1994) conducted a study on the respiratory responses to the thermal degradation products of thermoplastics. They measured nRD_{50} values at processing temperatures between 200 and 300 °C for four resins: polyacrylonitrile-polybutadiene-polystyrene, polypropylene-polyethylene copolymer, polypropylene homopolymer and plasticized poly(vinyl chloride). The respective nRD_{50} values were 21.10, 3.51, 2.60 and 11.51 mg m^{-3}. Based on these findings, the authors recommended exposure limits of 0.63, 0.11, 0.08 and 11.51 mg m^{-3} (0.03 nRD_{50}). Although such studies yield valuable practical information relevant to certain specific workplace activities, Muller and Black (1995) cautioned against the routine use of extreme generation conditions. They investigated the PCSI potential of various samples of indoor materials (including carpets, ceiling tiles and wall coverings) that were ventilated at either 23 or 70 °C. Using mouse plethysmography procedures (nRD_{50} measurements) they found irritation at 70 °C, but not 23 °C. They concluded that increasing the environmental temperature increased the concentration of chemical emissions, which accounted for the sensory irritant effects. They consider that ambient temperature generation conditions were more appropriate for assessing emissions in relation to such indoor situations.

In the workplace or general environment, exposure to several different materials may occur simultaneously. Hempel-Jørgensen *et al*. (1999) produced ocular irritation by interactive additive effects. Although, in the context of use of nRD_{50} measurements to assign OEGs, PCSI materials probably act on the same trigeminal chemoreceptors, several considerations suggest that a predictive approach by an additive process may not always be reliable. For example, competition of the same chemoreceptors may result in a decrease in breathing rate that is less than that predicted on the basis of an additive consideration. Thus, Cassee *et al*. (1996) found that sensory irritation in rats resulting from exposure to mixtures of aldehydes (formaldehyde, acrolein and acetaldehyde) was more pronounced than that caused by each aldehyde separately, but less than the sum of the individual potencies. Competitive antagonism has been demonstrated for acrolein and formaldehyde (Kane and Alarie, 1978) and for cumene and *n*-propanol (Nielsen *et al*., 1988). Also, different materials may display different desensitization patterns, Cassee *et al*. (1996) measured the decrease in breathing rates in rats for formaldehyde, acetaldehyde and acrolein and for mixtures of these aldehydes. They found that sensory irritation was more pronounced for mixtures than for than for each compound tested separately, but less than for the sum of individual components. They concluded that the decrease in breathing rate as a result of exposure to mixtures of PCSI substances could be predicted with a model for competitive antagonism. Threshold responses for odour and nasal pungency (in anosmics), and eye irritation were measured by Cometto-Muñiz *et al*. (1997) for single chemicals (1-propanol, 1-hexanol, ethyl acetate, heptyl acetate, 2-pentanone, 2-heptanone, toluene, ethyl benzene and propyl benzene) and for various component mixtures of them. They found various degrees of stimulus agonism for the three sensations when testing mixtures. Also as the number of components and the degree of lipophilicity of the compounds in the mixtures increased, so did the degree of agonism. Synergistic agonism characterized the eye irritation response for the most complex and most lipophilic mixtures. Korpi *et al*. (1999) reported that microbial volatile organic compounds may have some synergistic effects for sensory irritation, and also if a particular component of a mixture is much more potent than the other components of a mixture it may dominate the PCSI effect. In addition to the use of such information for determining OEGs in the workplace, it may also be of value for investigating situations such as the 'sick building' syndrome, and the authors suggest that the combined action of many relatively nonreactive substances at concentrations below their respective PCSI threshold could induce irritation in mucosae.

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Responses of the Kidney to Toxic Compounds

Edward A. Lock

CONTENTS

1 INTRODUCTION

The mammalian kidney is an extremely complex organ, both anatomically and functionally, and plays an important role in the control and regulation of homeostasis. In this regard it has a key role in the regulation of extracellular fluid volume and electrolyte composition. The kidney is also the site of synthesis of hormones and certain vasoactive prostaglandins and kinins that influence systemic metabolic function. For example, renin, the stimulus for the formation of angiotensin, and aldosterone are formed in the kidney. 25-Hydroxyvitamin D_3 undergoes hydroxylation specifically in the kidney to 1,25-dihydroxyvitamin D_3 which plays a key role in promoting bone resorption and calcium absorption from the gut. Erythropoietin, an important stimulus for erythrocyte formation in the bone marrow, is also synthesized in the kidney.

A toxic insult to the kidney may affect some or all of these functions, but, in general, it is markers of excretory function such as creatinine or blood urea nitrogen and the presence of glucose, proteins or enzymes and electrolytes in urine which are commonly monitored as indicators of renal dysfunction.

2 RENAL STRUCTURE AND FUNCTION

The kidney can be divided into two major anatomical areas—the cortex and the medulla. The cortex forms the major part of the kidney and receives most of the blood supply and, hence, nutrients. Thus, when a foreign chemical enters the bloodstream, a high percentage will be delivered to the cortex and, hence, have a greater chance of altering cortical function than medullary. Some chemicals, however, will be delivered to the medulla and, because of the anatomy of the vasa recta and loops of Henle, can become trapped by the countercurrent system in this region of the nephron. Thus, a foreign chemical could achieve relatively high concentrations in the medulla although the blood flow is relatively poor.

The functional anatomy of the kidney is based on the nephron structure, which has three separate elements, the vasculature, the glomerulus and the tubular component (**Figure 1**). All nephrons have their major vascular components and glomeruli in the cortex. The proximal convoluted tubules (pars convoluta) are located in the cortex, with the straight portions of the proximal tubules (pars recta) extending into the outer stripe of the outer medulla (**Figure 2**). Those nephrons whose glomeruli are close to the cortical surface (cortical nephrons) send their loops of Henle down into the inner stripe of the inner medulla, while those with glomeruli close to the medulla (juxtamedullary nephrons) send their loops of Henle deep into the medulla (**Figure 2**). For a more detailed consideration on renal structure and function, see Brenner (2004), Sands and Verlander (2004) or Alpern and Hebert (2008).

Each anatomically distinct part of the nephron has a specific function or functions, all of which can

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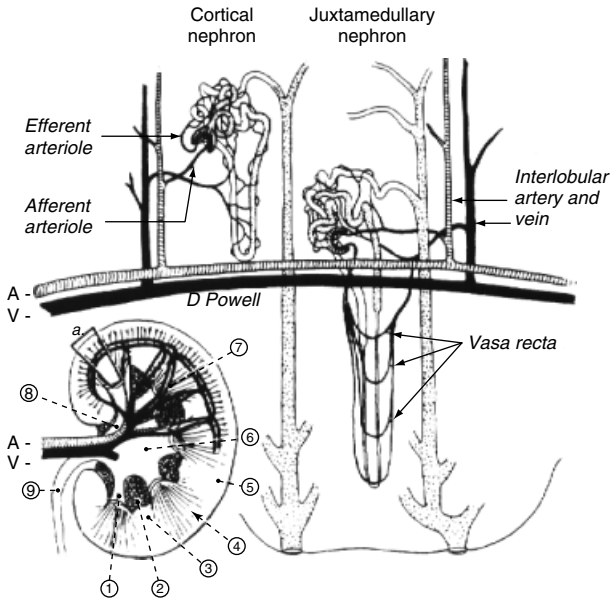

Figure 1 A saggital section of a human kidney is illustrated diagrammatically (lower left). Numbers (1)–(9) indicate the following: (1) minor calix, (2) fat in sinus, (3) renal column of Bertin, (4) medullary ray, (5) cortex, (6) pelvis, (7) interlobar artery, (8) major calix and (9) ureter. The letter A indicates the renal artery, and the letter V indicates the renal vein. Insert (a) from the upper pole is enlarged to illustrate the relationships between the juxtamedullary and the cortical nephrons and the renal vasculature. (Reproduced from Tisher, 1976. © Elsevier.)

be perturbed by a nephrotoxic insult. The vascular component serves to deliver oxygen and metabolic substrates to the nephron for maintenance of its function, while certain end products of metabolism and other materials are delivered to the tubule for excretion. The blood supply also enables reabsorbed materials and those synthesized in the kidney to be returned to the systemic circulation.

The glomerulus contains a specialized capillary network, which is relatively porous and acts as a selective filter of components from plasma. On the basis of molecular weight and net charge, certain materials and chemicals will be filtered into the tubular lumen, while others will be retained in the circulation. The tubular part of the nephron selectively reabsorbs the majority, about 98%, of the salts and water in the filtrate. In a normal healthy kidney there is almost complete reabsorption of filtered glucose and amino acids and selective elimination of end or waste products of metabolism. The proximal tubule is also able to actively secrete certain chemicals into the urine; the excretion of some organic compounds and the elimination of hydrogen and potassium ions occur primarily via this route (see Moe *et al*., 2000; Sica and Schoolwerth, 2000; Wright, 2005).

Depending on the size of a toxic insult, chemicals may produce changes in renal function that are mild and reversible or which are permanent, and if severe enough they may cause death. For example, nephrotoxicity may be expressed as a minor perturbation in tubular reabsorption, such as a mild or transient glucosuria or proteinuria, as a decreased concentrating ability (i.e. polyuria) or, following a more severe insult, as acute renal failure associated with anuria and elevations in creatinine and blood urea nitrogen.

3 SUSCEPTIBILITY OF THE KIDNEY TO TOXIC INSULT

The two kidneys comprise about 1% of the body weight, but receive about 25% of the cardiac output, and about one-third of the plasma water that reaches the kidney is filtered. Maintenance of renal function requires delivery of large quantities of oxygen and metabolic substrates to the kidney. Thus, the kidney, especially the pars recta of the proximal tubule, is particularly susceptible to agents that produce cellular anoxia—for instance, a decrease in blood pressure or blood volume, as in shock, or haemorrhage (Glaumann and Trump, 1975; Venkatchalam *et al*., 1978). Similarly, dehydration, due to either increased heat output or decreased water intake, or a chemical which causes a decrease in plasma volume (Lock, 1979) can lead to a marked alteration in renal function.

Potentially toxic chemicals present in the bloodstream will be delivered to the kidneys in large quantities, especially to the cortex, which receives about 80% of the total renal blood flow. This, together with the ability of the kidney to concentrate tubular fluid, may enhance the toxic effect on the proximal tubular cells, by generating a high concentration of the chemical in the tubular lumen. In addition, if a chemical which has been filtered at the glomerulus and concentrated within the tubule is then reabsorbed, by a passive or active mechanism, it will pass through the cells of the nephron at relatively high concentrations and could potentially lead to intracellular toxicity. Finally, many organic chemicals undergo active transport from the blood into proximal tubular cells and then diffuse into the tubular lumen. Thus, the proximal tubular cells can be exposed to higher concentrations than those present in plasma. This active transport occurs in all three segments of the proximal tubule, although considerable variation exists from segment to segment depending on the species (Wright, 2005; Hiasa *et al*., 2006).

The renal medulla receives a much lower blood flow and therefore receives relatively less potential toxic chemical via the bloodstream. However, as the chemical passes down the nephron into the medulla, the countercurrent mechanisms may lead to a chemical becoming

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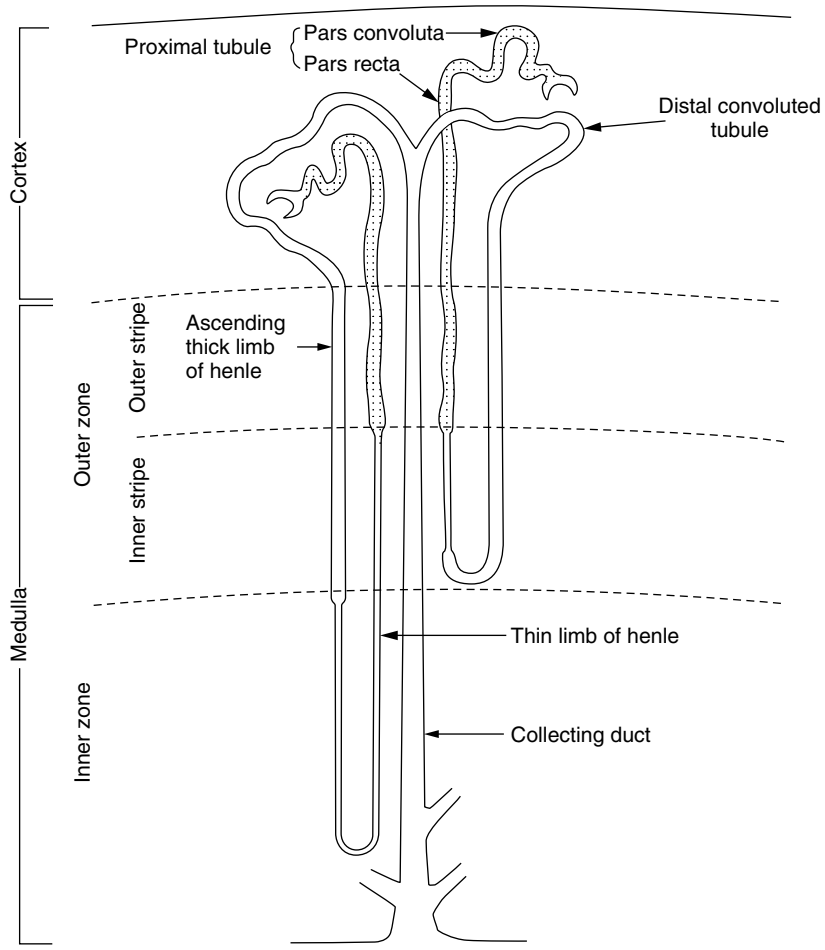

Figure 2 Diagram of the structural organization of the mammalian kidney to demonstrate the relationships between the various segments of the nephron and the zones of the kidney, especially the medulla. (Reproduced from Tisher, 1976. © Elsevier.)

concentrated in this region and the papilla to a concentration many times greater than that in the plasma.

Once a chemical has become concentrated in a renal cell, it may act directly or require further metabolism to produce a toxic response. A direct-acting chemical presumably acts by interfering with important metabolic events—for instance, inhibition of mitochondrial function or of key enzymes involved in energy metabolism. Alternatively, the chemical may be converted to a reactive species that may bind covalently to critical sites in proteins or initiate lipid peroxidation leading to cellular damage. In the latter case, the chemical could have already undergone metabolism in another organ and the stable metabolite enters the kidney where further metabolism to generate a reactive species occurs.

Most of the common enzymes involved in the metabolism of foreign compounds, such as cytochrome(s) P450 and glutathione S-transferases, are present in renal tissue, although the specific activities of these enzymes are usually lower than those found in the liver (Lock and Reed, 1998; Rankin and Valentovic, 2004; Korashy *et al*., 2004; Pavek and Dvorak, 2008). However, the nephron has a very marked cellular heterogeneity and there are major differences in the relative amounts of certain enzymes in the different regions of the nephron (Guder and Ross, 1984; Lock and Reed, 1998). Thus, any measurement of enzyme activities in whole kidney as opposed to renal cortex or medulla, or isolated glomeruli or proximal tubular cells can grossly underestimate the metabolic capabilities of these regions of the kidney. As a foreign chemical passes down the nephron, it may undergo metabolism by a number of different enzymes. For example, paracetamol (acetaminophen), on entering a proximal tubular cell, can undergo oxidation by cytochromes P450, whereas once it has entered the medulla, it may undergo co-oxidation via the prostaglandin H synthase pathway (Tarloff, 1997). Frequently the intrarenal location of injury represents the site of accumulation of either the chemical or its metabolite, or the location of the enzymes which are responsible for activating it.

The kidney is also under the influence of the sympathetic nervous system (DiBona, 1994), and

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chemicals which have a direct action on renal sympathetic nerves can alter renal vascular resistance or renin secretion and influence renal function *per se*.

4 MEASUREMENT OF THE EFFECT OF CHEMICALS ON RENAL FUNCTION

A number of noninvasive techniques are available to assess whether a chemical has had a marked effect on the kidney in both experimental animals and man. A battery of simple tests has evolved which can be applied to urine to give an indication of renal injury. These measurements can be conducted on a temporal basis during feeding studies in experimental animals, or in man exposed to a potentially harmful compound in the workplace, or during drug therapy. The various advantages and disadvantages of these techniques will not be discussed in detail in this chapter. Readers are referred to the following articles and papers therein: Bonventre, 2007; Vaidya *et al*., 2008; Lock and Bonventre, 2008. Traditionally a battery of measurements has been used for both experimental animals and man, which includes plasma creatinine and urea, urine volume, osmolality, pH and the excretion of the electrolytes Na^+ and K^+. The presence of glucose or excess protein in urine and changes in urinary sediment all indicate abnormalities in renal function. However, many of these markers of renal injury are rather insensitive and only change after significant kidney injury and then with a substantial time delay. The increased excretion of specific proteins or enzymes of renal origin in urine can indicate abnormality of function. Certain enzymes that increase in the urine can be of postrenal origin—for instance the bladder—and as such are not indicative of renal damage. *N*-acetyl-β-glucosaminidase (NAG), a proximal tubule lysosomal enzyme, has been extensively studied and has proven to be a sensitive, persistent and robust indicator of tubular injury. More recently, a number of novel proteins have been identified in urine which have potential as markers of renal injury in experimental animals and humans. These proteins include kidney injury molecule-1 (Kim-1), human neutrophil gelatinase-associated lipocalin, interleukin-18, cystatin C, clusterin, fatty-acid-binding protein and osteopontin (Vaidya *et al*., 2008; Dieterle *et al*., 2008). Kim-1 has been examined in preclinical and clinical studies and appears to be an excellent early diagnostic indicator of kidney injury when compared with many of the conventional biomarkers, for example plasma creatinine, blood urea nitrogen, glycosuria, increased proteinuria or increased urinary NAG (Zhou *et al*., 2008; **Figure 3**). Sensitive high-throughput assays have been developed for Kim-1, which is an important requirement for clinical use (Vaidya *et al*., 2008). Proteomic technology with mass spectrometry has enabled the identification of proteins that may also be useful as markers of renal injury (Bandara *et al*., 2003; Witzmann and Li, 2004; Betton *et al*., 2005; Korrapati *et al*., 2007). In an analogous manner, gene expression profiling has also provided insight into predictive markers of nephrotoxicity. For example, kidney gene expression profiling in male Sprague–Dawley rats with 64 nephrotoxic or non-nephrotoxic compounds identified a gene signature consisting of 35 genes which predicted future development of renal tubule injury. These gene changes occurred 76% of the time before the injury was detected histologically or was detected by clinical chemistry (Fielden *et al*., 2005). Thus genomic data can be more sensitive than traditional methods for the early prediction of chemically induced renal injury and hence is a valuable tool in preclinical research and development.

Once an indication of renal dysfunction has been observed, it may be necessary to examine in more detail this effect on renal function and to quantify the response. More specific information indicating whether the insult has occurred to the proximal tubule and/or the glomerulus can be gained by characterizing the proteins excreted in urine on a molecular weight basis, low-molecular-weight proteinuria being indicative of a tubular site of injury, while excretion of high-molecular-weight protein (>80 000) is indicative of glomerular injury (Stonard, 1987; Lauwerys and Bernard, 1989; Emeigh-Hart and Kinter, 2004). Further information on renal function can be obtained by measuring: (i) renal clearance of inulin or creatinine to determine glomerular filtration and (ii) renal clearance and excretion of *p*-aminohippuric acid to determine renal plasma flow, from which renal blood flow can be estimated. Alternatively, radiolabelled microspheres or an electromagnetic flowmeter may be used to specifically measure renal blood flow in experimental animals. Imaging of the kidneys in man and experimental animals is also available (Grenier *et al*., 2003; Dieterle *et al*., 2008) and has been used to identify analgesic nephropathy (Elseviers *et al*., 1995). High-resolution ^{1}H nuclear magnetic resonance (NMR) spectroscopy is now widely used to detect abnormal patterns of metabolites in plasma and urine to help identify renal injury (Williams *et al*., 2003; Lenz *et al*., 2004; Portilla *et al*., 2006), and this, in conjunction with high-performance liquid chromatography–time-of-flight mass spectometry (HPLC-TOF/MS) and pattern recognition techniques, can provide valuable information regarding the probable site of toxic action of a chemical in the nephron. **Figure 4** shows ^{1}H NMR spectra from rats dosed with hexachloro-1,3-butadiene, showing increased excretion of glucose and amino acids indicative of proximal tubule damage and for rats dosed with propyleneimine, showing increases in acetate, succinate, trimethylamine *N*-oxide (TMAO), dimethylamine (DMA) and *N*-*N*-dimethylglycine (DMG) associated

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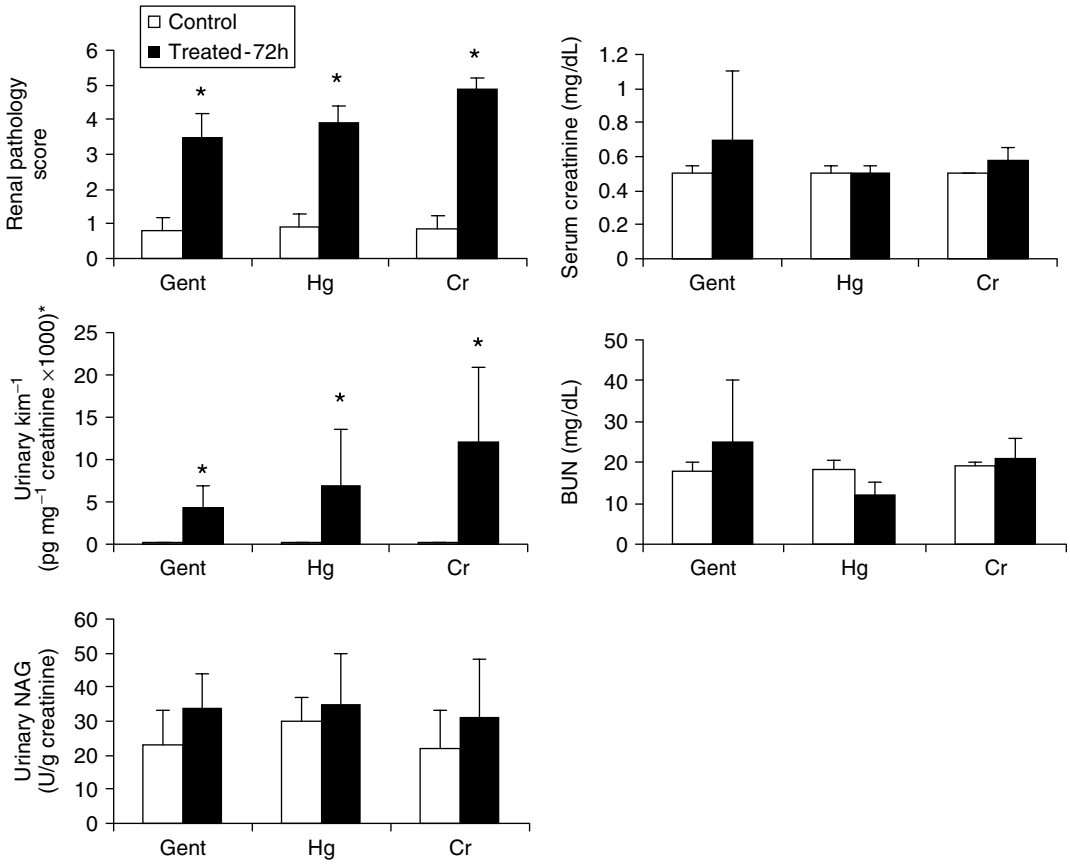

Figure 3 Comparison of urinary Kim-1 with routinely used biomarkers blood urea nitrogen (BUN), creatinine, NAG and nephropathy score for tubule injury 72 hours after gentamicin (100 mg kg^{-1}, sc); $HgCl_2$ (0.25 mg kg^{-1} iv) or potassium dichromate (5 mg kg^{-1}, sc) administration. Urinary NAG and Kim-1 are normalized to urinary creatinine. Asterisks indicate statistically significant difference ($p < 0.05$) compared to control group ($n = 6$ from each group). (Reproduced from Zhou *et al.*, 2008. © Oxford University Press.)

with papillary necrosis (Gartland *et al.*, 1989) For recent reviews on the use of the technique and its applications see Beckonert *et al.* (2007) and Coen *et al.* (2008).

These techniques will enable the detection of abnormal renal function *in vivo*; however, it can be difficult to ascertain whether this malfunction is a direct effect of the chemical, or secondary to altered renal haemodynamics. The direct toxic effect of chemicals may be evaluated *in vitro* by adding the chemical or its metabolite(s) directly to renal cortical slices or isolated proximal tubular cells/tubules in suspension or in primary culture (Zalups and Lash, 1996 and references therein; Lash, 1998). Our understanding of transport systems which enable organic molecules to enter and exit renal tubule cells is now much greater. Advances in molecular biology have identified and cloned many renal transport systems that transport organic cations (organic cation transporters; OCTs), organic anions (organic anion transporters; OATs) and amino acids into renal tubule cells (Wright, 2005; Anzai *et al.*, 2006; Nigam *et al.*, 2007; Bröer, 2008). Several substrates for these transport systems, such as tetraethylammonium (TEA), *p*-aminohippurate (PAH) or aminoisobutyric acid have been used as indices of nephrotoxicity in cell preparations or renal cortical slices (Kacew, 1987). Leakage of lactate dehydrogenase, loss of intracellular adenosine triphosphate (ATP), altered transport of the nonmetabolized sugar α-methyl-glucose, changes in mitochondrial membrane potential and stress protein responses (van de Water *et al.*, 2006) have also been used to indicate cytotoxicity.

Histopathological examination of the kidneys following exposure can identify structural alterations that may have occurred and will also provide valuable information on the area affected. For instance, light microscopy can identify selective damage to the nephron caused by chromium (pars convoluta), gentamicin (pars convoluta and pars recta) (**Figure 5**), 4-aminophenol (pars recta) or propyleneimine (papilla). Light microscopy can also provide information concerning the appearance of protein casts, lysosomal involvement, cellular regeneration and repair or the presence of crystals or stones in the kidney or urine.

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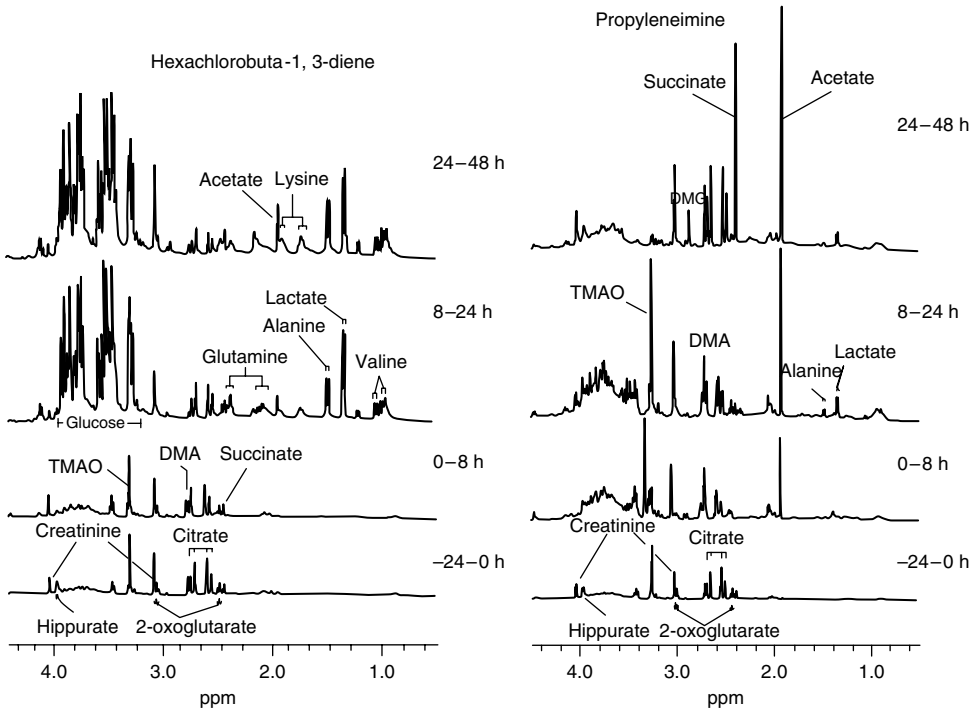

Figure 4 400 MHz ^{1}H NMR spectra of urine from rats before and after dosing with 200 mg kg^{-1} hexachloro-1,3-butadiene or 20 μL kg^{-1} propylene imine. DMA, dimethylamine; TMAO, trimethylamine *N*-oxide; DMG, *N*-*N*-dimethylglycine. (Reproduced with permission from Gartland *et al.*, 1989 © ASPET.)

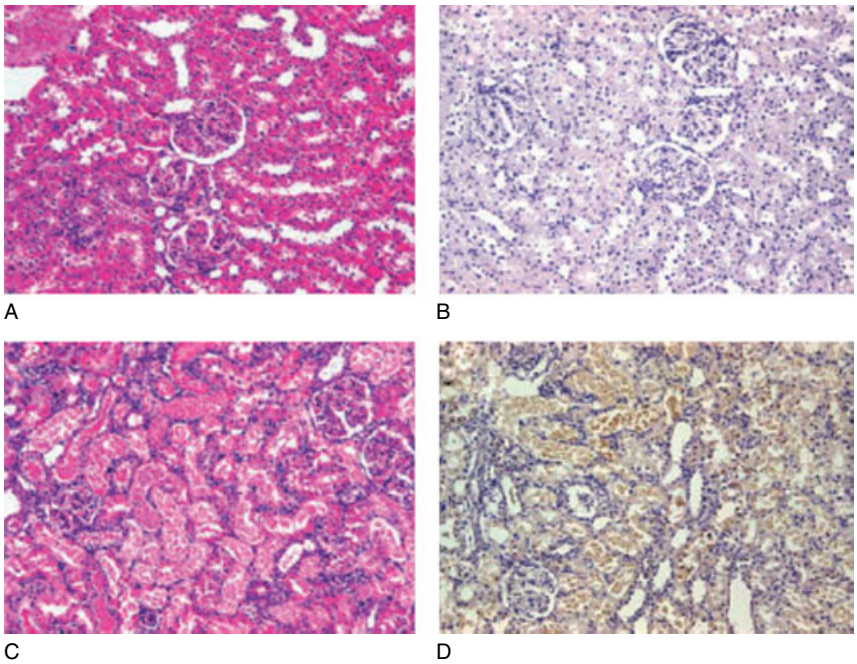

Figure 5 Photomicrographs of renal lesion and Kim-1 immunoreactivity in rats 72 hours following gentamicin, 100 mg kg^{-1}, sc; (A and C) haematoxylin and eosin stained sections; (B and D) immunoperoxidase staining for Kim-1. (A and B) Control saline-dosed showing no renal lesion or immunoperoxidase staining for Kim-1. (C and D) Gentamicin treatment: necrotic epithelial cells in proximal tubules showing positive Kim-1 immunoreactivity. Magnification × 200. (Reproduced from Zhou *et al.*, 2008. © Oxford University Press.)

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Histochemical, immunocytochemical (**Figure 5B** and **D**) and *in situ* hybridization techniques are also valuable in evaluating the response of the kidney to a toxic insult. Depending on the objective of the study, electron microscopy can be used to gain information concerning the subcellular localization of the tubular injury or to probe the glomerulus or papilla for changes that may have occurred following exposure to a nephrotoxin. Changes in mitochondria can be easily identified, as can proliferation of smooth endoplasmic reticulum or alterations in the other organelles such as peroxisomes.

Thus, there is a large battery of tests, many noninvasive, some only requiring small blood samples, which can be used to assess alterations in renal function. In experimental animals these findings can be supported by histopathology and *in vitro* studies with renal tissue.

5 CHEMICALLY INDUCED RENAL INJURY

This section will discuss certain specific nephrotoxic compounds. These chemicals may act either directly or require metabolism to produce the ultimate nephrotoxin. This metabolism may occur solely in the kidney or may involve initial biotransformation in an extrarenal organ followed by activation in the kidney. In some of these examples alterations in renal function may be secondary to changes in blood pressure or blood volume, or to hormonal or neural effects. It is not the intention of this section to discuss all known chemicals which have been reported to cause renal injury. Instead the focus will be on areas where there is some understanding of the mechanisms of nephrotoxicity.

5.1 Heavy Metals

Several heavy metals are nephrotoxic, since the kidney concentrates them prior to excretion and several of them are potent inhibitors of metabolic processes (Barbier *et al*., 2005; Sabolić, 2006). However, several mechanisms exist which protect the kidney against heavy metals—for example, the presence of metallothionein and other high-affinity metal-binding proteins, and the compartmentalization of the metals into lysosomes. The precise mechanism of metal transport into proximal renal tubular cells is not fully understood. Metals can enter proximal tubular cells by endocytosis following the binding of the metal itself or a metalloprotein complex such as Cd–metallothionein to the brush-border membrane (Foulkes, 1988). Transport can also occur via the divalent metal transporter (DMT-1) (Ferguson *et al*., 2001; Abouhamed *et al*., 2006), which is located on the endosomes/lysosomes, and toxic divalent cations, such as Cd^{2+}, Pb^{2+}, Co^{2+}, Ni^{2+} and Pt^{2+}, can enter renal cells by this route. Recent studies using RNA interference to knock down DMT-1 in renal tubule cells, showed some protection against the cytotoxicity produced by Cd-metallothionein-1, confirming a role for this transporter in metal toxicity (Abouhamed *et al*., 2007). Once inside the cell the metal can then be released from the protein–metal complex by lysosomal degradation. Metals may also enter renal cells following conjugation with glutathione to form cysteine or *N*-acetylcysteine conjugate and/or cystine and *N*-acetylcystine conjugates, which can enter renal cells by specific transport systems located on brush border (amino acid transporters) and basolateral membranes (organic anion transporters) (**Figure 6**) (Bridges and Zalups, 2005 and references therein). The intracellular distribution of the metal will then depend on the presence of various high-affinity binding sites or sinks within the cell (Cain, 1987; Fowler, 1989; Zalups and Lash, 1994).

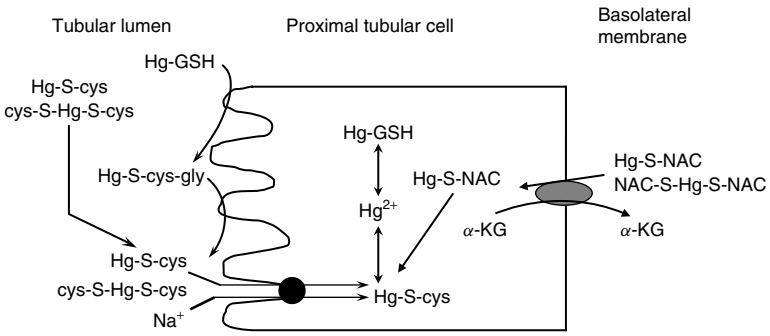

Figure 6 Proposed routes of entry of mercury into proximal renal tubular cells. Hg-cysteine (Hg-S-cys) and Hg cystine (cys-S-Hg-S-cys) in the tubular lumen, can enter via an Na-dependent amino acid transport system, while the *N*-acetylcysteine conjugate of Hg (NAC-S-Hg) and Hg di-*N*-acetylcystine conjugate (NAC-S-Hg-S-NAC) in the plasma can enter via the basolateral membrane organic anion transport system OCT1, in exchange for α-ketoglutarate. (Reproduced from Bridges and Zalups, 2006. © American Chemical Society.)

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Low doses of a number of heavy metals produce a similar response—for example, leakage of glucose and amino acids into urine and diuresis. If the dose of metal is increased, then renal tubular necrosis occurs which can lead to renal shutdown, a marked elevation in blood urea and ultimately the death of the animal. The histological pattern of injury is one of necrotic proximal tubules with dilation of the tubular lumen, which contains proteinaceous casts. This necrosis is thought to be due to a combination of ischaemia secondary to vasoconstriction and a direct cytotoxic action of the heavy metal, since renal ischaemia produced by temporary clamping of the aorta or sustained hypotension causes renal tubular necrosis which is mainly localized to the pars recta region (Glaumann and Trump, 1975; Kriesberg *et al*., 1976; Venkatchalam *et al*., 1978). The fall in blood pressure is thought to trigger the release of renin, which activates angiotensin, a potent vasoconstrictor. The site of renal necrosis produced by ischaemia is the same as that seen with several heavy metals, which suggests a vascular component in the acute renal failure. However, chronic salt loading of rats, which depletes intrarenal renin prior to administration of a heavy metal, can protect against the functional impairment without affecting the extent of necrosis (DiBona *et al*., 1971; Flamenbaum *et al*., 1973), which indicates a role for the direct action of heavy metals on the tubular epithelium. There is also frequently a good correlation between the renal localization of a heavy metal and the site of morphological damage. On the basis of the above it seems plausible to conclude that the nephrotoxicity of heavy metals occurs by two distinct mechanisms: (i) acute renal failure mediated through the release of renin, which can be prevented by depletion of intrarenal renin and (ii) proximal tubular necrosis, which seems to be due to accumulation of the metal in the proximal tubular cells.

5.1.1 Inorganic Mercury

Extensive data are available on the nephrotoxicity of mercuric chloride ($HgCl_2$), primarily because of its use as a model compound to produce acute renal failure in experimental animals. Functional impairment probably results from both vasoconstriction and a direct cytotoxic effect of the metal. Small nephrotoxic doses of $HgCl_2$ produce a selective necrosis histologically located in the pars recta of the proximal tubule (Rhodin and Crowson, 1962; Gritzka and Trump, 1968). The location of the lesion in this region is consistent with the localization of the metal (Taugner *et al*., 1966). However, as the dose of $HgCl_2$ is increased, the injury extends into the pars convoluta (Rhodin and Crowson, 1962). The basic biochemical mechanism whereby $HgCl_2$ produces renal cellular damage is unclear. $HgCl_2$ will readily react with the thiol groups in proteins and enzymes (Webb, 1966), in the latter case, causing inhibition of cellular function. It has, however, been difficult to identify *in vivo* those proteins most sensitive to mercury (Vallee and Ulmer, 1972). The earliest pathological changes following $HgCl_2$ are loss of brush-border membranes, dispersion of ribosomes and the clumping of smooth membranes in the cytoplasm (Ganote *et al*., 1975; McDowell *et al*., 1976). These changes are followed by the appearance of vacuoles in the cytoplasm and clumping of nuclear chromatin. Rupture of the plasma membrane and mitochondrial changes are late changes associated with the onset of renal necrosis. Histochemical studies have shown that enzyme activities associated with the brush-border membranes—for example, alkaline phosphatase and 5′-nucleotidase—were decreased as early as 15 minutes following $HgCl_2$ administration (Zalme *et al*., 1976). Thus, the changes following $HgCl_2$ are first associated with the brush-border membranes and apical vacuole and only later with mitochondria and other structures within the cell. The protein Kim-1, which is located on brush-border membranes, has been shown to be a sensitive marker of renal injury in rats exposed to a low dose of $HgCl_2$ (Zhou *et al*., 2008; **Figure 3**). The uptake of mercury from the tubular lumen probably occurs as cysteine (Hg-S-cys) or cystine (cys-S-Hg-S-cys) complexes via an Na^+-dependent amino-acid transport system (**Figure 6**). In addition, homocysteine or homocystine conjugates of mercury, if formed, may also enter via this amino-acid transport system. Mercury as cys-S-Hg-S-cys, Hg-S-NAC and NAC-S-Hg-S-NAC are also substrates for the organic anion transporter, primarily OCT1 and enter proximal tubular cells by this route (**Figure 6**) (see reviews by Bridges and Zalups, 2005; 2006). The precise intracellular targets for mercury have not been ascertained, the resultant cell toxicity probably results from a cascade of events, including a reduction in cellular glutathione, ascorbate and vitamin E making the cells more susceptible to oxidative stress, plus inhibition of critical thiol residues in mitochondria leading to reduced ATP synthesis (Diamond and Zalups, 1998 and references therein).

5.1.2 Organomercurials

The rate of decomposition of organomercurials to form Hg^{2+} appears to reflect the relative nephrotoxicity of these chemicals, the ranking order (from least to most toxic) being $MeHg^+ < EtHg^+ < PhHg^+ <$ MeoEtHg $< Hg^{2+}$ (Magos, 1982). The alkyl mercurials, which decompose quite slowly, can produce renal injury, although their primary action is on the nervous system. For example, the victims of the ethylmercury epidemics in Iraq showed polyuria or oliguria with urinary casts and excretion of albumin (Jalili and Abbasi, 1961). However, in the large epidemics following methylmercury exposure, the kidneys of even the most severely affected people were spared (Bakir *et al*., 1973). Nevertheless,

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chronic administration of methylmercury will produce renal damage in rats (Magos and Butler, 1972; Mitsumori *et al*., 1984) and mice (Mitsumori *et al*., 1990). Chronic administration of methylmercury to rats produced ultrastructural damage to the pars recta of the proximal tubule, which included proliferation of smooth endoplasmic reticulum, degeneration of mitochondria and cellular necrosis. These lesions are also seen following inorganic mercury exposure to rats (see above), which supports the view that it is the release of inorganic mercury that is responsible for the renal injury. Methylmercury also has a high affinity for thiol groups and readily reacts with glutathione and cysteine, and the cysteine conjugate is a molecular mimic for the amino acid methionine, whereby it can enter renal cells on an amino-acid transport system (Bridges and Zalups, 2005; 2006).

5.1.3 Mercury-Induced Glomerulonephritis

Mercury has been reported to produce an immunologically mediated glomerulonephritis in both man and experimental animals (Druet *et al*., 1987a; 1987b). The cause of this glomerulonephritis is not well understood and different mechanisms may operate depending upon species and strain. Administration of $HgCl_2$, $1\,mg\,kg^{-1}$ three times a week, to the Brown Norway strain of rat produces an autoimmune response with an increase of circulating IgE, IgG and anti-glomerular-basement membrane antibodies; these latter antibodies can be found deposited along the glomerular capillary wall and this deposition is associated with the occurrence of marked proteinuria and nephrotic syndrome. This autoimmune disease in the Brown Norway rat is not dose-dependent, since lower doses induce the same response, nor does the route of administration (oral, topical or parenteral) influence the response. Also important is the fact that several mercurials ($HgCl_2$, methylmercury or various pharmaceuticals containing mercury, such as thimerosal) all induce this response to various degrees. The autoimmune response is under genetic control, the Brown Norway rat being the only strain out of 22 tested which responded. However, glomerular lesions have also been induced with $HgCl_2$ in various other strains of rats and genetically susceptible mice, the immune response being different from that seen in the Brown Norway rat. In these strains it was characterized as an immune-complex-type glomerulonephritis with the presence of antinuclear antibodies. $HgCl_2$ induces in mice an autoimmune syndrome with T-cell-dependent polyclonal B cell activation and hypergammaglobulinaemia, which is associated with a dose- and H-2-dependent production of autoantibodies targeting the 34 kDa nucleolar protein fibrillarin and causing systemic immune-complex deposits (Havarinasab *et al*., 2007). This response is also seen with methylmercury and ethylmercury in the form of thimerosal, which are converted to the immunogen Hg^{2+} in the body. See Rowley and Monestier (2005) for a review of this topic.

5.1.4 Cadmium

Damage to proximal renal tubules is a characteristic feature of long-term, but not acute, cadmium exposure in both humans and experimental animals (Lauwerys *et al*., 1980; Goyer, 1989; Fels *et al*., 1994 and references therein). Acute doses of Cd^{2+} accumulate predominantly in the liver, whereas following chronic exposure in the diet it is the kidney which ultimately accumulates the highest concentration of Cd^{2+}. Cd^{2+} or Cd^{2+}–protein complexes accumulated by hepatic parenchymal cells induce the synthesis of metallothionein, a low-molecular-weight cysteine-rich protein (Kagi, 1993; Klaassen *et al*., 1999), which binds cadmium very avidly. This Cd^{2+}–metallothionein complex is then thought to be very gradually released from the liver and taken up by endocytosis into renal proximal tubular cells, where free Cd^{2+} can be generated due to lysosomal degradation of the complex. The kidney is able to accumulate large concentrations of Cd^{2+}–metallothionein without obvious damage, until a critical concentration of between 100 and 200 µg $Cd^{2+}\,g^{-1}$ renal cortex is attained, at which renal injury is initiated (Friberg, 1984). Proximal tubular cells can synthesize metallothionein in response to a rise in intracellular Cd^{2+} concentration and the onset of renal injury may reflect a saturation of all the available binding sites in the cell (both constitutive and induced). The toxic species which precipitates the renal damage is almost certainly free (nonmetallothionein-bound) cadmium. It is known that Cd^{2+} *per se* is a very potent inhibitor of enzymes and biochemical pathways (Vallee and Ulmer, 1972; Webb, 1977), whereas Cd^{2+}–metallothionein is not.

Administration of Cd^{2+}–metallothionein to rats produces a very marked nephrotoxicity at much lower doses than Cd^{2+} itself. Similarly administration of Cd^{2+} complexed to either glutathione or cysteine produces very selective toxicity to the pars recta of the proximal tubule, suggesting that the metal complexed in this form may be actively transported into renal cells, analogous to that described for mercury (Bridges and Zalups, 2005). Cd^{2+} can also complex with glycine and thereby reduce its uptake in renal cells (Tang *et al*., 2006). In these acute models considerably more Cd^{2+} is delivered to the kidney than in the low-level chronic situation such that the defence mechanism(s), for example, metallothionein, may not be adequately induced to afford protection; see Cain (1987), Klaassen and Liu (1997) and references therein. Recent studies with metallothionein transgenic mice, which have increased renal metallothionein content, or with metallothionein null mice have shown them to be as susceptible to

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Cd^{2+}–metallothionein-induced renal injury as control mice (Liu *et al*., 1996; Klaassen and Liu, 1997). These findings indicate that metallothionein appears to play little or no protective role in acute Cd^{2+}–metallothionein nephrotoxicity. More recent studies, using Cd^{2+}-resistant MT(−/−) (metallothionein knockout) and CdR revertant cell lines has indicated a role for the T-type calcium channel Cacnα1G in uptake of cadmium (Leslie *et al*., 2006). Thus Cd^{2+} can enter renal cells by a number of routes, via the DMT-1 transporter and T-type Ca^{2+} channel, as a glutathione-derived conjugate via the apical and basolateral membrane, and via endocytosis.

5.1.5 Other Metals

Two other metals which are nephrotoxic should be mentioned—these are chromium and lead. Acute necrosis of the proximal convoluted tubule has been reported in man following exposure to hexavalent chromium (Cr^{6+}) (Franchini *et al*., 1978; Jao *et al*., 1983) and following chronic exposure in the workplace (Wang *et al*., 1994). Various studies have confirmed these findings in rodents. The primary site of action of Cr^{6+} is on the convoluted portion of the proximal tubule (Evan and Dail, 1974; Berndt, 1975), which is different from that seen with mercury. These morphological findings are supported by functional studies where, for example, glucose reabsorption, a function of the convoluted part of the proximal tubule, is severely affected, leading to marked glucosuria (Berndt, 1975). As with the other metals, the earliest morphological change following Cr^{6+} administration is to the brush-border membrane (Evan and Dail, 1974; Kirschbaum *et al*., 1981). How the metal is transported into renal cells is not understood, but there have been some studies indicating that glutathione may play a role (Standeven and Wetterhahn, 1991). Little recent information has been reported on the mechanism of nephrotoxicity of Cr^{6+}; however, the urinary biomarker Kim-1 does appear to be a sensitive and early indicator of Cr^{6+}-induced renal injury (Zhou *et al*., 2008; **Figure 3**).

Lead is probably the most abundant nephrotoxic metal and because of industrial exposure there is considerable clinical data. Lead-induced nephrotoxicity is characterized morphologically by the presence of lead intranuclear inclusion bodies, karyomegaly, cytomegaly and ultrastructural changes in mitochondria, primarily in the pars recta of the proximal tubule. These changes are accompanied in severe cases by functional changes in glucose, amino acid and phosphate reabsorption (Goyer, 1982). Chronic exposure of experimental animals to lead salts produces a similar spectrum of renal changes to those seen in man (Goyer and Rhyne, 1973). The majority of lead present in blood is located in the red cell; only lead bound to proteins or ligands which are filterable at the glomerulus is available for uptake into renal cells. Like other metals, lead is taken up by endocytosis via the DMT-1 transporter into renal proximal tubular cells, where free Pb^{2+} can be generated due to lysosomal degradation of the protein complex. See Bridges and Zalups, 2005 for a more detailed discussion on transport systems for lead into renal cells. Studies in male rats have shown that lead is bound to the protein α_{2u}-globulin (Fowler and DuVal, 1991), which is synthesized in the liver and transported to the kidney (see later) where a proportion is taken up into renal proximal tubular cells. A lead-binding protein in human kidney has recently been identified as diazapine-binding inhibitor, which has similar properties to α_{2u}-globulin, but is not a member of the same gene family (Fowler and Squibb, 1997). Once inside the cell, any free metal will initially bind to certain high-affinity lead-binding proteins which are present in the kidney in high concentrations. These proteins are thought to carry lead into the nucleus, where *de novo* synthesis of a unique acidic protein results in metal precipitation to form the classical lead intranuclear inclusion bodies (Fowler, 1998).

Mitochondria are extremely sensitive to lead and, following chronic *in vivo* administration, mitochondrial swelling, which is associated with decreased respiratory control, has been reported (Goyer and Krall, 1969; Fowler *et al*., 1980). Certain enzymes involved in haem biosynthesis are also very sensitive to lead—in particular, δ-aminolaevulinic acid dehydratase. Inhibition of this enzyme *in vivo* results in urinary excretion of δ-aminolaevulinic acid and forms the basis of biological monitoring for lead.

5.2 Antineoplastic Agents: Cisplatin

The platinum antitumour drugs—for example, cisplatin (*cis*-dichlorodiamine platinum II)—are widely used for the treatment of a range of cancers (Loehrer and Einhorn, 1984; Rosenberg, 1985), but nephrotoxicity is frequently a side effect, which limits the dosage (Borch, 1987; Arany and Safirstein, 2003; Yao *et al*., 2007). Clinical manifestation of renal functional impairment includes elevation in blood urea and creatinine, some proteinuria and enzymuria and, in severe cases, the presence of cells and casts in the urine. Electrolyte disturbances are also common, particularly hypomagnesaemia, which may be related to impaired renal tubular absorption (Goldstein and Mayor, 1983; Litterst and Weiss, 1987). Histopathology of the human kidney showed focal tubular necrosis primarily to the distal tubule and collecting ducts, with some dilatation of the convoluted tubules and the presence of casts (Gonzalez-Vitale *et al*., 1977; Dentino *et al*., 1978). In experimental animals cisplatin produces marked impairment of renal function (Arany and Safirstein, 2003); however, the onset of this renal lesion is delayed.

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Following a single dose to the rat, histopathological alterations were minimal over the first two days, but by day 3 changes to brush-border membranes occurred selectively in the pars recta of the proximal tubule. By day 5 the predominant pattern of injury was widespread necrosis to the pars recta of the proximal tubule, which by day 7 had started to show extensive regeneration (Dobyan *et al*., 1980). No histopathological changes were seen in the distal tubule, in contrast to the findings in man.

The mechanisms underlying cisplatin nephrotoxicity (Arany and Safirstein, 2003; Hanigan and Devarajan, 2003; Bonegio and Lieberthal, 2004; Yao *et al*., 2007) and the basis of the delayed onset of toxicity are not fully understood. The platinum moiety *per se* may not be responsible for the nephrotoxicity, as the trans isomer of cisplatin is not nephrotoxic (Daley-Yates and McBrien, 1985). Platinum complexes are characterized by their slow rates of ligand substitution in comparison with other metal complexes. When cisplatin is dissolved in water, the more labile chloride ligands are displaced in a stepwise fashion to form aquated complexes. In plasma at a chloride concentration of 110 mM, cisplatin would be expected to exist predominantly as the neutral dichloro complex. In contrast, the aquated species would be expected to dominate at the lower chloride concentration of the cytosol. An important property of the aquated platinum complexes is that the water ligands are far more reactive than the chloride ligands and are readily replaced by a variety of biological nucleophiles. Cisplatin itself, as well as the aquated species will readily react with thiol groups in proteins (enzymes) and with glutathione, cysteine and methionine. Thus, a metabolite of cisplatin rather than the platinum moiety itself may mediate the nephrotoxicity of the drug. Support for the nephrotoxicity of a glutathione-derived conjugate comes from studies showing that inhibition of γ-glutamyltransferase in rats and mice will prevent cisplatin-induced renal injury (Hanigan and Devarajan, 2003); however, the role of other enzymes responsible for the further processing of glutathione conjugates is not clear.

What is known is that Pt from cisplatin accumulates in the kidney via the organic cation transporter in rats (rOCT2) (**Figure 7**) and humans (hOCT2). However, cisplatin is not a substrate for the multidrug and toxin extrusion transporter in rats (rMATE1) or humans (hMATE1 or hMATE2-K; **Figure 7**), suggesting cisplatin can be readily transported into renal tubule cells, but not out (Yokoo *et al*., 2007).

Cisplatin causes apoptosis in renal tubule cells and many studies have examined the cascade of events, such as caspase activation and cytochrome C release (Arany and Safirstein, 2003; Hanigan and Devarajan, 2003; Yao *et al*., 2007). Mitochondria seem to be an important intracellular target for cisplatin (Zhang and Lindup, 1993) and inhibition of oxidative phosphorylation (**Figure 5**) may be the primary event, with loss of intramitochondrial

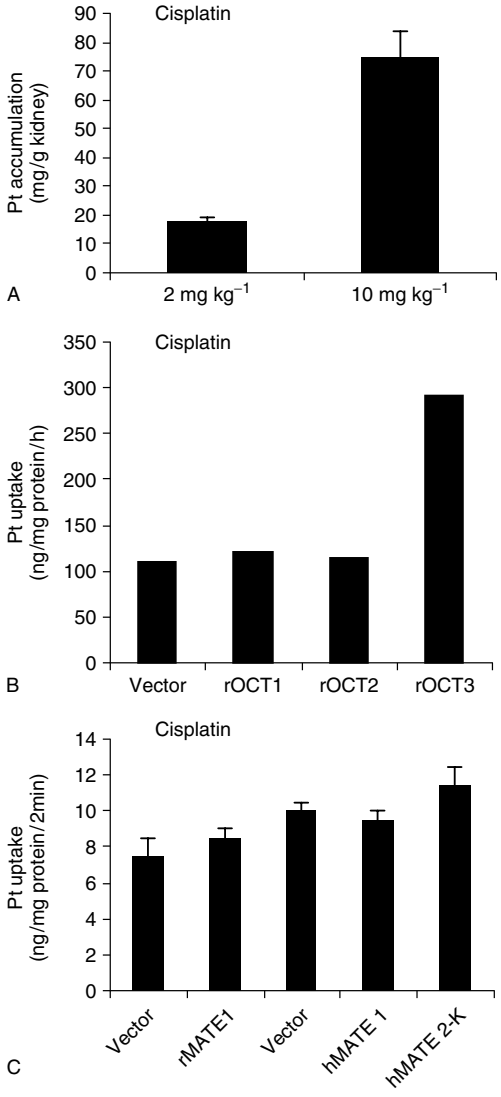

Figure 7 Cisplatin accumulation in rat kidney and uptake in human embrionic kidney (HEK) 293 cells expressing various transporters: (A) Renal platinum concentration 48 hours after 2 or 10 mg kg^{-1} cisplatin; (B) uptake of cisplatin (500 μM) by HEK 293 cells expressing the empty vector, rOCT1, rOCT2 or rOCT3 for one hour; (C) uptake of cisplatin (500 μM) by HEK 293 cells expressing the empty vector, rMATE1, hMATE1 or hMATE-2K for two minutes. (Reproduced from Yokoo *et al*., 2007. © Elsevier.)

glutathione and generation of reactive oxygen species a subsequent phenomenon (Kruidering *et al*., 1997). One of the earliest responses in renal tubule cells to cisplatin is activation of the MAPK (mitogen-activated protein kinase) cascade and stress-response genes, followed later by genes associated with cell cycle and inflammation (Arany and Safirstein, 2003; Yao *et al*., 2007; Wang *et al*., 2008; Suzuki *et al*., 2008).

DNase I knockout mice are significantly protected against cisplatin nephrotoxicity and it appears that endonuclease G (endo G) is also involved in mediating

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cell death as gene silencing of endo G *in vitro* protects against cell death (Yin *et al*., 2007). Selenium (Baldew *et al*., 1989) and a number of thiol ligands, such as glutathione, diethyldithiocarbamate (Borch and Markman, 1989), 4-methylthiobenzoic acid (Boogaard *et al*., 1991a) and metallothionein (Boogaard *et al*., 1991b) have been shown to afford some protection against the nephrotoxicity produced by cisplatin. Similarly, administration of cisplatin in hypertonic saline reduced the nephrotoxicity and lowered the kidney platinum concentrations in both rat (Litterst, 1981) and human (Borch and Markman, 1989). Thus, the data suggest that the retention of platinum or an aquated platinum complex in renal tubular cells and its reactivity with cellular nucleophiles, in particular in the mitochondria, may account for the nephrotoxicity. Numerous other agents have been shown to ameliorate cisplatin nephrotoxicity and the reader is referred to a recent review by Ali and Al Moundhri (2006).

5.3 Immunosuppressive Agents: Cyclosporine

Cyclosporine is a highly lipophilic, cyclic undecapeptide of fungal origin with potent immunosuppressive activity. It is widely used in solid organ transplantation. The major clinical problem associated with cyclosporine usage is nephrotoxicity, which is manifested as a decrease in glomerular filtration rate. This effect is, in part, due to altered renal haemodynamics; several studies in experimental animals (Murray *et al*., 1985; Barros *et al*., 1987; Whiting and Thomson, 1989) and man (Curtis *et al*., 1986) have shown that cyclosporine causes an increase in renal vascular resistance and a reduction in renal blood flow. Cyclosporine causes both acute and chronic toxicity and these may occur by different mechanisms. Acute nephrotoxicity is a functional abnormality related to a local imbalance of vasoconstrictor and vasodilator mediators of which there are many. The mechanisms underlying the altered renal function are still not entirely clear but the renin–angiotensin–aldosterone system seems to play a pivotal role with studies focussed on factors such as eicosanoids, endothelin and nitric oxide (see Cattaneo *et al*., 2004; Charney *et al*., 2004). There is also a growing body of evidence suggesting a role for free radicals in acute cyclosporin nephrotoxicity, where functional changes are accompanied by increases in malondialdehyde, lipid hydroperoxides, radical oxygen species and depletion of glutathione (Cattaneo *et al*., 2004).

Morphological studies of experimental acute cyclosporine nephrotoxicity have shown early sublethal cellular changes confined to the pars recta of the proximal tubule. However, whether these degenerative changes are a direct result of the chemical or are secondary to the ischaemia is currently unclear (Myers, 1986; Mihatsch *et al*., 1989).

Acute cyclosporine nephrotoxicity is related to the concentration of the circulating drug. Although it is still unclear as to whether the unchanged drug or metabolite is responsible for the toxicity, most information supports the view that it is the parent compound (see Burke *et al*., 1989). There is good evidence that alteration of the hepatic metabolism of cyclosporine in both man and experimental animals can affect the extent of renal functional impairment. For example, inducers (e.g. phenobarbitone) or inhibitors (e.g. ketoconazole) of cyclosporine metabolism will either lower or raise the cyclosporine circulating blood level and thereby reduce or potentiate the nephrotoxicity (Burke *et al*., 1989).

In vitro studies, in which cyclosporine has been added to renal cells, have indicated that the chemical can have a direct action on cellular systems. Cyclosporine will prevent the uptake of glucose by LLC-PK_1 cells (Scoble *et al*., 1979), which suggests that the glycosuria seen *in vivo* (Grieve *et al*., 1990) could be due to a direct effect of the chemical. Cyclosporine is a potent inhibitor of the mitochondrial matrix enzyme peptidyl-propyl cis–trans isomerase (cyclophilin) and thereby inhibits mitochondrial membrane transport (Griffiths and Halestrap, 1991). Inhibition of renal mitochondrial peptidyl-propyl cis–trans isomerase will disrupt the Ca^{2+}–calmodulin–calcineurin signalling pathway which may be relevant to the mechanism of nephrotoxicity.

Chronic cyclosporin nephrotoxicity is characterized by an irreversible and potentially progressive nephropathy (Hall *et al*., 1985; Mihatsch *et al*., 1989; Mason and Moore, 1997). Morphologically the nephropathy is characterized by a diffuse interstitial fibrosis or striped fibrosis with glomerular and arteriolar thrombi (Mihatsch *et al*., 1989). The haemodynamic consequences are a persistent renal vascular resistance associated with a marked decline in glomerular filtration rate and renal blood flow, and systemic hypertension. Angiotensin II may play a role in cyclosporin-induced chronic nephrotoxicity, at least in animals, while cyclosporin stimulates renal and systemic overproduction of TGF-β (transforming growth factor β). This cytokine plays a major role in the generation of renal fibrosis by directly stimulating extracellular matrix components and reducing collagenase production, leading to renal scarring (Islam *et al*., 2001).

5.4 Therapeutic Agents

5.4.1 Aminoglycosides

Nephrotoxicity related to aminoglycosides is still a major limitation in their clinical use. Kahlmeter and Dahlager (1984) reviewed over 10 000 patients in clinical

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trials with aminoglycosides and found a frequency of nephrotoxicity resulting from gentamicin and tobramycin of about 14%, and from netilmicin and amikacin of 9%. Similarly, Hall *et al*. (1983) reviewed cases of hospital-acquired acute renal failure and showed that 11% of over 2000 cases were attributable to aminoglycosides. In more recent surveys using elevation of serum creatinine as the marker of renal impairment, lower incidences of renal injury were found (Blaser *et al*., 1995), although others consider that nephrotoxicity is still a complication in 10–20% of therapeutic courses (Swan, 1997; Martínez-Salgado *et al*., 2007).

The aminoglycosides consist of two or more amino sugars which are cationic at physiological pH joined by a glycosidic linkage to a hexose nucleus (**Figure 8**). The clinically relevant aminoglycosides are gentamicin, tobramycin, netilmicin, amikacin, kanamycin, streptomycin and neomycin C. These antibiotics are primarily excreted by glomerular filtration. A small amount of the cationic drug binds to anionic phospholipids (primarily phosphatidylinositol) located on the brush-border membrane of the proximal tubule, and the bound aminoglycoside then enters the renal tubular cell by endocytosis and is stored in secondary lysosomes. This process leads to the accumulation of the antibiotic in proximal tubular cells (Cojocel, 1997 and references therein), where it can persist for days. Location of the drug in the pars convoluta and pars recta of the proximal tubule is in agreement with the histopathological location of necrosis in this region of the cortex. Ultrastructural examination of the renal cortex showed marked hypertrophy of the lysosomes in the proximal tubules and the presence of concentric lamellar material (myeloid bodies) within these organelles (Kosek *et al*., 1974; De Broe *et al*., 1984).

The earliest functional changes are alterations in renal concentrating ability, proteinuria and enzymuria and in acid–base balance (Kaloyanides, 1984; Cojocel, 1997; Zhou *et al*., 2008). In addition to renal tubular necrosis, functional and ultrastructural changes occur in the intraglomerular mesangial cells following gentamicin (Cojocel, 1997; Martínez-Salgado *et al*., 2007).

The biochemical events leading to renal tubular necrosis are believed to be initiated by the binding of the cationic aminoglycoside to negatively charged phospholipid bilayers and to the protein megalin a multiligand, endocytic receptor which is abundant in renal proximal tubule cells (Rougier *et al*., 2004; Nagai and Takano,

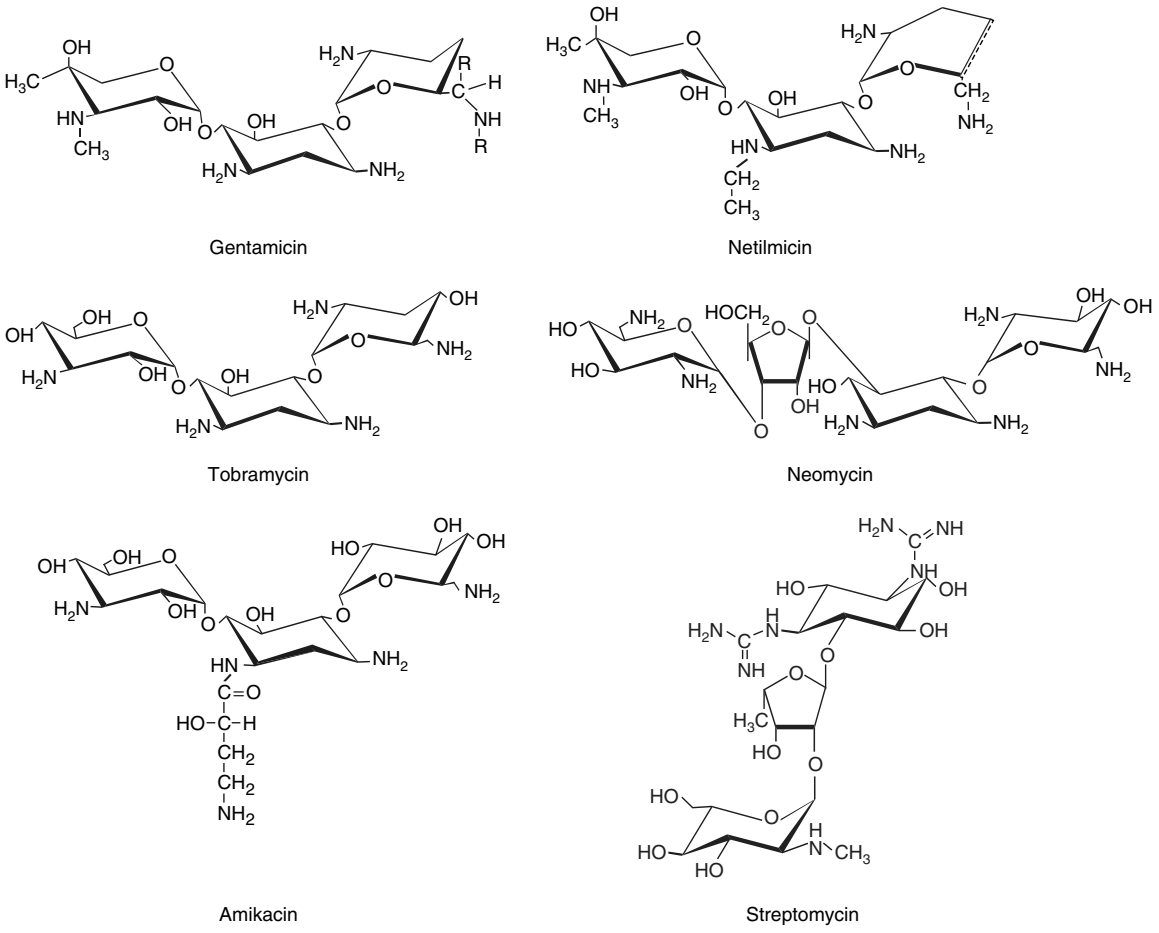

Figure 8 Structural formulae of some aminoglycosides used in clinical practice.

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2004). This impairs the degradation of phosphatidylinositol by binding to phosphatidylinositol-4,5-biphosphate and preventing its metabolism to the triphosphate (Kaloyanides and Ramsammy, 1989). The binding of the aminoglycosides also alters the activation and redistribution of the protein kinase C complex. Impairment of phosphoinositol metabolism probably results in altered Ca^{2+} membrane transport, which can lead to cellular injury and slow repair to damaged cell membranes. Calcium has been shown to inhibit the binding of gentamicin to renal membranes, and calcium loading can protect against the renal injury produced by this drug (Humes *et al*., 1984). Aminoglycosides also compete with Ca^{2+} for binding to megalin (Nagai *et al*., 2001), which may reduce the accumulation of the aminoglycoside by megalin-mediated endocytosis. Administration of an anionic polypeptide such as poly-L-aspartic acid with gentamicin will protect against the nephrotoxicity (Ramsammy *et al*., 1989; Swan *et al*., 1993). It is believed that poly-L-aspartic acid binds gentamicin, thereby displacing it from negatively charged membrane lipids and relieving the inhibition of phospholipid metabolism. Recent studies in megalin knockout mice has shown that in the absence of megalin the concentration of gentamicin in the kidneys is drastically reduced, strongly indicating that megalin-mediated endocytosis is the major pathway for the accumulation of aminoglycosides in the kidney (Schmitz *et al*., 2002). Many strategies have been tried to protect against gentamicin nephrotoxicity, see the review by Ali (2003).

Gentamicin inhibits oxidative phosphorylation in renal cortical mitochondria *in vitro* (Simmons *et al*., 1980). This may be due to altered mitochondrial calcium transport, which can influence mitochondrial respiration. At concentrations which are found in proximal tubular cells, gentamicin will induce the release of iron and enhance the generation of hydrogen peroxide by isolated mitochondria (Walker *et al*., 1999) implicating a role for free radicals in aminoglycoside nephrotoxicity. More recent studies examining changes in gene expression in the kidney after gentamicin have shown increases in oxidative stress genes in primary rat proximal tubule cells 24 hours after exposure (Suzuki *et al*., 2008) with some of these changes seen *in vivo* in the kidney (Wang *et al*., 2008). Genes associated with regeneration, such as Kim-1, IGFBP-1 (insulin-like growth factor binding protein 1), neutrophil gelatinase-associated lipocalin and osteopontin, were also markedly elevated five days after gentamicin exposure (Wang *et al*., 2008).

Thus the following cascade of events is still thought to occur (Tulkens, 1986; **Figure 9**), the major new information being the binding of the drug to megalin. The drug is filtered at the glomerulus and enters the tubular lumen, where it binds to megalin. These complexes undergo endocytosis, thereby developing high intracellular concentrations of the antibiotic, and become incorporated into lysosomes and inhibit phospholipid metabolism. Interaction with mitochondria can lead to generation of reactive oxygen species that can alter cellular function and lead to necrosis. The balance between the repair of the cellular injury and the extent and duration of the necrosis determines the extent of the renal failure.

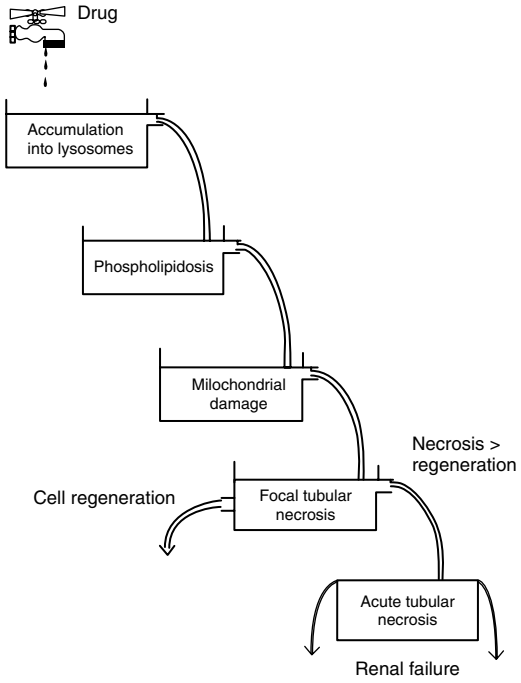

Figure 9 The aminoglycoside nephrotoxicity cascade, which occurs during exposure to low therapeutic doses of aminoglycosides. It is important to note that there is a threshold for each event; once this is reached it can induce the next event. For example, acute tubular necrosis develops only after the degree of focal necrosis outweighs the compensatory regeneration. (Reproduced from Tulkens, 1986. © Elsevier.)

5.4.2 β-Lactam Antibiotics

The β-lactam antibiotics include the penicillins, cephalosporins, carbapenems and several structurally related compounds. At least two cephalosporins (cephaloridine and cephaloglycin) and a carbapenem (imipenem) have proved to be highly nephrotoxic, while several other cephalosporins and carbapenems have mild to moderate nephrotoxicity.

The cephalosporins have two side-group substituents on the β-lactam structure, designated R_1 and R_2 (**Figure 10**). Those that are nephrotoxic do not share common or even similar R_1 or R_2 groups, although no cephalosporin with $R_2 = H$ is nephrotoxic. One common feature of the nephrotoxic cephalosporins is a comparatively unstable bond between the β-lactam ring and the R_2 substituent, which favours release of the R_2

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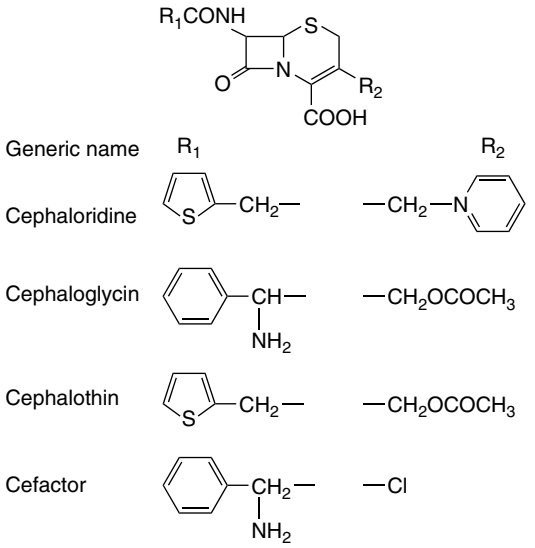

Figure 10 Structure of some cephalosporin antibiotics.

group. The acylating potential of the leaving group is an important factor in determining nephrotoxicity (Tune, 1986; 1997).

Cephaloridine causes renal tubular injury in both experimental animals and man which is characterized by a decreased glomerular filtration rate, glycosuria, enzymuria and proteinuria. These changes are frequently accompanied by histological evidence of necrosis to the proximal convoluted tubule (Silverblatt *et al*., 1970). Cephaloridine accumulates in the renal cortex to a much greater extent than in other organs (Wold and Turnipseed, 1980; Tune, 1986) and the extent of the accumulation is species dependent. Following administration to rabbits, guinea pigs and rats, the renal cortical cephaloridine concentration was highest in rabbit and lowest in rat. These findings parallel the susceptibility to nephrotoxicity.

Many cephalosporins are substrates for the organic anion transport system (OATs). A range of cephalosporins dose-dependently inhibit organic anion uptake into cells stably expressing human OATs and rat OAT2 (Takeda *et al*., 2002; Khamdang *et al*., 2003). Human OAT1, OAT2 and OAT3 are located on the basolateral membrane of the proximal tubule, while human OAT4 and rat OAT2 are located on the brush-border membrane. Cephaloridine decreases the viability of cells stably expressing human OAT1, OAT2, OAT3 and OAT4 and the cytotoxicity can be prevented by probenecid, except in the cells expressing OAT2 (Khamdang *et al*., 2003). Competitive inhibition of OATs by probenecid in the rabbit protects against the effects of cephalosporins (Tune and Hsu, 1990). Some cephalosporins, such as ceftizoxime and cefazolin, require ATP for transport and are substrates for the multidrug resistance-associated protein 4 (MRP4). Studies in MRP4 knockout mice have shown higher concentrations of the antibiotics in the kidney, indicating a role for this transporter, as well as OATs in the tubular secretion of some cephalosporins (Ci *et al*., 2007). Thus, not only transport into a renal cell, but also its rate of efflux into the tubular fluid is an important factor in determining the toxicity. With different β-lactams, the renal concentration can vary by several orders of magnitude and it is the area under the concentration–time curve (AUC) in the tubular cell that is important with regard to toxicity. For example, β-lactams that are rapidly and efficiently transported across the basolateral membrane and then rapidly transported from the cell into the tubular lumen have comparatively low AUCs—for example, cephalothin and cefaclor. Lower rates of secretion can be caused either by restricted movement from the cell into the tubular lumen giving very high AUCs (e.g. cephaloridine) or by little or no secretory transport into the cell, resulting in very low intracellular antibiotic concentrations (e.g. ceftazidime). As a consequence of these widely different intracellular AUCs, cephaloridine is very toxic and ceftazidime nontoxic to the kidney (Tune and Hsu, 1990).

The nephrotoxic β-lactams share two important properties, one or both of which are lacking in the nontoxic β-lactams (Tune, 1986; 1997). The first, as discussed above, is the ability to concentrate within cells in the proximal tubule and the second is their acylating potential. For example, the nephrotoxic β-lactam cephaloglycin covalently binds to proteins within renal tubular cells to a far greater extent than do the nontoxic β-lactams, such as cephalothin (Browning and Tune, 1983). Consistent with this is the finding that cephaloridine depletes glutathione levels in the renal cortex (Kuo *et al*., 1983). Prior treatment with agents that deplete glutathione will potentiate the nephrotoxicity of cephaloridine, which suggests that glutathione plays a protective role by scavenging the acylating moiety (Kuo and Hook, 1982). It has been postulated that cephaloridine can generate superoxide anion via a redox cycle, catalysed by NADPH–cytochrome P450 reductase (Kuo *et al*., 1983; Goldstein *et al*., 1986). The superoxide anion formed ultimately leads to lipid peroxidation (Cojocel *et al*., 1985), and the consequent oxidation of reduced glutathione in the renal cortex (Kuo *et al*., 1983). In support of the lipid peroxidation hypothesis are the findings that animals fed diets deficient in vitamin E or selenium are more susceptible to the nephrotoxicity of cephaloridine. The lipid peroxidative injury mechanism has been shown to be limited mainly to cephaloridine.

Several lines of study suggest that the toxic cephalsporins and imipenem produce their injury by an action on mitochondria: (i) respiration is reduced in mitochondria isolated from animals dosed with the nephrotoxic β-lactams; (ii) the mitochondrial respiratory toxicity is an early event occurring 0.5–1 hour after administration of a single dose; (iii) it is associated with a marked

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depletion of cortical ATP by 1.5 hours and (iv) it precedes the first indications of ultrastructural damage by five hours. The resulting lesion resembles that produced by ischaemic injury to the kidney (Tune, 1986). Subsequent studies have indicated that cephaloridine can selectively acylate and inactivate the transport systems that carry anionic substrates into the mitochondrial matrix (Tune *et al*., 1988; 1989; Tune and Hsu, 1990). Cephaloridine also inhibits mitochondrial accumulation of carnitine and its fatty acyl esters (Tune and Hsu, 1994), however cephalosporins which lack structural homology with cephaloridine, and hence carnitine, have no effect on mitochondria or the transport of carnitine or acylcarnitine (Tune, 1997). Cephaloridine also inhibits mitochondrial cytochrome C oxidase (Kiyomiya *et al*., 2002), and enzymes involved in glutamine metabolism (Guitton *et al*., 2005).

5.5 Industrial Chemicals

5.5.1 Chloroform

Chloroform is both hepatotoxic and nephrotoxic in most mammalian species, including man (Pohl, 1979; Davidson *et al*., 1982). The magnitude of chloroform-induced nephrotoxicity varies with species, and in mice there are unique sex and strain differences. Male ICR (imprinting control region) mice are susceptible to chloroform-induced renal injury, while female mice are resistant (Smith *et al*., 1983; 1984). The renal lesions induced in male mice by acute doses of chloroform include swelling of the tubular epithelium, increased renal weight, marked necrosis of the proximal tubular epithelium and the presence of tubular casts (Hewitt, 1956). Changes in renal function include glucosuria, proteinuria, an elevation of blood urea nitrogen and a decreased secretion of organic anions and cations (Plaa and Larson, 1965; Kluwe and Hook, 1978; Rush *et al*., 1984).

The mechanism of chloroform-induced nephrotoxicity has recently been shown to be similar to that established in the liver—namely generation of phosgene (see Smith, 1986, for a review). Studies with renal cortical slices from rats (Paul and Rubinstein, 1963) and rabbits (Bailie *et al*., 1984), and renal microsomes from male, but not female, mice (Smith and Hook, 1984) have demonstrated that [^{14}C]-chloroform is metabolized to [$^{14}CO_2$], a known degradation product of phosgene. Furthermore, studies in male mouse renal homogenates showed that phosgene can be trapped by two molecules of glutathione to form diglutathione-dithiocarbonate, which undergoes further metabolism to 2-oxothiazolidine-4-carboxylic acid (OTZ) (Branchflower *et al*., 1984; Pohl *et al*., 1984). Administration of chloroform to male mice causes renal glutathione depletion (Branchflower *et al*., 1984), which suggests that phosgene is also formed *in vivo*. The metabolism of chloroform requires NADPH and oxygen and can be inhibited by carbon monoxide and metyrapone (Smith and Hook, 1984). Thus, in the kidney, chloroform can undergo oxidative dechlorination catalysed by cytochrome P450 2E1 (Bailie *et al*., 1984) to give phosgene. This can covalently bind to nucleophiles (glutathione or cellular macromolecules) at or near the site of generation and cause cellular injury.

The marked sex and strain difference in chloroform-induced nephrotoxicity in the mouse appears to be related to the rate of metabolism via cytochrome P450 (Pohl *et al*., 1984). The concentration of cytochrome P450 is about fivefold greater in the kidneys of male as compared with female mice (Smith *et al*., 1984). Castration of male mice converted their pattern of cytochrome P450 expression to that seen in females (Henderson *et al*., 1990) and reduced their susceptibility to chloroform (Smith *et al*., 1984). Similarly, testosterone pretreatment of female mice resulted in a suppression of the female cytochrome P450 profile and induction of the male pattern (Henderson *et al*., 1990), thus rendering them susceptible to the nephrotoxic effects of chloroform (Smith *et al*., 1984). These findings, plus those of Clemens *et al*. (1979), suggest that the androgen-induced renal susceptibility to chloroform is mediated via the androgen receptor, which may control the expression of cytochrome P450 genes. Confirmation that metabolism of chloroform in the mouse kidney is responsible for the toxicity comes from elegant studies using mice, where cytochrome P450 reductase has been specifically deleted from the liver (Fang *et al*., 2008). Mice with deleted hepatic cytochrome P450 reductase were more sensitive to chloroform-induced nephrotoxicity compared to wild-type mice, indicating renal metabolism of chloroform is responsible for the nephrotoxicity *in vivo*.

In other species, the primary target organ for chloroform is the liver, with nephrotoxicity being either absent or only mild (Kluwe, 1981). This suggests that perhaps the cytochrome P450 which can metabolize chloroform to phosgene is either absent or present in low concentrations in the kidney of these species. Following chronic low-level exposure to chloroform, an increased incidence of renal tumours has been reported in male rats and, in one study, in male mice (see Davidson *et al*., 1982). The mechanism of renal carcinogenicity is thought to result from cytotoxicity and compensatory cell proliferation in the proximal tubules (Larson *et al*., 1994; 1995; Golden *et al*., 1997).

5.5.2 Haloalkenes

Hexachloro-1,3-butadiene (HCBD) is a byproduct formed during the manufacture of chlorinated solvents. The kidney appears to be the primary target for HCBD toxicity in rats, mice and other mammalian species (Lock, 1988). In the rat HCBD produces a well-defined

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lesion in the pars recta of the proximal tubule, the earliest morphological changes occurring in the mitochondria (Ishmael *et al*., 1982). The morphological changes are associated with renal functional impairment such as glucosuria, proteinuria and loss of concentrating ability (Lock, 1988).

Treatment of rats with inducers or inhibitors of hepatic and/or renal cytochrome P450 prior to HCBD administration had little or no effect on the nephrotoxicity, indicating that activation of HCBD by cytochrome P450 was not responsible. HCBD administration to male rats causes a depletion of hepatic, but not renal, glutathione, studies *in vitro* with rat liver microsomes and cytosol indicated that HCBD underwent direct conjugation with glutathione to form *S*-(1,2,3,4,4-pentachloro-1,3-butadienyl) glutathione (PCBD-GSH) (Wolf *et al*., 1984). Administration of PCBD-GSH, or its further metabolites, the cysteine conjugate (PCBD-CYS) or mercapturate (PCBD-NAC), or bile from a HCBD-treated rat, all produced necrosis to the pars recta of the proximal tubule, identical to that seen with HCBD (Nash *et al*., 1984; Ishmael *et al*., 1986). These data suggest that HCBD undergoes conjugation with glutathione in the liver, and is then eliminated in bile. In the bile and gastrointestinal tract it can undergo further metabolism to PCBD-CYS. Following enterohepatic circulation, it may be delivered unchanged to the kidney or may be *N*-acetylated in the liver prior to renal uptake (**Figure 11**). Metabolism of PCBD-GSH may also occur in the brush border of proximal tubular cells to afford PCBD-CYS, prior to renal uptake. For more detail on the intraorgan metabolism of glutathione and of HCBD-derived glutathione conjugates see Dekant *et al*. (1990), Commandeur *et al*. (1995) and Lash (1997).

The susceptibility of the proximal tubule to glutathione-derived conjugates of HCBD appears to be related to their ability to accumulate in that part of the nephron. PCBD-NAC appears to enter proximal tubular cells via the OAT system (Lock *et al*., 1986; Pombrio *et al*., 2001) and treatment of rats with probenecid completely prevents the accumulation and nephrotoxicity of HCBD or PCBD-NAC (Lock and Ishmael, 1985). Once accumulated within the cell, PCBD-NAC undergoes deacetylation to PCBD-CYS and then becomes covalently bound to renal macromolecules (**Figure 11**). The enzyme responsible for the activation of PCBD-CYS, and indeed other halolkene cysteine conjugates, is cysteine conjugate β-lyase. Metabolism of cysteine conjugates by this enzyme results in the formation of pyruvate, ammonia and an electrophilic mercaptan moiety, which will readily react with thiols (glutathione) and intracellular proteins (see reviews by Cooper *et al*., 2002; Anders, 2004). These latter events are believed to account for the cytotoxicity and carcinogenicity of HCBD. The critical proteins with which the reactive moiety interacts have not been identified, but PCBD-CYS impairs mitochondrial function *in vitro* (Jones *et al*., 1986; Schnellmann *et al*., 1989a), which leads to changes in mitochondrial membrane potential and intracellular calcium levels. Morphological evidence suggests that the mitochondria are early markers of renal damage (Ishmael *et al*., 1982). The localization of cysteine conjugate β-lyase in the mitochondria, as well as the cytosol (Stevens, 1985; Lash *et al*., 1986), plus the recent finding of a high-molecular-weight cysteine conjugate β-lyase with strong activity in the pars recta of the rat nephron, as well as in mitochondria (Abraham *et al*., 1996; Kim *et al*., 1997), may explain the selective localization. A sulfoxide metabolite of the mercapturate of HCBD has been identified in rat urine following administration of HCBD and studies with this metabolite showed it was cytotoxic to kidney cells *in vitro*, and, further, that inhibition of cysteine conjugate β-lyase did not afford any protection (Birner *et al*., 1998). Administration of the sulfoxide metabolite of the mercapturate of HCBD to rats also

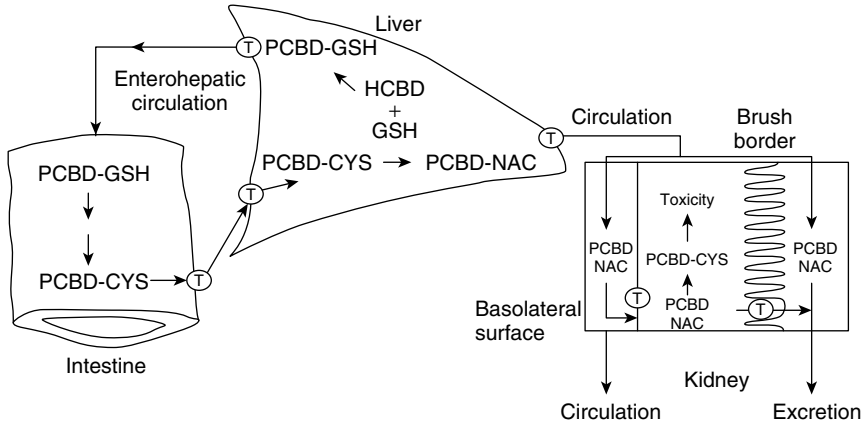

Figure 11 Interorgan co-operativity and metabolism of hexachloro-1,3-butadiene. For a more detailed scheme with supporting evidence see Commandeur *et al*. (1995). T represents a transport process.

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produced nephrotoxicity, indicating a possible role for a β-lyase-independent mechanism of renal toxicity (Birner *et al*., 1998).

Several halogenated chemicals undergo metabolism to form glutathione conjugates which can then be processed to cysteine conjugates: examples include trichloroethylene to *S*-1,2-(dichlorovinyl)-L-cysteine, tetrachloroethylene to *S*-1,1,2-(trichlorovinyl)-L-cysteine and tetrafluoroethylene to S-1,1′,2,2′-(tetrafluoroethyl)-L-cysteine. It has also been shown that certain anaesthetics undergo degradation with the bases in the carbon dioxide absorbents in the anaesthesia circuit; examples include trichloroethylene to dichloroacetylene, halothane to 2-bromo-2-chloro-1,1-difluoroethylene and sevoflurane to 2-(fluoromethoxy)-1,1,3,3,3-pentafluoro-1-propene (Compound A). These degradation products form glutathione conjugates, which are then processed to cysteine conjugates: dichloroacetylene to *S*-1,2-(dichlorovinyl)-L-cysteine and halothane to *S*-(2-bromo-2-chloro-1,1-difluoroethyl)-L-cysteine, while sevoflurane forms diastereoisomers, *S*-[2-(fluoromethoxy)-1,1, 3,3,3-pentafluoropropyl]-L-cysteine and *S*-[2-(fluoromethoxy) -1,3,3,3-tetrafluoro-1-propenyl]-L-cysteine. These halogenated cysteine conjugates are all substrates for renal cysteine conjugate β-lyase and result in renal injury (see Odum and Green, 1984; Dekant *et al*., 1986; Green *et al*., 1997; Anders, 2004; 2005; 2008).

5.5.3 Petroleum Hydrocarbons

Acute exposure to unleaded petrol and a variety of light hydrocarbons present in petrol produces a nephropathy in male rats characterized by: (i) an excessive accumulation of protein (hyaline droplets) in epithelial cells of the proximal tubule, (ii) accumulation of casts at the corticomedullary junction and (iii) evidence of mild tubular regeneration (Swenberg *et al*., 1989). This nephropathy only occurs in male rats; female rats and mice of either sex do not show any renal pathology. A number of chemicals present in unleaded petrol when tested alone have been shown to produce the nephropathy (Halder *et al*., 1985) and in particular 2,2,4-trimethylpentane and decalin have been used as model compounds. Certain other industrial chemicals (1,4-dichlorobenzene, isophorone), natural products (d-limonene) and pharmaceuticals (levamisole) also produce this male-rat-specific nephropathy. Chronic exposure of male rats to unleaded petrol, 1,4-dichlorobenzene, isophorone or d-limonene ultimately leads to the induction of a low incidence of renal adenomas and carcinomas (Swenberg *et al*., 1989; Hard and Whysner, 1994; Lehman-McKeeman, 1997; Lock and Hard, 2004).

Studies on the mechanism of pathogenesis have shown that the protein which accumulates in the proximal tubular cells is α_{2u}-globulin, a low-molecular-weight protein (18 700 daltons) that is synthesized in the liver of adult rats and is freely filtered at the glomerulus (see Swenberg *et al*., 1989; Lehman-McKeeman, 1997 for references). Female rats excrete less than 1% of the α_{2u}-globulin that male rats excrete (Vandoren *et al*., 1983). The chemical itself, or a metabolite, has been shown to bind reversibly to α_{2u}-globulin (Lock *et al*., 1987; Lehman-McKeeman *et al*., 1989; Borghoff *et al*., 1991; Lehman-McKeeman and Caudill, 1992) and this chemical–protein complex is then thought to be taken up by the proximal tubular cells (primarily in the S_2 segment) by endocytosis. These complexes appear to be quite resistant to, or impair, lysosomal degradation, which leads to accumulation as polyangular droplets. Lysosomal overload is thought to lead to individual cellular necrosis, which is followed by repair and regeneration (Short *et al*., 1989; Dietrich and Swenberg, 1991b). It has been suggested that a sustained increase in renal cell proliferation can promote initiated cells to form preneoplastic foci and lead to renal neoplasia (Swenberg *et al*., 1989). Strong support for this hypo thesis comes from studies in the National Cancer Institute (NCI) Black–Reiter strain of male rat which cannot synthesize α_{2u}-globulin and is refractory to the nephrotoxicity and carcinogenicity (Ridder *et al*., 1990; Dietrich and Swenberg, 1991a; 1991b). Recent studies with decalin, propylene glycol mono-*t*-butyl ether and Stoddard solvent IIC indicated that the relationship between the extent of α_{2u}-globulin nephropathy and cell proliferation following 90 days exposure does not correlate well with the extent of renal tumour incidence at two years, the latter being lower than expected. The tumour incidence does, however, show some correlation with the extent of α_{2u}-globulin nephropathy-associated linear mineralization of the papilla and with the severity of chronic nephropathy (Doi *et al*., 2007). These findings do not undermine the hypothesis of a male-rat-specific phenomenon, but do suggest that in addition to the renal injury caused by the accumulation of the male-rat-specific protein, other changes occurring in the kidney following chronic exposure may also contribute to renal tumour formation. Other species that do not synthesize the protein do not develop the toxicity. Man does not synthesize α_{2u}-globulin and therefore it is reasonable to conclude that α_{2u}-globulin nephropathy is unique to male rats. This is supported by detailed studies with other human proteins of the same superfamily as α_{2u}-globulin, which do not bind the xenobiotics or their metabolites (see Lehman-McKeeman, 1997). These findings provide compelling evidence that α_{2u}-globulin nephropathy is unique to male rats and unlikely to be relevant to humans (**Figure 12**), and that chemicals such as d-limonene are unlikely to be carcinogens in humans (Hard and Whysner, 1994).

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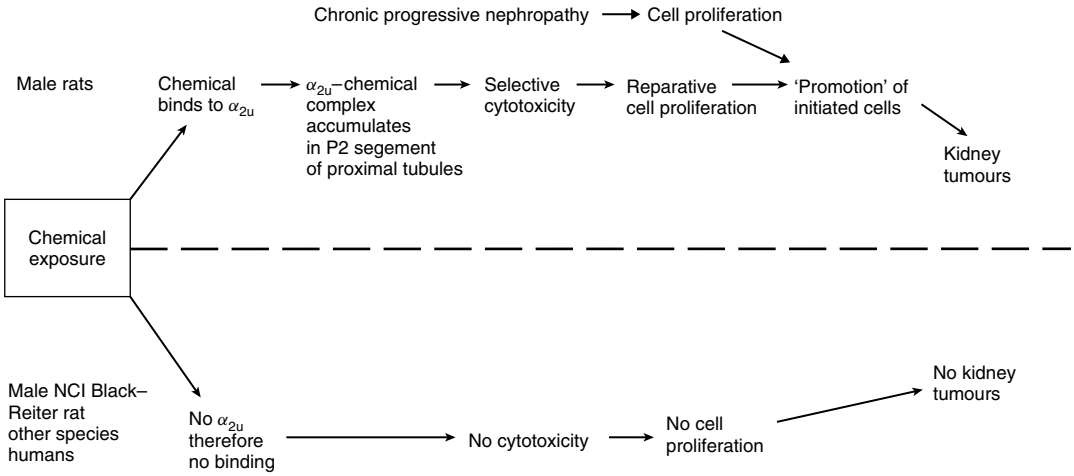

Figure 12 Proposed mechanisms for α_{2u}-globulin chemically induced renal tumours in male rats and its relevance to humans and other animals. The nephrotoxic chemical or metabolite reversibly binds to α_{2u}-globulin, altering the structure of the protein and decreasing its lysosomal catabolism in S_2 segment renal epithelial cells. This results in α_{2u}-globulin accumulation, lysosomal overload and cytotoxicity. Sustained increases in compensatory cell proliferation associated with chronic exposure result in greater 'fixation' of spontaneous DNA change and promotion of such initiated cells to renal tumours. Chronic progressive nephropathy may also contribute to the cell proliferation and promotion of initiated cells. Absence of the protein (NCI Black–Reiter rat) or absence of binding of the chemical or its metabolites to human proteins of this family means renal cell injury does not occur, along with all the downstream consequences.

5.6 Other Chemicals

It is not possible in this chapter to review all chemicals that cause renal injury; there are, however, some others worthy of note and the reader is are referred to recent reviews. Balkan endemic nephropathy is a chronic tubulointerstitial disease described only in some rural parts of southeastern Europe (Stefanovic and Radovanovic, 2008). The basis for this disease is not understood, but certain mycotoxins have been implicated, such as ochratoxin A, which is a potent renal carcinogen in the rat (Mally *et al*., 2007). The herbal drug aristolochic acid, derived from *Aristolochia* spp., has been associated with the development of a novel nephropathy and urothelial cancer in humans (Arlt *et al*., 2002) and there is increasing evidence to indicate that aristolochic acid is a significant risk factor in Balkan nephropathy (Arlt *et al*., 2007; Grollman *et al*., 2007; Shibutani *et al*., 2007).

Paracetamol (*N*-acetyl-4-aminophenol; acetaminophen) is a widely used analgesic and antipyretic drug. It is the major active metabolite of phenacetin, which has been used in analgesic mixtures and implicated in the aetiology of analgesic nephropathy (Bach and Bridges, 1985; Schnellmann, 1998; De Broe, 2004). Paracetamol at high doses also causes renal tubule necrosis in experimental animals and humans. The mechanism of toxicity is believed to be analogous to that in the liver, namely formation of *N*-acetyl-*p*-benzo-quinoneimine, which reacts with glutathione (**Figure 13**), once glutathione becomes depleted it will then acylate proteins, causing renal tubule injury (Josephy, 2005; Mazer and Perrone, 2008). Other mechanisms have also been proposed, such as the deacetylation of paracetamol to produce 4-aminophenol, which is about five times more nephrotoxic than paracetamol itself (see Fowler *et al*., 1991 and references therein). 4-Aminophenol can undergo one-electron reduction and hydrogen abstraction to form the 4-aminophenoxy radical, which in turn may undergo further oxidation to form 4-benzoquinoneimine, which will react with glutathione and acylate proteins (**Figure 13**). Paracetamol-glutathione-derived conjugates may also play a role in renal injury as the cysteine conjugate of paracetamol has been shown to deplete renal, but not hepatic glutathione and further that prior administration of cysteine conjugate potentiates the nephrotoxicity of a subsequent dose of paracetamol (Stern *et al*., 2005).

Bromobenezene has been shown to undergo metabolism to form glutathione conjugates, which are nephrotoxic see Monks and Lau (1997), Lau and Monks (1997), Van Vleet and Schnellmann (2003).

Ethylene glycol is known to cause renal injury in experimental animals and humans; the toxicity is ascribed to the terminal metabolite oxalic acid, which precipitates in the tubule lumen in the form of calcium oxalate crystals, causing physical injury to the renal tubule cells (Corley *et al*., 2005).

Radiological procedures requiring intravascular administration of iodinated contrast media is becoming a common source of nephropathy, and studies to try and ameloriate this condition and to identify risk factor is of increasing importance (Kaperonis *et al*., 2004; Pannu

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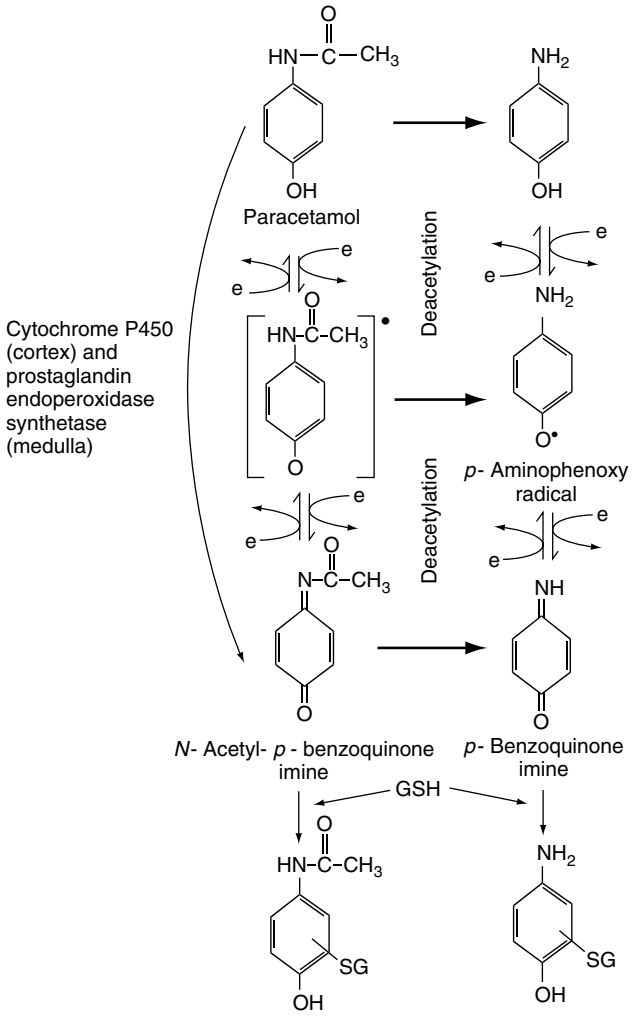

Figure 13 Proposed pathways of activation of paracetamol and 4-aminophenol.

et al., 2006; Toprak, 2007). The possible mechanism whereby injury occurs is unclear, but hyperosmolality and direct cytoxicity of some of these agents to renal cells in culture as been reported (Heinrich *et al*., 2005).

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Allergic Asthma and Rhinitis: Toxicological Considerations

Jean F. Regal

CONTENTS

1 INTRODUCTION

1.1 Definitions

Toxicity to the immune system can involve suppression of the normal immune response with a reduced ability to defend against pathogens and cancerous cells. Conversely, immunotoxicity can involve immunoenhancement, leading to reactions against self (autoimmunity) or reactions to normally innocuous substances (hypersensitivity). In the case of hypersensitivity, exposure to a xenobiotic results in the initiation of a specific immune response leading to sensitization with the potential for immune-mediated tissue damage (hypersensitivity) and the normal sequel of inflammation. For the purposes of this contribution, we will use the terms allergy and hypersensitivity interchangeably as requiring a specific immune response, whether humoral or cell-mediated immunity is involved. By strict definition, however, the term allergy is defined as a 'hypersensitivity reaction initiated by specific immunological mechanisms', whereas the term hypersensitivity does not require involvement of a specific immune response (Johansson *et al.*, 2004).

Allergic reactions in general are divided into two phases: (i) The induction or sensitization phase, characterized by immune system recognition of allergen as foreign, resulting in generation of allergen-specific antibody and/or allergen-specific T lymphocytes. (ii) The effector phase, characterized by allergic symptoms due to allergen exposure of a sensitized individual. The induction phase is responsible for the latent period between initial allergen exposure and the appearance of allergic symptoms in the effector phase. The presence of a latent period is evidence that a specific immune response is required.

1.2 Types I–IV Hypersensitivity

In the 1950s, Gell and Coombs divided allergic reactions into four basic mechanistic types; Types I–III were transferred by serum, that is, antibody mediated, and Type IV required transfer of lymphocytes to transfer the reactivity, that is, cell-mediated immunity. With the explosion of information in immunology, this classification has expanded to include three subdivisions under the Type IV hypersensitivity reaction: Th1 (T-helper Type 1), Th2

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(T-helper Type 2) and CD8 (CD—cluster of differentiation) (Janeway *et al*., 2008). Clinically, most allergic diseases involve a combination of these mechanisms to describe the pathophysiology of the disease.

Type I hypersensitivity (immediate hypersensitivity) is mediated by cytophilic antibody (IgE in human), bound to Fc (crystallizable fragment of immunoglobulin) receptors on mast cells and basophils. Atopic is a term referring to those individuals with a genetic predisposition to produce IgE (immunoglobulin E) antibody in response to ordinary exposure to allergens commonly encountered in the environment. Allergen crosslinking of IgE antibody bound to Fc receptors leads to an explosive release of mediators within minutes of exposure. This includes mediators preformed and secreted from the mast cell, such as histamine, as well as those immediately synthesized (arachidonate metabolites, platelet activating factor, etc.). These mediators are of primary importance in the immediate allergic reaction. In addition, the release of numerous cytokines, chemokines and adhesion factors from mast cell activation leads in part to the delayed reactions some 6–10 hours later. Type I hypersensitivity is a major mechanism leading to the acute symptoms of allergic asthma and allergic rhinitis.

Type II hypersensitivity is mediated by IgG (immunoglobulin G) antibody directed towards cell-surface antigens (e.g. drugs or haptens bound to a cell surface). The IgG antibody leads to clearance of the cell by Fc receptors on macrophages or to lysis of the cell in conjunction with complement. Haemolytic anaemia and thrombocytopaenia are examples of pathological reactions caused by Type II hypersensitivity.

In Type III hypersensitivity, specific antibody formed in response to soluble antigens leads to the formation of immune complexes. Small immune complexes formed in antigen excess can lead to deposition of the immune complexes in tissues, along with complement activation and neutrophil infiltration. Hypersensitivity pneumonitis or allergic alveolitis is an example of a Type III hypersensitivity reaction in the lung leading to compromised lung function (Ismail *et al*., 2006). Mechanistically, it is differentiated from Type I hypersensitivity in its more delayed time course in days and the lack of participation of IgE.

Type IV hypersensitivity is referred to as cell-mediated immunity because it requires the transfer of T lymphocytes to mimic the pathology. Currently, Type IV hypersensitivity is divided into pathology mediated by CD4+ Th1, CD4+ Th2 and CD8+ cells (Janeway *et al*., 2008). CD4+ cells recognize extracellular antigens presented in the context of major histocompatibility complex (MHC) II molecules, with subsequent activation of macrophages, chemokine and cytokine release and inflammation characterized by neutrophil influx. Mosmann *et al*. (1986) described differentiation of two primary types of CD4+ cells, Th1 and Th2. Exposure to allergen can lead to polarized immune responses of either the Th1 or Th2 type, depending on the extracellular milieu. IL-4 (IL—interleukin) and IL-13 favour the differentiation of Th2 lymphocytes, and IL-12 favours differentiation of Th1 lymphocytes. Th1 lymphocytes are associated with contact hypersensitivity reactions in the skin, and Th2 lymphocytes favour IgE production by the B cell, leading to Type I hypersensitivity reactions. CD8+ T cells will attack cells with modified intracellular proteins on their cell surface, presented in the context of MHC I antigens. Delayed type hypersensitivity refers to the Th1 and CD8 responses that can take 24–48 hours to develop after the individual is sensitized. As knowledge of immunology increases, the role of the inflammatory Th17 cell and Treg cells in immune-mediated disease will also be revealed.

This classification of allergic reactions is only intended to act as a guide or outline for the determination of mechanisms that are operating clinically. Allergic disease in response to either chemical or protein allergens can occur using any of these mechanisms, and most disease states are some combination of these hypersensitivity types. Thus, they should not be considered mutually exclusive, but should be used as a guide to focussing mechanistic studies and therapeutic interventions. The remainder of this section will focus on allergic asthma and allergic rhinitis as examples of allergic disease that can be caused by toxicants and that involve both Type I and Type IV allergic reactions in the acute and chronic stages, respectively (**Figure 1**).

2 ALLERGIC ASTHMA

2.1 Description and Clinical Features

Asthma accounts for approximately 5000 deaths per year in the United States, a figure that is well below the ~2 25 000 deaths caused by chronic obstructive pulmonary disease (National Heart Lung and Blood Institute, 2007). However, the true cost of asthma is in morbidity of the disease. In the USA, 4–8% of the population has asthma, and the toll on quality of life, missed work and missed school days is tremendous. The prevalence in children is greater than in adults and the cause of the increased prevalence over the last 20 years is unclear. Besides environmental causes, asthma can also be caused by workplace exposures (Bernstein *et al*., 2006). Estimates indicate that approximately 9–15% of adult asthma is due to occupational exposure (Mapp *et al*., 2005). Primary and secondary prevention measures to limit allergen exposure and prevent sensitization and symptoms, respectively, have met with variable success (Institute of Medicine, 2000). Asthma cannot be cured, but with insight into mechanisms of the disease, our attempts to control it therapeutically have improved.

Asthma is a lung disorder characterized by cough, wheezing, chest tightness and breathlessness. The effector

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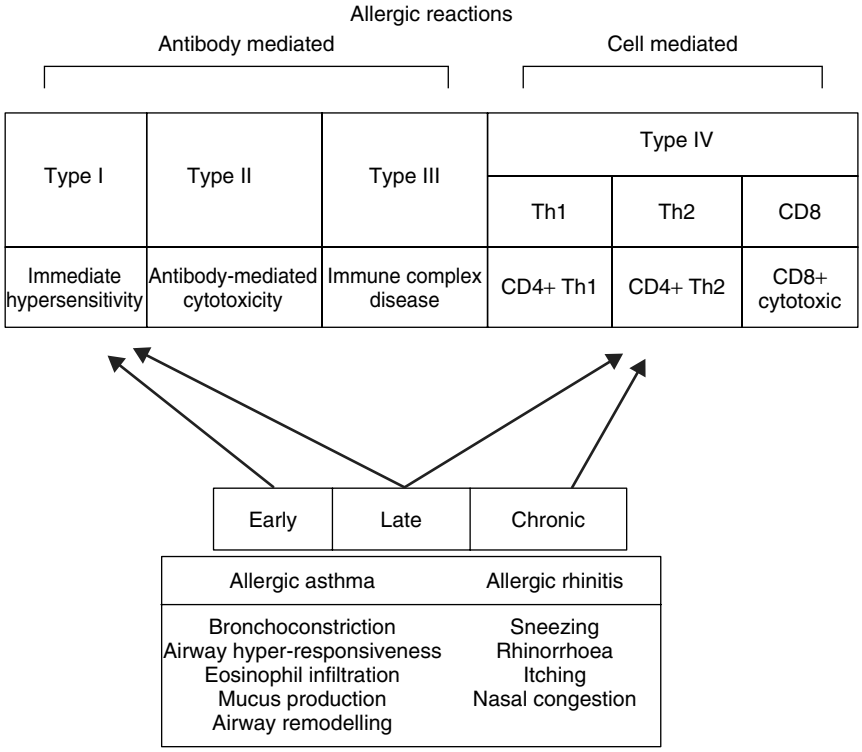

Figure 1 Allergic reactions are classified as Type I–IV, with most disease states involving a combination of mechanisms. Allergic asthma and rhinitis involve mechanisms described in Type I and Type IV hypersensitivity, depending on the time after allergen exposure and the allergen. Involvement of Th1 and CD8+ cells may also contribute to allergic asthma, depending on the allergen and severity of the disease.

phase of asthma is characterized by reversible airway obstruction, airway hyper-responsiveness, inflammation in the lung characterized by eosinophilia, mucus production and airway remodelling (National Asthma Education and Prevention Program, 2007). Numerous cell types are involved, including immune and inflammatory cells, as well as fibroblasts, airway epithelial cells and airway smooth muscle cells (Holgate, 2008). Asthma is a heterogeneous lung disorder with some patients presenting with all of the above-mentioned components, and others presenting with only a subset. Multiple asthma phenotypes have been described and Wenzel (2006) has categorized them in physiological terms (severe, treatment resistant), by inflammatory type (eosinophilic or neutrophilic) or by trigger (allergic, occupational, aspirin induced, exercise induced). Not all asthma is triggered by allergen exposure. Asthma is divided into allergic (extrinsic) and nonallergic (intrinsic) asthma, and estimates indicate that anywhere from 50 to 80% of asthma is triggered by allergen (Arbes *et al*., 2007). The focus of this chapter will be allergic asthma, but regardless of whether the asthma trigger is allergen or not, symptoms of asthma are the same and pharmacologic treatment is similar.

Development of asthma is not limited to inhalation exposure to allergen. The immune system is readily accessible, and sensitization by routes such as the gastrointestinal tract, bloodstream or skin can also result in allergic asthma. For example, animals sensitized by dermal exposure with trimellitic anhydride (Zhang *et al*., 2004) or toluene diisocyanate (Farraj *et al*., 2007) develop increased airway hyper-responsiveness, a symptom of asthma, when challenged with allergen by the respiratory route. Even dermal exposure with diisocyanates in the sensitization and challenge phase results in asthma symptoms, that is, airway hyper-responsiveness in the lung (Selgrade *et al*., 2006). Individuals with food allergy can respond with an asthmatic reaction to ingestion or even inhalation of the offending substance (James and Crespo, 2007). In addition, these individuals will have a positive skin-prick test response to the allergen. Thus, the immune system is sensitized and the location of the effector response is dictated by many factors, including the site of allergen challenge.

Allergens are often classified as respiratory allergens or contact allergens. This terminology does not dictate the route of exposure but is intended to reflect whether allergen exposure will result in respiratory pathology (respiratory allergen) or dermal pathology (contact allergen), regardless of route of exposure in the sensitization or challenge phase. A number of allergens can clearly cause both respiratory tract and dermal pathology. For example, latex exposure by skin and/or

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inhalation can result in skin or respiratory tract pathology in different individuals (Hamann *et al*., 2004; Nettis *et al*., 2004; Vandenplas, 1995). Dental workers exposed to methacrylates by inhalation or by skin contact can develop asthma and skin hypersensitivity (Dearman *et al*., 2007; Jaakkola *et al*., 2007). Human exposure to acid anhydrides and diisocyanates primarily results in asthma (Bardana, 2008; Zeiss *et al*., 1977; 1990; 1992), but in animal models, these molecules can cause both skin and respiratory tract symptoms (Herrick *et al*., 2003; Lauerma *et al*., 1997). Reports of allergic contact dermatitis to diisocyanates have increased as uses for the compounds have expanded and occupational dermal exposure has increased (Bello *et al*., 2008; Goossens *et al*., 2002; Stingeni *et al*., 2008). Multiple factors dictate the allergenicity of xenobiotics, and predicting substances that result in allergic reactions in the lung and elsewhere is an area of active investigation.

Our focus is allergic asthma. However, there is evidence that many allergens may cause symptoms of asthma by a combination of allergic and nonallergic mechanisms. For example, plicatic acid is a component of Western red cedar (*Thuja plicata*) that activates the complement system resulting in mediators that can cause asthma symptoms (Chan-Yeung *et al*., 1973). However, it is also clear that plicatic acid exposure initiates a specific immune response that is responsible for allergic asthma due to Western red cedar (Chan-Yeung *et al*., 1980). Toluene diisocyanate in general requires a latent period to induce asthma, indicating a specific immune response is required. However, toluene diisocyanate may also cause asthma symptoms in the absence of sensitization by stimulating the release of Substance P and other neuropeptides, as well as inhibiting the enzyme neutral endopeptidase to prolong their actions (Mapp *et al*., 1991; Nadel, 1991; Sheppard *et al*., 1988). Hexamethylene diisocyanate activates peripheral blood monocytes in humans in the absence of a specific immune response, altering gene/protein expression and potentially contributing to a nonallergic component of the diisocyanate respiratory pathology (Wisnewski *et al*., 2008). Trimellitic anhydride exposure initiates a specific immune response in animal models, but trimellitic anhydride–protein conjugates can also activate the complement system in the absence of specific antibody and contribute to asthma-like symptoms (Fraser *et al*., 1995; Larsen *et al*., 2001).

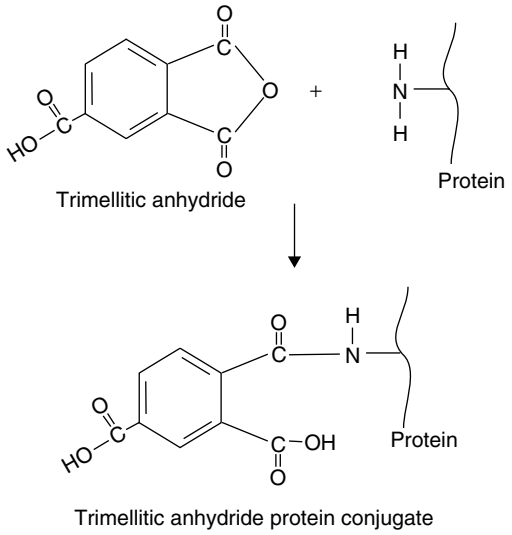

Figure 2 The low-molecular-weight allergen trimellitic anhydride acts as a hapten and reacts with side chains of amino acids, including amino groups, to form a hapten protein conjugate that is recognized by the immune system.

2.2 Sensitization Phase

Both genetics of the individual as well as characteristics of the chemical or protein are important in determining if sensitization occurs and allergic disease develops. For example, not all individuals exposed to ragweed pollen will become sensitized and/or develop allergy symptoms. The properties of chemicals and proteins that determine whether or not they will result in an allergic response are a subject of intense investigation (see **Laboratory Recognition of Potential Xenobiotic Respiratory Sensitizers**). An allergen is defined as an antigen that causes allergic disease (Johansson *et al*., 2004). Development of an allergic response initially requires sensitization or engagement of a specific immune response, and this process differs for low- and high-molecular-weight allergens. In the case of low-molecular-weight allergens, the substance is too small to be recognized by the immune system itself so it must react with a larger molecule, most often a protein, to form a hapten protein complex. As illustrated in **Figure 2**, trimellitic anhydride, a known cause of occupational asthma, reacts with side groups on proteins, including amino groups, to form a hapten protein conjugate. The immune system then responds to the hapten, or to new antigenic determinants formed when the hapten reacts with the protein (Zeiss *et al*., 1980), to initiate a specific immune response and sensitization. A limited study of five chemicals by Hopkins *et al*. (2005) suggests that allergens that cause contact dermatitis via a cell-mediated immune response preferentially react with cellular proteins, whereas substances such as trimellitic anhydride that cause primarily respiratory allergy have a greater tendency to react with extracellular proteins. Thus, the preferred reactivity of low-molecular-weight chemicals with particular proteins is an area of future investigation to predict chemicals that may result in skin vs. respiratory allergy. For protein allergens, recent studies have demonstrated that they are distributed to relatively few protein families and have a limited number of biochemical functions (Radauer *et al*.,

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2008). A large proportion of the protein allergens have enzymatic or metal ion, lipid or actin binding activity, and these activities may contribute to their allergenicity (Hammad and Lambrecht, 2008; Radauer *et al*., 2008; Vermaelen and Pauwels, 2005).

The lung is exposed to numerous substances with each breath, and inhalation of most foreign substances does not lead to an immune response, that is, a state of tolerance ensues. However, inhalation of some substances results in the bypassing of the normal tolerogenic mechanisms in the lung and establishment of allergic disease. Secondary properties of molecules such as irritancy or enzyme activity have been hypothesized as being important in leading to sensitization by inhalation exposure and ultimately allergic disease (Kheradmand *et al*., 2002; Mapp *et al*., 2005; Radauer *et al*., 2008). Enzymes used in the detergent and food industry are known to cause asthma with inhalation exposure (Kirchner, 2002; Schweigert *et al*., 2000). A major allergenic component of the common environmental allergen house dust mite (Der p 1) has cysteine protease activity and can cleave the tight junction protein occludin, leading to increased permeability of the epithelial barrier and access of the allergen to the dendritic cells for antigen processing and presentation (Janeway *et al*., 2008). Because of secondary signals associated with such molecules, the dendritic cells are activated to migrate to the local lymph node where they present the antigen in conjunction with MHC molecules to a CD4+ T cell (Hammad and Lambrecht, 2008; Vermaelen and Pauwels, 2005), which differentiates into a Th2 type cell and produces Th2 cytokines, IL-4, IL-5 and IL-13. These cytokines influence T cell differentiation and B cell function with the end result that the B cell becomes an IgE antibody-producing cell. In contrast, other allergens such as the protein ovalbumin are taken up by dendritic cells, but the cells are not stimulated to migrate to the local lymph node for presentation to the T cell—a state of tolerance to the inhaled allergen ensues. If exposure to allergens such as ovalbumin occurs in conjunction with a 'danger' signal such as damage to the epithelium, addition of a protease (Kheradmand *et al*., 2002) or stimulation of toll receptors on the epithelium by microbial factors (Eisenbarth *et al*., 2002), then the dendritic cell is stimulated to migrate to the local lymph node to present the antigen and initiate the specific immune response. Low-molecular-weight allergens such as acid anhydrides and isocyanates conjugated to protein are readily recognized as foreign by the dendritic cell. The ability of these molecules to bypass the normal tolerogenic mechanisms may relate to their irritative properties or their ability to cause epithelial disruption and access to the subepithelium.

Sensitization itself is not a guarantee that respiratory symptoms will occur with subsequent allergen exposure. In the detergent industry, a positive skin-prick test indicating sensitization does not require removal of the worker from exposure, or predict that the worker will develop respiratory symptoms (Schweigert *et al*., 2000). In the general population, it is estimated that 30% of the individuals are atopic and produce high concentrations of IgE. However, asthma prevalence in the general population is 10–12%. Thus, atopy is considered a risk factor for the development of asthma (Hammad and Lambrecht, 2008).

2.3 Effector Phase

2.3.1 Clinical Characteristics

In the effector phase of asthma, symptoms are divided into acute and chronic phases. The acute reaction to allergen (**Figure 3**) involves both an early phase (within minutes of allergen challenge) and a late phase (maximizing at 6–12 hours). Chronic asthma can develop over days and is likely the result of persistent and repetitive allergen exposure, with chronic inflammation and long-term changes in the lung, that is, airway remodelling. The late phase may be indistinguishable

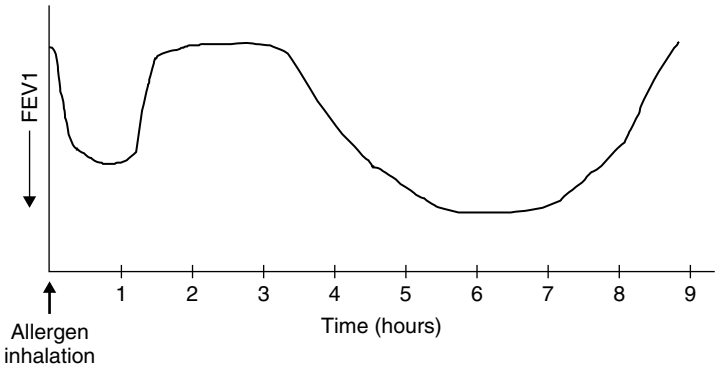

Figure 3 Airway obstruction in allergic asthma—immediate and late-phase reaction. After allergen exposure, airway obstruction occurs in phases in allergic asthma with an immediate phase occurring within minutes and a late phase hours later. Clinically, airflow limitation is measured as the forced expiratory volume in 1 second (FEV1).

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from the chronic phase, and individuals may present with any combination of phases. The mechanism and mediators for each of these phases have commonalities and differences. The early-phase response is characterized by wheezing and airflow limitation due to allergen challenge, and is generally responsive to therapy with bronchodilating β_2 agonists. The initial bronchoconstriction can resolve and is then followed 6–12 hours later with a late-phase reaction characterized by continued airflow limitation. Airflow limitation in the early phase is primarily due to airway smooth muscle contraction or bronchoconstriction. In the late phase, besides airway smooth muscle contraction, increased mucus production, increased microvascular permeability and swelling of the airway wall secondary to eosinophilic inflammation can also contribute to airflow limitation. In some asthmatics, neutrophilic inflammation predominates. Airway hyper-responsiveness is also a factor in airflow limitation in the late and chronic phases of asthma. Airway hyper-responsiveness refers to an exaggerated airway constriction to known smooth muscle constrictors such as cholinergic agonists and histamine and is expressed as a PC20 (the provocative concentration of agonist that causes a 20% decrease in the forced expiratory volume at 1 second (FEV1)). Asthmatic airways have a lower PC20 for methacholine and respond with significant constriction to concentrations of agonist that have minimal effects on airways from control subjects (National Asthma Education and Prevention Program, 2007). In the chronic allergic inflammation stage of asthma, structural changes can occur, that is, airway remodelling. These changes may make the lung less responsive to the bronchodilating effects of β_2 agonists. Airway remodelling includes thickening of the basement membrane, mucosal oedema, hypertrophied and hyperplastic smooth muscle and subepithelial fibrosis.

Historically, asthma is considered an airway smooth muscle disease, but the current asthma paradigm centres on inflammation with airway obstruction and airway hyper-responsiveness resulting (National Asthma Education and Prevention Program, 2007). The mainstay of therapy is bronchodilating β_2 agonists and inhaled anti-inflammatory steroids. A single agent to target a single mediator (antihistamine, antileukotriene, etc.) has generally been ineffective in treatment of asthma, whether due to low- or high-molecular-weight allergens.

2.3.2 Animal Models

Numerous animal models have been used to study mechanisms of the effector phase of asthma. No animal spontaneously develops asthma, and each species has its advantages and disadvantages (Johnson and Luster, 2006; Zosky and Sly, 2007). In modelling asthma in rodents, we are looking for similarities to the human in both the immune (Mestas and Hughes, 2004) and respiratory system (Finkelman and Wills-Karp, 2008). This is especially difficult in developmental studies of asthma because the immune and respiratory system mature at different times comparing rodents and humans. Optimally an animal model involves cytophilic antibody and mast cells (IgE and IgG1 in mice and guinea pigs), eosinophil infiltration into the lung, increased airway resistance in response to allergen (early and late phase) and increased airway hyper-responsiveness in response to a bronchoconstrictor such as methacholine. Realistically, most animal models assess the eosinophil infiltration into the lung and airway hyper-responsiveness to methacholine days after multiple allergen challenges. In addition, the immediate bronchoconstrictor response may be assessed within minutes of allergen exposure. Monitoring late-phase bronchoconstriction 6–12 hours after allergen challenge has been technically difficult. In addition, attempts to mimic chronic asthma and airway remodelling have met with limited success due to the long-term nature of the experiments. Clearly animal models are meant to predict the pathophysiology in the human, and the usefulness of the animal model is only established with direct testing of the predictions in the human.

Airway hyper-responsiveness is measured clinically by determining the concentration of methacholine that results in a 20% reduction in FEV1. Events in the lung that can result in a reduction in FEV1 include, but are not limited to, narrowing of small and/or central airways or plugging or closure of small airways and alveoli. Thus, airway hyper-responsiveness involves a number of changes in the lung, and has in general been attributed to alterations in airway smooth muscle function. In animal models, different physiological mechanisms, beyond simple airway narrowing, are operative in causing airway hyper-responsiveness (Wagers *et al*., 2007). However, in general, measurement of airway hyper-responsiveness in animal models has concentrated on measuring increased airway resistance in response to methacholine administration (Finkelman and Wills-Karp, 2008). Since airways are the primary site of airflow limitation, a single compartment model to measure increased total lung resistance in a tracheotomized animal is used to reflect increased airways resistance. Other more complex techniques and models have been developed measuring lung impedance to estimate the airway resistance and the tissue resistance contribution to total lung resistance (Wagers *et al*., 2002). Direct measures of airway resistance require control of respiratory frequency and the use of an anaesthetized animal. Development of unrestrained barometric plethysmography allowed use of an unanaesthetized animal to measure the pressure change inside a box as an animal breathes. Analysis of the breathing pattern then generates a dimensionless parameter called enhanced pause (Penh) that reflects changes in inspiratory and expiratory timing. Hamelmann *et al*. (1997) demonstrated that the change in Penh paralleled the increase in airway resistance in adult Balb/c mice, and thus Penh has

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been used in numerous studies as an indicator of airway hyper-responsiveness in animal models. However, in a number of instances, Penh changes do not reflect more direct measures of airway resistance and caution needs to be used in over-interpreting the information provided (Bates *et al*., 2004; Finkelman, 2008; Finkelman and Wills-Karp, 2008; Mitzner *et al*., 2007). Penh measures changes in breathing patterns that suggest changes in airway calibre, but it is not a measure of pulmonary mechanics.

2.3.3 IgE and Mast Cell Involvement

As knowledge of immunology has grown, the view of asthma as simply Type I hypersensitivity and mast cells has been replaced by a view of asthma as a combination of Type I hypersensitivity and Th2 Type IV hypersensitivity (**Figure 1**). In general, asthma in humans is associated with increased allergen-specific IgE. The work of Ishizaka *et al*. (1966) was instrumental in establishing the importance of IgE and the mast cell in immediate hypersensitivity reactions. IgE was identified as the important reaginic antibody responsible for transfer of allergic reactions in human skin, in isolated cells and in animal models. IgE binds to Fcε receptors (Fcε–Fc receptor for IgE) on mast cells and basophils. Subsequent crosslinking of IgE molecules by allergen results in a rapid secretion of preformed mediators from granules, including histamine. In addition, arachidonate metabolites are immediately synthesized by the mast cell and released (Liu *et al*., 1990). IgE has a very high affinity for FcεRI on mast cells, basophils and dendritic cells. A lower affinity IgE FcεRII is also present on numerous cell types such as platelets and macrophages and is thought to play an important role in mobilizing a variety of cells and mediators in allergic reactions (Kraft and Novak, 2006). IgE binding to FcεRI and FcεRII is not limited to immune cells. Stimulation of FcεRI and FcεRII on airway smooth muscle cells leads to release of substances that are chemotactic for eosinophils (eotaxin and IL-5) as well as increases in intracellular calcium, suggesting activation of contractile elements (Gounni, 2006). Clear evidence in humans regarding the importance of IgE in asthma comes from the effectiveness of therapy with anti-IgE (omalizumab) in inhibiting both the early and late-phase asthmatic reactions to inhaled allergen as well as benefitting chronic persistent asthma (**Figure 1**; Holgate *et al*., 2005; Strunk and Bloomberg, 2006). However, increases in IgE are not readily apparent in all allergic asthmatics, including asthma initiated by low-molecular-weight allergens such as toluene diisocyanate. Using animal models, the importance of IgE in the asthmatic reaction is also unclear. Systemic anaphylaxis can occur in mice deficient in IgE (Oettgen *et al*., 1994), and passive sensitization experiments indicate that the immediate bronchoconstrictor response to allergen is dependent on allergen-specific IgG1, not IgE (Crosby *et al*., 2002). Numerous studies in mouse models of asthma using transgenic animals and antibody depletion techniques have clarified that different mechanisms are operative depending on the protocol for sensitization and challenge. If mice are sensitized repeatedly by inhalation with ovalbumin, the airway hyper-responsiveness that develops is dependent on IgE and mast cells with minimal IL-13 involvement. However, mice sensitized with ovalbumin with alum adjuvant develop airway hyper-responsiveness that is independent of IgE and largely dependent on the Th2 cytokine IL-13 (Finkelman and Wills-Karp, 2008). As might be predicted by the heterogeneous nature of asthma, multiple mechanisms are likely operative, and differ amongst individuals.

2.3.4 T Cell Involvement

Not all aspects of allergic asthma are explained by IgE and mast cells, and allergic eosinophilia can develop in the lungs of B cell-deficient mice (Korsgren *et al*., 1997). Increasing evidence indicates that T cells, particularly Th2 cells, are also very important for the development of the late and chronic phases of the asthmatic reaction. Th2 lymphocytes are operative both in the sensitization and effector phases of the asthmatic response. In the sensitization phase, allergen exposure can result in a polarized immune response in some individuals, leading to differentiation of CD4+ T cells to the Th2 type. IL-4 and IL-13 favour differentiation to the Th2 phenotype and IL-12 favours differentiation to the Th1 phenotype. Th2 cells produce cytokines such as IL-4, IL-5, and IL-13 that facilitate the production of IgE antibody by the B cell. The factors that dictate whether an immune response develops a polarized Th2 component leading to IgE production and lung pathology is unclear but has revolved around the 'hygiene hypothesis', which suggests that environmental factors limiting exposure to microbial challenges may result in the skewing of the immune response to a Th2 response (National Asthma Education and Prevention Program, 2007). In the effector phase, activation of the mast cell by allergen crosslinking of IgE molecules leads to production of Th2 cytokines by the mast cell, in addition to histamine, platelet activating factor and leukotrienes. All of these mediators play an important role in the ensuing late phase and chronic response (Brusselle *et al*., 1995; Foster and Chan, 1991; Gulbenkian *et al*., 1992; Watson *et al*., 1993). Besides activating mast cells in the effector phase, allergen activates antigen-specific Th2 cells and the elaboration of IL-4, IL-5 and IL-13 (Cousins *et al*., 2008; Larche *et al*., 2003). These CD4 T cell mediators are important in the late phase and chronic response as demonstrated in mouse models using anti-CD4 antibody depletion of T cells (Hogan *et al*., 1998; Larche *et al*., 2003). Evidence also indicates that the number and type of mast cells is

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under T cell control, with a Th2 phenotype stimulating proliferation of mast cells and promoting their survival (Boyce, 2003).

The involvement of other T cell lineages besides Th2 cells has been considered in the pathophysiology of asthma. For example, the production of Th2 cytokines does not necessarily correlate with the ability of a particular diisocyanate to increase airway hyper-responsiveness (Selgrade *et al*., 2006). Evidence from animal models is conflicting regarding the role of Th1 cells and/or cytokines in allergic asthma, with some studies indicating that Th1 cells increase the severity of the allergic response in the lung (Hansen *et al*., 1999; Randolph *et al*., 1999), and other studies suggesting that Th1 cells decrease the lung pathology (Huang *et al*., 2001). Certainly increased numbers of Th1 cells are observed in the allergic lung, and the involvement of Th1 cells may increase with the severity of the asthma (Holgate, 2008). In animal models of isocyanate-induced occupational asthma, a mixed Th1/Th2 phenotype has been observed (Matheson *et al*., 2005a; 2005b). In addition, studies have provided evidence that CD8+ T cells also influence allergic inflammation, both in the toluene diisocyanate model (Matheson *et al*., 2005a; 2005b) as well as in models of protein allergy to ovalbumin (Hamelmann *et al*., 1996). The Th lineages are increasingly complex (Chatila *et al*., 2008), and a role for pro-inflammatory Th17 cells and T regulatory cells cannot be discounted.

The recognition from animal studies that Th2 cytokines were critical mediators of asthmatic events led to the introduction of therapies targeting Th2 cytokines in humans, particularly IL-4 and IL-5. However, the results in humans of such monotherapies were particularly disappointing (Finkelman and Wills-Karp, 2008). A broader approach targeting multiple cytokines may be more effective. Numerous cell types are activated in the lung, including epithelial cells, mast cells, macrophages, T cells and eosinophils (Larche *et al*., 2003). IL-4 and IL-13 have been implicated in multiple pathologies of asthma and interact with multiple cell types including Th2 cells, mast cells and eosinophils, resulting in production of chemokines such as eotaxin and MCP (monocyte chemoattractant protein) that attract inflammatory cells to the lung (Zimmermann *et al*., 2003). IL-4 Rα is a common signalling chain for both the IL-4 and IL-13 receptors, and recent studies targeting this receptor show promise in alleviating asthma symptoms in both mouse models and clinical trials (Gavett *et al*., 1997; Karras *et al*., 2007; Wenzel *et al*., 2007).

The importance of eosinophil infiltration in the development of airway hyper-responsiveness is debated. Some studies dissociate the two events (Hayes *et al*., 1993; Milne and Piper, 1994; Pretolani *et al*., 1994; Regal *et al*., 2006), and other studies show a clear association (Djukanovic *et al*., 1990; Ishida *et al*., 1989). Changes in neuroregulation and alterations in airway structure due to inflammation and airway remodelling have been suggested as causes of airway hyper-responsiveness, but no general consensus regarding the mechanism is evident (Leguillette and Lauzon, 2008; National Asthma Education and Prevention Program, 2007).

2.4 Asthma in Response to High- and Low-Molecular-Weight Allergens

Whether the allergen is low or high molecular weight, asthma symptoms are similar and therapeutic strategies the same. Comparing gene expression profiles in mice sensitized and challenged with different allergens including ovalbumin, *Aspergillus* antigen, or the low-molecular-weight chemical allergen trimellitic anhydride revealed substantial differences in expression profiles (Greene *et al*., 2005; Regal *et al*., 2007), suggesting that allergens cause asthma symptoms via different mechanisms. Differences in allergens may also account for a portion of the heterogeneity seen in asthma. Mechanistic differences in low-molecular-weight allergens are also exemplified by trimellitic anhydride and toluene diisocyanate, two known causes of occupational asthma. They are both reactive molecules and act as haptens to induce an immune response. Studies by Zeiss (Zeiss *et al*., 1977; 1990; 1992; Zeiss, 2002) indicated that trimellitic anhydride can cause a variety of immunologically mediated lung diseases, including allergic asthma associated with IgE. Trimellitic anhydride-induced asthma in the mouse is also associated with elevated IgE (Regal *et al*., 2001). In contrast, allergen-specific IgE is not consistently detected in toluene diisocyanate-induced asthma (Mapp *et al*., 2005; Pronk *et al*., 2007). This lack of detectable IgE has been noted with toluene diisocyanate as well as other low-molecular-weight allergens, and plausible explanations that have been investigated include: (i) Non-IgE antibody is critical for toluene diisocyanate-induced asthma. (ii) Local production of IgE antibody is critical and not detected by measurement of serum antibody. (iii) Cell-mediated immunity is a greater component of the response than humoral immunity. (iv) Assays evaluating allergen specific antibody do not measure the important antigenic determinants responsible for IgE production. Certainly evidence in animal models suggests that antibody other than IgE mediates some components of the allergic asthmatic response. Also, evidence in humans suggests that toluene diisocyanate-specific IgG is associated with the asthmatic response (Park *et al*., 1999), not allergen-specific IgE. Other studies have suggested that allergen-specific IgG is a measure of exposure rather

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than a critical determinant of the allergic response (Mapp *et al*., 2005). The state of sensitization to a given allergen is often assessed by allergen-specific serum antibody or skin-prick test. However, an immune response and class switching to IgE production can occur locally in the nasal or bronchial mucosa (Chvatchko *et al*., 1996; Takhar *et al*., 2005; 2007), so that systemic indicators such as circulating antibody may not always be evident. It is also possible that T cell activation plays a more prominent role than antibody, and some studies have shown increases in both Th1 and Th2 cells in the blood after inhalation challenge in occupational asthma with low-molecular-weight allergens (Mamessier *et al*., 2007). Development of assays that can reliably measure allergen-specific antibody has been difficult for low-molecular-weight chemicals in particular, because with most reactive chemicals, a wide variety of hapten protein conjugates are formed, and the critical antigens formed *in vivo* may not be formed under *in vitro* conditions used to develop the assays (Ott *et al*., 2007). The methodology for preparation of conjugates has not been standardized and can clearly influence the detection of antibodies in patients (Campo *et al*., 2007). Thus, much work is needed to establish allergen-specific antibody assays for different occupationally relevant allergens, and to determine if the antibody being assayed is critical to the development of allergic asthma.

Individuals with occupational asthma do not always recover after removal from exposure to the offending agent (Piirila *et al*., 2008), and this has led to the idea that low-molecular-weight chemical exposure results in asthma that is irreversible. A recent review of some 40 studies revealed variable outcomes after removal of asthmatics from exposure (Rachiotis *et al*., 2007). Surprisingly, the prognosis for recovery after removal from exposure to high-molecular-weight allergens such as snow crab was considerably poorer when compared to the low-molecular-weight allergens studied. Thus, strategies for allergen avoidance need to be carefully considered, depending on the allergen and the available evidence.

2.5 Causes of Allergic Asthma

Numerous substances can act as allergens and cause allergic asthma. Selected high- and low-molecular-weight substances are listed in **Table 1**. A more comprehensive listing of substances with references and likely occupational exposures can be found in recent reviews (Health and Safety Executive, 2008; Malo and Chan-Yeung, 2006; Mapp *et al*., 2005). Many of these substances may also cause Type I or Type IV hypersensitivity reactions in the skin, as well as Type II or Type III hypersensitivity reactions.

Table 1 Examples of low- and high-molecular-weight allergens

Low-molecular-weight allergens	High-molecular-weight allergens
Anhydrides	Laboratory animals
Diisocyanates	Mites
Plicatic acid	Natural rubber latex
Methacrylates	Wheat proteins
Platinum salts	Enzymes
Penicillins	Snow crab
Reactive dyes	Egg proteins
Formaldehyde	Moulds

2.6 Secondary Effects of Toxicants

Besides acting as allergens and triggering allergic asthma, toxicants can contribute indirectly to sensitization as well as to exacerbation of asthma symptoms. Because of airway hyper-responsiveness in asthmatics, toxicants that are not allergens can cause bronchoconstriction in asthmatic airways though they would not cause bronchoconstriction in a normal airway. The link between air pollution and increased numbers of emergency-room visits for asthmatics is well established (National Asthma Education and Prevention Program, 2007). In addition, toxicants can indirectly contribute to asthma by enhancing sensitization. For example, IgE production in response to allergens can be increased by coexposure to substances such as lipopolysaccharide (Eisenbarth *et al*., 2002), particulate matter or environmental tobacco smoke (Gilmour *et al*., 2006). Particles from wood smoke and road traffic can also act as adjuvants and increase the IgE response to ovalbumin administration (Samuelsen *et al*., 2008). Some of these effects may reflect epigenetic changes as evidenced by a study of Liu *et al*. (2008). Diesel exhaust enhanced IgE production in response to the allergen *Aspergillus,* and also resulted in hypermethylation of the IFN-γ promoter (IFN—interferon), and hypomethylation of the IL-4 promoter in CD4+ T cells, suggesting a new paradigm to explain environmental influence on the polarization of Th1/Th2 cytokine profiles and IgE production.

3 ALLERGIC RHINITIS

3.1 Description and Clinical Features

Allergic rhinitis is inflammation of the nasal mucosa due to an adaptive immune response. Allergic rhinitis is not associated with mortality, but clearly has a very negative effect on quality of life. In the United States, estimates indicate that 17% of the population is affected by allergic rhinitis (Greiner and Meltzer, 2006), and economic losses

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due to healthcare costs and lost work time for allergic rhinitis are thought to surpass the costs of allergic asthma (Bousquet *et al*., 2003; Lamb *et al*., 2006). Symptoms of allergic rhinitis often precede those of allergic asthma, but not all who have allergic rhinitis go on to develop asthma. Amongst patients who have allergic rhinitis, 20–50% of them have allergic asthma. Amongst patients with asthma, estimates indicate that anywhere from 50 to 100% also have rhinitis (Gaugris *et al*., 2006; Malo *et al*., 1997; Scadding *et al*., 2008). In a Finnish study, the risk of asthma was found to increase three-fold in individuals who had developed allergic rhinitis in the workplace (Karjalainen *et al*., 2003). Knowledge of the mechanisms and markers of allergic rhinitis may be useful in predicting exposures that may lead to asthma, with the end result of reducing morbidity and mortality from asthma as well.

Inhalation exposure of xenobiotics generally involves exposure to the nasal mucosa and the lung. The upper respiratory tract includes the nasal passages, nasopharynx, sinus and larynx. The function of the nasal passages is to filter out large particles and allergens and warm and humidify the air. If nasal passages are obstructed, then mouth breathing ensues with increased exposure of the lungs to substances that are normally eliminated in the upper respiratory tract. The airway mucosa is continuous from the nasal passages to the lower airways and the term 'united airway concept' is used to reflect the fact that inflammation in the nasal mucosa can have an effect on asthma, and asthma symptoms can worsen allergic rhinitis (Baraniuk, 2001; Bousquet *et al*., 2003).

Symptoms of allergic rhinitis include itchy eyes and nose, with sneezing, rhinorrhoea, lacrimation and nasal congestion. Just as with allergic asthma, there is an induction phase responsible for the latent period as the immune system is sensitized. A subsequent allergen exposure then results in the symptoms of allergic rhinitis. Rhinitis can also occur in the absence of a specific immune response and in this case is called nonimmunologic or nonallergic rhinitis. As with asthma, rhinitis symptoms in any given individual can be a combination of allergic and nonallergic components. The substances that are associated with allergic asthma (**Table 1**) can also cause allergic rhinitis.

As with asthma, allergic rhinitis can be divided into an early phase that occurs within minutes, a late phase 6–12 hours later, and chronic rhinitis. The early phase with sneezing, rhinorrhoea and nasal obstruction is attenuated by H1 histamine receptor antagonists, suggesting a critical role for histamine in causing the symptoms. However, the acute nasal obstruction is only partially relieved with H1 histamine receptor antagonists (Greiner and Meltzer, 2006). This is consistent with the fact that the vasodilatory properties of histamine are due to stimulation of both H1 and H2 receptors. Treatment with a combination of H1 and H2 antagonists does not relieve nasal congestion appreciably more than use of an H1 antagonist alone, suggesting that mediators other than histamine are critical for nasal obstruction. Nasal obstruction in rhinitis is due to dilation of venous sinusoids, leakage of post capillary venules with fluid exudation, and mucus secretion. Vasoconstrictors such as pseudoephedrine can reverse the vasodilation, but adverse effects associated with use of these drugs limit their usefulness for chronic treatment (Greiner and Meltzer 2006; Scadding *et al*., 2008). The most effective treatment to date for symptoms of allergic rhinitis is intranasal administration of anti-inflammatory steroids, indicating the key role for inflammation in the pathology.

3.2 Animal Models of Allergic Rhinitis

Animal models have been extensively used for the study of asthma, but are not as well established for studies of allergic rhinitis, particularly for measurements of nasal obstruction. Rodent nasal passages differ from humans in the shape of the nasal turbinates, the airflow patterns as well as the fact that the sensory neuroepithelium in rodents is especially well developed for olfactory function (Wagner and Harkema, 2007). For the early phase of allergic rhinitis, investigators have used the number of nasal rubbings or sneezing as an indicator of the severity of the disease (Al Suleimani *et al*., 2007; Hiromura *et al*., 2007). Magnetic resonance imaging (MRI) scans have been used to assess thickening of the nasal mucosa as an indicator of chronic allergic rhinitis (Hellings *et al*., 2001). Nasal lavage can detect increased eosinophils in animals after repeated intranasal administration of allergen (Al Suleimani *et al*., 2007; Hellings *et al*., 2001). Histopathology looking for inflammation and eosinophil infiltration has been most extensively used. Limitations of this technique include the lack of standardization of sites sampled in the complex upper respiratory tract, as well as the length of time it takes to prepare the nasal passages for sectioning because of the bony structures.

Measurement of nasal obstruction is an important end point given its central role in human allergic rhinitis. Intranasal pressure has been used as an indictor of early-phase nasal obstruction in the guinea pig (Al Suleimani *et al*., 2007). In the mouse, Miyahara *et al*. (2005) used a forced oscillation technique to determine total resistance in the upper respiratory tract of the Balb/c mouse. Repeated allergen challenge resulted in increased nasal resistance, and this change was paralleled by a decrease in respiratory frequency. Because of this parallel, measurements of respiratory frequency in whole body plethysmography were also used by Miyahara *et al*. (2005; 2006) as an indicator of nasal obstruction in their mechanistic studies. Unrestrained whole body barometric plethysmography, or measurement of Penh, has also been used as an indicator of nasal obstruction (Johnson *et al*.,

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2007; Kishi *et al*., 2007; Wagner and Harkema, 2007). If small volumes of allergen are delivered intranasally in an unanaesthetized mouse, the allergen is largely confined to the nasal mucosa, resulting in no detectable inflammation in the airways (Miyahara *et al*., 2005; 2006). Under these conditions in an obligate nose breather such as the mouse, the change in Penh will reflect the changes in the upper respiratory tract because the lower airways are not contributing to the obstruction. The same concerns that were raised for the use of Penh as an indicator of airway obstruction apply to the use of Penh for the measurement of nasal obstruction. Penh is measuring changes in breathing patterns and is not a measure of airway mechanics.

3.3 Pathophysiology of Allergic Rhinitis

As with allergic asthma, IgE production and mast cell mediator release are thought to be central to the allergic rhinitis mechanism, with an additional contribution by Th2 cells to the late and chronic phases. Mice lacking the FcεRI receptor do not develop an early nasal obstruction in response to ovalbumin and the late-phase nasal obstruction is also reduced, suggesting that IgE interaction with FcεRI is essential to these responses (Miyahara *et al*., 2005). Continued studies indicated that reconstituting the mice with bone marrow-derived mast cells restored the early-phase response, but not the late-phase response, indicating that FcεRI on mast cells are important for the early-phase response, and suggesting that FcεRI on some other cell type besides the mast cell is important for the late-phase response (Miyahara *et al*., 2008). IL-13 was important for the late-phase response but not the early-phase response (Miyahara *et al*., 2006). In ovalbumin-sensitized and challenged mice, IL-21 administration inhibited sneezing and eosinophil infiltration due to decreased IgE production by the B cell and decreased eotaxin production by fibroblasts (Hiromura *et al*., 2007).

Studies of allergic rhinitis using low-molecular-weight allergens are limited. Chemical exposures are difficult because of the irritant potential of many of the vehicles necessary for dissolving the chemical and the difficulty in conducting inhalation exposures. Farraj *et al*. (2004) used an acetone/olive oil vehicle to intranasally instil trimellitic anhydride, dinitrochlorobenzene or oxazolone into the mouse. Trimellitic anhydride administration resulted in a significant increase in the mRNA (messenger RNA) for Th2 cytokines and total serum IgE, suggesting that the mechanism for trimellitic anhydride parallels that of ovalbumin. Lung inflammation in this model was not apparent, suggesting that trimellitic anhydride did not distribute to the lower respiratory tract because of the viscosity of the acetone/olive oil vehicle.

Johnson *et al*. (2007) developed an allergic rhinitis model due to toluene diisocyanate inhalation in C57Bl/6 mice. Chronic inhalation exposure to 50 ppb toluene diisocyanate vapour for four hours per day for 12 consecutive weekdays resulted in eosinophils in the nasal mucosa, decreased respiratory frequency and increased Penh suggesting nasal obstruction, mucosal thickening, increased total serum IgE, increased toluene diisocyanate-specific IgG and a mixed Th1/Th2 cytokine response with increased IL-4, IL-5, IL-13 and IFN-γ mRNA in the nasal mucosa. No lung inflammation was apparent, suggesting that any Penh changes were due to nasal obstruction rather than increases in airway resistance. More prolonged exposure to toluene diisocyanate vapour will result in pulmonary inflammation and immune-mediated effects in the lung with a mixed Th1/Th2 cytokine profile (Matheson *et al*., 2005a; 2005b). Studies of Tsumuro *et al*. (2006) and Kishi *et al*. (2007) in a Brown Norway rat model of toluene diisocyanate-induced allergic rhinitis demonstrated increases in Penh that peaked at one hour after toluene diisocyanate intranasal challenge with a suggestion of a later-phase Penh response at four hours. Using antagonists, cysteinyl leukotrienes and thromboxane were important mediators of both the early- and late-phase Penh response, but contributed little to the immediate sneezing response. However no direct evidence is presented in this rat model to indicate that the measured change in Penh reflects changes in nasal resistance. Respiratory frequency increased in these animals with an increase in Penh, as opposed to decreases in respiratory frequency seen with increased nasal resistance in the mouse model (Miyahara *et al*., 2005). Thus, species differences exist and indirect measures of nasal resistance need to be confirmed using direct measures of nasal resistance.

4 SUMMARY

Allergic asthma and rhinitis are informative examples of immunotoxicity. Toxicants can operate as allergens to evoke a damaging immune response in the respiratory tract, or can influence the pathophysiology by enhancing sensitization to allergens and exacerbating airway hyper-responsiveness. A wide variety of allergens have been identified that can cause allergic asthma and rhinitis, and studies clearly indicate that mechanisms vary and include both antibody and cell-mediated components to explain the respiratory tract pathology.

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Ophthalmic Toxicology

Bryan Ballantyne

CONTENTS

1 INTRODUCTION

Materials may come into contact with the eye either by direct surface contact from solids, liquids and airborne materials, or via the circulatory system following systemic absorption by another route of exposure. In addition, and related mainly to physicochemical properties, materials coming into direct surface contact with the eye may be absorbed into the systemic circulation and exert toxic and/or pharmacological effects at sites remote from the eye. Thus, the eye may

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be a target of, or route for, toxic effects under various exposure conditions, as follows.

1. Direct local (topical) contact of a material with the surface of the eye (cornea, conjunctiva and palpebral surface) that may result in different tissue responses depending on the physical and chemical nature of the material. These may include:
   (a) a peripheral chemosensory effect
   (b) injury and/or an inflammatory reaction of the eye and its surrounding structures (local irritation)
   (c) a sensitization reaction (allergic conjunctivitis)
   (d) possible injury or functional changes to deeper ocular tissues, if materials penetrate surface structures at the site of contact.
2. Depending mainly on physicochemical properties of the material, there may be absorption into the systemic circulation through surface periocular vessels, or nasal and alimentary mucosa after drainage through the nasolacrimal duct. If of high toxicological or pharmacological potency, the absorbed parent material and/or metabolites may cause postabsorption systemic effects remote from the eye. This is descriptively referred to as transocular systemic toxicity.
3. Absorption of materials into the systemic circulation following exposure by routes other than direct ocular contact can result in systemic exposure of the eye to parent material and metabolites. Systemic toxicity to the eye and its adnexa may result.

This chapter reviews the above general aspects of toxicology as related to the eye, and gives illustrative examples of specific target sites for toxic effects, including iris, ciliary body, aqueous humour, lens, retina and extraocular muscles.

2 LOCAL IRRITANT EFFECTS ON THE EYE

2.1 General Considerations

Injuries to the eye resulting from local contamination with materials of various chemical categories are common in occupational, domestic, group recreational and general environmental situations (Saunders *et al*., 1996; Safranek *et al*., 2007; Teir, 1984). In the USA, eye injuries account for 3.3% of all occupational injuries resulting in lost workdays. One study of work-related eye injuries in the USA recorded that during 1999 there were about 280 000 work-related eye injuries treated in hospital emergency departments, with foreign bodies in the eye and chemical burns being the most common types of ocular injuries (Xiang *et al*., 2005). Occupational eye injuries from chemicals can, and should be, minimized by the use of appropriate precautionary and protective measures. However, domestic incidents are usually due to the use, handling, and storage of materials in an uncontrolled manner. For example, in one study it was found that 84.4% of eye injuries from chemicals occurred in the home, with predominant accidental exposures in children; the proportion of industrial chemical eye injuries in the series was 14.2% (Kersjes *et al*., 1987). In general, domestic chemical eye injuries are not usually a serious medical problem (Palmborg, 2005). This was also borne out by a Swedish Poison Information study that found chemically-induced eye injuries in the home setting are usually not a serious medical problem, with the majority of cases having no or mild symptoms, and advice to undertake water irrigation is sufficient. However, in spite of knowledge of the risks together with the availability of protective measures, occupational eye injuries often show more severe symptoms. US Poison Control Center reports indicate that there are more than 100 000 cases of chemical eye injuries annually, which accounts for about 6.5% of all Poison Control Center consultations (Litovitz *et al*., 1993). Similar proportionate values are seen in other countries that have good records systems, for example Sweden (Palmborg, 2005). In a detailed prospective follow-up interview study of ocular chemical injuries reported to the San Francisco Bay Area Region Poison Control Center, it was noted that the modes of exposure were quite varied, but the four most common substance groupings were personal care/medicinal products (15%), solvents (11%), strong acids or bases (7%) and hypochlorite bleaches (7%). The most common exposure patterns were by splash or drip, followed by spray can or aerosol (Saunders *et al*., 1996). With respect to occupational eye injuries, in 2002 there were 42 286 such injuries reported to the US Bureau of Labor Statistics (Harris, 2004); of these, 4811 (11.4%) were chemically induced. One cause of recreationally induced eye irritation and injury is exposure to swimming pools with inadequately controlled water chemistry; for example with respect to chloramines and free chorine (Petersson *et al*., 2008; Safranek *et al*., 2007).

Both the time to onset and the severity of chemically induced ocular injury vary with different chemical groupings and certain specific chemicals. For example, rapid onset and severe eye injury occurs with alkaline materials (Smally *et al*., 1992), whereas certain dyestuffs (e.g. gentian violet) may be associated with a latency of several days before the onset of severe eye injury (Ballantyne *et al*., 1973). Although the potential to cause eye injury with liquids and solids is well known, it should also be appreciated that exposure to many irritant vapours or gases can produce injury to the eye; examples include tetramethylbutanediamine (Ballantyne *et al*., 1992), 2-formyl-3,4-dihydro-2*H*-pyran (Myers *et al*., 1993) and acrolein (Hathaway *et al*., 1991). One

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example of vapours causing occupationally hazardous ocular effects is that resulting from exposure to the vapour of certain amines resulting in a condition known as glaucopsia, in which there is transient increase in corneal thickness causing blurring of vision. This condition, which is of practical occupational significance, is discussed later in more detail in Section 7.2.2.

Local contamination of the eye with chemicals or formulations may occur accidentally or, as with ophthalmic medicinal preparations, be deliberate. It follows that there is a need to know what is the potential for chemicals and formulations to produce eye injury by acute and repeated topical contamination of the eye, in order that (i) appropriate warnings can be given in product safety literature and on labels, (ii) advice given about first aid and medical management of eye injuries, and (iii) recommendations be developed for protective and precautionary measures when using or handling a particular material or formulation. In the special case of ophthalmic medicinal products, it is necessary to ensure that the in-use preparation does not cause structural and/or functional injury to the superficial and/or deep structures of the eye by acute and repeated deliberate ocular application (discussed further in Section 2.4). Additionally, it is also necessary to determine if medicinal products applied topically to the eye can be absorbed into the circulation and result in systemic toxicity (see Section 6.3).

2.2 Tests for Eye Irritating Potential

For many years the testing of chemicals and formulations for their potential to cause local injury and irritation to the eye by topical contamination was a simple procedure and usually conducted as an acute study. Following exposure of the eye to the test material (as solid, liquid or airborne material) the macroscopically visible ocular and periocular tissues were examined periodically for signs of inflammation and/or injury, and the effects noted were given subjective scores and noted as a function of time after exposure. In addition to recording macroscopic indications of eye irritation, ancillary methods have been developed and used to increase sensitivity for the detection of injury and to allow objective quantitation of the response. Additionally, and because of test-animal discomfort and stress associated with the conventional eye irritating test procedure, several alternative approaches for the determination or prediction of the eye irritating potential of chemicals have been proposed and developed. This work on alternatives to conventional eye irritation testing has been encouraged and facilitated by the expressed views of many professional toxicologists and by the opinions of several informed animal rights organizations. As a consequence, the numbers of conventional eye irritation tests that have been conducted have been progressively and significantly reduced over the past few years. Also, the sensitivity and specificity of alternative methods for assessing eye irritating potential have been investigated in detail. The following sections are devoted to a description of the conduct and interpretation of standard eye irritation test procedures, and to an evaluation of alternatives that have been proposed for assessing the eye injuring potential of chemicals and formulations.

2.3 *In Vivo* Eye Irritation Tests

Most studies have been conducted as single dose (acute) procedures, although repeated exposure studies have been carried out where this has been dictated by the use pattern of the test material. Although clinical reports of eye injury to humans have been published for many decades, it was not until the early 1940s that eye irritation testing became formalized as an animal laboratory procedure (Draize *et al.*, 1944). The basis of macroscopic *in vivo* eye irritation tests, and variants on such tests, is exposure of the eye to the test material followed by periodic sequential inspection of the eye for signs of ocular and periocular inflammation and injury, in order to note the onset, progression, severity and resolution of the eye effects. With respect to the last of these considerations, it is essential to know if any induced ocular injury is irreversible (permanent) or reversible (heals). If reversible there should be a determination of the time for healing to occur, and if first aid or medical treatment measures (e.g. eye irrigation) or the use of ocular anti-inflammatory agents are of therapeutic benefit. The more important elements of conventional *in vivo* eye irrigation studies are summarized and discussed below. There are differences in detail, with specific protocols recommended by various regulatory agencies and conducting contract laboratories (see Ballantyne, 1999; Daston and Freeberg, 1991).

2.3.1 Selection of Test Species

Some structural and functional differences between the human eye and those of common laboratory animals require that caution is necessary when extrapolating the results from laboratory animal eye irritation studies to the human. Most studies have been conducted using the rabbit as a test animal, for the following reasons:

1. Ease of handling.
2. Relatively large surface area of the eyeball and periocular tissues available for inspection.
3. There exists a large background literature on the effects of chemicals and drugs on the rabbit eye, allowing comparative evaluations.
4. The species is recommended in many regulatory agency protocols.

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Disadvantages include the following:

1. The rabbit has a nictitating membrane. The significance of this is debatable. Some authorities believe that the nictitating membrane removes irritant materials from the surface of the eye, but others consider that it acts as a tap (Buehler and Newmann, 1964).
2. The tearing mechanism in the rabbit is less effective than in the human (Buehler and Newmann, 1964), a factor of importance in relation to the degree and duration of contact of foreign material with the surface of theeye.
3. The pH and buffering capacity of the aqueous humour differ between man and rabbit (Carpenter and Smyth, 1946), which could explain why the rabbit is more susceptible to chemically induced iritis.
4. The rabbit has a slow blink reflex (Mann *et al*., 1948).
5. Both the thickness and the histology of the cornea differ between rabbit and man (Carpenter and Smyth, 1946). The rabbit corneal thickness averages 0.37 mm and that of the human 0.5 mm (Marzulli and Simmon, 1971). Histological differences include the rabbit corneal epithelium being several layers thinner, and Bowman's membrane is an order of magnitude thinner; both of these factors facilitate the penetration of materials through the rabbit cornea. Also, the area of the eye occupied by cornea is 25% in the rabbit, but only 7% in humans.
6. The rabbit eye is more readily anaesthetized by irritants than is the human eye, and hence more susceptible to reduction of protective blinking (Daston and Freeberg, 1991). Comparative studies using a variety of substances have shown that the eye of primates responds differently compared to that of the rabbit; the latter species usually has a more marked reaction (Buehler and Newmann, 1964). Comparative studies on the rabbit, monkey and human have demonstrated that the ocular reactions in the monkey are more akin to that of the human (Beckley *et al*., 1969). Thus, for critical purposes, such as the testing of ophthalmic preparations for use in humans, confirmatory studies on the results from animal (rabbit) tests may need to be undertaken in nonhuman primates.

2.3.2 Material to be Tested

For the majority of purposes it is usually recommended that the material be tested as 0.1 ml of liquid or 100 mg of solid, which is placed in the inferior conjunctival sac. In some cases, for example if it is suspected that the material will produce severe eye injury, then it is appropriate that 0.01 or 0.005 ml or mg of test material be used initially. It should be noted that 0.1 ml is close to the maximum volume that can be accommodated in the inferior conjunctival sac of the rabbit eye, and hence the use of smaller volumes has been proposed to be more appropriate for comparison with the practical aspects of splash contamination of the human eye. For example, it has been shown that the volume capacity of the human eye is 0.01 and 0.03 ml, with and without blinking, respectively (Wright and Megers, 1962; Misham *et al*., 1966). Using different volumes of several chemicals of varying irritant potential that were applied to the rabbit cornea, Griffith *et al*. (1980) determined the dose–response relationship for irritancy and compared the findings with the known human ocular irritancy to the chemicals used. They found that a dose volume of 0.01 ml produced irritation in the rabbit eye which was consistent with that observed in human, and used this volume in a low volume eye test (LVET). Subsequently, other investigators confirmed the reliability of the LVET for determining eye irritating potential (Allgood, 1989; Freeberg *et al*., 1984; 1986a; Walker, 1985; Williams, 1985). Also, it was demonstrated that the LVET correlates with the conventional Draize procedure (Blein *et al*., 1991). Cormier *et al*. (1995) reviewed consumer eye irritation comments (1985–1992) on more than 70 commercial products and found that the LVET correlated well with experience for human eye data. They also examined intra- and inter-laboratory reproducibility with the LVET and found it to have similar degrees of reproducibility to the Draize procedure (Cormier *et al*., 1996). Results from 70 parallel Draize and LVET tests indicated a strong positive association between the tests for corneal, iridial and conjunctival scores (r = 0.60, 0.73, 0.68, respectively; $p < 0.0001$).

For exposure to gas, vapour, aerosol or smoke, appropriate exposure chambers with sampling and analytical facilities are required. Since acute ocular irritancy with aerosols may vary with particle size (Punte *et al*., 1963), it is important that samples be collected for measurements of particle sizes as well as for substance concentration measurements. Also, for practical purposes, it may be necessary to measure ocular irritation as a function of particle size. In order to reduce the numbers of animals used, eye irritation observations should be routinely incorporated into acute and repeated-exposure general toxicology studies involving airborne materials.

When formulations are tested, it is ideal to know if eye injury is due to the active ingredient alone, to vehicle, or the formulation. However, in order to reduce the number of tests necessary, from a product safety viewpoint only the formulation may need to be tested. Clearly, and also for product safety reasons, if a formulation causes eye injury it may be required to determine if the in-use dilution of the formulation is irritant (Ballantyne and Swanston, 1977). It follows that if a material is required to be tested in solution, then a nonirritant solvent should be used; for example saline, low-molecular-weight molecular weight (MW) polyethylene glycol (PEG), propylene glycol or glyceryl triacetate (Ballantyne *et al*., 1972).

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Based solely on physical and/or chemical characteristics, some regulatory authorities may consider that there is sufficient information from these characteristics to allow an assumption that the material will produce a positive result on rabbit eye irritation testing. In such cases the material may be classified as having a potential to cause eye injury, and there is no requirement for standard eye irritation testing. For example, many regulatory authorities allow classification of a material as an eye irritant if its pH is <2.0, or >11.5. However, pH alone may not be a sufficient criterion for classification, and buffering capacity may be an important additional factor. For example, Neun (1993) conducted eye irritation studies with solutions of pH 11.5 having alkalinities of 0.5, 1.0 and 2.0 mEq g^{-1} using bicarbonate buffer systems. The results showed that the maximum 'Draize score' and median days to resolution indicated that the solutions with low buffering capacity are not necessarily irritant or corrosive at a pH considered to indicate a high irritant potential. The investigator suggested that with materials of extreme pH, at least knowledge of the buffering capacity is also needed.

2.3.3 The Test Procedure

Most studies are conducted as acute (single-exposure) procedures. Control and test animals for eye irritation studies need to be acclimated for about one week before testing to ensure recovery from any stress caused by transportation, including corneal dehydration. Animals are housed in cages designed to prevent accidental eye injury, including the use of wire mesh floors in place of sawdust or wood chippings. On the day before testing, animals should be examined to ensure the absence of ocular abnormalities.

Studies have been conducted to determine the smallest number of animals that can be used to adequately characterize the eye irritating potential of chemicals. DeSousa *et al*. (1984) examined the ability of two-, three- and five-animal subsets to accurately predict the outcome of six-animal tests for a large number of petrochemicals, and found that the three-rabbit subsets were 93% accurate in predicting the six-rabbit eye irritation tests. A similar finding was obtained by Talsma *et al*. (1988), and also confirmed by Bruner *et al*. (1992). It was found by Berdasco *et al*. (1966) that the number of animals can be reduced in standard ocular irritation tests, especially if a sequential approach is adopted using *in vitro* tests and structure-activity considerations first. However, if the findings from a particular three-animal test show a wide variability, then it may be necessary to undertake additional testing. For example, Dalbey *et al*. (1993) conducted a detailed evaluation of the influence of animal numbers on irritation classification criteria, and found that a significant number of incorrect classifications were made on the basis of the three-rabbit subsets. The authors advised that investigators should carefully evaluate the effects on accuracy of results before reducing the numbers of animals in a given eye irritation study.

Test materials are usually placed in the inferior conjunctival sac (larger amounts), or on the surface of the cornea, after which the eyelids are gently held together for a few seconds. One eye of each rabbit is used for test material instillation, and the contralateral eye serves as a control. It is generally considered that application of the test material to the surface of the cornea, rather than instillation into the inferior conjunctival sac, is a more practical method for mimicking the actual exposure conditions, with the exception of ophthalmic medicinal preparations that may deliberately be instilled into the inferior conjunctival sac (Chan and Hayes, 1985). Special devices for applying measured amounts of test material to the eye have been described (Buehler and Newmann, 1964; Battista and McSweeney, 1965).

After applying the test material, eyes are periodically and sequentially examined, under standard conditions of illumination, to determine and record the nature, severity, time to onset, duration and possible resolution of any induced ocular and periocular injury and/or inflammation (see Sections 2.3.4 and 2.3.5). The principal ocular and periocular lesions that should be recorded are as follows: excess lacrimation, blepharitis (hyperaemia and oedema), inflammation of the conjunctivae and nictitating membrane (injection, congestion, chemosis and sloughing), corneal injury (opacity/ulceration, vascularization, and area of involvement) and iritis (injection and congestion). In addition to macroscopic evidence for inflammation and injury, the eye should also be examined for any pharmacological effects that may have been produced, for example mydriasis and cycloplegia with anticholinesterase (antiChE) agents (Ballantyne, 2006a). Additionally, and with potentially biologically potent materials, the test animals should be inspected for signs of systemic toxic and/or pharmacological effects following transocular absorption. Periodic inspection of the eye following contamination is essential for extrapolation to possible in-use clinical situations, and for comparative evaluations between different materials and formulations. The original Draize protocol specified that eyes should be inspected at 1, 24, 48 and 96 hours, and if necessary at seven days post instillation. Other protocols specify alternative inspection periods. However, early inspections are essential to detect initial signs of injury and their progression. A minimum of one week of observations is required to ensure that any latent injury is detected.

The use of local anaesthesia has been proposed as a means to alleviate discomfort in eye irritation tests. However, some authorities believe that local anaesthetics may exacerbate ocular irritation, There is also evidence that local anaesthetics, to variable degrees, may delay healing of the cornea and themselves produce corneal injury, and hence potentiate ocular irritation (Daston and

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Freeberg, 1991). Also, local anaesthetics inhibit the blink reflex. Corneal injury produced by local anaesthetics was reviewed by Etter *et al*. (1992), who noted that oxybuprocaine experimentally produced a concentration-related increase in corneal permeability. Additionally, Lapalus *et al*. (1990) found that oxybuprocaine was cytotoxic to corneal epithelial cells *in vitro*. However, Choksi *et al*. (2007), based on a study with 97 proprietary formulations, found the use of 0.5% (w/v) tetracaine hydrochloride as a pretreatment in the *in vivo* Draize rabbit eye test had no effect on the variability of rabbit irritancy classification, and they consider their findings support the routine use of this local anaesthetic.

Most standard recommendations for the first-aid management of chemically induced eye injury include the use of extensive ocular irrigation (Herr *et al*., 1991). Whilst postexposure irrigation of the eye generally reduces or prevents eye irritation, in some cases increased ocular irritation has been noted following water irrigation (Gaunt and Harper, 1964). Because of this, some regulatory authorities have recommended that subgroups of animals be used to assess the efficacy of eye irrigation on the development and severity of chemically induced eye injury. Other therapeutic procedures which reduce or prevent ocular injury can be incorporated into conventional eye irritation studies, for example the use of anti-inflammatory preparations (Ballantyne *et al*., 1976).

2.3.4 Scoring Ocular Lesions

Several schemes have been proposed for the grading and scoring of ocular lesions observed in standards eye irritation tests. That introduced by Draize has been the one used most frequently (Draize *et al*., 1944; Draize, 1959), particularly for product development. The Draize system assigns values to effects seen on the cornea, iris and conjunctiva, usually at various specified periods (**Table 1**). There are three major concerns with this scoring system:

1. Only a limited number of effects in the three tissues are described. Thus, the cornea is graded separately for opacity and area of involvement, the iris is considered as an entity, and there are separate grades for conjunctival hyperaemia, chemosis and discharge. The limited number of effects recorded may give an incomplete description of the ocular reaction to a chemical.
2. The range of grades differs with the effect recorded. Thus corneal effects, both opacity and area of involvement, are graded 0–4; the iris is graded 0–2; and effects on the conjunctiva are graded 0–3 for discharge and hyperaemia, and 0–4 for chemosis. This nonuniformity in grading can lead to difficulties in comparative interpretations.
3. Results are not reported as scores as seen, but as arithmetically derived, biased (weighted) numbers calculated from the score as seen. For example, corneal scores are a product of grade for opacity and area of involvement, with a bias factor of five; iris is scored for five times the grade observed; and the conjunctival score is twice the sum of grades for hyperaemia, chemosis and discharge. Thus, for a possible total maximum score of 100 pints, 73% is derived from the corneal score, 18% from the conjunctival score and 9% for the iris. Thus, this nonuniformity in scoring results in a bias ratio of 8 : 2 : 1 with respect to the cornea, conjunctivae and iris.

The Draize approach was modified by The US Food and Drug Administration (FDA) for enforcing the Hazardous Substances Labeling Act; The FDA system does not differentiate between opacity and area of involvement of the cornea, and the scores are presented as the actual grades observed and are not arithmetically biased total scores (**Table 2**). To facilitate the scoring of injury between different laboratories and to obtain better reproducibility, colour photographs equivalent to the scores were published by the FDA (1965). Ballantyne and Swanston (1977) developed a more detailed scoring system (**Table 3**) using a large number of effects to be recorded, which included lacrimation, blepharitis, chemosis, conjunctival hyperaemia, iritis, keratitis, and corneal neovascularization; in order to obtain a more uniform approach, each effect is recorded on a six-point scale (0–5). Different scoring systems have been developed by other workers (see Ballantyne, 1999).

2.3.5 Recording, Presentation and Interpretation of Ocular Irritation Tests

The conventional eye irritation study is a subjective evaluation of macroscopic observations, and therefore it is hardly surprising that there have been considerable inconsistencies between the results obtained from differing laboratories, even using the same test protocol (Russell and Hoch, 1962; Weil and Scala, 1971). Such differences are due to a variety of factors which may include: variable methods of training in different test laboratories; the use of groups with different sized test animal populations; and differences in age, strain and sometimes the gender of animals used. Many effects seen, such as pannus formation, keratoconus, conjunctival haemorrhages and corneal neovascularization, may not readily lend themselves to scoring. Such effects are, however, functionally significant, and are usually tabulated separately or as footnotes.

The numerical systemic recording and presentation of scores involves tabulating as a function of the time of inspection. Values should be recorded for individual animals (e.g. **Table 4**) as well as group mean scores

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Table 1 Draize system for the scoring of ocular lesions[a]

Cornea	
(A) *Opacity—degree of density* (most dense area taken for reading)	
No opacity	0
Scattered or diffuse area, iris details clearly visible	1
Easily discernable translucent areas, iris details slightly obscured	2
Opalescent areas, iris details not visible, pupil size barely discernable	3
Opaque, iris not visible	4
(B) *Area of cornea involved*	
One quarter or less, but not zero	1
Greater than one quarter, but less than one half	2
Greater than one half, but less then three quarters	3
Greater than three quarters, up to whole area	4
Corneal score **= A × B × 5 (maximum total score = 80)**	
Iris	
(A) Normal	0
Folds above normal, congestion, swelling, circumcorneal injection, iris reacting to light	1
No reaction to light, haemorrhage, gross destruction	2
Iris score **= A × 5 (maximum total score = 10)**	
Conjunctivae	
(A) Vessels normal	0
Vessels definitely injected above normal	1
Diffuse, deep crimson red, individual vessels not readily discernable	2
Diffuse beefy red	3
(B) No chemosis	0
Any swelling above normal (includes nictitating membrane)	1
Obvious swelling with partial eversion of lids	2
Swelling with lids about half closed	3
Swelling with lids about half closed to completely closed	4
(C) No discharge	0
Any amount of discharge different from normal	1
Discharge with moistening of the lids and hairs adjacent to lids	2
Discharge with considerable moistening around the eyes	3
Conjunctival score **= (A + B + C) × 2 (maximum total score = 20)**	
Total possible maximum score **(cornea + iris + conjunctiva) = 110**	

[a]Reproduced with permission from Draize *et al*., 1944. © American Society of Pharmacology and Experimental Therapeutics.

with ranges (e.g. **Table 5**). In some published papers and reports only cumulative scores have been presented. In view of variability between animals and resultant interpretational difficulties, this is a practice to be avoided and prevented by reviewers. Scores may also be presented graphically, providing that the range of responses is not large, and used to compare the effect of differing concentrations of the same material or to compare the effects of the same dose of differing materials (e.g. **Figure 1**). Additionally, dose–response histograms (Macrae *et al*., 1970) can be used to demonstrate the relative proportions of animals having different reactions at each inspection period and hence permit a ready appreciation of the rate of development and resolution of eye injury, as well as group variability (e.g. **Figure 2**).

Several problems may be encountered in the interpretation of the Draize scoring system. Thus, scores are not usually recorded as the grade of effects seen, but as biased numbers derived by calculating arithmetic means using different biases for each eye component recorded. Because of this method of scoring, identical values may result from different eye lesions. For example, since the

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Table 2 Scoring system for grading ocular lesions according to the US Food and Drug Administration

Cornea	
No ulceration or opacity	0
Scattered or diffuse areas of opacity; details of iris visible	[1][a]
Discernable translucent areas; iris slightly obscured	2
Nacreous areas, details of iris not visible; pupil barely discernable	3
Complete corneal opacity; iris not discernable	4
Iris	
Normal	0
Markedly deepened folds, congestion, swelling, moderate circumcorneal injection (any or a combination), iris still reacting to light	[1][a]
No reaction to light; haemorrhage; gross destruction	2
Conjunctivae	
[A] Vessels normal	0
Some vessels with definite injection	1
Diffuse crimson red; individual vessels not discernable	[2][a]
Diffuse beefy red	3
[B] No swelling	0
Any swelling above normal	1
Obvious swelling with eversion of lids	[2][a]
Swelling with lids about half closed	3
Swelling with lids more than half closed	4

[a]The figures in parentheses indicate the lowest grade considered positive.

cornea is graded separately for the degree of opacity and area of involvement, and because this is presented as a biased product, a diffuse minor keratitis affecting the whole of the cornea may obtain a score identical with that produced by localized punctate lesions in one quadrant. This emphasizes the requirement to record each effect individually.

There have been attempts to reduce scoring to a single numerical value for the rating of irritancy. Since these schemes involve further arithmetic manipulation of the score, they give values even more remote from the actual biological response. Several authors have given a scale of rating; for example, Kay and Calandra (1962) described seven ratings from minor to severe. Such rating schemes usually have fixed standards and do not give any indication of the nature, severity and/or duration of ocular lesions. Hence, whilst such ratings may have some value in product development, they are not sufficiently precise and representative for regulatory purposes or publication of data, and do not provide a clinical description of eye injury to be expected from contamination of the eye with a particular chemical.

Schemes where precisely defined grades of effects are presented as a function of time permit a ready appreciation of the onset, duration, resolution, severity and nature of adverse effects on the eye. The FDA system requires that results should be presented in this way and defines, for regulatory purposes, what constitutes a positive response. Thus, a tissue is regarded as having a positive reaction if there is, at any reading, opacity or ulceration of the cornea, iritis, obvious chemosis with partial eversion of the lids or a deep crimson red appearance to the conjunctivae with individual vessels not being discernable. A material is considered irritant if four in a test group of six rabbits exhibits a positive reaction in any ocular tissue. A material that has produced lesions of the cornea and/or iris that have not cleared by the seventh day post-treatment is considered a severe eye irritant.

In a critical review of the Draize rabbit eye test, York and Steiling (1998) stressed the subjective judgment inherent in the procedure, and noted that the most important factors causing variability in results are group size and the fact that the methods for assessment cannot reflect the complex nature of the irritation reactions and the use of an algorithm is not valid and leads to misrepresentation of the true biological response. They suggested the establishment of a scientifically valid factor that encompasses the complexities of the total *in vivo* response, and that recovery could be a factor. They also suggested the generation of controlled human data to provide a benchmark for irritation against which Draize data could be compared.

2.3.6 Ancillary Methods to Eye Irritation

In order to obtain a more definitive evaluation of the presence and nature of ocular injury in standard eye irritation tests, several ancillary procedures have been proposed and adopted. The most relevant of these are summarized below.

2.3.6.1 Fluorescein Staining

The normal corneal epithelium presents a barrier to fluorescein, but when breached it allows fluorescein to penetrate into the underlying stroma. Hence the detection of

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Table 3 Equal grade scoring system for ocular lesions in eye irritation tests[a]

Effect	Grade	Observations
Lacrimation	0	Normal
	1	Slight excess at lower tarsal margin
	2	Slight wetting of periorbital hair
	3	Obvious wetting of lid margins and periorbital hair
	4	Gross wetting of periorbital hair
	5	Gross wetting of hair with fluid running down face
Blepharitis	0	Eyelids normal
	1	Slight reddening of lid margins
	2	Diffuse redness and slight swelling of lid margins
	3	Diffuse redness with moderate swelling of lids
	4	Diffuse bright redness with marked thickening and deformity of lids
	5	Diffuse bright redness with marked thickening and irregular deformity of lid margins; contracted palpebral fissure
Chemosis	0	No swelling of conjunctivae or nictitating membrane
	1	Minimal swelling with no eversion of lids
	2	Moderate swelling with eversion of lids
	3	Moderate swelling with moderate eversion of lids; swollen tissue encroaching over periphery of cornea
	4	Marked swelling and eversion with tissues almost completely obliterating the cornea
	5	Gross swelling and eversion of lids with complete obliteration of cornea
Congestion of conjunctivae and nictitating membrane	0	Vessels normal
	1	A few injected vessels
	2	Diffuse and moderate injection of blood vessels
	3	Diffuse and marked dilation, but individual vessels still discernable
	4	Diffuse and gross dilation with individual vessels barely discernable
	5	Diffuse intense redness
Sloughing	0	Absent
	1	Patchy detachment of film
	2	Diffuse detachment of fine membrane
	3	Diffuse detachment of fine film with slight contamination of lacrimal fluid
	4	Obvious stripping of membrane with moderate contamination of lacrimal fluid
	5	Gross detachment of membrane and gross contamination of lacrimal fluid
Iritis	0	Normal iris
	1	Slight injection of iris blood vessels
	2	Slight thickening of folds with diffuse and moderate injection; still reacting to light
	3	Moderate thickening of folds with diffuse and moderate injection; slight reduction in response to light
	4	Diffuse and gross erythema with obvious thickening; sluggish reaction to light
	5	No reaction to light; distorted pupil; gross inflammatory change with haemorrhages
Keratitis	0	Normal cornea
	1	Just detectable haziness of cornea
	2	Obvious opacity of cornea (local or general) but iris clearly visible
	3	Local or general opacity of cornea almost obscuring iris
	4	Local or general opacity of the cornea totally obscuring the iris
	5	Gross opacification of the cornea with deformity and/or ulceration

(*continued overleaf*)

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Table 3 (*continued*)

Effect	Grade	Observations
Vascularization of the cornea	0	Avascular
	1	Localized ingrowth of vessels for 1–2 mm
	2	Generalized ingrowth of vessels for 1–2 mm
	3	Generalized ingrowth of vessels for 3–4 mm
	4	Ingrowth of vessels to within 1–2 mm of centre of cornea, but without confluence
	5	Complete vascularization of cornea with confluence of vessels at centre of cornea

[a]After Ballantyne and Swanston (1977).

Table 4 Results from individual animals in a rabbit eye irritation study with 5% (w/v) 1-chloroacetophenone (CN) dissolved in polyethylene glycol 300, and scored by the method of Draize *et al*. (1944)

Animal number	Tissue scored	1 h	24 h	48 h	72 h	96 h	7 d
1	Cornea	0	30	45	45	60	60
	Iris	0	5	5	5	5	5
	Conjunctiva	14	14	14	10	10	5
	Total	14	49	64	60	75	71
2	Cornea	0	15	15	30	15	15
	Iris	0	5	5	5	5	5
	Conjunctiva	12	12	12	10	8	4
	Total	12	32	32	45	28	24
3	Cornea	0	30	45	45	45	30
	Iris	0	5	5	5	5	5
	Conjunctiva	16	14	12	10	6	2
	Total	16	49	62	60	55	37
4	Cornea	0	30	45	45	45	45
	Iris	0	5	5	5	5	5
	Conjunctiva	14	14	18	14	12	2
	Total	14	49	68	64	62	52
5	Cornea	0	30	30	45	45	45
	Iris	0	5	5	5	5	5
	Conjunctiva	16	14	16	10	8	6
	Total	16	49	51	60	58	56
6	Cornea	0	30	30	45	55	45
	Iris	0	5	5	5	5	5
	Conjunctiva	16	12	10	10	8	2
	Total	16	47	45	60	68	52
Mean scores[a]	Cornea	0	27.5	35.0	42.5	44.2	40.0
	Iris	0	5.0	5.0	5.0	5.0	5.0
	Conjunctiva	14.7	13.3	13.7	10.7	8.7	3.7
Mean total scores[b]		14.7	45.8	53.7	58.2	57.9	48.7

[a]Maximum tissue scores: cornea = 80, iris = 10, conjunctiva = 20.
[b]Maximum possible total score = 110.

fluorescein staining of the cornea is a valuable guide to early corneal injury. One or two drops of 2% fluorescein are applied directly to the cornea and washed off a few seconds later with isotonic saline. The injured cornea retains more dye than the intact epithelium, and appears a brighter yellow in colour. This is facilitated by examination under ultraviolet (UV) light, or using a blue filter in a slit-lamp biomicroscope. Yellowish green areas stained by fluorescein indicate regions where the dye has penetrated epithelial lesions and diffused into normal tissue. Rose Bengal has been recommended to stain areas of degenerate epithelial cells and Alcian Blue for the demonstration of mucous.

2.3.6.2 *Corneal Permeability Studies*

The presence of fluorescein in aqueous humour may give an indication if corneal permeability has been increased

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Table 5 Mean and range of scores for a group of six rabbits in an eye irritation study using 5 mg solid 1-chloroacetophenone (CN)[a]

Effect	Time of inspection post-treatment								
	10 min	1 h	6 h	1 d	2 d	3 d	4 d	7 d	14 d
				Lacrimation					
Mean	1.8	2.8	2.7	2.4	1.7	2.3	1.8	1.2	0.6
Range	1–3	2–4	2–3	1–3	1–2	1–3	1–3	1–2	0–2
				Blepharitis					
Mean	1.1	2.0	2.1	2.7	3.0	3.0	2.9	2.5	1.0
Range	1–2	All 2	1–4	2–4	2–4	2–4	2–4	2–3	0–3
				Chemosis					
Mean	1.9	3.0	3.3	2.7	2.3	2.2	1.6	1	0
Range	1–3	2–5	3–4	2–4	2–3	1–3	1–2	0–2	
				Hyperaemia					
Mean	2.1	2.5	2.3	3.2	3.2	3.1	3.0	2.4	0.9
Range	2–3	2–3	2–3	3–4	3–4	3–4	2–4	1–4	0–2
				Sloughing					
Mean	0	0	0	1.7	2.0	1.5	1.0	0.9	0.3
Range				1–4	1–3	1–2	0–3	0–2	0–3
				Iritis					
Mean	0	0	0.3	1.4	1.6	1.7	1.5	1.2	0
Range			0–2	1–2	1–2	1–3	1–3	0–3	
				Keratitis					
Mean	0	0	1.9	2.1	2.4	2.8	3.1	3.9	4.0
Range			0–2	2–3	2–4	2–4	2–4	2–5	1–5
				Corneal vessels					
Mean	0	0	0	0	0	0	0	0.5	2.3
Range								0–1	0–4

[a]Scored by the method of Ballantyne and Swanston (1977; see **Table 3**).

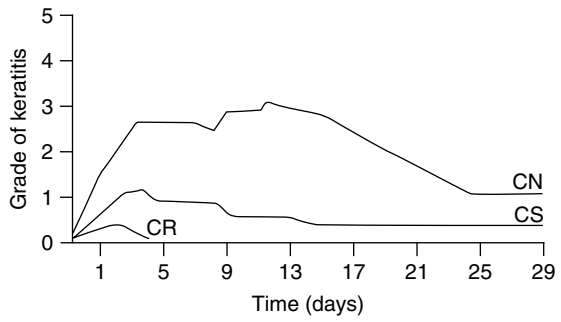

Figure 1 Comparative graphical representation of the average grades plotted as a function of time for corneal injury from dosing rabbits (10 per group) with 0.1 ml of 5% solutions of 1-chloroacetophenone (CN), 2-chlorobenzylidene malononitrile (CS), or dibenz [b.f]-1,4-oxaxepine (CR). Corneal injury scored by the method described by Ballantyne and Swanston (1977; see **Table 3**).

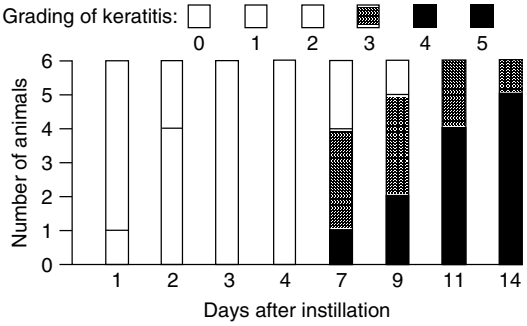

Figure 2 Response–duration histogram for the development of corneal injury in the rabbit eye following instillation of 2% Crystal Violet in water. Scoring system after that of Ballantyne and Swanston (1977; see **Table 3**).

since, as noted above, corneal epithelial damage may increase the rate of penetration of fluorescein through the cornea. An increased accumulation of fluorescein in the anterior chamber can be detected by a 'fluorescein flare' in the aqueous humour using a blue filter in the slit-beam path of a biomicroscope. Measurement of fluorescein in aqueous humour has been used as a basis for a quantitative evaluation of corneal permeability (Maurice, 1967; 1968). Sulforhodamine B has been preferred to fluorescein because of its lower lipid solubility at physiological pH, and hence its decreased permeability in the undamaged eye, and also because the red wavelength emitted by rhodamine allows better quantitation (Maurice and Singh, 1986). Changes in fluorescein concentration in aqueous humour can be quantified using a fluorophotometer attached to a slit-lamp

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biomicroscope; this permits an objective test for eye injury (Easty and Mathalone, 1969). Etter and Wildhaber (1985) used fluorimetry to quantify corneal permeability in mice following treatment with a series of surfactants, and found a good agreement between fluorescein penetration and *in vivo* irritancy. The use of corneal permeability studies as an objective sensitive method for the early evaluation of corneal injury is discussed in more detail in Section 3.2.3.

2.3.6.3 Slit-Lamp Biomicroscopy

Slit-lamp examination of the eyes is a valuable aid for the detection of early and minimal changes in the cornea, and allows a more precise evaluation of the structural condition of the cornea, iris, lens and aqueous humour; for example, the presence of a flare in aqueous humour due to excess protein (McDonald *et al*., 1972).

2.3.6.4 Confocal Microscopy

Confocal microscopy allows optical sectioning through intact living tissues such as the cornea and lens with enhanced resolution and contrast compared to conventional microscopy, and is without preparation artefacts. Coupled with rhodamine 123 fluorescence staining, this approach can be used to study the distribution and morphology of mitochondria.

2.3.6.5 Measurement of Corneal Thickness

The *in vivo* measurement of corneal thickness (pachymetry) is an objective method for determining injury to the cornea, particularly in the early stages of the injury process. Although it can be used as an ancillary method to supplement eye irritation studies, it is an approach that can stand alone for the objective quantitative detection and evaluation of corneal injury. This is discussed in detail in Section 3.2.1.

2.3.6.6 Corneal Epithelium Healing Rate

Following injury of the corneal epithelium, re-epithelialization occurs and corneal thickness increases; toxic substances, however, may retard the normal rate of healing. It has been suggested that measurement of corneal thickness and healing rate can be used as a method for the evaluation of potential eye irritants, particularly with ophthalmic preparations which may be applied therapeutically to the injured eye. The method involves creating a standard epithelial wound using a trephine and stripping the corneal epithelium. Test material is then applied to the corneal surface. The contralateral eye is also de-epithelialized and used as an untreated control. Corneal healing rate is followed by fluorescein staining, photographing the wounded area and assessing its size by planimetry. Corneal thickness is measured before and sequentially after wounding. The technique has been described in detail by Ubels *et al*. (1982). Several investigations have shown that chemicals of various categories delay return of corneal thickness to normal and retard epithelial healing rate (Fujihara *et al*., 1993; Green *et al*., 1989; Ubels *et al*., 1982).

2.3.6.7 Histology

At the end of the observation period of an eye irritation study it is useful to sacrifice animals for removal of the treated eyes to allow a detailed histological examination to confirm, or otherwise, that healing has occurred. In some studies, sequential sacrifices over the inspection period are conducted to allow a determination of the pathogenesis of ocular lesions.

2.4 Management of Chemically-Induced Eye Injuries

Standard eye irritation tests may have provision in the protocol for determining some aspects of appropriate first aid and medical management of the material under test. For example, although it is standard practice to recommend that chemically contaminated eyes are irrigated (Herr *et al*., 1991; Sharma, 2004), in a few instances irrigation with water has been shown to result in increased eye irritation and may lead to an exacerbation of the corneal injury (Gaunt and Harper, 1964). Thus, some protocols have specified that subgroups of animals be used to determine the efficacy of eye irrigation on the development, severity and resolution of eye injury. As discussed earlier, other therapeutic procedures can be incorporated into conventional *in vivo* eye irritation studies; for example, the use of anti-inflammatory agents (Ballantyne *et al*., 1976). Monitoring of such procedures may involve simple direct macroscopic visual determination of efficacy, or more sophisticated approaches such as measurement of aqueous humour prostaglandins (Spampinato *et al*., 1991).

When topical medicinal preparations are recommended for use in the treatment of local ocular injury it is clearly important to determine if such materials themselves have any potentially adverse effects on the eye which could interfere with the healing process or even exacerbate the injury. For example, over-the-counter (OTC) ophthalmic decongestion preparations may by themselves be a cause of conjunctivitis (Soparker *et al*., 1997). Such decongestion preparations usually contain an α-adrenergic vasoconstrictor (e.g. naphthazoline, tetrahydrozoline or phenylephrine), and some contain H_1 blockers. These materials have been shown to produce conjunctival hyperaemia, follicular conjunctivitis and/or blepharoconjunctivitis. This evaluation of the safety of materials that may come into direct contact with the eyes also applies to the possible development of corneal and/or conjunctival injury and of aseptic endophthalmitis (toxic anterior segment syndrome) caused by disinfectants such

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as glutaraldehyde and *o*-phthalaldehyde used to sterilize ophthalmic surgical instruments (Ayaki *et al*., 2007; Ballantyne, 2004a; Mamalis *et al*., 2006).

To reduce the potential for chemically induced eye injuries, personal protective eyewear such as goggles, face shields, safety glasses and, with occupational situations, full-face respirators should be used when an eye hazard exists. Contact lenses do not provide protection (Peate, 2007). The eye protection chosen for a specific work condition depends on the nature and extent of the hazard, the circumstances of exposure, other protective equipment used and personal vision needs allowing for sufficient peripheral vision (NIOSH, 2009).

3 ALTERNATIVES TO CONVENTIONAL EYE IRRITATION TESTS

3.1 General Considerations

Criticism of eye irritation tests has come from concerned scientists, informed individuals and self-interest groups. The primary cause for such criticism is an appreciation of the discomfort and deliberate induction of injury to the eye produced in standard tests, and the apparent trivial misuse of the approach for testing products such as cosmetic formulations. Such informed pressure, compounded by uninformed and media comments, together with the concern expressed by several practising toxicologists, led to a proliferation of investigations in attempts to modify conventional approaches in order to reduce discomfort, or to replace eye irritation tests with alternative methods to detect the ocular injuring potential of chemicals. As a consequence, there has been a proliferation of publications in which investigators report what they considered to be suitable alternatives with the ability to predict *in vivo* ocular injury. Some of these approaches have merit in being biologically and mechanistically based, but others displayed a lack of understanding of basic biological principles as applied to toxicological problems and of the mechanisms of induction of eye injury by chemicals. It is still generally believed that no one method, particularly *in vitro*, can reliably predict eye irritating potential for a differing series of chemical classes. Several methods, taken together, may assist in the prediction of eye irritating potential, but ultimately for potentially high risk situations the results (particularly if negative) may require confirmation by *in vivo* procedures (Secchi and Deligianni, 2006). Total reliance on many of the currently used alternative procedures is not likely to be conclusively helpful in, for example, litigation involving human eye injury from a material judged to be nonirritating by these procedures. Also, and paradoxically inhibitory to the development and validation of alternative procedures, many regulatory authorities, whist supporting the requirement for such alternative approaches for ocular irritancy testing, still will not accept results from alternative procedures for the purposes of human health hazard evaluation and related regulatory actions.

Table 6 Groups of alternative approaches to conventional *in vivo* eye irritation testing

In vivo threshold irritant methods
Isolated ocular preparations
Isolated nonocular preparations
In vitro cytotoxicity assays
Nonbiological methods
Analogy

Alternatives to conventional *in vivo* eye irritation testing can conveniently, though generally not mechanistically, be divided into *in vivo* alternatives, *in vitro* alternatives and the use of nonbiological models (**Table 6**).

3.2 *In Vivo* Threshold Alternatives

These methods, whilst using whole animals, are aimed at significantly reducing discomfort to animals by employing lower doses of the test material and by increasing the sensitivity for the detection of injury by the use of noninvasive techniques. Most approaches have the advantage of allowing an objective quantitative evaluation of the monitor for ocular injury.

3.2.1 Measurement of Corneal Thickness (Pachymetry)

In vivo measurement of the thickness of the cornea is a particularly sensitive method for the early detection of corneal injury. Corneal thickness, hydration and transparency are inter-related, with corneal thickness and hydration being linearly related (Medbys and Mishima, 1966). Increase in cornel thickness following acute chemical injury to the eye indicates the development of oedema within the corneal stroma, which may reflect damage to the epithelium, endothelium or limbic vessels (McDonald *et al*., 1983). Such damage facilitates the passage of water into the stroma, causing an increase in corneal thickness. Corneal oedema can result, for example, from epithelial and/or endothelial damage, increase in intraocular pressure (IOP) and inhibition of Na^+-K^+-ATPase (adenosine triphosphatase); such factors may be found in various types of chemically induced corneal injury (Chan and Hayes, 1985). Measurement of corneal thickness has several advantages, which include:

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1. Quantitative data are obtained which may be subjected to statistical analysis for comparative evaluation of different materials.
2. The method is noninvasive and causes the minimum of discomfort to animals.
3. Increase in corneal thickness usually occurs at test material concentrations below those producing macroscopic evidence for eye irritancy.
4. Examination of concentration–response curves allows prediction of irritant potency.

Corneal thickness may be measured *in vivo* by a simple optical device attached to a slit-lamp biomicroscope (optical pachymetry; **Figure 3**), or by means of an ultrasonic probe (ultrasonic pachymetry), both of which show good reproducibility (Chan and Hayes, 1985; Martins *et al*., 1992). The ultrasonic pachymeter uses a signal that penetrates corneal tissue, and from which the thickness is calculated based on corneal density and the change in reflectance at the junction of cornea and aqueous humour. In a detailed evaluation of methods and conditions for measuring corneal thickness, Myers *et al*. (1998) found that the best method was by the use of an ultrasonic pachymeter in rabbits that had been preconditioned to remain calm with mild restriction (a 'cat bag'). Sedation was found not to be necessary with these conditions. The use of local anaesthesia of the eye resulted, by itself, in a decrease in corneal thickness. Comparing the effect from an irritant alone with that resulting from irritant plus local anaesthesia demonstrated

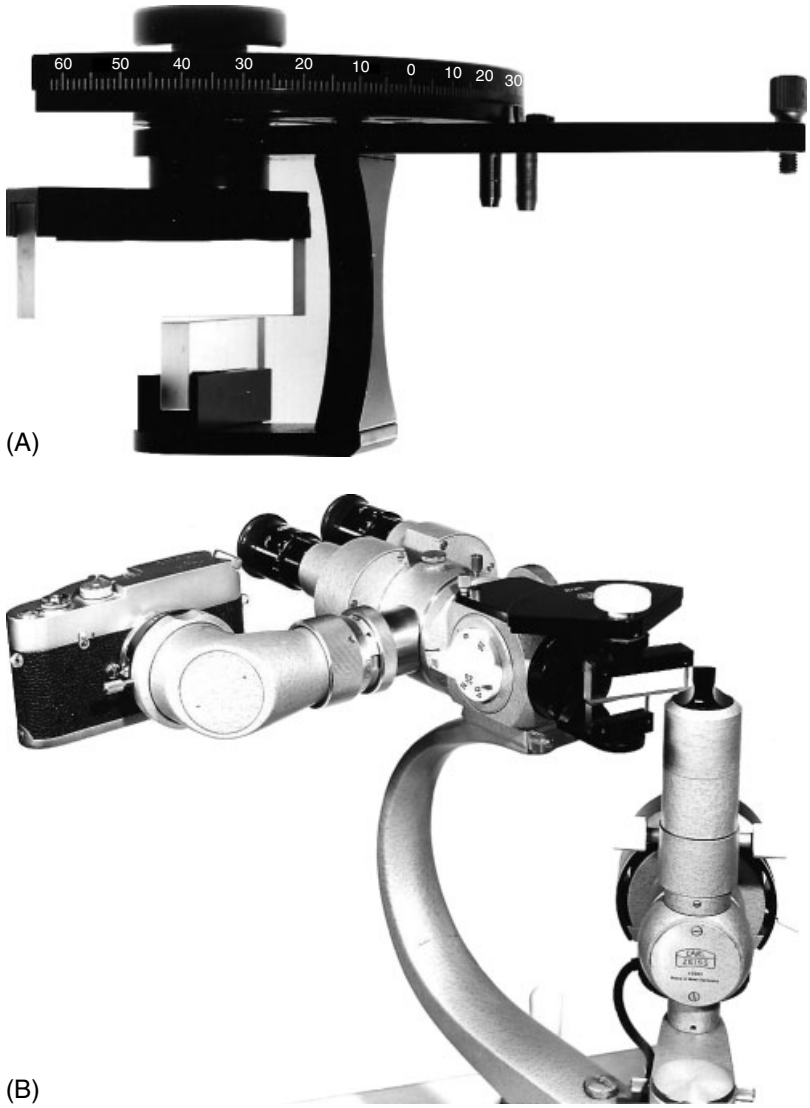

Figure 3 (A) Optical pachymeter. The slit beam from a biomicroscope is passed through two parallel-aligned glass blocks, which results in an image of the slit beam being visible on the surface of the corneal epithelium and a second image on the endothelium. One of the plates is rotated until the two images align, and the degree of rotation necessary to produce alignment is proportional to the thickness of the cornea (epithelial surface–endothelium distance). (B) Optical pachymeter mounted on slit-lamp biomicroscope.

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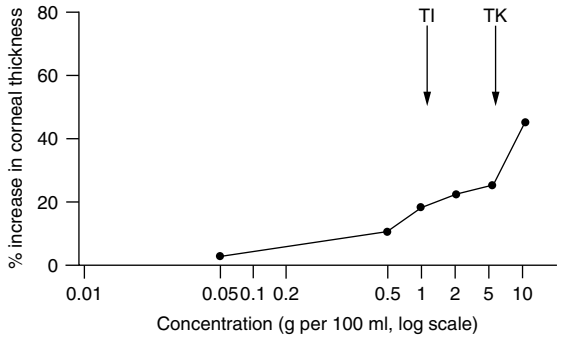

Figure 4 Peak increases in corneal thickness plotted as a function of the concentration of dibenz(b.f)-1,4-oxazepine (CR) in polyethylene glycol 300 applied to the rabbit cornea. Increase in corneal thickness occurs at concentrations below those at the threshold for irritation to the conjunctiva (TI, threshold for irritation) and even lower than those producing macroscopic evidence of corneal injury (TK, threshold for keratitis). (After Ballantyne, 1999.)

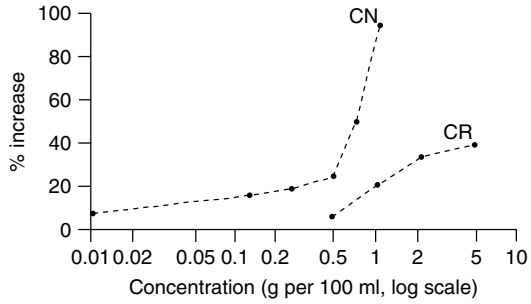

Figure 5 Increase in corneal thickness as a function of the concentration of 1-chloroacetophenone (CN) and dibenz(b.f)-1,4-oxazepine (CR) applied to the rabbit eye. For CN, the more irritant material, increase in thickness can be measured at lower concentrations than for the less irritant CR. Also, at any given concentration, the proportionate increase in corneal thickness is greater for CN than for CR. (After Ballantyne, 1999.)

that the use of topical anaesthesia may substantially affect the response of the cornea to a point of confounding both the corneal thickness and subjective Draize evaluations. It follows that if topical anaesthesia is used, the findings should be interpreted with caution because of the effect of local anaesthetics *per se* on corneal thickness, and since they may alter corneal permeability (Mauger, 1992; Nam *et al*., 2006). The rat was found to be a less suitable model for corneal thickness measurements. For example, and unlike the rabbit, corneal thickness in untreated eyes showed a variation with time. Although the rat cornea showed an increase in corneal thickness following the topical application of an irritant material, the inherent variability in corneal thickness made interpretation of the findings more difficult.

Several studies have shown a good predictive correlation between corneal thickness increase and ocular irritant potential. Thus, Burton (1972) found that measured changes in corneal thickness agreed well with subjectively assessed eye irritation, and that persistent corneal injury was associated with a greater degree of corneal thickening; Conquet *et al*. (1977) and Morgan *et al*. (1987) reached similar conclusions. Jacobs and Martens (1989) also found that a good correlation existed between mean proportionate corneal swelling and corneal opacity and hyperaemia scores. Using ultrasonic pachymetry on gently restrained animals, Myers *et al*. (1998) demonstrated a good correlation between corneal thickness and macroscopically assessed ocular irritation, and confirmed that measurement of corneal thickness is a useful and sensitive objective method for assessment of eye irritating potential of chemicals.

Increases in corneal thickness are concentration related (Ballantyne *et al*., 1975; 1976), and can be measured at concentrations less than those causing macroscopic evidence of irritant effects on the cornea or conjunctiva (**Figure 4**). The method can also be used to detect corneal thickness changes by exposure to vapours (Ballantyne *et al*., 1976; Chun and Ballantyne, 1997). With minor irritants, increases in corneal thickness are small and the concentration–effect curve has a plateau around those concentrations just producing macroscopic evidence of eye injury; moderate eye irritants produce a concentration-related increase in corneal thickness above the irritant threshold, and severe irritants produce concentration-related linear increases in corneal thickness up to the macroscopically visible irritation concentration and then show an abrupt increase with a marked change in the slope of the concentration–effect curve (Ballantyne *et al*., 1975; **Figure 5**). Thus, the shape and slope of the concentration–effect curve can in many cases be used to predict the potential severity of eye irritation of a test material. Since increases in corneal thickness can be measured at concentrations significantly below those causing a macroscopic irritant effect, the procedure may be carried out with the minimum of discomfort to the test animal. Also, Martins *et al*. (1992) demonstrated a high sensitivity with good correlation and reproducibility between corneal thickness and clinical observations for irritancy, and they consider that the method may be used with low application volumes without loss of sensitivity. The technique of confocal microscopy through focussing (CMTF) was developed to permit quantitation of the depth and thickness of different tissue layers and structures of the cornea (Li *et al*., 1996; Maurer and Jester, 1999). In this technique automated rapid focussing through the cornea at high speed allows the generation of a z-axis profile, and allows a three-dimensional reconstruction of the corneal thickness. Using a series of surfactants, Maurer *et al*. (1997) applied the test materials to the rabbit eye and assessed epithelial cell size, epithelial layer thickness, total corneal thickness and depth of

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keratinocyte necrosis. They found significant differences in the area and depth of injury with different surfactants of varying irritancy. They noted that observations taken at three-hour postinstillation can be used to distinguish different degrees of ocular irritation. The method can also be applied to *in vitro* preparations and used to assess and quantitate human eye injury.

3.2.2 Measurement of Intraocular Pressure

An increase in IOP can result from a variety of causes, including obstruction of the aqueous outflow tract, increase in aqueous humour production, and an increase in aqueous humour solute concentration (Chan and Hayes, 1985). It is also known that when the eye is exposed to an irritant material there is an increase in IOP followed by a return of the pressure to preirritant values; the magnitude of the increase in IOP and its duration usually depend on the severity of the irritant response (Ballantyne *et al*., 1977; Walton and Heywood, 1978). IOP has been measured by cannulation of the anterior chamber, but this procedure has many disadvantages; these include the development of leaks, that general anaesthesia is necessary, it is not suitable for long-term measurements and it is invasive (Ballantyne *et al*., 1977). More convenient are tonometric methods, which involve either measuring the amount of deformation of the cornea produced by a standard force applied to the cornea (indentation tonometry) or measuring the force necessary to produce a standard degree of corneal flattening (applanation tonometry). Indentation tonometry has several disadvantages compared to applanation methods, which include:

1. The applied indenting force displaces fluid, stretching and increasing tension in the wall of the eye, and thus producing an artefactual increase in IOP.
2. Most indentation tonometers have been designed for human clinical use with the patient in the supine position. Thus when used with experimental animals there may be considerable handling and restraining problems to align the eye and tonometer, which may lead to compression of cervical blood vessels and release of catecholamines, both of which may artefactually increase IOP (Perkins, 1965).
3. Instruments generally have footplates averaged for the human eye, and this may lead to errors in reading with animal use.

In view of the above disadvantages, applanation is the preferred method for measurement of IOP in experimental animal situations. Pneumotonometry is now available as a noninvasive, readily conducted technique for the measurement of IOP, and is reliable and reproducible (Avilla *et al*., 2005). The rabbit is a suitable animal model because of the large amount of documented information available concerning the influence of topically applied irritant materials on IOP.

Table 7 Values for intraocular pressure (IOP) in various species

Species	IOP value (mm Hg)	Reference
Rhesus monkey	17.1 ± 3.0 SD[a]	Schiavo (1973)
Beagle dog	19.0 (range 10.0–31.0)	Heywood (1971)
New Zealand white rabbit		
Male	15.77 ± 1.28 SD	Ballantyne *et al*. (1977)
Female	16.00 ± 1.46 SD	Ballantyne *et al*. (1977)

[a]SD: standard deviation.

In order to permit the continual recording of changes in IOP, McLaren *et al*. (1995) described a telemetric method that used a fluid-filled catheter inserted into the anterior aqueous chamber through an opening near the limbus. The method was limited by the fact that the tip of the catheter rotates, producing corneal and iris irritation. Subsequently, Percicot *et al*. (1996) used a method based on implantation of a catheter into the posterior chamber to allow long-term measurements of IOP. The procedures involved implantation of a miniaturized pressure transducer radiotelemetry system into the cervical subcutaneous (sc) tissues, from which a cannula was tunnelled subcutaneously to the superior conjunctival sac and inserted behind the corneoscleral junction to a midvitreous position. They used the preparation to study IOP in ocular normotensive animals (Schnell *et al*., 1995) and also investigated the effect of antiglaucoma eye drops in the chronic ocular hypertensive rabbit model, induced by posterior chamber injection of α-chymotrypsin (Percicot *et al*., 1996).

Values for IOP are available for several species as shown in **Table 7**. A normal frequency distribution is observed with IOP measurements in various strains and species (e.g. **Figure 6**). A circadian rhythm has been described for IOP in the rabbit (Ballantyne *et al*., 1977) and the rat (Moore *et al*., 1995). It follows that in experimental situations and for comparative evaluations, treatments and measurements should be made at similar times in the different groups, including the controls.

Increases in IOP with a given material depend on the total dose applied to the eye; that is, the response will vary with both the volume applied to the eye and the concentration of active ingredient (**Figure 7**). It follows that for a comparative evaluation of IOP changes, the conditions should be similar with respect to dosing. Also, if materials are to be tested in solution, then solvents that do not produce an effect on IOP should be used; examples include saline, PEG 300, glyceryl

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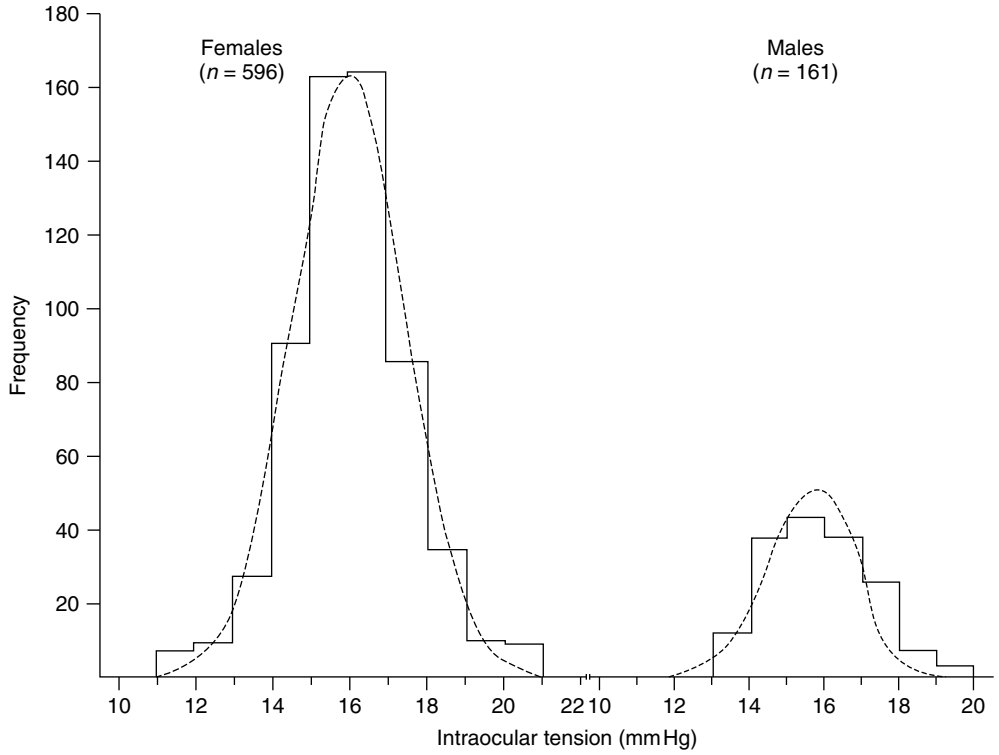

Figure 6 Frequency histograms to demonstrate the distribution of intraocular pressures in 596 female and 161 male New Zealand white rabbits. The broken line is the normal distribution with a mean ± SD of 16.00 ± 1.46 mm Hg for females and 15.77 ± 1.28 mm Hg for males.

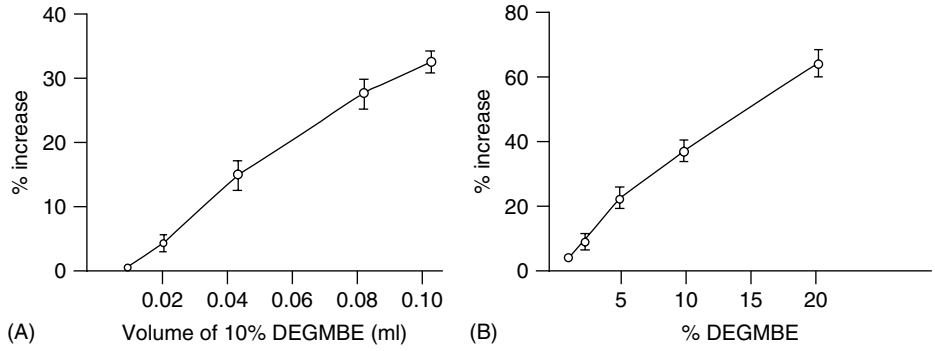

Figure 7 Increases in intraocular pressure (IOP), measured at 10 minute postapplication, produced by diethylene glycol monobutyl ether (DEGMBE). (A) Increase in IOP as a function of the volume of test material (10% DEGMBE) applied. (B) Increase in IOP following application of 0.1 ml of different concentrations of DEGMBE. In both instances the increases in IOP are dose dependent. (Data after Ballantyne, 1984.)

triacetate, tri(2-ethylhexyl) phosphate, corn oil and 1,1,1-trichloroethane (Ballantyne *et al*., 1972).

Increases in IOP are usually measurable within a few minutes of contaminating the eye with an irritant material. The time for IOP to return to control values depends on the concentrations of material applied, its irritant potential and the ability to penetrate the cornea to produce deeper structural and functional injury; times may vary from about an hour to several days. It follows that measurements should be made early after contaminating the eye and followed by sequential measurements. In general, the increase in IOP at a specific time after exposure is greater the more irritant the material (**Figure 8**). Also, as with corneal thickness measurements, mild to moderate irritant materials produce a linear increase in IOP with incremental increases in concentration, whereas with severely irritant materials at low concentrations there is a shallow dose–response curve which shows an abrupt change to a steep curve at higher concentrations (**Figure 9**). Thus, comparison of the magnitude of

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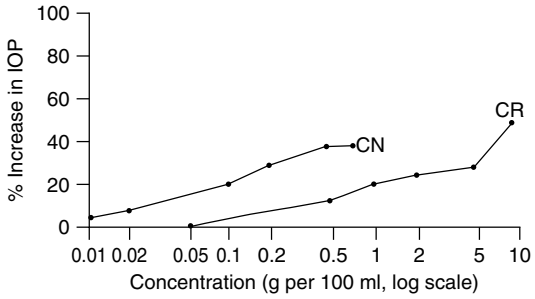

Figure 8 Proportionate increases in intraocular pressure (IOP) in the rabbit eye as a function of concentration from instilling 0.1 ml volumes of various concentrations of solutions of 1-chloroacetophenone (CN) or dibenz[b.f]-1,4-oxazepene (CR). The magnitude of increase in IOP is concentration-dependent, and for the more irritant CN, increases are measurable at lower concentrations than those for the less irritant CR.

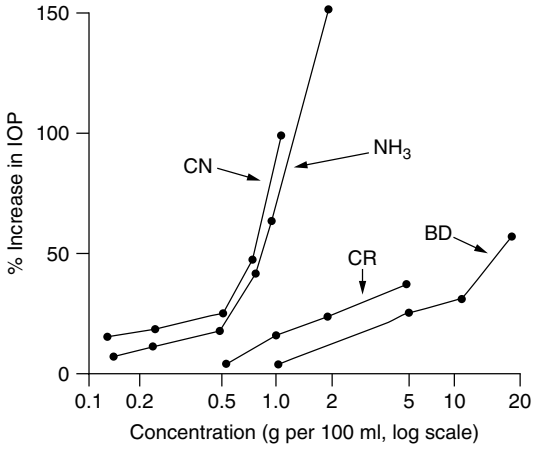

Figure 9 Peak increases in intraocular pressure (IOP) measured at about 10 minutes, as a function of concentration of material applied to the eye. Comparison of 1-chlororacetophnone (CN), aqueous ammonia (NH_3), diethylene glycol mono-*n*-butyl ether (BD), and dibenz[b.f]-1,4-oxazepine (CR). For BD and CR, increases in IOP begin to occur at higher concentrations than for CN and NH_3 and are essentially linear with concentration and have shallow slopes. With CN and NH_3, for which increases in IOP occur at lower concentrations, the initial part of the curve is shallow and then abruptly becomes steep; these two materials are severe irritants.

changes in IOP and the slopes on the dose–response curves can be used to predict the likely severity of irritation that will be produced by a given material.

3.2.3 Corneal Permeability Studies

Corneal permeability studies are sometimes used as an ancillary to standard eye irritation tests as discussed in Section 2.3.6.2, but with sensitive analytical methods the approach can be adopted as an alternative for the detection of early corneal injury, and thus for prediction of eye irritation. For example, Etter and Wildhaber (1984) described a method for use in conscious restrained mice based on measurement of fluorochrome penetration following brief topical contact with test material. As an extension, Maurice and Brooks (1995) described the use of a mouse corneal permeability procedure on the eyes of animals left *in situ* immediately following nitrous oxide euthanasia. Penetration was assessed using the fluorochrome sulforhodamine. The test involved applying a 'drop' of test material to the epithelium, which was washed off one minute later, and then a drop of fluorophore was applied to the epithelium for a further one minute. Measurement of fluorophore penetration was undertaken using a specially adapted fluorometer in which the proptosed eye was inserted into a 3 mm diameter circular opening in the equipment. They found the method to be reproducible and, in general, to give a good agreement with the known irritant potential of a wide range of materials. The investigators claimed the following advantages for the method: simple, rapid, gives an objective numerical assessment, economic, uses a biologically relevant tissue, and uses euthanized animals. Possible interpretive problems include that the process is limited to corneal epithelium and ignores deeper corneal structures, it measures only an immediate response, it is indirect, and it ignores the possible contribution of lacrimation. However, the authors consider that the approach may be of value for screening proposes.

3.3 *In Vitro* Isolated Ocular Tissues

3.3.1 Enucleated Eyes

A method was described by Burton *et al.* (1981) that involved the direct application of test substance to the cornea of rabbit eyes removed immediately after sacrifice of the animal, and subsequent evaluation of the cornea for swelling, opacity and fluorescein staining, followed by histological examination of the eye. The eye was mounted on a clamp in a temperature-controlled superfusion chamber with isotonic saline being dripped on to the corneal surface. After a 30–45 minute equilibration period, test material was applied to the cornea and then washed off. Eyes were subsequently inspected macroscopically, and then with a slit-lamp biomicroscope, corneal thickness was measured and fluorescein staining observed. Price and Andrews (1985) measured corneal thickness in enucleated rabbit eyes and found the method to be a reliable predictor of eye irritation potential. In a comparative study of the isolated ocular preparation in several laboratories, Koëter and Prinsen (1985) validated the test system against Draize eye results using 34 compounds. Whittle *et al.* (1992)

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found that the predictive value of the test and the consistency of results between different laboratories were good. A 10 second exposure allowed most irritant substances to be identified, and a 60 second exposure increased the corneal response and could therefore be useful for testing products of low irritancy. In a study conducted under the auspices of the Commission of the European Communities (CEC, also known as the European Commission, EC), involving 10 laboratories examining 21 reference compounds, it was found that there was consistency in the findings and across the various laboratories, and in general the results predicted the correct grades of the compounds (CEC, 1991). Prinsen and Koëter (1993) examined the suitability of the chicken eye in the enucleated eye test, because of the ready availability of tissues from chicken slaughterhouses. Using 21 compounds, and assessing injury by corneal thickness measurements, fluorescein retention and corneal opacity, they found the chicken eye results to correctly classify ocular irritation according to EC classification. Subsequent experience (Prinsen, 1996) with 44 test materials showed an excellent correlation with a standard *in vivo* rabbit eye irritation test (0.1 ml). Since nonirritant and severely irritant compounds were correctly identified, it was suggested that the chicken enucleated eye test could be useful as a screen for these two groups, which would not require further *in vivo* testing. Pérez-Rivera *et al*. (2008) also found that the chicken enucleated eye test correlated well with historically available animal *in vivo* and clinical eye irritation data.

3.3.2 Isolated Cornea and Corneal Epithelial Cell Lines

Gautheron *et al*. (1992) used the isolated cornea from bovine eyes. The preparation was held in place in a specially designed holder having compartments in front of and behind the mounted cornea, and incubated in a chamber. Corneal injury was assessed by opacity, and measured by light transmission and fluorescein permeability from the anterior to the posterior chamber of the corneal holder. The results demonstrated a good assessment of ocular irritating potential. Vanparys *et al*. (1993) tested 50 materials in a bovine corneal opacity–permeability preparation, and used opacity measurements and permeability to sodium fluorescein as the monitors. They obtained a 77% concordance using a four-level irritation classification (nonirritant, and mild/moderate/severe irritation), and a 95% correct classification using two categories (nonirritant and irritant). Because many alternative methods to *in vivo* eye irritation tests do not consider the question of reversibility, Gilotti *et al*. (2007; 2008) used a modified excised porcine cornea model to assess irritancy and its reversibility. Corneas in culture were treated one day after excision with undiluted ethanol, 3% sodium dodecyl sulfate (SDS), 1 N sodium hydroxide, or 1% benzalkonium chloride (BAC). The area of damage was visualized by brief fluorescein staining under UV light. With the exception of sodium hydroxide, the area of damage decreased over time, and histology demonstrated re-epithelialization. Decrease in fluorescein retention area correlated with time to recovery as determined by previously published Draize scores, and the investigators concluded that this approach may be of value since it has the potential to determine recovery after initial ocular injury.

Working on the basis that an irritant chemical may cause disruption of epithelial tight junctions, Xu *et al*. (2000) used bovine corneal culture models to assess epithelial responses to surfactants and for possible use for predicting ocular irritancy in humans. The approach was based on a belief that disruption of tight junctions could trigger cell stress-response modulated transcription factors such as AP-1 and NF-HB. They assessed the effects of SDS and BAC on corneal epithelial permeability and DNA-binding activity of AP-1 and NY-κB in cultured bovine corneas. Epithelial barrier integrity was assessed by a surface biotinylation method (Chen *et al*., 1997). Both SDS and BAC induced tight-junction disruption and increased permeability of corneal epithelium as assessed by surface biotinylation in a concentration and time-related manner. Increased DNA-binding activity was detected when corneas were treated with surfactants causing minimal to mild ocular irritation, indicating epithelial stress response. Also, exposure to SDS and BAC at concentrations causing severe ocular irritancy resulted in a decrease in DNA-binding activity of the transcription factors. These findings indicate that a combination of isolated corneal culture and measurements of corneal epithelial permeability and DNA-binding of stress response transcription factors following exposure to chemicals has the potential to be used as a mechanistically based *in vitro* isolated ocular tissue alternative for eye irritancy prediction.

Working with a series of six organosilicone compounds having different functional groups, Cassidy and Stanton (1997) obtained a correct classification using opacity and permeability measurements. They also found these monitors to accord with the histology of the cornea. Eurell *et al*. (1991), in preliminary work with cryomicrotome sections of rabbit and human corneas, used iso-electric focussing to assess effects on corneal protein profiles. They found that acetic acid and ammonia solutions produced a similar acid-base effect on a common rabbit and human corneal protein band, and thus aid in the extrapolation of findings. Kruszewski *et al*. (1995) described the use of a cell line grown from human corneal epithelium, which was grown as a three-dimensional culture on a collagen membrane. Effects on the preparation were measured using fluorescein retention. Using 12 chemicals they found a good

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correlation with Draize ratings and, supported by subsequent studies (Kruszewski *et al*., 1997). Schneider *et al*. (1997), described a complex *in vitro* corneal preparation prepared by establishing primary and subcultures of endothelial, stromal and epithelial cells from the foetal pig eye. A three-dimensional *in vitro* corneal model was established in culture plates by sequentially adding endothelial cells, stromal cells and epithelial cells. The cytotoxic response of the preparation was assessed by mitochondrial activity using an MITT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. Based on calculated E_{50} values (concentration causing 50% inhibition of mitochondrial capacity) they found a good correlation between E_{50} values and the Draize classification.

Using a rabbit corneal cell line (SIRC), Takahashi *et al*. (2008) developed an easy-to-perform short time exposure (STE) cytotoxicity test, with cell viability as the end point for irritation potential. Exposure time was five minutes, and when compared with Draize values there was good correlation. Comparing results from three laboratories, the investigators found the STE cytotoxicity test to have good reproducibility and transferability.

3.3.3 Isolated Lens

Using lenses cultured *in vitro,* several studies have shown a correlation between toxicity and lens optical function, and that with several materials there is a much higher sensitivity in detecting irritancy at lower concentrations than by the standard corneal irritation test (Bantseev *et al*., 2003; Sivak *et al*., 1992; 1994). This implies that the *in vitro* isolated cultured lens system can be used as a predictive and more sensitive indicator of eye irritation than the *in vivo* eye irritation test. On the basis that the lens is embryologically derived from the same source as corneal and conjunctival epithelium, Sivak *et al*. (1992) used an automatic scanning laser system to monitor spherical aberration and transmission of the lens in culture. Using five alcoholic test materials, the investigators considered that this method of measuring lens damage compared favourably with standard Draize scores for irritation. Bantseev *et al*. (2003) used the cultured bovine lens to investigate the effects of the surfactant SDS on lens optical properties by automated scanning laser system, and mitochondrial integrity as assessed by confocal microscopy. Loss of sharp focus was evident immediately following exposure, and loss of lens transparency, increased lens weight and increased axial length were seen at 24 hours postexposure. Confocal analysis at 24 hours postexposure showed a concentration-related increase in the numbers and lengths of mitochondria in lens epithelial and superficial cortical fibre cells. Thus, the results of this study demonstrated a correlation between lens optical properties and metabolic functions, and indicate this approach is a sensitive *in vitro* model of ocular chemical toxicity. The cultured bovine lens technique has been shown to be of value for assessing eye irritation potential of consumer products (Wong *et al*., 2003).

3.4 Isolated Nonocular Preparations

3.4.1 Chorioallantoic Membrane

The chorioallantoic membrane (CAM) of the hen's egg is a highly vascular tissue and has been used to determine the irritant response to substances as a predictor for eye irritation. It is a borderline *in vitro/in vivo* system and does not conflict with ethical and legal standards (Leupke, 1985). Basically the test involves the use of fresh fertile eggs, from which the shell is gently removed from around the air cell on day 10 of incubation. The vascular CAM is exposed by removing the inner egg membranes, and test substance is dripped on to the membrane. The blood vessels and albumin are subsequently scored for irritant effects (hyperaemia, haemorrhage, coagulation) at 0.5, 2 and 5 minutes post-treatment. Details of the methodology and scoring have been presented by Leupke (1985), who found a good correlation with Draize irritation scoring. Blein *et al*. (1991) found the method to be too sensitive with undiluted materials, but with a 10-fold dilution there was a reasonable correlation with *in vivo* eye irritation results, and under these conditions the method gave good discrimination between mild, moderate and severe irritants. Bagley *et al*. (1989) found that the CAM assay for surfactants correctly classified these materials, and they showed the method had a high sensitivity, specificity and predictive value.

Martins *et al*. (1992) used a combined approach of the excised bovine eye and CAM assay to assess the eye irritating potential of chemicals; corneal injury was assessed by opacity, fluorescein stain and epithelial detachment. The combined assay was developed to simulate both corneal injury (bovine eye) and mucosal response (CAM). They found a limited correlation using this dual approach, with fewer than 40% of the substances tested being in agreement with *in vivo* results. In contrast, Van Erp and Weterings (1990) found a good correlation between the combined assay and *in vivo* results. Problems that have been described with the CAM procedure have included variations in the application of the test material and the use of rinsing, which can damage the membrane. Gilleron *et al*. (1996) modified the procedure by using a test substance applicator and microscopic examination. With 46 compounds they obtained a sensitivity of 80% and a concordance with the Draize test of 80.4%.

3.4.2 Other Tissues

A good correlation was found between bovine erythrocyte haemolysis, blocking of ileal contractions, and *in vivo*

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Table 8 Examples of end-point measurements used in cytotoxicity assays as alternatives for eye irritation tests

Method	Basis/end point	Reference
Microphysiometry	Metabolic rate measurement	Bruner *et al*. (1991a; 1991b)
Uridine uptake inhibition	Membrane damage	Shopsis and Sathe (1984)
Neutral red uptake	General cytotoxicity	Borenfreund and Puerner (1987)
Neutral red release	Cell membrane injury	Rohde (1992)
Leucine incorporation	General cytotoxicity	Sina *et al*. (1992)
Total protein	General cytotoxicity	Riddell *et al*. (1986)
Fluorescein leakage	Cell membrane injury	Rohde (1992)
Colony-forming efficiency	Lethal cytotoxicity	North-Root *et al*. (1982)
MTT dye reduction	Mitochondrial damage	Sina *et al*. (1992)
Crystal violet staining	Lethal cytotoxicity	Itagaki *et al*. (1991)
Alkaline phosphatase release	Membrane injury	Scaife (1985)
Intracellular ATP	General metabolic toxicity	Kemp *et al*. (1985)

eye irritation by various surfactants (Muir *et al*., 1983). However, the biological basis of this approach makes its use for predicting eye irritating potential somewhat suspect.

3.5 Cytotoxicity Assays

Cytotoxicity assays measure loss of some cellular, or intercellular, structure and/or functions, including lethal cytotoxicity. They are generally simple to perform, reproducible and have clearly defined end points. In view of these considerations, several investigators have used cytotoxicity assays as the basis for the development of *in vitro* 'predictive' alternative approaches to eye irritation studies. However, predictability based on comparisons with *in vivo* standard eye irritation tests may be variable for a variety of reasons, including the fact that assay systems are continually exposed, whereas *in vivo* there are biological protective mechanisms in operation. Some assays may not be universally capable of detecting all chemical classes of irritants because of the end point used.

A variety of cell lines have been used, including corneal epithelial cells, lung fibroblasts, Chinese hamster ovary (CHO) cells, canine renal cells, HeLa cells, and microorganisms. In each case where nonocular cell lines have been employed, investigators have attempted to justify the reason for choosing the line used. For example, the use of canine renal cells was justified by Shaw *et al*. (1991) on the basis that the integrity of corneal epithelial cells depends on tight and desmosomal junctions, which are also seen in canine renal epithelial cells and form an impermeable barrier. They are thus considered appropriate to detect chemicals that may cause an increase in transepithelial permeability. These investigators used fluorescein leakage to assess injury to tight junctions, and neutral red release to detect renal membrane injury. A considerable number of differing end points have been used in cytotoxicity assays, some examples of which are listed in **Table 8**. A multiplicity of cytotoxicity assays have been described, and some illustrative examples are discussed below.

3.5.1 Crystal Violet Staining

Itagaki *et al*. (1991) employed a simple technique with lethal cytotoxicity as the end point, using cultured HeLa cells or SIRC cells (established line of rabbit corneal cells) in the presence of serial dilutions of the test material. After incubation, crystal violet (methyl violet) was used to stain residual viable cells. The IC_{50} was calculated: that is, concentration of test material inhibiting growth of cells by 50%. Using various surfactants they found a good correlation between the IC_{50} and the maximum *in vivo* eye irritation scores for the materials tested.

3.5.2 Silicon Microphysiometry

The silicon microphysiometer is a light-addressable sensor device, which can be used to measure the rate of acidic metabolite production from cells placed in a biosensor flow chamber. The end point calculated is the MRD_{50}; that is, the concentration of test material required to reduce the metabolic rate by 50% (Bruner *et al*., 1991b). Mouse fibroblasts have been used as the test cell. Bagley *et al*. (1992) found the MRD_{50} for a variety of materials to correlate with the maximum average score for *in vivo* eye irritation. Rougier *et al*. (1992), working with 41 commercial products, established MRD_{50} values in mouse fibroblasts and found a good correlation with Draize scores (r^2 = 0.81–0.91, depending on the class of the material). Catroux *et al*. (1993a; 1993b) also obtained a good correlation using surfactants and commercial products (r = 0.89). Bruner *et al*. (1991a; 1991b) also obtained excellent agreement between Draize rating and MRD_{50} for surfactants using human epidermal keratinocytes. This method appears to have good promise.

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3.5.3 Microtox Test

This test utilizes changes in luminescence from *Photobacterium phosphoreum*, which is generated through a process linked to respiration by reduced nicotinamide adenine dinucleotide (NADH) and flavin mononucleotide (Bulich, 1979). Light output is measured photometrically before and after addition of the test substance, and an EC_{50} value calculated; that is, concentration of test substance causing a 50% reduction in light emission. Bagley *et al*. (1992) found that, in general, test substances with the highest *in vivo* irritation gave the lowest EC_{50} values.

3.5.4 Plasminogen Activation

This assay uses the release of plasminogen activator from primary rabbit corneal epithelial cells as the quantitative index of toxicity. Some studies have shown a good correlation with known irritant potential (Bagley *et al*., 1994).

3.5.5 Neutral Red Uptake Assay

In this procedure cells, often mouse fibroblasts or CHO cells, are exposed to the test substance and then to neutral red (toluylene red). Retention of neutral red indicates cell viability. Bagley *et al*. (1992) found that, in general, the concentration of test material required to reduce neutral red uptake decreased as the *in vivo* irritant potential of the test material increased. Blein *et al*. (1991) found that correlations with materials of extreme pH were underestimated due to buffering in the culture medium, and volatile materials were also underestimated, probably because of loss of material.

Several intertest comparisons have been conducted. For example, Sina *et al*. (1992) compared leucine incorporation, MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) dye reduction and neutral red uptake in corneal epithelial cells and Chinese hamster lung fibroblasts. None of the end-point target cell combinations accurately predicted *in vivo* eye irritation in this series, but the MTT dye reduction method gave the overall best correlation. In a review, Christian and Diener (1996) noted that the neutral red assay appears to have merit for ranking potential ocular irritations, especially weak ones. The method is easily standardized, but interlaboratory variation occurs. Highly soluble simple substances appear to be more reliably predicted than complex pharmaceutical substances.

3.5.6 Comment on Cytotoxicity Assays

Cytotoxicity assays give an assessment of the effect of the test material on a particular aspect of cell or intercellular function or of lethal toxicity. They therefore give a measure of potential to cause cell and tissue injury, and as such may be used as a screen for predicting tissue injury, including eye injury. Appropriate choice of the test cell and end-point indicator for certain chemical classes may give a reasonable prediction of the potential for eye irritation. The choice of the cytotoxicity assay, or assays, should at least in part be determined by past experience, the likely mechanism for a positive response and the chemistry of the material tested.

3.6 Nonbiological Approaches

One example of nonbiologically-based approaches is the use of synthetic protein membranes, based on the fact that protein denaturation may be a contributory factor in corneal injury with several materials. The reactive component is a synthetic protein-globulin matrix. In one series of studies, the predictability of ocular irritation was 89% and in another 93% (Soto and Gordon, 1990).

3.7 Prediction by Analogy

3.7.1 Computer-Based Modelling

Structure–activity and structure–property analyses have been recommended for many years as possibly being predictive in ocular irritation, and used in the early stages of assessment procedures. The initial processes looked at chemical structure and class in terms of known Draize scoring, and used any correlations as a basis for predicting eye irritating potential with materials having the same chemical structure or class features. Following this simplistic approach, there have been several proposals that computer-based structure–activity relationships (SARs) could be used as a basis for predicting the eye irritating potential of chemicals. Quantitative structure–activity relationship (QSAR) analysis is a method used to relate the magnitude of a particular property (in this case ocular irritation) to one or several physicochemical and/or structural features of a molecule. The initial QSAR models to predict eye irritation were limited to a series of chemical analogues for which the dependent property (ocular irritation) was derived from a set of intramolecular descriptors based on an assumed common mechanism of action. Since eye irritation assessments are frequently sought for structurally diverse compounds, the standard QSAR approach is somewhat limited in utility in applications that estimate ocular irritation for diverse classes of chemicals. For example, Sugai *et al*. (1990) used a QSAR model to analyse the correlations between chemical structure and eye irritation in rabbits. They claimed 86.3% accuracy in classifying with respect to eye irritancy, and believe that the QSAR is of value in predicting irritating potential. Barratt (1997) described a QSAR for

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the eye irritating potential of neutral organic compounds based on the perturbation of ion transport across the cell membrane being related to dipole movements of the causative agent, the model parameters chosen were log P_{ow} (log octanol–water partition coefficient) and the internal axes R_y and R_x (representing the cross sectional area of the molecule). The results provided support for the validity of the QSAR model.

It has been suggested that a better predictive estimate of ocular irritation with chemically diverse materials is possible if the QSAR model includes receptors linked to the irritation response. Also, uptake and diffusion of an irritant into the corneal epithelium may be a significant related factor, and thus interactions between the irritant molecule and corneal epithelial cell membranes could be a determinant, and form the basis for a predictive approach known as membrane-interaction quantitative structure–activity relationship (MI-QSAR). Kulkarni *et al*. (2001) simulated the uptake and interaction of various solute molecules with a model phospholipid membrane. In these simulations the estimated membrane–solute interaction properties from the molecular simulations are added to the intramolecular physicochemical property descriptors. Their findings demonstrated that inclusion of parameters to model membrane interactions of potentially irritant chemicals provides a significantly better prediction of eye irritation for structurally diverse compounds than does modelling based solely on physicochemical properties of chemicals. The investigators noted that the MI-QSAR models can be used with high reliability to classify compounds of low and high predicted eye irritation. However, the value of a MI-QSAR model depends on the number, nature and reliance of the QSAR descriptors. Thus, in a study by Li *et al*. (2005), 13 structurally diverse drugs of known *in vivo* eye irritation potential were chosen for comparison in a MI-QSAR analysis for investigating the relationship between corneal permeability and ocular irritation. The investigators found no meaningful relationship between *in vivo* eye irritation potency and corneal permeability, with an R^2 of 0.0232 between predicted values of log P_{cornea} versus known *in vivo* eye irritation potency. It was suggested that the difference in findings between their study and that of Kulkarni *et al*. (2001) was, in part, due to differences in the two QSAR models, with no descriptor related to reactivity potency in the Li *et al*. (2005) model.

Although the SAR approach could be a useful adjunct with chemicals in a class of known irritancy, there still exits the possibility for litigation based on a product safety assessment consisting solely of QSAR analysis.

3.7.2 Prediction Based on Skin Irritation Analogy

It has been suggested that materials shown to be primary skin irritants should be regarded as being eye irritants, and therefore do not require to be tested for ocular irritation *per se*. Whilst such an approach is overcautious in that some skin irritants have been shown not to be irritant to the eye, the converse is of more concern since materials might be mistakenly classified as nonirritant to the eye. Several studies have shown that there may not be a good correlation between skin- and eye-irritating potential, and that some eye irritants are not skin irritants (Dalbey *et al*., 1993; Rhodes, 1987). Kennedy and Banerjee (1992) studied 72 fibre finish materials and found a 10.5% error in predicting that a severe skin irritant was a severe eye irritant, and commented on a poor predictive association between skin and eye irritancy for the compounds they investigated. Williams (1984) reviewed 60 materials that were severe irritants or corrosive to the skin; of these 36 were severe eye irritants, 6 moderate eye irritants and 18 mild or nonirritant. He concluded that to suggest materials are eye irritants on the basis of their skin irritating potential could be misleading. There is thus no simple relation between skin and ocular irritancy, and in several cases it may be misleading to attempt to predict one from the other (Daston and Freeberg, 1991).

3.8 General Review Comments on Alternative Tests

Currently, no single alternative test or combination of them can completely and unequivocally predict the eye irritating potential of all test substances. Indeed it has been suggested that expectations for a uniform test should be discouraged (Reinhardt, 1990). However, with an appropriate tiered scheme there may be significant reductions to *in vivo* eye irritation testing. Approaches to such schemes have been proposed (Jackson and Rutty, 1985; Reinhardt, 1990), and they should follow a sequential approach to include:

1. Consideration of the physical and chemical characteristics of the material being tested, along with comparison of materials in the same chemical class whose *in vivo* eye irritating potential are known.
2. Use of appropriate cytotoxicity studies, based on known predictivity for specific chemical classes.
3. Confirmation of potential for corneal injury using *in vitro* enucleated eye and/or corneal preparations.
4. In critical cases, such as high volume chemicals or therapeutic ophthalmic preparations, ultimate confirmation may be necessary by *in vivo* studies with objective evaluations.

On the basis of a comparison of several alternative methods with 37 materials, Sina *et al*. (1995) concluded that screening for chemicals with diverse structure and properties is best conducted using mechanistically based

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as opposed to cytotoxic assays. In a review of alternatives, Christian and Diener (1996) came to the following conclusions:

1. None of the methodologies has been accepted for regulatory classification purposes.
2. No single *in vitro* assay is considered capable of replacing the conventional *in vivo* eye irritation test.
3. The greatest potential methods (at that time) for screening were the hen's egg CAM test, the chicken or bovine enucleated eye test, neutral red or plasminogen activation assays for cytotoxicity, and the silicon microphysiometer.
4. Choosing a specific test method(s) will depend on the material to be tested.

In a review article, Hutak and Jacaruso (1996) also suggested that no single test system will supplant animal testing. The method to be chosen will depend on the physical and chemical characteristics of the material to be tested and the sensitivity and specificity of the method. The continuing comparative evaluation of animal eye irritation tests against alternative methods and approaches will refine an acceptable alternative method or 'test battery'.

4 PERIPHERAL CHEMOSENSORY EYE IRRITATION

Chemical stimulation of corneal and conjunctival sensory nerve receptors will cause local pain or discomfort and the associated reflexes of increased lacrimation and blepharospasm. These effects are biologically protective in that they give warning of exposure to a potentially noxious material and provide some degree of protection against further exposure. However, pain, excess lacrimation and blepharospasm also impair vision and are distracting; they therefore predispose to physical accidents and impede efficient working conditions. Thus considerations on peripheral sensory effects on the eye can be important, for example, in assessing workplace safety, and may need to be taken into account in assigning workplace exposure guidelines (Kuwabara *et al*., 2007). Also, from a product safety viewpoint, it is important to know if there is an adequate margin (of safety) between the exposure conditions causing sensory irritation of the eye and those leading to eye injury. It is relevant to know that some materials may give little, or no sensory warning on contact with the eye, for example bis(2-chloroethyl)sulfate, dimethyl sulfoxide and methyl bromide. Clearly with such chemicals there is a need to emphasize the requirement for protective and precautionary measures.

Objective aspects of the sensory irritant response, for example blepharospasm, can be investigated in experimental animals. However, subjective responses (e.g. discomfort and pain) require carefully controlled studies in human volunteer subjects. Peripheral chemosensory irritation in general, and of the eye in particular, is discussed in detail in **Peripheral Chemosensory Irritation: Fundamentals, Investigation and Applied Considerations**; see also Ballantyne (2006b).

5 THE EYE AS A TARGET ORGAN FOR SYSTEMIC TOXICITY

5.1 General Considerations

Many materials, including drugs and industrial chemicals, can produce structural and/or functional injury following systemic distribution of the material and/or its metabolites after absorption from routes of exposure remote to the eye. Some illustrative examples are listed in **Table 9**. As shown in this table, a diversity of toxicopathological effects can be produced at different anatomical sites in the eye. With some substances there are highly site-specific effects, and with others a series of sites in and related to the eye may be affected by the same chemical. With multiple site systemic ocular toxicity, the combination and severity of lesions varies with different specific chemicals and with different chemical classes. Additionally, the same lesion complex may be produced by a multiplicity of differing absorbed chemicals. A good example of this is seen in some drug treatments in the human leading to the Stevens–Johnson syndrome, first described in 1922, in which there is severe bullous erythema multiforme with mucosal involvement of the mouth, eyes, gastrointestinal tract, lower respiratory tract, vagina and urethra (French, 2006; Schofield and Rees, 2006). Most authorities consider that the Stevens–Johnson syndrome and toxic epidermal necrolysis are different manifestations of the same disease (Parrillo and Parrillo, 2008). Ocular involvement usually occurs in the form of acute and late ocular manifestations and complications (Yip *et al*., 2007), and is present in 43–81% of patients treated, depending on the nature of the therapy and the particular study (Robin and Dugel, 1988). The ocular manifestations are characterized by an acute inflammatory reaction, which may result in scarring and obstruction of the nasolacrimal ductules and loss of goblet cells, leading to a later complication of the 'dry eye' syndrome and trichiatic lashes (Yip *et al*., 2007). There may be corneal opacification, with vascularization, keratinization and attenuation. Also reported are anterior uveitis, scleritis and conjunctival cicatrization (De Rogas *et al*., 2007; Parrillo and Parrillo, 2008). The Stevens–Johnson

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Table 9 Examples of chemicals and drugs producing systemic toxicity to the eye

Chemical	Effect
Methanol	Retinopathy Retrobulbar optic neuropathy
Butyl 2-chloroethyl sulfide	Retinopathy
Imipramine	Retinopathy
Chloroquine	Retinopathy Corneal opacities
Methylhydrazine	Corneal endothelial injury
Chlorpropamide	Corneal opacities Lens opacities
Methylphenidate	Cataract Glaucoma
Dimethyl sulfoxide	Corneal opacity
Methylphenyltetrahydropyridine	Retinopathy
Oxygen	Retrolental fibroplasia
Methotrexate	Blepharoconjunctivitis Optic neuropathy
Corticosteroids	Cataract Glaucoma Visual field defects
Chlorpromazine	Keratopathy Cataract Pigmentary retinopathy
Ethambutol	Optic neuropathy
Aconitine	Myelo-optic neuropathy
Amiodarone	Keratopathy Lens opacities Optic neuropathy
Penicillamine	Ptosis and diplopia
Tamoxifen	Corneal deposits Retinopathy
Disulfiram	Optic atrophy
Metronidazole	Optic neuritis
Retinoids	Blepharoconjunctivitis Optic disc hyperaemia

syndrome is an immune-complex-mediated hypersensitivity condition with various known aetiologies which include drugs, infections (viral, bacterial and fungal) and malignancies. Over 200 drugs have been reported to produce the Stevens–Johnson syndrome, including sulfonamides, penicillins, ampicillin, isoniazid, carbamazepine, anticoagulants, salicylates, antiretrovirals, phenylbutazone and cocaine (Cac *et al*., 2007; Chia and Leong, 2007; Fraunfelder, 1989; Hofbauer *et al*., 2000; Metry *et al*., 2001; Mockenhaupt *et al*., 2005; Power *et al*., 1995; Roujeau *et al*., 1995; Tilden *et al*., 1991).

With any specific chemical, the potential to produce ocular injury, its site(s) and its severity, may have markedly differing conditions for its genesis. Major factors determining the potential of absorbed chemicals, and metabolites, to induce systemic toxicity to the eye are as follows:

1. Total absorbed dose and its determinants, notably the frequency, duration and magnitude of the environmental exposure dose.
2. The route of exposure, depending in part on whether the parent molecule or its metabolite(s) is responsible for the ocular injury.
3. The biotransforming capacity of the eye. Watkins *et al*. (1991) reviewed the xenobiotic transforming capacity of ocular tissues and experimentally extended the biochemical database. The role of ocular metabolism in the mechanism of ocular toxicity from xenobiotics is, in general, poorly understood but clearly is a factor to be considered in all cases of ocular toxicity.

It is clear from the above discussion that observations for systemic injury to the eye are required in general toxicological studies, particularly, though not exclusively, for materials that are to be used medicinally, as food additives, and chemicals with a potential for exposure domestically and occupationally. The necessity to undertake, at least, routine examination of the eye in general toxicology studies has been stressed by Barnett and Noel (1969). Systemic ocular effects can often be suspected in general toxicology studies, providing that the protocol allows for examination of the eye. Investigations into specific aspects of ocular injury may require that satellite or additional studies be conducted with appropriate detailed monitoring procedures (Peiffer *et al*., 2000). Depending on the lesion(s) produced, monitoring may include, for example: slit-lamp biomicroscopy, histology and cytohistochemistry, transmission and scanning microscopy, angiography, tonometry, pachymetry, electroretinography, visual evoked potentials (VEPs) and electro-oculography. Electroretinography is a valuable functional supplement to histopathology in investigations of retinal pathology (Maertins *et al*., 1993). Also, isolated tissue preparations have been employed, for example the retina and lens (Chiou, 1992). Specific ocular lesions may require the use of a range of investigational techniques. For example, studies of lenticular toxicity have included the use of various forms of microscopy, biochemical investigations and metabolic studies. Morphological and biochemical changes may be followed sequentially by magnetic resonance imaging and spectroscopy (Schliech *et al*., 1985), tryptophan fluorescence spectroscopy (Lerman and Moran, 1988) and scanning lens monitoring (Mitton *et al*., 1990). Lens biochemical changes that have been investigated include aldose reductase, sorbitol dehydrogenase, phosphofructokinase, glutathione reductase, glutathione (GSH), NADH, NADPH (reduced nicotinamide adenine dinucleotide phosphate), calcium, cholesterol, phospholipids and protein (Chiou, 1992).

In some instances, laboratory experimental studies may not be able to detect certain subjective aspects of systemic ocular toxicity, and these can only be

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discovered on detailed clinical examination of exposed humans. For example, using this approach it has been shown that colour recognition and discrimination is affected by exposure to a number of chemicals, especially to industrial organic solvents. Thus, workers exposed to styrene vapour may develop dyschromatopsia (Gobba *et al*., 1991; Iregren *et al*., 2005; Triebig *et al*., 2001), and styrene vapour concentrations of the order of 25 ppm can lead to impairment of colour vision (Fallas *et al*., 1992). It was concluded by Triebig *et al*. (2001) that styrene-induced colour vision dysfunction is reversible after an exposure-free interval of four weeks. With styrene, the current US threshold limit value (TLV), TWA_8 (time-weighted average concentration for an 8-hour workday, and a 40-hour workweek) of 20 ppm with short-term exposure limit (STEL) of 40 ppm (ACGIH, 2007) may not give adequate protection against adverse effects of styrene vapour on colour vision. There is clinical evidence that acrylamide monomer may cause slightly reduced light sensitivity and reduced colour discrimination (Mapp *et al*., 1977; Goffeng *et al*., 2008). Perchloroethylene vapour exposure also leads to colour vision deficits (Cavalleri *et al*., 1994). Defective colour discrimination, mainly blue-yellow, was found in 12 of 15 workers exposed to *n*-hexane by Raitta *et al*. (1978), who also examined carbon disulfide-exposed viscose workers and found impaired colour discrimination occurred more often in the workers than in nonexposed controls (Raitta *et al*., 1981). The adverse effects on colour vision resulting from exposure to *n*-hexane were confirmed by Chang (1990), and to carbon disulfide by Vanhoorne *et al*. (1996), who also described the presence of retinal microaneurysms. Although there is clinical evidence for toluene producing a neurotoxic effect on visual pathways by high concentration vapour exposures (Keane, 1978; Toyonaga *et al*., 1989), the evidence for this occurring at lower concentrations is equivocal (Muttray *et al*., 1995; 1999), suggesting that there is a threshold for the effect of toluene on the visual system (Schäper *et al*., 2004). This should be of the order of the lowest reported concentrations with significant differences between exposed individuals and nonexposed control; these values are 8.4 and 35.8 ppm, respectively, by Campagna *et al*. (2001) and 42 ppm by Cavalleri *et al*. (2000). At a then current exposure concentration of 26 $\pm$ 21 SD ppm, and after pre-exposures of about 43 ppm for 22 years, Schäper *et al*. (2004) found no effects of toluene on colour vision. These findings suggest that the current US TLV at a TWA_8 of 50 ppm provides sufficient protection against possible disturbance of colour vision (ACGIH, 2007). It has also been demonstrated that occupational exposures to multiple volatile solvents sufficient to cause the condition of chronic solvent encephalopathy also results in significantly impaired colour discrimination in the presence of otherwise normal visual function, suggesting that the effect may be mediated via higher level visual processing, possibly colour selective regions of the cerebral cortex (Päällysaho *et al*., 2007). The possible relationship between exposures to organic solvents and impairment of colour vision has been reviewed in detail by Gobba and Cavalleri (2003), Iregren *et al*. (2002), Lomax *et al*. (2004) and Paramei *et al*. (2004).

5.2 Examples of Substances Producing Systemic Toxic Effects on the Eye

The following examples of substances producing systemic ocular toxicity demonstrate the wide range of chemical substances that can produce such effects, and the wide spectrum of ocular pathology that can be induced.

5.2.1 Antineoplastic Agents

Several cancer chemotherapeutic agents have been reported to adversely affect ocular structure and function. Thus, a high proportion of patients on carmustine develop retinopathological effects including pigment abnormality, infarction, periarteritis and macular oedema (Chiou, 1992; Kupersmith *et al*., 1992; Shingleton *et al*., 1982). High doses of tamoxifen produce retinopathy with refractile opacities in the macular and perimacular areas (Gerner, 1989; Bourla *et al*., 2007), keratopathy (Vinding and Nielsen, 1983), corneal subepithelial opacities, cataract (Inada *et al*., 2005) and colour vision loss (Salomão *et al*., 2007). Long-term low dose tamoxifen use (20 mg day^{-1}) was associated with corneal depositions in three-quarters of the patients (Muftuoglu *et al*., 2006; Paganini-Hill and Clark, 2000). It has been reported that the keratopathy of tamoxifen is reversible, appears to be dose related, and is attributed to the cationic and amphiphilic nature of the molecule (Dua *et al*., 1996). Like other retinotoxic drugs, such as chloroquine and imipramine, tamoxifen is amphiphilic. It has been postulated that amphiphilic materials bind with polar lipids, causing abnormal metabolism of such lipids and a resultant accumulation in lysosomes (Lullman *et al*., 1975); that is, drug-induced phospholipidosis. Once within lysosomes, the materials, and metabolites, bind to cellular lipids and form complexes, leading to the accumulation of lysosomal inclusions, eventually observed as depositions. Retinal histopathology shows lesions in the neuroretina (nerve fibre layer and inner plexiform layer), with the paramacular region being particularly susceptible, possibly because of its highly vascularized proximity (Kaiser-Kupfer *et al*., 1981). In general, the incidence of retinopathy is low (Tang *et al*., 1998). 5-Fluorouracil has been reported to cause photophobia, excess lacrimation, ocular irritation, blepharoconjunctivitis, keratitis, punctual-canalicular

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stenosis and cicatricial ectropion (Caravella *et al*., 1981; Eiseman *et al*., 2003). Vincristine has produced ptosis and diplopia due to cranial nerve paresis (Vizel and Oster, 1982). Ocular complications of systemic cancer chemotherapy are not uncommon, affecting various components of the eyeball, optic nerve and extraocular muscles, and have been discussed in several reviews (Fraunfelder and Meyer, 1983; Imperia *et al*., 1989; Al-Tweigeri *et al*., 1996).

5.2.2 Ethambutol

Ethambutol, an antitubercular drug (Innes and Reid, 2006), produces an optic nerve toxicity that may be reversible in some patients, although the possibility for optic neuropathy reversibility is controversial (Chan and Kwok, 2006). The possibility that the lesion is irreversible is suggested by a study from Tsai and Lee (1997). They reviewed 10 consecutive cases with severe visual defects due to ethambutol, and in which the drug had been discontinued. Only five patients experienced improvement of their vision after one to three years of follow-up, with older patients having a poorer prognosis. The majority of patients had received a dosage in the range of 13–18 mg kg^{-1}, and therefore the authors questioned the existence of a 'safe dosage', which is likely to be small. They also questioned the use of ethambutol as a routine first line of defence for the treatment of tuberculosis. On the basis mainly of the ocular toxicity of ethambutol, Sivakumaran *et al*. (1998) had recommended that the daily dose should be 15 mg kg^{-1} (rather than 25 mg kg^{-1}) and that the drug should be discontinued if optic neuropathy develops. That reversibility is variable was suggested by the observations of Goyal *et al*. (2003), who noted ocular toxicity in 10% of the patients they examined and, when it occurred, visual recovery was in the first six to eight weeks after stopping ethambutol treatment; visual recovery was complete in only one patient, and partial in two as judged by visual fields, contrast sensitivity, and VEPs remaining abnormal. That ethambutol ophthalmic toxicity affects retinal elements as well as the optic nerve was shown by multifocal electroretinogram (ERG) studies by Kardon *et al*. (2006) and Behbehani *et al*. (2005a). This is supported by the clinical and electrophysiological findings of Vistamehr *et al*. (2007), who also demonstrated that ethambutol toxicity to the eye is expressed as a neuroretinopathy. Using ERG, electro-oculogram (EOG) and visual field testing, they detected retinal pigment epithelial changes, macular oedema and retinal haemorrhages. ERG findings included decreased amplitude and abnormal wave pattern. In patients with ethambutol-induced optic neuropathy it is possible to obtain some assessment of the degree of optic nerve damage just after the onset of visual loss by measuring a decrease in retinal nerve fibre layer thickness using optical coherence tomography (Chai and Foroozan, 2007). Clinically, there is evidence that in a few cases of ethambutol-induced optic neuropathy it may subsequently progress to involve the optic chiasma, causing a bitemporal hemianopia (Lim, 2006).

Early onset toxicity with ethambutol has been suggested to be due to an idiosyncratic reaction (Karnik *et al*., 1985; Kahana, 1987). Delayed onset optic neuropathy has been associated with zinc depletion (Chatterjee *et al*., 1986; Schild and Fox, 1991). In rat, studies on the mechanism of ethambutol retinal toxicity by Heng *et al*. (1999) found specific lesions to retinal ganglion cells *in vitro* and *iv vivo*, and that endogenous glutamate is required for full expression of ethambutol toxicity, and also that glutamate antagonists prevented ethambutol-mediated cell loss. The drug caused a decrease in cytosolic calcium and an increase in mitochondrial calcium, with an increase in mitochondrial membrane potential. The investigators suggested that the visual loss associated with ethambutol may be mediated through an excitatory pathway, in that ganglion cells are rendered sensitive to normally tolerated levels of extracellular glutamate. Also, they noted that ethambutol perturbs mitochondrial function, and its toxicity may be associated with decreased ATPase activity and mitochondrial energy homeostasis. They suggested that glutamate antagonists might be of use to limit the side effects seen with ethambutol. Yoon *et al*. (2000) used rat primary retinal cultures to study the mechanism of ethambutol-induced ocular toxicity, and obtained findings at variance to some of those noted above. Exposure of cultures to ethambutol for 24–48 hours induced cytoplasmic vacuolar changes and neuronal loss, with the vacuolar changes being partially reversible on termination of ethambutol exposure. Thy-1(+) neurones were more vulnerable than γ-aminobutyric acid (GABA) neurones. Glutamate antagonists, an antioxidant (trolox), or cycloheximide, did not attenuate either the vacuolar changes or neurone loss. A cell-permeant zinc chelater, TPEN (*N*, *N*, *N′*, *N′*-tetrakis(2-pyridylmethyl)-ethylenediamine), markedly attenuated vacuolar degeneration and neuronal loss, whilst the addition of zinc augmented both of these effects. Thus, in this study ethambutol-induced retinopathy was mediated not by excitatory processes or zinc deficiency, but by a mechanism requiring intracellular zinc. Additionally, features of the ethambutol-induced cytolethality were not consistent with those of apoptosis.

A few cases of Leber's hereditary optic neuropathy have been reported which were apparently precipitated by ethambutol, leading to a proposal that the drug could be a risk factor for this optic neuropathy (Ikeda *et al*., 2006). Also it has been reported that the primary mitochondrial DNA mutation at the nucleotide (nt) site nt-11778 in Leber's hereditary optic neuropathy has been shown to be present in patients with ethambutol-induced optic neuropathy. As a follow-up to this association, Hwang *et al*. (2003) studied patients with ethambutol-induced

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optic neuropathy for the presence of mitochondrial DNA mutation at nt-11778, nt-14484, nt-3460, nt-15257, nt-9438 and nt-9804. None of the patients were found to have any of these mutations, thus excluding an association between ethambutol-induced optic neuropathy and Leber's hereditary optic neuropathy mutations.

Ethambutol optic neuropathy has been reviewed by Fraunfelder *et al*. (2006).

5.2.3 Amiodarone

Amiodarone, a di-iodinated benzofuran used in the treatment of certain cardiac arrhythmias, produces intracytoplasmic lamellar deposits in the cornea and lens, and more rarely the retina and optic nerve (Ikäheimo *et al*., 2002; Ingram *et al*., 1982; Mäntyjärvi *et al*., 1998). Common ocular symptoms in patients on amiodarone include coloured haloes around lights and visual blurring. Some regard these ocular complications as not presenting a significant threat to vision (Ingram, 1983), although as discussed below other authorities take a more cautious approach. Frequent ocular signs are 'cat whisker' corneal epithelial opacities (verticillata) and lens opacities, often without impairment of visual acuity (Mäntyjärvi *et al*., 1998; Nielsen *et al*., 1983). Ingram (1983) investigated the occurrence of complications of long-term therapy, and found that keratopathy developed in 98% of patients, but only 6% of the affected individuals developed visual symptoms (photophobia, haloes and visual blurring). Uçakhan *et al*. (2005) found that corneal deposits were detectable using slit-lamp biomicroscopy and confocal microscopy by two months after the onset of amiodarone treatment, and deposition was correlated with duration and cumulative dose. Unilateral optic disc oedema, presenting with blurred vision, has been reported in a patient on amiodarone (Eryilmaz *et al*., 2000), as have bilateral optic nerve swelling with variable reduction in visual acuity or visual fields (Garrett *et al*., 1988; Johnson *et al*., 2004; Nagra *et al*., 2003; Nazarian and Jay, 1988). In one series of case descriptions (Johnson *et al*., 2004), most instances of optic neuropathy commenced within 12 months of starting amiodarone treatment, with a median onset of four months. Optic neuropathy is detected in only a small proportion of amiodarone-treated patients, and is reversible in about half of patients after discontinuing treatment (Turdamambetova *et al*., 2005). In a clinical study by Domingues *et al*. (2004), involving measurement of VEPs, there was a statistically significant difference ($p < 0.001$) between amiodarone-treated patients and age-matched controls with respect to pattern visual evoked potential latency (L-VEP) and amplitude of visual evoked potential (a-VEP). However, there was no significant correlation between prolonged L-VEP and duration of amiodarone ($r = 0.07$). After a one-year follow-up there was an average increment in L-VEP of 1.96 ms and a-VEP measurements showed a decline of 0.69 μV. In a group of patients having moderately high dosages of amiodarone (600–800 mg day^{-1} for up to 18 months), lens opacities were found in half of the patients; these opacities were punctate anterior subcapsular and within the papillary aperture (Flack *et al*., 1983). Confocal microscopic examination of the cornea in patients with amiodarone-induced keratopathy showed the presence of highly reflective, bright intracellular inclusions in the epithelium, particularly in the basal cell layers. With advanced keratopathy, bright microdots were seen within the anterior and posterior stroma and on the endothelial cell layer, with an irregular appearance to the stromal nerve fibres (Ciancaglini *et al*., 2001; Uçakhan *et al*., 2005). Ultrastructural examination of corneal and conjunctival biopsies from patients with amiodarone keratopathy demonstrated lipid deposits in lysosome-like intracytoplasmic inclusions in the epithelium, conjunctival fibroblasts, and vascular endothelium (D'Amico *et al*., 1981). It was proposed that the changes were a consequence of the cationic amphiphilic properties of amiodarone, which induces a generalized intracytoplasmic accumulation of polar lipids. A more widespread distribution of intracytoplasmic membrane-bound bodies, similar to myelin, was described by Ghosh and McCulloch (1984); they were present in corneal epithelium and endothelium, conjunctiva, lens, iris, ciliary body, choroid and retina.

5.2.4 Aconitine

Aconitine is the major constituent of aconite, a herbal remedy used in the Far East. Kim *et al*. (1991) showed that the material causes a myelo-optic neuropathy in rabbits. Principal findings were a delay to onset and peak latency of VEP, with reduction in amplitude, and histopathological evidence of myelin injury of the visual pathway, spinal cord and peripheral nerves. Retinal and optic nerve effects of aconitine given systemically (intraperitoneal (ip)) were studied in rabbits by means of VEP, ERG and electron microscopy (Kim and Kim, 1990). VEP amplitude was decreased, and optic nerve ultrastructure showed disorganization of the lamellar structure of the myelin sheath, diffuse mitochondrial swelling and vacuolization of myelinated nerve fibres. There were no significant ultrastructural changes in the retina.

5.2.5 Ethanol

Ingestion of ethanol can result in a reduced IOP (Grant, 1969; Peczon and Grant, 1965), and also affects extraocular muscle activity. Acute ethanolic intoxication results in a variety of visual disturbances, including nystagmus, transient convergent strabismus, diplopia, esophoria, and possibly transient change in colour vision (Bittencourt and Wade, 1980; Hogan and Linfield, 1983; Wilson and Mitchell, 1983). Pursuit movements of the eye

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are markedly affected by ethanol, with saccadic movements being slowed within 30 to 60 minutes of ethanol ingestion; smooth following movements are impaired and replaced by jerky movements, thereby reducing visual acuity through temporary loss of macular vision (Wilkinson *et al*., 1974). It has been reported that 50 ml of ethanol as a minimum is required to induce nystagmus (Howells, 1956). Colour vision tests on normal subjects after consumption of ethanol have shown a specific effect on blue-sensitive cones (Zrenner and Riedel, 1988). Acute administration of ethanol also can also affect the rate of recovery from exposure to bright lights (Adams and Brown, 1978; Sekuler and MacArthur, 1977). Acute dosing with ethanol has been shown to modify the ERG in rabbits, primates and humans (Knave *et al*., 1974; Textorious *et al*., 1985; Van Norren and Padmos, 1977). An effect on the c-wave of the ERG suggested that there was involvement of the retinal pigment epithelial cells (Knave *et al*., 1974; Skoog *et al*., 1975). Ethanol appears to retard dark adaptation of retinal cone cells (Van Norren and Padmos, 1977). A study of the effects of ethanol on functional magnetic resonance imaging (fMRI) during a visual perception test was conducted by Calhoun *et al*. (2004). Ethanol caused a dose-dependant decrease in activation amplitude over much of the visual perception network, and the anterior and posterior cingulate, precuneus and middle frontal areas. There was a dose-dependent activation in the insula, dorsolateral prefrontal cortex and precentral regions.

In rat studies, peroral (po) dosing with 1% ethanol for eight weeks produced an optic neuropathy (Taneda *et al*., 1988). In humans, chronic ethanolic intake can cause a toxic amblyopia that may be related to vitamin B complex deficiency (Grant and Schuman, 1993). Characteristically, there is bilateral onset of visual clouding, with a papillomacular distribution that is horizontal; the peripheral field usually remain normal (Grant and Schuman, 1993). Disturbance of colour vision has been reported in chronic alcoholism, sometimes with undisturbed visual acuity, and tending to disappear on abstinence from ethanol (Francq *et al*., 1979; Swinson, 1972). Pattern visual evoked potential (PVEP) recordings were conducted in chronic alcoholics by Nazliel *et al*. (2007), who found that detection of PVEP in asymptomatic chronic alcoholics might be useful for the early detection of central nervous system (CNS) changes and for follow-up progress of patients.

Foetal alcohol syndrome is characterized by prenatal and postnatal growth delay, craniofacial abnormalities, hypotonia, CNS deficits, and there is particular concern that ocular malformations may occur in the children of mothers who abuse alcohol during pregnancy. Up to 90% of children suffering from foetal alcohol syndrome have eye abnormalities (Hinzpeter *et al*., 1992), with two malformations being typical: hypoplasia of the optic nerve and increased tortuosity of retinal arteries (Chan *et al*., 1991; Ribeiro *et al*., 2007; Strömland, 1985; Strömland, 1987; Strömland and Pinazo-Duran, 2002). Also common are shortness of the palpebral fissures, ptosis, epicanthus, blepharoptosis, microphthalmus, coloboma, and convergent or divergent strabismus (Grant and Schuman, 1993; Hinzpeter *et al*., 1992; Ribeiro *et al*., 2007; Strömland, 2004a; 2004b). Glaucoma has resulted from abnormalities of the anterior chamber angle (Martin and Rabineau, 1990). Strömland (1987) has suggested that the ocular effects have shown a development timing indicating the risk to be present from early gestation to complete development. Ocular complications of foetal alcohol syndrome have been reviewed by Grant and Schuman (1993) and Irvine (2003).

5.2.6 Drugs of Abuse

There is a significant incidence of ocular complications in drug abusers (Gastaud *et al*., 1989; McLane and Carroll, 1986; Urey, 1991). For example, marijuana causes conjunctival hyperaemia, blepharospasm, diplopia and decreased IOP (Colasanti *et al*., 1984; Green, 1982; Green and McDonald, 1987; Hepler *et al*., 1972). Aerosolysed crack cocaine has been associated with corneal ulceration (Ghosheh *et al*., 2007; Pilon and Scheiffle, 2006; Sachs *et al*., 1993). Bilateral retinal artery occlusion has been reported following smoking of crack cocaine, possibly due to abrupt intense vasospasm (Catenacci and Tuckler, 2005; Wallace *et al*., 1992). Causes of noninfectious retinal artery complications following the use of illicit drugs given intravenously (iv) have been discussed by Raspiller *et al*. (2005) under two major mechanisms, namely microparticle embolization and arterial vasospasm. Embolization of microparticles causing obliteration of retinal vessels results from the presence of bulking filler materials such as talc (magnesium silicate) and flour, or from tablets that have been crushed and injected after partial dilution and filtration; tablets that have been associated with talc retinopathies include methylphenidate, pentazocine, codeine, methadone and meperidine. Following iv injection there is embolization to the pulmonary circulation, and particles $<5\,\mu m$ are not retained in the lung and may further embolize. Arterial vasospasm can affect retinal arteries directly or the cerebral circulation with indirect effects on the retina due to emboli resulting from disruption of atheromatous plaques; active ingredients responsible for vasospasm include cocaine, amphetamines and quinine. Peripheral retinal neovascularization has been described following iv drug abuse, including heroin and cocaine (Tran and Ilsen, 2007).

5.2.7 Butyl 2-Chloroethyl Sulfide

In the rat, systemically absorbed butyl 2-chloroethyl sulfide causes a swelling of the inner retinal layers, mitochondrial swelling and disarray of the outer segments of photoreceptors. Radiolabelled material is found in

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the retina, choroid, sclera, lens and cornea. Structural effects are preceded by an increase in thiobarbituric acid reactive products, which is an indicator of lipid peroxidation (Klein *et al*., 1991). Systemic exposure to the material produces metabolic and histological changes consistent with oxidative stress and lipid peroxidation.

5.2.8 Sildenafil Citrate

Sildenafil citrate (Viagra) is used in the treatment of male impotence based on its major inhibitory effect on cyclic guanosine monophosphate (c-GMP)-specific phosphodiesterase (PDE) 5 in the corpus cavernosus and subsequent facilitation of penile erection. The drug also exerts a lesser inhibitory effect on retinal PDE 6, which controls the level of c-GMP in the retina (Laties and Fraunfelder, 1999; Marmor and Kessier, 1999). Histological studies by Vatansever *et al*. (2003) using male Wistar rats dosed with sildenafil citrate (8 mg kg^{-1} day^{-1}, po) on alternate days over four weeks, showed that this treatment caused vascular dilation and congestion in the choroid; 3.44 $\pm$ 1.68 µm versus a control value of 1.78 $\pm$ 1.36 µm ($p < 0.001$). The retina showed no histopathology. There is a general opinion that sildenafil does not affect visual acuity, visual fields or contrast sensitivity (Laties and Sharlip, 2006). However, some men using sildenafil have experienced bluish haze vision, increased light sensitivity and blurry vision (Laties and Sharlip, 2006; Marmor and Kessier, 1999). Transient, mild impairment of colour discrimination can occur at the time of peak plasma concentration (Laties and Sharlip, 2006). Clinical observations have shown several other, but much rarer, side effects associated with the use of sildenafil; these include nonarteritic anterior ischaemic optic neuropathy developing within minutes to hours after ingestion (Akash *et al*., 2005; Bella *et al*., 2006; Carter, 2007; Cunningham and Smith, 2001; Pomeranz *et al*., 2002) and serous macular detachment with perimacular vitelliform deposits (Quiram *et al*., 2005). Relevant to the use of sildenafil by men with established glaucoma is a study by Grunwald *et al*. (2001), who demonstrated that in patients with chronic open-angle glaucoma given a 100 mg dose of the drug, there was no change in IOP. In healthy human adult volunteer subjects it was found that a 50 mg dose of sildenafil caused a significant increase in retinal arterial and venous diameters 30 minutes postdosing (5.8%, $p < 0.001$), with diameters returning to control values within 120 minutes (Pache *et al*., 2002).

5.2.9 Chloroquine Salts

Chloroquine derivatives are used as antimalarials and have been employed in the treatment of rheumatoid arthritis and lupus erythematosus (Jamshidzadeh *et al*., 2007; Sweetman, 2005). Chronic administration of chloroquine can produce fine corneal deposits that are visible in the corneal epithelium on biomicroscope examination, particularly in the region of the palpebral fissure, but are generally of little functional significance and resolve on cessation of treatment or reducing the dosage (Grant and Schuman, 1993). Although these deposits do not usually cause any visual disturbances, occasionally they may result in a slightly reduced visual acuity. Ultrastructural studies have shown the cytoplasmic inclusions in the epithelial cells to be similar to those produced by amiodarone and other cationic amphiphilic drugs (Section 5.2.3). Chloroquine keratopathy has been classified as a drug-induced phospholipidosis (Pulhorn and Thiel, 1976). More significant is that long-term dosing with chloroquine can result in the appearance of a retinopathy that, with high dosages, can seriously interfere with vision; this is discussed in more detail at Section 7.7.4.

5.2.10 Chlorpromazine

A phenothiazine drug, chlorpromazine like many other antipsychotic drugs can cause lens and corneal opacities with long-term treatment (Rasmussen *et al*., 1976; Sharpe and Potts, 2006). Confocal microstructural analysis of the cornea following a 20-year period of chlorpromazine therapy (mean daily dosage 400 mg day^{-1}), revealed irregular hyper-reflective deposits on the posterior surface of the endothelium, which ranged from 1 to 70 µm in diameter; otherwise the endothelium was normal (Phua *et al*., 2005).

5.2.11 Corticosteroids

It is well known that corticosteroids when applied topically to the eye can result in ocular hypertension and secondary open angle glaucoma (Skuta and Morgan, 1996). Increased IOP by topical application has been recorded for cortisone, hydrocortisone (Goldman, 1962), prednisone, prednisolone (Frandsen, 1964), β-methasone (Becker and Mills, 1963), dexamethasone (Armaly, 1963), triamcinolone (Becker and Mills, 1963), tetrahydrotriamcinolone and flurandrenolide (Brubaker and Halpin, 1975). It is now appreciated that in addition to such ocular effects being produced by local application of corticosteroids to the eye, they can also be produced with several corticosteroids by a systemic toxic effect to the eye following absorption by dosing at sites remote from the eye (Carnahan and Goldstein, 2000). Thus, ocular complications occur with corticosteroids when given by the oral route and they can also occur, probably to a lesser extent, when steroids are delivered by the inhalation but not nasal route. The use of inhaled glucosteroids has significantly improved the management of asthma (Vergnenègre *et al*., 2008), and intranasal steroids have been an important route of dosing for the management of rhinitis

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(Mabry, 1992). Other routes of exposure and absorption to corticosteroids that have been associated with ocular hypertension and open-angle glaucoma include oral and periocular skin and topical application to the face (Skuta and Morgan, 1996).

Inhaled and topically applied steroids may be absorbed through the nasal mucosa, oropharynx and lung, and by these routes have limited hepatic first-pass metabolism. Increased concern has been expressed over possible systemic adverse effects resulting from the use of inhaled and topically applied nasal corticosteroids. Case reports have indicated that the systemic absorption of steroids may result in development of posterior subcapsular cataracts, ocular hypertension and open-angle glaucoma (Dreyer, 1993; McGhee *et al*., 2002; Opatowsky *et al*., 1995). In a case-controlled study, Garbe *et al*. (1997) found that prolonged (greater than three months) dosing with inhaled steroids increased the risk of ocular hypertension or open-angle glaucoma. They did not observe an increased risk with topical nasal steroids, possibly because of the lower dosage by this route. Recommended dosages for intranasal administration do not exceed $400\,\mu g\ day^{-1}$, whereas inhaled steroids are recommended in doses of $1500-2000\,\mu g\ day^{-1}$ (Brogden *et al*., 1992). Several other studies have assessed the likelihood for development of ocular complications by various routes and modes according to age. There is good evidence that those using oral corticosteroids are at increased risk for the development of cataract (Derby and Maier, 2000). The use of inhaled steroids by children with asthma is not a major concern for the development of cataract or ocular hypertension (Behbehani *et al*., 2005b). For individuals older than 40 years, the risk of cataract development from the use of inhaled steroids was increased (Jick *et al*., 2001). In another study it was also found that the use of inhaled steroids by adults older than 49 years was associated with an increased risk for posterior subcapsular cataract development (Cumming *et al*., 1997). The risk of cataract in those using intranasal steroids is generally considered not to be increased (Derby and Maier, 2000). Differential absorption of various steroids appears to be a less important determinant since, for example, with flunisolide the systemic bioavailability is 39% of the administered dose by inhalation and 49% following intranasal dosing (Pakes *et al*., 1980). Garbe *et al*. (1997) suggest that those receiving inhaled steroids should be monitored by IOP measurements. Although there is still discussion about the possible mechanism by which corticosteroids increase IOP, it is generally considered that there is increased resistance to aqueous humour outflow (Becker and Mills, 1963; Kass and Johnson, 1989). It has been proposed that corticosteroids inhibit glycosaminoglycan metabolism, which leads to the accumulation of polymerized glycosaminoglycans in the trabecular network (Kass and Johnson, 1989).

Cumming *et al*. (1997) described an association between the use of inhaled steroids and the development of posterior subcapsular and nuclear cataracts, and have described the implications with respect to prevention and management (Cumming and Mitchell, 1999). Steroid-induced posterior subcapsular cataracts are associated only with steroids possessing glucocorticoid activity (James, 2007). In a self-reported population study with 2446 responders having long-term glucocorticoid use ($\geq$60 days) the incidence of cataracts was 15% (Curtis *et al*., 2006).

5.2.12 Organophosphate Anticholinesterases: Saku Disease

Attention was drawn to the potentially hazardous effect on the human eye from uncontrolled exposure of humans to pesticides, including organophosphate (OP) antiChE materials, by observations in the 1950s and 1960s, notably in children, that there was a probable correlation between a broad range of ocular complications and exposure to OP pesticides sprayed in fields in the Saku region of Japan. Under such circumstances the routes of exposure were probably multiple, inducing topical eye contact, inhalation, and possibly, though to lesser extents, percutaneous and peroral doses following swallowing of contaminated food and/or saliva. The range of adverse effects for Saku disease included blurring of vision, progressive myopia and astigmatism. Blood cholinesterase (ChE) inhibition, altered ERG, optic nerve atrophy, optic disc oedema and retinal degenerative changes (Dementi, 1994; Ishikawa, 1972; 1978; Ishikawa and Ohto, 1972; Ohto, 1974). Schoolchildren from Saku had a 65% incidence of optic neuritis and/or chorioretinal atrophy (Ishikawa *et al*., 1971). The OP insecticides applied near the regions of reported cases included dichlorvos (2,2-dichlorovinyl dimethyl phosphate (DDVP)), diazinon (*O*,*O*-diethyl *O*-2-isopropyl-6-methylpyrimidin-4-yl phosphorothioate), *O*-ethyl-*O*-*p*-nitrophenyl thiophosphonate (EPN), fenitrothion (*O*,*O*-dimethyl *O*-4-nitro-*m*-tolyl phosphorothioate), fenthion (*O*,*O*-dimethyl *O*-4-methylthio-*m*-tolyl phosphorothioate), malathion (diethyl (dimethoxyphosphinothioylthio)succinate), parathion (*O*,*O*-diethyl-*O*-4-nitrophenyl phosphorothionate), parathion-methyl (*O*,*O*-dimethyl *O*-4-nitrophenyl phosphorothioate)- and trichlorfon (dimethyl (*RS*)-2,2,2-trichloro-1-hydroxyethylphosphonate). Similar large studies on the potential for antiChEs to produce oculotoxic effects by occupational and environmental exposure have been documented from outside Japan; see Section 7.7.4. Also, several experimental studies with OPs accord with much of the ocular toxicity reported, and several epidemiological studies on the affected populations did establish a reasonable probability of an association between an increased incidence of adverse ocular effects and the greater use of OPs in Japan (Dementi, 1994; Ishikawa, 1973; Ishikawa and Miyata, 1980; Tamura and Mitsui, 1975). There also exists the

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possibility that there is an increased susceptibility of Oriental populations to antiChE-induced ocular adverse effects.

5.3 Developmental Ocular Toxicity by Maternal Exposure

A specialized aspect of ocular systemic toxicity is the investigation of developmental defects that may occur during gestation as a consequence of maternal exposure, which may result in foetal exposure to the absorbed molecule or its metabolite(s). One known example with humans, foetal alcohol syndrome, has been mentioned above (Section 53.5.2.5). An experimentally produced dramatic example of ophthalmic teratogenesis in the rat foetus is provided by nickel carbonyl. Sunderman *et al*. (1975) demonstrated that exposure to the vapour of nickel carbonyl for 15 min day^{-1} on gestational day (GD) 7 or 8 was sufficient to produce ocular abnormalities, including anophthalmia or microphthalmia, with a low incidence of extraocular abnormalities. Since nickel carbonyl can cross the alveolar membrane and blood–brain barrier, they considered it could also cross the maternofoetal barriers. However, ocular teratogenesis may be species dependent. Thus, in a developmental toxicity study in Syrian hamsters with nickel carbonyl, Sunderman *et al*. (1980) dosed pregnant animals (0.06 mg Ni l^{-1} 15 min^{-1}) on GD 4, 5, 6, 7 or 8, sacrificed them on GD 15, and found evidence for nickel carbonyl being teratogenic and embryotoxic, but with only a low incidence of ocular abnormalities. Exposure on GD 4 or 5 resulted in cystic lungs and exencephaly, but only one instance of anophthalmia; exposure on GD 6 or 7 caused a low incidence of fused ribs and hydronephrosis. Ocular teratogenesis has also been demonstrated in *Xenopus laevis* embryos exposed to a medium containing nickel chloride (180 µmol l^{-1}); the predominant lesion was loss of pigmented epithelial cells with secondary agenesis of the outer segments of photoreceptors and focal growth of neuroretina through gaps in the destroyed pigmented epithelium. Coloboma and cataract were also noted (Hauptman *et al*., 1991).

Cocaine use by pregnant women is known to be associated with a variety of structural and functional abnormalities in the offspring; these include perinatal mortality, craniofacial abnormalities, brain infarction, motor inability, seizures, hyperactivity, hydronephrosis and delay of growth (Chasnoff *et al*., 1986; 1988; Bingol *et al*., 1987; Dow-Edwards, 1991). Additionally, a wide spectrum of effects has been noted with cocaine, both experimentally and in human cases; these have included optic hypoplasia, anophthalmia, coloboma, retinal dysgenesis and retinal gliosis (Mahalik *et al*., 1980; Ferriero *et al*., 1989; Silva-Araujo *et al*., 1994). In an experimental study involving prenatal exposure of rats to cocaine (Mendez-Armenta *et al*., 1997), detailed histopathological observations in the retina were described, which included interstitial oedema, necrosis, hyperchromatic ganglion cells, reduced numbers of retinal cells, and alterations in the photoreceptor and bipolar cell layers. They concluded that cocaine produced alterations in the development and organization of neuroblasts in all layers of the retina.

For systemically absorbed substances, and with respect to the genesis of lens abnormalities, access of material to the lens depends on whether exposure is during the prenatal or postnatal period. Prenatally, the developing lens has a blood supply, which begins to degenerate on the first postpartum day, and is complete before adulthood is reached. The adult lens has no local blood supply and thus access of cataractogenic substances to the lens is via the aqueous humour or, to a lesser degree, through the vitreous humour. Cataractogenesis can be induced *in utero* by a serotonin antagonist, RG 12195 (Lerman *et al*., 1995), but the adult lens is resistant to the cataractogenic effect. RG 12195 is a selective 5-HT_3 antagonist that had been proposed for use in the management of nausea and emesis associated with cancer chemotherapy. Pregnant rats were given the material by gavage over GD 6–17, at dosages of 0 (control group), 1, 10 and 100 mg kg^{-1} day^{-1}. Postnatally, lens opacities were detected in the pups of the high dosage group. A repeat study conducted with dosages of 0, 10, 30, 60 and 100 mg kg^{-1} day^{-1} showed a dosage-related incidence of nuclear cataracts in the 60 and 100 mg kg^{-1} day^{-1} groups (respective litter incidences were 6 and 45%). In a subsequent perinatal/postnatal developmental toxicity study, RG 12195 was dosed from GD 15 to postpartum day 21, at dosages of 5, 20 and 80 mg kg^{-1} day^{-1}; lens opacities occurred in the high dose group. Since the common treatment period for all these groups was GD 15–17, and because the major organogenesis period for the lens is GD 11–15, it was suggested that the sensitive period for cataract induction by RG 12195 in the rat might have been close to GD 15. In the rat, lens capsule formation begins on GD 18, and the blood supply to the lens starts to degenerate on postpartum day 1 and is complete by 21 days. It is of interest to note that the serotonin agonist lysergic acid diethylamide (LDS) is also cataractogenic *in utero* (Hanaway, 1969), and that serotonin itself has been reported to produce reversible lens opacities in rats following acute ip dosing (Dietze and Tilgner, 1973). Additionally, *p*-chlorophenylalanine, a 5-hydroxytryptamine (5-HT) biosynthesis inhibitor, induced cataracts in weanling rats (Gralla and Rubin, 1970), and low tryptophan diets induce cataracts in weanling rats (Bunce *et al*., 1978). Other materials known to produce cataracts *in utero* include the chlorinated hydrocarbon insecticide, mirex (Rogers and Grabowski, 1983), azothioprene (Tuchmann-Duplessis and Mercier-Parot, 1964) and phenytoin (Wagner *et al*., 1983).

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Evidence for teratogenic effects, including ocular developmental abnormalities, based on epidemiological studies requires care with interpretation. For example, public concern followed media reports (Paduano *et al*., 1993) of alleged clusters of anophthalmia and microphthalmia that might have been linked with exposure to the fungicide benomyl (methyl 1-(butylcarbamoyl) benzimidazol-2-yl carbamate). Developmental studies in rats had shown that benomyl can induce anophthalmia and microphthalmia (Hoogenboom *et al*., 1991), but apparently only at doses approximately 1000 times greater than that expected to occur with farm workers handling the material (Mariman, 1998). Subsequent to the allegation of clusters of ophthalmic abnormalities, Dolk *et al*. (1998) investigated the geographical variation in anophthalmia and microphthalmia in England over the period 1988–1994. They found an overall prevalence of anophthalmia and microphthalmia of 1.0 per 10 000 live births. Regional variations in prevalence did not reach statistical significance. However, prevalence was higher by about two-fold in rural compared with urban areas. Whilst they did not specifically state that there was no association between benomyl exposure and ocular developmental defects, they state that there is very little evidence to support the presence of strongly localized environmental exposures of causing clusters of children to be born with anophthalmia or microphthalmia. In an Italian ecological study (Spagnolo *et al*., 1994), there was no evidence for an association between the regional use of benomyl and the prevalence of anophthalmia or microphthalmia. Also, a Norwegian study (Kristensen and Irgens, 1994), using a cohort of farming families, found only one case of anophthalmia/microphthalmia from the potentially exposed families. Official statements have recorded that low dose exposures to benomyl do not constitute a risk to humans (Ministry of Agriculture, Fisheries and Food, 1992; WHO, 1993).

6 THE EYE AS A PORTAL FOR SYSTEMIC TOXICITY (TRANSOCULAR TOXICITY)

6.1 General Comments

Most materials do not, or are unlikely to, produce systemic toxicity by topical application to the eye (Brown and Muir, 1971), principally because of the small volume that can be accommodated on the surface of the eye and in the conjunctival sac, even following deliberate instillation. Additionally, lacrimation and blepharospasm may reduce the degree of contamination and dosing. However, with materials of high toxicity or pharmacological potency, topical contamination of the eye with a sufficient material may result in enough material being absorbed to produce systemic effects. This may be a consequence of accidental (unintended) contamination of the eye with xenobiotics, and sometimes as a result of the deliberate introduction of drugs onto the eye for intended local therapeutic effects; examples of chemicals producing systemic effects by either accidental or deliberate ocular contact are given in Section 6.3. In addition to systemic toxic and unwanted pharmacological effects, such as those discussed as examples in Section 6.3, that may result from transocular absorption of xenobiotics, the deliberate use of the transocular route as a means to deliver dugs for systemic distribution has received therapeutic interest; this is further discussed in Section 6.4. Some sources for exposure having a potential for systemic toxicity may be unexpected or unusual. For example, Nir *et al*. (1992) investigated Kohl, an eye cosmetic used in Israel, which is frequently applied to infants. They found the lead content of samples of Kohl varied from 17.3 to 79.5%; blood concentrations of lead were high in infants that had been exposed to Kohl (11.2 vs. 4.3 $\mu g\ dl^{-1}$ for controls; $p < 0.001$).

In some instances, the potential for absorption of a material following topical application to the eye can be quantitatively assessed by identification of the parent compound and/or metabolites in body fluids. For example, with tropicamide (*N*-ethyl-3-hydroxy-2-phenyl-*N*-(pyridin-4-ylmethyl) propanamide), which is an anticholinergic muscarinic antagonist producing transient mydriasis, adverse effects have not been frequently reported but a myasthenia gravis-like syndrome has been described following topical application to the eye (Meyer *et al*., 1992). Plasma concentrations of tropicamide, and muscarinic receptor occupancy, have been investigated following the ocular administration of two 40 µl drops of 0.5% tropicamide into the inferior conjunctival sac of eight women (Vuori *et al*., 1994). The material was rapidly absorbed, with a measured peak plasma concentration of 2.8 ± 1.7 SD ng ml^{-1} at 5 minutes postinstillation. There was rapid plasma clearance, with the tropicamide concentration at 60 and 120 minutes being 0.46 ± 0.51 ng ml^{-1} and <240 pg ml^{-1}, respectively. There was a maximum tropicamide plasma receptor occupancy of around 8%. The authors stated that the low affinity of tropicamide for muscarinic receptors, and the negligible receptor occupancy in plasma, explains the low incidence of side effects following topical application of tropicamide eye drops.

6.2 Sites of Absorption

Materials contaminating the eye will come into contact with conjunctival blood vessels, then pass via the nasolacrimal drainage system into the nasal cavity, and thence through the naso-pharynx and be swallowed. The

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relative contribution of the conjunctivae, nasal mucosa and alimentary tract to the total absorbed dose may vary with the chemical species and formulation.

Absorption can occur through the conjunctival blood vessels, and this may be facilitated if materials also produce a conjunctival hyperaemia. Absorption from this site is also influenced by the rate of lacrimation, blepharospasm, and patency of the nasolacrimal drainage system. In many cases the nasal mucosa, with its large surface area and high vascularity, may be a major site of absorption of materials applied to the eye. It is well known that many pharmacologically active materials can be absorbed following introduction into the nasal cavity (Chien, 1985). For drugs having high systemic pharmacological activity when given by nasal dosing then this may offer a convenient route for dosing, and is discussed in more detail in Section 6.4. Hydrophobicity may be an important determinant for nasal mucosal absorption; for example, the hydrophobic drugs alprenolol and propranolol are well absorbed through the nasal mucosa (Duchateau *et al*., 1986; Hussain *et al*., 1980) whereas hydrophilic metaprolol is not (Duchateau *et al*., 1986). The rate of drainage of material from the conjunctival sac into the nasal cavity depends on a number of factors that include the rates of lacrimation and blinking, both of which may be increased by irritant materials. Also, the volume of material instilled into the sac will be a determinant of the amount transferred. Lacrimation and instilled fluid dynamics in the rabbit eye in relation to drainage from the eye have been investigated by Chrai *et al*. (1973). Since the drainage capacity of the eye exceeds the normal rate of lacrimation, drops placed in the conjunctival sac may be rapidly transferred to the nasal cavity (Shell, 1982). Reducing the rate of drainage may decrease systemic absorption. For example, Zimmerman *et al*. (1984) studied the effects of eyelid closure and manual nasolacrimal occlusion on the absorption of topically applied timolol and on the permeation of the eye to topically applied fluorescein. They found, with both eyelid and nasolacrimal occlusion, that the plasma concentration of timolol was significantly reduced and the anterior chamber fluorescein increased in comparison with ordinary eye drop procedures. For timolol, Chang and Lee (1987) found that the nasal mucosa was about 2.5 times more effective than the conjunctival mucosa in contributing to the total systemic absorption. That materials applied topically to the eye may reach the alimentary tract and be absorbed there is indicated by several studies. Thus, Wilensky *et al*. (1967) found that after ecothiophate iodide and isofluorophate were instilled into the eye, the greatest inhibition of ChE outside the eye was in the intestine. Also, in humans, antiChEs applied to the eye produced symptoms of intestinal distress (Humphreys and Holmes, 1963). The systemic absorption of epinephrine (adrenaline) and dipiveprin applied topically to the eye was studied by Anderson (1980), who found that a high concentration of material was present in the wall of the intestine and in the faeces. She suggested that the alimentary tract is not only a major elimination route, but also probably a major absorption pathway.

6.3 Examples of Substances Producing Systemic Toxicity by Transocular Absorption

6.3.1 General Comments

Whilst the potential for industrial and domestic chemicals to produce systemic toxicity following topical contamination of the eye is well known, the main human experience comes from the use of ophthalmic preparations deliberately applied to the eye. Some examples of differing types of materials are given to illustrate the variability in systemic toxicity that may be produced by transocular absorption of topically applied materials of high biological activity. They draw attention to the need for detailed monitoring of toxicological studies with materials that may be deliberately applied to the eye, or which may accidentally contaminate the eye. For such materials there is a need for precautionary statements to be developed.

6.3.2 Cyanides

Low MW cyanides can readily diffuse across biological membranes and systemic toxicity may occur by their instillation into the conjunctival sac. So potent are they by this route of exposure that they can produce mortality from which it is possible to calculate LD_{50} (LD—lethal dose) values by instillation into the conjunctival sac. **Table 10** compares the acute lethal toxicity of HCN and NaCN by instillation into the conjunctival sac of female rabbits with other routes of exposure in this species. It demonstrates that by the transocular route, at least in laboratory mammals, these cyanides are lethally potent, with HCN being as lethally toxic by conjunctival instillation as it is when given by iv injection, and more toxic than by the peroral and percutaneous routes.

6.3.3 Timolol

L-Timolol is a nonselective β-adrenoceptor antagonist used in the treatment of glaucoma, and usually available in preparations containing 0.25–0.5%. The use of timolol eye drops has been associated with a variety of potentially serious adverse effects to various body systems (**Table 11**). It has been shown that the oxidation of timolol exhibits genetic polymorphism, and those with low metabolism have higher plasma timolol concentrations and intensified β-blockade (Huupponen *et al*., 1991). That systemic absorption occurs following ocular instillation of timolol has been shown in several studies.

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Table 10 Acute lethal toxicity of hydrogen cyanide (HCN) and sodium cyanide (NaCN) by different routes of dosing to female rabbits[a]

Route	HCN		NaCN	
	LD_{50} (95% CL)[b] (mg kg^{-1})	Time to death (min)[c]	LD_{50} (95% CL)[b] (mg kg^{-1})	Time to death (min)[c]
Transocular	1.04 (0.96–1.13)	4–6	5.06 (4.4–6.10)	4–10
Intravenous	0.59 (0.55–0.65)	2.5–5.5	1.23 (1.11–1.34)	2–12
Intraperitoneal	1.95 (1.60–2.60)	6–9	2.79 (2.48–3.09)	5–26
Peroral	2.56 (2.14–3.05)	30–50	5.11 (4.62–5.66)	13–26
Percutaneous	6.89 (6.43–7.57)	25–260 h	14.62 (13.75–15.35)	1.75–5.75 h

[a]Data from Ballantyne (1983) and Ballantyne 1987.
[b]CL: Confidence limits.
[c]Percutaneous times in hours.

Table 11 Systemic adverse effects associated with the topical application of timolol to the eye and its transocular absorption

System	Effect
Cardiovascular	Arrhythmias
	Bradycardia
	Cardiac failure
	Hypotension
	Raynaud's phenomenon
Pulmonary	Asthma (exacerbation)
	Bronchospasm
CNS	Confusion
	Depression
	Dizziness
	Hallucinations
	Aggravation of myasthenia gravis
Gastrointestinal	Diarrhoea
	Nausea
	Vomiting
Genital	Impotence
	Decreased libido
Skin	Alopecia
	Urticaria
	Maculopapular rash
Metabolic	Hyperkalaemia
	Alerted diabetic hyperglycaemic response

Thus, Kaila *et al*. (1985) instilled 20 µl of 0.5% timolol into the conjunctival sac of six volunteers, and found that at 8 min postinstillation all but one subject had plasma timolol concentrations of >25 pg ml^{-1}. They also found that nasolacrimal occlusion reduced, but did not eliminate, systemic absorption of the drug (Kaila *et al*., 1980). Chang and Lee (1987) found that 75% of a 25 µl dose appeared in the blood steam of rabbits, and that the nasal mucosa was more effective than the conjunctivae in contributing to systemic absorption of timolol. Recommendations have been proposed to reduce the incidence of adverse effects from topical ocular application of timolol; these have included reduced medication, eyelid closure, and nasolacrimal occlusion (Fraunfelder and Meyer, 1987). Improved systemic safety has been shown by using a timolol hydrogel (0.1%) compared with an aqueous solution of timolol (0.5%); peak heart rate, head-up tilt test results and plasma concentrations of timolol were significantly lower with the hydrogel formulation (Uusitalo *et al*., 2006). The absorption of ocular topical timolol appears to be dependant on time of day, with the absorption at noon in the rabbit being about twice that measured at 6 a.m., 6 p.m. or 12 p.m. (Ohdo *et al*., 1991). Using a γ-scintographic method to monitor changes in precorneal solution retention, Lee *et al*. (1996) examined the basis for the time dependence. They found that precorneal solution drainage was slowest at noon, but that, in contrast, there was no statistically significant time-of-dosing influence on systemic timolol absorption following intranasal dosing. They concluded that changes in precorneal solution drainage might be the major factor underlying the diurnal incremental changes in ocular and systemic timolol absorption in the rabbit.

6.3.4 Sulfonamides

Several cases of Stevens–Johnson syndrome have been described following the use of ophthalmic preparations containing sulfacetamide (Rubin, 1977; Sewell *et al*., 1992). In some of these cases there may have been previous exposure to oral sulfonamides.

6.3.5 Chloramphenicol

Cases of aplastic anaemia have been reported from the use of topical chloramphenicol ophthalmic preparations (Abrams *et al*., 1980; Carpenter, 1975; Fraunfelder *et al*., 1982; Rosenthal and Blackman, 1965), some of which were fatal (Abrams *et al*., 1980; Fraunfelder *et al*., 1982). The response has been recorded for use of both drops and ointment. Fraunfelder (1989) suggested that physicians should carefully weigh the risk–benefit ratio for 27 lethal events that may occur for 1 in 30 000–50 000 cases, and proposed that chloramphenicol be used only for cases

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with organisms resistant to other antibiotics. At the time, Rayner and Buckley (1996) stated in a review that the available evidence for a relationship between ocular chloramphenicol and the development of aplastic anaemia was not conclusive. Currently, it is generally believed that haematological adverse effects from chloramphenicol are a reversible dose-dependent bone marrow depression with doses of $\gg 4\,g\,day^{-1}$ or cumulative dose $>25\,g$, and severe aplastic anaemia in 11 : 25 000–40 000 treatment regimes, unrelated to dose, duration of therapy or route of administration (Todd *et al*., 2006).

6.3.6 Cyclopentolate

Cyclopentolate (2-dimethylaminoethyl 2-(1-hydroxy cyclopentyl)-2-phenylacetate), a potent anticholinergic drug, is used to produce rapid mydriasis and cycloplegia. Signs of CNS involvement, usually developing within minutes or less than an hour, include ataxia, dysarthria, disorientation, hallucinations, amnesia and drowsiness, The effects are particularly notable in children, although they have been described with adults (Awan, 1976; Shihab, 1980). Gastrointestinal effects (vomiting, distension and ileus) were noted in premature twin neonates given cyclopentolate eye drops for eye examination after receiving oxygen. Blood samples taken 24 hours after the instillation of the eye drops gave plasma concentration values of 2 and $22\,\mu g\,ml^{-1}$ (Bauer *et al*., 1973). In a study involving 40 patients and a double blind trial with 35 subjects, Birkhorst *et al*. (1963) showed a statistically significant incidence of reactions.

6.3.7 Ecothiophate Iodide

Ecothiophate iodide (2-diethoxyphosphorylsulfanylethyl-trimethyl-ammonium iodide) is an antiChE used in the treatment of glaucoma. Long-term use of ecothiophate iodide eye drops can lead to inhibition of erythrocyte AChE (acetylcholinesterase) and serum BChE (butyrylcholinesterase), which raised a suggestion that there is a need for periodic blood ChE (cholinesterase) measurements in those receiving topical ocular antiChE treatment (Leopold, 1984). Humphreys and Holmes (1963) investigated 24 patients receiving ocular ecothiophate iodide and found a direct correlation between symptoms (nausea, diarrhoea, abdominal cramps and weakness) noted in 38% of patients, and decrease in blood ChE. Diarrhoea may be the fist indication of systemic toxicity from ecothiophate iodide eye drops (Markman *et al*., 1964). More serious indications of a cholinergic effect include vomiting, muscle weakness, fasciculations, excess lacrimation, hypersalivation, seizures, miosis, bronchospasm, bradycardia and cardiac arrest (Hiscox and McCulloch, 1965; Hallett and Cullen, 1972). A case report of severe cholinergic poisoning resulting from the use of ecothiophate iodide eye drops has been described by Manoguerra *et al*. (1995), in which the patient presented with profound muscle weakness, nausea and dysphagia, for which an initial diagnosis of myasthenia gravis was made. Subsequent investigations showed marked decreases in erythrocyte and plasma ChE activities. The symptoms of the patient spontaneously reversed after the ecothiophate iodide eye drops were discontinued. Asthmatic patients may experience an exacerbation of bronchospasm (Prakash and Rosenow, 1990), which may be accentuated by the coadministration of timolol (Gerber *et al*., 1990).

6.3.8 Proparacaine

Proparacaine is a topical local anaesthetic used to alleviate discomfort during eye examinations. Cydulka and Betzelos (1980) reported a case of tonic-clonic convulsions in a 28-year-old woman following the instillation of two drops of 0.5% proparacaine hydrochloride.

6.3.9 Atropine

Children may experience potentially serious systemic toxicity following the use of atropine eye drops to cause mydriasis. Induced systemic effects include flushing, tachycardia, agitation and hyperthermia (Sydow *et al*., 2008).

6.4 Therapeutic Applications of Transocular Absorption of Drugs

Transocular absorption is finding practical and potential applications in therapeutics. Thus several therapeutic substances when applied topically to the eye may be absorbed in amounts sufficient to exert pharmacological effects. For drugs showing high biological activity through the nasal route and which may have a high first-pass hepatic metabolism by the peroral route, the nasal mucosa may offer a useful therapeutic route of exposure. For example, Pillion *et al*. (1992) found that glucagon eye drops, containing 0.25% saponin, produced a rapid and dose-related increase in blood D-glucose in the rat. Chiou *et al*. (1990) also showed that glucagon may be readily absorbed following ocular instillation, and that this may be facilitated by permeation enhancers. Similar work with insulin demonstrated that whilst plain insulin eye drops had little effect on blood glucose concentrations, when combined with permeation enhancers the blood glucose concentration was markedly reduced in both normal and hyperglycaemic diabetic rabbits and rats (Chiou and Chuang, 1989; Yamamoto *et al*., 1989; Chiou *et al*., 1991; Pillion *et al*., 1991). As an extension of this work, Chiou and Li (1993) investigated the long-term delivery (twice daily for three months) of insulin by the transocular route in rabbits using 0.5% BZ-9 or Brij-78 as absorption

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enhancers. They found no evidence for the development of tolerance, local irritation or allergic responses. In normoglycaemic human volunteer studies, in which eight subjects received 50 µl of sterile saline solutions containing various concentrations of porcine insulin, no adverse effects to the anterior segment of the eye were detected by slit-lamp microscopy (Bartlett *et al*., 1994). Developmental work has been conducted on the transocular route as a possible means for dosing with oxytocin and vasopressin (Chiou *et al*., 1991). Although poorly absorbed as such by the ocular route, the addition of polyoxyethlene-20-stearyl ether markedly enhances their absorption.

Other experimental approaches to investigating the potential for delivery of therapeutic agents by the transocular route have included the use of parenteral ophthalmic antimuscarinic agents in acute antiChE poisoning. Thus, Bryant *et al*. (2006) studied the effect of a 5-minute pretreatment of Sprague Dawley rats with ophthalmic atropine sulfate (10 mg kg^{-1}) or homatropine sulfate (20 mg kg^{-1}) followed by the sc injection of dichlorvos (2,2-DDVP; 10 mg kg^{-1}). Survival rates for rats pretreated with ophthalmic homatropine and atropine sulfate were 100% ($p < 0.001$) and 90% ($p < 0.01$), respectively, compared to saline controls (20% survival). For comparison, pretreatment with atropine given by ip injection was 100%. The investigators concluded that pretreatment with parenteral ophthalmic homatropine or atropine sulfate was equal to standard ip pretreatment with atropine in preventing OP lethality in the rat model for acute OP poisoning, and shows promise as an alternative for antidotal therapy. In a further study they investigated the efficacy of the anticholinergic agents tropicamide and cyclopentolate in a similar rat acute OP poisoning protocol with 15 mg kg^{-1} dichlorvos given sc (Bryant *et al*., 2007). Survival in rats receiving pretreatment with ophthalmic tropicamide (20 mg kg^{-1}) and with cyclopentolate (20 mg kg^{-1}) was 90% ($p < 0.001$) for both anticholinergics compared to saline controls (10% survival). It was considered that the results indicated that pretreatments with tropicamide or cyclopentolate were promising as alternative sources of antidotal therapy for acute OP poisoning.

7 OPHTHALMIC TOXICITY AT SPECIFIC MORPHOLOGICAL AND FUNCTIONAL SITES

7.1 General Comments

Chemicals and/or their metabolites may produce injury at various sites in the eye following topical contamination or systemic absorption. Some chemicals produce mixed ocular lesions and others may result in pathologies at specific sites within the eye (Table **9**). General toxicology studies, particularly by repeated exposure conditions, can generally detect any potential for ocular pharmacological effects and for ocular injury at various anatomical sites, providing that appropriate monitoring is undertaken to detect morphological and functional lesions. In this section, examples of chemically induced structural and functional injuries at specific locations and on ocular physiological functions are discussed.

7.2 Cornea

The majority of instances of injury to the cornea by xenobiotics relate to injury that expresses itself in the form of an inflammatory response, and sometimes may be visible macroscopically. Such corneal lesions may be the result of topical contamination of the eye, or from systemically induced ocular toxicity (Hollander and Aldave, 2004). Additionally, more subtle effects may occur and also toxicity may result by other mechanisms. For example, and discussed below, ocular phototoxicity is not common but when it occurs can be of occupational significance. Likewise transient minor corneal epithelial oedema from exposure to low concentrations of vapour from certain chemicals can be a nuisance effect. Examples of corneal lesions resulting from phototoxic reactions, from low vapour concentration exposures and as a consequence of systemic toxicity are presented below.

7.2.1 Phototoxic Keratoconjunctivitis

In this process there is excitation of electrons in the causative sensitizing molecule(s), usually aromatic compounds, by UV radiation (320–400 nm). Phototoxic keratoconjunctivitis has been described from ocular exposure to coal tar pitch fumes with subsequent exposure to sunlight (Curtis and Greenberg, 2005). Coal tar pitch is a mixture of high MW aliphatic and aromatic hydrocarbons. Clinical presentation is as an increasing discomfort, burning sensation and pain in the eye, excess lacrimation, and photophobia, with conjunctival hyperaemia and central corneal abrasions; signs and symptoms develop within minutes to hours after ocular exposure to UV radiation. Treatment usually requires ophthalmic antibiotics and cycloplegics.

7.2.2 Glaucopsia

One practical example of a minor transient effect on the cornea, but which is of significant practical occupational significance, is that of glaucopsia, which is also referred to as blue-grey haze. In this condition there is a transient disturbance of vision resulting from the development of corneal epithelial oedema and associated microcysts following exposure to the vapour of certain

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Table 12 Chemicals known to be a cause of occupational glaucopsia

Chemical	Reference
N-ethylpiperidine	Jones and Kippling (1972)
Morpholine	Albrecht and Stephensen (1988)
N-Methyl morpholine	Dernehl (1966)
N-Ethyl morpholine	Dernehl (1966)
Dimethylamine	Munn (1967)
Diethylamine	Grant and Schuman (1993)
Dimethylethylamine	Warren and Selchan (1988)
Triethylamine	Åkesson *et al*. (1985)
Dimethylaminoethanol	Page *et al*. (2003)
Di-isopropylamine	Grant and Schuman (1993)
3-Dimethylaminopropylamine	Spaeth and Leopald (1964)
Dimethylisopropanolamine	Page *et al*. (2003)
Tetramethylethylenediamine	Mastromatteo (1965)
Tetramethyl-1,3-butanediamine	Mastromatteo (1965)
Ethylenediamine	Grant and Schuman (1993)
Triethylenediamine	Dernehl (1966)
tert-Octylamine	Munn (1967)
Bis[2-(dimethylamino)ethyl]ether	Ballantyne (2005)

industrial chemicals, notably aliphatic, alicyclic and heterocyclic amines (Ballantyne, 2004b). Chemicals cited as causing glaucopsia are listed in **Table 12**. Following a latent period of about 30 minutes to a few hours, clinical features include a blurring or haziness of vision followed by a more marked difficulty with visual discrimination and accompanied by a partial loss of colour discrimination, resulting in objectives assuming a blue-grey appearance. This may be followed by the development of haloes around bright objects. In more severe cases, photophobia may develop (Grant and Schuman, 1993). Macroscopic examination of the eyes during an episode demonstrates diffuse corneal cloudiness (Dernehl, 1966) and mild conjunctival injection (Jones and Kippling, 1972). Slit-lamp biomicroscopy reveals diffuse corneal epithelial oedema with subepithelial small vesicular collections of fluid (microcysts), but the endothelium appears normal. Exposure to high concentrations of causative chemicals may result in a slight decrease in objectively assessed visual acuity, and contrast sensitivity may be reduced at lower concentrations (Järvinen and Riihimäki, 1999). Pachymetry reveals an increase in corneal thickness, the magnitude of which is concentration related. On cessation of exposure to the causative vapour, vision returns to normal within a few hours without any permanent ocular sequelae.

The pathogenesis of glaucopsia involves a local effect of the causative vapour on the superficial cornea with diffusion of material into the epithelium, causing epithelial oedema, subepidermal microcysts and fluid accumulation in the superficial cornea. These changes cause scattering of light and blurring of vision. Corneal epithelial oedema is characterized by large increases in the forward scattering of light (Feuk and McQueen, 1971). With some materials it is possible that systemically absorbed chemical may be excreted in lacrimal fluid and in this manner contribute to the local corneal changes of glaucopsia. For example, in studies involving exposure of human volunteer subjects to triethylamine (TEA) vapour, the average lacrimal fluid TEA concentration was 41 times higher than the serum concentration (Järvinen *et al*., 1999). However, in another study in which volunteer subjects were exposed to TEA vapour the lacrimal fluid concentration of TEA was inversely proportional to lacrimal fluid volume, suggesting that increased lacrimal fluid TEA may have been a consequence of local solubility in the fluid (Åkesson and Skerfving, 1990). Also, there were no ocular symptoms following an acute oral dose of TEA, for which the pharmacokinetic constants were C_{max} of 1.17 µmol l^{-1} at t_{max} of 45 minutes, and plasma $t_{1/2}$ ranging from 2.4 to 3.5 hours (Åkesson *et al*., 1989). For experimental purposes, the rabbit is a suitable animal model to study the effects of vapours on corneal morphology. Thus, for example, studies on the effects of vapour from bis[2-(dimethylamino)ethyl]ether (DMAEE) showed that by corneal pachymetry it was possible to follow increases and subsequent decreases in corneal thickness, and to define a no-effects vapour concentration between 4.6 and 8.8 ppm (Ballantyne and Chun, 1996; Chun and Ballantyne, 1997).

Although glaucopsia is transient and does not result in any permanent effects on vision, the condition causes a temporary nuisance disturbance of vision which may be incapacitating and impair efficient and safe working conditions. This may secondarily cause a difficultly with reading initially, and subsequently result in a predisposition to physical accidents and impair the ability to undertake coordinated and skilled tasks. For example, this could result in potentially dangerous situations with driving a motorized vehicle because of the impairment of skilled movements and, with dusk or night driving, the hazard may be exaggerated by the brightness and haloes around oncoming lights (Ballantyne, 2004b). Most chemicals producing glaucopsia are also peripheral chemosensory irritant (PCSI) materials, but for a given material glaucopsia develops at vapour concentrations below those known to cause a PCSI effect, and PCSI occurs at concentrations below those acutely inducing corneal inflammation, and hence glaucopsia may be a more sensitive index of ocular effects (Ballantyne, 2004b;2005). It should be noted, however, that with longer term repeated exposure the vapour concentration causing ocular inflammatory change may be reduced compared with acute exposure conditions, and come closer to that causing

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glaucopsia. Thus, glaucopsia is of considerable industrial hygiene interest, and the visual effects may be taken into consideration when establishing a permissible exposure standard with a chemical producing this effect. As an example, based on correlation between measured DMAEE vapour concentrations and clinical symptoms and signs the American Conference of Governmental Industrial Hygienists (ACGIH) have adopted an 8-hour TWA_8 of 0.05 ppm with a STEL of 0.25 ppm as being appropriate for protection against visual disturbances (ACGIH, 2001; 2007). This is significantly lower than the vapour concentration causing acute PCSI effects on the eye (68 ppm; Chun and Ballantyne, 1997), but close to that concentration causing mild keratitis by subchronic vapour exposure (1.25 ppm; Losco *et al*., 1996). Also citing vision as a basis, ACGIH have adopted a TWA_8 of 1.0 ppm with STEL of 3.0 ppm for TEA (ACGIH, 2007). Contact lenses may act as a reservoir for some chemicals capable of causing glaucopsia, and thus prolong exposure. It has been advised specifically that contact lenses should not be worn when there is likely to be exposure to the vapour of TEA (Järvinen *et al*., 1999).

7.2.3 Corneal Injury from CGS 24565 Dosed Perorally

CGS 24565 was originally evaluated as a potential hypolipidaemic agent; chemically it is (11 R)-*N*, 15-dideoxo-1-deoxy-1,15-epoxy-11-hydroxy-4-0-methyl -8-0-(2,2-dimethyl-1-oxopropyl)-3-[4-{(2,4,6-trimethyl-phenyl)methyl}-1-piperazinyl]rifamycin. For a three-month po toxicity study CGS 24565 was dosed at 10, 50 or 300 mg kg^{-1} day^{-1} to beagle dogs by gelatine capsule. At week 12 there were bilateral adnexal and corneal changes at the high dose. Corneal changes were characterized as posterior stromal oedema, and stromal infiltrates in the area of Decement's membrane. These changes were interpreted as representing permanent and irreversible changes, being confirmed by subsequent ophthalmoscopic examination at weeks 15 through 26. Light microscopy confirmed irreversible corneal neovascularization, vacuolar degeneration of keratocytes, and polymorph infiltrates in the region of Decement's membrane. These findings terminated further development of CGS 24565 as a therapeutic agent (Schiavo and Bentley, 2000).

7.3 Iris

Effects on iris function are illustrated by exposures to vapour from OP compounds. The miosis caused by such exposures usually starts during exposure, and the degree and duration of miosis are generally related to the exposure dose, although this is not necessarily the general effect. Thus, certain concentrations of antiChEs in contact with the eye may cause extreme miosis but affect accommodation only to a moderate extent (Erickson-Lamy and Grant, 1992). For example, human volunteers exposed to sarin (isopropyl methyl phosphonofluoridate) vapour had marked miosis but only 2.3 D of induced accommodation compared with a potential maximum of 7–10 D (Moylan-Jones and Thomas, 1973). If marked, the inability of the pupil to fully dilate in darkness may persist for several weeks and the affected subject may complain of 'dim' vision and periocular pain (Sidell, 1994; Rengstorff, 1985). Stewart *et al*. (1968) found that topically applied sarin caused a decrease in light sensitivity that correlated with a decrease in pupil size, and proposed that miosis alone was responsible for the decreased light sensitivity. However, Rubin *et al*. (1957) found that topical application of sarin did not decrease dark adaptation, but inhalation of the vapour with the eyes protected decreased dark accommodation in the absence of miosis (Rubin and Goldberg, 1958). Also, systemic dosing with atropine, which penetrates the blood–brain barrier, reversed this decrease (Rubin and Goldberg, 1958). These findings suggest that cholinergic mechanisms proximal to the pupil, such as those in the retina or visual pathways, are responsible for the visual changes.

On occasion, contact with antiChEs can cause iritis that is usually associated with conjunctival hyperaemia. This complication responds to cholinergics (Aldridge *et al*., 1947), but in some cases the response to anticholinergics is slow despite treatment with mydriatics, corticosteroids and 2-pyridine aldoxime methiodide (2-PAM) (Becker *et al*., 1959). Iritis with the formation of posterior synechiae after long-term antiChE treatment has been reported (Kadin, 1963; Leopold, 1984; Werhahn and Schnarr, 1987). Morphological changes that have been reported in humans as a consequence of repeated application of antiChEs are iris cysts of the papillary border (Grant and Schuman, 1993; Werhahn and Schnarr, 1987). Typically they are multiple and 0.1–1.0 mm in diameter, and may develop within 1 to 40 weeks (Abraham, 1954). Pupillary cysts may interfere with vision, particularly in association with extreme miosis (Werhahn and Schnarr, 1987). Histologically, Straub and Conrads (1955) found that in cystic areas two posterior epithelial layers of the iris were separated in some places, forming fluid-filled cysts. This accords with the suggestion that the pathogenesis of the cysts is a result of miosis pinching the iris pigment epithelium against the lens, with retention of fluid between the layers producing cysts. The simultaneous use of phenylephrine drops with antiChEs can enlarge the pupil slightly and reduce the tendency to form iris cysts (Abraham, 1967; Chin *et al*., 1964; Ellis, 1985).

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7.4 Ciliary Body

AntiChEs topically applied to the eye can result in various changes in accommodative capacity of the eye (see Ballantyne, 2006a). In glaucoma, contraction of the ciliary muscle reduces the resistance to outflow and antiChE eye drops potentiate this action (Becker *et al*., 1959; Bito, 1968). In addition to pharmacological and toxic actions on the ciliary body from chemicals applied topically to the eye, there are several examples of systemic toxicity to the ciliary body. Examples are those of the OPs disulfoton and fenitrothion, briefly presented below.

7.4.1 Disulfoton

Susuki and Ishikawa (1974) described ultrastructural changes in the ciliary body of dogs chronically dosed orally (by capsule) with disulfoton (ethylthiodemeton; *O*,*O*-diethyl *S*-[2-(ethylthio)ethyl]-phosphorodithioate) at doses of ~0.5 to ~1.5 mg kg^{-1} day^{-1}, five days a week for two years. All dogs developed myopia within a year. Ultrastructure of the ciliary muscle cells showed membranous meshwork structures, considered to be the cause of the myopia by the investigators. Tokoro *et al*. (1973) studied dogs dosed with disulfoton for two years at doses of 5, 10 and 15 mg kg^{-1} day^{-1}. Myopic changes were noted, the onset of which was related to the degree of erythrocyte AChE inhibition. Chronic peroral studies in dogs at doses in the range 0.5–1.5 mg kg^{-1} day^{-1} resulted in decreases in refractive error and corneal curvature suggestive of myopia (Kawamura *et al*., 1974; Otsuka and Tokara, 1976), increase in IOP, and decreased erythrocyte AChE (Tokoro *et al*., 1973).

7.4.2 Fenitrothion

Fenitrothion (*O*,*O*-dimethyl *O*-4-nitro-*m*-tolyl phosphorothioate), was dosed orally (capsule) to Beagle dogs at 10, 20 and 100 mg kg^{-1} $week^{-1}$ for one year (Ishikawa and Miyata, 1980). Myopia was significantly increased in dosed versus control animals by nine months of dosing, and was still present at one year after the cessation of dosing. Lens thickness and IOP were also increased. Histology of the ciliary body showed muscle fibre swelling at all doses, and was morphologically similar to that seen with disulfoton (Section 7.4.1). Both erythrocyte AChE and serum BChE were slightly decreased during the dosing period but returned to normal three months after the cessation of dosing. Ciliary body AChE was significantly reduced.

7.5 Aqueous Humour and Intraocular Pressure

Aqueous humour is secreted by the ciliary processes and passes behind the ciliary muscle into the posterior compartment of the aqueous chamber, then flows between the fibres of the suspensory ligament into the anterior aqueous chamber, and is then reabsorbed into the canal of Schlemm. The trabecular network of the canal is opened, and reabsorption of aqueous humour is facilitated by contraction of the sphincter pupillae of the iris and the ciliary muscle, both of which have a cholinergic innervation. Thus, antiChE agents can pharmacologically cause an accumulation of acetylcholine (ACh) at the sphincters of the iris and ciliary body, resulting in their contraction, producing miosis and ciliary spasm with accommodation for near vision, and facilitating aqueous humour reabsorption and decrease in IOP. The increased IOP in glaucoma can be reduced by the use of antiChE drugs, due to their ability to increase cholinergic activity in the sphincter muscles of the iris and ciliary body. They can therefore be used for the emergency treatment of acute narrow-angle glaucoma and in long-term therapy for chronic wide-angle glaucoma (Koelle, 1994). They are generally not recommended for the long-term treatment of narrow-angle glaucoma because they may produce hyperaemia of the iris and trabecular network (Ellis, 1985). Since it was noted clinically that the continual use of long-acting antiChEs resulted in the development of lenticular opacities (see Section 7.6.8), they have generally been replaced by drugs operating through other mechanisms. These include parasympathomimetic agents (e.g. pilocarpine), which cause a contraction of the ciliary body, relaxation of the lens zonules and forward movement of the lens (Hung *et al*., 1995; Yang *et al*., 1997), and β-adrenergic receptor blocking agents (e.g. timolol) that decrease the rate of production of aqueous humour (Coakes and Brubacker, 1978; Wang *et al*., 1997). Although antiChEs reduce IOP in the normal and glaucomatous eye, a paradoxical transitory increase in IOP may occur in some patients as a result of breakdown of the blood–aqueous humour barrier (Erickson-Lamy and Grant, 1992). This can also be demonstrated experimentally in the rabbit eye. Thus, application of physostigmine, neostigmine or diisopropyl phosphorofluoridate (DFP) to the rabbit eye initially causes hyperaemia of the iris, an increase in IOP and an increased capillary permeability, permitting the entry of proteins that may result in an aqueous humour flare (Bárány, 1947; Von Sallam and Dillon, 1947), which may be prostaglandin-mediated (Erickson-Lamy and Grant, 1992).

Angle-closure glaucoma is probably precipitated in eyes with a shallow anterior aqueous chamber and narrow angle, which can be accentuated to the point of angle closure by miosis and anterior movement of the lens.

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Cases have been described in which young individuals have developed angle-closure glaucoma (François and Verbraeken, 1977; François and Verbraeken, 1978; Jones and Watson, 1967). In these cases, glaucoma was a consequence of the iris closing the anterior chamber angle and obstructing the outflow of aqueous humour. However, contraction of the ciliary muscle probably also had a role by loosening the lens zonules and permitting the lens and iris to move forward (Erickson-Lamy and Grant, 1992). Treatment with anticholinergic drugs was effective in these cases, probably as a result of tightening of the lens zonules resulting from ciliary muscle contraction.

7.6 Lens and Cataractogenesis

7.6.1 General Comments

Cataracts may be detected in general toxicology studies providing sufficient detail is given to ocular examination is such studies. For this and other reasons it is desirable that the protocol for a repeated exposure study should allow for routine ocular examinations by a veterinary ophthalmologist. Specific studies on the determinants and mechanisms of cataractogenesis can be carried out *in vivo* and *in vitro* using a variety of specialized approaches. These include various forms of microscopy, biochemical measurements and metabolic studies; these may be followed by magnetic resonance imaging and spectroscopy (Schliech *et al*., 1985), tryptophan fluorescence spectroscopy (Lerman and Moran, 1988), scanning lens monitoring (Mitton *et al*., 1990) and isolated lens preparations with biochemical analyses. Biochemical changes in the lens that have been investigated include aldose reductase, sorbitol dehydrogenase, phosphofructokinase, glutathione reductase, GSH, NADH, NADPH, calcium, cholesterol, phospholipids and protein (Chiou, 1992).

Cataract formation may result from the penetration of materials applied topically to the eye, with diffusion of materials into the anterior aqueous chamber, or be a consequence of systemic toxicity from materials, or their metabolites, absorbed at sites remotes from the eye. In some cases, such as corticosteroids, both local and systemic routes may be effective (see Section 5.2.11). Also, as noted in Section 5.3, with respect to systemic toxicity to the lens there may be differences in lens access depending on whether exposure is prenatal or postnatal. Postnatally, the cataractogenic material must be capable of penetrating the blood–aqueous humour barrier, and thus the necessity for slow excretion of the material and/or long-term repeated exposure, except with potent cataractogenic materials. The likelihood for the development of a cataract, and its nature, varies between species, which is possibly related to differences in metabolic activity in the lens. Also, with a given material the potential for development of a cataract and its time frame to develop may also vary between species, and thus comparative evaluations and extrapolations need to be undertaken with considerable thought.

The mechanisms involved in the development and pathogenesis of a cataract may be complex, and are often variable between different xenobiotics. The cataractogenic effects may be due to the parent absorbed (or penetrating) material or to metabolites formed distally to the eye. In other cases, the parent molecule may require metabolic activation in the lens to a locally acting metabolite. A common aetiological factor in the genesis of opacities often includes changes in osmotic pressure caused by electrolyte shifts, resulting in errors of refraction or lens protein denaturation. Major factors that appear to be involved in some cataractogenic processes include oxidative stress (Vinson, 2006), lens hydration, inhibition of Na^+-K^+-ATPase and calpain activation. This last mechanism involves proteolysis by Ca^{2+}-dependent calpains, which cleave proteins into fragments that decrease lens transparency. It is of interest to note that several photosensitizing agents have been implicated as being cataractogenic. Thus, methoxsalen (8-methoxypsoralen) causes anterior cortical cataracts in mice and guinea pigs having UV exposure (Cloud *et al*., 1960; 1961). Other substances that have been associated with photosensitization-induced cataractogenesis include nafoxidine, haematoporphyrin and bleomycin (Millichamp, 1992).

Several examples of cataractogenic substances, determinants for their activity, and possible modes of action are given below.

7.6.2 Naphthalene

Naphthalene is a well-known cataractogenic material, probably with a dihydrodiol metabolite as the major factor in cataract formation (Xu *et al*., 1992a; 1992b). This metabolite accumulates in the lens of naphthalene-fed rats (Xu *et al*., 1992a) and induces cataracts in rat lenses in culture (Xu *et al*., 1992b). An aldose reductase inhibitor (ALO1576) inhibited both the cataractogenesis and the accumulation of naphthalene dihydrodiol in naphthalene-fed rats (Xu *et al*., 1992b). It was speculated that the site of action of AOL1576 might be lens naphthol dihydrodiol dehydrogenase. Studies by Sato (1993) suggested that aldose reductase inhibitors prevented naphthalene cataractogenesis by inhibiting aldose reductase. Lou *et al*. (1996) fed rats both galactose and naphthalene, or incubated rat lenses in cultures containing high concentrations of galactose and naphthalene dihydrodiol. They found that both cortical and perinuclear cataracts developed. With naphthalene-fed rats, an aldose reductase inhibitor prevented cataract formation. One inhibitor, sorbinil, containing a spirohydantoin group, was less effective,

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and tolrestat, containing a carboxyl group, was ineffective. With an *in vitro* dual cataract model (galactose and naphthalene), tolrestat prevented the galactose cataract, but did not protect against the naphthalene cataract; ALO1576 prevented both cataracts. It was concluded that the prevention of naphthalene cataracts resulted from an inhibition of the conversion of naphthalene dihydrodiol to 1,2-dihydroxynaphthalene, and that the effect of aldose reductase inhibitors cannot be explained by the dihydrodiol dehydrogenase activation of aldose reductase.

The toxic effects of naphthalene have been suggested as being due to oxidative stress caused by the generation of reactive oxygen species (ROS) during its Phase I metabolism by cytochrome P450 and redox cycling of its metabolite, 1,2-naphthoquinone (Doherty *et al*., 1985; Wells *et al*., 1989). Naphthoquinone metabolites generated during cytochrome P450-mediated metabolism of naphthalene have a higher cataractogenic potential than the parent naphthalene (Wells *et al*., 1989). Consistent with the role of cytochrome P450 mechanisms in naphthalene cataractogenesis is the fact that P450 inhibitors prevent naphthalene-related cataract formation whilst P450 inducers augment the cataractogenic process with naphthalene. It has been shown that naphthalene causes DNA damage and lipid peroxidation (Bagchi *et al*., 1998), which is consistent with ROS involvement in the mechanism of its toxicity. Additionally, prevention of naphthalene cataractogenesis by pro-cysteine drugs and depletion of GSH in the lens during naphthalene-induced cataractogenesis also accords with the concept that oxidative stress is a causative factor in naphthalene cataractogenesis (Rathbun *et al*., 1996; Wells *et al*., 1989). Because of its known antioxidant effects and inducer effects on GSH-linked detoxification pathways, Pandya *et al*. (2000) studied the effect of curcumin, a constituent of turmeric, on naphthalene cataractogenesis. They found that rats treated with naphthalene and kept on a low dietary concentration of curcumin (0.005% w/w) had significantly less opacification of the lens than rats treated only with naphthalene. The authors noted that it was probable that curcumin protects against naphthalene-induced cataractogenesis through a combination of its antioxidant properties and its effects on GSH-linked pathways. Additionally, they were able to demonstrate that naphthalene-initiated cataract is accompanied by apoptosis of the lens epithelial cells, and that this apoptotic effect is attenuated by curcumin.

7.6.3 Acetaminophen

Cataracts were produced by acetaminophen (paracetamol) when injected into mice having cytochrome P450 mono-oxygenases induced by pretreatment with 3-methylcholanthrene or β-naphthoflavone, but they did not occur in mice not having such pretreatment (Schichi *et al*., 1978). The induction of cataracts by acetaminophen has been attributed to the generation of a reactive metabolite, probably *N*-acetyl-*p*-benzoquinonimine (Dahlin *et al*., 1984). Cataract formation in rabbits has also been demonstrated using high doses of acetaminophen, although the material is not hepatotoxic in this species. Conversely, with D2 mice, acetaminophen in high doses is hepatotoxic but not oculotoxic (Lubek *et al*., 1988). They suggested that cataractogenesis might involve a mechanism different to that causing liver injury. Zhao and Schichi (1995) investigated acetaminophen cataractogenesis in mice that were either cytochrome 450 inducer responsive or nonresponsive. They found acetaminophen to be cataractogenic in C57BL/6 mice (responsive strain) pretreated with β-naphthoflavone, but not in DBA/2 mice (nonresponsive). They therefore considered that the induction of cytochrome P450 enzymes, which metabolically activate acetaminophen, is essential for cataractogenesis. Subsequent observations, using light and electron microscopy, showed that tissue injury occurs first to the ciliary epithelium, and then the iris, corneal epithelium and lens, with mitochondria being the major target subcellular organelle. These findings suggest that the cytotoxic metabolite of acetaminophen passes through the ciliary epithelium to the aqueous humour and is then transported to the lens, and that inhibition of mitochondrial metabolism contributes to the acetaminophen-induced cataractogenesis.

7.6.4 Serotonin Antagonists

Certain serotonin (5-HT) antagonists are cataractogenic. For example, the 5-HT_3 antagonist SDZ ICT 322 (indole-3-carboxylic acid scopine ester), a gastric motility stimulant, has been reported to induce posterior subcapsular lens opacities in a 26-week feeding study in the rat (Langle *et al*., 1993). Histologically the lens fibres were grossly and irregularly fragmented, and there was marked vacuolation of the lens epithelium. The reduced GSH/oxidized GSH ratio was reduced, suggesting a possible role of oxidative stress in the cataractogenic process. Cataractogenesis can also be induced *in utero* by at least one serotonin antagonist, RG 12195 (Lerman *et al*., 1995; see Section 5.3).

7.6.5 Tamoxiphen

Tamoxiphen, a nonsteroidal antioestrogen, has been shown to be cataractogenic in the rat (Furr and Jordan, 1984). There is evidence that the lens opacity cased by tamoxiphen is caused by blockade of chloride channels in the lens fibre cells (Zhang *et al*., 1994). That chloride channel activity is necessary for maintenance of adequate cell hydration is indicated by the fact that removal of extracellular chloride, or addition of channel blockers, both result in lens opacities (Zhang and Jacob, 1994). An *in vitro* comparison was made between tamoxiphen and ICI 182,780 a structural analogue of oestrogen.

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Whereas tamoxiphen caused maximum inhibition of chloride channel activity at 10 μM, ICI 182,780 had no effect up to 30 μM (Zhang *et al*., 1995). Light transmission measurements with the lens showed that tamoxiphen caused a dose-related decrease in transmission from 5 to 100 μM, but ICI 182,780 only caused a light transmission decrease at >10 μM, and significantly less than occurred with tamoxiphen at comparable doses. These findings suggest that the channel-blocking and lens-opacification effects of tamoxiphen are independent of antioestrogenic activity.

7.6.6 4-Hydroxynonenal

Studies with 4-hydroxynonenal (4-HNE) may be relevant to the aetiology and pathogenesis of senile and diabetic cataract formation, particularly in relation to the role of free radical generation and lipid peroxidation. Free radicals initiate lipid peroxidation to produce lipid peroxides in certain types of cataract (Babizhayev and Costa, 1994). One highly reactive β-unsaturated aldehyde formed is 4-HNE (Benedetti *et al*., 1980), which is known to be cataractogenic (Srivastava *et al*., 1996). The highly reactive α,β-unsaturated region of 4-HNE can react with the sulfhydryl groups of GSH and proteins, imidazole group of histidine, and amino groups of lysine (Uchida and Stadtman, 1994). A resultant modification of membrane protein and lipid can alter membrane fluidity (Choe *et al*., 1995), which can be the basis for cell injury leading to lens opacity. Srivastava *et al*. (1996) showed that, *in vitro*, pretreatment with butylated hydroxytoluene (BHT) attenuates the cataractogenic effect of 4-HNE, suggesting that BHT-mediated induction of lens epithelium glutathione-5-transferases may be an important component of the protective effect.

7.6.7 Tacrolimus

An interesting indirect cause of cataract formation is seen with tacrolimus, an immunosuppressant, which may be cataractogenic through a diabetogenic effect. It is generally considered that diabetic cataract involves the accumulation of sorbitol, produced through the action of aldose reductase on glucose. Treatment with an aldose reductase inhibitor suppresses the development of diabetic cataract (Tsuji *et al*., 1990). Cataract formation was noted from tacrolimus in a 52-week rat oral study (Ohara *et al*., 1992). It has also been demonstrated to be diabetogenic in the rat (Hirano *et al*., 1992). In a study in which rats were dosed orally with tacrolimus at 0, 2, 5 or 10 mg kg^{-1} day^{-1} for 13 weeks, cataracts developed in the high dose group (26%) with increased sorbitol and reduced GSH (Ishida *et al*., 1997). They also measured an increase in blood glucose with glucose intolerance and decreased insulin. Coadministration of zenarestat, an aldose reductase inhibitor, decreased the incidence of cataract and decreased lens sorbitol. The findings suggest that tacrolimus-induced cataract in the rat is a consequence of increased sorbitol accumulation, and secondary to the diabetogenic action of tacrolimus.

7.6.8 Anticholinesterases

The long-term use of antiChEs for the treatment of glaucoma has resulted in the development of changes in the transparency of the lens and the development of opacities (Chiou, 1999; Harrison, 1960). In general, antiChEs dosed perorally do not result in the development of lens change (Lieberman *et al*., 1971), which may be a consequence of high concentrations of materials reaching the anterior aqueous chamber through the cornea following topical application and/or related to metabolism following peroral dosing. The development of lens changes is age related, with the elderly being more susceptible, and children showing changes only rarely (Axelsson and Nyman, 1970; Chamberlain, 1975; Harrison, 1960; Pietsch *et al*., 1972; Wolter and Lee, 1978). Slit-lamp biomicroscopy of the eye after several months of daily applications of antiChE eye drops demonstrates anterior and posterior subcapsular vacuoles or small opacities in around 50% of patients (Erickson-Lamy and Grant, 1992). Some patients are resistant to the adverse effects of antiChEs on the lens, and maintain normal visual acuity and lens transparency after many years of treatment (Deroetth, 1996; Shaffer and Hetherington, 1966). Also, eyes treated with pilocarpine before antiChEs are protected to some degree from the effects of antiChEs on the lens (Nordmann and Gerhard, 1970; Shaffer and Hetherington, 1966).

Several experimental studies have been conducted on the possible pathogenesis of antiChE-induced lens opacities. With rabbit lens in culture, Michon and Kinoshita (1968a; 1968b) found that concentrations of antiChEs sufficient to inhibit all lens ChE did not produce changes in transparency, but at 1000 times this concentration caused anterior, posterior and equatorial vacuoles to appear associated with increased lens permeability, increased Na^+ content and decreased K^+. Ecothiophate iodide produced these changes without altering lens metabolism, but demarcarium bromide caused anaerobic metabolism to predominate (Michon and Kinoshita, 1968a; Michon and Kinoshita, 1968b). A 50% inhibition of O_2 consumption was reported for the pig, rabbit and human lens treated with paraoxon. Rabbit eyes chronically exposed to ecothiophate iodide eye drops do not show macroscopic eye changes, but Härkönen and Tarkkanen (1970; 1976) found a decrease in ATP and lactate but no change in glycogen, and Firth *et al*. (1973) found no change in lens GSH. Daily applications of 0.25% ecothiophate iodide eye drops to the primate eye produced anterior and posterior opacities within 2–14 weeks (Kaufman and Bárány, 1975; Kaufman *et al*., 1977). Pretreatment iridectomy increased the incidence

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of opacities, but addition of atropine to the ecothiophate iodide eye drops reduced the number of opacities and delayed their development. It has also been demonstrated that ecothiophate iodide eye drops applied daily to primates produced subcapsular opacities and also caused swelling of the anterior cortex of the lens (Albrecht and Bàràny, 1979; Philipson *et al*., 1979).

7.7 Retinal Toxicity

7.7.1 General Comments

As shown in **Table 9** and discussed in Section 5.2 a variety of materials or metabolites can produce toxic effects on the retina by reaching the eye systemically. Some materials having a potential to produce retinotoxic effects may do so as a component of a general toxic effect, but others may have relatively specific effects with the retina being a target tissue. Thus, the effects may be limited to the retina with some substances, but with others materials may be associated with other intraocular pathologies. Also some materials or their metabolites may produce highly selective lesions within the retinal elements. For materials having an effect at more than one distinct anatomical site in the eye, the mechanism responsible for retinopathy may be primary, secondary, or of unknown causation. Some retinotoxic effects have been a consequence of patient management, such as hyperoxia of prematurity (Section 7.7.2), or of long-term medicinal dosing, as with hydroxychloroquine in the management of systemic lupus erythematosis or rheumatoid arthritis (Section 5.2.9).

Most guidelines for repeated exposure toxicity studies include provision for examination of the eye by ophthalmoscopy and histology, and hence some retinotoxic effects may be detected in conventional toxicology studies. However, the sensitivity of ophthalmoscopy for retinal lesions may be low if not carried out by an experienced ophthalmic veterinarian. Also, general pathologists reviewing histology sections in routine toxicology studies may not detect some of the more subtle retinal cytological lesions, which could be of toxicological significance. A variety of techniques of differing degrees of sophistication are available to detect, or confirm, a retinotoxic effect. These include retinoscopy, papillary light reflex, fluorescein fundus angiography, electroretinography and light and electron microscopy. Electroretinography is now well established as a noninvasive clinical procedure for investigating functional retinal defects (Armington, 1974; Shirao and Kawasaki, 1995). However, the above methodologies have differing diagnostic limitations and sensitivities for lesion detection. For example, Maertins *et al*. (1993) compared various approaches to the assessment of amoscanate-induced retinopathy, and found that ophthalmoscopic fundus examination did not yield any characteristic correlates. Time-dependent and dose-dependent effects were found using ERGs and light microscopy, with signs of retinal changes occurring earlier and more distinctively with ERGs. Animal models for human retinopathy with specific chemicals may not be available, or may be less sensitive than in humans. This is illustrated with methanol (see Section 7.7.2).

Reports of retinotoxic effects based on clinical reports need a detailed interpretive evaluation, because of the possible influence of a multiplicity of other possible operative factors in a patient. For example, sparsomycin, an antibiotic produced by *Streptomyces sparsogenes* and *S. cuspidosporus* (Owens *et al*., 1962; Argoudelis and Herr, 1962) apparently produced blurred vision in two of five patients in a Phase 1 clinical study. This was attributed to degeneration of the pigment epithelium, and resulted in the study being discontinued (Close and McFarlane, 1964; McFarlane *et al*., 1966). However, interest continued in the use of sparsomycin and its derivatives, notably ethyldeshydroxy-sparsomycin (EdSm), because of their ribosomal peptidyl transferase centre blocking activity (Goldberg and Mitsugi, 1966), and ability to enhance the antitumour activity of cisplatin (Theocharis and Coutogeorgopoulos, 1992; Zylicz *et al*., 1986). Studies in rats and monkeys, carried out under the auspices of the National Cancer Institute (NCI), have been conducted using histology and ERGs as monitors for ocular toxicity. Abnormal ERG activity was seen only in moribund rats; neither species showed any histological abnormality in the retina (NCI, 1967). Additionally, Hofs *et al*. (1995) found no evidence for a refractive dysfunction due to sparsomycin or EdSm, based on histology and measurement of opsin and rhodopsin activity.

7.7.2 Methanol Retinopathy

Methanol is a well-known cause of visual dysfunction and blindness in humans, in whom it produces optic nerve and retinal lesions. Rats and other nonprimates may not simulate the same ocular toxicity of methanol; however see below for description of a rodent model for methanol-induced retinal and optic nerve toxicity. In humans the ocular toxicity may be variable, for example, retinal histopathology has been observed in the absence of optic nerve pathology (Fink, 1943), and conversely optic nerve demyelination has been observed without retinal lesions (Sharpe *et al*., 1982). Differing findings have also been produced in primates. Thus, optic nerve lesions have been demonstrated after perorally dosed methanol (Baumbach *et al*., 1977), but other studies have shown retinal injury by ERG and histology, but without optic nerve involvement (Khosla *et al*., 1983). In mammals the metabolism of methanol involves sequential conversion to formaldehyde, formic acid and carbon dioxide. It has generally been believed that the accumulation of formate has been responsible for

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both the ocular and the systemic toxicity of methanol, including acidosis (McMartin *et al*., 1978; Martin-Amat *et al*., 1978). The metabolism of formate to carbon dioxide is mediated through a tetrahydrofolate-dependent pathway (Eells *et al*., 1982), and it has been demonstrated that folic acid depletion in the rat increased formate accumulation (Eells, 1991; Murray *et al*., 1991). In this way, the sensitivity of the rat to the ocular toxicity of methanol can be increased, and thus the rat may be used as a laboratory model (Eells, 1991; Murray *et al*., 1991). A number of suggestions have been advanced in order to explain the retinopathy of methanol, including retinal ischaemia secondary to optic nerve (disc) oedema (Hayreh and Weingerst, 1980), and effects on bipolar cells or Müller cells (reviewed by Garner and Lee, 1994). There has been speculation concerning the possible role of cytochrome oxidase inhibition in the optic nerve, leading to stasis of axoplasmic flow (Baumbach *et al*., 1977). In a major study by Garner and Lee (1994) involving a detailed comparison of ERG oscillatory potentials in folate-sufficient and folate-deprived rats, there was no evidence for retinal ischaemia in methanol-induced visual system toxicity. The basic causation of methanol-induced retinopathy remains unclear, as does the possible inter-relationship between optic nerve and retinal toxicity.

As noted above, rats and other nonprimate species may not serve as a suitable model for studying the mechanism of methanol toxicity for the more sensitive human and nonhuman primates. The greater sensitivity of humans and nonhuman primates to methanol toxicity is a consequence of the limited capacity of primates to oxidize, and hence detoxify, formic acid (Eells, 1992; Eells *et al*., 1983). Thus the syndrome of methanol toxicity in primates consists of formic acidaemia, metabolic acidosis and serious visual impairment. Nonprimate species are usually resistant to the accumulation of formate and the associated metabolic and visual toxicity. Eells *et al*. (2000) developed a nonprimate model of methanol toxicity, using rats in which formate oxidation had been selectively inhibited by treatment with nitrous oxide (Eells *et al*., 1983; 1996; Seme *et al*., 1999). Subanaesthetic concentrations of nitrous oxide inactivate methionine synthase (Deacon *et al*., 1980), reducing the production tetrahydrofolate, the cosubstrate for formate oxidation, thus allowing the accumulation of formate to toxic concentrations following dosing with methanol. Nitrous oxide treatment selectively reduces hepatic tetrahydrofolate concentrations in rats to concentrations that are in the region of those measured in humans and nonhuman primates. This rodent model closely replicates the metabolic and neurotoxic effects characteristic of acute human methanol intoxication, and developed formic acidaemia, metabolic acidosis, and visual toxicity. Visual dysfunction was demonstrated as reductions in the ERG and flash-evoked cortical potential, and occurred coincident with blood formate accumulation (Eells, 1992; Eells *et al*., 2000). Histology demonstrated mitochondrial disruption and vacuolation in the retinal pigment epithelium, photoreceptor inner segments and optic nerve. The functional and morphological characteristics in the methanol-intoxicated rats are consistent with the suggestion that formate acts as a mitochondrial toxin in the retina and optic nerve.

7.7.3 Hyperoxic Retinopathy

There has been considerable concern and interest in the retinopathy that accompanies prenatal neovascularization in premature infants: retrolental fibroplasia. Hyperoxia, resulting from the management of prematurity, has long been recognized as a prime causative factor. Also, increased arterial CO_2 has been suggested as an additional risk factor (Bauer, 1982; Holmes *et al*., 1994; 1997a; Wolbarsht *et al*., 1983). Using a neonatal rat model for oxygen-induced retinopathy, Holmes *et al*. (1997b) found that exposure to high concentrations of CO_2 resulted in an increased incidence and increased severity of neovascularization (85%) compared with a hyperoxia group exposed to low concentrations of CO_2 (57%). These findings suggest that hypercarbia may be an additional risk factor in the retinopathy of prematurity. In a further study, Holmes *et al*. (1998) found that 19% of rats in high inspired CO_2 conditions (10% CO_2, 21% O_2) developed preretinal neovascularization, compared with 14% in a pure hypercarbia group (10% CO_2, 12.5% O_2). These findings support the concept that hypercarbia is also a risk factor for the retinopathy of prematurity, and the authors stated that hypercarbia may also be an independent cause of retinopathy.

7.7.4 Chloroquine Retinopathy

Chloroquine is used an antimalarial, and has been employed as an immunomodulator in rheumatoid arthritis, has a selective effect on lysosomes and shows a marked affinity for pigmented tissues. Retinopathic changes occur first in the perifoveal area, shown as a change in granularity of the retinal pigment cell layer. At this stage, a subtle change in paracentral retinal function can be demonstrated, whilst central visual acuity may still be entirely normal. Subsequently a 'bull's eye' pattern and thinning of retinal pigment occurs around the fovea in the macular area. This often results in an annular scotoma within 2° to 3° of fixation (Grant and Schuman, 1993). Changes in peripheral pigmentation and peripheral visual fields occur at later stages. When retinopathy is diagnosed early and chloroquine therapy discontinued, visual loss may not recover, although in a small proportion of cases recovery may occur. Dosage appears to be a major risk factor in retinopathy, and generally retinopathy occurs with doses of >250 mg daily for chloroquine phosphate and >200 mg chloroquine sulfate

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to a total >100 g chloroquine base (Marks, 1982), or a maximum dose of 4 mg kg^{-1} day^{-1} (Gottlieb, 1990). There have been many suggestions as to how to monitor patients taking chloroquine or its derivates for the early detection of retinopathy. These have included instructing the patient in how to test their own central vision with a grid chart, coupled with review by an ophthalmologist at six to nine-month intervals. The latter should include biomicroscopy and detailed visual field testing. ERG and EOG are generally considered as adjuncts to biomicroscopy and visual field evaluations, rather than as prime indicators of incipient retinopathy (Henkind *et al*., 1964; Percival and Behrman, 1969). Fluorescein fundus angiography can be used to demonstrate the macular retinopathy (Kearns and Hollenhorst, 1966), but may not be of use for the early detection of retinopathy (Oosterhuis and Boen-Tan, 1969).

Histopathological examination of autopsy specimens from patients with chloroquine retinopathy demonstrated rod and cone destruction, with evidence for pigment migration from the retinal pigment epithelium to accumulate in cells in the nuclear and plexiform layers of the retina, with the perifoveal area being particularly affected (Bernstein, 1967; Wetterholm and Winter, 1964). Ultrastructural observations (Ramsey and Fine, 1972) revealed the retinal ganglion cells to contain membranous cytoplasmic bodies resembling those seen in drug-induced phospholipidosis, and being present in the inner nuclear layer and inner segments of the photoreceptor cells.

Several studies have described a pigment retinopathy that may be irreversible and associated with permanent loss of vision in a dose-dependent manner (Kraff *et al*., 1990; Meier-Ruge, 1965; Okun *et al*., 1963). Although in general dose dependent following peroral dosing, the variability in presentation is possibly due to perturbations in drug bioavailability. Since serum chloroquine concentrations are about four-fold greater following ip dosing compared with po doing (Zhang *et al*., 2003), in order to facilitate an increased rate of drug absorption Gaynes *et al*. (2008) studied retinal light and electron microscopical histopathology in mice following ip chloroquine dosing (10 mg kg^{-1}) daily for 62 days. They found all treated animals developed a well-defined chloroquine retinopathy during the treatment period, suggesting that ip chloroquine dosing facilitates retinopathy due to heightened drug absorption and bioavailability. Thus increased rate of tissue accumulation and bioavailability of chloroquine leads to an increased risk of retinal injury. The increased rate of chloroquine accumulation in the retina may lead to an increased lysosomotrophic effect due to the inability of the lysosome to compensate for a chloroquine-induced elevation of pH through re-acidification of the intralysosomal contents (Reasor and Kacew, 2001). The histopathological lesions produced by chloroquine have been described for several animal species. For example, in the mouse, the outer retinal layers show a loss of the outer plexiform layer and of photoreceptors and photoreceptor nuclei; loss of Müller cells and the presence of membranous cytoplasmic bodies in the inner retina; and focal atrophy with loss of nuclei and pigmentation in the pigmented epithelium. In humans on long-term chloroquine therapy, scanning laser polarimetry has shown that the retinal nerve fibre layer is significantly reduced, and correlated with chloroquine daily dosage (Bonanomi *et al*., 2006).

The details of the mechanism for the production of chloroquine retinopathy are not clearly understood, although progressive phospholipidosis may be a contributory factor (Hallberg *et al*., 1990; Mahon *et al*., 2004; Toler, 2004). Chloroquine is lysosomotrophic, and this may lead to loss of the degradative function of the lysosome, and can block the action of aliphatic esterases including phospholipase A_1 and A_2, resulting in an increase in the phospholipid content in the lysosome (Hostetler *et al*., 1985). Such disruption of lysosomal function has been demonstrated to occur in retinal neurones and the retinal pigmented epithelium (Mahon *et al*., 2004).

7.7.5 Anticholinesterase Retinopathy

7.7.5.1 General Comments

The retina has AChE and cholinergic activities (Hutchins, 1987), and choline uptake and conversion to ACh is documented (Atterwill *et al*., 1975). Also, it is relevant to note that ACh and its agonists cause ERG effects that include an initial increase followed by a decrease in b-wave amplitude, and antiChEs have a similar effect. However, retinal neurotransmission is complex, and at least five neurotransmitters have been identified: GABA, glycine, dopamine, idolamine and ACh. Thus, the potential for the biochemical pathogenesis of retinal lesions may be complex. Boyes *et al*. (1994) noted that several studies have demonstrated retinal degeneration in photoreceptors in the outer nuclear layers. Since these are devoid of cholinergic innervation, it is possible that if OPs are producing this effect they do it indirectly, such as by altering blood flow or its distribution. Examples of documented retinal effects produced by antiChEs are presented below.

That uncontrolled and potentially hazardous exposures to antiChEs can result in adverse effects on the human eye has been discussed with specific reference to Saku disease in Section 5.2.12. Several subsequent human clinical and epidemiological studies outside Japan have also drawn attention to a potential for retinal toxicity in groups having occupational or incidental (due to proximity) exposure to pesticides, including antiChEs. As with Saku disease, exposure could be via a quantitatively variable combination of routes, including local eye contact, inhalation, percutaneous and peroral. Several

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examples of documented accounts of multiple uncontrolled or poorly controlled exposures leading to regional occurrences of optic toxicity follow.

A cross-sectional study of Indian workers spraying fenitrothion (*O*,*O*-dimethyl *O*-4-nitro-*m*-tolyl phosphorothioate), demonstrated macular degeneration in 16% of the workers (Misra *et al*., 1985). Morse *et al*. (1979) described ocular toxicity, including a 22% incidence of macular degeneration, in workers exposed to methomyl ((1-methylsulfanylethylideneamino) *N*-methylcarbamate) in a pesticide manufacturing plant. Fluorescein angiography suggested that the macular lesion was a consequence of injury to the pigment epithelium. In a general cohort study on the possible relationships of retinal degeneration to pesticide application by licensed applicators in Iowa and North Carolina, Kamel *et al*. (2000) found that retinal degeneration was associated with fungicide use (odds ratio of 1.8; 95% confidence interval 1.3–2.6). Risk increased with cumulative days of fungicide use (p for trend was 0.011). Retinal degeneration was also related to the use of organochlorine and carbamate insecticides, but these associations were less consistent. In a subsequent follow-up of this Iowa/North Carolina study, the potential for eye injury in the wives of pesticide applicators was investigated, since they may be incidentally and/or indirectly exposed through carry-home contamination or spray drift from nearby fields (Kirrane *et al*., 2005). Self-reported cross-sectional data obtained by questionnaire between 1993 and 1997 from 31 173 wives were used. Self-reported retinal degeneration was associated with the wife's fungicide exposure.

Many studies have demonstrated that antiChEs can produce abnormal ERG alterations and/or retinal structural damage. Thus, in the cat, chlorphenvinphos ([2-chloro-1-(2,4-dichlorophenyl)ethenyl] diethyl phosphate) at dosages in the range 1–16 mg kg^{-1} increased a-wave amplitude, and at dosages >4 mg kg^{-1} increased b-wave amplitude (Takeda *et al*., 1976). The retina of dogs dosed chronically (two years) with disulfoton (0.5–1.5 mg kg^{-1}) was examined ultrastructurally (Uga *et al*., 1977). Degenerative changes were seen, principally in pigment epithelial cells in the area around the papilla, and myelin-like material was observed in retinal cells. Wistar rats dosed with chlorpyriphos (diethoxy-sulfanylidene-(3,5,6-trichloropyridin-2-yl)oxy-phosphorane) showed ERG changes of decreases in a-wave and b-wave amplitudes of >50% and increased latencies of 10–20%, which were associated with inhibition of plasma, erythrocyte and brain ChE activities. Neuropathological lesions included optic nerve demyelination, partial necrosis of pigmented epithelial cells, and cytoplasmic oedema and degeneration of ciliary muscle myofilaments (Kono *et al*., 1975; Mukuno and Imai, 1973). Although some studies have indicated that there may be a causal relationship between retinal AChE inhibition and the subsequent development of retinopathy, other repeated exposure studies have suggested that such an AChE inhibition may not be a major aetiological factor in the production of retinal toxicity. Thus, in a subchronic (six-month) study in which beagle dogs were given daily po doses of ethyl parathion (2.4, 7.9 or 794 µg kg^{-1} day^{-1}) by gelatine capsule, there was inhibition of plasma, erythrocyte and retinal ChEs at the high dose, but no evidence for functional impairment of ocular function during the six-month period as assessed by ophthalmoscopy, slit-lamp biomicroscopy, refraction, tonometry, ERG and ocular histology (Atkinson *et al*., 1996). In a chronic po study (one year), beagle dogs were dosed with disulfoton (*O*,*O*-diethyl *S*-[2-(ethylthio)ethyl]-phosphorodithioate) in the diet, with average daily consumptions of 0.00, 0.015, 0.121 and 0.321 mg kg^{-1} (Jones *et al*., 1999). Erythrocyte, plasma, corneal and brain ChEs were significantly decreased at 0.121 and 0.321 mg kg^{-1} day^{-1}; the no-effect level (NOEL) for ChE inhibition was 0.015 mg kg^{-1} day^{-1}. However, there were no adverse clinical neurological effects and no ophthalmological findings by ERG, refractivity, tonometry or pachymetry.

A few, more detailed, examples of retinopathy from specific antiChEs follow.

7.7.5.2 Diazinon Retinopathy

In what was essentially an environmental study, Hamm *et al*. (1998) investigated the effect of the OP diazinon on AChE activity and embryonic retinal cells in the teleost (*Oryzias lapites*); concentrations used were 1.8×10^{-5}, 4.4×10^{-5} and 8.8×10^{-5} M. They found that diazinon significantly inhibited AChE in a dose-related manner in whole embryos and in homogenates of retinae. Histology demonstrated that as the retina underwent differentiation into distinct cells, small foci of necrotic cells appeared within the inner nuclear layer, and isolated pyknotic cells were seen in the ganglion layer. Quantitation of the necrotic calls by image analysis revealed a dose-related increase in the number and area of these lesions that was statistically significant at 8.8×10^{-5} M diazinon. Enzyme histochemistry localized AChE activity to regions corresponding to those of sites of necrosis.

7.7.5.3 Fenthion Retinopathy

Various studies have been conducted on the retinotoxic potential of fenthion. Imai (1975a; 1975b) demonstrated ERG changes in rats given acute im injections of 5, 25 and 50 mg kg^{-1} fenthion. At the low dose there was an increase in the amplitude of the a- and b-waves that persisted up to 10 days postinjection; thereafter latency and peak times were lengthened and did not return to normal until 40 days postinjection. At the mid-dose, the a- and b-waves increased, and by postinjection day 4 the latency and peak times increased; the amplitudes

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recovered by 60 days, but the peak time remained increased. At the high dose the a- and b-wave amplitudes were decreased and the latency and peak times were increased; no recovery of amplitudes was noted. At doses $>50\,mg\ kg^{-1}$, fenthion produced dose-dependent ERG changes, a decrease in retinal ChE activity, fundoscopic evidence of retinal degeneration and histopathological changes in the pigmented epithelium and sensory retina (Imai, 1977; 1978; Imai *et al*., 1983; Miyata *et al*., 1973). Imai (1975a; 1975b) studied the effects on the ERG and retinal ChE in rats of acute sc dosages of fenthion in the range 0.0005–500 mg k^{-1}. All rats at $500\,mg\ kg^{-1}$ died. At 50 and $100\,mg\ kg^{-1}$, subnormal ERG activity was obtained; $25\,mg\ kg^{-1}$ was a transitional dose (ERG normal), and at $<25\,mg\ kg^{-1}$ the ERG was supernormal. Minimal change was seen at the lowest dose of $0.005\,mg\ kg^{-1}$, and thus a NOEL for the ERG was not obtained. Retinal and cerebellar AChE activity was inhibited in a dose-related manner in the $0.5–100\,mg\ kg^{-1}$ range. Thus, ERG changes were observed at acute fenthion doses lower than causing inhibition of retinal AChE activity. Imai (1975a; 1975b) also studied the effects of acute sc dosages of fenthion in rats at a more restricted range of 0, 5, 25 and $50\,mg\ kg^{-1}$. At $5\,mg\ kg^{-1}$ supernormal ERGs were recorded that increased until 10 days postdosing and then decreased to normal by two months. A transitional ERG was confirmed at $25\,mg\ kg^{-1}$, and at $50\,mg\ kg^{-1}$ a subnormal ERG was obtained that was still present at 66 days postdosing. Measured serum BChE activity decreased to approximately 15% at four days postdosing. Retinal AChE activity was decreased for up to four days, and in the high dose group the ERG changes corresponded with the retinal AChE recovery curve. A repeated exposure study was conducted with fenthion ($50\,mg\ kg^{-1}$) given sc every four days to Long Evans black rats for one year (Imai, 1977; Miyata *et al*., 1979; Uga *et al*., 1979). By three months the ERG was subnormal, and at nine months the ERG a-waves disappeared. Fundoscopy revealed pallor of the papilla. Histology demonstrated disappearance of the pigmented epithelial layer, and outer granular layer. Ultrastructural examination showed a complete disappearance of photoreceptor cells.

7.7.5.4 Tacrine Retinopathy

Tacrine (1,2,3,4-tetrahydroacridin-9-amine) is a ChE inhibitor of potential use in Alzheimer's disease, based on the ability of the compound to facilitate cholinergic function in the CNS via direct stimulation of M- and N-receptors through AChE-inhibition (Avery *et al*., 1997; Newhouse *et al*., 1997). Since ACh plays a basic role in visual function (Famiglietti, 1983; Hutchins, 1987; Ross *et al*., 1985), and because some visual symptoms and optic nerve degeneration occur in Alzheimer's disease patients (Hinton *et al*., 1986), and decreased ACh found in the CNS may also occur in the retina (Strenn *et al*., 1991), it was considered appropriate by Alhomida *et al*. (2000) to investigate the effects of tacrine on human retinal AChE activity. *In vitro* studies on human retinal homogenates showed a concentration-dependent inhibition of AChE activity by tacrine, with an IC_{50} (concentration causing 50% inhibition) of approximately 50 nM. The Michaelis–Menten constant (K_m) for the hydrolysis of acetylthiocholine iodide by retinal AChE was 0.12 mM, which increased in the presence of tacrine. The V_{max} was determined to be $2.23\,\mu mol\ h^{-1}$ mg $protein^{-1}$, and was decreased by treatment with tacrine. Dixon and Lineweaver–Burk plots, and their secondary replots, indicated that the inhibitory process of tacrine on human retinal AChE was a combination of competitive and noncompetitive processes.

7.8 Extraocular Muscles

Toxic and pharmacological effects may involve extraocular muscles as a result of certain materials being applied topically to the eye and with some materials reaching them from the systemic circulation. Those materials causing myotoxic effects following local topical applications do so because they have the appropriate physicochemical characteristics for penetration to the periocular area, and are of sufficient potency to cause effects from the comparatively small amounts attaining the extraocular muscles by this route. Effects from topical application are well demonstrated by antiChEs, in part because of the very high AChE activity in the extraocular muscles (Hikita *et al*., 1973; Mukuno and Imai, 1973; Mukono *et al*., 1973). Thus, antiChE eye drops used in the treatment of glaucoma commonly cause a few minutes of eyelid twitching.

Systemic toxicity to the extraocular muscles has also been demonstrated with antiChEs. For example, Mukuno and Imai (1973) dosed dogs long term with disulfoton and found decreased extraocular muscle ChE activity, and ultrastructurally there was degeneration of nerve fibre bundles, neuromuscular junction, and muscle fibres, with mitochondrial degenerative changes. Dogs dosed with disulfoton in the range $0.5–1.5\,mg\ kg^{-1}\ day^{-1}$ had dosage-related inhibition of AChE and BChE in the extraocular muscles, and dose-dependent decreases in retinal ChE activity (Hikita *et al*., 1973). Suicidal poisoning using OPs has been reported to cause bilateral third or sixth nerve paralyses in 8/200 patients and facial nerve paralysis in 12/200 (Wadia *et al*., 1974).

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Cutaneous Toxicology

Steven J. Hermansky

CONTENTS

1 INTRODUCTION

This chapter describes the general principles of the toxicological responses of the skin to cutaneously encountered xenobiotics. This discussion will be augmented by an abbreviated description of the methods used in the predictive testing currently used to identify potential cutaneous irritants and sensitizers. However, because of the large amount of information available, there will be no attempt to make these discussions complete. Several comprehensive references are available (Hobson, 1991; Marzulli and Maibach, 1991c; Chilcott and Price, 2008).

When addressing the effect of chemical substances on the skin, there are several fundamental factors to be considered. These include such variables as humidity, temperature, air currents (wind), exposure to radiation (including light), nutrition of the organism, friction, pressure, trauma (lacerations) and, perhaps, electromagnetic current. These variables influence the overall appearance and function of the skin, as well as the response of the skin to toxic insult. Physical agents (friction, pressure, electrical currents and trauma), thermal factors (excessive heat and cold), and living organisms (bacteria and parasitic insects) produce pathological responses in the skin. Literature often deals with toxic responses of the skin to chemicals as a series of discrete, mechanistically based entities. In reality, there can be several concurrent, pathological processes that influence the immediate response of the skin to chemicals. This confounds the diagnosis and treatment of the skin ailments. For example, chemical irritants have been shown to increase the allergic contact dermatitis response and, thus, may have profound effects on the

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diagnosis of skin ailments (McLelland *et al*., 1991). Furthermore, physical trauma to the skin, such as pressure or friction, can alter the response of the skin to chemical irritants, while the converse is also true (Susten, 1985; Lebwohl and Herrmann, 2005). Genetic factors can also influence an individual's susceptibility to irritation following chemical exposure (Ishii *et al*., 1990; Farage *et al*., 2006; McGrath and Uitto, 2008).

This chapter will deal primarily with the effects of chemicals, natural or synthetic, on the skin and will not, in general, address the interaction of the resulting toxicological responses with other pathological processes. Interesting developments are occurring in the realm of thresholds of skin effects, both irritant and sensitization, that may change many aspects of risk assessment and management of skin diseases in the near future (Safford, 2008). The European REACH (Registration, Evaluation and Authorization of Chemicals) project requires the review and registration of thousands of chemicals and products and will also contribute to a significant revolution in the global management of chemicals and risk assessment (Louekari *et al*., 2006). As such, the entire area of toxicology, including toxic responses of the skin, is a rapidly developing and evolving field.

Toxic responses of the skin are of significant importance to the organism because the skin is one of the largest organs of the body and possesses functions vital to survival. In humans, the skin comprises approximately 10% of the normal body weight. The skin is constantly exposed to environmental conditions and consequently possesses several important functions related to the interaction of an organism with its surroundings. These include protection from adverse conditions, regulation of the body temperature and a role in the retention or loss of body water (Rongone, 1987; Norlen, 2006). Several biochemical functions have also been attributed to the skin, including metabolism, melanin production, and protein and lipid synthesis and metabolism (Rongone, 1987; Lerner and McGuire, 1961; Johnson and Fusaro, 1972). The skin is also known to act as a storage depot for glucose (primarily when blood glucose concentrations are elevated).

The skin displays several toxicological responses to chemical and/or physical insult. Responses produced by insults of vastly different mechanisms and resulting from fundamentally different physiological and structural changes in the skin often appear outwardly to be very similar. Therefore, the clinical classification of skin ailments has historically been by morphology (appearance) rather than by mechanism. The functional changes produced by the insult to the skin and the impact of these changes on the behaviour and survival of the organism are not well understood. Mechanistic research, often employing *in vitro* skin equivalent models (see later in this chapter), into these changes is a rapidly evolving field (Roberts and Aptula, 2008; Uchino *et al*., 2008).

A significant number of environmentally related diseases and injuries involve the skin. Skin problems constitute approximately 34% of all occupational diseases, but may be as high as 70% in some occupations, including healthcare workers (Mathias, 1985; Hogan and Lane, 1986; Suskind, 1990; Kampf and Loffler, 2007). These statistics are based on reported incidences of skin diseases and injuries and generally omit laceration, burns and reactions of short duration. Thus, actual numbers have been estimated to be anywhere from 10- to 50-fold higher than medically reported (Mathias, 1985).

In addition to occupational hazards, surveys have indicated that greater than 20% of women and 10% of men claimed to have experienced adverse reactions to cosmetics and toiletries in the preceding five years (DeGroot, 1987; Orton and Wilkinson, 2004). Generally, the incidence of nonoccupation adverse reactions of the skin to xenobiotics is unknown. However, owing to the potential for exposure to a wide variety of chemicals, it can be speculated that nonoccupational exposure to xenobiotics results in a significant amount of adverse reactions. The cosmetic industry recognizes these reactions, and is constantly attempting to minimize the risk of sensitization and irritation (DeGroot *et al*., 1988).

2 STRUCTURE OF THE SKIN

The skin is a heterogeneous organ that consists of two different layers derived from separate germ-cell lines (**Figure 1**). The thin, outermost layer, or epidermis, is comprised primarily of loose connective tissue. The innermost layer or dermis is of variable thickness that contains both connective and adipose tissue. Normal skin also contains appendages, blood vessels and neuronal components.

2.1 Epidermis

This outer layer of the skin is a stratified cellular tissue derived from the ectoderm and has no direct blood supply. Therefore, the epidermis must be completely penetrated and the dermis injured for bleeding to occur. The epidermis receives all of it nutrients from the dermis. The primary cells of the epidermis (keratinocytes) are constantly evolving and forming a cornified, protective barrier or stratum corneum (the horny scales). The stratum corneum is several layers thick and composed of flattened keratinocytes that are no longer viable or metabolically active. The intercellular spaces in the

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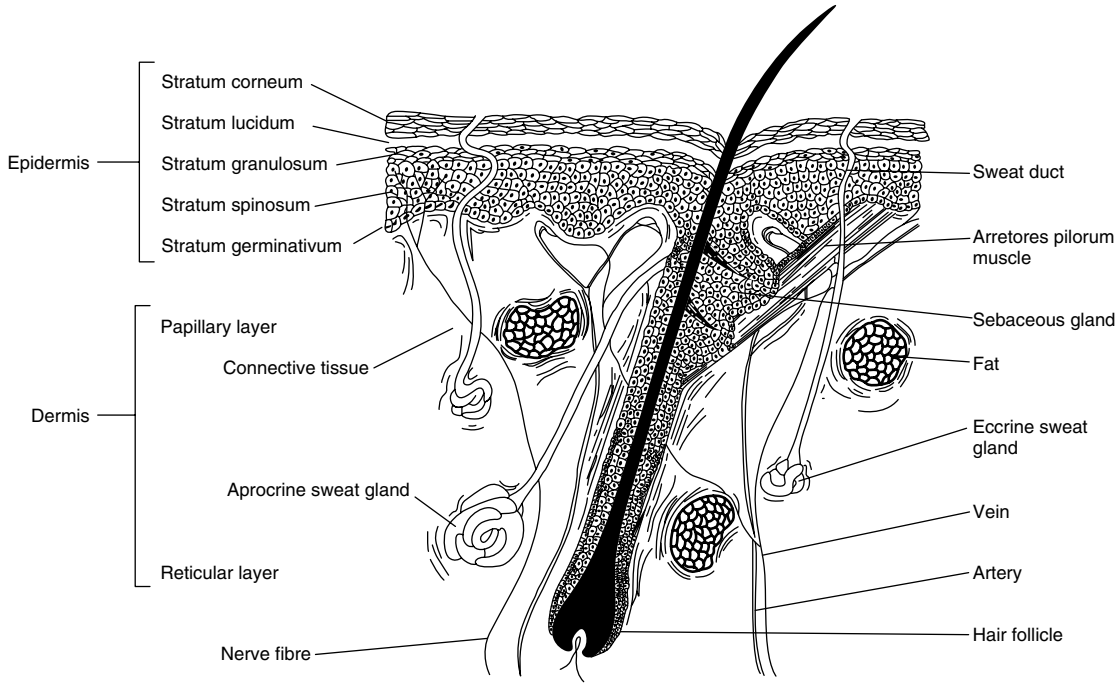

Figure 1 Schematic representation of the structure of the skin.

stratum corneum are filled by a matrix of materials including ceramides (a class of lipids that do not contain glycerol). This combination of cells and intercellular components plays a key role in the protective nature of the epidermis.

The primary keratinocyte proteins are keratin and filaggrin, that together contribute approximately 80–90% of the mass of the epidermis. Recently, a major breakthrough in the genetics of skin barrier diseases was achieved by the sequencing of the genes of the epidermal differentiation complex (EPC) and identification of loss-of-function mutations of these genes. Filaggrin plays an integral part of the epidermis in engineering and maintaining the barrier function (Rodriguez *et al*., 2008). Mutations that result in significant changes in the structure and function of profilaggrin and, ultimately, filaggrin represent an important risk factor in allergic disease (McGrath, 2008). These discoveries and advancements will ultimately add important aspects to genetic counseling and management of skin diseases.

Beneath the stratum corneum are several layers of viable, extremely metabolically active keratinocytes that form the remainder of the epidermis. Lying adjacent to the dermis is the innermost or basal cell layer (the stratum germinativum), that consists of a single layer of columnar epithelial cells. Presumably, keratinization begins in this layer by synthesis of fibrous prekeratins. When the cells of the basal cell layer divide, they travel outward to become part of the prickle cell layer (the stratum spinosum), which is several cell layers thick. By the time the cells reach the outermost areas of this layer, aggregated filaments extend the length of the cell. As the cells move closer to the surface of the skin, they form protein aggregates and keratohyalin granules and increase in size to form the stratum granulosum, which is two to four cell layers thick. In this layer, the number of cytoplasmic granules increase and the nuclei of the cells either break apart or are dissolved and the cells die.

Between the outermost layer (the stratum corneum) and the stratum granulosum lies the stratum lucidum, which varies in size and appearance. The stratum lucidum is most prominent in thick skin such as that found in the ball of the foot. Eleidin, presumably a product resulting from the transformation of keratohyalin, is found in this layer and is transformed into keratin as the stratum corneum is formed. The stratum corneum, which is continually being worn away, is constantly replaced in this manner. In the stratum corneum, the keratinocytes are filled with a filamentous network of proteins, including keratin and filaggrin. These proteins are buried in an array of mucus and lipids encapsulated by the chemical-resistant cell membrane. It has been estimated that it takes 26–28 days for a cell from the basal cell layer to progress through the layers of the skin to the outmost region of the stratum corneum (Rongone, 1987).

Approximately, 10% of the cells of the epidermis are Langerhans cells, which are dendritic cells primarily responsible for antigen recognition (Emmett, 1991; Kalish, 1991; Roberts and Aptula, 2008). New research is expanding the understanding of the role and biological importance of the Langerhans cells. These cells are now known to interact with viruses and play a role in viral

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immunity and infection including HIV, herpes simplex and varicella-zoster virus (Cunningham *et al*., 2008).

2.2 Dermis

Beneath the thin, protective epidermis is the much thicker dermis. This inner layer of the skin is derived from the mesoderm and is composed of connective tissue, fibroblasts, collagen and elastic fibres. The dermis serves as a supporting unit for the epidermis and constitutes 90–95% of the mass of human skin. The dermis is composed of two layers. The papillary layer (so named because of its prominent papillae) is the outer, thinner layer that contains a finer network of collagen fibres than the inner or reticular layer.

Capillaries are prominent around the papillae of the outer layer of the dermis. The exchange of nutrients and waste products between the blood and epidermis occurs by diffusion through the large surface area of this section of the dermis. Although not completely understood, it appears that the dermis may also serve as a reservoir of nutrients for the epidermis.

The predominant cell in the dermis is the fibroblast, which synthesizes fibrous proteins and several granular substances including hyaluronic acid and mucopolysaccharides. The dermis also contains several other cell types including macrophages, histiocytes, mast cells and fat cells. Blood vessels, nerve fibres and nerve endings that are associated with the skin are also located in the dermis.

2.3 Appendages Associated with the Skin

Two types of skin, differentiated by their structure and cutaneous appendages are recognized. Glabrous skin, found only in the palms and soles, has a thick, highly protective stratum corneum as well as sweat glands and encapsulated nerve endings. Hairy skin, found throughout the rest of the body, has a relatively thin stratum corneum and contains hair follicles, sweat glands and sebaceous glands but does not contain encapsulated nerve endings. Sweat glands secrete an aqueous solution (sweat) that aids the body in cooling by evaporation. There are two types of sweat glands. The eccrine sweat glands are located over the entire surface of the body and have ducts that lead directly to the epidermal surface. Secretion by the eccrine sweat glands is under autonomic control and sweating is induced by emotional or thermal stimuli or in response to certain tastes. Apocrine sweat glands are located only in the area of the axilla, genitalia and nipples and have a duct that leads to a hair canal. The secretory product of the apocrine sweat glands is initially odourless, but it acquires odour following degradation by bacteria found on the surface of the skin. There is no function of the apocrine sweat glands in humans except for possible communication within or between species (as a sex attractant or as a territorial marker).

Sebaceous glands are generally associated with hair follicles and, as with hair follicles, are found over the entire surface of the body except for the glabrous skin of the palms of the hands and soles of the feet. Sebaceous glands are under hormonal control and excrete sebum, a mixture consisting primarily of lipid breakdown products, into the hair canal. The process of sebum secretion, termed holocrine secretion, does not account for all lipids found on the surface of the skin. Some lipids are contributed by the desquamation of keratinocytes from the stratum corneum. Therefore, the quantity of lipids on the surface of the skin is dependent on the local concentration and activity of the sebaceous glands, as well as the amount of desquamation in the area. In areas where the sebaceous glands are numerous and actively secreting sebum, nearly 90% of the lipids on the surface of the skin can be derived from sebum. The primary function of sebum is as an antibacterial agent, but the lipid components may also act as a waterproofing compound (primarily in hairy mammals) and may prevent the loss of water from the epidermis in humans (Monteiro-Riviere, 1991; Schittek *et al*., 2008).

Hair follicles have three primary layers, including the inner root sheath, outer root sheath and the connective tissue sheath. Hair follicles undergo regular phases of growth (anagen) and atrophy (catagen) separated by periods of inactivity (telogen). The phases of hair growth are generally recognized to be under complex hormonal control. The cells of the deepest part of the hair follicle (sometimes termed the germinal matrix) are one of the most metabolically active areas of the body (Emmett, 1991). Therefore, the response of the skin to cutaneously encountered xenobiotics may be affected by the amount and growth of hair present at the site of contact. For this reason, hair growth should be synchronized within animals in a cutaneous toxicity study. This is best accomplished by clipping all animals the day before treatment (and at regular intervals during a repeated study), which induces a new hair growth cycle in all animals. This is especially important in studies of tumour initiation and promotion where metabolism can be critical to the outcome of the study (Monteiro-Riviere, 1991).

The nails and hair are important to the forensic toxicologist in the identification of poisonings, particularly involving arsenic (Blanke and Poklis, 1991). Biomonitoring, or the process of inferring chemical exposure through measurement of exceedingly low levels of chemicals in biological systems, is increasingly playing a role in the evaluation of environmental exposures. Data involving nails, hair, urine and other biological samples collected from humans is being used more frequently by researchers to measure and evaluate exposure to various

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chemicals (Esteban and Castano, 2008). Unfortunately, these data are often misused by the media, regulatory agencies and legislative bodies, who misinterpret exposure, regardless of how low, as an indicator of toxicity. As such, the importance of biomonitoring, including that using hair and nails, is likely to expand in the future.

Piloerection, where the hair tends to stand vertical to the plane of the skin, is an important effect of some neuroactive agents. This condition is produced following stimulation of the adrenergic receptors and resulting contraction of the arrectores pilorum muscle (Weiner, 1980). Other than these specific examples, these appendages are not generally of major concern to the toxicologist and will not be addressed further in this chapter.

3 FUNCTION OF THE SKIN

As stated earlier, the skin is the primary location where an organism interfaces with the physical, chemical and biological environments (Suskind, 1977; Proksch *et al*., 2006). It is vulnerable to toxic effects from agents encountered in the environment, especially those that may be absorbed into and through the skin. Thus, the primary function of the skin is not absorption, but rather the protection of the body (Zesch, 1987; Suskind, 1990; Rodriguez *et al*., 2008).

The keratinized stratum corneum, which exists as a layer of loosely packed dead epidermal cells, acts as a diffusion barrier against the absorption of chemicals into the skin (Dugard, 1987; Rawlings, 2003). Chemicals encountered by the intact skin may slowly pass into and then through this diffusion barrier and be gradually released into the body, eventually producing toxicity. The intact stratum corneum also provides a significant defence against the loss of water from the body. Additionally, the dermis contains an abundance of water that is systemically available during times of water deprivation. The dermal blood supply is substantially larger than that required for the metabolic activity of the skin. Therefore, radiation of heat from the body, controlled by the rates of blood flow to the skin, as well as the evaporation of sweat, aid in the thermal regulation of the body. Elastic and collagen fibres provide the skin with a physical barrier against trauma (Suskind, 1990) and a barrier to invasion by micro-organisms (related, in part, to the secretory products of the sebaceous glands).

Melanin, produced by melanocytes, is found in varying concentrations in the skin. Following production by the melanocyte, the melanin is transferred to keratinocytes in a specialized organelle (the melansome). These organelles are normally broken down in light-skinned people, but not in dark-skinned people. It is unknown if pigmentation plays a significant role in modifying chemical damage to the skin. However, melanin certainly plays a role in protection against ultraviolet radiation (Emmett, 1991). A dark-skinned person may be protected from the damaging effects of the sun similar to a light-skinned person who has applied a sun protection factor (SPF) 4 sunscreen. However, this is not sufficient protection to prevent skin damage after significant sun exposure and, as with fair-skinned individuals, persons of colour should be encouraged to protect themselves from overexposure to the sun. Finally, melanin is also known to act as an oxygen scavenger and may decrease the concentration of mutagenic and carcinogenic reactive oxygen species found in the skin (Nordland *et al*., 1989).

The skin, particularly the epidermis, is capable of metabolizing significant amounts of chemicals. This can have a protective effect in metabolizing potentially harmful xenobiotics to less toxic metabolites, or a detrimental effect by activating less toxic compounds to more toxic metabolites (for example, proximate carcinogens can be metabolized to highly reactive ultimate carcinogens). The metabolic activity of the skin may also produce harmful results by changing an innocuous chemical into a hapten and inducing allergic contact dermatitis. Thus, the metabolizing capability of the skin is important to the pharmacology and toxicology of externally contacted xenobiotics and cannot be discounted in the evaluation of the potential health effects of environmental compounds.

The maintenance of an intact and fully functional skin is essential to survival. Alteration of the skin by any of several mechanisms, induced by physical, biological or chemical means, can have serious implications for the health and wellbeing of the organism.

4 DERMATOLOGICAL EFFECTS OF TOXIC AGENTS

Cutaneous exposure of humans or animals to a xenobiotic substance may result in a toxicological reaction or dermatitis (defined as any pathological process of the skin). Toxic agents can include medicinal compounds, food constituents, environmental agents (natural or synthetic contaminants) and a host of other sources. Exposure can be accidental, suicidal or homicidal (Espinoza and Fenske, 1988). Effects of cutaneous exposure may be apparent immediately or may develop after days, weeks or years, and the severity may vary from mild ‘subjective irritation’ where no gross or histological changes are observed (for example, the burning and stinging experienced following a topical application of astringents) to life-threatening effects (Bruner, 1991). By a wide margin, the most common reactions to agents that contact the skin are inflammatory

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reactions to irritants, allergens and photosensitizers (Suskind, 1990).

In general, a chemical substance must be absorbed into at least the outermost layers of the skin to produce a reaction and, as discussed above, the skin (especially the stratum corneum) has evolved several protective mechanisms to retard the movement of chemicals. One of the most important mechanisms retarding the movement of chemicals in the skin is the complex mixture of stratum corneum lipids (Bouwstra *et al*., 2001). The most characteristic features of the stratum corneum lipid composition are its heterogeneity, presence of a high proportion of saturated lipids, large amounts of cholesterol and complexity of ceramide chemistry (Rawlings, 2003). Cholesterol modulates the mobility of the lipids and is important to the barrier function of the skin.

In spite of the complexity of the lipids that comprise much of the stratum corneum, the rate and extent of absorption and subsequent skin reaction depend on several externally determined or extrinsic factors and two primary intrinsic factors determined by characteristics of the skin (Dugard, 1983; Bouwstra *et al*., 2001; Fluhr *et al*., 2008). The extrinsic factors are often related to the chemistry of the substance in contact with the skin and include chemical concentration, molecular size, ionization and polarity (which affect lipid solubility), p*K* and pH of the chemical (Wester *et al*., 1985; Berner *et al*., 1988).

Environmental variables are also considered extrinsic factors and can play an important role in the irritation potential of a chemical. For example, low environmental humidity or increased humidity and warmth secondary to occlusion may enhance the irritation potential of a chemical (Rothenborg *et al*., 1977). Other extrinsic properties may be related to the vehicle in which the chemical is dissolved or suspended when it contacts the skin. These factors can include the partition coefficient of the chemical between the stratum corneum and the vehicle, antioxidants and preservatives within the vehicle, the corrosive properties of the vehicle and several other properties of the vehicle that affect the chemical or the skin (Zesch, 1987; Suskind, 1990). New research is shedding additional light onto the important contribution of the vehicle to the effectiveness and irritation potential of topical drugs and cosmetics (Gloor, 2004; Surber and Smith, 2005). Intrinsic factors are related to the properties of the skin and are primarily concerned with a diffusion constant for the chemical and the thickness (number of cell layers) of the stratum corneum. Intrinsic factors can be related to genetic background, age, sex, race, concomitant disease states, neurological factors, dietary state of the individual and medication (Mathias, 1987), as well as the location in the body where the chemical contact occurs.

It is generally recognized that the skin reactivity of children may be higher than in adults (Marcussen, 1963). Conversely, the skin of the elderly is often dry and irritated (itchy) and may be slower to react to an irritant (or the irritation may be less perceptible) than in younger adults. The mechanisms of these age differences are poorly understood, but the decreased water content and altered lipid composition may be important in the decreased reactivity of elderly skin (Elias, 1981). Furthermore, epidermal proliferation tends to be decreased in the elderly resulting in changes to the stratum corneum and overall epidermal thickness.

Clinically, black (African American) skin appears to be less irritable than white skin (Weigand and Gaylor, 1974). However, comparisons between black and white skin irritation based on visual evaluation of skin changes may not be valid because of the difficulty in identifying erythema in black skin. Black skin may contain a thicker (more cell layers) stratum corneum and altered electrical skin resistance as compared with white skin (Lammintausta and Maibach, 1988). Grimes *et al*. (2004) demonstrated differences between black and white skin only for the degree of photodamage and hyperpigmentation, but no differences for all other measures evaluated, including sebum concentration, pH, moisture or transepidermal water loss (barrier function). Thus, differences in irritability may be more perceived than real.

While it has been suggest that women are more susceptible to skin irritation than men (especially to soaps and detergents), this is most likely related to the greater exposure to irritants (both in frequency and quantity) and not to any real difference in skin irritability. However, clinical experience has suggested that women may have an increased susceptibility to skin irritation during the premenstrual period (Lammintausta and Maibach, 1988; Farange *et al*., 2005).

Most skin irritation appears to be due to solids (primarily powders) and liquids that contact the skin. Skin irritation from contact with airborne particles or vapours is possible, but rarely described. This may be due to the fact that irritation from airborne substances may be missed clinically (Lammintausta and Maibach, 1988) except in cases of occupational exposures where this issue is becoming more recognized. Most skin irritation resulting from contact with a liquid or powder is attributed to chemical reactions, but the physical characteristics (including sharp or rough edges) of the particle within a powder or suspension may play an important role in the irritability of the substance. The repeated damage to the outer layers of the skin by such particles can also significantly increase concurrent chemically induced irritation, which can confound the diagnosis and treatment of skin ailments.

While the diagnosis of dermatological reactions and/or toxicity is difficult because of the similar appearances of many types of skin changes, it is most useful for this discussion to classify these effects by their mechanism of action: cutaneous immunologically mediated, or mediated by unknown mechanisms.

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Table 1 Selected irritant-induced responses that may be observed

Exfoliation: dandruff-like scales
Excoriations: superficial scratches or cracks on the skin
Fissures: deep cracks in the surface of the skin
Ulcer: open sore
Erythema: reddened skin
Oedema: raised, swollen skin
Eschar: scab formation
Ecchymosis: haemorrhage into the skin or bruising
Necrosis: areas of dead skin

4.1 Irritant Dermatitis

Irritant dermatitis resulting from contact of the skin with chemicals is the most common form of xenobiotic-induced skin irritation. Nevertheless, the exact nature of the injury is not well defined, although research into the biochemistry of normal and irritated skin is demonstrating important differences (Agner and Serup, 1987; Lebwohl and Herrmann, 2005). In general, the concentration of the chemical, circumstances of contact (for example, occlusive conditions and length of time of exposure) and presence of other physically acting agents may be more important than how deeply the offending agent diffuses into the skin (Zesch, 1987). The current status of the stratum corneum and, therefore, the effectiveness of the skin barrier also play an important role (Kampf and Loffler, 2007). Virtually any chemical substance, under the appropriate conditions, can produce cutaneous irritation. In both humans and laboratory animals, erythema (redness) and oedema (swelling) are the most common signs of contact irritation (especially in animals). Vesiculation, scaling and thickening of the epidermis can also be observed (**Table 1** lists a number of irritant-induced clinical responses that may be observed on the skin).

4.2 Chemical Burns

Following contact of the skin with an irritant, a continuum of effects occurs, but two general patterns of reaction can be identified. The first involves the contact of skin with the corrosive agent of sufficient strength (often an acidic or alkaline solution) to cause an active and rapid response (often rapid cell death). In this 'primary irritation', pain, heat and erythema are often observed clinically. Microscopic changes can include varying degrees of tissue destruction, vascular permeability changes (often resulting in oedematous changes) and inflammatory cell infiltration. The extreme of this type of injury is often described as a 'chemical burn' and the exact appearance of the lesion (both on gross and microscopic observation) varies with the offending agent. Eliminating the chemical from contact with the skin is imperative to limiting the amount of tissue damage. This is usually best accomplished by repeatedly washing the affected area with copious amounts of soap and water. However, some xenobiotics may react vigorously with water (for example, calcium oxide), that could result in further tissue damage.

Knowledge of the chemistry of the compounds that may contact the skin is useful to the prevention of serious injury. Specific therapeutic interventions, based on the chemistry of the compounds, are required for many cutaneous irritants. Therefore, it is important for industrial health and safety personnel and medical practitioners, as well as individuals handling these substances to be aware of the potential dangers and recommended treatments in the case of accidental exposure. Every employer in the USA is required by the US Labor Code, Section 6390, to maintain a material safety data sheet (MSDS) for each substance located and used at the work site (Adams, 1990). These documents provide information on the precautions for the use of a substance and recommendations for treatment in case of accidental exposure. Importantly, the MSDS is not a comprehensive reference and substances in mixtures at a concentration of less than 1% are not required to be listed at all. Nevertheless, the MSDS indicates initial emergency procedures and usually lists a telephone number to gain additional information. All employees should be trained on the proper method of retrieving and reading an MSDS in their workplace.

4.3 Cumulative Dermatitis

At the other end of the cutaneous irritation scale is repeated contact with the skin with mildly to moderately irritating substances (often termed marginal irritants). This type of chemical contact often results in slow, insidious changes in the skin that may result in thickening with or without wrinkling and/or changes in pigmentation. Many of the changes observed are the indirect effects of cutaneous inflammation and may not result from direct cellular damage caused by the chemical. Frequent and extensive exposure to a marginal irritant may produce a form of desensitization known as 'hardening' (Rothenborg *et al*., 1977; Lammintausta and Maibach, 1988). This reaction appears to be chemical specific and the biological mechanisms are unclear, but thickening of the skin following repeated chemical exposure may be an important factor. New research has demonstrated differences in the lipid composition of the stratum corneum after experimental induction of hardening revealing a possible biochemical mechanism to this phenomenon (Heinemann *et al*., 2005).

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Table 2 Selected microscopic changes that may be observed following exposure to irritants

Herperkeratosis: thickening or overgrowth of the stratum corneum
Parakeratosis: retention of the nuclei in a thickened stratum corneum
Acanthosis: increased thickness of the epidermal layer of the skin
Epidermitis: inflammation, as evidenced by the presence of inflammatory cells, in the epidermis
Dermal oedema: presence of excess intercellular fluid in the skin
Dermatitis: inflammation, as evidenced by the presence of inflammatory cells, in the dermis
Folliculitis: inflammation of the hair follicles
Ulceration: loss of outer layer(s) of the skin, exposing the dermis or subcutaneous tissue
Congestion: swelling of the small blood vessels supplying the skin resulting in decreased blood flow to the affected area
Hydropic degeneration: presence of excess entracellular fluid within the cells of the epidermis
Dermal haemorrhage: presence of blood (fluids and cells) in the extravascular space of the dermis
Dermal fibrosis: abnormal formation of fibrous connective tissue

Microscopic changes that occur following repeated contact of the skin to marginal irritants include thickening of the entire epidermis (hyperplasia) and hyperkeratosis (thickening of the stratum corneum). This type of pathological change may be referred to as cumulative insult dermatitis and the cause may be difficult to elucidate because of the many factors usually involved and the often low acute irritancy of the offending agent(s).

A list of some common microscopic changes that may be observed following exposure to irritants is presented in **Table 2**.

4.4 Characterization of Skin Irritation

The concept of irritation or inflammation has a very long history, mainly because of its close relationship to the perennial human problem of infection. Egyptian scrolls, dated perhaps as early as 2650 BC, have several references to a word indicating inflammation that is associated with wounds (Ryan and Manjo, 1977). It was not until medical advances in the late nineteenth and early twentieth centuries that the separation of the inflammatory response from infections occurred.

The current description of the response of the skin to an irritant has evolved over the past 100 years and continues to change as advances in technology allow further insight into the process. One of the most important concepts, initially grasped in the later 1800s, is that the inflammatory response is a process and not a state.

In 1889, Julius Cohnheim described inflammation as a series of changes to the affected area, including redness, swelling, pain, warmth and altered function. Each of these descriptions was accompanied by a discussion of microscopic changes and causative factors that have been modulated by modern medicine, but not significantly altered. While these descriptions were intended to describe general inflammation, they describe skin inflammation in response to irritation equally well.

Attempts in the past century to further characterize skin irritation have led to greater awareness of the causes of the irritation and specific cutaneous changes following contact with an irritant. It is now clear that the initial changes in the skin can be related to a chemical mediator (for example, histamine, serotonin or the kinins) released into the vasculature in response to chemical contact or direct vascular damage cause by various mechanisms (e.g. a thermal or chemical burn).

Several authors have attempted to characterize the sequence of events following the contact of a chemical irritant with the skin. Steel and Wilhelm (1966; 1970) described skin irritation produced by organic solvents in terms of three phases of the response. These authors considered the first phase of irritant inflammation to be an increased permeability and blood flow to the region between zero and two hours. The increased blood flow and permeability appeared to occur simultaneously. Inflammatory cells generally infiltrated the area within five minutes. The initial erythematous response faded rapidly and vascular permeability slowly returned to normal by 90 minutes. The second phase of irritation occurred between two and 10 hours after exposure and was defined as a return to normal vascular permeability, accompanied by leucocytosis and the return of erythema (increased blood flow to the area). The third phase of the irritant response occurred some time between 10 and 36 hours and consisted of increased vascular permeability.

More recent studies do not support the concept that chemical irritation can be separated into distinct phases (Patrick *et al*., 1985; Agner and Serup, 1987). The irritation of the chemical and the resulting effects on the skin appear to be related to the specific irritant applied. Furthermore, skin irritation appears to be produced by multiple mechanisms resulting in variable patterns of response. Advances in analytical testing and *in vitro* modelling of the skin structure are providing additional information on the baseline changes that occur during and after the appearance of skin irritation. This research continues to provide examples of cellular and molecular changes that occur in response to skin irritation (Fluhr *et al*., 2008; Rawlings, 2003).

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As indicated earlier, the ability of a compound to provoke irritation is related to the chemical stability, purity, unique chemical properties and current condition of the skin barrier (Mathias, 1987). Several factors, including sunlight, temperature and oxidation (extrinsic factors) may alter the chemical, rendering it more or less irritating. Furthermore, alterations of intrinsic factors within an individual or population may contribute to the apparent irritability of a chemical (Agner and Serup, 1989a). Variations of these factors are especially important with marginal irritants and can partially account for the wide range of reported reactions to certain compounds.

4.5 Skin Sensitization

4.5.1 Definition and Description

Skin sensitization (allergic contact dermatitis) is the result of an elaborate interaction between the complicated pathophysiological mechanisms of Type IV cell-mediated immunity and environmental sensitizers (allergen). Characteristic structural changes in the skin are induced following contact of the skin with an allergen (Schmidt, 1989). While skin changes due to irritant dermatitis are generally produced by a direct interaction of the chemical with the constituents of the skin, allergic dermatitis results when the chemical elicits an immune reaction that, in turn, results in changes in the skin. The diffusion of the compound into the skin is often considered the determining parameter in whether a compound will produce allergic dermatitis (Zesch, 1987), but many factors are at play and the ability of a compound to covalently bind to a carrier protein may be the major determinant of skin sensitization potential (Roberts and Aptula, 2008). In any event, the structure and reactivity of the chemical are as important as the potential for exposure to the skin.

Chemicals that are potential allergens are continually being developed and introduced into the human environment. Therefore, allergic contact dermatitis can be expected to continue to be a dermatological problem, especially in the workplace. Allergens may be simple chemicals (haptens) that must interact with a chemical component of the organism before becoming allergenic or they may be complicated chemicals or biological substances that elicit the allergenic response without chemical modification. Allergic contact dermatitis remains one of the most common occupational diseases that may become debilitating if the causative agent is not identified and exposure controlled (Nethercott and Holness, 1989; Slavin and Ducomb, 1989; Roberts and Aptula, 2008). While the condition is notable because of the extremely low concentration of chemical required to induce a reaction, new research is defining threshold levels for various chemicals with regard to development of allergic contact dermatitis (Arts *et al*., 2006).

Efforts to define the mechanism of T-cell mediated immune response of skin sensitization have been ongoing for nearly a century, with a continuing incomplete understanding of this process (Roberts and Aptula, 2008). Several excellent summaries of current understanding are available (Roberts *et al*., 2007; Rustemeyer *et al*., 2006). The current understanding of the mechanism of skin sensitization was briefly summarized by Roberts and Aptula (2008) as follows:

> *A sensitizing chemical reacts with skin protein in the epidermis so as to make it antigenic. The antigenic protein is processed by Langerhans cells in the epidermis and these Langerhans cells are consequently stimulated to migrate to a lymph node where they present the antigen to naïve T-cells. T-cells with receptors able to specifically recognize the antigen are stimulated to proliferate and circulate throughout the body. Sensitization has now been induced, i.e. the subject is now sensitized. These events take place during the induction stage of a sensitization test, and are collectively referred to as the induction phase or afferent phase.*
>
> *On subsequent exposure to the same sensitizer, or a second sensitizer, or second sensitizer cross-reactive with the first, reaction with protein and processing of the resulting antigenic protein by Langerhans cells again occurs, after which the antigen presented by the Langerhans cells is recognized by the circulating T-cells, triggering a cascade of biochemical and cellular processes that produce the clinical sensitization response. These events take place at the challenge stage of a sensitization test and are collectively referred to as the elicitation phase or efferent phase.*

The period between initial contact with the causative agent and the development of skin sensitivity (the induction period) may be as little as two or three days for strong sensitizers like poison ivy, or as long as several years for a weak sensitizer like chromate. Following a short sensitization period, if sufficient allergen remains from the initial contact, a spontaneous reaction may occur at the site of exposure. This is demonstrated by the occasional presence of an allergic reaction following the first exposure to poison ivy. Once the sensitivity to an allergen has been established, it generally persists for many years. Following the initial development of sensitivity, the time between re-exposure to the causative agent and the occurrence of clinically observable effects is generally between 12 and 48 hours, but may be as short as 4 hours or as long as 72 hours. Thus arises the term 'delayed hypersensitivity' (Slavin and Ducomb, 1989). For reasons that are not entirely clear, but possibly related to particular

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intrinsic factors, an individual may be repeatedly exposed to an allergen and not develop sensitivity to the substance for many years, while other individuals develop sensitivity following a single or very short exposure period to the same substance. The time between initial exposure and development of sensitivity is termed the 'refractory period' (Emmett, 1991).

Acute contact dermatitis is characterized by papules and sharply demarcated erythema. Blisters are also frequently produced following the release of cytotoxic compounds by white blood cells attracted to the affected site. Therefore, the vesicle fluid is not antigenic and contact of this fluid with unexposed areas of the skin will not result in additional dermatitis (Slavin and Ducomb, 1989). Oedema may also occur in areas of loose tissue.

Acute (short-term) and chronic (long-term) allergic contact dermatitis are gradations of the same condition. As allergic contact dermatitis increases in duration (usually from repeated exposure to the causative agent), the prevalence of blisters are gradually replaced by the formation of a crust (scaling) and/or lichenification (thickening of the epidermis). Erythema may or may not persist during chronic allergic dermatitis. The key element in the formation of subchronic or chronic allergic dermatitis is recurrent exposure to the causative agent. If the agent can be identified and exposure eliminated, the disease state will resolve. Unfortunately, because of the wide range of potential exposure to antigenic compounds, including metals and metal-containing compounds, cosmetics, deodorants, clothing dyes, food additives, adhesives, oils, plants or animals and their products (lanolin) and fragrances to name just a few, this is often an extremely difficult, if not monumental, task. The success of corticosteroids in treating allergic contact dermatitis has generally eliminated this condition from the list of serious disorders. However, owing to the difficulty of identifying and eliminating allergens, as well as the prevalence of potential allergens, this skin reaction is a potentially serious disease that will continue to be a major cause of cutaneous eruptions (Menne and Nieboer, 1989; Sicherer and Leung, 2008). Furthermore, exposure via the skin to potentially allergenic chemicals has also been suggested as a major route of sensitization for respiratory allergy (Kimber and Cumberbatch, 1992).

4.5.2 Chemistry of Haptens

Haptens are low-molecular-weight environmental chemicals that form covalent bonds with cutaneous carrier proteins or other cellular macromolecules. When this carrier molecule is a normal endogenous entity, as is usually the case, the resulting chemical complexes are no longer identified by the system as 'self'. Therefore, this now foreign molecule is perceived as an intruder (the allergen) and elicits an allergic reaction. Examples of commonly occurring haptens include metals (nickel, cobalt and chromium), quinones, aldehydes and acrylates (Benezra, 1987; DeGroot, 1987).

The majority of haptens that are skin sensitizers have electrophilic properties. Thus, they are able to accept electron pairs from nucleophiles such as amino (NH_2) and thiol (SH) groups found in cutaneous proteins (Benezra, 1987). New useful information is being developed into the chemistry of haptens and use of structure–activity relationships based on the reactivity of the compound and resulting hapten–protein binding (Karlberg *et al*., 2008; Gerberick *et al*., 2008). These efforts are important to the continuing efforts to investigate the molecular basis of allergic contact dermatitis and development of new methods to identify the potential allergenicity of new compounds.

The potential allergenicity of many compounds is dependent on interaction of the compound with other biological processes and is not purely dependent on chemical reactivity. Some potential haptens must be transformed by metabolism or reaction with light before they are able to bind to cutaneous proteins and become allergenic. These compounds constitute a special class of allergic sensitizers and are often termed 'prohaptens'. A unique subgroup of prohaptens that must react with light to become allergenic belong to the class of photoreactive chemicals termed 'photosensitizers' or 'photoallergens' (see below). When photoallergens react with light, they often form free radicals that rapidly react with cutaneous proteins, forming the allergen. Other allergens may contain long, hydrophobic side chains that appear to form hydrophobic bonds within cell membranes. Examples of compounds that are this type of allergen include lanolin and the extremely allergenic pyrocatechols found in the *Rhus* genus of plants that includes poison ivy.

4.6 Light-Induced Cutaneous Toxicity

The most biologically active spectrum of light is found from 290 to 700 nm. This spectrum can be further defined as ultraviolet or UV (290–400 nm) and visible (400–700 nm). The UV spectrum is generally further divided into UV-A (315–400 nm), UV-B (280–315 nm) and UV-C (220–280 nm). UV-C wavelengths do not naturally occur on the surface of the earth, as this spectrum is absorbed in the stratosphere, predominantly by ozone. Generally, the longer the wavelength, the deeper the light penetrates into the skin (Stern, 1986; Kornhauser *et al*., 1991). The wavelengths of light found in the UV-B and UV-A spectra are generally considered the primary source of toxic changes in the skin. The specific wavelengths responsible for a particular biological response are termed the 'action spectrum' for that effect (Kaidbey, 1991). The action spectra for several light-induced cutaneous reactions have been clearly identified.

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There are several effects that result from the exposure of the skin to light, whether the light is natural or manmade (thus, the UV light found in tanning booths is no safer than UV light created by the sun). In some cases xenobiotics play a role in these effects, while in others, the interaction of light with the normal components of the skin is responsible. In either case, adverse reactions of the skin to light (UV or visible) is termed photosensitization (Emmett, 1991).

4.6.1 Photosensitization Not Related to Xenobiotics

The exposure of the unprotected skin to UV light (from sunlight or artificial sources) can result in several toxic responses of the skin. These include short-term, generally reversible effects such as sunburn (erythema) and tanning (enhanced pigment darkening) as well as long-term, generally irreversible effects such as premature skin ageing (actinic elastosis) and the development of skin cancer.

Exposure of the skin to UV-B radiation can selectively alter the immunological function of the skin (photoimmunotoxicity) and result in light eruptions with or without the intervention of an exogenous xenobiotic. Polymorphous light eruption is the most common photodermatosis not involving an exogenous xenobiotic, with estimates of incidence as high as 10–20% of the general population in the USA and Europe (Honigsmann, 2008). Skin interaction with light can also alter the immune response to contact allergens and micro-organisms (such as Herpes simplex types I and II). Furthermore, the altered immunological function induced by UV-B light exposure can facilitate the development and growth of skin cancer. Altered immune response related to UV-B light exposure, associated with concurrent chemical exposure, may result in a complex relationship to light and chemicals on the alteration of immune system function (Krutmann and Elmets, 1988; Marrot and Meunier, 2008).

4.6.2 Photosensitization Related to Xenobiotic Exposure

Xenobiotics localized within the skin can interact with light (generally UV-A but, perhaps, visible as well) and produce adverse reactions in the skin in many ways (Stein and Scheinfeld, 2007). These include phototoxicity, photoallergy, depigmentation, induction of endogenous photosensitizers and induction of photosensitivity disease states (Emmett, 1979). Of these, the most frequently observed are phototoxicity and photoallergy (Emmett, 1991) with phototoxicity being more prevalent than photoallergy (Stein and Scheinfeld, 2007).

4.6.2.1 Phototoxicity

Phototoxicity is defined as a nonimmunological, light-induced dermatitis to a photoactive chemical (Marzulli and Maibach, 1991a). The skin response to phototoxicity is likened to an exaggerated sunburn. After local absorption or distribution to the skin following systemic absorption from distant sites, the chemical reacts with light and the resulting light-altered compound produces skin irritation. It is possible that many phototoxic reactions are caused by the production of free radicals that can cause lipid peroxidation and localized inflammation (Hayes, 1989). As with any chemical irritant, erythema, oedema and desquamation may occur with or without hyperpigmentation. The affected area of the skin is generally limited to areas exposed to light (i.e. areas not covered by clothing). The severity of phototoxic reactions is usually dose-related. Furthermore, phototoxic reactions can be elicited in most individuals who are exposed to an adequate amount of the compound and the appropriate action spectrum. A list of phototoxic agents is presented in **Table 3**.

Table 3 Selected phototoxic agents

Tetracyclines and sulfonamides (antibiotics)
Furosemide and chlorothyazide (diuretics)
Chlorpromazine (antipsychotic agent)
Anthracene (constituent of many dyes)
Porphyrins (animal and plant respiratory pigments)
Psoralens (photochemotherapy agents for psoriasis)
Cadmium sulfide (colorant in tattoos)
Bergamot oil (essential oil)
Certain dyes
Coal tar
Fragrance ingredients (perfumes)

4.6.3 Photoallergy

Photoallergy is similar to allergic contact dermatitis with the exception that the xenobiotics must react with light prior to becoming allergenic. The role of light in the production of a photoallergen is most likely to be one of two types of reactions. The absorption of light by the chemical may, in itself, produce a potent allergen. Conversely, the absorption of light by the chemical may produce a reactive intermediate (hapten) that combines with cellular constituents resulting in an altered cellular component that is an allergen (Emmett, 1991). Clinically, photoallergy generally presents as dermatitis on light-exposed areas that often spreads to areas not exposed to light (i.e. photoallergy may occur in areas covered by clothing). The severity of the photoallergic reaction may not appear to be dose-related (the skin reactions may be very severe with relatively low exposure) and the reaction does not necessarily develop in all individuals exposed to the offending agent and appropriate action spectrum. A list of potential photoallergenic agents is presented in **Table 4**.

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Table 4 Selected potentially photoallergic agents

Sulfonamides (antibiotics)
Phenothiazines (antipsychotic agents)
Coumarins (anticoagulants)
Anilides (components in medicines and dyes)
Fragrance ingredients (perfumes)

4.7 Cutaneous Carcinogenesis

Skin cancer is the most common form of cancer in humans and is becoming an increasingly important public health issue (Rigel, 2008). The principal cause of skin cancer in humans is UV radiation (Suskind, 1990). There are three primary types of skin cancer: carcinomas of the epidermal-basal and squamous cells, sarcomas of the mesodermal elements, and melanomas. Squamous cell carcinomas are most often associated with chronic exposure to UV light, and causation of nonmelanoma skin cancer with exposure to UV radiation, whether natural or artificial, has been well established (Hogan and Lane, 1986; Rass and Reichrath, 2008). The relationship between melanoma and UV radiation is less clearly defined (Berwick *et al*., 2008; Moan *et al*., 2008).

The production of chemicals that may cause skin cancer has been known for more than 200 years (Potter, 1963), and it is apparent that several chemicals modulate the carcinogenic effects of UV light (Emmett, 1973). Additionally, a past history of trauma or frostbite to the light-exposed area may also play a role in the development of skin cancer (Hogan and Lane, 1986; Rustin *et al*., 1984). Prevention and early detection are the foundations of skin cancer treatment. Chemical carcinogenesis is covered in other areas of this book and will not be addressed in detail here.

4.8 Acne-Like Eruptions

Acne is a well known effect of several cutaneous toxins (Espinoza and Fenske, 1988). Testosterone, chlorinated compounds and topical steroids are known to produce eruptions in humans similar to acne vulgaris. Although the potential of cosmetics to cause acne may be somewhat exaggerated (Jackson, 1991), a skin lesion termed 'acne cosmetica' has been described for acne-like eruptions caused by cosmetics. Like acne vulgaris, these reactions are initiated by the proliferation of the epithelium of the sebaceous gland and formation of keratin cyst resulting in that development of the pustule filled with fatty compounds and other products of sebaceous origin.

4.9 Atopic Dermatitis

Atopic dermatitis is a chronic dermatitis characterized by intense itching and excessive skin dryness that predisposes the patient to bacterial and viral infections (Wollenberg and Klein, 2007). Recent interest in this relapsing skin disease has been sparked by reports of its increasing prevalence and contribution to increasing health care costs. Various studies reveal that atopic dermatitis has a cause of multiple origins with the activation of complex immunologic and inflammatory pathways (Ou and Huang, 2007). Other studies suggest a complex interaction between the environment, chemical irritants, genetics and the autonomic nervous system (Cicek *et al*., 2008). Changes in the autonomic nervous system may result in decreased activity of the sweat glands contributing to the hallmark dry skin of atopic dermatitis. In any event, this increasingly common and recurring condition demands significant attention by the affected individuals, as well as the entire medical community. Further research into the potential for chemical irritants to contribute to the development or severity of this disease is required.

4.10 Chloracne

The skin reaction termed 'chloracne' may be one of the most sensitive measures of exposure to specific toxins known (Tindall, 1985; Poland and Glover, 1977; Pelclova *et al*., 2006). Chloracne, with accompanying, specific epidermal changes, is the most characteristic and frequently observed lesion resulting from primate, including human, exposure to the halogenated aromatic hydrocarbon class of toxins (Crow, 1970; Taylor, 1974). Owing to the specific cause of the lesion, chloracne is rarely observed in clinical practice (Zugerman, 1990) although the assumed dioxin intoxication of the Ukraine president, Victor Yushchenko, in 2004 garnered considerable media attention and public interest in this form of skin lesion (Pape and Stahlmann, 2007). In humans, this 'hallmark' of chlorinated hydrocarbon exposure is characterized by prominent, dense, large blackheads (comedones), abscesses and skin-coloured cystic lesions (Taylor, 1979). Almost every follicle in the affected area may be involved. The skin of the face, frequently areas near the eyes and behind ears, is usually involved first. Severe chloracne may involve the trunk, arms, legs, face, neck and back, is frequently refractory to treatment and scarring may be severe. Chloracne may appear as early as one to three weeks after the first exposure or may not manifest itself for several months. Mild cases clear up spontaneously within a few months, while severe cases persist for as long as 30 years (Scientific Review Committee of the American Academy of Clinical Toxicology, 1985). The lesion is so specific that

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if there is not medical history of chloracne, the likelihood of significant exposure or adverse health effects from halogenated aromatic hydrocarbons, specifically 2,3,7,8-tetraclorodibenzo-*p*-dioxin (TCDD), is considered remote (Council on Scientific Affairs, 1982).

5 EVALUATING CHEMICALS FOR POTENTIAL ADVERSE EFFECTS ON SKIN

It is essential that the toxicologist, healthcare professional and industrial hygienist understand the importance of the 'prior-to-use safety assessment' as the cornerstone of control of cutaneous hazards. This necessitates the evaluation of initial acute toxicity screening in animals followed by a tier of tests for safety dictated by the potential for and conditions of human exposure to the chemical. The tier assessment includes single and repeated exposure of laboratory animals and human subjects. Therefore, a discussion of the techniques of such safety assessment follows. While preclinical testing (*in vivo* or *in vitro* alternatives to animals) should always be conducted with new chemicals or new mixtures of chemicals before human exposure, human testing will be discussed first.

5.1 Human Testing

The diagnostic or predictive tool used most often for irritant or allergic contact dermatitis is patch testing (Slavin and Ducomb, 1989; Marzulli and Maibach, 1991b; Agin *et al*., 2008). The test is always performed on normal skin that is free of injury or disease. In predictive testing with untested chemicals, it is best to start with diluted solutions of the chemical and utilize an unoccluded test site. The first application should be limited to 30 minutes or one hour to minimize the risk to the subjects (Patrick and Maibach, 1991). As with most *in vivo* cutaneous techniques, comparison of the results between tests is extremely difficult because of the subjective nature of evaluating cutaneous irritation, the effect of environmental and genetic differences between individuals and groups, the effect of the specific vehicle, the application technique and the exposure period. Human studies with untested chemicals should always be conducted and monitored by medical personnel with ample experience in the appropriate discipline and informed consent must be obtained under these circumstances.

Irritant or sensitization patch testing is done for many reasons either in a clinical setting or in a laboratory setting. Diagnostic patch testing is done in the clinical setting to help identify pre-existing chemical sensitivities in a person with recurrent signs of skin irritation, thereby helping the affected individual avoid the offending agent in the future. Predictive patch testing is done in the laboratory setting with human volunteers to help prospectively identify skin irritants and sensitizers. In both cases, the general methods of applying patches, attention to detail and the importance of using well-trained technicians to observe and interpret the results are similar (Mowad, 2006).

Patch-testing procedures have the potential to produce adverse effects. For example, irritant patch tests causing serious irritation may rarely result in scarring or permanently altered pigmentation of the site. Rarely, the patch-testing procedure alone may be the cause of sensitization to a chemical. This has the potential to alter the lifestyle of the participants for the rest of their lives. Therefore, the necessity of diagnostic or predictive patch testing must be carefully evaluated before studies are initiated.

5.1.1 Sensitization Patch Testing

Whether for predictive or for diagnostic purposes, for patch testing to be a reasonably reliable tool in the identification of contact allergens, meticulous attention to detail must be maintained. The method involves the application of a small amount of one or several suspected allergens in relevant concentrations and in appropriate vehicles. The occurrence of the disease is then documented, in miniature, at the site of application. A standardized procedure for diagnostic patch testing was developed in Scandinavia and has been widely used in diagnosing contact allergies (Hjorth, 1991). Generally, a single application is sufficient for diagnosis of an allergic contact dermatitis. However, in predictive tests as part of pre-exposure testing, several occlusive patches are applied for one or two days as an induction to sensitization, followed by an approximate two week rest period and then challenge with a patch at a different skin site (Marzulli and Maibach, 1973). There are several alterations to these basic procedures for predictive tests in humans (Marzulli and Maibach, 1991b).

The most common site for diagnostic patch testing is the back, but the lateral or inner side of the upper arm, the thighs or the legs may also be used (Hjorth, 1991). A small aluminum cup fixed to a strip of tape (the Finn chamber) is a patch type used frequently throughout the world (Adams, 1990). A patch composed of a cellulose allergen-bearing disc attached to a polyethylene-coated aluminum paper backing is also often used (Slavin and Ducomb, 1989). Using these patches, 50–55 diagnostic tests can be performed on the back of the patient at the same time. For proper diagnostic evaluation, the chemical-treated patches must remain on the skin for at least 24 hours, but often are left on the skin for 48–96 hours owing to the latency of many skin reactions.

Following the exposure period, the patch is removed and 20–30 minutes allowed to elapse prior to the evaluation of the sites to allow nonspecific, mechanical irritation

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to subside. A positive reaction can include erythema, oedema, itching and, perhaps, vesicles at the application site. Local reactions that are irritant in nature will subside after several hours while a true allergic reaction will persist for several days (Slavin and Ducomb, 1989). The test sites should also be evaluated 7–10 days after patch removal as some reactions do not occur for 96 hours to a week after exposure.

Both false-positive and false-negative reactions occur, as several factors can influence the result. These include both intrinsic and extrinsic factors. For example, chemical concentration, current treatment with anti-inflammatory medications, UV light exposure, reaction to the adhesive tape used in the patch and hyperexcited skin states can alter the results of a diagnostic or predictive patch test (Oxholm and Maibach, 1990). The clinician or researcher can minimize these errors in diagnosis through appropriate training and qualification of technicians and use of follow-up testing for questionable results (Orton and Wilkinson, 2004). Furthermore, new techniques to aid the clinician or researcher to improve the diagnostic or predictive nature of the sensitization patch test are being developed. These include incorporation of biomarkers such as cytokine or IgE responses to the test protocols (Sicherer and Leung, 2008).

Because diagnostic patch testing is done primarily in patients with known chemical sensitivities, results of diagnostic testing do not necessarily represent the incidence of allergy in the general population (Agin *et al*., 2008). Thus, the potential of a chemical to induce sensitization in the general population should be based on an overall assessment of predictive patch testing combined with the incidence of positive findings observed in diagnostic patch testing.

Photopatch testing involves similar techniques to patch testing except that the treated site is exposed to a source of UV light following the application of the test substance.

5.1.2 Irritant Patch Testing

The potential of a chemical to produce cutaneous irritation is difficult to evaluate because all chemicals are irritating under some conditions. Indeed, merely increasing the water content of the skin by occlusion with impermeable tape often results in mild to moderate irritation (Olson, 1991). The changes in the skin resulting from the application of a mild irritant may be difficult to detect on gross examination, but they may result in significant alteration in function. The continually increasing focus on cleanliness in the home and workplace resulting in increased exposure to irritants such as water, detergents and alcohol-based solutions (Pedersen *et al*., 2005) has the potential to alter the prevalence of skin irritation in the general population. All of these exposures have the potential to influence the overall health of the skin and potential for irritation and new products must be evaluated for the potential to produce skin irritation prior to being marketed. The irritant patch test procedure is the most frequently used method to evaluate the irritant potential of new chemicals and products.

Original study designs for irritant patch testing in humans recommended the use of application periods of up to 48 hours (Kooyman and Snyder, 1942; Draize *et al*., 1944; Justice *et al*., 1961; Rostenberg, 1961). Currently accepted study designs have shorter exposure times and a four hour application period has been adopted by the National Academy of Sciences (1977). Patches, bandages or chambers are utilized to apply the test substance to the skin. Increasing the degree of occlusion will generally increase the severity of irritation and the use of highly occlusive tape to hold the patch in place can significantly affect the results of a study. Accordingly, the area of chemical exposure should be clearly marked to differentiate irritation or skin damage produced by the tape from that produced by the test chemical.

While it may be sufficient to predict the irritant potential of some compounds of limited human exposure, utilizing a single exposure study design, compounds proposed for consumer use are often tested utilizing cumulative (repeated exposure) irritation assays. Some chemicals that are nonirritating following a single contact with the skin may be highly irritating following repeated contact with the skin, especially in areas of friction or repeated motion such as the interior surface of the elbow or behind the knee. A consumer product that has been reported to be irritating can be separated into individual ingredients and the irritating component identified by the use of cumulative patch testing. The study design for these assays, including length of exposure period and number of treatments, varies between investigators (Patrick and Maibach, 1991). The specific study design (duration of contact, concentration of chemical, location of exposure, etc.) used in cumulative patch testing is often customized to the expected exposure pattern or suspected irritability and toxicity of the chemical.

Several modifications of patch testing have been developed for specific applications. The scarification test, in which the epidermis is abraded using the bevel of a needle, was developed to evaluate the irritability of compounds that may be applied to damaged skin (Patrick and Maibach, 1991). The soap chamber test was developed to compare the potential for soaps to produce drying and flaking, which are effects normally produced using conventional patch testing methodology (Frosch and Kligman, 1979). Flex washing tests or elbow crease washing tests do not involve the use of a patch and have been used by some investigators primarily for evaluating the irritation produced by soaps and detergents. The behind the knee test has been used and shown to be a reliable alternative to irritant patch and in-use clinical testing (Farage, 2006). These tests have been used as a replacement for the arm immersion test originally

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proposed by Kooyman and Snyder (1942). Many other variations of the irritant testing have been utilized for miscellaneous purposes and, undoubtedly, more will be developed in the future.

Patch tests often over- or underestimate the irritant potential of the chemical. This is as a result of a wide variability in the susceptibility of individuals to develop irritation (intrinsic factors) as well as the multitude of possible exposure conditions, especially with a consumer product (extrinsic factors). Thus, the selection of subjects for testing and conditions of exposure must be controlled and carefully evaluated when assessing the results of irritant patch testing. Finally, it is important to confirm the acceptability of new topical therapies through in-use clinical testing of the product under the conditions of intended use in the marketplace. In-use studies involve distribution of the proposed product to a group of people representing the likely population of end-users and evaluating their skin and overall wellbeing before, during and after the use of the product as directed. These studies provide the most accurate information regarding the likely skin irritant effects of the product when used by the population at large.

5.1.3 Evaluation of Patch Test Results

Patch tests are generally evaluated visually by assigning grades of irritation to the occluded area. The clinician focuses on the centre of the patch for purposes of reproducible and accurate grading. The grades, based primarily on erythema, generally consist of a 0 for no response and a 1–4 or 5 for increasingly inflamed sites. This scale does not include vesicular, bullous or eschar formation. Therefore, more detailed grading systems with up to 16 possible grades have been proposed (Patrick and Maibach, 1991). Assigning too many descriptors of the test site can lead to confusion and reduce the ability of the test to clearly identify irritants of clinical and biological significance. Thus, efforts intended to increase the power of a simple patch test may actually reduce the overall ability of the test to identify irritants important to the clinician. These efforts are discouraged and should be carefully considered before implementation.

Several other mechanisms of evaluating the results of patch testing have been developed. These are briefly discussed below.

5.2 Animal Testing

When animals are used as models for human exposure to chemicals, the differences and similarities between species are important factors to consider. While the relationship of animal dose–response data to human toxicity is not always clear, an understanding of the potential of toxicity is essential to the development of human exposure guidelines. An important objective is to establish a relationship between animal data and epidemiological findings. This task is extremely difficult because of the poor availability of reliable data on the toxicity and irritability of most chemicals in humans. When both animal data and epidemiological findings exist for a chemical, the correlation of the information can aid in the assessment of laboratory data as it relates to humans.

5.2.1 Sensitization Testing in Animals

Historically, the most widely accepted animal model for predictive sensitization studies has been the guinea pig (Middleton, 1978). Tests using guinea pigs are now being widely replaced by the mouse local lymph node assay (LLNA) (discussed below). Because the toxicologist will be called upon to review data generated in older guinea-pig tests, this chapter will include general descriptions and principles of the tests for reference. There are varied guinea-pig methods used to evaluate the sensitization potential of a chemical, but most have not been used in many years. Only those tests the toxicologist is most likely to encounter will be further discussed.

To assess the potential sensitization properties of a chemical, guinea pigs are treated with an initial dose or several doses of the chemical (the induction phase) by intradermal and/or cutaneous application. Following a sensitization or incubation phase with no chemical treatment of approximately two weeks, the animals are treated with a second dose or series of doses of the same test chemical (the challenge phase). The chemical concentration and application site are often different between the induction and challenge phases. Sensitization is evaluated by examining the skin reaction following the challenge phase compared with any skin reaction immediately following the induction phase. The difference in the reaction between the two applications accounts for nonspecific skin irritation caused by the chemical. The relevance of the results of the tests to human exposure must be evaluated based on the severity and repeatability of the biological properties of the reaction. Often the number of animals that respond is a more important indicator of the sensitizing potential of a chemical than the severity of individual reactions. Practical experience is the best guide in this endeavour, as laboratory experiments may be conducted under extreme or unrealistic conditions. A fundamental understanding of the test procedures, along with the potential for false-positive and false-negative findings is also required for toxicologists to appropriately understand these tests.

Draize *et al.* (1944) were the first to describe standardized irritation and sensitization tests and several iterations to their original method were proposed. These early tests had several drawbacks, including a high incidence of false negatives with weak sensitizers (Marzulli

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and Maguire, 1982). Furthermore, the tests recommended consistent induction methods without regard to the use pattern or exposure potential of the chemical (Klecak, 1991).

The Buehler test was designed to reproduce a human patch test in animals and, therefore, allows variation of conditions to optimize the detection of moderate to strong sensitizers prior to testing in humans. This test utilizes induction and challenge phases of occluded epidermal doses of the test substance, which may be an allergenic chemical or a final product formulation. The results of this test can be very difficult to read and interpret, especially when low concentrations of test chemicals are used. This is primarily because of the high incidence of inflammatory reactions in both control and treated animals. This test may result in false-negative results, but these problems may be related to poor study conduct and may not be attributable to the design of the test (Robinson *et al*., 1989; 1990). Performed correctly, the Buehler test is relatively expensive, but is considered adequate to detect weak sensitizers (Botham *et al*., 1991).

While the Buehler test only applies the test article to the intact surface of the animals, many tests use a combination of injections and topical application of the test article as well as chemical means to increase the immunological response of the test animals. Freund's complete adjuvant (FCA; a mixture of heat-killed *Mycrobacterium tuberculosis*, paraffin oil and mannide monooleate) has been used to increase the immunological response of the guinea pig in a variety of tests. Most of these test methods are rarely used today, with the notable exception of the maximization assay discussed below. The use of FCA is intended to aid in the detection of weak sensitizers and is injected intradermally at different sites during the induction phase (Henningsen, 1991). Criticism of the methods employing FCA abound, including the open wounds produced at the site of injection as well as intradermal injection of the test article thereby bypassing the effect of the stratum corneum to limit the absorption of potential sensitizers. Thus, the use of FCA may cause the sensitizing potential of the test chemical to be overestimated.

The guinea-pig maximization test is the most widely used guinea-pig test in Europe and is generally considered to be very sensitive (Botham *et al*., 1991). As noted above, this test is being rapidly replaced by the LLNA (see below). The induction phase of the guinea-pig maximization test employs the simultaneous injection of FCA alone, test material in saline and test material in FCA into three different locations in close proximity to each other. This is followed seven days later by epicutaneous application of the test substance on a filter paper. The filter paper is occluded and held in place with tape and an adhesive bandage. The filter paper and bandaging are then left in place for 48 hours. The challenge phase, conducted with the test substance applied epicutaneously for 24 hours, is performed two weeks after the epidermal induction phase. The maximization test is considered to have good predictive value in humans, but does not lend itself well to use with final product formulations (Klecak, 1991). The maximization test may result in false-positive results. Indeed, the original classification scheme for the test did not allow for a test substance to be classified as a nonsensitizer; the lowest rating (0) corresponded to a weak sensitizer (Botham *et al*., 1991).

5.2.1.1 Local Lymph Node Assay (LLNA)

While guinea pigs remained the industry standard for evaluation of the sensitization potential of a compound for decades, the mouse has been studied extensively as a model for delayed-type hypersensitivity (Nakano and Nakano, 1978; Tanaka, 1980; Johnson *et al*., 1984). In 1968, the technique of measuring the swelling of the mouse ear as an indicator of delayed-contact hypersensitivity was developed (Asherson and Ptak, 1968). This mouse ear assay was further developed into a quantitative method in which cellular proliferation of draining lymph nodes was identified as an end point of sensitization.

The LLNA is a quantitative test developed using this end point and is based upon the biology of sensitization. This test is considered by many authors and regulatory agencies to be an acceptable alternative animal model to the guinea-pig tests in predicting the sensitization potential of chemicals in humans (Kimber *et al*., 2002). The LLNA is an *in vivo* method and, therefore, does not eliminate the use of animals. However, the LLNA provides certain advantages with regard to both scientific progress and animal welfare. Most importantly, the test studies the induction phase of skin sensitization and provides quantitative data suitable for dose–response assessment. There have been some questions raised regarding the potential for the test to produce false positives, especially when the test article is a skin irritant. Thus, it is important to carefully evaluate test results.

The basic principle underlying the LLNA is that sensitizers induce a primary proliferation of lymphocytes in the lymph node draining the site of chemical application (Gerberick *et al*., 2007). This proliferation is expected to be proportional to the dose applied combined with the potency of the allergen. The study design involves application of either 25 µL of the test article, or the positive or negative control to the dorsal surface of each ear on three consecutive days, followed by two days of rest (no treatments). On the sixth day of the study, the animals are injected with 20 µCi of ^{3}H-methyl thymidine or another suitable indicator of cell division and the animals are euthanized exactly five hours later. The auricular lymph nodes for each ear are removed and the degree of cell proliferation is analysed. A greater degree of cell proliferation in the local lymph nodes is considered an indicator of a greater degree of sensitization potential of the test article. Extensive international efforts have demonstrated consistency and repeatability of this assay to the degree

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that this assay is now generally accepted as a validated alternative to the guinea-pig models (Basketter *et al*., 2002).

5.3 Irritation Testing in Animals

It is generally recognized that human skin and the skin of other animals are different and do not always react to chemicals in the same manner. Overall, the skin of laboratory animals is more easily irritated than the skin of humans, although this is not always the case (Phillips *et al*., 1972; Olson, 1991). Animal testing will reliably detect moderate to strong irritants but the degree of irritation often correlates poorly with the degree of skin response in humans. Therefore, comprehensive experimental protocols are required to establish the degree of risk to humans. Comprehensive animal testing can also aid in the identification of adequate labelling of chemical containers and suggest appropriate protective clothing for use in the workplace. Irritation testing in animals (especially acute, single-dose studies) is rapidly being replaced by *in vitro* techniques (discussed below). Animal studies to evaluate skin irritation continue to be most appropriate in some circumstances and will continue to be of historical importance and, therefore, are discussed below.

A low acute oral or inhaled toxicity may indicate that extensive cutaneous testing is not required, as the skin is generally a more effective barrier to absorption than the intestinal tract or lungs. This generalization is not always true and the degree of human exposure and chemistry of the compound must be carefully considered when making testing decisions. For obvious reasons, the evaluation of the compound for irritability in animals does not need to be performed with strongly alkaline or acidic substances. Severe skin irritation can be presumed with these compounds and specific precautionary measures recommended without the results of animal testing. Only limited irritation testing may need to be performed when the substance will not come into direct and close contact with the skin (Gelbke, 1987).

5.3.1 Selection of Animal Species

Selection of the proper species and strain of animals for use in a toxicology study of any design is very important to the usefulness (predictive value) of the results (Rao and Huff, 1990). The albino rabbit remains the species of choice in acute and repeated applications irritation testing. Several authors (Davies *et al*., 1972; Motoyoshi *et al*., 1979; McCreesh and Steinberg, 1991) have addressed the use of other species including guinea pigs, hairless mice, albino rats and miniature swine in cutaneous irritation testing. Considerable interspecies variability has been identified. The skin of rabbits is generally identified as the most sensitive of the species tested, while human skin is one of the least sensitive. The reactivity of the skin of the albino rat is considered to be similar to the reactivity of human skin. Thus, the albino rat is often utilized in repeated cutaneous toxicity studies.

5.3.2 Single Application Irritation Testing (Draize Test)

The test described by Draize *et al*. (1944), or slight modifications of this test, is the most widely used test for predicting the potential skin irritation of chemicals and chemical mixtures. The authors divided the various elements of irritation into distinct categories for grading (**Table 5**). In this test, the hair is clipped from the back of a rabbit and four distinct areas for the application of test substances are identified. Two of the four areas are abraded by making four epidermal incisions in the appropriate areas. All four areas are covered with gauze that is held in place with adhesive tape and the test substance is applied to the appropriate area under the gauze. The entire trunk of the rabbit is wrapped in impervious cloth or plastic to hold the patches in place

Table 5 Evaluation of skin reactions as described by Draize *et al*. (1944)

Erythema and eschar formation	
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation	4
Total possible erythema score	4
Oedema formation	
Very slight oedema (barely perceptible)	1
Slight oedema (edges of area well defined by definite raising)	2
Moderate oedema (area raised approximately 1 mm)	3
Severe oedema (raised 1 mm and extending beyond area of exposure)	4
Total possible oedema score	4
Total possible score for primary irritation	8

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and decrease the evaporation of volatile test substances. The rabbits generally remain wrapped for 24 hours after treatment and are evaluated for irritation at the time of unwrapping, and at 24 and 48 hours after being unwrapped. Generally, four test substances are evaluated in a series of six rabbits.

The Draize test for dermal irritation has been criticized for various reasons. The original purpose of the test was to identify substances that would not be irritating to human skin and was primarily designed to eliminate false negatives. While the occurrence of false positives is undesirable in the development of new consumer products, they are infinitely preferable to the occurrence of false negatives that could lead to disastrous results when an undetected irritant is added to consumer products. Several aspects of the standardized Draize test, including the abrasion of the skin and time of exposure, are disputed. This has resulted in several modifications to the method. As with the evaluation of patch testing in humans, the subjective nature of the evaluation of the treatment areas results in significant variations between laboratories, as well as between technicians (Weil and Scala, 1971). This impedes the comparison of results between laboratories and makes the job of the risk assessor more difficult. This variability of the results of test patching in animals as well as humans has hindered the identification of alternative methods, although these issues are being overcome.

5.3.3 Repetitive Application Irritation Testing

The use of repeated cutaneous applications over at least 7–14 days appears to be better able to predict the irritability of a test substance than a single application. This is especially true if the material is designated for use in a consumer product and will remain in contact with the skin for extended periods or if there will be repeated applications of the material. Additionally, the repeated application test to assess irritability can be combined with an assessment for systemic toxicity by the cutaneous route. In this case, three or more groups of 5–10 animals per sex (generally albino rats or rabbits) are used, with each group receiving a different dose of the test substance daily for two, three, four or 13 weeks (weekend doses may be omitted). An additional group of animals is handled similarly, but treated with the vehicle or, when no vehicle is used, with water to serve as a control group. The fur is removed from the back of the animals with a veterinary clipper and the test chemical is applied directly to the back. The animal is then wrapped with an occlusive dressing and returned to its home cage for a period of six hours. The wrapping is removed and the back gently wiped with a damp or dry cloth. The animals are observed daily for signs of cutaneous irritation (**Table 1**) and graded for the presence of erythema and oedema (**Table 5**). At termination of the study, both treated and untreated skin is collected and evaluated microscopically for signs of irritation. A list of common microscopic changes that may be observed following the application of irritating test substances is presented in **Table 2**.

Table 6 Clinical pathology measurements that may be affected by cutaneous irritation

Decreased values
Haemoglobin concentration
Haematocrit
Erythrocyte count
Serum calcium concentration
Serum inorganic phosphorus concentration
Serum creatinine concentration
Increased values
Total leucocyte count
Neutrophil count
Platelet count (rabbit only)
Serum globulin concentration (rabbit only)
Serum glucose concentration (rat only)
Values that are variably affected
Mean corpuscular volume (MCV)
Mean corpuscular haemoglobin (MCH)
Total serum protein concentration
Serum albumin concentration

An evaluation of repetitive application studies using mild to severe irritants indicates that several haematology and clinical-chemistry parameters may be affected by chemically induced skin irritation (Weaver *et al*., 1990; 1991; 1992; Hermansky *et al*., 1995). These changes, summarized in **Table 6**, do not appear to be related to systemic toxicity of the chemical, but correlate well with the degree of cutaneous irritation. The relationship of these parameters to cutaneous irritation is not clear, but may be related to the vascular and fluid balance alterations that may occur following cutaneous irritation. Regardless of the cause of these changes, their consistent occurrence emphasizes the relationship of the skin to the overall health of the organism. It is also essential that the risk assessor be aware of these irritation-induced changes when evaluating the findings of repeated cutaneous application studies.

6 METHODS OF EVALUATING *IN VIVO* IRRITATION RESULTS

As discussed above, the interpretation of both human and animal cutaneous testing relies heavily on visual scoring of areas of the skin treated with the test substance. The subjectiveness of visually graded results in human

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patch testing and acute and repeated exposure studies in animals makes it very difficult to compare tests graded by different individuals and laboratories. This confounding experimental variable has led to complications for the toxicologist and risk assessor in evaluating the potential human hazard of cutaneous exposure to chemicals.

Therefore, methods other than visual scoring or grading of skin inflammation have been developed to aid in the interpretation of patch testing in dark-skinned humans, as well as in an attempt to decrease the subjective nature of visually grading skin irritation in all tests. Blood flow to the skin must generally increase three-fold or greater to be detected visually and reactions produced by mild or moderate irritants or weak sensitizers may not be detected by visual evaluation alone. Finally, the importance of differentiating an allergic reaction from an irritant reaction, especially in human diagnostic patch testing, cannot be overstated and methods of greater sensitivity than visual grading may increase the ability to differentiate these reactions.

Several methods developed for the evaluation of patch testing results have been examined in considerable detail. Contact thermography is the imaging of the temperature of the area of the skin where an irritant has been applied (Ring, 1986). The methodology is based on the premise that the greater the degree of irritation, the higher the relative temperature of the affected skin (that is, irritation and inflammation are fundamentally related). However, contact thermography has shown that different irritants produce lesions of different temperatures in humans. For example, croton oil produces a warm lesion while sodium lauryl sulfate results in the formation of a cold lesion (Agner and Serup, 1988). Because changes in the temperature of the affected skin are now considered a useful method for quantifying the degree of irritation, use of infrared thermography has shown promise in the differentiation of allergic and irritant responses, but more research is needed (Baillie *et al*., 1990).

Another method of evaluation of skin inflammation caused by irritant or allergic dermatitis is laser Doppler flowmetry or velocimetry (Staberg *et al*., 1984). The instrument is equipped with a hand-held probe that emits a laser light onto the skin and measures blood flow through the area. This technique is generally considered more sensitive than visual scoring in detecting erythema in humans (Wahlberg and Wahlberg, 1984). However, the technique often produces poor correlation with visual scoring. This is based partially on the dependence of visual scoring on parameters other than blood flow (i.e. blistering), as well as the fact that mild irritants often produce low increase in blood flow (Gawkrodger *et al*., 1991).

Transepidermal water-vapour loss, measured with an evaporimeter (Lammintausta *et al*., 1987), has also been used to evaluate patch tests. The technique, which evaluates the function of the stratum corneum as a barrier to water loss, is generally more sensitive to irritant dermatitis (indicating epidermal damage) than to allergic dermatitis. The technique has, therefore, been evaluated in humans as a method of differentiating irritant and allergic dermatitis. However, the variable severity of the allergic reaction does not allow this test to differentiate between allergic and irritant responses in all cases (Agner and Serup, 1989b).

Another technique that has been used in the evaluation of skin irritation is the erythema index, which quantifies the inflammatory response by comparing the reflection of red and green light from the skin (Diffey *et al*., 1984). Skin thickness measurements have also been used to evaluate oedematous reactions (Olson, 1991). Based on the observations that repeated exposure to irritants often results in an increased roughness and brittleness of the skin, measurements of physical changes to the cells of the stratum corneum following exposure to irritants have been evaluated (Olson, 1991). The loose, outer layer of cells of the stratum corneum are obtained from humans by noninvasive techniques (washing the site) and the cells are evaluated for shape, size and morphology under a light microscope. Because of the greater availability of intact skin samples, this technique appears to have more promise in animal testing.

To the risk assessor, a quantitative, objective measure of cutaneous inflammation remains a highly desirable goal. Alternative methods for evaluating inflammation in animals include many of the same methods used for evaluating the results of irritation patch testing in humans. However, owing to several factors, including thick and variable patterns of fur growth in most species (especially rabbits), these measurements have generally been unsatisfactory in animals and further increases the attractiveness of alternative (nonanimal) models.

Because of the availability of intact animal skin samples at study termination (following euthanasia), other methods of evaluating irritation, not currently practical for humans, have been developed for use in animal irritation studies. Evaluations of morphometry (the measurement of structural forms) on cells obtained from the full thickness of skin can be utilized in animals. Measurements of the average total cell volume, nuclear size and shape, as well as calculated parameters (e.g. the nuclear/cytoplasmic ratio) can be obtained with this method. Additionally, special fluorescent stains can be utilized to observe and grade the roughness of the surface of the skin.

Several biochemical markers have been evaluated as potential indicators of the degree of irritation. These include enzyme activity, tissue or serum concentration of prostaglandins and/or leucotrienes, and acute phase reactant proteins (Olson, 1991). Ideally, as with any clinical test, the biological sample should be easy to

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obtain, objectively measured and the results should be consistent and repeatable. The most frequently cited biomarker evaluated for skin irritation is interleukin 1 alpha (IL-1α) (Gibbs *et al*., 2002). Results of studies with the above markers, including IL-1α, have shown some success with specific compounds or classes of compounds, but have generally been unsuccessful in identifying markers of inflammation that have broad applicability (Fulzele *et al*., 2007). As techniques and instrumentation improve, acceptable biochemical markers for evaluating cutaneous inflammation may be identified, resulting in enhanced evaluation and interpretation of the assessment of cutaneous irritation, preferably, in animals, humans and nonanimal, nonhuman (*in vitro*) techniques.

7 *IN VITRO* ASSAYS

The *in vivo* procedures employing animals used by the toxicologist and risk assessor to measure the potential of a material to produce cutaneous irritation have been increasingly challenged. The validity of these evaluations as they relate to humans, as well as the use of animals in these procedures, has been questioned. As a result, attempts to measure the irritant potential of test substances using *in vitro* techniques have significantly increased (Vinardell and Mitjans, 2008). Several technological developments have made the *in vitro* growth of skin into a well differentiated and complex artificial organ with relevant structural and/or functional activity possible. While a complete description of the methods and applications of these techniques is beyond the scope of this chapter, a description of the general principles is warranted because of the increasing importance of these *in vitro* techniques. It appears that cutaneous irritation is an area where alternative methods to whole-animal predictive testing may soon play a substantial role in risk assessment (Van den Heuvel and Fielder, 1990; Spielmann *et al*., 2007). Political pressures, especially in Europe, are driving development of alternative methods for risk assessment due to the huge numbers of chemicals involved in programs such as REACH and the seventh amendment to the Cosmetics Directive that mandates the elimination of animal testing for cosmetics (Hartung *et al*., 2004).

The purpose of developing *in vitro* techniques to predict the potential irritability of a test substance can be: (i) to evaluate specific aspects of the irritation or sensitization process, (ii) to provide supplemental information to *in vivo* evaluations for the risk assessor or (iii) to replace an *in vivo* procedure (Hobson and Blank, 1991). Furthermore, *in vitro* tests may be more cost effective and, if designed and used properly, provide more objective and meaningful results than whole-animal studies.

In reducing the number of animals used for toxicological experiments and predictive testing, *in vitro* methods have particular value (Gad, 1990; Purchase, 1990). The primary ethical consideration for the toxicologist, industry and governmental agencies is the protection of human health. Therefore, the change from *in vivo* to *in vitro* techniques requires a comprehensive process of evaluation of the performance characteristics of the *in vitro* methodology. This process of confirming the effectiveness of a new procedure' is termed 'validation'. The validation of any new toxicological technique, whether or not it be an *in vitro* process, requires an exhaustive comparison of the new technique with the results obtained in the previous or concurrent studies using established techniques of evaluation as well as comparison with existing relevant epidemiological data in humans. The selection of the test substances for the validation work must be done carefully and should represent a wide range of both chemical and toxicological classes. Based on the legal and ethical implications, this validation and the subsequent move from *in vivo* to *in vitro* techniques must be a careful, deliberate process (Van den Heuvel and Fielder, 1990; Hartung, 2006). The final acceptance or rejection of the *in vitro* test depends on the ability to utilize the results and the confidence in the decisions based on the information provided by the procedure (Soto and Gordon, 1991).

A validation scheme for alternative methods has been devised and includes three stages (Scala, 1986). The first stage develops a mechanistic relationship between the alternative assay and the end point of interest in the target organ and can involve the evaluation of up to a hundred chemicals. The accuracy and dependability of the assay procedures are determined in the second stage. Therefore, problems with the methodology are detected, evaluated and, if possible, corrected during this stage. The third and final stage is performed after the new assay has gained considerable regulatory and scientific acceptance. This phase involves many laboratories and the testing of up to more than 1000 chemicals. Several *in vitro* skin techniques are in this third and final phase of validation as alternative models.

Ideally, the validation of an *in vitro* technique should be performed, at least partially, using human data. There are generally little or no human toxicological data available on enough compounds to evaluate and validate an *in vitro* technique satisfactorily. However, there is a substantial amount of data available on the cutaneous irritability of a multitude of chemicals and chemical mixtures. Researchers attempting to develop new *in vitro* techniques can access this information and compare the results of their models with the known findings of these chemicals in humans (Bason *et al*., 1992). Indeed, individual reputations and the future of entire companies have been staked in the attempt to develop a reliable and economical *in vitro* alternative to predictive irritation testing in animals.

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7.1 Types of *In Vitro* Alternative Methods

Several different types of *in vitro* models have been developed to predict the cutaneous irritancy and sensitization potential of compounds (Parish, 1986; Hobson and Blank, 1991; Vinardell and Mitjans, 2008). Structure–activity and biochemical models currently appear to have limited value, but increased mechanistic knowledge and technological advances may increase the usefulness of these models in the future. Computerized structure–activity models may have some applications, but have not received a great deal of industrial or regulatory acceptance for predicting skin irritation or sensitization. These models generally depend on the correlation of a toxic end point (in this case, cutaneous irritation or sensitivity) with specific chemical functional groups and/or a measured parameter of the molecule (e.g. rotational freedom of the functional group within the molecule). Based on an accumulated database of known chemicals, untested chemicals are evaluated for the potential to produce the toxic end point by comparison with the known chemicals of similar structural features. Obviously, these models rely heavily on the molecular interactions involved in production of the toxic end point. As the knowledge of molecular mechanisms of irritation and sensitization increases, the use of these databases for structure–activity modelling will also increase in accuracy and dependability. This methodology will undoubtedly have greater use in the future and scientific interest in structure–activity models continues to be intense.

The biochemical events involved in the production of an irritant response are exceeding complex and not well understood. It is clear that irritants from different chemical classes produce cutaneous changes leading to inflammation by vastly different mechanisms. Thus, development of an accurate structure–activity model for irritation of a wide range of chemicals is a monumental task. Similarly, production of a biochemical model to accurately predict the potential irritancy of chemicals and chemical mixtures is a considerably difficult undertaking. Current biochemical models emphasize the interaction between the irritant and an endogenous molecule of the skin. The interaction would, presumably, be required to initiate the irritant response. However, the identification of even one of the probably many biochemical events responsible for initiating irritation is difficult. Current biochemical models utilize mixtures of carefully selected organic chemicals organized into separate sections to simulate layers of the skin (Gordon *et al*., 1989). The test substance is added to the system and several parameters, including chemical reactivity, are evaluated. These biochemical models are rapidly being replaced by newer *in vitro* models using reconstructed skin models (El Ghalbzouri *et al*., 2008).

Mechanistically based computer or biochemical models used to predict the irritancy potential of a specific class of chemicals may provide valuable information in the selection of new chemicals for future work within that class. These models also add useful information on the mechanisms of irritation and inflammation and are currently being used to a limited extent in pharmacology and toxicology.

Cell-culture methodology has been adapted to *in vitro* irritation assays involving both cutaneous and ocular irritancy. Potential irritancy is evaluated by measuring the cytotoxicity of the chemical in the cell culture. Owing to the many cell varieties found in normal skin, the major complication encountered with this methodology has been the selection of the appropriate cell type(s) for use in the system. Furthermore, the effect of the stratum corneum in protecting viable cells of the epidermis from irritants is not taken into account by the cell-culture model (Hobson and Blank, 1991). Several cell types have been evaluated as models for cutaneous irritation with significant differences being identified (Gajjar and Benford, 1990).

Many of the problems with cell-culture systems are overcome by utilizing skin tissue models. Skin tissue models are represented by two major classes: (i) epidermal slices obtained from either human or animal sources and (ii) reconstructed human skin models. Epidermal slices are generally obtained from either human cadavers or from slaughtered animals and have limited utility due to availability. Furthermore, slaughtered animal skin is not always considered to provide acceptable alternatives to animal testing (Oliver and Pemberton, 1985). Thus, the reconstructed skin (or skin equivalent) models are becoming a focus of much effort to identify an alternative assay to human skin. These assays are 'manufactured' using cultured, normal human-derived epidermal keritinocytes cultured on an artificial membrane to form a highly differentiated model of the human epidermis. Once mature, the cultured human epidermis demonstrates morphological similarities to normal human epidermis, with multiple layers, including a well-developed stratum corneum. The cultured skin is also metabolically and mitotically active, with a lipid profile similar to normal human skin. Furthermore, characteristic markers of human skin and skin irritation can be readily identified and measured (Hoffman *et al*., 2005), These systems show promise as successful alternatives to *in vivo* skin irritation testing especially to replace single-dose (acute) studies (Hobson and Blank, 1991; Spielmann *et al*., 2007).

Several of these reconstructed skin models are currently commercially available. Two of the best known and most studied are EPISKIN (SkinEthic Laboratories, Nice, France) and EpiDerm (MatTek Corporation, Ashland, Massachusetts, USA). Both of these reconstructed skin models have successfully completed extensive validation studies by the European Centre for the Validation of Alternative Methods (ECVAM)

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Table 7 Functional performance standards for reconstructed human-skin models intended for use in the irritation testing of chemicals

Viability	As measured by MTT or other metabolically converted dyes demonstrating that control tissues are stable in culture for the duration of the test exposure period.
Morphology	Histological examination of the reconstructed skin model should demonstrate human epidermis-like multilayer structure including a stratum corneum.
Barrier Function	The tissue stratum corneum and its lipid composition should be sufficient to resist the rapid penetration of cytotoxic marker chemicals, such as solium dodocyl sulfate (SDS), under standardized testing conditions.
Reproducibility	Testing of specific reference chemicals, as defined by the OECD, using the reconstructed skin model should demonstrate reproducibility over time and between laboratories.
Quality Control	Prior to acceptance for skin-irritation testing, each batch of the reconstructed skin model must meet defined production release criteria including measures of tissue viability and barrier function.

Adapted from OECD draft guidance, *In Vitro Skin Irritation: Human Skin Model Test.*

(Spielmann *et al*., 2007) and were recommended by the study authors as potential replacements for the Draize rabbit skin irritation test.

Essentially, chemical testing for skin irritation with these reconstructed models is a combination of standard skin irritation testing, in which the chemical is directly applied to the surface of the 'skin' and cell culture techniques. Prior to using the *in vitro* cultures, the cell viability is measured using 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or thiazolyl blue reduction testing. The resulting changes to the reconstructed skin model are measured by microscopic observation as well as biochemically through the release of cellular markers such as IL-1α and additional MTT reduction assays for cell viability. The predictive value of changes in the concentration of biochemical markers, as well as structure may not be as important as cell viability as measured by the MTT reduction assay. Because of the sensitivity of these models to environmental conditions and variations in test methods, the conduct and interpretation must be carefully transferred and validated within laboratories. As these assays continue to gain acceptance, this process will likely become more routine and less burdensome. In fact, the Organization for Economic Coordination and Development (OECD) has proposed a set of performance standards for reconstructed skin models. These functional performance standards are summarized in **Table 7**. Continued development of the reconstructed skin models and wider validation efforts is expected in the near future.

In summary, these assays clearly show promise as a replacement for the use of animals in skin irritation testing. Additional reconstructed skin models including those containing dendritic cells as possible *in vitro* skin sensitization assays (Uchino *et al*., 2008) and oral mucosal models (Maharamzadeh *et al*., 2007) are being developed and validated. The international regulatory acceptance of these assays, as well as other techniques such as the silicon microphysiometer (Parce *et al*., 1989; Bruner *et al*., 1991) remains to be seen.

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Ototoxicity

Andrew Forge, Ruth Taylor and Ernest S. Harpur

CONTENTS

1 INTRODUCTION

The inner ear contains the end organs of hearing, in the cochlea, and of balance, in the vestibular system. It detects mechanical stimuli—sound vibrations in the cochlea, motion and changes in head position in the vestibular system—and converts them into neural signals. The term ototoxicity is used to refer to the process by which a number of therapeutically useful drugs and certain environmental agents such as industrial solvents cause damage to these peripheral end organs. Agents which cause hearing impairment or balance dysfunction through effects on the respective neural pathways and centres are not considered in this chapter.

Histologically, the inner ear is composed of: sensory epithelia; ion-transporting epithelia that maintain the environment in which the sensory cells function; nonspecialized epithelia; neurons; and connective tissue. Cells in connective tissues have a role in maintaining homeostasis. Broadly, agents that damage the inner ear may act on the sensory epithelia, the ion-transporting epithelia or both. Hearing loss is the common effect of ototoxins. Many affect the vestibular system as well but there are few agents known to be exclusively vestibulotoxic.

An understanding of the basic structure and function of the organs of hearing and balance and of the methods which have been used, or are currently available, for their study is necessary for full comprehension of ototoxicity. Thus, we begin with an account of the anatomy and physiology of the inner ear and then summarize techniques by which each of these may be studied. We attempt to explain why the inner ear is a selective target, focussing on the kinetics of ototoxic drugs in inner ear fluids and tissues, and current understanding of the physiological and biochemical mechanisms underlying ototoxic processes.

2 ANATOMY AND PHYSIOLOGY OF THE INNER EAR

The inner ear consists of a system of membranous canals (the membranous labyrinth) enclosed in bony channels in the base of the skull (**Figure 1**). In humans and higher primates, the inner ear is contained within the temporal bone that is fused with the base of the skull and is reputed to be the densest bone in the body. In other mammals, the inner ear, together with the middle ear, is contained in an auditory bulla that is not fused with the skull and thus can be relatively easily isolated (**Figure 2**).

The bony channels of the inner ear are filled with a fluid, perilymph, which has a high sodium ion (Na^+) content. The perilymphatic compartment connects to the subarachnoid space via the cochlear aqueduct. The fluid within the membranous canals, endolymph,

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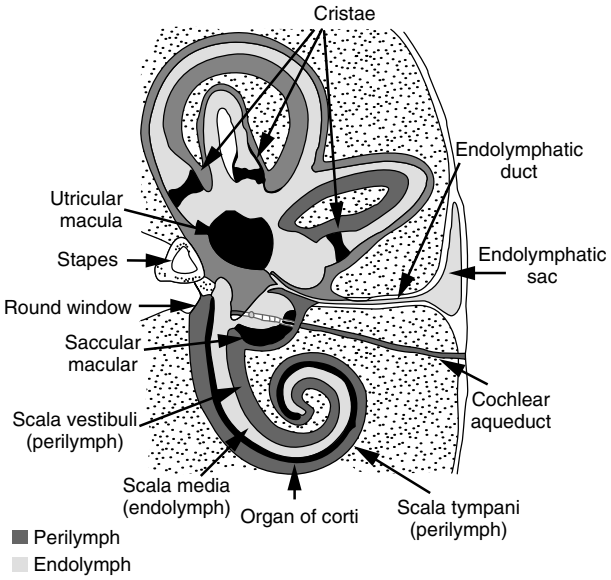

Figure 1 Diagrammatic representation of the human inner ear.

has a high potassium ion (K^+) concentration (about 140 mM). In the cochlea, but not the vestibular system, endolymph also has a high positive resting electrical potential of about +80 mV, the endocochlear potential (EP). The endolymphatic compartment terminates in the endolymphatic sac via the endolymphatic duct. In the cochlea, the canals coil in a spiral around a central axis, the modiolus, which contains the cochlea's nerve and blood supply. The number of turns of the spiral from base to apex varies with species. The endolymphatic canals and perilymphatic channels are continuous with those of the vestibular system, in which there are two sac-like chambers, the saccule and utricle, and the three semicircular canals.

2.1 The Cochlea

In the cochlea, the membranous canal is triangular in cross-section (**Figure 3A, B**) and creates three cochlear compartments (scalae). The lumen of the canal, which forms the scala media, contains endolymph. The scala vestibuli, above the scala media, and the scala tympani below it, contain perilymph. They connect at the apical end of the cochlear spiral at the helicotrema so that there is a continuous perilymphatic space (**Figure 1**). At the basal end of the cochlea, there are two openings through the bony wall from the middle ear space. The oval window over the scala vestibuli is filled with the stapes footplate that delivers sound vibrations displacing perilymph around the spiral. The round window over the scala tympani is covered with an elastic membrane that is displaced outwards and inwards in parallel with stapes movements inwards and outwards by sound vibrations.

The tissues of the cochlea form the walls of the membranous canal. The partition between the scala tympani and the scala media is formed of the acellular basilar membrane, and the sensory epithelium of the cochlea, the organ of Corti, that sits upon it (**Figure 3**). The basilar membrane is freely permeable to perilymph so that the cell bodies and nerves of the sensory epithelium are exposed to perilymph. The border between the perilymphatic and endolymphatic compartments is created by tight junctional sealing at the apical end of the epithelial cells. Hence the apical surfaces of the cells of the organ of Corti are bathed in K^+-rich endolymph, while the cell bodies are bathed in Na^+-rich perilymph. The scala media is separated from the scala vestibuli by the Reissner's membrane.

Along the lateral wall of the scala media is the stria vascularis, an ion-transporting epithelium that is responsible for the production and maintenance of endolymph.

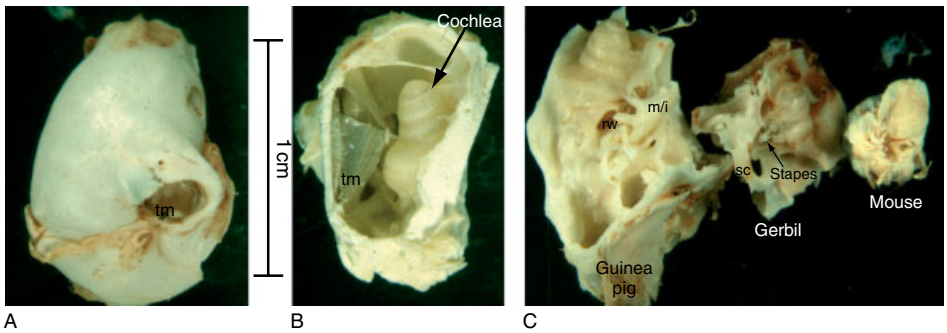

Figure 2 Isolated auditory bullae. (A) Intact bulla from guinea pig. The tympanic membrane (tm, ear drum) covers the opening into the middle ear space. (B) Guinea pig bulla opened to show the cochlea. The tympanic membrane is cone shaped, projecting into the middle ear space. The tip of the tympanic membrane cone is fixed to the first of the ossicles (bones of the middle ear). (C) Opened bullae of guinea pig, gerbil and mouse to show relative sizes. rw—round window, at base of the cochlea; m/i—malleus and incus, two of the middle ear bones which in humans are separate bones, but which are fused together in the guinea pig; sc—semicircular canal, one of three, which are part of the vestibular system.

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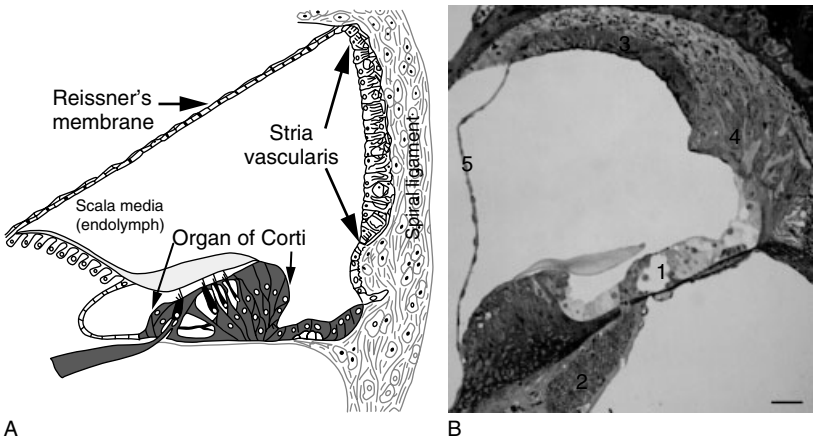

Figure 3 (A) Diagram of a cross-section of a single cochlear turn to illustrate positions of the various tissues. (B) Section of a single turn of the cochlea from a mouse: toluidine blue-stained plastic section. Scale bar 4 µm. The numbers refer to possible sites where pathological alterations might be observed. 1—organ of Corti (there is hair cell loss on this cochlea); 2—auditory nerves; 3—stria vascularis; 4—spiral ligament: the number is located in the approximate position where fibrocyte loss has been noted in some conditions; 5—Reissner's membrane: there is some evidence of distension towards the scala vestibuli in this section, but this may be a preparation artefact.

The stria vascularis rests on the spiral ligament that sits upon the lateral bony wall of the cochlea.

2.1.1 The Organ of Corti

The organ of Corti (**Figure 4**) is composed of sensory 'hair' cells (HCs) and various types of supporting cells. Each HC is separated from its neighbours by intervening supporting cells so no two HCs contact each other. This arrangement creates a regular mosaic of cells at the apical surface of the organ of Corti, that is called the reticular lamina (**Figure 4**). HCs, the mechanosensory cells of hearing and balance organs in all vertebrate classes as well as the neuromasts of the lateral line of fish and aquatic forms of amphibian, generally are elongated in shape and innervated at the their basolateral surface. They are characterized by the presence of an organized bundle of erect projections at their apical end. This hair bundle is formed of rows of 'stereocilia' that increase in height in one particular direction across the cell apex (**Figure 4C**). Stereocilia are not true cilia, but are modified microvilli, each one formed of closely packed parallel filaments of actin (**Figure 4D**). In the apical cytoplasm of the HC is a meshwork of actin filaments, the cuticular plate, into which the rootlets of the stereociliary microfilaments are crosslinked, providing support for the stereocilia. The apical surfaces of the HCs are bathed in endolymph, into which the stereocilia project, whilst the body of the cell is surrounded with perilymph (**Figure 3**). Deflections of the hair bundle stimulate the HC. Deflections towards and away from the longest row of stereocilia regulate the opening and closing of nonselective cation channels (Hudspeth, 1989). Fine tip-links stretching upwards from the distal end of a shorter stereocilium to the side of the longer stereocilium behind (Pickles *et al*., 1984) are thought to act as gating elements controlling the opening of the 'transducer' channel (Hudspeth, 1989; Gillespie, 1995).

In addition to the tip link, the stereocilia in an individual hair bundle are connected by a variety of other extracellular crosslinks (Goodyear *et al*., 2005). One function of these lateral connections is to couple the stereocilia mechanically so that the bundle moves as a unit when deflected. The crosslinks may also play crucial roles in development and maintenance of the hair bundle. Mutations in genes that encode hair bundle crosslinking proteins cause hearing impairment and balance dysfunction, highlighting the importance of the crosslinks to HC activity, and being extracellular they might be considered likely sites of action for potentially ototoxic agents.

In the organ of Corti, the HCs are arranged in parallel rows along the length of the spiral and there are two types (**Figure 4**): a single row of inner hair cells (IHCs) and three to five rows (depending on the species) of outer hair cells (OHCs). In the CBA/Ca mouse strain (whose hearing acuity is maintained for most of its life span and is thus regarded as a 'normal' mouse strain for auditory investigation (Zheng *et al*., 1999)) there are on average 725 IHC and 2300 total OHC along the length of the organ of Corti (Ding *et al*., 2001). In the guinea pig, there are approximately 2000 IHCs and 7000 OHCs (Thorne and Gavin, 1984), while in humans there are about 3000 IHCs and 9000 OHCs (Wright *et al*., 1987). Overlying the organ of Corti and the HCs is another acellular, fibrous structure, the tectorial membrane. The longest stereocilia of the OHC are embedded in the underside of the tectorial membrane. The coupling of the stereocilia to the tectorial

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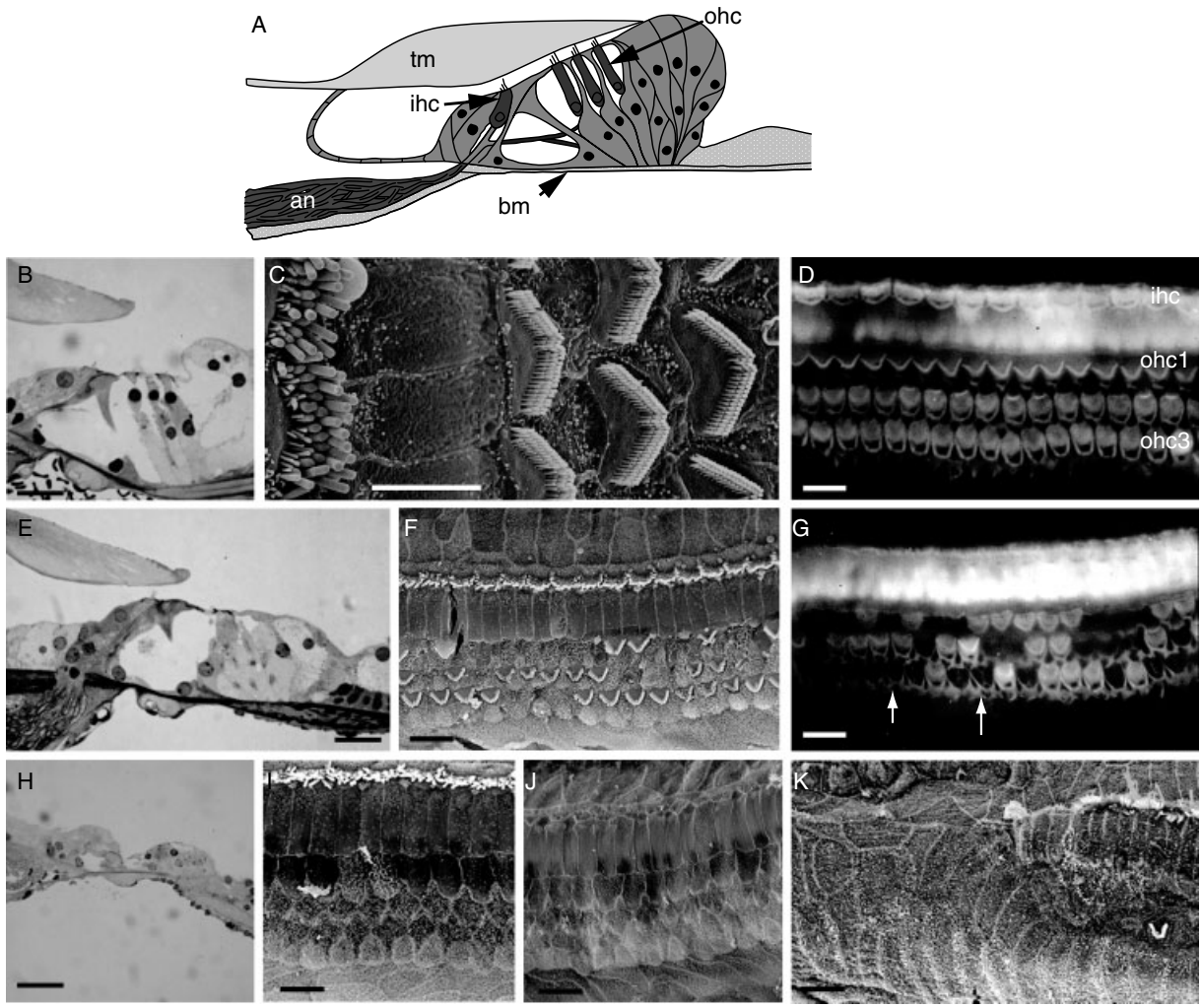

Figure 4 The organ of Corti. Illustration of different examination methods and of aminoglycoside-induced hair cell loss. Scale bars: (B) and (D–K) 20 μm, (C) 5 μm. (A) Diagram to illustrate the architecture. ihc—inner hair cell; ohc—outer hair cell; tm—tectorial membrane; bm—basilar membrane; an—auditory nerve. (B, E, H) Toluidine blue-stained plastic sections. The tectorial membrane tends to shrink away from the apical surface of the organ of Corti during tissue processing. (C, F, I, J, K) Scanning electron microscope (SEM) images of the apical (endolymph-facing) surface, the reticular lamina. Tectorial membrane removed. (D, G) Fluorescence microscopy of phalloidin-stained whole-mount preparations to show the apical surface (phalloidin interacts with actin and so highlights the actin-rich structures at the HC apex). ihc—inner hair cells; ohc1, ohc3—first and third row of outer HCs. (B, C, D) Normal, undamaged organ of Corti. SEM (B) and phalloidin-stained (C) whole mount show that each HC displays a bundle of stereocilia, organized in a W shape on each OHC and a wide curve on IHCs. In each hair bundle the stereocilia are organized in rows that increase in height from the innermost to outermost row (B). The surfaces of supporting cells intervene between each HC. (E–K) Stages during HC loss induced by an aminoglycoside, and subsequent reorganization of the sensory epithelium. (E) Section showing loss of all three OHCs, but persistence of IHCs. (F, G) Early stage of aminoglycoside-induced HC loss. SEM (F) and phalloidin labelling of whole mount (G) show scattered loss of OHC. Remaining OHCs are scattered along the organ of Corti, and SEM (F) reveals some signs of ongoing degeneration. The locations from which HCs have been lost are filled by expansion of the apical surfaces of supporting cells. Arrows in G indicate typical 'scar' formations, where labelling of actin associated with cell junctions reveals where all the supporting cells surrounding an individual HC expand into the lesion site to close it. IHCs are unaffected at this stage. (H) Section showing near complete loss of almost all the elements of normal organ of Corti and its replacement by a simple, flat epithelium. (I, J, K) SEM of stages in HC loss subsequent to that shown in (F) and (G). (I) All OHC lost, but IHC remain. (J) IHC as well as OHC lost. (K) Initiation of replacement of the elements of the severely damaged organ of Corti (to the right of the figure) with a simple epithelium (to the left), an area similar to that shown in section in (H).

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membrane provides a mechanism for stimulating the HC during sound reception.

During sound stimulation, vibration of the stapes footplate displaces fluid along the scala vestibuli and scala tympani, resulting in displacements of the basilar membrane and the organ of Corti towards and away from the scala media. A relative shear motion between the tectorial membrane and the reticular lamina is thereby produced. The consequent deflection of the stereocilia alternately towards and away from the longest row of stereocilia as the basilar membrane is displaced up and down modulates a K^+ current flowing through the HCs from the K^+-rich endolymph at the apical surface to the Na^+-rich perilymph around the HC body. The opening of the transducer channels as the stereocilia are deflected towards the longest row leads to increased K^+ entry, resulting in depolarization of the HC; deflection in the opposite direction closes the channels and the HC becomes hyperpolarized. The potential changes stimulate HC activity. The large positive EP (+80 mV), coupled with a negative resting potential inside the HC, provides a driving force for the current flow increasing the sensitivity of the system (Davis, 1965). The alternating changes in HC potentials in phase with the sound vibrations can be recorded extracellularly from electrodes close to the round window as the cochlear microphonic (CM) potential.

The two HC types in the cochlea have different roles. The IHC is the primary receptor cell. IHCs are directly innervated exclusively by afferent fibres. Each IHC synapses with several different fibres, and about 90–95% of the total afferent innervation to the cochlea terminates on IHCs (Spoendlin, 1973). These Type 1 auditory neurons are myelinated where they exit the sensory epithelium and are bipolar neurons, their cell bodies localized in the spiral ganglia, located just inward of the sensory epithelium all along the cochlear spiral. The changes in electrical potential of IHCs upon stereociliary deflection lead to neural excitation. The activity of the auditory nerve upon sound stimulation recorded from extracellular electrodes as the compound action potential (CAP) is a measure of the neural output from the cochlea that derives primarily from IHC stimulation.

OHC, on the other hand, have a modulatory role. They have an extensive direct efferent innervation, about 80% of the efferent innervation to the cochlea terminating on OHCs. Only 5% of the cochlea's afferent neurons innervate OHC; these Type 2 neurons are thinner than the Type 1 neurons that innervate the IHCs. They are unmyelinated and pseudomonopolar, with their cell bodies also located in the spiral ganglion, and one Type 2 afferent neuron innervates several different OHCs (Jagger and Housley, 2003). Recording of the neural activity of individual cochlear afferent nerves in response to sound, which represents the output from a single IHC, shows that each neuron is extremely sensitive to one particular frequency, its 'characteristic frequency'. This 'tuning' of the neurons to a particular frequency is physiologically vulnerable and it has been found that when OHCs are lost but IHCs remain intact there is a loss of sharp neural tuning and in the ability to detect sounds lower than 60 dB in sound pressure level (i.e. there is a severe hearing impairment but loud sounds can be heard). It is believed that, *in vivo,* OHCs actively modulate the movement of the basilar membrane in response to sound, leading to amplification of the signal reaching the IHC, and increasing sensitivity (by about 60 dB) (Ashmore and Kolston, 1994; Dallos, 1996). The changes in OHC potential in response to sound drive this active response which is mediated by a 'motor' protein, called prestin (Zheng *et al*., 2000) that is unique to OHCs and is highly concentrated in their lateral plasma membranes.

The EP is thought to provide the power for the 'cochlear amplifier' function of the OHCs (Ruggero and Rich, 1991; Dallos, 1996). Thus, loss of EP will result in a hearing impairment because of a loss in amplification. This activity of the OHC results in the emission of acoustic signals from the ear (otoacoustic emissions, OAEs) in response to stimulating sounds (Kemp, 1978; Dallos, 1996). OAE recording provides a noninvasive, sensitive, objective means to assess cochlear function, which merely involves the insertion of a small probe consisting of a microphone and loudspeaker assembly into the external ear canal. It measures the activity of OHCs. CM recorded extracellularly is also dominated by the responses of the OHCs. There are three times as many OHCs as IHCs, and the resting intracellular potential of OHCs is much lower (−70 mV) than that of IHCs (−40 mV), so that CM potentials are much greater in OHCs than IHCs.

There are a number of systematic dimensional variations of the basilar membrane and organ of Corti along the length of the spiral, which affect the mechanical properties of the system such that for different frequencies of the sound stimuli, maximal vibration of the basilar membrane occurs at different locations: sounds of particular frequency cause maximal stimulation at a particular place; a place–frequency relationship or tonotopic organization along the organ of Corti. High-frequency (high-pitched) sounds cause maximum displacement, and thus stimulation, of the HCs at the base of the cochlea, and low frequencies are detected at the apical end (Pickles, 1988). This means that differential damage to the organ of Corti along its length will be reflected in differential loss of frequency perception; if HCs at the basal end of the cochlea are affected, a high-frequency hearing loss results but the ability to detect low frequencies may be unimpaired.

2.1.2 The Stria Vascularis and Spiral Ligament

The stria vascularis is the ion-transporting epithelium of the cochlea. It is composed of three cell types and

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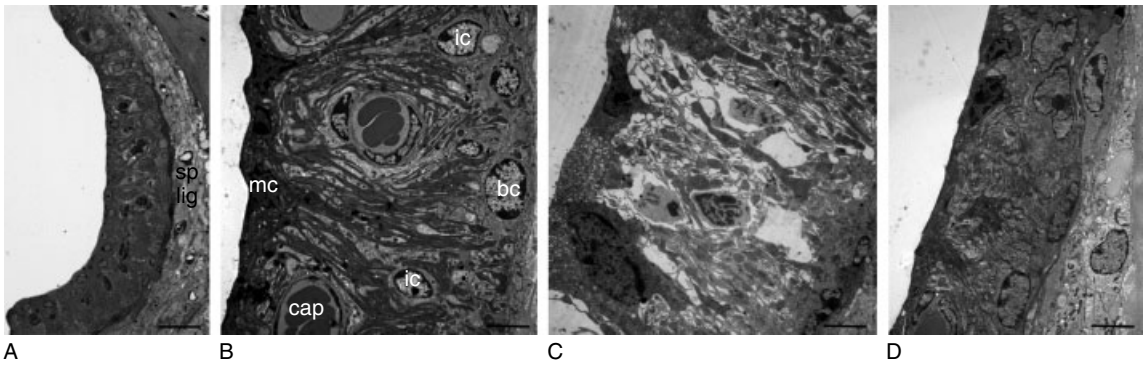

Figure 5 The stria vascularis, to illustrate normal histological features and some possible pathologies. (A) Light microscopy of toluidine-blue-stained section of plastic-embedded normal stria vascularis. sp lig—spiral ligament. (B) Transmission electron microscopy of thin section of mouse stria vascularis. mc—marginal cell, ic—intermediate cell, bc—basal cell, cap—capillary. Note the extensive basal infolds of the marginal cells. (C) Oedema in stria as a consequence of the effects of the loop diuretic, furosemide. Extracellular spaces are grossly enlarged, but the overall architecture of the tissue is undisturbed. (D) Atrophy of stria, 12 weeks after treatment with an aminoglycoside (kanamycin). The stria is much reduced in thickness, principally due to atrophy of the marginal cells, which show a great reduction in the extent of the basal infoldings seen in the normal tissue (B). Scale bars: (A) 20 µm, (B–D) 5 µm.

encloses its own capillary blood supply (**Figure 5A, B**). The stria vascularis is not only responsible for the maintenance of cochlear endolymphatic K^+, but also for the generation and maintenance of the EP. Damage to the stria vascularis will result in a loss of EP. The generation of EP is dependent upon the recycling of K^+ passing through the HCs from endolymph to perilymph and from perilymph back to endolymph via the stria vascularis (Wangemann, 2002). The endolymphatic compartment is lined by marginal cells that actively transport K^+ into endolymph. The basolateral membrane of the marginal cells is extensively infolded and contains high levels of Na^+-K^+-ATPase (adenosine triphosphatase) (Kuijpers and Bonting, 1969; Souter and Forge, 1998) and a Na-K-2Cl co-transporter (Ikeda *et al*., 1997), which are responsible for the entry of K^+ into the marginal cell, while KCNQ1 channels in the apical membrane provide an exit for K^+ from the marginal cell into endolymph (Wangemann, 2002). Tight junctions between marginal cells separate the stria cells from the endolymph. The intermediate cells are enclosed entirely within the stria and are melanocytic cells. The basal cells separate the stria vascularis from the underlying spiral ligament, which is freely permeable to perilymph. A complex network of tight, occluding junctions between adjacent basal cells acts to seal the intercellular spaces of the stria from the perilymph in the underlying spiral ligament. Thus, there is no direct diffusion pathway between the stria and either perilymph or endolymph, entry of oxygen and nutrients (and ototoxins) to the stria deriving from the enclosed vasculature. Numerous gap junctions connect adjacent basal cells, basal cells and cells in the spiral ligament, and basal cells and intermediate cells (Forge, 1984; Kikuchi *et al*., 1995; Forge *et al*., 2003).

The spiral ligament beneath the stria is part of the perilymphatic compartment. The ligament is formed of several subpopulations of fibrocytes, one of which is rich in Na^+-K^+-ATPase (Spicer and Schulte, 1996). These cells take up K^+ from the perilymph in the extracellular spaces of the ligament. K^+ is then passed through cells of the ligament via gap junctions between fibrocytes, and then into the basal cells of the stria vascularis via the gap junctions between strial basal cells and ligament fibrocytes. Basal cells are connected to strial intermediate cells also by gap junctions (Forge *et al*., 2003), and EP is generated by electrogenic K^+ passage across the membrane of the intermediate cells via channels composed of KCNJ10 (Kir 4.1) into the extracellular spaces of the stria (Marcus *et al*., 2002). The activity of the marginal cell Na^+-K^+-ATPase and the Na-K-2Cl co-transporter maintain the low levels of K^+ in the intrastrial spaces necessary for EP to develop, while the tight junctional sealing between marginal cells, between basal cells and between endothelial cells of the capillaries prevents dissipation of the potential. The marginal cell itself is 'electrically transparent', so that the potential generated in the intrastrial space extends into the endolymphatic space.

As well as a role in K^+ recycling, the various subpopulations of spiral ligament fibrocytes are involved in other aspects of cochlear homeostasis, including pH balance (Spicer and Schulte, 1996). Damage to the spiral ligament can therefore be detrimental to cochlear function more generally, and death of certain subpopulations of spiral ligament fibrocytes, along with death of OHC and permanent hearing deficits, has been noted both following exposure to excess noise and with ageing (Hequembourg and Liberman, 2001; Wang *et al*., 2002). Death of OHC in particular can be triggered by failures to maintain the

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physiological environment within the cochlea. Mutations in genes encoding proteins whose activity is necessary for maintenance of the characteristics of endolymph that are localized in cells of the lateral wall can cause deafness, and progressive loss of HCs is often apparent (Steel and Kros, 2001; Boettger *et al*., 2002; Cohen-Salmon *et al*., 2002; Rozengurt *et al*., 2003). These proteins that are involved in maintaining the physiological environment of the cochlea are potential targets for xenobiotics.

2.2 The Vestibular System

The sensory epithelia of the vestibular system are the maculae of the utricle and the saccule, and the cristae which are located within swellings, the ampullae, at one end of each semicircular canal (**Figure 1**). The vestibular neuroepithelia are organized in two-dimensional arrays of HCs and supporting cells, but unlike the organ of Corti, the vestibular sensory epithelia are compact tissues with no large extracellular spaces, and the basement membrane, equivalent to the basilar membrane of the organ of Corti, sits directly upon connective tissue. The intercellular spaces of the vestibular sensory epithelia are continuous with the perilymphatic compartment, while the apical surface is bathed in endolymph. The maculae of the utricle and saccule are overlaid by an acellular fibrous membrane upon which sits a mass of calcium-containing, crystal-like structures, the otoconia, while the cristae of the semicircular canals are overlaid by a gelatinous structure, the cupula. As in the organ of Corti, the HCs are stimulated by deflections of their hair bundles, which in the vestibular system are initiated by changes in the relative position of the head. The utricular and saccular maculae detect static changes relative to the direction of the force of gravity. The cristae detect linear acceleration.

There are two types of vestibular HC. Type 1 HCs are flask shaped, with the basolateral membrane entirely enclosed in a single afferent nerve calyx. In certain respects they resemble cochlear IHCs. Type 2 vestibular HCs are cylindrical and innervated by afferent nerves with bouton-like endings, as well as directly by efferent nerves. Thus, they superficially resemble cochlear OHC. The two vestibular HC types also differ in the types of ion channel along their basolateral membrane which shape the cells' receptor potentials in response to the transduction currents that arise from deflection of the stereocilia (Eatock and Rusch, 1997). The hair bundles of vestibular HCs consist of, in addition to the ranked rows of stereocilia, a single true cilium—the kinocilium—that is located behind the row of longest stereocilia. (A kinocilium is present in hair bundles of the HCs in all other vertebrate classes and is present in the immature hair bundles of the mammalian organ of Corti, but it regresses during cochlear maturation to remain only as the basal body in the mature IHC and OHC.) The positions of the kinocilium and the longest stereocilia define the morphological and functional polarity of the hair bundle, and in the maculae of the vestibular organs there is an abrupt change in bundle polarity along the 'striola', a region running within the middle of the epithelium. Hair bundles on one side of the striola are oriented at 90° to all those on the other: in the utricular macula the kinocilia face each other across the striola; in saccular maculae the rows of shortest stereocilia are facing across the striola. In the maculae, the two HC types are differentially distributed with reference to the striola. Type 1 cells predominate across the striolar regions while Type 2 HCs are more prevalent towards the peripheries. In the cristae, the bundles of all the HCs are oriented in the same direction across the epithelium, but there is a similar differential distribution of the two HC types: Type 1 HCs predominate at the crest of the saddle-shaped structure (the central region), while there is a much higher proportion of Type 2 HCs down the skirts (the peripheral region).

Vestibular endolymph is maintained by the cells of the dark cell regions located around the utricular macula and at the base of the saddle-shaped cristae (but not in the saccule). The dark cell regions consist of a single layer of cells directly overlying connective tissue. The dark cells are equivalent to the marginal cells of the stria vascularis, and actively transport potassium from the perilymph, which surrounds their basolateral surfaces, into the endolymph, which bathes their apical surfaces. There is no separation of the dark cells from the perilymphatic spaces of the underlying connective tissue equivalent to that created by the strial basal cells of the cochlea, and there is no potential in the vestibular endolymphatic compartment equivalent to EP.

3 ACCESS OF OTOTOXINS TO THE INNER EAR

The complete enclosure of the inner ear structures within the bone means that they are not exposed directly to potentially damaging environmental agents. Access to the perilymphatic compartment of the inner ear is possible from the middle ear cavity via the membrane covering the round window at the base of the cochlea. The round window membrane is a three-layered structure, with two cell layers separated by a fibrous layer, but it acts like a semipermeable membrane (Salt and Plontke, 2005). Transfer of substances into perilymph across the round window membrane is mainly passive. After entry into the perilymphatic compartment of the inner ear, an agent would arrive in the vestibule, with access to the basal end of the cochlea (**Figure 1**). Subsequent drug distribution is somewhat complicated, influenced by a relatively slow diffusion rate away from the site of entry and localized

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clearance rates (Salt and Plontke, 2005). Computerized modelling of drug distribution is being used to assess these parameters. Bacterial toxins associated with middle ear infections, as well as ototoxic drugs, may potentially enter the inner ear across the round window. Although this is rare for the former, application of ototoxic agents, in particular aminoglycoside antibiotics, to the middle ear cavity, thereby enabling entry of the drug to the inner ear, is used as a clinical procedure to ablate HCs in the vestibular system in cases of severe balance dysfunction such as that resulting from unilateral Ménière's disease (Blakley, 1997). This approach is also a potential means for introducing therapeutics that may inhibit the effects of agents that normally cause damage to the inner ear, or indeed agents that might stimulate the regeneration of lost HCs. For these reasons interest in the pharmacokinetics of the entry and distribution of drugs in inner ear fluids has been rekindled (Salt and Plontke, 2005).

Access to perilymph via the cochlear aqueduct from the cerebrospinal fluid (CSF) is also possible and this may be a route of entry again for bacterial toxins, such as those associated with meningitis. However, the cochlear aqueduct is not patent in many individuals, and tends to close as an individual ages. Ototoxic agents reach the inner ear predominantly through the blood supply. There are, however, restrictions on the entry of agents to the fluids of the inner ear. Perilymph is not simply an ultrafiltrate of blood plasma, nor does it derive from CSF. The composition of perilymph is different both from CSF and from blood plasma, and the composition of perilymph in the scala vestibuli differs from that in the scala tympani (Wangemann and Schacht, 1996). This indicates that perilymph is produced and circulated locally and that there is a so-called 'blood–perilymph barrier'. Glucose entry, for example, requires facilitated diffusion through glucose transporters (Ito *et al*., 1993; Wangemann and Schacht, 1996). This may limit access of potentially damaging agents to the perilymphatic compartment. However, relatively little is known about the characteristics of this blood–perilymph barrier, and of which and how substances can cross it, but again as possibilities for therapeutic interventions to prevent or ameliorate deafness have become apparent there is further interest in research in this area (Salt and Plontke, 2005). Nevertheless, if entry to perilymph is gained then, because the basilar membrane is freely permeable, there is direct access to the lateral membranes of the HCs, to their synaptic regions and the nerve fibres (**Figure 3B**).

Entry to endolymph in the internal compartment of the inner ear is even more restricted and endolymph composition is tightly controlled (Wangemann and Schacht, 1996). The principal boundaries between endolymph and perilymph, formed by selectively permeable membranes and tight junctions between adjacent cells, appear to be Reissner's membrane, at the level of the basal cells in the stria vascularis, and the network formed by the apical surfaces of the HCs and adjacent supporting cells in the organ of Corti. If an agent were to penetrate these boundaries, however, it would have access to the hair-bearing (apical) ends of the HCs.

4 METHODS OF STUDY

4.1 Physiology and Function

Techniques for assessing the activity of the inner ear have largely been developed for the cochlea. Physiological investigation of the vestibular system is more difficult to perform especially with experimental animals. In the context of ototoxicity this is probably not a significant drawback. There are no agents known to affect only the vestibular system without also being potentially damaging to the cochlea, and chemical agents which are cochleotoxic also have effects in the vestibular system. (Noise, of course, which some define as an ototoxic agent, predominantly affects the cochlea.)

The functioning of the cochlea can be assessed at a number of different levels. Auditory cues detected by the cochlea evoke responses at higher brain centres which can be monitored through the specific reflexes which are elicited (the Preyer reflex) or by behavioural audiograms; the electrical activity associated with the passage of neural signals in the brainstem can be monitored, or the biomechanical activity of the cochlea itself can be evaluated. Electrophysiological testing can provide further detailed analysis.

4.1.1 The Preyer Reflex and Behavioural Response Audiometry

The Preyer reflex is a twitching of the external pinna of the ear in response to sound. The test can be refined by presenting tones of differing frequencies and differing intensities, but clearly there is a subjective element in determining whether there is a response, and the twitching is elicited only at quite high sound pressure levels so that quite significant losses in hearing acuity can accrue before any effect on the reflex is noticeable. Nevertheless, the Preyer reflex is used as a first-level screen for hearing loss, particularly in strains of mutant mouse. For this routine screening a simple device—the 'click box'—has been devised, (Kiernan and Steel, 2000) which, at the push of a button, emits high-intensity (loud, about 90 dB), very high-frequency signals that are within the higher auditory frequency range of mice but which are inaudible to humans because they are above their auditory frequency range (i.e. they are ultrasonic to humans).

More sophisticated and sensitive estimation of the ability of an animal to 'hear' can be obtained through behavioural response audiometry to derive behavioural audiograms (Stebbins *et al*., 1981). For these, an animal is

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trained to perform some task when sounds are presented. The threshold of the response, that is the lowest sound pressure level at which a response is elicited, can be determined for a number of different frequencies over the frequency range to which the animal is sensitive. Any change in the threshold concomitant with the administration of some ototoxic agent can then be evaluated. Such procedures have the advantage that repeated testing of the same animal is possible, allowing preadministration and postadministration thresholds to be compared and the progression of alteration to be followed. However, the procedure is obviously time consuming, especially in the initial training, and is not applicable for many species such as guinea pigs, which in other ways are extremely good animals for ototoxic investigation but are impossible to train. On the other hand, procedures that involve training to respond to sounds are beginning to prove valuable in assessing animal, especially rat, models of tinnitus (Kaltenbach *et al*., 2005; Puel and Guitton, 2007). Tinnitus is the perception of sound in the absence of a sound signal. It is a reported side effect of some ototoxins and may accompany the development of deafness. However, its existence generally requires self-reporting and thus is usually almost impossible to identify in animals.

4.1.2 Auditory Brainstem Responses (ABR)

The stimulation of the auditory nerve following reception of a sound in the cochlea leads to successive stimulations of a number of centres along the auditory neural pathway in the brainstem to the auditory cortex. The electrical activity associated with such stimulations can be recorded with electrodes placed on the skull as a succession of waves of different amplitudes and latencies. The recording of these evoked electrical potentials is a noninvasive procedure that can be used repeatedly in an individual subject, thereby allowing examination of the progression of a hearing impairment following an ototoxic insult. The test involves presentations of sounds of different intensities at a variety of frequencies that cover the frequency range of the animal, and estimation of the lowest sound pressure level that provokes a just-detectable response, the threshold, at each tested frequency. It has been shown that permanent increases in the frequency thresholds of the auditory brainstem responses (ABRs) (louder sounds are necessary to elicit the response, indicating hearing impairment) correlate with loss of HCs at corresponding locations in the cochlea, and that it is possible to monitor transient, that is recoverable, changes in hearing acuity using this procedure (Canlon *et al*., 1993; Zheng *et al*., 1999). ABR reveals the neural output from the cochlea, which it has been argued is the most meaningful measure for rapid assessment of the functional effects of potentially ototraumatic agents (Liberman, 1990), and ABR testing has become the routine procedure for assessing auditory function and identifying hearing impairment in animals.

4.1.3 Otoacoustic Emissions (OAEs)

Activity of the OHCs, induced by sound stimulation, modulates the movement of the basilar membrane and leads to the emission of sounds from the ear, known as otoacoustic emissions. OAEs can be detected in the external ear canal some microseconds after the stimulating input signal (Kemp, 1978). Because OAE recording is a noninvasive procedure that involves only the insertion of a small probe consisting of a microphone and loudspeaker assembly into the external ear canal, it can be used repeatedly and reproducibly in individual subjects and has clinical application especially for testing cochlear function in newborn babies. There are a number of different types of OAE signal depending upon the stimulus parameters used, and for studies of animals the most useful is the distortion product otoacoustic emission (DPOAE). By using a variety of stimulating frequencies, DPOAEs of different frequencies, that is, emissions from different regions of the cochlea, can be obtained, enabling assessment of the active mechanical responses along the length of the cochlea. Recording of DPOAEs has been shown to be a sensitive, reproducible method for assessment of ototoxic effects in the cochleae of animals (Brown *et al*., 1989) and, like ABR testing, is becoming a widely used procedure for auditory assessment of animals in a variety of conditions including ototoxicity, noise trauma and with ageing (Martin *et al*., 2007).

Since OAEs are produced by OHC activity, any changes in OAE amplitude can be attributed either to damage to OHCs themselves and/or to impairment of the stria vascularis affecting EP generation and thus the driving force for the electromotile responses of OHCs that produce OAEs. Loss of OAEs will also be reflected in an increase in ABR threshold, since loss of the OHC activity that produces the OAE will result in loss of cochlear amplification. On the other hand, if OAEs are normal but ABR thresholds are increased, then the hearing impairment so revealed can be attributed to a defect at the level of the IHC or its synapses with the afferent innervation. Consequently the combined use of the two noninvasive procedures, ABR and OAE recording, can provide some diagnostic information on possible sites of damage in the cochlea.

4.1.4 Electrophysiological Activities of the Cochlea

More detailed assessment of the site of a functional lesion in the cochlea can be obtained from monitoring the resting EP or the sound-evoked electrical potentials that derive from the activity of particular parts of the transduction pathway. EP can be monitored via a potassium-filled glass electrode inserted into the scala

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media, either through the round window membrane and the basal coil of the organ of Corti, or through the lateral wall, in anaesthetized animals. Alterations of the EP reflect an effect in the stria vascularis, and because the EP provides a driving force for current flow through the HCs as well as the power that drives the 'cochlear amplifier', a decline in the level of EP decreases the sensitivity of the organ of Corti to sound stimuli. The sound-evoked responses—CM potential and CAP—can be recorded remotely from (usually silver ball) electrodes placed near the round window. CM derives from the modulation of current flow through the HCs during transduction that produces an alternating extracellular electrical potential, which mimics exactly the original sound stimulus, that is, the frequency and polarity of CM follows precisely that of the stimulus (hence 'microphonic'). By varying the frequency of the acoustic stimulus, the CM originating from different regions of the cochlea can be assessed. The sound level at each frequency required to produce a constant CM output, or the amplitude of CM in relation to sound intensity at each frequency, can be measured. CM recorded extracellularly is dominated by the responses of OHCs and thus monitors the health of those cells. Neural excitation following sound stimulation results in the generation of action potentials in the auditory nerve. Remote electrodes placed close to the round window record the gross activity of all the afferent neural units responding to the particular sound stimulus as the CAP. Since the vast majority of those nerves innervate IHCs, the CAP is a measure of their activity.

The recording of EP, CM and CAP are invasive techniques. Generally, these procedures are used acutely and the animal has to be sacrificed after the recording has been made. This means that in examination of ototoxic agents, preadministration and postadministration levels of these potentials, or the progression of any alteration, cannot be made unless this occurs over a time period sufficiently short for the animal to be maintained under anaesthesia, which may be the case with some agents such as diuretics which produce acute effects on EP. It also means determination of abnormality must be made by comparison with untreated control subjects. However, these disadvantages can be overcome by permanently implanting a round window electrode for recording CM and CAP (Aran and Darrouzet, 1975). With such a procedure, long-term, continuous electrophysiological assessment of the effects of ototoxic agents in individual conscious animals can be undertaken.

4.2 Procedures for Examination of Cochlear Tissues

In most mammals the auditory bulla can be relatively easily isolated from the base of the skull and opened to expose the cochlea (**Figure 2B, C**). Breaking open the round window and removing the stapes from the oval window at the base of the cochlea and removing a small piece of bone from its apex provides access for direct perfusion of an appropriate fixative by gentle injection through these openings, before immersion of the opened bulla in fixative. It is also possible to use intravital perfusion of the whole animal with fixative prior to isolation of the bulla, opening the cochlea and then immersing the opened bulla in fixative, but generally fixation may not be as good as after direct perfusion.

The most straightforward means for assessing the neuroepithelia of the inner ear is to examine the features at the surface in whole mounts of the tissue. Following fixation, the bone enclosing the inner ear can be picked away and the neuroepithelia dissected out. Decalcification after fixation may assist dissection. The organ of Corti can be removed in segments, usually sequentially from apex to base. It can then be examined by either phase-contrast or differential interference contrast (DIC) microscopy, or by fluorescence microscopy following staining with fluorescently conjugated phalloidin. Phalloidin interacts with filamentous actin so the stereocilia and cuticular plates, actin-rich structures at the HC apex, are intensely labelled. The actin associated with the junctional complexes between the hair and supporting cells is also labelled, so the cell borders at the tissue surface are outlined (**Figure 4D, G**). Immunohistochemical procedures can also be applied directly to the whole mount preparations. Alternatively scanning electron microscopy (SEM) can be employed to examine the apical surface of the organ of Corti (**Figure 4B, F**) or vestibular sensory epithelia (Davies and Forge, 1987). These techniques can be used to draw up a 'map' depicting the position of each HC along the entire length of the organ of Corti (a 'cytocochleogram') (Viberg and Canlon, 2004), or across the vestibular sensory epithelia (Forge and Li, 2000). The extent and location of damaged or missing HCs can be determined. In the case of the organ of Corti, this can be related to physiological data from the same cochlea. SEM not only provides data for the construction of cytocochleograms, but also allows assessment of some of the earliest events in the progression of damage to the HCs, or of relatively minor alterations, especially to the stereociliary bundle, which would not be visible by light microscopy but may have quite significant functional implications. For example, with high-resolution SEM it is possible to identify the loss of the 'tip-links' between stereocilia, which can occur without there being any other significant alterations to the stereociliary bundle (Osborne and Comis, 1990; Assad *et al*., 1991), or the consequences of effects on the lateral crosslinks between stereocilia, or other components of the stereocilia, that can result in various distortions of the hair bundle (Bryant *et al*., 2005).

An alternative view can be obtained by examination of sections of the cochlea. After opening the bulla to expose

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the cochlea, and fixation, the inner ear is decalcified. The entire, decalcified inner ear can be embedded and sections cut parallel to the modiolus. When sections of the entire cochlea are taken, the tissues are retained in their original relationship and examination can be made not only of the organ of Corti (**Figure 4B, E, H**) but also other tissues which may be sites of action for ototoxic agents, or which are affected as damage following the initial ototoxic insult progresses (**Figure 3B**). Thus, as well as the presence or absence of HCs and the overall architecture in the organ of Corti, it is possible to examine for pathologies other than, or in addition to, HC loss that may account for an observed hearing impairment: for example, loss of, or injury to, afferent neural cell bodies in the spiral ganglion; swelling or atrophy of the stria vascularis; loss of spiral ligament fibrocytes; swelling or collapse of Reissner's membrane that may occur as a consequence of interference with the mechanisms that maintain the volume of cochlear fluids. It is also possible to obtain 'cytocochleograms' from cochlear sections by examining serial sections, reconstructing the length of the cochlear spiral and determining the absolute distance from the apex or base of any particular HC and of the other tissues in its vicinity. This technique is far more laborious than surface examination of whole mounts but gives a more complete picture of the overall pathology (Liberman, 1990).

4.3 Methods to Examine the Direct Effects of Ototoxic Agents

Many studies of the effects of ototoxic agents involve examination of structure and/or function after systemic application of the agent, a situation which mimics clinical conditions, particularly with regard to the important aspect of their distribution to cochear and vestibular tissues. However, such a regimen has disadvantages when investigating cellular or molecular mechanisms, not least that it is difficult to separate the initial interaction of the agent from the ensuing sequelae. Thus for mechanistic investigations, methods that permit the direct exposure of the tissue or cells of interest, either *in situ* or *in vitro*, have an important role to play.

Perfusion of the fluid spaces of the inner ear with ototoxic substances provides one means of assessing direct effects (Nuttall, 1981; Puel *et al*., 1990). In an anaesthetized animal, the cochlea is exposed and two small holes are made in the bony wall over the perilymphatic scalae. A solution of salts of similar composition to perilymph and containing the substance under study is gently pumped into the cochlea through one hole and flows out from the other. The effects can be determined by simultaneous monitoring of CM, CAP and/or OAE, and the reaction terminated at some specified point to examine the attendant structural correlates after perfusion of fixative.

To examine direct effects of ototoxic agents at a cellular level, HCs can be isolated and their morphological and physiological responses following exposure to agents of interest examined in the short term, over a few hours (Zajic and Schacht, 1987; Karlsson and Flock, 1990; Shehata *et al*., 1991; Clerici *et al*., 1993; Sha *et al*., 2001a). Explants maintained in organotypic culture, however, provide a means to examine over quite prolonged periods *in vitro* the responses to damaging agents of neuroepithelia in which the normal tissue architecture is retained. There are difficulties with culturing the mature organ of Corti, but the immature organ of Corti from early postnatal altricial rodents such as mice and rats (animals which are born deaf because the latter stages of cochlear maturation occur postnatally) can be maintained in organotypic culture and the differentiated HCs show responses to stimulation and to ototoxic agents broadly similar to those of HCs in mature tissue *in vivo* (Richardson and Russell, 1991; Richardson *et al*., 1997). Such cultures have been used to examine the effects of a number of different ototoxic agents as well as to test for agents that may prevent ototoxic damage (Richardson and Russell, 1991; Kotecha and Richardson, 1994; Zheng and Gao, 1996; Kopke *et al*., 1997). The mature vestibular organs can also be maintained in organotypic culture for some weeks, and show responses to ototoxins similar to those that occur *in vivo* (Li and Forge, 1995). Indeed, the vestibular sensory epithelia from humans, obtained during certain operative procedures, can be maintained in organotypic culture (Warchol *et al*., 1993), and this provides a rare opportunity for direct experimental studies of human inner ear tissues. In addition, inner ear tissues from nonmammalian vertebrates can be used to explore the effects of ototoxic agents upon HCs. The molecular and biochemical characteristics of all HC types are broadly similar, and the vulnerability of HCs to particular damaging agents is conserved across vertebrate classes. Thus, explant cultures of the inner ears from chickens have been used extensively to explore the molecular basis of HC susceptibility to ototoxins (Matsui *et al*., 2002; 2004; Mangiardi *et al*., 2004). The neuromast organs of the lateral line in fish also offer a potential model. HCs in the neuromasts of zebra fish are susceptible to aminoglycoside antibiotics in much the same way as are mammalian HCs (Williams and Holder, 2000). This has led to the possibilities of using the fish lateral line as a model for screening for agents that kill HCs (Ton and Parng, 2005; Ou *et al*., 2007; Chiu *et al*., 2008), and employing the power of zebra fish genetics to determine the molecular basis of susceptibility and resistance to ototoxic damage. The lateral line organs of living larval zebra fish are easily visualized by light microscopy, and particular fluorescent dyes, for example DASPEI (2-(4-dimethylamino) styryl)-*N*-ethyl pyridinium iodide),

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are preferentially taken up into viable neuromast HCs. This provides an easy means to gain accurate counts of HC numbers and of the extent of HC death in response to potentially damaging agents.

5 EFFECTS AND ACTIONS OF OTOTOXIC DRUGS

A diverse range of therapeutically useful drugs and some environmental agents have been reported to be ototoxic (**Table 1**). For some of these chemicals the evidence for ototoxicity is limited; sometimes it derives from individual case reports, where other confounding factors may have contributed to the observed effect, and there are no confirmatory experimental studies in animals. The adverse effects of these agents vary between relatively minor reversible deficits to permanent functional impairment. Some, exemplified by the 'loop' diuretics and erythromycin, exert acute effects in the stria vascularis resulting in temporary hearing loss (temporary 'threshold shift' (TTS)). Others, such as salicylate and quinine, predominantly produce reversible impairment of HC function resulting in a TTS, which is often accompanied by tinnitus (the perception of sound in the absence of an auditory signal). These symptoms are completely relieved upon withdrawal of the drug. The third, and most significant, group of ototoxic agents cause death of the HCs and permanent hearing loss (permanent threshold shifts (PTSs)). Aminoglycoside antibiotics and *cis*-platinum, as well as organic solvents, fall into this category. Excess noise and ageing also cause HC death, and the effects of ototoxic drugs may afford models of the damaging activity of these nonchemical agents. In the cochlea, aminoglycosides, *cis*-platinum (Platinol-AQ, cisplatinum II, *cis*-diamminedichloroplatinum, *cis*-dichlorodiammine platinum II or *cis*-DDP) and trimethyltin (TMT) affect OHCs predominantly and high frequency (basal) regions of the cochlea are initially affected. They can also produce strial pathologies. Whereas the effects of aminoglycosides and *cis*-DDP generally develop only after repeated administration, effects of TMT become rapidly apparent after a single administration. Toluene and trichloroethylene, like aminoglycosides, damage OHCs following chronic exposures, but mid frequencies rather than high frequencies are initially affected. Carboplatin is of note because, although in many species the ototoxicity is similar to that of *cis*-DDP, in chinchillas at least it causes damage preferentially to IHCs rather than OHCs (Hofstetter *et al*., 1997). In fact it is the only agent yet identified that appears to cause

Table 1 Compounds known to be, or implicated in, ototoxicity (those discussed in detail in the chapter are indicated in bold lettering)

Classification	Compounds	Usual effects (for compound classes where ototoxicity is a frequently reported side effect)
Aminoglycoside antibiotics	Amikacin, dibekacin, dihydrostreptomycin, framycetin, gentamicin, kanamycin, neomycin, netilmicin, ribostamycin, sisomicin, streptomycin, tobramycin	Permanent hearing loss (progressive; high to low frequencies) Vestibular dysfunction
Macrolide antibiotics	Erythromycin, azithromycin, clarithromycin	Temporary hearing loss
Other antibiotics	Ampicillin, capreomycin, chloramphenicol, colistin (polymyxin E), minocycline, polymyxin B, rifampicin, vancomycin, viomycin	
Antitumour agents	***cis*-Platinum,** carboplatin, actinomycin, bleomycin, nitrogen mustards (e.g. mustine), misonidazole	Permanent hearing loss
Anti-inflammatory agents	**Salicylate (Aspirin),** fenoprofen, ibuprofen, indomethacin, naproxen, phenylbutazone	Temporary hearing loss and tinnitus?
Antimalarials	**Quinine,** chloroquine	Temporary hearing loss
Loop diuretics	Bumetanide, ethacrynic acid, frusemide (furosemide), piretanide	Temporary hearing loss
Iron chelators	Desferrioxamine	
Beta blockers	Practolol, propranolol	
Contraceptives	Medroxyprogesterone	
Industrial chemicals	Trimethyltin, toluene, trichloroethylene, styrene, xylene	Permanent hearing loss

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damage preferentially to the IHCs. This has made it a useful tool to examine cochlear physiology (Trautwein *et al*., 1996; Liberman *et al*., 1997), but relatively little has been discovered about the basis of this selective action, which seems to be species specific, and it will not be discussed further. In mammals, the functional deficits resulting from the HC losses caused by these agents are permanent because, unlike the sensory epithelia in the inner ear in birds and other nonmammalian vertebrates (Cotanche, 1999; Staecker and Van De Water, 1998; Stone *et al*., 1998; Taylor and Forge, 2005), the organ of Corti does not spontaneously regenerate HCs to replace those lost, although the mammalian vestibular organs possess some capacity to regenerate HCs (Forge *et al*., 1993; 1998; Kopke *et al*., 2001).

The occurrence and extent of ototoxicity is to some degree dependent upon the dosing regime (or exposure conditions), but is compounded by the status of the patient receiving the drug, and multiple drug regimes. Malnourishment may increase sensitivity as may stress produced by infection (Forge and Schacht, 2000). Drug interactions can also result in much greater damage than would be expected from single drug regimes. A particular example is the interaction between loop diuretics and aminoglycosides that produces rapid, extensive HC loss (Taylor *et al*., 2008) and profound deafness. A number of ototoxic agents, including aminoglycosides, polypeptide antibiotics and antineoplastics, are also nephrotoxic, so that possible damage to the kidney may result in reduced drug clearance and higher serum levels potentially increasing the risk to the inner ear. In addition, certain genetic factors may predispose to ototoxin-related damage. Individuals under similar conditions and with similar drug-dosing regimes differ in their sensitivity to ototoxic side effects. Some of the mutations associated with such predisposition to drug-induced hearing loss have been identified, such as the 'A1555G' mutation in a mitochondrial gene that results in enhanced sensitivity to aminoglycosides (Prezant *et al*., 1993; Usami *et al*., 1998). For these reasons it is not always possible to predict a likely effect following administration of a potentially ototoxic drug.

The classification of ototoxic chemicals into the three broad groups as defined above provides a basis for illustrating the selective action of these different agents upon the inner ear.

5.1 Agents Affecting the Ion-Transporting Epithelia

Those agents whose primary site of action is on the ion-transporting epithelia, the stria vascularis in the cochlea and the vestibular dark cells adversely affect endolymph composition and, in the case of the stria vascularis, the EP. Such agents, exemplified by the loop diuretics, generally cause acute, completely reversible affects after a single drug administration (Rybak *et al*., 1991), although some cases of permanent deafness have been attributed to diuretic administration (Rybak, 1988). The stria vascularis has one the highest rates of oxidative metabolism in the body (Wangemann and Schacht, 1996), with oxygen delivered from the intraepithelial blood supply, so agents that induce anoxia or ischaemia will affect strial activity. Aminoglycoside antibiotics (Forge *et al*., 1987) and *cis*-DDP (Laurell and Engstrom, 1989), may induce permanent strial pathologies after prolonged drug administration although this does not necessarily correlate with effects on EP (see later).

All loop diuretics, that is those diuretics whose principal site of action is in the ascending limb of the loop of Henle, including ethacrynic acid, furosemide (frusemide), bumetanide and piretanide, produce a transient hearing loss across most of the frequency range. This usually occurs following intravenous administration of large doses. The effects are rapid in onset, within minutes or hours, and persist for some hours, but are usually completely resolved within one day if the drug is discontinued. Repeated diuretic administration does not appear to cause permanent damage to the inner ear.

Histological studies of the temporal bones from patients who have died while on diuretic treatment (Matz, 1976; Arnold *et al*., 1981) have shown extensive oedema and swelling of the stria vascularis. Experimental studies on animals have confirmed the stria as the principle site of action (**Figure 5C**), and that diuretics produce a rapid, reversible decline in EP. In experimental studies, EP is seen to decline within seconds of intravenous injection, or minutes following intraperitoneal injection, falling from the usual $+80\,\text{mV}$ to negative values as low as about $-40\,\text{mV}$, the K^+ diffusion potential (Pike and Bosher, 1980; Forge, 1981; Lee and Harpur, 1985). The rate of decline and the level of suppression are dependent upon the dose of drug administered (Rybak *et al*., 1991). The EP then recovers over a period of a few hours but takes several hours to return to normal levels after a single administration. In parallel with the decline in EP an extensive oedema occurs; the extracellular spaces become grossly enlarged (**Figure 5C**) and strial thickness can almost double. This oedema is also reversible, resolving within about two to four hours, prior to complete recovery of EP (Pike and Bosher, 1980; Forge, 1981). The decline in EP correlates with suppression of CM and CAP (Forge and Brown, 1982) and of OAEs, a finding that provides evidence that EP is the power that drives the active mechanical responses of the organ of Corti in response to sound (Ruggero and Rich, 1991). This illustrates the importance of EP maintenance to the cochlear amplification mechanism and why damage to the stria will cause hearing impairment.

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The rapid onset of their effects suggests that the diuretics gain direct access to their site of action through entry from the strial vasculature. This mode of entry would give direct access to the basolateral membranes of the marginal cells. The development of oedema suggests that diuretics inhibit the ion-transporting processes: ions accumulating in the extracellular spaces would be confined by the tight junction sealing between basal cells and those between marginal cells, resulting in osmotic uptake of fluid. In the kidney, diuretics act on a Na-K-2Cl co-transporter. Using an *in vitro* perfusion system, Marcus *et al*. (1987) established that in both the vestibular dark cell layer (Marcus and Marcus, 1989) and isolated strial marginal cell epithelia preparations (Wangemann *et al*., 1995) diuretics act at the basolateral membranes of the dark cells and marginal cells to affect Cl^- transport by interaction with a Na-K-2Cl co-transporter. The ENaC3 (epithelial sodium channel 3)-type Na-K-2Cl co-transporter is expressed in the lateral wall of the cochlea (Ikeda *et al*., 1997). It is currently thought, therefore, that the diuretics reversibly inhibit the action of an electroneutral Na-K-2Cl co-transporter, in the basolateral membranes of strial marginal cells and vestibular dark cells, leading to inhibition of ion transport into endolymph.

Na^+-K^+-ATPase is also present at high concentration on the basolateral membrane of marginal cells. Inhibition of this might have a similar effect as inhibition of the co-transporter. Potassium cyanide, along with its other actions in the body, causes TTS with a symptomatology similar to that of loop diuretics, thought to result from inhibition of marginal cell Na^+-K^+-ATPase (Tawackoli *et al*., 2001). Macrolide antibiotics such as erythromycin also produce effects similar to those of diuretics: generally TTS (Brummett, 1993) of the order of about 50 dB maximum across all frequencies (McGhan and Merchant, 2003) following intravenous administration of a large dose; in animals, a rapid, dose-dependent decline in EP—though not as great a suppression as with diuretics (Kobayashi *et al*., 1997); an action on the basolateral side of the marginal cells affecting ion transport by these cells shown in isolated tissues maintained *in vitro* (Liu *et al*., 1996); and, consistent with inhibition of ion transport in the stria vascularis, sections of temporal bone from patients who have died during a course of erythromycin therapy have shown extensive oedema of the stria vascularis (McGhan and Merchant, 2003). However, the precise site of action of the macrolides in the stria vascularis has not yet been identified.

5.2 Agents Causing Reversible Effects on HCs

Salicylates and quinine cause TTS across most of the detectable frequency range, and tinnitus. Dizziness is reported as a symptom of their use, but there have been no experimental studies of their possible effects on the vestibular system. The ototoxic effects of these agents are generally reversible when treatment ceases; salicylates are rarely associated with permanent hearing loss (Garetz and Schacht, 1996). Although it has been reported that excessive doses of quinine or chloroquine may result in permanent deafness (McKenzie *et al*., 1968; Toone *et al*., 1965), quinine-induced ototoxicity is also usually completely reversible both in healthy volunteers and in patients with malaria (Tange *et al*., 1997).

Salicylates produce TTS across all frequencies simultaneously, indicating effects along the entire cochlear spiral. The shifts are usually no more than about 40–60 dB and almost equal across the frequency range or somewhat greater at higher frequencies. These symptoms are completely reversible within one to three days following withdrawal of the drug and usually develop only at the high dosage levels used in treating rheumatoid arthritis, 2–5 g day^{-1} (Garetz and Schacht, 1996). Salicylate enters perilymph rapidly after systemic administration, peak levels in perilymph being reached one to two hours after injection. There is a linear relationship both between the concentration in perilymph and the serum concentration (Jastreboff *et al*., 1986; Boettcher *et al*., 1990), and between the deterioration of CAP threshold and the perilymph salicylate concentration. Thus, the degree of TTS is quantitatively related to salicylate plasma concentration. These findings indicate that salicylate penetrates the blood–perilymph barrier readily, but the mechanisms of entry have not been examined.

Experimental studies in animals have shown that salicylate inhibits CM responses and CAP only at low stimulus intensities; responses to stimuli above about 60 dB appear to be unaffected. The tuning of responses of individual nerves to their characteristic frequency also is lost (Stypulkowski, 1990; Kujawa *et al*., 1992). In addition, OAEs are reversibly suppressed in humans (Long *et al*., 1986; Martin *et al*., 1988), and animals (Stypulkowski, 1990; Kujawa *et al*., 1992). These findings suggest effects on OHCs and inhibition of the activity that produces signal amplification in the cochlea. EP is unaffected following salicylate administration (Puel *et al*., 1989; Stypulkowski, 1990), and effects of salicylates on cochlear vasculature and the activity of the stria vascularis in generating EP are not considered to be a significant factor in their ototoxicity. Rather, OHCs appear to be the primary target of salicylate following its entry into the perilymph and access to a site of action on the OHC basolateral membrane. Investigation of basilar membrane mechanical responses to sound stimulation during salicylate perfusion *in vivo* (Murugasu and Russell, 1995) have confirmed direct effects on OHCs that adversely influence their active responses, and thus, auditory sensitivity. Salicylates inhibit electrically driven motile responses of isolated OHCs (Shehata *et al*., 1991; Tunstall *et al*., 1995). It is these responses that *in vivo* are

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thought to underlie the cochlear amplification mechanism (Ashmore and Kolston, 1994; Dallos, 1996) and that are driven by the prestin motor protein, unique to the OHC and packed into its basolateral plasma membrane (Dallos and Fakler, 2002). There is evidence that reversible interactions with anions produce conformational changes in prestin that result in the reversible electrically driven changes in OHC length (Oliver *et al*., 2001). Salicylate, an anion, appears able to interact with prestin and block this electromotility (Oliver *et al*., 2001).

Likewise, quinine, at therapeutic doses of about 200–300 mg day^{-1}, can cause threshold shifts at all frequencies, effects which are usually entirely reversible, disappearing upon withdrawal of the drug (Tange *et al*., 1997). As with salicylate, quinine enters perilymph rapidly (Alvan *et al*., 1991), reaching perilymph concentrations directly related to serum concentrations, and direct perfusion of quinine into the perilymphatic space produces decreases in CM and CAP, the magnitude of which is related to perilymphatic concentration of the drug between 10 and 100 μM (Puel *et al*., 1990). OAEs are also affected but EP is unchanged (Puel *et al*., 1990; McFadden and Pasanen, 1994), which considered together indicate a direct effect on OHCs. Consistent with this, electron microscopy of the organ of Corti in quinine-treated animals showed reversible morphological damage exclusively in OHCs (Karlsson *et al*., 1991). Isolated OHCs exposed to quinine undergo slow length changes (Karlsson and Flock, 1990), which, if they occurred *in vivo*, might decouple stereocilia from the tectorial membrane and reduce sensitivity to stimulation. However, these *in vitro* responses were elicited only at concentrations of quinine of between 4 and 5 mM, much higher than those which affect electrophysiological activity of the organ of Corti. In addition, unlike salicylate, quinine affects CM and CAP responses across all stimulus intensities, not just the lower ones, indicating a site(s) of action different from salicylate and not confined to the cochlear amplifier. It has been reported that at the lowest concentrations at which effects can be detected, about 0.05 mM, quinine does not affect OHC responses, OAE or CM, but produces a reversible elevation in threshold for the CAP, which derives from stimulation of IHCs, without affecting neural tuning (Mulheran, 1999; Zheng *et al*., 2001). Tuning derives from the activity of OHCs that produces amplification. This suggests that the initial site of action of quinine may be on the IHCs, synaptic transmission at the base of IHCs, or/and upon the spiral ganglion neurons themselves, with OHCs affected only at higher concentrations. An effect of quinine at the HC synapse could also explain the vertigo, as the ready entry of the drug into perilymph would enable it to gain access to the basolateral membranes of vestibular HCs. It has been found that quinine and its derivatives, such as chloroquinine, block nicotinic acetylcholine receptors (nAChRs) composed of $\alpha 9\alpha 10$ subunits (Ballestero *et al*., 2005), which is the predominant acetylcholine receptor subunit composition of HCs in both chicks and mammals (Fuchs, 1996; Jagger *et al*., 2000). Acetylcholine is the predominant efferent neurotransmitter in the cochlea. Efferent fibres synapse both with the afferent neurons of IHCs just below the HC body, modulating the responses of those afferent neurons to stimulation, and also directly with OHCs whose activity is regulated by the efferent system. Thus, at least some of the effects of quinine may result from its action at these efferent synapses. Nevertheless, since primarily CAP alone is affected at lower drug concentration, the initial effects of quinine may be at the level of the generation of action potentials by IHCs (Ballestero *et al*., 2005).

At present, it is not clear whether the tinnitus, which often accompanies the hearing impairment associated with salicylate and quinine, results from the same sites and modes of action, or represents action(s) at other yet-unidentified sites. The release of efferent control of afferent responses that may occur with quinine could lead to an increase in the spontaneous afferent neural activity (i.e. neural stimulation in the absence of an acoustic signal) that could be perceived as a sound signal. With salicylate, the alterations of cochlear mechanics resulting from inhibition of electromotility may affect the mechanical relationship of the OHCs and IHCs in such a manner as to stimulate activity in the IHCs. It has been shown that following systemic salicylate administration there is an increase in the spontaneous firing rate of auditory neurons (Evans and Borerwe, 1982). Although this may result from direct effects upon the afferent synapses, in other systems salicylates usually decrease and eventually block neural excitation (Neto, 1980). The perceived tinnitus in humans following systemic salicylate treatment is reported to be of high frequency, but as salicylate in perilymph has access to the entire organ of Corti and causes threshold shifts across most of the frequency range, it remains to be explained why it is only neurons which respond normally to high frequencies that show an increased spontaneous firing rate (Evans and Borerwe, 1982; Stypulkowski, 1990).

5.3 Agents that Cause Permanent Hearing Loss and Balance Disorders

The third, broad group of agents are those whose administration causes permanent hearing impairment and/or balance dysfunction resulting from death of the sensory HCs. The ototoxicity of most of these agents usually develops only after repeated systemic administration; single systemic application does not generally produce side effects.

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5.3.1 Aminoglycoside Antibiotics

The aminoglycoside antibiotics constitute the clinically most important group of ototoxic agents (see Forge and Schacht, 2000 for a detailed review). They are toxic to HCs in all vertebrate classes. Indeed the nematocyst cells in marine and aquatic invertebrates, which are mechanotransducing cells thought to be evolutionarily related to vertebrate HCs, are also affected by aminoglycosides (Watson *et al*., 1997). Although all aminoglycosides are potentially both cochleotoxic and vestibulotoxic, the different aminoglycosides exhibit differences in their toxic potential and organ preference. Assessment of the degree and time course of suppression of CM following cochlear perfusion with different aminoglycosides (Schacht, 1986), and analysis of the extent of damage to cultured explants of the organ of Corti directly exposed to different aminoglycosides (Kotecha and Richardson, 1994) have established similar rank orders of cochleotoxic potency for different aminoglycosides. These have indicated that neomycin is the most toxic; gentamicin, kanamycin and tobramycin less so, and amikacin and netilmicin least toxic, but such differential toxicity may not apply in a clinical setting. Streptomycin and gentamicin are considered more vestibulotoxic than cochleotoxic to humans, whereas amikacin and neomycin are primarily cochleotoxic in the human inner ear. The reasons for such preferences are not known, but it is not related to any site-specific uptake mechanism or drug levels in the tissues (Forge and Schacht, 2000). There are also species differences in susceptibility to the different aminoglycosides (Harpur, 1987), of which dihydrostreptomycin provides the most striking example (Hawkins *et al*., 1977; Hawkins and Johnsson, 1981; Stebbins *et al*., 1981). In early clinical use dihydrostreptomycin proved to be markedly toxic to the human cochlea. However, the patas monkey (*Erythrocebus patas*) seems to be the only laboratory animal with a cochlea which is sensitive to the toxic action of dihydrostreptomycin. A significant feature of the response of the patas monkey to dihydrostreptomycin (Hawkins *et al*., 1977) is the fact that the hearing loss progresses long after the cessation of drug administration, resembling the situation in man. Streptomycin also produces hearing loss in patas monkeys when given at a dose which has been shown not to be toxic in man when administered for prolonged periods (Hawkins and Johnsson, 1981). However, in the pigtail macaque monkey (*Macaca nemestrina*) treated with large doses of dihydrostreptomycin for a prolonged period, there was only a very modest shift in threshold at the highest frequency tested, and less than 10% of the HCs were absent at the basal end of the cochlea (Hawkins *et al*., 1977).

The effects of aminoglycosides usually become manifest only after days or weeks of parenteral treatment. Single systemic administrations are not normally damaging to the inner ear, although topical application of a single dose of the drug to the middle ear cavity can almost immediately initiate the progressive damage observed after chronic systemic treatment (Wersäll *et al*., 1969; Forge *et al*., 1998). The severity of the effects increase progressively with time, continuing after drug administration has been stopped; insidiously, in some cases, hearing impairment may not even begin until after treatment has ceased. The initial effect in the cochlea is a hearing loss confined to the high frequencies (Fausti *et al*., 1984) indicating HC damage in the most basal region of the cochlea. The hearing loss then continues progressively to include successively lower frequencies, indicating a spread of the damage to HCs apicalwards along the organ of Corti spiral and involving frequencies in the human speech range to cause a permanent communication disability. A bilateral loss of 20 dB at two or more adjacent test frequencies is accepted as a hearing impairment (Wright, 1998). However, most clinical audiometry usually covers the frequency range only from about 8 kHz downwards, whereas in the average person the high-frequency limit is about 16–18 kHz, so that the initial ototoxic effects of the drug will be missed unless high frequency audiometry is used. Vestibular damage from systemic aminoglycoside administration results in severe unsteadiness which becomes worse in the dark. Perception of unreal movement, usually elicited by head motion, can also occur. Objective clinical assessment is, however, difficult and limited (Wright, 1998).

The degree of vestibular or auditory impairment with aminoglycosides may depend upon the drug itself, the unit or total dose, the route and period of administration and the patient's age or pathological state; for example, the risk is substantially increased by impaired renal function (Jackson and Arcieri, 1971). The situation is further complicated in that the aminoglycosides, to varying degrees, are nephrotoxic. A predisposition to aminoglycoside ototoxicity is also conferred by mutations in the gene that encodes 12s ribosomal RNA of mitochondria. The 'A1555G' missense mutation (an adenosine to guanosine substitution at base position 1555) (Cortopassi and Hutchin, 1994; Fischel-Ghodsian *et al*., 1997), a thymidine to cytosine mutation at 1095 (Zhao *et al*., 2004), cytosine to thymidine missense at 1494 (Chen *et al*., 2007b) and a cytosine insertion at position 961, all appear to confer particular sensitivity to aminoglycosides. In some cases profound hearing loss may occur after only a single parenteral injection rather than the usual situation where hearing loss occurs only after chronic administration. The presence of these mutations in the mitochondrial chromosome has been identified through maternal inheritance patterns. The A1555G mutation is prevalent in Chinese, Arab–Israeli, Japanese and North American families (Prezant *et al*., 1993; Usami *et al*., 1998). A carrier rate of 17% of those developing aminoglycoside-induced hearing loss has been estimated. However, the mutations appear to affect only the cochlea's sensitivity to aminoglycosides; there is no

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enhanced effect on the vestibular system, and no other organ of the system is affected. The reasons why only cochlear HCs are affected or how the mutation enhances susceptibility are currently not known.

Aminoglycosides may also present a problem for patients with tuberculosis or cystic fibrosis. These antibiotics were first developed for use against tuberculosis and are still part of combination drug therapy against it. However, it has been estimated that as many as 80% of patients receiving aminoglycosides chronically to treat tuberculosis develop ototoxic side effects (Brouet *et al*., 1959). Cystic fibrosis patients also receive aminoglycosides on continuous, or frequently repeated, regimes to combat pneumonia. The incidence of hearing loss in these patients has been recorded as at least 16% using audiometric testing (Mulherin *et al*., 1991), but more sensitive measures using OAEs to examine OHC activity suggest a much higher proportion (Mulheran and Degg, 1997). A further consideration is that aminoglycoside antibiotics can cross the placenta. Thus there is a potential to cause deafness in the foetus. Studies of development in animals have suggested a 'critical period' in development, when sensitivity to the ototoxic agent is greatest, at around the time of the onset of auditory function during development. The existence and timing of a critical period in humans has not been identified, but based on anatomical findings comparing development of the human inner ear with that of experimental animals it has been estimated that a critical period for the human cochlea may be present in about week 18–20 of gestation. An ototoxic action of aminoglycosides during intrauterine life of human embryos has been reported (Rasmussen, 1969).

5.3.1.1 Pharmacokinetics

There is a linear relationship between serum concentration of aminoglycoside and the perilymph concentration, at least for gentamicin (Federspil, 1981) and ribostamycin (Harpur *et al*., 1981), but aminoglycosides enter perilymph relatively slowly, the peak concentration after extravascular injection to guinea pigs occurring much later in perilymph (about four hours) than in serum (about 15–30 minutes). Additionally, clearance from perilymph is delayed and the drug persists in the inner ear for some time. The half-life of aminoglycoside in the inner ear has been estimated as more than 30 days (Tran Ba Huy *et al*., 1986), whereas half-life in serum is approximately 3–5 hours. Harpur and Gonda (1982) studied the full kinetic profile of ribostamycin in perilymph and serum after a single dose and after the last of 14 doses under conditions of unchanged renal function. There was no accumulation of the drug in perilymph despite a long half-life (15 hours) after a single dose. This was attributable to a marked increase in the rate of transfer from perilymph to serum after multiple dosing. This indicates an effect of the drug on the cochlea that enhances its elimination from perilymph, but how this occurs is not known. The peak level reached in perilymph after multiple dosing has been reported to be about 50–250 μM (Harpur and Gonda, 1982; Ding *et al*., 2003). As pointed out by Brummett and Fox (1982), the concentration of aminoglycosides in perilymph associated with complete HC destruction following chronic drug administration is much lower than the cytotoxic drug concentration *in vitro*. They concluded that the cytotoxic effect of aminoglycosides on the HCs is highly selective.

Aminoglycosides also enter endolymph, but only after a prolonged period following entry into perilymph. Careful analyses of perilymph and endolymph during and after constant aminoglycoside infusions (Tran Ba Huy *et al*., 1983b; 1986) revealed drug in perilymph 45 minutes after the start of an infusion, with a continuing rise for up to 48 hours, but no drug was detectable in endolymph until after 10 hours and then there was very little further accumulation. This suggested that aminoglycosides probably enter endolymph from perilymph rather than directly from the strial vasculature. After termination of a 48 hour infusion, the drug persisted in endolymph for up to 15 days without significantly declining. It was thought that the slow elimination of the drug might be accounted for by slow release of the drug from binding sites in cochlear tissues. Redistribution of the drug into secondary cellular compartments was evidenced by the complex multiphase elimination kinetics. The half-lives of elimination from cochlear tissues were extremely long, varying from 10 hours to 30 days depending on the duration of drug administration. This may suggest that redistribution of the drug into susceptible cells, or cellular compartments, was an important factor in the development of the delayed toxicity. However, there are no obvious differences between different aminoglycosides in their ability to enter endolymph (Henley and Schacht, 1988), indicating that differential ototoxicity is not related to differential uptake of aminoglycosides into endolymph. Furthermore, *in vivo* pharmacokinetic studies (Dulon *et al*., 1986) of a number of drugs with varying vestibulotoxic and cochleotoxic potentials indicate that their toxicity is not related to different tissue concentrations. Dulon *et al*. (1986) found similar cochlear and vestibular drug levels for amikacin, gentamicin and netilmicin—drugs which exhibit quite different cochleotoxic and vestibulotoxic potentials.

The persistence in perilymph and delayed entry to endolymph may account for the delayed effects of aminoglycosides. The delayed responses and the progressive effects may result from slowly increasing endolymphatic concentrations of the drug to critical levels that can cause damage. Several pieces of evidence from a range of experimental models suggest that aminoglycosides affect HCs through access from their endolymphatic (apical) surface (see below).

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5.3.1.2 Location and Nature of Lesions

Aminoglycoside-induced HC death occurs in a distinct pattern. In the organ of Corti, in line with the pattern of hearing loss, HCs in the basal (high-frequency) coil are affected first, damage spreading progressively apicalwards with time and with increasing dosage. OHCs are more sensitive than IHCs (**Figure 4F, G**). IHCs do not usually appear to die until all the OHCs in their immediate vicinity are lost (**Figure 4E, I, J**). IHCs in mice are particularly resistant to damage. IHCs can persist for months after there has been complete loss of all OHCs (Taylor *et al*., 2008). The reasons for the apparent differential sensitivity between OHCs and IHCs are not yet known, but the delay before IHCs are affected may suggest that their loss is a secondary event that occurs as a consequence of OHC loss, and is unrelated to the initial ototoxic events (Taylor *et al*., 2008). Following death of the IHCs, the spiral ganglion neurons which innervate them begin to die (Hawkins and Johnsson, 1981; Wersäll, 1981). This is probably because survival of the innervation requires neurotrophic factors derived from the IHCs, identified as NT-3 (neurotrophin 3) and BDNF (brain-derived neurotrophic factor) (Ylikoski *et al*., 1993; Ernfors *et al*., 1995), as well as continuing stimulation (normally provided by IHC activity). Perfusion of neurotrophic factors into the cochlea following aminoglycoside administration has been shown to prevent loss of the auditory neurons (Ernfors *et al*., 1996).

The death of each HC is accompanied by expansion of the supporting cells around them to close the lesion and effect tissue repair (Forge, 1985; Raphael and Altschuler, 1991; Li *et al*., 1995; Meiteles and Raphael, 1994). Supporting cells are not usually affected by aminoglycosides (or other ototoxins). Following the initial lesion closure by expansion of the supporting cells (**Figure 4F, G**), reorganization of the sensory epithelium may continue for some time after HC loss, and ultimately the crest of cells that normally constitutes the organ of Corti becomes replaced by an apparently simple cuboidal-like epithelium across the basilar membrane (**Figure 4H, K**). It is not at present clear whether this arises from de-differentiation of those supporting cells that normally surround the HCs in the intact organ of Corti, or their replacement by some other cell type. The pattern of HC loss observed following aminoglycoside exposure—OHC death spreading from base to apex with delayed IHC death—is also seen in many other pathologies in which HC death occurs, including *cis*-DDP ototoxicity (Laurell and Bagger-Sjoback, 1991a; Kaltenbach *et al*., 1997), ageing (Wright *et al*., 1987), as a consequence of the effects of various different genetic mutations that are associated with deafness (Steel and Kros, 2001; Boettger *et al*., 2002; Cohen-Salmon *et al*., 2002; Rozengurt *et al*., 2003) and even with noise, where effects on HCs at a location that relates to the frequency of the damaging sound are accompanied by a base–apex spread along the organ of Corti, of initially OHC death and then of IHC (Wang *et al*., 2002).

In the vestibular system aminoglycoside-induced HC loss is seen initially in the central regions of the epithelia, that is, at the crests of the saddle-shaped cristae and across the 'striola' along the middle of utricular and saccular maculae; HC loss then spreading progressively towards the peripheries (Lindemann, 1969). There is also differential susceptibility among the vestibular organs; in an individual subject, cristae show greater HC loss than the utricle, which in turn shows more extensive damage than the saccule (Wersäll, 1981). This differential susceptibility conforms to a differential uptake of aminoglycoside between the two HC types; immunohistochemistry has shown preferential uptake of gentamicin into the Type 1 HCs (Lyford-Pike *et al*., 2007). The Type 1 HCs predominate on those regions where damage is initiated and are thought to be more susceptible to aminoglycoside-induced damage than the Type 2 vestibular HCs. It is of note that the Type 1 HCs have an exclusively afferent innervation and other similarities to cochlear IHCs, but are the more susceptible of the two vestibular HC types, whereas the more resistant Type 2 HCs have a direct efferent innervation resembling OHCs, the more susceptible HC type in the cochlea. This implies that the HC innervation patterns do not have an influence upon susceptibility of HCs to the toxic effects of aminoglycosides.

Unlike the organ of Corti, where lost HCs are not replaced, there is some evidence for regeneration of HCs in the mammalian utricle (Forge *et al*., 1993; 1998; Lopez *et al*., 1997), but the extent to which this occurs is limited, and only a proportion of the lost HCs may be replaced by new ones (Forge *et al*., 1998). Regeneration of HCs is extensive and can lead to complete recovery of damaged sensory epithelia in birds and other nonmammalian vertebrates (Stone *et al*., 1998; Cotanche, 1999; Taylor and Forge, 2005). The replacement HCs derive from the supporting cell population, which is unaffected by injuries that kill HCs, through initiation of cell division amongst the supporting cells that is stimulated by loss of the HCs (Stone *et al*., 1998; Cotanche, 1999), and/or through direct nonmitotic transdifferentiation, or 'phenotypic conversion' of supporting cells into HCs (Li and Forge, 1997; Taylor and Forge, 2005).

5.3.1.3 Mechanisms of Toxicity

The susceptibility of all HC types in all vertebrate classes emphasizes that HCs are specific targets of the aminoglycosides, and studies using radioactively or fluorescently labelled aminoglycosides administered to animals have shown that the drug is indeed taken up specifically into HCs in the mammalian cochlea (Hiel *et al*., 1992; 1993). The common feature of all HCs is the transduction apparatus at their apical poles (Hudspeth, 1989; Bryant *et al*., 2005). Direct application of aminoglycoside to explant cultures of the organ of Corti from neonatal mice,

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in which the transduction mechanism works normally, though the HCs are not fully mature, (Geleoc and Holt, 2003) results in blockage of the transduction channel (Marcotti *et al*., 2005). Blocking of the transduction channels by aminoglycoside has also been observed in the lateral line systems of fish and amphibians *in vivo* (Kroese and van den Bercken, 1982), and in bullfrog vestibular HCs *in vitro* (Kroese *et al*., 1989). Indeed aminoglycosides are one of the few known blockers of the HC transduction channel, the molecular characteristics of which are currently unknown. Further evidence that an interaction of aminoglycoside with the transduction channel is a crucial element in determining both the cellular selectivity of the response and the progression of injury is demonstrated by the resistance to aminoglycosides of HCs in a strain of mutant mouse with a defect in an unconventional myosin, myosin VIIa (Richardson *et al*., 1997). Myosin VIIa localizes to the apical ends of HCs in both the auditory and vestibular organs in all vertebrates (Hasson *et al*., 1997), and the mutant mice show disorganized hair bundles at their apical ends (Self *et al*., 1998). HCs in organotypic cultures of the organ of Corti prepared from homozygous mutant animals were unaffected by exposure to aminoglycoside, and radiolabelled drug did not enter the cells, which remained undamaged. In contrast in cultures prepared from the heterozygous littermates, the drug entered HCs, but not supporting cells, and killed them (Richardson *et al*., 1997). Myosin VIIa has been found to play an important role in the operation of the gating elements, such as the tip-link, that regulate the opening of the transduction channel in response to sound stimulation (Kros *et al*., 2002). In HCs in which myosin VIIa is defective, opening of the transduction channels is impeded, so that the channels are normally closed, and open only with extreme deflections of the hair bundle (the equivalent of very loud sounds). Since the transduction channels are closed in the HCs in these mutant animals, the failure of entry of aminoglycoside suggests that the drug enters the HC via the transduction channel. Studies of the entry of the styryl fluorescent dye FM1-43 into HCs (Gale *et al*., 2001) support this contention. FM1-43 is of similar size to aminoglycoside (molecular weight of FM1-43 is 452, of kanamycin is 484), is of a similar, elongated shape, and like aminoglycosides FM1-43 is a cation. It is taken up specifically into HCs after short exposures of explant cultures and *in vivo* after systemic application (Meyers *et al*., 2003; Taylor *et al*., 2008). It enters through the transduction channel (Gale *et al*., 2001; Meyers *et al*., 2003; Marcotti *et al*., 2005) and it blocks aminoglycoside entry, protecting HCs from the drug-induced damage (Gale *et al*., 2001). Entry of aminoglycoside from endolymph across the HC's apical membrane has also been inferred from the presence of immunolabelled gentamicin in vesicles in the apical cytoplasm of chicken HCs shortly after the end of a course of systemic drug treatment (Hashino *et al*., 1997). Entry via the transduction channels could explain the faster and greater uptake of aminoglycoside into OHCs in the basal cochlear coil in comparison with those at the apex, that has been observed following systemic application of radioactively labelled gentamicin (Hiel *et al*., 1992; 1993). There are differences in the probability of the open state of the transduction channels that match the differential uptake; in OHCs in the basal coil the probability of transduction channel opening is 50%, but the open-state probabilities decrease towards the cochlear apex (Russell and Kossl, 1992). IHC transduction channels have a 5% open-state probability. However, in explant cultures of the early postnatal mouse organ of Corti directly exposed to radioactively labelled gentamicin, radiolabelling, which is confined only to the HCs, is almost as intense in IHCs as it is in OHCs, indicating entry of drug into both HC types; but IHC are not killed by the aminoglycoside when OHC are (Richardson *et al*., 1997). Furthermore, it is not clear that there are differences between vestibular HC types and across the vestibular sensory epithelia in transduction-channel-open probability that match the differential HC loss observed in those organs. Nor is it easy to explain in terms of transduction-channel-open probability the differential vulnerability of HCs along the cochlea in response to nonchemical damaging agents, such as noise and ageing, in a pattern similar to that of aminoglycosides. Thus, while an affinity between aminoglycosides and the HC transduction channel that enables the drug to enter through that channel could account for HC specificity in all vertebrate classes, other, or additional, factors are likely to be important for the toxicity.

A second molecular mechanism proposed to explain the damage to HCs caused by aminoglycosides is a specific, irreversible interaction of the drugs with the cell membrane phospholipid, phosphatidylinositol-4,5-bisphosphate, (PIP_2) (Schacht, 1986). PIP_2 is located on the cytosolic side of the plasma membrane and, thus, would be accessible only after entry into the cell. The inner ear, neural tissue and the kidney (the tissues susceptible to aminoglycoside toxicity) show a much more active metabolism of phosphoinositides than do other tissues such as the liver or lung, and it has been demonstrated *in vivo* that neomycin inhibits the turnover of phosphoinositides in both the kidney and the inner ear. Furthermore the interactions of the aminoglycosides with lipid bilayers containing PIP_2 produce alterations in bilayer conformation that are unique in comparison with those that occur with other anionic phospholipids, indicating a specific interaction (Forge *et al*., 1989). In addition, changes in the surface pressure of monomolecular films formed of different phospholipids on addition of aminoglycoside have indicated not only a specific binding of the drug to PIP_2, but that the strength of binding of different aminoglycosides matches the rank order of ototoxic potencies (Schacht and Weiner, 1986). That is, neomycin induces

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the greatest change in surface pressure, indicating the strongest interaction, with other aminoglycosides inducing relatively lower changes in parallel with their relative cochleotoxic potencies. Aminoglycosides such as neamine and spectinomycin which are not ototoxic (nor nephrotoxic) and do not cause injury to HCs (Kotecha and Richardson, 1994) do not appear to interact with PIP_2 as assessed in monomolecular films (Schacht and Weiner, 1986).

However, binding of aminoglycoside to PIP_2 does not of itself explain how the HC is killed. It has been shown that following systemic administration, gentamicin can remain inside HCs for as long as 11 months without injury ensuing (Dulon *et al*., 1993). Isolation of the undamaged organ of Corti and its maintenance in short-term culture has revealed that differential sensitivity to damage of HCs along the organ of Corti is inherent in the tissue even in the absence of any specific extraneous potentially damaging agent (Sha *et al*., 2001a). Upon isolation of strips of organ of Corti, OHCs from the basal coil die within two to three hours in maintenance medium, but those at the apex can survive for more than six hours. IHCs survive for more prolonged periods, and supporting cells for up to 24 hours. The survival of basal coil OHCs can, however, be enhanced by addition to the maintenance medium of free radical scavengers, including *N*-acetylcysteine, salicylate and glutathione (Sha *et al*., 2001a). This supports the hypothesis that one trigger for HC death is the production of excess free radicals, and indicates that basal coil HCs are more susceptible to such damage than those at the apex. In line with this notion, the levels of glutathione, one of the natural cellular free radical scavenger systems, are higher in HCs at the cochlear apex than in those at the base (Sha *et al*., 2001a). Previous work (Hoffman *et al*., 1988) had shown that depletion of glutathione by administration of buthionine sulfoxamine rendered the cochlea very sensitive to ototoxic damage, and the sulfhydryl-containing radioprotectant, WR 2721, provided some protection against kanamycin-induced ototoxicity in the guinea pig (Pierson and Moller, 1981). In agreement with these earlier suggestions, coadministration of glutathione with aminoglycoside attenuates both the histopathological and functional consequences of aminoglycoside injury in guinea pigs (Garetz *et al*., 1994), though this is most effective in nutritionally deficient animals (Lautermann *et al*., 1997). Aminoglycoside-induced HC death is also markedly reduced in transgenic mice that overexpress superoxide dismutase 1, one of the enzymes involved in detoxifying free radicals in cells (Sha *et al*., 2001b). The release of free radicals in the inner ear coincident with aminoglycoside treatment has been observed in both chick HCs after gentamicin exposure (Hirose *et al*., 1997) and the mammalian organ of Corti after kanamycin administration (Takayama *et al*., 1997), and the products of free radical damage can be localized by immunohistochemistry to HCs during the early stages of the progression of injury following chronic kanamycin treatment in mice (Jiang *et al*., 2005). These observations provide further support for a role of free radicals in the ototoxic action of aminoglycoside.

Aminoglycosides can chelate iron (Fe^{3+}) (Priuska and Schacht, 1995; Priuska *et al*., 1998), the iron–aminoglycoside complex becoming a source of free radicals, and the iron–aminoglycoside complex may be an initiator of the HC death (see Forge and Schacht, 2000 for review). In the presence of an electron donor, Fe^{3+} can be converted to Fe^{2+}, with subsequent generation of the hydroxyl radical ($OH^{\bullet}$), regeneration of Fe^{3+} and then successive cycling through the same pathway generating further toxic $OH^{\bullet}$ radicals (the Fenton reaction; Evans and Halliwell, 1999). Arachidonic acid has been identified as a preferred electron donor (Priuska *et al*., 1998), and arachidonic acid is one of the fatty acids of which PIP_2 is composed. Therefore, the strong binding that occurs between aminoglycosides and PIP_2 may be a significant factor in the generation of the ototoxic response as the drug–phosopholipid interaction would bring the aminoglycoside in close proximity with arachidonic acid (Sha and Schacht, 1997). Supplemental iron has been shown to enhance aminoglyocoside ototoxicity *in vivo* (Conlon and Smith, 1998). More significantly, coadministration of iron chelators with aminoglycoside can completely prevent aminoglycoside-induced ototoxicity *in vivo* in guinea pigs, preserving both the HCs and auditory function in the cochlea (Song and Schacht, 1996; Song *et al*., 1997) and HCs in the vestibular organs (Song *et al*., 1998; Forge and Li, 2000). Deferoxamine (DFO) provides significant attenuation of the ototoxicity in vestibular organs (Forge and Li, 2000), but complete protection of cochlear HCs is afforded by 2,3-dihyroxybenzoate in combination with mannitol (Song *et al*., 1997; 1998). Interestingly, salicylate (aspirin), which was shown from *in vitro* experiments to enhance survival of HCs in short-term culture (Sha *et al*., 2001a), is a monohydroxybenzoate. It has both iron-chelating and free radical-scavenging properties and, as discussed in the preceding section, is able to enter cochlear perilymph rapidly and thus, crucially, to be available in the right place potentially to be effective in protecting HCs. It has been shown to ameliorate HC loss and hearing impairment when coadministered with aminoglycoside in animal models (Sha and Schacht, 1999). A preliminary clinical trial undertaken in China has now proven the principle of using salicylate as a therapeutic intervention to protect against aminoglycoside ototoxicity in clinical conditions (Sha *et al*., 2006). In a group of about 400 patients prescribed daily gentamicin for two weeks for a variety of ailments, the coadministration of three aspirin tablets three times a day (a total dose of salicylate below that which induces the temporary hearing impairment discussed in the previous section)

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reduced the incidence of a clinically significant hearing loss from 13% in the group of patients who received only gentamicin to 3% in those who also received the aspirin.

Free radicals are one initiator of programmed cell death leading to apoptosis (Hockenberry *et al*., 1993; Evans and Halliwell, 1999). There is considerable evidence that aminoglycosides cause death of HCs by inducing apoptosis (Forge, 1985; Forge and Li, 2000; Forge and Schacht, 2000; Jiang *et al*., 2006). The morphological characteristics of cells undergoing apoptosis have been observed in cochlear HCs of guinea pigs affected by gentamicin (Forge, 1985) and mice affected by kanamycin (Jiang *et al*., 2006; Taylor *et al*., 2008), and in vestibular HCs following systemic gentamicin treatment (Li *et al*., 1995). In explant cultures, both of the mammalian (Forge and Li, 2000) and avian (Matsui *et al*., 2002; 2003) utricular maculae, incubation in pan-caspase inhibitors prevented gentamicin-induced HC death. Inhibitors of the *c-Jun-N-terminal kinase* (JNK) cell stress pathway also attenuated cochlear HC loss not only in explant cultures exposed to gentamicin (Pirvola *et al*., 2000), but also *in vivo* following continuous delivery of the inhibitor from osmotic pumps to the perilymphatic space in neomycin-treated animals (Wang *et al*., 2003). These results implicate the classical caspase-dependent apoptotic pathway triggered by intracellular stress factors that initiate the intrinsic cell death pathway in the HC death (Forge and Van De Water, 2008). Detection of the activation of caspase-3, the principle effector of apoptosis, in aminoglycoside-damaged avian cochlear and vestibular HCs in explant culture, and of the activated initiator capsase-9 in mammalian and avian vestibular organs, supports that conclusion (Matsui *et al*., 2003; Cunningham *et al*., 2002). Activated caspase-3 has also been identified in the kanamycin-damaged organ of Corti in conditions that result in rapid loss of all OHC (Taylor *et al*., 2008), but other work has indicated that the cell death pathways in damaged HCs following chronic treatment protocols which resemble the more usual clinical conditions of aminoglycoside use may be more variable (Jiang *et al*., 2006). There was no indication of activation of caspase-3 but evidence of the involvement of non-caspase-dependent apoptotic pathways. This increasingly detailed understanding of the programmed cell death pathways that are activated, and how they are triggered, is leading to investigation of a variety of procedures for preventing aminoglycoside-induced damage (Rybak and Whitworth, 2005). Such therapies may have wider value since apoptosis triggered in response to excess free radical production is now thought to be a common mechanism of HC death in a variety of conditions (Rybak and Whitworth, 2005; Lynch and Kil, 2005).

5.3.1.4 *Effects of Aminoglycosides on the Stria Vascularis*

Although HCs are the primary site of aminoglycoside action, there is evidence that the stria vascularis also is affected in the initial stages of the response of the cochlea to these drugs. In experimental studies, in animals immediately following the end of a course of aminoglycoside treatment alterations to the stria can be seen at the same time as the earliest effects in the organ of Corti are apparent (Forge and Fradis, 1985), and a decrease in the volume of the stria vascularis in human temporal bones obtained within two weeks of aminoglycoside treatment has been observed (Kusunoki *et al*., 2004). However, as both the organ of Corti and stria show drug-induced abnormalities at these earliest stages, it is not possible to determine the relative chronology of these events, or whether effects in the stria and the organ of Corti are related or occur independently. The lateral wall of the cochlea has been shown to contain a high level of PIP_2, higher in fact than that of the organ of Corti (Schacht, 1986) and, thus, it is possible that the stria may be directly susceptible to the drugs if, as discussed above, this phospholipid serves as the target for aminoglycoside action. There is no direct evidence for an interaction between aminoglycosides and strial tissues, but the use of Texas Red-tagged gentamicin has shown that the aminoglycoside enters strial marginal cells as rapidly as it does into HCs (Dai and Steyger, 2008).

The observed decrease in thickness of the stria is due to an atrophy almost exclusively of marginal cells (Forge *et al*., 1987). Some cells are lost by a process which shows morphological attributes of apoptosis, but the majority of marginal cells remain, but with much reduced volume. Such alteration might be expected to affect EP and the ionic profile of endolymph, but EP appears to be maintained at close to normal levels for up to 4 weeks after the end of aminoglycoside treatment, and is only slightly reduced 12 weeks after treatment when there is significant decrease in strial thickness (Hellier *et al*., 2002). Thus the functional consequences of the effects of aminoglycoside ototoxicity on the stria are not clear. Obviously, the reorganization in the organ of Corti resulting from the loss of HCs and their replacement by supporting cells that occurs following aminoglycoside treatment will have profound effects on cochlear physiology. This might be expected to influence the activity of the stria, but as yet there is no indication of whether and how there might be an inter-relationship between effects in the stria and in the organ of Corti.

Interestingly the changes to the stria and a coincident lack of effect on EP that occur with aminoglycosides are similar to those seen with ageing in a gerbil model (Schulte and Schmeidt, 1992). Here there is significant thinning of the stria, but EP is little affected until the stria is less than one-third its normal volume, when there is a catastrophic loss of EP. These results might suggest that there is significant redundancy in the stria; EP and

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organ of Corti activity can be maintained even when there has been considerable strial injury. Thus, it may be that the stria can sustain injury for some time without a noticeable effect on auditory function, but in this 'compromised' state it might be less able to resist further insults. Consequently a sudden loss of auditory function might ensue in conditions which would otherwise not be expected to cause hearing loss.

5.3.2 cis-Platinum

cis-Platinum (*cis*-DDP), is used to treat various tumours of soft tissue. Like the aminoglycosides, it is nephrotoxic as well as ototoxic (reviewed in Rybak *et al*., 2007). Some audiometric studies have reported that 75–100% of patients suffer some degree of hearing impairment. *cis*-DDP induces a progressive loss of HCs, the extent of which correlates with the dose of drug administered (Hoeve *et al*., 1988). This occurs following repeated injections of relatively low drug doses (1 mg kg^{-1} daily) administered by intramuscular, intraperitoneal or subcutaneous routes, and after a single, large intravenous dose (10–12 mg kg^{-1}) (Laurell and Engstrom, 1989; Laurell and Bagger-Sjoback, 1991a; Laurell and Bagger-Sjoback, 1991b). The pattern of HC damage in the cochlea also resembles that of the aminoglycosides; the first (innermost) row of OHCs is the first to show damage, followed by the second and third rows, and HCs in the basal coil of the cochlea are preferentially affected with damage spreading progressively apicalwards (Laurell and Bagger-Sjoback, 1991a; Laurell and Bagger-Sjoback, 1991b). As with aminoglycosides, the IHCs appear to be relatively resistant to damage following *cis*-DDP administration (Kaltenbach *et al*., 1997). These histopathological findings correlate with assessment of auditory function in both animals and patients, which show an initial high-frequency hearing loss measured by ABR, and suppression of OAEs indicating an effect on OHCs (Sie and Norton, 1997; Allen *et al*., 1998). However, the amplitudes of the CAP and ABR are reduced to a greater extent than is CM, which has revealed that *cis*-DDP may have direct effects upon the spiral ganglion cells themselves (van Ruijven *et al*., 2005), and effects in the stria vascularis are also apparent. Differing dosing regimes may influence which tissue is most affected (Rybak *et al*., 2007). *cis*-DDP also causes HC loss from the vestibular organs in a manner similar to that resulting from aminoglycosides (Wright and Schaefer, 1982; Cunningham, 2006).

There have been very few studies of the pharmacokinetics of *cis*-DDP in the inner ear or of the mechanisms by which it is taken up into cells. It has been reported (McAlpine and Johnstone, 1990) that a rapid deterioration in the auditory nerve response threshold is produced when *cis*-DDP is present in the scala media at concentrations of about 5 μM, but no effect is apparent with perilymphatic perfusion at drug concentrations of less than 3 mM. This suggests that one possible site of the drug action is at the apical end of the HCs. *cis*-DDP has been shown to be able to block transduction currents in isolated HCs from chicken (Kimitsuki *et al*., 1993), and from the characteristics of the response to a single systemic administration in guinea pigs a similar conclusion has been drawn. Thus, it may be that like aminoglycosides an affinity of *cis*-DDP for the HC transduction channel may underlie specific action upon HCs, but there is no conclusive evidence for this.

Nevertheless, it is now clear that the cytotoxicity of *cis*-DDP upon HCs results from the action of free radicals (Rybak *et al*., 2007). *cis*-DDP ototoxicity correlates with a decrease in cochlear glutathione and significant decrease in the activity of glutathione peroxidase, glutathione reductase and superoxide dismutase in cochlear tissues (Ravi *et al*., 1995). Why this occurs is not altogether clear, though it may be due to direct binding of *cis*-DDP to enzymes through the sulfhydryl groups, or *cis*-DDP-dependent depletion of essential cofactors (Rybak *et al*., 2007). This interference with antioxidant systems in the cochlea may lead to accumulation of free radicals and the consequent death of the HCs. Ototoxic doses of *cis*-DDP also lead to the up-regulation of NOX3, a particular isoform of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase that is normally highly expressed in the inner ear. The activity of this enzyme naturally produces reactive oxygen species (ROS), but upon exposure to *cis*-DDP, significant enhancement of superoxide was observed and this has been shown to occur in cochlear tissue (Mukherjea *et al*., 2006). The formation of hydrogen peroxide (H_2O_2) from superoxide radicals has been observed in cochlear tissue (Dehne *et al*., 2001), and H_2O_2 can be converted to $OH^{\bullet}$, which reacts with fatty acids to form 4-hydroxynonenal (Evans and Halliwell, 1999). Superoxide also reacts with nitric oxide to form peroxynitrite, which upon reaction with proteins forms nitrotyrosine (Evans and Halliwell, 1999). Immunohistochemistry has identified 4-hydoxynonenal, but not nitrotyrosine in HCs of *cis*-DDP-treated animals (Lee *et al*., 2004), indicating that generation of $OH^{\bullet}$ may have a critical role in *cis*-DDP-induced death of HCs.

In support of the conclusion that generation of excessive free radicals follows from the effects of *cis*-DDP in the cochlea, agents such as (+)-methionine and 4-methylbenzoic acid, which enable maintenance of glutathione levels or preserve the activity of associated enzyme systems, are highly effective in preventing the ototoxic effects of *cis*-DDP *in vivo* (Campbell *et al*., 1996; Rybak *et al*., 1995). Furthermore, these agents also prevent the HC loss that normally follows from direct exposure to the drug in organotypic cultures of neonatal organs of Corti (Kopke *et al*., 1997). This organotypic culture system also proved of value to screen a range of antioxidant molecules to identify those that are effective in preventing *cis*-DDP ototoxicity. More detailed discussions of agents that are being investigated for their

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value on preventing *cis*-DDP-induced HC death can be found elsewhere (Rybak and Whitworth, 2005; Rybak *et al*., 2007).

5.3.2.1 Effects of cis-Platinum on the Stria Vascularis

cis-DDP causes a decline in EP, indicating an action in the stria, and has been reported to cause strial atrophy, but the effects appear to vary depending upon the dose of drug administered (Laurell and Engstrom, 1989; Klis *et al*., 2000). Following a single, large intravenous dose ($10-12.5\,mg\ kg^{-1}$), EP begins to decline within one day and becomes permanently lost in parallel with the loss of HCs that also ensues. However, with lower doses of the drug given repeatedly (e.g. daily injections of about $2\,mg\ kg^{-1}$ for 10–14 days) by subcutaneous or intramuscular routes, EP does not show any immediate alteration. Ultimately, strial atrophy develops several days or weeks after the end of the chronic treatment. The contrasting responses to the different dosing regimens suggest that effects in the stria and in the organ of Corti are independent of each other and that the stria may be less susceptible to damage from *cis*-DDP than are the OHCs. Immunolabelling for platinated DNA, that results from interaction of *cis*-DDP with DNA, in cochleae from animals treated with ototoxic levels of the drug, has shown positive labelling in the marginal cells in the stria vascularis and in some fibrocytes of the spiral ligament (van Ruijven *et al*., 2005). The identification of TUNEL (terminal-dUTP-nicek end labelling)-positive nuclei (Alam *et al*., 2000) and of activated caspase-3 (Watanabe *et al*., 2003) in marginal cells indicates that *cis*-DDP triggers apoptosis in these cells. The TUNEL-positive cells were present in all three turns of the cochlea in *cis*-DDP treated animals, even when the HC damage was confined to the basal coil. This again suggests the stria vascularis as a separate target of action from the organ of Corti. Free radical damage is the likely cause of cell death in the stria vascularis, but unlike the organ of Corti, there is some evidence that nitrotyrosine as well as 4-hydoxynonenal are generated during *cis*-DDP intoxication of the stria, potentially pointing to some difference in the toxic action in the two tissues. Apoptosis is also evident amongst spiral ligament fibrocytes in cell culture when exposed to *cis*-DDP (Liang *et al*., 2005). This was shown to be a consequence of block of the large conductance potassium (BK) channels in those cells by the drug. However, whether this occurs *in vivo* has not been verified.

5.3.2.2 Effects upon the Spiral Ganglion Neurons

Electrophysiological assessment of sound-evoked responses (CM and CAP) following *cis*-DDP administration has shown that there can be large elevations of CAP thresholds concomitant with relatively small elevations in CM threshold (van Ruijven *et al*., 2004). Since IHCs appear to be intact, these discrepancies suggest an effect at the level of the auditory nerve. Detachment of the myelin sheaths from Type 1 spiral ganglion cells (those that innervate IHCs) has been observed and appears to progressively increase in extent in parallel with loss of OHCs. This suggests that the injuries to the HCs and to spiral ganglion cells are separate phenomena.

5.3.3 Trimethyltin (TMT)

TMT is quite extensively used in a number of industries and is a known neurotoxic agent, but it has been shown to have direct ototoxic effects at doses far lower than those which are neurotoxic (Fechter *et al*., 1986; 1992). Studies in rats and guinea pigs (Crofton *et al*., 1990; Clerici *et al*., 1991; Hoeffding and Fechter, 1991) have shown that it produces effects in the cochlea generally similar to the 'classical' pattern as identified with aminoglycosides and *cis*-DDP; that is, hearing loss beginning at high frequencies which correlates with a loss of OHCs in the basal coil and spreading towards lower frequencies with time.

However, unlike the drugs, TMT rapidly produces pronounced effects after a single intraperitoneal injection. A profound decrease in CAP across all frequencies tested occurs within 30 minutes of injection, and depression of CM, indicative of effects at the OHCs, occurs within 6 hours (Fechter *et al*., 1992). The CAP recovers across much of the lower and middle frequency range with time (Hoeffding and Fechter, 1991), but high-frequency hearing loss persists and OHCs in the basal coil die. These observations indicate that TMT rapidly enters perilymph, gaining access to the HCs and their innervation, and that it acts at the perilymphatic side of the organ of Corti. They also suggest that TMT has two separate sites of action; first, an acute, reversible effect on afferent neurotransmission at the IHCs, noted from the effect on CAP, along the entire cochlea; second, an ultimately fatal effect on OHCs.

That TMT has direct effects on OHCs has been demonstrated in studies with isolated cells (Clerici *et al*., 1993), studies which provide a good illustration of how a direct interaction of a putative ototoxin with a presumed cochlear target can be performed relatively quickly. At concentrations between $30\,\mu M$ and $1\,mM$, TMT caused a dose-dependent shortening of the HCs that was not reversed during a 30 minute TMT-free rinse. Such an effect *in vivo* would result in changes in the micromechanical responses of the organ of Corti to sound and a decrease in auditory sensitivity. TMT also disrupted the lateral plasma membrane of many OHCs. Subsequent studies have shown that incubation of isolated OHCs with TMT leads to release of Ca^{2+} from intracellular stores (Liu and Fechter, 1996), although whether this is a result of direct interference of TMT with calcium

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sequestration, or an indirect effect deriving from more generalized damage, is not clear.

5.3.3.1 Effects of TMT on the Stria Vascularis

Following TMT administration, the stria vascularis becomes reduced in thickness, strial capillary diameter increases but the density of radiating arterioles and collecting venules decreases (Fechter and Carlisle, 1990). These changes, observed six weeks after TMT treatment, are most marked in the middle and apical cochlear turns, whereas OHC loss at this time is most marked in the basal coil. It has therefore been suggested that the effects in the stria are separate from those in the organ of Corti, although EP has not been measured during the acute phase following TMT administration. TMT is thought to uncouple oxidative phosphorylation. Since the strial activity is dependent upon a high level of oxidative metabolism, these effects on the stria may be a consequence of this activity (Fechter and Carlisle, 1990).

5.3.4 Organic Solvents

Exposure to high concentrations of organic solvents induces acute, reversible narcosis and neurotoxicity, but the possibility of ototoxic damage was initially recognized from the unusally high number of workers in the chemical industry who showed a hearing loss, and from case reports of hearing deficits in solvent abusers (Morata *et al*., 1994). The effects of solvents may be exacerbated by concurrent exposure to high noise levels, or the converse; noise-induced hearing loss may become more pronounced with concomitant exposure to certain solvents, thus presenting a significant occupational hazard (Morata and Lemasters, 1995; Morata, 1998). Vestibular dysfunction may also follow exposure to organic solvents (Morata *et al*., 1995) but there is evidence that this results from specific effects on vestibulo-oculomotor pathways in the vestibulocerebellum rather than from effects in the inner ear (Morata *et al*., 1995). This would be classified as neurotoxicity rather than ototoxicity according to the definitions used in this chapter.

A number of different aromatic solvents have been implicated in ototoxicity (Morata *et al*., 1994), and in a comparative study of 21 different solvents, conducted in rats, 8 were identified as ototoxic: toluene, *p*-xylene, ethylbenzene, *n*-propyl benzene, styrene, α-methylstyrene, *trans*-β-methylstyrene and allylbenzene (Gagnaire and Langlais, 2005). Trichloro ethylene has also been reported to be ototoxic (Crofton *et al*., 1994; Fechter *et al*., 1998). The features of the ototoxicity caused by all of these agents are similar and are exemplified by the effects of toluene. Specific cochleotoxic effects of toluene were first observed in studies in rats exposed by inhalation (Pryor *et al*., 1984). ABR and behavioural audiometry revealed auditory deficits after repeated exposure to high doses for periods of 2–16 weeks. Similar results were subsequently obtained after subcutaneous administration. Concomitant with the the occurrence of permanent hearing loss, OHCs are lost (Pryor *et al*., 1984; Campo *et al*., 1997). Suppression of OAEs by toluene confirms the OHCs as a site of injury *in vivo* (Johnson and Canlon, 1994a). However, unlike the majority of ototoxins discussed above, the characteristic features of damage caused by toluene are: (i) it is the mid-frequency ranges of hearing that are affected rather than the high frequencies (Johnson and Canlon, 1994a; Crofton *et al*., 1994), which coincides with HC loss in the middle and apical turns of the organ of Corti (Johnson and Canlon, 1994b; Campo *et al*., 1997); and (ii) damage spreads from the third (outermost) row of OHCs inwards to involve subsequently the second and maybe the first row of OHCs, the reverse of the pattern of damage seen with aminoglycosides (although, as with the aminoglycosides, the IHCs are spared) (Campo *et al*., 1997). Additionally, the supporting cells, especially the third (outermost) row of Deiters' cells, are affected (Campo *et al*., 1997). These features are characteristic of all the solvents implicated in ototoxicity (Crofton *et al*., 1994; Fechter *et al*., 1998; Gagnaire and Langlais, 2005; Campo and Maguin, 2007). The cell bodies of the afferent neurons may also be a target of trichloroethylene (Fechter *et al*., 1998). However, there are species differences in ototoxic susceptibility to solvents. While rats have proved to be sensitive, organic solvents do not cause damage in the inner ears of guinea pigs (Campo and Lataye, 1993; Fechter, 1993; Lataye *et al*., 2003) or chinchillas (Davis *et al*., 2002). This obviously raises questions about the use of particular species of animals as models for the likely conditions in humans. However, it has been argued that the way solvents are distributed and metabolized in rats is similar to the human and thus that these animals are an appropriate model (Campo and Maguin, 2007).

Since organic solvents are minimally water soluble, their distribution in the inner ear is unlikely to be determined by entry into the fluid spaces. It is their distribution in the tissue that is significant. The pattern of damage across the organ of Corti, from outside to in, has suggested that the solvents reach the inner ear from the vasculature of the stria vascularis or the spiral prominence region just below it and then pass through the tissues to the organ of Corti (Campo *et al*., 2001; Campo and Maguin, 2007). The cuboidal cells of the outer sulcus, the epithelial cells that connect the epithelia of the lateral wall to the organ of Corti along the basilar membrane (**Figure 1**) may be a major transport route (Campo *et al*., 2001). Upon reaching the organ of Corti, the supporting cells of the organ of Corti, the Hensen's cells that form the outer ridge of the sensory epithelium and the Deiters' cells that surround the HCs, may then become injured. Deiters' cells, in particular those in the outermost row,

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have been reported to be the most vulnerable cells in the organ of Corti following styrene administration (Chen *et al*., 2007a). The supporting cells are involved in the reuptake of K^+ from around the HCs, and damage to these cells may therefore result in excessive K^+ levels around the OHC that would lead to their death. The effects of the solvents may be upon the membranes of the supporting cells and of the HCs themselves. Loss of membrane integrity and cell swelling, perhaps as a consequence of membrane damage, have been described as early events in the progression of damage in the organ of Corti, and it has been contended that the principle mode of cell death in the organ of Corti is necrosis (Campo and Maguin, 2007). However, the death of Deiters' cells that occurs following styrene administration was reported to be apoptotic and to be triggered through both the intrinsic intracellular stress pathway as well as through the cell death receptor pathway (Chen *et al*., 2007a).

However, why damage is initiated in the middle-frequency region of the cochlea is not clear. In this regard it is perhaps of note that the stria vascularis in the middle and apical cochlear turns is affected by TMT (Fechter and Carlisle, 1990), perhaps indicating some characteristic of the vascular pathways and blood flow that may influence solvent distribution.

5.3.5 Other Ototoxic Agents

Good evidence for the ototoxicity of many of the other drugs listed in **Table 1** is relatively sparse. Vancomycin has been reported to cause transient hearing loss and/or tinnitus, but many of these clinical reports derive from cases in which it has been used in combination with other potentially ototoxic drugs. The few experimental studies which have been performed suggest no loss of HCs or permanent hearing impairment from systemic administration of polypeptide antibiotics even at very high doses (Brummett, 1993). Viomycin has been reported to be predominantly vestibulotoxic following chronic treatment regimes (Daly and Cohen, 1965; Nakayama *et al*., 1991), and animal studies have confirmed that, after repeated systemic injections of relatively high doses, viomycin causes HC death in the vestibular sensory organs in a pattern similar to that seen with aminoglycosides (Kanda and Igarashi, 1969). Chloramphenicol has been shown to cause irreversible hearing loss following infusion into the middle ear cavity in animals (Morizono and Johnstone, 1975), presumably gaining access to the perilymph following uptake across the round window membrane, but clinical reports of hearing loss following use of chloramphenicol are rare. Polymyxin B, when perfused through the perilymphatic spaces, caused an almost immediate decline in CM followed shortly after by a decline in EP, suggesting separate effects on both the organ of Corti and the stria vascularis (Komiya and Tachibana, 1990). However, the rarity of clinical reports in which an ototoxic effect can be attributed directly to polymyxin B suggests the use of this antibiotic does not present a significant risk to the inner ear. Desferrioxamine (deferoxamine mesylate; DFO) binds iron and is used in patients with β-thalassaemia to remove excess iron from the serum. In support of iron chelation as a means to ameliorate aminoglycoside toxicity, in cultured explants of inner ear sensory epithelia, DFO attenuates aminoglycoside-induced HC loss (Forge and Li, 2000). However, repeated high-dose systemic administration of DFO to patients has been reported to cause high-frequency hearing loss in about 20–40% of those receiving long-term therapy (Olivieri *et al*., 1986; Chiodo *et al*., 1997; Karimi *et al*., 2002). In experimental studies in birds, high-dose chronic treatment with DFO produced HC loss in the high-frequency region of the auditory organ in a pattern similar to that caused by gentamicin (Ryals *et al*., 1997), suggesting DFO may kill HCs in a pattern similar to that caused by aminoglycosides. On the other hand, other clinical studies failed to identify a direct ototoxic effect of DFO (Masala *et al*., 1988; Cohen *et al*., 1990), and experimental studies with a mammalian model (chinchilla) could find no effects on cochlear physiology following long-term systemic treatment (Shirane and Harrison, 1987). The reasons for these apparent discrepancies have not been resolved. Differences between experimental models may derive from the differences in susceptibility between species, known to be the case for aminoglycosides (Forge and Schacht, 2000), and differing treatment regimes and/or patient groups may account for differences in clinical reports (Porter *et al*., 1989; Karimi *et al*., 2002).

The reports of ototoxicity following the use of other agents listed in **Table 1** are often anecdotal, and there are no rigorous, well-controlled clinical studies or experimental studies in animal models to confirm and define the ototoxic effects.

5.3.6 Ototoxic Interactions

Both epidemiological studies of the incidence of hearing loss in man and experimental studies in animals have indicated that noise exposure in conjunction with an ototoxic agent may cause more extensive damage than with either agent alone. This is true for noise combined not only with organic solvents (Morata *et al*., 1994; Morata, 1998) as pointed out above, but also noise and aminoglycosides (Brummett and Fox, 1992). Indeed, exposures under conditions in which neither noise nor the aminoglycoside alone would be expected to cause damage can result in marked auditory impairment and HC loss when the two agents are presented together. It has been observed that animals reared in a sound-attenuated room do not take up gentamicin as extensively as animals raised in the environment of a normal animal facility (Hayashida *et al*., 1989). This could result from increased uptake through the transduction channel; in the presence

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of sound stimulation, transduction channels will be in the open state more frequently, thereby enabling increased entry of the aminoglycoside than would be the case in a quiet environment. With increased sound exposure this effect will be enhanced. Thus, with exposure to noise, aminoglycoside uptake to a 'critical' level necessary to cause HC death would be reached more quickly. High sound pressure levels also lead to the generation of a variety of free radical species (Yamashita *et al*., 2004; Henderson *et al*., 1999). In combination with those produced by the interaction of aminoglycoside with cochlear tissues, levels of free radicals exceeding those with which the natural free radical scavenger systems can normally deal may be achieved more quickly than when either agent is presented alone. However, there have been no systematic studies of the mechanisms by which these combined exposures exert their synergistic effects.

Clinical experience and animal studies have also shown that coadministration of a single dose of loop diuretic with a single systemic administration of aminoglycoside, neither of which alone would cause HC loss, produces significant cochlear damage (Brummett, 1981a). All aminoglycoside antibiotics have been found to interact with the loop diuretics (Brummett, 1981a), as has *cis*-platinum (Brummett, 1981b). Administration of an intravenous or intraperitoneal dose of ethacrynic acid, frusemide or bumetanide shortly before or shortly after a single, nonototoxic dose of kanamycin rapidly produces depression of cochlear function which is permanent and associated with extensive HC destruction in guinea pigs or mice (West *et al*., 1973; Brummett *et al*., 1975; Prieve and Yanz, 1984). In mice, subcutaneous injection of kanamycin followed after about 40 minutes (the time of peak plasma levels of the kanamycin after subcutaneous injection (Wu *et al*., 2001)) with a single intraperitoneal injection of bumetanide causes almost complete loss of all OHC within 48 hours of the injections (Taylor *et al*., 2008). The stria vascularis is also affected, becoming progressively thinner through loss of marginal cells. This protocol has provided a model for examining cochlear pathologies in mice, which are particularly resistant to the effects of many agents that cause cochlear damage. The effects of the interaction are generally confined to the cochlea; no significant loss of HCs is apparent in the vestibular system which appears to remain unaffected even at prolonged periods of time after all OHC have been lost from the organ of Corti (McFadden *et al*., 2002; Taylor *et al*., 2008).

Ethacrynic acid markedly increases the penetration of aminoglycosides into endolymph (Tran Ba Huy *et al*., 1983a) and enhances uptake of gentamicin into cochlear HCs (Dai and Steyger, 2008). Bumetanide also enhances the uptake of FM1-43 into cochlear HCs (Taylor *et al*., 2008). The potentiation of cochleotoxicity following systemic administration of both agents therefore likely arises because the diuretic enhances uptake of the aminoglycoside from the systemic circulation into endolymph. It is perhaps noteworthy that the interaction is only manifest when the time between administration of the two agents is relatively short, that is, the diuretic is administered no more than one hour before or two hours after the aminoglycoside (Brummett, 1981a) and that the vestibular system is not affected following the combined administration of single doses of these drugs. The time scale covers the time over which EP is significantly reduced by the diuretic. The reduction in EP to negative values from the normally high positive potential may encourage entry of the cationic aminoglycoside (and FM1-43, or potentially *cis*-DDP) into endolymph. Since there is no equivalent of EP in the vestibular system and the diuretic does not cause a reduction of the positive diffusion potential of the vestibular endolymph, enhancement of aminoglycoside uptake into vestibular HCs would not occur. In line with the absence of HC loss in the vestibular system with combined diuretic–aminoglycoside treatment, FM1-43 uptake into vestibular HCs is not enhanced by coadministration of diuretic (Taylor *et al*., 2008). However, the combination may have also have effects additional to those of either drug alone. Mulheran and Harpur (1998) found that adminstration of a diuretic–aminoglycoside combination quite rapidly caused a significant increase in the CAP threshold without there being adverse effects on either EP or CM. This may suggest that the primary site of action of the two drugs in combination is on the inner HC and/or the afferent synapse.

5.4 Closing Considerations

The inner ear is histologically complex, with a variety of different tissue types serving a diverse range of activities necessary to maintain the optimal physiological environment for detection and transduction of sensory information. All of these are potential targets of ototoxic agents. It should be noted that at the threshold of hearing the deflections of the hair bundles that produce HC responses are of the order of 0.4 nm, within the range of atomic dimensions. Given this level of sensitivity it is surprising how relatively few are the agents that cause significant functional impairment in the inner ear. Although the primary effect of several agents is to cause HC death, damage to other tissues may also occur. Atrophy of the stria vascularis, mainly as a consequence of death and atrophy of marginal cells, is observed as a consequence of the effects of aminoglycosides and of *cis*-platinum, and with noise and ageing, both of which cause HC loss, there is associated loss of a particular subpopulation of spiral ligament fibrocytes (Hequembourg and Liberman, 2001; Wang *et al*., 2002). While the effects of *cis*-platinum on the stria vascularis

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appear to be direct and independent of effects on HCs, with other damaging agents the extent to which the initiation and progression of HC loss is related to damage to other cochlear tissues, or vice versa, is not clear. Damage to the stria vascularis or spiral ligament is likely to affect cochlear homeostasis, which will have adverse effects on HCs. This might be expected to contribute to the characteristics of the hearing impairment resulting from exposure to other ototoxins, noise or ageing. This emphasizes the potential importance of analysing the entire cochlea rather than only the sensory epithelium to determine the possible cause of an observed hearing impairment or in assessing the ototoxic potential of any chemical agent.

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Cardiac Toxicology

Lalita A. Bharadwaj

CONTENTS

1 INTRODUCTION

One of the most important organ systems required for maintaining homeostasis in the human body is the cardiovascular system. The cardiovascular system is the transport system of the human body and its functions are to deliver oxygen, nutrients and other substances to cells and to remove waste products of cellular metabolism. The cardiovascular system is composed of three basic components: the heart, the blood vessels and the blood. The heart weighs less than one pound in an adult, is the size of a fist, lies obliquely in the mediastinum and serves as a pump imparting pressure to blood, thereby establishing a pressure gradient that is required for blood to flow to the tissues of the human body. The blood vessels serve as the passageways through which blood is distributed from the heart to all parts of the body. Blood vessels also redirect blood back to the heart and form a closed-circuit circulatory system. Finally, the blood, the third component of the cardiovascular system, serves as the transport medium within which nutrients, oxygen and waste products are dissolved or suspended. Numerous control mechanisms regulate and integrate the diverse functions and component parts of the cardiovascular system, so that blood is supplied to body areas according to tissue-specific needs. These essential control mechanisms ensure a constant internal homeostatic environment that surrounds each body cell, regardless of differing demands for nutrients or removal of waste products. The normal functioning of the cardiovascular system is essential for life. Toxicity to the component parts of the cardiovascular system can lead to devastating irreparable damage to the normal functioning of all systems of the human body. Toxicity can also result in sudden death due to cardiac arrest.

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Diseases of the cardiovascular system are the main cause of death in the UK and Canada and account for approximately 200 000 and 75 000 deaths each year, respectively (British Heart Foundation, 2006; British Heart Foundation Promotion Research Group, 2005; Heart and Stroke Foundation of Canada, 2004). Diseases of the cardiovascular system are associated with dysfunction of the component parts of the cardiovascular system; namely the heart, the blood vessels of the heart and body, and the blood. Currently the main forms of cardiovascular disease (CVD) are divided into coronary heart disease (CHD) and stroke. About half (48%) of all CVD deaths are associated with CHD and approximately one quarter with stroke. CVD not only affects the elderly, but is also the third leading cause of premature death under the age of 75. In 2006, CVD resulted in just over 53 000 premature deaths in the UK alone (British Heart Foundation, 2006).

Extensive clinical and statistical studies have identified several factors that increase the risk of CVD. The major risk factors contributing to the high prevalence rate of CVD in Canada and the UK are associated with behavioural risk factors. Behavioural risk factors are considered factors in one's personal life that can be eliminated or reduced through lifestyle or behavioural changes (World Health Organization, 2002; Sanderson *et al*., 2009). Some behavioural risk factors include: smoking, physical inactivity, high blood pressure, dyslipidaemias, obesity and diabetes. Although the risk of CVD is primarily associated with lifestyle factors, there are several factors that contribute to an increased risk of CVD that cannot be controlled. These include age, gender, family history and ethnicity. More recently there has been a growing interest in the role of low-level chronic exposure to environmental pollutants in the cause of CVD. Environmental factors are considered key determinants of CVD. Although lifestyle choices, such as smoking, diet and exercise, are viewed as major environmental influences, the contribution of pollutants and environmental chemicals is less clear. There is a growing body of research evidence that suggests that exposure to pollutants and chemicals could elevate the risk of CVD (O'Toole *et al*., 2008). Epidemiological studies report that exposure to fine particles present in ambient air is associated with an increase in cardiovascular mortality and statistically significant relationships between particulate air pollution and ischaemic heart disease, arrhythmias and heart failure have been reported (Puett *et al*., 2008).

It has been known for decades that certain chemicals; both industrial and pharmaceutical, can specifically affect the cardiovascular system. Chemicals that target the heart are classed as cardiotoxins and will be specifically discussed in this chapter. Cardiotoxins exert their effects on the heart both directly and indirectly. The degree of their effects on the heart is mainly associated with dosage and exposure period (Wu, 2008). Cardiotoxins can induce functional or structural effects on the heart. Chemicals can produce functional alterations in the heart by affecting its normal physiological function. For example, cardiotoxic chemicals may alter the normal pumping action of the heart by inducing arrhythmias. Cardiotoxins can selectively induce structural damage to the heart by altering its cellular components. Cardiotoxins may affect the architectural structure of the heart through direct alterations of the myocardial cells. Structural toxicants may directly affect the cardiomyocyte membrane integrity, ion transport and energy supply systems. In general, cardiotoxicity results as a secondary consequence of pharmaceutical/industrial chemical overdoses. Functional alterations in the heart can occur after a single exposure or they may last only during the exposure period to the cardiotoxic compound. More permanent structural and functional changes result as side effects, after administration of an overdose or as a result of the initiation of an inflammatory response following repeated long-term exposures.

A few well-known cardiotoxic agents are chemically classed as plant- and animal-derived toxins, heavy metals, aliphatic alcohols and hydrocarbons, aldehydes, gases (CO), carcinogens and pharmaceutical agents utilized in the treatment of cancer, high blood pressure (antihypertensive drugs), arrhythmias and depression (tricyclic antidepressants), and anaesthetics (such as bupivacaine and halogenated hydrocarbon anaesthetics). Environmental pollutants, including particulate matter, polyaromatic hydrocarbons and metals are suspected to be associated with CVD; however, the contribution of environmental contaminant exposures to CVD is still incompletely understood.

In summary, cardiotoxins lead to the development of heart muscle damage and eventually result in the loss of normal heart function. The heart becomes weaker and is not as efficient in pumping and therefore circulating blood; the main function of the heart. Cardiotoxins affect the normal function of the heart through a number of indirect and direct mechanisms that result in biochemical and electrophysiological alterations. Direct toxic effects include those where the cardiomyocyte is directly affected. These effects on the cardiomyocyte include alterations in the plasma membranes ability for ion transport, and the ability of the cardiomyocyte to generate force and energy. Generally, chemicals influence the electrical and contractile properties of the heart. Chemicals may affect the heart through their effects on heart rate (chronotropic), conductivity (dromotropic), excitability (bathmotropic) or contractility (ionotropic). Indirect damage can occur as a result of the cardiomyocytes' sensitivity to hypoxia, and loss of homeostatic mechanisms of the body responsible for acid–base and electrolyte balance. In general, chemicals can depress oxygen uptake and interfere with cardiac energy metabolism (carbon monoxide), interfere with normal calcium homeostasis and interfere

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with cardiac contraction and impulse conduction (metals, β-adrenergic agents), induce direct damage, resulting in myocardial necrosis (antihypertensives), produce cardiac arrhythmias (respiratory tract irritants) and interfere with nucleic acid synthesis (anticancer agents). The mechanisms of cardiotoxicity are complex and differ substantially amongst the classes of chemicals known to induce cardiotoxicity. In this chapter, several cardiotoxic chemicals will be overviewed, along with their mechanisms of toxicity.

2 OVERVIEW OF THE HEART

2.1 Structure and Function of the Heart

The heart is composed of three layers: the pericardium, the myocardium and the endocardium (**Figure 1**). The pericardium is a double-walled membranous sac that encloses the heart. It acts as a physical barrier, anchors the heart in the mediastinum and protects the heart from infection and inflammation arising from the pleural cavity and lungs. The myocardium is composed of cardiac muscle. Cardiomyocytes, the cells of the cardiac muscle are composed of long, narrow fibres that contain bundles of longitudinally arranged myofibrils, a nucleus, superabundance of mitochondria, sarcoplasmic reticulum (SR) and a plasma membrane or sarcolemma that encloses the cell. Cardiomyocytes, due to their unique features and arrangement in the myocardium, are able to transmit action potentials quickly from cell to cell and maintain high levels of energy synthesis to maintain blood pressure and the regular pumping action of the heart; essential for the circulation of nutrients and gases to the cells of the human body. There are two specialized types of cardiac muscle cells. A majority of cardiac cells that make up the myocardium are contractile cells. These cells perform the mechanical work of imparting pressure to blood for circulation through the body. The cardiac muscle is divided into two separate networks, one atrial and one ventricular. Within these networks, the cardiomyocytes are in close physical contact through transverse thickenings of the sarcolemma or intercalated discs. These discs function to allow action potentials to pass from cell to cell very rapidly and efficiently, thus each network, atria and ventricles, contract as one functional unit. There is no direct physical contact between atrial and ventricular myocardium, thus a specialized conduction system is present to facilitate and coordinate the electrical excitation from the atria to the ventricles to ensure synchronization between atrial and ventricular pumping, and a normal heart rate.

There are a small number of cardiac cells, autorhythmic cells, that do not contract, but are specialized for initiating and conducting action potentials. These cells are responsible for the excitation and contraction of the contractile cells of the myocardium. They are found in specific locations in the myocardium called nodes. These include the sinoatrial node (SAN) and atrioventricular node (AVN), the AV bundle (bundle of His) and the Purkinje fibres. The third and final layer, the endocardium, is the internal lining of the myocardium and is comprised of connective tissue. The endocardium is continuous with the lining of the endothelium that lines the blood vessels of the circulatory system of the body and thus creates a continuous closed-circuit system.

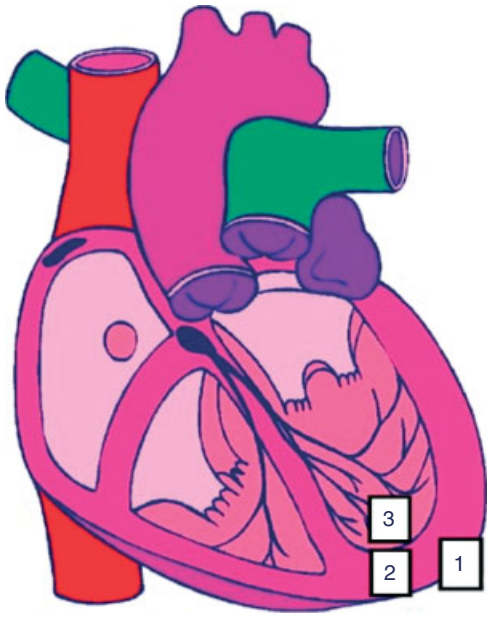

Figure 1 Wall of the heart. This section of the heart shows the (1) pericardium, (2) the myocardium and (3) the endocardium. (Reproduced with permission from John Ross, © 1999.)

2.2 Blood Flow Through the Heart

The heart is the centre of the cardiovascular system. Generally, the cardiovascular system is divided into two separate vascular loops. These include the pulmonary circulation, which carries blood between the heart and the lungs, and the systemic circulation, which carries blood between the heart and organ systems.

The heart is divided into right and left halves and is composed of four chambers; the right and left atria, and right and left ventricles (**Figure 2**). The atrial and ventricular chambers of the heart are separated by electrically nonconductive fibrous tissue. These chambers are essential in facilitating the movement of blood from the heart to the body. The right heart chambers pump deoxygenated blood through the pulmonary

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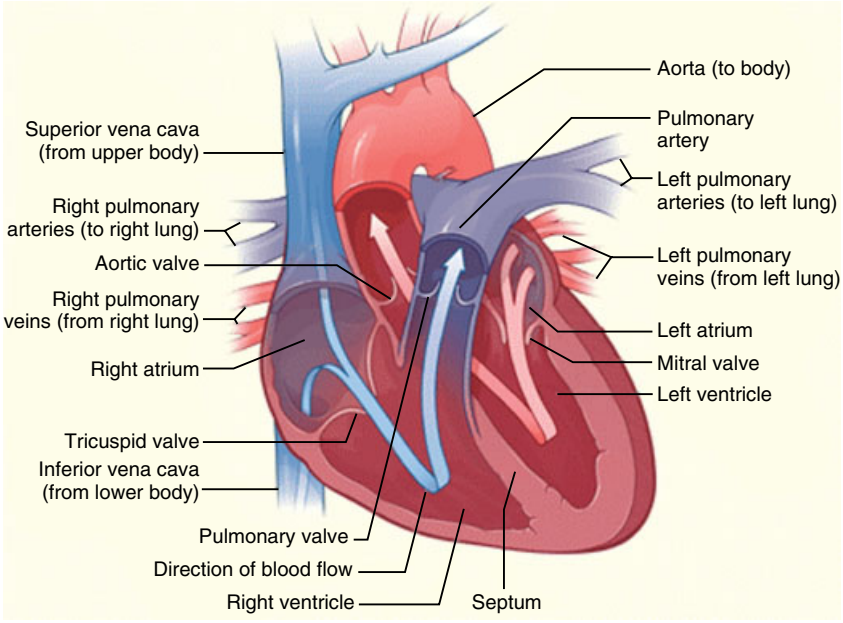

Figure 2 Chambers of the heart. The heart has four chambers: the left atrium, the right atrium, the right ventricle and the left ventricle. During diastole, blood flows into the atria, atrioventricular valves are pushed open and blood begins to fill the ventricles. Atrial systole ejects blood remaining in the atria out into the ventricles. During ventricular systole, the ventricles contract, pushing blood out through semilunar valves into the pulmonary artery (right ventricle) and the aorta (left ventricle). (From *http://www.nhlbi.nih.gov/ . . . /arr/arr_howheartwork.html*.) (Reproduced from U.S. Department of Health and Human Services.)

circulation and the left heart pumps blood through the systemic circulation. The right heart generally consists of structures located on the right side of the heart. These include the right atrium, AV valve (tricuspid valve) and ventricle, the pulmonary semilunar valve and the pulmonary artery. The left heart consists of the pulmonary vein, left atrium, AV valve (mitral valve) and ventricle, the semilunar aortic valve and the aorta.

The opening and closing of valves, controlled by the blood pressure changes in the heart chambers, and the contraction and relaxation of the myocardium control the movement of blood through the heart. During relaxation of the myocardium, or diastole, deoxygenated blood continuously flows through the great venous vessels (inferior and superior vena cava) and the coronary sinus to the right atrium, and oxygenated blood flows from the pulmonary veins into the left atrium. During diastole, blood passively flows from the atria into the ventricles through the opened AV valves, and ventricular pressure rises above atrial pressure, resulting in the closure of the AV valves. During contraction of the myocardium, or systole, ventricular pressure rises above arterial pressure, the semilunar valves open and blood is forced from the ventricles into their respective arteries.

2.3 The Normal Heart Rate

The continuous pumping (beating) action of the heart is a result of the rhythmic contraction (systole) and relaxation (diastole) of the myocardium of the heart wall. In a normal adult the heart beats an average of about 70 beats per minute. The continuous rhythmic beating of the myocardium is highly dependent upon the transmission of electrical impulses or cardiac action potentials through the cardiac muscle. Heart rate can be altered by changes in autonomic nervous system activity. For example, increased parasympathetic activity can slow the heart rate below normal resting (bradycardia is defined as a resting heart rate below 60 beats per minute) and an increased sympathetic activity will augment the heart rate above normal (tachycardia is defined as a heart rate of above 180 beats per minute). Cardiac muscle is capable of generating action potentials without nervous stimulation as a result of specialized cardiac muscle that is part of the heart's specialized conduction system. A complete heartbeat, or cardiac cycle, consists of the contraction (systole) and relaxation (diastole) of both atria and ventricles.

3 THE CONDUCTION SYSTEM

The conduction system is composed of specialized muscle tissue that generates and distributes electrical impulses that stimulate cardiac muscle to contract (**Figure 3**). These include the SAN, a small specialized region in the right atrial wall located near the opening of the superior vena cava; the AVN, a small bundle of specialized cardiac muscle cells located at the base of

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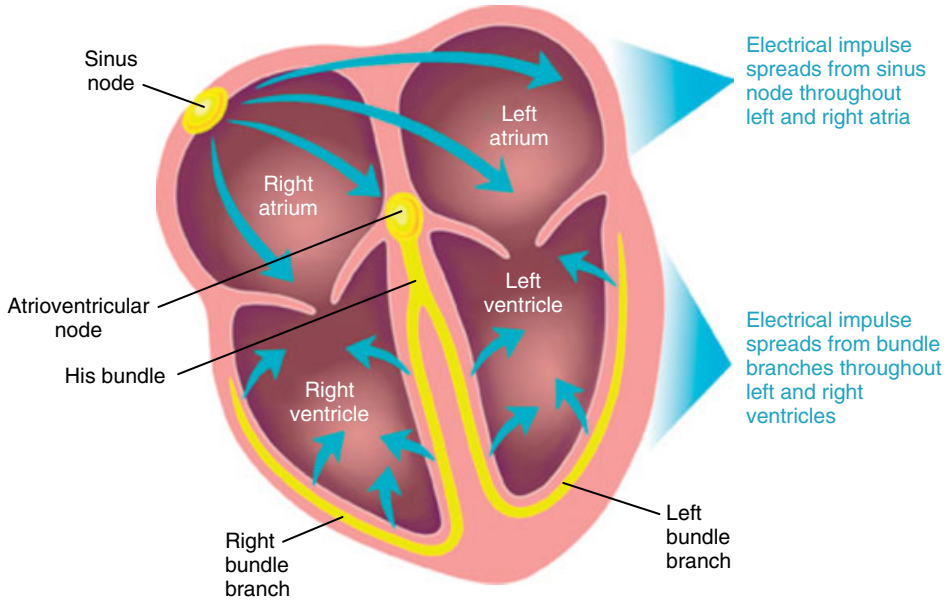

Figure 3 Specialized cardiac muscle cells in the wall of the heart rapidly conduct an electrical impulse throughout the myocardium. Signals are initiated at the pacemaker (sinoatrial node; SAN) and spread to the rest of the atrial myocardium and to the atrioventricular node. The AVN then initiates signals that are conducted throughout the ventricles by way of the bundle of His and Purkinje fibres. (From *http://www.bioen.utah.edu/faculty/sri/Lab8_humancardiovascular.htm*.) (Reproduced with permission from Marquette Electronics, © 2006.)

the right atrium just above the junction of the atria and ventricles; the AV bundle (bundle of His), a tract of specialized cells that originates at the AVN and enters the interventricular septum, where it divides to form the right and left bundle branches, and travels down the septum to the tip of the ventricular chambers and back towards the atria along the outer walls; the Purkinje fibres, small fibres that emerge from the bundle branches and pass into the myocardium of the ventricles.

The sinoatrial node, also known as the pacemaker sets the basic pace for the heart rate, generating about 100–110 action potentials per minute. The average pace of an adult heart is approximately 72 beats per minute. The SAN is under vagal control at rest. Vagal tone brings the resting heart rate down to the normal rate of 60–72 beats per minute. The SAN generates action potentials faster than other components of the conduction system and therefore sets the beating rhythm of the heart. Once the SA node initiates an action potential, the impulse spreads quickly over both atria and causes them to contract (P wave on an electrocardiogram (ECG)). The action potential originating in the SAN is transmitted from the atria to the ventricular myocardium through fibres travelling to the AVN. The action potential is then transmitted to the bundle of His, the left and right bundle branches and finally to the Purkinje fibres of the heart wall to result in contraction of the ventricular muscle (QRS wave on an ECG).

Chemicals may disturb the conduction system of the heart and lead to alterations in heart rate, cardiac impulse origin and conduction velocity. Arrhythmias (dysrhythmias) or deviations from 'normal sinus rhythm' may manifest as a change in heart rate (tachycardia or bradycardia), a disturbance in impulse formation or conduction (AV block) or as premature contractions arising from areas within the heart and outside the normal conduction sequence, as a result of augmented excitability and/or re-entry excitation. In summary, alterations in the conduction system can manifest as dysfunctions in heart rate (chronotropic) contractility (inotropy), conductivity (dromotropy) excitability/irritability (bathmotropy) and in the ability of the heart to relax (lusitropy).

4 THE CARDIAC ACTION POTENTIAL

Movement of ions into and out of the cell creates an electrical difference across the cell membrane called the membrane potential. The resting membrane potential of non-nodal myocardial cells is between −80 and −90 mV. These include the atrial and ventricular myocytes, and the Purkinje cells of the heart. The resting membrane potential of the SAN and AVN are between −50 and −60 mV and −60 and −70 mV respectively. During depolarization, the inside of the cell becomes less negatively charged. The various phases of the cardiac action potential are related to changes in the permeability of the cell membrane to ions, primarily to sodium and potassium. Typically, normal myocardial cell depolarization and repolarization occurs in four phases.

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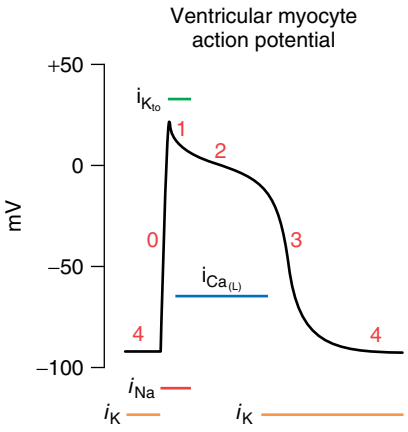

Figure 4 Ventricular action potential. Cardiac contractile cells remain at a resting membrane potential of −90 mV until excited. The rising phase of the action potential is caused by fast sodium influx and the falling phase by a fast potassium efflux. The plateau phase of the action potential results from a slow influx of calcium coupled with a marked decrease in potassium permeability. (Image obtained from Cardiovascular Physiology Concepts, Dr. Richard E Klabunde, PhD at *http://www.cvphysiology.com/Arrhythmias/A004.htm*.) (Reproduced with permission from Klabunde, © 2008.)

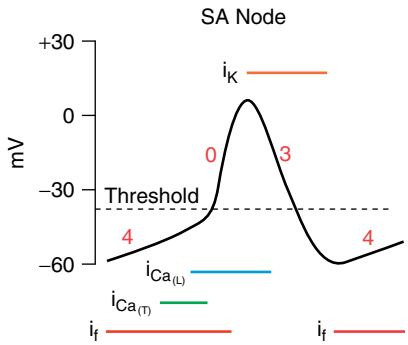

Figure 5 Nodal action potential. The membrane potential of cardiac autorhythmic cells does not remain constant between action potentials. Following an action potential, the membrane slowly depolarizes to a threshold as a result of inactivation of potassium channels, followed by the opening of transient calcium channels. At the same time, less potassium is leaving the cells and sodium continues to leak into the cell, and the cell slowly depolarizes to the threshold. Once the threshold is reached, longer-lasting L-type calcium channels open. There is a rapid influx of calcium, resulting in the initiation of an action potential. The fall back to resting membrane potential results from rapid potassium efflux upon activation of the potassium channels. (Figure obtained from Cardiovascular Physiology Concepts Richard E. Klabunde, PhD *http://www.cvphysiology.com/Arrhythmias/A004.htm*.) (Reproduced with permission from Klabunde, © 2008.)

Phase 4 of the non-nodal myocardial cells (**Figure 4**) represents the resting membrane potential of the cells. The resting membrane potential is maintained during this phase because of the movement of potassium ions through potassium channels. In Phase 4, positive potassium (K^+) ions are moving out of the cell, making the membrane potential more negative inside. At the same time there is no movement of sodium (Na^+) or calcium (Ca^{2+}) into or out of the cell. This is primarily due to the closure of fast sodium channels and (L-type) slow calcium channels. When non-nodal myocardial cells are rapidly depolarized to their threshold voltage of about −70 mV, there is a rapid depolarization (Phase 0) that is caused by an inwardly directed movement of Na^+ into the cell. This occurs through the opening of fast Na^+-channels on the sarcolemma. At the same time, potassium channels close and movement of K^+ out of the cell ceases. Therefore, the membrane potential moves towards a more positive value and away from its resting membrane potential of −80 to −90 mV.

Phase 1 represents an initial repolarization that is of short duration and is caused by the outward movement of K^+. This movement is primarily due to the opening of a unique K^+ channel located in the myocardial membrane. At approximately the same time as K^+ is moving outward, and when the membrane potential has depolarized to approximately −40 mV, slow Ca^{2+} channels open, calcium moves into the cells and Phase 2 or the plateau of the action potential results. Repolarization, or Phase 3, of the myocardial action potential occurs when the movement of K^+ out of the cell increases due to complete opening of the K^+ channels and closing of the Ca^{2+} channels.

In summary, the action potential in nonpacemaker cells of the myocardium is primarily determined by the changes in fast Na^+, slow Ca^{2+} and K^+ channels and the movement of these ions across the sarcolemma.

Cells within the SAN and the AVN are characterized as having no true resting potential and have the ability to generate regular, spontaneous action potentials. Nodal action potentials, unlike non-nodal myocardial cells, are divided into three phases (Phases 4, 0 and 3) (**Figure 5**). Phase 4 of the action potential in these cells is considered the phase of spontaneous depolarization (pacemaker potential) or the period where an action potential is triggered once the membrane potential reaches a threshold between −40 and −30 mV. Phase 4, starts when the membrane potential is about −60 mV. At this membrane potential, slow Na^+ channels are open and there is a slow inward movement of Na^+ into the cell. Additionally, during Phase 4 there is a slow decline in the outward movement of K^+, as the K^+ channels, responsible for Phase 3 or repolarization, continue to close. This fall in K^+ conductance (gK^+) also contributes to the ability

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for spontaneous depolarization. The slow inward movement of Na^+ causes the membrane potential to begin to spontaneously depolarize resulting in the initiation of Phase 4 of the pacemaker action potential. Phase 0 is the depolarization phase of the action potential. The depolarizing current in these myocardial cells is primarily a result of the movement of Ca^{2+} through slow Ca^{2+} channels. The movement of Na^+ through fast Na^+ channels is responsible for the depolarization of the nonpacemaker myocytes of the heart. In fact, there are no fast Na^+ channels operating in nodal cells. As a result these cells depolarize more slowly and pacemaker action potentials are sometimes referred to as 'slow-response' action potentials. Phase 0, when depolarization begins and the membrane potential reaches approximately -50 mV, the transient or T-type Ca^{2+} channels open and Ca^{2+} moves into the cells. The movement of Ca^{2+} into the cells results in movement of the membrane potential to a more positive value. As the membrane continues to depolarize and moves towards a more positive, value, a second Ca^{2+} channel opens. Opening of this channel occurs around a membrane potential of -40 mV. These Ca^{2+} channels are called the long-lasting or L-Type Ca^{2+} channels. The L-Type channels open and result in the movement of more Ca^{2+} into the cell until the action potential threshold is reached (usually between -40 and -30 mV). Thus, Phase 0 or depolarization in nodal tissue is primarily caused by an increased Ca^{2+} conductance (gCa^{2+}) through the L-type Ca^{2+} channels that begin to open towards the end of Phase 4. The movement of Ca^{2+} through the L-type Ca^{2+} channels is not rapid, and thus the rate of depolarization (slope of Phase 0) is much slower than that found in other cardiac cells (e.g. the nonpacemaker cells, the atrial and ventricular myocytes, and the Purkinje cells). Phase 3, or repolarization, follows phase 0. The movement back towards the resting membrane potential in Phase 3 is a result of the movement of K^+ ions out of the cell. Once the cell is completely repolarized at a membrane potential of about -60 mV, the cycle is spontaneously repeated. Phase 3 or repolarization occurs as K^+ channels open (increased conductance, gK^+). This results in an increasing outward-directed, hyperpolarizing K^+ current. At the same time, the L-type Ca^{2+} channels close, gCa^{2+} decreases and the inward depolarizing Ca^{2+} currents diminish, resulting in the re-establishment of the resting membrane potential.

The normal initiation and propagation of myocardial action potentials is essential for the proper functioning of the heart. Alterations in ion movements, membrane potential or transmission of cardiac action potentials through the heart muscle can lead to severe disturbances in the normal cardiac rhythm and contractile ability of the heart. It is well characterized that some chemical agents cause disturbances in the normal formation and conduction of cardiac action potentials by their alteration of normal ionic gradients and fluxes across the membrane of cardiomyoctes. For example, some metals, such as barium, affect the normal formation of cardiac action potentials. Barium, because of its charge (2^+), can carry currents across the membrane in place of calcium ions, thus reducing the normal resting membrane potential of the cell. When the resting membrane potential is reduced or modified to a more positive value, the cells are more likely to have an increased automaticity. Due to a reduction in resting membrane potential, the cell is more readily excitable and will depolarize prior to its normal time course. This results in a premature impulse formation and contraction of myocardial cells, resulting in both alterations in normal heart rate (or arrhythmias) and contraction.

Antiarrhythmic drugs are known to alter the conduction of action potentials in non-nodal tissue of the heart. These drugs block the specific ion channels important in the normal generation of action potentials in non-nodal tissue. Sodium-channel blockers, such as quinidine, can inactivate the fast sodium channels and reduce the rate of depolarization (decrease the slope of phase 0) of non-nodal tissue, thus reducing the propagation rate of the action potential, and slowing conduction and ultimately contraction of the heart. Calcium-channel blockers, such as verapamil and diltiazem, affect Phase 2 of the action potential. Verapamil blocks the slow inward calcium current, an action that decreases the concentration of intracellular calcium and thus contractile activity of the heart decreases. Potassium-channel blockers delay Phase 3, or repolarization, by blocking the potassium channels. When the action potential of a cardiac cell fails to return to the resting level along its normal timeframe, a second action potential may arise (early after depolarization). In this case, where repolarization is delayed, a second depolarization may occur prematurely, and this can lead to premature contractions of the heart and altered heart rhythms ultimately affecting the blood supply to the systemic and pulmonary circulations, and oxygen to the body tissues.

Chemicals may also alter impulse conduction in nodal tissue. Chemicals, such as cardiac glycosides, may delay the propagation of electrical impulses in the conduction tissue of the heart. For example, digitalis glycosides will increase the refractory period in the AVN and thus decrease impulse conduction in this section of the conduction system. If the effect is severe enough, conduction will be blocked at the AVN. Chemicals may also modify the sensitivity of the conduction system to endogenous agents. For example, halogenated hydrocarbons, such as chloroform, have been shown to sensitize the heart to the effects of catecholamines and sympathomimetic agents. Thus, in the case of a previous exposure to halogenated hydrocarbons, the normal catecholamine effects (increased heart rate, force of contraction) are exaggerated at lower internal concentrations. Additionally, as the catecholamine dosage is increased, there is risk for the development of rhythm

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(tachycardia) and contraction (premature contractions) dysfunctions.

5 EXCITATION–CONTRACTION COUPLING

Excitation–contraction coupling (ECC) is the process whereby a cardiac action potential triggers a myocyte to contract. When a myocyte is depolarized by an action potential, calcium ions enter the cell, through L-type calcium channels located on the sarcolemma, during Phase 2 of the action potential. Calcium triggers a subsequent release of stored calcium from the SR through calcium-release channels. The intracellular calcium concentration is increased. Free intracellular calcium binds to a protein, troponin-C (TN-C), which is part of a regulatory complex attached to thin contractile filaments (actin myofilaments). The binding of calcium to TN-C induces a conformational change in the regulatory complex and a site on actin is exposed and able to bind to the myosin head of the thick contractile filament. This binding results in the hydrolysis of adenosine triphosphate (ATP), which supplies energy for a conformational change to occur in the actin–myosin complex, resulting in cross-bridge cycling (actin and myosin filaments slide past each) and contraction of the cardiomyocyte. Cross-bridge cycling occurs as long as the cytosolic calcium remains elevated. At the end of Phase 2, calcium entry into the cell slows and calcium is sequestered by the SR by an ATP-dependent calcium pump (sarcoendoplasmic reticulum calcium-ATPase), thus lowering the cytosolic calcium concentration; actin-myosin interaction stops and the myocardial cell relaxes.

Contraction of the myocardium is highly dependent on the availability of ATP and calcium. Mechanisms that enhance the concentration of cytosolic calcium increase the amount of ATP hydrolysed and the force generated by the actin and myosin interactions, as well as the velocity of shortening. Physiologically, cytosolic calcium concentrations are influenced primarily by β-adrenergic stimulation. β-adrenergic stimulation increases the force and shortening velocity of contraction (i.e. positive inotropy) and increases the rate of relaxation (i.e. positive lusitropy). Mechanisms that alter ATP availability will also impact the normal contraction of the myocardial cell.

Chemical agents can alter the force of myocardial contraction by affecting any of the steps involved in the ECC process. Generally, agents that alter intracellular calcium levels interfere with the autonomic nervous system control of the heart, alter metabolic processes for energy production/utilization, oxygen and nutrient delivery and the process of ECC will impact the force of myocardial contraction. Additionally, disturbances in the integrity of the myocardial cell membrane, ion movements and membrane-bound enzyme activity will alter the propagation and duration of the myocardial action potential and thus alter contractile strength. Alterations in the normal function of myofilaments of the conduction system will also alter contraction, leading to alterations in force and development.

6 THE NORMAL ELECTROCARDIOGRAM

Electrical impulse transmission through the conduction system generates currents that can be detected on the surface of the skin and recorded using an electrocardiograph. The recording of the sum of all cardiac potentials (a full cardiac cycle) is called an electrocardiogram or ECG (**Figure 6**). This type of recording is performed to evaluate symptoms of heart disease, to detect abnormalities in heart rate or conduction and to evaluate effectiveness and side effects of therapeutic agents. There are three very distinct waves recorded during each cardiac cycle. The first is called the P wave and it represents atrial depolarization. The atria contract a fraction of a second after the P wave begins. The second wave, called the QRS wave or complex represents the sum of all ventricular muscle cell depolarization. This wave begins as a downward deflection, then continues as an upward triangular wave and finishes as a downward wave. The ventricles undergo contraction shortly after the QRS complex begins. The third wave, the T wave indicates ventricular repolarization.

There are several intervals and segments on the ECG and they provide information on the rate of conduction. The PR interval, measured from the beginning of the P wave to the beginning of the QRS wave, is a measure of the conduction time from the beginning of atrial excitation to the beginning of ventricular excitation or the time necessary for impulses to be transmitted from the SA node through the atrium, AVN and His–Purkinje system. The PR interval normally ranges from 0.12 to 0.2 seconds. The ST segment represents the time during which the ventricles are contracting and emptying. The QT interval lasts approximately 0.4 seconds and is sometimes referred to as the electrical systole of the ventricles. The TP interval represents the time when the heart is in diastole and ventricular filling is taking place.

7 CARDIOTOXIC CHEMICALS

Chemicals known to induce cardiotoxicity can be classed chemically or by therapeutic usage. Aliphatic alcohols, aldehydes, glycols, halogenated alkanes, heavy metals and biotoxins, as well as cardiovascular, central nervous system and chemotherapeutic agents have all been identified as cardiotoxic chemicals. Cardiotoxicity is highly

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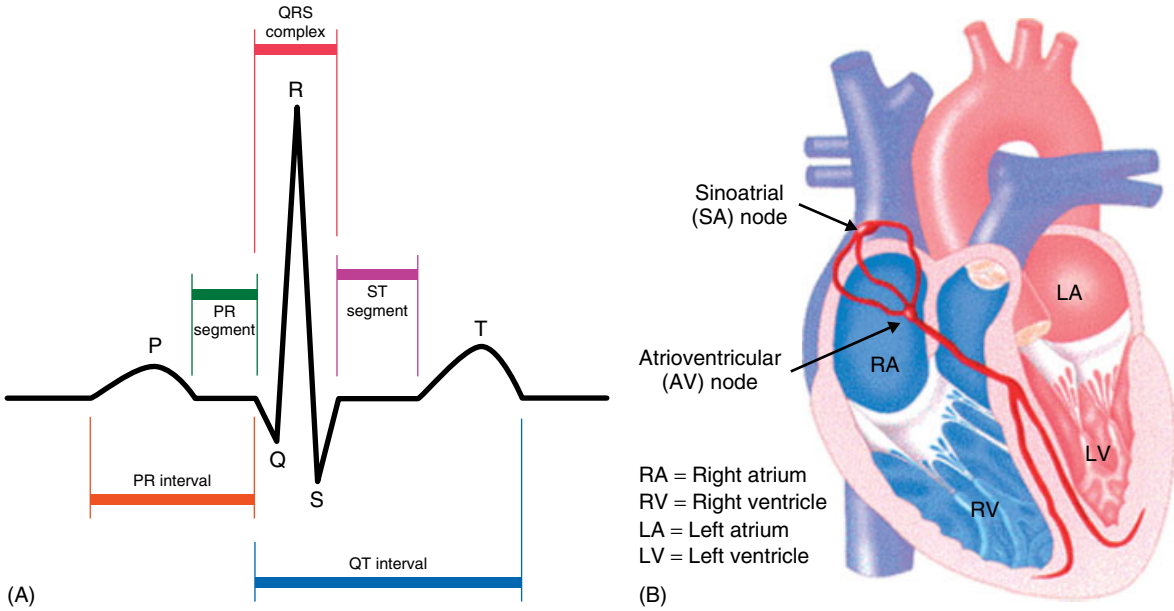

Figure 6 A normal electrocardiogram of a single heartbeat illustrating the electrical changes that complement the cardiac cycle. Three waves accompany the cardiac cycle and these include the P-wave, a small upward deflection indicating atrial depolarization, the QRS-wave, indicating ventricular depolarization or the spread of electrical impulses through the ventricles and the T-wave, indicating ventricular repolarization. (Reproduced with permission from Encyclopedia Britannica, © 2008.)

dependent on dose, route magnitude, and frequency and duration of exposure. Cardiotoxicants can be placed into the basic categories of pharmaceuticals, industrial chemicals and natural products. Pharmaceuticals with cardiotoxic action include antineoplastic, anaesthetics, psychotropics and antibiotics. In general, pharmaceutical cardiotoxic effects are seen only when the dose exceeds the therapeutic range.

Many pharmaceutical agents have been withdrawn from the market or severely restricted to specific indications because of unexpected adverse events, including fatalities. Cardiac abnormalities are one of the major causes of withdrawal of drugs or restriction in their labelling. Among these unintended effects, drug-induced arrhythmogenic death is the most dramatic. In 2004, rofecoxib (Vioxx), a nonsteroidal anti-inflammatory drug marketed by Merck & Co. to treat osteoarthritis and chronic pain was withdrawn due to concerns about increased risk of heart attack and stroke associated with long-term high-dosage use. The mechanism proposed to be associated with rofecoxib cardiotoxicity was the suppression of prostacyclin, a vasodilator and anticlotting agent.

Cisapride (Propulsid), a gastrointestinal pharmaceutical used to treat nocturnal heartburn as well as a variety of other gastrointestinal disorders was withdrawn in 2000, due to its association with acquired long QT syndrome and ventricular arrhythmias. The cardiotoxic effects were suspected to be due to blockade of one or more types of K^+ channel currents in the human heart, resulting in dysrhythmias and cardiac arrest. Commonly known pharmaceutical agents that alter heart function will be discussed in the following section.

8 PHARMACEUTICAL AGENTS

8.1 Chemotherapeutic Drugs

Chemotherapeutic drugs are used in the treatment of malignant tumours. They include anthracyclines such as doxorubicin and daunorubicin and alkylating agents. The anthracycline class is the best known of the chemotherapeutic agents that cause cardiotoxicity. Alkylating agents such as cyclophosphamide, ifosfamide, cisplatin, carmustine, busulfan, chlormethine and mitomycin have also been associated with cardiotoxicity.

Anthracycline antibiotics such as doxorubicin (i.e. adriamycin) and daunorubicin are some of the most effective chemotherapeutic agents used in the treatment of cancer and have been used for over 30 years. These drugs are used effectively in the treatment of leukaemias, lymphomas and adenocardinomas. However, the utility of these pharmaceuticals is limited by cumulative, dose-related, progressive myocardial damage that eventually leads to congestive heart failure (CHF) (Shan *et al.*, 1996). The incidence of anthracycline-induced heart failure represents approximately 1% of patients with

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advanced heart failure due to ventricular systolic dysfunction (Praga *et al*., 1979). A number of risk factors may predispose a person to cardiotoxicty under chemotherapy. These are cumulative dose (anthracyclines, mitomycin); total dose administered during a day or a course of treatment (cyclophosphamide, ifosfamide); rate of administration (anthracyclines, fluorouracil); schedule of administration (anthracyclines) and other factors, such as age, gender and history of pre-existing cardiovascular disorders (Von Hoff *et al*., 1979). Anthracyclines are well-known cardiotoxins and are known to induce arrhythmias, left ventricular failure and cardiomyopathy following long-term usage (Lenaz and Page, 1976).

Anthracycline-induced cardiotoxicity has been classified into three distinct types. First, acute or subacute injury can occur immediately after treatment. This rare form of cardiotoxicity may cause transient arrhythmias, a pericardial/myocardial inflammatory syndrome or acute failure of the left ventricle. Second, anthracyclines can induce chronic cardiotoxicity resulting in cardiomyopathy (Ferrans, 1978; Von Hoff *et al*., 1977; Bristow *et al*., 1978a; Friedman *et al*., 1978; Haq *et al*., 1985). This is a more common form of damage and is considered the most important. Chronic anthracycline-induced cardiomyopathy characteristically presents within one year of treatment. In a series of more than 3900 patients treated with anthracycline, Von Hoff *et al*. (1977) noted that CHF secondary to anthracycline-induced chronic cardiomyopathy occurred 0–231 days after the completion of anthracycline therapy. Finally, late-onset anthracycline cardiotoxicity may manifest years to decades after anthracycline treatment and is increasingly being recognized, causing late-onset ventricular dysfunction and arrhythmias (Schwartz *et al*., 1987).

Acute and subacute cardiac toxicity, which occur immediately after a single dose of an anthracycline or a course of anthracycline therapy, are uncommon under current treatment protocols. However, several distinct, early cardiotoxic effects of anthracyclines have been described. First, electrophysiologic abnormalities may result in nonspecific ST and T-wave changes, decreased QRS voltage and prolongation of the QT interval. Sinus tachycardia is the most common rhythm disturbance, but arrhythmias, including ventricular, supraventricular and junctional tachycardias, AV and bundle-branch block have been reported (Steinberg *et al*., 1987; Lenaz and Page, 1976; Ferrans, 1978; Von Hoff *et al*., 1977; Bristow *et al*., 1978a).

The incidence of CHF secondary to doxorubicin (anthracycline)-induced cardiomyopathy depends on the cumulative dose of the drug. At total doses of less than $400\,mg\,m^{-2}$ body surface area, the incidence of CHF is approximately 0.14%; and this incidence increases to 7% at doses of $550\,mg\,m^{-2}$ body surface area and to 18% at doses of $700\,mg\,m^{-2}$ body surface area (Von Hoff *et al*., 1979). The rapid increase in clinical toxicity observed at doses greater than $550\,mg\,m^{-2}$ body surface area has made a 550 mg dose the empiric limiting dose for doxorubicin-induced cardiotoxicity (Von Hoff *et al*., 1979; Praga *et al*., 1979). Doses in excess of $1000\,mg\,m^{-2}$ body surface area can be well tolerated by some patients (Henderson *et al*., 1989; Bristow *et al*., 1978a), however appreciable decreases in left ventricular ejection fraction have been documented at doses as low as $300\,mg\,m^{-2}$ body surface area (Bristow *et al*., 1978a; Cortes *et al*., 1975). Histopathologic changes characteristic of doxorubicin-induced cardiotoxicity have been observed at doses as low as $183\,mg\,m^{-2}$ body surface area, which is one third less than the conventional limiting dose. In general, a substantial proportion of patients will experience anthracycline-induced cardiac damage while receiving standard treatment regimens, whereas others can tolerate cumulative doses twice as large as the conventional limiting dose (Friedman *et al*., 1978). Age remains a significant risk factor to anthracycline cardiotoxicity, with patients older than 65 years having triple the risk of doxorubicin-related CHF compared with younger patients at the same cumulative dose (Von Hoff *et al*., 1979).

It is hypothesized that the toxicity of anthracyclines is associated with oxidative stress, loss of mitochondrial integrity, disrupted calcium homeostasis and altered gene expression leading to apoptosis (Doroshow, 1983; 1991; Dorowshow *et al*., 1980; Ito *et al*., 1990; Rajagopalan *et al*., 1988). Anthracycline-induced cardiac injury has been based upon studies in animals and cell cultures. Although anthracycline-induced injury appears to be multifactorial, a common denominator among most of the proposed mechanisms is cellular damage mediated by reactive oxygen species. However, it is not clear why this mechanism would be preferential to inducing cardiotoxic effects.

Anthracyclines have been shown to induce membrane damage via lipid peroxidation in all tissues, including the heart (Doroshow, 1983). The formation of ROS is induced by the quinone moiety of anthracyclines; however, oxidative stress is also known to occur via induction of nitric oxide synthase, leading to nitric oxide and peroxynitrite formation. This latter mechanism of oxidative stress has been linked to nitration and inactivation of key enzymes in the heart that include myofibrillar creatine kinase, which is a critical energetic controller of cardiomyocyte contractility. Myofibrillar creatine kinase has been demonstrated to be a particularly sensitive target of peroxynitrite-induced nitration and inactivation. Anthracyclines are also known to cause impairment of membrane binding, assembly and enzymatic activity of mitochondrial creatine kinase, although the consequences of this impairment are not clear (Olson *et al*., 1974; Kusuoka *et al*., 1991; Holmberg and Williams, 1990).

In the heart, like other tissue, anthracyclines intercalate into nucleic acids, causing suppression of

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DNA, RNA and protein synthesis. Some transcriptional regulatory proteins that appear important for regulation of cardiac-specific genes appear particularly susceptible to anthracyclines. Myocyte cell death by both apoptosis and necrosis has also been implicated in anthracycline-induced cardiotoxicity. It is proposed that a net loss of cells' apoptotic and necrotic mechanisms contributes to its cardiotoxicity and heart failure. Recently *in vivo* studies have demonstrated that anthracyclines have a selective inhibition of cardiac muscle gene expression for actin, troponin, myosin light-chain 2 and the M isoform of creatine kinase *in vivo*. Other suspected theories for anthracycline-induced myocardial damage include free-radical-mediated myocardial injury (Doroshow, 1983; Rajagopalan *et al*., 1988), myocyte damage from calcium overload (Holmberg and Williams, 1990), disturbances in myocardial adrenergic function (Wakasugi *et al*., 1992), release of vasoactive amines (Bristow *et al*., 1983) and cellular toxicity from metabolites of anthracycline therapeutics, such as doxorubicin.

Anthracyclines also induce changes in adrenergic function and impart abnormalities in Ca^{2+} handling, both of which are critical components in the regulation of cardiac function. Down-regulation of myocardial β-adrenergic receptors, resulting in adrenergic dysfunction, has also been demonstrated in anthracycline-induced ventricular dysfunction (Tong *et al*., 1991; Robison and Giri, 1986; Fujita *et al*., 1991). However, the extent to which each of these mechanisms contributes to the dose-dependent heart failure in anthracycline-treated patients remains somewhat controversial.

It has also been hypothesized that proinflammatory cytokines may be directly relevant to anthracycline -induced cardiac injury. Recent reports have indicated that circulating proinflammatory cytokines may be linked to anthracycline-induced ventricular dysfunction and cardiomyopathy (Ehrke *et al*., 1986). Doxorubicin has been shown to induce the release of tumour necrosis factor from macrophages and of interleukin-2 from monocytes (Shi *et al*., 1993).

In summary, the cause of anthracycline-induced cardiotoxicity is multifactorial; however, a large body of evidence points to two major mechanisms: calcium overload and free-radical-mediated myocyte damage. Increased oxygen-radical activity generated through the semiquinone moiety of the doxorubicin molecule can cause lipid peroxidation and cell injury. Additionally, anthracycline-induced intracellular calcium overload may also lead to myocyte death. Doxorubicin has been shown to activate the calcium-release channel across the SR and causes calcium influx into the myocyte. Free-radical-induced cell-membrane damage has been demonstrated with calcium influx, suggesting that these two cellular mechanisms of anthracycline-induced cardiotoxic may be linked.

8.2 Digitalis Glycosides

Cardiac glycosides have been used for centuries as therapeutic agents for the treatment of heart diseases and are one of the most commonly described cardiac drugs. Cardiac glycosides, known as a class of digitalis, are widely used in the pharmaceutical treatment of CHF and atrial tachyarrhythmias (The Digitalis Investigation Group, 1997). Digitalis derivatives are found in several plants, including oleander, foxglove and lily of the valley. Digoxin and other cardiac glycosides exert their effects on myocardial contractility through their effects on ion homeostasis.

In patients with heart failure, cardiac glycosides exert a positive inotropic effect by indirectly increasing intracellular calcium levels through inhibiting the plasma membrane Na^+-K^+ ATPase. This inhibition results in an increase in intracellular sodium, which in turn inhibits Na^+/Ca^{2+} exchange and ultimately leads to an increase in calcium levels and a positive inotropic effect. The therapeutic index of digitalis is narrow and thus patients are susceptible to arrhythmias in cases where the therapeutic window is compromised. Although digoxin-induced arrhythmias are a common clinical issue in patients being treated for heart disease, the underlying cellular mechanisms of these arrhythmias are yet to be elucidated.

There are several theories that have been proposed regarding the arrhythmic effects of digoxin. The toxic arrhythmogenic effects of cardiac glycosides are said to be due to a combination of direct and indirect effects on the myocardium (Kemmerer, 2008). It is proposed that digoxin induces arrhythmias by causing oscillatory fluctuations in cardiac resting membrane potential. It is proposed that digoxin increases the rate and amount of Ca^{2+}-induced calcium release from the cardiac SR vesicles (McGarry and Williams, 1993) and as a result induces an oscillatory fluctuation in cardiac resting membrane potential, an increase in myocardial automaticity and ultimately the development of cardiac arrhythmias. Both systolic and diastolic intracellular calcium concentrations increase during digitalis-induced arrhythmias leading to the notion that calcium overload contributes to the overall arrhythmogenic effects of digitalis. It is believed that spontaneous cycles of calcium release and reuptake ensue resulting in afterdepolarizations (abnormal depolarizations of cardiac myocytes that interrupt Phases 2, 3 or 4 of the cardiac action potential and lead to cardiac arrhythmias) and aftercontractions (Hauptman and Kelly, 1999).

There is some evidence to suggest that increased cardiac sympathetic activity is involved in digitalis-induced cardiotoxicity. Experimental studies demonstrate that stimulation of central α-adrenoceptors depresses cardiotoxicity (Chen *et al*., 1991; Thomas and Stephen, 1991), whereas β-adrenoceptor blockers (Mest *et al*.,

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1995; Nakanishi *et al*., 1992) can reduce these arrhythmias. Other proposed mechanisms of toxicity include the involvement of nitric oxide (Altug *et al*., 1999), cyclo-oxygenase products (Mest *et al*., 1981; Moffat *et al*., 1986; 1987), ROS (Schlieper and Tawfik, 1987; Ark *et al*., 1999; Tian *et al*., 2003) and magnesium (Kafiluddi *et al*., 1989).

In summary, digitalis glycosides alter the normal contractile and rhythmic function of the heart. They alter impulse formation and conduction through their effect on ion homeostasis. Cardiac glycosides indirectly increase intracellular calcium levels through inhibition of the plasma membrane Na^+-K^+ ATPase resulting in a positive inotropy. Additionally the magnitude of the resting membrane potential is reduced and in myocardial automaticity is increased, leading to increased susceptibility for arrhythmias.

8.3 Antiarrhythmic Drugs

Antiarrhythmic drugs affect the heart by directly or indirectly altering membrane ion conductance across the membrane of the myocardial cell. Antiarrhythmic drugs such as the fast Na^+-channel blockers (Class I), which include lidocaine, procainamide, quinidine and phenytoin impair intraventricular conduction. These agents can be responsible for life-threatening arrhythmias after either an accidental intravenous administration or enhanced absorption from an injection site. Accidental intravenous injection in humans or experimental injection in laboratory animals has been shown to result in a variety of cardiac tachyarrhythmias, including the common occurrence of ventricular fibrillation. The mechanisms by which Class I antiarrhythmics produce cardiac rhythm disturbances are incompletely characterized, but have been assumed to result from excessive blockade of Na^+ channels. However, interactions with Ca^{2+} channels, K^+ channels and SR-associated calcium channels, as well as many other ion channels and enzymes have been demonstrated (MacMahon *et al*., 1988; Hondeghem and Katzung, 1977; Bolger *et al*., 1987; Castle, 1990; Kindler *et al*., 1999; Tovey *et al*., 1998). Any of these actions on membrane-associated ion channels could potentially trigger arrhythmias and account for cardiotoxicity.

8.4 Tricyclic Antidepressants

Cyclic antidepressants have been used in the treatment of major depression since the late 1950s. Currently, cyclic antidepressants are also utilized in the treatment of chronic pain syndromes and for migraines. Cyclic antidepressants were identified to have a low threshold for toxicity following reports of overdose shortly after their use in the 1950s. Although in the past 10 years, the prescription of cyclic antidepressants has declined significantly with the arrival of selective serotonin reuptake inhibitors (SSRIs), they remain second only to analgesics as the most common drugs implicated in overdose fatalities. The most commonly prescribed cyclic antidepressants include the following: amitriptyline, desipramine, imipramine, nortriptyline, doxepin and clomipramine (Hong *et al*., 1974; Coull *et al*., 1970; Curry *et al*., 1971).

Significant adverse effects are generally seen at doses of greater than $20\,mg\,kg^{-1}\,d^{-1}$, in aged patients with pre-existing heart disease, and in situations where drug administration is uncontrolled long term (Freeman *et al*., 1969; Rasmussen, 1965). The toxic effects of cyclic antidepressants are primarily related to the pharmacologic effects of these drugs (Cairncross and Gershon, 1962). Cyclic antidepressants exhibit a number of pharmacologic effects that include anticholinergic and antihistamine activity, α-adrenergic blockade, inhibition of norepinephrine and serotonin reuptake and blockade of fast sodium channels in myocardial cells, resulting in quinidine-like effects on the heart (Robinson, 1965; Williams and Sherter, 1971; Rosenbaum *et al*., 1969; Sacks *et al*., 1968). The most serious adverse effects of cyclic antidepressant toxicity are due to central nervous system effects and cardiovascular instability. Depressed mental status is generally caused by the antihistamine and anticholinergic properties of cyclic antidepressants. Life-threatening cardiovascular complications are due to impaired conduction from fast sodium-channel blockade. The impairment decreases the slope of Phase 0 depolarization, widens the QRS complex, and prolongs the PR and QT intervals, resulting in alterations in cardiac conduction. Impaired cardiac conduction may lead to heart block and unstable ventricular arrhythmias or asystole. Cyclic antidepressants have also been shown to directly depress myocardial contractility and induce profound hypotension, due to vasodilatation from direct α-adrenergic blockade in serious poisoning situations.

8.5 Antihistamines

Antihistamines are the most widely used drugs in the treatment of allergic reactions of all kinds. Their main action is to counter the effects of histamine, one of the chemicals released in the body when there is an allergic reaction. The antihistamine drugs, known as H1 blockers, block the action of histamine on certain receptors, known as H1 receptors. Several clinically useful drugs (such as chlophenamine (formally chlorpheniramine), brompheniramine, triprolidine, hydroxyzine and diphenhydramine), able to antagonize the effects of histamine at the level of the H1 receptor, were developed in the late 1940s and early 1950s.

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Following the development and success of these early antihistamines (first-generation antihistamines), hundreds of new antihistamine drugs (second-generation antihistamines) were synthesized and marketed with outstanding success. The more recently introduced antihistamines include: acrivastine, astemizole, cetirizine, ebastine, levocarbastine, loratadine, fexofenadine, ketotifen and oxatomide. These second-generation antihistamine drugs had improved selectivity for histamine receptors and lack sedative effects (Taglialatela *et al*., 2000).

Despite the enormous therapeutic effects of these drugs (both first- and second-generation antihistamines) in the treatment of allergies, in the mid 1980s several reports appeared in the literature indicating the occurrence of cardiotoxicity after the administration of astemizole or terfenadine, two of the most successful second-generation antihistamines on the market (Soldovieri *et al*., 2008). Astemizole and terfenadine were withdrawn from the market in several countries because they caused the rare occurrence of *torsades de pointes* (TdPs) (Paakkari, 2002). TdPs is a potentially fatal polymorphic ventricular arrhythmia that occurs in the setting of a marked prolongation of the QT interval either in patients taking intentional or accidental overdoses of these two antihistamines or in insubjects with predisposing CVD (Taglialatela *et al*., 2000). The metabolic products of astemizole, desmethyl astemizole and norastemizole are cardiotoxic, whereas fexofenadine, the active metabolite of terfenadine is devoid of cardiotoxicity. Terfenadine and astemizole were shown to cause a prolonged QT-interval in the ECG (Paakkari, 2002). This effect was soon demonstrated to be associated with an impaired repolarization of cardiomyocytes due to a selective blockade of cardiac potassium currents (Paakkari, 2002; Soldovieri *et al*., 2008).

8.6 Aliphatic Alcohols

Ethanol consumption has been associated with a variety of CVD. In the 1950s evidence began to emerge to suggest that alcohol has a direct toxic effect on the myocardium. Over the last 25 years research has been conducted to characterize the cardiotoxicity associated with alcohol consumption and it is now well recognized that the heart is a primary target for the damaging effects of excessive ethanol consumption (Urbano-Marquez *et al*., 1989). Upon autopsy, individuals who have consumed excessive amounts of alcohol have been observed to have cardiac enlargement, often with thickened regions of the endocardium and other pathological and histological symptoms of heart failure, such as interstitial fibrosis, hypertrophy and atrophy of myocytes, together with focal regions of myocardial necrosis (Teragaki *et al*., 1993). Alcohol use has been shown to have numerous effects on the cardiovascular system. It has been associated with arrhythmias, heart failure, cardiomyopathy, hypertension and stroke. The most prevalent form of alcoholic heart disease is cardiomyopathy, which is characterized by depressed cardiac output, reduced myocardial contractility and dilation of all four chambers of the heart (Richardson *et al*., 1986). Alcoholic cardiomyopathy develops primarily after prolonged periods of sustained and daily heavy drinking, generally exceeding a period of 10 years.

Ethanol has been shown to induce structural changes that often precede functional alterations in the heart. An increase in left ventricular mass is a common structural change that often precedes contractile alterations. Impairment of cardiac function appears to develop incrementally with increasing ethanol consumption until overt alcoholic cardiomyopathy manifests itself. Progression to cardiomyopathy is associated with substantial ventricular and atrial dilation, as well as mitral valve regurgitation, which may be associated with ethanol-induced papillary muscle insufficiency (Teragaki *et al*., 1993; Nakanishi *et al*., 1990). Ethanol is also known to cause rhythm effects of the heart and there are a number of abnormalities that may be associated with resulting arrhythmias seen in alcoholics. Alcoholics often have atrial dysrhythmias and these may be attributed to effects on conduction times and increases in action potential refractory periods (Teragaki *et al*., 1993; Regan, 1978).

Reports on the short-term effects of ethanol ingestion are somewhat contradictory. Some studies indicate that acute ethanol ingestion induces a direct negative inotropic and chronotropic effect on the myocardium (Thomas *et al*., 1994). However, others have reported no change or enhancement of rhythm and contractility. The most common observation following acute ethanol ingestion is a modest increase in heart rate, which is assumed to be a secondary consequence of the action of ethanol on autonomic regulation of the heart (Patel *et al*., 1997). Acute ethanol intake has been shown to increase systolic time in healthy volunteers. Under conditions of autonomic blockade, increases in systolic times are said to be associated with reduced myocardial contractility and blood ethanol levels of approximately $110\,\mathrm{mg\,dl^{-1}}$ (or 20 mM) (Ahmed *et al*., 1973; Child *et al*., 1979).

Investigations into the cellular mechanisms of the acute ethanol affects on contractile function have been mainly based on experiments in animal models. *In vivo* experimentation on the effects of ethanol on cardiac contractility, in animals as well as in humans, is complicated due to the indirect effects ethanol has on other elements of the cardiovascular system. However, a number of studies in dogs have demonstrated that ethanol, at blood levels between $70–310\,\mathrm{mg\,dl^{-1}}$, causes decreased contractile function (Mendoza *et al*., 1971). The acute effect of ethanol is more pronounced under conditions of autonomic blockade (Child *et al*., 1979). *In vitro* investigations of acute ethanol affects on isolated cardiac muscle

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preparations have indicated that ethanol concentrations of 20–200 mM depress cardiac contractility (Hirota *et al*., 1976). Ethanol concentrations of 70 mM have been shown to decrease the rate and tension development in rats, ferrets and canines and in human foetal cardiac muscle preparations (Gimeno *et al*., 1961; Kobayashi *et al*., 1979; Richards *et al*., 1989). Animal studies have linked acute ethanol exposure with direct negative inotropic effects on the heart and these appear to be independent of the neuronal and vascular effects of ethanol.

There are several theories that have emerged regarding the mechanisms of reduced contractile function following acute ethanol exposures. These include the interference of ECC. A number of steps in the ECC have been proposed as targets of ethanol action. These include the ion channels that are responsible for the generation and conduction of action potentials, the release of stored Ca^{2+} from the SR required for contraction, the resequestration of stored Ca^{2+} for myocardial relaxation and finally the activation of contractile proteins by Ca^{2+} (Richards *et al*., 1989; Thomas *et al*., 1991).

The effects of chronic alcohol consumption on cardiac function have been examined in a variety of animal models. These include the rat, mouse, dog, turkey and rhesus monkey. Structural, metabolic and functional alterations in cardiac function have been observed in experimental animal studies of chronic ethanol exposure. Experimental studies of chronic ethanol intake in animals have demonstrated structural abnormalities of the heart. The effects observed include a characteristic increased ratio of heart weight to body weight, pronounced left ventricular dilation, reduction in the total number of myocytes comprising the left ventricle, distention of the mitochondrial matrix, swelling of the SR and T-tubules, dehiscence of intercalated discs, disruption of the myofibrillar structure and accumulation of lipid droplets (Segel *et al*., 1975; Thomas *et al*., 1980; Bing, 1982). Chronic ethanol feeding in animal studies has also demonstrated an influence on the metabolic processes of the heart. The most pronounced effect demonstrated is on the capacity of the myocyte for oxidative metabolism. Numerous studies have reported a reduced efficiency of mitochondrial oxidative phosphorylation (Bing, 1982). It has been reported that the reduced oxidative metabolism of heart mitochondria is associated with a reduction in fatty acid oxidation and increase in triglyceride deposition in the heart tissue (Bing, 1982). In addition, ethanol metabolites have been shown to affect cardiac contractile function. The best characterized of these metabolites is acetaldehyde, which has been demonstrated to decrease action potential duration in Purkinje fibres, inhibit Na^+/K^+-ATPase activity in sarcolemma and enhance catecholamine release at concentrations of >0.4 mM (Sorrell and Tuma, 1987).

Ethanol metabolites, such as fatty acid ethyl esters, that are a result of nonoxidative ethanol metabolism, have been shown to accumulate in the hearts of ethanol-fed animals (Lange, 1991). These metabolites have been suggested to disrupt sarcolemma functions. A variety of long-term ethanol feeding studies in animals have demonstrated clear and consistent depressive effects on cardiac contraction and increase in the vulnerability of the heart to fibrillation following acute ethanol administration.

In summary, ethanol has acute negative inotropic effects on the heart, which are mediated by direct action on the cardiac muscle. Some theories have arisen in regard to the mechanisms of cardiotoxity induced by acute ethanol exposures, but the alteration of intracellular calcium seems to stand out as a major mechanism for cardiac dysfunction following acute exposures. The chronic effects of long-term frequent episodes of ethanol consumption on cardiac function is assumed to be a result of the cumulative effects of the cardiac actions of this agent ultimately leading to degeneration of contractile function and cardiomyopathy. The mechanism of cardiac damage produced by chronic alcohol exposure remains unclear. However, several theories have arisen based on clinical and experimental evidence derived from human and animal studies. Proposed mechanisms of toxicity include: the generation of toxic metabolites, interference with normal metabolic processes, alterations in mitochondrial structure, alteration in calcium metabolism and glutathione content, inhibition of protein synthesis, fatty acid ester accumulation, inhibition of calcium–myofilament interactions and disruption of cell membrane structure.

8.7 Hydrocarbons

A variety of hydrocarbons, with and without halogen substitution, have long been known to sensitize the heart to adrenaline-induced arrhythmias (Himmel, 2008; Hermann and Vial, 1935; Garb and Chenoweth, 1948; Hays, 1972; Reinhardt *et al*., 1973). For example, chlorofluorocarbons (CFCs) have been demonstrated to produce ventricular arrhythmias. CFC exposure studies in dogs have demonstrated the ability of this hydrocarbon to sensitize the myocardium to exogenous adrenaline. Reinhardt *et al*. (1971) exposed dogs to varying concentrations of CFC-12 for periods of 0.5–10 minutes and found that a concentration of CFC-12 in air of 250 g m^{-3} (5%) sensitized the heart to an intravenous dose of adrenaline (8 µg (kg body weight)$^{-1}$). Similar findings were observed in rabbit exposure studies; however, the arrhythmogenic thresholds of CFCs are known to be dependent upon the animal species and concentration of exogenous adrenaline (Lessard and Paulet, 1986).

The arrhythmogenic potency of CFCs, derived through inhalation studies, has been shown to be dependent on the

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degree of fluorination. It has been shown that as fluorination increases within a homologous series of CFCs, the arrythmogenic potency decreases. For example, for the chlorofluoromethanes, the arrhythmogenic potency of CFC-11 seems to be greater than that of CFC-12. A similar pattern has been observed in fully halogenated ethanes (CFC-113 > CCF-114 > CFC-115) (Reinhardt *et al*., 1971; 1973; Clark and Tinston, 1972a; 1972b; Wills, 1972). Through animal studies it appears that a critical blood level of CFC is needed to cause cardiac sensitization. It has been hypothesized that differences in cardiac sensitization among various CFCs may reflect differences in absorption characteristics rather than mechanisms of action at the molecular level (Jack, 1971; Taylor *et al*., 1971; Clark and Tinston, 1972a; Azar *et al*., 1973). Similarities in the lowest venous blood concentrations associated with cardiac sensitization in various animal studies suggest that these CFCs act in a similar and nonspecific manner in causing arrhythmias. This hypothesis is somewhat supported by basic similarities in cardiac sensitization caused by these and other halo-substituted hydrocarbons.

Inhalational exposure studies in animals have demonstrated mixed results regarding the effects of CFCs on heart rate. Through these numerous studies it appears that any observed changes induced by an inhalational exposure to CFC is highly dependent on the use of anaesthetic in experimental design, the CFCs, CFC concentration and animal species. For example, inhalation of CFC-12 in a gas mixture given to anaesthetized animals has been reported to cause bradycardia in mice (Aviado and Belej, 1974), rats (Doherty and Aviado, 1975) and dogs (Flowers and Horan, 1972). No change in heart rate has been observed in monkeys (Belej *et al*., 1974), rats (Friedman *et al*., 1973; Watanabe and Aviado, 1975) and cats (Harris *et al*., 1971). However, tachycardia has been induced in anaesthetized monkeys and unanaesthetized rats (Aviado and Smith, 1975; Watanabe and Aviado, 1975).

Chlorofluorocarbon gases may exert their effects on cardiac rhythm in two ways; directly on the myocardium and indirectly through sensitization of the ventricular myocardium to endogenous catecholamines (Taylor *et al*., 1971; Aviado and Belej, 1974). The latter theory is consistent with the blocking of CFC-induced arrhythmias by propranolol. Electrophysiological analysis of CFC-induced effects on different cardiac cells indicates that arrhythmias are a result of nonspecific impairment of the membrane properties and notably the inhibition of transmembrane ionic currents (Lessard and Paulet, 1985; 1986).

Several investigators have attempted to determine if CFCs affect oxidative phosphorylation. However, the CFCs, CFC-12 and CFC-114 were shown not to affect oxygen consumption or oxidative phosphorylation in mitochondria isolated from the liver, lung, brain, heart or kidney of exposed rats (Griffin *et al*., 1972). Subsequent *in vitro* studies conducted with liver and heart mitochondria supported the *in vivo* findings, also indicating no effects on either oxidation or phosphorylation (Griffin *et al*., 1972).

In summary, hydrocarbons have long been known to sensitize the heart to adrenaline-induced arrhythmias; however, the mechanisms of action are not well elucidated, fully investigated and understood. Further studies are required to determine the exact mechanisms of action, although it is proposed that hydrocarbons, halogenated or not, seem to induce cardiac sensitization through a similar mechanism of action.

8.8 Volatile Substance Abuse

Volatile substance abuse (VSA), referred to as glue sniffing, inhalant abuse, solvent abuse, is the deliberate inhalation of volatile substance for the purposes of achieving intoxication, has been reported in many part of the world and occurs most commonly in adolescents, individuals living in remote and rural communities, and in individuals whose occupations provide access to these substances (Flanagan and Ives, 1994). Solvents from contact adhesives, including toluene and gasoline, in addition to halogenated solvents and volatile organic hydrocarbons (aliphatic hydrocarbons, alkyl halides, alkyl nitrites, aromatic hydrocarbons, ethers and ketones), such as those present in cigarette-lighter refills, aerosol propellants, halocarbon fire extinguishers and inhalational anaesthetics are chemical agents that are utilized by volatile substance abusers (Flanagan and Ives, 1994; Linden, 1990). In the UK, the most common types of substance abused today are butane lighter fuel and aerosol inhalers (Harris, 2006). There are also a large number of deaths annually occurring in the UK as a result of VSA. Substances that are inhaled for the purpose of recreational self-intoxication have the ability to cause asphyxia, arrhythmias, cardiovascular depression, neurological dysfunction and mucosal, pulmonary and skin irritation following acute exposures. The major risk following acute poisoning is sudden death. Arrhythmias leading to cardiac arrest are thought to be the major causes of sudden death in cases of acute poisoning. Like CFCs, the aliphatic hydrocarbons found in solvents utilized by solvent abusers can sensitize the myocardium to the effects of catecholamines, which can then predispose exposed individuals to tachydysrhythmias, which can result in syncope or sudden death.

Deaths occurring from VSA can be classified as a result of either direct or indirect toxic mechanisms (Harris, 2006). Direct toxic effects are thought to account for over half of all VSA-associated deaths in the UK and are thought to be associated with mechanisms related to

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cardiac arrhythmia, vagal inhibition, respiratory depression and anoxia (Shepard, 1989).

Cardiac arrhythmia is considered the most common cause of direct death in individuals who are termed solvent abusers (Harris, 2006). Cardiac arrhythmias arise due to the process of cardiac sensitization. Inhaled volatile compounds sensitize the myocardium to the effects of circulating catecholamines. Intravenous administration of epinephrine, in attempted emergency resuscitations, has been associated with cardiac arrest in VSA users admitted to emergency wards (Lawrence, 1987).

8.9 Metals

Human exposures to heavy metals have been associated with a variety of diseases, including cardiovascular disorders. Cadmium, lead, arsenic, cobalt, manganese, nickel and barium have all been associated with CVD.

Arsenic is an environmental toxicant, which has been used by physicians for more than 2000 years to treat diseases ranging from cancer to syphilis and tuberculosis. Arsenic trioxide has also been shown to be clinically effective in treating acute promyelocytic leukaemia. However, its use clinically has been restricted due to its cardiotoxic effects (Freeman, 2007). Arsenic is also an element found in multiple compounds commonly encountered in the environment. Human exposure to inorganic and organic arsenicals can occur from a variety of sources, including occupational, medical and environmental (Lee and Fraumeni, 1969; Watanabe *et al*., 2004; Westervelt *et al*., 2001). Whether exposure occurs from medical therapy (as with the use of arsenic trioxide in cancer treatment) or accidental malicious acute poisoning, the cardiac effects of high-level acute arsenic exposure are well documented (Westervelt *et al*., 2001; Hall and Harruff, 1989; Little *et al*., 1990). Arsenic exposure is associated with direct myocardial injury, cardiac arrhythmias and cardiomyopathy (Benowitz, 1992; Goldsmith and From, 1980). Arsenic trioxide has been shown to cause QT prolongation, TdPs and sudden death. The exact mechanism by which arsenic trioxide causes myocardial toxicity remains unclear, although it is believed that arsenic produces extensive organ damage by combining with protein thiol groups. Earlier reports predicted that oxidative stress might play a crucial role in cardiac and vascular abnormalities in different types of CVDs (Haidara *et al*., 2006; Dhalla *et al*., 2000; Bashir *et al*., 2006). Emerging research also indicates that apoptosis of terminally differentiated cardiomyocytes may contribute to the development of myocardial infarction (MI) and CHF. Apoptosis is known to play an important role in the pathogenesis of a variety of CVDs, however, the role in acute and chronic myocyte loss associated with arsenic exposures remains to be fully elucidated.

Excessive amounts of cobalt are known to be cardiotoxic. Congestive cardiomyopathy has been reported in people who drank large quantities of beer to which cobalt had been added as a foam stabilizer (Morin and Daniel, 1967; Kesteloot *et al*., 1968; Sullivan *et al*., 1969) and in those receiving oral cobalt therapy (Manifold *et al*., 1978). Although cobalt exposure is associated with cardiotoxicity, the mechanism for this toxicity remains unclear.

Cobalt given as a daily subcutaneous injection of cobalt chloride for eight days at doses of 1, 5, 20 and 50 mg $(\text{kg bw})^{-1}$, produced a marked and dose-dependent accumulation of cobalt in the myocardium and a decrease in manganese-superoxide dismutase. Myocardial acute cobalt cardiotoxicity may involve a reduction of intrinsic scavengers resulting in an increased vulnerability to oxygen free-radical toxicity. Cobalt cardiotoxicity may also be attributed to its antagonistic action towards the Ca^{2+} ion, as well as its ability to form complexes with macromolecules. Cobalt interacts with sulfhydryl groups to impair thiol-enzyme activities (Alexander, 1972). In a dog model, cobalt myocardial toxicity was characterized by vacuolation and loss of myofibres (Sandusky *et al*., 1981a) with histochemical evidence of severe mitochondrial damage (Sandusky *et al*., 1981b). The effects observed were hypothesized to be related to cobalt–thiol group interaction resulting in citric acid cycle malfunction (Jarvis *et al*., 1992) and a decrease in myocardial energetics.

Lead exposure has also been associated with an increased incidence of CHD, stroke, peripheral arterial disease (Schober *et al*., 2006; Lustberg and Silbergeld, 2002; Menke *et al*., 2006; Navas-Acien *et al*., 2004) and with cardiovascular function abnormalities, such as left ventricular hypertrophy and alterations in cardiac rhythm (Cheng *et al*., 1998; Schwartz, 1991). The link between low-level lead exposure and hypertension has been intensively studied for many years and an association between blood pressure, increases risk of atherosclerosis and cardiovascular mortality has been reported (Rossi, 2008; Patrick, 2006). Animal studies support the possibility that lead exposure promotes hypertension and accelerates CVD. Chronic treatment of rats with low levels of lead in the drinking water has been shown to elevate blood pressure (Marques *et al*., 2001). This increase in blood pressure has been linked to the ability of lead metal to increase ROS generation, to inactivate nitric oxide synthase and to a decrease in nitric oxide.

Like lead, cadmium has also been shown to increase atherosclerosis in animal models (Subramanyam *et al*., 1992; Revis *et al*., 1981) and environmental exposure to cadmium has been suggested to be particularly toxic to heart and blood vessels, in part because of the ability of cadmium to increase free-radical generation. Overall, metals that diminish antioxidant defences or increase radical production could increase the risk of CVD.

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9 AIR POLLUTION

Environmental factors are considered key determinants of CVD. Although lifestyle choices, such as smoking, diet and exercise are viewed as major environmental influences, the contribution of pollutants and environmental chemicals is less clear. Accumulating evidence suggests that exposure to air pollutants and chemicals in ambient air could elevate the risk of CVD. One only has to look back at history to remember the sudden rise in mortality rates and the estimated 4000 premature deaths that resulted in 1952 as a result of the great smog in London, England. In December 1952 on days 7 and 8, smoke and sulphur dioxide concentrations rose from 0.49 to 4.46 mg m^{-3} and 0.41 to 3.83 mg m^{-3} respectively. It was estimated that 4000 deaths had occurred as a result of the great smog and the main causes for these deaths were associated with respiratory disease and CVD. Respiratory diseases such as emphysema and bronchitis were reported to account for approximately 59% of the deaths recorded during the time of the great smog. CVD was reported to account for approximately 22% of the deaths recorded. Many current epidemiological studies report that exposure to fine particles present in ambient air is associated with an increase in cardiovascular mortality (Yang and Omaye, 2009; Puett *et al*., 2008; Simkhovick *et al*., 2008). Statistically significant relationships between particulate air pollution and ischaemic heart disease, arrhythmias and heart failure have been reported. An elevated risk for acute MI (Lanki *et al*., 2006; Peters *et al*., 2001) and cardiorespiratory symptoms (de Hartog *et al*., 2003) has been reported in relation to air pollution. Animal studies show that exposure to ambient air particles increases peripheral thrombosis and atherosclerotic lesion formation. Exposures to arsenic, lead, cadmium, pollutant gases, solvents and pesticides have also been linked to increased incidence of CVD. Mechanistically, these effects have been attributed to changes in the synthesis or reactivity of nitric oxide that may be caused by environmental oxidants or increased endogenous production of reactive oxygen species. Alveolar inflammation induced by particles may either directly or via oxidative stress lead to systemic inflammation with increased levels of blood coagulability, progression of atherosclerosis and destabilization or even rupture of vulnerable plaques, resulting in acute ischaemic events (Brook *et al*., 2004; Seaton *et al*., 1995) and myocardial infarct.

However, future studies are required to identify the contribution of individual pollutants, as well as mixtures to specific aspects of CVD and myocardial injury. Additionally, the underlying physiological and molecular mechanisms of cardiotoxicity associated with exposures to environmental contaminants need to be further elucidated.

10 CONCLUSIONS

Impaired pump function is the common end point of cardiotoxicity. Toxicity generally results due to an injury to the heart muscle and/or an alteration in the conduction system of the heart, leading to electrical disturbances that result in arrhythmias. The myocardium is very vulnerable to damage as it has no regenerative capacity and injury to the myocardium can occur as a result of indirect and direct toxic mechanisms. Some general mechanisms of injury to the myocardium of the heart include interference with ion homeostasis (Na^+, K^+, Ca^{2+}), damage through altered or insufficient blood supply to the heart muscle, oxidative stress, as a result of ischaemia/reperfusion injury, organellar dysfunction, as a result of lack of ionic homeostasis, alterations of metabolism and mitochondrial injury.

Alterations in ion channels can occur as a result of a primary alteration in ion channels, signalling or calcium-handling proteins, structural changes in the myocytes or extracellular matrix and changes in the activity of the neurohumoral system. Inhibition of Na^+/K^+-ATPase leads to an increase in intracellular calcium and a decrease in conduction velocity; Na^+-channel blockade decreases conduction velocity, K^+-channel blockage decreases repolarization and Ca^{2+}-channel blockade (L-type channel, calcium release) can lead to a decrease in AV conduction.

Pro-oxidant/antioxidant balance determines the degree of myocardial oxidative stress. Lipid peroxidation can impair the sarcolemma, cause mitochondrial and SR dysfunction and alter calcium homeostasis. These oxidative effects contribute to arrhythmias, myocardial stunning and myocyte death.

Cytotoxic calcium overload caused by SR dysfunction, sarcolemmal injury, Na^+/K^+-ATPase inactivation or sympathetic activation is another common mechanism of cardiotoxicity. It results in the breakdown of high-energy phosphates, blebbing of plasma membrane, activation of Ca^{2+}-dependent phospholipases and neutral proteases, Ca^{2+}-activated DNA fragmentation and Ca^{2+} activation on oncocytic and apoptotic cell death. Apoptosis predominates soon after toxicant-induced injury and is a prominent feature in heart failure. Overall, cardiotoxicity occurs as a result of affects on biochemical, structural and functional abnormalities induced by overexposure to cardiotoxic agents.

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Hepatotoxicity

Richard H. Hinton, Paul Grasso and Timothy C. Marrs

CONTENTS

1 INTRODUCTION

The liver has many metabolic roles and is, accordingly, affected by a very large number of xenobiotics. However, the liver also has an immense capacity for self-repair, so that the majority of lesions observed in the liver reverse rapidly on cessation of treatment. Nevertheless the liver's defences can be overwhelmed. Liver failure from paracetamol (acetaminophen) overdose is familiar to most clinical toxicologists, whereas repeated liver damage, for example from ethanol, may lead to cirrhosis. Also, while primary liver cancer is rare in Western Europe and North America, it is common in other parts of the world. Non-dose-dependent side effects on the liver affecting up to 5% of patients are common with many classes of drug and may be sufficiently severe to require cessation of treatment. Hence understanding hepatotoxicity is vital to both clinical and academic toxicologists.

In any discussion of hepatotoxicity, it is necessary to remember Paracelsus' dictum that it is the dose that makes the poison. One of the major roles of the liver is to protect the body against naturally occurring toxins present in, for example, plants, or produced by the body's own intestinal flora. Exposure to small amounts of such materials results in changes in the liver which permit efficient removal of the toxin. These changes are called adaptive because they do not compromise the ability of the liver to perform its other vital functions. As the dose of the compound is increased there may come a point when other functions are compromised and this is defined as the toxic phase of the response. However, before considering this and other aspects of the action of chemicals on the liver, it is necessary to discuss briefly the structure and metabolic roles of the liver.

2 ANATOMY AND PHYSIOLOGY OF THE LIVER

The liver is a large organ making up about 3.5% of the body weight of an adult rat or 2% of the body weight of an adult human. The overall shape of the liver differs markedly between species. In rats and mice, the liver is divided into several distinct lobes; the human liver, on the other hand, is divided into two rather poorly differentiated lobes. The liver lies immediately under the diaphragm, is covered in a thin capsule and is supported mechanically by attachments to the diaphragm and by the blood vessels. The liver has a complex blood supply. Approximately 80% of the blood is derived from the portal vein (PV). This drains the stomach, duodenum, small intestine and colon. The remaining 20% of the blood supply comes from the hepatic artery. Blood leaves the liver by the very short hepatic veins which join the ascending vena cava. The liver acts as an exocrine gland, secreting bile. This bile is conducted down the extrahepatic bile duct (BD) into the intestine. In the majority of mammalian species, but not in the rat, a portion of the bile secreted from

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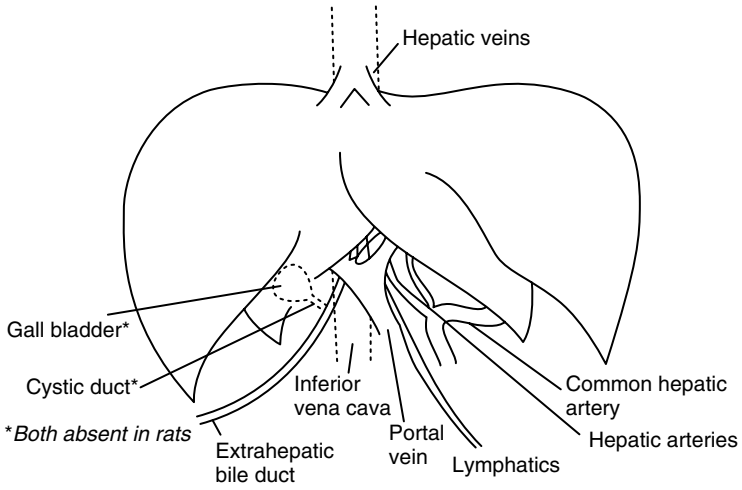

Figure 1 Diagram showing the blood supply to the liver and the drainage of bile and lymph.

the liver is stored and concentrated in the gall bladder, a blind-ended sac attached to the BD. As will be seen later in this chapter, the organization of the bulk of the liver substance, the parenchyma, means that no lymph is formed in most of the liver. Lymph is, however, formed in the connective tissues of the portal tracts and is drained by a duct which connects with the lymphatics that drain the intestine and from there it passes to the thoracic duct. The PV, hepatic artery, extrahepatic BD and the major lymphatics all enter the liver substance at a single point, the porta hepatis. The hepatic veins, however, leave at different points, the number and location of which vary with the species. These various connections are summarized in **Figure 1**.

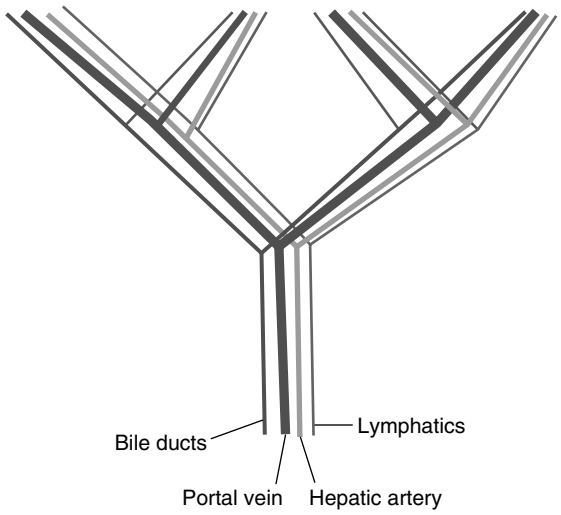

Figure 2 Diagram showing branching of the portal tree within the liver substance. Note that even the smallest branches contain branches of all four types of vessel.

2.1 Organization of Vessels and Nerves within the Liver

As mentioned in the last section, the PV, the hepatic artery, the extrahepatic BD and the major liver lymphatics enter the liver together at the porta hepatis. Once within the liver, the vessels branch to form the portal tree (**Figure 2**). At all branch points there is bifurcation of all types of vessel. This has two consequences. First, all branches of the portal tree, from the largest to the smallest, contain all four types of vessel (**Figure 3**). Second, there are few anastomoses between vessels. Hence any blockage of a branch of the hepatic artery, which provides oxygenated blood to the liver, will result in destruction of an area of the liver (hepatic infarct), blockage of a branch of the PV will result in a similar lesion known as a pseudo-infarct or Zahn's infarct, which is distinguished by atrophic cells, whereas blockage of a branch of the BD will result in local cholestasis. Hepatic infarcts and pseudo-infarcts are normally associated with thrombosis at distant sites and are rare in experimental animals, although, as discussed in a later section, local cholestasis may play a role in BD proliferation, but this is purely speculative.

The organization of vessels within the portal tract is more complex than is apparent at first sight. As will be seen from **Figure 3**, each portal tract contains a single branch of the PV and of the hepatic artery, but may contain several BDs of varying diameter, and several lymphatics. Each BD is surrounded by a group of blood vessels termed the peribiliary plexus which are supplied from the hepatic artery and which will eventually drain into the PV. This is shown in **Figure 4**. The result is a 'contraflow' system which is assumed to be concerned with the resorption of nutrients lost into the bile, although this is also a matter for speculation (Jones *et al*., 1980). Also, bile acids absorbed by cholangiocytes recycle via

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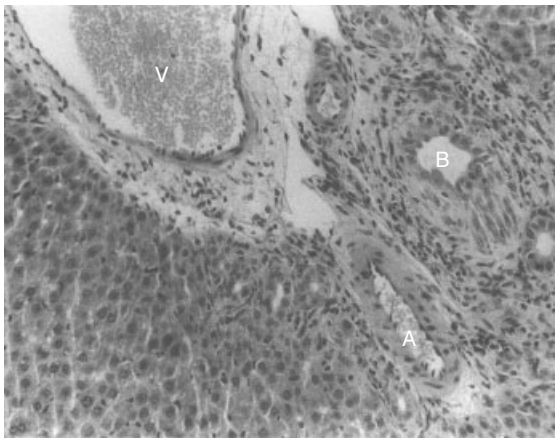

Figure 3 Light micrograph of a middle-sized branch of the portal tree showing branches of the portal vein (V) and the hepatic artery (A) and several bile ducts (B) of various size. The lymphatics are not shown, as these small, thin-walled vessels cannot be identified with any certainty under the light microscope.

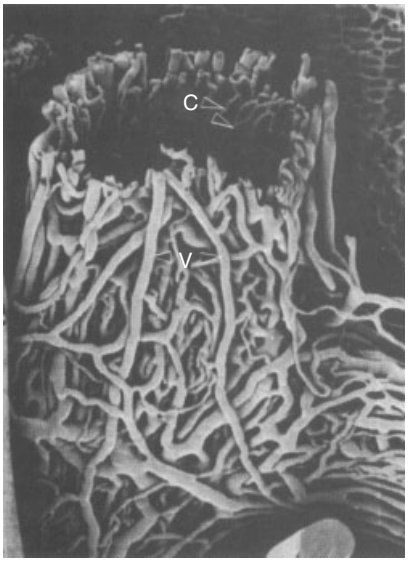

Figure 4 Scanning electron micrograph of a corrosion cast of the peribiliary plexus of inner blood capillaries and outer venules in the rabbit. The network of vessels is most prominent in the larger ducts, but extends into the smallest of the portal canals. (Reproduced with permission from Ohtani, 1979. © ISCH.)

the peribiliary plexus back to hepatocytes for resecretion into bile (Xia *et al*., 2006). In some species, such as humans and rats, the BDs themselves branch, often forming blind-ended pouches (Yamamoto *et al*., 1985) which may be involved in bile storage and modification.

As the portal tree divides, the vessels gradually become smaller. From the smallest veins (terminal hepatic venules), small vessels bud off and pass to the

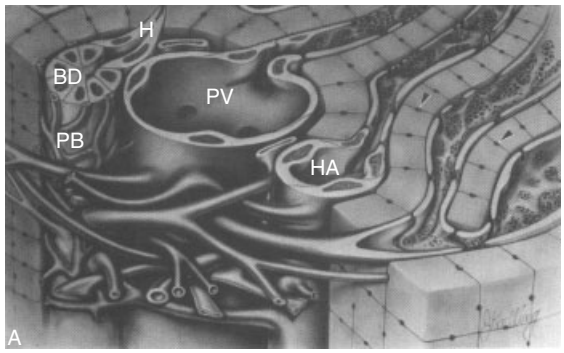

Figure 5 Diagram showing the principal features of a portal canal and connections to the parenchyma. Portal veins (PVs) and hepatic arteries (HAs) empty either directly or via small branch vessels. Bile duct (BD) lining cells contact hepatocytes directly, the junction zone being termed the canal of Hering (H). (Reproduced from Jones, *et al*, 1980. © Springer.)

edge of the portal tract, discharging their blood into the capillaries, which, in the liver, are termed sinusoids (**Figure 5**). Similar branches probably arise from the terminal hepatic arterioles, but a part of the flow from the hepatic artery is believed to pass through the peribiliary plexus before reaching the parenchyma. As is discussed below (Section 2.3.1.1), bile is first formed by discharge into channels between hepatocytes, these channels being called bile canaliculi (BC). These empty directly into BDs, as shown in **Figure 5**. Lymphatic vessels are only found within the portal tracts and around the larger branches of the hepatic veins, so that no connections with the parenchyma are necessary.

The liver is only lightly innervated, and the success of liver transplants shows that innervation is not necessary for liver function. Most fibres are unmyelinated and form part of the autonomic nervous system. It is generally assumed that these fibres, which run in the portal tracts up to their smallest branches, are involved in the regulation of blood flow and possibly of bile flow (Ballantyne, 1978). However, small fibres have been found in the parenchyma contacting hepatocytes (Friedman, 1982) and, because hepatocytes are electrically coupled through the gap junctions, it is reasonable to assume that all hepatocytes may be influenced by signals from the sympathetic nervous system, although the effect of this is obscure.

2.2 Organization of the Hepatic Parenchyma

The bulk of the liver is composed of a single type of cell, the hepatocyte. These are assembled into sheets (sometimes termed muralia), each a single cell thick, which

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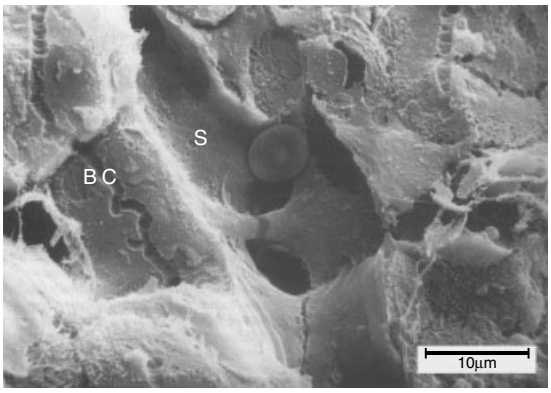

Figure 6 Scanning electron micrograph showing the bile canaliculi (BC) and the sinusoids (S). Tight junction complexes, not visible at this magnification, separate the two types of vessel from each other. (Courtesy Dr S. Singh and Miss D. Chescoe.)

bifurcate and fuse to give a most complex network. Through this network run the liver capillaries, termed sinusoids. Between cells within the wall run small branching channels called bile canaliculi (**Figure 6**). The relationship between these vessels is shown schematically in **Figure 7**.

The liver sinusoids are lined by endothelial cells. Unlike the endothelia of normal capillaries, these cells do not form a continuous barrier, but are penetrated by fenestrations (**Figures 8** and **9**), which allow a free exchange of proteins between the blood within the sinusoids and the space of Disse (perisinusoidal space), which lies between the endothelial cell and the hepatocyte. There is no distinct basement membrane in the undamaged liver, but some collagen fibres are present and the scant extracellular matrix which is laid down by Ito cells appears to play a role in signalling in the liver and may be rapidly expanded on liver injury (Bissell, 1998). Within the sinusoids are Kupffer cells, fixed macrophages which form attachments both to the walls of the endothelial cell and, by means of processes pushed through the fenestrations, to the hepatocytes. The principal role of the Kupffer cells is to remove particulate material which may have been absorbed from the intestine, and their position and structure, particularly the numerous processes which may extend right across sinusoids, are adapted to this role.

As blood enters the parenchyma from the portal tract and is drained through branches of the hepatic vein, hepatocytes lying close to portal tracts receive blood with a much higher oxygen content than hepatocytes lying close to the central veins. This is in turn reflected in differences in function and a marked variation in sensitivity to certain toxins. It is thus unfortunate that there is no generally agreed system for naming the different zones. The two most common nomenclatures

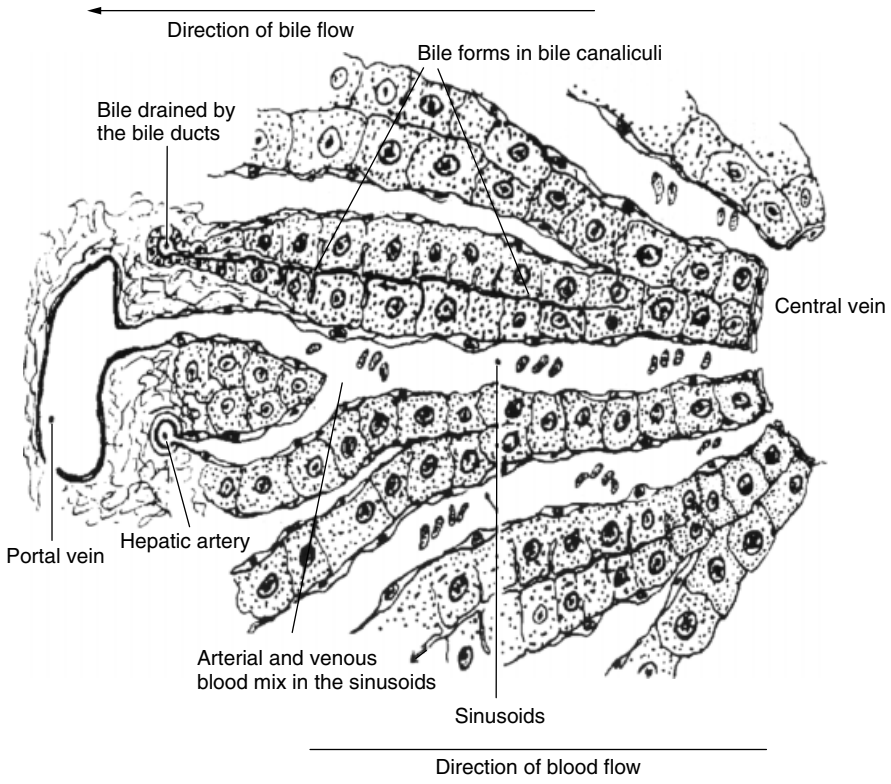

Figure 7 Drawing to show the relationship between the sinusoids and the bile canaliculi in the liver parenchyma. Adapted from Ham (1974).

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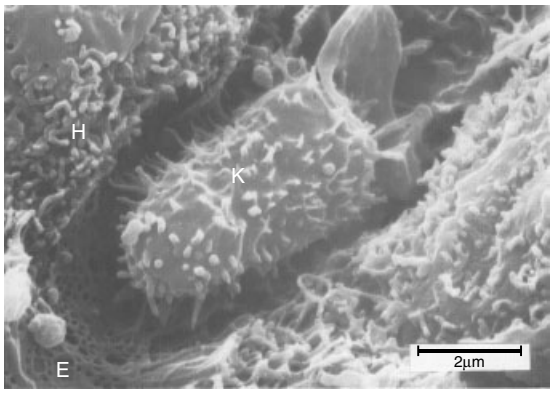

Figure 8 Scanning electron micrograph showing the relationships between hepatocytes (H), sinusoid endothelial cells (E) and Kupffer cells (K), showing a sieve plate. (Courtesy Dr S. Singh and Miss D. Chescoe.)

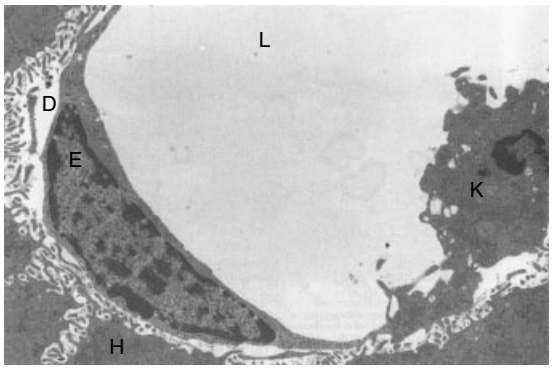

Figure 9 Transmission electron micrograph showing the relationship between hepatocytes (H), sinusoid endothelial cells (E), Kupffer cells (K), the space of Disse (D) and the sinusoid lumen (L). (Courtesy Dr S. Singh and Miss D. Chescoe.)

are shown in **Figure 10**. Neither is ideal for toxicologists. A concept of cylinders (circles in thin section) encircling the portal tract to define a periportal area and branches of the hepatic vein to define a centrilobular area corresponds most closely with the distributions of lesions within the liver (**Figure 11**) and with differences in oxygen tension.

2.3 Structure and Function of the Different Types of Cell in the Liver

2.3.1 Hepatocytes

2.3.1.1 Structure of Hepatocytes

Hepatocytes (**Figure 12**) form approximately 90% of the volume of the liver parenchyma, but are only 60% of the total cell number. They are large cells, approximately cuboidal in shape. The nucleus or nuclei (in rats about half the cells are binucleate diploid cells, the remainder being mostly mononucleate tetraploids) is normally placed centrally; markedly eccentric nuclei are only seen in severely damaged cells. Approximately 20% of the cytoplasm consists of mitochondria, which are actually not spheres or blunt cylinders, as they appear in thin section, but long and often branching tubular structures. Scattered amongst the mitochondria are peroxisomes which normally form only 2% of the volume of the cytoplasm. Normal rat liver peroxisomes can be identified by the crystalloid core, which consists of uric acid oxidase, but this is absent from primates, including humans. Hepatocytes contain a highly developed endoplasmic reticulum which, as usual, is continuous with the outer nuclear membrane. In rodent liver, rough-surfaced endoplasmic reticulum elements may either be wrapped around mitochondria, an arrangement especially associated with periportal cells, or arranged in small stacks, an arrangement characteristic of cells in the centrilobular region. At high magnifications it is seen that the ribosomes are arranged with great regularity along most rough endoplasmic reticulum (RER) elements. Partial loss of ribosomes (RER degranulation), leading to a 'moth-eaten' appearance, is an early sign of hepatotoxicity.

While the RER consists predominantly of sheets of membrane, which, on ultrathin sectioning, gives the characteristic paired membranes, the smooth-surfaced endoplasmic reticulum is predominantly arranged as tubules which, on sectioning, give small circles of ellipses of membrane which are often difficult to identify. In well-fed animals, smooth-surfaced endoplasmic reticulum is largely associated with deposits of the storage polysaccharide glycogen. On treatment with many chemicals, the smooth endoplasmic reticulum proliferates with, in extreme cases, masses accumulating in the centre of the cell marginalizing other organelles.

The Golgi apparatus in hepatocytes is small and, in undamaged cells, invariably located close to the bile canaliculus. The stack of membranous sacs (dictyosomes) of the Golgi apparatus is, however, merely the centre of a much more complex series of membranes (**Figure 13**). The central sacs are connected to a complex of membranous tubules which interleave with elements of the smooth endoplasmic reticulum. Closely associated with the Golgi apparatus in function are the membranes of the late endosome compartment, where proteins taken up by endocytosis are sorted. The final stages of sorting preceding exocytosis also appear to be associated with 'extreme trans-Golgi' elements or trans-Golgi network, which do not form part of the main membrane stack. The Golgi apparatus also forms the centre for a system of microtubules which act as 'railroads' for transferring vesicles to or from the sinusoidal surface of the cell. The lysosomes are believed to bind to this network because in normal hepatocytes they are always located close to the

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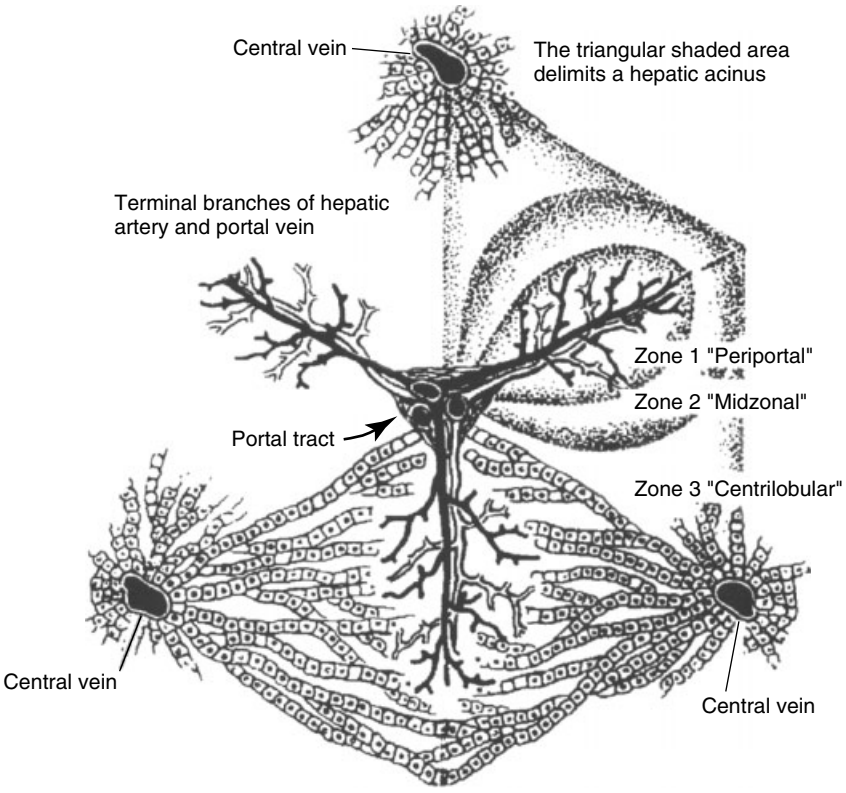

Figure 10 Diagram of the simple liver acinus, small terminal branches of the hepatic artery and portal vein branch from the portal tract. These deliver blood into sinusoids which drain into the central vein. Zone 1 (also known as the periportal zone) receives well-oxygenated blood and nutrient-rich blood from the portal tracts. As the blood passes through the parenchyma, both nutrient and oxygen concentrations fall (Zone 2 or mid-zonal area), being lowest around the central veins (Zone 3 or centrilobular zone). Adapted from Ham (1974).

Golgi apparatus. The plasma membrane of the hepatocytes is divided into two domains. As mentioned above, bile is discharged into small channels between hepatocytes, the BC (**Figure 12**). At the edge of the BC the membranes of the hepatocytes are sealed together by tight junctions, which almost entirely prevent the passage of both soluble and membrane-bound proteins into or out of the bile canaliculus by diffusion between the cells. Close to the BC are the intermediate junctions (zonula adherens), which provide an anchoring point for actin microfilaments, some of which are organized to form a contractile belt around the bile canaliculus (the terminal web). Away from the bile canaliculus the cells are joined by 'spot welds' formed by desmosomes. These act as anchorage points for the intermediate filaments of cytokeratin which cross the cell, giving it mechanical strength and which also anchor the nucleus in place.

2.3.1.2 Functions of Hepatocytes

Hepatocytes have many functions. The liver serves both as an exocrine and an endocrine gland. The exocrine secretion of the liver is bile. The formation of bile is a complex topic (Burwen *et al*., 1992; Crawford, 1996). The critical factor in bile formation is the secretion of bile acids and bile salts, detergents important for the emulsification of fat in the intestine (Stamp and Jenkins, 2008). It should be noted that bile acids are highly bioactive compounds, for example genotoxicity has been reported in some cases (Hardie, 2008).

Of the other major components of bile, water appears to enter passively across the tight junctions whereas phospholipids and cholesterol are extracted from the bile canalicular membrane by the detergent action of the bile salts. In some species, such as rat and dog, IgA antibodies are secreted into bile by a process involving uptake of the IgA at the sinusoidal surface of the cell, sorting in the endosome compartments and release into bile by exocytosis. Bile also has an excretory function. Conjugates, both of endogenous materials such as bilirubin and hormones, and of xenobiotics, are secreted into bile in a process that will be described later in this section. There is also, as mentioned earlier, a slow discharge of lysosomes into bile.

The liver, in its second role as an endocrine gland, secretes almost all the major proteins of plasma with the exception of the immunoglobulins. In addition, the liver plays a central role in lipid metabolism, taking up chylomicron remnants in the blood from the intestine and repackaging this and newly synthesized lipid with a new group of proteins to form very low density lipoprotein

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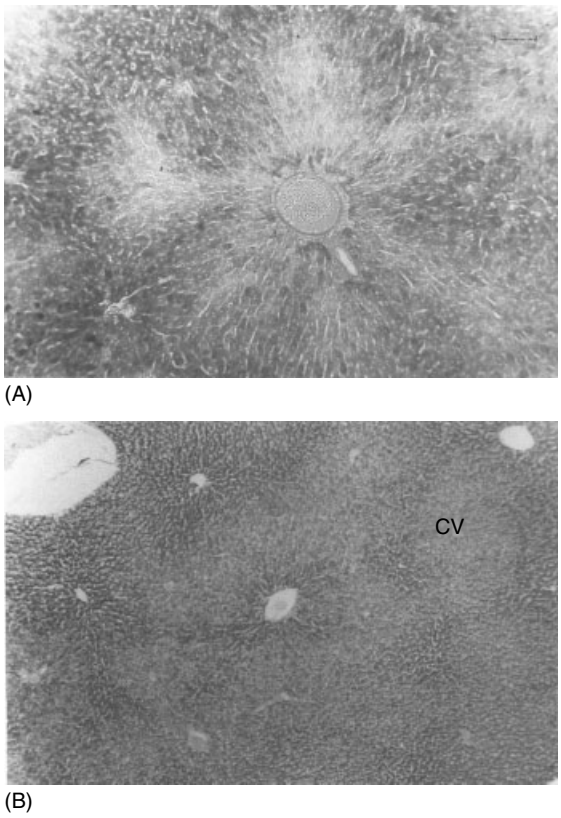

Figure 11 Micrographs showing loss of glycogen from (A) the periportal area and (B) the centrilobular zone. Animals were treated respectively with chlorpromazine (Mullock *et al*., 1983) and fenofibrate (Price, 1985). Glycogen loss is indicated by pale staining.

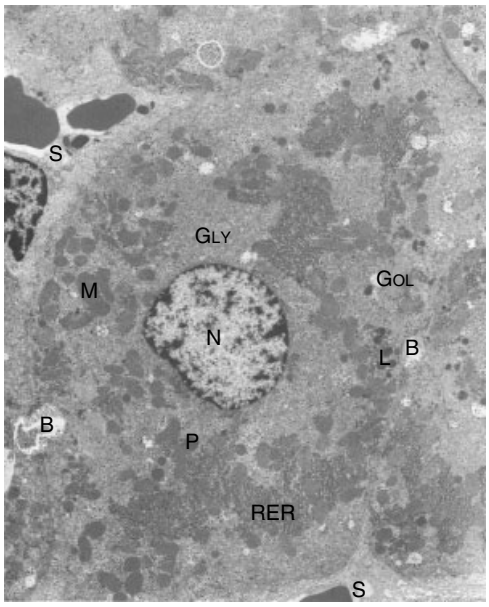

Figure 12 Electron micrograph of the normal structure of a hepatocyte, showing the large centrally placed nuclei (N), rough (RER) and smooth (S) endoplasmic reticulum, mitochondria (M) and peroxisomes (P). Note that the lysosomes (L) and Golgi apparatus (GoI) are located close to the bile canaliculus (B). Gly indicates areas of glycogen traversed to smooth endoplasmic reticulum. (Courtesy Dr S. C. Price.)

(VLDL) particles, which are then exported from the liver. The transport of proteins through the exocytic pathway is especially sensitive to change in intracellular ATP. This is reflected in the accumulation of fat in the damaged liver and in the fall in plasma proteins and disturbances in blood clotting that follow long-standing liver damage.

In addition to forming the bulk of the plasma proteins, the liver is also responsible for their recycling. Most proteins in plasma are glycoproteins, possessing a terminal sialic acid on their sugar side chains. Removal of this sialic acid exposes a galactose residue, which binds avidly to a receptor on the hepatocyte plasma membrane. Following binding, the proteins are taken up by endocytosis and transferred to lysosomes for digestion. Nonglycosylated plasma proteins, such as albumin, are taken up by using other, less well-characterized receptors. The liver is also responsible for recycling old red blood cells, this task being carried out principally by Kupffer cells. Aged red blood cells are recognized because the removal of sialic acid residues from their cell surface glycoproteins reveals galactose residues, which are recognized by a receptor. Following binding, the cells are taken up by phagocytosis and their proteins digested. The haem residues are passed to hepatocytes for reuse or degradation.

In addition to these roles, the liver acts as the centre of intermediary metabolism in the body. The role of the liver in the metabolism of exogenous lipid has already been discussed. In addition, the liver provides a central store of sugar, in the form of glycogen, and is also a major site for the conversion of sugars to lipids and for conversion of amino acids to sugars and lipids. This may have marked consequences. For example, in rats, the metabolic demands of 8 result in a 30% increase in liver weight, exacerbating the toxicity of liver-enlarging agents, such as butylated hydroxytoluene (BHT) (McFarlane *et al*., 1997). In addition, the liver is the main, or in the case of humans almost the sole, site of *de novo* cholesterol synthesis. The result is that liver damage can cause marked disturbances in endocrine function, for example liver cirrhosis results in feminization in men and masculinization in women (Johnston and Alberti, 1985). See review by Foresta *et al*. (2008).

The final function of the liver is in the modification and excretion of a variety of hydrophobic compounds. These reactions involve both endogenous body constituents, such as haem or steroids, and nutrients absorbed with the diet or produced by the body's own microflora. In general, the physiological role of the reactions is to prepare the compounds for excretion. The enzymes

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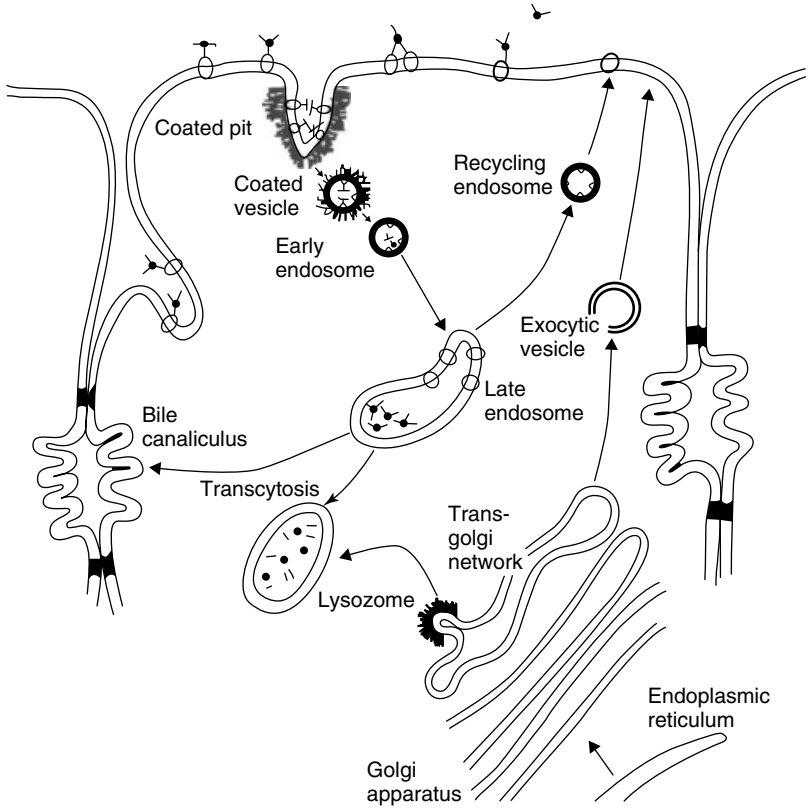

Figure 13 Scheme showing the relationship between the central stack of the Golgi apparatus and associated elements.

involved are conventionally divided into two groups (see **Biotransformation of Xenobiotics**). Phase 1 reactions involve chemical modification of the reactant, most often by oxidation, whereas Phase 2 reactions are biosynthetic, generally involving conjugation with a hydrophilic moiety such as glucuronic acid, sulfate or glutathione. Under most circumstances, the role of these enzymes is protective and results in the removal of potentially harmful materials from the body. However, under some circumstances, the metabolites are markedly more toxic than the parent compound.

By far the most important group of enzymes involved in Phase 1 reactions are the cytochromes P450 (CYPs). These are a large group of enzymes which catalyse a wide variety of reactions (**Table 1**).

The cytochromes P450 are classified into families and subfamilies according to their amino acid sequences, for example CYP 4A1 is the first member of subfamily A of family 4. There is, however, only limited correlation between the classification of the cytochromes P450 and their substrate specificities (**Table 2**), and while there are structural analogies between the P450s of different mammalian species it is unclear how far these correlate with the function of the enzymes (Ioannides, 1996; Lewis and Ito, 2008). The number of isoforms of cytochrome P450 is very large. Some are present constitutively, whereas others are only synthesized in response to inducing agents. Thus the response of animal populations to a xenobiotic may be altered by prior exposure to other chemicals and the situation is further complicated by the fact that outbred populations, such as humans, may show considerable genetic polymorphism. See review by Guengerich (2002).

Although there are several other Phase 1 drug-metabolizing enzymes in the liver, there is little evidence that they play a significant role in the pathogenesis of liver damage. Again, although the liver is the principal location of the Phase 2 drug-metabolizing enzymes listed in **Table 3**, these reactions generally remain, 'as intended', purely protective; the metabolism of aromatic amines (see later) is a notable exception. It should be noted, however, that there are marked variations in levels of Phase 2 enzymes between species of animals and even among individuals (**Table 4**) and that this may result in major differences in toxicity. For example, the chromone FPL 52757 (a candidate antiasthmatic drug) is markedly hepatotoxic in the dog and mildly hepatotoxic to some individual humans, but has no effect on other species because it is not detoxified by N-acetylation; it causes BD necrosis (Eason *et al*., 1982; 1990).

Many of the materials metabolized in the liver are excreted into the bile. In addition, bile contains bile acids and bile salts, present in the bile, play a vital role in digestion Secretion of bile salts from the liver and

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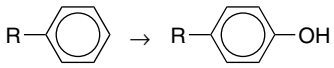
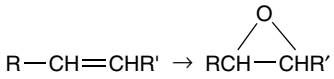
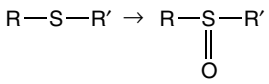
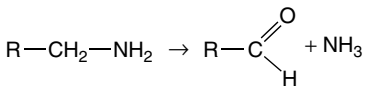
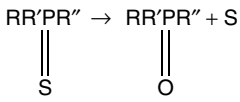
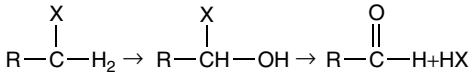
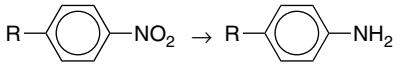
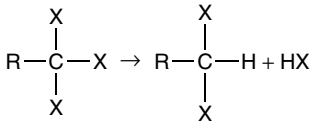
Table 1 Examples of reactions catalysed by cytochromes P450

(A) Oxidative mode	
Aliphatic hydroxylation	$RCH_2CH_3 \rightarrow RCH_2CH_2OH$
Aromatic hydroxylation	
Epoxidation	
N-Oxidation	$R{-}NH{=}R' \rightarrow R{-}NOH{-}R'$
S-Oxidation	
Dealkylation[a]	$R{-}X{-}R' \rightarrow R{-}XOH + R'H$
Deamination	
Desulfuration	
Oxidative dehalogenation	
(B) Reductive mode	
Azo reduction	$R{-}N{=}N{-}R' \rightarrow RNH_2 + R'NH_2$
Aromatic nitroreduction	
Reductive dehalogenation[b]	

adapted from Sipes and Gandolfi (1991).
[a]Where X may be O, NH or S.
[b]Where X is any halogen.

absorption from the intestine comprise the enterohepatic circulation of bile acids. Understanding of the mechanisms by which these compounds enter bile has advanced markedly and dedicated bile-salt transporters in hepatocytes and enterocytes are responsible for the unidirectional transport of bile salts in the enterohepatic cycle (Steiger and Meier, 1998; Pellicoro and Faber, 2007; Stamp and Jenkins, 2008). In the hepatocytes, selection occurs both at the sinusoidal surface of the cell and at the bile canalicular surface. Bile acids are specifically taken up at the sinusoidal surface of the cell by the sodium taurocholate cotransporter. Organic anions may also be transported by nonspecific sodium-independent 'organic anion transport protein(s)'. The driving force for these has not been established. Monoanionic bile acids are secreted into bile by the ATP-dependent 'bile-salt efflux pump', whereas dianionic bile salts, glutathione conjugates and at least some glucuronic acid conjugates are transported into bile by the rather broad-specificity MDR (multiple drug resistance) transporters, again using ATP as energy source. Transfer is also mediated by the related MRPs (multidrug resistance-associated proteins). There would appear to be at least six of these in the human genome, but not all have been fully characterized (see reviews by Alrefai and Gill, 2007; Kosters and Karpen, 2008).

2.3.2 Sinusoid Endothelial Cells

The smallest blood vessels in the liver, the sinusoids, are lined by endothelial cells specialized in structure to assist in the liver's major roles of removal of waste materials from the blood and of blood formation. In the majority of tissues, the capillaries form a continuous barrier preventing blood components from coming directly into contact with the cells of the tissue. However, in the

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Table 2 Specificities of major isoforms of cytochrome P450 showing the preferred substrate conformations (clearly a wider range of compounds can be metabolized than are specified here)

Isoform	Preferred substrate
1A1	Polyaromatic hydrocarbons
1A2	Polyaromatic hydrocarbons with amine substituents, multiringed heterocyclic aromatic compounds
2B	V-shaped molecules with one or more nonfused aromatic rings
2D	Similar to 2B but with a basic nitrogen at a specific distance and orientation from an aromatic ring
2E	Small molecules, not necessarily planar
3A	Large molecules, not necessarily planar, some show cross-specificity with 1A1 and 2B
4A	Carboxylic acids and their esters, usually with one or two unfused aromatic rings at a certain distance from the carboxylate group

Guengerich (2002) should be consulted for further details.

live, the endothelial cells which line the smallest blood vessels are pierced by fenestrations about 100 nm in diameter, sometimes called sieve plates (**Figure 8**), which allow all components of the plasma, but not blood cells, to make direct contact with the hepatocytes. It is for this reason that the smallest blood vessels in the liver are termed sinusoids, not capillaries. Apart from the possession of sieve plates, the sinusoidal endothelial cells present few features of interest to toxicologists. As with other endothelial cells, there would appear to be active uptake of material by pinocytosis, but the nature of the materials absorbed is not known. The cells contain only small numbers of mitochondria and the endoplasmic reticulum is not well developed. The Golgi apparatus and the lysosomal system account for a considerably greater proportion of the cell volume than in hepatocytes. It is believed that sinusoid endothelial cells play an important part in cell–cell communication: these cells are active in the secretion of cytokines, eicosanoids (i.e. prostanoids and leukotrienes), endothelin-1 and nitric oxide (Kawada, 1997; Kmieć, 2001).

2.3.3 Kupffer Cells

Kupffer cells are fixed macrophages which are found within the sinusoids (**Figures 8** and **9**). They are attached both to the endothelial cells and, via processes extending through the fenestrations in the endothelial cells, to hepatocytes. Like all macrophages, Kupffer cells are actively phagocytic, removing particulate material, such as bacteria or bacterial fragments (endotoxins), which may have entered the blood from the intestine. Kupffer cells are also largely responsible for the recycling of old red blood cells and cooperate with hepatocytes in the metabolism of haem. The structure of Kupffer cells is similar to that of other macrophages. Phagolysosomes are prominent and numerous filamentous elements are present. There are a fair number of mitochondria, but the endoplasmic reticulum is poorly developed and consists mainly of rough-surfaced elements.

It is becoming increasingly evident that, in addition to their protective role, Kupffer cells have other roles (Laskin *et al*., 2001). Kupffer cells are heavily involved in the signalling systems of the liver and, like other macrophages, Kupffer cells secrete a range of cytokines. Some of these, such as IL-1, are principally concerned with signalling to the immune system, but others, such as tumour necrosis factor (TNF)-α, act on hepatocytes and other cells; in this case IL-6 induces synthesis of the protective acute-phase proteins (Scotte *et al*., 1996) while TNF-α, at low concentrations, causes hepatocytes to prepare for division (Webber *et al*., 1998), but causes apoptosis of hepatocytes at high concentrations (Kunstle *et al*., 1997). In addition, Kupffer cells and endothelial cells are the major source of the mitotic inhibitory factor,

Table 3 Phase 2 drug metabolizing enzymes

Reaction	Substrates
Glucuronidation	*O*-Glucuronides formed from alcohols, carboxylic acids, unsaturated ketones, hydroxylamines *N*-Glucuronides formed from carbamates, arylamines, aliphatic tertiary amines, sulfonamides *S*-Glucuronides from aryl thiols and dithiocarbamic acids *C*-Glucuronides from 1,3-dicarbonyl systems
Sulfation	Only *O*-sulfates are formed; phenols, catechols, hydroxylamines, steroids are substrates
Methylation	Aliphatic and aromatic amines, *N*-heterocycles, mono- and polyhydric phenols, sulfydryl-containing compounds
Acetylation	Many including aromatic primary amines, hydrazines, hydrazides, sulfonides and certain primary aliphatic amines
Amino acids	Carboxylic acid residues including arylacetic acids, aromatic carboxylic acids, aryl-substituted and bile acids. The amino acid(s) employed varies with species
Glutathione	Enzymic substitution reactions with alkyl, aryl and aralkyl halides. Enzymic addition reactions with alkenes, aryl and alkyl epoxides. Nonenzymic addition reactions with many free radicals and reactive intermediates

Adapted from Sipes and Gandolfi (1991).

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Table 4 Species defects in foreign compound metabolism

Reaction	Defective species
Aliphatic amine *N*-hydroxylation	Rat, marmoset
Arylacetamide *N*-hydroxylation	Guinea pig
Arylamine *N*-acetylation	Dog[a]
Glucuronidation	Cat, lion, lynx, Gunn rat
Sulfation	Pig, opossum, brachymorphic mice
Hippuric acid formation	African fruit bat
Mercapturic acid formation	Guinea pig

[a]A proportion of the human population shows unusually low rates of *N*-acetylation and this may have significant effects on the toxicity of certain drugs.
Source: Sipes and Gandolfi (1991).

transforming growth factor (TGF)-β, although when stressed, for example in the late stages of liver repair, production is predominantly in hepatocytes (Bissell *et al*., 1995). This may also be true with other cytokines. In addition, as discussed below, interchange of signals between Kupffer cells and Ito cells is believed to play an important role in liver fibrosis. See review by Tiegs (2007).

2.3.4 Hepatic Stellate Cells (Ito Cells)

Hepatic stellate cells, also known as Ito cells, are found tucked in between hepatocytes. Their structure and functions have been reviewed by Kawada (1997), Hautekeete and Geerts (1997) and Senoo (2004). Hepatic stellate cells are modified fibroblasts, one of whose roles in the undamaged liver is to maintain the sparse extracellular matrix of the space of Disse and to store vitamin A. Hepatic stellate cells are small cells compared with hepatocytes. The 'fat' droplets, which are their most prominent feature, contain a 40% solution of vitamin A in triglyceride. Hepatic stellate cells play pivotal roles in vitamin A homeostasis. These cells express, on their cell surface, specific receptors for retinol-binding protein, a binding protein which is specific for retinol: the cells take up the complex comprising retinol and for retinol-binding protein by receptor-mediated endocytosis (Senoo *et al*., 2007). Stellate cells have a well-developed RER and Golgi apparatus and are the source for hepatocytes growth factor (HGF), which is one of the two major mitogens which act on hepatocytes. Their most prominent role in liver damage, however, is when they differentiate into myofibroblast-like cells and actively form Type 1 collagen and secrete TGF-β. The transition between the two states of the cell is regulated by a network of signals coming from Kupffer cells, damaged hepatocytes, endothelial cells, platelets and inflammatory cells. It is thus clear that hepatic stellate cells play an important role in the development of liver diseases, in particular those which involve fibrosis (see Henderson and Forbes, 2008).

2.3.5 Other Cell Types of the Liver

Most biochemical tests are carried out on liver homogenates and, when interpreting these, one must bear in mind that blood vessels make up about 5% of the hepatic parenchyma so that blood plasma and cells may contribute significantly when measurements are made on the homogenates. Furthermore, even the normal liver in experimental animals generally shows some small inflammatory foci and these may increase greatly in treated animals, again emphasizing the necessity for biochemical studies on poorly characterized hepatotoxins to be accompanied by histological examination of the tissue. Blood cells and inflammatory cells cannot, however, be thought of as normal components of the liver. There is, however, a final cell type, the pit cell, whose status is less clear. Pit cells are rarely observed, although there are about 500 000 per gram of rat liver (Wisse *et al*., 1989). These cells are located on, or embedded in, the endothelial lining and possess numbers of small granules. Isolated pit cells have natural killer (NK) activity (i.e. they recognize and kill tumour cells *in vitro*) and morphologically they resemble a population of NK cells, the large granular lymphocytes in the blood, although the two populations do not seem to be identical. Cells considered morphologically to be pit cells probably include a proportion of NK T cells. The morphology and function of pit cells has been reviewed (Nakatani *et al*., 2004).

2.4 Development of the Liver

2.4.1 The Embryonic Liver

The liver develops as a fused pair of diverticulae of the foregut. Clusters of cells grow from this into a mesenchymal stroma in which has developed a plexus of small blood vessels. The intimate relationship between blood vessels and hepatocytes is thus established from the very origins of the organ. Throughout foetal life, the liver consists of sheets of hepatocytes two cells thick, unlike the single cell muralia found in adult life. The development of the blood supply to the foetal liver is extremely complex (MacSween and Scothorne, 1987). The development of the ductular system is equally complicated, but is of more relevance to toxicologists. BC between hepatocytes are seen very early in development. The BDs themselves develop much later. It has been suggested (MacSween and Scothorne, 1987) that BD lining cells differentiate from immature hepatocytes through contact with the connective tissue of the developing portal tracts,

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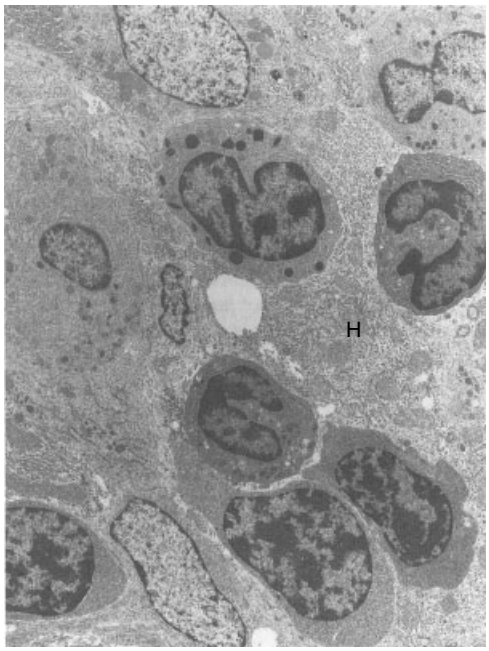

Figure 14 Electron micrograph of the liver of a foetal rat showing very large numbers of immature blood cells between the hepatocytes (H). (Courtesy Dr S. C. Price.)

whereas mature hepatocytes differentiate from cells in contact with the vascular endothelium and with the sparse collagenous framework which these lay down. This view is supported by some direct explantation experiments (Shiojiri, 1984).

Hepatocytes in the developing liver are highly differentiated and are functional, producing plasma proteins. Drug-metabolizing enzymes are inducible, although the constitutive expression is small. In addition to its adult role, the liver takes on a second temporary role as the main organ for haematopoiesis. Cells deriving from the yolk sac settle in the liver and rapidly dominate the histological picture (**Figure 14**) (McGrath and Palis, 2008). All cell lineages are represented, although erythropoietic activity is dominant (Moore and Johnson, 1976). In humans, the role of the liver as the major haematopoietic organ passes to the bone marrow in the fifth month of gestation, but in rats and mice this change occurs in the first three weeks after birth.

2.4.2 Changes in the Liver with Age

There are only minor structural or ultrastructural changes in the liver after birth. The proportion of connective tissue increases to accommodate the increasing weight of the organ. In rats, blood flow through the liver diminishes over the first 12 months of life (Van Bezooijen, 1984). In addition, there is a marked increase in BD hyperplasia with age in the rat, possibly as a consequence of spontaneous infections. In both humans and experimental animals the 'age pigment' lipofuscin, which is thought to represent lysosomes filled with an indigestible material consisting largely of peroxidized lipid, accumulates in hepatocytes in later life. There is also a marked increase in nuclear ploidy and in the number of binucleate cells which occur in mice and, to a lesser extent, in some strains of rats (Johnston *et al*., 1968; Grice and Burek, 1984). In mice, ageing animals show significant numbers of hexadecaploid nuclei. Of much more significance to toxicologists are significant metabolic alterations, especially in the enzymes which metabolize xenobiotics.

As noted in the previous section in connection with the embryo/foetus, the liver of a newborn rat is underdeveloped and, in particular, has much less drug-metabolizing capacity than normal liver. Different drug-metabolizing enzymes develop at different rates (Ioannides, 1996). Cytochromes P450 IA develop before birth, reach maximum activity before weaning (three weeks) and decline thereafter (Lum *et al*., 1985). Total cytochrome P450 and the amount of mRNA for the phenobarbital-inducible isoenzymes CYP 2B1, 2B2, 2C3 and 3A1 reach adult levels approximately three weeks after birth and then stabilize (Omiecinski *et al*., 1990). However, the activity of benzphetamine N-demethylase, which is markedly inducible by phenobarbital, does not stabilize in rats until the animals are over seven weeks of age (Lum *et al*., 1985). It is unclear whether this is due to other, unidentified, late-developing isoforms of cytochrome P450 catalysing this reaction or to age-dependent variations in the half-life of the P450 proteins.

In addition to changes in the constitutive expression of genes for drug-metabolizing enzymes, there are also changes in their inducibility. Many, but not all isoforms of cytochrome P450 are inducible in the foetal rat (Marie and Cresteil, 1989). Following birth there are also marked changes in inducibility. The phenobarbital-induced form (CYP 2B2) is highly inducible two weeks after birth (Giachelli and Omiecinski, 1987), but the degree of inducibility declines later, as constitutive expression increases. The main methylcholanthrene-inducible P450 isoforms (CYP 1A) show high constitutive expression early in life, but later this constitutive expression declines and the inducibility increases (Ioannides and Parke, 1990).

Changes in hepatic enzyme levels also occur in the ageing animal. Both constitutive expression of drug-metabolizing enzymes and their inducibility decline with age (Schmucker and Wang, 1981). Superoxide dismutase and catalase activity also decrease (Mote *et al*., 1990) and there is a reduced response to peroxisome proliferators (Hinton *et al*., 1986). The hyperplasia which is so marked in young rats treated with phenobarbital or peroxisome proliferators is much reduced in the ageing animal (Schmucker and Wang, 1981; Hinton *et al*., 1986). There is thus, in ageing animals, a generalized decrease in the capacity of the liver both to metabolize xenobiotics and to protect itself against damaging

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products of metabolism such as superoxide free radicals.

2.5 Adaptive Changes in the Liver

Because of its many roles, the liver is continuously changing and this affects even its histological appearance. Glycogen is stored following meals and used at night. Fat accumulates transiently and drug-metabolizing enzymes, alter depending on the composition of the diet. In the small animals normally used in toxicology studies even the size of the liver shows significant variation. For example, a 'normal' rat, mouse or chicken liver is significantly larger than that of their germ-free counterparts (Gordon, 1959). Accordingly, it is necessary, before considering the action of hepatotoxins, to identify the changes in the liver that occur naturally in the course of an animal's life.

One would not expect major changes in bile secretion in the normal life of an animal. Changes in the synthesis of plasma proteins will occur following haemorrhage and, more importantly for toxicologists, as a result of inflammation in any part of the body. In the former case there is a compensatory increase in the synthesis of normal blood proteins. In the latter case a reaction termed the 'acute-phase response' occurs, and the liver synthesizes and secretes into the plasma a distinct range of proteins, the acute-phase respondents, some of which are barely detectable in normal plasma (Kushner, 1982), in addition to the normal plasma proteins. A prominent feature of acute-phase reaction is the alteration of gene expression in hepatocytes (Fulop, 2007). The intermediary metabolism in the liver is affected by pancreatic, adrenal, pituitary, thyroidal and sex hormones, among others (Van Thiel, 1982). The interactions are extremely complex and result in, *inter alia,* the establishment of marked diurnal rhythms (Belanger, 1988; Feuers and Scheving, 1988). This emphasizes the need for strict standardization of conditions in toxicological studies. Generally, however, although hormones at natural levels will not cause morphological change in the liver apart from affecting glycogen content, marked changes are found in the liver during lactation. In rats the weight of the organ increases by over 30% and this is associated with a marked increase in lipogenesis (Williamson, 1980).

The most marked changes in the liver occur in response to xenobiotics. The ranges of variety may seem bewildering, but some logic is appearing. Inducible genes in eukaryotes are controlled by regulatory elements which may lie either before or after the coding area. Transcription of the genes occurs when regulatory protein molecules bind to these sequences. Each gene will normally have a range of regulatory elements and interactions may be positive or negative. Induction of enzymes to metabolize small hydrophobic molecules usually involves binding of the inducing agent to the regulatory protein. This produces a conformational change which permits the protein to bind to DNA, initiating transcription. Thus a single control element may control several genes. The response of the rat liver to agents, such as clofibrate, which induce peroxisome proliferation, typifies this phenomenon. These agents induce a range of enzymes (Mannaerts and van Veldhoven, 1993), including the group responsible for fatty-acid oxidation in the peroxisomes, cytosolic epoxide hydrolase, cytochromes P450 of the 4A family, especially CYP 4A1 and bilirubin-glucuronyl transferase (Magdalou *et al*., 1993), in addition to a range of other enzymes associated with lipid metabolism (it will be noted that these together form a tool kit for degrading and excreting peroxidized fatty acids). These enzymes are induced in parallel. In addition to increasing the metabolic capacity of the existing cells, there is also a burst of cell division, which may result in a doubling of the liver weight. The fundamental process is transactivation of nuclear receptors, typified by peroxisome proliferation associated receptor-α (PPAR-α) (Rettie and Kelly, 2008). These effects all appear to be caused by binding of the inducing agent to PPAR-α, which then binds to the appropriate genes; there are significant species differences in this process (Johnson *et al*., 2002) (see also Section 3.2.5.2.3). The situation as regards inducers of microsomal enzymes can be complicated. Two receptors appear to be specialized for binding to xenobiotics. Best understood is the aryl hydrocarbon (Ah) receptor. Compounds which bind to this induce CYP 1A1 and a range of other enzymes, and are associated both with hepatic injury and immunotoxicity (Whitlock, 1999; Moorthy, 2008). It is interesting to note, however, that the increased activity of CYP 1A2 caused by Ahs is not due to changes in transcription, but is probably associated with increased stability of the mRNA (Kawajiri and Hayashi, 1996). Less well understood are the receptors by which other classes of xenobiotic-metabolizing cytochromes P450 are induced. In the case of CYP 2E1, regulation may affect the translation of the gene, the half-life of the mRNA and the half-life of the protein, although, in the case of xenobiotics, regulation appears to occur at the post-translational level (Ronis *et al*., 1996). The CYP 2B subfamily and the CYP 3 family of cytochromes P450 are both regulated at the transcriptional level. A possible receptor regulating the induction of at least some of the CYP 3 family has been identified (Kliewer *et al*., 1998). Like the PPARs, thyroid hormone, vitamin D and retinoic acid receptors (Lemberger *et al*., 1996), this pregnane X receptor binds to DNA only after forming a heterodimer with the retinoid X receptor. With regard to the regulation of the CYP 2B subfamily, the structural diversity of xenobiotics displaying CYP 2B-inducing ability, the apparent lack of clearly defined structure–activity relationships within certain structural classes and the absence

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of enantioselectivity in the induction process makes it difficult to rationalize the existence of a receptor for this induction process, however it is now thought that the constitutive active receptor (constitutive androstane orphan nuclear receptor) (CAR) is involved in the regulation of CYP 2B (Guengerich, 2002; Corcos and Berthou, 2008). A number of factors are responsible for activating transcription of the genes coding for CYP 2A proteins, including hepatocyte nuclear factor 4, octomer transcription factor 1 and others (see Raunio *et al*., 2008). Nuclear receptors regulating CYPs have been reviewed (Yoshinari *et al*., 2008).

It is thus clear that regulation of the enzymes involved in metabolism is extremely complicated. It is clear that there may be regulation both of transcription and of the rate of degradation both of mRNAs and of the proteins. It would seem almost certain, as with PPAR-α, that the other receptors will induce not single enzymes, but 'tool kits', including both Phase 1 and Phase 2 metabolizing enzymes; certainly coordinated induction is seen. In addition, while compounds which bind to the PPARs are sufficiently different in structure from other xenobiotics for there to be little cross-reaction with other receptors, it would seem that microsome inducers can often bind to more than one receptor. Thus, for example, phenobarbital, which is a strong inducer of the CYP 2B subfamily, also induces, albeit weakly, the CYP 3A subfamily. This complexity, coupled with the differences between species, means that the accurate prediction of induction and of routes of metabolism will remain impossible for some period of time.

2.5.1 Adaptive Changes and Regulatory Toxicology

Liver hypertrophy raises a problem in risk assessment undertaken by regulators in that it is necessary to consider whether a pure adaptive change should be considered adverse. If it is considered that such changes are not adverse, it is necessary to define adaptive changes. The Joint FAO/WHO Meeting on Pesticide Residues (JMPR) concluded that no single effect is generally sufficient to support a determination that liver hypertrophy is adaptive or adverse and that it would be needful to look at morphology, clinical chemistry and other end points to decide (FAO/WHO, 2006). (See also Andrew, 2005.)

3 LIVER TOXICITY

3.1 Diagnosis of Toxic Changes in the Liver

Diagnosis of liver damage depends principally on the gross pathology and on histological examination of the tissue.

Gross pathology is often neglected, especially in research laboratories. For those laboratories doing work intended for submission to regulatory authorities, the data to be gathered at necropsy will usually be specified in guidelines and will almost always include the liver weight. The most common changes observed are liver enlargement, which can, of course, be confirmed by weighing the liver, and changes in colour. The most common of these changes is when the liver assumes a fawny colour and feels greasy on cutting. These changes are generally associated with accumulation of fat in the liver and may be confirmed by examining frozen sections stained with specific stains for neutral fats such as Oil Red O. In humans and to a lesser extent in large experimental animals, ultrasound imaging has proved invaluable in the diagnosis of large-scale changes in the liver architecture, but these techniques are much less useful in small animals such as rats. The significance of liver enlargement is discussed later in this chapter. Other changes that may be seen at necropsy include masses, which may be single or multiple, and haemorrhage.

There is no single guide to the histopathology of the livers of experimental animals and it is usually necessary to consult both textbooks of general pathology, such as that by McGee *et al*. (1992) and Majno and Joris (2004), descriptive texts, such as that by Greaves (1990), and atlases, such as that by Gopinath *et al*. (1987). Special stains may be useful in the diagnosis of some types of liver damage. Loss of glycogen and certain types of fat accumulation are useful early indicators of liver damage and may be confirmed by staining with the periodic acid Schiff technique and with Oil Red O, respectively. Pigmented inclusions may be characterized by stains for iron (Perls' Prussian Blue) and lipofuscin (Schmorl's stain). The initial stages of fibrosis may require confirmation with a reticulin stain. Mallory bodies are eosinophilic inclusions characteristic of human alcoholic liver disease and a number of other conditions which predispose to cirrhosis; they are now known to consist of bundles of intermediate filaments. There is, however, no connection between these and the development of cirrhosis in experimental animals. Mallory bodies are found, for example, in the livers of mice treated with griseofulvin, colchicine or dieldrin (Meierhenry *et al*., 1981; Stumptner *et al*., 2001; Zatloukal *et al*., 2007) and should probably be regarded simply as a marker of hepatocellular damage.

Electron microscopy frequently provides the earliest indications of toxic changes in hepatocytes, degranulation and vacuolation of the RER being a particularly useful indicator. The electron microscope is also invaluable in detecting proliferation of smooth endoplasmic reticulum, peroxisomes and mitochondria, although minor changes in these organelles should not, in themselves, be taken as indicators of hepatotoxicity. Mitochondrial damage is also readily detected under the electron microscope, but careful comparison with control sections is needed as these organelles are exquisitely sensitive to

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post-mortem change. Comprehensive guides to ultrastructural pathology has been produced by Ghadially (1982) and Cheville (2009). However, electron microscopy is now little used outside of research laboratories because of the time needed for tissue preparation and section cutting, and the difficulties in interpreting micrographs of anisotropic tissues such as the liver.

Biochemical tests are much less valuable for the diagnosis of liver damage, although biochemical studies on the liver itself may be invaluable in determining the mechanism of change. In particular, increases in drug-metabolizing enzymes do not, in themselves, indicate damage to the liver. A fall in hepatic glucose-6-phosphatase activity, which parallels a reduction in glycogen, or in reduced glutathione indicates stress on the liver. ATP levels can be used to quantify acute toxic stress, but are not useful in subacute or chronic studies.

Whereas measurement of enzymes and other proteins in blood is important in the diagnosis of human liver disease, such measurements have limited value in work with small laboratory animals. In general, the same enzymes are measured in studies on experimental animals as are measured for the diagnosis of liver disease in humans. It should be noted that γ-glutamyl transpeptidase is a poor marker for cholestasis in rat, whereas secretory IgA and free secretory component provide excellent markers in rats, but do not change in humans (see also **Clinical Chemistry in Toxicity Testing: Scope and Methods**).

3.2 Action of Toxins on the Liver

In 1964, a review of experimental toxic injury to the liver by Rouiller attempted to draw up a comprehensive list of hepatotoxins (Rouiller, 1964). Even then this list covered nine published pages. Advances in our knowledge of the deleterious effects of natural and synthetic chemicals make compilation of a similar list impossible within the bounds of a book such as this. Accordingly, it is necessary to group hepatotoxins. Pathologists have grouped toxins according to the part of the lobule affected (i.e. centrilobular, midzonal and periportal), but, as will be seen, this may differ between species. Ideally toxins should be grouped according to their mechanism of action, but this is often not understood. We have accordingly adopted a hybrid system grouping toxins which do not require metabolic activation by mechanism of action, but discussing toxins which require metabolic activation as a group.

3.2.1 Acute Hepatotoxicity

In 1979, the US Public Health service issued guidelines for the detection of the hepatotoxicity of drugs and chemicals (Davidson *et al*., 1979). In the guidelines, hepatic lesions were divided into two groups. Type I lesions are 'predictable, dose and time dependent, occurring in most, if not all, subjects exposed to appropriate doses of the causative chemical; the lesions are usually readily reproducible in animals'. Type II lesions are 'unpredictable, dose and time independent, occurring sporadically and often becoming apparent only after monitoring a large number of exposed individuals; the lesions are not usually reproducible in animals'. Fortunately, it is unusual for Type I lesions to affect humans except in serious accidents or deliberate ingestion. However, Type II lesions occur with a large number of pharmaceutical agents. We shall, therefore, deal first with the sporadic hepatotoxicity observed in humans before we consider the effects likely to be observed both in humans and in experimental animals.

3.2.1.1 Sporadic Hepatotoxicity in Humans

Drug-induced liver effects are major impediments to the introduction of new drugs into clinical use and are major causes of drug withdrawal (Antoine *et al*., 2008). Hepatic side effects are observed following treatment with a large number of drugs spanning all therapeutic classes, as well as with other xenobiotics. There would appear to be two distinct lesions. The first, and more common, is intrahepatic cholestasis. This is especially associated with phenothiazines, for example chlorpromazine (van Ommen and Brown, 1955), but is also found in many other classes of drug (see also Section 3.2.10). The presenting symptom is generally jaundice with elevation of serum aminotransferases. The incidence varies markedly between compounds. Liver biopsies show portal-tract inflammation, sometimes with a high eosinophil count, which suggests some involvement of the immune system. The condition reverses rapidly when the compound is withdrawn. Much more serious are compounds, such as halothane, which, very occasionally, induce symptoms resembling those of viral hepatitis. In this case there is massive damage, which may be fatal, and cases have been reported since the early 1960s (Inman and Mushin, 1974). The cause is thought to be a hypersensitivity reaction against a conjugate of the drug with a hepatocellular protein (Neuberger, 1998). Even if the acute lesion is not lethal, chronic damage may ensue, although generally the condition does ameliorate with time.

3.2.1.2 Toxins and Requiring Activation by Specific Enzymes

3.2.1.2.1 Agents Affecting Lipid and Lipoprotein Metabolism

The role of the liver in detoxifying potentially dangerous chemicals requires that hepatocytes have an exceptional capacity to take up xenobiotics. When one remembers the numerous metabolic roles of the liver it is not surprising that it is affected by inhibitors of the basic pathways of metabolism, particularly those affecting lipid

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metabolism. As has been mentioned earlier, the liver plays a central role in fat metabolism, both repackaging dietary lipid from the intestine for use by peripheral tissues and engaging in the synthesis *de novo* of fatty acids from sugars and amino acids. It would seem that these processes are especially sensitive to metabolic changes, because accumulation of large droplets of fat in the liver (steatosis) is amongst the most common signs of acute hepatotoxicity (see review by Postic and Girard, 2008).

It should be noted that fat accumulation in the liver does not always indicate liver damage. Following partial hepatectomy there is a massive importation of fat into the liver, but there is no evidence of hepatocyte misfunction (Bucher and Malt, 1971). In rats fed high-fat diets (Ashworth *et al*., 1961) or made hyperphagic by hormonal manipulation (Meyer and Hartoft, 1960), and in rats treated with the peroxisome proliferators discussed later, fat generally accumulates in numerous small droplets, an exception being the perfluorinated fatty acids, where lipid accumulates in the midzonal area of the lobule.

While the 'metabolic fat' discussed in the previous paragraph accumulates in small droplets and is principally found in the periportal zone, the action of hepatotoxins often results in accumulation of lipid in large droplets and principally in the centrilobular zone. It is clear that there is more than one mechanism for accumulation of fat in the liver but it seems likely that interference in the formation of VLDL particles plays a major role (Zimmerman, 1978). The protein synthesis inhibitor cicloheximide (cycloheximide) induces the development of a fatty liver that is correlated with loss of apoprotein B, which forms the core of the VLDL particle (Mori, 1983; Janero *et al*., 1984; Gibbons *et al*., 2004). In animals treated with ethionine, which decreases hepatic ATP levels, apparently by sequestering adenine (Okazaki *et al*., 1968), the lipoprotein particles are found in the endoplasmic reticulum, but not in the Golgi apparatus (Mori, 1983). Transfer from the endoplasmic reticulum to the Golgi apparatus is known to be exceptionally sensitive to low ATP levels (Farquehar and Palade, 1981). Colchicine interferes even further along the export pathway, causing depolymerization of the microtubule network that guides secretory vesicles from the Golgi apparatus to the plasma membrane (Mori, 1983). It would appear that many other toxins also cause steatosis by interfering with lipoprotein metabolism. Puromycin, like cicloheximide, is an inhibitor of protein synthesis. A similar mechanism was proposed for tetracycline toxicity but later work suggests interference in the assembly of lipoprotein particles (Deboyser *et al*., 1989). Choline deficiency, treatment with orotic acid and administration of phosphorus also inhibit the release of VLDL particles, by mechanisms that are not fully understood.

Inhibition of lipoprotein export is, of course, only one mechanism responsible for accumulation of fat in the liver. Fat accumulation is also caused by necrogenic toxins, such as carbon tetrachloride (see later). Fat accumulation also occurs in animals treated with valproic acid (Olson *et al*., 1987) and, at high doses, with perfluorodecanoic acid and related compounds (Borges *et al*., 1993). In these cases, fat accumulation appears to result from inhibition of fatty-acid oxidation. Fat accumulation also occurs in the human liver following alcohol abuse, but the mechanism remains obscure (Andrews and Snyder, 1991).

3.2.1.2.2 Effects on Lysosomes

Compounds may affect lysosomes in two ways. First, indigestible material may reach lysosomes and accumulate there, causing enlargement and interference in cellular function. Second, compounds may cross the lysosomal membrane and be trapped as a result of the difference in pH between lysosomes and the cytoplasm. The former route is rarely a major problem for toxicologists. Up to 5% of liver lysosomes are discharged into the bile each day (Godfrey *et al*., 1981) so, in general in the liver, materials do not accumulate in lysosomes. However, it would appear that there are circumstances in which fusion of lysosomes with the bile canalicular membrane is prevented and enormously enlarged lysosomes may accumulate in patients with haemochromatosis, Wilson's disease and the various lysosomal disorders (Watts, 1986). It would also appear that spontaneous changes may occur, because in ageing animals, including humans, lipofuscin granules, lysosomal residual bodies containing material with the staining properties of polymerized, peroxidized lipid, accumulate and this process may be accelerated in experimental animals by treatment with 'peroxisome proliferators' (Hinton *et al*., 1986) and with an imidazole antifungal drug (Nishikawa *et al*., 1984). Enlargement and labilization of hepatocyte lysosomes has also been observed in rats treated with the food dye Neutral Red (Abraham *et al*., 1967), although the mechanism of action has not been determined. Accumulation of material in the lysosomes of Kupffer cells may result in reticuloendothelial blockade and even in cell death (see later).

The acidity of the interior of lysosomes is below pH 5, whereas the cytosol is essentially neutral, having a pH of 7.2. As a result, weak bases may be trapped in lysosomes. Taking, as an example, a simple amine with a pK of 8.2: at pH 7.2, 10% of the molecules of this material will be in the nonionized form and therefore capable of dissolving in phospholipid membranes. Such a material may diffuse across the lysosomal membrane but, at the pH of the interior of the lysosome, less than 0.1% of the compound will be in the nonionized form, effectively trapping it within the lysosome. Such weak bases accumulate in lysosomes and, as they concentrate, water enters the lysosomes to maintain an osmotic balance, resulting in a marked distention of these organelles. The type of compound here is monensin (an ionophore antibiotic), which has

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been much used in cell biology experiments, but similar effects will be produced by any compound with a p*K* between 7 and 9.5. Damage by this mechanism requires a high concentration of the compound and, because of the other biological activities of amines, this type of lysosomotrophy is rarely a practical problem, although some such effect may contribute to the antimalarial effects of quinine derivatives (Abraham *et al*., 1967).

3.2.1.2.3 Other Metabolic Inhibitors

Although the principal symptom of the action of many direct hepatotoxins affects fat metabolism, there are numerous exceptions. Galactosamine, for example, is capable of mimicking galactose to the extent that it is converted into uridine diphosphate (UDP)-galactosamine, but the galactosamine cannot be donated to glycoproteins. Thus uridine is effectively sequestered and the resulting depletion of UTP- and uridine triphosphate (UDP)-sugars has major metabolic consequences. Depending on the dose, the result is either acute hepatitis (with poor recovery) or chronic hepatitis and cirrhosis (Medline *et al*., 1970). Other compounds affect different systems. Phalloidin, a fungal toxin found on lupin products, prevents the depolymerization of fungal microfilaments. This results, by mechanisms that remain uncertain, in cholestasis, biliary hyperplasia, centrilobular hepatocellular necrosis and cirrhosis (Read, 1985; Peterson, 1990). The fungal toxin, phomopsin, is directly cytotoxic, but also interferes with cell division, preventing normal liver repair (Kelly, 1985). Another group of compounds, exemplified by griseofulvin, 3,5-diethoxycarbonyl-l,4-dihydrocollidine, inhibit ferrochelatase, resulting in an increase in porphyrin deposits (De Matteis *et al*., 1987; De Matteis and Marks, 1996). Similar deposits are seen in the livers of animals treated with hexachlorobenzene (a fungicide banned globally under the Stockholm Convention on persistent organic pollutants) and the antiarthritic agent 3-[2(2,4,6-trimethylphenyl)thiothyl]-4-methylsydnone (Greaves, 1990; McNamee and Marks, 1996), and in humans with a hereditary deficiency of ferrochelatase (Bloomer, 1988). On the basis of studies in mice, Sinclair *et al*. (2000) concluded that CYP 1A2 was essential in the processes leading to hexachlorobenzene-induced uroporphyria. Alteration in the metabolism of porphyrins is also implicated in the toxicity of metals, including lead and arsenic (see **Haematology and Toxicology** and the next section).

3.2.2 Hepatotoxicity of Metals, Near Metals and Their Compounds

Several metals are toxic to the liver. In some cases this is a reflection of their general toxicity. High doses of a number of transition metals result in liver damage, possibly from redox cycling. The effects of copper are especially notable. Liver damage from excess copper has been reported in experimental animals, domestic animals and in humans (Stokinger, 1981). Sheep are particularly susceptible (Kelly, 1985). Wilson's disease (Sternlieb and Scheinberg, 1985), a hereditary condition in humans probably involving a defect in the transfer of copper from hepatic lysosomes to bile, produces all the symptoms of copper intoxication. Bedlington terriers also show a hereditary disease with symptoms of copper intoxication, which probably involves a defect in copper excretion (Su, 1982).

Other transition elements are capable of causing liver damage. Salts of iron, cobalt and mercury cause liver damage in experimental animals under extreme conditions and, as with copper, hereditary human diseases, notably haemochromatosis, cause symptoms of iron intoxication at normal dietary levels (Powell, 1985). In general, however, the liver is less susceptible to transition-metal toxicity than other tissues, such as the kidneys. This is not because sulphur-binding metals such as copper, zinc, gold, bismuth and mercury do not reach the liver, but because the metals induce binding proteins, called metallothioneins, which sequester the metals resulting in their inactivation and, in the case of the liver, exit from the tissue (Ham, 1986).

Several of the less common metals and semimetals are hepatotoxins. Beryllium may induce granulomatous lesions in the liver, although its main toxic action is directed against the lung (Stokinger, 1981). Arsenic and its compounds induce hepatocellular damage, indicated by fatty change, particularly in the centrilobular zone. Electron microscopy showed vesicularization of the endoplasmic reticulum and intracellular damage indicated by an increase in autophagosomes (Ishinishi *et al*., 1980) and mitochondrial swelling (Fowler *et al*., 1977). Biochemical studies suggest that interference in the assembly of haemoproteins and redox cycling consequent on the release of free haem may contribute to the toxicity (Albores *et al*., 1989). Fatty liver is also induced by antimony administration (Stokinger, 1981).

Lanthanides cause hepatic changes (Arvela, 1979), although this is of limited practical importance as these elements are not well-absorbed by the gut. The lighter members of the series localize to the liver and, in large amounts, induce a fatty liver, whereas the heavier elements show less preference for the liver and cause focal necrosis without fatty change. Electron microscopy shows changes in the nuclei and the endoplasmic reticulum after treatment with the lighter lanthanides, but there is much less indication of systematic morphological changes after administration of the heavier elements in the series. The similarity in ionic radius of calcium and the lanthanides, first pointed out by Lettvin *et al*. (1964), suggests a possible mechanism of toxicity. There are, indeed, effects of lanthanides on other tissues, which can be directly related to calcium antagonism but, as discussed by Arvela (1979), there are many other interactions which may explain the hepatotoxicity. It is of interest, for example, that hepatic damage caused by

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praseodymium is antagonized by silybin, an agent which also antagonizes the RNA-polymerase inhibiting toxins phalloidin and α-amanitin (Tuchweber *et al*., 1976). In addition to these effects some lanthanides affect Kupffer-cell function (see later).

The metalloid selenium is both an essential nutrient (it is a component of the active site of enzymes, *inter alia* glutathione peroxidase and thioredoxin reductase) and a potential toxicant. Excess doses of selenium cause liver atrophy, necrosis and haemorrhages in both experimental (Rouiller, 1964) and farm animals (Hogberg and Alexander, 1986), although liver effects are not particularly prominent in humans (ATSDR, 2009). In general, selenites are more toxic than selenates or selenium salts (see review by Alexander, 2007).

3.2.3 Toxins Requiring Metabolic Activation

In **Biotransformation of Xenobiotics**, Timbrell and Marrs point out that, in a number of cases, metabolism of a xenobiotic increases rather than reduces the toxicity. As the liver is the major site for xenobiotic metabolism, damage by 'active metabolites' plays an important role in chemically induced liver damage: thus even in 1964, Rouiller was able to list over 100 chemicals capable of causing acute or subacute damage to the liver. Since that time, many more chemicals capable of causing liver damage have been identified, but there is no agreement on their classification. The major reason why our understanding of acute hepatotoxicity is so poor, in spite of the efforts which have been made, is the sheer number of the parameters which have to be considered. These factors will include the identity and localization of the enzymes which produce the active metabolite, the nature and stability of the active metabolite, and secondary effects either due to 'message-passing' between liver cells or direct effects on cells other than hepatocytes.

3.2.3.1 Enzymes Involved in the Formation of Active Metabolites

It is a commonplace among toxicologists to say that chemical warfare is nothing new—plants and fungi have been engaged in it for millenia. Accordingly, animals have evolved very elaborate defence systems to detoxify xenobiotics and coevolution of plants and animals has resulted in significant differences in species; for example, the cat family, which are obligate carnivores, are notoriously deficient in some detoxication systems (see **Biotransformation of Xenobiotics**). As has already been mentioned, the highest concentration of xenobiotic-metabolizing enzymes is in the liver. Human liver contains numerous isoforms of cytochrome P450 and many isoforms of glutathione-S-transferase (Vermeulen, 1996). It is noteworthy that the spectrum of cytochromes P450 in human liver differs markedly from the spectrum found in rats. In addition, there may be marked differences between individuals. Thus poor metabolism of certain drugs is associated with polymorphism in isoforms of cytochrome P450 (CYP 1A2, CYP 2C18, CYP 2D6 and CYP 3A5), while it has been suggested that the 40% of the population which are deficient in the μ forms of glutathione-S-transferase may be more susceptible to certain carcinogens and an increased incidence of lung cancer has, indeed, been reported (Vermeulen, 1996). Hence, to summarize, we can state that: (i) xenobiotic-metabolizing enzymes differ both between individuals and between species and (ii) compounds may often be metabolized by more than one route (e.g. testosterone may be hydroxylated at several positions depending on which isoform of cytochrome P450 is involved). Hence variations in the balance of xenobiotic-metabolizing enzymes between species and between individuals may result in differences in the generation of active metabolites and hence in damage to the liver.

Although the majority of 'active metabolites' are formed by the action of isoforms of cytochrome P450, other enzymes may be involved. Probably the most important here are the glutathione-S-transferases and glucuronyl-, sulfo- and acetyltransferases. These enzymes are generally considered as protective, but metabolism of certain halogenated hydrocarbons, such as 1,2-dibromobenzene may result in the formation of the extremely reactive episulfonium ions which may enter the nucleus and bind to DNA (**Figure 15**). It should be noted that, in this case, the normal assumption that cytochromes P450 make toxic metabolites and glutathione-S-transferases destroy them is reversed, as dibromobenzene may also be metabolized by the cytochromes P450, especially CYP 2E1, to the stable bromoacetic acid. The toxicity of dibromobenzene will hence be modulated upwards by inducers of glutathione-S-transferase and downwards by inducers of CYP 2E1. Likewise, formation of glucuronate, sulfate or acetate conjugates is normally a final step in preparing a compound for excretion, but, in the case of certain aromatic amines, the hydroxylamine formed by the action of a cytochrome P450 is stable, but the conjugates are unstable and potent alkylating agents (see **Toxicokinetics of Xenobiotic and Metabolite Distribution and Excretion**). It must be stressed, however, that these are the exceptions that prove the rule; in general, active metabolites are formed by the cytochromes P450 and detoxified by glutathione-S-transferases.

3.2.3.1.1 Differences between Areas of the Liver Lobule

Generally, hepatotoxins have their principal effects on a particular portion of the liver lobule. In the majority of cases, damage is found principally in the centrilobular zone, but the effect of certain toxins, exemplified by allyl alcohol, is on the periportal hepatocytes. There are three reasons for these differences. First, there is the change in oxygen tension across the lobule; second,

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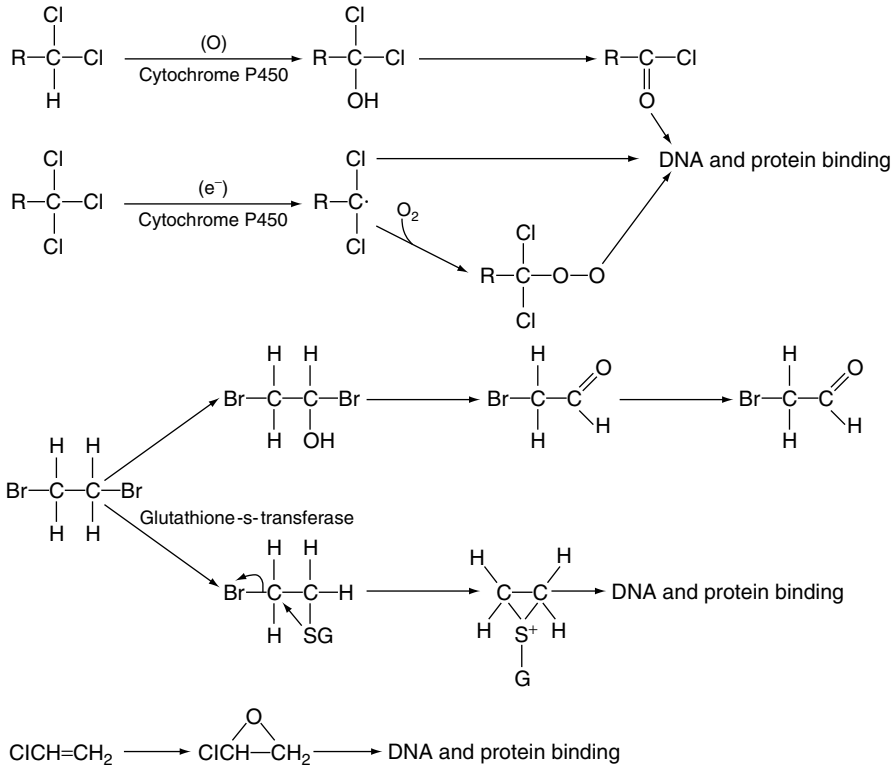

Figure 15 Scheme for the oxidation of haloalkanes and haloalkenes.

the enzymes involved in xenobiotic metabolism are not evenly distributed, for example in the absence of inducing agents most cytochromes P450 are located in the centrilobular zone of the liver; and also, when oxygen tension is low, the cytochromes P450 may catalyse reductive reactions, and these are responsible, for example, for the reduction of azo dyes and for the activation of the cytotoxic agents adriamycin and mitomycin C (Vermeulen, 1996). The factors discussed in the last paragraph mean that the majority of toxins which require metabolic activation, affect the centrilobular hepatocytes more than the periportal cells. However, there are exceptions to this rule. First, metabolism may not involve the cytochromes P450. Thus, for example, allyl alcohol is metabolized by oxidation to acrolein by alcohol dehydrogenase, and this reaction occurs more readily in regions of high oxygen tension leading to periportal toxicity. In other cases the reasons for zonal differences in toxicity are less clear. For example, the pyrrolizidine alkaloid retrorsine causes centrilobular necrosis in rats, mice and guinea pigs, periportal necrosis in hamsters and focal necrosis in chickens and monkeys (White *et al.*, 1973).

In addition to interspecies variation affecting the site of liver damage, alterations may also be seen if pretreatment has resulted in induction of a particular isoenzyme or group of isoenzymes. It is, of course, obvious that induction of an isoform of P450 which generates active metabolites will enhance toxicity, while induction of an alternative path of metabolism is likely to be protective and that the contrary will be the case with inhibitors. However, sometimes pretreatment with a substrate for the isoform responsible for producing the active metabolite may be protective. An example here is that a small dose of carbon tetrachloride protects against a subsequent larger dose, the reason being that the carbon tetrachloride is acting as a 'suicide substrate', the metabolite bonding to, and inactivating, the enzyme. This will reduce the liver's ability to metabolize the subsequent dose, hence less active metabolite will be formed and there will be less damage.

In addition to altering the amount of damage, preinduction may also affect the localization of damage, a phenomenon called 'floating necrosis'. This is exemplified by the action of the sesquiterpenoid, ngaione. This produces midzonal lesions in untreated rats, periportal lesions in rats treated with phenobarbitone and centrilobular lesions in rats pretreated with inhibitors of cytochrome P450 (Kelly, 1985). In a similar way, the effects of cocaine in the uninduced rat are centrilobular, but may be shifted by induction to be midzonal and even to the periportal zone (Powell *et al.*, 1991). The reason is unknown, but two factors appear to contribute to this phenomenon. First, while cytochromes P450 in uninduced rats are concentrated in the centrilobular zone, after induction, the distribution becomes more even and there may even be accumulation in the periportal area. Second, the fact that necrosis is seen in a band rather than across the lobule suggests that, in these cases, optimum

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formation of the toxic metabolite occurs preferentially in an oxygenated zone, provided that the concentration of the appropriate cytochrome P450 is sufficiently high.

3.2.3.1.2 The Nature of the Active Metabolite

The active metabolites formed in the liver will vary in their reactivity and this will, in turn, determine the type of damage which occurs. This is true even where the active metabolite is a free radical. It is frequently assumed that these are the 'mad axemen' of the intracellular world, but in fact the free radical most commonly formed in the body, the superoxide radical, is too weak to cause lipid peroxidation (it can, however, be transformed to the hydroxyl radical, which most certainly can). Inversely correlated with the reactivity of the active metabolite will be its half-life. For example, 1-aminobenzotriazole is metabolized by a range of cytochromes P450 and produces a metabolite which reacts with the P450 molecule itself, inactivating the enzyme, and this phenomenon extends to organs other than the liver (Knickle and Bend, 1992). There is, however, practically no damage to the liver, probably because there is no significant release from the enzyme. In the case of carbon tetrachloride, the free radical produced by its metabolism does bind to and destroy the cytochromes P450 to some extent. However, it is also released from the enzyme, causing severe damage to the interior of the cell. The metabolite is, however, too short lived to enter the nucleus in significant amounts and hence there is no genotoxicity (see review by Weber *et al*., 2003). Mutagenic carcinogens such as dimethylnitrosamine, on the other hand, both cause cytoplasmic damage and enter the nucleus, binding to the DNA. Finally, the active metabolites produced from the pyrrolizidone alkaloids are so long lived that they not only damage the vasculature of the liver, but also pass to the lungs, causing damage there (WHO, 1988). Hence the damage which an active metabolite causes will be determined both by its reactivity, in particular whether it is potent enough to cause lipid peroxidation or bind to DNA, and also by its half-life in the environment of the cell interior

3.2.3.1.3 Influence of Nonhepatocytes

It has long been known that the body's response to tissue damage involves, not only the damaged cells themselves, but also other cell types, but details of the message-passing system operating in the liver remain obscure. The best characterized example of cooperation is between Kupffer cells and hepatocytes. Here it has been shown that interference in Kupffer cells by administration of gadolinium chloride reduces the liver damage caused by carbon tetrachloride (Edwards *et al*., 1993), paracetamol (acetaminophen) (Laskin, 1989), cadmium (Kayama *et al*., 1995) and allyl alcohol (Przybocki *et al*., 1992), while activation of Kupffer cells enhances the toxicity of carbon tetrachloride (Sipes *et al*., 1989). The mechanism by which the Kupffer cells influence hepatocytes is not known. Kupffer cells are known to produce TNF-α and TGF-β, both of which are capable of inducing apoptosis in hepatocytes and, given the liver's powers of regeneration and the risk of damage to DNA in severe cell injury, it would seem logical that damaged cells should be removed and, indeed, an increase in apoptotic cells has been reported in damaged livers (Gopinath *et al*., 1987). However, El-Hassan *et al*. (2003) have shown that, while inhibitors of caspases (proteases involved in apoptosis) inhibit the later stages of paracetamol toxicity, the relationship between hepatocyte damage and apoptosis is not simple. Marked cell–cell interactions are observed first in liver enlargement, where HGF, one of the major hepatocyte mitogens, is secreted by stellate cells, while fibrosis in the liver is controlled by a message-passing system involving hepatic stellate cells and Kupffer cells. It is also clear that sinusoid endothelial cells are also involved in message passing, but the nature and toxicological implications of this remain obscure.

3.2.4 Chronic Damage to the Liver

3.2.4.1 Fibrosis Including Cirrhosis

Repair of the liver parenchyma normally occurs by regeneration. However, repeated injury, for example by alcohol abuse in humans or repeated carbon tetrachloride administration in experimental animals, may eventually cause fibrotic changes in the parenchyma. In addition, repair by fibrosis may be observed in the small spontaneous granulomatous lesions observed in laboratory rats and in similar lesions induced by highly immunogenic materials, such as bacterial cell walls or pig intestinal alkaline phosphatase (PAP) (Dijkhuis *et al*., 1989). There is strong immunohistochemical evidence that the fibroblast-like cells in small PAP-induced granulomas are derived from stellate cells (Dijkhuis *et al*., 1989). Fat stellate cells synthesize collagen and proliferate when stimulated by factors derived from Kupffer cells (Zerbe and Gresner, 1988) and it is extremely likely that these cells will be stimulated in viral infections or by foreign carbohydrates or proteins. Repair by fibrosis may also be observed following administration of single doses of certain compounds, notably the pyrrolizidine alkaloids (WHO, 1988), which cause livestock poisoning worldwide, one plant responsible being common or tansy ragwort (*Senecio jacobaea*). Comfrey (*Symphytum officinale* L.) is a source of exposure in man. The reason why pyrrolizidine alkaloids induce fibrosis is unknown, but, as discussed earlier, the acute effect of these compounds is a haemorrhagic necrosis associated with damage to branches of the hepatic vein, changes frequently described in farm animals, including horses, and in humans (e.g. Dimande *et al*., 2007) (see also Section 3.2.3.1.1 and COT, 2008). Damage associated with blood clots is normally repaired by fibrosis in extrahepatic tissues and it is possible that this is also

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the case in the liver; it has also been suggested that the fibrosis is related to the biotransformation of alkaloids by hepatic microsomal enzymes (Stickel and Seitz, 2000).

It is also not clear why repeated insults to the liver by carbon tetrachloride, ethanol and other compounds result in repair by fibrosis. It could be that fibrosis requires that the repeat insult should occur before the earlier damage is fully repaired. In the early stages of injury to hepatocytes, fibrosis is observed together with an increase in reticulin fibres in the space of Disse. This in turn is associated with metaplasia of the hepatic stellate cells to a myofibroblast morphology.

There would thus appear to be two factors which may contribute to the development of further fibrotic change on continued insult. First, it appears that the sparse reticulin network in the space of Disse plays the role of a basement membrane in guiding liver regeneration and changes to these reticulin fibres associated with collapse of the parenchyma may prevent repair by regeneration. Second, as proliferation of all cells depends on secretion of growth factors from neighbouring cells, changes in the secretion of these factors or in the sensitivity of the target cells may change the balance between regeneration of hepatocytes, and proliferation and transformation of fat-storing cells. Thus, for example, the transformation of stellate cells to myofibroblasts is controlled by, amongst other things, factors secreted by Kupffer cells and hepatocytes, while the myofibroblasts secrete TGF-β, a negative growth factor for hepatocytes (Hautekeete and Geerts, 1997; Bissell, 1998). This is controlled by the bone-morphogenetic protein (BMP)-7, an important antagonist of the actions TGF-β (Gressner *et al*., 2008). It is important to stress the word balance; repair by regeneration continues in parallel with development of fibrosis and plays a critical role in the development of the cirrhosis.

The processes which lead from fibrosis to cirrhosis are much better understood (McGee *et al*., 1992; Bataller and Brenner, 2005; Friedman, 2008). With continued bouts of cell death and fibrous repair, the fibrous septa extend and form links between adjacent central veins, and between portal tracts and central veins. Capillaries develop in these tracts and enlarge to form veins, allowing blood to flow directly from the portal tract to the central vein, thereby bypassing the parenchyma. This results in further cell death followed by further fibrous repair. The condition is self-sustaining and the liver is reorganized into nodules of cells separated by connective tissue septa. The circulation in the nodules is highly disturbed so that foci of cell loss alternate with areas of regeneration. Overall, however, there is a progressive loss of liver substance leading finally to hepatic failure and death.

3.2.5 Hepatocarcinogenesis

Primary liver cancer of humans is not particularly common in North America or Europe, but it is much more common in east and southeast Asia and sub-Saharan Africa, amongst the aetiological factors being alcohol and aflatoxins (Kew, 2002; Gomaa *et al*., 2008). Hepatocellular adenomas and carcinomas are frequently observed in experimental animal studies. These tumours may be induced both by genotoxic and nongenotoxic agents. Furthermore, any treatment which causes chronic hepatocellular damage or which results in a persistent increase in the size of the liver is likely to promote the action of a genotoxic carcinogen and, in fact, many of these regimens will actually increase the incidence of liver cancer on their own. In the past there has been much emphasis on determining whether particular chemicals are genotoxic. Protocols using a combination of several promoters, such as that described by Moslen *et al*. (1985), have been devised to increase the sensitivity of assays for liver carcinogenesis. Emphasis has also been placed on the role of promoters (Ames *et al*., 1987; Cohen and Ellwein, 1991).

The stages in the development of liver cancer appear to be the same in spontaneous tumours and in tumours induced by genotoxic and nongenotoxic carcinogens. The first detectable changes are the development of foci of altered cells (Pitot, 1990). These are small groups of cells, sometimes visible using conventional staining techniques, but generally only detectable by histochemical techniques. The early foci are extremely heterogeneous in their enzyme composition. These early foci do not appear committed to tumour development and, under some experimental conditions (see later), may regress completely. It is nevertheless thought that some foci of altered cells do progress to the second stage of liver carcinogenesis, the development of distinct hyperplastic foci that compress the surrounding tissue. Small hyperplastic foci can, again, reverse completely, but larger foci develop into adenomas and, in some cases, carcinomas. The morphology of liver carcinomas is very varied, but tumours induced by nongenotoxic agents generally remain well differentiated, even in metastases, while those induced by the more potent genotoxins may be very anaplastic.

The incidence of cancer in the liver, as in many other tissues, is influenced by many factors (see above). Conventionally, discussion is divided between considerations of initiators, that is compounds where a single treatment is sufficient to start the processes which lead to cancer, and promoters, compounds not carcinogenic on their own, but which increase the incidence of cancer following treatment with an initiator. This is simplistic when applied to the liver because the classic hepatic 'promoters' are almost all carcinogens in their own right when administered to mice over a lifetime. We consider the compounds under two major headings: compounds which appear to act as direct mutagens and 'nongenotoxic' carcinogens, which in turn are split into compounds which cause chronic liver damage and compounds which cause persistent liver enlargement. It should be noted,

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however, that both classes of nongenotoxic hepatocarcinogen strongly promote the action of genotoxic agents and theoretical considerations suggest that the enlarged or damaged liver which results from the action of nongenotoxic hepatocarcinogens may be especially susceptible to the action of genotoxic agents.

3.2.5.1 Mutagenic Hepatocarcinogens

Examples of aromatic amines which increase the incidence of cancer in humans and experimental animals are given in **Table 5**. The majority of the compounds contain two aromatic rings; aromatic amines with a single ring either do not increase cancer incidence or are very weak carcinogens. It is also noted that there is a marked interspecies variation in the target organ, with liver cancer generally being found only in mice. The mechanism of action (King *et al*., 1988) appears to involve N-oxidation by cytochromes CYP 1Al and CYP 1A2 to form the corresponding hydroxylamine. Once formed, these can yield the reactive nitrenium ion at an acidic pH or by oxidation (**Figure 16**). Alternatively, there may be further metabolism to form the *N*-hydroxy sulfate or *N*-hydroxy acetyl derivatives. Spontaneous decomposition of these again results in the formation of nitrenium ions, and of DNA and protein adducts. Acetylation or sulfation appears an absolute requirement in the case of N-acetylated or N-arylated amines. It is likely that the greater potency of the polycyclic aromatic amines, and their derivatives, as carcinogens results from their ability to facilitate the formation of nitrenium ions by derealization of its excess electrons. It should be noted that the cytochromes P450 which oxidize aromatic amines have marked structural specificity (Williams and Weisburger, 1991). For example, 1- and 3-acetylaminofluorene are not carcinogenic, unlike 2-acetylaminofluorene. The three corresponding hydroxylamines are equally carcinogenic. The difference arises because the liver is unable to metabolize the 1- and 3-isomers. As there are marked interindividual variations in the human population in the ability to N-hydroxylate arylamines, it would seem likely that there will be equally marked differences in sensitivity to these compounds.

Nitro analogues of carcinogenic aromatic amines also lead to tumour formation (Williams and Weisburger, 1991). These compounds may be reduced by rather weakly active mammalian enzymes and by gut bacteria to hydroxylamines, which can then be further metabolized as described in the previous paragraph. As the nitro reductases are much less specific than the cytochromes P450 which oxidize aromatic amines, it appears possible that these compounds may be more widely toxic. In practice, this does not seem to be the case. Whereas 1-hydroxyaminonaphthalene is carcinogenic, 1-nitronaphthalene is not, presumably because the rate of reduction of the nitro group to the carcinogenic hydroxylamine is much lower than the rate of reduction of the hydroxylamine to an amino group. 1-Naphthylamine is not oxidized by CYP 1Al and so there can be no re-formation of the hydroxylamine, whose steady-state concentration is therefore extremely low. However, the effects of equivalent nitroaryl and aminoaryl compounds may sometimes be very different: 2,4-diaminotoluene is a potent carcinogen, whereas 2,6-diaminotoluene is consistently negative (Cunningham *et al*., 1991). 2,6-Dinitrotoluene is, however, a much more potent carcinogen than 2,4-dinitrotoluene (King *et al*., 1988). As discussed earlier, part of the explanation may lie in the fact that 2,4-diaminotoluene is a potent hepatotoxin

Table 5 Organs affected by carcinogenic aromatic amines in different species

	Mouse	Rat	Dog	Human
Benzidine	Liver	Mammae	Bladder	Bladder
	Zymbal gland	—	—	—
4-Aminobiphenyl	Liver	Mammae	Bladder	Bladder
	Bladder	Intestine	—	—
	Endothelia	—	—	—
2-Naphthylamine	Liver	?Bladder	Bladder	Bladder
3,3′-Dichlorobenzidine	Liver	Mammae	Bladder	—
	—	Zymbal gland	Liver	—
	—	Leukaemia	—	—
4,4′-Thiodianiline	Liver	Liver	—	—
	Thyroid	Thyroid	—	—
	—	Ear canal	—	—
	—	Uterus	—	—
o-Anisidine	Bladder	Bladder	—	—
4-Chloro-*o*-phenylenediamine	Liver	Bladder	—	—
p-Cresidine	Bladder	Bladder	—	—
	Liver	Liver	—	—
	Nasal cavity	Olfactory neuroblastoma	—	—

IARC (1982; 1987).

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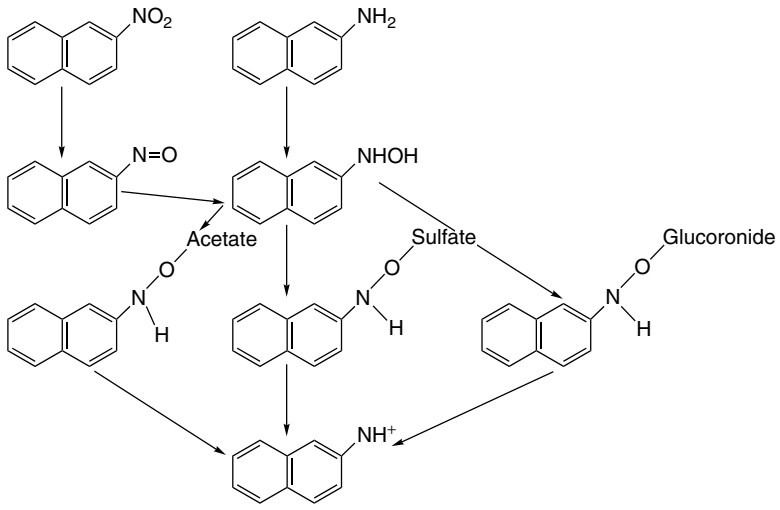

Figure 16 Scheme for the metabolism of aromatic amines.

and hence increases cancer incidence by a nongenotoxic mechanism. All in all, the varying toxicities of aromatic amines and nitro compounds show the difficulty of predicting toxicity from chemical structures, even when the metabolic pathways linking the compounds are well understood.

Aromatic azo compounds can be divided into two distinct groups (Williams and Weisburger, 1991). In the first group are simple compounds consisting of two aromatic groups joined together by an azo bond. These compounds include hepatocarcinogens such as dimethylaminoazobenzene (Butter Yellow) (IARC, 1975a). Carcinogens in this group have an amino group attached to one of the aromatic rings and are metabolized in exactly the same way as the amines described above. The azo group bridging the two rings allows efficient delocalization of the electrons in the nitrenium ion. The carcinogenicity of these compounds is influenced by two factors. First, the azo bond may be reduced, yielding noncarcinogenic or very weakly carcinogenic monocyclic aromatic amines. Second, compounds containing polar constituents such as sulfonic acid residues, are usually not carcinogenic.

Complex azo dyes consist of polycyclic structures generally linked by more than one azo group. With the simple azo dyes, the reductive splitting of the azo group is a detoxification reaction and simple monocyclic amines are released, but with the complex materials, the same reaction releases polycyclic aromatic amines which, as seen earlier, may be potent carcinogens affecting both the liver and other tissues.

The *N*-nitroso compounds of interest to toxicologists are derivatives of secondary amines, amides or ureas; *N*-nitroso derivatives of primary amines are not stable. They are readily formed in the digestive tract by the reaction of a secondary amine with nitrite, which is formed in the body from nitric oxide. *N*-Nitroso compounds are almost all potent carcinogens. The target organ, however, differs between species and between compounds. Nitroso derivatives of alkylureas, alkyl amides and esters are exceedingly potent carcinogens, but normally the liver is not the major target organ. These unstable compounds decompose spontaneously, yielding alkylating agents, on contact with aqueous solutions, especially of a neutral or alkaline pH. *N*-Nitroso derivatives of linear secondary amines such as dimethylnitrosamine or of cyclic amines such as morpholine require metabolism and are thus much more likely to affect the liver. Such *N*-nitrosamines are activated by hydroxylation on a carbon adjacent to the nitrogen (**Figure 17**). This results in cleavage of the carbon–nitrogen bond yielding first a diazonium and then a carbonium ion, both of which are potent alkylating agents. Although such metabolism is theoretically possible for all *N*-nitrosamines in the livers of all species, there is, as already mentioned, a marked variation in sensitivity. Discussion of the reasons for these differences may be found in monographs by Bartsch *et al*. (1987) and O'Neill *et al*. (1991).

Metabolism of dialkylhydrazines can yield products identical to those produced by *N*-nitrosamines (**Figure 17**). As substituted hydrazines are more uncommon than secondary amines both in nature and in industry, much less is known about their toxicity. However, there are indications that there is likely to be the same variation in target organ as with the *N*-nitrosamines (Williams and Weisburger, 1991). Probably the most important compound in this class is cycasin, a compound found in cycad flour. Cycasin is the glucoside of methylazoxymethanol: the glucose is removed by bacterial hydrolysis in the gut, releasing methylazooxymethane, a powerful carcinogen affecting the liver, kidney and intestinal tract (Williams and Weisburger, 1991).

It is generally assumed that toxins are, in some way, unnatural compounds. This is certainly not the case. There are few direct benefits to plants or fungi

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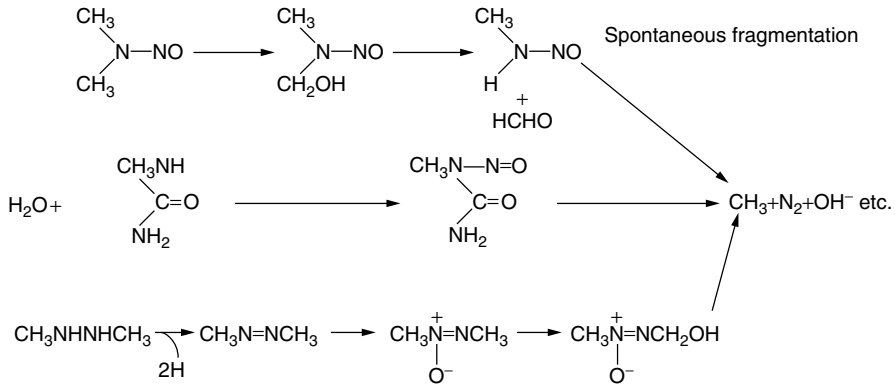

Figure 17 Scheme for the metabolism of *N*-nitrosamines and dialkylhydrazines.

in being eaten, with the result that both groups have developed chemical deterrents. Many of these compounds are very potent hepatotoxins (**Table 6**). These materials are commonly complex polycyclic hydrocarbons with a wide variety of substituents and there are few common features apart from their ability to be metabolized to electrophiles. The mechanisms by which they cause liver damage and liver cancer are the same as the synthetic chemicals discussed earlier. The more potent hepatotoxins were considered in some detail by Kelly (1985) and structure–activity relationships in general were discussed by Ashby and Tennant (1988).

3.2.5.2 Nonmutagenic Hepatocarcinogens

A considerable number of the chemicals which increase the incidence of tumours in the liver give no evidence for mutagenicity in either prokaryotic (Ashby and Tennant, 1988; Ashby *et al*., 1989) or eukaryotic (Williams *et al*., 1989) tests. The mechanism by which they increase cancer incidence is not known. On initial examination most fall into four distinct groups, namely agents which cause chronic damage to the liver, agents which cause proliferation of peroxisomes, agents which cause proliferation of smooth endoplasmic reticulum, and possibly agents which cause proliferation of mitochondria. However, as discussed at the end of this section, it is possible that, with three of the groups, the underlying superficial dissimilarities belie an underlying connection: persistent enlargement of the liver (Grasso and Hinton, 1991). The first group, however, is mechanistically distinct.

3.2.5.2.1 Agents Causing Chronic Liver Damage

There is a close association between chronic tissue damage and the induction of cancer (Butterworth *et al*., 1991; Grasso *et al*., 1991). Eschenbrenner and Miller (1945) showed that chloroform increased liver cancer at doses which were hepatotoxic, but not at lower doses.

Table 6 Natural products that show marked hepatotoxicity

Chemical	Chemical class	Source species	Action
Methylazoxymethanol	Aglycone of glycoside	Cycadales (plants)	Alkylating agents
Ngaione	Furanosesquiterpenoid	Myoporaceae (plants)	Zonal necrosis
Trematoxin	Glycoside	Ulmaceae (plants)	Periportal necrosis
Aflatoxins	Bisfuranocoumarins	*Aspergillus* (fungi)	Alkylating agents
Phomopsin	Not characterized	Moulds on *Lupinus* spp.	Mitotic arrest
Sporidesmin	Not characterized	Moulds on dead ryegrass	Cholestasis
'Many compounds'	Pyrrolizidine alkaloids	Many plants	Alkylating agents Cirrhosis Endothelial damage[a]
Lantadenes (identical to Icterogenin)	Triterpenes	*Lantana camera* *Lippia rehmanni*	Cell enlargement Cholestasis

These plant and fungal products have been responsible for widespread loss of cattle and, especially, sheep. The list is intended to demonstrate the range of sources and effects on the liver and is in no way exhaustive. In particular it should be noted that where soil contains abnormal amounts of trace components such as selenium there may be marked concentrations in growing plants and resultant toxicity.
[a]Petasitenine also induces endothelial cell tumours.
Kelly (1985).

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More recently, the link between cell proliferation and carcinogenesis has been emphasized by Klaunig *et al*. (1986), who demonstrated that rats treated with chloroform by gavage or in the drinking water showed the same very small amount of DNA binding, but liver damage only developed in rats treated by gavage and it was only these animals which developed tumours. In an earlier paper (Grasso and Hinton, 1991), references were cited showing an association between liver damage and tumour induction in animals treated with carbon tetrachloride, tetrachloroethylene, 1,1,2,2-tetrachlorethane or paracetamol (acetaminophen), all agents known to be nonmutagenic. Ethionine should probably be added to this group (Farber, 1963). Selenium is another possible member of this class of carcinogen (IARC, 1975b) (see review by Rayman, 2005). At nontoxic doses, selenium has anticarcinogenic properties (Combs and Clark, 1997; Yu *et al*., 1997; Burns *et al*., 2008), but, as noted earlier, selenium is markedly hepatotoxic at high doses and at these doses tumours have been observed (Fishbein, 1987).

3.2.5.2.2 Compounds Causing Mitochondrial Proliferation

Methapyriline and certain close analogues (Reznik-Schuller and Lijinsky, 1981) cause marked proliferation of mitochondria, liver enlargement in short-term studies and cancer in the longer term. The effects seem to be specific to rats (see Copple *et al*., 1992). It is of interest that other close analogues of methapyriline cause peroxisome proliferation (Reznik-Schuller and Lijinsky, 1982). Although methapyriline is negative in most *in vivo* and *in vitro* mutagenicity tests, there is binding to cell proteins and suppression of mitochondrial protein and DNA synthesis (Lijinsky and Muschik, 1982; Copple *et al*., 1992). It has been shown that there is a sustained increase in the rate of cell division (Cunningham *et al*., 1995), suggesting that these compounds should be classified as inducers causing sustained damage to the liver.

3.2.5.2.3 Compounds Causing Peroxisome Proliferation

Proliferation of peroxisomes in the liver of rats and mice treated with a number of lipid-lowering drugs was noted in the 1960s, but it was only in the early 1980s, when a connection with increased cancer incidence was noted (Cohen and Grasso, 1981; Reddy and Lalwani, 1984), that much interest was taken in these compounds. Since then compounds in this group have been extensively studied. The results have been extensively surveyed by the European Chemical Industry Ecology and Toxicology Centre (ECETOC, 1992) and in books edited by Gibson and Lake (1993) and Reddy *et al*. (1996). The phenomenon of induction of peroxisome proliferation extends to compounds other than antilipidaemic drugs, including phthalate esters of branched-chain alcohols, typified by di-(2-ethylhexyl)phthalate (DEHP) (Howarth *et al*., 2001), and the herbicide dicamba (Espandiari *et al*., 1995).

Although there are marked variations in potency between different peroxisome proliferators, the development of hepatic changes are remarkably uniform. There are major species differences in the response to peroxisome proliferators (Gonzalez and Shah, 2008). Thus marked peroxisome proliferation is only found in old-world rodents (family Muridae). There are marked differences even within this family, with rats and mice responding much more vigorously than hamsters. It is unlikely that other species are totally nonresponsive. The most potent peroxisome proliferators are reported to have some effect on rhesus monkeys (Lalwani *et al*., 1985), but xenobiotic-induced peroxisome proliferation is not generally seen in humans; however, marginal effects have been reported for some fibrate hypolipidaemic agents such as clofibrate and cyprofibrate (Hinton and Grasso, 2000). Humans express much less PPAR-α, which is required for peroxisome proliferation, than mice (see reviews by Green, 1995; Lake, 1995; Klaunig *et al*., 2003). It is reasonable, however, to conclude that peroxisome proliferation is probably metabolically important to rats and mice, but is vestigial outside this family of animals. Neither animal studies nor exceedingly comprehensive epidemiological surveys (Committee of Principal Investigators, 1978) in human populations have given any evidence that these materials increase cancer incidence in species where peroxisome proliferation is not observed and it is generally believed that any marked peroxisome proliferation is predictive of a carcinogenic potential in rodents, but that this does not automatically indicate a hazard to humans.

Several studies have been carried out on the development of hepatic changes in rats treated with peroxisome proliferators (Mitchell *et al*., 1985; Price *et al*., 1986; ECETOC, 1992). These changes appear to be mediated by binding of the compounds to specific nuclear receptor(s) (Green, 1993; Reddy and Chu, 1996; Espandiari *et al*., 1998) (see also **Nongenotoxic or Epigenetic Carcinogenesis**). The earliest change is an accumulation of small lipid droplets similar to those found in hyperphagia which are possibly associated with alterations in fatty-acid metabolism (Mitchell *et al*., 1986). Messenger RNAs for a specific cytochrome P450, CYP 4A1, and for the peroxisomal proteins (Milton *et al*., 1990) accumulate within a few hours of treatment. Consequent on these changes are a burst of cell division, which occurs two to three days after first administration of diets, and the accumulation of specialized peroxisomes lacking a uricase-rich core, smooth endoplasmic reticulum and a specific, cytosolic, epoxide hydrolase (Meijer and de Pierre, 1987). Peroxisome proliferators are further discussed in **Toxicity of Peroxisome Proliferators**.

In Section 2.5, the distinction was made between adaptive and toxic changes. It is likely that the changes mentioned above are essentially adaptive, permitting, for example, oxidation of peroxidized fatty acids. Treatment

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for one month or more results in changes clearly indicative of toxicity, namely the accumulation of enlarged lysosomes filled with material with the staining properties of lipofuscin and, at least in some cases, an increase in cell turnover (Price *et al*., 1986; Cattley *et al*., 1987). Dose–response experiments suggest that with each compound there is a distinct threshold for these latter effects, suggesting they represent the result of some form of metabolic overload. More prolonged treatment results in the development of foci of altered cells. These foci differ from those formed spontaneously or in response to microsomal inducers such as phenobarbital in that they lack γ-glutamyl transpeptidase and foetal-type glutathione-S-transferase activity (Rao *et al*., 1982; Sato, 1989). These foci and at least some of the hyperplastic nodules which develop on more prolonged treatment reverse on withdrawal of the compound (Mompon *et al*., 1987), but if treatment is continued, well differentiated hepatocellular carcinomas appear, which occasionally show metastases, particularly to the lungs. The mechanism of carcinogenesis is not fully understood, but is generally believed to be associated with liver enlargement rather than oxidative stress (Belury *et al*., 1998; also see below).

3.2.5.2.4 Compounds Inducing Microsomal Oxidases

Whereas compounds causing proliferation of peroxisomes may be treated as a single group, this is not the case for compounds which induce microsomal enzymes. Furthermore, there has been considerable controversy about the mechanism of carcinogenesis by certain compounds, such as the food dye safrole, which are potent inducers of microsomal oxidases, but are also weakly mutagenic in certain *in vitro* tests. In the following discussion we will attempt to group the compounds, but the classification is very tentative.

First, it should be noted that phenobarbital (IARC, 1987) and other compounds, such as BHT (Inai *et al*., 1988), which induce cytochrome CYP 2B isoforms almost invariably increase the incidence of liver cancer in mice when administered at high doses. These compounds all cause massive liver enlargement, are potent promoters of the action of genotoxic carcinogens, and give negative results in both *in vivo* and *in vitro* mutagenicity tests. In general, these compounds do not increase cancer incidence in species other than mice, although Rossi *et al*. (1977) did report an increase in rats following three years administration of phenobarbital. It is generally believed that with this group of compounds an increase in liver tumours in mice alone does not indicate any risk to humans. This attitude is supported by studies showing high spontaneous rates of initiation of proto-oncogenes in the livers of strains of mice susceptible to liver cancer (Anderson *et al*., 1992).

At first sight, the polyhalogenated aromatic and alicyclic hydrocarbons would appear to form a second, group but, in fact, these compounds fall into two or more distinct subclasses. The first group is formed by TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) and the planar polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs) and related compounds. These induce a group of enzymes, controlled by the Ah locus, which induce cytochrome CYP 1Al and 1A2 (Nebert and Gonzalez, 1987). These compounds increase the fat in the liver and cause alterations in the RER, changes which are not observed with the nonplanar PCBs, which are phenobarbital-type inducers (Kohli *et al*., 1979). TCDD, PCBs and PBBs all increase the incidence of liver cancer (IARC, 1987). It is of interest that another group of inducers which act on the Ah locus, the polycyclic aromatic hydrocarbons, neither affect liver size nor cause any increase in liver tumours, although they are potent carcinogens.

The second group of halogenated hydrocarbons which increase the incidence of liver tumours are heavily substituted alicyclic compounds. The following have been assessed as hepatocarcinogens (IARC, 1979a; IARC, 1987): 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 2,4,6-trichlorophenoxyacetic acid (2,4,6-T) (obsolete herbicides), aldrin/dieldrin, mirex, photomirex, chlordecone hydrate, lindane, chlordane and toxaphene (organochlorine insecticides). These compounds induce a poorly characterized spectrum of cytochrome P450s. They also produce massive liver enlargement and it is of interest that, whereas α-,β-,γ- and δ-hexachlorocyclohexane were all effective inducers of cytochromes P450, only the γ-isomer (lindane) was a hepatocarcinogen in mice (FAO/WHO, 2003), and that it is by far the most potent in causing liver enlargement (Grasso and Hinton, 1991). There is generally little liver damage apart from some accumulation of fat, although in mice treated with dieldrin, Mallory bodies accumulate in the cytoplasm (Meierhenry *et al*., 1981).

With two hepatocarcinogens, the food dyes Ponceau MX (Grasso and Gray, 1977) and safrole (Crampton *et al*., 1977), the increase in microsomal enzymes reverses on prolonged administration of the compound. Electron microscopic examination, however, shows continued proliferation of the endoplasmic reticulum (ER) and enlargement of lysosomes. Such 'hyperplastic, hypoactive' ER has also been observed in rats treated with dieldrin, but not in mice treated with the same material (Tennekes *et al*., 1979) and it is likely that the carcinogenicity of Ponceau MX and safrole is attributable to other factors (see IARC, 1976 for a review of the carcinogenicity of safrole).

There is no evidence for a direct mechanistic relationship between induction of microsomal oxidases and development of cancer. A common feature to all the compounds discussed in this section is continued liver enlargement with little liver damage. This is also observed with other groups of nongenotoxic hepatocarcinogens and will be discussed later. It should also

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be noted that most of the compounds discussed in this section are only weak carcinogens when administered on their own, but all are potent promoters of the action of genotoxic carcinogens.

3.2.5.2.5 Hormones

Certain types of combined oral contraceptive appear to increase very slightly, but significantly, the risk of cancer incidence in human patients (IARC, 1987). The tumours produced are typical of those induced by nonmutagenic hepatocarcinogens in experimental animals being normally benign, well differentiated and, in some cases, they appear to regress on cessation of treatment (Klatskin, 1977). In animal trials, increases in liver tumours have been observed with ethinyloestradiol, the progestins, ethynodiol diacetate, norethisterone and norethynodrel, as well as various oestrogen–progestin combinations (IARC, 1979b), but there is no evidence for mutagenicity. Several oestrogens are capable of promoting the action of mutagenic carcinogens (IARC 1979b). The best-characterized hormone which induces liver cancer is the synthetic progestin cyproterone acetate. This increases the incidence of liver tumours both in mice and in rats (Tucker *et al*., 1996). The actions of cyproterone acetate have been studied extensively by Schulte-Hermann and his colleagues (see Grasl-Kraupp *et al*., 1998). This agent is a potent inducer of the CYP 3A subfamily of cytochromes and causes marked liver enlargement. There is some evidence that human liver cells have the capacity to activate cyproterone acetate to genotoxic intermediates (Kasper, 2001; Brambilla and Martelli, 2002).

3.2.5.2.6 Other Compounds or Treatments

The incidence of liver cancer, like that of other tumours, in rodents is affected by diet. Increased incidences have been found in rats induced to eat a hypercaloric diet, whereas the incidence of tumours falls when food intake is restricted (Cheney *et al*., 1983; Pollard and Luckert, 1989). The incidence of liver tumours is also increased in rats receiving a choline-deficient diet (Mikol *et al*., 1983). The increase is not caused by the associated liver damage, as an increase in hepatic tumours is also found in rats following transient choline deficiency in early life (Ghosal *et al*., 1987). Choline deficiency results in hypomethylation of DNA (Wainfen *et al*., 1989). DNA methylation is important for maintaining the accuracy of postsynthetic repair and cell differentiation, and it is thought that DNA hypomethylation, in itself, increases the risk of cancer (Rogers, 1995).

3.2.5.3 Liver Enlargement and Liver Remodelling by Apoptosis and Carcinogenesis

Although it was believed in the early 1980s that all carcinogens acted directly on the cell's genome, by the end of that decade it had become generally accepted that there were carcinogens which did not cause mutations in short-term prokaryotic or eukaryotic test systems, and that these fell into two major groups: agents which caused persistent tissue damage and those which produced tissue enlargement without causing a sustained increase in the rate of cell division (Grasso *et al*., 1991; Hildebrand *et al*., 1991). Into the latter group fell, not only xenobiotics, but also a considerable number of hormones, for example thyroid adenomas are found when there is sustained elevation of thyroid-stimulating hormone (TSH), pancreatic acinar cell cancer when there is elevation of cholecystokinin, and carcinoid tumours when there is persistent elevation of gastrin. The response of the liver to inducers of microsomal and peroxisomal enzymes resembles the responses induced by the trophic hormones. There is a burst of cell division which is complete by about three days of the initiation of treatment. Enlargement of the postmitotic cells results in the tissue weight continuing to rise for a few more days, but then tissue weight stabilizes and the half-life of the cells returns to its normal value.

In normal circumstances, the liver weight is carefully controlled and, if it falls, due to toxic damage or to surgical removal of some of the liver, it rapidly returns to its original value. It is thus clear that the body is able to measure accurately the liver weight (or more precisely the liver: body-weight ratio) and induce mitosis or apoptosis to make any suitable adjustments. It was thus felt that the compounds which cause liver enlargement worked by affecting this control mechanism so that, in the presence of the compound, liver weight was controlled at a higher value than normal, but when the compound was withdrawn, the control point reverted to its normal value. Unfortunately, more recent studies suggest that this simple and elegant hypothesis is incorrect.

The control of liver regeneration following partial hepatectomy has been reviewed by Michalopoulos (2007). Repair of the liver following partial hepatectomy follows a similar time course to chemically induced liver enlargement. There is a rapid shift of hepatocytes from G_0 to G_1, DNA synthesis in hepatocytes peaks at around 24 hours and mitosis at around two days. Although the signalling pathways leading to cell division are not known for certain, it would appear that TNF-α plays an important part in the process. Levels of this cytokine rise within minutes of partial hepatectomy. TNF-α induces IL-6. This would appear to play a vital role in regeneration as liver regeneration is inhibited in IL-6 knockout mice. TNF-α *in vitro* can induce synthesis of the HGF in stellate cells and also of the nuclear factor CCAAT/enhancer binding protein β (C/EBPβ). HGF is a paracrine mitogen acting on hepatocytes, and could be the proximate effector of cell division. Obviously there have to be factors which stop further cell division when the liver cell number has been restored. Expression of IL-10, which down-regulates TNF-α, occurs soon after partial hepatectomy, while synthesis of TGF-β, which

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inhibits hepatocyte division, begins about 24 hours after partial hepatectomy.

It has already been mentioned that the time course for hepatocyte DNA synthesis and replication is similar in liver repair and in chemically induced enlargement. However, when liver enlargement is induced by cyproterone acetate or nafenopin, there is no increase in expression of the 'first early' genes *c-myc*, *c-fos* and *c-jun*, although there is following partial hepatectomy or induction of liver enlargement by lead nitrate or ethylene dibromide (Coni *et al*., 1993). Further, when liver enlargement is induced by cyproterone acetate, there are no changes in C/EBPα or β, whereas both change following partial hepatectomy (Skrtic *et al*., 1997). Other authors report no increases in mRNA for TNF-α, TGF-α or HGF following treatment with a peroxisome proliferator (Ohmura *et al*., 1996), and no increase in TGF-β or its receptors in rats treated with the peroxisome proliferator, nafenopin, or with cyproterone acetate (Grasl-Kraupp *et al*., 1998), although Jirtle *et al*. (1991) reported a sustained increase in TGF-β in rats treated with phenobarbital and Oberhammer *et al*. (1996) reported an increase following treatment with cyproterone acetate at doses sufficiently high to induce apoptosis. It would therefore seem that, whereas the time courses of cell division and DNA synthesis in repair and chemically induced liver enlargement are similar, there are fundamental differences in mechanism and there may also be differences between the effects of different classes of inducer.

These differences between the control of liver enlargement and of liver repair emphasize that the enlarged liver is in an abnormal state. In the enlarged liver a continued drive to cell division is balanced by an antimitotic mechanism, which, in the past, was believed to come from the action of TGF-β. It is generally believed that tumours induced by genotoxic carcinogens arise from mutations in genes involved in the transduction of positive growth factors, such as *H-ras*. However, in the enlarged liver there is a second group of genes involved, those which stop the chemically induced cell division. The importance of these genes is shown by the fact that the preneoplastic lesions may be totally removed by apoptosis when treatment ceases. The influence of the negative growth factors is also emphasized by the increased rate of apoptosis in preneoplastic and neoplastic lesions (Grasl-Kraupp *et al*., 1998). It would thus seem likely that, as discussed by Andersen *et al*. (1995), the increased rate of liver cancer in rats treated with agents which cause liver enlargement is due to mutation in the genes which limit cell division which allows clones of cells to divide, and that these cells differ from normal hepatocytes in some way, as they are selectively removed when the liver remodels after withdrawal of the compound from the diet (Schulte-Hermann *et al*., 1995).

3.2.6 Toxic Damage to Sinusoid Endothelial Cells

Sinusoid endothelial cells may be damaged by direct-acting liver toxins or as a result of the release of toxic metabolites from neighbouring hepatocytes. The destruction of sinusoid endothelial cells will cause haemorrhage or, if there is also damage to hepatocytes, haemorrhagic necrosis. There are few toxins which appear to act directly on the endothelium. Sinusoidal endothelial cells do, however, appear to be affected by allyl formate earlier than any other cell types (Haenni, 1964), and light microscopic studies suggest a similar lesion may be produced by urethane (Doljanski and Rosin, 1944) and alloxan (du Bois, 1954). Pyrrolizidine alkaloids cause destruction of the endothelial cells of sinusoids and branches of the hepatic vein, apparently due to a metabolite produced in the hepatocytes (WHO, 1988). In this case, the damage to endothelial cells is clear because of the effects on the venous endothelium, but it is likely that similar, but milder, 'by-stander' damage will occur with other toxins. Certain genotoxic carcinogens, such as thioacetamide and Butter Yellow cause enlargement and hyperactivity of endothelial cells (Simon and Rouiller, 1963).

The numbers of tumours arising from endothelial cells (angiosarcomas) can be increased by certain compounds. The most notable of these is vinyl chloride, which, in animals, produces a range of tumours, including angiosarcomas, especially in the liver. In humans, exposure to vinyl chloride is associated with an increase in a range of tumours, in addition to hepatic angiosarcomas (IARC, 1987). Vinyl chloride is a potent mutagen and it is not known why exposure should be associated with this particular tumour. Vinyl chloride is metabolized in hepatocytes by P450 isoenzymes which, as discussed earlier, gives rise to products with protein- and DNA-binding capacity that are detoxified by glutathione. There is no obvious reason why the sinusoid endothelial cells should be the targets, although evidence now points to action involving the formation of genomic DNA adducts (see **Nongenotoxic or Epigenetic Carcinogenesis**).

Angiosarcoma in humans has also been associated with exposure to arsenic (Lander *et al*., 1975). Most early effects of arsenic are, however, on hepatocytes and these have been discussed earlier. Angiosarcomas have also been found in patients treated with colloidal thorium dioxide (thorotrast) which was, at one stage, employed as an X-ray contrast medium (Swarm, 1967) and is probably associated with accumulation of the radioactive material in the neighbouring Kupffer cells (discussed in the next section).

3.2.7 Toxic Damage to Kupffer Cells

Particulate material taken up by Kupffer cells that is not digestible by lysosomal enzymes may be retained in the liver for very long periods. If the material is radioactive

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Table 7 Compounds that cause bile duct necrosis and bile duct proliferation without hepatocellular damage

Compound	Species	Reference
α-Naphthyl isothiocyanate	Rats, mice	Goldfarb *et al*. (1962)
Sporidesmin	Sheep[a]	Kelly (1985)
FPL 52757	Dogs[b]	Eason *et al*. (1982)
Dibutyltin	Rats, mice[c]	Barnes and Magee (1958)
Arsenic (III) or (V) compounds[d]	Rats, dogs	Byron *et al*. (1967), Ishinishi *et al*. (1980)
Tilidine fumarate	Rats	McGuire *et al*. (1986)
Oxamniquine	Rats[e]	Gregory *et al*. (1983)
Methylenedianiline	Rats	Kanz *et al*. (1992)

[a]Direct injection of the alkaloid into rabbit gall bladder also produces a lesion (Worker, 1960).
[b]Bile duct damage in rats may be produced by retrograde infusion (Eason *et al*., 1982).
[c]Development of the lesion probably depends on interaction with pancreatic enzymes (W. N. Aldridge, personal communication).
[d]Arsenic (III) and (V) compounds also induce damage to hepatocytes in the centrilobular area.
[e]Not dogs, mice or hamsters and probably (Chvedoff *et al*., 1984) not humans.

this may have serious consequences. Colloidal thorium dioxide (thorotrast) was used as an X-ray contrast medium up to 1945. Thorium is radioactive, emitting alpha particles. Patients exposed to thorotrast retained thorium in the liver for many years and have developed a range of tumours both in the liver and at other sites of accumulation. Liver tumours have included tumours arising from hepatocytes (hepatomas), from BD lining cells (cholangiomas) and from endothelial cells (angiosarcomas) (Swarm, 1967). Uptake of 'indigestible' material may also result in loss of Kupffer-cell function and reticuloendothelial blockade, for example gadolinium chloride (Bouma and Smit, 1989), dextran sulfate (Laskin, 1989) and carrageenen (Abraham *et al*., 1972) are taken up by Kupffer cells and inhibit their function.

Kupffer cells may also modulate the effects of xenobiotics on hepatocytes. This may occur through processing of the toxin; for example, it appears that the uptake of colloidal beryllium into Kupffer cells and the subsequent release of the free ion play a significant role in the toxicity of this metal (Skilleter, 1987). Alternatively, the interaction may be complex; for example, inactivation of Kupffer cells attenuates the toxicity of paracetamol (acetaminophen) (Laskin, 1989) and of allyl alcohol (Przybocki *et al*., 1992), whereas activation of Kupffer cells enhances the toxicity of carbon tetrachloride (Sipes *et al*., 1989). In the brain, much of the damage following ischaemic injury results from the secondary effects mediated through glial cells and it is interesting to speculate that inappropriate release of mediators from Kupffer cells may, likewise, exacerbate hepatic damage.

3.2.8 Toxic Damage to Bile Duct Lining Cells

A small number of compounds are specifically toxic to the BD lining cells (**Tables 7** and **8**). This does not appear to be due to a special susceptibility of these cells to the action of the toxin, but rather to the concentration of compounds as they pass down the biliary tree. Toxic changes are, therefore, normally confined to middle- and large-sized ducts. In two cases, the mycotoxin, sporidesmin (Mortimer, 1963; Kelly, 1985), and the antiasthmatic, FPL 52757 (Eason *et al*., 1982), toxicity is due to the parent compound. However, with other compounds, such as α-naphthyl isothiocyanate (ANIT), the actual toxin is a metabolite produced by the hepatocytes (Horky *et al*., 1971; Connolly *et al*., 1988b). The pattern of change is consistent with a toxin, possibly a glutathione conjugate (Carpenter-Deyo *et al*., 1991) escaping from the BDs and, indeed, electron microscopy shows opening of the tight junctions between BD lining cells (Connolly *et al*., 1988a). In addition to damage to the BD lining cells there is also marked portal oedema and haemorrhage, presumably from damage to the small

Table 8 Compounds that cause damage to hepatocytes and bile duct proliferation

Compound	Reference
Ethionine	Farber (1963)
Tannic acid	Korpassy (1961)
Thioacetamide	Ambrose *et al*. (1949)
Flectol H	Panner and Packer (1961)
Allyl alcohol and allyl formate	Haenni (1964)
Butter Yellow[a]	Buyssens (1962)
Pyrrolizidine alkaloids	WHO (1988)
Stilbamidine	Seager and Castelnuovo (1947)
Aflatoxin A_1	Busby and Wogan (1984)
Coumarin[a]	Evans *et al*. (1989)

[a]These compounds increase the incidence of cholangiocarcinoma in rats.

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blood vessels of the peribiliary plexus. In mice there is destruction of the gall bladder epithelium (Connolly *et al*., 1988b). Acute lesions heal rapidly; in rats total biliary necrosis produced by ANIT resolves within seven days with restoration of the normal architecture (Plaa and Priestley, 1976). Chronic treatment with such agents will lead to lethal liver failure if the dose is sufficient to prevent repair of the vessels, but if repair is possible, BD proliferation occurs.

Some di- and trialkyltin salts also cause BD damage, an example being di-*n*-butyltin dichloride (Merkord *et al*., 2001). The lesion is only found in species in which there are anastomoses between the BD and the pancreatic ducts (WHO, 1990), and mainly consists of an ascending cholangitis that affects initially the extrahepatic portion of the BD (Barnes and Magee, 1958). The mechanism of damage is not understood.

Arsenical compounds (III or VI) produce centrilobular damage to hepatocytes and damage to the biliary tree. The organic arsenical candidate chemical-warfare agent, dichloro(2-chlorovinyl)arsine (lewisite) has been observed to produce changes in the liver and epithelial necrosis (and even transmural necrosis) of the gall bladder in experimental animals (Inns *et al*., 1988; Inns and Rice, 1993; Marrs and Maynard, 2007).

3.2.9 Bile Duct Proliferation

3.2.9.1 Idiopathic or Infectious

It should be noted that spontaneous BD proliferation occurs in hamsters and in many strains of rat in the second year of life (Greaves, 1990), and that BD changes, up to and including cholangiocarcinoma, are sometimes observed in ageing dogs and cats (Kelly, 1985). The lesions cannot be distinguished from those consequential on BD damage described in the next section. Marked BD proliferation may follow infection with certain liver flukes, such as *Fasciola hepatica*, which may infest the BDs. It is believed that the changes following such infestations result from damage to the duct by the parasite (Kelly, 1985) and hence have a similar pathogenesis to the lesions described in the next section.

3.2.9.2 Consequences of Bile Duct Damage

Repeated exposure to toxins affecting the BDs results in marked proliferation. With chronic treatment, the initial damage caused by the toxin resolves by fibrosis. After a short time, heavily vascularized immature connective tissue is seen between the ducts. The BDs are embedded in dense connective tissue. Histochemical staining shows that the blood vessels of the peribiliary plexus no longer abut directly on to the basement membrane of the duct, but are separated from it by a ring of connective tissue (Kelly *et al*., 1991). It would seem that development of this type of BD proliferation involves two processes. First, the process exemplifies the 'rule' that acute injuries tend to heal by regeneration, but that chronic injuries heal by fibrosis. However, the proliferation of ducts and the separation of the vulnerable blood vessels from the ducts suggest that this is an adaptive change that increases the ability of the liver to excrete cytotoxic materials into the bile.

3.2.9.3 Proliferation Associated with Periportal Damage

Hyperplasia of the BDs and ductules occurs with a number of hepatotoxins, including ethionine, tannic acid, thioacetamide, allyl formate, 4-dimethylaminoazo benzene (Butter Yellow), lasiocarpine and stilbamidine. With these compounds, unlike the materials discussed in the previous section, damage to BDs appears proportional to the degree of hepatocellular damage (Rouiller, 1964). In other cases, exemplified by the antioxidant BHT, a very mild BD proliferation is observed at doses below those required to cause frank periportal necrosis (Powell *et al*., 1986). In some cases BD proliferation with these agents resembles that described in the previous section, namely well-defined BDs often surrounded by fairly dense connective tissue, grouped within distinct portal tracts. In other cases a different pattern is observed. In the minimal lesion, small BDs with little associated connective tissue appear to grow into the parenchyma. With other agents (Fausto *et al*., 1987), exemplified by the hepatocarcinogens ethionine, 2-acetylaminofluorene and 3′-methyl-4-dimethyl aminoazobenzene, the proliferating cells no longer form distinct ducts, but are found as strings of so-called oval cells. These have now been isolated and found to be a mixed population, a portion of which are committed BD lining cells, while others have the potential to differentiate into either hepatocytes or BD lining cells (Fausto, 1990). It is thought possible that these cells, which possibly arise from a small population of stem cells present in the normal liver, are associated with the process of carcinogenesis (Fausto, 1990). An increased prevalence of BD proliferation and portal sclerosis were noted in female rats in the mid- and high-dose 80 ppm after 52 and 104 weeks of treatment with fentin (triphenyltin), a fungicide (FAO/WHO, 1992). Cyhexatin (tricyclohexylhydroxystannane), an acaricide, produced BD hyperplasia in both sexes in a two year feeding study in rats (FAO/WHO, 1979). The relationship between these lesions and those seen with alkyl tins is unclear.

3.2.10 Cholestasis

The word cholestasis means simply the absence of bile flow and covers several distinct pathological conditions. Firstly, there is mechanical obstruction of the extrahepatic duct from either gall stones (cholelithiasis) or external pressure, for example carcinoma of the head of the pancreas. Secondly, there is obstruction of intrahepatic BDs, normally as a result of inflammation. Finally, there exist changes in hepatocytes, which may interfere

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with the initial secretion of bile into the BC, either by inhibiting the secretion of bile acids and bile salts (the fluid component of bile enters by passive diffusion) or by altering the permeability of the tight junctions which prevent materials passed into the bile canaliculus from returning to the plasma. In experimental animals, treatment with agents causing biliary obstruction results in proliferation of BDs and focal necrosis in the liver parenchyma. Treatment with cholestatic agents which affect hepatocytes, on the other hand, does not generally cause damage to hepatocytes or BD lining cells, although bile plugs may be observed in BC and ducts.

The most common form of gall stone is composed of cholesterol and calcium salts, often arranged in alternating layers (Karran *et al*., 1985; Thomas, 2008). Pigment stones also occur, especially in Asiatic countries, and consist largely of salts of bilirubin (Leuschner *et al*., 1994). The two types of stone are thought to form by different mechanisms. Biliary cholesterol is dissolved in the interior of mixed micelles formed by phospholipids, and bile acids and salts. Normal human bile is technically supersaturated in cholesterol, but this does not normally crystallize. It is thought that crystallization may be provoked by the formation of nucleation centres, short-term fluctuations in cholesterol secretion and changes in the stability of bile micelles with changes in bile salt composition (Heaton, 1985). Formation of pigment stones is associated with infection of the biliary tree: the β-glucuronidase secreted abundantly by bacteria such as *Escherichia coli* breaks down bilirubin glucuronides and causes precipitation of free bilirubin (Karran *et al*., 1985). There is probably a threshold to this process, as normal bile contains an inhibitor of β-glucuronidase (Godfrey *et al*., 1981). Cholelithiasis is not normally associated with the administration of xenobiotics, but an increase in gall stones has been reported in patients treated with clofibrate and possibly similar drugs (Committee of Principal Investigators, 1978; Backes *et al*., 2007).

The normal cause of intrahepatic biliary obstruction in experimental animals is damage to BD lining cells. Compounds causing such damage have been considered earlier. There are, however, a few materials which appear to cause intrahepatic obstruction by a distinctive mechanism. Geeldikkop is a photosensitizing disease of sheep in South Africa and there have been similar reports from other countries, including Australia (Glastonbury *et al*., 2008). Geeldikkop appears to result from an interaction between sporidesmin (a mycotoxin) and a component of puncture vine (*Tribulus terrestis*) and the BD becomes blocked with crystalline material of unknown composition (Kelly, 1985). Similar crystals have been observed in sheep intoxicated by other phytotoxins. It has been suggested that the spontaneous BD proliferation observed in some strains of ageing mice may also be associated with obstruction by a crystalloid material (Lewis, 1984). Treatment of rats with benzofuran also causes symptoms of intrahepatic cholestasis but, in this case, there is no evidence of damage to the BD (Connelly, 1988). At autopsy, gross dilation of the extrahepatic BD was noted and it would appear possible that spasm of the sphincter of Oddi may play a role in inducing cholestasis with this material.

Whereas intrahepatic obstructive jaundice is a relatively minor problem in experimental animals, it is a major problem in clinical practice in humans (Read, 1985). The condition was described in the early 1950s with chlorpromazine (van Ommen and Brown, 1955), and the response to phenothiazines is typical of this lesion (see also Section 3.2.1.1). This adverse effect is confined to a small minority of patients who may show marked hypersensitivity. Patients affected are jaundiced, and liver biopsies show portal tract inflammation, sometimes with a high eosinophil content, and some damage to hepatocytes. An immunological mechanism has long been suspected, but cannot be proven. It is of interest that BD lining cells are a major target in autoimmune disease and that, following liver transplantation, the BDs are especially liable to rejection (Welsh and Male, 1989). Investigation of these conditions has been hampered by the fact that, even after immunological manipulation, it has proved impossible to reproduce the condition in experimental animals.

As mentioned earlier, the formation of bile is a complex process and it is therefore not surprising that there are many classes of compound that induce intrahepatic cholestasis by interference with bile secretion (Oelberg and Lester, 1986; Kukongviriyapan and Stacey, 1991; Plaa, 1991). The classic agents, in this case, are anabolic and contraceptive steroids. The mechanism of action of these compounds remains obscure. There is a lag phase of approximately 12 hours before the establishment of cholestasis. There are some indications for both interference in the uptake of bile acids into hepatocytes and for alterations in membranes which might affect tight-junction structure. It remains unclear how far the mechanism of cholestasis by steroids correlates with the mechanism of cholestasis by D-ring glucuronide conjugates of oestrogens or testosterone. Unlike the parent steroids, the effect of these compounds is immediate and clearly dose dependent. In this case there is evidence for a direct competition with bile acids at the bile canalicular plasma membrane.

In addition to steroids and their glucuronides, many other classes of compound induce cholestasis at the canalicular level. The monohydroxy bile acid, lithocholate, has a cholestatic effect, probably after conjugation with taurine. Studies show that taurolithocholate inhibits taurocholate uptake into hepatocytes (Schwenk *et al*., 1977), but cytological examination of the livers of treated animals shows dilation of the Golgi apparatus and loss of microvilli from BC, which would appear

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to be independent events. The reason why the immunosuppressive drug ciclosporin (cyclosporin A) can induce cholestasis is equally obscure, but this may be related to inhibition of transport of bile acids (Kukongviriyapan and Stacey, 1991). Cholestasis following the intake of manganese is observed in both humans and experimental animals. It has been demonstrated that the mechanism requires interaction of manganese and bilirubin at the bile canalicular membrane, but why, given that bilirubin excretion is responsible for only a tiny proportion of bile flow, this should result in cholestasis remains obscure. A mechanism involving hydroxy-3-methylglutaryl coenzyme A reductase and cholesterol 7α-hydroxylase has been proposed (Akoume *et al*., 2003; Goering, 2003).

The compounds mentioned in the last few paragraphs are only a small proportion of those capable of causing cholestasis through their action on hepatocytes. As the compounds described are 'the best understood' it will be clear how limited is our present knowledge of the mechanisms of cholestasis at the canalicular level, despite the availability of animal models. It is to be hoped that the development of *in vitro* techniques, such as the use of hepatocyte doublets and microanalytical techniques may assist solution of these problems in the future. Xenobiotic-induced cholestasis has been reviewed (Mohi-ud-din and Lewis, 2004).

The reduction of bile flow is a serious toxicological problem, but an increase in bile flow (choleresis) is without any serious consequences. Passage of water into bile appears to occur by passive diffusion across the tight junctions between hepatocytes. Normally this is governed by the rate of secretion of bile acids and bile salts. The administration of large doses of compounds whose metabolites are excreted into bile will result in an increase in bile flow and, often, dilation of BC. On their own, these are changes without long-term implications.

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Toxicology of the Exocrine Pancreas

John R. Foster

CONTENTS

1 INTRODUCTION

The exocrine pancreas is a medium-sized organ lying below the stomach and attached, via mesenteric connective tissue, between the duodenum and the spleen. It has a primary function in the production and secretion of digestive enzymes, under neurohormonal control, into the small intestine via a complex, anastomosing, ductal system which empties into the duodenum, either in combination with the common bile duct, as in man and the rat, or, in species such as the pig, as a separate system each of which opens into the duodenum via a separate duct. Approximately 60% of the human population possesses an additional, accessory pancreatic duct, which empties directly into the duodenum without fusing with the bile duct.

The possession of extremely large amounts of digestive enzymes requires safety measures to avoid their premature activation within the organ and consequential autodigestion, but anatomical variances, disturbances of metabolism and secretion in both the pancreas and the liver, and in particular unfavourable lifestyle choices, such as alcoholism, can rapidly induce life-threatening disease in the organ.

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In the context of toxicology, changes, which in other organ systems would lead to adaptation, can rapidly progress to wholesale organ failure and death in the pancreas.

Clinical and experimental evidence suggests that the physiology and function of the exocrine pancreas intimately determines its vulnerability. A complete understanding of the features of the organ that make it susceptible to toxicity requires an appreciation of the evolutionary and anatomical links between the pancreas, the gastrointestinal tract and the liver.

Because of the potentially vast scope of this chapter, the bibliography largely relates to the section on toxicology. Individual references on other aspects of pancreatic disease can be found in alternative reviews on the pancreas (Cano *et al*., 2007; Howat and Sarles, 1979; Go *et al*., 1993); monographs on pancreatitis (Grigorescu and Grigorescu, 2005; Behrman and Fowler, 2007; Lee and Enns, 2007; DiMagno and DiMagno, 2006; Nair and Lawler, 2007; Talukdar *et al*., 2006; Braganza, 1991; Kingsnorth, 1996) and pancreatic cancer (Koorstra *et al*., 2008; Hart *et al*., 2008; Moossa, 1980; Scarpelli *et al*., 1987; Hezel *et al*., 2006), and in the journal series *Current Opinion in Gastroenterology* (Braganza, 1990; Fernandez-Zapico *et al*., 2003; Weber and Adler, 2003).

2 NORMAL PANCREAS

2.1 Embryology/Phylogeny

The mammalian arrangement of the hepatopancreatoduodenal complex is a relatively recent phylogenetic development compared to that of the drug-metabolizing enzyme systems (**Figure 1**).

The pancreas develops through two evaginations of the foregut endoderm during the fifth week of gestation in man. It is ventral, initially bilobed, and grows out from the lateral aspect of the hepatic bud. Duct systems appear in each outgrowth by the sixth week and open into the gut tube. By the seventh week the duodenum rotates to become a retroperitoneal organ. The two rudimentary glands of the pancreas then fuse, so that the dorsal bud forms the bulk of the gland while the ventral bud forms the inferior aspect of the 'head' and the uncinate process.

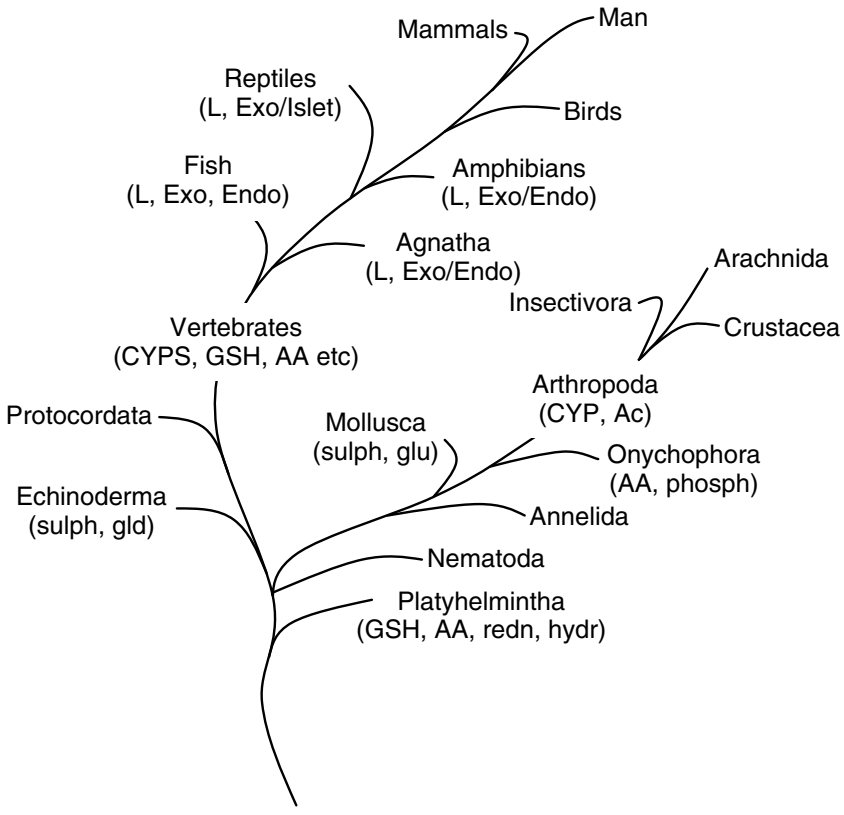

Figure 1 Phylogenetic tree showing that xenobiotic enzymes were present in animals well before the components of the liver and pancreas separated. Conjugating reactions with glutathione (GSH), amino acids (AA), sulfate (sulph), glucose (glu) and glucuronic acid (gld). Primitive degradation reactions are present with reduction (redn), and hydrolysis (hydr); cytochrome P450 dependent oxidation (CYPs) and acetylation (Ac). L = liver; Exo = exocrine pancreas; Endo = endocrine pancreas; Islet = islet arrangement. (Reproduced with permission from Braganza, 1991. © Manchester University Press.)

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The duct of the larger dorsal bud (duct of Santorini) opens directly into the gut through a tiny orifice on the minor duodenal papilla; while, a fraction distally, the major papilla is the entry point for the joint bile duct and duct of the ventral pancreas (duct of Wirsung). In 10–80% of humans these two ducts may fuse into a common channel, the ampulla of Vater; smooth muscle surrounding the lower end of each duct, and of the ampulla, forms the sphincter of Oddi. During the seventh week of gestation the duct systems of the pancreas fuse, and the section of dorsal duct between the site of fusion and the minor duodenal papilla usually withers away. In approximately 5% of individuals fusion fails to occur and gland drainage relies largely on Santorini's duct. This condition in man, 'pancreas divisum', is recognizable by endoscopic retrograde cholangiopancreatography (ERCP).

Pancreatic acini, the secretory units, bud off the distal-most cells of the elongating duct system. A lobular arrangement is seen in man in the fourth month and acinar cells are functional a month later, as shown by abundant rough endoplasmic reticulum (RER) and the development of zymogen (enzyme precursor) granules. As morphological differentiation occurs so does the transcription and translation of those genes involved in the production of the digestive pancreatic enzymes. Prior to the appearance of zymogen granules (ZGs), very small amounts of carboxypeptidase, amylase and chymotrypsin can be detected within the gland, but as ZGs appear morphologically during development there is a surge in the transcription of the messenger RNA (mRNA), followed by the appearance of large amounts of the protein, for all of these digestive enzymes within the organ. In man, therefore, enzyme secretion begins in the 26th week of gestation, coincident with the emergence of the trypsinogen activator, enterokinase, in the duodenum.

Endocrine cells are present as single cells within the ductal epithelium from which they undergo extensive proliferation, and during the last trimester they begin to organize into islet-like clusters and undergo additional remodelling and maturation after birth before assuming the adult islet arrangement (Habener *et al*., 2005).

2.2 Applied Anatomy

The pancreas lies deep in the upper abdomen, usually in a transverse plane at the level of the first lumbar vertebra. The pancreas in man has a head, a neck, body and a tail. The hepatic portal vein is formed posterior to the neck of the pancreas by the union of the superior mesenteric and splenic veins (**Figure 2**). The pancreatic head lies within the curve of the duodenum and is usually seen encompassing the lower part of the common bile duct. The body and tail extend to the left, across the vertebral column and the tail ends at the spleen. The body lies superior to the duodenal-jejunal flexure.

In laboratory animals the organ is more diffuse, but the rough divisions can still be made out. The gland is surrounded by a thin condensation of retroperitoneal connective tissue and no definite capsule can generally be made out, as is seen in other abdominal organs.

The pancreatic duct begins in the tail and runs towards the head region where it fuses with the bile duct before emptying into the second part of the duodenum at the greater duodenal papilla. A second, accessory pancreatic duct drains part of the head which empties separately into

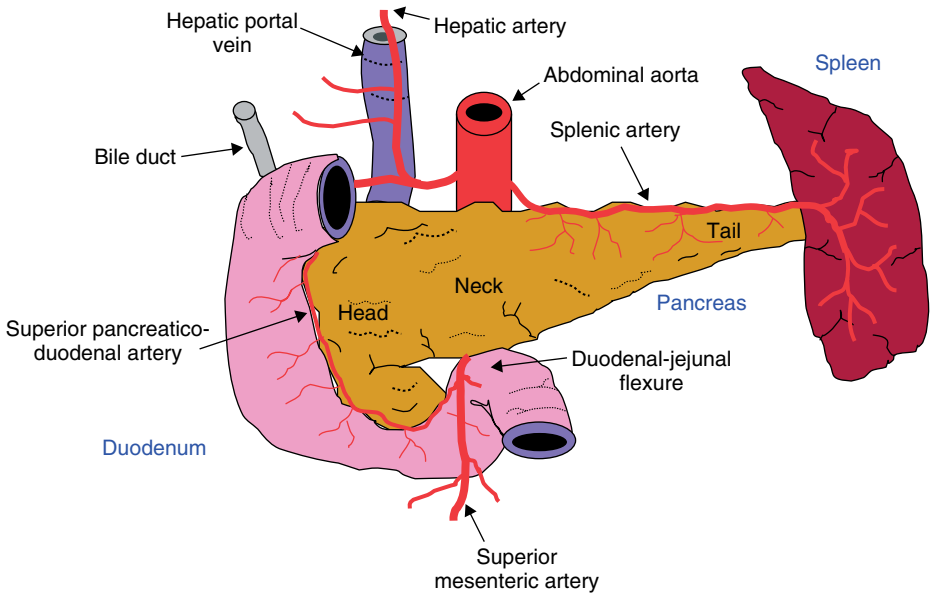

Figure 2 Anatomical relationship between the pancreas, the spleen and the duodenum showing the major blood vessels.

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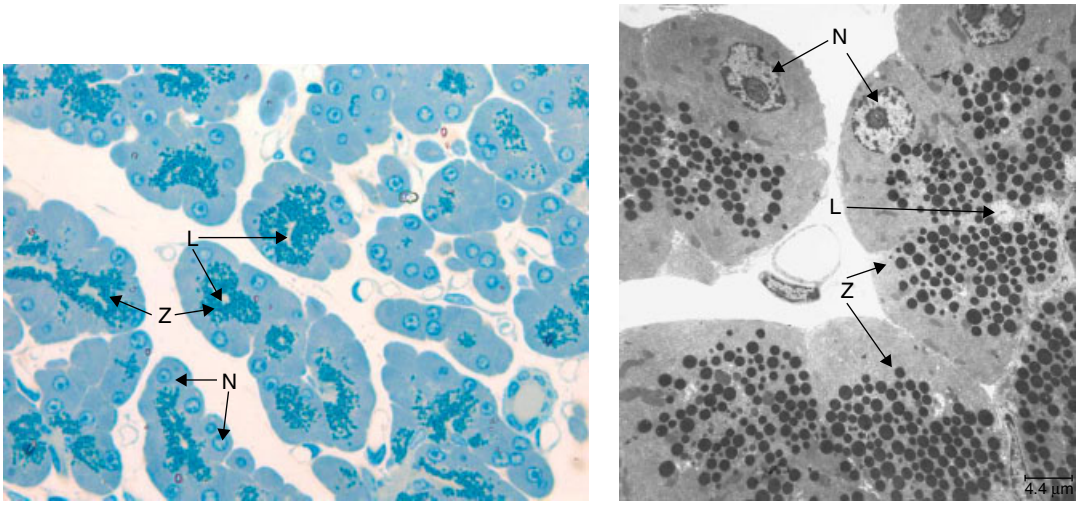
the duodenum proximal to the greater duodenal papilla. The exocrine pancreatic secretions flow from the tail towards the head before emptying into the duodenum.

The pancreas is closely related to a number of important blood vessels: behind it lie the aorta, the inferior vena cava, the superior mesenteric artery and the portal vein; along its upper border are the hepatic and splenic arteries. The pancreatic head receives its blood supply via the anterior and posterior superior pancreaticoduodenal arteries (from the hepatic artery via the gastroduodenal artery) and by the anterior and posterior inferior pancreaticoduodenal arteries (from the superior mesenteric). The remainder of the organ is supplied by branches of the splenic artery. Venous drainage is into the hepatic portal vein. Lymphatics on the surface of the pancreas lead into a complex system of lymph nodes.

The pancreas has extrinsic and intrinsic innervation and autonomic control is critical in regulating exocrine secretion. Parasympathetic innervation is effected via the vagal nerves, which act upon ganglion cells within the pancreas. These neurones in the ganglia are capsaicin-sensitive and contain substance P, calcitonin-gene-related peptide and low levels of norepinephrine and dopamine (Love *et al*., 2007; Buijs *et al*., 2001) and they lie within the connective tissue being most abundant in the head and neck regions. The ganglion cells are also innervated by the axons from the enteropancreatic neurones of the stomach and duodenum allowing local reflux control by the gastrointestinal tract, which almost certainly signals the mechanical and physicochemical states of the stomach and proximal small intestine to coordinate pancreatic secretion (Noble and Liddle, 2005). In contrast to the pancreatic ganglia, pancreatic duct cells have been shown to contain large amounts of epinephrine and 5-hydroxytryptophan, and moderate amounts of norepinephrine (noradrenaline) and dopamine (Yi *et al*., 2004). Pancreatic enzyme secretion is thought to be under the control of cholecystokinin (CCK) and serotonin and the vago-vagal reflex activating the cholinergic post-ganglionic neurons of the pancreas. Evidence suggests that at least for CCK in man, regulation occurs via the vagal cholinergic pathway rather than it acting directly on the ganglia cells, which were found not to express functional CCK receptors (Owyang and Logsdon, 2004).

2.3 Microanatomy

The exocrine pancreas is a lobular, compound tubuloalveolar gland with the lobes being separated by thin connective tissue that is continuous with that surrounding the outside of the gland. A variable proportion of the gland's blood supply passes directly to pancreatic islets and hormone-enriched blood may then enter acinar lobules before draining into veins. An insular-acinar 'portal' circulation would favour zonality of pancreatic exocrine function, in the same way as the simple hepatic acinus describes a microcirculatory functional unit in the liver. This concept would rationalize the higher levels of pancreatic enzymes in 'peri-insular acini', which display changes associated with hyperactivity. The acini are irregular oval or elongated glands comprised of a single layer of pyramidal-shaped cells with the apex of the pyramid defining the lumina of the acini while the broad base of each cell lies on a basement membrane and sparse connective tissue stroma (**Figure 3A**). The nucleus lies at the base of each acinar epithelial cell surrounded by abundant RER (**Figure 3B**). The ZGs are arranged at the



A B

Figure 3 (A) Light micrograph of pancreatic acini in a 2 µm thick resin section stained with Toluidine Blue ×63 magnification. The small black granules are the zymogen (Z) with the nuclei (N) and acinar lumina (L). (B) An electron micrograph of pancreatic acini showing the same features. ×2500 magnification.

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apex of each cell and appear as black oval bodies by electron microscopy.

The apical cytoplasm of the acinar cells is packed with large round ZGs (0.8–1 μm in diameter), which appear eosinophilic, in contrast to the RER, which appears basophilic, in haematoxylin and eosin-stained histological sections. Pancreatic duct cells can be seen as flattened cells with pale cytoplasm and while the larger ducts are totally lined with these cells, the beginning of the duct system can be observed where small intercalated ducts are half lined by acinar cells and half by clearly designated ductular epithelial cells.

2.4 Structure–Function Relationships

The RER manufactures intramembranous digestive, and lysosomal, enzymes. The luminal plasma membrane projects microvilli consisting of bundles of microfilaments containing actin, myosin, tropomyosin, β-actinin and villin. The microvilli are anchored to a terminal web, an extension of the inner leaflet of the plasma membrane. Freeze-fracture studies show that the bulk of ZGs are located near the apical plasma membrane in readiness for exocytosis, but also that vesicles impinge against the basolateral membrane of the acinar cell, apparently for discharge directly into the interstitial space.

Ductal cells are equipped to secrete a bicarbonate-rich fluid: they have abundant mitochondria, immunostainable carbonic anhydrase activity in the cytoplasm and a generous amount of the cystic fibrosis transmembrane regulator (CFTR) protein at the luminal domain. The 'fuzzy coat' lining ductal epithelium is caused by mucus secreted by interspersed cells.

2.5 Acinar Cell

2.5.1 Enzyme Synthesis and Growth Regulation

In addition to the reference sources mentioned above, useful information can also be obtained from the reviews by Johnson (1987), Musa (1989) and Case (1990). High rates of amino acid influx in the rat pancreas are mediated by at least four transporters in the basolateral membrane, while amino acid efflux is mediated by at least two further transporters and activated by transexchange with extracellular amino acids. Diet and endocrine status are among many factors that interact to alter enzyme synthesis profiles. Recent work in rats shows that at least some of those changes are brought about by signal transduction mechanisms akin to those involved when the regulated secretory pathway is activated by occupancy of specific plasma membrane receptors.

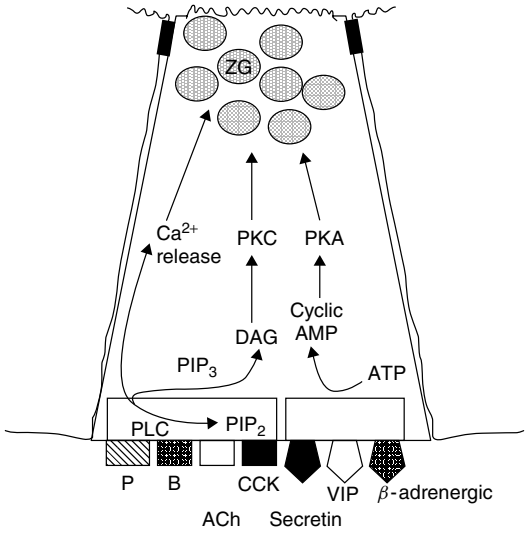

Figure 4 Plasma membrane receptors and intracellular chemical messengers involved in signal transduction by the regulated secretory pathway in the acinar cell. ZG: Zymogen granule; PKC: protein kinase C; PKA: protein kinase A; DAG: diacylglycerols; PIP_3: inositol 1,5-triphosphate; PIP_2: Inositol 4,5-bisphophate; AMP: andenosine monophosphate; ATP: adenosine triphosphate; PLC: phospholipase C; P: pirenzepine receptor; B: the PAF receptor antagonist drug, BN52021; CCK: cholecystokinin; VIP: vasoactive intestinal peptide; ACh: acetylcholine.

These membrane receptors fall into two main classes (**Figure 4**), of which one mainly accommodates secretin and VIP (vasoactive intestinal peptide) and the other accommodates mainly CCK (cholecystokinin-pancreozymin) and acetylcholine (ACh). The CCK receptor has high affinity (K_d for CCK-8, 65 pM) and low affinity (K_d, 15 nM) subtypes. Synthetic CCK receptor antagonists include glutamic acid derivatives (e.g. CR-1392, CR-1409, CR-1505 from Rotta Research Laboratories, Milan, Italy), benzodiazepine derivatives (e.g. L-364, 718 from Merck, Sharp & Dohme Research Laboratories, West Point, Pennsylvania, USA) and a series of CCK analogues. The acinar cell membrane also has receptors for somatostatin, insulin, epidermal growth factor, platelet-activating factor (PAF) (Nigam *et al*., 1996) and pirenzepine (low affinity, M2B subtype). Occupation of the CCK-type receptor increases the synthesis of several proteolytic enzymes, acting preferentially via increased cytoplasmic Ca^{2+}.

Hyperthyroidism extends the effect to include heightened synthesis of amylase and lipase. Occupation of the secretin-type receptor increases lipase synthesis by causing activation of adenylate cyclase (AC), which generates cyclic adenosine monophosphate (AMP) from adenosine triphosphate (ATP).

Not surprisingly, factors that stimulate pancreatic enzyme synthesis and secretion also promote pancreatic growth, but it is not known whether these three

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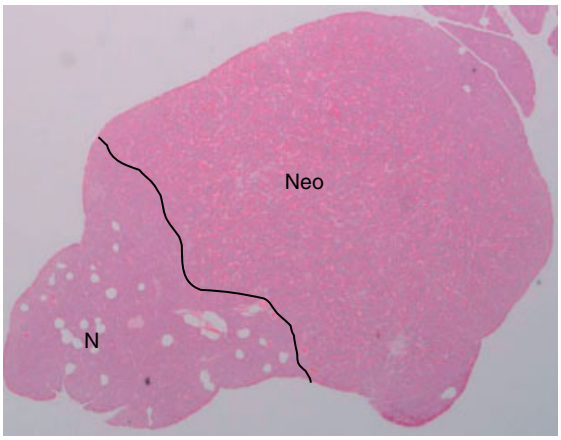

Figure 5 Light micrograph of pancreas from rat given a trypsin inhibitor for 12 months. The line shows the margin of the neoplastic nodule on the right hand side (Neo) from the normal pancreas (N) on the left. Magnification × 63.

effects are regulated via a common messenger, or whether enzyme discharge triggers synthesis and subsequent hypertrophy [enlargement of the cell] and/or hyperplasia (cell proliferation). Growth can be induced by administering CCK (or similar peptides such as caerulein) to rats, or by promoting its endogenous release by lowering the level of pancreatic proteases in the small intestinal lumen. This can be done by feeding with soya flour, which contains trypsin inhibitors, treatment with a synthetic inhibitor of serine proteases, such as Camostate, or by pancreatoduodenal diversion. The relevance of this feedback loop in humans is questionable, however. Pancreatic growth can also be evoked by hormones (glucocorticoids, insulin, epidermal growth factor), the bile-salt-binding resin cholestyramine, occlusion of the main pancreatic duct, pancreatic trauma and dietary deprivation of essential amino acids followed by free access. Chronic induction of exocrine pancreatic growth in rodents has been shown to result in the development of benign pancreatic exocrine tumours (**Figure 5**).

2.5.2 Regulated Secretory Pathway

Proteins synthesized in the RER are transported vectorially to the cisternal space through signalling transport sequences. Thereafter transport is believed to follow the ZG packaging and exocytosis model, in which lysosomal and food digestion enzymes are largely separated from each other in different, membrane-bound, compartments. As in other secretory cells, signal transduction is accomplished through a system of intracellular messengers (**Figure 4**). Occupation of the CCK-type receptor leads, via a G-protein (guanine nucleotide-binding protein), to activation of phospholipase C (PLC) producing inositol 1,5-triphosphate (PIP_3) and diacylglycerols (DAG from precursor inositol 4,5-bisphosphate (PIP_2), preferentially. Those chemical messengers mobilize Ca^{2+}, probably from the RER, or activate protein kinase C (PKC), respectively (**Figure 4**). The rise in cytosolic Ca^{2+} activates calmodulin (CAM) and thereby protein kinase (PK), and phosphatase (PP). Studies suggest that biological free radicals and free radical oxidation products (FROPs) provide a further network of intracellular signal transducers in a variety of secretory cells (Remacle *et al*., 1995). Examples of such reactive species include the nitric oxide radical, derived from arginine (Moncada *et al*., 1991), and derivatives of lipid oxidation, such as 4-hydroxynonenal, isoprostanes, isoleukotrienes and the lysophospholipid PAF (Nigam *et al*., 1996).

The methylation of membrane phospholipids, by transfer of methyl groups from methionine via sulfadenosyl methionine (SAMe) also seems to be important for signal transduction in a variety of cells. Thus, it has been shown that the binding of catecholamine to its receptor activates methyl transferases, resulting in methylation of phosphatidylethanolamine to generate phosphatidylcholine. In the process, membrane fluidity increases, allowing lateral mobility of the β-adrenergic receptor, so that it can interact with a G-protein coupling factor and AC to generate cyclic AMP, as in **Figure 4**. *In vitro* studies also showed that when SAMe was present in the incubation medium, treatment with PLC was accompanied by a methylating-enzyme-facilitated flip-flop realignment of membrane phospholipids with increased membrane fluidity. The potential application of these findings to signal transduction in the acinar cell is unknown.

It is also not known exactly how ZGs are shunted to the apical plasma membrane, but regulated exocytosis involves fusion of the granules with the apical plasma membrane mediated by opposing membrane complexes of soluble *N*-ethylmaleimide-sensitive fusion-protein attachment protein receptor (SNARE) proteins, for example, syntaxins (Weiss *et al*., 2008). In acinar cells syntaxin is present on the apical membrane and enters the ZG during exocytosis. The SNARE protein VAMP8/endobrevin was found to be enriched on the membrane of ZGs, and VAMP8 knockout mice contained approximately three times as many ZGs than control acinar cells (Wang *et al*., 2004). It also seems probable that microtubule/myosin phosphorylation is involved in the process and that membrane proteins in the ZGs, apical membranes or both are necessary for the fusion event, and that G-proteins, other Ca^{2+} dependent proteins and probably also CFTR (Trezise *et al*., 1992; Kopelman *et al*., 1995) interact to facilitate enzyme discharge. It is also apparent that free thiol groups, and as yet uncharacterized protein thiols, play critical roles in exocytosis, in a process that involves the formation, and maintenance, of an exocytosis transmembrane pore (Braganza and Chaloner, 1995; Weiss *et al*., 2008).

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Considering the potentially lethal cocktail of proteases contained within the acinar cells, the pancreas has evolved systems to prevent premature activation of its contained enzymes and autodigestion. The most potent enzymes (proteolytic, phospholipase A_2) are synthesized as precursor enzymes or zymogens, which require hydrolysis in order for activation to occur. The proenzymes are constrained within membrane-enclosed granules, while the enzyme activators tend to be located in other organs, for example, the trypsinogen activator, enterokinase is located in the duodenum. In addition, a low-molecular-weight trypsin inhibitor, the pancreatic secretory trypsin inhibitor-1 (PSTI-1), is cosecreted within the ZG and can trap as much as 20% of potential trypsin activity (Bartelt *et al*., 1977), and should any trypsin become prematurely activated within the gland, it quickly activates an inhibitor-resistant protease, mesotrypsin, which in turn inactivates trypsinogen and other zymogens to help curtail the activation cascade (Szmola *et al*., 2003).

2.5.3 Nonregulated Secretory Pathway

There is evidence for a constitutive, nonregulated facility for protein discharge from acinar cells through pathways that seem to be preferentially located in the basolateral membrane, and that lead directly into the interstitium and bloodstream (**Figure 6**). As a backup mechanism, plasma contains safeguards against premature activation of pancreatic proteases that enter via the 'endocrine' route. These include the α_1 protease inhibitor (α_1PI) whose primary function is to protect against leucocyte-derived elastase (Karnaukhova *et al*., 2007) and which completely inactivates proteases and also binds to proelastase.

2.6 Ductal Cells

2.6.1 Bicarbonate Secretion

Secretin and VIP are the main stimuli of ductal bicarbonate secretion, although other agonists, notably CCK, can potentiate their action. Signal transduction involves cyclic AMP acting in concert with CFTR to increase the open probability of a chloride channel on the luminal plasma membrane. The active secretion of bicarbonate into the duct system results from the coordinated action of a chloride/bicarbonate exchange protein spanning the luminal membrane, an intracellular carbonic anhydrase, which generates carbonic acid from carbon dioxide that has diffused into the cell, and three proteins in the basal plasma membrane (sodium–potassium exchanger, hydrogen sodium antiporter, potassium channel) (Szucs *et al*., 2006). Sodium appears to enter

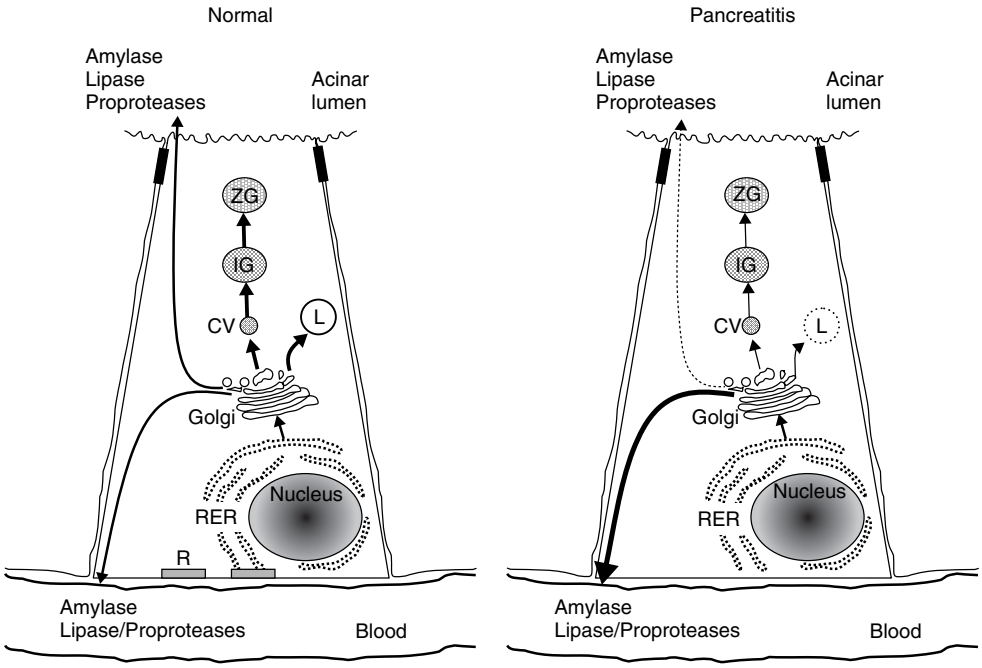

Figure 6 Nonregulated vesicular pathway into the interstitium via the basolateral membrane: a minor secretion route in the normal acinar cell (Arvan and Castle, 1987), but seemingly used preferentially in pancreatitis. R = plasma membrane receptor; N = nucleus; RER = rough endoplasmic reticulum; Golgi = Golgi stacks; L = lysosome; CV = condensing vacuole; IG = intermediate granule; ZG = zymogen granule. (Reproduced from Braganza, 1992. © Oxford University Press.)

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the duct via the intercellular fluid and through the spaces between the ductal epithelial cells.

2.6.2 Mucus Secretion

Different cells secrete highly sulfated mucin, weakly sulfated neutral mucin, sialomucin or sulfomucin, and, following mucin synthesis, glycosylation occurs in the Golgi apparatus. Thereafter glycoproteins are packaged into granules and propelled along microtubules for exocytosis. Cholinergic agonists and prostaglandins are secretagogues for mucus secretion, while bile salts may owe their effectiveness to a local response mediated by receptors on the cell surface.

2.7 Pancreatic Juice

2.7.1 Phases of Secretion

Interdigestive secretion varies with the phases of upper gastrointestinal motility. The digestive pattern is evoked by food, hormonal and neural stimuli. The coordination between secretion and motility may help to protect the intestinal lining from irritation by pancreatobiliary secretions and to ensure that duodenal pressure does not exceed that in the pancreatic duct.

2.7.2 Composition of Pancreatic Juice

The human pancreas secretes between 0.7 and 2.5 l of pancreatic juice each day, which has a pH of 7.5–8.8 and which is isotonic with serum. Its bicarbonate concentration rises with flow rate to a maximum of 130–150 mmol l^{-1} at a rate of 300 ml h^{-1}. The sum of bicarbonate and chloride is constant at around 154 mmol l^{-1}. Sodium is the principal cation (139–143 mmol l^{-1}), and the concentration of calcium varies between 2.2 and 4.6 mmol l^{-1}. Digestive enzymes account for most of the high protein content, 4.8–5.3 g l^{-1}, while traces of albumin, globulin and mucoprotein account for the remainder. There are more than 20 different enzymes, including inactive precursors of serine proteases (three forms of trypsinogen, two of chymotrypsinogen, two of proelastase and kallikreinogen) and exopeptidases (four forms of procarboxypeptidase, and prophospholipase A2) and lipase, colipase, cholesterol ester hydrolase, amylase, RNase and DNase. The concentration of lipids is very low, 5.2 mg l^{-1}. Other constituents include lysosomal enzymes, lactoferrin and a 'pancreatic stone protein (PSP)', that is, nowadays called lithostatin, which helps to solubilize calcium and has bacteriostatic potential (Weiss *et al*., 2008).

3 EXOCRINE PANCREAS: TARGET FOR TOXICITY FROM DRUGS AND CHEMICALS

3.1 Chemicals in Non-Neoplastic Pancreatic Disease: Human

3.1.1 Acute Pancreatitis

Table 1 is a compilation reviewing the evidence linking exposure to drugs and chemicals with pancreatic disease. It is largely taken from the textbook by Braganza (1991), but updated with the more recent reviews on the subject by Badalov *et al*. (2007), Dhir *et al*. (2007) and Trivedi and Pitchumoni (2005), along with more recent information from case reports uncovered by computer-assisted searches. The interpretation of a proposed link with chemical exposure is frequently confounded by the presence of concurrent disease and the pancreatic lesion, for example, the connection between azathioprine, Crohn's disease and pancreatitis, and the three-way relationship between HIV infection, pancreatic lesions and treatment with pentamidine or didanosine (Schwartz and Brandt, 1989). It can be difficult to unravel the interaction between pancreatic ischaemia-reperfusion injury and the toxicity, to pancreatic acinar cells, of drugs such as cyclosporin A. There is evidence that this drug accelerates pancreatic acinar cell injury in caerulein-treated rats (Hirano *et al*., 1991) although it was also shown to decrease the severity of pancreatitis in a rat model most probably through its anti-inflammatory effect in inhibiting neutrophil infiltration into the damaged pancreas (Scott *et al*., 1992).

Table 2 is an attempt to clarify the underlying principles governing drug-induced acute pancreatitis, in the absence of a confounding influence, from pharmacological properties (Smith, 1973; Bowman and Rand, 1980; Schenkman and Kupfer, 1982; Halliwell and Gutteridge, 1985; Hodson and Levi, 1987), by analogy with drug toxicity in the liver (Plaa and Hewitt, 1982; Timbrell, 1982; Cohen, 1986). Evidence exists for the presence of drug-metabolizing activity within the pancreas of animals (Scarpelli, 1989) and enzymic activation is one likely mechanisms in the genesis of some forms of acute pancreatitis. This analysis has been forced in some way, while appreciating that any single agent can potentially injure the pancreas in more than one way, as exemplified by the toxicity of ethanol. The role of ethanol in the development of chronic pancreatitis is discussed in the next section.

With regard to injury from bioactivated drug metabolites, a case report of methyldopa-associated severe acute pancreatitis, affecting ectopic as well as entopic pancreas, is described, that defies any alternate explanations other than the generation of toxic metabolites of the drug

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Table 1 Drugs and human pancreatitis[a]

Definite	Probable	Possible	Questionable
No other factor	−	±	−/?
Temporal sequence	+	+	±/?
Rechallenge	+	−	−/?
Frusamide	—	Angiotensin-converting enzyme (ACE) inhibitors	Amphetamines
Methyldopa	—	Paracetamol	β-blockers
Metronidazole	—	L-Asparaginase	Cimetidine
Oestrogens	—	Calcium excess	Cholestyramine
Tetracycline	—	Chlorthalidone	Clofibrate
Valproic acid	Pentamidine	Cyclosporine	Clonidine
	Nitrofurantoin	Cytosine-Ara	Cyproheptidine
	Sulfonamides	Corticosteroids	Dextropropoxyphene
		Erythromycin	Diazoxide
		Ethacrynic acid	Enalapril
		Indomethacin	Growth hormone
		Isoniazid	Histamine
		6-Mercaptopurine	Isoretinoin
		Procainamide	Meprobromate
		Rifampicin	Phenformin
		Stibogluconate	Warfarin
		Salicylates	
		Sulindac	
		Theophylline	
		Vinblastine	

[a] −, no information. +, clear information. ±, conflicting evidence?, questionable interpretation from insufficient information. −/?, some articles with insufficient information. +/?, articles giving questionable information. (Reproduced with permission from Braganza, 1991. © Manchester University Press.)

Table 2 Possible mechanisms of drug toxicity in human acute pancreatitis

Mechanism	Drugs
Impeded protein synthesis/transport/secretion	Alcohol, L-asparaginase, azathioprine, caerulein/CCK, calcium infusion, cobalt chloride, colchicines, corticosteroids, cytosine-Ara, didanosine, DL-ethionine, 6-mercaptopurine, pentamidine, phenylalanine, puromycin, sulfonamides, tetracycline, theophyline, vinblastine.
Cytochrome P450 induction	Alcohol, corticosteroids, isoniazid, meprobromate, rifampicin, valproic acid.
Toxic metabolites via cytochrome P450	Alcohol, azathioprine, indomethacin, isoniazid, methyldopa, metronidazole, nitrofurantoin, oestrogens, paracetamol, sulindac, valproic acid.
Toxic metabolites via other route	Chlrorthiazide, chlorthalidone, ethacrynic acid, procainamide, sulfonamides.
Increased triglycerides	Alcohol, β-blockers, corticosteroids, isoretinoin, oestrogens.
Impeded drainage	Alcohol, dextropropoxyphene, diphenoxylate.
Haptene formation	Alcohol, 5-aminosalicylic acid, isoniazid, cyclosporin, procainamide, sulfonamides.
No clues	Amphetamine, cholestyramine, cimetidine, clonidine, cyproheptidine, diazoxide, enalapril, histamine, phenformin, salicylates, warfarin.

(Bembow, 1988). The principles of pharmacogenetics (Idle and Smith, 1979) could explain why the most persuasive case reports linking frusemide (furosemide) and pancreatitis involved American Negroes (Buchanan and Cane, 1977), while other negroid races have been reported to possess higher intrinsic levels of certain cytochromes P450. Prior induction of cytochromes P450 by an extrinsic agent, such as grapefruit juice or St John's wort can be equally important in determining the toxicity or otherwise of a drug metabolically activated within the liver or pancreas (Azuma *et al*., 1998; Lee *et al*., 2006). This connection is recognized in

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the case of paracetamol (acetaminophen)-, or carbon tetrachloride-associated acute liver toxicity. Reports on drug or chemically related acute pancreatitis sometimes mention alcohol, but seldom, if ever, whether the patient was a smoker or whether the diet contained substantial amounts of C18 : 2 fatty acids. Antioxidant status is also generally not reported in these case histories. Finally a case report that has a bearing on the pathogenesis and treatment of drug-induced pancreatic damage is worth discussing. This report concerned a man who died within 12 hours of consuming a large amount of theophylline, and who showed all the neurological manifestations ascribed to blockade of adenosine receptors (Burgan *et al*., 1982). The authors concluded that he did not have pancreatitis at autopsy because there was no histological evidence of inflammation. His blood amylase level was 10 times the upper limit of normal five hours after tablet ingestion and had doubled within the next three hours, increments that typically accompany the pancreastasis phase of experimental pancreatitis. Blockade of adenosine receptors on plasma membranes of pancreatic acinar cells (Yamagishi, 1986) would be expected to compromise cell stores of SAMe and ATP (**Figure 7**).

There are several case reports of acute pancreatitis after intentional or accidental exposure to organophosphate insecticides in children (Weizman and Sofer, 1992) and adults following inhalation (Hsiao *et al*., 1996) or cutaneous exposure (Marsh *et al*., 1988), with agreement that excessive cholinergic stimulation of pancreatic acinar cells is the damaging mechanism (**Table 3**).

There are several reported associations between inhaled chemicals and the induction of pancreatitis, including dimethylformamide (Chary, 1974), pentachlorophenol (as part of a wood preservative which also contained zinc naphthanate) (Cooper and MacCaulay, 1982) and trichloroethylene, volatile petrochemical products and diesel/petrol exhaust fumes (Braganza *et al*., 1986). It is recognized that many aliphatic/halogenated hydrocarbons undergo bioactivation, and hence that prior enzyme induction has the potential to magnify any tissue damage (Sato *et al*., 1980; Strubelt, 1980; Plaa and Hewitt, 1982; Koop *et al*., 1985). As little as 30 g alcohol may thus precipitate an attack of pancreatitis some 10 hours later, following exposure to metabolically activated chemicals in the home or work environment. A genuine link between inhaled hydrocarbons and acute pancreatitis would rationalize the observation that postoperative pancreatitis, after halogenated hydrocarbon anaesthetics, occurred even when the operation was nowhere near the gland (Howat and Sarles, 1979; Go *et al*., 1993).

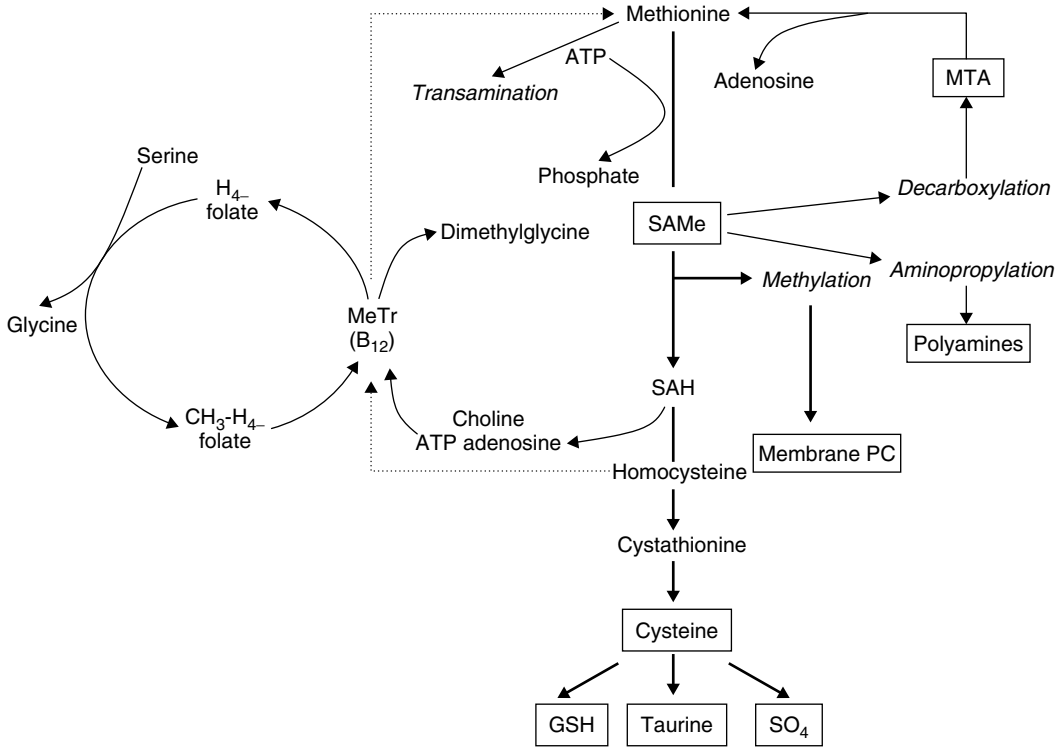

Figure 7 Pathways of methionine metabolism. SAMe = sulfadenosylmethionine; MTA = methylthioadenosine; SAH = sulfadenosylhomocysteine; GSH = glutathione; SO_4 = inorganic sulfate; CH_3-H_4-folate = methyltetrahydrofolate; Me Tr = methyl transferases. (Reproduced with permission from Braganza, 1991. © Manchester University Press.)

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Table 3 Acute pancreatitis: agents/routes

	Human	Experimental
Ductal	Migrating gallstone	Duct clamp ± secretion
	Pancreas divisum	Duodenal obstruction ± infection[a]
	ECRP	—
	Ampullary cancer	Retrograde bile ± enzyme[a]
	Duodenal diverticulum	Prograde bile ± aspirin/ethanol[a]
	Afferent loop obstruction	—
Arterial	Hypothermia	Ischaemia/reperfusion
	Translumbar aortography	Oleic acid infusion into artery
	Abdominal aneurism repair	Glass beads into splenic artery[a]
	Vasculitis	
	Serum triglycerides $>20\,\text{mmol l}^{-1}$	
Acinar	Trauma	CDE diet[a]
	Virus (mumps, HIV, hepatitis B)	Drugs
	Hypercalcaemia; porphyria	Chemicals
	Chronic renal failure	
	Inflammatory bowel disease	
	Surfeit after fast	
	Anorexia nervosa	
	?Pregnancy	
	Total parenteral nutrition	
	Essential fatty acid deficiency	
	Reye's syndrome	
	Haemolytic uraemic syndrome	
	Drugs and chemicals	
Mixed	Alcoholic debauch	Pancreas allograft[a]
	Cardiopulmonary bypass	Supramaximal CCK
	Pancreas-renal transplant	—
	Insecticide	Insecticide
	Scorpion bite	Scorpion bite

[a]Typically haemorrhagic.

3.1.2 Chronic Pancreatitis

Alcoholism, cyanogenic glycosides, drugs such as azathioprine, oestrogens and sodium valproate, and high fat/protein diets are among the accepted environmental risk factors. Unfortunately there is no reproducible animal model of alcoholic pancreatitis and hence detailed investigation of the aetiology still remains to be achieved. The current dogma for the human disease is that there is no threshold for alcoholic toxicity to the human pancreas and that among patients with chronic pancreatitis, 'the logarithm of risk increases linearly in terms of the mean amount of alcohol consumed each day' (Howat and Sarles, 1979). A major problem has been the difficulty in providing laboratory animals with a nutritionally balanced diet while ethanol is administered (Go *et al.*, 1993). When this is accomplished, biochemical changes akin to those in alcoholics with or without chronic pancreatitis do occur after some 12 months, for example, increased amounts of pancreatic zymogens with increased fusion between zymogen and lysosomal compartments (Singh, 1992). The introduction of the rat gastric infusion model (Horn and Tsukamoto, 1993) has made it easier to dissociate the effects of alcohol from those of high fat intake, the former leading to hypogranulation and apoptosis of pancreatic acinar cells with additional dietary fat exaggerating the effects. The combination also affected the way in which the pancreas subsequently responded to CCK, inhibiting the binding of the hormone to receptors and reducing the pancreatic secretory response. At least in the rat, CCK is a major mitogen for the exocrine pancreas and interference with its action could inhibit regeneration of pancreatic cells following an insult.

While not diminishing the usefulness of these animal studies they have limited applicability to the clinical situation since they are unable to take into account the complex lifestyle and generally inadequate dietary intake that alcoholics engage in where it is known that protein intake and smoking are significant risk factors in the development of alcoholic pancreatitis (Howat and Sarles, 1979).

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Ethanol is largely metabolized by cytosolic dehydrogenases, but protracted exposure brings the microsomal cytochrome P450 2E1 system into play. The dehydrogenase pathway generates fatty acid ethyl esters while the cytochrome P450 pathway generates ethinyl radicals and reactive oxygen species (ROS). Both systems lead to the production of the highly toxic acetaldehyde metabolite. Acetaldehyde has been shown to inhibit mitochondrial function and to form adducts with constituents of the cytoskeleton that could jeopardize secretion, provoke a reversal in secretory polarity and/or form adducts with constituents of the plasma membrane to generate chemotactins (Lieber, 1988; Werner *et al*., 1997; Israel, 1997). These effects would be compounded by changes in CCK-receptor binding and ethanol-induced hypertriglyceridaemia (**Table 2**).

In the case of alcoholic hepatotoxicity, the emphasis has shifted away from mitochondrial damage and on to cytochrome P450 2E1-mediated production of reactive intermediates from drugs/chemicals to which an individual is simultaneously exposed. This mechanism is attractive for alcoholic damage to the exocrine pancreas also, not least because it could rationalize the resistance to ketosis when patients become diabetic, because ketones would be more rapidly processed. Computer-assisted discriminant analysis of factors contributing to accelerated theophylline clearance in patients from Manchester, UK (Acheson *et al*., 1989) identified cigarette usage as the major influence, with a contribution from dietary protein and other factors, irrespective of whether or not the patients were alcoholic.

In regard to nonalcoholic disease, it is interesting to note that many of the drugs, incriminated in chronic pancreatitis, can be metabolized to reactive intermediates. Furthermore, recent studies of European patients suggest an aetiological connection between regular inhalation of volatile chemicals, usually in the occupational environment, and chronic pancreatitis in nonalcoholics (Dossing *et al*., 1985), as well as in exalcoholics (Braganza *et al*., 1986). Those chemicals included paints, solvents and thinners, diesel/petrol fumes, degreasing agents and dyes. The connection was confirmed in a large case-control study in Manchester, UK (McNamee *et al*., 1994). A similar range of chemicals emerged in several patients with nongallstone recurrent acute pancreatitis or pancreatic cancer (Kemppainen and Puolakkainen, 2007) (**Figure 8**).

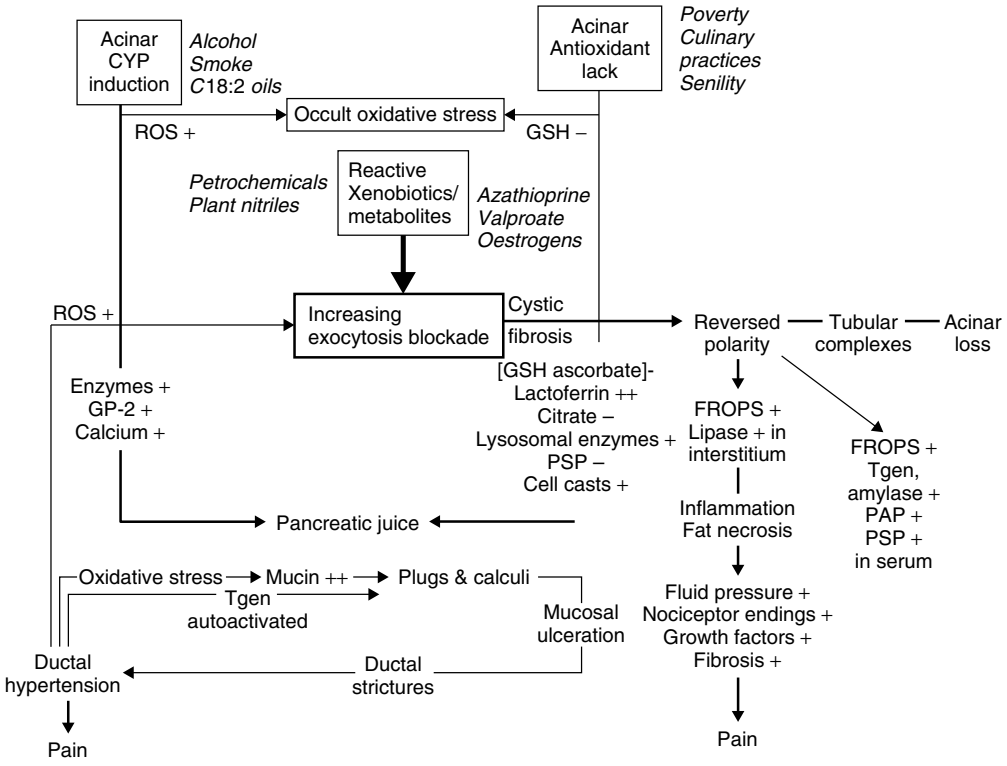

Figure 8 Mechanism for the interaction of chemicals and drugs with the pancreas in the induction of pancreatic pain. CYPs, cytochrome P450 monooxygenases; ROS, reactive oxygen species; GSH, glutathione; FROPs, free-radical oxidation products; T-gen, trypsinogen; PAP, pancreatitis associated protein; PSP, pancreatic stone protein; HOCl, hypochlorous acid; GP-2, glycosylphosphotidylinositol-anchored protein from the zymogen granule membrane; +, increased;−, decreased; [], predicted change not yet proven. (Reproduced from Braganza, 1996. © Oxford University Press.)

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An animal model of pancreatitis has been achieved by feeding young Syrian hamsters for six months with a corn-oil-enriched diet, supplemented with either β-naphthoflavone or phenobarbital, to induce cytochromes P450 1 or P450 2, respectively. The hepatobiliary and pancreatic changes associated with chronic pancreatitis were reproduced in this model and high concentrations of FROPs were found in bile, with excess deposits of lipofuscin and pancreatic lipoatrophy. These results are consistent with a chronic elevation of ROS accompanying the development of pancreatitis (Rutishauser *et al*., 1995). The hamster was chosen for these experiments because of their high intrinsic rate of drug metabolism and because this species is peculiarly vulnerable to pancreatic carcinogenesis by nitrosamines that require bioactivation for genotoxicity (see below).

With regard to tropical, nonalcoholic, chronic pancreatitis as is seen in the Far East, it has for long been accepted that hydrogen cyanide released from the dietary staple cassava (manioc, tapioca) is a major determinant. Cassava contains the cyanogenic glycosides, linamirin (2-((-D-glucopyranosyl-oxy)isobutyronitrile)) and lotaustralin (methyl linamirin). However, the acute administration of hydrogen cyanide to rats results in death from respiratory failure and diabetes, but not in chronic pancreatitis. It has been suggested that, based upon the findings of damage to other organs in an experimental setting, a possible alternative explanation for this disease residing in a non-cyanide-generating chemical in cassava (Ballantyne, 1987). This hypothesis is supported by reports that in rats, another plant nitrile, 1-cyano-2-hydroxy 3-butene(nitrile), caused biphasic changes akin to those found in human tropical pancreatitis, namely, early hypersecretion of pancreatic juice and bile followed by loss of pancreatic nonprotein thiols and ZGs, with vacuolation of the acinar cell cytoplasm and inflammatory oedema (Maher *et al*., 1991). Further, it has been shown that subtle differences in nitrile structure spared the pancreas, but caused liver and kidney damage (Wallig *et al*., 1988). The dissociation between the changes due to reduced protein intake and those due to the nitrile, and the additive effect when these problems coexisted, was demonstrated in another study which confirmed that the pancreatic acinar cell was the primary target, despite descriptions of mucin-choked ducts in the human disease (Geldof *et al*., 1992).

One area where such changes in the ductal system have been described is in Madras in southern India, where chronic pancreatitis is relatively common (Mohan *et al*., 2003). A study of xenobiotic exposures among these people revealed that many patients with chronic pancreatitis were in regular close contact with smoke (from firewood, vehicle emissions or cigarettes) and petrochemical products, especially kerosene fumes, from lamps or cookers (Braganza *et al*., 1990). These links, if validated by case-control studies, could rationalize cardiomyopathy in those patients, considering that similar effects

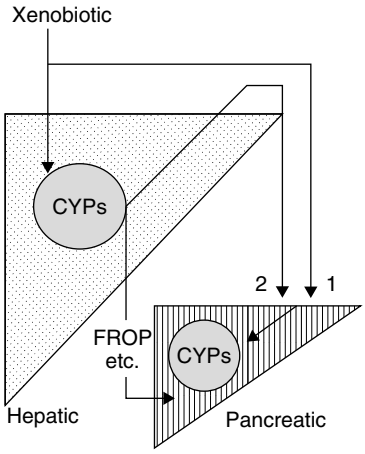

Figure 9 Possible routes of pancreatic injury from toxic metabolites of drugs/chemicals. See text for discussion. (Reproduced with permission from Braganza, 1991. © Manchester University Press.)

accompany kerosene toxicity in experimental animals (Rai and Singh, 1980), and also the decline in disease incidence in Kerala, India, in the past few years in line with increasing electrification. Although there are no reports of kerosene metabolism by the pancreas, studies of the liver strongly suggest the generation of highly toxic metabolites (Starek and Kaminski, 1982), including acrolein. The regular use of C18:2 fatty acids in cooking oil (linseed, peanut) would facilitate prior enzyme induction, as would any polycyclic aromatic hydrocarbons in smoke, and in situations of malnutrition where antioxidant content would be suboptimal, tissue damage could occur from exposure to nitriles or kerosene.

We thus conclude that the template for chronic pancreatitis in **Figure 9** accommodates many of the experimental and clinical observations to date, not least the low prior antioxidant status in some African communities, where chronic pancreatitis is endemic (Segal, 1998; Segal *et al*., 1995). A number of reports on dietary insufficiencies now endorse the oxidative stress philosophy behind the development of chronic pancreatitis (Wesson *et al*., 2008; Mishra, 2007; Merentie *et al*., 2007; Schoenberg *et al*., 1995).

3.1.3 Experimental Non-Neoplastic Injury

Individual references are given in **Table 4**. Some agents (asterisked) generate toxic metabolites, including oxygen radicals, organic intermediates or both. It is unclear why excess corn oil in the diet can, of its own, cause cytochromes P450 1 induction in hepatocytes of Syrian hamsters after six months, but this is associated with an increase in hepatic lipid peroxidation and spotty pancreatic lipoatrophy (Rutishauser *et al*., 1995). The degree of enzyme induction was similar to that achieved

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Table 4 Chemicals and non-neoplastic experimental pancreatic injury

Chemical[a]	Injury[b]	System[c]	Species	Reference
Abrin	3,5	i, v	Rat	Barbieri *et al*. (1979)
Acetaldehyde*	3	i, iv	Rat, dog	Majumdar *et al*. (1986)
Allyl alcohol*	3	i	Rat	Nizze *et al*. (1979)
Lysine, phenylalanine	1,3	i	Rat	Kitajima and Kishino (1985), Bieger and Kern (1975)
Aminomethyl-α-carboline	4	i	Rat	Takaya *et al*. (1985)
Azathioprine*	5	ii	Dog	Broe and Cameron (1983)
β-Adrenergic agonists	5	i	Rabbit	De Dios *et al*. (1989)
Bendamustin*	4	i	Rat, mouse	Horn *et al*. (1985)
Bile salts	1	ii	Cat	Hong *et al*. (1988)
Caerulein, CCK, and so on	1	i, iv	Several	Go *et al*. (1993)
Carbon tetrachloride*	2,3,4	i	Rat	Veghelyi *et al*. (1950a)
Chlorothiazide*	1,3	i	Mouse	Cornish *et al*. (1961)
Cobalt chloride	5	i	Rat	Kern and Kern (1969)
Corn oil (20%)*	4	i	Hamster	Parker *et al*. (1986)
Corticosteroids*	1,3,4	i	Rabbit	Benscombe and Lazarus (1956)
1-Cyano-2-hydroxy-3-butene	3,6	i	Rat	Wallig *et al*. (1988)
Cyclosporin	5	i	Rat	Mueller *et al*. (1988)
Cythion*	3	i	Teleost	Wallig *et al*. (1988), Ray and Bhattacharya (1984)
Diazinon*	1,3	i	Guinea pig, dog	Frick *et al*. (1987)
Dibutyl dichloride	1	i	Rat	Merkord and Hennighausen (1989), Sparmann *et al*. (1997)
2,4-Dinitrophenol*	1,3	i, iv	Rat	Letko *et al*. (1989)
Endrin*	4	i	Teleost	Datta and Ghose (1975)
DL-Ethionine*	1–6	i, iv	Several	Go *et al*. (1993), Rutledge *et al*. (1987)
Ethanol*	2–6	i, iv	Several	Go *et al*. (1993), Wilson *et al*. (1986)
Fenofibrate	3	i	Rat	Price *et al*. (1986)
Galactose	3	i	Rat	Putzke and Bienengraber (1967)
1,2,4,5,7,8-Hexachloro(9H) xanthene	3	i	Guinea pig	DeCaprio *et al*. (1987)
Modofinil	5	i	Rat	Chariot *et al*. (1987)
Monosodium glutamate	5	i	Rat	Lee and Sheen (1988)
Oleic acid	1	li	Cat	Sanfey *et al*. (1986)
Palmitoylpentachlorophenol	3,6	i	Rat	Ansari *et al*. (1987)
Pancreatic enzymes	1	ii	Cat	Hong *et al*. (1988), Letko *et al*. (1989)
Paraquat*	3,4	i	Carp	Benedeczky *et al*. (1986)
Petrochemicals*				
Crude	4	i	Chick	Nwokolo and Ohale (1986)
Diesel oil	3	i	Trout	Poirer *et al*. (1986)
Tetracycline	5	i, iii	Pigeon	Tucker and Webster (1972)
Puromycin	1,3	i	Rat	Longnecker (1991)
Toxin: paradysentery	3,4	i	Rat	Veghelyi *et al*. (1950a)
Trichothecine (mycotoxin)	3	i	Pig	Pang *et al*. (1986)
Triethyl citrate	6	i	Rat	Ohtaki *et al*. (1985)
Uraemia toxins	3,5	i	Rat	Krempien and Grosser (1987)
Scorpion venom	1	i	Several	Williams *et al*. (1982), Murthy *et al*. (1989)
Sand viper venom	1,3	i	Rat	Hodhod *et al*. (1989)
Vinblastine/vincristine	3,4,5	i	Mouse	Nevalainen (1975)

[a]Asterisk items can generate reactive oxygen species and/or reactive intermediates.
[b]Pattern of injury −1 = acute pancreatitis; 2 = chronic pancreatitis; 3 = toxic (microvesiculation ± lipofuscin ± membrane blebbing ± dilated ER ± leakage of LDH, and so on; 4 = degenerative (loss of polarity ± tubular complexes ± cysts ± atrophy; 5 = reduced protein and/or phodpholipid synthesis or secretion; 6 = immune, vascular, apoptotic P450 mediated, and so on.
[c]System used for observations: i = whole animal; ii = isolated perfused gland; iii = gland slices; iv = dispersed acini; v = subcellular fraction.
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following phenobarbitone or ß-naphthoflavone given with a low-corn-oil diet in these experiments, and those reported by others (Scarpelli, 1989).

The pancreas seems to be much more sensitive than the liver to damage from some toxic metabolites, judging by studies with carbon tetrachloride. where 0.07 g per 100 g body weight was sufficient to induce pancreatic lesions, while considerably higher doses were needed to induce hepatic injury. The pancreatic lesions occurring were reported to be similar to those of human chronic pancreatitis (Veghelyi *et al*., 1950a), except that features compatible with enzyme induction (**Table 5**) were lacking. Differences in the distribution of pancreatic cirrhosis nodules in different glands were ascribed to possible differences in blood supply (Veghelyi *et al*., 1950a; Veghelyi and Kemeny, 1962), which in turn could reflect variations in islet-acinar 'portal' circulation, especially now that certain cells in pancreatic islets have been shown to contain easily inducible cytochromes P450 (**Figure 10**).

The changes in the pancreas brought about by the long-term administration of hypolipidaemic agents are especially interesting because, in similarity to the effects seen after dietary methionine deprivation followed by refeeding, acinar cells metamorphose into fully functional hepatocytes (Rao *et al*., 1986). Not only do these findings underline the common ancestral lineage of the liver and pancreas from the evolving foregut (Braganza, 1986; 1988a), but they also show that repair mechanisms, after any form of toxic insult in either tissue can potentially result in its conversion to the alternate cell type (Rao *et al*., 1986; 1989). When pancreatic injury is caused by interference with protein synthesis, it is hardly likely that antioxidants will find use in prophylaxis or treatment. However, where a reactive intermediate is involved, glutathione (GSH) precursors and/or vitamin C could be useful.

4 CHEMICALS IN CANCER OF THE PANCREAS

4.1 Human Pancreatic Cancer

Cigarette smoke has the clearest association with the development of human pancreatic cancer. It is recognized that each intake of cigarette smoke delivers approximately 1015 free radicals in the gas phase (e.g. oxides of nitrogen) and 1014 free radicals in the tar phase (e.g. from benzo[a]pyrene) (Lu *et al*., 2008). Smoke contains large numbers of toxic chemicals, but it also has two main classes of potentially genotoxic chemicals—polycyclic aromatic hydrocarbons (Freudenthal and Jones, 1976; Coggins and Gaworski, 2008) and nitrosamines (Lijinsky, 1987; Bacsik *et al*., 2007), and several potential epigenetic agents (Williams, 1984; Upham *et al*., 2008), including catechols and phenolic compounds.

Several occupations, and linked chemical exposures, have been implicated in the development of pancreatic cancer (**Table 6**), but for each positive epidemiological study there are several others that do not support a connection (Alguacil *et al*., 2003; Bond *et al*., 1985; Hanis *et al*., 1982; Hotz *et al*., 1990; Rushton and

Table 5 Pancreas: target for chemical toxicity

	Liver	Pancreas
Compatible with enzyme induction	↑ Size of hepatocytes	↑ Size of acinar cells
	↑ Smooth endoplasmic reticulum	↑ Rough endoplasmic reticulum
	↑ Very-low-density lipoprotein (VLDL) to serum	↑ Protein to PJ
	↑ Phospholipid to bile	↑ Calcium to PJ
	↑ Drug metabolism	
	$BSPk_1$	
	Antipyrine clearance	? Pancreatic contribution
	Theophylline clearance	
	Urine D-glucaric acid (DGA)	
	↑ Cytochromes P450	↑ Cytochromes P450
Suggests oxidative stress and mobilization of natural antioxidants	↑ Microvesicular fat	↑ Microcirculation
	↑ Lipofuscin	↑ Lipofuscin
	↑ FROPs in serum	'Pancreastasis'
	↑ FROPs in bile	'Tubular complexes'
		↑Lysosomal enzymes in pancratic juices (PJ)
		↑Lactoferrin in PJ
	↑ Ferroxidase 1 in serum	↑ Mucin in PJ

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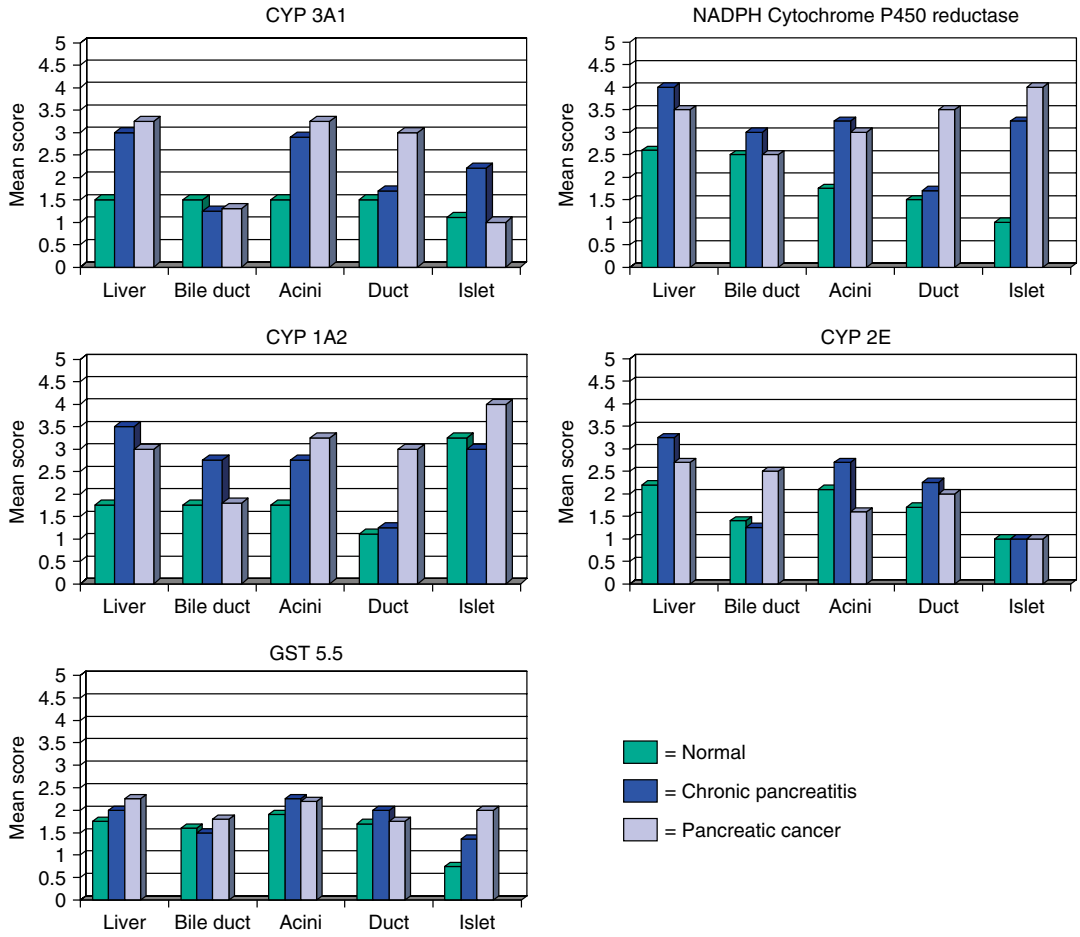

Figure 10 Summary of immunohistochemical findings using a panel of monospecific antibodies against different drug-metabolizing enzymes in subjects with and without exocrine pancreatic disease. (Reproduced with permission from Foster, *et al*., 1993.) CYP = cytochrome P450; Acini = pancreatic acini; Duct = pancreatic duct activity; Islet = islet activity; GSH 5.5 = glutathione S-transferase 5.5; AP = acute pancreatitis; CP = chronic pancreatitis; PAHS = polycyclic aromatic hydrocarbons.

Alderson, 1981; Clapp, 2006). In their review Pietri and Clavel (1991) concluded that despite these reservations there does appear to be a significant risk from the chemical and petroleum industries.

The fractionation of petroleum gives rise to many of the substances associated with pancreatic cancer (**Table 6**). This process yields natural gas (C1–2), used for fuel and in the chemical industry; liquefied or bottled gas (C3–4), used as fuel gas for the synthesis of rubber compounds and in the petrochemical industry; petroleum ether (C4–5), for solvents and as an anaesthetic; gasolines (C6–l0) in cleaning fluids, solvents and for refining stock; kerosenes (C5–l6), used as jet, tractor and gas turbo-fuels, and in lamps and stoves in underdeveloped countries; gas-oil (C9–l6), used as diesel or furnace oil; lubricating stocks (C > 17), for white oils, lubricating oils and greases; waxes (C > 20), for ceiling wax and bottoms (C > 20), used as heavy fuel oil, road oil and asphalt. Suspicion that chronic pancreatitis in tropical zones may increase the risk for pancreatic cancer (Balakrishnan, 1987) would be rationalized if smoke and kerosene fumes were to be involved, and the mutagenicity of airborne particles from a kerosene heater has been demonstrated (Yamanaka and Maruoka, 1984). Similarly, it is not difficult to see why chronic pancreatitis in temperate zones may be a forerunner of pancreatic cancer, since aliphatic/aromatic halogenated hydrocarbons in solvents, degreasing agents, paint thinners and so on, seem to be involved in each condition (**Figure 11**) (McNamee *et al*., 1994). These hydrocarbons, whose potential genotoxicity is recognized (Schwartz and Brandt, 1989), are ubiquitous (Aviado *et al*., 1976; Nicholson and Moore, 1979) in the polluted atmosphere of industrialized and traffic-congested cities.

Other suspected risk factors for pancreatic cancer could be rationalized through promotion of cytochromes P450 activity. Thus, the increased susceptibility of American blacks may be due to a higher intrinsic level of certain cytochromes P450 (Idle and Smith, 1979). The interaction between genetic and environmental factors

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Table 6 Occupational exposure to chemicals and human pancreatic cancer

Chemical	Reference
Benzene	Wocka-Marek *et al*. (1987)
Benzidine	Mancuso and El-Attar (1967)
β-Naphthylamine	Li *et al*. (1969)
Coal tar	Registrar General's Decennial Supplement (1958)
	Redmond *et al*. (1976)
Coal pitch	Turner and Grace (1938) Dorken (1964)
Dichlorodiphenyltrichlroroethane (DDT)	Garabrant *et al*. (1992)
Dry cleaning solvents	Lin and Kessler (1981)
Ethylene chlorohydrin	Benson and Teta (1993)
Methylene chloride	Friedlander *et al*. (1978)
	Ott *et al*. (1985)
	Hearne *et al*. (1990)
Paints, varnishes and other petroleum	Lin and Kessler (1981)
products	Norell *et al*. (1986)
	Thomas *et al*. (1982)
Plastics	Selenskas *et al*. (1995)
Printing industry	Lloyd *et al*. (1977)
Rubber workers	Monson and Fine (1978)
	Delzell *et al*. (1981)
Stone mining and wood industries	Partanen *et al*. (1994)

influencing cytochromes P450 may explain the occurrence of pancreatic cancer in siblings and kindreds with hereditary chronic pancreatitis. It would also rationalize the report of pancreatic cancer in a chemical worker and his son who were exposed to vinyl chloride and other substances (Reiner *et al*., 1977). The association between high-fat diets and pancreatic cancer may reflect the steady increase in consumption of polyunsaturated fatty acids (PUFA), especially C18:2, in corn-oil by Western societies in the past 50 years (Hollander and Tarnawski, 1986). Continued suspicion that alcohol may be a risk factor is understandable now that its inducing effect on cytochrome P450 2E1 is appreciated. It is hardly surprising that these potential interactions could not be dissected out in the study relating expression of different pancreatic cytochromes P450 (**Figure 10**) and exposure to xenobiotics (Foster *et al*., 1993). Failure or inability to document prior exposure to enzyme inducers or antioxidant status could also explain discrepant results concerning chemicals and human pancreatic cancer in some studies (Li *et al*., 1969; Hoar and Pell, 1981; Mack and Paganini-Hill, 1981; Gold *et al*., 1985).

4.2 Experimental Pancreatic Cancer

The inhalation route of xenobiotic entry, which may be very relevant to the pathogenesis of human pancreatitis and also pancreatic cancer, has only rarely been used in experimental studies in laboratory animals. One such study (Malley *et al*., 1995) used the 1,1-dichloro-2,2,2-trifluoroethane, a potential substitute for the ozone-depleting hydrocarbons, and showed the induction of exocrine pancreatic cancer when given by inhalation exposure to rats for two years. This compound led to the development of acinar cell adenomas at dose levels down to 300 ppm in males, while also inducing acinar hyperplasia, but not neoplasia, in females. It was also found to be a potent hypolipidaemic agent and peroxisome proliferator in rodent liver. Although the relationship between this hepatic effect and the pancreatic lesions was unclear, several other compounds in the class of hypolipidaemic agents, for example, nafenopin and clofibrate (Reddy and Rao, 1977; Longnecker, 1983), also induce the development of pancreatic cancer in the rat.

Experimental studies on the development of pancreatic cancer have largely focussed on a ductal phenotype tumour as produced by the nitrosamine, *N*-nitroso-bis-(2-hydroxy-propyl) amine (BHP), in hamsters (Go *et al*., 1993; Pour and Lawson, 1984), and an acinar phenotype tumour from *O*-diazoacetyl-L-serine, azaserine, in rats (Mäkinen *et al*., 1998; Longnecker *et al*., 1984) (**Table 7**). Transgenic mouse models have also been described involving either an elastase enhancer/promote–SV40 T-antigen construct (Ornitz *et al*., 1987), or an elastase enhancer/promoter–myc oncogene construct (Sandgren *et al*., 1990). The former has an acinar phenotype, while the latter may have a ductal or mixed phenotype.

The spectrum of cancer precursor lesions in the BOP and azaserine models includes multiple neoplasias and neoplastic precursor lesions. They generally show the full spectrum of lesions from the atypical acinar cell focus, consisting of cells with increased nuclear size, prominent nucleoli, increased mitotic index, cytoplasmic

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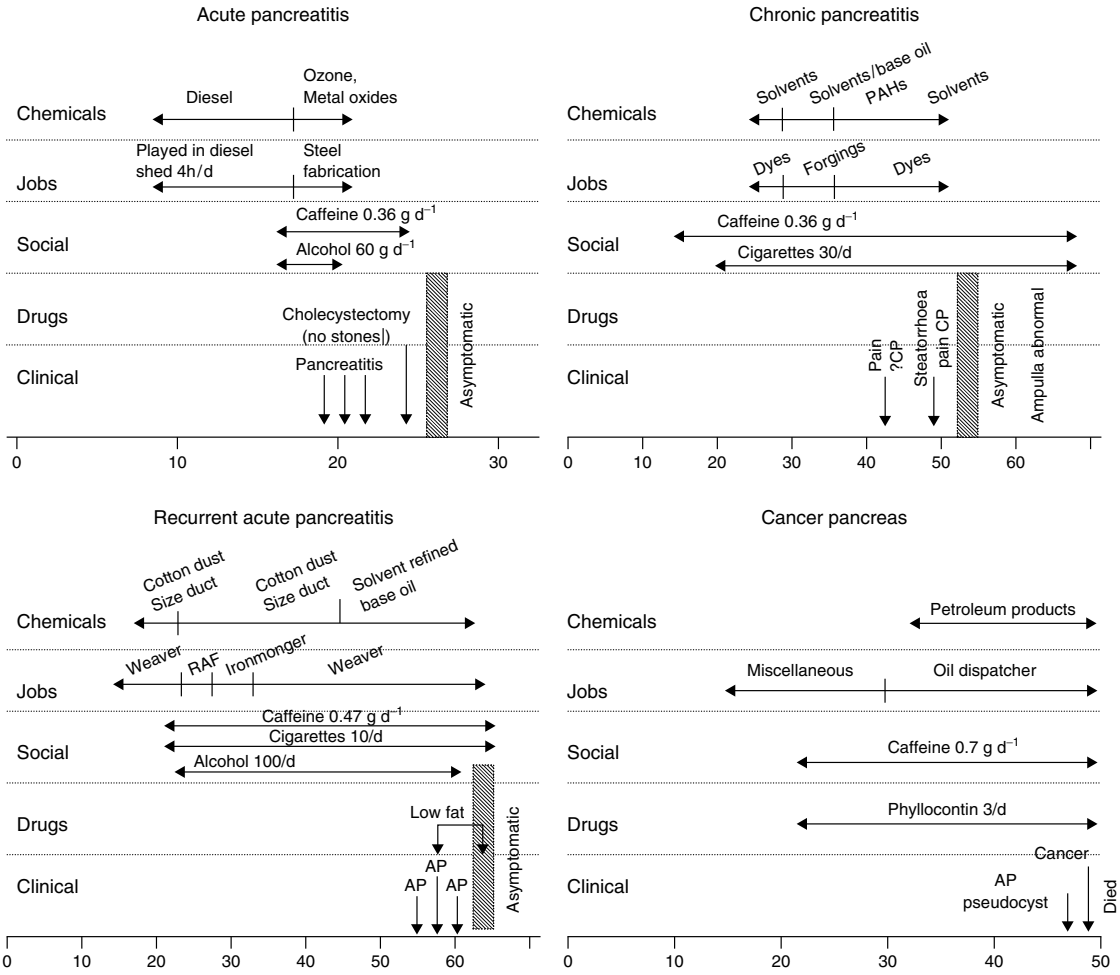

Figure 11 Examples of xenobiotic exposure histories in four patients in Manchester, UK. RAF = Period in the UK Royal Air Force; AP = acute pancreatitis; CP = chronic pancreatitis. The numbers on the x axis are years.

basophilia and reduced zymogen content, the atypical acinar cell nodule, similar in all respects to the focus except for its larger size, the acinar cell adenoma with size more than 3 mm and a tendency to capsule formation, the cystic ductal complex with one or more luminal spaces lined by flattened epithelium containing some cells that possess ZGs, the tubular ductal complex with small lumina and finally, carcinoma-*in-situ*. The last three lesions and papillary hyperplasia are characteristic of BOP-induced lesions in hamsters, whereas the first three are typical of cancer in rats following exposure to many carcinogens (Go *et al*., 1993).

Several oxidized derivatives of dipropylnitrosamine (**Figure 12**) can produce pancreatic cancer in hamsters, but BOP is the most pancreas-specific and carcinogenic potency lies in the order MOP (*N*-nitrosomethyl-2-oxopropylamine) > BOP > HPOP (*N*-nitroso-bis-2-(hydroxypropyl)-2-oxopropyl)amine) > BHP (*N*-nitroso-bis-2-hydroxypropylamine) (**Table 7**). HPOP exists as a tautomeric mixture of the open chain and cyclic forms (**Figure 12**). It is not surprising therefore that the cyclic form, *N*-nitroso-2,6-dimethylmorpholine (NDMM), which is a derivative of morpholine, also has carcinogenic potential. It resembles the endocrine cell toxin streptozotocin, which may help to rationalize the variable effects on exocrine cancer yield when this drug is given along with BOP. Structure–activity relationships have been defined for these nitrosamines (Moossa, 1980; Pour and Lawson, 1984). Thus, the presence of a keto or hydroxy group in the α-position on an aliphatic chain proved to be a prerequisite for pancreatic carcinogenicity, while the addition of a second α-group increased activity and specificity for the pancreas. The replacement of a 2-oxo chain with a methyl group diminished pancreas specificity, as did prolongation of the aliphatic chain, while carboxylation at the 3-position was associated with a complete loss of toxicity to the pancreas. HPOP and MOP seem to be more proximate carcinogens, but the ultimate carcinogen is unclear. The bulk of BOP metabolism occurs in the liver by a microsomal cytochrome-P450-dependent system, and to a much lesser extent by a cytosolic system, which utilizes NADH or NADPH (Boux *et al*., 1983). There is agreement that

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Table 7 Main animal models for pancreatic cancer

Model	Hamster	Rat
Carcinogen	BOP	Azaserine
Tumour phenotype	Ductal	Acinar
Tumour histogenesis	?	Acinar
Proximate carcinogen	HPOP, MOP, other	Diazoacetate
Activator	P450 dependent	Pyridoxyl dependent
Location	Microsomal/cytosolic	?
GSH/GSSG change	19-fold/14-fold	?
DNA damage	O^6-methylguanine	N^7-carboxymethylguanine
Potential liver contribution	+++	?
Promoters' effects	Similar (see text)	Similar (see text)
Effect of BOP		
Pancreas cancer yield	>80%	Nil
Metabolizing capacity		
Liver microsomes	+++++/+	±/++
Cytosol NADH/NADPH	+/+	±/±
Pancreas microsomes	±/+	ND/ND
Cytosol NADH/NADPH	±/+	ND/+
DNA damage		
Whole organ	++++	+
Duct/acinar cell	+++/+	+/+
DNA repair efficiency		
Whole organ	?	?
Duct/acinar cell	+/+++	++++/+++++
Mutagenicity		
Via liver S9	?	?
Via acinar cell	+	+
Via pancreas	+	++
Protein synthesis reduction	Nil	Nil
Effects of azaserine		
Pancreas cancer yield	Nil	>80%
Metabolizing capacity	+++	+++
DNA damage		
Whole organ	+++	+++
Duct/acinar cell	−/++	−/+++
DNA repair efficiency	?	?
Mutagenicity (via acinar)	+	+
Protein synthesis reduction	+	++

substantially less BOP is metabolized in the pancreas, and that exocrine cell cytosol may be the predominant site, with little (Kokkinakis and Scarpelli, 1989) or no (Boux *et al*., 1983) contribution from microsomes.

These data suggest that carcinogenic metabolites produced in the liver may enter the gland by way of the bloodstream, and in certain circumstances through bile reflux (Ruckert *et al*., 1981). This concept, which has also been proposed for human pancreatic disease (Braganza, 1986; 1988a), is supported by the greater damage to pancreatic DNA after *in vivo* treatment of animals with BOP and MOP than occurs following *in vitro* incubation of isolated pancreatic cells (Curphey *et al*., 1987), and the observation that interruption of the hepatic blood supply reduced the DNA-damaging effect of MOP *in vivo* (Schaeffer *et al*., 1984). The DNA-damaging effects were accompanied by a 14-fold increase in GSH and 19-fold increase in oxidized glutathione (GSSG) in the pancreas after BOP treatment (Toyooka *et al*., 1989). BOP was found to damage DNA in cells isolated from the whole pancreas, hence largely acinar, in doses as low as $0.5\,\mu g\,ml^{-1}$ (Levin *et al*., 1984), while HPOP was marginally less potent.

Damage to duct cells was substantially higher than to acinar cells when these were examined separately (Lawson and Nagel, 1988) and DNA repair efficiency was also much lower in ductal cells. Several specific methylated and hydroxypropylated DNA bases were identified after BOP and HPOP treatment. However, levels of O6-methyl guanine best reflected their relative carcinogenic potency (Kokkinakis and Scarpelli, 1989); while O6 and N7-alkyl guanine adducts persisted much longer in ductal than acinar cells (Bax *et al*., 1990).

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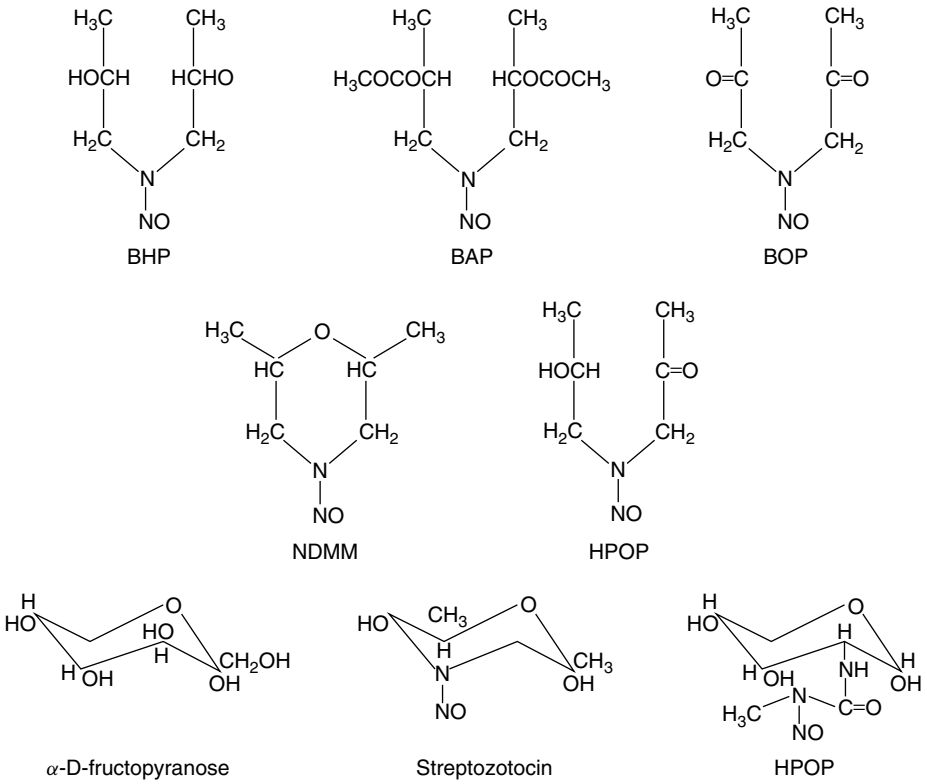

Figure 12 Chemical structures of some oxidized derivatives of dipropylnitrosamine and, for comparison, chemical structure of islet cell toxins such as streptozotocin. For abbreviations see **Table 8**.

Mutagenicity does not seem to have been tested via liver fractions. Studies of pancreatic S-9 fractions from control and polychlorinated-biphenyl-induced hamsters showed mutagenicity in strain TA100 by BOP and HPOP but not by NDMM or BHP (Mori *et al*., 1986). NDMM was mutagenic in a study with strain TA1535 as detector (Scarpelli *et al*., 1980); and use of Chinese hamster V79 cells as target showed that BOP was a more potent mutagen than HPOP (Mangino *et al*., 1985). However, carcinogenic or mutagenic potential was not reflected in protein synthesis, which was unchanged by BOP treatment (Zucker *et al*., 1988). Finally, it is noteworthy that treatment of pregnant animals with BOP caused a different spectrum of tumours in maternal and foetal tissues (Pour, 1986).

Azaserine (**Figure 13**) produces precursor lesions of the acinar phenotype in rats. As in the case of BOP, this carcinogen requires metabolic activation, but seemingly via a pyridoxal-dependent reaction yielding diazoacetate. The structurally similar 6-diazo-5-oxo-L-norleucine (DON) was found to be considerably less effective (Lilja *et al*., 1981). Metabolic activation of azaserine results in N7-carboxymethylguanine, in addition to the more usual O6 guanine adduct (Go *et al*., 1993) and the degree of pancreatic DNA damage is the same in *in vitro* as is seen *in vivo*, indicating that it is the pancreas itself that is metabolically activating the chemical and that another organ, such as the liver, is not required for carcinogenic activation (Curphey *et al*., 1987).

From the information in **Table 7**, it is clear that the species specificity of BOP and azaserine is best explained in terms of differences in pancreatic DNA damage/repair efficiency. With regard to ductal versus acinar phenotype, the facts are consistent with the hypothesis that the acinar cell may be the cell of origin for tumour induction in both species (Moore *et al*., 1983), but that species-dependent factors influence subsequent development so that the tumour acquires features of duct cells in hamsters, but retains acinar cell features in rats. **Tables 8** and **9** summarize information regarding other pancreatic carcinogens.

There is considerable literature evidence for promotion of pancreatic cancer by many factors including hormones (Skett, 1987) and dietary factors, and the gender of rats, whether or not neutered, and sex hormone replacement therapy have been shown to influence the development of azaserine-induced pancreatic cancers and precursor lesions (Longnecker and Sumi, 1990). Diets rich in soya flour have been shown to result in the development of exocrine pancreatic cancers in rats after two years (McGuiness *et al*., 1980), and an 8–10-fold stimulation in DNA synthesis was observed within as little as 48 hours (Woutersen *et al*., 1991). This effect was tracked down to the ability of soya-rich flour to inhibit trypsin in the small intestine, resulting in the feedback release of

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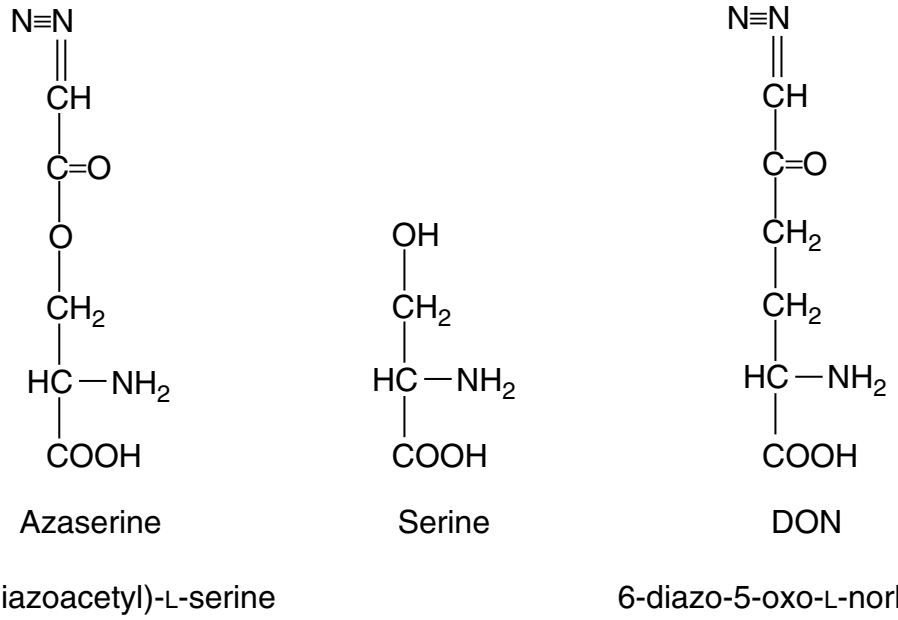

Figure 13 Chemical structure of the amino acid, serine, the genotoxic chemical analogue, azaserine and the related compound, DON (6-diazo-*S*-oxo-L-norleucine).

Table 8 Pancreatic cancer and genotoxic chemicals

Nitrosamine	Abbreviation	Species
N-Nitroso-bis-2-hydroxypropylamine	BHP	Hamster, rat
N-Nitroso-bis-2-oxypropylamine	BOP	Hamster
N-Nitroso-bis-2-(hydroxypropyl)-2-oxopropyl)amine	HPOP	Hamster, rat
N-Nitrosodimethylamine	DMN	Rat
N-Nitroso-2,6-dimethylmorphine	NDMM	Hamster
N-Nitrosomethyl-2-oxopropylamine	MOP	Hamster
N-Methyl-*N*-nitrosourea	MNU	Guinea pig, human
N-Methyl-*N*-nitrosourethane	MNUT	Guinea pig
N-Nitrosomethyl (2-oxopropyl)propylamine	NOPPA	Guinea pig
N^d-(*N*-Methyl-*N*-nitrosocarbamoyl)-L-ornithine	MNCO	Hamster, rat
4-(Methylnitrosoaminol)-1-(3-pyridyl)−1-butanone	NNK	Rat
Tobacco specific	—	Rat
Other		
2-Acetylaminofluorene	AAF	Rat
Azaserine	—	Rat
4-Hydroquinoline-1-oxide	4-HAQ	Rat

excess CCK that leads to hypertrophy and subsequently hyperplasia of the pancreatic exocrine tissue (Kato *et al*., 1994). Enrichment of the diet with corn oil achieved a similar outcome in rats (Dowling *et al*., 1987).

When a single injection of azaserine was given at the start of a 15-month experiment to compare the modifying effect of low fat intake, 5% corn oil, and high fat intake, 25% corn oil, the number of atypical acidophilic acinar cell foci increased from 472 per pancreas to 963 respectively, with a fivefold increase in adenomas and a sixfold increase in microcarcinomas in the high-fat group (Woutersen *et al*., 1991). High-corn-oil diet had a similar impact on the course of nitrosamine-induced carcinogenesis in hamsters (Go *et al*., 1993). It is not known exactly how corn oil facilitates pancreatic carcinogenesis, and factors such as the heightened release of CCK from the small intestine and direct induction of cytochrome P450 1 isoenzymes have been proposed as playing a significant role in promoting initiated cells.

Another area that cannot be covered in detail in the current review concerns the burgeoning literature on the influence of dietary antioxidant composition and experimental cancer. This is potentially of great importance,

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Table 9 Literature behind chemicals inducing experimental pancreatic cancer

Chemical	Species	References
Acrolein	Rat	Lijinsky and Rueber (1987)
Aflatoxin	Rat	Go *et al*. (1993)
2-Amino-5-niitrophenol	Rat	Irwin (1988)
Azinphos-methyl	Rat	Moossa (1980)
Azo dyes: yellow 3	Rat	Plankenhorn (1983)
Benzo[a]pyrene	Several	Moossa (1980)
1,3-Butadiene	Rat	Owen *et al*. (1987)
Chlorendic acid	Rat	National Toxicology Program (1987a), National Toxicology Program (1987b)
Chlorinated alkanes	Rat	National Toxicology Program (1986)
Clofibrate	Rat	Go *et al*. (1993)
2,6-Dichloro-*p*-phenylenediamine	Rat	McDonald and Boorman (1989)
1,1-Dichloro-2,2,2-trifluoroethane	Rat	Malley *et al*. (1995)
Dimethylaminobenzene	Rat	Moossa (1980), Watari (1985)
7,12-Dimethylbenz[a]anthracene	Rat	Go *et al*. (1993), Moossa (1980)
Dimethylhydrazine	Rat	Moossa (1980)
4-Fluoro-4-aminobiphenyl	Rat	Moossa (1980)
N-2-Fluorenylacetamide	Rat	Moossa (1980)
2,7-Fluorenylenebisacetamide	Rat	Moossa (1980), Hoch-Ligeti *et al*. (1985)
Gabapentin	Rat	Sigler *et al*. (1995)
2-Mercaptobenzthiazole	Rat, mouse	Dieter (1988)
Methylazoxymethanol acetate	Guppy	Fournie *et al*. (1987)
3-Methylcholanthrene	Mouse	Moossa (1980)
Nafenopin	Rat	Go *et al*. (1993)
Nitrofen	Rat	Go *et al*. (1993)
Toluene	Rat, mouse	National Toxicology Program (1986)

not least because antioxidants, such as pyrrolidine dithiocarbamate (PDTC) and vitamin E have been shown to enhance the antitumour efficacy of chemotherapeutic agents such as 5-fluoruracil and doxorubicin (Kastan, 1997).

4.3 Drug-Metabolizing Enzymes

The function of the drug-metabolizing system within the organs of the body, but particularly within the liver has been covered in detail in earlier chapters of this textbook. However, it is pertinent to review some of the key features that impact on the pancreas being a target for metabolically activated drugs and industrial chemicals. Briefly, the system acts to convert lipophilic chemical species that easily traverse membranes, into more polar, hydrophilic derivatives that are more easily eliminated from cells and from the body as a whole. The process is usually described in two phases and while in Phase I, an electrophilic group is inserted into the drug or chemical molecule, during Phase II the substituent group is conjugated with an amino acid, the tripeptide GSH or glucuronic acid, for example, which allows excretion via either the biliary tract or the urinary system. The various enzymes involved are collectively termed drug-metabolizing enzymes, but, in fact, they metabolize a wide range of xenobiotics and also endogenous intermediaries such as sterols, prostaglandins, bile acids, bilirubin, cholesterol and so on. The terminal component of the Phase I system, cytochrome P450, exists in multiple molecular forms that are classified by gene families (numbered 1–21), then subfamilies (A–E) and finally individual genes (numbered 1–13) (Nebert *et al*., 1987; Guengerich, 1989). It is widely recognized that the intrinsic level, and inducibility, of certain isoenzymes is genetically determined (Idle and Smith, 1979).

Studies of hepatocytes show that a xenobiotic traverses the plasma membrane along pathways shared by bilirubin and other organic anions, such as bromosulfophthalein (BSP) and, likewise, is transported by GSH-S-transferase B to the smooth endoplasmic reticulum for Phase I reactions. The ingredients are oxygen, NADPH, the flavoprotein NADPH cytochrome P450 oxidoreductase, the haemoprotein cytochrome P450, membrane phospholipids (Wade, 1986) and the trace element selenium (Correia and Burk, 1976). Phospholipids are vital because they hold substrate—enzyme complexes in optimal configuration, while stabilizing the active site region of the cytochrome. On insertion of activated oxygen into the lipophilic substrate, a hydroxylated polar metabolite is generally produced, which dissociates and

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moves into the aqueous environment of the cell where, if required, it becomes more polar through conjugation with GSH, glucuronic acid, sulfate, bile acids and so on. Polarity, size and charge are just some of the characteristics that determine the route of excretion, whether into bile or urine (Smith, 1973).

4.4 Pancreatic Drug-Metabolizing Enzymes

The contribution of drug-metabolizing enzymes in extrahepatic tissues in general, and in the pancreas in particular, to the overall disposition of xenobiotics *in vivo* is considered to be small, even in those organs where enzyme induction is known to occur, such as the lung and kidney. Nevertheless, drug metabolism in extrahepatic sites may be toxicologically very significant (reviewed in Braganza, 1991; Go *et al*., 2005; Pavek and Dvorak, 2008). Pancreatic cytochromes P450 are known to exist in adults of many species and their distribution within acini or ducts varies between different animal species (Baron *et al*., 1983). Pancreatic mono-oxygenases are also inducible in animals and in man (Weisburger, 2002; Weibkin *et al*., 1984; Foster *et al*., 1993). Immunohistochemical methods seem to be more effective in demonstrating these drug-metabolizing systems in the healthy pancreas than the measurement of enzyme activities, possibly due to the heterogeneous nature of the tissue and the discontinuous distribution of the enzymes concerned (Norton *et al*., 1995; Anderson *et al*., 1997).

4.5 Enzyme Induction is a Double-edged Tool

It is paradoxical that a defence reaction, enzyme induction, may have deleterious consequences. This threat arises because the cellular load of oxygen radicals inevitably increases when an increased metabolic load is placed on an organ, and more importantly because certain chemicals undergo bioactivation to highly toxic species, so that prior enzyme induction amplifies the risk of tissue damage. If antioxidants are simultaneously denied, through dietary manipulation, the risk increases further. Selenium deprivation causes newly synthesized haem to be directed, and hence lost, down the bilirubin pathway, instead of being incorporated into cytochrome P450 (Correia and Burk, 1976). It is noteworthy that not all cytochrome-P450-inducers are primarily metabolized by that system. Thus, alcohol is largely metabolized via cytosolic dehydrogenases, and cytochrome P450 2E1 assists only when intake increases substantially over long periods (Go *et al*., 2005). However, even small doses of alcohol are potent inducers of that subfamily, so that they increase the yield of, and hence potential damage from, reactive intermediates of chemicals to which the individual may be simultaneously exposed and which are processed by the same system (Sato *et al*., 1980; Strubelt, 1980; Koop *et al*., 1985). Such potentially competing chemicals include nitrosamines, paracetamol (acetaminophen) oestrogens, ketones, and aliphatic and halogenated hydrocarbons.

4.6 Pancreatic Disease: Casualty of Detoxification

4.6.1 Evidence Linking Enzyme Induction in the Liver and the Pancreas

Table 5 summarizes observations in patients from Manchester, UK, which are collectively best interpreted as indicating liver, as well as pancreatic, enzyme induction and include aspects that have been discussed in previous reviews (Uden *et al*., 1990a; Braganza, 1986; 1988b; 1991; 1996). Although attractive as a hypothesis, there is little direct support for the idea that genetic polymorphism may underlie susceptibility to chronic pancreatitis among alcoholics, even though a polymorphism in the *SPINK1* gene has been associated with a threefold increased risk of developing alcoholic chronic pancreatitis (Hanck *et al*., 2003; Liddle, 2006) and although pharmacogenetic studies showed no differences between patients and controls with regard to debrisoquine hydroxylation status (P450 2 subfamily) (Acheson, 1988), there has been reported a link between a polymorphism in the UDP glucuronosyltransferase (UGT1A7) gene and increasing risk for both pancreatic cancer and chronic pancreatitis (Ockenga *et al*., 2003). Since this enzyme is concerned with Phase 2 drug metabolism it is highly likely that drug exposure in such patients might lead to a compromised elimination profile and subsequent toxicity. DNA analysis indicated that the genotypes of GSH-S-transferase T1 and M1, or cytochrome P450 2E1 did not influence disease susceptibility (Frenzer *et al*., 1997; Yang *et al*., 2001).

Pharmacokinetic studies have been more informative. They showed a modest increase in antipyrine clearance (P450 2 subfamily), but unequivocal increase in theophylline clearance (P450 1 subfamily) (Acheson *et al*., 1989; Braganza, 1996). These conclusions applied to most, if not all patients, with chronic pancreatitis, some 50% with gallstone-related acute pancreatitis, the majority with recurrent (nongallstone) acute pancreatitis, and the few with pancreatic cancer who reported relatively early. Increased theophylline clearance was also found in patients with nonalcoholic chronic pancreatitis

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at Madras, South India (Chaloner *et al*., 1990). However, the clearance values in those patients were much lower than in the Manchester patients, which underlined the need to consider antioxidant status, rather than enzyme induction alone.

Ultrastructural studies from Europe showed an increase in the volume density of smooth endoplasmic reticulum in hepatocytes (Soames *et al*., 1988) and in the volume density of both rough and smooth endoplasmic reticulum in pancreatic acinar cells (Tasso *et al*., 1973) in patients with chronic pancreatitis. Immunocytochemistry of surgically obtained tissue fragments, using a panel of monospecific antibodies against different cytochromes P450, provided evidence of enzyme induction (Foster *et al*., 1993).

A few other studies have been published on immunohistochemical localization of drug-metabolizing enzymes in the normal human pancreas (McManus *et al*., 1987; Sasano and Sasano, 1988) but not, to our knowledge, in the diseased gland.

4.6.2 Evidence for Oxidative Stress in Liver and Pancreas

Table 5 also summarizes observations pointing to oxidative stress in liver and pancreas of British patients with chronic pancreatitis. Recent studies give clear evidence of heightened free radical activity in African patients with alcoholic disease (Gut *et al*., 1994) in whom iron overload could not be incriminated (Segal *et al*., 1996).

4.6.3 Evidence for Micronutrient Antioxidant Insufficiency

Three sets of observations suggested that a lack of dietary antioxidants may be relevant to the pathogenesis of the disease. In patients admitted with acute-on-chronic pancreatitis there is frequently a rapid, early-phase, disappearance of injected bromosulfthalein (BSP), together with high levels of bilirubin in secretin-stimulated bile. Additionally a high lactoferrin content of pancreatic juice can also be observed, especially in tropical, nonalcoholic, pancreatitis. The first two findings are reminiscent of those that accompany enzyme induction in selenium-deficient animals (Correia and Burk, 1976; Diplock, 1984) while the third reminds us that lactoferrin has appreciable antioxidant potential (Braganza and Worthington, 1995).

Home dietary inventories in patients with idiopathic chronic pancreatitis, who had not changed their diets or lifestyles between attacks, revealed significantly lower intakes of selenium and vitamin C than in age, and gender-matched, controls. Female patients also ingested lower amounts of many other antioxidants (**Figure 14**). Selenium was the best discriminator overall, and consideration of this factor, alongside theophylline clearance, a marker of cytochrome P450 1 induction, gave a discriminant line (**Figure 15**) to separate data from controls (with higher intakes of selenium for any given level of theophylline clearance) and chronic pancreatitis patients (Rose *et al*., 1986). Data from patients with acute pancreatitis were in the borderline zone. A similar demarcation emerged when blood 9,11 LA' and selenium concentrations replaced theophylline clearance and selenium intake, respectively, in a discriminant analysis (Uden *et al*., 1988; 1992), which suggested that this trace element may help to curb the isomerization pathway of free radical attack on lipids.

When these studies were extended to include a group of enzyme-induced controls, that is, patients with epilepsy on treatment with anticonvulsant drugs, the lower intakes of methionine and vitamin C emerged as the key factors in identifying patients with chronic pancreatitis (Uden *et al*., 1988). These findings strongly suggested the potential importance of reactive intermediates from xenobiotics in the development of chronic pancreatitis, compared with ROS in several animal models of acute pancreatitis and in their human counterparts (**Table 3**). GSH is a major defence against reactive electrophilic chemical species and the rate-limiting component for its synthesis, cysteine, draws on the essential amino acid methionine for its source (**Figure 7** and **Figure 16**). A previous study from Sweden had implicated a breakdown in the methionine trans-sulfuration pathway in patients admitted with an alcohol-induced exacerbation of chronic pancreatitis (Martensson and Bolin, 1986).

In a clinical trial where the diet was supplemented with essential nutrients, the combination of delivered methionine, as a precursor for GSH, along with vitamin C and selenium as GSH refurbishers (Uden *et al*., 1990b; Braganza, 1991) was shown to be effective in reducing symptoms of recurrent acute pancreatitis. SAMe was ineffective on its own, or when combined with β-carotene and selenium (Bilton *et al*., 1994). It is also interesting to note a recent report that in families with hereditary chronic pancreatitis, vulnerable members are earmarked by their poor antioxidant status, and antioxidant supplements to bolster GSH ameliorated symptoms (Prasad *et al*., 1996). Along the same lines, there is persuasive evidence to support the concept that a deficiency in pancreatic GSH is involved in tropical chronic pancreatitis and that the gland may be damaged by the concurrent ingestion of cyanogenic glycosides (Wallig *et al*., 1988).

These observations find parallels in animal studies, where selenium deficiency caused dilatation of the RER in pancreatic acinar cells, followed by cell differentiation and replacement fibrosis, as was also caused by a deficiency of zinc (Go *et al*., 1993). Copper deficiency, too, tended to result in damage to the acinar cells selectively, causing them to transdifferentiate into hepatocyte-like cells when the normal diet was resumed some eight

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weeks later (Rao *et al*., 1988). The effects of experimental methionine deficiency were shown to vary with degree of deficiency and duration. Hence, when administered carbon tetrachloride, a mild protracted deficiency resulted in pancreatic fibrosis (Veghelyi *et al*., 1950a; Veghelyi and Kemeny, 1962), whereas a more severe deficiency accelerated frank toxicity (Veghelyi *et al*., 1950a) causing vacuolation of pancreatic acinar cells and cystic dilatation of acini, while profound deficiency, along with dietary choline depletion, resulted in haemorrhagic pancreatic necrosis (HPN) (Rao *et al*., 1976).

4.6.4 Integrated Scheme

The control of the symptoms of pancreatitis by antioxidant supplementation is powerful evidence that the aberrations seen may be linked to disease pathogenesis. **Figure 9** integrates the three sets of aetiological factors—cytochromes P450 inducers, factors decreasing micronutrient antioxidant availability, xenobiotic sources of reactive intermediates—into a template for the pathogenesis of chronic pancreatitis.

Further, it seems that the gland can be damaged from reactive intermediates generated *de novo* through induced pancreatic cytochromes P450, and also from stable metabolites that are generated via induced hepatic cytochromes P450, if they entered the gland in refluxed bile or in the bloodstream (**Figure 8**). This deduction finds echoes in experimental pancreatic carcinogenesis (see below).

5 ABNORMAL PANCREAS: OBSERVATIONS AND TRADITIONAL INTERPRETATIONS IN MAN

5.1 Diseases and Definitions

Acute pancreatitis, chronic pancreatitis and pancreatic cancer are the main acquired diseases of the exocrine pancreas in man. The gland is affected in kwashiorkor and by the ageing process, it can be damaged *in utero* in the congenital disease, cystic fibrosis, and is involved in the inherited metal storage diseases, haemochromatosis and Wilson's disease (Klöppel and Heitz, 1984; Braganza, 1991; 1992).

Pancreatitis, inflammation of the exocrine pancreas, usually manifests in man as a severe attack of upper abdominal pain accompanied by a sharp rise in blood levels of pancreatic enzymes. Spontaneous and rapid resolution follows in some 60% of patients, but other patients develop a local complication, commonly a pseudocyst, or deteriorate with multiple organ dysfunction syndrome (MODS). The death rate of around 20% has

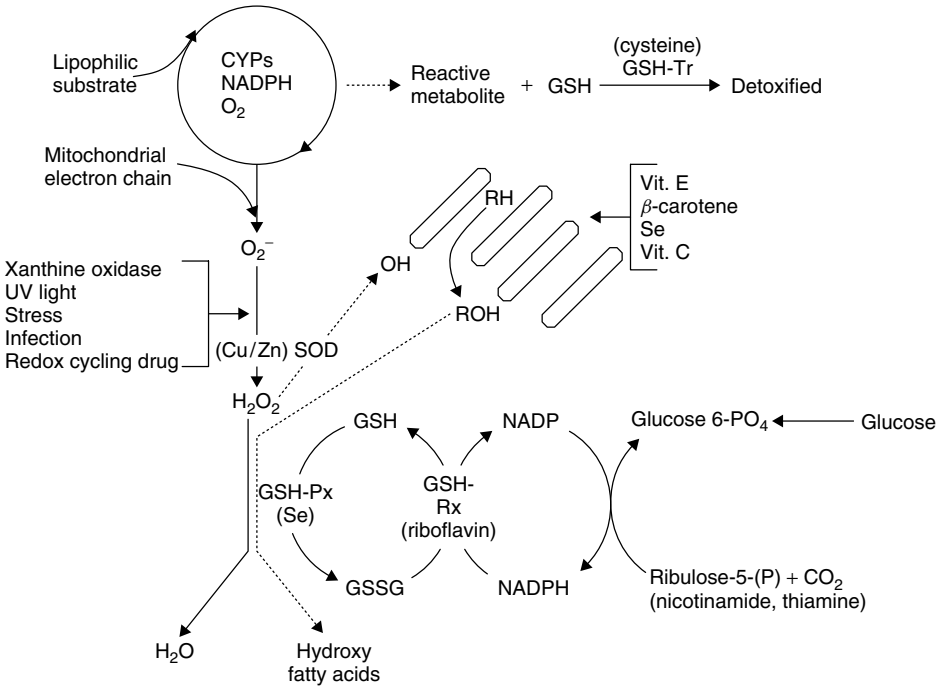

Figure 14 Schematic representation of free-radical producing and quenching mechanisms in cells. (Reproduced with permission from Braganza, 1991. © Manchester University Press.) CYPs: cytochromes P450; GSH; reduced glutathione; GSSG: oxidized glutathione; GSH-Px: glutathione peroxidase; GSH-Px: glutathione reductase; GSH-Tr: glutathione-S-transferase; SOD: superoxide dismutase. (Reproduced with permission from Braganza, 1991. © Manchester University Press.)

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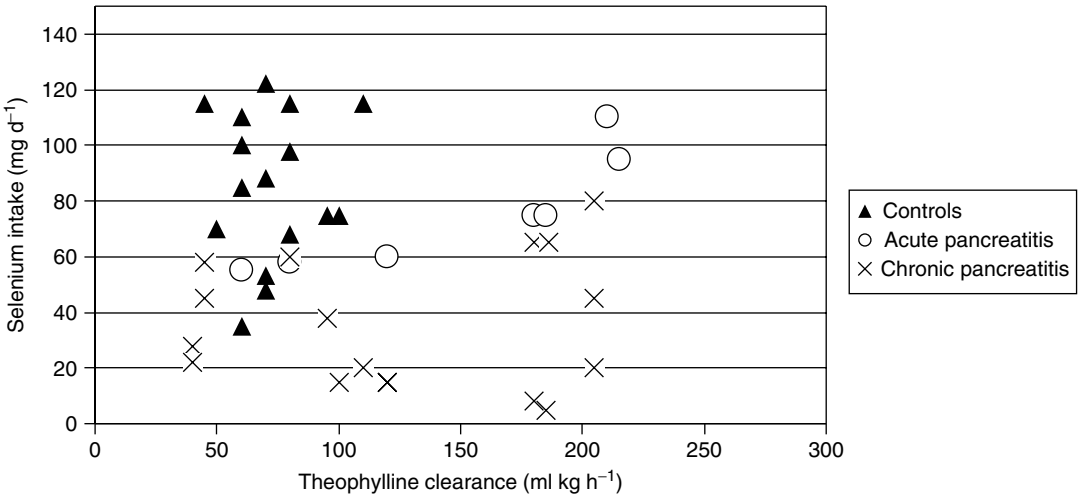

Figure 15 Discriminant analysis of theophylline clearance and selenium intake data.

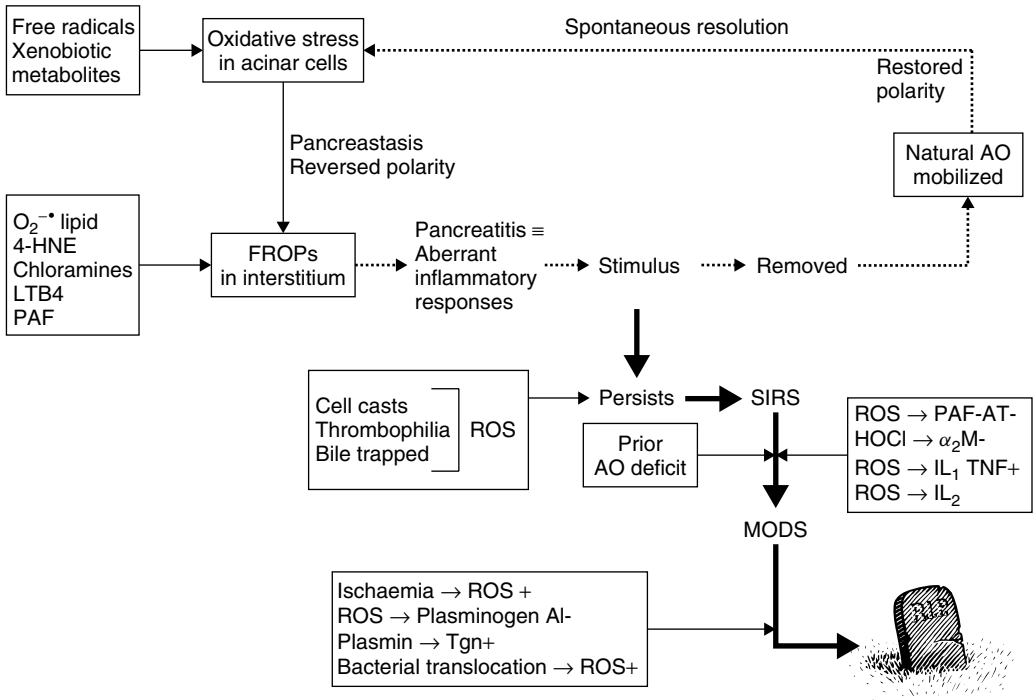

Figure 16 Proposed evolution of human acute pancreatitis. AO: antioxidants; α2 M: α2-macroglobulin; FROPs: free-radical oxidation products; 4-HNE: 4-hydroxynonenal; HOC1: hypochlorous acid; IL: interleukin; LTB4: leucotriene B4; O_2^- lipid: superoxide-altered lipid; PAF: platelet activating factor; PAF-AT, platelet activating factor-acetylhydrolase; plasminogen-A1: inhibitor of plaminogen activator; ROS: reactive oxygen species; Tgn: trypsinogen; TNF: tumour necrosis factor; ↑, up-regulation; ↓, down-regulation; MODS: multiple organ dysfunction syndrome; SIRS: systemic inflammatory-response syndrome.

not changed in the past 20 years. About half of the deaths occur within the first week, while the remainder die during the second week when the devitalized pancreatic bed becomes overrun by colonic bacteria (Braganza and Chaloner, 1995).

Pancreatitis may strike a gland that was previously normal and will return to normal when, and if, it recovers from the active lesions of oedema and/or haemorrhage and/or necrosis. The term 'acute pancreatitis' describes this situation and the same term is applied if recovery is complete following future attacks (**Figure 16**). Alternatively the attack, or recurrence, may occur against a background of chronic destruction, in which case the pathology is proof, after the superimposed lesions of active damage, and associated abdominal pain, which punctuate the clinical course of the disease, have healed.

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The term 'chronic pancreatitis' describes this development. The established disease has the characteristic triad of steatorrhoea, intraductal calculi and diabetes (Külling *et al*., 2003). It is noteworthy that pancreatic cancer may present with, or be complicated by, acute or chronic pancreatitis, while the chronic pancreatitis-like lesions of cystic fibrosis occasionally manifest as a painful attack.

Chronic pancreatitis is damaging and, although it does not usually result in death directly, reduces lifespan from associated problems such as diabetes and maldigestion. The cardinal problem in pancreatic cancer is the long presymptomatic period of tumour growth. Death follows within six months of the first symptom in most patients; hence mortality statistics give incidence, as well as prevalence rates. Between 1940 and 1980 the age-adjusted death rate from pancreatic cancer doubled in the UK, trebled in the USA and quadrupled in Japan, but there has been a levelling off in mortality rate thereafter.

5.2 Acute Pancreatitis

5.2.1 Aetiological Factors

Gallstones are the overriding risk factor. They are present in some 50% of acute pancreatitis patients in the USA and Europe, but in a much higher proportion in South America, and in more than 75% of patients over the age of 80 years, irrespective of geography (Frossard *et al*., 2008). The composition of gallstones varies widely, from almost pure cholesterol stones through to almost pure pigment stones (Braganza, 1988a). The 'migrating gallstone theory' regards transient obstruction to drainage of pancreatic fluid, when a tiny gallstone migrates from bile duct into the duodenum, as the cause of acute pancreatitis (**Table 3**). This explanation is supported by the ease with which oedematous pancreatitis is produced experimentally when the pancreatic duct is clamped transiently, and even more easily when secretin is simultaneously infused.

Certain agents seem to initiate the human disease by compromising the pancreatic microcirculation, while others may do so by interfering with the metabolism of the acinar cell. Experimental models are available to simulate these situations (**Table 3**). The acinar-ignition route of clinical pancreatitis leading to a severe outcome is nicely mimicked when young mice are reared on a diet that deprives them of the essential fatty acid, choline, and the essential sulphur amino acid, methionine, (CDE dietary model).

The following conditions may damage the pancreas by more than one route, sequentially or concurrently: an alcoholic debauch (which increases duodenal and sphincter pressure while also affecting the metabolism of pancreatic acinar cells), cardiopulmonary bypass surgery (which could compromise the pancreatic microcirculation, but also involve prolonged exposure to volatile anaesthetics which might be the true precipitant), combined pancreas-renal transplantation (when the same considerations apply with the additional possibility of drug-mediated toxicity) and pancreatic hyperstimulation, as may occur following exposure to the insecticide diazinon, or the bite of a scorpion (which not only increases ductal pressure but also alters acinar cell metabolism).

5.2.2 Pathophysiology and Progression of Acute Pancreatitis

It is generally assumed that an attack of pancreatitis represents autodigestion of the pancreas by its prematurely activated enzymes, notably elastase and phospholipase A2. The finding of these enzymes in the pancreas or ascitic fluid of animals with established HPN, or of 'activation peptides', released from trypsinogen and other precursor enzymes, in urine from animals and patients with severe disease, has been accepted as proof of this philosophy. A popular theory for premature activation of trypsinogen in patients with gallstone pancreatitis is depicted in **Figure 17**. However, it fails to explain why the experimental transpancreatic passage of bile, via an intestinal loop for many months, results in the complete activation of pancreatic enzymes, but not HPN. Another theory revolves around bacterial toxins present in infected bile or duodenal fluid, but once again definitive evidence of infection is lacking at the time of presentation in most patients. Proposals to overcome these objections include activation of trypsinogen by lysosomal cathepsin B, or altered calcium compartmentalization in pancreatic acinar cells.

Upon recovery, 'tubular complexes' appear within the pancreas. These represent dedifferentiated acini—also termed redifferentiated, transdifferentiated, retrodifferentiated or phenotypically modulated acini (Bockman, 1995). These complexes disappear in oedematous pancreatitis, but in HPN they persist until the acini are completely effaced.

5.3 Chronic Pancreatitis

5.3.1 Aetiological Factors

This subject has been extensively reviewed recently (Nair and Lawler, 2007; Behrman and Fowler, 2007; DiMagno and DiMagno, 2006; Talukdar *et al*., 2006). In developed countries the disease is regarded as synonymous with alcoholism (Apte and Wilson, 2003; Hanck *et al*., 2003; Sand *et al*., 2007), yet alcohol on its own is a weak risk factor, with some 17 years of drinking more than 150 g alcohol daily usually preceding the first symptom, such that the peak age at presentation is in the third or fourth decade. The paradox remains that a few patients report

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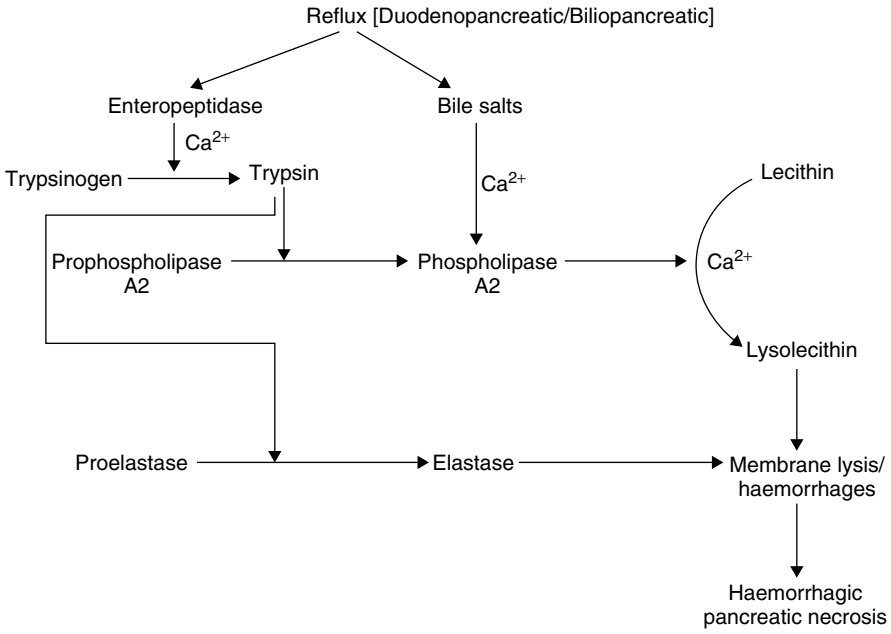

Figure 17 Traditional explanation for gallstone related pancreatitis.

with the disease after drinking less than 50 g alcohol daily for less than two years, which is said to indicate that there is no threshold for alcohol toxicity to the pancreas and/or that certain individuals are genetically predisposed (Hanck *et al*., 2003) and/or that pancreatic injury reflects the sum of toxicities from alcohol, cigarette smoke and diets that either contain too much or too little fat and protein (Sand *et al*., 2007; Oruc and Whitcomb, 2004). In Brazil, it is claimed that 100% of patients have alcoholic disease, whereas only 45% of cases in Manchester, UK, fall into this category when it is defined as the consumption of more than 50 g of ethanol per day for 12 months preceding the first symptom.

Hyperparathyroidism, surgery, chronic renal failure, renal transplantation, inflammatory bowel disease, endoscopic retrograde cholangiopancreatography and exposure to certain drugs/chemicals are other accepted risk factors, but the interaction is not straightforward. In contrast, the autosomal recessive disease cystic fibrosis is the surest cause of chronic pancreatitis.

Another inherited condition, transmitted in an autosomal dominant pattern with incomplete penetrance, is rare but well documented. Several siblings may be affected and the first symptoms occur in childhood, with 20% of patients going on to develop pancreatic cancer. In this group a mutation in the cationic trypsinogen gene at locus 7q35 has been identified recently and it has been suggested that resistance to proteolysis of trypsin underlies the vulnerability to developing chronic pancreatitis (Whitcomb *et al*., 1996). There is no ready explanation when the disease develops in family members without an ancestral history, or when it follows the alcoholic pattern in one member and is idiopathic in another.

The same disease, but with much higher prevalence, familial clustering, presentation in the first decade and accelerated course to pancreatic calculi and diabetes, is found in several countries of tropical zones, for example, southern India (Balakrishnan, 1987) where it is known as 'tropical calcific pancreatitis'. These peculiarities suggest the disease-promoting effect of indigenous genetic and environmental factors (Braganza, 1991). There is a good geographic match between the distribution of the disease and the growth/consumption of cassava (tapioca, manioc), which contains cyanogenic glycosides, which led to the hydrogen cyanide toxicity theory, namely, that there is inadequate detoxification of this poison in malnourished people because of low dietary protein and hence low sulphur amino acid intake. The main problems with this attractive theory are that certain populations who subsist on cassava do not develop chronic pancreatitis, that among residents of the tropical belt who do, there are many who have not consumed cyanogenic foods in excess, and that, whereas some patients develop the full-blown disease, others have insulin-requiring diabetes, but without calcific chronic pancreatitis. More recently a link has been made to a mutation in the serine protease inhibitor, Kazal type 1 (*SPINK1*) gene that encodes for an inhibitor of intrapancreatic trypsin activity (Bhatia *et al*., 2002).

Various animal models for alcoholic pancreatitis have been introduced over the last 20 years and they have proved valuable in helping to understand critical factors and mechanisms of the human condition (Oruc and Whitcomb, 2004). They generally fall into one of two camps, the Lieber and DeCarli (1986) model and the Tsukamoto *et al*. (1988) model. Whereas the former uses alcohol added to a nutritionally defined liquid diet

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and where alcohol exposure is discontinuous, the latter uses catheters implanted into the stomach to permit a continuous infusion of, and hence exposure to, ethanol plus liquid diet. Neither model is accurate in its depiction of the mode of exposure in the human population, but each has produced the morphological characteristics of chronic ethanol-induced pancreatitis.

A rat model with many of the characteristic features of chronic pancreatitis in man was developed in the rat (Puig-Divi *et al*., 1996) using an intraductal injection of trinitrobenzene sulfonic acid, which is reported to reproduce both the morphological changes seen in the pancreas together with the hallmark 'pain' observed during the onset and progression of the human disease (Winston *et al*., 2005). Animal models generally vary in their similarity to the clinical presentation and progression of the human form of chronic pancreatitis and while some will mimic the histological picture of fibrosis and tubular complexes, similar to that of the human disease, more natural methods of producing the disease, showing the full clinical spectrum, including abdominal pain, pancreatic failure and vascular and gastrointestinal complications, have not generally been reproduced (Hirschfield and Gimson, 2007). On the other hand various simplified animal models can be instructive for helping to dissect this complex syndrome and build a more realistic understanding of the disease in man.

5.3.2 Pathophysiology and Progression of Chronic Pancreatitis

The classical mode of presentation of chronic pancreatitis is with an attack of acute pancreatitis, followed by further attacks (**Figure 6**), with a steady erosion of exocrine secretory capacity, although the rate of progress is unpredictable and varies between patients. Most of them develop background pain of increasing intensity but this is multifactorial, with inflammatory oedema and irritation of nociceptive nerve endings in the interstitium being prominent components. Viable acinar cells may be a prerequisite, in that pancreatic pain tends to disappear when more than 90% of acini are lost. There is a correlation between chronic pancreatitis and the future development of pancreatic cancer (Lowenfels *et al*., 1993; Hart *et al*., 2008).

Between attacks, secretory profiles initially show increased amounts of pancreatic proteins, including lysosomal enzymes, but there is a reversal in the normal 2:1 ratio of cationic to anionic trypsinogen in patients who go on to develop pancreatic calculi. Later secretory changes include an increase in lactoferrin and mucoprotein in the pancreatic secretion. The nidus of a calculus has been shown to consist of shed cells around which protein fibrils, calcium, mucin and lactoferrin aggregate to form a matrix for deposition of calcite (calcium carbonate). As time goes by, there is a steady reduction in both protein and bicarbonate secretion, and with each attack of pancreatitis, increasing groups of acini die. Tissue lysis and remodelling is facilitated by activation of prourokinase at the plasma membrane aspect of acinar cells (Freiss *et al*., 1997), with overexpression of transforming growth factors and activation of the epidermal growth factor receptor. Alongside these changes, acinar cells dedifferentiate to form tubular complexes before they are finally replaced by fibrous tissue. Islets may show nesidioblastosis (hyperactivity), even when patients are frankly diabetic.

Because the earliest abnormality seen in chronic pancreatitis at the light microscopic level is the ductal protein plug, because ducts become more distorted over time, and ductal calculi and strictures may develop, ductal elements are traditionally regarded as the focus and ductal obstruction as the cause of the disease. The ductal theory is undermined by the discovery of free anastomoses between the lumina of the pancreatic ducts and the acini. Nevertheless, a possible role for bile reflux in initiating ductal strictures in the 'head' of the gland is suggested by the rare histological finding of bile pigment throughout the duct system or sequestered within the pancreatic parenchyma (Braganza, 1986; Sandilands *et al*., 1990).

5.4 Cancer of the Pancreas

5.4.1 Aetiological Factors

Established risk factors include old age, high fat/protein diets, genetic predisposition and tobacco smoke, with the latter estimated to be responsible for >30% of all cases in the UK (Oliveira-Cunha *et al*., 2008; Hart *et al*., 2008). A series of reports suggests that exposure to certain volatile industrial chemicals may also be involved. Alcoholism remains under suspicion as a risk factor. Familial background of pancreatic cancer is suggested by the susceptibility of American Negroes and the occurrence of the disease in siblings, kindreds with hereditary chronic pancreatitis, and patients with ataxia telangiectasia.

5.4.2 Pathophysiology and Progression of Pancreatic Cancer

Pancreatic ductal cells are generally regarded as being the source of most human pancreatic cancers, but there is some evidence that acini can undergo metaplasia to tubular/ductal complexes and these cells may be the true progenitor in a number of cases (Bockman, 1995). Most tumours in man lie in the 'head' of the gland, a predilection that is not entirely explained by its relatively greater amount of ductal epithelium. It has been

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suggested that reflux of a biliary carcinogen or procarcinogen directly, or via the duodenum, into the pancreatic duct may explain this propensity. A tumour developing in the head of the pancreas can rapidly obstruct the pancreatic and/or common bile duct and accelerate any pancreatic damage through partial blockage of pancreatic outflow following neurohormonal stimulation. Pain, diabetes and weight loss are commonly associated with pancreatic cancer, as is recurrent thrombosis of peripancreatic and systemic veins and the expression of tissue-type plasminogen activator in the plasma membrane of cancer cells has been described and may be a compensatory phenomenon (Steven and Al-Ahmad, 1984).

Orthotopic models of human pancreatic cancer, whereby a human pancreatic cancer cell line is implanted at, or near to, the pancreas of severe combined immunodeficient mice have provided excellent systems for assessing novel chemotherapeutic agents against the disease (Céspedes *et al*., 2006), but have probably provided little additional information about the natural history and progression of pancreatic cancer.

Cultured human pancreatic explants have also provided a tool to study certain aspects of the human disease. Biochemical studies have shown the DNA-damaging effects of certain nitrosamines, and formaldehyde, on the pancreatic cells, while immunohistochemical studies indicate the persistence of O6-methyl guanine in target cell nuclei after treatment with alkylating nitrosamines.

Acquisition of the cancer phenotype involves activation of oncogenes and, in the case of the pancreas, the c-K-ras oncogene seems to be preferentially activated by mutation at codon 12 in >90% of cases in the UK (Oliveira-Cunha *et al*., 2008; Agbunag and Bar-Sagi, 2004), with very high incidences of inactivation of thep16/RB1 (>90% of cases), p53 (~75%), DPC4 (55%) and BRCA2 tumour-suppressor genes (Jimeno and Hidalgo, 2006; Wenger *et al*., 1999; Lebedeva *et al*., 2006; Hruban *et al*., 2000). Oncogenes, in turn, cause increased expression of receptors for growth factors on plasma membranes of cells, leading to activation of the growth-promoting ornithine pathway. Other characteristic features include loss of certain cell surface proteins and increased expression of others, notably a plasminogen activator-like protease, along with increased expression of an intracellular multidrug-resistance (MDR) gene product P-glycoprotein (Goldstein *et al*., 1989), and an early increase in the ratio of lysosomal to digestive hydrolases in pancreatic juice.

Studies of experimentally induced pancreatic carcinogenesis have proved invaluable in understanding the evolution of human pancreatic cancer (Moossa, 1980; Scarpelli *et al*., 1987; Go *et al*., 1993). As with most induced cancers, a multistep evolutionary pattern is accepted (Hirst and Balmain, 2004). Genotoxic agents, whether chemical, virus or other, damage nucleophilic oxygen, as well as the nitrogen atoms of the DNA bases. The most reactive groups in DNA are the N-7 of guanine and N-3 and N-7 of adenine. DNA damage is generally rapidly repaired by specific enzymes, but multiple 'hits', epigenetic agents (tumour promoters) and genetically determined defects in DNA repair, are just some of many factors that favour conversion of a DNA lesion to a mutation.

6 PANCREAS: TARGET FOR OXYGEN RADICAL TOXICITY

6.1 Oxidative Stress in Experimental Acute Pancreatitis

6.1.1 Oedematous Pancreatitis

Time-course analysis of experimental models shows that whatever the inciting agent or its route of attack may be (**Table 3**), and whether the disease pursues a self-limiting or life-threatening pattern, the pivotal problem is a metabolic blockade to exocytosis, that follows close on the heels of a burst of free radical activity. Inflammation is a secondary event, so 'pancreastasis' is a better descriptor than pancreatitis for the earliest metabolic aberration (Braganza, 1988a; 1988b; 1990; 1991; Braganza and Chaloner, 1995).

The early events following induction of pancreatitis in most models can be rationalized as an attempt by the cell to get rid of pent-up secretions and to combat oxidative stress:

1. The normal secretory products of the gland are rerouted via the basolateral membrane, draining off into the lymphatics and into the bloodstream.
2. There is excessive fusion of the lysosomal and zymogen compartments, demonstrable as cytoplasmic vacuoles by microscopy, and within these spaces trypsinogen is activated by cathepsin B en route to its complete degradation and without any spill-over into the systemic circuit, as can be tracked by analysing urine for 'activation peptides' (Leach *et al*., 1992).
3. The cell switches off its synthesis of elastase, instead showing up-regulation of stress-activated PK with over-expression of mob-1 and mcp-1 chemokine genes (Grady *et al*., 1996; 1997) and a particular brand of acute phase protein that has bacteriostatic potential, the pancreatitis-associated protein (McKie *et al*., 1996). The changes seen in chemokine gene expression, implicate nuclear factor κ B (NFκB), released from its precursor peptide

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by the burst of free-radical activity (Schreck and Baeuerle, 1991).

There is little doubt regarding the initiating burst of oxidant damage, and it has been directly demonstrated by chemiluminescence measurements in a model of hyperstimulation and by electron spin resonance spectroscopy in the CDE dietary model. Further, pretreatment with superoxide dismutase and catalase confers protection in several models, whereas the administration of oxidizing agents caused a sharp reduction in pancreatic secretion in the isolated perfused pancreas preparation (Braganza, 1991; Kingsnorth, 1996). However, the precise source of the free-radical burst in pancreatic acinar cells is unclear, as is the precise cause of the exocytosis blockade.

In theory, there are many ways in which oxidative stress might interfere with exocytosis (van der Vliet and Bast, 1992): by activating guanylate cyclase and altering the balance between G- and Gs-proteins), by interfering with microtubular transport systems and actin, by reducing membrane fluidity through production of 9,11-lauric acid, by attacking vulnerable enzymes in the methionine trans-sulfation pathway and thereby reducing membrane phospholipid methylation (**Figure 7**). It may be that different mechanisms operate in different animal models. However, observations in the CDE dietary model, viewed alongside more recent observations in caerulein-induced pancreatitis (Luthin and Grendell, 1994), suggest that interference in the methionine trans-sulfation pathway may be the critical factor. Studies of hepatocytes treated with DL-ethionine show that this analogue follows the metabolic pathway of the natural amino acid methionine, but does not readily donate its ethyl group in the way that SAMe donates its methyl group; the adenine moiety is thus trapped, depleting cells of ATP and jeopardizing protein and PIP_3 synthesis. The additional deprivation of the choline would remove the choline-betaine pathway that contributes to the limited capacity to resynthesize methionine from homocysteine.

6.1.2 Free Radicals Do Not Rationalize Haemorrhagic Pancreatic Necrosis

The zymogens of human pancreatic proteases (Guyan *et al*., 1988), and also phospholipase A2, are not activated, nor is pancreatic trypsin inhibitor destroyed, upon exposure to high doses of the primary or secondary metabolites of oxygen. Hence, it is very unlikely that oxygen free radicals directly activate pancreatic zymogens to initiate HPN. Instead, many observations suggest that activated phagocytes may be the primary source of elastase and phospholipase A2 in HPN (Braganza, 1988b), and also the main cause of MODS, through frustrated phagocytosis coupled with premature immunosuppression (Braganza, 1988b; 1991; Rinderknecht, 1988; Braganza and Chaloner, 1995). In this scheme (**Figure 6**), the discharge into the pancreatic interstitium of FROPs, together with PAF and tumour necrosis factor (TNF), produced in excess by the injured acinar cell, are envisaged as causing the early degranulation of mast cells (Hollender *et al*., 1983) with early activation of the endothelium. PAF, initially from the acinar cell and then from the mast cell, seems to serve as a second relay station, such that neutrophils are bombarded and respond aberrantly. These enzymes, together with chemokines, hypochlorous acid (HOCl), complement factors and kinins, flood the bloodstream and can be detected as a consequence.

The amplitude of the initial free-radical burst did not distinguish between mild and severe disease in animal models. Hallmarks of severe disease are an unremitting blockade of exocytosis, the presence of irritant material, such as bile salts or cell casts (**Table 3**), death of injured cells by necrosis rather than apoptosis, hordes of neutrophils, truncation of capillaries with platelet thrombi and premature immunosuppression (Braganza, 1988b). In this highly proinflammatory, procoagulant state, trypsinogen and pancreatic phospholipase A2 are ignited, most probably by way of plasmin and thrombin.

6.2 Oxidative Stress in Human Acute Pancreatitis

6.2.1 Pancreastasis and Phagocyte-Activation Sequence

Prospective studies, in situations where pancreatitis is expected, collectively confirm that the template for the evolution of acute pancreatitis shown in **Figure 16** is applicable to the human disease. Thus, ERCP causes pancreatitis by the ductal ignition route (Kemppainen *et al*., 1997; Messmann *et al*., 1997), pancreas transplantation (Büsing *et al*., 1993), coronary artery bypass grafting (Fernandéz del Castillo *et al*., 1991) and aortic aneurysm repair (Gullo *et al*., 1996) exemplify ischaemia-reperfusion injury to the pancreas, and haemolytic uraemic syndrome and Reye's syndrome offer the opportunity to study pancreatitis from metabolic derangements in the acinar cell (Durie *et al*., 1985) (**Table 3**).

The early burst in free-radical activity is suggested by the ultrastructural findings in pancreatic acinar cells during the ischaemic phase of pancreas transplantation. The exocytosis blockade with reversal in secretory polarity is also clearly demonstrated in pancreas transplants. The presymptomatic increase in trypsinogen with appearance of trypsin-inhibitor complexes some 48 hours later were striking findings in children with the stated metabolic problems. This sequence was accelerated in patients undergoing ERCP, when trypsinogen peaked at six hours with trypsin/antitrypsin complexes notable at

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24 hours and rising. Finally, studies of ERCP pancreatitis indicated an increase in interleukin-6 beginning one hour after the procedure and increasing to maximal levels after 24–48 hours, with the elicited increase in C-reactive protein evident from 12 hours and peaking at 72 hours.

In day to day practice, the time lag between first symptoms and diagnosis of acute pancreatitis usually exceeds 15 hours and may be as long as 72 hours. At this stage, admission blood samples show clear evidence of oxidative stress with marked depletion of several antioxidants, notably plasma GSH, ascorbate and selenium, but with preservation of red cell GSH and ATP levels. From the earliest sampling point there are high concentrations in plasma of neutrophil elastase-α_1PI complexes, markedly higher in patients who go on to have a severe outcome. By 48 hours this subgroup shows evidence of inappropriate trypsinogen activation from the presence of anionic trypsin-inhibitor complexes in serum, the activation peptide of trypsinogen and especially of carboxypeptidase B in urine (Gudgeon *et al*., 1990; Kemppainen *et al*., 1997; Appelros *et al*., 1998). Phospholipase A2 profiles show an early increase in the synovial-type isoenzyme and the pancreatic isoenzyme mimic from certain phagocytes (Kingsnorth, 1996), with the later appearance of the indigenous pancreatic isoform. It is now accepted that acute pancreatitis epitomizes the systemic inflammatory response syndrome (SIRS), the natural progression of which is death from MODS (**Figure 16**) (Braganza and Chaloner, 1995).

Whereas daily treatment with *N*-acetylcysteine, in a handful of patients with MODS undergoing haemodialysis, produced dramatic results (Braganza, 1991), in a second study *N*-acetylcysteine, together with another GSH precursor, SAMe, had no impact in all-comers, when treatment was restricted to the first 24 hours in a controlled trial (Sharer *et al*., 1995). Similarly, enthusiastic early reports of the benefit of a PAF antagonist (Kingsnorth, 1996) are now more muted with the realization that the drug is most useful if given within the first six hours to patients who might appear only moderately ill. Treatment with the PAF antagonist (lexipafant) was associated with lower blood concentrations of interleukin-8 than in the untreated group, but without any reduction in plasma concentration of neutrophil elastase-α_1PI complexes. Therefore the benefit from lexipafant may have stemmed from its antioxidant function, in that partially oxidized lipid fragments such as isoleukotrienes and isoprostanes exert their toxic effect via the PAF receptor (Nigam *et al*., 1996).

6.2.2 Recurrent (Nongallstone) Pancreatitis

If a burst of free-radical activity underlies an isolated attack of pancreatitis, then it seems reasonable to assume that further bursts underlie successive attacks (**Figure 18**). These are predictable in patients with a congenital deficiency of the enzyme lipoprotein lipase, in whom high levels of triglycerides, of the order of $20-100\,\mu mol\,l^{-1}$, are the rule. Attacks typically begin in childhood. Many patients are characterized as recurrent acute pancreatitis with normal exocrine secretory capacity between attacks, but a few may develop chronic pancreatitis with a steady reduction in exocrine secretory reserve and increasing background pain (**Figure 18**). Daily oral administration of an antioxidant combination, including methionine as a precursor for GSH, along with vitamin C and selenium as GSH refurbishers has been shown to prevent exacerbations of pancreatitis in these patients. The successful formulation also contained vitamin E and β-carotene but blood antioxidant profiles before and after supplementation showed that levels of the lipid antioxidants were in the high-normal range pretreatment, whereas the concentration of GSH in red cells and levels of GSH, vitamin C and selenium in plasma/serum were subnormal pretreatment and normalized thereafter (Heaney *et al*., 1999).

6.2.3 Oxidative Stress in Other Pancreatic Diseases

With regard to the development of pancreatitis in patients with cystic fibrosis, a significantly higher frequency of a mutation in one copy of the CFTR gene has been found in patients with chronic pancreatitis than among unrelated partners of patients with cystic fibrosis in the absence of chronic pancreatitis (Quesnel *et al*., 1998). Furthermore the highest frequency of the mutation was found among nonalcoholic pancreatitis. It was also shown that CFTR is present in pancreatic acini and that the normal CFTR protein is involved in vesicle trafficking and macromolecule targeting, over and above its role in ion transport (Quesnel *et al*., 1998). Loss of the normal function of CFTR protein in cystic fibrosis has the potential to prevent exocytosis, setting in motion the train of events leading to chronic pancreatitis and, further, that in cystic fibrosis carriers, the smaller quota of wild-type protein makes them more vulnerable to oxidative stress from unquantifiable xenobiotic exposures, for example, through passive smoking (Childers *et al*., 2007). There is some evidence that frustrated phagocytosis may contribute to the lung injury seen in cystic fibrosis, when neutrophil-derived oxidants wipe out the α_1PI shield in the airways (Bilton, 1992; Vandivier *et al*., 2006; Morris *et al*., 2005).

Oxidative stress has been documented in kwashiorkor (Golden and Ramdath, 1987; Dreosti, 1991) and seems to represent the combined effects of poor dietary antioxidant intake coupled with increased free-radical load from infective agents, dietary/atmospheric contaminants, strong ultraviolet rays and other sources. Iron-catalysed free-radical production plays an important part in the tissue injury of haemochromatosis and it is likely that copper-catalysed reactions are similarly involved in

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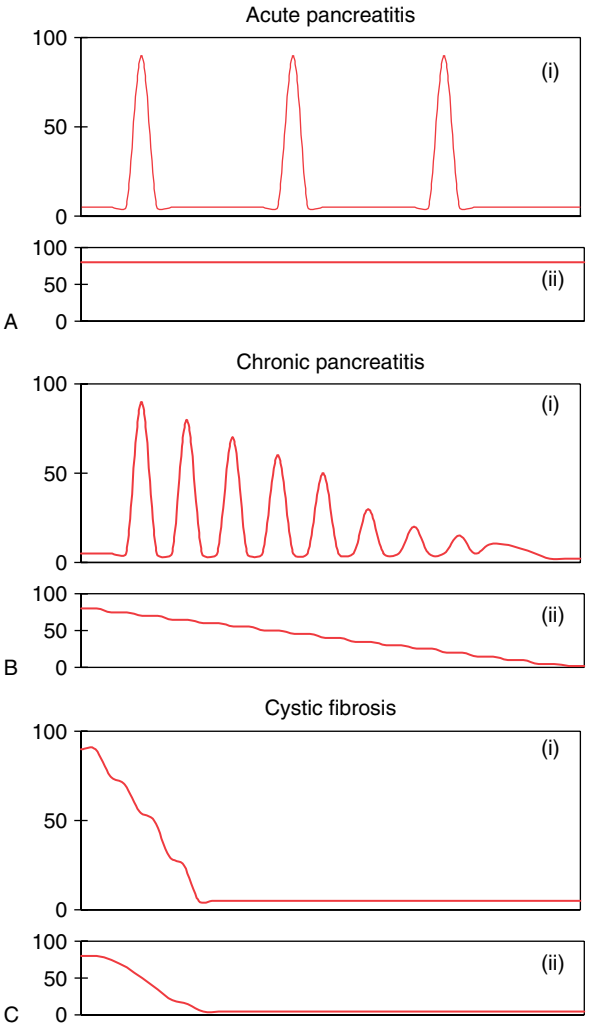

Figure 18 Exocrine pancreatic disease. Profiles of blood enzymes and pancreatic secretory capacity in: (A) acute pancreatitis, (B) chronic pancreatitis and (C) cystic fibrosis. (i) = enzyme levels of trypsin, elastase, lipase and (ii) = pancreatic function. (Adapted from Uden *et al*. (1990a).)

Wilson's disease (Langner and Denk, 2004; Kitzberger *et al*., 2005).

6.3 Summary: Target for Toxicity

An overriding goal of laboratory animal studies, in all aspects of toxicology, is to relate any adverse findings with drugs and chemicals to a possible risk to man after either intentional or accidental exposure. It is now clear that the expression of toxicity *in vivo* depends not upon broad similarities between species, but rather upon specific absorption, excretion, pharmacological and metabolic properties of the animal and/or the compound in question, over and above the question of dose. The concentrations and efficiencies of xenobiotic activating and detoxifying enzyme systems differ markedly between outwardly healthy laboratory animals and man (Anderson *et al*., 1997). In many cases the differences are quantitative rather than absolute (Garner *et al*., 1984) with human tissues generally showing lower amounts of activating enzyme systems (Foster *et al*., 1993).

In terms of the pancreas, the physiological control of function in rodents and man is similar in some respects, but not in others (Longnecker, 2004). The species factor is extremely important and while there is clear evidence of feedback control on CCK release by dietary components in the rat, this is a hotly debated issue in man. In contrast the hamster may be closer to man than the rat in regard to drug-metabolism studies, because of the high intrinsic rate of drug metabolism which rationalize the ease with which ductal adenocarcinomas, so similar to human pancreatic cancer, develop upon exposure to certain nitrosamines. Recent reports of rat pancreatic cancer, including a ductal phenotype lesion from a tobacco-specific nitrosamine (Rivenson *et al*., 1988; Pour and Rivenson, 1989; Hoffman *et al*., 1991; Adsay *et al*., 2000) are of particular interest because of the strong link between cigarette smoking and the human disease, and also because of the recent finding of aromatic amine and nitro aromatic hydrocarbon-adducts in eight among 29 DNA samples from organ donors (Anderson *et al*., 1997). It is unlikely to be a coincidence that many of the drugs and chemicals incriminated/suspected in the aetiology of exocrine pancreatic disease require bioactivation for their toxicity. The paradox that the pancreas bears the brunt of injury in chronic pancreatitis, even though the liver is clearly involved (**Table 5**), may result from a much greater endowment of GSH and other detoxification systems in the liver. It may also be that smoking provides an inhalation exposure route that would optimize the pancreas, with its rich arterial blood supply, being exposed to any noxious chemicals present in the smoke.

Observations in patients with alcoholic chronic pancreatitis have shown that overt signs of liver damage may be delayed for a decade after the first symptom of pancreatic damage (Howat and Sarles, 1979). Involvement of liver cytochromes P450 in the pancreatic cancer setting may explain why hepatic metastases are often present at the time of clinical presentation in pancreatic cancer in man. The spectrum of pancreatic injury inducible by the same toxin, for example, DL-ethionine or ethanol, underlines the need to consider dose, species, confounding factors and a multiplicity of potential tissue-damaging agents, including products of activated phagocytes and the immune response. Finally, the mystery of ketone resistance in diabetics with chronic pancreatitis, especially in tropical zones, may be resolved if induced cytochromes P450 2E are implicated, as this isoenzyme family seems to be important in processing ketones.

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Table 10 Summary: A 'radical' approach to pancreatic disease

Disease	Free radicals			Antioxidants		Oxidative stress		
	Load	Source	Type	Supply	Type	Pattern	Speed	Degree
Acute Pancreatitis								
(i) *Oedematous*								
Ductal artery	↑	XO	O_2 (1)	N or ↓	Vit C			
Xenobiotics	↑	CYPs	O_2 (1)		+	Episodic	Rapid	Mild
Idiopathic	↑	Mixed	+		GSH	Often		
Mixed	↑	Mixed	Organic		Items	Fatal		
(ii) *HPN*	↑↑	PMN/MO	O_2 (2)	↓↓				Severe
Chronic pancreatitis								
(i) *Alcoholism*	↑↑	ADH		N or ↓	Vit C	Constant		
		CYP 2E						
(ii) *Idiopathic*	↑↑	CYPs		N or ↓	±	+ Bursts	Gradual	Moderate
Cigarette smoke		CYP 1A2			Methionine			
Volatile chemicals		CYP 2E1			±			
C18:2 oil/fat		CYP 1	O_2 (1)		Selenium			
(iii) *Tropical*	↑↑		+	↓↓	Vit C	Constant		
Wood smoke		CYP 1	Organic		±			
Cassava		CYP?			β-carotene		Rapid	Severe
Kerosene		CYP?			± SAA			
C18:2 oil		CYP 1			± Selenium			
Cancer pancreas	↑↑	CYP 3A1	O_2	N or ↓	?	Constant	Gradual	Moderate
		CYP 1A2	+					
		CYP 2E	Genotoxins					
Cystic fibrosis								
(i) *Childhood*	↑	CFTR	O_2 (1)	N	Vit C	Constant bursts	Gradual	Mild
(ii) *Adult*	↑↑	PMN/MO	O_2 (2)	Varied	±		Rapid	Severe
		Pseudomonas	O_2 (1)		Selenium			
Kwashiorkor	↑↑	Mixed	O_2 (2) organic	N or ↓	All	Constant	Rapid	Severe
Metal storage	↑	Fenton reaction	O_2 (1)	?	?	Constant	Gradual	Moderate

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7 HORIZONS

The bulk of information presented in this chapter suggests that the regulated secretory pathway in pancreatic acinar cells is exquisitely sensitive to oxidative stress, and that this vulnerability plays a major part in each of the acquired pancreatic diseases (Braganza, 1991). There are clues as to the pathogenesis of oxidative stress in these diseases as well as in certain congenital diseases (**Table 10**) (Braganza, 1991). The predominance of cytochromes P-450 as a source of excessive free-radical activity should not come as a surprise when one considers that this is a very primitive acquisition in phylogenetic terms (**Figure 1**). Hence, evidence of chemical damage to pancreatic acinar cells in lower vertebrates, for example, the toxicity of weed killers in teleosts, or diesel oil in trout (**Table 4**), should not be disregarded as irrelevant to human pancreatic disease. The similarity in patterns of pancreatic damage from toxic metabolites or antioxidant deficiency, for example, vacuolation of cytoplasm and dilated endoplamsic reticulum on exposure to many toxins, as well as from isolated trace metal deficiency, or production of'pancreatic hepatocytes' by a particular regimen of BOP treatment or copper deficiency, can be reconciled with the realization that each manoeuvre would precipitate oxidative stress. These factors can probably be considered as components of disease aetiology (Rothman, 1986), insofar as pancreatic disease is concerned (**Table 10**). The inhalation route may be especially important with regard to the second and third components, while the first component has implications for treatment and, more importantly, for prophylaxis.

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Skeletal Muscle Toxicology

M. Alexander Kenaston, Ellen M. Abramson, Matthew E. Pfeiffer and Edward M. Mills

CONTENTS

1 INTRODUCTION

Skeletal muscle is the workhorse for all voluntary movements of limbs and tissues, and accounts for approximately 45% of body weight in adult males (~35% in females). Humans have approximately 630 skeletal muscles. Skeletal muscle is also the most metabolically demanding tissue in the body and uses roughly half of all the energy consumed. Given these properties, muscle toxicity (myotoxicity) and dysfunction can result in a variety of detrimental metabolic disturbances and even life-threatening sequelae for the patient. Because of its broad clinical coverage and significance, myotoxicity induced by environmental (poisons, drugs and microgravity) and endogenous (hormones, temperature) insults has been the subject of intensive research interest for decades. Insights gained from experimental and clinical research in skeletal muscle toxicology have contributed

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greatly to the understanding of normal muscle physiology, and to the clinical management of drug-induced and idiopathic myopathy syndromes. However, skeletal muscle toxicology has received comparatively little attention in textbooks of toxicology.

The enigmatic adage *form follows function* is particularly suited to skeletal muscle. Muscle cells are designed to contract and relax in a homeostatic process to enable coordinated, purposeful movements such as walking or winking, and the manipulation of external objects. During embryonic development, multinucleated skeletal muscle fibres arise from the fusion and differentiation of multiple skeletal muscle stem cell precursors or myoblasts into elongated, cylindrical muscle fibres (a single muscle fibre = myocyte) specialized for contraction. A single muscle is composed of multiple parallel muscle fibres ranging in diameter from 10 to 100 μm, and up to several centimeters in length. Within myocytes, intracellular contractile elements, referred to as myofibrils, contain highly structured thick actin and thin myosin filaments. Upon excitation by motor neurons at the neuromuscular junction, an influx of extracellular Ca^{2+} and concomitant accumulation of intracellular Ca^{2+} from endoplasmic reticulum (ER; 'sarcoplasmic reticulum' (SR)) stores in the muscle fibre results in a complex series of molecular events that culminate in the rearrangement of the thick and thin filaments and a shortening, or contraction, of the fibre.

The unique architecture, the major role of Ca^{2+} for contraction, and the great metabolic demands of skeletal muscle endow its sensitivity to damage by toxins and in many cases underlie the multiorgan systemic sequelae of muscle pathology (myopathy). To be discussed in more detail below, skeletal muscle-mediated damage to distant organs most prominently involves myoglobinaemia induced by the release of myoglobin from necrotic oxidative myocytes with leaky outer membranes. Because of the size of skeletal muscle, even a small amount of myotoxicity per total muscle mass can result in clinically significant myoglobinaemia and resultant toxicity to renal glomeruli. Drugs, hormones or poisons that induce myopathies can be subdivided into two main categories: (i) agents that induce skeletal muscle breakdown, or *rhabdomyolysis* (*rhabdomyo* = striped muscle; *lysis* = breakdown), and (ii) agents that induce contractile dysfunction or failure. Because contractile toxicities result largely from xenobiotic-induced damage to peripheral and/or central neuronal components of movement control and only indirectly affect muscle, this chapter will be focussed on agents that directly induce damage to skeletal muscle structure and function, and mechanisms of action if known. However, it should be noted that skeletal muscle toxicity secondary to neurotoxicity is an important contributor to diverse skeletal muscle pathologies, as proper innervation of muscle by motor neurons is essential for both the development and growth of muscle, and also in the maintenance of skeletal muscle architecture and function. In this chapter, we will review essential concepts dealing with skeletal muscle toxicology and will provide an overview of experimental methodologies, clinical markers and management of muscle intoxication and pathology. Finally, we will survey the diverse range of skeletal muscle toxins and their mechanisms of action, with a particular emphasis on pharmacogenetic considerations where appropriate.

2 OVERVIEW OF SKELETAL MUSCLE STRUCTURE AND PHYSIOLOGY

2.1 Normal Muscle Development and Anatomy

Myoblasts are mononucleated primitive myocyte precursors that are committed to terminal differentiation into large, cylindrical, multinucleated skeletal myocytes referred to commonly as 'myotubes'. *In vivo,* complete myoblast differentiation requires innervation by a motor neuron. Once innervated, developing myotubes rapidly increase the expression of contractile proteins, or myofibrils, that mediate the shortening of the muscle fibre in response to neuronal excitation. Another feature of innervation-induced maturation is the development of distinct sets of muscle fibre types distinguished primarily on the basis of their metabolic profiles. In this regard, these fibre types are determined by the kind of innervating lower motor neuron and can be distinguished by colour—some are deep red, some are white. As will be discussed below, the red fibres are highly oxidative, slow to contract and have high endurance, whereas the white fibres are more glycolytic, contract rapidly and are more easily exhausted. From a clinical toxicology perspective, agents that induce damage to red fibres can lead to more multiorgan pathologies than those that damage white fibres because of the high concentration of myoglobin in red fibres. As will be discussed in more detail throughout this chapter, myoglobinaemia is arguably the most life-threatening complication of skeletal muscle intoxication by drugs, hormones and poisons.

Skeletal muscles are connected to the skeleton with thick fibrous tendons, and typically span joints to function in a lever motion with the attached bones. A single skeletal muscle is composed of many fibre bundles referred to as fascicles, and is encased in an outermost layer of connective tissue called the fascia, or epimysium, which forms the tendons at the ends of the muscle. Inside the fascia, fascicles are similarly bundled together with an internal layer of connective tissue, the perimysium. Each individual fibre within a fascicle comprises a single elongated multinucleated myocyte. Muscles contain between 10 000 and 1 million individual

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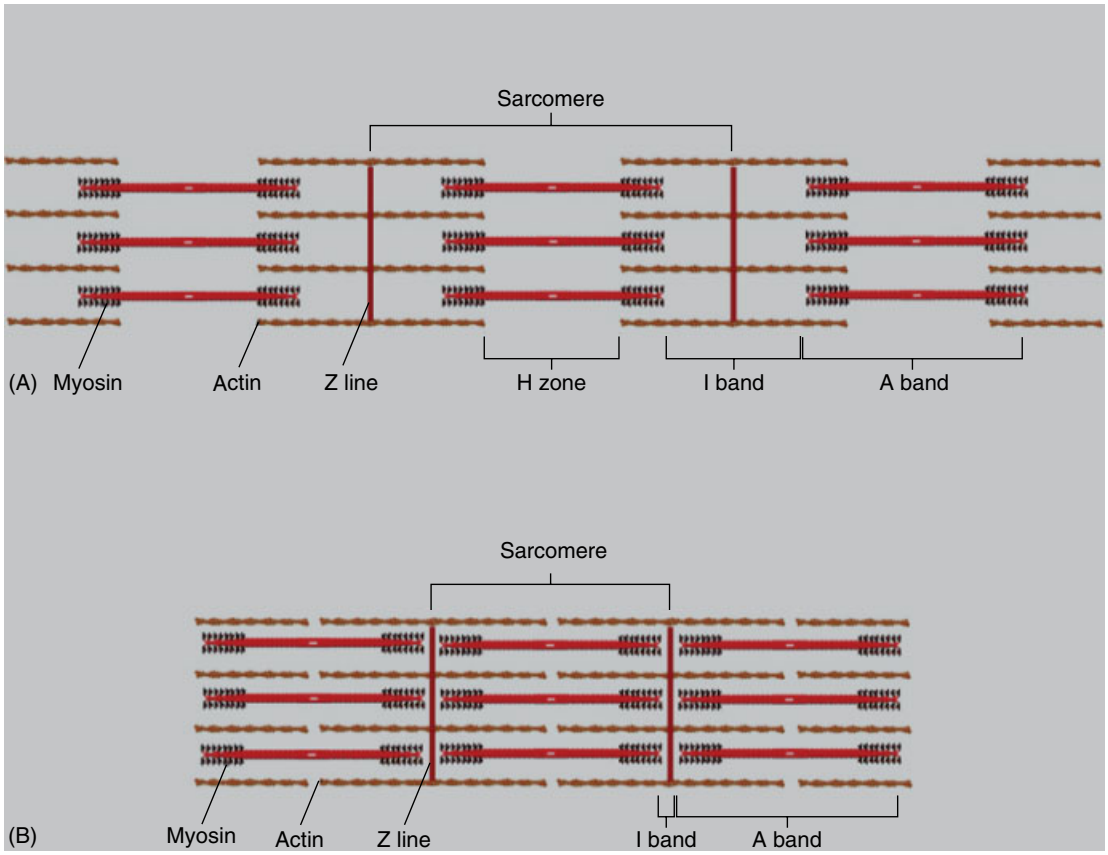

Figure 1 Structural organization of myofibre contractile filaments. (A) Relaxed muscle; (B) contracted muscle.

muscle fibres, each of which contains numerous myofibrils, the intracellular contractile elements comprising 80% of the volume of muscle cells. Myofibrils are long, cylindrical structures approximately 1 μm in diameter, and span the entire length of the muscle. Each myofibril is composed of the thin actin and thick myosin filaments that essentially slide together to mediate contraction in response to a rise in intracellular calcium (Sherwood, 2007). As depicted in **Figure 1**, these filaments exhibit a partially overlapping arrangement that give skeletal muscle its unique appearance. Using electron microscopy to observe a single myofibril reveals a patterned structure with alternating bands of light (I band) and dark (A band) that are perpendicular to the long axis of the fibre. The I bands are the portions of thin actin filaments that do not overlap with the thick myosin filaments, whereas the A bands consist of the overlapping portions of actin and myosin filaments. At the junctions of the A and I bands, the outer plasma membrane of the muscle fibre extends into the fibre interior forming the transverse tubule (T tubule) system that is critical for propagating contractile signals efficiently throughout the muscle fibres. This banding pattern is what gives skeletal muscle its striped or 'striated' appearance. Hence the term 'striated muscle' was coined. Within each I band is a dark-appearing line perpendicular to the fibres, referred to as a 'Z line'. It is that portion of the myofibre in between two Z lines that defines the sarcomere, the smallest functional unit of muscle capable of contraction. These structural features of a myofibril are summarized in **Figure 1**.

2.2 Fibre Types

Skeletal muscle fibres can be subdivided into three main classes: slow and fast oxidative fibres (Type I and IIa, respectively), and fast glycolytic (Type IIb). Fast glycolytic fibres exhibit nearly 10-fold faster contraction rates compared to slow oxidative fibres. Thus, these contractile elements are also termed 'fast twitch'. No skeletal muscle in the body is composed of one fibre type, but different muscles contain different proportions of fibre types. As summarized in **Figure 2**, oxidative fibres are so named for their large dependence on mitochondrial oxidative phosphorylation for adenosine-5′-triphosphate (ATP) production, whereas glycolytic fibres rely most on glycolysis for ATP synthesis. Not surprisingly, glycolytic fibres contain relatively few mitochondria, but oxidative muscle fibres have an abundance of these organelles. Mitochondria in skeletal muscle fibres lie in the intracellular spaces between myofibrils, along with glycogen, a source of

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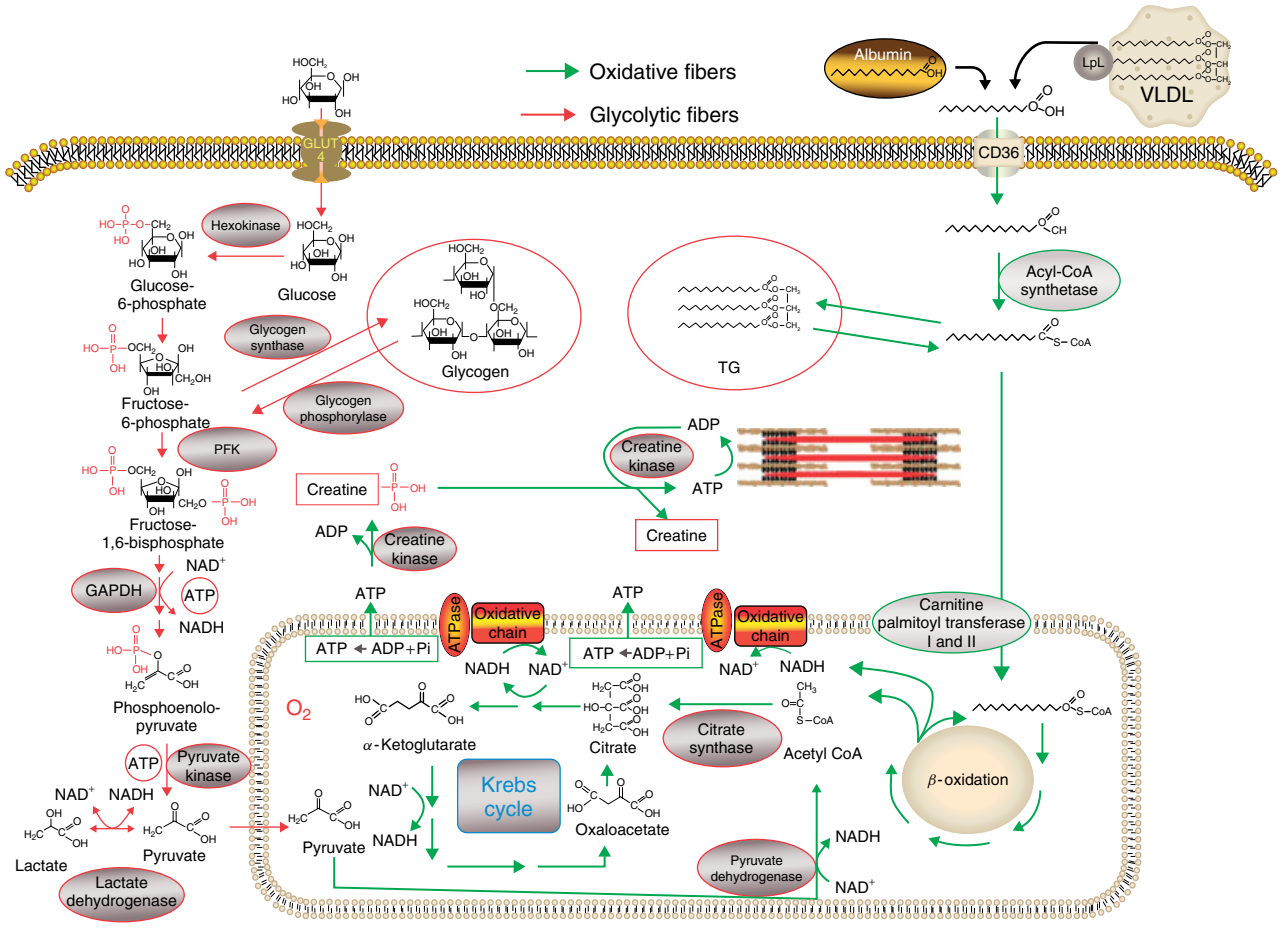

Figure 2 Glucose, fatty acid and energy metabolism in skeletal muscle. Glycolytic and oxidative muscle fibres have the alternative primary energy substrates of glucose (red arrows) and fatty acids (green arrows), respectively. Extracellular glucose enters the cell through plasma membrane glucose transporters (GLUTs) and is subsequently phosphorylated by hexokinase. After conversion to fructose and then pyruvate, it is transported into mitochondria for entry into the Krebs' cycle as acetyl CoA. Alternatively, glucose can be stored as the polysaccharide glycogen. Fatty acids are carried in the bloodstream via binding proteins such as albumin or as constituents of lipoproteins. Free fatty acids enter myocytes through plasma-membrane fatty acid transporters such as CD36, and translocate to the mitochondria after carnitine attachment via the carnitine palmitoyl transferase system. Through β-oxidation, free fatty acids are metabolized to acetyl CoA and oxidized during the Krebs' cycle. Free fatty acids that accumulate within the cell can also be stored as triglycerides (TGs). ATP that is produced through oxidative phosphorylation is exported into the cytosol where it is used to drive muscle contraction or can alternatively be stored as phosphocreatine. Abbreviations used: PFK—phosphofructokinase; GAPDH—glyceraldehyde-3-phosphate dehydrogenase; ATP—adenosine-5′-triphosphate; ADP—adenosine diphosphate; LpL—lipoprotein lipase; VLDL—very low-density lipoprotein; NAD+/NADH— nicotinamide adenine dinucleotide.

stored glucose for ATP synthesis. Oxidative fibres also contain high myoglobin concentrations as a strategy to maximize oxygen uptake from the bloodstream for its delivery to mitochondria for ATP production. This property endows oxidative fibres with their red colour. In contrast, glycolytic fibres have low myoglobin levels, and their colour is pale/white. Because oxidative fibres can generate several fold more ATP per molecule of glucose compared to fast fibres they are more suited for endurance work, and fatigue at a slower rate compared to glycolytic fibres.

2.3 The Neuromuscular Junction and Excitable Membranes

The membrane systems of muscle are highly specialized for muscle contraction. The outer plasma membrane makes physical contacts with motor neuron axon terminals, is electrically excitable and encloses multiple myofibrils. As mentioned above, protrusions of plasma membrane into the muscle fibre occurring at regularly spaced intervals (at each sarcomere) by T tubules

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efficiently propagate nervous impulses to muscle fibres deep within the muscle. When a central command for voluntary movement is coordinated by movement centres in the brain (basal ganglia, cortex and cerebellum), it is delivered via spinal and motor neuronal efferents to a muscle via action potentials that are propagated along the motor neuron axon terminals, leading to the vesicular release of the neurotransmitter acetylcholine (ACh). The specialized connections between nerve and skeletal muscle, referred to as neuromuscular junctions, are composed of axon terminals of motor neurons, and the motor end plate, the region of the sarcolemma that interacts with the motor neuron. A single motor neuron innervates a single motor end plate. High concentrations of acetylcholine receptors (AChRs) localized to the end plate are made possible by the increased surface area created by deep folds or fenestrations in the end plate plasma membrane. Binding of ACh to AChR on the end plate leads to the influx of Na^+ into and efflux of K^+ from the intracellular compartment of the muscle fibre. As the result of large amounts of Na^+ entry and the increased positive charge, ion flux thereby changes the resting electrical potential across the plasma membrane of the muscle fibre from the resting state at -80 mV, to a depolarized state at -15 mV. With sufficient nerve impulses and complex integration, the release of ACh and membrane depolarization at the contact points across the fibre will be propagated as an electrical signal and penetrate deep into the muscle via the T tubule membranes. It is this event, the depolarization of the plasma membrane and T tubule system, that initiates the contractile process (Rhoades and Pflanzer, 1989). Termination of the neural component of muscle contraction is regulated mainly by the enzyme acetylcholinesterase (AchE), which degrades ACh in the neuromuscular junction. Agents that induce motor neuronal death or block neuromuscular synaptic transmission lead to paralysis and loss primarily of Type II fibres; on the other hand, agents that induce excessive neuronal firing at neuromuscular junctions, or those that induce membrane damage that renders skeletal muscles leaky to sodium and potassium, can profoundly enhance muscle contractile responses and lead to sustained, painful and toxic muscle contractions or tetanus, some cases of which can be followed by neurotransmitter depletion and fatigue, and even death. **Table 1** outlines the major effects of neuromuscular junction perturbation by biologic or chemical toxins.

2.4 The Sarcoplasmic Reticulum and the Molecular Mechanism of Muscle Contraction

At the heart of both the contractile response and many, if not most, skeletal muscle toxicities is the intracellular second messenger calcium. The plasma membrane and its series of interconnected T tubule membranes are in close proximity to a specialized intracellular membrane structure, the sarcoplasmic reticulum, which is a form of endoplasmic reticulum. The ER of nonmyocytes and the SR of myocytes is a major intracellular storage depot for calcium. Release of stored calcium from the SR into the myocyte cytoplasm (sarcoplasm) is the fundamental link between neuronal excitation of the muscle and contraction. Similarly, dysregulation of calcium movement across the SR membrane is the mechanistic basis for skeletal muscle toxicity induced by a vast array of drugs and toxins. Agents that induce calcium handling abnormalities comprise the largest and most diverse group of compounds associated with skeletal muscle toxicity and pathology. As shown in **Figures 1** and **2**, at each junction of the A and I bands of the filaments within the myocyte or muscle fibre, the plasma membrane protrudes into the muscle forming the T tubule. The T

Table 1 Agents that alter myoneural transmission

	Source	Mechanism of action and symptoms of exposure
Neuromuscular blockers that induce paralysis		
Alpha toxins	Snake venoms	Inhibition of ACh receptors. Weakness, fatigue, paralysis, respiratory failure, death
Botulinum toxin	*Clostridium botulinum*	Blockade of vesicular ACh release. Weakness, respiratory failure, death
β bungarotoxin	Snake venoms	Blockade of vesicular ACh release. Mild muscle weakness
Neuromuscular stimulants that induce hypercontraction or spasm		
Alpha latrotoxin	Black Widow spider	Induces a pore in the myocyte membrane that is permeable to Na^+ and K^+, leading to membrane depolarization. Initial effects are muscle spasms followed by depletion of ACh, respiratory failure and death
Organophosphate	Pesticides, chemical warfare agents	Inhibition of acetylcholinesterase. Seizures, neuropathy, weakness, respiratory failure, death

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tubule extends into the interior of the muscle, perpendicularly to the filaments. Since the plasma and T tubule membranes are continuous, excitation by motor impulses at the neuromuscular junctions leads to action potentials across these membranes, and the activation of membrane protein voltage-gated calcium channels (VGCCs). Via mechanisms that are not completely understood, it is primarily the activation of the VGCCs in the plasma membrane which leads to the opening of the SR Ca^{2+} channel commonly known as the ryanodine receptor (RyR). The plant toxin ryanodine binds to RyR and locks it in an open conformation. Many of the VGCC in the sarcoplasm physically interact with VGCC in the plasma membrane in regions of the SR that come in close proximity to the outer plasma membrane or T tubule membrane. Upon activation of a sufficient number of RyR by activated VGCC, RyR not in close association with VGCC also open, and calcium stored in the SR is released into the cytosol or sarcoplasm, where it activates the sliding filament mechanism of muscle contraction wherein the actin and myosin filaments slide along each other in a manner that shortens the sarcomere in an ATP-dependent reaction. Contraction is terminated by the reuptake of Ca^{2+} from the sarcoplasm back into the SR via the ATP-dependent calcium pump sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA). As will be discussed below in the next section, the RyR is probably the most widely studied molecule in skeletal muscle toxicology. Mutations in the gene encoding RyR in humans predispose individuals to hyperthermia and skeletal muscle breakdown induced by volatile anaesthetics. This sometimes-fatal condition, commonly referred to as malignant hyperthermia (MH), is one of the first-described 'toxicogenetic' disorders where toxicity requires a genetic mutation. A summary of the molecular mechanisms of calcium-induced muscle contraction and calcium homeostasis in a muscle fibre sarcomere is depicted in **Figure 3**.

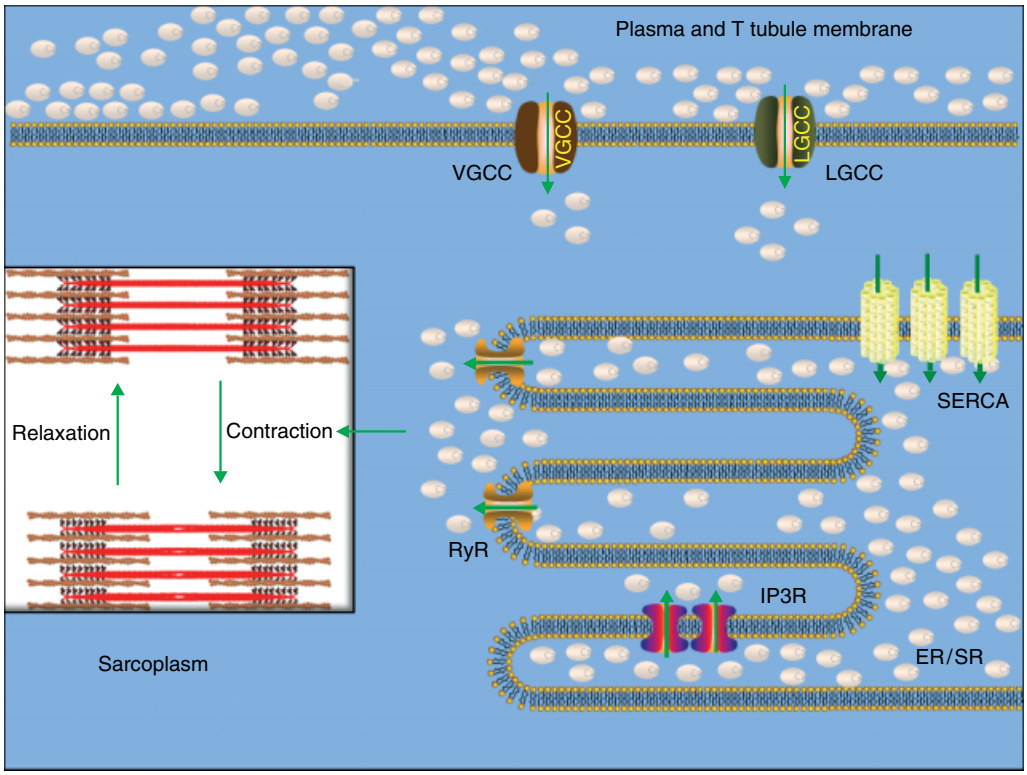

Figure 3 The role of calcium in muscle contraction. The ryanodine receptor (RyR) is an intracellular calcium transporter situated within the membrane of the sarcoplasmic reticulum (SR), similar to the inositol triphosphate receptor (IP3R) found in the endoplasmic reticulum (ER) of some cells. The sarcoplasmic reticulum is a specialized reservoir for calcium storage within muscle cells. Voltage-gated (VGCC) or ligand-gated calcium channels (LGCCs) initially allow extracellular calcium ions to enter the cell cytosol. These receptors are present on transverse tubules (T tubules), which are invaginations of the plasma membrane that come into contact with the SR. Because these ion channels are in close proximity to RyR/IP3R receptors on the SR/ER, a robust positive-feedback cascade is initiated. The small rise in cytosolic Ca^{2+} levels causes activation of the RyR receptor in a calcium-activated calcium release mechanism. Calcium stores from the SR/ER are liberated, rapidly increasing cytosolic Ca^{2+} concentrations and initiating muscle contraction. Calcium ions then re-enter the ER/SR through the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA).

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3 Laboratory Methods (*In Vivo* and *In Vitro*) for Investigating Skeletal Muscle Injury, and Biomonitors for Skeletal Muscle Toxicity

3.1 Introduction

The body of research on skeletal muscle diseases, particularly responses to toxic insults, has markedly advanced in the preceding 30 years. As such, the battery of tests available to the clinician and the number of research protocols available to the scientist have grown as well. Biochemical tests used to evaluate serum samples for skeletal muscle enzymes are readily available in most clinical laboratories. Many healthcare institutions and research facilities have dedicated electrophysiology laboratories capable of assessing contractile and conduction abnormalities. Histological analyses using light microscopy, electron microscopy and immunohistochemistry are well-established techniques for evaluating muscle biopsy samples. The isolation of intact skeletal muscles for tissue culture or myocyte isolation is a routine practice in research laboratories. In addition, numerous animal models of skeletal muscle-relevant disease states have been developed for better understanding pathophysiologic mechanisms of muscle injury and disease.

3.2 Biochemical Evaluation

Established protocols exist for the management of emergency myopathies that develop acutely and require immediate diagnosis and intervention. The cornerstone of identification and treatment of many myopathies is the array of biochemical tests which are used to gauge muscle injury through evaluation of venous blood flow. The most appropriate candidates for assessment of myopathy are enzymes that are usually confined to the intramyofibrillar space. The presence of large quantities of these enzymes in the systemic vasculature indicates that a substantial injury to the skeletal muscle has occurred. Many enzymes, including lactate dehydrogenase (LDH), aspartate transaminase (AST) and alanine transaminase (ALT), can be elevated after damage to skeletal muscle (Haschek and Rousseaux, 1998). However, the best marker of skeletal muscle injury is creatine kinase (CK). Although CK, like many other enzymes, can be elevated with a variety of disorders, it is possible to differentiate the affected area through exclusion of alternative sources. CK catalyses a reversible enzymatic reaction that liberates a phosphate group from phosphocreatine and thereby provides an essential energy reservoir for the production of ATP within myocytes. The Oliver–Rosalki method for estimating enzyme activity utilizes the reverse CK reaction of ATP synthesis from creatine phosphate and adenosine diphosphate (ADP) (Rosalki, 1967; McPherson and Pincus, 2006). Second and third reactions are used to generate glucose-6-phosphate and nicotinamide adenine dinucleotide (NADH), respectively. The NADH produced is subsequently measured as the indicator of CK levels. Enzyme measurements are typically expressed in units of activity per litre or per millilitre, with the normal range being $30–200\,\mu l^{-1}$ (Daniels, 2002; McPherson and Pincus, 2006; Barohn, 2008). The test for CK activity does have some limitations due to its short plasma half-life, making it possible for a patient with active myopathy to have relatively normal levels of plasma CK. This is discussed in further detail in section 4 'Clinical Management of Skeletal Muscle Toxicity (Especially Rhabdomyolysis)'.

In addition to the CK assay discussed above (i.e. total CK), a more-specific test can be performed to better determine the origin of the released enzyme. The mature CK protein contains a combination of two different subunits which have been found to be tissue-specific in their expression, 'M' for muscle and 'B' for brain. Therefore, three CK isoenzymes exist; CK-MM is primarily expressed in skeletal muscle, CK-MB is found predominantly in cardiac tissue, and CK-BB is found in brain and intestines (Haschek and Rousseaux, 1998; McPherson and Pincus, 2006; Barohn, 2008). Typically, in order to differentiate between different CK isoenzymes, an immunoassay approach is employed. This strategy is useful in assessing total CK, followed by the specific isoenzyme contribution to the total CK measurement. First, the total CK enzyme activity is measured, followed by the addition of antibodies specific to the CK-M subunit. By binding to the 'M' enzyme subunits, these antibodies would theoretically abolish the enzyme activity of the CK-MM isoenzyme and decrease the activity of CK-MB by half (Barohn, 2008). This technique also has its limitations, as unexpected aberrations in CK isoenzymes do occur. For instance, CK-MB can be elevated in patients with skeletal muscle myopathy that do not have an injury to cardiac tissue (Kiely *et al*., 2000). Other immunoassays, such as the detection of skeletal troponin I (sTnI), are being explored for their utility in diagnosing skeletal muscle-specific injury (Simpson *et al*., 2005). sTnI exists as two isoforms, ssTnI and fsTnI, which are produced in slow twitch and fast twitch fibres, respectively. This detection method has not been developed into a commercial assay for implementation in clinical laboratories.

Another specific marker for skeletal muscle injury is the haem-containing protein, myoglobin. Perhaps most importantly, myoglobin can have a direct toxic effect on the kidneys (see 'Clinical Management of Skeletal Muscle Toxicity section, below). The detection

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of myoglobin is highly time dependent because it is efficiently cleared from the plasma (Beetham, 2000). Therefore, it is more appropriate to measure urine myoglobin, which is usually first evident as a urine colour change from yellow to dark red/brown. Drastically elevated myoglobinuria represents a condition that requires immediate treatment intervention because of the possibility of acute renal failure (ARF). The most rapid test is a qualitative assessment using inexpensive urine test strips. A urine sample is applied to a test strip containing hydrogen peroxide and the tetramethylbenzidine colour reagent. After interaction with the peroxidase activity of the haem in haemoglobin or myoglobin, the reagent will produce a detectable change in colour (McPherson and Pincus, 2006). One confounding factor to interpreting the qualitative test results is that haemoglobin will also be detected (in addition to myoglobin). Therefore, these findings must be considered in the context of other results such as urine erythrocytes and serum CK. A positive urine myoglobin test, together with absent urine erythrocytes and a high serum CK is indicative of skeletal muscle injury. An alternative procedure uses immunoassay techniques to identify and quantify myoglobin in urine. Although there is a small degree of interference from haemoglobin, this test is much more specific for myoglobin (McPherson and Pincus, 2006).

3.3 Electrophysiological Evaluation

Exposure to xenobiotics can induce changes in the contractility and nerve conduction of skeletal muscles. One tool to investigate these alterations is electromyography (EMG), which evaluates the electrical activity of the muscles by comparing amplitude, number and duration of impulses (Acosta *et al*., 1997). Primarily, this technique is employed in order to determine if symptoms are myogenic or neurogenic (Daniels, 2002). Two superficial or needle electrodes are used. The patient is then asked to either contract or relax a given muscle or muscle group. The trained electrophysiologist can make determinations on the nature of the pathology by comparing the spontaneous and voluntary electrical potentials. Nerve conduction studies and needle EMG studies can also be conducted using skeletal muscle tissue obtained through a needle or punch biopsy (Barohn, 2008).

3.4 Microscopic Evaluation

Several groups have published protocols for the microscopic evaluation of skeletal muscle toxicity in animals (Ownby *et al*., 1976; Suarez-Kurtz and Eastwood, 1981; Melo and Ownby, 1996). After exposure of the organism to a toxin, the muscles are harvested and fixed in glutaraldehyde, dehydrated in acetone, and embedded in a resin such as paraffin. Sections are then obtained using an ultramicrotome in either a longitudinal or cross-section depending on the desired perspective. The sections can then be stained with azure II, methylene blue, trypan blue or haematoxylin and eosin for visualization. As the slides are reviewed under a light microscope, abnormal findings such as presence of vacuoles, aggregation of cells, amorphous appearances, cell necrosis and cell oncosis (death by cell swelling) are noteworthy. Muscle biopsies from human patients are used for microscopic examination. Typical findings during myopathy are central nuclei, hypertrophic fibres, split fibres, degenerating fibres and regenerating fibres (Barohn, 2008). Inflammatory myopathies in particular are characterized by infiltrates of mononuclear cells in the connective tissue of the epimysium and perimysium (Barohn, 2008). Immunohistochemistry and electron microscopy for prepared tissue samples can also easily be performed. Histochemistry can be employed in order to identify a particular protein using immunostaining. Scanning and transmission electron micrographs have been used to visualize autophagic vacuoles and morphology of intracellular organelles, respectively.

3.5 Tissue and Cell Culture

The utility of *in vitro* tests for skeletal muscle toxicity has been well documented. Experimental systems exist to transiently maintain a muscle tissue explant (i.e. *ex vivo*) or grow a confluent monolayer of contracting, differentiated myotubes (*in vitro*). Extraction of an *ex vivo* tissue sample can consist of an entire muscle group, muscle strip or single cell preparation. Maintaining a tissue in culture requires immersion in a temperature-controlled media bath which will closely replicate the endogenous conditions *in vivo* (Acosta *et al*., 1997). Of particular importance are the nutrient and oxygen demands of the tissue. The tissues are usually superfused with a modified Krebs buffer which is maintained at 37 °C, pH 7.4 and has been supplemented with glucose, sodium, potassium, calcium, magnesium and chloride ions to recapitulate their physiologic concentrations (Melo and Ownby, 1996). This system has many advantages, including ease of modification of the constituents of the perfusate, and convenient serial sampling of the perfusate. Because the oxygen-carrying capacity of perfusion media is unknown, some researchers have supplemented the buffers with haemoglobin. Another limitation of the system is the uncertainty of the penetration of oxygen and nutrients into the interior of the tissue explants. Creating thin cross-sections of muscle or treatment with detergents at low concentration (e.g. Triton X-100 0.1%) are strategies to increase access of nutrients to the intramyocellular compartment. The greatest obstacle to the use of tissue

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explants is the rather temporary cell viability that is achieved.

Cultured myocyte systems to evaluate toxicity have been widely used since first being introduced in the 1970s (Wenzel *et al*., 1970). The two most commonly used cell lines, C2C12 and L6, were both developed by Yaffe and colleagues from mouse and rat, respectively (Yaffe, 1973; Yaffe and Saxel, 1977). Cells are also routinely isolated from human muscle biopsies (Sarabia *et al*., 1990). Isolation of myoblasts requires separation from the other cells of the skeletal muscle such as myotubes, myofibroblasts, erythrocytes and leukocytes. Isolation protocols utilize the ability of cells other than myoblasts to adhere to cell culture vessels (Acosta *et al*., 1997). After a series of digestions by proteases and/or collagenases, the homogenate can be filtered to isolate intact cells, which can then be washed and retrieved by centrifugation. After plating in a cell culture vessel, the supernatant can be moved to a new vessel at predetermined time points in order to isolate a particular cell variety. Myoblasts are usually the last cells to attach and are easy to distinguish from myofibroblasts via light microscopy. Myoblasts can be induced to differentiate into mature myotubes by changing their growth media (usually Dulbecco's Modified Eagle Medium with 10% foetal bovine serum) to media containing 2% equine serum. The differentiating cells will form multinucleated myotubes that have characteristic changes in their protein expression patterns and have the ability to contract. Cultured myocyte systems can be immortalized through viral transduction to create a renewable cell line that can be cryogenically preserved. This homogenous system is also useful because it can be used to study a specific cell type without the potentially confounding presence of others. Similarly to *ex vivo* tissues, serial sampling of the media is accomplished easily. The major disadvantage and criticism of cultured myocyte systems is that they fail to duplicate the *in vivo* environment in all aspects, making extrapolation of results difficult (Haschek and Rousseaux, 1998).

3.6 Animal Models

Numerous experimental models exist to recapitulate human disease states in animals. Gross examination of skeletal muscle should be performed at necropsy with particular emphasis on the presence of lesions and alterations in size, shape, consistency and colour of the tissue (Haschek and Rousseaux, 1998). Animal models exist for genetic disorders (e.g. glycogen storage disease, MH), toxin-induced myopathies (e.g. rhabdomyolysis, critical illness myopathy), as well as neuropathies (Gonzalez *et al*., 1998; Chander *et al*., 2003; Rich and Pinter, 2003; Rucker *et al*., 2004). The vast majority of skeletal muscle myopathy models are in the mouse or rat; however some researchers have used rabbits for studying rhabdomyolysis, inclusion body myositis and botulinum-induced toxicity (Piepmeier *et al*., 1995; Gonzalez *et al*., 1998; Chen *et al*., 2008). The ability of the researcher in controlling the experimental conditions and interpreting the data in the context of human disease is critical with the use of animal models.

4 CLINICAL MANAGEMENT OF SKELETAL MUSCLE TOXICITY

4.1 Introduction

The aetiology of skeletal muscle toxicity has a diverse set of possible mechanical, molecular, biochemical, genetic or pharmacological origins (reviewed in Guis *et al*., 2003). However, there are many similarities in the clinical and laboratory findings between different types of skeletal muscle toxicities. The hallmark finding of skeletal muscle toxicities tends to be disruption of the sarcolemmal membrane surrounding myofibrils. This determination is traditionally not made by visualizing sarcolemmal rupture via microscopy or histology, but rather by assessing serum levels of intracellular myocyte proteins. The disruption of this membrane can lead to pain, electrolyte disturbances, abnormal muscle function and muscle cell lysis. Potentially more serious complications include muscle necrosis, renal failure, shock and even death. Skeletal muscle toxicities can present as syndromes with an inconsistent array of symptoms, making assessment and diagnosis particularly challenging. The clinician treating these disorders must be experienced with musculoskeletal examination, associated diagnostic observations and medical management of the patient.

4.2 Clinical Presentation

The most important and obvious clinical symptom of myopathy is muscle pain and/or weakness, which is seen in the majority of skeletal muscle toxicities. Also, unlike electrolyte disturbances and laboratory test deviations, these symptoms can be easily assessed by the clinician or reported by the patient. The principal patient report will be pain or tenderness in the affected area. The area in question can be a muscle group or compartment or a more focal region. The most frequently affected areas are the extremities and lower back (Finnish Medical Society, 2007).

Assessment of a patient with musculoskeletal symptoms begins with a medical history and basic physical examination which includes a test for muscle pain

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and muscle strength/muscle weakness (Barohn, 2008). Joints, bones, tendons and muscles should be individually palpated to isolate the painful area. Determining the origin of the pain can assist the clinician in differentiating between neurological and musculoskeletal pain. A typical musculoskeletal examination would also include passive and active range of motion for affected muscle groups. In addition, a motor and sensory neurological examination should also be performed. Techniques for neurological examination include isolating the involved nerve group (e.g. deep versus superficial reflexes, fasciculations and cranial nerves). Motor and sensory deficits that follow a pattern consistent with myopathy rather than neuropathy should be identified.

The clinical presentation of a patient with musculoskeletal toxicities can be deceivingly similar to other metabolic, nervous system or genetic disorders. Differential diagnosis is a crucial part of the initial patient assessment. The patient assessment should be focussed on excluding other possible causes of symptoms such as traumatic injuries (e.g. crush injuries), bacterial infections, heatstroke, genetic disorders (e.g. CPT (carnitine palmitoyl transferase) deficiency) and autoimmune diseases (e.g. Guillain-Barré syndrome).

4.3 Diagnostic Observations

In addition to the physical findings mentioned above, clinical laboratory tests can be used to support the diagnosis of skeletal muscle injury. As mentioned previously, loss of integrity of the sarcolemmal membrane is a cardinal feature of myopathies. This results in release of the cellular contents, which can then be detected in the systemic blood flow. In addition to serum indicators, histological examination of muscle biopsies can provide valuable evidence needed for diagnosis.

The most appropriate and most sensitive indicator of skeletal muscle injury is a test for CK, also known as phosphocreatine kinase or creatine phosphokinase. Creatinine kinase is an enzyme involved in supplying the muscle with phosphate needed to make energy as ATP. Myosin uses ATP to travel along actin filaments and contract muscles. The energy pool devoted to this process is stored as phosphocreatine, which can then be rapidly liberated by CK to generate ATP when needed. This enzyme is found in the cytoplasm and mitochondria of cells in skeletal and heart tissue. Certain isoenzymes, such as CK-MB are most highly expressed in the heart, and therefore a ratio of total CK to CK-MB is used to differentiate between skeletal muscle versus cardiac muscle injury. Serum CK levels are expressed in units of enzyme activity over litres of blood (U l^{-1}). The basal levels of serum CK are negligible (30–200 U l^{-1}); therefore elevated levels are sensitive indicators of muscle damage. CK levels that are five-fold higher than the normal range are indicative of the most severe form of myolysis: rhabdomyolysis. Serum CK level is also useful for monitoring muscle injuries because it has a predictable clearance from the systemic blood flow, provided normal renal function is present. Onset of elevated CK levels is 12 hours following muscle injury, while peak levels can occur 24–36 hours postinsult. The levels usually decline within three to five days if the muscle injury has resolved. Failure of the CK levels to return to baseline indicates a continuing myopathic condition. A serum CK level greater than 5000 U l^{-1} can indicate impending or current renal failure, although other tests such as serum or urine myoglobin may be more useful for that purpose (Ward, 1988).

Myoglobin is a monomeric haem protein with high binding affinity for oxygen. It is the protein that gives meat its red colour. Although structurally related to haemoglobin, it is only present within muscle cells and therefore a useful marker for muscle injury. Serum levels of myoglobin are less useful than serum CK because the half-life is only approximately 1–3 hours and can therefore lead to false negatives if not collected at the appropriate time (Beetham, 2000; Barohn, 2008). However, myoglobin excreted through the kidneys will colour the urine dark red and is therefore more easily detected. Presence of urine myoglobin can be assumed in a patient with haematuria whose urine tests negative for red blood cells using conventional urine test strips. Confirmation is performed either microscopically by a urine myoglobin assay or via electrophoresis and radioimmunoassay.

During muscle injury that results in cell lysis, chemicals such as phosphates, potassium and urine acid are released in addition to proteins (e.g. CK and myoglobin) (Haschek and Rousseaux, 1998). Electrolyte disturbances are a common finding in skeletal muscle toxicities. Elevations in phosphates, potassium and uric acid are most frequently seen. This can result in severe acidosis, especially in the presence of concomitant renal failure. Damage to myocytes also causes release of sarcoplasmic calcium stores, creating precipitates in the cytosol in addition to lowering the serum calcium level. If severe, the hyperkalaemia and hypocalcaemia can cause cardiovascular complications such as arrhythmias.

Specialized tests such as EMG and histology are sometimes used to confirm diagnoses as well as exclude other potential conditions. EMG findings are typically unremarkable, because they primarily evaluate conduction problems. Muscle biopsies may be taken and subjected to electron microscopy, immunohistochemistry and, potentially, genetic studies. Muscle biopsy samples from patients with severe myopathy may show an irregular distribution of myocytes or necrotic areas of cell death.

Rhabdomyolysis is important to mention independently of other conditions of skeletal muscle damage because it is relatively common and one of the most

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severe forms of myopathy. Rhabdomyolysis is characterized by a triad of symptoms; myasthenia/myalgia, elevated serum CK levels and myoglobinuria (Ward, 1988; Beetham, 2000; Barohn, 2008). As swelling accumulates at the site of muscle injury, systemic hypovolaemia and a rise in blood pressure is seen. Although all forms of skeletal muscle toxicity involve some cellular damage, rhabdomyolysis is defined by the release of toxic intramyocellular substances into the bloodstream. The most severe complication of rhabdomyolysis is renal failure caused by acute tubular necrosis (ATN) (Finnish Medical Society, 2007). The release of myoglobin from damaged skeletal muscle quickly exceeds the capacity of serum binding proteins to carry it. The kidneys will begin to clear the myoglobin once its concentration exceeds $230\,mg\,l^{-1}$ (about three times basal levels) (Coco and Klasner, 2004). Acidosis caused by release of phosphate, potassium and uric acid can cause the myoglobin to dissociate into globulin and the iron haem protein ferrihaemate, usually below pH 5.6. Ferrihaemate is a direct toxin for the renal tubular epithelium, in addition to causing myoglobin casts which precipitate within the kidney and cause obstruction. Subsequently, renal failure, shock and disseminated intravascular coagulation are all potential outcomes with rhabdomyolysis.

4.4 Clinical Management of Symptoms

The goals in managing a patient with myopathy are to determine and remove the initial cause of the injury, ensure hydration and correct electrolyte imbalances (Better and Stein, 1990). The fluid balance within the myocytes as well as between the associated musculoskeletal compartments and the systemic vasculature must be corrected with intravenous fluid administration. Hydration with isotonic fluids such as normal saline should begin as early as possible. By diluting elevated electrolytes and myoglobin, aggressive hydration can ameliorate metabolic acidosis and assist in alkalinization of the urine (Coco and Klasner, 2004). Urine alkalinization via administration of intravenous sodium bicarbonate can be used to prevent renal cast formation by titrating urine pH to 6.5. Its use should be carefully monitored as it can exacerbate hypocalcaemia. The osmotic diuretic mannitol can also be used to cause dieresis which may be effective in raising urine pH (Brown *et al*., 2004). Urine output should be carefully monitored using a Foley catheter, in addition to urine pH levels and acid-base status (Bertorini, 1997).

The most common electrolyte disturbances seen in myopathies are hypocalcaemia, hyperkalaemia and hyperphosphataemia. The hypocalcaemia initially is due to sequestration of calcium within the myocytes; however, in later stages of myopathy liberation of that pool can cause increases of systemic calcium and subsequent hypercalcaemia. Hypocalcaemia can be left untreated unless cardiac arrhythmias or seizures are present. The patient should, however, be carefully monitored for late-stage hypercalcaemia (Coco and Klasner, 2004). Hyperkalaemia can be treated by shifting plasma potassium into the intracellular compartment. This can be accomplished by coadministration of glucose and insulin, using inhaled β-agonists, or potassium exchange resins such as Kayexalate (sodium polystyrene sulfonate) (Brown *et al*., 2004). Hyperphosphataemia is usually treated by hydration and dieresis. Conditions resistant to appropriate interventions such as severe anuric renal failure, persistent electrolyte abnormalities or metabolic acidosis may require dialysis (Brown *et al*., 2004).

5 MYOTOXIC AGENTS AND THEIR MECHANISMS OF ACTION

5.1 Overview

The most common expression of drug-induced direct muscle toxicity is rhabdomyolysis, a diffuse, noninflammatory breakdown of skeletal muscle. In most cases, the precise mechanisms of xenobiotic or hormone-induced skeletal muscle breakdown are poorly defined, but it is apparent that mitochondrial dysfunction, energy (ATP) depletion and disruption of ionic gradients (e.g. calcium, protons, potassium, sodium) underlie the vast majority of myopathic responses. No matter the upstream intracellular toxic signalling events, agents that induce rhabdomyolysis ultimately cause sarcolemmal breakdown leading to the spilling of the intracellular contents of myocytes into the bloodstream. As to be discussed in further detail below, serum levels of intracellular skeletal muscle enzymes such as CK and myoglobin are useful quantitative markers of muscle damage both in clinical cases and in experimental animal models of muscle toxicities. In addition, accumulation of myoglobin in the bloodstream (myoglobinaemia) is a major cause of renal failure and death induced by skeletal muscle toxins. Interestingly, a diverse array of compounds induce parallel muscle breakdown and hyperthermia. From a clinical perspective, the most common myotoxicities are associated with exposures to agents that also induce concomitant hyperthermia. From a biological viewpoint, information gleaned from understanding the mechanisms of thermoregulatory myotoxins may shed light on a mystery in human biology, dealing with the mechanisms by which the body generates heat, and the role of muscle in thermogenesis.

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5.2 Hyperthermia-Inducing Rhabdomyolytic Agents

Skeletal muscle fibres are especially sensitive to destruction by a pathologic rise in body temperature. Given its metabolic prominence, skeletal muscle is also a main site for heat production in the human body, but the extent to which hyperthermia and skeletal muscle breakdown are causally related, or rather are epiphenomena, is unclear. One of the least understood aspects of human metabolic physiology involves the actual mechanism(s) and cellular sources of heat generation in response to physiologic inducers such as bacterial toxins for fever, or in response to hyperthermia-inducing agents such as amphetamines, serotonin reuptake inhibitors (antidepressants) and levothyroxine, to name a few. Not all agents that induce rhabdomyolysis induce hyperthermia. Interestingly though, most if not all compounds, including physiologic bacteria-derived fever mediators capable of raising body temperature, universally share a common capacity to induce rhabdomyolysis. Accumulating evidence from studies on two main muscle poisons, volatile anaesthetics and amphetamine-type drugs, implies a causal link between thermogenic mechanisms and rhabdomyolysis. These links are based upon the identification of hyperthermia mediators RyR (the target of volatile anaesthetics) and uncoupling protein 3 (UCP3, the target of amphetamines), both of which regulate thermogenic ionic cycles (see below) and are enriched in skeletal myocytes. **Table 2** includes a list of agents that induce concomitant hyperthermia and rhabdomyolysis, along with those agents that induce myopathic responses independently of effects on body temperature.

5.3 Skeletal Muscle in Temperature Homeostasis and Drug-Induced Hyperthermia Syndromes

Body temperature in warm-blooded animals is regulated by a complex and highly orchestrated series of central neuronal and peripheral physiologic mediators, and is integrated mainly by the hypothalamus in the brain. Brain neurotransmitters serotonin, norepinephrine and dopamine are the primary mediators of temperature regulatory neurotransmission, with serotonin being the most important. Prothermogenic changes in the levels of these central hypothalamic neurotransmitters in thermoregulatory neurons in the hypothalamus lead to activation of the peripheral sympathetic nervous system and the release of norepinephrine, the primary thermogenic neurotransmitter, at peripheral organ sites and into the bloodstream. Norepinephrine interactions with α adrenergic receptors in cutaneous vessels lead to heat conservation by vasoconstriction. Interactions of norepinephrine with the β_3 adrenergic receptor lead to metabolic heat production, though the mechanisms and tissue sites are not well

Table 2 Agents that induce myopathic responses

Major agent-class/syndrome	Drug subtypes/examples
Co-inducers of rhabdomyolysis and hyperthermia	
Bacterial pyrogens (fever)	Lipopolysaccharides, endotoxin
Adrenergic agents (indirect mitochondrial uncouplers)	Phenylethylamines including amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA); cocaine, monoamine oxidase inhibitors
Direct mitochondrial uncouplers	Salicylates, pentachlorophenol, dinitrophenol
Neuroleptic malignant syndrome	Serotonin-increasing drugs such as tricyclic antidepressants; dopamine modulators such as olanzapine, metoclopramide, promethazine
Anticholinergic hyperthermia	Plant 'belladonna' alkaloids, antispasmodics, antiulcer drugs, acetylcholine receptor antagonists
Malignant hyperthermia	Volatile anaesthetics, depolarizing muscle relaxants
Serotonin syndrome	Hallucinogens, serotonin reuptake inhibitors
Thyrotoxicosis	Thyroid hormone, levothyroxine
Inducers of myopathies not associated with hyperthermia	
Endocrine disrupters	Anabolic steroids, dexamethasone, vitamin D, parathyroid hormone dysregulation, growth hormone intoxication
Lipid-lowering compounds	Statin drugs, fibrate analogues, niacin
Alcohol intoxication	Acute and chronic alcohol ingestion
Lysosomal disrupting agents	Chloroquine, amiodarone
Potassium-disrupting agents	AChE inhibitors, diuretics
Microtubule-disrupting agents	Colchicine, vincristine, phalloidin
Neuromuscular blockers	AChE inhibitors

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defined. In human and animal studies, infusion of norepinephrine into isolated brown adipose tissues (BATs) and skeletal muscle induces thermogenesis (Astrup *et al*., 1984; Astrup, 1986). Thus, it is likely in humans, which lack BAT, that the sympathetic nervous system activates thermogenic mechanisms in muscle (Astrup *et al*., 1984). In addition, the precise molecular mechanisms for the selective sensitivity of muscle to undergo breakdown in response to body heat accumulation are unclear. Hyperthermia is distinguished aetiologically from fever by its lack of regulation, or rather, its escape from normal temperature regulatory mechanisms. Fever, on the other hand, is not generally myotoxic, and involves a more moderate and highly regulated rise in body temperature in response to infection.

The sum inefficiency of all metabolic reactions in the body generates a sufficient amount of heat to keep the body warm at neutral ambient temperatures (~25 °C). This type of heat production is referred to as *obligatory thermogenesis*. On the other hand, inducible metabolic reactions are required to raise body temperature in response to cold exposure, infection or thermoregulatory toxins. In this type of *facultative* thermogenesis, as will be discussed below in more detail, three metabolic mechanisms have been identified that are able to generate sufficient heat to rapidly raise body temperature: (i) exercise or skeletal muscle contraction (shivering, tetany); (ii) futile proton cycling in mitochondria; and (iii) futile RyR-induced calcium cycling in the SR. Each of these processes, when overactive, is associated with significant skeletal muscle damage and morbidity in humans.

5.4 Thermogenic Mechanisms Involving Futile Ionic (Ca^{2+}, H^{+}) Cycling

Facultative thermogenesis in man is poorly understood, but in most, if not all types of significant heat production in cells, the futile transport and flux of ions such as calcium and protons is the main thermogenic mechanism. The term *futile* is meant not to imply that ionic flux is meaningless. Rather, the active, energy-requiring transport of protons out of the mitochondrial matrix during oxidative phosphorylation, and the pumping of calcium ions into the SR, are processes that are seemingly 'wasted' by the leak or passive diffusion of these ions back into the respective cellular compartments from where they were originally pumped. Of these, only proton cycling and leak in mitochondria is established as the physiologic generator of heat production in the cells of endothermic organisms. Futile calcium cycling, as discussed in more detail below, is only thought to contribute to pathologic thermogenesis induced in the pharmacogenetic syndrome MH.

In rodents and hibernating mammals, the two organs established to generate sufficient heat for the quick increase of body temperature are skeletal muscle and BAT. One view of skeletal muscle thermogenesis, at least in a physiologic sense, is that shivering accounts for its main capacity to generate heat and participate in body temperature regulation. However, shivering is only short lived during infective-fever-associated thermogenesis, and many toxic thermogenic responses, including those induced by mitochondrial uncouplers (e.g. dinitrophenol), serotonin reuptake inhibitors, amphetamines and thyroid hormone (TH) excess do not involve a significant shivering component, and therefore appear to involve heat-producing mechanisms apart from contraction. Contraction-independent heat production in skeletal muscle can be invoked by diverse compounds, suggesting the capacity for similar mechanisms to exist for physiologic functions. Unlike shivering or contraction-induced heat production, BAT thermogenesis doesn't involve work by cells *per se,* but rather employs a futile cycle of mitochondrial proton flux to convert energy stored in mitochondria to heat. Essentially, much like the heat produced by short-circuiting an electrical device (e.g. automobile battery), proton leak short-circuits the pH and proton gradient across the inner mitochondrial membrane and generates heat. Unlike white fat, BAT is a mitochondria-rich fat tissue, and is established as the thermogenic mediator of cold-adaptation thermogenesis in some mammals. Mitochondrial uncoupling protein 1 (UCP1), localized to the inner mitochondrial membrane of brown adipocytes, is the molecular mediator of BAT-induced heat production (Nicholls and Rial 1999). The proton gradient provides the energy necessary for ATP production by the F1/F0 ATPase. As protons are released into the mitochondrial matrix, by flowing through the proton pore formed by the ATPase, the energy released from proton flux along its concentration gradient is consumed for the phosphorylation of ADP to ATP. In contrast, proton leak induced by UCPs uncouples this proton gradient from ATP synthesis through the provision of an alternate pathway for proton entry into the mitochondrial matrix. However, since proton-leak-generated energy is not linked to another energy-consuming process, the energy is released as heat. The 'futile cycle' component of mitochondrial uncoupling involves the continuous pumping of protons from the matrix to the inner membrane space for the creation of the mitochondrial electron-transport proton gradient, followed by the successive leak of these protons back into the matrix. Much like the excessive and thermogenic cycling of calcium into and out of the SR that accounts for malignant hyperthermia (to be discussed below), UCP-induced heat production involves a thermogenic futile cycle of proton extrusion from, and leak back into, the mitochondrial matrix.

One challenge to our understanding of human thermoregulation is that adult humans, like most large

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animals, have minimal amounts of BAT, and no similar physiologic thermogenic mechanism in humans has been identified. Thus, human BAT depots are regarded as thermogenically irrelevant due to the small aggregate size of the tissue in man. In 1997 it was discovered that human skeletal muscle is enriched in UCP3, a UCP1 homologue linked to skeletal muscle thermogenesis and toxicities in experimental animal models (Vidal-Puig *et al*., 1997; Mills *et al*., 2003). Consistent with the idea that humans may have evolved a similar pathway of thermoregulation in muscle as for the BAT of rodents, sympathetic nervous system stimulants activate skeletal muscle thermogenesis and shut down brown fat thermogenesis (Rusyniak *et al*., 2008). Likewise, a recent series of reports demonstrated that certain of the substituted amphetamines induced hyperthermia and rhabdomyolysis through the activation of UCP3, a skeletal muscle-enriched proton leak regulator. UCP3-null mice were shown to be almost completely resistant to a range of thermogenic doses of the amphetamine 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy), and MDMA failed in these studies to induce lethality in UCP3-null mice at doses that were lethal to wild-type mice and can induce rhabdomyolysis and death in humans (Mills *et al*., 2003). Similarly, rats administered β adrenergic inhibitors designed to inhibit the activation of upstream signalling pathways regulating UCP3 activity were protected from rhabdomyolysis induced by amphetamine-type psychostimulants (Sprague *et al*., 2004a; 2005). More work is needed to understand the relationships between UCP3 and diverse other myotoxic responses, and the mechanisms by which some drugs elicit UCP3 activation in skeletal muscle. However, excessive uncoupling of mitochondrial ATP synthesis and thermogenesis in skeletal muscle resulting from UCP3 activation appears to be critical for both the thermogenic and myotoxic responses to all amphetamine drugs, and therefore represents a new target for the development of strategies of intervention and treatment for certain skeletal muscle toxicities. **Figure 4** provides an overview of proton cycling in mitochondria and its relationships to thermogenesis and oxidative phosphorylation.

The mitochondrial electron transport chain is energized by the intake of fuel substrates derived from food and their oxidation (combustion) in the mitochondrial matrix to liberate electrons used in the oxidation and reduction of mitochondrial respiratory complexes I–IV in the inner mitochondrial membrane. Electrons passed from fuel substrates to electron donors NADH and FADH2 (flavin adenine dinucleotide) are passed from complexes I and II, respectively, to complex III and on to complex IV by the membrane redox carrier coenzyme Q (CoQ) and the soluble protein, cytochrome C. Electrons at complex IV are passed to molecular oxygen to form water (oxygen consumption). As electrons pass along complexes I, III and IV, conformational changes lead to the pumping of protons from the matrix to the inner membrane space. These protons can flux back into the matrix via the F1/F0 ATPase, where the energy from proton flux is consumed for ATP production. Alternatively, protons can 'leak' back into the matrix by UCP activation. Since this process isn't coupled to an energy consuming reaction, the energy from proton flux is liberated as heat. This is the most significant thermogenic mechanism in warm-blooded animals, and in experimental models

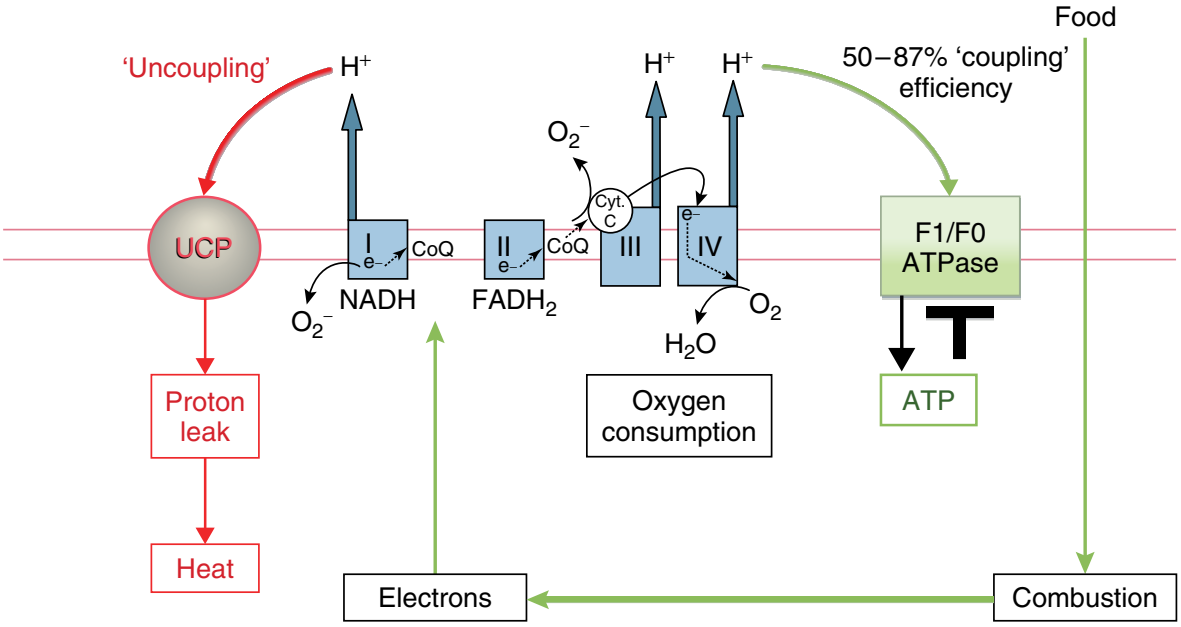

Figure 4 Thermogenic uncoupling of the mitochondrial electron transport chain. Abbreviations used: CoQ—coenzyme Q; Cyt. C—cytochrome C.

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is associated with amphetamine-induced muscle breakdown and hyperthermia by the skeletal muscle-enriched UCP3.

5.5 Rhabdomyolytic Agents that Induce Hyperthermia

5.5.1 Volatile Anaesthetics: Inducers of the Pharmacogenetic Syndrome, Malignant Hyperthermia

Halogenated anaesthetic drugs and muscle relaxants, including all gaseous anaesthetics, and the neuromuscular-blocking skeletal muscle relaxant succinylcholine, induce a rare, genetically mediated autosomal dominant syndrome of skeletal muscle-derived hyperpyrexia referred to as malignant hyperthermia (MH) (Stowell, 2008). MH is a highly lethal condition that is caused by skeletal muscle heat production and leads to diffuse necrotic skeletal myocyte death and leakage of muscle-derived ions, myoglobin and CK into the bloodstream. Hyperthermia induced during MH is associated with intravascular coagulation and pulmonary oedema, and myoglobinaemia efficiently damages renal glomeruli, leading to renal failure. In many cases, renal failure and hyperthermia precipitate multiorgan failure and death. In this toxic syndrome, it appears that the loss of intramyocyte levels of ATP, heat accumulation and high levels of intracellular calcium combine to form a triad of toxicities that culminate in the loss of sarcolemmal integrity and the release of myoglobin from necrotic muscle fibres.

Though several mutations are linked to MH susceptibility, the most important loci are found in the RyR of the sarcoplasmic reticulum (Girard *et al*., 2008). MH susceptibility mutations are generally associated with an increased extracellular calcium-induced intracellular calcium release response from the SR that is unmasked when patients are administered MH-triggering agents such as general anaesthetics or muscle relaxants. In these cases, the genetic lesion and drug administration causes excessive RyR-dependent calcium cycling across the SR membrane. This ionic flux is thought to give rise to the excessive skeletal muscle thermogenesis associated with MH syndrome, and the overwhelming of the body's thermoregulatory homeostatic mechanisms, and hyperthermia. Moreover, because MH is generally caused by myocyte calcium accumulation, intoxication is also associated with profound muscle contraction, which is both thermogenic and metabolically exhaustive to the muscle (ATP depletion) as well as the rest of the body. MH syndrome can easily usurp oxygen from the rest of the body, and combined with hyperthermia and rhabdomyolysis is a highly life-threatening toxic response associated with multiorgan failure, disseminated intravascular coagulation, coma and death. Parenteral administration of the RyR inhibitor dantrolene is the only approved treatment for MH (Kobayashi *et al*., 2005). Combined with screening for susceptibility mutations and the avoidance of anaesthesia in susceptible individuals, the use of dantrolene has dramatically decreased the overall mortality rates from MH worldwide from near 80% in the 1960s to roughly 10% today.

During an MH event, excess calcium in the cytoplasm leads to ATP-dependent activation of SERCA pumps to limit excess toxic calcium accumulation in the cytosol of myocytes. ATP is also consumed during the high-calcium-induced contractile responses of skeletal muscle during MH. Thus, hyperthermia, ATP depletion and oxygen consumption each likely contributes to generalized necrotic skeletal muscle breakdown during MH. MH is associated with a significant release of intracellular constituents including myoglobin into the bloodstream, and can rapidly induce kidney and other multiorgan failures, and death, without rapid interventions. Common agents which cause or exacerbate RyR-dependent malignant hyperthermia include the plant-derived alkaloid ryanodine, halothane, 4-chloro-*m*-cresol, caffeine and succinyl choline.

5.5.2 Sympathomimetic Drugs (Amphetamines, Cocaine)

The abuse of sympathomimetic drugs such as MDMA (Ecstasy) and methamphetamine (METH) has steadily increased worldwide into what was cited by the United Nations in a 2003 report as a worldwide 'public health crisis'. The two most dangerous side effects of exposure to these psychostimulant drugs are hyperthermia and rhabdomyolysis. Sequelae of intoxication downstream of muscle breakdown and hyperthermia include, as in MH cases, myoglobinaemia, myoglobinuria, ARF and disseminated intravascular coagulation. However, because sympathomimetics mimic the actions of sympathetic nervous system catecholamine neurotransmitters, norepinephrine and epinephrine, their toxic actions also include increased vasoconstriction and heart rate. These effects can lead to increased blood pressure and adverse myocardial events. Most importantly for skeletal muscle, hyperthermia in the setting of vasoconstriction can lead to a double-edged sword of heat accumulation and muscle ischaemia (reduced blood flow). Amphetamines increase metabolic heat production (through mitochondrial uncoupling and proton flux) and block heat dissipation by constricting vessels, decreasing both muscle perfusion and heat dissipation from cutaneous vessels.

Not surprisingly, given the clinical similarities and outcomes, it has long, and erroneously, been assumed that all disorders of skeletal myotoxicity that are associated with hyperthermia are due to MH mechanisms (Girard

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and Urwyler, 2007). Biophysical mechanisms of thermogenesis, and therefore skeletal muscle breakdown, do appear to be similar in cases of amphetamine toxicity to MH-induced rhabdomyolysis and toxic calcium cycling. Recent evidence indicates that the core mechanisms in sympathomimetic-induced skeletal muscle breakdown also involve ionic cycling. However, MDMA- and METH-induced thermogenesis appears to employ mitochondrial proton leak rather than sarcoplasmic calcium flux (Sprague *et al*., 2003; 2004b). A recent report showed that dantrolene, the RyR inhibitor that blocks RyR-induced calcium flux and is a treatment for MH, fails to affect MDMA hyperthermia in rodents (Rusyniak *et al*., 2004). On the other hand, as mentioned above, mice deficient in skeletal muscle-enriched UCP3 fail to mount a significant thermogenic response to MDMA in experimental conditions, and are protected from MDMA-induced death (Mills *et al*., 2003). In contrast to MH, where anaesthetics appear to have direct-acting effects on the sarcoplasmic reticulum RyR molecule, activation of proton leak by UCPs in MDMA intoxication appears instead to be indirect (Sprague *et al*., 2003). MDMA initiates thermoregulatory derangements and ultimately skeletal muscle breakdown downstream of its activation of norepinephrine-activated β_3 adrenergic receptors, localized to diverse tissues in the periphery. In rodents, MDMA induces a massive increase in plasma norepinephrine that precedes the onset of hyperthermia and skeletal muscle breakdown and renal failure (Mills *et al*., 2004). If β_3 adrenergic receptors are antagonized with the β_3 adrenergic receptor-antagonist, carvedilol (used in cardiovascular medicine), MDMA hyperthermia and rhabdomyolysis are effectively prevented and fully reversed. β_3 adrenergic receptors liberate UCP, activating free fatty acids most importantly in fat and muscle cells, and evidence points to a mechanism involving these intracellular fatty acid messengers in the pathology of MDMA-induced hyperthermia. What aren't clear are the precise locations (either white fat cells or skeletal myocytes, or both) of the β adrenergic receptors most significant in the myotoxicity induced by amphetamines, and the precise mechanisms wherein the free fatty acids are coupled to mitochondrial UCP3 activation. What is clear is that β_3 adrenergic receptors, free fatty acids, and most importantly mitochondrial UCP3 and proton flux distinguish MDMA-induced hyperthermia and rhabdomyolysis from calcium-mediated, anaesthesia-induced MH rhabdomyolytic syndromes.

5.5.3 Thyroid Hormone Intoxication

The most important endocrine regulator of body temperature is thyroid hormone, which acts by binding to intracellular steroid/TH receptor transcription factors and activating the gene expression of a plethora of metabolic regulatory genes in cells. The endogenous thyroid hormones, thyroxine (T4) and triiodothyronine (T3), regulate numerous biological pathways that control the overall metabolism of carbohydrates, proteins and fats, as well as the distribution and regulation of other endocrine hormones. Due to the prominent role THs play within the body, changes in their levels are capable of causing a wide variety of symptoms and health problems, namely related to metabolism. When thyroid tissue becomes over-stimulated due to antibody-mediated autoimmune reactions (Graves' disease), cancer or an imbalance of thyroid stimulating hormone, supraphysiologic and toxic concentrations of T3, T4 or both are released into the bloodstream. This hyperthyroid state induces weight loss, arrhythmias and dyspnoea, but can often lead to thyrotoxic myopathies and hyperthermia (Ramsay, 1966; Engel, 1972). Like other myopathies, the initial symptom of thyrotoxic myopathy involves muscle weakness, and the symptoms of frank thyrotoxicosis (a severe form of hyperthyroidism that is acutely life threatening) include rhabdomyolysis and severe body heat accumulation. Weakness can occur gradually and usually appears in the shoulders, neck and hips. If not reported, weakness can sometimes spread to the eyes and throat, potentially impairing speaking, breathing and vision (Puvanendran *et al*., 1979). If left untreated, chronic and acute cases can result in permanent muscle fibre damage and possibly rhabdomyolysis (Alshekhlee *et al*., 2002). Evidence suggests that it is the TH-induced up-regulation of metabolic respiration, β-oxidation and protein degradation that often leads to the muscle weakness and eventual muscle breakdown/atrophy associated with hyperthyroidism (Bennett and Huston, 1984; Celsing *et al*., 1986; Fukui *et al*., 2001). Muscle biopsies have also revealed an alteration in muscle fibre type from Type 1 oxidative slow-twitch fibres to Type 2 glycolytic fibres. This is attributed to the up-regulation of genes, such as the myosin heavy-chain gene, transcribed by the thyroid nuclear hormone receptor (Olson *et al*., 1991). Interestingly, one of the metabolic proteins induced strongly by thyroid hormones is the thermogenic UCP3, the target of MDMA-induced hyperthermia and rhabdomyolysis (Bouillaud *et al*., 2001). Hyperthyroidism has been shown to lead to dramatically enhanced MDMA thermogenesis, and hypothyroidism leads to a loss of hyperthermia induced by MDMA, and a loss of UCP3 expression (Sprague *et al*., 2003). Though the molecular mechanisms still remain to be fully elucidated for a variety of thermogenic rhabdomyolytic syndromes, interesting links are being established between UCP3 and a variety of myotoxic thermal dysfunction-inducing agents.

There have also been reports linking TH dysregulation to the muscle disorder known as periodic paralysis. Periodic paralysis is considered a genetic disease, occurring predominantly in Asian ethnicities (Okinaka *et al*., 1957; Crane, 1960; McFadzean and Yeung, 1967). This syndrome is characterized by recurring and temporary

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muscle fatigue due to malfunctions in potassium channels resulting in hyperkalaemia (Tinker and Vannatta, 1987; Kung, 2006). This channelopathy is triggered by high-carbohydrate diets, stress and starvation. Not only does hyperthyroidism affect overall metabolism, but it is also known to increase the activity of sodium/potassium ATPases, which contributes to hyperkalaemia and inhibited muscle contraction (Lin and Akera, 1978; Lam *et al*., 1989; Chan *et al*., 1991). Once a patient becomes euthyroid through drug treatment or thyroidectomy, periodic paralysis ceases to occur.

5.6 Myopathic Agents Not Associated with Significant Induction of Hyperthermia

5.6.1 Endocrine Disrupters

- **Growth hormone:** One of the most common syndromes associated with the pituitary gland is acromegaly, a disease wherein an individual produces excess growth hormone. It is often the result of a pituitary adenoma, but more recently has been associated with growth hormone abuse by bodybuilders. This condition causes skeletal muscle hypertrophy in both Type I and Type II muscle fibres. Myolysis has been confirmed by elevations in serum creatinine kinase (Wolf *et al*., 1978). Actinomyosin ATPase levels are reduced and membrane excitability is also decreased; this commonly results in myofibrillary vacuolization, apoptosis and tissue degeneration (Ruff and Weissmann, 1988; Frustaci *et al*., 1999). The exact mechanism by which this occurs remains unclear, but it is believed to involve nitric oxide (Sacca *et al*., 2003).
- **Corticosteroid intoxication and deficiency:** Corticosteroids are steroid hormones synthesized in the adrenal cortex that are essential regulators of immune, stress and inflammatory responses, and also play fundamental roles in metabolic and catabolic reactions throughout the body. Corticosteroids are generally subdivided into two classes: glucocorticoids and mineralocorticoids. Glucocorticoid hormones such as cortisol modify lipid, carbohydrate and protein metabolism to promote energy storage and stress responses. They also play roles in preventing inflammation by reducing cytokine production and secretion, as well as inducing apoptosis of cytokine-producing cells. Mineralocorticoids, the most common being aldosterone, control electrolyte and water homeostasis by modifying sodium retention in the kidney nephron. Consistent with their metabolic functions, excessive or high-dose corticosteroid use often causes a generalized metabolic shift towards energy storage in patients. This is evident by fat redistribution to the trunk in affected individuals. Corticosteroid myopathy is typically localized to the extremities with the legs being the preferential site for progressive weakness and muscle wasting, with only rare cases of myalgia. This corticosteroid-induced atrophy is marked by CK elevation and rhabdomyolysis primarily in Type II fibres (Dekhuijzen and Decramer, 1992; Fernandez-Sola *et al*., 1993).

Glucocorticoids have been demonstrated to increase cytoplasmic protease activity in skeletal muscle, which increases the rate of muscle damage as well as inhibiting repair mechanisms (Lang *et al*., 2001b). This effect is potentiated by glutamine synthetase, the major enzyme that produces glutamine from glutamate. Under conditions of high circulating glucocorticoid levels, intramuscular glutamine levels drop initially, resulting in decreased protein synthesis. This effect is followed by enhanced function of glutamine synthetase, which activates catabolic pathways and further exacerbates proteolysis (Shoji *et al*., 1974; Max *et al*., 1988).

Clinical manifestations of corticosteroid deficiency-induced myopathy are often observed in patients suffering from Addison's disease, a condition involving insufficient hormone secretion of the adrenal gland. In these patients, muscle biopsy and CK levels appeared normal, but they displayed a pronounced weakness with occasional paralysis (Ruff and Weissmann, 1988). These effects were found to be caused by impaired carbohydrate metabolism and alterations in serum electrolytes, especially hyperkalaemia (Van Dellen and Purnell, 1969).

5.6.2 Acetylcholinesterase Inhibitors

As mentioned above, ACh is the major neurotransmitter that drives skeletal muscle movement in response to peripheral nervous system (PNS) stimulation. In the PNS, ACh is released by neurons at neuromuscular junctions, resulting in the opening of ligand-gated Na^+ channels, depolarization, calcium-induced sarcoplasmic release of calcium and, finally, muscle contraction. After neuronal firing and release of ACh at the junction, ACh is rapidly metabolized by AChEs. AChE inhibitors (AChE-Is) are compounds that inhibit this enzyme. Common AChE-Is include venoms, pesticides and drugs to combat Alzheimer's disease and myasthenia gravis.

The progression of acute AChE-I poisoning has been well studied in humans. Initially, involuntary fasciculation is observed, followed by weakness, myalgia, rhabdomyolysis, and finally the development of subjunctional necrotic lesions, with Type I fibres being preferentially affected (Laskowski *et al*., 1977; Gupta *et al*.,

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1986). As is commonly observed during rhabdomyolysis, serum CK levels are increased, and myocyte ATP levels are decreased (Gupta and Dettbarn, 1987). In cells, AChE-I toxicity is expressed as mitochondrial swelling, vacuolization, sarcomeric hypercontraction, myofilament breakdown and Z-band fragmentation. Necrosis induced by AChE-I toxicity was observed to originate near the muscle endplate; it is postulated that this effect is initiated by hyperstimulation and increased contractile activity of individual fibres (Gupta *et al*., 1987). This would lead to the rapid consumption of ATP stores, which may in turn exacerbate calcium release from sarcoplasmic Ca^{2+} stores, resulting in the activation of PLA_2 (phospholipase A_2) and Ca^{2+}-activated proteases, causing aberrant signalling and the degradation of macromolecules necessary for cell structure and function.

Treatments for AChE-I poisoning include AChR antagonists such as atropine, AChE phosphorylating/activating drugs such as pyridine-2-aldoxime methyl chloride (2-PAM), and in extreme cases anti-AChE receptor antibodies are administered to block ACh binding (Laskowski *et al*., 1977; Patterson *et al*., 1988).

5.6.3 Amiodarone

Amiodarone is a controversial antiarrhythmic agent that is used for the acute treatment of sudden arrhythmias, or is administered chronically to suppress existing arrhythmic conditions. It has a relatively long half-life and has been found to preferentially accumulate in muscle tissues (Patterson *et al*., 1988). It is considered a class III antiarrhythmic agent as its primary mechanism of action is to prolong Phase 3 of the cardiac action potential. This activity is most likely an effect of amiodarone blocking potassium export channels, which in turn stalls membrane repolarization.

Despite the fact that the most common complications associated with amiodarone are neuropathic in nature, skeletal muscle myopathies are also common. The majority of myopathies resulting from amiodarone administration typically arise when it is used in conjunction with other drugs, especially those of the statin family. Amiodarone myopathies are generally mild and are most commonly described as progressive and persistent muscle weakness similar to myasthenia gravis. This weakness is believed to be due to decreased depolarization in muscle fibres, and therefore the diminished capacity for contraction. There is typically no myolysis associated with amiodarone treatment. Amiodarone is structurally similar to, and also loosely mimics, thyroid hormone in its ability to bind the nuclear thyroid receptor (Franklyn *et al*., 1985). Though the mechanisms for myopathic effects are not clear, it is likely that its activity in the regulation of TH receptors may account for at least some of its effects on skeletal muscle.

5.6.4 Amphiphilic Drug Myopathy

Amphiphilic drugs contain both hydrophilic and hydrophobic regions. Many drugs included in this category are psychotropic compounds such as chlorphentermine, chlorcyclizine, l-chloro-amitriptyline, triparanol, quinacrine (Mepacrine), iprindole or imipramine, as well as chloroquine (CQ), hydroxychloroquine (HCQ) and doxorubicin (Drenckhahn and Lullmann-Rauch, 1979; Kuncl and Wiggins, 1988). Myopathies caused by these drugs generally present as muscle tissue restructuring as denoted by fibre branching and subfibre clustering. Increased vacuolization and myofibrillary necrosis have also been associated with amphiphilic compounds (Drenckhahn and Lullmann-Rauch, 1979). The two regions of these drugs have discrete mechanisms by which they promote myotoxicity. The nonpolar hydrophobic regions insert into myofibre membranes and induce permeabilization leading to electrolyte and osmotic imbalances, and ultimately, necrotic death of the muscle fibre. In contrast, the polar hydrophilic regions of amphiphilic toxins tend to interact with lysosomes in muscle cells where they cause perturbations in lysosomal pH. The core of lysosomes is generally maintained at a pH of 4.5, which is necessary for the proper function of the digestive enzymes that mediate lysosomal functions. Changes in intralysosomal pH inactivate these enzymes and prevent lysosomal degradation and recycling of cellular macromolecules such as lipids, proteins and carbohydrates. Lysosomal dysfunction leads to mucolipidosis, a condition characterized by the intracellular accumulation of waste and debris in myocyte vacuoles (Plante *et al*., 2008). This vacuolization impedes cellular function and myofibrillary contraction.

5.6.5 Emetine

So named, emetine is a potent 'emetic' drug that is often used clinically for the removal of harmful substances from the stomach, and is often abused by bulimic patents for weight control. Emetine is an alkaloid that acts by irritating the gastric mucosa. This in turn leads to stimulation of the medullary chemoreceptor trigger zone (CTZ), likely via serotonin signalling. The CTZ communicates with the area postrema, which then signals the induction of vomiting. While acute use is considered safe, chronic administration of emetine has been linked to several complications, the most common of which are cardiac and skeletal muscle myopathies.

Researchers have discovered that in rats, chronic dosing of emetine caused severe muscle atrophy, with decreases in muscle weight and contraction strength. While atrophy was most common in Type II fibres, decreases in myofibrillar ATPase and NADH tetrazolium

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reductase were also shown in all three fibre types, indicating muscle injury. These animals also showed occasional necrotic and splitting fibres, and it was determined that these effects were not due to neuromuscular aberrations (Blasco *et al*., 1976).

Similar research also indicated that much of this atrophy could be due to apoptosis and autophagy. Chronically dosed rats showed progressive ultrastructural abnormalities in their muscle tissues much like in the above findings. Myofibrillar changes observed included rod formation, Z-line streaming, contractile clumping, myofilament loss and mitochondrial degeneration. It was also found that these myofibres showed extensive vacuolization, membrane proliferation, sarcoplasmic abnormalities and increased lysosomal activities, which are indicative of autophagy (Bindoff and Cullen, 1978; Stam and van Crevel, 1989).

Findings made using immunological cell lines revealed that emetine treatment induced phosphatidylserine exposure, DNA fragmentation and mitochondrial depolarization. It was also shown that emetine resulted in the activation of caspase-3, -9 and -8, but that these effects could be prevented by either over-expressing Bcl-2 or knocking-out caspase-8. These data indicate that if emetine is causing apoptosis in muscle cells, it is most likely through the mitochondrial pathway (Moller and Wink, 2007). Emetine-induced myopathy is reversible upon discontinuation of the drug, and no other treatment exists for emetine poisoning.

5.6.6 Ethanol Myopathy

Ethanol was initially implicated in muscle injury in the early 1800s, and the injurious effects of ethanol on skeletal muscle occur after both acute and chronic exposure (Hed *et al*., 1962; Pittman and Decker, 1971). Acute ethanol myopathy generally occurs after bouts of excessive, or binge, drinking. Chronic ethanol-induced myopathy may occur when lower doses are administered long term (Pittman and Decker, 1971). Symptoms of these conditions are often observed as generalized muscle pain, swelling, rhabdomyolysis, cramping or noninflammatory atrophy. Ethanol, myopathies are commonly associated with or potentiated by degrees of malnutrition and dehydration (Fahlgren *et al*., 1957; Haller and Drachman, 1980). As with many myopathies, those induced by ethanol are diagnosed by elevated levels of serum CK and myoglobinuria. Alcohol myopathy is considered a multifactorial disease state and is often accompanied by other conditions including vitamin deficiency, electrolyte imbalance, metabolic derangement, endocrinopathy, oxidative stress, gene dysregulation and altered signal transduction (Urbano-Marquez and Fernandez-Sola, 2004).

5.6.7 Acute Alcohol Myopathies

Acute alcohol myopathies preferentially affect Type I (slow-twitch) fibres (Haller, 1985). Tissue analysis revealed myofibrillar necrosis, irregularities in sarcomeric banding patterns, mitochondrial disruption and increased cellular vacuolization (Hed *et al*., 1955; Klinkerfuss *et al*., 1967; Martinez *et al*., 1973; Martin *et al*., 1982). Since acute ethanol myopathies are associated with large doses of alcohol, membrane disruption is a major contributor of the disease state. While ethanol exposure does not affect the stoichiometry of membrane components, it does directly alter the fluidity of membranes, especially those of the SR. Ethanol causes the acyl chains of membrane phospholipids to become disordered, and may even trigger dissociation of cholesterol from the membrane during extended exposure (Goldstein and Chin, 1981; Ferguson *et al*., 1984). Under more extreme conditions cell lysis may occur along with inflammatory responses (Preedy *et al*., 1989). Increased membrane fluidity is also in part mediated by the disruption of ion transport mechanisms for sodium, potassium and calcium, resulting in a decrease in the cellular resting state potential (Blachley *et al*., 1985). In addition, acute ethanol exposure increases passive calcium permeability in the SR, which is a probable cause of the negative muscle inotropic effects observed in cases of ethanol intoxication (Ohnishi, 1985).

Several metabolic effects have also been associated with acute alcohol myopathies. These effects include a decrease in glucose uptake and utilization, glycogen depletion, excess lactate production, and increased gluconeogenesis and glycolytic activities (Perkoff, 1971; Hed *et al*., 1977; Chui *et al*., 1978; Cook *et al*., 1988; Peters *et al*., 1996). Protein metabolism has not been well studied in the acute condition, but some reports show that protein synthesis is decreased along with amino acid transport (Rubin, 1979; Rubin and Rottenberg, 1982). Effects on mitochondria include structural derangements, uncoupled oxidative phosphorylation and decreased levels of Ca^{2+} ATPase in myofibres (Lange and Sobel, 1983; Martin *et al*., 1984). Hypokalaemic myopathies have been observed after acute alcohol abuse and generally present as weakness or periodic paralysis. They are thought to be derived from ischaemia induced by vasoconstriction. This condition is exacerbated by exercise (Knochel and Schlein, 1972). Acute ethanol use has also been linked to alterations in gene expression, which could contribute to the development of myopathies. Gene microarrays performed on rats treated with ethanol showed altered expression of over 400 genes (Shankar *et al*., 2006). Genes with altered expression included those involved in metabolism, apoptosis and gene expression.

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5.6.8 Chronic Alcohol Myopathies

Unlike acute alcohol myopathies, chronic consumption of ethanol typically affects Type II (fast-twitch) fibres (Lang *et al*., 1999a). Roughly 50% of high-dose alcohol abusers develop a skeletal myopathy (Estruch *et al*., 1993). Gradual muscle atrophy is the most pronounced metabolic aberration found in chronic alcohol abusers (Lang *et al*., 1999b). This muscle wasting is typically described by a decrease in fibre size, but not fibre number (Peters *et al*., 1985). The metabolic effects of ethanol may be mediated either directly through alterations in membrane fluidity or receptor binding, or indirectly through the generation of secondary mediators such as inflammatory cytokines, stress hormones and reactive oxygen species (ROS).

Skeletal muscle comprises 40% of human body weight and acts as a considerable site for protein synthesis and storage. Chronic ethanol consumption has been shown repeatedly to impair protein synthesis in muscle, particularly that of myofibrillar proteins (Lang *et al*., 1999b; Reilly *et al*., 2000). The eukaryotic initiation factors (eIFs) are responsible for binding of t-RNA (transfer RNA) to the 40 S ribosomal subunit to form the 43 S preinitiation complex. Alcohol does not appear to directly alter the abundance or modification of these complexes (Lang *et al*., 2001a). It does however appear to impair the activity of the mammalian target of rapamycin (mTOR), a key regulatory kinase in many signal transduction pathways. One of the functions described for mTOR is the phosphorylation and inactivation of the eIF-4E binding protein (4E-BP1), which functions to sequester eIF-4E (Gingras *et al*., 2001). Insulin-like growth factor-1 receptor (IGFR-1) also mediates protein synthesis by phosphorylating ribosomal protein S6. Decreased IGFR-1 activation has been observed in skeletal muscle preparations from chronic alcohol users (Kumar *et al*., 2002). Mitochondrial metabolism is also altered in patients presenting with chronic ethanol myopathies. In particular, β-oxidation of fatty acids is markedly decreased (Lange and Sobel, 1983). Even though Type II fibres are more heavily reliant on glucose, and not fats (and mitochondrial respiration) for ATP production, these factors may potentiate the disease state by inhibiting energy production required for muscle repair and function.

ROS have also been implicated in alcoholic myopathy. ROS are most commonly generated in the mitochondrion as a by-product of oxidative phosphorylation where electrons that pass along the respiratory complexes slip away and react with molecular oxygen, forming partially reduced oxygen species. Since ethanol has been shown to damage mitochondria, it is likely that this is the cause of increased ROS generation observed after alcohol exposure. ROS, such as superoxide and hydrogen peroxide, indiscriminately damage cellular macromolecules such as DNA, RNA, lipids and proteins and extensive damage has been shown to induce apoptosis. Rats fed ethanol showed a marked increase in antioxidant enzymes, a sign of increased oxidative stress.

Chronic alcohol consumption has been shown to induce apoptotic death in several cell types including Type II muscle fibres (Fernandez-Sola *et al*., 1996; Narula *et al*., 1996; Brooks, 2000; Kelkar *et al*., 2002). Ethanol may trigger both the intrinsic (mitochondrial) and extrinsic (death receptor-mediated) apoptotic pathways (Molina *et al*., 2003). The extrinsic pathway is most likely activated by the up-regulation of the Fas receptor, while activation of the intrinsic pathway is probably caused by either mitochondrial damage, altered gene expression or by disrupting calcium concentrations (Narula *et al*., 1996; Trump and Berezesky, 1996; Lu *et al*., 2002). Ethanol-induced mitochondrial membrane damage causes the release of cytochrome C, a major constituent of the apoptosis-promoting apoptosome complex, which is responsible for activating caspase-3, the cell's major effector caspase. Increased intracellular calcium levels have also been implicated in release of cytochrome C from mitochondria via opening of the mitochondrial permeability transition pore by the voltage-dependent anion channel (VDAC). Levels of proapoptotic genes such as *BAX* are also up-regulated in alcoholic myopathies (Narula *et al*., 1996). Cytosolic Bax translocates to the mitochondrion where it facilitates VDAC opening. These findings indicate that both acute and chronic ethanol myolysis and atrophy are multifactorial disease states with several molecular mechanisms contributing to the apoptotic and necrotic loss of myocytes and cellular dysfunction.

5.6.9 Potassium-Based Myopathies

Potassium is an important electrolyte that plays several important roles in the muscle, the primary of which is to maintain membrane potential and aid in the membrane depolarization process. Myopathy may result under conditions of either hyperkalaemia or hypokalaemia, and appears to affect both Type I and Type II fibres equally (Brumback *et al*., 1995).

- **Hyperkalaemia:** Increased circulating potassium may be caused by dietary overabundance, potassium-sparing diuretics, nonsteroidal anti-inflammatory drugs (NSAIDs) and ACE (angiotensin -converting enzyme) inhibitors (Knecht *et al*., 1985). Excess serum potassium leads to membrane depolarization, which activates membrane sodium channels, resulting in the influx of extracellular sodium, but not enough to propagate a membrane potential, causing these channels to become intractable. Without proper Na^+ channel function, the threshold required for mounting an action potential increases, which prevents further depolarization. The most common clinical symptom of hyperkalaemia is

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generalized muscle weakness, with bicarbonate therapy being the most common treatment. Bicarbonate serves to increase serum Na^+ concentration, which then reactivates Na^+ channels and motor function.

- **Hypokalaemia:** Circulating potassium levels may be decreased due to dietary insufficiency, heavy fluid loss or the loop diuretic, furosemide. Pathology of the condition often presents as myalgia and cramping, with paralysis, tetany and rhabdomyolysis in more severe cases. Diminished potassium levels cause skeletal muscle membrane hyperpolarization due to the inability of cells to import sufficient potassium to restore membrane potential to a 'normal' resting state. The consequence of these actions is a reduced response to stimulus, which may lead to atrophy under chronic conditions (Mastaglia, 1982). Treatment typically consists of potassium supplementation and treatment or discontinuation of the aforementioned causes.

5.7 Lipid-Lowering Drugs

5.7.1 Statins

Statin drugs are among the most widely prescribed drugs worldwide. Statins are a group of 3-hydroxy -3-methylglutaryl coenzyme A reductase (HMG CoA reductase) inhibitors, considered the most effective lipid-lowering drugs on the market. They function by blocking the first, rate-limiting enzymatic step during *de novo* cholesterol biosynthesis, thus preventing the generation of mevalonate. Inhibition of cholesterol synthesis results in the activation of proteases which cleave the membrane-bound sterol regulatory element binding protein, leading to its translocation to the nucleus and the up-regulation of a variety of proteins/enzymes, including the LDL (low-density lipoprotein) receptor. The increase of LDL receptors on the liver membrane results in the subsequent binding and clearance of both LDL and VLDL, and decreased circulating LDL and VLDL cholesterol levels. The major side effect of statins, however, is the tendency to develop myopathic symptoms and, in extreme cases (7.8% of patients), rhabdomyolysis and consequently kidney failure. Less severe myopathies involve muscle pain and muscle weakness and do not involve a significant rise in CK levels (Paoletti *et al*., 2002; Dirks and Jones, 2006).

While the mechanism for muscle toxicity is not fully understood, the more extreme cases of myopathy and rhabdomyolysis are often due to combination therapies with drugs such as fibrates, cyclosporine A and niacin (Boger, 2001; Hodel, 2002). These agents inhibit the drug metabolizing enzyme cytochrome P450 3A4 (CYP3A4), the protein responsible for the breakdown of the majority of statin drugs. This causes the statins to remain in the bloodstream longer, thus altering their pharmacokinetic and pharmacodynamic properties related to absorption, distribution, metabolism and excretion of the drug (Paoletti *et al*., 2002). Current research shows that statins are capable of inducing apoptosis in a variety of cell lines including vascular smooth muscle cells, and skeletal myoblasts and myotubes (Blanco-Colio *et al*., 2002; Johnson *et al*., 2004; Erl, 2005). Myocyte apoptosis is a likely contributor to the myopathic side effects of statins. Interestingly, inhibition of enzymes downstream of the HMG CoA reductase in the cholesterol biosynthetic pathway, such as squalene synthase, fails to induce apoptosis, suggesting it is not depletion of cholesterol itself that is resulting in the induction of apoptosis. Studies suggest that statin-induced apoptosis is, rather, linked to an alternative mevalonate metabolic pathway related to the synthesis of isoprenoids (Guijarro *et al*., 1998; Laufs and Liao, 2003). Isoprenoids are important lipid moieties required for both ubiquinone biosynthesis and for the post-translational modifications of the membrane-associated proteins Rho, Ras and Rac—key players in cell fate decisions involving cell growth and death. Treatment with statins does not alter ubiquinone levels but does affect the isoprenylation, farnesylation or geranylgeranylation of Rho and Ras in L6 muscle cells and vascular smooth muscle cells, thus altering their ability to translocate to the plasma membrane and be activated (Johnson *et al*., 2004; Matzno *et al*., 2005). This inhibition of Rho and Ras is also associated with an increase in intracellular calcium that causes the activation of calpains, triggering the translocation of the proapoptotic protein Bax to the mitochondria, and the activation of the mitochondrial death pathway via caspase-9 and caspase-3 (Sacher *et al*., 2005).

5.7.2 Fibrates

Fibrate derivatives, while less effective than statins at lowering LDL, are also considered hyperlipidaemic agents for their ability to raise HDL (high-density lipoprotein) and lower triglyceride (TG) levels (Duriez, 2003). The mechanism by which fibrates treat hyperlipidaemia is attributed to their ability to act agonists for peroxisome proliferator-activated receptors (PPARs), specifically PPARα (Matzno *et al*., 2006). PPAR isoforms belong to the nuclear hormone receptor superfamily, and their activation results in the up-regulation of a variety of lipid metabolism genes. While the fibrate derivative, gemfibrozil, has been the only fibrate reportedly associated with causing rhabdomyolysis, all the fibrates are capable of causing some muscle toxicity resulting in palpitation-induced muscle pain, cramps, myalgia and weakness. When used in combination with statins, risk

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of more severe induction of rhabdomyolysis is reported (Guis *et al*., 2003). As mentioned above, combination therapies with fibrates and statins lead to drug metabolism issues, as each drug class is metabolized by CYP3A4, and each drug class can induce myopathic responses upon monotherapeutic use. Likewise, drugs such as cyclosporine A, which inhibit CYP34A, could also lead to dangerous myopathic symptoms when used together with fibrates. Less clear are the mechanisms by which fibrates induce myolysis when administered alone. Recent studies show treatment of L6 rat skeletal myoblasts with clofibrate caused apoptosis in a dose-dependent manner within 24 hours (Matzno *et al*., 2006). Clofibrate is one of the more traditionally prescribed hyperlipidaemic agents and is broken down to the free acid derivative clofibric acid. Treatment of L6 rat skeletal myoblasts resulted in the rapid release of calcium from the SR followed by the extended elevation of calcium levels due to the influx of extracellular calcium. This triggers the cleavage and activation of caspase-12 in a calpain-independent manner. Activation of caspase-12 leads to the activation of caspases-9 and -3 and the induction of DNA fragmentation and apoptosis (Matzno *et al*., 2006). The ability of clofibrate to induce apoptosis by a slightly different mechanism than statin-induced apoptosis supports the higher risk of skeletal myopathy when the two drugs are used in concert; however several mechanistic questions remain unclear that could provide safer combination drug therapies in the future.

Because mechanisms of myopathic muscle cell death induced by statins remain to be further elucidated, still no treatment exists that allows patients sensitive to statin-myopathy to persist in receiving statin therapy should a clinically significant myopathic response occur. Unfortunately for these patients, especially given the great effectiveness of these agents in the treatment of hyperlipidaemia and the prevention of cardiovascular events, the only way to treat statin myopathy is via cessation of the drug treatment.

5.7.3 Niacin

Another commonly prescribed lipid-lowering agent associated with rare myopathies is niacin (nicotinic acid/vitamin B_3). Like statins and fibrates, niacin prevents the production of VLDL and lowers overall cholesterol secretion from the liver while sparing cardioprotective HDL levels (Gharavi *et al*., 1994). Very few cases of myopathy have been reported in patients using niacin alone, and in those rare cases the mild symptoms ceased once drug administration was stopped. In contrast, more severe cases of myotoxicity have been attributed to the coadministration of niacin with either fibrates or statins. Excessive consumption of alcohol while taking niacin may also predispose a patient to more extreme myopathies (Ballantyne *et al*., 2003; Bays and McGovern, 2003).

5.8 Lysosomal/Vacuolar Myopathies

5.8.1 Chloroquine/Hydroxychloroquine

CQ and its hydroxylated version, HCQ are widely used as antimalarial agents and in the treatment of connective tissue diseases such as rheumatoid arthritis. These agents rarely induce myopathy alone, but in combination with other drugs can elicit muscle and nerve toxicities. Termination of CQ or HCQ treatment will usually result in the reversal of muscle toxicity (Richards, 1998; Stein *et al*., 2000). Most cases of CQ- or HCQ-induced myopathy are referred to as lysosomal or vacuolar myopathies due to the accumulation of lysosomal vacuoles within the skeletal muscle. Initial signs of CQ myopathy are easily confused with the disease symptoms themselves, including weakness in the proximal limbs and areflexia. CK levels are usually normal and lysosomal myopathic symptoms usually do not present themselves until 6 months to even 10 years later (Estes *et al*., 1987; Sghirlanzoni *et al*., 1988). While the myotoxic mechanisms remain largely undefined, CQ and HCQ are cationic, amphiphilic compounds with long half-lives that appear to affect the autophagy pathway and subsequent protein degradation. Research has also shown in rat models that treatment with CQ causes an increase in the activity of lysosomal proteolytic enzymes such as cathepsins and acid hydrolases (Tagerud *et al*., 1986; Sugita *et al*., 1987).

5.9 Microtubule Disrupting Agents

5.9.1 Colchicine

Colchicine continues to be the standard long-term treatment for gout. It is also used to treat other diseases such as primary biliary cirrhosis, amyloidosis and Mediterranean fever (Chattopadhyay *et al*., 2001). Colchicine is able to treat gout due to its ability to block the formation of uric acid crystal deposits by inhibiting the synthesis of lactic acid. Colchicine is also a microtubule disrupting agent that binds to tubulin and prevents microtubule polymerization. This property has generated some interest in the use of colchicine as a potential chemotherapeutic agent due to the necessity for microtubules in the creation of the mitotic spindle required for the completion of mitotic cell division. However, there is currently no means of preventing the toxic effects on nontumorigenic cells. Colchicine induces vacuolar myopathy, indicated by the accumulation of lysosomal and autophagic vacuoles within the non-necrotic muscle fibres. Likewise, colchicine has also been found to inhibit endosomal/lysosomal trafficking in a variety of cell lines. Microtubules play a critical role in developing skeletal muscle by directing and securing AChRs (Kuncl

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et al., 2003). Thus, scientists have been interested in defining the mechanisms by which colchicines disrupt the microtubule-dependent receptor trafficking process. Several studies have recently determined that colchicine inhibits AChR exocytosis, providing a likely explanation by which colchicine disrupts ACh signalling and induces muscle weakness and skeletal muscle myopathy (Kuncl *et al*., 2003).

5.9.2 Vincristine

Another microtubule disrupting agent, vincristine, is often used as an antineoplastic drug. The main side effect associated with vincristine treatment is axonal peripheral neuropathy; however, myopathy can also occur (Sieb and Gillessen, 2003). Similar to colchicines, accumulation of lysosomes and autophagosomes is often observed in vincristine-treated skeletal muscle fibres. Due to the smaller amount of vincristine-induced myopathy research, it has yet to be determined if the same inhibition of exocytosis as seen with colchicine treatment also occurs with vincristine treatment (Bradley *et al*., 1970).

5.10 Mitochondrial Myopathies

5.10.1 Zidovudine (AZT)

Patients infected with human immunodeficiency virus (HIV) are often more susceptible to myopathy due to a variety of disease- and treatment-related reasons. Approximately 20% of patients treated with the nucleoside reverse transcriptase inhibitor, zidovudine, displayed myopathic symptoms such as myalgia, fatigue, overall muscular weakness and elevated CK levels, and duration and dose of treatment can also dictate the occurrence and severity of symptoms. Zidovudine helps to treat HIV infection by substituting for the viral reverse transcriptase. Unfortunately, that also makes zidovudine a good substitute for the mitochondrial DNA polymerase γ, thereby disrupting replication of mitochondrial DNA, which leads to a decrease in the mitochondrial respiratory proteins and subsequent mitochondrial dysfunction (Gold *et al*., 1990; Dalakas *et al*., 1994). Both muscle biopsies and experiments in cultured human muscle cells revealed vacuolization, lipid accumulation, aberrant cristae and ragged-red fibres often associated with abnormal mitochondria and myopathy (Masini *et al*., 1999). It is often difficult to differentiate between HIV-induced myopathy, T cell-mediated inflammatory myopathy, and the mitochondrial myopathy induced due to zidovudine treatment; however, discontinuation of zidovudine treatment or L-carnitine supplementation can sometimes result in an almost full reversal of symptoms (Gold *et al*., 1990; Dalakas *et al*., 1994; Masini *et al*., 1999).

5.11 Necrotic Myopathy/Other

5.11.1 Venom

Envenomation by a variety of species, from snakes and insects to fish and mushrooms, is known to cause mild-to-severe myotoxicity. Snakes can be categorized into two main groups. The elapids (cobras and coral snakes), which reside mainly within Africa and Southern Asia contain venom that causes neurotoxicity. The viperids (rattle snakes, water moccasins and copper heads), on the other hand, inhabit North and South America, and their venom is known to cause necrotic myopathy, including the more severe compartment syndrome, rhabdomyolysis and myoglobinuria. Viperid venom contains a variety of components that promote the spreading of the venom, as well as digestive enzymes including phospholipase A_2 and other specific myotoxins capable of acting both locally and systemically. A combination of necrotic and intact fibres can be visualized in the muscle biopsies of snake-bite victims (Rossi *et al*., 1989; Mebs and Ownby, 1990; Bush and Jansen, 1995; Jolles *et al*., 1998; Spiller and Bosse, 2003).

Insect venom can also cause myotoxicity distant from the wound. Most insect bites and stings cause very little myopathy; however, certain arthropod's venom can elicit severe reactions in the body including rhabdomyolysis and, in rare cases, death. Arachnids (spiders and scorpions) such as the brown recluse, the redbacked spider and the mouse spider, often exert more neurotoxic effects on their victims; still general muscle aches and acute rhabdomyolysis along with some necrotic lesions have been described in some cases (Gala and Katelaris, 1992; Muller, 1993). The most severe cases usually involve the honey bee or larger attacks by the Africanized honey bee. Multiple stings from wasps can also induce mild rhabdomyolysis (Franca *et al*., 1994; Vetter *et al*., 1999). What is not clear is whether these intoxications result in direct muscle injury by chemical components in the venoms, or rather are secondary to fevers produced during the acute immunologic reactions to venom exposure.

Eating certain vegetation or animals can also result in skeletal muscle toxicity. The mushroom known as *Amarita phalloides* contains the myotoxin phalloidin that binds directly to tubulin and prevents microtubule polymerization. Phalloidin is often conjugated to fluorochrome molecules and used to visualize microtubules in cell culture experiments (Westendorf, 1999). Ingestion of certain types of fish has also triggered rhabdomyolysis. This condition is referred to as Haff disease. The first described cases of Haff (*Haff* is the German word for lagoon) disease occurred around the Baltic Sea. Other fish that have since been found to cause Haff disease are the tropical blue humphead parrot fish and the buffalo fish found in the USA (Buchholz *et al*., 2000). Although the

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Table 3 Summary of rhabdomyolytic agents and their mechanistic and clinical properties

Type of myopathy	Cause	Mechanism	Signs and symptoms	Clinical and diagnostic findings
Necrotic	Lipid lowering drugs: statins, fibrates and niacin	Statins: inhibit the synthesis of isoprenoids, disrupting Rho and Ras pathways leading to apoptosis Fibrates: cause elevated levels of calcium and the activation of calpains and caspase-12 leading to apoptosis Niacin: affect the metabolism of fibrates and statins when coadministered Co-treatment of fibrates, statins and niacin with one another as well as cyclosporine A or alcohol inhibits cytochrome P450 (CYP34A) preventing the proper metabolism and excretion of the drug	Muscle pain/ weakness/cramps, rhabdomyolysis, renal failure	Elevated CK levels, Myoglobinuria
	Venom: snakes and insects	Venom contains combination of digestive enzymes and muscle-specific toxins	Pain, compartment syndrome, rhabdomyolysis, myoglobinuria	Combination of necrotic and intact muscle fibres. Elevated CK levels in more severe cases
	Corticosteroid excess	Increases cytoplasmic protease activity in skeletal muscle and inhibits glutamine synthesis, reducing protein synthesis	Muscle weakness/wasting, rhabdomyolysis, occasional myalgia	Elevated CK levels, atrophy of Type II fibres, lowered intramuscular glutamine levels, fat redistribution to lower extremities
	Alcohol abuse, acute/chronic	Acute: overall dehydration and malnutrition. Disrupts phospholipid membranes and other metabolic pathways; hypokalaemia due to vasoconstriction, altered gene expression	Muscle pain/swelling/ cramping, rhabdomyolysis	Elevated CK levels, myoglobinuria, necrotic Type I fibres with irregular sarcomeric banding, irregular mitochondria, vacuolization
		Chronic: impairs protein synthesis (e.g. myofibrillar protein), impairs mTOR function and damages mitochondria, resulting in increased apoptosis	Muscle wasting, gradual muscle fibre atrophy, rhabdomyolysis	Elevated CK levels, decrease in Type II fibre size, elevated ROS levels, decreased IGFR-1 activity, myoglobinuria
	Opiates	Unknown	Rhabdomyolysis, compartment syndrome, renal failure	Myoglobinuria, necrotic muscle fibres (see focal myopathy)
	Amphetamines (MDMA)	Increase in norepinephrine, increased mitochondrial uncoupling	Rhabdomyolysis, compartment syndrome, renal failure	Elevated CK levels, myoglobinuria, increased levels of free fatty acids, elevated temperature

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Table 3 (*continued*)

Type of myopathy	Cause	Mechanism	Signs and symptoms	Clinical and diagnostic findings
Lysosomal/ vacuolar	Amphiphilic drugs: chloroquine/ hydroxychloro-quine, amiodarone	Hydrophobic region: disrupts myofibre membrane causing electrolyte/osmotic imbalance Hydrophilic region: interacts with lysosomes disrupting protein and lipid degradation. Accumulation of vacuoles impedes further cell function and muscle contraction	Proximal muscle weakness, rhabdomyolysis	Accumulation of lysosomal and autophagic vacuoles within muscle fibres. CK levels normal
Potassium-based	Hyperkalaemia (caused by diuretics, NSAIDs and ACE inhibitors)	Excess serum potassium results in membrane depolarization without the ability to repolarize	Generalized muscle weakness	Atrophy of Type I and Type II fibres
	Hypokalaemia (caused by dietary insufficiency or extreme dehydration)	Causes skeletal muscle membrane hyperpolarization. Reduced response to stimulus	Myalgia, cramping, periodic paralysis, tetany, rhabdomyolysis	Atrophy of Type I and Type II fibres. Elevated CK levels
Antimicrotubular	Colchicine and vincristine (can also be considered lyso-somal/vacuolar myopathies)	Stably bind to tubulin preventing microtubule polymerization. Disrupt microtubule-dependent receptor trafficking. Possibly alter acetylcholine signalling	Proximal muscle weakness	Accumulation of lysosomal and autophagic vacuoles within muscle fibres
	Amarita phalloides phalloidin	Binds directly to tubulin and prevents microtubule polymerization		
Mitochondrial	Zidovudine	Disrupts the mitochondrial DNA polymerase γ causing a decrease in mitochondrial proteins, leading to mitochondrial dysfunction	Myalgia, fatigue, overall muscle weakness, rhabdomyolysis	Muscle biopsies reveal vacuolization, lipid accumulation, ragged-red fibres, abnormal mitochondria with aberrant cristae
Endocrine	Thyroid	Hyper: up-regulates metabolic respiration, β- oxidation and protein degradation. Altered gene expression (myosin heavy-chain), increased activity of Na/K ATPase.	Muscle weakness of shoulder neck and hips, permanent muscle fibre damage, rhabdomyolysis, periodic paralysis	Alteration in muscle fibre type in favour of more Type II glycolytic fibres
		Hypo: alters metabolic processes, effects β-adrenergic receptors and catecholamine expression	Severe muscle cramps/spasms/ weakness	Atrophy of fast-twitch Type II fibres and increase in slow-twitch Type I fibres
	Parathyroid/Vitamin D disorders	Both hyperparathyroidism and Vitamin D intoxication cause desensitization of calcium-regulated proteins (e.g. troponin).	Muscle fatigue	Elevated calcium levels

(*continued overleaf*)

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Table 3 (*continued*)

Type of myopathy	Cause	Mechanism	Signs and symptoms	Clinical and diagnostic findings
		Both hypoparathyroidism and Vitamin D deficiency prevent efficient calcium reabsorption (hypocalcaemia). Effects calcium-regulated neurotransmission and muscle contraction	Muscle cramps and tetany, pain and tingling in the face, legs, hands and feet	Low serum calcium levels
	Pituitary (acromegaly)	Excessive production of growth hormones altering gene expression and resulting in skeletal muscle hypertrophy	Rhabdomyolysis	Hypertrophy of both Type I and Type II fibres; mild increase in CK levels, vacuolization within fibres, elevated NO levels
Other	Acetylcholinesterase inhibitors	Causes hyperstimulation and increased activity of individual fibres resulting in the rapid consumption of ATP stores and excess calcium leading to aberrant signalling, protein degradation and induction of apoptosis	Involuntary fasciculation, muscle weakness, myalgia, rhabdomyolysis	Increase in CK levels, decrease in cellular ATP levels, mitochondrial swelling, sarcomeric supercontraction, vacuolization, Z-band fragmentation
	Emetine	Induces apoptosis	Severe muscle atrophy/ weakness, decrease in muscle weight and contraction strength, rhabdomyolysis	Atrophy of Type II fibres, decrease in myofibrillar ATPase and NADH tetrazolium in all three fibres; occasional necrotic fibres, mitochondrial degeneration, vacuolization, sarcoplasmic abnormalities, increased autophagy and lysosomal activity
	Food poisoning: quail (hemlock poisoning), fish (Haff disease)	Unclear	Rhabdomyolysis, acute renal failure	Myoglobinuria
	Focal	Chronic intramuscular injections	Muscle pain, abscess formation, rhabdomyolysis	Focal necrosis. Mild elevation in CK Levels

toxin and mechanism of poisoning from these fish are still uncertain, other fish- borne illnesses are thought to be due to the fish's ingestion of ciguatoxin, a toxin emitted by coral reefs (Gillespie *et al*., 1986; Stommel *et al*. 1991). Similarly, consumption of quail has also led to cases of rhabdomyolysis and myoglobinuria, but similar to Haff disease, the origin of the myotoxic compounds is dietary. European migratory quail which reside primarily in Mediterranean countries feed on seeds from the hemlock plant (*Conium maculatum*). Hemlock plants contain the neurotoxin coniine. When consumed by humans and some livestock this can cause a breakdown of the PNS and is frequently associated with rhabdomyolysis and ARF (Rizzi *et al*., 1991; Papadimitriou *et al*., 1996).

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5.11.2 Opiates

Derived from the opium poppy (*Papaver somniferum*), opiates include all the alkaloid narcotics morphine, codeine, thebaine and papaverine, as well as synthetic derivatives heroin and hydrocodone that elicit their main effects from the central nervous system (Aggrawal, 1995). Morphine and codeine are often utilized for medical/anaesthetic purposes, but are still considered highly addictive and lethal. The semisynthetic opiate, heroin, derived from morphine, due to its abuse as a recreational drug is often linked with the more severe opiate-induced side effects (Aggrawal, 1995). Heroin is typically taken intravenously, and the medical problems are typically more neurological in nature; however there have been rare cases of heroin-induced myopathies such as rhabdomyolysis and compartment syndrome followed by renal failure (Vitris *et al*., 1991; Sahni *et al*., 2008). Heroin-associated myopathies have also been described when users inject the drug intramuscularly, however it is still unclear whether muscle damage arises from general focal myopathy or the toxic effects of the adulterants in the injection solution (Weber *et al*., 2000). Studies have shown that injection of pure heroin into the soleus muscle of rats causes myopathic changes and necrosis (Pena *et al*., 1990; 1993).

5.12 Endocrine Myopathies

5.12.1 Hypothyroidism

Hypothyroidism involves the deficiency of circulating levels of thyroid hormone, and is most often caused by the loss of TH synthesis secondary to iodine deficiency. Like the over-production of thyroid hormone, the lack of TH can also result in myopathic symptoms (Martin *et al*., 1994; Kisakol *et al*., 2003). However, TH deficiencies affect muscles differently from hyperthyroidism. Patients often experience severe muscle cramps and spasms secondary to fibre type switching. Hypothyroidism can lead to atrophy of fast-twitch fibres and increased expression of slow-twitch fibres (Modi, 2000). TH deficiency leads to decreased expression of β adrenergic receptors and levels of neurotransmitter catecholamines (adrenaline), and these events can contribute to muscle weakness (Monzani *et al*., 1997). Treatment of hypothyroidism with levothyroxine generally leads to complete cessation of myopathic events, and full recovery.

5.12.2 Vitamin D Intoxication

Vitamin D is a prohormone produced in the skin upon exposure to sunlight. It is converted to its active form, calcitriol within the liver and kidneys and binds the vitamin D receptor (VDR) (Jones *et al*., 1998). When activated, VDR acts as a transcription factor that up-regulates the expression of transport proteins such as TRPV6 and calbindin, which promote the reabsorption of calcium within the intestines (Bouillon *et al*., 2008). Vitamin D intoxication is rare; only excessive exposure to certain rodenticides has been demonstrated to induce vitamin D toxicity. Vitamin D intoxication does not directly target muscle tissue, but can sometimes lead to myopathic symptoms secondary to changes in circulating calcium levels (Vieth, 1990).

5.12.3 Hypoparathyroidism

Hypoparathyroidism occurs when the parathyroid glands are lost due to autoimmune disorders or surgical removal. The reduction in parathyroid hormone (PTH) leads to hypocalcaemia, a potentially lethal condition characterized by low circulating levels of calcium. Myopathic symptoms are common, and include muscle cramps and tetany. Other symptoms such as pain and tingling in the face, legs, hands and feet are due to a combination of neuropathy and myopathy linked to both calcium-regulated neurotransmission at neuromuscular junctions, and calcium-regulated muscle contraction (Yamaguchi *et al*., 1987; Barber *et al*., 2001). Intravenous calcium is typically administered in severe cases. Vitamin D_3 supplementation, as well as the synthetic PTH drug, teriparatide can be used to treat hypoparathyroidism.

Table 3 summarizes rhabdomyolytic agents and provides details on the mechanism of action, clinical presentation and diagnostic findings for each.

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Cartilage and Bone as Target Tissues for Toxic Materials

Alan B.G. Lansdown

CONTENTS

1 INTRODUCTION

Cartilage and bone are not commonly regarded as sites of toxic action by environmental chemicals, food additives or drugs. They develop from anlages in the primitive mesenchyme to become highly organized and complex tissues providing a structural framework for the body, and functional rigidity with attachments for musculoskeletal action. In their developmental state and in adulthood, both tissues are metabolically active and subject to age-related structural variations determined by genetically regulated patterns of mitosis and differentiation, hormonal action and nutrition (Urist *et al*., 1968; Dunnill *et al*., 1967). Developmental profiles involving programmed sequences of cell proliferation, migration and functional differentiation are subject to natural cell loss (apoptosis) and numerous biochemical changes, with each event being potentially vulnerable to chemical or physical insults (Exton-Smith *et al*., 1969). For the toxicologist, it is essential that cartilage, and more importantly bone, should not be considered in isolation, but as integral parts of the body with key roles in mineral homeostasis.

Cartilage and bone are specialized connective tissues composed of cells (chondrocytes and osteocytes respectively) embedded in intercellular matrices permeated by a network of collagen fibres (Jackson, 1970; Williams and Warwick, 1980; Davies, 1998). The two tissues become anatomically and functionally distinct and differ greatly in their cytological profiles, physical properties, vascularization, growth, and regeneration patterns following injury. Whereas cartilage predominates in early stages of skeletogenesis in all mammals, much of this chondroskeleton becomes ossified with advancing age to be replaced by bone (Owen, 1970). Intrinsic factors, physiological changes or toxicological interventions at this stage may lead to aberrations in growth and functional capacity in later development (Pappas, 1968). In adulthood, residual cartilage persists in the articular surfaces of long bones, intervertebral discs, costal regions, nasal cartilages, the xiphoid protuberance of the sternum, larynx and trachea, and certain elements of the skull. These tissues become specialized to fulfil functional roles: hyaline cartilage, white collagen-rich fibrocartilage and yellow elastic fibrocartilage are recognized. To an anatomist, white fibrocartilage and yellow fibrocartilage

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are more specific, distinct and stable than hyaline cartilage which is subject to wide variations in composition and age-related changes (Williams and Warwick, 1980).

Cartilaginous elements in the axial (vertebral) and appendicular skeletal regions of the early skeleton are largely ossified and replaced by bone as maturation progresses. The modulation of this process is not fully appreciated but can be expected to involve hormonal changes, up-regulation of cytokines and growth factors and genetically programmed sequences involving apoptosis and cell death. Multinucleate osteoclast cells of reticuloendothelial origin are central to this process and in regulation of mineral homeostasis. Osteoclasts found in close association with bony surfaces are characterized by numerous lysosomal inclusions with an array of hydrolytic and proteolytic enzymes including acid phosphatase and cathepsin D involved in cartilage breakdown and in the remodelling of articular and other cartilages, and regulation of mineral homeostasis later in life (Weston *et al*., 1969). Impairment of lysosomal physiology or inactivation of lysosomal hydrolases by pathological or toxicological influences is a potential cause for teratogenesis and skeletal abnormality (Beck and Lloyd, 1963). Muscular action and movement is critical in developmental stages of articular cartilages, such that agents evoking musculoskeletal or neuromuscular paralysis at critical phases in prenatal development impair specialization and subsequent joint development (Drachman and Sokoloff, 1966). Cartilages in the larynx and trachea should be included in the present discussion under hyaline cartilage, but other than age-related metastatic mineralization, these tissues are rarely implicated as sites for toxic action.

Bones of the axial and appendicular skeletons preformed in cartilage are correctly termed 'endochondrial' bones to distinguish them from certain elements of the vault of the skull (e.g. frontal, parietal, occipital bones) which develop from osteoblasts differentiating as mesenchymal condensations in the suprachondral regions of the primitive chondrocranium. These 'membrane' bones ossify and anastomose, ultimately replacing the cartilaginous elements of the chondrocranium. This means that in skull development, any hereditary or toxic influence acting to deform the chondrocranium or impair the osteogenesis or movement of the membrane bones will have an adverse effect on skull development, resulting in such conditions as craniorachischisis, exencephaly, anencephaly and persistent fontanelles (Kokich *et al*., 1982).

Correctly, dentine in teeth is included in a discussion of the toxic effects of xenobiotic agents in bone. Dentine resembles bone in its mineral content (up to 70% calcium hydroxyapatite by weight), but it is structurally different and mechanisms controlling its morphogenesis are distinct (Fitzgerald, 1969). Dentine is more stable than compact bone and resistant to toxic change (Moss, 1968), but theoretically any toxic material impairing collagen or hydroxyapatite biosynthesis in early odontoblast differentiation is a potential cause for abnormal tooth development and dental decay (Rogers, 1967). Dentine is vulnerable to certain bone-seeking ions like cadmium and strontium which interact with and displace calcium from the hydroxyapatite matrix.

Mineral nutrition and hormonal regulation are of fundamental importance in the early development and functional maturation in cartilage and bone. Structural development in the mammalian skeleton is a function of genetical factors, maternal nutrition and health, and conditions in the microenvironment (pO_2, temperature, pressure, etc.). Additionally, cation and anion balances (notably calcium : phosphate ratios) are highly relevant in developing bone, ossification, maintenance of functional rigidity, and repair following injury. Ionic balances between calcium and zinc, magnesium and calcium, and magnesium and iron are relevant in tissue function (Lansdown, 1995). Nutrition is an imprecise science and cellular demands for vitamins, fats, carbohydrates, amino acids (proteins), minerals and electrolytes differ greatly according to the age, sex and state of health of an individual. Inherent metabolic inhibitors or toxic factors impairing the availability of key nutrients are potential causes for deformity in cartilage, bone and other tissues (Rao and Knapka, 1998; Ionnides, 1998). Accumulating biochemical evidence points to the role of metal ion carrier proteins including metallothionein, calmodulin and caeruloplasmin in the metabolism and regulation of mineral elements in the mammalian body and their transport to target tissues following gastrointestinal absorption (Lansdown, 2002a; 2002b; Lansdown *et al*., 2007). The bivalent metal ions are highly inter-reactive in metabolic systems, and excess of one ion is liable to impair availability of another through competitive ionic binding at carrier protein or cell membrane receptors.

Toxicological studies conducted as a mandatory part of most safety evaluation programmes commonly use rodent species as predictive models. Although the biology, physiology and pathology of the rat differ greatly from the human, its predictive value for agents liable to be hazardous in humans is recognized (Rhodes, 1999). It is noteworthy that whereas the human skeleton develops to maturity and is then subject only to normal homeostatic events related to mineral metabolism, the rat skeleton continues to grow throughout most of an animal's life and is appreciably more sensitive to hormonal changes, or pathophysiological responses to idiopathic or toxic agents. Active osteogenesis persists in the metaphyseal growth plates of major long bones until about 12 months of age (or 30–50% of expected lifespan) according to strain and nutritional sufficiency. Nevertheless, a large proportion of current knowledge on cartilage and bone physiology has been derived from experimental studies in the rat, and the rat has been the choice species in unravelling the complexities of toxic action in these tissues.

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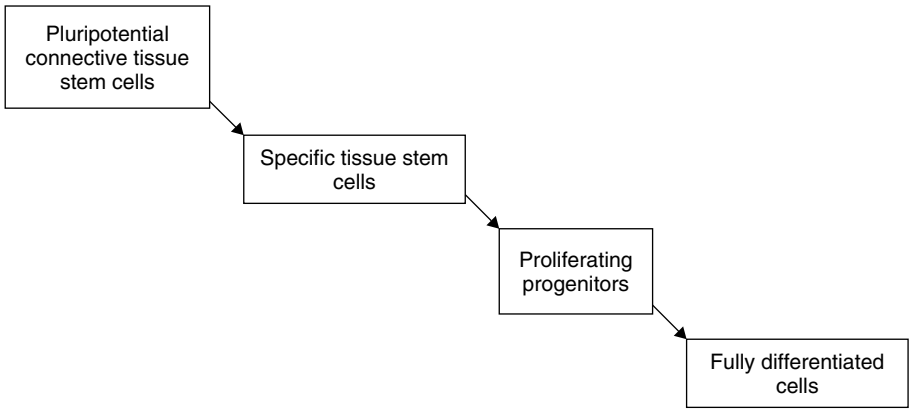

Figure 1 Differentiation of connective tissues. (Reproduced from Owen, 1970 © Elsevier.)

2 DEVELOPMENT, STRUCTURE AND FUNCTION

2.1 Prenatal Growth

Cartilage and bone in the mammalian skeleton have a common origin in the mesenchyme of the early embryo (Owen, 1970; Hall, 1970). From early studies by Maximow, Maureen Owen (1970) proposed the paradigm of a small pool of pluripotent cells emerging in primitive mesenchyme with a capacity to differentiate along predetermined pathways (**Figure 1**). Under the influence of ill-defined indigenous factors, these 'stem cells' give rise to all connective tissues, including chondroblastic and osteoblastic progenitor cell lines which undergo defined differentiation patterns.

Growth factors are variously implicated as 'messengers' in tissue proliferation, and repair. Recombinant DNA technology has allowed isolation of these peptides in a pure form, thereby permitting analysis of amino acid sequences, appreciation of their specific action in undifferentiated tissue, and their modulation of morphogenic sequences in regeneration and repair (Roberts *et al*., 1990). Further, whilst major macromolecules synthesized in early primordia of both cartilage and bone are possibly similar, the relative concentrations of glycosaminoglycans, water and other constituents vary greatly as tissues develop under the influence of different and specific control mechanisms and the nature of the surrounding 'medium' (Urist *et al*., 1968; Jackson, 1970). As tissues age, so their sensitivity to growth factors and intrinsic regulators change. Whilst negative-feedback mechanisms and growth factors are widely implicated in the normal homeostasis in the skin and epithelial tissues and their responses to injury, their contribution in skeleton genesis is entirely speculative (Scarano and Augusti-Tocco, 1967).

The cartilaginous phase in endochondrial bone development provides a template for subsequent ossification patterns and the ultimate configuration of limb bones, ribs, vertebrae and some bones of the skull. Development in endochondrial bones is viewed as a progressive and overlapping three step sequence involving:

1. Chondrogenesis—involving differentiation of chondroblasts, initial synthesis of cartilage matrix and collagen fibres.
2. Maturation and hypertrophy of cartilage, differentiation of a perichondral sheath, infiltration of osteoclasts (chondroclasts) leading to cartilage breakdown, differentiation of osteoblasts in association with vascular tissue.
3. Maturation of osteoblasts, deposition of calcium hydroxyapatite and formation of actively ossifying trabeculate bone. Cartilage becomes eliminated (other than at metaphyseal growth plate zones and at articular surfaces.

Growth profiles in typical endochondrial bones (e.g. tibia or femur) are illustrated in **Figure 2**. From early condensations of chondroblasts in presumptive mid-shaft or diaphyseal zones (**Figure 3**), subsequent growth extends proximally and distally, with the appearance of three cytologically and behaviourally distinct regions:

1. The central or diaphyseal region comprising a cuff of actively ossifying tissue with an advanced degeneration of cartilage, and marrow cavity differentiation.
2. Terminal (proximal and distal) epiphyseal regions composed of mature cartilage permeated by vascular channels with foci of osteoblast infiltration and early mineralization.
3. Metaphyseal growth plate regions separating the diaphyseal and epiphyseal zones of ossification/mineralization (**Figure 4**).

Collagen is an essential feature of growing bone and functional maturation of perichondral and periosteal sheaths, such that inherited, nutritional or toxic defects in collagen fibre crosslinking are a cause of deformity.

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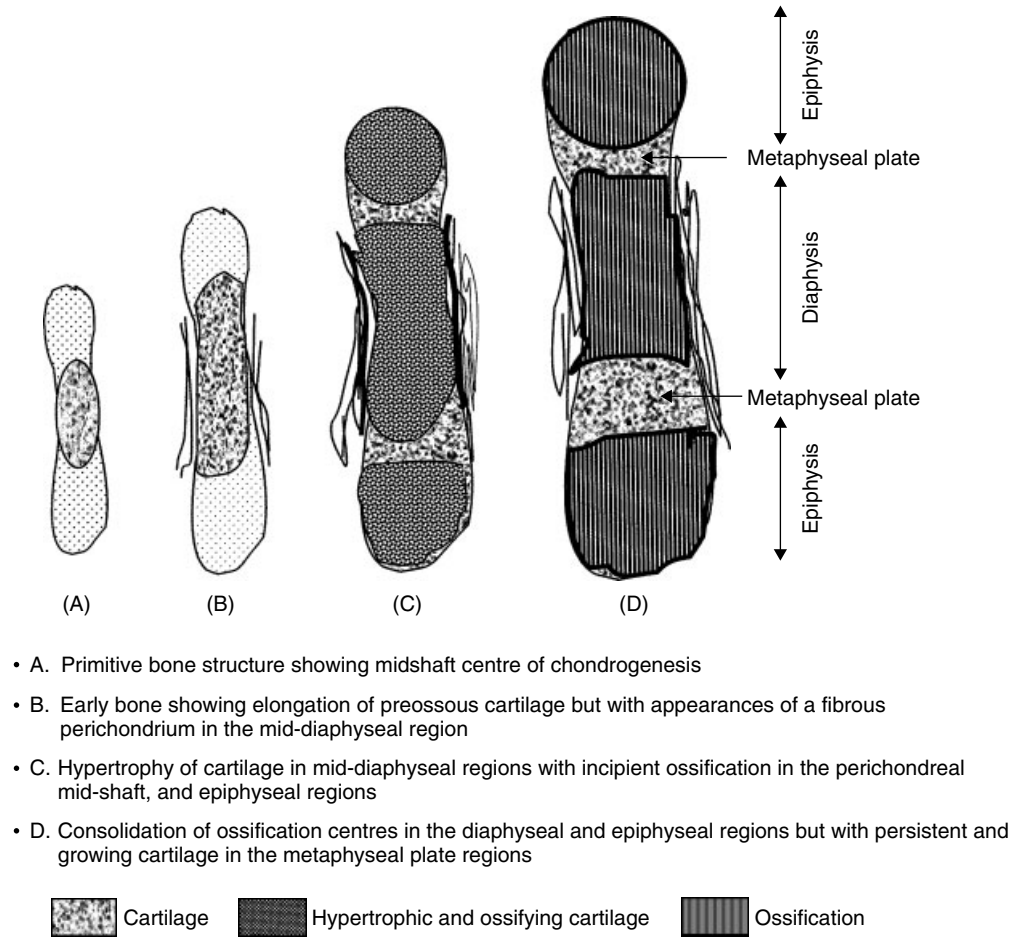

Figure 2 Differentiation patterns of a typical long bone (endochondrial) illustrating the initial condensation of chondroblasts, perichondrium/periosteum and perichondral ossification and epiphyseal ossification.

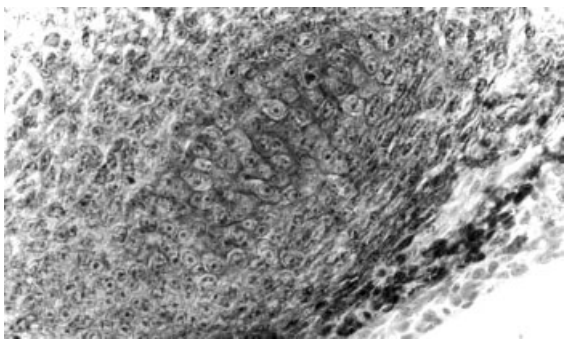

Figure 3 Early development of diaphyseal cartilage of a long bone, with secretion of chondroitin sulfate matrix. Ill defined contours and lack of fibrous perichondral tissues. ×40 objective.

Growth in the diaphyseal cuff becomes robust with increased mineralization on the surface but with further deposition of osteoid and bony trabeculae on internal or endosteal surfaces lining the marrow cavity (**Figure 5**). Ossification in epiphyseal regions is characterized by infiltration by vascular tissues, hypertrophy and lysis of cartilage matrix and fusion of centres of mineralization. The part played by infiltrating vascular tissues in the resorption of cartilage in the appearance of foci of mineralization in epiphyseal regions is controversial. Whilst blood vessels are essential in mineral nutrition at sites of ossification, circumstantial evidence suggests that vascular tissues *per se* may be involved in cartilage degeneration.

Ossification in periosteal and endosteal surfaces of the diaphysis and in epiphyseal regions is active through the latter part of foetal life and persists well into the postnatal period. On the other hand, growth, differentiation and mineralization persist in the metaphyseal plate regions through until adulthood with ossifying cartilage columns remaining vulnerable to wide-ranging physiological and toxic factors (**Figure 6**). This is illustrated by abnormalities in the long bones in young sheep presenting with pathological changes in metaphyseal chondrocytes and bizarre patterns of ossification and mineralization (**Figure 7**) (Duffell *et al*., 1985). The aetiology of these abnormalities is unknown, but irradiation, manganese deficiency and infection may have been implicated. In this case, there was a tendency for the metaphyseal

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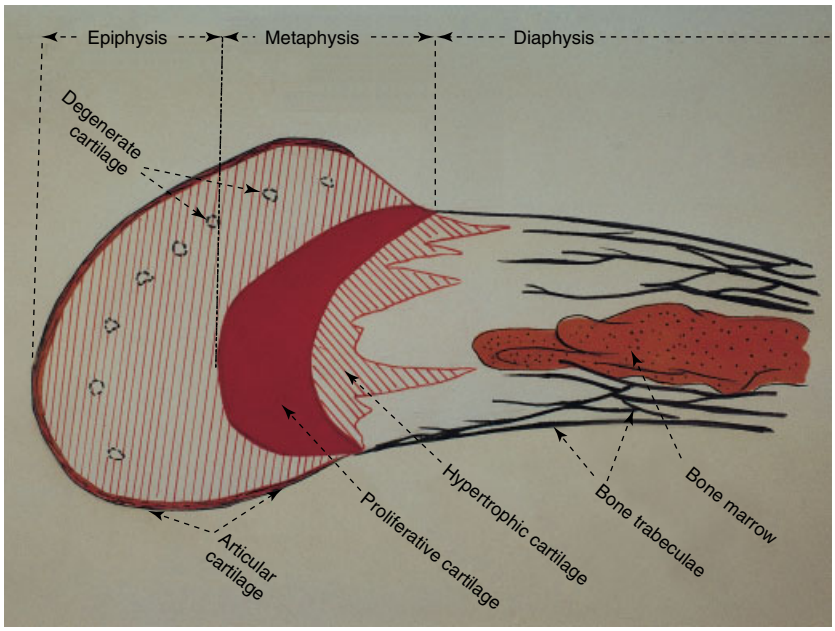

Figure 4 The general morphology of a typical long bone illustrating zones of ossification and growth.

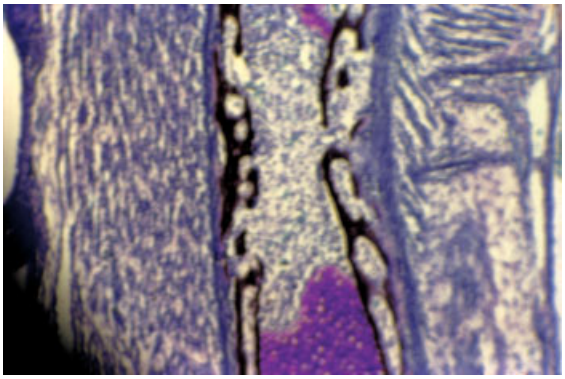

Figure 5 Mid-diaphysis of a rat femur in late gestation illustrating the trabeculae of ossification, marrow cavity and receding cartilage matrix (×10 objective, toluidine blue-silver impregnation).

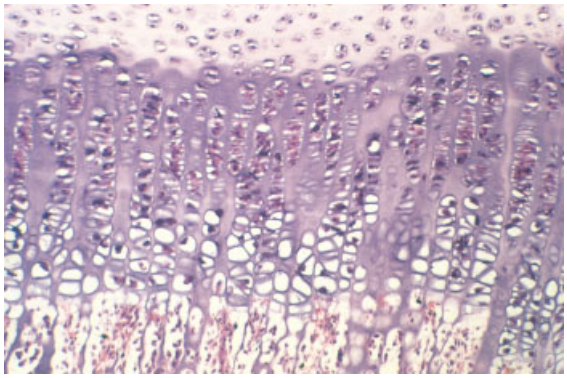

Figure 6 Epiphyseal plate region of a postnatal sheep (?six to eight weeks) illustrating columns of chondrocytes and chondrocyte hypertrophy in region of mineralization in diaphysis (×10 objective, haematoxylin and eosin (H and E)).

plate to separate from the epiphyses. The limbs of these animals were dystrophic and the undefined condition was fatal.

Whilst the developmental pattern of membrane bones is well documented, the action or interaction of exogenous or indigenous factors in the differentiation of osteoblasts and tissue modelling is largely unclear. It is tempting to implicate chemotactic gradients, oxygen tensions, cell-to-cell contacts, cytokines and growth factors, all of which are implicated in tissue repair and morphogenesis (Hall, 1967; Owen, 1970; Slavin, 1996; Kingsnorth and Slavin, 1991). Membrane bones of the skull are largely immature at birth. They are separated by mesenchyme, and vulnerable to a wide variety of conditions affecting general growth and movement, mineral balances and deficiencies, and aberrant tissue inter-relationships. In the human baby, closure of the membranous spaces or fontanelles between the major skull bones is incomplete at birth, but continued growth, expansion and ossification of surrounding membrane bones normally leads to full closure within the first year of postnatal life (Ford, 1956). Deformities are commonly documented in teratological studies, particularly where individuals are exposed to deforming agents like hypervitaminosis A in early-to-mid pregnancy (Kalter, 1968). The literature is replete with reports of spontaneous, idiopathic or chemically induced exencephaly, cleft palate and anencephaly, but mechanisms are still equivocal.

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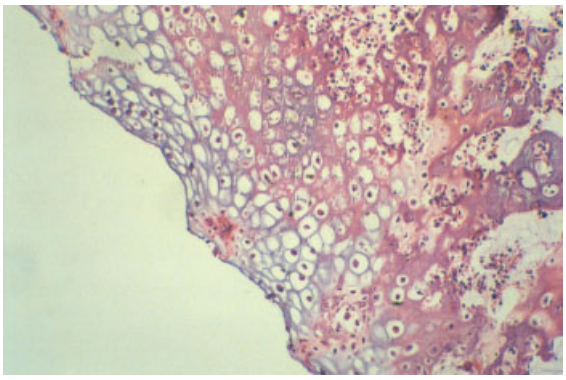

Figure 7 Chondrodystrophy in the metaphyseal plate region of a postnatal lamb illustrating aberrant cartilage columns, irregular ossification and disordered morphogenesis (×10 objective, H and E).

2.2 Collagen and Elastic Tissue

Collagens are the most prevalent proteins in animal tissues (Davies, 1998). They are essential components of all connective tissues including bone and cartilage and are obligatory in the normal structure of intercellular matrices (Miller and Gay, 1992). The density and distribution patterns of collagens vary widely throughout the body according to the tissue and its location, age and physiological function (Davies, 1998). Thirteen types of collagen are recognized, of which eight (Types I, II, V, VI, IX, X, XI and XII) are found to a greater or lesser extent in hyaline cartilage and/or bone. The structure and biochemical constitution of at least five of the fibre-forming collagens in bone and other connective tissues have been elucidated, but this information is likely to be valid for all other molecular species of collagens (Grant and Jackson, 1976; Prockop *et al*., 1976; Bailey, 1978; Herring, 1968). The major collagen type in cartilage is collagen II, other collagens, I and III, are typically prevalent in the dermis and vascular tissues, and Type IV in basement membranes (Jackson, 1970). Collagenesis is modulated by growth factors and cytokines and is vulnerable to genetical abnormalities, environmental toxins, and nutritional factors leading to imbalances in the enzyme cofactors copper, ferrous iron and manganese (Bailey, 1978; Phillips and Wenstrup, 1992).

Collagens are extracellular polysaccharide α-chains held in the form of a triple helix by covalent bonds and intermolecular linkages which can be broken by heat or chemical denaturing agents. Proline and lysine residues are hydroxylated in the triple helix domain in defining the final structure of collagen fibres, and assays of either provide suitable assays for collagenesis in normal growth and tissue maturation, as well as markers for metabolic disease and defective repair systems following injury (Firschein, 1967; 1969; Öberg *et al*., 1969). Proline estimation is a suitable marker for changes more associated with mineralization and bone repair (Shtacher and Firschein, 1967). Glycine is present in collagen molecules and is repeated at a one-in-three sequence between constituent amino acids of chain sequences. In rare inherited conditions like osteogenesis imperfecta, where cysteine substitutes for glycine in collagenesis, bones are fragile, collagen chains do not intertwine, and dense packing characteristic of normal bone is absent (Davies, 1998). In normal chondrogenesis, specific proline and lysine residues are hydroxylated by the copper-containing enzymes prolyl oxylase and lysyl oxidase, which also depend upon ferrous iron, ascorbic acid, molecular oxygen and α-ketoglutaric acid as cofactors. Further, glycolysation of hydroxylysine and synthesis of various collagen types require manganese-dependant transferases. These transferases are bound to the rough endoplasmic reticulum, and hydroxylation commences on nascent polysaccharide chains attached to membrane-bound ribosomes. Glycolysation involving galactosyl and glucosyl transferases is a further process integral to collagen formation and maturation.

Elastin is a component of the so-called yellow fibroelastic cartilages of the ear and laryngeal cartilages (Cox and Peacock, 1977). Like collagen, elastin synthesis involves the cupro-enzymes—lysyl oxidase and prolyl oxylase; but according to Partridge (1962; 1966), the reactions in elastin formation are 'more complete'. The histogenesis of elastin is a two-stage process involving an initial deposition of glycoprotein framework followed by impregnation (Bailey, 1978; Davidson *et al*., 1992). Collagen and elastin are vulnerable to copper-deficiency conditions and agents like the lathyrogens (β-aminopropionitrile) which inhibit lysyl and prolyl oxidases (Lenaers *et al*., 1971).

2.3 Dentine

Dentine forming the major component of teeth is a hard, avascular tissue composed largely of crystalline hydroxyapatite, water and calcium phosphates. Human dentine consists of 72–75% inorganic matter, 2% organic (including 0.08% by weight of hexosamine and equivalent amounts of glucosamine and galactosamine) and 5% water (Clark *et al*., 1965; Herring, 1968). Unlike bone, which is subject to remodelling and age-related changes, dentine is a stable structure. Tooth enamel contains a high mineral content (96% hydroxyapatite). Both tissues are robust and resistant to toxic changes except in cases of severe calcium or vitamin D deficiency, abnormal parathyroid function or xenobiotic agents acting to displace calcium or zinc. Although documentation is limited, it is expected that as in bone, the hydroxyapatite moiety in dentine and enamel will bind

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bone-seeking metals including lead and the radioactive isotopes of strontium (^{89,90}Sr) (Curzon and Spector, 1980; Johnson *et al*., 1970). The extent to which tooth or enamel provides a reservoir from which toxic metals are released into the circulation is not well documented.

Fluoride is commonly regarded as being important in the health and stability of teeth. Dentifrices containing fluoride are widely marketed as a nutritional supplement for care of teeth and gums (Mellberg and Ripa, 1983; Mason, 2000). The World Health Organization 1994 recognized that use of fluoridated dental cream made a major contribution in the dramatic decline in dental caries in the developing world. Today, the so-called anticavity dentifrices contain fluoride salts at 1000–1500 ppm (Mason, 2000).

2.4 Bone

Bone is a rigid form of connective tissue with a major role in mineral homeostasis in the body. Three principle cell types are found in mature bone—osteoblasts and osteocytes of mesodermal origin, and osteoclasts derived from reticuloendothelial precursors. Osteoblasts are primarily responsible for bone growth, including synthesis of bone matrix, osteoid, and maintenance of bone mass. They differentiate in perichondral, endochondral or intramembranous regions but predominate in metaphyseal plate regions during phases of active bone growth. Osteocytes, on the other hand, are functionally mature bone cells with limited mitotic capacity but some potential to secrete matrix (Marcus, 1987; Osdoby *et al*., 1987). Osteoclasts are rich in hydrolytic enzymes and serve the dual role of resorbing bone and recirculating bone minerals in bone remodelling (Stevenson and Evans, 1981). Osteoblasts are embedded in an organic matrix which is impregnated with 'bone salts' (also referred to as 'lime salts') including calcium, zinc, magnesium, chloride, phosphate, hydroxyl, fluoride, citrate and carbonate, which confer hardness and rigidity (Biltz and Pellegrino, 1969). Bone structure and mineral metabolism is modulated by circulating concentrations of Ca^{2+}, Mg^{2+}, phosphate and vitamin D (D_3), and hormonal interactions involving oestrogens, parathyroid hormone, calcitonins and growth hormone.

A precise knowledge of the normal constituents of bone, their variations with age, sex and racial variation is an important basis for appreciating pathological changes attributable to disease, physiological influences and toxicological action. Mammalian bone is a complex of organic materials, mineral salts and water; wide interspecies variations exist; individual species display certain distinguishing chemical characteristics (Biltz and Pellegrino, 1969). Water is a major constituent of bone and a reciprocal relationship exists between the water content of the tissue and the level of mineralization. Analytical studies have shown that the 'average dry bone' of mammals comprises of 93.75% by weight of organic and inorganic material and 6.25% 'crystalline' water (Biltz and Pellegrino, 1969). Robinson and Elliot (1957; 1958) analysed dog bone and reported 13 volumes per cent of crystalline water, 38% organic and 49% mineral salts. As noted above, collagen fibres are an essential constituent of bone matrix, but unlike some other collagens, bone collagens tend to be more stable in the presence of denaturing agents and are resistant to swelling in the presence of acids, possibly reflecting a higher level of fibrillar crosslinking.

Calcium and phosphate are the principle minerals in normal bone growth and mineralization. Experimental studies in growing rat bone illustrate that Ca : P ratios are critical in the mineralization process, but vary according to the age of the tissue and the metabolic state of osteoblasts (Bernhardt *et al*., 1969). Phosphate levels are higher during the active growth phase, whereas increased calcium is a feature of the mineralization process. Magnesium is an essential element in bone growth and a major constituent of bony tissue, but is well known to interact with and suppress calcium in many systems including gastrointestinal absorption and in metal carrier protein-mediated metabolic processes (Lansdown, 2002a). Clark and Bélanger (1967) demonstrated a complex inter-relationship between magnesium, calcium and phosphate in dietary studies in rats, supporting the view that magnesium modulates calcium homeostasis in bone development. They presented evidence showing that bone is capable of sequestering relatively large amounts of Mg^{2+} and can enhance bone calcium and phosphate balances. Histological studies revealed that animals with the highest calcium phosphate balance and reduced bone ash (i.e. those fed 0.5 or 1.0% magnesium) had abnormal bone structure with pathological changes involving epiphyseal cartilages, diaphyseal osteocytes and matrix indicative of osteolytic change. Since magnesium is a 'surface-limited ion' its increase in bone concentration may be explained largely by absorption to the surface of hydroxyapatite crystals, but some ion is possibly absorbed within the crystal matrix. Ossification and hydroxyapatite synthesis was inhibited in metaphyseal growth plate regions.

Oestrogens are well known causes of increased bone mineral turnover and accelerated trabecular bone loss in women and natural or artificial menopause (Fentiman *et al*., 1989). The anti-oestrogenic therapeutic, tamoxiphen, prescribed for treating advanced breast cancer, is without significant effect in bone density or calcium balance (Fentiman *et al*., 1989). Calcitonin (thyrocalcitonin), a hormone secreted by C cells of the thyroid, acts to protect mature bone against resorption and has a major therapeutic role in treating human conditions

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including Paget's disease, osteoporosis and other disorders of calcium metabolism (Hosking, 1985; De Deuxchaisnes, 1983). Oestrogens possibly exert a protective influence against lathyrogenic agents, which, as discussed below, impair collagenesis and elastic tissue formation and are a cause of skeletal abnormalities, but the mechanisms are unclear (Simmons *et al*., 1965; Berntsen, 1968).

The structure and chemical composition of bone matrix in mammals and birds has been subject to extensive electron-microscopic, microchemical and *in vitro* analysis in the past 40 years (Moss, 1968; Herring, 1968). Bowness (1968) reviewed the role of ground substance in mineralization and proposed a conjectural model stressing the importance of mucopolysaccharides as inducers and modulators for calcium deposition. Although chondroitin sulfate inhibited mineralization in preosseous cartilage, his experiments demonstrated a rapid increase in synthesis of sulfated mucopolysaccharides and mucoprotein macromolecules in the vicinity of areas of active endochondrial calcification (and possibly other calcifying areas). These intercellular polysaccharide–protein complexes possibly provide a medium for the preferential accumulation of calcium in relation to sodium, potassium and magnesium; even so they retain a relatively low level of phosphate. Phosphate accumulates more in fibrous tissue compartments of the intercellular space and in cellular compartments in the form of organic and inorganic salts. Early evidence suggests that calcium accumulates in preosseous cartilage possibly as a preliminary to mineralization (Bonucci, 1969; Herring, 1970). Whilst differences exist in the structure and chemical composition of bone according to age, sex, nutrition and health status within a species, the observations are compatible with and reveal a structural complex involving glycoproteins, acidic lipids, peptides with a potential role in nucleation, and ion transport in active mineralization (Herring, 1970).

X-ray diffraction and ultrastructural studies show that the amorphous and crystalline mineral salts of mature bone contain calcium hydroxyapatite $Ca_5(PO_4)_3OH$ (or more commonly shown as two molecules $Ca_{10}(PO_4)_6(OH)_2$ as the largest single component). Taylor *et al*. (2000) have used inelastic neutron scattering (INS) spectroscopy to determine the complex molecular structure of calcium hydroxyapatite. INS is especially sensitive to 'vibrations of groups with hydrogen' to resolve the substitutions at the hydroxyl group site in bone, and the authors have shown that the extracellular matrix of bone comprises largely calcium hydroxyapatite as an extracellular protein, but emphasize that the composition of this mineral is controversial. In their conjectural model, the hydroxyapatite crystal viewed in the a–b plane and observed along the c-axis shows hydroxyl moieties lying in a channel formed by calcium ions at the intersection of four unit cells; Taylor *et al*. (2000) discussed the capacity of carbonate ions to substitute hydroxyl groups which, in ox bone at least, occurs at a rate of approximately 60%. Importantly, these authors point out that the sensitivity of the INS technique opens up new possibilities in monitoring changes in the composition of bone due to ageing and pathological conditions (including osteoporosis), presumably including those attributable to binding of xenobiotic ions like lead, aluminium and ^{89,90}strontium.

Calcium hydroxyapatite has been subjected to numerous biochemical studies, and the ability of organic diphosphates, fructose biphosphate, pyrophosphate, imidodiphosphate and 3-phosphoglycerate to inhibit the conversion of amorphous calcium phosphate into the apatite complex has been extensively examined, and putative mechanisms of action evaluated (Fleisch *et al*., 1969; Williams and Sallis, 1979). These chemical studies suggest that strong inhibitors require at least one phosphate group and another acidic group (phosphate or carboxylic acid group) at some other position. Phosphonoformate and phosphocitrate were thus shown to be powerful inhibitors of calcium hydroxyapatite synthesis, probably through their profound ability to chelate Ca^{2+}.

Calcium hydroxyapatite crystals in normal human bone are needle-like, with their long axes lying parallel to fibres of the collagen network, but the precise relationship between the two is unclear. Uncertainty exists also regarding the positioning and interaction of chloride, citrate, carbonate and fluoride ions in normal bone and their availability to bind xenobiotic ions like lead, radium and other 'bone-seeking cations'. The role of strontium in human nutrition and bone development and physiology and development is equivocal, but its capacity to interact with and displace calcium in skeletal tissues is well documented (Lansdown *et al*., 1972; Sutton *et al*., 1970; Colvin *et al*., 1972).

3 NUTRITIONAL ASPECTS OF SKELETAL DEVELOPMENT

Cartilage and bone develop through prenatal and postnatal life in keeping with the growth and maturation of the body, with differentiation patterns subject to interaction between genetical factors, hormones and nutritional factors (Underwood and Mertz, 1987; National Research Council, 1995). In each case, histological profiles and chemical composition of the tissues are consistent with age, sex, hormonal status, and the overall state of health and nutrition of the body (Visco *et al*., 1991; Dunnill *et al*., 1967). Radioisotope studies using L-phenylalanine, L-leucine, ^{35}S-labelled chondroitin sulfate and ^{75}Se-salts have variously been employed in experimental and *in vitro* studies to monitor growth and patterns of mucopolysaccharide, mucoprotein and protein synthesis. They serve as a measure of growth rates under normal and

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pathological (toxic) conditions (Campo *et al*., 1967). With advancing skeletal maturity, bone consolidates to achieve a more compact structure with metabolically active regions persisting in the highly vascular periosteal and endosteal regions where osteoblasts and osteoclasts regulate mineral accumulation and mobilization. ^{14}C-proline and ^{48}Calcium are available as indicators of collagen formation and bone metabolism respectively (Firschein and Miller, 1966).

Many toxic materials or clinical situations capable of influencing growth patterns in cartilage or bone in perinatal or postnatal development act through impairing the availability of essential nutrients (notably minerals) or by inhibiting essential biosynthetic events through impairment of enzyme systems, particularly those like lysyl oxidase which depend on metal cofactors (Kagan and Li, 2003; Kosonen *et al*., 1997). Whilst nutritional requirements and recommendations are published for humans and some experimental species, variations reflect the date of the study and current opinion. Nutritional factors as potential causes of impaired growth and development in cartilage and bone are summarized as follows:

- general and severe malnutrition (e.g. kwashiorkor, marasmus, major protein-energy insufficiency)
- deficiency in one or more key nutrients (e.g. calcium, zinc, copper)
- nutrient imbalances (excess of one nutrient inhibits essential metabolic events dependant upon others)
- dietary materials with minimal or dubious nutrient value which impair uptake and metabolism of key nutrients.

Nutritional requirements vary according to the developmental state of the tissue and biosynthetic events in progress at the time (**Table 1**). Calcium, zinc, magnesium, vitamin A and phosphate have a major role in bone structure and function throughout life, whereas silicon, sulphur-containing amino acids and micronutrients are of greater importance in early active growth phases and in differentiation (Food and Drug Administration, 1997; Carlisle, (1982; 1988); Lansdown and Williams, 2007). In each case, recognised nutrients fulfil Underwood's (1977) criteria, namely that each is necessary for one or more key physiological or biochemical events in development and maintenance of structure and function.

Whereas defects in growth resulting from nutrient deficiencies in adulthood can in many cases be rectified by dietary correction or hormonal supplementation (Talmage, 1969; Stevenson and Evans, 1981), defects involving impaired mineral metabolism occurring in prenatal and postnatal growth are unlikely to be corrected by nutrient supplementation later in life. Minerals like calcium, zinc, magnesium and phosphate exist in bone in stable or structural forms or as labile moieties from which they may be mobilized to compensate for mineral deficiencies in other tissues through parathyroid action (Talmage, 1969).

3.1 Proteins and Amino Acids

Protein-calorie malnutrition presents major public health problems in many technically underdeveloped parts of the world, and conditions of marasmus and kwashiorkor are well documented. When general malnutrition or undernutrition is imposed during pregnancy, protein becomes a limiting nutrient, implying that close similarities exist between the effects of malnutrition and protein deficiency states (Hurley, 1977). Severe long-term deficiencies are common causes of fatality, whereas lesser levels of chronic protein or key amino acid deprivation are more likely to evoke growth retardation and defects in the maturation and function of tissues developing at the time (Apgar and Everett, 1991). The brain is particularly susceptible to malnutrition and nutrient deficiencies. Numerous studies are available discussing the complications of marasmus as a clinical cause of muscular wasting, and retardation in postnatal growth and development (Scrimshaw, 1974; Scrimshaw and Behar, 1961).

The role of chronic protein deficiency in the aetiology and pathogenesis of kwashiorkor-related conditions is incompletely characterized, and animal models are not entirely satisfactory in researching the biochemical changes of severe protein deficiency in children (Scrimshaw and Young, 1976). Enwonwu and Sreenby (1970) studied biochemical changes in rats fed low dietary protein (0.5%) for up to 12 weeks and demonstrated body weight loss, subcutaneous oedema, fatty liver and atrophy in spleen and pancreas similar to that seen in kwashiorkor children. Serum analyses showed significant depression in levels of essential and nonessential amino acids, including those with key roles in cartilage and bone growth, but no skeletal abnormalities were reported. The absorptive properties of the intestinal mucosa are initially increased to accommodate dietary protein deprivation, but later there was an onset of body weight loss, degenerative changes and atrophy (Worthington and Boatman, 1974). Guinea pigs which were considered to be a good representation of malnourished children, also showed severe growth retardation without involvement of skeletal tissues following low protein diets for three to four weeks (Enwonwu, 1973). In contrast, when pregnant mice were infected with a pancreatotropic strain of *Coxsackie virus B*, severe exocrine pancreatic insufficiency lead to impaired dietary protein utilization; offspring were severely growth retarded and exhibited skeletal malformations and abnormal ossification patterns (Lansdown, (1976a; 1976b)).

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Table 1 Major nutrients in growth and development of mammalian bone and cartilage

Nutrient	Biological function	Deficiency	Excess
Vitamin A	Mucopolysaccharide synthesis	Cartilaginous deformity, impaired ossification	Cleft palate, multiple skeletal deformities
Vitamin C	Maintains intercellular matrices in cartilage, bone and teeth; modulates mineral absorption and metabolism; collagen synthesis	Degenerative changes in tissues, mineral deficiency	—
Vitamin D (D_3)	Modulates growth and mineral content of bone, promotes calcium uptake and metabolism	Defects in mineralization in bone and teeth	—
Calcium	Bone cartilage growth and structural integrity	Defects in mineralization and skeletal rigidity, osteoporosis	Hypercalcaemia, metastatic mineralization in soft tissues
Zinc	Mitotic activity, enzyme cofactor	Impaired growth and survival	Minimal
Copper	Mitotic activity, crosslinking in collagenesis and elastic tissue formation	Impaired growth and structural deformities linked to collagen disorder	Neurological abnormalities and muscular pathology
Magnesium	Enzyme cofactor, mitochondrial function, calcium metabolism	Increased mineralization, impaired growth	Mineral imbalance
Iron	Enzyme cofactor, mineral absorption, oxygen/energy metabolism	Impaired growth and survival	Mineral imbalance
Manganese	Enzyme cofactor in cartilage development	—	—
Silicon	Collagenesis, glycosaminoglycan synthesis in matrices	Structural weakness	Not known
Phosphate	Growth and maturation in cartilage, mineralization in bone and teeth	Impaired skeletal growth and structural weakness	—
Sulfate	Mucopolysaccharide synthesis in cartilage and bone matrices	Impaired skeletal growth, defects in ossification	—
Chloride	Mineralization	Mineralization defects	—
Fluoride	Bind to hydroxyapatite, stabilizes bone structure and strength	Reduced bone and tooth resistance to degeneration	—
Protein/amino acid	General growth and development in all tissues of cartilage, bone and teeth	Stunting and delayed growth	—
Oxygen	Mineralization	Reduced bone structure	—

Numerous clinical reports illustrate how protein and/or calorie insufficiency leads to structural defects in ossifying elements of the skull and appendicular skeleton in human babies. Impaired collagen synthesis is a frequent complication (Frandson *et al*., 1954; Reddy *et al*., 1972; Krishnamachari and Iyengar, 1975). Frequently, damage induced in late prenatal or early postnatal stages is irreversible and catch-up growth does not occur even when protein-sufficient diets with growth hormone supplement are provided (Toews and Lee, 1975). On occasions, protein insufficiency may not be the principle cause of skeletal abnormality, but human babies exposed to malnutrition or other forms of growth retardation *in utero* fail to achieve catch-up growth in prenatal life even where nutrient levels are optimal (Dickerson and Hughes, 1972).

Herring (1968) discusses in detail the chemical composition of cartilage, dentine and bone matrix, with specific reference to collagen, protein-polysaccharides, glycoproteins, lipids and peptides. Analysis of the fibrillar structure of collagen and the amino acid composition of tropo-collagen chains and the intermolecular and intramolecular crosslinking has enabled a clearer vision of the amino acid sequences and their relative importance in molecular stability and physiological significance. Protein-polysaccharides of bone and

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Table 2 Variations in amino acid composition of galactosaminoglycan and mucoproteins of bovine scapular cartilage (residues per 1000)

Amino acid	Resting cartilage	Transforming zone	Ossifying zone
Cysteic acid	11.8	14.2	9.8
Aspartic acid	94.7	82.6	92.0
Threonine	47.3	42.7	46.0
Serine	47.3	51.3	49.1
Glutamic acid	124.2	136.7	113.5
Proline	100.6	82.6	73.6
Glycine	112.4	125.3	14.3
Alanine	112.4	114.0	17.4
Valine	65.1	68.4	79.8
Isoleucine	35.1	37.0	39.9
Leucine	71.0	74.1	89.0
Phenylalanine	29.6	28.5	39.9
Lysine	35.5	42.7	61.3
Histidine	59.2	59.8	55.2
Arginine	53.3	39.9	39.9

From Lindenbaum and Kuettner (1967).

cartilage are deemed to be highly heterogeneous with regard to the protein : polysaccharide ratios. Interest is also shown in the role of glycoproteins in bone and connective tissue development, and in Herring's (1968) opinion, concentrations are much higher than at one time thought. Further, amino acid concentrations of protein-polysaccharide complexes differ in cartilage according to its level of proliferative activity or onset of ossification (**Table 2**). Sulphur-containing cysteine is relevant in mucopolysaccharide synthesis and production of cartilage and bone matrix (Simmons, 1968). Experimentally, uptake of ^{35}S in murine long bone provided an accurate assessment of growth and mucopolysaccharide synthesis and revealed daily rhythms. It is also clear that deficiency in key amino acids can be as damaging as more generalized patterns of protein deficiency (Zamenhof *et al*., 1974; Hurley, 1977).

The relative importance of individual amino acids in cartilage and bone development has been examined in experimental studies with diets lacking lysine, proline, and so on (Hurley, 1977). Experimental studies have commonly demonstrated that deficiency in one or more key amino acids at vulnerable stages in development is a cause of severe growth retardation and decreased viability, without obvious effects on skeletal development. However, administration of the specific leucine antagonist—hypoglycine A—to pregnant rats did lead to syndactyly, and defects in skull ossification (Persaud and Kaplan, 1970). Ethionine is an antagonist for methionine, and prenatal exposures lead to a marked reduction in general body development and bone growth (Lee *et al*., 1955). On the other hand, excesses in single amino acids can be detrimental to foetal development; mostly the effects are manifest in terms of retarded growth and reduced foetal survival, with skeletal abnormalities reflecting growth retardation rather than actual pathological changes in chondrogenesis or ossification.

3.2 Vitamins

Excesses and deficiencies of vitamins A, C and D, niacin, folic acid and riboflavin are well-documented causes of structural deformities and pathological changes in prenatal or postnatal skeletal growth. Teratological studies illustrate how specific inhibitors including 6-aminonicotinamide, galactoflavin, α-methylpteroylglutamate and 5-bromo-2-deoxyuridine impair prenatal growth and viability, with reduction in skeletal ossification and abnormal skeletal patterns. Deficiencies in key vitamins or administration of specific vitamin inhibitors in experimental studies have commonly been related to a generalized pattern of deformities including cleft palate, defective ossification in the vault of the skull, micrognathia and impaired ossification in long bones (Chamberlain and Nelson, 1963; Hurley, 1977).

Vitamin A (retinol/retinoic acid) is an essential nutrient in all higher animals and correct levels are critical in normal epithelial cell growth and foetal development (Kalter, 1968; Maden, 1998). It is an example of an essential nutrient which is teratogenic if present at suboptimal or supraoptimal concentrations. In developing and adult mammals, vitamin A analogues interact with nuclear receptors including steroid and thyroid hormone receptors. These vitamin A receptors are involved in up-regulating gene transcription factors including genes modulating cell growth and differentiation in prenatal growth. Excess vitamin A affects mesenchymal tissue

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specifically and has variously been associated with abnormalities in cell proliferation, cell migration and differentiation leading to chondrodystrophies in cephalic and axial bones with manifestations of craniorachischisis, anencephaly and spina bifida (Marin-Padilla, 1966; Kochhar, 1968; Morriss and Steele, 1974). Cellular shrinkage and oedema formation were observed. The ossifying elements of the skull appear to be particularly vulnerable to injury (Cohlen, 1953; Masi *et al*., 1966; Giraud, 1968; Kalter, 1968; Morriss, 1972). Excesses of vitamin A and related retinoids led to degenerative changes through their action in labilizing lysosomal enzymes (notably hydrolases and phosphatases) in developing tissues (Goodman *et al*., 1974).

Research on retinoids has thrown light on their action in remodelling of bone, including an activation of osteoid resorption through enhanced osteoclastic activity leading to metastatic mineralization (Cunliffe and Miller, 1984). Turton *et al*. (1985) demonstrated that bone exposed to retinoid compounds exhibited greater radiolucency and showed a reduction in diaphyseal width and increased tendency to fracture. Histological sections revealed increased osteoclastic activity, reduced osteoid in mid-shaft regions, but increased mineral deposition in endosteal regions and in zones of ossification in hyperplastic chondrocyte columns of the metaphysic (Teelmann, 1989). Other changes including osteocytosis, hyperostosis and ossifying spondylitis have been recorded in response to retinoid toxicity (Clark, 1970; Cho *et al*., 1975; Mahtle and Berger, 1982).

Vitamin D (or its metabolically active form, D_3), 1,25-dihydroxycholecalciferol, exhibits a major role in the uptake and mobilization of calcium and phosphate in bone and other tissues (Ornoy *et al*., 1968; 1969; Talmage, 1969). It may function as a trophic hormone in calcium uptake by up-regulating the genetically determined calcium-binding proteins, calcium channels, membrane receptor sites and cell membrane permeability to the trace metals zinc, magnesium and iron, all of which are known to interact with calcium-binding receptors (Friedlander *et al*., 1977; Cole *et al*., 1999; Lansdown, 2002a). Calcium uptake in the intestine is a relatively inefficient process in which only about 30% reaches the systemic circulation. In normal individuals, calcium absorption is proportional to 'available calcium' in the circulation and free binding sites on the calcium-binding proteins (calbindin, calmodulin, S-100 proteins, etc.). Vitamin D_3 controls the reversible calcium–calbindin complex and transfer of Ca^{2+} to the Ca^{2+}-Mg^{2+}-ATPase pump for transfer to the peripheral circulation. Vitamin D_3 may mimic the action of parathyroid hormone in regulating extracellular calcium through calcium-sensing receptors (CaSRs) and act synergistically with it (Goldhaber, 1963). Mutations in CaSR genes are implicated in familial hypercalcaemia and hypocalcaemia. Either condition is a potential cause for abnormalities in cartilage and bone formation. Thus hypocalcaemia resulting from low vitamin levels is a cause of rickets in children and retarded bone growth affecting long bones, skull bones and costal cartilages and bone (Sevastikoglou *et al*., 1970). Hypophosphataemia is additionally a cause of impaired mineralization and pathological changes in regions of active bone/cartilage growth. Tooth formation and structure is at risk of demineralization (Rosen *et al*., 1974). The influence of vitamin D_3 on parathyroid hormone secretion is equivocal, but activation of enzymes including alkaline phosphatase and citrate metabolism may be involved (Sevastikoglou *et al*., 1970). High levels of systemic vitamin D or D_3 are toxic and may lead to demineralization of bone and the withdrawal of 'lime salts' (Carlsson, 1952). Additionally, hypervitaminosis D accentuates magnesium uptake (Ornoy *et al*., 1968). Calcitonin suppresses calcium mobilization from bone in cases of hypervitaminosis D or parathyroid hormone and is a recognized therapy for Padget's disease characterized by an up-regulation of osteoclasts and accelerated bone resorption (Siris *et al*., 1980).

Vitamin C (ascorbate) may not exert a direct influence on the development or physiological function of cartilage or bone, but is necessary for the intestinal absorption of several trace metal nutrients upon which those tissues depend. Thus, iron uptake relies on adequate dietary sources of vitamin C, and in cases of hypovitaminosis, resulting anaemias are associated with indolent skin wounds, impaired carriage and metabolism of oxygen radicals, impaired mitochondrial respiration in growing and repairing tissues, dyscollagenesis and wide-ranging conditions reflecting oxygen deficiency (Lansdown, 2001).

3.3 Minerals

Underwood (1977) identified at least 21 minerals as trace elements and essential nutrients in the mammalian body. Most exhibit a role in the structural integrity or physiological function of cartilage and bone, and deficiency symptoms are documented. With the exception of calcium and phosphate, minimal effective concentrations for normal healthy tissue at any age are normally low, but may vary according to the age, the developmental state of the tissues and the state of health of the individual. Calcium, zinc, magnesium, copper, silicon, iron, manganese, chloride, fluoride, sulfate and phosphates are variously implicated in the growth, structure, homeostasis and physiological function of cartilage and bone. Additionally, elements including lead, silver, strontium and aluminium with no recognized trace metal

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status, are occasionally located in bone bound structurally or covalently within hydroxyapatite crystals. As in the skin and related tissues, metal ions interact; they compete for receptor sites in the gastrointestinal mucosa, and competitive binding occurs on metal ion carrier proteins in the circulation and in growing and metabolically active tissues (Lansdown, 1995). The importance and biological implications of cahederins, calmodulins and S-100 proteins in calcium metabolism, and metallothioneins in zinc and copper uptake and mobilization have been reviewed (Lansdown, 2002a; 2002b; Lansdown *et al*., 1999; 2007). Imbalances between elements of nutritional importance, and interaction with xenobiotic ions (including radioactive and stable forms) are significant causes of structural deformity in growing bone and impairment of normal homeostatic mechanisms in adult tissues (Lansdown, 1995). The clinical and experimental implication of strontium, aluminium, lead, beryllium and iron in calcium metabolism and manifestation of rachitogenic changes is discussed below.

3.3.1 Calcium and Phosphate

Calcium uptake and mineralization in bone is influenced by excess concentrations of the anions fluoride, phosphate and phosphonates (Clark and Bélanger, 1967) Many studies have been conducted to evaluate the influence of changes in calcium–phosphate balance in growing bone and to demonstrate that whereas low phosphate favours mineralization, high phosphate suppresses calcium uptake and hydroxyapatite binding, leading to increased mobilization of labile calcium and osteoporotic symptoms (**Figure 8**) (Spencer *et al*., 1975). In a similar way, polyphosphonates and diphosphates impair calcium hydroxyapatite formation leading to structural damage in the bone matrix (Francis, 1969; Cram *et al*., 1971). Fleisch *et al*. (1975) suggested that pyrophosphates and ATPase in calcifying calf cartilages may modulate Ca^{2+} and mineralization in cartilage matrix vehicles and function as a physiological regulator of calcium homeostasis. In contrast, fluoride is probably beneficial in improving the structure and physicochemical properties of the calcium hydroxyapatite in bone and teeth, possibly through enhancing calcium binding. Shambaugh and Petrovic (1968) showed that although increased fluoride did not increase the overall dimensions of major long bones it did improve their flexibility and tensile strength. The mechanisms are presently unclear.

According to Capen (1999), the sensitivity of the parathyroid chief cells to calcium appears not to be fixed but may change during development and ageing and in response to disease processes. In this case, the negative feedback system regulating release of calcium from skeletal tissues breaks down and is influenced by circulating levels of vitamin D, adrenergic catecholamines and phosphate (Wongsurawat and Ambrecht, 1987). Additionally, urinary phosphate excretion is significantly higher in older rats compared to young adult animals fed equal dietary phosphate (Lee *et al*., 1984).

Numerous alkaloids and environmental chemicals show an ability to bind calcium in biological systems, occasionally with toxic or functional implications in bone or cartilage. Thus, alizarin dye from the madder plant (*Rubia tinctorum*), found in Mediterranean regions, binds calcium and can provide a useful nontoxic marker for bone growth and development in experimental studies (**Figure 9**). Steckoll *et al*. (1971) described red-stained bones of human skeletons recovered from archaeological sites in Qumran near the Dead Sea where madder is used as a vegetable food. The dye

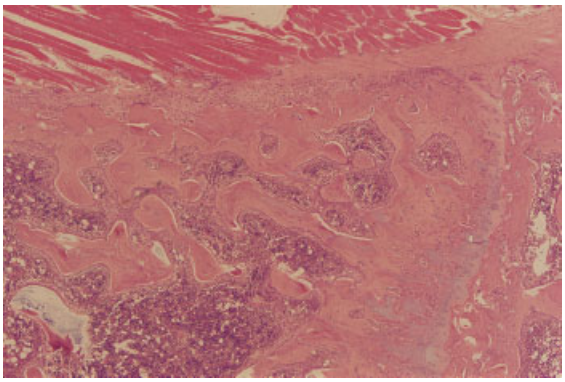

Figure 8 Idiopathic osteoporosis in a rat femur showing loss of bone trabeculae with increased osteoblastic activity (×10 objective, H and E).

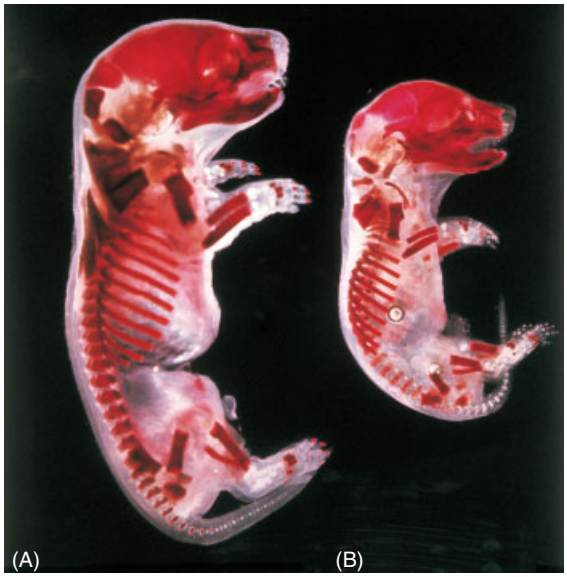

Figure 9 Alizarin red S-stained foetal skeletons of rats illustrating the growth-retarding influence of low-protein diets on bone growth and maturation. (A) 12% protein w/v in diet and (B) 1% protein.

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selectively stained osteogenic parts of the skeleton (especially the medullary cavity) and other sites of calcium deposition. Purified calcein preparation (DCAF) and tetracycline are also absorbed by bone and provide useful markers for bone growth in experimental studies (Rahn and Perrin, 1970; Rasmussen, 1975). Unlike the relatively nontoxic plant dyes, tetracycline is associated with toxic effects in embryonic and adult tissues (Rolle, 1967). Skeletogenic toxicity is manifest through increased uptake of calcium in osteoplastic and osteogenic tissues leading to premature ossification. In cultured bone explants tetracycline was shown to depress collagenesis by inhibiting proline uptake and proto-collagen formation, possibly through a competitive interaction of ferrous iron in the oxidation process (Halme *et al*., 1969). Alternatively, impairment of the cupro-enzymes, lysyl hydroxylase or prolyl hydroxylase, is involved. In teratological studies, tetracycline and other antibiotics binding or otherwise impairing the metabolism of calcium cause skeletal abnormalities with delayed ossification in rats dosed in early to mid gestation (Filippi and Mela, 1957a; 1957b; Murphy, 1965; Lejour-Jeanty, 1966). Deformities involved also included skull bones, cleft palate and long bones.

3.3.2 Strontium

Strontium is an enigma in human and animal nutrition and its role as a trace element in the mammalian body with a possible action as an enzyme cofactor or structural component in mineralization in bone is equivocal. Although early studies suggested that strontium might have a trace metal value in modulating early mineralization in bone (Johnson, 1973; Johnson *et al*., 1970), this is not substantiated. Chemically, strontium is similar to calcium and interacts with it in several biological systems, notably at receptor sites on cell membranes, carrier proteins and hydroxyapatite (Rygh, 1949; Harrison *et al*., 1959; Baud *et al*., 1968; Treffitt and Sutton, 1969). In bone, strontium fixation on bony surfaces occurs initially by a physicochemical process involving ionic exchange followed by progressive diffusion of the Sr^{2+} into the hydroxyapatite crystal structure (Harrison *et al*., 1959). This process may be modulated by dietary phosphate which favours calcium uptake (Colvin *et al*., 1967), but results of X-ray diffraction and extraction studies indicate that strontium substitutes *iso*morphically for calcium in hydroxyapatite and forms a complex tentatively identified as—$Sr_6H_3(PO_4)_5 \cdot 2H_2O$. In teeth, calcium and strontium exhibit a reciprocal relationship in enamel but with incisor areas highest in calcium.

Although hydroxyapatite possibly discriminates against strontium (Rygh, 1949), experimental evidence suggests that it is absorbed against a concentration gradient and that the rate of uptake reflects the Ca : Sr balance in tissue fluids; calcium : strontium ratios in bone resembling serum concentrations. Experimental studies also suggest that strontium can substitute for calcium in adult and foetal bone without obvious pathological change other than a change in tissue mass (Johnson, 1973; Lansdown, 1969). However, high dietary strontium levels are potentially toxic and have been shown to inhibit body growth leading to reduced bone and tooth ash (Johnson, 1973; Curzon and Spector, 1980; 1981). In severe cases, excessive strontium is a documented cause of 'strontium rickets', which may involve a deactivation of vitamin D (or its active form D_3) or competitive binding of calcium receptors, leading to calcium displacement and impaired mineralization. Strontium is known to suppress calcium-binding proteins and gastrointestinal absorption and is a further recognized cause of skeletal pathology and congenital bone deformity (Colvin *et al*., 1972). Strontium is absorbed by tooth surface enamel from soluble salts in drinking water, but there is conflicting evidence implicating it as a cause of dental caries or other pathological changes (Curzon and Spector, 1980).

Greater toxicological problems derive from exposure to radioactive strontium ($^{89,90}Sr$) where the radioactive emissions are the primary cause of skeletal dysgenesis. Herring (1969) emphasized that whereas many isotopes, including ^{90}Sr, locate in sites of active mineralization, ^{90}yttrium as a breakdown product of ^{90}Sr concentrates particularly on the surface of bone where ion exchange is active and where osteoblastic and osteoclastic activity is dormant. Other radioisotopes, including the bone seeking metals americium and plutonium, also concentrate in resorbing and resting bone surfaces but tend to be endosteal rather than periosteal in distribution (Vaughan, 1956). Radioactive emissions of bone-seeking elements including americium, plutonium, radium, yttrium and thorium are also potential causes of abnormal bone formation and mineral metabolism (Priest *et al*., 1983). Yttrium and thorium have been identified causes of impaired sialoprotein synthesis as a potential route to aberrant mineralization in bone (Williams and Peacocke, 1965). Radioisotopes of these and some other metals displace calcium and are a potential cause of degenerative and neoplastic changes, but in each case the pathology is largely attributable to the β-emissions rather than the mere displacement of the calcium ion, or disturbances in homeostatic mechanisms. Carcinogenicity is a recognized hazard following exposure to strontium and several other radioactive nuclides.

3.3.3 Zinc, Copper and Magnesium

Zinc and copper are essential trace minerals necessary for many key biochemical events maintaining health throughout life (Ma and Betts, 2000). Magnesium is present in all living cells but highest concentrations are found in bone ($1100\,\mu g\,g^{-1}$ tissue in normal human adult) (Schroeder *et al*., 1969). These elements are

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active biochemically and form structural components or cofactors for a large number of enzymic reactions involved in tissue morphogenesis and homeostasis. Zinc and copper are similar in induction and binding to metallothionein I and II carrier proteins, which modulate their uptake and intercellular distribution patterns (Lansdown, 2002a). Magnesium readily forms complexes with, and acts as an activator or catalyst for, a large number of enzymic reactions, notably those involved in the phosphorylation of ATP and oxidative phosphorylation (Wacker and Parisi, 1968). In cartilage and bone biology, magnesium and calcium interact, such that magnesium acts as a modulator of calcium uptake, tissue binding and excretion (Clark, 1969). Calcium–magnesium balances are central in controlling mineral homeostasis in bone.

Zinc is essential as a constituent or cofactor in more than 70 metalloenzyme systems including matrix metalloproteinases, alkaline phosphatase, RNases and DNases, involved in the development and maintenance of cartilage and bone (Lansdown *et al*., 2007). Hypozincaemias resulting from the chelating action of histidine, phytate, plant fibres, cyclohexamide or actinomycin D are potential causes of impaired bone growth and mineralization (Yamaguchi and Takahashi, 1983; Yamaguchi and Yamaguchi, 1986). Epidemiological studies by Prasad, of children in Middle Eastern countries fed high levels of plant fibre and phytates in vegetables, have shown that severe zinc deficiency syndromes were manifest as profound body growth retardation and tissue maturation, with defects in many organ systems including bone (Prasad, 1978; Lansdown *et al*., 2007). Cadmium, lead and alcohol in the diet reduce intestinal absorption and availability of zinc, and lead to symptoms of hypozincaemia (Yamaguchi and Yamaguchi, 1986; Eckhardt *et al*., 1981).

Copper is present at very low levels in living cells but is best recognized for its role as a cofactor in the metalloenzymes lysyl and prolyl oxidases involved in the crosslinking of collagen fibres, cytochrome oxidase, dopamine hydroxylase, superoxide dismutase and enzymes related to ferrous iron metabolism. It is bound in albumin complexes in the circulation, and more particularly to the specific carrier protein, caeruloplasmin. Copper deficiencies in early chondrogenesis leading to impaired development of the collagen-rich perichrondral sheath are a cause of impaired ossification and subsequent bone development. Copper deficiency leads to an excess of soluble collagen (Lansdown, 1995). Menkes' syndrome is a rare lethal mutation characterized by an inability to absorb or metabolize copper. It is a known cause of multiple defects of which neurological changes, epidermal changes and 'kinky hair' syndrome, vascular damage and impaired skeletogenesis are documented. Excessive copper is toxic in early development in the brain and liver but may be alleviated by the chelating agent penicillamine. Penicillamine has been examined as a potential antifibrotic therapeutic on account of its ability to block reactive aldehydes in collagen crosslinking (Nimni, 1980).

Collagen and elastic tissue crosslinking are inhibited by lathyrogenic agents including β-aminopropionitrile and α-amino acetonitrile, isolated from food plants (e.g. *Lathyrus odorata*). These lathyrogens are documented causes of mandibular, tooth and other skeletal abnormalities in cattle and experimental animals (Lenaers *et al*., 1971; Ohshima *et al*., 1989; Keles *et al*., 2005). Lathyrogenic agents have been shown experimentally to evoke a wide range of skeletal abnormalities including periodontal defects, mechanical strength of mandibles and long bones, mid-facial bone structure and calcium content in bone ash (Bar and Hurwitz, 1974; Kemm, 1975; Ohshima *et al*., 1989; Keles *et al*., 2005). They selectively impair the action of copper-dependant lysyl and prolyl oxidases leading to dyscollagenesis (Herring, 1968). As in states of dietary copper deficiency, the process may be modulated by growth factors, and cytokines, as seems to be the case in wound healing (Davidson *et al*., 1992). Resulting collagens are thermally unstable in the triple helix configuration and are subject to increased turnover; proline analogues investigated therapeutically in the treatment of fibrotic conditions can also be expected to impair collagen and elastic tissue formation in preosseous cartilage and synthesis and repair of fibroelastic cartilages.

3.3.4 Manganese

Manganese is a minor trace element important in chondrogenesis and bone development, and in the mineralization of otoliths in the middle ear (Tsai and Everson, 1967; Gamble *et al*., 1971). Manganese acts as a cofactor in phosphorylases and phosphotransferases involved in mucopolysaccharide synthesis in chondrogenesis. Structural deformities, poor growth and abnormalities in otolith formation are tell-tale features of manganese deficiency (Leach and Muenster, 1962). Recent views suggest that these symptoms reflect changes in circulating cholesterol and glucose concentrations. Other deficiency signs including reproductive failure, depressed growth and anaemia are nonspecific (Greger, 1999). Skeletal deformities in manganese deficiency are permanent but Greger suggests that there is an urgent need to identify accurate markers for manganese status. Serum manganese concentration with lymphocyte manganese-dependant superoxide dismutase and possibly serum arginase activity may be suitable for monitoring manganese deficiency, whereas neurofunctional tests with brain MRI scans are available to detect excessive or toxic exposure to the metal. Neurological symptoms are an occasional

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health risk in workers consuming manganese fumes occupationally.

In veterinary medicine, dietary manganese deficiency is associated with a condition known as 'perosis' in long bones. Affected tissues exhibit a diaphyseal thickening, possibly as a result of increased alkaline phosphatase activity (Wolbach and Hegsted, 1953; Leach *et al*., 1969). It is unclear to what extent manganese impairs calcium or zinc metabolism in this malformation. Investigative studies in deformed sheep identified pathological abnormalities in the columns of hyperplastic cartilages and ossifying metaphyses of limb bones (**Figure 7**) (Duffell *et al*., 1985). The changes resembled those associated with manganese deficiency, but this was not established. Earlier, manganese deficiency was suspected as a contributory cause of a skeletal deformity in turkeys, the so-called 'TY-65' syndrome in which young turkeys presented with abnormalities in the metaphyseal plate region of major long bones (Wise *et al*., 1973).

4 TOXIC DAMAGE IN CARTILAGE AND BONE

A xenobiotic material may invoke structural damage and functional changes in cartilage and bone at any stage from early differentiation through to old age. The type and severity of the damage will be a reflection of the nature of the toxic insult, the vulnerability of the target tissue or cell at the time of exposure, and the genetical susceptibility of the tissue. In the prenatal and perinatal periods, developing tissue in any organ is appreciably more susceptible to injury than in adulthood, and at a time of high biosynthetic activity, tissue differentiation and mitotic activity and differentiation (Lansdown, 1987). Thus toxic action and manifestations of structural and physiological damage in prenatal life commonly reflect impaired growth and cellular interaction in differentiation, incomplete or defective macromolecular syntheses in intercellular matrices (including collagenesis and elastic tissue formation), abnormal morphogenic tissue movements and defective intertissue relationships. The status of maternal health and nutrition has a major bearing on foetal development, such that any condition, chemical exposure, microbiological infection or physical insult (anoxia, X-irradiation, hyperthermia, etc.) in/on the mother is a potential cause of foetal abnormality, substandard postnatal growth and reduced survival.

Skeletal tissues become vulnerable to a different range of toxic changes in postnatal life as mitotic activity and postmitotic cell migration and morphogenic events diminish and as the tissues of appendicular and axial structures achieve their expected adult proportions. At this stage, we consider:

1. Functional change
   (a) impairment in the regulatory mechanisms for bone as a physiological source of minerals (Ca, Zn, Mg, etc.)
   (b) obstruction in the ability of bone matrix to absorb xenobiotic ions thereby protecting vulnerable soft tissues
   (c) reduction in the capacity of tissues to repair following fracture or other injury
2. Pathological damage
   (a) impairment in vascular function → tissue death, nutritional deficiency
   (b) osteoporosis, osteodystrophy → bone weakness, vulnerability to fracture
   (c) metastatic mineralization in cartilages → osteoarthritis
   (d) mutagenic change → bone/cartilage carcinogenesis.

In each case the severity of tissue response is influenced by genetical factors, hormonal changes (oestrogens, parathyroid), nutrition (phosphates, vitamins D, C) and general metabolic health.

A wide range of environmental chemicals, agrochemicals and drugs have a capacity to influence cartilage and bone at one or more stages, leading to transitory or irreversible damage. Unlike soft tissues, including liver and kidney, where minor changes in structure and function of adult tissues are readily detected by serum enzyme changes, secretion and excretion patterns, subtle changes in skeletal morphology are less readily appreciated other than through biopsy with chemical analyses, or X-radiography. In toxicological studies, it is now common practice to examine key bones for histological changes after nine months (subacute) or two years (chronic) (Lansdown, 1987). Where, on the rare occasions manifestations of damage are detected, they may result from direct action of a test material on metabolically active chondrocytes, osteocytes or cells of the periosteum or endosteal surfaces. Alternatively, they may result by an indirect means, namely through the action of drugs, pharmacological or toxic agents on vascular pathways, nerve conduction and neuromuscular activity, which have a bearing on the health and function of musculoskeletal tissues.

5 TERATOGENIC MECHANISMS IN SKELETAL DYSGENESIS

Skeletal abnormalities are commonly reported in teratological studies. Virtually every teratogen having a direct or indirect influence on prenatal or perinatal body growth is a noted cause of defective skeletal development. This is illustrated by personal studies on salicylates, bone-seeking minerals, anaesthetic gases and infections

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in pregnancy. Principle mechanisms of skeletal deformity and/or pathological changes involving cartilage or bone include:

1. Impaired differentiation and function of chondrocytes.
2. Abnormal cartilage formation and maturation; defective formation of ground substance (glycosaminoglycan) of bone matrix.
3. Impaired function and regulation of osteoclasts and their degradative enzymes.
4. Abnormalities in mineralization and ossification.
5. Impairments in physiology and general body growth in the foetus/embryo.
6. Impairments in maternal health leading to abnormalities in the intrauterine environment (an indirect effect).

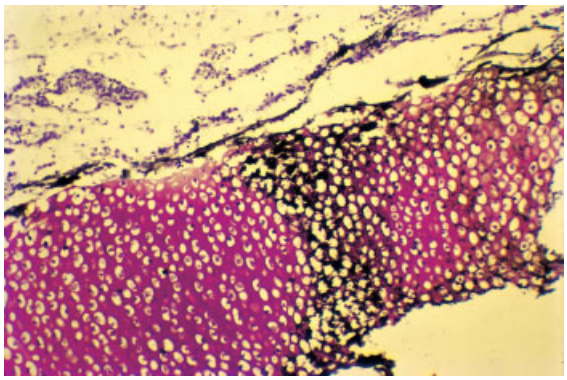

Figure 10 Aberrant ossification patterns in the femur of a rat foetus (12 days gestation) following maternal exposure to sodium salicylate, illustrating impaired cartilage and chondrocyte hypertrophy. Note disorganized perichondrium (×10 objective, toluidine blue and silver impregnation).

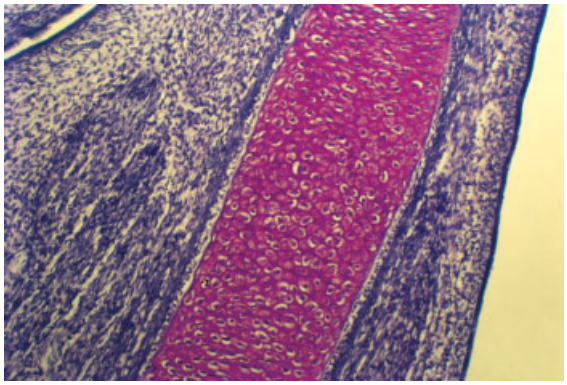

Figure 11 Normal preosseous cartilage in the rat femur after 10 days gestation (×10 objective, toluidine blue).

Putative teratogens including chemical, physiological or biological (bacteria, viruses, etc.) may exhibit site-specific effects or multifocal sites of toxic action. Manifestations of toxic action vary according to species, time and duration of exposure and genetic factors (as in induction of cleft palate). Cleft palate, as frequently described in teratological literature, has a complex aetiology and may be associated with cleft lip (Shah and Halperin, 1982). In human babies with orofacial clefts, isolated cleft lip and isolated cleft palate each account for approximately 20–30% of cases, with the remaining 50% being represented by combined cleft lip and cleft palate. Certain strains of mice are particularly susceptible to spontaneous cleft palate, a feature which has been used to advantage in studying the genetical sensitivity to cortisone teratogenicity (Loevy, 1962; Pinsky and Di George, 1965).

Patterns of structural deformity in endochondrial bones are illustrated by reference to site-specific teratogens like salicylates and cadmium with known biochemical action on cartilage matrix (Lansdown, 1969; 1970; Foulton *et al*., 2000; Larsson and Piscator, 1971). Experimental studies demonstrate that such toxins impair the biosynthesis of chondroitin sulfate and other mucopolysaccharides through blocking sulfation patterns, and are potential causes of abnormal development and maturation in long bones of the limbs, vertebrae and ribs (**Figure 10**).

The chondrogenic phase of all long bones is characterized by elaboration of an intercellular matrix composed largely of mucopolysaccharides like chondroitin sulfate. These mucopolysaccharides exhibit a metachromatic staining reaction in response to toluidine blue and related dyes; the intensity of the metachromasia serving as a guide to the maturity and normality of the cartilage (**Figure 11**) (Lansdown, 1968). In normal chondrogenesis, cellular maturation in mid-shaft regions is associated with more intense metachromasia and hypertrophy of chondroblasts. As site-specific teratogens, salicylates impair the sulfation of polysaccharides leading to defective cartilage matrix and subsequent osteogenic phases. Thus in experimental studies, foetal rats exposed to high maternal doses of sodium salicylate exhibited major abnormalities involving limb bones, ribs, vertebrae and skull bones. In each case cartilage matrix showed reduced staining and abnormal ossification patterns (Lansdown, 1970).

Cadmium, on the other hand, concentrates more in cranial regions of exposed rat foetuses, leading to cleft lip and facial deformities (Holmberg and Ferm, 1969). Preosseous cartilage was abnormal and impaired growth led to abnormal fusions and mesenchymal derangement (Mulvihill *et al*., 1970). Cadmium is severely cytotoxic and induces inflammatory changes, but its principle biochemical effect is the induction and binding of metallothioneins, thereby impairing the zinc and copper-dependent events (Margoshes and Vallee, 1957). Cadmium is known to evoke vascular hypertension and is commonly regarded as an antimetabolite in biological systems (Schroeder *et al*., 1967). Vitamin A (retinoates,

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EDTA (ethylenediaminetetraacetic acid)) and penicillamine are other causes of cartilage abnormalities and defects in mineralization (Shah, 1979; Hendrickx and Binkerd, 1979).

It is customary in teratological studies to demonstrate patterns of ossification in foetal skeletons using the red dye alizarin red, which binds calcium hydroxyapatite in skeletal tissues (**Figure 9**). Although the underlying mechanisms for deformity may not be known, skeletal preparations stained with alizarin red dye allow appreciation of such features as:

1. the extent of ossification in the various elements of the skeleton—limbs, vertebrae, ribs, skull
2. presence of abnormal centres of ossification
3. absence of centres of ossification
4. abnormal fusions and sutures.

Whereas retarded prenatal development is frequently a consequence of impaired maternal health, abnormal fusions, deletions and growth of additional vertebrae, ribs and digits can be attributed to deviations in intercellular contact or other defects within the skeletal blastema modulated by genetic or idiopathic factors (Lansdown, 1976a). Rodent and rabbit species commonly used in laboratory studies show a strain-related incidence of structural abnormalities in bone (Lansdown, 1987). Thus the mouse is of limited value in view of its known susceptibility to cleft palate (Loevy, 1962). The incidence of 'spontaneous' abnormalities in rabbits is slightly higher than in rodent strains, but in view of its susceptibility to known human teratogens like thalidomide, this is not considered to be a major disadvantage (Gibson *et al*., 1966; Stadler *et al*., 1983; Lansdown, 1987).

Pathologically, materials capable of inducing specific structural abnormalities in cartilage or bone through prenatal exposure may act during initial transformation of mesenchymal cells in the early blastema (the induction phase), elaboration and synthesis of chondroitin sulfate in the cartilage matrix (preosseous phase), or during the differentiation of osteoblasts and mineralization in mid-shaft and endochondral sites (osseous phase). Once centres of ossification become established, teratogenic agents may influence subsequent skeletal growth through toxic action in metaphyseal growth plates, impaired chondroblastic action (impaired breakdown of preosseous cartilage matrix) or defective mineralization patterns. Impairment in the induction phase of chondrogenesis in long bones and vertebrae is a common cause of limb and spinal deformity. Defects in the ossification of membrane bones of the skull are commonly associated with impaired closure of fontanelles or other forms of craniofacial abnormality (Kokich *et al*., 1982).

The highly toxic alkylating agent, nitrogen mustard, is an illustration of a teratogen that targets mesenchymal cells (Sweeney and Watterson, 1969; Salzgeber, 1979). It is a cause of mutagenic damage, mitotic inhibition and necrosis in preosseous cartilage, leading to severe deformities in limb bones, vertebrae and rib cage.

Hypoxia is an acknowledged cause for circulatory disturbances leading to oedema, imbalances in anion–cation distribution, and changes in sugar, lactate and free amino acid concentrations in the developing embryo/foetus (Grabowski, 1966). Oxygen tension in the induction phase of early development is critical in osteoblast differentiation (Hall, 1967). Calcium is absorbed more readily by chondroblasts, which in due course hypertrophy and undergo premature mineralization. Other illustrations of materials that impair oxygen balances in early bone formation include trypan blue dye, dinitrophenol, lactate and calcium chloride (Grabowski, 1964; Kaplan and Johnson, 1968). Whilst mechanisms of subcellular action differ, vascular changes, rarefaction of proliferating cell populations, blisters and local oedema through hypervitaminosis A, bis-azo dyes and nutritional deficiency of linoleic or pantothenic acids are recorded causes of impaired oxygen metabolism leading to skeletal abnormalities in experimental animals or human babies (Marin-Padilla, 1966; Turbow, 1966; Morriss and Steele, 1974; Sandor and Amels, 1975).

Mucopolysaccharide synthesis is a target for several bone-seeking teratogenic agents, including hypervitaminosis A, salicylates and cortisone, although mechanisms differ. Vitamin A possibly acts by impairing the sulfation of polysaccharides (Wolbach, 1947; Fell and Mellanby, 1952; Wolf and Varandani, 1960; Pasternak and Thomas, 1968). Experimental studies suggest a strong relationship between the concentration of vitamin A and the developmental sensitivity and genotype of the tissue (Kochhar, 1967; Nolen, 1969; Robens, 1970; Biddle and Fraser, 1976). Biochemical and teratological studies demonstrate that salicylates also impair chondrogenesis and osteogenesis, acting by inhibiting key enzymes involved in the sulfation of polysaccharides, and advancing the hypertrophy of cartilage matrix (Whitehouse and Bostrom, 1961; Larsson and Bostrom, 1965; Grisolia *et al*., 1968; McArthur and Smith, 1969; Somogyi *et al*., 1969). Foetal deformities in pregnant rats exposed to high levels of sodium salicylate or salicylic acid in early- to mid-gestation are related closely to the concentration of salicylate, the gestational age of the foetus and genotype (Warkany and Takacs, 1959; Larsson *et al*., 1963; Ericksson, 1971). Abnormal cartilage in long bones and vertebrae were shown histologically to lead to impaired ossification (**Figure 12**) (Lansdown, 1970). In older foetuses, high salicylate has been associated with excess ossification with occasional metastatic mineralization, possibly resulting from an inhibition of oxidative phosphorylation (Whitehouse, 1963; Cotty and Harris, 1968). Deformed human babies have been reported in mothers consuming high levels of aspirin during pregnancy, but where the doses were at suicidal levels (British

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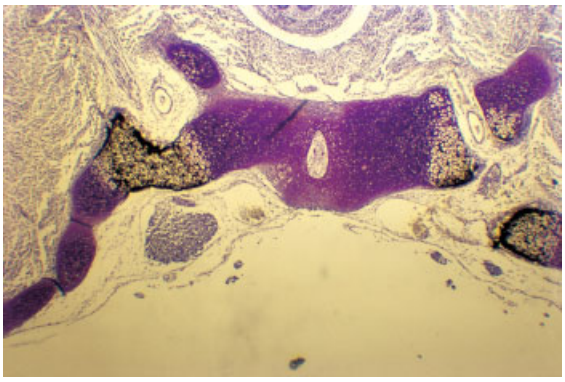

Figure 12 Chondrodystrophy associated with abnormal ossification in the mid-dorsal region of a 13-day rat foetus exposed to high levels of sodium salicylate *in utero* (×2.5 objective, toluidine blue and silver impregnation).

Medical Journal, 1963; Jackson, 1948). A further illustration of the osteogenic influence of salicylate is seen in teeth. Berry and Nickols (1979) showed that salicylates impair developmental patterns, although the actual effect on odontoblasts is not clear.

A commonly described and often contentious skeletal abnormality in experimental and clinical teratology is cleft palate formation (Burdi, 1977). Cleft palate is a serious medical problem in clinical management and patient care, but equally disturbing is its association with a high incidence of other medical problems involving soft tissues—neurological abnormalities, congenital heart disease, miscellaneous hernias and delayed maturation (Shah and Halperin, 1982). Iron-deficiency anaemias were reported in 10% of 304 patients with cleft palate; haemoglobin levels of $<11.4\,\mathrm{g\,dl^{-1}}$ were possibly attributable to poor dietary habits. Cleft palate occurs with a high incidence in certain strains of mice, and has been described in numerous experimental studies in which animals have been dosed *in utero* with high doses of vitamin A, aminopterin, thalidomide, diazepam (Valium), the environmental defoliant 2,4,5-trichlorophenylacetic acid (2,4,5-T) and cortisone (Fogh-Andersen, 1971; Safra and Oakley, 1976; Nanda and Romeo, 1978). Underlying mechanisms suggested for cleft palate formation include defects in chondrogenesis, and inhibition in the horozontalization of lateral palatal shelves and their fusion in the midline (Asling *et al*., 1960; Loevy, 1962; Ross and Walker, 1967). Impaired mucopolysaccharide synthesis, delays in mitosis and postmitotic cell migration, induced myopathy, or impairment in lysosomal enzyme degradation of prechondrous mesenchyme may be contributory in each case (Burdi *et al*., 1972; Shah and Halperin, 1982).

Toxic and teratogenic factors influencing the development and functional maturation of cartilages and bone late in prenatal life and postnatally act mainly through impairment of essential nutrients, alterations in mineral metabolism or through inhibiting essential biosynthetic events. They may be generalized or target a particular tissue depending upon the nature of the deforming influence, the time of exposure and the developmental state of the tissue. Continued growth and maturation in cartilage and bone depend upon a regular source of nutrients (**Table 1**). Lucille Hurley (1977) emphasized the special importance of nutrition in the perinatal period and noted that malnutrition leading to low birth weight is a common cause of congenital abnormality and permanent anatomical aberration. Whereas at one time there was a commonly held belief that foetuses *in utero* were protected from malnutrition or nutritional stress by the maternal organism, early experimental observations by Hale (1937) showed that piglets born to sows deficient in vitamin A were low in birthweight and displayed a high incidence of malformation and reduced postnatal viability. Although there is extensive epidemiological evidence to demonstrate that nutritional deprivation and malnutrition as in kwashiorkor is a cause of structural abnormality, low birth weight and reduced neonatal survival, much of our present knowledge on the importance of vitamins, minerals, amino acids and proteins in prenatal and postnatal growth derives from controlled and reproducible experimental studies in animal models (Hurley, 1968; O'Dell, 1968). Although some subtle and some major interspecies differences will preclude a direct extrapolation of results in animal models to humans, it can be safely assumed that the biochemical mechanisms involved in tissue morphogenesis in bone, cartilage and soft tissues will be similar in all mammals, and that studies conducted in rodents, rabbits, pigs and so on, will be of reliable predictive value for humans.

6 ACCUMULATION OF METALS IN BONE: A PROTECTIVE MECHANISM?

The principle mineral components of normal mammalian bone include calcium hydroxyapatite and various bone salts, including magnesium, phosphate, citrate, fluoride, hydroxyl and chloride (McLean and Urist, 1969; Urist *et al*., 1968). These mineral salts vary in concentration but confer hardness and rigidity to the bone. They are mostly soluble in mineral acids and assayed by atomic absorption spectrometry following prolonged immersion in dilute solutions. Calcium hydroxyapatite crystals are of central importance in the uptake and release of anions and cations. Thus, citrate ions and carbonates are probably arranged on the surface of the crystals, but carbonates may substitute for phosphate ions within the crystal structure. Even in histologically normal trabeculate or dense bone, the calcium hydroxyapatite crystal structure is not stable and is subject to variations, with fluoride substituting for hydroxyl groups

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and bone-seeking cations replacing calcium (Samachson, 1968).

The ability of hydroxyapatite and mucosubstances in bone to concentrate and bind metal ions has several implications in physiological and toxicological terms, notably in regulating the uptake and mobilization of ions with nutrient value, like calcium and magnesium (Herring, 1969). The greatest proportion of the body calcium load is bound in bone, partly as a structural component and partly as a labile moiety. This labile calcium is available as a multifunctional ion, as enzyme cofactor, tissue modulator or physiological factor in many tissues in the body. It is mobilized in cases of nutritional or other forms of deficiency by hormonal modulation (Lansdown, 2002a). Profound bone-seeking metals like cadmium deplete labile calcium, and calcium in the structural matrix, leading to osteoporosis, particularly in patients subject to postmenopausal changes (Åkesson *et al*., 2006). Sodium also is a normal constituent of bone, but as Biltz and Pellegrino (1969) found, concentrations found in mammalian bone show no constant correlation with the total mineral content. Bone is a major source of labile zinc but its role in providing a reservoir for zinc in cases of deficiency is less clear (Lansdown *et al*., 2007). Regulation of zinc concentration in body fluids is controversial but serum concentrations seem to relate to circulating levels of carrier proteins and hormones (e.g. parathyroid hormone); dietary factors may be involved. Of greater toxicological significance is the capacity of bone, dentine and cartilage to concentrate and bind metal ions with questionable or no acknowledged physiological or nutritional role in the body (e.g. lead, cadmium, silver, aluminium), and which are recognized toxins in key soft tissues like liver and kidney. Analysis of the mineral content of bone through iliac crest biopsies provides an indication of exposure to such metals and the concentrating ability of hydroxyapatite or other constituent of cartilage or bone (Lindh *et al*., 1980). The assay of bones from workers in Sweden using atomic absorption spectrometry, neutron activation analysis and X-ray emission analysis in a proton microprobe showed that workers exposed to lead had about five-fold greater levels of bone-bound lead. Elsewhere, bone samples of prehistoric North American Indians analysed for lead and cadmium showed appreciably lower concentrations of the metals than seen in modern day populations in Europe and the USA (Ericson *et al*., 1991). Thus, ratios of lead to calcium ($8.4 \pm 4.4 \times 10^{-7}\,\mu g\,g^{-1}$ dry weight) were many times lower than seen in modern populations ($210–350\,\mu g\,g^{-1}$). Analytical studies provide information on the mineral content of tissues and offer the opportunity to calculate ionic balances as an index of toxicity.

Increasing use of xenobiotic metals in medical devices and concerns over environmental pollution have resulted in an increasing availability of techniques to monitor low levels of metal in tissue. We now have the ability to monitor xenobiotic metals concentrated in human bone and appreciate imbalances in physiological concentrations of key metals like calcium, zinc and magnesium leading to pathological conditions, notably osteoporosis; the stability of xenobiotic metal hydroxyapatite complexes; release of xenobiotic metals from bone; and their metabolism via carrier proteins to vulnerable soft tissue.

Information compiled so far in relation to such metals as aluminium, silver, cadmium and lead provides an incomplete profile of the inherent risks associated with environmental contamination and indicates the need for further biochemical and pathological investigation. Aluminium is used extensively in industrial processes but its value in renal dialysis is controversial. Berlyne *et al*. (1972) assessed the toxicity of several aluminium compounds including aluminium hydroxide in rats dosed orally and parenterally, and identified increased aluminium deposits in bone. They did not report histological changes but noted that tissue and plasma aluminium concentrations were increased two-fold in uraemic animals, consistent with their unpublished human studies. D. J. Sherard (unpublished observations, 1972) disclaimed these experimental studies and maintained that they were a misrepresentation of normal routes of human exposure to aluminium in diuresis, and that the observations were at variance with his own clinical experience. He reported no evidence of bone disease in patients exposed to aluminium through renal dialysis for up to six years. Elsewhere, aluminium-related osteomalacia dystrophy was observed in two long-term studies of dialysis patients; aluminium possibly displaced calcium in hydroxyapatite (Ward *et al*., 1978; Parkinson *et al*., 1979). The authors reported that the aluminium content of bone correlated well with levels of osteoclast activity in iliac crest biopsies, and that bone volume correlated with aluminium content (Cournot-Witmer *et al*., 1979). X-radiography revealed phalangeal erosions, subperiosteal resorption in long bones, and severe osteomalacia. Clinicians have questioned also whether high aluminium levels in bone may be correlated with phosphate or fluoride concentrations (Parsons *et al*., 1971). In teeth, a stable chemical balance seems to exist between the aluminium content and the fluoride. More recent work suggests that the risks identified in experimental studies are insufficient to justify replacing aluminium hydroxide in uraemic therapy (Thurston *et al*., 1972; Verberckmoes, 1972).

Silver is a less contentious metal and so far has not been associated with clinical evidence of toxicity in bone or other tissues (Lansdown, 2002c; 2006; Lansdown and Williams, 2004). Silver has enjoyed widespread use as a broad-spectrum antimicrobial substance in medical devices including orthopaedic prostheses, bone cements, surgical pins and dental materials. The silver compounds used in medical devices ionize in the presence of

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body fluids to release biologically active Ag^+, which readily binds receptors on cell surfaces, and induces and binds the zinc and copper carrier proteins, metallothioneins I and II (Lansdown, 2002b). Earlier biochemical evidence that Ag^+ evokes release of Ca^{2+} from the sarcoplasmic reticulum vesicles isolated from rat muscle homogenates through activation of calcium channels or calcium ion pump mechanisms involving magnesium (Tupling and Green, 2002; Prabhu and Salama, 1990), suggests that silver ion absorbed by bone may trigger release of labile calcium and lead to pathological changes. However, no evidence has been seen to establish that Ag^+ displaces Ca^{2+} or activates Mg^{2+} in bone leading to osteoporotic or physiological changes. Human osteoblasts exposed *in vitro* to a novel nanosilver bone cement exhibited normal behaviour and formed cellular processes indicating good biocompatibility (Alt *et al*., 2004). In another study, morphological alterations were reported in rat osteosarcoma-derived (osteoblast-like) cells exposed to silver, gold, palladium, copper or nickel–titanium alloy wire as used in dental materials (Cortizo *et al*., 2004). Silver and copper wires with an exposed area of $0.157\,cm^2$ were cytotoxic to cultured osteoblasts, whilst other elements appeared to be biocompatible. Cells exposed to silver were smaller than in controls but some mitoses and apoptotic bodies were observed. Copper wire evoked more profound cytopathic changes, ranging from total loss of viability to abnormal cytoplasmic inclusion, impaired growth and loss of cellular integrity. Spadaro *et al*. (1979) evaluated silver-containing polymethyl methacrylate as used in bone cements, by implanting samples intramuscularly in rabbits. Tissues surrounding implant sites were morphologically normal and exhibited only a mild foreign-body reaction, indicative of good biocompatibility of the material *in vivo*.

The toxicology of lead in bone and cartilage is complex and still imperfectly understood, but substantial evidence exists to show that lead interacts with and may displace key mineral elements in bone, notably calcium, zinc, copper, iron and magnesium, which contribute to bone structure and exhibit central roles in cell metabolism, proliferation and homeostasis (Moore, 1979). Settle and Patterson (1980) emphasized the strong affinity of lead for calcium receptors and claimed that biochemical systems regulating calcium metabolism 'operate passively' on some other elements including lead and barium, and reflect small differences in their chemical properties. Whilst experimental evidence suggests that lead inhibits uptake of calcium and displaces it from bone (Gruden *et al*., 1974), dietary calcium, in the rat at least, can modulate lead concentrations in bone and its mobilization to soft tissues (Vega *et al*., 2002). Experimental studies suggest that lead efflux from bone is related to bone resorption during lactation. It seems that a homeostatic balance is achieved between calcium and lead, and that this is regulated by calciotropic and pregnancy-related hormones (Silbergeld, 1991). Lead uptake and metabolism is sensitive to vitamin D and parathyroid hormone, and is influenced by dietary concentrations of sodium citrate, vitamins C and D, certain amino acids, proteins, fats and sugars, and mineral salts including Ca^{2+}, Mg^{2+} and phosphate (Moore, 1979; Smith *et al*., 1978). Lead is a cumulative poison, and uptake from the intestine and through respiration occurs independently of the body and tissue levels and against concentration gradients. No recognized feedback mechanism seems to exist to limit lead absorption as occurs with calcium and most trace metals. As with other metals, lead is metabolized in the form of a carrier-protein complex. A proportion of this lead will be excreted via the skin, hair, nail, urine and faeces, but as much as 90% may be deposited in bone (Moore, 1979). In one clinical study, 68 men exposed occupationally to lead showed increased serum concentrations of 1,25-dihydroxyvitamin D (Mason *et al*., 1990). Hypervitaminosis correlated well with blood lead and lead concentrations in tibial biopsies.

Somervaille *et al*. (1988) discussed the value of X-ray fluorescence assessment of tibial lead concentrations as a guide to occupational exposures. Differing views are expressed as to the true toxicological significance of lead bound in bony tissues (Rabinowitz *et al*., 1977; Rosen and Markowitz, 1980; Rosen, 1983). Pounds and Rosen (1986) considered that bone was not only a place of storage for lead, but a target tissue for lead toxicity, but this has to be ratified clinically. Lead is readily taken up by osteocytes, and concentrates in hydroxyapatite matrix from which it is released to evoke potentially toxic and irreversible damage in many soft tissues, notably the brain and kidney (Emmerson and Thiele, 1960; Rosen, 1985; Silbergeld, 1991). Lead locates in osteoblast and osteoclast mitochondria which are vulnerable to lead-induced swelling and depressed respiratory activity (Goyer and Krall, 1969). Further studies are required to investigate other intracellular pathways of lead metabolism and toxicity in bone cells and how these are influenced by hormones, drugs and nutrients (zinc, magnesium iron, etc.). Some authors believe that much of the lead bound in bone is bio-inert and unavailable for metabolism to vulnerable tissues (De Mechele, 1984). This argument is fuelled by the observation that only part of this lead is available for chelation by EDTA (ethylenediaminetetraacetic acid) or other cytoprotective agents (Pounds and Rosen, 1986). In sites of toxic action, lead exhibits a strong affinity for sulfhydryl (–SH) groups, lysine residues, and hydroxyl (–OH) groups, and it binds to and inhibits a variety of proteins including enzymes.

Lead pollution from traffic fumes and water systems is a potential cause of tooth decay. Researchers in Spain have suggested that lead absorbed from fumes interacts with the minerals of tooth enamel and that exposed children and individuals with tooth

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decay displayed more than three times the level of blood lead than those with normal healthy teeth (Pearce, 1997). The concentration of lead in bone has been shown to correlate with levels of nephropathy, lead in teeth and neurophysiological development in children (Emmerson, 1963; Needleman *et al*., 1979).

Cadmium is a profound bone-seeking element, and high exposure is a known cause of bone damage and osteoporosis (Åkesson *et al*., 2006). A study of women's health in the Lund area of Sweden revealed that menopause, smoking, body-weight status and hormone replacement therapy affect blood and bone cadmium levels. Patients of 53–64 years of age exposed to low-levels of cadmium exhibited a significant interaction between cadmium and menopause; increased cadmium excretion was associated with negative effects on bone mineral density. Experimental studies in rat emphasize the strong interactions between cadmium and calcium; cadmium administered orally in diet or by parenteral injection impaired calcium absorption and metabolism leading to osteoporotic and osteomalacic changes in bone (Itokawa *et al*., 1974). In part, this may be attributable to cadmium-induced renal damage and impairment of phosphate and calcium metabolism (Friberg, 1984; Kawamura *et al*., 1978). Histological analysis of femurs of cadmium-fed hypocalcaemic rats revealed reduced cortical osseous tissue, whereas in calcium-sufficient cadmium-treated animals, the metaphyseal plate was thinned and zones of mineralization disturbed, and trabecular bone formation was abnormal (Itokawa *et al*., 1974). Osteoclast activity was also prominent in femurs of rats fed low levels of calcium and 100 ppm cadmium in diet.

7 CARCINOGENICITY

The toxicology of cartilage and bone rarely extends to a consideration of the potential carcinogenic or mutagenic effects of environmental contaminants and industrial chemicals in skeletal tissue. Spontaneous bone tumours are rare in rodents and are seldom an issue in toxicological studies (Luz and Gössner, 1994). In an evaluation of 368 chemicals, the International Agency for Research on Cancer (IARC) identified 26 chemicals for which carcinogenicity in humans was unequivocal, and a further 221 for which evidence of carcinogenicity in animals provided some evidence for putative carcinogenicity in man (Tomatis *et al*., 1978; International Agency for Research on Cancer (IARC), 1980; US DHHS, 2005). Importantly, many of the reports scrutinized fail to recognize that bone or cartilage in any part of the body is a target for putative carcinogens or that either tissue is a potential site for metastases (Haseman and Lockhart, 1993). However, the IARC has discussed the hazards associated with radioactive metals for which unequivocal risk of carcinogenicity is indicated in experimental studies in rodents (Litvinov and Soloviev, 1990).

External local or whole-body radiation with α-, β- or γ-emitters is a potential cause of osteogenic or osteoblastic sarcoma in rats (Litvinov, 1964; Litvinov and Soloviev, 1990). Osteogenic tumours were reported in rats exposed orally, parenterally or by intraosseous route to bone-seeking radionuclides including phosphorus (^{32}P), calcium (^{45}Ca), strontium (^{89,90}Sr), barium (^{149}Ba), radium (^{226}Ra) and uranium (^{238}U), which by their systemic nature appear to evoke pathogenic and potentially neoplastic changes in all bones of the skeletal system (Litvinov and Soloviev, 1990). Radiation-induced tumours commonly occur in the regions of active endochondrial ossification at the interface between the metaphysis and diaphyseal regions of immature long bones. Changes tend to be more prevalent in spongy bone where the metabolically active tissues of the endosteum, periosteum and around Haversian canals are particularly vulnerable to β-emissions. A typical example is seen in a study where young Wistar rats were exposed to 3.7 MBq strontium (^{90}Sr) and developed pathological changes in areas of medullary osteogenic sarcoma within 8–10 months of intramuscular injection (Graf *et al*., 1970). In early carcinogenesis, normal osteogenic profiles were markedly disturbed and retarded, but bone formation predominated over resorption. Progressive degenerative and dysplastic changes preceded frank neoplasia, manifest by abnormal bone trabeculae and osteoid, with deposits of dystrophic collagen and amorphous protein.

Induction and severity of carcinogenic change is directly related to the radioactivity emitted by the element administered, exposure time, and the age and physiological activity of the bone area exposed. At high doses, β-emitting isotopes are potentially fatal to animals before tumours develop. Luz and Gössner (1994) noted that the majority of α-particle induced osteosarcomas in mice are of vertebral origin, but unusually in the case of β-emitter ^{90}Sr exposure, tumours tend to be spinal following high doses of radiation, and predominantly in diaphyseal regions of long bones at low levels (Nilsson, 1962; Nilsson, 1970). Occasionally, these murine osteosarcomas induced by ^{90}Sr incorporate a chondroblastic element which is rarely the case with α-emitters. The mechanism is unclear.

Bone tumours have been observed less frequently following parenteral or oral administration of nonradioactive carcinogens, including 7,12-dimethylbenzo[b]phenanthrene (DMBA), 1-(2-hydroxyethy)-1-nitrosourea, vinyl chloride and 3,3-dichlorobenzene (Mirvish *et al*., 1987; Viola *et al*., 1971; Pliss, 1965). Waalkes *et al*. (1991) have extensively reviewed the carcinogenicity of cadmium in experimental and human studies. They reported bone tumours (mainly osteogenic sarcomas) in young Fischer F344 rats following single subcutaneous injections (30–70 mM) of cadmium chloride). In other

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experiments, they reported osteogenic sarcomas induced in the ear cartilages of 14 of 168 Wistar rats which had nickel and copper ear tags inserted (Waalkes *et al*., 1987). Nickel is an acknowledged soft-tissue carcinogen, whereas copper is an essential trace metal with a role in cell proliferation and collagenesis (US DHHS, 2005). Although the work has not been repeated, it suggests that carcinogenesis in this case is in a two-stage form, where nickel is the inducer and copper is the promoter. In other respects, bone tumour promotion in rats at least is achieved by local external irradiation and/or by administration of growth hormone or thyroxine (Litvinov and Soloviev, 1990).

Beryllium is a nonmetallic element known to induce tumours in experimental animals and following occupational exposures in humans (Berlin *et al*., 1994). In the latter situation, lung tumours are a particular hazard (Smith and Suzuki, 1980), but osteosarcomas are reported in rabbits following intravenous or intramedullary administration of metallic beryllium, or beryllium oxide, carbonate, phosphate or silicate, and zinc beryllium silicate (Fodor, 1977). Beryllium oxide injected intravenously (1%) in rabbits was shown to concentrate in bone marrow throughout the skeletal system and cause local haematopoietic damage. Irregular osteogenesis with fibrosis was reported prior to appearance of pleomorphic medullary tumour formation in metaphyseal regions of long bones (Fodor, 1977). There was little evidence of osteoclastic activity, and bone breakdown was minimal. No human cases of beryllium-induced bone tumours have been seen.

The possible mechanism for osteosarcoma induction in rats by separating metaphyseal growth plate regions from periosteal tissues by cellophane film is unclear (Korobko, 1966; Studitski, 1966); it is unclear whether the osteosarcomas were induced by the cellophane film *per se,* or by the mere physical act of disturbing tissue 'balances'. The carcinogenicity and mutagenicity of substances leaching from the cellophane film remains to be ratified.

More direct evidence for the carcinogenicity of xenobiotic materials in bone or cartilage is provided by experimental studies where test materials have been instilled or injected into the medullary cavity of long bones or into articular cartilages of rats. Thus, the multifocal carcinogens DMBA and benzo[a]pyrene induced high yields of osteogenic sarcomas when injected into the medullary cavity of femurs (Litvinov and Soloviev, 1990; Moritani, 1977). Further, this tumour incidence was promoted by feeding animals rachitogenic (low vitamin D) diets. Other experiments demonstrate that intraosseous administration of the carcinogenic metals chromium and nickel can be a cause of osteosarcoma (Guelstein, 1965). A copper-chelated *N*-hydroxy-2-acetyl aminofluorine administered in a similar manner into rat femur was shown to evoke osteosarcoma, chondrosarcoma and haemangiosarcoma (Stanton, 1967).

In an attempt to identify the carcinogenic properties of metal alloys used in prosthetic devices, Lewis *et al*. (1995) and Sunderman (1989) administered metal powders by intra-articular route in Fischer F344 rats. Whereas no neoplastic activity was identified in animals treated with cobalt–chromium–molybdenum (CoCrMo) or titanium–aluminium–vanadium (TiAlV) complexes, either at injection sites or at distant locations, poorly developed primary pleomorphic sarcomas were seen in animals injected with nickel subsulfide. (Nickel subsulfide is listed as a material for which sufficient evidence exists for carcinogenicity (respiratory tract) to humans through industrial exposure (US DHHS, 2005)). Sunderman (1989) considered that sarcomas at implantation sites were a potential, albeit rare, problem with implantation of prostheses, and that surgeons should select prostheses on the basis that they are not subject to corrosion or mechanical failure and are safe in the presence of human tissue.

Whereas there is still no conclusive evidence for the carcinogenicity of cadmium in human bone or cartilage, Friberg *et al*. (1974) reported that over 20% of women aged over 50 years in the Jintzu River region of Japan developed leg pains and bone tenderness following exposure to high cadmium levels in soil. Their bones were deformed and susceptible to fracture, possibly due to serious malnutrition, but cadmium toxicity is probably implicated. Cadmium is a cause of chondrodystrophy and abnormal bone in embryonic and neonatal skeletal structures (Mulvihill *et al*., 1970; Holmberg and Ferm, 1969). More recent studies of cadmium poisoning in the Shipham (UK) area have failed to demonstrate the carcinogenicity of cadmium in bone/cartilage at greatly increased body cadmium levels (Philipp, 1985).

8 DISCUSSION

Cartilage and bone are vulnerable to toxic damage and structural deformation at any stage from early prenatal life through to adulthood. Toxic agents targeting skeletal tissue may exert their influence by a direct action on vulnerable cells at periods of high proliferative activity or differentiation, or they may influence their local environment by impairing nutrients or regulatory mechanisms modulated by hormones, cytokines and growth factors. The damage or structural changes seen in each case reflect the nature and severity of the toxic insult, its target tissue(s) and biosynthetic pathways, and the age and developmental state of the individual. Commonly, toxic changes or structural defects arising prenatally in periods of maximal differentiation can be expected to be reflected in subsequent phases of development and the definitive structure and function of the musculoskeletal system in the adult. Deformities in long bone, vertebral and skull development induced at later stages will

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more frequently involve transitory or progressive alterations in mineral metabolism and the functional capacity of the tissues. Carcinogenic changes in bone and cartilage are not widely discussed, but are a recurrent problem in people exposed occupationally and environmentally to certain bone-seeking radioisotopes and toxic metals like beryllium. These substances are mutogenic and bind strongly to the hydroxyapatite moiety of the bone matrix.

My long-standing interests in mineral metabolism and in the action and interaction of metals in biological systems lead me to conclude that many toxic agents, potentially toxic to bone and possibly cartilage at some stage, act through blocking the availability and metabolism of key minerals such as calcium, zinc and magnesium. Bivalent metals, in particular, interact and compete for cellular receptors at sites of gastrointestinal absorption, carrier proteins in the circulation, and on cell membranes. Imbalances in trace metal ions, attributable to impairment in absorption, blockage of metabolic channels by ionic interaction or inactivation of enzyme systems, are potential causes of developmental abnormality, functional and pathological change. Investigative studies in intact and *in vitro* cell systems show that cartilage and matrix vesicles play a central role in mineralization, and that agents like salicylates and cadmium which impair mucopolysaccharide synthesis and chondrocyte maturation are causes of defective mineralization (Fleisch *et al*., 1975).

The ability of bone to concentrate toxic metals like lead without exhibiting obvious pathological changes, except following chronic exposure, has several clinical complications. Bone provides a reservoir from which toxic agents are released to invoke injury in vulnerable soft tissues, even though mechanisms of metal binding are frequently unclear and the circumstances and modulation of ion release into the systemic circulation are equivocal. Lead, for example, has been extensively studied as an environmental contaminant and as a cumulative neurotoxin. It shows a strong tendency to accumulate in bone and is released progressively with potential risks of neurodegeneration with impaired intellectual development (Moore, 1979; Carson *et al*., 1974). Further studies are required to evaluate the metabolic and toxic consequences of chronic exposure to aluminium, strontium and other environmental metals on long-term bone damage using newer and more sensitive analytical techniques.

The implications of silicon in collagenesis and bone formation are unclear. Although silicon is regarded by some regulatory authorities as an essential trace element, published views on its biochemical role in the body are based on research conducted 50 years ago (Carlisle, 1982; 1988; Lansdown and Williams, 2007). Presently, although nutritionists list silicon as a trace metal, accurate daily requirements are not known. A second contentious nutrient is strontium. Daily requirements for human health are not documented and its role in bone metabolism is equivocal. Interestingly, a strontium salt (strontium ranelate) (Protelos®, Servier Laboratories Ltd.) is listed as a therapeutic means of reducing bone resorption in patients with postmenopausal osteoporosis and to reduce the risk of vertebral injury and hip fracture (BNF, 2007).

Review of the literature suggests that the full extent of toxic injury to bone and cartilage is not fully appreciated and that information is widely scattered and incomplete. Whilst it is common practice to analyse skeletal growth and development in teratological studies by alizarin-staining techniques, structural and functional damage in bone and cartilage in medium to long-term toxicological studies is rarely analysed. Histological examination in representative long bones will show up evidence of osteoid resorption or rarefaction in cartilage and bone matrix, as characterized by parathyroid disease or hypovitaminosis D, but more subtle changes in mineral content are not appreciated other than through bone ashing and analytical studies using atomic absorption spectrometry. This suggests that guidelines in routine toxicological investigation might be revised as and when biochemical evidence suggests that an environmental substance is likely to target bone or bone mineral metabolism.

Clinical studies of cartilage and bone largely concentrate on age-related and genetically induced deformities and implications of hormonal changes leading to osteoporosis. Development of thyrocalcitonin-like drugs to alleviate reduction in bone mass in conditions like Paget's disease is ongoing, and therapies including salcatonin (a synthetic or recombinant salmon calcitonin) have been introduced to lower plasma calcium levels in patients with hypercalcaemia or postmenopausal osteoporosis (BNF, 2007). Other drugs available include teriparatide (a recombinant form of parathyroid hormone) and cinacalcet (Mimpara®, Amgen Ltd.). A further category of therapies aimed at reducing bone mass include the bisulfonates, which are adsorbed to the hydroxyapatite matrix and act to stabilize the rate of bone growth and inhibit mineral turnover in osteoporotic states.

Studies of clinical bone disease and mechanisms of toxicology affecting skeletal tissue will continue to be of interest to palaeontologists studying the prevalence of diseases in ancient civilizations. Not only have the observations of ancient bones retrieved from the Qumran region near the Dead Sea revealed the dietary habits of people living in the area at the possible time of writing of the Dead Sea Scrolls, but they have helped to identify environmental conditions and prevalence of disease affecting bone at the time, as much as modern day orthopaedic clinicians and laboratory scientists (Steckoll *et al*., 1971). The red dye from the madder plant (Ca^{2+} *Rubia tinctorum*) is recorded as being one of the most important natural dyestuffs in the ancient Mediterranean region (Löw, 1924). Other analyses of ancient bones have illustrated patterns of exposure to toxic materials like lead and cadmium in early times. It is interesting to see from

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the limited information available that exposure to these elements was many fold lower than that seen in more recent analyses.

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Toxicology of the Endocrine System

Robert W. Kapp Jr. and John A. Thomas

CONTENTS

1 INTRODUCTION

Information flow which permits physiological responses between cells, tissues and organs is critical to survival and homeostasis of living organisms. The primary pathways for these physiological data are the nervous and endocrine systems. These pathways operate independently and cooperatively in many complex ways in order to optimize the organism's ability to function. The nervous system is usually directly connected to the affected organ and sends electrical impulses directly to the affected organ in much the same way a hard-wired landline telephone would send signals. The nervous system controls actions that need immediate attention and are rapid in nature, such as heartbeat, skeletal movements and breathing. The endocrine system is generally not directly connected to the affected organ and sends chemical messages over a distance as a mobile telephone would send and receive signals over a distance. The hormone gland produces a discreet chemical message that can be read by a specific chemical receptor in the regulated organ at some distance away, which switches a particular activity on or off. The endocrine system controls actions that are usually slower and are critical,

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but do not need immediate attention, such as cellular growth and development and metabolism.

The concept of the distinction between endocrine and nervous system controls has become less rigid in recent years as we have gained knowledge about multicellular organism physiology. Over the years it has been shown that the chemical and nervous system control of bodily functions works in concert with the immune system to maintain a delicately balanced 'milieu intérior' or homeostasis (Kapp and Thomas, 2008). There are many areas of crossover, where both systems have some influence. For instance, important controlling endocrine glands, such as the hypothalamus, are derived from neural tissue. The hypothalamus produces antidiuretic hormone (ADH) and oxytocin and various hypothalamic releasing hormones—which are considered to be neural hormones, since the hypothalamus is derived from neural tissue. Also, the adrenal medulla is neurological in origin and its chromaffin cells produce norepinephrine (noradrenaline), which is chemically identical to the norepinephrine produced by nervous system transmitters. Catecholamine receptors located throughout the body in various tissues react the same to norepinephrine no matter where it originated. Further distinctions can be noted in the simple definition of a hormone as a substance produced by a ductless gland, secreted into the blood to be carried to some distant target to produce an effect. This is true for a vast majority of hormones, however, it is now known that some hormones act within the very tissue that they regulate and circumvent the need to move through the blood stream. Hormones that act upon the same secretory cells are termed autocrines while those that act upon adjacent cells are termed paracrines.

Hormones are very specific in nature binding to a specific receptor in much the same way a key fits into a lock. At any place and time there may be a multitude of hormones or 'keys' present at the location of the 'locks', but only one specific hormone fits and activates a hormone receptor. Therefore, even though hormones reach all parts of the body, only target cells with compatible receptors are equipped to respond. Once a receptor and a hormone bind, the receptor carries out the hormone's instructions, generally by altering the cell's existing proteins, building new proteins or turning off production of new proteins. These actions create hormonally mediated chemical reactions throughout the body and maintain internal stability for survival. The endocrine system regulates virtually all critical biological processes from conception through to old age, including, but not limited to, metabolism, development of the brain, nervous system, bone structure, reproductive glands, reproductive function, blood sugar levels, water and electrolyte balance, red blood cell production and control of functions such as circulation, digestion and adsorption.

Over 50 hormones have been specifically identified in mammals to date and there are probably others that have not yet been identified (US EPA, 2006). There are several categories of hormones, including bioamine-derived, polypeptides, proteins, steroids and fatty acids (Kapp and Thomas, 2008). These hormone classes differ in their general molecular structures, which changes their chemical properties in such a way as to affect whether or not the hormone can enter a target cell and how they can modulate the various activities of that cell. Steroid and fatty-acid molecules enter the target cell's cytoplasm or the nucleus by virtue of their compatible chemical properties. The subsequent hormone–receptor complex binds with the appropriate nuclear DNA and results in the regulation of specific hormone responsive genes. Bioamine-derived hormones are similar to some of the basic proteins and can enter the target cell, where they can interact with specific receptor proteins located at specific sites within the nuclear DNA. Polypeptide and protein hormones are chains of amino acids of varying length, which cannot enter the cell as previously described, but regulate the cellular activity by interacting with specific cell-surface receptors (Hiller-Strumhöfel and Bartke, 1998). Signal transduction by cell-membrane-bound receptors occurs either via intracellular second messengers (e.g. cyclic adenosine monophosphate (cAMP), calcium or phosphatidylinositol metabolites), or through mechanisms such as phosphorylation of serine, threonine or tyrosine residues of intracellular kinases and other enzymes (Leung and Steele, 1992).

2 CONTROL OF HORMONAL ACTIVITY

In order to maintain homeostasis, the mechanisms that control the production and secretion of hormones must be closely managed within a very tight range. To achieve this, many hormones are produced that not only regulate various metabolic functions, but regulate each other in complex negative feedback systems. Transport of hormones to target tissues can involve carrier plasma proteins such as sex-hormone-binding protein (SHBP) that specifically, and with high affinity, bind the hormone while it is transported through the circulatory system. These carrier proteins can be a limiting factor in that they often possess low capacities (Grishkovskaya *et al.*, 2000). Albumin, on the other hand, has a high capacity to bind hormones for transport, but has low specificity, hence limiting the transport of some biogenic amines, steroids, thyroid and some polypeptide hormones.

Once a hormone has been transported to the target by the carrier proteins, it must interact with the receptor at the target site to have a biologic effect. In order to have a biologic effect, the hormone must become unbound from the carrier protein. A bound hormone is not able to exert an effect at the receptor site, membrane surface or present in the cytoplasm. In order to permit exposure of the needed amount of hormone, there exists a delicate

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equilibrium among the bound hormone (B), the plasma protein (P) and unbound or free hormone (F) which is expressed in the following equation (Porterfield, 2001):

$$F \times P = [B] = [F] \times [P] \div [B] \quad (1)$$

The free hormone is the critical moiety for activation, as well as for deactivation in feedback control. Although there is some evidence to suggest that hormonal receptor internalization may play a role in some processes, most polypeptide hormones interact with cell-membrane-surface receptors. In contrast, steroid and thyroid hormones interact with intracellular receptors. Transport into the cell may be aided by cell membrane transporters (e.g. megalin), and once inside the cell, the steroid or thyroid hormone receptor complexes are transported to the nucleus, where they may modulate gene expression by binding to certain DNA regulatory sequences.

Overall, the endocrine system utilizes two primary control systems to respond to physiological changes: (i) internal controls from the various concentrations of the hormones themselves and (ii) nervous system override of the self-regulating chemical system. The primary function of the nervous control override is to respond to external stimuli (e.g. fight-or-flight-type situations), which results in a hormonal readjustment. The continuous self-regulation is a complex physiologic hierarchy of chemically mediated controls which modulates the homeostasis of the endocrine system through a series of negative feedback loops, where the output of a hormone controls the input in a simple inverse relationship. As the concentration of the hormone increases in the system, the hormone itself decreases the call for the hormone, in much the same way as a thermostat regulates room temperature. As the temperature increases, the thermostat turns off the heating unit until the temperature drops to a predetermined point, whereupon the signal is once again sent to the heating unit to increase heat. In its simplest form, hormonal regulation is the involvement of a single endocrine gland, which is activated and deactivated as in this thermostat example. On the other hand, complex hormonal regulation is another level of control which includes the 'master endocrine gland' or anterior pituitary (adenohypophysis), as well as the target receptors. The anterior pituitary is frequently referred to as the 'master endocrine gland' because it controls a number of other glands and target cells throughout the body. For example, the anterior pituitary produces a number of hormones, including trophic hormones that regulate the activity of other endocrine glands such as thyroid-stimulating hormone (TSH), which regulates the thyroid gland, and adrenocorticotrophic hormone (ACTH), which regulates the adrenal cortex. Also included as trophic hormones are luteinizing hormone (LH) and follicle-stimulating hormone (FSH) which regulate the release of steroid hormone in the ovaries and testes and stimulate the maturation of eggs and sperm, respectively. The secretory releases of these endocrine glands feed back to decrease the hormonal releases of the previously noted hormones, thereby regulating the system to within a narrow range needed for normal homeostasis. A negative feedback loop inhibits an endocrine pathway whereas a positive feedback loop enhances or augments an endocrine response. Hence, the constant feedback from the target organ glands to the hypothalamus and pituitary ensures that the level of hormone governing hormonal activity remains within the boundaries necessary for homeostasis. Interestingly, these negative feedback boundaries can change at different physiological stages in life, thus creating varying sensitivities to hormone levels to permit—for instance—sexual maturation. On the other hand, positive feedback boundaries and sensitivities are altered in female menstrual cycles where increasing oestrogen levels temporarily stimulate, rather than inhibit, LH release from the pituitary and hypothalamus, resulting in enhanced oestrogen levels leading to ovulation, the formation of the corpus luteum and progesterone release. The general relationships within the adenohypophyseal-hypothalmic axis are depicted schematically in **Figure 1**.

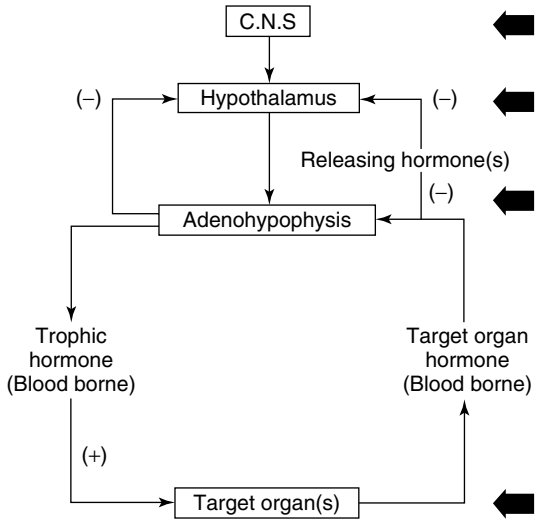

Figure 1 Hormonal feedback system between the adenohypophysis and endocrine target organs: (+) sign denotes hormonal stimulation, (–) sign denotes an inhibitory action and (←) arrows denote common sites of potential interference by toxic agents.

That the concentration of a hormone can be determined by three critical factors: (i) rate of production, (ii) rate of delivery and (iii) rate of elimination. The rate of hormone production is affected by many factors, and is controlled by feedback loops. Feedback loops can further be modulated by other endocrine systems that are pulsatile, cyclical or are stimulated through other mechanisms. The integration of various feedback loops gives rise to a complex cascade of endocrine

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responses to particular stimuli. The rate of hormone delivery is affected by the rate of blood flow to the target receptor. Lastly, the rate of elimination is affected by two factors: (i) the body's ability to metabolize and excrete the hormone from the bloodstream and through renal elimination and (ii) the inherent physiological rate of degradation of the hormone. Some hormones have short biological half-lives while others have long biological half-lives. Protein binding can affect a hormone residence time in the blood. The constant feedback from the target organ glands to the hypothalamus and pituitary gland creates a steady state where the given hormone remains within appropriate boundaries. Therefore, the negative feedback system generally operates, whereby hormones that are released by the target organ glands reach a predetermined level, they essentially affect a cessation of hormone release by the hypothalamus or pituitary.

It has been shown that the physiological state of the organism can change the level of the hormone trigger or the sensitivity of the negative feedback system. For instance, sexual maturation occurs with the successive reduction in sensitivity of both the hypothalamus and the pituitary to the natural gonadal steroid hormones resulting in enhanced gonadal hormone release.

3 ENDOCRINE DISRUPTION

Some drugs and chemicals have been shown to affect the endocrine system in ways that can lead to metabolic disruptions, developmental anomalies and reproductive dysfunction (see **Endocrine Disruption in Toxic Responses**; Gornall *et al*., 1986; Thomas, 1996). An endocrine disruptor is defined as 'an exogenous agent that interferes with the production, release, transport, metabolism, binding, action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes' (Kavlock, 1999; US EPA, 2008). With this recognition of the effects of oestrogen-like environmental chemicals in the environment, the potential for detrimental changes to the endocrine system in intact animals could be of considerable concern. These exposures could result in perturbations to the finely tuned endocrine system that could bring about changes in maturation, development and regulation of various critical bodily functions that would put the organism at risk for survival. It is believed that there are four categories of adverse effects that could be mediated by endocrine disruptors, including cancer, immunological effects, neurological effects, and reproductive and developmental effects (US EPA, 1996).

There appears to be ample evidence that endocrine disruptors have affected changes in laboratory animals, as well as selected animals in the wild such as birds, alligators and so on (Aulerich *et al*., 1973; Broley, 1958; Gilbertson *et al*., 1991; Guillette *et al*., 1995; Mason *et al*., 1986). The effects in humans have not been as clearly elucidated and are scientifically controversial (Kavlock, 1999). In a recent publication, the US Environmental Protection Agency (EPA) stated: 'The seriousness of the endocrine disruptor hypothesis and the many scientific uncertainties associated with the issue are sufficient to warrant a coordinated federal research effort' (US EPA, 2006). It is anticipated that future developments in endocrine disruption detection will include additional hormone assays that may be added to standard hormonal assays.

4 ENDOCRINE MODULATION

Specific target organs of the endocrine system in addition to the foetus and the neonate can by affected by external chemicals and drugs (Thomas, 1989). Many of these target organs are modulated by secretions from the anterior pituitary, which can also be perturbed by external chemicals. Various chemicals have been shown to be toxic to the female reproductive system and male accessory sex organs. Organs such as the thyroid gland, the adrenal gland, the testes, the ovaries and the pancreas can also be affected by certain external chemicals and drugs. Drugs and synthetic steroids have been shown to alter glandular internal secretions directly or by modifications in the level of trophic hormone secretion from the adenohypophysis. Unanticipated effects of drugs on an endocrine organ can interfere with the assessment of a diagnostic test. Approximately 90% of endocrine toxicities appear to occur in the adrenal gland, the gonads and the thyroid gland (Ribelin, 1984; Colby, 1988; Thomas and Thomas, 2000). Some endocrine target organs have specific localized cell populations that appear to be more susceptible to toxins than others and may lead to multiple changes in hormonal balance. Chemically induced modifications in pituitary function can affect target end-organs. Similarly, chemically induced changes in hepatic microsomal enzyme systems can alter the secretions of sex steroids (Thomas and Thomas, 2000).

Toxic effects on gonads and teratogenesis are important aspects of endocrine aberrations brought about by external chemicals and drugs (Thomas and Thomas, 2000). It has been shown that reproductive toxicity (see **Developmental Neurotoxicity**) and developmental toxicity (see **Developmental Toxicology**) often involve the endocrine system. It should be noted however, that there are other nonreproductive endocrine target organs that can be affected by external chemicals and drugs. Chemicals and drugs can adversely alter the thyroid, adrenals, testes, ovaries and pancreas internal secretions either directly with their internal secretions or by interfering with trophic hormone secretion by the adenohypophysis.

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Table 1 Hormone assay and endocrine functions for the trophic hormones of the anterior pituitary gland and their respective hypothalamic releasing hormones

Adenohypophyseal trophic hormones		Hypothalamic releasing hor mones	
Abbreviation	Full identification	Abbreviation	Full identification
ACTH	Adrenocorticotrophic hormone	CRH	Corticotrophic-releasing hormone
		VP	Vasopressin
TSH	Thyroid-stimulating hormone	TRH	Thyrotrophin-releasing hormone
FSH	Follicle-stimulating hormone	FHR	Follicle-stimulating hormone-releasing hormone
LH	Luteinising hormone	LRH	Luteinizing hormone-releasing hormone
GH	Growth hormone	SRH,GRH	Somatotrophin-releasing hormone
		SRIF[a], GIH	Somatotrophin-release inhibiting factors
Prl	Prolactin	PIH	Prolactin-inhibiting hormone
		PRH	Prolactin-releasing hormone
MSH	Melanocyte-stimulating hormone	MRH	Melanocyte-stimulating hormone-releasing hormone
		MRIH	Melanocyte release-inhibiting hormone

[a]Somatostatin.
Source: Thomas and Thomas (2000).

Because of the fact that many chemicals can change the adenohypophyseal hormone releases, it is important to take that into account when assessing pituitary function. Drug-induced changes in pituitary function tests can lead to misinterpretations and therapeutic mistakes (Thomas and Thomas, 2000).

Chemically induced changes in sex steroids can affect pancreatic secretions of insulin. Stress results in an increased secretion of glucocorticoids and can also affect changes in insulin secretions and can affect a decrease in ACTH levels. The pituitary gonadotrophins FSH and LH can be altered by agents that act directly on the testes and the ovaries.

Some drug-induced changes in the endocrine system balance can be therapeutic, such as use of synthetic progestational steroids to suppress pituitary gonadotrophins, which affords millions of women with a chemical means of birth control. Similarly, the suppression of excessive endogenous hormone secretion can be exploited therapeutically, as in the case of synthetic adrenocorticoids reducing ACTH levels.

Insight into pituitary target organ feedback systems is of value when attempting to predict the effects of potentially toxic agents on specific endocrine target organs (Thomas and Keenan, 1986). Generally, chemically induced changes that affect pituitary/target organ relationships are seldom manifest after a single exposure to a toxic agent. Rather, compounds that can exert deleterious effects upon the endocrine system generally require longer exposure scenarios, usually coupled with repeated administrations. For instance, agents that cause hepatic microsomal enzyme induction usually require seven or more days of exposure before affecting a measurable change in the endocrine system.

Hormones of the adenohypophysis and their hypothalamic releasing hormones are shown in **Table 1**. The trophic hormones being released from the adenohypophysis are relatively high-molecular-weight substances that are frequently proteins or glycoproteins. These glycoprotein hormones are often involved in the modulation of metabolism and reproduction.

Other specific endocrine cell types found in the adenohypophysis are responsible for the synthesis of the trophic hormones such as corticotrophs (ACTH), gonadotrophs (FSH and LH), thyrotrophs (TSH), somatotrophs (growth hormone (GH)) and lactotrophs (prolactin (Prl)). It has been shown that there are a number of environmental factors which can affect hypothalamus activity, such as light, darkness, temperature variations and stress. These factors can produce either increases or decreases in trophic hormone secretions from the adenohypophysis, which include both long-loop hormonal feedback systems (i.e. individual target organ secretions) and short-loop feedback systems (i.e. the trophic hormone itself affects its own secretion by modulating hypothalamic or inhibiting factors (see **Figure 1**).

Chemicals, including certain classes of therapeutic drugs, can interfere with the release of trophic hormones

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or can perturb their synthesis (Thomas and Thomas, 2000). Other agents exert inhibiting effects at various sites within the adenohypophyseal target-organ system, resulting in the inhibition of the biosynthesis of target-organ hormone secretions. The sensitivity of the each site of action varies depending upon the concentration and toxic moiety in play. For example, environmental stress can and does affect the secretory activity of certain hypothalamic releasing hormones, which subsequently alter pituitary–target-organ relationships. Rapidly dividing meiotic cells in the gonads are frequently more vulnerable to chemical changes than slower dividing somatic cells. Indirectly, some toxic agents are known to bind to circulating blood proteins, which results in alterations in the critical ratios of bound and unbound levels of target organ hormones, subsequently modifying the sensitive pituitary–target-organ relationship.

The physiological hierarchy that exists within the endocrine system is a series of complicated and interwoven feedback loops that constantly regulate each other. The feedback is the modulation of output by an end-product, such as a hormone or a metabolite. Other hormonal loops can be modulated leading to pulsatile, cyclic waves, such as hormonal diurnal rhythms. The integration of all these sundry feedback loops give rise to a complex cascade of endocrine responses which can be difficult to separate, but act in concert with one another to maintain homeostasis.

One of the complex feedback systems within the body is the hypothalamic-pituitary-adrenal (HTPA) axis. The HTPA axis is a complex set of direct influences and feedback interactions between the hypothalamus, the pituitary gland and the adrenal or suprarenal gland that controls reactions to stress and regulates an assortment of body processes, including digestion, the immune system, sexuality and behaviour. The paraventricular nucleus of the hypothalamus produces ADH (which is also known as vasopressin) and corticotrophin-releasing hormone (CRH). ADH is a water-conservation hormone and acts as an antidiuretic. It is released when the body is dehydrated and has significant water-conserving effects on the kidneys. It is also a potent vasoconstrictor. The release of CRH is affected by stress, the concentration of cortisol and also by the diurnal cycle. The level of cortisol rises quickly upon wakening. The concentration of cortisol peaks within an hour and then slowly drops over the course of the day until it stabilizes at its lowest concentration during the middle of the sleep cycle. Aberrations in the diurnal cortisol cycles have been associated with insomnia (Backhaus *et al*., 2004) and chronic fatigue syndrome (MacHale *et al*., 1998). Cortisol appears to act within the brain at mineralocorticoid and glycocorticoid receptors, as well as the hippocampus, which is an important controlling factor in the HTPA axis. CRH and ADH act synergistically to regulate the anterior pituitary by stimulating the secretion of ACTH from the corticotrope cells. ACTH in turn stimulates the secretion of glucocorticoids (primarily cortisol biosynthesis from cholesterol). The cortisol inhibits, via a negative feedback mechanism, both the hypothalamus and the pituitary gland, subsequently reducing the production of CRH and ADH. Interestingly, the local effects of cortisol include up-regulation of enzymes which are precursors to the synthesis of epinephrine (adrenaline) and norepinephrine, resulting in a positive feedback to the pituitary increasing the breakdown of proopiomelanocortins (POMCs) into ACTH.

Another feedback system is the hypothalamic-pituitary-thyroid (HPT) axis, which regulates metabolism via feedback loops between the hypothalamus, the pituitary and the thyroid gland. The hypothalamus senses low circulating levels of thyroid hormone and responds by releasing thyrotrophin-releasing hormone (TRH), stimulating the pituitary to synthesize TSH, which ultimately stimulates the thyroid to produce thyroid hormone. The thyroid hormone itself then turns off the production system at levels that are within the range sensed as normal by the hypothalamus.

The hypothalamic-pituitary-gonadal (HPG) axis is yet another feedback system that combines the effects of the hypothalamus, the pituitary gland and the gonads and is a critical part in the development and regulation of various organs, such as the reproductive and immune systems. The HPG axis is regulated normally through negative feedback by hydrophobic and hydrophilic hormones. Gonadotrophin-releasing hormone (GnRH) is secreted from the parvicellular neurons which ultimately reaches the anterior pituitary triggering the gonadotrophs to release LH and FSH. FSH and LH in turn act directly upon the gonads producing sex hormones. Hence, this hormonal release within the HPG axis is also a negative feedback mechanism between the hypothalamus, the pituitary and the gonads.

The classic concept of the endocrine system is that endocrine cells release a hormone that is transported to a receptor site in a target tissue, where the hormone binds to the receptor site and exerts a biological effect. Endocrine systems in general encompass those tissues that release a hormone that is transported through the bloodstream to a receptor cell in a target tissue. However, the advent of new techniques in tissue cell cultures and molecular biology has permitted the identification of several intercellular signalling pathways that do not transport hormones via the general circulatory system to the target tissue. Examples of paracrine systems are various growth factors and inflammatory mediators such as arachidonic acid metabolites, complement factors, cytokines, clotting factors and somatostatin. Examples of autocrine systems include cytokine interleukin-1 found in monocytes and peripheral membrane protein in T cells.

Many classification schemes have been devised for describing hormones. Some classify hormones by their

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Table 2 Categories of hormones

Category	Example(s)
Biogenic amines	Epinephrine
Polypeptides	Thyroid-releasing hormone
Proteins	Insulin, growth hormone
Steroids	Oestrogens, androgens
Thyroid hormones	Thyroxine
Fatty acids	Prostaglandins

chemical source or according to their solubility characteristics, and others define hormones according to chemical composition (Baxter *et al*., 2004; Porterfield, 2001). One classification system that is recognized in the literature divides hormones into six general chemical categories, including biogenic amine hormones, polypeptide hormones, protein hormones, steroid/sterol hormones and fatty-acid hormones (see **Table 2**).

Bioamine-derived hormones include catecholamines such as epinephrine, norepinephrine, serotonin (indoleamine) and histamine (imidazoleamine). They are hydrophilic in nature and are similar to compounds found in the nervous system that can exert local effects in tissues. Biogenic amines are small, biologically active, modified single amino acids. They act primarily as neurotransmitters and are capable of affecting mental functioning.

Polypeptide hormones are short amino-acid chains, while protein hormones are composed of many amino acids and polypeptides together in a single molecule and are generally hydrophilic in nature. These types of hormones can range in length from three amino acids (e.g. TRH) to lengths of just under 200 amino acids (e.g. GH). They can be composed of two or more subunits (e.g. gonadotrophins) or may be linked by disulfide bonds (e.g. insulin). These hormones are synthesized in the endoplasmic reticulum.

Steroid/sterol hormones are derived from cholesterol, contain a skeleton with a cyclopentanoperhydrophenanthrene ring (four connected rings) structure and are hydrophobic in nature. Steroid hormones are generally synthesized in the adrenal cortex, the placenta and the gonads. Because they are fat soluble, these hormones can readily cross cell membranes and have intracellular receptors, which assist in the transport across the membrane. Once into the cytoplasm, steroid hormones bind to specific metalloprotein receptors forming a steroid–receptor ligand complex, which permits further migration into the nucleus. Upon penetration into the nucleus, the ligand complex binds to specific DNA sequences and induces transcription of its target genes. Examples of steroid hormones include the sex steroid hormones, corticosteroids, mineralocorticoids, vitamin D and retinoic acid. Recent evidence suggests that megalin, a member of the low-density-lipoprotein receptor superfamily of endocytic proteins, is a critical facilitator of steroid entry into cells (Birn *et al*., 2005).

Thyroid hormones are intermediate molecular-sized group of hormones that fall in between biogenic amine hormones, and short polypeptide hormones. Thyroxine (T_4) and triiodothyronine (T_3), are tyrosine-based hormones produced by the thyroid gland. Thyroxine contains four iodine atoms and is produced by attaching iodine atoms to the ring structures of tyrosine molecules. Thyroxine is converted to the active T_3 (three to four times more potent than T_4) within cells by deiodinases (5′-iodinase). The major form of thyroid hormone in the blood is thyroxine (T_4). The ratio of T_4 to T_3 released in the blood is roughly 20 to 1. The thyroid hormones are further processed by decarboxylation and deiodination to produce iodothyronamine (T_1a) and thyronamine (T_0a).

Fatty-acid hormones or eicosanoids are generally derived from polyunsaturated fatty acids such as linoleic acid and phospholipids, with the most prominent of these being arachadonic acid. Eicosanoids are made by oxygenation of 20-carbon essential fatty acids (EFAs)—either omega-3 ($\omega - 3$) or omega-6 ($\omega - 6$). They exert complex control over many bodily systems, mainly in inflammation or immunity. Other groups of hormones in the fatty-acid class include prostaglandins, prostacyclin, leukotrienes and thromboxanes, lipoxins, isoprostanoids and hydroxylated fatty acids (Soberman and Christmas, 2003). They are synthesized throughout the body and their primary effects are paracrine and autocrine in nature through both surface and nuclear receptors (Porterfield, 2001). These hormones are rapidly inactivated by being metabolized, and are typically active for only a few seconds.

5 BIOCHEMICAL ASSESSMENT

Assessing endocrine function is an important aspect of determining the mechanisms involved with endocrine toxicity. Measurement of hormonal metabolite levels in blood can reveal important information regarding the site of endocrine toxicity. Potential sites of toxicity include the site of hormone synthesis and release, the receptor site, the intracellular signalling pathway sites, the entire pathway of transport from the endocrine gland to the target cell, including the hormone carrier proteins and other factors which could alter the bound status of the hormone during transport and/or release.

Endocrine system evaluation may reveal much about the pathophysiology of endocrine toxicology, yet biochemical assessment methods have limitations when examining toxicological mechanisms. For example, perturbation of some factors within the specific endocrine mechanism being examined may not be detected by

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simply measuring the level of circulating hormone. One can only get a macroscopic view of the toxic mechanisms in play due to the complex interplay of regulatory and counter-regulatory feedback loops, as well as a host of other variables, such as diurnal cycles, stress, changes in binding coefficients at carrier protein sites and changes in the physiology of intracellular signalling pathways (Gardner and Nissenson, 2004).

Until the 1960s, the measurement of hormones was limited to *in vivo* bioassays. An *in vivo* bioassay determines the biological activity of a hormone by noting its effect on a live animal or isolated organ preparation. Historically, bioassays have used hypoglycaemia to measure insulin, bone growth to measure GH and ovarian weight change to measure gonadotrophins. The primary drawbackof *in vivo* endocrine bioassays is that they are often difficult to quantify The discovery of monoclonal antibody techniques greatly enhanced the accuracy and methodology of measuring hormones. Subsequently, antibody-based competitive protein-binding assays such as radioimmunoassay (RIA) were developed with the availability of radioactive, or tracer hormones.

In vitro bioassays employ endocrine-responsive tissue-cell-culture lines that can assess the amount of biologically active hormone in sera. The hormonal activity of the sera can be determined by measuring a cellular response to a particular hormone. Hormonally induced changes in adenylate cyclase activity have been used, but more recently, changes in intracellular calcium levels, phosphoinositol metabolites and protein phosphorylation have been used to assess hormonal activity. Other *in vitro* bioassays can examine changes more distal to the receptor/signal-transduction mechanisms, and note changes in enzymatic activity or steroidogenesis. Finally, some *in vitro* bioassays can assess the mitogenic responses to a given hormone.

A problem with *in vitro* bioassays is that there may be coexisting stimulatory/inhibitory substances in the sera which can confound the observed response. For this reason, it is difficult to design a valid bioassay without using fractionated serum, which requires full characterization of an endocrine-responsive cell line.

5.1 Radioimmunoassay

With the development of the RIA, Yalow and Berson (1959) were able to measure nanomolar concentrations of hormones. Immunoassays are based upon the fact that specific proteins can distinguish three-dimensional structures at the molecular level by tightly binding to those specific molecules produced by a biological organism in a highly complex mixture, providing a means to measure these proteins in a controlled situation. The process involves mixing known quantities of radioactive antigen (frequently labelled with gamma-radioactive isotopes of iodine attached to tyrosine) with antibody to that antigen, then adding unlabelled antigen and measuring the amount of labelled antigen displaced. Initially, the radioactive antigen is bound to the antibodies. When unlabelled antigen is added, the two compete for antibody binding sites. The higher concentrations of unlabelled antigen bind to the antibody, displacing the radioactive variant. The bound antigens are subsequently separated from the unbound ones.

An RIA uses radioactive isotopes to identify these specific proteins. RIAs allow measurement of biological materials not previously detectable by chromatographic or spectrophotometric techniques (Krumm, 1994). They are also used for hormones that are unable to fix complement when bound to antibodies or they can be used to identify crossreacting antigens that compete and bind with antibodies.

Generally, RIAs are divided into two categories: competitive inhibition and noncompetitive inhibition. The competitive assays use a single specific antibody which is fixed to a surface and a corresponding analogue of the protein to carry the label. The protein in the sample competes with the labelled analogue for binding positions on the antibody. Once the unbound analogue is separated, the amount of label remaining is inversely related to the amount of bound protein. Competitive inhibition of radiolabelled hormone–antibody binding by unlabelled hormone (either as a standard or an unknown mixture) is the principle of most RIAs. A standard curve for measuring antigen (hormone) binding to antibody is constructed by placing known amounts of radiolabelled antigen and the antibody into a set of test tubes. Varying amounts of unlabelled antigen are added to the test tubes. Antigen–antibody complexes are separated from the antigen and the amount of radioactivity from each sample is measured to detect how much unlabelled antigen is bound to the antibody. Smaller amounts of radiolabelled antigen–antibody complexes are present in the fractions containing higher amounts of unlabelled antigen. Usually a standard curve is constructed that measures the percentage of radiolabelled antigen bound with the concentration of unlabelled antigen present. Competitive assays are compatible with a wide variety of proteins and are used for the majority of low-molecular-weight proteins.

Noncompetitive inhibition assays utilize two specific antibodies to 'capture' the protein being examined. One antibody (termed the *capture protein*) is immobilized to a surface and a second (termed the *label protein*) carries the label. In this assay, the protein being examined is bound simultaneously by both the capture and label proteins. At completion of the assay, the unbound label protein is separated and measured and the remaining label is measured and is directly proportional to protein concentration in the sample. Noncompetitive assays are generally limited to those proteins of sufficient size to

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be able to bind two materials simultaneously, typically proteins and microorganisms.

Although several methods exist for the separation of antigen–antibody complexes, two methods are most commonly employed in RIAs. These are the following: (i) The double-antibody technique, which precipitates the antigen–antibody complexes out of solution by utilizing a second antibody binding to the first antibody. Although other means of antigen–antibody precipitation exist, they can sometimes chemically alter antigen–antibody binding properties. The drawback to the double-antibody technique is expense, which makes this technique uneconomical for RIA screening procedures. (ii) The dextran-coated activated charcoal technique involves the addition of dextran-coated activated charcoal to the sample followed by immediate centrifugation that absorbs free antigen and leaves antigen–antibody complexes in the supernatant. This technique works best when the molecular weight of the antigen is 30 kDa or less. Also, sufficient carrier protein must be present to prevent adsorption of unbound antibody.

The RIA technique permits the determination of hormone concentration in a sample of protein or urine. Various factors, such as ionic strength and pH can affect the antigen binding and subsequent accuracy of the test, therefore controlled conditions are necessary when assessing the standard and the test sample. Other drawbacks to the RIA include the fact that some RIAs lack specificity, which is generally due to nonspecific crossreactivity of the antibody. Since the assays utilize radioactive materials, any laboratory performing RIAs must be regulated by the Nuclear Regulatory Commission (NRC). Notwithstanding the complexities associated with the RIA and monoclonal antibody methodologies, these are the techniques of choice in the examination of trophic hormones. RIAs are sensitive, requiring minute sample amounts compared to bioassays.

5.2 Enzyme-Linked Immunosorbent Assay (ELISA)

Because the radioactivity associated with RIA posed a health threat, a safer alternative was sought. A suitable alternative to RIA would substitute a nonradioactive signal in place of the radioactive signal. It was found that when certain enzymes (such as peroxidase) react with appropriate substrates (such as 3,3′,5,5′-tetramethylbenzidine), they can result in changes in colour, which can be used as a signal; however, the signal has to be associated with the presence of an antibody or antigen. Hence the enzyme has to be linked to an appropriate antibody. This linking process was independently developed by Stratis Avrameas and G.B. Pierce (Lequin, 2005). This development resulted in the enzyme-linked immunosorbent assay (ELISA), which produces an end-product that is nonradioactive and can be assessed with a spectrophotometer. This change eliminated the complex handling and disposal issues involved with radioisotopes, as mandated by the NRC for safety and control measures. ELISA is comparable to the immunoradiometric assay (IRMA), except that an enzyme tag is attached to the antibody instead of a radioactive label. The hormone is bound to the enzyme-labelled antibody, and the excess antibody is removed for IRMAs. Since it is necessary to remove any unbound antibody or antigen by washing, the antibody or antigen has to be fixed to the surface of the container, that is, an *immunosorbent* has to be prepared (Wide and Porath, 1966). After excess antibody has been removed, or the second antibody containing the enzyme has been added (two-site assay), the substrate and cofactors necessary are added in order to visualize and record enzyme activity. The level of hormone present is directly related to the level of enzymatic activity. The sensitivity of ELISAs can be enhanced by increasing the incubation time for producing substrate. Occasionally, the substrate formed may yield a colour change, so that detection of the hormone being measured can be determined visually.

5.3 Immunoradiometric Assays (IRMA)

IRMAs are similar to RIAs in that both use a radiolabelled substance in an antibody–antigen reaction. The radioactive label in the IRMA assay, however, is attached to the antibody instead of the hormone. This assay is based upon the reversible and noncovalent binding of an antigen by a specific antibody labelled with a radioisotope, and excess of antibody, rather than a limited quantity, is present in the assay. All the unknown antigen becomes bound in IRMA rather than just a portion, as in RIA; IRMA assays are more sensitive. There are basically two types of IRMA assays: (i) In the one-site assay, the excess antibody that is not bound to the sample is removed by addition of a precipitating binder. (ii) In the two-site assay, a hormone with at least two antibody-binding sites is adsorbed onto a solid phase, to which one of the antibodies is firmly attached (either the walls of the assay tube itself or to beads that are added to the biological sample in assay buffer). After binding to the antibody is completed, a second antibody labelled with ^{125}I is added to the assay. This ^{125}I antibody reacts with the second antibody-binding site composed of antibody-hormone-labelled antibody. In contrast to RIA and similar competitive protein-binding assays, the amount of hormone present is directly proportional to the amount of radioactivity measured in the assay (Bennett and Cells, 1992; Dumon *et al.*, 1996).

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5.4 Enzyme-Multiplied Immunoassay Technique (EMIT)

The enzyme-multiplied immunoassay technique (EMIT) is a type of immunoassay in which the ligand is labelled with an enzyme replacing the radiolabels, and the enzyme–ligand–antibody complex is enzymatically inactive, allowing quantitation of unlabelled ligand. The antibody binding alters the enzyme characteristics, allowing for measurement of hormone without separating the bound and free components (i.e. homogeneous assay). EMIT assays are used in drug monitoring of urine, but because of lack of sensitivity have not been used to assess hormones. No extraction is required and the assay can be completed within a few minutes (Foltz *et al*., 1980). The enzyme is attached to the hormone/drug being tested. This enzyme-labelled antigen is incubated with the sample and with antibody to the hormone/drug. Binding of the antibody to the enzyme-linked hormone either physically blocks the active site of the enzyme or changes the protein conformation so that the enzyme is no longer active. Once antibody binding occurs, the enzyme substrate and cofactor are added and enzyme activity can be measured. If the sample contains hormone or drug, it will compete with enzyme-linked hormones for antibody binding, the enzyme will not be blocked by antibody, and more enzyme activity will be measurable (Johnson, 1988).

5.5 Monoclonal Antibodies (mAb)

Monoclonal antibodies (mAb or moAb) are antibodies that are identical because they are produced by one type of immune cell that are all clones of a single parent cell. Given most any substance, it is possible to produce antisera containing a variety of polyclonal antibodies to create monoclonal antibodies that specifically bind to that substance. These monoclonal antibodies can then serve to detect or purify that substance. This has become an important tool in biochemistry, molecular biology and medicine. Many hormones can now be assessed using monoclonal antibody techniques. Antibodies against hormones have been used in many types of RIAs and radioreceptor assays; but polyclonal antisera can create some nonspecificity problems, such as cross-reactivity and variation in binding affinity. Therefore, it is desirable to produce a group of antibodies that selectively bind to a specific region of the hormone (i.e. antigenic determinant). In the past, investigators produced antisera to antigenic determinants of the hormone by cleaving the hormone and immunizing an animal with the fragment of the hormone containing the antigenic determinant of interest (e.g. ACTH immunization with the 24 amino-acid N-terminal of the hormone). This approach solved some problems with crossreactivity of antisera with other similar antigenic determinants, but problems were still associated with the heterogeneous collection of antibodies found in polyclonal antisera.

A process of producing monoclonal antibodies involving human–mouse hybrid cells was first described by Schwaber and Cohen (1973) and the process was further developed by Georges Köhler and César Milstein (Köhler and Milstein, 1975). The concept was to use a line of myeloma cells that had lost their ability to secrete antibodies, fuse these cells with healthy antibody-producing B-cells and select for the successfully fused cells. Because propagation of a single antibody-producing cell *in vitro* does not occur, Köhler and Milstein used spleen cells from an immunized mouse and fused them with myeloma cells (malignant lymphocytes) in the presence of a reagent that causes the cells to fuse (e.g. Sendai virus of polythylene glycol). The fused cells or hybridomas share characteristics of their antibody-producing cells from the spleen by continuously synthesizing identical antibodies and could be infinitely propagated *in vitro* using tissue-culture techniques. The production of monoclonal antibodies offered investigators a homogeneous collection of antibodies that could selectively bind to a specific antigenic determinant with the same affinity.

Further complications arise because unfused spleen cells do not survive in tissue culture media. To separate unfused myeloma cells from hybridomas, the cells are placed in hypoxanthine, aminopterin and thymidine (HAT) medium. Myeloma cells used in the production of monoclonal antibodies lack the enzyme hypoxanthine-guanine-phosphoribosyltransferase (HGPRT), whereas hybridomas contain the enzyme (contributed by fused spleen cells). Because the main pathway of DNA synthesis is blocked by aminopterin, only cells containing HGPRT can utilize hypoxanthine and synthesize DNA in order to propagate. Thus, unfused myeloma cells are eliminated in HAT medium because they lack HGPRT. Once the hybridomas have been isolated from unfused cells, they are separated into individual colonies and the type of antibody they produce is characterized. Identification of a hybridoma clone that is producing a specific antibody to the antigen of interest allows for harvesting large quantities of the monoclonal antibody. While monoclonal antibodies offer a highly sensitive, specific method for detecting antigen, sometimes increasing monoclonal-antibody specificity compromises affinity of the antibody for the antigen. Monoclonal antibody techniques provide a means of producing a specific antibody for binding antigen. This technique is useful for studying protein structure relations and has been used for devising specific RIAs.

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5.6 Gene Expression

Gene expression is the process in which the inheritable information in the DNA sequence in a gene is made into a gene product, for example, protein or RNA. Several steps in the gene expression process may be modulated, including the transcription step and the post-translational modification of a protein. The effect of an endocrine modulator or disruptor can influence gene expression via transcriptional control. By increasing or decreasing the rate of gene transcription it is possible to modify gene expression and by quantitating the amount of messenger ribonucleic acid (mRNA) for a given gene, it is possible to measure the effect on transcriptional gene expression (Shupnik, 1995). Since mRNA represents a relatively small fraction of RNAs in the cell, methods have been developed which are sensitive and specific for detecting changes in mRNA levels and can distinguish between transfer ribonucleic acid (tRNA) and ribosomal ribonucleic acid (rRNA).

Suppression (or suppressive) subtractive hybridization (SSH) is a technique that uses the polymerase chain reaction (PCR) to quickly compare the expression of mRNA from different samples, which can show the relative difference in the concentration of these molecules and can be used to identify mRNAs that are differentially expressed between tissue or cell types under different hormonal or environmental conditions (Sambrook *et al*., 1989; Munir *et al*., 2004). SSH is a method which allows for the rapid isolation of differentially distributed nucleic acids, where the substrates in the process are nucleic acids (tester component) which contain specifically expressed sequences that are to be extracted (target component) and nucleic acids that are utilized for comparison of the target molecules (driver component). The tester is subsequently combined with the driver to form a 'tester–driver' hybrid which is the nontarget component. 'Tester–tester' hybrids and single-stranded tester molecules are also formed, which represent the fractions amplifying the target molecules. After completion of hybridization, the target and nontarget fractions are separated and quantified.

5.7 Polymerase Chain Reaction

The PCR is another molecular biology technique for enzymatically replicating specific regions of DNA or complementary DNA (cDNA) template without using a living organism. PCR is thought to have been conceived by Mullis in 1983. The technique allows a small amount of the DNA molecule to be amplified many times by using a pair of oligonucleotide primers (usually ranging from 10 to 40 kb in length, flanking a DNA sequence of interest—approximately 100–500 kp in length), a thermostable DNA polymerase (an enzyme that catalyses the elongation of DNA from a template) and deoxynucleotide triphosphates (dNTPs, reaction buffer and magnesium (Innis *et al*., 1990). Oligonucleotide primers are first designed to be complementary to the ends of the sequence to be amplified, and then are mixed in molar excess with the DNA template and deoxyribonucleotides in a buffer. Following heating to denature the original strands and cooling to promote primer annealing, the oligonucleotides each bind to a different strand of the target fragment. The primers are positioned so that when each is extended by the action of a DNA polymerase, the newly synthesized strands will overlap the binding site of the opposite oligonucleotide. The cycles of PCR are performed in a machine called a *DNA thermal cycler*, which can be adjusted to optimize the amplification of DNA, depending on a number of variables (e.g. the length and nature of the DNA sequence, the source and quality of the DNA template, and the application of the procedure by heating and cooling the internal reaction tubes within a precise temperature range for each reaction step). DNA templates can be derived from genomic DNA (e.g. human, animal, bacterial or viral), or RNA that has been converted to a DNA template using reverse transcriptase (reverse transcriptase polymerase chain reaction (RT-PCR)). PCR can be employed to detect mRNA for hormones, growth factors, polypeptides, receptors and other proteins involved with the endocrine system.

5.8 *In Situ* Hybridization and Immunochemistry

In situ hybridization (ISH) is a type of hybridization that uses a labelled complementary DNA or RNA strand or probe (a fragment of DNA of variable length usually 100–1000 bases long, used to detect the presence of nucleotide sequences in DNA or RNA samples that are complementary to the sequence in the probe) to localize a specific DNA or RNA sequence in a portion or section of *in situ* tissue. Tissue samples are treated to increase permeability, permitting the desired probe to enter the cell. The samples are fixed, embedded and thinly sectioned prior to hybridization. Hybridization is permitted at elevated temperatures and then the excess probe is removed. A complementary probe is labelled, thus permitting the precise location and quantification of the probe remaining in the tissue. Other methods of signal detection include fluorescence (e.g. fluorescent *in situ* hybridization (FISH)). FISH is a cytogenetic technique used to detect and localize specific DNA sequences on chromosomes. Fluorescence microscopy is utilized to localize specific DNA sequences on chromosomes. For instance, a probe is constructed and is tagged with fluorophores with specific targets. The preparation is embedded and subsequently examined under the fluorescence microscope. Low abundance mRNAs can

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be detected by *in situ* PCR, which amplifies the amount of nucleic acid target in the tissue. This technique is useful for detecting hormone or receptor subtypes that are biologically unique, by combining immunogenic features with related biological molecules.

Immunochemistry is a process of detecting and localizing synthesized proteins in cells of a tissue section utilizing antibodies (Watkins, 1998; Ramos-Vara, 2005). The antibody is tagged with a colour-producing tag. Primary monoclonal or polyclonal antibodies are then visualized with a secondary marker, such as fluorochrome-conjugated secondary antibody or streptavidin-biotin labels. Selection of a fluorochrome depends on microscopy wavelength and filters, the stability of the signal, the type of the tissue being examined and whether there is a need for double-labelling. This technique is widely used in basic research to help characterize the distribution and localization of biomarkers in different parts of a tissue. Immunohistochemical detection of tissue antigens often lacks a degree of sensitivity.

The *indirect method*, on the other hand, involves an unlabelled primary antibody (first layer) which reacts with tissue antigen, and a labelled secondary antibody (second layer) which reacts with the primary antibody. This method is more sensitive due to signal amplification through several secondary antibody reactions with different antigenic sites on the primary antibody. The second layer antibody can be labelled with a fluorescent dye or an enzyme.

Some markers are known for specific cancers such as CEA (carcinoembryonic antigen) (Hammarstrom, 1999), which is used for identification of adenocarcinomas. CD15 has been identified as a marker for Hodgkin's disease (Xue *et al*., 2000). Another useful marker is CD117 which is also called KIT or the C-kit receptor. CD117 is a marker for gastrointestinal stromal tumours (GISTs) (Edling and Hallberg, 2007).

Immunohistochemistry can be a powerful complement to ISH, but it has limitations, such as a lack of antibody specificity, denaturation of the antigen during fixation and cell membrane permeability.

5.9 Signal Transduction

Signal transduction refers to any process by which a cell converts one kind of signal or stimulus into another type of signal. Most processes of signal transduction involve an ordered sequence of biochemical reactions inside the cell, which are carried out by enzymes which are activated by second messengers and result in a signal-transduction pathway. These processes can last only a few milliseconds, as in the case of ion flux, or for hours or even days relative to gene expression. The number of proteins and other molecules that are involved in the signal transduction increases as the process emanates from the initial stimulus, resulting in a signal cascade which begins with a relatively small stimulus that elicits a large response. This is referred to as amplification of the signal. Besides affecting hormone receptor activation, some compounds can inhibit inactivation of signal-transduction pathways, such as phosphodiesterase inhibitors like caffeine and theophylline. Sildenafil and tadalafil are selective inhibitors of Type V phosphodiesterase, which degrades the cyclic guanosine monophosphate (cGMP) in the corpus cavernosum leading to vascular engorgement in erectile dysfunction (Francis and Corbin, 1999).

Most signal transduction involves the binding of extracellular signalling molecules (or ligands) to cell-surface receptors that face outward from the plasma membrane and trigger events inside the cell. It has also been shown that intracellular signalling cascades can be triggered through cell-substratum interactions, as in the case of integrins, which bind ligands that occur in the extracellular matrix. Steroids are another example of extracellular signalling molecules that may cross the plasma membrane due to their lipophilic or hydrophobic nature (Beato *et al*., 1996). After binding to their receptors, some of the hormones can act to promote steroid-responsive genes (Hammes, 2003). Within multicellular organisms, there are many small molecules and polypeptides that coordinate a cell's individual biology as it relates to the maintenance and survival of the organism as a whole. These molecules can be classified as follows: hormones Sugden *et al.* (2004), growth factors (Carpenter and Cohen, 1990), extracellular matrix components (Rensing, 1972), cytokines (Schroder *et al*., 2004), chemokines (Chung *et al*., 1995), neurotransmitters (Kistler *et al*., 1982) and neurotrophins (Wiesmann and de Vos, 2001). Hormones are a generic class of molecules able to initiate signal transduction, these include insulin (a polypeptide), (Adams *et al*., 2000), testosterone (a steroid) (Roy and Chatterjee, 1995) and epinephrine (an amino acid derivative; in essence, a small organic molecule) (Small *et al*., 2003). Common endocrine signal-transduction mechanisms include activation of cyclic AMP, activation of cGMP, activation of serine, tyrosine and threonine kinases, activation of phosphoinositides, release of intracellular calcium and control of receptor-operated ion channels.

In multicellular organisms, many different signal-transduction processes are required for coordinating the behaviour of individual cells to support the function of the organism as a whole. Sensing of both the external and internal environments at the cellular level relies on signal transduction. Many disease processes, such as diabetes, heart disease, autoimmunity and cancer, arise from defects in signal-transduction pathways. Environmental stimuli may be molecular in nature, as described above or more physical, such as light striking cells in the retina of the eye (Burns and Arshavsky, 2005), odorants

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binding to odorant receptors in the nasal epithelium, (Ronnett and Moon, 2002) or bitter and sweet tastes stimulating taste receptors in the taste buds (Wong *et al*., 1996), further highlighting the critical importance of signal transduction to homeostasis in the multicellular organism. Cellular responses to extracellular stimulation that require signal transduction include activation of genes, (Lalli and Sassone-Corsi, 1994), alterations in metabolism and the stimulation or suppression of locomotion (Bornfeldt *et al*., 1995).

Receptors can be roughly divided into two major classes: (i) intracellular receptors which must cross the cell membrane to initiate signal transduction and (ii) cell-surface receptors which span the plasma membrane of the cell, with one part of the receptor on the outside of the cell (the *extracellular domain*), and the other on the inside of the cell (the *intracellular domain*). In this case the signal transduction occurs as a result of stimulatory molecule or the binding of a ligand to its extracellular domain; the ligand itself does not pass through the plasma membrane.

6 ENDOCRINE RECEPTORS

Many receptors for endocrine target organs are ligand-dependent transcriptional regulators that fall into the superfamily of proteins that include oestrogens, androgens retinoic acid, thyroid hormone and vitamin D. These hormones have a molecular weight between 300 and 400 Da and can permeate the cell's plasma membrane. Once inside the cell, these hormones bind to specific proteins in the cytoplasm or nucleus which are known as receptors. These intracellular proteins are specific for each protein and are concentrated at the appropriate endocrine target organ. Thus, male sex accessory organs, such as the prostate gland possess a high density of androgen receptors (ARs) while the female reproductive organs contain a high density of oestrogen receptors (ERs). The binding of the hormone to plasma receptor initiates a cascade of responses that will ultimately alter the activity of the target endocrine organ. This mechanism is termed the second-messenger hypothesis. In this hypothesis the hormone is the first messenger which contains certain information for the target organ receptor. The binding of the hormone subsequently interacts with other cellular components either to activate or suppress transcription of a target organ gene in a promoter and the cell-specific event. Sunderland discovered cAMP and showed that it had an intermediate role in many hormonal functions (Kresge *et al*., 2005). It appears that there are several types of receptors found in hormone regulation, including G-protein (guanine nucleotide-binding protein) receptors, ion channel receptors, receptors containing tyrosine kinase, receptors associated with tyrosine kinase, steroid receptors, ERs, ARs and thyroid receptors (TRs).

6.1 G-protein-coupled Receptors (GPCRs)

G-protein-coupled receptors (GPCRs), also known as seven transmembrane receptors (because they usually span the membrane seven times) or G-protein-linked receptors (GPLRs), comprise the largest protein family of transmembrane receptors that sense molecules outside the cell, and activate internal signal-transduction pathways and, ultimately, cellular responses. G-proteins were discovered by Alfred G. Gilman and Martin Rodbell in 1994 (Lodish *et al*., 2000). G-protein-coupled receptors function as chemical switches, alternating between an inactive guanosine diphosphate (GDP)- and active guanosine triphosphate (GTP)-bound state, ultimately regulating downstream cell processes. They are activated by an external signal in the form of a ligand or other signal mediator, which causes a conformational change in the receptor, causing activation of a G-protein to release GDP. This, in turn, causes the uptake of GTP, resulting in activation or deactivation of adenylyl cyclase, forming cAMP. The G-protein hydrolyses the GTP almost immediately, which then decreases the G-protein's affinity for the catalytic subunit and ends the cycle. Receptor-activated G-proteins are bound to the inside surface of the cell membrane. They consist of G_α and tightly associated $G_{\beta\gamma}$ subunits. There are four main families for G_α subunits: $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q/11}$ and $G_{\alpha 12/13}$. These groups differ primarily in effector recognition, but share a similar mechanism of activation.

$G_{\alpha s}$ stimulates the production of cAMP from adenosine triphosphate (ATP). The direct stimulation of the membrane-associated enzyme adenylate cyclase causes the cAMP to act as a second messenger that interacts with and activates protein kinase A (PKA). PKA can then phosphorylate downstream targets. $G_{\alpha i}$ inhibits the production of cAMP from ATP. $G_{\alpha q/11}$ stimulates membrane-bound phospholipase Cβ, which then cleaves phosphatidylinositol biphosphate (Pip2) into two second messengers, inositol triphosphate (ip3) and diacylglycerol (DAG). $G_{\alpha 12/13}$ are involved in Rho family GTPase signalling and control cell-cytoskeleton remodelling, therefore regulating cell migration. $G_{\beta\gamma}$ sometimes also have active functions, for example, coupling to L-type calcium channels (a type of voltage-dependent calcium channel) (Filmore, 2004).

6.2 Ion-Channel Receptors

Ion-channel receptors are regulated by ligands and are usually very selective to one or more ions, like Na^+, K^+, Ca^{2+} or Cl^-. Such receptors located at synapses convert the chemical signal of a hormone directly and very quickly into the receptors that regulate the flow

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of ions through specific channels in the membrane. The most common are calcium channels, for example the calcium-calmodulin system. Calcium/calmodulin directly activates calcium/calmodulin-dependent protein kinase I by binding to the enzyme and indirectly promotes the phosphorylation and synergistic activation of the enzyme by calcium/calmodulin-dependent protein kinase I kinase. In this system a hormone binding to the ion channel receptor opens the calcium channels permitting the calcium concentration to rise. Frequently calcium binds to calmodulin as it transits the membrane (Chin and Means, 2000).

6.3 Receptors Containing and Associated with Tyrosine Kinase

Receptors containing tyrosine kinase usually consist of ligand-binding domains containing a transmembrane receptor with a tyrosine kinase domain protruding into the cytoplasm. This receptor contains tyrosine kinase as an integral part of the receptor. When the activating hormone binds to the receptor, the tyrosine kinase is activated, resulting in two effects: autophosphorylation that can potentiate the signal and phosphorylation of the tyrosine residues on adjacent proteins, thereby regulating their activity.

Receptors associated with tyrosine kinase recruit to a receptor following hormone binding and are involved in a number of signalling cascades, principally those involved in cytokine signalling (Manning *et al*., 2002).

6.4 Steroid Receptors

Steroid hormone receptors are intracellular receptors (usually cytoplasmic) that create signal transduction for steroid hormones. Steroid hormone receptors are part of the nuclear receptor family that include a group of homologous structured receptors that bind to nonsteroid ligands such as thyroid hormones and vitamin A, as well as to vitamin D, and orphan receptors. Steroid hormones, such as the oestrogens, bind to receptors in the cell cytoplasm and are translocated to the nucleus, where they can activate a series of complex biochemical events. In most cases the uptake into the nucleus is covered up by heat shock proteins (HSPs) which bind the receptor until the hormone is present. Most inactive steroid receptors are sequestered in a complex with HSP90 (Smith and Toft, 1993). Steroid receptors are composed of six domains based upon amino acid similarity and mutational analysis (Ekena *et al*., 1996). The AB domain contains a hormone-independent transcriptional activating function, while the conserved C domain is the site of DNA binding. The D domain is a hypervariable region while the E and F domains are conserved ligand-binding domains of the steroid receptor.

6.5 Oestrogen Receptors (ERs)

The ER is a member of the nuclear hormone family of intracellular receptors which is activated by the hormone 17β-oestradiol (Dahlman-Wright *et al*., 2006). The main function of the ER is as a DNA-binding transcription factor which regulates gene expression. There are two different forms of the ER, usually referred to as α and β, each encoded by a separate gene. Oestrogen receptor α (ER-α), also known as NR3A1 (nuclear receptor subfamily 3, group A, member 1), is a nuclear receptor which is activated by oestrogen (Tanaka *et al*., 2003). The ER is a ligand-activated transcription factor composed of several domains important for hormone binding, DNA binding and activation of transcription. Oestrogen receptor β (ER-β), also known as NR3A2 (nuclear receptor subfamily 3, group A, member 2), is a nuclear receptor which is also activated by the sex hormone oestrogen (Koehler *et al*., 2005).

While the biological importance of the α and β ERs remains elusive, there do appear to be differences in their anatomical distribution. ER-α is expressed in the uterus, pituitary, gonads and selected other organs. ER-β is found in the lungs, bladder, brain, gonads and prostate. Both receptors have a high binding affinity for oestradiol and diethylstilboesterol. Both receptors will also bind to several weak oestrogens and other xenobiotics (Thomas, 1998). An E-screen assay (for oestrogenic activity) has been proposed using the MCF-7 human breast cell line (Soto *et al*., 2006). While the assay is fairly sensitive, it is not specific. Oestrogen structure–binding-affinity relationships and affinity-labelling and site-directed mutagenesis studies on ER α and β have provided more insight into the interaction between the receptor and the ligand (Anstead *et al*., 1997). The ultimate action of the ligand–receptor complex is endocrine modulation of the transcription of specific target organ genes.

6.6 Androgen Receptors (ARs)

The AR, also known as NR3 C4 (nuclear receptor subfamily 3, group C, member 4), is a type of nuclear receptor which is activated by binding of either of the androgenic hormones testosterone or dihydrotestosterone (Roy *et al*., 1999). The AR primary function is as a DNA-binding transcription factor which regulates gene expression (Matsumoto *et al*., 2007). However, the AR also has additional functions independent of DNA binding (Heinlein and Chang, 2002; Fix *et al*., 2004). Androgens mediate androgen-induced male sexual development and function (Zhou *et al*., 1994), and directly

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regulate many biological processes, which are mediated by a nuclear receptor that exhibits four major activities, including steroid binding, DNA binding, transactivation and nuclear localization (Lindzey *et al*., 1994). The primary mechanism of action for ARs is direct regulation of gene transcription. When androgen binds to the AR, it affects a conformational change in the receptor causing dissociation of HSPs, dimerization and transport from the cytoplasm to the cell nucleus, where the AR dimer binds to a specific sequence of DNA known as a hormone response element. ARs interact with other proteins in the nucleus, resulting in increased or decreased regulation of specific gene transcription. Increased transcription produces more messenger RNA, ultimately increasing the production of the specific protein being regulated. ARs interact with certain signal-transduction proteins in the cytoplasm and can cause rapid changes in cell function independent of changes in gene transcription, such as changes in ion transport. Regulation of the signal-transduction pathways by these cytoplasmic ARs can indirectly lead to changes in gene transcription.

The AR can be studied using a three-dimensional quantitative structure–activity model that might be important for the identification of environmental contaminants with potential endocrine-disrupting properties (Waller *et al*., 1996). Thus, chemicals that have avidity for the AR can be identified for their toxicological potential upon the endocrine system.

6.7 Thyroid Receptors (TR)

The TR is a type of nuclear receptor that is activated by the binding hormone (Flamant *et al*., 2006). The TR plays critical roles in regulation of metabolism, heart rate and organism development (Yen, 2001; Harvey and Williams, 2002). TRs are transcription factors that regulate gene transcription either positively or negatively after binding thyroid hormones (e.g. thyroxine). TR interacts with transcriptional corepressors. There are three forms of the TR that are known to bind to the thyroid hormone, $\alpha 1$, $\beta 1$ and $\beta 2$:

1. TR-$\alpha 1$ (high expression in cardiac and skeletal muscles)
2. TR-$\beta 1$ (expressed in brain, liver and kidney)
3. TR-$\beta 2$ (expression limited to the hypothalamus and pituitary).

Certain mutations in the thyroid hormone receptor are associated with thyroid hormone resistance (Olateju and Vanderpump, 2006), which is a rare syndrome where the thyroid hormone levels are elevated, but the TSH level is not suppressed, or not completely suppressed, as would be expected. The most common cause is mutations of the β form of the thyroid hormone receptor, of which over 100 different mutations have been documented (Refetoff and Dumitrescu, 2007; Refetoff, 2008).

7 ENDOCRINE TARGET ORGAN TOXICITY

Perturbation of a target organ by a drug or chemical can be mediated through the central nervous system (CNS) by interfering with trophic hormone secretion or by a chemical or drug acting directly upon the particular target organ. Thus, there are both indirect (e.g. via the adenohypophysis) and direct-acting sites of action. In addition, hepatic microsomal enzyme systems responsible for endogenous steroid metabolism can also be affected by xenobiotics.

7.1 Adrenal Glands

The adrenal glands are located in the thoracic abdomen situated on their anterosuperior aspect of the kidneys. In humans, these glands are found at the level of the 12th thoracic vertebra and receive their blood supply from the adrenal arteries. They are responsible for regulating the stress response via the synthesis of corticosteroids and catecholamines. The adrenal glands are each separated into two distinct structures—the adrenal medulla and the adrenal cortex. The adrenal cortex and the adrenal medulla are a functional unit and together manifest a capacity to manage changes in both internal and external environmental conditions.

The adrenal medulla is the central core of the adrenal gland, surrounded by the adrenal cortex. The medulla consists of neurons that are part of the sympathetic branch of the autonomic nervous system and is chiefly populated with chromaffin cells, which are the source of the catecholamines, epinephrine and norepinephrine, fast-acting hormones that signal an immediate physiological change and are derived from the amino acid tyrosine. The medulla releases these neurotransmitters into the blood instead of at a synapse. Therefore, although it is part of the nervous system, the medulla functions as an endocrine gland. These chemicals are part of the fight-or-flight response initiated by the sympathetic nervous system. The adrenal medulla is also the main source of dopamine, which is a catecholamine closely related to epinephrine and norepinephrine that functions in the brain, including important roles in behaviour and cognition, motor activity, motivation and reward, inhibition of milk production, sleep, mood, attention and learning.

The adrenal cortex is located along the perimeter of the adrenal gland and manages stress responses at a slower pace through the production of mineralocorticoids and glucocorticoids, such as aldosterone and cortisol,

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as well as androgens from cholesterol (Pikuleva, 2006). The adrenal cortex is composed of three tissue layers. The external layer is termed the zona glomerulosa which secretes mineralocorticoids such as aldosterone as part of the renin-angiotensin system regulating the blood pressure. The intermediate layer is termed the zona fasciculata, and produces cortisol, which is involved in the response to stress, increases blood pressure, hyperglycemia and suppression of the immune system. The production of steroid hormones by the adrenal cortex is triggered by the secretion of ACTH by the anterior pituitary gland. These hormones act at the level of gene expression to affect fundamental biochemical processes. Mineralcorticoids affect the body content of sodium and potassium by acting on the kidney to promote the reabsorption of sodium ions (Na^+) into the blood. Water follows the salt and this helps maintain normal blood pressure. Glucocorticoids affect body fuel metabolism by stimulating gluconeogenesis in the liver: the conversion of fat and protein into intermediate metabolites that are ultimately converted into glucose. Cortisol and the other glucocorticoids also have an anti-inflammatory effect on the body by depressing the immune response—especially cell-mediated immune responses—hence they function to help the body respond to injuries.

The principal mineralocorticoid in humans is aldosterone although deoxycortisosterone exhibits mineralocorticoid activity. Cortisol, the major glucocorticoid produced in humans, has some mineralocorticoid activity. Aldosterone modulates body electrolytes and promotes the renal absorption of sodium in the ascending portion of the loop of Henle in the distal tubule and in the collecting tubule. Aldosterone facilitates the urinary excretion of potassium and hydrogen ions. Mineralocorticoid deficiencies produce a rise in extracellular fluid potassium concentration and reductions in sodium and chloride content, which can lead to diminished cardiac output and death.

Cortisol or hydrocortisone, corticosterone and cortisone display varying degrees of glucocorticoid activity. Cortisol is probably the most important human glucocorticoid. It is essential for life, and regulates or supports a variety of important cardiovascular, metabolic, immunologic and homeostatic functions. The glucocorticoids stimulate gluconeogenesis and decrease glucose utilization. Cortisol stimulates several processes that collectively serve to increase and maintain normal concentrations of glucose in blood. These effects include stimulation of gluconeogenesis in the liver, resulting in the synthesis of glucose from nonhexose substrates such as amino acids, and glycerol from triglyceride breakdown. It further mobilizes amino acids from extrahepatic tissues—which serve as substrates for gluconeogenesis (Freeman, 2004). Glucocorticoids appear to interfere with the transport of amino acids into extrahepatic cells and combined with continuing protein catabolism in these cells, results in an increase in plasma amino acids. This increase in plasma amino acids levels and their subsequent transport into the liver may promote gluconeogenesis. Cortisol also conserves glucose by inhibiting the uptake of glucose in muscle and adipose tissue. It also stimulates the fat breakdown of adipose tissue. The fatty acids released by lipolysis are used to produce energy in muscle, while the released glycerol provides additional substrate for gluconeogenesis. In many steroidogenic cells, these responses often arise from the stimulation by an appropriate trophic hormone acting through the cyclic AMP second-messenger signalling pathway. The focus for this acutely regulated step in steroidogenesis appears to be determined by the transfer of the substrate cholesterol to the inner mitochondrial membranes. Cholesterol needs to be transferred from the outer mitochondrial membrane to the inner membrane where cytochrome P450scc enzyme is located, to split off the cholesterol side chain, which is the first enzymatic step in steroid synthesis. The aqueous phase between these two membranes cannot be crossed by the lipophilic cholesterol, unless certain proteins assist in this process, which is primarily mediated by the action of a steroidogenic acute regulatory protein (StAR) on the peripheral benzodiazepine receptor (PBR), causing the movement of Cl^- out of the mitochondria, bringing the outer and inner mitochondrial membranes together. The StAR is a transport protein that regulates cholesterol transfer within the mitochondria, which is the rate-limiting step in the production of steroid hormones (Alpy and Tomasetto, 2006; Stocco *et al.*, 2005).

7.1.1 Toxicological Effects

The adrenal cortex is vulnerable to chemical lesions because the adrenal cortical cells contain a high concentration of lipid cells which are used as substrate in the production of steroid hormones. The lipophilic toxic compounds tend to accumulate in these lipid-rich cells of the adrenal cortex. Additionally, the adrenal cortical cells contain many enzymes (e.g. cytochrome P450 family) which can metabolize these xenobiotic compounds. These compounds result in direct toxic effects by covalent interactions with macromolecules or via oxygen activation generating free radicals (Capin, 2008). Many substances can cause lesions in the adrenal gland (See **Table 3**). Such lesions may be localized and in specific areas of the gland. Chemically induced changes can cause specific biochemical changes. Carbon tetrachloride causes adrenocortical necrosis, but its site of toxicity is in the zona reticularis which is the innermost layer of the adrenal cortex. Spironolactone, a mineralocorticoid antagonist, causes functional lesions in the zona fasciculata which is directly on top of the zona reticularis. Dichlorodiphenyltrichloroethane (DDT) metabolites (e.g. 3-methylsulfonyl-1,1-dichloro-2,2-bis (*p*-chlorophenyl) ethylene (3-methylsulfonyl-DDE))

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Table 3 Agents known to cause lesions to the adrenal gland

Adrenal cortex	Adrenal medulla
Adriamycin	Acrylonitrile
Aminoglutethimide	ACTH
4-Aminopyrazolo (3,4-d)pyrimidine	Alloxan
Cadmium	Blocadren
Carbon tetrachloride	Chlordecone
Chloramphenicol	*o*-Chlorobenzylidine
Chlordane	Cysteamine
Chloroform	Dichloromethane
Chlorphentermine	7,12-Dimethyl-benzanthracene
Chlorpromazine	Oestrogens
Copper	Growth hormone
Cyclosporin	Interleukin-2
Cyproterone	Lactitol
o,*p*′-DDD	Lactose
Danazol	Malathion
Dichlorvos	Mannitol
7,12-Dimethyl-benzanthracene	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyrine (MPTP)
Etomidate	Neuroleptics
5-Fluorouracil	Nicotine
Kepone	Pyrazole
Ketoconazole	Reserpine
Nicotine	Retinol acetate
Phenobarbital	Sorbitol
Polychlorinated biphenyls	Thiouracil
Spironolactone	TSH
Tamoxifen	Thyroid hormones
TCDD	1,1,2-Trichloroethane
Tetrahydrocannabinol	Xylitol
Toxaphene	

Source: Kapp and Thomas (2008); Colby and Longhurst (1992) and Thomas and Keenan (1986).

exert their adrenotoxic effects by specifically binding to a nonextractable residue in the zona (Colby and Longhurst, 1992; Colby *et al*., 1996; Lindhe *et al*., 2002). Hexadimethrine bromide causes toxic damage to the zona glomerulosa, which is the most superficial layer of the adrenal cortex, lying directly beneath the adrenal gland's capsule, and ischaemic necrosis of the zona fasciculata and reticularis.

Acrylonitrile produces adrenal necrosis in experimental animals (Szabo and Sandor, 1997). The acute adrenocorticolytic effect of a single exposure to acrylonitrile rapidly causes adrenal haemorrhage and necrosis. Interestingly, cysteamine and pyrazole produce quantitatively similar lesions to those produced by acrylonitrile. Repeated injection of 7,12-dimethylbenz[a]anthracene (DMBA) produces mammary tumours in experimental animals and results in slow progressive adrenal necrosis (Fu *et al*., 2005). In contrast to the single dose administration of other adenocorticolytic agents (e.g. acrylonitrile and cysteamine), multiple injections of thioguanine are necessary to produce haemorrhagic necrosis of the adrenal cortex.

Chemicals may directly or indirectly affect adrenal cortical secretions. It has been shown that the response of the adrenal gland to ACTH may be compromised by chemically induced changes in membrane receptors, cyclic nucleotide levels, protein synthesis and other biochemical processes involved in stimulating steroidogenesis. Changes in biochemical processes in adrenal cortical secretions produced by chemicals can be mediated by several different mechanisms. The adrenal cortex is highly vascularized, and has a high concentration of cytochrome P450 and lipids providing a milieu conducive to assimilating and sequestering fat-soluble toxins. Xenobiotics can undergo detoxification during metabolism, resulting in potentially more toxic intermediates and may contribute to the overall toxicological actions of a compound. Differences in adrenal metabolism of xenobiotics among species may account for some differential toxicity noted among various chemicals. Hence, the adrenal xenobiotic-metabolizing P450 isoenzymes may also contribute to the overall toxicological actions of a compound. Triaryl phosphates (e.g. tricresyl phosphate (TCP) and butylated triphenyl posphate (BTP)) cause inhibition of cholesterol ester hydrolase. Cholesterol acyltransferase inhibitors are also adrenotoxic. Agents that inhibit acyl-CoA:cholesterol acyltransferase, an enzyme that catalyses the esterfication of cholesterol have cytotoxic action on the adrenal gland (Chang *et al*., 2006).

7.2 Gonads

The gonads include the ovaries and testes and provide two major functions: (i) each produces germ cells or gametes (e.g. spermatozoa in the testes and ova in the ovaries; (ii) the gonads synthesize steroid sex hormones which are critical for the development and functioning of the reproductive organs. In addition these hormones produce secondary sex characteristics such as facial hair in males and breast development in females. There are three basic types of sex hormones: (i) oestrogens (e.g. oestradiol) which produce feminizing effects; (ii) progestogens (e.g. progesterone) that prepare and maintain the uterus for pregnancy and (iii) androgens (e.g. testosterone) which

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produce masculinizing effects. In addition to these basic reproductive functions, these sex hormones are active in other critical bodily functions, such as carbohydrate and lipid metabolism, bone growth and the cardiovascular system.

7.2.1 Ovaries

In females, the ovaries are located internally on the posterior wall of the pelvis lateral to the position of the uterus. They are structurally supported by three ligaments—the suspensory ligament, the broad ligament and the ovarian ligament. The ovaries are in close proximity to the fallopian tubes, which participate in the reproductive process by transporting the ovum from the ovary to the uterus. Hormonal modulation of the female reproductive system is quite complex and involves many neuroendocrine factors and hormones that act in concert to produce mature gametes. Hormone modulation incorporates a series of cyclic events and is influenced by the adenohypophysial-hypothalamic-gonadal axis. Successful reproduction in the female depends upon highly coordinated actions of numerous biochemical processes, including gametogenesis, sperm–ovum interactions and the processes of implantation, embryo development and parturition. The ovaries, not only produce eggs for reproduction, but also secrete female sex hormones. Ovarian function is dependent upon the stimulatory effects of the FSH and LH. These two hormones are secreted from cells in the anterior pituitary called gonadotrophs. Most gonadotrophs secrete only LH or FSH, but some appear to secrete both hormones. FSH regulates the development, growth, pubertal maturation and reproductive processes of the human body. FSH initiates follicular growth which affects the granulosa cells. There is a concomitant rise in inhibin B, and FSH levels then decline in the late follicular phase. At the end of the luteal phase, there is a slight rise in FSH that seems to be of importance to start the next ovulatory cycle. Ovulation of mature follicles on the ovary is induced by a large burst of LH secretion known as the preovulatory LH surge. Residual cells within ovulated follicles proliferate to form corpora lutea, which secrete the steroid hormones progesterone and oestradiol. Progesterone is necessary for maintenance of pregnancy, and, in most mammals, LH is required for continued development and function of corpora lutea. The main role of the oestrogens is to coordinate the normal development of the female genitalia and breasts. As females move through adolescence, oestrogens promote the growth of the uterus, vagina and breasts. In addition oestrogens determine the pattern of fat deposition throughout the body, which results in the typical female shape. In the adult female, the primary functions of oestrogens include regulation of the menstrual cycle and control of libido. During pregnancy oestrogens also contribute to the regulation of lactation.

Progestogens are also produced by the ovaries during various phases of the menstrual cycle as well as during pregnancy. In preparation for pregnancy, progestogens cause changes in the uterine lining and promote development of the mammary glands in preparation for lactation.

7.2.1.1 Toxicological Effects

The ovaries are a target organ for chemical injury (Mattison *et al*., 1990). Granulosa cells, which form a single layer around the oocyte, produce steroids and many growth factors, which interact with the oocyte during development. FSH stimulates these cells to convert thecal cell androgens to oestradiol with aromatase. In addition, post ovulation, the granulosa cell produces progesterone which assists in pregnancy maintenance. These granulosa cells also possess many sites of vulnerability to chemical or heavy metal insults. Agents that are gonadotrophin antagonists can injure gonadotrophin receptors or can interfere with receptor activity. The thecal cells may also be targets for chemical or heavy metal injury (Chrysikopoulos, 2000). Agents that impair cell proliferation, migration and communication may affect thecal cell function; however, there is a paucity of data about thecal cell vulnerability to chemical insults. Oocytes are similarly vulnerable to chemical injury, especially by lead, cadmium and mercury (Mattison *et al*., 1990). In general, heavy metals have been shown to affect ovarian function (Thomas and Thomas, 2000).

During menopause, oestrogen production from the ovaries is eventually terminated, which can result in various symptoms, such as hot flushes, heart palpitations, anxiety, depression and osteoporosis. Hormone replacement therapy (HRT) involves the medical management for treatment for surgically menopausal, perimenopausal and postmenopausal women, which is based upon the assumption that this therapy may prevent discomfort and health problems caused by diminished circulating oestrogen and progesterone hormones. The treatment generally involves a series of drugs designed to artificially boost hormone levels to help prevent some of these symptoms. The main types of hormones involved are oestrogens, progesterone and sometimes testosterone. Menopausal hormone therapy once seemed the answer for many of the conditions women face as they age. It was thought that hormone therapy could prevent heart disease, osteoporosis and cancer, while improving women's quality of life. In 2002 however, findings emerged from clinical trials that showed this was not the case. The oestrogen-plus-progestin therapy increased women's risk for heart attacks, stroke, blood clots and breast cancer. It also doubled the risk of dementia and did not protect women against memory loss. However, the therapy had some benefits: it reduced the risk for colorectal cancer and bone fractures. The findings come from the Women's Health Initiative (WHI), launched in 1991 by the National Institutes of Health to test

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ways to prevent a number of medical disorders in postmenopausal women (WHI, 2008). The objective of this women's health research initiative was to conduct medical research into some of the major health problems of older women. In particular, clinical trials were designed and funded that address cardiovascular disease, cancer and osteoporosis. It consisted of a set of clinical studies on hormone therapy, diet modification and calcium and vitamin D supplements; an observational study and a community prevention study. A recent review of the WHI data indicates that there was a greater risk of fatal and nonfatal malignancies occurred after intervention with HRT, while the risk index was 12% higher in women randomly assigned to HRT compared with placebo (Heiss *et al*., 2008). There are other publications which minimize the findings of the WHI (Goldman, 2004).

An increasing number of studies have shown that certain risks are associated with combinations of progestins and equine oestrogens (Smith *et al*., 2004). According to a report citing early findings and reported at a recent American Academy of Neurology meeting, hormone therapy may help protect against dementia, even though it raises the risk of mental decline in women who do not take the drugs until they are older. Because these have been used most commonly and for the longest time, there are many more studies of these forms of hormones than of some of the newer forms with newer delivery systems, and therefore the most is known about these kinds (Knopman *et al*., 2007). Whether or not such risks exist with other forms of oestrogens and progestins, and other delivery systems, remains to be seen.

7.2.2 Testes

Male mammals have two testicles, which are located below the penis and are generally contained within an extension of the abdomen called the scrotum. The collecting tubules inside the testicle join together to form a tube called the epididymis which transports spermatozoa. The epididymis becomes the spermatic cord which continues to form a short tube called the ejaculatory duct. It is this duct that opens into the urethra (the tube from the bladder to the end of the penis), just above the prostate gland. Hormonal modulation of the male reproductive system is much less complex than that of the female. The testicles are homologous to the ovaries in that they produce gametes (but in this case spermatozoa) and they secrete a sex hormones (e.g. testosterone). It is possible to perturb the male reproductive system indirectly at the hypothalamic-adenohypophysis axis or directly at the level of the testes. As in the female, the functions of the testicles are stimulated by the gonadotrophin hormones produced by the anterior pituitary—LH (also referred to as the interstitial cell stimulating hormone (ISCH) in males) and FSH. These gonadotropic hormones modulate selected cellular processes with the testes, with FSH stimulating spermatogenesis and LH (ICSH) stimulating the production of testosterone in the Leydig cells. The principal androgenic steroid produced by the testes is testosterone, whose main function is to stimulate the development and growth of the male genital tract. Additionally, testosterone promotes protein generation (anabolic activity) which leads to enhanced muscle mass. In the foetus, testosterone ensures the development of the male genitalia. During puberty testosterone enhances the growth of the male sex organs and promotes other male characteristics such as deepening of the voice, growth of facial, pubic and body hair, enhanced muscle size and strength, enhances bone growth and strength, promotes sperm production and maintains the male libido. Testosterone binds to sex-hormone-binding-globulin (SHBG) and appears to work in conjunction with other carrier proteins to control the equilibrium between the free and bound androgens (Kapp and Thomas, 2008). While the gonadotrophins play an important physiological role in maintaining the integrity of the male reproductive system, there are a number of important local testicular factors including Sertoli cells, Leydig cells and germ cells. There are also peritubular cells and endothelial cells, which are located in the vascular system of the testes. These subpopulations of cells found throughout the mammalian testes are subject to some degree of local regulation by factors such as peptide growth factors, cytokines, preopiomelanocortin derivatives, neuropeptides and steroids (Foote and Berndtson, 1992; Foster, 1992; Ewing, 1992; Spiteri-Grech and Neischlag, 1993; Weinbauer *et al*., 2001).

7.2.2.1 Toxicological Effects

Xenobiotics, certain drugs and heavy metals can injure different gonadal cell populations and/or biochemical processes and they may act directly or indirectly through the CNS or possibly a combination of the two. The typical general characteristics of chemicals that are gonadotoxicants include: increase in lipophilicity, affinity for the AR, molecular weight <400 and the ability to cross the blood–testes barrier. Nutrition can play a significant role in maintaining the metabolic integrity of the testes and the male accessory sex organs and hence can affect the toxicity of a material in the testes. Heavy metals appear to affect different sites within the testes including the germ cell, Leydig cell and the Sertoli cell (see **Table 4**). Heavy metals—with the exception of lead—act directly upon the testes to cause damage to the germ cell (Thomas and Thomas, 2000). Cadmium, on the other hand, acts at several sites, but most frequently injures the endothelial cells of the vascular supply of the testes. **Table 5** shows many drugs/chemicals and relevant biological activities that can affect the secretion of the Leydig (interstitial) cell androgens (e.g. testosterone and dihydrotestosterone)

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Table 4 Cellular site of action of heavy metals on the male reproduction system

Metal	Hypothalamic/ adenohypophyseal effects	Testicular toxicity evidence			Mechanisms
		Germ cells	*Leydig cells*	*Sertoli cells*	
Cadmium	None	Some direct action. Possibly secondary	Some direct action. Possible secondary	Some direct action. Possibly secondary	Hypoxia/ischaemia of the endothelial cells
Chromium	None	Direct cellular action	No data	No data	Unknown
Cobalt	None	Some direct action. Possible secondary	No data	Some direct action. Possible secondary	Toxicity due to general hypoxia
Lead	Possible FSH & LH suppression	Direct cellular action	No data	Some direct action. Possibly secondary (?)	Endocrine and paracrine toxicity
Platinum	None	Direct cellular action	Direct cellular action	Direct cellular action	Inhibits DNA synthesis
Vanadium	None	Direct cellular action (?)	No data	No data	Unknown
Zinc	None	Deficiency causes cellular toxicity	Some direct action. Possible secondary	Deficiency causes cellular toxicity	Toxicity due to deficiency

Source: Thomas and Thomas (2000).

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Table 5 Chemicals and drugs producing Leydig cell hyperplasia and neoplasia

Agent	Agent class or biological activity
Cadmium	Heavy metal
Carbamazepine	Anticonvulsant/analgesic
Cimetidine	Histamine receptor blocker
Chlomiphene	Treatment of infertility
Cyclophosphamide	Antineoplastic
Diethylstilboestrol	Synthetic hormone
Dimethylformamide	Industrial use (tannery, leather goods, metal dyes)
Diltiazem, verapamil, nefedipine, telodipine	Calcium channel blockers
Diuron	Herbicide
Finasteride	5-α Reductase
Flutamide	Antiandrogen
Guanadrel	Adrenergic blocker
Gemfibrozil	Hypelipidaemic
Hydrazine reserpine	Antihypertensive
Mesulergine	Dopamine (D2) agonist/antagonist
Methoxychlor	Pesticide with oestrogen properties
Metronidazole	Antiprotozoal
Methylcholanthrene	Experimental carcinogen
Nafarelin	Luteinizing hormone-releasing hormone analogue
Nitrosamine	Industrial and food uses
Oestrogen	Hormone
Oxolinic acid	Antimicrobial
Perfluorooctanoate	Industrial ingredient (plasticizers, lubricant/ wetting agents)
Procymidone	Systemic fungicide
Spironolactone	Diuretic
Vidarabine	Antiviral

Source: Thomas and Thomas (2000).

(Thomas and Thomas, 2000). The mechanism of toxicity is generally competitive antagonism in the case of those substances that possess some inherent hormonal activity (e.g. oestrogen, diethylstilbestrol, spironolactone). In some instances the metabolite and not the xenobiotic itself is the testicular toxin. **Table 6** summarizes some of the indirect testicular toxins. In general, these gonadotoxins interfere with spermatogenesis and, depending upon the dose and exposure, can produce varying degrees of sterility. Both diethylhexyl phthalate (DEHP) and its metabolite monoethylhexyl phthalate (MEHP) are injurious to germ cells. It is also believed that these phthalates may interfere with Sertoli cell function.

Table 6 Metabolites which are toxic to the testes

Parent compound	Metabolite
Acrylamide (industrial chemical)	*N*-Methylacrylamide, *N*-isopropylacrylamide
Amiodarone (antiarrhythmic drug)	Desethylamiodarone
Benomyl (antifungal)	Carbenolazim
Cephalosporin analogues (antimicrobial drug)	*N*-Methyltetrazolethiol
Dibromochloropropane (nematocide)	Dichloropropene derivatives
Diethylhexyl phthalate (fungicide)	Monethylhexyl phthalate, 2-ethylhexanol
Ethylene glycol monethyl ether (solvent)	2-Methoxyacetaldehyde
n-Hexane (solvent)	Hexane-2,5-dione
Vinclozolin (fungicide)	Butenoic acid derivative and an enanilide metabolite

Source: Thomas and Thomas (2000).

7.3 Thyroid Gland

The thyroid gland is located on the anterior spinal chord between vertebrae C-5 and T-1. Thyroid hormones regulate the metabolism of almost every organ in the body. Normal thyroid function is a prerequisite for normal growth and development, the development of the nervous system and the normal functioning of the nervous, cardiovascular and gastrointestinal systems. Thyroid hormone serves to increase the metabolism of most body tissues. For instance, thyroid hormone stimulates the production of those proteins involved in heat generation. This is critical for maintaining body temperature in mammals. Thyroid hormone also enhances metabolic processes of carbohydrates, lipids and proteins that help generate energy necessary for bodily functions. Thyroid hormone is also necessary for the normal development of teeth, skin and hair follicles.

The thyroid produces two structurally related hormones—triiodothyronine (T_3) and thyroxine (T_4). Both of these hormones are iodinated derivatives of tyrosine and both are referred to as 'thyroid hormone'. In addition, the thyroid follicles which produce these hormones contain a protein termed thyroglobulin. Parafollicular cells or C cells which secrete calcitonin are found throughout the thyroid gland. Calcitonin is critical in the regulation of vitamin D, bone mineralization and

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the decrease in the calcium absorption process. While T_4 represents about 90% of the hormone produced by the thyroid gland, T_3 is the more active of the two. Interestingly, much of the T_4 is converted to T_3 in target organs such as the liver and kidneys.

The secretory process of the thyroid gland is under the influence of the adenohypophyseal-hypothalamic system and is mediated by TSH. TSH production is controlled by a TRH, which is produced in the hypothalamus and transported to the anterior pituitary gland, where it increases TSH production and release. When the thyroid is functioning normally it is termed euthyroidism. Hypersecretion of the thyroid gland leads to the condition termed thyrotoxicosis which can cause increased height growth and increased speed of all the body's organs and intestines. Undersecretion of the thyroid gland or hypothyroidism can lead to a severe skin and tissue disorder termed myxoedema. Congenital hypothyroidism results in cretinism and is characterized by mental retardation. Thyroid hyperplasia is also known as a goitre and may be caused by autoimmunity or a nutritional deficiency of iodine.

Thyroid cell growth and function are regulated by hormones and growth factors that bind to cell surface receptors that are coupled via the G-proteins, Gs and Gq, to the adenyl cyclase and phospholipase C signal-transduction systems (Wettschureck and Offermanns, 2005). G-proteins function as 'molecular switches', alternating between an inactive GDP- and active GTP- bound state, ultimately going on to regulate downstream cell processes. G-proteins were discovered by Gilman and Rodbell (Roche, 1996). They found that when a hormone like adrenaline was bound to a receptor, the receptor did not stimulate enzymes like adenylate cyclase directly. Instead, the receptor stimulated the production of a G-protein, which then stimulated the adenylate cyclase to produce a second messenger, cyclic AMP. The binding of TSH to its receptor results in activation via the G-proteins of the andenylyl cyclases and phospholipase C signalling pathways (Popov *et al*., 1997).

7.3.1 Toxicological Effects

Knowledge of thyroid gland biochemistry is important to understanding endocrine toxicity. **Table 7** shows the drugs and chemicals that interfere with the secretion of thyroid hormones. T_3 and T_4 are synthesized in several steps as shown and toxic agents can inhibit synthesis at several sites (Thomas and Thomas, 2000; Kester *et al*., 2004; Atterwill and Flack, 1992). These agents can be categorized by their mechanisms of toxic action. **Table 8** shows that drugs such as salicylates, anticoagulants and phenyltoin can change thyroid function by affecting T_4 uptake, ^{131}I uptake and thyroid-binding globulin (TBG) concentrations. Propylthiouracil (PTU), a drug used in the treatment of hyperthyroidism, decreases both T_3 and T_4.

Table 7 Drugs and hormones that interfere with the secretion of thyroid hormones

Blockage of iodide trapping	Blockage of iodide oxidation	Mechanism not established
Chlorate	Amphenone	Acetazolamide
Hypochlorite	Carbimazole	Chlorpromazine
Iodate	Cobalt	Chlortrimeton
Nitrate	Methimazole	Thiopental
Perchlorate	*p*-Aminosalicylate	Tolbutamide
Thiocyanate	Phenylbutazone	
	Phenylindanedione	
	Propylthiouracil	
	Resorcinol	

Source: Kapp and Thomas (2008).

Phenoxyisobutyrate derivatives such as clofibrate produce histological changes in the thyroid gland. Occupational cyanide exposure can impair thyroid function and is a known goitrogen (ATSDR, 2006).

Lithium has been shown to cause hypothyroidism by several proposed mechanisms. It may act directly by inhibiting the iodine-concentrating capacity and by interfering with the biosynthesis of iodotyrosine and iodothyronines. It could also inhibit the secretion of thyroid hormones by stabilizing follicular cell microtubular systems (Henry, 2002; Greller, 2007). Chlorine dioxide used for the bleaching of wood pulp, bleaching of flour and for the disinfection of some municipal drinking-water systems may decrease T_4 levels (IRIS, 2000) Amiodarone, an antiarrhythmic drug used to treat various forms of tachyarrhythmias, has been shown to cause both hyperthyroidism and hypothyroidism, since iodine constitutes 37.3% of its molecular weight (Meek and Smallridge, 2001). Amiodarone is also structurally similar to thyroxine which contributes to the effects of amiodarone on thyroid function. Amiodarone has specific inhibitory effects on agonist-stimulated functions in thyroid cells, which may interfere with TSH receptor interactions. (Batcher *et al*., 2007).

Many agents can affect the binding, distribution and metabolism of thyroid hormones, but infrequently cause permanent alterations in thyroid function, as evidenced by **Tables 7** and **8**. Prolonged thyroid hormone alterations caused by xenobiotics may lead to follicular cell carcinogenesis (Atterwill and Cockburn, 1997; NCI, 2008). Xenobiotic-induced thyroid tumours may be due to increased circulating levels of TSH, which may be the result of increased metabolism of T_4 via heparin enzymatic conjugation with uridine diphosphate glucuronosyl transferase (UGT) which is a family of enzymes that catalyse the addition of sugars to lipids and other apolar xenobiotics (Bock and Köhle, 2005). Increases in

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Table 8 Chemicals that modify thyroid activity

Chemical	T_3 uptake	^{131}I uptake	Thyroid-binding globulin concentration
Aminosalicylic acid	—	↓	—
Anabolic steroids	↑	—	↓
Anticoagulants (dicoumarol and heparin)	↑	—	—
Anti-inflammatory agents (phenylbutazone)	↑	↓	—
Indocyanine green (casrdiogreen)	—	↓	—
Corticosteroids	—	↓	↓
Phenytoin (DPH)	↑	—	—
Oral contraceptives	↓	—	↑
Lithium carbonate	—	↑	—
Phenothiazines	—	↓	↓
Salicylates	↓	—	—
Sulfonamides	—	↓	—
Cyanide	↓	—	↑

Source: Thomas and Thomas (2000).

UGT activity might be a useful biomarker for tumorigenic changes in thyroid hormone levels subsequent to exposure to TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) (Matsuura *et al*., 2001). The thyroid hormone-decreasing effects of TCDD are primarily extrathyroidal and mediated by a marked reduction of hepatic T_4 UGT activity (Schuur *et al*., 1997; ten Tusscher and Koppe, 2004).

Several plant toxins are considered natural goitrogens. Vegetables such as mustard, radish, horseradish, cress, cabbage, Brussels sprouts, kohlrabi, kale, cauliflower, broccoli, turnip, swede and rapeseed contain glucosinolates which are a class of organic compounds that contain sulphur, nitrogen and a group derived from glucose. They occur as secondary metabolites of many plants of the order *Brassicales* and also the genus *Dryetes* (Bones and Rossiter, 1996). Glucosinolates can be metabolized into thiocyanates and isothiocyanates, which are potent natural goitrogens. Further, garlic, onions and chives contain 5-substituted cysteine sulfoxides, which are also natural goitrogens.

Certain herbicides such as chlorinated phenylureas, biscarbamates, substituted uracils, pyridazinones and the biphenyl ethers can produce changes in thyroid gland activity. Nitrofen, a diphenyl ether herbicide, has been associated with congenital diaphragmatic hernia (CDH), and may interfere with thyroid hormone secretion (Kling *et al*., 2005). Apparently, nitrofen reduces follicular size and colloidal density in the thyroid (Christian and Trenton, 2003). Triazines are also herbicides that suppress the thyroid. Amitrole is chemically related to the triazines and also can promote an antithyroid action. This mechanism appears to be through the inhibition of thyroid peroxidase (TPO), which produces a decrease in thyroid hormone levels which, in turn, causes hyperplasia of the thyroid. As with other antithyroid compounds, amitrole can induce thyroid tumours (Durham, 2002). Aminothiazole is a heterocyclic amine that has a direct action upon the thyroid and acts by inhibiting T_4 synthesis and acceleration its deiodination. 2-Aminothiazole can be used as a thyroid inhibitor in the treatment of hyperthyroidism and it has antibacterial activity (Uchikawa *et al*., 1996). The herbicide 2,4-D can decrease serum protein-bound iodine. Fungicides such as nabam, zineb and ziram can also inhibit iodine uptake by the thyroid. Dithiocarbamate pesticides are toxic to the thyroid and appear to act upon TPO (Marinovich *et al*., 1997; WHO, 1970). The fluorinated herbicide thiazopyr causes an increase in thyroid follicular cell tumours in rats by increasing the amount of circulating TSH. This increase in TSH appears to stimulate the thyroid follicular cells resulting in follicular cell hypertrophy, hyperplasia and ultimately neoplasia (Holz *et al*., 1997).

Polychorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs) also affect thyroid gland metabolism causing goitres and hypothyroidism. This mechanism may be related to the ability of PCBs to cause developmental neurotoxicity (Miyazaki *et al*., 2004). Several polyhydroxyphenols, including resorcinol, catechol, hydroquinone, phloroglucinol and orcinol have been shown to inhibit TPO (Linsay and Gaitan, 1989).

Some chemicals, such as thioamine drugs, act directly upon the thyroid gland. In addition to these direct-acting antithyroidal agents, some drugs affect liver thyroid hormone-conjugating enzymes. Drugs that induce

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hepatic microsomal P450, for example, phenobarbital and β-naphthoflavone can cause hepatocellular and thyroid follicular cell hypertrophy (Johnson *et al*., 1993). Some drugs alter thyroid function indirectly through the CNS or the adenohypophysis (Philip *et al*., 1999). Glucocorticoids also impair basal and TRH-stimulated TSH levels (Pekary *et al*., 2006). Phenytoin sodium is a commonly used antiepileptic drug which decreases the TSH response to TRH by enhancing the cellular uptake and metabolism of T_4 (Nader, 2004). Acetylsalicylic acid and L-DOPA (3,4-dihydroxy-L-phenylalanine) can also suppress TSH responsiveness by displacement of thyroid hormones from TBG (Daminet, *et al*., 2003).

7.4 Pancreas

The pancreas is located in the posterior to the stomach in close proximity to the duodenum. The pancreas has two distinct functions: (i) It acts as an exocrine organ by producing digestive enzymes and an alkaline fluid, and secretes them into the small intestine through a system of exocrine ducts. The digestive enzymes include trypsin, chymotrypsin, pancreatic lipase and pancreatic amylase, and are produced and secreted by acinar cells of the exocrine pancreas. (ii) It acts as an endocrine organ which made up of a million cell clusters called islets of Langerhans (Hellman *et al*., 2007). There are four main cell types in the islets which can be classified by their secretion, including α cells, which secrete glucagon, β cells, which secrete insulin, δ cells, which secrete somatotrophin and F cells (PP cells), which secrete pancreatic polypeptides (PP). In addition ε cells of the pancreas have also been identified as releasing ghrelin, an appitite-promoting peptide (Prado *et al*., 2004).

The exocrine pancreas constitutes the majority of the pancreas and is regulated by both neural and endocrine controls. During periods between digestion, little secretion takes place, but as food enters the stomach and, as chyme flows into the small intestine, pancreatic secretion is strongly stimulated. Trypsin and chymotrypsin are proteases which are synthesized by the pancreas. Interestingly, they are packaged as inactive precursors and the secretory vesicles also contain a trypsin inhibitor to keep them inactive until they are safely out of the pancreas and the ducts. The proteases are converted to their active forms by enterokinase, which is located in the intestinal mucosa.

The endocrine pancreas produces hormones in the islets of Langerhans which are directly secreted into the blood stream. The types of cells and their secretions are shown in **Table 9**. The two primary hormones released for the pancreas are insulin and glucagon. Insulin is the primary regulator for carbohydrate homeostasis by controlling the blood glucose levels. Insulin also regulates the uptake of amino acids and electrolytes, muscle tone and the release of triglycerides. Glucagon complements glucose regulations through glucogenolysis (the release of glycogen stores) and gluconeogenesis (the biosynthesis of additional glucose).

A primary concern in testing for toxicity of an experimental drug is the potential for the compound to interfere with the normal functioning of the pancreatic α cells. Often pancreatic toxicity is heralded by hyperglycaemia. The relationship between insulin and carbohydrate, fat and protein metabolism is evident in diabetes mellitus. Insulin is an anabolic hormone that promotes sequestration of carbohydrate, fat and protein storage depots throughout the body. Its actions are exerted primarily on skeletal muscle, liver and adipose tissue. On the other hand, the actions of glucagon are counter-regulatory to insulin by producing gluconeogenesis and glucogenolysis in the liver. The interplay between these two hormones contributes to homeostasis in glucose concentrations and thus to the availability of

Table 9 Cells of the islets of Langerhans and their hormones

Cell type	Location	% of total cells	Hormone	Function
α-cell	Periphery of islets	10–15%	Glucagon	Carbohydrate metabolism—stimulates glycogen to glucose
β cell	Throughout islets	70–80%	Insulin and amalin	Insulin—manages removal and use glucose from the blood
				Amalin—contributes to glycaemic control
δ cell	Periphery of islets	3–5%	Somatotrophin	Hormone which inhibits various releases from the anterior pituitary and the GI tract
F (PP) cell	Head of pancreas	<1%	Pancreatic polypeptide	Regulates the pancreas secretion activities (endocrine and exocrine); also affects hepatic glycogen levels and gastrointestinal secretions
ε Cell	Throughout islets	<2%	Ghrelin	Stimulates growth hormone secretion; regulates energy balance via hunger

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Table 10 Class I drugs commonly associated with acute pancreatitis (>20 cases reported and one + rechallenge)

Drug	Function
Asparaginase	Chemotherapeutic
Azathioprine	Immunosuppressant
Furosemide	Diuretic
6-Mercaptopurine	Immunosuppressive
Mesalamine	Anti-inflammatory
Oestrogens	Hormone replacement
Opioids	Analgesic
Pentamidine	Antimicrobial
Steroids	Immunosuppressant
Sulfamethoxazole	Bacteriostatic drug
Sulindac	Nonsteroidal anti-inflammatory drug (NSAID)
Sulfasalazine	Sulfa anti-inflammatory
Tetracycline	Broad-spectrum polyketide antibiotic
Valproic acid	Anticonvulsant and mood-stabilizer

Source: Eltookhy and Pearson (2006); Trivedi and Pitchumoni (2005).

metabolic energy to all cells. Glucagon acts in concert with other fuel-mobilizing hormones to balance the metabolic effects of insulin.

7.4.1 Toxicological Effects

Acute pancreatitis typically is an inflammation of the pancreas that involves the surrounding tissue. Generally, gallstones account for about 30–60% of the cases, while excessive use of alcohol accounts for about 15–30% of the cases. Less common causes include hypertriglyceridemia, hyperparathyroidism, trauma, pancreatic tumours, endoscopic retrograde cholangiopancreatography (ERCP) and postabdominal and nonabdominal surgical procedures (Eltookhy and Pearson, 2006). Drug-induced pancreatitis (DIP) is less common, but it still accounts for up to 5% of the cases found in the general population (Greenberger and Toskes, 2008). There is no clear evidence that drugs that cause acute pancreatitis also cause chronic pancreatitis (Trivedi and Pitchumoni, 2005). Acute pancreatitis is frequently accompanied by increased level of pancreatic enzymes—notably amylase. The mechanism of cellular inflammation in pancreatitis is not understood, but several factors may contribute. Factors that stimulate primary sensory neurons include hydrogen ions, heat, leukotrienes, arachidonic acid metabolites, bradykinin and proteases such as trypsin, all of which may participate in the generation of acute pancreatitis (Liddle and Nathan, 2004). The cellular and molecular mechanisms involved in sensory nerve activation within the pancreas are complex; however, a key factor to the development of the damage to the pancreas often occurs as the result of digestive enzymes leaking into the pancreas and starting to digest the organ itself. This sets up inflammation, and when the inflammation settles, the scarring process distorts the pancreas, making further attacks of inflammation likely. Thus a vicious cycle develops. As previously noted, drug-related episodes of pancreatitis account for about 5% of the pancreatitis case reports found in the literature. Most of the evidence for drug-induced pancreatitis is anecdotal, thus making definitive classifications difficult at best. There are numerous complicating factors that add to the complexity of making the determination, so, many authors have classified drug association with pancreatitis based upon the number of cases reported in the literature. Based upon that assumption, Trivedi and Pitchumoni (2005) proposed a classification which categorizes drugs into Class I, II or III. Class I drugs are associated with pancreatitis and have at least 20 reported cases of acute pancreatitis with at least one case with a positive rechallenge (see **Table 10**). Class II drugs are associated with pancreatitis and have >10 but <20 reported cases of acute pancreatitis with or without positive rechallenge (see **Table 11**). Class III drugs are all medications implicated in pancreatitis, for example, Class

Table 11 Class II drugs commonly associated with acute pancreatitis (>10 but <20 cases reported)

Drug	Function
Acetaminophen (paracetamol)	Analgesic and antipyretic
Carbamazepine	Anticonvulsant and mood stabilizer
Cisplatin	Chemotherapeutic
Cytarabine	Chemotherapeutic
Cyclopenthiazide	Chemotherapeutic
Enalapril	Angiotensin converting enzyme (ACE) inhibitor
Erythromycin	Macrolide antibiotic
Hydrochlorothiazide	Diuretic
Interferon-α	Chemotherapeutic
Lamivudine	Nucleoside analog reverse transcriptase inhibitor (nRTI).
Octreotide	Synthetic somatostatin
Oral contraceptives	Birth control
Phenformin	Antidiabetic
Rifampicin (rifampin)	Bactericidal antibiotic

Source: Eltookhy and Pearson (2006); Trivedi and Pitchumoni (2005).

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Table 12 Class III drugs commonly associated with acute pancreatitis (all drugs implicated)

General functional category	Drug
Antimicrobial agents	Abacavir, alatrofloxacin, amoxicillin, ampicillin, atovaquone, azithromycin, ceftriaxone, cidofovir, ciprofloxacin, clarithromycin, dapsone, demeclocycline, didanosine, delavirdine, doxycycline, efavirenz, enfurvirtide, ertapenem, fluconazole, foscarnet, ganciclovir, indinavir, isoniazid, lamivudine, levofloxacin, metronidazole, minocycline, monocycline, nelfinavir, nitrofurantoin, norfloxacin, quinolones, quinpristin/dalfopristin, penicillin, ribavirin, rifapentine, ritonavir, saquinavir, stravudine, trovafloxacin, trimethoprim/sulfamethoxazole, voriconazole, zalcitabine, zidovudine
Anti-inflammatory agents	Celecoxib, codeine, colchicine, diclofenac, hydrocodone, ibuprofen, ketoprofen, ketorolac, meloxicam, sulinac, mefenamic acid, morphine, nabumetone, naproxen, oxycodone, piroxicam, rofecoxib
Antineoplastic agents	Alemtuzumab, asparaginase, capecitabine, cyclophosphamide, doxorubicin, estramustine, gefitinib, ifosfamide, imatinib, levamidole, methotrexate, oxaliplatin, paclitaxel, pegasparaginase, tamoxifen, thalidomide, trastuzumab, vinblastine, vinorelbine
Cardiovascular agents	Amiodarone, amlodipine, ateriolol, benazepril, bendroflumethiazide, candesartan, captopril, chlorothiazide, chlorthalidone, enalapril, ethracrynic acid, fosinopril, furosemide, hydrochlorothiazide, irbesartan, lisinopril, losartan, methyldopa, metolazone, mexilitine, moexipril, perindopril, prazosin, polythiazide, quinapril, ramipril, thiazide, trandolapril
Gastrointestinal agents	Azathioprine, balsalazide, cimetidine, diphenoxylate, famotidine, granisetron, mercaptopurine, mesalamine, olsalazine, omeprazole, ondansetron, pantoprazole, phenolphthalein, rabeprazole, ranitidine, sulfasalazine
Synthetic hormones	Cortisone, danasol, dexamethasone, fludrocortisone, methylprednisolone, octreotide, prednisolone, prednisone, somatrophin, conjugated oestrogens, ethinyl oestradiol, oestradiol
Hyperlipidaemia agents	Artorvaststin, bezafibrate, fenofibrate, fluvastatin, gemfibrozil, lovastatin, pravastatin, simvastatin
Immunomodulating agents	Cyclosporine, glatiramer, interferon β-1b, interferon γ-1b, mycophenolate, sirolimus, tacrolimus, thalidomide, peginterferon α-2B
Neuropsychiatric agents	Aripiprazole, bupropione, carbamazepine, citalopram, clozapine, divalproex sodium, escitalopram, felbamate, fluoxetine, gabapentin, lamotrigine, mertazapine, olanzapine, paroxetine, quetiapine, risperidone, rivastigmine, sertraline, sumatriptan, tacrine, topiramate, valproic acid, venlaflaxine, zolmitriptan
Thrombolytic agents	Aspirin, alteplase, anagrelide, dipyridamole, reteplase, streptokinase
Miscellaneous agents	Acitretin, alendronate, calcitrol, clomiphene, contrast media, doxercalciferol, etanercept, finasteride, glimepiride, glyburide, gold, granulocyte colony stimulating factor (G-CSF), infliximab, isotretinoin, metformin, MMR vaccine live, montelukast, penicillamine, pergolide, pilocarpine, propofol, propylthiouracil isotretinoin, repaglinide, riluzole, rivastigmine, ropirinole, tacrine, tretinoin

Source: Eltookhy and Pearson (2006); Trivedi and Pitchumoni (2005).

I, Class II and all others with $\leq$10 reported cases (see **Table 12**).

Pancreatitis appears to be induced by a number of different mechanisms and the effects can be direct or indirect. In general, direct effects include toxicity- and hypersensitivity-type reactions. Indirect effects include, but are not limited to, ischaemia, thrombosis and increased viscosity of pancreatic juices (Frazee, 2004). The correlation between antibiotics and pancreatitis is poorly understood and has been speculated to be due to immune-mediated reactions, toxic metabolites and increased pressure of the sphincter of Oddi by gallbladder contractions (Blomgren *et al*., 2002). DIP is reported to be 40% higher in antibiotic treatment of opportunistic infections associated with human immunodeficiency virus (HIV) (Trivedi and Pitchumoni, 2005). Management of these infections is complicated by the fact that there are a plethora of drugs that have been reported to cause acute pancreatitis and are yet necessary for the patient.

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Table 13 Diabetogenic agents

Agent	Function	Susceptible species
Alloxan	Experimental diabetes induction	Several
Cyproheptadine	Antihistamine; antiseroton-ergic	Rodents
Hexamethyl-melamine	Antineoplastic	Rat
Pyriminil (Vacor)	Rodenticide	Human
Streptozocin	Experimental diabetes induction; antineoplastic	Several
Verapamil	Calcium-channel blocker	Human

Source: Thomas and Thomas (2000).

Alloxan destroys pancreatic β cells and leads to experimental diabetes mellitus in rodents. Alloxan has avidity for rodent pancreatic β cells and concentrates in this organ. It is rapidly metabolized to dialuric acid, a product that undergoes autoxidation to yield peroxide, superoxide anion and free radicals. Streptozocin is also a β-cell toxin whose toxic properties reside in its *N*-methylnitrosourea moiety. Streptozocin is also an alkylating agent. Nitroso agents such as streptozocin and cycasin induce long-lasting damage to both the β cells and the neurons (Eizirik *et al*., 1996). Both alloxan and streptozocin can damage pancreatic DNA. Streptozocin also impairs reproductive function by causing a reduction in LH secretions by the pituitary (Bestetti *et al*., 1997).

Agents effective in producing hyperglycaemia are referred to as diabetogenic substances. The common diabetogenic substances are listed in **Table 13**. The list covers a broad biological spectrum of chemicals that are able to cause destruction of the β cells of the pancreas. Although alloxan was one of the first agents used to produce β cell necrosis, it has largely been replaced by streptozocin to produce experimental insulin-dependent diabetes. Cyproheptadine (CHP), pentamidine and hexamethylmelamine are capable of suppressing the function and altering the morphology of insulin-secreting cells. CHP is an antihistaminic and antiserotonergic agent which acts as a 5-HT_2 receptor antagonist. 5-HT_2 receptors are receptors for the neurotransmitter and peripheral signal mediator serotonin, also known as 5-hydroxytryptamine or 5-HT. CHP also blocks calcium channels. CHP and its analogues appear to suggest that the inhibition of insulin synthesis known to be elicited by CHP treatment of RINm5F cells (insulin-producing clonal cells) and intact animals involves alterations of initiation-factor phosphorylation leading to a decrease in insulin synthesis and of stored insulin in insulin-producing cells (Fischer, 1996; Hawkins and Fischer, 2004). Pentamidine is an antimicrobial used for prevention and treatment of pneumocystic pneumonia, a severe pneumonia often seen in patients with HIV. The exact mechanism of its antiprotozoal action is unknown (though it may involve reactions with ubiquitin), despite the fact that it is a basic therapeutic modality (in concurrence with multiple antifungal medications) when treating *Acanthamoeba* infections in immunocompromised patients (Nguewa *et al*., 2005). Hexamethylmelamine is used to treat refractory ovarian cancer. Although hexamethylmelamine structurally resembles an alkylating agent, it has not been found to have alkylating activity *in vitro*. The mechanism by which exerts its anticancer effect is unknown, but there is some evidence that it may inhibit DNA and RNA synthesis (Hansen and Hughes, 1991). Diphenylhydantoin (DPH) is a commonly used antiepileptic which exerts inhibitory effects upon insulin secretion. The mechanism of action of DPH is also not well characterized, however extensive research strongly suggests that its main mechanism is to block frequency-, use- and voltage-dependent neuronal sodium channels, therefore limiting repetitive firing of action potentials (Ebert *et al*., 1997). Diazoxide is an antihypertensive which also has an inhibitory effect upon insulin secretion. Diazoxide acts as a potassium-channel activator causing local relaxation in smooth muscle by increasing membrane permeability to potassium ions, thus switching off the voltage-gated calcium ion channels, which inhibits that action potential (Korotkov *et al*., 2006).

Thiazolidinediones (TZDs)—also referred to as glitazones—and related compounds are a group of active ingredients that augment the action of insulin in insulin-resistant states. TZDs act by binding to peroxisome proliferator-activated receptors (PPAR)s, a group of receptor molecules inside the cell nucleus, specifically *PPAR*γ (gamma). The normal ligands for these receptors are free fatty acids (FFAs) and eicosanoids or oxygenated EFA signalling molecules. When activated, the receptor migrates to the DNA, activating transcription of a number of specific genes. This class of oral antidiabetic agents decrease hyperglycaemia and hyperinsulinaemia by increasing insulin-sensitivity in target tissues.

Oral hypoglycaemia drugs such as sulfonylureas act by increasing insulin release from the β cells. They are used therapeutically for diabetes mellitus Type II (formerly termed noninsulin-dependent diabetes mellitus (NIDDM, or adult onset diabetes). Cyclosporine is an immunosuppressant drug widely used in postallergenic organ transplant to reduce the activity of the patient's immune system and subsequently the risk of

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organ rejection. Cyclosporine is thought to bind to the cytosolic protein cyclophilin (immunophilin) of immunocompetent lymphocytes, specifically the T-lymphocytes. This complex of cyclosporine and cyclophylin inhibits calcineurin, which under normal circumstances is responsible for activating the transcription of interleukin-2. It also inhibits lymphokine production and interleukin release and therefore leads to a reduced function of effector T-cells. It does not affect cytostatic activity (Pritchard, 2005). Cyclosporine also has an effect on mitochondria by preventing the mitochondrial permeability transition (PT) pore from opening, thus inhibiting cytochrome c release, which is a potent apoptotic stimulation factor (Borel, 2002).

Cholecystokinin (CCK), a peptide hormone of the gastrointestinal system responsible for stimulating the digestion of fat and protein and enhancing insulin secretion, can be antagonized by loxiglumide, a CCK A receptor antagonist. This may, in turn reduce the level of metabolic stress on the acinar cells and has been shown to mitigate the severity of acute pancreatitis. CCK receptors can be found in the gall bladder and pancreatic membranes.

Vanadate has been demonstrated to interact chemically with the hydrogen peroxide formed by amine oxidases resulting in the generation of peroxovanadium. This material can mimic virtually all the biological effects of insulin (Nolte *et al*., 2003).

Benzylamine, a substrate of semicarbazide-sensitive amine oxidase, has been shown to stimulate glucose uptake and inhibit lipolysis in human fat cells (Iglesias-Osma *et al*., 2004). Galparan is a chimeric peptide that can powerfully stimulate reversible insulin secretion acting at a distal site in the stimulus-secretion coupling of the β cell (Östenson *et al*., 1997). Chemicals such as cobalt can selectively destroy pancreatic α cells. There are species differences with regard to α-cell destruction. The injection of cobalt chloride has been shown to produce degranulation and vacuolization of the α cells in rabbits, dogs and guinea pigs (Mobasheri *et al*., 2006).

Some drugs, such as alloxan and streptozocin produce destruction of β cells leading to diabetogenic states, while others cause pancreatitis. Hence, both endocrine and exocrine functions are vulnerable to inflammation and chemically induced necrosis. The possible mechanisms of some DIP are listed in **Table 14**. Diuretic-induced changes in electrolytes appear to be correlated with acute pancreatitis—especially in association with hyperglycaemia—the state produced by a higher than normal level of glucose in the blood. Tetracyclines may exert direct pancreatotoxicity: azathioprine—an immunosuppressant which acts to inhibit purine synthesis necessary for the proliferation of cells—suppresses the immune system, yet also has a direct cytotoxic action (Konstantopoulou *et al*., 2005). Valproic acid is an anticonvulsant and mood-stabilizing drug, primarily in the treatment of epilepsy and bipolar disorder which exerts direct cytotoxic effects (Admirant *et al*., 2006). L-Asparaginase, an enzyme that catalyses the hydrolysis of asparagine to aspartic acid and is used to treat acute lymphoblastic leukemia, has been shown to be directly correlated to acute pancreatitis (Appell *et al*., 2007).

Table 14 Possible mechanisms of drug-induced pancreatitis

Mechanism	Drugs implicated
Pancreatic duct construction	Indomethacin, salicylates (via prostaglandin inhibition), opiates
Immune suppression	Steroids, azathioprine
Cytotoxic effect	Azathioprine, colaspase (L-asparaginase)
Arteriolar thrombosis	Oestrogens
Osmotic effects	Contrast media
Pressure effects	Contrast media
Metabolic effects (ionic changes)	Thiazides
Direct cellular toxicity	Sulfonamides, furosemide (frusemide), chlorothiazide
Possible hepatic mechanism (via free radicals)	Paracetamol (acetaminophen), tetracyclines

Source: Thomas and Thomas (2000).

Although some drugs can cause acute pancreatitis, their mechanisms of toxicity are poorly understood. In others some underlying aspects of the pathobiology of experimental acute pancreatitis are known. Animal models of pancreatic carcinoma have been induced by chemical carcinogens and by transgenic methods. Hence drugs or chemicals not only exert short-term inflammatory responses in the pancreas, but also their chronic effects may lead to tumourigenesis. Pancreatic tumourigenesis may be caused by several mechanisms (Obourn *et al*., 1997; Esposito *et al*., 2007; Maitra and Hruban, 2008). These suggested mechanisms are CCK receptor agonism, typsin inhibition, alteration in gut fat content, cholestasis and altered bile flow/composition, many based upon a genetic predisposition germ-cell and somatic-cell gene mutations. Drugs that produce peroxisome proliferation also produce pancreatic acinar cell hyperplasia/adenocarcinomas (Obourn *et al*., 1997; Vitale *et al*., 2007; Pogue-Geile *et al*., 2006).

Numerous agents and proposed mechanisms induce disorders of glucose metabolism. The most important are those that produce hypoglycaemia (**Table 15**) and those that produce hyperglycaemia (**Table 16**). The effects of a

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Table 15 Agents that produce hypoglycaemic and possible mechanisms of action

Decreased counter-regulatory responses	Increased insulin secretion	Increased insulin sensitivity
ACE inhibitors	β sympathomimetrics	ACE inhibitors
β blockers	Acetylsalicylic acid	β blockers
Ethanol	Disopyramide	Human insulin-like growth factor
Octreotics	Ethanol	
	Pentamidine	
	Quinine	
	Sulfonylureas	
	Cotrimoxazole (trimethoprin-sulfamethoxazole)	

Source: Thomas and Thomas (2000).

Table 16 Agents that produce hyperglycaemia and possible mechanisms of action

Increased counter-regulatory responses	Reduced insulin secretion	Decreased insulin sensitivity
β sympathomimetrics	β blockers	β blockers
Diazoxide	Diazoxide	Diuretics
Growth hormone	Diuretics	Corticosteroids
Corticosteroids	Octreotics	

Source: Chan *et al*. (1996).

drug on glucose metabolism may be due to a combination of factors involving the secretion and action of insulin, as well as counter-regulatory hormones and responses. **Table 15** delineates some possible hypoglycaemic mechanisms. The most important therapeutic agents causing hypoglycaemia include insulin, the biguanides, the thiazolidinediones and the sulfonylurea drugs. Mechanisms that can produce hyperglycaemia are shown in **Table 16**. The adverse metabolic effects of a drug and its underlying mechanisms may provide important insights into the control of glucose regulation.

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Immunotoxicology

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CONTENTS

1 OVERVIEW

For the purposes of risk assessment, immunotoxicity is defined as unintended modulation of immune function. Depending on dose, duration of exposure, mode of action and biopersistence, immune system effects may be detectable at the molecular or cellular level (altered markers of exposure) and/or at the organism level (altered function, adverse health effects). As depicted in **Figure 1**, suppression of immune function is associated with increased rates of infection and some types of cancer, whereas inappropriate modulation of the immune system may result in allergic and autoimmune responses.

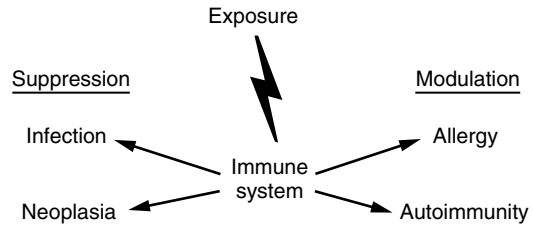

Figure 1 Xenobiotic exposure and immunocompetence.

1.1 Function of the Immune System

The immune system is a complex set of cellular and soluble mediators that protects the body against infectious agents and certain types of tumour cells via adaptive and innate responses. Adaptive responses require recognition of foreign antigens via the complex interaction of cell-surface molecules, production of growth factors, lymphocyte proliferation (clonal expansion) and implementation of effector mechanisms that ultimately mediate destruction of the foreign threat. The response is very specific, but is rather slow (4–10 days to the peak response). In contrast, innate responses do not require antigen recognition or clonal expansion and thus provide a rapid response (<24 hours) to infection.

Although most immune responses are beneficial, hypersensitivity reactions and autoimmune diseases are examples of inappropriate responses that cause morbidity and may be fatal. The diseases are believed to require genetic susceptibility and an environmental trigger. Hypersensitivity responses include four distinct types of immune responses. Allergy (Type I or immediate hypersensitivity) is characterized by the production of antibodies (typically IgE, immunoglobulin E) against normally innocuous molecules, including pollen, components of food, drugs and insect venoms. These antibodies attach to receptors on mast cells and eosinophils and trigger the release of potent proinflammatory and vasoactive compounds that cause smooth muscle contraction, swelling and inflammation. The most extreme manifestation of Type I responses is anaphylaxis, in which bronchoconstriction, airway oedema and cardiovascular effects may cause death. Type II and Type III responses are often associated with autoimmune disease; antibodies bind to host cells or tissues (Type II) or form complexes with antigens and accumulate in tissues (Type III), triggering inflammation and, in some cases, cytotoxic responses that destroy host tissues. Type IV responses are mediated by thymus-derived lymphocytes (Section 1.2) that respond to modified host proteins (e.g. allergic contact dermatitis to metals) or to exogenous proteins that are recognized as foreign (e.g. poison ivy). Autoimmunity is characterized by an immune response directed against host tissues that, for a variety of reasons, are not recognized as 'self'. This may be the result of a failure to remove self-reactive cells (Section 1.2), or failure to develop regulatory cells that limit immune system activity. Although some degree of autoreactivity is not uncommon, a robust response to self invariably leads to tissue damage and, in extreme cases, death. Although autoimmune diseases have been extensively studied, the initiating events are poorly understood. Hypersensitivity and autoimmunity are discussed in greater detail later in this chapter.

1.2 Cells and Tissues of the Immune System

Immune cells are located throughout the body, either in discrete organs, such as the bone marrow, spleen, thymus and lymph nodes or in diffuse accumulations of lymphoid and myeloid cells, as are found in association with the skin, lung and gastrointestinal tract, strategic locations for detection of entering pathogens and exogenous proteins (**Figure 2**). Nonspecific effector cells include macrophages (production of proinflammatory mediators, phagocytosis of pathogens and dead cells), natural killer (NK) cells (contact-dependent killing of certain tumour cells and pathogens) and polymorphonuclear (PMN) leukocytes (phagocytosis of bacteria). Lymphocytes are the prime cellular effectors of adaptive responses and are classified by their tissue of origin and subdivided based on function or maturity. Bone marrow-derived lymphocyte progenitor cells that migrate to and mature in the thymus are referred to as T lymphocytes or T cells; B lymphocytes or B cells are also derived from the common lymphocyte progenitor population and take their name from the Bursa of Fabricius, the site of B cell maturation present only in avians. In all other vertebrates, B cells migrate to and mature in bursal equivalents, including the spleen and lymph nodes. T cells that assist in and amplify other immune responses are known as T helper (Th) cells or CD4+ T cells, because they express CD4 antigen (CD - cluster of differentiation) on the cell

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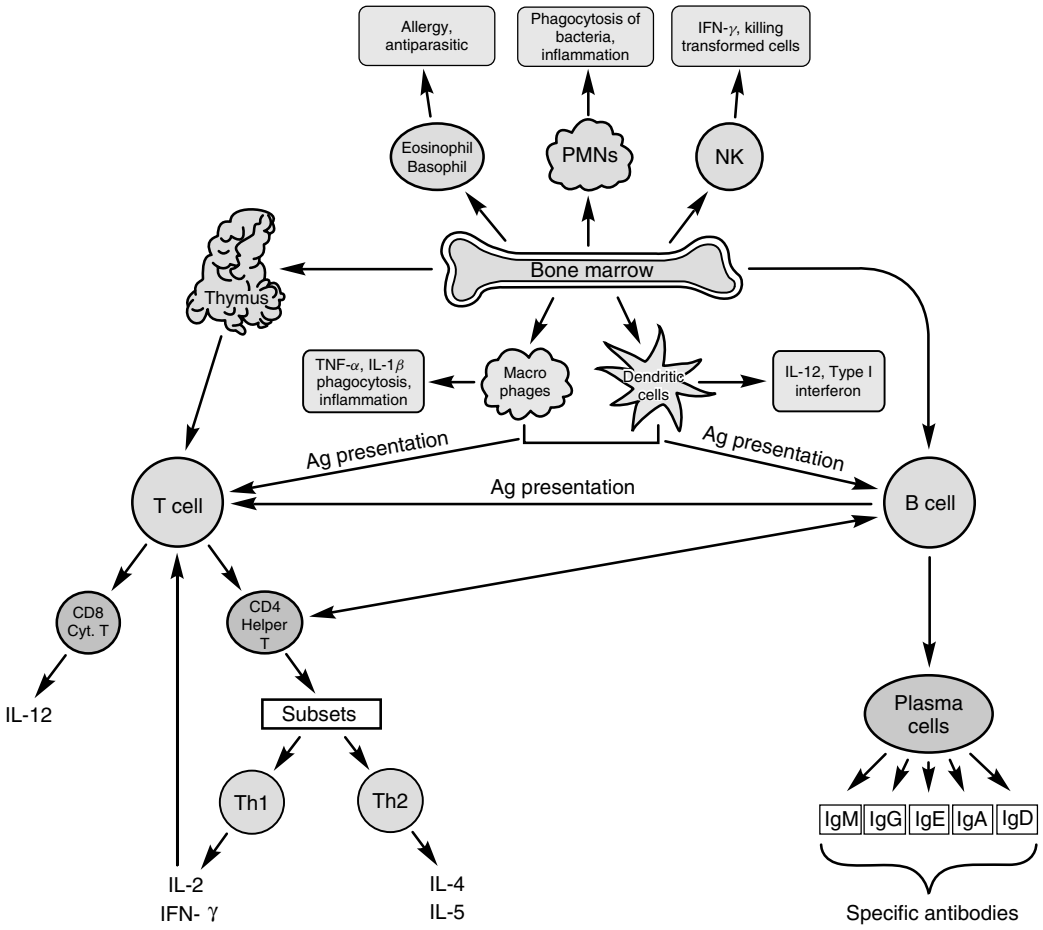

Figure 2 Cells and mediators of the immune system.

surface. Subpopulations expressing surface CD8 antigen down-regulate other immune responses (T suppressor cells; Ts) or act as effectors to destroy infected or neoplastic cells (T cytotoxic cells; Tc).

The bone marrow is the major haematopoietic organ in adult mammals. It is located within the central cavity of the major long bones, as well as in the core of the spongy bones of the sternum, skull and vertebrae. The marrow contains a complex mixture of stem cell progenitors that differentiate into cells of the erythroid, myeloid, platelet and lymphoid lineages. Compounds that target the bone marrow cells, certain malignancies and other processes that compromise marrow function are associated with profound alterations in immune function.

The thymus is the site of T cell maturation and selection. Selection is critical to establishing and maintaining tolerance to constitutively expressed 'self' proteins (antigens) present on somatic cells. Most (>95%) immature T cells that migrate to the thymus are eliminated, because of either inefficient or overzealous recognition of host antigens. Failure to remove these autoreactive clones is a contributing factor in autoimmune disease. Compounds that target the thymus may cause significant suppression of cellular and humoral immune function by disrupting maturation of T cell progenitors.

The spleen contains periarteriolar lymphoid sheaths (T cell areas), adjacent follicles (B cell areas), the marginal zone within the white pulp and large numbers of NK cells in the red pulp, which serves as a high-volume filter for the circulatory system. Follicle cellularity and germinal centre development have been reported to be the most sensitive histopathological predictors of immunotoxicity (Germolec *et al.*, 2004), because these compartments consist of highly active structures, formed as a direct result of immune activation that stimulates extensive lymphocyte proliferation and differentiation.

Lymph nodes consist of the cortex, composed of follicles and germinal centres (the B cell zone) and the paracortex (the T cell zone). Lymph nodes act as filters for the lymphatic and circulatory systems, trapping infected or damaged host cells, and can serve as a local source of activated immune cells in response to infection or inflammation. As discussed above for the spleen, germinal centres are distinct, highly active structures, formed as a direct result of immune activation, and thus would be susceptible targets for chemical-induced insult. Specialized lymphoid tissues

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associated with the skin and mucosal surfaces are discussed in Section 2.

1.3 The Innate Immune Response

Cells of the innate immune system are 'early responders' and arrive at the site of infection within hours. Pathogen-associated molecular patterns (PAMPs), structural and genetic components of infectious agents, are expressed by microbes and recognized by innate immune system cells via pattern recognition receptors (PRRs), including membrane-bound toll-like receptors and cytosolic NOD (nonobese diabetic)-like receptors. Ligation of PRRs activates phagocytic cells to engulf bacteria and to release proinflammatory molecules that activate other cells and attract new cells to the site of infection. PRRs on the surface of dendritic cells (DCs) play a key role in stimulating antigen processing and presentation to lymphocytes, thus driving the development of the adaptive response. In addition to phagocytic myeloid cells, specialized lymphocytes known as NK (natural killer) cells recognize and bind to some types of tumour cells and destroy the targeted cell by release of granules containing lytic enzymes. A variety of soluble mediators contribute to innate responses as well, including the complement cascade (lysis of cells and augmented phagocytosis of bacteria) and cytokines (modulation of the inflammatory response). Innate responses are well conserved phylogenetically; the same or very similar responses that mediate resistance to infection in invertebrates act as a first line of defence in mammals to destroy similar classifications of pathogens.

1.4 Induction of the Adaptive Immune Response

1.4.1 Antigen Recognition, Processing and Presentation

When substances from infectious agents or altered host cells are recognized as foreign, a complex series of events is initiated that culminates in an immune response. Protein antigens are captured, processed to peptide fragments, and presented to lymphocytes bearing antigen-specific surface receptors by professional antigen presenting cells (APCs) (macrophages, B cells and dendritic cells), triggering cytokine production, gene rearrangement, cell proliferation, differentiation and maturation into cells responsible for effector function and immunological memory. Triggering of this process depends on concurrent binding of processed peptide to antigen receptors and host-specific proteins of the major histocompatibility complex (MHC) to receptors on the responding lymphocytes. Polymorphisms in MHC proteins can alter antigen presentation and recognition, leading to specific nonresponsiveness ('tolerance') to an antigen, or enhanced responsiveness to foreign or self-antigens, resulting in allergy or autoimmune disease. A lack of surface MHC antigens, as occurs on certain neoplastic cells, acts as a trigger to initiate cell lysis by NK cells of the innate immune system.

1.4.2 Cell-Mediated Responses

Recognition of antigens and co-stimulatory molecules on APCs by cell-surface receptors on naive T cells initiates a highly regulated signalling cascade that culminates in clonal expansion of effector and memory cells. The circumstances of antigen processing and antigen presentation ultimately control whether the resulting clones are helper (Th) or effector T cells (Tc or regulatory T cells; Treg). Th cells produce cytokines that regulate immune function and can be further subdivided into subpopulations which assist other cells (Th1) or which stimulate and perpetuate antibody responses (Th2). Th2 cytokine production predominates in newborns and is also associated with increased allergy and asthma. Soon after birth there is a switch to the Th1-dominated adult phenotype, important in eliminating certain bacteria and viruses.

1.4.3 Antibody Synthesis

Circulating naive B cells encounter antigen in lymph nodes or tissue-associated lymphoid tissues and become activated. B cells recognize antigen via membrane-bound antibody/immunoglobulin (Ig) molecules that act as antigen receptors. Crosslinking of Ig molecules on the cell surface initiates a signal transduction cascade that, with the appropriate stimulus from cytokines, leads to activation, proliferation and differentiation into antibody-secreting plasma cells. Considerable heterogeneity in the composition and arrangement of amino acids in the Ig binding site makes recognition of a large number of widely diverse antigens possible. Five classes of antibodies—IgM, IgG, IgE, IgA and IgD—have been described based on the characteristics of their heavy chain polypeptides, and each of these antibody classes has distinct expression patterns and functional properties. IgM, the first antibody to be produced, is generally of low affinity; however, its pentameric conformation allows it to bind effectively to multivalent antigens such as bacterial cell-wall polysaccharides, and it plays a significant role in the activation of the complement system. IgG is the most abundant isotype in serum and is important in the neutralization of bacterial toxins, opsonization of pathogens for phagocytosis and complement-mediated cell lysis. IgA is important in immunity at the mucosal surfaces of the body and protects via binding and crosslinking of pathogens. IgE binds with high affinity to mast cells, leading to the release of inflammatory mediators involved in the clearance of parasites and allergic

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hypersensitivity responses. Although IgD is present on the surface of all naive B cells, it is generally lost during maturation and its function is not well defined.

1.5 Resistance to Infection

Infectious organisms fall into two categories: those that only survive or multiply inside host cells (viruses and facultative intracellular organisms) and bacteria that multiply outside host cells (e.g. *Streptococcus, Staphylococcus, Haemophilus*). In the earliest stages of infection, all bacterial pathogens may be controlled by nonspecific defences. However, organisms may replicate quickly and cause an overwhelming infection if not rapidly controlled. If innate immunity is compromised by chemical exposure, bacteria may proliferate too rapidly for innate immunity to be effective. Individuals at the extremes of age are more susceptible to these organisms because innate and adaptive immune functions are compromised in these populations (Burns-Guydish *et al*., 2005; Roberts *et al*., 2005). Likewise, a majority of heritable or acquired conditions that alter antigen presentation, T helper cell activity or antibody responses also predispose to infection (Buckley, 2003).

Extracellular pathogens are eliminated by phagocytic cells of the innate immune system after being coated by antibody. Antibodies also bind to and neutralize bacterial products (e.g. diphtheria toxin, *botulinum* toxins). Human exposure to certain environmental chemicals prior to immune system maturation has been associated with increased susceptibility to infection with extracellular bacteria (Section 3). The primary protective response to intracellular bacteria is mediated by products of Tc cells that in turn activate phagocytic cells to kill internal bacteria, or, in the case of viral infection, are directed against the infected host cell, leading to lysis of the infected cell. Infection with obligate intracellular pathogens are more commonly observed in individuals with defects in cellular immunity.

2 LOCAL IMMUNE RESPONSES

2.1 Mucosal-Associated Lymphoid Tissue (MALT)

Lymphoid tissues are classified as either primary or secondary organs based on their functional differences. Primary lymphoid organs include bone marrow and thymus, where haematopoietic stem cells and undifferentiated lymphocytes acquire their basal phenotype, and secondary lymphoid organs include the spleen, lymph nodes and mucosal-associated lymphoid tissue (MALT). MALT is a diffuse network of lymphoid tissues found on the surface of organs that encounter exogenous challenges such as microbes and soluble antigens, interconnected via the lymphatic system. Mucosal surfaces are prominent in the gastrointestinal, respiratory and urogenital tracts; the presence of MALT on these susceptible areas allows a localized response to a foreign antigen that results in immunological memory or tolerance. Although not part of the MALT system, the skin also has unique localized immune function (Section 2.1.4).

MALT is organized in multiple functional compartments including lymphoid follicles and the overlying follicle-associated epithelium, which contains microfold/membranous (M) cells, epithelial cells that allow the transport of micro organisms and soluble molecules across the epithelium, giving lymphoid tissues access to luminal antigens. Collections of MALT are covered by a mucous layer that provides a physical and chemical barrier against pathogens, as well as local concentrations of antibodies and other host defence molecules including lysozymes, complement, collectins and defensins.

Toll-like receptors (TLRs) are found on a number of cell types in the MALT, including macrophages, DCs and epithelial cells, and provide a link for signalling APCs to respond to an invading pathogen. In addition, signalling via TLRs in MALT can modulate potentially damaging proinflammatory responses, down-regulate immune responses and establish antigenic tolerance (Abreu *et al*., 2001).

MALT contains large numbers of intraepithelial lymphocytes (IELs) that are situated in the mucosal epithelium and, together with the epithelial cells, form an immunological barrier. The majority of IELs are CD3+ T cells expressing either $\alpha\beta$ or $\gamma\delta$ T cell receptor (TCR), approximately 80% belonging to the CD8 subset. Cells bearing the $\gamma\delta$ TCR are essential for the induction and regulation of antigen-specific IgA (Fujihashi *et al*., 1996), the dominant antibody isotype of the mucosal immune system. IgA and IgM are synthesized by plasma cells and transported to and released from the mucosal surface by immature epithelial cells that express polymeric immunoglobulin receptors on their basolateral surfaces.

2.1.1 Gut-Associated Lymphoid Tissue (GALT)

Multiple lymphocyte-rich areas are present in the intestine, including Peyer's patches, isolated lymphoid follicles, and lymphoglandular complexes which make up the gut-associated lymphoid tissue (GALT). These structures are randomly distributed throughout the intestinal tract with the greatest density in the jejunum. However, the size, number, distribution and composition of the Peyer's patches can vary among species and strain (Bruder *et al*., 1999). Ingested antigens can move through the intestinal lumen via M cells in the Peyer's patches without inducing

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an immune response, a mechanism called oral tolerance. The maintenance of immunologic tolerance, one of the unique features of the GALT, inhibits inflammatory responses to commensal organisms and ingested antigens, thereby preventing autoimmunity and food allergy.

2.1.2 Nasal-Associated Lymphoid Tissue (NALT)

In humans, the lymphoid structures termed Waldeyer's ring and the adenoids comprise the nasal-associated lymphoid tissue (NALT), whereas in rodents NALT is composed of paired lymphoid aggregates in the caudoventral portion of the left and right nasal passages at the entrance to the nasopharyngeal duct. APCs such as macrophages and dendritic cells are scattered throughout NALT but are less numerous than in other mucosal immune tissues such as the Peyer's patches.

2.1.3 Bronchial-Associated Lymphoid Tissue (BALT)

There is a great diversity of bronchial-associated lymphoid tissue (BALT) among species, and BALT is normally not found in dogs, cats and hamsters, whereas the largest BALT is found in rabbits (Pabst and Gehrke, 1990). Regardless, BALT can be induced in mice by pathogen exposure and has some characteristics similar to other species (Moyron-Quiroz *et al*., 2004). T and B lymphocytes located in the BALT generate cytotoxic and antibody-mediated reactions that rid the lung of pathogens while maintaining the delicate tissues for oxygen exchange.

2.1.4 Skin

Although the skin is not typical MALT, it does provide a barrier from environmental factors and is the site of local immune responses. Specialized APCs, termed Langerhans' cells (LCs), are situated in the epidermis and dermis. When antigen is encountered, the cells migrate to draining lymph nodes, and present the processed antigens to T cells, resulting in differentiation and activation. LCs play a pivotal role in regulating the balance between immunity and peripheral tolerance.

2.2 Toxicant Insult to MALT

The mucosal surfaces and skin are the first sites of contact with foreign antigens and chemicals. Toxicant exposures can cause the breakdown of mucosal immunity by either altering immune responses or decreasing tolerance, and may result in local or systemic effects. For example in the gut 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has been reported to target the GALT, resulting in a marked decrease in IgA secretion and impaired oral tolerance to ovalbumin (OVA) (Kinoshita *et al*., 2006). Cyclophosphamide given orally has been shown to suppress antibody production in the GALT but also in the spleen (Kuper *et al*., 2003).

Chronic inhalation of ozone has been reported to damage the respiratory tract-associated lymphoid tissues. Ozone induces lesions in the nasopharyngeal epithelium and increases mucus production, suggesting NALT damage and possible dampening of the mucosal immune response (Harkema *et al*., 1997), whereas in the BALT it has been shown to suppress lymphocyte function (Jakab, 1993). These decrements have also been associated with increased susceptibility to respiratory infections with pathogens such as *Listeria monocytogenes*, which requires T cell-mediated immune responses in order to clear the organism (Steerenberg *et al*., 2004). NALT and BALT are also known as important sites for the induction of mucosal tolerance, and chemical exposures have been shown to alter this process. For example, exposure to diesel exhaust particles has been shown to elicit profound adjuvant effects in humans and animal models of respiratory allergy, and may play a role in overcoming tolerance to normally innocuous proteins.

Exposure to a number of toxicants and naturally occurring substances can damage the protective properties of the skin. Suppression or inappropriate modulation of the skin's immune response can alter its barrier function, making it a potential route of entry for pathogens. For example, multiple doses of UV increase susceptibility to microbial, fungal and viral pathogens and alter LCs migration and function (Kripke *et al*., 1990). Chemical exposure may also activate the skin immune system, resulting in hypersensitivity responses in the skin (Section 6).

3 IMMUNOSUPPRESSION AND RESISTANCE TO DISEASE

3.1 Introduction

Defects in immune function may be primary (inherited) or follow accidental or deliberate exposure to industrial chemicals, drugs, radiation, stress or infectious agents (acquired). Suppression may range from mild and transient to profound and long lasting, depending on the nature of the defect, and, in the case of drugs and xenobiotics, the duration of exposure and rate of clearance, and the stage of immune system maturity during exposure. Mild suppression may only be detected by laboratory tests, whereas moderate suppression may be expressed as an increased incidence or severity of common, community-acquired infections. Severe suppression, as observed in AIDS and certain primary immunodeficiency diseases, is typically associated with opportunistic

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infections that do not usually pose a threat to the general population, and is an unlikely consequence of exposure to xenobiotics in the environment (Luebke *et al*., 2004). However, the range of 'normal' function is broad in humans, and is influenced by gender, age, genotype, nutritional status and the use of therapeutic and recreational drugs, factors that account for most of the variability reported in mean immune values. The consequences of exposure to potential immunotoxicants are thus the product of host immunocompetence at the time of exposure, immunotoxicant potency and the dose and virulence of infectious agents encountered following exposure. As such, exposures that do not cause biologically significant changes in immune function in some individuals may be sufficient to render others more susceptible to infection.

3.2 Mechanisms of Immunosuppression

Acquired immunodeficiency may be the result of reduced cell numbers, reduced or inappropriate cell activity or a combination of effects. Mechanisms of suppression include developmental arrest, metabolic pathways blockade, abnormalities in cytokine synthesis or secretion, disrupted signalling pathways, impaired DNA synthesis and lymphocyte proliferation and/or failure of normal apoptotic mechanisms. Complete suppression of immune function or haematopoiesis is unlikely to occur following exposure to chemicals not specifically designed to target the immune system, because the immune system is self-renewing and there is functional overlap in most host-protective responses. However, if an immune defect occurs in cells that cannot be replaced, (e.g. certain bone marrow cells), persistent suppression effects typically follow.

Examples of immunosuppression secondary to reduced cell numbers include the effects of xenobiotics on bone marrow function, and the effects of corticosteroids on immature cells. Polycyclic aromatic and aryl hydrocarbons decrease production of stromal cell-derived growth and differentiation factors critical for pluripotent immune cell progenitors, thus reducing the supply of B cell and T cell precursors (Jensen *et al*., 2003). Increased hypothalamic–pituitary–adrenal axis activity in response to chemical or physical stressors, or the therapeutic use of corticosteroids, results in apoptosis of immature lymphocytes, reducing the pool of available naive cells. Corticosteroids also reduce the production of many cytokines, including those associated with both inflammation and antibody synthesis, by interfering with gene expression or reducing the stability of cytokine mRNA (messenger RNA), thus suppressing immune-cell function. Other immunotoxicants (e.g. the benzene metabolite benzoquinone, TCDD, lead) reduce cell function by inhibiting critical transcription factors, reducing or preventing normal activity, but not necessarily leading to cell death.

3.3 Increased Human Susceptibility to Disease with Mild to Moderate Immunosuppression

Both infectious and neoplastic diseases are associated with immunodeficiency, although infectious diseases are more likely to be identified in short-term epidemiological studies and may appear to be more common due to differences in latency. Infections are common at the population level but occur relatively infrequently in individuals, with one to two episodes reported per year. Therefore, detecting xenobiotic-related increased rates of infection in epidemiological studies is difficult, thus limiting the number of reports that demonstrate an increased susceptibility in exposed humans. Immunodeficiency is also associated with an increased incidence of certain virally induced tumours, such as non-Hodgkin's lymphomas and tumours of the skin (Penn, 2000). Suppression of cell-mediated immunity has been associated with higher incidences of skin cancers, leukaemias and lymphoproliferative disorders in transplant patients. Severe immunosuppression, as seen in patients with AIDS, has been associated with Kaposi's sarcoma and Epstein–Barr virus-associated B cell lymphomas.

3.3.1 Adult Exposure to Environmental Chemicals

Increased rates of infections have been reported in children and adults following accidental or occupational chemical exposure. Pesticide exposure has been associated with increased incidence of infection and autoimmune disease, although the power of many studies is limited by small numbers of self-selected subjects. Living near a dump site containing organochlorine pesticides, volatile organic compounds and metals, was associated with a higher incidence of *herpes zoster* (reactivated herpes infection causing shingles), but not other infectious diseases (Arndt *et al*., 1999). Occupational exposure to lead has been associated with an increased incidence of influenza infections in a group of lead-refinery workers (Horiguchi *et al*., 1992) and similarly, welding fumes with respiratory bacterial infections (Antonini *et al*., 2003).

3.3.2 Chronic Stress

It is well established that chronic stress associated with divorce, bereavement or caregiving for Alzheimer's patients results in low-to-moderate degrees of immunosuppression and increased infectious disease incidences

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(Kiecolt-Glaser *et al*., 2002). For example, the rate of respiratory infections and clinical colds in healthy subjects administered nasal droplets containing respiratory syncytial virus or coronavirus increased in a dose-responsive manner with increasing degrees of psychological stress (Cohen *et al*., 1991). Furthermore, rates of seroconversion following immunizations with hepatitis B vaccine were negatively correlated with the level of stress in students under academic pressure. Even more striking were the effects on pneumococcal vaccine responses in Alzheimer's caregivers, where a significant decrease in antibody titre occurred in current caregivers compared to controls over the six-month period following immunization (Glaser *et al*., 2000). Immune testing in chronically stressed individuals indicates that increased rates of infection correlate with altered lymphocyte populations, even though values are within normally reported ranges.

3.3.3 Correlation of Infections with Immune System End Points

Prospective studies in patients receiving haematopoietic stem cell transplantation (HSCT) provide an excellent model to identify quantitative relationships between immune end points and disease. In these individuals, immunodeficiency can persist for well over a year due to pregrafting radiation treatment, and is manifested as decreases in primary antibody responses and delayed hypersensitivity responses (Ochs *et al*., 1995). The incidence of infections exceeds 80% during the first two years postengraftment, with 50% of the patients having three or more infections, and opportunistic infections predominate (Ochs *et al*., 1995). Infections that occur in the first month following transplant are most likely due to deficiencies in granulocytes, and later infections appear to be due to deficiencies in CD4+ T cells and B cells. HSCT patients also manifest deficiencies in humoral immunity, presumably due to insufficient T cell help, rather than direct B cell deficits. Studies in organ transplant patients have also provided insights into the long-term consequences of moderate immunosuppression. Infection rates range between 65 and 70% during the first six months post-transplantation (Sia and Paya, 1998). With the advent of long-term monitoring, an increased incidence in cancer has also been noted in this population.

4 DEVELOPMENTAL IMMUNOTOXICITY (DIT)

4.1 Ontogeny of the Immune System

Immune development begins with haematopoiesis; the resultant pluripotent progenitor cells develop into myeloid or lymphoid lines after colonization of the liver, bone marrow, spleen and thymus. Initial lineage-restriction begins within sites of lymphopoiesis in the foetal liver; further differentiation and maturation into specific lymphocyte subpopulations occurs in the thymus (T cells) or bone marrow (B cells). Most types of T and B cell are functionally immature at birth and do not attain full maturity for several weeks, months or years into postnatal life, depending on the species.

Organogenesis of the lymphoid system is independent of initial lymphopoiesis in the liver and bone marrow. Thymus-derived lymphocyte development, including positive and negative T cell selection, is not completed until after the thymic medulla forms. Once in the thymus, thymocytes mature into CD4+ or CD8+ thymocytes that migrate to the periphery as functional Th and Tc lymphocytes, respectively (Holladay and Smialowicz, 2000). B lymphocyte development involves immunoglobulin gene rearrangement, which diversifies the assortment of antigen receptors, and selection by self-antigens; immature B cells that are stimulated by self-antigens are removed or inactivated. T and B cell maturity is achieved, in part, after antigens are encountered, and a pool of memory T and B cells in secondary lymphoid tissues is formed (Holsapple *et al*., 2003). Cells of both the innate (granulocytes, macrophages, eosinophils and NK cells) and adaptive (T and B cells) immune system are also functionally immature at birth.

The foetus is essentially an allograft that the mother's immune system must tolerate or risk rejection. Therefore, during pregnancy, the maternal immune system is biased towards the Th2 pattern of cytokine expression to ensure an adequate maternal antimicrobial humoral response, and reduced proinflammatory Th1-type reactions that may lead to foetal rejection. The predominant foetal phenotype is also Th2-biased, which is likely from *in utero* exposure to antigens that cross the placenta and prime foetal T cells (Kay, 2001). As exposure to microbes increases after birth, the cytokine profile shifts to Th1-mediated responses. Exposure to environmental chemicals that skew the Th1/Th2 balance, particularly air pollutants, has been suggested as a contributing factor in the increased incidence of allergic disease.

4.2 Sensitive Windows in Immune System Development

The requisite programmed events of immune-system development increase susceptibility to agents that may disrupt one or more critical steps. According to Landreth (2002), five windows of sensitivity exist in the developing rodent immune system. In the prenatal period, the immune system is especially vulnerable to insult during stem cell formation, liver development

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(as a haematopoietic organ), and colonization and establishment of the bone marrow and thymus. In the postnatal period, sensitivity to insult is increased during maturation to immunocompetence, and during the establishment of immune memory.

4.2.1 Chemical Modulation of the Developing Immune System

Several environmental agents have been implicated as developmental immunotoxicants. These include chlorinated compounds such as TCDD and PCBs (polychlorinated biphenyls), lead, mercury, organotins, pesticides (e.g. chlordane, heptachlor and hexachlorobenzene) and therapeutics (e.g. diazepam, diethylstilbestrol and dexamethasone). Although developmental exposure to these agents produces a myriad of effects, animal data indicate that timing of exposure determines the immunotoxicological outcome (**Table 1**). For example, if lead exposure occurs throughout gestation, delayed-type hypersensitivity (a functional measure of cell-mediated immunity) is decreased; if exposure is during the first half of gestation, it is unaffected (Dietert *et al*., 2002).

Evidence that developmental immunotoxicity (DIT) is a concern in the human population is provided by the well-documented association between PCB exposures and infections in children from China, Japan, the Netherlands and Canada. Studies of accidentally exposed populations in Japan (Yusho) and China (Yu-Cheng) suggested an association of PCBs, their thermal breakdown products (quaterphenyls) and polychlorinated dibenzofurans with immune abnormalities and increased infections. Developmental PCB exposure was associated with recurrent respiratory infections in the Yusho and Yu-Cheng study populations, as were recurrent episodes of otitis media in the Yu-Cheng population and in infants in arctic Quebec, Canada with high levels of organochlorine exposure. In Dutch preschool children, PCB levels in breast milk (non-ortho and coplanar PCBs) were also associated with increased recurrent otitis media and other symptoms of respiratory infection. In these children, the body burden of PCBs at age 42 months was associated with a higher prevalence of recurrent otitis media and chicken pox. The impact, if any, of background levels of these chemicals on disease burden has not been determined.

Table 1 Selected examples of efficacy of testing in adult rodents to detect immunotoxicity following exposure during immune system development[a]

Exposure	Reported effects	NOAEL/LOAEL	Adult vs. developmental testing
		Diethylstilbestrol	
PND 1–5	↓Antibody, NK activity	One dose level (2.2 mg kg^{-1})	Adequately detect LOAEL, but not lifetime persistence
Adult, 5 day	↓Antibody, DTH	0.2/2.0 mg kg^{-1} day^{-1}	
		Diazepam	
GD 14–20	↓Resistance to infection	One dose level (1.25 mg kg^{-1})	Adequately detect LOAEL, but not persistence
Adult, 3 day	↓Antibody, DTH	2.0/4.0 mg kg^{-1} day^{-1}	
		Lead	
GD 14–21	↓DTH, ↑IgE	None/250 ppm DTH, 100 ppm IgE	Adequately detect LOAEL, but not effects on IgE
Adult, 3 wk	↓DTH	128 ppm/512 ppm	
		2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	
GD 14	Thymic atrophy, ↓DTH	1.0/3.0 μg kg^{-1}	Adult testing did not detect suppression that occurred in offspring
Adult, once	↑Antibody synthesis	1.0/3.0 μg kg^{-1}	
		Tributyltin oxide	
Preweanling	↓NK cell activity	None/5.0 mg kg^{-1} day^{-1}	Adult testing did not detect suppression that occurred in offspring
Adult, 10 day	↑Antibody synthesis	1.25/2.5 mg kg^{-1} day^{-1}	

[a]See (Luebke *et al*., 2006) for additional information. GD–gestation day, PND–postnatal day, DTH–delayed type hypersensitivity, LOAEL–lowest observed adverse effect level, NOAEL–no observed adverse effect level.

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4.3 Evaluation of Developmental Immunotoxicity

Rodents are commonly used to assess the potential effects of toxicants on immune system development, although the timing of particular developmental landmarks during gestation and immediately postpartum differs significantly from that in humans. Because rats and mice have relatively immature immune systems at birth, significant developmental events occur after birth (Holladay and Smialowicz, 2000). Thus, timing of exposure relative to birth is an important element of experimental design, and must be taken into account when extrapolating rodent data to potential windows of increased sensitivity in humans.

Data generated in a number of laboratories have demonstrated that the effects of chemicals on the developing immune system may last longer and/or occur at lower doses than effects on adults. It has therefore been suggested that assessment of immunotoxicity in adult animals may not adequately reflect the severity or the persistence of the effects of developmental exposure. A DIT study must consider when a toxicant is administered and for how long, as well as the end point being evaluated. Rodents are considered appropriate models for humans in DIT testing, as long as immune system ontogeny is considered when interpreting the resulting data (Holsapple *et al*., 2003). In general, DIT studies utilize the same end points as those evaluated in adult immunotoxicity studies (Holsapple *et al*., 2003). However, as most traditional immune end points are difficult to assess in rodents less than three weeks of age, DIT studies are usually conducted after weaning.

5 AUTOIMMUNITY

5.1 Autoimmune Diseases

Autoimmune diseases result from a breakdown of immunological tolerance, leading to immune responses against self-molecules. The mechanisms for this self-reactivity are the same as those associated with responses to foreign antigens, including activation of the innate and adaptive immune systems, excessive production of inflammatory mediators, activation of T lymphocytes or the generation of antibodies with specificity for self-antigens. In many instances the events that initiate the immune response to self are unknown; however, intrinsic factors (e.g. specific gene polymorphisms, gender-related hormones and age) and extrinsic factors (e.g. lifestyle, exposures to certain drugs, chemicals and infectious agents) have been shown to be associated with the induction, development or exacerbation of autoimmunity. Autoimmune disorders can affect virtually any site in the body, and present as a spectrum of diseases ranging from organ-specific, in which antibodies and T cells react to self-antigens localized in a specific tissue, to systemic, characterized by reactivity against a specific antigen or antigens present in various tissues. Recent estimates suggest that 3–5% of the general population suffer from autoimmune diseases, and there is epidemiological evidence that the prevalence of certain autoimmune diseases is increasing in industrialized countries (reviewed in World Health Organization, 2006). In addition, there is evidence that a number of common health problems, such as atherosclerosis, schizophrenia, inflammatory bowel disease, autism and aspects of male and female infertility may have an autoimmune component. Women have a significantly higher risk of developing an autoimmune disease than men. A female predominance is observed in the most common autoimmune diseases (thyroiditis, scleroderma, systemic lupus erythematosus (SLE), multiple sclerosis (MS), rheumatoid arthritis (RA)). However, there appears to be a higher risk among men for some autoimmune diseases, such as ankylosing spondylitis and adult-onset diabetes.

5.2 Mechanisms of Autoimmunity

Lymphocytes that recognize self-antigens with high affinity undergo negative selection in the bone marrow (B cells) and thymus (T cells) and are eliminated via apoptosis. However, autoreactive B and T cells constitute a normal part of the immune cell pool and natural autoantibodies are observed in sera from normal, healthy individuals. The presence of these cells presents a low-grade risk for autoimmune disease in most individuals because recognition of self-antigens occurs with low affinity and can be controlled by peripheral tolerance, a post-thymic control mechanism that limits antigen-specific activity via the regulatory interactions of a variety of cell types and soluble mediators. A good example of one such regulatory check is the requirement for two signals from APCs, one antigen-specific and a second nonspecific signal, for lymphocyte proliferation. In the absence of a co-stimulatory signal, self-reactive cells are rendered unresponsive (anergic). However, in some instances, these autoreactive cells can recognize self-antigens in situations that overcome anergy and make the antigens appear immunogenic. One such example is cytokine-mediated polyclonal activation following infection or chemical-induced inflammation. In these cases, the inflammatory stimulus or a microbial protein may serve as an adjuvant and tolerance can be broken.

Following activation, regulation of beneficial immune responses is mediated by a number of inhibitory pathways that balance the positive and negative aspects of immune

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system activation. Intrinsic defects or chemical agents that modify these regulatory pathways may lead to failure in restoring normal immune homeostasis and contribute to the pathogenesis of autoimmune disease. Changes in apoptotic pathways, leading to inappropriate cell death or survival, or disturbances in the clearance of apoptotic cells, have been suggested as the underlying mechanisms for several autoimmune diseases including RA, SLE and Hashimoto's thyroiditis. Dysfunction of regulatory NK T cell or T cell activity due to altered cytokine production, and/or deletion or mutation of cell-surface molecules, has been described for several autoimmune diseases. Organ-specific autoimmune diseases are typically characterized by cell-mediated responses directly affected by autoreactive CD8+ Tc cells or indirectly via release of proinflammatory cytokines and other soluble mediators by activated CD4+ T cells and macrophages. In contrast, systemic autoimmune diseases are frequently characterized by specific autoantibodies, which can cause injury via activation of complement, blocking or stimulating cell-surface receptors or by aggregation into immune complexes that activate nonspecific inflammatory responses.

5.2.1 Genetic Predisposition

Familial aggregation and laboratory animal studies suggest a clear association between genetics and essentially all autoimmune diseases. Concordance rates between identical twins range from approximately 9 to 40%, depending on the disease. Limited concordance may be explained by nonidentity in immune repertoires due to T cell receptor and Ig gene recombination, variations in receptor assembly, and somatic mutation. Additional evidence indicates that environmental factors also contribute to disease aetiology (Section 5.2.2). Any gene coding for products that are involved in the induction and maintenance of self-tolerance and in regulating immune effector functions as well as organ-specific functions may be involved in defining individual susceptibility. The most clearly established genetic association is with specific alleles within the MHC gene complex (Rose and Mackay, 2006). Polymorphisms in regulatory regions of genes coding for immunoinhibitory receptors, such as CTLA-4 (cytotoxic T lymphocyte antigen 4), which down-regulate T-cell activation, have also been shown to be important in disease susceptibility. Gene polymorphisms that affect the function or the level of expression of regulatory or effector molecules of inflammation, fibrosis or other pathologic processes involved in autoimmune disease development have also been observed. Examples of these include systemic sclerosis (TGFβ1, TGFβ2, TGFβ3), juvenile idiopathic arthritis (IL-1α), RA (IL-4, TNF-α), SLE (IL-10, TNF-α), Sjögren's syndrome (IL-10), juvenile idiopathic inflammatory myopathies (IL-1 Rα) and Wegener's granulomatosis (IL-10). Finally, polymorphisms in genes associated with nonimmune parameters, such as drug-metabolizing enzymes, may result in differential susceptibilities to drug or chemical-induced autoimmunity. This is likely the result of changes in the generation of protein adducts and covalently modified antigens through the use of alternative metabolic pathways. In the majority of autoimmune diseases, a multigenic process with multiple susceptibility loci working in concert has been suggested.

5.2.2 The Role of Environmental Factors in Autoimmune Diseases

Environmental factors have been shown to be important triggers for expression of autoimmunity and have been suggested to both induce onset and modulate disease severity (**Table 2**). Lifestyle factors such as diet, smoking, therapeutic and recreational drug use, infection with certain bacteria and viruses and exposure to UV radiation and environmental chemicals have all been implicated in the pathogenesis of autoimmune diseases (Heindel *et al*., 1999).

For some diseases, the causal link between infection and autoimmunity is well established. Many peptide fragments of microbial agents are homologous with host proteins, and the induction of an immune response to these antigens results in cross-reactivity with self-antigens and the induction of autoimmunity. The best example of this 'molecular mimicry' is a membrane protein on the β-haemolytic streptococcus bacterium, which has a high degree of homology with cardiac myosin. Antibodies that target the bacterium also cross-react with cardiac muscle and induce rheumatic fever. Antibodies to *Yersinia enterocolitica*, a bacterium normally associated with food poisoning outbreaks, cross-react with a variety of thyroid antigens, and increased levels of antibodies to *Yersinia* have been demonstrated in patients with Graves' disease or autoimmune thyroiditis. Microbial pathogens can also serve as adjuvants by nonspecifically stimulating innate immunity, inducing the production of soluble mediators and co-stimulatory molecules important in the perpetuation of the immune response.

For other environmental factors, the associations with autoimmunity have been suggested from epidemiologic studies and/or animal models or *in vitro* studies. Many of the chemical and therapeutic agents that have been linked to autoimmunity act through a chemical-induced alteration of self-peptides (Heindel *et al*., 1999). Metals such as mercury induce autoimmune disease via the creation of new high-affinity binding sites for MHC molecules. Drugs such as penicillin and halothane induce reactions in which antigen-specific T cells provide help

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Table 2 Chemicals and drugs associated with autoimmunity[a]

Autoimmune syndrome	Compound
Haemolytic anaemia	α-Methyldopa Penicillins Sulfa drugs
Hepatitis	Halothane Interferon-α Ethanol Halothane
Scleroderma/systemic sclerosis	Vinyl chloride Trichloroethylene Spanish toxic oil Tryptophan Silicone Interleukin-2 Diphenylhydantoin
SLE or lupus-like syndrome	Aromatic amines Chlorpromazine Formaldehyde Hydralazine Interferon-γ Isoniazid Procainamide Silica Trichloroethylene
Thyroiditis	Iodine Lithium PCBs, PBBs
Thrombocytopaenia	Interferon-α Rifampicin Quinidine Iodine
Vasculitis	Allopurinol Silica Tetracyclines

[a]Adapted from Rose and Mackay (2006). This is not meant to be a comprehensive review, but is meant to provide illustrative examples of the types of compounds associated with autoimmunity. PBB—polybrominated biphenyl.

to antibody-producing B cells that recognize chemically modified proteins but not the native form of the self-protein.

5.3 Animal Models of Autoimmune Diseases

Animal models of autoimmunity have been used to explore both molecular mechanisms and therapeutic interventions for a variety of autoimmune diseases (Germolec, 2005). With the advent of transgenic and knockout mice, the number of genetically predisposed, autoimmune models has significantly increased (Boyton and Altmann, 2002). In the genetically predisposed models, mild to severe syndromes spontaneously develop, and are frequently due to alterations in genes encoding for MHC molecules, immune receptors or cytokines. The lupus-prone MRL mouse and the NOD model for insulin-dependent diabetes are two excellent examples of models where point mutations have been used to elucidate the role of specific genetic loci in the disease process.

In models where autoimmunity is induced by exposure to chemical or biological agents, foreign substances are used to initiate the autoimmune disease state. These may include biological substances such as bacterial or viral antigens, environmental chemicals or therapeutic drugs. One of the more commonly employed models used in toxicology studies is the Brown Norway (BN) rat model, in which the animals are injected with low doses of $HgCl_2$. Although the chemical exposure produces no overt signs of toxicity, the rats develop an immunologically mediated disease characterized by T cell-dependent polyclonal B cell activation, autoantibodies to specific components of the glomerular basement membrane and proteinuria similar to that observed in humans with autoimmune glomerulonephritis.

Immunization with purified self-antigens can elicit autoimmune responses, particularly when adjuvants are administered in conjunction with self-proteins. A frequently used model of this type, experimental autoimmune encephalomyelitis, is induced in laboratory rodents by injection of myelin basic protein in the presence of Freund's complete adjuvant. The resulting pathology is a T cell-mediated autoimmune disease characterized by lymphocytic infiltration in the central nervous system and destruction of the myelin nerve sheath with resultant paralysis, similar to that observed in patients with MS.

In each type of model the development and severity of symptoms has multiple components, in that the presence of the disease and its progression can be influenced by age, hormonal and/or environmental factors. In addition, there is a tendency for more than one autoimmune disorder to occur in several of the individual models. Nevertheless, a number of syndromes similar to those clinically observed in humans can be mimicked in animal models.

6 HYPERSENSITIVITY REACTIONS

6.1 Introduction

Hypersensitivity reactions represent abnormal adaptive immune responses to normally innocuous exogenous agents and/or altered host proteins commonly referred to as allergens. These reactions often involve a lag period between the initial encounter with the offending

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agent and the hypersensitivity response. Once sensitized, the host immune system mounts one or more of the four distinct hypersensitivity responses (Section 1.1). Secondary exposure to the agent elicits an aggressive immune response resulting in tissue inflammation and destruction that may be life threatening, as in the case of systemic anaphylaxis.

6.2 Respiratory Hypersensitivity

6.2.1 Clinical Condition and Pathogenesis

The respiratory system serves the vital function of supplying oxygen and removing waste carbon dioxide from tissues with the upper airways filtering and conditioning the air for the lungs where gas exchange occurs. Respiratory hypersensitivity affects many aspects of airway function, ranging from a bothersome runny/itchy nose to disconcerting wheeze and shortness of breath and even fatal airway constriction. Allergen exposure manifests as an early phase, a late phase or dual responses, which are the result of isolated or combined Type I and Type IV hypersensitivity reactions. The early-phase response is primarily driven by allergen-specific IgE antibody, produced following sensitization, which is bound to surface receptors of mast cells and basophils residing in circulation and respiratory tissue (D'Amato, 2006). Inhalation of allergen results in crosslinking of IgE and activation/degranulation of mast cells, leading to the release of preformed mediators such as histamine and leukotrienes. These molecules, among others, elicit the effector responses of rhinitis in the upper airways (sneezing, itching, rhinorrhoea and severe nasal obstruction) or asthma in the lower airways (dyspnoea, wheeze, coughing and chest tightness).

Hypersensitivity reactions in the airways often involve an extended late-phase cell-mediated response that occurs several hours after inhalation of allergen. Mediators released during the early-phase response as well as activated lymphocytes producing cytokines and chemokines orchestrate the recruitment of effector inflammatory cells to the airways. Eosinophils, basophils, mast cells, neutrophils, lymphocytes, plasma cells and macrophages infiltrate the airway tissue and lumen to varying degrees, depending upon the nature of the allergen and the site of allergen exposure. These cells become activated in the tissue and liberate mediators aimed at destruction and elimination of the 'foreign' allergen. Unfortunately, these same mediators cause damage to host tissues resulting in the pathology and symptomatology of asthma and rhinitis.

The early- and late-phase reactions culminate in the pathological changes characteristic of respiratory hypersensitivity diseases. Cellular inflammation of the upper airways results in mucosal thickening, vasodilation and oedema, all culminating in nasal obstruction. Inflammation can also lead to epithelial changes such as goblet metaplasia and excessive mucus production, also contributing to obstruction. In severe and chronic cases, denudation, extensive metaplasia, hyperplasia and/or atrophy of the epithelium may occur, dramatically affecting the respiratory, olfactory and air conditioning/filtering functions of the upper airway. When the hypersensitivity reaction occurs in the lower airways, similar pathological changes in the airway epithelium and cellular inflammation ensue, which contributes to bronchoconstriction and congestion of the lung airspaces. In addition, chronic hypersensitivity responses in the lung, as observed in asthma, can lead to thickening and fibrosis of the basement membrane as well as hypertrophy of the smooth muscle layer. Together, these remodelling events contribute significantly to reduction in effective gas exchange and airway hyper-responsiveness or 'twitchy airways' that characterize asthma (Lloyd and Robinson, 2007).

6.2.2 Environmental/Occupational Exposures Contributing to Airway Hypersensitivity

Many of the compounds that we encounter on a daily basis, either through the indoor and outdoor environments or occupational atmospheres, can lead to sensitization and subsequent hypersensitivity responses in the upper and lower airways (**Table 3**). Common environmental causes include pollen from trees and grasses, fungal spores, house dust mites, cockroaches as well as pet dander and saliva. Environmental triggers of hypersensitivity reactions tend to be high-molecular-weight (HMW) agents capable of eliciting an immune response following antigen processing. Airway hypersensitivity reactions elicited by workplace exposures can be directed towards HMW and low-molecular-weight (LMW) agents. Prominent examples of HMW agents include flour proteins and enzymes responsible for Baker's rhinitis/asthma, latex proteins encountered by healthcare workers, proteins from dander and urine causing airway hypersensitivity in animal care workers, and enzymes used in a variety of industries including dry-cleaning. Airway sensitization and disease could also occur following occupational exposure to LMW chemicals, although these compounds tend to be too small to elicit an immune response directly and instead will haptenize host proteins, producing neo-epitopes capable of eliciting an immune response. Common examples include diisocyanates (e.g. toluene diisocyanate) and acid anhydrides (e.g. trimellitic anhydride) in the paint and plastics industry, metals (e.g. platinum salts and beryllium), and LMW compounds encountered in the wood working/manufacturing industry (plicatic acid) (Johnson and Luster, 2006). This discussion has listed many of the well-established mediators

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Table 3 Agents that contribute to hypersensitivity responses

Hypersensitivity response	Molecular weight	Environmental agents	Occupational agents
Respiratory	HMW	Pollen from trees and grasses, fungal spores, house dust mites, cockroaches, pet dander and saliva	Wheat and other grain proteins, enzymes for baking, laundry and dry-cleaning enzymes, latex proteins, proteins from dander and urine, fish and shellfish allergens, vegetable gums
	LMW	Fragrance chemicals	Diisocyanates, acid anhydrides, platinum salts, beryllium, nickel, cobalt, aluminium, plicatic acid and other chemical sensitizers in wood dust, amines, aldehydes, resin acids, hair dressing chemicals
Skin	HMW	Penicillin, polymyxin, bacitracin	Latex proteins, animal-associated proteins
	LMW	Nickel, gold, mercury, fragrance chemicals, lanolin, terpenes, acrylates, thimerosal, urushiol	Nickel, chromium salts, diisocyanates, acid anhydrides, dyes, resin acids, acrylates and methacrylates, aldehydes, thiuram
Food allergy	HMW	Proteins from milk, eggs, fish, shellfish, tree nuts, peanuts, wheat, and soya beans	Egg, milk and wheat from food processing, latex proteins
	LMW	Natural food colourings, synthetic food colourings	Carmine, epigallocatechin gallate from processing of green tea

of airway hypersensitivity; however, it is important to note that many more have been recognized, and additional environmental and occupational airway sensitizers are being identified.

6.3 Hypersensitivity Responses in the Skin

6.3.1 Clinical Condition and Pathogenesis

The skin serves as an important barrier between body tissues/fluids and the external environment. Hypersensitivity reactions involving the skin can compromise this barrier, increasing host susceptibility to infectious agents and toxic compounds. Such reactions are usually directed towards LMW compounds that are capable of penetrating the complex stratification of the skin and result in delayed (24–48 hours) cell-mediated responses (Type IV hypersensitivity). However, immediate hypersensitivity reactions in the skin, commonly referred to as immunologic urticaria and atopic dermatitis, can also occur following sensitization and subsequent skin exposure to HMW proteins capable of eliciting specific IgE production, as in the case of contact allergy to natural rubber latex (Toraason *et al*., 2000). Clinical symptoms observed following secondary exposure to the skin allergens include pruritus and stinging sensations that are accompanied by vesicular and bullous lesions and/or pigment alterations at the site of allergen contact. Inflammatory pustules and secondary bacterial infections may accompany these rashes, which can be progressive and destructive if further allergen exposure is not prevented.

LMW contact allergens penetrate the skin and bind to host proteins that are subsequently phagocytized and processed by LCs residing in the epidermis (**Figure 3**). These cells migrate to draining lymph nodes and present the allergens to T cells, leading to differentiation and clonal expansion (Cavani *et al*., 2007). Proteins capable of inducing immunologic urticaria undergo a similar process of inducing host sensitization, although initial exposure to these allergens can occur through routes other than skin, such as in the diet. In the case of immunologic urticaria, secondary skin contact with the allergen leads to crosslinking of allergen-specific IgE on mast cells, which causes degranulation and release of cytotoxic granule proteins and histamine that mediate a local immediate-type hypersensitivity reaction (Type I). In contrast, re-exposure to contact allergens eliciting a delayed-type response (Type IV) results in the recruitment and activation of specific T cells that liberate proinflammatory cytokines and chemokines that, in turn, attract additional inflammatory cells to the skin, thus contributing to the growing erythema and induration. These events begin shortly after allergen exposure but the symptoms only become evident around 12–48 hours following contact. Down-regulatory mechanisms then take over, including the production of IL-10 by Treg (Cavani *et al*., 2007), leading to the

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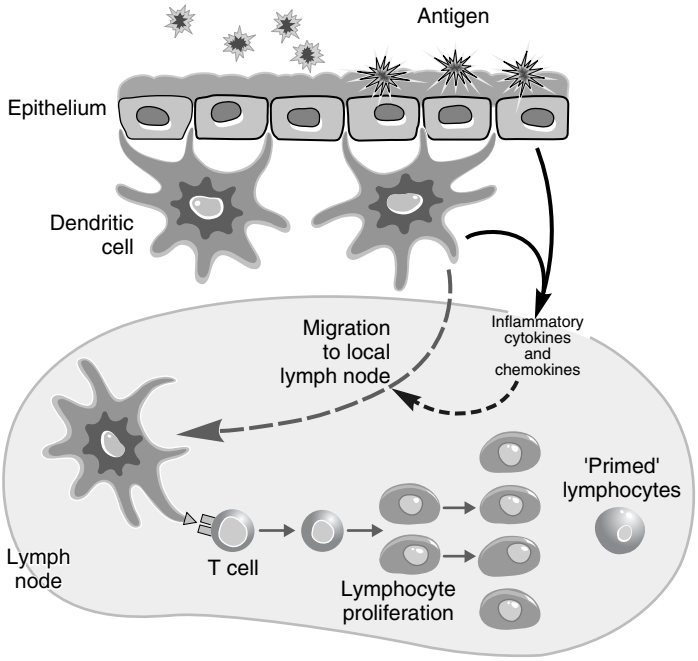

Figure 3 Antigen processing and presentation.

resolution of the cellular infiltrates, with the exception of memory T cells that will respond to subsequent exposure.

6.3.2 Environmental/Occupational Exposures Contributing to Hypersensitivity Responses in the Skin

Contact allergens of environmental origin include compounds found in clothing (dyes and resins), cosmetic/hygiene products (lanolin, fragrance terpenes, acrylates in fingernail coatings), antibiotics (polymyxin, bacitracin), preservatives (formaldehyde, thimerosal), and plant products, including urushiol, the chemical responsible for the response to 'poison ivy'. Metals commonly used in the manufacture of jewellery and keys and also in the dental industry, such nickel, gold and mercury, can cause hypersensitivity responses in the skin. The majority of agents responsible for immunologic urticaria are common food products including dairy, numerous fruits (e.g. apricot, apple, peach, olive) and nuts (e.g. peanuts, sesame seeds), meats (e.g. beef, chicken, turkey), seafood (e.g. codfish, clams, shrimp), and vegetables (e.g. beans, cabbage). In addition to skin reactions, these food products can also induce respiratory effects, gastrointestinal effects and anaphylaxis depending on the degree of sensitization. Allergic reactions in the skin are often related to LMW chemical exposure in the workplace. Examples include hypersensitivity responses in cement workers (chromate), autobody painters (diisocyanates, epoxy resins, hardeners), woodworkers (abietic acid), plastics manufacturers (acid anhydrides, acrylates) and healthcare workers (latex, thiuram, methacrylates).

6.4 Hypersensitivity Reactions towards Foodstuffs (Food Allergy)

6.4.1 Clinical Condition and Pathogenesis

Food allergy is a relatively new topic of interest to toxicologists. Advances in biotechnology, particularly the genetic modification of food crop plants, have raised concerns regarding dietary exposure to novel antigens of unknown allergenicity. Food allergy results from undesirable immune responses directed towards dietary proteins. Under normal circumstances, oral exposure to novel proteins leads to a state of immune tolerance. However, for reasons that remain largely unknown, some immunogenic dietary proteins are recognized aggressively by the immune system and act as allergens. Therefore, basic research to understand mechanisms of immune tolerance may also provide valuable insight into the complex aetiology of food allergy. Subsequent exposure to the protein allergen elicits an allergic response resulting in a range of digestive problems such as nausea, vomiting, diarrhoea and abdominal pain. The allergic response is not restricted to the digestive system and can manifest as respiratory problems (allergic rhinitis and asthma), skin problems (hives, atopic dermatitis, immunologic urticaria) and systemic anaphylaxis. The underlying immunopathogenesis of food allergy usually

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involves IgE-mediated Type I hypersensitivity responses that are mechanistically similar to those addressed above for respiratory and skin immediate hypersensitivity responses. However, some dietary proteins can cause cell-mediated (Type IV) immune responses to food that are frequently delayed or chronic, and include food protein-induced enterocolitis, proctitis and enteropathy, as well as coeliac disease.

6.4.2 Environmental/Occupational Exposures Contributing to Food Allergy

According to the US Food Allergen Labelling and Consumer Protection Act of 2004, there are eight major food groups that account for more than 90% of food allergies: milk, eggs, fish, shellfish, tree nuts, peanuts, wheat and soya beans, although over 160 other foodstuffs have been shown to contain allergenic proteins. The incidence of allergy to specific foods tends to vary geographically due to dietary exposure levels and is increasing due to altered food preparation habits and introduction of non-native foods to regional diets. Food allergy is more prevalent in children, with allergies to milk, eggs, soya and wheat frequently being outgrown whereas sensitivities to peanuts, tree nuts, fish and shellfish often persist into adulthood.

It is generally assumed that allergies to food proteins are initiated and provoked via the oral route. However, there is evidence for allergic sensitization by other routes of exposure, such as the respiratory tract and skin (James and Crespo, 2007; Strid *et al*., 2005). Inhalation and cutaneous exposure to food antigens most frequently causes sensitization in occupational settings where food processing generates dust or airborne food particles (e.g. wheat allergy following inhalation in bakers; seafood allergy following skin exposure in processors). Importantly, these alternate routes of exposure may circumvent the natural development of oral tolerance to food proteins. Another concern is cross-reactivity between respiratory/skin allergens and food proteins. The best known example of this is the association between birch pollen allergy and allergy to certain fruits such as apples or peaches. Latex allergy in healthcare workers, which may be acquired by sensitization through the skin or respiratory tract (Toraason *et al*., 2000), is associated with reactions to papayas, bananas, avocados and other fruits and vegetables.

6.5 Genetics and Hypersensitivity

Genetic variation plays a critical role in determining interindividual differences in susceptibility to hypersensitivity diseases. Hypersensitivity responses tend to be more frequent in individuals with atopy, the genetic predisposition to produce high levels of IgE. In addition, genetic polymorphisms, especially those within immune response and MHC genes, have been shown to influence the development of allergic diseases caused by environmental and occupational allergens (Yucesoy *et al*., 2007).

6.6 Assessment of Hypersensitivity Reactions

6.6.1 Assessing Respiratory Hypersensitivity

Assessment of respiratory hypersensitivity reactions in humans involves comprehensive pulmonary function testing, airway hyper-reactivity to cholinergic stimuli and/or histamine, serum antibody responses, and skin prick testing with common aeroallergens. These diagnostic procedures can help confirm disease and also provide insight into the cause, although they often do not definitively identify the allergen responsible, especially in the case of occupational exposures. Specific inhalation challenge testing with the suspect agent can be used to link clinical outcomes to the exposure agent. Although no validated experimental testing methods have been established, numerous animal models have been developed to identify and study the mechanisms of respiratory hypersensitivity, including models of asthma and rhinitis (Johnson and Luster, 2006; Johnson *et al*., 2007).

6.6.2 Assessing Hypersensitivity Responses in the Skin

Allergic responses in the skin are diagnosed in patients through clinical examination and detailed exposure history and timeline. It is often difficult to distinguish between irritant and allergic manifestations of dermatitis, and skin biopsies are rarely performed in a diagnostic setting. A powerful tool for identification of the offending allergen is skin patch testing (Mowad, 2006). Several animal models have been proposed for the detection of skin sensitizers/allergens. The local lymph node assay (LLNA), guinea pig maximization test (GPMT) and the mouse ear swelling test (MEST) have all been validated for the detection of skin allergens (Section 8.5).

6.6.3 Assessing Allergenicity of Novel Food Proteins

Transgenic food crops as well as alterations to native diets pose new challenges for risk assessment. Genetic engineering changes the expression of proteins in the crop, which may alter the dose and/or exposure milieu for new or normally tolerated proteins. A major obstacle to overcome is the lack of definitive characteristics for

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allergen, including food allergen, identification. However, allergenic proteins tend to be heat stable, glycosylated, 10–70 kDa in size, and resistant to degradation by acid and digestive enzymes. Though many allergens resist degradation, the association is not absolute; some allergens are highly labile, whereas nonallergenic proteins may be stable. Therefore, allergenicity assessment is currently a weight-of-evidence approach including *in silico* analysis of structural and amino acid sequence homology (recommended eight contiguous amino acids) with known protein allergens, assessment of heat stability, and determination of resistance to digestion in simulated gastric fluid (Kimber *et al*., 2007). Due to limitations of these methods, current research efforts are focussing on development and validation of new tools including structural databases, serum screening techniques and animal models.

Rodent models of food allergy have capitalized on the fact that the majority of food allergens induce IgE-mediated responses. Therefore, the BN rat, BALB/c mouse and C3H/HeJ mouse have been used for the majority of testing due to their inherent skew towards a Th2 allergic phenotype and high IgE production (McClain and Bannon, 2006). These models have focussed mainly on peanut, OVA and milk proteins, and have demonstrated high levels of IgE production indicative of sensitization. However, lack of standardization due to variable use of adjuvant, varying routes of exposure and even uncontrolled prior life-stage exposure to the target protein make screening for novel protein allergens very difficult (de Jonge *et al*., 2007). Rodent models tend to replicate the IgE response to food allergens seen in humans but often fail to present similar clinical symptoms. Other models using dogs and pigs are available that consistently demonstrate many clinical characteristics of human food allergy including respiratory involvement, digestive problems and even anaphylaxis (McClain and Bannon, 2006).

7 MOLECULAR MECHANISMS OF IMMUNOTOXICITY

7.1 Linking Immunotoxicants and Cellular Responses

In light of the fundamental role that the immune system plays in the maintenance of the health of the individual, the interaction of xenobiotics and regulatory mechanisms within the immune system has become an area of intense interest. Recent technologies allowing genome- and proteome-wide analysis of immune responses upon exposure to xenobiotics do not provide a complete picture of immunotoxicity since there may not be a direct correlation between gene and protein expression and biological function. Because of the potentially profound effects resulting from disruption of the immune system's balance, there exists a need to understand how xenobiotics can alter the genetic, cellular, biochemical and molecular mechanisms underlying immune function.

7.2 Signal Transduction

Activation of the immune response following antigen–receptor binding is mediated by signalling pathways critical to regulation of cellular function and immune system-associated genes. Cell-associated enzyme families, including tyrosine protein kinases, protein kinase C (PKC) and mitogen-activated protein kinases (MAPKs), phosphorylate transcription factors that up-regulate gene expression or proteins that alter cellular function without affecting gene expression. Other signalling pathways that are activated by oxidative damage and other types of cellular stress result in protein phosphatase activity that ultimately controls apoptotic pathways, cell activation and cellular differentiation.

The family of MAPKs, including c-Jun N-terminal kinase (JNK), p38 and extracellular signal-related kinases (ERKs), are all involved in stress signalling in immune cells following acute cell injury. The brominated flame retardant, tetrabisphenol A, stimulates the immune function of mussel haemocytes *in vitro* through kinase-mediated cell signalling (e.g. MAPKs and PKCs) thereby altering microbicidal activity towards *E. coli*, such as lysosomal enzyme release, phagocytic activity and extracellular superoxide (O_2^-) production (Canesi *et al*., 2005). Cadmium, a well-known carcinogenic and immunotoxic metal, also induces a calcium–ROS–JNK–caspase-3 signalling pathway (ROS—reactive oxygen species), leading to apoptosis of murine macrophages. Moreover, cadmium-induced alterations in calcium signalling regulate phosphorylation/dephosphorylation of JNK and p38, as well as the modulation of signal transduction pathways for proliferation, mitochondrial activity and necrosis (Kim and Sharma, 2004).

Cellular stress in immune cells also activates signal transduction pathways that are associated with survival pathways such as ERK and protein kinase B (PKB, also known as Akt). For example, 2-cyano-3-hydroxy-*N*-(4-trifluoromethylphenyl)-butenamide, which is an active metabolite of leflunomide (a clinical treatment for RA), decreases the number of viable human primary mast cells through apoptosis. Its mechanism of action includes inhibition of Akt via a concentration-dependent inhibition of PDK1 (pyruvate dehydrogenase kinase, isozyme 1) phosphorylation,

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which controls Akt activation and the downstream molecules caspase-3 and caspase-9 (Sawamukai *et al*., 2007).

Heat shock proteins (hsps) also play a critical role in the adaptive response of immune cells to stress. Upon entering the draining mediastinal lymph nodes, *Bacillus anthracis* (anthrax) endospores germinate and bacteria quickly multiply, causing overwhelming and lethal bacteraemia. Studies by During *et al*. (2007) in both human neutrophils and HeLa cells suggest that anthrax lethal toxin blocks both p38 MAP kinase and hsp27 phosphorylation, resulting in inhibition of actin-based motility and reduced neutrophil chemotaxis.

7.3 Transcription Factors—Gene Expression

Xenobiotics may cause dysregulation of gene expression via direct effects on transcription, modulation of intracellular signalling cascades, or by altering the storage, synthesis or release of extracellular signalling molecules. Some toxic chemicals may directly regulate transcription of specific target genes by the activation of receptors that function as ligand-activated transcription factors. Perhaps the best-studied ligand-activated transcription factor in the immune system is the glucocorticoid receptor. When activated with glucocorticoids (or dexamethasone) this receptor transitions into the nucleus, binds to glucocorticoid response elements and inhibits the transcriptional activation of numerous genes, including proinflammatory and anti-inflammatory cytokines. Another well-characterized ligand-activated transcription factor is the aryl hydrocarbon receptor (AhR). Following binding to high-affinity ligands such as polyhalogenated aromatic hydrocarbons (i.e. TCDD and PCBs), the AhR translocates to the nucleus and heterodimerizes with aryl hydrocarbon receptor nuclear translocator (ARNT). Binding of this activated complex to dioxin response elements on target genes (e.g. IL-2) leads to transcriptional regulation of the immune response. It remains to be determined how other ligand-activated transcription factors such as the pregnane X-receptors and constitutive androstane receptors, which are xenosensors of endocrine disruptors (i.e. environmental oestrogens) may affect the immune system.

Alternatively, gene transcription may be affected by crosstalk of intracellular receptors with signal transduction networks. For example, activation of the AhR leads to disruption of the NF-κB (nuclear factor κB) and AP-1 (activator protein 1) signalling pathways in immune cells (Tian *et al*., 2002; Nebert *et al*., 1993). NF-κB is a ubiquitous transcription factor that regulates the expression of many immune-related genes and inflammatory cytokines. Although primarily considered to be proinflammatory in nature, activation of NF-κBs also results in anti-apoptotic (prosurvival) signalling—and the delicate balance between the two functions must be tightly regulated.

7.4 Cytokines

The immune system is regulated by a variety of soluble polypeptide mediators termed cytokines, which are produced by cells of the immune system, as well as other nonimmune cells within the local microenvironment. Cytokines that regulate and maintain normal immune homeostasis, and play a vital role in the development of an immune response may be altered by exposure to toxicants such as organophosphate pesticides, heavy metals and endocrine disruptors (Li, 2007; Hemdan *et al*., 2007; Chalubinski and Kowalski, 2006). During innate immune responses, cytokines are primarily secreted by phagocytic cells and NK cells, but in adaptive immune responses they are mainly produced by APCs and lymphocytes. Although often described separately, crosstalk between the innate and adaptive immune systems is frequent, and cytokines represent a major means of communication between the two.

Cytokines exhibit a number of general biological effects that are important to understand when considering their roles in immunotoxicology. A single cytokine may be pleiotropic in action, for example affecting a number of different cell types or targets (**Table 4**). Similarly, cytokines may act back upon the cell source that secreted them in an autocrine manner and/or on neighbouring cells in a paracrine manner. Cytokines also exhibit multiple effects on different cell types, often with synergistic or opposing results. This frequently means that cytokines act as molecular messengers to coordinate the interplay between, and control of, different component cell types in the immune response—an action that is of great importance in the amplification of the immune response.

In normal immune responses, cytokines are released by, and act upon, cells which are in close proximity to one another such that detrimental bystander effects are limited. In contrast, during acute and/or chronic inflammatory conditions, cytokines may be released in sufficient quantity such that systemic effects are observed. The balance between production of a satisfactory immune response or tissue damage also depends on homeostatic mechanisms. Continuous cell stimulation may result in sustained production of soluble mediators that induces a chain of responses, which if left unchecked may be damaging. The short half-life of cytokines provides evidence that under normal conditions most soluble mediators are rapidly eliminated by a variety of different

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Table 4 Selected proinflammatory and anti-inflammatory cytokines of interest to immunotoxicology

Cytokine	Source	Function
	Proinflammatory	
Interleukin 1 (IL-1)	Macrophages, dendritic cells, B cells	Inflammatory mediator Increases production of other cytokines
Interleukin 2 (IL-2)	Activated T cells	Growth and activation of T and B cells Growth and activation of NK cells
Interleukin 6 (IL-6)	Macrophages, dendritic cells, B cells, activated T cells	Multiple effects on T cells Myeloid cell development Regulation of acute phase proteins
Interleukin 12 (IL-12)	Macrophages, dendritic cells, B cells	Differentiation of Th cells Stimulates production of IFN-γ
Interferon gamma (IFN $-\gamma$)	T cells, NK cells, epithelial cells, fibroblasts	Increases MHC expression Enhances CTLs, NK and macrophage activity Stimulates production of IL-1 and TNF-α
Tumour necrosis factor alpha (TNF-α)	Macrophages, dendritic cells, lymphocytes, mast cells	Increases MHC expression Activates macrophages Enhances tumour cell killing
	Anti-inflammatory	
Interleukin 4 (IL-4)	Mast cells, T cells, stromal cells, basophils	Growth and differentiation of B cells Inhibition of Th1 cells
Interleukin 10 (IL-10)	Macrophages, dendritic cells, T cells, B cells	Regulation of macrophage and Th cells Stimulates B cell proliferation
Interleukin 13 (IL-13)	Macrophages, T cells	Stimulates B cell differentiation and IgE production
Transforming growth factor beta (TGFβ)	Macrophages, megakaryocytes, chondrocytes	Inhibits cytokine production and activity Inhibits B cell proliferation Stimulates wound healing

processes, thereby ensuring their limited bioactivity. However, toxic agents that act on either the mediators themselves or the cells that produce them may cause damaging immune responses such as hypersensitivity, chronic immunodeficiency or autoimmune conditions.

8 HAZARD IDENTIFICATION AND RISK ASSESSMENT OF IMMUNE SYSTEM TOXICITY

8.1 Hazard Identification

Guidelines for evaluating the potential for chemicals and drugs to induce immunosuppression or hypersensitivity have been published by a number of agencies, including the US Food and Drug Administration, the US Environmental Protection Agency, the Organisation for Economic Cooperation and Development and the World Health Organization, and are available on the agencies' web sites. There currently are no regulatory testing guidelines for autoimmunity.

8.2 Approaches for Detecting Immunosuppression

Immunotoxicologists have applied tiered panels of assays to identify suppressive immunomodulatory agents in laboratory animals or via focussed epidemiological studies. The configurations of testing panels vary, but typically include assessment of one or more of the following: (i) lymphoid organ weights and histopathology; (ii) quantitative assessment of lymphoid tissue cellularity and peripheral blood haematology; (iii) immune cell function at the effector or regulatory level; and/or (iv) host resistance studies involving infectious or neoplastic challenge. The first tier is usually a screen for immunotoxicity, while subsequent tiers consist of more specific or confirmatory studies, host-resistance studies or in-depth mechanistic studies. At present, most information regarding these models comes from the US National Institute of Environmental Health Sciences,

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National Toxicology Program (NIEHS/NTP) (Luster *et al*., 1988) and the Netherlands National Institute of Public Health and the Environment (RIVM) (van Loveren and Vos, 1992). Recently, a database consisting of over 50 compounds, including environmental, industrial and pharmacologic agents evaluated in one or more tiers of the NIEHS/NTP panel was analysed in an attempt to improve the accuracy and efficiency of screening for immunosuppression. Although some datasets had limitations, a number of important conclusions were drawn from these data (Luster *et al*., 1988; 1992).

- The T-dependent antibody response (TDAR) has good predictive value, while assessment of as few as two or three other immune parameters may successfully predict immunotoxicants in mice. However, commonly employed gross measures, such as lymphoid organ weights were somewhat insensitive as measures of immunotoxicity.
- A good correlation existed between changes in functional tests and altered host resistance. However, immune changes were often observed in the absence of detectable changes in host resistance, suggesting that immune tests are, in general, more sensitive than host-resistance assays.
- No single immune test was identified which could be considered highly predictive for altered host resistance in mice. However, combining several immune tests increased the ability to predict host-resistance deficits, in some cases to 100%.
- Most immune-function—host-resistance relation ships followed a linear rather than threshold model, suggesting that even small changes in immune function may theoretically translate into a deficit in host resistance. However, because of the variability in the responses, it was not possible to establish linear or threshold models for most of the chemicals.

8.3 Species and Dose Considerations

Although the pharmacokinetics of chemicals may differ between experimental animals and humans, rodents have proven to be useful models for examining the immunotoxicity of non-species-specific compounds. The exposure route should parallel the most probable route of human exposure, at dose levels likely to establish a distinct dose–response curve as well as a no-observable-adverse-effect-level (NOAEL). Immune system changes observed at overtly toxic dose levels should be interpreted cautiously since stress and malnutrition are known to impair immune responsiveness. Inclusion of a positive control group, exposed to a well-characterized immunosuppressant, is also important to validate the robustness of the assay.

8.4 Evaluation of Immune Function

A detailed list of tests and methods used to screen compounds, evaluate resistance to infection or neoplastic challenge or determine mode/mechanism of action is beyond the scope of this chapter. Reference works (Burleson *et al*., 1995) are an excellent source of detailed protocols and discussions of assay merits and shortcomings. The response evaluated by the TDAR is usually considered to be the best single indicator of immune function, probably because it requires intact macrophage (antigen processing), Th cell (source of stimulatory cytokines) and B cell (differentiation into antibody-producing plasma cells) function. Human antibody studies have generally been limited to measuring titres to common vaccine antigens. Studies of primary immune responses in newborns and young children in conjunction with established vaccination programs (such as against measles, diphtheria, tetanus and poliomyelitis) may offer a significant opportunity to assess chemical-induced alterations in immune status in populations with identified chemical exposure.

Cellular immunity is traditionally thought of as reactions mediated by T cells, exclusive of the Th component of antibody responses. Cytokines released by antigen-specific T cells amplify inflammatory responses, down-regulate normal immune responses to prevent tissue damage, affect contact-dependent killing of altered host cells and regulate the activity of self-reactive cells. Because clonal expansion of antigen-specific cells is critical to immune function, the proliferative capacity of T cells has been used as an *ex vivo* correlate of clonal expansion, although the predictive value of the assay is limited (van Loveren and Vos, 1992). The mixed lymphocyte response, an *in vitro* proliferative response to foreign antigens, rather than a polyclonal activator, is now preferred as a functional correlate of T cell expansion. Cytotoxic T lymphocytes (CTLs) play a central role in destroying chemically or virally modified host cells and neoplastic cells bearing tumour antigens. CTL function is typically assessed by culturing antigen-primed T cells, generated either *in vivo* or *in vitro*, with labelled tumour cells or foreign lymphocytes, and measuring label release.

Innate immune function may or may not be included in tiered testing panels, although the functional status of macrophages may be assessed as phagocytic activity, release of soluble mediators, and bactericidal or tumouricidal activity. Lysis of labelled tumour cells is the most common measure of NK cell function, and a variety of methods are available to assess the function of neutrophils, including chemotaxis, phagocytosis and the respiratory burst.

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8.5 Evaluation of Allergic Contact Dermatitis

Guinea pigs were traditionally used to test the sensitizing potential of chemicals, but animal costs, sensitivity issues and subjectivity of the assay end point led to the development of other assays (Burleson *et al*., 1995). The MEST is similar to the guinea pig assay in that both immune sensitization and elicitation of an immune response phases are required. In this assay, a compound is applied to the ear pina and evaluated by measuring changes in ear thickness following challenge. An alternative test is the LLNA in which the test material or appropriate control is applied topically in three successive daily applications to both ears of the test species, usually the mouse. Cell proliferation is subsequently measured in the lymph nodes draining the ears. At least one concentration of the test chemical must produce a three-fold increase or greater in lymphocyte proliferation in the draining lymph nodes of test animals compared with vehicle-treated control mice to be considered a positive.

8.6 Immunotoxicity Risk Assessment

As of 2008, formal guidance for immunotoxicity risk assessment has not been published, although efforts are underway in the US and Europe to develop guidelines. However, the basic risk paradigm of hazard identification, dose–response assessment, exposure assessment and risk characterization is applicable for immunotoxicity risk assessment. Confidence that the data reflects immunotoxicity can be judged with criteria similar to those described for end points such as cancer, as follows:

- Clear evidence of immunotoxicity: Dose-related changes in function (e.g. antibody synthesis or resistance to disease) or effects in multiple end points that suggest biological plausibility (i.e. alterations in NK cytotoxicity and NK cell numbers).
- Some evidence of immunotoxicity: No change in functional parameters but statistically significant, dose-related adverse changes in lymphoid tissue, histopathology or peripheral blood leukocyte counts.
- Equivocal evidence of immunotoxicity: Marginal deficits in immune parameters that may be chemically related (e.g. statistically significant changes in one or more parameters at middle or low doses, but not at high doses, in the absence of other supportive data).

Regardless of the end point being measured, data generated to assess immunotoxicity must be considered *in toto*, including dose responsiveness, general indications of toxicity, appropriateness of the test methods and the historical predictive value of the data.

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Mutagenesis

Anthony Lynch

CONTENTS

1 INTRODUCTION

'Mutagenesis' is defined by the Collins English Dictionary as the origin and development of mutation, and a 'mutagen' is a substance or agent that can induce genetic mutation. In a recent edition of the BBC Radio 4 series 'In our Time', the broadcaster Melvyn Bragg introduced a programme on Genetic Mutation (BBC archive at *www.bbc.co.uk/radio4/history/inourtime*) by referring to the lines written by the British geneticist J. B. S. Haldane, which he penned while lying mortally ill with cancer:

Cancer's a funny thing:
I wish I had the voice of Homer
to sing of rectal carcinoma,
which kills a lot more chaps, in fact,
than were bumped off when Troy was sacked…

Bragg went on to say that 'Haldane knew better than most that many cancers and many other diseases are caused by genetic mutation. Indeed, to understand mutation fully may explain the ravages of illness and even unlock the secrets of ageing. But mutation, so often a destroyer of life, is also its creator. Without it, the variety of living things on earth simply wouldn't exist. It is, in the Darwinian view of life, the raw material of evolution'. The aims of this chapter are to provide an overview of the organization of the genetic material, DNA, and describe the mechanisms by which mutations arise in DNA (mutagenicity) and the links between mutation and the role they play in heredity diseases and cancer; and also other multifactorial diseases and ageing. The chapter goes on to describe the discipline of genetic toxicology, its history and the development of assays for the measurement of mutations in model organisms. It culminates in a description of the standard regulatory battery of *in vitro* and *in vivo* tests used to assess the potential genotoxic liability of a novel test agent, plus additional supplementary tests that can be used to investigate further the underlying biological mechanisms resulting in genotoxicity, and thereby improve hazard characterization and/or risk assessment. The chapter continues with a discussion on data interpretation and current concepts of risk assessment in genetic toxicology and concludes with an outline of the challenges faced by genetic toxicology in the new Millennium, and describes developments within the discipline

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to meet these challenges. Other chapters of this volume are inextricably linked to this subject, including those on metabolism (see: Cytogenetics (**Cytogenetics**), Genetic Toxicology Testing and its Relevance to Human Risk and Safety Evaluation (**Genetic Toxicology Testing and its Relevance to Human Risk and Safety Evaluation**), Carcinogenicity and Carcinogens that are also Genotoxic Carcinogens (**Carcinogenesis and Carcinogens that are also Genotoxic**), Reproductive Toxicology and Developmental Toxicology (**Reproductive Toxicology** and **Developmental Toxicology**) and Regulations (Part 10)).

2 GENETICS, DNA STRUCTURE AND FUNCTION

Genetics is the study of inheritance, and the phrase genome, coined by Professor Hans Winkler in the 1920s, is the term used to describe the entirety of the hereditary information for a specific organism which is encoded by the genetic material, deoxyribonucleic acid (DNA). Consisting of gene sequences, regulatory sequences and structural sequences, the DNA contains all the basic information necessary to construct and control cell function and thus may be thought of as the Lego® of life.

The DNA is located in the nucleus of the cell and contains four different nucleotides: the larger purine bases, adenine (A) and guanine (G), consisting of two aromatic rings, and the smaller pyrimidine bases, cytosine (C) and thymine (T), consisting of a single aromatic ring (**Figure 1**). The bases are paired together in a precise fashion by hydrogen bonds: adenine with thymine (A:T) and cytosine with guanine (C:G); thus they connect the sugar phosphate backbone of the molecule, like the runs of a ladder, resulting in the characteristic double-helix structure of DNA (**Figure 1**), first proposed by the Nobel Prize laureates, Watson and Crick (1953). One consequence of the conserved base pairing in the double-helix structure is that each strand is the exact reverse of the other. The original 'coding strand' is called the sense strand, whilst the opposite strand is known as the antisense strand.

In prokaryotic organisms, such as bacteria, DNA is organized into a single molecule, normally a closed circle, which is not complexed with protein. In eukaryotic

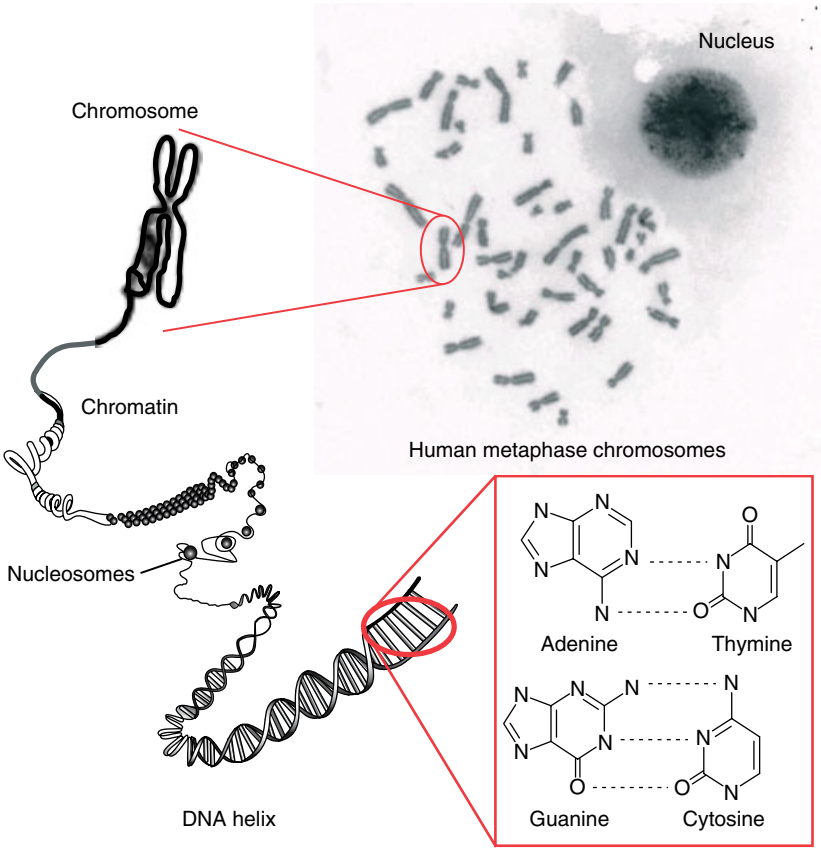

Figure 1 DNA structure and organization within the cell. The complement of 46 metaphase chromosomes (human karyotype) is shown lying next to an intact nucleus. One chromosome is blown up to reveal the degree of chromatin condensation and associated nucleosome structures involved in the organization of eukaryotic DNA. The red box shows the molecular structure of the nucleoside bases which form the 'struts' of the DNA helix, and the bonds between the pyrimidine and purine bases (dashed lines).

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organisms, however, DNA is organized into higher-order structures called chromosomes, which are comprised of DNA complexed with proteins called histones to form chromatin. Human chromatin consists of approximately 2 m of DNA coiled around a series of nucleosomes, with each nucleosome consisting of histones H2A, H2B, H3 and H4 as heterodimers that form a core unit. The histone tails carry a positive charge and are capable of interacting with the polyanionic back bone of DNA, contributing to nucleosome stability (Yap and Zhou, 2006). The nucleosomes are themselves tightly packaged with other proteins, to produce chromosomes which are parcelled into the nucleus (**Figure 1**). Surprisingly, even in this form chromatin retains a large degree of functionality (Richmond, 2006). The cells of each species contain a distinct number of chromosomes in their genomes; for humans this number is 46, arranged as 23 pairs (**Figure 1**), whilst for Chinese hamsters this is 22, arranged as 11 pairs. Thus each species has a characteristic compliment of chromosomes, known as a karyotype. Contained within the length of each chromosome are the genes. Each gene codes for, and controls the production of, a specific polypeptide or protein.

2.1 Genetic Code, Genes and Gene Regulation

The individual bases in DNA represent the fundamental units of genetic language in all living organisms (akin to the alphabet of human languages), whereas the linear sequence of bases within the entire genome represents the complete instruction manual. To be of any use, however, the information contained in the genome must be deciphered by living cells via the genetic code. This is the set of rules by which information encoded in genetic material is translated into polypeptides and proteins (amino acid sequences). Specifically, the code defines a mapping between trinucleotide sequences or triplets in the DNA, called codons, and amino acids, via an RNA intermediate, and is regulated by the processes of transcription and translation (see below). Each triplet codon specifies a single amino acid within the genetic code.

A structural gene may be defined as a linear sequence of codons which codes for a functional polypeptide or protein; that is, a linear sequence of amino acids. Individual polypeptides/proteins may have structural, enzymatic or regulatory roles within the cell or organism. In prokaryotic organisms, genes are made up of simple nucleic acid sequences which are transcribed directly into messenger RNA (mRNA) and then translated into proteins. In contrast, genes in eukaryotic organisms are organized into 'intragenic regions' or introns and 'extragenic regions' or exons. Discovered by the 1993 Nobel Prize laureates, Roberts and Sharp, and coined by the biochemist Walter Gilbert, the term exons refers to nucleic acid sequences that are transcribed into mRNA and eventually code for amino acids in polypeptides and proteins, whereas introns are DNA sequences which are found between exons, but are not transcribed (Gilbert, 1978). Genes which contain introns are known as interrupted genes. Simple eukaryotic organisms such as yeasts have very few interrupted genes, whereas in higher-order species, like mammals, almost every gene has an intron. The number and DNA sequence length of individual exons that code for different proteins vary. Some proteins are derived from genes with only three or four exons, but others may have many more. Introns sometimes allow for alternative splicing of a gene, so that several different proteins which share some sequences in common can be translated from a single gene. For example, Nogo proteins are a group of polypeptides belonging to the reticulon family (Oertle and Schwab, 2003). Cloning of the *Nogo* gene showed three major transcripts, referred to as Nogo-A, -B, and -C, originated by alternative splicing (Nogo-A vs. Nogo-B) or differential promoter usage (Nogo-C vs. Nogo-A/B). The control of mRNA splicing is performed by a wide variety of signalling molecules.

The expression of structural genes is regulated by a special set of codons, organized as 'promoter' sequences. The promoter sequence is a regulatory region of DNA, generally located upstream to a gene that provides the initial binding site for RNA polymerase, the enzyme that synthesizes the RNA from the coding region of the gene, and promotes transcription of the gene (see below). Different promoter sequences have different affinities for RNA polymerase, due to specific DNA sequences called response elements, which are recognized by proteins known as transcription factors. These factors bind to the promoter sequences, recruiting RNA polymerase. In eukaryotic organisms, some sets of structural genes with linked functions have a single promoter, and their coordinate expression is controlled by another regulatory gene called an operator. A group of such genes is called an operon. The activity of the operator is further controlled by a protein called a repressor, since it stops the expression of the whole operon by binding to the operator sequence, preventing RNA polymerase from binding to the promoter. Repressors can be removed by relevant chemical signals or in a time-related fashion.

In eukaryotic organisms, promoters are extremely complex and diverse and can be difficult to characterize, particularly in higher species. Indeed, the expression of a particular gene may be regulated by the concerted action of both *cis*- and *trans*-acting elements. The promoter sequence may be considered as the principal *cis*-acting regulatory element which promotes transcription of, and is physically adjacent to, the coding sequences of the gene, although some *cis*-acting regulatory elements that moderate gene expression may be located up to several kilobases upstream from the transcriptional start codon ATG; for example, the haemoxygenase-1 (*hmox-1*) gene promoter contains regulatory sequences ~5 kb

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upstream of the site for the start of transcription (Elbirt and Bonkovsky, 1999). *Trans*-acting factors affect the expression of genes that may be physically located very far away, sometimes 3′-prime from the gene promoter, as with the *GADD45* (growth arrest and DNA damage) gene (Zheng *et al*., 2000), and, on occasion, even on different chromosomes. Promoters may be classified according to the type of gene expression control they provide and may be considered tissue and/or development specific, constitutive (where gene expression is seen in virtually every tissue irrespective of cell type and/or developmental stage and/or environmental factors) or inducible (where expression may be modulated by environmental and/or temporal factors, including chemical exposures). Examples of the last of these are the aryl hydrocarbon inducible promoters of the cytochrome P450 gene family.

2.1.1 Transcription and Translation

The DNA of each protein-coding gene is transcribed into a template molecule of the related polymer RNA, known as messenger RNA(mRNA), by RNA polymerase. Although similar to DNA, mRNAs are single stranded, and possess the base uracil instead of thymine, and the sugar ribose rather than deoxyribose. These molecules act as short-lived copies of the genes being expressed. The mRNA in turn is translated on the ribosome into an amino acid chain or polypeptide. The process of translation requires transfer RNAs (tRNAs) specific for individual amino acids with the amino acids covalently attached to them, guanosine triphosphate as an energy source, and a number of translation factors. tRNAs have anticodons complementary to the codons in mRNA, and can be 'charged' covalently with amino acids at their 3′ terminal CCA ends. Individual tRNAs are charged with specific amino acids by enzymes known as aminoacyl tRNA synthetases, which have high specificity for both their cognate amino acids and tRNAs. The high specificity of these enzymes is a major reason why the fidelity of protein translation is maintained.

The standard genetic code is shown in **Table 1** and shows each amino acid specified by each of the 64 codons. For example, the codon AAA represents the amino acid lysine, and ACA and ACU represent threonine (standard three-letter designations, Lys and Thr, respectively). There are $4^3 = 64$ different codon combinations possible with a triplet codon of three nucleotides. In reality, all 64 codons of the standard genetic code are assigned for either amino acids or stop signals during translation. If, for example, an RNA sequence, AUGCCCUUU is considered and the reading-frame starts with the first A (by convention, 5′ to 3′), there are three codons, namely, AUG, CCC and UUU, each of which specifies one amino acid

Table 1 The standard genetic code

mRNA codon	Amino acid	mRNA codon	Amino acid	mRNA codon	Amino acid	mRNA codon	Amino acid
AAA	Lysine (Lys)[a]	ACA	Threonine (Thr)	AGA	Arginine (Arg)	AUA	Isoleucine (Ile)
AAC	Asparagine (Asn)	ACC	Threonine (Thr)	AGC	Serine (Ser)	AUC	Isoleucine (Ile)
AAG	Lysine (Lys)	ACG	Threonine (Thr)	AGG	Arginine (Arg)	AUG[b]	Methionine (Met)
AAU	Asparagine (Asn)	ACU	Threonine (Thr)	AGU	Serine (Ser)	AUU	Isoleucine (Ile)
CAA	Glutamine (Gln)	CCA	Proline (Pro)	CGA	Arginine (Arg)	CUA	Leucine (Leu)
CAC	Histidine (His)	CCC	Proline (Pro)	CGC	Arginine (Arg)	CUC	Leucine (Leu)
CAG	Glutamine (Gln)	CCG	Proline (Pro)	CGG	Arginine (Arg)	CUG	Leucine (Leu)
CAU	Histidine (His)	CCU	Proline (Pro)	CGU	Arginine (Arg)	CUU	Leucine (Leu)
GAA	Glutamic acid (Glu)	GCA	Alanine (Ala)	GGA	Glycine (Gly)	GUA	Valine (Val)
GAC	Asparagine (Asp)	GCC	Alanine (Ala)	GGC	Glycine (Gly)	GUC	Valine (Val)
GAG	Glutamic acid (Glu)	GCG	Alanine (Ala)	GGG	Glycine (Gly)	GUG	Valine (Val)
GAU	Asparagine (Asp)	GCU	Alanine (Ala)	GGU	Glycine (Gly)	GUU	Valine (Val)
UAA[c]	Stop	UCA	Serine (Ser)	UGA	Stop	UUA	Leucine (Leu)
UAC	Tyrosine (Tyr)	UCC	Serine (Ser)	UGC	Cysteine (Cys)	UUC	Phenylalanine (Phe)
UAG[d]	Stop	UCG	Serine (Ser)	UGG	Tryptophan (Trp)	UUG	Leucine (Leu)
UAU	Tyrosine (Tyr)	UCU	Serine (Ser)	UGU	Cysteine (Cys)	UUU	Phenylalanine (Phe)

[a] Standard three-letter designations are shown in parenthesis.
[b] Also known as the start of translation codon.
[c] Also known as the ochre codon.
[d] Also known as the amber codon.

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(i.e. methionine, proline and phenylalanine). This RNA sequence will be translated into an amino acid sequence, three amino acids long. From **Table 1** it can be seen that the genetic code has redundancy but no ambiguity. For example, although codons AAA and AAG both specify lysine (redundancy), neither of them specifies any other amino acid (no ambiguity). The codons encoding one amino acid may differ in any of their three positions. For example the amino acid glutamic acid is specified by GAA and GAG codons (difference in the third position); the amino acid leucine is specified by UUA, UUG, CUU, CUC, CUA, CUG codons (difference in the first or third position); while the amino acid serine is specified by UCA, UCG, UCC, UCU, AGU, AGC (difference in the first, second or third position).

A position of a codon is said to be a four-fold degenerate site if any nucleotide at this position specifies the same amino acid. For example, the third position of the threonine codons (ACU, ACC, ACA and ACG) is a four-fold degenerate site, because all nucleotide substitutions at this site are synonymous, that is, they do not change the amino acid. Only the third positions of some codons may be four-fold degenerate. A position of a codon is said to be a two-fold degenerate site if only two of four possible nucleotides at this position specify the same amino acid. For example, the third position of the glutamic acid codons (GAA, GAG) is a two-fold degenerate site, so is the first position of the leucine codons (UCA, UCC, CCU, CCC, CCA, CCG). In two-fold degenerate sites, the equivalent nucleotides are always either two purines (A/G) or two pyrimidines (C/U), so only transversional substitutions (i.e. purine to pyrimidine or pyrimidine to purine, see below) in two-fold degenerate sites are nonsynonymous. A position of a codon is said to be a nondegenerate site if any mutation at this position results in amino acid substitution. There is only one possible three-fold degenerate site where changing three of the four nucleotides has no effect on the amino acid, while changing the fourth possible nucleotide results in an amino acid substitution. This is the third position of an isoleucine codon: AUU, AUC or AUA all encode isoleucine, but AUG encodes methionine. There are three amino acids encoded by six different codons: serine, leucine and arginine. Only two amino acids are specified by a single codon; one of these is the amino acid methionine, specified by the codon AUG, which also specifies the start of translation; the other is tryptophan, specified by the codon UGG. The degeneracy of the genetic code is what accounts for the existence of silent mutations (see below).

These properties of the genetic code make it more fault-tolerant for point mutations. For example, in theory, four-fold degenerate codons can tolerate any point mutation at the third position, although codon usage bias restricts this in practice in many organisms; two-fold degenerate codons can tolerate one out of the three possible point mutations at the third position. Since transition mutations (i.e. purine to purine or pyrimidine to pyrimidine) are more likely than transversion mutations (i.e. purine to pyrimidine or vice versa), the equivalence of purines or that of pyrimidines at two-fold degenerate sites adds a further fault tolerance.

2.1.2 The Cell Cycle

The majority of somatic (nonreproductive) cells in the body undergo cycles of cell growth and cell division resulting in the formation of daughter cells. The totality of events leading to, and including cell division, is known as the cell cycle (**Figure 2**), and consists of four recognized stages:

- **G1 (gap 1):** the cell enlarges and prepares to replicate the DNA
- **S-phase (DNA synthesis):** the complement of chromosomes is duplicated
- **G2 (gap 2):** there is little cellular activity during this phase; the cell synthesizes RNA and proteins required for mitosis
- **M-phase (mitosis):** the complement of chromosomes separates to produce two new, identical daughter cells.

The new daughter cells may either enter G1 immediately, or alternatively, may undergo a rest period (termed G0) until presented with a stimulus to replicate. Cells at the G1 phase may re-enter the G0 rest period up to a point where the cell becomes committed to undergoing division, called the restriction point. Cells may undergo up to 30–45 divisions during a lifetime (depending on cell type), after which the cells age (a process termed senescence), enter a period of crisis and then die.

2.1.2.1 DNA Replication (S-phase)

Double-stranded DNA has a unique property in that it is able to make identical copies of itself when supplied with precursors, relevant enzymes and cofactors. The first step in DNA replication requires that the two strands of DNA are uncoiled, in an unzipping process, as the hydrogen bonds are broken. This results in single-stranded DNA and exposure of free base pairs, to which new complementary deoxyribonucleotide triphosphates bind under the control of a DNA polymerase enzyme, forming new base pairs and hence a new strand of DNA. Once again, because base pairing is always the same, the new strand is a reverse copy of, or complementary to, the original, producing two exact copies of the original double helix. The period of the cell cycle where DNA replication occurs is referred to as S-phase (or DNA synthesis).

2.1.2.2 Cell Division (M-phase)

Mitosis and cytokinesis together define the mitotic (M)-phase of the cell cycle, the division of a cell into

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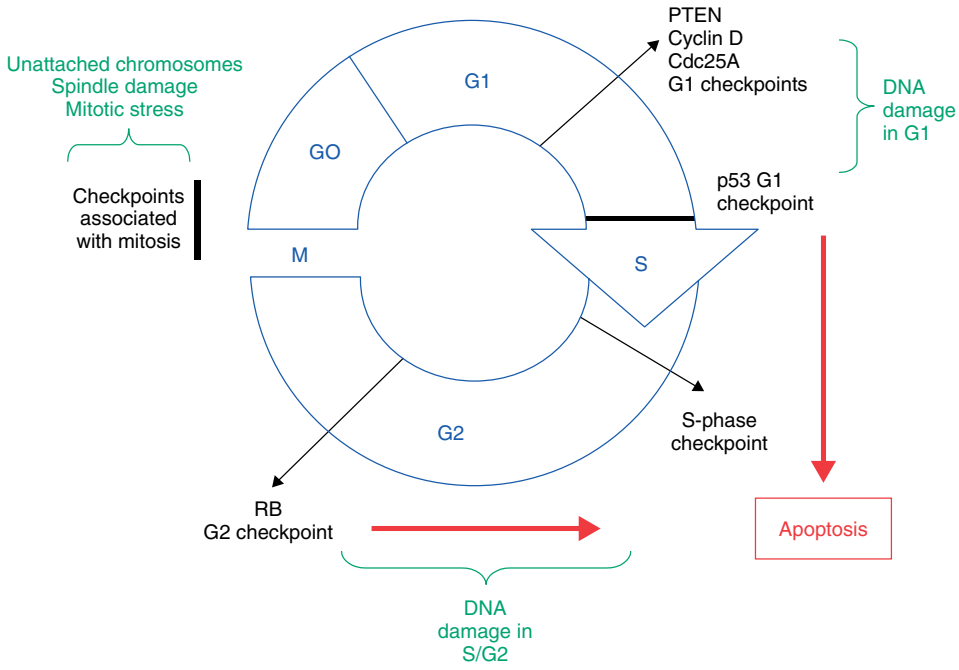

Figure 2 The cell cycle. The cell cycle is divided into G1 (cell growth 1), S (DNA synthesis), G2 (cell growth 2) and M (mitosis). DNA or genomic damage results in cell cycle delay through the activation of cell cycle checkpoints. If the damage is repaired then the cycle resumes. If there is too much damage then the cell may undergo apoptosis (programmed cell death). The cyclins, for example Cdc25A and cyclin D, plus the tumour suppressor proteins p53, RB and PTEN contribute to cell cycle control in mammalian cells. Loss or mutation of genes encoding these proteins encourages cell proliferation and the deregulation of cell cycle blocks in response to genetic damage.

two daughter cells, each with the genetic equivalent of the parent cell. Mitosis is the process by which the chromosomes in the cell nucleus are divided in order to generate two, haploid, daughter nuclei. The process of mitosis in mammalian cells is complex and highly regulated. The sequence of events is divided into phases, termed prophase, metaphase, anaphase and telophase. During prophase, nuclear chromatin material condenses to form chromosomes, the nuclear envelope disassembles and the centrosomes migrate to opposite poles of the cell. The mitotic spindle apparatus is then assembled under the control of centrosomes, which function as microtubule organizing centres at opposite poles of the spindle. The microtubule fibres from opposing poles of the mitotic spindle attach to one another or to the chromosomes via kinetochores, complex protein structures located at the centromeres of chromosomes. During metaphase, the chromosomes become aligned at the metaphase plate, an imaginary line that is equidistant from the two spindle poles. This even alignment is due to the counterbalance of the pulling powers generated by opposing kinetochores and centrosomes. At anaphase, the proteins that bind sister chromatids together are cleaved, allowing them to separate, and the spindle apparatus pulls the sister chromatids to opposite poles of the spindle. Mitosis is concluded with telophase, when sister chromatids arrive at the opposite poles of the spindle, decondense and become enveloped by new nuclear membranes to form the daughter nuclei. Mitosis is generally followed immediately by cytokinesis, where the two daughter nuclei, cytoplasm, organelles (including the centrosomes) and cell membrane are divided equally between the two daughter cells. An interactive animation showing the various stages of animal cell mitosis may be viewed at *www.cellsalive.com/mitosis.htm*.

2.1.2.3 *Cell Cycle Regulation*

Regulation of the cell cycle involves steps crucial to the cell, including detecting and repairing genetic damage, and provision of various checks to prevent uncontrolled cell division. The 2001 Noble Prize for Physiology was shared by Hartwell, Hunt, and Nurse for the discovery of the key molecular events that control the cell cycle (for an illuminating account of this work see Nurse, 2002). There are two main classes of regulatory molecules, cyclins and cyclin-dependent kinases (CDKs), which control progress through the cell cycle (reviewed by Nurse, 1997; also see Malumbres and Barbacid, 2005; Besson *et al*., 2008). Many of the genes encoding cyclins and CDKs are conserved among eukaryotic organisms, but in common with other biological systems, the organization of cell-cycle control systems is more elaborate in higher-order species (Sánchez and Dynlacht, 2005). The two main checkpoints are the G1/S checkpoint and the G2/M checkpoint. G1/S transition is a rate-limiting step

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in the cell cycle and is also known as the restriction point, as the cell cannot proceed to the next phase until checkpoint requirements have been met. Cell-cycle checkpoints are used by the cell to monitor and regulate the progress of the cell cycle, thereby ensuring that various phases are completed before progress can continue and/or to repair DNA damage and prevent damaged or incomplete DNA from being passed on to daughter cells.

The proteins encoded by the tumour suppressor genes *TP53* and retinoblastoma (*Rb*) play an important role in triggering both the G1/S and G2/M checkpoints (**Figure 2**). The p53 protein, voted molecule of the year in 1993 by the journal *Science*, is a transcription factor that becomes activated when the cell is subject to various types of DNA damage and/or other cellular stresses; for example membrane damage, oxidative stress, osmotic shock, heat shock, nucleotide depletion and deregulated oncogene expression. Once activated, p53 plays a pivotal role in inducing cell cycle arrest and activating DNA repair enzymes. The cell cycle arrest provides time for the repair of DNA damage before the cell replicates its DNA (G1/S) or prior to mitosis (G2/M), where unrepaired damage can be lethal to the cell or lead to the formation of deleterious mutations. Checkpoint-arrested cells resume cell cycle progression once damage has been repaired, whereas cells with unrepairable DNA lesions undergo permanent cell cycle arrest or programmed cell death (apoptosis), which may also be initiated by p53. The signalling pathways associated with p53 and DNA damage surveillance, cell cycle arrest and apoptosis are highly elaborate and beyond the scope of this chapter; therefore the reader is referred to the following reviews: Meek (2004); Helton and Chen (2007); Chumakov (2007); Schmitt *et al*. (2007); Branzei and Foiani (2008).

Surprisingly, the centrosome is increasingly being recognized as a key player in regulating cell cycle events, such as orchestrating entry into mitosis, anaphase onset, cytokinesis, G1/S transition, and monitoring DNA damage. Recently, the centrosome has also been described as a hub where regulatory complexes, such as kinases, phosphatases and other cell cycle regulators accumulate to coordinate multiple cell-cycle-specific functions. Dysfunctional centrosomes are associated with various diseases including cancer, Alström's syndrome (Online Mendelian Inheritance in Man™ (OMIM™) database reference #203800; see **Box (1)**), various neurological disorders, and several types of infertility (reviewed by Schatten, 2008). In addition, there are a number of other key structural and protein components which are pivotal for mitotic fidelity; for example, the spindle checkpoint and G(1) postmitotic checkpoints which monitor for gross cell division aberrations and thereby prevent the likelihood of aneugenicity and/or polyploidy (reviewed by Decordier *et al*., 2008).

Box 1 Mendelian Inheritance in Man™ database

Founded in the 1960s by the eminent human geneticist, Dr. Victor McKusick (John Hopkins University), who remains the current curator, the Mendelian Inheritance in Man™ (MIM™) database catalogues all known human genes and genetic disorders and focusses on the relationship between phenotype and genotype. The online version, OMIM™ (at *www.ncbi.nlm.nih.gov*) was set up in 1985 and contains full-text and referenced overviews of all known Mendelian disorders and over 12 000 genes. OMIM™ is updated daily, and the entries contain many links to other genetics resources. For an illuminating account of his life-works and origins of medical genetics as a discipline, the reader is referred to an autobiographical review by McKusick (2006).

Therefore, the control of the cell cycle, DNA replication and mitosis are highly complex process which are critical to the maintenance of the fidelity of DNA and genomic stability. Disregulation of the cell cycle (McDonald and El-Deiry, 2000) or DNA synthesis (McCulloch and Kunkel, 2008) or mitosis (Schatten, 2008), for example via mutation in p53, cyclins or DNA polymerases and/or disruption to centrosome function and so on, may result in genomic instability, and is associated with increased cancer susceptibilities. For example, mutations in p53 are common in many human cancers and thus p53 is considered to be a key 'gatekeeper' in multicellular organisms, protecting genomic stability, and thereby suppressing cancer (Helton and Chen, 2007). As such, p53 is often described as 'the guardian of the genome' (see below).

2.2 Gene Mutation, Mutagenesis and Mutagenicity

The concept of gene mutation (from the Latin word *mutare*, to change) was originally developed by one of the first geneticists, Hugo Marie de Vries, following his rediscovery of Gregor Mendel's laws on heredity in the 1890s, and based on his observations on the evening primrose, *Oenothera* (de Vries, 1900). However, it was later discovered that much of what de Vries was describing in terms of his evidence had nothing to do with what is now known as genetic mutation. To the modern geneticist/genetic toxicologist, the term '*gene mutation*' is used to mean a detectable permanent (heritable) change within a single gene or its regulatory sequences, and 'mutagenesis' is the term used to describe the process by which this occurs. The term 'gene mutation' is therefore usually applied to point mutations; that is,

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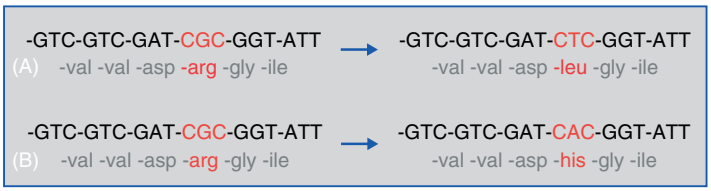

Figure 3 Single base substitution mutations. (A) Single base substitution (guanine replaced by thymidine) in the fourth codon, resulting in a transversion mutation (a mutation in which a purine/pyrimidine is exchanged for a pyrimidine/purine base pair or vice versa); (B) single base substitution (guanine replaced by adenine) in the fourth codon, resulting in a transition mutation (in which a purine is exchanged for another purine nucleotide or a pyrimidine by another pyrimidine nucleotide).

qualitative changes within a single gene involving one or a few nucleotide bases in the DNA sequence (see below). However, it is also common to find the term 'gene mutation' applied more loosely, referring to larger changes involving deletion of chromosomal fragments and/or whole chromosomes (also known as aneuploidy) and even to changes in whole chromosome sets (polyploidy).

Mutagenicity is the capacity to induce permanent changes within genes or their regulatory sequences. The changes may be single base substitutions, insertions, deletions and so on (see below). Sometimes, the term is used more loosely to describe *genetic toxicity (or genotoxicity)*; that is, a broader term that refers to any deleterious change in the genetic material regardless of the mechanism or fate of the induced change. Thus genotoxicity may also encompass nonpermanent and/or premutagenic lesions in the DNA, such as DNA adducts (see below), or may reflect cellular responses, such as the induction of DNA repair systems or cell cycle delay (see above), that are used to infer the presence of DNA damage either directly (DNA adducts) or indirectly (induction of DNA repair or cell cycle delay). However, since DNA damage *per se* may or may not result in permanent genetic changes, it is recommended that specific terminology is used where appropriate, depending on context.

Gene mutations may be characterized in various ways, depending on their molecular make-up or genetic consequences.

2.2.1 Single Base Substitutions (Point Mutations)

Gene mutations can occur when one base is substituted for another, leading to a single base substitution (also known as a point mutation). There are basically two types of base substitution; the first is where one purine base or one pyrimidine base is substituted for another; this type of base substitution is called a transition mutation. In comparison, the substitution of a purine base for a pyrimidine base or vice versa is called a transversion mutation. Viewed in another way, the orientation of purine and pyrimidine bases remains the same in a transition mutation but is reversed in a transversion mutation (**Figure 3**). Both types of base substitution are observed within mutated genes.

Because of the redundancy in the genetic code (see above), about a quarter of all possible base substitutions produce silent (or synonymous) mutations that do not result in amino acid replacements. Single base substitutions, however, may lead to codon changes within the coding sequence of a gene that result in a nonsynonymous mutation, which can occur in one of three main ways:

- **Missense mutations** result from single base substitutions that lead to the insertion of an 'inappropriate' amino acid into the polypeptide during translation. Most single base substitutions will often have no functional effect and are termed conservative substitutions. Nonconservative substitutions, however, have a dramatic effect on protein function depending on the context of the amino acid change within the polypeptide sequence. For example, if the changed amino acid involves the active centre of an enzyme this can lead to a gross reduction in, or indeed, complete loss of, enzyme function. Alternatively, if the amino acid change affects protein folding and its three-dimensional tertiary structure, then the formation of a mutant protein may result, with a similar outcome in loss of biological activity. Many of the abnormal haemoglobins associated with human sickle cell disease and thalassaemias are the result of missense mutations.
- **Nonsense mutations** are single base substitutions which result in the formation of a new inappropriate terminator (or nonsense) codon (see **Table 1**) within the 'reading frame' of the gene. This can lead to the premature termination of gene transcription. The polypeptide formed from such mutated genes will be truncated, that is, shorter than normal, and usually inactive, although on rare occasions the truncated protein may acquire a new and/or novel function (i.e. 'gain-of-function' mutation).
- **Frameshift mutations** arise from the deletion or insertion of nucleotide bases in the DNA that result in a change in the 'reading frame' of a gene (**Figure 4**). A deletion involves the loss of one or

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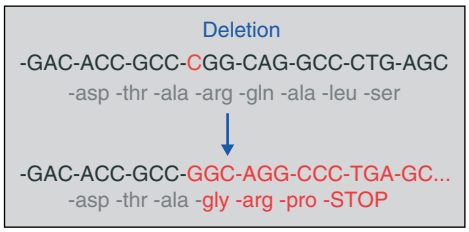

Figure 4 Frameshift mutation. Single base deletion at cytosine in the fourth codon results in a change of the downstream reading frame and introduction of a premature stop codon to produce a nonsense mutation.

more nucleotides, and if a deletion occurs within a coding sequence that involves one, two, or a larger number of bases which is not a multiple of 3, the deletion will result in a change in the 'reading frame' of the gene. Larger deletions may result in partial or whole gene deletions, and may arise through unequal crossover between repeat DNA sequences; for example, as seen in the human genetic disease hereditary and sensory neuropathy Type 1, or chromosomal damage. An insertion, in contrast, involves the addition of one or more nucleotides into the DNA sequence. Again, if the insertion occurs in a coding sequence and involves one, two, or a larger number of bases that is not a multiple of 3, the deletion will result in a change in the 'reading frame' of the gene. Frameshift mutations tend to have a dramatic effect on the translation of the affected gene, as most amino acids after the point of the insertion or deletion of bases will differ from the wild-type in the resulting mutant polypeptide. Very often a new terminator codon is introduced into the reading frame downstream from the point of the insertion or deletion, so, again, a truncated polypeptide will result. As with nonsense mutations, frameshift mutations usually result in polypeptides that are inactive, although, again on rare occasions, they may be 'gain-of-function' mutations.

Single base substitutions, deletions and insertions may also occur in key promoter elements, and these may impact on the transcriptional regulation of a gene, sometimes resulting in reduced and/or total loss of gene expression. Occasionally, mutations result in 'gain-of-function', either as a result of increased levels of gene expression or the development of a new capacity. All types of mutation that result in an altered polypeptide or gene expression, no matter how they arise, can, in turn, have a marked effect on the phenotype of the affected cell (most often due to the loss of enzyme/protein function and the resulting biological sequelae). Much use of phenotypic changes is made in mutagenicity tests. Sequence variants with no obvious effect upon phenotype may be termed polymorphisms.

The vast majority of base substitutions, frameshift mutations, deletions and insertions occur spontaneously, through errors in DNA replication, repair or mitotic division, although they can be induced by exposure to environmental mutagens such as ultraviolet light, ionizing radiation (IR) (i.e. cosmic rays and/or nuclear radiation) and/or genotoxic chemicals. Although the molecular mechanisms resulting in mutation induction maybe specific to a particular environmental genotoxin, the potential hazards associated with environmental exposure to genotoxins capable of inducing different types of genetic mutation are equivalent. Somatic mutations may cause adult-onset disease, such as cancer, but these mutations cannot be transmitted to offspring. A mutation in the germline can be transmitted to future generations, unless it affects fertility or survival into adulthood. It is estimated that each individual carries up to six recessive mutant alleles that in a homozygous state would have serious biological consequences, that is, result in lethality or severe genetic disease (see below).

2.2.2 Chromosome Abnormalities

These may be divided into structural and numerical abnormalities. Structural chromosomal rearrangements result from chromosome breakage (also known as clastogenicity) and, on occasion, subsequent reunion of the chromosomes in a different configuration. Structural chromosomal rearrangements can be balanced or unbalanced. In balanced rearrangements the chromosome compliment is complete, with no loss or gain of genetic material, for example the reciprocal translocation of genetic information from one chromosome to another. They are generally harmless, with the exception of rare cases where one of the breakpoints may adversely impact the expression of important regulatory genes; for example, Philadelphia chromosome, a diagnostic marker of chronic myeloid leukaemia, is associated with a translocation in the *BCR-ABL* oncogene which transforms cells (Sessions, 2007). An unbalanced rearrangement, as the name suggests, usually results in a net loss of genetic material (and rarely gain) and is associated with more adverse genetic consequences. Numerical chromosomal abnormalities involve the gain or loss of one or more whole chromosomes, which is known as aneuploidy, or the addition of one or more haploid complements of chromosomes, which is known as polyploidy. The types of chromosome abnormalities and mechanisms by which they arise are described in more detail in **Cytogenetics**.

2.2.3 DNA Adducts

The reaction of a chemical genotoxic carcinogen with DNA, either directly or after metabolic activation, typically involves covalent binding of an electrophilic carcinogen with a nucleophilic site in DNA, to form

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a DNA adduct. Guanine is by far the most prevalent target in DNA, and adducts have been described at several sites within the guanine molecule (e.g. N7, C8, O6 and exocyclic N2 positions), although adducts have been reported for all of the bases present in DNA. Such adducts can be small, such as the simple addition of ethyl and methyl groups associated with DNA alkylation or oxidized DNA bases such as O6-MeG, or they can be bulky, owing to reaction with large polycyclic organic molecules such as the metabolites of benzo(a)pyrene or aflatoxin B_1 (e.g. aflatoxin B_1 and the 8,9-dihydro-8-(*N*′7-guanyl)-9-hydroxy-aflatoxin B_1 adduct), or a combination of small and bulky adducts such as those associated with reactive oxygen species (Phillips, 1997). Fundamental to the advances made in the last two decades has been a vast effort to elucidate the chemical structures of DNA adducts. Adduct species formed via chemical reactions with naked DNA *in vitro,* as well as in experimental biological model systems, have been characterized using various sensitive analytical techniques mass, fluorescence and nuclear magnetic resonance spectrometry, high-pressure liquid chromatography, thin-layer chromatography (TLC), and so on (reviewed by Sharma and Farmer, 2004). Further information, on a molecular level, has been obtained by subjecting purified adducts to nuclear magnetic resonance and other methods to determine stereospecificity and three-dimensional structure. For a review of the field over the past two decades see Delaney and Essigmann (2008).

Adducts can form chemical links between adjacent nucleotide bases on the same DNA strand (intrastrand crosslinks) and can form interstrand crosslinks between each strand of double-stranded DNA. If a DNA adduct involves the nitrogen or oxygen atoms involved in base pairing, and the adducted DNA is not repaired, base substitution can result. Some unrepaired alkylated bases are lethal, owing to interference with DNA replication, while others, such as O6-methylguanine (O6-MeG) lead to mutation if unrepaired. These differences indicate that not all DNA adducts are equivalent. In fact, some adducts appear not to interfere with normal DNA functions or are rapidly repaired, others are mutagenic and yet others are lethal. DNA adduct levels, measured at any single point in time, reflect tissue-specific rates of adduct formation and removal, which depend upon genotoxic carcinogen exposure and activation, DNA repair, adduct instability and tissue turnover (Poirier *et al*., 2000). As such, DNA adduct levels have been used as biomarkers of exposure to potential genotoxic carcinogens in a wide variety of human occupational and environmental exposure studies. The correlations of DNA adducts, however, with cytogenetic biomarkers of genetic damage (such as chromosome aberrations or micronuclei (MN)) or indeed target organ tumourigenicity, are invariably complex, with examples of both positive correlation and no correlation whatsoever (reviewed by Gyorffy *et al*., 2008).

Some compounds that have a flat, planar structure, such as the anthracyclins, can intercalate between the DNA strands of the DNA helix and bind reversibly but noncovalently to DNA. The most extensively studied DNA intercalating agents are acridine and its derivatives. The intercalated molecules may interfere with DNA replication or the activity of repair enzymes and cause additions and deletions of base pairs, and are therefore frameshift mutagens, especially in bacteria and bacteriophage, but do not otherwise show a wide range of mutagenic properties. Recent advances in technology for detecting intercalation (Snyder, 2007) suggest this may be a property of a wider range of chemicals than previously recognized. For example, quite a number of flavonoids appear to intercalate into DNA. However, their mutagenic properties may be dominated by the fact that many of them are also able to inhibit topoisomerase II enzymes, and are therefore likely to be clastogenic (Ferguson and Denny, 2007).

Protein adduct formation is considered to be a valuable surrogate for DNA adduct formation since many chemical carcinogens bind to both DNA and protein in blood with similar dose–response kinetics. Haemoglobin and serum albumin are the proteins of choice because they are readily accessible, more abundant than DNA, and have known rates of turnover. Since protein adducts are not removed by active repair processes, chemically stable adducts of haemoglobin or serum albumin provide a more precise measure of dose integrated over the lifespan of the proteins in question (reviewed by Farmer and Shuker, 1999). Thus protein adducts have been used as biomarkers of exposure to potential genotoxic carcinogens in a variety of human occupational and environmental exposure studies, for example with heterocyclic amines, dietary genotoxic carcinogens present in cooked red meat (Lynch *et al*., 1991; 1993).

2.3 DNA Repair

The integrity of the genome is crucial for its function. However, DNA in living cells is inherently unstable and it is constantly attacked by damaging agents that threaten its integrity. These agents can arise from cellular metabolic processes or can be induced by environmental factors. Insults such as hydrolysis, oxidation, alkylation, and mismatch of DNA bases, are endogenous sources of DNA damage; IR, ultraviolet radiation (UVR), and many kinds of chemical agents are common sources for exogenous DNA damage.

DNA damage can lead to severely impaired cellular functioning, which might cause cell death, or can induce permanent changes in the DNA sequence, contributing to oncogenesis, premature ageing and severe genetic disorders (Hoeijmakers, 2001; van Gent *et al*., 2001). Cells have developed complex signal transduction,

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cell-cycle-checkpoint and repair pathways to respond to DNA damage and promote genomic stability. About 150 genes associated with DNA repair have been identified in humans to date (Wood *et al*., 2005). This is not surprising, considering that DNA molecules can be damaged in many ways and repair systems must be able to recognize and deal with each type of damage.

Exposure of mammalian cells to genotoxic agents activates an intricate network of mechanisms collectively known as the DNA-damage response. This response includes DNA repair and DNA-damage signalling pathways that alert the cell to the presence of DNA damage and coordinate the appropriate response (Iliakis *et al*., 2003; O'Driscoll and Jeggo, 2006). The DNA-damage response leads to slowing or arrest of cell-cycle progression at defined checkpoints, and DNA repair, but also to marked alterations in numerous physiological processes, such as an increase in cellular levels of deoxyribonucleotides, changes in chromatin structure at the site of DNA damage, changes in gene-expression profiles, and probably also in protein synthesis, degradation and trafficking (Jackson, 2002; Rouse and Jackson, 2002; Shiloh, 2003).

Different DNA-repair pathways exist that are essential for cells to efficiently respond to DNA damage. These pathways include: (i) the direct reversal pathway, (ii) the mismatch repair (MMR) pathway, (iii) the nucleotide excision repair (NER) pathway, (iv) the base excision repair (BER) pathway, (v) the homologous recombination (HR) pathway, and (vi) the nonhomologous end-joining (NHEJ) pathway. In 1994, the journal *Science* voted the 'DNA repair enzyme' molecule of the year!

2.3.1 Direct Reversal of DNA Damage

Direct reversal of the DNA damage is in most cases not possible due to thermodynamic or kinetic reasons. In some cases, however, the DNA damage can be reversed directly. These repair reactions are direct one- or two-step processes, and do not require multiple proteins like the repair pathways.

2.3.1.1 Photoreactivation of Pyrimidine Dimers

UV light induces the formation of covalently linked dimers of cytosine and thymine bases which are adjacent to each other on the same strand of DNA. Two common UV products are the cyclobutane pyrimidine dimer (CPD) and, at one-third the frequency of that, the 6,4 photoproduct (6,4 pyrimidine-pyrimidone).

The enzyme photolyase binds to complementary DNA strands and is able to split the pyrimidine dimer (Sancar, 2003). This reverse reaction can occur when the enzyme is activated by energy absorption from blue or UV light (300–500 nm). Photolyases are flavoproteins, and consist of two light-harvesting factors, FADH2 (in all photolyases) and, as a second cofactor, depending on the class, either a pterin in folate photolyases or a deazaflavin in deazaflavin photolyases. The pyrimidine dimer is cleaved by an electron transfer of the reduced FADH2 (Sancar, 2003). Photolyases are found in prokaryotes and lower eukaryotes, but not in placental mammals.

2.3.1.2 Direct Reversal of O6-alkylguanine

Alkylating agents can transfer methyl or ethyl groups to various O or N sites in guanine, thereby modifying the base and interfering with its pairing with cytosine during DNA replication. The O6 site in the guanine has the highest mutagenic potential and is, besides the N2 position, highly selectively alkylated by substances that react via an SN1 mechanism (Margison *et al*., 2002). The most abundant environmental alkylating substance is dimethylnitrosamine, which is formed during food preparation. Besides environmental substances, there are also alkylating substances, which are used as chemotherapeutics for cancer treatment (e.g. procarbazine, carmustin).

The methyl group at the O6 site of guanine is removed by the DNA repair enzyme O6-methylguanine-DNA methyltransferase (MGMT) via a one-step methyl transfer reaction. During this reaction the methyl group is transferred from the alkylated base onto an internal cysteine residue within the active centre of the alkyl-transferase. This results in the restoration of the guanine within the genomic DNA. The alkyl group transfer leads to irreversible inactivation of the transferase and targets it for ubiquitylation and proteasome-mediated degradation. This so-called 'suicide reaction' implies that the cells' capacity for repairing O6-guanine lesions depends on the pre-existing MGMT levels in the cell, or the rate at which cells can resynthesize MGMT.

DNA alkyltransferases are ubiquitously found in prokaryotic and eukaryotic organisms (Wood *et al*., 2005). The human MGMT acts in a similar way to the Ogt protein of *E. coli*, which is constitutively expressed in this micro-organism. O6-MeG lesions, which are not repaired by MGMT, may mispair with thymine during the DNA replication. In the following DNA replication cycle, thymine will pair with adenine. By this mechanism the O6-MeG lesions cause distinctive G–C to A–T point mutations, which are believed to be the driving force of the carcinogenic effects of alkylating agents. In the event of failure of direct reversal repair, these mispair lesions can potentially be repaired by the MMR pathway.

2.3.1.3 Repair of Single-Strand Nicks

In this case the damage is the presence of single-strand nicks having free 5′-phosphates and 3′-hydroxyls. Nicks with other configurations or nicks accompanied by additional backbone or base damage require more complex processing prior to repair.

DNA ligase repairs single-strand nicks by catalysing phosphodiester bond formation using adenosine

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triphosphate (ATP) or nicotinamide adenine dinucleotide (NAD^+) as energy donor.

2.3.2 Mismatch Repair (MMR)

Mismatch of bases (e.g. G–T or A–C) is a common DNA lesion due to a failure in proofreading during DNA replication. Another common lesion is the incorporation of uracil (normally found in RNA) instead of thymine.

DNA MMR is a highly conserved process from prokaryotes to eukaryotes. In *E. coli* a number of genes have been discovered, which when mutated lead to hypermutation. Thus the gene products of these genes are called MutS, MutL and MutH. These proteins interact with each other as homodimers or heterodimers/trimers and are the key players in the detection of a DNA mismatch and its preparation for repair. They determine the exact position of the mismatch on the daughter strand, induce the strand separation and the recruiting of an exonuclease, which excises the mismatched base after the separated daughter strand has been nicked. There are human homologues of MutS (Msh2, Msh3 and Msh6) and MutL (Mlh1 and Pms1), but not MutH. The endonuclease function of MutH in prokaryotes leads to the above-mentioned DNA nicking prior to strand separation. In humans this function is accomplished by MutL homologues (Kunkel and Erie, 2005).

MMR factors also seem to be involved in the physiological function of immunoglobulin (Ig) diversification, an essential process for immunity. The Ig diversification is achieved by somatic hypermutation (SMH), as well as recombination in the heavy chain variable (VDJ) region and class-switch recombination (CSR), two processes mediated by NHEJ. Studies with MMR mutants in mice have suggested an implication of the MMR pathway in SMH and also CSR, since Msh2, Msh6 and ExoI mutants display reduced levels of MMR in CSR and SMH (Kunkel and Erie, 2005).

2.3.3 Nucleotide Excision Repair (NER)

The NER pathway plays an important role in the repair of different kinds of DNA damage caused by UVR and mutagenic chemicals, and its impairment in humans is associated with growth defects, excessive UV sensitivity, and, in certain cases, increased skin cancer. Discovered in 1964, the NER apparatus represents the sole enzyme system responsible for the removal of bulky DNA adducts and is universally found in all free-living organisms, including mycoplasmas. In addition to bulky adducts, the enzyme system repairs many other lesions that do not distort the helix, including O6-MeG and other methylated bases.

In NER, an enzyme system hydrolyses two phosphodiester bonds, one on either side of the lesion, to generate an oligonucleotide carrying the damage. The excised oligonucleotide is released from the duplex, and the resulting gap is then filled in and ligated to complete the repair reaction. This nuclease activity, unique to DNA repair, is called excision nuclease (exinuclease). The enzyme activity of exinuclease results from sequential and partly overlapping activities of several polypeptides that bind to DNA and utilize the energy released by ATP hydrolysis to deform (kink and unwind) the DNA and eventually excise the lesion by dual incisions.

A side effect of this wide substrate range is that exinuclease even 'excises' mismatched nucleotides; however, in contrast to the MMR process, the exinuclease will excise a mismatched base from either strand and can actually cause mutation fixation rather than mutation avoidance. Fortunately, this activity is rather inefficient so that mutations caused by this side reaction do not significantly contribute to the mutation load of cells.

Only three proteins (UvrA, UvrB and UvrC) are required in NER in the prokaryotes, whilst more than 30 proteins are involved in the mammalian NER (Truglio *et al*., 2006). Although prokaryote and eukaryote exinucleases perform the same function and share remarkably similar excision mechanisms, the subunits comprising the exinuclease activity share very little homology.

Two NER subpathways have been identified: the global genome NER (GG-NER) that repairs DNA damages throughout the genome, and the transcription -coupled NER (TC-NER) that is specific for detecting and removing damages within actively transcribed genes.

During the multistep process of the NER pathway, the DNA lesion is recognized and the oligonucleotide containing the lesion is removed. The gap is then filled with the ligation of a new oligonucleotide which is complementary to the opposite DNA strand. It has been discovered that a factor essential for transcription (TFIIH) is recruited to the damage site and involved in NER. This fact has created considerable interest, particularly with regard to the finding that there is preferential repair of the transcribed strand in expressed genes. In fact, early work on gene-specific repair showed that essential or active genes were preferentially repaired compared with inactive genomic regions or the bulk of the genome (i.e. 99% noncoding); for example, mouse and human fibroblasts have identical survival curves to UV irradiation *in vitro*, however, most of the pyrimidine dimers are removed from human cells after 24 h, whereas only 20% are removed from mouse cells. This suggests mouse cells preferentially repair those dimers essential to survival. Therefore, preferential repair reflects the cell's ability to survive DNA damage.

NER is involved in three human genetic syndromes: xeroderma pigmentosum (XP, OMIM reference *611153), Cockayne syndrome (CS, OMIM #216400) and trichothiodystrophy (TTD, OMIM #601675). These syndromes are characterized by neurodegeneration, increased cancer frequency and ageing (Thoms *et al*., 2007). XP patients are sensitive to short sun exposures

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and have a greater than 1000-fold increased skin cancer risk that usually develops at an average age of 10 years old. XP individuals may also develop neurological abnormalities. Mutations in different genes coding for proteins involved in the NER pathway are responsible for causing XP: these mutations are located within the genes *XPA, XPB, XPC, XPD, XPE, XPF* and *XPG*. The mutations in *XPC* or *XPE* impact only the GG-NER pathway, whereas mutations in the remaining genes affect both GG-NER and TC-NER (Thoms *et al*., 2007).

CS is an autosomal recessive genetic disease and it is very rare in humans (Thoms *et al*., 2007). CS patients are also sensitive to sunlight but they do not have predisposition for skin cancer. The disease is characterized by growth retardation, cognitive impairment and ophthalmologic disorders. CS individuals have a very short lifespan and usually die in the first or second decade of life. The causes of CS are mutations within two proteins that are essential for DNA damage recognition in NER. TTD is also rare and inherited as an autosomal recessive disorder. The clinical features of TTD are brittle hair and nails, dwarfism and ataxia (Thoms *et al*., 2007). Many mouse models for NER mutations have been studied. Mice that carry a homozygous mutation for XP genes are viable, except the *XPD* mutants that show embryonic lethality (Nakane *et al*., 1995; Harada *et al*., 1999; Itoh *et al*., 2004; Yoon *et al*., 2005). Most of these mutants are UV sensitive and have an increased predisposition to UV-induced skin cancer.

2.3.4 Base Excision Repair (BER)

Base damages, the most common insult to cellular DNA, are repaired by the BER pathway (Wilson and Bohr, 2007). BER includes a short-patch BER subpathway that replaces a single nucleotide, and a long-patch subpathway during which 2–13 nucleotides are incorporated.

Initially the damaged base is removed by glycosylases such as Ogg1 and Mutyh. The glycosylases catalyse the hydrolysis of the *N*-glycosylic bonds linking the bases to the deoxyribose-phosphate backbone of DNA, leaving an abasic sugar in the DNA called an apurinic or apyrimidinic site (AP site), depending on the nature of the base removed. Then the endonuclease APE1 digests the DNA strand at the apurinic or apyrimidinic site. DNA polymerase β (Pol β), in the case of the short-patch BER subpathway, or Pol β and/or Pol ε or δ, in the case of the long-patch BER subpathway, fills the gap by incorporating nucleotides in the DNA strand. Then the complex XRCC1/ligase III carries out the strand ligation during the short-patch subpathway. Other proteins are involved in the long-patch subpathway for the DNA synthesis step, and ligase I carries out the strand ligation. Examples of BER include removal of uracil, hypoxanthine and methyladenine from DNA; however BER has a limited substrate range because the DNA glycosylases that initiate the repair process are in intimate contact with the lesion during catalysis.

In the past there was only a small amount of evidence to support the involvement of BER in human disorders or in cancer. However, a human genetic disorder linked to defective BER has recently been identified. This autosomal recessive disorder, called MUTYH-associated polyposis (MAP, OMIM reference #608456), is characterized by a biallelic germline mutation of the *MUTY* gene (OMIM reference 604933), and causes multiple colorectal adenomas and carcinomas (Cheadle and Sampson, 2007). The main function of the glycosylase MUTYH in the BER is the excision of adenines misincorporated opposite 8-oxoG when oxidative DNA damage occurs. When this damage is not repaired properly, G:C $\rightarrow$ T:A mutations are induced. These mutations are typically found in the adenomatous polyposis coli (*APC*) gene in MAP tumours.

In recent years also, mouse models have been generated and have shown that knockouts of individual glycosylases are viable, which is explained by partial redundancy between different glycosylases. Ogg1$^{-/-}$ mice were normal (Klungland *et al*., 1999; Ocampo *et al*., 2002). Mutyh$^{-/-}$ mice developed intestinal tumours after a long latency (Sakamoto *et al*., 2007). The double knockout Ogg1$^{-/-}$ Mutyh$^{-/-}$ mice show a stronger phenotype: they have elevated cancer susceptibility and shorter lifespan with 50% of double mutants developing lung tumours, lymphomas and sarcomas by 15 months of age (Xie *et al*., 2004).

On the other hand, inactivation of BER proteins which function downstream of the glycosylases induce embryonic or postnatal lethality, highlighting the vital importance of the process as a whole. Murine homozygous mutants for LigI, Lig III and Xrcc1 die before birth (Petrini *et al*., 1995; Puebla-Osorio *et al*., 2006; Tebbs *et al*., 1999), whilst homozygous mutants for Pol β die immediately after birth (Gu *et al*., 1994; Sugo *et al*., 2000).

2.3.5 Homologous Recombination and Nonhomologous End Joining

DNA double-strand breaks (DSBs) are considered to be the most dangerous form of DNA damage (Hoeijmakers, 2001; Khanna and Jackson, 2001; Rich *et al*., 2000; van Gent *et al*., 2001). They are generated when the two complementary strands of the DNA double helix are broken simultaneously at sites that are sufficiently close to one another that base pairing and chromatin structure are insufficient to keep the two ends juxtaposed (Jackson, 2002). Thus, with DSBs, DNA loses physical integrity and information content on both strands, making repair more difficult to perform than for other types of DNA damage in which the complementary strand is used as a template. Moreover, the biochemical configuration and

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the structure of broken DNA ends can be very diverse and they may require processing prior to ligation.

DSBs can be generated following exposure of cells to exogenous agents or can arise during endogenous processes. IR, such as X- and γ-rays, is probably the most significant exogenous agent inducing DSBs. Other exogenous agents that can induce DSBs are radiomimetic or chemotherapeutic drugs, such as bleomycin, neocarzinostatin, etoposide and other topoisomerase inhibitors (Povirk, 1996). DSBs are generated also by endogenous agents such as reactive oxygen species (ROS) produced during cellular metabolism (Lieber *et al*., 2003). Metabolically generated ROS species are unlikely to give rise to the clustered damage observed with IR; nevertheless, they produce DSBs that are likely associated with damaged bases and sugars (Pouget and Mather, 2001). Furthermore, DSBs are produced during programmed chromosomal rearrangements, such as meiosis to initiate recombination between homologous chromosomes, VDJ and immunoglobulin isotype CSR (Khanna and Jackson, 2001). Physiological DSBs can be also generated when the replication machinery meets nicks or other types of lesions, from mechanical stress on the chromosome, or at the termini of chromosomes due to defective metabolism of telomeres (d'Adda di Fagagna *et al*., 2004; Mathieu *et al*., 2004).

There is experimental evidence supporting a causal relationship between DSBs, genomic instability and carcinogenesis. Indeed, incorrect repair of DSBs may cause loss or amplification of chromosomal material or translocations. These events can lead to tumourigenesis if, for example, they are associated with the inactivation of tumour suppressors or activation or deregulation of proto-oncogenes (Jackson, 2002; Khanna and Jackson, 2001). Many cancers of lymphoid origin exhibit chromosomal rearrangements that have arisen as a consequence of the defective DSB repair of V(D)J recombination intermediates (Vanasse *et al*., 1999). Besides, mutations in factors involved in DSB signalling and repair lead to chromosomal instability (CIN) and to increased cancer predisposition in humans and in animal models (Ferguson and Alt, 2001; O'Driscoll and Jeggo, 2006).

The two main pathways to repair DSBs are HR and NHEJ (Jackson, 2002; Lieber *et al*., 2003; Valerie and Povirk, 2003). A third pathway, single-strand annealing (SSA), can be considered as a variant of HR. HR and NHEJ are largely distinct from one another and function in complementary ways. Their fundamental differences are the requirement for a homologous template DNA and the fidelity of DSB repair. A review of the early events in the mammalian response to DNA DSBs has recently been published by Riches *et al*. (2008).

HR relies on extensive sequence homology and involves copying the missing information from an undamaged homologous chromosome. In principle, this is error free and occurs without the loss of genetic information. Conversely, NHEJ does not require an undamaged DNA molecule and joins the broken DNA ends using little or no sequence homology. In most cases, this pathway results in the loss of a few nucleotides at the broken DNA ends. Hence, NHEJ is typically error prone and is an imperfect process from the standpoint of preserving genomic information. SSA is similar to HR and is dependent upon homologous sequences flanking the break site. Also this process leads to loss of genetic material since the nonhomologous sequences are removed and, after annealing of the homologous sequences, ligation completes the reaction.

HR and NHEJ pathways are highly conserved throughout eukaryotic evolution, but their relative importance differs from one organism to another. Simple eukaryotes such as yeasts rely mainly on HR to repair DSBs, while in higher eukaryotes NHEJ is the predominant DSB repair pathway (Jackson, 2002). This difference could be related to their genome organization. The genomes of multicellular eukaryotes have a substantial fraction of repetitive DNA (about 40% of the human genome). Therefore, the search for a homologous template DNA during HR is insurmountable when the break occurs in the portion of the genome that is repetitive, and homology partners might be chosen inappropriately, except during late S, G2 and M phases, when a sister chromatid is optimally positioned physically (Lieber *et al*., 2003). Indeed, the relative contribution of HR and NHEJ in mammalian cells differs depending on the stage of the cell cycle. Cells defective for HR, such as *RAD54* mutants, show a relatively flat IR-sensitivity pattern during the cell cycle, and they are more IR sensitive only during the late S to G2 phases (Sonoda *et al*., 2006). Instead, cells defective for NHEJ are extremely sensitive in the G1 and early S-phases (Sonoda *et al*., 2006). This indicates that NHEJ functions throughout the cell cycle and is the predominant mechanism for DSB repair during G0, G1 and early S-phases, whereas HR is most efficient during the late S and G2 phases of the cell cycle. However, these pathways are not mutually exclusive, and repair events that involve both HR and NHEJ have been detected. Moreover, they are not necessarily independent, and the coordinated action of both pathways is necessary in order to repair a DSB with minimal error. In addition to its crucial role during G0, G1 and early S-phases, the NHEJ pathway is essential to repair DSBs that occur during V(D)J recombination and Ig CSR in developing lymphocytes.

2.4 Human Genetics and the Consequences of Mutation

The human gene pool is known to carry many deleterious genes acquired from preceding generations, and over

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11 000 traits or monogenic (single gene) genetic disorders have been described that follow recognized patterns of Mendelian inheritance. It is clear that these arise as a result of DNA changes affecting particular chromosomes or genes. They can be grouped as follows:

1. Autosomal dominant inheritance, in which a change in only one of a pair of alleles is sufficient for the condition to be expressed.
2. Autosomal recessive inheritance in which both alleles must be mutated for the trait to become manifest.
3. Sex-linked inheritance, which may also be recessive or dominant, where the mutant gene is on a sex chromosome and will be expressed at high frequency in males (XY), and at a much lower frequency in females (XX), if the gene is X-linked and acts in a recessive manner.
4. Genomic imprinting.
5. Polygenic and multifactorial inheritance, in which the condition results from the interaction of several genes and may include an environmental component.

Each type of inherited defect is discussed in more detail below, with examples describing the impact each has on the human population; more detailed discussions can be found in reputable human genetics textbooks, such as Emery's *Elements of Medical Genetics* (Turnpenny and Ellard, 2005). Another source of information is the OMIM™ database (**Box 1**). At a molecular level, the germline alterations identified in Mendelian diseases include a broad variety of DNA changes, from 'microlesions' to 'gross lesions'. The microlesions constitute the largest category of DNA sequence alterations (52 027 out of 59 613, or 92.3%). These include single base-pair missense/nonsense and splicing mutations or small deletions among 2229 genes listed in the Human Gene Mutation Database (HGMD; *www.hgmd.org*) as of August 2008. The remainder (4586 or 7.7%) are 'gross lesions', including gross deletions (3452), repeat variations (149), gross insertions/duplications (545) and complex rearrangements (440).

2.4.1 Autosomal Dominant Inheritance

Genetic conditions resulting from autosomal dominant inheritance usually only manifest in individuals carrying both a normal allele and abnormal or mutant allele, that is, in the heterozygous state (an allele is an alternative form of a gene found at the same locus on homologous chromosomes). It is often possible to follow dominantly inherited disorders through many generations of a family. The most common example of a monogenic autosomal dominant disorder is familial hypercholesterolaemia (FH, OMIM reference #143890), which can result from mutations in either the low density lipoprotein receptor gene (*LDLR*, OMIM *606945), or the apolipoprotein B-100 gene (*APOB*, OMIM #144010), or the recently identified proprotein convertase subtilisin/kexin type 9 gene (*PCSK9*, OMIM *607786) (Austin *et al*., 2004). These patients have abnormally high serum cholesterol levels, which increases the risk of coronary heart disease. Homozygotes (who have two copies of the mutant allele) usually die from cardiac infarction in the third decade of their lives, while about 50% of heterozygotes die of ischaemic heart disease before the age of 60.

2.4.2 Autosomal Recessive Inheritance

Genetic conditions resulting from autosomal recessive inheritance are usually only manifested in individuals with two copies of the mutant allele, that is, they are homozygous recessive, whereas individuals heterozygous for the mutant allele are healthy *carriers* of the trait but show no features of the disorder. In most cases, affected individuals are likely to be 'compound heterozygotes'; that is, they will have inherited one mutant allele from one parent and another, different mutant allele, from the other parent. In other words, they will carry two mutations at the same locus, unless their parents are related, when they are likely to be homozygous for the same mutation by decent, inherited from a common ancestor. Thus genetic disorders due to recessive genes are normally rare as the prevalence of the condition depends on the frequency of carriers in any one population.

One of the most common examples of an autosomal recessive genetic disorder is cystic fibrosis (CF, OMIM reference #219700), which is caused by mutations of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene (Riordan *et al*., 1989). More than 1400 mutations associated with CF have been described (Flume and Stenbit, 2008), with some mutations resulting in milder forms of the disease (see Turnpenny and Ellard, 2005, for a more in-depth account). The disease has a high prevalence in Western European Caucasian populations, with an incidence of 1 in 2000 live births, and accounts for 1–2% of admissions to children's hospitals. The prevalence, however, is lower in American Africans (1 in 15 000) and American Asians (1 in 31 000). The clinical features of CF are characterized by a generalized disorder of the mucus-secreting glands of the lungs, pancreas, mouth and gastrointestinal tract, as well as the sweat glands, as a result of disregulation of the CFTR protein which plays a principal role as a chloride channel transporter in epithelial cell membranes. As a result, mucus production is more viscous than normal and this leads to an increased susceptibility for infection by pathogenic micro-organisms. Chronic lung disease caused by recurrent infection eventually leads to fibrotic changes in the lungs, with secondary cardiac failure, a condition known as *cor pulmonale*. Although antibiotics and physiotherapy have been very effective in increasing the average life expectancy of affected children, from less than 5 years in 1955 to around 30

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years at present, CF remains a significant clinical burden (Flume and Stenbit, 2008).

2.4.3 Sex-Linked Inheritance

Sex-linked inheritance refers to patterns of inheritance shown by genes located on the sex chromosomes. Genes located on the X chromosome are X-linked, whilst those on the Y chromosome are Y-linked. This leads to special patterns of inheritance, particularly with recessive genes. Genetic disorders associated with X-linked recessive inheritance are usually only manifest in males, as the affected individual will be hemizygous for the mutant allele present on his single X chromosome. Such genetic diseases are transmitted to affected males by heterozygote healthy carrier females, as well as by affected males, to their obligate carrier daughters, with a consequent risk to male grandchildren through these daughters (Turnpenny and Ellard, 2005). In other words, a female with a recessive allele on the X will not show the syndrome, as is the case for normal recessive alleles. An affected male, however, with such a gene on his X chromosome will not be able to mask it with the normal copy, as this is not present on his Y chromosome.

One of the most common examples of an X-linked recessive disorder is Duchenne muscular dystrophy (DMD, OMIM reference #310200), the most common form of muscular dystrophy with a prevalence of 1 per 3500 live male births, which is due to mutation in the dystrophin gene (Koh *et al*., 1987). The clinical features are characterized by progressive weakness and wasting of certain muscles which usually manifest as an awkward gait, inability to run quickly and propensity to fall. The muscle fibres undergo necrosis, to be replaced by fatty and fibrous tissue. Most boys are wheelchair bound by the age of 10, and the majority die by 20 years of age, usually due to heart failure (for further information see Turnpenny and Ellard, 2005).

Genetic disorders resulting from X-linked dominant inheritance are manifest both in females and in males, and although uncommon, they superficially resemble autosomal dominant traits because affected females have a one in two chance of transmitting the condition to their daughters and sons. There is, however, an important difference because an affected male will transmit the trait to all of his daughters but none of his sons (as sons inherit their single X chromosome from their mothers). An example of an X-linked dominant disorder is Charcot-Marie-Tooth disease Type 1X (CMT1X, OMIM reference #302800), a clinically and genetically heterogeneous group of hereditary motor and sensory peripheral neuropathies, caused by mutations in the gap junction beta 1 (*GJB1*) gene, located on the X chromosome. The gene encodes the gap junction protein connexin 32, although the natural history of CMT1X is poorly understood (Shy *et al*., 2007).

Genetic disorders resulting from Y-linked dominant inheritance, which affect males only, are rarer still. Examples include mutations in the 'deleted in azoospermia' family of genes (*DAZ* genes, OMIM reference 400003), which encode highly conserved RNA-binding proteins that are essential for gametogenesis and male fertility (Reynolds and Cooke, 2005), and the 'sex determining region-Y' gene (Su and Lau, 1993), which encodes a transcription factor that is a member of the high mobility group (HMG)-box family of DNA-binding proteins (Harley and Goodfellow, 1994). Mutations in the 'sex determining region-Y' gene (*SRY*, OMIM 480000) give rise to XY females with gonadal dysgenesis (Swyer syndrome, OMIM 306100) and translocation of part of the Y chromosome containing this gene to the X chromosome in XX males (OMIM 278850).

2.4.4 Genomic Imprinting

Generally, individuals inherit two allelic copies of every gene, a maternal copy and a paternal copy, and both alleles are expressed equally at most loci. The genomic imprinting of genes represents an exception to this rule and refers to maternally and/or paternally inherited genes which contain specific epigenetic modifications, for example via DNA methylation at cytosine bases and/or chromatin remodelling via histone acetylation, which result in heritable changes in gene expression that are not coded in the DNA sequence itself. Such epigenetic modifications result in differential gene expression patterns in the offspring. Thus, individual germline marks can control allelic-specific silencing or activation of imprinted genes, and sometimes neighbouring genes also, which can lead to clusters of imprinted transcripts. In the simplest scenario, only one of the two alleles at an imprinted gene locus is expressed. In other cases, an imprinted locus can include a variety of maternally expressed, paternally expressed and bi-allelically expressed transcripts (reviewed by Ubeda and Wilkins, 2008). Imprinted genes have been associated with a wide range of diseases; evidence for epigenetic abnormalities at imprinted loci have been observed in cloned mammals (Jaenisch *et al*., 2005), and their disruption has been reported in several human genetic disorders, such as imprinted in Prader-Willi syndrome (IPW, OMIM *601491) and Angelman syndrome (AS, OMIM #105830) and also in various cancers (see below).

2.4.5 Polygenic and Multifactorial Inheritance

Simple monogenic modes of inheritance represent the tip of the iceberg of disease where gene mutations play a part, and many disorders demonstrate familial clustering that does not follow Mendelian patterns of inheritance. Examples include common congenital malformations such as cleft lip, anencephaly, spina bifida and

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also common diseases of both childhood and adulthood, for example asthma, diabetes mellitus, hypertension and ischaemic heart disease, various degenerative neurological disorders, and so on. Although all show some genetic determination, other factors, including environmental ones, are implicated in their aetiology, and therefore these disorders are said to show multifactorial inheritance. In addition, many gene loci can be involved in the aetiology of common diseases; that is, they are polygenic. Each individual gene may have only a small effect and act with varying dominance—that is, some genes have low penetrance and are only expressed in some individuals, and therefore the genetic contribution is not always straight forward. In other words, some genes play a more important part than others (see Turnpenny and Ellard, 2005, for further discussion).

An example of multifactorial inheritance is Type 1 (insulin-dependent) diabetes mellitus (IDDM, OMIM 222100), a disorder of glucose homeostasis which results from the destruction of the insulin-producing β cells of the pancreas due to an autoimmune response. Family studies have indicated that predisposition to the condition is polygenic. The principal genes leading to susceptibility are within the major histocompatibility complex (MHC) HLA class II loci and a promoter polymorphism of the insulin gene. It is clear, however, that even a sufficient complement of susceptibility polymorphisms at disease-associated genes, both inside and outside the MHC, does not always lead to the development of disease at a clinical level, as genetically identical twins are often much less than 100% concordant (reviewed by Todd, 1999). Nongenetic factors are also associated with the disease—in particular, infection with specific viruses. There is an increased prevalence of Type I diabetes in congenital rubella patients, and there is an increased frequency of Coxsackie B viral antibodies in newly diagnosed diabetics compared with controls (Field *et al*., 1987). It is possible that viral infection is a stress that catalyses the pathological process which leads to Type I diabetes, without being a direct component.

The previous discussion illustrates the bewildering complexity surrounding the nature of just one multifactorial disease. The genetic mechanisms underlying most multifactorial diseases are poorly understood. Molecular epidemiology research is beginning to unravel some of the mysteries (reviewed by Dekker and van Duijn, 2003), particularly with the establishment of large population-based biobanks, such as UK Biobank (*www.ukbiobank.ac.uk*). Nevertheless, there remains much to elucidate.

2.4.6 Reproductive Effects

Approximately 15% of all conceptions result in spontaneous miscarriages, and chromosome abnormalities are present in 40–50% of all first trimester pregnancy losses (although this figure is likely to be higher if miscarriages in unrecognized pregnancies were to be taken into account). In newborn infants, congenital abnormalities are present in 2–3% of births, of which 50% may be exclusively attributed to genetic factors. In developed countries, genetic disorders and congenital malformations together account for 30% of all childhood hospital admissions and 40–50% of all childhood deaths. Genetic conditions are also responsible for the impairment of male and female fertility (Turnpenny and Ellard, 2005). The contribution of environmental exposures to this human genetic burden is unknown.

What is clear is that if a potent genotoxin is able to cross the placental barrier, it is very likely to interfere with differentiation of the developing embryo and thus possess teratogenic potential. Of the 40 or so recognized human teratogens, about a quarter have sufficient information to establish that they are clearly genotoxic, 5 of which are anticancer drugs: busulfan, ciclophosphamide (cyclophosphamide), cytarabine, mercaptopurine (6-mercaptopurine) and daunorubicin/doxorubicin (Bishop *et al*., 1997). These compounds display a wide variety of genotoxic mechanisms and it is very likely that a particular human teratogen will produce its effects by several different mechanisms. However, mutagens form only one class of teratogens, and a large proportion of 1000+ teratogenic agents identified in animal models are not mutagenic. Alternative mechanisms of teratogenesis include cell death, retarded differentiation, vascular insufficiency and inhibited cell migration (see **Developmental Toxicology**).

2.4.7 Ageing

Genetic instability is widely thought to be involved in the process of ageing, and a large body of evidence has been gathered in recent years which shows that nuclear DNA damage, gene mutations and cytogenetic damage, such as translocations, insertions, dicentrics and acentric fragments, accumulate with age in mammalian cells (Vijg, 2000; Karanjawala and Lieber, 2004). In humans, there are several genetic disorders associated with phenotypes characterized by shortened lifespan and/or premature ageing, for example, ataxia telangiectasia (AT, OMIM #208900), Bloom's syndrome (BLM, OMIM #210900), Cockayne syndrome Type A (CSA, OMIM #216400), Hutchinson-Gilford Progeria Syndrome (HGPS, OMIM #176670), TTD (OMIM #601675), Werner's syndrome (WRN, OMIM #277700), and so on. Disorders caused by these mutations are termed segmental progeroid syndromes, because they accelerate some, but not all, signs of normal ageing. Many of the heritable mutations giving rise to the symptoms of accelerated ageing in these patients partially or wholly inactivate proteins that sense or repair DNA damage (see above). This finding suggests

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that failure to maintain genomic integrity underlies at least some ageing phenotypes.

For example, WRN patients probably represent the best model for premature ageing in adult humans, and the disease is characterized by premature greying, cataracts, loss of subcutaneous fat, skin atrophy, osteoporosis, diabetes, atherosclerosis and malignancies (Goto, 1997). The defective gene in WRN, the *RECQL2* gene, encodes a helicase which possesses a DNA exonuclease domain and is believed to play a role in the maintenance of overall genomic stability. The RecQ family of DNA helicases is of particular interest since several human RecQ helicases are defective in diseases associated with premature ageing and cancer, and are believed to play a role in the maintenance of genomic stability through their catalytic activities and protein interactions in various pathways of cellular nucleic acid metabolism, with an emphasis on DNA replication and repair (reviewed by Brosh and Bohr, 2007).

HGPS, in contrast, is a rare childhood progeria syndrome characterized by a striking precocious senility. Death from coronary artery disease is frequent and may occur before 10 years of age. HGPS is caused by mutations in the lamin A (*LMNA*) gene, the product of which, lamin A, is an essential nuclear matrix protein underlying the inner nuclear membrane required for nuclear organization and shape, DNA replication and repair (Eriksson *et al*., 2003). Mutations in the gene are believed to have a devastating effect on nuclear architecture and function that is responsible for the premature ageing seen in HGPS. Interestingly, certain severe forms of WRN are reported to be associated with mutations in the *LMNA* gene also.

Mouse mutants defective in genome maintenance, like their human counterparts, often display segmental progeria and exhibit the characteristics of early onset of ageing. For example, mouse models with mutations in DNA excision repair that affect both NER and transcription coupled repair (TCR) show symptoms of accelerated ageing; for example, CSA and CSB null mice, and mice with a defect in the NHEJ gene, *Ku80* (*Ku86*), *AT* and *ReqQ* helicase genes. However, $wrn^{-/-}$ mice have no obvious ageing phenotype, except on a p53 null background (Lombard *et al*., 2000), possibly because the role and regulation of the *WRN* gene differs between mouse and human (reviewed by Hasty *et al*., 2003).

Cellular senescence, a phenotype characterized by cell cycle arrest, is often used as an *in vitro* model of cellular ageing (Ben-Porath and Weinberg, 2004; 2005). In many primary human cells, replicative cell senescence is related to progressive telomere shortening associated with cell passage (Harley *et al*., 1990). Telomeres are the repetitive sequences of 5–15 kb in length, consisting of 5′-TTAGGG-3′ tandem repeats and specialized proteins, which cap the ends of linear eukaryotic chromosomes, protecting them from degradation, fusion and recombination. The 3′ overhang of the repeat is arranged in a T-loop structure that protects the individual chromosome ends from end to end chromosome fusion and prevents the single strand being recognized as a double-strand break, triggering the cell DNA repair system (Griffith *et al*., 1999). Under normal cell division and chromosome replication, the telomere region shortens because DNA polymerase uses RNA primers that are degraded after elongation (Moyzis *et al*., 1988). Once a telomere has shortened beyond a critical threshold, after about 25–40 cell passages (depending on cell type), the DNA ends become structurally unstable. This leads to *TP53*/*Rb* activation, resulting in either replicative senescence or cell crisis, leading to cell cycle arrest (**Figure 2**). Interestingly, HGPS-affected individuals have abnormally short telomeres, and mutations in DNA repair genes which are associated with premature ageing also confer premature senescence, for example, WRN cells senesce prematurely in culture.

Among the various sources of somatic DNA damage (see above), ROS, such as superoxide anion, hydroxyl radical, hydrogen peroxide, nitric oxide, and so on, which are the natural by-products of oxidative energy metabolism in the cell, are thought to be one of the prime causes of ageing. ROS can cause lipid peroxidation, protein damage, and several types of DNA lesions, including single- and double-strand DNA breaks, DNA-adducts and DNA strand crosslinks. Longer-lived animal species generally show higher resistance to oxidative stress compared to shorter-lived species (Kirkwood and Holliday, 1979), and lifespan extension has been observed in various genetically modified animal models with increased resistance to ROS, such as $p66^{SHC}$ mice and insulin-like growth factor Type 1 receptor (IGF-1 R) knockout mice (reviewed by Hasty *et al*., 2003). Indeed, caloric restriction, an intervention that extends lifespan in many organisms, is also believed to decrease ROS production (Barja, 2004). In addition, levels of DNA adduct 8-oxoguanine (oxo8dG), a major product of oxidative damage to DNA, accumulate with age (Hamilton *et al*., 2001). The ROS theory of ageing is reviewed in detail by Balaban *et al*. (2005).

In conclusion, the accelerated ageing symptoms in humans and mice with genetic defects in genome maintenance strongly suggest that genome instability, driven by oxidative damage, is a primary cause of normal ageing. Moreover, the contention that nuclear DNA is an important target of age-related change is supported by evidence that nuclear DNA damage and mutations accumulate with age. While ROS are likely to be one important source of this damage, there are numerous other cellular and environmental sources of damage, and the impact of such lesions may be enhanced by age-related defects in DNA repair capacity. Accumulation of mutations in critical genes may be one general mechanism by which compromised DNA repair could contribute to ageing. In addition, p53–Rb mediated senescence and apoptosis are also likely contributors to ageing, since both

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of these respond to DNA damage and protect against cancer by eliminating severely damaged/senescent terminally differentiated cells that no longer function. Thus the sum of these effects, particularly in adult stem cells, may lead to accelerated degenerative changes and provide links to the physiology of ageing (Kenyon and Gerson, 2007).

2.5 Cancer and Carcinogenesis

Cancer (or neoplasia) represents a vast medical problem and as a cause of death is second only to cardiovascular disease. According to Cancer Research UK there were 153 491 deaths from cancer in the United Kingdom in 2005. More than 1 in 3 people will be expected to develop some form of cancer during their lifetime and ~285 000 new cases of cancer are diagnosed every year (*http://info.cancerresearchuk.org*). In terms of human prevalence, the most common cancers are those of the lung, bowel, breast and prostate; these account for more than half of all new cases and nearly half of all cancer mortalities. Evidence for the existence of cancer can be dated back as far as prehistoric times, having been found in the skeletons of ancient peoples, such as Egyptian mummies.

The term 'cancer' derives from the Latin for crab—due to the resemblance of the swollen veins around a tumour to a crab's limbs. Cancer is not a single disease; there are over 200 pathologically distinct neoplastic entities (each depending on the cell/tissue-type of origin). Nevertheless, all cancers are generally characterized by uncontrolled abnormal cell growth, which eventually forms a cell mass, or tumour. Tumour development, or tumourigenesis, generally occurs over a period of years, and cancer incidence is more prevalent in aged populations. Sooner or later, the tumour will generally invade and destroy surrounding normal tissues, and cells from the tumour can spread (metastasize) through the bloodstream or lymphatic system to start new tumours in other parts of the body.

The process of carcinogenesis, that is, the change in cells from normal, controlled cell division and differentiation to cells that are transformed, dividing without check, and are undifferentiated or abnormally differentiated, does not appear to occur as a single step. In other words, transformation is a multistage process and involves a sequence of events from tumour cell initiation, to promotion, malignant conversion and progression. Evidence for this comes from *in vitro* studies (e.g. cell transformation studies), animal models and clinical/epidemiological observations and, in particular, the long latency period between cell initiation and the appearance of a tumour in the target tissue and the progression to metastatic disease (see **Carcinogenesis and Carcinogens that are also Genotoxic**).

2.5.1 Cancer Genetics

All cancers are genetic in origin and involve an accumulation of inherited and somatic cell mutations in specific cancer genes such as oncogenes, tumour suppressor genes, DNA repair genes and other cancer susceptibility genes (see below) and/or epigenetic changes affecting cancer gene regulation. The disease, at the genetic level, is often characterized by progressive genomic instability, somewhat reminiscent of ageing in some respects, and the acquisition of particular cancer cell phenotypes, such as cell growth deregulation and immortalization, de-differentiation, angiogenesis and metastasis. For example, the majority of metastatic tumours show abnormal karyotypes and genomic alterations involving chromosome copy number gains and/or losses; these impact differential gene expression and cellular development. Many altered regions are thought to be the location of novel candidate tumour suppressor genes and/or oncogenes. A small proportion of cancers are predisposed by inherited germline mutations in specific cancer susceptibility genes, such as Li-Fraumeni syndrome (OMIM reference #151623), which is associated with mutations in the *TP53* tumour suppressor gene, (OMIM *191170), retinoblastoma (mutations in the *RB1* gene, OMIM +180200) and familial adenomatous polyposis (FAP) of the colon (OMIM #175100), which is associated with mutations in the *APC* gene, (OMIM *611731).

2.5.2 Oncogenes

An oncogene is a protein encoding gene, which, when deregulated, participates in the onset and development of cancer. Genetic mutations resulting in the activation of oncogenes increase the chance that a normal cell will develop into a tumour cell. A proto-oncogene is a normal gene that can become an oncogene due to mutations or increased expression. Proto-oncogenes code for proteins that help to regulate cell growth and differentiation, and are often involved in signal transduction of mitogenic signals and transcriptional regulation of downstream genes in the nucleus. Upon *activation*, which generally involves mutation at a single allele because they are gain-of-function mutations, a proto-oncogene (or its product) becomes a tumour inducing agent, that is, an oncogene (see **Table 2**).

2.5.3 Activation of Oncogenes

A proto-oncogene can become activated to an oncogene by a relatively small modification of its original function. There are three basic activation types:

- A mutation within a proto-oncogene can cause a change in the protein structure, causing
  - an increase in protein (enzyme) activity and/or
  - loss of gene regulation.

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Table 2 Genomic fidelity and cancer susceptibility genes

Category	Pathways affected	Examples
Oncogens	Growth factors and mitogens, signal transduction kinases, nuclear transcription factors . . .	*EGFR, VEGFR, RAS, RAF, SRC, WNT, FOS, JUN, MYC, ERK . . .*
Tumour suppressor genes	Cell cycle regulation coupled with DNA damage surveillance	*TP53, RB, PTEN, APC, BRCA1 . . .*
Cell cycle control	Cyclin-dependent kinase inhibitors	*WAF1, Kip1* and *Kip2 . . .*
DNA repair genes	Various DNA repair pathways: BER, NER, MMR . . .	*Ogg1, Mutyh, MSH, PMS, XPA . . .*
Apoptosis genes	Balance between pro- and anti-apoptotic pathways	*BCL2, DAPK . . .*

- An increase in protein activity, caused by
  - an increase in gene expression
  - an increase of protein stability, prolonging its existence and thus its activity in the cell
  - a gene duplication resulting in an increased amount of protein in the cell.
- A chromosomal translocation causing
  - inappropriate or constitutive gene expression, that is, in the wrong cell type or at the wrong time; for example, *c-Abl* in chronic myaloid leukaemia.

Over-expression of growth factor receptors is a frequent occurrence in cancer and has been observed in many tumour types, including colorectal cancer (CRC) and adult astrocytoma. In the latter, both PDGFRα and EGFR dimerise once activated by ligand binding. This initiates autophosphorylation of cytoplasmic domains stimulating the Ras/Raf/Map pathway and activating PI3-kinase. PI3-kinase then phosphorylates phosphatidylinositol-4,5-diphosphate (Pip2) to phosphatidylinositol-3,4,5-triphosphate (Pip3). The cellular levels of Pip3 are regulated by phosphatase and tensin homologue (PTEN). Pip3 is bound to the intracellular cytoplasmic membrane, where it recruits proteins with pleckstring homology domains, including Akt, a well-known anti-apoptotic and survival factor (reviewed by Kohn, 1999; Mirza *et al*., 2003).

2.5.4 Tumour Suppressor Genes

Tumour suppressor genes encode for proteins which generally inhibit tumorigenesis, and their existence was originally inferred from experiments where tumour cells were fused with normal cells in culture that resulted in nontumourigenic hybrids. Subsequent loss of specific individual chromosomes or specific regions of a chromosome in the hybrid, and derived from the normal parental cell, resulted in the re-emergence of the tumorigenic phenotype. Numerous tumour suppressor genes have since been identified, and the functions of the associated proteins fall into several categories:

1. The activities of the cyclin-dependent kinase (CDK) family are in turn regulated by cyclin-dependent kinase inhibitors (CDIs), which are essential for continuing the cell cycle. If these genes are not expressed the cell cycle will arrest, effectively inhibiting cell division (Fero *et al*., 1998).
2. Cell cycle regulation and DNA damage surveillance: the cell cycle can be arrested by tumour suppressor genes of the p53 and Rb pathways that are activated by aberrant proliferative stimuli or DNA damage (reviewed by Kohn, 1999; Mirza *et al*., 2003). After cell cycle arrest, there is an opportunity to rectify DNA damage where necessary. The role of some members of the p53 and Rb pathways is to maintain low levels of inactive *TP53* in the absence of genomic damage allowing normal cell cycle function.
3. If the DNA damage is irreparable, the cell enters senescence or undergoes apoptosis (programmed cell death), preventing replication of the damaged genome (Malumbres and Barbacid, 2001). Apoptosis in multicellular organisms involves a series of biochemical events leading to a characteristic cell morphology and death, characterized by a variety of morphological changes, including blebbing, changes to the cell membrane such as loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation (see **Cytogenetics**). Mutations in genes regulating apoptosis can shift the balance between pro- and anti-apoptotic influences, and may contribute to genomic instability, if cells carrying DNA damage are permitted to survive. Such cells which survive past their 'use-by-date' are capable of replication and they and their progeny are subject to increased genomic instability, increasing the likelihood of the cell becoming cancerous or diseased (Schmitt *et al*., 2007).
4. Some tumour suppressor genes encode for proteins involved in cell adhesion which prevent tumour cells from dispersing, block loss of contact inhibition, and inhibit metastasis. These proteins are known as metastasis suppressors.

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The majority of tumours have mutations in multiple tumour suppressor genes, causing major disruptions to the p53 and Rb pathways (Hahn and Weinberg, 2002) and/or apoptosis pathways or both (**Table 2**). Tumour suppressor genes generally require mutations in both alleles before a particular gene can contribute to tumourigenesis. In other words, both maternal and paternal copies of the gene must be inactivated for the tumour to develop, and in many spontaneously arising tumours, individual chromosomes or specific regions of a chromosome are lost or deleted which contain putative tumour suppressor genes; that is, these tumours demonstrate loss of heterozygosity (LOH). As such, mutant tumour suppressor alleles are usually recessive, whereas mutant oncogene alleles are typically dominant. The first tumour suppressor protein discovered was the Retinoblastoma protein (pRb) in human retinoblastoma (Fung *et al*., 1987). This provided evidence for the classical 'two-hit' hypothesis first proposed by A.G. Knudson, following his observation that the age of onset of retinoblastoma followed second-order kinetics, implying that two independent genetic events were necessary (Knudson, 1971). He recognized that this was consistent with a recessive mutation involving a single gene, but requiring biallelic mutation.

Another important tumour suppressor is the p53 tumour suppressor protein encoded by the *TP53* gene. Homozygous loss of p53 is found in 70% of colon cancers, 30–50% of breast cancers and 50% of lung cancers. Mutated p53 is also involved in the pathophysiology of leukaemias, lymphomas, sarcomas, neurological tumours and many others. Interestingly, certain p53 mutations can function as a 'dominant negative', meaning that a mutated p53 protein can prevent the function of the normal protein copy from the unmutated allele (Baker *et al*., 1990), and represents a notable exception to the 'two-hit' rule for tumour suppressors. Other tumour suppressor genes which are exceptions to the 'two-hit' rule are those which exhibit haploinsufficiency (i.e. when a single functional copy does not produce enough of a gene product to bring about the normal 'wild-type' condition, leading to an abnormal or diseased state). An example of this is the p27Kip1 cell cycle inhibitor, in which mutation of a single allele causes increased carcinogen susceptibility (Fero *et al*., 1998).

The activation of *TP53* transactivates many genes with varied functions, including those involved in apoptosis and inhibitors of the pRb pathway preventing cell proliferation (**Figure 5**). The transcription of $p21^{WAF-1}$ is activated by *TP53* and inhibits the pRb pathway. *MDM2* transcription is also initiated by *TP53*, which itself acts as a negative regulator of *TP53*. Cell cycle progression is also regulated through the release of E2F transcription factors by pRb phosphorylation, which initiates $p14^{ARF}$ transcription. This protein inhibits MDM2, preventing p53 degradation, consequently increasing p53 cellular concentration and inducing cell cycle arrest or apoptosis (Mirza *et al*., 2003). The pRb pathway controls cell cycle progression from G1 to S-phase. The release of E2F transcription factors through pRb phosphorylation initiates the transcription of S-phase genes. Mitogenic stimuli up-regulate CCND1, CCND2 and CCND3, which bind CDK4 or CDK6. This complex phosphorylates the pRb protein, causing the release of E2F transcription factors. Phosphorylated pRb also modulates chromatin structure (Zhang and Dean, 2001). CDK inhibitors, $p15^{INK4b}$ and $p16^{INK4A}$, compete with the cyclins to bind CDK4 or CDK6, blocking complex formation and the pRb pathway (Hahn and Weinberg, 2002).

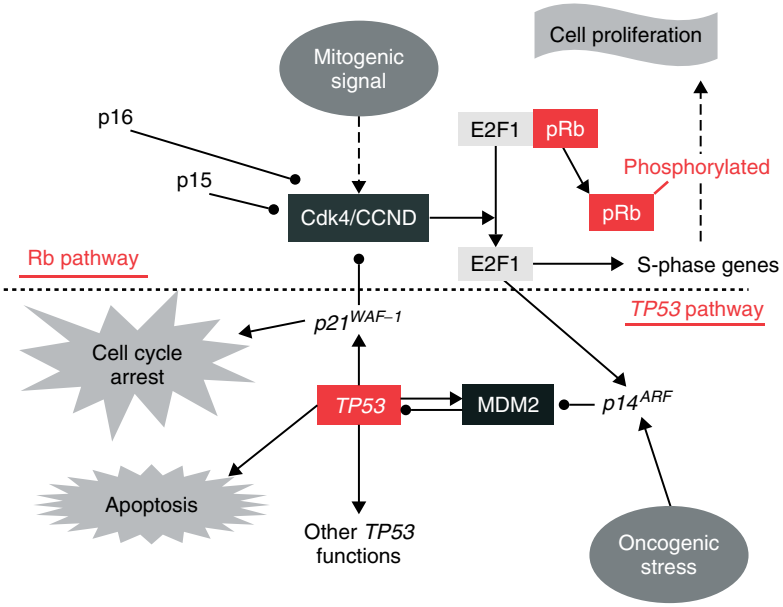

Figure 5 *TP53* and Rb pathways.

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PTEN encodes a tumour suppressor lipid phosphatase, which negatively regulates the oncogenic, proliferative and anti-apoptotic promoting characteristics of Akt (Li *et al*., 1997). The *PTEN* gene, the second most frequently deleted tumour suppressor gene (after *TP53*), encodes a protein pivotal to DNA-damage-induced apoptosis and cell cycle arrest, with a role in cyclin D down-regulation and subsequent inhibition of G1–S-phase transition (**Figure 2**). Thus PTEN acts by opposing the action of PI3K, which is essential for anti-apoptotic, pro-tumorigenic Akt activation (Yin and Shen, 2008). Other examples of tumour suppressor genes include *APC* in colorectal cancer (see below) and *BRCA1* in breast cancer.

2.5.5 Genomic Instability

It has long been suggested that multistage carcinogenesis requires a mutator phenotype (Loeb, 1991). This idea is now supported by examples wherein defects in several pathways that determine the fidelity of DNA, including replication, mitotic division and cell cycle progression result in increased genomic instability and increased susceptibility to cancer (see **Table 2**). For example, microsatellite instability (MSI) is a feature in approximately 15% of human colorectal cancers and is also found in gastric and endometrial tumours. MSI is caused by defective DNA MMR, which leads to profuse insertions and deletions in microsatellites and is associated with increased mutation prevalence (Røyrvik *et al*., 2007). While cancer is the most cited example, a number of other diseases are also associated with defects in DNA fidelity maintenance and genomic stability. These include Alzheimer's disease, CS, Parkinson's disease, Friedreich's ataxia, Huntington's disease, TTD, a number of progressive neuropathies and multiple mitochondrial wasting diseases and ageing (see Schmitt *et al*., 2007; McCulloch and Kunkel, 2008 and references therein).

Chromosomal instability is also a feature of most human cancers. Mutations in CIN genes increase the rate at which whole chromosomes or large parts of chromosomes are lost or gained during cell division. CIN causes an imbalance in chromosome number (aneuploidy) and an enhanced rate of LOH, which is an important mechanism of inactivating tumour suppressor genes. A critical question in cancer biology is whether CIN is an early event in carcinogenesis and thus a driving force of tumourigenesis or whether it is a later event associated with metastasis (Michor *et al*., 2005).

2.5.6 Cell Immortalization

Cell immortalization is a further event that contributes to malignant growth of neoplastic cells. In most cases, this is thought to be achieved through the maintenance of chromosome telomeres. As described above, telomere shortening is associated with replicative cellular senescence. Deletion of *TP53* significantly attenuates cellular effects of telomere dysfunction, but only during the earliest stages of genetic crisis (Chin *et al*., 1999). Tumour cells evade replicative senescence through the activation of telomerase, a riboprotein complex that adds TTAGGG repeats to the telomeres, preventing shortening (Bocchetta and Carbone, 2004). It has been suggested that over 85% of human cancers have measurable levels of telomerase activity, allowing indefinite proliferation (Shay and Bacchetti, 1997). Tumours without telomerase activity are thought to maintain telomere length through a process known as alternative lengthening of telomeres (ALT). This involves nonreciprocal recombination between telomeres of different chromosomes (Bryan *et al*., 1997).

2.5.7 Angiogenesis

Angiogenesis is the formation of new blood vessels from those that already exist and is controlled through a balance of proangiogenic and antiangiogenic factors. A high level of angiogenesis occurs during embryonic development but is limited in adults to the menstrual cycle and tissue repair/remodelling. This complex physiological process is now recognized as an essential component of tumour development and progression (Folkman, 1990). During tumorigenesis the angiogenic signal balance is pushed towards new vessel growth to create a vascular network that facilitates nutrient and waste product exchange and thus allow tumour growth to exceed 1–2 mm^3 (Folkman, 1990). Furthermore, the extent of tumour vascularity correlates with increasing tumour malignancy. Indeed, tumour cells that are more than 10 cell layers from a blood vessel are hypoxic and it is this lack of oxygen that inhibits tumour growth (Evans *et al*., 2001); such tumours also show areas of necrosis linked to extensive tissue hypoxia.

A plethora of growth factors, cytokines and respective receptors are involved in angiogenesis causing basement membrane degradation, endothelial cell migration, proliferation and tubular formation (reviewed by Folkman, 2002). The most prominent factor is VEGFα, an endothelial cell mitogen that binds to the tyrosine kinase receptors VEGFR-1 and VEGFR-2 and initiates endothelial cell proliferation, migration and development of new blood vessels (Kanno *et al*., 2000). Significantly, the expression of this factor is mediated by hypoxia-inducible factor 1 (Hif1), a factor stabilized under hypoxic conditions (Forsythe *et al*., 1996).

2.5.8 Epigenetics

In normal cells, epigenetics can be defined as the mechanisms that preserve inheritable gene expression patterns and functions without altering the genome sequence. The epigenetic network is complex but can be summarized into four tiers of regulation via: DNA methylation, chromatin remodelling, histone modification, and noncoding

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RNAs (ncRNAs). It has become increasingly evident that major disruptions to the epigenetic network are common in the vast majority of tumours, and promoter hypermethylation is recognized as the most common epigenetic alteration in tumours causing loss or down-regulation of tumour suppressor gene expression (Esteller, 2006).

The methylation of cytosine residues located within dinucleotide CpG repeats does not occur randomly within the genome, but clusters in CpG-rich regions known as CpG islands that are primarily located at the 5′ end of genes. These islands often span the promoter, untranslated region and exon 1, indicating the beginning of a gene sequence. In the DNA of normal cells, 5-methylcytosine (5mC) constitutes 0.75–1% of all nucleic acid bases and 3–4% of methylated cytosines (Paz *et al*., 2002). Furthermore, in normal cells, methylation controls the gene expression of imprinted genes, X-chromosome genes in the female, germline specific genes and tissue-specific genes (Feinberg *et al*., 2002). In comparison, CpG dinucleotides are often unmethylated in normal cells and hypermethylated in tumour cells, silencing gene expression (Esteller, 2005).

Hypomethylation has also been documented in tumour cells and has been attributed to tumorigenesis. In such cases, tumour cells have been found to have 20–60% less genomic 5mC than normal cells, occurring mainly at coding regions and introns, compared to hypermethylation, that is usually found at promoter regions. Reduced levels of 5mC are thought to invoke chromosome instability, reactivation of transposable elements and loss of imprinting, promoting mitotic recombination and LOH (Esteller, 2005). It has also been suggested that hypomethylation increases with tumour malignancy (Fraga *et al*., 2004).

Genes that control global genome methylation have been investigated in many tumours and cancer. A family of genes with DNA methyltransferase activity including *DNMT1, DNMT2, DNMT3a, DNMT3b* and *DNMT3L*, are thought to play a role in the maintenance of promoter region methylation. Differential expression of only *DNMT1* in colon cancer cells was shown to have little effect on global methylation. However, when both *DNMT1* and *DNMT3a* were down-regulated, methyltransferase activity was lost and methylation was reduced to less than 5% of the original level (Rhee *et al*., 2002).

Tumour suppressor genes are frequently hypermethylated in tumours, with significant consequences that provide cells with a selective advantage (**Table 3**). The cell cycle inhibitor $p21^{WAF-1}$ is hypermethylated in a variety of cancers, allowing cells to proliferate and escape senescence (Merlo *et al*., 1995). Other genes involved in tumour cell cycle control found to be hypermethylated include the *Rb* gene and $p15^{INK4b}$. Regulation of *Rb* gene methylation status has also been linked to chromatin conformation at the promoter region, suggesting that several epigenetic mechanisms are simultaneously involved (De La Rosa-Velazquez *et al*., 2007). Several genes in the *TP53* network, including $p16^{INK4A}$ and *TP73,* have been found to be hypermethylated. Methylation, and consequent silencing of $p16^{INK4A}$, allows uninhibited MDM2 to induce *TP53* degradation (Esteller *et al*., 2001). Furthermore, genes of the APC/β-catenin, E-cadherin and Wnt signalling pathways are often methylated in tumours, including *APC, E-cadherin, H-cadherin, FAT* and *SFRP1* (Esteller *et al*., 2001; 2000; Graff *et al*., 1995; Paz *et al*., 2003; Toyooka *et al*., 2001).

Alterations in methylation patterns also affect DNA repair mechanisms in tumours. Silencing of the mismatch DNA repair gene, *hMLH1* , has been found in colorectal, endometrial and gastric tumours in conjunction with

Table 3 Examples of genes hypermethylated in colorectal cancer, and their molecular consequences

Gene	Function	Location	Tumour profile	Consequences
$p16^{INK4a}$	Cyclin-dependent kinase inhibitor	9q21	Multiple types	Entrance in cell cycle
$p14^{ARF}$	MDM2 inhibitor	9q21	Colon, stomach, kidney	Degradation of p53
hMLH1	DNA mismatch repair	3p21.3	Colon, endometrial, stomach, glioblastoma	Frameshift mutations
MGMT	DNA repair of 06-alkylguanine	10q26	Multiple types	Mutations, chemosensitivity
LKB1/STK11	Serine/threonine kinase	19p13.3	Colon, breast, lung	Unknown
RARβ2	Retinoic acid receptor	3p24	Colon, lung, head and neck	Vitamin sensitivity
FAT	Cadherin, tumour suppressor	4q34–35	Colon	Dissemination
APC	Inhibitor of β-catenin	5q21	Colon	Activation of β-catenin route
SFRP1	Secreted frizzled-related protein-1	8p12–p11	Colon	Activation of Wnt signalling
COX-2	Cyclooxygenase-2	1p25	Colon, stomach	Anti-inflammatory resistance
GATA-4	Transcription factor	8p23–p22	Colon, stomach	Silencing of target genes
GATA-5	Transcription factor	20q13	Colon, stomach	Silencing of target genes
DAPK	Pro-apoptotic	9q34.1	Lymphoma, lung, colon, glioblastoma	Resistance to apoptosis

Adapted from Esteller (2005).

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microsatellite instability (Fleisher *et al*., 1999). Furthermore, the methylation status is a good predictive marker of response to chemotherapy with alkylating agents (Fukushima *et al*., 2005; Soejima *et al*., 2005).

The role of chromatin remodelling and histone modification as mechanisms involved in tumour and cancer development is only just emerging. The regulation of chromatin structure and transcription is often influenced by post-translational modification of histone tail residues, affecting the accessibility of transcription factors to DNA. This includes methylation, acetylation, phosphorylation and ADP-ribosylation (Vitolo *et al*., 2000). A specific group of transcription factors are involved in chromatin structure remodelling allowing other transcription factors access to DNA regions (Thomas and Chiang, 2006). Alterations in chromatin structure can also occur through ATP-dependent nucleosome remodelling (Johnson *et al*., 2005). Histone acetyltransferases (HATs), histone methyltransferases (HMTs) and histone deacetylases (HDACs) are all involved in a proposed aberrant histone modification signature for cancer (Fraga and Esteller, 2005). A large study by Ozdag *et al*. illustrated that six different cancer types had a unique pattern of histone modifier gene expression compared to that of the corresponding normal tissue or other cancers (Ozdag *et al*., 2006).

The extent of ncRNA involvement in epigenetics is largely unknown. Small regulatory ncRNAs include microRNAs (miRNAs) and small interfering RNAs (siRNAs), which are approximately 22 nucleotides in length and suppress translation in two ways. miRNAs undergo nonperfect pairing with target mRNA preventing translation, and siRNAs pair perfectly with target mRNA causing degradation by the RNA-induced silencing complex. siRNAs are also involved in the silencing of chromatin and chromosome dynamics (Mattick and Makunin, 2006).

Recent discoveries suggest that the role of ncRNAs in disease is broader than initially understood, involving the control of gene expression through chromatin architecture and transcription (Mattick and Makunin, 2006). miRNA expression profiles have been used to classify human cancers (Lu *et al*., 2005), and disrupted miRNA target genes have been characterized in pituitary adenomas (Bottoni *et al*., 2007). Furthermore, miRNAs have been found to be disrupted in adult primary glioblastoma (GBM) through microarray analysis. In the primary GBM investigated, nine miRNAs were significantly up-regulated and four were significantly down-regulated. When the tumours were clustered according to the expression profile similarity of the miRNAs, a clear distinction could be seen between normal controls and GBM samples, suggesting a role for miRNAs in GBM development (Ciafre *et al*., 2005).

The role of somatic cell mutations and epigenetic changes in cancer may be exemplified by CRC or cancer of the bowel. The third most common form of cancer in the UK, with an incidence of approximately 44 persons per 100 000 population (see *http://info.cancerresearchuk.org*), the majority of sporadic CRCs are believed to develop from 'benign' adenomas. The adenomas are thought to arise from aberrant crypt foci (ACF) in colorectal mucosa, the earliest known morphological precursors to CRC. ACF can be subclassified as dysplastic, heteroplastic (nondysplastic) and mixed types. The progression of an ACF through to small adenoma and on to malignant carcinoma (**Figure 6**) is thought to take between 5 and 10+ years, but only a fraction of ACF and small proportion of adenomas proceed to malignant disease (reviewed by Suehiro and Hinoda, 2008). The genetic changes associated with the adenoma–carcinoma sequence in CRC were first described by Vogelstein *et al*. (1988). The Vogelstein model of CRC (**Figure 6**) describes the acquisition of somatic mutations in various cancer genes (tumour suppressor genes, oncogenes, DNA repair genes and other cancer susceptibility genes) in a predictable linear sequence from initiated stem cells through to benign tumour and frank metastatic disease. Thus the Vogelstein model has been used to provide a genetics-based working hypothesis of the carcinogenic process involving gene mutations associated with initiation and progression of neoplastic cells in the adenoma–carcinoma sequence (reviewed by Cho and Vogelstein, 1992). Over the past two decades, the general principals of the Vogelstein model have been extended to various other cancer types, for example, neurological cancers (Karakoula *et al*., 2008) and chronic myelogenous and acute lymphoblastic leukaemias (Karakosta *et al*., 2005).

In part, the Vogelstein model was based on the genetic analysis of tumours from patients with FAP (OMIM #175100), a rare genetic disorder where individuals have a predisposition for early onset CRC as a result of inherited mutations in the tumour suppressor gene, *APC* (adenomatous polyposis coli). Patients with FAP typically develop hundreds to thousands of colorectal adenomas, which begin to appear in adolescence (Petersen *et al*., 1991), and by the age of 35 years 95% of individuals have polyps (Evans *et al*., 1993). A mouse model of FAP, called Min (multiple intestinal neoplasia) has also been described and was originally identified in a pedigree established from mating a C57BL/6 J (B6) male treated with ethylnitrosourea with an AKR/J (AKR) female (Moser *et al*., 1990). Heterozygous B6-Min mice typically develop ~30 intestinal tumours (mostly adenomas) and usually die within four months of birth; *Min* homozygotes die *in utero* (Moser *et al*., 1990). As such, both FAP and Min are considered to have a common aetiology, that is, analogous genetic predisposition and similar morphology of adenomatous polyps, and Min is therefore regarded by many researchers as a model of human FAP and CRC.

However, there is extensive heterogeneity within the mutational spectrum of families with FAP, with over 826

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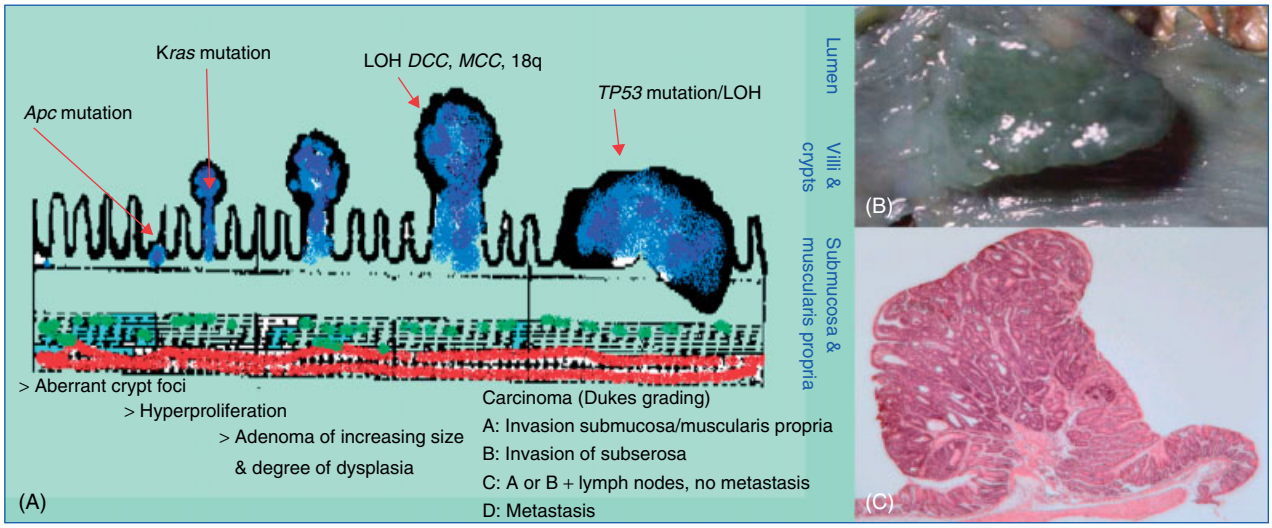

Figure 6 The adenoma–carcinoma sequence model in colorectal cancer. (A) Schematic of adenoma formation and associated genetic changes over time. Mutation in *Apc* is considered an initiating mutation in this model, and progression is characterized by the acquisition of additional mutations in key cancer-susceptibility genes, such as the oncogene *ras*, and loss of heterozygosity in tumour suppressor genes such as deleted in colorectal cancer (*DCC*) and mutated in colorectal cancer (*MCC*) genes and often *TP53* at the carcinoma stage; (B) adenoma in colon of Apc^{Min} mouse; (C) histopathological section of colon adenoma from Apc^{Min} mouse.

different germline mutations identified (Beroud *et al*., 2000), and significant genotype–phenotype correlation exists between the locations of *APC* germline mutations and polyposis severity (Soravia *et al*., 1998; van der Luijt *et al*., 1996). In contrast, mice heterozygous for *Min* inherit a single specific mutation in *Apc*, the murine orthologue of the human tumour suppressor gene *APC*. The nonsense mutation results from a T → A transversion in nucleotide 2549 of *Apc*, converting codon 850 from leucine (TTG) to a stop (TAG) codon (Su *et al*., 1992). Mahmoud *et al*. (1997) concluded that the resulting truncated Apc protein exerts a dominant-negative effect which is associated with changes in the growth characteristics of preneoplastic tissue, including decreased apoptosis and proliferation and decreased enterocyte migration. As such, the codon 850 mutation present in Min is more likely to be representative of the 'severe end' of the mutational spectrum seen in FAP, a conclusion consistent with experimental evidence for a dominant-negative effect of APC gene products associated with severe polyposis in humans (Dihlmann *et al*., 1999).

In addition, the B6-Min mouse strain carries additional genetic susceptibility factors which strongly influence the severity of the phenotype. For example, *Mom1* ('modifier of *Min*-1') accounts for ~50% of the genetic variation in tumour frequency across different mouse strains (Dietrich *et al*., 1993). The secretory phospholipase (*Pla2g2a*) gene is considered a good candidate for *Mom1,* which is a semidominant modifier of both polyp number and size (MacPhee *et al*., 1995; Cormier *et al*., 1997). Another modifier of *Min* (*Mom2*) has also been reported and acts in a dominant fashion, significantly reducing the incidence of colorectal adenomas (Silverman *et al*., 2002). Background strain can also affect colorectal adenoma frequency. For example, B6-Apc^{Min} mice crossed with the CAST inbred strain exhibit a total absence of colorectal adenomas; an observation independent of *Mom1* status (Koratkar *et al*., 2002). Thus it is clear that strain-specific genes influence susceptibility to intestinal adenomas. Transgenic mouse models of FAP, such as $Apc^{\Delta 716}$ (Oshima *et al*., 1995) and Apc^{1638N} (Fodde *et al*., 1994) also demonstrate varying cancer susceptibilities. For example, $Apc^{\Delta 716}$ mice develop hundreds (250–650) of small intestinal adenomas, whereas Apc^{1638N} develop only five to six tumours per mouse, predominantly small intestinal adenomas and carcinomas (Yang *et al*., 1997).

In mammals, the loss of *APC* tumour suppressor gatekeeper function is associated with the initiation of intestinal tumorigenesis (Nakamura *et al*., 1991). Functional loss of *APC* has been shown or proposed to arise from several different mechanisms: mutation in *APC*, nondisjunction, homologous somatic recombination, and epigenetic silencing. In FAP, somatic mutations resulting in LOH at *APC* are determined by the site of the germline mutation. For example, FAP patients with germline *APC* mutations within codons 1194–1392 show mainly allelic loss in their colorectal adenomas, whereas LOH in other FAP patients tends to occur by truncating mutations in the 'mutation cluster region' located at codons 1286–1513 in exon 15 (Lamlum *et al*., 1999). These results suggest that different *APC* mutations provide cells with different selective advantages, with mutations close to codon 1300 providing the greatest advantage.

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Genetic analysis of adenomas in Min mice also shows 100% LOH of the wild-type *Apc* allele on mouse chromosome 18. In contrast to FAP, however, research clearly shows that homologous somatic recombination rather than chromosome loss is the primary cause of adenoma formation in the Min mouse model (Haigis and Dove, 2003). Thus neoplastic growth in the Min mouse can be initiated in the absence of genomic instability, and polyp development is likely to occur independently of environmental factors known to influence disease progression in humans with FAP (Tischfield and Shao, 2003). Somatic mutations in the *Apc* gene in Apc^{1638N} mice are also associated with the entire loss of mouse chromosome 18. Interestingly, the mouse orthologues of *MCC* (mutated in colorectal cancer) and *DCC* (deleted in colorectal cancer), two putative tumour suppressor genes implicated in human CRC, also map to mouse chromosome 18. In contrast to FAP patients, however, mutations in the *K*-, *N*- and *H-ras* genes or exons 7 and 8 of the *TP53* gene are uncommon in these mouse models. These studies suggest that genetic inactivation of *Apc* is an initiating event in murine as well as human intestinal tumorigenesis, but thereafter, tumour growth and progression follow different mutational pathways in these two species (Smits *et al*., 1997). As such, there are distinct differences, in terms of genetics, molecular carcinogenesis and tumour pathogenesis, between mouse models of CRC and the human inherited condition, FAP. Moreover, until comparatively recently, there was a failure to recognize that sporadic CRC is in fact a multipathway disease, comprising disparate subgroups with particular clinical, pathological and molecular features, with underlying genetic/genomic instability. Thus the oversimplification of the evolutionary pathway characterized by the Vogelstein model may have, in part, confounded the identification of risk factors associated with sporadic CRC, whether genetic, constitutional or lifestyle related (reviewed by Jass, 2007).

2.6 Environmental Exposures, Mutagenesis, Cancer and Heritable Genetic Effects

A small proportion of cancers are predisposed by inherited germline mutations in specific cancer susceptibility genes, such as Li-Fraumeni syndrome (*TP53*) and FAP (*APC*). Such mutations may occur spontaneously, owing to rare errors in DNA replication and cell division. Environmental factors, however, contribute significantly to the majority of cancer aetiologies, and exposure to many different environmental agents is associated with increased cancer risk; for example, exposure to viruses (e.g. acute transforming retroviruses), ionizing and nonionizing radiations absorbed by DNA (e.g. X-rays, ultraviolet light in sunlight) or particular chemical species capable of covalently interacting with DNA or the fidelity of the cell division apparatus. For example, human epidemiological studies demonstrate significant associations between ultraviolet exposure from sunlight and skin cancer, cigarette smoke exposure and lung cancer, and aflatoxin B_1 contamination in food and liver cancer. These associations are also true for many cancers linked to industrial exposures, for example vinyl chloride exposure and liver cancer in the plastics industry. In the majority of human cancers, however, the differentiation between genetic and environmental factors is not always obvious and it is not clear which genetic changes or environmental exposures are causative.

There is also good evidence to show that inherited mutational changes in humans, although individually rare, collectively are responsible for a significant proportion of genetically determined diseases and heritable (congenital) malformations. However, to date, no adverse heritable genetic effects have been documented in humans following exposure to mutagens, including children born to people exposed to very large doses of radiation and/or mutagenic drugs, and so on (Sankaranarayanan, 1990). For example, epidemiological and other investigations have failed to find convincing associations between exposure, germline mutagenicity and heritable malformations in the offspring of survivors exposed to IR at Hiroshima and Nagasaki (Otake *et al*., 1990), Chernobyl fallout (Little, 1993) or mutagenic cancer therapies in general (Boice *et al*., 2003), although for female childhood cancer patients the transgenerational effects of treatment may include restricted foetal growth and early births among their offspring (Signorello *et al*., 2006). These contrast markedly with the identification of human mutagenic carcinogens.

With regard to the induction of *de novo* (new) mutations into the gene pool there are many reasons why environmental causes of heritable mutation, as evidenced by human genetic disease, are difficult to discern. According to Elespuru and Sankaranarayanan (2007), among them are: (i) Very large sample sizes are required to detect small increases in individual, rare, Mendelian disorders. As such, there has been a lack of statistical power in historical epidemiological studies which have set out to investigate associations between exposure to individual mutagens and increases in specific human genetic traits (deleterious or otherwise). (ii) The fact that most well-monitored disease-causing germ cell mutations are not of *de novo* origin but are inherited (i.e. low 'signal-to-noise' ratio). (iii) An inability, at least in early studies, to distinguish newly arising mutations from inherited ones. (iv) A tradition of monitoring congenital anomalies as surrogates, when germ-cell mutations attributable to disease, inherited or *de novo*, are but a tiny fraction of visible birth defects (again, low 'signal-to-noise' ratio). (v) Lack of practical ways to associate the time of origin of mutations with environmental exposures. (vi) Lack of appropriate, large-scale

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studies of the relationship between common genetic damaging agents, such as tobacco smoke or certain medicines, and adverse nonsomatic genetic outcomes.

Advances in current knowledge about the genetic basis of human genetic diseases, mutagenic mechanisms and the actions of environmental mutagens, however, suggest that the assessment of the human health impact of germ cell mutations may now be feasible (Elespuru and Sankaranarayanan, 2007). For example, *molecular epidemiology* is beginning to characterize the types of mutations found in specific genetic diseases, using the knowledge gained from the human genome project and data on the relationships between exposure to environmental agents and the particular DNA sequence alterations they cause. In addition, the development of more practical assays for germline mutagenesis, for example transgenic gene mutation assays (see below), and the use of mouse minisatellite mutations (Dubrova *et al*., 1993) and repetitive DNA sequences as mutational targets (Bouffler *et al*., 2006), plus insights gained from population-based genetic screening in personalized medicine, mean that where once it was considered to be almost impossible to trace back germline mutations to specific causative agents in the past, this may now become feasible in the future.

3 GENETIC TOXICOLOGY

3.1 History of the Discipline

As eluded to by Bragg, it has been known for several hundred years that exposure to particular chemicals or complex mixtures can lead to cancer in later life (Doll, 1998). It has also been recognized that chemicals can also induce heritable changes in man, and these may potentially lead to diseases in the next generation.

There had been accumulating evidence throughout the twentieth century that such changes can arise as a consequence of damage to DNA and the resulting mutations. Prior to the Second World War, the only mutagen known was radiation. For example, Muller (1927) showed that X-rays could induce mutations in the germ cells of the fruit fly *Drosophila melanogaster,* and for a short period it was thought that the mutation process required the high activation energy that could only be provided by radiation (Crow, 1989). The first published report of a chemical mutagen was for allyl-isothiocyanate from mustard oil (Auerbach and Robson, 1944). The discovery that mustard gas was also a potent mutagen (Auerbach and Robson, 1946) was based on the observation that the skin blistering observed in people exposed to mustard gas was similar to the skin lesions caused by radiation exposure. The discovery of other chemical mutagens soon followed, for example urethane, ethylene oxide, epichlorohydrin, and so on.

The realization that the integrity of human DNA is essential for the future of the species and the health of its individuals and that exposure to particular human-made chemicals and exposure to radiation, particularly following the use of the atom bombs at Hiroshima and Nagasaki, could result in increases in heritable defects in human children, led to calls for controls of known hazards and the identification and control of new hazards. Another factor was the impact of drugs, particularly antibiotics, in reducing infant mortality and disease. Thus, the relative contribution made by heritable diseases has increased dramatically in the latter half of the twentieth century.

Novel chemicals were recognized as representing an additional potential source of damage to DNA (directly or indirectly via effects on DNA replication and repair, mitosis, etc.). This concept slowly emerged in the 1950s and 1960s with the growing number of studies showing that a variety of human-made chemicals were mutagenic in various test systems, along with improved understanding of the basis of genetics. Several seminal conferences were held around this time with a focus on chemical mutagens and their potential risk to future generations. For example, in 1963 Muller stated that humans were being exposed to a great number of new chemicals, such as food additives, drugs and pesticides, not encountered by previous generations, and that humans were not specifically adapted to such chemicals by natural selection (reviewed by Wassom, 1989).

Genetic Toxicology became recognized as a formal discipline in May 1969 when the Environmental Mutagen Society (EMS) was founded in the USA under the chairmanship of Professor Alexander Hollaender (Oak Ridge National Laboratory) and a group of interested geneticists (as recounted by Wassom, 1989). In the following year the European Environmental Mutagen Society (EEMS) was formed under the Presidency of Professor Fritz H. Sobels (University of Leiden, the Netherlands). The primary thrust of these societies was towards estimating risks to germ cells. However, this was broadened in the 1970s when evidence began to accumulate that carcinogenicity and mutagenicity were linked. Indeed, early analyses of rodent carcinogens and noncarcinogens suggested that almost all carcinogens were also mutagens (Ames *et al*., 1973). From 1973 onwards various national expert committees were formed to advise governments on what approach could be taken to screen new chemicals for potential heritable or carcinogenic risks, and thus appropriate testing was recommended to minimize the risk of genotoxicity. From 1976 onwards, key methods papers were written for what were to become mainline tests in the various national guidelines. The EMS monographs edited by Alexander Hollaender *(Chemical Mutagens, Principles and Methods for Their Detection)* were very influential in this regard. Various other countries also began to issue national guidelines for mutagenicity testing, including Italy (Comma, 1977), the USA (US

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EPA, 1982) and the UK (DHSS, 1981). In 1984 the European Economic Community issued 'recommendations' for all new drugs at the Product Licence Application stage (EEC, 1984). Practical handbooks on mutagenicity testing procedures were published, in the UK, firstly by Parry and Venitt (1984) and subsequently in the 1990s by the UK Environmental Mutagen Society. The series of books entitled 'Basic Mutagenicity Tests', 'Supplementary Mutagenicity Tests' and 'Statistical Evaluation of Mutagenicity Test Data', edited by David Kirkland and published by Cambridge University Press, were very influential at the time (Kirkland, 1989; 1990; Kirkland and Fox, 1993). Nowadays, these recommended procedures have been augmented by a series of publications from the International Workshop on Genotoxicity Testing Procedures (IWGTP).

The first IWGTP Workshop was held in Melbourne in 1993 (as a two-day satellite meeting of the Sixth International Conference on Environmental Mutagenesis). Organized by David Kirkland (UK), Mike Selby (USA) and Toshio Sofuni (Japan), the focus of the meeting was towards standardizing recommendations on genotoxicity test procedures around the world. At the time, there was a growing realization that many countries and regions of the world had introduced detailed recommendations or requirements on test methodology, but independently of any other guidelines, thereby leading to a diverse range of recommendations. For industry this often meant tests had to be performed more than once to modified protocols in order to provide data acceptable to different countries, leading to wasted resources and time. For regulatory agencies it meant often trying to evaluate data from protocols with which they were unfamiliar and by methods with which they did not necessarily agree (Kirkland, 1994). Follow up workshops have been held on a regular basis since the inaugural meeting and the various recommendations have been published in peer-reviewed journals (see **Table 4**).

In the 1990s, the harmonisation of genotoxicity testing requirements for pharmaceuticals was also underway as a result of the International Conference on Harmonisation (ICH) program (Müller *et al*., 1999). This process brought together regulatory authorities from Europe, Japan and the USA, and experts from the pharmaceutical industry in these three regions. Areas of disharmony were identified and differences in regulation with respect to genotoxicity in the three regions were discussed. The guidelines that emerged were an evolution of pre-existing guidelines in Japan and the European Union. At the same time as the ICH process was occurring, the OECD (Organisation for Economic Co-Operation and Development) also updated a number of its genotoxicity guidelines. Both processes influenced each other, resulting in similar recommendations.

Today it is not possible to register a new drug, food additive, and so on, or transport a new industrial chemical within the major industrialized nations, without providing basic mutagenicity information, and, therefore, it has become necessary to determine whether widely used chemicals or potentially useful new chemicals possess the ability to damage DNA. The discipline of genetic toxicology exists to achieve this aim. In industry, such information may be used to discard a new chemical if a safer alternative can be found, to control or eliminate human exposure for a mutagenic industrial compound, or for a drug, to proceed with development if benefits clearly outweigh risks. Data concerning the mutagenicity of a chemical have become part of the basic toxicological information package. They are needed for decision making and to reduce risks that might otherwise be unforeseen.

The current paradigm for regulatory genetic toxicology involves a tiered strategy towards testing, and this has been largely adopted throughout all sectors of industry. The minimum genotoxicity package generally consists of (i) an *in vitro* bacterial assay for gene mutation, (ii) an *in vitro* mammalian cell assay for gene mutation and/or cytogenetic damage, and (iii) an *in vivo* mammalian assay for cytogenetic damage (**Figure 7**). In the pharmaceutical industry, the ICH guidelines for genotoxicity (ICH Topic S2A *Guidance on Specific Aspects of Regulatory*

Table 4 Reports of the International Workshop on Genotoxicity Testing (IWGT)

Workshop (location and date)	Primary publication of reports
Reports on the International Workshop on Standardisation of Genotoxicity Test Procedures (Melbourne, Australia, February 1993)	*Mutation Research,* **312**, 195–318, 1994
Reports on the International Workshop on Standardisation of Genotoxicity Test Procedures (Washington DC, USA, March 1999)	*Environmental and Molecular Mutagenesis,* **35**, 159–263, 2000
Reports on the International Workshop on Standardisation of Genotoxicity Test Procedures (Plymouth, UK, June 2002)	*Mutation Research,* **540**, 119–181, 2003
Reports of the 4th International Workshop on Genotoxicity Testing (IWGT) (San Francisco, USA, September 2005)	*Mutation Research,* **627**, 1–117, 2007

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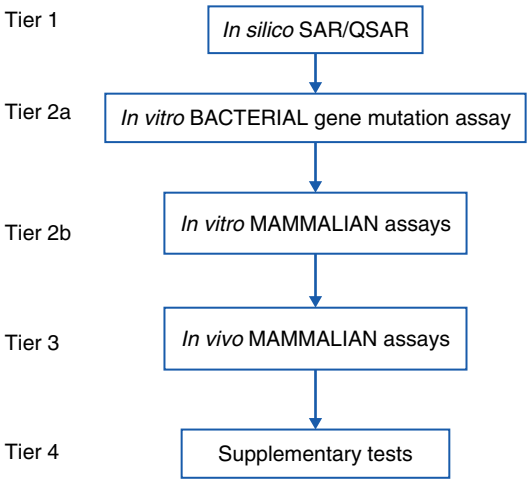

Figure 7 Tiered approach to genotoxicity testing. The tiered approach involves an initial evaluation of potential drug candidates without any biological experimentation whatsoever (Tier 1). Selected compounds are then evaluated for genotoxicity using a simple *in vitro* bacterial system (Tier 2a), usually the Ames test. This is followed by further testing using an *in vitro* mammalian cell system (Tier 2b); for example, a chromosome aberration assay or mouse lymphoma assay. The use of these assays permits highly genotoxic compounds to be filtered, and thereby reduces potential toxicity issues in later development (Tier 3). The tiered approach promotes the 'clean development' of new compounds or drug candidates, which reduces the need for additional *in vivo* investigative studies using supplementary tests (Tier 4).

Genotoxicity Tests for Pharmaceuticals; and S2B *A Standard Battery of Genotoxicity Testing of Pharmaceuticals*) describe both the philosophy and types of assays that comprise the testing regimen recommended by regulatory agencies for the assessment of new drugs. The ICH guidelines recommend that *in vitro* studies should be completed before commencing Phase I clinical trials in humans, and the complete battery of tests should be complete by the time Phase II clinical studies begin (Lynch and Connelly, 2003).

A comprehensive description of the individual test systems used for screening new compounds for genotoxic activity is described in the subsequent sections of this chapter. The strategies, study designs and terms described below largely reflect the custom and practice in those laboratories of which the author has experience. However, similar approaches are used throughout the pharmaceutical, consumer product, chemical and contract research industries for genetic toxicology. By default, development of a drug intended for oral administration in the clinic is assumed, as this is by far the predominant route in clinical practice. Evaluation of the genotoxicity of a test agent given by non-oral routes requires some adaptations, but the principles of testing remain the same.

3.2 The Standard Test Battery

3.2.1 *In Silico Assessment of Genotoxicity and Carcinogenicity*

Recent advances in combinatorial chemistry and high-throughput screening technologies have led to a massive explosion in the numbers of potential drug candidates being produced at the earliest stages of drug discovery. Consequently there is a greater need to acquire alternative methods for the prediction of toxicity in order to be able to cut these numbers rationally to manageable proportions. Computer-based or *in silico* 'expert systems' for predicting genotoxicity have been developed in order to screen new active substances for potential chemical activity prior to any biological testing (Greene, 2002). These systems fit broadly into two categories, although there is an overlap between them. The first comprises mathematical models, in which correlations are sought between chemical structure and biological activity, and the second includes predictive models, which rely on existing knowledge in the form of rules describing historical data (Dearden, 2003). The application of computational tools for reducing the attrition rate of compounds during drug discovery by predicting adverse drug reactions in preclinical development has recently been reviewed by Muster *et al.* (2008).

3.2.1.1 *Quantitative Structure–Activity Relationships (QSAR)*

The assumption supporting the use of quantitative structure–activity relationships (QSARs) for the prediction of toxicity is that 'biological activity may be described as a function of chemical constitution'. This was first proposed by Crum-Brown and Frazer (1868), and latterly has been driven by the work of Corwin Hansch (for a historical review see Rekker, 1992). In the main, one of the most successful applications of QSAR has been genotoxicity, primarily for well-defined congeneric series of chemical structures.

Rules-based expert systems rely on existing knowledge about chemical structures and their associated toxicological potential, rather than forming empirical relationships. It is fair to state that no system routinely outperforms another. Localized QSAR models for highly conserved congeneric series provide the optimum concordance values, although rules-based systems are applicable to a wider range of chemical entities. The most commonly used systems are summarized below.

- DEREK (Deductive Estimation of Risk from Existing Knowledge; LHASA UK Ltd.) (*www.lhasalimited.org*) is a knowledge-based system for the qualitative prediction of a range of toxic end points (principally mutagenicity, carcinogenicity and skin sensitization). The system has a rules base consisting of molecular substructures (toxophores)

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which have been associated with a toxic outcome. A novel structure is presented to the system, and any toxophores are returned with corresponding descriptive text for the user to review. An example of a DEREK genotoxic alert is shown in **Figure 8**.

- COMPACT (Computer-Optimized Molecular Parametric Analysis of Chemical Toxicity) (*http://ukpmc.ac.uk/articlerender.cgi?artid=633320*) may be used to predict the capability of a compound to act as a substrate for cytochrome P450. Structural characteristics are used to determine the ability of a molecule to fit into the enzyme binding site, and electronic characteristics are used to determine the likelihood of oxidative metabolism. Expert knowledge is then required in order to assess the toxicological significance of such metabolism, for example, activation of a test article to electrophilic metabolites capable of reacting with DNA.
- OncoLogic (LogiChem Inc.) (*www.epa.gov/nrmrl/std/mtb/pdone/tfs11.pdf*) is a PC-based program for the prediction of carcinogenicity based on human expert knowledge. Predictions are made using a hierarchical decision tree, with four subsystems available for the prediction of fibres, metals or metal-containing compounds, polymers and organic chemicals. The organics module is the most extensive, with in excess of 40 000 discrete rules derived from over 10 000 compounds. The system requires the user to specify the chemical class of the query molecule. It then follows a series of predefined rules to produce a mechanism-based evaluation of carcinogenic potential.
- M-CASE ('Multiple Computer Automated Structure Evaluation'; MultiCASE Inc.) (*www.multicase.com/index.html*) analyses the structural fragments of the compounds in a 'training' database to identify biophores, that is, those fragments responsible for the toxicological end point (such as carcinogenicity and mutagenicity) for which data are contained in the database. It compiles a dictionary containing the contribution of each biophore to the particular toxicological outcome. The bioactivity

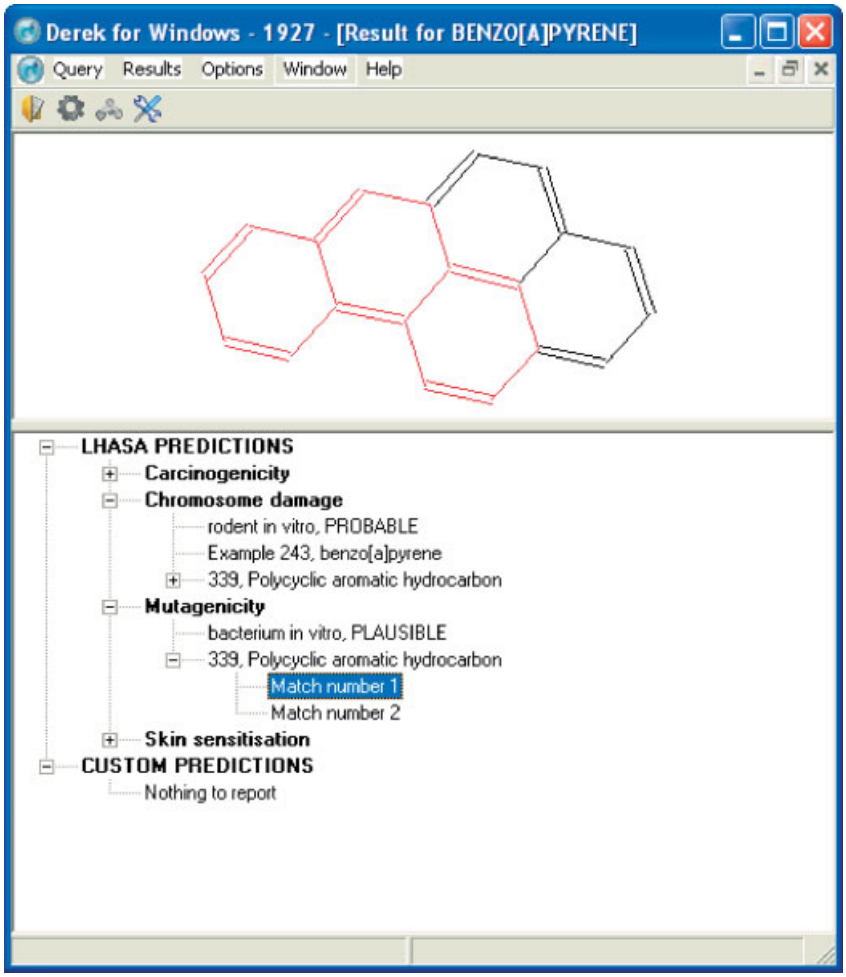

Figure 8 *In silico* predictions of genotoxicity. Computer software, such as DEREK (Deductive Estimation of Risk from Existing Knowledge; LHASA UK Ltd., Leeds, UK) may be used to predict genetic toxicity via chemical structure; in this case benzo(a)pyrene (B(a)P).

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of any compound not in the training database is predicted from the sum of the contributions of the biophores that it contains.

Recently, Mayer *et al*. (2008) evaluated 650 compounds from the Carcinogenic Potency Database (CPDB) to assess the predictivity of OncoLogic (Version 4.1) and MCASE (Version 3.1) with respect to Ashby–Tennant structural alerts (Ashby and Tennant, 1994) and genetic toxicity testing, individually and in combination. The sensitivity of the methods for predicting carcinogens and the specificity for predicting noncarcinogens was examined. Potent carcinogens, defined as those with TD_{50} values (i.e. the dose resulting in 50% of animals with tumours) of less than $6.25\,mg\,kg^{-1}$ body weight day^{-1}, were then examined separately. The study concluded that SAR analysis programs and structural alerts perform well for compounds with low human exposure levels and have the potential to supplement the results of routinely requested genetic toxicity tests in a weight-of-evidence approach in predicting carcinogenicity.

All of these systems, however, have their own strengths and weaknesses; for example, in rules-based systems the most important attributes are the quality and quantity of the knowledge databases supporting the predictive algorithms. Unfortunately, these are frequently biased towards certain chemical classes and to compounds with positive toxicological outcomes, such that nontoxic compounds are often under-represented. Attempts have been made to form noncongeneric models, but the multiplicity, and often uncertainty, of biological end points makes this approach far more complex (Benigni and Richard, 1996). Therefore, when new chemical structures with unknown toxicological potential are analysed the systems are liable to provide inaccurate predictions.

However, the predictive accuracy of these databases has improved in recent years as they have become more comprehensively populated with different chemical classes and as the rules influencing the knowledge-based systems have been refined. In the author's laboratory, QSAR assessments are routinely performed on all development compounds prior to any 'wet testing'. *In silico* assessments are also used to prioritize the testing of drug intermediates, impurities and degradents with genotoxic alerts (see below). This has helped to reduce drug development costs by reducing late phase attrition. As the prediction has been exhaustively optimized for early safety-relevant end points such as genotoxicity, future activities are likely be focussed on more idiosyncratic toxicities in patients, by making these tools more relevant to human disease.

3.2.2 In Vitro Assays

Genetic toxicology is one area in which the strength of *in vitro* testing is particularly apparent. Many genotoxins require metabolic activation, and *in vitro* assays facilitate control over the provision of a bioactivating system, usually a microsomal enzyme-containing preparation from the liver of rats treated with a wide spectrum cytochrome P450 inducer (see below for a more comprehensive overview). Microbial or cellular systems often also permit concentrations to be used that could not be achieved in the plasma of animals or humans. This is important in evaluating a compound's *potential* for genotoxicity, which is relevant to particularly susceptible individuals, or to tissues in which the compound accumulates to very high local concentrations. *In vitro* studies are also useful in investigating mechanisms of genotoxic action that would be extremely difficult to pursue in animals. Compounds shown to be potent genotoxins in *in vitro* assays can be—and generally are—eliminated from further development at that point. Those that are negative, or in which a risk–benefit estimate remains favourable, still require *in vivo* assessment, which is mandatory for drug and chemical registration.

In the *in vitro* assays, cells (bacterial and mammalian) are treated with test agents in the presence and absence of exogenous mammalian oxidative metabolizing system (S9-mix), to mimic mammalian metabolism (Garner *et al*., 1972). A phenobarbital/5,6-benzoflavone or aroclor 1254-induced rat liver postmitochondrial fraction (S9) may be used as an exogenous oxidative metabolizing system (McGregor *et al*., 1988). The S9 fraction may be obtained commercially, for example, MolTox™ (Molecular Toxicology Inc., USA) or generated 'in house'. Every batch of rat liver S9 fraction should be checked for sterility, protein content, ability to convert known promutagens to bacterial mutagens and cytochrome P450 enzyme (e.g. alkoxyresorufin-O-dealkylase) activity. Batches of S9 may be stored as frozen aliquots at $-80\,^{\circ}C$ prior to use and should be thawed and mixed with appropriate cofactors to produce S9-mix when required.

3.2.2.1 Bacterial Mutation Tests

The study of mutation in bacteria and bacterial viruses has had a fundamental role in genetics, particularly in relation to the understanding of biochemical pathways, identifying DNA as the hereditary material, and providing insights into gene structure, regulation, and the basis for mutagenesis, and so on. Early studies on the biosynthesis of amino acids in the bacteria *Escherichia coli* (Yanofsky, 1971) and *Salmonella typhimurium* (Ames, 1971), using mutant strains unable to grow in the absence of specific amino acids, were the very basis of the bacterial 'reverse' mutation assay. For an illuminating insight into the early development of these assays the reader is referred to reviews by MacPhee (1989) and Mortlemans and Zeiger (2000).

This pioneering work eventually resulted in what is commonly known as the Ames *Salmonella*/microsome mutagenicity assay (or simply *Salmonella* test; Ames

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test); a short-term bacterial 'reverse' mutation assay specifically designed to detect a wide range of chemical substances that can produce genetic damage that leads to gene mutations (Ames *et al*., 1975; Green, 1978; Maron and Ames, 1983). The test employs several histidine-dependent (auxotrophic) *Salmonella* strains, each carrying different mutations in various genes in the histidine operon. These mutations act as hot spots for mutagens that cause DNA damage via different mechanisms. When the *Salmonella* tester strains are grown on a minimal media agar plate containing a trace of histidine, only those bacteria that revert to histidine independence (his+), by acquiring a second mutation that directly reverses or suppresses the original mutation in the histidine operon, are able to form colonies. In other words, the second mutation restores prototrophy, that is, the ability for the affected cell to grow in the absence of the relevant amino acid, histidine. The absence of the essential amino acid in the culture medium provides the selective pressure which enables a small number of mutant colonies to grow from a few individual bacteria within a population of millions of unmutated cells (that are unable to grow). This simple concept underlines the great strength of the bacterial 'reverse' mutation assay, since the number of spontaneously induced revertant colonies per plate is relatively constant, whereas when a mutagen is added, the number of revertant colonies per plate is increased, usually in a concentration-dependent manner.

The *Escherichia coli* WP2 tryptophan reverse mutation assay detects trp(−) to trp(+) reversion in comparison. All WP strains carry the same AT base-pair mutation at a critical site within the *trpE* gene which blocks the biosynthesis of tryptophan prior to the formation of anthranilic acid (Bridges *et al*., 1967). The assay is currently used by many laboratories in conjunction with the Ames *Salmonella* assay for screening chemicals for mutagenic activity. In general, the *E. coli* WP2 assay procedures are the same as those for the Ames *Salmonella* assay (detailed below), with the exception that limited tryptophan instead of limited histidine is used. The WP2 strains are used as a substitute for, or as an addition to *Salmonella* strain TA102 which also carries an AT base pair at the mutation site (reviewed by Mortlemans and Riccio, 2000).

The relative simplicity and speed of the Ames *Salmonella* and *E. coli* WP2 assays, in addition to the qualitative relationship (relatively high specificity and sensitivity) between mutation in bacteria and rodent carcinogenicity, for most classes of DNA-reactive compounds, has promoted their worldwide adoption as an initial screen to determine the mutagenic potential of new chemicals and drugs. Over the years, many validation studies have been performed to determine the reproducibility of test results on an intralaboratory and interlaboratory level, and also the sensitivity and correlation of the Ames test with animal carcinogenicity studies. It has indeed been established that there is a high predictivity of a positive mutagenic response in the Ames test for rodent carcinogenicity (Zeiger, 1985; 1987). The test therefore is in many instances used as a first screen to determine the mutagenic potential of new chemicals and drugs. The Ames *Salmonella*/*E. coli* WP2 test is also used for submission of data to regulatory agencies for registration or acceptance of many chemicals, including drugs and biocides. National (COM, 2000) and international (OECD, 1997a) guidelines have been established for performing these mutagenicity assays and to ensure uniformity of testing procedures (reviewed by Gatehouse *et al*., 1990; Mortlemans and Zeiger, 2000; Mortlemans and Riccio, 2000).

An alternative approach is to use bacteria to detect 'forward mutations' such as the acquisition of resistance to a toxic chemical (e.g. an amino acid analogue) or an antibiotic (such as streptomycin). Although genetic systems which detect forward mutations have an apparent advantage, in that a wide variety of genetic changes (e.g. point mutation, deletions, insertions) may lead to a forward mutation, the spontaneous mutation rates tend to be relatively high (Gatehouse *et al*., 1990). Therefore, if a particular mutagen causes rare specific changes, these changes may be lost against the background of more common events. Forward mutation systems in bacteria, however, are no longer commonly used in regulatory genetic toxicology.

3.2.2.1.1 Tester Strains

The most widely used strains are those developed by Professor Bruce Ames and colleagues, which are mutant derivatives of the organism *Salmonella typhimurium*. Each strain carries one of a number of mutations in the operon coding for histidine biosynthesis. In each case the mutation can be reverted either by base-change or by frameshift mutations. The genotypes of the commonly used strains are listed in **Table 5** along with the DNA sequence at the site of the original mutation in the relevant histidine gene. With the exception of the TA102 strain, all the other strains contain G–C base pairs at the site of the histidine mutation, and this has some effect on the selectivity of the mutagens detected, that is, those that act preferentially on these bases.

Many carcinogens (or their metabolites) are large molecules that are unable to cross the outer cell wall of wild-type *Salmonella*, therefore Ames introduced *rfaE* mutations (deep rough) into the *Salmonella* tester strains, which results in defective lipopolysaccharide, and increases the permeability of the cell wall to bulky hydrophobic chemicals (Ames *et al*., 1973). In addition, bacteria possess several major error-free DNA repair pathways which can repair DNA damage without generating mutations. By removing the UV endonuclease component B (*uvrB*) gene, Ames was able to increase the sensitivity of the tester strains to mutagens by several

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Table 5 *Salmonella* and *E. coli* strains commonly used in the Ames test

Strain	Genotype[a]	Nature of his mutation	Enzyme loss	Reversion events	Reference
TA97	*hisD6610 hisO1242 rfa* Δ *gal chlD bio uvrB* (pKM101)	+2CG frameshift		Frameshift mutagens; for example substituted triazines, phenothiazines	Levin *et al*. (1982)
TA98	*hisD3052 rfa* Δ *gal chlD bio uvrB* (pKM101)	ICR364-OH induced -1GC frameshift mutation	L-histidinol dehydrogenase	Frameshift mutagens; for example aromatic nitroso compounds, aflatoxin B_1, benzo[a]pyrene	Isono and Yourno (1974)
TA100	*hisG46 rfa* Δ *gal chlD bio uvrB* (pKM101)	Spontaneous G → T missense base mutation	PR-ATP pyrophosphorylase	Variety of suppressor mutations based on a subset of bp substitutions	Ames (1971)
TA102	*hisG428* (pKM101) *rfa* Δ *gal E* (pAQ1)	Ochre C → T missense mutation	PR-ATP pyrophosphorylase	All possible transitions and transversions	
TA104	*hisG428* (pKM101) *rfa* Δ *gal E* (pAQ1)	Ochre C → T missense mutation		All possible transitions and transversions	Koch *et al*. (1996)
TA1535	*hisG46 rfa* Δ *gal chlD bio uvrB*	Spontaneous G → T missense base mutation		See TA100	
TA1537	*hisC3076 rfa* Δ *gal chlD bio uvrB*	ICR364-OH induced +1 C frameshift mutation	Imidazoleacetol phosphate transaminase	Frameshift mutagens; for example ICR-191, 9-aminoacridine, and epoxides of PAHs	Isono and Yourno (1974)
TA1538	*hisD3052 rfa* Δ *gal chlD bio uvrB*				

[a]*rfa*—deep rough; *gal E*—UDP galactose 4-epimerase; *chlD*—nitrate reductase (resistance to chlorate); *bio*–biotin; *uvrB*—UV endonuclease component B; Δ—deletion of genes following this symbol; PAHs—polycyclic aromatic hydrocarbons; pAQ1—a plasmid carrying the *hisG428* gene; pKM101–a plasmid carrying the *mucA8* genes that enhance error.

orders of magnitude (note the deletion through *uvrB* also includes the nitrate reductase (*chl*) and biotin (*bio*) genes, hence the genotypes described in **Table 5**).

DNA interstrand crosslinking agents (e.g. mitomycin C, psoralens, UVR), however, require an intact excision repair pathway to generate mutations (see Kondo *et al*., 1970). Thus, it is useful to include a strain with an intact excision repair pathway in the test battery. *Salmonella typhimurium* TA102 is such a strain. The relative loss in sensitivity to other types of mutagen due to the *uvrB*+ background is offset by the presence of multicopies of the *hisG428* mutation on plasmid pAQ1 in this strain.

The *E. coli* WP2 trp E series possess a terminating ochre mutation in the *trp E* gene, which codes for anthranilate synthetase, an enzyme late in the biosynthetic pathway for tryptophan. The ochre mutation involves an A–T base pair (Bridges *et al*., 1967). Reverse mutation can take place at the original site of mutation or in the relevant tRNA loci, and thus all types of transition and transversion can be detected. Although error-prone repair is present, strains possessing *ruvrA* mutations and mutator plasmids (pKM101 or R46) have advantages, including the detection of some frameshift mutagens. In addition to being effective general strains for mutagen detection, studies by Wilcox *et al*. (1990) have shown that a combination of *E. coli* WP2 lip E (pKM101), which has a functioning excision repair system for the detection of cross-linking agents, and *E. coli* WP2 ir-p E urrA (pKM 101) can be used as alternatives to *Salmonella* TA102 for the detection of oxidative mutagens. The *E. coli* strains have the advantage of lower spontaneous mutation rate and are somewhat less difficult to use and maintain.

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During the 1990s, an improved understanding of the molecular process of mutagenesis and advances in biotechnology led to the development of engineered bacterial strains for specific areas of genotoxicity research. In particular, the introduction of genes for the enzymes involved in mutagen activation and the processing of DNA damage resulted in a number of new tester strains. For example, bacterial strains expressing specific bacterial enzymes (e.g. *N*-acetyltransferases and nitroreductases) were constructed for the metabolism/detection of nitroaromatic compounds (nitropyrenes) in complex mixtures (Watanabe *et al*., 1990). Also, modifications to DNA repair capacity were introduced, for example *S. typhimurium* TA1535 with inactivated MGMT. Consequently, this strain is extremely sensitive to the mutagenic action of direct acting alkylating agents (Yamada *et al*., 1995). Recently, eukaryotic P450s have also been expressed in bacteria, as reviewed by Guengerich *et al*. (1996). The expression of human P450 enzymes in bacterial cells has made possible the development of mutagenicity assays where metabolic activation takes place within the internal milieu of the target cell (reviewed by Josephy, 2002). At present, these systems do not appear in general use.

Many chemical mutagens and carcinogens generate DNA lesions which block replication, often because of their bulky character, and result in the induction of the SOS response. This response, in turn, increases mutagenesis and survival by allowing replicative bypass of the DNA lesions. As the induction of the SOS response strongly correlates with DNA damage, spectrophotometric assays based on the expression of reporter genes (β-galactosidase (*lacZ*) or luciferase (*LUC*)) transcribed from an SOS promoter have been developed. The SOS chromotest (Quillardet and Hoffnung, 1993) and the Umu test (Oda *et al*., 1992) are such examples. The Ames II test (Gee *et al*., 1994) developed in the Bruce Ames laboratory at the University of California, Berkeley, is based on the same principle as the traditional test, and is a liquid microplate version which offers several advantages over the original, including a higher-speed format, new strains, colorimetry, easy handling and the possibility of automated plating and plate reading. The assay requires lower amounts of test chemical and shows good correlation with the traditional assay (Gee *et al*., 1998). The assay is based on the use of strains TA7001–TA7006 and TA98. Like the traditional strains, the genetic backgrounds of the TA700X strain series have been modified to improve the sensitivity of their reversion. Individually, these strains are designed to revert by only one specific base-pair substitution out of all possible changes. Thus, when mixed, all base-pair substitution mutations can be represented in one culture. The TA98 strain is used for the detection of frameshift mutations. The Ames II test is available commercially from Xenometrix (see *www.aniara.com*).

3.2.2.1.2 Bacterial Reversion Tests: Practical Aspects

The protocol most widely used is the plate incorporation assay (described briefly below). Treat and plate tests and fluctuation tests have also been used (reviewed by Tweats and Gatehouse, 1999). A comprehensive description of the Ames/*Salmonella* test is provided by Mortlemans and Zeiger (2000) and the *Escherichia coli* WP2 tryptophan reverse mutation assay by Mortlemans and Riccio (2000).

In the current OECD, ICH and Committee on Mutagenicity (COM) Guidelines, the following base set of bacterial tester strains is recommended:

- *S. typhimurium* TA98, TA100, TA1535
- *S. typhimurium* TA1537 or TA97 or TA97a
- *S. typhimurium* TA102 or *E. coli* WP2 uvrA or *E. coli* WP2 uvrA (pKM101).

As mentioned earlier, strains TA102 or *E. coli* WP2 uvrA are included to detect point mutations at AT sites. To detect crosslinking agents specifically, it is suggested that the repair-proficient *E. coli* strain WP2 (pKM101) might be selected. Whichever strain is selected, the genetic compositions of the tester strains are fundamental to the operation of these tests and therefore should be assured.

3.2.2.1.3 Storage and Checking of Tester Strains

It is paramount that tester strains are regularly checked to ensure their characteristic genetic features are maintained, particularly if there is cause for concern, for example, extreme fluctuations of the spontaneous mutation values between experiments. These checks should include:

- Amino acid requirement (i.e. histidine for *S. typhimurium* strains; tryptophan for *E. coli* WP2 strains).
- Sensitivity to diagnostic mutagens using pairs of strains designed to give either a strong or weak response.
- Resistance to ampicillin for strains carrying pKM101 and tetracycline for strains carrying pAQ1.
- Regular monitoring of background mutation rates and induced mutation rates with reference mutagens (i.e. 'positive controls').

The importance of these checks together with careful storage and cell culture maintenance and response to reference mutagens can not be over-emphasized: failure to apply them can result in much wasted effort.

3.2.2.1.4 Ames *Salmonella*/Plate Incorporation Method

Plate incorporation tests are carried out essentially as described by Maron and Ames (Maron and Ames, 1983), and should be conducted with and without external

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metabolic activation. In short, the test involves preparation of the appropriate amount of bacterial culture, test article and S9-liver fraction (or S9-buffer as appropriate) in sterile glass tubes (maintained at 37 °C) to which is added molten top agar (maintained at ~45 °C) supplemented with limiting amounts of biotin and the required amino acids (histidine or tryptophan). The final mixture is quickly poured over Vögel Bonner plates and allowed to set. The plates are then inverted and incubated for approximately 48–72 hours at ~37 °C in the dark before scoring for revertant colonies.

Before carrying out the main test, it is necessary to conduct a preliminary toxicity test, performed to the same basic protocol as the mutation test, except that instead of scoring the number of mutants on, for example, minimal media plates with limiting amounts of the required amino acid, the number of survivors is scored on fully supplemented minimal media. This preliminary toxicity test will also demonstrate excess growth, which may indicate the presence of histidine or tryptophan or their precursors in the test material, which could make testing for mutagenicity impracticable by this method. Sterility check plates should also be prepared and run in parallel with both toxicity and main tests.

In the main test, the upper treatment level should be 5 mg plate^{-1} for freely soluble, nontoxic compounds. Where toxicity is observed at lower concentrations, the highest concentration tested should show evidence of significant toxicity; this may be detected by a reduction in the number of revertants or a diminution of the background lawn (see below). For compounds that are relatively insoluble in the test system, the lowest precipitating concentration (determined by eye at the end of the incubation period) should be used as the maximum test concentration, up to 5 mg plate^{-1}. It is recommended that a minimum of three plates per dose be used and at least five analysable concentrations be selected for scoring, although the concentrations obtained may be strain specific in the event of variable strain toxicity. Vehicle and positive control compounds (**Table 6**) should be tested in parallel, and, generally, two standard plate incorporation tests are performed to support a submission of data to regulatory authorities for the registration of chemicals, drugs and biocides, although a single, well-conducted test may be appropriate (ICH S2B: A Standard Battery for Genotoxicity Testing of Pharmaceuticals, 1997 and revision expected in 2009).

3.2.2.1.5 Scoring of Plates

Before scoring the treatment plates it is important to review the vehicle control and sterility-check plates in order to confirm that the assay has been performed correctly. It is important to monitor the number of mutant colonies present on untreated or vehicle controls. Acceptable ranges are shown in **Table 7**. Deviations in background reversion frequencies should be viewed with suspicion and investigated, as it is possible that strain cross-contamination has occurred or media quality has been compromised, and so on (see above on checking of tester strains). In addition, the toxicity of the various treatment plates should be determined in order to select the appropriate plates for scoring; this requires the evaluation of bacterial lawns on the agar plates after the incubation period, rather than a quantitative assessment of cell survival.

The addition of limiting amounts of the required amino acid to the top agar allows the bacteria to undergo approximately six to eight cell divisions. In many cases, this limited growth is essential for mutagenesis to occur by allowing fixation of the mutational lesions. The bacterial revertants (pre-existing and induced) will continue to grow in the absence of the essential amino acid and will give rise to visible colonies. These are easily scored against a slightly hazy-looking background lawn which is made up of microcolonies of auxotrophic bacteria, that is, those that are dependent on the required amino acid. Examination of the background lawn in the absence of toxicity will reveal the presence of densely packed

Table 6 Positive control compounds used in the standard plate incorporation Ames test

Strain	Positive control, concentration in μg plate^{-1}	Vehicle
Without S9		
S. typhimurium TA98	2-Nitrofluorene (2NF), 1	DMSO
S. typhimurium TA100, TA1535	Sodium azide (NaAz), 2	Water
S. typhimurium TA1537	ICR-191, 1	DMSO
S. typhimurium TA102	Mitomycin-C (MMC), 0.5	Water
E. coli WP2 (pKM101) and WP2 uvrA (pKM101)	4-Nitroquinoline-1-oxide (4NQO), 5	DMSO
With S9		
S. typhimurium TA98	Benzo(a)pyrene (B(a)P), 10	DMSO
S. typhimurium TA100, TA1535, TA1537 and *E. coli* WP2 (pKM101)	2-Aminoanthracene (2AAN), 5	DMSO
E. coli WP2 uvrA (pKM101)	2-Aminoanthracene (2AAN), 10	DMSO
S. typhimurium TA102	2-Aminoanthracene (2AAN), 20	DMSO

DMSO—dimethyl sulfoxide.

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Table 7 Spontaneous revertant control values in the Ames test

Strain	Number of revertants (range)	
	With S9	Without S9
TA97[a]	100–200	75–200
TA98	20–50	20–50
TA100	75–200	75–200
TA102	20–400	100–300
TA104	300–400	200–300
TA1535	5–20	5–20
TA1537	5–20	5–20
TA1538	5–20	5–20
E. *coli* WP2 uvrA (pKM101)[b,c]	15–50, 35–160	35–160

[a]Mortlemans and Zeiger (2000).
[b]Wilcox *et al*. (1990).
[c]Mortlemans and Riccio (2000).

microcolonies which form a uniform layer. However, when a chemical is toxic there may be 'thinning' or complete absence of the background lawn compared to the vehicle control for that strain. A reduction in the number of revertant colonies to levels below the spontaneous reversion level may also be seen on occasion and this may reflect toxicity too, but could also indicate antimutagenic properties of the test article. A complete absence of the background lawn indicates a high level of toxicity with the inability of the bacteria to grow and form a lawn. Such toxic concentrations should not be used, as colonies may appear which are not true revertants, but are instead surviving auxotrophic cells. These colonies will be visible to the naked eye. If necessary the phenotype of any questionable colonies (pseudo-revertants) should be checked by plating the bacteria onto histidine- or tryptophan-free medium, as appropriate.

The presence of the background bacterial lawn may be recorded using the following general assessments:

- **Very slight toxicity:** very slight thinning (up to 10% loss) of the background bacterial lawn and increase in the size of the lawn microcolonies compared to vehicle control.
- **Slight toxicity:** slight thinning (up to 30% loss) of the background bacterial lawn and increase in the size of the lawn microcolonies compared to vehicle control.
- **Clear toxicity:** marked thinning (30–70% loss) of the background bacterial lawn and increase in the size of the lawn microcolonies compared to vehicle control.
- **Severe toxicity:** lack of background bacterial lawn on over 80% of the plate, lawn colonies visible to the naked eye.
- **Toxic:** complete killing of all the test bacteria.

Plates are also evaluated (by eye) for the presence of test compound precipitation at the end of the incubation period. The number of bacterial colonies per plate is scored, usually with the aid of an automatic colony counter; however, where necessary the plates may be scored manually, for example, plates with large amounts of colonies or heavy precipitate. Bacterial mutation plates may be stored at 2–8 °C for up to one week prior to scoring.

3.2.2.1.6 Expression and Interpretation of Results

The number of colonies per plate is recorded and mean values calculated for each concentration of test compound used. Mean values may also be expressed as a ratio of the mean concurrent vehicle control value (i.e. fold increase). The values for vehicle control of number of revertants for each strain must be within or close to appropriate ranges determined from the laboratory historical control data, and positive control cultures should show clear, unequivocal, positive responses. There should be an absence of confounding technical problems such as contamination, outliers, excessive toxicity or unacceptable numbers of lost plates. Tests that do not fulfil these criteria should be rejected and not reported.

If the data for any test agent treatment concentration shows a response greater than or equal to two times the concurrent vehicle control value (strains TA98, TA100, TA102, WP2 uvrA (pKM101) and WP2 (pKM101)), or greater than or equal to three times the concurrent vehicle control value (TA1535 and TA1537), in conjunction with a concentration-dependent response, the result is considered positive. Where the data for any strain shows a concentration-dependent response, approaching the two- or three-fold threshold as detailed above, but does not exceed it, or the two/three-fold increase in an appropriate strain is obtained only at the highest concentration tested, or there is an isolated increase with evidence of toxicity to the bacterial strain at the next highest test dose, then the result may be equivocal and further testing may be required for clarification, as follows:

- The test may be repeated using the strain(s) and a narrowed-down concentration range with which the initial positive result was observed in order to assess/confirm reproducibility.
- A second preincubation-type test (Yahagi *et al*., 1975) may be conducted (see below) or alternatively a different metabolic activation system may be used, for example using a higher concentration of S9-liver mix, or S9-liver fraction from an alternative species (if appropriate).
- Testing in additional bacterial strains (e.g. WP2 (pKM101) or TA102) can also be considered.

A number of statistical programs have been developed for analysing *Salmonella* mutagenicity data, and all have their strengths and weaknesses (see review by Mahon

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et al., 1989). It should be recognized that variation within experiments does occasionally produce weak positive results that are not obtained in repeat assays. In addition, experimental variability of unknown origin may occur from time to time, yielding a stronger positive result that, similarly, is not confirmed in repeat experiments. In such cases the components of the test system should be thoroughly checked. If no further positive result is obtained the test compound should be regarded as nongenotoxic.

3.2.2.1.7 Modification of the Plate Incorporation Method

The liquid preincubation assay (also known as the Yahagi test) is a modification of the standard plate incorporation assay and involves exposing the tester strains for a short period of time in a small volume containing the test agent with buffer or external metabolic activation prior to plating on minimal agar medium (Yahagi *et al*., 1975). It is believed that the modification renders the test more sensitive than the standard assay for the detection of some classes of genotoxic carcinogens, for example nitrosamines and azo dyes. This is because short-lived mutagenic metabolites have a better chance of reacting with the tester strains in the small volume of the preincubation mixture compared to when the incubation mixture is plated immediately on minimal defined agar plates. In addition, the smaller preincubation volume results in a higher effective concentration of S9-liver fraction and cofactors in the external metabolic activation mix. All procedures are exactly as described in the standard plate incorporation test with the following modifications: sterile glass tubes are prepared and maintained at 37 °C before adding the appropriate amount of the required test compound, vehicle or positive controls and S9-liver fraction metabolic activation mix for a fixed period of time (usually 20–60 minutes). The top agar is added and the final mixture is poured over Vögel Bonner plates. The plates are then inverted and incubated for approximately 72 hours at ∼37 °C in the dark before scoring for revertant colonies. All positive control final concentrations are the same as described in the standard plate incorporation test with the exception of WP2 uvrA (pKM101), for which the concentration of 4NQO is reduced to 1 μg plate^{-1}.

3.2.2.1.8 Fluctuation Tests

The fluctuation test, originally devised by Luria and Delbruck (1943) was further developed as a genotoxicity assay in the 1970s (reviewed by Hubbard *et al*., 1984) and more recently by Flückiger-Isler and Kamber (2007). In the fluctuation test the number of mutants in a series of small independent replicate cultures is determined. Amino acid-requiring bacteria are treated in a suitable medium either with or without a metabolizing system, with the chemical to be tested and with a trace of the required amino acid. As for the Ames test, this trace allows a few generations of growth, which allows mutation expression following the initial DNA damage. This mixture is then divided into a large number of aliquots, for example into 50 test tubes or the 96 wells of a microtitre tray. When the trace of amino acid is consumed, only cells that have reverted at the target gene, thus restoring biosynthesis of the relevant amino acid, can grow. Test tubes or wells containing such a revertant become turbid and the medium in the well or tube becomes acid, as a result of acid release during growth. A pH indicator such as bromocresol purple or bromothymol blue can highlight such changes. The pH of the medium will drop into the range 5.2–6.8 in such cultures, causing a change of the indicator to yellow. A statistically significant, reproducible increase in the number of positive wells or tubes indicates a mutagenic response. The preferred methods of statistical analysis have been reviewed in Robinson *et al*. (1989).

For a description of other types of bacterial reversion assays or more exotic modifications of the Ames test, for example the desiccator assay for volatile liquids and gases, and so on, the reader is referred to Tweats and Gatehouse (1999) and the review of the Ames/*Salmonella* assay by Mortlemans and Zeiger (2000), respectively.

3.2.2.2 Mammalian Cell Mutation Assays

3.2.2.2.1 Mouse Lymphoma Assay

The mouse lymphoma assay (MLA) using the thymidine kinase (*tk*) gene is the most widely used of the various *in vitro* mammalian cell gene mutation assays. There are currently two equally acceptable methods for performing the assay, one using soft agar medium for cloning and enumeration of mutants and the other using liquid medium and 96-well microwell plates (Cole and Arlett, 1984).

The MLA detects gene mutations and chromosomal damage in proliferating mammalian L5178Y mouse lymphoma cells *in vitro*. The assay selects for mutations at the *tk* locus, an autosomal gene of moderate size (11–13 kb), located on chromosome 11 in the mouse genome, which encodes for a pyrimidine salvage enzyme involved in a biochemical pathway for nucleic acid breakdown products. The mouse lymphoma L5178Y cell line is heterozygous for thymidine kinase, that is, $tk^{+/-}$. When L5178Y ($tk^{+/-}$) cells are grown in culture medium containing the toxic base analogue, 5-trifluorothymidine (TFT), only mutant ($tk^{-/-}$) cells are able to survive. Thus the assay selects for forward mutation at the *tk*-locus (**Figure 9**).

TFT-resistant, L5178Y ($tk^{-/-}$) mutant colonies may be characterized as large colonies or small (slow-growing) colonies (**Figure 9**). Molecular analysis has indicated that large colonies generally arise from cells subject to genotoxic events which occur within the *tk* gene locus (i.e. intragenic lesions such as base-pair substitutions, frameshift mutations and/or small deletions, etc.). These

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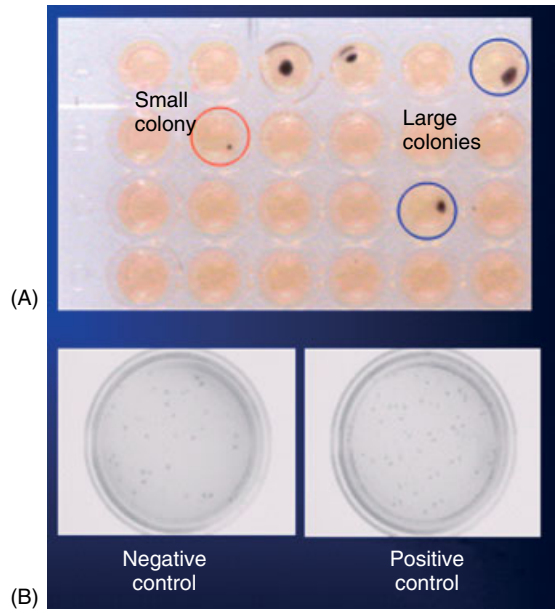

Figure 9 Mouse lymphoma assay. (A) Microwell-plate version; (B) agar plate version (note 'pin-prick' colonies).

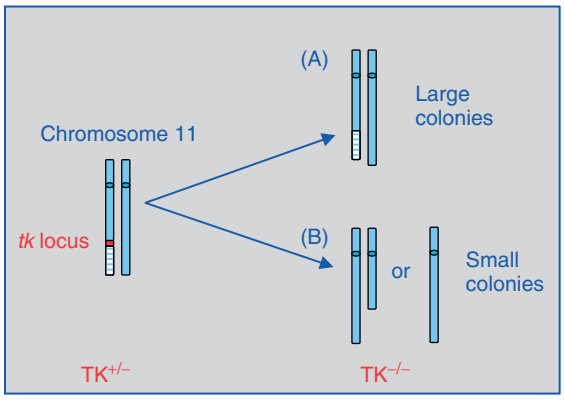

Figure 10 Colony sizing in the mouse lymphoma assay. The thymidine kinase locus is proximal to a number of genes essential to cell growth (located at the end of the q-arm of chromosome 11). (A) Intragenic mutation at the *tk* locus (i.e. point mutations or small deletions) result in large colony formation; (B) large deletions or chromosome loss (extragenic mutations) result in small colony formation (due to the loss of the essential growth genes).

affect only the *tk* gene and result in significant reduction or total loss of gene expression and/or thymidine kinase enzyme function. In contrast, small colony $tk^{-/-}$ mutants are derived from cells with much larger aberrations on chromosome 11b, the chromosome homologue that carries the wild type (active) *tk* gene, which may arise from chromosomal recombination, chromosomal deletion (clastogenic) or chromosomal loss (aneugenic) events (Applegate *et al*., 1990; Moore *et al*., 1985). Thus, in cell lines heterozygous for the *tk* allele, both mutations in the *tk* gene, and chromosomal events involving the gene, may be detected by selecting *tk* mutants in culture medium containing TFT (**Figure 10**). Therefore, mutant ($tk^{-/-}$) colony sizing in the MLA may provide information regarding genotoxic mechanism (gene mutation, clastogenicity and/or aneuploidy).

The MLA, like the majority of mammalian gene mutation systems, requires colony formation to detect mutants; this has the benefit of allowing further analysis of selected clones to verify the mutational origins of the LOH events leading to TFT resistance, for example by DNA sequencing or the use of molecular probes.

3.2.2.2.2 The Test System

In the MLA, a preliminary cytotoxicity test is generally followed by three independent mutation assays: two in which cells are treated for 3–4 hours in the presence and absence of exogenous mammalian oxidative metabolizing system (S9-mix), to mimic mammalian metabolism (Amacher and Turner, 1982; McGregor *et al*., 1988), and a third in which cells are treated for 24 hours in the absence of S9-mix. The purpose of the 24 hours exposure arm is to account for compounds that induce cell cycle delay or cell division aberrations. Such compounds often induce aneuploidy and these are not always detected using a short 3–4 hour exposure period (Moore *et al*., 2002).

The test methodology is based on established procedures (Clive *et al*., 1979; 1995; Cole *et al*., 1990; Moore *et al*., 1985) and the assay should be conducted in accordance with the general principles of current regulatory guidelines (OECD, 1997e; ICH S2B: A Standard Battery for Genotoxicity Testing of Pharmaceuticals, 1997). In addition, the recommendations of the MLA workgroup of the IWGTP should also be employed to ensure consistency in results and data interpretation. The MLA workgroup, chaired by Dr. Martha Moore (National Health and Environmental Effects Research Laboratory, US Environmental Protection Agency) since its inception at the IWGTP meeting in Washington DC in the spring of 1999, consists of experts from Japan, Europe and the USA. The MLA workgroup has been responsible for the production of several documents relating to the conduct of the MLA, which specifically address the following: (i) recommendations for an appropriate cytotoxicity measure, (ii) issues related to the use of a 24-hour treatment time (including the ability of the assay to detect aneugens), and (iii) criteria for data acceptance and appropriate approaches to data evaluation (Moore *et al*., 2000; 2002; 2003; 2006; 2007).

The description of the assay below is based on the microwell method protocol that is used in the author's laboratory and is based on the recommendations of the MLA workgroup. Other protocols may include minor deviations to the protocol described below. Any major deviations in the agar method are included, were appropriate, for completeness.

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3.2.2.2.3 Cell Line and Culture

L5178Y ($tk^{+/-}$) mouse lymphoma cells were originally isolated by Dr. Donald Clive (Burroughs Wellcome Co., USA). Cultures may be obtained from established cell banks (e.g. The European Collection of Cell Cultures: see *www.hpacultures.org.uk*). Cells should be karyotyped on receipt and stocks should be prepared and stored frozen in liquid nitrogen. Each batch of frozen cells should be purged of L5178Y ($tk^{-/-}$) mutants, checked for spontaneous mutant frequency (MF) and genetic stability and also be *Mycoplasma* free. Cell media and culture conditions have been described by Moore and Howard (1982). Culture media should be supplemented with heat-inactivated horse serum in order to eliminate a factor present in other sources of animal sera which degrades TFT (Moore and Howard, 1982).

3.2.2.2.4 Rationale for Dose Selection

The maximum concentrations tested in the MLA are defined by current guidelines (see above) and are restricted to 5000 $\mu g\,ml^{-1}$ or 10 mM for freely soluble compounds, or the limit of solubility or toxicity, whichever is lowest. If compound solubility is a limiting factor, the maximum concentration tested is generally set as the lowest level at which compound precipitate is observed by eye at the end of treatment. Vehicle controls, diluted to the same extent as the test-compound solutions, and a positive control should be used. In the author's laboratory, methylmethane sulfonate (20 $\mu g\,ml^{-1}$ for 3 hour, and 5 $\mu g\,ml^{-1}$ for 24 hour treatments) and benzo(a)pyrene (3 $\mu g\,ml^{-1}$) are generally used as positive controls in the absence and presence of S9 activation, respectively.

3.2.2.2.5 Mammalian Oxidative Metabolizing System (S9-mix)

A phenobarbital/5,6-benzoflavone (Elliot *et al.*, 1992) or aroclor 1254 (Amacher and Turner, 1982) induced rat liver postmitochondrial fraction (S9) may be used as an exogenous oxidative metabolizing system in the short (3–4 hour) treatment arm of the MLA, but not the 24-hour treatment arm. Cole *et al.* (1990) noted that S9 itself (or culture medium in which S9 has been preincubated) maybe mutagenic in the MLA when used for prolonged periods (18–24 hours) or at high concentrations (50 μl S9 ml^{-1}), and therefore S9 is not included in the 24-hour treatment arm of the MLA.

3.2.2.2.6 Preliminary Cytotoxicity Test and Main Test

A preliminary cytotoxicity test is generally conducted with the test agent using a standard range of concentrations to define toxicity limits (unless solubility or existing toxicity data indicate otherwise), in the presence (3-hour treatment) and absence (3-hour and 24-hour treatments) of metabolic activation (S9-mix). Single cultures are generally acceptable. The relative suspension growth (RSG) values are calculated as described below and these are used to determine the main test concentrations to be used. Based on the results of the preliminary cytotoxicity test, a minimum of five test concentrations are generally selected for testing in the MLA, both in the presence and absence of S9-mix. A range of concentrations, from nontoxic to appropriate dose-limiting levels should be selected, and the concentration range may be specific for each treatment arm of the study (i.e. 3 or 24 hours duration of exposure and presence/absence of S9-mix). As fluctuations in osmolality of more than 50 mOsm kg^{-1} (Scott *et al.*, 1991) or extremes of culture-medium pH (Brusick, 1986) are associated with increased mutant frequencies, these parameters may be measured, on a case-by-case basis (e.g. if the final concentration in the medium is anticipated to be ~10 mM or the molecular weight of the test compound is unknown), and subsequently used to define the limit concentration. In the author's laboratory, testing is routinely limited to those compounds with a molecular weight of less than 200, based on previous experience.

3.2.2.2.7 Expression Period

Following treatment with the test agent, L5178Y cell cultures should be maintained in an incubator at 37 °C for a period of two days to allow time for any residual thymidine kinase enzyme and wild-type RNA to degrade in $tk^{-/-}$ null mutants. This ensures sufficient time has passed so that the mutant phenotype (i.e. TFT resistance) may be expressed. During this time, cell densities ($<1 \times 10^6$ cells ml^{-1}) are maintained by subculturing. From observations of cell growth and recovery during the expression period, four to five test concentrations plus vehicle and positive controls are selected for plating to assess viability (cloning efficiency (CE)) and TFT resistance (mutant assessment).

3.2.2.2.8 Plating for Viability

At the end of the expression period, cell densities should be determined using a Coulter counter or haemocytometer, and selected cultures should be adjusted to contain 10 000 cells ml^{-1} (except for the preliminary cytotoxicity test, where this adjustment is not made). Cultures are then adjusted to 8 cells ml^{-1} and aliquots transferred to two 96-well microtitre plates (0.2 ml $well^{-1}$, averaging 1.6 cells per well). The plates are placed in a humidified incubator and maintained for a minimum of three days (viability test) or one to two weeks (mutation assays) at 37 °C with 5% (v/v) CO_2 in air. Wells containing viable clones (i.e. ≥50 cells per clone) may be identified under a microscope or by eye, and counted.

3.2.2.2.9 Plating for Determination of Mutant Frequency (MF)

At the end of the expression period, cell densities are determined using a Coulter counter or haemocytometer,

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and selected cultures adjusted to 1×10^4 ml^{-1} with culture media containing TFT (3 μg ml^{-1}), and transferred to four 96-well microtitre plates (0.2 ml well^{-1} at 2×10^3 cells per well). The plates are then placed in a humidified incubator and maintained for one to two weeks at $37 \pm 1\,^{\circ}$C, with $5 \pm 0.5\%$ (v/v) CO_2 in air. Wells containing mutant clones may be identified, either under a microscope or by eye, and counted. Only colonies of >50 cells should be scored. Where toxicity is a limiting factor, the lowest concentration that provides a relative total growth (RTG) value in the range 10–20% will normally be selected as the maximum treatment concentration for the scoring of mutant frequency. Where this is not possible then the highest treatment concentration selected for scoring MF should result in a RTG value as close to this range as possible. In these circumstances, the validity of the data should be assessed in accordance with the principles discussed by Moore *et al*. (2002). In addition, where one or more treatment concentrations provide the same or similar toxicity levels, only one of these treatment concentrations will normally be scored for MF, provided that at least four treatment concentrations are scored overall.

3.2.2.2.10 Colony Counting and Sizing

If increases in MF compared with the vehicle control are observed, the distribution of large and small colonies may be determined for the vehicle and positive controls and for those concentrations of test compound showing increases in mutant colony frequency. The criterion for sizing colonies is based on morphology and is generally <1/4 well diameter for small colonies and >1/4 well diameter for large colonies (Moore *et al*., 1985; 2000).

'Suspension growth' (SG) is a measure of the growth in suspension during treatment and the expression period. SG is calculated as follows:

$$\text{Suspension growth} = a \times b \times c \qquad (1)$$

where

$$a = \left(\frac{D_0 \text{ post-treatment cell count}}{\text{Pretreatment cell density}}\right)$$

$$b = \left(\frac{D_1 \text{ cell count}}{\text{Cell count set up on } D_0 \text{ post-treatment}}\right)$$

$$c = \left(\frac{D_2 \text{ cell count}}{\text{Cell count set up on } D_1}\right)$$

NB for three-hour treatments assume that $a = 1$.

Usually the denominators for b and c are 2×10^5 cells ml^{-1}. However, if cytotoxicity causes the cell count to be lower than 2×10^5 cells ml^{-1} following treatment and/or if the cells do not grow during part of the expression period, it can be lower. In these cases, the respective cell count values have to be entered into the calculation above.

RSG is a measure of the cell growth in suspension during treatment and the expression period, relative to the mean vehicle control suspension growth.

RSG is calculated as follows:

$$RSG\,(\%) = \left(\frac{\text{Individual SG value}}{\text{Mean control SG value}}\right) \times 100 \qquad (2)$$

Viability is the measure of the cells' ability to clone; that is, *cloning efficiency* (CE).

CE is calculated as follows: for microtitre plate tests, calculations are based on $P(0)$, the proportion of wells in which a colony has not grown:

$$P(0) = \left(\frac{\text{Number of wells with no colony}}{\text{Total number of wells}}\right) \qquad (3)$$

The CE for each culture is calculated according to the following:

$$CE = \left(\frac{-\ln P(0)}{\text{Number of cells per well*}}\right) \times 100 \qquad (4)$$

*Number of cells per well is 1.6, on average, on all viability plates.

Relative total growth (RTG) is the measure of cytotoxicity relative to the vehicle control and takes into account all cell growth and cell loss during the treatment period and the two-day expression period (RSG), and the cells' ability to clone two days after treatment (viability).

$$RTG = RSG \times \left[\frac{\text{Individual viability value}}{\text{Mean control viability value}}\right] \qquad (5)$$

Mutant frequency (MF) is calculated as follows:

$$MF = \frac{-\ln P(0) \text{ for mutant plates}}{\text{Number of cells per well*} \times \left(\frac{\text{viability}}{100}\right)} \qquad (6)$$

*Number of cells per well is 2000, on average, on all mutant plates.

Mutant frequencies for each treatment concentration of test compound will be expressed as a ratio of the concurrent mean vehicle control mutant frequency (i.e. fold increase). Small and large colony mutant frequencies will be calculated in an identical manner, using the relevant number of empty wells for small and large colonies.

3.2.2.2.11 Acceptance Criteria and Data Evaluation

The acceptance criteria for the negative/vehicle control parameters for the soft agar and microwell methods of performing the MLA using 3–4 and 24-hour treatments as recommended my the MLA workgroup are shown in **Table 8**.

The background MF for the MLA shows a broad range of values (**Table 8**), which on occasion may produce a

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Table 8 Assay acceptance criteria for the MLA

Measure	Agar method	Microwell method
MF	$35–140 \times 10^{-6}$	$50–170 \times 10^{-6}$
CE (%)	65–120	65–120
SG[a]	8–32	8–32
SG[b]	32–180	32–180
GEF	90	126

[a] 3 h treatments.
[b] 24 h treatments.

'statistically' positive dose–response within the normal range of the background MF for some experiments. Therefore, a biologically relevant approach to MLA data evaluation has been recommended. This requires that the induced MF exceeds a value based on the global background MF, which is unique for each MLA method (agar or microwell). This value is known as the global evaluation factor (GEF). The basic rationale for this approach can be found in Moore *et al*. (2003). In addition, statistical analysis should be applied to determine whether there is a dose-related increase in MF. The adoption of this procedure disregards small increases in induced MF, judged by experts in the assay to be of little or no toxicological significance, but includes statistical analysis to evaluate the presence of a positive dose-related trend. In numerical terms, the GEF has been defined as the mean of the negative/solvent MF distribution plus one standard deviation, and GEF values for the agar and microwell versions of the assay are described in **Table 8**.

To meet the GEF criterion for a positive result then a test culture must have an MF of at least $50 + 126$ (microwell GEF) $= 176 \times 10^6$, or $35 + 90$ (agar GEF) $= 125 \times 10^6$ in the microwell and agar versions of the assay, respectively. In addition, an appropriate statistical trend test should be applied to determine whether there was a positive dose-related increase in MF. This approach should be applied in all experiments, that is, those that use single or replicate cultures for each data point. A test-agent response in an experiment is positive if both the induced MF for any treatment meets or exceeds the GEF and a positive trend test is obtained. A test-agent response is clearly negative if both the trend analysis and the GEF are negative. Occasionally, either (but not both) the GEF or the statistical analysis may indicate a positive response and these situations should be evaluated on a case-by-case basis. It is generally advisable to conduct one or more additional experiments to better define the assay response (particularly in the 30–10% RTG cytotoxicity range).

In approaching the evaluation of MLA experiments the following points should be borne in mind:

1. **Does the experiment meet all assay acceptance (quality control) criteria?** If it does not, the experiment should be declared invalid and repeated. This is particularly important if the data appears to be negative. 'Negative' experiments that do not meet the assay acceptance criteria should not be included in the overall evaluation of a chemical test article.
2. **Has the cytotoxicity range of the test article been adequately covered?** Generally this requires the testing of test-agent concentrations that cover the range between 100 and 10% RTG. A more detailed set of recommendations is reported in Moore *et al*. (2002).
3. **Is the observed response positive, negative, or equivocal?** Lastly, determine whether the data from a single experiment is definitive or whether additional experiments are required to confirm the result. It is generally necessary to adjust the dose selection for any confirmatory experiment(s), particularly when the result is equivocal (see Moore *et al*., 2002).

As ever, biological relevance should be a major factor in data evaluation and it is recommended that all studies be considered in their entirety before reaching a final evaluation of a test agent.

3.2.2.2.12 Chromosome Aberration Assay

Chromosome aberrations and related events are associated with many human genetic diseases and there is substantial evidence that chromosomal damage is also associated with the process of carcinogenesis, both in humans and experimental animal models (see above). The chromosome aberration (CA) assay detects structural chromosome aberrations in cultured mammalian cells (Preston *et al*., 1981), generally using traditional cytogenetic methods and light microscopy. The test is used to screen for possible mammalian mutagens and carcinogens and may be used as an alternative to the MLA in the standard genotoxicity test battery (**Figure 7**). Briefly, the test involves exposing cell cultures to a test article both with and without metabolic activation. At predetermined intervals that are specific for each cell type (representing ~1.5 cell cycles), the cell cultures are treated with a metaphase-arresting substance (e.g. Colcemid® or colchicine), harvested, stained, and metaphase cells are analysed microscopically for the presence of chromosome aberrations. The methodology describing the conduct of the test can be found in OECD (1997b), and is explained in detail in **Cytogenetics**.

An agent that produces structural changes in chromosomes is known as a clastogen, and the term clastogenicity refers to the capacity to induce structural chromosomal aberrations. Structural aberrations may be of two types, chromosome or chromatid, and many compounds that are positive in this test are mammalian carcinogens; however, there is not a perfect correlation between this test and carcinogenicity (see Section 4 below). The mechanisms by which the different types of structural aberrations arise are described in more detail in **Cytogenetics**. Studies may be conducted in established cell lines or primary cell cultures. For example, the CHL (Chinese hamster lung) (Ishidate and Sofuni,

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1985) and CHO (Chinese hamster ovary) (Galloway *et al*., 1987) rodent cell lines are routinely used. The cells are selected on the basis of growth ability in culture, stability of the karyotype, chromosome number, chromosome diversity and spontaneous frequency of chromosome aberrations. The use of human peripheral blood lymphocytes is also recommended because these cells maintain a stable karyotype as short-term (primary) cultures (Evans, 1976), and there is increasing evidence to suggest that the use of human cell lines reduces the frequency of irrelevant positive results (Kirkland *et al*., 2007). Increases in numerical chromosome aberrations, particularly polyploidy, can be detected but the assay is not specifically designed to evaluate aneuploidy or polyploidy. Indeed, it has been considered that an increase in polyploidy may indicate that a chemical has the potential to induce numerical aberrations, as the majority of definitive aneugens evaluated as part of a European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) research programme induced polyploidy *in vitro* (Aardema *et al*., 1998). However, particularly *in vitro*, polyploidy is induced by many types of insult and the end point does not necessarily reflect a potential for aneuploidy (Mitchell *et al*., 1995; Galloway, 2000). Therefore, positive results for *in vitro* polyploidy have to be interpreted with much caution (Müller *et al*., 1999). Indeed, in a recent study by Muehlbauer *et al*. (2008), the polyploidy end point was shown not to be a reliable indicator for aneugenic potential in structural CA assays in lymphocytes, as it clearly over-predicts the frequency of truly aneugenic compounds.

3.2.2.2.13 Micronucleus Test

The *in vitro* micronucleus test is a genotoxicity assay for the detection of micronuclei (MN) in the cytoplasm of interphase cells grown in cell culture, first described by Countryman and Heddle (1976). MN present in interphase cells can result from acentric chromosome fragments (i.e. those lacking a centromere) or whole chromosomes that are unable to migrate to the poles during mitosis, but only in cells that have undergone cell division during or after exposure to a test article. The predominant induction of either type of micronucleus can be used to classify chemical activity according to the underlying mechanism of action (MOA), that is, aneugenicity (chromosome loss) or clastogenicity (structural chromosome aberrations). The convincing demonstration of the presence of whole chromosomes in MN has been made possible through the availability and application of suitable molecular cytogenetic technologies. For example, the combination of centromeric and telomeric probes using fluorescence *in situ* hybridization (FISH) (Doherty *et al*., 1996) or kinetochore probes using immunocytochemical labelling of kinetochore proteins (Lynch and Parry, 1993) for the detection and quantification of chromosome loss, and chromosome-specific centromere probes for the detection and quantification of nondisjunction has now been successfully used to analyse the aneugenic activity of an extensive range of chemicals. Thus, the *in vitro* MN test can also provide information on the mechanisms of chromosome damage and micronucleus formation, and many compounds that are positive in this test are mammalian carcinogens (for reviews see: Parry and Sorrs, 1993; Kirsch-Volders, 1997; Parry and Parry, 2006; Fenech, 2007). However, like the chromosome aberration test there is not a perfect correlation between the *in vitro* MN test and carcinogenicity (Kirkland *et al*., 2005; 2006; Matthews *et al*., 2006a; 2006b).

Briefly, the test involves exposing cell cultures to a test article both with and without metabolic activation. At predetermined intervals that are specific for each cell type, the cell cultures are harvested, stained, and interphase cells are analysed for the presence of MN using microscopy. MN represent damage that has been transmitted to daughter cells, whereas chromosome aberrations scored in metaphase cells may not be transmitted. Because MN in interphase cells can be assessed relatively objectively, laboratory personnel need only determine whether or not the cells have undergone division and how many cells contain an MN. As a result, the preparations can be scored relatively quickly and analysis can be automated by flow cytometry (Bryce *et al*., 2008; Collins *et al*., 2008a) and/or image analysis (Diaz *et al*., 2007). This makes it practical to score thousands instead of hundreds of cells per treatment as in the *in vitro* chromosome aberration test, increasing the statistical power of the assay. The *in vitro* MN test has been used in a variety of established rodent cell lines (CHO, V79, CHL, and L5178Y) and in primary human lymphocytes. Protocols have been described with and without the use of the actin polymerization inhibitor cytochalasin B, which inhibits cytokinesis, resulting in the formation of binucleated cells (Fenech and Morley, 1986). The addition of the cytochalasin B prior to the targeted mitosis allows for the identification and selective analysis of MN frequency only in those cells that have completed one mitosis, because such cells are binucleate (reviewed by Fenech, 2007). The *in vitro* MN test has been used in academia and industry for hazard identification as an alternative/replacement of the *in vitro* structural chromosome aberration test, or as a back-up test to elucidate MOA (aneugenicity vs. clastogenicity) for risk assessment purposes (see Section 4 below). The test has already gained widespread international interest, as it offers significant advantages over the assessment of chromosome aberrations, since slide reading is more objective and quicker, resulting in a much higher throughput. Metaphase analysis, by contrast, is very tedious, time consuming and has a low throughput. A limitation of the test is that the assay does not provide information about the types of structural chromosome aberrations and/or polyploidy.

In the past decade, several studies have been performed to support the validity of the *in vitro* MN test for incorporation into the standard regulatory genotoxicity test

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battery as an alternative to the *in vitro* structural chromosome aberration test. These include, in particular, the international validation studies coordinated by the Société Française de Toxicologie Génétique (SFTG) (Aardema *et al*., 2006; Clare *et al*., 2006; Lorge *et al*., 2006; Oliver *et al*., 2006; Wakata *et al*., 2006) and the reports of the International Workshop on Genotoxicity Testing (IWGT) (Kirsch-Volders *et al*., 2000b; 2003a). A high correlation (>85%) between the *in vitro* structural chromosome aberration test and the *in vitro* MN test has also been observed (Miller *et al*., 1997; 1998). More recently, a formal validation for the test has been carried out by the European Centre for the Validation of Alternative Methods (ECVAM). This consisted of a retrospective validation of the existing data (reviewed by Corvi *et al*., 2008), which concluded that the *in vitro* MN test is reliable and a relevant genotoxicity assay that can be used as an alternative to the *in vitro* structural chromosome aberration test. Indeed, recent revisions of international regulatory guidances, such as the new European Chemicals Regulation, REACH (registration, evaluation, authorization of chemicals) and ICH2 for pharmaceuticals (see below) have recommended the adoption of the *in vitro* MN test as an alternative to the structural chromosome aberration assay and/or MLA. A draft OECD guideline for the conduct of *in vitro* MN test (TG 487) for regulatory purposes is currently under review and is likely to be adopted in 2009. It remains to be seen, however, how quickly the test is accepted by regulatory authorities as an alternative system in the standard test battery.

The methodology describing the conduct of the *in vitro* MN test and the mechanisms by which MN arise are described in more detail in **Cytogenetics**.

3.2.3 In Vivo Assays

3.2.3.1 The Rodent Micronucleus Assay

The micronucleus assay (Matter and Schmid, 1971; Heddle, 1973; Schmid, 1975) is an *in vivo* test that detects structural (clastogenicity) or numerical (aneuploidy) chromosomal damage that arises from acentric chromosomal fragments or lagging whole chromosome(s) that fail to incorporate into daughter nuclei after nuclear division in proliferating somatic cells. MN are well-characterized biomarkers of genetic damage that are commonly evaluated in lymphocytes and erythrocytes due to the ease of obtaining blood samples. The target cell in the bone-marrow micronucleus test is the developing erythroblast. During the formation of enucleated erythrocytes, at the final cell division of the erythroblast, the nuclear contents fragment in a process termed karyorrhexis, in a process similar to apoptosis (Hristoskova, 2004). The fragments are collectively expelled from the cell, contained in a plasma membrane. This is an efficient process, but occasionally some material is left behind in the immature erythrocyte. In haematology, these fragments are termed Howell-Jolly bodies, and constitute at least a part of the background frequency of MN seen in cells from untreated animals (the other component arising from spontaneous structural and numerical chromosomal aberrations).

The rodent *in vivo* micronucleus assay, using either bone marrow or peripheral blood, is often the default assay for regulatory genetic toxicology assessment *in vivo*. The test methodology, which is described in detail in **Cytogenetics** and **Genetic Toxicology Testing and its Relevance to Human Risk and Safety Evaluation**, should be conducted in accordance with the general principles of current national and international regulatory guidelines (OECD, 1997c; ICH S2A: Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals, 1996; ICH S2B: A Standard Battery for Genotoxicity Testing of Pharmaceuticals, 1997; Richold *et al*., 1990).

Experience has shown that the assay has proved useful for the detection of agents that induce chromosomal damage as:

- the end point is easy to score,
- it provides good statistical power as many cells can be scored for the presence of MN, and
- there is evidence that chromosomal breakage always leads to micronucleus formation; thus, if the micronucleus test is negative (and the compound and/or its metabolites can be shown to reach the bone marrow), chromosomal aberrations in this tissue can be ruled out.

The converse, however, is not always true, since indirect physiological mechanisms, for example the stimulation of haematopoiesis or decreases in core body temperature, can also result in small increases in micronucleus frequency in the bone marrow, above control values, and these may sometimes account for a positive micronucleus assay. Thus it is important to exclude the possibility of nonrelevant mechanisms which do not indicate genotoxic hazard when considering whether or not a test agent has induced chromosomal breakage, or indeed whether or not the micronucleus assay is the most appropriate end point for assessment (Tweats *et al*., 2007).

Nevertheless, the rodent bone marrow micronucleus test is perceived as having relatively high specificity for the detection of genotoxic carcinogens; that is, false positives are apparently rare, although the sensitivity is only considered moderate, in that not all genotoxic agents or genotoxic metabolites necessarily reach the bone marrow in sufficient quantities to produce a detectable genotoxic response. Thus, with the regulatory genotoxicity test batteries currently in use, the results of *in vivo* genotoxicity tests play a vital role in risk and hazard assessments. Increases in micronucleated bone-marrow cells are given great regulatory weight, indicating that the test compound is likely to be a genotoxic carcinogen.

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3.2.3.2 *Unscheduled DNA Synthesis Assay*

The unscheduled DNA synthesis (UDS) assay, using primary rat hepatocytes, was first developed by Professor G. Williams (1976; 1977), and the method provides a means of assessing test-agent-induced nuclear DNA excision repair, as determined by the amount of radiolabelled (usually tritium) thymidine incorporated into the nuclei of cells that are not in S-phase (Rasmussen and Painter, 1966). Thus, the UDS assay provides indirect evidence of the DNA-damaging ability of a test agent, as it is assumed that the induction of DNA repair would have been preceded by DNA damage. The UDS assay can be performed either *in vitro* or *in vivo/in vitro*; however, the UDS assay does not, in itself, indicate if a test agent is mutagenic, because it provides no information regarding the fidelity of DNA repair, nor does it identify DNA lesions repaired by mechanisms other than DNA excision repair (see Section 2.3 on DNA repair).

In July 1997, the OECD guideline for the *in vivo* UDS assay was ratified (OECD, 1997d). The UDS assay is usually conducted using rats, although other species may be used if justified. OECD methodology recommends that two dose levels are selected on the basis of preliminary toxicity testing, with the highest dose defined as the maximum tolerated dose (MTD). At least three animals per group (typically males only) are administered the test agent (generally once, by gavage), and each assay generally consists of three test-article doses, one vehicle control and one positive control. According to the OECD guideline, two sampling times should be used. For the early (2–4 hours) sampling time only one positive control (*N*-nitrosodimethylamine, (NDMA)) is recommended. The second group of animals is sacrificed 12–16 hours after treatment. The liver is generally used for analysis because, under normal circumstances, there is a low proportion of hepatocytes in S-phase, although theoretically, any tissue with a low proportion of replicating cells may be used. The liver is also the site of first-pass metabolism for chemicals administered orally or by intraperitoneal injection, although the latter is not a recommended route of exposure in the UDS assay because it could potentially expose the liver directly to the chemical.

Cultures of hepatocytes are prepared and incubated in medium containing tritiated thymidine (for ~18 hours) and slides are prepared and processed for autoradiography using standard techniques (Butterworth *et al*., 1987; Kennelly *et al*., 1993). At least 100 cells per animal are examined, and both nuclear and cytoplasmic grains should be counted to determine the net nuclear grain count (i.e. cytoplasmic grains subtracted from nuclear grains). Chemicals inducing a significant increase in mean net nuclear grain count for at least one treatment group are considered to have induced UDS and are therefore deemed positive (**Figure 11**). Additional recommendations for the performance of UDS assay *in vitro* and *in vivo* are reviewed by Madle *et al*. (1994). Although the *in vivo* UDS assay protocol takes into account the pharmacokinetic and pharmacodynamic properties of a test agent when assessing its genotoxicity, contemporary regulatory experience suggests that the UDS assay is relatively insensitive to all but the most potent genotoxins (Kirkland and Speit, 2008). As such, the rat-liver UDS assay has all but fallen from favour in recent times and is no longer considered the default second *in vivo* assay in the context of most modern regulatory genotoxicity testing regimens. Nevertheless, the UDS assay may still be useful as a follow-up assay to investigate putative genotoxic agents that require metabolic activation as the liver is the target organ.

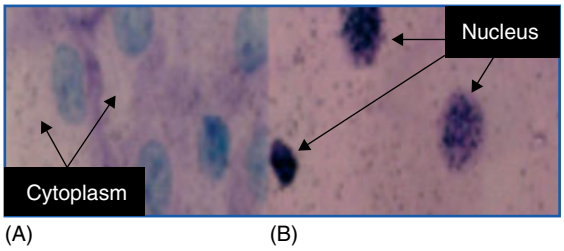

Figure 11 Unscheduled DNA synthesis assay. (A) Control liver; (B) treated liver (note significant net grain counts present in nucleus).

3.2.3.3 *Comet Assay*

The Comet assay (also known as the single cell gel electrophoresis assay) is a simple, rapid and sensitive technique for measuring and analysing DNA strand breakage within individual mammalian cells. The methodology, originally developed by Ostling and Johanson (1984) and modified by Singh *et al*. (1998), combines the simplicity of biochemical techniques for detecting DNA single-strand breaks (strand breaks and incomplete excision repair sites), alkali-labile sites and DNA crosslinks, with the single-cell approach typical of cytogenetic assays, and can be applied to both *in vitro* and *in vivo* systems (recently reviewed by Collins, 2004; Collins *et al*., 2008b).

The Comet assay is based on the ability of negatively charged loops/fragments of DNA to be drawn through an agarose gel in response to an electric field. The degree of DNA migration can be correlated to the extent of DNA damage induced within the cells (Olive *et al*., 1990). The profile of the cells and the degree of DNA migration may be scored on a photomicrograph or captured and examined using an image analysis system. The resulting images look like a 'comet', with a distinct head, comprised of intact DNA, and a tail, consisting of damaged or broken pieces of DNA; hence the name Comet assay (**Figure 12**). The use of alkaline conditions of pH >13 enables alkaline-labile sites as

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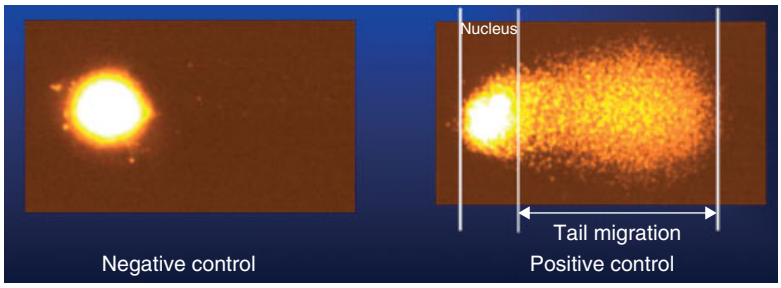

Figure 12 Comet assay. (A) Cell from control liver showing intact nucleus; (B) treated liver (note migration of DNA fragments to form comet tail).

well as single-strand and double-strand DNA breaks to be expressed during electrophoresis, thus increasing the sensitivity of the assay (Singh *et al*., 1998).

The main advantages of the Comet assay include: (i) the collection of data at the level of the individual cell, allowing more robust statistical analyses; (ii) the need for only a small number of cells per sample; (iii) sensitivity for detecting DNA damage; and (iv) use of any eukaryote single cell population both *in vitro* and *in vivo*, including cells obtained from exposed human populations and aquatic organisms for ecogenotoxicological studies and environmental monitoring (Collins *et al*., 2001; Dixon *et al*., 2002; Jha, 2004). In combination with the fluorescence *in situ* hybridization techniques (Comet-FISH), the application of this assay has also been extended to determine sequence- or gene-specific damage and repair (McKenna *et al*., 2003), as well as being of possible diagnostic use (Kumaravel and Bristow, 2005). In addition, the assay is being used in translational research to assess whether tumour radio-sensitivity (Fisher *et al*., 2007) and chemo-sensitivity (Smith *et al*., 2007) can be determined. This would allow clinicians to tailor the management of individual patients, adjusting cancer therapy to those for whom it will be of most benefit and reducing the likelihood of patients receiving toxic, and as such, ineffective therapy (McKenna *et al*., 2008).

The Comet assay has demonstrated potential as a powerful tool for assessing chemically induced DNA strand breaks in a wide range of tissues (Sasaki *et al*., 2000). In this capacity, the method has distinct advantages over conventional *in vivo* mutagenicity tests, such as the rodent bone marrow micronucleus test or the liver UDS test. For example, the Comet assay is recommended by the UK COM as an appropriate test for assessing the genotoxic potential of short-lived reactive mutagens at their site of contact or as a supplementary *in vivo* test for investigation of genotoxicity in tissues other than the bone marrow (COM, 2000). In these circumstances, the bone marrow or liver may not be the first tissue exposed to the test article or may not be the target for a particular test agent. As such, the Comet assay provides an important alternative for the identification of site-of-contact clastogens and/or for those compounds that act in organs other than the bone marrow or liver. Moreover, in a recent review of appropriate follow-up testing *in vivo*, Kirkland and Speit (2008) reported that the Comet assay detected almost 90% of bone marrow micronucleus test-negative or equivocal carcinogens and concluded that the Comet assay should play a more prominent role in regulatory testing strategies than the UDS test.

At present there are no OECD guidelines available for the Comet assay and the use of the *in vivo* Comet assay for evaluation of genetic toxicity within a regulatory framework is still under evaluation, although an international interlaboratory ring trial is underway to develop a validated assay for OECD ratification under the auspices of JaCVAM (Japanese Center for the validation of alternative methods). Moreover, recent revisions of international regulatory guidances such as ICH2 for pharmaceuticals (see below) have recommended the Comet assay as alternative to the UDS assay as a follow up second *in vivo* assay to the rodent bone marrow micronucleus test. Protocols that have been designed in accordance with the recommendations of the IWGTP *in vivo* Comet assay workgroup should be adopted (Tice *et al*., 2000; Burlinson *et al*., 2007) and detailed protocols may be found in the literature (Fielder *et al*., 1993; Lovell *et al*., 1999; Hartmann *et al*., 2003; 2004) or on the Comet assay web site (*www.cometassay.com*).

3.2.3.3.1 The Comet Assay: Practical Aspects

In the *in vivo* Comet assay, a minimum of three animals per group (typically males only) are administered the test agent, generally once, by gavage, although multiple-dosing regimens, usually 24 hours apart, can be used if justified. In the author's laboratory, the *in vivo* Comet assay is usually conducted in rats, although other species, such as mouse, have been used on occasion. The degree of DNA damage within specific tissues or cells may be investigated in any tissue or organ from which single-cell suspensions can be prepared. Typically these are the liver, stomach, bone marrow and peripheral blood lymphocytes. The tissues are usually sampled 2–6 and 16–26 hours after the last dose, but when the protocol involves multiple dosing only the 2–6 hour sample

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is required. Briefly, cell suspensions are embedded in agarose gel on glass microscope slides and treated with a combination of detergent and high-salt solution to rupture the cell membranes and cause cell lyses, extract the nuclear proteins and leave the supercoiled DNA in a nucleus-type structure, called the nucleoid. Subsequently, the supercoiled DNA is left to relax and 'unwind' in a strongly alkaline buffer. As DNA carries a net negative charge, the nonsupercoiled loops and single-strand fragments migrate towards the anode on electrophoresis whilst DNA strand breaks result in further migration into the gel. A discussion on electrophoresis conditions may be found in the review by Collins *et al*. (2008b). Following electrophoresis, the DNA is neutralized and stained using a nucleic acid-specific dye, such as propidium iodide. In the author's laboratory, the cells are visualized and quantitated using fluorescence microscopy linked to an image analysis system.

The extent of DNA migration maybe scored in three ways (manually or using customized imaging software):

- **Tail length:** defined as a measurement from the point of greatest intensity within the comet head, to the end of the fluorescing tail.
- **Tail % intensity (% tail DNA):** defined as the percentage of the total DNA present within in the comet tail.
- **Tail moment:** defined as the product of the tail length and the fraction of total DNA present within the comet tail (i.e. % tail DNA).

According to Collins *et al*. (2008b), % tail DNA covers the widest range of damage and is linearly related to break frequency across most of the range (based on calibration experiments with X-ray or gamma irradiation where ~0.2–10 Gy equivalents roughly produce 0.06–3 breaks per 10^9 daltons (i.e. one hundred to several thousand breaks per human cell). Therefore, tail intensity is recommended as the parameter of choice.

3.2.3.3.2 Cytotoxicity Assessment

It has been well recognized that comet images resulting from excessively damaged cells, that is those that are necrotic or apoptotic, are characterized by small or nonexistent heads and large diffuse tails; these are sometimes referred to as 'ghost' comets (Fairbairn *et al*., 1995). Cell death is associated with increased levels of DNA strand breaks, and therefore high levels of cytotoxicity can confound the interpretation of the Comet assay. In the author's laboratory, therefore, sections of tissue are reserved for the histopathological examination of necrosis and apoptosis. These are analysed only when a test article is considered positive in the Comet assay in order to exclude any potentially confounding cytotoxic doses that may affect interpretation of the data. Further information regarding viability is discussed by Collins *et al*. (2008b).

3.2.3.4 Transgenic Mutation Assays

Several transgenic rodent (TGR) (mouse and rat) mutation assays have been developed during the past two decades to study mutagenesis *in vivo*. A comprehensive review of transgenic rodent gene mutation (Tg-GM) assays and their potential use in a regulatory context has recently been published by Lambert *et al*. (2005). Notes on guidance for routine testing have also been defined by the IWGTP and are described by Thybaud *et al*. (2003). The performance of Tg-GM assays with respect to predicting rodent carcinogens has recently been reviewed (Kirkland and Speit, 2008) and these authors concluded that Tg-GM assays should play a more prominent role in regulatory testing strategies as appropriate follow-up testing *in vivo*.

3.2.3.4.1 The Development of Transgenic Rodent Gene Mutation (Tg-GM) Assays

Compared to existing regulatory assays for measuring *in vivo* genotoxicity, which are generally limited to bone marrow, peripheral blood and liver, Tg-GM assays provide a significant advance in our ability to rapidly assess spontaneous and test-agent-induced mutations *in vivo* in virtually any tissue or organ, and they allow comparative studies between them. Tg-GM assays close the 'gene mutation–chromosome aberration' paradigm *in vivo*, and furthermore, these assays provide a relatively simple means to analyse mutations at the molecular level. This can supply mechanistic information about mutagens; for example, in cases where a chemical agent causes only a marginal increase in mutation frequency, a unique mutation spectrum may determine whether the agent is positive or negative. Investigations using repeat doses may provide data with immediate relevance to carcinogenicity bioassays, and, moreover, they may also provide a novel opportunity for studying the effects of nongenotoxic carcinogens on endogenous mutation induction in target tumour tissues. Finally, they may improve on current methods for the assessment of heritable damage in germ cells (historically determined by the specific-locus and dominant lethal (DL) tests) and, therefore, they have the potential for reducing animal usage.

All of the available Tg-GM animal models have been genetically engineered on the common principle of using specific prokaryotic 'reporter' genes stably inserted into the host animal genome. These transgenes can be easily recovered from target tissues, using various shuttle vector technologies or gene capture, and analysed for mutations in suitable bacterial assays. The prokaryotic transgenes are not transcribed and are nonpathogenic to the host animal. A general outline of the steps involved in mutation analysis in Tg-GM assays is shown in **Figure 13**.

3.2.3.4.2 Mouse Models and Reporter Transgenes

The following section describes the animal strains, the reporter transgenes and basis for mutation detection used in the various TGR models developed for gene mutation

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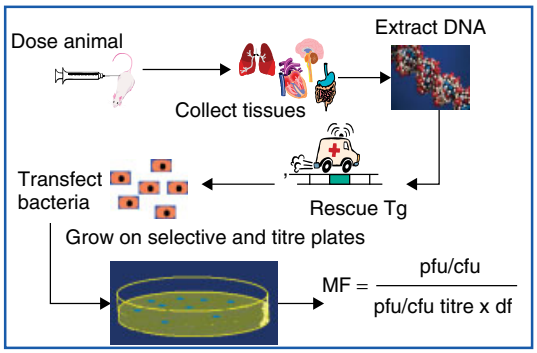

Figure 13 Gene mutation analysis in transgenic assays. pfy—plaque forming units; cfu—colony forming units; df—dilution factor.

analysis *in vivo* to date. Two Tg-GM animal models (Muta™Mouse and BigBlue®) are sold commercially, however, only Muta™Mouse is currently available in the UK. As with any other rapidly changing scientific endeavour, it is likely that new 'second generation' Tg-GM models will be proposed and developed in the future, for example through crossing separate transgenic strains to yield 'double transgenic' animals.

3.2.3.4.3 Muta™ Mouse

Originally described by Gossen *et al.* (1989), this was the first transgenic strain developed for the analysis of gene mutations *in vivo*. This system features genomic integration of a bacteriophage λ shuttle vector containing the bacterial *lacZ* gene. The λgt10-*lacZ* shuttle vector is approximately 47 kb in length and is flanked by *cos* sites. Within the Muta™Mouse genome, the λgt10-*lacZ* shuttle vector resides as a concatamer of approximately 40 copies (Blakey *et al.*, 1995), which is located on chromosome 3 (Swiger *et al.*, 1994). After isolation of genomic DNA from the transgenic organs/tissue of interest, the λ genomes are excised by, and packaged with, a bacteriophage λ packaging extract. The resulting phage particles are then used to transfect a lacZ $^{(del)}$ *E. coli* strain in the presence of X-gal. This produces blue plaques on a bacterial lawn for phage with wild-type *lacZ* and clear plaques for mutant *lacZ* following overnight incubation. In addition, the exact sequence alteration in the *lacZ* transgene can be determined by DNA sequencing.

This system has been improved significantly by using a selection assay in lieu of colour screening. This makes it possible to assay large numbers of phage particles, whereby only mutant particles form plaques (Dean and Myhr, 1994). This is based on the ability of LacZ to metabolize phenyl-β,D-galactoside (P-gal) into galactose. In *gal E* host cells (carrying a plasmid containing the *gal T* and *gal K* genes), this accumulates as UDP-galactose, which is toxic to the cells. However, phage particles with mutant *lacZ* are able to transfect the bacterial host cells and form plaques.

The genotoxic carcinogen, B(a)P has been shown to induce gene mutations in various organs (forestomach, spleen, colon and glandular stomach) in Muta™Mouse when given orally at doses of 75 and 125 mg kg^{-1} body weight day^{-1} for five consecutive days (Hakura *et al.*, 2000). These B(a)P doses were also shown to be carcinogenic in Muta™Mouse (Hakura *et al.*, 1998).

3.2.3.4.4 BigBlue®

First described by Kohler *et al.* (1991), this model utilizes the bacterial *lacI* gene integrated into a bacteriophage λ shuttle vector, as a target reporter gene. The construct is present in approximately 40 copies per cell genome, integrated at a single locus in a head-to-tail arrangement. The B6 C3F1 mouse strain was the first to be engineered, that is, the same genetic background as used in the National Toxicology Program. In addition, a transgenic BigBlue® rat and rat cell line have been constructed (Dycaico *et al.*, 1994).

After isolation of genomic DNA from a tissue, the λ genomes are excised by, and packaged with, a bacteriophage λ packaging extract. When used to transfect *E. coli lacZ* bacteria grown on X-gal LB-plates, phage particles with a wild-type *lacI* gene will produce a clear bacteriophage plaque, while those that have sustained a mutation in the *lacI* gene will produce a blue plaque. The ratio of blue plaques to clear plaques determines the MF. In addition to the determination of mutant frequencies, the exact sequence alteration can be determined by DNA sequencing.

A BigBlue® hemizygous p53$^{+/-}$ double transgenic mouse strain is available (Taconic, Germantown, NY) which permits the analysis of gene mutations in a short-term cancer model.

3.2.3.4.5 Gpt-Δ

First described by Nohmi *et al.* (1996), this assay is useful for the simultaneous detection of point mutations and deletion mutations. This system uses a bacteriophage λ EG10 construct with integrated plasmid pYG144. The plasmid has the *E. coli gpt* gene flanked by two loxP sequences. When the rescued phages are transfected into *E. coli* YG6020 expressing Cre recombinase, the phage DNA is converted into plasmid pYG142 carrying the chloramphenicol resistance gene and the *gpt* gene, which is sensitive to point mutations. The *gpt* mutants can be positively detected as colonies when grown on plates containing chloramphenicol and 6-thioguanine (6-TG). The EG10 DNA carries a Chi site along with *red* and *gam* genes, so that wild-type phages display Spi+ (sensitive to P2 interference) phenotype. Mutant phages lacking both the *red* and *gam* genes (i.e. Spi−), can be positively selected as plaques that grow in P2 lysogens of *E. coli*, such as XL1 Blue MRA (P2) cells.

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3.2.3.4.6 SupF

A transgenic model carrying the *supF* amber suppressor tyrosine tRNA gene as a reporter was developed by Leach *et al*. (1996). The *supF* gene was selected because the tRNA gene offers a target sensitive to mutations at almost every base pair, it has a short coding sequence (85 bases) which makes it highly amenable to DNA sequencing, and there is an extensive *supF* mutagenesis database. To control for locus-specific effects, three independently derived transgenic strains were created, each containing approximately 80–100 copies of the *supF* gene per cell genome integrated in a head-to-tail arrangement at different loci.

After isolation of genomic DNA from a tissue, the λ supF genomes are excised and packaged into phage. These are used to infect *E. coli* C1a lacZ125 (am) cells grown on X-gal plates. Phage particles with a wild-type *supF* gene produce blue plaques, while those with a mutation in the *supF* gene will produce clear plaques. Mutation frequency is determined by the ratio of clear plaques to blue plaques. Detection is possible because mutants in the *supF* gene do not suppress amber mutations in the *lacZ* gene in the host cell. Again, the exact DNA sequence alteration can be determined by DNA sequencing.

3.2.3.4.7 λ cII

The use of the bacteriophage lambda cII gene as a mutational target in transgenic animals was first described by Jakubczak *et al*. (1996). This system uses the *cII* gene in a forward, positive-selection system and can be used with all transgenic systems based on the integration of bacteriophage λ, for example BigBlue® (Papp-Szabó *et al*., 2003) and Muta™Mouse (Smith *et al*., 1999).

The cII protein induces the expression of the *cI* and the integrase genes. The products of these genes are required for integration of the λ DNA into the bacterial genome, and this results in lysogeny. A mutation in the *cII* gene results in growth of λ particles and lysis of the cell. The cII gene is only 294 base pairs long and provides a convenient target for analysis of mutations by DNA sequencing.

3.2.3.4.8 LacZ Plasmid Assay

The development of a lacZ plasmid-based transgenic mouse model, which uses gene capture to rescue the reporter transgene, has been reported by Boerrigter *et al*. (1995).

Isolated genomic DNA from transgenic tissue is digested with *Hin*dIII restriction enzyme to release the pUR288 plasmid. The vector is purified and enriched by gene capture using LacI repressor magnetic beads, and the plasmids are circularized by T4-ligation. Competent $lacZ^{(del)}$ bacteria are transformed by electroporation, and lacZ mutants are grown using a positive-selection assay similar to that used in the Muta™Mouse assay. Mutant clones can be picked to make plasmid preparations and further characterized by Southern analysis following electrophoresis, or by sequencing the *lacZ* gene. Analysis of spontaneous and X-ray (5×0.5 Gy)-induced mutant *lacZ* genes showed that 40–50% of all mutations were caused by large deletions. The possibility of detecting a broad spectrum of mutations in the lacZ plasmid-based transgenic mouse is an obvious advantage for studying *in vivo* mutagenesis.

3.2.3.5 Performance of Tg-GM Assays and Regulatory Use

Bacteriophage-based Tg-GM assays such as Muta™ Mouse and BigBlue® have been shown to yield very low responses to clastogenic (chromosome-breaking) agents such as methyl methane sulfonate (MMS) and mitomycin-C, which predominantly induce large deletions in DNA, and there is concern that Tg-GM assays may be insensitive to the detection of such mutations (Suzuki *et al*., 1993; 1997; Lynch *et al*., 2008a). This is most likely due to the fact that phage particles are unable to package vectors less than 40 kb or greater than 52 kb in size (Gossen *et al*., 1995) and therefore vectors containing DNA deletions of 5–10 kb or more cannot be rescued from genomic DNA. Thus, if the majority of DNA lesions induced by a test article are a result of large DNA deletions (i.e. >10 kb), then it is unlikely that they would be detected in any Tg-GM assay.

3.2.3.6 Other In Vivo Mutation Tests

Insect tests, such as those in the fruit fly *Drosophila melanogaster*, for example the sex-linked recessive lethal test and the somatic mutation and recombination test (SMART) assays which include (i) the wing mosaic system and (ii) white eye/white-coral eye mosaic system (reviewed by Wurgler *et al*., 1984; de Mitchell and Combes, 1984) are no longer widely used in commercial genotoxicity testing. The same is true for the majority of *in vivo* mammalian tests developed in the 1950s and 1960s such as the mouse somatic spot test (Russell and Major, 1957; Russell, 1984) and the mouse specific locus (SL) test (Russell, 1951; Carter *et al*., 1956; Russell *et al*., 1989), although the dominant lethal (DL) test (Bateman, 1966; Holmstrom *et al*., 1993) is still an option cited in some regulatory guidelines, for example ICH. The DL test has been recommended for use in a strategic sense, to determine whether a test agent which is genotoxic in somatic cells is also genotoxic in germ cells.

The rodent assays enjoyed popularity in the 1960–1980s and were included as part of the GENETOX program (see **Box 2**) of reports (*http://toxnet.nlm.nih.gov*), but they have fallen from favour in more recent times. This is mostly due to the large numbers of animals required and also the perceived lack of sensitivity of the end points measured. Although still of interest to the academic geneticist, they are no longer considered relevant to modern standard regulatory genotoxicity testing regimens and are not commercially

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available. However, it is conceivable that a DL test could still be requested by a regulatory agency if there was sufficient concern regarding the potential for germ cell mutagenesis.

Box 2 The GENETOX program

The US Environmental Protection Agency's GENETOX program is a multiphased effort to review and evaluate the existing literature in assay systems available in the field of genetic toxicology. The first phase of the GENETOX program selected assay systems for evaluation, generated expert panel reviews of the data from the scientific literature and recommended testing protocols for the systems. Phase II established and evaluated the database of chemical genetic toxicity data for its relevance to identifying human health hazards. The ongoing phase III continues reviewing and updating chemical data in selected assay systems. Currently, data exist on over 4000 chemicals in 27 assay systems. The review data are published in GENETOX, a toxicology data file of the National Library of Medicine's (NLM) Toxicology Data Network (TOXNET®). The review and analysis components of GENETOX comprise 45 published papers, and several others are in preparation.

3.2.3.7 Rodent Dominant Lethal Assay

A DL mutation is one occurring in a germ cell which does not cause dysfunction of the gamete, but which is lethal to the fertilized egg or developing embryo. Thus the DL effects cause embryonic or foetal death, and induction of a DL event after exposure to a test agent indicates that the substance has affected germinal tissue of the test species. DLs are generally accepted to be the result of chromosomal damage (structural and numerical anomalies) but gene mutations and toxic effects cannot be excluded.

Generally, rats or mice are used as the test species. Strains with low background dominant lethality, high pregnancy frequency and high implant numbers are recommended. Male animals are exposed to the test substance and mated to untreated virgin females. In a typical experiment, the number of males in each group is usually sufficient to provide between 30 and 50 pregnant females per mating interval. Concurrent positive (e.g. cyclophosphamide or ethyl methane sulfonate (EMS)) and negative (vehicle) control groups should be included. The various germ-cell stages can be tested separately by the use of sequential mating intervals. The females are sacrificed after an appropriate period of time and the contents of the uteri are examined to determine the numbers of implants and live and dead embryos.

The calculation of the DL effect is based on comparison of the live implants per female in the treated group to the live implants per female in the control group. The increase of dead implants per female in the treated group over the dead implants per female in the control group reflects the postimplantation loss. The postimplantation loss is calculated by determining the ratio of dead to total implants from the treated group compared to the ratio of dead to total implants from the control group. Preimplantation loss can be estimated on the basis of corpora lutea counts or by comparing the total implants per female in treated and control groups.

Several treatment schedules are available. The most widely used requires single administration of the test substance. Other treatment schedules, such as treatment on five consecutive days, may be used on a case-by-case basis if justified. Normally, three dose levels are used with the highest dose producing signs of toxicity (e.g. slightly reduced fertility and slightly reduced body weight) or limit dose ($2\,g\,kg^{-1}$) or limit of solubility.

Test performance: Individual males are mated sequentially at appropriate predetermined intervals to one or two virgin females. Females should be left with the males for at least the duration of one oestrus cycle or alternatively until mating has occurred as determined by the presence of sperm in the vagina or by the presence of a vaginal plug. The number of matings following treatment should be governed by the treatment schedule and should ensure that germ-cell maturation is adequately covered. Females should be sacrificed in the second half of pregnancy and uterine contents examined to determine the number of implants plus live and dead embryos. The ovaries may be examined to determine the number of corpora lutea.

Data reporting and analysis: Data should be tabulated to show the number of males, the number of pregnant females and the number of nonpregnant females. The results of each mating, including the identity of each male and female, should also be reported individually. For each female, the dose level and week of mating and the frequencies of live implants and of dead implants should be enumerated, with clear recording of early and late deaths. If preimplantation loss is estimated, it should be reported. Preimplantation loss can be calculated as the difference between the number of corpora lutea and the number of implants or as a reduction in the average number of implants per female in comparison with control matings.

Interpretation of results: There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of DLs. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points. A test substance which does not produce either a statistically significant

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dose-related increase in the number of DLs or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system. Both biological and statistical significance should be considered together in the evaluation.

A positive DL assay suggests that under the test conditions the test substance may be genotoxic in the germ cells of the treated sex of the test species. A negative result suggests that under the conditions of the test the test substance may not be genotoxic in the germ cells of the treated sex of the test species.

Several other rodent assays are capable of monitoring germline mutation. These include traditional assays such as the morphological SL test and the heritable translocation (HT) assay, and more modern assays such as the expanded simple tandem repeat (ESTR) and TGR mutation assays have been developed. The performance of these assays has recently been reviewed by Singer *et al*. (2006).

3.2.4 Supplementary Tests

3.2.4.1 Gene Reporter Assays

The 'traditional' regulatory genetic toxicology assays described above have one particular attribute in common; they detect the end product of a genotoxic event (for example, a gene mutation, DNA fragmentation, chromosome aberrations, etc.). The advent of mature molecular biology technologies in the 1990s coupled with an increased understanding of DNA damage and repair has led to the development of assays which allows genetic toxicologists to detect the cells' molecular response to genotoxic stress, which may be applied as a surrogate biomarker for the traditional end points.

The importance of the cellular response to genotoxic stress is exemplified by the discovery of a number of proteins encoded by key oncogenes and tumour suppressor genes that are central to the mammalian response to DNA damage (see above). Some of these proteins, such as p53, BRCA1, c-MYC and AP-1, are transcription factors with well-established roles in the response to genotoxins and in human carcinogenesis. Monitoring the activity of these pivotal genes, via gene and/or protein expression or through the expression of the downstream genes they transactivate or repress has the potential to identify genotoxic carcinogens, and possibly even nongenotoxic carcinogens. By coupling promoter sequences from these pivotal genes to reporter gene technology such as chloramphenicol acetyltransferase (CAT), LacZ, LUC and Green Fluorescent protein (GFP) it is possible to monitor the cellular response to various genotoxic stresses (Bullock and Gorman, 2000; Gorman *et al*., 1982; An *et al*., 1982; Alam and Cook, 1990; Prasher *et al*., 1992; de Wet *et al*., 1987). The standard regulatory genotoxicity tests are designed to detect and quantify rare genetic events, whereas gene reporter assays monitor the response of the entire population of cells exposed to a genotoxic stress. Reporter gene assays are not expected to take the place of the current regulatory assays; rather they complement them, allowing the genetic toxicologist to better understand the data they obtain from the regulatory assays (Todd *et al*., 1995; Walmsley, 2005; Hastwell *et al*., 2006). A key advantage of the reporter gene assays is the opportunity for these tests to be deployed early in new chemical entity development where the amount of compound is limited, and/or a large number of compounds are to be screened. (For a more detailed review of reporter genes the reader is directed to Alam and Cook, 2003).

3.2.4.1.1 Xenometrix CAT-Tox (L) assay

The CAT-Tox (L) assay consists of 14 recombinant cell lines derived from the human hepatoma cell line, HepG2, each stably transfected with a unique stress response gene promoter or synthesized consensus response element (**Table 9**) fused to the *CAT* reporter gene (Todd *et al*., 1995). Each of the 14 lines has been derived from genes that respond to a variety of cellular injuries whereas the parental cell line, HepG2, is included for the measurement of cytotoxicity. The CAT-Tox (L) assay provides a molecular profile of cellular stress and toxicity by measuring the activity of the 14 stress gene promoter–*CAT* fusions, and uses a 2×96-well microplate format which requires only milligram quantities of test compound; generally five concentrations are tested and results are available within 24–48 hours. Using the CAT ELISA system the fold induction of each of the *CAT* fusions maybe described as a three-dimensional histogram, a 'XenoMatrix'. Todd *et al*. (1995) have shown that the CAT-Tox (L) assay is able to detect subtle differences in mechanisms of action among closely related compounds.

3.2.4.1.2 The Yeast GreenScreen® GC (Genotoxicity-Cytotoxicity) Biosensor

Central to the GreenScreen® GC biosensor assay (Gentronix Ltd., Manchester, UK) is a RAD54-yEGFP DNA damage response reporter. This reporter utilizes the promoter of the yeast *S. cerevisiae* DNA-damage inducible gene *RAD54* to drive the production of the yEGFP (yeast enhanced GFP) reporter (Walmsley *et al*., 1997). The response to DNA damage and the induction of the *RAD54* promoter in yeast is well characterized (Tan *et al*., 1999). *RAD54* encodes a structural element of the HR repair pathway and is transcriptionally up-regulated in response to a broad spectrum of genotoxins. This assay has been designed as a high-throughput assay based on a 96-well plate format which requires only milligram amounts of compound, and results are available within ~16–20 hours (Cahill *et al*., 2004). Extensive validation studies demonstrate that the GreenScreen® GC assay exhibits high specificity (i.e. compounds with positive results are

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Table 9 Stress gene promoter–*CAT* fusion constructs used in the CAT-Tox (L) assay

Promoter–fusion constructs	Endogenous gene product	Function
CYP1A1	Cytochrome P450 IA1	Phase I biotransformation enzyme
GST Ya	Glutathione *S*-transferase *Ya* subunit	Phase II biotransformation enzyme
HMTIIA	Metallothionein IIA	Sequestration of heavy metals
FOS	*c-fos*	Member of *AP-1* transcription factor complex
XHF	Collagenase	Inflammatory mediator
HSP70	70 kDa heat shock protein	Helps to refold or sequester damaged proteins (protein chaperone)
GADD153	153 kDa growth arrest and DNA damage protein	Involved in cell cycle regulation and response to genotoxic agents
GADD45	45 kDa growth arrest and DNA damage protein	Involved in cell cycle regulation and response to genotoxic agents
GRP78	78 kDa glucose-regulated protein	Endoplasmic reticulum protein chaperone and also responds to DNA damage and calcium ionophores
Response element–fusion construct	**Name**	**Function**
XRE	Xenobiotic response element	Binding site for Ah receptor–planar aromatic hydrocarbon complexes
NF-κB-RE	NF-κB response element	Binding site for NF-κB transcription factor (oxidative stress)
CRE	cAMP response element	Binding site for CREB protein
p53RE	53 kDa protein tumour suppressor response element	Binding site for p53 transcription factor (DNA damaging agents)
RA-RE	Retinoic acid response element	Binding site for retinoic acid–retinoic acid receptor complexes

Adapted from Todd *et al*. (1995).

very likely to be genotoxic carcinogens), but relatively low sensitivity (Cahill *et al*., 2004; Van Gompel *et al*., 2005; Knight *et al*., 2007). This most likely reflects the differences between yeast and mammalian cells with regard to the cellular responses to genotoxic stress, and where in yeast the HR repair pathway is not induced in response to certain genotoxic events, such as aneuploidy. However, the GreenScreen® GC assay does cover a number of eukaryotic targets not present in bacterial cells (chromatin and DNA-metabolizing enzymes acting on chromatin, etc.), and therefore identifies some compounds missed by the Ames test. Knight *et al*. (2007) suggest that as part of early screening, a test battery of the Ames test ± S9 metabolic activation and a GreenScreen® GC assay would provide a firm basis to prioritize compounds

Table 10 Results of the standard genotoxicity assays for eight known rodent carcinogens that cause protein and DNA adducts

Compound	Adduct	Ames	*In vitro* mammalian tests		*In vivo* mammalian assay	
			Mouse lymphoma	Chromosome aberrations	Micronucleus	Chromosome aberrations
Butadiene	Hb/DNA	+	−	+	+	+
Vinyl chloride	DNA	+		+	+	
Aflatoxin	Hb/DNA	+	+	+		+
Benzo[a]pyrene	Hb/Alb/DNA	+	+	+	+	+
4-Aminobiphenyl	Hb/Alb/DNA	+		+	+	
Ethylene oxide	Hb/DNA	+				
NNK	Hb	+		+	+	

Hb—haemoglobin; Alb—albumin; NNK—*N*′-nitrosonornicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

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for development, reducing the number of hazardous compounds proceeding to regulatory genotoxicity assays, including animal tests, while not producing misleading positive results which can lead to the rejection of valuable compounds.

3.2.4.1.3 The Mammalian GreenScreen® HC (Human Cell) Biosensor

In the GreenScreen® HC assay (Gentronix, Manchester, UK), like the GreenScreen® GC assay, a DNA damage response promoter is used to drive the transcription of a reporter gene. Due to the more complex gene expression regulation in higher eukaryotes, however, a number of cis-acting regulatory elements have also been utilized in the construction of the biosensor (Hastwell *et al*., 2006). The GreenScreen® HC assay uses the transcriptional response of the of the human *GADD45a* gene in the human lymphoblastoid cell line TK6. *GADD45a*, the first gene to be identified as a target of p53 (Kastan *et al*., 1992), plays a fundamental role in maintaining genomic stability following genotoxic stress, with roles in cell cycle regulation and DNA repair, and emerging roles in apoptosis (Gao and Richardson, 2001; Zhan, 2005). Exposure of the modified TK6 cells to a genotoxin induces the *GADD45* promoter and expression of *GFP* reporter; thus cells fluoresce green when subjected to genotoxic stress. The GreenScreen® HC assay is also based on a 96-well plate format and requires milligram amounts of compound; data are available within 48 hours. Validation studies have shown that the GreenScreen® HC assay has both high sensitivity and specificity for genotoxic carcinogens, and unlike other *in vitro* mammalian genotoxicity assays, is not prone to false-positive prediction of *in vivo* genotoxicity/genotoxic carcinogenicity (see below). The assay has been transferred to several laboratories, and has been shown to be robust and reproducible (Billinton *et al*., 2008). An adaptation to the GreenScreen® HC assay that uses flow cytometry and allows for the inclusion of S9-mix has recently been described (Jagger *et al*., 2009).

3.2.4.2 DNA and Protein Adducts

Various analytical methods have been used to investigate the mechanism of carcinogen activation and monitor human exposure to environmental carcinogens, since carcinogen-adduct measurement provides a integrated biomarker of carcinogen intake, metabolic activation and delivery to the target macromolecule in target tissues. Protein adducts are not repaired and are considered primarily as exposure monitors, but DNA adducts may give further information about the mutagenic significance of the exposure. Indeed, such DNA damage is recognized as an early event in the carcinogenic process. As such these techniques have been used for measuring environmental, occupational and dietary exposures to a variety of genotoxic compounds in human populations (reviewed by Wild and Pisani, 1998; Sharma and Farmer, 2004; Farmer *et al*., 2005). Occasionally, these assays are used as supplementary tests to the standard genotoxicity battery to help elucidate whether a test agent is a DNA-reactive genotoxin or not. **Table 10** lists several compounds that require metabolism to various reactive intermediates and are positive in the standard battery of genetic toxicology assays, which covalently bind to proteins and/or DNA to form adducts.

3.2.4.2.1 ^{32}P-postlabelling Assay

The ^{32}P-postlabelling assay is one of the most common methods used for the detection of DNA adducts. Nuclear DNA from cells treated in culture *in vitro* or from target organs and tissues from exposed animals or individuals may be analysed. The procedure involves the enzymic digestion of DNA to 3′-mononucleotides and labelling with T4 polynucleotide kinase using (γ-^{32}P)ATP followed by chromatographic separation of the labelled adducts, usually by two-dimensional TLC (**Figure 14**) but the use of high-pressure liquid chromatography and mass spectroscopy have also been reported (reviewed by Sharma and Farmer, 2004). The limits of sensitivity of the standard ^{32}P-postlabelling assay range from 1 adduct in 10^8 to 1 in 10^9 nucleotides (i.e. 2–3 orders of magnitude higher than reported background mutation frequencies). The sensitivity of the assay may be increased to 1 adduct in 10^{10} or 10^{11} nucleotides by adduct enrichment techniques, which

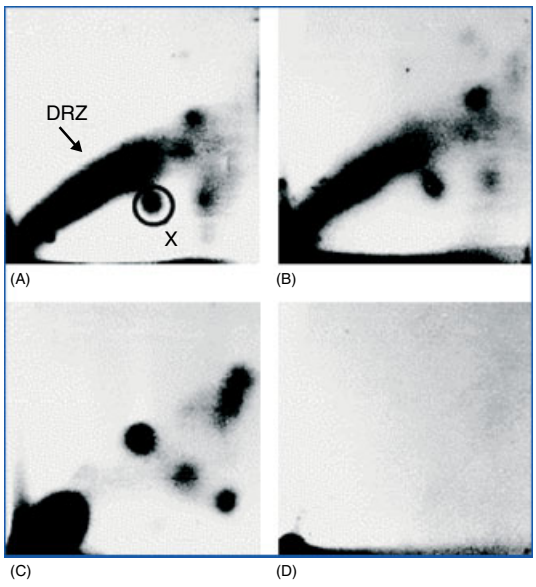

Figure 14 ^{32}P-postlabelling assay. Autoradiograms of ^{32}P-postlabelled human DNA isolated from the placenta of individuals environmentally exposed to benzo(a)pyrene (B(a)P): (A) human placenta with diagonal radioactive zone (DRZ), that is, where DNA adducts are not completely resolved by thin-layer chromatography. X signifies a major adduct; (B) human placenta; (C) B(a)P rat liver standard; (D) rat liver negative control.

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remove normal nucleotides from the digest before ^{32}P-labelling (Phillips and Castegnaro, 1993). Enrichment is normally achieved using 1-butanol extraction or nuclease P1 digestion (which dephosphorylates normal nucleotides and prevents them from being substrates for T4 polynucleotide kinase and ^{32}P-phosphorylation). The choice of enrichment techniques, however, needs scientific justification and some prior knowledge of the type of adduct likely to be produced.

3.2.4.2.2 Interpretation of ^{32}P-postlabelling Assay Results

The reaction of chemical carcinogens with DNA, either directly or after metabolic activation, typically involves covalent binding of an electrophilic carcinogen with a nucleophilic site in DNA. Guanine is by far the most prevalent target, although adducts have been reported for all bases (Farmer and Shuker, 1999). Thus the demonstration of DNA adducts in the ^{32}P-postlabelling assay suggests a direct (DNA-reactive) genotoxic mechanism. However, the DNA adduct levels in a particular cell type and/or tissue, measured at any point in time, will reflect specific rates of adduct formation and removal, which depend upon carcinogen activation, DNA repair, adduct instability and tissue turnover (Poirier *et al*., 2000).

The disadvantage of the ^{32}P-postlabelling assay is that the chemical identity of a particular adduct is not easily determined without the use of standards, which after post-labelling, can be compared with unknown lesions to help interpretation. In the absence of a chemical standard the interpretation of assay results may be complex, for example, with the investigation of a new chemical entity. This is because, without further characterization of the DNA adduct itself, one cannot be sure of the origin of the DNA adduct. Additional studies, for example the removal of the DNA adduct from TLC plate and mass spectroscopy analysis, or the use of a radioisotope-labelled standard, may be required to demonstrate that the adduct complex contains the chemical in question.

In contrast, the absence of DNA adducts in a ^{32}P-postlabelling assay may imply that the chemical in question is non-DNA reactive or simply not amenable to detection under the conditions of the assay. Many factors may impact the performance the assay, such as chemical instability, resistance to enzymic digestion or phosphorylation, poor recovery using standard enrichment techniques or inappropriate chromatography procedures. These, in turn, may influence assay sensitivity and reliability. All of these factors should be considered when the ^{32}P-postlabelling assay is used as a supplementary test for regulatory purposes.

3.2.4.2.3 Protein Adducts

Protein adduct formation is considered to be a valuable surrogate for DNA adduct formation, as many chemical carcinogens bind to both DNA and protein in blood with similar dose–response kinetics, and the levels of adduct formation are determined by external dose. Although there is no evidence to suggest that the formation of protein–carcinogen adducts is in any way associated with carcinogenesis, the presence of such adducts nevertheless demonstrates both exposure and activation. Indeed, the use of blood proteins as molecular dosimeters has distinct advantages over the use of DNA. Blood proteins (haemoglobin and albumin) are readily obtainable and relatively long lived. Haemoglobin has a lifetime of 120 days in humans whereas albumin has a biological half-life of ~45 days. Their metabolic activity is also very limited and they lack repair mechanisms to remove covalently bound adducts. Since protein adducts are not removed by active repair processes, chemically stable adducts of haemoglobin or serum albumin provide a more precise measure of dose integrated over the lifespan of the proteins in question (reviewed by Farmer *et al*., 2005). Furthermore, chronic exposure to carcinogens leads to accumulation of protein–carcinogen adducts, which in the case of 4-aminobiphenyl increase to plateau values 30-fold those seen after an acute dose. Adducts of other genotoxic carcinogens also reach steady state levels during chronic exposure, and these properties facilitate the detection of protein–carcinogen adducts following chronic exposure to low levels of carcinogen. These advantages have been exploited by several groups to evaluate human exposure to a variety of carcinogenic alkylating and arylating agents (Neumann, 1988). The application of mass spectrometry analysis for the detection of acid-labile (sulfinamide) protein adducts produced by carcinogenic aromatic amines has also been reported (Lynch *et al*., 1991; 1993). Using such analytical techniques, the lowest limits of sensitivity of protein adduct measurements can be below 1 pmol adduct per g protein.

3.2.4.2.4 Covalent Binding to DNA or Protein

Another method of measuring DNA and/or protein adducts is to use radioisotope-labelled compounds and measure covalent binding by liquid scintillation counting (Davies *et al*., 1995; Lynch *et al*., 1991). This is a well-established technique, but requires the specific chemical synthesis of radiolabelled molecules, usually with tritium or stable isotopes (such as ^{14}C or ^{17}N). The significance of low-level binding observed in these assays is often difficult to interpret since some low-level activity measured may not be due to covalent binding to DNA.

3.2.4.3 Alternatives to the Rodent Bioassay

3.2.4.3.1 SHE Cell Transformation Assay

The Syrian Hamster Embryo (SHE) cell transformation assay (SHE assay) is a short-term *in vitro* test that measures morphological cell transformation, a biological end point which is considered to model carcinogenicity in the two-year rodent bioassay (LeBoeuf *et al*., 1990; 1996). Uniquely, the SHE assay is capable of detecting both genotoxic and nongenotoxic rodent carcinogens (LeBoeuf *et al*., 1996) and has an impressive empirical

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correlation (>80%) with predicting the outcome of the rodent bioassay (Myhr and Zhang, 2000; Mauthe *et al*., 2001). In recent years, this empirical correlation alone has promoted some regulatory agencies to request short-term carcinogenicity tests, and specifically *in vitro* cell transformation tests like the SHE assay, to aid in the risk assessment of *in vitro* genotoxicants (Bigger, 2003).

On closer inspection, however, the impressive empirical correlation figures appear to be based on data biased in terms of the ratio of rodent carcinogens to noncarcinogens tested in the SHE assay. Therefore many more noncarcinogens require testing to provide a more accurate assessment of performance of the SHE assay. In addition, and aside from the fact that evaluation of morphological cell transformation *in vitro* remains subjective, the intrinsic variability seen in the SHE assay and lack of repeat testing in the existing data set is also a cause for concern. Moreover, the effect of pharmacologically active agents, particularly at high *in vitro* test concentrations, in the complex mixture of embryological cells is unknown. Similarly, although the mechanism of tumour formation in an *in vivo* rodent assay can in some cases be understood with respect to the pharmacological activity of the compound, no similar mechanistic understanding exists for the SHE assay. On the contrary, it is not understood how chemicals that induce species/sex/tissue-specific tumours through well-studied mechanisms (such as peroxisome proliferation in liver tumours, or compounds that induce thyroid tumours through the proliferative effects resulting from increased thyroxine clearance) should be positive in the SHE transformation assay (Ashby, 1997). Thus, without a better understanding of the differences in the mechanisms of morphological transformation and rodent tumours, many consider it premature to use empirical correlation alone for making regulatory decisions (Farmer, 2002).

For these reasons the SHE assay is rarely used in the pharmaceutical industry as a regulatory tool for the assessment of carcinogenic potential. Nevertheless, the SHE cell assay has been positioned as a predictive tool for genotoxic and nongenotoxic rodent carcinogenicity, and is more typically employed by industries outside of the pharmaceutical industry, like environmental and consumer products, where testing in animals and/or the development of products that induce rodent tumours is unacceptable. For example, the SHE assay may be used to discriminate between rodent carcinogens and rodent noncarcinogens for certain classes of chemicals like aromatic amines where the SHE assay has been more predictive than the bacterial reverse mutation test (Kerckaert *et al*., 1998). Moreover the impact of REACH legislation in the EU and the imminent drafting of an OECD guideline for the SHE assay may lead to the resurgence of the SHE assay outside of the pharmaceutical industry. Further research, however, will be required to establish the relevance and reliability of the SHE assay and elucidate the mechanisms underpinning SHE cell morphological transformation if it is to be anything other than a 'black box' predictor of carcinogenicity (Harvey *et al*., 2005).

At present the SHE assay does not remove the need for either a traditional rodent carcinogenicity study or assessment in an alternative carcinogenicity model. The future of the SHE assay will be dependant on a number of factors, not least a formal validation study to establish its reliability to predict carcinogenicity. The establishment of official guidelines for the conduct of the assay would be a prerequisite prior to any industry validation. Ideally in the future cell transformation assays should attempt to use human cells rather than primary rodent tissue, to enable a more representative model of human carcinogenesis.

3.2.4.3.2 Short-Term Carcinogenicity Assays

The substantial cost (financial and high number of animals required) and duration (approximately 3.5 years to final report) of lifetime carcinogenicity studies, using the two-year rodent bioassay, has been the subject of extensive debate and concern, along with doubts about the assay's reliability (as results are frequently considered to be of questionable relevance for human safety). This led the scientific community to search for suitable, shorter term alternatives to the standard rodent bioassay in the 1990s. The aim was to develop assays in animal models which allowed more rapid investigations of potential carcinogenicity by reducing the latency period of tumour development, reducing animal numbers, cost, time and improving the interpretation of rodent cancer bioassay data.

Several mouse models were developed bearing genetic manipulations to reduce tumour latency for the assessment of carcinogenesis (**Table 11**). These included tumour suppressor gene knockout mouse models, for example $p53^{+/-}$ hemizygous (Harvey *et al*., 1993) and XPA null mice (de Vries *et al*., 1997), and mouse models transgenic for additional copies of specific (proto)oncogenes such as TgHras2 (Yamamoto *et al*., 1997), Tg.AC (*ras*) (Hansen *et al*., 1995) and OncoMouse® (*myc, ras* and *neu*) (Stewart *et al*., 1984). Four of the genetically modified models ($p53^{+/-}$, XPA null, TgHras2 and Tg.AC) were the subject of extensive multilaboratory validation studies which were conducted under the auspices of the International Life Sciences Institute (ILSI) (reviewed in detail in a special issue of *Toxicological Pathology* **30**, 2002). In contrast, OncoMouse® was the subject of extensive worldwide legal wrangling over patent rights in the 1990s (now resolved), which held up the scientific development of the model. In addition, the neonatal mouse model (ICH guideline) was also investigated as a potential alternative model in the ILSI trials along with the SHE assay.

Generally, these models reduce the duration of testing to approximately 26 weeks. Although the issues of biological significance and predictiveness are far from resolved, the performances of the four models used in the

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Table 11 Mouse strains used in short term carcinogenicity bioassays

Mouse model	Genotype and phenotype	Reference
The $p53^{+/-}$ hemizygous mouse	C57BLK/N6 × FVB mouse background with one copy of the tumour suppressor gene *p53* 'knocked out'; an increased incidence of cancer	Harvey *et al*. (1993)
Tg.AC mouse	FVB/N mouse strain with four copies of an activated Harvey *ras* oncogene coupled to a zeta-globin promoter and an SV40 viral polyadenylation sequence with an increased incidence of skin cancer	Hansen *et al*. (1995)
Tg.Hras2 mouse	Hemizygous for multiple copies of the human *c-Ha-ras* gene with enhancer/promoter. Activated *ras* (following chemical insult) initiates expression of genes controlling cell proliferation	Yamamoto *et al*. (1997)
The $XPA^{-/-}$ (null) mouse	Deletion of both copies of the Xeroderma pigmentosum gene, a DNA repair defect with an increased incidence of skin cancer	de Vries *et al*. (1997)
OncoMouse®	Transgenic mouse carrying an activated *myc* oncogene under the control of the mouse mammary tumour virus promoter	Stewart *et al*. (1984)

ILSI trial have been reviewed by Pritchard *et al*. (2003). The $p53^{+/-}$, TgHras2 and Tg.AC models all showed good concordance with the rodent bioassay but all missed some human carcinogens when the models were used to examine 99 chemicals/drugs. When an alternative model was used in combination with the rat bioassay, no human carcinogens were missed.

Nowadays, regulatory authorities may suggest the use of one or more of these transgenic models when a positive *in vitro* genotoxicity assay is obtained. For example, in the pharmaceutical industry such an *in vitro* positive finding may result in an 'on hold' status of a drug's clinical development and may prevent repeat-dose clinical studies in man until it is resolved. A negative result in an alternative carcinogenicity test may be used to allay concerns and permit continued clinical development. The regulatory acceptance for these models, however, still varies somewhat between Europe and the USA. For example, European regulators do not support the use of alternative models by default but feel more science is required and that genotoxic compounds can be detected by many standard tests. The FDA on the other hand has suggested the use of different transgenic models depending on the nature of the initial positive result. For nongenotoxins Tg.AC or TgHras2 is suggested, for genotoxins the $p53^{+/-}$ mouse or mouse neonatal assay or a SHE assay is recommended. The alternative models are not without their limitations; for example, the TgHras2 model has a high background incidence of lung tumours, as well as a myopathy, which may limit its use in some cases. Standard rodent carcinogen bioassays (in the rat) are still required as there are more historical data on carcinogenic mechanism in rats and more compounds are positive in 'rat only' than in 'mouse only' studies. Because of this, the development of new murine models raises additional concerns, and limited experience with transgenic mice increases the risk of results of debatable human relevance, but which nonetheless imperil or delay regulatory approval. Nevertheless, regulatory pressure to use such models is increasing, and results are beginning to influence interpretative thinking.

3.2.4.4 *Photogenotoxicity Testing*

It is well known that solar UVR induces DNA damage (Cleaver and Crowley, 2002) and that UVR exposure is the major aetiological cause of skin cancer in humans (Melnikova and Ananthaswamy, 2005). It has also been known for many years that some molecules can absorb light that is either visible or UV and become photoactivated. This can lead to a number of biologically relevant toxicities, including allergy, irritation, genotoxicity and carcinogenicity, when applied to the skin directly or systemically (Gocke, 2001). For example, certain drug classes such as fluoroquinolone antibiotics and psoralens are known to enhance photocarcinogenesis in animal models (Gocke *et al*., 1998; Dunnick *et al*., 1991), and there is now good evidence that patients treated with PUVA (Psoralen plus UVA) have a small, but real increased risk of squamous cell carcinoma (Lim and Stern, 2005). In addition, some molecules may greatly enhance the carcinogenic effects of UVR. As such, photosafety testing has become a regulatory requirement for pharmaceutical or cosmetic (dermatological and sunscreen) products that absorb light or UVR between the wavelengths of 290 and 700 nm in the electromagnetic spectrum and are applied either topically, or locally, and/or reach the skin or eyes via systemic exposure.

In Europe, the conditions for the photosafety evaluation of pharmaceuticals and cosmetics are described by

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The European Medicines Agency (formerly the European Agency for the Evaluation of Medicinal products (EMEA)) Committee for Proprietary Medicinal Products (CPMP) 'Notes for Guidance on Photosafety Testing' or the 'Notes of Guidance of the EU Scientific Committee on Cosmetics and Non-Food Products (SCCNFP)', respectively. In the USA, the Federal Drug Administration (FDA) Centre for Drug Evaluation and research (CDER) guidances serve the same purpose (and cover both drugs and consumer products such as sun-screens). These guidance documents describe the strategy for evaluating the photosafety of products prior to review by regulatory authorities. In Europe, testing may include an assessment of acute phototoxicity (photoirritation) (Lasarow *et al*., 1992; Holzhutter, 1997), photoallergy, photogenotoxicity and photocarcinogenicity (for a review see Maurer, 1987).

In terms of photogenotoxicity testing, the main objective has been to make an assessment of the potential of a compound to turn into a photochemical carcinogen upon activation with UV or visible (solar-simulated) radiation. Several *in vitro* photogenotoxicity assays, such as the photo-Ames, photo-chromosome aberration and photo-Comet assays have been described in the literature and are based on standard 'dark' versions of *in vitro* regulatory assays used for genotoxicity assessment (for reviews see Meunier *et al*., 2002; Brendler-Schwaab *et al*., 2004, Kersten *et al*., 1999). In contrast, in its Guidance for Industry and subsequent paper by Jacobs *et al*. (2004), the FDA clearly state that they have no requirement for a separate photogenotoxicity test, and focus much more on results of the phototoxicity test using the 3 T3-NRU (Neutral Red uptake) assay. If the latter is positive, they recommend labelling to indicate that the test agent may cause photoirritation in humans and for individuals to avoid exposure to the sun.

In recent years, experience of industry suggests that the *in vitro* photosafety (phototoxicity/photogenotoxicity) assays are over-sensitive and that the photogenotoxicity assay in particular may also lack specificity. This is based on the lack of biological plausibility for the response of certain chemicals in the photogenotoxicity assays (Lynch *et al*., 2008b), reports of reproducibility issues (Kasper *et al*., 2005) and identification of a new phenomenon, termed pseudophotoclastogenicity (Dufour *et al*., 2006). As a consequence, the EMEA safety working group have proposed a revision of the CPMP guidance on Photosafety Testing in 2008 to address these concerns.

The points for consideration will focus on the following:

1. A refinement of the criteria for deciding whether photosafety testing is required, as these are considered rather unspecific and apparently result in testing of too many new pharmaceuticals. Therefore, a better prediction of possible photobiological properties or lack thereof is required.
2. Currently the guideline stipulates a parallel approach to photosafety testing; that is, phototoxicity, photoallergy and photogenotoxicity. A tiered approach, where photoallergy and photogenotoxicity testing would usually not be required if the compound in question is clearly negative in an initial *in vitro* phototoxicity study would provide a more suitable and efficient testing strategy.
3. Oversensitivity and the occurrence of 'pseudo-effects' with *in vitro* models recommended by the current guideline, and in particular photogenotoxicity. The use of some test models for regulatory purposes can no longer be justified and these need to be replaced by more appropriate approaches.
4. The question of timing of photosafety evaluation during drug development.

4 INTERPRETATION OF GENETIC TOXICOLOGY DATA

The assessment of the potential genotoxicity (and carcinogenicity) of a new chemical entity is an essential part of product development in the pharmaceutical, chemical and consumer product industries. Compound-related genotoxicity and carcinogenicity findings are assessed and are considered as part of the risk–benefit continuum based on intended use (including clinical indication for drugs) and anticipated human exposure, and are regulated accordingly (**Figure 15**).

As mentioned above, the minimum regulatory genotoxicity package generally consists of (i) an *in vitro* bacterial assay for gene mutation, (ii) an *in vitro* mammalian cell assay for gene mutation and/or cytogenetic damage, and (iii) an *in vivo* mammalian assay for cytogenetic damage. The aim is to assess the potential of a compound to induce genetic damage, directly or indirectly, by various mechanisms. A negative result in all three assays is usually sufficient to provide evidence for a lack of genotoxic potential, although additional testing may be required for substances with structural chemical alerts, or when there is evidence of the carcinogenicity of structurally similar compounds. Assays producing equivocal results should be repeated. More testing is also needed if one or more of the assays produces a positive result, but the nature of the findings is insufficient to drive immediate termination of development. A risk assessment is made using the results of the complete package, taking into account the significance and limitations of each test. Thus a single positive result may reveal a theoretical hazard, but cannot automatically be interpreted as an unacceptable genotoxic risk, especially if *in vivo* studies are negative. Indeed, it is increasingly accepted that a positive result should not be considered in isolation, and that a weight-of-evidence approach considering

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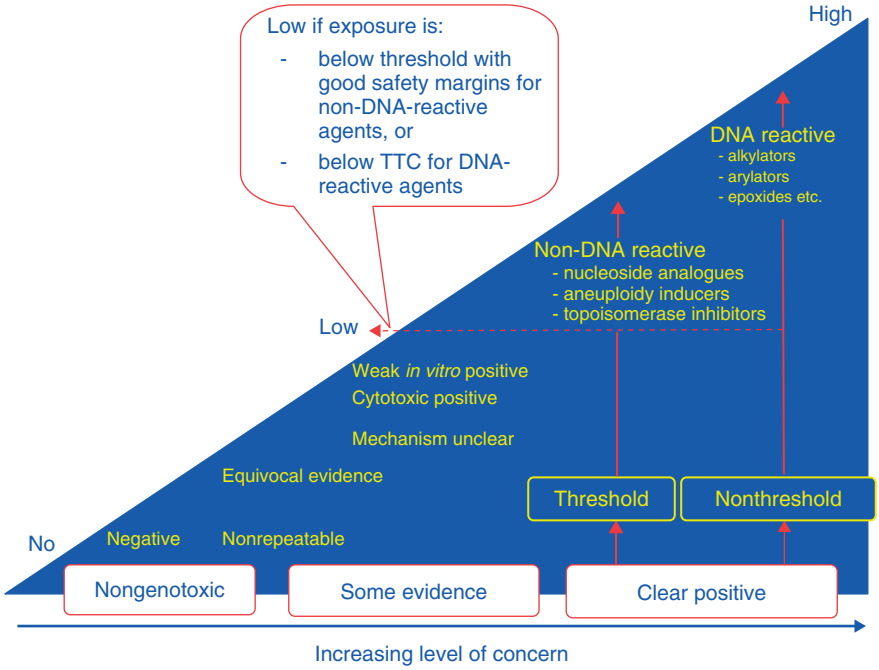

Figure 15 Risk–benefit continuum for genotoxicity. TTC: threshold of toxicological concern (see main text). (Figure adapted from Tweats and Gatehouse (1999).)

all pertinent data should be preferred. As part of this weight-of-evidence approach, information on the mode of action, kinetics, and the extent of human exposure is useful for risk assessment (US Environmental Protection Agency (EPA), 2005a; Dearfield and Moore, 2005; Jacobson-Kram and Jacobs, 2005; Thybaud *et al*., 2007a; 2007b). This may include further investigations using one or more of the supplementary tests described above to provide a mechanism-based risk assessment. In addition, the option to test whether the compound induces tumours in animal models may also be considered. Any decision should be made on a case-by-case basis, with scientific justification. For example, threshold-based arguments may be used for non-DNA reactive compounds, such as aneugens, that is, compounds that induce chromosome loss (aneuploidy), and therefore risk assessments may be based on safe margins of exposure in the patient population.

4.1 Threshold Mechanisms in Genetic Toxicology

The ECETOC has defined an 'absolute' threshold as a 'concentration below which a cell would not "notice" the presence of the chemical'; that is, a threshold assumes that a biological effect only occurs at concentrations above a certain threshold concentration of a chemical. In other words, 'the chemical is present but does not affect the cellular target'. However, the precise characterization of such a threshold is difficult to prove experimentally (Schneiderman *et al*., 1979). In contrast, a 'pragmatic' threshold can be considered as a concentration below which any effect is considered biologically unimportant (Lutz, 1998), and may be defined, in part, with the help of statistical tests (Lovell, 2000). For example by determining the concentrations where increases do not exceed the range of responses seen in the negative control in a well-conducted series of experiments. Although a threshold may be defined by statistical modelling, the existence of such a threshold must be justified from biological arguments and not just from statistical ones. Biological mechanisms and effects have to exist which change abruptly or cause a 'break' of the process when the 'effective' concentration is surpassed (Edler and Kopp-Schneider, 1998).

Genotoxic chemicals which interact directly with DNA have historically been thought to abide by 'the single hit, single target' hypothesis, first proposed by Knudson (1971), whereby a cancer-causing mutation may result from a single interaction causing a nucleotide base change (reviewed by Kirsch-Volders *et al*., 2003b). It is therefore postulated that such chemicals do not exhibit a threshold for genotoxicity. From a regulatory perspective, this has been interpreted such that it can be assumed there is no safe level of DNA damage, however low this is within the DNA. In contrast, for chemicals that do not interact directly with DNA but cause inactivation or modification of redundant or multiple cellular targets, the converse is held to be the case; that is, these chemicals have a threshold dose above which a toxic response

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is produced. Examples of non-DNA-damaging genotoxic substances include those that target the mechanical components required for chromosome segregation (e.g. microtubules, kinetochores, centrioles), DNA metabolism and/or synthesis (e.g. topoisomerases, DNA polymerases, imbalanced nucleotide pool) or DNA repair (e.g. polymerases, endonucleases, ligases). The demonstration and acceptance (by the scientific and regulatory community) of the concept of a threshold of genotoxic activity can have important economic implications for the use of individual chemicals and drugs. The unambiguous demonstration of a threshold of genotoxic activity indicates that a compound will not produce mutations (or chromosomal effects) below a critical exposure level, thus reducing the potential for the induction of cancer or congenital abnormalities at these exposures.

For example, spindle poisons, which disrupt the mitotic machinery, are widely accepted as having no observed effect levels (NOELs) for the induction of aneuploidy (COM, 2000), since multiple targets need disabling before an effect is seen (Elhajouji *et al*., 1995; 1997). Potential thresholds have been established for several aneugens including colchicine, mebendazole, nocodazole, nitrobenzene, benzonitrile, benomyl and its major active metabolite carbendazim, all of which are spindle inhibitors (Elhajouji *et al*., 1995; Grawé *et al*., 1998; Bentley *et al*., 2000; Decordier *et al*., 2002; Bonacker *et al*., 2004). Another example is topoisomerase inhibitors. In mammalian cells, the interaction of topoisomerase II poisons with DNA through the formation of a topoisomerase/DNA/drug cleavage complex results in the biological lesion that is ultimately believed to be responsible for the induction of clastogenicity and/or cytotoxicity (Fortune and Osheroff, 2000). It is this indirect mechanism of DNA damage, therefore, that is generally held for the genotoxicity of topoisomerase II poisons and provides the biological mechanism which underpins the threshold concept for this class of compound. Potential thresholds have been established for several topoisomerase inhibitors using the *in vitro* micronucleus assay and statistical modelling, including etoposide, doxorubicin, genistein and ciprofloxacin (Lynch *et al*., 2003).

More recently, the concept of threshold mechanisms for direct DNA damaging agents, including some alkylating agents, has been postulated (Jenkins *et al*., 2005). The premise is based on the fact that mammalian cells have a number of cellular defences that provide protection to the cell and may limit permanent DNA damage (e.g. DNA repair, chemical detoxification, physical barriers such as cellular and nuclear membranes and redundant targets, etc.). These homeostatic mechanisms and cytoprotective processes are particularly important in the low-dose exposure range, and may prevent and/or limit induced damage from becoming permanent so long as the cellular defences do not become saturated. This can result in a NOEL; that is, the experimental concentration below which no statistically significant increase in mutations is detected. For example, Doak *et al*. (2007) have shown that MNU (methylnitrosourea) and ENU (ethylnitrosourea) display linear dose-responses for chromosomal damage and point mutations (determined by the micronucleus and hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) gene forward mutation assays, respectively), whereas MMS and EMS have nonlinear dose-responses containing a range of nonmutagenic low doses. More recently, Muller *et al*. (2008) showed that EMS demonstrated a threshold for genotoxicity in animal studies *in vivo*. Based on these data, threshold arguments were successfully used by the pharmaceutical company, Roche, to support a risk assessment made to the regulatory authorities for an absence of risk to patients exposed to EMS present as an impurity in a batch of the HIV drug, Viracept (nelfinavir).

The nonlinearity in the dose-response for certain alkylating agents such as EMS is thought to be due to homeostatic maintenance by DNA repair, which is efficient at low doses of compounds that primarily alkylate N^7-G and rarely attack oxygen atoms. Indeed, all organisms exist in environments which lead to low levels of DNA damage being induced, and they have evolved to cope with this. Thus, for many people, the concept that thresholds (or NOELs) exist and that organisms can tolerate low levels of DNA damage is self-evident, and therefore, the logical extension of these arguments is the existence of pragmatic thresholds for certain DNA-reactive genotoxins with regard to carcinogenicity. These ideas, however, still remain controversial, but nevertheless, this work, and other arguments (see Bolt and Degen, 2004) has led to the possible distinction of four groups of carcinogens:

- nonthreshold genotoxic carcinogens (for low-dose risk assessment, a linear nonthreshold (LNT) model appears appropriate);
- genotoxic carcinogens, for which the existence of a threshold cannot be sufficiently supported (in these cases the LNT model is used as a default assumption, based on scientific uncertainty and the precautionary principle);
- genotoxic carcinogens for which a practical threshold is supported; and
- nongenotoxic carcinogens and non-DNA-reactive carcinogens (e.g. spindle poisons and topoisomerase inhibitors). For these compounds an absolute threshold is associated with a clearly founded no-observed-adverse-effect level.

The current regulatory viewpoint remains that thresholds have to be proven on a 'case-by-case' basis. This point of view may well change as more data become available from chemicals with similar modes of action. It is again important to point out that DNA-damaging agents and non-DNA-damaging agents are mechanistically distinct and are likely to be treated differently in regulatory terms.

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4.2 Threshold of Toxicological Concern (TTC)

Genotoxic or carcinogenic impurities in drugs are considered to represent a risk to patients, with no associated benefit. The regulation of the levels of genotoxic and carcinogenic impurities in drugs to ensure they impart no risk or have an acceptable level of risk can be challenging as it is widely accepted that complete avoidance of such impurities in drugs is unrealistic (McGovern and Jacobson-Kram, 2006).

The importance of controlling impurities in drug substance and drug products is well established. The thresholds for identification, reporting and qualification of impurities are described in the ICH Q3A/B/C Guidance. While the scope of this regulatory guidance is restricted to new drug products, it also offers a framework for the assessment of drug-related impurities in later clinical development. The ICH Q3 guidance describes the process for assessing the general, genetic and special toxicity of impurities in drug substance. For example, ICH Q3A recommends that for a drug substance where the clinical dose is less than 2 g day, impurities that are at a level of 0.05% must be reported, impurities that are at a level of 0.1%, or represent a total dose of 1 mg (whichever is lower) must be identified, and finally impurities that are at a level of 0.15%, or represent a total dose of 1 mg (whichever is lower) must be qualified.

Typically, qualification requires general toxicology studies (from 14 to 90 days) and genetic toxicology studies (including an assessment of the potential to induce point mutations and chromosome aberrations) on either the impurity or with parent drug substance containing appropriate levels of the impurity. The guidance for residual solvents is more specific, in that it describes a toxicology-based risk assessment approach that defines acceptable limits, known as a permissible daily exposure (PDE), for individual solvents. This PDE approach considers the NOEL from the most relevant toxicity studies alongside various risk factors to account for extrapolation between species, variability between individuals, and so on. Importantly, while these PDEs for individual solvents appear precise, the standard errors around them in terms of mg day^{-1} are likely to be large.

A recognized shortcoming of the ICH Q3 regulatory guidance is that the genetic toxicology qualification of drug substance impurities is highly insensitive. For example, impurities that are potent mutagens (e.g. with similar potency to MMS) may go undetected in a standard genetic toxicology study if present in drug substance at the 0.15% qualification threshold. Likewise, the calculation of a PDE for genotoxic agents is considered to be problematic, as current dogma assumes that DNA-reactive genotoxic agents represent some degree of risk even at very low levels of exposure (i.e. there is no safe dose). In response to this issue, the European Medicines Agency's Committee for medicinal products for human use (CHMP) issued a new guidance in January 2007, to address the Toxicological assessment of genotoxic impurities and the determination of acceptable limits for such impurities in active substances (European Medicines Evaluation Agency, 2006).

The CHMP guidance applies to all new active drug substances in development and, in certain cases, existing drug substances if there is a cause for concern. The main focus of the guidance is on the assessment of direct-acting DNA-reactive substances (as the default assumption for such substances is that they are likely to be genotoxic carcinogens). Although indirect-acting genotoxic substances that can be demonstrated to act via a biologically meaningful threshold were also considered. The CHMP proposed that for the latter class of compound, if sufficient experimental data was available, a PDE approach could be adopted to determine acceptable levels of an impurity in a drug. However, it was recognized that demonstration of such thresholds can often be problematic, and in most cases the default assumption for a genotoxic impurity would be that it was a nonthresholded genotoxic carcinogen.

In considering the control of direct-acting DNA-reactive impurities, the CHMP stated that ideally such substances should be controlled to levels 'as low as reasonably practicable (ALARP)' but also accepted that in many cases this was not technically possible; it is estimated that ~20–25% of all pharmaceutical synthetic routes contain DNA-reactive substances (Delaney, 2007). In such cases, the CHMP argued that the acceptable levels of such direct-acting DNA-reactive impurities in a drug would require justification from a toxicological perspective (i.e. calculation of an acceptable daily intake using rodent carcinogenicity data). However, the CHMP also recognized that in the majority of cases this type of comprehensive toxicology data would not be available for potentially DNA-reactive drug-related impurities. Therefore, the CHMP proposed a pragmatic, risk-based approach to address this situation, that involved limiting DNA-reactive impurities in a drug substance/product to a level known as the threshold of toxicological concern (TTC).

The regulatory paradigm known as the TTC was originally developed by the FDA as the threshold of regulatory concern and was defined as a level of any unstudied chemical that would not pose a risk of significant carcinogenicity or other toxic effects (Threshold of Regulation for Substances Used in Food-Contact Articles (Final Rule), 1995). The TTC figure was derived following a series of linear extrapolations involving the doses of known rodent carcinogens that induced tumours in half of the test animals (i.e. TD_{50} mg $kg^{-1}day^{-1}$) to doses of 0.15 μg day^{-1} that would be equivalent to less than a one in a million (1×10^{-6}) upper bound lifetime risk of cancer; also known as a 'virtually safe dose' (Rulis, 1989;

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Munro, 1990; Kroes and Kozianowski, 2002). Some adjustments around this original TTC level based on structural class, recognition of the conservative nature of the linear extrapolation model and its application to pharmaceutical development resulted in the CHMP defining a new TTC figure for genotoxic impurities in drugs of $\sim 1.5\,\mu g\,day^{-1}$ that was associated with a one in one hundred thousand (1×10^{-5}) upper bound lifetime risk of cancer. It is important to recognize that the risks associated with such low levels of genotoxic impurities are vanishingly small. For example they are much lower than the annual risk of death by natural causes (above the age of 40), by road accident, playing soccer or homicide in the UK (Calman, 1996).

The CHMP accepted that in certain cases that application of the TTC for genotoxic impurities will not always be appropriate and that higher levels may be acceptable depending on the seriousness of the clinical indication (e.g. for life-threatening conditions), or if human exposure to the genotoxin will be greater from other sources (Delaney, 2007) or if the duration of exposure to the impurity is limited. The last of these considerations (i.e. duration of exposure) has been the subject of much debate and it is now recognized that a 'staged TTC' approach, in which acute exposure to a genotoxic substance at levels greater than the TTC is considered to be of comparable risk to chronic exposure to the levels equal to the TTC (Müller *et al*., 2006). The concept clearly has wider implications given that short-term exposures to high concentrations of an environmental genotoxin are a regular cause of concern in areas outside the pharmaceutical industry (Bos *et al*., 2004).

In summary, risk–benefit considerations made for parent drugs cannot be extended to genotoxic impurities in drug substance/product. Recent CHMP guidance, which is aimed at the control of genotoxic impurities in drug substance/product, are based on the concept of TTC. The assumptions used in the TTC are extremely conservative and the TTC may significantly over-estimate the risk associated with low levels of genotoxic substances. Nevertheless, and perhaps more interestingly, the CHMP guidance has reintroduced the concept that for DNA-reactive carcinogens, there will be acute and chronic exposure levels that could be considered to be associated with an acceptable carcinogenic risk. This is an important paradigm shift from the prevailing dogma of the 'no safe dose' which resulted from 'the single hit, single target' hypothesis (Knudson, 1971), given the risks associated with exogenous exposure to agents that can induce carcinogenicity are a source of great personal and social relevance (European Medicines Evaluation Agency, 2007).

4.3 Cytotoxicity

Regulatory guidelines stipulate the maximum test concentration required in the *in vitro* mammalian genotoxicity assays as the lowest of $5\,mg\,ml^{-1}$, 10 mM, the limit of solubility, or the threshold of cytotoxicity. The methods for cytotoxicity used in genotoxicity assays and defined by OECD and ICH guidelines a decade or more ago, are now undergoing rigorous scientific scrutiny in concert with the development of more accurate technologies. One of the main reasons for this new scrutiny is the high frequency of positive *in vitro* findings in the genotoxicity test batteries with agents found to be noncarcinogenic in rodents and thought not to pose a carcinogenic health hazard to humans. For example, Kirkland *et al*. (2005; 2006) recently reported that 75–95% of known noncarcinogens were positive in one or more of the standard *in vitro* genotoxicity assays. In this analysis, the false-positive rate was highest in mammalian cell tests such as the chromosome aberration assay in Chinese hamster cells (the analysis was based on over 700 chemicals with rodent carcinogenicity data). These data, subsequently confirmed by Matthews *et al*. (2006a; 2006b) using FDA and EPA databases, demonstrate that positive results from *in vitro* mammalian genotoxicity assays do not accurately predict rodent carcinogenicity or human carcinogenic risk.

The need to reconsider the evaluation of positive findings in mammalian genotoxicity assays *in vitro* and their impact on risk assessment has been highlighted by regulatory authorities (US Environmental Protection Agency (EPA), 2005a; 2005b; Dearfield and Moore, 2005; Jacobson-Kram and Jacobs, 2005; FDA (Food and Drug Administration) CDER (Center for Drug Evaluation and Research), 2006) and organizations such as the IWGT (Thybaud *et al*., 2007a), ECVAM (Kirkland *et al*., 2007) and the Health and Environmental Sciences Institute (HESI), which is the global branch of ILSI (Thybaud *et al*., 2007b). One of the re-occurring themes is the rationale for the selection of the highest *in vitro* concentration, including the required levels of cytotoxicity currently prescribed by various regulatory guidelines for *in vitro* tests. It follows that accurate cytotoxicity assessments are critical to improve the scientific rigour of these *in vitro* assays to ensure accurate human risk assessment. In addition, recent evidence suggests that rodent cell lines, and in particular Chinese hamster cells, produce more positive results in the *in vitro* structural chromosome aberrations assay compared with human cell lines (Hilliard *et al*., 2007) and therefore the choice of cell type may lead to positive results not relevant to humans (Kirkland *et al*., 2007).

Rodent cell lines are exceptionally sensitive to cytotoxicity-related chromosomal damage (compared with human cells) because of relaxed cell cycle regulation. For example, CHO cells uniquely continue

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to undergo cellular growth during DNA synthesis inhibition and the degree of aberrant growth is directly related to the degree of subsequent cytotoxicity and cell death (Kung *et al*., 1990a; 1990b), implying that the mechanisms for clastogenicity and cytotoxicity are intrinsically linked in these cells. The sensitivity of CHO cells therefore makes them more susceptible to artefactual increases in chromosomal damage. This may also account for the increased sensitivity of rodent cells in cytogenetic assays compared with human cells.

Cytotoxicity in cultured mammalian cells is a function of the methods used to estimate it (Fellows and O'Donovan, 2007). The use of traditional end points such as mitotic inhibition and/or final cell counts as a percentage of control to assess toxicity often underestimates toxicity (Armstrong *et al*., 1992; 2000; Kirkland, 1992; Galloway, 2000; Greenwood *et al*., 2004; Fellows and O'Donovan, 2007). Cell counts can be misleading; for example, in cultures seeded at 0.5×10^6 cells per culture, if controls double to 1×10^6 during the experiment, a treated culture in which cell division is totally shut down, that is, complete failure to grow during the expression period, will have a cell count 50% of control at the end of the experiment. This underestimation is exacerbated with increasing toxicity and is a particularly confounding issue for compounds shown to produce cell cycle arrest *in vitro*. For example, it is well known that chromosome aberrations are often associated with cytotoxicity and DNA synthesis inhibition (Galloway *et al*., 1998; Hilliard *et al*., 1998) which can be induced *in vitro* by compounds that do not directly damage DNA. It is also recognized that chromosome aberrations may occur under conditions that would not be relevant *in vivo* (Kirkland and Muller, 2000).

Measures of cytotoxicity such as relative population doubling (RPG) and relative increase in cell counts (RICC), that evaluate population growth during the treatment period, may provide more suitable end points for use, as they are sensitive to all cytotoxic compounds regardless of MOA, and are equally sensitive in human and rodent cell lines (Galloway *et al*., 2000; Fellows *et al*., 2008; Bromfield, 2009). Greenwood *et al*. (2004) have shown that several non-DNA-reactive chemicals and metabolic poisons would not have given positive chromosome aberration results if the 50% cytotoxic concentration had been chosen based on a reduction in population doubling rather than a reduction in cell count, and that, using this measure, no important DNA-reactive genotoxicants would be missed. Similarly, data presented at a recent ECVAM workshop (Kirkland *et al*., 2007) by Kirchner demonstrated that relative cell count, mitotic index and reduction in population doublings produce quite different concentrations for 50% toxicity. These observations confirm that accurate assessment of cytotoxicity is critical for assay validity (Fellows *et al*., 2008) and therefore this is likely to be an area of ongoing refinement in the coming years.

5 THE FUTURE

Following a 30-year period of regulatory guideline convergence and a decade or so of stability, the current genetic toxicology paradigm is now undergoing a period of revision, with the possibility of divergence in regulatory guidelines, as different industrial sectors respond to varying regulatory and/or societal/political demands specific for each business. For example, in Europe the Seventh Amendment to the Cosmetics Directive (2003/15/EC) will prohibit the use of animals for safety testing of cosmetics ingredients from 2009/2013, which is driving the rapid development of *in vitro* human tissue genotoxicity assays, such as the skin micronucleus assay. The new European Union chemical legislation (EC 1907/2006) concerning the REACH will require genotoxicity testing of ~30 000 existing chemicals and is responsible, in part, for driving refinements in *in silico* testing and the robustness of *in vitro* assays. These diverse imperatives are likely to have a significant impact, particularly in regulatory genotoxicity testing, and drive future changes in the discipline.

For example, in the pharmaceutical industry, the revision of the ICH guidelines is currently underway and these are likely to be implemented in 2009. The revised draft guidance makes use of experience accumulated over more than 10 years with the existing S2A and S2B guidance. There is clear recognition worldwide among regulatory agencies of an urgent need to update the guidance

1. with strategies to address the high frequency of positive results in the mammalian cell genotoxicity assays *in vitro* (for the reasons described above), and
2. to include options for newer assays now that substantial experience has been gained, such as the *in vitro* micronucleus assay and the Comet assay for DNA strand breaks.

The revised ICH S2 guidelines therefore reinforce the upper toxicity limits for CA of 50% toxicity, and for MLA of 80% reduction in RTG. They also support the reduction in the top concentration for *in vitro* mammalian cell assays from 10 to 1 mM, based on several lines of reasoning:

1. The 1 mM limit maintains a component of hazard identification, being above known blood plasma and tissue levels for pharmaceuticals.
2. Most physiological processes and K_m values for enzymes, and so on, are well below 100 μM.
3. Review of the data compiled by the ICPEMC (International Commission for Protection against Environmental Mutagens and Carcinogens) to support the original 10 mM limit confirms that *in vivo* clastogens are detectable at 1 mM or less *in vitro*, and that compounds of concern for potential human genotoxicity are detectable at 1 mM or below.

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4. Experience in pharmaceutical companies confirms that compounds that may be considered 'relevant' positives based on knowledge of mechanism, DNA damage detected in other assays, or *in vivo* positive results, are positive at 1 mM or less.
5. Finally, the 1 mM limit is supported by data reviews when the whole battery is taken into account; for example, a mutagen such as EMS, which induces chromosome aberrations in the mM range, is recognizable by its structure and is clearly positive in the Ames test, so EMS would not be 'missed' by omitting the *in vitro* mammalian cell assay or limiting it to 1 mM.

The revised ICH S2 guidelines also extend the standard battery to include an option that comprises an Ames test and two *in vivo* tests, the *in vivo* micronucleus assay and *in vivo* Comet assay for DNA damage, which is the strategy that is currently in use by the US National Toxicology Program (**Box 2**). Existing ICH guidance (S2A and S2B) and the FDA's guidance (FDA (Food and Drug Administration) CDER (Center for Drug Evaluation and Research), support the concept that two *in vivo* genotoxicity assays, in appropriate tissues and with documented exposure, outweigh the positive results in mammalian cell assays *in vitro*, such as CA and MLA. Based on this concept, it follows that an effective test battery comprising an Ames test and two *in vivo* end points should be equally acceptable. Furthermore, the revisions promote the integration of *in vivo* genetic toxicity studies into existing toxicology studies where possible, thus saving animals and taking advantage of the exposure and other toxicological information generated in the toxicity study. The guidance contains stringent criteria for establishing that the dose level is high enough to maximize sensitivity *in vivo*. At present, there is sufficient information to support this integrated approach for the micronucleus assay but there is limited experience with the Comet assay, and therefore the guidance notes that protocol adaptations may be needed to allow integration. If repeat-dose toxicology studies are not appropriate for genotoxicity assays then the option to revert to the standard battery may be followed. Additional reductions in animal use are enabled by eliminating the requirement for concurrent positive controls in every *in vivo* assay, provided the laboratory has demonstrated appropriate competence with the assay and reproducible results with positive controls. Also, a reduction in nonrelevant positive results in the *in vitro* assays is expected to reduce animal use in follow-up testing. It will be interesting to see the impact these revisions will have on reducing the high frequency of nonrelevant positive results in the new genetic toxicology test battery.

5.1 Translational Medicine and Biomarkers of Genetic Damage

Different cytogenetic end points have been employed to assess the burden of genetic damage, particularly for human populations. However, these methods have traditionally suffered from the limited number of countable cells and potential individual differences in analysing cells (both in terms of investigator bias and interindividual variability). They are also influenced by confounders such as age, gender, overall health status, immune status, smoking habits and other environmental exposures, and so on, and therefore have not provided reliable translational biomarkers of individual genetic damage.

One of the most promising new developments for a translational biomarker of individual genetic damage has been the automated measurement of MN in reticulocytes, that is, immature, RNA-containing erythrocytes. Since the spleen eliminates micronucleus-containing reticulocytes in humans, previous data concerning this end point were limited to splenectomized individuals (Schlegel and MacGregor, 1984; Tucker *et al*., 1993; Zuniga *et al*., 1996; MacGregor *et al*., 1997; Smith *et al*., 1990). Now, methods have been found to enrich very young reticulocytes from peripheral blood samples before they are trapped by the spleen (Abramsson-Zetterberg *et al*., 2000; Dertinger *et al*., 2003; 2004). The assay relies on the identification, selection and evaluation of the youngest fraction of reticulocytes, that is, those less than 48 hours old, representing ~10% of circulating reticulocytes. These cells are identified by the expression of a specific cell surface marker, the transferrin (CD71) receptor, which is active for only a few hours after the young reticulocyte enters the peripheral circulation. The assessment of micronucleated reticulocytes in the youngest fraction of reticulocytes mitigates against the effects of active splenic removal of micronucleated cells in various species, including rat (Schlegel and MacGregor, 1984; MacGregor *et al*., 2006; Hayashi *et al*., 2007), dog (Harper *et al*., 2007) and human (Dertinger *et al*., 2003; 2004; 2007), although this means that the 'memory' of the biomarker is relatively short with respect to acute exposures. *Plasmodium berghei*-containing erythrocytes may be used as a calibration standard (Dertinger *et al*., 2000), and the method has been validated to GLP standards as an alternative cytogenetic end point in the mouse (Torous *et al*., 2005), rat (Torous *et al*., 2000; 2003; MacGregor *et al*., 2006; Hayashi *et al*., 2007) and dog (Harper *et al*., 2007). In all three species, the analysis of micronucleated reticulocytes correlates with microscopic scoring of MN from bone marrow samples or peripheral blood. Moreover, automation using flow cytometry greatly reduces the labour of scoring, permits the evaluation of far more cells (20 000 compared with 2000 is

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Table 12 Frequency of micronucleated reticulocytes in normal human adult, juvenile and paediatric patients and following various therapeutic interventions

Patients	%MN-reticulocytes	References and comments
Healthy Cancer	0.12 (0.01–0.36) Significant increase on days 1–4	Abramsson-Zetterberg *et al*. (2000)
Healthy Cancer	0.09 ± 0.06 (0.01–0.20) Significant increase on days 2–4	Dertinger *et al*. (2003)
Healthy	0.11 ± 0.06	Dertinger *et al*. (2004)
Healthy Cancer	0.12 ± 0.06 Significant increase on days 2–4	Dertinger *et al*. (2007)
HIV mothers and Infants	Off treatment Mother: 0.12 ± 0.02 Infant: 0.17 ± 0.02 On HIV treatment >10-fold increase	Witt *et al*. (2007)
Children with sickle cell disease	Off treatment 0.22 ± 0.13 On hydroxyurea treatment 3-fold increase	Harrod *et al*. (2007)
Thyroid cancer patients treated with Radioiodine therapy (100–700 mSv)	Pretreatment 0.13 ± 0.05 Post-treatment (121 mGy) 0.56 ± 0.16	Stopper *et al*. (2005), Grawé *et al*. (2005)

standard), and this in turn provides greater reproducibility than microscopy.

Importantly, micronucleated reticulocytes have been used as a biomarker of cytogenetic damage in various human clinical studies. A summary of the human data (**Table 12**) shows that there is high consistency in the mean spontaneous frequency of micronucleated reticulocytes in normal human adult, juvenile and paediatric populations and that the baseline frequencies are similar to those previously reported for rodents and dogs (Harper *et al*., 2007). Despite interindividual variation in human populations, the intra-individual variation is low (Abrahamsson Zetterberg *et al*., 2000). Indeed, not only is the variation over time in the same individual generally substantially lower than the interindividual variation, but also that obtained for different types of chromosome aberrations determined in the same individual over time (Abramsson-Zetterberg *et al*., 2000; Andersson, 1993). Human exposure to genotoxic agents has been demonstrated to produce significant increases in mean micronucleated reticulocyte frequencies in blood samples obtained from various treatment groups. For example, cancer patients receiving various chemotherapy treatments (Abramsson-Zetterberg *et al*., 2000; Grawé *et al*., 2005; Stopper *et al*., 2005; Dertinger *et al*., 2007); HIV infected mothers and their infants receiving antiretroviral therapy to prevent vertical transmission of the HIV virus (Witt *et al*., 2007); and children with sickle cell disease receiving hydroxyurea treatment (Harrod *et al*., 2007). Furthermore, micronucleated reticulocyte frequencies are negatively correlated with folate status (Abramsson-Zetterberg *et al*., 2006) and positively correlated with smoking (Offer *et al*., 2005). Thus, the assessment of micronucleated reticulocytes in peripheral blood provides an opportunity for a translational biomarker of acute genetic damage, from preclinical species into the clinic and/or for human biomonitoring studies. Whether or not this will lead to an increase in the assessment of genetic damage in human remains to be seen. A debate as to the impact of low levels of acute genetic damage with respect to long-term risk in humans will be required beforehand.

6 CONCLUSION

The assessment of the potential genotoxicity (and carcinogenicity) of a new chemical entity is an essential part of product development in the pharmaceutical, chemical and consumer-product industries. Genetic toxicology is a complex science because there are many different types of genetic damage, caused by a variety of different genotoxic agents and chemicals. The challenge for genetic toxicology is to provide tests which are accurate. For safety assessment there has to be a high degree of certainty that mutagenic carcinogens will be detected and human exposure limited. For hazard assessment there has to be a high degree of certainty that noncarcinogens are not misclassified, as this can lead to

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scare stories, needless concern about chemicals that are safe and rejection of potentially valuable compounds. During the process of development, compound-related genotoxicity and carcinogenicity findings are assessed and are considered as part of the risk–benefit continuum based on intended use (including clinical indication for drugs) and anticipated human exposure, and are regulated accordingly.

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Cytogenetics

Diana Anderson, Adolf Baumgartner and Eduardo Cemeli

CONTENTS

1 INTRODUCTION

There are various types of cytogenetic change which can be detected in chromosomes; in particular, structural chromosome aberrations (CAs), sister chromatid exchanges (SCEs), and numerical changes which could result in aneuploidy, polyploidy or micronuclei (MN). CA assays are used to detect the induction of chromosome breakage (clastogenesis) in somatic and germinal cells by direct observation of chromosomal damage during metaphase analysis, or by indirect observation of MN. CAs detected with these assays are mostly lethal to the cell during the cell cycle following the induction of the damage. The presence of CAs, however, indicates a potential to induce more subtle and therefore transmissible chromosomal damage, which survives cell division to produce heritable cytogenetic changes. Cytogenetic damage is usually accompanied by other genotoxic damage, such as gene mutation.

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1.1 Cytogenetic Damage and its Consequences

Structural and numerical chromosomal aberrations in somatic cells are involved in the aetiology of neoplasia (Beckmann *et al*., 1997); while in germ cells they can lead to perinatal mortality, dominant lethality or congenital malformations in the offspring (Chandley, 1981; Anderson *et al*., 1999; Marchetti and Wyrobek, 2005) and some congenital tumours (Anderson *et al*., 1999; Nomura *et al*., 2004). Over 80% of all structural chromosomal aberrations in humans occur *de novo* and are of paternal origin (Thomas *et al*., 2006). Chromosome defects arise at the level of the individual chromosome or at the level of the chromosomal set, so affecting number of chromosomes.

1.2 Individual Chromosome Damage

Damage to individual chromosomes consists of breakage of chromatids, which must result from a discontinuity of both strands of the DNA in a chromatid. How mutagens produce chromosome breakage is complex and not fully understood, but DNA lesions which are not in themselves discontinuities will produce breakage of a chromosome as a consequence of their interference with the normal process of DNA replication. Important in making the initial DNA lesion are reactive oxygen and nitrogen species, which can produce DNA damage either directly or indirectly via lipid peroxidation and formation of etheno-DNA adducts (Martinez *et al*., 2003). In haploid micro-organisms and prokaryotes chromosome breaks are usually lethal, but in diploid eukaryotes this is not so. In these organisms chromosome breaks may reconstitute in the same order as a result of nonhomologous end joining or homology-directed repair of DNA double-strand breaks, resulting in no apparent cytogenetic damage (van Gent and van der Burg, 2007). However, unrepaired or misrepaired breaks could result in CAs (Iliakis *et al*., 2004) and thus most of these aberrations may contribute to mutagenesis and carcinogenesis (Hanawalt *et al*., 2003). DNA damage may even result in cell death (apoptosis) at the next or following mitoses—if, for example, unrepaired chromosomal fragments are introduced into the zygote via an affected germ cell, the embryo may die at a very early stage from a dominant lethal mutation (Fukamachi *et al*., 2001). It has been shown that oocytes can tolerate via their repair capacity only $<8\%$ sperm DNA damage (Ahmadi and Ng, 1999). Open DNA ends upon chromosome breaks are exposed to nucleolytic attacks (Fisher and Zakian, 2005) and prone to loss of genetic material, hence the necessity to efficiently repair at any cost. When chromosome breaks are then rejoined in a different order from the original one, chromosomal rearrangements are the consequence (Bryant, 2004). Hence, chromosome-type and chromatid-type aberrations but not SCEs can predict cancer risk (Norppa *et al*., 2006). There are various types of chromosomal rearrangements (ISCN, 2005):

Chromosomal exchanges are a result of the rearrangement of segments between two chromosomes due to one or more breaks in each of these chromosomes and, depending on the position of the centromeres in the rearranged chromosomes, different configurations will result (see **Figure 1** for the most common aberrations).

1. Asymmetrical exchanges arise when one of the rearranged chromosomes carries both centromeres while the other carries none. These chromosomal rearrangements are known as a *dicentric* (*dic*) and an *acentric* (*ace*) fragment. The somatic or germ cell carrying this anomaly usually dies, death being caused by segregation difficulties of the dicentric fragment or the loss of the acentric fragment at cell division. Such a chromosomal rearrangement contributes to dominant lethality. However, cells carrying a dicentric fragment could in $\leq 50\%$ of the cases progress past mitosis, causing various other disruptions via bridge–breakage–fusion events (Gisselsson *et al*., 2000; Krishnaja and Sharma, 2004).
2. *Reciprocal translocations* (*t*), which are symmetrical exchanges, occur when each rearranged chromosome carries just one centromere. This allows the zygote to develop normally, but when such heterozygotes form germ cells at meiosis, about half of their gametes will be genetically unbalanced, since they have deficiencies and duplications of chromosomal material (Benet *et al*., 2005). The unbalanced gametes which survive produce unbalanced zygotes, which results in death shortly before and after birth, or congenital malformations (Munne, 2002).

Robertsonian translocations (rob) originate through centric fusion of two acrocentric chromosomes (chromosomes 13, 14, 15, 21 or 22) to produce a single metacentric or submetacentric chromosome by deleting the short arms. These rearrangements are very common in humans (Bandyopadhyay *et al*., 2002). When Robertsonian translocations are produced in a germ cell through a breakage and fusion in the short arms, as a consequence of loss of the derived acentric fragments a genetic deficiency can result. Some Robertsonian translocations are able to survive but others pose a risk. In heterozygotes, the two arms of the derivative chromosome may pair with the two separate homologous chromosomes at meiosis but segregate in a disorderly manner. Some of the resulting germ cells lack copies (nullisomy) or carry two copies (disomy) of one or the other of the two chromosomes involved, which results in monosomic or trisomic embryos. Monosomic embryos die early but trisomic embryos, which carry three copies of a chromosome, can survive to birth or beyond. If chromosome 21 is involved in the translocation, it can form a translocation trisomy

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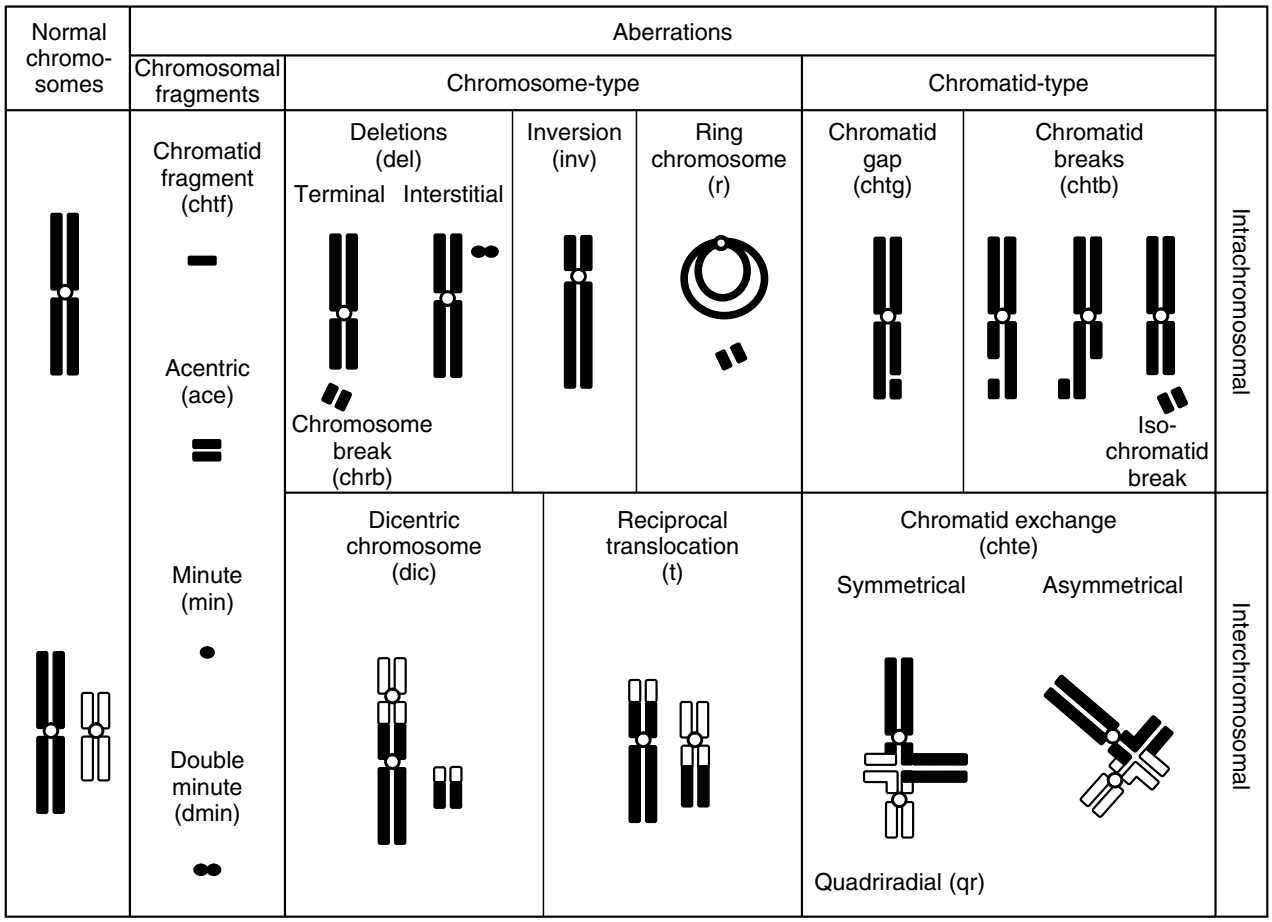

Figure 1 Overview of frequent chromosome-type and chromatid-type aberrations after a clastogenic or chemical insult. These aberrations can occur within a single chromosome (intrachromosomal) or between two or even more chromosomes (interchromosomal). The chart also shows the designations for chromosomal fragments being mostly generated after chromosomal damage. Note that even if the aberrations for a terminal deletion and an isochromatid break seen under the microscope are the same, the mechanisms to form such aberrations (chromosome break vs. chromatid break) are different. A more detailed description of various other aberrations and their abbreviations can be found in An International System for Human Cytogenetic Nomenclature (ISCN, 2005).

and produce inherited Down's syndrome (this differs from nondisjunctional Down's syndrome trisomy).

Ring chromosomes (r) can either derive from one or from more than one chromosome. For the latter possibility, the ring chromosome can even carry several centromeres. However, a ring chromosome does not necessarily need to have a centromere. For instance, a ring chromosome with one centromere normally originates from two subtelomeric breaks on either arm within the affected chromosome deleting the terminal chromosomal material as an acentric fragment. Bridge–breakage–fusion events of ring chromosomes as well as dicentrics, during the cell cycle may even further contribute to structural aberrations and genetic heterogeneity (Gisselsson *et al.*, 2000), especially in the germ line.

Inversions (inv) arise when two breaks occur in the same chromosome. For *paracentric inversions* these breaks are located on the same arm; for *pericentric inversions* the breaks occur on either side of the centromere. The portion between the two breaks is detached and becomes reinserted in the opposite way to its original position, that is, the gene order is reversed. This need not cause a genetic problem in somatic cells, but imbalanced gametes could result in congenital malformation or foetal death.

Deletions (del) and deficiencies (loss of chromosomal material) are produced interstitially when two breaks arise close together in the same chromosome. The two ends of the chromosome join when the fragment between the breaks becomes detached. Terminal deletions on the other hand can cause the loss of a chromosome end. At the next cell division the unattached piece of chromosomal material is likely to be lost. Large deletions may contribute to dominant lethality. Small cryptic deletions are difficult to distinguish from point mutations. Deletions may uncover pre-existing recessive genes. If one gene that is essential for survival is uncovered, it can act as a lethal in a homozygote and as a partial dominant in a heterozygote.

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Various types of cytogenetic events can lead to neoplasia in humans, such as reciprocal translocations or nonreciprocal rearrangements with chromatin loss, deletions or duplication of whole chromosomes or chromosome segments (Gilbert, 1983; Solomon *et al*., 1991; Albertson, 2006). Cytogenetic aberrations have been found in over 45 000 human neoplasms and tumours linked to specific structural balanced rearrangements (Croce, 1987; Mitelman *et al*., 2004). Such changes can cause the elimination of tumour suppressor genes, resulting in malignancy, or activation of proto-oncogenes (Suhardja *et al*., 2001; Yang *et al*., 2007). The mechanisms by which proto-oncogenes become activated to their oncogenic forms in tumour cells include, as well as single-point mutations, deletions, inversions and translocations of gene material between chromosomes creating for instance fusion genes, as well as gene amplification (O'Connell, 2003; Mitelman *et al*., 2004; Peter *et al*., 2005; Albertson, 2006). The consequence of this genetic change may be the altered production of an otherwise normal gene product (Cox and Goding, 1991). This can occur either by increasing the expression rate of the gene (transcriptional activation) or by post-transcriptional stabilization of the messenger RNA (mRNA) or the final protein product. Both mechanisms have been seen in Burkitt's lymphoma cells and in mouse plasmacytomas induced by pristane (CAS 1921-70-6) treatment. In these tumours the normal cellular MYC (c-Myc) gene, which is involved in the control of cell proliferation, is translocated to the immunoglobulin locus (Hecht and Aster, 2000; Boxer and Dang, 2001; Janz, 2006). This initiates a dysregulated expression of the MYC proto-oncogene at 8q24.21 (Rui and Goodnow, 2006) and causes elevated levels of the MYC protein or inability to switch off the gene at the appropriate time, and finally uncontrolled cell proliferation (Gartel and Shchors, 2003). Thus, chromosome rearrangements may induce neoplasia by activating a potential oncogene that is a proto-oncogene (Bos, 1992).

Another mechanism operates in the case of human chronic myeloid leukaemia (CML). A proportion of cases of this disease carry the 'Philadelphia chromosome'. This term depicts the derivative chromosome 22 resulting from a reciprocal t(9;22)(q34;q11) translocation (ISCN, 2005; Jorgensen and Holyoake, 2007). As a consequence the ABL1 (c-ABL) proto-oncogene on chromosome 9 (9q34.1) becomes joined with the BCR region on chromosome 22 (22q11.23) resulting in the expression of a fusion protein encoded by both DNA sequences (Shtivelman *et al*., 1985; Jorgensen and Holyoake, 2007). The exact mechanism of this fusion protein causing cell transformation was poorly understood (Maru, 2001); however, in recent years the molecular underlying mechanism became clear and led to the development of specific BCR-ABL tyrosine kinase inhibitors like imatinib mesilate (Gleevec™) or dasatinib (Sprycel®), which allow a targeted approach in treating CML (Melo *et al*., 2003; Panigrahi and Naithani, 2006; Steinberg, 2007).

The tumour state can also be inherited. Most hereditary predispositions affect only one particular cell type, however; the genes with the relevant germ-line mutation are not cell-type specific and thus increase the risk to unrelated cell types (Bignold, 2004). Retinoblastomas and osteosarcomas, for instance, develop in children who inherit a defective chromosome 13 from one parent. The defect has been shown to involve a deletion or mutation in the RB1 gene at the 13q14.2 locus (Issing *et al*., 1993; Houdayer *et al*., 2004). Tumours arise when the normal copy of the same gene on the other chromosome is lost or mutated in early childhood. This demonstrates that the gene when present in a functional state has suppressive effects on the development of tumours. Genes on chromosome 11 (e.g. WT1 at 11p13 or H19 at 11p15.5) are also frequently lost or epigenetically modified and thus silenced in tumours or in children with the Tay–Sachs familial variant of Wilms' tumour (Koufos *et al*., 1985; Fukuzawa *et al*., 1999). The predisposition *per se* is due to the inheritance of a defective allele of a tumour suppressor (Rohde *et al*., 1997).

1.3 Chromosome Set Damage

Accuracy of chromosome replication and segregation of chromosomes to daughter cells require accurate maintenance of the chromosome complement of an eukaryotic cell (Ghosh *et al*., 2006). Chromosome segregation in meiosis and mitosis is dependent upon the synthesis and functioning of the proteins of the spindle apparatus and upon the attachment and movement of chromosomes on the spindle. The kinetochores attach the chromosomes to the spindle and the centrioles are responsible for the polar orientation of the division apparatus (Taylor *et al*., 2004; Bloom, 2005). Sometimes such segregation events proceed incorrectly and homologous chromosomes separate, with deviations from the normal number (aneuploidy) into daughter cells or as a multiple of the complete karyotype (polyploidy). When both copies of a particular chromosome move into a daughter cell and the other cell receives none, the event is known as nondisjunction (Duesberg *et al*., 1999; Hassold *et al*., 2007).

Aneuploidy in live births and abortions arises from aneuploid gametes during germ cell meiosis. Trisomy or monosomy of large chromosomes leads to early embryonic death. Trisomy of the smaller chromosomes allows survival but is detrimental to the health of an affected person (Nagaishi *et al*., 2004); for example Down's syndrome (trisomy 21), Patau syndrome (trisomy 13) and Edward syndrome (trisomy 18) (Altug-Teber *et al*., 2007). Sex chromosome trisomies (Klinefelter's syndrome and XXX syndromes) and the sex chromosome monosomy (XO), known as the Turner syndrome, are also compatible with survival.

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Aneuploidy in somatic cells is involved in the formation of human tumours (Duesberg and Rasnick, 2000; Bignold, 2007). The 'Mitelman Database of Chromosome Aberrations in Cancer' gives a detailed overview of how chromosomal aberrations relate to tumour characteristics (Schaefer *et al*., 2001; CGAP, 2008). An abnormal number of chromosomes seems to be the primary cause of the genomic instability in neoplasms, due to destabilization of the karyotype and the genes (Atkin, 2003; Duesberg *et al*., 2004). Up to 10% of tumours are monosomic or trisomic for a specific chromosome as the single observable cytogenetic change. Most common among such tumours are trisomies 8, 9, 12 and 21 and monosomy for chromosomes 7, 22 and Y (Dey, 2004).

Thus, chromosome changes can have severe consequences in cells and whole organisms, and this present chapter will be concerned with cytogenetic assays which measure gross chromosome changes in human somatic and germ cells *in vitro* and *in vivo*. Various regulatory guidelines have been developed to measure cytogenetic damage at the national level; for example by the US Environmental Protection Agency (EPA), the UK Environmental Mutagen Society (UKEMS), at the European level in the Official Journal of the European Community (OJEC), but most importantly at the international level of the Organisation for Economic Cooperation and Development (OECD). The OECD currently has 30 member states and the membership includes many of the world's major industrial nations. However, it is important to note that a number of significant states are not members, including the BRIC countries (Brazil, Russia, India and China): of course, this does not prevent contract houses in India and Brazil being able to perform studies to OECD guidelines. The pharmaceutical industry has agreed a harmonized approach through the acceptance and implementation of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and the ICH issues guidelines for pharmaceuticals in three major economic regions: Europe, Japan and the USA. The veterinary equivalent to the ICH is the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) and the objective of the VICH is the issuance of guidelines for veterinary pharmaceuticals. The OECD, ICH and VICH guidelines constitute the three major sets of internationally harmonized genotoxicity guidelines in regulatory use.

2 *IN VITRO* CYTOGENETICS ASSAYS

2.1 Introduction

The *in vitro* cytogenetic assay is a short-term mutagenicity test for detecting chromosomal damage in cultured mammalian cells, for example human lymphocytes. For cultured cells, techniques most frequently used for cytogenetic evaluation are the micronucleus assay (MNA), the chromosome aberrations test (CAT) and the sister chromatid exchange test (SCET). MNA provides information on DNA damage, cytostasis and cytotoxicity, while CAT also covers DNA damage, but additionally it might reveal the precise chromosomal rearrangement which has occurred. However, metaphase spreads are required for the evaluation of these rearrangements using transmissive light microscopy. These classical techniques can be combined with modern fluorescence *in situ* hybridization (FISH) methods like spectral karyotyping (SKY) in order to provide a more detailed assessment. For instance, if CAT is combined with FISH, specific regions of a chromosome can be pinpointed and the rearrangements can be tracked. In MNA, two types of micronuclei are generated: those originating (i) from acentric chromosomal fragments and/or (ii) from nondisjunction of whole chromosomes. The use of FISH on MNA preparations discerns whether the genetic material within the micronucleus is due to clastogenesis (acentric fragments) or aneugenesis (mis-segregated chromosomes bearing a centromere). Matsuoka and colleagues proposed a simple way to distinguish aneugens from clastogens, based on the capability of aneugens to induce a larger number of polynuclear and mitotic cells than clastogens (Matsuoka *et al*., 1999). MNA is a system originally developed for the detection of genetic material in interphase cells that does not migrate to the corresponding poles. It also measures other events such as nucleoplasmic bridges (NPBs), nuclear buds (NBUDs), cell death (necrosis or apoptosis) and the nuclear division index (NDI). In recent years, MNA has evolved into a 'cytome' assay (see **Figure 2**) for comprehensively measuring a chromosomal instability phenotype and altered cellular viability caused by genetic defects and/or nutritional deficiencies and/or exogenous genotoxins (Fenech, 2006). As stated in the OECD guidelines 473 and 487 (OECD, 1997a; 2007) for testing chemical compounds, MN represent damage that has been transmitted to daughter cells whereas CA scored in metaphases may not be transmitted. Because MN in interphase cells can be assessed relatively objectively, laboratory personnel need only to determine whether or not the cells have undergone division and how many cells contain MN. As a result, the preparations can be scored relatively quickly and analysis can be automated. This makes it practical to score thousands instead of hundreds of cells per treatment, increasing the statistical power of the assay. Finally, there is the potential to detect aneuploidy-inducing agents that are more difficult to study with CAT, although MNA does not allow identification of polyploidy (OECD, 2007). Current effort is being addressed to validate a fully automated system for scoring MN. It is worth taking into account

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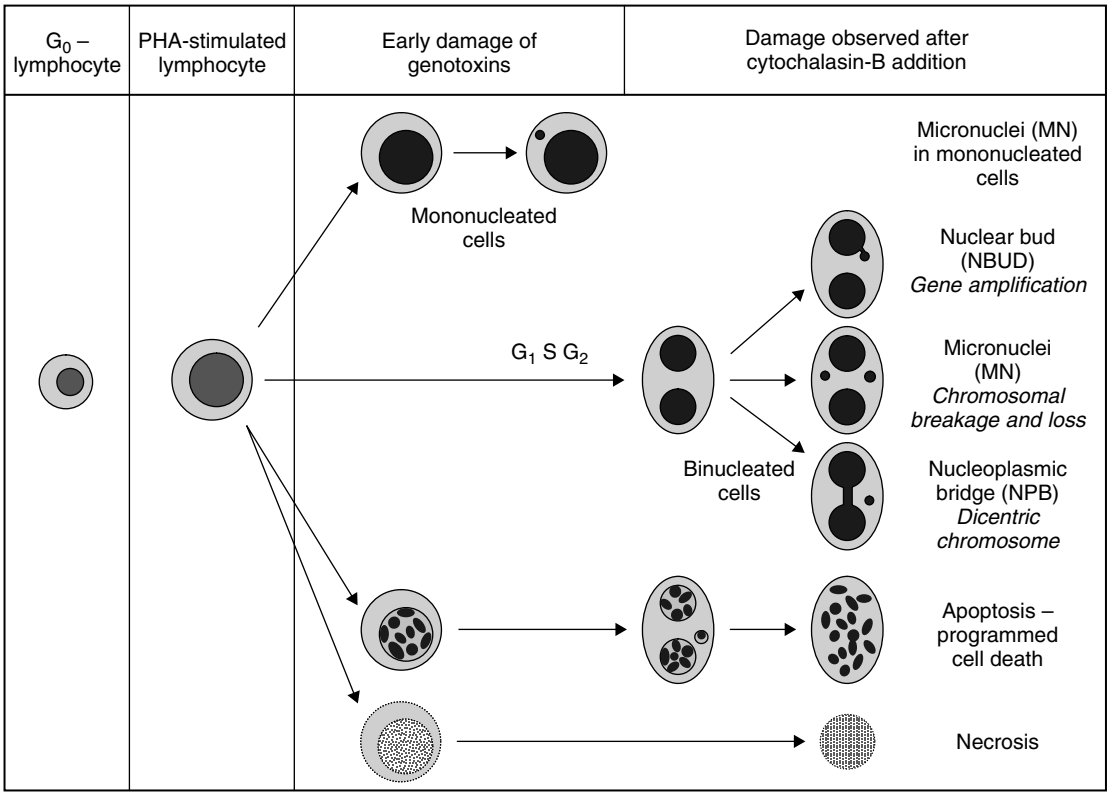

Figure 2 Based on the cytome assay (Fenech, 2006) this outline shows possible chromosomal damage after a genotoxic treatment. As cytochalasin-B does not allow cytokinesis but only the generation of daughter nuclei, the damage seen in binucleated cells is MN resulting from chromosomal breakage and loss, nucleoplasmic bridges due to dicentric chromosomes, and nuclear buds as a consequence of gene amplification. Additionally, MN in mononucleated cells can be observed as well as apoptotic and necrotic events.

that these assays can be used as complementary and/or as a substitute for each other.

Since MNA and CAT are complementary, it has been considered whether it is necessary to use both simultaneously for testing purposes. Kirkland *et al*. (2005) state that there is evidence that the majority, if not all, clastogens are detected by the induction of MN, or are positive in the Ames test, or are inducing mutations in the mouse lymphoma assay. All suspected aneugens, however, induce MN *in vitro* when tested according to current rigorous recommendations. Thus, there is no reliable scientific basis to include both CAT and MNA in addition to the Ames test and mouse lymphoma assay. Since MNA detects both aneugens and clastogens, it alone could complement the Ames test and mouse lymphoma assay (Kirkland *et al*., 2005).

Despite the high correlation observed *in vitro* between CAT and MNA there is no formal validation for the MNA *in vitro*. Thus, the European Centre for the Validation of Alternative Methods (ECVAM) has recently performed a retrospective evaluation (Corvi *et al*., 2008). Two publications fulfilled the criteria established to evaluate the reliability of the test and these were (i) the German ring trial (von der Hude *et al*., 2000) and (ii) the *in vitro* MNA with Chinese hamster V79 cells: results of a collaborative study with 26 chemicals (Lorge *et al*., 2006). It was concluded that the MNA is reliable and relevant and can be used as an alternative method for CAT. Consequently, there are concerns over whether this conclusion can be extrapolated *in vivo*. Hence, the situation *in vivo* is rather unclear and as yet no one has provided a definitive answer.

The cytogenetic techniques mentioned herewith, namely, MNA, CAT and SCET are employed for assessment of biomarkers of exposure. For years, the outcomes of cytogenetic monitoring studies have been assessed to ascertain whether there could be a positive relationship between higher frequencies of CAs or MNs and prediction of cancer. A great variety of results have been observed, and some studies displayed a positive relationship with cancer (Rossner *et al*., 2005; Norppa *et al*., 2006; Boffetta *et al*., 2007; Bonassi *et al*., 2007; 2008). However, this was strongly dependent on the end point, because one end point provided a positive outcome while the other was negative (Hagmar *et al*., 1998a; 1998b; Norppa *et al*., 2006). Occasionally, negative correlations were due to a lack of statistical

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power or lack of follow-up studies. Another issue is the presence of confounding factors. Gender, age, smoking habits, diet, occupational exposure and in-house levels of radon, among other factors, certainly influenced these results. Thus, an extensive questionnaire is necessary when monitoring (Carrano and Natarajan, 1988). Some of these confounding factors such as age and gender are known to increase the frequency of MN and CA (Surralles *et al*., 1996; 1999; Fenech, 1998). However, other factors such as smoking still provide contradictory data (Fenech *et al*., 2003; Mateuca *et al*., 2006). Mostly lymphocytes are employed as a reporter/surrogate tissue for monitoring, although current effort is being addressed in exfoliated cells (Fenech *et al*., 2007; HUMN, 2008) and reports are already available using buccal cells for monitoring (Pastor *et al*., 2002; Martinez *et al*., 2005; Thomas *et al*., 2007; 2008).

2.2 Cell Types

Established cell lines, cell strains or primary cell cultures may be used for cytogenetic evaluation of chromosomal damage, for example Chinese hamster cells, Syrian hamster embryos, mouse lymphoma L5178Y cells, human or other mammalian peripheral blood lymphocytes. The particular cell system must be validated and consistently sensitive to known clastogens. The United States EPA indicates that the cells employed for *in vitro* CAT must be selected on the basis of stability of the karyotype, chromosome number, chromosome diversity and spontaneous frequency of CAs (EPA, 1998a). Likewise, the background frequency of MN will influence the sensitivity of the assay, as it is recommended that cell types with a low, stable background frequency of micronucleus formation should be used (OECD, 2007).

When human peripheral lymphocytes are used for MNA they should be obtained from young, healthy, nonsmoking individuals with no known recent exposures to genotoxic chemicals or radiation. If cells from more than one donor are pooled for use, the number of donors should be specified (OECD, 2007). It has to be borne in mind that the MN frequency increases with age and this trend is more prominent in females than in males (Norppa *et al*., 2006). On the other hand, Chinese hamster cell lines have a small number of large chromosomes (11 pairs). Chinese hamster ovary (CHO) cells in which there has been an extensive rearrangement of chromosome material, and the chromosome number may not be constant from cell to cell, are frequently used (Doppalapudi *et al*., 2007; Tayama *et al*., 2008). Polyploidy, endoreduplication and high spontaneous CA frequencies can sometimes be found in these established cell lines, but careful cell culture techniques should minimize such effects. Therefore, cells should be treated in their exponential growth phase when cells are in all stages of the cell cycle.

2.3 Solvents, Positive and Negative Controls

When the solvent of a test compound differs from the culture medium or water, the solvent by itself, a mixture of solvent and S9 mix as well as an untreated control without this particular solvent have to be used as negative controls. Since cultured cells are normally treated in their usual growth medium, the solubility of the test material in the medium should be ascertained before testing. Various organic solvents are used, such as dimethyl sulfoxide (DMSO), dimethylformamide, ethanol or acetone. However, where possible, water should be used with the consideration that water greater than 10% (v/v) can be toxic because of nutrient dilution and osmolality change. The solvent/vehicle should (i) not react with the test substance, (ii) be incompatible with the survival of the cells and (iii) should not interfere with S9 activity at the concentration used (OECD, 2007). Extremes of pH can be clastogenic. It has been shown that low pH (pH 6.6) leads to CA and SCE in CHO cells and that these effects are S-phase dependent (Morita, 1995). Osmolality conditions must be maintained (Kirkland, 1992).

A known clastogen should always be included as a positive control. When metabolic activation via S9 mix is used, a positive control chemical known to require metabolic activation should also be used to ensure that the system is functioning properly. Without metabolic activation, a direct-acting positive control chemical should be used (**Table 1**).

2.4 Treatment of Cells

Cultured cells *per se* might not be able to activate various potential clastogens. For this reason, the effects of metabolic activation might be determined by supplementing the treatment regime with S9 mix or by using genetically engineered cells which express biotransformation enzymes (Parry *et al*., 1995). In the latter case, the choice of the cell line used should be scientifically justified (Doherty *et al*., 1996). The phase of the cell cycle during which they are treated poses variations regarding the sensitivity towards clastogens, and thus, the number of MN, CA and SCE induced varies. For this reason, continuous treatment is preferable. This, however, is not a problem when treating in the absence of S9 mix, but treatment over extended periods of time is not advisable in the presence of S9 because it becomes cytotoxic (O'Hare and Atterwill, 1995). Treatments of about three hours are usually carried out. However, cells may be treated with chemicals either continuously up to harvest time or for a short time followed by washing and addition of fresh medium to allow cell cycle progression. When metabolic activation is used, S9 mix should not exceed 1–10% (v/v) within the culture

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Table 1 Recommended reference chemicals for MNA and CAT (OECD, 2007)

Category	Chemical	CAS number
1. Direct acting clastogens	Cytosine arabidinose	147-94-4
	Mitomycin C	50-07-7
2. Clastogens requiring metabolic activation	Benzo(a)pyrene Cyclophosphamide	50-32-850-18-0
3. Aneugens	Colchicine	64-86-8
	Vinblastine	143-67-9
4. Negative substances	Di(2-ethylhexyl)phthalate	117-81-7
	Nalidixic acid	389-08-2
	Pyrene	129-00-0
	Sodium chloride	7647-14-5

medium (EPA, 1998a). It has been shown that the S9 mix is clastogenic in CHO cells and mouse lymphoma cells (Cifone *et al*., 1987; Kirkland *et al*., 1989) but not in human lymphocytes, where blood components, for example catalase, can inactivate active oxygen species which could cause chromosome damage. When S9 mix from animals treated with other enzyme-inducing agents, such as phenobarbital/beta-naphthoflavone, is used, clastogenesis may be minimized (Kirkland *et al*., 1989).

Prior to testing, it is necessary to determine the cytotoxicity of the test material, in order to select a suitable dose range for the *in vitro* cytogenetic assay both with and without metabolic activation. The range most commonly used determines the effect of the agent on the mitotic index (MI) in CAT and the NDI, replication index (RI) or cytochalasin-B proliferation index (CBPI) in MNA. For CAT, the highest dose should inhibit mitotic activity by approximately 50% (EPA, 1998a; ECCD, 2000), 75% (Scott *et al*., 1990) or exhibit some other indication of cytotoxicity. If the reduction in MI is too great, insufficient amounts of cells can be found for chromosome analysis. At least 1000 cells per culture are required for the preliminary study in order to determine the MI. For MNA, the CBPI, RI or NDI should be determined from at least 500 cells per culture following the method of Eastmond and Tucker (1989). If treatments are performed without cytochalasin-B, then it is necessary to provide evidence that the cells being scored have proliferated in the culture (EPA, 1998a). Large numbers of publications provide this value as CBPI (Decordier *et al*., 2007; Paiva *et al*., 2008; Yuzbasioglu *et al*., 2008) or NDI (Colognato *et al*., 2007; Yavuz-Kocaman *et al*., 2008). The formula to calculate the NDI is provided by Fenech's protocol in the cytokinesis-block micronucleus cytome assay (Fenech, 2007). The lowest value possible is 1.0, which is only obtainable if all cells fail to divide; however, if all viable cells complete one cell division, then the value is 2.0. The value can only be greater than 2.0 if a substantial proportion of the cells have completed more than one nuclear division (Fenech, 2007). Fenech also describes a more accurate assessment of nuclear division status and provides a modified formula (Fenech, 2000). The inclusion of necrotic and apoptotic cells in the total number of cells scored at higher toxic doses of chemicals provides more accuracy since it can be expected that a proportion of the cells become nonviable. It is therefore important to note that both the binucleated (BN) cell ratio and the NDI are overestimated if necrotic and apoptotic cells are not included when scoring cells (Fenech, 2000).

According to the OECD guidelines, the highest concentration should aim to produce $50 \pm 5\%$ cytotoxicity (OECD, 2007). It is stated in all guidelines that if no cytotoxicity or precipitate is observed, the highest test concentration should correspond to 0.01 M, 5 mg ml^{-1} or 5 µl ml^{-1}, whichever is the lowest and, moreover, the chemical must be tested up to the maximum solubility in the treatment medium and not just the maximum solubility in stock solutions (Scott *et al*., 1990; EPA, 1998a; OECD, 2007).

A minimum of three doses of the test material should be used—the highest chosen as described above, the lowest on the borderline of toxicity and an intermediate one. Up to six doses can be managed satisfactorily, and this ensures the detection of any dose response and that a toxic range is covered. Cells from only three doses need to be analysed.

2.5 Treatment Schedule

Duplicate cultures should be applied to all concentrations investigated, also being strongly recommended for negative/solvent cultures. Single cultures might be acceptable if minimal variations are found when compared to historical data.

For MNA, Fenech (2007) provides a protocol described in a stepwise fashion and it also incorporates the material and equipment required for its performance.

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For complementary information, the OECD guideline 487 is recommended (OECD, 2007). The performance of CAT must be in compliance with the EC Commission Directive 2000/32/EC–B.10 (ECCD, 2000), the OECD guidelines (OECD, 1997a) or the EPA recommendations (EPA, 1998a), which also supplies detailed protocols. Despite the comprehensive information provided in the above-mentioned publications and guidelines, some remarks are necessary. Firstly, the treatment schedule for cell lines and primary cells might differ somewhat from that of lymphocytes which require mitogenic stimulation to begin the cell cycle (Fenech *et al*., 2003). Secondly, it has to be borne in mind that most aneugens and clastogens exert their effects in a short time (3–6 hours) in the presence and absence of S9 (Lorge *et al*., 2006), followed by removal of the test substance and a growth period of 1.5–2 cell cycles. Cells are sampled at a time equivalent to 1.5–2.0 times the cell cycle; however, the sampling time might be extended if it is known or suspected that the test substance might disrupt the cell cycle. If treatment is extended, the presence of S9 mix could be reduced unless compensated for. In the case of MNA, options are offered to allow treatment of the cells with the test chemical in the absence or presence of cytochalasin-B (OECD, 2007). For CAT, a repeated test should include an additional sample at approximately 24 hours if a first experiment produced negative results (OJEC, 1984).

Taking into account the differential sensitivity of the cell towards the chemical depending on the phase of the cell cycle, and therefore variation in the outcome, the most efficient approach for MNA is to test lymphocytes 44–48 hours after phytohaemagglutinin (PHA) stimulation (Fenech, 2007). This is also the case for CAT. This is because cell cycle synchronization has dissipated.

2.6 Scoring Procedures

Prior to scoring, slides should be coded, randomized and then scored 'blind'. Scoring should only be carried out by an experienced observer who knows the criteria. Matching more than one scorer is also advisable. A detailed description of the criteria for scoring with the cytokinesis-block MNA using isolated human lymphocyte cultures was published in 2003 by Fenech and colleagues participating in the Human MicroNucleus (HUMN) project (Fenech *et al*., 2003; HUMN, 2008). However, the most updated criteria for MN scoring have been presented by Fenech (2007), which indicates several parameters to be considered: (i) number of viable mononucleated, binucleated, multinucleated cells per 500 cells scored; (ii) number of apoptotic cells per 500 cells; (iii) Number of necrotic cells per 500 cells; (iv) number of MN in at least 1000 BN cells; (v) frequency of BN cells containing MN in at least 1000 BN cells; (vi) frequency of BN cells containing NPBs in at least 1000 BN cells; and (vii) frequency of cells containing NBUDs in at least 1000 BN cells.

For CA, the classification and nomenclature is based on that established by the International System for Human Cytogenetic Nomenclature (ISCN, 2005). Metaphase cells should be sought under low-power magnification and those with well-spread, that is, nonoverlapping, clearly defined nonfuzzy chromosomes, should be examined under a high-power microscope with oil immersion. It is acceptable to analyse cells with total chromosome numbers or that have lost one or two chromosomes during processing. In human lymphocytes ($2n = 46$) 44 or more centromeres and in CHO cells ($2n = 22$; range 21–24) 20 or more centromeres can be scored. Chromosome numbers can be recorded for each cell, to give an indication of aneuploidy. Only cells with increased chromosome numbers (above 46 in human lymphocytes and 24 in CHO cells) should be considered in this category, since decreases can occur as artefacts through processing steps. Two hundred cells (100 from each of two replicates) should be scored per treatment group. This number can be reduced when high numbers of aberrations are observed. When ambiguous results are obtained, there may be further 'blind' reading of these samples. Although the purpose of the test is to detect structural CAs, it is important to record polyploidy and endoreduplication (EPA, 1998a).

2.7 Data Recording

For MNA, the following information should be included on a score sheet (Fenech, 2007): (i) name of the person scoring the slides; (ii) code number of each slide; (iii) number of BN cells scored; (iv) frequency of MN in 1000 BN cells; (v) frequency of BN cells containing MN in 1000 BN cells; (vi) frequency of BN cells with NPBs in 1000 BN cells; (vii) frequency of BN cells with NBUDs in 1000 BN cells; (viii) frequencies of viable mono-, bi-, tri- and tetranucleated cells, necrotic and apoptotic cells in a total of 500 cells; and (ix) NDI.

Optional additions to the score sheet include (a) the distribution of BN cells with 0, 1, 2, 3 or more MN in 1000 BN cells and (b) the coefficient of variation for duplicate estimates of the above parameters.

Likewise, the information required for CAT must contain the slide code, scorer's name, date, cell number, number of chromosomes and aberration types. These should include chromatid and chromosome gaps, deletions, exchanges and others. From the score sheets, the frequencies of various aberrations should be calculated and each aberration should be counted only once. A space for the Vernier reading, comments and a diagram of the aberration should be available. Recording microscope coordinates of cells is necessary and allows verification of abnormal cells. For cells with aberrations, a photographic record is also useful.

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2.8 Presentation and Interpretation of Results

The guidelines clearly state the information that should be included in a test report (OECD, 1997a; 2007; EPA, 1998a; ECCD, 2000). Similarly, Fenech's review (2007) provides the information that a manuscript based on the MNA must contain.

In cytogenetic assays the absence of a clear positive dose-response relationship at a particular time frequently arises. This is because a single common sampling time may be used for all doses of a test compound. CA yields can vary markedly with post-treatment sampling time of an asynchronous population, and increasing doses of clastogens can induce increasing degrees of mitotic delay (Scott *et al*., 1990). Additional fixation times should clarify the relationship between dose and aberration yield. In the case of Chinese hamster fibroblasts, for instance, a number of chemicals which gave negative responses with a fixation time of 24 hours became positive at 48 hours (Ishidate, 1988).

When it comes to the determination of a positive result, the biological relevance of the results should be considered first. Considerations such as obtained values falling within historical records or published literature can provide guidance. Adequate statistical methods must be applied, but must not be the only determinant of a positive response (OECD, 2007). In some instances, it might be worth considering an increase of the statistical power, by performing further testing. Where CA yields are on the borderline of statistical significance above control values, the inclusion of gaps could be useful (Anderson and Richardson, 1981). Further details on this approach may be found in the UKEMS guidelines (Scott *et al*., 1990).

3 *IN VIVO* CYTOGENETICS ASSAYS

Damage induced in whole animals can be detected in *in vivo* chromosome assays in either somatic or germinal cells by examination of metaphases or the formation of micronuclei. The micronucleus test in erythroblasts can also detect whole chromosome loss or aneuploidy in the absence of clastogenic activity and is considered comparable in sensitivity to chromosome analysis (Tsuchimoto and Matter, 1979; OECD, 1997b; EPA, 1998b). Rats and mice are generally used for *in vivo* studies, with the mouse being employed for bone marrow micronucleus analysis and the rat for metaphase analysis, but both can be used for either. Mice are cheaper and easier to handle than rats, and only a qualitative difference in response has been found between the species (Albanese *et al*., 1988). Chinese hamsters are also widely used for metaphase analysis because of their low diploid chromosome number of 22. However, there are few other historical toxicological data for this species.

3.1 Somatic Cell Assays

3.1.1 Metaphase Analysis

Metaphase analysis can be performed in any tissue with actively dividing cells, but bone marrow is the tissue most often examined. Cells are treated with a test compound and are arrested in metaphase by the administration of colcemid or colchicine at various sampling times after treatment. Preparations are examined for structural chromosome damage. Because the bone marrow has a good blood supply, the cells should be exposed to the test compound or its metabolites in the peripheral blood supply, and the cells are sensitive to S-dependent and S-independent mutagens (Topham *et al*., 1983; OECD, 1997c; EPA, 1998c).

Peripheral blood cells can be stimulated to divide even though the target cell is relatively insensitive (Newton and Lilly, 1986). It is necessary to stimulate them with a mitogen since the number of lymphocytes which are dividing at any one time is very low. Cells are in G_0 when exposure is taking place, so they may not be sensitive to cell-cycle-stage-specific mutagens and any damage might be repaired before sampling.

3.1.2 Micronuclei

The assessment of micronuclei is considered simpler than the assessment of metaphase analysis. This assay is most often carried out in bone marrow cells, where polychromatic erythrocytes (PCEs) are examined; damage is induced in the immature erythroblast and results in a micronucleus outside the main nucleus, which is easily detected after staining as a chromatid-containing body (OECD, 1997b). When the erythroblast matures, the micronucleus, the formation of which results from chromosome loss during cell division or from chromosome breakage forming centric and acentric fragments, is not extruded with the nucleus. Micronuclei can also be detected in peripheral blood cells (MacGregor *et al*., 1980). In addition, they can be detected in liver (Tates *et al*., 1980; Braithwaite and Ashby, 1988) after partial hepatectomy or stimulation with 4-acetylaminofluorene, or they can be detected in any proliferating cells.

3.1.3 Germ Cell Assays

The study of chromosome damage is highly relevant to the assessment of heritable cytogenetic damage. Many compounds which cause somatic cell damage have not produced germ cell damage (Holden, 1982) and, so far, all germ mutagens have also produced somatic damage. As tests for germ cell cytogenetics are not prominent in regulatory guidelines, Kirkland contacted 12 regulatory agencies in 1987, sending detailed questionnaires, and 7 responded (Kirkland, 1987). The germ cell tests are rarely requested as a matter of course. When they are

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indicated, a dominant lethal test (most often) or heritable translocation test (sometimes) would be seen as most useful, not the mouse specific locus assay, due to large animal numbers. As most widely used germ cell cytogenetic assays do not detect inherited mutations or effects on meiotic processes, they were not readily accepted as alternatives. Preston (1994) considered the future of germ cell cytogenetics. It is possible to assess numerical and structural alterations in the male in differentiating spermatogonia, spermatocytes and postmeiotic cells (at the first cleavage division), and for the female in oocytes and the zygote. He predicted that advances would be made in the identification of specific alterations through FISH of interphase spermatozoa in humans (Baumgartner *et al*., 1999). The Comet assay has also allowed the study of effects in human sperm (Anderson *et al*., 2003; Schmid *et al*., 2007).

With the reduction in use of animal studies in recent years, much of the basic research in germ cell systems was carried out two or three decades ago. Germ cell data, however, are still needed for genetic risk estimation, and testing can be performed in male or female germ cells. The former are most often used, owing to systemic effects in females. Testing in the male is performed in mitotically proliferating premeiotic spermatogonia, but chromosomal errors in such cells can result in cell death or prevent the cell from passing through meiosis. Damage produced in postmeiotic cells, the spermatids or sperm are more likely to be transmitted to the F_1 progeny (Albanese, 1987). In females it is during early foetal development of the ovary that oogonial divisions and prophase stages of meiosis up to arrested diplotene stage occur. Therefore, testing necessitates the use of pregnant mothers at suitable gestation stages. The arrested dictyate oocyte stage is the most commonly tested stage in the adult female. To test other stages during the first or second meiotic divisions demands the use of oocytes undergoing ovulation, which occur naturally or are hormone stimulated. It is thus more difficult technically to test female germ cells.

3.2 Heritable Chromosome Assays

Damage may be analysed in the heritable translocation test, which involves the examination in male F_1 animals of diakinesis metaphase I spermatocytes for multivalent association (Leonard, 1973; 1975; Cattanach, 1982). Fertilized ova can also be analysed by cytogenetic analysis (Albanese, 1987), or early embryos can be examined by metaphase analysis (Hansmann, 1973). Such techniques are demanding technically, and the heritable translocation assay requires large numbers of animals to attain appropriate sample sizes. Chromosome damage in fertilized ova or early embryos may not be compatible with survival after birth, so it is only the heritable translocation assay which provides absolute evidence of induced heritable effects. In foetal mice which have been exposed transplacentally after exposure of the mother, tissues can be assessed for micronuclei or metaphase analysis. Cole and colleagues scored MN in polychromatic erythrocytes in the liver or peripheral blood of foetal mice (Cole *et al*., 1981). Such methods have been reviewed and are used to investigate factors which might affect embryos (Henderson, 1986).

3.3 Experimental Design—General Features of *In Vivo* Assays

3.3.1 Treatment of Animals

A control group dosed with a vehicle used routinely or an untreated control will be needed in addition to vehicle controls. Water soluble materials can be administered in distilled water or isotonic saline, and the pH may require adjustment if there is a marked deviation from physiological pH. Various substances other than water can be used as controls. Although DMSO is used within *in vitro* studies, it is not wise to use it in animal studies. It is locally irritant and toxic, and increases the penetration through cell membranes, so altering absorption and distribution patterns. Methyl cellulose or carboxymethyl cellulose (0.5% up to 2%, w/v) can be used as a water-based vehicle for homogeneous suspensions obtained after milling. For those substances that are not miscible with water, corn (maize) oil can be used, but this could affect absorption rates. Tween 80 may also aid in the suspension of non-wettable materials. Routes of administration, such as gavage, intraperitoneal (ip), intradermal or intravenous (iv), can be used with these agents, but for inhalation routes an experienced inhalation toxicologist is required to advise on the most appropriate action.

It is usually necessary to determine the toxicity of the test substance unless this is available from the scientific literature. The test substance is required for regulatory purposes to be tested at the maximum tolerated dose—that is, a dose which produces some signs of toxicity such as hypoactivity, piloerection, ataxia, ptosis or a change in the body weight. Toxicity of the animals is usually observed over a 14 day observation period. In the case of the MNA, to determine toxicity to the cells, the ratio between polychromatic and normochromatic erythrocytes (normocytes or NCEs) can be measured and, in the case of metaphase analysis, MIs. These values are measured over a period up to 72 hours.

The highest dose, when toxicity is not evident, ranges from 2 to 5 g kg^{-1}, according to different regulatory guidelines (Richold *et al*., 1990). A single dose level is considered adequate for screening purposes, but for more extensive evaluation, several dose levels are required. Dose values should not normally exceed 10 ml kg^{-1} by the ip and iv route or 20 ml kg^{-1} by the oral route. There

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is often a 10-fold separation between the upper and lowest dose, and there is also an intermediate dose. Sometimes the lowest dose is selected as the most appropriate for man. Less than 2-fold separation factor for doses should not be considered.

In terms of the route of exposure, there is a need to maximize the chance of absorption or to mimic human exposure. In a collaborative study, the oral and ip routes were compared and found to be equally sensitive when toxicity is accounted for (Hayashi *et al*., 1989), and it was argued that the ip route and the route relevant to human exposure could be used sequentially in the evaluation of a compound (Shelby, 1986). However, Ashby (1985) argued against the ip route as being suitable. In general, the oral route is preferable, and dermal or subcutaneous routes are not used unless it is clear that the compound is absorbed or used by these routes (Ashby, 1985).

A concurrent vehicle control group should be included in each experiment at each sampling time in order to compare the treated groups. Untreated controls can be induced when a nonstandard vehicle is used. A positive control group should be included to confirm the sensitivity of the assay and check on the scoring. A sufficient dosage of the positive control substance is required to check on any reduced assay sensitivity.

Assays are usually performed only once, but if equivocal results are obtained, another study design or alternative dosage route may be considered for sensitivity optimization.

There are various requirements for animal husbandry and welfare, and, in the UK, project and personal licences are mandatory for animal experimentation. Various safety precautions are necessary to protect personnel from exposure to suspect mutagens and carcinogens (Ehrenburg and Wachtmeister, 1977; Waters, 1980), and special waste disposal procedures should be observed.

3.3.2 Rodent Micronucleus Test

The rodent micronucleus test can be used as a sensitive screen to assess hazard or for more extensive/qualitative hazard determinations to aid in risk assessment. Rats and mice can be used and methods are available to avoid contaminating mast cell granules in rats, which stain in a similar way to micronuclei. Fluorescent stains can be used (Hayashi *et al*., 1983; MacGregor *et al*., 1983) as well as haematoxylin and eosin (Pascoe and Gatehouse, 1986). For good-quality preparations, the use of cellulose columns is recommended (Romagna and Staniforth, 1989). Romanowsky-type stains, such as May-Grunwald/Giemsa (Schmid, 1976) or Wright's stain (Albanese and Middleton, 1987) are routinely employed for mouse micronuclei.

It is suggested in various international guidelines (OECD, 1997b; 2007; 1997c) that five males and five females be used per group, although only a few quantitative differences in clastogenic responses have been shown to exist between sexes (Henry *et al*., 1980; CSGMNT, 1986). The use of two sexes has been debated. The EEC, OECD and EPA recommend the use of both sexes but the UKEMS (Richold *et al*., 1990) and Japanese Guidelines (JMHW, 1984) suggest the use of males only. This obviously reduces animal numbers, and Lovell and colleagues state that with a group size of seven, there is not a significant loss in test sensitivity if 2000 PCEs are read (Lovell *et al*., 1989).

Since no qualitative differences exist in response to clastogens, even though different strains of rats and mice have different spontaneous frequencies of micronucleated polychromatic erythrocytes (MPEs) (CSGMNT, 1988), there is no preferred strain. It is appropriate, however, that the spontaneous incidence should not exceed 0.4% (i.e. 4 per 1000 PCEs) (Topham *et al*., 1983). Young animals of 6–8 weeks for mice and 8–10 weeks for rats should be used, to avoid fat deposition in the marrow, since fat in older animals interferes with the clarity of staining.

The experimental unit is the animal and not the cell, but if less than 500 cells are examined clusters of cells might be formed (Albanese and Middleton, 1987; Mirkova and Ashby, 1987). Regulatory guidelines generally require 1000 PCEs scored per animal. Mirkova and Ashby (1987) favoured the reading of 2000 PCEs. However, the minimum number of PCEs to score per animal depends upon the spontaneous frequency of micronuclei; the lower the frequency the greater the number of PCEs per animal requiring analysis to detect a specified level of increase in MPEs. Two thousand PCEs should be scored if the control incidence is less than 0.2%. Confirmation of equivocal results could be achieved by extending numbers of PCEs read before a repeat study. For positive controls, the group size can be smaller (no fewer than three animals), using an optimal sampling time with a dose to monitor sensitivity. To determine toxicity, the PCE : NCE ratio is examined, and alterations in the ratio result from the inhibition of division or maturation of nucleated erythropoietic cells or replenishment of marrow with peripheral blood. A total of 1000 erythrocytes (PCEs and NCEs) should be examined.

For the micronucleus test, either a single-dose study with two harvest times at 24 and 48 hours after treatment or a multiple-dose study with two or three doses 24 hours apart, followed by a single harvest time 24 hours after the last dosing, is considered acceptable (Richold *et al*., 1990). A 72 hour sampling time was initially required, based on the results with 7,12-dimethylbenzanthracene (Salamone and Heddle, 1983), but responses can be shown at earlier times (Ashby and Mirkova, 1987). Most regulatory authorities, however, require three sampling times, and 24 hours is suitable to detect most chemicals which induce micronuclei. It was not necessary to sample the marrow of treated animals earlier than 19–24 hours after dosing (MacGregor *et al*., 1987), since the lifespan of an immature or RNA positive erythrocyte is between

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10 and 20 hours in both rat and mouse (Salamone and Heddle, 1983).

Usually both femurs are removed and cleared of extraneous muscle. From the proximal end the shafts are aspirated with a needle and the marrow flushed out with serum to obtain a homogeneous cell suspension. The cells are concentrated by centrifugation at 800–1000 rpm for 5 min. A small drop of suspension is spread on the smear-free slide and pulled with a cover glass (Schmid, 1976). Another method is to push the marrow onto the slide with a pin inserted in the epiphysial (distal) end. The marrow is mixed with serum to disperse the cells, using the edge of a second slide (Salamone and Heddle, 1983). Alternatively, a fine sable paint brush in physiological saline is inserted into the distal end of the femur and drawn across the slide for up to four strokes with fewer cells at the end of strokes (Albanese and Middleton, 1987).

The scored elements are the micronucleated cells and not the number of micronuclei. The majority of MN are circular but some are oval or ring shaped; doubtful shapes should not be scored. Cells which are orange/red after staining should be classed as mature (NCEs); those which are bluish as immature (PCEs). The frequency of micronuclei in NCEs should be 0–1 per 1000. For each animal the number of PCEs, NCEs, micronucleated PCEs and micronucleated NCEs as well as PCE : NCE ratio should be recorded. It is not necessary to record Vernier readings for micronucleated cells. The micronuclei arise from anaphase lag of chromosome fragments, bridged translocations or detached whole chromosomes. Detailed recommendations on the type of statistical analysis to be carried out on data generated in this assay have been proposed (Lovell *et al*., 1989). The positive and negative control values should fall within the historical control range for the laboratory. If the test material has been administered at the highest dose and there is a negative response, the test material should be classified as negative under the test conditions. No-effect levels could be used in risk evaluation.

3.3.3 Rodent Bone Marrow Metaphase Analysis

Most bone marrow metaphase assays have been carried out in the rat, although mouse and Chinese hamster are also used. The assay is conducted at doses up to the maximum tolerated dose (MTD) or for nontoxic substances 2 or 5 g kg^{-1}, depending on the regulatory authorities. A dose that causes a 50% reduction in the MI or some other index of cellular toxicity in a preliminary toxicity assay is also used. A single dose may be used for screening purposes but for hazard assessment at least three doses should be used. A dose relevant to man is sometimes included and an intermediate between it and the MTD. Other protocols, including a 10-fold and one-third of the MTD have been used (Anderson and Richardson, 1981) but the UKEMS (Richold *et al*., 1990) recommended that dose levels should be separated by approximately 2-fold intervals. The OECD guideline 475 (OECD, 1997c) recommends five males and five females but the UKEMS (Richold *et al*., 1990) recommends seven males per test group only. The OECD (OECD, 1997c) recommends that 50 cells per animal be scored (500 per test group) and the UKEMS (Richold *et al*., 1990) also recommends that this number is scored.

It was recommended that cells be sampled at 6, 24 and 48 hours after a single dose administration. Cells were also sampled at 6 and 24 hours after a multiple administration. The UKEMS debated the use of a 48 hour sampling and suggested that it should only be used when there was evidence of mitotic delay. This can be determined during the preliminary toxicity assay. OECD guideline 475 (OECD, 1997c) now recommends only two samplings at 24 and 48 hours and after multiple dosing, sampling between 18 and 24 hours and between 36 and 48 hours.

A mitotic arresting agent such as colchicine or colcemid is dosed to the animals at 2–4 hours prior to sampling. Methods for satisfactory stained metaphase spreads have been described (Adler, 1984). All slides should be coded and randomized. Various readings should be taken of aberrations and recorded for future reference and Good Laboratory Practice audits. The classification criteria of Scott *et al*. (1990) can be used and all aberrations should be described fully in the raw data. For each animal, the number of cells scored, the number of cells with each aberration type, the frequency of aberrations including and excluding gaps should be determined. Recommendations on the statistical analysis of this assay have been made by Lovell *et al*. (1989). The positive and negative control values should fall within the acceptable range for the laboratory for a substance to be considered positive or negative under the conditions of the study.

3.3.4 Germ Cell Cytogenetic Assays

Either mouse or rat can be used but the mouse is generally the preferred species. Normally such assays are not conducted for routine screening purposes.

Spermatogonial metaphases can be prepared by Evans' air-drying technique (Evans *et al*., 1964) for the first and second meiotic metaphase (MI and MII) in the male mouse. This method is not so suitable for rat and hamster. The numbers of spermatogonial metaphases can be boosted if, prior to hypotonic treatment, the testicular tubules are dispersed in trypsin solution (0.25%). At least one month between treatment and sample should be allowed to pass in the mouse to allow treated cells to reach meiosis. It was established that 11 days and 4 hours was required for spermatogonial cells to reach preleptotene and 8 days and 10 hours to reach zygotene (Brook and Chandley, 1986). It takes 4 hours for cells to move from MI to MII but test compounds

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can alter this rate. A search for multivalent formation can be made at MI for the structural rearrangements induced in spermatogonia. Using electron microscopy, it became possible to examine the synaptonemal complex at pachytene (Cawood and Breckon, 1983). Errors of segregation should be searched for at the first meiotic division in the male mouse, MII cells showing 19 (hypoploid) and 21 (hyperploid) chromosomes (Brook and Chandley, 1986). Various groups describe assays in the female mouse and procedures used for inducing ovulation by hormones and treatment of specific stages of meiosis (Hansmann and El-Nahass, 1979; Brook, 1982; Brook and Chandley, 1985).

There are still some differences between ICH and OECD guidelines that must be addressed (Kirkland, 1998; 2002). Examples are: When are differences and toxicity metabolism between males and females sufficient that both sexes need to be used in *in vivo* testing? What is considered sufficient exposure of target tissue for a valid *in vivo* test? When is absorption considered sufficiently low that *in vivo* testing would be considered appropriate? What efforts need to be taken to ensure a compound does not possess a structural alert?

4 SISTER CHROMATID EXCHANGE ASSAYS

SCEs occur between both sister chromatids of replicating chromosomes. They result in a change in morphology of the chromosome at homologous loci, which involves breakage and reunion.

4.1 Historical View

The first visualization of SCEs happened in the late 1950s in plant cells, using radioactive tritium and autoradiography (Taylor, 1958). Then, with the addition of the DNA base analogue 5′-bromodeoxyuridine (BrdU), in combination with Hoechst dye 33258 staining, it was possible to clearly discern the sister chromatids (see **Figure 3**), and to reveal SCEs (Latt, 1973). Later, the Giemsa staining was reported to detect SCEs (Korenberg and Freedlender, 1974). However, the standard method for the visualization of SCEs became the combination of fluorescence plus Giemsa (FPG) (Perry and Wolff, 1974). The FPG procedure is recommended in view of the fact that stained slides can be stored, and microscope analysis is simpler. Alternatives to this method have appeared over the years, for example: utilization of an antibody binding to BrdU-substituted DNA following denaturation (Pinkel *et al*., 1985), such as biotin-16-2′-deoxyuridine-5′-triphosphate (biotin-dUTP) (Wojcik *et al*., 2004), use of DAPI (2-(4-amidinophenyl)-1H -indole-6-carboxamidine) (Lin and Alfi, 1976; Lin *et al*., 1976) or propidium iodide (Tucker *et al*., 1986) in combination with low doses of BrdU. SCE scoring is a time-consuming technique, and the necessary precautions have to be taken to work with BrdU. However, the concentrations of BrdU added to

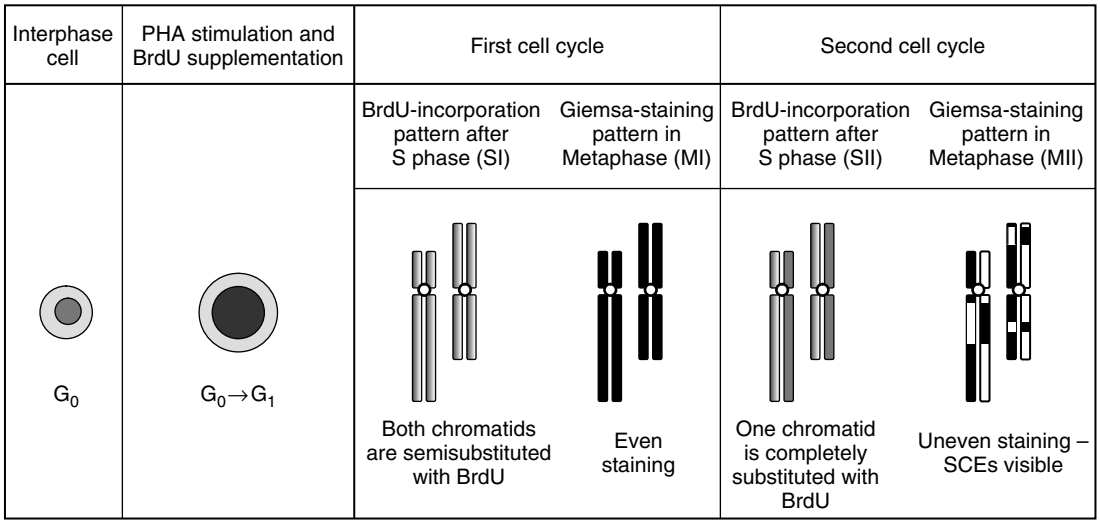

Figure 3 SCEs can be visualized in the second mitotic metaphase as the assay is based on the incorporation of BrdU in the newly synthesized DNA strands during S Phase (semiconservative replication). After PHA stimulation, lymphocytes progress into the first cell cycle. In the first mitotic metaphase the DNA strands of both chromatids are semisubstituted with BrdU leading to an even staining of the chromosomes. In the second mitotic metaphase one chromatid remains semisubstituted with BrdU while the other is fully substituted. This leads to an uneven staining where one chromatid becomes darker than the other. SCEs become visible only then after interchromatid exchanges of similar regions on each chromosome producing 'harlequin' chromosomes.

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the culture medium have to be very low not to induce mutagenic effects. Thus, assays which need very low concentrations of BrdU are advantageous.

In general, the methodology developed to reveal the presence of SCEs is based on the semiconservative replication hypothesis (Meselson and Stahl, 1958). Therefore, the requirement to observe SCEs at metaphase is that cells must past through S phase (Wolff *et al*., 1974).

4.2 Mechanism(s) of SCE Generation

Based on the fact that genotoxins linearly induced SCEs and single-gene mutations, it was possible to affirm that SCEs are related to mutational events (Carrano *et al*., 1978). Also research used repair-deficient mutants which, after treatment with genotoxins, yielded an increase in SCEs (Thompson *et al*., 1981). This explains the inability of the cell to repair efficiently replication-blocking lesions, which result in fork collapse and initiation of homologous recombination to repair the broken DNA replication fork upon encountering a nick or a gap in the parental strand (Wilson and Thompson, 2007). In recent years, the underlying molecular mechanisms generating SCEs have become clearer. SCEs seem to result from a mechanism involving the RAD51 dependent double-strand repair (Gonzalez-Barrera *et al*., 2003). Other investigators, however, also claim a nonhomologous end joining (NHEJ) mechanism of repair as an SCEs mediator (Painter, 1980; Bryant and Helleday, 2006). Interestingly, SCEs provide a different and alternative end point when compared to CAs and MNs, since the mechanisms underlying them are dissimilar.

BrdU, a base analogue, by itself contributes to the generation of SCEs (Stoilov *et al*., 2002). This might be due to the highly reactive bromine atom which interacts with agents like ionizing and UV radiation, so enhancing the frequency of SCEs (Wojcik *et al*., 2004). This is likely to be due to the formation of single-strand breaks (SSBs) or alkali-labile sites (ALSs) (Dillehay *et al*., 1984). In addition to compounds generating SSBs and ALSs, other SCE inducers are alkylating agents, base analogues, S phase dependent compounds and inhibitors of fork progression such as mitomycin C. Furthermore, conditions which increase the burden of SSBs, like XRCC1 deficiency (Thompson *et al*., 1982), inhibition of poly(ADP-ribose) polymerase 1 (PARP-1) (de Murcia *et al*., 1997), or inhibition of DNA synthesis (Degrassi *et al*., 1989), contribute to the generation of SCEs.

The baseline SCE frequencies in human and mouse peripheral lymphocytes are 3.6 and 2.4 SCEs per cell per generation (Tucker *et al*., 1986), being invariant at the lowest BrdU levels in humans. Taken together, it is evident that SCEs are an integral part of DNA replication, even in the absence of agents known to induce SCEs. Further, SCEs occur at the replication points (Latt and Loveday, 1978). This is due to a maximal SCE generation at the beginning of the replication which decreases as replication progresses.

4.3 Experimental Design

The SCET is based on the breakage within the sister chromatids and the subsequent exchange of regions within these chromatids. This process is conservative and 'virtually' an error-free process because there is generally no alteration of genetic information during the reciprocal exchange via homologous recombination (Wilson and Thompson, 2007). Visualization, however, requires one chromatid being bifiliarly substituted with BrdU, while the other sister chromatid is monofiliarly substituted. This test has been correlated with recombinational repair, promotion of point mutations, gene amplification, cytotoxicity and estimation of cell division.

Established cell lines, primary cell cultures or rodents may be used. Detailed information on the performance of SCE *in vitro* and *in vivo* assays may be obtained in reviews on SCET (Latt *et al*., 1981; Perry *et al*., 1984; Perry and Thompson, 1984). Guidelines are also available for *in vitro* (OECD, 1986; EPA, 1996a) and *in vivo* (EPA, 1996b) SCE assays. The *in vitro* methods will be briefly explored here.

Either monolayer or suspension cultures can be employed, as well as human lymphocytes. Human fibroblasts are less suitable because of their long cell-cycle duration. When using monolayer cultures, the cultures are set up the day before BrdU treatment so that the cells will be in exponential growth before the addition of BrdU or the test compound. A number of experimental designs can be carried out with SCET (Wojcik *et al*., 2004). SCEs can be visualized by growing cells for either two rounds of replication in the presence of BrdU or for one round with BrdU and the next without. If BrdU is added after cells were treated with a DNA-damaging agent, the effect on SCEs can only be analysed in the second post-treatment mitosis. Analysis of the first post-treatment mitosis requires cells, monofiliarly labelled with BrdU, being treated. Whichever the procedure, the cells are allowed to undergo the equivalent of two cell cycles before cell harvest. A spindle inhibitor such as colchicine or colcemid is introduced for the final 1–2 hours of culture to arrest cells in metaphase, after which the cells are harvested and chromosome preparations are made by routine cytogenetic techniques. Peripheral blood cultures are established in medium containing BrdU and PHA. Colcemid is added 1–2 hours before harvest and the cells are harvested between 60 and 70 hours post PHA

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stimulation. Cell harvest and slide preparations are conducted according to routine cytogenetic methods.

In the absence of metabolic activation, BrdU and the test agent can be added simultaneously and left for the duration of BrdU labelling. Shorter treatments should be used in the presence of metabolic activation or to avoid synergistic effects with BrdU; then, cells can be pulse treated, for example for 1 hour before BrdU addition (Edwards *et al*., 1993). If metabolic activation is not required, treatment is best conducted during the final 24 hours of culture, or if metabolic activation is required, a pulse exposure may be employed to treat cultures at the first S phase at around 24–30 hours, or at 48 hours for an asynchronous population.

The number of concentrations and cultures, use of duplicates, extended cultures when negatives are found, positives recommended, use of negatives and so on follow the same principles as for MNA and CAT, and these have been detailed previously. However, there are some differences: (i) The concentration of organic solvents for the test compound should not exceed 0.8% (v/v), as higher concentrations could lead to slight elevations in the SCE level (Perry *et al*., 1984). (ii) Exposure of cells to fluorescent light during the culture period leads to photolysis of BrdU-containing DNA and a concomitant increase in SCE frequency (Wolff and Perry, 1974). Consequently, SCE cultures should be kept in the dark and manipulated under subdued light conditions such as yellow safe light. Furthermore, media used in SCE assays should be stored in the dark, since certain media components produce reactive SCE-inducing intermediates on exposure to fluorescent light (Monticone and Schneider, 1979). (iii) Even for an apparently unambiguous positive response with a greater than two-fold increase in SCEs over the background level at the highest dose, and with at least two consecutive dose levels with an increased SCE response, a repeat study is necessary to show a consistent response (Perry *et al*., 1984).

Regarding scoring, slides should be read coded and randomized. The quality of differential staining will determine the ease and accuracy of SCE scoring, and, to eliminate variation, results from different observers should occasionally be compared. Furthermore, to avoid observer bias, matched scorers should have slides from different treatment groups equally distributed among them, as with all cytogenetic studies.

5 HUMAN MONITORING

People exposed to environmental mutagens can be examined for chromosome damage in peripheral blood lymphocytes. Briefly, a sample of blood is taken per individual (about 10 ml), and chromosome preparations are made in the usual way (Evans and O'Riordan, 1975) from the lymphocytes, which have been stimulated into division by a mitogen such as PHA. This is because lymphocytes are normally resting in G_0 stage. A detailed questionnaire is also taken from the individual at the time of blood sampling, relating to lifestyle habits. These could act as confounding factors in study interpretation when increases in chromosome damage are found. Such exogenous lifestyle factors include smoking, drinking alcohol and caffeine, therapeutic drug usage, X-ray examinations, recent viral infections and so on. Endogenous factors (age, gender, etc.) are also taken into account. Such human monitoring questionnaires and appropriate study designs have been described by the International Commission for Protection Against Environmental Mutagens and Carcinogens (ICPEMC) (Carrano and Natarajan, 1988), and many human monitoring studies have been described in a special issue of Mutation Research (Anderson, 1988). International Programme on Chemical Safety (IPCS) guidelines for the monitoring of the genotoxic effects of carcinogens in humans have also been described in a special issue of Mutation Research (Albertini *et al*., 2000).

6 THE HUMAN GENOME

One of the most exciting developments in cytogenetics was the launching of the Human Genome project in 1986 by the Department of the Environment in the USA. The ultimate goal of the project was to analyse the structure of human DNA, to determine the location of all human genes on chromosomes. In April 2003 the draft sequencing of the complete human genome finally finished after one and a half decades (NCBI, 2008a); however, the exact number of genes remains still unknown.

6.1 Mapping and Sequencing the Human Genome

The human genome (build 35) consists of up to 20 000–25 000 genes located on the 23 pairs of chromosomes covering 99% of the euchromatic genome, that is 2.85 billion nucleotides interrupted by only 341 gaps (IHGSC, 2004). By 2006, the completion of the DNA sequence was announced for the last of the different 24 human chromosomes (Muzny *et al*., 2006; Scherer *et al*., 2006). The resulting sequence is a generic sequence representative of humans in general.

The homologue chromosomes from each pair derived either paternally or maternally consist of a DNA–protein complex, which essentially encodes the genes, among other functional elements. The basic DNA structure is represented by a double-stranded helix composed of a linear array of nucleotides carrying four different bases

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(adenine, A, thymine, T, cytosine, C and guanine, G). These bases are precisely paired, opposite to each other (A-T and G-C), and the consecutive order of these bases along the DNA strand determines the information content of a particular gene or piece of DNA. The length of the genes varies, ranging from about 2000 to 2×10^6 bp. Sequencing determines the order of the nucleotides or base pairs in a DNA molecule, while mapping is the process which determines the spacing and position of genes, or other genetic landmarks on chromosomes, relative to one another. There are two types of maps, genetic and physical, which differ in the methods used to construct them and in the measurements used to determine the distance between genes.

Up to 25 000 human genes embedded within 3 billion base pairs have been mapped so far. The National Center for Biotechnology Information (NCBI) database holds today the information on 39 954 genes, including hypothetical genes and open reading frames (ORFs) (NCBI, 2008b). It is presumed that functionally important DNA is conserved among humans. Also, DNA regions, such as genes involved in genetic disease, are still of particular interest. Now that the human genome has been fully sequenced many scientific questions might soon be answered in detail concerning gene regulation, gene function, chromosomal organization, noncoding DNA types, gene sequence variations in diseases, multigene diseases, genomics as well as tanscriptomics.

6.2 Genetic Maps

Genetic maps can be used for the identification of genes associated with genetic disease and other biological properties, and are also used to guide in the construction of physical maps. Genetic maps are constructed by determining the frequency with which two marker genes are inherited together. Those that lie close together are inherited together more frequently than those genes that are further apart. Genetic studies of families determine how frequently two genes are inherited together. The distance between genes is measured in centimorgans (cM). Two markers are 1 cM apart when during transmission from parents to children they are separated 1% of the time. The average distance for a centimorgan is about 106 bp in physical length.

6.3 Physical Maps

The numbers of nucleotide pairs constitute the units of physical length of the distance between sites on physical maps. These can be constructed in different ways derived from recombinant DNA techniques, which allow the isolation and cloning of DNA fragments and the identification of specific sequence markers on DNA, and determine the distance between and order of such chromosome markers.

A position or locus within the genome can be specified by a sequenced genomic clone, an mRNA or sequence-tagged site (STS) or expressed sequence tags (ESTs) marker (Lee *et al*., 2005). Databases at the NCBI (USA) or the UCSC (University of California Santa Cruz, USA) hold the information on millions of these markers. To generate a physical map, a collection of cloned pieces of DNA comprising a complete chromosomal segment or chromosome and the order of cloned pieces may be used. Constructing these overlapping clone sets, known as 'contigs', was one of the key elements in the sequencing endeavour of the Human Genome project; particularly, bacterial artificial chromosome (BAC) clones have been used (McPherson *et al*., 2001). For instance, the human (male) BAC library RPCI-11 consists of over half a million clones with known sequence/end-pair sequence and chromosomal position, covering the genome more than 32 times (Osoegawa *et al*., 2001), which makes them ideal to be used as DNA probes for modern molecular cytogenetic techniques. For large quantity sequencing, BACs have crucial advantages over earlier used clones like cosmids, plasmids, P1-derived artificial chromosomes (PACs) or yeast artificial chromosomes (YACs). They have an ideal insert length of about 200 kb, a small sized vector, they are stable bacterial clones and the BAC DNA is easy to isolate. During the last two decades sequencing became faster and cheaper due to new techniques (von Bubnoff, 2008) and therefore the construction of physical maps became more inexpensive, rapid and routine procedure.

6.4 Model Organisms

Information derived from studies of the biology of model organisms is useful in the interpretation of data obtained in studies of humans and in understanding human biology. In addition to the known human genome, other complete genomes from lower and higher organisms have been fully sequenced. Those identified as useful for comparative purposes are the bacterium *Escherichia coli*, the yeast *Saccharomyces cerevisiae*, the fruit fly *Drosophila melanogaster*, the worm *Caenorhabditis elegans*, the zebra fish *Danio rerio* and the laboratory mouse *Mus musculus*. These organisms' genomes have already been fully sequenced and their sequence can be viewed on the publicly available databases such as NCBI.

7 MOLECULAR CYTOGENETICS

Since the development in the 1970s of chromosomal staining techniques to accurately evaluate numerical abnormalities and structural aberrations, cytogenetics has

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continuously progressed in recent years towards more precise and sophisticated techniques using the advantages gained in molecular biology as well as the knowledge assessed in the Human Genome project.

7.1 FISH and SKY

For the classical staining techniques, dyes like Giemsa have been intensively used, preferably binding to heterochromatic regions, hence resulting in a banding pattern (Bayani and Squire, 2004), which shows 300–850 bands depending on the degree of condensation and time in mitosis (Craig and Bickmore, 1993; ISCN, 2005). With the known longitudinal length of our complete genome of about 3000 Mb, each band at the highest resolution stands for approximately 3.5 Mb; thus, leaving the detection threshold for structural aberrations in typical metaphase preparations at 5–10 Mb. With modern molecular cytogenetic techniques it is now possible to target specific areas of interest using fluorescently labelled DNA probes or to extend the detection towards all chromosomes individually when looking at the whole chromosome set, by introducing differential chromosome staining.

Evaluation of chromosomal damage by FISH has been extensively carried out and improved since this technique was introduced for the classification and detection of chromosome abnormalities in the mid 1980s (Pinkel *et al*., 1986). The variety of fluorescently labelled DNA probes now ranges from whole chromosomal painting probes, chromosome arm or band probes to locus specific gene or sequence probes including repetitive probes for satellites and DNA repeats. In contrast to classical Giemsa staining techniques, more than one colour can be employed and targeted at the area of interest on a chromosome by choosing a suitable probe DNA, for example from the vast selection of BAC clones available. With the successive development of more sophisticated FISH applications by either using an interferometer or a set of optical single band-pass filters, the whole genome can now be evaluated on metaphases with one set of whole chromosome painting probes labelled with different fluorochromes using SKY (Schrock *et al*., 1996) or multiplex FISH (Speicher *et al*., 1996). Especially by targeting interphases (interphases FISH) or stretched DNA strands (fibre FISH) the resolution increased to 5 kb to 2 Mb, depending on the methodology used (Speicher and Carter, 2005). Thus, with these cytogenetic techniques at hand it is possible to evaluate for example cryptic translocations (Barbouti *et al*., 2002) which were hidden and not detectable using classical methods.

7.2 CGH and Array CGH

Comparative genomic hybridization (CGH) is another technique also based on FISH but using a different approach when detecting DNA gain and loss throughout the whole genome (Kallioniemi *et al*., 1992). In brief, isolated genomic DNA from normal tissue and from a tumour is differently labelled with fluorochromes and then hybridized onto metaphases, which are in contrast to the above FISH techniques only used as a hybridization matrix. The information about numerical abnormalities, deletions and amplifications of genetic material is entirely assessed through the ratio of the two fluorescent 'probes'. Utilizing the information the Human Genome project provided as well as microarray techniques together with CGH, a new method was developed: array CGH (Solinas-Toldo *et al*., 1997). For array CGH, the target for of the probe, namely the labelled tumour DNA, is not a metaphase spread but thousands of mapped clones that are spotted on a microscope slide (Fiegler *et al*., 2003). By selecting single-nucleotide polymorphism (SNP) array probes extending up to 100 000 SNPs per array, genotyping is possible as well as the evaluation of loss of heterozygosity (LOH) (Huang *et al*., 2004). These microarray methods either based on glass slides or silicon have the potential to rapidly and accurately screen the genome, for example for oncogenes or tumour suppressor genes in cancers, extending the information accessible from classical methods exponentially (Kashiwagi and Uchida, 2000). It has probably never been easier or cheaper to detect cryptic aberrations routinely for cytogenetic diagnostics on a large scale (Hwang *et al*., 2005; Horsley *et al*., 2006).

Modern cytogenetics has shown the power to detect cryptic aberrations and LOH on a molecular level never seen before. By combining classical cytogenetics with the outstanding variety of molecular cytogenetics, comprehensive high-resolution analysis of complex chromosomal rearrangements becomes possible (Speicher and Carter, 2005), helping to shed new light on inherited diseases and cancer.

8 EPIGENETICS

Any transgenerational phenotype would require transmission through the germ line. However, it has been demonstrated that the exposure of a pregnant rat transiently to endocrine disruptors (the antiandrogenic fungicide, vinclozolin, and the pesticide, methoxychlor) caused a spermatogenic cell defect and subfertility in the F1 generation and all subsequent generations (F1–F4). The critical exposure period was at the time of sex determination, and the transgenerational phenotype was transmitted through the male germ line (Anway *et al*., 2005). A DNA sequence mutation or chromosomal event was not involved since there was no decline of such events in subsequent generations. This suggested an epigenetic mechanism involvement due to the frequency of the phenotype, and two genes were identified in the sperm

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that had altered methylation patterns associated with the phenotype; epigenetics appears to involve altered DNA methylation (Anway *et al*., 2005; 2008). Altered methylation of imprinted genes has also been shown to promote disease states (Jiang *et al*., 2004) and when the animals were one year of age additional diseases had developed such as cancer, prostate disease, kidney disease and immune cell defects. According to Skinner, epigenetics is the next layer of complexity beyond the DNA sequence and is likely in future to be a much more important factor in biology than at present realized (Skinner, 2007).

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Genetic Toxicology Testing and its Relevance to Human Risk and Safety Evaluation

Elisabeth Lorge

CONTENTS

1 INTRODUCTION

Genotoxicity testing was initially set up to measure any toxic effects resulting in inheritable damage to DNA. For some human carcinogens, a correlation was found between carcinogenicity and genotoxicity assessed by the Ames test (Ames and McCann, 1976). Genotoxicity is a major concern in toxicity as it may cause carcinogenicity and/or genetic diseases. Therefore genotoxicity has become a part of the toxicity regulatory assessment.

Over 30 years of practice in genotoxicity, various tests have been developed, validated and refined. In the 1980s, a large variety of genotoxicity tests emerged from laboratories working on genetics, involving such experimental systems as *Drosophila* or yeasts, with protocols defined in the guidelines of the Organisation for Economic Co-operation and Development (OECD). All these tests were relevant to genotoxicity research but only a few were simple, suitable and reliable enough to justify collaborative efforts on validation and better definition of their profile of detection. Then it was time to refine and improve the protocol of the most widely used tests and monitor experimental conditions more satisfactorily. The standard minimal three-test battery issued by the International Conference on Harmonisation (ICH) for genotoxicity tests for pharmaceuticals, the S2B Guideline, was defined in 1997 for the genotoxic assessment of new pharmaceuticals (ICH, 1997). After 10 years of increasing experience with this core battery in regulatory genotoxicity testing, the time has come to think on what has been learned from this experience. More is known about the limitations of genotoxicity tests and new tools have been developed. For 30 years, different aspects of genotoxicity testing have been evaluated and debated in different groups. Now, various worldwide organizations have taken the lead in improving genotoxicity testing, relying on genotoxicity expertise; that is, the International Workshop on Genotoxicity Testing (IWGT), the European Centre for the Validation of Alternative Methods (ECVAM), the Interagency Coordinating Committee on the Validation of Alternative Methods (ICVAM), the Japanese Center for the Validation of Alternative methods (JaCVAM), the Health and Environmental Sciences Institute (HESI), the ICH and the OECD. The main concerns now are to develop relevant strategies

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and adequate complementary tests to the minimal battery in the case of genotoxicity- or carcinogenicity- positive findings in preclinical studies, and to have a better understanding of the different mechanisms likely to induce a genetic change (FDA, 2006). Clear and precise guidelines for human risk assessment, relying on the entire database from all areas of Research and Development, are also being drawn up.

2 AN OVERVIEW OF GENOTOXICITY TESTS

2.1 Recent Advances in Common Genotoxicity Test Methods

The current ICH standard battery for genotoxicity testing of pharmaceuticals has been in use since 1997 (ICH, 1997). It includes a test for gene mutation in bacteria, an *in vitro* test with cytogenetic evaluation of chromosomal damage with mammalian cells, or an *in vitro* mouse lymphoma tk (thymidine kinase) assay and an *in vivo* test for chromosomal damage using rodent haematopoietic cells. This standard minimal battery of genotoxicity tests has been widely recognized as suitable for detecting genotoxic compounds since 1997 and, when negative, as sufficient for demonstrating the absence of genotoxicity. However, improvements have been made to the genotoxicity tests in recent years. New tests or new protocols have been validated. Although the standard battery is sufficient for assessing negative genotoxicity, however, the relevance of *in vitro* positive results has been addressed and it was demonstrated that this current battery is prone to give false *in vitro* positive results. Finally, in the case of positive results in this battery or in rodent carcinogenicity bioassays, additional tests should be conducted, but the appropriate tests and strategies needed for follow-up testing have to be defined more precisely. This section will give an inventory of the standard tests in the minimal battery with their improvements and possible diversifications.

2.1.1 The Bacterial Reverse Mutation Assay

The test for gene mutation in bacteria, namely the Ames test (Ames *et al*., 1973; 1975), has been used for years and is the oldest test used for genotoxicity assessment. The *Salmonella typhimurium* strains used are histidine deficient due to base substitutions or frameshift point mutations. They all come from the Bruce Ames collection of strains, originally designed to study the histidine operon, and are highly sensitive to reversion using most chemical mutagens of various types (Ames and McCann, 1976). Strains TA1535, TA1537, TA98 and TA100 will detect mutagens which are able to induce reversion of these mutations at the guanine–cytosine sites within the histidine gene. Additional strains such as *Salmonella typhimurium* TA102 or *Escherichia coli* WP2 uvrA or WP2 uvrA (pKM101), both tryptophan deficient, will be able to detect point mutations at the adenine–thymine sites (Levin *et al*., 1982). *Salmonella typhimurium* TA102 or repair-proficient WP2 *Escherichia coli* (pKM101) will be used to detect crosslinking agents (Mortelmans and Zeiger, 2000; Gatehouse *et al*., 1990; Maron and Ames, 1983).

Few changes have been made to the standard protocol over the years (OECD, 1997a). A metabolic activation, generally provided by the Aroclor 1254-induced rat liver microsomal fraction (S9) added with cofactors (S9 mix), is needed to metabolize chemicals via cytochromes P450 (see Section 3.3). In the plate incorporation method, a preparation of the tested compound is mixed with the tester strain, S9 mix, when required, and top agar, and poured on agar plates. After two or three days' incubation at 37 °C, colonies are counted. In the preincubation method, the bacteria are exposed to the compound with S9 mix in liquid medium at 37 °C. This procedure is presumed to increase the availability of reactive compounds to the bacteria (Mortelmans and Zeiger, 2000).

This test is the very first step in genotoxicity evaluation and, although it is performed on a prokaryote, it is recognized as being the most robust of the genotoxicity tests.

2.1.2 In Vitro Mammalian Cell Genotoxicity Assays

The most commonly used *in vitro* mammalian genotoxicity assays are either the L5178Y mouse lymphoma cell mutation assay or the *in vitro* chromosome aberration test, as recommended in the ICH S2B guideline (ICH 1997; OECD, 1997b; 1997c). The *in vitro* micronucleus assay is also often used as a screening or additional test to the minimal standard battery. It has recently been regarded as validated by the ECVAM (2006), and an OECD guideline is in preparation. It could be used in the future for *in vitro* genotoxicity assessment on mammalian cells as an equivalent to the mouse lymphoma cell mutation assay or the *in vitro* chromosome aberration test.

For any *in vitro* mammalian cell genotoxicity test, the recommendation is to expose cells for approximately 1.5 normal cell cycles without metabolic activation in addition to a short treatment time. Such a long treatment time is expected to allow compounds interacting with the cell cycle to be detected more efficiently (nucleotide analogues or aneuploidy inducers), or is recommended in cases where a high exposure level was impossible to achieve in short treatments. For any *in vitro* genotoxicity test, an exogenous metabolic activation system is required

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for short treatments unless the cells used are proved to be metabolically competent.

2.1.2.1 The In Vitro Chromosome Aberration Test

The *in vitro* chromosome aberration test is designed to detect clastogenic activity on cultured mammalian cells. Structural chromosome aberrations are recorded after cell exposure to the tested compound. Various cell types can be used, provided cells are actively dividing at the time of treatment, are karyotypically stable and have a low spontaneous frequency of chromosome aberrations. For example, they could be mitogen-stimulated human lymphocytes in primary cultures, or Chinese hamster ovary (CHO) or Chinese hamster lung (CHL) established cell lines (Galloway *et al*., 1994).

The advantage of the *in vitro* chromosome aberration test is that it can identify the type of chromosomal aberration induced and can also be performed on normal human cells, but it is tedious, and time consuming.

Lymphocytes from whole blood or isolated lymphocytes are induced to divide in culture by a mitogen (phytohaemagglutinin). Cells are exposed to the test compound after the completion of 48 hours culture, so that each cell division stage will be represented in the cultures of asynchronous cells. For cell lines, actively growing cells are seeded at a density so that they will not reach confluence before harvest. Cells are exposed to the test compound for a short treatment period with and without metabolic activation, and sampling will take place after a recovery period of about 1.5 cell cycle length after the treatment has started. If negative results are obtained, cells will be exposed for a long, continuous period until sampling (about 1.5 normal cell cycles after the start of treatment). Longer treatment times (e.g. 48 hours until sampling) or longer recovery periods could be useful in detecting compounds which cause the cell cycle to slow down. Before harvest, cells are exposed to a metaphase-arresting substance (e.g. colchicine) so that the cultures will contain a large number of metaphase-arrested cells to allow individual chromosomes to be examined. Using this protocol, chromosomes will be examined at the post-treatment metaphases (Scott *et al*., 1990; Obe and Beek, 1982). Cells are prepared, fixed, spread on slides and stained for examination under a microscope. Analysed concentrations are selected on the basis of cytotoxicity, generally measured by the mitotic index; the highest concentrations inducing at least 50% cytotoxicity (see Section 3.2). Clastogenic activity is measured by scoring the number of abnormal metaphases and the number of breaks. Good standards for slide scoring are crucial for an accurate evaluation of clastogenicity (Savage, 1975). Chromosome and chromatid breaks, as well as chromosome or chromatid exchanges, should be recorded separately so that a specific cell stage effect can be detected if further investigations are deemed useful in elucidating a mechanism of action. Breaks are distinguished from gaps, achromatic lesions where DNA remains undamaged, by the evidence of a discontinuity in the chromosome or chromatid (Bauchinger *et al*., 1976). As gaps do not result from genetic events but reflect protein modifications along the chromatin, they are related to cytotoxicity. Therefore, they are excluded from the recording of abnormal metaphases. It can also be useful to deduce, from the type of aberration observed, the number of primary breaks theoretically needed to produce the observed structural aberration (Savage, 1975). It allows quantification of aberrations, taking their gravity into account. Generally, numerical aberrations (polyploidies or endoreduplications) are recorded concurrently with the evaluation of clastogenicity (see Section 3.4) to give an indication of possible aneugenic effects.

2.1.2.2 The L5178Y Mouse Lymphoma Cell $tk^{+}/_{-}$ Mutation Assay

The mouse lymphoma cell mutation assay is more straightforward than the chromosome aberration test, less time consuming and able to detect some aneugens, whilst the chromosome aberration test gives only indications of aneugenic effects. In the mouse lymphoma cell mutation assay, the mutation frequency at the tk locus on the L5178Y cell line is measured by the number of 5-trifluorothymidine-resistant (TFT^{R}) colonies, obtained by forward mutation from the parental type ($tk^{+}/_{-}$) to the mutant form ($tk^{-}/_{-}$). After a two-day expression period after treatment, cells are cloned in a selective medium and allowed to form mutant colonies after several days' incubation at 37 °C.

The mouse lymphoma assay was initially developed as the soft agar version (Clive *et al*., 1972; 1979). The protocol of the mouse lymphoma assay has gradually been improved over the years and is now widely used in genotoxicity assessment of pharmaceuticals. First, the microwell method based on the fluctuation test was developed (Cole *et al*., 1983; Oberly *et al*., 1997) to avoid interferences due to metabolic cooperation between mutant cells and to make the detection of mutant colonies statistically more reliable. The agar cloning method is still acceptable as there are insufficient experimental data to prefer one method over another. The colony sizing added value to the test as it was shown to be a good indicator of clastogenicity (Davies *et al*., 1995; Moore and Doerr, 1990). Indeed, the size of the mutant colonies is known to be related to the type of induced mutation, small colonies resulting mainly from large deletions or chromosome rearrangements, and large colonies resulting mainly from point mutations or small deletions. Adding a 24 hour treatment without metabolic activation to a negative 3–4 hour treatment has been shown to be useful in detecting nucleotide analogues as well as aneuploidy inducers (Honma *et al*., 1999; Moore *et al*., 2007).

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Good standards for variability and statistical analysis are extensively described throughout the reports of the successive workshops including IWGT work groups (Clive *et al*., 1995; Clements, 2000; Moore *et al*., 2000; 2002; 2003; 2006; 2007). The assessment of cytotoxicity as well as acceptability and positivity criteria were also debated and agreed during these workshops. Relative total growth (RTG), based on cloning efficiency at time of mutant selection and cellular suspension growth over the two-day expression period, was definitively recommended as the appropriate measure for cytotoxicity (Moore *et al*., 2000). Positive results obtained at RTG <10% should be cautiously interpreted, as cytotoxicity-related genotoxicity effects could be induced at such a cytotoxicity (ICH, 1995). Acceptable values for suspension growth were set at 8–32 for 3–4 hour treatments and 32–180 for 24 hour treatments, and the limits for cloning efficiency at the time of mutant selection were set at 65–120%. The mutation frequency in the negative controls must be between 50 and 170 mutant colonies per 10^6 cells for the microwell version of the test and between 35 and 140 mutant colonies per 10^6 cells for the cloning agar version. In addition to the evaluation of the magnitude of an increase in mutation frequency over the negative control, the assessment of a positive response was improved by the use of the global evaluation factor (GEF). It represents the value by which the induced mutation frequency should exceed the negative control to consider a positive result as biologically relevant. Based on interlaboratory surveys, the GEF (Moore *et al*., 2003) should be 126 mutant colonies per 10^6 cells for the microwell version of the test and 90 mutant colonies per 10^6 cells for the cloning agar version.

2.1.2.3 *The In Vitro Micronucleus Test*

Micronuclei may originate either from acentric chromosome fragments resulting from chromosomal rearrangements or from breakages; that is, typically clastogenic effects produced by initial mutagenic events. They can also originate from whole chromosomes unable to migrate to the cell poles during the last stages of cell division under the action of an aneugen. Then micronuclei can be observed in the cells at the next interphase stage. When using primary cell cultures, like mitogen-stimulated human lymphocytes from whole blood or isolated lymphocytes (Elhajouji *et al*., 1994), cytochalasin B (an actin assembly inhibitor inducing a cytokinesis block) is added prior to the next interphase, so that cells which had completed one division appear as binucleated cells (cytokinesis-block method; Fenech, 1993). If cell lines are used, the use of cytochalasin B is not mandatory, provided cells are actively dividing. Cytotoxicity is evaluated by different means depending on whether cytochalasin B is used or not: cytokinesis-block proliferation index (CBPI) when cytochalasin B is used, or increased cell counts (ICCs) or population doubling (PD) when cytochalasin B is not used, based on cell counts (see Section 3.2).

The *in vitro* micronucleus test was first used in genotoxicity in the 1970s. Experience of this system was first gained in studies on human lymphocytes mostly performed for biomonitoring purposes (Fenech and Morley, 1985; Norppa *et al*., 1993a; Van Hummelen and Kirsch-Volders, 1992). Since then, due to its simplicity, it has been widely used in genotoxicity testing, especially as a screening tool for pharmaceutical research. Automated and miniaturized versions of the test have been developed for this purpose (Van Hummelen *et al*., 1995; Frieauff *et al*., 1998; Verhaegen *et al*., 1994; Nesslany and Marzin, 1999).

The use of human lymphocytes, especially for screening tests, may be limited by the availability of the cells and by the variability between human donors. Therefore, a wide variety of cells are also used to detect the induction of micronuclei in various fields of investigation, like environment or tissue-specific carcinogenesis, for example, using different cell types such as hepatocytes or keratinocytes. Suitable cell types for the *in vitro* micronucleus test also include cell lines, which are chosen for their growth capabilities and their karyotypic stability.

Data from this test have been accumulated from the literature (Garriott *et al*., 2002) and through several collaborative validation studies (Elhajouji and Lorge, 2006; Lorge *et al*., 2006; Matsushima *et al*., 1999; von der Hude *et al*., 2000). These studies have shown the accuracy of the *in vitro* micronucleus test for detecting clastogens and aneugens, and have confirmed that different cell lines are suitable for scoring micronuclei in addition to human lymphocytes (Clare *et al*., 2006): CHO cells (Aardema *et al*., 2006), CHL cells (Wakata *et al*., 2006), V79 hamster cells (Miller *et al*., 1998), and the L5178Y mouse lymphoma cell line (Oliver *et al*., 2006), allowing comparisons with the mouse lymphoma assay, and, more recently, the human TK6 lymphoblastoid cell line (Zhang *et al*., 1995; Le Fevre *et al*., 2007). A standard protocol was designed at the IWGT meetings (Kirsch-Volders *et al*., 2000; 2003a). Now the *in vitro* micronucleus assay is recognized as validated by the ECVAM (2006), and a specific OECD guideline is in preparation (OECD, 2008).

In addition to the use of the *in vitro* micronucleus assay as a standard test of the regulatory battery, the *in vitro* micronucleus test is used to discriminate aneugens from clastogens, by means of complementary techniques such as fluorescence *in situ* hybridization (FISH) with centromeric DNA sequences (Migliore *et al*., 1993) or kinetochore-specific staining (Norppa *et al*., 1993b). This is a valuable tool which is used to gain a better understanding of the mechanisms of aneuploidy (Fenech, 2007) and to determine thresholds for aneuploidy (see Section 3.6) when a compound is found positive in an *in*

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vitro genotoxicity test and needs further complementary genotoxicity studies.

2.1.3 In Vivo Genotoxicity Assays

In vivo genotoxicity assays are crucial in determining the relevance for the organism of negative or positive findings obtained in *in vitro* experiments. In the whole animal, where adsorption, distribution, metabolism, detoxification and excretion are fully functional, *in vitro* positive results may not be confirmed in *in vivo* testing or, conversely, positive results could be found in the animal but not detected in *in vitro* experiments. Both situations could be due to inadequate metabolism in *in vitro* tests, irrelevant levels of exposure, differences in specificity, sensitivity or cytotoxicity between *in vitro* and *in vivo* test systems, or due to multistep indirect genotoxicity mechanisms not efficient in *in vitro* systems. Therefore, *in vitro* and *in vivo* test systems are complementary.

2.1.3.1 The Rodent In Vivo Erythrocyte Micronucleus Assay

Micronuclei arise during the anaphase step of mitosis as a result of the loss of a fragment of chromosome (clastogenicity) or the loss of a whole chromosome (aneuploidy). As micronuclei are expelled into the cytoplasm, they can easily be seen in the erythrocytes, which lack a main nucleus. The *in vivo* micronucleus assay was initially performed on bone marrow (Schmid, 1975; Heddle *et al*., 1983; Richold *et al*., 1990), which is easily exposed to the test substance or its derivatives. Bonne marrow is collected from animals receiving the test substance at different times after one or more (generally two) daily treatments. Bone marrow cells are then suspended, spread on slides and stained with May-Grünwald Giemsa to distinguish polychromatic (immature) erythrocytes from normochromatic (mature) erythrocytes. DNA-specific fluorescent stains (e.g. Acridine Orange) are also increasingly used because they make it easier to distinguish between true micronuclei and artefacts (Hayashi *et al*., 1983; Tinwell and Ashby, 1989). Erythrocytes are analysed for the presence of micronuclei (OECD, 1997d).

Due to a clastogenic or an aneugenic effect, the frequency of micronucleated polychromatic erythrocytes is increased. A clastogenic effect is revealed by an increase in micronucleated polychromatic erythrocytes starting about eight hours after bone marrow exposure to the test substance. An aneugenic effect is characterized by an increase in micronucleated polychromatic erythrocytes as early as about six hours after administration, proving that the test substance or its derivatives had interacted with the mitotic process. A clastogenic or aneugenic effect may be observed up to 48 hours after administration, depending on the pharmacokinetic properties of the test substance or on any inhibition of the cell cycle it induces (Hart and Hartley-Asp, 1983). Due to its simplicity and the possibility of scoring a high number of cells per animal (2000 polychromatic erythrocytes recommended), the micronucleus test is the most widely used *in vivo* test, compared to the *in vivo* chromosome aberration test, considered equally appropriate for *in vivo* genotoxicity assessment in the current ICH S2 guideline.

Except for specific purposes, young adult animals are used. For a long time, the *in vivo* micronucleus assay has been performed mainly on mice. However, as a species widely used in toxicology assessment, with data on toxicology, toxicokinetics and pharmacokinetics, rats are more often used for the *in vivo* micronucleus assay in pharmaceutical development (Krishna *et al*., 1991). Choosing the species used in general toxicity studies, the micronucleus test can also be integrated into repeated toxicity studies. Using the same animals for toxicity and genotoxicity purposes has the advantage of reducing the number of animals needed for toxicity studies. Additionally, in some cases, repeated treatment may increase exposure to the tested substance, and in any case will be closer to human exposure conditions. Prospective studies have already been performed for feasibility (Garriott *et al*., 1995; Hamada *et al*., 2001). Recommendations on practical aspects of a repeated integrated micronucleus assay using the same animals are under evaluation. Another major concern being evaluated is the choice of doses, because in repeated dose studies the highest tolerated dose should be, in a number of cases, lower than the maximum tolerated dose in acute treatment when the test compound exhibits toxic effects. The criteria for appropriate dose level acceptability are currently being refined.

In terms of integrating the micronucleus test into repeated toxicity studies, it might be preferable to examine polychromatic erythrocytes from peripheral blood rather than erythrocytes from bone marrow. Blood is easier to collect, and could be sampled at different times during the study without interfering with the conduct of a toxicity study. Using polychromatic erythrocytes from peripheral blood has proved satisfactory on mice where the spleen is unable to remove micronucleated erythrocytes from peripheral blood (CSGMT, 1995); therefore, steady state frequencies of micronucleated erythrocytes are similar in bone marrow and peripheral blood. In contrast to mice, rats selectively remove micronucleated erythrocytes from peripheral blood due to the spleen function (Schlegel and MacGregor, 1984), as in humans and dogs. This may theoretically have an impact on the ability to detect micronucleated erythrocytes in rat peripheral blood. This is being evaluated by the ICH revision working group, as previous studies have experimentally shown that most clastogens and aneugens are detected using rat peripheral blood erythrocytes (Hamada *et al*., 2001). The development of the micronucleus assay using peripheral blood and the use

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of fluorescent staining promoted the automated detection of micronuclei by flow cytometry (Hayashi *et al*., 2000). Another acceptable method for automated scoring of micronuclei is image analysis, provided it is appropriately validated (Romagna and Staniforth, 1989).

Many variations of the initial erythrocyte micronucleus assay are expected in the next few years, improving the predictivity of *in vivo* genotoxicity testing further (Hayashi *et al*., 2007).

2.1.3.2 The In Vivo Mammalian Bone Marrow Chromosome Aberration Test

The *in vivo* chromosome aberration test (Preston *et al*., 1987; Tice *et al*., 1994) is performed on the bone marrow of animals as in the *in vivo* micronucleus assay, but, after animals have received a metaphase-arresting agent, cells are prepared as they are in an *in vitro* chromosome aberration test (see previous sections) (OECD, 1997e). Generally, rats or Chinese hamsters are preferred to mice, whose chromosomes are small and of the same morphology. The analysis, as in the *in vitro* assay, is tedious and time-consuming and only the presence of polyploidies or endoreduplications will give an indication of a potential aneuploidy, whilst the *in vivo* micronucleus test detects aneugens. However, the *in vivo* chromosome aberration test is useful when the *in vivo* micronucleus assay is prone to give false positive results (see Section 2.2) or if, for example, the hamster is a much better model of human metabolism than rats or mice.

A variation of the test consists in collecting lymphocytes from animals receiving the test compound, inducing mitogenesis of these lymphocytes in cell culture and then obtaining metaphase spreads on slides. Such a method could be used in toxicology studies as lymphocytes are easy to collect at any time during a study (Flammang *et al*., 2007).

2.2 The Limitations of the Genotoxicity Tests

2.2.1 Bacterial Reverse Mutation Assay

Although *Salmonella typhimurium* TA102 or repair-proficient WP2 *Escherichia coli* (pKM101) will allow detection of crosslinking agents, the Ames test is poor at detecting clastogens that induce large DNA deletions because if the target sequence in the histidine or tryptophan gene of the tester bacteria is deleted, reversion to auxotrophy (histidine or tryptophan independence) cannot occur. The test is very specific, but subsequently not very sensitive. The highest tested concentrations will be limited for antibiotics and will be inefficient for specifically mammalian-cell-acting agents, such as topoisomerase inhibitors, for example. In fact, it is frequently observed that negative compounds in the Ames test could be revealed as positive in mammalian genotoxicity tests (see the Section 2.2.2).

On the other hand, attention should be paid to molecules able to free some histidine or tryptophan in the selective medium, hence artefactually increasing the number of apparent revertant colonies when using *Salmonella typhimurium* or *Escherichia coli* histidine or tryptophan mutants (Grüter *et al*., 1991).

2.2.2 In Vitro Mammalian Cell Genotoxicity Assays

The limitations of the *in vitro* mammalian test systems have been addressed for a long time (Zeiger, 1997). The difficulty in assessing the relevance of these systems compared to carcinogenicity data were often pointed out because carcinogenicity bioassays are not always the most relevant reference, and very few data exist on human carcinogenicity.

It is known that mouse lymphoma L5178Y cells might be theoretically more susceptible than other cells to genotoxic damages as they produce a dysfunctional p53 protein (Storer *et al*., 1997). In a recent compilation work, the limits of the *in vitro* mammalian cell assays were addressed (Kirkland *et al*., 2005). It was shown that the *in vitro* chromosome aberration test and the mouse lymphoma assay were lacking specificity, and that the current ICH genotoxicity battery is prone to give false *in vitro* positive results.

Of course, some of the Ames negative compounds found to be positive in at least one *in vitro* mammalian genotoxicity test are truly positive, but only detected on mammalian cells. Others may result from extreme culture conditions. Extreme pH and osmolality have been known for a long time to induce artefactual effects such as chromosome breakage (Brusick, 1986; Scott *et al*., 1991; Kirkland and Mueller, 2000; Moore and Brock, 1988). Therefore, it is recommended that pH and osmolality should be systematically checked and should not vary by more than 0.5 pH unit or 50 mOsm kg^{-1} compared to the culture medium in any of the *in vitro* mammalian cell systems ($tk^{+}/_{-}$ mouse lymphoma assay, *in vitro* chromosome aberration or *in vitro* micronucleus assay). So, cells are exposed to the compound under physiological conditions and the culture conditions will not be responsible for false positive responses.

However, extreme conditions or mammalian-specific effects do not account for all of the excess positive genotoxic responses seen in the *in vitro* mammalian cell assays. The questions addressed in the essential review of Kirkland *et al*. (2005) and the ECVAM workshop (Kirkland *et al*., 2007) are the adequacy of the current standard battery and the relevance of the high doses chosen on the basis of such a nonphysiological cytotoxicity of 90% for the mouse lymphoma assay, for example, or set at 10 mM for the noncytotoxic compounds.

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2.2.3 In Vivo Micronucleus Assay

Modified erythropoiesis and changes in body temperature have been shown to interfere with micronuclei production. It is now well established that a chemically-induced decrease in body temperature, or bleeding or haemolysis, leading to accelerated erythropoiesis, may artefactually increase the spontaneous level of micronuclei.

As early as in 1985, the effects of the stimulation of erythropoiesis on the frequency of micronucleated erythrocytes were reported in peripheral blood of mice (Steinheider *et al*., 1985). The effects of erythropoietin or bleeding (Suzuki *et al*., 1989a; Hirai *et al*., 1991) on the frequency of bone marrow micronucleated erythrocytes were reported in the literature. Further studies extensively demonstrated the role of erythropoietin in the production of micronucleated erythrocytes in mice, without any concomitant genotoxic effect on the induction of chromosome aberrations in bone marrow cells (Yajima *et al*., 1993a; 1993b; 1993c; Jones and Fos, 2003), showing that it was due to a specific nongenotoxic effect on erythropoietin-receptive cells.

The mechanism of this increase in micronucleated cells is based on the acceleration of differentiation and multiplication of erythroblasts in response to bleeding, chemical-induced haemolysis or erythropoietin stimulation, making DNA repair less efficient, chromosome missegregations and disturbance of the enucleation process more frequent when cells are rapidly dividing (Suzuki *et al*., 1989b; Hamada *et al*., 2001). It has also been established in rats (Hamada *et al*., 2001; George *et al*., 1990).

These data were considered sufficiently convincing by authorities or scientists involved in the process of regulatory changes in genotoxicity testing. Indeed, in regulatory assessment, a recent example was given with 2-hydroxy-1,4-naphtoquinone, the natural dye ingredient of Henna (Marzin and Kirkland, 2004). Positive responses in mice bone marrow micronucleus assays were attributed to haematotoxicity. Therefore, this compound was regarded as safe for human use. The regulation position can also be illustrated by the issues debated at the recent IWGT (Tweats *et al*., 2007a), where erythropoiesis was clearly reported as a cause of nonrelevant *in vivo* positives, with the need to carry out another test.

Hypothermia has also been shown to induce increases in the frequency of micronucleated bone marrow erythrocytes in mice (Asanami and Shimono, 1997; 2000; Asanami *et al*., 1998; Hamada *et al*., 2001; Guzman *et al*., 2004). The mechanism is still unclear. An explanation is that hypothermia indirectly induces erythropoietin stimulation. Therefore, accelerated erythropoiesis results in enucleation anomalies as shown above. Because these micronuclei are produced from fragmentation of the interphase nucleus, hypothermia should have no effect on chromosomal aberrations. At the IWGT meeting in September 2005 (Tweats *et al*., 2007a), hypothermia was clearly reported as a cause of nonrelevant *in vivo* positives.

In conclusion, when a compound is suspected to act on erythropoiesis before the *in vivo* genotoxicity evaluation, directly or through hypothermia, it will be preferable to use the *in vivo* chromosome aberration test on bone marrow rather than the *in vivo* micronucleus test to evaluate the genetic potential of the compound, as it is less prone to produce irrelevant results in that case. Indeed, the *in vivo* chromosome aberration test can be used equally to the *in vivo* micronucleus assay without any justification, as both are considered equivalent by authorities (ICH, 1997), provided there is a test in the battery for the detection of aneuploidy.

Conversely, some compounds have been found to be positive only *in vivo*. The genotoxicity assessment of such compounds has been extensively reviewed by the working group of the IWGT (Tweats *et al*., 2007b). Differences in metabolism *in vivo* and *in vitro*, higher exposure *in vivo* in the target cells and alteration in cell lines of normal regulation pathways may account for these apparent discrepancies. Most of these cases were predictable considering the pharmacological activity, metabolism and pharmacokinetics of the tested compounds.

2.3 Additional Genotoxicity Tests and Other Tools

2.3.1 Tests for Measuring DNA Primary Lesions

Before transformation of a DNA lesion in an inheritable stable modification, an interaction between the genotoxin and DNA can be detected. Tests able to measure these primary lesions can be useful for discriminating between DNA-reactive genotoxins and indirect genotoxins. However, the results from these tests should be cautiously interpreted in relation to the possibility of thresholds, because these primary alterations are normally repaired and do not always result in permanent genetic alterations.

2.3.1.1 The UDS Test

In the standard *in vivo* unscheduled DNA synthesis (UDS) test, animals receive the test compound and the liver is collected for analysis. Liver cells are suspended and seeded onto plates for primary cultures in the presence of radiolabelled thymidine, a precursor of DNA synthesis. If DNA lesions have occurred, they are susceptible to be repaired by the excision-repair system, and a subsequent incorporation of radioactivity in the nuclei will be detected by autoradiography (Ashby *et al*., 1985; Butterworth *et al*., 1987; Kennely *et al*., 1993; OECD,

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1997f). The UDS test was at first developed *in vitro* on primary cultures of treated hepatocytes. Now, the *in vivo* UDS test is widely used to detect test chemicals that would be missed in other genotoxicity tests and is recommended for additional testing by the current ICH S2 guideline (ICH, 1995). It is one of the tests recommended as follow-up for genotoxicity testing in relation to tumour findings in rodent carcinogenicity bioassays (Kasper *et al*., 2007) and is useful for testing the hypothesis of a non-DNA reacting genotoxic compound. The *in vitro* UDS on treated hepatocytes can also be used as an additional test to the standard battery (Madle *et al*., 1994). Compared to the *in vivo* UDS assay on liver, the *in vitro* UDS assay will help to assess the relevance of *in vitro* positive results.

The UDS test has the advantage of displaying Phase I and Phase II metabolic capacities, using whole animal or isolated liver cells, which are more representative of the real situation. In addition, metabolites are formed at, or very close to, the presumed site of genotoxic action, allowing the detection of various procarcinogens (Anderson *et al*., 1997; Wolterbeek *et al*., 1993).

2.3.1.2 The Comet Assay

In the Comet assay, isolated cells prepared from either cell cultures (*in vitro* Comet assay) or from any organs of treated animals (*in vivo* Comet assay) are prepared as a cell suspension, mixed with agarose and spread on slides. After an *in situ* cell lysis and DNA unwinding, slides are subjected to an electric field for electrophoresis of fragmented DNA, and stained with a DNA-specific fluorochrome. The technique has been standardized using an alkaline buffer (pH $>$ 13) for DNA unwinding and electrophoresis (Hartmann *et al*., 2003). Under the action of the electrophoretic field, DNA fragments will migrate from the nucleus according to their size, forming a DNA tail more, or less, extended (Comet tail). Slides are analysed under the microscope for the percentage of DNA in the tail, tail length and moment. An image analysis system is recommended for this analysis. The Comet assay detects single- and double-strand DNA breaks, resulting from oxidative damage, DNA–DNA, DNA–proteins crosslinks and alkali-labile sites. However, this pattern of detection of DNA lesions is not restricted to primary events resulting in clastogenicity because abasic sites, related to gene mutation, can also be detected. The Comet assay has the advantage of being applicable to any cultured cell type *in vitro* or to cell suspensions prepared from any organ. For this reason, the *in vivo* Comet assay is very useful in assessing the possibility of a compound which tested positive in the *in vitro* mammalian cell systems acting through DNA damage, and in addressing the mode of action of a compound inducing tumours in a rodent carcinogenicity bioassay when it is negative in the *in vitro* genotoxicity battery (Hartmann *et al*., 2004). Using any organ also provides a surrogate to the *in vivo* bone marrow micronucleus test in case local exposure is higher than systemic exposure. Compared to the UDS test, the Comet assay could be more sensitive in that it better detects small lesions than the UDS test and is not restricted to lesions repaired via an excision-repair process. The cytotoxicity has been a matter of concern for the Comet assay, but the confounding effects of apoptosis on genotoxicity-induced DNA breaks can be avoided now by the improvement of experimental conditions and scoring (Hartmann and Speit, 1997; Kim *et al*., 2002; Rundell *et al*., 2003). However, indirect cytotoxic mechanisms may lead to positive effects in the Comet assay (Andersson *et al*., 2003) although often associated to high cytotoxicities, so that the Comet assay could be less specific than the UDS test when evaluating the mode of action of non-DNA reacting compounds. Therefore it is necessary that a cytotoxicity assessment should be done on isolated cells in addition to histopathological examinations for the selection of the top dose used in genotoxicity assessment (Burlinson *et al*., 2007).

Many reviews have been published and a standardized protocol has been established at the IWGT of 1999 and 2005 (Tice *et al*., 1990; 2000; Brendler-Schwaab *et al*., 2005; Burlinson *et al*., 2007). Collaborative validation works have been completed or are ongoing, especially on the *in vivo* Comet assay (Sasaki *et al*., 1998; 2002).

The *in vivo* Comet assay is used at the moment for mechanistic studies and widely recognized by authorities as an additional test to the standard genotoxicity battery. It could be, in the future, increasingly used at the first steps of genotoxicity evaluation.

2.3.1.3 DNA Adducts

The formation of DNA adducts can be measured using the radiolabelled test compound and measuring its covalent binding to DNA. On the contrary, in the ^{32}P-postlabelling assay, the DNA is radiolabelled and exposed to the unlabelled tested compound. After DNA digestion, modified nucleotides are detected by two-dimensional chromatography (Randerath *et al*., 1981; Gupta, 1985). The method is highly sensitive, therefore very useful for assessing human exposure to a known compound (Randerath *et al*., 1989), even at a low exposure level. However, it is less robust for studying the possible genotoxicity of an unknown compound where modified nucleosides often need to be identified to demonstrate a real effect on DNA (Reddy, 2000). In addition, the high sensitivity of detection of primary DNA lesions makes it very sensitive to the variations of experimental conditions. Indeed, many factors, including environmental factors, are able to induce a positive signal in the highly sensitive ^{32}P-postlabelling assay (Moller and Wallin, 1998; De Bont and Van Larebeke, 2004; Li *et al*., 1995; Reddy, 2000), due to the production of reactive endogenous species, that is typically an indirect genotoxicity effect.

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Therefore, the ^{32}P-postlabelling assay is less suitable than adducts measured with the radiolabelled compound to demonstrate a direct effect on DNA. It was recognized about that primary DNA alteration that 'their relationship to mutations is not precisely known' (ICH, 1995). Whilst the mechanisms involved in the UDS test and the Comet assay are better understood, the relevance of the ^{32}P-postlabelling results for mutations remains questionable.

2.3.2 Transgenic Rodent Mutation Assays

The transgenic rodent mutation assays are the only assays available for the *in vivo* detection of gene mutation in any tissue. Mutations on the transgene integrated in the genome of the treated animal are revealed after its extraction and integration in appropriate bacteria. The general principle is described in Gossen *et al*. (1989). These models are very useful for assessing the relevance of *in vitro* positive results and also for improving cancer risk assessment. Various transgenic models are available. The best known models are Muta™ mouse, Big Blue® mouse and rat, *LacZ* plasmid mouse and the *gpt* delta mouse. According to the transgene, they are able to detect punctual gene mutations or small deletions. The specifications of the protocol have been addressed in the successive IWGTs held in 1999 and in 2005 (Heddle *et al*., 2000; Thybaud *et al*., 2003). An extensive review has been published by Lambert *et al*. (2005), OECD (2009).

2.3.3 Short-Term Carcinogenicity Assessment

A number of carcinogenic compounds have a nongenotoxic mode of action. As a part of toxicity studies, but emerging with the development of genotoxicity tests, models have been developed to address the possibility of carcinogenic effects in the first steps of pharmaceutical development, long before the rodent carcinogenicity bioassays are performed. Indeed, the results of the carcinogenicity bioassays are available at the late stages of development, after the clinical studies have started, and an early assay predictive of carcinogenicity would be useful.

2.3.3.1 The In Vitro Cell Transformation Assays

For this purpose, *in vitro* cell transformation assays were developed in the 1980s (Barrett *et al*., 1979; 1984). They consist of the identification of foci of transformed cells in a culture of normal cells receiving the test compound. Either embryonic Syrian hamster embryo (SHE) cells (Barrett and Lamb, 1985) or immortalized cell lines are used, namely, BALB/c 3T3 or C3H10T1/2 cell lines (Combes *et al*., 1999; LeBoeuf *et al*., 1999).

Collaborative studies have shown a certain correlation with rodent carcinogenicity bioassays, especially with the SHE cell transformation assay, after improvements of the conditions of experiments and the scoring (Gibson *et al*., 1995; LeBoeuf *et al*., 1996; Zhang *et al*., 2004). These studies showed approximately 80% concordance between the results of the SHE cell transformation assay and carcinogenicity on a series of genotoxic and nongenotoxic compounds (see OECD, 2007 for a review). However, this good performance was contradicted by a comparative assay where only one out of five carcinogenic compounds was identified as such by the SHE cell assay (Harvey *et al*., 2005). Carcinogenicity is typically a multistep and multifunctional process, which seems difficult to model simply by the use of a short-term *in vitro* assay. Therefore, insufficient data and knowledge of the mechanisms involved in *in vitro* transformation are available at the moment to recommend using cell transformation assays as early predictors of carcinogenesis.

2.3.3.2 The In Vivo Transgenic Tumour Models

In vivo transgenic animals were developed for predicting the results of the two-year rodent carcinogenicity bioassay. These animals, excessively sensitive to carcinogens, are able to develop tumours after six months of exposure to a carcinogen instead of the two-year exposure needed in a traditional rodent bioassay. Two main models are used, the p53^{+}/$_{-}$ knockout mouse, whose tumour suppressor gene is dysregulated (Storer *et al*., 2001), and the ras-H2 mouse, whose *ras*-proto-oncogene is dysregulated (Usui *et al*., 2001).

Based on the International Life Sciences Institute (ILSI) HESI collaborative evaluation works (Robinson and MacDonald, 2001; MacDonald *et al*., 2004; Cohen, 2001) and on IWGT recommendations (Storer *et al*., 2003), these models have been extensively reviewed (for a review, see Pritchard *et al*., 2003; Jacobson-Kram *et al*., 2004; Goodman, 2001). *In vivo* transgenic tumour models are especially useful to assess the relevance of positive findings in *in vitro* genotoxicity assays or to assess the possibility of a nongenotoxic mode of action when tumours are found in the traditional two-year rodent bioassay. These models are now accepted by authorities to study the mechanism of action of nongenotoxic carcinogens.

2.3.4 High-Throughput Screening Tests

Screening tools were designed to assess genotoxic effects in pharmaceutical development as early as possible, using small amounts of chemicals, so that new potential products could be chosen more easily. With the production of large series of new chemical entities, miniaturized genotoxicity tests are needed and have been developed in pharmaceutical companies or are available on the market

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(Reifferscheid and Heil, 1996; Reifferscheid *et al*., 2005). They are reductions of the standard Ames test using smaller plates and regulatory strains (Flamand *et al*., 2001) or cultures in suspension of mixed strains representative of the regulatory strains, like the Ames II™ test (Fluckiger-Isler *et al*., 2004). These screening tests are highly predictive of the results obtained with regulatory Ames tests but, they require lower amounts of test chemicals. Therefore they can be used at the early steps of development. It is essential to add a test using mammalian cells to a screening battery, as the bacterial test will not be able to detect genotoxins acting through a mammalian cellular pathway and will miss a number of clastogens. For example, the *in vitro* micronucleus test using mouse lymphoma L5178Y cells cultured in microplates can also be used as a miniaturized version (Nesslany and Marzin, 1999). Both Ames and *in vitro* micronucleus tests often constitute a minimal screening battery but other genotoxicity tests can be used, which need lower amounts of test chemicals or have different end points (Johansson *et al*., 2004; Marple *et al*., 2004). Among the tests requiring very little compound, the GreenScreen tests, based on a gene promoter induction by DNA damage, was developed on yeast (Van Gompel *et al*., 2005) and, better, on human cells (Hastwell *et al*., 2006). Depending on the chemical class of the compound or its expected mechanism of action, all or part of these methodologies can be used. This battery of screening tests, using minimal amounts of compound, gives pharmacologists crucial information at the very early stages of development so that new nongenotoxic backups can be found and fewer compounds are dropped during their preclinical development.

2.3.5 Structure–Activity Relationships

The study of structure–activity relationships is a field of interest in genotoxicity (Ashby *et al*., 1989; Kalopissis, 1991; Rosenkranz *et al*., 1991; Benigni, 1991; Snyder and Smith, 2005). Many attempts have been made to correlate genotoxic activity with chemical structure with the hope of predicting genotoxicity and carcinogenicity from the structure. It is not an easy exercise, because the genotoxic activity of a chemical depends not only on the presence of radicals identified to be at risk, but also on other radicals present in the structure susceptible to modulate a chemical reactivity. The three-dimensional conformation of the chemical is also a major decisive characteristic. However, the structure–activity relationships of a series of chemically related compounds are fruitful to identify structures at risk in a given family of compounds and to give alerts on some chemical structures. In any case, the study of structure–activity relationships should not replace experiments in genotoxicity studies, and a presumptive reactivity will need to be confirmed in a genotoxicity experiment. The use of databases and model softwares is expanding, for identifying structures of interest in drug research or for identifying alert-structures in toxicology, especially at the early steps of genotoxicity screening (Muster *et al*., 2003). The widely used softwares are DEREK and MultiCase (Snyder *et al*., 2004), which allow an in-house elaboration of a dedicated database for the chemical classes of interest. The study of structure–activity relationships is growing in the screening set of genotoxicity tests to help to predict the genotoxicity of impurities possibly contaminating the compound (Glowienke *et al*., 2005).

3 EVALUATION OF GENOTOXICITY

3.1 Evaluation of Results

When evaluating the results, we should be able to detect a positive effect, even if it is weak, above a measurable background of spontaneous mutations, whatever the genotoxicity test used. Several criteria are used to define an assay as positive. The magnitude of the effect above a negative control value, estimated either by a statistical analysis or by an induction factor, is not predominant because it depends not only on the potency of the compound but also on the system and its interaction with the compound at high doses. Other indications such as the reproducibility for weak or equivocal positive responses will be important, together with the dose–effect relationship. Historical data of both negative and positive control values of the laboratory will be useful for monitoring the variability between experiments, with the use of limits for concurrent control values based on historical values. Some recommendations have been made specifically for several genotoxicity tests (Krishna *et al*., 2000; Gatehouse *et al*., 1994). Historical data will also be a reference for assessing the biological relevance of a genotoxicity result (Mitchell *et al*., 1990).

3.2 Evaluation of Cytotoxicity

Based on the assumption of a linear dose–effect relationship for genotoxic effects, the highest cytotoxicities are required for the top doses of genotoxicity tests. However, high cytotoxicity, required for the highest tested concentrations, may also account for some irrelevant *in vitro* positive findings. In the mouse lymphoma assay, where the surviving cell population is exposed to a majority of dying cells releasing reactive oxygen species in the medium, it is not surprising that the DNA of surviving cells is damaged. Such positive responses are regularly seen, quantitatively and qualitatively correlated with high cytotoxicity. The possibility of irrelevant positive results due to cytotoxicity has also been pointed out for the *in*

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vitro chromosome aberration test (Hilliard *et al*., 1998; 2007), where the minimal level of required cytotoxicity at the top concentration was arbitrarily set at more than 50%. Therefore, the level of cytotoxicity needed in the *in vitro* genotoxicity tests is being questioned and has to be taken into account in the evaluation of results (Galloway, 2000; Kirkland and Mueller, 2000; Mueller and Kasper, 1995; Kirkland *et al*., 2005; Fellows and O'Donovan, 2007).

Given the importance of the cytotoxicity evaluation in the genotoxicity assessment, it is crucial that the measurement of cytotoxicity accurately reflects the proportion of the cell population which was unable to be hit by a mutagen, because the cell died, was dysfunctional or unable to divide. The measurements of cytotoxicity are designed to take into account these different aspects of cytotoxicity. They range from cell counts to cloning efficiency or mitotic index but none of these parameters takes into account all aspects of cytotoxicity. In the mouse lymphoma assay, the measurement of cytotoxicity has actually been improved, taking into account the short-term and the long-term cytotoxicity. For the *in vitro* chromosome aberration test, the calculation of a PD, taking into account both cell death and inhibition of cell division, has been proposed to improve the assessment of cytotoxicity (Greenwood *et al*., 2004). The use of the PD has also been shown accurate in the *in vitro* micronucleus assay in the absence of cytochalasin B (Kirsch-Volders *et al*., 2003a; Fellows *et al*., 2008; Lorge *et al*., 2008). When using cytochalasin B, the cytotoxicity relies commonly on CBPI which also takes into account cell death and inhibition of cell division (Kirsch-Volders *et al*., 2004).

3.3 Metabolic Activation

In *in vitro* test systems, an exogenous metabolic activation system is needed in cases where genotoxic metabolites could be produced by the compound. The most widely used system is the Arochlor 1254 induced rat liver microsomal fraction (S9) added with cofactors (S9 mix). A combination of beta-naphthoflavone and phenobarbital is also being used to gradually replace Arochlor (Elliott *et al*., 1992; Johnson *et al*., 1996). The S9 is presumed to contain a large panel of cytochromes. It is important that this preparation, whose composition might vary from one preparation to another, is checked for efficacy before use in genotoxicity studies. It has been shown that, provided the cofactor mix is adequate and not limiting, the amount of S9 will not be a major source of variation in metabolic efficacy (Majeska and McGregor, 1992). Rat induced liver S9 has proved to be effective in metabolizing most promutagens into mutagens (Clive *et al*., 1979). However, species differences are known between rodents, dogs, monkeys and humans. For example, some compounds such as nitrosamines are metabolized into the genotoxic active metabolite more effectively by hamsters, even when cytochromes have been induced (Amacher *et al*., 1982). Nevertheless, these differences are mostly quantitative not qualitative. It might be more realistic for pharmaceuticals to use human liver S9 instead of rodent induced liver S9, which could produce specific metabolites not relevant to human metabolism. Human S9 gave satisfactory results compared to rodent induced liver S9 (Hakura *et al*., 2005). However, human S9 could not be routinely used in genotoxicity assessment due to interindividual variations, but could be considered as a complement for risk assessment. Whatever the origin of the metabolic activation systems used in genotoxicity testing, it is an imperfect system which could produce irrelevant metabolites or, on the other hand, might be unable to produce relevant metabolites for humans. Negative and positive genotoxicity results with metabolic activation should be considered together with metabolic profiles in various species and the measurement of metabolites in plasma, in species used for toxicology assessment and in humans.

3.4 Aneuploidy

Aneuploidy is an irregular variation in the haploid chromosome number due to loss or gain of chromosomes. It represents a genotoxic and carcinogenic risk because additional or missing chromosomes will cause genomic instability or change the chromosome balance of the cell, which might be able to modify gene expression, for example reactivation of oncogenes which are normally repressed. Aneuploidy has been shown to be related to the induction of tumours (Haesen *et al*., 1993; Kolstad *et al*., 1996). Therefore, it is important that aneuploidy should be assessed for a full evaluation of the genotoxic potential of a test compound. For a long time, no standard *in vitro* genotoxicity test has been suitable for detecting aneugens, which might induce numerical chromosome aberrations. The *in vivo* and *in vitro* chromosome aberration tests, which are suitable for detecting chromosome structural aberrations, are not particularly appropriate for detecting aneuploidy. Numerical aberrations recorded in the chromosome aberration tests, that is polyploidy and endoreduplication, are not strictly indications of aneuploidy, but they may have a common origin (Mitchell *et al*., 1995). Cells arrested in metaphase by spindle poisons are able to produce both polyploidy and chromosome loss or gain, that is typically aneuploidy. However, polyploidy could also result from interference with other stages of the cell cycle, for example during telophase, inhibiting cell membrane formation at the end of mitosis, thus producing a binucleated cell (e.g. with cytochalasin B). Polyploid cells are usually produced in safe organs as a part of maturation or differentiation of

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normal cells (e.g. myoblasts) without any relationship to tumour induction. Consequently, polyploidy alone does not signal a genotoxic effect but should be verified by exploring the possibility of aneuploidy induction in a more appropriate test such as the micronucleus assay, which can also detect clastogens. Endoreduplication is a more complex phenomenon related to a disturbance in cell cycle regulation resulting in multiple copies of chromosomes, and as such, a form of polyploidy. Several S-phase cycles occur before the chromosome migrates to the poles of the dividing cell, so that metaphasic chromosomes contain multiples of two chromatids. The consequences of endoreduplication remain unclear. It is sometimes found in tumour cells but endoreduplication is also seen in normal organisms where it could be a means of increasing gene level expression. Therefore, scoring polyploidy and endoreduplication in the chromosome aberration test will not give an accurate assessment of aneuploidy and may also give signals which do not relate to a genotoxic alert.

Aneuploidy can be directly assessed by the *in vivo* and the *in vitro* micronucleus test. Aneugens will induce an increase in the number of micronucleated cells. The micronuclei induced by an aneugen will generally be larger in size than when induced by a clastogen. However, if there is a need to discriminate between aneuploidy and clastogenicity, centromere labelling can be used to discriminate accurately between micronuclei with centromeres, corresponding to a chromosome, and micronuclei lacking a centromere, corresponding to a chromosome fragment. Centromere labelling can be achieved either by immunolabelling of kinetochores with antibodies labelling specifically the kinetochore proteins (Lynch and Parry, 1993), or by FISH of DNA probes labelling specifically the DNA repetitive sequences of the centromere (Bentley *et al*., 2000; Marshall *et al*., 1996). The human antikinetochore antibody is convenient for several species as kinetochore proteins are well-preserved proteins among species. DNA centromeric probes are available for most cell types and species. These methods can be applied *in vivo* and *in vitro* and will be very useful for the evaluation of thresholds, as aneuploidy is typically a threshold mechanism (see Section 3.6).

3.5 Non-DNA-Reacting Mutagens

Over decades of genotoxicity evaluation of candidates for pharmaceutical development, the number of unique *in vitro* positive compounds has increased, without significantly changing either the way genotoxicity tests were conducted or the intended therapeutic axes. However the concepts being developed now for discovering new active pharmaceuticals involve new cellular targets. This probably results in a diversification of the possible interactions of a chemical with the different cell functions, including interactions resulting in DNA damage. In the last few years, a number of publications on the non-DNA-reactive genotoxic compounds, that is indirect mutagens, have shown that these complex interactions may account for a number of *in vitro* genotoxic positive results in mammalian cells.

Many possible targets for non-DNA-reacting mutagens have been listed (Kirkland and Mueller, 2000). To give some examples, they are: interferences with DNA repair and replication proteins, DNA precursors imbalance, lipid peroxidation, depletion in glutathione, nuclease release from lysosomes and probably many others. Any event which contributes to increasing or releasing endogenous reactive species or interferes with all the stages of cell division is susceptible to induce DNA alterations by an indirect mechanism. Aneuploidy is a special case of indirect mechanism because the ultimate target is not DNA, but it is the best known and most documented example of indirect genotoxicity (Kirsch-Volders *et al*., 2003b). There are some examples of experimentally demonstrated relationships between lysosomal breakdown or nuclease release and induction of chromosome breakage for example (Bradley *et al*., 1987). The obviously irrelevant *in vitro* genotoxic activity of sodium chloride has been shown to result from an indirect mechanism (Seidel *et al*., 2004). Another example of genotoxic effects induced through an indirect mechanism is that of carbon tetrachloride, recently shown to induce DNA adducts via oxidation and lipid-peroxidation-derived products (Eastmond, 2008). Few pharmaceuticals have been demonstrated inducing genotoxicity through an indirect mechanism (Mueller and Kasper, 1995; 2000). Spindle poisons, nucleosides and topoisomerase inhibitors have been selected for their pharmacological properties to react with a cell target likely to induce indirect genotoxicity effects. However, few pharmaceuticals have been shown to induce an unexpected and pharmacologically unrelated indirect genotoxicity. Paracetamol (acetaminophen) was shown to induce a thresholded metabolic overload (Bergman *et al*., 1996). For most pharmaceuticals showing positive findings in *in vitro* genotoxicity, the possibility of an indirect mode of action remains unknown because the compound was dropped without complementary investigations. Therefore, the number of unique *in vitro* positive compounds due to indirect mechanisms is likely to be underestimated and may partly account for the excess of positive results in *in vitro* genotoxicity testing (Kirkland *et al*., 2005).

For risk assessment in routine genotoxicity evaluation, it is essential to distinguish between thresholded effects, due to indirect mechanisms of genotoxicity, and nonthresholded effects, due to DNA-reacting mutagens. Pharmaceuticals are complex compounds having multiple cell targets. In the case of an indirect effect, the absence of a direct effect on DNA as well as a mechanism explaining the induction of mutations by an indirect effect

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should be demonstrated. Investigating the indirect mechanisms of genotoxicity is now a part of the current thinking on the improvement of genotoxicity tests, their suitability, the possible sources of false positive results, and the appropriate complementary tests which can be added to the minimal battery so that actual genotoxic activity presenting a potential hazard for humans can be correctly assessed. These issues are examined by the IWGT, the ECVAM, the HESI, and the ICH.

3.6 Thresholds

Up to the 1990s, it was generally admitted that any amount of a compound found genotoxic, even at very high concentration in *in vitro* test systems compared to human plasma levels, was likely to present a risk when administered at a therapeutic dose. This concept relied on the irreversibility of genotoxic events. However, it has been shown at first for non-DNA-reacting compounds that thresholds account for observed nonlinear genotoxic responses. Thresholds have been measured for aneugens (Elhajouji *et al*., 1995; 1997; 1998; Morales-Ramirez *et al*., 2004), reflecting that as long as the concentration of aneugen is not sufficient to alter mitotic spindle such that the chromosome migration is disturbed, no effect will be induced by the aneugen. To generalize, indirect mechanisms of genotoxicity are thresholded mechanisms and thresholds can be measured (Kirkland and Mueller, 2000; Kirsch-Volders *et al*., 2003b). Practical thresholds have been measured for various genotoxic compounds (Asano *et al*., 2006) and the possibility of a threshold for direct-acting DNA compounds has been addressed (Jenkins *et al*., 2005). The concept of a 'threshold of toxicological concern' was introduced in the European Medicines Agency guideline on the limits of genotoxic impurities (EMEA, 2007). It derived from the Food and Drug Administration (FDA) guidance for food-contact materials (FDA, 1995) and was based on the estimated risk of inducing cancer in humans after a lifetime exposure to a carcinogen. Identifying and taking into account thresholds for genotoxic compounds will help to improve risk assessment for humans.

3.7 Strategy for Human Risk Assessment

To help to assess the relevance of *in vitro* positive results, to avoid producing irrelevant results, or to understand the mode of action of a compound, and finally evaluate the risk for humans, additional tests to the standard battery may be useful. An alternative *in vitro* chromosome aberration test or micronucleus assay on cell types other than L5178Y cells will indicate whether cell specificity is involved in an *in vitro* positive response, as well as possible aneugenic effects. *In vivo* experiments, that is, micronucleus assays after long-term exposure or in relevant target organs, that is, in most cases in liver for pharmaceuticals, in addition to toxicokinetic and pharmacokinetic data, will attest to the predictive value of *in vitro* positive results for *in vivo* situations. The *in vivo* Comet assay was also found to be very helpful in assessing *in vivo* genotoxicity in the appropriate organs detected as targets in other toxicity studies. Finally, short-term carcinogenicity tests (p53, rasH2) will increase confidence in *in vivo* negative results before the standard rodent bioassays are initiated for the assessment of carcinogenicity. Examples of decision trees have been drawn up to help in making decisions during the toxicity evaluation of a new pharmaceutical (Thybaud *et al*., 2007a; 2007b; Kasper *et al*., 2007; Kirkland *et al*., 2006; Lorge *et al*., 2007).

4 CONCLUSION

The improvements brought to the experimental genotoxicity testing as well as to the strategy of results evaluation will be useful in moving from hazard identification towards an integrated risk for cancer and genotoxicity. Over the past 30 years, genotoxicity evaluation has become a multidisciplinary science. Each compound is a specific case with an original mode of action defined on the basis of genotoxicity results, integrating the toxicity profile, the chemical and pharmaceutical properties of the compound; constantly evaluated and updated, re-assessing the risk–benefit at each step of the development.

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Carcinogenesis and Carcinogens that are also Genotoxic

Douglas McGregor

CONTENTS

1 INTRODUCTION

Cancer as a cause of death is not negligible at any age, but it is primarily a terminal illness in aged populations; therefore, because population age-structure is commonly shifting towards the elderly, the prevalence of cancers will increase even if there is no change in the age-standardized incidences. Carcinogenesis is the process by which these ultimately malignant tumours develop, and oncogenesis refers to the changes involved in the development of any kind of tumour, including benign tumours. A carcinogen, therefore, is a risk factor causally related to an increase in cancer incidence or prevalence. Cancer epidemiologists study the factors that lead people either to develop clinically diagnosed cancer or to die of cancer. In the latter case, the event is entered into mortality statistics; but in both cases, cancer epidemiology is almost always the study of malignancies. Other neoplasms may have been present within the individual that were not recorded because either they were never recognized (e.g. histology was limited to major lesions observed at autopsy) or they did not contribute to the cause of death. To an experimental oncologist, who will make detailed examination of a large number of organs and tissues from all of the animals in his experiment, a carcinogen is not necessarily an agent causing a pathological phenomenon resulting in death. A carcinogen in this experimental context is an agent which increases the incidence of any neoplasm irrespective of whether it is lethal, potentially lethal or benign. In extreme circumstances, a carcinogen may be defined also as an agent that increases the incidence of certain types of preneoplastic change and may even include certain types of hyperplasia. This degeneration in definition has been justified on the basis that had animals lived longer, or could have tolerated a higher dose, then progression to neoplasia malignancy would have occurred (Interagency Regulatory Liaison Group, Work Group on Risk Assessment, 1980). On the other hand, although a morphological

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continuum may be evident from the histological evaluation of preneoplastic and neoplastic lesions, it is not axiomatic that all preneoplastic or benign lesions progress to malignancy. Hyperplastic lesions may not progress to neoplasia and may be reversible (Eustis, 1989). Furthermore, certain types of tumour in laboratory animals, for example Leydig cell tumours in rats, are almost never malignant and are extremely rarely a cause of death. In this particular example, the difference between hyperplasia and adenoma is based exclusively on size and an arbitrary decision rule.

The information upon which evaluations of carcinogenic potential are made comes from epidemiology and laboratory animal experiments. Frequently, the animal experiments conducted with high-volume production chemicals, or chemicals which are important for some other reasons, give the first indications that special care may be necessary in handling the chemical because it is carcinogenic, but this is not always so. On other occasions, the primary information may come from observations upon human disease patterns. This chapter is a description of some of the problems associated with carcinogenicity experiments and a discussion of what the results might mean, particularly for those substances that are so-called genotoxic carcinogens. 'So-called' because the term is misleading: its use is based on the tacit assumption that if a chemical possesses both genotoxic and carcinogenic properties then genotoxicity is its mode of carcinogenic action. This is not necessarily so and is a topic that will be discussed below; however, carcinogens that clearly act through some mode of action not involving an inherent genotoxic property are discussed in **Nongenotoxic or Epigenetic Carcinogenesis**.

2 CARCINOGENESIS

The multistep, multifactorial process by which normal cells are thought to develop into cancerous cells involves the loss of genomic stability and the sequential acquisition of genetic alterations (Loeb and Loeb, 2000; Gray and Collins, 2000; Eyfjord and Bodvarsdottir, 2005). The somatic mutations found in cancers are either 'drivers' or 'passengers' (Greenman *et al*., 2007). Driver mutations are those which are causally involved in the neoplastic process and are selected for during tumourigenesis. Passenger mutations are neutral and nonlethal; therefore they are retained by chance during cell population expansion. Proto-oncogenes and tumour suppressor genes have been identified as the main mutational targets (Bishop, 1991; Weinberg, 1991), and carcinogen-specific mutational patterns have been observed in these genes in tumours of laboratory animals (Balmain and Brown, 1988) as well as in human cancers (Harris, 1992; Semenza and Weasel, 1997). This association suggests a mechanistic link between carcinogen exposure, genetic alterations and cancer (Hussain and Harris, 1999). The highly increased tumour incidence in subjects with defects in nucleotide excision repair supports the key role of DNA alterations in the process of cancer development (Stary and Sarasin, 2002). Moreover, organ and cell-type specific differences in DNA repair capacity have been demonstrated to correlate with site of tumour formation under a variety of experimental situations (Goth and Rajewski, 1974; Kleihues and Margison, 1974; Swenberg *et al*., 1984).

3 GENETIC FACTORS IN CARCINOGENESIS

An association of abnormal mitotic metaphase chromosomes with cancer cells has been known for more than a century, but chromosomal aberrations seemed to exhibit no specific pattern with particularly neoplastic diseases and it was far from clear whether the aberrations were causative or secondary events arising during tumour development. The first documentation of a nonrandom chromosomal aberration with a neoplastic disease was the reciprocal translocation between chromosomes 9 and 22 (t(9,22)), giving the Philadelphia syndrome, seen in chronic myeloid leukaemia (Nowell and Hungerford, 1960). Since then, it has been shown that specific reciprocal translocations are prevalent in leukaemias and lymphomas and they also occur in 3% of all neoplasms (reported by Solomon *et al*., 1991). More than 100 recurrent translocations have been described (Mitelman *et al*., 1990). In addition, progression towards increased malignancy appears to be characterized by increased genetic instability, manifest as translocations, deletions, chromosomal breaks, ploidy changes and nondisjunctions.

The genetic instability of tumour cells is well documented, but the mechanisms involved are not yet defined. They may involve mutations in DNA repair and synthesis genes. Evidence for this mechanism in man comes from instability genes in association with inherited chromosomal fragility syndromes, such as xeroderma pigmentosum and ataxia telangiectasia. Comparison of point mutation rates in tumorigenic and nontumorigenic cells does not reveal any marked difference, whereas the rate of gene amplification is clearly higher in tumorigenic cells. This was demonstrated about 20 years ago in the amplification of base sequences encoding the CAD protein (a multifunctional enzyme: carbamoyl phosphate synthase, aspartate transcarbamylase and dihydroorotase). Amplification of this gene cannot be demonstrated in normal cells from man, hamster or rat (frequency $<10^{-9}$), whereas amplification occurs in tumorigenic cells from each of these species at a frequency of about 10^{-4} (Tisty *et al*., 1989; Tisty, 1990).

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3.1 Dominant Oncogenes

The finding that the avian retroviral *src* gene had evolved from a captured cellular gene (Stehelin *et al*., 1976) was to guide much future research and the development of the concept of oncogenes. These are dominantly acting mutant forms of normal cellular genes (proto-oncogenes) which play a physiological growth-regulating role (reviewed by Bishop, 1987), many of them having protein kinase activity, particularly for the phosphorylation of tyrosine residues.

When mouse NIH 3 T3 fibroblasts are transfected with DNA from human tumours, the result is malignant transformation of the fibroblasts. Oncogenes were isolated from these cells (Shih *et al*., 1981) and were found to be homologues of retroviral transforming genes (Der *et al*., 1982). Over 100 oncogenes have been discovered since the early 1980s by use of these transfection assays. However, only a few of the oncogenes have a demonstrated involvement in human carcinogenesis, although, in an altered form, they function as oncogenes in laboratory animals and *in vitro* experimental systems. Apart from a few viruses, such as human papilloma virus (HPV) and hepatitis B virus (HBV), which may introduce foreign DNA, genetic alterations in human neoplasia entirely result from changes in structure and/or function of the cell's own genome. The proto-oncogenes are known or suspected to code for proteins acting as growth factors, growth factor receptors, secondary messengers that transduce signals through the cytoplasm, and transcription factors that regulate gene expression within the nucleus. In addition, the bcl-2 gene product appears to be localized on mitochondrial membranes and functions, not by stimulating growth, but by preventing the programmed cell death (apoptosis) of B lymphocytes.

The c-*myc* family and c-*ras* of proto-oncogenes are clearly important in human carcinogenicity, whereas, at present, no major role in human cancer has been found for *fos, myb, scr* or *jun*, which are central to normal mammalian cell growth and differentiation (Nowell, 1991). Nevertheless, oncogenes have been reported in 10–30% of human cancers (Slamon *et al*., 1984), and some less prominent oncogenes could act in collaboration with those that are more manifest. Exposure to some tumour promoters may cause transient increases in *fos, jun, sis* and *myc* expression; and in the case of c-*fos*, this transient expression in NIH 3 T3 cells gives a protein that activates or induces the synthesis of functions that accelerate mutation frequency (van den Berg *et al*., 1991). Oncogenes are capable of cell immortalization and transformation *in vitro,* and normally control a wide variety of cellular functions, such as proliferation, differentiation, morphology, intercellular communication and motility (Barbacid, 1987; Bishop, 1987). Their functions are affected by the cytogenetic processes mentioned earlier. For example, the t(9,22) translocation of chronic myeloid leukaemia results in the activation of the c-*abl* proto-oncogene, while t(8,14), t(2,8) and t(8,22) translocations in Burkitt's lymphoma all result in deregulated c-*myc* expression. There are many examples of proto-oncogenes located close to chromosomal breakage points in leukaemia and other neoplasms.

The prevalence of oncogenes in particular human tumours has its counterpart in chemically induced animal tumours. Some of these animal oncogenes show specific genetic changes involving growth factor receptors (*neu*), signal transducing systems (*ras*) or nuclear DNA-binding proteins (*myc*). The *ras* proto-oncogene mutations are particularly important and result in gene activation or amplification. The mutations are often consistently carcinogen specific (e.g. GT→TA transitions at the second base of codon 12 in H-*ras*, induced by methylating agents such as NMU (*N*-nitroso-*N*-methylurea (*N*-methyl-*N*-nitrosourea)) and MNNG (*N*-methyl-*N′*-nitro-*N*-nitrosoguanidine); AT→TA transversions at the second base of codon 61 in H-*ras*, induced by DMBA (dimethylbenz[*a*]anthracene)), but several polycyclic aromatic hydrocarbons other than DMBA do not induce consistent changes in the *ras* oncogene expressed in the induced tumour (Balmain and Brown, 1988).

Mutations in *ras* are, in some cases, very early events in carcinogenesis, but proto-oncogene activation may also occur late. In a transgenic mouse carrying *pim*-1, a single dose of ENU (*N*-ethyl-*N*- nitrosourea (*N*-nitroso-*N*-ethylurea)) induced lymphomas, most of which expressed K-*ras* or N-*ras*. However, the data suggested that at least some of the *ras* mutations were late events and therefore not induced by the NMU treatment (Breuer *et al*., 1991). Both NMU and ENU also induce gliomas and schwannomas in which *neu* is activated by specific TA → AT transversions within the transmembranal domain. However, adducts formed by NMU or ENU which might lead to this type of transversion have not been identified, so either the adduct is very minor but biologically important, or the activation of *neu* occurs late in tumour development (Balmain and Brown, 1988). Currently, this type of observation is sparse, but they could be important for the interpretation of data in which oncogene expression is found in advanced neoplasms.

3.2 Tumour Suppressor Genes

Malignant transformation cannot be described in terms of proto-oncogene activation. Familial studies have shown that mutations associated with loss of function are important in neoplasia and these are strongly supported by experimental work. Fusion of mouse fibroblasts with carcinoma cells, or fusion of two varieties of sarcoma, resulted in cells which were initially nontumorigenic (Harris *et al*., 1969). As the hybrid clones were cultured

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in vitro, there was reversion to the malignant phenotype, which was always associated with loss of specific chromosomes. In mouse, the re-emergence of malignancy was strongly associated with the loss of chromosome 4 (Evans *et al*., 1982). In human/rodent cell hybrids, the reappearance of malignancy was also associated with the loss of certain human chromosomes, particularly chromosome 11 (Klinger and Shows, 1983). Powerful evidence for the insufficiency of oncogene activity for the malignant phenotype is the finding that the hybrid of normal fibroblasts fused with EJ bladder carcinoma cells, which contain an activated H-*ras* gene, are nontumorigenic, even though H-*ras* gene expression continued at high levels (Noda *et al*., 1983). Similarly, the tumorigenicity of K-*ras*-transformed mouse cells is suppressed when they are fused with nontumorigenic mouse cells, although the K-*ras* gene continues to be expressed at high levels (Craig and Sager, 1985). It also emerged from this kind of experiment that fusion of a transformed bladder epithelium cell line with differentiated normal bladder epithelial cells resulted in suppression of tumorigenicity, whereas fusion with undifferentiated fibroblasts did not (Summerhayes and Franks, 1979; Cowell, 1980; Cowell and Franks, 1984). Thus, factors were present in normal, differentiated cells which suppressed malignancy. The concept arose that these factors were coded for in antioncogenes, or tumour suppressor genes. A number of these genes have been reported for a variety of human tumours. Tumour suppressor genes are probably more interesting than oncogenes, since loss of genetic material occurs far more frequently than does activation of a gene function.

The *TP53* suppressor gene (tumour suppressor gene located on the short arm of human chromosome 17 and coding for the phosphoprotein p53) may be the most frequently involved gene in human carcinogenesis, and it is certainly one of the more frequently studied. Indeed, a TP53-specific database is available at the International Agency for Research on Cancer (IARC) (*http://www-p53.iarc.fr/index.html*). TP53 is contained within 16–20 kb of DNA in the short arm of human chromosome 17 at position 17p13.1. This gene codes for a nuclear phosphoprotein that plays a critical role in safeguarding the integrity of the genome. It is mutated or part of its regulatory circuit is functionally inactivated in a proportion of almost all human cancers (**Figure 1**). TP53 protein is a sequence-specific DNA-binding transcription factor that is normally at low levels in cells. Stress signals, such as DNA damage, can stabilize and activate TP53. The critical event leading to the activation of TP53 is the phosphorylation of its N-terminal domain. The N-terminal transcriptional activation domain contains a large number of phosphorylation sites and can be considered as the primary target for protein kinases transducing stress signals. This activation and stabilization results in a large increase in the half-life of the protein, which therefore accumulates in cells. Upon activation, TP53 binds to the enhancer or promoter elements of downstream target genes and regulates their transcription. Four cellular elements are involved in a regulatory circuitry: TP53, murine double minute-2 (Mdm2), $P14^{ARF}$ and E2F-1. These are organized in two interactive feedback loops. In the loop formed of TP53 and Mdm2, TP53 activates Mdm2 transcription, and Mdm2 negatively regulates TP53 by promoting TP53 ubiquitination and degradation. In the other loop, E2F-1 activates ARF (acute renal failure protein) transcription, and $P14^{ARF}$ facilitates the proteolytic degradation of E2F-1. The two loops interact in two ways. $P14^{ARF}$ interacts with Mdm2, thereby inhibiting Mdm2-mediated ubiquitination and degradation of TP53 and this, in turn, results in the stabilization of TP53 (reviewed in Jin and Levine, 2001).

The majority of *TP53* mutations in human tumours occur in the evolutionarily highly conserved domains in exons 5–8. The missense mutations are predominantly GT → TA transitions and > 95% code amino acids that are entirely conserved in mouse, rat, monkey and man (Hollstein *et al*., 1991). In squamous-cell carcinoma of the skin, however, UV-specific CC → TT double-base and dipyrimidine C → T substitutions are also found which apparently do not occur in *TP53* of internal organs (Brash *et al*., 1991). All mutations of this gene so far examined have lost the ability to suppress transformation. Some of these may be dominant negative mutations that inhibit the function of normal TP53 by complex formation, while others not only have lost suppressor activity, but also can act, with the p21 product of *ras*, as dominant, cooperating oncogenes. Interaction between the products of oncogenes and tumour suppressor genes has been described. In the case of wild-type TP53 protein, inactivation can presumably result from binding with the oncoproteins derived from several tumour viruses, for example, SV40 (simian virus 40) large T antigen, adenovirus E1B protein and papilloma virus E6 protein, as well as specific cellular gene products, for example Mdm2. Similar inactivation can occur with retinoblastoma gene product, $p105^{RB}$, which may play a role in transcriptional regulation (Mitchell, 1991) and is a binding target of the protein from transforming gene *E1A* of adenovirus-12 (Whyte *et al*., 1988; 1989), of SV40 large T antigen (DeCaprio *et al*., 1988) and of HPV-16 E7 protein (Dyson *et al*., 1989). These DNA tumour virus oncoproteins may, therefore, mediate transformation by inactivating the *RB* gene by forming a complex with $p105^{RB}$.

3.3 Genomics

There has been an enormous amount of study of individual tumour suppressor genes and oncogenes, but even that effort is fast becoming over-shadowed by what is now possible following the sequencing of the human

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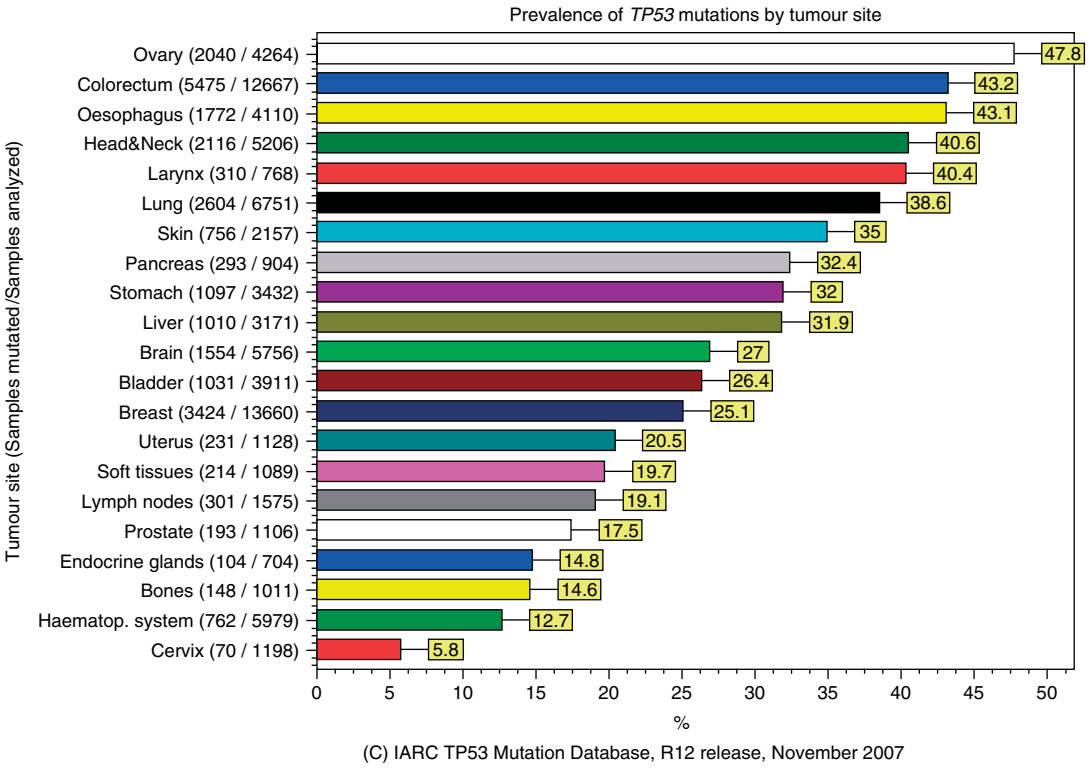

Figure 1 Prevalence of *TP53* mutations in human cancers. (Reproduced with permission from IARC.)

genome. Recently developed technologies have permitted the investigation and analysis of large fractions of the genomes of cancer cells to levels of detail that would have been unthinkable a decade ago. In spite of these advances that have made the identification of genetic alterations in cancers a goal that is attainable, it remains much more difficult to understand the precise role of these changes in the carcinogenic process. Previously, emphasis was placed on studying the very commonly occurring mutant genes of tumours (e.g. *TP53, APC* and *KRAS* in particular), but the data accumulated using the new technology show that the vast majority of mutations in cancers do not involve these genes. The data support the view (Beerenwinkel *et al*., 2007) that a large number of mutations, each associated with a small fitness advantage, drive tumour progression. Also, pathways, rather than individual genes, appear to govern the course of tumourigenesis (Vogelstein and Kinzler, 2004). The particular genes that seem to contain the driver mutations are quite different in different tumours, most mutated genes occurring in <5% of tumours. These differences in pattern across the genome may be responsible for the differences in characteristics observed in the individual tumours.

Analysis of exons representing 20 857 transcripts from 18 191 genes in DNA isolated from 11 breast and 11 colorectal tumours showed that the genomic landscapes of breast and colorectal cancers were composed of a handful of high-frequency mutated genes. In colorectal cancer these were *TP53, APC, KRAS* and, to a lesser extent, *Pik3CA* and *FBXW7*, while in breast cancer these were *TP53* and *Pik3CA*. In both organ cancers there was a much larger number of genes that were mutated at low frequency (Wood *et al*., 2007). However, most of the circa 80 DNA mutations that alter amino acids in typical cancers occur at very low frequency, and most of these changes may be harmless. There are <15 mutations likely to be responsible for tumour initiation, progression and maintenance (Wood *et al*., 2007).

It is generally assumed that, although these genetic changes are being observed in malignancies, they arise at all stages of the carcinogenic process, and it appears reasonable to assume that agents acting through genotoxic mechanisms are responsible for these changes. For carcinogens operating through a nongenotoxic mechanism it is assumed that there is a dose threshold below which an administered compound may not give a carcinogenic response, thus leading to a nonlinear dose–response relationship. Although for genotoxic carcinogens there may be a dose-threshold response due to a number of different protective mechanisms acting at different levels, it remains the default position that nonthreshold dose–response relationships exist for genotoxic carcinogens.

By analogy with the 'single hit' model of action of ionizing radiations (Lea, 1946), it has been proposed that, no matter how little it may be, any interaction of

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direct-acting genotoxic chemicals with the genetic material poses a finite probability of generating a response (McMichael and Wooward, 1999). Studies on covalent binding of substances that are both genotoxic and carcinogenic to DNA show a linear dose–response relationship in the low-dose range, with no indication of a threshold (Neumann, 1980; Dunn, 1983; Lutz, 1987; Beland *et al*., 1988). This might be thought to suggest a linear decrease of genotoxicity, and eventually of cancer risk, at low doses, and implies that exposure to even a single molecule of a genotoxic substance could produce DNA damage and thereby some degree of risk. However, a DNA adduct does not in itself have genetic consequences, but it needs to be fixed into a mutation through DNA replication. The probability that a DNA adduct becomes fixed is dependent on the rates of DNA repair and cell proliferation, both of which may be influenced by dose; consequently, there are likely to be deviations from linearity (Lutz, 1990). It is to be noted that a relatively high level of DNA damage is normally produced by physiological processes (Beckman and Ames, 1997). This suggests that the contribution of very low doses of substances that are both genotoxic and carcinogenic to background damage may be negligible. The saturation or overload of repair capacity at high doses (Pegg and Dolan, 1987) may result in increased mutation incidences and tumour yields. Moreover, chemicals which stimulate the rate of cell division or reduce cell cycle delay (which is required for DNA repair) enhance the fixation of mutations from primary DNA lesions. As an example, the stimulation of cell proliferation is believed to be responsible for the nonlinearity of bladder tumour induction in mice treated with 2-acetylaminofluorene (2-AAF) (Cohen and Ellwein, 1990), and liver tumours in rats treated with *N*-nitrosodiethylamine (Peto *et al*., 1984). As the high doses applied in carcinogenicity bioassays usually elicit significant toxicity with regenerative cell proliferation in target organs, simple linear extrapolation from experimental data to effects at low doses may lead to a considerable overestimation of the true incidence. Moreover, cancer is acknowledged to be a multistep process, where sequential steps consist of different genetic alterations driving towards the progressive transformation of normal cells into malignant derivatives (Hanahan and Weinberg, 2000). Because cancer is the result of multiple, independent genetic alterations, the incidence of cancer is theoretically expected to rise as a polynomial function reflecting the number of independent events required. This expectation also applies when the acquisition of increased mutability speeds up the entire process (Lengauer *et al*., 1998). This may result in a 'practical' threshold for carcinogenesis induced by genotoxic chemicals, similarly to other toxic effects arising from multiple molecular interactions (Kirsch-Volders *et al*., 2000). The possibility that DNA repair may cope successfully with low levels of DNA damage has been advocated as a putative mechanism for a biological threshold for genotoxic effects (Purchase and Auton, 1995). According to Preston and Williams (2005), most, if not all, chemicals that are DNA reactive in rodent species are DNA reactive in humans, although there may be quantitative differences. In addition, it is reasonable to assume, also as a qualitative conclusion, that the form of the dose–response curve for DNA-reactive chemicals is similar across species. This conclusion encompasses the important point that linearity is not the rule for DNA-reactive chemicals, but rather the default: nonlinear forms are observed and predicted (US Environmental Protection Agency, 2005).

4 THE GENOTOXICITY OF CARCINOGENS

Chemicals which increase carcinogenic risks have been categorized as 'genotoxic' and 'nongenotoxic', a process which can have effects upon the manner in which the chemicals are handled in the regulatory arena. Butterworth (1990) has defined a genotoxic agent as one for which a *primary biological activity* of the chemical or a metabolite is alteration of the information encoded in the DNA. Chemicals exhibiting such activity can usually be identified by assays that measure reactivity with DNA, induction of mutations, induction of DNA repair or cytogenetic effects (see **Mutagenesis**; **Cytogenetics**). Many of these assays have been considered to be predictive of carcinogenicity. Many human cancers are associated with exposure to such genotoxic chemicals and the formation of DNA adducts is an important early step in their mode of action. The key events that follow (Preston and Williams, 2005) are shown in **Table 1**.

Nongenotoxic chemicals are those that lack genotoxicity as a primary biological activity. While these agents may yield genotoxic events as a secondary result of other induced toxicity, such as forced cellular growth, their primary action does not involve reactivity with the DNA (see **Nongenotoxic or Epigenetic Carcinogenesis**).

Two assumptions are made in formulating this distinction: that genotoxic carcinogens are biologically active at all doses, and that nongenotoxic carcinogens have biologically significant effects only above a particular threshold dose. This division arises because genotoxic chemicals interact directly with nuclear DNA, of which there is basically a single copy within each cell (although there may be multiple copies of particular genes), while nongenotoxic chemicals react with non-DNA molecules, of which there are many copies. Thus, damage to non-DNA molecules will have biologically important consequences only when a sufficiently large proportion is inactivated so that first-order kinetics and their reactions are no longer followed. The validity of this division is questionable.

A chemical that interacts with DNA will also react with other nucleophiles, and these latter reactions are quantita-

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Table 1 Key events for tumour development: DNA-reactive modes of action

1.	Exposure of target cells (e.g. stem cells) to ultimate DNA-reactive and mutagenic species—in some cases this requires metabolism.
2.	Reaction with DNA in target cells to produce DNA damage.
3.	Misreplication on damaged DNA template or misrepair of DNA damage.
4.	Mutations in critical genes in replicating target cell.
5.	These mutations result in initiation of new DNA/cell replication.
6.	New cell replication leads to clonal expansion of mutant cells.
7.	DNA replication can lead to further mutations in critical genes.
8.	Imbalanced and uncontrolled clonal growth of mutant cells may lead to preneoplastic lesions.
9.	Progression of preneoplastic cells results in emergence of overt neoplasms, solid tumours (which require neoangiogenesis) or leukaemia.
10.	Additional mutations in critical genes as a result of uncontrolled cell division results in malignant behaviour.

Reproduced from Preston and Williams, 2005. © Taylor and Francis Group.

tively dominant and could be as important in the carcinogenic process as the reactions with DNA. For no chemical is its mode of carcinogenic action fully understood; assumptions are always made that have greater or lesser validity. Consequently, the term used by the European Food Safety Authority (EFSA) (2005)—chemicals that are both genotoxic and carcinogenic—is less presumptive (although more clumsy) than the common term 'genotoxic carcinogens', which assumes a genotoxic chemical is a carcinogen *because* it interacts with DNA. The carcinogenic response might directly involve DNA damage, but this is seldom—if ever—sufficient to explain the response. Genotoxicity in general terms does not, by itself, prove that the carcinogen is acting through a genotoxic mechanism. It does not even provide evidence for this mechanism, although it does strongly suggest its plausibility. Also, genotoxicity can sometimes be found in the apparent absence of carcinogenicity.

A small number of agents (eight chemicals and one physical-chemical entity) have been evaluated by the IARC for which there is *evidence suggesting a lack of carcinogenicity in experimental animals* (italics as used by IARC). They are all listed in **Table 2**. Note that, of these, only caprolactam was classified in IARC Group 4; all others were classified in IARC Group 3. Indeed, since there were no epidemiological data available at the time of the evaluation, it could be argued that this classification of caprolactam was a demonstration of optimism rather than based on evidence from exposed people as well as laboratory animal experiments. Even though there have been no significant results from genotoxicity tests *in vivo* with caprolactam, significant results were obtained repeatedly in tests for chromosomal aberration and aneuploidy induction *in vitro* with human lymphocytes (Howard *et al*., 1985; Norppa and Jarventaus, 1989; Sheldon, 1989). However, for four of these nine agents, there were available reports of genotoxicity, not only *in vitro*, but *in vivo*.

In addition, styrene can induce lung tumours in mice following inhalation, gavage or intrauterine exposure. Mouse lung tumour incidence is increased following inhalation of 20 ppm styrene atmospheres, whereas the inhalation of even 1000 ppm styrene atmospheres is not carcinogenic to rats. Styrene is metabolized to styrene oxide and can form DNA adducts in mouse lung Clara and non-Clara cells; however, no O^6-guanine adducts were found in the lungs of mice exposed to 160 ppm or of rats exposed to 500 ppm, whereas O^6-guanine adducts were found in the liver of rats exposed to 1000 ppm styrene ($40/10^8$ nucleotides) (Otteneder *et al*., 1999). Hydroquinone is an example of a chemical that has clear genotoxic properties and is carcinogenic to the kidneys of male rats, but genotoxicity appears to play no role in the neoplastic response (reviewed in McGregor, 2007).

Nevertheless, genotoxicity is at least evidence that a chemical interacts with biological molecules to induce an adverse response. The demonstration of genotoxicity *in vivo* indicates that the responsible chemical is bioavailable and that it has the potential to interfere with biological processes, the disruption of which may play multiple roles in neoplasia, including initiation. Biological elements in this process could include, for example, some cell population death with subsequent proliferation of a fraction of the survivors, or loss of communication and homoeostatic control between cells within a tissue.

From another point of view, some carcinogens that are nongenotoxic may be involved in reactions which generate radicals and other charged species. The metabolism of xenobiotics usually involves the cytochrome P450 system, in which electron transfer to the xenobiotic substrate occurs with varying efficiency. Active oxygen species are inevitable products of this inefficiency, so, while neither the xenobiotic nor any of its metabolites might be involved in reactions with DNA, the potential for DNA damage always exists.

Many of the schemes of epigenetic modes of action that have been developed from observations on rodents and man involve disturbance of hormonal regulating networks, while others involve enzyme induction or enzyme inhibition and other expressions of toxicity. It is also clear that a property common to the proposed

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Table 2 Genotoxicity *in vivo* of agents evaluated by IARC as showing *evidence suggesting a lack of carcinogenicity* in experimental animals

Chemical	Last IARC reference	Genotoxicity *in vivo*	Genotoxicity references
γ-Butyrolactone	Vol. 71, 1999	None	
Caprolactam	Vol. 71, 1999	None	
2,4-Dichlorophenol	Vol. 71, 1999	None	
Dimethylformamide	Vol. 71, 1999	SCE, human lymphocytes	Seiji *et al*. (1992)
		Chromosomal aberrations, human lymphocytes	Koudela and Spazier (1981)
			Berger *et al*. (1985)
Estazolam	Vol. 66, 1996	None	
Methyl methacrylate	Vol. 60, 1994	Chromosomal aberrations, rat bone marrow	Fedyukovich and Egorova (1991)
Surgical silicone female breast implants	Vol. 74, 1999	None	
Toluene	Vol. 71, 1999	Micronucleus test, mouse bone marrow	Mohtashamipur *et al*., (1985; 1987)
		Micronucleus test, rat bone marrow	Roh *et al*. (1987), Dobrokhotov (1972)
		Chromosomal aberrations, rat bone marrow	Lyapkalo (1973), Roh *et al*. (1987), Popp *et al*. (1992)
		DNA strand breaks, human lymphocytes	Bauchinger *et al*. (1982)
		SCE, human lymphocytes	Schmid *et al*. (1985)
		Chromosomal aberrations, human lymphocytes	Pelclová *et al*. (1990), Nise *et al*. (1991)
Vinyl toluene	Vol. 60, 1994	Micronucleus test, mouse bone marrow	Norppa (1981)

SCE—sister chromatid exchange.

mechanisms of action is the persistent stimulation of cell populations to divide, as a hyperplastic response either to toxicity or to mitogenesis; inhibition of apoptosis may also play a role. In many examples, the mode of action has been reasonably well established, up to the point where there is a cell population increase. Why hyperplasia should ever result in neoplasia is not well established, but the assumed mechanism until now has been that a shortened cell cycle time reduces the time for repair of 'background' damage to DNA and increases the probability of mutation. Thus, evidence of genetic toxicity can be important, even when an epigenetic scheme is reasonably supported by sound experimental evidence. For this reason, the IARC has consistently required that there should be no genetic toxicity associated with a substance if an epigenetic mechanism of carcinogenesis is to be accepted.

While intermitotic DNA damage and inefficient repair may be parts of the process, it is known that relatively short cell cycle times are also characteristic of several normal cell populations; for example, dermis, intestinal epithelium, haematopoietic tissues and, not least, throughout the development of embryos and early foetuses. Recognition of another possible process has been emerging from studies in molecular biology and protein chemistry. If a chemical reacts with DNA, then it is highly probable that it also reacts with various amino acids in proteins and peptides that, together with DNA and many other types of molecules, constitute chromatin. These proteins, including histones, are often involved in normal gene regulatory function. It has even been proposed that there is a 'histone code', based upon patterns of acetylation, methylation, phosphorylation and ubiquitination of basic amino acid histone tails protruding from the nucleosomes, that enables other proteins to recognize specific regions of the genome (Strahl and Allis, 2000). This may explain the link between the covalent chemical modification of histone proteins and the epigenetic regulation of gene activity. These patterns, probably coordinating with the methylation patterns that are a feature of CpG islands commonly associated with promoter regions (Antequera and Bird, 1993), determine whether particular genes are expressed or not. Interference with the activity or function of the methyl transferases, acetylases and deacetylases, and so on, may cause inappropriate phenotypic changes, which could include the silencing of repair genes or of tumour suppressor genes. Very important consequences of inappropriate methylation result when certain DNA repair enzymes are silenced, particularly *MLH1* (Nakagawa *et al*., 2001),

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which leads to an increased microsatellite instability, and *MGMT*, which seems to precede mutations by occurring in premalignant polyps that do not yet harbour gene mutations (Estelier, 2000; 2001). Post-translational modifications of histones are involved in regulation of chromatin structure and gene activity. These modifications include the core histones H2A, H2B, H3 and H4, which have been extensively studied, and more recently H1, the linker histone, which is not only phosphorylated, but is now recognized as containing a range of phosphorylation, acetylation and methylation sites, as well as sites of ubiquitination and of lysine formylation residues (Wisniewski *et al*., 2007). This recent change in our knowledge of linker histone variations has been brought about by the use of functional proteomic techniques, principally mass spectrometry, to characterize these modifications, although the functionality of many of these sites remains to be determined (Godde and Ura, 2008). A proper understanding of their significance has still to emerge. For some investigators, the tendency for histone modifications to co-occur in groups has suggested that they are not creating an intricate code at all, although an alternative function of these modifications has not been suggested (Rando, 2007). For other investigators, the existence of combinations of structural modifications in histones has suggested that they are associated with distinct gene readouts, for example the methylated histone H3, H3K4me2, coincides with elevated expression levels only in combination with acetylation, while H3ac association with expression is reduced if there are other modifications to the histone (Fischer *et al*., 2008). Where these new avenues of research will lead is unknown, but it is becoming increasingly clear that the informational changes in normal cells that result in neoplastic transformation may be just as subtle as the changes that occur in normal differentiation and development, that is, far more subtle than would be expected from a blunt instrument like direct DNA damage and repair. It is incontrovertible that mutations are involved in neoplasia, but the way in which these occur may not be as obvious as hitherto assumed.

5 THRESHOLDS OF EFFECTS

For most toxic processes, excluding genotoxicity, it is generally assumed that there is a threshold of exposure below which no biologically significant effect will be induced (Dybing *et al*., 2002).

For genotoxic carcinogens where there is no dose threshold, a range of extrapolation models have been developed to estimate carcinogenic potency from animal data. The models include the One Hit, the Linearized Multistage Linearized Multistage model, the Weibull model, Logit model and Probit models. The simplest model is the 'One Hit' model, which leads to linear extrapolation and assumes the carcinogenic mechanism of action is a single event. The more complex models use the assumption of multiple events and interactions leading to a carcinogenic event. These models are described widely in the literature (Edler *et al*., 2002; European Food Safety Authority (EFSA), 2005; Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment (COC), 2004).

The demonstration of a threshold is normally not possible, even when nongenotoxic mechanisms are involved. Attempts to do so have fallen short of their objective because, although there might indeed be a threshold, statistical analysis of the data is also consistent with the absence of a threshold. As an example, Wanibuchi *et al*. (2006) considered that the dose response for GST-P (glutathione-*S*-transferase placental form) foci in the liver of rats administered very low doses of 2-amino-3,8-dimethylimidazol[4,5-*f*] quinoxaline (MeIQx) was consistent with such a threshold. Unfortunately, the graphical representations presented were not optimal (**Figure 2**). Later analysis showed that the 95% confidence interval associated with the curve included zero and so a threshold cannot be demonstrated (Slob, 2007). Erroneous graphical representations have occurred on a number of occasions (e.g. Waddell, 2002; 2003a; 2003b; Waddell *et al*., 2006) in this area of thought, always leading to the unjustified conclusion that a threshold has been demonstrated. If a legitimate graphical representation is used for cancer data, for example log-log scales, then it can be readily appreciated that it is impossible to demonstrate a zero response. This has been done in **Figure 3**, where cancer data from two very large experiments, one with *N*-nitrosodiethylamine (Peto *et al*., 1991a; 1991b), the other with 2-AAF (Farmer *et al*., 1980), are presented in which responses in the very narrow experimental ranges are shown on a log scale, as are the doses, presented as numbers of molecules of each carcinogen per kilogram body weight per day. A linear model was used and the 95% confidence limits are also drawn for extrapolations down to an incidence of one-in-a-million. It is interesting that there would appear to be several thousand molecules of carcinogen per kilogram body weight per day, even at this low extrapolated response. This dose expression can be further manipulated to give approximate numbers of molecules per cell. While one can feel justifiably uncomfortable with such a presentation, these graphs at least give some substance to the threshold discussion that would otherwise give free reign to the default assumption that as little as one molecule of carcinogen per DNA molecule is sufficient to initiate the carcinogenic process. Furthermore, the possible intervention of repair and other protective processes have not been taken into account. Since the possible threshold is in an area of the dose–response curve that is not experimentally

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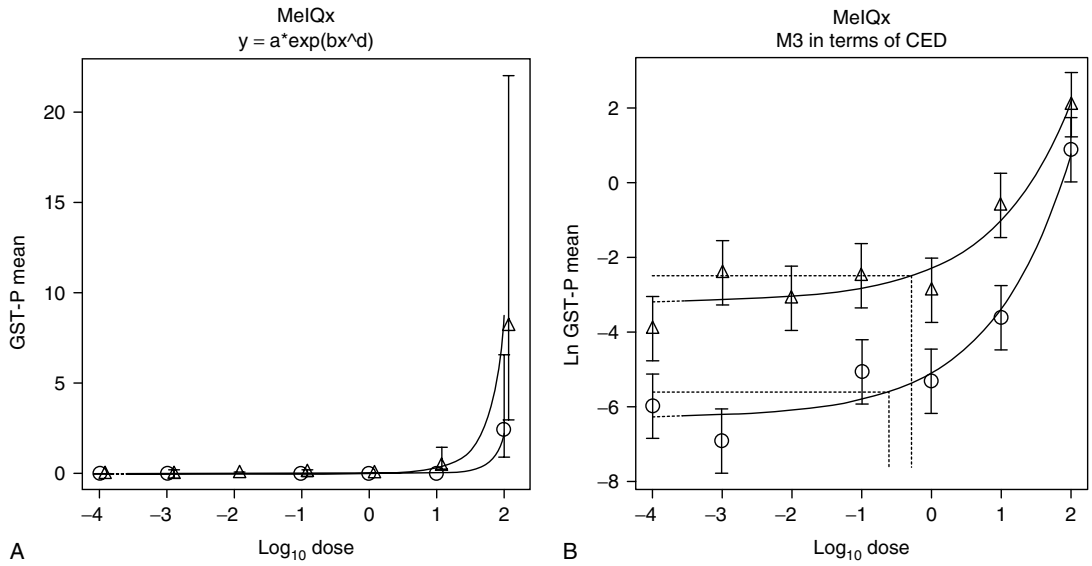

Figure 2 Original graphic presentation (A) and representation on log-log scales (B) of the frequencies of GST-P foci in the livers of rats treated with 2-amino-3,8-dimethylimidazol[4,5-*f*]quinoxaline (MeIQx). (Reproduced from Slob, 2007. © Sage Publications.)

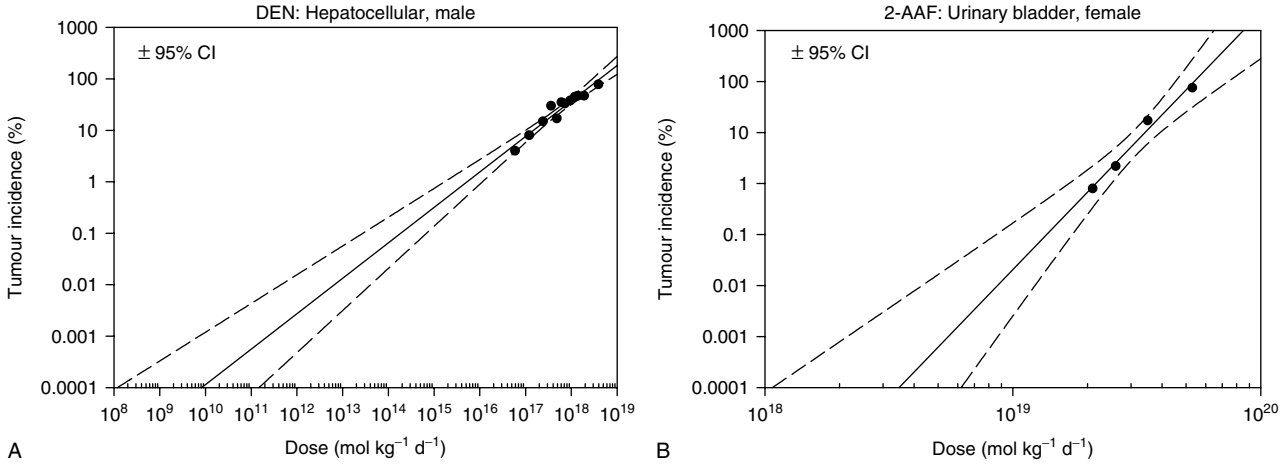

Figure 3 Extrapolations of tumour incidences to unobservable levels (1 in a million) following treatment of male rats with *N*-nitrosodiethylamine (DEN) (A) and female mice with 2-acetylaminofluorene (2-AAF) (B). (Data from Peto *et al*., 1991a; 1991b (A) and Farmer *et al*., 1980 (B).)

observable, it must be conceded that a threshold, or a practical threshold, might exist even if it cannot be demonstrated.

Even though the existence of a threshold cannot be proved or disproved experimentally, the presence of homoeostatic and cytoprotective mechanisms, and the abundance of cellular targets, mean that a minimum degree of interaction of the substance with the critical sites or their occupancy must be reached in order to elicit a toxicologically relevant effect (Dybing *et al*., 2002). Below this critical (threshold) level of interaction, homoeostatic mechanisms would be able to counteract any perturbation produced by xenobiotic exposure, and no structural or functional changes would be observed. In this context, some properties of Mdm2, an important component of the TP53 circuitry (see Section 3.2), following treatment with genotoxic substances are interesting. Phosphorylation of TP53 at Ser15 and Ser20 inhibits the interaction of TP53 and Mdm2, thereby resulting in an accumulation of TP53 (Meulmeester *et al*., 2005). Some early Mdm2 phosphorylations can also lead to TP53 stabilization (Shinozaki *et al*., 2003). DNA damage is one stimulus that results in such stabilization of TP53 and it has been found that nanomolar concentrations of benzo[*a*]pyrene, mitomycin C, etoposide and 5-fluorouracil can induce Mdm2 phosphorylation, while phosphorylation of TP53 requires higher concentrations (Malmlöf *et al*., 2008). Particularly interesting was the

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discovery that higher concentrations of benzo[*a*]pyrene (7–10 μM), as well as exposure to the more mutagenic dibenzo[*a*,*l*]pyrene induced TP53 Ser15 binding and a histone, γH2AX, but did not induce Mdm2 binding. This outcome demonstrates that the Mdm2 response versus the TP53 and γH2AX responses in chromatin are mutually exclusive. Clearly, Mdm2 is a sensitive biomarker for at least some types of genotoxic activity. In addition, polycyclic aromatic hydrocarbon-induced Mdm2 binding to chromatin reflects repairable damage, whereas chromatin binding of TP53 Ser15 and γH2AX indicates more persistent DNA damage. There is certainly much more work to be done in this area, but there does seem to be a possible means for separating doses of at least some genotoxins into those that induce readily repairable DNA adducts, and doses that overwhelm the capacity for repair. If so, this would surely indicate a practical, if not an absolute, threshold.

Most, if not all, chemicals that are DNA reactive in rodent species are DNA reactive in humans, although there may be quantitative differences. In addition, it is reasonable to assume, also as a qualitative conclusion, that the form of the dose–response curve for DNA-reactive chemicals is similar across species (Preston and Williams, 2005).

6 RODENT CARCINOGENICITY TESTS

6.1 Test Design

The basic issues in chronic toxicity study design have frequently been described (e.g. Sontag *et al*., 1976; International Agency for Research on Cancer (IARC), 1980; 1986; International Life Sciences Institute (ILSI), 1997; Rhomberg *et al*., 2007); therefore, only some particularly troublesome issues will be mentioned here and the reader is directed to the references cited for more detailed discussion.

It is important to bear in mind that two-year studies are very expensive, take far longer than two years from design stage through to even the first steps in interpretation, and for these reasons the identification of carcinogens is usually only one of the objectives. A notable exception is the US National Toxicology Program (NTP) carcinogenicity assays in rats and mice, in which other systemic effects of a compound have been of secondary interest and there has certainly been no attempt to identify doses that are apparently without effects.

The rodent carcinogenicity test is usually designed as a hypothesis-generating experiment. As a generality, these tests should be followed by confirmatory experiments in which the hypothesis is tested. The scientific desirability of independent verification of a result, be it positive or negative, is, however, frequently lacking from these carcinogenicity data, often for reasons which are justifiable in terms of either animal welfare or—at first sight—finance, but not in terms of scientific method. The use of different sexes, strains and species does not form a basis on which data responsibility can be judged. Many 'standard' carcinogenicity assays have been subsequently repeated because the results from the first experiment could not be interpreted confidently. The multiple pairwise comparisons made in such experiments can result in apparent associations between tumour incidence and treatment which are statistically significant. Furthermore, if the investigators choose to analyse only the increases in tumour incidence, then any random nature of such associations is masked and it becomes more difficult to override them with other considerations.

Moderate control tumour incidences (e.g. 15–25%) can allow the statistically significant demonstration of both increases and decreases in tumour incidence. Partly because of prejudice, the presence of a decrease in tumour incidence (which occurs in many experiments) has often been viewed as an interpretative problem, whereas an increase can be more readily attributed to the action of the chemical under test.

If, however, in animals of the control group incidences of site- or tissue-specific tumours are low, then only increases in tumour incidence are likely to reach statistical significance, while if control group incidences are very high, then only significant decreases can be expected. In the latter case, the animal strain would probably be considered as inappropriate for this kind of experiment.

The currently accepted design for carcinogenicity testing is for rats and mice to be exposed for about two years. Rarely is any other species used and there are efforts to find ways of eliminating one of them—most likely mouse. For each species, 50 males plus 50 females per group are dosed with a vehicle or the test agent in that vehicle. Daily observations are made and, if animals become moribund during the experiment, then they are killed so that their tissues are not lost through autolysis. All animals are subjected to autopsy and almost 40 different tissues are taken from each animal for histological examination. All observations are recorded, summarized and analysed.

The objective is to expose a statistically acceptable number of animals to the highest dose that they will tolerate without reducing their lifespan for reasons other than tumour development. A series of preliminary toxicity tests is conducted, beginning by following some form of single-dose protocol. This initial study is followed by a 2-week and then a longer (4- or 13-week) toxicity study, the purpose of which is to identify a minimally toxic dose, as manifested by reduced body weight gain, reduced food intake, altered appearance or altered behaviour patterns and non-neoplastic histology observed at the end of the experiment. This is the so-called maximum tolerated dose (MTD) determination.

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6.2 Maximum Tolerated Dose

There seems to be general agreement that this measurement is necessary, but there are differences over its definition or what should be done with it. Twenty-seven different guidelines were described by the International Life Sciences Institute for the selection of doses in chronic toxicity/carcinogenicity studies (International Life Sciences Institute (ILSI), 1984). Among these, the most commonly used definition is that adopted by the US National Cancer Institute (NCI) (Sontag *et al*., 1976) and the US National Toxicity Program. This states: 'The MTD is defined as the highest dose that can be predicted not to alter the animals' normal longevity from effects other than carcinogenicity.' In practical terms, the MTD is the dose which, in the three-month study, 'causes no more than a 10% weight decrement, as compared to the appropriate control groups, and does not produce mortality, clinical signs of toxicity, or pathological lesions (other than those that may be related to a neoplastic response) that would be predicted to shorten an animal's natural life span.' Ideally, this is precisely what should happen in the carcinogenicity test, but in reality the prediction of the MTD made on the evidence of sequential toxicity studies may be incorrect. In the carcinogenicity test, animals may die too early, thereby invalidating the study, or the predicted toxicity may not be realized, which invites the criticism that higher dose levels could have been used.

Other considerations in establishing the MTD value are metabolism and pharmacokinetics. Disproportionate changes in these parameters with increasing dose may signal saturation of metabolic pathways that are dominant at lower—and, therefore, more probably encountered—human dose levels.

It has been proposed, however, that metabolic and pharmacokinetic considerations should not be used to establish the MTD, but only to determine a lower dose level (International Agency for Research on Cancer (IARC), 1980; 1986). The use of inappropriate metabolism as a reason for reducing the importance of a carcinogenic response has been criticized (Haseman, 1985) on the grounds that it is up to the protagonists of this opinion to demonstrate '. . . explicitly how this overload produced carcinogenic effects.' It is indeed necessary to have a sound basis for introducing the argument of inappropriate metabolism and it would be preferable for this inappropriateness to be demonstrated in advance of the carcinogenic experiment; but the mechanism (as opposed to mode of action) leading to a carcinogenic response is not known in advance for any chemical at any dose level. It would seem to be sufficient to demonstrate a plausible metabolic overload mode of action supported by a reasonable amount of data. The evidence should be documented in a formal manner, as proposed in various publications, particularly those supported by the International Programme for Chemical Safety (IPCS) (Sonich-Mullin *et al*., 2001; Boobis *et al*., 2006).

Discussion of the MTD is important because it is clearly needed for the demonstration of a carcinogenic response in many instances. In the US NTP feeding studies (1979–1983), 62% (8/13) of chemicals were judged to be carcinogenic on the basis of effects observed only at the MTD; at 0.5 × MTD, no carcinogenic effects occurred (Haseman, 1985). The basic problem is that the rodent carcinogenicity bioassay as currently conducted is insensitive: in relation to human populations, the numbers of rodents exposed are exceedingly low. Hence, it is readily conceded that some means must be used to compensate for the low statistical power inherent in the assay and that the use of high dose levels may be one device by which this end can be achieved. At the same time, however, the use of such high dose levels does raise legitimate concerns regarding the mechanisms and relevance of carcinogenesis in these experiments.

It appears that the MTD is not required to demonstrate the carcinogenicity in rodent studies of those compounds known to be human carcinogens. Apostolou (1990) listed the appropriate rodent carcinogenicity data and doses for 13 human carcinogens to demonstrate that the lowest observed effective doses range from 0.005 × MTD to 0.5 × MTD. Since, in many cases, the MTD was not known but was assumed to be the highest tested dose, the real MTD fractions may be even lower than these estimated fractions in some cases. A conclusion one may reach from these data is that a carcinogen of potency sufficient to allow its detection at <0.5 × MTD in rodent tests is required before the relatively insensitive epidemiological methods permit detection in human populations. It would be wrong to conclude, however, that a substance showing a carcinogenic effect only at the MTD in the standard bioassay is not a human carcinogen. All that can be said of these compounds is that they may not be recognizable as human carcinogens by epidemiology, and this must remain a moot point unless we can demonstrate that the mechanism of tumour induction in rats or mice is dependent upon the minimally toxic effect.

6.3 Pathology

The primary information for carcinogenicity evaluation is generated by pathologists. These professionals, like any other group of professionals, vary in their training and experience, and these are characteristics which may influence the evaluation in a number of ways. Some of these are listed below.

1. Differences in terminology may be important when considering controversial lesions.

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2. Lack of consistency throughout a study is likely when a pathologist has only recently become involved with rodent carcinogenicity. Training is usually in a clinical situation, where each animal is unique and there is not the same need for consistency as there is in a rodent carcinogenicity study consisting of 500 animals.
3. Unfamiliarity with the observed lesion in that particular species may cause problems in interpretation.

There is often, among nonpathologists, a suspicion that pathology is highly subjective and that pathologists are uncertain of their own discipline, as shown, for example, by a great reluctance on their part to evaluate tissues without prior knowledge of the treatment. Such criticism is unfair and, indeed, demonstrates a lack of understanding of biology in general and pathology in particular. Certainly, there is the possibility that treated animals might be examined more thoroughly than the controls and that knowledge of treatment of the animal might influence the terminology used to diagnose a lesion. But randomized, 'blind' slide reading is not the solution in the initial, routine evaluation of histological slides. If slide reading were 'blind', then there would be a loss of information about the controls, particularly in long-term studies, where many lesions in senile animals will be found. The other, sometimes stated, objection to 'blind' slide reading—that autopsy data are also important in diagnosis—is correct, but can be overcome through suitable coding of the information.

Possible bias introduced by knowledge of treatment can be corrected in several ways, but the use of a two-stage process would seem to be most efficient:

1. An initial evaluation is performed with full knowledge of the animal's history, including treatment.
2. A second evaluation of specific lesions is then carried out. This should be done blind, either by the same pathologist or, preferably, by the same and a second pathologist.

Differences in evaluation between pathologists should always be discussed by them to resolve these differences; they may be due to subtle differences in diagnosis and do not indicate incompetence in one of the pathologists. It is quite unacceptable for a study sponsor to shop around until he finds a pathologist who gives—for whatever reason—the result he is looking for without giving an opportunity for interaction with all of the other evaluators. Sometimes these diagnoses are given years apart, during which time understanding of the pathogenesis of lesions may change or there may be a change in the accepted definition of a particular lesion. In these circumstances even the first pathologist may not arrive at the same conclusion as he did some years ago.

Evaluation of the data is not purely a statistical exercise. A number of important factors should be considered, as follows: (i) dose–effect relationship; (ii) a shift towards more anaplastic tumours in organs where tumours are common; (iii) earlier appearance of tumours; and (iv) presence of preneoplastic lesions.

The language used to describe the carcinogenic response has masked its complexity and presents a stumbling block to its understanding among nonhistopathologists. Benign or malignant neoplasms do not arise without some precursor change within normal tissue. An important concept in carcinogenicity evaluation is that of neoplastic progression, which was derived from studies on skin tumours (Berenblum and Shubik, 1947) and expanded to a number of other tissues (Foulds, 1969; 1975). There is, on many occasions, a far from clear distinction between normal and hyperplastic tissue, between hyperplasia and benign neoplasia, and between benign and malignant neoplasia.

Hyperplasia and benign and malignant neoplasia are convenient medical terms with prognostic significance. Hyperplasia can occur either as a regenerative response to injury, with no neoplastic connotations, or as a sustained response to a carcinogenic agent. It is an increase in the number of normal cells retaining normal intercellular relationships within a tissue. This normality may break down, resulting in altered growth patterns and altered cellular differentiation, a condition which may be described as atypical hyperplasia or presumptively as preneoplastic lesions. There are found as sequelae of hyperplasia: (i) persistence without qualitative change in either structure or behaviour; (ii) permanent regression; (iii) regression, with later reappearance; and (iv) progression to develop new characteristics indicating increased probability of malignancy. The last of these is the least likely to occur in experimental multistage models, such as in mouse skin or rat liver, where large numbers of hyperplastic lesions may occur, but notably fewer carcinomas develop from them.

Benign neoplasms in most rodent tissues apparently arise in hyperplastic foci, for example, squamous-cell papillomas of the skin and forestomach. Furthermore, these papillomas seldom demonstrate autonomous growth and their progression to squamous-cell carcinomas is even rarer (Burns *et al*., 1976; Colburn, 1980). This decisive progression to carcinoma, when it occurs, provides powerful evidence for the multistage theory of carcinogenesis: the new, malignant cells arising as a focus within the papilloma or even in an area of hyperplasia, since the papilloma is not a necessary intermediate stage. In other organs, benign neoplasia is usually characterized by well-differentiated cell morphology, a fairly uniform growth pattern, clear demarcation from surrounding tissues and no evidence of invasion. The progression towards malignancy involves anaplasia (loss of differentiation) and pleomorphism (variety of phenotypic characteristics within the neoplasm). These changes

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may be focal in an otherwise benign neoplasm and may vary in degree and extent. Evidence of invasion of the surrounding tissues or of metastasis are not essential characteristics of malignancy, although their presence strengthens the diagnosis.

6.4 Grouping of Tumours for Analysis

The grouping together of certain tumour types can be an asset to statistical analysis, but it must be done carefully, with full appreciation of the biology and whatever is known of the pathogenesis of the lesions. Grouping for analysis of all animals showing neoplasia, irrespective of the tumour type, is inappropriate because the incidence in most treatment control groups can be very high and, in US NTP studies, approaches 100% in rats and 50–70% in mice (**Table 3**).

There may be similar incidences of tumours in ageing people, but the real prevalence of tumours in human populations is uncertain. In the USA, it has been found that when autopsies are conducted, over one-third reveal previously undiagnosed cancers (Silverberg, 1984). A single type of neoplasm, renal adenoma, is present in 15–20% of all adult kidneys (Holm-Nielsen and Olsen, 1988), although it is unclear whether these 2–6 mm foci of proliferating tubular and papillary epithelium represent small carcinomas or benign precursors of renal cell carcinomas. Irrespective of the significance of these lesions in human pathology, the presence of similar foci in a rodent carcinogenicity experiment would trigger the recording of renal tumour-bearing animals and, hence, their consideration in the statistical and pathological evaluation processes.

The independent analysis of every different diagnosis in rodent studies would also mask significant effects in many cases, while enhancing them in others. Benign and malignant neoplasms of a particular histogenesis are often grouped because the one is seen as a progression from the other. However, this grouping may result in a nonsignificant difference from the controls because there has been an acceleration of progression towards malignancy, the incidence of benign neoplasms decreasing while the malignant neoplasms increase. Guidelines are available for 'lumping' or 'splitting' tumour types (McConnell *et al*., 1988), but in using them, the basis for the classification of neoplastic lesions should be clarified, especially when data generated over several or many years are coupled, since diagnostic criteria and ideas regarding tumour histogenesis may have changed. Reliance on tabulated results alone can lead to serious misinterpretation by those not closely connected with a particular study. For this very important reason, the pathology and toxicology narrative should be full and clear. If it is not, then there will always be doubts about future interpretations, even if these doubts are not, in reality, justified.

6.5 Historical Controls

The use of historical control data, to either support or refute an apparent treatment-related response, assumes that there is a greater stability in the accumulated data variation than in the much smaller concurrent control group and that the experiments performed over a period (perhaps several years) were done under identical conditions with animal stocks which did not vary. If these conditions were to be met, then the larger historical control group would provide a basis for increasing the

Table 3 Tumour-bearing animals in control groups from rodent carcinogenicity studies

Species, strain and sex	Number of control animals	Percentage with tumours in controls		
		Malignant	Benign	Total
B6C3F1 mice				
Male	1692	42	35	64
Female	1689	45	33	64
F344 rats				
Male	1596	55	95	98
Female	1643	38	76	88
Osborne-Mendel rats				
Male	50	26	68	78
Female	50	12	80	88
Sprague Dawley rats				
Male	56	9	36	39
Female	56	30	68	79

Haseman, unpublished summary of US NTP data.

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power of the assay. When a higher incidence of a tumour that is rarely seen in untreated controls (e.g. brain tumours in rats) is found in treated animals, or when an unusually low incidence of a tumour type that normally has a variable and often high incidence is found in controls with respect to treated animals (e.g. mammary tumours in rats), a comparison with historical control data may provide a clearer insight into the possible biological significance of the finding. Various factors can influence the incidence of spontaneous tumours in rodents (van Zweiten *et al*., 1988), but it is clear that flaws in the randomization process are not commonly considered. An unexpected incidence of tumours in a control group might suggest some bias in the distribution of the animals to their groups at the beginning of the experiment. If such bias has been properly eliminated, then the unexpected incidence should also have occurred in the treated groups. The absence from the treated group of an anomalous low incidence indicates a treatment effect. Thus, a high tumour incidence in a treatment group claimed to be within the historical control range and therefore not attributable to treatment only indicates that, in the absence of randomization bias, if the control group tumour incidence had been in the expected range, then the treatment group incidence would have been higher. However, it is probable that, in most situations, it cannot be assumed that conditions have remained constant over years and it cannot be assumed that animal stocks have not changed with time.

Outbred strains, such as Sprague Dawley (SD) and Wistar rats, are genetically variable. The strain characteristics vary both with time and with the colony from which they are derived. MacKenzie and Garner (1973) showed how SD rats from different suppliers differed substantially in their incidence of spontaneous neoplasms. It has also been shown that Wistar rats from four colonies exhibit substantial genetic differences (Yamada *et al*., 1979) and there appears to be considerable overlap in the genetic characteristics of SD and Wistar rat strains, which cannot be reliably distinguished from each other. A further important characteristic of Wistar rats is that the degree of heterozygosity (a measure of inbreeding) varied in the Yamada *et al*. (1979) investigation from 0% (i.e. this supply of 'outbred' Wistar rats was fully inbred) to 38%. Thus, at most, the outbred strain had only one-third of the heterozygosity that was expected of a fully outbred population and the variability in this value would, in most cases, be unknown because it is not normally checked.

The arguments for the use of outbred as opposed to inbred stocks for certain types of study are two-fold (Gill, 1980): (i) they serve as better models for human populations, which are heterogeneous; and (ii) heterogeneity is particularly useful for first-level screening.

Both proffered reasons, however, are similar and refutable by the same argument. An experiment with outbred animals that would truly mimic a human population would require very large numbers. Because only a portion of the exposed animal population might be responsive, the sensitivity of the assay performed could be low unless this factor was incorporated into the design. However, since the heterozygosity of so-called outbred strains is both low and variable, the modelling objective is confounded from the start. While it is not the only outcome, one important result from the use of outbred animals is that a biological unknown factor is introduced which can lead to difficulties in reproducing an effect with the 'same' outbred strain, adding to confusion in the literature, and the total unreliability of carefully accumulated historical control data to which reference can be made with well-recognized statistical methods.

Many of these problems should be overcome, theoretically, by the use of isogenic strains. Where these are used and—an important caveat—the environmental conditions have been constant and well-controlled, historical control data may be useful, particularly in the assessment of rare events, such as the occurrence of uncommon neoplasms. Isogenic strains are the result of brother–sister mating for >20 generations and F_1 hybrids between such strains. No strain can become fully inbred, but for all practical purposes they can be considered as a stock of genetically identical individuals which can be characterized and, within similar environmental conditions, they can be expected to exhibit the same phenotype over time and in different laboratories (Festing, 1990). However, in spite of the expected lower variance in isogenic strains, when tumour incidence is considered the results are disappointing; there are for example, substantial differences in the experiences of laboratories using F344 rats (Tarone *et al*., 1981; **Table 4**).

The reliability of the historical data is increased when variables are minimized by considering experiments in the same laboratory, with the same strain of animals from the same source and kept under the same husbandry conditions, and where the pathology is evaluated by the same pathologist. Peer review can aid pathology evaluation when different pathologists have been involved in the studies. Also, reliability increases when the data are derived from studies temporally close (e.g. three to five years) to the study in question. Even when historical control tumour data are peer-reviewed and re-examined with uniform and currently acceptable criteria applied, differences in the incidences of certain tumours do occur with time (van Zweiten *et al*., 1988). These authors also point out that the incidence of non-neoplastic changes in shorter studies (e.g. 13 weeks) also varies. In general, the best controls are those concurrent with treatment; resorting to historical controls should be done with great caution and only to support an apparent effect, not to refute one.

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Table 4 Tumour and leukaemia incidences in F344 rats from six laboratories

Tumour type or site	Sex	Percentage of rats with tumours at different laboratories[a]						
		1 $n = 8$	2 $n = 7$	3 $n = 8$	4 $n = 22$	5 $n = 11$	6 $n = 16$	Overall
Lymphoma-leukaemia	M	23.6** (4–54)	10.4 (4–13)	19.0** (6–38)	22.7 (5–45)	11.8 (0–20)	9.1 (0–20)	16.9**
Pituitary	M	18 (7–34)	17* (0–29)	30 (22–40)	31 (10–65)	12.8** (0–33)	7.5 (0–21)	20.3**
Adrenal phaeochromocytoma	M	14** (6–26)	16 (13–21)	5 (0–12)	11 (0–30)	9 (0–21)	8 (0–20)	10.7**
Mammary fibroadenoma	F	22 (12–32)	24** (8–38)	9 (4–20)	17 (5–30)	6 (0–15)	11 (0–20)	15.5**
Endometrial stromal polyp	F	15** (4–31)	25 (10–33)	18 (8–38)	12* (0–30)	10* (0–30)	9.9** (0–30)	14.9**

[a]No. of control groups in each laboratory.
Values in parentheses are the ranges observed over the separate experiments.
*Significant heterogeneity, $p < 0.05$.
**Significant heterogeneity, $p < 0.01$.
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6.6 Evaluation of High-Dose Effects

Given at a sufficiently high dose level, any chemical will induce biological damage. This is an understanding in toxicology that is beyond dispute. This position was tentatively advanced a step further by several people as a result of the examination of carcinogenicity data from tests conducted at the MTD. It gave rise to the notion that carcinogens might be recognizable by their systemic toxicity, although there is not necessarily a causal connection. The possible tautology of the correlation was discussed in the literature (Bernstein *et al*., 1985; Crouch *et al*., 1987) and the experimental evidence for the hypothesis was restricted to a small number of chemicals. Thus, among 99 chemicals tested for carcinogenicity and site-specific toxicity in long-term rodent studies by the NTP, only seven of the 53 which were shown to be carcinogenic exhibited target organ toxicity that could have been causal to the observed neoplasms (Hoel *et al*., 1988). Nevertheless, correlations as high as 0.79 were calculated for mouse LD_{50} and TD_{50} values (TD_{50} is that chronic dose rate in mg kg^{-1} body weight per day that would give one-half of the animals tumours within some standard experimental time (the 'standard lifespan' for the species)) among the NCI/NTP carcinogens (Zeise *et al*., 1986), although the correlation was reduced to 0.73 when non-NCI/NTP data were included in the analysis (Metzger *et al*., 1989).

The effects upon the physiology of rodents of life-long exposure to doses in the neighbourhood of the MTD may be expected to be so severe that some perturbation of the ageing process incidence is inevitable (Salzburg, 1989). In the case of a pharmaceutical substance given at a dose which inhibits almost all of some particular aspect of biological activity, the animal is driven into altered equilibrium as a consequence of the changed biochemical milieu. The toxicity engendered by such treatment may be a response to the desired, but exaggerated, pharmacology; but drugs seldom—perhaps never—have a single action and there may be substantial biological effects which were not designed into the drug. These effects will be of an unspecified nature characteristic of any chemical, and so concern regarding effects on ageing could be extended to any substance submitted to a lifespan, high-dose animal experiment. Since the development of most neoplasms is age related, tumour incidence would be one of the factors affected, the response being manifest as either an increase or a decrease in tumour yield. Among 170 NCI studies published as of 1980, 97% showed statistically significant dose-related modifications in at least one species or sex and 70% of them showed statistically significant dose-related modifications of tumour patterns that involved one type of tumour increasing with dose, while another type of tumour was decreasing with dose (Salzburg, 1983). The same situation prevails with the more recent NTP data, at least up to 1986 (Salzburg, 1988). Theoretically, high-dose effects on tumour incidence need not be primarily on the organ or tissue in which the change is found, particularly if hormonal control mechanisms are involved.

In an attempt to accommodate these conflicting responses within experiments, Salzburg (1989) used principal component analysis to describe a tumour profile, which is a weighted combination of site-specific tumours; nonparametric techniques are then used to compare these tumour profile scores across groups. However, this combination technique has been criticized by Haseman (1990) for having no biological significance

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and for being misapplied to experiments in which the resulting differences in tumour profiles were due to survival differences and data coding errors. Nevertheless, the problem of increases and decreases in the incidence of different tumour types is one which must be faced and not simply brushed aside, although interest in this issue appears to have waned in recent years.

6.7 Identification of Noncarcinogens

Since the proof of a negative statement is philosophically impossible, then the identification of chemicals which can be said to have no carcinogenic potential is also impossible. The most that can be said regarding carcinogenic inactivity is that activity has not been demonstrated within the operational definition that has been selected. This operational definition includes a testing programme restricted to rodents and a statistical power which varies from experiment to experiment according to the number of animals at risk in control and treatment groups from particular tumour types. While rejection of the null hypothesis is always presented in reports, the power of the experiment to demonstrate its rejection is seldom, if ever, presented when no significant treatment effect has been shown. This is not identical with asserting that all chemicals are carcinogens, although this may also be correct, provided that the circumstances in which carcinogenicity becomes recognizable can be defined. This kind of reasoning may lead one to conclude that there is no strict dividing line separating carcinogens from noncarcinogens, and that epidemiological or laboratory studies of a particular power may fail to demonstrate an effect, whereas a higher-power study may do so.

There is a very high prevalence of carcinogens described in the open literature today, in contrast with the situation to be found 20 years ago. Gold *et al*. (1989a) reported a prevalence of 51% among 955 chemicals tested and entered into the Carcinogen Potency Database (CPDB). This included the NCI/NTP data, where the prevalence was 48% among 251 chemicals (currently the prevalence is 54% among 301 chemicals). Yet, in 1969, Innes *et al*. reported their results with 120 chemicals, largely pesticides and industrial chemicals, in which the prevalence of carcinogens was only 9%. It is plausible that the 9% prevalence of carcinogens in the Innes *et al*. (1969) study is partially due to low statistical power, low doses and short observation time.

6.8 Transgenic Animals in Carcinogenicity Testing

Advances in molecular biology have opened up the possibility of approaches to carcinogenicity testing differing from more traditional approaches. Specifically, the transfer of new or altered genes to the germ-line of mammals has produced transgenic animals that can then be used in short-term *in vivo* tests for carcinogenicity, and may be useful for research into the characterization of critical, genotoxic events in carcinogenesis. In looking for alternatives to lifespan studies (that are so expensive and often provide results that are contentious), much consideration has been given to models involving proto-oncogenes and tumour suppressor genes. These classes of genes have been highly conserved in evolution and are therefore likely to be similar in man and inbred strains of rats and mice. Also, they are known to have key roles in the early and late stages of carcinogenesis in both man, where the aetiology is usually unknown, and in rats and mice used in experiments, where there is a high probability that the causative agent is the test substance administered.

A number of transgenic and knockout models are available and the results obtained so far with three—Trp53+/−,Tg.AC (zeta globin-promoted v-Ha-*ras*) and CBF_1-Tg-H*ras*-2 (originally *ras*H2) mice—indicate that the models can be useful if the outcomes are suitably interpreted.

Heterozygous *p53*-deficient mice (with one wild-type and one null allele) on a C57BL/6 genetic background have a low spontaneous tumour incidence up to 12 months of age. Mice that are homozygous for the null allele generally have a high incidence of spontaneous tumours within the first three to six months of life. Thus, induced deletion of the active allele in the heterozygote will lead to an increase in tumour incidence. This model responds to carcinogens that are genotoxic, although responses are not restricted to genotoxins.

Tg.AC mice on an FVB/N genetic background carry multiple copies of a v-Ha-*ras* oncogene fused to the promoter of the mouse zeta globin gene. An important characteristic of the model is that the skin has the characteristics of genetically initiated skin, and untreated skin appears normal in comparison with the skin of the wild-type FVB/N parent strain. The incidence of spontaneous skin papillomas in untreated mice is very low to zero in a standardized protocol of dermal exposure for 20–26 weeks. Skin papilloma is the most commonly reported response, but other target sites are affected by both mutagenic and nonmutagenic carcinogens. Specific activation of the expression of the transgene is the critical event underlying responses in the model. Since induction of transgene expression can be achieved either by treatment with known carcinogens or tumour promoters or by full-thickness wounding of the skin, 'false-positive' responses might be induced by a chemical that induces some epidermal toxicity; such responses have not been observed, but the possibility should be recognized.

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Table 5 Performance of transgenic assays for carcinogenicity as judged against the National Toxicology Program (NTP) carcinogenicity test results in rats and mice

Assay	a. Positive for carcinogens	b. Positive for noncarcinogens	c. Negative for carcinogens	d. Negative for noncarcinogens	Sensitivity*	Specificity**
Trp53+/−	7	0	16	12	7/23 30%	12/12 100%
Trp53 (genotoxins)	7	0	4	5	7/11 64%	5/5 100%
Tg.AC	14	2	14	10	14/28 50%	10/12 83%
*ras*H2	15	0	8	9	15/23 65%	9/9 100%

*Sensitivity = a/(a + c).
**Specificity = d/(b + d).

CBF_1-Tg-H*ras*-2 transgenic mice carry five or six copies of a human c-Ha-*ras* proto-oncogene which is expressed in both tumours and normal tissues.

These three transgenic models were originally evaluated in several laboratories, and each model has been used in at least two laboratories to test chemicals for carcinogenicity (Tennant *et al*., 1998). The available data from 26-week tests indicate that these transgenic models with neoplastic end points could be considered appropriate for identifying carcinogens and tumour promoters. Nevertheless, the limited range of chemicals evaluated to date left the uncertainty that agents that act through pathways other than those altered in the transgenes may not be detected, and the possibility that noncarcinogens may be identified as carcinogens. A more recent evaluation (Pritchard *et al*., 2003) has permitted a larger number of chemicals to be tested in these three models, although, by the standards set by *in vitro* predictive models, the numbers remain low. In **Table 5** it can be seen that, when evaluated against the US NTP standard two-species assay for carcinogenicity, the specificities of these assays are high, whereas their sensitivities are low. Thus, a significant response in one of them is indicative that the chemical would have a high probability of also producing a significant response in a two-year assay. Unfortunately, a nonsignificant response would give little assurance of a similar result in our traditional carcinogenicity tests. This outcome is highly reminiscent of some of the genotoxicity tests that were developed as short-term tests predictive of carcinogenicity (McGregor *et al*., 1999).

7 CARCINOGENIC RISK ASSESSMENT AND MANAGEMENT

These concluding paragraphs may appear ludicrously brief, but my intention is not to describe and discuss the various ways in which regulatory authorities treat data from experiments for carcinogenic potential in animals or the rarer, more complex data from human epidemiology. My intention is only to make a very few general statements.

Firstly, if risk assessment is separate from risk management and risk assessment is a purely scientific process while risk management includes not only these conclusions, but also the contemporary wishes and opinions of society, then this difference should be fully recognized. Currently, there is a tendency for risk assessors to step beyond their strict limits of scientific evaluation and include, notably, the 'precautionary principle'. Of course, this should be a part of safe-guarding human health as far as possible, but there is a danger that the principle can be invoked twice: once in risk assessment and again in risk management. The outcome of this double application is not precautionary, but overly protective.

Secondly, and following from the first, risk assessors must state their evaluations and reasons for them very clearly and unambiguously. When a text is read by another person, it will frequently be interpreted in a sense different from that intended by the writer. It is, therefore, necessary to try to reduce this margin for different interpretations as much as possible.

Thirdly, carcinogenesis is a complex and incompletely understood process. It is therefore necessary to keep an open mind in order to stand any chance of reaching an understanding. Dogma has no place in risk assessment—and neither do default assumptions. If these are to be used in the protection of human health, it is the task of the risk managers to implement them, although it remains the duty of the risk assessors to indicate where they could be reasonably considered.

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Nongenotoxic or Epigenetic Carcinogenesis

Christopher J. Powell and Sir Colin Berry

CONTENTS

1 INTRODUCTION

For most, if not all, tumours, heritable changes in the genetic material provide *the* fundamental alteration in cellular biology which results in the production of a neoplasm. However, it is clear that there are many mechanisms by which this process may occur and that epigenetic events, either acting alone or in concert with genetically determined events, may produce tumours. This process, of epigenetic carcinogenesis, is best considered in terms of specific examples, as generalizations are difficult in this field, despite the frequency with which they are employed. Nevertheless, it is reasonable to say that in *many* examples of epigenetic carcinogenesis, the affected tissues show a predictable sequence of changes, including hypertrophy, hyperplasia and a loss of uniformity of cellular response, with focal hyperplasia, adenoma and

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possible progression to adenocarcinoma as a consistent sequence.

1.1 Definition

The term 'nongenotoxic' is usually applied to chemicals which cannot be shown to interact directly with, or cause damage to, DNA in a number of short-term screening tests, but which none the less are capable of producing tumours in laboratory rodents by the conclusion of a bioassay.

This definition is pragmatic, and is used because it is neither practicable, desirable nor appropriate to subject a chemical to all possible types of genotoxicity test in order to exclude DNA reactivity. Moreover, some types of DNA damage are not detected by present test methods and there are examples of difficulties of interpretation where genotoxicity testing *in vivo* has given different results to those obtained *in vitro* (see **Mutagenesis**; **Cytogenetics**; **Genetic Toxicology Testing and its Relevance to Human Risk and Safety Evaluation**). Generally, *in vivo* tests are considered more relevant for human-risk assessment because they allow for the complexity of tissue distribution, kinetics and metabolism. Rather unhelpfully, some authors continue to interpret nongenotoxic as meaning 'all chemicals which are not mutagenic in bacterial test systems' (Lee *et al*., 1995), a source of confusion for those unfamiliar with this subject.

1.2 Bioassays and Mechanistic Studies

Normally, an animal carcinogenesis bioassay would not be performed with a chemical that was clearly positive in genotoxicity tests *in vivo* and, in this sense, a carcinogenesis bioassay is a test for nongenotoxic carcinogenicity. Consequently, for a chemical of commercial interest, the potential or actual existence of a nongenotoxic mechanism only comes to light towards the end of a development programme, which in the case of a new drug is after four to five years of intensive research and the expenditure of several tens of millions of pounds, dollars or euros. For commercial reasons it is therefore often worth investigating whether a nongenotoxic mechanism, which operates in a rodent at high exposure levels, is likely to be relevant for humans. This type of mechanistic study has most frequently been performed in the rat rather than the mouse, because of: (i) the greater biological significance attached to positive results in the former species and (ii) the perception that the mouse may be too small to be practicable for mechanistic studies—a view certainly not relevant for molecular studies.

2 GENERAL FEATURES OF NONGENOTOXIC CARCINOGENS

Certain general statements can be made about many nongenotoxic carcinogens. However, with what can be described as a 'mixed bag' end point reached by many routes, there are many exceptions. The general characteristics include:

- They are nonmutagenic
- They show no evidence of other direct chemical reactivity with DNA
- There are no common chemical structural features between chemicals
- Dose-threshold effects are clearly evident
- The effects of a particular compound are usually limited to one organ and/or species
- Carcinogenic potency is lower than that of genotoxic chemicals.

A large number of the chemicals tested for carcinogenicity in rodent bioassays by the National Toxicology Programme (NTP) in the USA have been characterized as nongenotoxic carcinogens, the most common target organ for these chemicals being the liver (**Figure 1**), with a variety of other glandular tissues as less frequent targets.

2.1 Mechanisms

Comparatively few examples of this process have been studied in sufficient detail to justify the claim that a 'nongenotoxic mechanism' is well understood. A number of mechanisms have been identified (**Table 1**), several of which are considered in more detail in the examples hereafter.

3 THYROID CARCINOGENESIS

Hormonal feedback loops, changes in thyroid hormone synthesis, altered cellular receptor activity, variation in rates of hormone release and metabolism, and direct interference with cell function may all operate to produce the sequence of growth leading to neoplasia in the thyroid gland. Thyroid neoplasia may develop following any stimulus which results in prolonged and excessive thyroid stimulating hormone (TSH) secretion.

Bircher (1910) first reported thyroid nodules in the rat due to iodine deficiency, and Axelrod and Leblond (1955) used a low-iodine diet to induce thyroid neoplasia in this species, with the occurrence of pituitary tumours in the test animals, suggesting over-stimulation of that gland.

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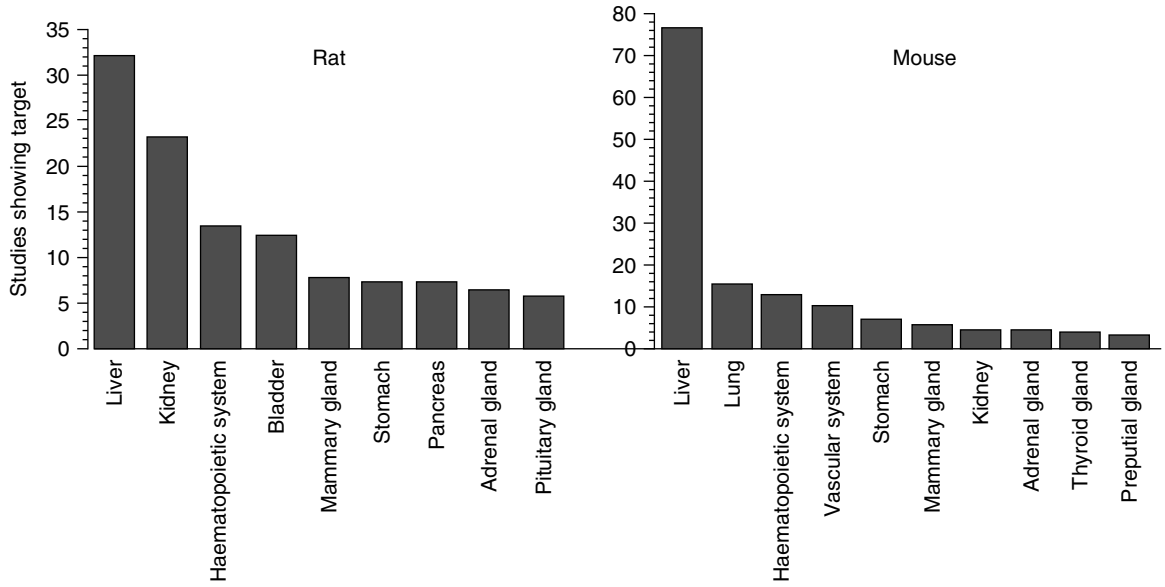

Figure 1 Tissue target sites for nonmutagenic carcinogens. Data from 175 positive NTP carcinogenicity studies. (Reproduced from Gold et al, 1993. © Elsevier.)

Table 1 Proposed mechanisms of nongenotoxic carcinogenesis

Mechanism	Reference
Chronic stimulation of cell proliferation via chronic cytotoxicity or persistent supraphysiological levels of trophic hormones	Melnick *et al*. (1993)
Inhibition of cell death (apoptosis) in DNA-damaged cells	Schulte-Hermann *et al*. (1995)
Impairment of DNA-replication fidelity	Umar and Kunkel (1996)
Inhibition of replication error or unscheduled DNA repair, that is, mutator phenotype	Kaufmann and Kaufman (1993)
Inhibition of gap-junctional intercellular communication	Yamasaki (1995)
Persistent immunosuppression	Cockburn and Krupp (1989)
Altered methylation status of genes controlling cell growth or differentiation	Jones and Gonzalgo (1997)
Dysregulation of karyokinesis, producing aneuploid cells	ECETOC (1997)

Subsequent experiments produced tumours following. TSH administration (Sinha *et al*., 1965). In man, excessive production of TSH occurs in chronic iodine deficiency or following subtotal thyroidectomy, and may be accompanied by neoplasia. The sequence is most clearly evident in dyshormogenetic goitres, where failure of the production of thyroxine leads to pronounced pituitary drive. Increased production of TSH due to inadequate levels of thyroid hormone production may be induced by any of the following failures of the mechanisms of normal thyroid metabolism in dyshormogenesis:

- Failure of iodine trapping
- Peroxidase deficiency, resulting in the failure of intrathyroidal iodine binding
- Failure of iodotyrosine coupling
- Dehalogenase deficiency leading to failure of deiodination of iodotyrosine residues
- Production of abnormal iodoproteins due to defects in thyroglobulin synthesis.

In humans with this type of thyroid disease, the gland may become very large and glands with weights of several hundred grams were not uncommonly found in the past. Nodules are almost invariably present, and microscopically, hyperplasia, often with many cells with pleomorphic nuclei, is inevitable. It is important to note that, rather like some experimental lesions, the progression of these changes to truly malignant behaviour with metastasis is rare (Crooks *et al*., 1963).

In animals, experimental work has shown that the pattern of stimulation of the gland by TSH is also critical, both duration and continuity being important. In general, a period during which the TSH level is allowed to return to normal has a protective effect with regard to tumour production. Goitrogen-induced alterations in the normal pattern of thyroid growth occur in three phases (Stringer *et al*., 1985). There is initially a rapid growth

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phase (four to eight weeks), followed by a period in which growth is limited, but where the important change of loss of normal response of thyroid follicular cells to normal growth mechanisms occurs (see below). This is followed by the appearance of multiple follicular cell proliferation (tumours).

In the initial rapid growth phase, cell proliferation begins at concentrations within the normal physiological range of TSH levels, but growth responsiveness continues to well above this. In the plateau phase, a growth response can still be obtained to non-TSH stimuli (wounding), but the proliferative response to TSH is lost, although the functional response is preserved. These results were obtained in dissociated cells and were therefore not attributable to locally acting factors dependent on tissue organization. Naturally occurring goitrogens are found in cabbage leaves, Brussels sprouts, turnips and mustard. Goitrin (L-5-vinyl-2-thiooxazolidone), the active goitrogen in turnips, is about as active as propylthiouracil (Haynes and Murad, 1985), and although TSH production may be increased after exposure to naturally occurring goitrogens, a role in thyroid carcinogenesis in man seems unlikely.

3.1 Effects of Drugs

It is simple to identify the steps in thyroid hormone synthesis and release, and to identify the drugs likely to affect the processes involved. However, there is a need for caution, since extrinsic factors may affect target cell sensitivity and the pattern of exposure will affect the outcome dramatically. When the effects of the various factors acting on the demand for, and degradation of, thyroid hormone are added (see **Figure 2A**), a more complex picture generally emerges *in vivo.* It is important to note, however, that the simultaneous administration of goitrogenic drugs (such as thiouracil or thiourea) and thyroid hormone has been shown to prevent the development of follicular cell neoplasia.

Some highly specific effects have been characterized. Aminoglutethimide was widely used as an anticonvulsant until reports of the development of goitre in children on the drug appeared. The drug acts directly on the thyroid cells by reducing the production of thyroxine and diiodotyrosine. The drug does this by inhibiting iodine organification in a concentration-dependent manner, with no effect on I^- uptake by the cells. The related compounds glutethimide, nitroglutethimide and acetylaminoglutethimide had no effect, which suggests that the effect depended on a free amino group in the phenyl ring of the molecule (Brown *et al*., 1986). The increase in thyroid gland size is thus triggered by a very clear metabolic block. Blockage of effective thyroid function may be produced by more than one mechanism. Bromide inhibits the uptake of iodine by the thyroid gland and inhibits the oxidation of iodide to iodine, and thus the incorporation of iodine into tyrosine residues. Bromide also inhibits the coupling of tyrosine residues to thyronine and causes an increase in NADH cytochrome c reductase activity (van Leeuwen *et al*., 1983).

In chronic toxicity studies in rodents, thyroid enlargement and histological changes suggestive of hyperactivity are commonly observed, frequently in association with hepatic hypertrophy and the induction of hepatic xenobiotic-metabolizing enzymes. An increased incidence of thyroid follicular epithelial tumours is often subsequently encountered when animals are continuously exposed to the chemical in lifespan carcinogenicity studies. The underlying mechanism of hormonal dysregulation and adaptation is now well documented (Saito *et al*., 1991; Johnson *et al*., 1993; Wilson *et al*., 1996).

When a xenobiotic is metabolized in the liver via conjugation with glucuronic acid prior to excretion in the bile, the uridine-5′-diphospho-glucuronosyl transferase (UDP-glucuronosyl transferase) responsible is often induced by two- to fivefold, an adaptive response enhancing the rate of biliary clearance of the chemical. Several isoforms of UDP-glucuronosyl transferase exist, but when the type induced is the isoform also responsible for the glucuronidation of thyroxine, there is a simultaneous increase in the rate of excretion of both the xenobiotic and T_4 (Barter and Klaassen, 1994). The resulting drop in circulating T_3 and T_4 levels reduces feedback inhibition on hypothalamic TRH production, in an attempt to restore circulating levels of thyroxine. Release of TSH from the adenohypophysis of the pituitary is thus increased, stimulating both thyroxine secretion from, and cell proliferation in, the follicular epithelium (**Figure 2B**). When elevated levels of TSH are sustained, nodular hyperplasia and, ultimately, neoplasia of the follicular epithelium result. The demonstration of increased rates of biliary thyroxine excretion *and* an increased level of circulating TSH are key to confirming this mechanism of nongenotoxic carcinogenesis. Diurnal variation in TSH levels necessitates careful control of measurement, otherwise biologically significant changes, which may be only 30–50% above basal levels, can be undetectable.

The rat is unusually sensitive to chemicals which enhance hepatic excretion of thyroxine for two reasons. First, the molecular weight threshold for biliary excretion of xenobiotics is lower in the rat than many other species and so more xenobiotic molecules of moderate molecular weight are excreted in the bile than they are in man. Second, plasma protein binding and transport of T_3 and T_4 differ from that of other species, including even the mouse. Rats effectively lack thyroid-binding globulin and, in consequence, rely far more on pre-albumin for transporting T_3 and T_4 (**Table 2**). A significantly larger circulating pool of unbound or free T_3 and T_4 thereby

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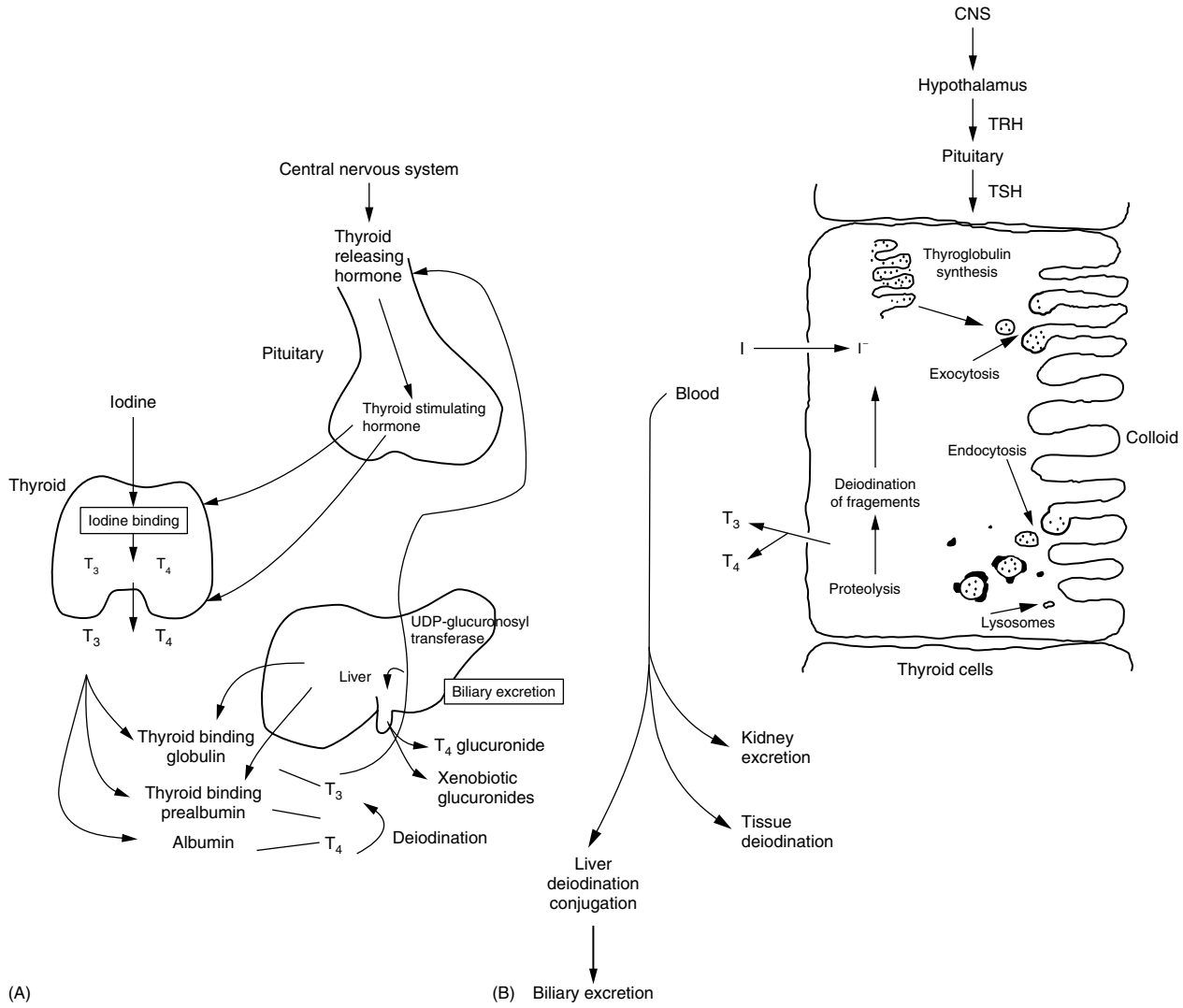

Figure 2 The regulation, transport and excretion of thyroxine. (A) The liver is the site of synthesis of plasma transport proteins and a major route of excretion. (B) Within the follicular epithelium, thyroglobulin is synthesized and secreted into follicles for storage, then reabsorbed, catabolized and excreted as T_3 and T_4.

allows changes in the rate of metabolism or clearance to readily influence plasma levels, that is, there is a smaller reservoir of T_3 and T_4 to buffer changes in circulating levels.

3.2 Support for the Model and Conclusions

In an attempt to predict the oncogenicity of a number of nongenotoxic compounds in a number of organs at early stages in a bioassay (7, 28 and 90 days), Elcombe *et al.* (2002) examined biochemical changes in thyroid-related markers in rodents. They found that TSH levels were increased when T_4 levels were diminished (T_3 was less well correlated) and the thyroid labelling index was closely related to TSH levels. It appears that there is a critical level of T_4 that must be reached before TSH increases. These observations add further weight to the mechanism of action (MOA) described above. In addition, thiazopyr, which was the original case study used to illustrate the 2000 IPCS framework for this mode of carcinogenic action, has been revisited as a case study to illustrate the additional guidance provided in the new 2005 IPCS framework for evaluating the human relevance of animal modes of carcinogenic action (Dellarco *et al.*, 2006).

If human exposure to chemicals of this type is at substantially lower levels than that causing thyroid hyperplasia in the rat, and studies in other species do not suggest effects on thyroid proliferation, epigenetic effects in the rat do not portend a human carcinogenic risk. There is considerable public anxiety about the effects of pesticides in the food chain, and some of these compounds, including the dithiocarbamates (ethylene

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Table 2 Species differences in thyroid-hormone-binding plasma proteins (% hormone bound)

		Thyroxine-binding globulin	Albumin	Thyroxine-binding prealbumin	Total protein binding (%)	Unbound (%)
Man	T4	62.5	9.9	27.7	100	0
	T3	62.5	19.9	14.8	97.2	2.8
Rat	T4	1	6	80	87	13
	T3	3	32	42	77	23
Mouse	T4	60	40	0	100	0
	T3	65	25	0	90	10

Note that because the rat effectively lacks thyroid-binding globulin, a far higher proportion of T_3 and T4 circulate unbound and are available for metabolism and excretion. After McClain *et al*. (1989) and McClain (1994).

bis-dithiocarbamates) and triazoles, are goitrogenic and carcinogenic in animals under appropriate conditions of high and continuous dosage (O'Neil and Marshall, 1984; see also **Toxicology of Pesticides**). The food colouring erythrosine (used in maraschino cherries) is also a thyroid carcinogen in this mode of dosing. The clear evidence of threshold effects and the large safety margins emphasize the theoretical nature of the risk in this well-understood example of carcinogenesis.

4 PEROXISOME PROLIFERATION AND NEOPLASIA

Peroxisomes are single-membrane-bound organelles which increase in number dramatically in the rodent liver during exposure to certain lipid-lowering drugs and phthalate-ester plasticizers (Reddy and Lalwani, 1983). The increase in liver size accompanying this change is mediated by binding of the chemical to the peroxisome proliferator-activated receptor (PPAR) and is directly related to increased synthesis of membranes and smooth endoplasmic reticulum (Rao and Reddy, 1996). Total DNA in the liver increases in animals exposed to peroxisome proliferators over a period of days and will persist for weeks (Moody *et al*., 1977). It seems probable that the increase in cell division is in response to the metabolic demands placed on the liver, but the rapidly proliferating liver cells may be more vulnerable to specifically induced (oxidative) damage. The compounds producing peroxisome proliferation are generally not genotoxic and their ability to induce liver tumours in rats and mice after high-dose exposure is of questionable significance for man.

Many enzymes are associated with peroxisomes, including those responsible for the β-oxidation of long-chain fatty acids. Fatty acyl-CoA oxidase is the first and rate-limiting enzyme in the β-oxidation of fatty acids. It uses molecular oxygen and produces hydrogen peroxide. In man, the β-oxidation system has been most studied in the syndrome of sudden infant death, where deficiencies are apparently involved in a small proportion of cases (see Berry, 1989). The induction of hydrogen-peroxide-generating peroxisomal enzymes and the failure of epoxide-metabolizing enzymes (cytosolic epoxide hydrolase, glutathione-S-transferase (GST), microsomal epoxide hydrolase) to increase *pari passu,* was implicated as a key DNA-damaging event, in the initiation of hepatocarcinogenesis (Reddy *et al*., 1986).

This mechanism was questioned because the differential responses of parts of the system which produce and metabolize peroxides, and which prevent activated oxygen-dependent DNA damage, are capable of definitive study only in an intact cellular system with the correct concentration and compartmentalization of the various enzymes and cofactors (Bentley *et al*., 1987; 1988). When DNA damage was sought *in vivo,* excess production of hydrogen peroxide was not associated with oxidative damage, because no strand breaks in hepatic nuclear DNA, unscheduled DNA synthesis in hepatocytes or induction of the formation of micronuclei in the liver were found.

Studies in mice with functional deletion of the gene for fatty acyl-CoA oxidase now fundamentally question the role of peroxisomes in hepatocarcinogenesis. Fatty acyl-CoA oxidase null mice ($AOX^{-/-}$ knockout) develop fatty livers and, subsequently, marked peroxisome proliferation (Fan *et al*., 1998). Even though the peroxisomes of these mice are quite incapable of β-oxidation, the mice still develop high levels of hydrogen peroxide in the liver and hepatocellular carcinoma by 15 months of age. Other sources of oxidizing damage are, therefore, thought to be important, possibly including inflammatory cell-generated hydrogen peroxide.

The significance of the PPAR receptor in hepatocarcinogenesis was revealed in another experiment with genetically engineered mice. PPAR null mice failed to increase hepatic DNA labelling in response to a diet containing 0.1% Wy-14 642 (a potent peroxisome proliferator) and, whereas wild-type mice all developed multiple hepatic tumours within 11 months, none of the PPAR null mice did (Peters *et al*., 1997). Thus, receptor-mediated alteration of gene expression and/or cell proliferation appear necessary for carcinogenesis.

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While the mechanisms of peroxisome proliferator carcinogenesis in rodents remain uncertain, it is clear that humans express much less PPARα subtype in their liver than rodents and thus, in humans, carcinogenic effects are unlikely to occur at low levels of exposure.

5 THE THIAZOLIDINEDIONES AND VASCULAR NEOPLASMS

Haemangiosarcoma is rare in man and relatively common in domestic and experimental animals. The tumour occurs in about 1% and 5% of control rats and mice, respectively, in NTP studies, and more than 20 agents have been associated with induction of vascular neoplasms (some causing haemangiosarcoma incidences of more than 80%). Since vascular proliferation is an essential part of many continuously running bodily processes, including wound healing and repair, and endometrial cycling, it is clearly a system, like others in the body, where proliferative capacity exists and is closely regulated.

Regulatory concerns have been raised relating to certain thiazolidinediones (antidiabetic and anti-inflammatory drugs) which act via the PPARγ receptor. Troglitazone binds to the PPARγ receptor, resulting in a conformational change that activates the transcriptional regulatory activity of the receptor and correlates with its antidiabetic actions (Berger *et al*., 1996). Troglitazone and several other PPARγ/PPAR dual agonists induce haemangiosarcomas in mice (Herman *et al*., 2002). Muraglitazar, pioglitazone and rosiglitazone also bind and activate PPARγ, but do not produce haemangiosarcoma; therefore, PPARγ binding appears to be necessary, but not sufficient, to produce haemangiosarcoma (Tannehill-Gregg *et al*., 2007; Waites *et al*., 2007).

5.1 Angiogenesis

Angiogenesis is mainly an adaptive response to local hypoxia and is dependent on the local accumulation of hypoxia inducible factors (HIFs) which are composed of α and β subunits of a heterodimeric transcription factor. These are normally continuously degraded by oxygen-dependent processes (the von Hippel–Lindau (vHL) protein (see below) acts by recognition of specific proline residues (Pro 402, Pro 563) in the α subunits, which are hydroxylated and degraded by E3 ubiquitin ligase), but in the absence of adequate oxygen levels the α subunits are stabilized and form heterodimers with HIF β, which is not oxygen sensitive. These transcription factors then activate a number of angiogenic genes, including those for vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (FGFb) and hepatocyte growth factor (HGF) in descending order of importance. VEGF-A, a specific endothelial cell mitogen, acts locally and systemically at tyrosine kinase growth factor receptors (VEGFR-1 and 2) to stimulate endothelial cell mitogenesis and recruit endothelial progenitor cells.

The downstream effects of these growth factors are complex. Connective tissue growth factor (CTGF) is a heparin-binding growth factor produced by fibroblasts after transforming growth factor β (TGFβ) activation and promotes angiogenesis in wound repair, tissue regeneration and skin fibrosis (Igarashi *et al*., 1998). In mesenchymal tumours, CTGF messenger RNA (mRNA) is expressed in fibroblasts of dermatofibromas, some dermatofibrosarcomas, angiolipoma, angiomyeloma and pyogenic granuloma, but not angiosarcoma, suggesting that benign fibroblasts and/or vascular endothelial cells have the capability to express CTGF mRNA when activated, but that expression is lost in malignant tumours. The CD34 antigen (Lanza *et al*., 2001), a glycoprotein involved in cell adhesion, is found to be expressed only in dermatofibrosarcomas (which are locally recurrent tumours) and angiosarcomas. The CD34 antigen is also expressed in multipotent stromal cells (MSCs) from adipose tissue, but not in bone marrow MSCs (Lazennec and Jorgensen, 2008) suggesting that it is a good marker of 'de-differentiation'.

5.1.1 Abnormalities of Angiogenic Regulation

There are a number of clinical syndromes in man in which abnormalities of the vasculature and neoplasia (including vascular and nonvascular tumours) are both significant features. In these syndromes, an inter-relationship between the changes leading to tumour formation in both epithelia and the vasculature are common and are apparently genetically determined. These conditions are informative about the potential role of factors assumed to be significant in an MOA for the production of haemangiosarcoma, in the sense that they exclude a number of what appear to be 'obvious' stimuli. They include syndromes (vHL, Chuvash polycythaemia) associated with mutations in the vHL gene which controls the stability of the HIFs, thus regulating VEGF-mediated angiogenic responses, and the hamartomatous polyposis syndromes, Bannayan-Riley-Ruvalcaba (BRR) syndrome and the Cowden syndrome (Marsh *et al*., 1999; Cho *et al*., 2008). From study of these conditions it is clear that the unrestrained action of HIFs cannot alone produce haemangiosarcoma; in the Chuvash syndrome, the stabilization of the potent angiogen HIFα with its persistent action induces polycythaemia, but not neoplasia (Gordeuk *et al*., 2004). Although these genetic disorders may shed light on vascular proliferation and angiogenesis, the vascular tumours associated with these are benign.

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5.1.1.1 The Target Cells

Recent advances in the understanding of tumour angiogenesis have highlighted the important roles of circulating, bone-marrow-derived progenitor cells, including both endothelial and monocyte/macrophage lineage cells. These findings suggest that haemangiosarcomas may arise, not only from transformation of tissue-resident endothelial cell populations, but also from circulating progenitors or adult stem cells recruited from bone marrow, or possibly also from extramedullary sites of haematopoiesis, such as the liver and spleen. The recent report by Tang *et al*. (2008) suggests that pericytes in fat are possibly the stem cells for some vascular and mesenchymal tumours (they express PPARγ, CD34 and PDGFRβ).

Recruitment, proliferation, localization and differentiation of the different cell types that regulate angiogenic processes is complex and provides ample opportunity to allow species, strain and individual animal variation in angiogenic responses. Research on canine haemangiosarcoma has shown that tumour-restricted expression of proinflammatory and angiogenic genes clusters in the haemangiosarcoma of Golden Retrievers and is distinct from haemangiosarcoma of other dogs, suggesting heritable factors mould phenotypes in sporadic, naturally occurring tumours.

5.2 Mechanisms of Action in the Production of Vascular Tumours

In man, the established causes of malignant vascular tumours—following thorotrast injection (Kojiro *et al*., 1985), after vinyl chloride exposure, in the arms of women with lymphoedema following radical breast surgery (Abbott and Palmieri, 2008) in human herpesvirus 8 (HHV-8) infection and in certain hereditary syndromes—differ widely in pathogenetic mechanisms. As examples, Kaposi's sarcoma (Barillari and Ensoli, 2002), a spindle cell endothelial-cell sarcoma, occurs at a 310-fold higher incidence in the HIV-infected population. It is caused by HHV-8 infection, and is associated with elevated levels of cytokines (IL-1, IL-6, IL-8), HHV-8-encoded vIL-6 and other factors (leukaemia inhibitory factor, oncostatin M and cardiotropin) secreted by a spindle-cell component and acting via gp130 receptors on endothelial cells to induce subsequent proliferation (Amaral *et al*., 1993). Latency-associated nuclear antigen (LANA) is found in the nuclei of this tumour, but not in benign human tumours or angiosarcomas (Schmidt and Zietz, 2005). However, vinyl chloride (see below) acts via a genotoxic mechanism, as does thorotrast, as an α-emitter.

The importance of VEGF signalling pathways in angiogenesis and haemangiosarcoma is highlighted by the finding of mutations in the phosphatase and tensin homologue (PTEN) gene in canine and human haemangiosarcomas. PTEN is a tumour- and angiogenesis-suppressor gene which inhibits angiogenesis due to its lipid phosphatase activity on substrates in the phosphoinositide 3 (PI3) kinase pathway downstream from VEGF receptor signalling (Eng, 2003). PTEN mutations are found in other common tumours, including endometrial and prostatic carcinomas, gliomas and melanoma, and in 80 and 60% of patients with Cowden and BRR syndromes, respectively.

5.3 Regulatory Issues

Haemangiosarcomas in rodents have become an increasingly important issue in evaluating the significance of the results of carcinogenicity studies of a variety of classes of pharmaceutical agents. These tumours, primarily in mice, have been seen with many PPAR dual agonists, some (but not all) calcium-channel blockers, antipsychotics, phosphodiester 5 (PDE-5) inhibitors, dipeptidyl peptidase 4 (DPP-4) inhibitors, antiarrythmics, antisense compounds, nitric oxide releasers, agents causing haemolysis and VEGF inducers. The pharmaceutical agents producing these effects are not genotoxic in standard *in vitro* and *in vivo* genotoxicity assays, and they are not DNA reactive. It is reasonable to assume, therefore, that their mode of action must involve an increase in cell proliferation, but the absence of a, MOA results in significant uncertainty in the assessment of human risk and regulatory decision-making.

Several questions remain regarding haemangiosarcoma induction. Is the target cell that gives rise to the sarcomas the endothelial cell itself or a precursor stem cell arising from the bone marrow or fat? Why are the tumours found in certain tissues in mice (predominantly liver, spleen and bone marrow), but in subcutaneous adipose tissue in humans? Does the inducing chemical act directly on cells that become transformed or do they act on other cell types, producing a biological effect leading to the release of growth factors or other substances which then affect the precursor cells?

5.4 Potential Modes of Action

Haemangiosarcoma clearly arises by a number of mechanisms.

5.4.1 Genotoxicity

Vinyl chloride, an inducer of haemangiosarcomas in both rodents and man, appears to work through genotoxic mechanisms. The mode of action involves the formation of genomic DNA adducts. In rodent studies, molecular dosimetry of DNA adducts in the liver has

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been examined after vinyl chloride treatment and showed that 7-(2′-oxoethyl)guanine (7OEG) was the major DNA adduct detected (98% of adducts). N^2,3-Ethenoguanine and 3,N^4-etheno-2′-deoxycytidine were also found (1% of adducts). N^6-Etheno-2′-deoxyadenosine was present at even lower concentrations. The persistence of these four adducts showed that while the 7OEG adduct had a half-life of 62 hours, the other three etheno adducts were highly persistent, having half-lives of 30 days or more (see Morinello *et al*., 2002).

5.4.2 Nongenotoxic Mechanisms

There is presently no unifying hypothesis for a nongenotoxic MOA for the many compounds that induce haemangiosarcoma.

2-Butoxyethanol increases haemangiosarcomas following chronic inhalation exposure and, in mice, oncogenesis appears to be related to haemolysis and the induction of oxidative damage in Kupffer cells (iron accumulation and oxidative damage resulting in release of growth factors—Corthals *et al*., 2006). More recent data suggests a possible role for HIF-1α and VEGF in the carcinogenetic process following butoxyethanol exposure.

Carbaryl also induces haemangiosarcomas in the mouse in a dose-related manner. In a mouse two-year carcinogenicity study where 80 CD-1 mice per group were given diets providing 0, 100, 1000 and 8000 ppm carbaryl, there was an increase of liver tumours in females and kidney tumours in males at 8000 ppm (a dose exceeding the maximum tolerated dose) and also an increased incidence of vascular tumours in both sexes at 8000 ppm. An increase of vascular tumours (statistically significant) was still observed in male mice at the mid dose (1000 ppm). At the low dose of 100 ppm, the incidence of vascular tumours in male mice was increased compared to the control, but without statistical significance; no clear dose–effect relationship was obtained (see FAO/WHO, 1997). Carbaryl also failed to induce haemangiosarcomas or other tumours at doses up 4000 ppm in heterozygous p53 knockout mice, suggesting a nongenotoxic mode of action (Bigot-Lasserre *et al*., 2003).

Cohen *et al*. (1990) assessed the human relevance of mouse endothelial cell tumours induced by troglitazone (a low affinity PPARγ agonist developed to treat Type II diabetes) that produced an increased incidence of haemangiosarcomas—mainly in adipose tissue in mice, but not rats. They assessed endothelial cell proliferation using a dual label of Ki-67 (cell proliferation) and CD-31 (an endothelial cell marker) in mice, rats and man. Mice were shown to have a higher basal endothelial cell proliferation rate than rats or humans and have a higher background incidence of vascular tumours. *In vivo*, troglitazone induces endothelial cell proliferation in mice after four weeks of dosing. Using *in vitro* experiments with troglitazone, therapeutic levels were shown to be cytotoxic to human cells, while mitogenic to mouse microvascular endothelial cells. This differential *in vitro* response suggests that troglitazone would not stimulate a mitogenic endothelial response in man (see also Ohnishi *et al*., 2007).

The PPAR nuclear receptor forms a heterodimer with the retinoid receptor (RXR) and retinoids have been shown to induce haemangiosarcomas in mice. 4-Hyroxyphenyl retinamide (4-HPR) (Fenretinide) induces a 100% incidence of haemangiosarcomas at several sites in mice, but like the PPAR agonists, most of the tumours induced are in adipose tissue. Other retinoids, retinyl acetate and etretinate, are also mouse haemangiosarcomagens. They bind to and activate retinoic acid receptors (RARs) on target genes and thus regulate gene transcription. Evidence based on *in vitro* investigations demonstrates that they negatively regulate cell growth and angiogenesis, but it is possible that this might be through RAR-independent pathways.

Several possible factors have also been demonstrated to be involved in the haemangiosarcomas induced in mice by pregabalin, which binds to the $\alpha_2\delta$ subunit of voltage-gated calcium channels. Most of these tumours occurred in the usual tissues involved in this form of mouse neoplasia, namely liver, spleen and bone marrow. The data indicate that the initial event is the induction of hypoxia at high doses, leading to a cascade of events, including the overproduction of megakaryocytes and platelets, with consequent release of various endothelial growth factors, including VEGF and PDGF.

Early events in this process in mice are increases in HCO_3 and blood pH, and corresponding decreases in respiratory rate and minute volume, which are all tightly linked physiological responses. Extensive changes in mouse bone marrow occur, and these are consistent with effects of hypoxia and/or alkalosis and are characterized by erythropoiesis, megakaryopoiesis, increased macrophages, with erythrophages and clusters of haemosiderin-laden macrophages suggestive of macrophage activation. Extramedullary haematopoiesis is also evident in the spleens of pregabalin-treated mice. Peripheral erythrocyte and platelet counts are increased in association with bone-marrow changes, and platelet activation is also increased.

5.4.3 Possible Mechanisms of Action: A Synthesis

A document produced by the Health and Environmental Sciences Institute (HESI) as a result of a Reston (Virginia) meeting cosponsored by HESI and the Society of Toxicology suggests that there are two main contenders for an MOA (SOT, 2008).

Adipocytes are a rich source of growth factors, including those that stimulate angiogenesis, and studies with angiogenesis inhibitors have demonstrated that

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angiogenesis is a necessary requirement for fat-pad growth (Hutley *et al*., 2001; Kershaw and Flier, 2004; Fukumura *et al*., 2003; Tang *et al*., 2008). A key component of a proposed MOA for PPARγ agonist induction of haemangiosarcoma is that adipocytes release angiogenic growth factors, which would stimulate endothelial cell proliferation.

The proposed HESI MOA includes the following major components: (i) PPARγ agonists bind to the PPARγ receptor in adipocytes and stimulate their proliferation, leading to the release of angiogenic growth factors and/or a decrease in antiangiogenic growth factors; (ii) dysregulated angiogenesis occurs due to PPARγ agonists initially inhibiting endothelial cell growth, resulting in local tissue hypoxia which occurs in the presence of a net angiogenic environment, leading to selection of endothelial cells which can proliferate and (iii) a selective growth stimulus leads to clonal endothelial cell expansion and oncogenesis. Accessory cells, such as macrophages, could also contribute to an angiogenic stimulus via interleukins (e.g. IL-1, IL-6).

An alternative hypothesis is that the target organ (i.e. fat pad) provides an angiogenic growth stimulus that recruits circulating endothelial stem cells from the bone marrow and these stems cells seed the target organs, leading to the formation of haemangiosarcoma.

5.5 Conclusions

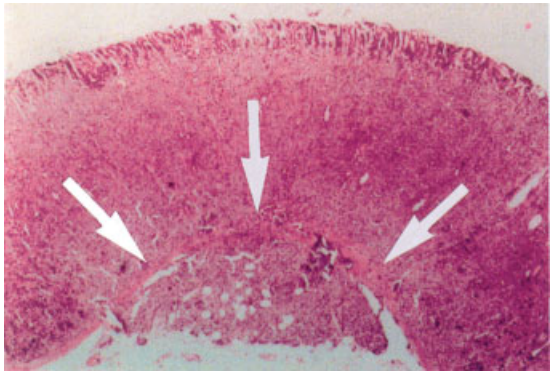
There is a need for better management and control of Type II diabetes mellitus and the PPARγ-receptor-active compounds appeared likely to make a considerable contribution to this problem, encouraging research into this regulatory issue. However, as so often in toxicology, the resolution of the scientific questions is not only a challenging scientific problem but one that, if it can be resolved, may inform us about many processes in the important phenomenon of angiogenesis.

6 CARCINOID TUMOURS INDUCED BY GASTRIC ANTISECRETORY DRUGS

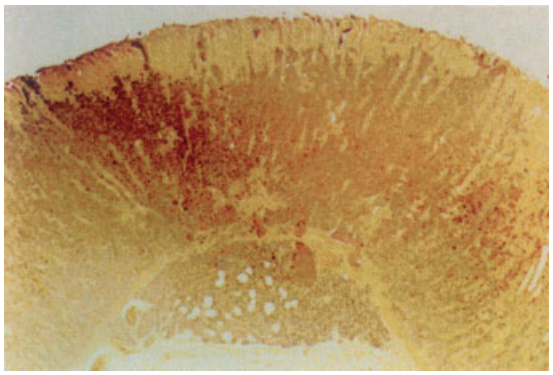
Carcinoid tumours are tumours of the diffuse neuroendocrine cell populations which are dispersed in many glandular tissues, particularly the gastrointestinal tract (see review by Oberg, 2002). Carcinoid tumours have an extremely low spontaneous incidence in humans and in all commonly used strains of rat and mouse.

6.1 Drugs Responsible

The more potent members of the highly commercially successful group of drugs which inhibit gastric acid secretion (including the histamine H_2 antagonists, loxtidine, tiotidine, oxmetidine, sufotidine. SKF93479, ICI 162, 846, BL-6341 and proton-pump inhibitors such as omeprazole) induce carcinoid tumours in rodent carcinogenesis bioassays (Berlin, 1991; Poynter and Selway, 1991). High doses of these drugs, persistently administered, *first* induce generalized hyperplasia of the gastric glandular mucosa, followed by diffuse and focal hyperplasia of enterochromaffin-like (ECL) cells and, in a significant proportion of cases, invasive carcinoid tumours of ECL cells (**Figure 3**). It is generally accepted that this group of drugs are nongenotoxic (Gatehouse *et al*., 1988).



(A)



(B)

Figure 3 Invasive carcinoid tumour in the fundic mucosa of a female rat induced by loxtidine (50 mg kg^{-1} day^{-1}). (A) Stained with haematoxylin and eosin, luminal surface uppermost, showing a solid invasive tumour which has penetrated the muscularis propria (arrowed). (B) A serial section, stained by the Grimelius method for endocrine cells, shows a diffuse carcinoid tumour within the gastric epithelium (dark cells), confirming the submucosal tumour as a carcinoid. (Photographs courtesy of Dr.S. A. M. Selway, Glaxo Wellcome.)

6.2 Mechanism

The relevance to humans of such rodent carcinoid tumours provoked fierce controversy in the mid 1980s

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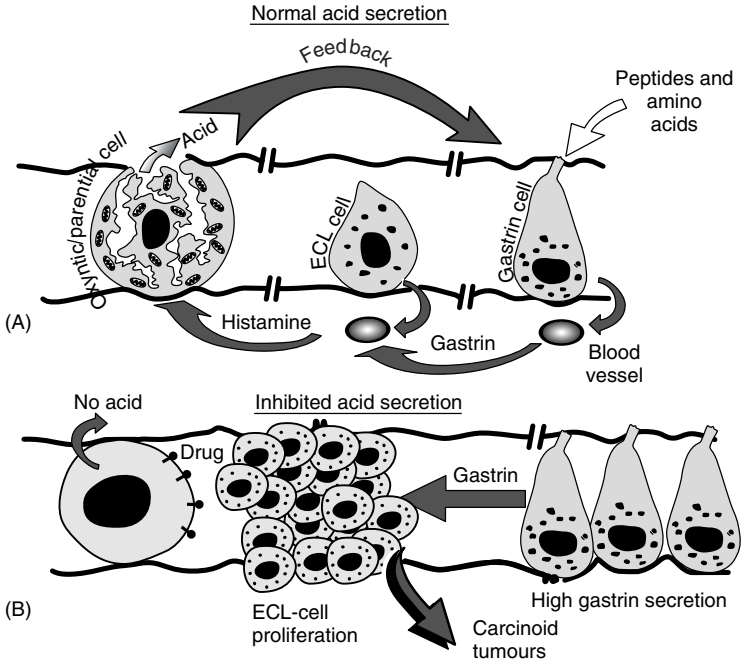

Figure 4 (A) The normal mechanism regulating acid secretion from oxyntic cells in the gastric glandular mucosa. (B) In the presence of antisecretory drugs, the absence of acid feedback inhibition on gastrin secretion causes hypergastrinaemia and sustained proliferation of enterochromaffin-like (ECL) cells.

and remains a matter of some debate. In response, substantial efforts were expended to identify components of a nongenotoxic mechanism, and to determine whether these effects were likely to occur in humans. Normally, acid secretion from parietal cells in gastric glands inhibits gastrin production from G-cells. The inhibition of acid secretion (by antisecretory drugs) removes this feedback inhibition on G-cells, causing hypergastrinaemia and a modest increase in the number of G-cells. Persistent hypergastrinaemia exerts a trophic effect on ECL cells, causing diffuse hyperplasia, areas of nodular hyperplasia and, eventually, carcinoid tumours which invade local lymph nodes and submucosal tissue (**Figure 4**). The initial proliferative response in ECL cells can occur very rapidly and has been observed after as little as two days, in studies with some antisecretory drugs. The degree of hypergastrinaemia which rodents develop is substantially greater than occurs in humans taking clinical doses of these drugs and, when sustained, is proportional to the ultimate incidence of carcinoid tumours (Poynter and Selway, 1991). Marked hypergastrinaemia also characterizes several human conditions in which carcinoid tumours develop: Zollinger–Ellison syndrome, pernicious anaemia and atrophic gastritis (Dayal and Wolfe, 1991).

Carcinoid tumours have also been induced in the rat following long-term administration of the hypolipidaemic fibrate drugs (Spencer *et al*., 1989), which, subsequently, were also shown to have antisecretory activity in this species, but not in the mouse or marmoset. In the rat, surgical resection of the region of the gastric mucosa largely responsible for acid secretion (partial corpectomy) induces hypergastrinaemia and generalized mucosal hypertrophy with ECL-cell hyperplasia. Although not exposed to more than normal diet, 23% of these rats eventually developed carcinoid tumours at two years (Mattsson *et al*., 1991). The gastrin hypothesis may not fully account for the development of carcinoid tumours, as *in vitro* experiments have shown that carcinoid tumours express receptors for a number of growth factors including IGF-1, TGF_Q and EGF (Nilsson *et al*., 1993) and so several mitogens could contribute to ECL cell proliferation.

In terms of human relevance, It is important to remember that the carcinoid tumours that occur in human atrophic gastritis and that are presumably gastrin driven, are small (<1 cm) and slow growing—if they grow at all (Harvey *et al*., 1985).

7 FORESTOMACH TUMOURS IN THE RAT

Spontaneous tumours of the upper gastrointestinal tract are rare in rats, but a large number of compounds of diverse chemical structure will produce hyperplasia in the forestomach with papillary change and the subsequent development of squamous carcinoma. This sequence has been well documented for fatty acids by von Griem (1986), in which class of compounds, the smaller the

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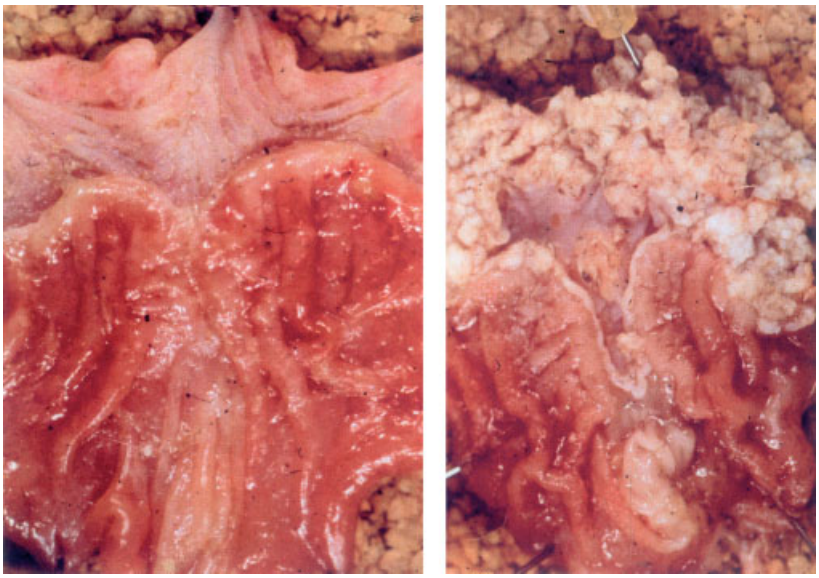

Figure 5 The rat stomach mucosal aspect. (A) In a young untreated rat, showing a clear demarcation between the lower (reddish) mucosal epithelium and the upper (paler) keratinized epithelium of the forestomach. (B) Following two weeks of exposure to 4.8% butyric acid, hyperplasia has caused a diffuse papillomatous thickening of the forestomach.

number of carbon atoms, the greater is the effect: propionic acid (C3) producing hyperplasia, papilloma and carcinoma; butyric acid and valeric acid (C4 and C5), hyperplasia and papillomas only (**Figure 5**) and lauric acid (C12), no significant change. The shorter-chain acids which stimulate epithelial proliferation may be carcinogenic after an appropriate duration of administration; this was not always adequately explored. The antioxidant, butylated hydroxyanisole (BHA), which is widely used as a food additive, produces this sequence in rats, mice and hamsters. Cell proliferation is greatest in the squamous epithelium immediately adjacent to the glandular mucosa (the limiting ridge) and this is also reported to be the site at which most tumours develop.

Although some of the compounds producing this effect produce an initial epithelial necrosis in the forestomach (Lauriault *et al*., 1990), it has been suggested that this change may not be necessary for the later development of hyperplasia (Toledo, 1965). The oesophagus is not usually affected, but cell kinetic studies have shown increased cell proliferation in some species. To illustrate the difference between the effects of genotoxic and nongenotoxic compounds in the same system, aristolochic acid, a phenanthrenecarboxylic acid obtained from the medicinal plant *Aristolochia clematis* L which is genotoxic, produces the sequence of hyperplasia, papillomatosis and squamous carcinoma in the rat (Mengs, 1983) and the mouse (Mengs, 1988), but with this compound adenocarcinoma of the forestomach also develops, together with tumours in other tissues and organs. It is possible in the mouse to achieve 100% tumour incidence. A comparison of the effects of this type of compound in animals with and without a forestomach has been made: the changes observed with BHA in rodents are not seen in dogs (Tobe *et al*., 1986) or monkeys (Iversone *et al*., 1986).

8 LIVER TUMOURS IN THE RAT AND MOUSE

More nongenotoxic mechanisms have been invoked for hepatocarcinogenesis than for most other tissue targets; these include sustained stimulation of cell proliferation, toxin-induced selection for a growth-dysregulated phenotype, down-regulation of gap-junctional intercellular communication, inhibition of apoptosis and altered methylation patterns for genes controlling growth and differentiation.

The significance of a stimulus to cell growth as a major factor in epigenetic carcinogenesis is clearly illustrated by the lesions found in the rodent liver as a result of the adaptive growth induced by xenobiotic compounds. The compounds vary widely in type, phenobarbital, steroid hormones, such as norethisterone, organochlorine pesticides such as dichlorodiphenyltrichloroethane (DDT), hexachlorocyclohexane (HCH) and also Ponceau MX being examples. In general, in the early stages of treatment all these agents produce liver enlargement by a combination of hypertrophy and hyperplasia of cells, with increases in total DNA content, parenchymal DNA synthesis and mitotic activity. In contrast, the antihistamine methapyrilene does not cause marked liver

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hypertrophy, but provokes a significant and sustained increase in cell proliferation, a regenerative response to chronic cytotoxicity (Powell *et al*., 1994). For many chemicals there has frequently been no evidence of toxic injury to liver cells on conventional morphological examination, and Schulte-Hermann (1979) has suggested that liver tumour formation may be the result of promotion of tumours from pre-existent tumorigenic lesions, as a result of prolonged and excessive stimulation of growth. Newer, more sensitive techniques, such as cumulative cell labelling via implanted miniosmotic pumps, frequently reveal increased hepatocyte turnover in situations where this was not previously detectable.

The apparent similarity of growth response conceals differences in cellular response. A diversity of xenobiotic-metabolizing enzymes can be induced, both microsomal and cytosolic, but no particular pattern of enzymes or magnitude of induction has been related to the neoplastic process. The only safe generalization is that during tumour progression neoplastic hepatocytes tend to acquire a more fetal phenotype, but this is not a distinguishing feature of nongenotoxic carcinogens. Early phenotypic changes are themselves of major significance if they alter the metabolic handling of other xenobiotics. Enzyme induction in the liver is nonuniform and this phenotypic diversity can allow selection pressure to favour the emergence of clusters of hepatocytes (foci) which are less metabolically disadvantaged from continuous exposure to a high level of a xenobiotic. This is consistent with the observation that most, but not all, foci have high levels of protective or defensive metabolizing enzymes such as GST-P or GGT (γ-glutamyl transferase) (**Figure 6A**). At least initially, resistance to toxicity probably confers only a modest or slight degree of growth advantage and is largely reversible. The suggestion that the cells of the altered foci represent an 'initiated' population (Schulte-Hermann, 1987) is speculative and not entirely consistent with the relative abundance of foci in the rat liver compared with most strains of mouse, despite which most mouse strains develop a higher spontaneous incidence of hepatic neoplasia. It may be more appropriate to consider altered foci as cells with a reversible phenotypic change, favouring subsequent selection for genetic changes.

Inhibition of apoptosis has been proposed as a mechanism of nongenotoxic carcinogenesis on the basis that cells with damaged DNA would be prevented from dying and replication could then 'fix' spontaneous mutation or DNA damage, allowing defective cells to accumulate. The observation that apoptosis increases when hypertrophy-inducing hepatocarcinogens (such as phenobarbitone (phenobarbital), nafenopin or butylated hydroxytoluene) are withdrawn, and is suppressed when the chemical is readministered, has prompted the conclusion that these chemicals suppress apoptosis *per se* (Roberts *et al*., 1995; Schulte-Hermann *et al*., 1995).

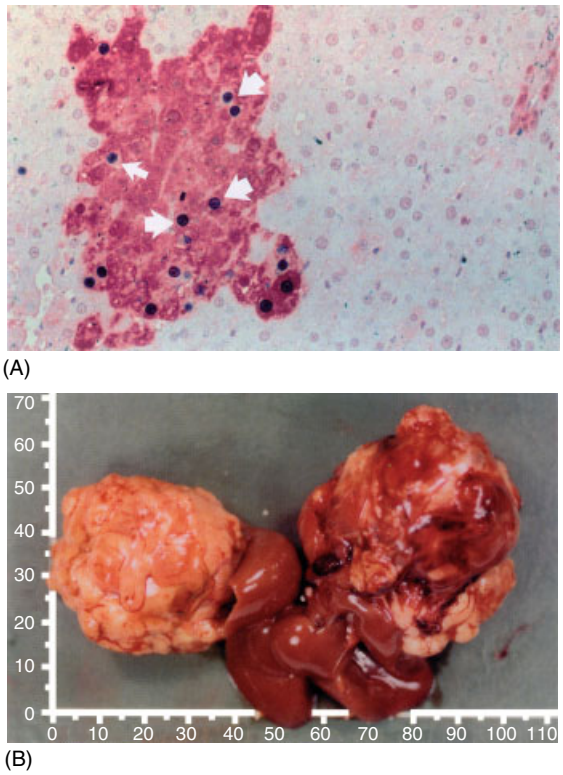

Figure 6 Methapyrilene-induced hepatocarcinogenesis. (A) Preneoplastic focus of altered hepatocytes, after six months of exposure to 400 ppm, identified by immunostaining for placental form of GST (also stains bile ductules, top right). Simultaneous staining for bromodeoxyuridine incorporation shows a higher level of cell proliferation in the focus (arrowed dark nuclei) than the surrounding liver. (B) Two large hepatic tumours induced by a further 12 months of exposure (18 months total) to the same dose of methapyrilene.

This conclusion is flawed by the fact that: (i) involution of liver hypertrophy, consequent to a decreased metabolic demand, cannot be distinguished from the removal of a primary suppression of cell death and (ii) inhibition of apoptosis is not the major cause of liver hypertrophy when these chemicals are first administered. Withdrawal of chemicals which selectively favour growth of preneoplastic foci (i.e. phenobarbital) understandably increase apoptosis in the foci, because the differential growth advantage has been removed. This does not, however, prove that the growth advantage arose from suppression of apoptosis, it merely reflects that growth advantage, from whatever mechanism, has been removed. More convincing support for this hypothesis would result if it could be demonstrated that apoptosis increased on withdrawal of a hepatocarcinogen which did not rapidly induce microsomal enzymes and liver hypertrophy or if lower levels of apoptosis could be convincingly shown during continuous administration of

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nongenotoxic carcinogens. At variance with the suppression of apoptosis hypothesis is the observation that preneoplastic lesions or adenomas induced by nongenotoxic carcinogens often have conspicuously increased levels of apoptosis. The study of Bursch *et al*. (2005) confirmed that apoptosis was not a major cause of strain difference in tumour susceptibility in C3 h/He, B6 C3F1 and C57BL/6 J mice; it is of more significance in the rat.

Examination of the activities of cells from later stages on the neoplastic process, that is liver nodules (adenoma), or carcinomas (**Figure 6B**) has shown them to be altered in metabolic capacity, expression of or responsiveness to growth factors and/or growth inhibitors, levels of cell proliferation or cell death, ploidy or the expression of cellular oncogenes, such as *myc, Jos, raf* and many others. As many malignant neoplasms have gross chromosomal rearrangement or partial deletion and duplication, it is clear that some change during the process of carcinogenesis enhances the likelihood of subsequent genetic change, with the development of a mutator phenotype or genetic instability.

9 SEX HORMONES AND ANTAGONISTS

This group contains an enormous diversity of chemical structures, some of which are potent carcinogens in animals, and includes a number with proven, although low-potency, carcinogenicity in humans, including the oestrogenic hormones used as oral contraceptives and antioestrogenic compounds used prophylactically and therapeutically for hormone-responsive tumours (Grisham, 1996). Many compounds, for example ethinyloestradiol, cyproterone acetate and tamoxifen, reproducibly induce liver tumours in rodents (Greaves *et al*., 1993; Tucker *et al*., 1996), and had for a long time been considered nongenotoxic until more recent studies refuted this. For instance, cyproterone acetate induces unscheduled DNA synthesis in hepatocytes (Demi *et al*., 1993), DNA adducts by ^{32}P postlabelling (Kerdar *et al*., 1995) and sister chromatid exchanges, while tamoxifen produces DNA adducts when studied by ^{32}P postlabelling methods (Carthew *et al*., 1995). Numerous animal studies have shown that oestrogen administration can increase tumour yield if given after a single dose of a genotoxic carcinogen (IARC, 1987). For reasons which are complex, the endocrine tissues of rodents appear to be peculiarly sensitive to chemical carcinogenesis.

In a review of the lesions occurring in the rat pituitary gland, Attia (1985) found that the majority of ageing rats displayed hyperplasia of prolactin-producing (PRL) cells with the morphological criteria of high secretory activity. Somatotrophin-producing (STH) cells showed evidence of similar functional hyperactivity and there was an increased relative proportion and apparent secretory activity of adrenocorticotrophin-producing (ACTH) cells. Around half of the animals in this study had pituitary adenomas which were assigned to cell types on morphological criteria alone; the tumours were not characterized immunocytochemically or electron microscopically. Studies which include the use of current pathological methodology show that many of these tumours are likely to be prolactinomas, probably induced as a result of the changes in oestrogen production which occur in ageing rats (Lu *et al*., 1979). Prolactin has a pronounced proliferative effect on rat mammary epithelial cells *in vivo* and *in vitro* (Nandi *et al*., 1984), and this change probably underlies the increased incidence of mammary tumours in ageing rats. Indeed, it has been known for many years that the administration of prolactin to normal rodents produces an increase in RNA synthesis in the mammary glands, followed by increased DNA synthesis and cellular proliferation (Simpson and Schmidt, 1971).

10 BLADDER CARCINOGENESIS IN THE RAT

In rats, a number of compounds act to produce bladder neoplasia, with a marked difference in incidence in the two sexes, the lesions being almost exclusively seen in males. The silicates which occur in many rat diets are precipitated in the urine under certain conditions and irritate the mucosa, a change apparently more common in males, owing to the large amounts of macromolecules excreted in their urine. Hence there is a background of proliferative change against which other factors may act; urothelial transitional cell hyperplasia occurs and may be defined as an area (focal or diffuse) at least three to four cells thick in an inflated bladder, whereas normal bladders have a cell thickness of one or two flattened cells.

10.1 2-Phenylphenol and its Sodium Salt

An example of a compound which operates in this way is 2-phenylphenol (*o*-phenyl phenol; OPP) and its sodium salt (SOPP). There is an additional point that two different mechanisms appear to act at different doses. Metabolic studies have shown that these compounds are converted into the sulfate and glucuronide conjugates at doses up to around 50 mg kg^{-1}; the conjugates are then excreted in the urine (Nakao *et al*., 1983; Reitz *et al*., 1983). At doses above 200 mg kg^{-1}, hydroxylation occurs, with conversion into 2,5-dihydroxybiphenyl, which is subsequently conjugated. Studies at a very high dose (600 mg kg^{-1} OPP; Reitz *et al*., 1983) showed that quinone compounds were formed via active intermediaries (semiquinones) in the rat. There are large sex

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differences in handling these compounds; in males eight times as much 2,5-dihydroxybiphenyl was formed as in females. The pathway via semiquinones has not been identified in dogs, cats or following dermal application in man, but the doses used may well not have revealed it (Savides and Oehme, 1980; Harke and Klein, 1981).

The tumours of the urinary tract which have occurred after exposure to OPP and SOPP have been observed predominantly in male rats. There appears to be a no-effect level of 250 mg kg^{-1} day^{-1}, but a clear dose-related increase in incidence is seen above this level (Hiraga and Fujii, 1981; 1984), which declines above 500 mg kg^{-1} day^{-1} for OPP and less markedly above 1000 mg kg^{-1} day^{-1} for SOPP. Data on the binding of metabolites to macromolecules in the urinary tract (Reitz *et al*., 1984) provide evidence for the suggestion that the 2,5-dihydroxybiphenyl cannot be the oncogenic agent, an argument supported by noting that considerable amounts of the 2,5-compound are formed below doses of 200 mg kg^{-1} and transformed into conjugates; none of these metabolites is therefore likely to be the carcinogen. However, injection of 0.05 or 0.1% 2-phenyl-l,4-benzoquinone resulted in evident hyperplasia of the bladder epithelium within five days. It has been suggested that saturation of the detoxification mechanism resulting in the production of free 2,5-dihydroxybiphenyl and its oxidation product, 2-phenyl-1,4-benzoquinone, allows the excretion of a bladder irritant in significant amounts at high doses in feeding studies. However, there is also evidence of the influence of other factors. Both OPP and SOPP are irritants, and this may be an adequate explanation of bladder mucosal hyperplasia; in addition, there is clearly an effect attributable to changes in pH.

In a study by Fukushima *et al*. (1986), male F344 rats were given OPP or SOPP at 2% in the feed for 24 weeks. Groups of treated animals also received ascorbic acid (AS), sodium ascorbate (SA), acid saccharin (SAC), sodium saccharin (SSAC), hippuric acid (HI) or sodium hippurate (SHI) at 5% in the feed. The urinary sodium concentration was increased in all animals receiving sodium salts in the diet and in SOPP-treated animals. The pH of the urine increased after SOPP, SA and SSAC and the urine osmolarity was decreased by SOPP, SA and SHI. OPP decreased the osmolarity, but did not affect urinary sodium concentrations. Histopathologically, the bladders showed epithelial thickening (4–8 cell epithelial thickness) at 8, 16 and 24 weeks after SOPP, and papillary and nodular changes at 16 and 24 weeks. Treatment with other sodium salts provoked some hyperplasia at 8 and 16 weeks, but no papillary or nodular changes; these changes had regressed by 24 weeks. The authors concluded that a combination of raised urinary pH and sodium acted as a promoting agent for SOPP; SHI raised urinary sodium, but not pH and was without effect.

Fujii *et al*. (1987) performed an essentially similar study on male F344 rats, using $NaHCO_3$ to raise the urinary pH and NH_4Cl to lower it. Both OPP and SOPP were examined (at 1.25 and 2% in the diet, respectively). Changes were seen in all groups and can be summarized as follows: hyperplasia of the bladder epithelium occurred with OPP, OPP and $NaHCO_3$, and SOPP. SOPP with NH_4Cl had no significant effect. Tumours were significantly increased after OPP (12/31), SOPP (22/31) and OPP plus $NaHCO_3$ (20/31). Only three tumours were seen in 31 rats after SOPP and NH_4Cl. The oncogenic effects of OPP were promoted in alkaline urine; those of SOPP were inhibited in acidic urine. The authors' view was that findings that suggested that SOPP was more carcinogenic than OPP related to the greater alkalinity of the former.

These studies have been considered in detail, since they shadow the well-known story of SSAC and bladder neoplasia (see review and comment: Cohen and Ellwein, 1990; IARC, 1999; Weihrauch and Diehl, 2004). This common route of development seen for a number of compounds with a clear dose-, sex- and species-specific effect typifies epigenetic carcinogenesis.

11 IMMUNOSUPPRESSORS

All immunosuppressant drugs, when tested adequately in animal carcinogenicity studies, are likely to increase tumour yield by suppression of normal immunosurveillance, a nongenotoxic mechanism. It is reported that certain immunosuppressors have not tested positive in the rat bioassay, but, when survival is limited by target organ toxicity, such tests can have poor sensitivity. The consensus, nonetheless, is that immunosuppressive drugs are carcinogenic and the International Agency for Research on Cancer has designated ciclosporin (cyclosporine) and azathioprine as human carcinogens (Class 1) (IARC, 1997; 1998). It is likely that all immunosuppressants will prove to be human and animal carcinogens, irrespective of whether they have *in vivo* genotoxic potential. Recent information also suggests that certain immunosuppressants may inhibit DNA damage-repair enzymes, an additional nongenotoxic mechanism.

12 FOREIGN BODY OR INJECTION-SITE CARCINOGENESIS

Extensive experimentation has shown that rats or mice, implanted subcutaneously with foreign bodies, such as plastic films (PVC, polythene, etc.), glass, stainless steel or gold, develop a vigorous fibrosing reaction around the implant and, ultimately, malignant connective tissue tumours. If the same materials are implanted in a powdered form, no tumours result, demonstrating that

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Table 3 Examples of nongenotoxic chemicals and their target tissues

Nongenotoxic carcinogen	Target tissue
Oestrogenic hormones	Liver/endometrium
Histamine H2-receptor antagonists and proton-pump inhibitors	Enterochromaffin-like (ECL) cells, stomach
Various chemicals which induce hepatic glucuronosyl transferase	Thyroid follicles
Peroxisome proliferators: phthalates, fibrates and so on	Liver hepatocytes
Immunosuppressants: cyclosporin and so on	Various tissues, particularly lymphoid
Various chemicals inhibiting the catabolism of α_{2-}microglobulin	Kidney
Butylated hydroxyanisole, C_3–C_5 fatty acids and a variety of other chemicals	Squamous epithelium of forestomach
Various chemicals causing liver hypertrophy and persistent compensatory hyperplasia	Liver hepatocytes
Various chemicals causing a persistent increase of cell turnover in the urothelium	Bladder
Subcutaneously implanted inert plastic films and foreign bodies	Connective tissue

carcinogenesis is not due to chemicals leaching from the implant, but to the physical shape of the implanted material (Moizhess and Vasiliev, 1989). This effect has also been termed 'solid-state carcinogenesis'. Larger, more angular implants produce a more vigorous fibrosing reaction and a larger tumour yield than do smaller more regularly shaped objects. Thin films of inert plastic, which induce neoplasia when implanted intact, do not do so when implanted after being repeatedly perforated. A substantial body of experimental evidence shows that foreign body carcinogenesis in rodents is related to the extent of inflammation and fibroplasia (local trauma) at the site of implantation and this is determined by physical features of the implanted object, including surface charge, angularity, surface texture, surface wettability and size (Brand *et al*., 1976). In this type of carcinogenesis, there is no exogenous chemical damage to or interaction with DNA, and so this is a nongenotoxic mechanism. Examples of nongenotoxic chemicals and their target tissues are given in **Table 3**.

A similar mechanism seems to operate when repeated subcutaneous or intraperitoneal injections of irritant materials, such as solutions of low pH or high tonicity, provoke a localized fibrosing reaction. These too can eventually result in malignant connective-tissue tumours (Grasso and Golberg, 1966). Administration of identical, nonirritant solutions (i.e. pH 7.0) has been reported not to cause tumour induction.

Although the subcutaneous tumours induced have been described using varying terminology, most would now be described as mesenchymal tumours, fibrosarcomas or even mixed fibrous histiocytomas. Variants with smooth muscle, osteoid, lipoid or myxoid differentiation illustrate the puripotent nature of mesenchymal cells. No one type of implant has consistently induced tumours of a particular type, and factors such as the strain of animal or diagnostic criteria account for some of the variation. Almost all tumours develop within or adjacent to the fibrous capsule which envelopes the foreign material, but subsequently, tumours invade locally and a proportion metastasize to the liver, lung or other tissue sites.

Humans have not been reported to develop the same persistent fibroplasia around implanted materials and few connective-tissue tumours have been observed at sites of prosthetic implants. Daily subcutaneous administration of peptide hormones in solutions at pH 4.0 or less to rats or mice have produced metastasizing tumours at the injection site, but in regular human use, the same drugs have not provoked injection-site reactions. Almost nothing is known about the reason for species differences in injection-site reactions to foreign bodies. However, the cat can develop mesenchymal tumours at the interscapular site of vaccination (Doddy *et al*., 1996) and, as in rodents, these tumours are preceded by focal and persistent fibroplasia. It is plausible that persistent fibroplasia is sustained by the local release of mitogenic growth factors, such as fibroblast growth factors, and that species differences in the regulation or activity of these peptides could account for the disparity.

12.1 Mesothelioma

On the micro scale, one of the best known and most widely studied examples of foreign body carcinogenesis is asbestos-induced pleural and peritoneal mesothelioma. This distinctive and highly lethal tumour can be induced in experimental animals and man. While asbestos is not mutagenic in bacterial tests, several forms of asbestos fibre give positive results when tested using *in vitro* or *in vivo* tests for genotoxicity (IARC, 1987). Experimental animals exposed to asbestos by inhalation, orally or by intraperitoneal injection develop an increased incidence of tumours at a variety of sites, including abdominal tumours, colonic polyps and mesenteric haemangiomas. The reader is referred to more specialist texts (IARC, 1988) for details of the mechanism of tumour induction

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and details of the pathogenesis. Asbestos fibres require particular aerodynamic characteristics to be respirable, but it is their resistance to intracellular biodegradation, which has been related to the localized generation of reactive oxygen species, that are believed to initiate DNA damage and cell transformation.

13 NEW SHORT-TERM TESTS FOR NONGENOTOXIC CARCINOGENS

The substantial cost and time required to perform a rodent bioassay, combined with the frequency with which the results are considered not to be relevant for human safety, has prompted many prototype alternatives. There is considerable enthusiasm, not to say political pressure in some circles, for a six month transgenic or knockout mouse study or a 12 month newborn mouse bioassay to be used in preference to one of the two currently used lifetime carcinogenicity bioassays. A substantial multinational programme to investigate the performance of several such mouse models, Tg.AC, TgHras2 (Yamamoto *et al*., 1997), activated oncogene, $XPA^{-/-}$ (De Vries *et al*., 1997), p53 heterozygote (Harvey *et al*., 1993), inactivated DNA repair or tumour suppressor, and newborn mouse high background of cell proliferation, is underway. Early indications are that certain multitarget, trans-species carcinogens, such as glycidol, are poorly detected. Another limitation is that in some animals the transgene or knockout is not stable (it reverts to normality), and failure to monitor this has compromised some of the recent multilaboratory testing.

On inadequate evidence, the Tg.AC mouse has been suggested, by some, to be a suitable model to detect nongenotoxic carcinogens, whereas the p53 heterozygous or newborn mouse was recommended for testing of mutagenic, nonclastogenic chemicals. In the Tg.AC mouse, four copies of an activated Harvey *ras* oncogene coupled to a zeta-globin promoter and an SV40 viral polyadenylation sequence are located in tandem on chromosome 11 of an FVB/N strain mouse (Hansen *et al*., 1995). The transgene is primarily expressed in skin, the target tissue, with dermal application as the normal route of administration. Only limited data have been published on the performance of the Tg.AC model, but ethyl acrylate, a nongenotoxic carcinogen targeting the squamous forestomach, failed to test positive, whereas two nongenotoxic noncarcinogens (resorcinol and rotenone) did. It has been argued that as the metabolizing capability of skin is limited there may be inadequate exposure to metabolites which are formed on systemic exposure.

Although fascinating experimental tools, the models currently being researched require considerably more fundamental investigation before they could be considered suitable for human-risk assessment. It is unrealistic to anticipate that a uniform protocol of six months duration and 10–20 animals per group will be optimal, or even suitable, for each transgenic or knockout model. The observation that the genetic background onto which a transgene is inserted or a p53 allele is deleted can significantly affect biological response is an obvious cause for concern. It is well understood that humans with Li–Fraumeni syndrome (p53 heterozygotes), or xeroderma pigmentosum, ($XPA^{-/-}$) have a greater probability of developing cancer, and whether results based on such mouse models are better predictors of general human risk than a conventional mouse bioassay is questionable.

14 CONCLUSIONS

As new tests for genotoxic potential are developed, or existing tests are refined and adapted to new target tissues (see **Mutagenesis**), a number of the chemicals currently considered to be nongenotoxic will require reassessment.

One criticism of the most common underlying mechanism in nongenotoxic carcinogenesis is that increased cell proliferation in normally differentiated cells is neither equivalent to nor synonymous with the dysregulated, autonomous proliferation that characterizes neoplasia. Whether exposure of persistently proliferating cells to high levels of a chemical or its metabolites predisposes them to a spontaneous genotoxic event is difficult to confirm. The key question is whether lower exposure levels have a linear relationship with effect, or whether a real biological threshold needs to be surpassed. Except for the case of thyroid tumours which result from administration of exogenous TSH, there are few experimental models in which to examine these relationships. It has been observed that 'increased cell proliferation is neither necessary nor sufficient for chemical carcinogenesis'. While the polemic on mitogenesis vs. mutagenesis continues, it is advisable to consider that there may be a less than distinct boundary between genotoxic and nongenotoxic carcinogenesis, even if this is a little unhelpful in the regulation of chemical safety.

What is the importance of this form of carcinogenesis? Epigenetic neoplasia has a great significance in that the resolution of the mechanisms involved in the instances described above, and in others not considered here, has provided data of considerable significance in our understanding of cell growth and proliferation. In practical terms, the stimulus to resolve some of the issues has come from regulatory toxicology; it is certain that the identification of a mechanism for an adverse effect greatly facilitates risk/benefit

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analyses and often permits much lower safety factors to be set—some neoplastic end points may simply not occur in man. Understanding of neoplasia in this work makes both a practical and a theoretical contribution.

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NOTES

Colin Berry worked on this update alone. Christopher Powell only wrote the original article.

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Structure–Activity Relationships for Carcinogenic Potential

Raghuraman Venkatapathy, Nina Ching Y. Wang, Todd M. Martin, Paul F. Harten and Douglas Young

CONTENTS

1 INTRODUCTION

The primary objective of testing chemicals for carcinogenicity is to identify the carcinogenic potential in animals, and to extrapolate the relevant risk to humans. The choice of data for use in assessing the potential for carcinogenicity includes (i) experimental human carcinogenicity data, (ii) information from an animal species with biological responses that are most similar to those of humans, and (iii) data from the most sensitive animal species/sex/strain combination when (i) and (ii) are not available. Due to a lack of experimental human carcinogenicity data for many chemicals and because carcinogenicity in mice and rats often predicts carcinogenicity in humans for most chemicals (Ashby and Paton, 1993), carcinogenicity experiments almost always involve using animals, and consist of short- and long-term studies on rats and/or mice. In a typical carcinogenicity assay, one or more groups of rodents of each sex (typically 50 animals of each sex of Fisher rats and/or B6C3F1 mice) is exposed to multiple doses of the test chemical (generally ranging from 0 mg kg body weight^{-1} day^{-1} to the maximum tolerated dose (MTD)) for two years (104 weeks). At the end of the exposure duration, animal tissues are examined histopathologically for the presence of lesions at any of 59 different organ sites. Based on the results of these examinations, the carcinogenicity of the chemical for each sex/species combination is generally classified as Clear Evidence, Some Evidence, No Evidence, Equivocal Evidence or Inadequate Study (NTP, 2009). In addition, the carcinogenic potency of the chemical for each sex/species is also calculated from the experimental dose–response data (see **Dose-Response Analysis in Experimental Toxicology and Risk Assessment**).

1.1 Measures of Carcinogenic Potency

The carcinogenic potency, or the dose at which chemicals cause carcinogenicity in a test animal, is generally measured using the tumorigenic dose rate (TD_{50}) or the oral slope factor (OSF). TD_{50} is defined as that chronic dose-rate in mg of chemical per kg body weight per day, which, at the end of their standard life span, would induce tumours in half the test animals with respect to the control animals (Peto *et al.*, 1984). Other equivalent measures of

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the tumorigenic dose rate include the tumorigenic concentration rate (TC_{50}; used to represent carcinogenic potency due to inhalational exposure) or TD_{05}/TC_{05} (chronic dose/concentration that induces tumours in 5% of an exposed population). The US Environmental Protection Agency (EPA) uses an OSF (inhalational risk unit in the case of inhalational exposure) as a measure of carcinogenicity (US EPA, 2005). Slope factor is defined as an upper bound, approximating a 95% confidence limit, on the increased cancer risk from a lifetime exposure to an agent. This estimate, usually expressed in units of proportion (of a population) affected per $mg\,kg^{-1}\,day^{-1}$, is generally reserved for use in the low-dose region of the dose–response relationship, that is, for exposures corresponding to risks less than 1 in 100 (US EPA, 2005).

Both TD_{50}s and slope factors are widely used as measures of carcinogenic potency, with slope factor generally being the more conservative of the two (NRC, 1993). In general, TD_{50}s do not provide target-organ-specific dose response; they can be determined either for a particular category of neoplastic lesion or for all tumours (Peto *et al*., 1984). Sometimes, the TD_{50} can also be defined even if some of the control animals get tumours. In this aspect, the TD_{50} can be considered to be similar to the dose–response characterization of an acute toxic effect, and, hence, is not reflective of cancers from true environmental exposures. On the other hand, OSFs provide information on cancer at a specific target organ resulting from prolonged exposure to a chemical contaminant. Therefore, TD_{50} is conservative from an acute exposure point of view, while slope factor is conservative from a chronic or lifetime exposure point of view.

1.2 Disadvantages of Animal Testing in Determining the Carcinogenic Potential of Chemicals

In the absence of human carcinogenicity data, long-term animal bioassays for carcinogenicity or short-term mutagenicity tests are regularly used to determine whether chemical agents are capable of inducing cancer in humans (NRC, 1983). In conducting the animal bioassays, it is generally necessary to expose a relatively small group of animals to doses that far exceed potential human exposure levels in order to detect the presence of tumours. Hence, the results of the carcinogenicity bioassays need to be extrapolated from the high dose observed in animals to a lower dose that is more relevant to human exposure. Besides high dose levels, three other important disadvantages of current bioassays are that (i) testing covers a substantial portion of the life span of the test species, (ii) the bioassays are extremely expensive, and (iii) there are ethical and social issues involved with animal testing (Lewis, 1992; Gombar, 1998). In addition, as hundreds of new chemicals are being synthesized every year, innovative approaches are needed to determine the carcinogenicity of new chemicals as well as the existing chemicals that currently lack carcinogenicity data. In the case of predicting carcinogenic potency, these disadvantages can be circumvented by either using mathematical equations that relate the carcinogenic potency of a chemical to other measures of toxicity such as the LD_{50} (lethal dose that produces mortality in 50% of an affected population) and the MTD (highest daily dose of a chemical that does not cause overt toxicity in a 90-day study in laboratory mice or rats), or quantitative structure–activity relationships (QSARs) that relate carcinogenic potency to physicochemical properties of the carcinogens. Similarly, in the case of predicting whether a given chemical is a carcinogen, either the mutagenicity of a chemical or structure–activity relationships (SARs) that relate the carcinogenicity of a chemical to its physicochemical properties and/or chemical structure (in the form of structural alerts (SAs)) may be used.

2 ALTERNATIVE MEASURES FOR PREDICTING CARCINOGENIC POTENCY

Several authors have reported high correlations between the TD_{50} and other measures of toxicity such as the MTD and LD_{50} for chemicals in the Carcinogenic Potency Database (CPDB) (Bernstein *et al*., 1985; Crouch *et al*., 1987; Gaylor and Chen, 1986; Parodi *et al*., 1982; Portier and Hoel, 1987; Rieth and Starr, 1989a; 1989b; Zeise *et al*., 1984; 1985; 1986). The correlation between carcinogenic potency and alternative measures of toxicity such as the LD_{50} and MTD has been shown to take the form

$$\log(1/\mathrm{TD}_{50}) = m\log(1/T) \qquad (1)$$

where T is an alternative measure of toxicity, and the slope, m, is approximately equal to 1.

The above-mentioned equation implies that the potency and the toxicity differ by a factor of 10^{m-1}, and is without a proportionality constant (intercept = 0).

Travis *et al*., (1990a; 1990b; 1991) published three papers pertaining to the correlation of carcinogenic potency with short-term toxicological testing. Their original publication (Travis *et al*., 1990a) showed that short-term batteries of tests (mutagenicity and acute toxicity, $r = 0.83$) are more reliable than test batteries comprised solely of mutagenicity tests ($r = 0.40$) in predicting carcinogenic potency. Subsequently, Travis *et al*. (1990b) combined all the toxicological data (mutation, toxicity, reproduction and subchronic and chronic tumourigenicity) and found a correlation coefficient of

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$r = 0.87$ for 156 mouse carcinogens. Travis *et al*. (1991) studied the prediction of carcinogenic potency of known mouse carcinogens using short-term toxicological data. The ultimate purpose of their work was to determine the classes of toxicological data that either singularly, or in combination, best predict the carcinogenic potency of known mouse carcinogens. The authors concluded that: (i) mutation data alone were poor predictors of carcinogenic potency, and (ii) batteries of different classes of short-term toxicological data were excellent predictors of the carcinogenic potency of known mouse carcinogens. The results of the mutation data alone are not surprising given the failure of the mutagenicity tests to separate known carcinogens from known noncarcinogens by a variety of investigators (Tennant *et al*., 1987; Ashby and Tennant, 1988; Ashby *et al*., 1989).

Goodman and Wilson (1992) developed a series of mathematical equations between the tumour dose (log ($1/TD_{50}$)) and the MTD (log (1/MTD)) for several structurally diverse mutagens and nonmutagens from two different data sources for rats and mice. The first data set, tabulated by Ashby and Tennant (1988), consisted of 222 structurally dissimilar chemicals from the National Cancer Institute/National Toxicological Program (NCI/NTP) database that were mutagenic to *Salmonella*. The second data set consisted of 245 chemicals that had tested positive for mutagenicity in various *Salmonella* strains, and for which quantitative information in terms of the mean number of revertant colonies per plate at each dose level was available in the literature (Haworth *et al*., 1983; Dunkel *et al*., 1985; Zeiger *et al*., 1988). The authors found that for the rat bioassays, the variance of the tumour dose was larger, and the correlation between the TD_{50} and the MTD was weaker for mutagens ($r = 0.74$) than nonmutagens ($r = 0.85$), thereby suggesting that the relationship between the two measures was generally stronger for nonmutagens than for mutagens. In addition, the authors reported no significant differences in the correlation for various statistically significant cut-off values for the tumour dose (probability, $p < 0.01$, <0.025, <0.05 and <0.1) for both mutagens and nonmutagens. Moreover, the authors did not find any significant differences when the level of dose (low, medium and high) as a measure of separating the chemicals in their database was considered ($r = \sim 0.85$ for all levels).

Krewski *et al*. (1993) demonstrated a good correlation ($r = 0.952$ for a multistage model, $N = 191$) between TD_{50} and MTD. Krewski *et al*. (1989) also demonstrated a similar correlation ($r = 0.941$ for a multistage model, $N = 263$) between the OSF, an alternative measure of carcinogenic potency, and MTD.

Part of the correlation between the two measures may be due to a bias that is inherent in a carcinogenicity bioassay. A higher dose is generally more likely than a lower dose to cause cancer in experimental animals. However, a few researchers have suggested that the carcinogenicity observed at the high dose may be due to the toxicity and not due to cancer (Ames *et al*., 1987; Fischer *et al*., 1988). For example, chemicals may be carcinogenic at high doses due to their cytotoxicity via the production of free radicals or increased cell proliferation (Ames, 1989; Fischer *et al*., 1988). For several nongenotoxic chemicals, evidence suggests that carcinogenicity occurs only when the dose is high enough to produce a significant amount of tissue damage at a target site (Goodman and Wilson, 1992). However, toxicity alone may not be a sufficient condition for carcinogenicity since some chemicals tested in rodent bioassays produce site-specific toxic effects following chronic administration at the MTD without showing any evidence of carcinogenicity at any site (Hoel *et al*., 1988). In addition, even if there is a statistical correlation between the carcinogenic potency and alternative measures of toxicity, investigators have concluded that those correlations, and possibly the potency, are in someway determined by the bioassay design or the mathematical and statistical methods used to estimate potency rather than by inherent biological properties of the chemicals (NRC, 1983).

As a case in point, Venkatapathy *et al*. (2009) correlated the TD_{50} of 467 chemicals obtained from the CPDB with alterative measures of toxicity such as the lowest observed adverse effect level (LOAEL), LD_{50} and MTD (due to gavage and feed/water). To preserve consistency, the authors evaluated the alternative measures using TOPKAT®, a commercial QSAR software for toxicity estimation. **Table 1** provides the summary statistics for the correlation between TD_{50} and the alternative measures for the CPDB chemicals. The authors noticed a distinct lack of correlation between carcinogenic potency (for both TD_{50} and OSF) and the alternative measures. The results of the analysis by the authors suggest that alternative measures of toxicity may not be used to estimate the carcinogenic potency of the chemicals in the CPDB. However, the authors noted that a combination of the three alternative measures along with other experimental or easily estimated variables such as mutagenicity and octanol–water partition coefficients may be used to develop classification and regression trees (CART), which may in turn be used to predict the carcinogenic potency of chemicals. Hence, in order to increase the predictive ability, QSAR models with OSF or TD_{50} as the dependent variables and physicochemical properties of the chemicals as independent variables may be necessary to predict the carcinogenic potency of chemicals. These QSAR models have the added advantage in that the carcinogenic potency predictions would depend on the structure of the chemical alone and not on experimental toxicity data of the alternative measures. Hence, the carcinogenic potency can be estimated even for chemicals that lack experimental toxicity data in the literature.

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Table 1 Summary statistics of the correlation between carcinogenic potency and alternative measures of toxicity

Alternative measure	Equation	Statistics
LD_{50}	$\log(1/TD_{50}) = 0.922\ (\pm 0.189)\ \log(1/LD_{50}) + 1.96\ (\pm 0.458)$	$N = 423$; $r^2 = 0.179$; adj-$r^2 = 0.177$; $F = 91.5$
MTD-gavage	$\log(1/TD_{50}) = 0.814\ (\pm 0.130)\ \log(1/\text{MTD-gavage}) + 1.41\ (\pm 0.446)$	$N = 372$; $r^2 = 0.290$; adj-$r^2 = 0.288$; $F = 150.9$
MTD-F/W	$\log(1/TD_{50}) = 0.838\ (\pm 0.130)\ \log(1/\text{MTD-F/W}) + 1.06\ (\pm 0.481)$	$N = 369$; $r^2 = 0.306$; adj-$r^2 = 0.304$; $F = 161.8$
LOAEL	$\log(1/TD_{50}) = 0.783\ (\pm 0.147)\ \log(1/\text{LOAEL}) + 1.16\ (\pm 0.558)$	$N = 363$; $r^2 = 0.232$; adj-$r^2 = 0.230$; $F = 109.4$

F/W—feed/water.

3 STRUCTURE–ACTIVITY RELATIONSHIPS FOR CARCINOGENICITY

Predictive toxicological approaches provide the means to estimate the carcinogenic and noncarcinogenic toxicity of a wide variety of chemicals in the absence of experimental toxicity data. Such approaches include (Doull *et al*., 2007): (i) qualitative SAR methods; (ii) QSAR methods; (iii) expert systems; (iv) biologically-based models such as 2D or 3D receptor modelling, comparative molecular field analysis (CoMFA), hologram QSAR (HQSAR), binding, and ligand SAR; and (v) integrative models that incorporate or combine both chemical and biological information. In general, these toxicological approaches describe correlations between various physicochemical properties of a chemical (usually referred to as descriptors of molecular structures of chemicals) and their observed or predicted biological activities. Such relationships assume a common mechanism behind the biological activity of a structurally/functionally related set of chemicals (Basak *et al*., 1995). Hence, differences in the chemical structures that induce the same biological effect, such as carcinogenicity, and their associated descriptors can then be mapped to changes in activity through mathematical equations. The resulting equation can then be used to calculate the carcinogenicity of new chemicals.

3.1 Types of (Q)SAR Models

SARs for predicting the carcinogenicity of chemicals are either qualitative in nature or expert-system based. In the former case, there is a qualitative relationship between the observed biological activity (such as whether a test chemical is a carcinogen or not) and a chemical and/or its substructure (such as its 1- and 2-atom fragments). A substructure associated with the presence of the biological activity may also be referred to as a structural alert. SARs are generally able to qualitatively estimate the toxicity of a given chemical as positive, negative or indeterminate. In the case of expert systems, experts in various toxicological end points review the experimental data on a case-by-case basis and make judgements regarding the toxicity potential for any given chemical. Expert systems may be classified as knowledge based (rules are based on human expert knowledge), induction rule based (based on artificial intelligence, neural networks, machine learning or data mining to automatically derive the rules) or hybrid (rules are initially based on human expert knowledge; machine can 'gain experience' or learn new rules). For example, Ashby (1985) introduced a set of 19 SAs for predicting potential carcinogens and noncarcinogens. These include: alkyl esters of either phosphonic or sulfonic acids; aromatic nitro groups; aromatic azo groups; aromatic rings *N*-oxides; aromatic mono- and di-alkylamino groups; alkyl hydrazines; alkyl aldehydes; *N*-methylol derivatives; monoalkenes; N and S β-haloethyl; *N*-chloroamines; propiolactones and propiosultones; aromatic and aliphatic aziridinyl derivatives; aromatic and aliphatic substituted primary alkyl halides; derivatives of urethane (carbamates); alkyl *N*-nitrosoamines; aromatic amines (including their *N*-hydroxy derivatives and the derived esters); aliphatic and aromatic epoxides; and polycyclic aromatic hydrocarbons.

A QSAR is a mathematical model between a quantifiable biological activity (such as carcinogenic potency) and one or more physicochemical properties of the chemical (also referred to as molecular descriptors) using various statistical methods such as regression analysis, principal component analysis (PCA) or factor analysis. In general, such relationships may be classified as either mechanistic or correlative. Correlative (or statistical) models try to find associations between molecular descriptors (physicochemical properties), toxicological data and a wide variety of chemical structures by statistical means, which are then used to predict the toxicity of a test chemical. Mechanistic models, on the other hand, are developed using human expertise on known mechanisms of action or are limited to a congeneric series of chemicals with the assumption that congeneric chemicals have similar mechanism(s) or mode(s) of action.

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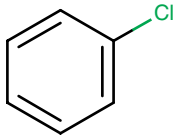
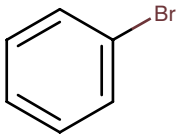
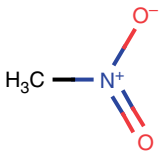
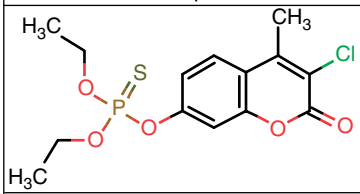
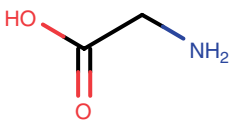
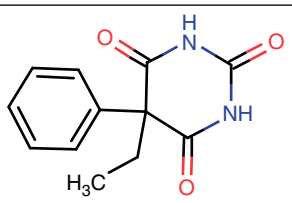
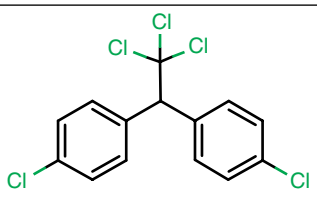
Other approaches to predicting the toxicities of chemicals include read-across and chemical-analogues. Read-across is a nonformalized approach in which end-point information for one or more source chemicals is used to make a toxicity prediction for another chemical based on some form of similarity (Worth *et al.*, 2007). Read-across can either be qualitative or quantitative, depending on the whether the data being used to make the prediction are qualitative or quantitative in nature. To estimate the properties of a given substance, read-across can be performed in a one-to-one manner (one analogue is used to make a single prediction) or in a many-to-one manner (two or more analogues are used to make a single prediction).

In rare cases, when existing toxicity data are inadequate for determining a toxicity value for a chemical of interest, a chemical analogue for the test chemical is identified using either known mechanisms of toxicity or using a SAR/QSAR-based approach. The choice of the appropriate analogue is based on similarity between the test chemical and the analogue. The toxicity value of the analogue may then be used as the toxicity value for the test chemical. The similarity of chemicals used in the analogue selection process may be either structural or functional. Structural similarity between chemicals, as the name implies, refers to chemicals that have a similar chemical structure or backbone. Functional similarity, on the other hand, refers to chemicals that have similar functionality or activity. In such a case, the two chemicals may have either similar physicochemical properties, properties that follow the same trend, or they may cause similar health effects and/or have the same mechanism of action. **Table 2** shows examples of the two kinds of similarity.

3.1.1 Advantages/Disadvantages of the Different Types of Models

As with statistical models, there are both advantages and disadvantages with mechanistic and expert-system-based models. The correlative models use unbiased assessment of the data to generate relationships and make toxicity predictions; however, these models provide no insight on the mechanistic nature of chemicals and are capable of generating relationships that have little biological plausibility. In addition, the predictive capability of the model is dependent upon the quality of the data used in model development. For these reasons, correlative models have to be carefully validated prior to use. The advantage of a mechanistic model is that it is based on an understanding of mechanism of action to determine the activity of a given chemical, which is generally done by using human input. However, such systems are restricted to human knowledge and are not capable of providing insight into new relationships or discovering new insights

Table 2 Structural similarity vs. functional similarity

Structurally similar chemicals	Chlorobenzene	Bromobenzene
		
	Chloromethane	Nitromethane
		
Functionally similar chemicals	Coumaphos	Glycine
		
	Phenobarbital	DDT
		

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and/or novel associations between structure and toxicity. As a result, such models are biased towards current ideas on mechanism of action. The most serious drawback of these systems is that the rules, sometimes in thousands, relate only to 'positivity', and assume that all chemicals that are not governed by any of the rules are 'negative'. This generally leads to a large number of false positive predictions.

3.2 Commercial/Noncommercial Software for Predicting Carcinogenicity

Various commercial and noncommercial (Q)SAR software programs purport to predict the carcinogenicity of noncongeneric series of chemicals (Dearden *et al*., 1997; Lewis *et al*., 1992; 1993; Richard, 1994; 1998; Wang and Milne, 1993). These programs may be broadly classified as expert or knowledge based, or statistically based. Examples of knowledge-based systems include Deductive Estimation of Risk from Existing Knowledge (DEREK, LHASA Ltd., Leeds, UK); HazardExpert (Compudrug Ltd., Budapest, Hungary); OncoLogic (Logichem Inc., Boyertown, PA, USA); and Computer Optimised Molecular Parametric Analysis of Chemical Toxicity (COMPACT, University of Surrey, Guildford, UK). Examples of statistically based systems include Automatic Data Analysis using Pattern Recognition Techniques (ADAPT, Pennsylvania State University, Philadelphia, PA, USA); Discovery Studio TOxicity Prediction by Komputer Assisted Technology (DS TOPKAT, Accelrys Software, Inc., San Diego, CA); Computer-Automated Structure Evaluation (CASE) and Multiple Computer-Automated Structure Evaluation (MultiCASE) (MultiCase Inc., Cleveland, OH, USA); (Q)SAR Application Toolbox (Organisation for Economic Co-operation and Development (OECD)); Laboratory of Mathematical Chemistry (LMC), Bourgas, Bulgaria); Quantitative Structure-Activity Relationships Expert System (QSAR-ES, MultiCase Inc.; US Food and Drug Administration (FDA)); Lazy Structural Activity Relationships (lazar, in silico toxicology, Freiburg, Germany); Prediction Activity Spectra for Substances (PASS, Poroikov and Filimonov, 2005); and COmmon REactivity PAttern (COREPA, Bourgas Prof. Assen Zlatarov University, Bourgas, Bulgaria). Patlewicz *et al*. (2003) and Benigni (2005) provide recent reviews on the ability of the QSAR models referred above to predict the mutagenicity and carcinogenicity of chemicals.

The software programs mentioned above predict the carcinogenicity of chemicals based on their structure alone, and have been used and validated by regulatory agencies including the Danish EPA, US EPA, US FDA, Health Canada, European Chemicals Bureau and the UK Health and Safety Executive (HSE), academia and industry alike because of their ease of use and rapid application. Commonly used software programs to predict the carcinogenicity of chemicals are described in more detail below.

3.2.1 Computer-Automated Structure Evaluation (CASE)/MultiComputer Automated Structure Evaluation (MCASE/MultiCASE)

The CASE and MultiCASE methodology, and the resulting commercial software, including MCASE, MCWeb, MC_NET, MC4PC, CaseTox, ToxLite and METAPC were developed by Klopman *et al*. (Klopman, 1984; 1992; Klopman and Rosenkranz, 1991; MultiCASE Inc., *www.multicase.com/*). These software programs can automatically identify molecular substructures and/or fragments that have a high probability of being responsible for an observed biological activity such as carcinogenicity for a set of tested chemicals, provided the training set that was used to develop the model contains both active and inactive chemicals (i.e. chemicals that are both carcinogens and noncarcinogens in the case of carcinogenicity). New, untested molecules can then be submitted to the program either through a structure-drawing program or as an MDL Molfile (an MDL Molfile is a file format formerly created by MDL, Inc., and currently owned by Symyx Technologies, Inc. (*www.symyx.com*), for holding information about the atoms, bonds, connectivity and coordinates of a molecule) (Dalby *et al*., 1992), and an expert prediction of the potential activity of the new molecule can be obtained. CASE- and MultiCASE-based products provide the user with the capability of adding experimental data.

A modified version of the CASE and MCASE program called MultiComputer-Automated Structure Evaluation-Expert System (MCASE-ES) was developed through a cooperative agreement between the FDA's Centre for Drug Evaluation and Research, and MultiCASE (Matthews and Contrera, 1998). MCASE-ES was built using a larger training set of substances to identify all possible functional groups that may be responsible for the biological activity of the training set, and has a broader range of applicability. In addition, MCASE-ES contains an expanded training set of drug substances, and was specifically designed to filter out single-sex or single-species carcinogens, and thus has the ability to focus on trans-species and transgender carcinogens. Substances that are organometallic or with a molecular weight >5000 Da cannot be evaluated by MultiCASE products, thus limiting its usefulness with regard to polymers and catalysts (Contrera *et al*., 2007).

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Table 3 MultiCASE modules for carcinogenicity

Module ID	Description	No. of compounds
A06	All data from miscellaneous carcinogenicity databases	208
A0E	CPDB mouse carcinogenicity	633
A0D	CPDB rat carcinogenicity	742
A0C	CPDB rodent carcinogenicity	430
A0K	Inducing glutathione S-transferase positive preneoplastic lesions in rat livers	100
A0L	Mouse two-stage skin carcinogenicity	206
A01	*N*-Nitroso compounds—5% daily intake in rats	64
A0S	Cell transformation—Balb/C 3T3	183
A0T	TCDD + PAH binding to aryl hydrocarbon hydroxylase receptor	148
A0H	NTP female mouse carcinogenicity	286
A0F	NTP female rat carcinogenicity	287
A0I	NTP male mouse carcinogenicity	286
A0G	NTP male rat carcinogenicity	314
A08	NTP mouse carcinogenicity	319
A09	NTP rat carcinogenicity	316
A07	NTP rodent carcinogenicity	313
A6C	Inhibition of metabolic cooperation for cultured cells	251
A0J	Human carcinogenicity IARC	628
AX1	Hepatocarcinogenicity male rat	474
AX2	Hepatocarcinogenicity female rat	441
AX3	Hepatocarcinogenicity male mouse	434
AX4	Hepatocarcinogenicity female mouse	429
AF1	FDA rodent carcinogenicity male rat (nonproprietary)	1179
AF2	FDA rodent carcinogenicity female rat (nonproprietary)	1170
AF3	FDA rodent carcinogenicity male mouse (nonproprietary)	1069
AF4	FDA rodent carcinogenicity female mouse (nonproprietary)	1076
AFU	FDA male/female rat and mouse (nonproprietary)	1374
AFV	FDA male/female rat (nonproprietary)	1229
AFW	FDA male/female mouse (nonproprietary)	1090
AG1	FDA rodent carcinogenicity male rat (proprietary)	1346
AG2	FDA rodent carcinogenicity female rat (proprietary)	1338
AG3	FDA rodent carcinogenicity male mouse (proprietary)	1224
AG4	FDA rodent carcinogenicity female mouse (proprietary)	1232
AGU	FDA male/female rat and mouse (proprietary)	1549
AGV	FDA male/female rat (proprietary)	1397
AGW	FDA male/female mouse (proprietary)	1246

TCDD—tetrachlorodibenzo-*p*-dioxin; PAH—polycyclic aromatic hydrocarbon.

MCASE and CaseTox contain more than 180 modules that cover various areas of toxicology and pharmacology, including acute toxicity in mammals, absorption, distribution, metabolism and excretion (ADME), various adverse effects, antibacterial and pharmacological, carcinogenicity, cytotoxicity, developmental toxicity and teratogenicity, ecotoxicity, biodegradation and bioaccumulation, enzyme inhibition, genetic toxicity, and skin, eye irritations and allergies. There are 36 modules in the carcinogenicity area including CPDB rat and mice carcinogenicity, NTP and FDA rodent carcinogenicity, hepatocarcinogenicity and human carcinogenicity. **Table 3** lists the names of the 36 modules under carcinogenicity, and the number of test chemicals that were used to build the modules.

The CASE and MultiCASE methodology use probability assessment to determine whether structural fragments are associated with carcinogenicity. To achieve this, both carcinogens and noncarcinogens are split into structural fragments up to a certain path length (2–10 atoms). Each of these fragments is associated with a confidence level and a probability of carcinogenicity or noncarcinogenicity that is derived from the distribution of these biophores (a fragment whose functionality is

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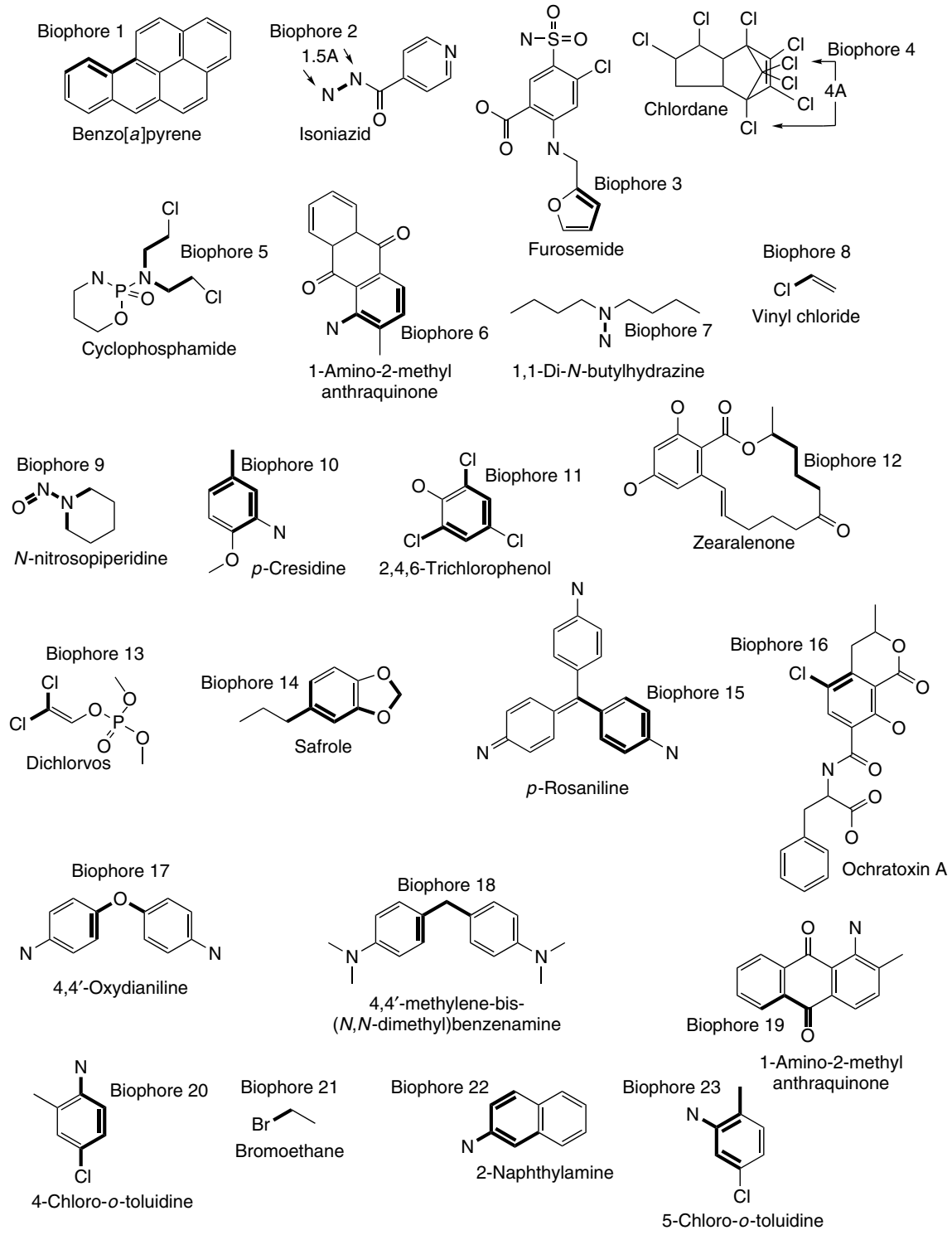

Figure 1 Biophores (bonds that are dark) that predict the carcinogenicity of chemicals in rats. (Reproduced with permission from Cunningham *et al*., 1998. © Elsevier.)

associated with the largest number of carcinogens and the smallest number of noncarcinogens in a training set that contains an equal number of carcinogens and noncarcinogens) and biophobes (the opposite of a biophore). Examples of biophores for predicting carcinogenicity in rats and mice are shown in **Figures 1** and **2**, respectively.

The fragments are then combined to give an equation of the following form (Klopman and Rosenkranz, 1994):

$$\text{CASE units} = a[\text{Fragment 1}] + b[\text{Fragment 2}] + \cdots + \text{Constant} \quad (2)$$

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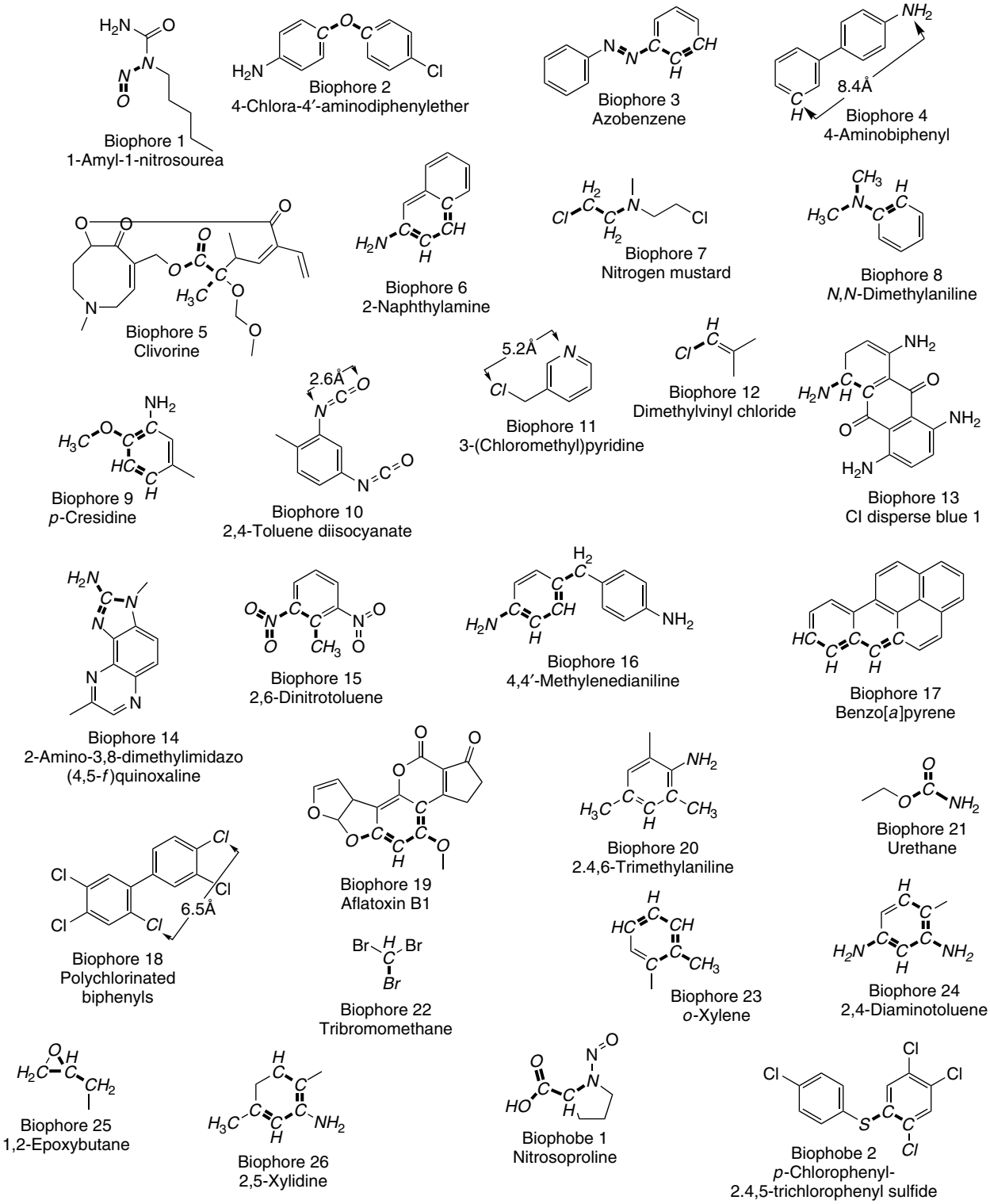

Figure 2 Biophores that predict the carcinogenicity of chemicals in mice. Each end point and species have their own biophores. (Reproduced with permission from Cunningham *et al*., 1998. © Elsevier.)

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Based upon this relationship, chemicals are designated as carcinogens, marginal or chemicals with weak carcinogenicity, or noncarcinogens.

Cunningham *et al*. (1998a; 1998b) studied the prediction of MultiCASE for carcinogenicity in rats and mice for chemicals in the CPDB. For mice, the authors observed a concordance rate (number of correct predictions divided by the number of total predictions) of 70%, a sensitivity (per cent correct positive predictions) of 63%, and a specificity (per cent correct negative predictions) of 78% using the overall SAR model. The corresponding values for rats were 64, 55 and 73%, respectively. The authors concluded that the poor predictivity of the rat SAR model when compared to the mouse SAR model may be indicative of a more variable response to chemical carcinogens for rats than for mice. The authors also suggest that MultiCASE SAR models for rat and mice were able to make relatively accurate predictions of carcinogenicity for chemicals of unknown activity.

3.2.2 Computer Optimized Molecular Parametric Analysis of Chemical Toxicity (COMPACT)

COMPACT is a methodology developed by Lewis *et al*. (1992). COMPACT can be used to predict the potential of a chemical to act as a substrate for one or more of the cytochromes P450 (P4501, P4502B, P4502E and P4504; Parke *et al*., 1990; Lewis, 1996). COMPACT can also be used to identify chemicals which have the potential to bind to receptors involved in the induction of cytochromes P450 and peroxisome proliferation (Dearden *et al*., 1997), and is based on the hypothesis that the structural characteristics of a molecule determine its ability to fit into the appropriate binding site on an enzyme (molecular shape such as area, depth, etc.) whereas its electronic characteristics (chemical reactivity) determine whether the resulting enzyme–substrate complex can be activated for oxidative metabolism. Thus, a combination of structural and electronic parameters is used to predict whether a particular molecule is likely to act as a P450 substrate. Lewis (1996) and Dearden *et al*. (1997) provide more information on the methodology behind COMPACT.

Lewis *et al*. (1990; 1993) evaluated the rodent carcinogenicity of chemicals tested by the NTP using COMPACT. The authors indicate that the methodology gave a concordance of 72% in a study containing 40 chemicals (Lewis *et al*., 1990) and a degree of correlation of 92% in a study containing 100 chemicals (Lewis *et al*., 1993). Brown *et al*. (1994) evaluated the rodent carcinogenicity of 80 NTP chemicals (56 carcinogens; 24 noncarcinogens) using COMPACT; the authors note that the program predicted both carcinogens (71%) and noncarcinogens (67%) with a similar level of accuracy, leading to an overall concordance of 70%.

3.2.3 Deductive Estimation of Risk from Existing Knowledge (DEREK) for Windows

DEREK is a knowledge- or rule-based expert system that was originally created by Schering Agrochemicals in the UK in 1986, and subsequently developed and marketed by LHASA Ltd. (School of Chemistry, University of Leeds, UK) and Harvard University (Boston, MA, USA) in collaboration with industry, academia and user groups. DEREK is able to predict toxicological end points such as skin sensitization, respiratory sensitization, irritancy, corrosivity, mutagenicity, carcinogenicity, teratogenicity, neurotoxicity, lachrymation, methaemoglobinaemia and anticholinesterase activity, and is based on an analysis of the chemical structure alone. Toxicological predictions for a given end point are generally based on the following criteria (Maslankiewicz *et al*., 2005): SAs, species, toxicity data, toxicity end point and physicochemical properties of chemicals.

To use the system interactively, molecular structures are entered into DEREK either via the built-in chemical editor program or by importing Molfiles or SDfiles (Dalby *et al*., 1992). The program compares structural features in the test chemical with the toxicophores described in its rule base, highlights toxicophores (or biophores), and provides a justification for each prediction in terms of the rules fired, the mechanistic or historical basis for the rule, and any supporting literature used in rule development. In addition, end-point-specific information is also included in the output. For example, in the case of carcinogenicity, additional information may be in the form of organ or species specificity, where sufficient supporting data exist. However, the system reports that no toxicophores have been identified in the structure in the case none of the rules were applied.

DEREK has several rule bases, which generally consist of descriptions of molecular substructures (SAs), which have been associated with toxic end points on the basis of existing knowledge (Dearden *et al*., 1997). An SA in DEREK consists of a toxicophore along with associated references, opinions, comments and examples. The rules are developed and monitored by LHASA Ltd. and its collaborators including industry, academia and user groups, and are based on sets of related chemicals rather than on specific chemicals. In addition, the rules may be based on generic 'use' categories of chemical such as dyes and solvents, or classes of organics such as nitroaromatics and organophosphates. A majority of the rules are derived from mechanistic organic chemistry. New DEREK rules are established using a detailed review of published sources of toxicological, mechanistic and chemical data. Reasoning rules have the following formula (Maslankiewicz *et al*., 2005):

$$\text{If } [\textit{Grounds}] \text{ is } [\textit{Threshold}] \text{ then } [\textit{Proposition}] \text{ is } [\textit{Force}] \quad (3)$$

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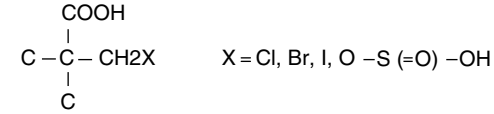
RULE 27:
RISK Mutagenicity, Carcinogenicity
TOXOPHORE: Alkylating agent
REMARKS:

This rule describes the mutagenicity and carcinogenicity of alkylating agents (alkyl halides, sulphinates and sulphates) according to the following toxicophores:

(I) C-Cl,Br,I (II) C-O-S=O but not C-O-S(=O)-OH

Where C is a primary or secondary alkyl carbon atom, which may only be neopentyl if it is a beta-halo carboxylic acid or its sulphinate, sulphonate or sulphate equivalent of



This is based on the observed mutagenicity, at pH 7.4 of 3-chloropivalic acid, which results from intramolecular cyclisation to a mutagenic beta-lactone.

References: (1) J Ashby, RD Callander and D Gilman, Mut. Res. 140:71 (1984);
(2) EL Tan, PA Brimer, RL Schenley and AW Hsie, J. Tox. Env. Health 11: 373 (1983).

Figure 3 DEREK reference screen for Rule 27 that is triggered for the mutagenicity and carcinogenicity prediction of haloacetic acids. (Reproduced with permission from Richard, 1998. © Elsevier.)

where *[Grounds]* is the evidence to be considered by the reasoning rule, *[Threshold]* is the level above which the grounds must be for the proposition to be assigned the force, *[Proposition]* is the outcome of the reasoning rule, and *[Force]* is the likelihood of the reasoning rule outcome.

For example, **Figure 3** shows a sample reference screen for a DEREK rule supporting the prediction of mutagenicity/carcinogenicity for monohalogenated acetic acid, along with a description of the structural requirements of the rule, mechanistic rationale and supporting references from the literature. The DEREK rule base for carcinogenicity contains more than 49 rules.

Marchant (1996) and the DEREK Collaborative Group (1996) evaluated the ability of DEREK to predict the carcinogenicity of 30 chemicals that were bioassayed as part of the second NTP comparative exercise. The authors report that DEREK had an accuracy of approximately 43%. After the exercise, additional rules were added into the DEREK carcinogenicity knowledge base.

3.2.4 HazardExpert

HazardExpert is a rule-based system developed by Compudrug (Budapest, Hungary) using known toxicophores or biophores collected from the literature or through *in vivo* experiments. The knowledge base in HazardExpert was developed based on the list of toxic fragments reported by more than 20 experts. HazardExpert predicts a range of toxicities including mutagenicity, carcinogenicity, teratogenicity, oncogenicity, irritation, sensitization, immunotoxicity and neurotoxicity. In addition to toxicity, HazardExpert also estimates toxicokinetic effects such as bioaccumulation and bioavailability that are based on predicted physicochemical properties (primarily the octanol–water partition coefficient, log P, and the acid-dissociation constant, pK_a). HazardExpert in combination with its sister product, MetabolExpert (a system for predicting metabolites), can be used to predict the toxicity of both the parent compound and its metabolites.

To use HazardExpert, a user selects a chemical from the HazardExpert database, defines the species, route of administration, dose level and duration of exposure. If the test chemical is missing in the database, the user must enter it into the database using the attached chemical editing interface or by adding the metabolites predicted by MetabolExpert before selecting the chemical. Upon execution, substructures that exert a positive or negative effect with respect to the chosen toxicity are identified based on known toxicophores stored in the toxic fragments knowledge base. HazardExpert provides the user with the means to add and/or modify rules and their related references through the knowledge maintenance module. Toxicity estimation results including the toxicity classification are displayed to the user as histograms.

Dearden *et al*. (1997) evaluated the performance of HazardExpert for its ability to predict human and animal carcinogenicity on 192 chemicals from the International Agency for Research on Cancer (IARC) monographs. The authors report that approximately 55% of the chemicals were predicted by HazardExpert within ±1 of the IARC classification when the 'high' exposure condition was chosen, compared to about 75% when the 'low' exposure condition was chosen. The authors also report the lack of important fragments in the toxic fragments knowledge base, thus leading to incorrect predictions for benzene, vinyl chloride, organophosphates, polyaromatic hydrocarbons, organometallics and isocyanates. In a second study, Brown *et al*. (1994) evaluated the rodent

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carcinogenicity of 80 NTP chemicals (56 carcinogens and 24 noncarcinogens) using HazardExpert. The authors report that HazardExpert was found to be good at identifying noncarcinogens (81% specificity), but was poor at identifying carcinogens (36% sensitivity), with an overall concordance of 51%.

3.2.5 OncoLogic

OncoLogic™ is a knowledge-based expert system developed by LogiChem Inc. (Boyertown, PA, USA) in cooperation with the US EPA's Structural Activity Team. The software predicts potential carcinogenicity of chemicals by applying rules of mechanism-based SAR analysis, functional analysis and expert judgement. The rules were developed based on data from a variety of sources including IARC Monographs, NCI/NTP bioassay reports and other publicly available data and information from EPA files (US EPA, 2008).

To make a carcinogenicity prediction, OncoLogic makes use of mechanism-based SAR analysis, which involves comparison with structurally related compounds with known carcinogenic activity, identification of structural moieties or fragments that may contribute to carcinogenic activity through a perceived or postulated mechanism, and evaluation of the modifying role of the remainder of the molecule to which the structural moiety/fragment is attached (OncoLogic, *www.epa.gov/oppt/newchems/tools/oncologic.htm*). Currently, OncoLogic has subsystems that can evaluate fibres, metals, polymers, and 48 classes of organic chemicals. Each subsystem has a fixed, hierarchical, decision-tree structure, consisting of rules of the 'if-then-else' type, and proceeds to a carcinogenicity evaluation based on the structural information entered, and on answers to program queries provided by the user. The organics subsystem is the largest of the four subsystems, and consists of over 40 000 rules derived from the examination of more than 10 000 organic chemicals. If the chemical of interest cannot be placed into one of the chemical classes listed, either the Miscellaneous Class or the Functional Analysis component has to be used to evaluate the chemical.

The carcinogenicity evaluation of a chemical begins when the user chooses one of the predefined chemical classes, and enters the test chemical by either selecting structural templates or by drawing in structures within the constraints of the chosen class. Upon execution, the program makes a prediction of the carcinogenic potential of the chemical (low, marginal, low–moderate, moderate, moderate–high or high) and produces a detailed report which conveys the mechanism-based expert reasoning underlying the evaluation, including the general considerations applicable to the class of chemicals, the rules specific to the test chemical, and rules that apply to the test chemical's reactivity and/or metabolites.

Mayer *et al*. (2008) evaluated the predictive ability of OncoLogic and MCASE for 301 chemicals tested under the NTP. The authors reported that MCASE and OncoLogic had similar concordance values (83 and 88%, respectively), and that OncoLogic had a lower percentage of false negatives. However, the authors also reported that MCASE had broader applicability as it was able to analyse more chemicals in general than OncoLogic. Benigni and Zito (2004) compared the predictive ability of 19 different SAR models for 30 new chemicals tested under the NTP. The authors found that OncoLogic had the best prediction accuracy of 65% among the 19 models.

3.2.6 (Q)SAR Application Toolbox

The OECD (Q)SAR Application Toolbox is open-source software that is currently being developed through a collaborative effort between the Danish EPA, European Chemicals Bureau (ECB), Environment Canada, Ministry of Economy, Trade and Industry of Japan (METI Japan), MultiCASE Inc., LMC, OECD, Syracuse Research Corporation (SRC) and the US EPA. The Toolbox provides several computational methods and information technologies for the application of (Q)SARs, or a category approach for filling data gaps that are necessary for hazard and risk assessment. The toolbox currently contains three tracks—(Q)SAR track, the category track and the flexible track (OECD, 2007).

The (Q)SAR track provides the user with approaches to fill the data gaps using (Q)SAR models. Within this track, the user is able to input target chemical(s), collect available information from databases, and select reliable (Q)SAR models to predict toxicities of chemicals for various health end points. To make a prediction for a particular end point, the user may choose a model from a list of available models and their performance statistics. The category and flexible tracks provide the user with the ability to fill the data gaps by making use of analogues. The Toolbox provides the user with the ability to form a group of chemicals similar to the target chemical using a set of rules based on chemical functionality and structural characteristics. Read-across, trend analysis and (Q)SARs predictions may also be used to assess the missing end points and their reliability for the target chemical. The main difference between the category and flexible tracks is that the user has the ability to jump across functionalities in the flexible track (OECD, 2007).

Currently, depending on the availability of data, the (Q)SAR Application Toolbox is able to make qualitative and quantitative predictions and/or develop (Q)SAR models for various health end points such as aquatic toxicity, carcinogenicity, mutagenicity, eye irritation, skin sensitization, acute toxicity, immunotoxicity, neurotoxicity, biodegradation and bioaccumulation.

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The Application Toolbox was created in two phases. A beta version of the Toolbox that emphasised a technological proof-of-concept was released in March 2008. The first phase of the project that developed a more comprehensive Toolbox that fully implemented the capabilities of the beta version while adding functionalities to the built-in databases, and an expansion of the QSAR and SA libraries was launched as (Q)SAR Application Toolbox version 1 in November 2008, which was later updated to version 1.1 in December 2008.

The OECD started the second phase of the project, upgrading the first version to a more comprehensive Toolbox, in November 2008. The long-term goal of the Phase 2 project is to ensure that the 'categories approach' to filling data gaps works uniformly for all discrete organic chemicals and for all regulatory endpoints. Areas of work in Phase 2 include information technology, chassis development and additional functionalities, database compilation, (Q)SAR library, and expert system compilation and training (OECD, 2009). The Phase 2 activities are scheduled over four years (2008–2012). The OECD anticipates that new versions of the Toolbox will be released periodically; version 2.0 is expected to be released in 2010, while version 3.0 is expected to be released in 2012 (OECD, 2009).

3.2.7 TOPKAT

Discovery Studio Toxicity Prediction by Komputer Assisted Technology (DS TOPKAT), originally developed by Health Designs, Inc. (Rochester, NY) and currently marketed by Accelrys Software Inc. (San Diego, CA), is a PC-based modular software for the prediction of a wide variety of health end points. The predictions made by TOPKAT can either be quantitative or qualitative in nature. The qualitative models in TOPKAT provide dichotomous output (yes/no) for NTP rodent carcinogenicity, FDA rodent carcinogenicity, weight of evidence rodent carcinogenicity, Ames mutagenicity, developmental toxicity potential, skin sensitization, skin irritancy, ocular irritation and aerobic biodegradability (Accelrys, 2001). The NTP and FDA rodent carcinogenicity modules are comprised of four models, one each for male rats, male mice, female rats and female mice. The quantitative models provide point estimates for LOAEL, LD_{50}, lethal concentration (LC_{50}), effective concentration (EC_{50}), MTD and octanol–water partition coefficient (V log P) along with 95% confidence limits for each (Accelrys, 2001). Each TOPKAT module consists of a specific database of carefully screened chemicals, and several chemical subclass-specific cross-validated QSAR models for predicting a specific toxicity end point. In order to assess the end-point specific toxicity for any given chemical structure, the software employs appropriate bulk, electronic and transport attributes that are presumed to be responsible for the biological activity of the molecule (Gombar, 1998; Purcell *et al*., 1973).

To make a carcinogenicity prediction, the user has to enter the chemical structure in SMILES (Simplified Molecular Input Line Entry System; Weininger, 1988) format in TOPKAT and select a model as the prediction module. The software first screens the chemical structure against the model substructural library to determine adequate coverage, and automatically chooses the appropriate chemical class-specific QSAR submodel to generate the toxicity prediction. A prediction is considered successful when the results of the substructure analysis satisfy the validation criteria for both the univariate and multivariate procedures that are built into the model, and the descriptor values are within the model domain (Gombar, 1998; Moudgal *et al*., 2003; Gombar and Enslein, 1996).

Prival (2001) used TOPKAT to predict the results of the rodent carcinogenicity bioassays conducted by the NTP (Research Triangle Park, NC, USA). The author found that the positive and negative predictivity and overall concordance figures ranged from 40 to 64%. In addition, Prival (2001) found that TOPKAT predicted a higher proportion of negatives (80%) than were actually found in the NTP studies (52%).

Cotterill *et al*. (2008) compared experimental toxicity of approximately 780 chemicals to those made by TOPKAT for various health end points. Of the 65 chemicals for which carcinogenicity data were available, TOPKAT was able to accurately classify 9 of 11 chemicals as carcinogenic (82%). Of the remaining 54 chemicals that were noncarcinogenic, TOPKAT accurately classified 40 chemicals (74%), while 2 were classified as indeterminate and the remaining 14 chemicals were false negatives.

3.3 SARs and QSARs for Predicting Carcinogenicity and Carcinogenic Potency

In general, QSARs for predicting carcinogenicity have been mostly limited to chemical congeners, that is, chemicals belonging to a certain class such as aromatic amines, in anticipation that these chemicals will have the same mechanism of action (Benigni and Giuliani, 1996; Cronin and Dearden, 1995; Debnath *et al*., 1994; Hansch, 1991; Leo *et al*., 1995; Passerini, 2003). However, a few non-class-specific QSARs for predicting the carcinogenicity of chemicals have also been developed over the past few years. A number of authors have developed QSARs to predict the mutagenicity of aromatic amines (Benigni *et al*., 2003; Chung *et al*., 1997; Colvin *et al*., 1998). Yuta and Jurs (1981) used the ADAPT software to discriminate between carcinogens and noncarcinogens among 157 aromatic amines using topologic and

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geometric descriptors. The authors found that the total number of rings was the most significant descriptor that differentiated between carcinogens and noncarcinogens.

Dunn and Wold (1981) used the SIMCA (soft independent modelling of class analogy) pattern recognition method to classify the carcinogenicity of 50 *N*-nitroso chemicals that were divided into three classes depending on whether they required metabolic activation (41 chemicals) or not (9 chemicals). The chemicals that required metabolic activation were divided into two classes depending on neutral or electron-donating substituents on the amine nitrogen (27 chemicals) or electron-withdrawing substituents (14 chemicals). The authors reported that the SIMCA procedure correctly classified 44 of the 50 (88%) chemicals.

Ashby and Tennant (1988) analysed 222 chemicals tested by the NTP to determine relationships between chemical structure and carcinogenicity, and mutagenicity. They discovered a 90% concordance between SAs based on DNA-reactivity and *Salmonella* mutagenicity. In a later study on 301 NTP chemicals, Ashby and Tennant (1991) classified each of the compounds as either structurally alerting or nonstructurally alerting. They found that most of the rodent carcinogens (64%) were structurally alerting and that 84% of the structurally alerting carcinogens analysed were mutagenic. However, the authors also observed a low concordance between DNA reactivity and carcinogenicity.

Purdy (1996) developed a hierarchical model consisting of QSARs mainly based on chemical reactivity to predict the rodent carcinogenicity of organic chemicals. The hierarchical model had a classification rate of 96% in a training set of 306 chemicals and 90% in a test set of 301 chemicals. Vračko (1997) studied the carcinogenic potency of 45 benzene analogues using artificial neural networks and three types of descriptors—geometrical structures of molecules described by the three-dimensional coordinates of atoms, geometrical structures in combination with atomic charges and energy of the frontier molecular orbitals. The author reported that while the model was able to describe the training set ($r > 0.9$), the predictive ability was quite poor.

Gini *et al*. (1999) developed an artificial neural network-based QSAR model for predicting the TD_{50} of nitrogen-containing aromatic chemicals. Thirty-four descriptors were calculated and principal components used to reduce the number of descriptors while retaining 85% of the variance in the descriptors. A four-neuron model using all chemicals yielded a cross-validated correlation of 0.69, which increased to 0.82 after removing 12 outliers.

Benigni *et al*. (2000) developed QSAR models for grading the carcinogenic potency of nonheterocyclic aromatic amines for rats and mice. Among the various descriptors in the QSAR model, the authors found that the carcinogenic potency was influenced by increasing hydrophobicity. However, the authors also found that the QSAR models for carcinogenic potency were inadequate in describing the difference between carcinogenic and noncarcinogenic amines. Hence, the authors developed four discriminant models based on electronic and steric parameters for male/female rats/mice to differentiate between carcinogenic and noncarcinogenic aromatic amines (Franke *et al*., 2001). The discriminant functions showed classification rates between 83.3 and 96% for the chemicals considered in their study.

Choo *et al*. (2001) compared the predictive ability of three different three-dimensional QSAR (3D-QSAR) methods (CoMFA, HQSAR and comparative molecular similarity indices analysis (CoMSIA)) for predicting the anticarcinogenic activity of 28 diarylsulfonylureas. The authors reported that all three 3D-QSAR methods had a good predictive ability in terms of external cross-validated correlation coefficient values between 0.63 and 0.74. The authors also indicated that while HQSAR and CoMSIA had slightly lower predictive ability than CoMFA, the two methods process data at a much faster rate than CoMFA as HQSAR does not require the generation of a 3D structure while CoMSIA does not require superposition of the chemical structures.

Contrera *et al*. (2003) used MDL-QSAR software (MDL, San Ramon, CA) to develop SARs to predict the rat carcinogenicity of 86 pharmaceuticals and 22 organic chemicals. The SAR model was developed using a test set of 1275 pharmaceuticals and organic chemicals. The authors report that the model had good coverage (93%), concordance (73%), sensitivity (74%) and specificity (72%) for the 108 chemicals in the validation set. In addition, the model was able to accurately predict 72% of noncarcinogenic compounds and compounds with carcinogenic findings.

Helguera *et al*. (2005) developed a discriminant model that was based on a topological substructural molecular design approach (TOPS-MODE) to predict the carcinogenic and noncarcinogenic activity of 189 chemicals. The authors found that their model classified approximately 76% of the chemicals accurately. The authors suggest that hydrophobicity increased the carcinogenic activity of chemicals while dipole moment had the opposite effect. Helguera *et al*. (2007) also developed a QSAR model using TOPS-MODE and other descriptors calculated using the Dragon software to predict the carcinogenic potency of 35 chemicals that contained a nitroso group. The authors indicate that models developed using TOPS-MODE descriptors was able to account for 83% of the experimental variance while other models developed using Dragon descriptors could account for less than 79% of the experimental variance. The authors suggest that QSARs developed using TOPS-MODE can help in forming a mechanistic basis for the prediction of carcinogenic potency through the identification of SAs.

Valerio *et al*. (2007) evaluated the ability of MDL-QSAR and MDL's rodent carcinogenicity module

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to predict the carcinogenic potential of 123 naturally occurring chemicals in the human diet. The authors indicate that MDL-QSAR was able to correctly predict 97% of the naturally occurring chemicals with experimental high-risk rodent carcinogenic potential and 53% with experimental low-risk rodent carcinogenic potential. A similar study of 108 pharmaceuticals by Contrera *et al*. (2003) indicated that MDL-QSAR had excellent coverage (93%), good sensitivity (72%) and specificity (72%) for rodent carcinogenicity.

Venkatapathy *et al*. (2007) developed QSARs to predict the rat carcinogenic potency of 448 chemicals from the CPDB (*http://potency.berkeley.edu/cpdb.html*) and the Integrated Risk Information System (IRIS) database (US EPA, 2008). Approximately 4000 descriptors were calculated using CAChe™, AMPAC™/CODESSA™ (Semichem Inc., Kansas City, MO), Dragon (Talete SRL, Milano, Italy) and Molecular Connectivity (Molconn-Z; eduSoft LC, Ashland, VA). Results of the analysis indicated that the harmonic mean of tumour doses in molar units gave the best correlations using fewer descriptors. The authors also found that QSARs based on sex-specific TD_{50}/OSF and mechanism-specific TD_{50}/OSF have slightly higher predictive abilities than nonspecific or global QSAR models. In addition, QSARs developed using a combination of two-dimensional and three-dimensional descriptors gave higher correlations than QSARs developed using only two-dimensional descriptors.

Martin *et al*. (2008a) used a hierarchical clustering (HC) approach to predict the carcinogenicity of several noncongeneric sets of chemicals from the CPDB (Gold, 2009). The HC approach (Martin *et al*., 2008b) used Ward's method (Romesburg, 1984) to divide an experimental toxicity training set into a series of structurally similar clusters (defined in terms of 2D and 3D descriptors) in the hope that the congeners within each cluster possessed the same mode of action. The authors used genetic algorithms to generate several statistically valid QSAR models for each cluster. The toxicity for a given query compound was estimated using the average of the predictions from the cluster models whose chemicals were the most structurally similar to the query compound. Martin *et al*. (2008a) reported that the HC approach achieved cross-validation concordances of 60–65%.

While commercial and noncommercial (Q)SAR models are able to successfully predict the carcinogenic potential and carcinogenic potency of a wide variety of chemicals, there may be a few chemicals (e.g. new chemicals and/or drugs) that may fall outside the prediction domain of currently available models. In such cases, (Q)SAR models may have to be developed to adequately cover the prediction domain of the new chemical.

3.4 Developing (Q)SAR Models to Predict the Carcinogenicity or Carcinogenic Potency of Chemicals

The general procedure for developing a (Q)SAR model for predicting the carcinogenicity or carcinogenic potency of a wide variety of chemicals is outlined in the steps below.

3.4.1 Choosing the Training Set

The values of the dependent variables for the chemicals, such as the carcinogenicity potential or tumour dose, are gathered from public databases. Since a good QSAR model is highly dependent on the quality of the data used in the model construction process, the data should be chosen from a well-documented and vetted source such as the databases from the NTP or the US EPA. In general, the carcinogenicity studies should have followed good laboratory practice (GLP), and have been conducted according to internationally agreed-upon guidelines such as those published by the US EPA (2005) or the OECD (1981). For example, the criteria used by IRIS (US EPA, 2008) to determine the choice of data sets to use in the quantification of carcinogenic potency include (i) the use of human data, (ii) use of animal studies in which the biological responses in animals resemble those in humans if (i) is not available, (iii) use the most sensitive animal species/strain/sex combination if (i) and (ii) are not available, (iv) use the same route of exposure as that of human exposure, (v) estimate overall risk using tumours at sites that have elevated responses, and (vi) combine benign tumours with malignant tumours, unless the benign tumours are not considered to have potential progress to the associated malignancies of the same histogenic origin (McConnell *et al*., 1986). In addition, robust (Q)SAR models are highly dependent on a high degree of similarity between the target chemical and the chemicals in the training set. Hence, in order for the (Q)SAR models to have a broad applicability, the training set should include an adequate number of each diverse set or class of chemicals. **Table 4** illustrates potential sources of experimental toxicity data that may be used to develop (Q)SARs for predicting the carcinogenicity/carcinogenic potency of chemicals.

3.4.2 Calculating Descriptors

Carcinogens may either be genotoxic or nongenotoxic (see **Carcinogenesis and Carcinogens that are also Genotoxic** and **Nongenotoxic or Epigenetic Carcinogenesis**), and have a propensity to be an electrophile, generate electrophilic intermediates (Miller and Miller, 1977) or involve reversible binding onto active sites in the receptors. Hence, descriptors that are commonly chosen for (Q)SAR modelling include

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Table 4 Sources of carcinogenicity data for building (Q)SAR models

Database name	Source	Type of data	Structures	Downloadable	Free	URL
CCRIS (Chemical Carcinogenesis Research Information System)	US National Library of Medicine	Qualitative	Names	No	Yes	*http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CCRIS*
ChemIDplus	US National Library of Medicine	Qualitative, quantitative	Names, structures	No	Yes	*http://chem.sis.nlm.nih.gov/chemidplus/*
CPDB (Carcinogenic Potency Database)	University of California, Berkeley	Qualitative, quantitative	Names, structures	Yes	Yes	*http://potency.berkeley.edu/cpdb.html*
DSSTox (Distributed Structure-Searchable Toxicity)	US Environmental Protection Agency	Qualitative, quantitative	Names, structures	Yes	Yes	*www.epa.gov/ncct/dsstox/index.html*
Genetic Toxicity, Reproductive and Development Toxicity, and Carcinogenicity Database	Center for Drug Evaluation and Research, US FDA	Qualitative	Names, SMILES	Yes	Yes	*www.fda.gov/Cder/Offices/OPS_IO/genrepcar.htm*
HSDB (Hazardous Substances Data Bank)	US National Library of Medicine	Qualitative, quantitative	Names	No	Yes	*http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB*
IARC (International Agency for Research on Cancer) Classifications Database	International Agency for Research on Cancer, Lyon	Qualitative	Names	No	Yes	*http://monographs.iarc.fr/ENG/Classification/index.php*
IRIS (Integrated Risk Information System)	US Environmental Protection Agency	Qualitative, quantitative	Names	No	Yes	*www.epa.gov/iris*
ISCAS (Informatics and Computational Safety Analysis Staff) Carcinogenicity Database	Center for Drug Evaluation and Research, US FDA	Qualitative, quantitative	Names, structures	Yes	No	*www.fda.gov/Cder/Offices/OPS_IO/icsas.htm*
ISSCAN (*Cancerogeni chimici: strutture e dati sperimentali*)	Istituto Superiore di Sanità, Rome	Qualitative, quantitative	Names, structures	Yes	Yes	*www.iss.it/ampp/dati/cont.php?id=233&lang=1&tipo=7*
Leadscope Toxicity Database	Leadscope	Qualitative, quantitative	Names, structures	No	No	*www.leadscope.com/db_tox_product.php*
National Toxicology Program	National Cancer Institute, USA	Qualitative, quantitative	Names, structures	Yes	Yes	*http://ntp-apps.niehs.nih.gov/ntp_tox/index.cfm*
RTECS (Registry of Toxic Effects of Chemical Substances)	National Institute for Occupational Safety and Health, USA	Qualitative, quantitative	Names, structures	Yes	No	*www.cdc.gov/niosh/rtecs/RTECSaccess.html*
TOXNET (TOXicology data NETwork)	US National Library of Medicine	Qualitative, quantitative	Names	No	Yes	*http://toxnet.nlm.nih.gov/*

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quantum chemical variables such as LUMO (energy of the lowest unoccupied molecular orbital), log P (octanol–water, blood–tissue or a similar partition coefficient), molecular area and volume, molar refractivity index and steric energy. Descriptors for statistical models include the mechanistic descriptors, in addition to those that may or may not have a mechanistic basis such as information indices (Kier and Hall, 1986). In general, the descriptors may be categorized as experimental quantities, spectroscopic data, substituent constants (electronic, hydrophobic, steric), parameters derived from molecular modelling and quantum chemical computations, graph theoretical indices, and variables that describe the presence or absence of certain substructures or fragments.

Descriptors that describe the electronic states responsible for any given end point may be calculated using descriptor-generator programs such as ADAPT, TSAR (Accelrys), Molconn-Z, CODESSA™ GRID (Molecular Discovery, Ponte San Giovanni, Italy), E-Calc, EPIWIN, Dragon and other computational chemistry programs such as CAChe™ and Gaussian. Todeschini and Consonni (2000) provide a comprehensive compilation of molecular descriptors that may be used to develop (Q)SAR models for various health end points.

3.4.3 Developing the (Q)SAR Model(s)

Once a list of potential descriptors is calculated, several statistical methods may be used for generating the final (Q)SAR equation. These may include multiple linear regression, partial least squares (PLS), artificial neural networks, genetic algorithms, logistic regression (for SARs), classification and regression trees, simple linear regression, stepwise multiple linear regression, and principal components analysis.

Various commercial and noncommercial software that provide built-in tools to calculate descriptors as well as develop (Q)SAR models are available. Commonly used software programs are briefly described below.

3.4.3.1 CODESSA™ PRO

CODESSA™ (Comprehensive Descriptors for Structural and Statistical Analysis) PRO (University of Florida, Gainesville, FL) is a comprehensive program for developing QSARs by integrating all necessary mathematical and computational tools to calculate a large variety of molecular descriptors on the basis of the 3D geometrical structure, develop regression equations, carry out cluster analysis of the experimental data and molecular descriptors, interpret the developed models, and predict property values for any chemical compound with known molecular structure.

3.4.3.2 Discovery Studio QSAR

DS QSAR (Accelrys Software, Inc., San Diego, CA) provides easy access to molecular descriptors such as Extended Connectivity Fingerprint (ECFP) descriptors, physicochemical properties and quantum chemical descriptors. The software provides a wide variety of modelling techniques such as Bayesian models, multiple linear regressions, PLS and genetic functional analysis to develop the models.

3.4.3.3 MDL QSAR

MDL QSAR (MDL, San Ramon, CA) is a comprehensive QSAR modelling system that lets scientists establish reliable QSARs, create new calculators for *in silico* screening, and generate new compound libraries based on results—accelerating the discovery of lead compounds in drug and agrochemical research. MDL QSAR provides over 400 built-in 2D and 3D molecular descriptor calculators for rapid processing of thousands of molecules. In addition, MDL QSAR allows users to use third-party calculators through the built-in interface, or import previously calculated descriptor values.

3.4.3.4 Molecular Operating Environment (MOE)

MOE (Chemical Computing Group, Quebec, Canada) provides a suite of applications for manipulating and analysing large collections of chemicals, calculating over 300 molecular descriptors and building QSAR models using linear, probabilistic and decision-tree methodologies.

3.4.3.5 (Q)SAR Application Toolbox

The OECD (Q)SAR Application Toolbox is open-source software that provides several computational methods and information technologies for the application of (Q)SARs, or a category approach for filling data gaps that are necessary for hazard and risk assessment.

3.4.3.6 Scigress Explorer

Scigress Explorer (formerly named CAChe™; Biosciences Group, Newton, MA) is a desktop molecular modelling software package that can draw and manipulate chemical structures, calculate physicochemical properties, and develop QSAR equations using multiple linear regressions.

3.4.3.7 Sybyl QSAR with CoMFA

Sybyl QSAR with CoMFA (Tripos, St. Louis, MO) module builds statistical and graphical models that relate the properties of molecules (including biological activity) to their structures. The software builds the QSAR models by organizing structures and their associated data into Molecular Spreadsheets™, calculating molecular descriptors, and performing sophisticated statistical analyses that reveal patterns in structure–activity data. These models may then be used to predict the properties or activity of novel compounds.

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3.4.3.8 Tsar

Tsar and Tsar-3D (Accelrys Software, Inc., San Diego, CA) are fully integrated QSAR packages for library design and lead optimization. Tsar's chemical spreadsheet interface may be used to explore 2D or 3D physicochemical properties to analyze for those properties that promote activity, identify chemical analogues and develop predictive models for the activity of interest.

3.4.4 Model Validation

The International Council of Chemical Associations (ICCA) and the European Chemical Industry Council (CEFIC) proposed a set of principles to evaluate the validity of (Q)SAR models (OECD, 2009). According to the established principles, QSARs should:

1. be associated with a defined end point of regulatory importance;
2. take the form of an unambiguous algorithm;
3. have a defined domain of applicability;
4. be associated with appropriate measures of goodness of fit, robustness and predictivity; and
5. have a mechanistic basis, if possible.

The most demanding way to validate the predictive ability of a model is by external validation, which consists of making predictions for an independent set of data not used in the model calibration (Eriksson *et al*., 2003). In the past decade, the NTP conducted two exercises for the prediction of carcinogenicity (Bristol *et al*., 1996). In these exercises, toxicity values were predicted prior to the release of the experimental carcinogenicity data (true external prediction). In the first NTP exercise, the QSAR approaches based solely on information from molecular structure yielded somewhat low prediction accuracies (in the range of 50–65%). One can achieve prediction concordances of 50% simply by randomly selecting compounds to be carcinogenic or not. This was in contrast to the high internal validation statistics reported for these methodologies (Benigni and Zito, 2004). In the second NTP exercise, the best-performing QSAR methodologies achieved prediction accuracies ranging from 60 to 65% (Benigni and Zito, 2004). The NTP prediction exercises illustrate the importance of external validation in establishing the predictive ability of cancer models.

If no external validation set is available, cross-validation may be used instead. Cross-validation is performed by dividing the training set into a number of groups and then developing a number of parallel models from reduced data with one of the groups deleted (Eriksson *et al*., 2003). It should be noted that increasing the number of cross-validation groups to N (the number of compounds in the training set) is not recommended because the Q^2 cross-validation statistic becomes too similar to R^2 (Eriksson *et al*., 2003). There have been several recent studies that have employed cross-validation to evaluate the predictive ability of cancer QSAR methodologies (Contrera *et al*., 2003; Valerio *et al*., 2007).

3.4.5 Applicability Domain

Every QSAR model can formally predict the toxicity (i.e. carcinogenicity) for any compound for which chemical descriptors can be calculated. However, since the models are based on chemicals from a given training set (that cover only a small fraction of the entire chemistry space), an applicability domain for each model should always be defined. This restriction reduces the likelihood of making predictions for compounds that differ substantially from those in the training set (Zhu *et al*., 2008). Generally, there is no universal method of defining the applicability domain. In the carcinogenicity estimation method developed by Contrera *et al*. (2003), a unique cluster (and model) is constructed for each test chemical using the most similar chemicals from the training set. Predictions are only made if there are a minimum number of chemicals in the training set that have a certain degree of similarity with the test compound. Several methods for defining the applicability domain for toxicity models are available in the literature (Eriksson *et al*., 2003; Zhu *et al*., 2008).

3.5 Predictive Ability of Carcinogenicity (Q)SAR Models

In QSAR modelling, it is easy to manipulate data such that an apparently good model can be formulated (i.e. by removal of outliers or by over-fitting the dataset with a large number of descriptors). Such an inappropriate model often arises when one is merely interested in the fit of the model to the underlying data (i.e. in terms of internal validation statistics such as R^2). The problem with internal validation statistics is that they do not provide an indication of the predictive ability of the model. Prediction validation is one way to assess model adequacy for new compounds (Eriksson *et al*., 2003).

Most of the QSAR models for cancer consist of multiple linear regression models containing a small number of molecular descriptors (Passerini, 2003). The descriptors in the models (usually energies of the highest occupied molecular orbital (HOMO) or the lowest unoccupied molecular orbital (LUMO) and the octanol–water partition coefficient) are typically selected manually based on mechanistic considerations. Due to the small size of the training sets, cross-validation is usually not performed. If cross-validation is performed, it must be done carefully. Typically, the model for each cross-validation training set is constructed by recalculating the coefficients for descriptors appearing

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in the overall model (Franke *et al*., 2001). This approach can overestimate the predictive ability of the overall model since the descriptors were selected based on knowledge of the entire dataset instead of the corresponding training sets. A possible solution is to use a genetic algorithm approach to develop new models for each cross-validation training set. The advantage of this approach is that each model is constructed only with the knowledge of its respective training set. The descriptors appearing in the different models might be slightly different, but this approach should provide a conservative estimate for the predictive ability that can be expected from a multilinear regression model fit to the entire dataset (Martin *et al*., 2008a).

4 USE OF 3D-QSAR MODELS TO INCREASE PREDICTIVE ABILITY

In the past, it was typical to characterize toxicity of a chemical without describing and/or understanding the actual target(s) at the molecular level or cellular level. Toxicity is often observed and then recorded at the whole animal level (systemic toxicity) and organ level (end-point toxicity). Even though toxicity at the organ level can provide valuable information (e.g. liver toxicity), SARs/QSARs can be best developed at molecular level with elucidated interactions with macromolecules (e.g. DNA, protein, etc.). Advances in QSAR technology allow researchers to take advantage of these potential chemical–macromolecule interactions as part of QSAR development, namely, 3D-QSAR. Three-dimensional QSAR includes the spatial relationship between a chemical and a potential target. Inclusion of this additional parameter can strengthen the existing (Q)SAR, which relies solely on physicochemical properties of a chemical.

A 3D-QSAR depends on the application of force-field calculations, which requires three-dimensional structures, for example protein crystallography, and molecule superposition (alignment). It is typically used for studying the interactions between a receptor and its ligands. This approach normally uses computed potentials such as the Lennard-Jones potential (Lennard-Jones, 1931), which account for two distinct forces (attractive and repulsive) for a pair of neutral atoms or molecules, rather than experimental constants. Three-dimensional QSAR is different from 2D-QSAR in that it is concerned with the overall molecule rather than a single substituent or fragment. Both the steric and electrostatic fields are taken into consideration for potential target–chemical interactions (Leach, 2001).

Early developers of 3D-QSAR include the Hopfinger group (Potenzone *et al*., 1977) and the Marshall group (Marshall *et al*., 1979), who were the first to investigate the contribution of three-dimensional shape in the analysis of a molecule. Cramer *et al*. (1988) proposed that biological activity could be related to the shape-dependent steric and electrostatic fields of molecules. The authors developed a new method based on PLS and cross-validation, CoMFA, in order to develop models for biological activity predictions instead of a single regression line. CoMFA has since become one of the most popular 3D-QSAR techniques. CoMFA is now widely used to derive quantitative models for protein-binding affinities. Its underlying concept is that a group of congeneric compounds used in a study should act or cause toxicity via the same mechanism and, most likely fit into a common pharmacophore. Even though CoMFA has been applied to drug discovery specifically to eliminate hits with undesirable toxicity, it can also be applied to toxicology if a common target or similar mechanism is known or assumed (Liu *et al*., 2005).

Assuming or knowing chemicals have a common target (e.g. DNA, protein), molecular modellers can use the 3D-QSAR approach for toxicity prediction, or in this case, DNA as a common target for predicting cancer potency. For chemicals that are predicted to be mutagens based on both *in vitro* and *in vivo* assays, one may assume that these chemicals either bind or interact with DNA in either covalent or noncovalent fashion. Docking (*in silico* receptor–ligand binding simulation) may be performed as the initial step to provide the optimal binding orientation (Lengauer and Rarey, 1996). Subsequently, a 3D-QSAR can be generated using either commercial software such as CoMFA in Sybyl (Tripos Inc., MO).

Recently, higher dimensional QSARs such as 4D- (ensemble of conformers), 5D- (induced-fit models) and 6D-QSARs (solvation scenarios) are now possible. These models can provide even more predictive powers (Vedani *et al*., 2006). It would be of great interest to apply these new techniques towards existing 3D-QSAR models, thus making more reliable predictions of carcinogenicity and carcinogenic potency.

The robustness and the predictive ability of (Q)SARs may generally be increased through the use of 3D or higher order QSAR models, which generally requires the modelled chemical as well as the target (protein, DNA, etc.) to have 3D coordinates. One disadvantage of using models that require 3D coordinates is the amount of time required to generate the 3D coordinates as well as the time required to study the interactions between the chemical and the target. One approach to circumvent this problem is to use a grid or distributed computing platform such as the Berkeley Open Infrastructure for Network Computing (BOINC) to perform these computer-intensive calculations.

BOINC (*http://boinc.berkeley.edu/*) is a software program that relies on a network of users who donate the idle time on their computers to run applications that study issues such as climate change, extraterrestrial life, drug discovery and treating cancer. BOINC has several

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ongoing projects that study the chemical structures that cause cancer, develop drugs that may help treat cancer or study target sites that are implicated in causing cancer. For example, Rosetta@Home (Schueler-Furman *et al*., 2005) use the BOINC infrastructure to model protein structures and interactions to understand diseases and find cures. In addition, Rosetta@Home are also developing methods for cutting DNA at specific target sites in the genome that are implicated in cancer. After these sites are cut, the DNA could potentially be repaired using an unmutated copy of the gene, thereby eliminating the progression of cancer (Rosetta@Home, *http://boinc.bakerlab.org/rosetta/rah_medical_relevance.php*). Other BOINC projects that study cancer include Folding@Home (*http://folding.stanford.edu/English/FAQ-Diseases*) and World Community Grid (WCG, *www.worldcommunitygrid.org/projects_showcase/hcc1/viewHcc1Main.do*). The predictions made by the BOINC models may then be used to supplement the predictions made by the (Q)SAR models for carcinogenicity and carcinogenic potency.

5 USE OF -OMICS IN (Q)SARs FOR CARCINOGENICITY

Recent technological advances in -omics fields (genomics, proteomics, metabonomics, transcriptonomics, lipomics, etc.) have generated vast amounts of biological data that can be potentially considered as descriptors and subsequently be utilized as part of (Q)SAR model development. In addition to the three common -omics (genomics, proteomics and metabonomics), a new field named toxicogenomics (see **Toxicogenomics and the Evolution of Systems Toxicology**) provides the analysis of a particular toxin or chemical substance on both cellular and tissue level that can be identified and characterized based on a profiling of its known effects on genetic material. This recent technology can serve in a role similar to DNA testing in the forensic identification of individuals. Application to carcinogenicity and/or mutagenicity is therefore possible.

These -omics techniques may also be used as diagnostics to predict adverse effect(s) of chemicals in the environment at low dose. Some suitable measurable markers (biomarkers) that are correlated to the adverse effect(s) in clinical trials and animal studies can be developed as part of mode of action analysis and eventually for SAR analysis. Using both expression profiling and biomarker identification, it is theoretically possible to accumulate enough data for developing a (Q)SAR for a particular chemical class. **Figure 4** summarizes the whole process of integrating -omics data as part of model development. While this approach is not currently implemented, it has great potential.

Traditionally, a descriptor of a chemical is based on physiochemical properties and/or structural properties. With the new -omics data, one can use quantitative expression level (up- or down-regulation) of a particular macromolecule, cluster/family of macromolecules, or assembly of a complex, as an indicator of biological process. These newly generated data can help a QSAR modeller to integrate chemical and biological information into the final QSAR equation, which reflects both the biological and chemical space of chemicals in the training set (**Figure 4**). The final QSAR equation then can be

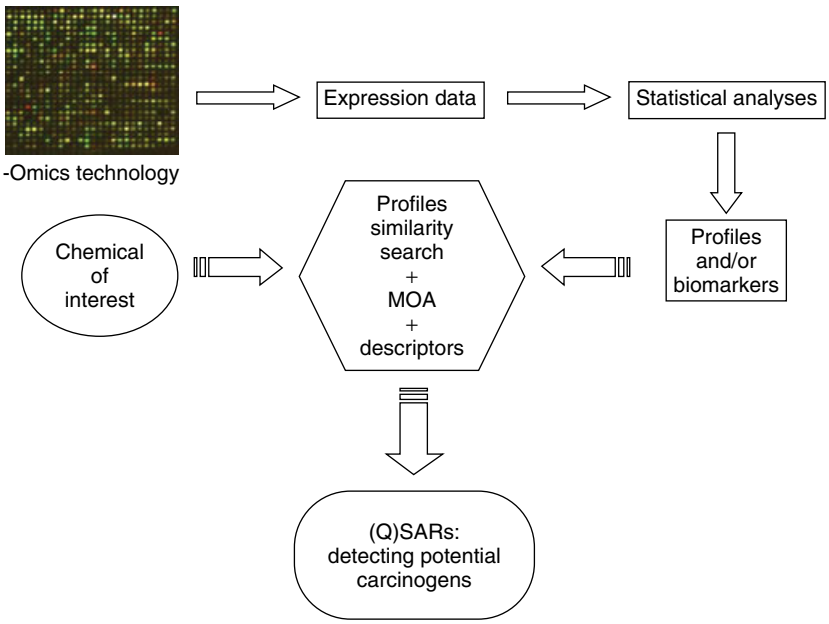

Figure 4 A schematic for developing (Q)SAR models using -omics data.

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used for predicting the carcinogenicity of new chemicals of interest that are not part of the original training set, and these chemicals should also be predetermined to be inside the applicability domain of the QSAR model.

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Short-term Tests for the Determination of Genotoxic and Carcinogenic Potential of Xenobiotics

Alok Dhawan

CONTENTS

1 INTRODUCTION

Genetic toxicology includes studies of DNA damage (genotoxicity), mutations in DNA (mutagenicity) and cancer formation (carcinogenicity), as well as consequences of DNA damage and repair (see **Mutagenesis**, **Carcinogenesis and Carcinogens that are also Genotoxic**). The main aim of genotoxicity tests is to address gene mutations and chromosome damage. Genetic toxicology has been used for hazard identification, in regulatory toxicology testing since the 1997s and is now being considered for integrated risk assessment (Thybaud *et al*., 2007). It is now an intrinsic component of safety testing for the approval of drugs and animal health products, and also a regulatory tool used by the United States Environmental Protection Agency (USEPA) for environmental exposures.

Genetic toxicology testing started with studies in *Drosophila melanogaster*, demonstrating increased mutation frequency after exposure to X-rays, and later by exposure to chemicals. Similar responses upon exposure to X-rays were observed in mice, leading to the concept that radiation and chemicals could induce mutations in *Drosophila*, as well as mammalian cells (Pitot and Dragan, 2001). Ames (1971) developed a mutation assay in a bacterium—*Salmonella typhimurium*, called the Ames test. This was used to screen large number of chemicals for their mutagenic potential and to label them as potential mutagens and carcinogens. A number of other *in vitro* tests were also developed during the same period. The history of chemical genotoxicity testing has been outlined by Zeiger (2004).

Humans are exposed to a wide range of chemicals—including industrial chemicals, solvents, pesticides and other new chemical entities (NCEs), as well as ionizing radiation, which may lead to DNA damage. About 10^3–10^6 DNA breaks take place per cell per day. However, due to the efficient DNA repair mechanism, most of the DNA damage gets repaired. Damage that is persistent may lead to alterations, like mutations in genes, structural and numerical chromosomal damage, and recombination (see **Cytogenetics**). These changes may cause adverse health effects, including cancer, as well as affect the incidence of heritable mutations in man, and may be transferred to the progeny.

Alterations in DNA that lead to DNA damage/mutations are covalent binding (adduct formation leading to base-pair substitution mutations), crosslinking (intra- or interstrand, producing DNA or chromosomal breaks), intercalations (between the base pairs of DNA

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leading to insertion/deletion mutations) or breakage (DNA strand breaks, unrepaired DNA damage causing chromosomal aberrations (CAs)). Indirect genotoxicity is caused by alterations in spindle fibre or kinetochore proteins (leading to incorrect chromosomal segregation) and incorporation of DNA analogues (leading to strand breaks).

It is therefore important to understand the genotoxic potential of chemicals for risk assessment and hazard prediction to safeguard human and environmental health. Hence, adequate test systems that detect alterations in the DNA due to chemicals and/or radiation, have to be developed. Compounds that give positive results in such tests have the potential to be human mutagens/carcinogens.

There are nearly 200 *in vivo* and *in vitro* short-term assays that have been developed to test for genotoxicity in bacteria, plants, insects and mammals. Various strategies have been employed for the identification of a chemical as genotoxic, mutagenic and/or carcinogenic using different test systems (**Figure 1**). Work-groups of the European Union (EU), Organization for Economic Co-operation and Development (OECD) and International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) have defined a standard battery of tests (Kramer, 1998), which need to be conducted for a compound. MacGregor *et al*. (2000) reported the various recommendations from different countries for *in vivo/in vitro* genotoxicity testing. The genotoxicity testing strategies for most regulatory agencies in the USA, Canada, the UK, the EU, Japan and other countries are similar, however, certain variations exist (Cimino, 2006; Jena *et al*., 2002).

A tiered approach is usually employed for the assessment of genotoxicity of chemicals. A standard battery of test includes both *in vitro* as well as *in vivo* tests/assays. The ICH has recommended a bacterial mutagenicity test, an *in vitro* test for chromosomal damage in mammalian cells or mouse lymphoma assay (MLA) (for those compounds that cannot be adequately tested in bacterial systems and are relevant in mammalian cells) and an *in vivo* test for chromosomal damage in rodent haematopoietic cells, for providing information on additional factors such as metabolism and excretion of compounds (International Conference on Harmonization, 1999). The current regulatory scheme of the USEPA (US Environmental Protection Agency, 2005) consists of three levels, where the first level tests consist of a test for gene mutations in bacteria (*Salmonella*), and mammalian cells (MLA) and an *in vivo* test for CA/micronucleus (MN) assay. The second level tests are then conducted *in vivo* in the gonadal (testicular) cells and the third level tests are carried out in the offsprings of the exposed animals (US Environmental Protection Agency, 2005). However, to reduce animal experimentation, the guidelines from the Committee on

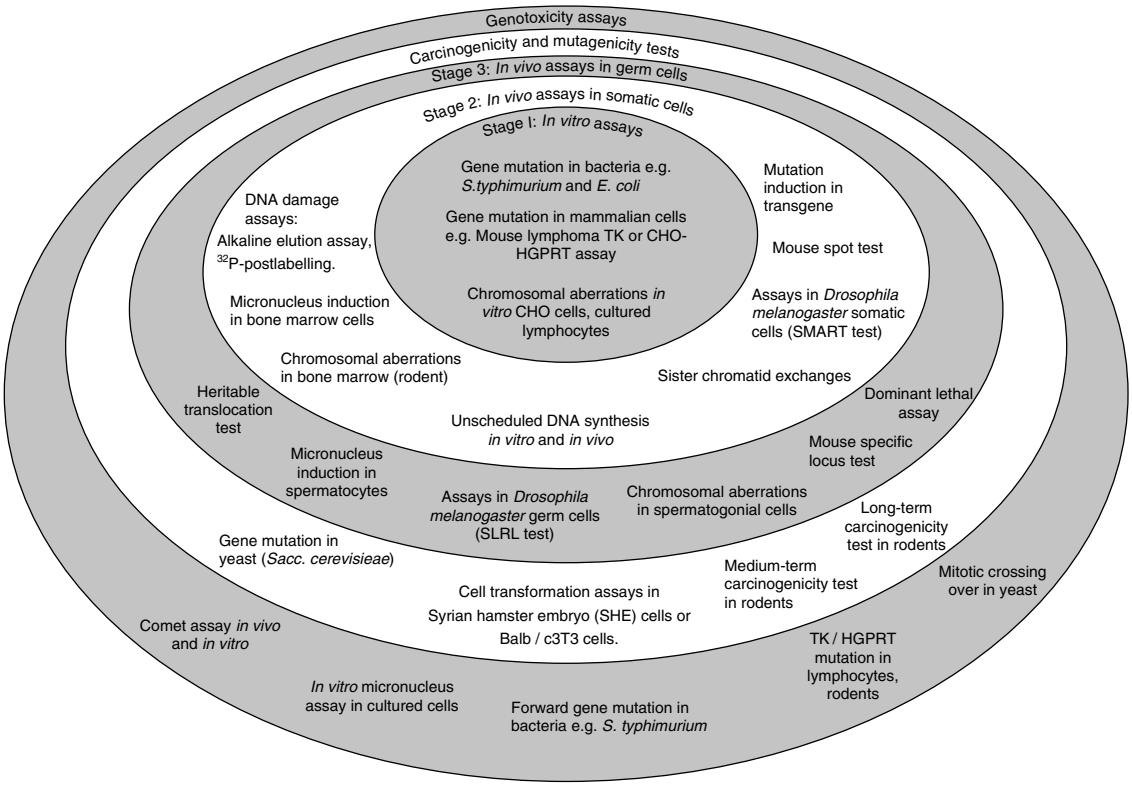

Figure 1 Strategy for testing of genotoxicity, mutagenicity and carcinogenicity. (Reproduced with permission from Bajpayee *et al*., 2005. © Informa Healthcare.)

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Mutagenicity, UK (2000) have added a test for clastogenicity (CA/MN assay) in cultured mammalian cells in the first stage (Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM), 2000). The second stage includes *in vivo* tests for cytogenetic damage in somatic cells (rodent bone marrow MN or CA assay), while additional tests, such as the Comet assay, unscheduled DNA synthesis (UDS) and ^{32}P-postlabelling may also be conducted, and the third stage consists of *in vivo* tests in germ cells, as well as intergenerational tests (Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM), 2000).

A positive result in the second tier *in vivo* genotoxicity tests (Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM), 2000) or data from structure-activity relationship (SAR)/long-term bioassay leads to the chemical being labelled as a potential carcinogen while if *in vivo* genotoxicity data are negative the chemical is evaluated as a potential nongenotoxic carcinogen (Cimino, 2006).

The current genetic toxicology tests being used cannot assess all types of genetic damage that may be relevant for carcinogenicity. Hence, new tests are being developed to characterize the potential carcinogenicity of chemicals. The advances in molecular biology have helped the tests in monitoring at the single gene or chromosome level, as well as evaluating the response of the entire genome. These include the use of microarrays, for example, array CGH (competative genomic hybridization) and electrochemiluminescent arrays (Gant, 2007; Hvastkovs *et al*., 2007; Kim *et al*., 2005), real-time polymerase chain reaction (PCR) (Soberón *et al*., 2007) and *in silico* (Hayashi *et al*., 2005; Mazzatorta *et al*., 2007) tools for prediction of genotoxicity and carcinogenicity of chemicals. These newer tests are cost effective, more sensitive, provide a broad range of responses and permit high throughput for the identification of genotoxicity/carcinogens.

2 TESTS FOR GENOTOXICITY AND CARCINOGENICITY

Well-characterized genetic assays are conducted for evaluation of the genotoxic/carcinogenic potential of chemicals. Depending on the length of time required for the completion of the assay, the tests have been designated as long- (12–24 months), medium- (2–8 months) and short- (several weeks) term assays.

Long-term tests include the chronic two-year rodent bioassay as the 'gold standard' for determining potential carcinogenic activity of a chemical. It has been used to detect human carcinogens by the International Agency for Research in Cancer (IARC; Vainio *et al*., 1991). The bioassay detects complete carcinogens and tumour promoters, as well as cocarcinogens. It requires observation of the test animal for a major portion of its life span for the development of neoplastic lesions, during or after exposure to various doses of a test substance by an appropriate route (Bannasch *et al*., 1986). However, this assay is time-consuming, costly and requires adequate physical facilities and qualified personnel.

Medium-term tests (e.g. newborn mouse model), are used as an alternative to the chronic two-year bioassay, and neoplasia in various tissues in mice, for example, liver, lung and lymphoid organs, are recorded over a period of two to eight months (Fujii, 1991).

Due to advancements in the field of molecular biology and combinatorial chemistry, a large number of chemicals are now being synthesized and are ready for release. However, to keep abreast with this rapid growth of chemicals, and to assess their genotoxic/carcinogenic potential, new and sensitive tests need to be employed, which can provide reliable results in a shorter time. Thus, short-term tests were developed to identify carcinogens by assessing *in vitro* or *in vivo* mutations in DNA (**Table 1**). The short-term tests are also used for mechanistic evaluation and thus help in risk assessment (see **Genetic Toxicology Testing and its Relevance to Human Risk and Safety Evaluation**). These assays may take from one day to a few weeks for completion.

Use of short-term tests is based on the assumptions that: (i) the ultimate cause of cancer is due to some mutational changes in DNA (carcinogens have been shown to induce mutations and impaired DNA repair leads to cancer) and (ii) the similarity of the genetic code and protein synthesis between organisms, allows extrapolation between species (Ramel, 1983). Since none of these tests alone can predict genotoxicity/carcinogenicity of a compound, it has been recommended that a battery of tests needs to be conducted before a compound is labelled as a mutagen and/or carcinogen. These results are then considered equivalent to the predictive value of a long-term animal bioassay.

In vitro short-term tests provide a rapid, inexpensive, highly sensitive, controlled and reproducible tool for the assessment of genetic damage (Kluwe, 1995). They also provide an alternative to the use of animals. However, *in vitro* tests cannot fully mimic the *in vivo* environment, and the exposures given may be higher than those taking place *in vivo*. Hence, an *in vivo* test for genetic damage should be a part of the test battery to provide a test model in which factors like absorption, distribution, metabolism and excretion are included, which may influence the genotoxic activity of a compound (International Conference on Harmonization, 1999). There may also be compounds which are difficult to test in the *in vitro* systems, but can cause genetic alterations *in vivo*, which have been reviewed by Tweats *et al*. (2007).

This chapter reviews the various short-term tests for assessment of the genotoxic and/or carcinogenic potential of chemicals.

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Table 1 Short-term tests for genotoxicity assessment

Tests	End point	Regulatory agency recommendation[a]
***In vitro* bacterial assays**		
Mutation in *Salmonella typhimurium* (Ames test)	Reverse or forward mutation in specific bacterial strains, detects base-pair, frameshift mutations, oxidizing and crosslinking agents	OECD (471), ICH, COM
Mutation in *Escherichia coli*	Reverse mutation in WP2, WP2*uvr*A strain, detects oxidizing and crosslinking agents	OECD (471)
Mitotic recombination, mitotic crossing over or mitotic gene conversion in yeast	Conversion of heterozygous alleles to homozygous state	OECD (481)
***In vitro* mammalian assays**		
Mouse lymphoma thymidine kinase (TK)	Mutation in TK gene, detects point mutations and chromosomal deletions	OECD (476), ICH, COM
Chinese hamster ovary (CHO) and V79 hypoxanthine guanine phosphoribosyl transferase (HGPRT)	Mutation in HGPRT gene, detects point mutations and chromosomal deletions	OECD (476), COM
Chromosomal aberrations	Structural and numerical alteration in chromosomes, detects clastogenicity in cell lines (CHO, V79, etc.) and cultured lymphocytes	OECD (473), ICH, COM
Micronucleus	Detects clastogenicity and aneugenicity appearance of micronuclei in cell lines (CHO, V79) and cultured lymphocytes	OECD (draft)
DNA repair	Unscheduled DNA synthesis (UDS), detects repair activity by thymidine incorporation in lymphocytes	OECD (482), ICH, COM
Comet assay; alkaline elution assay; ^{32}P-postlabelling.	Detects DNA damage, alkali labile sites, covalent DNA adducts, oxidative lesions in lymphocytes or cell lines (CHO, V79, etc.)	ICH, COM
Induced neoplastic transformation of cells	Syrian hamster embryo (SHE) cells, Balb/c3T3	OECD (draft)
***In vivo* mammalian assays**		
Mouse spot test	Somatic cell mutation, detects coat colour variation	OECD (484), COM
Mouse specific-locus test	Germ cell mutation, coat colour and ear size change	COM
Dominant lethal assay	Death of fertilized egg in mammalian implanted species	OECD (478), COM
Mutation induction in transgenes	Mutation in *LacZ* gene, *LacI* gene in mouse or rat	ICH, COM
Chromosomal aberrations	Visible structural and numerical alteration in karyotype, detects clastogenicity in bone marrow cells, spermatogonial cells of mice/rats	OECD (475,483), ICH, COM
Sister chromatid exchanges	Visible exchanges of differentially labelled sister chromatids in lymphocytes	OECD (479),

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Table 1 (*continued*)

Heritable translocation test	Translocation induced in germ cells in mice	OECD (485), ICH, COM
Micronucleus test	Detects clastogenicity and aneugenicity in bone marrow cells of rodents	OECD (474), ICH, COM
Rodent liver unscheduled DNA synthesis induction	UDS in primary rat hepatocytes, detects repair activity by thymidine incorporation outside S phase	OECD (486), ICH, COM
DNA damage	Comet assay, alkaline elution assay, ^{32}P-postlabelling, for DNA damage in lymphocytes or cell lines (CHO, V79, etc.)	ICH, COM
Assays in *Drosophila melanogaster*	Somatic and germ cells mutations	OECD (477)

[a]OECD, Organization of Economic Cooperation and Development; number in parenthesis denotes the OECD guideline number for the test. ICH, International Conference on Harmonization. COM, Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment, UK.

3 SHORT-TERM TESTS FOR GENOTOXICITY AND CARCINOGENICITY

In vitro and *in vivo* assays for evaluating genotoxic/carcinogenic potential include tests for gene mutation, cytogenetics and DNA damage, which are discussed in detail below.

3.1 Gene Mutation Assays

These assays rely on the selection of specific traits to detect mutations and are conducted both *in vitro* and *in vivo*. The assays can detect point mutations, frameshift and base-pair substitutions, and small deletions in the genome. They are simple, rapid and inexpensive and thus are prominently used in genetic toxicology.

3.1.1 Mutation Assays in Bacteria

Bacteria have been widely used to study the mechanisms of DNA damage (mutagenicity) as well as for short-term screening tests for potential carcinogens (Venitt *et al*., 1986). They possess elaborate mechanisms for responding to DNA damage. Several methods have been developed which detect reverse or forward mutations.

Bacteria lack the metabolic capabilities of mammalian systems, therefore metabolic activation which converts certain compounds (promutagens) into reactive species (mutagens) is supplied exogenously. The mammalian metabolizing system widely used is the postmitochondrial fraction (S9) from liver homogenate of rats treated with arochlor 1254, benzo[a]pyrene (B[a]P) or a mixture of β-naphthoflavone and phenobarbital.

3.1.1.1 Reverse Mutation Assays

The most widely used reverse mutation assay, known as the Ames test, was developed by Ames (1971) using the *Salmonella typhimurium* bacterium. It is the preferred *in vitro* primary screen for gene mutation and detects most of the human mutagens and carcinogens (Ames *et al*., 1973a; 1973b; 1975; Maron and Ames, 1983). The assay is based on the premise that a substance mutagenic to the bacterium is likely to be a carcinogen in laboratory animals, and hence, presents a risk of cancer in humans.

Bacteria already mutated at an easily detectable locus (e.g. histidine) are tested with compounds that produce a second mutation, nullifying the first mutation; thereby the bacterial strain reverts to normal. Bacterial strains mutated at the histidine locus, do not synthesize histidine and thus die when plated on an agar medium lacking histidine. Different bacterial strains (**Table 2**) are treated with varying concentrations of the test compound in the absence and presence of the exogenous metabolic activation system. When a mutation occurs in these cells that reverts the original *his* mutation, the reverse-mutated cells (revertants) grow on a histidine-deficient agar plate and form a visible colony after 48 hours of incubation (**Figure 2**). A reproducible dose-dependant response in at least one tester strain indicates a positive result, suggesting that the substance is a mutagen and could be a carcinogen as well. If no increase in mutant colonies is seen after testing various strains under different culture conditions, the test chemical is considered to be nonmutagenic. Modifications to the assay for testing gaseous and volatile liquid compounds, as well as solvents and pure compounds, have been discussed (Mortelmans and Zieger, 2000).

The sensitivity of the Ames assay to mutagens and specific classes of chemicals (Josephy *et al*., 1997; McCann *et al*., 1975) has been enhanced through strain engineering. Several specially constructed strains of

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Table 2 Bacterial strains used in the Ames test and their characteristics

Bacterial strain	Target DNA sequence	Target allele	Plasmid and other characteristics	Mutation detected	Primary mutations
S. typhimurium TA 97a	CCCCCC GGGGGG	*his*D6610	pKM101 (Ap^r), *rfa, Δuvr B*	Frameshift	C or G deletion
S. typhimurium TA 98	CGCGCGCG GCGCGCGC	*his*D3052	pKM101 (Ap^r), *rfa, Δuvr B*	Frameshift	GC or CG deletions and Complex frameshift
S. typhimurium TA 100	CCC GGG	*his*G46	pKM101 (Ap^r), *rfa, Δuvr B*	Base-pair substitution	GC → AT (ts) AT → GC (ts) TA → GC (tv)
S. typhimurium TA 102	CAAGTAAGAGC GTTCAT TCTCG	*his*G428	pKM101 (Ap^r) and pAQ1 (Tc^r), *rfa*	Base-pair substitution, oxidative and crosslinking mutagens	AT → CG (tv) AT → TA (tv)
S. typhimurium TA 104	TAA GC ATT and CG	*his*G428	pKM101 (Ap^r), *rfa, Δuvr B*	Base-pair substitution oxidative and crosslinking mutagens	GC → AT (ts) GC → TA (tv) AT → GC (ts) AT → CG (tv) AT → TA (tv)
S. typhimurium TA 1535	CC GG	*his*G46	None, *rfa, ΔuvrB*	Base-pair substitution	GC → AT (ts) AT → GC (ts) TA → GC (tv)
S. typhimurium TA 1537	CCCCC GGGGG	*his*C3076	None, *rfa, ΔuvrB*	Frameshift	C or G deletion
E. coli. WP2 *uvr*A	AT TA	*trpE*	None, *uvr A*	Base-pair substitution and crosslinking	GC → AT (ts) GC → TA (tv) AT → GC (ts) AT → CG (tv) AT → TA (tv)
E. coli. WP2 *uvr*A (pKM101)	AT TA	*trpE*	pKM101, *uvr A*	Base-pair substitution and crosslinking	GC → AT (ts) GC → TA (tv) AT → GC (ts) AT → CG (tv) AT → TA (tv)

ts, transition; tv, transversion; *rfa* increases the permeability of cell wall to large molecules; *uvrB* deletes excision repair; plasmid pKM101 encodes *mucAB* gene that participates in SOS repair system; (Ap^r) ampicillin resistance selection of plasmid, (Tc^r) tetracycline resistance selection of plasmid.
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Salmonella typhimurium, for example, TA1535, TA1537, TA97/TA97a, TA98, and TA100, TA102 and TA104 (Bonneau *et al*., 1991; Levin *et al*., 1982; Ohe *et al*., 1999; Venitt *et al*., 1984; **Table 2**) exist for use in the assay. The strains can detect changes at guanine-cytosine (G-C) or adenine-thymine (A-T) base-pair sites within target histidine genes. Using different strains in the assay helps to assess the specific mutagenic mechanism of the compound. The tester strains have been modified with deep rough (*rfa*) mutation which eliminates the polysaccharide side chain of the lipopolysaccharide on the bacterial surface, making the bacteria more permeable to the test chemicals. Deletion of the excision repair system (Δuvr) confers sensitivity to many mutagens while the plasmid pKM101 carrying the *mucAB* gene confers sensitivity to compounds which act via the SOS system. The TA1535 set (TA1535, TA1536, TA1537, TA1538), is most sensitive to mutagenesis and is recommended for general testing for mutagens and carcinogens *in vitro* (Ames *et al*., 1973b). The compounds suspected to be oxidative mutagens may be detected by strains TA102 and TA104.

Plasmids encoding for mutagenesis and metabolic function (e.g. specific enzymes) provide broad substrate specificity and high mutagenic sensitivity towards chemicals (Carroll *et al*., 2002; Josephy *et al*., 1997; Mortelmans and Zieger, 2000; Suzuki *et al*., 1998). Specific and sensitive detection of the genotoxicity

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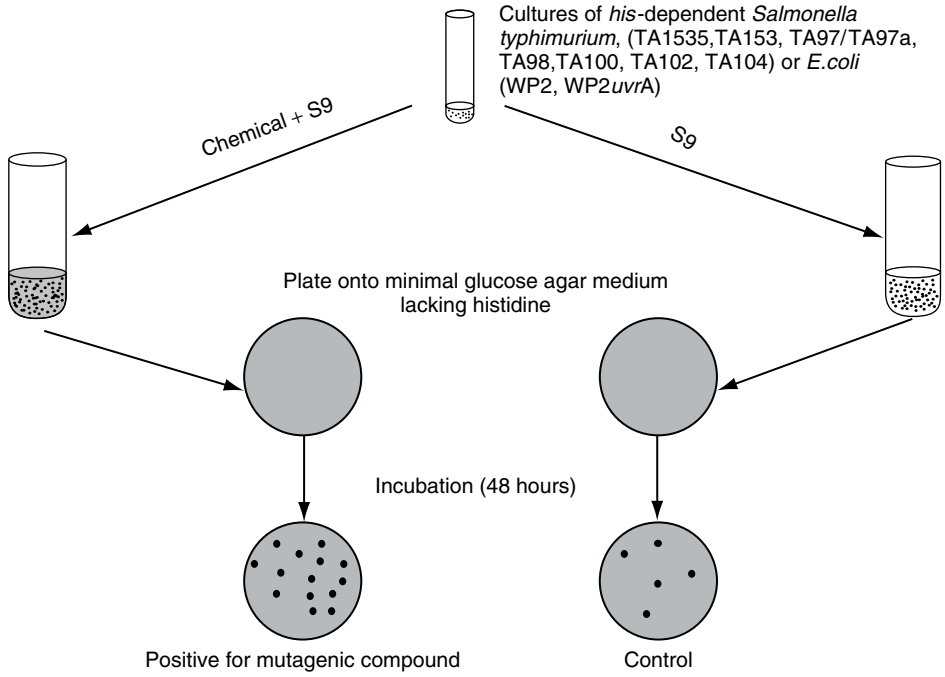

Figure 2 Schematic representation of Ames test.

of B[a]P and other polycyclic aromatic hydrocarbons (PAHs) in complex mixtures with the help of the engineered *S. typhimurium* strain, YG5185, (carrying a plasmid encoding *E. coli* DNA polymerase IV), is several times higher than standard strains (Yamada *et al*., 2006). A new bioluminescent strain of *Salmonella*, developed by Aubrecht *et al*. (2007), employs bioluminescence for identifying histidine-independent revertant cells, and provides an economical, high throughput tool for assessment of mutagenicity.

Along with *Salmonella* strains, the bacterial tryptophan reverse mutation assay with *Escherichia coli* WP2 strains is also recommended (Gatehouse *et al*., 1994; Mortelmans and Riccio, 2000). Inclusion of the DNA-repair-proficient strain, *E. coli* WP2 and WP2 (pKM101), and excision-repair-deficient strains *E. coli* WP2 *uvr*A for detection of crosslinking agents (Barrueco and de la Peña, 1988; Ohta *et al*., 2002; Wilcox *et al*., 1990; **Table 2**) has been accepted by the international guidelines in place of the *Salmonella* strain TA102, because both carry an AT base sequence at the target site which leads to the detection of transitions and transversion (Mortelmans and Riccio, 2000).

A bacterial reverse mutation assay has been designed utilizing the *E. coli lacZ*$^-$ strain. *E. coli* uses lactose as a carbon source, and each strain carries a *lacZ* allele encoding for an inactive β-galactosidase protein, which upon mutation results in *lacZ*$^+$ cells that can grow in a medium lacking lactose. The assay can detect base-pair substitution and frameshift mutations (Josephy, 2000).

It has been established that there is a high predictivity of a positive mutagenic response in the Ames test for rodent carcinogens (McCann *et al*., 1975; Mortelmans and Zieger, 2000). International guidelines have been developed for this assay (e.g. OECD 471; ICH) to ensure uniformity of testing procedures. The extensive database on the assay justifies its inclusion as the initial screening test for detection of mutagens (Seifried, 2008). However, bacterial assays may not provide sufficient information for the assessment of genotoxicity of some compounds. These include compounds that are highly toxic to bacteria (e.g. some antibiotics), those which would interfere with mammalian cell-specific systems (e.g. topoisomerase inhibitors, nucleoside analogues or certain inhibitors of DNA metabolism) or those which may be clastogens and do not produce mutations (heavy metal). Hence, the Ames test should be used with a second test which detects clastogenicity as well as gene mutations and in mammalian cells, for example MLA (Seifried, 2008).

3.1.1.2 Forward Mutation Assays

These assays are less extensively used than the reverse mutation assays. Some mutagenic compounds may not revert the point mutations in the bacterial strains and thus not be detected in the reversion assay. Also, a specific mutagen may have a weak effect on the genome, but cause large number of revertants. These shortcomings led to development of forward mutation assays (Ruiz-Rubio *et al*., 1984). The L-arabinose forward mutation assay in *Salmonella typhimurium* was developed by Ruiz Vazquez *et al*. (1978); mutation at any of at least three different genes (*ara* A, *ara* B or *ara* D) blocks the utilization of arabinose as a carbon source

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and leads to the accumulation of the toxic intermediate L-ribulose-5-phosphate. Bacterial growth is thus inhibited in the presence of L-arabinose and a carbon source unable to repress the *araBAD* operon. The assay has been used to study the genotoxic potential of river and tap water (Vahl *et al*., 1997; Shen *et al*., 2003). Mutagenicity of nitro-containing compounds can be assessed in a single strain of the *Salmonella typhimurium* BA bacterium that has both the *ara*D531 allele and *his* auxotrophy, allowing for quantitative comparison of induction of forward mutations (to L-arabinose resistance) and reverse mutations (Jurado *et al*., 1994).

Miller *et al*. (2005) have developed a forward mutation assay in *Salmonella* based on resistance to 5-fluorouracil (FU) as a sensitive alternative screen to the Ames assay for detecting a variety of mutagens, including highly toxic compounds that may not be assessed in the standard Ames assay. The assay has been validated with a variety of mutagens and nonmutagens (Vlasakova *et al*., 2005). The mutational target was found to be the uracil phosphoribosyl transferase (*upp*) gene, which, when functional, converted FU to the toxic intermediate 5-fluorouridine monophosphate, thereby inhibiting the wild-type cells. The use of a single strain allows for higher-throughput and reduces the amount of compound required. The highly parallel nature of culture handling/dilution and use of standard microtitre plates also offers the possibility of assay automation (Glaab *et al*., 2005).

3.1.2 Mutation Assays in Mammalian Cells

3.1.2.1 Gene Mutation In Vitro

In vitro cell mutation assays in mammalian cells for possible mammalian mutagens and carcinogens use gene mutations at two loci: thymidine kinase (TK) and hypoxanthine guanine phosphoribosyl-transferase (HGPRT), as end points that confer resistance to toxic chemicals (DeMarini *et al*., 1989). The assays which use these gene mutations are the TK +/− Mouse lymphoma L5178Y assay (Clive *et al*., 1979) and the Chinese hamster ovary (CHO)-HGPRT assay (O'Neill *et al*., 1977; Li *et al*., 1987). Cell lines used in these tests are selected due to the stability of their spontaneous mutation frequency (Aaron *et al*., 1994). CHO and L5178Y mouse lymphoma cells are commonly used since their mutants can be easily isolated. These assays are based on forward mutations and cover a wide range of mutagenic lesions (point mutations involving deletions, base substitutions, frameshift and rearrangement). Mutations at the selected gene result in a loss of phenotype, for example growth in the presence of a toxic compound, and its expression is compared in treated and untreated cells. Forward mutation to drugs like 8-azaguanine, 6-thioguanine, 5-bromo-2-deoxyuridine are widely used as genetic markers. Proliferating cells are exposed to the test substance, both with and without metabolic activation with the mammalian liver postmitochondrial fraction (S9) and concurrent positive and negative (solvent or vehicle) controls are included in each experiment. The phenotypic expression of newly induced mutants in TK requires at least two days and at least six to eight days in the HGPRT assay.

3.1.2.1.1 Mouse Lymphoma Assay (MLA)

This assay, developed by Clive *et al*. (1979), is widely used for detecting mammalian *in vitro* gene mutations and is recommended by regulatory agencies (ICH, USEPA) for genotoxicity testing. L5178Y *tk*+/− 3.7.2 C cells are heterozygous at the TK locus (*Tk1*) on chromosome 11 of mouse (Chen *et al*., 2002). The TK enzyme is used for incorporating pyrimidines in the salvage pathway of DNA synthesis. The functional TK allele thus allows toxic pyrimidine analogues, for example, bromodeoxyuridine (BrdU), fluorodeoxyuridine (FdU) and trifluorothymidine (TFT), to be incorporated into nucleotides, leading to inhibition of cellular metabolism and cytotoxicity in normal cells (**Figure 3**). Forward mutation ($TK^+ \rightarrow TK^-$) in the cells due to a chemical compound inactivates the TK allele conferring resistance to these analogues, and mutants proliferate, forming colonies (Clements, 2000; Moore *et al*., 2000).

There are two versions of the assay—soft agar and micro-well methods (Honma *et al*., 1999a; Moore *et al*., 2003; Oberly *et al*., 1997; Omori *et al*., 2002), and both have been adopted by ICH guidelines (Mitchell *et al*., 1997). Short (3–6 hours) as well as long treatment durations (24 hours) have also been recommended by the International Workgroup on Genotoxicity Testing (IWGT) for conducting an MLA (Moore *et al*., 2007). Short treatments may be insufficient for detecting some clastogens and spindle poisons, which may be cell-cycle dependent. Treatment of cells for more than one cell cycle may be required to see their effect (Honma *et al*., 1999; Moore *et al*., 2002; 2007). A set of 990 compounds reviewed by the workgroup on the MLA, revealed that less than 2% of the compounds were mutagenic at 24 hours treatment time, while the percentage of compounds positive for short-duration exposure was lower (Moore *et al*., 2007). This led to the recommendation and consensus of the workgroup that a long duration of exposure should be applied in cases of compounds which yielded a negative response with 3–4 hours treatment. Mutagenesis in MLA yields small and large colonies, which points to different degrees of genetic damage (Blazak *et al*., 1989) and differential gene expression (Han *et al*., 2006). The small colonies grow slowly and are associated with mutations compatible with viable cells, as compared to gross CA analysis, and thus present a significant risk for human carcinogenicity or mutagenicity (Moore and Doerr, 1990). Molecular analysis and studies with different chemicals, like spindle poisons (colchicine; Honma *et al*., 2001) and alkylating agents (ethylnitrosourea; Chen *et al*., 2002) have shown that

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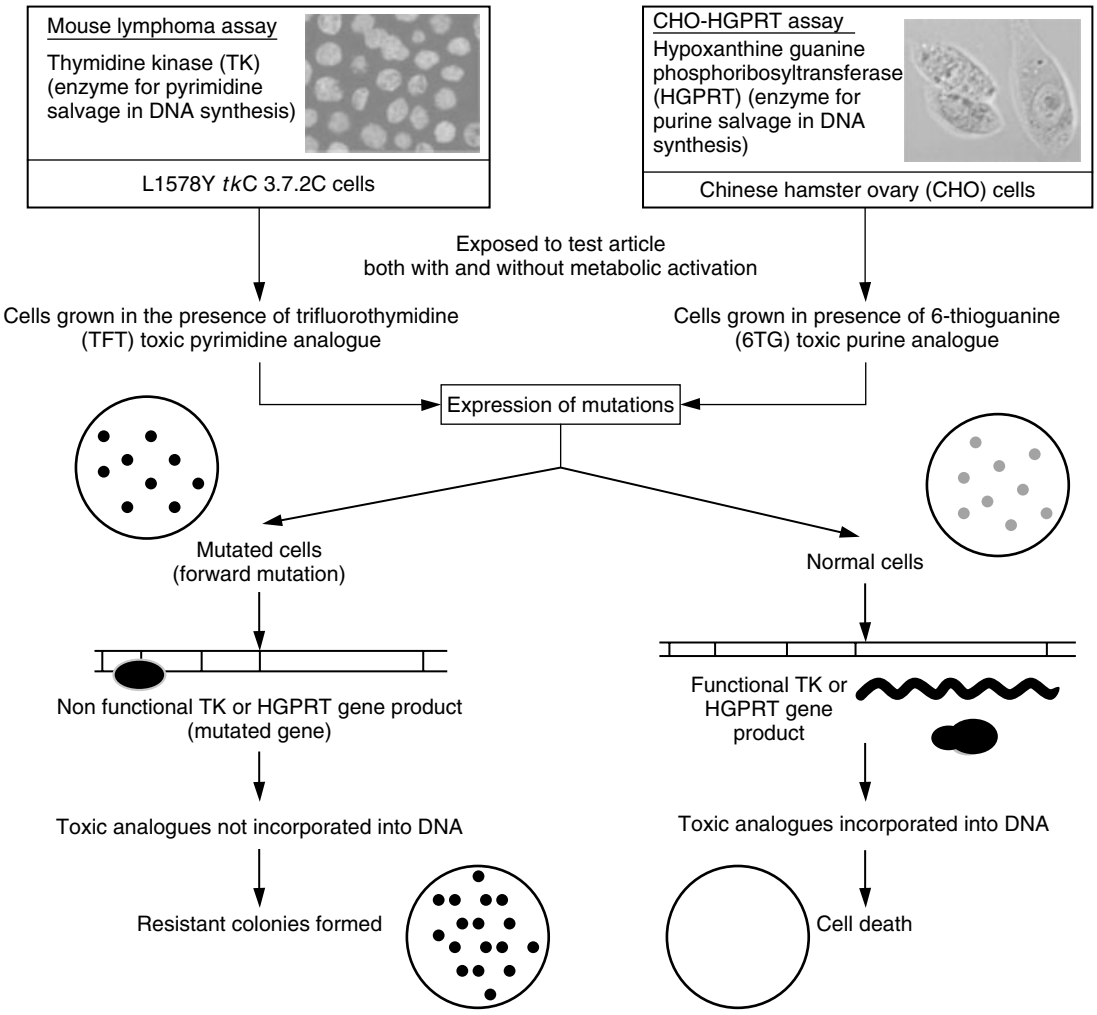

Figure 3 Schematic representation of the *in vitro* mammalian cell mutation assays.

the MLA is suitable for detecting a range of mutagenic events, which has led to its inclusion in regulatory testing.

3.1.2.1.2 Hypoxanthine Guanine Phosphoribosyltransferase (HGPRT) Assay

This assay, widely used for mutagenicity testing, is usually conducted in CHO cells and uses the target gene at the *hgprt* locus on the X chromosome (Li *et al*., 1987). The HGPRT enzyme is important for purine salvaging and will incorporate toxic analogues, for example, 6-thioguanine or 8-azaguanine into DNA, resulting in cell death or inhibition of cell growth. A forward mutation results in cells with an inactive gene product that permits the survival of the cells and formation of colonies in the presence of toxic analogues (**Figure 3**). The use of suspension cultures and soft agar cloning in the CHO assay provides a sensitive test for the identification of mutagens (Oberly *et al*., 1990) and is a viable alternative to the traditional monolayer procedure (O'Neill *et al*., 1977). A modification of this assay permits treatment in suspension and soft agar cloning comparable to the MLA. This methodology eliminates the risk of metabolic cooperation and the trauma of trypsinization. In addition, a larger population of cells can be treated and cloned for mutant selection (Oberly *et al*., 1993).

3.1.2.2 Gene Mutation Assays In Vivo

The analysis of mutations in somatic or germ cells in suitable tissues is carried out after *in vivo* treatment in rodents and also employs cells from humans exposed to chemicals.

3.1.2.2.1 Hypoxanthineguanine Phosphoribosyltransferase (HPRT) Assay in Human and Rodent Cells

The hypoxanthineguanine phosphoribosyltransferase (HPRT) assay employing human peripheral blood lymphocytes has been developed and validated for the understanding of *in vivo* somatic cell mutagenesis (Albertini, 1985). The HPRT enzyme helps salvage purines for DNA synthesis, however, it can also utilize

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toxic analogues, rendering them cytotoxic to the normal cells. A mutation in the *hprt* gene leads to the mutated cells growing in the presence of these toxic analogues (e.g. 6-thioguanine (6-TG)). Lymphocytes from blood collected from control and exposed populations are cultured with and without 6-TG, and assayed for positive selection by either autoradiography or cloning, as recommended by the International Programme on Chemical Safety (IPCS) guidelines (Albertini *et al*., 2000). The increased *hprt*-mutated T-lymphocytes in blood of an exposed population demonstrate a genotoxic effect. Peripheral blood T-cells from melanoma patients show increased *hprt* mutant frequencies when compared to controls (Albertini, 2001). The assay is, however, limited in human studies due to confounding factors such as ethics, genetic differences, gender, age and lifestyle (dietary and smoking status), which affect the recovery of 6-thioguanine-resistant clones (Jones *et al*., 1995) and need to be accounted for during the analysis. Also, sampling time has to be considered to obtain and optimally detect mutant T-lymphocytes, since the blood cell population is periodically renewed (Cole and Skopek, 1994).

HPRT is also finding use for *in vivo* mutations in germinal cells (Albertini, 2001). The HPRT assay using rodents has been developed in lymphocytes from spleen, serving as a surrogate model for *in vivo* tissue-specific mutation studies. The assay in humans and rodents is capable of detecting a range of mutations including base-pair substitutions, chromosomal recombinations, inversions and deletions (Albertini *et al*., 1997; 2000; Cole and Skopek, 1994), induced by promutagens as well as direct-acting mutagens (Aidoo *et al*., 1997; Casciano *et al*., 1999; MacGregor *et al*., 2000). However, since only one copy of the *hprt* gene is present, a mutation would result in loss of heterozygosity, and thus large deletions, chromosomal recombination and nondisjunction cannot be detected by the assay (Dobrovolsky *et al*., 1999).

Most carcinogens have been shown to induce *hprt* mutations in lymphocytes of rats. Caloric restriction in ageing rats has been shown to reduce accumulation of spontaneous *hprt* mutation, thereby leading to decreased tumour incidence and retardation in ageing (Aidoo *et al*., 2003).

Though the assay has been widely used in genotoxicity testing, a detailed performance assessment is necessary for it to be used for routine testing.

3.1.2.2.2 Transgenic Animals for *In Vivo* Mutagenesis

In vivo mutation assays developed in transgenic mice, with reporter genes, help in the measurement of spontaneous and chemically induced mutations in different tissues (Mirsalis *et al*., 1995; Lambert *et al*., 2005; Provost *et al*., 1993). These assays allow for rapid screening of frequency of mutants/mutations in DNA from somatic and germ tissues of the animal (Singer *et al*., 2006; Thybaud *et al*., 2003). The assay is accepted by the COM guidelines for the assessment of *in vivo* mutagenesis (Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM), 2000) and efforts are being made towards formulation of OECD guidelines (Lambert *et al*., 2005). The transgenic mouse mutation assays are lambda phage-based, which employ the bacterial *lacZ* or *lacI* gene as a reporter gene for mutations (Schmezer and Eckert, 1999) and are available as two commercially available transgenic mice named Muta Mouse and Big Blue Mouse (Kohler *et al*., 1990; Nohmi *et al*., 2000), respectively (**Figure 4**). Other systems are also available for testing larger deletions not detected by the *lac* systems, for example, *LacZ* plasmid mouse and *gpt* delta rodents (Lambert *et al*., 2005; Nohmi *et al*., 2000).

In the *lacI* system two transgenic mice (C57BL/6 and B6C3F1) and a rat, (Big Blue transgenic rat system in Fischer rats), have been constructed with each cell containing multiple copies (30–40) of a bacteriophage lambda shuttle vector (λLIZα), at a single locus on chromosome 4 (Dycaico *et al*., 1994). These vectors contain a *lacI* gene as a mutational target. The mice are exposed to the test compound and then DNA is extracted from the tissue of interest (Heddle *et al*., 2003). The shuttle vector is recovered from genomic DNA by *in vitro* packaging and infected into *E. coli* cells, which are then plated onto dishes to form plaques or colonies. The mutated plaques are selected on the basis of their ability to cleave a chromogenic substrate X-Gal. The wild-type phages encode for the *lacI* repressor, which suppresses the action of the enzyme, hence the normal plaques appear colourless. A mutation in the *lacI* gene inactivates the *lac* repressor protein and allows functional expression of β-galactosidase, which cleaves X-Gal, giving a blue colour to the mutant plaques. A significant increase in the mutant frequencies gives a positive response for the test article.

The *lacZ* CD2F1 (BALB/C_DBA2) mouse has the *lacZ* gene in a single EcoRI site of a λgt10 vector (Gossen *et al*., 1989). The principle of recovering the vector and colorimetric detection of mutants is similar to the *lacI* system, however in this system, the β-galactosidase enzyme is active in the wild-type (Heddle *et al*., 2003). Therefore the wild-type cells form a blue plaque and the mutants are colourless. Positive selection of the mutants may also be carried out on *E. coli* host, plated on P-Gal, which is otherwise toxic to cells carrying functional *lacZ* (Vijg *et al*., 1996).

The mutations scored in the *lacI* and *lacZ* transgenic systems consist primarily of base-pair substitution mutations with a few frameshift mutations and small insertion/deletions. A positive response in the assay shows the compound to be genotoxic *in vivo.* Since the sensitivity of the assay is lower as compared to other *in vitro* tests, a positive response is considered more serious,

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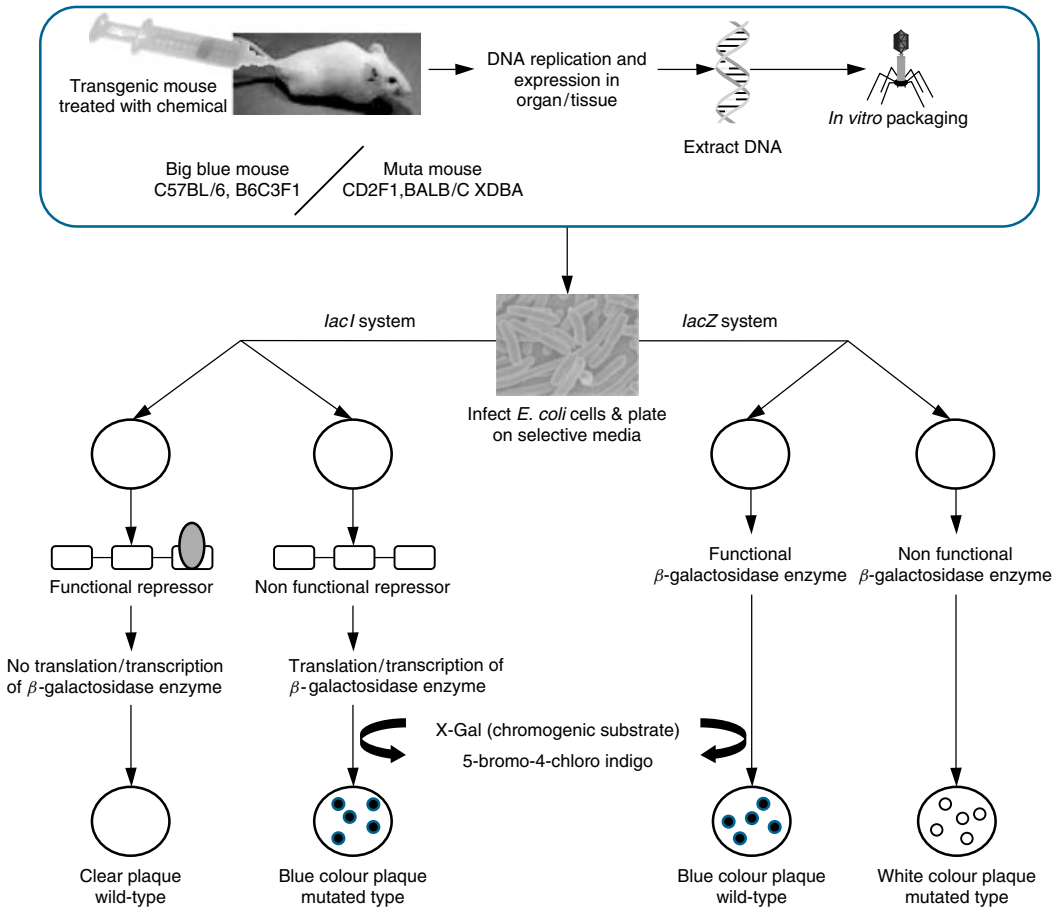

Figure 4 Schematic representation of *in vivo* transgenic rodent assay for mutagenicity.

especially for humans (Nohmi *et al*., 2000). Also mutations induced in the parent germ cells are transmissible to the F1 offsprings (Barnett *et al*., 2002).

The advantages of the transgenic system have been reviewed (Lambert *et al*., 2005). These include flexibility of route of exposure for chemicals, assessment of mutations in any tissue of rodents (due to the neutrality of the transgene) at all stages of development, ease of analysis of the mutations at the molecular level (for deducing the mechanism of mutation induction by different chemicals, and comparison of mutagenesis at different loci), high reproducibility of results in interlaboratory studies and use of the 3Rs of toxicity—reduction (animals can be used for germ/somatic cell mutagenesis, along with other *in vivo* tests like CAs), refinement (in test procedures) and replacement (use of transgenic cell lines). However, constraint in detecting some deletions/insertions, high spontaneous mutation frequency and cost of rodents and consumables are some of its drawbacks (Lambert *et al*., 2005).

Other transgenic models, developed for detecting specific genetic alterations for studying the mechanism of carcinogenesis are promising tools as *in vivo* alternatives to the two-year rodent assay (Storer, 2000; Tennant *et al*., 1995; 1996). Due to their genetic predispositions, these models have increased susceptibility to carcinogens and rapid development of cancer (~24–26 weeks; significantly shorter than the chronic rodent bioassay). These short-term *in vivo* models include the rasH2 transgenic mouse, Tg.AC transgenic mouse and p53 knockout mouse (**Figure 5**). These models have been recommended for cancer testing by the ICH, and the US Food and Drug Administration (US FDA), for new drug applications (Schmidt, 2002). The p53, Tg.AC and rasH2 models are genetically stable (genetic specificity remains in successive generations of breeding), have flexible dosing options and are able to produce a wide spectrum of tumours, depending upon the type of chemical exposure. These assays are hence rapid, reduce the number of animals used in testing and use of an appropriate model gives an accurate human response, aiding in regulatory risk assessment and identification of the mechanism underlying carcinogenesis (Pritchard *et al*., 2003).

The Tg.AC mouse developed by Leder *et al*. (1990), carrys a v-Ha-*ras* oncogene fused to the zeta-globin gene promoter. The v-Ha-*ras* transgene has double point mutations at codons 12 and 59. The altered gene expression (activation) of the transgene shows genetically initiated skin as a target for tumorigenesis (Cannon *et al*.,

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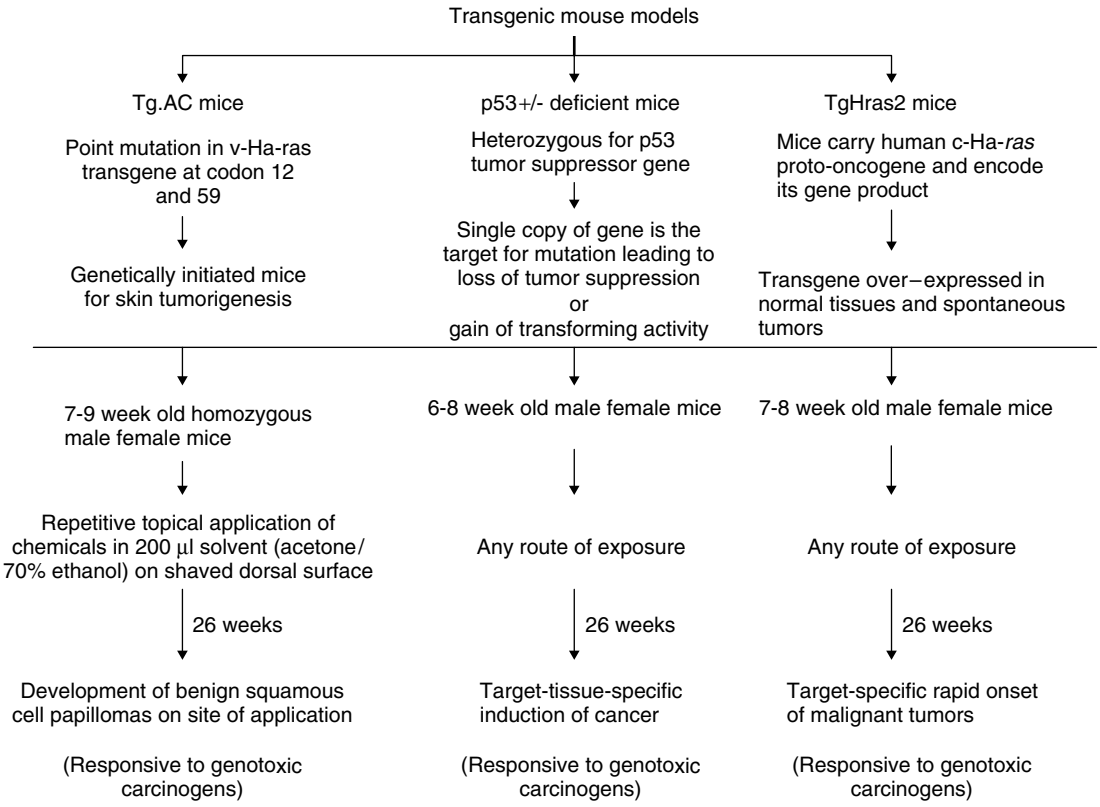

Figure 5 Short-term tests for carcinogenicity in transgenic mouse models. (Reproduced with permission from Bajpayee *et al.*, 2005. © Informa Healthcare.)

1997; Storer, 2000). Repeated application of the skin tumour-promoting agent leads to the development of benign papillomas, which may become malignant. This model has been used for the study and differentiation of initiating agents, tumour-promoting agents and complete carcinogens. Heterozygous p53 +/− deficient mice have a single functional allele and an inactivated null allele of the p53 gene. The mice become nullizygous upon inactivation of the wild-type functional allele due to mutation on carcinogen exposure and develop a high incidence of neoplasia (Tennant *et al.*, 1995). The TgHras2 mice carry a human protooncogene c-H-*ras* and have an endogenous promoter for the c-H-*ras* gene (Yamamoto *et al.*, 1997). The transgene is overexpressed in normal tissues and tumours, for example papillomas and lymphomas, develop in the animal. It has been used to study the effect of carcinogens in Japan (Yamamoto *et al.*, 1998).

The transgenic models are sensitive and specific, providing a better platform for carcinogenicity evaluation *in vivo*.

3.1.2.2.3 Mouse Spot Test

This genetic assay is a rapid and inexpensive test for the detection of somatic cell mutations and has been recommended by the OECD guidelines (OECD 484). Coat colour variation in mice occurs due to mutation in the loci controlling hair colour, resulting in the loss of the heterozygosity. The assay is widely used for the detection of a number of genetic alterations ranging from chromosomal damage to gene mutations (Styles and Penman, 1985). *In utero* treatment of mouse embryos is carried out at a time when pigment precursor cells migrate to the dermis. Mutant clones are recognized by the coloured patches (brown, grey or yellow) of hair at two weeks after birth. Mouse assays detecting genetic instability at endogenous loci predict hazardous effects of an environmental agent and/or genetic predisposition to cancer (Reliene and Schiestl, 2003).

3.1.2.2.4 Mutations in Germ Cells

Genotoxicity tests for monitoring germline mutations induced by chemicals (Russell *et al.*, 1981), which may pose a risk for heritable effects in future generations, are the mouse-specific locus test, mouse heritable translocation assay and dominant lethal assay. These tests are accepted as third-tier tests for gonadal mutations and intergenerational assays (Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM), 2000), while the latter two are also recommended by the OECD guidelines (OECD 485 and 478, respectively).

The mouse-specific locus test detects single base-pair substitutions to insertions/deletions (Favor, 1999) and

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intralocus changes to multilocus deletions (Ehling, 1989). Normally heterozygous offsprings with the wild-type phenotype are produced from chemically treated homozygous mice mated with homozygous recessive tester mice. The tester mice are homozygous for seven loci controlling coat colour and the size of external ear. Simple and rapid screening of F1 offsprings with the recessive phenotype due to mutations in the parent is possible (Russell and Russell, 1996).

The mouse heritable translocation test detects reciprocal translocation in chromosomes, which is the mutual exchange of fragments of two nonhomologous chromosomes (Generoso *et al*., 1980). Several protocols can be used to generate mouse mutations with reciprocal translocations, to understand the biological functions of mammalian genes (Stubbs *et al*., 1997).

Genetic alterations, such as clastogenicity or aneuploidy (structural or numerical CAs) in germ cells *in vivo,* are detected by the dominant lethal assay. The test attributes embryonic death to chromosomal breakage in parent germ cells. Induction of a dominant lethal event after exposure to a test substance indicates that the substance has affected germinal tissue of the test species (Ehling *et al*., 1978). Normal female rats mated with chemically treated males are sacrificed at 14–16 days of pregnancy, and the live/dead embryos from the implants counted. The postimplantation loss from the treated and control groups is compared by determining the ratio of dead to total implants. The preimplantation loss by counting the total implants per female in treated and control groups can also be compared (Brewen *et al*., 1975). The post-/preimplantation loss and fertility rate were recommended as the main parameters to be assessed by the Working Group on Mammalian Germ Tests (Adler *et al*., 1994). A schematic method of data presentation was proposed, which enables the most pertinent assay data and parameters to be viewed and considered simultaneously (Ashby and Clapp, 1995).

Most chemicals/compounds that are recognized as *in vivo* somatic cell mutagens are considered to be genotoxic carcinogens and potential germ-cell mutagens (Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM), 2000). Few compounds are germ-cell-specific mutagens and not somatic mutagens, for example *N*-hydroxymethylacrylamide (NHMA), a mouse carcinogen (Witt *et al*., 2003). The development of transgenic rodent assays and the expanded simple tandem repeat (ESTR) assay, have added value to the detection of germline mutations induced by chemical and radiological agents. The result of these assays is comparable to the specific locus test (Singer *et al*., 2006). The mechanisms of induction of dominant lethal mutations and heritable translocations show that they are dependent on the stage at which the germ cell is treated (Adler, 2000; Generoso *et al*., 1982), with spermatids being most sensitive to induction of dominant lethal mutations (Barnett and Lewis, 2003).

3.2 Assays for Chromosomal Abnormalities

In vivo as well as *in vitro* assays are available for determining the adverse effects of chemicals on the structural and/or numerical makeup of chromosomes. These assays use direct visualization of chromosomal preparations under a microscope. Chemicals/compounds can lead to breakage, translocation insertions/deletions, causing structural changes (clastogenicity) or numerical changes (aneuploidy), which can be studied by analysing CAs in metaphase cells or MN formation (see also **Cytogenetics**). Cultured mammalian cells are most often used for *in vitro* studies, while rodent bone-marrow cells are utilized for *in vivo* studies.

These assays are used as screening tests, along with bacterial mutagenesis assays in the standard battery of tests for determining genotoxicity of chemicals/compounds. The COM guidelines recommend the use of *in vitro* assays for chromosomal alteration in the first stage and the *in vivo* assays in the second stage as follow-up to positive *in vitro* tests. These assays are also recommended for use by other international regulatory agencies like USEPA and ICH (reviewed by Cimino, 2006).

The tests for chromosomal abnormalities include mitotic recombination in yeast, *in vitro* and *in vivo* CA, MN and sister chromatid exchange (SCE) assays.

3.2.1 Mitotic Recombination Assay

The *Saccharomyces cerevisiae* mitotic recombination assay is used to measure gene conversion (unilateral DNA transfer within a gene) or crossing-over (exchange of DNA segments between genes), for assessing the genotoxicity of chemicals (Freedman and Jinks-Robertson, 2002; Zimmermann, 1984). It has been recommended by OECD guidelines (OECD 481). Similar mitotic recombination events in *S. cerevisiae* are induced by double-strand breaks and associated with low and high transcription conditions, showing that transcription promotes DNA breaks (Gonzalez-Barrera *et al*., 2002). A novel sensitive and highly reproducible assay in a diploid construct of *S. cerevisiae* has been developed, to allow efficient detection of mitotic recombination events and forward mutations, as well as chromosome loss or nondisjunction (Schafer *et al*., 2008).

Cells are treated with mutagens in solvents, in the presence or absence of metabolic activation. The colonies are then counted on nonselective or selective media after four to six days. Spell and Jinks-Robertson (2004) have

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described statistical analyses for calculating the true mean of recombination events, and have discussed their application for the regulation of homologous recombination and to avoid the interference of a spontaneous recombination event in culture.

3.2.2 Chromosomal Aberration

A cytogenetic assay for chromosomal abnormalities using metaphase analysis is one of the currently recommended *in vitro/in vivo* tests for the genotoxic/mutagenic potential of a substance. Chemicals that induce structural changes in the chromosome are known as 'clastogens', while those causing changes in the number of chromosomes are 'aneugens'. These compounds cause alterations in the DNA, which may be processed during replication and/or DNA repair into the various types of aberrations visible in chromosomes at the metaphase stage of cell division.

CAs may be present as the whole chromosome or as single chromatids and include lesions such as breaks (deletion/fragments) or rearrangements (inversion/translocation and exchanges). Chromosome-type aberrations arise from alterations in the unreplicated 'G1' chromosome, while chromatid-type aberrations are formed from lesions arising during or after the 'S' phase. Majority of the chemical clastogens are S-phase dependant and induce aberrations only in cells that pass the S phase after exposure. Clastogens acting in the early S phase, lead to higher number of CAs, probably due to limited error-free repair of primary DNA lesions in the cell. Unbalanced chromatid-type aberrations are frequently lethal because both chromatids are affected, leading to structural chromosome damage in both daughter cells. Cytogenetic alterations include shortening of telomeres, formation of dicentric chromosomes, transfer of telomeric DNA to the homologous chromosomes, inactivation of the centromeres, endoreduplication of chromosomes, and other structural and numerical chromosome abnormalities (Obe *et al*., 2002; Pathak *et al*., 1994). Double-stranded breaks in DNA lead to the formation of chromosome translocations or rearrangements commonly seen in cancer cells. Chromosome breaks and aneuploidy cause a hypermutable state, which may lead to the development of cancer (Fenech, 2002a; 2002b). Most cancer cells show aneuploid states, pointing to the fact that aneuploidy destabilizes the karyotype, leading to preneoplastic and ultimately cancer cells (Fenech, 2002a; 2002b).

The *in vitro* CA is used as a first tier test for screening of compounds for their genotoxicity and is accepted by international regulatory agencies USFDA, USEPA and ICH. The assay is conducted according to the OECD guidelines (OECD 473). Cells with stable, well-defined karyotypes, short cell cycles, low chromosome numbers and clearly visible chromosomes are ideal for cytogenetic analysis. The CHO cells and human peripheral lymphocytes have been used extensively for *in vitro* testing (Evans, 1984; Galloway *et al*., 1994; Preston *et al*., 1987a). Cells are treated during a sensitive period of the cell cycle, for example, the S phase, arrested in the metaphase of the first mitotic cycle using a spindle inhibitor and aberrations scored under a microscope (**Figure 6**). The assay may be affected by artifacts or experimental conditions, such as extreme culture conditions (Scott *et al*., 1991), highly cytotoxic concentrations, extreme pH and genotoxicity of the metabolic activation system (Kirkland *et al*., 1989), leading to false positive results. The study should, therefore, be extended till at least some cytotoxicity is observed (Galloway, 2000).

In vivo CA is conducted in mice and hamsters. Animals are treated with the compound for a specified time and then treated with a metaphase-arresting compound, for example colchicines, two hours before sacrifice (Preston *et al*., 1987b). Cells from bone marrow are then collected for cytogenetic analysis (Tice *et al*., 1994). CAs are counted under a microscope from Giemsa-stained metaphase spreads prepared on slides. The *in vivo* CA test is used in the first tier (US Environmental Protection Agency, 1997; International Conference on Harmonization, 1999) or second tier (Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM), 2000) of genotoxicity testing to analyse if the chemical is an *in vivo* somatic-cell mutagen, and is conducted according to the OECD guidelines (OECD 475).

CAs have also been employed in human biomonitoring studies (Au *et al*., 2001; Au, 2003; Wolff, 2007) as a biomarker of DNA damage (Albertini, 2000). IPCS guidelines for the planning, performing and conduct of the assay in blood lymphocytes for monitoring human genotoxicity have been reviewed by Albertini *et al*. (2000). An association has long been shown between the level of CAs in human peripheral lymphocytes and the risk of cancer (Bonassi *et al*., 2000; Hagmar *et al*., 2004).

The analysis for aberrations is time-consuming and use of fluorescent *in situ* hybridization (FISH) (Johnson *et al*., 1998) or chromosome painting, to highlight the alterations in the number of copies of selected chromosomes (Natarajan, 2001), has also been made. FISH allows increased resolution of the detection of aberrations and leads to a better understanding of the mechanisms of formation of chromosome aberrations (Natarajan and Boei, 2003). These methods are efficient tools for tumour diagnosis. Specific chromosomal translocations have been shown by FISH painting in mouse plamacytoma (MPC), human Burkitt lymphoma (BL) and other B-cell-derived tumours (Szeles, 2002). Spectral karyotyping and multicoloured or multiplex-fluorescence *in situ* hybridization (m-FISH) allow for visualization of

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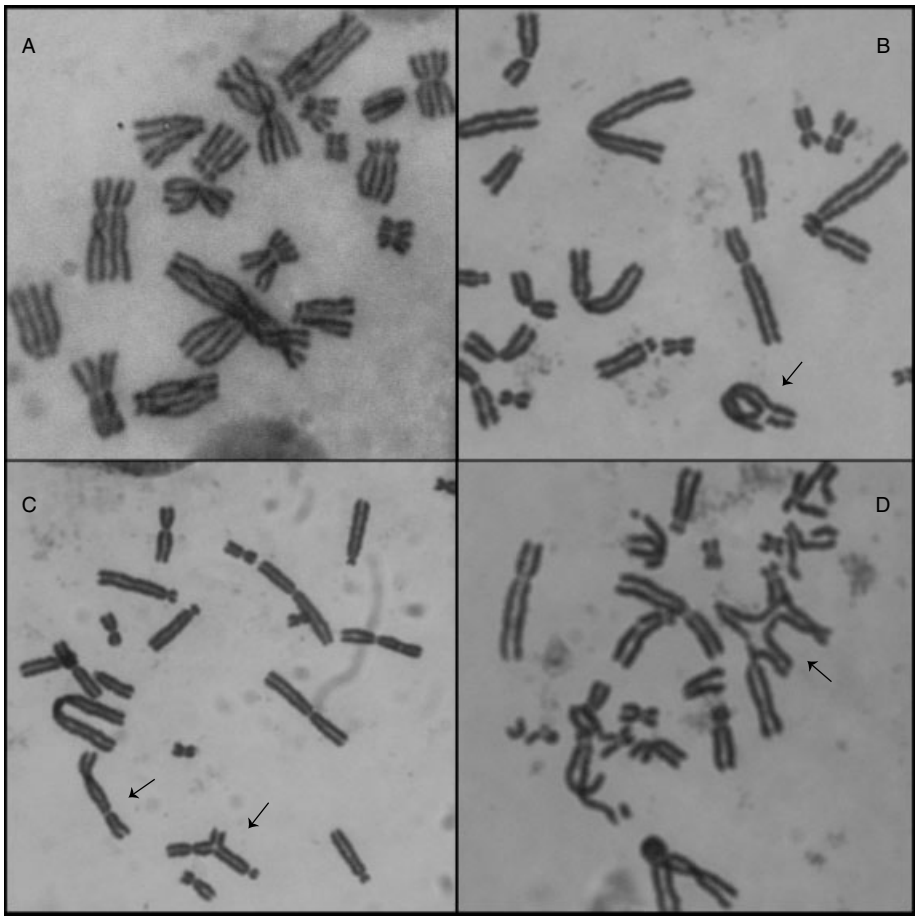

Figure 6 Chinese hamster ovary (CHO) cells treated with ethyl methanesulfonate showing chromosomal aberrations: (A) endoreduplication; (B) centric rings; (C) dicentric interchange and (D) reciprocal translocation.

all chromosomes of a metaphase in a single hybridization step, which enables screening for aberrations and the comprehensive analysis of complex karyotypes (Tchinda *et al*., 2003; Brizard *et al*., 2004; Hande *et al*., 2005).

3.2.3 Micronucleus Assay

Micronucleus is an extranuclear chromatin-containing body formed during mitosis and made of either chromosomal fragments or whole chromosomes (Schmid, 1975). The MN assay can be conducted both *in vitro* (in cell cultures and primary cultures of lymphocytes) and *in vivo* (in bone marrow or blood cells of rodents) for detection of both aneuploidy and clastogenicity (**Figure 7**). MN formation in peripheral blood lymphocytes in human populations occupationally exposed to genotoxic compounds is a valuable cytogenetic biomarker (Bolognesi *et al*., 2004), and widely used in human biomonitoring studies (Albertini *et al*., 2000; Costa *et al*., 2006; Neri *et al*., 2003).

In vivo MN assay is usually conducted in the erythrocytes from bone marrow of rodents according to the OECD guidelines (OECD 474) and the method of Schmid (1975). The animals are treated with the chemical (single or multiple dosing) and bone marrow collected after animal sacrifice. Cell smears are prepared on slides, stained with Giemsa and counted under a microscope (**Figure 8**). A large number of chemicals have been studied using this method for the assessment of genotoxicity of chemicals, including clastogens and spindle poisons (Hayashi *et al*., 1994; Sato and Tomita, 2001). This assay finds its place in the first tier (US Environmental Protection Agency, 2005; International Conference on Harmonization, 1999) or the second tier (Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM), 2000) of the genotoxicity testing battery. The *in vivo* MN assay has also been conducted using other tissues, for example, liver (Igarashi and Shimada, 1997; Müller-Tegethoff *et al*., 1997). FISH can also be utilized in the *in vivo* MN test to help discriminate MN of clastogenic and aneugenic origins (Attia *et al*., 2003). The use of flow cytometry for analysis of MN has helped in making the assay high throughput (Kissling *et al*., 2007; Hayashi *et al*., 2000). The IWGT *In Vivo* MN Assay Working Group has recently discussed issues related to the performance of the assay (Hayashi *et al*., 2000) and reached a consensus on the use of automated scoring using flow

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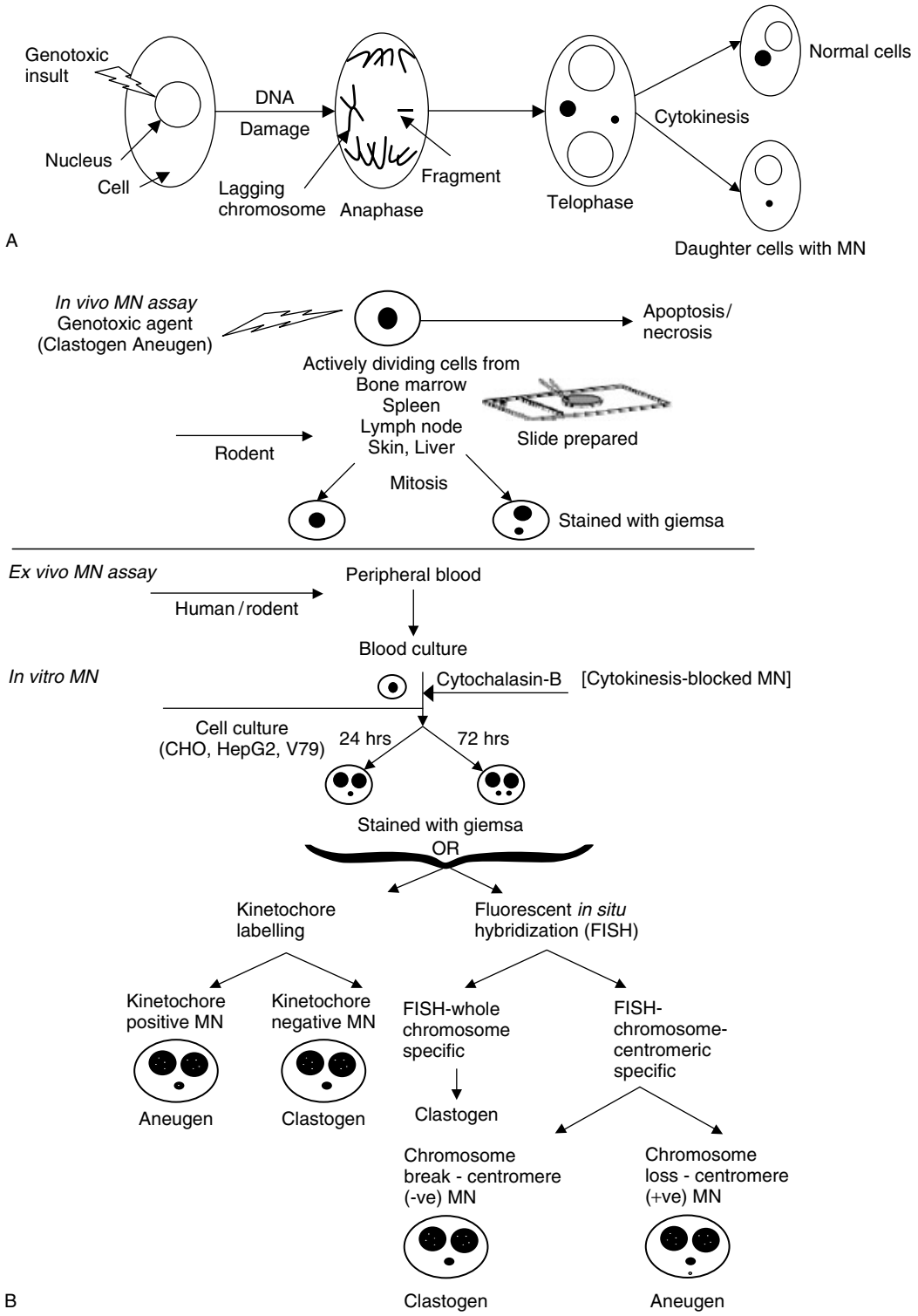

Figure 7 Schematic representation of: (A) micronucleus formation; (B) micronucleus assay.

cytometry (Hayashi *et al*., 2007). The use of blood reticulocytes from young rodents was found to be acceptable for conducting an MN assay, while an MN assay in liver cells from young rodents could be used as a good alternative methodology for genotoxicity assessment (Hayashi *et al*., 2007). The single-dose rat bone marrow MN assay has enhanced sensitivity compared to the subchronic rat dominant lethal assay, thus the rodent bone marrow MN assay can be used as a predictor of possible germ-cell mutagenicity of chemicals (Hayashi *et al*., 2000; Tinwell *et al*., 2001). The IWGT also have reviewed compounds, for example, which are specifically detected in *in vivo*

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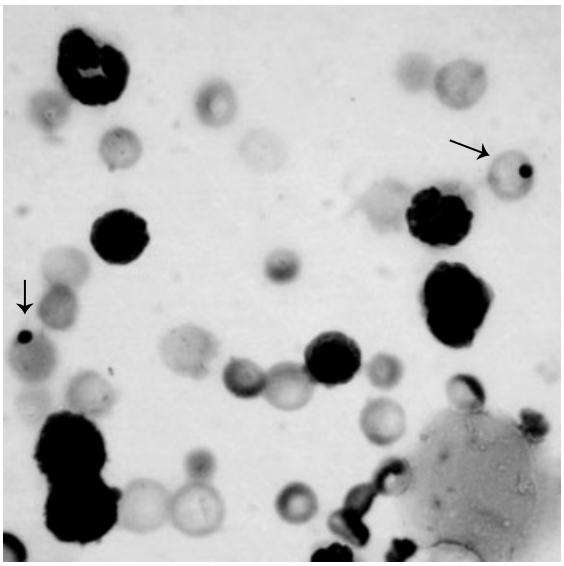

Figure 8 Mouse erythrocyte showing micronucleus.

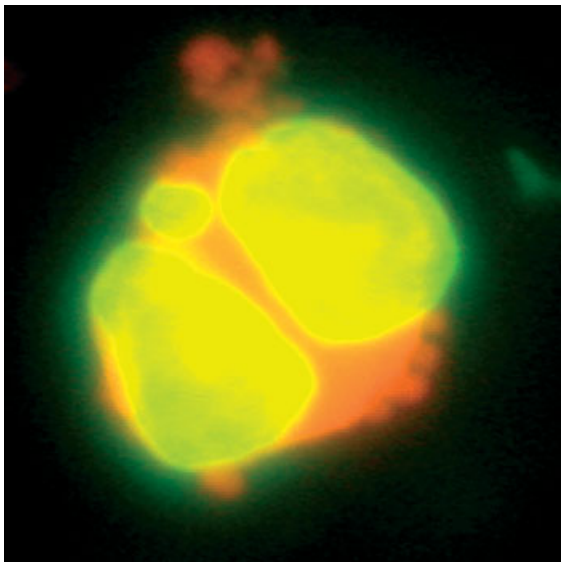

Figure 9 Chinese hamster ovary (CHO) cell treated with ethyl methanesulfonate showing a micronucleus in a binucleate cell.

tests and give a negative response *in vitro*, and have formulated a decision tree for the evaluation of such chemicals (Tweats *et al*., 2007).

The *in vitro* cytokinesis-blocked micronucleus (CBMN) assay is the most widely used assay for genotoxicity assessment (Fenech and Morley, 1985; Kirsch-Volders *et al*., 1997; Rosefort *et al*., 2004) and human biomonitoring (Bonassi *et al*., 2001; 2007; Kirsch-Volders and Fenech, 2001). The MN assay can also be used as a biomarker for cancer (Bonassi *et al*., 2005). The *ex vivo*/*in vitro* CBMN assay for human biomonitoring was validated by the Human MicroNucleus (HUMN) Working Group, which has discussed variations in the experimental and confounding factors influencing the MN frequency (Fenech, 2002a; 2002b; Fenech *et al*., 1999; 2003a; 2003b; Kirsch-Volders *et al*., 2000; 2003). In this assay, cells are treated with the test chemical and cytochalasin-B, an inhibitor of contractile filaments, which prevents the cell cycle at the cytokinesis stage, thus preventing division of the cytoplasm. Hence bi- and multinucleate cells are obtained and the MN are counted in binucleate cells (**Figure 9**). Dicentric and centric-ring chromosomes that arise in the anaphase are visible in CBMN assays as nucleoplasmic bridges (NPBs) due to inhibition of cytokinesis (Thomas *et al*., 2003). Evaluation of NPBs provides a valuable measure of chromosome breakage/rearrangement that would not be available in the MN assay. The analysis of MN can also be carried out with the help of flow cytometry in CHO cells (Wagner *et al*., 2003) and cultured lymphocytes (Nusse and Kramer, 1984).

The nature of any MN formed can be determined with use of FISH, using human pancentromeric DNA probes (Iarmarcovai *et al*., 2006), kinetochore and centromeric staining (Dhawan *et al*., 2003; Lynch and Parry, 1993; Natarajan, 1993; **Figure 10**), which help to categorize the test compound as a clastogen or aneugen (Parry *et al*., 2002; Parry and Parry, 2006), contributing to the sensitivity and specificity of the assay (Mateuca *et al*., 2007). The time of treatment and sampling in relation to the cell cycle influence the formation of MN. Since the majority of clastogens give rise to chromosomal damage during the 'G1' and S phases, leading to formation of micronuclei in subsequent cell cycles, at least one cell cycle is allowed between treatments and harvesting of cells. At shorter sample intervals, MN may escape detection, while longer sampling time may lead to loss/dilution of MN in the cells. Micronuclei induced by dysfunction of spindle or S-phase-independent clastogens can be seen at shorter sampling intervals than S-phase-dependant chemicals.

Discrimination between mutagens inducing DNA breakage (clastogens) or chromosome loss/nondisjunction (aneugens) contributes to the high specificity of the MN assay. COM guidelines (Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM), 2000) and the IWGT have suggested the inclusion of the *in vitro* MN assay as an alternative test to the *in vitro* CA. Protocols for human primary lymphocytes and cell lines were validated and harmonized (Kirsch-Volders *et al*., 2003) and are now in the final phases of validation by the European Centre for the Validation of Alternative Methods (ECVAM) and of acceptance in the OECD guidelines (Mateuca *et al*., 2007). The MN assay is as sensitive as the CA, having a high concordance with the *in vitro* CA, has good predictivity for cancer, is rapid, less resource intensive, inexpensive, automated and has a higher statistical power (Mateuca *et al*., 2007). The performance of three *in vitro*

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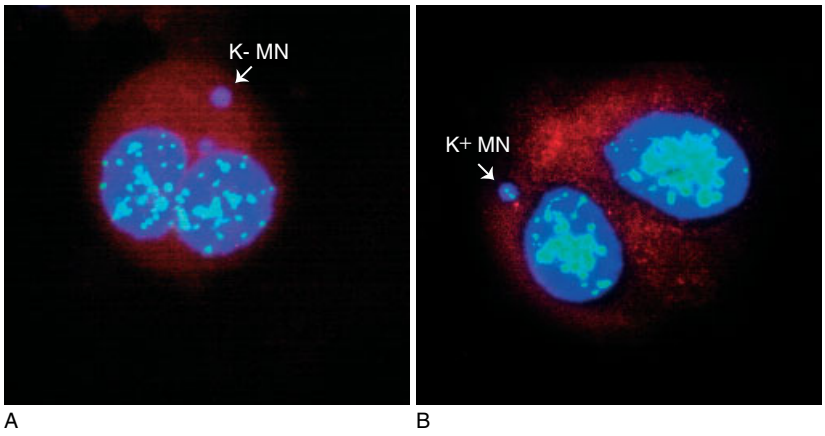

Figure 10 Human lymphocytes treated with doxorubicin, depicting kinetochore (A) negative and (B) positive micronuclei.

tests: MN assay, CA and MLA were also compared for 700 chemicals, where the MN assay gave 84% concordant results with MLA, and had a better predictivity than MLA (Lorge *et al*., 2007; Kirkland *et al*., 2005).

3.2.4 Sister Chromatid Exchange Assay

SCEs represent a symmetrical exchange in chromatid segments within a single chromosome at homologous loci, which involve DNA breakage and reunion during cell division in mammalian cells and are end products of homologous recombination (Helleday, 2003). There is no altered chromosome morphology and differentially stained or labelled sister chromatids are detectable in the SCE assay (Tucker *et al*., 1993), however the mechanisms that mediate SCE are not understood. SCE *per se* is not lethal or cytotoxic, but has been extensively used as an end point in cytogeneticity/mutagenicity testing.

An SCE assay can be performed *in vivo, in vitro* and *ex vivo* (Perry and Evans, 1975) and is conducted according to the OECD guidelines (OECD 479). Lymphocytes from animals or humans can be removed from an exposed population and cultured in presence of BrdU (*in vivo-in vitro* SCE), and cell lines are used for *in vitro* studies (Sayed Aly *et al*., 2002), while *in vivo* SCE is performed on animals implanted with BrdU tablets, or given a continuous infusion of BrdU (OECD 479). Exposure to chemicals is given in the presence or absence of S9 metabolic activation systems in the early S phase and allowed to pass through one cell cycle. The detection of SCE involves pretreatment of cells with agents, that is, BrdU and H^3-thymidine, which are incorporated into the DNA and induce SCE. The removal of SCE-inducing damage is seen by a reduction in SCE frequency with time between exposure and sampling of cells for analysis *in vivo*. Thus these times are important determinants in the final yield of SCE.

A large number of chemicals have been shown to increase SCE frequency in animal/human cells *in vivo* (Rodriguez-Reyes and Morales-Ramirez, 2003) as well as *in vitro* (Mahata *et al*., 2004; Poma *et al*., 2003; Yüzbaşioğlu *et al*., 2008). The observations suggest that SCE inducing damage can be removed before the S phase and that formation of SCE is closely linked to the replication process.

Many SCE-inducing agents are mutagens. The majority of induced SCEs do not represent recombinational/mutational events, but a fraction of exchanges occur at nonhomologous loci and these unequal SCEs could represent mutational events or chromosomal deletion, or be involved in DNA amplification.

3.2.5 Chromosomal Alterations in Germ Cells

Genetic risk due to exposure of chemicals in mammalian germ cells (female and male), may be analysed using cytogenetic end points, such as structural and numerical CA, micronuclei and SCEs. The metaphase analysis is difficult and time consuming, but more feasible in male (spermatogonia, spermatids) than female germ cells (oocytes). The use of FISH has also been made for detection of CAs and MN (Russo, 2000). Germ-cell MN assays in rodent spermatids permits the analysis of chromosomal damage during meiosis. Aneuploidy assays in mouse genetic models have been developed to identify risks of chemical exposures or age in rodent germ cells (Eichenlaub-Ritter, 2005). These models have structural chromosomal rearrangements, which predisposes them for aneuploidy, and thus are a tool for detecting aneugens.

3.3 Tests for Primary DNA Damage

The evaluation of DNA damage and repair due to exposure to chemicals has been used in short-term tests for genotoxicity studies. These tests utilize nonreplicative DNA synthesis and involve the measurement of DNA strand breakage by the alkaline elution method,

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detection of DNA adducts through ^{32}P-postlabelling and the Comet assay.

3.3.1 Unscheduled DNA Synthesis

DNA repair mechanisms such as excision repair can be used for the detection of chemically induced DNA lesions. The damaged segment of the DNA in mammalian cells is removed by excision enzymes and DNA repair synthesis occurs using the opposite strand as the template. This repair can be detected by autoradiography, after incorporation of H^3-thymidine, as UDS, that is, DNA synthesis outside the S phase of the cell cycle (Madle *et al*., 1994).

In vitro UDS is carried out on primary rat hepatocytes, which are sensitive to the effect of DNA-damaging chemicals, and is conducted as recommended by the OECD guidelines (OECD 482), as well as those of the US Environmental Protection Agency (1998). These cells do not require metabolic activation and are capable of metabolizing procarcinogens to the active carcinogen (McGregor and Anderson, 1999). Since these cells are epithelial in origin, monitoring of genetic damage is relevant to the *in vivo* situation. The autoradiographic method of *in vitro* UDS was found to be sensitive for detecting genotoxic damage by mutagens and pro mutagens in human hepatoma cells HepG2 (Valentin-Severin *et al*., 2004). Many chemicals that are positive for UDS *in vitro* are also positive in the Ames test, but not all are rodent carcinogens. The use of *in vitro* and *in vivo* UDS as supplemental tests in genotoxicity testing of hair dyes has been recommended by the European Scientific Committee on Cosmetics and Non-Food Products (SCCNFP) guidelines and its inclusion in the test battery has been discussed (Kirkland *et al*., 2005).

The rodent liver (*in vivo*) UDS assay is an established test for evaluating the genotoxicity (DNA damage and repair) induced by chemicals in the liver and is appropriate for compounds that require metabolic activation to express genotoxic activity (Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM), 2000). Use of *in vivo* UDS has been recommended (OECD 486) independent of the results of *in vitro* UDS and as a follow-up to negative results in the standard *in vivo* tests (Kirkland *et al*., 2005).

Ex-vivo UDS assay is designed for the complex patterns of metabolic activation, detoxification uptake distribution and excretion of chemicals (Mirsalis and Butterworth, 1980). The hepatocytes from rats are isolated at two points, that is, 2–4 hours and 12–16 hours after *in vivo* treatment, and cells allowed to undergo *in vitro* UDS. The detailed procedure has been reviewed by Casciano (2000). The positive results from UDS performed *in vivo* or *in vitro* are highly predictive of rodent carcinogenicity. DNA repair capacity and its effect on cancer susceptibility in humans is an important area of investigation (Berwick and Vineis, 2000). UDS in human leucocytes has become a popular tool for estimating an individual's DNA-repair capacity and sensitivity to mutagens (Pero and Ostlund, 1980). Nucleotide excision repair (NER) is differentially expressed in human tissues. A unique culture system in primary cultures of lymphocytes and foreskin fibroblasts to measure NER capacity using the the UDS functional assay has been carried out to study the baseline DNA repair in normal human cells (Kelly and Latimer, 2005; Latimer *et al*., 2003). This helps in evaluation of the effects of 'mutator' genes as aetiological factors in the development of cancer (Latimer *et al*., 2003).

The UDS assay has also been conducted in male germ cells (spermatogonia, meiotic spermatocytes and early spermatid stages) and its correlation with other heritable mutation tests indicates that the *in vivo/in vitro* spermatocyte DNA-repair assay is useful in predicting the mutagenic potential of chemicals in male germ cells (Inoue *et al*., 1993; Sotomayor *et al*., 1996; 1999; Sotomayor and Sega, 2000).

3.3.2 Single Cell Gel Electrophoresis/Comet Assay

Conventional genotoxicity assays, such as CAs and MN, are time-consuming, cumbersome and require pooled cell populations, besides requiring skilful personnel. A simple, rapid, sensitive, reliable, inexpensive and visual microgel electrophoretic technique, to assess quantitatively and qualitatively the DNA damage in single cells, was developed by Ostling and Johanson (1984). This technique has since been modified and improved for the detection of different types of DNA damage (double- and single-strand breaks) and has now become a state-of-the-art technique known as the single-cell gel electrophoresis/Comet assay (Olive *et al*., 1990; Singh *et al*., 1991). The Comet assay has gained wide acceptance and popularity during the last two decades as a short-term test to measure the level of DNA damage and repair *in vitro* and *in vivo*, as well as in human biomonitoring studies. Its ability to evaluate DNA damage in nonproliferating cells makes it a useful tool to work on any eukaryotic cell.

The assay is conducted according to the Comet assay guidelines (Tice *et al*., 2000; Hartmann *et al*., 2003) and is recommended as an *in vivo* test in the second stage of genotoxicity testing (Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM), 2000), while the IWGT workgroup has recently made recommendations for the assay to be accepted by international agencies (Burlinson *et al*., 2007). The *in vitro* Comet assay has been proposed as an alternative to cytogenetic assays in early genotoxicity/photogenotoxicity screening of drug candidates (Witte *et al*., 2007). Cells of interest are embedded in agarose on a microscope slide and lysed

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under high salt conditions. The DNA is subjected to unwinding and electrophoresis under neutral or highly alkaline conditions (pH $\geq$ 13). The migrated DNA after neutralization is stained with an appropriate dye, for example ethidium bromide, propidium iodide, YOYO-1, Hoescht 33258, 4′,6-diamidino-2-phenylindole (DAPI) or acridine orange, and visualized under a fluorescence microscope (**Figure 11**). The DNA damage is then scored using software, or by visual scoring (Kumaravel *et al*., 2009). Several parameters can be monitored by the software, however the most frequently used are tail DNA (%), tail length (μm) and tail moment (arbitrary units).

The assay has been extensively used in evaluating DNA damage in different models, ranging from prokaryotes (bacteria) to human beings (Dhawan *et al*., 2009). The assay is a sensitive biomarker for monitoring ecogenotoxicity using sentinel species such as earthworms (Di Marzio *et al*., 2005), molluscs (Lee and Steinert, 2003) and fish (Dhawan *et al*., 2009; Winter *et al*., 2004). The studies in *Drosophila* have shown it to be a good alternative to animal models for the assessment of *in vivo* genotoxicity of chemicals using the Comet assay (Mukhopadhyay *et al*., 2004; Siddique *et al*., 2005a; 2005b). It is a valuable tool in fundamental DNA damage and repair studies (Speit and Hartmann, 2005; Olive and Banath, 2006), genotoxicity testing (Møller, 2005) and human biomonitoring (Basaran *et al*., 2003; Kassie *et al*., 2000; Møller, 2000, 2006a; Palus *et al*., 2003). The assay has also been used for dietary intervention studies (Glei *et al*., 2005; Møller *et al*., 2004; Wilms *et al*., 2005) and clinical studies (Wynne *et al*., 2007; McKenna *et al*., 2008). White blood cells are the most frequently used cell type for Comet assay in human biomonitoring studies (Angerer *et al*., 2007; Møller, 2006b), however other cells have also been used, for example, buccal (Szeto *et al*., 2005), nasal (Mussali-Galante *et al*., 2005), sperm (Delbes *et al*., 2007; Schmid 2007; Singh *et al*., 2003), epithelial (Graham-Evans *et al*., 2004) and placental cells (Augustowska *et al*., 2007). The Comet assay has also been used for detecting genotoxicity in plant models (Dhawan *et al*., 2009; Gichner *et al*., 2006) with cells from leaves (Gichner and Plewa, 1998), stems and roots (Vajpayee *et al*., 2006). The Comet assay in plants can be incorporated as a test for *in situ* environmental monitoring.

The assay is versatile in the detection of single- and double-strand breaks, DNA crosslinking (Singh, 2000), apoptosis (Singh, 2000) and necrosis (Fairbairn *et al*., 1995). The specificity and sensitivity of the assay is increased by use of lesion-specific enzymes (Collins, 2004), hence, broad classes of oxidative DNA damage, alkylations, and ultraviolet-light-induced photoproducts can be detected as increased amount of DNA in the tail (Giovannelli *et al*., 2003; Møller, 2006a). The Comet assay has also been coupled with FISH (Comet-FISH) for evaluating DNA-specific damage and repair (Glei *et al*., 2009; McKenna *et al*., 2008; Spivak *et al*., 2009).

Although the Comet assay is a useful technique, the variability in results from different laboratories, interpretation of the results and lack of validated studies are some of its disadvantages.

3.4 Cell Transformation Assays

Induction of phenotypic alterations that are indicative of neoplasia in cell cultures is defined as cell

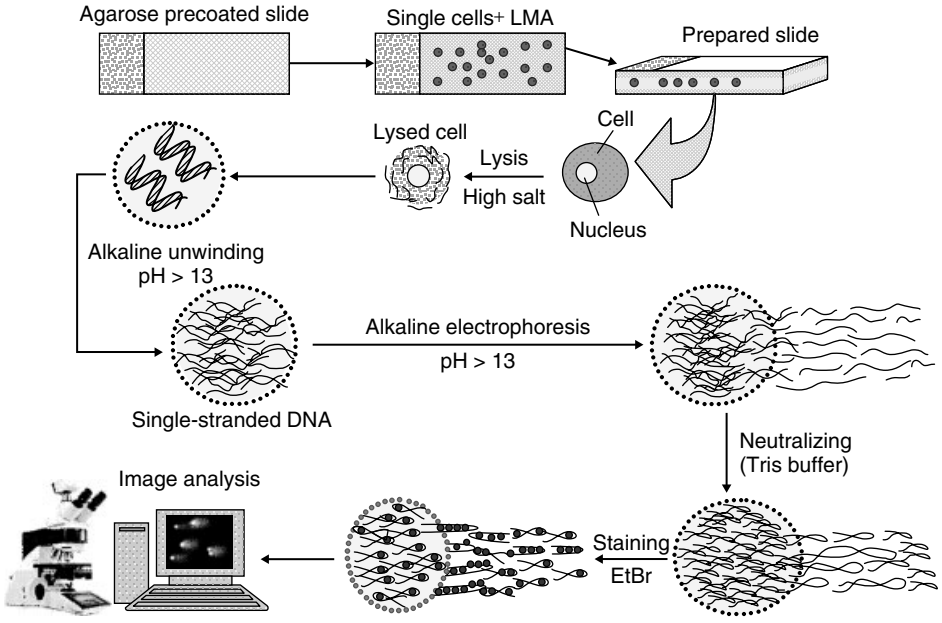

Figure 11 Comet assay protocol.

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transformation (Barret *et al*., 1986; LeBoeuf *et al*., 1999). The mammalian cell culture (*in vitro*) transformation assay is based on the assumption that neoplastic transformation in the cultured cells occurs by the same mechanism as neoplastic alterations of cells *in vivo*. These assay systems are important short-term tests, used to study the multistage process of neoplastic transformation, and therefore the mechanism of action of genotoxic and nongenotoxic carcinogens (Yamasaki *et al*., 1996).

Morphological transformation of fibroblast cultures shows loss of growth inhibition and cell-to-cell orientation. These cells also have the capacity to produce tumours in immunosuppressed animals. The Syrian hamster embryo (SHE) and Balb/c cell systems are frequently used in this assay.

3.4.1 Syrian Hamster Embryo (SHE) Cell Transformation Assay

This assay was developed by Berwald and Sachs (1965) for detecting the carcinogenic potential of chemical, physical and biological agents (Engelhardt *et al*., 2004; Isfort, 2000; Yamaguchi and Tsutsui, 2003). The assay is conducted in primary cultures of SHE foetal cells (12–14 days of gestation) to test. Alkylating agents, polyaromatic hydrocarbons, nitrosamines and amides, aromatic amines and so on have been tested for their ability to produce morphological transformations in this assay. An OECD guideline has also been drafted for the assay (OECD 965).

The SHE cells are plated onto culture dishes and, after obtaining small colonies, the cells are treated with test chemicals for 24 hours or for the entire period of the study. Colonies are allowed to grow for a week after removal of the test chemical. The resultant colonies formed are fixed with methanol and stained with Giemsa (Barrett *et al*., 1986).

The assay provides the advantage of employing normal diploid cells as target cells, which have a wider range of metabolic capabilities than other cell lines, allowing procarcinogens to convert into the active form, and thus it does not require any exogenous metabolic activation. The assay is also rapid and scoring for transformed cells is possible within 10 days from plating.

The early stages of cells are used for the study, but to reduce culture variations and cell variations from different embryos, cryopreserved SHE cells are preferred. Primary cultures are screened for transformation and highly susceptible batches of the cells are cryopreserved for later use. Normal clones have cells growing in a monolayer in an organized pattern and minimal cell stacking at confluency. Morphologically transformed colonies contain cells in an extensive random-orientation stacked growth with criss-crossing at the periphery and centre. The SHE cell assay has been developed at low pH (6.7) for reducing/eliminating the problems associated with the standard assay (Harvey *et al*., 2005; Kerckaert *et al*., 1996). The pH 6.7 SHE transformation assay allows separation of the cell transformation process into two phases, initiation and promotion, as in *in vivo*. This allows chemicals found to be positive in the traditional SHE cell transformation assay to be further classified as initiators or promoters. Cigarette smoke condensate was found to act at both the initiation as well promotion stages of transformation (Breheny *et al*., 2005).

3.4.2 BALB/c3T3 Mice Cell System

The BALB/c3T3 clone, developed by Aaronson and Todaro (1968), is a spontaneously immortalized cell line derived from a mouse embryo with high sensitivity to chemical transformation, and has been utilized for screening potential carcinogens. The assay is being validated by the ECVAM. Chemicals with short half-lives can be screened on this cell line and a dose–response curve can be obtained with relatively a small number of plates having a large number of cells per plate. The carcinogenic potential of a compound tested is assessed by comparing the number of foci in treated and untreated cells. The interpretation of results is better with statistical analysis by Fisher's exact test rather than the t-test (Ponti *et al*., 2007).

Actively growing cells are treated with chemicals with or without activation and then plated onto dishes at an appropriate cell density and the transformation of cells observed. Positive transformed foci of the BALB/c3T3 cells show basophilia, multilayering of the cells, random orientation and invasion into the monolayer (Fitzgerald *et al*., 1989). The *in vitro* transformation assay of BALB/c3T3 cells has been improved with the use of T medium (modified DME/F-12) supplemented with insulin, transferrin, ethanolamine and sodium selenite (ITES) and 2% foetal calf serum (FCS), causing a five-fold higher transformation frequency and earlier appearance of transformation foci (Kajiwara and Ajimi, 2003; Tsuchiya and Umeda, 1995). This assay is useful as a short-term test for detecting potential carcinogens and shows a concordance of 73.5% with rodent bioassay. The sensitivity and reproducibility of the transformation assay could be enhanced with the use of a low concentration of serum in the growth medium after treatment (Kajiwara *et al*., 1997). In the BALB/c3T3 clone, Bhas 42 cells, transfected with the v-Ha-*ras* gene, are the initiated cells in the two-stage transformation paradigm for detecting tumour promoters, and the end point of the assay is the induction of transformed foci (Ohmori *et al*., 2004). The *Fusarium* mycotoxins, fumonisin B_1 and T-2 toxin, are positive in the Bhas 42 promotion assays, which supports their tumour-promoting activity observed in two-stage carcinogenesis tests in experimental animals (Sakai *et al*., 2007).

The only disadvantage of this cell system is that this subclone BALB/c3T3 has a relatively unstable karyotype

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and its properties in culture may also change. Thus, maintenance of cell stock with the original properties is important.

3.5 Assays in *Drosophila melanogaster*

Drosophila melanogaster has been widely used in genetic toxicology as an alternative to animal models for the identification of potential carcinogens, and also for studies of the mechanisms of mutagenesis of chemicals (Vogel *et al*., 1999).

Detection of a variety of genetic damage in germ cells of *Drosophila* is possible because of short generation time (10–12 days) and a broad range of suitable reporter genes. Earlier the sex-linked recessive lethal (SLRL) mutation, an *in vivo* forward mutation assay, was used for detecting both point mutations and small deletions in the germ line of the insect. These mutations, occurring in the X chromosome of *Drosophila*, are phenotypically expressed in the mutant males. Most of the database of chemicals is based on this forward assay. The SLRL assay has been used to study adaptive response of germ cells to alkylating agents, where the response was demonstrated in larvae, but not in adult flies (Savina *et al*., 2003).

The SLRL germ line tests were, however, costly and have been replaced with assays of mitotic recombination, and mutations in somatic tissues (Wurgler *et al*., 1984), allowing for evaluation of a large number of chemicals. The somatic mutations in eye or wing disc cells have been validated, are well established and include the simple and rapid wing-spot test or the wing somatic mutation and recombination test (SMART). Larvae are exposed to the chemical through feeding or inhalation and if loss of heterozygosity occurs in the heterozygous recessive wing markers, (multiple wing hair (*mwh*) and flare (*flr*)), it leads to formation of clones. Various end points such as mitotic recombination, point mutations, deletions, nondisjunction and chromosomal loss can be detected with a single wing-spot phenotype comprising either *mwh* or *flr*. Twin-spot phenotypes, with adjacent *mwh* and flr areas, give direct proof for the recombinogenic property of the test compound (Zimmering *et al*., 1997). This test is highly sensitive and has been validated for distinct types of genotoxic compounds that damage the DNA. The wing-spot test in *Drosophila* is suited to the detection of recombinogenic activity of genotoxic chemicals (Spano *et al*., 2001), and has been used to assess the mutagenic activity of food colourants (Demir *et al*., 2008), airborne particulate matter (Dihl *et al*., 2008), herbicides (Fragiorge *et al*., 2008; Kaya *et al*., 2004), fungicides (Osaba *et al*., 2002) and the genotoxicity of Vitamin A (Klamt *et al*., 2003).

Another test is the white/white$^+$ eye mosaic system for homologous recombination, which is also based on the loss of heterozygosity. In this method, the larvae are exposed to the chemical, and the light spots in the eye of adult females are monitored (Vogel, 1992). Point mutations and deletions which inactivate the white$^+$ allele, CAs and recombination give rise to the expression of white spots in the female genotypes. Parallel detection of these aberrations is possible with eye tissue as the target (Vogel and Nivard, 2000). The assay has been used to detect both mitotic recombination and X chromosome loss, induced by antineoplastic drugs (Rodriguez-Arnaiz *et al*., 2004).

D. melanogaster has also been used as an *in vivo* model for assessment of genotoxicity using the alkaline Comet assay (Bilbao *et al*., 2002; Siddique *et al*., 2005a,b). The mid-gut and brain ganglia cells of larvae have been used in a modified Comet assay for the genotoxicity assessment of a pesticide, cypermethrin (Mukhopadhyay *et al*., 2004).

3.6 Plant Bioassays

Hazardous chemicals (pesticides, industrial waste) may adversely affect the ecosystem, hence *in situ* monitoring of environmental pollutants becomes a necessity. Plants have also been used for the detection of genetic hazards from exposure to genotoxicants in the environment, particularly direct-acting compounds. Short-term plant bioassays utilizing higher plants have the advantage of being rapid, reliable, inexpensive and easy to conduct. These assays detect a wide range of genotoxic end points (CAs, gene mutations) arising in mitotic and meiotic cells from exposure to simple chemicals, as well as complex mixtures (Grant, 1994; Maluszynska and Juchimiuk, 2005). However, promutagens such as nitrosamines, heterocyclic amines and PAHs cannot be detected in these bioassays. Longer life-cycles and major differences in biochemical/pharmacokinetic parameters in plants and mammalian systems are, however, the drawbacks.

The most widely used test systems are conducted in *Allium cepa* (anaphase aberration and MN assays with meristematic root-tip cells), *Vicia faba* (MN and CA assays in root-tip cells) and *Tradescantia paludosa* (MN assay in the meiotic cells and stamen hair mutation tests). Other plants used for the bioassays are *Arabidopsis thaliana*, *Glycine max*, *Hordeum vulgaris* and *Zea mays*. These plant bioassays have been reviewed for their performance in assessing the genotoxicity of chemicals (Ma *et al*., 2005).

Root tips from the *Allium* (onion) bulb and seedlings of *Vicia* are treated with the chemical or environmental sample, fixed and stained, and micronucleated cells counted under the microscope. The CA test in *Vicia*

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root-tip meristem is a reliable short-term bioassay for studying clastogenicity of chemicals, as well as environmental samples (Kanaya *et al*., 1994). *Vicia* has the advantage of possessing six large chromosomes, which are suitable for cytogenetic analysis and, unlike mammalian cultures, no sterile conditions are required for the assay. These cytogenetic assays have been used for determining the genotoxicity of disinfectants in drinking water (Monarca *et al*., 2005), soil samples (Cotelle *et al*., 1999), radioactive isotopes (Minouflet *et al*., 2005) and industrial effluents (Abdel *et al*., 2007).

The *Tradescantia*-micronucleus (Trad-MCN) bioassay is a rapid, efficient and reliable short-term bioassay for detecting clastogens, used in the International Programme on Plant Bioassays (IPPB) under the auspices of the United Nations Environment Programme (UNEP). It measures chromosome damage as the indicator of the carcinogenic properties of screening carcinogens in gaseous, liquid and solid forms. It is specially qualified for *in situ* monitoring of ambient pollutants. Genotoxicity monitoring of metal contamination in soils (Majer *et al*., 2002), genotoxic compounds in air (Carreras *et al*., 2006; Isidori *et al*., 2003; Klumpp *et al*., 2006), drinking-water disinfectants (Monarca *et al*., 2005), and soil lechates (Lah *et al*., 2008) has been carried out using this bioassay. Young inflorescences are treated with the environmental sample, and flower buds with the early tetrad stage of meiotic pollen cells are selected for scoring of the micronuclei (Ma *et al*., 1994). Test results are obtained within 24–48 hours after the exposure, either on site or in the laboratory.

The *Tradescantia* stamen hair mutation assay is a simple, inexpensive approach for laboratory/*in situ* monitoring of liquid and gaseous mutagens, radiation and radioactive materials (Minouflet *et al*., 2005). It is conducted in mitotic stamen hair cells, which are heterozygous for colour change from blue (dominant) to pink (recessive) (Ma *et al*., 1994). A mutation event would produce a pink cell surrounded by blue cells, which are evaluated for mutagenic frequency.

The *Zea mays* assay is a very good plant bioassay for assessing chromosome damage, both in mitosis and meiosis, and for somatic mutations induced by chemicals and radiations (Grant and Owens, 2006).

Rapid and sensitive detection of DNA damage induced by chemicals/heavy metals in plants has been carried out using single-cell gel electrophoresis/Comet assay (Dhawan *et al*., 2009). Assays have been conducted in fungi (Banerjee *et al*., 2008) and algae (Aoyama *et al*., 2003), as well as higher plants like *Vicia faba* (Menke *et al*., 2000), tobacco (Gichner *et al*., 2007) and *Bacopa monneiri* (Vajpayee *et al*., 2006).

The tests results in plant systems have a positive correlation with mammalian assays and are highly sensitive to predicting carcinogenicity, and hence it has been recommended that plant bioassays be used as alternatives for screening in the first tier of genotoxicity testing.

3.7 Current Status of Short-term Tests

A large number of chemical entities being produced each year have to be evaluated for their genotoxic potential before being released for use by humans. These chemicals are tested using an *in vitro* battery of tests recommended by regulatory agencies like ICH, USEPA and USFDA. The *in vitro* tests being used, that is, the Ames test, MLA, *in vitro* MN and chromosomal assay, are highly sensitive for identifying potential genotoxic carcinogens and mutagens (80–90% of rodent carcinogens are detected). However, 75–90% of rodent noncarcinogens are also reported as positive in one of the standard tests leading to a high level of 'false positives' that are not relevant to humans. Kirkland *et al*. (2005, 2006) have examined data for over 700 chemicals that had genotoxicity and rodent carcinogenicity. The analysis with a larger database covering a wider variety of chemical classes demonstrates that reliable cancer health risk determinations cannot be made on the basis of *in vitro* findings alone (Aardema 2007; Thybaud *et al*., 2007). The *in vitro* and *in vivo* clastogenicity tests have been reviewed by Cimino (2006) and Witte *et al*. (2007). The sensitivity of each assay was high and concordant results were obtained (Ames + MLA + CA = 84.7%). The MLA provides concordant results to the *in vitro* CA, however the MLA is preferred due to its simplicity and sensitivity by most of the regulatory agencies (Cimino, 2006; Honma *et al*., 1999b). The *in vitro* MN assay is suggested to be used as an alternative to the *in vitro* chromosome aberration test or the MLA (Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM), 2000; Lorge *et al*., 2007) due to its simplicity, and its ability to analyse aneuploidy along with clastogenicity. A strong agreement of Comet assay and chromosome aberration test (CA test) results was demonstrated, indicating that the Comet assay can be used as a predictive test for the outcome of the CA test, as used for regulatory purposes (Hartmann *et al*., 2003) and it could be used as a alternative to the *in vitro* clastogenicity tests.

However, the predictive value and specificity of *in vitro* mammalian clastogenicity assays was very low for rodent carcinogenicity (Kirkland *et al*., 2005; 2006). The large number of false positives in the *in vitro* assays has raised concern requiring revision of testing methods (Witte *et al*., 2007), as well as follow-up with an *in vivo* assay.

4 RECENT ADVANCEMENTS IN GENETIC TOXICOLOGY STUDIES

Genetic toxicity tests are being improved and modified to meet the challenges of modern-day drug discovery and chemical development. Genomics, proteomics and

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high-throughput screening have added to the sensitivity, specificity and reliability of the test systems. The advancements in genotoxicity studies using molecular biology have made it possible to characterize the potential carcinogenicity of chemicals. These tests include flow cytogenetics, application of microarrays (arrayCGH, electrochemiluminescent arrays) for genotoxicity assessment (Gant, 2007; Hvastkovs *et al*., 2007; Kim *et al*., 2005), real-time PCR (Soberón *et al*., 2007), randomly amplified polymorphic DNA (RAPD; Noel and Rath, 2006) and *in silico* tools (Hayashi *et al*., 2005; Mazzatorta *et al*., 2007) for prediction of genotoxicity and carcinogenicity of chemicals. These newer tests are cost effective, sensitive and permit high throughput for the identification of carcinogens.

Flow cytogenetics has immense potential in assessment of genotoxicity of compounds at low concentrations, and has evolved into a rapid, sensitive technique for analysis of DNA content. The technique involves the use of flow cytometry to assess cytogenetic parameters like MN. Flow cytometry has been used for analysis of cellular DNA content, phenotypic expression of markers, drug transport, calcium flux, proliferation and apoptosis.

Flow cytometry is a laser-based technology that is used to measure biochemical and molecular characteristics of single cells and its constituents (nuclei and other organelles) as they flow in a liquid medium past an excitation source (Cram *et al*., 1998; Jaroszeski and Radcliff, 1999). Flow cytogenetics is used in aneuploidy studies by estimating DNA content in cells (Pajor *et al*., 1998), karyotyping of cells in patients with aberrant chromosomes (Fiegler *et al*., 2003), evaluating MN *in vitro* — in CHO cells (Wagner *et al*., 2003) and cultured lymphocytes (Nusse and Kramer, 1984) — and *in vivo* — in reticulocytes in human blood (Abramsson-Zetterberg *et al*., 2000) and mouse erythrocytes (Cammerer *et al*., 2007) — as well as in studying chromosomal abnormalities, DNA fragmentation and *hprt* variants in the human population (Kotova and Grawe, 2002).

The *in silico* approach involves reactivity, structure, mechanism, activity metabolic data and the use of algorithms with activity relationships for prediction of a broad range of toxicological end points, including carcinogenicity, genotoxicity, hepatotoxicity and various other end points for different classes of compounds. Development of statistical and expert systems helps in genotoxicity predictions. Commercially available *in silico* tools, including DEREK, QSAR, MCASE, ADMEWORKS, Meteor (for prediction of the metabolic fate of xenobiotics), Vitic (structure searchable toxicity database) are widely used in the assessment of genotoxicological end points. Some of these *in silico* systems have been applied to a large number of chemicals using *Salmonella*/microsome assay results from CGX and ECJ databases. With the use of these approaches we get high-throughput results with greater sensitivity, specificity and high concordance (Kirkland *et al*., 2005; Hayashi *et al*., 2005). Genetic toxicology data has accumulated during the last few decades from *in vitro* as well as *in vivo* mutagenicity and carcinogenicity tests. This data, along with the knowledge of structure–activity properties of chemicals, has helped in the creation of computational quantitative structural toxicity relationship (QSTR) models (see also **Structure-Activity Relationships for Carcinogenic Potential**). Such QSTR models provide robust statistical analyses, and the development of neural networks help in predicting the genotoxicity of NCEs, thereby screening them rapidly and avoiding animal testing (Snyder and Smith, 2005). The combination of SAR models and inductive databases for the Ames test has also been proposed (Mazzatorta *et al*., 2007). However, the challenge is to predict organ toxicity as well as toxicity in whole organisms. It will also be an appreciable advancement in this field to predict long-term toxicity effects in chronic treatments.

Microarrays, such as arrayCGH, have been developed for genotoxicity assessment, epigenetic assessment in developmental and transgenerational toxicology and micro RNA (miRNA) assessment in all toxicology types, but particularly developmental toxicology (Gant, 2007). Electrochemiluminescent arrays for high-throughput *in vitro* cytochrome P450-activated genotoxicity screening have also been developed (Hvastkovs *et al*., 2007). These arrays help in the estimation of the relative properties of different enzymes to produce genotoxic metabolites. Complementary DNA (cDNA) microarrays have also been developed with mouse cDNA chips, to evaluate similarities and differences in the gene-expression profiles for genotoxic and carcinogenic compounds (Kim *et al*., 2005). The results provide valuable information for the evaluation of potential genotoxicity using gene-expression profiling in mouse lymphoma cells.

With these advancements in the field of genetic toxicity testing, it is likely that fewer potent genotoxicants will get released into the environment, and the validated short-term tests for mutagenicity and carcinogenicity will help to develop better interventional strategies for human cancers.

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Epigenetic Mechanisms and their Toxicological Significance

Rebecca E. Watson

CONTENTS

1 INTRODUCTION

With the sequencing of the human genome, a great deal of attention has been paid to various sequence polymorphisms and mutations, and which of these are linked to various toxic outcomes. However, the maintenance of normal patterns of genetic expression is a multidimensional process, and DNA sequence is only one of those dimensions. Another, often overlooked dimension involves epigenetic mechanisms that regulate gene expression without altering the DNA sequence itself. Although it should be recognized that an epigenetic alteration in and of itself does not necessarily lead to a toxicologically significant effect (just as a mutation might or might not have a meaningful impact), the end result of certain epigenetic changes can easily be as, or more, pronounced than one would expect from established mutagens. It stands to reason that an agent that decreases the expression of a gene through epigenetic means would ultimately have a similar impact to a mutation that decreased the expression or function of the gene. Thus, when evaluating possible factors contributing to gene dysregulation, toxicologists should be careful to consider both genotoxic and epigenetic modes of action. Conversely, careful manipulation of epigenetic mechanisms to up- or down-regulate gene expression could aid in the treatment of certain disorders. Current information regarding the major epigenetic mechanisms and their impact on human health is presented herein.

2 DEFINING EPIGENETICS

The definition of epigenetics has changed considerably over the past several decades, and the first major definition of epigenetics was proposed well before what we currently understand as the field of epigenetics was acknowledged. In 1942, C. H. Waddington defined epigenetics as 'the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being' Waddington (1942). The current definition of epigenetics refers to the study of reversible, heritable genetic mechanisms by which a gene's phenotype is altered without changing

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the underlying DNA sequence. Such mechanisms basically behave to amplify or decrease gene expression. As is the case with DNA sequence, epigenetic patterns are inheritable and tend to be maintained from cell to daughter cell in the adult organism. However, unlike most changes to the DNA sequence itself, epigenetic mechanisms are reversible. The overall effect is that epigenetic mechanisms act as a form of malleable 'genetic memory' whereby patterns of gene expression are typically maintained through replication but can be altered in response to certain stimuli. Of interest to toxicologists is determining both what types of stimuli influence epigenetic mechanisms and what the consequences might entail.

3 EPIGENETIC MECHANISMS

This review will focus on DNA methylation, histone acetylation, histone methylation and microRNAs. Additional epigenetic mechanisms such as ATP (adenosine triphosphate)-mediated chromatin remodelling complexes and histone phosphorylation are also biologically important, but less well characterized, and will not be discussed here.

3.1 DNA Methylation

Methylation of the 5′ site of the cytosine base of DNA occurs predominantly (but not exclusively) at the CpG dinucleotide (cytosine–phosphate–guanine) of eutherian mammals, and approximately 4% of cytosines throughout the genome are methylated (Ehrlich *et al*., 1982). In general, DNA methylation serves to silence gene expression by directly blocking access to transcription factors and/or by recruiting CpG-binding proteins that contribute to chromatin condensation, further limiting transcription factor accessibility (Curradi *et al*., 2002). When examining the impact of DNA methylation on transcription of a particular gene, the focus is usually on the methylation status at a CpG island in the promoter region of that gene. CpG islands are defined as stretches of DNA around 200 bp long that are typically unmethylated and contain a greater density of the CpG dinucleotide than found in the rest of the genome (Gardiner-Garden and Frommer, 1987). Approximately 60% of mammalian genes are potentially regulated by a promoter region CpG island (Antequera and Bird, 1993). Although methylation at these sites is of considerable biological significance, it should be noted that the bulk of DNA methylation is not located in CpG islands, but at transposable elements throughout the genome (Walsh and Bestor, 1999; Robertson and Jones, 2000). Overall, about 70% of mammalian CpG sites are methylated, mainly at repetitive sequences (Yoder and Bestor, 1996). Although at first glance, this appears to contradict the above statistic of the percentage of methylated cytosines in the genome, this percentage is consistent, because throughout the genome, the CpG dinucleotide is present at a much lower frequency than would be expected with a random distribution of nucleotides (Sved and Bird, 1990). Methylation of transposable elements and viruses throughout the genome serves a protective function by silencing the expression of DNA which could otherwise compromise genetic integrity (Carnell and Goodman, 2003; Vivekanandan *et al*., 2008).

Deoxyribonucleic acid methyltransferases (DNMTs) catalyse the reaction whereby a methyl group from *S*-adenosylmethionine (SAM), the proximate methyl group donor, is added to the 5′ site of the cytosine residue. The three major methyltransferases are DNMT1, DNMT3a and DNMT3b. DNMT1 binds preferentially to hemimethylated DNA and is the predominant maintenance methylase whereby patterns of DNA methylation are inherited from cell to daughter cell (Okano *et al*., 1999). DNMTs 3a and 3b are predominantly active during development, and function as *de novo* methyltransferases. Levels of SAM are influenced by dietary components that play a role in the one-carbon folic acid pathway as will be discussed in detail in a subsequent section. In general, DNA methylation serves as a relatively stable epigenetic mark and is transferred to daughter cells upon replication; however in some cases demethylation may occur. Demethylation may occur passively through replication without maintenance methylation, or actively via demethylase activity (Cervoni *et al*., 1999).

3.2 Histone Acetylation and Methylation

Histone acetylation and methylation alter the conformation of chromatin around the histones. Acetylation of the lysine residues of amino terminal histone tails by histone acetyltransferases (HATs) reduces the positive charge of lysine, and thereby decreases the affinity of the histone for negatively charged DNA (as reviewed in Clayton *et al*., 2006). The result is a looser chromatin conformation, termed euchromatin. Euchromatin is more easily accessible by RNA polymerase and various transcription factors and is therefore considered relatively transcriptionally active. When histone deacetylases (HDACs) remove the acetyl groups from the chromatin, the positive charge of the lysine residues is unopposed, and thus, the affinity of the chromatin for the DNA is increased (Nakao, 2001). Tightly wound, unacetylated, transcriptionally repressed chromatin is termed heterochromatin. Acetylation of residue 9 on the N-terminus of histone 3 (H3K9) in particular has been well characterized and is

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tightly linked to transcriptional activation (Sinha *et al.*, 2006).

Histone methylation is a process whereby histone methyltransferase (HMT) adds one to three methyl groups to lysine or arginine residues of the histone amino terminal tails. Depending on the histone methylation site, the effect may be to enhance or suppress levels of transcription in the surrounding DNA. For instance, methylation of H3K9 inhibits acetylation of the H4K16 site associated with active chromatin (Nishioka *et al.*, 2002). Methylation at H3K27 is also repressive, and is particularly important during development (Reik, 2007). Conversely, methylation of H3K4 facilitates transcription, and appears to inhibit H3K9 methylation and promote H3 acetylation (Fischle *et al.*, 2003).

3.3 Interactions between DNA Methylation and Histone Modifications

DNA methylation and histone modifications are mutually reinforcing processes, such that the histones of highly methylated DNA are modified in a manner that condenses the chromatin. Hypoacetylated H3 and H4 histones are associated with highly methylated DNA and vice versa (Schübeler *et al.*, 2000). Various proteins can bind to methylated cytosines, and recruit additional proteins which impact histone conformation. These mechanisms work in concert to render the DNA in the region transcriptionally inactive, as illustrated in **Figure 1**. The best characterized example of this is the methylcytosine binding protein 2 (MeCP2) binding to the mSin3A transcriptional repressor and HDACs (Nan *et al.*, 1998). In this model, DNA methylation serves as the first epigenetic mark; however, there is evidence to suggest that histone modifications can impact DNA methylation status. Cervoni and Szyf (2001) showed that administration of a HDAC inhibitor to a human cell line increased histone acetylation and was associated with active DNA demethylase activity.

3.4 Micro RNA

Although microRNA (miRNA) is less often described as an epigenetic mechanism, miRNA molecules are involved in the regulation of various genes and are of considerable interest for their therapeutic potential. MicroRNA expression is controlled by tissue-specific transcription factors and serves to modulate various processes necessary for proper differentiation and development (Negrini *et al.*, 2007). As will be discussed in greater detail, altered levels of miRNAs are frequently reported in human cancers.

MicroRNAs consist of small segments of RNA that inhibit post-transcriptional gene expression (Chuang and Jones, 2007). Their formation and mode of action is illustrated in **Figure 2**. MicroRNA production begins with a 60–70 bp nuclear-derived primary microRNA (pri-miRNA) transcript with a stem–loop structure. The pri-miRNA interacts with a microprocessor complex consisting of Drosha and Pasha proteins to form a shorter

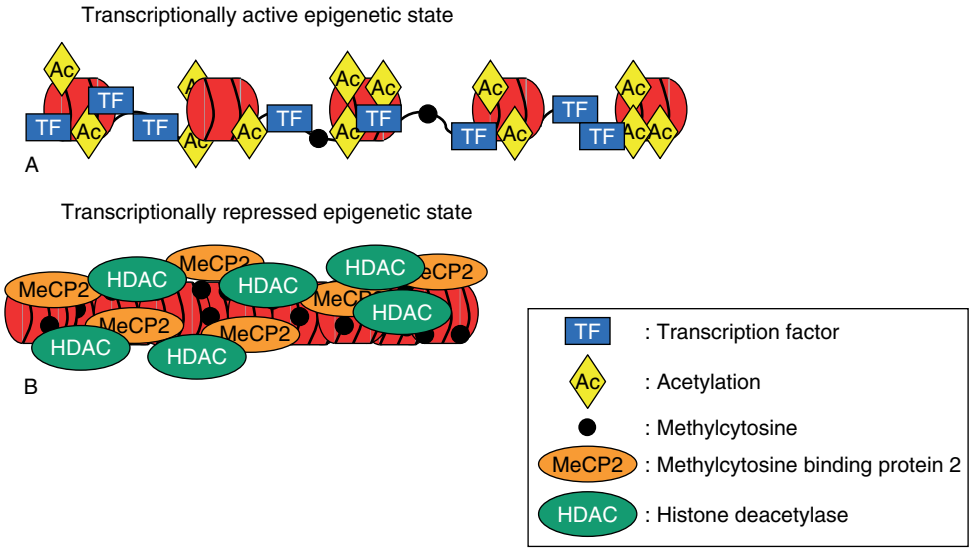

Figure 1 The role of DNA methylation and histone modifications in transcriptional silencing. In (A) the DNA is sparsely methylated and the histones are acetylated, facilitating access to transcription factors. In (B) the DNA is methylated, and methylcytosine binding protein 2 (MeCP2) binds to the methylcytosine bases. MeCP2 recruits histone deacetylases, condensing the chromatin. The overall effect is to silence gene transcription.

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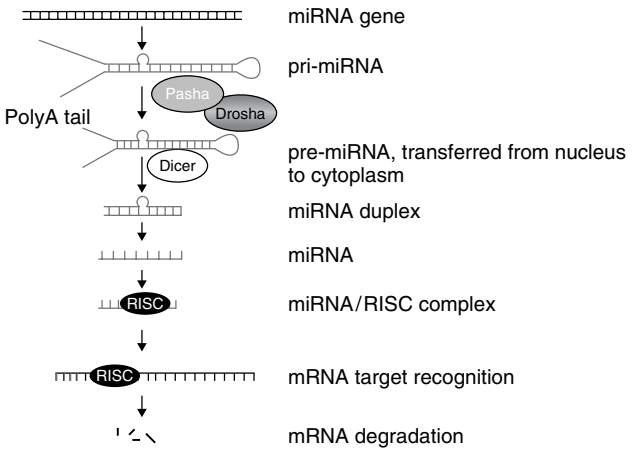

Figure 2 MicroRNA (miRNA)-mediated transcriptional control. In this illustration, a pri-miRNA (~70 bp) with a hairpin loop structure and a polyadenine (PolyA) tail is produced from a miRNA gene in the nucleus. Pasha and Drosha proteins bind to the pri-miRNA, forming a microprocessor complex with nuclease properties. The result of this interaction is a shortened pre-mRNA molecule that is transported into the cytoplasm. The dicer endonuclease protein binds to the pre-miRNA in the cytoplasm, removing the stem–loop structure and producing an miRNA duplex approximately 22 bp long. A single stand of the duplex binds to the RISC (RNA-induced silencing complex), which directs the binding of the miRNA to the mRNA. Binding of this complex to mRNA leads to degradation of the molecule.

pre-miRNA molecule that is transported to the cytoplasm (Denli *et al*., 2004). Once in the cytoplasm, the pre-miRNA is acted upon by the Dicer endonuclease to produce a ~22 bp miRNA duplex molecule (Denli *et al*., 2004). One strand in this duplex then enters a complex with an RNA-induced silencing complex (RISC) and binds to a complimentary mRNA (messenger RNA) target (Tang, 2005). Argonaute proteins within this complex then facilitate the cleavage and degradation of the mRNA target (Diederichs and Haber, 2007).

4 THE EPIGENETICS OF DEVELOPMENT AND REPRODUCTION

Epigenetic processes undergo dynamic changes during early development, and therefore, the epigenome is likely to be particularly sensitive to chemical and/or nutritional factors that affect the epigenome during this time. Epigenetic changes during development are necessary for two major goals: (i) to reprogramme the epigenome for the next generation; and (ii) to orchestrate gene expression patterns necessary for viable, healthy offspring. Epigenetics also regulates reproductive processes. In assisted reproductive technologies, subtle changes in epigenetic mechanisms might underlie an increased incidence of certain diseases, an issue that is of considerable clinical importance given the increased demand for these procedures. Additionally, the role of epigenetics in the normal function of the ovary serves as an excellent example of how hormonal stimuli can influence epigenetic processes in a manner that allows for cyclical, specific functions.

A general illustration of epigenetic changes occurring throughout development is presented in **Figure 3**. Gametes have sex-specific epigenetic patterns, with sperm DNA typically more heavily methylated than in the oocyte (Mertineit *et al*., 1998). The paternal pronucleus is demethylated within the zygote prior to the first cellular division, while the maternal genome is passively demethylated (Mayer *et al*., 2000). This begins the process whereby the epigenome is largely 'reprogrammed' (Brandeis *et al*., 1993). However, epigenetic marks responsible for the proper expression of imprinted genes, as well as intracisternal A particles (IAPs) are likely spared from this wave of demethylation, making transgenerational inheritance of particular epigenetic mechanisms possible (Oswald *et al*., 2000; Reik, 2007). Afterwards, *de novo* DNA methylation, as well as methylation of H3K27, occurs just prior to implantation, silencing expression of various genes. It should be noted that methylation of H3K27 imparts somewhat transient silencing properties, while DNA methylation is thought to silence gene expression in a more stable manner (Reik, 2007). Gene-specific histone acetylation, demethylation of H3K27 and/or methylation of H3K4 then allows for the expression of genes at the appropriate time for normal development (Reik, 2007). In mice, it has been shown that an additional wave of demethylation occurs in the primordial germ cells shortly prior to entering the gonads, erasing much of the epigenome for the subsequent generation (Hajkova *et al*., 2002).

The importance of epigenetic mechanisms during development has been underscored by research demonstrating that the lack of components necessary for these processes reduces viability of the offspring. Normal patterns of methylation and histone conformation are crucial for normal development and the maintenance of a differentiated state (Reik *et al*., 2001; Rideout *et al*., 2001). Murine embryos lacking DNMT1, 3a or 3b die *in utero* or soon afterwards, likely because lack of these proteins results in patterns of gene expression that are incompatible with life (Okano *et al*., 1999). In the oocyte, the methyltransferase DNMT3L appears to be required for maternal-specific methylation patterns required for imprinted genes (Hata *et al*., 2002). MicroRNA's involvement in development is not yet well characterized, but it is known that embryonic stem cells without the Dicer endonuclease needed for miRNA processing fail to differentiate (Bernstein *et al*.,

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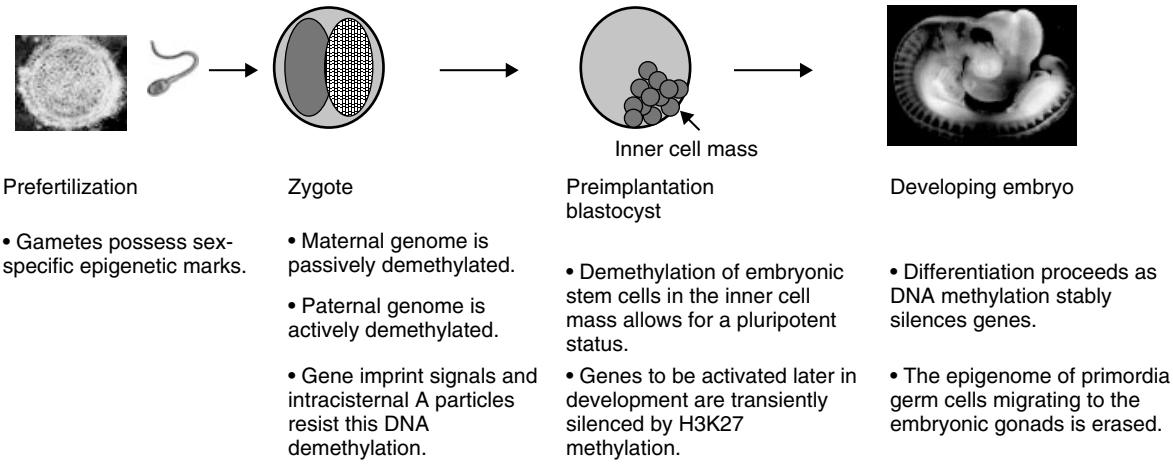

Figure 3 Reprogramming of the epigenome.

2003; Kanellopoulou *et al*., 2005). In addition, specific miRNAs, including miR-196 regulate homeobox genes that encode vital developmental transcription factors (Mansfield *et al*., 2004).

Recent studies have shown that altering natural reproduction occasionally appears to adversely affect developmental outcome, and this might be due to subtle changes in epigenetic processes. Normal patterns of DNA methylation appear to be more reliably established when an animal is naturally conceived with egg and sperm compared to using nuclear transfer techniques. It has been suggested that a lack of normal waves of epigenetic changes during development might contribute to developmental failure of cloned animals, and aberrant methylation patterns have been reported in cloned cows and mice in which aberrant expression of imprinted genes typically regulated by epigenetic processes is commonly observed (Cezar *et al*., 2003; Ohgane *et al*., 2008). Similarly, in humans conceived by assisted reproductive technologies, there is a small, but significant increase in the risk of diseases characterized by aberrant imprinting such as Angelman syndrome (AS) (Cox *et al*., 2002), Beckwith–Wiedemann syndrome (DeBaun *et al*., 2003) and transient neonatal diabetes (Lawrence and Moley, 2008).

The role of histone conformation in regulating ovarian function demonstrates how hormone-induced epigenetic changes allow for the proper timing of gene expression and hormone release needed for a cyclical process. The LH (luteinizing hormone) surge preceding ovulation has been shown to be regulated by an epigenetic-mediated increase in expression of the steroidogenic acute regulatory (STAR) protein (Christenson *et al*., 2001). Stimulation of monkey and murine granulosa cells with hCG (human chorionic gonadotrophin) has been shown to increase the acetylation status of H3K9 and decrease H3K9 methylation in the promoter region of STAR, increasing expression and stimulating ovulation (LaVoie, 2005). In porcine cells, FSH (follicle-stimulating hormone) was found to stimulate H3K9/K14 acetylation, increasing STAR expression. Acetylation was repressed by the STAR transcriptional repressor EGF (epidermal growth factor) (Rusovici *et al*., 2005).

5 IMPRINTING

Epigenetic mechanisms are necessary for genetic imprinting, a non-Mendelian genetic process whereby gene expression occurs in a paternal- or maternal-specific fashion (Li *et al*., 1992). It is estimated that approximately 100–200 genes are imprinted (Murphy and Jirtle, 2003). Imprinting is of interest to toxicologists because a loss of imprinting has been associated with various disease states (Feinberg *et al*., 2002). Furthermore, imprinted genes are likely to be particularly sensitive to toxic insults because the functionally haploid expression of these genes negates the normal protection received from diploid gene expression (Murphy and Jirtle, 2003).

Igf2 (insulin-like growth factor 2) is the classic example of a paternally expressed imprinted gene. DNA methylation at a particular imprint control region (ICR) on the paternal allele blocks the binding of the vertebrate enhancer blocking protein CTCF, which would otherwise inhibit access of an enhancer protein to the Igf2 promoter region (Bell and Felsenfeld, 2000). On the maternal allele, the ICR DNA is unmethylated, permitting CTCF binding, and forming a block between enhancers located distally to the CTCF binding region and the Igf2 promoter region (Bell and Felsenfeld, 2000). The end result is that the paternal Igf2 allele is transcribed, and the maternal allele is silenced. Maintenance of chromatin conformation associated with imprinted genes is likely to be tightly associated with DNA methylation patterns (Murphy and Jirtle, 2003).

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6 EPIGENETICS AND DISEASE

In general, epigenetic processes are protective of the genome, directing patterns of gene expression in a manner that is beneficial to the organism. However, when these processes are altered, the result can lead to widespread dysregulation of gene expression patterns, which, in turn, can lead to disease. It is important to recognize that not all epigenetic malfunctions are the underlying cause of a disease; changes in epigenetic mechanisms might occur secondarily to other toxic outcomes, and/or may not, in and of themselves, be associated with an adverse clinical outcome. That said, epigenetic mechanisms have been shown to be probable underlying factors in the development of particular diseases including, but not limited to, cancer and neurological disorders.

6.1 Cancer

Epigenetic mechanisms are almost always altered in some manner during carcinogenesis. Typically, the epigenetic change associated with cancer involves one of the following outcomes (reviewed in Watson and Goodman, 2002a): (i) decreased expression of tumour suppressor genes; (ii) increased expression of transposable elements or oncogenes that compromise genetic integrity; (iii) alterations to normal patterns of gene imprinting; and/or (iv) an increase in cytosine-to-thymine transitions secondary to increased incidence of deamination of 5-methylcytosine to thymine. Neoplastic tissue is typically characterized by an overall reduction in global, or overall, levels of DNA methylation with increased methylation status and decreased histone acetylation in the promoter regions of specific tumour suppressor genes (Robertson and Jones, 2000; Ehrlich *et al*., 2002). The mechanisms underlying the increased methylation at certain regions with a decrease in other regions is poorly understood, but might be due to sequence-specific activity of particular DNMTs and/or histone-modifying enzymes. Tumour suppressor genes shown to be silenced by DNA methylation and/or histone deacetylation include p16, E-cadherin, O^6-methylguanine methyltransferase (Esteller, 2003). DNA hypomethylation and histone acetylation of the promoter regions of oncogenes and subsequent up-regulation of these genes is infrequently described in carcinogenesis, but appears to underlie the increased expression of raf in mouse liver tumours (Ray *et al*., 1994). Finally, the increased prevalence of CT (charge-transfer) transitions in the p53 tumour suppressor is likely due to an increased amount of methylcytosine in the region (Schumutte *et al*., 1996). Differences in the extent of epigenetic alterations observed after administration with a tumour-promoting dose of phenobarbital have been shown to be associated with strain differences in cancer susceptibility. Following treatment, the C57 tumour-resistant mouse strain exhibited a slight decrease in global methylation status and a slight increase in DNA methylation in CpG island regions. In contrast, in the B3H/He tumour-sensitive mouse strain, a pronounced decrease in global methylation status with increased methylation in CpG island regions was observed (Counts *et al*., 1996; Watson and Goodman, 2002b). Thus, susceptibility to carcinogenesis appears to be inversely related to the ability to maintain normal patterns of DNA methylation.

A number of proteins that contribute to histone modifications have also been linked to carcinogenesis, including the Enhancer of Zeste homologue 2 (EZH2), which methylates the K9 and K27 residues on H3, and functions as an oncogene (Kuzmichev *et al*., 2002). Increased levels of EZH2 are associated with poor prognosis in breast cancer and lymphoma (Kleer *et al*., 2003). One of EZH2's particularly notable properties is its links to DNA methylation and miRNA. EZH2 recruits DNMT activity to EZH2-targeted genes (Viré *et al*., 2006). MicroRNA-26a targets and inactivates EZH2 mRNA (Wong and Tellam, 2008). Similar cooperative actions are likely to occur with numerous other specific epigenetic factors.

MicroRNAs are becoming increasingly recognized as being involved in carcinogenesis, as various interactions with tumour suppressors and oncogenes are being revealed (Negrini *et al*., 2007). In human cancer specimens, various miRNAs have been deregulated (Negrini *et al*., 2007). The let-7 family of miRNAs is involved in the down-regulation of the ras oncogene; therefore, when let-7 miRNA is down-regulated, ras expression increases (Johnson *et al*., 2005). Members of the let-7 family of miRNAs have been found to be down-regulated in various cancer types, including 50% of lung cancers (Takamizawa *et al*., 2004). In the same vein, microRNAs 15a and 16-1 regulate the antiapoptotic BCL2 oncoprotein (Negrini *et al*., 2007). Down-regulation of these miRNAs is associated with a reduction in apoptosis and an increased susceptibility to carcinogenesis (Negrini *et al*., 2007). MicroRNA 15a and/or 16-1 have been shown to be down-regulated in over 50% of chronic lymphocytic leukaemia cells (Cimmino *et al*., 2005). MicroRNA can also present a problem if its expression results in activation of a particular oncogene. Translocation of the mir-142 regulatory element has been shown to serve as an activator of the MYC proto-oncogene in B cell leukaemia (Gauwerky *et al*., 1989; Negrini *et al*., 2007).

Overall, epigenetic mechanisms appear to be particularly well suited for the promotion phase of the initiation, promotion and progression model of carcinogenesis (Pitot and Dragan, 1994). In this model, initiation typically involves an irreversible, heritable alteration to a cell such that it has a growth advantage over

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surrounding cells in the promotion stage. The promotion stage then provides a favourable environment for the initiated cell to clonally expand, whereby additional alterations confer additional growth advantages to subclones of cells. In the progression stage, malignant conversion occurs. Promoting agents such as tobacco and phenobarbital facilitate the clonal expansion of initiated cells (Schulte-Hermann *et al*., 1990). An important feature of the promotion stage is that it is reversible, and the dose–response relationship for promoters exhibits a threshold (Pitot and Dragan, 1994). Epigenetic mechanisms in particular appear to be prone to act as underlying mechanisms in the promotion stage of carcinogenesis because they provide reversible means by which gene expression can be increased or decreased in a manner that confers a growth advantage in response to various stimuli. In SENCAR (sensitive to mouse carcinogenesis) mice initiated with DMBA (dimethylbenzanthracene) and promoted with several doses of cigarette smoke condensate (CSC) for various amounts of time, increased DNA methylation in CpG-rich regions of skin DNA was observed in a dose and time-dependent manner. Furthermore, when skin was left unpromoted for six weeks prior to harvesting, most of the methylation increases were no longer detected, suggesting that the altered patterns of DNA methylation no longer provided a growth advantage once the promoting agent was withdrawn (Watson *et al*., 2003).

6.2 Neurological Disorders

Specific patterns of DNA methylation and histone acetylation are necessary for normal brain development and function. Conversely, particular abnormal epigenetic patterns have been implicated in the aetiology of various types of neurological/developmental disorders including Rett syndrome, Prader–Willi syndrome (PWS) and AS, as well as Beckwith–Wiedemann syndrome. Errors in the epigenetic mechanisms responsible for maintaining imprinted genes appear to be a particularly common finding with some of these disorders.

With respect to the role of epigenetics in normal brain function, an illustrative example of how epigenetic changes are necessary for crucial neurological function is in the maturation of astrocytes. In this process, DNA methylation is present in the promoter region of the glial fibrillary acidic protein (GFAP) gene on embryonic day (E) 11.5 in the mouse, preventing the binding of the STAT3 transcription factor (Takizawa *et al*., 2001). By E 14.5, methylation at this region is no longer present, allowing for the binding of STAT3, and the transcription of GFAP, a gene necessary for the differentiation of neuroepithelial cells into the astrocytes (Takizawa *et al*., 2001). Histone acetylation has been shown to be necessary for normal patterns of cognition and synaptic cognition, and has been shown to be altered in Huntington's and Parkinson's diseases (Abel and Zukin, 2008).

Neurological disorders associated with errors in epigenetic machinery tend to result in mental deficits coupled with various forms of developmental abnormalities. Rett syndrome, an X-linked form of mental retardation afflicting mostly females, is tightly linked to mutations in the gene for the MeCP2 protein (Nan and Bird, 2001). Some autistic individuals have also been reported to have decreased levels of MeCP2 (Nagarajan *et al*., 2006). Altered patterns of imprinting are clearly implicated in some mental disorders such as PWS and AS, as well as Beckwith–Wiedemann syndrome. Both PWS and AS are characterized by severe mental deficits and developmental abnormalities. The aetiology of these syndromes is linked to alterations in a 2 Mb domain in the 15q11–q13 region of DNA at which a variety of imprinted genes are clustered. PWS results from microdeletions in this region on the paternal allele, and AS results from microdeletions at this site on the maternal allele (Shemer *et al*., 2000). The end result is to disrupt paternal- and maternal-specific imprinted gene expression (Mann and Bartolomei, 1998). Beckwith–Wiedemann syndrome is characterized by developmental growth disorders and an increased prevalence of Wilms' tumour. This condition is typically accompanied by an increase in Igf2 expression, due to alterations to normal patterns of imprinting (Issa and Baylin, 1996).

7 NUTRITIONALLY INFLUENCED EPIGENETIC CHANGE

The impact of nutrition in DNA methylation is much more thoroughly defined than the role of nutrition in either histone conformation or miRNA. It has long been recognized that DNA methylation is dependent upon methyl group donor nutrients and cofactors in the one-carbon metabolic pathway (Poirier, 2002). Several components of this pathway, including choline, methionine, zinc, B_{12}, betaine and folic acid, are predominantly obtained from dietary sources, such as leafy green vegetables, eggs and meats. In addition, soya products appear to impact the epigenome in a manner that encourages healthy patterns of gene expression. The effects of these nutrients on the epigenome and associated heath consequences are presented in **Table 1**.

The one-carbon metabolic pathway is presented in **Figure 4**. DNMTs catalyse the reaction whereby methyl groups from SAM are made available to various methyl group acceptors, including DNA. The end result of this reaction is a methylated acceptor and *S*-adenosylhomocysteine (SAH) (Bottiglieri *et al*.,

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Table 1 A selection of nutrients that alter the epigenome

Nutrient	Impact on epigenome	Outcome of epigenetic alterations
Components of the one-carbon metabolic pathway • Folic acid • Methionine • Choline • B_{12} • Betaine	• Groups in this provide necessary components for DNA methylation to occur. • Deficiency of these nutrients reduces DNA methylation status. • Increased levels of methyl group donor nutrients in the maternal diet increase methylation status of a target intracisternal A particle in the offspring in an agouti mouse model (Wolff *et al*., 1998; Waterland and Jirtle, 2003).	• Deficiencies in choline, methionine and folic acid have been linked with various cancer types including hepatocarcinoma, colon cancer (Counts *et al*., 1996; Dela Pena *et al*., 2007; Kim, 2005). • Some studies indicate that excess folic acid could increase breast cancer risk (Kim, 2006; Stolzenberg-Solomon *et al*., 2006; Kotsopoulos *et al*., 2003); some studies do not support a link (Larsson *et al*., 2007; Ericson *et al*., 2007).
Soya products • Genistein • Soya protein isolate • Lunasin	• Aid in maintaining a healthy SAM/SAH ratio (Le Leu *et al*., 1998). • Decrease DNA methylation status promoter regions of tumour suppressor genes and inhibit methyltransferase (Fang *et al*., 2007). • Increase DNA methylation status at intracisternal A particles when administered during gestation (Dolinoy *et al*., 2006). • Lunasin has been shown to selectively disrupt histone acetylation/histone deacetylation dynamics in cells in the process of transformation (De Lumen, 2005).	• Epidemiological and animal studies have demonstrated that dietary soya is associated with a decreased incidence of various types of cancers including breast, colon and prostate (Zhou *et al*., 2007; Oba *et al*., 2007; McCormick *et al*., 2007). Soya's impact on hypomethylating and re-activating tumour suppressor genes might play a role in the chemopreventive effects.

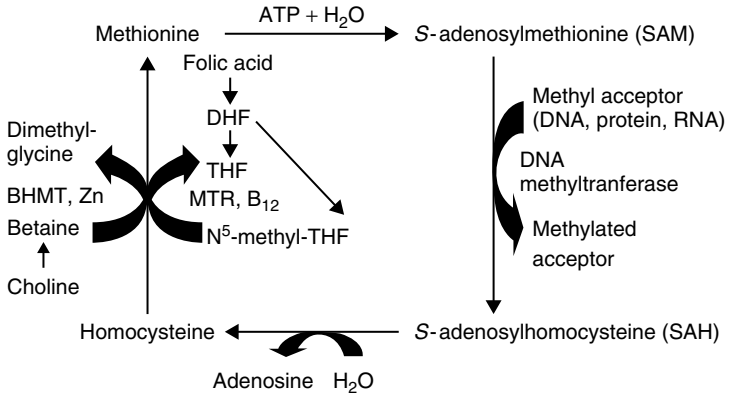

Figure 4 The one-carbon metabolic pathway. DHF—dihydrofolate.

2000). In a reversible reaction, SAH is hydrolysed to homocysteine, a precursor for methionine. There are then two pathways by which a methyl group can be added to homocysteine to synthesize methionine. In one pathway, methionine is produced in a B_{12}-and-folic-acid-dependent reaction catalysed by methionine synthase (MTR), and involving the transfer of a methyl group from N^5-methyl tetrahydrofolate (N^5-methyl-THF) (Van den Veyver, 2002). In the second pathway, a methyl group from betaine can be coupled to homocysteine in a reaction catalysed by betaine homocysteine methyltransferase (BHMT), which requires zinc as a cofactor. BHMT is only available in the mammalian liver and kidney (Finkelstein and Martin, 1986). In this reaction, choline

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is a precursor for betaine (Zeisel and Blusztajn, 1994). Following either of the above pathways, methionine is hydrolysed to SAM (Van den Veyver, 2002).

Many studies have indicated that deficiencies in nutrients involved in the one-carbon pathway are harmful. For instance, a choline and/or methionine-deficient diet leads to global hypomethylation of liver DNA, and an increase in spontaneous hepatocarcinoma (Counts *et al*., 1996; Dela Pena *et al*., 2007). Folic-acid deficiency has been linked to an increased risk of colon cancer, neural tube defects and cardiovascular disease (Michie *et al*., 1998; Kim, 2005). Fewer studies have addressed the adverse effects associated with elevated levels of methyl group donor nutrients, but there have been recent conflicting reports of whether excessive folic acid increases the risk of breast cancer. Some epidemiological reports have indicated that women who consume folic acid supplements of $\geq 400\,\mu g\,day^{-1}$ have a 20% or more increase in breast cancer risk compared to unsupplemented women (Kim, 2006; Stolzenberg-Solomon *et al*., 2006). Similarly, in rats, a deficiency in dietary folic acid beginning at weaning was associated with inhibition of *N*-methyl-*N*-nitrosourea-induced breast cancer in Sprague Dawley rats (Kotsopoulos *et al*., 2003). However, a meta-analysis by Larsson *et al*. (2007) indicated that there is no clear link between folic acid intake and breast cancer, and high folate intake was associated with a decrease in breast cancer in postmenopausal women in a study by Ericson *et al*. (2007). An increased amount of methyl group donor nutrients in the maternal diet have been shown to impact the methylation status of an IAP controlling coat colour in the A^{vy} (agouti viable yellow) mouse model, with increased IAP methylation associated with a darker coat colour (Wolff *et al*., 1998; Waterland and Jirtle, 2003).

Soya products have also been shown to alter epigenetic patterns, although details of the underlying mode of action are not yet fully understood. Soya protein has been shown to help maintain normal patterns of DNA methylation and maintain a healthy SAM/SAH ratio (Le Leu *et al*., 1998). Soya and its isoflavone components have been shown to inhibit methyltransferase activity and reduce the DNA methylation status of tumour suppressor genes, reactivating expression (Fang *et al*., 2007; Raynal *et al*., 2008). In addition, administration of genistein to pregnant mice has been shown to increase the DNA methylation status of an IAP in the A^{vy} agouti mouse model (Dolinoy *et al*., 2006). Epidemiological and animal studies have demonstrated that dietary soy is associated with a decreased incidence of various types of cancers including breast, colon and prostate (Zhou *et al*., 2007; Oba *et al*., 2007). Soya protein and/or soya isoflavones in the maternal diet of the rat can reduce the incidence of colon and mammary carcinogenesis in the F1 generation (Xiao *et al*., 2005; Su *et al*., 2007). It is possible that the chemopreventive effects are due, in part, to soya-mediated hypomethylation and reactivation of tumour suppressor genes, particularly in light of the fact that epigenetic changes are plausible underlying factors in transgenerational effects, such as those reported in Xiao *et al*. (2005) and Su *et al*. (2007), as will be discussed in greater detail. The reason for the soya-product-mediated decreased methylation in some areas of the genome and the increase in others might be due to activity on a particular type of methyltransferase that works selectively at promoter region CpG islands. Lastly, certain soya products are also thought to impact histone conformation. For example, lunasin, a cancer-preventive soya-derived peptide, appears to selectively kill cells in the process of being transformed by binding to deacetylated core histones, disrupting the dynamics of histone acetylation–histone deacetylation and contributing to cell death (De Lumen, 2005).

8 EPIGENETIC ALTERATIONS ASSOCIATED WITH XENOBIOTICS

Various xenobiotics have been shown to impact patterns of DNA methylation and histone conformation, and a summary of these is presented in **Table 2**. It should be recognized that with the exception of certain chemotherapy drugs, the role of epigenetics in mediating the toxic outcomes associated with these chemicals is unclear. Further research in this area is needed to determine if epigenetic perturbations underlie the effects of these xenobiotics and/or whether they could serve as useful biomarkers of exposure.

Certain chemotherapeutic drugs alter epigenetic status in a manner that could plausibly underlie the therapeutic and toxicological effects by reversing certain epigenetic marks characteristic of cancer. The cytosine analogue 5-aza-2′-deoxycytidine (dAzaC) irreversibly binds to and inactivates methyltransferases, thus decreasing DNA methylation status (Avramis *et al*., 1989). This drug targets the methylation status of rapidly replicating cells. As is the case with many other chemotherapeutic drugs, administration of dAzaC during pregnancy is teratogenic, perturbing DNA synthesis and contributing to embryonic death or low foetal weight (Rogers *et al*., 1994). Similarly, the antileukaemic adenosine analogues, 2-chloro-2′-deoxyadenosine (cladribine) and 9-arabinosyl-2-fluoroadenine (fludarabine), inhibit DNMTs (Wyczechowska and Fabianowska-Majewska, 2003). Trichostatin A is used as a HDAC inhibitor experimentally, and HDACs are a particularly promising group of therapeutic drugs (Yoshida *et al*., 1990).

Nonchemotherapeutic drugs shown to alter the epigenome include certain metals, CSC, phenobarbital, bisphenol A and high doses of trichloroethylene

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Table 2 A selection of xenobiotics that alter the epigenome

Chemical	Impact on epigenome
5-Aza-2′-deoxycytidine (decitabine)	Irreversibly binds to and inhibits methyltransferases (Avramis *et al*., 1989)
2-Chloro-2′-deoxyadenosine (cladribine)	Inhibits DNA methyltransferases (Wyczechowska *et al*., 2000)
9-Arabinosyl-2-fluoroadenine (fludarabine)	
Arsenic	Methyl group acceptor; reduces global methylation (Vahter, 2008)
Bisphenol A	Decreases DNA methylation at intracisternal A particle (Dolinoy *et al*., 2006)
Chromium, nickel	Increase DNA methylation levels, chromatin condensation (Salnikow and Zhitkovich, 2008)
Cigarette smoke condensate	Increases DNA methylation at GC-rich regions (Watson *et al*., 2003)
Phenobarbital	Decreases global DNA methylation status, and increased methylation in GC-rich regions (Counts *et al*., 1996; Watson and Goodman, 2002b)
Trichloroethylene	At high doses, these reduce the DNA methylation status of oncogenes, increasing their expression (Tao *et al*., 2000)
Dichloroacetic acid Trichloroacetic acid	
Trichostatin A	Experimental histone deacetylase inhibitor (Yoshida *et al*., 1990)
Valproic acid	Demethylates DNA and increases histone acetylation (Detich *et al*., 2003)
Vinclozolin	Transient exposure contributes to transgenerational increases in DNA methylation (Anway *et al*., 2005)

(TCE), dichloroethylene (DCE) and trichloroacetic acid (TCA). Nickel and chromium have recently been shown to increase DNA methylation levels and favour a condensed chromatin conformation (Salnikow and Zhitkovich, 2008). Arsenic is a methyl group acceptor, effectively decreasing the amount of methyl groups available for DNA methylation. Some of arsenic's adverse effects (particularly associated with early life exposure) on immune suppression and neurological function are proposed to be at least partially due to hypomethylation (Vahter, 2008). CSC leads to increases in GC (guanine–cytosine)-rich and gene-specific methylation patterns in tumour and nontumour tissue, as well as global hypomethylation in skin papillomas in the SENCAR mouse (Watson *et al*., 2003). Valproic acid, a drug used for epilepsy and mood stabilization, has been found to trigger a decrease in DNA methylation and increase histone acetylation in a replication-dependent manner via recruiting of a methylcytosine-binding protein/demethylase complex, representing a rare example of chemically-induced active demethylation (Detich *et al*., 2003). Phenobarbital is associated with decreased global methylation and increased methylation in CpG rich regions (Counts *et al*., 1996; Watson and Goodman, 2002b). Bisphenol A in the maternal diet was shown to decrease the methylation status of an IAP in the F1 generation (Dolinoy *et al*., 2007). Finally, administration of high doses of TCE, DCE and TCA have been shown to reduce the DNA methylation status of the promoter region of c-jun and c-myc oncogenes, and increase expression of these genes (Tao *et al*., 2000).

9 EPIGENETICS IN THE DEVELOPMENTAL ORIGINS OF ADULT DISEASE

Given the number of confounding factors involved, making a clear link between maternal diet and adult disease is extraordinarily difficult in humans. However, there are suggestions that this does in fact occur, and the means by which this takes place may be epigenetic-mediated. In a study by Lucas (1991), premature babies were fed a standard infant formula, a fortified formula or breast milk for approximately one month. When these individuals were examined fifteen years later, there were major group differences in the leptin/fat mass index and the allergy status. Breast feeding has been associated with lower blood pressure and a reduced incidence of some immune-related diseases (Schack-Nielsen and Michaelsen, 2006). Furthermore, the children of women that were pregnant during the Dutch famine were found to exhibit glucose intolerance, and a more atherogenic lipid profile as adults (Rosebloom *et al*., 2006). Thus, there is precedence for the belief that maternal diet can impact adult human health, and epigenetic mechanisms, by way of serving as a type of genetic memory, can potentially play a key role in this phenomenon.

Although DNA methylation patterns are potentially reversible, patterns developed *in utero* tend serve as relatively stable templates for thousands more daughter cells in a rapidly proliferating cell population (Reik, 2007). Thus, epigenetic mechanisms are likely to be

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particularly sensitive to the availability of methyl group donor nutrients during embryonic development, when the epigenome is first being established. Exposure to carcinogenic substances later in life could then result in selection for cells that have methylation patterns more typical of precancerous and cancerous tissues.

Several animal studies have shown that maternal diet can alter offspring DNA methylation patterns in a long-lasting manner. In studies by Wolff *et al.* (1998) and Waterland and Jirtle (2003), supplementation of the maternal diet with a combination of methyl group donor nutrients increased methylation at an IAP inserted into an agouti gene governing coat colour. In Dolinoy *et al.* (2006), a similar finding was found after the maternal diet was supplemented with genistein. DNA methylation patterns remained static in the adult animals up to at least 100–150 days of age, even though the animals were placed on control diets postweaning (Waterland and Jirtle, 2003; Dolinoy *et al.*, 2006). Feeding rats a protein-restricted diet leads to hypomethylation of PPARα (peroxisome-proliferator-activated receptor alpha) and glucocorticoid receptor (GR) promoters and increased expression of these genes (Lillycrop *et al.*, 2005). Finally, it appears that maternal behaviour can perhaps impact epigenetic mechanisms that in turn impact offspring patterns of gene expression. Pups of rats that demonstrated low levels of nurturing behaviour had an increased stress response correlating with hypermethylation of the glucocorticoid receptor in the hippocampus (Weaver *et al.*, 2004). Furthermore, there is evidence for multigenerational effects of agents that alter epigenetic processes. The F1 and F2 generations of rats fed a protein-restricted diet exhibited hypomethylated GR and PPARα DNA (Burdge *et al.*, 2007a; 2007b). Transient exposure of pregnant rats to vinclozolin or methoxychlor induced a decrease in spermatogenic capacity in the F1–F4 generations (Anway *et al.*, 2005). These changes correlated with altered DNA methylation patterns in the germ line (Anway *et al.*, 2005).

If similar long-lasting and transgenerational effects hold true in humans, this would have a considerable impact on our current understanding of the importance of maternal nutrition and chemical exposure during gestation and early development. A relevant example of this is the current recommendation that pregnant women take a supplemental amount of folic acid. Although this practice clearly reduces the incidence of neural tube defects (Centers for Disease Control and Prevention (CDC), 2008), the effects of this methyl donor nutrient on the epigenome are poorly understood and may have long-lasting effects which may be beneficial or detrimental. This is of particular concern if many pregnant women take a 'more is better' approach towards folic acid supplementation, when the long-term risks associated with this practice are unknown.

10 THERAPEUTIC APPROACHES TARGETING EPIGENETIC MECHANISMS

An enhanced understanding of epigenetic processes as well as the nutrients and chemicals that affect them has resulted in therapeutic approaches that work through actions on the epigenome. In addition, aberrant epigenetic processes can serve as biomarkers of disease development or susceptibility, particularly in cancer.

The ability to hypomethylate DNA underlies the anticarcinogenic activities of dAzaC, cladribine and fludarabine (Wyczechowska *et al.*, 2000). Genistein re-activates tumour suppressor genes in leukaemic cells in mice (Raynal *et al.*, 2008), and in human breast cancer cells (King-Batoon *et al.*, 2008). HDAC inhibitors have also been shown to have considerable promise as chemotherapeutic agents (Zhu and Otterson, 2003), including trichostatin A, sodium butyrate, depsipeptide and valproic acid. A synergistic therapy with HDAC and dAzaC has been shown to be particularly effective in the induction of apoptosis, differentiation and cell growth arrest in a number of cancer cell lines (Zhu and Otterson, 2003). HDAC inhibitors have also been shown to ameliorate some of the adverse effects associated with various neurological disorders, including Huntington's and Parkinson's diseases (Abel and Zukin, 2008).

As previously discussed, it is also possible that nutritional supplementation can counteract the action of agents that cause various effects on the epigenome, with folic acid and genistein shown to counteract the hypomethylating effects of bisphenol A-induced hypomethylation in the F1 generation (Dolinoy *et al.*, 2007). Similarly, methionine administration prevented hypomethylation induced by injection of doses of TCE, DCA (dichloroacetic acid) and TCA (Tao *et al.*, 2000).

The use of microRNAs in therapeutic strategies to alter patterns of gene expression is attractive largely due to the potential of having some degree of control over the specific gene target. MicroRNA has shown some promise for use in chemotherapeutic strategies. Administration of miR-15a and miR-16-1 led to apoptosis induction, and hence a reduction in carcinogenic potential, in a human chronic lymphocytic leukaemia cell line (Cimmino *et al.*, 2005). Conversely, targeting miRNAs that inhibit apoptosis, such as miR-21 can be accomplished by antioligonucleotide constructs (Chan *et al.*, 2005). To date, miRNA has not been used in the clinic, but use of these approaches delivered via viral vectors, liposomes and nanoparticles is currently being investigated (Negrini *et al.*, 2007).

Epigenetic markers can also be used as biomarkers to predict various untoward outcomes, an approach that has shown considerable potential in the early detection of cancer. Hypermethylation of the p16 tumour

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suppressor gene in the sputum and/or plasma identified 92% (46/50) of lung patients studied (Liu *et al*., 2003), and methylation of the promoter regions of p16 and MGMT (0^6-methylguanine-DNA methyltransferase) tumour suppressor genes has been detected in the sputum DNA of all patients with squamous cell carcinoma of the lung up to three years prior to clinical diagnosis (Palmisano *et al*., 2000). Furthermore, a blood test for the loss of normal patterns of Igf2 imprinting has shown to be effective in the early detection of colon cancer (Cui *et al*., 2003).

11 FUTURE DIRECTIONS

In the past decade, our knowledge of the impact of epigenetic mechanisms on a plethora of biological processes has been greatly expanded. Just as the DNA sequence has been determined, investigators are now at work on describing the human epigenome. New computational techniques using sequence information to identify imprinted genes in the human genome have been successful (Luedi *et al*., 2007), and characterization of the healthy and cancer methylome using a variety of molecular techniques is underway (Jacinto *et al*., 2008). The goal of these endeavours is to identify additional genes influenced by epigenetic mechanisms in a manner that, if altered, could compromise human health. Such research will continue to increase our awareness of a variety of issues pertinent to public health, including the importance of particular nutrients and chemicals, the labile nature of the epigenome during early development, and the role of epigenetic mechanisms in imparting a long-lasting impact on patterns of gene expression.

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Reproductive Toxicology

Norbert Makori, Satoru Oneda, Patricia R. McElhatton, Jennifer M. Ratcliffe and Frank Sullivan

CONTENTS

1 INTRODUCTION

Broadly, reproductive toxicology (or toxicity) is defined as any adverse effect on any aspect of adult male or female sexual function and fertility, or on the conceptus or on lactation, which would interfere with the production or development of normal offspring which could be reared to sexual maturity, capable in turn of reproducing the species. In its more narrow definition, reproductive toxicology is divided into two major classes: (i) adverse effects on reproductive ability or capacity in adult males and females, that is, effects on sexual behaviour and fertility and (ii) adverse effects on maintenance of pregnancy (embryofoetal loss) and developmental outcome of the offspring, that is, developmental toxicity. The former is assessed in both males and females as exposure of a toxicant applies to both, while the latter is assessed in only females and their concepti/offspring (teratogenicity and postnatal evaluations), as exposure only occurs during pregnancy, where the male is used only for mating purposes and is not exposed to the reproductive toxicant.

1.1 Terminology

- Reproductive toxicology: Occurrence of physiologically adverse responses or alterations involving female or male reproductive systems resulting from exposure to chemical or biological agents. The responses or alterations may involve the reproductive organs and related endocrine system, or may affect pregnancy outcomes.
- Fertility: Natural capacity to conceive (females) or induce conception (males).
- Fertile: Capable of breeding or reproducing within or above normal for a given species.
- Infertility: No fertility for a specified period, either temporarily or permanently (sterility).
- Fecundity: The potential reproductive capacity of an organism or population, measured by the number of gametes (e.g. in animals). Producing large litters is regarded as a component of fecundity.
- Teratogen: An agent that alters the normal development of an embryo or foetus that can subsequently result in either embryo/foetal death or a malformation(s) in the newborn.

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- Teratogenicity: The ability of a teratogen to cause gross structural malformations in the developing foetus.
- Behavioural teratogenicity: The ability of a teratogen to affect the foetus in such a way as to result in neurobehavioural disorders in the offspring following prenatal exposure of pregnant females to a teratogen.
- Developmental toxicity (see **Developmental Toxicology**): Occurrence of adverse effects on the developing organism as a result of exposure prior to conception (either parent), during prenatal development, or postnatal, including up to the time of sexual maturity. Common manifestations of developmental toxicity include, but are not limited to: (i) death of the developing organism, (ii) structural (macroscopic or microscopic) abnormality, (iii) delayed or altered growth and (iv) functional deficiency (US EPA, 1998).
- Developmental neurotoxicity (DNT; topic dealt with in detail in **Developmental Neurotoxicity**): Potential, functional and morphological hazards to the nervous system which may arise in the offspring from exposure of the mother during pregnancy and lactation.
- Developmental immunotoxicity (DIT): Development and function of the immune system assessed postnatally, for detection of adverse effects following perinatal immunotoxicant exposure.

2 SCREENING TESTS IN ANIMAL MODELS FOR REPRODUCTIVE TOXICOLOGY

The screening tests will be discussed in four sections, dealing with: (i) drugs (small molecules or biopharmaceuticals), (ii) pesticides, (iii) food additives and (iv) industrial chemicals.

2.1 Drug Testing

The objective of systemic reproductive toxicity testing of drugs (pharmaceuticals) or drug candidates in laboratory animal models is to predict if a drug would adversely affect the ability to achieve and maintain pregnancy and normal development of offspring.

Many drugs that are harmless to the mother can impair development of a human embryo. This led to the recognition of the need for a testing procedure to detect any potential toxicity. A classic example is thalidomide that had beneficial maternal outcomes, but had altered normal embryonic development, resulting in an abnormal offspring. This led to the introduction of legal requirements in many countries for the testing of potential new drugs before release to the general public for use. A major aim was to prevent a repetition of the teratogenic effects of thalidomide and so, not unnaturally, detailed reproductive tests were included as part of the testing battery. In 1966, under the direction of Dr. Lehmann, the United States Food and Drug Administration (US FDA) published guidelines for a three-segment study for drug testing for adverse effects on fertility and pregnancy. This proved to be a classic design, which was used until recently, with only minor modifications, throughout the world (Sullivan, 1988). In 1992, new guidelines for the testing of drugs were agreed internationally (ICH, 1992 and revisions thereafter).

2.1.1 *Three-Segment Reproduction and Teratogenicity Study*

In general for rodents and rabbits, three dose levels plus controls are used in each segment. In nonhuman primates (NHP), a minimum of two dose levels plus control is routinely used, but up to three dose levels is not uncommon.

2.1.1.1 *Fertility Studies (Segment I)*

Fertility and general reproduction performance studies fundamentally test for toxic responses that may be a sequelae of treatment with the test substance before mating takes place, during the mating period, at preimplantation and until completion of implantation (gestation day (GD) 6 in rats and GD9 in NHP) (Dimitriadis *et al*., 2003). It is worth mentioning that the process of embryo implantation itself plays a major role in embryogenesis and the outcome of pregnancy. Parameters evaluated include libido (rats and monkeys), effects on oestrous (rats), effect on menstrual cycle length (monkeys) and implantation (dead and resorbing foetuses in rats).

2.1.1.1.1 Segment I Study Design in Rats

In rats, male animals are treated for 60–80 days to cover the whole period of spermatogenesis. The female rats are pretreated for 14 days to cover three oestrous cycles. The female animals are then mated with the treated males (combined Male and Fertility Study, FDA Segment I and International Conference on Harmonization (ICH) S5), treatment of the males and females continuing during the mating period. In rats, treatment of the females continues either through to GD6 or throughout pregnancy, followed by necropsy and evaluation at GD13 or GD21 (**Figure 1**).

In stand-alone female or male rat fertility studies, the test article is administered only to the females or males, the untreated sex acting only to demonstrate if the treated sex's fertility was impaired.

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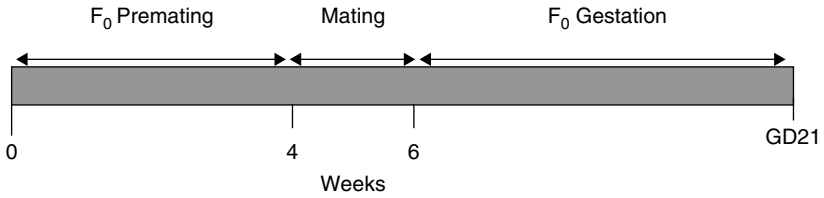

Figure 1 Standard combined study design for investigation of fertility in male and female rats.

2.1.1.1.2 Segment I Study Design in Nonhuman Primates

The use of NHP (specifically the cynomolgus monkey, *Macaca fascicularis*) in reproductive toxicology testing, including fertility (male and female), teratogenicity and peri- and postnatal toxicity, has become increasingly commonplace in recent years because of tremendous growth in biopharmaceuticals being tested for human use. Subsequently, standardized study designs have been established in order to meet international requirements for reproductive toxicity testing using NHP to be in line with ICH Guidelines (ICH, 1994).

In fertility studies using the monkey model (male and female), mating is not normally required (unlike in rodent studies) and as such, fertility or reproductive function assessments are incorporated in a chronic toxicology study design. Animals assigned to such studies must be sexually mature (generally > four years), regularly menstruating females, and males with total testicular volume of at least 20 ml (10 ml per testis) and that can ejaculate on electrostimulation. In males, the ejaculates should have adequate numbers of sperm (mean concentration of $600 \times 10^6\,\mathrm{ml}^{-1}$ (range 150–1012) and mean total count of 131×10^6 per ejaculate (range 50–260)), be of good quality, that is, highly motile and with minimal dysmorphologies. Prestudy screening for hormones is optional (baseline data is obtained during acclimation before animal dosing), and only testosterone levels may be measured. Overall, male reproductive function assessments during the study include testicular volume measurements (using a caliper), semen analysis (preferably automated, CASA (computer assisted semen analysis), e.g. IVOS Hamilton Thorpe Inc.), hormonal analysis (testosterone and luteinizing hormone (LH)) and histopathology of the testis and male reproductive accessory glands (seminal vesicles, prostate gland and the bulbourethral glands). Semen collection methodology is an important consideration for good semen quality (VandeVoort, 2004). Direct penile electrostimulation is the preferred method. This method was first developed by Mastroianni and Manson (1963) using metal foil electrodes and square wave pulses. With the use of EKG gel electrodes, the risk of burn injury is drastically reduced (Sarason *et al*., 1991). Animals are not anaesthetized for this procedure, but rather are trained for chair restraint. This method has become common for semen collection and analysis in chronic toxicology studies. To assure the best collection of semen, the following must be taken into consideration: training and acclimation of animals to the restraint method and the procedure (a minimum of four weeks required), and the operator must be calm. Semen collection should be performed at intervals, not only for animal welfare, but also ejaculate quantity and quality, even though it has been reported that collection can be performed several times in a row without ill effects (Settlage and Hendrickx, 1974). As has been reported by VandeVoort, 2004 and based on author experience, early morning collection time produces the best samples. It is likely that in the late morning the male may have already masturbated to the point of depleting sperm numbers in the ejaculate. In order to accurately determine the important variable related to fertility in males, the total sperm count (number of spermatozoa per ejaculate) rather than sperm concentration (number of spermatozoa per unit volume of semen) is taken into account. Moreover, total sperm count reflects testicular volume (Handelsman *et al*., 1984; Andersen *et al*., 2000), and is therefore a measure of total testicular sperm output (MacLeod and Wang, 1979). This correlation is directly related to the chances of pregnancy after coitus (Cooper *et al*., 2007). For hormone analysis, samples are also collected in the morning and at a consistent time throughout the study period. For LHs, a total of three samples are normally collected at 30 minute intervals (±5 minutes) at each time-point to measure moment-to-moment changes in circulating concentrations of the hormone (Ramaswamy *et al*., 2007).

In females, at least two normal menstrual cycles (mean 28 days (range 25–35 days) must be recorded prior to assigning an animal to a study. Hormonal measurements include progesterone and 17β-oestradiol, at several time-points during one or two menstrual cycles for baseline and each study period (menstruation days (MDs) 2, 9, 13, 16, 19, 23, 27 and 30, and every five days thereafter until the onset of the next menstrual cycle).

2.1.1.2 *Teratology Studies (Segment II)*

Maternally mediated developmental toxicity may occur as a consequence of either direct drug (or metabolite) exposure to the embryo after crossing the placental barrier or toxicant-induced alterations in maternal physiology at dosages not causing overt maternal toxicity, but affecting the conceptus.

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Segment II of the study is the 'teratogenicity' or embryotoxicity segment, in females only (males are used for breeding purposes only), and is still essentially included in the new ICH guidelines for rats and rabbits.

2.1.1.2.1 Rats and Rabbits

For most small molecules and industrial chemicals, a teratology study is normally carried out in two species, usually rats and rabbits. Mated animals are treated during the period of organogenesis (days 6–15 in rats, 6–18 in rabbits) and the pups are delivered by Caesarian section (C-section) on day 20 for rats (the day before expected parturition for examination, 21 day gestation period) or on gestation day 28 for rabbits (32 days normal gestation period). The harvest of foetuses before the normal natural delivery dates is necessary in order to prevent loss of deformed foetuses by cannibalism, a normal phenomenon common in these species. The pups are examined for gross, soft tissue and skeletal defects by the use of appropriate dissection and staining techniques.

2.1.1.2.2 Nonhuman Primates

The cynomolgus monkey is the model of choice because of availability of extensive teratology background data. The rhesus monkey may also be used when the test article is crossreactive only in this species. The common marmoset (*Callithrix jacchus*) is rarely used and there is no reproductive background data generated in a regulated (Good Laboratory Practice; GLP) environment. In addition, marmosets have germ-line chimerism (when the sperm and egg cells of an organism are not genetically identical to the adult organism that gave rise to the sperm or egg) and they can carry the reproductive cells of their twin siblings, because of placental fusion during development (Ross *et al*., 2007), thus making this species unsuitable for teratology studies. No guidelines have been established for use of NHP in teratology studies. However, the designs take into consideration ICH S5(R2) (Detection of Toxicity to Reproduction for Medicinal Products and Toxicity to Male Fertility) and ICH 6 (Preclinical Safety Evaluation of Biotechnology-Derived Products, 1997) and are now routinely used. In addition, the embryofoetal developmental timeline is longer than in rodents or rabbits and is comparable to humans (O'Rahilly, 1988), with first trimester between GD0 and 50, second trimester GD50 to 100 and third trimester between GD100 and natural delivery at GD165 (±10 days).

In teratology studies using the monkey model, typically 12–16 animals are assigned to a group and dosed, starting between GD20 and 50 for small molecules or GD20–139 for biopharmaceuticals. Ultrasound monitoring is performed on a regular basis to monitor embryofoetal viability.

At day 100 (typically for small molecules) or day 140 (for biopharmaceuticals), foetuses are collected by C-section and evaluated for external, visceral and skeletal malformations (example details in Martin *et al*., 2007). The diagram below outlines the general scheme for a teratology study in NHP using the traditional small molecule design as an example (**Figure 2**).

The incidence of embryofoetal loss ranges from 0 to 25%, with a mean of 12% (Hendrie *et al*., 1996; Martin *et al*., 2007).

2.1.1.3 Peripostnatal Studies (Segment III)

The study design aims to detect any adverse outcomes developmentally and/or functionally on the offspring. Pregnancy period outcomes may also be detected (e.g. abortions) because the treatment of the female starts at implantation (GD6 for rats, GD20 for NHP), or in rats towards the end of organogenesis (~GD15), to lactation and weaning (postnatal day (PND) 21). Detailed study designs and regulatory requirements for using rats can be found in the ICH Guidelines. Segment III study designs in NHP are not included in any guidelines because the need for testing using this species is recent due to the emergence of biopharmaceuticals. In designing Segment III studies using NHP, the ICH guidelines are taken into consideration. Dosing starts on GD20 up to GD50 for small molecules and up to natural delivery (±165 days) for biopharmaceuticals. Typically, no dosing of

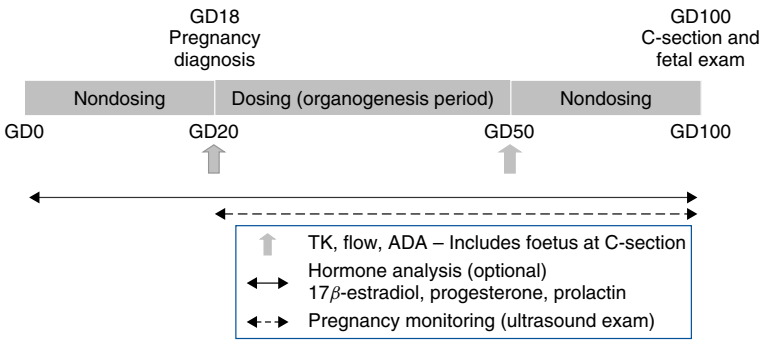

Figure 2 Standard teratology study design in the cynomolgus monkey. In-life parameters measured routinely for animals health monitoring include clinical signs, body weight, food consumption and haematology. ADA = anti-drug antibodies; TK = toxicokinetics; flow = flow cytometry. (Reproduced with permission from Gerhard *et al*., 2007. © Waxmann Verlag.)

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Table 1 Cynomolgus monkey foetal growth and heart rate measurement time-points

Gestation day	Crown–rump length (CRL <)[a]	Femur length (one side, FL)	Biparietal diameter (BPD)	Heart rate (HR)
GD31 (±1 d)	X	—	—	—
GD37 (±1 d)	X	—	—	—
GD45 (±1 d)	X	—	—	—
GD53 (±1 d)	—	—	X	X
GD70 (±2 d)	—	X	X	X
GD90 (±2 d)	—	X	X	X
GD110 (±2 d)	—	X	X	X
GD130 (±2 d)	—	X	X	X
GD165 (±10 d)	—	X	X	X

[a]Equivalent to 'greatest length' at these stages of embryo development; X = measured; — = not measured.

the maternal animals or infants is performed following delivery. The infant is raised naturally by the maternal animal and is evaluated for approximately six months.

In both the Segment II and Segment III studies, detailed embryofoetal monitoring is carried out throughout pregnancy. Parameters monitored by ultrasound at various time-points are shown in the following chart (**Table 1**) (see Tarantal and Hendrickx, 1998 for additional parameters).

Segment III studies in rats are carried out on pregnant animals treated either during the last part of gestation (in rats) or throughout gestation in nonhuman primates, NHP (GD20–165).

2.1.1.3.1 Rats

Dosing continues through parturition until weaning. This is to examine whether the drug has any adverse effects on parturition or lactation which have not been detected in the Segment I study. Higher doses may be used in this segment than in the Segment I study.

2.1.2 International Conference on Harmonization (ICH) Guidelines

Although the US FDA guidelines were widely accepted and used for testing drugs, advances in knowledge and increasing interest in adverse effects of chemical exposure during pregnancy on postnatal development led to a number of changes and additions to the test protocols. In particular, there were small, but significant, differences between US/European guidelines and those recommended in Japan, and the number of postnatal studies required to fulfil all the requirements became unwieldy and led to the recommendation of a new set of guidelines for the testing of new medicinal products under the auspices of the ICH in 1992 (ICH, 1993; 1994; Manson, 1994). The underlying theme of the new guidelines is flexibility; to define the major areas of concern to be investigated with respect to developmental toxicity and to give wide scope for the experimenter to design a series of tests, suitable for the specific drugs investigated. Factors specifically relevant in the design of the actual testing strategy include: (i) anticipated drug use in relation to reproduction, (ii) the form and routes of administration intended for humans and (iii) making use of any existing data toxicity, pharmacodynamics, kinetics and similarities with other compounds in structure/activity.

The specified aim of the studies is to choose a combination of studies, which will allow exposure of male adults and all stages of development from conception to sexual maturity. To allow for detection of immediately delayed effects, it is recommended that exposure should be through the whole of one complete life cycle, that is, conception in one generation through to conception in the following generation. For convenience of testing, the guidelines divided this into six stages:

1. Premating to conception
2. Conception to implantation
3. Implantation to closure of the hard palate
4. Closure of the hard palate to the end of pregnancy
5. Birth to weaning
6. Weaning to sexual maturity.

It is emphasized that whatever study design is chosen by the investigator, all of these stages should be performed without omissions, and that where a number of separate studies are performed, there should be an overlap of at least one day in the exposure periods of related stages. Other guidelines address studies which are designed primarily for the detection of effects, and state that when an effect is detected, then further studies to characterize the nature the response have to be designed on a case-by-case basis.

When positive results are observed in any part of reproduction studies, this will normally lead to further studies being carried out to investigate the mechanism of action. The effects observed may be extensions of

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pharmacological actions which might be expected at high doses used in the toxicity tests, but would no longer be expected at the lower doses used clinically. Sometimes the actions may be exerted on rodent-specific aspects of reproductive physiology and would not be a problem to humans. Differences in results between rats and rabbits may be due to metabolites which are produced in one species, but not another. It is then important to know which metabolites are produced in humans and to be sure that these are adequately tested in the animal studies; it may sometimes be necessary to synthesize adequate quantities of human-specific metabolites and for animal tests to be repeated using these.

2.2 Pesticide Testing

The safety evaluation of pesticides for reproductive toxicity has to take into consideration several factors which are not relevant for drug testing. By their nature pesticides are toxic compounds since their use is to kill target species, which may be plants, insects, fungi and so on, although they may have very low toxicity for mammals. First consideration has to be given to the safety of workers handling these chemicals. Depending on their use, the exposure of operators may be considerable and this has to be taken into account in the final risk assessment of safety in use and the need for protective clothing to be worn. For chemicals used for crops there have to be residue data so that exposure of the general public consuming the food can be calculated. For chemicals used on amenity lands such as parks, gardens and playing fields, consideration has to be given to exposure of the public using the facilities. The environmental ecological impact must also be examined so that studies may have to be carried out to look for effects on beneficial species such as earthworms and bees, and also on fishes, birds, predators and so on. Reproduction tests on these latter species are outside the scope of this chapter, but are of great environmental interest.

For tests related to operator exposure, it is common to carry out tests equivalent to the Segment I ad II tests described above for drugs. These cover possible affects on male and female fertility and developmental toxicity. For pesticides leaving significant residues on food, it is usual to carry out a multigeneration study to test for long-term or cumulative effects in adults or effects on young developing animals. In such tests animals are treated continuously with the chemical, usually at three dose levels administered in the diet, though several generations. Several different designs of multigeneration studies have been used for pesticides testing. The original design proposed by the US FDA for testing food additives (Fitzhugh, 1968) which is a three-generation, two-litter test, has been widely used. This involves treating young male and female rats with three dose levels of the test substance, plus a control group, and allowing them to produce two litters, F1a and F1b. Selected pups from the second (F1b) litters are reared to maturity, still under treatment, and in turn are allowed to produce two litters (F2a and F2b). Again, selected pups from the second (F2b) litters are reared to maturity, still under treatment, and allowed to produce two litters (F3a and F3b). The second (F3b) litters are then subjected to full pathological examination to examine for any affects of long-term exposure. An immense amount of information is obtained in such studies, not only on the different aspects of fertility and pregnancy, but also on possible affects on growth and development of the young animals.

In Europe, in an attempt to reduce the numbers of animals involved without significant loss of information, it is common to perform this test using only two generations with two litters in each. The new European legislation covering nonagricultural pesticides (Biocides Directive, 1998; Biocides Directive, Revision of Directive 98/8/EC, 2008) recommends that the tests carried out should be those in Annex V of Directive 67/548/EEC, which are, in practice, identical with the Organization for Economic Cooperation and Development (OECD) test guidelines. There is a new OECD draft guideline for a two-generation reproduction toxicity test (OECD, 1996). In the USA, a modified test using two generations with one litter in each, but with a large number of end points being assessed, is proposed for pesticide testing for the US EPA (US EPA, 1998). Recently, designs have been used where a third litter is produced in one or two of the generations with the dams killed just before delivery for examination of the foetuses so that the test also includes a teratology element.

2.3 Food-Additive Testing

The reproductive toxicity test requirements for the testing of food additives are less well defined in Europe than for the other classes of chemicals considered above (EEC, 1989; DHSS, 1982), but in general the test methods of the OECD are acceptable. In the USA, however, the FDA have published an extensive account of the types of test which may be required depending on the use, amount, frequency and duration of exposure expected (US FDA, 1982), and an extensively revised final version (Redbook II) was published in 2000 (US FDA, 2000). In general, fertility and developmental toxicity are tested using segments modified so that the period of chemical exposure, especially in the teratology test, is extended to include most of gestation. Consideration also has to be given to whether the metabolism of the chemicals may alter on chronic administration, so that more than one dosing regimen may have to be used. For food-additive

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chemicals, where there is likely to be prolonged or extensive exposure, multigeneration studies are normally performed using one of the designs discussed above, usually a two-generation, two or three-litter test.

2.4 Industrial Chemicals Testing

In most industrialized countries, there are now testing requirements for industrial chemicals when these are introduced in significant amounts. In Europe, for example, under the requirements of the laws generally known as the 'Dangerous Substances Directive' (EU, 2008) there is a stepwise system which permits regulatory authorities to require sequential testing for fertility, teratogenicity and multigenerational effects, as the tonnage of chemical produced per year, or *in toto*, reaches certain critical values. There has been good international cooperation under the auspices of the OECD to establish mutually acceptable guidelines for conduct of reproductive toxicity studies, which have acceptance worldwide. As these are regularly updated, interested readers should obtain the most recent versions from the OECD.

The US National Toxicology Program has developed a test protocol for the evaluation of the reproductive toxicity of chemicals using a continuous breeding study design (Chapin *et al*., 1997; Lamb, 1985). The protocol was originally developed for mice as a cost-effective alternative to the more conventional regulatory breeding studies, but has also been employed successfully with the rat as the test species. The outline design is that after a one-week pretreatment period, males and females are housed as breeding pairs in individual cages and allowed to mate continuously during a 14 week testing period. Exposure to the test substance (usually in the feed or drinking water) is continued throughout the study, and the litters are removed from the cage immediately after parturition. Usually four to five litters will be produced in such a study. After the cohabitation breeding period, the pair is separated and the last litter is raised to weaning. This study design allows several litters to be examined per generation and adverse effects, which may not be observed in the first mating, may become evident in later mating. Other short-term testing or screening protocols have been published (Harris *et al*., 1992; Linder *et al*., 1992).

3 MECHANISM OF ACTION OF CHEMICALS AFFECTING REPRODUCTION

When any adverse effects are detected in any of the above screening tests, then extrapolation to humans involves analysis of the mechanisms and sites of action of the chemicals causing the effects. Some of the factors which have to be taken into account in analysing effects in males and females are considered in detail below, together with detailed descriptions of the types of toxic effects which can be produced in males and females.

3.1 Effects on the Male Reproductive System

3.1.1 Introduction

Exposure to a toxic agent may affect the male reproductive system at various stages of life: during foetal development of the gonads, due to exposure of the pregnant mother; during the quiescent prepubertal period before active spermatogenesis begins and postpubertal, during the long period of activity of spermatogenesis, which, although a gradual decline in testicular function occurs after middle age, continues into old age in man (Lipshultz and Howards, 1983). The two critical end points of concern in male reproductive toxicology are: (i) the production of sufficient sperm which are capable of fertilizing an egg and (ii) the production of sperm with normal chromosome number and structure, and genetic material. A toxic agent may produce alterations in spermatogenesis via an effect on the hypothalamo-pituitary-testicular axis, or affect accessory sex gland function or sexual function (libido, potency, ejaculation), with the end result that fertilization fails to occur. A toxin may, however, cause genetic or chromosomal damage to the germ cell, so that if a sperm carrying damaged genetic material fertilizes an egg, foetal death or a structural or functional abnormality in the newborn occurs (which may not become apparent until later life or even the subsequent generation).

In Western countries, about 15% of couples are clinically infertile (i.e. have not conceived after one year of unprotected intercourse), although estimates vary widely in different countries (Belsey, 1984). An estimated 30% of clinical infertility in couples has been attributed to the male, with a further 20% due to a combination of male and female factors (Lipshultz and Howards, 1983). A considerable proportion of infertility (perhaps 30%) is idiopathic, that is, cannot be explained by diagnosed genetic, anatomical or organic conditions in either partner. There is increasing concern about the possible role of environmental chemicals that cause endocrine disruption as possible aetiological factors in the decline in sperm counts which have been reported in different populations (Carlsen *et al*., 1992). Some 15–20% of recognized pregnancies end in spontaneous abortion and a further unknown number of conceptions are absorbed before pregnancy is diagnosed; approximately 50% of recognized first trimester abortions and about one-third of all recognized abortions up to 28 weeks are abnormal,

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according to Warburton *et al*. (1980); the frequency in early abortions is thought to be considerably higher. Approximately 6% of prenatal deaths and 0.6% of live births are chromosomally abnormal; further, an estimated 1% of infants carry a gene for autosomal dominant disease, of which possibly 20% are new mutations (Wyrobek, 1989). It is not known, however, what proportion of these abnormalities are contributed by the male or could be attributable to toxic exposures. Nevertheless, a number of agents have been identified experimentally as affecting one or more aspects of male reproduction and mechanisms of toxicity elucidated by which they exert their effect; a number of agents have also been studied in humans. Below we first briefly describe the physiology of male reproduction, then outline the main methods by which reproductive toxicity can be studied in animals and man and finally describe mechanisms and site of action of toxic agents, using examples from the experimental, clinical and epidemiological literature.

3.1.2 Physiology of the Male Reproductive System

3.1.2.1 The Hypothalamo-Pituitary-Testicular Axis

In the hypothalamus, neuroendocrine neurons secrete gonadotrophin-releasing hormone (GnRH) into the hypophyseal portal system, where it is carried in the blood to the adenohypophysis or anterior pituitary, where it stimulates the release of the gonadotrophic hormones, LH and follicle-stimulation hormone (FSH). Prolactin is also released from the anterior pituitary under the control of dopamine released from neuron terminals in the median eminence. In contrast to the action of GnRH, the presence of dopamine inhibits the release of prolactin. In turn, the hypothalamic neuroendocrine neurons contain receptors for monoamines (noradrenaline, dopamine, serotonin), and these are affected by the CNS transmitters (and agents which affect these transmitters). The target of action of both LH and FSH is the testis; although the primary actions of each hormone are on different testicular cells (see below), there is increasing evidence that their functions may be more complex and overlapping than previously thought. The functions of prolactin in males is less clear; it appears that prolactin may potentiate the effect of LH in the testis (for general reviews, see, e.g. Hansson *et al*., 1981; Lobl and Hafez, 1985; Cooke and Sharp, 1988; Johnson and Everitt, 1988; Knobil and Neill, 1994).

3.1.2.2 The Testis

The mammalian testis consists of two major compartments: the seminiferous tubules, in which spermatogenesis takes place, and the interstitial compartment. The interstitial space contains Leydig cells, which produce testosterone and other androgens under the influence of LH. These cells are closely associated with blood vessels and lymphatics which facilitate transport into the seminiferous tubules. Most of the circulating testosterone produced by the testis is bound to plasma proteins. Androgens are responsible for controlling spermatogenesis, the growth and secretory activity of accessory sex glands, somatic masculinization, male behaviour and various metabolic functions. *In utero*, androgen secretion by the developing foetal testis, critically in gestational weeks 4–6 in humans, is essential for sexual differentiation of the gonads; androgens control both the regression of the Mullerian duct (which forms female internal genitalia), the development of Wolffian ducts into the epididymis, vas deferens, seminal vesicle and ejaculatory duct and the development of the urethra, penis, scrotum and prostate.

There are two major cell types in the seminiferous tubules: the spermatogonial cells and the Sertoli cells. Sertoli cells have numerous functions, principally: (i) the nutrition of developing cells, (ii) the synthesis of androgen-binding protein, which permit the binding of testosterone within the tubular fluid of the adluminal compartment (note that the Sertoli cells themselves also have androgen receptors on them), (iii) synthesis of inhibin, which is probably involved in feedback regulation of both LH and FSH in the pituitary, (iv) secretion of fluid into the lumen of the tubule and (v) maintenance of the blood–testis barrier (BTB). The BTB, first demonstrated by Setchell *et al*. (1969), consists principally of specialized Sertoli-cell-to-Sertoli-cell junctions within the seminiferous epithelium. The layers of myoid cells surrounding the seminiferous tubule epithelium also appear to retard the passage of molecules. Permeability to compounds appears to be determined primarily by molecular size (Dixon, 1983; 1985; Pereira and Garcia e Costa, 2007; Gallegos-Avila *et al*., 2007; Wong *et al*., 2006). Molecules smaller than about 0.36 nm or MW $\sim$ 150 (e.g. water, urea) are transported easily. The rate-limiting factor for transport across the BTB is lipid solubility (Dixon, 1983). The permeability characteristics appear to be similar to those of the blood–brain barrier, membranes in the gastrointestinal tract, mammary gland and aqueous humour. (For general reviews of testicular function and the BTB, see, e.g. Burger and de Kretser, 1981; D'Agata *et al*., 1983; Schulze, 1984; Guo *et al*., 2007).

3.1.2.3 Spermatogenesis

Spermatogonia undergo several mitotic divisions in the basal compartment of the seminiferous tubule (separated from the adluminal compartment of the Sertoli cells). One population of dividing spermatogonia become stem cells, which will go on to become spermatozoa, while the rest form a replacement population of spermatogonia. The last mitotic divisions of spermatogonia (after a characteristic number of divisions in different species) result in primarily spermatocytes, which push past the tight

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Sertoli cell junctions and enter the adluminal compartment, where they continue to divide meiotically into secondary spermatocytes and finally round spermatids. Thus, meiotically dividing spermatocytes and subsequent stages of spermatozoal development are protected by the BTB provided by the Sertoli cells, whereas the spermatogonia are not. Cohorts are differentiating at varying times; there is a net continuous release of mature spermatids from the tubule. One cycle of the seminiferous epithelium (the time between successive entries into spermatogenesis of any given Type A spermatogonia) is remarkably constant in different species (e.g. 12 days in rats, 16 days in human), as is the total time for spermatogenesis (the spermatogenic cycle), which is about four times the length of the seminiferous cycle. The *rate* of spermatogenesis does not appear to be affected by the action of exogenous agents. This regularity allows the identification of the precise stage of spermatogenesis affected by, for example, experimental administration of a toxin. Agents can selectively cause degeneration of a given population of germ cells and thus eliminate subsequent stages of cells.

Spermiogenesis is the 'packaging' process by which round haploid spermatids become spermatozoa, and this occurs in close association with Sertoli cells. Immature spermatozoa (incapable of movement and fertilization) undergo maturation in the epididymis under androgen stimulation. It usually takes about 20 days from late spermatid stage to ejaculation in man. During passage through the epididymis, the sperm chromosomes are condensed and packed into the sperm head complete with a cap, and the acrosome which contains the digestive enzymes (including hyaluronidase and acrosin) required to facilitate sperm penetration into the ovum. A midpiece containing mitochondria and a tail are developed. In some species, for example, the mouse, sperm morphology is highly characteristic of the species, but in man, depending on the classification used, up to 40% of the sperm may be 'abnormal', without the oval head and usual tail shape (Belsey *et al*., 1980). In most mammalian species, there is a high degree of redundancy in sperm production, that is, some 10 000 to 100 million sperm are ejaculated. According to early work by Cohen (1975; 1977), at least part of this redundancy may be due to errors in crossing over of chromosome material during meiosis, which render the sperm unsatisfactory for fertilization, since he found a correlation between chiasma frequency in different species and sperm numbers in the ejaculate. Clearly, there is also considerable wastage due to structural or functional deficiency in many sperm, which reduces their viability, motility and ability to reach and penetrate the ovum. Sperm have to penetrate the cervical mucous of the female and combat the possible effect of sperm antibodies in the female tract before proceeding to the ovum. They must also reside for some period of time (usually several hours, depending the on species) in the female tract to become 'capacitated' or capable of fertilization (Yanagimachi, 1981).

The seminal fluid consists of epididymal fluid and secretions of the accessory sex glands, principally from the prostate (30% in man) and seminal vesicles (60% in man), but also from the bulbourethral (Cowper's) glands and ampulla. The biochemical composition of the seminal fluid and its volume may affect the viability and motility of the spermatozoa after ejaculation, but in complex ways that are not clearly understood, including presence of mRNA (for reviews, see, e.g. Burger and de Kretser, 1981; Mann and Lutwak-Mann, 1981; Burger *et al*., 1989; Almog *et al*., 2008; Lambard *et al*., 2004).

3.1.2.4 Libido, Potency and Ejaculatory Function

In man, psychological factors are of critical importance in determining libido, but neurological integrity of the nervous system is also important, as centrally acting agents can affect libido in man and mating behaviour in mammals (see below). Potency in man is under parasympathetic nervous control; endocrine factors are less involved. The sympathetic nervous system controls ejaculation. In both cases, psychological factors may be involved in disturbances of these functions (for a review, see, e.g. Benson and McConnell, 1983; Jern *et al*., 2008).

3.1.3 Methods for Assessing the Effect of Toxic Agents on the Male Reproductive System

Studies of toxic agents and their mechanisms of effect on male reproduction can be divided into: (i) experimental (usually using the rat, mouse or rabbit), (ii) clinical (usually infertility clinic patients or patients undergoing specific drug treatments) and (iii) epidemiological (studies of groups of men exposed occupationally or environmentally to some agent of concern). Clearly, a greater number of reproductive end points and mechanisms can be studied in the laboratory than in the clinic or field setting. Examples of the techniques used in the laboratory include gross pathology and histology of the testes and accessory sex glands (US EPA, 1998; OECD, 1996). Sperm analysis may be carried out to measure sperm viability, motility, swimming speed and morphology, and computer-assisted methods are now widely used (Working and Hurtt, 1987; Slott *et al*., 1991; Chapin *et al*., 1992; Sloter *et al*., 2006). The rabbit (Williams, 1993) and NHP are useful animal models for such studies, since repeated semen samples can be obtained for sequential analysis. Very useful overviews of methodology have been published (Chapin and Heindel, 1993; WHO, 1999).

Studies in the mouse indicate that sperm shaping is under genetic control in this species, and that exposure to known mutagens increases the frequency of abnormal sperm shapes (Wyrobek *et al*., 1983a; Narayana *et al*.,

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2002). There is also a correlation between induction of sperm abnormalities and an increase in germ cell mutations, as tested by the dominant lethal, specific locus and heritable translocation tests (see below), suggesting that, in the mouse at least, abnormal morphology may predict for germ-cell mutagenicity (Wyrobek *et al*., 1983a). However, other reviewers have argued that sperm shape does not necessarily predict for chromosomal or DNA damage within the sperm, particularly as damage to Sertoli cells may result in secondary morphological abnormalities (Russell and Shelby, 1985; Working, 1989). Chromosomal analyses of male germ cells have been developed, which have been shown to detect germ-cell mutagens (Working, 1989). Dixon and colleagues have used a method of measuring unscheduled DNA synthesis in sperm (which would not normally occur after the first replications of DNA before mitotic divisions in the spermatogonia) using incorporation of radiolabelled thymidine into sperm DNA following experimental mutagen treatment (Lee and Dixon, 1978). It has been shown to occur in spermatogonia and leptotene, zygotene, pachytene and diplotene stages in spermatocytes and round spermatids, but not in elongated spermatids and spermatozoa, indicating that DNA mutation induced in these stages cannot be repaired (Working, 1989). The swimming ability, capacitation and fertilization capacity of mammalian sperm have also been studied *in vitro* (Katz *et al*., 1989; White *et al*., 1990). Assays of seminal fluid to assess accessory sex gland function (e.g. levels of fructose and zinc) and assays of gonadotrophic hormones in blood and urine may also be conducted.

To determine the effect of a toxic agent on reproductive performance, mating behaviour (e.g. frequency of copulation) may be measured, usually in rodents (Chubb, 1993). To determine the effect of treatment on the various stages of spermatogenesis, serial mating of treated males with untreated females is conducted and the number of implantations, foetal deaths or litter sizes determined; the effect of agents affecting the epididymal spermatozoa will be seen in the first mating and effects on stem-cell spermatogonia in the last matings (Dixon, 1983). One of the most common tests of chromosomal damage in the germ cell in mammals is the dominant lethal mutation test (Bateman and Epstein, 1971; Ehling *et al*., 1986). A dominant lethal mutation is one which results in the death of the early conceptus, and may be due to chromosome breakage or nondisjunction, or irreparable fatal breaks in DNA strands in meiotic or postmeiotic cells. The mutation occurs prior to fertilization and, theoretically, could kill the zygote at any time during its development. However, in mice, for example, death is rarely seen after 10 days (half the gestational age) (Bateman and Epstein, 1971). It is not clear what the period of vulnerability is in humans, although recent studies of karyotyped human abortuses suggest that most lethal mutations are expelled in the first trimester of pregnancy (Warburton *et al*., 1980), often, as in the case of all monosomies except XO, too early to be detected. To determine the heritability of nonlethal chromosomal and genetic defects, heritable translocation and specific locus mutation tests and multigeneration tests in, for example, mice may be conducted (Flamm and Dunkel, 1989). Behavioural and developmental tests of late effects of toxic exposure of the male on viable off-spring have also been utilized.

In the clinical setting, investigations of infertility or the reproductive toxicity of drugs in humans may include a battery of tests on semen quality, including sperm count, viability, motility, velocity and morphology and seminal fluid analyses (Belsey *et al*., 1980; Amann, 1981; Dixon, 1983; Eliasson, 1983; Lipshultz and Howards, 1983; WHO, 1999); FSH, LH and testosterone levels in serum (Swerdloff and de Kretser, 1983); sperm-cervical mucus interaction assays (Kremer and Jager, 1976); *in vitro* fertilization assays using zona pellucida-free hamster eggs (Rogers, 1989; WHO, 1999); physical examination and testicular biopsy; psychological evaluation and measurement of time to conceive in the individual couple (Lipshultz and Howards, 1983).

The epidemiological study of reproductive toxins presents considerable methodological difficulties, principally those of obtaining an adequate sample size, of being able to measure or estimate exposures or effects accurately and of being able to take into account other exposures or risk factors (e.g. age) which could explain an apparent association between exposure to a toxic agent and an affect of reproductive function (Bloom, 1981; Vouk and Sheehan, 1983; Lockey *et al*., 1984). Further, a number of the tests which have been used in the clinical setting to diagnose the infertility of individuals are not suitable for epidemiological studies, for reasons of expense, practicality and/or unknown predictive value of the tests for infertility in populations (e.g. *in vitro* fertilization, sperm-cervical mucous interaction). Relatively few studies of reproductive outcomes (e.g. spontaneous abortions, birth defects) in partners of exposed men have been conducted, partly owing to the large sample sizes required and the difficulty of taking into account maternal factors and exposures. For example, approximately 170 pregnancies in each of an exposed and nonexposed group, representing about 2000 person years of exposure, are needed to detect a twofold increase in foetal deaths due to an exposure. The use of live birth rates to detect changes in fertility in exposed groups of men has been attempted, but is an insensitive measure of subfertility and presents problems of determining a standardized expected live birth rate (adjusted for, e.g. parity, race), particularly if existing vital statistics have to be used.

Wilcox, Baird and Weinberg have developed a more sensitive measure of time to conception (Baird *et al*., 1986), which requires about 55 pregnancies in each of an exposed and nonexposed group to detect a 50% decrease in the probability of conceiving in any one noncontracepting menstrual cycle. This may still mean

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that more than 700 person years of exposure per group must be studied (depending on the pregnancy rate) to obtain the required number of pregnancies. Reliance has therefore been placed on studies of semen quality in exposed men. For example, techniques of semen analysis using mobile laboratory equipment have allowed the study of semen characteristics requiring fresh samples (e.g. sperm motility) to be evaluated in the occupational setting (Schrader *et al*., 1987).

The principal problems in the epidemiological study of male reproductive toxicology remain: (i) determining the predictive value of experimental data in laboratory animals for humans; (ii) determining the biological significance of alterations in semen quality in humans to male fertility and subsequent pregnancy outcomes and (iii) improving the methodologies for studying exposure to toxins on semen parameters and reproductive outcomes.

3.1.3.1 *Mechanisms of Sites of Action of Male Reproductive Toxins*

Several types of toxic agents will be considered here, which may be usefully classed into one of the following groups: physical agents (e.g. ionizing radiation); pharmaceutical (therapeutic) agents; recreational and illicit drugs; industrial or environmental chemicals, including agricultural chemical, and biological, naturally occurring toxins. Although the modes of action of some chemicals or classes of agents have been discovered, it must be emphasized that the mechanisms of action of many agents known to affect one or more aspect of male reproduction have not been elucidated, and may involve multiple sites of action and complex activity.

Some general mechanisms of toxicity may be discussed first. A toxic substance may act in one of more of the following ways:

1. An alteration in chromosome number or structure (e.g. translocations, deletions or insertions of parts of chromosomes) or a gene mutation in the germ cell may occur. Although assessment of chromosomal abnormalities and genetic mutations in human sperm has not been possible until very recently, some data suggest that perhaps 2% of human sperm are aneuploid (i.e. have numerical abnormalities) and 6% have structural chromosomal abnormalities (Martin, 1985; Martin, 2008). In a recent study, it was found that microdeletion and sex chromosome aneuploidy (47, XXY) rates in somatic cells are approximately 3.2 and 4.7%, respectively, while sperm aneuploidy rates are approximately 9.22 and 47% (Cinar *et al*., 2008). It is possible that sperm with abnormal chromosomes are somehow selected out in the female tract before arrival at the ovum or they cannot fertilize the egg. If fertilization does take place, most chromosomal abnormalities would be expected to result in early, or occasionally late, foetal death through a dominant lethal effect, for example, virtually all monosomies and most trisomies are fatal. If the foetus survives, it may have a congenital abnormality or a genetic disorder which may be expressed at birth, later in life or in the next generation. Certain chromosomal abnormalities are entirely due to a paternal contribution (e.g. XYY), whereas in others the contribution is less, for example, 30% for Down's syndrome (trisomy 21); about 50% of mutations of small segments of DNA (minisatellite mutations) are thought to be due to the male. The role of toxic exposures in the induction of genetic abnormalities is unknown.
2. Spermatogenesis and normal sperm viability, motility and morphology may be affected by either direct cytotoxic action on spermatogonial cells or later sperm cells (including agents which interfere with normal meiosis, for example, spindle poisons such as benomyl), an effect on Leydig cells or Sertoli cells, or an effect on the hormonal release and feedback of the hypothalamo-pituitary-testicular axis. Effects on accessory sex gland function secretions may also affect normal sperm function after ejaculation and seminal fluid volume. The net result may be impairment of fertility, due to low sperm numbers, their failure to survive in the female tract or to swim up to the ovum, or their failure to fertilize due to a morphological abnormality or a functional deficit, such as failure to capacitate.
3. An effect on sexual behaviour (reduced mating frequency or loss of libido, impotence or ejaculatory function) may occur, mediated by an effect on central nervous system (CNS) or autonomic nervous system function.

Reproductive toxins may act directly because of structural similarity to an endogenous compound (e.g. agonists or antagonists of endogenous hormones), or because of general chemical reactivity (e.g. alkylating agents); these toxins usually have several sites of action (Mattison and Thomford, 1989; Fukushima *et al*., 2005). Other toxins may require metabolic activation before exerting their effects (e.g. cyclophosphomide, 1,2-dibromo-3-chloropropane (DBCP), polycyclic aromatic hydrocarbons (PAHs)). Finally, some toxins act indirectly by inducing or inhibiting enzymes involved in steroid synthesis or clearance or by interfering with neuronal control of hormone levels or sexual function (Mattison and Thomford, 1989).

It is clear that in order to exert a toxic effect at a given site of action, the toxin or its metabolite must reach the site of action in sufficient concentration to overcome the detoxification and repair mechanisms in the cells of the tissue (Lee and Dixon, 1978). Apart

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from general aspects of the pharmacokinetics of absorption, distribution, metabolism and excretion of a toxic agent, several specific aspects of the male reproductive system's response to insult affect toxicity. These include: (i) the resistance offered by the BTB to testicular toxins; (ii) local and systemic biotransformation (activation, detoxification) of chemicals and (iii) the capacity of spermatogenic cells to repair cell damage and damage to DNA (Lee and Dixon, 1978; Dixon, 1983).

Both the testis and the liver contain mixed function oxidase systems which can metabolize (activate or detoxify) chemicals, and also enzymes which are capable of conjugating toxic chemicals for elimination (Lee and Dixon, 1978). In general, the elimination of numerous xenobiotic and endobiotic toxic chemicals requires concerted function of Phase I and II enzymes, as well as the membrane-spanning drug transporters (Sonoda *et al*., 2003). Mixed function oxidase activity appears to be found principally in the interstitial tissue of the testis, whereas conjugating enzymes, such as epoxide hydrase and glutathione-S-transferase, are found mainly in the tubules. In general, the levels of these enzymes are considerably lower than in the liver, but may be important in the local biotransformation of xenobiotics that penetrate the BTB. Certain compounds, such as direct-acting alkylating agents can affect cells without activation, but several testicular toxins, such as dibromochloropropane, PAHs, hydrazines and cytoxan, require metabolic activation first (Lee and Dixon, 1978). There may also be differences in the capacity of the testis to metabolize a given toxin at different stages of spermatogenesis, which may be one explanation for differences in sensitivity of different spermatogenic stages (Bateman and Epstein, 1971).

The timing of exposure is also of critical importance in determining toxicity. If exposure occurs *in utero*, normal sexual differentiation may be interrupted, resulting in anatomical abnormalities in the male gonads. Unlike in the female, however, where the complement of ova are developed *in utero*, the male gonad does not start to develop mature germ cells from the seminiferous epithelium until puberty, when spermatogenesis begins, so that prepubertal exposures would be expected to be of significance only if the testicular seminiferous cords of Leydig cells were irreparably damaged. Exposures to exogenous hormones such as sex steroids, if they delayed the onset of puberty and normal spermatogenesis, would be of importance if administered peripubertally. Once spermatogenesis has begun, the timing of exposure may alter the effect observed, depending on the stage(s) of spermatogenesis exposed and the agent involved. For example, in rodents, early spermatids appear to be two to three times more sensitive to the effects of ionizing radiation on mutation induction than epididymal sperm (Bateman and Epstein, 1971), but late spermatids and epididymal sperm are more sensitive to the effects of alkylating agents.

3.1.3.2 Examples of Toxins and Their Mechanisms and Sites of Action

3.1.3.2.1 Toxins Which May Affect Germ Cell Chromosomes, Genes and DNA or RNA Synthesis

Toxins that can interfere with normal meiosis and the accompanying numerical and structural rearrangement of chromosomes, that subsequently cause chromosomal or genetic damage or that interfere with DNA synthesis or replication are likely to be especially toxic to rapidly proliferating cells such as germ cells (Wudl and Sherman, 1983). Some oligopeptide antibiotics, such as netropsin and distamycin A, form stable complexes with DNA and inhibit DNA and RNA synthesis (Kornberg, 1980). Similarly, certain planar polycyclic aromatic compounds, such as acridine and actinomycin D, intercalate with the DNA molecule and disrupt its structure and function. Alkylating agents, among other actions, covalently bind to DNA base molecules or DNA-binding proteins, for example ethylnitrosourea is a potent mutagen, as shown by the specific locus test in the mouse, and induces reversible sterility in this species (Russell *et al*., 1979; Kennedy and O'Bryan, 2006). Other agents shown to be mutagenic in the mouse-specific locus test include cyclophosphomide, methyl, ethyl and propyl methanesulfonate, procarbazine and triethylenemelamine (Russell *et al*., 1981). A number of cancer chemotherapy drugs, such as niridazole, appear to suppress the meiotic division of spermatocytes (Mann and Lutwak-Mann, 1981).

Certain heavy-metal ions, such as mercury, lead and cadmium, can bind to phosphate, sulfhydryl and imidazole groups in proteins and disrupt the activity of a number of enzymes, such as DNA polymerase. These metals are generally cytotoxic, as they also interfere with the action of mixed function oxidases and phosphorylases, and may affect the transport of ions across cell and mitochondrial membranes.

There are a large number of chemicals which have been tested for dominant lethal mutations in rodents. According to a review of the literature by Green (1985), 65 chemicals could be considered positive in this test. Joffe and Soyka (1981) have also reported data showing that certain compounds, such as morphine, methadone, alcohol and lead, increase the frequency of postnatal mortality in rodents; the mechanism of this effect has not been elucidated. Owing to the methodological difficulties discussed above, there are no cytogenetic data on the induction of chromosomal or genetic mutations by these agents in human sperm, however. Several have been shown to affect sperm quality (see below). Only a few agents have been studied in relation to spontaneous abortions, heritable birth defects or later effects in man, mainly due to methodological difficulties, such as the large samples sized required, as discussed above.

Although some effects have been reported in man, none of the studies have provided conclusive evidence

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of a paternally mediated chromosomal or genetic effect on the foetus or offspring. Infante *et al*. (1976) reported an increase in spontaneous abortions among wives of men exposed to vinyl chloride. In another study by Lindbohm *et al*. (1991), specific mutagenic exposures were evaluated, involving paternal exposure to ethylene oxide, rubber chemicals, solvents used in refineries and solvents used in the manufacturing of rubber products. The study found an increased relative risk of spontaneous abortion, with the risk being higher among wives of rubber-products workers than among unexposed men. A number of early studies of lead-exposed men suggest an increased rate of abortion among their wives (Rom, 1976). Anaesthetic gas exposure has also been linked to abortion in wives (Tomlin, 1979), although not in other studies (Knill-Jones *et al*., 1975). There have been very few studies of paternal exposures and birth defects; a number of investigations using malformation registry data have not shown consistent associations (Barlow and Sullivan, 1982). Specific exposures studied include exposure to 2, 3, 7, 8-tetrachlorodibenzodioxin (TCDD) in Agent Orange used in Vietnam, reported by Erickson *et al*. (1984), who found no association with malformations and waste anaesthetic gas exposure among anaesthetists by Knill-Jones *et al*. (1975), who reported a significant increase in minor malformations, but this has not been confirmed in other studies. Gardner *et al*. (1989) reported a significant increase in childhood leukaemia associated with occupational radiation exposure of the fathers; this is the first case where a link between a paternal exposure to radiation and childhood cancer has been demonstrated.

3.1.3.2.2 Toxins Which Affect Spermatogenesis and Sperm Quality

There have been a large number of experimental studies of drugs, narcotics and chemicals on sperm parameters, including sperm count, viability, motility, velocity, morphology and *in vitro* fertilizing capacity (see reviews by Wyrobek *et al*., 1983a; 1983b; Pasqualotto *et al*., 2004). It is well known that different toxins may act selectively on different stages of spermatogenesis, affecting different germ-cell populations (Parvinen *et al*., 1993). It is often not easy to distinguish, however, even in experimental studies, whether observed effects on spermatogenesis are caused by direct effects on germ cells, damage to Leydig or Sertoli cells or effects on endocrine function, or by combinations of these effects. According to Bernstein (1984), there are a number of classes of organic compounds for which structure–activity relationships have been shown to predict for spermatotoxicity, and which have in fact been shown to affect sperm quality in animals (Wyrobek *et al*., 1983a). These include: (i) straight-chain two-and three-carbon compounds containing halogen atoms (e.g. ethylene dibromide, dibromochloropropane, halothane); (ii) organochlorine compounds with a symmetrical chlorinated carbon bridge (e.g. dichlorodiphenyltrichloroethane (DDT), methoxylchlor, heptachlor, chlordane and hexachlorophene pesticides); (iii) nitrobenzenes (e.g. 4-nitrobenzamide, dinoseb); (iv) certain organophosphates (e.g. the pesticides dichlovos, trichlorfon); (v) aminobenzenes containing sulfonic, carboxylic or hydroxyl solubilizing groups (e.g. the drug sulfasalazine, used to treat ulcerative colitis) and (vi) the glycol ether family of solvents, especially the methyl and ethyl forms. In addition, heavy metals and some of their compounds (Barlow and Sullivan, 1982; Davies, 1983); alkylating agents, antibiotics and antimetabolites used as cancer chemotherapeutic agents, such as cyclophosphamide, chlorambucil, adriamycin, cytosine arobinoside, vinblastine (Davies, 1983; Kinsella, 1989); other halogenated PAHs (e.g. polychlorinated and polybrominated biphenyls) and a number of other miscellaneous chemicals and biologically occurring toxins, for example, gossypol, are spermatotoxic (Davies, 1983).

A number of these agents have also been reported to affect semen quality in man, including the range of cancer chemotherapeutic drugs and the drugs prednisone, sulfasalazine (Wyrobek *et al*., 1983b); the recreational drugs alcohol, marijuana and, more equivocally, tobacco (Wyrobek *et al*., 1983a; Pasqualotto *et al*., 2004); the industrial chemicals carbon disulfide (Lancranjan, 1972), lead (Lancranjan *et al*., 1975; Assennato *et al*., 1986); dibromochloropropane (Whorton *et al*., 1977); ethylene dibromide (Ratcliffe *et al*., 1985; 1987); chlorodecone (kepone) (Cannon *et al*., 1978); chloroprene (Sanotskii, 1976) and the glycol ether 2-ethoxyethanol (marginally) (Ratcliffe *et al*., 1989). Physical agents shown to affect spermatogenesis in man include ionizing radiation (Kinsella, 1989). With radiation exposure, spermatogonial cells are the most sensitive, showing morphological and quantitative changes at doses as low as 10 cGy. Spermatocytes are functionally damaged at 200–3000 cGy, when they cannot complete maturation division, and spermatids at approximately 600 cGy; recovery of testicular function is directly related to the dose received, taking up to five years at doses of approximately 600 cGy, in some cases (Kinsella, 1989). Heating of the testis has also been shown to affect sperm count in man and other species reversibly; epididymal sperm seem to be most susceptible to short periods of an increase in temperature of even 2–3°C, such as may occur with repeated hot-tub use. Prolonged heating of the scrotum may affect earlier stages of spermatogenesis (Procope, 1965). Loss of sperm may be partly mediated by a decrease in androgen-binding protein synthesis (Rich and de Kretser, 1977). Environmental chemicals, including endocrine disruptors, have been suggested as factors in the decline in sperm count and the increasing incidences of hypospadias and testicular cancer observed in many countries (Ansell *et al*., 1992; Carlsen *et al*., 1992; Giwercman and Skakkebaek, 1992; Sharpe, 1993).

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3.1.3.2.3 Toxins Which Affect Sexual Behaviour

The assessment of libido and potency presents difficulties in humans, particularly when studied epidemiologically, since reliance must be placed largely on self-reported symptoms, and there is no good animal model (other than, e.g. mating frequency or ejaculatory capacity) for this complex area of sexual function. In humans, a number of drugs, which act on the CNS or the autonomic nervous system have been reported to affect libido, erectile potency or ejaculatory function (Woods, 1975). The two largest categories of drugs are antihypertensives and psychoactive drugs (Editorial, 1979). Antihypertensives may affect both erectile potency and ejaculation. Antidepressants may cause impotence via an anticholinergic action (tricyclics) or peripheral ganglionic blockage (monoamine oxidase inhibitors). Certain sedatives and tranquillizers, such as benzodiazepines and phenothiazines, notably thioridazine, may act at several sites, by both decreasing libido at a central level and inhibiting autonomic nerve transmission at a peripheral level to impair ejaculation. It is possible that hypothalamic and pituitary function is also disturbed by phenothiazines, according to Woods (1975). Centrally acting drugs of abuse, including heroin, marijuana, alcohol, lysergic acid diethylamide (LSD) and amphetamines, have all been reported to affect libido and potency in men, particularly after prolonged use. The use of anabolic steroids was reported to decrease libido and potency (together with other spermatotoxic effects in male athletes in one study) (Frasier, 1973). Some industrial chemicals have been reported to affect libido and/or potency, notably carbon disulfide, boric acid, lead and the pesticide kepone (chlordecone) (Barlow and Sullivan, 1982), but findings are not consistent.

3.1.3.2.4 Toxins Which Affect Endocrine Function

Disruption of endocrine secretions, binding, feedback control or target activity can be affected by action at several sites. Often it is not clear, when an alteration in hormone activity is observed, whether this is the result of a primary effect on hormone secretion or a response to testicular damage or some other mechanism. Cimetidine, used in the treatment of peptic ulcers, appears to act by competing with dihydrotestosterone for androgen receptors in the testis and accessory sex glands (Winters *et al*., 1979), resulting in decreases sperm count, gynaecomastia, increased prolactin secretion and decreased prostate and seminal vesicle weight. The persistent DDT metabolite *p*, *p*′-DDE (dichlorordiphenyldichloroethane) has been shown to be a potent androgen receptor antagonist (Kelce *et al*., 1995). LH receptors in Leydig cells have been shown to be depleted in rats exposed to ionizing radiation *in utero* (Rich and de Kretser, 1979). Men working on the production of oral contraceptives were found to have gynaecomastia (Harrington *et al*., 1978); these exogenous steroids are thought to act by suppressing gonadotrophic hormone secretion. Some of the chlorinated PAHs (e.g. polychlorinated biphenyls (PCBs), hexachlorophene) may act by either oestrogen agonism or by inducing mixed function oxidases which increase the clearance of endogenous steroids (Mattison, 1981); this may explain their observed effect on spermatogenesis.

There is one striking example of the effect of exogenous oestrogenic hormone exposure occurring *in utero* and affecting foetal gonadal development. Diethylstilbestrol (DES), a synthetic oestrogenic drug widely prescribed to pregnant women in the 1940s and 1950s to prevent miscarriage, premature delivery and toxaemia, exerted a dramatic effect on male foetal gonadal development by antagonizing the activity of foetal testosterone. The key aspect of the toxicity of DES is its lack of binding to maternal steroid-binding protein or conjugation in foetal liver, processes which protect the developing male genital tract from damage by maternal oestrogens from the placenta and ovaries (Gill, 1989). Numerous structural and functional abnormalities of the reproductive system have been observed in the male offspring, including epididymal (spermatocoele) cysts, testicular hypoplasia, cryptorchidism, hypospadias, abnormal semen parameters and primary infertility (Gill *et al*., 1979; Stenchever *et al*., 1981; Whitehead and Leiter, 1981; review by Gill, 1989). There are conflicting reports of testicular cancer in exposed males, perhaps due to methodological limitations (Gill, 1989).

3.1.3.2.5 Toxins Which Affect Accessory Sex Gland Function

There are relatively few examples of toxins which directly affect these tissues. α-Chlorohydrin and 6-chloro-6-deoxy sugars affect glucose metabolism of epididymal spermatozoa, and at high doses α-chlorohydrin damages the epithelium of the cauda epididymus, causing spermatoceles (Zanefeld and Waller, 1989). Sulfasalizine, probably acting via an active metabolite, affects the fertilizing capability of sperm in rodents and man, apparently by inhibiting the acrosome reaction, either by an effect on epididymal function, so that sperm maturation is impaired, or by a direct effect on the sperm (Zanefeld and Waller, 1989). Hexachlorophene has been found to impair ejaculation in rats by causing fibrosis of the prostate (Gellert *et al*., 1978). Toxins which can impair the motility of spermatozoa *in vitro* could, if found in the secretions of the seminal vesicles or prostate, affect the motility of the sperm after ejaculation. Imidazoles, such as the oral antifungal agent ketoconazole, have been shown to be transported into seminal fluid and to immobilize sperm (Zanefeld and Waller, 1989).

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3.2 Effects on the Female Reproductive System

3.2.1 Introduction

Reproduction in the female mammal involves a complex series of inter-related steps under hormonal control via the hypothalamo-pituitary-ovarian axis. These steps involve follicular development, ovulation, ova transfer, fertilization, transport of the conceptus, and its subsequent implantation, and development culminating in parturition. All of these processes are important and are the summation of many systemic, local, cellular and molecular interactions, which still remain poorly understood.

Because of the complexity of the reproductive system and the species differences involved, a description based on the human female will be used with additional information given on other species where appropriate. Basically, female reproduction will be described under the following headings: general physiology of the female reproductive tract, the hypothalamo-pituitary-ovarian axis, the ovary, including ovarian cycles, and the various ways in which these systems can be adversely affected.

3.2.2 General Physiology of the Female Reproductive System

The female reproductive tract consists basically of paired gonads, or ovaries, and a system of hollow ducts, the oviducts, for transport of eggs to the exterior. The oviduct is modified in several ways according to the species and its characteristic mode of reproductions. In humans, the oviducts consist of paired uterine tubes (Fallopian tubes) connected to a single mid-line uterus terminating in a vagina. All of these components are inter-related and are sensitive to hormonal changes, which are controlled by the hypothalamo-pituitary-ovarian axis. This hormonal controlling system is also responsible for the development of secondary sexual characteristics, for example, mammary gland development, fat disposition and, especially in humans, hair distribution and voice changes (Takizawa and Mattison, 1983; Johnson and Everitt, 1988; Knobil and Neill, 1994; Plowchalk *et al*., 1993; Osorio and Windham, 1997).

3.2.2.1 The Hypothalamo-Pituitary-Ovarian Axis

The hypothalamus is a complex structure involved in the control of body temperature, integration of autonomic activity and the release of GnRH. GnRH is carried in the blood via the hypophyseal portal system to the anterior pituitary gland. The anterior lobe of the pituitary contains a variety of cells which release gonadotrophic hormones, FSH, LH, prolactin and other hormones. Prolactin release is under the control of dopamine, released from neurons in the median eminence. Whereas GnRH stimulates the release of FSH and LH, dopamine inhibits the release of prolactin. The target organ for FSH and LH is the ovary. The role of prolactin is less clearly defined and its actions are more species specific. In some rodents, for instance, it has the ability to maintain the corpus luteum (CL) and there is some evidence in humans that it may be involved in luteolysis and in lactation (Lunenfeld and Insler, 1993; Ryan, 1993).

The release of GnRH from the hypothalamus is controlled by a number of feedback loops, principally from the hormones produced by the ovary (Yen, 1991). Although the exact mechanisms and site of action are not fully understood, it is generally thought that tonic release of FSH is involved in follicle growth. As the follicle grows it secretes increasing amounts of sex steroids, predominantly oestrogen, which has negative feedback effect on the hypothalamic pathways resulting in decreased release of FSH from the pituitary (Sadow *et al*., 1980; Feder, 1981a; McCann, 1981; Ben-Menaham and Naor, 1994; Insler and Lunenfeld, 1993).

In some mammals, other factors such as temperature and light intensity, also affect hormone secretion, presumably via other CNS mechanisms (Elliot and Goldman, 1981; Komisaruk *et al*., 1981).

3.2.2.2 The Ovary

The ovary plays a central role in that it controls the production of gametes and sex steroids, which are regulated via the hypothalamic-pituitary pathways. The principal site of action of these ovarian hormones is the uterus, which responds by undergoing cyclical changes which enable conception and foetal development to occur (Ryan, 1993; Paul, 1995).

3.2.2.2.1 Ovarian Development

The ovary develops from the germinal ridge and descends into the pelvis in the early part of foetal life. The germ cells or oocytes, are formed before birth and develop to the primary oocyte stage having undergone the first phase of meiosis (Ross and Schreiber, 1978; Feder, 1981c; Larsen, 1993). They remain in this resting oocyte–follicle complex until they are stimulated by gonadotrophin release at the onset of puberty (Moor and Warnes, 1979) to produce the characteristic mammalian Graafian follicle. Thus, any agents that damage the oocytes will accelerate the depletion of this resting pool and may lead to a reduction in fertility. At maturity, the ovary undergoes cyclical activity of a biphasic nature during which follicles undergo growth, maturation and regression.

In humans it is estimated that in excess of half a million follicles are present at birth; many of these die (atresia) and those that survive are continuously reduced in number. It is estimated that fewer that 500 will be ovulated and fewer still will be fertilized to produce offspring (Sadow *et al*., 1980; Page *et al*., 1981).

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These ovarian cycles are species specific; some of the similarities and differences are described in the next section (Johannisson, 1990; Adashi, 1991).

3.2.2.2.2 Ovarian Cycles

Ovarian cycles, that is, oestrous cycles, and menstrual cycles in the higher primates and humans, vary in their length and frequency of occurrence. Their duration may be a few days, that is, in the mouse, or several weeks, as humans and the horse. The term 'oestrus' refers to the period when the female is most receptive to the male, that is, 'in heat', which usually coincides with high levels of circulating oestrogen and subsequent stimulation of sexual excitement and behaviour patterns (Feder, 1981b). The human female does not exhibit oestrus behaviour as such, even though changes do occur in the reproductive organs, but there is no clear correlation between the secretion of oestrogens and sexual drive or libido.

Mammals having a succession of oestrous cycles, for example, rodents, are called polyoestrous. In others, such as the goat, the cycles are limited to a particular season of the year, that is, the breeding season. The interval between cycles is of relative quiescence and is termed 'anoestrus'. Other mammals, such as cats, dogs and ferrets, are monoestrous. They have one long sustained oestrus during the spring and occasionally another in late summer (Perry, 1971; Elliot and Goldman, 1981). In humans and the high primates such as Old World monkeys and apes, the cycles occur at approximately monthly intervals and are termed menstrual cycles (*mensis* = month). These cycles differ from those of most other mammals in that there is external loss of blood, that is, menstruation at the end of each cycle.

In most mammals, ovulation occurs spontaneously under hormonal control via the hypothalamo-pituitary-ovarian pathways, either during or just after oestrus. However, in some monoestrous animals such as the dog, the additional stimulus of mating is required to trigger ovulation, that is, induced ovulation.

This complex series of events involved in the hormonal control of follicle maturation, ovulation, luteinization and luteolysis will be illustrated by reference to the human menstrual cycle. Significant species differences will be discussed where appropriate (Sadow *et al*., 1980; Feder, 1981a; 1981b; Page *et al*., 1981; Taubert and Kuhl, 1993).

3.2.2.2.3 Menstrual Cycle

The complex process by which a single dominant follicle is selected for ovulation is not well understood (Bahr *et al*., 1977; Takizawa and Mattison, 1983; Greenwald and Terranova, 1988; Espey, 1989). However, it is generally accepted that tonic release of FSH from the pituitary stimulates follicular growth. As the follicle grows, it secretes sex steroids, predominantly oestrogen at this stage. As oestrogen levels increase, they eventually trigger off a reflex discharge of FSH and LH from the anterior pituitary, which results in the release of a preovulatory LH surge. Oestrogen also has a negative feedback on the hypothalamic-pituitary pathways to decrease the amount of FSH released. Following the LH surge, there is an increase in LH concentration in the follicular fluid, which is associated with oocyte maturation, preovulatory progesterone secretion by the granulose cells and the stimulation of prostaglandin (PG) synthesis. Ultimately this results in the rupture of the dominant follicle and the release of the oocyte, that is, ovulation occurs (Chaslow and Pharriss, 1972; Delforge *et al*., 1972; McNatty *et al*., 1975; Gore-Langton and Armstrong, 1988; Lunenfeld and Insler, 1993; Tsafriri and Braw-Tal, 1993). In some mammals there is a slight blood-stained discharge associated with ovulation, which is different from menstruation.

Following ovulation, the remaining follicles become atretic. The dominant follicle collapses and the granulose cells begin to proliferate to form the CL. In humans, the main hormone secreted by the CL for about 8–10 days is progesterone. If fertilization occurs, the conceptus, even prior to implantation, is thought to secrete substances which are recognized by the mother and prevent the breakdown (luteolysis) of the CL (Heap *et al*., 1979). In the cow, PG synthesis is suppressed by embryonically derived pregnancy-specific proteins (Goding, 1974). Similarly in the pig, the blastocyst produces oestrogens which lead to maternal recognition of the conceptus and also inhibit PG synthesis. However, in primates such as monkeys, apes and man, the conceptus secretes human chorionic gonadotrophin (hCG) which supports the CL and maintains a suitable endometrium.

If fertilization does not occur, the CL undergoes luteolysis and its endocrine function ceases. Luteolytic mechanisms tend to be species specific. In rodents, rabbits, cattle, sheep and pigs, PG via the utero-ovarian circulation plays a major role. PG-induced vasoconstriction may also be a contributory factor (Karim, 1972; Cole and Cupps, 1977). In contrast, the primates do not seem to control luteolysis in this way. Although the mechanisms are poorly understood, it seems that alterations in the concentrations and inter-relationships of various hormones, sex steroids, gonadotrophins, prolactin and PG initiate a feedback loop to the hypothalamus and trigger off the release of gonadotrophins, for example, FSH and LH, to initiate the next cycle (Feder, 1981b; Takizawa and Mattison, 1983; Richardson, 1986; Johnson and Everitt, 1988).

3.2.2.3 Oviducts/Fallopian Tubes

The anatomy of the tubes is species specific and related to the way in which ovulation and fertilization occur. The tubes are the site of fertilization and their rich autonomic nerve innervation assists egg retention. The lumen of each tube is bathed with fluid, whose composition and direction of flow are under the control of sex steroids and the autonomic nervous system and varies at different stages of the cycle. In general, egg transport from the

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ovary to the uterus seems to be a cumulative effect of coordinated cilial motility, muscle contractions and subsequent fluid flow. Tubular mobility is controlled by the synergistic effects of oestrogen and progesterone, and in humans during the luteal phase it is towards the uterus (Sadow *et al*., 1980; Page *et al*., 1981; Takizawa and Mattison, 1983; Villalon *et al*., 1991; Verdugo and Villalon, 1993).

3.2.2.4 The Uterus

The anatomy and physiology of the uterus are also species specific. In many species, the uterus acts simply for the transfer of sperm, whereas in others, such as rodents, cows and pigs, it acts as a sperm reservoir.

In humans, the uterus undergoes hormonally dependent alterations in the endometrium during the cycle to prepare the uterus for implantation and subsequent development of the conceptus (Johannisson, 1990). During the preovulatory phase, the follicular-derived oestrogen increases uterine blood flow and stimulates endometrial development. After ovulation, the synergistic actions of oestrogen and progesterone complete this process. The effects of progesterone on the oestrogen-primed endometrium are pleiotropic, causing an increase in protein synthesis and microvilli formation. There is increased vascularity, permeability and discharge of secretory products into the endometrial lumen of the glands which enables the conceptus to implant (Johnson and Everitt, 1988; Dockery *et al*., 1990; Kim-Bjöerklund *et al*., 1991).

The time taken for the conceptus to implant varies with the species and is generally not known. However, it is known that receptivity for implantation has a limited time span in rodents at five to six days and in humans at about seven days (Psychoyos, 1962; Edwards, 1982). The myometrium, on the other hand, remains quiescent for most of pregnancy. However, towards the end of pregnancy, it develops a coordinated pattern of contractions which assist in the expulsion of the foetus at parturition. There are many and varied theories as to the mechanisms controlling this, but on the whole it is accepted that PG synthesis and degradation is one of the key control factors (Porter and Finn, 1977; Yen, 1978; Challis and Lye, 1986; Johnson and Everitt, 1988).

3.2.3 Mechanisms of Action of Reproductive Toxins

As successful reproduction in the female relies on a complex system of hormonally controlled inter-related events, toxic insults at any one stage of this process can have profound effects on both the mother and the foetus. Agents may act directly on germ cells so that conception does not occur, or they may cause severe anomalities resulting in early abortions. Some of the abortuses have been shown to have chromosomal damage. Alterations in sex rates may occur, but interpretation of existing data is difficult. Outcomes involving the prenatal period may involve low birth weights, stillbirths, neonatal deaths, chromosomal damage or gross structural malformations.

Just as there are species-specific differences in the physiology and hormonal control of the reproductive processes, there are also differences in response to toxins. This may in part be explained by the interspecies differences in the pharmacokinetic responses, that is, absorption, distribution, metabolism and excretion of a particular toxin. Other factors, such as gender differences and duration of exposure to the toxin, are also involved.

The principal action of toxins is to alter, in some way, the normal functioning of cells, organs or organisms. Toxins may inhibit ovulation, ova transport, fertilization and implantation by altering the environment within the oviduct. In order for toxins to exert such effects they must have an adequate distribution to the target organ. Alternatively, they may have a much wider range of effects which may be nonspecific and may act at many sites within the organism (Dixon, 1980; Mattison, 1981; Mattison, 1983; Kamrin *et al*., 1994).

3.2.3.1 Mechanisms of Toxicity

Toxins may act directly or indirectly. Toxins may have a direct action because they bear structural similarities to an endogenous substance, for example, hormones, or because of their chemical reactivity, for example, alkylation agents. Others may act indirectly by requiring various metabolic processes prior to exerting their toxic effect or by metabolism to an active metabolite, which may then exert its effect by direct mechanisms. In addition, some indirectly acting toxins exert their effect by altering the controlling mechanisms, for example, inhibition of gonadotrophin release or enzyme-induction inhibition (Forman *et al*., 1996; Daston *et al*., 1997; Gray *et al*., 1997).

Toxins may exert their effect by more than one mechanism of action. The halogenated PAHs, for example, PCBs or PBB (polybrominated biphenyls), behave in such a way, in that they may act indirectly by induction of microsomal mono-oxygenases or transferases or directly because of their steroid hormone agonist properties (Doull, 1980; Neal, 1980; Moses, 1993; Kippen, 1996).

3.2.3.2 Directly Acting Toxins

Toxins which have structural similarities to biologically active molecules are generally agonists or antagonists of endogenous hormones. Oral contraceptives belong to this group in that they act predominantly by suppression of gonadotrophin release (Chasan-Taber *et al*., 1997). Workers who manufacture oral contraceptives are at risk from this type of reproductive-endocrine toxicity (Harrington *et al*., 1978; McDiarmid, 1993). There is also evidence that occupational exposure to antineoplastic drugs is associated with an increased incidence of

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infertility (McDiarmid, 1993; Valanis *et al*., 1997). Exposure to other occupational and environmental substances which have oestrogenic or progestogenic activity may inhibit gonadotrophin release or ovarian function via the hypothalamus and pituitary pathways. Other chemically reactive compounds may be nonspecific in their site of action. This category includes the alkylating agents, the toxic effects of which have been discussed above.

3.2.3.3 Indirectly Acting Toxins

Indirectly acting toxins are substances that may require metabolic activation and the metabolite thus formed may itself be chemically reactive or may mimic an endogenous protein. Oxidation by microsomal mono-oxygenases is one of the mechanisms involved in removing hydrophobic xenobiotics from the body. It has been shown that the ovary has microsomal mono-oxygenases, epoxide hydrases and transferases capable of metabolizing many xenobiotics (Heinrichs and Juchau, 1980). The polar metabolites thus formed may undergo conjugation or be excreted directly. However, some of these metabolites are chemically reactive and are capable of interacting with cellular macromolecules in the same way as exogenously administered substances. Cyclophosphomide (Koyama *et al*., 1977; Lentz *et al*., 1977), DES (Metzler and McLachlan, 1978), ethanol (Ouellette *et al*., 1977; Sullivan and McElhatton, 1986) and the PAHs (Felton *et al*., 1978; Nerbert, 1981) are examples.

Other indirectly acting toxins may affect enzymes, that is, induce or inhibit enzyme systems, which in turn may stimulate or inhibit steroid production or excretion. As the reproductive system is controlled by hormonal feedback loops, substances which alter the steroid balance in any way may have a profound effect on the entire reproductive process. Rodent studies have shown that some of the polyhalogenated hydrocarbons, for example, DDT, PCB, PBB, behave in this way. Some toxins may actually enhance rather than impair fertility. Oral contraceptives, for instance, exert fertility regulation via feedback inhibition of gonadotrophin secretion, and xenobiotics that stimulate clearance of the oestrogenic and/or progestogenic component in the oral contraceptive will cause a decrease in circulating level and consequently may increase the probability of ovulation (reviewed by Mattison, 1983).

3.2.3.4 Interference with Detoxification

Detoxification mechanisms are the methods by which biological organisms respond to toxic insults. Some of these mechanisms have shown to be present in the ovary. They tend to have a relatively broad-based specificity, and may involve conjugation hydrolysis by epoxide hydratase in order to decrease the concentration of the toxin, or metabolism to a less toxic or more easily excreted metabolite and thus limit the activity of the toxin in the body. Impaired detoxification due to the nature of the substrate or enzyme deficiencies within the organism will enhance the toxic effects.

3.2.3.5 Repair

Despite the presence of detoxification pathways within the cell, cellular or organ damage can still occur. In such a situation, attempts are made to repair the damage by various mechanisms. The repair may simply involve alteration in protein synthesis to replace nonfunctioning proteins destroyed by the toxic insult, or more sophisticated repairs may be required if the DNA itself has been damaged, involving excision and subsequent replacement of the damaged DNA region. There is some evidence to suggest that mature oocytes are able to repair damage within the oocyte DNA prior to and after fertilization. However, it is not known whether the oocyte has other types of repair mechanism (Lehmann and Bridges, 1977; Dixon, 1980; Mattison, 1983).

A more detailed account of some of the reproductive toxins and their proposed modes of action is described below.

3.2.4 Sites and Mechanisms of Action of Some Reproductive Toxins

3.2.4.1 The Hypothalamo-Pituitary-Ovarian Axis

Chemicals can act at several levels, on the hypothalamus, pituitary, the endocrine and ovulatory function of the ovary or on the tissues in the reproductive tract, and frequently may act at several sites (Miller and Bellinger, 1993; Paul, 1993; Welch, 1993).

The mode of action of the chemical may give some indication as to whether the toxic effects it produces are reversible or not. Drugs and other chemicals that alter gonadal function directly may well produce infertility via genetic effects, which are irreversible, whereas those producing alterations in the nerve pathways in the hypothalamus, for instance, may cause disruption of fertility, which is reversible.

Ketoconazole, a broad-spectrum antifungal drug, inhibits steroid synthesis by inhibition of cytochrome-P450-dependent enzyme systems, giving rise to a number of endocrine abnormalities (Loose *et al*., 1983). In women, spironolactone acts as an oestrogen antagonist, which may explain the reported menstrual disturbances associated with its use (Levy *et al*., 1980).

Certain CNS drugs such as tranquillizers, barbiturates, narcotics and ‘social’ drugs, such as marijuana, have an inhibitory effect on hypothalamic pituitary function by inhibiting gonadotrophin secretion (McDiarmid, 1993). In animal studies, effects, such as suppression of ovulation, oestrus and fertility, have been reported. Thus, the primary endocrine effect of inhibition of gonadotrophins induces a secondary effect on ovarian steroid synthesis,

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resulting in adverse effects on growth, development and function of the accessory organs.

As the nerve pathways to the hypothalamus are adrenergic and dopaminergic, drugs which alter catecholamine levels, either by inhibiting CNS activity, for example anaesthetics, sedatives, tranquillizers and analgesics, or by stimulating the CNS, for example psychotropic drugs, antidepressants and hallucinogens, can also affect the hypothalamic-pituitary control of gonadotrophin release. Such actions may result in alterations in the secretions of FSH, LH and prolactin, which are important for follicle growth, oocyte maturation, ovulation and maintenance of the CL. However, the CNS and autonomic effects of such drugs are often difficult to distinguish. Similarly, nitrous oxide in high concentrations has been reported to reduce fertility among women employed in dentistry, but the mechanism of action is undetermined (Rowland *et al*., 1992). Drugs of abuse, such as cocaine (crack), ecstasy, cannabis, mescaline and alcohol may also have adverse effects on reproduction, such as decreased fertility, an increase in spontaneous abortions, intrauterine growth retardation (IUGR) and postnatal behavioural problems in the offspring, but these effects are less well documented and their exact mechanisms of action are not known. The study of social drug use and of drugs of abuse in pregnancy is complex because it has been shown, for example, that heavy smokers are often also heavy drinkers and caffeine consumers. Similarly, smoking and alcohol consumption are common in addicts, which, combined with their general lifestyle and poor nutrition, makes identification of the individual contributions of each substance to any one adverse effect difficult.

Some of the drugs which have been studies more extensively are discussed below and have been reviewed by Rosen and Johnson (1982), Abel (1983), Gibson *et al*. (1983), Smith (1983), Armstrong (1986), Boobis and Sullivan (1986), Johnson and Everitt (1988), Clark and Weinstein (1993), Schardein (1993), Ellenhorn *et al*. (1997) and Gilstrap and Little (1998).

3.2.4.2 Major Drugs of Addiction

Adverse reproductive effects, such as a decreased sexual desire, menstrual irregularities, infertility and increased foetal loss have been reported (Wallach *et al*., 1969; Wieland and Yunger, 1970; Finnegan, 1981; Liel *et al*., 1993). The adverse effects on early foetal and postnatal development following intrauterine exposure have been well documented (Ostrea and Chavez, 1979; Sullivan and McElhatton, 1986; Fulroth *et al*., 1989). This has been partly attributed to altered function of the hypothalamo-pituitary axis and partly to altered ovarian functions. Some of the narcotics are reported to cause a significant decrease in LH levels, which may disrupt oocyte maturation and ovulation and interfere with the FSH-LH feedback loops to the hypothalamus.

3.2.4.3 Marijuana

Marijuana is a complex group of drugs derived from the plant *Cannabis sativa*. Recent animal tests and human studies have indicated that chronic, sustained exposure is associated with reproductive dysfunction (Peterson, 1980; Abel, 1983; Hollister, 1986; Ellenhorn *et al*., 1997). A study of marijuana users has reported that these women had shortened menstrual cycles and a higher proportion of these cycles was anovulatory or had shorter luteal phases (Kolodny *et al*., 1979). The psychoactive component is Δ^a-tetrahydrocannabinol (THC) and it is thought that it inhibits the secretion of FSH and LH via a hypothalamic route with secondary effects on the ovary. However, the Kolodny *et al*. study did not confirm these effects as regards gonadotrophins or progesterone, but found a significant decrease in serum prolactin. Experimental studies have shown that the duration of gonadotrophin depression is dose related and persists for up to 24 hours after peak levels of the drug have occurred in the circulation. Furthermore, these effects can be reversed by giving leutinizing hormone releasing hormone (LHRH). THC has also been shown to depress oestrogen production and the LH surge and thus inhibit ovulation in rhesus monkeys. One small double-blind study in 16 women has shown that marijuana smoking significantly depressed LH levels for up to 120 minutes when compared with a placebo (Mendelson *et al*., 1986). Rodent studies have also indicated that there is a decrease in serum prolactin, possibly by THC inhibiting its release from the pituitary. Other studies indicate a decrease or absence of oestrogen and gonadotrophins, but a marked rise in progesterone (Smith *et al*., 1980). It would seem that the data within a species are variable and that there are species-specific differences.

3.2.4.4 Alcohol

The effects of alcohol on female reproduction have been studied in terms of its possible teratogenic effects on the offspring, producing the foetal alcohol syndrome (Jones and Smith, 1975), and also a wide range of other adverse effects, such as increased spontaneous abortions (Harlap and Shiono, 1980), IUGR (Little, 1977), overall failure to thrive postnatally and some mental retardation (Streissguth *et al*., 1978; Mello *et al*., 1989). As alcohol has significant pharmacological effects via the central nervous system in adults, it is probable that the hypothalamic-pituitary pathways and thus reproductive hormone control may also be adversely affected. Whether adverse effects on hormones occur in the developing foetal brain is not known (Root *et al*., 1975). Chronic use of alcohol has been associated with various obstetric and gynaecological problems, such as menstrual disorders, recurrent miscarriages and infertility. However, there is no clearly defined evidence to assess whether alcohol consumption is the cause of such problems (Abel, 1983; Boobis and Sullivan, 1986; Liel *et al*., 1993; Olsen *et al*., 1997).

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Studies in which female rats consumed alcohol showed atrophy of the ovaries, Fallopian tubes and uteri, and the ovaries from these rats had fewer well-developed follicles, corpora lutea and secretory granules (Van Thiel *et al*., 1978). It has been shown that infusions of alcohol in female rats cause inhibition of spontaneous LH release, but no inhibition of LH release by LHRH, so that ovulation may be affected by inhibition of LH release (Kieffer and Ketchel, 1970; Blake, 1974).

3.2.4.5 Gonadal Dysfunction Resulting from Cancer Chemotherapy

Alkylating agents (e.g. cyclophosphamide, chlorambucil, busulfan, nitrogen mustard) the vinca alkaloids (e.g. vincristine, vinblastine, vindesine) and radiation produce varying degrees of gonadal dysfunction, which are not only dose related, but also associated with the age at which the exposure occurs. In women, irreversible sterility resulting in premature menopause and sexual dysfunction occurs at higher doses than in males. Lesser degrees of gonadal injury are associated with subfertility, but normal sexual function. Age at the time of exposure is an important factor in that children apparently sustain less gonadal damage for a given dose than do adults (Liel *et al*., 1993; Schardein, 1993).

The ovaries of prepubertal girls are more resistant than those of adults to toxicity induced by cytotoxic chemotherapy, in that the majority who survive the illness go on to have normal menstrual cycles and serum gonadotrophin levels. Furthermore, post-mortem examination of some of the girls whose illness proved fatal showed that their ovaries were normal (Arniel, 1972; Pennisi *et al*., 1975; Lentz *et al*., 1977). In a study of prepubertal, leukaemic children who died, cytotoxic therapy (usually prednisone, vincristine and occasionally methotrexate, 6-mercaptopurine, cytosine arabinoside and l-asparaginase) was shown to inhibit follicle development in the ovaries, as opposed to ova destruction, which is sometimes seen in adults receiving similar therapy (Himelstein-Braw *et al*., 1978).

In mature women, treatment with cytotoxic drugs has variable effects on gonadal function and fertility. Age-dependent factors and also dose and duration of treatment often determine the degree of toxicity observed. However, insufficient data are available to determine critical dose levels for a given age group (Warne *et al*., 1973). Teenagers, for instance, may sustain impaired fertility, whilst serum hormone levels and menstrual cycles remain normal, whereas women in their 20s may develop clinical ovarian dysfunction, the reversibility of which is often dependent on the particular drug regime. Abnormal ovarian function may manifest itself in terms of irregular or anovulatory cycles, alteration in serum gonadotrophins and oestradiol levels, sometimes resulting in premature menopause (hot flushes, insomnia, irritability, depressed libido) and menopausal levels of oestradiol and gonadotrophins. The intensity of such symptoms is often age related in that they tend to be worse in younger women (Chapman, 1983). Although the critical age at which most women undergoing such treatment are likely to develop ovarian failure is not known, multiple drug exposure does seem to increase the risk. For instance, more than 80% of women over the age of 25 years developed complete and irreversible ovarian failure after six cycles with MOPP (mechlorethamine, oncovine (vincristine), procarbazine and prednisone) or MVPP (mustine, vinblastine, procarbazine and prednisolone) (Chapman *et al*., 1979b; Schilsky *et al*., 1981).

Cyclophosphamide alone may produce a reversible form of ovarian dysfunction or complete ovarian failure, in which amenorrhoea, abnormal hormone levels or ova destruction may occur (Kumar *et al*., 1972; Uldall *et al*., 1972; Rose and Davis, 1977). Cyclophosphamide seems to have an affinity for attacking the resting or small oocytes. As these are present throughout the life of the female in the same metabolic state, it cannot account for the age-dependent differences in sensitivity. However, there is evidence that the pathways for detoxification of the reactive metabolites do change with age and this may be a key factor (reviewed by Mattison, 1981).

Busulfan use has also been associated with permanent amenorrhoea, a side effect which also seems to be age related (Belohorsky *et al*., 1960; Schulz *et al*., 1979). Autopsy data from a small number of women treated with busulfan have shown signs of ovarian atrophy (Belohorsky *et al*., 1960; Heller and Jones, 1964).

Neither the vinca alkaloids nor nitrogen mustard alone seem to be associated with ovarian dysfunction. However, there have been occasional case reports of women who developed either reversible or permanent amenorrhoea after vinblastine therapy (Sobrinho *et al*., 1971).

There are many secondary effects, which follow on from ovarian toxicity, leading to early menopause, such as a greater risk of developing osteoporosis, cardiovascular disease and uterine cancer. To some extent these effects can be ameliorated by giving sequential hormone therapy or replacement therapy with oestrogen and progesterone (Gambrell, 1978; Chapman *et al*., 1979a; 1979b; Nordin *et al*., 1980; Paganini-Hill *et al*., 1981; Whitehead *et al*., 1981).

6-Mercatopurine, the active metabolite of azathioprine, has been shown in experimental rodent studies to reduce the fertility of female offspring exposed *in utero* (Gross *et al*., 1977; Reimers *et al*., 1980). It seems to act by causing premature ovarian failure, but whether the mechanism is oocyte destruction *per se* or inhibition of oogenesis is not known. The reproductive toxicity of this group of drugs was reviewed by Schardein (1993).

3.2.4.6 Ovotoxicity

PAHs are ubiquitous environmental pollutants. This group includes substances produced by the combustion of

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fossil fuels contained in car exhaust fumes, smoke stack emissions and cigarette smoke. The PAH group have been implicated as carcinogens, neurotoxins, hepatotoxins and reproductive toxins. They produce their reproductive toxicity by a variety of mechanisms including induction of microsomal monooxygenases, which alter hormone production and clearance, and also metabolism to active intermediates and hormone agonist activity (Mattison *et al*., 1983; Paul, 1993).

PAHs require conversion to reactive intermediates via microsomal cytochrome-P450-dependent mono-oxygenase and epoxide dyrase before they can exert their toxic effects within any biological system. Such converting enzymes are widely distributed throughout the body and are found in high levels in the liver and gonads. It has been demonstrated that in order to cause oocyte destruction, PAHs need to be distributed to the ovary and converted into one or more active intermediate(s) that cause oocyte destruction by either a direct action on the oocyte or by an indirect route via toxicity to the granulose cells that support the oocyte within the follicle (Mattison and Ross, 1983). It is thought that differences in ovarian metabolism may account for strain and species differences in sensitivity to PAH-induced oocyte destruction. Treatment during pregnancy is capable of destroying the oocytes of a female foetus whilst *in utero* (Felton *et al*., 1978).

The susceptibility of NHP and humans to oocyte destruction by PAHs is not known. It has been shown, however, that women who smoke one or more packs of cigarettes per day have a menopause about two years earlier than nonsmokers (Jick *et al*., 1977). The mechanism suggested is that the PAHs in cigarette smoke destroy the oocytes at an earlier age.

3.2.4.7 Oestrogenic Chemicals

Many other halogenated hydrocarbons, including pesticides and fungicides such as DDT, aldrin, 2,4,5-trichlorophenoxyecetic acid (2,4,5-T), PCB, PBB, 2,4-dichlorophenoxyacetic acid (2,4-D), heptachlor, chlordane and hexachlorophene, can interfere with mammalian reproduction in two main ways, namely oestrogen agonist activity and induction of microsomal mono-oxygenase activity responsible for production and clearance of steroid hormones (Mattison, 1981; Barlow and Sullivan, 1982; Bulger and Kupfer, 1983; Safe, 1984; Moses, 1993; Schardein, 1993; Osorio and Windham, 1997).

Treatment of neonatal rats with DDT has led to a dose-dependent premature vaginal opening culminating in an anovulatory syndrome with similarities to the human polycystic ovary disease. These effects, including the inhibition in the rise of gonadotrophins following ovariectomy, are attributed to the oestrogen agonist activity of DDT. The sites of action are thought to be the vagina and the hypothalamus; the neonatal treatment is believed to alter the patterning of the hypothalamus (Welch *et al*., 1969; Bitman and Cecil, 1970; Gorski, 1971). Similar results have been reported for PCB (Gellert *et al*., 1978).

By induction of the mono-oxygenase system, both DDT and PCB can increase the length of the oestrous cycles and decrease the frequency of implantation in the sexually mature mouse. Similar reproductive toxicity has been observed in other species (Ringer *et al*., 1972; Linder *et al*., 1974; Hansen *et al*., 1975; Kihlstrom *et al*., 1975; Spencer, 1982). A direct relationship between enzyme activity and inhibition of uterine weight response to oestrone has also been shown (Welch *et al*., 1971). There is also indirect evidence in primates that the halogenated polycyclics can induce mono-oxygenase activity and thus impair fertility by similar mechanisms (Kolmodin *et al*., 1969; Allen and Lambrecht, 1978).

Methoxychlor (bis-*p*-methoxy-DDT) is much less oestrogenic, less toxic, less persistent and has a shorter half-life than DDT in mammals. The reduced toxicity seems to be related to its rapid metabolism to more polar derivatives (Bulger and Kupfer, 1983; Schardein, 1993).

Kepone (chlordecone), a polychlorinated polycyclic aromatic compound, also has oestrogenic action on the murine uterus (Huber, 1965) and quail oviduct (Eroschenko and Wilson, 1975). There are conflicting data concerning its potential for reproductive toxicity in mice and rats in terms of teratogenicity (Ware and Good, 1967; Chernoff and Rogers, 1976; Khera *et al*., 1976) and postnatal survival and development (Gaines and Kimbrough, 1970; Chu *et al*., 1981). There have been no reports of effects in human pregnancy. However, it has been reported to cause loss of libido and sperm damage amongst manufacturing workers.

3.2.4.8 Genetic Abnormalities and the Effects of Irradiation

During the preimplantation stage, the embryo is susceptible to toxic insults which may cause mutations. If the damage is not repaired or compensated for, it may result in early embryonic death. Radiation and chemical toxins have a wide range of effects on the preimplantation embryo, for example, alterations in membrane permeability, disruption of the mitotic spindle and enzyme activity leading to cell disruption and possibly death. These mutations can be inherited via the germ cells or they can occur in somatic cells during embryogenesis. The nature of these abnormalities varies from specific point mutations in the genome to chromosome derangement, altering both numbers and structural arrangements. The majority of chromosome abnormalities occur during meiosis and gametogenesis, although some do occur during preimplantation development (Dean, 1983; Brent *et al*., 1993; Liel *et al*., 1993).

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It has been known for many years that germ cell loss may be due to exposure to either radiation or chemicals. Sensitivity is often species specific and may be dependent on the stage of germ-cell maturation (Oakberg and Clark, 1964) in addition to the age of the animal. There seems to be no general rule to determine radiosensitivity across the species. Oocytes from juvenile mice have the highest sensitivity known for any mammalian cell. There is less information available on the effects of chemical exposure when compared with radiation, but the increasing use of chemotherapy in humans is adding to our knowledge of such effects, as discussed above.

3.2.4.9 *Ionizing Radiation*

High radiosensitivity in the ovarian germ cells has been demonstrated in the mouse, prenatal squirrel monkey and prenatal pig (Dobson *et al*., 1978; Erickson, 1978). Although in the adult pig, germ cells are not particularly sensitive, exposure to continuous gamma radiation at 1 rad day^{-1} throughout gestation results in severe oocyte deficiency in the newborn (Erickson, 1978). Similar studies in dogs (Andersen *et al*., 1961) and cows (Erickson and Reynolds, 1978) have shown that in these species also there are no serious effects on the germ cells of adults. The effects in squirrel monkeys have been reviewed by Dobson and Felton (1983). Prenatal exposures of 0.7 rad day^{-1} can completely kill off the germ cells, but in the adult the same exposure causes a less dramatic germ-cell loss. In other primates, such as the rhesus monkey and Bonnet monkey, the results of experimental studies have been variable, but on the whole, high sensitivity seems lacking (Baker and Beaumont, 1967; Andersen *et al*., 1977). Very little data are available in the human female. However, it would seem that exposures of approximately 400 rad are associated with induction of sterility, with larger doses being required in younger women (Baker, 1971; Baker and Neal, 1977). There have been reports of ovarian failure following abdominal radiotherapy, including treatment for childhood cancer (Shalet *et al*., 1976; Stillman *et al*., 1981), but this has not precluded the occurrence of pregnancies (Gans *et al*., 1963; Vuksanovic, 1966).

There is significant concern about the extrapolation of the animal data, which are species- and strain-specific, to humans. Some studies have shown that the mechanism of action may be different (e.g. the extreme sensitivity of murine oocytes may be due to the vulnerability of the plasma membrane, not adverse effects on DNA)—that is, it is a nongenetic type of effect. Thus, the genetic risks or radiation in humans, especially when it involved exposure of the primordial oocytes, may be overestimated by referring to mouse data unless this is taken into account (reviewed by Dean, 1983; Dobson and Felton, 1983).

3.2.4.10 *Psychosexual Function*

While it is widely accepted that drugs and other chemicals can affect male libido or cause impotence, the possibility that such effects can occur in women has been largely overlooked. In most mammals and subhuman primates, sexual activity is closely linked to ovulation, and thus it is difficult to find an animal model for sexual dysfunction occurring in women. Drug-induced changes in female libido have been associated with the use of oral contraceptives, tranquillizers, monoamine oxidase inhibitors, heroin, methodone, cannabis, cytotoxic drugs and alcohol (Heinrichs and Gadallah, 1983; Griffin, 1986).

Studies by Chapman and her colleagues (Chapman *et al*., 1979a; 1979b) have reported the occurrence of ovarian failure in women treated with cytotoxic drugs, including MVPP for Hodgkin's disease. About 92% (34) of these women reported a decrease in libido; 82% had menstrual irregularities or amenorrhoea and a constellation of other menopausal-type symptoms.

Oral contraceptives have also been associated with decreased libido; however, the mechanism is not established, although some studies associate it with pill-induced depression. It has been shown that oral contraceptives abolish the hormone-controlled mid-cycle peak in female-initiated sexual activity (Adams *et al*., 1978). Cyproterone acetate, which is an antiandrogen used to treat severe acne and hirsutism in women, is also used in some cases as a contraceptive, and this too is associated with loss of libido in women.

Danazol, a drug which is used in the treatment of endometriosis and other menstrual disorders, inhibits pituitary gonadotrophin secretion and is frequently reported to cause loss of libido in women (Westerholm, 1977). The antiobesity drug, fenfluramine, when used in high doses of around 240 mg day^{-1}, produces loss of libido as its main side effect. It is reported that at this dosage 85% of women treated may suffer loss of libido (Connell, 1977).

4 MISCELLANEOUS DRUG AND CHEMICAL EFFECTS

4.1 Oral Contraceptives

The use of oral contraceptives has proved to be one of the most effective forms of reversible fertility control in use today. The composition and actions of these 'pills' have been reviewed recently (Belfield, 1997; Guillebaud, 1997). Currently there are basically two main types: the combined type, containing an oestrogen and a progestogen, with efficacy in the range of 97–99%, and a progestogen-only type, with lower efficacy. In order to eliminate some of the side effects associated with the high oestrogen content of the combined preparations, phased formulations containing a progestogen and variable amounts of oestrogen have been introduced.

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There are several types of combined pills available, for example, monophasic, biphasic and triphasic. Monophasic pills are the most widely used. They come in packets of 21 identical tablets, which are taken for 21 consecutive days. The pills are then stopped for seven days, during which a withdrawal bleed usually occurs.

The hormone content of the phasic pills varies throughout the cycle. They aim to provide a lower total dose of hormones over each cycle. The different strengths of pill must be taken in the right order for 21 consecutive days as marked on the packet, followed by seven pill-free days.

There are also 'every day' (ED) pills for those who find it easier to take pills every day without a break. Of the 28 pills, seven are inactive. There are also monophasic, biphasic and triphasic varieties of ED pills.

In addition, there is a postcoital pill, containing levonorgestrel 500 µg (UK formulation), which is used in such a way that high dosages of each of the hormones are attained within 12 hours.

4.1.1 Combined Oral Contraceptives

The combined preparation is considered to be the most effective for inhibiting ovulation. The effects of ovarian hormones on gonadotrophin release via the hypothalamic-pituitary axis have already been discussed. The predominant effect of oestrogen is to inhibit the release of FSH, whilst the continued action of progesterone inhibits the release of LH. Thus, ovulation may be inhibited by either inhibiting the ovulatory stimulus or by preventing follicle growth; both of these actions have been observed in experimental studies. However, the orally active progestogens used in the pill cannot be directly equated with progesterone *per se*, because some are inherently oestrogenic or androgenic and some purely progestational, and consequently they may inhibit ovulation in different ways. Measurements of circulating FSH and LH have shown that the oestrogen and progestogen combination inhibits both hormones, resulting in stable plasma FSH and LH and the absence of early follicular FSH and mid-cycle FSH-LH peaks. The combined preparation may also have a direct effect on the genital tract in that they alter the stage of development of the endometrium, thus making it unsuitable for implantation (Briggs, 1976). There are also changes in the viscosity of cervical secretions. At the time of ovulation these secretions are usually copious and watery and provide a good environment for sperm. Under the influence of progesterone, these secretions become thick and mucoid in nature and provide a hostile environment. Although little is known about the coordinated movement of the Fallopian tubes, uterus and cervix in the transport of eggs and sperm, it is generally accepted that the correct hormonal environment is essential for fertilization and implantation to occur.

4.1.2 Progestogen-Only Pills (POPs)

These preparations have lower efficacy than the combined type (pregnancy rates range from 1 to 10%) and have been associated with a higher incidence of menstrual irregularities. Progestogen-only pills (POPs) do not always prevent ovulation and are thought to be effective in only 15–40% of cycles. However, they do provide a suitable alternative for those women in whom high oestrogen levels are to be avoided, and it is a very effective method when taken correctly and consistently.

Low doses of progestogens may cause structural changes in the endometrium and alter the consistency of the cervical mucus without causing menstrual disruption. There are varying degrees of inhibition of FSH and LH and ovulation and this may be associated with the reduced efficacy seen with this type of pill. When a daily administration regime is used, menstruation does occur, but the length of the cycle and the duration of the bleeding period vary considerably. Long-acting progestogens, for example, norethisterone oenanthate and medroxyprogesterone acetate, are often given at two- and three-monthly intervals, respectively, and after an initial period of irregular bleeding give way to amenorrhoea and an atrophic endometrium. The main advantage is that there are no 'user failures' and the efficacy rate is 99–100%.

Although there may be some initial impairment of fertility for some months after oral contraception has stopped, this is of a transitory nature in the majority of women (Vessey *et al*., 1978). However, in a small number of women there is more permanent infertility (Fletcher, 1986).

4.2 Diethylstilbestrol (DES)

DES is of interest in that it is a reproductive toxin with two possible mechanisms of action. It is an oestrogen agonist, which causes changes in both the male and female reproductive tract in rodents (McLachlan and Dixon, 1977; Boylan, 1978; Vorherr *et al*., 1979) and in humans has been reported to cause an increased risk of adenocarcinoma of the vagina in females exposed prenatally (Herbst *et al*., 1971; 1975; O'Brien *et al*., 1979). A few cases have been reported of breast cancer in men treated with DES for metastatic prostatic carcinoma (International Agency for Research on Cancer (IARC), 1979). Some of the women exposed to DES *in utero* have vaginal abnormalities and there is evidence that they have irregular menstrual cycles and dysmenorrhoea and may be less fertile (Barnes *et al*., 1980; Herbst *et al*., 1980). Abnormal development of the uterus with a small hypoplastic T-shaped endometrial cavity has been seen in some of these women (Kaufman *et al*., 1977; Haney, 1987).

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4.3 Industrial Chemicals

The potential of industrial chemicals to cause reproductive toxicity has been reviewed by Barlow and Sullivan (1982), John *et al*. (1984), Schardein (1993), Paul (1993), Kippen (1996) and Solomon (1997). There is also growing evidence of reproductive effects associated with occupational and environmental agents, for example, germ-cell damage, embryotoxicity and infertility. The complex relationship between the underlying pathogenic processes and the difficulties of assessing prospective and retrospective data have been carefully reviewed by Joffe (1997).

The major effects reported in female workers are as follows. Menstrual irregularities and other gynaecological disorders have been associated with exposure to aniline, benzene, chloroprene, formaldehyde, glycol ethers, inorganic mercury, PCBs and toluene, but in many instances the lack of data precludes a critical risk evaluation being made (Zielhuis *et al*., 1984; Commission of the European Communities, 1986; Rosenberg *et al*., 1987; Snow, 1994; Osorio and Windham, 1997; Paul, 1997).

Recently, issues have been raised concerning possible reproductive disorders in communities living near hazardous chemical sites (Savitz *et al*., 1997; Scialli *et al*., 1997). Various parameters were measured in women, namely menstrual dysfunction, infertility, pregnancy loss, lactation disorders and pregnancy outcome.

Occupational exposure limits have been set for some dangerous chemicals in the working environment (Hunter *et al*., 1997). However, these are only a tiny fraction of the chemicals to which workers are exposed, but it is encouraging that an ongoing priority work programme is in place to assess other chemicals.

Other substances have been associated with infertility or spontaneous abortions (Paul, 1997). These include anaesthetic gases in both males and females, ethylene oxide, arsenic, lead and some of the substances mentioned above, that is, aniline, formaldehyde and benzene (Figá-Talamanca, 1984; Lemasters *et al*., 1985; Sullivan and Barlow, 1985; Kline, 1986; Winder, 1987; Friedman, 1988; van der Gulden and Zielhuis, 1989; Paul, 1995; Rosenberg *et al*., 1997).

Factors such as decreased foetal growth, low birth weights and poor postnatal survival have been associated with exposures to carbon monoxide, PCBs, vinyl chloride, toluene and formaldehyde (Fletcher, 1986; Tabacova, 1986; Lemasters *et al*., 1989).

Teratogenic effects and transplacental carcinogenesis have been omitted from this section as they are described elsewhere.

4.4 Effects of Exercise

Although there have been reports associating strenuous exercise with adverse effects on the reproductive system, such as delayed menarche, menstrual irregularities or amenorrhoea, a cause–effect relationship is difficult to establish (Terjung, 1979; Cumming and Rebar, 1983). There seems to be a complex interplay of physical, hormonal, nutritional, environmental and psychological factors. One of the underlying concerns is whether or not so-called exercise-induced reproductive dysfunction is truly reversible once vigorous exercise has stopped.

The amenorrhoea associated with exercise is generally thought to be hypothalamic in origin (Speroff, 1981), but data are accumulating to suggest that other circulating hormones may also be involved (Shangold *et al*., 1981). In a review by Cumming and Rebar (1983), it is postulated that exercise-induced amenorrhoea is caused by alterations originating in the periphery as well as from CNS abnormalities. Furthermore, they suggest that the amenorrhoea produced is reversible.

Adverse effects of strenuous exercise once pregnancy has been established have also been reported. These seem to be related to the complex physiological adjustments required in terms of metabolic, endocrine, respiratory and circulatory responses in both the mother and the foetus (Lotgering *et al*., 1985).

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Developmental Toxicology

John M. DeSesso and Calvin C. Willhite

CONTENTS

1 BACKGROUND AND MORPHOLOGY

Human embryonic development is a critical period of life and one during which insults can have severe impacts on the quality and length of life. Surprising to many, an embryo's wellbeing may actually be compromised decades *before* conception by insults that occurred during the fourth to sixth weeks of gestation of its parents. At this time in the parents' development, spherical primordial germ cells appear in the yolk sac and migrate to the presumptive gonad region during embryonic folding. During the folding process, the embryo establishes orientation, becomes segmented and develops three-dimensional architecture, as part of the yolk sac is incorporated into the embryo proper. The primordial germ cells then extend protozoan-like cytoplasmic processes (pseudopodia) and migrate along the dorsal mesentery from the hind gut (former yolk sac wall) to the medial aspect of the early urogenital ridge. At gestational week 6, the primordial germ cells (destined to differentiate into the primary oocytes of the genetic female and spermatogonia of the genetic male) become incorporated into the primary sex cords; at this time in gestation the gross morphology of the gonads of both sexes is identical. At birth, the 700 000 to two million primary oocytes of the genetic female have completed the first prophase of their first meiotic division and enter into the dictyotene (resting) stage where they remain in 'suspended animation' for up to 40 years or more. Chromosome damage may accumulate in these cells over time as evidenced by the increased incidence of genetic disease in children (e.g. trisomy 21) born to older (>45 years) mothers (1 : 50 live births) compared to that in younger (<29 years) mothers (1 : 2000). Thus, it is from the time of the appearance of the primordial germ cells during the parents' embryonic development that defines the earliest period sensitive to developmental insult.

Developmental toxicity is manifested in several ways. These include death of the embryo/foetus, structural malformations, retarded growth and functional alterations (that may not be evident until some time after birth). This wide diversity of end points can be induced by an equally broad spectrum of causes or mechanisms. Among these are conditions associated with chromosomal damage (e.g. ovarian dysgenesis in Turner syndrome XO females),

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neurological deficits (e.g. ethanol-induced behavioural disorders) and frank physical (structural) malformations (e.g. thalidomide-induced phocomelia). The present chapter will restrict its contents to the origin and pathogenesis of selected structural malformations in humans, including some of the maternal factors that have been shown to contribute to or play a role in their aetiology. Given the vast numbers and diverse types of congenital defects (Wise *et al*., 1997) and the many authoritative texts and reviews, the present chapter is limited to examples that introduce the reader to the discipline.

At the outset, it must be appreciated that some 5% of all live-born infants have one or another defect (including mental retardation) (Behrman and Vaughan, 1987). Unlike many other facets of toxicological sciences, developmental toxicology must be addressed at several levels of biological organization. Furthermore, one must take into account the developmental stage of the embryo and its susceptibility at the time of exposure, in addition to the frequency and magnitude of exposure to the agent. The pathogenesis of congenital malformations begins with descriptions of the ultimate lesion (e.g. agnathia, micrognathia) and works backwards to describe any changes in numbers of cells, movement and fate of embryonic tissues (e.g. reduced migrating cranial neural crest and reduced pharyngeal arch) to changes in the timed intercellular signalling and intracellular regulatory circuits that determine basic embryonic pattern formation (e.g. anteroposterior and dorsoventral directions, segmentation and limb organization) that likely contribute to the ultimate defect. The fundamental patterns followed in human embryogenesis have been conserved over at least 540 million years and these patterns are shared with those used by *Drosophila, Xenopus* and other chordates (including birds) (Gans and Northcutt, 1983; National Research Council, 2000).

1.1 Monsters, Trolls, Mermaids, Villains and Ghosts

There is perhaps no other science save astronomy that predates recorded history more so than teratology (the study of monsters). Records of congenital malformations date from antiquity and they have been described by cultures all over the world. These conditions are not necessarily products of the industrial revolution; they are not necessarily due to modern medicine or consumer products nor do they necessarily result from exposure to obscure, unique or novel synthetic environmental contaminants. For instance, maternal infection with the single-stranded RNA rubella virus responsible for German measles caused more birth defects—including deafness, cataracts and profound mental retardation (not to mention foetal deaths)—in the United States in one year than all of the cases of thalidomide embryopathy (~10 000) recorded during its entire career worldwide (Webster, 1998). Some of the most destructive teratogens (an agent that induces congenital defects in the embryo) were recognized in ancient civilizations and some (e.g. ethanol) remain on pharmacy and grocery shelves to this very day (Willhite *et al*., 1988). Teratology and teratogens are inextricably intertwined into the fabric of contemporary society. The prospect of teratogenesis presents ethical dilemmas in clinical practice, tort claims and medical insurance; sensational stories in the popular press and personal decisions to abort pregnancy often stem from an innate fear of birth defects.

Ample archaeological evidence exists to show that congenital malformations are nothing new (Keenleyside and Mann, 1991; Mann *et al*., 1992; Mann and Owsley, 1989). Ancient people developed explanations for all kinds of natural phenomena ranging from the origin of the planet, to geothermal activity, to the existence of rare and unusual animals and human beings. Clay and marble sculptures, pictographs and woodcuts that date back at least as far as 6500 BC, provide evidence of the birth of ancient malformed humans (Warkany, 1971; 1977). That these depictions are not those of the imagination can be seen from the fact these creations are anatomically correct (Weisman Collection, 1965). Just as in contemporary society, ancient cultures sought reasons why an otherwise normal pregnancy gave rise to such an unusual or gruesome outcome. Today we retain many of these same figures, explanations and depictions in fairy tales, popular literature, in the cinema, in commerce and in legislation, but most people have no idea of their origins or the relations between distant oracles, modern myths and congenital defects.

When a child is born with an anomaly, the parents *always* look for an event or series of events to explain the condition. When explanations are not forthcoming, a reason *will inevitably be devised and applied*—regardless of the evidence. Early cultures practised infanticide, purposely neglected deformed neonates or leaving them to die from exposure (Hrdlicka, 1908; Friedman and Padula, 1988). In some parts of the world today, abnormal infants are sacrificed or abandoned as a result of a belief they arose from the supernatural (Lenz, 1988). In the Western world, and even among the most sophisticated and educated parents and consumers, ignorance, superstition and fear coexist with naturalistic and scientific views of the world. Nowhere is this more evident than a mother's fear of exposure to therapeutic drugs, pesticides, hair dyes, paints, varnishes or solvents, or perhaps trace levels of unidentified, exotic or difficult-to-pronounce industrial or environmental chemicals (Koren *et al*., 1989) that might lead to development of what appears to be an alien being *in utero* (**Figures 1** and **2**).

Pregnant women believe their risk of giving birth to an infant with major malformations is 25%—very

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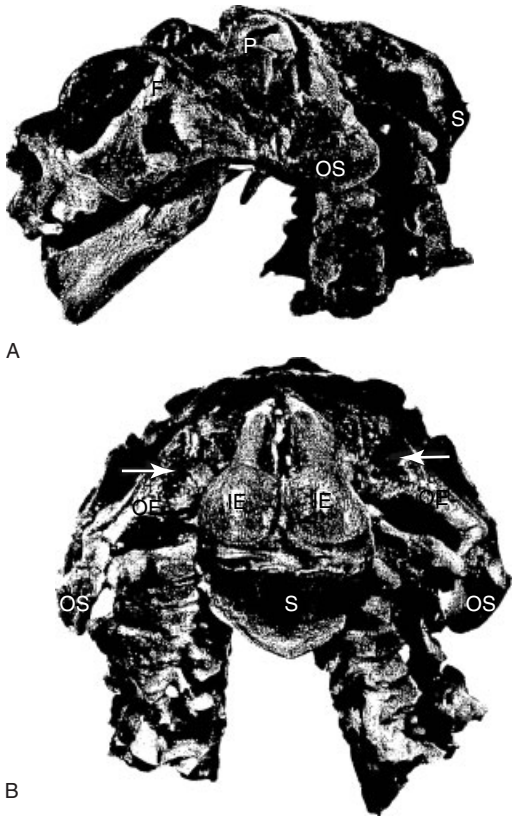

Figure 1 (A) Lateral view of 33-week-old female anencephalic cephalothoracopagus twins with fused skull. P: petrous temple; OS: outer supraoccipital; S: midline supraoccipital; F: frontal. (B) Posterior view of the same skull showing fusion of the vertebral columns. IE: inner occipital; OE: outer exoccipital; OS: outer supraoccipital; S: midline supraoccipital. Arrows show outer hypoglossal canals. (Reproduced from Herring and Rowlatt, 1981. © John Wiley & Sons, Inc.)

near the risk seen with thalidomide (Koren *et al*., 1989; 1993). Lessons from history show that this belief is reinforced by sensational articles in the popular press. In his text *Wunder, Wundergeburt and Wundergestaldt in Einblattdrucken des funfzehnten dis archtzehnten Jahrunderta* (published in 1543), E. Hollander recounts the story of a Polish child born with 'fiery eyes, Satan's tail and the head of a cat, webbed digits, a trunk and dog heads at the forearms'. This preposterous description appeared in illustrated pamphlets and books, and these were printed and distributed then reprinted and redistributed so many times that the account became accepted as fact (Warkany, 1971; 1977). These stories are really not all that different from today, where 10 popular 'family' and 'science/health' magazines had misleading and sensational accounts that exaggerated the risk of birth defects. More than half of these articles were inaccurate, 46% were intentionally alarmist, 8% were false and only 5% provided even a reasonable account of health risk (Gunderson-Warner *et al*., 1990).

The Babylonians (who lived along the Tigris River about 4000 years ago) were among the first to record unusual humans and the local Babylonian authorities considered these rare individuals evidence of divinity. In a world inhabited by witches, demons, soothsayers, wizards and sorcerers, malformed infants were thought to be evil or the work of the Devil. Babylonian records detail the predictive power of each type or combination of malformations. Belief in that divine power was then followed by the Egyptians, Romans, Greeks and Syrians, and it was adopted by the Europeans, all in the belief that if one could only understand the cause of the condition, this would be key to unlocking the future, divining the hidden and controlling events. Since only a deity possessed true knowledge, the oracle settled for the belief that understanding the reasons behind exceptional animals and humans would predict the future. As a case in point, the Roman, Quintus (Cicero's brother), described the birth of a conjoined (dicephalic) girl and concluded this foretold of 'sedition among the people and adultery in the home'. Further, 'When a woman gives birth to an infant that has the heart open and that has no skin [extrathoracic ectopia cordis], the country will suffer from calamities' and 'When a woman gives birth to an infant whose upper lip overrides the lower, the people of the world will rejoice for the troops'. In some instances, the birth of a monster was taken as a sign the gods were angry at the mother herself or at the whole of society because of a decline in 'family values' (Warkany, 1971; 1977).

1.1.1 Monsters

There are few examples of terata more striking than conjoined twins and the cyclops.

Conjoined dicephalic figurines dating from 500 BC to 800 AD have been recovered from Mexican and Central American graves and from the South Pacific and southern Turkey (Weisman Collection, 1965). Conjoined twins can be craniopagus (cranial union only), pyopagus (joined at the lower spine), thoracopagus (union at the upper half of the thorax), cephalopagus (dicephalics with anterior union at the upper half of the body), parapagus (dicephalics with heads joined side-by-side), ischiopagus (anterior union at the lower body), omphalopagus (anterior union at mid-trunk) (Spencer, 1992) and rachipagus (dorsal union of the entire head and trunk) (Spencer, 2005). Among the more notable, but nowadays unrecognized, conjoined twins are the pair Janus, the two-faced (dicephalic) Roman god of gates and doorways (**Figure 3**). Agni, the Hindu god of fire, is also dicephalic and Brahma (the Creator) has four heads so he can see in all directions at the same time. Dicephalics are not limited to ancient history, for we have drawings and written accounts, from 1790 of the 'Two-Headed Boy of Bengal' (Bondeson and Allen, 1989) to the more

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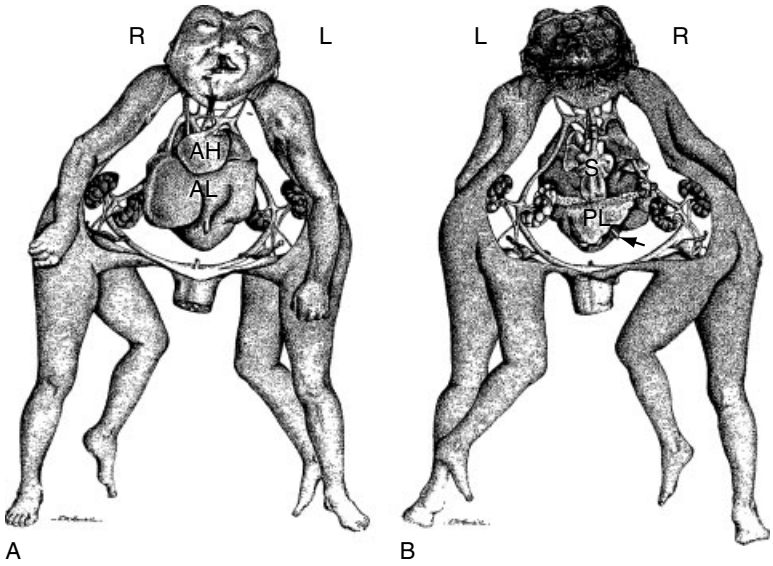

Figure 2 Anterior (A) and posterior (B) drawings of the right (R) and left (L) conjoined twins depicted in **Figure 1**. Note the anterior liver (AL), posterior liver (PL), single oesophagus (E) and stomach (S) and the anterior heart (AH) with double aorta. (Reproduced from Herring and Rowlatt, 1981. © John Wiley & Sons, Inc.)

Figure 3 The Roman craniopagus Janus, god of gates and doorways, change and transition, beginnings and endings. His ability to look forward to the future and back to the past brings us the month of January. He also brings us the word 'janitor', a term for the guards and custodians of doors, hallways and gates. (Reproduced with permission from New York Public Library Picture Collection.)

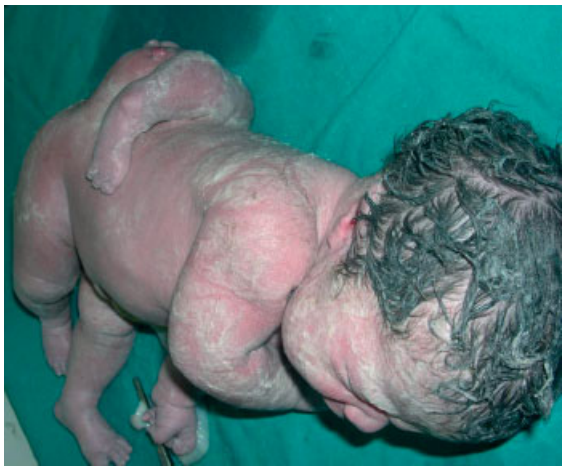

Figure 4 Anterior view of 38-week live female infant at delivery. Note the parasitic sacral twin with irregular lower limbs and left foot. Ultrasound and magnetic resonance imaging found no relation with the spinal cord of the otherwise healthy infant. The parasite was surgically excised and neonatal follow-up at nine months was normal. (Reproduced from Gul *et al*., 2004. © BioMed Central.)

recent accounts of parasitic craniopagus (Lotfy *et al*., 2006).

The physical descriptions of the Brahman Triad (Shiva the Destroyer, his wife Devi and Vishnu the Preserver) are strikingly similar to that of Betty Lou Williams (Albany, Georgia), Jean Libbera (Rome, Italy) and Ernie Defort (Manitoba, Canada) all of whom were born with parasitic twins, a rare (**Figure 4**) but well-documented condition (Gul *et al*., 2004). According to Homer, the carnivorous Scylla lived at the Strait of Messina and she had the face and breasts of a woman but six heads with 12 legs and feet (to assist her in scaling the high Sicilian rocks). Complete duplication of the lower body (dipygus) has been seen in contemporary times, as in the case of Myrtlye Corbin who lived in Lincoln County, Tennessee. Parasitic twins and the *fetus in fetu* (a full or partially developed parasitic twin that is completely enveloped by

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the otherwise normal sibling) were also reported in the late twentieth century (including the case of Sanju Bhagat who at 39 years of age underwent surgery to remove his vestigial brother from his abdomen).

By the late 1950s, cinematic techniques advanced with the stop-motion creatures invented by Harryhausen (1958) who used his ball-and-joint armatures (Dynamation) to bring the fantastic Technicolor cyclops in *The 7th Voyage of Sinbad* to life. Unlike his hard-working predecessors, Argos, Steropes and Brontes (the offspring of Uranus and Gaia) or Polyphemus (the son of Poseidon and Thoosa) of Homer's *Odyssey*, the irritated cyclops whom Sinbad had the misfortune to stumble across sprouted a single horn atop his skull, his lower torso took on the furry hindquarters of a goat and he preferred his captives well-done over an open spit. Rather than a malformation (partial or complete fusion) of the eye(s) *per se*, cyclops represent the most extreme manifestation of holoprosencephaly, a condition characterized by failure of the midline neural plate structures to differentiate and separate.

Holoprosencephaly ranges from comparatively mild abnormalities of the midface (flat nose with anosmia due to absent olfactory bulbs or tracts) to a single cerebral ventricle with a single eye and orbit. At least 48 holoprosencephalic variants have been described in human beings, including those presenting with microcephaly, ocular hypotelorism or hypertelorism, unilateral or bilateral cleft lip, coloboma and minimal facial dysmorphia from cebocephaly (hypotelorism with single nostril nose) to ethmocephaly (normal orbits with a single proboscis). Holoprosencephalic features are seen in 1 : 250 pregnancies and at least some facets are seen in 1 : 16 000 newborns (Ming and Muenke, 1998). The severity of the condition is described as lobar, semilobar and alobar (Kokich *et al*., 1982). True cyclopia (alobar holoprosencephaly) presents with either a median orbit and eye, fused eyes (synophthalmia) or absent eyes (anophthalmia). The ethmoid and middle portions of the sphenoid are absent. The absence of the ethmoidal cartilage is responsible for the related structural defects of the face. The cyclops may have no discernible trace of a nose or it may possess a blind-ended proboscis above or below the eye (**Figure 5**). In some cases, derivatives of the cranial neural crest (e.g. mandible) are absent or the cyclops may have a jaw, but only a single central incisor.

Holoprosencephalic conditions are the most common brain anomalies in humans. These occur at rates between 0.56 to 0.63 per 10 000 live births and they are seen in as many as 40 per 10 000 spontaneous abortions (Cohen, 1989). A great many epidemiological studies have been published, including those that focussed on the influence of pregnancy history, maternal disease (e.g. diabetes), tobacco and ethanol consumption, paternal exposure to herbicides, metals and pesticides, prematurity, twinning and birth weight. Of these, only reduced birth weight was associated with the condition and this was due to the fact that most severely malformed infants (regardless of the defect) are often significantly growth retarded. More of the affected infants are females (60%) than males and ~60% are afflicted with other structural malformations (notably polydactyly in 32%, branchial arch defects in 16% and CNS (central nervous system) defects in 16%). Among survivors with serious terata, mental retardation is usually severe.

Figure 5 Foetal rabbit with cyclopia.

The incidence of cyclopia is increased in foetuses with trisomy 13, but, overall, about 50% of the cases have a normal karyotype and 20–50% display one or another chromosomal anomaly. Autosomal recessive inheritance has been seen in the majority of cases with partial trisomy 13 in 70%, deletion at 13q in 10% and the deletion 18p syndrome in 10% of all cases. Foetuses with trisomy 18, dup(3p), del(7)(pter → q32), as well as those with deletions at 1q, 2p, 5q, 5p, 7q, 9p, 11q, 18p and trisomy 20 and 22 or anomalies of the sex chromosomes (e.g. 47,XXX; 47XY, +B, 46 XY/47,XY) are at increased risk. Medical geneticists have also identified an autosomal dominant form of holoprosencephaly with incomplete penetrance and the possibility of X-linked inheritance has been put forward (Cohen, 1989).

From the first anecdotal reports to the epidemics in Idaho, cyclopia among herds of sheep represented a substantial, endemic economic loss experienced by ranchers in the Intermountain West. In the early 1960s, research sponsored by the US Department of Agriculture found that when pregnant ewes ingested a subalpine range plant known as California false hellebor (*Veratrum californicum*) on the 14th day of gestation (corresponding

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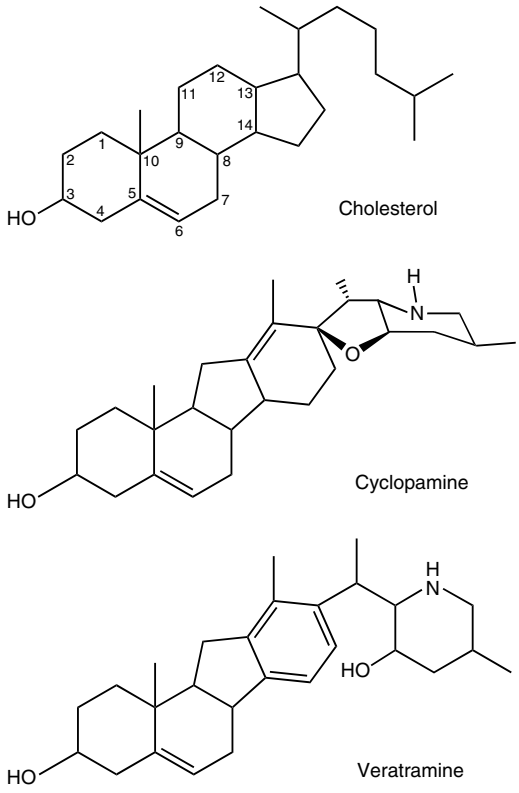

Figure 6 Chemical structures of cholesterol, cyclopamine and veratramine. The C3-OH is shown at the left in all three drawings and the double bond at C5 and C6 is required for activity. When cyclopamine encounters acid in the stomach of rabbits and other monogastric animals, the furan ring is hydrolysed to the –OH of veratramine and the teratogenic activity is destroyed. (Reproduced from Incardona *et al*., 2000. © Elsevier.)

to the neural plate stage), the full range of holoprosencephalic terata could be induced (Binns *et al*., 1963; 1964). Cyclopia and associated terata can also be induced in other ruminants (e.g. cattle, goats) depending upon the quantity of plant consumed and the gestational age at which exposure occurs (Binns *et al*., 1972). This large flowering perennial (commonly known as 'cow cabbage' or 'wild corn') synthesizes and accumulates a number of complex steroidal alkaloids including muldamine, veratramine, corvine, verstrosine, rubijervine, jervine, 11-deoxojervine and cycloposine. These molecules are steroids to which a furanopiperdine ring has been fixed. When 11-deoxojervine (also known as cyclopamine) (**Figure 6**), cycloposine (3O-glucosyl-11-deoxojervine) or jervine were fed to pregnant sheep, malformations identical to those observed after feeding the plant were induced. Structure–activity comparisons revealed that the presence of an intact tetrahydrofurylpiperidine ring was required for teratogenic activity. Oral administration of cyclopamine to pregnant rabbits failed to induce terata because cyclopamine is acid-labile and it is hydrolysed

Figure 7 Anterior view of the common troll.

in the monogastric gut to its inactive congener veratramine (**Figure 6**), in contrast to the ruminant, where the parent compound survives the gut intact. However, when oral cyclopamine was given on day 7 of pregnancy in achlorhydric rabbits after the gastric pH was increased by concomitant administration of sufficient bicarbonate, cyclopia was induced (Keeler, 1970).

1.1.2 Trolls

Scandinavian folklore speaks of the common troll, an ugly and dangerous heathen race of fearsome humanoid goblins. Carnivorous, dim-witted and cowardly, trolls dwell in caves, dark forests or under bridges, as in the Norwegian fairytale *Three Billy Goats Gruff*. Other accounts describe trolls as small, shy, thieving, evil dwarfs that have a nasty habit of carrying away valuables, small children and seducing young maidens (especially princesses) with the same magic found in the lyrical *March of the Trolls Op.54 No. 3* by Edvard Grieg. Today trolls are no longer ugly or shy, but they are whimsical. They wear cute little outfits and they have grown a great mass of long fuzzy hair over their rudimentary skull (**Figure 7**). Trolls have the large eyes (an illusion due to the reduced cranium) and skull of the anencephalic (**Figure 8**).

Anencephalus is part of a constellation of terata known collectively as 'neural tube defects' (NTDs) and these range from complete absence of the head (acephaly) to merely deformed or rudimentary spinal vertebrae covered by skin (occult spina bifida) that may have no clinical consequence (DeSesso *et al*., 1999). While the anencephalic always dies shortly after birth, children with spina bifida (**Figure 9**) are physically disabled with severe neurologic and muscular problems and (often) sphincter paralysis. NTDs arise during the fourth week of development. Damage to the embryonic mesoderm is reflected in the abnormal skeletal elements and interference with elevation and fusion of the neural folds is reflected in the abnormal neurological elements. These defects result from failure of the neural groove to close, a process that begins at day 22 in the central dorsal region and progresses to the caudal and cephalic regions.

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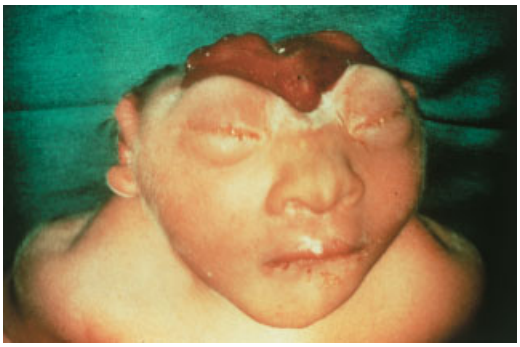

Figure 8 Anterior view term human anencephalic. Note the protruding and rudimentary brain, low-set ears, elevated nose and maxilla and the short neck. The short neck is due to malformations of the cervical vertebrae and the protruding maxilla is a consequence of lordosis and deformations of the sphenoid. (Reproduced from Willhite and Mirkes, 2005. © Elsevier.)

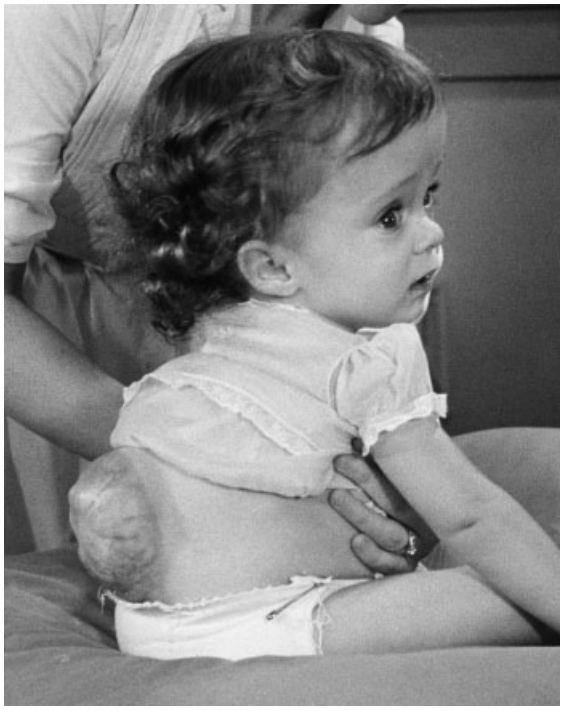

Figure 9 Infant with spina bifida. Note the protruding meningocele. (Reproduced with permission from the March of Dimes Foundation.)

Anencephalus and many forms of spina bifida arise from a similar mechanical failure, but differ in the location of initial embryonic damage. The anencephalic face (viscerocranium) and skull (chondrocranium) are rudimentary (**Figure 8**), but all of the bones of this portion of the lower skull are present (Marin-Padilla, 1991). Overall, the incidence of human anencephalus (termed exencephaly in rodents and lagomorphs) can vary by location, by time of year and by social class and race (Elwood and Elwood, 1980). In the United States, historical rates of anencephalus and spina bifida averaged 3.4 and 5.0 per 10 000 live and stillborn deliveries. Rates of anencephalus ranged from 0.8 to 18.4 per 10 000 live and stillborn deliveries in Southern France and Mexico, respectively; areas with high rates of anencephalus also have high rates of spina bifida. Some retrospective epidemiologic studies found seasonal distributions with higher rates among babies born in winter than in summer, but other studies found no such pattern. Families of unskilled manual workers had rates five times those of the professional class, and the incidence was sometimes higher in urban than rural areas, but there was no correlation with urban density. Historical rates in Northern Ireland, South Wales and the Vale of Glamorgan were among the highest in the world (5.2–11.5 per 10 000) and in England there was an apparent decline from west to east, but in Ireland the incidence was higher in the east compared to the west. Rates among those of Irish descent living in Boston were less than those of people who lived in Ireland. Italians and those of African descent had some of the lower NTD rates and Jewish children had very low NTD rates. Offspring of British women living in Quebec had elevated rates of anencephaly, but offspring of French women living in Quebec had elevated rates of spina bifida. Rates were generally higher in a first pregnancy and lower in second pregnancies, but about 95% of affected infants were born to mothers with no such history. NTD rates were high in women less than 20 years of age or greater than 35 years of age compared to ages between those values. The incidence among siblings was up to seven times that in the general population and the risk is increased to as high as 1 in 10 for those born to mothers with two prior NTD pregnancies. Risk of recurrence varies with time and individual risk is related to that for the local population. Studies of same-sex fraternal and identical twins found it rare that both twins were affected, but paternal age had no independent effect (Horowitz and McDonald, 1969).

1.1.3 Mermaids

Half-human, half-fish and always insidiously seductive, mermaids are among the most universal mythical creatures. Their fame increased after publication by Hans Christian Andersen of his 1836 tale *The Little Mermaid*, a book that was translated into many languages and became the subject of a successful animated Disney motion picture. Mermaids appear on Tarot cards, they appear on the corporate logo for the Starbucks chain of coffee houses and they appear on the Warsaw (Poland) coat-of-arms.

There are popular suggestions that the mermaid legend originated in the New World by sailors' observations of the manatee (*Trichechus manatus*), a large aquatic herbivorous mammal of the Order Sirenia that inhabits the

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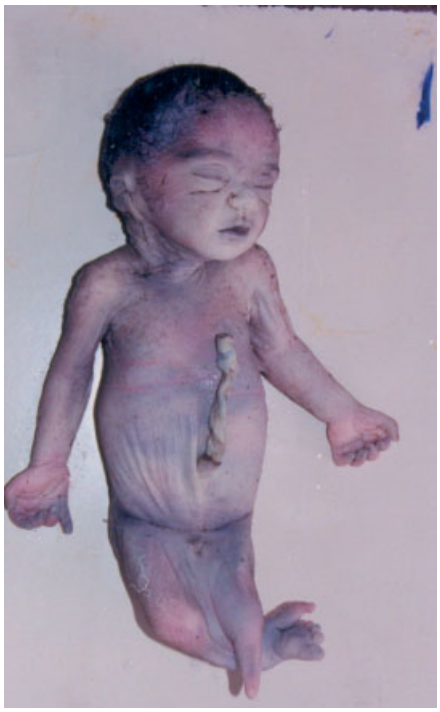

Figure 10 Anterior view of a male siren (sympodia) at 33 weeks showing partial fusion of the lower limbs to the level of the knees. Note the absence of the left ankle and foot, the twisted right foot (talipes equinovarus) with duplicated toes. (Reproduced with permission from Mysorekar *et al*., 2007. © MedKnow Publications.)

coastal waters of Florida (Cooper, 1992). Nonetheless, there are mariner tales dating from as far back as 1000 BC to the second century AD that speak of mermaids as water nymphs and sirens, those innocent and beautiful singing female water fairies prone to enchanting and distracting sailors, causing shipwrecks and dooming the crews. In spite of (or perhaps because of) her six heads, Scylla was the object of romantic desire by the Greek merman Glaucus. Olokun, sea king of the Yoruba (who live in the Midwestern part of Nigeria), is depicted with mudfish legs and the Hindu incarnation Matsya is a four-armed merman.

The incidence of sirenomelia (also known as sympodia or symmelia) (**Figure 10**) varies between 1.5 and 4.2 : 100 000 depending upon whether only live births or aborted foetuses are included in the calculation. The lower limbs are usually fused and may present as a single femur and tibia with the sole of the foot exhibiting fused toes. The foot is often oriented in a ventral position. There are cases where fully developed legs are joined only by cutaneous tissue. Sirenomelic foetuses commonly present with hypoplastic lungs and multiple defects of the heart, spine and abdominal wall, as well as absent genitalia, urinary bladder and anus. In very few cases, sirenomelia was evident along with NTDs (Rodriguez *et al*., 1991; Rodriguez and Palacios, 1992; Kulkarni *et al*., 1994). Sirenomelia is usually lethal due to hypoplastic lungs, cardiac defects and congenital absence of both kidneys, but there are reports of at least three cases with lesser malformations who survived (Murphy *et al*., 1992). Sirenomelia occurs more commonly in males (Sirtori *et al*., 1989) and in twin pregnancies (Temtamy and McKusick, 1978); there are usually no chromosomal abnormalities and there is no increased risk among sibling births (Tang *et al*., 1991).

1.1.4 Villains

Villains have common traits: ignorance, arrogance, greed and the propensity to reappear and repeat their same patterns of dangerous behaviour. There is likely no greater villain in developmental toxicology than methyl mercury (MeHg).

In the early 1900s, Shin Nichitsu (the former Japan Nitrogenous Fertilizer Company known since 1965 as the Chisso Corporation) established an ammonia fertilizer production facility on the shores of Kyushu Island in the Shiranui Sea (about 570 miles southwest of Tokyo). At that time, the residents of Kumamoto made their living primarily as subsistence fishermen and farmers, and they anticipated the economic prosperity they hoped would follow the opening of the plant. By 1932, the plant was successful and Shin Nichitsu expanded their capabilities to manufacture acetaldehyde as a chemical intermediate, a synthetic pathway that required the use of a mercuric ion catalyst (Hagemeyer, 1978). Industrial process water from the plant was discharged to Hyakken Harbour and thence into Minamata Bay. Ionic mercury is transported downward in the water column to sediments (Hurley *et al*., 1991) where it is converted by sulphur-reducing aerobic and anaerobic microorganisms to alkyl mercury (methylated forms) in both salt and freshwater ecosystems (Gilmour and Henry, 1991; Regnell and Tunlid, 1991). MeHg is lipophilic and it concentrates 10 000- to 100 000-fold as it moves up the ecological food chain into fish (particularly large predatory species) and shellfish (International Programme on Chemical Safety (IPCS), 1990).

On May 1, 1956 the first case of an 'unknown disease of the central nervous system' was recorded by the company's hospital staff. By the early 1960s Kumamoto University researchers concluded that the encephalopathy seen in Kumamoto residents was due to ingestion of sea foods containing mercury discharged by Shin Nichitsu (Takeuchi *et al*., 1962; Tsubaki and Irukayama, 1977). Factory discharge was rerouted in 1958 from the harbour to the Minamata river past the village of Hachimon and into the Shiranui Sea where those residents who also consumed local fish subsequently developed identical symptoms. In 1959 Shin Nichitsu began to compensate victims without admitting responsibility or the source of the problem. In 1965, mercury effluent discharged from acetaldehyde production to the Agano River by Showa

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Denko Company also induced MeHg encephalopathy among residents who consumed fish caught in the Niigata Prefecture.

Alkyl-mercury-induced toxic encephalopathy has a prolonged latency (usually several asymptomatic months to possibly years). The initial signs are characterized by slurred speech, numbness of the lips and limbs, loss or constriction of visual fields and muscular weakness that leads to progressive paralysis, blindness, coma and death (International Programme on Chemical Safety (IPCS), 1990). MeHg is readily taken up in the intestine; it crosses the placenta and human milk can contain high levels (Koos and Longo, 1976). Prenatal exposure to sufficiently high concentrations of MeHg (hair Hg $\geq 70\,\mu g\,g^{-1}$) presents a >30% risk for foetal encephalopathy—a condition that resembles cerebral palsy. Often the mothers of affected infants presented with little if any clinical signs of MeHg intoxication. At least 100 victims died and 17 babies were born with MeHg-induced encephalopathy; as of 1974 some 800 victims had been officially recognized. In some cases, the mothers were relatively unaffected, but their offspring were blind and were left mentally and physically debilitated (Matsumoto *et al*., 1965). The pivotal victim was Tomoko Uemara whose black and white photograph with her mother (Smith and Smith, 1975) was published in *Life Magazine*. This intimate and famous portrait titled *Tomoko Uemara in Her Bath* (1972) by W. Eugene Smith is considered one of the most important images ever in the history of photography (Overseas Press Club of America, 1974). Tomoko Uemara died in 1977.

In 1968, Chisso's acetaldehyde production ceased. The company denied all allegations and there were suggestions that the disease was contagious; the provincial government responded by imposing a ban on Minamata seafood in commerce, but permitted continued subsistence fishing in the bay. 'Single shareholder' resistance began with letter writing, nonviolent protests, tent cities and citizens each purchasing one Chisso share in an effort to disrupt the annual shareholders' meeting and corporate governance. In 1972 and apparently at the direction of the company, six Yakuza intimidated and beat Mr. Smith severely (Kaplan and Dubro, 2003). Mr. Smith continued to photograph the civil unrest, the Central Pollution Board meetings and victim presentations before the Environmental Disputes Coordination Commission; for his efforts, he was awarded the Robert Capa Gold Medal for the 'best published photographic reporting from abroad requiring exceptional courage and enterprise' (Overseas Press Club of America, 1974).

In 1973, the Kumamoto District Court ruled the company was negligent: 'We cannot find that the defendant took any of the precautionary procedures called for in this situation whatsoever... We cannot even find one measure taken by the defendant that was either adequate or satisfactory... The presumption that the defendant had been negligent from beginning to end in discharging wastewater from its acetaldehyde plant is amply supported'. As of March 2001, some 3000 people had been certified under Japan's Pollution-Related Health Damage Compensation Law as victims and had received income and funeral compensation, financial and medical assistance totalling ~144 billion yen. In 1975, Kumamoto Prefecture carried out dredging of Minamata Bay at a cost of 48 billion yen and in 1976 the Niigata Prefecture carried out dredging of sediments from Showa Denko's discharge points to the Agano River basin. Total ongoing costs for MeHg pollution control and prevention are 123 million yen per year and total damages amount to 12.6 billion yen per year (Ministry of the Environment, 2002).

Despite the infamous history, disregard for its hazards led to MeHg-induced morbidity in Brazil (Malm *et al*., 1995), French Guiana (Frery *et al*., 2001), the Philippines (Appleton *et al*., 2006), Mexico (Guentzel *et al*., 2007), New Zealand (Kjellstrom *et al*., 1986), Bolivia (Maurice-Bourgoin *et al*., 2000) and Quebec (McKeown-Eyssen *et al*., 1983), in addition to the 400 deaths as a result of MeHg poisoning in Iraq (Amin-Zaki *et al*., 1974). Environmental MeHg remains a threat to the public health to this very day (**Figure 11**).

1.1.5 Ghosts

Parents tell their children 'there are no such things as ghosts'; nonetheless, a scary story frightens young children, in spite of the admonitions of their parents. The same is true of adults, particularly when it comes to their livelihood, their investments in time, labour or life savings and their children. In an atmosphere of scientific ignorance or uncertainty, it is not uncommon that untested ideas, conjectures or hypothetical explanations come forward today—just as in ancient times—to explain the origin and cause of congenital disorders. These stories often appear or sound 'scientific' or have roots in the scientific literature. In the discussion that follows, a modern-day ghost story is recited as an example, but there are similar tales like those of depraved pharmaceuticals (Brent, 1995), demonized spray adhesives (Hook and Healy, 1976), plastics (Goodman *et al*., 2006; Willhite *et al*., 2008) and atmospheric radiation (Trichopoulos *et al*., 1987). Equally frightening (and uninformed or misinformed) exposure scenarios and the consequences of 'chemical rumours' are well known (Koren *et al*., 1989; 1993).

Our ghost story concerns the potato.

Coincident with the Great Depression of the 1930s there was an NTD epidemic in the United States with rates of five cases per 1000 pregnancies in Boston and Providence, but the incidence of the disease mysteriously declined over the next 50 years. In the early 1970s, researchers at the London School of Hygiene and Tropical Medicine noted geographic and temporal correlations between anencephalus and spina bifida and

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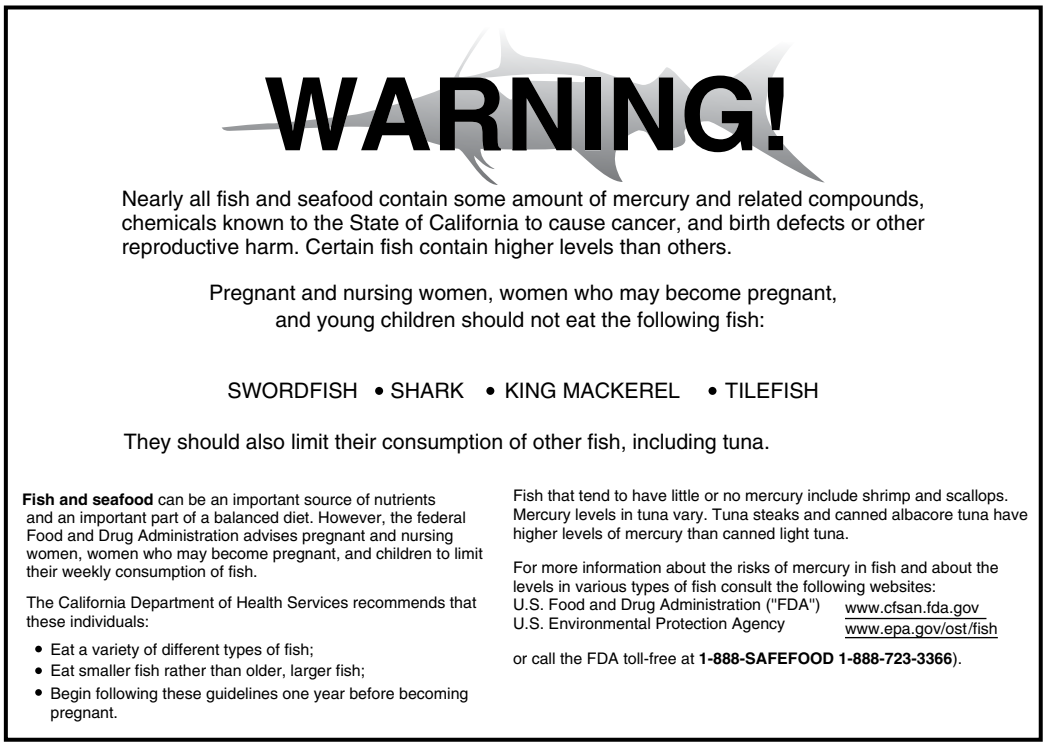

Figure 11 Methyl mercury warning sign posted in restaurants and markets.

found that areas with elevated anencephalus rates also had elevated rates of spina bifida. Year-to-year data indicated a seasonal peak in May and that poor mothers were affected about four times more often than wealthy mothers (Renwick, 1972; 1973; Renwick *et al*., 1974). At that time the epidemiology of anencephalus and spina bifida led Horowitz and McDonald (1969) to state: 'We conclude that all the available evidence indicates that environmental factors are responsible for NTDs but there are few clues to their nature. The tendency of some women to produce children with NTDs appears to be operative throughout the child-bearing period, since multiple cases in sibships appear to be randomly distributed in time. The characteristic must therefore be acquired before maternity, perhaps through nutrition or infection'.

Based on a strength of statistical association (p = 0.005) between the seasonal and geographical observations that fungal infestations of potatoes (blight), (*Phytophthora infestans*) which occurred just before harvest, and the seasonal and geographic rates of NTD, Renwick (1972; 1973) concluded that eating blighted potatoes caused NTDs and that avoidance of potatoes would reduce the incidence of NTDs: 'Most of the evidence for the potato-avoidance hypothesis for the prevention in the UK of 95% of spina bifida and anencephaly does indeed come from blight, that being the best-recorded and most important potato diseases. But the potato, like man, is subject to many diseases perhaps several being capable of eliciting a phytoalexin (antibiotic) response, which in most plants is relatively nonspecific, as it apparently is in the potato'. Renwick (1973) continued, 'Since teratogenesis probably occurs in the first month of pregnancy, the date of peak incidence points to maximal teratogenicity in May. In that month the over-wintered potatoes are at their worst quality'.

That an unassuming, economical dietary staple like the potato could cause birth defects became of immediate distress among potato farmers, processors, trade associations and purveyors who elevated their concerns to government agencies. No one knew whether the effect was due to potato storage conditions, whether one potato variety was more or less toxic than another, whether imported potatoes might be more or less dangerous than those grown locally, whether an unidentified agent was associated with the principal fungus (*Phytophthora infestans*) or to other fungi (e.g. *Erwinia carotovora*, *Alternaria solani*), to an unidentified toxic agent produced by the fungus, secondary infection by other opportunistic organisms or perhaps produced by the potato tuber itself due to fungal infestation, to variations in cooking conditions or inhaled cooking vapours or potato consumption habits—or worse—that unidentified potato and/or fungal toxin(s) accumulated in maternal tissues only to be slowly released into the mother's bloodstream during gestation (Renwick *et al*., 1974). All that was known for certain was that 'culinary pride'—being more or less prevalent in one country or geographical region than another—might lead to 'more frequent

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discarding of partly blemished potatoes' and those factors might account for differences in national NTD rates.

Governments in the United Kingdom and America responded by sponsoring feeding studies in animals using potatoes rejected by the potato industry or potatoes purposely infected under controlled conditions. Although indications of 'cranial osseous defects' were reported in four of 11 marmoset foetuses (small squirrel-like arboreal primates from Central and South America) whose mothers ate 'blighted' potatoes during gestation (Poswillo *et al*., 1972), there were no reproducible signs of potato blight-induced developmental toxicity in studies with other mammals. Studies in chicken embryos with purified cytochalasins and solanines from green potatoes found NTDs (Mun *et al*., 1975; Jelinek *et al*., 1976), but subsequent retrospective epidemiological evaluations found no differences in potato consumption patterns between mothers with NTD pregnancies and those with healthy pregnancies (Clarke *et al*., 1973). Nevin and Merrett (1975) then conducted a trial in Northern Ireland with 88 mothers matched for age, economic status, religion and parity, all of whom had previously given birth to an NTD child. Interviews at six to eight weeks gestation found 27 who were careful to avoid all potatoes and potato-containing foods and 61 who consumed potatoes. Of 23 pregnancies in the potato-free group that went to full term, there were two anencephalics; of the 56 full-term pregnancies in those who ate potatoes, there were two with spina bifida. There was no significant difference in NTDs between the potato eaters and the potato-free group. With the subsequent publication of anecdotal reports showing NTDs in potato-free pregnancies (Lorber *et al*., 1973), the obituary of the 'potato blight' hypothesis was essentially complete.

But that is not the end of our story. Recall that Horowitz and McDonald (1969) observed 'The characteristic must therefore be acquired before maternity, perhaps through nutrition or infection'. Earlier, Hibbard (1964) and Hibbard and Smithells (1965) examined mothers who had given birth to infants with NTDs and measured their folic acid status at term. These investigators found that mothers of NTD babies were five times more likely to have positive formimoglutamic acid elimination results than matched controls. In 1969, Lanzkowsky *et al*. (1969) and others began to assemble clues that dietary deficiencies, whether due to the dietary composition or due to impaired gastrointestinal absorption or impaired nutrient utilization, might contribute to development of NTDs. In 1976 Smithells *et al*. (1976) reported the results of their prospective study of maternal (N = 900) vitamin status during the first trimester and found six mothers who gave birth to infants with NTDs. In the affected pregnancies, both leucocyte and erythrocyte ascorbate and folate were reduced significantly compared to mothers with uneventful pregnancies. The focus on folic acid was prompted by observations from the 1950s of malformations in offspring of rodents given antibiotics to reduce their intestinal flora that normally synthesize folate (Evans *et al*., 1953; Giroud and Lefebvres-Boisselot, 1951), and by the increased foetal deaths and severe malformations in survivors exposed to the biochemical folate antagonist, aminopterin (Warkany, 1978).

In 1992, Czeizel and Dudas (1992) published one of the more important public health discoveries of the late twentieth century (Oakley, 1993). In a randomized controlled prospective blind trial of 4753 women planning their pregnancy, one group of 2052 received a trace element supplement and 2104 received a vitamin supplement with 0.8 mg folic acid. There were six NTD cases (four anencephalics, two of which also presented with lumbar or thoracic rachischisis, one spina bifida cystica and one lumbosacral spina bifida) in the group given the trace element supplement compared to zero NTD cases in those given the vitamin supplement. In 1992, the Scottish, Welsh and Northern Ireland Departments of Health and Social Services and their US and Australian counterparts responded by advising all adolescent and adult women capable of becoming pregnant to consume at least 400 µg of folic acid each day to prevent having an NTD pregnancy. Follow-up investigations by Werler *et al*. (1993a) and Werler and Mitchell (1993b) found a relative NTD risk of 0.4 among mothers who had consumed folate-containing vitamin supplements, an observation that led Oakley (1993; 2002) to conclude that up to 60% of all NTDs could be prevented by adequate dietary folate and that folate supplementation of diets in Mexico and northern China would reduce their very high NTD rates within one year to levels seen in the United States that required 50 years to achieve. Follow-up studies (Stevenson *et al*., 2000; Persad *et al*., 2002; Williams *et al*., 2005) put the reductions in NTDs at 50% in South Carolina, 54% in Canada and the reductions in spina bifida afforded by adequate dietary folate at 34% in non-Hispanic whites, 36% in Hispanics and 19% in non-Hispanic blacks, respectively. A community-based intervention trial with 400 µg synthetic folic acid per day carried out by Beijing Medical College reported an astounding 85% reduction in NTDs (Berry *et al*., 1999).

So as for the potato ghost, we find that it was not the presence of an unseen and unidentified toxin, or deposition and storage of an exotic or mysterious substance in the body that was the primary aetiologic agent, but that it was the *absence* of an essential nutrient that was responsible for the disease. Folates are present in citrus fruits and vegetables; dark leafy greens (spinach, broccoli, legumes) are particularly rich sources. While the specific dietary composition among mothers giving birth during the Great Depression or among the mothers in Scotland, England and Wales evaluated by Renwick (1973) is not known, it appears that the high financial cost of citrus and other fruits and vegetables—particularly during the winter months—prevented poor urban mothers from

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obtaining adequate folate compared to their wealthier or rural neighbours.

2 DEVELOPMENTAL BIOLOGY

To begin objective appreciation of the cause(s) and pathogenesis of any congenital defect, one must possess a basic understanding of normal embryonic development and should be familiar with the establishment of the body's architecture. The earliest steps an embryo takes define the anteroposterior and dorsolateral dimensions and determine body segmentation, steps that are known collectively as 'axis specification'. The fact that fundamental embryonic patterns are conserved from comparatively primitive protochordates to the higher-order chordates suggests vertebrates evolved from ancient notochordal organisms that resembled contemporary cephalochordates (perhaps similar to amphioxus) (Gans and Northcutt, 1983). These patterns have existed for at least one billion years. While the events in vertebrate embryogenesis are more numerous and are more complex than those in simpler animals, the basic chemical signals, pathways, patterns and responses seen at the molecular level are remarkably similar, and these have been conserved over hundreds of millions of years. With the basic organizational patterns remaining intact, one might argue that materials or factors which disrupt embryonic development in lower taxa could also possibly disrupt embryonic development in higher taxa, assuming all other things are equal.

The chordate 'body plan' involves the notochord (primary organizer), a hollow dorsal nerve, segmented muscles, an unsegmented trunk and a perforated pharynx. Humans and other mammals are members of the superclass Gnathostoma, a chordate class that includes reptiles, the cartilaginous and bony fishes, amphibians, mammals and birds. All of these species (along with their agnathous relatives) are members of the subphylum Vertebrata. Amphioxus and the other protochordates (e.g. tunicates) are members of the phylum Chordata, but they have a collagenous or chitin skeleton, their embryonic myotomes are staggered (where vertebrate myotomes are paired) and they lack gills and muscular aortic arches. In vertebrates, the notochord extends all the way to the basicranium, but in protochordates it is restricted to the rostral cephalic region. It is this difference that accounts for the fact vertebrates have a cartilaginous and calcified cranial skeleton; indeed, we should all be thankful for the differentiating cranial neural crest and sclerotomal mesoderm, as without their participation we should all be forever looking for our heads.

The early nervous system influences body shape, but the brain and spinal cord develop in somewhat different manners. To understand its establishment, we need follow it in three dimensions (Campbell *et al*., 1986; Copp *et al*., 1990; DeSesso *et al*., 1999; Gordon, 1985; Hamilton and Mossman, 1972; Morrissey and Mottet, 1980; Moore and Persaud, 2003; Sadler, 2006; Schoenwolf and Smith, 1990; Schoenwolf *et al*., 2008; Sulik and Sadler, 1993).

To follow the shape changes, we begin at fertilization, which, in humans, occurs in the ampulla of the uterine tube. This creates a new diploid organism, the zygote, which undergoes a rapid series of cellular divisions over the next four to five days as it traverses the uterine tube to reach the uterine cavity. By the time the zygote reaches the uterine cavity and begins to implant in the uterine wall, it is a sphere of cells surrounding a small cavity that is referred to as the blastocyst (**Figure 12**). The cells that line the inner cavity of the blastocyst differentiate into hypoblast cells which, in turn, induce the overlying cells to form the epiblast. The epiblast gives rise to the embryo proper. Approximately two weeks after fertilization, the epiblast and hypoblast form the bilaminar germ disc, with the epiblast facing the presumptive amniotic cavity and the hypoblast facing the primitive yolk sac cavity.

At the start of the third week, epiblast and hypoblast rearrange into the three primary germ layers (ectoderm, mesoderm and endoderm) from which the primordia of all tissues and organs arise. This process, termed ingression, begins with the appearance of the primitive streak, a crease in the epiblast of the caudal region (**Figure 13**). Some epiblast cells migrate medially towards the primitive streak and then move beneath the dorsal surface to make up deeper layers. As the epiblast cells migrate through the primitive streak, they acquire information concerning their fate; other epiblast cells subside directly beneath the surface epiblast cells. Most ingressing (or subsiding) cells become the mesoderm (the middle germ layer), but some intercalate with the hypoblast cells to form the endoderm (hypoblast cells *per se* give rise only to the extraembryonic yolk sac endoderm). The epiblast cells that do not ingress or subside become ectoderm.

At the cranial end of the primitive streak, a condensation of tissue (called the primitive node) appears and it surrounds an indentation (called the primitive pit). At days 17–20, ingression of epiblast cells through the primitive pit with subsequent cephalic migration in the midline, results in the formation of a transient tubular structure, the notochordal process (**Figure 14A**). The lumen of the notochordal process remains continuous with the lumen of the primitive pit. Shortly thereafter (days 20–23), the notochordal process becomes associated with the endoderm to form a new structure, the notochordal plate (**Figure 14B**). The notochordal plate subsequently separates from the endoderm (**Figure 14C**) and then segregates into: (i) a deeper rod-shaped epithelial structure (the definitive notochord formed by days 24–25) and (ii) a layer of cells that is incorporated into the floor plate of the neural tube (**Figure 14D**). Similar positional information signals emanate from the notochord and floor plate,

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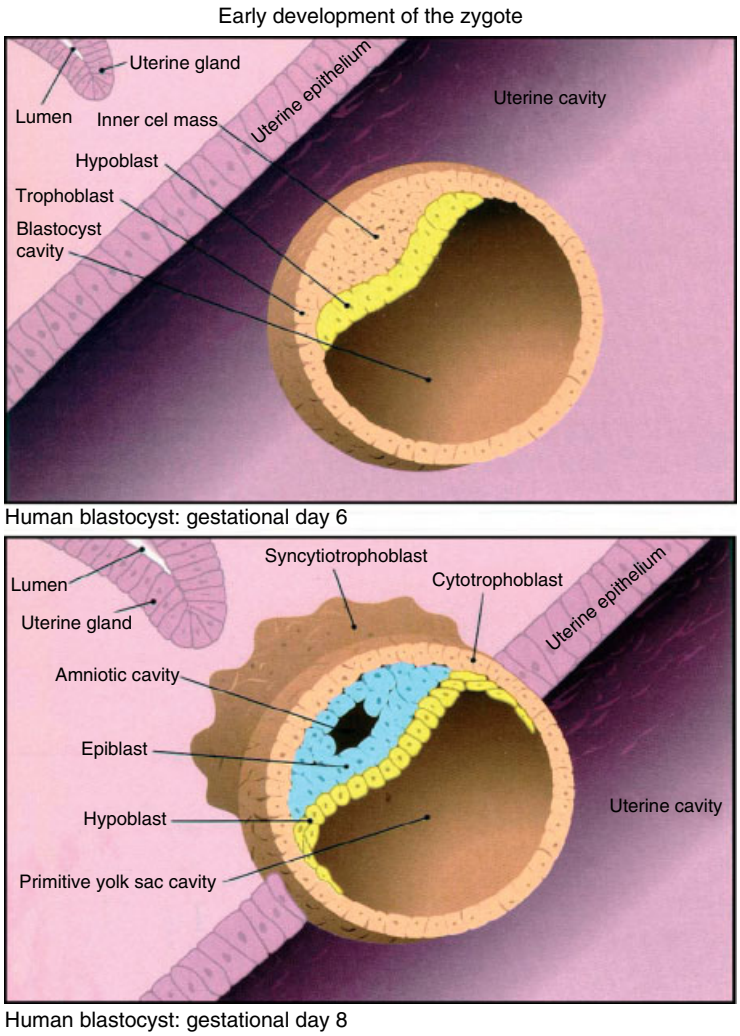

Figure 12 Diagrams that portray the human peri-implantation blastocyst. The upper panel depicts the blastocyst floating freely after hatching in the uterine cavity on gestational day (GD) 6. The major regions of the blastocyst are readily identified by their positions relative to the blastocyst cavity. The single layer of cells that composes the outer shell is the trophoblast, which gives rise to most of the foetal membranes, while the group of cells internal to the shell is the inner cell mass that develops into the embryo proper. The layer of the inner cell mass that is adjacent to the blastocyst cavity is the hypoblast. The lower panel shows the relationships of various parts of the implanting blastocyst on GD8. Note the layer of the inner cell mass above the hypoblast has differentiated into the epiblast. Together, the epiblast and the hypoblast make up the bilaminar embryonic disc. The space within the inner cell mass is the amniotic cavity. The nervous system develops from the epiblast cells shown at the inferior border of the amniotic cavity. (Reproduced from DeSesso *et al*., 1999. © John Wiley & Sons, Inc.)

providing inductive signals for the ventral portion of the nervous system (Tanabe and Jessell, 1996). The columnar neuroepithelium of the neural plate gives rise to all of the future brain and spinal cord.

Induction of the neural plate by the notochord and floor plate is the first step in neurulation. The notochord/notochordal process induce changes in the overlying ectoderm using cellular signals (morphogenic proteins); the epiblast that constitutes the presumptive ectoderm sends and receives the bone morphogenetic proteins 4 (*BMP-4*). As long as epiblast cells receive the *BMP-4* signal proteins, they will differentiate into ectoderm. If a different signal is present that inactivates *BMP-4*, then those cells will never become ectoderm. Inhibitory signals include *Noggin*, *chordin* (*Chd*) and *follistatin*; all of these proteins are made and released by the notochord and they signal a delimited region of the epiblast to differentiate into the slipper-shaped neural plate. If the cells over the notochord fail to receive the message to become ectoderm (i.e. because *Noggin, Chd* or *follistatin* are present and inhibit *BMP-4*), those cells become the neural plate. If the neural plate receives no other signals, it will develop into the brain. If, in the caudal part of the embryo, the somites (derived from

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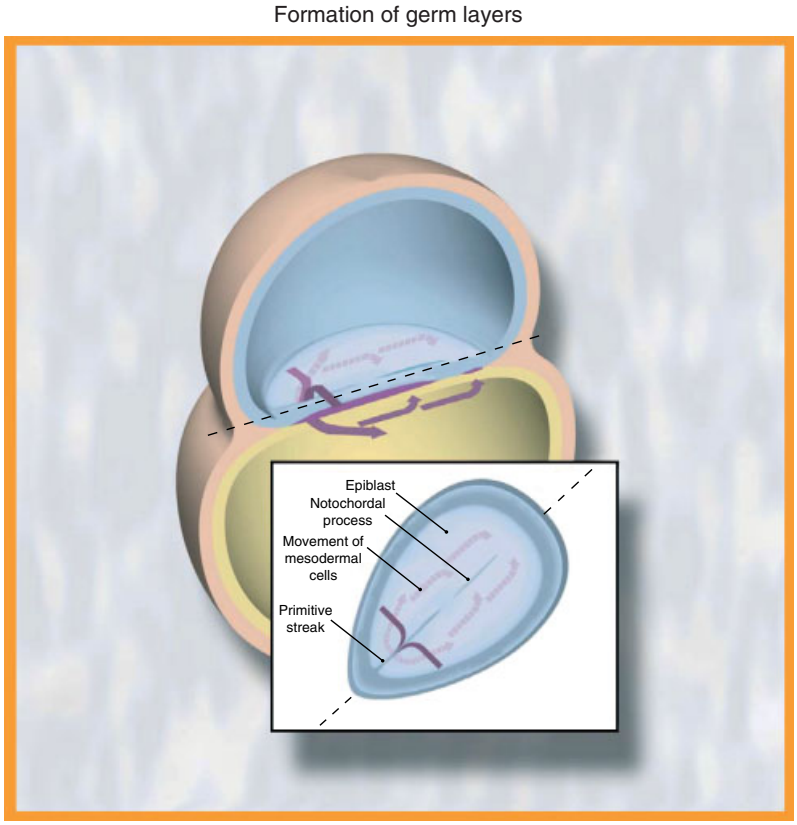

Figure 13 The formation of the human third germ layer (mesoderm) is illustrated. On GD15, a groove (primitive streak) appears in the caudal half of the embryo. Epiblast cells migrate medially to the primitive streak, where they ingress between the epiblast and endoderm and migrate laterally and cephalically to form the mesodermal layer. Epiblast cells that ingress through the cephalic point of the primitive streak migrate directly in the midline to form the notochordal process. The solid arrows demarcate the migration of epiblast cells; the dashed arrows depict the movement of the ingressing mesodermal cells. (Reproduced from DeSesso *et al*., 1999. © John Wiley & Sons, Inc.)

mesodermal cells that contribute to the formation of the vertebrae) secrete fibroblast growth factor (FGF), it specifies that the neural plate in that region shall become the spinal cord.

The neural plate is a cohesive structure whose cells are linked together by junctional complexes. There are structural changes that occur within the neuroepithelial cells that contribute to the formation of the neural groove and then the neural tube. Initially, the notochordal process emits signals that tell the cells of the cuboidal epiblastic ectoderm to change their shape to high columnar; as these cells migrate laterally and away from the central axis, they become truncated pyramids—not unlike building a stone arch. These cells then adopt shapes to create the cylindrical neural tube. As reviewed in Wallingford (2005), the different cell shapes are determined by:

- Actin filaments at the tops of these cells that cinch the crest and force the apex to become narrower than the base.
- Adhesion molecules located at specific apices of the neuroepithelial cells (facing the lumen of the future central nervous system) help these cells retain their new-found shape, while maintaining their positions relative to each other even as their orientation changes from a flat sheet into an arch.
- Microtubular polymerization along the apical–basal axis leads to increased cell height.
- The location of the nucleus within the cell causes the cell to bulge and changing their location changes the height and shape of these cells.

Initially, the neural plate appears as a flat teardrop, broad in the cranial aspect (presumptive brain) and tapering back towards the caudal end (presumptive spinal cord). As the embryo grows, the primitive node moves caudally, with resultant lengthening of the notochord as the latter continues to induce the neural plate. The edges of the neural plate gradually elevate, forming the neural groove; these changes result from continued induction of the caudal neural plate, mitotic divisions in the neuroepithelium, alterations in cell shape (the tall columnar neuroepithelial cells become short cylinders

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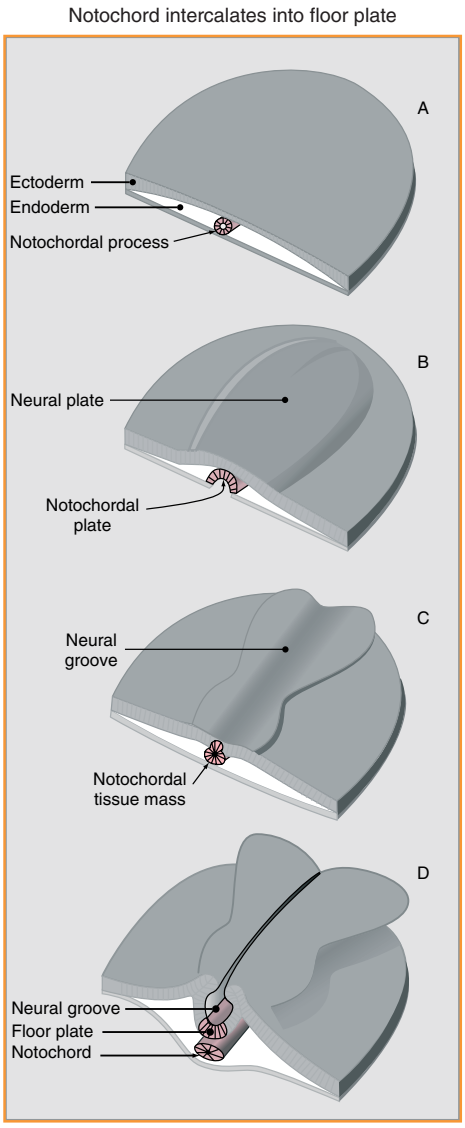

Figure 14 Sequence of diagrams that illustrates the notochordal and neuroepithelial tissues during human neurulation. Early influence of the notochordal process (shown in A) is important in differentiation of the neuroepithelium of the neural plate from the ectodermal epiblast (B). The notochordal process is intercalated into the endoderm as the notochordal plate (B), followed by its separation from the endoderm and reorganization into a tissue mass (C) that gives rise to the definitive notochord and contributes to the floor plate of the neural groove (D). (Reproduced from DeSesso *et al.*, 1999. © John Wiley & Sons, Inc.)

with wide tops) and positional rearrangements of the neuroepithelial cells.

The tall columnar neuroepithelial cells of the neural plate remain firmly attached to each other at the lumen (amniotic) surface and at the basal surface where they face the notochord and mesoderm. During the cell cycle, the nuclei of these cells move in between the mesodermal surface and the dorsal (luminal) surface. During interphase, the nucleus is located near the basal surface, but when the nucleus is ready to divide it is found at the lumenal surface. As the nucleus traverses the cell from basal to luminal surfaces, it causes the cell to bulge. Thus, the shape of the neural plate cells depends on the average location of nuclei among all of the neuroepithelial cells. For any given cell, when the nucleus is in the middle of the cell, the cell assumes a spindle shape with tapered ends; when the nucleus is at either end, the cell assumes a broad or bulbous shape at that end and it tapers towards the other end (**Figure 15**).

The process of rolling up the neural plate into a hollow neural tube is called neurulation. Generally speaking, the neural plate elevates into a 'U' shape, in part because the underlying paraxial mesodermal cells proliferate and push the overlying neuroepithelial cells upwards towards the dorsal aspect. The ridges of the 'U' continue bending towards the midline and fold over to form the neural tube. To understand this important step, a few details of this process follow. Prior to neurulation, the notochordal process is a hollow tube. As the epiblast-derived endoderm replaces the hypoblastic cells (forming the definitive embryonic endoderm), the notochordal process (found at the midline) is attached to the edges of the migrating hypoblast cells and it completes the lining over the yolk-sac cavity. The notochordal process opens up to form an arch (notochordal plate) and then attaches itself to the overlying neuroepithelium. The notochordal plate and the neural plate are connected such that the notochordal plate acts as the neural plate's anchor; differential growth and morphogenesis of the paraxial mesenchyme (which develops into blocks of tissue called somites) contribute to pushing up the two lateral edges of the neural plate while maintaining the anchored connection in the middle. This forms the neural groove (**Figure 15**). Some of the notochrodal cells are incorporated into the bottom of the neural plate and later form the floor of the neural tube. Then, the notochordal plate separates from the neural plate and forms the definitive notochord, a structure that not only generates cell signals, but also provides some rigidity and gives the embryo its axial symmetry.

During the latter part of the third and into the early part of the fourth week, the neural groove deepens with increasing elevation of the neural folds and the folds begin to fuse in the dorsal midline. This fusion process is initiated at several locations along the neural tube and fusion mechanisms vary with location (O'Rahilly and Müller, 1989). In some areas, the initial contact is at the surface ectoderm, with interdigitation of surface cells from the two sides fusing. In other areas, the surface and neural ectoderm from each side make simultaneous contact. Regardless which cells make initial contact, in the dorsal region of the fusing neural folds, the epidermal epithelium and neuroepithelium first delaminate from one

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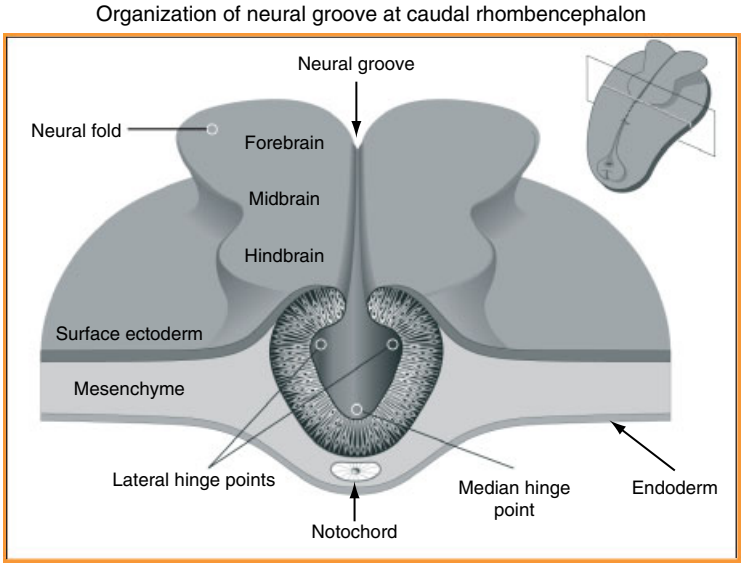

Figure 15 A cross-sectional diagram through the hindbrain of a human embryo during neurulation. There are two lateral hinge points at the region where the neural folds bend and approach each other in the midline and one median hinge in the floor plate that is associated with the notochord. Note the regional differences in position of the neuroepithelial cell nuclei; these help in cell wedging. The neural folds in areas that will form the forebrain, midbrain and hindbrain are of dramatically different sizes. Note the absence of interposing mesenchyme or neural crest cells between the surface ectoderm and the neuroepithelial cells prior to neural fold fusion in the cranial region. (Reproduced from DeSesso *et al*., 1999. © John Wiley & Sons, Inc.)

another providing a pre-existing source of each cell type used in closure (Martins-Green, 1988). The surfaces of the apposing ectodermal and neuroepithelial cells elaborate complex carbohydrates that aid in both recognition and preliminary fusion (Sadler, 1978; McLone and Knepper, 1986). In the rhombencephalon and perhaps at the neuropores, the neural tube is closed by a roof-like membrane that grows over the elevated, but still separated, neural folds (Van Allen *et al*., 1993). The diversity of cellular mechanisms used in this fusion may be one explanation for the different sensitivities of defects in various regions of the embryonic CNS (O'Rahilly and Müller, 1989).

Neural fold closure begins in the cervical region at somite 4 and progresses both rostrally and caudally. The head of the embryo is older than the tail due to the regression of the primitive streak. Because the head and tail are at different stages, the neural tube does not fold evenly along its axis. The right and left sides of the neural plate first make contact then fuse at what will be the nape of the neck (somite 4), and early fusion progresses along the somite regions. At one time, it was thought that from the initial point of contact, the tube 'zipped up' in both directions (i.e. towards the cranial and caudal neuropores), but in fact closure occurs at multiple locations and the large neuropores are only the last of several closure regions. In humans, the anterior neuropore closes 24–26 days after fertilization (20 somite stage) and the posterior neuropore closes at days 25–28 (26 somite stage); the anterior neuropore requires ~5.5 hours to close a gap of 0.3 mm compared with 27 hours to close the same size gap at the posterior neuropore (O'Rahilly and Müller, 1989).

Establishment of hinge points is one of the prominent changes in the neuroepithelium. In the cranial region, there are three such points: one in the midline (where the notochord adheres to the overlying neural plate) and two dorsolateral (**Figure 15**). In the caudal region, there is only one such point. The hinges are formed through alterations in neuroepithelial cell shape, termed wedging (**Figure 16**). The height of the cell in the median hinge decreases simultaneously with an increase in the height of cells lateral to the hinge point. The disparity in cell height accentuates the midline folding of the neural plate and results in a midline furrow. Elevation of the neural folds results in part from the configuration of cells at the hinge points, where they are narrower at their apices than at their base.

Cell wedging depends on the adherence of the neural plate to the notochord and it is related to alterations in cell-cycle length (Schoenwolf and Smith, 1990). The nuclei of the neuroepithelial cells move towards the apex for mitosis and move to the base during interphase; prolongation of the cell cycle with a longer duration of interphase results in a greater amount of time during which the nucleus is at the base and during which the cell is wedge-shaped. The prolonged cell cycle occurs at all three hinge points and in the median hinge, it is associated with juxtaposition of the neural plate and notochord.

The bending forces of cell wedging are supplied by microfilaments at the apices of the neuroepithelial cells.

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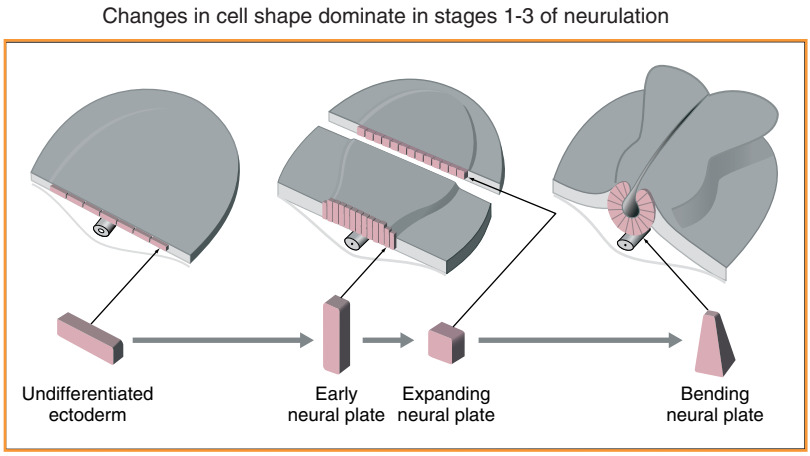

Figure 16 This sequence of diagrams illustrates the changes in shape that occur among neuroepithelial cells as human neurulation proceeds. In the diagram on the left, the undifferentiated epiblast is depicted as squamous cells that are subject to inductive signals with the adjacent notochord and mesenchyme to become the tall columnar cells of the neural plate (shown in the lower portion of the middle diagram). Subsequently, the tall columnar cells shorten themselves, thereby increasing the surface areas of their tops and bottoms and causing the neural plate to expand (upper portion of the middle diagram). With continued growth, the neuroepithelial cells exhibit asymmetric tops and bottoms; the lumenal surfaces become constricted, giving the cells the appearance of a wedge. Cellular wedging is a consequence of differential displacement of the nuclei (**Figure 15**) and it assists in formation of the neural groove (right). (Reproduced from DeSesso *et al*., 1999. © John Wiley & Sons, Inc.)

Synthesis and elaboration of the extracellular matrix, including hyaluronic acid (Schoenwolf and Fisher, 1983) and the cell adhesion molecules E- and N-cadherin (Newgreen *et al*., 1997), are import in expansion. As important as they may be, the forces of cell wedging are not the only drivers of neural fold elevation, as migration and expansion of the surface ectoderm and growth of the mesoderm beneath the neural plate all contribute to normal neural fold elevation (Schoenwolf and Smith, 1990).

The caudal part of the future spinal cord does not participate in the 'rolling up' of the neural plate (primary neurulation). This region is formed from the caudal eminence (in animals with tails this is significant, but less so in humans). Human embryos do have a tail bud that can be seen at the end of the fourth week (DeSesso, 2006) and it forms from the lower part of the pelvis (and in the tails of animals normally having one). Within the tail bud, condensation of undifferentiated mesenchymal tissue (caudal eminence) intercalates with overlying ectodermal cells to form a solid core (blastema). A lumen develops inside the blastema forming a hollow structure that fuses with the caudal end of the primary neural tube (week 6). In humans, the caudal eminence gives rise to a very small part of the neural tube incorporating (at most) spinal cord segments S4 and S5 and the coccygeal elements.

The vertebrate sensory and much of the autonomic system originate with the neural crest and neurogenic placodes. The neural crest is an ectodermal derivative that arises bilaterally along the length of the neural tube to form postganglionic autonomic motor neurons, pigment cells, some endocrine tissues and it contributes to the anterior neurocranium. As the body architecture advances, the neural crest cells migrate away as the neuroectoderm elevates and fuses to form the neural tube.

Vertebrate body organization depends upon development and differentiation of the cephalic epidermal placodes and the hypomere (the unsegmented lateral plate mesoderm found along the ventral aspect). The sensory organs of the head are derived exclusively from the placodes. The epidermal and dorsolateral placodes and the neural crest originate with the ectoderm; the dorsolateral portions of the anterior (cephalic) placodes participate in formation of the sense organs and adenohypophysis, and the ventrolateral portions give rise to the cranial sensory nerves (including the taste buds). Cells from the placodes and neural crest migrate through an extracellular mucopolysaccharide milieu; the data suggest these active organizing centres operate independently and they are derived from an ancestral protochordate. Thus, the principal difference between vertebrate chordates (like hagfish and humans) and the protochordates is the addition of a head. The unsegmented lateral plate mesoderm provides the foundation from which the musculature of the gut and heart derive; in contrast to the neural crest, placodes and special sense organs of the head, comparative data suggest that the hypomere and its skeletal, smooth and cardiac muscle derivatives evolved down an independent pathway from primitive myoepithelial cells (Gans and Northcutt, 1983).

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Commensurate with the extensive evolution of vertebrates from primitive metazoic species, numerous and complicated (albeit conserved) cell-to-cell signalling patterns and transcription control pathways came into existence. It is nothing short of remarkable that the basic framework and control systems at the level of the gene are so similar in nematode worms, arthropods and chordates. The increased complexity of higher-order animals comes with common mechanisms that activate and inactivate specific circuits and response profiles (e.g. proliferation, apoptosis, migration) that—under normal circumstances—are subject to strict, orchestrated regulatory controls in both space and time. These complex patterns of gene activation, feedback and repression are not only conserved across phyla, but are repeated over and over in embryonic construction of the more than 300 cell types found in the vertebrate body. The real differences between more primitive and more complex animals rest with the larger numbers of possible combinations, permutations and variations on the basic circuits that are conserved across apparently unrelated (and structurally diverse) animals.

Conserved within the inductive changes in embryonic tissues and spatial contortions that dictate development of a single spherical mass of cells into the segmented cylindrical body are the remarkable intracellular and intercellular regulatory circuits whose expression is repeated over and over as development progresses. These controls are choreographed and the cell signals (of which at least 17 are well described) used and the range of cell responses are common to arthropods, worms and vertebrates. Fourteen of these information-transfer pathways rely on signal activation and transduction, where an agonist combines with its transmembrane receptor, followed by conversion of multiple intermediates from inactive conformations to active conformations and these then activate various protein kinase enzymes that phosphorylate target proteins. Other information transfer pathways involve intracellular receptors (e.g. the retinoic acid family). The common embryonic signalling pathways (e.g. ANT-1, transforming growth factor β (TGFβ), Hedgehog (Hh), Notch-Delta, Cytokine and the Nuclear Receptor) and cell responses to those signals have multiple variants (e.g. the TGFβ family has at least 24 members, including the *Bmp*7, *TGIF*, *TGif2* and *Noggin*). These pathways have been conserved over diverse phyla because they 'were present and already functional in the pre-Cambrian common ancestor of those animals' (National Research Council, 2000).

Embryos use these regulatory circuits sequentially over both small (e.g. physically adjacent cells) and comparatively large (e.g. direction and extent of axon growth) distances to direct organization of whole tissues and organs. Distinct biochemical cascades generate messenger RNA (mRNA) and protein gradients (e.g. high at the anterior and low at the posterior) and these cascades control implementation of the three-dimensional (back-to-front and top-to-bottom) 'body plan'. In turn, these proteins activate positional 'gap' genes whose transcription yields 'gap' proteins that activate 'pair-rule' genes that, when transcribed, activate segment-polarity genes that, when transcribed, activate segmented homeotic or homeobox (*Hox*) genes (e.g. *Ubx* and *Antp*).

Members of the *Hox* gene family (including the Hhs sonic *Shh*, Indian *Ihh* and desert *Dhh*) share ~60 base pairs (termed the homeobox) and are expressed along stripes that correspond to both the anteroposterior body dimensions and their order on the chromosome ('colinearity'); these *Hox* gene anteroposterior patterns (stripes) are seen in *Drosophila*, frog and mouse embryos, but the patterns in mice are more numerous and more complicated than those in arthropods. During anteroposterior patterning in *Drosophila* embryos, gap proteins (also called transcription factors) activate eight pair-rule genes that are arranged in seven stripes arranged in sevenfold repeats. These eight segment-polarity genes are activated by combinations of at least two pair-rule proteins amounting to a total of 14 expression stripes. The 14 segment-polarity genes correspond to the 14 segments of the head, thorax and abdomen. Segment-polarity genes are activated only by pair-rule proteins; *Hox* genes are activated by pair-rule and segment-polarity proteins that correspond to eight domains in the head, thorax and abdomen. Following activation of *Hox* and segment-polarity genes, the pair-rule and gap genes are no longer expressed and their proteins are degraded. Dorsoventral development also employs transcription factors that in *Drosophila* originate with the Pipe protein, a transcription factor that is expressed on the future ventral part of the body and that is involved in mesodermal induction. The Pipe protein activates a series of cascades that ultimately hydrolyse the Spätzle protein to a peptide that binds the Toll transmembrane receptor found on one side of the fertilized egg. The activated transmembrane Toll receptor then activates a series of intracellular reactions that activate the Dorsal protein that, in turn, activates the *Twist* and *Snail* genes (whose transcription products activate gastrulation genes) that inhibit other genes (*Zen* and *Dpp*).

On the other side of the future torso, the inhibitory Dorsal protein is not expressed so that *Zen* and *Dpp* are expressed on the ventral side. On the lateral margins, Dorsal represses *Zen* and *Dpp* expression, yet the expression of Dorsal is not so great as to activate *Twist* and *Snail*. So it comes to be that the early body plan is comprised of three regions (dorsal, lateral and ventral) that arise as a result of expression of *Zen, Dpp* and *Sog*, the last of which encodes for a protein that diffuses into regions controlled by *Zen* and *Dpp* inhibiting their expression in regions giving rise to the future dorsal ectoderm. Interactions between *Hox* gene-expression-patterns control orientation and establishment of the neuroectoderm, neural tube (brain, spinal

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cord), somites (vertebrae), cranial neural crest (branchial arch cartilage, sensory ganglia, peripheral nerve Schwann cells, odontoblasts) and limbs in a manner akin to a central dispatching office that is under the control of a complex regulatory network, where independent gene action is discouraged and whose carefully orchestrated transcription products subsequently regulate genes within their individual regions over time and space.

Vertebrate *Shh* arises from the notochord (among the earliest recognizable features of the embryonic midline extending from the prochordal plate to the primitive node) and floorplate. *Shh* expression is required for induction of cells in the ventral neural tube, leading to development of the motor neurons, interneurons of the trigeminal nucleus, neural crest, pituitary gland, oligodendrocytes, the floor plate, the primitive foregut and the developing limb. There is a corresponding *Bmp* gradient that is highest in the dorsal roof and *Bmp* inhibitors arise from the notochord. Dorsal–ventral patterns are determined by the *Bmp* gradient and its activity is reduced by the Tsg (Twisted gastrulation or *Twsg* 1) and *Chd* secreted proteins that bind *Bmp* and prevent its signal; the signal is re-established when a metalloprotease (Tld) hydrolyses *Chd* from its complex with *Bmp* (Larrain *et al*., 2001). Together, the actions of the *Shh* and *Bmp* pathways specify the interneurons and motorneurons, and the *Tsg* and *Bmp* signals control chondrogenesis. Vertebrate *Shh* participates in establishment of the left–right body axis, beginning at the axial mesoderm and it controls CNS dorsal–ventral orientation all along the rostral–caudal plane (Roessler and Muenke, 2003). Among transgenic mice with reduced *Shh* expression, the floor plate fails to develop (Litingtung and Chiang, 2000).

The precursor *Shh* protein is a large peptide that undergoes post-translational autoprocessing to the biologically active form. Autoprocessing involves hydrolysis of the parent protein to a 45 kDa form that yields a 19 kDa amino-terminal and a 26 kDa carboxy-terminal peptide that is esterified with cholesterol molecules at the amino (*Shh*-Np) and carboxy (*Shh*-C) domains. The addition of cholesterol at the C-terminus apparently anchors *Shh* in the cell membrane. Esterification of *Shh* controls its diffusion and confers spatial restriction on the activity associated with the N-terminus. Free *Shh* binds the downstream genes, patched 1 (*Ptch1*), patched 2 (*Ptch2*) and smoothened (*Smo*), and the former inhibit *Shh* signalling in the ventral neural tube. Neural tube *Ptch* expression is upregulated by the *Shh* signal. Relatively high *Shh* and *Ptch* local concentrations are expressed close to the notochord and floorplate and their concentrations decline dorsally as distance from the notochord increases. When the biologically active form of *Shh* binds *Ptch*, the latter is inactivated and *Smo* increases; in the absence of *Shh, Ptch* inhibits *Smo* activity. *Smo* is a seven-pass (serpentine) transmembrane protein that supports *Shh*-directed patterns in the ventral neural tube and *Smo* is important because its actions release *Glis*, transcription factors that mediate *Shh* signal transduction. All three vertebrate *Glis* translocate to the nucleus where their zinc-finger domains bind and activate the promoter consensus sequence TGGGTGGTC in *Shh/Hh* target genes. Cytoplasmic *Glis* are controlled by sequestration with kinase (Fused and Fused suppressor [Su(fu)]) and kinesin-related (Cos-2) proteins to prevent accumulation of *Glis* in the nucleus. The transcriptional activator *Gli1* differs from its siblings in that its amino acid sequence outside the zinc finger is not homologous, and the N-terminal domains of *Gli2* and *Gli3* have repressor functions. The C-terminal domains have activator functions. Functional *Gli* genes are required for neural tube and interneuron development; *Gli2* and *Gli3* are expressed in the neural plate, whereas *Gli1* is found in the ventral neural tube. These genes participate in control of and gradations in *Shh* signalling in neural plate cells and they control *Hox* gene inhibition (Class I) or activation (Class II) along the dorso-ventral axis of the neural tube.

Finally, there is added complexity of the so-called 'cross-talk' between signalling pathways in early embryonic pattern formation and the existence of additional pathways that antagonize the activity of the basic pathways. For instance, the *FRzb*, *Cerberus* and *Sizzled* antagonists modulate the *Wnt* pathway and signals from the *Shh* pathway repress the activity of the Nodal *TGF β* pathways. Mice with defective *TGF β 2* genes are born with cardiac, lung, limb, and craniofacial malformations and NTDs, those with defective *Noggin* genes develop NTDs and mice with defective *Bmp7* genes develop hindlimb polydactyly and renal malformations (Dudley *et al*., 1995); mice missing one copy of *Bmp4* develop holoprosencephaly and craniofacial malformations (Zakin and De Robertis, 2004) and mice with homozygous null *Shh* genes are born with axial skeletal and limb malformations and cyclopia (Chiang *et al*., 1996).

3 PATHOGENESIS OF SELECT CONGENITAL MALFORMATIONS

There are at least 860 specific categories of structural defects (Wise *et al*., 1997) and among those some have multiple variants (Spencer, 1992). Consideration of the immense volume of literature dedicated to each necessitates restriction of the present discussion to the conditions described in the preceding section.

3.1 Conjoined Twins

Human conjoined twins are extremely rare (1 : 50 000 to 1 : 200 000 live births) and there are no reliable animal

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models for any of the many conjoined variants. There are ample reports of conjoined rodents, cats, rabbits, dogs, cattle, horses and lambs, but all of the specimens are apparently spontaneous and unrelated to any known xenobiotic or physical factor(s) (Willhite *et al*., 1985). Conjoined twins are always joined along the body axis (head-to-head, tail-to-tail, front-to-front, rump-to-rump or side-to-side) sharing the same anterior–posterior orientation. There are no accounts of twins having been joined solely by their limbs or with posterior structures of one joined with the anterior of the second or the dorsal side of one joined with the ventral of the other.

It has long been thought that conjoined twins result from incomplete separation ('fission') of the embryonic inner cell mass (Moore, 1977). More recent descriptions suggest that reunion ('fusion') of two separate embryos may occur, sometimes leaving only vestiges of one (Spencer, 1992). Parallel duplication of the notochord leads to parapagus twins. Parasitic rachipagus (fused double spinal columns) have been attributed to the dorsal union of two identical embryos 'having occupied directly opposite aspects of a single amniotic cavity and the union having occurred in the open neural folds before they united to close the neural tube' (Spencer *et al*., 1996).

3.2 Cyclopia

It is during the third and fourth week of gestation (often before a mother knows she is pregnant) that the human embryo is but a very small flat disc of cells and becomes organized into three layers: the dorsal-most ectoderm, the ventral-most endoderm and the intra-embryonic mesoderm. By this time, cells on the dorsal surface migrate from the early primitive streak towards the future head. By the end of the first month of human pregnancy, the anterior neural folds have completed their elevation and fusion. In short order, this single tube continues to grow and expand and three primary swellings (termed vesicles) appear: the forebrain (prosencephalon), the midbrain (mesencephalon) and hindbrain (rhombencephalon). By five weeks, the prosencephalon grows into two distinct regions: the anterior telencephalon (end brain) with two lateral bulges (primitive cerebral hemispheres) and the diencephalon (destined to give rise to the optic vesicles).

Varying degrees of failure in division of the prosencephalon into proper cerebral hemispheres account for the wide range of conditions known collectively as holoprosencephaly. In its most striking form, there is no recognizable forebrain, a single optic vesicle develops as an outgrowth and the face is severely distorted (**Figure 5**). Holoprosencephalic malformations have been associated with abnormal function of several gene families (e.g. *Six3*, *Zic2*, *TGIF*) (Wallis *et al*., 1999; Brown *et al*., 1998; Muenke and Beachy, 2000), but the most firmly established mode of action concerns interference with the mitogenic, inductive and survival functions of the *Hh* protein family (Belloni *et al*., 1996; Roessler and Muenke, 2003). Of the three *Hh* factors (Sonic, Indian, Desert), prechordal plate *Shh* has been most thoroughly studied and its proper function is required for induction of the ventral brain and spinal cord cells (Nanni *et al*., 1999).

Mutational analyses of 30 families having members with familial or sporadic holoprosencephaly revealed at least five mutations in the *Shh* exon 3 (Roessler *et al*., 1997). In the first multigenerational family, there was a GTG → GAG sequence change in the *Hh* gene that resulted in a single invariant amino acid substitution (Val224 → Glu224). All of the members of this family who displayed signs of holoprosencephaly exhibited this genotype and none of the normal members had this mutation. In the second family, the affected individuals and an unaffected father (who was a carrier of a GCG → ACG mutation) had an Ala226 → Thr226 substitution in the C-terminus of *Hh*. A 21 base-pair deletion was detected in two members of another affected family and this deletion segregated as an autosomal dominant; this deletion resulted in the loss of seven amino acids in an *Hh* processing protein. In yet another family, a GAG → TAG mutation (also linked to chromosome 7q36) resulted in premature termination of *Shh* at position 284 and in still another, there was a GCG → ACG substitution that coded for a Thr → Ala change at position 384. Reduced *Shh* activity in human ventral brain results in failure of *Shh* target gene regulation. Mis-sense mutations in *Shh* result in conformational changes that reduce *in situ* activity to the point that the downstream genes that normally respond to the *Shh* signal are not activated (Schell-Apacik *et al*., 2003). Relative failure to activate the downstream members of the *Shh*-induced cascade disrupts pattern formation in the midline brain and these lead to various degrees of failure of prosencephalon separation into the primitive cerebral hemispheres, leading to failures in pattern formation within the diencephalon. Based on these observations, Roessler *et al*. (1997) predicted that about 23% of familial and 0.005% of sporadic holoprosencephalics were associated with haploinsufficiency for the human *Shh* gene. Follow-up studies found that 37% of families with holoprosencephalic members had *Shh* mutations (Nanni *et al*., 1999), that mutations in *Ptch1* reduced the *Shh* signal (Ming *et al*., 2002) and that deletion of or single nucleotide polymorphisms in the *TGIF* gene (a 272 polypeptide homeodomain transcriptional repressor) that misfold the protein were also seen in human holoprosencephalics (El-Jaick *et al*., 2007).

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When the teratogenic activity of cyclopamine and its congeners was discovered, it was hypothesized (due to its structural resemblance to steroid hormones) that these compounds could compete with endogenous steroids and interfere with protein synthesis (Brown and Keeler, 1978). Although cyclopamine shares structural features with cholesterol (**Figure 6**), cyclopamine actions involve neither endogenous steroids, cholesterol metabolism nor intracellular transport (Incardona *et al*., 2000). Cyclopamine interferes with activation of the *Shh* pathway (Cooper *et al*., 1998; Incardona *et al*., 1998) by inhibiting *Shh* signal transduction in a concentration-dependent manner (Incardona *et al*., 1998). It does so not by interference with *Shh* or *Smo* biogenesis or processing (Incardona and Roelink, 2000), but as a result of binding at the *Smo* heptahelical domain to induce a conformation shift in that protein. The conformational shift induced by cyclopamine binding is similar to that induced by *Ptch* that places the membrane protein *Smo* into an inactive state and inhibits *Shh* signal transduction (Chen *et al*., 2002).

Confirmation of the *in vivo* actions of cyclopamine and the temporal nature of *Shh* pathway inhibition come from the studies of Cordero *et al*. (2004) who found that *Hh* blockade prior to *Shh* expression in the prosencephalon produced a single fused telencephalic vesicle in the chick embryo. Blockade of the *Hh* pathway with cyclopamine after *Shh* was expressed in the prosencephalon produced malformations of the face without forebrain defects; thus, Cordero *et al*. (2004) were able to reproduce the full range of variable midline defects of the brain and face that are characteristic of holoprosencephaly and demonstrated that pathogenesis of holoprosencephalic defects depends upon disruption of the *Hh* control pathway at different stages.

3.3 Neural Tube Defects

Although it has been assumed that anencephaly and spina bifida represent failure of closure of the anterior and posterior neuropores, respectively, clinical evaluations suggest that this concept is incorrect (Murakami, 1968; Warkany, 1971; Lemire *et al*., 1978; Golden and Chernoff, 1995). It appears more likely that human NTDs correspond to failure of neural tube closure in larger regions, rather than to only the much smaller neuropores. Extensive failure of closure at the neuropore and down towards somite 4 results in cranioschisis.

Due to the intricate timing and complex tissue interations during neurulation, the process is prone to error. Interruption of any of the aforementioned steps can precipitate faulty development. NTDs are multifactorial in origin and their cause has both genetic and environmental components (Fraser, 1976). Several lines of evidence support this view (DeSesso *et al*., 1999):

- The incidence of NTDs varies greatly among ethnic groups, suggesting genetics plays a role (Copp and Bernfield, 1994).
- The incidence displays a familial pattern among first-, second- and third-degree relatives (Carter, 1974; Copp and Bernfield, 1994).
- Cranial NTDs occur two to three times more frequently among females than males (Carter, 1974; Seller, 1987).
- Increased NTD risk is associated with defects in the gene for 5,10-methylenetetrahydrofolate reductase (*MTHFR*) (van der Put *et al*., 1995; Whitehead *et al*., 1995; Ou *et al*., 1996).

Emery (1986) argued that the human NTD data infer a 60% heritability. Genes other than *MTHFR* implicated as causal factors in human NTDs include those for folate receptor-α (Finnell *et al*., 1998; Trembath *et al*., 1999), *PAX3* (termed *Hup2* in humans) (McLone, 1998; Trembath *et al*., 1999) and others (Fleming *et al*., 1997) that are indispensable for neural tube closure.

Investigations with mutant mice (Copp and Bernfield, 1994; Spörle *et al*., 1996; Smith and Schoenwolf, 1997) led to a catalogue of spontaneous mutations and knockouts that induce NTDs (Harris and Juriloff, 1997). Given the similarities in chordate neurulation, there is optimism that identification of molecular entities that mediate normal and abnormal neurulation in mice (Goulding and Paquette, 1994; Lumsden and Krumlauf, 1996; Tanabe and Jessell, 1996; Fleming *et al*., 1997) will yield information of clinical relevance.

A great many agents (e.g. inorganic arsenic, trypan blue) have been used to study mechanisms responsible for the pathogenesis of NTDs (DeSesso *et al*., 1998; Morrissey and Mottet, 1980). In fact, any material that interferes with one of more of the numerous developmental processes involved in neurulation *if given at an effective delivered dose at the specific time of development when neurulation occurs* will likely elicit an NTD. Thus, the fact that many substances can induce NTDs in laboratory animals should come as no surprise. However, the prevalence of NTDs and their multifactorial nature precludes one from inferring that the mere existence of the defect must be the result of exposure to a particular material or agent.

3.4 Sirens

By the beginning of the fifth week of normal gestation, the two posterior limb buds become evident as a somatic mesodermal core and overlying ectoderm. The initial factors responsible for the siren remain largely unknown, primarily because the condition is so rare and its presentation is so variable. In some cases, there is complete absence of the caudal vertebrae, urinary

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tract, bladder and hindgut, but others present with a patent large intestine, urinary tract and traces of sacrococcygeal vertebrae. Genetic and xenobiotic manipulations in rodent and chick embryos (e.g. inorganic Pb, amphetamines, trypan blue) can induce caudal defects (e.g. haematoma, regression and dysplasia) in some cases, but until recently there was no reliable animal model of the mammalian siren. From studies at foetal autopsy, at least three explanations for merging, malrotation and defects of the lower limbs have come forward and these have provided some insight into pathogenesis of the siren.

Ordinarily, an early vascular plexus is present around the distal primitive streak and blood channels can be seen even before the somites are evident. Subsequently, the secondary yolk sac supplies embryonic nutrients through the developing embryonic cardiovascular system. These transient vitelline veins are continuous with a web of diffuse blood vessels that surround the yolk sac. Kampmeier (1927) advanced the hypothesis that 'vascular steal' developed and this resulted in a single umbilical artery that arose directly from the abdominal aorta. In this version, merging of the lower limbs follows transfer of blood from the caudal regions of the embryo through a single 'aortic-umbilical artery' (Stevenson *et al*., 1986). This hypothesis holds that reduced vascular perfusion results in reduced angiogenesis leading to disruption of the caudal mesoderm. Alternative hypotheses held that death and complete resorption of one of two monozygotic twins leads to sirenomelia (Kapur *et al*., 1991), that lateral compression of the amniotic folds was responsible or that induction of a localized caudal haematoma between days 13 and 22 was responsible (Barr, 1988). Chandebois and Brunet (1988) observed that a 'factor may stop the migration of the caudal mesodermal cells at the end of the primitive-streak stage'.

Sirens arise from abnormalities induced during establishment of the three germ layers (gastrulation) and prior to the appearance the body form (prior to the end of the third week of human development). Histologic examination of compound mutant $Tsg^{+/-}$; $Bmp7^{-/-}$ mouse embryos destined to become sirens found only a single abnormal umbilical artery arising directly from the dorsal aorta (Zakin *et al*., 2005). At this stage, the lateral plate mesoderm of control embryos was thickened in preparation for growth of the right and left hindlimb buds, but in the mutants, there was only a single ventral mesodermal thickening in the midline and the tailbud mesoderm was sparse. Reductions in the numbers of ventroposterior mesodermal cells due to reduced *Bmp7* signalling results in early fusion of the hindlimb buds and coelomic cavities. Cells of the posterior primitive streak were apparently specified correctly, but cells in the affected ventral posterior mesoderm were 'unable to either proliferate or survive' and the mutant hindlimb buds were fused as early as day 9.5; endodermal *Shh* expression in these mutants was reduced and corresponded to the structural reductions in the hindgut and genitourinary tract (Zakin *et al*., 2005).

4 EVALUATION OF ANIMAL DATA AND HEALTH RISK ASSESSMENT

Developmental toxicity studies ascertain whether a test agent administered to a pregnant mammal adversely affects her offspring. Although data from the mother are collected throughout the study and analysed as part of the assessment, four major end points of developmental toxicity are assessed in the offspring. These end points are: death, structural abnormalities (congenital malformations), altered growth and functional deficits (Harris and DeSesso, 1994). A biologically significant increase in the frequency of any of the four end points indicates the test agent disrupts embryonic development and that sufficiently high exposures may pose a potential developmental hazard for human beings. Standard developmental toxicity tests examine the possibility that a test substance may produce any of the first three manifestations of developmental toxicity. In the past, functional deficits were evaluated infrequently in routine testing, but protocols that include developmental landmarks, motor activity, startle response, a functional observational battery, routine histopathology, *in situ* brain perfusion and brain morphometry are now standard (United States Environmental Protection Agency (US EPA), 1998).

4.1 Testing Procedures

A conventional developmental toxicity assessment begins with a preliminary dose-range-finding or pilot study followed by the definitive toxicity study. The dose-range-finding study determines the exposures to be used in the definitive study. To do this, the range-finding study uses more dose levels with fewer animals per group than the definitive study. A typical range-finding study employs four to six dose groups of 8–10 pregnant animals (usually rats, mice and rabbits) each. A successful range-finding study identifies an exposure that causes minimal maternal toxicity (which will be the highest dose in the definitive study) and an exposure that elicits no adverse effects in the offspring (which will be the lowest dose in the definitive study). In-life maternal data collection requirements in the pilot study are identical to those of the definitive study, but pilot data collected at term are usually limited to gross examination and measures of foetal body weight. The definitive study determines whether or not a test material causes adverse effects in animals and if so, determines a no observable adverse effects level (NOAEL).

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4.2 Experimental Design

4.2.1 Route of Exposure

All regulatory agencies recommend that the route of test material administration should be the route by which humans are exposed (Health Canada, 1975; World Health Organization (WHO), 1984; US Environmental Protection Agency (USEPA), 1991a). In these studies, the route of exposure is usually oral (see **Peroral Toxicity**) unless the chemical or physical characteristics (e.g. vapour) (see **Inhalation Toxicity**) indicate a more relevant route of administration.

4.2.2 Dose Selection

The doses in the definitive developmental toxicity study are based on the results of the pilot study. Except when the biological, physical or chemical properties of the test material limit exposure, the highest dose should elicit signs of maternal toxicity (e.g. statistically significant reductions in maternal body weight or body-weight gain). If more than 10% of the treated dams die, the dose is too high. Doses that produce excessive (but nonlethal) maternal toxicity (e.g. convulsions) may also produce increased prenatal death and resorption (rodents) or increased spontaneous abortions (e.g. rabbits). Studies that experience excessive maternal mortality or prenatal loss have limited utility in determining health risk and usually must be repeated. Ideally, the range of doses in these studies should be set such that the highest exposure causes mild signs of maternal toxicity and the lowest dose elicits no adverse effects on the mother or her offspring.

4.2.3 Treatment Groups

The number of treatment groups should be sufficient to establish a dose–response relationship. At a minimum, three groups treated at different dose levels and a concurrent (usually the vehicle in oral studies) control group are required by US and international regulatory agencies. The numbers of animals per group must be large enough to provide sufficient statistical power for meaningful analyses. Most regulatory guidance suggests 20 pregnant rodents and 12–16 pregnant rabbits per group. For changes in body weight to be useful as a valid indicator of potential toxicity, the animals must be assigned randomly to treatment groups in a way that ensures that all dose groups (including the control) start with similar mean maternal body weights and variances. Although regulatory agencies do not often require a reference teratogen ('positive control'), one should be included in the study design for verification of laboratory proficiency.

4.2.4 Exposure Period

Developmental toxicity protocols require time-mating of healthy animals (Kimmel and Price, 1990; Organization for Economic Cooperation and Development (OECD), 1981; 2001; US Environmental Protection Agency (USEPA), 1985; US Food and Drug Administration (USFDA), 1996; 2000). Gestational day (GD) zero (0), the usual reference point for the beginning of gestation, is identified in one of the following ways, depending on the test species and method of breeding. GD 0 is: (i) the day the vaginal plug is observed (rats or mice) or (ii) the day sperm are found in vaginal lavage (rats) or (iii) the day mating was observed (rabbits) or (iv) the day artificial insemination was performed (rabbits). Exposure of the inseminated animals begins after implantation and continues throughout major organogenesis until closure of the hard palate and development of the genitalia (days 6–17 or 18 for rats and mice, 7–19 or 20 for rabbits and 5–14 for hamsters). When the test agent is not incorporated into drinking water or feed and the material is administered by gastric intubation, topical application or inhalation, dosing should be performed at the same time each day with no longer than two hours elapsing between treating of the first and last animals. The timing of exposure is critical because embryological schedules operate during narrow periods of time (DeSesso, 2006). This is especially true in rodents (e.g. hamsters) where gestational stages are compressed.

Current designs incorporate an extended exposure period compared to protocols outlined in historical regulatory guidance. Some designs continue treatment of pregnant animals from the beginning of organogenesis until Cesarean section near term. Such studies can find growth retardation (usually characterized by reduced foetal body weights) that may be missed in studies of short exposure durations. Extended dosing periods allow more thorough evaluation of organs like the heart, brain, lungs and gonads that undergo functional and morphological maturation after the end of major organogenesis. Alternatively, single-exposure studies that target a uniquely sensitive period of development (e.g. early primitive streak) can elicit frank congenital malformations, whereas increased duration of exposure results in higher absorbed doses and increased prenatal death. Thus, the route, frequency and duration of the exposure period may affect the results of a study, and those parameters must be stated carefully when interpreting the study and when comparing different studies.

4.2.5 Study Termination

Pregnant animals should be scheduled for sacrifice and Cesarean section just prior to delivery in order to prevent cannibalization of defective young. Study termination is usually scheduled for GD 21 in rats, GD 18 or 19 in mice and GD 29 in rabbits.

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4.2.6 In-Life Procedures and Data

Humane husbandry practices (National Institutes of Health (NIH), 1985; US Department of Agriculture (USDA), 1970; US Department of Agriculture (USDA), 1990) are required at all times (see **Animal Welfare in the Toxicology Laboratory**). Animals must be observed at least once daily for mortality, morbidity and clinical signs. This is usually accomplished at the same time as body weight is measured. Additional daily observations (shortly after exposures) may be necessary, especially if the test article is acutely toxic to the dam.

4.2.7 Maternal Deaths and Abortions

Pregnant animals can die or abort due to conditions unrelated to the test agent. Such conditions include disease, technical errors during dosing and environmental factors (e.g. hyperthermia). If a pregnant animal dies on test, the necropsy records should be inspected to ascertain the probable cause. For instance, a study wherein the dams were gavaged with an oil vehicle and the deceased exhibited inflamed (red) tracheal lining, pulmonary congestion, nasal discharge and pulmonary oedema, then the most likely cause of death was accidental intratracheal intubation or respiratory disease. Another example is that of a pregnant rabbit whose clinical signs include alopecia, anorexia and diarrhoea; these signs are consistent with a hairball lodged in the stomach (a common occurrence in rabbits). Therefore, any maternal deaths should be investigated, because they may not be associated with the test material, but may be the result of a spontaneous or iatrogenic episode unrelated to the toxicity of the test agent.

Abortions and total litter resorptions may also result from events unrelated to the toxicity of the test material (Chernoff *et al*., 1987; Schardein, 1987). Excessive noise, deviations in the light–dark cycles and rough handling can induce abortions in rabbits. While total litter resorption does not usually occur in rabbits, this can occur in rodents placed under stress.

4.2.8 Maternal Body Weights and Body Weight Gain

In the best designs, the pregnant animals are weighed daily. Alternatively, dams should be weighed according to the following schedule: GD0, GD5, each day of the exposure period; at three to five day intervals during the postdosing period and at study termination. Maternal body-weight gain during specific segments of gestation, especially during the exposure period, is generally a more sensitive indicator of maternal adverse effects than either the mean body weight at study termination or the mean body weight gain over the entire gestational period. The increased sensitivity of incremental body-weight changes is due to their easy detection and the likelihood that those changes will not be masked by a 'rebound' weight gain that can take place after exposure has ceased.

4.2.9 Clinical Signs

Clinical observations are significant qualitative indicators of toxicity. The pilot (range-finding) and other toxicological studies afford an opportunity to confirm clinical signs (e.g. tremors, hunched posture, dyspnoea) induced by exposure to the test agent. Clinical signs and body-weight reductions are the most reliable indicators of maternal toxicity. In many cases, clinical signs are more sensitive indicators of maternal intoxication than changes in maternal body weight.

Documentation of clinical signs includes identity of the observed effect, its time of onset, intensity and duration. Examples of important clinical observations include diarrhoea, excess salivation, discharges from the eyes or nose, hair loss (alopecia), listlessness and convulsions. Likewise, changes in respiratory rate, alertness, mucous membrane colour, urinary frequency and the colour of the urine should be recorded.

Behavioural observations (sometimes described as social interest, passive-avoidance, anxiety, aggression, time spent rearing, feeding or nest building) reported during daily observations are not as objective or quantifiable as clinical signs. These observations are frequently within the range of normal animal behaviour and are not necessarily treatment-related. While changes in behaviour should be recorded, it is not possible to predict whether behavioural changes are early signs of subclinical toxicity that could be manifest as objective clinical signs at higher doses unless a clear dose–response relationship can be demonstrated.

4.2.10 Food and Water Consumption

When the test material is administered in the feed or drinking water, food and water consumption must be measured daily in order to calculate the administered dose. Food and water consumption should also be monitored when the test material is suspected of causing palatability problems due to objectionable taste or odour or causing appetite or excretory effects. Altered food and water consumption after the start of test article exposure are end points that can be used to assess maternal changes, including body weight.

4.2.11 Necropsy Procedures and Data

Detailed descriptions of methodologies for examining offspring are available (Wilson, 1973; Manson and Kang, 1989; Tyl and Marr, 2006). Briefly, the mother is humanely killed and her gravid uterus, with the ovaries intact, is surgically excised and weighed. The corpora lutea on each ovary are counted and recorded for both rats and rabbits; however, in mice, due to the difficulty of differentiating luteal tissue from surrounding ovary, this is not necessary. The uterine contents are examined by incising the uterus along the antimesometrial border

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to open the lumen. The numbers and locations of implantation sites, resorptions, viable and dead foetuses are recorded. Viable foetuses are removed from the uterus, dissected free of their placentae and foetal membranes and weighed. They are then examined to ascertain their sex and evaluated for the presence of external malformations. Pilot studies require no further study of the offspring, but in the definitive study, one half of the viable foetuses are examined for internal (visceral) malformations by either dissection (Manson and Kang, 1989; Staples, 1974; Stuckhardt and Poppe, 1984) or by the freehand razor blade technique (Wilson, 1965). Skeletal structures are visualized in the remainder by clearing then staining with dyes that are specific for bone (alizarin red S; Staples and Schnell, 1968) or bone and cartilage (alizarin red S and alcian blue; Inouye, 1976). An alternative method subjects all foetuses to fresh, visceral dissections of the thorax and abdomen, after which either all foetuses are eviscerated and prepared for skeletal staining or one half are decapitated and the heads fixed in Bouin's solution for examination of the brain and skull by freehand razor sectioning.

4.3 Maternal Data

4.3.1 Confirmation of Pregnancy

When the uterus is examined at study termination, the presence of a conceptus and/or resorption sites is considered evidence of pregnancy. The pregnancy (conception) index for each treatment group can be calculated by dividing the number of confirmed pregnancies in that group by the number of females mated. The pregnancy index is used in the assessment of reproductive performance. Depression of this index relative to that of the control may indicate a possible effect, but *only* if treatment began *prior to mating and implantation*. Since treatment usually begins after implantation is complete, a low pregnancy index suggests maternal health was compromised (perhaps due to poor husbandry) or that the time for initial dosing was miscalculated (i.e. exposure began too early).

Pregnancy indices in timed-pregnant animals (especially mice) that are shipped from commercial suppliers are generally lower than those of animals bred in-house. If there is a significant difference in pregnancy indices among groups, the study may need to be repeated.

4.3.2 Number of Corpora Lutea

Corpora lutea are yellow sites (spots) that remain on the exterior surface of the ovary after the egg cells are expelled during ovulation. Since each corpus luteum contained a single ovum, the number of corpora lutea equals the number of ova that were available for fertilization. The number of implantation sites cannot exceed the number of corpora lutea in a given female. In contrast, when the corpora lutea outnumber the total number of implantations, (i.e. the number of foetuses plus resorption sites), this is evidence the litter experienced pre-implantation loss. Increased pre-implantation loss relative to control values in a standard developmental toxicity study *cannot* be a consequence of treatment because embryonic death occurred *prior* to initial exposure. Thus, the data must be audited to ensure that: (i) dosing did not begin prematurely (i.e. prior to completion of implantation) and (ii) there is no evidence of environmental stress (e.g. hyperthermia) in the animal facility.

4.3.3 Gravid Uterine Weight and Corrected Maternal Body Weight

The pregnant uterus and its contents are removed by transecting the vagina just inferior to the union of the uterine horns and cutting the mesentery that connects the uterine horns to the body wall. The weight of these organs is informative when individual animals bear fewer foetuses per litter than other animals having many foetuses per litter. If a control animal has many more pups than is typically expected in a litter, the foetal body weight within that litter is likely to be less than the usual mean foetal body weight; conversely, in litters with only very few (e.g. two or three) foetuses without a concurrent high number of resorptions, the average foetal body weight is frequently much greater than the norm. While pup weights in the two preceding examples may not be significantly different, often the gravid uterine weights are not. The gravid uterine weight provides a measure of the body weight gain of the pregnant female, regardless of how many foetuses she had in her litter.

Corrected maternal body weight is the terminal body weight of the dam at sacrifice minus the gravid uterine weight. Comparison of this measure among groups allows determination of whether test-compound-induced changes in body weight were caused primarily by toxicity in the dam or in the foetal-placental unit.

4.3.4 Numbers of Implantations, Resorptions, Living and Dead Foetuses

In rodents, prenatal mortality can be identified by resorbed embryos or dead foetuses ('foetal wastage'). Rabbits either resorb or abort their litters. Together, these findings are considered postimplantation loss. Foetal wastage can result from direct toxicity of the test substance, lethal malformations (whether spontaneous or treatment-related), maternal toxicity and/or environmental stress. When there is a positive dose–response relationship, conclusions regarding developmental toxicity have increased confidence. When one type of postimplantation loss predominates, it may be possible to deduce

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the time or gestational stage at which the exposure adversely affected the conceptus.

The number of live foetuses is recorded. This end point, when presented as the percentage of implantations per litter, provides a measure of an agent's ability to kill the embryo or foetus.

4.3.5 Organ Weights and Clinical Chemistry

Current regulatory guidelines do not address maternal organ weights and clinical chemistry data; however, dose-related changes in absolute and relative maternal organ weights (absolute organ weight divided by the corrected maternal body weight) are valuable in assessing potential maternal toxicity. For instance, the maternal liver may show increased weight and this can be a function of enzyme induction, fatty degeneration or hydropic change. Taken in concert with toxicokinetic data, this information can be used to identify the extent to which exposure to the test material adversely influenced maternal well-being and that determination is as important as assessing the potential developmental toxicity of the substance under study.

In some instances, clinical-chemistry parameters (e.g. haematology and enzyme markers) are reported (see **Haematology and Toxicology**). Changes in blood cells or changes in hepatic and other enzymes (see **Clinical Chemistry in Toxicity Testing: Scope and Methods**) can be useful in assessment of maternal health, especially in the absence of changes in gross measures like food consumption or body weight.

4.3.6 Foetal Data

Data must be collected and reported for all pups in the litter; reporting observations for only one, two or three members as representative of a whole litter is deceptive. Foetal body weight is a sensitive end point in developmental toxicity studies. Because the foetal weights vary within the litter, the parameter calculated is the mean foetal body weight expressed on a litter basis. The mean foetal body weight for each litter is determined, then a mean value of the litter means (sometimes called a grand mean) for each treatment group is calculated. This approach helps stabilize the variance and accounts for the impact of the maternal system on her offspring. Significantly reduced mean foetal body weights in treated groups compared to control values provide evidence for growth retardation. When the mean number of foetuses per litter is similar across treated and control groups, dose-related reductions in mean foetal body weight indicate treatment-related toxicity in the offspring. Sometimes, reduced foetal body weight is the only sign of developmental toxicity. When evaluating those data, two points must be considered; first, the mean number of pups per litter should be comparable because foetuses from litters with very few siblings tend to be heavier and second, the similarity of sex distribution within groups must be verified as males can weigh more than females and a disproportionate number of males/females may skew the findings.

The consequences of reduced foetal body weight in common laboratory animals are not known. When reduced foetal body weights are the only observation in a study, the interpretation is not necessarily clear-cut, as modest reductions in neonatal body weights are temporary. In reproduction studies, where the offspring are kept with the dams through weaning, those that were smaller at birth often increase their weight, size and maturation to the point that any appreciable differences between the control and treated groups are abolished. The outcome of differences in foetal body weights cannot be determined from a developmental toxicity study, but must be gleaned from growth and viability data from multigeneration reproduction studies.

Extremely small offspring present a different problem. If the body weight of a given foetus is two or more standard deviations less than the mean control foetal body weight (or 25–30% less than the historical mean control), the foetus is considered a 'runt' or 'stunted' and some laboratories classify these as malformed.

4.3.7 Foetal Examinations

Viable foetuses are examined for external, soft-tissue and skeletal alterations and all morphological differences from the normal configuration should be recorded. These differences are categorized as malformations, anomalies, variations, deviations or retardations (Palmer, 1977; Khera, 1981). Malformations are major anatomical changes that interfere with viability, health, vigour or quality of life. Exencephaly, cleft palate, absence of limbs or digits and cyclopia are examples of malformations (Wise *et al*., 1997). Anomalies include comparatively minor changes like the absence of nails and pliable carpal flexures in rabbits. Anatomical variations are common changes, like asymmetric sternebrae or bifurcated gall bladders in rabbits, that are found in control animals and that have little or no impact on health. Classifying a foetal change as a deviation, retardation or variation depends on the competence of the laboratory and a difference in judgments can lead to disagreements.

At study termination, the gravid uterus and foetuses must be handled with care. Poor laboratory practice can cause subcutaneous haemorrhages that have been mistaken for treatment-related effects. The gravid uterus and foetuses should be removed promptly from the dam and examined, as tissues left unattended become oedematous, foetuses develop carpal flexures and hyperextended or stiff joints, changes that have been mistaken for arthrogryposis. If the umbilical cord is cut carelessly, back flow of blood from the placenta can induce what appears to be a haemorrhage into the liver and abdominal cavity.

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Skeletal changes are diverse, even among control foetuses; for example, the presence of 12 or 13 pairs of ribs in rabbits and reduced ossification at sternebrae 5 in both rabbits and rodents are common. Minor alterations in skeletal patterns are called variations and these are due to transient delays in foetal maturation. These include reduced calcium deposition in phalanges and sternebrae, supernumerary ribs and wavy ribs (both of which can resolve during postnatal growth). Supernumerary ribs have been seen in rodents, in foetuses from dams that exhibited nonspecific maternal toxicity (Khera, 1985; 1987), and while these are not considered adverse findings, they can be indicative of maternal stress. To determine whether a significant, dose-related increase in variations relative to the concurrent control is treatment-related, the results are compared to historical control values. As rodent foetuses undergo rapid growth just prior to term, foetuses removed too early (e.g. GD19) show retarded ossification of the skeleton. Thus, mistakes in determination of conception or failures in timing can result in spurious conclusions.

Visceral (soft tissue) changes are not so easily observed as are those of the external body and skeleton. Ventricular septal defects, transposition of the great vessels, hydrocephalus, unilateral or bilateral renal agenesis, polycystic kidney and diaphragmatic hernia are examples of visceral changes that can only be observed upon foetal dissection. Knowledge of normal foetal anatomy is essential; in some instances, the transparent membranous region of the diaphragm has been mistaken as evidence of teratogenicity. The presence of a ventral pancreas or retroesophageal subclavian artery is considered a malformation in rats, but in rabbits these are normal. In rabbits, a bifurcated or duplicated gall bladder is normal because these result from slight shifts in the branching pattern of the hepatic diverticulum. Foetal dissections should be made with care so as not to damage the organs; jagged edges on any organ suggest poor laboratory technique.

4.4 Animal Data Interpretation

4.4.1 Maternal Toxicity

Since the highest dose in the study was designed to elicit mild maternal toxicity, it should be expected that the in-life phase of the definitive study will include signs of maternal toxicity. This requirement has led to several thoughtful papers on the interaction between maternal health and the health of her offspring (Khera, 1981; 1984; DeSesso, 1987; Daston, 1994; Hood and Miller, 2006). Animals in the high-dose group may experience death, increased abortions and/or resorptions, especially in rabbits. When exposures are too high and deaths and litter resorptions too great, there will be too few offspring for evaluation. Ideally, maternal signs will be manifest only at the highest dose, but when the dose–response relationship is steep, the difference between a dose that causes death and that which elicits foetotoxicity can be very small.

In most rodents, maternal body weight and weight gain are end points that can determine the maternal NOAEL. Reduced mean maternal body weights are usually the result of systemic poisoning or reduced food consumption. Rabbits are different in this respect in that they often lose weight during a normal pregnancy. When rodent body weights at the low dose are reduced, but that of the high-dose animals is no different from the concurrent control, this is usually a consequence of excessive maternal mortality at the high dose. Maternal body weight is less useful in determination of a treatment-related effect if a high proportion of the litters are dead.

Another parameter for assessment of maternal well-being is food consumption. In studies where the test material is incorporated into the feed, one must determine whether reduced food consumption is related to palatability or to systemic poisoning. To do this, a feed efficiency index (FEI; grams of food consumed/grams body weight gain) is calculated; if the FEI values for treated and control groups are similar, this suggests systemic toxicity was related to the body-weight reduction. Some rabbits reduce their food consumption just prior to parturition while they nest; abdominal alopecia can arise as they use their hair in nest construction.

4.4.2 Developmental Toxicity

Exposure to a test material is considered a potential developmental hazard when an increase in any of the adverse parameters compared to the spontaneous ('background') incidence can be demonstrated. The background incidence can be estimated from both the concurrent and historical control. When the end point is a frank congenital malformation, the agent can be considered a suspect teratogen.

Interpretation of the data can be confounded, since foetuses within a given litter exhibit similar changes, thereby increasing the apparent response. This tendency is called the 'litter effect' and it is thought to be due to the fact that all foetuses within a litter are exposed to the same maternal environment as their siblings, whereas the maternal environment varies from litter to litter within the same dose group. This leads to an important caveat for the interpretation of the results: due to the large numbers of offspring evaluated and the very large numbers of observations made per foetus, the findings may achieve statistical significance if the foetus is considered the sampling unit. Several statistical procedures (see **Statistics for Toxicology**) have been developed to address this problem (Weil, 1970; Gaylor, 1978; Gad and Weil,

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1986). The appropriate experimental unit is the number of treated females (US Environmental Protection Agency (USEPA), 1991a). To address the variation in foetuses per litter and the 'litter effect', these statistical procedures analyse incidence per litter.

Statistical analyses alone are *not* sufficient to judge whether or not an agent should be considered a developmental hazard. When the level of statistical significance is $p \leq 0.05$, one of every 20 comparisons will be significant due to chance alone. Because so many observations (e.g. all skeletal and visceral elements for 300 or more foetuses per dose) are made, one or more observations are likely to achieve statistical significance by chance.

One of the most important considerations is dose–response. A positive dose–response is strong evidence for causality and positive dose-related trends without attainment of statistical significance can be important. To illustrate, a rare malformation like spina bifida may occur at a low (but dose-related) rate, yet still be no different than the control. To understand the observation, the historical control rate for that condition in that species and strain must be consulted (see below).

Another aspect is that the end points may be related to each other such that the presence of one precludes another. For instance, embryonic death precludes finding growth retardation, malformation or functional deficit, and it reduces the numbers of potentially affected offspring. This occurs where malformations are seen at the low doses, but not in the high-dose group because the highest dose killed the offspring; alternatively, when foetotoxicity was not evident at the low- or mid-dose levels, but the highest dose elicits extensive postimplantation loss, the teratogenic potential may be masked. The preceding discussion notwithstanding, the mechanisms that underlie postimplantation loss are *not* always the same as those causing terata (i.e. the agent may increase embryo/foetal deaths, but it may not induce malformations) (Manson and Kang, 1989).

A third consideration is whether or not the dam experienced overt toxicity during organogenesis. Selective developmental toxicants are those that induce reproductive failure in the absence of maternal toxicity. When adverse effects are seen in litters from mothers that experienced notable toxicity, the adverse effects in the conceptus may have been produced indirectly. Maternal toxicity has been associated with production of wavy ribs, retarded ossification and reduced foetal body weight (Khera, 1985; 1987) and occasionally, adverse effects are seen at doses causing only minimal maternal toxicity. In these cases, the findings *cannot* be interpreted to have been caused by maternal toxicity; rather, the data suggest that both the embryo and the mother are susceptible to the exposure. Furthermore, the appearance of maternal toxicity does not guarantee that adverse effects in the conceptus will occur, because some agents elicit profound maternal poisoning, yet exert no apparent adverse effects on the offspring.

Finally, it must be understood that not all malformations are caused by the test agent. Practically every malformation occurs spontaneously (Palmer, 1977) and any particular malformation can be caused by many different chemical or physical agents. Every animal species (and strain) has a background incidence of spontaneous malformations. When rare terata are seen, examination of the laboratory or breeder's historical experience is required.

4.4.3 Historical Control Data

All facilities that follow Good Laboratory Practices maintain control incidence data (usually over the past 5–10 years) and evaluation of rare or unusual terata (e.g. extrathoracic ectopia cordis) or statistical relationships can be facilitated by comparisons of historical to concurrent data. When the incidence of an effect in the treated groups is within the historical range, the finding is likely due to chance. An additional advantage is that over time, historical control data can provide insight into changes caused by genetic drift, changes due to dietary constituents, season or even differences in technical competence.

4.5 Health Risk Assessment

4.5.1 Maternal Toxicity

Since the pregnant female provides her embryo with a physical environment, nutrition and mechanism for waste elimination, it follows that her physiological status affects the health of her offspring (DeSesso, 1987). Indeed, her health and environment may affect her offspring (Chernoff *et al*., 1987; Schardein, 1987). Where there is significant maternal toxicity, it is often difficult to distinguish effects mediated by compromised maternal health and those induced in the embryo itself (Khera, 1985; 1987). Because maternal toxicity can exert nonspecific adverse effects on the developing offspring, it is important to identify an NOAEL (or benchmark dose (BMD)) for developmental toxicity—preferably in the absence of compromised maternal health. Other than agents like ethanol, where the maternal and foetal toxic dose are essentially identical, the greatest regulatory concern rests with substances or physical agents (e.g. radiation) that cause severe effects in the offspring, but elicit few to no adverse effects in the mother. Examples of the latter are methyl mercury and thalidomide.

4.5.2 Predictive Value of Animal Data

Dose–response relationships (see **Dose-Response Analysis in Experimental Toxicology and Risk Assessment**) for the many different end points studied in developmental toxicity assays are often dissimilar because

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the mode of action is not always identical and high doses may preclude manifestations of effects seen at low doses. Unfortunately, the inference of human health risk from animal data is not straightforward (Kalter, 1965). The induction of a particular effect in one species (including humans) does not necessarily predict the outcome in another species. In many situations, humans are exposed to much lower concentrations than those used in laboratory studies and while positive results from adequately designed animal studies suggest the possibility of increased health risk for humans, the animal data cannot *predict* whether or not an exposure to a material or agent will induce reproductive failure in humans. Because laboratory mammals respond to known human teratogens (but not always in the same manner as that in humans), a biologically significant increase in any of the major end points in a developmental toxicity study is of concern and the concern is increased when the response is observed in more than one test species.

4.5.3 Animal Data and Human Teratogenicity

Assessing whether a clinical or environmental agent causes developmental toxicity in humans is neither rote nor trivial. Results from well-designed studies in laboratory mammals can alert one about the potential influence on human reproduction, but because of differences in physiology, systemic bioavailability by different routes of exposure, metabolic handling and disposition, margins of exposure and maternal status, interspecies extrapolation of results in animals to humans with widely divergent exposure conditions cannot be performed with certainty. Consequently, many human developmental toxicants have been discovered by perceptive physicians and astute biomedical scientists who recognized a tentative relation between exposure and adverse outcome. Often, the recognition comes from a case report or series of similar cases. Subsequent epidemiological studies analyse the incidence and trends of adverse pregnancy outcome in relation to exposure to investigate a possible causal relationship. While epidemiology studies examine the species of immediate concern, the most problematic aspect concerns verification of exposure; animal studies have the advantage of precise exposure, but have the troubling aspect that common laboratory species (including nonhuman primates) are only (distant) relatives of the species of concern.

Several criteria are used to examine the likelihood of a causal relationship between human exposure to an agent of interest and its potential developmental toxicity (Brent, 1986):

- Well-designed epidemiology studies consistently find an increased incidence of a particular developmentally toxic effect or set of developmentally toxic effects after exposure.
- When exposures are wide-ranging, secular trend data demonstrate a relationship between the expected developmental toxicity and exposure levels in humans.
- An animal model is (or can be) developed that mimics the human findings at doses that elicit neither maternal toxicity nor reduced food or water consumption.
- The signs and types of adverse developmental outcomes increase as exposures increase.
- The likely underlying mechanism(s) for induction of developmental toxicity are plausible and do not contradict the principles of biology.

While none of the above criteria are sufficient to prove the existence of a human teratogen, the greater the compliance with those criteria, the more compelling the rationale that supports a causal relationship.

5 ENDOCRINE DISRUPTION

'Endocrine disruption' (ED) is a term used to describe the toxicological activity of a wide range of chemicals that display agonist or antagonistic pharmacologic actions of the steroid hormones, substances that influence the biogenesis or metabolism of those hormones and substances that influence their cellular signals and associated gene expression (Phillips and Foster, 2008) (see **Endocrine Disruption in Toxic Responses**).

There is a popular notion that synthetic EDs found in the diet (Tyshenko *et al*., 2008), wastewater (Kumar *et al*., 2008), agricultural and livestock production, pulp and paper mills, mining and textile industries and consumer products (Ashby, 2001) are responsible for reduced sperm counts, altered sex ratios, precocious puberty and increased cancers (Sharpe and Skakkeback, 1993; Sharpe, 2001). These reports have been interpreted by some as due to interference with normal regulation of development by mimicking or otherwise antagonizing the actions of physiological steroids. Human maladies alleged to have been caused by environmental ED range from spontaneous abortions, infertility, mammary, uterine, ovarian and prostate cancer, obesity, diabetes and psychological disorders (reviewed in Phillips and Foster, 2008; Willhite *et al*., 2008) to systemic lupus erythematosus (Yurino *et al*., 2004). However, the results of rodent studies with environmental ED vary substantially and the hypothesis that 'low-dose' (pg to μg kg^{-1}) effects are responsible for these conditions often cannot be reproduced as the 'lack of attention to methodological details makes it difficult to reconcile different ED assay outcomes for the same chemical' (Ashby, 2001). Even the definition of the term ED is debated in that one often cannot differentiate between physiologic alterations and actual adverse health effects due to changes in endocrine status (Foster and Agzarian, 2008).

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Clinical literature dating from the early 1950s is rich with accounts of ED as a consequence of inadvertent iatrogenic exposures. Ingestion of either androgens (testosterone, methandriol, methyl testosterone, normethandrone) or natural (progesterone) or synthetic (ethisterone, norethindrone) progestins during the first trimester to control nausea and emesis, to maintain pregnancy to term or in pregnancy testing, respectively, have been unequivocally linked to masculinization of the female foetus (pseudohemaphroditism). Feminization of male foetuses due to prenatal progestogen exposure has also been reported (Schardein, 1980).

Overall, the risk of female masculinization associated with prenatal androgen and progestin exposure is <1%. The individual response is related to dose and gestational stage during which exposure occurred. As dose increased, the relative extent of female masculinization increased; exposure during gestational weeks 8–13 produced more complete labioscrotal fusion than at other times because differentiation of the genitalia occurs during that period. Affected infants present with an absent, closed or patent vagina; enlarged labia and clitoris; normal ovaries and Fallopian tubes; and (sometimes rudimentary) uterus. As these girls mature, there is usually total regression of the genital hypertrophy and they are fertile. Iatrogenic female pseudohemaphrodites who experienced transient androgenic hormone exposure *in utero* differ from those with spontaneous congenital adrenal hyperplasia who suffer from progressive virilization. Feminization of the male foetus is limited to hypospadia (Schardein, 1980), but even the tentative association between oral steroids and hypospadias could not be confirmed in follow-up epidemiology studies (Kallen *et al*., 1991).

Prenatal exposures to oral contraceptives (oestrogens with or without progestrogens) have long been suspected of damaging the offspring of women who consumed these drugs during gestation (Janerich *et al*., 1980; Khera, 1984). Overall, the data were conflicting and reports of increased rates of total (Janerich *et al*., 1980; Lammer and Cordero, 1986), skeletal (Kricker *et al*., 1986), cardiac (Hook, 1994; Rothman *et al*., 1979) and urinary tract (Li *et al*., 1995) terata appeared, but other studies (Bracken, 1990; Kallen *et al*., 1991; Oakley *et al*., 1973) found no such associations. Martinez-Frias *et al*. (1998) published a 20 year (1976–1995) case–control study of 20 388 infants with mild to major congenital defects compared to 19 981 normal infants (controls) using 600 different defect categories to investigate possible associations between steroid hormone use and congenital malformations. Exposed infants were those whose mothers consumed sex hormones at any time during the first trimester. Of the hormones used (oral contraceptives, oestrogen combinations, progestogen combinations, oestrogens + progestogens, gonadotropins or other sex hormones), only prenatal exposure to oral contraceptives or oestrogen combinations without progestogens showed significant associations; however, after considering the heterogeneity of the defects and taking into account potential confounding, there was no evidence for increased genital (e.g. hypospadias) or nongenital malformations in any of the exposure categories.

One of the most thoroughly studied EDs is zearalenone, a nonsteroidal oestrogenic mycotoxin found in bread, pie, pizza, corn chips, popcorn, pasta, cake, oatmeal, doughnuts, cow's milk, infant foods and beer, with beer containing some $100\,\mu g\,L^{-1}$ (International Programme on Chemical Safety (IPCS), 2000). The molecule is synthesized by *Fusarium graminearum*, *Fusarium culmorum* and *Fusarium crookwellense*. These fungi colonize corn, wheat, sorghum, rice and barley. Heavy precipitation encourages growth of the fungi; mean concentrations in cereal grains of $17\,\mu g\,kg^{-1}$ with peak levels up to $2000\,\mu g\,kg^{-1}$ have been documented. Dietary zearalenone is hyperoestrogenic, especially in pigs fed $\mu g\ kg^{-1}\ day^{-1}$ doses (Kuiper-Goodman *et al*., 1987). Signs of oestrogenic activity in young female swine include uterine enlargement, precocious mammary development, vaginal prolapse and vulvar swelling; males had reduced testicular size and enlarged mammary and preputial glands. When fed to mature female pigs at similar doses, the animals maintained constant oestrus, as evidenced by morphological changes in the endometrium; ovarian atrophy and pseudopregnancy have also been seen (Berger *et al*., 1981; Etiene and Jemmali, 1982; Kurtz *et al*., 1969). Multigeneration studies with rats fed zearalenone found reduced male and female fertility, reduced numbers of live offspring, increased prenatal death and absent eyelids in the survivors after doses as low as $100\,\mu g\,kg^{-1}\ day^{-1}$ (International Programme on Chemical Safety (IPCS), 2000). Zearalenone binding *in vitro* to oestrogen receptors is 1–10% of that for 17β-oestradiol and its relative potency in cultured cells is 2–5% that of 17β-oestradiol. There are no reports of human ED associated with zearalenone ingestion, perhaps because of the relatively small exposures ($\sim 0.2\,\mu g\,kg^{-1}\ day^{-1}$), efficient hepatic conjugation with α-D-glucose and elimination in urine as the glucuronide. In order to control exposure to zearalenone, a maximum tolerable intake of $0.5\,\mu g\,kg^{-1}\ day^{-1}$ based on the lowest adverse effect level (LOAEL) in pigs has been established (International Programme on Chemical Safety (IPCS), 2000).

Far from restricting the discussion of ED to reproductive hormones, there are few rodent teratogens more effective and reliable than the glucocorticoids (e.g. cortisol, cortisone, hydrocortisone). Long before the appreciation of xenobiotic-induced terata became common knowledge after the thalidomide experience, Fraser and Fainstat (1951) and Fainstat (1954) reported cleft palate in mice and rabbits after maternal cortisone and, like many other rodent teratogens, the dose required to elicit a response is strain-dependent (Diewert and Pratt, 1981; Hackney, 1980; Walker and Patterson, 1978). In general, the doses of corticosteroids required

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to induce cleft palate in rodents are equal to or less than those used clinically. Corticosteroids induce irreversible pathology within the basal epithelial cells (evidenced by chromatin clumping and swelling of the mitochondria and rough endoplasmic reticulum) of the vertically developing rodent palatal shelves. Ordinarily, the palatal shelves become horizontal and then fuse with one another over their entire length, but after corticosteroids exposure the shelves fail to fuse as the primary basal cells within the medial edge epithelium have disintegrated (Shah and Travill, 1976).

Given the alterations in the hypothalamic-pituitary-adrenal (HPA) axis seen after deficiency or supraphysiologic corticosteroid levels, their efficacy in treatment of acute and chronic inflammatory disease, allergic reactions, asthma, autoimmune disease and rheumatic disorders (Schimmer and Parker, 1996), in light of their teratogenic activity in animals and the case reports of cleft palate in cortisone-exposed humans (Harris and Ross, 1956; Doig and Coltman, 1956), there was increased concern for possible damage in human embryos after exposure to these compounds. At least 10 prospective cohort (Propert, 1962; Warrell and Taylor, 1968; Schatz *et al*., 1975; Heinonen *et al*., 1977; Mogadam *et al*., 1981; Mintz *et al*., 1986; Fitzsimons *et al*., 1986; Pollard *et al*., 1992; Laskin *et al*., 1997; Park-Wyllie *et al*., 2000; Gur *et al*., 2004) and three retrospective case–control (Czeizel and Rockenbauer, 1997; Rodriguez-Pinilla and Martinez-Frias, 1998; Carmichael and Shaw, 1999) studies of pregnancy outcome after prenatal therapeutic exposure to cortisone, hydrocortisone, prednisone, prednisolone, methylprednisolone, triamcinolone, flucortolone, dexamethasone, flumetasone, mazipredon and betamethasone have been conducted.

To understand the epidemiology of corticosteroids or any other material, it is important to note the baseline ('background') incidence of all malformations in live infants is ~5% (Behrman and Vaughan, 1987). The background rate for cleft palate is 1 : 2500 and that for cleft palate with or without cleft lip is 1 : 500–1000 (Harper, 1998). Although these studies examined large total numbers (e.g. 56 557 to 1 287 345) of liveborn infants (Czeizel and Rockenbauer, 1997; Rodriguez-Pinilla and Martinez-Frias, 1998), there were relatively few infants with maternal prenatal corticosteroid exposure (oral, spray or topical) during the first trimester (0.42–1.88%). As with many epidemiology studies, problems with potential recall bias and adjustments for confounding factors (e.g. maternal tobacco consumption, age, infection, disease, birth order, family history, hyperthermia, maternal use of antiepileptic and other drugs, including sex hormones) were acknowledged. Based on four cases of oral clefts with confirmed exposure during the first trimester (from a total of 1184 total cases of oral clefts), Rodriguez-Pinilla and Martinez-Frias (1998) concluded the risk of cleft lip was increased significantly (p = 0.014) with first trimester corticosteroid exposure. Others (Czeizel and Rockenbauer, 1997; Gur *et al*., 2004) found no increase in absolute risk-adjusted odds ratios and the Fraser and Sajoo (1995) survey of 468 exposed pregnancies concluded that 'treatment with corticosteroids in pregnancy presents little, if any, teratogenic risk to the foetus'. While reduced birth weight was identified as an adverse outcome (Reinisch *et al*., 1978), prenatal corticosteroids during weeks 24–34 represent standard intervention for prevention of spontaneous premature delivery that complicates 7–10% of all pregnancies. Antenatal glucocorticoids reduce infant mortality associated with respiratory failure secondary to inadequate pulmonary surfactant (Anonymous, 1994). The NIH (National Institutes of Health) panel concluded that prenatal corticosteroid therapy does not influence the risk for neurodevelopmental delay or disability (Anonymous, 1994).

6 MALE-MEDIATED DEVELOPMENTAL TOXICITY

Dozens of studies concerning paternal smoking (Wasserman *et al*., 1996), ethanol consumption (Little and Sing, 1987; Savitz *et al*., 1992), occupational 'pesticide' (Garcia *et al*., 1998; 1999; Lacasana *et al*., 2006; Savitz *et al*., 1997), solvents or other anthropogenic exposures (Hooiveld *et al*., 2006; Mageroy *et al*., 2006; Savitz *et al*., 1994) in relation to birth outcome have been published. There is no question that exposure of fertile male rats and male mice to potent mutagens (e.g. 1,3-butadiene, cyclophosphamide) can increase the number of dominant lethal mutations and produce malformations in the offspring of untreated females (Adler *et al*., 1994; Anderson *et al*., 1996; Jenkinson *et al*., 1987; Trasler *et al*., 1985). Male-mediated developmental toxicity in those instances is due to alkylation of germ cell DNA; however, there are no confirmed reports of male-mediated developmental toxicity in human beings after exposure to chlorambucil, cisplatin, bleomycin, 2-bromopropane or other similar genotoxic substances. In those cases, azoospermia, oligospermia, Leydig, Sertoli and germ cell (spermatogonia) toxicity, atrophy of the seminiferous tubules, reduced serum testosterone and elevated lutenizing hormone are seen in both humans and animals and the affected males are infertile. Affected females suffer amenorrhoea, atrophy of their ovarian follicles, arrested follicular development and reduced numbers of primary follicles (Boekelheide *et al*., 2004). Thus, while examples of male-mediated developmental toxicity in rodents can be identified, the findings of male-mediated developmental toxicity are rare, primarily because the outcome at lower doses is most often reduced fertility and/or other systemic complications (e.g. haematopoietic toxicity) (Boekelheide *et al*., 2004).

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Other than famine, epidemics and pestilence, perhaps only open and sustained armed conflict has more shaped human history. Since the 1950s, the United States and its allies prosecuted wars in Korea, Vietnam and the Arabian Gulf ('Desert Shield/Storm' and 'Iraqi Freedom'). Subsequent to each conflict, anecdotal accounts of increased birth defects in veterans' offspring appeared only to be followed by increased public and Congressional concern with reproductive toxicity among veterans (Friedman, 1984; National Academy of Sciences, 1997; 2003; Araneta *et al*., 2003; Gamboa, 2003). The more unpopular the war, the greater the level of concern.

There is no more contentious substance in this regard than 'Agent Orange'—a dioxin-containing herbicide sprayed multiple times over 2.6 million acres in Vietnam as part of 'Operation Ranch Hand'. Field and Kerr (1998) concluded 'the evidence from all available studies supports a causal contribution to defects in veterans' children from a paternally mediated genetic effect'—this despite the fact that chlorinated dibenzo-*p*–dioxins (2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDD) were neither mutagenic *in vitro* (Randerath *et al*., 1989) nor *in vivo* (Lopreino *et al*., 1982; Lim *et al*., 1987) and dioxins were unable to induce dominant lethality (Khera and Ruddick, 1973). Friedman (1984) reviewed the animal data and found that '2, 4, 5-T is more likely to be teratogenic than TCDD when the level of dioxin contamination of the herbicide is on the order of a few ppm. If the same dose were teratogenic in pregnant women, ingestion of 1.5–2.5 g/day of 2, 4, 5-T or 3–5 ml of Agent Orange would be necessary to produce an effect. In fact, there is no scientific rationale for such extrapolation from rodent to human, and these doses might better be considered those that would be teratogenic in a 110-pound mouse'. The National Academy of Sciences (1997) concluded: 'Laboratory studies of potential male-mediated developmental toxicity of TCDD and herbicides, specifically with regard to birth defects, are too limited to permit conclusions'.

Based upon the Wolfe *et al*. (1995), the Centers for Disease Control (1989) and the Erickson *et al*. (1984) epidemiology studies that identified increased spina bifida and other NTDs among the offspring of Vietnam veterans (five per 1000 and an odds ratio of 1.7, respectively), the National Academy of Sciences (1997) concluded, 'the exposure opportunity index based upon interview data was associated with an increased risk of spina bifida; for the highest exposure opportunity index the odds ratio was 2.7 (confidence interval = 1.2–6.2)'.

Wolfe *et al*. (1995) actually concluded:

"There was an increase in nervous system defects in Ranch Hand children with increased paternal dioxin, but it was based on sparse data. These data provide little or no support for the theory that paternal exposure to Agent Orange and its dioxin contaminant is associated with adverse reproductive outcomes."

Erickson *et al*. (1984) actually concluded:

"Vietnam veterans, in general, did not have an increased risk of fathering babies with defects (all types combined; relative risk estimate 0.97). However, for a few specific types of subgroups of defects the estimated risks were higher for subgroups of Vietnam veterans that may have had a greater likelihood of exposure to Agent Orange. These seemingly higher risks could be chance events, the result of some experience in the Vietnam service of the father or the result of some other unidentified risk factor."

In Australian Vietnam Veterans Validation Study (1999) identified 34 cases of spina bifida to bring the total there to 50 cases (as compared to 33 expected for the cohort). In 2003, the National Academy's Institute of Medicine believed that 'the available scientific literature provides limited/suggestive evidence of an association between exposure to herbicides (2, 4-D, 2, 4, 5-T and its contaminant TCDD, cacodylic acid and picloram) and spina bifida in offspring'.

In September of 1997, President Bill Clinton signed US Public Law 104–204 that provides Veterans Affairs Department (1997a; 1997b) healthcare, vocational rehabilitation and monthly monetary allowances for conditions related to spina bifida. For an affected child to qualify for those benefits, the birth father (or mother) must have served in Vietnam from January 9, 1962 up until May 7, 1975. The US Congress then directed the Department of Veterans Affairs to establish healthcare benefits and compensation 'for children with certain birth defects born to women Vietnam veterans' who entered the Republic of Vietnam between February 28, 1961 and May 7, 1975 (The Children of Women Vietnam Veterans Health Care Program). In December of 2003, the Veterans Benefit Act signed by President George W. Bush granted financial compensation and healthcare benefits to children with spina bifida born to a veteran who served in the Korean demilitarized zone between September 1, 1967 and August 31, 1971 and who was exposed to herbicides.

Examination of reports by 7924 US Army veterans found a clear 'dose–response' for 21 adverse health outcomes and self-reported herbicide exposure; however, other than chloracne and post-traumatic stress disorder, those associations were deemed not plausible because the biologic evidence showed little actual exposure to dioxin-containing materials in Vietnam (Decoufle *et al*., 1992). Subsequent studies with more than 1100 pregnancies with high occupational dioxin exposures 'indicate that TCDD is unlikely to increase the risk of low birth weight or preterm delivery through a paternal mechanism' (Lawson *et al*., 2004). Not one of the Agent Orange epidemiology studies upon which the spina bifida and herbicide conclusions (National Academy of Sciences, 1997;

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2003) were based accounted for maternal folate status, an omission common with recent analyses (Ngo *et al*., 2006).

7 SUMMARY AND CONCLUSION

We have seen the monsters, trolls, mermaids, villains and ghosts of developmental toxicology. We have also seen that ghost stories are as dangerous for the human foetus as are villains. To avoid these and other related mistakes, rigorous application of the principles of evidenced-based toxicology (Guzelian *et al*., 2005) guides the investigator into questions of causation and permits one to articulate clearly the limits of our knowledge and experience. There are many historical lessons in developmental toxicology (Warkany, 1971; 1977) and there is no end to contemporary conjecture and xenobiotic speculations that surround reproduction, particularly when the subject sounds as though it has a 'scientific' basis and it is dispensed repeatedly to a willing audience eager to seize upon one or another mysterious cause of impending permanent and debilitating disease (Willhite and Mirkes, 2005). Cardinal sins are committed by those who promote statistical associations as a basis for causal conclusions and by those who transfer animal data, tissue culture and laboratory measurements to human beings without application of the fundamental tenants of hazard identification, dose–response, exposure assessment and risk characterization that are necessary to evaluate and interpret that information (Harris and DeSesso, 1994; Presidential/Congressional Commission on Risk Assessment and Risk Management, 1997).

Those who disseminate rumour and perpetuate fear of intrauterine and neonatal damage prey upon public ignorance of pharmacology and developmental biology. Overwhelming fear of congenital defects and an innate, unshakable desire to identify perpetrators responsible for harming babies amplify the contemporary chemical superstitions that mirror the mysticism of the past. Unsupported conclusions, extrapolations and irrational anxiety surrounding chemical and physical agents in the world of developmental toxicology extract a heavy toll in human misery and suffering (Brent, 1995; Hook and Healy, 1976; Koren *et al*., 1993; Trichopoulos *et al*., 1987). In contrast, effective reductions in foetal and neonatal wastage can be realized by emphasizing public programmes to encourage consumption of folate supplements (Bell and Oakley, 2006)—especially by ethnic minorities (Williams *et al*., 2005)—and providing resources to combat maternal alcoholism, obesity and diabetes (Willhite and Mirkes, 2005).

It is becoming clear that traditional division of toxicology into disciplines like developmental toxicology, cell repair, regeneration and carcinogenesis are more likely a reflection of history than of intrinsic biological differences. For example, it is the deregulation (inappropriate activation or 'escape') of *Hh* genes normally active in partitioning of the prosencephalon and mesodermal differentiation in the gut that is associated with some of the most dangerous and lethal cancers (e.g. chronic pancreatitis and ductal pancreatic carcinoma, prostate carcinoma, small lung carcinoma, medulloblastoma and basal cell carcinoma) in adults (Kayed *et al*., 2006; Nakashima *et al*., 2006; Karhadkar *et al*., 2004; Morton and Lewis, 2007). Mutations in *Smo*, a gene that regulates *Shh*, are found in human basal carcinomas (Xie *et al*., 1998) and transient *Hh* activation is seen during normal epithelial regeneration and turnover in the adult lung and gut (Watkins *et al*., 2003; Beachy *et al*., 2004). It is the untimely expression of genes (like *Shh* that participate with other genes like *Smo* in normal embryonic patterning) that determine progenitor cell transformation and trigger invasive tumour metastasis; the greater the inappropriate *Hh* pathway activity in adult cells, the greater the metastatic response (Scott, 2003). It can be anticipated that advances in understanding genomic regulatory circuits will provide additional parallels between embryonic development and disturbances that give rise to neoplastic disease and other conditions in children and adults (Roessler and Muenke, 2003).

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Developmental Neurotoxicity

Susan L. Makris and Kathleen C. Raffaele

CONTENTS

1 INTRODUCTION

Developmental toxicity is defined as including effects that may result from either prenatal or postnatal exposure, may be manifested at any life stage, and may be expressed as functional deficits (OECD, 2008a; US EPA, 1991a). The developmental neurotoxicity (DNT) study is a specialized type of developmental toxicity study designed to screen for adverse effects of pre- and postnatal exposure on the development and function of the nervous system and to provide dose–response characterizations of those outcomes. This chapter focusses on the DNT guideline as a commonly used study design conducted in rodents and used in a regulatory context. While there are many other methods that can be used to assess various aspects of developmental neurotoxic potential, for example, the assessment of neurochemical changes, behavioural studies in nonmammalian species such as zebra fish, and human epidemiological studies, these methodologies are not discussed in this chapter. As well, since the DNT guideline has been primarily developed for the assessment of environmental toxicants, most of the information presented in this chapter is focussed on nonpharmaceutical issues.

2 BACKGROUND AND HISTORY

The field of developmental neurotoxicology evolved from the disciplines of neurotoxicology, experimental and development psychology, and developmental toxicology. It is based upon an extensive history of scientific research and regulatory consideration.

The evolution of a standardized DNT study protocol is rooted in scientific publications that began to appear in the early 1960s, but both the science and the study protocol have continued to develop over the past 40–50 years. A strong foundation for the development, implementation and validation of the DNT guideline is based upon extensive scientific literature. This includes studies evaluating the potential for physical, pharmaceutical and environmental agents to affect the development and function of the nervous system after prenatal and early postnatal exposure and provide dose–response characterization of those outcomes. **Table 1** lists some of the key critical contributions to the development of the DNT guidelines.

In a study conducted according to the standard Environmental Protection Agency (EPA) or Organization for Economic Cooperation and Development (OECD) DNT study guidelines (OPPTS 870.6300 or GL 426,

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Table 1 Historical contributions to the Developmental Neurotoxicity Guideline

Date	Event[a]	Summary
1960–1980s	Published research on developmental neurotoxicity and behavioural testing	Evidence that developmental exposure to chemicals and drugs can alter behavioural functioning in young and adult animals
1978–1984	Collaborative Behavioural Teratology Study (CBTS)	Study to examine intra- and interlaboratory reliability and sensitivity of behavioural test methods
1984	Cincinnati Test Protocol	Within-laboratory comparison of CBTS test protocol with the Cincinnati Test Protocol
1982–1985	Collaborative studies of the Japanese Teratology Society	Interlaboratory methods evaluations and assessment of six reference chemicals
1985–1988	European Interlaboratory Collaborative Study	Interlaboratory study to assess sensitivity of behavioural test procedures to detect neurotoxicity of methylmercury
1989	Williamsburg Workshop	Workshop to evaluate the qualitative and quantitative comparability of animal and human data for developmental neurotoxicity
1984–1997	Collaborative studies of the Japanese Teratology Society	Three interlaboratory studies using behavioural teratogens to evaluate a core battery of tests
1995	International Programme on Chemical Safety (IPCS) Collaborative Study	Interlaboratory study using neurotoxic chemicals to evaluate validity, reliability and measurement variability of tests
2000	International Life Sciences Institute Risk Science Institute (ILSI RSI) workshop on developmental neurotoxicity testing	Workshop to review EPA DNT behavioural test methods, pharmacokinetics and neuropathology
2003	Japanese Interlaboratory Study	Interlaboratory study using neurotoxic chemicals to determine sensitivity of behavioural measures
2003 (Sept)	Behavioral Test Methods Workshop	Expert workshop to address design, conduct and analysis of behavioural tests for neurotoxicity evaluation
2004–2008	ILSI RSI Working Group	Working group focussed on variability, statistical analyses, positive controls, identification and interpretation of treatment-related effects and application of developmental neurotoxicity testing to public-health protection

[a]Relevant references for each event are detailed in OECD, 2008b.

respectively), as illustrated in **Figure 1**, pregnant rats are administered the chemical orally from gestation day 6 through to postnatal day (PND) 10 or 21 (US EPA, 1998d; OECD, 2007). The offspring are therefore exposed to the chemical, via the maternal circulation and/or milk, during *in utero* and early postnatal development for approximately 25 to 40 days. Detailed clinical observations are conducted (outside of the home cage) on approximately half of the dams in each group, twice during gestation and twice during lactation, and maternal body weight is recorded at least weekly. The offspring are assessed for evidence of deficits in functional development. Litters are randomly standardized on PND 4 to yield four pups per sex per litter, and the pups are assigned to testing subgroups for neurobehavioural and neuropathological assessment. End points evaluated between birth and PND 60 include measures of physical development, reflex ontogeny, motor activity, motor function, sensory function, and learning and memory. Daily cage-side observations are conducted, and 10 pups per sex per group are examined outside the cage on days 4, 11, 21, 35, 45 and 60. Pups are counted and weighed individually at birth, on PNDs 4, 11, 17, 21 and at regular intervals postweaning. At a minimum, developmental landmarks recorded include the age of vaginal opening or balanopreputial separation. Motor activity is monitored

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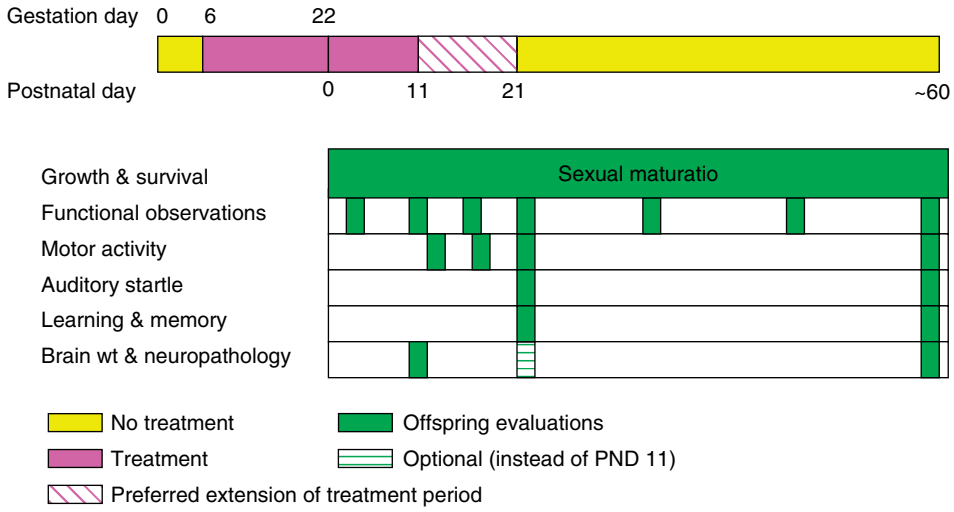

Figure 1 Developmental neurotoxicity study design (US EPA, 1998d). (Reproduced from U.S. Environmental Protection Agency.)

at early time points and at termination; the EPA guideline specifies the use of an automated activity recording apparatus on PNDs 13, 17, 21 and 60 (±2). Tests of auditory startle habituation (preferably using prepulse inhibition), and associative learning and memory are performed on the offspring around the time of weaning (day 21) and around day 60. At an early time point (PND 11 or 21) and at study termination, the offspring are subjected to extensive neuropathological examination, including simple morphometric analysis. One pup per sex per litter is killed on PND 11 or 21, and of these, 6–10 pups per sex per group are assigned to neuropathological evaluation; their brains are removed and immersed in an aldehyde fixative. At study termination, all remaining offspring are killed; 6–10 rats per sex per group are prepared for neuropathological evaluation with *in situ* transcardial perfusion of appropriate fixatives (paraformaldehyde and gluteraldehyde). Brain weight is recorded at both an early time point (PND 11 or 21) and at study termination (around PND 60). Qualitative neuropathological examination is conducted for the control and high-dose groups, and if a treatment-related finding is evident, the low- and mid-dose groups are also examined. Guidance is provided regarding the regions of the brain to be examined and the types of alterations upon which to focus, particularly emphasizing structural changes indicative of developmental insult. Simple morphometric analysis, performed on offspring killed at the early time point and at termination, is defined as consisting, at a minimum, of a reliable estimate of the thickness of major layers at representative locations within the neocortex, hippocampus and cerebellum.

3 DEVELOPMENT OF THE NERVOUS SYSTEM

3.1 Critical Windows of Nervous System Development

Temporal aspects of nervous system development are critical to assessing insult for use in life-stage-specific risk assessment (Makris *et al*., 2008). The concept of identifying critical windows of development in various organ systems for application to human-health risk assessment was explored in a workshop conducted by the US EPA (Selevan *et al*., 2000). Neurobehavioural outcomes were addressed by Adams *et al*. (2000). It was noted that specific adverse outcomes have been shown to be associated with particular windows of vulnerability for specific agents. The sensitive developmental windows are initiated prior to the formation of the neural plate and end at approximately the age of puberty, when nervous system maturation is predominantly complete. Many neuroactive agents are able to disrupt development at multiple time points during this wide period of potential sensitivity. The end points affected by a particular agent may also vary, dependent on the timing of exposure. Expression of the adverse response may occur during a particular stage of development, at the time of or following exposure and may not be observed except under specific conditions of measurement or following an environmental challenge.

The ontogeny of neurological development is temporally different between humans and the rodents that are typically used in DNT testing. In humans, there is extensive prenatal maturation of the nervous system, while in rodents much of the maturation process (e.g.

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myelination) continues postnatally. These developmental processes include proliferation, migration, differentiation, synaptogenesis, gliogenesis and myelination, and apoptosis (Rice and Barone, 2000). Regional growth rates, timelines of regional neurogenesis and myelination and functional ontogeny exhibit distinct cross-species differences. Thus there is no simple approach to precise correlation of the stage of neurological development in humans vs. test animals. For this reason, the most comprehensive approach to screening for developmental neurotoxic insult in test species has been to include exposures to the test substance during all stages of neurological development (i.e. throughout both prenatal and early postnatal life stages).

Nevertheless, some attempt has been made to compare the achievement of key events in human postnatal central nervous system (CNS) development to those in common laboratory animal species (rats, dogs and nonhuman primates) (Wood *et al*., 2003). The ontogeny of fine motor development and dexterity, sensory and reflex development, cognitive development, communication, social play, fear response and sleep cycles are addressed in the Wood *et al*. paper. Consideration of cross-species differences in the timing of these developmental milestones can be very useful in designing juvenile animal toxicity studies (US FDA, 2006), or in enhancing a standard guideline DNT study in order to address chemical-specific toxicity issues.

3.2 Special Vulnerabilities

Concerns about the vulnerability of the developing nervous system are firmly based in historical tragedies such as the Minamata Bay disaster with methylmercury, the well-documented effects of foetal alcohol syndrome (a very serious public-health problem), the consequences of lead exposure in children and the adverse consequences of *in utero* exposures to some neuroactive pharmaceuticals (e.g. phenobarbital, or antiepileptic agents such as phenytoin and valproic acid) (Weiss, 2000). The importance of screening and testing of pharmaceuticals and environmental toxicants for the potential to affect nervous system development has lead to legislation, such as the FDA Best Pharmaceuticals for Children Act (FDAMA, 2001), which mandates the testing of pharmaceuticals that are intended for paediatric use, and the Food Quality Protection Act (FQPA) (FQPA, 1996), an amendment to the Federal Insecticide Fungicide and Rodenticide Act (FIFRA). The implementation of these laws has resulted in increased testing for DNT. For example, the number of DNT studies that had been submitted to the US EPA Office of Pesticide Programs in support of pesticide registration increased from a total of nine in 1998 (Makris *et al*., 1998) to a current total of at least 73 (Makris *et al*., 2009).

While susceptibility of children can often be attributed to such factors as increased exposure due to child-specific behaviours, or to genetic polymorphisms, there are also a number of vulnerabilities to the developing nervous system that are either unique or enhanced in comparison to development in other systems. Due to the prolonged ontogeny of the nervous system (which continues postnatally for many years in humans), its complexity and the interconnections within the system, there are many opportunities for developmental insult (Bennett and Finnell, 1998). Adverse consequences of agent exposure can, in some cases, be observed as overt structural defects, for example, malformations such as spina bifida or exencephaly, or may be comprised of more subtle changes, for example, disruptions in synaptogenesis, histogenesis or cell–cell communication, leading to functional deficits that may be much more difficult to detect. There are a number of other reasons that effects on neurodevelopment can be difficult to identify. For example, the process of nervous-system development includes constant restructuring of the tissues, through mechanisms such as apoptosis and cell migration. This can provide an opportunity for compensatory development to occur, which might mask the consequences of the primary insult. In some cases, such adverse outcomes may not be observed until the system is challenged (e.g. by a pharmaceutical or toxicant exposure, or by the consequences of ageing) at a later time point. Studies conducted to screen for adverse outcomes in animals or humans may lack the power to detect effects, or they may not include the appropriate tests or parameters.

4 TESTING PARADIGM

4.1 Guideline Study: History of Development

The first regulatory protocol designed to evaluate DNT was developed and implemented by the US EPA in support of hazard evaluation for specific solvents (US EPA, 1986), and a DNT guideline applicable to the evaluation of both industrial chemicals and pesticides was finalized in 1991 (US EPA, 1991b). In 1998, the guideline was revised as part of a broader US effort to harmonize testing guidelines within the US EPA Office of Prevention, Pesticides, and Toxic Substances (OPPTS) and with the OECD (OPPTS 870.6300; US EPA, 1998d). A separate OECD DNT guideline, which includes several improvements to the original testing paradigm, was recently finalized (OECD TG 426; OECD, 2007). Differences between the US EPA and the OECD test guidelines are summarized in **Table 2**.

The process of guideline development, validation and refinement has been an intensive long-term effort over the

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Table 2 Differences between the US EPA and OECD DNT Test Guidelines

Testing element	US EPA (1998d)	OECD (2007)
Dosing period	Gestation day 6 through postnatal day 11 Note: PND 21 recommended[a]	Gestation day 6 through lactation (PND 21)
Direct dosing to preweaning pups	Not discussed Note: Recommended in some situations[a]	Should be considered for some situations
Minimum group size for pup behavioural assessments	10 per sex per dose for most tests	20 per sex per dose for most tests
Functional observations	Specific days recommended (PND 4, 11, 21, 35, 45 and 60)	Specifies assessment weekly preweaning and biweekly postweaning
Behavioural ontogeny[b]	Not discussed	At least two measures required
Motor and sensory function	Auditory startle habituation specified	Quantitative sampling of sensory modalities and motor functions specified, auditory startle habituation listed as example
Motor activity	Specific days recommended (PND 13, 17, 21 and around 60)	Specifies assessment one to three times preweaning, once during adolescence (around PND 35), and once for young adults (PND 60–70)
Neuropathology—number of animals	Six per sex per dose specified Note: 10 per sex per dose recommended[a]	10/sex/dose
Neuropathology assessment at early timepoint	PND 11, with immersion fixation Note: PND 21 accepted, with perfusion fixation[a]	Between PND 11 and 22, either perfusion or immersion fixation

[a]In general practice, EPA testing recommendations reflect procedural enhancements that have been incorporated into the OECD guideline.
[b]Behavioural ontogeny is not specifically required in the EPA guideline; however, two measures are required in the OECD guideline, one of which can be assessment of preweaning motor activity. Other examples provided in the guideline are righting reflex and negative geotaxis.

past 25 years, involving numerous scientists from various public and private sectors (e.g. government, industry, academia, nongovernment organizations, contract research organizations and public interest groups), with expertise in critical scientific areas (e.g. developmental toxicology, neurotoxicology and risk assessment). A number of pivotal meetings and collaborative studies have been conducted.

The formative history of the DNT guideline includes research on test method development, as well as efforts to characterize and document the sensitivity, reliability and performance of the test methods. Included among these efforts were a number of intralaboratory collaborative studies. A series of studies were conducted in the 1970s in which rats were developmentally exposed to a variety of materials and subsequently tested during postnatal development, using a battery of neurobehavioural tests (Butcher and Vorhees, 1979; Vorhees *et al*., 1979). In other laboratories, behavioural and histological batteries (focussing on sensory and motor function) were used in adult rodents that had been exposed to a wide variety of neurotoxicants (Pryor *et al*., 1983; Tilson *et al*., 1979). An extensive body of research (Gad, 1982; Irwin, 1968; Moser *et al*., 1988) examined the ability of the functional observational battery to detect and characterize the effects of drugs and environmental chemicals in adult and developing animal models. Subsequently, there were broad efforts to characterize the specificity of these test methods and the impact of factors at the level of the organism, as well as experimental factors (e.g. noise, species, strain, gender, test history) (Gerber and O'Shaughnessy, 1986; Levine and Butcher, 1990; MacPhail *et al*., 1989; Spencer *et al*., 1993). Overall, the collective work in this area documents and supports the consensus opinion that proper use and interpretation of the data derived from these test methods provides unique insight into the impact of xenobiotics on the developing and adult nervous system (Cory-Slechta *et al*., 2001; IPCS, 2001; Tyl *et al*., 2008).

Historically, there have also been a number of efforts to characterize the interlaboratory reliability and sensitivity of the neurotoxicology test methods that are included in

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the DNT study design. Learning and retention methods were compared among three laboratories by Butcher *et al*. (1979). Subsequently, the Collaborative Behavioral Teratology Study (CBTS) (Buelke-Sam *et al*., 1985; Kimmel and Buelke-Sam, 1985) and the 'Williamsburg Workshop' on qualitative and quantitative comparability of human and animal DNT (Kimmel *et al*., 1990) were conducted. Collectively considered, these efforts addressed various aspects of DNT study design and conduct, demonstrating a sound scientific basis for the test method and its use in hazard evaluation. Since the US EPA DNT guideline was published in 1991, efforts have been conducted to review and update methodologies used for neurotoxicology testing in general and for developmental neurotoxicology testing in particular. These include, for example, the IPCS collaborative study on neurobehavioural screening methodologies (MacPhail *et al*., 1997), an International Life Science Institute (ILSI) Risk Science Institute (RSI) workshop on developmental neurotoxicity and risk assessment (Cory-Slechta *et al*., 2001; Dorman *et al*., 2001; Garman *et al*., 2001; Mileson and Ferenc, 2001), a collaborative study on neurobehavioural screening in 11 Japanese laboratories (Okazaki *et al*., 2003), a behavioral test methods workshop (Slikker *et al*., 2005) and a series of reports from an ILSI/RSI expert working group on neurodevelopmental end points (Crofton *et al*., 2008; Fitzpatrick *et al*., 2008; Holson *et al*., 2008; Raffaele *et al*., 2008; Tyl *et al*., 2008). Descriptions of each of these efforts and their contributions to the scientific basis for DNT testing are summarized by Makris *et al*. (2009).

In the context of toxicological screening and testing to support human-health risk assessment and chemical regulatory activities, the DNT study is a critical tool, because it addresses information that is not covered by other EPA or OECD test protocols. It is the only test guideline that assesses functional, behavioural and anatomical evaluations of the nervous system at multiple time points and in test subjects that were exposed to a test substance during critical pre- and early postnatal periods of nervous-system development. Over the past two decades, this test method has been used extensively to screen a wide variety of chemicals (**Table 3**).

Table 3 Number of chemicals studied using the EPA DNT Guideline or draft OECD 426 Guideline[a]

Chemical class	Number of studies
Pharmaceuticals	3
Miscellaneous agents[b]	4
Solvents	7
Industrial chemicals	8
Positive control chemicals	15
Pesticides	73
Total	110

[a]As cited in OECD, 2008b.
[b]Food additives, cigarette smoke, dietary restriction and maternal separation.

4.2 Validity and Cross-Species Comparability

A recent review of the history and performance of DNT testing was conducted in support of finalizing and implementing the OECD DNT test guideline (TG 426) (Makris *et al*., 2009). As a result of this review, OECD member countries reached a consensus opinion that the DNT guideline was considered to be validated, in accordance with the recommendations of OECD Guidance Document No. 34 (OECD, 2005), which addresses the validation and regulatory acceptance of new or updated test methods for hazard characterization.

In this review, the history of the DNT guideline development (in the US and internationally) was addressed, and the scientific basis supporting the guideline was summarized in detail. This included a number of workshops and collaborative studies, which are highlighted in **Table 1**. The extensive effort that contributed to the development of the DNT guideline development yielded incontrovertible evidence of the sensitivity of the study in detecting effects on neurological development in animal test subjects for a broad array of neurotoxic chemicals and other agents. Research programmes and interlaboratory collaborative studies demonstrated reliability and comparability in test results for the guideline-specified measurements, due in great part to concerted corollary efforts leading to the standardization of testing protocols and methods of data analysis and interpretation.

Another critical issue addressed by Makris *et al*. (2009) was the cross-species comparability of developmental neurotoxic outcomes in animal test models vs. humans. Evidence for such comparability was examined in the 1989 workshop on the Qualitative and Quantitative Comparability of Human and Animal Developmental Neurotoxicity (i.e. the 'Williamsburg Workshop') (Kimmel *et al*., 1990). In this workshop, expert scientists from government, industry, public interest groups and academia reviewed a range of representative chemicals and environmental exposures including: drugs (cannabis, cocaine, methadone and phencyclidine), ethanol, the anticonvulsant phenytoin, methylmercury, lead, polychlorinated biphenyls and ionizing radiation. Based on available human and animal data for these known human developmental neurotoxicants, the workshop participants concluded that DNT methodologies were adequate for detecting DNT. A major conclusion of the workshop was that the DNT protocol would have identified each of the agents considered at the workshop as a potential developmental neurotoxicant (Francis *et al*., 1990), although the critical effects, and the dose at which the effects were observed, might vary across species. This

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concept has been further discussed by Schardein (1998). The predictive power of DNT guideline studies is largely attributed to the broad scope of neurobehavioural and neuropathological tests included in the study, that can screen neurological functions across multiple neurological domains (i.e. sensory, motivational/arousal, cognitive and motor). The laboratory-animal model serves as an adequate surrogate for humans because many of the biological and behavioural mechanisms underlying these neurological functions are shared between humans and laboratory animals. Wood *et al*. (2003) presents additional discussion of cross-species comparisons of postnatal CNS development, with a focus on functional measurements.

4.3 End Points and Issues

As described above, the current design of the regulatory DNT study assesses a broad spectrum of end points, in an attempt to identify the potential for a chemical to cause changes across many different neural systems. Due to the complexity and organization of the nervous system, it is not possible for changes in a single type of behaviour (or pathology in a single brain region) to adequately reflect dysfunction for the many individual systems of concern. Although one type of assessed behaviour may require intact functioning of multiple parts of the brain (e.g. motor activity involves control of motor systems as well as more cognitive aspects, for example those leading to habituation), each type of assessment also includes functions that may not be required for other types of behaviour (for example, hearing and auditory reflex circuits in auditory startle habituation). Each brain system also has its own normal developmental pattern, and the integration of various neural systems changes as each system matures, leading to differences in behavioural responses over time. For this reason, changes in a single parameter, or at a single assessment period, often reflect an adverse change in neural development even though later assessments of the same behaviour may look normal. It is often not the case that changes in nervous system development will lead to broad disruptions of behaviour on multiple types of end points. The ability to detect adverse effects will depend both on the type of behaviour assessed and on the sensitivity of the procedures used.

It is beyond the scope of this chapter to discuss in detail all aspects of the behavioural testing paradigms employed in evaluating DNT. For more detailed discussion of specific types of behaviour and their evaluation, the reader should refer to the neuropsychological literature (including some of the references cited below) and to the many other works in this area (Annau, 1985; Eckerman and Bushnell, 1992; Harry, 1994; Slikker and Chang, 1998; Weiss and O'Donoghue, 1994). In the discussion below, each type of procedure will be briefly described and some critical aspects of its use in this context will be mentioned.

In addition to behavioural evaluations, alterations in neurochemistry (for example changes in neurotransmitter levels, enzyme levels, ion-channel distribution or receptor levels) may be indicative of DNT. These types of methods have been extensively used for some compounds (Slotkin, 2004); interpretation of the findings in a regulatory context has been difficult, as the consequences of neurochemical changes with respect to functional effects on the whole organism is often not known. Discussion of these methods and their interpretation is beyond the scope of this chapter, however it is likely that their use will become more common as scientific understanding of the functional consequences of these types of changes increases.

In cases where a chemical or class of chemicals is known to cause a specific biological effect, for example, cholinesterase inhibition or thyroid hormone disruption, regulatory studies may also include measurement of these end points. Specific protocols have been developed and recommended by the EPA for these two categories of compounds (Makris, 2006; US EPA, 2005), but they will not be further discussed here. Consideration of these types of evaluations is also discussed in the OECD DNT guideline (OECD, 2007).

4.3.1 Relationship to Maternal/Foetal Paradigm

As a developmental study, the DNT guideline includes exposure to the mother and the foetus, both *in utero* and during lactation. This exposure period is designed to include the periods of most rapid brain development, although some aspects of brain development continue through adolescence, and studies with extended exposure have been conducted (Chapin *et al*., 1997). Because brain development continues after birth, it is important that the exposure to the offspring continue as well; for compounds that are not excreted in milk, it may be necessary to administer the compound directly to offspring after birth to assure continued exposure at levels of concern. It is important to be aware of the possibility that direct effects of the compound on the mother may impact neural development. In most cases, it is not possible to distinguish these effects from direct effects on the offspring. In any case, similar impact of maternal effects on offspring development would be of concern for substances to which humans are exposed, leading to the assumption that any adverse effects detected in the offspring are of concern, regardless of their ultimate cause.

4.3.2 Developmental Landmarks

Several landmarks or milestones of development are assessed in the DNT study. These include measures of

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birth weight and postnatal growth. Pup body weights are recorded by sex several times during the preweaning period and once or twice per month after weaning.

A number of other preweaning developmental landmarks may optionally be assessed. Age at ear pinna unfolding, upper incisor eruption and eye opening have been shown to be reliable measures of development (OECD, 2008a) and are commonly evaluated. Assessments commence just prior to the day that the event is typically expected to occur for the species being tested; this may vary by animal strain. The sample size evaluated (e.g. all pups on study vs. a subset of pups from each litter) and the manner in which the data are compiled and reported (e.g. as mean day of achievement per litter vs. the number of pups achieving the event during several discrete ranges of days assessed) can affect the sensitivity of these assays for identifying treatment-related effects. It is not unusual for preweaning developmental landmarks to be positively correlated with pup body weight.

The DNT guideline includes an assessment of the age of sexual maturity (puberty) in both males (age at balanopreputial separation) and females (age at vaginal patency). Typically, these events occur at approximately PND 40–45 in male rats and PND 30–35 in female rats (OECD, 2008a), although there may be some strain-related differences. It is important to record the body weight of the animals at the time that sexual maturity is achieved, since body mass is associated with age of puberty. Thus the interpretation of these data may be complicated by treatment-related effects on offspring growth.

The method used in calculating the age of the offspring may influence the assessment and interpretation of some developmental landmarks. Pup age may be calculated from the time of birth (i.e. postpartum age) or from the time of conception (i.e. postcoital age). The use of postcoital age has been shown to be a better predictor of the age that preweaning developmental landmarks are achieved than the use of postnatal age (Raimondo and Draghetti, 1990). This is particularly important when the duration of gestation is affected (either increased or decreased) by treatment, since this affects the stage of offspring development at the time of birth.

4.3.3 Functional Observations

The DNT guideline requires functional observations in both dams and offspring, at multiple time points. In the EPA DNT guideline, these assessments are less extensive than those required for the neurotoxicity screening studies in adult rats (US EPA, 1998c), and consist of evaluation in and outside the home cage for autonomic function, abnormal movements (including convulsions and tremors), posture and gait abnormalities, and unusual or abnormal behaviours. In addition, observations in offspring should be age-appropriate.

Presentation of data for these evaluations varies considerably across laboratories. It is important to look for trends across days, as well as differences in treatment groups for any given evaluation. It is also important to note that not all neural systems are fully developed at birth, and expected responses may differ for specific ages of animals. In our experience, this end point is not very sensitive to detecting treatment-related effects in DNT studies because of the limited nature of the observations; however, some chemical classes are known to produce effects on these end points (for example, tremors may be seen with exposure to pyrethroids). In those cases, failure to elicit effects on these end points during the dosing period may raise questions regarding the adequacy of the administered doses.

4.3.4 Auditory Startle

Auditory startle habituation is evaluated twice in offspring: once around the time of weaning and once towards the end of the study (in adult animals). In this paradigm, sound bursts are presented to the animal repeatedly, at regular intervals and whole-body motor response is measured. Over time, the response magnitude will decline, as the animal adapts to the repeated presentations. The neural circuits involved in this behaviour are well understood, and techniques exist that allow identification of the specific neural lesions leading to changes in this response (Adams, 1986; Buelke-Sam *et al*., 1985; Cassella and Davis, 1986; Crofton, 1992; Crofton and Sheets, 1989; Crofton *et al*., 1994; Davis, 1984; Geyer and Swerdlow, 1998; Ison, 1994; Koch, 1999; Marable and Maurissen, 2004; Muijser, 1994). Parameters evaluated in the DNT study include the peak response amplitude, latency to respond and change in response amplitude over repeated trials (habituation). Results are usually presented as mean response for five blocks of 10 trials each. In some studies, prepulse inhibition may also be evaluated; in that procedure, the startle stimulus is preceded by a prepulse of a pure tone, and the magnitude of response with and without the prepulse is compared (Crofton, 1992; Crofton *et al*., 1994). As might be inferred from the title, the use of a prepulse normally attenuates the response to the startle stimulus. Changes in the magnitude of this inhibition can be a sensitive indicator of a change in the function of the neural systems underlying this behaviour.

Auditory startle data should be evaluated by looking at differences in response amplitude among treatment groups, both with respect to mean values for each block of trials and differences in habituation rate, as these end points may respond independently to nervous system toxicity. Baseline response amplitudes can vary considerably among labs, and depend to some extent on initial settings and calibration of the equipment used (this is especially true for accelerometers, where the gain values may be set differently to accommodate the

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difference in weight of animals as they grow). However, within a laboratory or a study, the baseline values should be comparable between control and treated animals. Although it has been suggested that differences in animal weight (perhaps due to other types of toxicity) may confound interpretation of auditory startle data, in our experience this has not been the case.

In some cases, excessive variability may obscure treatment-related effects (Raffaele *et al*., 2008; Sette *et al*., 2004). Selection of parameters such as interstimulus interval, background noise and stimulus strength (i.e. loudness) can affect responses for this procedure (Crofton, 1992; Geyer and Swerdlow, 1998; Raffaele *et al*., 2008). Selection and control of these variables is very important to obtaining quality data.

4.3.5 Motor Activity

In the DNT guideline study, motor activity is evaluated four times in offspring: three times during lactation (postnatal days 13, 17 and 19), and once in adults. The evaluations are performed in an automated recording chamber, with a sufficient duration to ensure that habituation (a decrease in activity over time) will occur. The three evaluations during lactation allow assessment of the normal development of this behaviour. Both the activity levels (total activity over the duration of the record, usually ranging from 40 to 60 minutes per session) and pattern of activity (the change over time, recorded for consecutive (e.g. 5–10 minute) intervals within the longer session) have been shown to change during development in a reliable way (Ruppert *et al*., 1985), although the exact pattern may depend to some extent on the environment or animal strain. Evaluation of whether or not the measured behaviour is consistent with the expected developmental pattern allows an assessment of whether neural development is proceeding normally in treated offspring. Failure to see expected developmental patterns in control offspring may raise concern regarding the conduct of the testing, and may complicate interpretation of treatment-related changes.

Similarly to auditory startle habituation, motor activity levels and variability in this behaviour can be influenced by many environmental factors, including lighting, extraneous noises and odours, test-device size and configuration, animal handling, and so on. Thus, it is very important that all these factors be well controlled, in order to ensure that data are reliable. In addition, the recording chambers need to be configured so that they accurately record activity in young animals. For example, inappropriate placement of photocells (i.e. height and distance apart) could result in decreased sensitivity (recording of fewer beam breaks) for smaller animals.

Motor activity, like auditory startle habituation data, should be evaluated both with regard to total activity level (for the entire session, as well as for subsessions) and to habituation (the expected decrease in activity with continuing time in the chamber). These parameters can change independently; for example, the data in **Figure 2** show two groups with the same total activity levels, but very different patterns over time. In some types of chambers, females will have higher activity levels than males. In addition, different activity patterns have been seen with different types of devices and different types of measurement systems. Laboratories should be able to demonstrate stable behaviour in control animals over time (Crofton *et al*., 1991; Crofton and MacPhail, 1996; MacPhail *et al*., 1989; Moser *et al*., 1997). Large shifts in baseline within a given laboratory may raise concern about the reliability of the data for this end point.

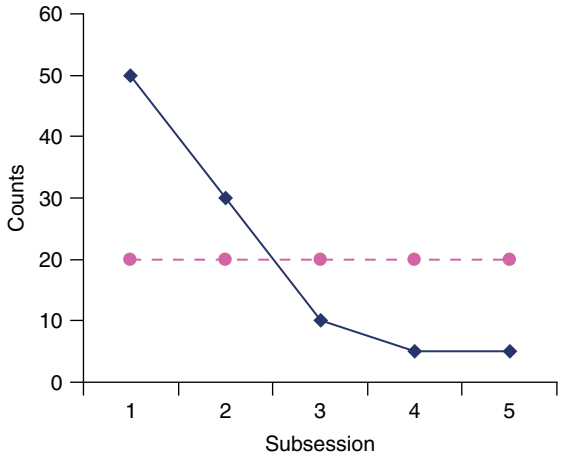

Figure 2 Each line represents activity levels for a group of animals, measured in five equal time intervals. Although the total activity levels for each group are equal (100 counts), the activity patterns are very different. The pattern represented by the diamond shapes shows the type of habituation typical for adult rats in most device types.

Failure of treated animals to demonstrate expected patterns of motor activity, both within a session and with respect to the expected developmental changes in activity patterns is indicative of a treatment-related effect. Treatment-related effects can result in either increases or decreases in activity; and some chemicals may cause both types of changes, depending on the exposure level (i.e. a U-shaped (or inverted-U-shaped) dose–response curve) or age at assessment (see additional discussion below of paradoxical dose–response curves).

4.3.6 Cognitive Testing

The test guideline requires an evaluation of 'learning and memory' at two time points: around the time of weaning and in adult animals (similar to assessments required for auditory startle habituation testing). The guidelines do not specify a particular type of assessment, except to state that both associative learning and retention should be evaluated; broad discretion is left to the testing laboratory regarding the particular procedure to be used.

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Recent guidance (OECD, 2008a) has also indicated that either different tests or different animals should be used at the two time points, in order to avoid confounding the results based on retention of previously learned procedures.

To date, studies performed for regulatory purposes have used a fairly limited group of testing procedures, consisting, for the most part, of single choice point position discrimination (T- or Y-) mazes, the Biel water maze, the Morris water maze or a shock-motivated passive avoidance procedure (Bammer, 1982; Brandeis *et al*., 1989; Bushnell, 1982; Crofton *et al*., 1993; D'Hooge and De Deyn, 2001; Green and Stanton, 1989; Lochry and Riley, 1980; Morris, 1984; Peele *et al*., 1990; Vorhees, 1987; 1997; Wier *et al*., 1989). Details of the protocols vary greatly among laboratories, and the sensitivity of some of the procedures has not been well documented (Raffaele *et al*., 2004; 2006).

In interpreting results of these evaluations, it is important to examine both the learning (change in performance across trials) and retention of the task. Learning is usually measured as a decrease in errors or latency to reach a goal, across trials or sessions. These data are easiest to evaluate when plotted in the form of a learning curve. Retention is usually evaluated by comparing the first trial during the retention session with the last trial of the learning session. As an illustration, the time for L6 to R1-1 or R2-1 can be compared in **Figure 3**. The degree of retention will depend on the strength of the initial learning as well as the length of the delay between the final learning trial and the first retention trial; a decline in performance across these two trials would be attributable to a lack of retention. It is very important that both aspects (learning and retention) be assessed, as many substances have been demonstrated to affect one but not the other. In addition, to the extent possible, data for multiple measures for performance (for example errors, latency or path to goal) should be examined, since different measures may reflect different aspects of task performance (cf. **Figure 4**, where the increase in errors with dose is much more apparent than the increase in latency, especially for the low-dose group). Latency data may in some cases be greatly affected by changes in motor behaviour (for example, swim speed may change or the animal may respond abnormally to a shock stimulus). Where available, control procedures (such as latency to traverse a straight channel or otherwise escape from an aversive stimulus) can help to sort out these types of effects. In the absence of appropriate control procedures, the only alternative is to assume the effect is related to treatment, even if the specificity of the deficit to learning or memory function is unclear.

As for other types of behaviour, it is important to assess the performance of control animals. First, it must be determined whether animals in the control group have demonstrated both learning and retention of the task. It is not possible to determine whether treated animals are impaired by comparing them against controls performing at or near chance levels. Conversely, the sensitivity of a particular task may be questionable if controls perform perfectly in every trial but the first. For such a task, a very severe impairment would likely be needed before a performance decrement could be detected. As with other types of tasks, high levels of variability may impede the sensitivity of the task to detect decrements, especially with respect to the power of the statistical analysis. In some cases, increasing the number of subjects may resolve this problem, but in other cases changes in the task parameters may be required.

Any change in task performance should be considered potentially adverse, at either time point. Potential effects of concern are not limited to a failure to learn or remember the task, but also include a change in the rate at which it is learned. Evaluation of a change in the

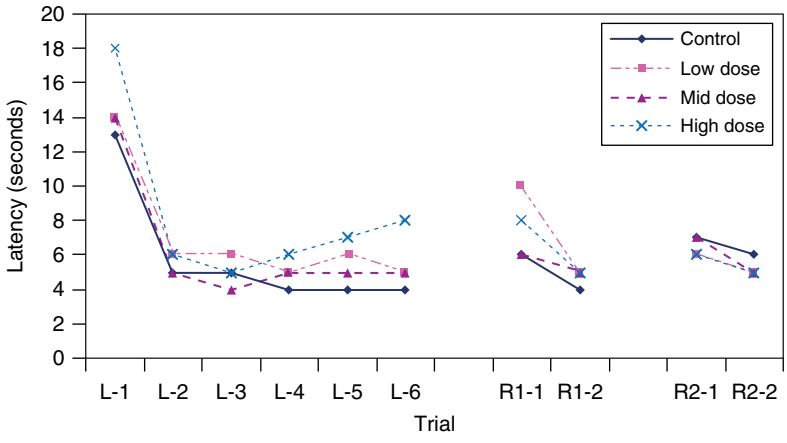

Figure 3 Learning curve for a water maze task, showing latency to goal for six learning trials on one day (L1–L6) and two sets of retention trials (R1 and R2) with a five day delay between each set of trials. Most groups show good retention (i.e. latency for trials R1-1 and R2-1 are similar to those for L-6). The low-dose group shows some decrease in retention for R1, but not for R2.

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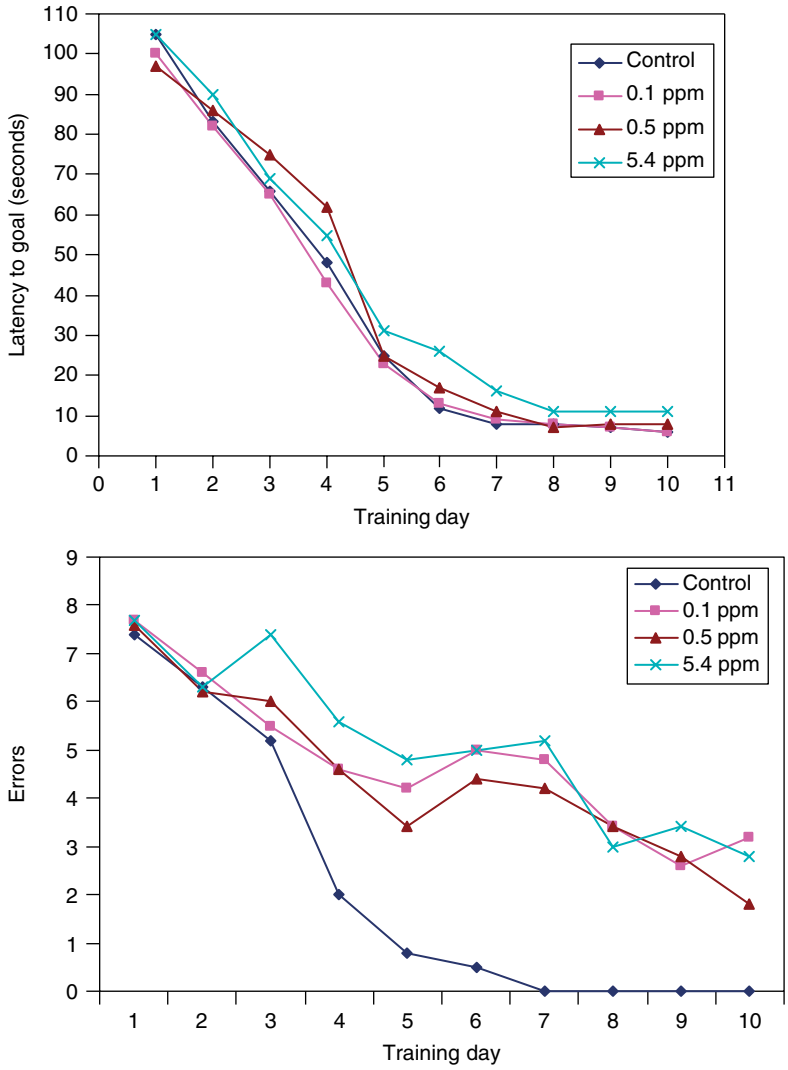

Figure 4 Learning curves for a water labyrinth test in rats, showing the changes in latency (a) and errors (b) over test days. The low-dose group shows no change in latency when compared with controls, but has an increase in errors starting with day 4. This figure also demonstrates the importance of examining the entire learning curve, as the mid- and high-dose groups show increased latency most prominently on day 4, with the mid-dose showing latencies similar to controls by day 5 and the high-dose latencies approaching control levels towards the end of training. Note that errors for all treated groups remain high through the end of training on day 10. (Data extracted from Malek *et al*., 2003b, p. 280.)

rate of learning requires that data be presented for all trials (or blocks of trials) sufficient to allow evaluation of the learning curve. Results reported as differences in errors/trial, averaged over the entire task or a single trials-to-criterion number (without indicating the pattern of errors (i.e. the sequence of 'failed' and correct trials) will not allow adequate assessment of the overall learning process). Although it is sometimes tempting to regard some types of changes (e.g. a decrease in latency to goal) as 'improvements' (and thus not of concern), this is rarely an appropriate interpretation. For example, decreased latency may result from hyperactivity; increased latency (which would result in improved performance in some types of avoidance tasks) could result from impaired motor abilities). As noted above, in many cases sufficient data are not available to determine whether changes in performance in 'learning and memory' tasks are due to specific cognitive deficits or to some other type of performance impairment. Appropriate control procedures (for example, activity levels or speed in a straight alley) may allow improved interpretations of these types of outcomes, but these types of data are often not available for guideline studies.

4.3.7 *Social Behaviours, Attention, Anxiety/Stress*

Changes in social behaviour, attention and anxiety/stress levels are not specifically evaluated in the current recommended guideline test battery, although the OECD

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guideline does recommend that they be considered in some situations. A number of procedures have been developed that assess anxiety and social behaviours (Branchi *et al*., 2001; Lapiz-Bluhm *et al*., 2008; Malatynska *et al*., 2007), and for the evaluation of different aspects of attention (Bushnell, 1998); some of these paradigms have been used in drug development (Kalueff *et al*., 2005; Malatynska *et al*., 2005).

4.3.8 Postmortem Observations (Brain Weight, Gross Pathology, Neuropathology, Including Morphometry)

The DNT guideline recommends three types of assessments for neuropathology: brain weight, qualitative neuropathology and quantitative neuropathology in the form of linear morphometric measurements of at least three brain regions (neocortex, hippocampus and cerebellum). Assessments are performed at least twice: once in pups (either on postnatal day 11 or 21) and once in adults. Neuropathology assessments performed after PND 11 should use perfused tissue; staining with, at a minimum, haematoxylin and eosin, is recommended. Adult assessments should include tissues from the peripheral nervous system, as well as the brain. Brain weight can be evaluated using either fresh or post-fixed tissue.

For developing animals, special attention should be paid to evaluating structural changes indicative of developmental insult. Examples of these types of changes include death of neuronal precursors, abnormal proliferation or migration, changes in size or shape of brain regions (or evidence of hydrocephalus) or abnormal differentiation. Cellular alterations and tissue changes should be evaluated in both young animals and adults.

Recent evaluation of submitted DNT data has demonstrated that all three types of neuropathologic evaluation provide important information about brain pathology, and that no one measure is reliably predictive of effects on the others (Raffaele *et al*., 2005). This result reinforces the importance of using multiple types of measures to more completely examine the different types of neuropathology that may result from developmental exposures. As might be anticipated, differences in brain weight were more commonly seen in assessments performed at the early time point. Although in some cases brain-weight decreases were seen concomitant with body weight decreases, it is important to note that body-weight decreases were also commonly seen in the absence of brain-weight decreases; a causal relationship between these two measures should not be assumed.

In spite of initial concerns regarding the difficulty of reliably assessing neuropathology, and in particular the conduct of linear morphometric measurements, in the smaller and less robust brain tissue of neonatal animals, experience has shown that these measurements can be reliably performed (Crofton *et al*., 2001) and are sensitive to detection of treatment-related effects (Raffaele *et al*., 2005). Evaluation of data submitted to the EPA indicates that changes in linear morphometric measurements are, in many cases, a more sensitive indicator than changes in brain weight or neuropathology, and that treatment-related changes in this measure are more likely to be seen at both assessment times than are changes in brain weight (Raffaele *et al*., 2005). It is possible that more detailed qualitative neuropathological assessments, using additional tissue-staining techniques and/or evaluation of more brain sections, would result in greater sensitivity than the techniques currently prescribed in the test guidelines. Regardless, it appears that all three types of assessments, and both time points, provide information critical to a thorough neuropathological assessment of the developing nervous system.

4.3.9 Exposure Issues

As noted above, in conducting or evaluating DNT data it is important to include consideration of the actual exposure to the developing organism. The brain and nervous system continue to develop and mature for a considerable period after birth; different neural systems have different maturation periods and patterns, and their sensitivity to developmental insult will vary accordingly (as discussed in more detail above). Exposure limited to the *in utero* period may fail to adequately evaluate potential adverse effects on the development of neural systems that mature after birth. This is especially critical when using data from rats to evaluate potential human DNT, since many systems that mature prenatally in humans mature postnatally in rats.

The optimal DNT study would include exposure during a period equivalent to that expected for the human population of concern. If postnatal exposure is anticipated, it is important to document postnatal exposure in the animal model. For compounds without adequate lactational transfer (i.e. those not excreted in the milk), direct offspring exposure may be needed. Initial concerns about possible adverse effects on the offspring due to direct dosing have not been substantiated (Makris *et al*., 2005; 2006; Moser *et al*., 2005; Zoetis and Walls, 2003). Pharmacokinetic studies evaluating lactational transfer and tissue levels in offspring and maternal animals provide valuable information regarding actual exposure levels that can be used to document offspring exposure and potentially determine the specific exposure levels leading to adverse responses in the offspring. This type of information is particularly useful in a regulatory setting, in determining exposure levels below which adverse effects would not be expected.

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5 EVALUATION AND INTERPRETATION OF DATA

A number of issues regarding interpretation of DNT data, including those related to evaluation of specific end points, have been discussed above. It is important to emphasize here that data need to be interpreted in the context of the developmental exposures and the apical assessments used in the screening battery approach. As discussed previously, development represents a moving target to toxic agents. As the various systems of the nervous system are generated and become mature, each time point presents new opportunities for different types of adverse effects to be caused or different manifestations of an earlier impact to become evident. In the context of a DNT study, this may result in apparently contradictory effects seen during sequential evaluations of the same end point (e.g. an increase in motor activity at one time point, a decrease at another). Given our limited understanding of the mechanisms behind changes in the measured behaviours, it is inappropriate to interpret these apparently contradictory findings as unrelated to treatment.

Related to the likelihood that the impact of exposure to neurodevelopmentally toxic compounds may manifest in different ways at different times, is the likelihood that the impact may vary among different behavioural end points. The DNT study was designed as a test battery with the goal of assessing a wide spectrum of functional consequences. Although there is some overlap in the neural correlates of the various behaviours, many unique systems are involved in different types of behaviour. Thus, adverse effects detected in only one type of test (e.g. motor activity) should be assumed to be related to treatment, even in the absence of effects on other types of test (e.g. auditory startle habituation, neuropathology, learning or memory).

Consistent with other types of toxicity, response to treatment may vary by sex, and an effect seen in only one sex should not be dismissed on that basis. In some cases, appropriate statistical analyses will demonstrate that both sexes have similar responses with statistical significance achieved only in one; in other cases differences in metabolism or hormonal status may be the source of variable responses by sex.

In interpreting the results of specific behavioural assessments, it is important to keep in mind the common occurrence of nonlinear (including U- or inverted U-shaped) dose–response curves for behavioural end points. One example of a compound causing this type of effect might be a stimulant, such as amphetamine, or an intoxicant, such as ethanol: motor activity increases following lower doses, but as the dose continues to increase the activity will start to decrease; ultimately the animal will lose consciousness and motor activity will cease altogether. Depending on which points on the dose–response curve were evaluated, this changing response might also appear to be a plateau (i.e. if doses at opposite points on the increasing and decreasing side of the curve were evaluated), or as an initial increase above normal followed by a decrease below normal (if doses sampled early and late phases of the curve). An example showing this type of response is depicted in **Figure 5**. These complex dose–response curves, when combined with the limited number of doses assessed in a typical DNT study, can make data interpretation difficult. However, it is important to consider the possibility of this type of effect, given the frequent temptation to dismiss effects that fail to show the paradigmatic linear dose–response relationship seen for many standard toxicology end points. Dose–response curves of unusual shape can also complicate attempts to mathematically model these data.

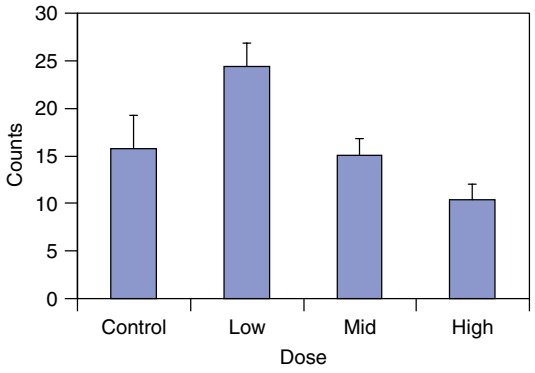

Figure 5 Wall-climbing counts during a three minute open-field motor activity evaluation in rats. These data demonstrate a typical U-shaped dose–response curve, with an increase in counts at the low dose, no change from control at the mid dose, and a decrease in counts at the high dose (changes at both the low and high dose were statistically significantly different from control). (Data extracted from Malek *et al*., 2003a, p. 164.)

Numerous groups have also emphasized the importance of using well-designed procedures and well-trained personnel in order to achieve reliable results in behavioural studies (Crofton *et al*., 2004; 2008; Slikker *et al*., 2005). In the absence of positive control information demonstrating the ability of the procedures to detect treatment-related changes in performance induced by agents known to impact a particular type of behaviour, the failure to detect a treatment-related effect of a test agent cannot be reliably interpreted as a negative result (Crofton *et al*., 2004; 2008). A series of reports evaluating methodologies and results from DNT studies submitted to EPA have found large differences in the stability and variability of control data from different laboratories, for a number of DNT end points (Crofton *et al*., 1991; 2004; Makris *et al*., 2005; 2006; Raffaele *et al*., 2003; 2004; 2005; 2006; Sette *et al*., 2004). These results reinforce the importance of careful evaluation and interpretation of results

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from each study with respect to the appropriateness of the procedures used, their sensitivity in detecting the effects of concern and the quality of the resulting data.

It is also important to note that not all types of effects on the nervous system will be detected by the types of behavioural screens included in the guideline DNT study. The lack of specific evaluations of social behaviour, anxiety or attention have already been noted above. In situations where previous information about a chemical (or structurally related chemicals), or knowledge about a mechanism of action, may indicate the potential for adverse effects unlikely to be detected by standard guideline procedures, it may be important to add evaluations, or conduct additional studies, that will specifically address those effects of concern. Two examples where such chemical- or class-specific testing has been used to enhance the evaluation of DNT include the evaluation of neuroreceptors and delayed behavioural outcomes for the pyrethroid pesticides deltamethrin and bioallethrin (Eriksson and Fredriksson, 1991), and the conduct of comparative cholinesterase studies for food-use organophosphorus pesticides, as required by the US EPA Office of Pesticide Programs (Makris, 2006). As another example, potential refinements in staining for neuropathological procedures for DNT testing are discussed in the OECD Guidance on Mammalian Reproductive Toxicity Testing and Assessment (OECD, 2008a). Another approach to DNT screening is to evaluate precursor changes, such as with thyroid-disrupting pesticides, where conduct of a comparative thyroid study (US EPA, 2005) might preclude the need to conduct a standard guideline DNT study.

Several recent efforts have reviewed the current DNT guideline, and have made recommendations for refinement of methodologies (Cory-Slechta *et al*., 2001; Dorman *et al*., 2001; Garman *et al*., 2001; Kaufmann and Gröters, 2006) and improving conduct and interpretation of the studies (Crofton *et al*., 2008; Fenner-Crisp *et al*., 2005; Holson *et al*., 2008; Raffaele *et al*., 2008; Tyl *et al*., 2008). In addition, the historical development and reliability of the DNT methodology has been reviewed (Makris *et al*., 2009). These efforts have generally agreed on the utility of the study and the reliability of the methods, and have provided specific recommendations for future improvement.

6 USE OF DNT IN HAZARD/SAFETY ASSESSMENT AND RISK ASSESSMENT

6.1 Pharmaceuticals and Food Additives

Preclinical screening of pharmaceuticals for adverse developmental outcomes (including DNT) is conducted according to guidelines published by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH, 2005). This guideline specifies that in a study for effects on prenatal and postnatal development, an assessment of physical development, sensory functions, reflexes and behaviour also be conducted in F1 offspring. While the guidance mentions several developmental landmarks that might be recorded, it does not make any specific recommendations regarding methods for assessing sensory functions, motor activity or learning and memory. A report by Middaugh *et al*. (2003) described a survey that was designed to evaluate the contribution of F1 neurobehavioural testing to hazard identification and characterization in safety-assessment studies. This survey included studies completed since 1990 in industrial laboratories in the US, Europe and Japan. Studies on 179 compounds were included in the analysis, and the majority (81%) of them were pharmaceuticals. Middaugh *et al*. (2003) found that even though the F1 behavioural end points were affected by test compounds less often than general toxicology parameters, these end points (along with other parameters) defined the no observed effect level (NOEL) in 15% (17/113) of the studies, and solely defined the NOEL in 2.6% (3/113) of the studies. Unfortunately, this study did not indicate whether the studies in which the behavioural end points defined the NOEL were for pharmaceuticals or some other type of agent. Nevertheless, Middaugh *et al*. (2003) concluded that the behavioural testing sometimes improved on the standard toxicological measures used in hazard identification, in part because it requires measurement of outcomes not assessed in other preclinical testing.

For preclinical testing of agents that are intended to be marketed as paediatric pharmaceuticals, testing in preweaning animals may be indicated. General guidance for such testing has been published (US FDA, 2006). Studies for known neuroactive agents may be designed to provide a general screen for neurological changes in multiple domains (e.g. as does the US EPA guideline DNT study) or may be designed to address specific parameters or concerns.

For food additives, which are regulated through the FDA Center for Food Safety and Nutrition (CFSAN) in the US, testing guidelines are published in the 'Redbook' (US FDA, 2000a; 2000b). Notably, however, a DNT guideline is not included. The need for DNT testing is considered on a chemical-by-chemical basis. Acrylamide is an example of a food additive for which a DNT study was conducted (Wise *et al*., 1995), although this study did not affect the acceptable daily intake (ADI).

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6.2 Agricultural, Industrial and Environmental Chemicals

As previously mentioned, in the context of toxicological screening and testing to support human-health risk assessment and chemical regulatory activities, the DNT study satisfies an information need that is not addressed by other test guidelines. It is quite notably recognized as the only available standardized test guideline that includes functional, behavioural and anatomical evaluations of the nervous system at multiple time points, and in test subjects that were exposed to test substance during critical pre- and early postnatal periods of nervous system development. The DNT study is an important tool in addressing concerns about the potential for increased risk of developmental neurotoxic outcomes following early life exposures to pesticides and other substances and the need for more rigorous assessment (Brent *et al*., 2004; NRC, 2000; Weiss *et al*., 2004).

The basic risk-assessment paradigm is comprised of four components: hazard identification, dose–response assessment, exposure assessment and risk characterization (NRC, 1983). The hazard identification and dose–response assessment includes the overall toxicological evaluation (for epidemiological as well as animal data). It characterizes the sufficiency and strength of the data set, discusses the confidence in the data, and may include some modelling of the toxicological outcomes to better characterize dose–response. Exposure assessment derives estimates of actual or potential human exposure, based upon various scenarios (e.g. environmental, occupational or residential). The risk characterization integrates the toxicology and exposure data and is used along with other factors (e.g. social, economic and engineering) in the process of risk management. The potential use of DNT data in risk assessment has long been recognized and has been described by Kimmel (1998).

Evaluations of the use of the DNT study in human health risk assessment have been conducted by the US EPA Office of Pesticide Programs (OPP). As noted in **Table 3**, the largest number of guideline DNT studies that has been conducted to date has been on pesticides. The reason for this preponderance of pesticide DNT studies is that the US EPA OPP has conducted a concerted effort over the past decade to obtain information on DNT for specific pesticides to satisfy the mandates of the US FQPA (FQPA, 1996). In addition, over the years, the EPA has participated in a continuing analysis of and discourse on the conduct of DNT studies, the interpretation of the data from these studies and their regulatory impact.

The first analysis that was conducted was a retrospective review of 12 DNT studies (Makris *et al*., 1998) that had been submitted to the EPA Office of Pollution Pesticides and Toxic Substance (OPPTS) in support of the registration and/or use of nine pesticides and three solvents. For the nine pesticides examined, the EPA analyses concluded that the NOEL for the DNT study was lower than that of the foetal NOEL from the prenatal developmental toxicity study (OECD, 2001a; US EPA, 1998a) for eight of the nine pesticides tested, and demonstrated an equivalent dose for one. The offspring NOEL for the DNT study was lower than the offspring NOEL for the reproduction study (OECD, 2001b; US EPA, 1998b) for six of the nine pesticides, and equivalent for one. Overall, in two of nine cases, the NOEL for DNT was lower than or equal to that for any adult or offspring end point from the prenatal developmental, reproduction or adult neurotoxicity (OECD, 1997; US EPA, 1998c) studies. Although this review was limited by the small database of DNT studies available in 1998, it clearly indicated that the DNT study, because of its unique end points, which are not examined in any other Test Guideline, enhances the detection of neurobehavioural and neuropathological effects in offspring following exposure during sensitive periods of neurological development. It also supports the conclusion that the DNT study is often identified as a sensitive study in a chemical database, as well as an important source of quantitative and qualitative information for risk assessment.

This analysis was presented to a FIFRA Scientific Advisory Panel (SAP) (US EPA, 1999), which also reviewed the use of the DNT study in risk assessment. The Panel agreed that DNT study results are appropriate for use to support acute and chronic dietary risk assessments and short- and intermediate-term occupational and residential risk assessments for pesticides. As previously noted, the DNT study utilizes exposures of approximately 25–40 days duration, during critical stages of nervous-system development. Even a single toxicant exposure during a critical developmental period has the potential to result in an adverse developmental outcome to the nervous system (Rodier, 1980; 1986; 1994; Rice and Barone, 2000). Therefore, OPP has used the DNT study outcomes in risk calculations for acute (single dose) reference doses (RfDs), and for short-term (1–30 days) and intermediate-term (1–6 months) nonoccupational exposures to pesticides. Although these particular risk calculations are not utilized by all EPA Offices and Programs, they are nevertheless especially applicable to risk assessments for children (Makris *et al*., 2008). Additionally, in accordance with common risk assessment practices used across the EPA (US EPA, 2002a) and in other regulatory programmes world-wide, the results from DNT studies have also been used for chronic risk assessments (i.e. in calculating a chronic RfD for lifetime exposure to a toxicant) when it has been shown to be the most sensitive study in the toxicology database.

To further pursue the issue of how the DNT study is being used in OPP risk assessment, an updated survey was conducted by Rowland *et al*. (2007). In this study, the specific use of the DNT study in the selection of

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end points and doses for the risk assessment was examined; this was a different approach than was taken by the 1998 retrospective analysis which compared the NOELs of DNT studies with other studies in the various chemical databases. The Rowland *et al*. analysis found that for 58 pesticide chemicals where a DNT study had been considered in the weight-of-evidence review of the toxicology database, the DNT study was utilized to select end points and doses for risk assessments for eight of those chemicals. Four studies were used to establish an acute RfD, four were used for a chronic RfD and six were used for short- or intermediate-term nondietary (e.g. residential) risk assessments. A single study was sometimes used for multiple risk-assessment scenarios. One particularly notable finding of this review was that for four of the eight DNT studies used to select end points and doses for risk assessment, the critical effects either included or were based solely upon offspring behavioural and neuropathological tests that are not evaluated in other guideline studies (i.e. motor activity, auditory startle habituation, learning and memory and morphometric analysis of the brain). Also, based on an examination of the doses and effects identified in the OPP DNT study reviews, in comparison to those used as points of departure in the most recent published OPP risk assessment for the same chemicals, 17 cases were identified where an end point from a DNT study could potentially be selected for use in one or more risk assessment scenarios in future risk assessment actions. The findings of the Rowland *et al*. (2007) review were concordant with the conclusions of the 1998 retrospective analysis by Makris *et al*. Further evidence was provided regarding the sensitivity of the DNT study in identifying adverse effects of chemical exposures during development, and the important role of DNT studies in human-health risk assessments.

In addition to using DNT data for regulatory decisions, some regulatory agencies have also, on a case-by-case basis, incorporated an additional database uncertainty factor into their regulatory decisions because of the absence of DNT data. The use of these uncertainty factors in risk calculations reflect regulator views that DNT data are valuable in establishing permissible exposure levels, and reducing uncertainties about the toxicity of the chemicals (US EPA, 2002a; 2002b).

7 FUTURE DIRECTION AND RESEARCH NEEDS

Diverse groups have advocated increased testing for DNT (Andersen *et al*., 2000; Grandjean and Landrigan, 2006; NRC, 1992; 1993; Nelson, 1986; OTA, 1990; Stein *et al*., 2002; Vorhees, 1986). Although the overall performance of the DNT test method and its ability to detect effects of concern from a regulatory perspective have been well established (Makris *et al*., 2009), there remains an intense focus on this test method. Some critics of the study argue that specific tests (e.g. assessment of cognitive and sensory dysfunction) are insensitive; on the other hand, others believe that the tests included in the DNT study are overly sensitive and have a high rate of false positives (Claudio *et al*., 1999; 2000; Cory-Slechta *et al*., 2001; US EPA, 1995; 2006). Recommendations have been made to include evaluations of end points not currently assessed in the DNT study; examples are social behaviour (Cory-Slechta *et al*., 2001), pharmacokinetics and neurochemistry (Andersen *et al*., 2000; Dorman *et al*., 2001), and latent changes (e.g. during senescence) that are attributable to developmental exposures (Cory-Slechta *et al*., 2001). Criticisms of the study complexity have been accompanied by the suggestion to delete some test components from the protocol (Li, 2005) or to utilize screening approaches that incorporate DNT testing into other protocols (Cooper *et al*., 2006; Ladics *et al*., 2005). Some critics of the DNT study say that for some end points (e.g. motor activity, morphometrics) variability is too high (CMA, 1987; Nolen, 1985; York *et al*., 2004), and others suggest that this *in vivo* test is not necessary to detect DNT (Balls and Combes, 2005). These various and diverse opinions demonstrate a need for ongoing scientifically based evaluation of the test method and the incorporation of appropriate revisions commensurate with advances in scientific knowledge and increased experience with the analysis, interpretation and use of the DNT study.

There are a number of efforts currently underway that are intended to review data from existing DNT studies, identify ways to refine the DNT test and, to the extent possible, reduce the number of animals used in screening for DNT, for example, by applying certain statistical approaches to the behavioural analysis (Chiarotti and Puopolo, 2000; Puopolo, 2004), or through the use of alternative tests (Lein *et al*., 2007). Reviews of historical and positive control data have demonstrated the need for more standardized reporting requirements (Crofton *et al*., 2004; 2008). Further retrospective reviews of control data have identified differences among laboratories in data quality and variability (Crofton *et al*., 1991; 2004; Raffaele *et al*., 2003; 2004; 2008; Sette *et al*., 2004), while another review of various neuropathology assessments conducted in DNT studies identified low variability for measures of brain weight, qualitative histopathology and quantitative (morphometric) evaluations of the brain (Crofton *et al*., 2001), further concluding that no one post-mortem measure is more sensitive than the others, with each providing important data (Raffaele *et al*., 2005). The outcome of these continuing efforts will be to enhance data interpretation, help refine requirements for future testing and guide and promote the development of new methods.

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While recognizing the need to refine and enhance the current DNT testing paradigm, there is also a driving need to apply new methodologies and approaches to DNT screening, in the spirit of the recent report on *Toxicity Testing in the 21st Century* (NRC, 2007). At this time, many thousands of chemicals which have a high potential for human exposure (e.g. high-production-volume chemicals, pesticide inert ingredients and antimicrobial pesticides) are lacking in the most basic toxicological screening data (NRC, 1984). Assessing potential neurotoxicological effects will be a major challenge for these chemicals, and new tools and methods are likely to be needed (Dix *et al*., 2007; Kavlock *et al*., 2008; NRC, 2007). As previously discussed, the current DNT guideline generates useful data for risk-assessment purposes. However, conducting this *in vivo* study is expensive and time-consuming. Additionally, it uses a relatively large number of naïve animals when conducted as a stand-alone study. Thus, an alternative approach is needed, that incorporates a validated first tier screening paradigm (e.g. a high-throughput *in vitro* screening battery) designed to rapidly assess large numbers of chemicals and predict their potential to cause DNT (Coecke *et al*., 2007; Lein *et al*., 2007; NRC, 2007; US EPA, 2006). When considered in conjunction with appropriate decision frameworks (Combes *et al*., 2003), data from these high-throughput screens could facilitate prioritization of further *in vivo* testing, for example, for substances identified as potentially hazardous under the European regulation on Registration, Evaluation, Authorisation and Restriction of Chemical Substances (REACH) (EC, 2006). Data generated by the current DNT test method will be important to the validation of high-throughput *in vitro* methodologies, by providing information on their utility and limits, as well as guidance on the potential use of data from these alternative methods in a risk-assessment context (Makris *et al*., 2009).

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Drug Toxicity in Neonates, Infants and Young Children

Imti Choonara

CONTENTS

1 INTRODUCTION

It is important to recognize that paediatric patients of all ages are at greater risk of drug toxicity than healthy adults. This is because of several key differences. Firstly, neonates, infants and young children are growing rapidly. Therefore, the organs associated with rapid growth may experience specific toxicities that are not apparent in adults. For example, corticosteroids may result in growth retardation in growing children, whereas this specific drug toxicity is not apparent in fully grown adults. Similarly, substances may have an adverse effect on the developing brain. Corticosteroids used to minimize chronic lung disease in the neonatal period are associated with an increased risk of cerebral palsy.

An additional problem in paediatric patients is that many of the medicines used have not been thoroughly evaluated scientifically before being used widely. Many of the medicines are used in a manner different to that for which they were authorized, that is, they are used in children of a younger age than have been studied previously or used at a different dosage or even administered by a different route to that for which the medicine received a marketing authorization. Such use is termed off-label drug use, and in association with the use of medicines that are unlicensed is a significant problem in children throughout the world (Conroy *et al*., 2000). The risk of drug toxicity in association with unlicensed and off-label drug use in children is greater than that associated with medicines used in the manner for which they were authorized (Turner *et al*., 1999). Unfortunately paediatric health professionals have no other option than to use these medicines in an off-label manner as otherwise children would be deprived of appropriate therapy. It is to be hoped that this situation will improve following European and American legislation which encourages the pharmaceutical industry to perform clinical trials in children (Choonara, 2007).

Another major predisposing factor is the different drug metabolism in paediatric patients of different ages. In general, oxidative processes involving cytochrome P450 enzymes are markedly decreased in neonates. Enzyme activity is dramatically reduced in the premature neonate in particular. Hence, clearance of many drugs is reduced in the neonatal period (de Wildt *et al*., 2003). CYP3A7 activity may compensate slightly for decreased CYP3A4 activity, but overall drug clearance is reduced. This has had major implications in relation to drug toxicity, as documented below.

Phase II metabolizing enzymes such as the UDP-glucuronyltransferases are decreased in neonates

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and infants. For certain medicines (e.g. paracetamol (acetaminophen)) there may be compensatory sulfation. The increased sulfation, however, does not fully compensate for the decreased glucuronidation, and clearance of paracetamol remains reduced in neonates, especially if preterm (Anderson *et al*., 2002). Although the changes in drug metabolism in young children may predispose to drug toxicity, in certain cases it can minimize drug toxicity. In the case of paracetamol, differences in drug metabolism in prepubertal children result in decreased hepatotoxicity following an overdose (Penna and Buchanan, 1991). As well as changes in enzyme activity involved with drug metabolism, there are major changes in relation to volume of distribution, protein binding and renal excretion. These are greatest in the preterm neonate and critically ill neonates and infants.

2 EPIDEMIOLOGY OF DRUG TOXICITY IN PAEDIATRIC PATIENTS

Adverse drug reactions (ADRs) are a significant problem in paediatric patients. A systematic review and meta-analysis of 17 prospective studies suggested that almost 1 in 10 children in hospital experience an ADR, and up to 2% of all paediatric hospital admissions are the consequence of ADRs (Impicciatore *et al*., 2001). More recent studies have shown a similar rate of ADRs occurring in children in hospital and in children admitted to hospital (dos Santos and Coelho, 2006; Fattahi *et al*., 2005). Most of the prospective studies of drug toxicity in paediatric patients have focussed on children. There have been relatively few studies looking at the incidence of ADRs in neonates. The few studies that have occurred have suggested that the incidence of ADRs in neonates on the intensive care unit is considerably higher (10–30%) (Aranda *et al*., 1982; Bonati *et al*., 1990).

Paediatric pharmacovigilance studies carried out in conjunction with regulatory authorities have been successful in identifying less frequent ADRs and, in particular, identifying fatalities (Choonara, 2006). Two studies in particular have reviewed large numbers of fatalities over a longer period of time. One study was performed in the UK and identified 331 fatalities that were suspected ADRs (Clarkson and Choonara, 2002). The group of medicines most likely to be associated with a fatality in this study were antiepileptic drugs. A review of suspected ADRs reported to the Food and Drug Administration in the USA involving infants and children under the age of two years reported over 700 deaths in a period of just over three years (Moore *et al*., 2002). In this study 41% of the deaths involved neonates. Drug toxicity may often not be suspected in a critically ill neonate with multiorgan failure, and hence the actual number of ADRs is likely to be higher.

3 PERCUTANEOUS DRUG TOXICITY

One of the earliest recognized ADRs was the development of methaemoglobinaemia due to aniline dye, which had been used to stamp the name of the institution on nappies (Rayner, 1886). As reported over a century ago, 17 newborn infants developed cyanosis after absorbing the dye percutaneously. Subsequently, there have been other cases of infants developing cyanosis due to percutaneous absorption of aniline dye (Kagan *et al*., 1949). By 1949, a total of seven different reports had identified 63 cases with 5 fatalities (Kagan *et al*., 1949). Many of these infants had a decreased level of consciousness and vomiting. The infants who died all developed pneumonia two to four weeks after developing acute symptoms in association with aniline toxicity.

This ADR illustrates the possibility of enhanced drug toxicity through percutaneous absorption in infants. In general, absorption of compounds is enhanced by issues such as the prolonged contact of a wet nappy with the perineum. Newborn infants have a higher surface-area-to-weight ratio than both children and adults. Therefore medicines that are administered topically result in greater relative exposure in newborn infants. Additionally, a relative increase in the water content of the dermis and a thinner stratum corneum in young infants facilitates transcutaneous diffusion of small molecules. There have been several other examples of percutaneous toxicity occurring both in newborn infants and in children. Antiseptic agents such as hexachlorophene (Powell *et al*., 1973), iodine (Chabrolle and Rossier, 1978) and ethyl alcohol (Püschel, 1981) have been associated with neurotoxicity, hypothyroidism and hypoglycaemia, respectively (**Table 1**).

Hexachlorophene had been used widely as an antiseptic agent in an attempt to reduce staphylococcal infections. Studies in newborn infants showed that, following use as an antiseptic agent on the skin, the compound was detectable in the plasma of newborn infants (Curley *et al*., 1971). Studies in rats had suggested that hexachlorophene may be neurotoxic. Unfortunately this was also shown to be the case in premature, newborn infants. Powell *et al*. (1973) described a spongiform myelinopathy in seven neonates. The infants who developed the spongiform myelinopathy were all premature, weighing less than 1.4 kg, and had been exposed to hexachlorophene washes on more than four occasions (Powell *et al*., 1973). Contamination of a baby powder containing talc with 6.3% hexachlorophene in France resulted in over 200 infants and young children becoming ill (Martin-Bouyer *et al*., 1982; Goutières and Aicardi, 1977). Many of the affected children developed an encephalopathy and 36 died.

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Table 1 Examples of percutaneous drug toxicity in paediatric patients

Drug	ADR	References
Aniline dye	Methaemoglobinaemia	Rayner (1886); Kagan *et al*. (1949)
Hexachlorophene	Neurotoxicity	Martin-Bouyer *et al*. (1982); Powell *et al*. (1973)
Iodine	Hypothyroidism	Chabrolle and Rossier (1978)
Ethanol and methanol	Cardiac arrest Hypoglycaemia Metabolic acidosis Respiratory depression Seizures	Giménez *et al*. (1968)
Ethanol	Hypoglycaemia	Püschel (1981)
Nicotine	Agitation Coma Dizziness Vomiting	Davies *et al*. (2001); Woolf *et al*. (1997)

Percutaneous toxicity is not restricted to the neonatal period. In Argentina it has been a widespread practice to apply alcohol-soaked cloths to the abdominal area of sick children. Giménez *et al*. (1968) described 48 children presenting with alcohol toxicity over a period of 14 months (Giménez *et al*., 1968). The mean age of these children was 11 months. Over half the children were exposed to ethanol and a smaller number methanol. There were a total of 14 deaths and the symptoms ranged through hypotonia, coma, respiratory depression, seizures and hypoglycaemia.

Transdermal nicotine is used in adults to help wean them off cigarette smoking. A study in the USA described reports received from Poison Centres in relation to children who had accidentally been exposed to transdermal nicotine patches (Woolf *et al*., 1997). None of the children came to harm and the symptoms were predominantly gastrointestinal weakness, dizziness or localized rashes. In the UK, there has been a case report of an eight-year-old boy who received a traditional topical remedy for eczema (Davies *et al*., 2001). The traditional remedy contained nicotine and the child developed acute nicotine poisoning (agitation, vomiting, fluctuating level of consciousness). He was admitted to hospital and received treatment for suspected acute meningoencephalitis until toxicology demonstrated high levels of nicotine and cotinine in both blood and urine (Davies *et al*., 2001). This case report is a reminder of the problems associated with traditional remedies.

4 ANTIBIOTIC TOXICITY IN NEONATES

4.1 Sulfonamides and Protein Binding

In 1956, neonates who received a combination of penicillin and sulfafurazole were found to have a significantly higher mortality that those who received oxytetracycline (Silverman *et al*., 1956). There was a higher incidence of kernicterus in the neonates who received the penicillin and sulfonamide combination. These infants developed seizures, opisthotonus, spasticity, oculogyric movements and poor feeding. On postmortem examination the pathological diagnosis of kernicterus was made on the basis of yellow staining of the brain. It is important to note that the authors were unable to provide an explanation for the toxicity observed. They were, however, concerned in relation to the increased mortality and were keen that clinicians knew of the dangers associated with this combination of antibiotics.

Subsequent *in vitro* studies showed that sulfonamides have a higher binding affinity than bilirubin for albumin (Odell, 1959). Studies in rats showed that sulfonamides increased the risk of kernicterus (Johnson *et al*., 1959). It was several years later that it was confirmed that sulfonamides increased the free fraction of bilirubin in the plasma in neonates (Dunn, 1964). The free bilirubin in the plasma crossed the blood–brain barrier, and its deposition in the brain resulted in kernicterus. As a result of the studies by Silverman *et al*. (1956), medicines that displace bilirubin, such as salicylates and ceftriaxone, are not used in ill neonates who are at risk of bilirubin encephalopathy.

4.2 Chloramphenicol

A few years after the reported mortality of sulfonamides in sick neonates, the grey baby syndrome was reported with the antibiotic chloramphenicol (Sutherland, 1959). Infants developed abdominal distension, vomiting, cyanosis, cardiovascular collapse, irregular respiration and subsequently died shortly after therapy with chloramphenicol was started. The following year, studies of the pharmacokinetics of chloramphenicol in

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neonates showed significantly higher plasma concentrations of chloramphenicol in neonates in comparison to levels in older children. The plasma half-life of chloramphenicol in neonates was shown to be 26 hours in comparison with a plasma half-life of 4 hours in children between the ages of four and five years (Weiss *et al*., 1960). The prolonged plasma half-life was due to the reduced conjugation of chloramphenicol to its glucuronide metabolite. Previous studies had shown that newborn infants have decreased conjugation of bilirubin with glucuronic acid, and this is the mechanism for the physiological jaundice that appears in many newborn infants (Brown *et al*., 1958). The high plasma concentrations of chloramphenicol which were responsible for the grey baby syndrome could be avoided by a reduction in the total daily dosage from $100\,mg\,kg^{-1}$ to $50\,mg\,kg^{-1}$.

The drug toxicity in neonates associated with both sulfonamides and chloramphenicol could have been prevented by a greater understanding of the capacity of neonates to handle and metabolize drugs.

5 DEXAMETHASONE THERAPY IN NEONATES, AND DEVELOPMENTAL DELAY

Corticosteroids (usually dexamethasone) have been used for the last 20 years to treat neonates who developed bronchopulmonary dysplasia (Grier and Halliday, 2003). These infants were often ventilator dependent, and dexamethasone was found to be beneficial in weaning the infants off the ventilator and reducing the risk of chronic lung disease (Halliday *et al*., 2003). In 1998 a follow-up study of these infants, at the age of two years, showed a significantly higher incidence of neurotoxicity in the neonates who received dexamethasone (Yeh *et al*., 1998). Over the next two years other investigators confirmed these findings (Kothadia *et al*., 1999; Shinwell *et al*., 2000).

Corticosteroids were known to increase the risk of infection, hyperglycaemia and hypertension, but the adverse neurological effects were unexpected. The neurotoxicity was associated with the early use of dexamethasone (Grier and Halliday, 2003). Subsequent studies have confirmed that dexamethasone used in the first few days of life has an adverse effect on the infant, which results in poorer performance in school-age children (Yeh *et al*., 2004). Dexamethasone use later in the neonatal period, however, does not appear to increase the risk of developmental delay (Halliday, 2003). The neurotoxicity associated with corticosteroids in neonates is a reminder that unexpected drug toxicity may occur with drugs that have been extensively used in children and adults. One therefore needs to be cautious with the use of all drugs especially in the sick, preterm infant.

6 SODIUM VALPROATE AND HEPATOTOXICITY

Sodium valproate is an effective antiepileptic drug that is widely used in adults and children. It was introduced in the USA in 1978 and shortly after its introduction was noted to have a hepatotoxic effect (Sussman and McLain, 1979). Within a few years there had been more than 20 fatalities associated with hepatotoxicity in patients receiving sodium valproate (Zimmerman and Ishak, 1982). A detailed retrospective review of 37 fatalities in the USA identified two main risk factors (Dreifuss *et al*., 1987). Children under the age of three years and patients receiving polypharmacy were at the greatest risk. Subsequent studies by the same group have confirmed that young age and polytherapy are the greatest risk factors and that developmental delay and coexisting metabolic disorders are also risk factors (Dreifuss *et al*., 1989; Bryant and Dreifuss, 1996). The risk of developing hepatotoxicity in children less than two years old on polytherapy in all three of these studies was 1 in 600. In contrast, the risk in an adult on monotherapy is less than 1 in 500 000 (Bryant and Dreifuss, 1996). Polytherapy is the most important risk factor, with adults on polytherapy having a 10-fold greater incidence of sodium valproate-induced hepatotoxicity than adults on monotherapy, that is, one in 50 000 (Bryant and Dreifuss, 1996).

The mechanism of the hepatotoxicity is unknown but is thought to be associated with alterations in fatty acid metabolism by sodium valproate or its metabolites. It is recognized that the metabolism of sodium valproate is different in young children. Additionally, the use of other anticonvulsants alongside the sodium valproate may result in the induction of certain metabolic pathways which predispose to the formation of toxic metabolites (Gopaul *et al*., 2003). One of the metabolites of sodium valproate, in particular, has been suggested as a cause of hepatotoxicity, and this is 4-ene valproate (Bryant and Dreifuss, 1996; Zimmerman and Ishak, 1982).

7 SALICYLATES AND REYE'S SYNDROME

Reye's syndrome was originally described in 1963 (Reyes *et al*., 1963). Children usually had a preceding viral infection and subsequent drowsiness which led to coma, hypoglycaemia, seizures and liver failure. The association between Reye's syndrome and salicylates was raised as a possibility two years later by an observant physician who noted that 15 of the 31 cases reported had received aspirin prior to admission (Giles, 1965).

In 1980, evidence supporting the association between Reye's syndrome and the use of salicylates during a

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preceding viral infection was published (Starko *et al*., 1980). During an outbreak of influenza A, seven children were admitted to hospital with Reye's syndrome. These seven children were compared with controls from the same class who did not have signs/symptoms compatible with the diagnosis of Reye's syndrome. All the children with Reye's syndrome took salicylates, whereas only 8 of the 16 controls took salicylates. The patients took a larger dose of salicylates as compared to those in the control group and the level of salicylate consumption was associated with the severity of the Reye's syndrome. Subsequent studies confirmed the association between salicylates and the development of Reye's syndrome in children who were experiencing a viral infection (Hurwitz *et al*., 1987; Hall *et al*., 1988).

The restriction of the use of salicylates as an antipyretic and analgesic in children aged 12 and under has led to a dramatic reduction in the incidence of Reye's syndrome (Belay *et al*., 1999). Several cases were subsequently reported of salicylates leading to the development of Reye's syndrome in children between the ages of 12 and 16 (McGovern *et al*., 2001; Clarkson and Choonara, 2002). In 2002 the regulatory agency in the UK issued advice to doctors to avoid the use of aspirin in children under the age of 16 years as an antipyretic (MCA/CSM, 2002). The mechanism of the toxicity is unknown. It is recognized, however, that both the administration of salicylates and a viral infection are required for the development of Reye's syndrome.

8 VIGABATRIN AND VISUAL FIELD DEFECTS

Vigabatrin is an antiepileptic drug which acts as a selective irreversible inhibitor of gamma-aminobutyric acid (GABA) transaminase. Vigabatrin was found to be particularly effective in the management of infantile spasms. In 1997 visual field constrictions were described in three adults receiving vigabatrin (Eke *et al*., 1997). By 1999 there had been several more cases reported and the regulatory authorities in the UK issued advice in relation to restricting the prescribing and also ensuring that regular monitoring was undertaken in patients receiving vigabatrin (MCA/CSM, 1999).

Vigabatrin is known to accumulate in the retina, and retinal degeneration has been reported in rats following therapy with vigabatrin (Sills *et al*., 2001; Butler *et al*., 1987). The pathophysiology in relation to the visual field defects is uncertain. It is, however, thought to be permanent, despite the occasional case report of reversible changes in visual fields (Versino and Veggioti, 1999).

A study in Finland suggested that the prevalence of visual field constriction in children was lower than that in adults (Vanhatalo *et al*., 2002). The Finnish study suggested that just under 20% of children exposed to vigabatrin experienced visual field constrictions. Studies in adults suggest that one in three patients receiving vigabatrin will develop visual field defects (Kalviainen and Nousiainen, 2001). The risk in men is twice that for women (Kalviainen and Nousiainen, 2001). Despite the risk of visual field defects, the benefits of vigabatrin for the management of young children with infantile spasms are considerable and it is still recommended for use in this patient group (BNFC, 2007; Nabbout, 2001).

9 PROPOFOL INFUSION SYNDROME

Propofol is an ultrashort-acting anaesthetic agent that is widely used for the induction and maintenance of anaesthesia in both adults and children. The short elimination half-life and rapid distribution kinetics of propofol allow patients to recover rapidly upon discontinuation of infusion with less toxicity than other parenteral anaesthetic agents (e.g. barbiturates, ketamine). Propofol has also been used as a sedative to facilitate prolonged mechanical ventilation in critically ill children.

To date, there have been reports of 24 children dying following the use of propofol for nonprocedural sedation (Parke *et al*., 1992; Bray, 1998; Wysowski and Pollock, 2006). These children developed severe metabolic acidosis, lipaemia and progressive cardiac dysfunction (hypotension, cardiac failure, arrhythmias). Many of the children subsequently developed multiorgan failure. As well as the 24 children reported in the medical literature, there were 21 deaths reported to the Food and Drug Administration in the USA (Wysowski and Pollock, 2006). These 21 American cases all involved children receiving propofol for nonprocedural sedation. The majority of cases of propofol infusion syndrome have occurred in children who have received propofol at doses above $4\,mg\,kg^{-1}\,h^{-1}$, and for periods of more than 48 hours (Bray, 1998). However, not all children who receive high-dose propofol for more than 48 hours develop the propofol infusion syndrome (Bray, 1999). The propofol infusion syndrome has also been described in adults. It is, however, less common in adults, like many of the other ADRs described in this chapter (**Table 2**).

The mechanism(s) associated with the production of metabolic acidosis by propofol in children without concomitant hypoxaemia is not known. It has been suggested that propofol at high doses disrupts fatty acid oxidation within the mitochondria (Wolf *et al*., 2001). Another case report described a 10-month-old infant who improved with continuous venovenous haemofiltration, and the authors have therefore postulated that a water-soluble metabolite of propofol is involved in the development of propofol infusion syndrome (Cray *et al*.,

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Table 2 Examples of adverse drug reactions that are more common in paediatric patients than adults

Drug/compound	Age group	ADR	Mechanism
Aniline dye	Neonates	Methaemoglobinaemia	Percutaneous absorption
Sulfafurazole	Neonates	Kernicterus	Protein-displacing effect on bilirubin
Chloramphenicol	Neonates	Grey baby syndrome	Impaired metabolism
Sodium valproate	Young children (<3 yr)	Hepatic failure	Abnormal metabolism?
Salicylate	Children	Reye's syndrome	Unknown Dose related?
Propofol	Children	Metabolic acidosis	Unknown Dose related?

1998). This group also described reduced cytochrome oxidase activity in muscle tissue.

10 EXCIPIENT TOXICITY

It is important to recognize that all drugs contain excipients. These are other substances that are added to a drug in order to increase its solubility, improve its stability or, in the case of oral medications, often to improve its palatability. Some of the major cases of drug toxicity that have occurred in paediatric patients have been in association with excipient toxicity. Diethylene glycol was used as a solvent to make sulfonamides more soluble in water. It was originally used in the USA in 1937 to prepare a solution of sulfanilamide. Unfortunately diethylene glycol is highly toxic, resulting in both hepatic and renal failure. The sulfonamide/diethylene combination was responsible for the deaths of at least 76 American children and adults (Geiling and Cannon, 1938).

It is disappointing that this historical tragedy has been repeated numerous times, with episodes of diethylene glycol contamination of medicines occurring in Argentina, Bangladesh, Haiti, India, Nigeria, South Africa and Spain (Woolf, 1998; Pandya, 1988). In many of the cases either all or a majority of the victims have been children. Diethylene glycol was used as a solvent for paracetamol and resulted in the death of 47 children in Nigeria (Okuonghae *et al*., 1992), 51 children in Bangladesh (Hanif *et al*., 1995) and 85 children in Haiti (O'Brien *et al*., 1998). In the most recent episode (Haiti), the paracetamol was accidentally contaminated with diethylene glycol. Glycerine was used as an excipient for the paracetamol formulation and it was the glycerine that was contaminated with diethylene glycol. In the cases in Nigeria and Bangladesh, it is unclear whether the diethylene glycol was a contaminant or whether it was used unwittingly by a small unregulated manufacturer. The importance of an adequate quality control of medicines for children in developing countries has been previously highlighted (Choonara, 1992; Woolf, 1998).

There have been several other examples of excipient toxicity, especially in neonates (**Table 3**). Benzyl alcohol is used for its antibacterial properties in ampoules of sodium chloride and water that are intended for intravenous administration. Metabolic acidosis, hepatic and renal failure and cardiovascular collapse have been described among premature newborn infants who were receiving multiple injections of sodium chloride for flushing catheters and bacteriostatic water in association with medicines that have been reconstituted (Brown *et al*., 1982; Gershanik *et al*., 1982). Both solutions contained 0.9% benzyl alcohol, which was postulated as the causative agent in neonates and young infants with a reduced capacity to metabolize and excrete this toxic 'inactive ingredient' in the doses that were unwittingly administered.

It has been suggested that benzyl alcohol may have had a far greater impact on both mortality and morbidity than the 10 deaths reported by Gershanik *et al*. (1982). A group in Oregon reported significant decreases in the mortality rate of preterm infants after the use of solutions containing benzyl alcohol was stopped (Hiller *et al*., 1986). The same group also described a significant decrease in intraventricular haemorrhage and subsequent development of cerebral palsy and developmental delay following discontinuation of benzyl alcohol (Hiller *et al*., 1986; Benda *et al*., 1986). Benzyl alcohol toxicity has also been described in association with its use as a preservative in antibiotic injections such as clindamycin (Hall *et al*., 2004).

In October 1983, intravenous vitamin E was being used in neonatal intensive care units in the USA in order to prevent retinopathy of prematurity. Within a few months, case reports of infants developing hepatic or renal failure in association with acoagulopathy were being described (Bodenstein, 1984; Centers for Disease Control and Prevention, 1984). By April 1984, the intravenous formulation of vitamin E had been withdrawn from the market following the death of 38 neonates

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Table 3 Specific examples of excipient toxicity

Error	Drug	Country	Reference
Diethylene glycol used as solvent	Sulfanilamide	USA	Geiling and Cannon (1938)
Contamination of glycerine by diethylene glycol	Paracetamol	Nigeria Bangladesh Haiti	Okuonghae *et al*. (1992) Hanif *et al*. (1995) O'Brien *et al*. (1998)
Contamination with diethylene glycol	Cough expectorant	India	Singh *et al*. (2001)
High concentrations of benzyl alcohol as preservative	Sodium chloride and water	USA	Brown *et al*. (1982) Gershanik *et al*. (1982)
Emulsifiers toxic?	Vitamin E	USA	Bodenstein (1984)

(Phelps, 1984). It was postulated that the emulsifiers used to make the vitamin E water miscible for intravenous use may have been responsible for the deaths (Phelps, 1984).

11 SUMMARY AND CONCLUSIONS

Neonates, infants and young children may experience the same drug toxicity as adults. They may also experience specific drug toxicity that adults do not experience. This may be associated with toxicity related to growth and development, for example the adverse effect of corticosteroids on growth or development of the brain following exposure in the early period of neonatal life. Alternatively they may develop a specific toxicity in relation to a condition that does not occur in adults, for example, Reye's syndrome following exposure to salicylates during the presence of a viral infection.

The altered drug metabolism within paediatric patients is an additional risk factor for drug toxicity. This is most marked in the neonatal period and is illustrated by the impaired metabolism of chloramphenicol which resulted in cardiovascular collapse. Similarly, the altered metabolism of antiepileptic drugs such as sodium valproate in children less than two years of age predisposes this particular age group to hepatotoxicity.

Percutaneous toxicity can occur at any age but is a greater problem in newborn infants who have a higher surface-area-to-weight ratio than older children and adults. Lastly, one needs to recognize that excipient toxicity has been a particular problem in both children and neonates. It is to be hoped that recent legislation within Europe (Choonara, 2007) will result in the pharmaceutical industry spending more money on research into medicines for children. Further, it is to be hoped that this will increase the evidence base for the use of medicines safely and effectively. It is also anticipated that this will improve the availability of formulations created specifically for paediatric patients, which will hopefully prevent major cases of excipient toxicity in the future.

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Toxicology of the Placenta

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CONTENTS

1 INTRODUCTION

Placental toxicology is a fascinating subject, as it deals with three components: mother, placenta and foetus. It encompasses knowledge of structural and functional changes in the placenta, placentation, implantation, embryotoxicity, foetal death, structural malformation, growth retardation and functional deficits. The late nineteenth and early twentieth century was a golden age for placental research, and Mathias Duval (1844–1907) was one of the pioneers in elucidating intricate details of placentas of different mammalian species, notably rodents (Pijnenborg and Vercruysse, 2006). This subject has received much attention from teratologists, toxicologists, pharmacologists, biologists and regulatory agencies since the disaster of thalidomide in the 1960s. An estimated 8000 babies were born with deformities by the use of thalidomide. Since then, a huge number of chemicals have been shown to produce birth defects. In later studies, the placenta has been found to have many enzymes, receptors and transmitters (Kanevsky *et al*., 1997; Hewitt *et al*., 2006), and thereby it has received enormous attention. By now, most researchers are well convinced that the majority of chemicals reach the foetus from the mother by crossing the placenta. Thus, the placental barrier is not a true full barrier, but prevents access of certain chemicals to the foetal circulation.

The placenta is a rapidly developing complex organ which interfaces two separate genomes: mother and foetus. This organ becomes further complicated by multiple pregnancies, which are common in human gestation and the rule in many species like cats, dogs, pigs and rodents (Benirschke and Masliah, 2001). Undoubtedly, the placenta is an evolutionary organ and unique in the sense, as it plays many roles in the development of the foetus by serving as the lung, gut, kidney and exocrine/endocrine glands. Its role in providing oxygen and nutrients to the developing foetus through maternal circulation is well established. Unfortunately, some of the toxic metals, such as lead (Pb) and cadmium (Cd), are also delivered to the foetus by the same mechanism as that of nutrients.

With regard to chemical toxicity, it is noteworthy that the placenta itself is a target organ for toxicity. In the assessment of placental toxicity of any xenobiotics, there are two major areas of concern: what the placenta does to xenobiotics and what xenobiotics do to the placenta (Myllynen *et al*., 2005; Pelkonen *et al*., 2006). In the former area, the major topics of concern are: the entry and possible storage of substances in placental cells and through the placenta, aided perhaps by various transporters and efflux pumps; the distribution and binding of compounds in placental cells; and biotransformation of substances by intracellular enzymes. Metabolic activation and production of reactive intermediates by placental enzymes link these areas with toxicodynamics of placental toxicants. In the latter area, effects of compounds on placental blood flow and vasculature and the presence of membrane and intracellular receptors, enzymes and other potential targets for xenobiotics are important areas of placental toxicity. Therefore, by having a wide range of activities, the placenta can modify the outcome of foetal toxicity of xenobiotics. In the toxicant-exposed mother, the chemical encounters the placenta before it reaches the foetus. It is likely that chemical-induced damage in the placenta will also appear in the foetus. Obviously, the health of the placenta seems as important as the health of the mother or the foetus.

Recently, with some chemicals such as methylmercury (MeHg), adverse effects on foetal growth or survival have been observed at doses that did not produce any toxicity in the mother. In addition to limited activity of metabolism and elimination, the foetus has a low protein binding capability and incomplete or partial blood–brain barrier (BBB). These few factors, among several others, suggest that risks to the foetus from chemical toxicity are far greater than that to the mother. It is beyond the scope of this chapter to discuss the placental toxicity of every single chemical; instead, this chapter is mainly focussed on drugs of abuse, alcohol, smoke, metals, pesticides, environmental toxicants and mycotoxins.

2 PHYSIOLOGICAL ROLE OF THE PLACENTA: STRUCTURE AND FUNCTION

The primary role of embryonic (foetal) membranes is to provide protection to the embryo and foetus. They also play a significant role in foetal nutrition, respiration and excretion. The placenta is formed from the foetal chorion and maternal endometrium. The umbilical cord, placenta, amnion and chorion are expelled after the birth of the foetus. For many years, the view that the placenta is a rejected tissue and may not be suitable for bioassay or biocomparative assays of drugs and evaluation of the cellular mechanisms of actions of drugs and chemicals has been popular among pharmacologists and toxicologists. Use of pregnant animals is considered the preferred model to assess the toxicological actions of chemicals. However, information obtained with animal models cannot be transferred easily to human situations. A significant progress was made in the 1990s in the use of human placental tissues in pharmacological and toxicological investigations due to several developments (Sastry, 1997): (i) Emphasis on alternative methods to animal use in life sciences. (ii) Relevance of living cells from human sources for screening drugs which are eventually used in humans. (iii) Formation of associations of scientists who use placentas in their investigations, and (iv) an increase in the use of abused drugs by pregnant women. Now several methods are available to use different parts of the placenta in screening drugs and

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chemicals and to study the molecular mechanisms for their pharmacological and toxicological effects.

2.1 Placenta Types and Functional Aspects

Mammalian placenta can be classified into four types based on the number of layers between maternal and foetal blood: (i) haemochorial (human, rat, rabbit, guinea pig); (ii) endotheliochorial (cat, dog); (iii) syndesmochorial (sheep, ruminants); and (iv) epitheliochorial (pig, horse). There are anatomical and functional differences among different types of placentas (Syme *et al.*, 2004). Due to practical and technical advantages, animal placentas have been used to study placental function and toxicology. Although qualitative similarities among several types of placenta are present, identity of function and effects of toxicants should not be assumed.

The human placenta is of the haemochorial type, in which the foetal tissue is in direct contact with the maternal blood (**Figure 1**). The membrane separating the maternal and foetal compartments is thin and consists of only three layers: syncytiotrophoblast, connective tissue and vascular foetal endothelium. The intensity of the passage of substances across the placenta is inversely proportional to the thickness of placental membranes. For example, intensity of Na^+ exchange across different types of placentas can be arranged in the following order: haemochorial > endotheliochorial > syndesmochorial > epitheliochorial. In all types of placenta, the intensity of Na^+ exchange increases with gestation, reaching a maximum shortly before term. In the haemochorial placenta, the intensity of exchange decreases at the end of gestation due to deposition of fibrinoid on the exchanging surface. Exchange involves not only physiological constituents, but also substances or elements which represent a pathologic risk for the foetus. Therefore, in assessing placental toxicology of chemicals, all physiological and functional variables should be taken into consideration.

2.1.1 Stages in the Development of Placenta and Dysmorphogenic Agents

The placenta is an important foetal accessory structure which brings maternal and foetal circulations and tissues into close relationship. Morphologically, it is partly of foetal origin (the trophoblast) and partly of maternal origin (from a transformation of the uterine mucosa). In humans, there are two stages for the development of placenta: (i) early embryonic period (of two months), followed by (ii) later foetal period (of seven months). The placenta is differentiated by the third month of pregnancy. During the embryonic period (the first

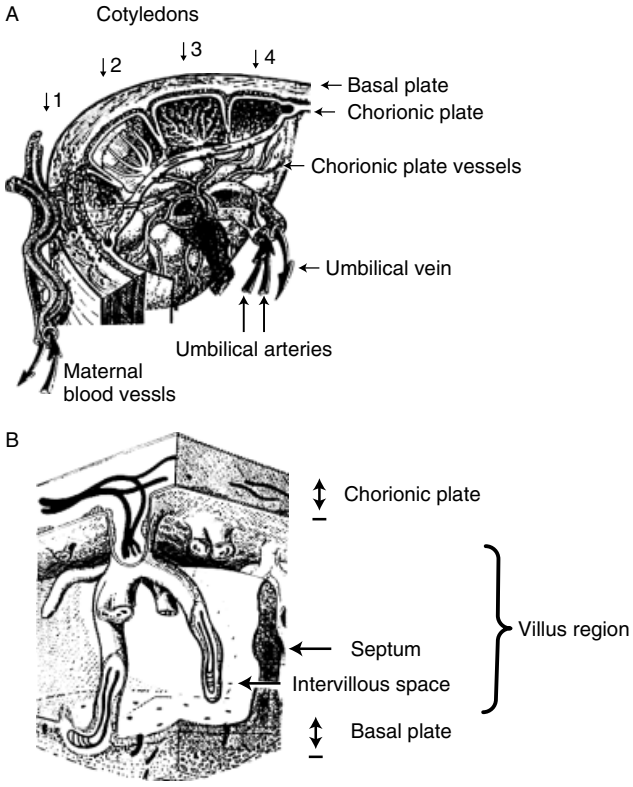

Figure 1 (A) A schematic drawing showing a cross-section of human term placenta. Four distinct cotyledons (1, 2, 3, and 4) are shown. In cotyledon 1, maternal and foetal blood flows are depicted. In cotyledon 2, anchoring villi to basal plate are shown. In cotyledons 3 and 4, all villi and blood flows are shown. (B) Schematic drawing of a single cotyledon, villi and other parts of the placenta. For details of human placental structure, standard books on human embryology should be consulted. (Reproduced from Tuchmann-Duplessis, 1973. © Springer.)

two months) of pregnancy, the embryo acquires definite form (morphogenesis), and the outlines of main organs (organogenesis) are defined (**Table 1**). By the end of the second month, the embryo becomes a foetus. During the foetal period (two to nine months), the organs undergo maturation at the histological (histogenesis) and functional levels. During this period, the foetus grows in size (crown–rump length), volume and weight (**Figure 2**). During the embryonic period, there is no placental barrier. Drugs and environmental chemicals have easy access to the developing embryo and interfere with different stages of morphological differentiation. Chemical- or drug-induced dysmorphogenicity has been recognized in experimental animals for many years. However, the clinical implications of these experimental results have been widely recognized since 1961 due to the discovery of thalidomide-induced phocomelia and other embryopathies. The critical exposure of the embryo occurs *in utero* when the sedative drug thalidomide (100 mg) is ingested by a pregnant woman in the fourth and sixth

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Table 1 Stages of morphogenesis and organogenesis during human embryonic period (60 days)[a]

Day	Embryo size (mm)	Primordial development Organ formation
0	—	Fertilization
6	—	Implantation
15	1.5	Gastrulation
17		Somites
18		Neural plate, auditory placode, heart primordium
21	2.5	
24		Neural tube, vessels, buccopharyngeal opening
26		Anterior neuropore closed, upper limb
28		Posterior neuropore closed, optic placode, pancreas, lower limb
30	4.5	Olfactory placode, septum formation in atria
35		Five vesicle brain
42		Hand (paddle)
45	17	
49		Four cavity heart
56		Separate digits
60	30	

[a]Summarized from Tuchmann-Duplessis *et al*. (1972). For detailed accounts on correlation of timing systems used for human embryos (one to eight weeks), one may consult Larsen (1993).

Table 2 Exchange of physiologic constituents and other substances across placenta

Substances which pass from maternal to foetal circulation (Maternal → Foetal)	Substances which pass from foetal to maternal circulation (Foetal → Maternal)
Oxygen	Carbon dioxide
Water + electrolytes	Water + urea
Carbohydrates	
Lipids	
Vitamins	
Hormones	Hormones
Drugs (certain)	
Viruses (majority)	

weeks of pregnancy. About 8000 victims of this teratogenic substance have been born (Rodin *et al*., 1962; Taussig, 1962).

During the foetal period, the fully formed placenta plays an important role in the maintenance of nutrition to the foetus and in the secretory and regulatory functions that are essential for the maintenance of pregnancy. All 'supplies' to the embryo and foetus—oxygen, water, electrolytes, nutrients, hormones and antibodies—must pass through the placenta (**Table 2**). Several enzymes involved in the four basic processes of drug biotransformation—oxidation, reduction, hydrolysis and conjugation—are present in the placenta. The placenta has a significant role to play in the synthesis of steroid and protein hormones and of several regulatory factors, including acetylcholine (ACh), opioid peptides, tachykinins, platelet activating factor (PAF) and prostaglandins. If the placenta is not functioning properly, it can be the limiting factor not only for foetal nutrition, but also for the maternal–foetal exchange of physiological constituents and foetal–maternal exchange of waste products that may represent a pathologic risk to the foetus. Some of the drugs ingested by the mother may enter the placenta and interfere with placental function and, thereby, foetal development. Some of the compounds may enter the placenta and be metabolized into toxic compounds and enter foetal circulation. Therefore, the human placenta can be a target organ for drug- and chemical-induced injury, especially through abused pharmacologically

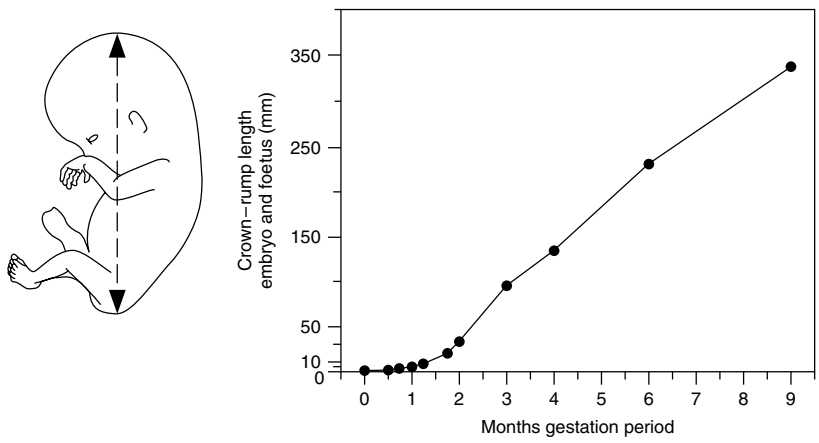

Figure 2 The relationship between gestation period and crown–rump length of human embryo and foetus. In foetal growth retardation both parameters will decrease.

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active agents (morphine, cocaine and components of tobacco smoke), which may affect placental function and cause foetal growth retardation and other adverse reproductive effects in humans.

2.2 Structure of Term Human Placenta

The placenta at term looks like a disc of about 20 cm in diameter, 3 cm in thickness, and weighs about 500 g (**Figure 1**). The umbilical vessels are seen through the amnion. The top part of the placenta is termed chorionic plate and contains layers of amnion, connective tissue, parts of cytotrophoblast and syncytiotrophoblast, which faces maternal blood. The umbilical vessels subdivide into chorionic plate arteries and veins and enter the villi. There are 20–30 large villus trunks that correspond to cotyledons, which are distinct lobes seen on the maternal side of the placenta. The lower part of placenta is termed the basal plate, which contains layers of syncytiotrophoblast, parts of cytotrophoblast, compact zone (decidua) and spongy zone. At the time of delivery, the placenta separates from the uterus at the spongy zone. Maternal blood enters the intervillous space through uterine spiral arteries and drains through uterine veins. Maternal blood spreads out in intervillous spaces and circulates between the branches of villus trees. The foetal blood enters villus trunk through umbilical arteries, flows through capillaries in the villi and returns to the foetus through umbilical veins. The exchange of substances from maternal blood to foetal blood takes place through villus membranes which contain three layers: syncytiotrophoblast, collagen or connective tissue layer, and foetal endothelial cells. There are two types of villi: floating villi which hang in maternal blood, and anchoring villi which are attached to basal plate at their tips.

The blood flow in the maternal and foetal circulations of placenta is very high, about 500 ml min^{-1}. This high flow rate favours foetal–maternal exchange. Maternal circulation results from a difference in pressure: high in the artery (70 mm Hg) and low in the intervillous space (10 mm Hg). Foetal circulation is carried out in a closed vascular system where the estimated pressure of 30 mm Hg is higher than that of intervillous space (10 mm Hg). This difference in pressure maintains the position of the villi and prevents their collapse.

2.3 Human Placental Tissues for Pharmacological and Toxicological Evaluation

There are several types of pharmacological preparations from human placenta which have been described by several investigators. These include (i) perfused single placental cotyledon; (ii) villus preparation; (iii) segments of umbilical, chorionic plate, and villus stem arteries and veins; (iv) trophoblast plasma membrane; (v) isolated receptors and transporters; and (vi) stem cells from umbilical blood. Several of these preparations have been described in detail in many papers and reviewed by Sastry (1996).

2.3.1 *Dually Perfused Human Placental Cotyledon*

Several animal models have been used to assess transplacental drug passage, but species differences in placental anatomy and physiology have prevented general acceptance of animal studies as to how well they relate to humans (Hamshaw-Thomas and Reynolds, 1985; Kennedy *et al*., 1986; Carson and Reynolds, 1992; Pelkonen *et al*., 2006; Gupta, 2007). A wide gap exists in our knowledge and understanding of the mechanisms and rates of transport of drugs and chemicals from (i) maternal to foetal and (ii) foetal to maternal circulations.

An isolated, dual-perfusion, human placental model was developed by Schneider *et al*. (1972) and has been revised by Schneider (1995). It has been well validated though studies of the transfer of several different substances. Experiments can be performed using either the closed (recirculating) method or the open (single pass or noncirculating) method. In the closed model, both the maternal and foetal perfusates are recirculated. After a 30 minute equilibration period, the drug (or a chemical) and antipyrine (10 μg ml^{-1}, 53 μM) are added to either the maternal or foetal reservoir and allowed to equilibrate for 3 hours. Samples (1 ml) are taken from the maternal and foetal circuits at 15–30 minute intervals for the duration of the experiment (3 hours), for the measurement of the drug (or chemical), antipyrine, lactate production and glucose consumption.

The recirculation studies are not useful to calculate meaningful clearance data because these data change over time as more drug is transferred and accumulates towards equilibrium between the circuits. Instead, data are expressed in terms of a percent transfer of the drug during the period of greatest transport (i.e. on the steepest aspect of the exponential decline curve of the donor circuit) (Johnson *et al*., 1995).

In open perfusions, after a period of equilibration, a steady state is attained. Maternal to foetal drug clearance (C1) can be calculated by the standard formula. Similarly, foetal to maternal clearances can also be calculated. Integrity and viability of placental tissue are monitored by lack of fluid shifts, partial pressures in both maternal and foetal circuits, lactate output, lactic dehydrogenase release and glucose consumption. Antipyrine is generally used as a freely diffusible marker to evaluate the placental transfer of drugs. These validating methods are standards for this system.

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The *in vitro* human placental model has been used to measure placental transfer of certain nutrients (e.g. insulin), vitamins (biotin), local anaesthetics used during pregnancy (e.g. bupivacaine), abused drugs (e.g. cocaine) and several other compounds (**Table 3**). This information is useful to assess the rate of transfer of substances into the foetal circulation after the drugs and other chemical substances are ingested by the mother. The perfusion model is also useful to determine the clearance of the drug and toxic chemicals from the foetal circulation after cessation of maternal exposure to these chemicals.

The *in vitro* human placental model is also useful to assess the effects of endogenous biogenic amines, exogenous amines and other chemical substances on foetal perfusion pressure. For example, the effects of several biogenic amines have been determined on perfusion pressures in human placenta. They may cause vasoconstriction or dilation (**Table 4**). The effects of new chemicals or toxicants on the perfusion pressure changes induced by biogenic amines and other endogenous substances can be studied.

2.3.2 Isolated Placental Blood Vessels

Isolated blood vessel strips from the rat aorta, porcine coronary artery and various other preparations have become useful for evaluating the vascular resistance caused by biogenic amines in pharmacological screening techniques. In recent years, techniques have been developed to use isolated placental blood vessels to evaluate the vascular resistance of biogenic amines. One advantage of placental blood vessels is the availability of human sources. As the placenta is devoid of innervation, direct effects of biogenic amines on their receptors can be evaluated.

As discussed in the previous section, perfusion of isolated human placentas *in vitro* through the umbilical artery has indicated that placental vasculature is sensitive to a variety of compounds with vasoactive properties.

Table 3 Placental transfer of substances using perfused placenta

Hormones and vitamins	Nutrients	Therapeutic agents	Abused agents
Insulin (Schenker *et al*., 1993)	Glucose (Hauguel *et al*., 1986; Haggerty *et al*., 2002)	Methohexital (Herman *et al*., 1993)	Morphine (Gerdin *et al*., 1990)
Biotin (Henderson and Schenker, 1995)	Amino acids (Schneider *et al*., 1972)	Fentanyl (Zakowski *et al*., 1993)	Cocaine (Schenker *et al*., 1993; Krishna *et al*., 1993; Simone *et al*., 1994)
Pyridoxal (B_6) (Henderson and Schenker, 1995)	Carnitine (Schmidt-Sommerfeld *et al*., 1985)	Bupivacaine (Johnson *et al*., 1995)	Nicotine (Sastry and Chance, 1994)
Thiamine (Henderson and Schenker, 1995)	Fatty acids (Haggerty *et al*., 2002; Herrera, 2002)	Lidocaine (Ala-Kokko *et al*., 1993)	Alcohol (Henderson and Schenker, 1995)
		Cimetidine (Schenker *et al*., 1987)	

Table 4 Effects of biogenic amines and other endogenous substances on perfusion pressure in isolated human placenta

Substance	Dose (μg)	Effect[a] (frequency of occurrence %[b])		
		Constriction[c]	Dilation[c]	Weak or absent
Acetylcholine (Sastry, 1991a; Sastry, 1991b; Sastry, 1993; Sastry, 1997)	2–500	11	27	56
	100	80	—	20
Epinephrine (von Euler, 1938)	2–500	73	—	27
	10–500	69	18	13
Norepinephrine (Ciuhta and Gautieri, 1964)	10–500	90	—	3
Histamine (von Euler, 1938)	5–20	50	—	50
	10–100	85	11	4
Serotonin (Ciuhta and Gautieri, 1964)	2–5	97	—	3
Adenosine (von Euler, 1938)	100–600	67	33	—
Vasopressin (von Euler, 1938)	2–10 IU	43	—	57
Prostaglandin (von Euler, 1938)	0.2–2 IU	100	—	—

[a]Frequency of occurrence indicates the number of placentas in which the affect will be observed in a total of 100 full-term placentas.
[b]Unless otherwise stated, summarized from Sastry and Sadavongvivad (1979) and Sastry (1996).
[c]Constriction and dilation are judged from the increase or decrease in perfusion pressures.

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This preparation provides useful information on the perfusion flow and overall resistance of the system. However, this preparation does not distinguish the sites of vascular resistance and permit quantification of the actions of drugs. Strips of umbilical arteries and veins have been examined for drug sensitivity, but these studies reflect only the sensitivity of cord vessels *per se* and not of the placental vasculature. Different regions of the placental vasculature tree exhibit different sensitivities to vasoactive peptides. Since the small arteries or arterioles of the placental vasculature are the most probable sites of placental vasculature resistance, it is necessary to compare sensitivities of resistance of vessels to biogenic amines with those of the larger arteries supplying the placenta.

The three regions of human placenta—(i) umbilical cord, (ii) chorionic plate, and (iii) villus stem arteries—are useful for pharmacological evaluation of biogenic amines and drugs. All of these preparations have been reviewed by Sastry (1996).

2.3.3 Trophoblastic Tissue

Several preparations from human trophoblastic tissue have been used to study the effects of drugs on processes in human placental trophoblast. The main parameters frequently used are uptake of amino acids, especially nonmetabolizable amino acids (e.g. L-aminoisobutyric acid), by the trophoblastic tissue, and release of biogenic amines (e.g. ACh) or opioid peptides (e.g. methionine enkephalin). These aspects have been reviewed by Sastry and Janson (1995), Sastry (1993) and Ahmed and Cemerikic (1995). The various types of preparations include (i) placental slices, (ii) dissected syncytiotrophoblastic tissue, (iii) microvillus membrane vesicles, and (iv) subcellular fractions including microsomes. The former three types of preparations are often used in pharmacological assays, while microsomes are used in drug metabolism studies.

2.3.4 Microvillus Plasma Membrane

A preparation has been described by several investigators in which surface microvilli of human term placenta are exfoliated from the syncytiotrophoblast cells (Smith *et al*., 1974; 1977; Fant and Harbison, 1981; Barnwell and Sastry, 1987). The exfoliated microvilli are further purified by differential centrifugation and washing. The microvilli (or membrane vesicles) are essentially plasma membrane. This preparation is enriched in the two marker enzymes of plasma membrane, alkaline phosphatase and 5′-nucleotidase, 24–25-fold when compared to the whole villus homogenate (Barnwell and Sastry, 1987).

The plasma membrane forms microvesicles, which have been used to measure amino acid uptake, to demonstrate the presence of muscarinic receptors by radioligand binding, and to establish the role of phospholipid-*N*-methyltransferase 1 (PMT1) in the uptake of amino acids. PMT1 is enriched about 1.5–2.0-fold in the microvillus plasma membranes when compared to its activity in whole villus homogenate. Inhibition of PMT1 resulted in the depressed uptake of the amino acid AIB (aminoisobutyric acid) by placental villi. This preparation will be useful to study the effects of drugs and toxicants on amino acid transport across the plasma membrane.

2.3.5 Cultured Human Placental Villus Tissue and Cells

There are several types of receptors on human placental cells: muscarinic, nicotinic and opioid receptors. It is difficult to study receptor down-regulation, up-regulation, desensitization and tolerance development against agonists in fresh placenta because the placenta will also be exposed to several other drugs administered to pregnant women. The villus trophoblast which is thoroughly washed free of drugs and maintained in culture for several days is more suitable for studying the different aspects of receptor regulation and the influence of toxic substances in placenta.

The availability of an *in vitro* purified trophoblast system would also facilitate studies on several aspects of trophoblast function, transport of nutrients between maternal and foetal circulation, secretion of steroid and protein hormones, transport of immunoglobulins from mother to foetus, and blockade of the rejection of the foetus by the mother. Isolated cytotrophoblasts and syncytiotrophoblasts facilitate molecular biological studies on placenta. Well-established cell lines from choriocarcinoma are useful in several of the above studies. Some investigations are focussed in developing these techniques: (i) culture of villus trophoblast for long periods (Cemerikic *et al*., 1994; 1995), (ii) isolation of cytotrophoblasts from human term placenta (Kliman *et al*., 1986; 1987; Daniels-McQueen *et al*., 1987; Lobo *et al*., 1987), and (iii) development of cell lines from choriocarcinoma (BeWo, JAr and JEG-3) cells (Loke and Hussa, 1987).

Each culture system has specific advantages and disadvantages. The choice of culture system is based upon the purpose for which it is used (Sastry, 1996). Techniques have also been developed to grow JAr cells as multicellular spheroids (White *et al*., 1988). The similarity of JAr choriocarcinoma cells in monolayer culture to normal human trophoblast cells in terms of morphology and hormone production has enabled investigators to use JAr cells, and other choriocarcinoma cell lines, to study the properties of the trophoblast *in vitro*—for example, the basal and stimulated production of placental protein and steroid hormone. However, hormone production is generally assessed for short periods of time in nearly confluent cultures or on frequently trypsinized cells, where normal functioning may have been impaired.

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Further, monolayer cultures are not suitable for the study of higher placental functions, such as implantation, where a three-dimensional arrangement of cells may be appropriate and necessary. These problems have limited the study of certain placental cell functions and have prevented the study of others. JAr cells which are grown as multicellular spheroids are more suitable for studying normal placental cell functions. Of special interest are those functions which may change with time or which may involve three-dimensional cell interactions, such as differentiation and implantation. The potential applicability of the spheroid system for studying higher-level functions of the trophoblast are (i) the long culture period, to study processes over time; (ii) the large size and free-floating status of spheroids; (iii) the three-dimensional nature of the spheroids, which stimulates *in vivo* conditions; and (iv) the large numbers of cells generated in each experimental trial. The spheroid culture system is also useful to study both acute and chronic effects of drugs and toxicants on trophoblast functions.

3 PLACENTAL BARRIER: A REALITY OR FALSE NOTION?

The term 'placental barrier' is a misnomer and a widely held false notion, since the placenta is not a true barrier for the transfer of most xenobiotics from mother to foetus. Instead, the placenta is the entry through which the foetus is exposed to chemicals. The placenta has been characterized as 'a lipid membrane that permits bidirectional transfer of substances between maternal and foetal compartments' rather than as a barrier. In humans, the placental barrier consists of the trophoblastic epithelium covering the villi, the chorionic connective tissue and the foetal capillary endothelium. The average thickness of the barrier at term is 3.5 μm, the average exchange area is approximately 11 m^2, and the placental blood flow rate is approximately 450 ml min^{-1} (Pacifici and Nottoli, 1995).

The placental architecture is composed of cells derived from foetal membranes. Placentation differs among domestic animals (epitheliochorial in pigs, horses and donkeys, and syndesmochorial in sheep, goats and cows), laboratory animals (haemoendothelial in rats, rabbits and guinea pigs), and man and monkeys (haemochorial or haemomonochorial) (Thomas, 1996). Anatomically, the placenta has also been described as zonary in the dog, bidiscoid in the monkey and multicotyledonary in the sheep. The placental thickness depends on the number of foetal and maternal cell layers. For example, the rat and the rabbit have a single layer of cells, primates and humans have three layers of cells and pigs and horses have six layers. It should be noted that the placenta is the first foetal organ to be exposed to exogenous substances.

There appear to be two common factors that are involved in transplacental transfer of toxicants: (i) physicochemical properties of the chemical and (ii) type of placenta. Any chemical with a molecular weight (MW) < 1000 readily crosses the placenta, and most pesticides, metals, mycotoxins, plant alkaloids and other xenobiotics have MW < 1000. Hence these chemicals are not restricted from reaching the foetus. However, the placenta poses a limited permeability barrier to chemicals with MW > 1000 (Morgan, 1997; Syme *et al*., 2004; Gupta, 2007). It is important to mention that metabolic processes in the mother or the placenta can degrade or biotransform high MW into low MW chemicals, thus allowing them to cross the placental barrier. Chemical properties, such as lipophilicity, polarity and degree of ionization, can also affect the placental barrier. Much more research needs to be done to substantiate these facts.

The other factor that predominantly influences the transplacental transfer of chemicals is the type of placenta. From the limited literature available, it appears that the placental barrier is partial and selective to some xenobiotics and is recognized in the simpler choriovitelline type of placenta present in rodents, and also in the chorioallantoic type present in higher mammals (Welsch, 1982; Juchau, 1995). In general, the more complex multilayered placenta of higher animals can make it more difficult for xenobiotics to gain access to the foetus.

So far, diffusion (simple or facilitated) has been the only proven mechanism by which drugs and toxicants cross the human placenta, although several animal studies have suggested a role for active transport or pinocytosis (Mihaly and Morgan, 1984). The rate and extent of transfer differ for various compounds (Welsch, 1982). The rate of diffusion is determined by the maternal–foetal drug gradient, uterine and umbilical blood flow, MW of drug/toxicant, protein binding, lipid solubility and degree of ionization. These factors also determine the time required for maternal/foetal equilibrium.

The evidence for placental transfer of chemicals stems from either direct detection of a chemical residue or its metabolite in the placenta, umbilical cord blood, embryo/foetus or the specific biochemical and morphological changes induced by a chemical toxicant in the placenta/foetus. Placental transfer of various classes of pesticides is described in our previous publications (Gupta, 1995; 2007; Pelkonen *et al*., 2006). Similar information on placental transfer of metals, mycotoxins and drugs has been reported by Eisenmann and Miller (1996), Rogers (1996), Gupta (1998) and Gupta (2007).

Indirect evidence for placental transfer of certain classes of chemicals, for example organophosphates

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(OPs) and carbamate (CM), has been substantiated by measuring the cholinesterase inhibition in foetal tissues (Cambon *et al*., 1979; 1980; Gupta *et al*., 1985; Gupta, 1995; Pelkonen *et al*., 2006). *In vivo* protein synthesis in the placenta, embryo and foetal tissues of rats following *in utero* exposure to parathion-methyl, and polydactylism in the forepaws of cat foetuses exposed to dimethoate are considered strong evidence for indirect placental transfer of OPs (Khera, 1979; Gupta *et al*., 1984).

In essence, the anatomical placental barrier for the majority of toxicants/drugs (including charged molecules such as (+)-tubocurarine, highly ionized salicylates, pesticides, metals, mycotoxins and narcotics) is a widely held false notion, since they cross the placenta and reach the embryo/foetus and, thereby, produce a variety of toxicological and teratogenic effects (Gupta, 1995; 2007; Eisenmann and Miller, 1996).

Recently, the metabolic placental barrier has drawn equal attention from physicians, toxicologists, pharmacologists and others. For example, human term placenta, by having a significant amount of butyrylcholinesterase (BuChE) activity, metabolizes cocaine and thereby serves as a metabolic barrier to protect the conceptus (Simone *et al*., 1994; Sastry, 1995c; Gupta and Sastry, 2000).

4 METABOLISM IN PLACENTA AND FOETUS

Metabolic pathways for both Phase I and Phase II reactions exist in both the placenta and the foetus. Phase I reactions include oxidation, reduction and hydrolysis, and Phase II reactions include conjugation of a chemical with endogenous moiety, such as glucuronic acid or sulfate. The capacity for metabolic processes is expected to vary with species, age and, more importantly, gestational stage. Xenobiotic metabolism in the placenta appears to be similar to that in maternal tissues, but the amount of metabolic activity is usually less. Still, the metabolic activity is adequate enough that the placental-foetal toxicity of chemicals can be significantly modified.

By now, it is well established that the placenta has an abundance of drug/xenobiotic metabolizing enzyme systems, that is, cytochrome P450. Cytochrome P450 exists in multiple forms with distinct, but generally overlapping, substrate specificities, and many of the isoforms are inducible by exposure to exogenous agents (Pasanen and Pelkonen, 1994; Juchau, 1995). The human placental mono-oxygenase system consists of at least one 'constitutive' and one 'inducible' form of cytochrome P450 for xenobiotic transformation. Although several isoforms of cytochrome P450 (P45011A1, P45019, P4501A1, P4503A7 and others) exist in the placenta, isoform P4501A1 appears to have the highest inducibility by xenobiotics. Induction of xenobiotic-metabolizing enzymes mainly takes place in the maternal liver, placenta and foetal liver, but it is not limited to these organs. In the placenta, several steroid hormones are formed and metabolized by cytochrome P450 systems, which may also participate in xenobiotic metabolism (Gonzalez, 1989). It should be mentioned that the human placenta lacks the marker activities for cytochrome P4503A enzymes, which are abundantly present in the liver of the adult human and foetus (Pasanen and Pelkonen, 1994).

The metabolites produced by biotransformation are usually less toxic or inactive, and thereby the xenobiotic-metabolizing enzymes in the placenta protect the foetus from potentially foetotoxic drugs and chemicals. For example, the human term placenta, by having a considerable amount of smooth endoplasmic reticulum and BuChE activity, metabolizes cocaine and thus protects the foetus from maternal cocaine abuse (Simone *et al*., 1994; Sastry, 1995c). It is important to note that the xenobiotic-metabolizing enzymes can also form metabolites which are more toxic than their parent compounds. For example, oxon metabolites of organophosphorus insecticides and epoxides of cyclodiene chlorinated hydrocarbons have a greater potential for foetotoxicity/teratogenicity than their parental forms. In addition, placental metabolism has the capacity to form carcinogenic–DNA adducts.

Xenobiotic metabolism in foetal tissues differs from that in adults, both qualitatively and quantitatively. Some of these differences were described in a review by Slikker and Miller (1994). In brief, (i) general foetal metabolic activity is lower than that in the adult of the same species, (ii) enzymes or isoenzymes (e.g. cytochrome P450) present in the foetus can be replaced by immunologically different isoenzymes in the adult animals, and (iii) enzyme activity generally increases with gestational age; however, the ontogeny of each individual enzyme may be different. With regard to similarities, the liver of the conceptus, and also of the adult, has the greatest xenobiotic-metabolizing activity compared with any other organs (kidney, lung and adrenal gland). Metabolism of the chemical compound may occur in the foetus, and this may lead to a prolonged foetal half-life if the metabolite is a more polar compound. The significance of the placental-foetal metabolism is immense and it is beyond the scope of this chapter to provide complete coverage. Therefore, for further details, readers are referred to some other comprehensive reviews (Pasanen and Pelkonen, 1994; Pelkonen, 1984; Juchau, 1995; Pelkonen *et al*., 2006).

Compared with Phase I, Phase II reactions (conjugation), including sulfation, acetylation and glucuronidation, in the placenta have not been studied in detail. One of the reasons is that the placenta contains low activities of these enzymes (Juchau, 1980), although active involvement of human placental glutathione transferase in detoxification of both endogenous and exogenous alkylating

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agents is well established. For further details on Phase I and Phase II reactions in placenta, readers are referred to a recently published review by Syme *et al*. (2004).

5 SUSCEPTIBILITY OF PLACENTA AND FOETUS TO CHEMICAL TOXICITY

The placenta has been described as a dynamic organ that interfaces two separate genomes (mother and developing foetus) during pregnancy (Slikker and Miller, 1994). Structural changes in the placenta evolve as the pregnancy advances and it plays numerous roles, acting as the foetal lung, gut, kidney, exocrine gland and endocrine gland (Juchau, 1995). Since the placenta serves as an exchanger between mother and foetus for nutrients, the health of the foetus seems to be dependent on the health of the mother and the placenta. Unfortunately, toxic chemicals also traverse by similar mechanisms. Eisenmann and Miller (1996) explained the mechanism to support this fact by stating that toxic metals (e.g. Cd or Pb) may enter the placenta and be concentrated there, or may be transferred to the foetus via a mechanism designed to transport nutrients, such as calcium. Further, because foetal demands for nutrients increase with gestation (Haggerty *et al*., 2002), the potential for placental-foetal intoxication with toxic metals may also increase with gestation (Gupta, 2007). It is important to mention that pregnancy is a state of oxidative stress arising from increased placental mitochondrial activity and production of reactive oxygen species (ROS), mainly superoxide anion. The interaction of superoxide with nitric oxide produces peroxynitrite, which exerts nitrosative stress. Placental oxidative/nitrosative stress can be further exacerbated by chemicals (certain metals, pesticides and mycotoxins) that are known to produce oxidative/nitrosative stress.

More than one mechanism exists by which toxicants are concentrated in the placenta and foetal tissues in greater quantities than in maternal tissues. In general, the large placental surface area comes in contact with a relatively large volume of maternal blood (required for normal placental function), and that makes the placenta vulnerable to toxicants (Eisenmann and Miller, 1996). The placenta has certain biomolecules (proteins, carbohydrates, lipids, nucleic acids/nucleotides and enzymes) which can either be the target of toxicity or can play a role in modification of toxicity. Being rich in proteins, the placenta may bioconcentrate chemical residues by means of protein binding and release them into the placental circulation and ultimately into the foetus.

Foetal tissue often accumulates chemical residues because of its inadequate capability for degradation and elimination. Incomplete or partial BBB in the foetus is another reason for its greater sensitivity. As a result, birth/congenital defects occur with the greatest frequency in the central nervous system (CNS) compared with any other organ system, when exposed *in utero* to toxicants such as MeHg, Pb, thalidomide, retinoids, alcohol and others.

In adults, the BBB normally restricts the entry of many solutes and chemicals from blood into the brain and can provide a significant level of protection, especially against neurotoxicants. The foetal BBB develops during pregnancy and, in humans, the BBB is not fully developed until the middle of the first year of life. During this prenatal as well as postnatal period with an incomplete BBB, some toxic agents, like Cd, that are very unlikely to enter the mature brain, may pass into the developing brain. Literature demonstrates that different classes of pesticides are able to change the permeability characteristics of the BBB in rats when administered during susceptible periods of the BBB development, and this effect may persist for varying periods after the cessation of exposure (Gupta *et al*., 1999). Therefore, altered BBB can render the nervous system more vulnerable to other toxicants that would otherwise not be able to pass the BBB.

The placenta and foetus, by having acetylcholinesterase (AChE) and other cholinergic elements, remains highly susceptible to OP and CM toxicity (Koshakji *et al*., 1974; Cambon *et al*., 1979; Gupta *et al*., 1984; 1985; Sastry, 1993; Simone *et al*., 1994; Pelkonen *et al*., 2006; Gupta, 2007). In addition, the placenta has drug-metabolizing enzymes (e.g. mixed function oxidase, cytochrome P450, glutathione transferase), which means that it can transform certain OPs of the 'thioate' group (e.g. parathion) to their 'oxon' analogues (e.g. paraoxon) and transform chlorinated hydrocarbons of the 'cyclodiene' group (e.g. aldrin and hepatochlor) to their 'epoxide' forms (e.g. dieldrin and hepatochlor epoxide). Similar metabolic pathways, although to a lesser extent, exist also in the foetal tissues (Pelkonen, 1984; Gupta, 1995; Harbison *et al*., 1995; Juchau, 1995).

These findings suggest that the placenta and the foetus have the capability of so-called 'lethal synthesis', since the OPs in the form of 'oxon', and cyclodienes in the form of 'epoxides' are much more toxic than their parental compounds. Pesticides with a mechanism(s) of enzyme inhibition, such as AChE-inhibiting OPs and CMs, adenosinetriphosphatase (ATPase)-inhibiting organochlorines and cytochrome P450-inhibiting OPs, make the placenta as susceptible as the mother or the foetus. The placenta has an oxidative mechanism and is, therefore, also predisposed to carcinogenesis by numerous aryl and aromatic hydrocarbons. In essence, the structure and function of the placenta are so unique that its susceptibility to chemical toxicity seems far greater than that of the mother. Also, any damage to the placenta caused by a toxic chemical is likely to be reflected in the embryo and foetus.

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6 FACTORS THAT CAN MODULATE PLACENTAL TOXICITY

Whether the toxicants affect only the mother, only the conceptus, only the placenta, or the whole unit of mother–placenta–foetus is not fully understood for any toxicant. Important factors which can modify the overall toxicity of a chemical during pregnancy include (i) maternal toxicity, (ii) placental transfer and (iii) placental-foetal metabolism.

6.1 Maternal Toxicity

Maternal toxicity has been defined as the transitory or permanent state of alteration in maternal physiology or behaviour with a potential to cause adverse effect in the offspring during embryo/foetal or postnatal development (Khera, 1987). In contrast, the term 'maternotoxic' commonly refers to test dosages, agents, signs/symptoms or data relevant to pregnancy without any connotation to its embryo-foetal implications.

Maternal toxicity-related factors, whether intrinsic (such as homeostasis alterations) or extrinsic (such as chemical and physical agents) at maternotoxic levels can produce deleterious consequences in foetal development by either altering maternal physiology or causing maternotoxic effects. Khera (1987) hypothesized that maternal effects caused by chemical agents may be among the factors causing congenital malformations, but found it impossible to test this hypothesis, since data on the maternal health in teratological studies are inadequate.

A strong relationship seems to exist between maternal toxicity and embryo-foetal toxicity/mortality and, therefore, maternal toxicity can be regarded as an aetiological factor for foetal malformation and embryo-foetal death. The common factors related to maternal toxicity include (i) route of drug/chemical exposure, (ii) maternal drug/chemical distribution, (iii) maternal drug/chemical metabolism, (iv) uterine blood flow, and (v) pH of the blood. Although the exact mechanism by which maternal toxicity factors are responsible for foetal toxicity/teratogenesis has remained obscure, alterations in placental function appear to be important. Hence it can be suggested that maternal toxicity plays a major role in adverse foetal outcome by modifying placental function.

6.2 Placental Transfer

Foetal growth directly depends on the nutrients crossing the placenta. Glucose, which is the principle carbohydrate crossing the placenta, is transported by facilitated-diffusion glucose transporters, according to concentration-dependent kinetics (Illsley, 2000), whereas amino acids are transported through energy-dependent processes, via selective amino acid transporters (Battaglia and Regnault, 2001). However, knowledge about placental transport of lipids is still scant. For the details on placental transport of nutrients, readers are referred to the review by Herrera (2002). The role and importance of placental transfer in relation to chemical toxicity have already been discussed in earlier parts of this chapter; some of the factors that are involved in trans-placental transport of chemicals which may alter placental toxicity are discussed here in brief.

The important placental transfer factors include placental blood flow, pH of the blood, placental permeability (passive or active transport system), placental maturity over gestation period (size, surface area and thickness), interspecies variation in placental morphology, lipid–protein content of the membranes, placental metabolism, plasma protein binding, lipid solubility and other physicochemical properties of toxicants (Welsch, 1982; Mihaly and Morgan, 1984; Slikker and Miller, 1994; Andersen *et al*., 2000; Syme *et al*., 2004). The factors related to placental morphology, physiology and metabolism seem to be interrelated, and with continuing change they become more complex as gestation advances.

Plasma protein binding (largely to the albumin fraction) appears to be the important factor that can modify placental toxicity, the reason being that only a chemical in a free form (i.e. unbound) can cross the placenta. It should be mentioned that protein binding is a reversible process and, therefore, the protein-bound chemical can become a free form and cross the placenta. Once the chemical has reached the foetus, either it remains free to produce deleterious effects or, once again, it can bind to proteins of blood and foetal tissue, a phenomenon described as the 'sink effect'. OP and CM insecticides and a toxic metal, Cd, are appropriate examples to substantiate this phenomenon. The protein binding factor has further implications in potentiation of OP or CM toxicity if the mother is pre-exposed to another OP that binds to esterases or proteins (Pelkonen *et al*., 2006; Gupta, 1995; 2007).

6.3 Placental-Foetal Metabolism

Placental-foetal metabolism is one of the major factors that can significantly modulate the placental toxicity of certain chemical toxicants. Metabolism occurs in all maternal, placental and embryonal/foetal tissues, but the capacity differs at all stages of gestation. During pregnancy, metabolism of the drug or toxicant is complicated by two major factors: (i) the pregnancy itself, since the general metabolic activity is low, which may lower the degradation of drugs or toxicants and thereby increase the toxicity; and (ii) pre-exposure or simultaneous exposure

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to other chemicals or environmental pollutants generally results in either reduced or enhanced metabolism and, consequently, altered placental toxicity. For example, previous exposure to drug-metabolizing enzyme inducers, such as enhanced mono-oxygenase activity by polychlorinated biphenyls (PCBs), or other similar environmental pollutants, can potentiate the toxicity of the 'thioate' type of OP insecticides. Cigarette smoking and chronic consumption of alcohol and drugs of abuse (e.g. steroid, cocaine, cannabis) are additional factors that can modify placental-foetal metabolism and placental toxicity (Sastry, 1995c; Gupta and Sastry, 2000; Syme *et al*., 2004).

7 ABUSED DRUGS AND PLACENTAL FUNCTION

There are several drugs which are abused by humans. These include nicotine (through tobacco smoking), morphine, cocaine and alcohol. If pregnant women are exposed to abused drugs or alcohol, their placental functions will be compromised. The common effect of these four drugs is intra-uterine foetal growth retardation (IUGR). All of the causes of IUGR are not well understood. There may be direct effects of drugs on the developing foetus, or indirect effects via influence on placental function. In IUGR, there is a decrease in all dimensions of foetal growth, indicating a common cause for the development of all organs of the foetus (**Figure 2**). One of the requirements for the growth and development of foetal organs is essential amino acids for protein synthesis and tissue formation. Therefore, effects of abused drugs on the placental transport of amino acids are emphasized. There is accumulating evidence that there may be abnormalities in the transplacental transport of amino acids, leading to poor foetal growth. Amino acid supply to the foetus and plasma amino acid concentrations are the strongest regulators of amino acid incorporation into protein and the major factors limiting protein breakdown (Pastrakuljic *et al*., 1999).

7.1 Maternal Smoking, Placental Function and Amino Acid Transport

Currently, tobacco exposure during pregnancy remains a major public health problem. It is well established that human placenta does not pose a metabolic barrier to nicotine transfer to the developing foetus. Maternal smoking in pregnancy is associated with two well-recognized effects—an increase in the frequency of spontaneous abortions, and low birth weight babies (Seller *et al*., 1992; Campbell, 1997; England *et al*., 2001; Zhao and Reece, 2005). The low birth weight may be related to decreased placental functions in smokers. The effects of maternal smoking and nicotine use on placental function have been reviewed (Sastry and Janson, 1995; Shiverick and Salafia, 1999). Maternal smoking alters many biochemical parameters in the maternal compartment (e.g. blood carboxyhaemoglobin), placental compartment (e.g. lowered oxygen consumption) and foetal compartment (e.g. decreased breathing movements). The final result of all these alterations is foetal growth retardation as indicated by the infant birth weight and crown–rump length (**Figure 2**).

Babies of smokers are, on average, 200 g lighter than babies born to comparable nonsmokers (Hasselmeyer *et al*., 1979). The whole weight distribution of babies of smokers is shifted downward compared with the weight distribution of babies of nonsmokers. Smoking-induced foetal growth retardation is not related to the characteristics of the smoker. The more the mother smokes, the greater the reduction in foetal growth. This foetal growth retardation may be related partially to disturbances in amino acid metabolism as indicated by depressed placental transport of amino acids in smokers.

The foetus is dependent on the placental transfer of amino acids from maternal to foetal circulation for its requirement of amino acids. The placental transfer is a two-step process: (i) active uptake of amino acids by placental syncytiotrophoblast cells from the mother's blood; and (ii) passive diffusion of amino acids from placental syncytiotrophoblast cells into umbilical blood. The first step is critical, and its efficiency could be compromised, resulting in depressed uptake into trophoblasts under placental hypoxic conditions induced by maternal smoking. Nicotine and tobacco smoke components (carbon monoxide, cyanides and nitrites) reduced active uptake of amino acids by isolated human placental villi (Rowell and Sastry, 1978; Barnwell and Sastry, 1983; Sastry *et al*., 1983; Sastry, 1984). Exposure of human placental villi to nicotine inhibited the uptake of α-aminoisobutyric acid (α-AIB), and decreased both maximum velocity (V_{max}, 71%) and Michaelis–Menten constant (K_m, 67%) for uptake of α-AIB (Barnwell and Sastry, 1983). Part of the inhibition (16%) is not reversible, which may be of significance in chronic smoking (Sastry, 1991a). Concentrations of several essential amino acids (valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine) and nonessential amino acids (aspartic acid, glutamic acid, glycine, alanine, arginine) in the placental villi of nonsmoking mothers were about 30–50% higher than those of smokers (Sastry *et al*., 1989b). Concentrations of threonine and phenylalanine were about 14–15% higher in the placental villi of nonsmokers than in those of smokers. According to these observations, maternal smoking decreases the uptake of amino acids by the placenta and thus the net transfer of amino acids from

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maternal to foetal blood. Further, the placental transfer of all essential amino acids is not affected equally, indicating that the nitrogen balance may be disturbed in the foetus. Therefore, foetal undernutrition for amino acids may provide partial explanation for foetal intra-uterine growth retardation in tobacco smokers.

7.2 Maternal Smoking and the Regulatory Mechanisms for Amino Acid Uptake by the Placental Trophoblast

α-AIB, a nonmetabolizable amino acid, has been used as a model amino acid to study the mechanisms of amino acid uptake by human placenta. Four regulatory mechanisms have been proposed for the cellular uptake of amino acids in the placenta: (i) gammaglutamyl cycle (GC) for the regulation of amino acid transport (Meister and Anderson, 1983; Payne and Payne, 1984); (ii) placental ACh release and amino acid transport coupling (Harbison *et al*., 1975; Rowell and Sastry, 1978; 1981; Sastry *et al*., 1983); (iii) phospholipid *N*-methylation in the plasma membrane (Barnwell and Sastry, 1987); and (iv) oxidative energy sources (Longo *et al*., 1973; Miller and Berndt, 1974).

The GC was postulated for amino acid transport by Meister and Anderson (1983). Gammaglutamyl transpeptidase (GGTP) plays a key role in the GC cycle. It is bound in the outside bilayer of the plasma membrane. Glutathione (gammaglutamylcysteinylglycine) is continuously secreted onto the cell surface, where GGTP transfers a glutamyl group to an incoming molecule of an amino acid. The products, gammaglutamylamino acid and cysteinylglycine, are reabsorbed into the cell. The bulk of the amino acid molecules are absorbed by independent transport systems. The number of these carrier molecules may be inversely related to the degree of absorption of gammaglutamylamino acid. During starvation of the cells for amino acids (or during low levels of extracellular amino acids), more transport carrier molecules are induced and incorporated into the plasma membrane. Thus, gammaglutamylamino acids may serve as transmembrane or environmental signals for the uptake of amino acids by cells (Payne and Payne, 1984). Cells can use the GC cycle for efficient recovery of cysteine. Human trophoblastic microvilli contain high levels of GGTP (Barnwell and Sastry, 1987). Maternal smoking during pregnancy decreases the activity and V_{max} of GGTP of the microvillus plasma membrane (Sastry and Horst, 1988; Horst and Sastry, 1988; Sastry *et al*., 1989a). This decreases the formation and absorption of gammaglutamylamino acids, signalling the cell to induce the synthesis of more amino acid transport carriers and to insert them into the plasma membrane. There is an increase in the uptake of α-AIB, mainly due to an increase in V_{max}, in washed placental villi from smokers. The induction of placental α-AIB transport sites during maternal smoking can be partially explained by depression of the GC cycle. However, in the presence of tobacco-smoke components, the increased carrier systems do not fully compensate for the depression of the GC cycle. The effects of maternal smoking on components of the GC cycle other than GGTP are not known.

Depression of the synthesis of placental ACh by inhibitors of choline acetyltransferase (ChAT) (Rowell and Sastry, 1981; Sastry *et al*., 1983) and inhibition of the conversion of membrane phosphatidylethanolamine to phosphatidylcholine by phospholipid *N*-methyltransferases (PMTs) (Sastry *et al*., 1983; Barnwell and Sastry, 1987) decreases amino acid uptake by placental villi. Therefore, it has been postulated that the placental cholinergic system and phospholipid *N*-methylation regulate amino acid uptake by the placenta. Both ChAT and the PMTs contain active -SH groups. The Cd ions in tobacco smoke inhibit these enzymes (Sastry, 1984; Sastry *et al*., 1985). The PMT activity of the placental plasma membrane is depressed in cigarette smokers (Horst and Sastry, 1989).

Oxygen consumption by placental slices from smoking mothers decreases in proportion to carboxyhaemoglobin (CoHb) concentrations in maternal blood (Tanaka, 1965). The rate of oxygen consumption in placental slices from nonsmoking mothers ($1.9\,\mu l\ mg^{-1}\ h^{-1}$) is about 30% higher than that in placental slices from smoking mothers ($1.3\,\mu l\ mg^{-1}\ h^{-1}$, at an 8% maternal CoHb concentration). These observations indicate that the energy-dependent processes of placental cells may be depressed, and that the formation and concentrations of cellular adenosine 5′-triphosphate (ATP) may be affected by hypoxia. All of the above mechanisms for the regulation of amino acid transport are ATP-dependent. Three ATP molecules are required for operation of the GC cycle and the uptake of one molecule of gammaglutamylamino acid. Another ATP molecule is required to form one molecule of acetyl coenzyme, a substrate for ChAT to form one molecule of ACh. Still another ATP molecule is required to form one molecule of phosphatidylcholine by phospholipid methylation. Therefore, all postulated regulatory mechanisms of amino acid transport in the placenta may be indirectly affected by maternal smoking. Not all babies of smokers are subjected to IUGR. The placentas of some smokers incorporate more amino acid transport systems into trophoblast plasma membranes, and compensate for the effects of smoking. If the placentas of some babies have a genetic defect for this purpose, those babies are subjected to IUGR due to smoking.

In a recent report, Zhao and Reece (2005) reported that the nicotine-induced embryonic malformations are, in part, a result of excessive cell death. Nicotine

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increases intracellular [Ca^{2+}] and ROS level, which play a role in nicotine-induced embryonic apoptosis and malformations.

7.3 Morphine Placental Opioid System and its Role in Amino Acid Transport

Different aspects of the human placental opioid system have been reviewed by Sastry (1995a) and Ahmed and Cemerikic (1995). Placental transfer of the three commonly used opioids (fentanyl, alfentanil and sufentanil) was reported by Giroux *et al*. (1997). When ingested by pregnant women, morphine and its analogues are known to retard intra-uterine foetal growth, the extent of which is related to the degree of exposure. This raises questions about the role of the placenta in morphine-induced intra-uterine growth retardation and about the occurrence and role of endogenous opioids. Generally, it is believed that enkephalins and endorphins may serve as neuromodulators and may regulate neurotransmitter or hormone release by positive or negative feedback systems. More specifically, enkephalins may regulate neuronal release of ACh and norepinephrine by negative feedback systems (Sastry, 1991b; 1995b). The occurrence of methionine enkephalin and β-endorphin in human placental villi has been demonstrated using sensitive and specific radioimmunoassays and bioassays. Immunoreactive corticotrophin, lipotrophin and β-endorphin occur in whole placental extracts. Dynorphin (1–8) was also identified in placental extracts by mass spectroscopy. The opioid receptor of the placenta was also purified and identified as the kappa subtype (Ahmed *et al*., 1989). When the floating villi, chorionic plate and basal plate of the same human placenta were extracted and analysed, the methionine enkephalin concentration was lower in the chorionic and basal plates. This suggests that the distribution of methionine enkephalin is similar to that of ACh in human placenta.

ACh occurs in high concentrations in the human placenta. Inhibition of synthesis of ACh in placenta inhibits amino acid uptake. ACh release, activation of a cholinergic receptor, Ca^{2+} influx and amino acid transport are linked to one another in the uptake of amino acids by the human trophoblast (Sastry, 1997). Atropine blocks ACh release and amino acid uptake by placental villi, indicating that ACh stimulates a muscarinic receptor and thereby regulates amino acid uptake by the trophoblast. Amino acid uptake by placental villi is also depressed in a Ca^{2+}-free medium and by morphine (Barnwell and Sastry, 1983). These observations indicate that, in morphine addicts, ACh-facilitated uptake of amino acids by the trophoblast is depressed. Therefore, IUGR in morphine addicts can be partially explained by depressed amino acid transport in the placenta. These studies have yet to be supported by analysis of amino acids in the placentas of morphine addicts and nonaddicts. Some compensatory changes have been reported to occur in the placental opioid systems of mothers who used morphine-like compounds during pregnancy. The number of kappa opiate receptors decreased in the placentas of mothers who used pentazocine or methadone during pregnancy. Morphine did not decrease ACh release from these placentas. These observations indicate that the negative feedback mechanism for the release of ACh is depressed or inactivated due to an inadequate number of kappa receptors in the placentas of opiate addicts. This is a compensatory change to improve ACh-facilitated amino acid uptake by the placentas of opiate-addicted mothers. If there are any other compensatory changes (e.g. a decrease in the synthesis of opioid peptides) in the opioid systems of opiate addicts, they have yet to be investigated.

7.4 Cocaine and Placental Amino Acid Transport

Cocaine abuse has become increasingly prevalent among certain urban populations. Cocaine readily crosses the placental barrier and enters the foetal circulation with the potential to cause adverse effects on the developing foetus. Studies with the perfused human placental cotyledon showed that rapid cocaine transfer exhibits the characteristic of passive transport consistent with its high lipid solubility and low MW (Schenker *et al*., 1993; Simone *et al*., 1994). Cocaine, as a weak base, is only 8–10% protein bound in human plasma, suggesting that the majority of the administered dose is available to equilibrate with the foetal circulation. In addition, most evidence indicates that cocaine decreases uteroplacental blood flow in rats as well as humans. Intra-uterine growth retardation, often associated with cocaine use, is assumed to result from chronic decreases in uteroplacental flow.

The subject of maternal cocaine use and IUGR was reviewed by Sastry (1995c). Investigators have focussed on various complications of pregnancy and malformations in infants, but consistent findings are foetal growth retardation and prematurity. In addition, cocaine may interfere with normal physiological processes in pregnancy, resulting in a higher incidence of spontaneous abortion, placental abruption and premature labour. Cocaine seems to interfere with placental amino acid transport in a similar way to nicotine and morphine. Cocaine acts as a Ca^{2+} antagonist in smooth muscle. It also decreases the release of ACh from placental villi (Sastry *et al*., 1977). This action may arise from the blockade of Ca^{2+} influx into the syncytiotrophoblast. Ca^{2+} is necessary for the release of ACh and the uptake

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of amino acids (Sastry *et al*., 1983). Cocaine depresses the uptake of neutral, acidic and basic amino acids by the human placenta and thus the placental transport of these amino acids (Barnwell and Sastry, 1983). Therefore, cocaine-induced foetal growth retardation also can be partially explained by depression of placental amino acid transport.

7.5 Alcohol and Placental Function

The effect of alcohol on placental functions and foetal toxicity has been reviewed by Henderson and Schenker (1995). Maternal ethanol consumption, either continuously throughout pregnancy or in a 'binge' pattern, may produce adverse and lasting or even fatal outcome. In the human foetus, the most consistent impact is growth retardation, with specific detrimental impacts on the developing CNS. The overall effect of alcohol is described as foetal alcohol syndrome (FAS) which is characterized by (i) growth retardation; (ii) CNS abnormalities (which may include abnormal brain morphology, neurological abnormalities, developmental and intellectual impairment); and (iii) the characteristic pattern of craniofacial abnormalities (Jones *et al*., 1973; Larroque, 1992). These are associated with sustained heavy maternal ethanol consumption (Forrest *et al*., 1992). In addition, several extensive epidemiological studies over the past decade have provided evidence that, even in the absence of growth deficits and developmental delay, subtle but lasting behavioural and intellectual dysfunctions may persist (Committee on Substance Abuse and Committee on Children with Disabilities, 1993). These 'foetal alcohol effects' (FAE) include hyperactivity, attention and fine-motor deficits and a constellation of psychosocial disorders. A question arises about the contribution of the placenta to overall FAS. According to Henderson and Schenker (1995), there is an enormous body of evidence supporting direct impact of ethanol and acetaldehyde, a metabolite of alcohol, on a variety of foetal tissues and their components, and such direct effects may ultimately be proven to be the origin of toxicity of ethanol towards the foetus. In view of the central role of the placenta in maintenance of foetal development and the well-documented effects of ethanol on its three primary functions (transport, metabolic and endocrine), there is ample evidence that alterations of a variety of placental functions may contribute partly to the toxic effects of ethanol on the foetus.

Alcoholism is frequently associated with malnutrition and this, combined with the lack of normal growth and development of FAS children, suggests that impaired nutrition contributes to this syndrome. Thus, several studies have focussed on the hypothesis that maternal ethanol consumption produces a suboptimal supply of nutrients needed for foetal growth. There is some evidence from animal experiments and *in vitro* experiments using human placental tissues that ethanol affects nutrient transport of amino acids, glucose, some vitamins (vitamin B_6, folate, thiamine) and some trace elements (Ca, Zn). During placental transfer a small amount of ethanol is metabolized to acetaldehyde, which is toxic to the embryo and foetus.

Alcohol influences the effects of biogenic amines on human placental vasculature and, therefore, placental blood flow. It also influences membrane fluidity of placental membranes and thereby influences responsiveness of the placental vasculature to biogenic amines.

8 METALS

Metals are ubiquitous in the environment, contaminating soil, water and air and, finally, the human food chain. As a result, metal pollution has been one of numerous environmental problems throughout the world. Some heavy metals, such as copper, iron, zinc, cobalt, manganese and selenium, are nutritional trace elements, whereas others, such as aluminium (Al), arsenic (As), Cd, Pb, mercury (Hg) and nickel (Ni), are metals of greatest toxicological concern. In excessive concentrations, metals from either category are known to exert a wide range of toxic effects. It is well recognized by now that maternal intoxication with certain metals in both man and laboratory animals may adversely affect pregnancy and the development of the conceptus. Placental toxicity of some important metals is discussed here in brief.

8.1 Aluminium

Aluminium is one of the three most abundant metals in the earth's crust. As a result, it contaminates food and water. Al is commonly used in several forms, including the chloride, nitrate, hydroxide (in stomach antacids), chlorhydrate (in deodorants), sulfate (in water treatment) and phosphide (in insecticidal grain fumigants). In addition, Al is also extensively used in cooking utensils, appliances, automotives, aircraft, construction and so on.

Al can be absorbed through oral (po), dermal, inhalation, subcutaneous (sc) and other parenteral routes, and concentrated in the liver, lungs, bone, kidney, spleen, heart, brain and muscles. Al is unequally distributed in these tissues (Yokel and McNamara, 2001). In addition, Al transplacentally traverses and accumulates in the foetal tissues in amounts that adversely influence foetal development (Yokel and McNamara, 1985). Studies using mice and rabbits revealed that the placenta contained four- to five-fold higher Al levels than the foetal or maternal tissues (Yokel and McNamara, 1985; Cranmer *et al*., 1986; Yumoto *et al*., 2000). However, the placenta of

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the guinea pig does not accumulate Al. Furthermore, placental accumulation of Al in mice and rabbits does not preclude accumulation in the foetal tissues. For the detailed toxicokinetics of Al, the review published by Yokel (1997) and Steinhausen *et al*. (2004) should be consulted.

Al is a nonessential element, as its absence in nutrition does not influence developmental growth. In the last two decades, however, Al has been recognized as an important toxic metal in experimental animals and humans (ATSDR (Agency for Toxic Substances and Disease Registry), 2007a).

Pregnancy in general enhances susceptibility to Al toxicity. Oral administration of Al during pregnancy in laboratory animals is known to cause a developmental syndrome which includes *in utero* death, delayed ossification of skeleton, malformations, growth restriction and developmental delay, at doses that also influence maternal weight gain. The severity of the effects is highly dependent on the form of Al administered.

Following oral administration, only a small fraction of Al is absorbed and, therefore, the degree of severity of developmental toxicity is minimized. Studies using high Al exposures obtained by intravenous (iv) or intraperitoneal (ip) administration report a developmental toxicity syndrome consisting of death and resorptions, skeletal and soft tissue abnormalities, low birth weights and growth retardation in rats, mice and rabbits (Wide, 1984; Yokel, 1997). The high incidence of resorptions was found to be significantly higher in pregnant rats treated with aluminium chloride at dose levels of 75 mg kg^{-1} on gestation days (GDs) 9–13, 100 mg kg^{-1} on GD 14–18 and 200 mg kg^{-1} on GD 9–13 (Benett *et al*., 1975). A high incidence of dead offspring was recovered from mothers treated with aluminium chloride at a dose level of 200 mg kg^{-1} on GD 9–13.

The above studies using rats, mice and rabbits indicate that Al can cause delays in skeletal development in pups. Gross foetal abnormalities include abnormal digits, wavy ribs, missing ribs, absence of xiphoid and poor ossification (particularly in the cranial bones, lower part of the vertebral column and long bones of the limbs). The high incidence of skeletal defects, and also poor ossification in foetuses of mothers treated with aluminium chloride, suggests that Al has an adverse influence on foetal bone formation. This could be due to the binding of Al with phosphate in the tissues, thus reducing the amount of phosphate in the tissues available for bone formation. Depletion of phosphate has also been linked to Al-induced foetal internal haemorrhage by causing failure of the blood clotting mechanism (Wide, 1984).

A number of studies have reported neurotoxicity and neurobehavioural changes in offspring following Al exposure of rabbits, rats and mice at doses of Al that did not produce maternal toxicity (Wide, 1984; Domingo, 1994; Yokel, 1997; Colomina *et al*., 2005). Exposure to aluminium nitrate or aluminium citrate can also produce similar developmental effects. Overall, developing conceptuses or pups are much more sensitive than adults to Al toxicity.

8.2 Arsenic

Arsenic occurs in many forms and, therefore, commonly contaminates the air, food and water. The major sources of human exposure to As include sea food, tobacco and pharmaceutical preparations. Individuals who work in smelters and industries and those involved in the production and use of As-containing pesticides are at especially greater risks. Paint pigments, fly papers and wood preservatives are also potential sources of As.

Both human and animal studies have shown that As crosses the placenta. In pregnant mice, Hood *et al*. (1987; 1988) examined the uptake, distribution and metabolism of sodium arsenite (2.5 µg kg^{-1}, po) and sodium arsenate (40 µg kg^{-1}, po) on GD 18. Maximum concentrations of As in the placenta were noted at 4 and 2 hours after administration of arsenite and arsenate, respectively. Corresponding concentrations in the foetal tissues appeared at 24 and 6 hours. Regardless of the form of As administered, >80% of the As is methylated (mono- and dimethyl-arsenic) in the foetus. In the foetal tissues, inorganic As is converted to the organic form(s) over time. In other words, methylation appears to be the major detoxification mechanism and, therefore, substantially reduces toxicity. Arsenic is completely eliminated from the placenta and foetus within 24 hours of exposure. For some reason, organic As does not seem to cross the placenta; instead, it is stored in the placenta.

Epidemiological investigations conducted in a few countries strongly support the evidence of placental toxicity in humans due to As. In a Swedish study, adverse reproductive outcomes (spontaneous abortions, congenital malformations and low birth weights) were observed in populations working in or residing near the Ronnskär copper smelter. Congenital malformations were higher in the occupationally exposed population. Similar findings were observed in a Hungarian study. In a study conducted in Bulgaria, pregnancy complications and rates of mortality at birth (due to malformations) were high in the smelter area. The average concentration of As in the placentas of exposed populations living near the copper smelter was 26.6 µg kg^{-1}, compared with 7.4 µg kg^{-1} in the placentas of those living in areas with no industrial sources of metal pollution. Reduced glutathione and increased lipid peroxidation in the placenta with As appeared to cause the pregnancy complications. Furthermore, As-induced genetic damage (such as chromosomal aberrations) seems to be the cause of malformations, spontaneous abortions and stillbirths in those who work in smelters or those living nearby (Nordström *et al*.,

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1978). In a case-control study conducted in Boston, no association between As in drinking water and congenital heart defects was found, although an association with coarctation of the aorta was noted. Owing to the small number of cases, this association might be due to random variation. In Bangladesh, As $\geq$0.10 ppm in drinking water was associated with adverse pregnancy outcomes in terms of spontaneous abortion and stillbirth. A study of 202 women from West Bengal, India, reported that exposure to As concentrations of $\geq$0.2 ppm in drinking water (approximately 0.02 mg As kg^{-1} day^{-1}) during pregnancy were associated with a six-fold increased risk of stillbirth (von Ehrenstein *et al*., 2006).

Animal studies (using hamsters, rats and mice) suggest that high doses of ingested As may be foetotoxic and teratogenic. There is evidence that sodium arsenate and sodium arsenite are embryotoxic (Chaineau *et al*., 1990). Common deformities include hypoplasia of the prosencephalon, somite abnormalities, and failure of development of limb buds and sensory placodes. In hamsters, a single iv injection of 20 mg kg^{-1} sodium arsenate, given on GD 8, resulted in 49% malformed and 84% either malformed or resorbed embryos on GD 13 (Holmberg and Ferm, 1969). Common malformations included exencephaly, encephalocele, cleft lip/palate, micro/anophthalmia and ear malformations. In similar studies, other investigators also found developmental defects in the ribs and kidneys. In rats, the spectrum of malformations by As is similar to that described for hamsters.

Placental/developmental toxic effects of As have been more extensively studied in mice than in rats or hamsters. Treatment of female mice with arsenite/arsenate on GD 9 produces the highest incidence of malformed live foetuses, whereas treatment on GD 11 or 12 produces the highest rate of resorption. The common external malformations include exencephaly, micrognathia, exophthalmia, anophthalmia, cleft lip, hydrocephalus, micromelia, ectrodactyly, open eyes, rib defects and vertebral defects. Oral administration of dimethylarsenic acid (100 mg kg^{-1}) in mice produces foetotoxicity (reduced weight, delayed ossification and cleft palate) and maternal toxicity. Mice and rats are much more sensitive than hamster to organoarsenicals, in terms of placental toxicity.

Although the exact mechanism involved in placental toxicity of As has not been explored, increased lipid peroxidation in the human placenta has been linked to As exposure. Increased lipid peroxidation appears to be due to excess production of superoxide and hydroxyl radicals. Arsenic is known to interact with protein sulfhydryl groups, and thereby inactivate target enzymes. Free sulfhydryls are essential for the function of a wide range of enzymes, including glutamic-oxaloacetic acid transaminase, pyruvate oxidase, monoamine oxidase, choline oxidase, glucose oxidase, urease, oxidoreductases and kinases. Arsenic accumulates in the mitochondria and thereby affects a number of enzymes, including those involved in mitochondrial respiration (Rogers, 1996). Arsenic inhibits succinic dehydrogenase activity and uncouples oxidative phosphorylation, which results in ATP decreases. Reduced ATP affects virtually all cellular functions (Na^+/K^+ balance, protein synthesis, etc.). In an *in vitro* study, arsenite proved to be teratogenic at concentrations between 3 and 4 μM, and embryolethal at higher concentrations, whereas arsenate had similar activity, but at concentrations 10 times higher than for arsenite (Chaineau *et al*., 1990). Induction of stress protein or heat shock protein (hsp) synthesis in the embryo has been explained as a common mechanism of teratogenesis by As.

In conclusion, inorganic arsenicals are much more potent toxic agents than organic arsenicals. Trivalent As is developmentally more toxic than pentavalent As. It is believed that compounds containing pentavalent As exert their toxic actions only after their conversion to the trivalent form. Foetotoxicity or teratogenicity due to As is of human concern only at the levels that are maternotoxic. In other words, the foetus is not more susceptible to As toxicity than the mother. For further details on As toxicity in general and placental toxicity in particular, readers are referred to the ATSDR (Agency for Toxic Substances and Disease Registry) (2007b).

8.3 Cadmium

Cd occurs naturally in the earth's crust. Most Cd used in the United States is extracted as a by-product during the production of other metals, such as Zn, Pb and Cu. The major sources of Cd exposure to humans include contaminated food, cigarette smoke and industrial pollution.

Although the biological function of Cd is unknown, its potential for causing developmental toxicity in humans and animals is well established. Correlation between maternal Cd exposure and low foetal birth weight has been established in epidemiological studies conducted in Russia and France. None of these studies has revealed any congenital malformations.

In laboratory animals, Cd has been shown to be a developmental toxicant by the oral, inhalation and parenteral routes. Cd crosses the placenta in both humans and animals with similar kinetic characteristics. High toxic concentrations of Cd accumulate in the human placenta, and then perturb the placental transport of essential elements such as Ca and Zn (Eisenmann and Miller, 1996).

From both humans and animal studies, it is clear that the placenta itself is a target organ for Cd toxicity. In low to moderate doses, Cd is sufficiently sequestered in the placenta, whereas in higher doses, Cd causes

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placental necrosis and foetal toxicity. Maternal exposure to Cd is known to cause ultrastructural changes in the placenta, especially trophoblast cell layer II (di Saint'Agnese *et al*., 1983). Changes include lysosomal vesiculation, nuclear chromatin clumping, nucleolar alterations and apparent mitochondrial calcification. Cd-induced placental necrosis occurs initially in trophoblast cell layer II and follows rapidly in the remaining trophoblasts.

Subcutaneous administration of cadmium chloride acetate or lactate to pregnant rats at a dosage of 0.04 mmol kg^{-1} between days 17 and 21 of gestation resulted in rapid, progressive placental destruction, especially in the pars foetalis (Parizek, 1964). Necrotic changes could often be seen within 6 hours. Complete destruction of the pars foetalis resulted in resorptions or delivery of dead conceptuses. Similar effects have been observed with other cadmium salts (Rogers, 1996).

In animal models, Cd has been shown to produce a variety of adverse reproductive outcomes. Maternal exposure to Cd during pregnancy can cause maternal toxicity, placental damage, impaired implantation, increased resorptions, reduced litter size, foetal growth retardation, congenital malformations in the foetus and embryonic/foetal death. The developmental toxicity of Cd during mid to late gestation involves both placental toxicity (reduced blood flow and necrosis) and inhibition of nutrient transport across the placenta (Rogers, 1996). Exposure during late gestation results in foetal death in rats, despite low levels of Cd entering the foetus (Levin and Miller, 1980). The increased toxicity of Cd to the pregnant rat may be due to renal failure subsequent to shock from placental haemorrhage.

The teratogenic potential of Cd has been proved in hamsters, rats and mice. Single iv injections of 2 mg kg^{-1} cadmium sulfate were administered to pregnant hamsters on GD 8. In addition to high incidence of resorption, live foetuses showed a high rate of malformations, including facial clefts, exencephaly, anophthalmia, limb defects and rib fusions (Ferm and Carpenter, 1967). Common malformations in the foetuses of rats and mice are club foot, dysplasia of facial bones and rear limbs, cleft palate, micrognathia, sirenomelia/amelia, delayed ossification of the sternum and ribs, microphthalmia/anophthalmia, cryptorchidism, gastroschisis and palatoschisis.

The placenta provides a remarkable barrier to Cd and, therefore, minimized foetal toxicity. This is partly due to the ability of Cd to induce synthesis of metallothionein (MT), a small protein rich in sulphur-containing amino acids; and synthesis in maternal tissues, as well as in the placenta. MTs retain Cd in maternal tissues and the placenta and, thereby, reduce Cd transport to the conceptus. To date, the best understood mechanism for developmental toxicity is the interaction between Cd and Zn, in which Cd substitutes for Zn in metalloenzymes. Cd interferes with Zn transfer across the placenta, possibly via MT induction in the placenta. Because of the high affinity of MT for Zn, MT sequesters Zn in the placenta, impeding transfer to the conceptus. Cd inhibits Zn uptake by human placental microvesicles, suggesting that Cd may also compete directly with Zn for membrane transport (Rogers, 1996).

Reduced uteroplacental blood flow, reduced nutrient transport and placental toxicity by Cd appear to be the major contributing factors for low foetal birth weight, foetal toxicity, malformations and death. Evidence suggests that Cd-induced impaired foetal growth is partly mediated through Zn deprivation, since maternal tissues and the placenta retain Zn. The direct effect of Cd on foetal growth also occurs because of Cd accumulation in the foetus. Mechanistic studies provide strong evidence of embryotoxicity by Cd (Fein *et al*., 1997). The accumulation of only small amounts of Cd could inhibit embryonic DNA and protein synthesis (Holt and Webb, 1987). Results of studies also suggest that Cd exposure significantly compromises the Ca handling ability of trophoblast cells. This effect is probably a consequence of alterations in subcellular cytosolic calcium binding properties.

8.4 Lead

Pb occurs naturally in the environment. The common sources of Pb exposure include Pb-based paint, batteries, gasoline, ceramics, soil/dust, caulking, roofing, ammunition and scientific and medical equipment. As a result, the whole environment (including food, water and air) is contaminated with low levels of Pb. Populations at greatest risk to the adverse health effects of Pb have been identified as pregnant women and their unborn children and preschool-age children.

Pb exposure in the pregnant woman usually occurs through the oral and/or inhalation route. After absorption, Pb is distributed to most of the tissues, but it deposits mainly in the skeleton, kidney and brain (primarily gray matter). Pb is known to cross the placenta in both humans and animals and the maternal/cord blood ratio for the lead ranges from 0.9 to 0.93 (ATSDR (Agency for Toxic Substances and Disease Registry), 2006). Accumulation of Pb occurs in the foetal brain owing to partial or lack of a BBB. In one study, the placenta from a Pb-exposed mother was found to have a greater than three-fold higher Pb level compared with the placenta from an unexposed mother (Loiacono *et al*., 1992). Pb also accumulates in the placenta in times of foetal stress.

In general, cord blood and placenta of exposed women have high Pb levels, which, consequently, are also reflected in the foetus. In fact, prenatal exposure to Pb is generally estimated through maternal and/or cord blood Pb concentrations. Studies suggest that a large maternal–foetal concentration gradient exists and the placenta poses a limited transplacental barrier. Also, a

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number of adverse maternal health conditions can affect the transfer of Pb to the foetus and/or the retention of Pb by the mother or the foetus.

At present, the exact mechanism involved in placental toxicity of Pb remains unknown, although Pb produces intoxication in general by interfering with protein/haemoprotein biosynthesis and by inhibiting membrane and mitochondrial enzymes (Eisenmann and Miller, 1996). Pb is also known to cause deficits in cholinergic, dopaminergic and glutamatergic functions (ATSDR (Agency for Toxic Substances and Disease Registry), 2006).

Following *in utero* exposure, Pb can have a direct effect on the developing conceptus. It is important to mention that the developing nervous system is the most sensitive target of Pb toxicity. Following low levels of Pb exposure in pregnant women, Pb has been associated with increased incidence of preterm births and reduced birth weight (Andrews *et al*., 1994). The other developmental effects include reduced gestational age and neurobehavioural deficits. Exposure to high levels of Pb can cause infertility, neonatal morbidity, miscarriages, spontaneous abortions and stillbirths. It has been shown that Pb can interfere with an early stage of development, such as the invasion of the fertilized ovum into the uterine wall.

Unborn children and young children appear to be particularly sensitive to Pb because of their greater sensitivity during development. At low doses, Pb does not produce congenital malformations. However, at high doses, Pb produces minor anomalies such as haemangiomas, lymphangiomas, cryptorchidism and tags and papillae on the skin. An association seems to exist between drinking water which contains Pb and congenital anomalies of the ear, face, neck and cardiovascular system. In addition, prenatal exposure to Pb can result in neurobehavioural deficits, reduced hearing acuity and endocrine disruption.

Developmental toxic effects of Pb have also been studied in experimental animals, including rats, mice, hamsters and chicks. *In utero* exposure to Pb can cause reduction in fertility and growth retardation. Embryotoxic and foetotoxic effects of Pb seem to be dependent on the exposure period of gestation. For example, when exposure occurs on GD 9, teratogenic effects are observed with few resorptions, compared with exposure on GD 16, with hydrocephalus and CNS haemorrhage (McClain and Becker, 1975). At maternotoxic doses, Pb can cause retarded skeletal development.

In mice, Pb is known to cause postimplantation mortality and skeletal malformations in foetuses. The common skeletal anomaly observed is the fusion of two or more cervical vertebrae (Jacquet and Gerber, 1979). In hamsters, malformations due to Pb exposure occur mainly in the tail, ranging from stunting to complete absence of the tail (Ferm and Carpenter, 1967). In general, common malformations observed in experimental animals are related to brain defects, neural tube defects and urogenital system and tail defects. It should be noted that Pb has a greater potential for neurotoxicity than for placental or developmental toxicity. Further details can be found in previous publications (Eisenmann and Miller, 1996; Rogers, 1996; ATSDR (Agency for Toxic Substances and Disease Registry), 2006; Gupta, 1998; 2007).

8.5 Mercury

Hg is a nonessential metal that occurs naturally in the environment and exists in several forms. Hg is released into the environment in the metallic or elemental form by natural processes. Common sources of Hg include industrial, chemical/mining, medical supplies, adhesives, ink, caulking, fungicides and interior/exterior paint. As a result, Hg is a widespread environmental contaminant that threatens aquatic ecosystems and human health.

The general population is exposed to Hg primarily from food and dental amalgam. In most foodstuffs, Hg is found in the organic form. Fish, marine mammals and some microorganisms convert elemental Hg to organic Hg, which accumulates in the food chain. It is of great interest to note that in humans, organic Hg can be converted to inorganic Hg.

It is now well recognized that Hg (in all forms) is toxic to humans as well as animals. There are numerous incidents in which Hg has been associated with developmental toxicity in humans. The first mass outbreak of MeHg poisoning occurred in Japan in the 1950s and 1960s following the consumption of heavily contaminated fish. The syndrome was referred to as 'foetal Minamata disease'. Over 90% of the total Hg in fish occurs in the form of MeHg. An even larger outbreak of MeHg poisoning took place in Iraq in 1971–1972. Poisoning resulted from consumption of bread loaves prepared from wheat seed grain treated with an MeHg-containing fungicide. These incidents, along with others, indicated that prenatal exposure to MeHg caused severe brain damage in the foetus and infants, whereas mothers were hardly affected. In other words, following *in utero* exposure to MeHg, the target organ is the foetal brain in humans and animals. This is partly due to accumulation of Hg in the foetal brain because of its high requirement for protein synthesis. Also, pregnant women appear to be more sensitive to MeHg toxicity than nonpregnant women.

MeHg is readily absorbed and distributed throughout the body with various concentrations in different tissues. In humans, brain MeHg levels can be as high as six-fold, compared with blood levels. This is in contrast to rats, which have a brain to blood ratio of 0.06, and mice with a ratio of 1.20. There is evidence that the human and rodent

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placenta, by having a metal- binding protein (metallothionein, a low-MW protein enriched with cysteine), presents some barrier to Hg (Yoshida *et al*., 2002). By using the Gray physiologically based pharmacokinetic (PBPK) model for MeHg, the placenta is modelled as four compartments with separate transfer constants for placental barrier and placental tissue transport. Organic and metallic Hg cross the placenta more readily than inorganic Hg. Consequently, MeHg and metallic Hg accumulate in the foetus, whereas inorganic Hg concentrates in the placenta. Metallic Hg, after crossing the placenta, can be oxidized to Hg^{2+} in foetal tissues. It has been shown that Hg^{2+} accumulates in the placenta and inhibits the foetal uptake of certain essential metabolites or analogues of these metabolites.

It is well evidenced from both human and animal studies that MeHg at a moderate to high level of exposure has a strong potential for neurotoxicity and developmental toxicity. Several studies suggest that prenatal exposure to MeHg can cause a widespread pattern of adverse effects on brain development and organization. Neuropathological findings in the victims of the Japan and Iraq incidents revealed that MeHg significantly altered the normal migration of neurons to the cerebellar and cerebral cortices during brain development. Other changes included diffuse and severe cortical damage with cytoarchitectural anomalies, such as ectopic cell masses and disorganized cell layers, altered oxidative stress, membrane function and signal transduction, decreased protein synthesis and changes in neurotransmission (Gilbert and Grant-Webster, 1995).

Both the Japan and Iraq incidents provided evidence that Hg-exposed pregnant women delivered infants with severe developmental and behavioural deficits, without any obvious symptoms of Hg poisoning in mothers during pregnancy. Common developmental defects due to Hg exposure are reduced birth weight, ataxia, retarded walking and limb deformities. Hg-induced neurobehavioural effects include severe mental retardation, sensory and motor deficits, cerebral palsy, plasticity, seizures, delayed speech, deafness and blindness. At present, human studies with MeHg indicate that maternal hair levels of 10–20 ppm may result in adverse effects on foetal outcome.

Several studies using animal models with high-dose MeHg exposure have described effects similar to those observed in humans. MeHg has been reported to be embryotoxic/foetotoxic and teratogenic in mice, rats, cats, guinea pigs and hamsters. In general, resorptions, dead foetuses and cleft palate are the most common findings. Some other developmental effects include generalized oedema, brain lesions, wavy ribs, asymmetric sternebrae and decreased ossification of parietal and occipital bones (Domingo, 1994).

In summary, both human and animal data suggest that prenatal exposure to sufficient amounts of Hg results in developmental toxicity. In addition, Hg has a strong potential for neurotoxicity and neurobehavioural toxicity. For further details, readers are referred to other publications (Eisenmann and Miller, 1996; ATSDR (Agency for Toxic Substances and Disease Registry), 1999; Gupta, 1998; 2007).

8.6 Nickel

Ni is primarily used in alloys because it imparts the properties of heat resistance, hardness and strength. Its salts are used in alloy steel, stainless steel, cast iron, alkaline batteries, ceramics, electroplating and pigments and as catalysts. The general population is exposed to Ni via food, water, air and tobacco smoke, at very low levels. Data suggest that Ni bioconcentrates in fish and aquatic organisms and, therefore, seafood can be a potential source of Ni exposure. Workers in the industries that produce, process or use Ni are usually exposed to higher levels. As a result, this population group is at greater risk.

Although Ni is an essential element for human and animal health, at higher levels Ni produces deleterious effects, ranging from minor allergic reactions to those as serious as abortions, birth defects and cancer. There is only one published epidemiological report, which provides inconclusive findings on the developmental toxicity of Ni in humans (Chaschschin *et al*., 1994).

Ni has not been studied for placental toxicity in as much detail as other metals. Exposure of pregnant rats to nickel oxide at a dosage of 1.6 mg m^{-3} for 23.6 h day^{-1} throughout gestation resulted in a decrease in foetal body weight, although the weight of the placenta and the number of foetuses remained unchanged (Weischer *et al*., 1980). Treatment of pregnant rats during early gestation with nickel chloride (8–16 mg kg^{-1}, intramuscular) and nickel sulfide (30 mg kg^{-1}, intramuscular) caused embryonic mortality at doses that did not cause maternal deaths, suggesting accumulation of Ni in foetal tissues and greater sensitivity of the foetal tissue to Ni toxicity (Sunderman *et al*., 1978). In another study, pregnant rats treated with 4 mg kg^{-1} ip Ni showed teratogenic malformations (Mas *et al*., 1985). In a multigeneration and multilitter study conducted in rats, Ni has been shown to adversely affect the time of gestation, birth weight and the number of pups surviving through lactation (ATSDR (Agency for Toxic Substances and Disease Registry), 2005).

Ni has also been studied for placental toxicity in mice and hamsters. Nickel chloride given to pregnant mice on GD 7–11 produces embryonic effects such as a higher incidence of resorptions, reduced foetal weight, delayed skeletal ossification and malformations, such as acephalia, ankylosis, club foot and skeletal anomalies. Embryonic tissues retained Ni at levels as much as 800 times higher than controls (Lu *et al*., 1979). With similar findings, Ni has also been proved

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to be embryotoxic and teratogenic in hamsters. Further details can be found in other publications (Domingo, 1994; ATSDR (Agency for Toxic Substances and Disease Registry), 2005).

9 INSECTICIDES

Insecticides of four classes (OPs, CMs, organochlorines and pyrethroids) are known to adversely affect the mother, placenta and conceptus in laboratory animals (Gupta, 1995; 2007; Pelkonen *et al*., 2006). Here, OPs and CMs are discussed together because their effects are similar.

9.1 Organophosphates and Carbamates

Both OPs and CMs, in general, exert overt toxicity of hypercholinergic preponderance by inhibiting the activity of AChE at cholinergic synapses and neuromuscular junctions. Inactivation of AChE occurs owing to phosphorylation by OPs and carbamylation by CMs. It is important to mention that the level of AChE activity is already low during pregnancy. As a result of AChE inhibition by OPs or CMs, accumulation of ACh occurs, which overstimulates the muscarinic and nicotinic ACh receptors. Muscarinic receptor-associated effects include miosis, salivation, lacrimation, urination, diarrhoea and tracheobronchial secretion. Nicotinic receptor-associated effects include tremors, muscle fasciculations and convulsions. In addition, AChE inhibition disrupts cell replication and differentiation, synaptogenesis and axonogenesis (Lauder and Schambra, 1999; Bigbee *et al*., 2000). Evidence also suggests that many of the pharmacological/toxicological actions of OPs and CMs are much more complex and have no direct relationship to AChE inhibition or the accumulation of ACh (Dettbarn *et al*., 2006; Gupta *et al*., 2007). Finally, death supervenes owing to paralysis of the respiratory centre.

Both OPs and CMs readily cross the placenta and act on the cholinergic and noncholinergic components of the developing nervous system and other vital organs (Gupta *et al*., 1984; 1985; Pelkonen *et al*., 2006; Gupta, 2007). Recent studies have shown that, in general, foetuses and young children are more sensitive, because they have lower than adult levels of detoxifying enzymes (paraoxonase or chlorpyriphos-oxonase) that activate OPs (Furlong *et al*., 2006; Holland *et al*., 2006). Furthermore, the developing organism appears to be much more susceptible to the induction of functional neural deficits (Rice and Barone, 2000; Eskenazi *et al*., 2007). Following prenatal exposure to OPs (quinalphos, dicrotophos (Bidrin), parathion-methyl and others), significant inhibition of AChE has been demonstrated in maternal, placental and foetal tissues of rats and mice (Bus and Gibson, 1974; Gupta *et al*., 1985; Srivastava *et al*., 1992). Similar results have been reported for CMs, including carbaryl, carbofuran, aldicarb and pirimicarb (Declume and Derache, 1977; Cambon *et al*., 1979; 1980). These studies revealed AChE inhibition as the major biochemical mechanism of toxicity. Furthermore, subchronic prenatal exposure to parathion-methyl in rats resulted in altered postnatal development of brain AChE and ChAT activities, and selected subtle alteration in behaviour (Gupta *et al*., 1985).

Prenatal exposure to OPs has been shown to affect protein synthesis both *in vivo* (Clouet and Waelsch, 1963; Gupta *et al*., 1984) and *in vitro* (Welsch and Dettbarn, 1971). The inhibitory effect on *in vivo* protein synthesis of parathion-methyl (administered throughout the period of organogenesis) was shown to be dose dependent, greater on day 19 than day 15 of gestation and more pronounced in foetal than in placental or maternal tissues of rats (Gupta *et al*., 1984).

It is important to mention that the mechanisms responsible for OP- or CM-induced embryonic/foetal development and teratogenesis appear to be different from those involved in general toxicity. Alkylation of nicotinamide adenine dinucleotide (NAD^+) coenzymes by OPs appears to be the major mechanism involved in the induction of teratogenesis (Schoental, 1977). Other investigators found altered levels of RNA, glycogen, sulfated mucopolysaccharides and calcium in the developing tibiotarsus (Ho and Gibson, 1972). No such studies have been reported with CMs.

Both OPs and CMs have been demonstrated to have potential for embryotoxicity, embryolethality, foetotoxicity and teratogenesis. In general, these effects vary depending upon the particular OP or CM involved. Embryolethality is encountered so often that the expression of teratogenesis is rarely seen. In humans, babies born to mothers exposed to OPs such as oxydemeton-methyl (Metasystox-R) and mevinphos (Phosdrin) showed cardiac defects (ventricular and atrial septal defects), stenosis of the pulmonary artery and a patent ductus arteriosus, bilateral optic nerve colobomas, microphthalmia of the left eye, cerebral and cerebellar atrophy and facial anomalies (Ogi and Hamada, 1965; Romero *et al*., 1989).

Many placental toxicity studies conducted in rats, mice, rabbits and hamster have failed to demonstrate any teratogenic response to OPs, although, in some studies, growth retardation and embryotoxicity have been noted at maternotoxic doses. However, in some other studies, OPs have been shown to produce developmental alterations in rats, mice, hamsters and rabbits. In an elegant study, Khera (1979) discovered polydactyly in foetuses of cats treated with dimethoate ($12\,mg\ kg^{-1}\ day^{-1}$) during day 14 to 22 of pregnancy (**Figure 3**). A wide species variability

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Figure 3 Cat foetus showing heptadactyly of right and hexadactyly of left forepaw. From cat exposed to dimethoate during day 14 to 22 of pregnancy. (Reproduced with permission from Khera, 1979. © Begell House.)

exists in sensitivity to OP-induced placental toxicity. Furthermore, variability exists in gestation period susceptibility.

Abundant recent literature shows that links between exposure to OP pesticides and suboptimal neurodevelopment are found in numerous animal studies (Eskenazi *et al*., 2004). It has been suggested that OP exposure may contribute to poorer neurobehavioural functioning in young animals by producing cellular deficits in their developing brains, particularly rich with cholinergic projections (Campbell *et al*., 1997; Eskenazi *et al*., 2004). Evidence exists that these deficits may result even with lower level exposure if it occurs during critical periods of brain development (Gupta *et al*., 1984; 1985; Eskenazi *et al*., 2004). Recent studies, where chlorpyrifos was administered to neonatal rats further indicated that neonatal exposure produces numerous and persistent deficiencies in cholinergic synaptic function, which can continue into adulthood (Slotkin *et al*., 2001; Slotkin and Seidler, 2007).

Among CMs, carbaryl is the only chemical which has been studied in detail for placental toxicity. Just like OPs, CMs have a greater potential for embryolethality and foetotoxicity, and that precludes an expression of teratogenicity. A study conducted with carbaryl in beagles showed dystocia due to atonic uterine musculature and evidence of terata in 21 of a total of 181 pups. Foetal abnormalities included abdominal-thoracic fissures with varying degrees of intestinal agenesis and displacement, brachygnathia, ecaudate pups, failure of skeletal formation and superfluous phalanges (Smalley *et al*., 1968). Carbaryl exposure during organogenesis produced terata in guinea pigs, but not in hamsters and rabbits (Robens, 1969). Other CMs, such as carbofuran, have proved to be teratogenic (Gupta, 1994).

9.2 Organochlorines

Insecticides of this group are classified into three subgroups: (i) dichlorodiphenylethanes (DDT(dichlorodiphenyltrichloroethane), dicofol, methoxychlor and perthane); (ii) chlorinated cyclodienes (aldrin, dieldrin, endrin, chlordane, endosulfan and heptachlor); and (iii) hexachlorocyclohexanes (BHC (benzene hexachloride), chlordane, lindane, mirex and toxaphene). The acute toxic signs associated with DDT and chlorinated benzene types of insecticides include paresthaesiae of the tongue, lips and face, apprehension, tremors and clonic-tonic convulsions. Stimulation of the CNS is the most prominent effect. The acute signs produced by cyclodienes include dizziness, nausea, vomiting, myoclonic jerking, motor hyperexcitability, convulsive seizures and generalized convulsions. It is important to mention that the epoxide metabolites are much more toxic than their parent cyclodiene compounds (Bondy *et al*., 2003).

The mechanism of action of organochlorine insecticides is not yet fully understood. The DDT-type insecticides alter the transport of sodium and potassium ions across axonal membranes, resulting in an increased negative after-potential and prolonged action potentials. As a result, repetitive firing and a spontaneous train of action potentials occur. Specifically, DDT inhibits the activation of sodium channels and the activation of potassium conductance. The mechanism of cyclodienes involved in hyperexcitation of the CNS and convulsions has been explained based on their structural resemblance to the bicyclic γ-aminobutyric acid (GABA) receptor antagonist picrotoxin. The mammalian GABA receptor is coupled to an intrinsic chloride ion channel and is the primary mediator of neuronal inhibition in the brain. Like picrotoxin, cyclodienes block the inhibitory action of GABA. Similar to other organochlorines, mirex and chlordecone (Kepone) also cause stimulation of the CNS, hepatic injury and induction of the mixed-function oxidase system.

Some of the organochlorine insecticides have the potential to induce placental toxicity. Many organochlorines and their metabolites are known to be found in placentas and to cross the placental barrier (Sala *et al*., 2001). In a recent report, Shen *et al*. (2005) documented the content of many organochlorine pesticides in human placentas. All placentas had measurable levels of some pesticides. The average number of pesticides found per placenta was 18.5 from a total of 27. In

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animal studies, pregnant Swiss mice exposed to lindane at different stages of pregnancy produced various toxicological effects, including foetotoxicity and reproductive failure (Sircar and Lahiri, 1989). Lindane exposure during early pregnancy (days 1–4) caused total absence of any implantation, during mid-pregnancy (days 6–12) caused total resorption of foetuses, and during late pregnancy (days 14–19) caused the death of all pups within 12 hours to 5 days after parturition. In addition, lindane can cause reproductive failure by causing a deficiency of steroid hormones (oestrogen and progesterone).

The organochlorines (such as methoxychlor, *o*-, *p′*-isomers of DDT, DDE (dichlorodiphenyldichloroethylene), DDD (dichlorodiphenyldichloroethane), dieldrin, toxaphene and endosulfan) have been associated with oestrogen-like effects in the reproductive system of laboratory animals. Exposure of female rats to methoxychlor before and during pregnancy can cause blockade of implantation, suppression of uterine decidualization, lack of corpora lutea and atresia of ovarian follicles (ATSDR (Agency for Toxic Substances and Disease Registry), 2002). Exposure during the preimplantation period of pregnancy blocks implantation, whereas exposure during the postimplantation period causes foetal resorption. Interference in the requisite hormonal milieu seems to be the major effect. Other effects of methoxychlor include oestrogenic influence on uterine preimplantation differentiation, ovum transport rate, luteal regression and postimplantation decidual growth. There are reports that dieldrin produced teratogenic effects, such as supernumerary ribs, with concomitant decrease in ossification centres in foetal hamsters (Chernoff *et al*., 1975; 1979; ATSDR (Agency for Toxic Substances and Disease Registry), 2002).

Exposure of pregnant rats to mirex has been shown to result in perinatal deaths due to persistent cardiovascular problems, such as first- to third-degree foetal heart blockade (Grabowski, 1983). Mirex also causes altered lens growth and cataracts, along with other biochemical, physiological and histological changes (Rogers and Grabowski, 1983).

9.3 Pyrethroids

Recently, the use of synthetic pyrethroids has increased tremendously because they possess such high insecticidal efficacy and low mammalian toxicity. Still, the risks to human and animal health exist from accidental exposure and environmental contamination. These insecticides are of two types. Type I pyrethroids are those which lack α-cyano moiety and give rise to the T-syndrome. This syndrome includes whole body tremors, incoordination, prostration, tonic-clonic seizures and death. Common examples of this type are pyrethrin I, allethrin, tetramethrin, resmethrin and permethrin. Type II pyrethroids are those which contain α-cyano moiety and cause the choreoathetosis/salivation (CS) syndrome. This syndrome is characterized by hyperactive behaviour, hunch backed posture, profuse salivation, tremors and motor incoordination, progressing to sinuous writhing movements. Common examples of this type of pyrethroid include cyphenothrin, cypermethrin, deltamethrin and fenvalerate.

Based on symptomatology, Type II syndrome involves primarily an action in the CNS, whereas with Type I syndrome, peripheral nerves are also involved. Intoxication by pyrethroids results primarily from hyperexcitation of the nervous system. This hyperexcitation is caused by repetitive firing and depolarization in nerve axons and synapses. Pyrethroids act directly through interaction with the sodium channel gating mechanism, thereby interfering with the generation and conduction of nerve impulses and inducing marked repetitive activity in various parts of the brain. Type I pyrethroids affect sodium channels in nerve membranes, causing repetitive neuronal discharge and a prolonged negative after-potential, the effects being similar to those produced by DDT. Type II pyrethroids produce an even longer delay in sodium channel inactivation, leading to a persistent depolarization of the nerve membrane without repetitive discharge, a reduction in the amplitude of the action potential and eventual failure of axonal conduction and a blockade of impulses.

Prenatal or early postnatal exposure of rats to pyrethroids (cypermethrin, fenvalerate and others) has been shown to produce significant neurochemical alterations in neonatal rats (Husain *et al*., 1991; 1992; Malaviya *et al*., 1993). Delayed maturation of the cerebral cortex occurs due to alterations in key enzymes of the neurotransmission process (MAO (monoamine oxidase), AChE and Na^+-K^+-ATPase). Prenatal exposure to these pyrethroids significantly delays differential responses in the levels of brain regional polyamines, and ontogeny of sensory and motor reflexes in offspring. Other biochemical/neurochemical effects of pyrethroids include impairment at the neurotransmitter receptor (dopaminergic, catecholaminergic and cholinergic) level.

The pyrethroids, compared with other groups of insecticides, have been less well studied for placental toxicity because pyrethroids are relatively less toxic. For example, permethrin at concentrations of 2000–4000 ppm showed only a weak to moderate influence on *in utero* foetal development. Female rats dermally exposed to cyhalothrin throughout pregnancy had offspring with delayed fur development, delayed ear and eye opening and delayed descent of the testes, but with no change in the age of vaginal opening. In adulthood, however, the sexual behaviour of both male and female rats exposed to cyhalothrin prenatally is no different from that of control animals (Gomes

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et al., 1991a; 1991b). Prenatal exposure of rats to a newer pyrethroid, deltamethrin, has been shown to increase the number of early embryonic deaths and foetuses with retarded growth, hyperplasia of the lungs, dilation of the renal pelvis and increased placental weight (Abdel-Khalik *et al*., 1993). For further details on placental toxicity of pyrethroids, readers are referred to Gupta (2007).

10 DIOXINS AND POLYCHLORINATED BIPHENYLS (PCBs)

10.1 Dioxins

Polychlorinated dibenzo-*p*-dioxins belong to a family of chlorinated aromatic compounds commonly referred to as dioxins. Although there are 75 different dioxin congeners, the term dioxin most often refers to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which is one of the most toxic synthetic chemicals ever known. These compounds are slowly metabolized in the body tissues and, as a result, they persist for a long period of time. It is important to mention that no individual dioxin occurs alone in the environment. In fact, exposure to dioxins usually occurs with a mixture of dioxin congeners along with dibenzofurans, chlorophenols, PCBs and other halogenated aromatic compounds. Hence the toxic effects observed in humans and animals are due to a mixture of chemicals, in which TCDD may be only one ingredient (Safe, 1990; Bursian, 2007). The toxicity of individual congeners varies as much as 1000-fold.

Humans are exposed to dioxins/TCDD by accidental, environmental or occupational means. Contaminated food and water are the major sources of human exposure. So far, there have been only a few studies that described the placental toxicity of dioxins/TCDD in humans. Whether TCDD crosses the human placental barrier is still a matter of conjecture. Human epidemiological studies revealed no clear evidence that TCDD exposure caused birth defects (Mastroiacovo *et al*., 1988; Stockbauer *et al*., 1988). However, high rates for several malformations (in the genitourinary tract, oral cleft, cardiovascular defects and hip dislocation) have been noted in a few studies (Stockbauer *et al*., 1988). A study conducted by the CDC (Centers for Disease Control and Prevention) (Stellman *et al*., 1988) revealed that wives of veterans who had been exposed to Agent Orange had significantly higher risks of having children with birth defects, including spina bifida, cleft lip, cleft palate and a variety of neoplasms. Among a variety of adverse effects, chloracne is the only proven consequence to TCDD exposure.

In animal studies, a variety of toxic effects of TCDD have been reported, which include weight loss, hepatotoxicity, porphyria, dermal toxicity, gastric lesions and haemorrhage, thymus atrophy, immunotoxicity, teratogenicity, reproductive failure, enzyme induction and vitamin A depletion (WHO, 1989; Dickson and Buzik, 1993). These effects vary greatly, depending on dose, species, strain, age and gender.

TCDD is considered one of the most potent embryotoxic and teratogenic chemicals in rats and mice (Safe, 1990; Bursian, 2007). In rats, TCDD is foetotoxic (subcutaneous oedema, intestinal haemorrhage, kidney anomalies, reduced foetal weight and increased foetal death rates) rather than teratogenic (WHO, 1989). In mice, however, teratogenic effects, such as cleft palate and hydronephrosis, have been reported with a single or repeated dose as low as $1-10\,\mu g\ kg^{-1}$ (Skene *et al*., 1989). In rats and mice, the foetotoxic and teratogenic effects are not observed at $0.1\,\mu g\ kg^{-1}\ day^{-1}$. TCDD has also been proved a teratogen in other species, including rabbits, chickens and monkeys (WHO, 1989).

The dioxins and related compounds are proved to be potent developmental toxicants by virtue of their ability to disrupt the actions of hormones and growth factors (Gasiewicz, 1997). Exposure of pregnant animals to dioxins at extremely low levels (doses that do not adversely affect the mother) leads to alterations in the reproductive system of the pups. Ikeda *et al*. (2002) reported that *in utero* TCDD exposure induces demasculinization in male offspring by inhibiting the aromatase activity in the brain during CNS development. Many of the developmental effects are detectable during very early stages, while other effects are not detectable until the offspring reach puberty. Sperm count is decreased in male offspring and their mating behaviour is subtly altered (Gray *et al*., 1997). Ohsako *et al*. (2002) demonstrated that giving pregnant Sprague Dawley rats a single oral dose of TCDD at $1\,\mu g\ kg^{-1}$ body weight on GD 15 resulted in significant decreases in the urogenital complex and ventral prostate weights and urogenital glans penis length of male rat offspring, when sacrificed on postnatal day 70. Testicular and epididymal weights were also lower than control group. Anogenital distance was significantly reduced. The same authors also reported that a single oral administration of TCDD ($12.5-800\,ng\ kg^{-1}$ body weight) to pregnant Holtzman rats on GD 15 caused a decrease in androgen receptor (AR) messenger RNA (mRNA) level in the ventral prostate during the prepubertal period. There are structural abnormalities in the external genitalia of the female offspring and delayed puberty in the male. In the more severely affected pups, fertility is reduced. These effects have been observed in both rats and hamsters. In another study, adult female rhesus monkeys fed a diet containing $0.5\,\mu g\ kg^{-1}\ day^{-1}$ TCDD for six months suffered hair loss, swelling of the eye lids, loss of lashes, irregularities in menstrual cycle, poor conception and abortion (IARC, 1977).

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In addition to the effects described above, the developing immune system appears to be particularly susceptible to TCDD (Skene *et al*., 1989). The immunosuppressive effects can be mediated through *in utero* exposure or by postnatal exposure via the mother's milk. Evidence suggests that both prenatal and postnatal exposure to TCDD can substantially reduce the delayed hypersensitivity and lymphoproliferative response for a prolonged period of time.

There is convincing evidence that all known toxic effects of TCDD are mediated via the aryl hydrocarbon (Ah) receptor. The Ah receptor is present in several tissues, including liver, lung, kidney and placenta of man and animals. In rats, the receptor has been detected in the liver, lung, brain, thymus, kidney, skeletal muscle and testes. The Ah receptor has also been detected in human B-lymphocytes and tonsils (Dickson and Buzik, 1993). TCDD binds to the Ah receptor-ligand complex. This complex is speculated to transform the receptor to a form that can translocate the nucleus, bind with higher affinity to specific DNA sequences and stimulate transcription (Landers and Bunce, 1991).

10.2 Polychlorinated Biphenyls (PCBs)

Unlike dioxins, which are unwanted by-products of certain industrial processes and combustion, PCBs are commercially synthesized and used in transformers and capacitors. PCBs represent a complex mixture of 209 unique compounds. Owing to their resistance to thermal degradation and their stability, they persist and bioaccumulate in the environment. Some of these chemicals persist in the environment for tens or hundreds of years, and in the body tissues for many years.

Two major PCB poisoning incidents occurred in the 1970s in which adverse effects were clearly observed in children born to women exposed to high levels of PCBs along with dioxin analogues and polychlorinated dibenzofurans. One incident occurred in Taiwan in 1979, in which many of the babies born had pigmentation abnormalities and problems with teeth and nails. They were small in size and were developmentally delayed, both physically and mentally. Another incident occurred in Seveso, Italy, in 1976, in which a group of humans was exposed to high levels of dioxin after an explosion at a herbicide plant. Children of these exposed women have not yet been examined for subtle developmental deficits, either structurally or functionally. High concentrations of dioxins and PCBs pose serious health problems in pregnant women and their conceptuses in several other parts of the world, such as around the Canadian Great Lakes, South Vietnam, the former Soviet Union and India.

A small number of PCBs are dioxin-like in their biological activity and can produce all of the toxic effects mentioned above for dioxin/TCDD. Some PCBs appear to be neurotoxic, with prenatal exposure to certain PCB mixtures resulting in altered behaviour in the offspring (Tilson *et al*., 1990). Many of the studies, both experimental and epidemiological, suggest that the unborn may represent a population at special risk because PCBs disrupt the endocrine system, which is so critical for rapid growth and development. For details of PCB toxicity, readers are referred to ATSDR (Agency for Toxic Substances and Disease Registry, 2000).

11 MYCOTOXINS

Mycotoxins are secondary metabolites of fungi, some of which are extremely toxic to man and animals. For most mycotoxins, the common substrates are human food and animal feed, especially cereals, resulting in a persistent threat to human/animal health. Several mycotoxins are toxic *in utero*, but only the most important mycotoxins are discussed here. In general, mycotoxins can produce placental toxicity by one or more of three common mechanisms. In brief, mycotoxins can interfere with hormonal activity, which can damage parental gametes, producing infertility or abnormal offspring if successful fertilization occurs. Mycotoxins may cause foetal malformations, especially during organogenesis, by interfering with foetal nucleic acid and protein biosynthesis. This interrupts normal cell differentiation and organogenesis, resulting in malformations. Mycotoxins can also affect the foetus indirectly by affecting vital maternal organs, such as the liver, reducing nutrient transfer to the foetus or increasing transfer of toxicants (Hayes, 1981; Lebepe-Mazur *et al*., 1995). In addition, some mycotoxins, such as fumonisin B_1, cause direct necrosis in the placenta and, consequently, embryolethality/foetotoxicity and abortions (Harrison *et al*., 1990; Floss *et al*., 1994a; 1994b; Gupta, 2007).

11.1 Aflatoxins

Aflatoxins are produced by the moulds, *Aspergillus flavus* and *Aspergillus parasiticus,* which commonly contaminate a variety of food and animal feed. The toxins are usually found as a mixture of aflatoxin B_1 (AFB_1), AFB_2, AFG_1 and AFG_2. Exposure to aflatoxins is typically by ingestion of contaminated foodstuff. Aflatoxins are mutagens, carcinogens and teratogens. Aflatoxin B_1 and G_1, with varying doses administered orally or ip to mice on GD 12 and 13, produced moderate retardation in foetal development, cleft palate and diaphragm changes. Aflatoxin G_1 also produces malformations of the kidneys (Roll *et al*., 1990). Exposure of pregnant rats to aflatoxin B_1 via the oral route has been shown to result

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in significant anti-implantational activity and loss of viability among the litters (Choudhary *et al*., 1992). From the limited studies conducted on laboratory animals, it appears that both aflatoxin B_1 and G_1 have strong potential for embryocidal, foetotoxic and teratogenic effects. Prenatal exposure of rats to aflatoxin B_1 resulted in delay of early response development, impaired locomotor coordination and impaired learning ability in the offspring of rats exposed to this mycotoxin during middle pregnancy, and the early gestational exposure appears to produce more effects than latter exposure (Kihara *et al*., 2000).

11.2 Fumonisins

Fumonisins are a group of mycotoxins (FA_1, FA_2, FB_1, FB_2, FB_3, FB_4 and FC_1) produced by *Fusarium* fungi, principally *F. moniliforme* and *F. proliferatum*. Only the B-series fumonisins have been confirmed as natural products. The A-series consist of *N*-acetyl derivatives, which may be produced during the isolation process. These mycotoxins are found in corn or corn-based food/feed and, therefore, contaminate human food and animal feed worldwide. It is well established that FB_1 and FB_2 are usually the most abundant and appear to be the most potent mycotoxins of this group.

Ingestion of fumonisin-contaminated food/feed has been linked to many organ-specific fatal diseases; for example, oesophageal cancer in man in the Transkei region in South Africa and Linxian county in China, hepatopathy and hepatocarcinoma in laboratory rats, porcine pulmonary oedema (PPE) in swine and equine leukoencephalomalacia (ELEM) or mouldy corn poisoning in equidae. Fumonisins additionally produce mild to fatal toxicity in the liver, kidney and heart of rats and horses. Among all species, the equidae appear to be the most sensitive because fumonisins severely damage the CNS of this species.

Published findings from experimental animal research, and anecdotal findings from field cases, suggest that fumonisins have a strong potential for developmental toxicity and teratogenicity. Purified FB_1 and *F. proliferatum* culture extracts have been shown to be embryotoxic when inoculated into incubated eggs (Javed *et al*., 1993a; 1993b). In addition to mortality, which depended both on the dose and on the time of its administration, day 1 or 10, embryonic changes occurred, including hydrocephalus, enlarged beaks and elongated necks.

Potential detrimental effects of fumonisin B_1 have been discovered in developing hamsters (Floss *et al*., 1994a; 1994b). FB_1 caused increased incidence of prenatal losses, including deaths and resorptions. At a dose of 12 mg kg^{-1}, all litters were affected and 100% of the foetuses were dead and resorbing, without clinicopathological evidence of maternal toxicity. Foetal resorptions were associated with a greater severity of placental necrosis. Placental tissues showed signs of degeneration and involution, focal distortion and necrosis of the trophoblastic layer, especially at the periphery, and recent haemorrhage and necrosis at the base of the placenta. Placentas in dams receiving a 12 mg kg^{-1} dose had extensive necrosis and loss of foetal capillaries in the haemochorial portion, with concomitant collapse of the stroma. Such changes were consistent with the embryolethal effects of FB_1. These data strongly suggest that FB_1 is a developmental toxicant capable of inducing prenatal deaths when administered during organogenesis in hamsters. External malformations, such as hooked or curled tail, ectrodactyly of the front and/or rear limbs, and cleft palate, were also observed. In fact, the incidence of curled tail malformation in foetuses from fumonisin-treated hamsters is dose related.

Developmental toxic effects of aqueous culture extract from *F. moniliforme* in CD1 mice are reported to be due to FB_1 (Gross *et al*., 1994; Reddy *et al*., 1995). These effects include maternal deaths, reduced maternal body weight gain, increased embryonic resorptions, reduced pup weights and foetal malformations, such as cleft palate, hydrocephalus and ossification deficits. FB_1 is also found to be foetotoxic in F344/N rats. Pregnant rats given FB_1 orally at a dose of 30 or 60 mg kg^{-1} from GD 8 to 12 caused significant impairment of ossification of the sternebrae and vertebral bodies in their foetuses (Lebepe-Mazur *et al*., 1995). It appears that FB_1 is foetotoxic to rats by suppressing growth and foetal bone development (**Figure 4**). However, in Sprague Dawley rats, there is very little or no evidence of embryotoxicity or foetotoxicity by FB_1, especially in the absence of maternal toxicity (Ferguson *et al*., 1997). An

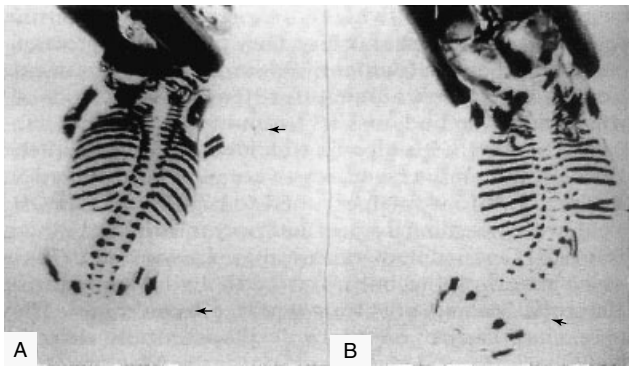

Figure 4 Skeletal development of (A) control and (B) fumonisin-treated rat pup. A = Pup from a control dam, with normally shaped ribs and normal ossification of the phalanges and vertebrae bodies. B = Pup taken from dam treated with 60 mg kg^{-1} fumonisin B_1, having underossification of the vertebral bodies of the cervical and lumbar regions as well as the phalanges. (Reproduced from Lebepe-Mazur *et al*., 1995. © American College of Veterinary Toxicologists.)

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experimental study with FB_1 in pregnant New Zealand white rabbits revealed no evidence of embryotoxicity or teratogenicity, despite the fact that the pregnant rabbits were sensitive to very small doses of FB_1 (LaBorde *et al*., 1997).

Pregnant mares exposed to fumonisins by consuming contaminated corn/corn-based feed showed late term abortions. In some cases, the surviving colts were behaviourally abnormal and slow learners. Suppressed growth, bone development and immune system appeared to be the major contributing factors (Gupta, 2007). Abortions have also been observed in pregnant sows consuming fumonisin-contaminated corn, and may be due to hypoxia resulting from maternal respiratory distress (Harrison *et al*., 1990).

Thus far, the mechanism of toxicity of fumonisins is not completely understood. Fumonisins have been demonstrated to inhibit sphinganine-sphingosine *N*-acetyl transferase (ceramide synthetase) activity and, consequently, increase the concentrations of sphinganine and the ratio of free sphinganine to sphingosine in target organs of many animal species (Wang *et al*., 1992; Riley *et al*., 1993). Accumulations of sphinganine may be responsible for cell death because long-chain bases such as sphinganine and sphingosine are known protein kinase C inhibitors and can be cytotoxic. It is suggested that an abnormal sphinganine/sphingosine ratio in the foetus in the presence of a normal ratio in the dam will attest to the unique sensitivity of the conceptus to fumonisin (Riley *et al*., 1993; Floss *et al*., 1994a; 1994b).

Finally, it is important to mention that human exposure to fumonisins in yellow and white corn meal, measured at 0.75–7.5 mg per 55 kg woman per day (14–140 µg kg^{-1}), suggests that fumonisins might be teratogenic to humans, but do not pose a severe risk. For further details on general toxicity and placental toxicity of fumonisins, readers are referred to Smith (2007) and Gupta (2007).

11.3 Ochratoxin A

Ochratoxin A (OTA) is produced by *Aspergillus* (primarily *A. ochraceus*) and *Penicillium* species (*P. viridecatum* and *P. cylopium*) of fungi. This mycotoxin has been encountered as a contaminant in a variety of feeds and in human food, including cereals and different kinds of beans, such as coffee, cocoa, soya, peanut and corn. OTA has been implicated as the aetiological agent of Balkan endemic nephropathy in humans and acute ochratoxicosis (nephropathy and hepatotoxicosis) in animal species. In addition to other toxic effects, such as carcinogenesis and immunotoxicity, this mycotoxin has been demonstrated to cause teratogenesis in rats (Mayura *et al*., 1982b). The main mode of action of OTA is inhibition of protein synthesis, although the toxin is also known to cause enhancement of lipid peroxidation, partial inhibition of ATP-dependent calcium uptake and inhibition of the cell-mediated immune response. OTA also inhibits the phosphorylase enzyme system, possibly by competing with 3′, 5′-cyclic AMP (adenosine monophosphate) for phosphorylase b kinase activity.

The placental transfer of OTA has been demonstrated in rodents and other species (Galtier, 1991). The relationship of OTA to foetal death in laboratory and domestic animals was established by Still *et al*. (1971). *A. ochraceus* cultures administered on GD 10 to groups of pregnant rats induced a large number of foetal deaths and embryo resorptions. Subsequent work in rats indicated that a high dose of purified OTA administered on GD 10 also caused a large number of foetal deaths and embryo resorptions. Compared with rats, placental transfer of OTA in sheep was found to be in trace amounts, resulting in very little or no evidence of foetal toxicity or teratogenesis. In a recent study, rabbits receiving a combination of OTA and aflatoxin B_1 showed antagonistic interaction, yet revealed a characteristic cardiac anomaly with a valvular defect at the auriculoventricular junction (Wangikar *et al*., 2005). For further details on OT toxicity, see Fung and Clark (2004) and Gupta (2007).

11.4 Rubratoxins

Rubratoxins A and B are mycotoxins produced by *Penicillium rubrum* and *P. purpurogenum*. Rubratoxin B is the principal toxin and it is a hepatotoxic agent. Experimental intoxication has been demonstrated in both laboratory and domestic animals. Typical signs of intoxication are hepatic failure, coagulopathy, haemorrhage of the gut mucosa, bloody faeces and death. Rubratoxin B_1 has been proved to be a potent mutagen and teratogen in mice (Wilson and Harbison, 1973). The teratogenic effects on near-term foetuses are varied and striking, including exophthalmos, missing ears, spina bifida, microphthalmia, anophthalmia, short tail, exencephaly and hydramnion.

11.5 Secalonic Acid D

Secalonic acid D (SAD), a toxic fungal metabolite, is produced by *Penicillium oxalicum*. SAD has been proved to be embryocidal/embryotoxic/foetotoxic and teratogenic in rats and mice. Pregnant CD1 mice treated with SAD at doses of more than 5 mg kg^{-1} ip on GD 7–15 showed a reduction in body weight gain of dams and an increase in resorptions of implanted embryos (Reddy *et al*., 1981). The resorption effect was nearly 100% at 15 or 9 mg kg^{-1} given in sodium bicarbonate, with or without dimethyl sulfoxide, respectively. Multiple

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gross, skeletal and visceral anomalies were noted in foetuses born to mothers receiving 10 mg kg^{-1} or more. Major malformations included cleft palate, cleft lip, open eyelids, missing phalangeal ossification centres and shortened mandibles. It is concluded that SAD is embryocidal and foetotoxic, as well as teratogenic, when given to female CD1 mice during pregnancy. In another study conducted on rats, SAD was injected as a single sc dose (25 mg kg^{-1}) on one of GD 6–10, 12 or 14, or 15 mg kg^{-1} SAD on GD 10. Both doses produced teratogenic and foetotoxic effects, although the effects were less marked with the lower dose (Mayura *et al*., 1982a). The highest number of resorptions, greatest depression of foetal body weight and largest number of malformations occurred when SAD was injected on day 10. It is indicated that anophthalmia (days 9 and 10), exencephaly (day 9) and defects in limbs, digits and tail (day 10) were the major gross malformations. Major skeletal defects involved the vertebrae and ribs. The main internal soft tissue defects were hydronephrosis (days 9 and 10), tracheo-oesophageal fistula and renal agenesis (day 10). Hence, it appears that exposure of rats to SAD on day 10 of pregnancy is the most sensitive period for foetotoxicity and teratogenicity.

11.6 Trichothecenes

The commonly encountered mycotoxins of this group include T-2 toxin, diacetoxyscirpenol (DAS) and deoxynivalenol (DON) (vomitoxin). T-2 and DAS are produced by *Fusarium sporotrichioides* and vomitoxin is produced by *F. roseum*. It is important to mention that trichothecenes are heat stable and are not destroyed by normal industrial processing (Creppy, 2002). These mycotoxins are of both human and animal health importance. There is evidence that suggests that trichothecenes cross the placental barrier and can cause both embryonic death and structural malformations when administered to laboratory animals during pregnancy (Francis, 1989). At maternotoxic doses, trichothecenes can produce embryotoxicity or foetal death in both mammals and birds, and only few frank congenital defects are observed in surviving foetuses, though anomalies in the nervous and skeletal systems have been noted. It is noteworthy that trichothecenes in general cause feed refusal in mammalian and avian species and this factor can minimize maternal/foetal toxicity. Trichothecenes can induce toxicity by multiple mechanisms, including inhibition of protein, RNA and DNA synthesis, enhanced lipid peroxidation, inhibition of mitochondrial and electron transport chain function, induction of apoptosis and modulation of immune responses. Only a brief description of trichothecenes' toxicity is given here, while the details can be found in a recent publication by Mostrom and Raisbeck (2007).

11.6.1 T-2 Toxin

One of the most toxic members of the trichothecene family is T-2 toxin, which is mainly responsible for mass poisonings in man (Forgacs and Carll, 1962) and farm animals (Hsu *et al*., 1972). It is well established that T-2 toxin crosses the placenta (Hayes, 1981). The only effect that has been reported in rats exposed prenatally to T-2 toxin is behavioural teratogenesis (Francis, 1989). Mice, compared with rats, have been studied in detail for placental toxicity of trichothecenes. There is a report that indicates that mice receiving a single dose of 3 mg kg^{-1} T-2 toxin on GD 7, 8, 10, 11 or 12, showed maternal toxicity with 17% mortality and whole litter resorptions (Rousseaux *et al*., 1985). Maternal death was typically due to placental haemorrhage, and surviving pups had major, minor and retardation defects of the skeletal system, and also exencephaly, abdominal defects and cleft palate.

In a comprehensive study (Stanford *et al*., 1975), female mice were treated with 0.5, 1 or 1.5 mg kg^{-1} T-2 toxin, ip on GD 7, 9, 10 or 11. Treatment on day 9 reduced the survival rate to 85% at 1 mg kg^{-1} and to 8% at 1.5 mg kg^{-1}, without any evidence of teratogenesis in surviving pups. Among the surviving pups of dams treated at 1 or 1.5 mg kg^{-1} on day 10, 37% were grossly malformed and 42% had skeletal malformations. Commonly observed malformations were exencephaly, congenitally open eyelids and retarded jaws. Treatment on day 11 resulted in greater embryotoxicity (75% deaths at 1 mg kg^{-1} and 100% deaths at 1.5 mg kg^{-1}), but the malformation rate among surviving pups was not greater than 13% at any dosage. In another study, female mice given T-2 toxin in feed at 5 and 10 ppm throughout gestation resulted in whole litter abortions in 67 and 100% of the dams, respectively (Francis, 1989). Delayed ossifications of the coccyx and growth retardation were the prominent effects. Overall, the observed effects were cumulative and dose dependent. In mice, concurrent prenatal exposure to T-2 toxin and rubratoxin B has been shown to result in an increased adverse effect on both foetal weight and mortality of the conceptus in comparison with either mycotoxin given alone (Hood, 1986).

11.6.2 Diacetoxyscirpenol (DAS)

There is one report which describes the placental toxicity of DAS in rats (Mayura *et al*., 1985). DAS at a dose rate of 2, 3 or 6 mg kg^{-1} ip on one of GD 7–11 caused no maternal toxicity. The highest dose caused 100% resorptions. With the lowest dose (2 mg kg^{-1}), the surviving pups showed a variety of malformations, including hydrocephaly and exencephaly.

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11.6.3 Deoxynivalenol (DON, Vomitoxin)

Vomitoxin is one of the most common contaminants of cereal grains world wide. In rats, feeding a vomitoxin-contaminated diet at 5 ppm throughout gestation does not adversely affect pregnancy or increase birth defects (Morrissey, 1984). In another study, the same author reported that vomitoxin does not produce teratogenesis even at a 20 ppm dietary level. However, at this dose it can decrease the rate of pregnancy. In a recent study, Collins *et al*. (2006) gavage fed pregnant Sprague Dawley rats daily with vomitoxin at doses of 0, 0.5, 1, 2.5 or 5 mg kg^{-1} body weight on GD 6–19. All females survived to caesarean section. At the highest dose, gravid uterine weights were significantly reduced, 52% of litters were totally resorbed, and average foetal body weight and crown–rump length were significantly decreased. The ossification of foetal sternebrae, centra, dorsal arches, vertebrae, metatarsals and metacarpals was significantly decreased. No adverse developmental effects were observed at the 0.5 or 1 mg kg^{-1} dose. Vomitoxin is considered a teratogen at 5 mg kg^{-1} day^{-1} in Sprague Dawley rats. In mice, intubation on GD 8–11 at doses of 2.5 and 5 mg kg^{-1} vomitoxin produced embryotoxicity, and at doses of 5 mg kg^{-1} or greater produced embryolethality (Khera *et al*., 1982). In mice, vomitoxin at a single dose of 10 mg kg^{-1} caused maternal mortality and 100% foetal mortality (Khera *et al*., 1982). Foetal mortality was 80% at 5 mg kg^{-1} and 72% at 2.5 mg kg^{-1}. With various dosing regimens, there is no evidence of damage to the placenta or teratogenesis by vomitoxin in mice. In essence, the trichothecenes at maternally toxic doses are also embryotoxic. Death of any embryo or foetus is the most common finding. In surviving pups, frank malformations (nervous system anomalies and skeletal malformations) are seen, but rarely. In rabbits, DON given daily on days 0 through 30 of gestation at increasing levels of 0.3–2.0 mg kg^{-1} body weight caused 100% foetal resorption at 1.8 and 2.0 mg kg^{-1} day^{-1} and reduced body weight in rabbit does (Khera *et al*., 1986). Dosages of 0.3 and 0.6 mg kg^{-1} body weight per day of DON did not produce adverse effects in rabbit foetuses at term and these doses were not maternotoxic. The authors concluded that DON did not produce a teratogenic response in rabbits. Minor malformations described as delayed ossification and unwithdrawn yolk sac were reported in chick embryos when hens were fed rations containing DON at 2.5 and 3.1 mg kg^{-1} diet (Bergsjo *et al*., 1993).

12 EPILOGUE

Placental toxicology is an important area of research that has been neglected in the past. The placenta, in addition to maintaining pregnancy, acts as an exchanger interfacing two separate genomes, that is, mother and foetus. It is the main entryway for drugs and toxicants to the unborn from the exposed mother. There is ample evidence that a variety of chemicals (including abused drugs, metals, pesticides and mycotoxins) with MWs of less than 1000 cross the placenta with little or no restriction. This indicates that the placental barrier is not a full barrier, which is a widely held false notion.

It is evident from research findings of the last three decades that the placenta is not a waste or rejected tissue; instead, it plays multiple roles during pregnancy. Compared with toxicology of other target organs, placental toxicology is much more difficult to understand owing to three major factors: (i) it deals with three components—mother, placenta and foetus; (ii) placental structure and functions vary widely among different species; and (iii) continuous changes occur in the structure and function of the placenta throughout the gestation period. Several chemical toxicants concentrate in the placenta, where they produce minor biochemical changes to severe necrosis. Consequently, these changes are also reflected in the conceptus. The placenta, by having an active metabolism, modifies the toxicity of some chemicals, to the placenta and to the foetus. In addition, the placenta has receptors for abused drugs, and therefore, maternal exposure to these substances results in altered placental and foetal functions. The human placenta has a true capacity to compensate for some loss of function due to abused drugs by induction of transport systems to amino acids and down-regulation of placental receptors. This compensation may occur if the pregnant mother abuses one drug at any rime. It is not known if such compensation occurs if the mother abuses several drugs simultaneously. In the years to come, intense efforts of toxicologists will explore novel mechanisms involved in placental toxicity to better understand teratological, neurotoxicological, reproductive and behavioural deficits.

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Air Pollution

Robert L. Maynard

CONTENTS

1 INTRODUCTION

Air pollution science has developed remarkably during the past 20 years: it is not too much to say that the subject has undergone a renaissance. Before discussing air pollutants in detail it will be interesting to consider three advances:

1. Long-term exposure to ambient particles mainly affects the cardiovascular system
2. For none of the common air pollutants can a threshold of effect be defined
3. Whilst particle mass concentration is universally monitored, particle number or surface area might better reflect effects on health.

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Not one of these widely accepted propositions would have been accepted 10 years ago. Added to these is the growing realization that very small particles—ultrafine or nanoparticles, may play an important role in causing effects on health. How can these changes in thinking be explained? The answer does not, as yet, lie in toxicology; on the contrary, the advances are due to the application of epidemiological methods on a large scale. Powerful new methods and a willingness to capitalize on 'natural experiments' and on the results of policy initiatives have pushed the field forwards. Epidemiological studies of the effects of air pollutants on health are now numbered in hundreds and meta-analytical techniques have raised confidence in their findings. It is disappointing to record that advances in toxicology—in understanding mechanisms of effects—have not kept pace and, to some, air pollutant toxicology has become a much less important field than it was in the past. Put starkly, toxicology can sometimes explain and support epidemiological findings, but it can seldom refute them. This has led to doubts about the veracity of some epidemiological conclusions. In general, such doubts have been displaced by the sheer weight of evidence. But the challenge to toxicology remains and is being met by innovative work using genetically modified animal models and specially concentrated preparations of ambient pollutants. Dramatic support, for example, for links between exposure to particles and the rapidity of development of atherosclerotic plaques has been produced by studies in rabbits susceptible to atherosclerosis. This work will be discussed later. All this has led to challenges to policy-makers in the air pollution area; at a time when levels of common pollutants are probably lower in developed countries than at any time since the Industrial Revolution, pressure to reduce levels further is at its greatest. In less developed countries a recognition that air pollution has a very significant effect on health is changing the pattern of industrial development. Few areas of toxicology, in its broadest sense, are having more impact on the way we live and work.

2 HISTORICAL BACKGROUND

Air pollution has been a problem since the discovery of fire. Burning fossil fuels inevitably generates pollutants: the blackened civic buildings of the Victorian era testify to this. But for a long period during and following the Industrial Revolution, air pollution was accepted as a cost, undesirable to be sure, of progress. Readers interested in the historical aspects of this subject should consult Brimblecombe's excellent monograph: *The Big Smoke: A History of Air Pollution in London Since Medieval Times* (Brimblecombe, 1987). The possible dangers to health of air pollutants were noted in the thirteenth century and stressed by Evelyn in the seventeenth. Evelyn's book *Fumifugium: or The Inconvenience of the Aer and Smoake of London Dissipated* (Evelyn, 1661) may be taken as the first text on air pollution and, as Lawther has pointed out, can be read with profit today (Lawther, 1961). But little was done and by the nineteenth century coal-smoke-laden fog was a feature of industrial England. Victorian authors like Sir Arthur Conon Doyle used fog as a backdrop for their novels and foggy London is linked in many readers' minds with Sherlock Holmes. Smog (meaning a combination of smoke and fog) was a term coined by Dr. H. A. Des Volux of the Coal Smoke Abatement Society in 1905 and has remained in use ever since. In London the Great Smog of 1952 brought matters to a head: at least 4000 'extra' deaths over a two week period and daily average concentrations of sulphur dioxide (SO_2) and particles of about 4000 $\mu g\ m^{-3}$ (the particle filters of monitoring apparatus were overloaded and results were therefore inaccurate) led to a public outcry and to the Clean Air Act of 1956. Equally importantly, the Medical Research Council (MRC) set up the Air Pollution Unit and research into the effects of air pollutants on health began. Lawther, Waller, Cummins and others undertook seminal studies that form an essential source of reference on the effects of coal-smoke air pollution. Sadly this work stopped in 1979 when it was erroneously believed that the problem of air pollution had all but disappeared. Work in the UK began again in the 1990s.

London was not the only city affected by air pollution, of course. In 1930 a serious episode in the Meuse Valley of Belgium claimed 60 deaths from a population of 6000 and in the United States, in Donora, near Pittsburgh, fog in 1949 killed 20 people and affected 43% of the population.

Reductions in coal-smoke pollution were fairly easily obtained: smoke-free fuel for domestic heating, the increasing use of gas and electricity, and taller chimneys for industrial sources all proved effective. But whilst coal smoke was being mastered, a second source was increasing: the motor vehicle.

Los Angeles in the early 1950s was affected by what was then a new form of 'smog'. No longer smoke and fog, but a mixture of traffic emissions and photochemically produced oxidants such as ozone (O_3) and peroxyacylnitrate produced by sunlight acting on primary pollutants. This 'smog' was strongly irritative to the eyes and lungs and O_3 concentrations of >500 ppm (1000 $\mu g\ m^{-3}$) were recorded. Such levels were sufficient to damage vehicle tyres made of natural rubber. Research in the US and other countries developed rapidly. Progress in research can be followed in the publications of the World Health Organization (WHO; 1987, 2000, 2006). Close reading of these reports reveals the slow move away from accepted thresholds of effect and suggests that a policy of progressive reduction should be adopted. This is not new: in the UK such an approach was advocated by the MRC Air Pollution Unit workers in the 1970s.

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In the early years of air pollution research, emphasis was placed on air pollution episodes. Lawther (1961) argued that much emphasis was misplaced and that more attention should be placed on the effects of long-term exposure. He was correct, though he argued that the key effects were likely to be respiratory. This is no longer believed: the key effects are on the cardiovascular system.

Modern epidemiological studies of air pollutants generate coefficients linking concentrations of pollutants with effects on indices of health. In the UK, a database set up to collate the results of short-term studies now contains about 10,000 coefficients. That air pollution damages health is no longer a subject for dispute: the extent to which it damages health is important, as are the mechanisms by which the effects are produced.

Recent work has focussed on the benefits to health that can be expected as a result of reductions in levels of air pollution. In the UK these have been calculated with some accuracy and form the basis of the Air Quality Strategy for England, Scotland, Wales and Northern Ireland. This has recently been published and runs to three volumes (Defra, 2007a; 2007b). Readers wishing to gain a grasp of modern air pollution control are directed to these volumes. Similar work is underway in other countries and the US Environmental Protection Agency (EPA) has published reviews of unparalleled completeness of effects of air pollutants on health. These are available on the US EPA web site at the following web site address: *http://www.epa.gov*. In the mid 1990s the EPA criteria document on particulate matter ran to three large volumes: these reports are obviously a key source. Fortunately they are available from the US EPA in CD format.

3 SOURCES OF AIR POLLUTION

3.1 Definitions

Air pollutants appear as particles or gases. Particles, including fine droplets of liquid, suspended in air and in a reasonably stable state, form an aerosol. Pollutants may be described as primary if directly emitted from sources, for example, vehicles emit nitric oxide (NO) or secondary if they are formed in the air, as is O_3. Some pollutants, such as particles, comprise both primary and secondary components. Particles range from 1 nm (10^{-9} m) diameter to about 100 µm diameter (1 µm = 10^{-6} m). Primary particles are formed at source: some are given off from a fire, for example. Secondary particles form in the air and begin as very small units (<5 nm diameter), but they soon grow and aggregate and form an important component of the ambient aerosol, with diameters between about 0.1 and 1 µm. Particles larger than about 1 µm are produced by mechanical processes, for example, wind-blown dust and sea spray. The terminology used to describe the ambient aerosol is complicated. Particles are sampled using size-selective (filtration) heads that reject particles above a certain size. This is described as the 'cut size'. If we imagine that all particles of less than 10 µm diameter are selected, collected and weighed, and the result expressed as mass per m^3 air sampled, we will have determined PM_{10}. Of course, the acceptance/rejection profile is not perfect at 10 µm diameter, but the acceptable level of imperfection is specified in the full definition of PM_{10}. In referring to diameter we actually mean aerodynamic diameter: the actual diameter of a sphere of unit density with the same aerodynamic properties as the particle in question (see below in Section 8 on particle toxicology). Similarly, $PM_{2.5}$ or $PM_{1.0}$ may be defined. Particles of diameter less than 2.5 µm are described as fine particles, those larger are described as coarse, those between 2.5 and 10 µm are described as the coarse fraction of PM_{10}. Particles of <100 nm diameter are described as ultrafine. Nanoparticles are described a little more precisely as particles with at least one dimension (width, length) of less than 100 nm. Thus, nanoparticles include rods and tubes, as well as spheroids. The distribution of particles in the air can be expressed in terms of number or mass. Very small particles dominate the former, larger particles dominate the latter. Typical PM_{10} levels for Central London today are 20–25 µg m^{-3}. Note that PM_{10} defines a concentration of size-selected particles, not the particles themselves. Thus, though the term $PM_{10's}$ is widely used, it is in fact a solecism. It will be obvious that PM_{10} includes $PM_{2.5}$, $PM_{1.0}$ and all smaller size fractions.

Gaseous air pollutants such as NO and SO_2 are formed when fossil fuels burn: the latter from sulphur in the fuel, the former largely from the combination of nitrogen and oxygen. NO is oxidized by oxygen (slowly) and O_3 (rapidly), to form nitrogen dioxide (NO_2) and higher nitrogen oxides (N_2O_4, N_4O_8). NO_2 is thus largely a secondary gaseous air pollutant though some (primary NO_2) is directly formed from fuel during combustion. O_3 is entirely a secondary pollutant and forms as a photochemical product in the air. O_3 formation can be represented by the following equations:

$$NO_2 + \text{sunlight} \rightarrow NO + O\cdot \quad (1)$$

$$O\cdot + O_2 \rightarrow O_3 \quad (2)$$

$$NO + RO_2 \rightarrow NO_2 + RO \quad (3)$$

ROO· (or RO_2) represents peroxyradicals provided by volatile organic compounds. In busy streets O_3 is destroyed by the following reaction:

$$NO + O_3 \rightarrow NO_2 + O_2 \quad (4)$$

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Table 1 Contribution of road transport (RT) and other modes of transport (OT) to selected pollutant emissions by percentage of total emissions for the United Kingdom in 2000, the United States in 1999, various European countries in 1999 and Delhi, India in 1995

Area	Carbon monoxide		Nitrogen oxides		NM VOC		Sulphur dioxide		PM_{10}		$PM_{2.5}$		PM_1	
	RT	OT	RT	OT	RT	OT	RT	OT	RT	OT	RT	OT	RT	OT
United Kingdom	69	11	42	11	24	4	1	3	18	6	24	5	30	7
United States	51	26	34	22	29	18	2	5	1.4[a]	2.2[a]	3.4[b]	—	—	—
France	41.5	—	51.4	—	25.5	—	4.9	—	11.3	—	—	—	—	—
Sweden	57.5	—	44.9	—	21.8	1.6	1.9	2.6	13.9	—	—	—	—	—
Delhi, India	85.5	—	82.4	—	84.1	—	39.0	—	15.6[b]	—	—	—	—	—

[a]Direct emissions only (i.e. does not include fugitive dust).
[b]Based on inventory for total suspended particulates.
Modified from World Health Organization (2006). Air Quality Guidelines. Global Update 2005. Particulate Matter, Ozone, Nitrogen dioxide and Sulphur dioxide.

This reaction is failing in some areas, as NO levels decline due to a reduction in emissions caused by the use of catalytic converters, and thus average urban O_3 levels are rising. Peak O_3 levels, on the other hand, are falling in urban locations in the UK.

Pollutants are described as being emitted from:

1. Point sources, for example, a power station
2. Line sources, for example, a motorway
3. Area sources, for example, an urban area.

Sources can also be classified as domestic, industrial, traffic-based, and so on. Inventories of pollution sources are available in developed countries and these form the basis of predictions of reductions of ambient concentrations consequent upon policy initiatives directed at specified sources.

Emissions may be calculated as:

$$\text{annual emissions} = \text{measure of activity} \times \text{emission factor} \quad (5)$$

Emissions may vary by source and by season: domestic heating generates more NO_2 in winter than in the summer. Detailed inventories have been devised for traffic, with vehicle emission factors being defined in terms of vehicle type and age. Inventories can be defined at a large scale (50×50 km grid squares), as used by the European Environment Agency, or smaller, for example, a 1×1 km grid square scale, as in UK urban sites. **Table 1** provides data on transport-based emissions in five countries.

A broader appreciation of sources of air pollutants in the UK is provided by **Table 2**.

Table 2 Sources of air pollutants in the United Kingdom

Source[a]	NO_x	SO_2	NH_3	Nonmethane VOC
Road transport	46.5%	—	—	36%
Domestic	4.5%	—	—	—
Industry	15.2%	31%	—	—
Power generation	22.6%	64%	—	13%
Other (non road) transport	9.1%	4%	—	—
Agriculture	—	—	89%	—
Solvents	—	—	—	18%
Fuel extraction	—	—	—	17%

[a]Only major and well characterized sources are shown. (World Health Organization, 2006).

In developed countries levels of air pollutants are, in general, low in comparison with the past, but this is not the case in rapidly industrializing, developing countries. In very large conurbations, such as Mexico City, a combination of extensive local industry, traffic and sunlight is producing high levels of both photochemical (O_3) pollution and particles. Detailed studies of effects on health in such areas reveal that air pollution remains a major threat. **Figures 1–4** are taken from the recent World Health Organization (2006) publication and provide a snapshot of conditions across the world. (**Figures 1–4** are reproduced by kind permission of the World Health Organization, 2006.)

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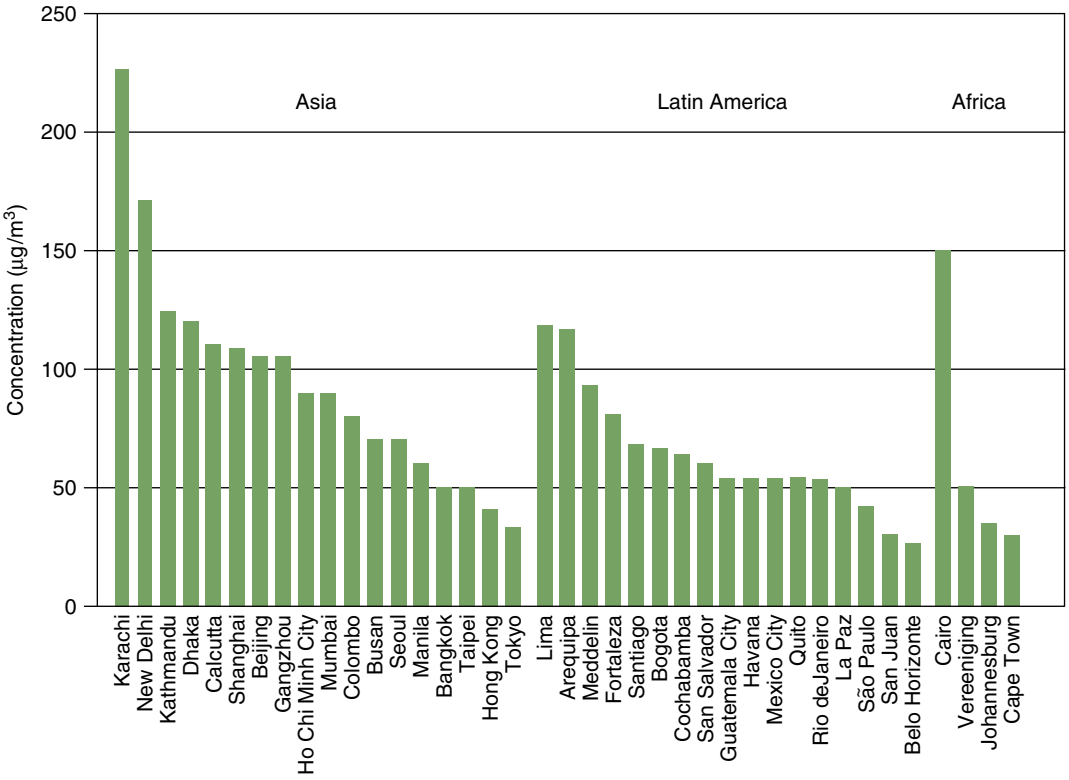

Sources: Bourotte et al. (7); US Environmental Protection Agency (8); Sivertsen & El Seoud (9); Sivertsen et al. (10); State Environmental Protection Agency (11); CAFE (12); Department of Environment and Heritage (13); Department of Environmental Affairs and Tourism (14); US Environmental Protection Agency (15).

Figure 1 Annual average PM_{10} concentrations observed in selected cities worldwide. (Reproduced from the World Health Organization, 2006.)

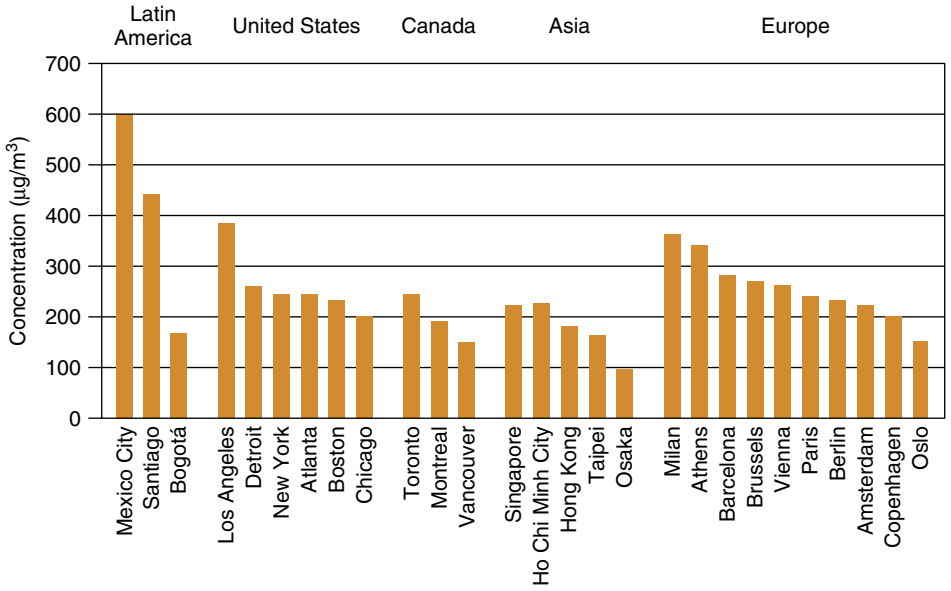

Figure 2 Highest (1 hour average) ground-level ozone concentrations measured in selected cities. (Reproduced from the World Health Organization, 2006.)

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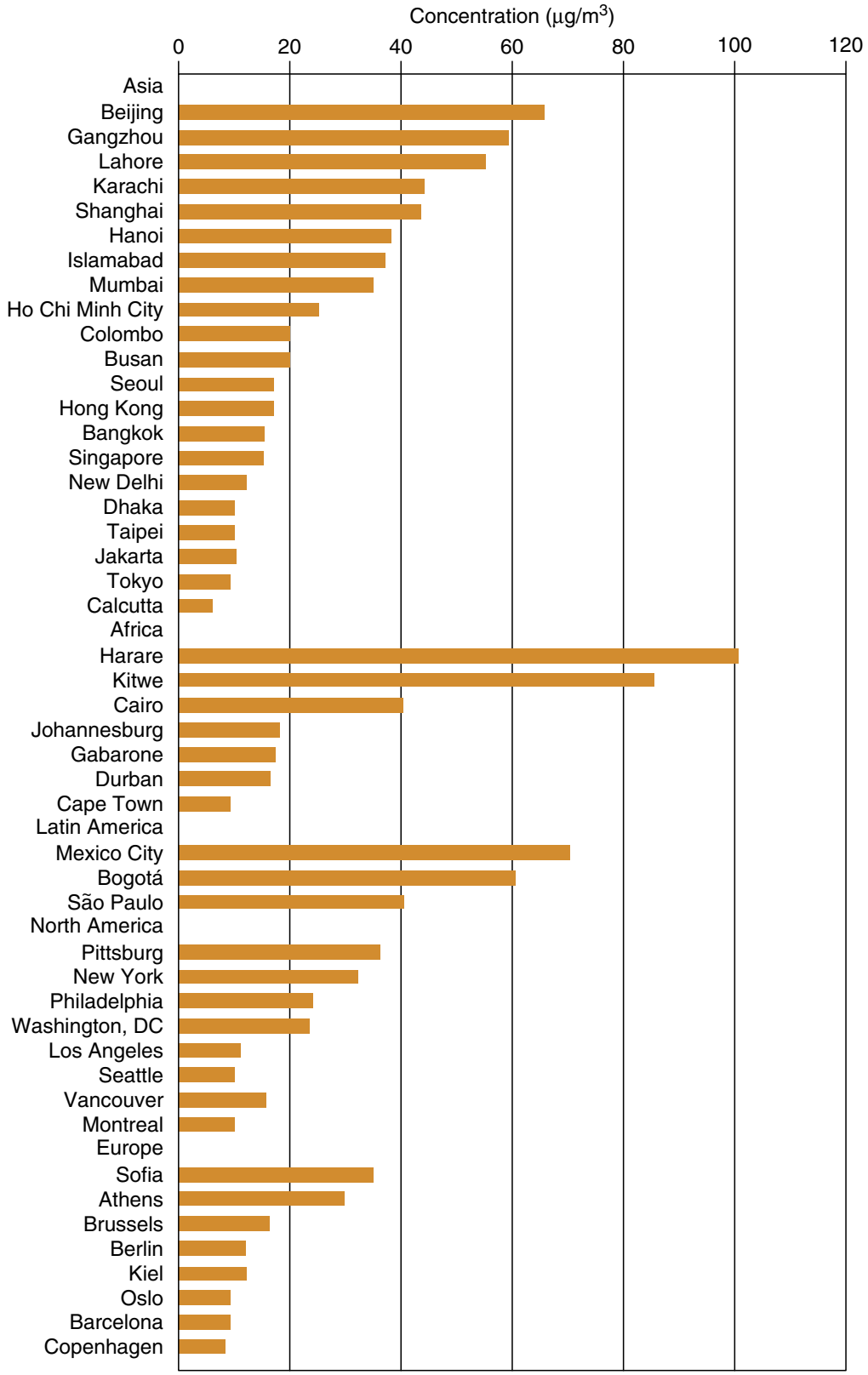

Figure 3 Annual average sulphur dioxide concentrations in 2000–2005 reported from selected cities worldwide. (Reproduced from the World Health Organization, 2006.)

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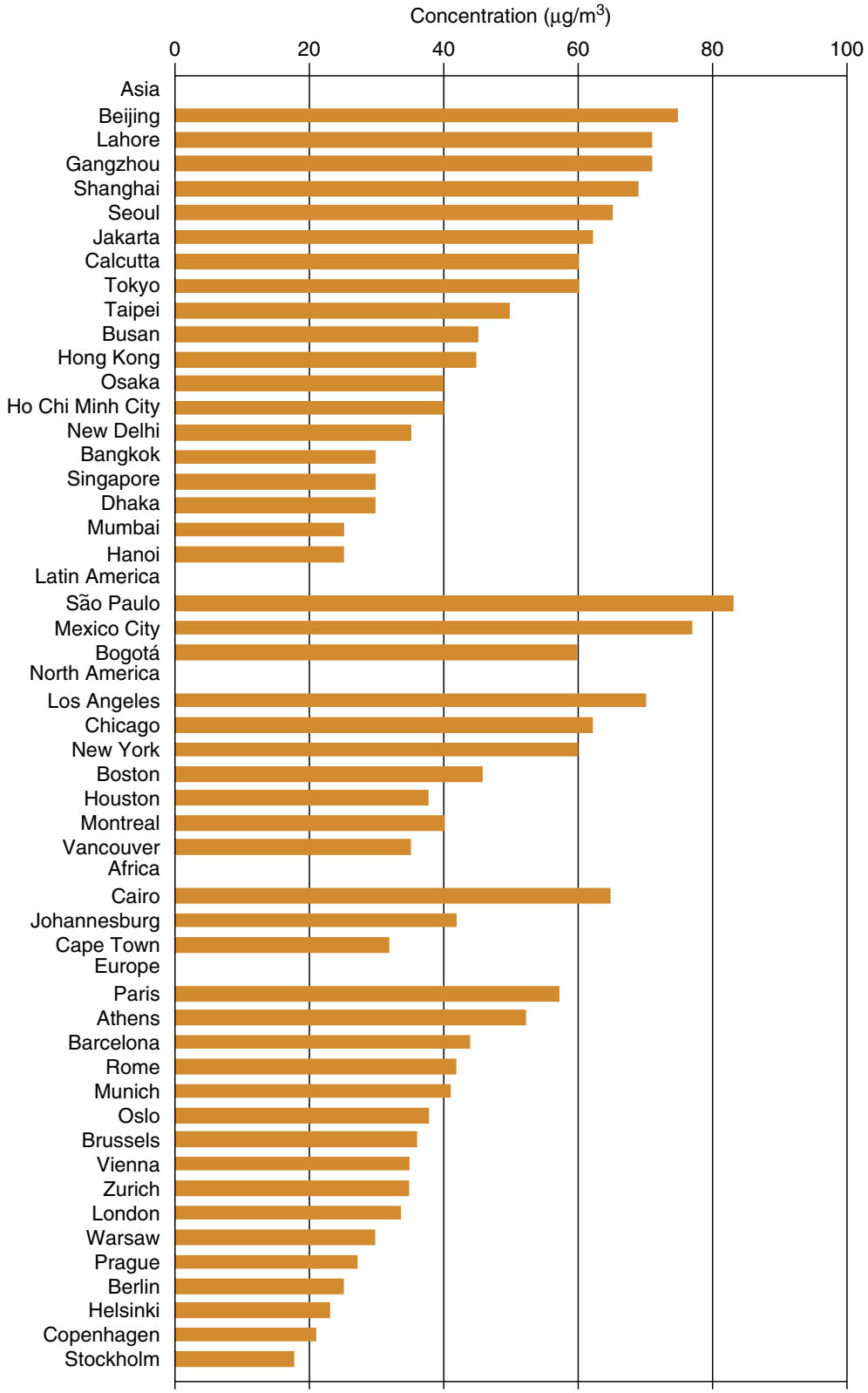

Figure 4 Annual average nitrogen dioxide concentrations in 2000–2005 reported from selected cities worldwide. (Reproduced from the World Health Organization, 2006.)

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4 EXPOSURE TO AIR POLLUTANTS

A notable feature of work on the health effects of air pollutants is that we know a good deal about concentration–response, less about exposure–response and still less about dose–response relationships. Ambient concentrations are fairly easy to measure, exposures much less easy and doses very difficult. Epidemiological studies focus on concentration–response relationships and these form the basis for assessing policy initiatives. It is accepted that dose is inevitably correlated with exposure–concentration, though the exact form of the concentration × time product that relates best to response has not been sufficiently explored. For irritant compounds, response is likely to be dependent more upon the concentration of material to which the subject is exposed than to the duration of exposure. In this case the Haber relationship:

$$Ct = K \tag{6}$$

may be modified as:

$$C^n t = k \tag{7}$$

where n = perhaps 3 or 4.

This has been explored at some length by the author elsewhere (Maynard, 2007).

That ambient concentration is related to exposure is also generally assumed, though there is less evidence for this. People spend up to about 90% of their lives indoors and yet we seek to relate outdoor concentrations of pollutants to effects on health. That this has been so successful is in some ways surprising! Indoor concentrations of pollutants generated outdoors tend to be lower than outdoor concentrations. On the other hand, concentrations of NO_2 and carbon monoxide may be greater indoors because of indoor sources. Particle concentrations indoors may be greater than those outdoors, but the composition of the ambient aerosols in the two environments may differ. Indoors, the so-called personal cloud of dust thrown up from furnishings and fittings may make a significant contribution to particle concentrations. Such particles are believed by some to be innocuous, though a firm basis for this belief is lacking. Patterns of indoor to outdoor exposure vary greatly from country to country: in developing countries, people spend less time indoors than is the case in developed countries. But indoor levels of pollution in developing countries can be very high, especially where wood and dried dung are used as fuel. The WHO Air Quality Guidelines (2006) noted that exposure to indoor air pollution associated with solid fuel use might be responsible for nearly 1.6 million excess deaths and about 3% of the global burden of disease. Detailed accounts of indoor air pollution are available (Spengler *et al*., 2001; United States Environmental Protection Agency, 1992; Department of the Environment and Heritage, 2001).

A valuable collation of data on indoor concentrations of particles was published in the WHO Air Quality Guidelines Global Update document (World Health Organization, 2006). This is reproduced with permission as **Table 3**.

It will be seen that indoor concentrations of particles can approach or exceed those of the London Smog of 1952, that is, 5000 $\mu g\ m^{-3}$. Lung cancer, chronic obstructive pulmonary disease (COPD) and airway irritation are associated with exposure to such concentrations.

Total exposure is calculated as the sum of exposures occurring in the series of microenvironments through which people pass each day. These have been studied and high exposures identified, for example, for commuters in busy traffic (van Wijnen *et al*., 1995; Zagury *et al*., 2000; Gómez-Perales *et al*., 2004; Krzyzanowski *et al*., 2005; Han and Naeher, 2006). It should be noted that dose depends on the volume of air inspired and particle or gaseous pollutant concentration in that air: cyclists may increase their dose by vigorous exercise.

Recalling that the majority of a person's time may be spent indoors, the relationship between personal exposure and outdoor concentration may be reduced to (or represented by) the relationship between indoor and outdoor concentrations. This has been studied in detail; the findings are variable (Landis *et al*., 2001; O'Neill *et al*., 2003; Janssen *et al*., 2005; Sarnat *et al*., 2005). In general, it is accepted that average personal exposure correlates well with outdoor concentrations on a day-to-day basis (Özkaynak and Spengler, 1996). Similarly, but on a longer timescale, average exposures in cities over a period of years correlates well with concentrations of pollutants measured in those cities at single site/fixed monitors. We know less about the distribution of individual exposures; this is difficult to measure on a large scale. Living near roads and low socioeconomic status is associated with increased personal exposures (Morello-Frosch *et al*., 2002; Green *et al*., 2004). Much effort has been put into thinking about errors that may be introduced into epidemiological studies by the use of approximations to true exposures. Two types of error have been considered. The first is the so-called 'classical' error that is common in studies where the measured pollution concentration may differ from the true concentration. Such an error is independent of true exposure levels and bias results 'towards the null'. The second type of error is the Berkson error. This occurs when a single value (exposure level) is assigned to many individuals. It is likely that individual exposures will vary around this value. This type of error is considered to introduce little or no bias. But all errors reduce study power and make detection of associations between air pollutants and effects on health more difficult (Steenland and

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Table 3 Comparison of particulate levels as determined in some recent studies in developing countries

Location	Averaging time/size fraction	Type of fuel	Mean levels/range (μg m^{-3})
Nepal (Pandey *et al*., 1990)	Cooking period/$PM_{2.5}$	Wood crop/ residues	8200 (traditional stove) 3000 (improved stove)
Garhwal, India (Saksena *et al*., 1992)	Cooking period/TSP[a] 24 h exposure/TSP	Wood/shrubs	4500 (GM)[a] 700–1690 (winter) 250–1130 (summer)
Pune, India (Smith *et al*., 1994)	12–24 h/PM_{10}	Wood	2000 (area) 1100 (personal)
Mozambique (Ellegard, 1996)	Cooking period/PM_{10}	Wood	1200
Rural Bolivia (Albalak *et al*., 1999)	6 h/PM_{10}	Dung	1830 (GM, indoor kitchens) 280 (GM, outdoor kitchens
Kenya (Ezzati *et al*., 2000a; 2000b)	Daily average exposure/PM_{10}	Mixed	1000–4800
Tamil Nadu, India (Balakrishnan *et al*., 2002)	Cooking period/ respirable fraction (d_{50} = 4 μm) Daily average exposure/respirable fraction (d_{50} = 4 μm)	Wood/agricultural waste Wood/agricultural waste	1307–1535 (GM, personal) 172–226
Guatemala (Albalak *et al*., 2001)	24 h/$PM_{2.5}$	Wood	1560 (GM, traditional stove) 250 (GM, improved stove) 850 (GM, LPG[a]/open fire combination)
Andhra Pradesh, India (Balakrishnan *et al*., 2004)	24 h/respirable fraction (d_{50} = 4 μm) Daily average exposure/respirable fraction (d_{50} = 4 μm)	Wood/dung/ agricultural waste Wood/dung/ agricultural waste	297–666 (kitchen area) 215–357 (living area) 431–467
Bangladesh (Dasgupta *et al*., 2004)	24 h/PM_{10}	Wood/dung/ agricultural waste	196–264 (personal) 60–1165 (area)

[a]TSP, total suspended particulate; LPG, liquid petroleum gas; GM, geometric mean.
Original references are provided in the WHO report (World Health Organization, 2006).

Savitz, 1997; Dominici *et al*., 2000; Heinrich *et al*., 2005).

5 EPIDEMIOLOGICAL APPROACHES

Epidemiological methods have been applied to three main types of problem in the air-pollution area.

5.1 Effects of Short-term Variations in Ambient Concentrations

Though the major sources of air pollutants vary but little from day to day, ambient concentrations vary considerably. This is because meteorological conditions vary markedly from day to day. This variation provides

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the temporal heterogeneity of concentration, and thus exposure, that is studied using time-series methods. A number of subgroups of this approach may be identified: classic time-series studies, panel studies and case-crossover studies. Details may be found in handbooks of epidemiological methods (Rothman and Greenland, 1998). The classic time-series study seeks to relate day-to-day variations in concentrations of pollutants to day-to-day variations in indices of ill health. The latter include daily death rates, hospital admissions, primary-care consultations or the consumption of therapeutic preparations. Consider daily death rates. These vary considerably depending on season and on meteorological conditions: in countries like the UK, day-to-day variation in temperature is a, perhaps the, major factor. Temperature is thus a major confounding factor and must be allowed for in the analysis. Day of the week may also be a confounding factor: fewer people are seen by doctors on Sundays than on Mondays. In the early years of time-series analysis a visual approach was taken: daily indices of ill health were plotted against daily levels of pollution and the plot scanned for associations. This approach was used with great success in London in the 1950s (Waller, 1991), but led to an erroneous conclusion that a threshold of effect existed for coal-smoke (particles and SO_2) air pollution. Sophisticated mathematical approaches have been designed in recent years and, despite problems with software (Dominici *et al*., 2002; Ramsay *et al*., 2003), these are now felt to be reliable. Such methods allow identification of very small changes, for example, in risk of death. An increase in PM_{10} of $10\,\mu g\ m^{-3}$ is associated with about a 0.6% increase in daily mortality. This is a very small effect indeed and led some commentators to question the findings. Questions have focussed on the adequacy of allowance for the effects of confounding factors. It has been noted that as methods improved the coefficient (slope of the line linking pollution concentration and daily death rate generated by the mathematical model that takes into account confounding factors and which uses sophisticated nonparametric smoothing functions) has fallen. This has led cynics to observe that with sufficient improvement in modelling, the effect attributed to air pollutants will disappear. This is not true: coefficients from around the world are stabilizing and multicentre studies have increased confidence in the findings. Reanalysis (Dominici *et al*., 2005), has confirmed the validity of the original findings, albeit that adjustments for a long undetected error in software have been necessary.

Time-series studies have a great advantage in that they need not adjust for factors such as socioeconomic conditions, diet and smoking, though these are known to have important effects on health. This is for the simple reason that such factors do not vary from day to day. In recent years the results of time-series studies have been collated (notably in the UK) and meta-analytical techniques applied. This has led to the production of so-called Forest plots; an example is shown in **Figure 5** below.

This figure and the dozens like it that are reproduced in the recent report on cardiovascular disease and air pollution (Department of Health, 2006) bear close study. It will be seen that the central estimates of the coefficient linking PM_{10} and cardiovascular mortality vary widely from study to study. It will also be seen that as the central estimate increases so, in general, do the 95% confidence limits around it. This may be due to publication bias: large coefficients tend to be reported and published even if their confidence limits are wide. Small studies, inevitably, tend to generate wide confidence limits due to their lack of statistical power. The figure shows as an unfilled diamond the meta-analysis-derived summary coefficient. Note that the confidence limits around this are narrow: narrower than those of the great majority of the studies that were included in the meta-analysis. This reflects the great statistical power generated by combining results from many studies. It will also be noted that the vast majority of studies show that PM_{10} is associated with an increase in cardiovascular mortality. The results are remarkably consistent. This consistency has been an important consideration in regarding the association as causal. Further features of the association and the use of tests for publication bias are discussed in the report on cardiovascular disease and air pollution. **Figure 6** below shows a second Forest plot: for cardiovascular mortality and daily NO_2 concentrations.

It will be seen that this closely resembles the plot for PM_{10} shown in **Figure 5**. Why is this? It could be that NO_2 is about as effective as PM_{10} in affecting the daily cardiovascular death rate or (and very importantly) it could be that NO_2 is closely correlated with PM_{10} and the apparent association with NO_2 is simply reflecting the real association with PM_{10} or vice versa. This is a very real problem. The studies used in the meta-analyses deployed single-pollutant models and the effects attributed, for example, to PM_{10} were not adjusted for possible associations with NO_2. If PM_{10} and NO_2 were found to be perfectly and invariably correlated with each other, the approach used in these analyses could not separate their effects. Multipollutant models are needed, but have been used, as yet, to a much lesser extent than single-pollutant models. There are technical reasons for this: incorporation of more variables is said to lead to instability of the models. No doubt this is so, but the toxicologist is left concerned that the epidemiologist may not, despite the power and sophistication of his methods, have identified the causal agent (or agents) linking daily variations in pollutant levels with daily impacts on health. An added complication is provided by effect modification. Studies show that the coefficient linking PM and daily deaths is larger in areas where NO_2 levels are high. NO_2 may be playing a synergistic role in addition to being a confounding factor and, perhaps, playing a role

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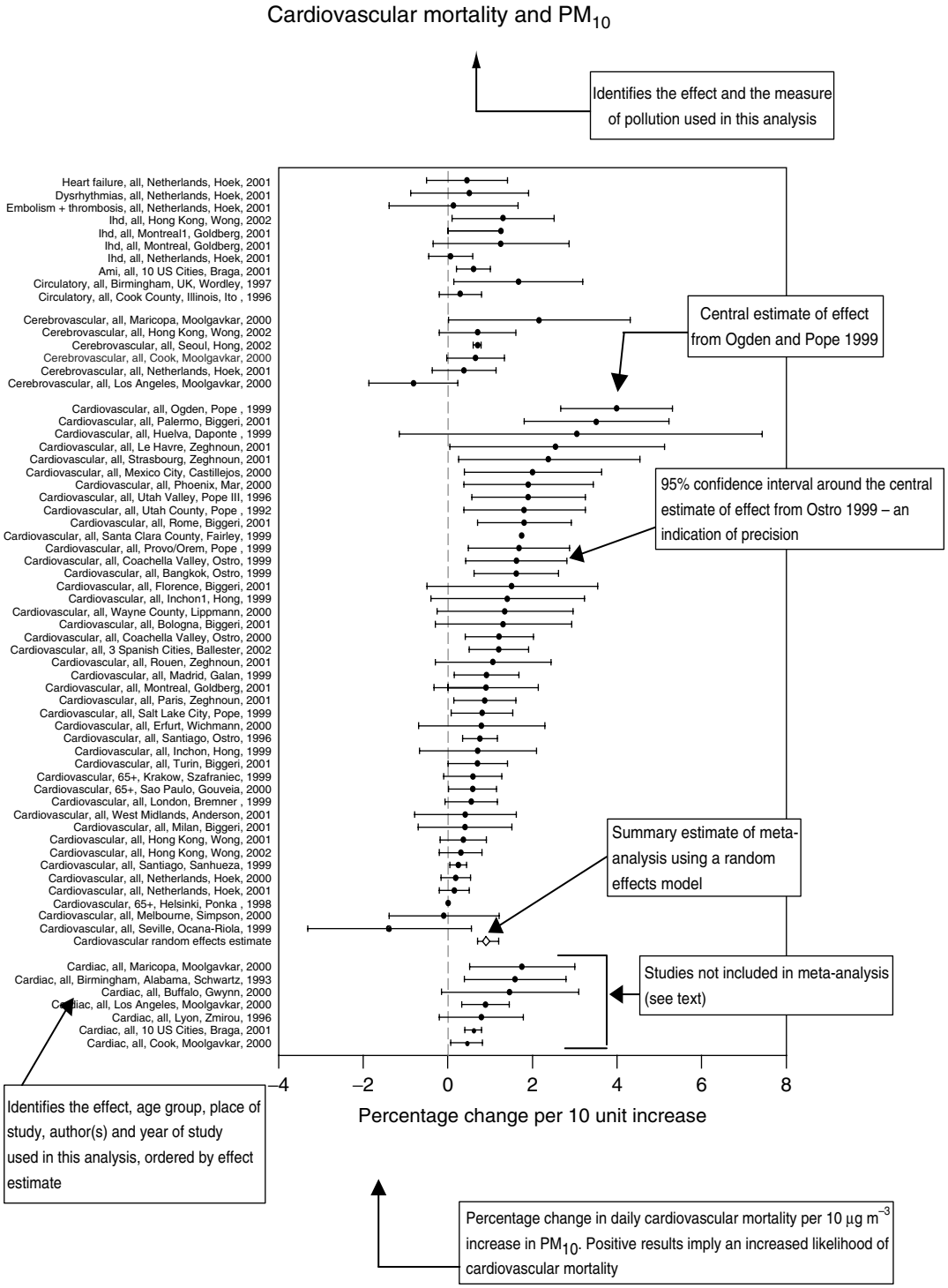

Figure 5 A Forest plot of cardiovascular mortality and PM_{10}. (Department of Health, 2006.)

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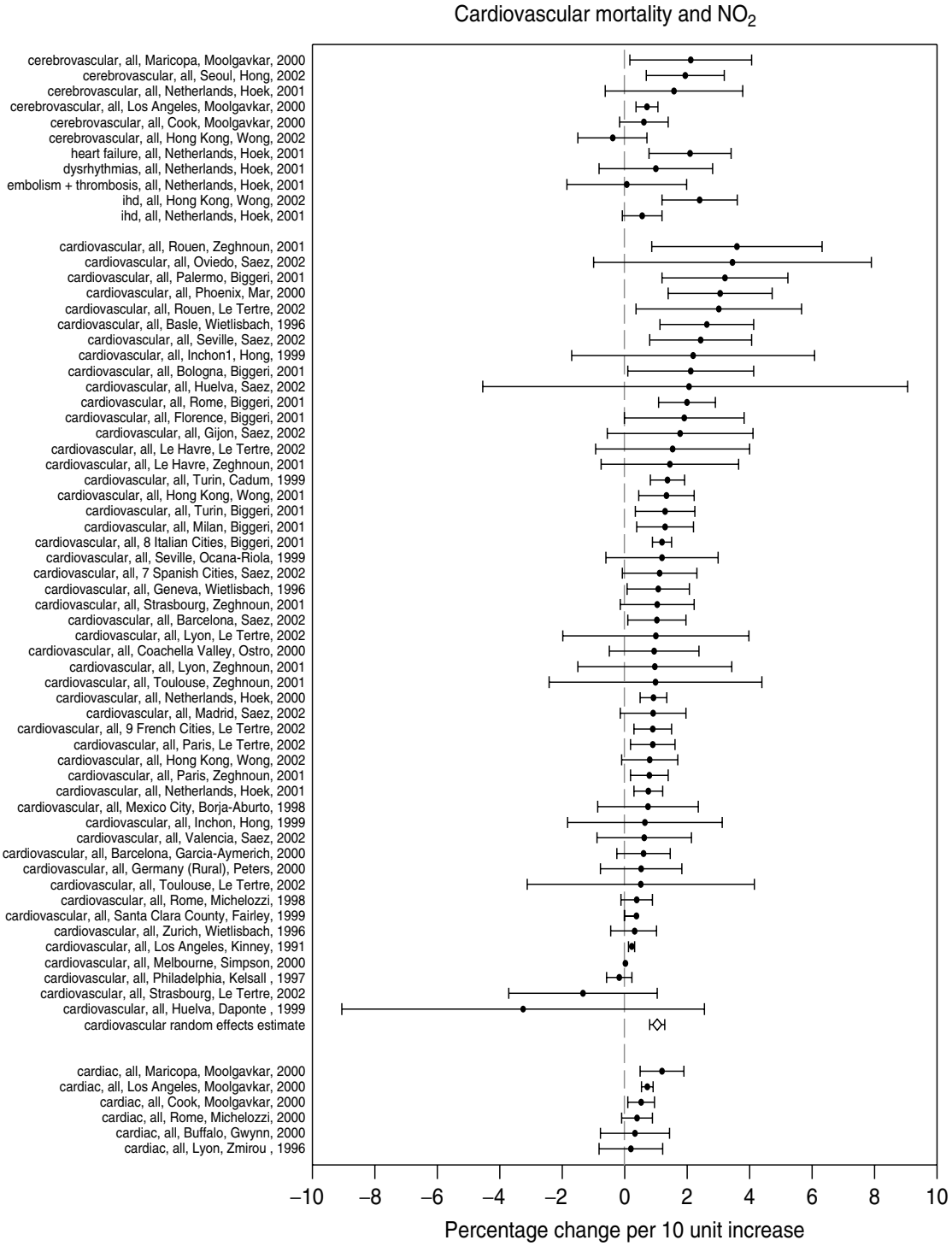

Figure 6 A Forest plot of cardiovascular mortality and daily NO_2 concentrations. (Department of Health, 2006.)

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of its own. We are some distance from unravelling such complexities.

5.2 Intervention Studies

Producing a sudden change in air pollutant levels is seldom possible. Four such events have been studied in some detail: the effect of banning coal sales in Dublin (Clancy *et al*., 2002); the effect of reducing the sulphur content of all oil burnt by power plants and road vehicles in Hong Kong (Hedley *et al*., 2002); the effect of a strike at the Utah Valley steel works (Pope, 1989; Pope *et al*., 1992) and the effect of changing transportation patterns in Atlanta in preparation for the Olympic Games (Friedman *et al*., 2001). In each of these studies a sudden, significant and positive effect on health was observed. In the case of the study in the Utah Valley, toxicological studies have shown that the toxicity of the ambient aerosol fell during the strike (Pope, 1989). Such studies provide excellent evidence to show that reducing levels of air pollutants improves health and conform with Bradford Hill's advice to use the results of natural experiments to assess the likelihood of associations being causal in nature (Hill, 1965).

5.3 Cohort Studies

It is now known that long-term exposure to air pollutants, specifically particulate matter, leads to a reduction in life expectancy. That this was likely may well have been suspected in the days of coal-smoke air pollution, but the question of the magnitude of the effect has recently been put beyond doubt. Studies from the United States and, now, further studies from Europe have shown that long-term average concentrations of fine particles ($PM_{2.5}$) are associated with an increased risk of death at all ages. The key studies were Dockery *et al*. (1993), Pope *et al*. (1995; 2002; 2004), Hoek *et al*. (2002) and Filleul *et al*. (2005). Dockery *et al*. (1993) studied six US cities (the Harvard Six Cities Study): work began in the early 1970s with recruitment of a cohort (8111 people aged 25–74) and the placing of pollution monitors. Thus, the study was truly prospective. In Pope *et al*. (1995; 2002; 2004) advantage was taken of the already extant American Cancer Society (ACS) Cohort (552 138 individuals from 151 US metropolitan areas from which data on $PM_{2.5}$ were available in 50 and on sulfates in all). Confounding factors were carefully considered. Unlike the case of time-series studies, control for individual confounding factors is critical in cohort studies. **Table 4** shows the level of care taken in the Six Cities Study; this was increased in the American Cancer Study.

The results in both studies were modelled using Cox's proportional hazards model and associations between long-term $PM_{2.5}$ levels (or sulfate) and deaths from 'all causes' (save accidents), cardiopulmonary causes and cancer derived. The increase in risk of death (mortality risk ratio) was defined in terms of the difference in long-term pollutant concentrations between the least and most polluted cities or areas in the series. Recent examination of all this work (Department of Health, 2007) has led to the following conclusions:

1. Long-term exposure to a pollutant increment of $10\,\mu g\ m^{-3}$ $PM_{2.5}$ is associated with about a 6% increase in risk of death, at all adult ages, from cardiovascular disease. The effect on deaths from respiratory disease is comparatively trivial.
2. For lung cancer (also per $10\,\mu g\ m^{-3}$ $PM_{2.5}$) the increase risk is about 14%.
3. For other causes of death the effect is zero.

These are simplified conclusions. The US cohort studies have been exposed to unparalleled reanalysis and examination. This was undertaken by a team of experts put together by the US Health Effects Institute (HEI). This report, published in 2000, runs to 421 pages plus appendices. This reanalysis has put doubt to rest: long-term exposure to particulate air pollutants increases the risk of death at all adult ages and shortens life expectancy. Further and equally searching examination of the evidence has been undertaken in the UK (Department of Health, 2006; Department of Health, 2007).

Work by Brunekreef (1997) and Künzli *et al*. (2000) has sought to estimate the extent of life shortening implied by the US cohort studies—in particular by the most recent update of the ACS study (Pope *et al*., 2004). In the UK, a method of life table computation has been developed (Miller *et al*., 1993; Department of Health, 2007). This allows an imaginary cohort (birth cohort or representative population cohort) to be followed for 100 years. The background risk of death at all ages is known from actuarial data and at each age group this is increased according to the results of the ACS analysis. The method is not mathematically complex, but it is arithmetically tedious. If a birth cohort is then considered for long-term exposure to $10\,\mu g\ m^{-3}$ $PM_{2.5}$ the estimated loss of life expectancy (averaged across the cohort) is seven to eight months.

It should be noted that the impact is expressed as an average across the entire population. It is likely that this is not, in fact, the real case: susceptible groups are probably much more affected than suggested by the averaged impact. Unfortunately, we do not know how these susceptible groups are comprised and thus a more refined calculation is impossible. The calculation, as it

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Table 4 Characteristics of the study population and mean air pollution and mean air pollution air levels in six cities[a]

Characteristic	Portage (Wis)	Topeka (Kansas)	Watertown (Mass)	Kingston-Harriman (Tennessee)	St Louis (Missouri)	Steubenville (Ohio)
No. of participants	1631	1239	1336	1258	1296	1351
Person-years of follow-up	21 618	16 111	19 882	17 836	17 715	17 914
No. of deaths	232	156	248	222	281	291
Deaths/1000 person-years	10.73	9.68	12.47	12.45	15.86	16.24
Female sex (%)	52	56	56	54	55	56
Smokers (%)	36	33	40	37	35	35
Former smokers (%)	24	25	25	21	24	23
Average pack years of smoking						
Current smokers	24.0	25.6	25.2	24.5	30.9	28.0
Former smokers	18.0	19.7	21.8	21.1	22.0	25.0
Less than high-school education (%)	25	12	22	35	45	30
Average age (yr)	48.4	48.3	48.5	49.4	51.8	51.6
Average body-mass index	26.3	25.3	25.5	25.1	26.0	26.4
Job exposure to dust of fumes	53	28	38	50	40	48
Total particles ($\mu g\ m^{-3}$)	34.1	56.6	49.2	49.4	72.5	89.9
Inhalable particles ($\mu g\ m^{-3}$)	18.2	26.4	24.2	32.5	31.4	46.5
Fine particles ($\mu g\ m^{-3}$)	11.0	12.5	14.9	20.8	19.0	29.6
Sulfate particles ($\mu g\ m^{-3}$)	5.3	4.8	6.5	8.1	8.1	12.8
Aerosol acidity ($nmol\ m^{-3}$)	10.5	11.6	20.3	36.1	10.3	25.2
Sulphur dioxide (ppb)	4.2	1.6	9.3	4.8	14.1	24.0
Nitrogen dioxide (ppb)	6.1	10.6	18.1	14.1	19.7	21.9
Ozone (ppb)	28.0	27.6	19.7	20.7	20.9	22.3

[a]Air pollution values were measured in the following years: total particles, sulphur dioxide, nitrogen dioxide and ozone, 1977 through 1985; inhalable and fine particles, 1977 through 1985; sulfate particles, 1979 through 1984 and aerosol acidity 1985 through 1988.
World Health Organization, 2006.

stands, forms the basis for calculating benefits to health expected to be delivered by the Air Quality Strategy for England, Scotland, Wales and Northern Ireland. Those wishing to know the details of such calculations are referred to UK official reports published by Defra (2007a; 2007b).

6 SUSCEPTIBILITY TO AIR POLLUTION

It seems self-evident that some individuals are more sensitive or susceptible to the effects of air pollutants than others. Indeed, much of the toxicological thinking is, or was, based on the log-normal dose–response curve

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described so well by Finney and used by him in his development of probits (Finney, 1971). It is less easy to say why sensitivity is distributed as it seems to be; we fall back on the assertion that it, like other variables (height, for example) is subject to 'normal' variation. One consequence of a normal distribution of sensitivity is that there is unlikely to be a true threshold of effect at a population level. This has come to be accepted in the field of air pollution as a result of epidemiological studies such as those described above. Even at very low ambient concentrations powerful techniques continue to demonstrate adverse effects. In the 1990s this acceptance led experts working on the WHO Air Quality Guidelines to move away from recommending guidelines in the traditional terms of a concentration and an averaging period, and to begin to regard the slope of the line linking concentration and response as the guideline. This was received with some dismay and it was argued that countries which had relied on the WHO Air Quality Guidelines as a basis for (or as) air quality standards were now without practical guidance. Some, including the present author, argued that a new approach to control of air pollutants was needed, indeed required, and that a policy of cost–benefit-tested reductions in concentrations was better than simply 'working down to a standard'. In the 2006 WHO update (World Health Organization, 2006) a compromise was agreed: slopes (coefficients) and numerical guidelines and targets for policy workers were recommended.

Some of the confusion regarding the distribution of sensitivity to air pollutants arises from conflation of thresholds at an individual level and at a population level. If a group of healthy people chosen at random is exposed in a controlled way to decreasing concentrations of SO_2 then a concentration at which nobody in the group shows a measurable increase in airway resistance (reflected as a fall in FEV_1 (forced expiratory volume in 1 s) or PEF (peak expiratory flow)) will be discovered. Unsurprisingly, if subjects with sensitive airways, for example, asthma sufferers, are studied, the threshold will be reached at a lower concentration than in the nonasthmatic subjects. Each person in the group will show a personal threshold and this may be fairly constant, day to day. The same would be true for O_3, NO_2 or SO_2 (Department of Health, 1991; Department of Health, 1992; Department of Health, 1993). The 'no observed effect level' for the group would be likely to be lowered as more and more subjects were studied. In addition the size of the response would be likely to fall as lower concentrations were studied. How large a response should be regarded as adverse? This has puzzled air pollution scientists for years. At one time the view was that if the response, say in terms of reduction in FEV_1, did not exceed that found day to day it should not be regarded as adverse (World Health Organization, 1987). Others have argued that variables such as FEV_1 would need to be reduced by 10% before the change could be regarded as significant in terms of health (American Thoracic Society, 2000). Neither approach now receives much support. It has been pointed out that a 10% reduction in a variable may not mean much to a normal subject, but may be very important in a subject in whom that variable is already depressed by disease. All this brings the epidemiological findings and the human (or animal) toxicological findings into conflict and the debate has become sterile.

So, can anything useful be said about susceptibility? The answer is, yes—the following groups are accepted as likely to be more susceptible (sensitive) than healthy young adults:

1. People with cardiopulmonary disease (Frampton *et al*., 2000; Gold *et al*., 2000; Peters *et al*., 2001)
2. People with asthma or COPD (Foster *et al*., 1993; Weinberger, 1993; Pope, 1991; Thurston *et al*., 1997; Slaughter *et al*., 2003; Chalupa *et al*., 2004)
3. Old people (Devlin *et al*., 2003)
4. Children (Gauderman *et al*., 2004)
5. Unborn babies (Šram *et al*., 2005)
6. Diabetics (Zanobetti and Schwartz, 2002; Zanobetti and Schwartz, 2001)
7. Subjects with GSTM1 (glutathione-S-transferase gene) deficiency (Gilliland *et al*., 2002; Romieu *et al*., 2004)
8. Subjects with the ZZ and null phenotypes for α-1-antitrypsin (Goss *et al*., 2004)
9. Smokers (Torres *et al*., 1997; Daigle *et al*., 2003).

The length of the list reflects its inutility! It might be hoped that if it were possible to identify groups at special risk then these groups could be provided with useful advice as to when episodes of high concentrations of air pollutants are expected as a result of meteorological forecasting. This has proved very difficult and in the UK, advice provided by the Committee on the Medical Effects of Air Pollutants (COMEAP) (web site address: *http://www.advisorybodies.doh.gov.uk/comeap*) has been limited.

Increased susceptibility is also often conflated with increased likelihood of high exposure. Those in urban areas are likely to be exposed to greater concentrations of air pollutants than those in rural areas and thus to be at greater risk of adverse effects. But this does not mean that urban dwellers are *per se* more susceptible to air pollutants than those living in rural areas.

7 INDIVIDUAL AIR POLLUTANTS

Air pollutants may be classified as 'classical' and 'the others'. Classical air pollutants are those that

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have attracted the greatest attention and which have been the subject of regulation. In 2000, the WHO Air Quality Guidelines (Second Edition) (World Health Organization, 2000) listed NO_2, O_3 and other photochemical oxidants, particulate matter and SO_2 as classical air pollutants. Many would add carbon monoxide, lead, benzene, 1,3-butadiene and polycyclic aromatic hydrocarbons (PAH compounds) to this list. Lead and carbon monoxide are considered elsewhere, as are carcinogens including benzene, butadiene and PAH compounds. Here we shall consider only those listed by World Health Organization (2000).

8 PARTICULATE MATTER

It is not possible to present a comprehensive account of all aspects of particulate air pollution in a few pages. For detailed accounts the reader is referred to the WHO Global Update (World Health Organization, 2006) to four UK reports (Department of the Environment, 1995; Department of the Environment, Transport and the Regions, 2001; Department of Health, 1995; 2006) and to the author's monograph (Holgate *et al*., 1999). In addition to these, *Particles in Our Air* edited by Spengler and Wilson (1996) remains a useful source. For a detailed, but accessible, account of aerosols in general, Hinds' book *Aerosol Technology* is unequalled (Hinds, 1999). Lippmann's book *Environmental Toxicants* (Lippmann, 2000) is also an essential source. For details of monitoring methods, the US EPA criteria documents provide excellent accounts and the recent UK publication *Particulate Matter in the United Kingdom* (Department for Environment, Food and Rural Affairs, 2005) presents a comprehensive one-country account in 444 pages. Here we will consider only three aspects of the subject.

8.1 Deposition and Fate of Particles in the Respiratory System

Inhaled particles are deposited on the surfaces of the air passages, from the nares to the alveoli by three major processes. Impaction describes the deposition of particles that, by virtue of their inertia, continue for a short time along their original path when the flow of suspending air changes direction. This causes particles to impinge on the walls of the airways especially at branch points and to be deposited. This form of deposition is dependent on the square of the diameter of the particle. Similarly dependent on the square of diameter is sedimentation: the settling of particles under the influence of gravity. This is important in the slow-moving air in the distal airways. Lastly, particles diffuse as a result of impaction of gas molecules on their surfaces. The diffusivity of a particle is dependent on the square root of the reciprocal of its diameter. Thus, large particles impact and sediment rapidly(d^2) and small particles diffuse rapidly ($\sqrt{1/d}$). If we think of particles between 1 nm and 20 μm in diameter we may define a deposition curve for the entire respiratory tract and another for the alveoli. Deposition is minimal at about 0.5 μm diameter. At this size particles are too small to sediment rapidly and too large to diffuse rapidly. A deposition nadir at 0.5 μm is a constant feature of mechanical filtration systems. The efficiency of respiratory deposition processes for small particles was demonstrated in 1870 by Tyndall who showed that light was not scattered by air emerging from the deep lung (Tyndall, 1870). Small particles are deposited, by diffusion, with great efficiency in the alveolar region. Alveolar deposition declines rapidly as particles of <10 nm are encountered; these particles do not reach the alveoli, they are efficiently deposited, by diffusion, in the upper airways. Particles of diameter greater than about 10 μm deposit in the upper airways and do not enter the thoracic airways. Thus, PM_{10} is based on the so-called thoracic convention and represents particles which might, often do, but need not, deposit in the lung. Particles of more than about 5 μm diameter stand little chance of depositing in the alveolar region. This is reflected by the respirable convention, which is centred at 4 μm diameter. A finer cut is provided by the high risk respirable convention that is centred at 2.5 μm and gives rise to $PM_{2.5}$ as a common measure of particulate air pollution. This latter convention is designed to represent particles likely to be deposited in the alveolar zone of the sick and infirm, and of children (Department of the Environment, 1996). Deposition curves need to be interpreted in terms of number of particles deposited, mass deposited and, perhaps, in terms of the total surface area of the particles deposited. Very small particles dominate in terms of particle number, particles between about 0.05 and 1.0 μm dominate in terms of surface area and mass is contributed by two major size fractions. It will be obvious that though the efficiency of particle deposition is high in the <0.1 μm (<100 nm) diameter region, such deposition will produce a low dose in mass terms. Much will depend on whether dose expressed as mass or dose expressed in terms of particle members is the more important.

Particles of soluble salts (and liquid droplets) dissolve on deposition in the lung and, it is thought, do little harm. Insoluble, or slowly dissolving particles are deposited, but then cleared from the lung. Rapid cilia-dependent clearance is much slower (see **Inhalation Toxicity**). Ambient particles contain both soluble matter (e.g. sodium chloride, nitrate and sulfate, ammonium sulfate), insoluble materials, including elemental carbon, metallic oxides (iron, nickel, vanadium, depending on source)

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and insoluble silicates from crustal sources. Reactions between acids and metallic oxides may lead to soluble metallic species being formed. This has recently been seen as a means by which material measured as 'sulfate', but which may contain acidic species, may contribute to particle toxicity (Department of Health, 2007).

8.2 Epidemiological Studies Linking Ambient Particles and Effects on Health

8.2.1 Time-Series Studies

The problem in describing the evidence in this area is that it is so extensive that preparing a summary is difficult. Time-series studies are common and the principles have been discussed above. Two major multicity studies, the NMMAPS (National Morbidity, Mortality and Air Pollution Study) in the USA and the APHEA (Air Pollution and Health: A European Approach) study in Europe (World Health Organization, 2006). For effects on mortality the following summary figures may be compared:

Europe: increase of 0.6% (95% CI: 0.4–0.8) per 10 μg m^{-3} PM_{10}

USA: increase of 0.21% per 10 μg m^{-3} PM_{10}.

A collection of the results from Canadian and US studies are shown in **Figure 7**.

It will be seen that the few studies using multipollutant models (1–4) show narrow confidence intervals (CIs): these are large, powerful studies. Note also that there is considerable heterogeneity in the results from different studies and that in some the results are not statistically significant, though in only eight instances does the central estimate suggest a reduction in mortality on exposure to particles. The reader will also see that the estimates regarding $PM_{2.5}$ are similar to, or perhaps a little lower than those for PM_{10}. This observation should put to rest the persistent but ill-informed assertion that measuring PM_{10} fails to provide data indicative of effects on health and that measuring $PM_{2.5}$ would provide such data.

Similar compilations have been prepared regarding effects of short-term exposure to particles on indices of morbidity. Hospital admissions have been studied and results from Canada and the USA are shown in **Figure 8**.

Again, the extraordinary richness of the air pollution database is shown. Clear effects on both cardiovascular and respiratory causes of admission to hospital are seen. For further work on indices of morbidity the reader is referred to the WHO report (World Health Organization, 2006).

8.2.2 Cohort Studies

Nine cohort studies relating to particles had been reported by October 2005: some are follow-ups to earlier work (Dockery *et al*., 1993; Pope *et al*., 1995; 2002; Abbey *et al*., 1999; McDonnell *et al*., 2000; Lipfert *et al*., 2000b; Burnett *et al*., 2000; Hoek *et al*., 2002; Filleul *et al*., 2005). Additional studies have now been reported (Laden *et al*., 2006; Jerrett *et al*., 2005; Enstrom, 2005). In general, the smaller spatial scale studies, some of which follow populations living adjacent to roads, report larger coefficients than the bigger cohort studies. Combining the results of cohort studies using meta-analytical techniques has not yet proved possible, but in the UK a Delphic approach to definition of plausibility intervals around a central estimate coefficient has been developed. This approach is described in the COMEAP report QUARK 2 and the report should be considered for details (Department of Health, 2007). Cohort studies have provided little information on the effects of long-term exposure to particles on indices of morbidity. The startling finding from the cohort studies has been the effect of long-term exposure to fine particles on cardiovascular mortality. Impacts broken down by smoking status are shown in **Table 5**. Note the effects on ischaemic heart disease, but also on dysrhythmias, heart failure and cardiac arrest. These findings have led to intriguing hypotheses of mechanisms of effect discussed below.

8.2.3 Toxicological Studies of Ambient Particles

Until fairly recently the toxicologist was left behind by the epidemiologist; this is now being rectified.

The most relevant toxicological studies undertaken in experimental animals and in human volunteers have involved exposure to concentrated ambient particles (CAPs). These are prepared by taking ambient air and exposing it to high centrifugal forces; a concentration of particles occurs in the outer regions of the rotating air mass and this is drawn off. Thus, for example, ambient concentrations can be raised by a factor of 10. Animals and volunteers have been exposed to these aerosol preparations. In addition, ambient particles can be collected and administered to animals by intratracheal instillation. Sixteen studies in volunteers were reported in the World Health Organization (2006) report. **Table 6** is extracted from a much larger table in that report.

From these and from animal studies the following conclusions have been drawn.

1. PM causes an inflammatory response in the lung

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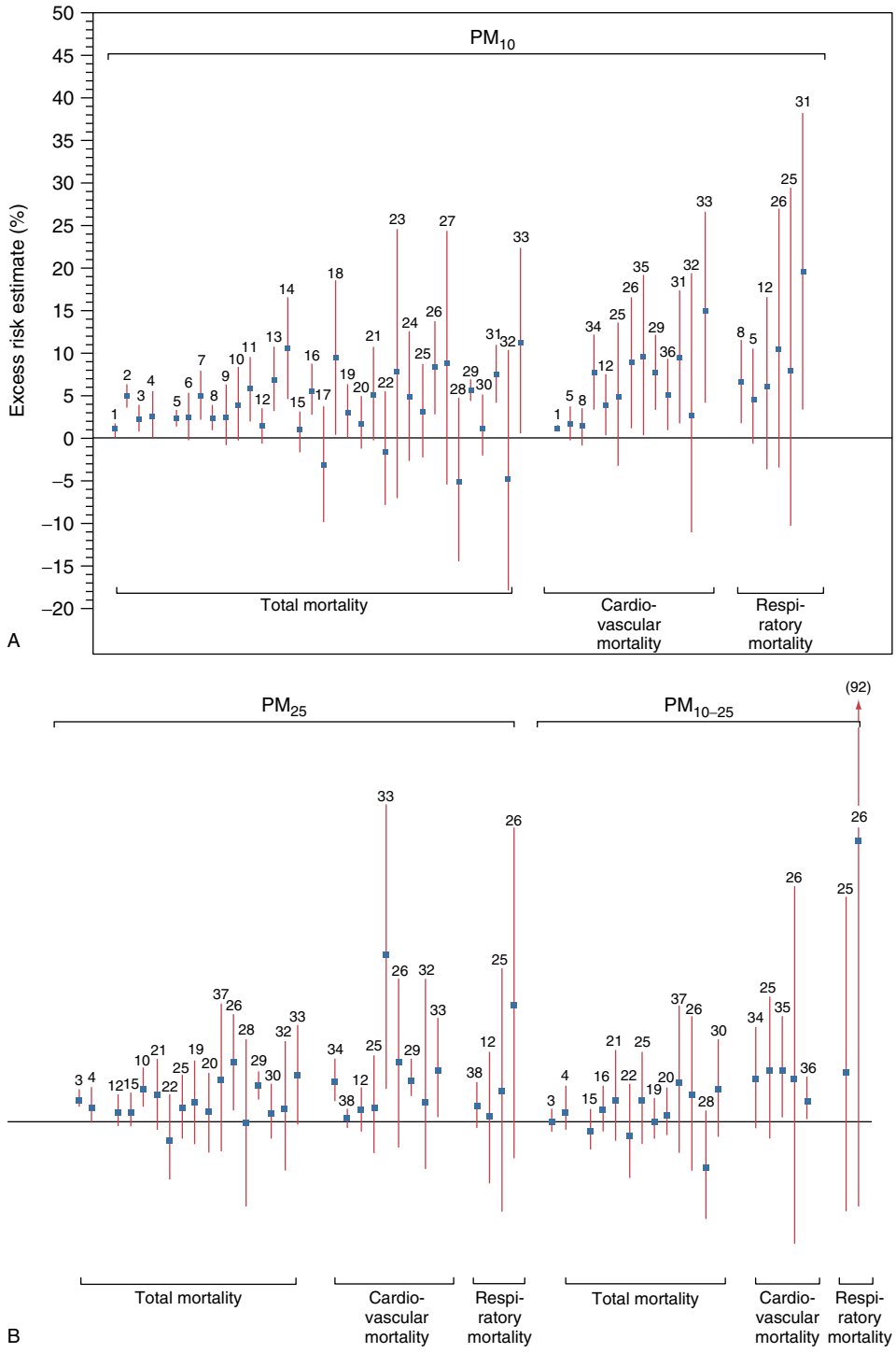

Figure 7 Excess risk estimates for total nonaccidental, cardiovascular and respiratory mortality in multipollutant (1–4) and single-pollutant models for Canadian and United States studies. (Reproduced from the World Health Organization, 2006.)

2. The inflammatory response is likely to be caused by the production of oxidative free radicals
3. Metal species may be involved in free radical formation
4. Transcription factors (e.g. NFκB and AP-1) may be involved, along with changes in cytosolic calcium concentration, in switching on genes controlling production of inflammatory cytokines
5. Changes in clotting factors and in small vessel reactivity may be induced
6. Changes in the autonomic control of the heart may occur.

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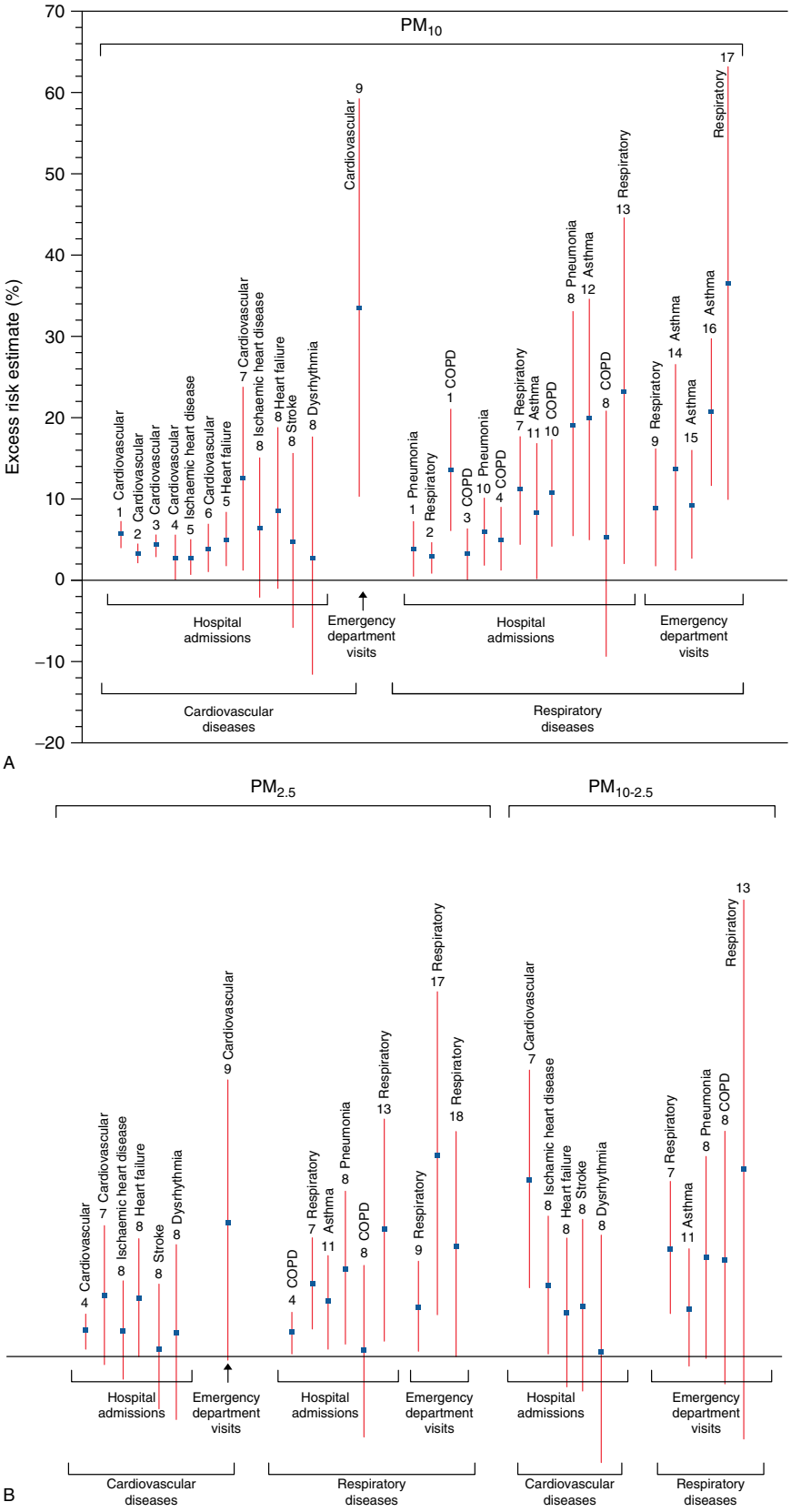

Figure 8 Excess risk estimates for hospital admissions and emergency department visits for cardiovascular and respiratory diseases in single-pollutant models from Canadian and United States studies, including aggregate results from one multi-city study. (Reproduced from the World Health Organization, 2006.)

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Table 5 Adjusted RRs and CIs stratified by smoking status for a 10 μg m^{-3} increase in $PM_{2.5}$ (average)

Cause of death	Never smokers	Former smokers	Current smokers
All cardiovascular diseases plus diabetes	1.11 (1.07–1.16)	1.09 (1.04–1.15)	1.16 (1.09–1.23)
Ischaemic heart disease	1.22 (1.14–1.29)	1.15 (1.07–1.23)	1.16 (1.07–1.27)
Dysrhythmias, heart failure, cardiac arrest	1.04 (0.95–1.15)	1.14 (1.00–1.29)	1.31 (1.12–1.52)
Hypertensive disease	0.88 (0.69–1.12)	1.05 (0.76–1.44)	1.57 (1.12–2.19)
Other atherosclerosis and aortic aneurysms	1.18 (0.90–1.55)	0.91 (0.70–1.19)	1.08 (0.84–1.40)
Cerebrovascular disease	1.03 (0.93–1.15)	1.01 (0.88–1.17)	1.01 (0.86–1.20)
Diabetes	1.01 (0.83–1.23)	0.86 (0.66–1.12)	1.26 (0.91–1.74)
All other cardiovascular diseases	0.86 (0.67–1.09)	0.83 (0.61–1.13)	0.83 (0.59–1.15)
Diseases of the respiratory system	1.03 (0.91–1.17)	0.89 (0.80–1.00)	0.85 (0.76–0.96)
COPD and allied conditions	0.96 (0.73–1.24)	0.86 (0.73–1.00)	0.81 (0.70–0.93)
Pneumonia and influenza	1.20 (1.02–1.41)	0.98 (0.80–1.20)	0.90 (0.69–1.18)
All other respiratory diseases	0.74 (0.56–0.97)	0.88 (0.68–1.16)	1.10 (0.76–1.60)

Table modified from Pope *et al*. (2004).

Key studies supporting these findings have been reported by: Batalha *et al*. (2002), Campen *et al*. (2001), Chapman *et al*. (1997), Costa and Dreher (1997), Gardner *et al*. (2000), Godleski *et al*. (2000), Gordon *et al*. (1998), Gurgeria *et al*. (2002), Kodavanti *et al*. (2000), Nemmar *et al*. (2001; 2002a; 2002b; 2003a; 2003b), Salvi *et al*. (1999), Suwa *et al*. (2002) and Vincent *et al*. (2001). All this has led to two key hypotheses. The first suggests that inhaled particles lead to destabilization of, or an increased rate of progression of atherosclerotic plaques in coronary arteries. Suwa and colleagues work (Suwa *et al*., 2002) in animals is key to this, as is the series of papers on APOE−/− mice reported by Lippmann's group (Chen and Hwang, 2005; Chen and Nadziejko, 2005; Gunnison and Chen, 2005; Lippmann *et al*., 2005a; 2005b; 2005c; Maciejczyk and Chen, 2005; Maciejczyk *et al*., 2005; Veronesi *et al*., 2005).

The second relates to changes in heart-rate variability (HRV). HRV refers to the variations in the beat-to-beat interval of the heart. Though the parasympathetic system slows the heart, it actually increases HRV; the sympathetic system has the opposite effect. Decreased HRV is a known indicator of impending heart failure in patients with impaired ventricular function (Department of Health, 2006). Evidence to show that inhaled particles can reduce HRV has been obtained in animals (Godleski *et al*., 2000) and in man (Devlin *et al*., 2003). Links between air pollution and arrhythmias have been reported by Peters *et al*. (1999) and Peters *et al*. (2000). The latter study looked at the frequency of firing of implanted defibrillators and found that, in patients susceptible to frequent arrhythmias, this was linked to ambient concentrations of particles. This is an exceptionally suggestive finding.

8.3 The Ultrafine Hypothesis

Seaton *et al*. (1995) proposed that ultrafine particles might underlie the effects of ambient particles and that the key effects of very low mass concentrations on the cardiovascular systems could thus be explained. A similar hypothesis was advanced by Oberdörster *et al*. (1995). Much of the work outlined above, linking particles with inflammation, with changes in intravascular clotting factors and the evolution of atherosclerotic plaques supports this idea. But epidemiological evidence of effects of ultrafine particles has been slow to accumulate; the question remains open. The ultrafine hypothesis has sparked a great interest in nanotoxicology (see **Nanotoxicology-The Toxicology of Nanomaterials**) and it is not possible to discuss the key evidence in any detail here. The reader is referred to an excellent review by Oberdörster *et al*. (2005).

8.4 Conclusion

Particles are important air pollutants—fine particles ($PM_{2.5}$) appear to be especially important. A vast amount

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Table 6 Review of studies with concentrated PM

Article title	Study design	Population	Exposure	Findings	Reference
Inhalation of fine particulate air pollution and ozone causes acute arterial vasoconstriction in healthy adults	Randomized crossover trial	25 healthy adults	2 h inhalation of approximately 150 μg m^{-3} fine CAPs plus ozone (120 ppb) vs. response to the inhalation of filtered air	CAPs plus ozone caused a significant brachial artery vasoconstriction compared with filtered air inhalation (−0.09 ± 0.15 mm vs +0.01 ± 0.18 mm, $P = 0.03$). No significant differences in flow-mediated dilatation (+0.29 ± 4.11% vs. −0.03 ± 6.63%, $P = 0.88$), nitroglycerine-mediated dilatation (+3.87 ± 5.43% vs. +3.46 ± 7.92%, $P = 0.83$), or blood pressure responses between exposures	Brook *et al*. (2002)
Exposure to concentrated ambient air particles alters haematological indices in humans	Clinical trial	20 young adults	Mean PM mass of 120.5 ± 14.0 μg m^{-3} for 24 h (n = 15) or filtered air (n = 5)	Exposure of health volunteers to CAPs was associated with decreases in both white blood cell count and lactate hydrogenase and increased concentrations of fibrinogen in the blood	Ghio *et al*. (2003)
Elderly humans exposed to concentrated air pollution particles have decreased heart rate variability	Clinical trial	10 elderly subjects 22 young subjects	Particle concentrations 6–10 times normal Chapel Hill air of $PM_{2.5}$	Elderly people experienced significant decreases in both time and frequency of heart rate variability immediately following exposure. No CAPs-induced changes in heart rate variability were found in young volunteers exposed to CAPs in a previous study	Devlin *et al*. (2003)
Exposures of elderly volunteers with and without chronic obstructive pulmonary disease (COPD) to concentrated ambient fine particulate pollution	Clinical trial	13 elderly volunteers with COPD and six age-matched healthy adults	200 μg m^{-3} CAPs <2.5 μm in diameter and filtered air for 2 h	A significant negative effect of CAPs on arterial oxygenation was more pronounced in healthy subjects. Peripheral blood basophils increased after CAPs in healthy people but not in COPD patients. In both groups, red cell counts increased slightly one day after exposure to filtered air but not to CAPs. Pre-exposure ectopic heartbeats were infrequent in health subjects but increased modestly during/after CAPs exposure relative to filtered air. Ectopic beats were more frequent in COPD patients, but decreased modestly during/after CAPs relative	Gong *et al*. (2004)

(*Continued overleaf*)

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Table 6 *(continued)*

Article title	Study design	Population	Exposure	Findings	Reference
				to filtered air. Heart rate variability over periods of several hours was lower after CAPs than after filtered air in healthy elderly subjects but not in COPD patients. Unexpectedly, individuals with COPD appeared less susceptible than healthy elderly individuals	Gong *et al.* (2004)
The role of soluble components in ambient fine particles-induced changes in human lungs and blood	Clinical trial	37 young healthy volunteers	Particle concentrations 6–10 times normal Chapel Hill air of $PM_{2.5}$	Sulfate/iron/selenium factor was associated with increased percentage of neutrophils in bronchoalveolar lavage fluid and a copper/zinc/vanadium factor with increased blood fibrinogen. The concentration of sulfate, iron and selenium correlated highly with PM mass ($R > 0.75$) while the correlations between PM and copper/zinc/vanadium were modest ($R = 0.2–0.6$)	Huang *et al.* (2003)

Modified from World Health Organization, 2006.

of evidence, both epidemiological and toxicological has accumulated to support these conclusions. A series of guidelines have been recommended by WHO. These are shown in **Tables 7** and **8**.

9 NITROGEN DIOXIDE (NO_2)

NO_2 is an irritant gas which, in high concentrations, can cause pulmonary oedema. Concentrations may reach 500 ppb (940 μg m^{-3}), though urban annual averages are much lower: 10–50 ppb (20–90 μg m^{-3}). In 1991, in London, hourly average concentrations exceeded 400 ppb (720 μg m^{-3}) during a cold period in early December and this was considered exceptional. Indoor concentrations often exceed those outdoors: gas cookers (stoves) in kitchens generate NO_2 and levels can exceed 1000 ppb (1880 μg m^{-3}). This has led to concerns about NO_2 as an indoor air pollutant: studies of children have shown that those living in homes using gas for cooking and heating are more liable to respiratory infections than are others (Hasselblad *et al.*, 1992; Chauhan *et al.*, 2003; Sunyer *et al.*, 2004). These studies have led to the view that long-term exposure to NO_2, can be dangerous, and in the early 1990s drove the thinking of experts advising WHO on air quality guidelines for NO_2. Since then a great deal of new data has appeared (Miller *et al.*, 1993).

NO_2 is a gas and 70–90% of inhaled NO_2 is absorbed by the respiratory tract. About 50% of that inhaled may be absorbed in the upper airways (Berglund, 1993; Kommission Reinhaltung der Luft in VDI und DIN, 1985; United States Environmental Protection Agency, 1993). Peak tissue dose along the airways has been modelled: as in the case of O_3, this occurs at the junction of the conducting airways and the gas-exchange zone. Exercise increases the tissue dose in the gas-exchange region of the lung.

9.1 Epidemiological Studies

The great problem in studying the effects of NO_2 is its close association with particulate air pollutants. When effects are found it is difficult to decide which pollutant represents the causal factor. In recent years, enthusiastic work on particles has led to NO_2 being ignored and some authorities argue that NO_2 should be regarded merely as a marker or index for the traffic-related pollution mixture. The position is complicated by the likelihood that if sources of fine particles are controlled, then sources of NO_2 will also be controlled. Seeking areas where NO_2 and PM are not closely correlated is important—but difficult.

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Table 7 Air quality guideline and interim targets for PM: annual mean

Annual mean level	PM_{10} ($\mu g/m^3$)	$PM_{2.5}$ ($\mu g/m^3$)	Basis for the selected level
WHO interim target 1 (IT-1)	70	35	These levels are estimated to be associated with about 15% higher long-term mortality than at AQG levels
WHO interim target 2 (IT-2)	50	25	In addition to other health benefits, these levels lower risk of premature mortality by approximately 6% (2–11%) compared to IT-1
WHO interim target 3 (IT-3)	30	15	In addition to other health benefits, these levels lower risk of premature mortality by approximately another 6% (2–11%) compared to IT-2
WHO air quality guidelines (AQG)	20	10	These are the lowest levels at which total cardiopulmonary and lung cancer mortality have been shown to increase with more than 95% confidence in response to $PM_{2.5}$ in the ACS study (Pope *et al*., 1995; 2002). The use of the $PM_{2.5}$ guideline is preferred.

Reproduced from the World Health Organization, 2006.

Table 8 Air quality guideline and interim targets for PM: 24-h mean

24 h mean level[a]	PM_{10} ($\mu g\ m^{-3}$)	$PM_{2.5}$ ($\mu g\ m^{-3}$)	Basis for selected level
WHO interim target 1 (IT-1)	150	75	Based on published risk coefficients from multicentre studies and meta-analyses (about 5% increase in short-term mortality over AQG)
WHO interim target 2 (IT-2)	100	50	Based on published risk coefficients from multicentre studies and meta-analyses (about 2.5% increase in short-term mortality over AQG)
WHO interim target 3 (IT-3)[b]	75	37.5	About 1.2% increase in short-term mortality over AQG
WHO air quality guidelines (AQG)	50	25	Based on relationship between 24-h and annual PM levels

[a]99th percentile (3 d/yr).
[b]For management purposes, based on annual average guideline values, the precise number to be determined on the basis of local frequency distribution of daily means.
Reproduced from the World Health Organization, 2006.

9.2 Time-Series Studies

Many studies have been reported. Stieb *et al*. (2002) have published a meta-analysis of 109 studies: 32 coefficients for NO_2 were included from single pollutant models and 15 from multi-pollutant models. The bottom-line result was for percentage increase in all cause mortality single pollutant models: 2.8% (95% CI: 2.1–3.5) per 24 ppb NO_2; multipollutant models: 0.9% (95% CI: −0.1 to 2.0) per 24 ppb NO_2.

European studies (Katsouyanni *et al*., 1996; 2001; Touloumi *et al*., 1997) have also been reported. These are worth considering: APHEA (1) (Katsouyanni *et al*., 1996): 1.3% increase in all cause deaths per 50 $\mu g\ m^{-3}$ NO_2 (1 hour maximum concentration) when adjusted for particles the coefficient fell to 0.6%.

The US study (NMMAPS) (Samet *et al*., 2000; Dominici *et al*., 2003) also found significant effects. Studies in this area are difficult to compare because:

1. Authors use different measures of NO_2 concentration, for example, 24 hour average or maximum 1 hour concentration in the day
2. Effects on day D may be related to concentrations on day D, day $D-1$, day $D-2$, and so on; this is described in the 'lag structure' of the analysis
3. Adjustment for other pollutants (or some of them) may or may not have been done.

However, it is accepted that daily variations in low concentrations of NO_2 affect daily death rates. Similar effects on indices of respiratory morbidity have been studied: hospital admissions for COPD (Anderson *et al*.,

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1997), asthma admissions (Sunyer *et al*., 1997), all respiratory admissions (Spix *et al*., 1998). COPD admissions were associated, asthma admissions were inconsistently associated and all respiratory admissions were not associated with NO_2. Similarly variable results have been found for cardiovascular admissions. The analysis by Metzger *et al*. (2004) provides a summary of the evidence on these and that of Peel *et al*. (2005) deals with respiratory admissions. It seems that NO_2 is related to hospital admissions for cardiorespiratory disorders. But control for covariate pollutants lowers the estimate of the impact of NO_2 and the coefficient linking NO_2 and effects may lose statistical significance. Of all the impacts, that on asthma admissions is perhaps the most certain. This has been studied in children.

9.3 Time-Series Studies of NO_2 and Asthma Morbidity in Children

Studies in London (Anderson *et al*., 1998; Atkinson *et al*., 1999a, b; Hajat *et al*., 1999), in Canada (Lin *et al*., 2003), in Australia and New Zealand (Barnett *et al*., 2005) and the USA (Peel *et al*., 2005) show that NO_2 is related to emergency admissions to hospital in children suffering from asthma. Associations with PM and O_3 were also found in several studies; NO_2 'survived' adjustment for copollutants better than others, for example, PM and O_3. Studies of panels of asthmatic children have supported these findings and an interaction between exposure to NO_2 and viral infections (a major trigger of asthma attacks in children—especially young children) is now accepted. However, a large European study, the PEACE (Pediatric Asthma Clinical Effectiveness) study (Roemer *et al*., 1998) failed to find associations between particulate air pollutants or NO_2 and indices of asthma morbidity.

9.4 Studies of Effects of Long-term Exposure to Outdoor Concentrations of NO_2

A number of studies have been reported. Here we focus on those that have had a large impact on thinking.

9.4.1 The South California Children Study

This study, reported by Gauderman *et al*. (2000; 2002) and McConnell *et al*. (2003) shows that children growing up in communities with raised levels of NO_2 (and nitrate-rich particulates) suffer a retardation in lung development. Decrements in FEV_1, MMEF (maximum mid expiratory flow) and PEF were recorded and, very importantly, these were shown to resolve in children who moved to less polluted areas. Details may be found in the original papers, but across a range of NO_2 concentrations varying by 35 ppb (difference between communities) FEV_1 decreased by -101 ml (95% CI: -165 to -35). Careful adjustment for other factors affecting indices of lung function was undertaken. These results are supported in adults by European work (Schindler *et al*., 1998). Lung function decrements sustained in childhood are likely to last throughout adult life; these findings are, therefore, potentially important.

9.4.2 Asthma, Respiratory Disorders and Atopy

Here the evidence is less clear cut. Consider a study from the Netherlands: a birth cohort of 3745 children was studied (Brauer *et al*., 2002). NO_2 concentrations at home addresses were modelled. These varied between 12 and 58 $\mu g\ m^{-3}$, annual average. Adjusted odds ratios for a 10 $\mu g\ m^{-3}$ increase in NO_2 were 1.13 (95% CI: 0.99–1.29) for wheeze and 1.18 (95% CI: 0.93–1.51) for asthma. NO_2 was highly correlated with PM. The California Children study referred to above looked at NO_2 and bronchitis and cough. Four year average NO_2 concentrations ranged from 4 to 38 ppb (very low concentrations). The odds ratios for bronchitis symptoms were: per 1 ppb NO_2: 1.02 (95% CI: 1.00–1.03).

These data seem less persuasive, to me, than those on lung function.

In addition to the above, a number of studies have found associations between low birth weight and preterm birth in areas relatively polluted with NO_2 (Wilhelm and Ritz, 2003; Dales *et al*., 2004; Lin *et al*., 2004).

9.5 Toxicological Studies of NO_2

Studies in animal models have not been helpful in explaining the effects seen in man at very low concentrations of NO_2. This section is divided into two parts: studies in animals and studies in man. *In vitro* studies of consumption of antioxidants (Halliwell *et al*., 1992; Kelly and Tetley, 1997) on membrane permeability (Devalia *et al*., 1993) and on the release of inflammatory mediators by culture cells (Ayyagari *et al*., 2004) have been reported. In general, high concentrations of NO_2 were used in these studies.

9.5.1 Animal Studies

Increased lipid peroxidation (a sign of free radical activity) has been reported in rats exposed to 4000 ppb

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(752 μg m^{-3}) NO_2 for 18 months (Sagai *et al*., 1984; Sagai and Ichinose, 1987). Damage to Type I alveolar cells and ciliated airway cells (Type II alveolar cells and Clara cells appear to be more resistant) has been shown in rats exposed to 340 ppb (640 μg m^{-3}) NO_2 (Department of Health, 1993). Long-term exposure to NO_2 induces emphysema-like changes in several species (Snider *et al*., 1985). Inflammatory responses have been reported, (Ichinose and Sagai, 1982) as has enhanced injury in mice sensitized to and then challenged with ovalbumin after exposure to NO_2 (Hussain *et al*., 2004). Effects on airway defence mechanisms against bacteria have been reported (Ehrlich and Henry, 1968; Ehrlich *et al*., 1977; Gardner *et al*., 1977). These may provide an explanation for the increased respiratory infections experienced by children living in gas-using homes, though the animals were continuously exposed to concentrations similar to those occasionally found in such homes.

9.5.2 Human Studies

Exposure to NO_2 causes changes in indices of lung function, but in normal subjects concentrations of about 1000 ppb (1880 μg m^{-3}) are needed to produce such effects. Asthmatic subjects are more sensitive than others and effects have been recorded on exposure for 2–2.5 hours to 300 ppb (520 μg m^{-3}) NO_2 (Avol *et al*., 1989; Bauer *et al*., 1986; Roger *et al*., 1990). But results amongst studies at this level—and higher levels—are inconsistent (Linn *et al*., 1985; 1986; Linn and Hackney, 1984). Also, the changes in indices of lung function at such levels of exposure are small and of debatable significance. Induction of symptoms in normal subjects and asthmatics requires exposure to much higher concentrations (Linn and Hackney, 1984). Studies involving exposure to NO_2 and subsequent bronchoalveolar lavage have shown that exposure to >1 ppm (1880 μg m^{-3}) can induce a brisk inflammatory response (Jörres *et al*., 1995; Sandström *et al*., 1992). At lower levels of exposure (600 ppb (1130 μg m^{-3}); four separate two hour exposures over six days) no such response was found (Boushey *et al*., 1988). Other studies have shown effects on subsets of lymphocytes but these, too, have used concentrations of NO_2 considerably greater than occur commonly in ambient air. Emphasis has been placed on effects on cytokines (IL-5, IL-10, IL-13 and ICAM-1). These Th2 cytokines might indicate a proallergic effect, but this is unproven (Pathmanathan *et al*., 2003).

9.6 Conclusion

NO_2 is a common air pollutant. Epidemiological studies have shown a range of associations with mortality and morbidity and, most surprisingly, with lung development in children. Indoor exposure is likely to exceed outdoor exposure. Consideration of the evidence led WHO to recommend the following Air Quality Guidelines: annual average concentration: 40 μg m^{-3}; 1 hour average concentration: 200 μg m^{-3}.

10 SULPHUR DIOXIDE (SO_2)

SO_2 is produced when fuel containing sulphur is burnt in air. In the days of coal-smoke smog, SO_2 was treated in combination with particles (often measured as 'black smoke' by a reflectance method), but more recently the effects of SO_2 alone have been considered. SO_2 dissolves rapidly in water and oxidation on the surface of particles—especially if catalytic metals are present—leads to the formation of sulphur trioxide and sulfurous and sulfuric acid. Droplets of sulfuric acid form in the air by primary nucleation—this is an unusual property of sulfuric acid vapour. Sulfuric acid is neutralized by combination with ammonia to produce ammonium bisulfate and sulfate ($NH_4HSO_4(NH_4)_2SO_4$)). Details can be found in the Medical Aspects of Air Pollution Episodes (MAAPE) report on SO_2, acid aerosols and particulates (Department of Health, 1992). SO_2 has been thought to be effectively removed on inhalation by the upper airway. As the concentration of SO_2 in inhaled air falls, so does the percentage absorbed and Amdur (1986) pointed out that upper airway removal of SO_2 might be inefficient at ambient concentrations. Ammonia found in the mouth, produced by bacteria, may be important in neutralizing acid aerosols. The following reactions occur in water:

$$SO_2 + H_2O \rightarrow H_2SO_3 \quad (8)$$

$$H_2SO_3 + H_2O \rightarrow HSO_3^- + H_3O^+ \quad (pK_a = 1.86) \quad (9)$$

$$HSO_3^- + H_2O \rightarrow SO_3^{2-} + H_3O^+ \quad (pK_a = 7.2) \quad (10)$$

Absorbed bisulfite is converted to sulfate:

$$HSO_3^- \rightarrow SO_4^{2-} \text{(catalysed by a molybdenum-dependent oxidase)} \quad (11)$$

Production of sulfate is limited in the lung and much more pronounced in the liver and kidney. HSO_3^- ions react with oxidized glutathione. Note HSO_3^- is a reducing agent: the coal-smoke smog was described as a 'reducing smog'.

SO_2 is a notably irritant gas that causes rapid bronchoconstriction at high concentrations. Asthmatic subjects are more sensitive to SO_2 than are other subjects. It is generally agreed that the lowest concen-

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Table 9 Estimated total (nonaccidental) mortality expressed as percentage excess deaths (95% CI in parentheses) per 50 μg m^{-3} increase in sulphur dioxide reported in recent multicity time-series studies and meta-analyses

Study	Estimate	Comment
APHEA 1 (Katsouyanni *et al*., 1997)	Western Europe: 2.9% (2.3–4.6) at the best lag between 0 and 3 d for each city	The effects of sulphur dioxide and PM were 'mutually independent'
12 European cities	Central and eastern Europe: 0.9% (0.2–1.5)	
APHEA 1 (Samoli *et al*., 2001; 2003)	Western Europe: 2.6% (2.1–3.1)	Restricting data range below 150 or 200 μg m^{-3} increased sulphur dioxide risk estimates
12 European cities, using national splines rather than sine/cosine to adjust for temporal trends	Central and eastern Europe: 0.7% (0.0–1.4)	
EMECAM (Ballester *et al*., 2002) (GAM[a] study), 13 Spanish cities	2.5% (0.3–4.9), average of lag 0 and 1 d	—
NMMAPS (Samet *et al*., 2000; Dominici *et al*., 2003) 90 largest US cities	1.1% (0.5, 1.7) at lag 1 d	Adding copollutants reduced the estimate by ~20% and widened confidence bands
Stieb *et al*. (2002; 2003)	Non-GAM[a]	—
Meta-analyses	Single pollutant (29 studies): 1.7% (1.2–2.3) With copollutant(s) (10 studies): 1.6% (0.6–2.5) GAM[a]: Single-pollutant (17 studies): 2.0% (1.3–2.6) With copollutant(s) (11 studies): 1.6% (0.8–2.4)	

[a]GAM stands for generalized additive model: this caused problems in some time-series studies due to a 'glitch' in the software.
Reproduced from the World Health Organization, 2006.

tration of SO_2 likely to cause effects on the airways of asthmatic subjects lies between 100 and 400 ppb (286–1144 μg m^{-3}), with effects towards the lower end of this range being unlikely (Linn *et al*., 1983). Recent work by Tunnicliffe *et al*. (2001) has shown that exposure to 200 ppb (572 μg m^{-3}) SO_2 can produce changes in indices of HRV. Oddly, the changes differed in normal and asthmatic subjects. No lung function changes were observed in either group. Amongst asthmatic subjects the high-frequency component of HRV was reduced—in a clinical setting this would be seen as a deleterious effect—its significance in asthmatic patients is unknown. Whilst considering the effects of SO_2 in man it is important to recall that on exposure to effective concentrations of SO_2 bronchoconstriction appears rapidly—generally within minutes. It does not then increase as exposure is continued. Thus this response is classically a C-dependent response, time (t) is relatively unimportant. Animal studies have contributed little to our knowledge of the effects of exposure to very low concentrations of SO_2. At 300 ppm (858 mg m^{-3}) ciliary slowing has been observed; in explanted human bronchial tissue this effect has been seen at 200 ppb (572 μg m^{-3}). Amdur (1986) showed that the effects of SO_2 were increased by simultaneous exposure of guinea pigs to ultrafine particles. It has been suggested that formation of H_2SO_4 on the surface of the zinc-rich particles may explain this effect.

10.1 Epidemiological Studies

10.1.1 Mortality Studies

Early work focussed on the SO_2–coal smoke mixture; the results of such work are now of limited relevance in developed countries where concentrations of SO_2 are generally low. Time-series studies have been undertaken and the results of a number of large studies and of a meta-analysis are shown in **Table 9**.

More important, perhaps, than the time-series approach has been the intervention study reported from Hong Kong

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Table 10 Summary table based on meta-analysis of time-series studies

Pollutant (24 h average)	N	Outcome measure	Assessment	Random effects (95% CI) (% change per 10 μg m^{-3})
SO_2	67	CV mortality	+	0.8 (0.6, 1.0)
SO_2	7	CV admissions	+	0.6 (0.1, 1.2)
SO_2	18	Cardiac admissions	+	2.4 (1.6, 3.3)
SO_2	10	IHD admissions	+	1.2 (0.5, 1.9)
SO_2	5	Heart failure admissions		0.9 (−0.1, 1.8)
SO_2	7	Cerebrovascular admissions		0.3 (−0.5, 1.1)

Reproduced from the World Health Organization, 2006.

Table 11 Sulphur dioxide air quality guidelines and interim targets to be achieved in improving air quality

	24 h average	10 min average
WHO interim target 1 (IT-1) (2000 guideline level)	125 μg m^{-3}	—
WHO interim target 2 (IT-2)	50 μg m^{-3} Intermediate goal based on controlling either: (i) motor vehicle, (ii) industrial emissions and/or (iii) power production; this would be a reasonable and feasible goal to be achieved within a few years for some developing countries and lead to significant health improvements (such as aiming for the guideline).	—
WHO air quality guidelines	20 μg m^{-3}	500 μg m^{-3}

Reproduced from the World Health Organization, 2006.

by Hedley *et al*. (2002). In this work the concentration of SO_2 fell from 44 to 21 μg m^{-3} but PM_{10} concentrations were unchanged. Now both 44 and 21 μg m^{-3} are low concentrations of SO_2; low in comparison with concentrations used in chamber studies and extremely low in comparison with historical concentrations in, for example, London. But effects were found: the average annual trend in death rate (increasing as the population ages) fell by 2.1% (respiratory deaths: 3.9%; cardiovascular: 2.0%). At the same time as SO_2 levels fell, so did the vanadium content of particles. Whether this was important is unknown, but the point is raised in view of Lippmann's perceptive work regarding the importance of nickel in particles in New York (Lippmann *et al*., 2006).

10.1.2 Morbidity Studies

The intractable problem of surrogacy has bedevilled work on SO_2 as on other air pollutants. Lippmann *et al*. (2006) noted:

> *Interestingly, many of the researchers, in their discussions when sulfur dioxide associations were found, did not often interpret these sulfur dioxide associations with mortality or morbidity as causal but rather as 'artefact', and suggested that sulfur dioxide was acting as a 'surrogate' for a source type. The reasoning offered for the sulfur dioxide levels being 'too low' to be causal was based on prior knowledge. This situation highlights a limitation of the observational studies, the 'surrogate' interpretation, that is, that sulfur dioxide represents a source type (e.g. coal-fired power plant), or a mixture that can affect health through a co-pollutant (e.g. PM), or through pollutants that it is converted into (i.e. sulfuric acid and sulfates). Yet another interpretation is that PM becomes more toxic when sulfur dioxide coexists and gets adsorbed onto PM surfaces.*
>
> *Source: (Reproduced by kind permission of the World Health Organization)*

These problems have stemmed from a lack of confidence in effects of SO_2 at very low concentrations—so low that certainly no effects would be expected in chamber studies using sensitive asthmatic subjects. We shall come back to this point later. Meta-analysis of time-series studies linking SO_2 with effects on the cardiovascular system has shown that clear effects occur. The results of the analysis are shown in **Table 10**.

Perhaps surprisingly Spix *et al*. (1998) failed to find a consistent association between SO_2 concentrations (24 hour averages) and respiratory admissions to hospital. Atkinson *et al*. (2001) did find such an association.

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Table 12 Selected studies relating ambient variations of ozone concentrations to short-term mortality

Location	End point	Other pollutants considered	Observed effect	Reference
London	Total mortality, mortality in people >70 years, and cardiovascular and respiratory mortality	Black smoke, sulphur dioxide and nitrogen dioxide	An increase of 8 h ozone from the 10th to the 90th percentile of the seasonal change (14–72 μg m^{-3}) was associated with increases of 3.5, 3.6 and 5.4% in all cause, cardiovascular and respiratory mortality, respectively	Anderson *et al*. (1996)
Rotterdam	All causes (excluding accidents)	Black smoke, sulphur dioxide and carbon monoxide	RR[a] 1.06 for a change of 67 μg m^{-3} ozone. Black smoke also exhibited positive association with mortality	Hoek *et al*. (1997)
Eleven cities in Europe	Respiratory, cardiovascular and digestive (used as control)	Sulphur dioxide, nitrogen dioxide and different indicators of PM	A 50 μg m^{-3} increase in 8 h ozone was associated with cardiovascular (RR 1.02) and respiratory (RR 1.06) mortality. Positive results for black smoke and sulphur dioxide	Zmirou *et al*. (1998)
Meta-analysis of studies conducted in several European cities	All causes (excluding accidents), respiratory disease and cardiovascular disease	—	A 10-μg/m^3 increase in ozone was associated with a RR of 1.003 (1.001–1.004) for all-cause mortality and 1.004 (1.003–1.005) for cardiovascular mortality. The RR for respiratory mortality was 1.000 (0.996–1.005)	Anderson *et al*. (2004)
Fourteen communities in the USA	All causes (excluding accidents), respiratory and cardiovascular disease	PM_{10}	A 20 μg m^{-3} increase in hourly ozone was associated with a 0.23% (CI 0.01–0.44) increase in the risk of death	Schwartz (2005)
Meta-analysis of 144 effect estimates from 39 time-series studies	All causes (excluding accidents), respiratory disease and cardiovascular disease	PM_{10} or $PM_{2.5}$	A 10 μg m^{-3} increase in ozone was associated with a 0.87% (0.55–1.18) increase in total mortality for all seasons and 1.34% increase (−0.45 to 3.17) in the warmer season. For cardiovascular disease, the same increment of ozone was associated with a 1.1% (0.68–1.53) increase during all seasons and a 2.45% increase (0.88–4.1) in the warmer season	Bell *et al*. (2005)

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Table 12 (*Continued*)

Location	End point	Other pollutants considered	Observed effect	Reference
Meta-analysis of 43 studies conducted in different parts of the world plus an additional analysis in seven cities in the USA	All causes (excluding accidents), PM_{10} or $PM_{2.5}$	—	A 0.39% increase (95% CI 0.26–0.51) in mortality per 20 μg m^{-3} increase in 1 h daily maximum ozone. No appreciable modification by including PM as co-pollutant in the models. Effects were larger for the warmer season	Ito *et al*. (2005)
Meta-analysis of 28 studies	All causes (excluding accidents), PM_{10}, or $PM_{2.5}$	PM_{10}, $PM_{2.5}$, sulphur dioxide, nitrogen dioxide and carbon monoxide	AA 0.21% increase (CI 0.16–0.26) in mortality per 10 μg m^{-3} increase in 1 h maximum ozone	Levy *et al*. (2005)

[a]RR, relative risk.
Reproduced from the World Health Organization, 2006.

10.2 Long-term Exposure Studies

Reanalysis of the ACS study (see above) by Krewski *et al*. (2000) showed that long-term concentrations of SO_2 were associated with all-cause mortality. Very interestingly, the results were not removed when adjustments for $PM_{2.5}$ and SO_4^{2-} were made. That SO_2, $PM_{2.5}$ and SO_4^{2-} covary in some areas is certain and separation of true causal factors by statistical analysis may be impossible. But the possibility that SO_2 may itself be playing a role remains. Less easy to interpret is the finding from the Adventist Health Study of Smog (AHSMOG) cohort (Abbey *et al*., 1999). In this study SO_2 was found to be related to lung cancer in males and females. That SO_2 is acting as a surrogate for some more obviously carcinogenic component of the ambient air pollution complex seems likely.

10.3 Conclusions

Thinking about SO_2 has changed dramatically during the last 10 or so years. It is now believed that daily variations in already low concentrations affect both deaths and morbidity (cardiovascular and respiratory). In addition, it seems clear from the study in Hong Kong that reducing concentrations of SO_2 is beneficial to health. All this has led WHO to recommend the following Air Quality Guidelines and targets, as shown in **Table 11**.

11 OZONE

We have already noted that O_3 is an outdoor air pollutant, indeed exposure indoors is unlikely because of the rate at which O_3 reacts with furnishings and fittings, and because there are no indoor sources. The last point is true if we except photocopiers and electric motors. O_3 is an unusual air pollutant in that concentrations are rising. In the UK this is due to a slow upward drift of the so-called hemispherical background concentration caused by increased production of volatile organic compounds by developed countries and, in urban areas, by a decline in production of NO by motor vehicles. O_3 concentrations tend to be higher in rural than urban areas, but can be high in suburban areas also. Concentrations in the UK seldom exceed 120 ppb (240 μg m^{-3}), though in Mexico City and São Paulo concentrations can exceed 200 ppb. Concentrations are higher in summer than winter and fall during the night. Peak levels are usual reached in the afternoon. In fact, two peaks can often be seen: the first due to local production and the second due to long-range transport. This is clearly shown by Lippmann (2000). The rather broad afternoon peak in O_3 concentrations and the fact that responses to O_3 are about equally dependent on C (concentration) and on t (duration of exposure) has led to the daily-peak-eight-hour-average concentration being used as a basis for the WHO Air Quality Guideline (World Health Organization, 2006). Personal exposure to O_3 is not closely correlated with outdoor O_3 concentrations and studies linking outdoor concentrations with effects on health are likely to underestimate the 'real' exposure–response relationship. The study of the effects of O_3 on health has been advanced by both studies in volunteers and epidemiological studies. The database is now vast: in the WHO Air Quality Guidelines published in 2006 a separate appendix was needed in which to present a synopsis of the evidence.

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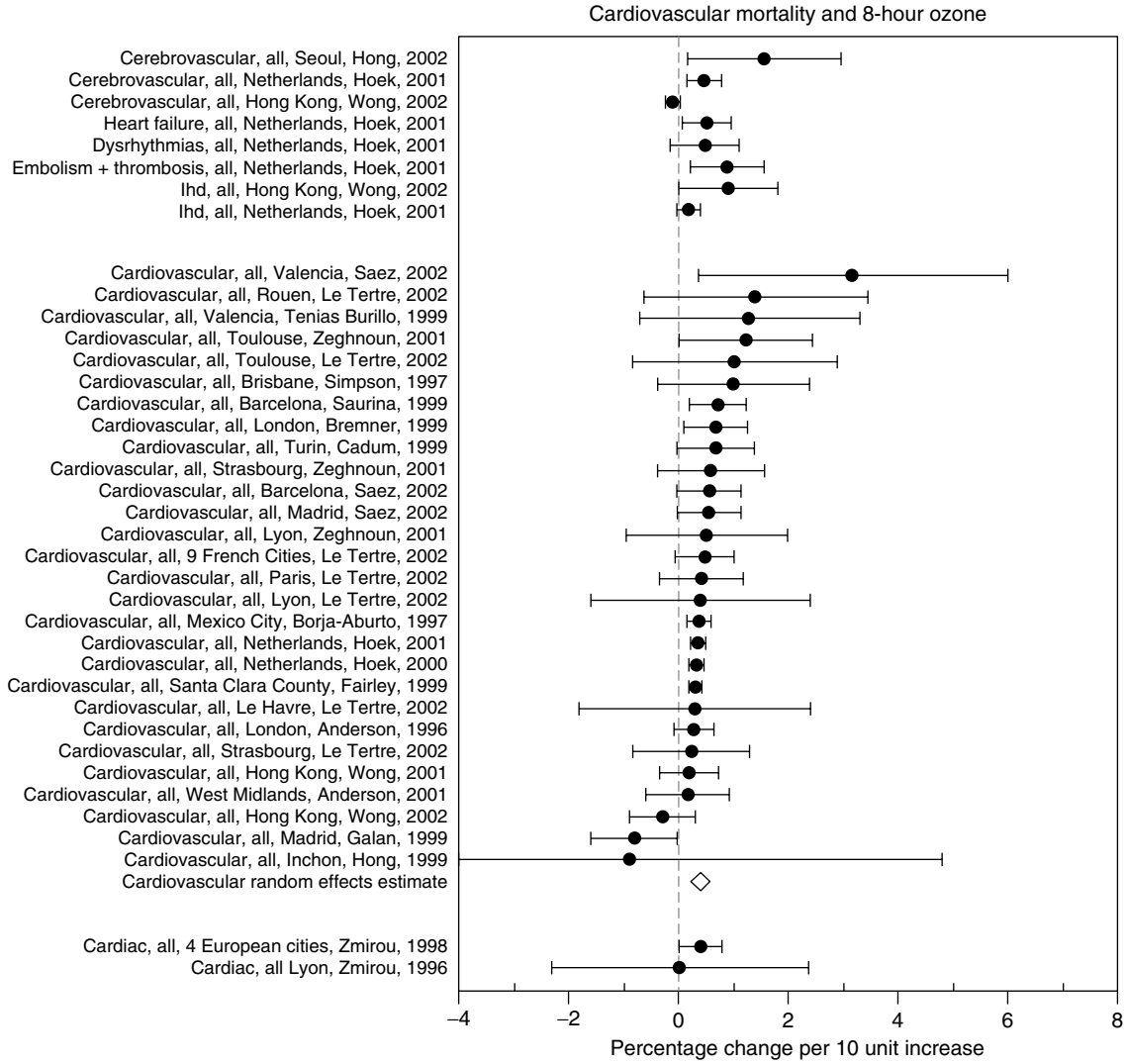

Figure 9 Cardiovascular mortality and eight hour ozone. (Reproduced from the World Health Organisation, 2006.)

11.1 Epidemiological Studies

Time-series studies have linked O_3 with mortality (respiratory rather than cardiovascular) and with indices of morbidity (respiratory and cardiovascular). The World Health Organization (2006) report listed 31 key studies and meta-analyses linking short-term exposure to O_3 and mortality. An abbreviated table is provided here (**Table 12**).

Once again, the weight and consistency of the evidence is impressive. The results of a meta-analysis undertaken from COMEAP in the UK are shown in **Figure 9**.

For morbidity the evidence is more mixed: many studies show no effect. **Figure 10** shows this as regards cardiovascular and one hour peak O_3 concentration.

The pictures for eight and 24 hour average O_3 concentrations are hardly more persuasive (see **Figures 11** and **12** below).

The results of these meta-analyses are summarized in **Table 13**.

Now this is surprising. The association between O_3 and cardiovascular mortality is firm, but associations with indices of cardiovascular morbidity are not. This casts some doubt on the causal nature of the association with cardiovascular mortality: the association is not supported by coherent results regarding other end points. For respiratory effects the picture is clearer: associations with respiratory mortality and indices of respiratory morbidity are firmly established. O_3 has also been shown to be associated with symptoms in asthmatic children (Gent *et al.*, 2003; Mortimer *et al.*, 2002).

11.2 Long-term Exposure to Ozone

Cohort studies are less helpful as regards O_3 than other pollutants. Extension of the ACS cohort study, see above, showed an association between long-term O_3

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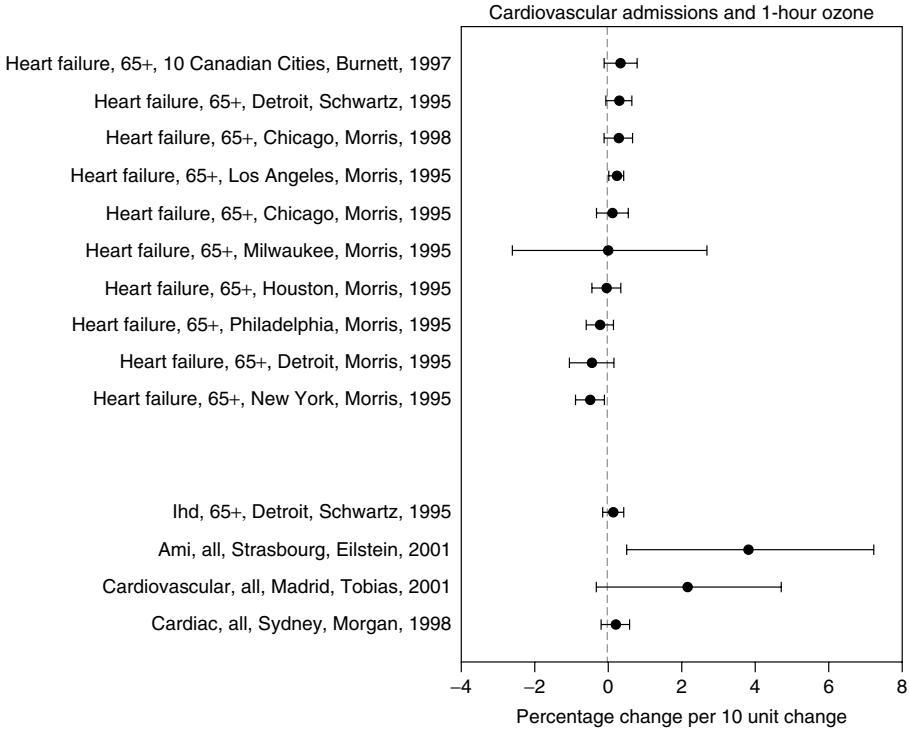

Figure 10 Cardiovascular admissions and one hour ozone. (Department of Health, 2006.)

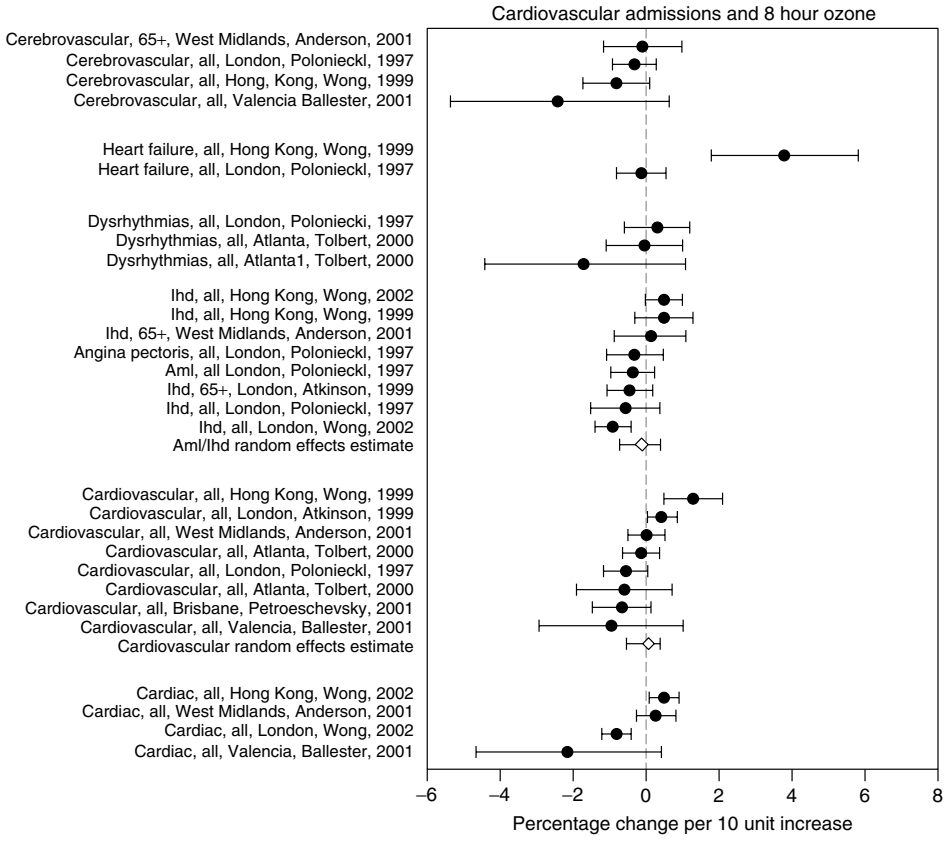

Figure 11 Cardiovascular admissions and eight hour ozone. (Department of Health, 2006.)

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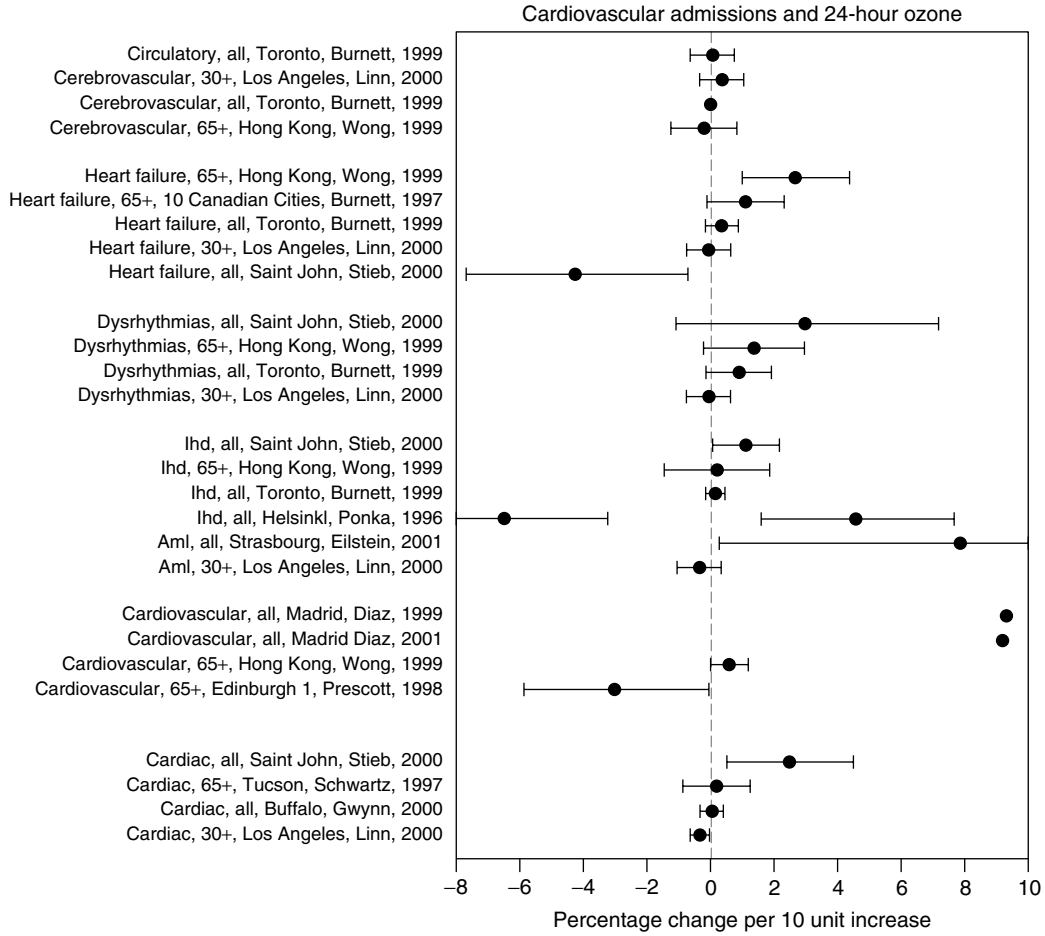

Figure 12 Cardiovascular admissions and 24 hour ozone. (Department of Health, 2006.)

Table 13 Summary table based on meta-analysis of time-series studies

Pollutant (24 h average)	N	Outcome measure	Assessment	Random effects (95% CI) (% change per 10 μg m^{-3})
O_3 8 h average	26	CV mortality	+	0.4 (0.3, 0.5)
O_3 8 h average	8	CV admissions		0.1 (−0.5, 0.4)
O_3 8 h average	6	IHD admissions		−0.1 (−0.7, 0.4)

CV, cardiovascular mortality; IHD, ischaemic heart disease.

concentrations and risk of death from cardiopulmonary disease (Pope *et al*., 2002). But a study of those people in the ACS cohort living in southern California failed to find this effect (Jerrett *et al*., 2005). The AHSMOG cohort study found an association with lung cancer in men (not in women) (Abbey *et al*., 1999). No association with lung cancer has been reported from the ACS cohort study.

Growing up in high O_3 areas seems to retard and reduce development of normal levels of lung function. One study reflects the effect: Galizia and Kinney (1999) studied first year students at Yale and stratified them according to O_3 levels in the areas in which they had grown up. FEF_{25-75} (forced midexpiratory flow) was 8.11% (range: 2.32–13.9%) higher in those growing up in the lowest as compared with the highest O_3 areas. The difference between lowest long-term (mean of daily one hour maximum) concentration was about 300 μg m^{-3} (150 ppb).

11.3 Toxicological Studies in Experimental Animals and Man

O_3 is a reactive oxidant gas, but is rather insoluble in water. Thus it penetrates deep into the lung and peak

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Table 14 Ozone air quality guideline and interim target

	Daily maximum 8 h mean	Effects at the selected ozone level
High level	240 μg m^{-3}	Significant health effects; substantial proportion of vulnerable population affected
WHO interim target 1 (IT-1)	160 μg m^{-3}	Important health effects; an intermediate target for populations with ozone concentrations above this level. Does not provide adequate protection of public health ***Rationale*** Lower level of 6.6 h chamber exposures of healthy exercising young adults, which show physiological and inflammatory lung effects. Ambient level at various summer camp studies showing effects on health of children. Estimated 3–5% increase in daily mortality[a] (based on findings of daily time series studies)
WHO air quality guideline	100 μg m^{-3}	This concentration will provide adequate protection of public health, though some health effects may occur below this level. ***Rationale*** Estimated 1–2% increase in daily mortality[a] (based on findings of daily time series studies). Extrapolation from chamber and field studies based on the likelihood that real-life exposure tends to be repetitive and chamber studies do not study highly sensitive or clinically compromised people or children. Likelihood that ambient ozone is a marker for related oxidants.

[a]Deaths attributable to ozone concentrations above an estimated baseline of 70 μg m^{-3} (based on 0.3–0.5% increase in daily mortality for 10 μg m^{-3} 8 h ozone.
Reproduced from the World Health Organization, 2006.

tissue doses (μg μm^{-2} surface) occur at the terminal bronchiole (Miller *et al*., 1993). The oxidative nature of O_3 leads to consumption of antioxidants, including glutathione, ascorbic and uric acids. O_3 also affects pain receptors in the airways and deep inspiration is impaired. Exercise increases the tissue dose of O_3 and thus lowers the concentrations needed to produce specified effects. The database on the toxicology of O_3 is vast (see above) and only the solidly established relationships and mechanisms are listed below. This is derived from the (World Health Organization, 2006) report. For detailed information the reader is referred to Lippmann (2000) and World Health Organization (2006) (Appendix listing studies on O_3) and, for older work, to the report published by the Advisory Group on the MAAPE in 1991 (Department of Health, 1991).

Established effects on lung function and inflammation are:

1. Short-term exposure impairs lung function
2. Chamber studies show that exposure to 80 ppb (160 μg m^{-3}) for 6.6 hours with exercise, causes a reduction in indices of lung function such as FEV_1 and thus reflects an increase in airway resistance
3. Asthmatics are more sensitive to O_3 than are other subjects
4. Inflammatory responses (increase in neutrophils, up-regulation of inflammatory cytokines and adhesion molecules accompany the changes in indices of lung function and may persist for some days following a single exposure
5. Exposure to O_3 increases airway responsiveness to other challenges
6. Studies outdoors show effects at lower levels of O_3 than those used in chamber studies. This may be due to coexposure to other pollutants.

11.4 Conclusions

Exposure to O_3 at ambient concentrations has significant effects on health. These reflect both short-term and long-term exposure. The evidence has led WHO to recommend the following targets and guidelines as shown in **Table 14**.)

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Indoor Air Quality

Rikke B. Jørgensen, Sten O. Hanssen, Jan V. Bakke and Ellen K. Jensen

CONTENTS

1 INTRODUCTION

Human physiology is not adapted to the climatic conditions found in temperate and polar latitudes, although parts of these regions have been populated for several 1000 years. The ideal ambient temperature for a naked person at rest is about 29 °C, a climatic condition found in the mountain and savannah regions of Africa, the probable origin of our ancient ancestors (Åstrand and Rodahl, 1986). Without clothing and shelter, man could be regarded as a tropical animal that can only survive in a narrow zone along the equator. When our forefathers migrated north, not only was proper clothing needed, but a protective climatic shield also had to be developed: housing and building technology were adapted to very challenging winter climates. Originally, we were also biologically adapted to continuous supplies of fresh outdoor air for breathing and keeping our bodies cool. The term 'indoor environment' is a consequence of the need for shelter against wild animals and, as mankind moved to more weather-beaten areas, against unfavourable outdoor climates. Indoor air quality, however, depends on a number of factors, including the outdoor air quality, the amount of fresh air provided indoors and the amount of air pollution derived from numerous indoor sources. On average, an adult male with a sedentary occupation will breathe about 15 m^3, or roughly 15 kg of air, drink 1.5 l or 1.5 kg of water, and eat about 0.75 kg of solid food per day. Hence, the weight of breathed air constitutes about 87% of the total mass of human exposure every 24 hours.

Clothing and a building envelope provide two vital levels of shelter between the human organism and its surroundings. Indoor environments are not only vital for our survival, health and wellbeing, but also constitute the greater part of human environmental exposure. The world's first Public Health Act was the British Act in 1848 (UK, 1848), which was a direct result of the *Report on the Sanitary Conditions of the Labouring Population and on its Means of Improvement* (Chadwick, 1842). Basic requirements for healthy built environments had been well established thanks to the health and hygiene movements during the 100 years before 1850. Their implementation over the next 100 years provided the conditions needed for the remarkable improvements

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in general health and living standards seen in modern welfare states. These are:

1. Dry building land and dry dwellings
2. Good cleaning and adequate ventilation
3. As much access as possible to sunlight and full daylight (bactericidal effect)
4. Smallest possible risk of accumulation of waste, dust and other pollutants by suitable choice of materials and design of interiors, furniture and furnishings
5. Fast and safe removal of all refuse and garbage by skillfully executed and maintained drains and sewers, rational cleaning methods
6. Abundant access to clean and pure water.

2 HUMAN HEALTH

2.1 Current General Population Exposure Time

People often spend less than 10% of their time outdoors. More than 90% is spent indoors, 65% at home, most of this usually in bedrooms, 20% in other premises outside homes and a further 5% in vehicles (WHO, 1999). The working population typically spends about 20% of its time at work, and an increasing proportion of employees have their work places in nonindustrial environments.

2.2 Vulnerable Groups

In a statement from the World Allergy Organization on World Allergy Day, 8 July, 2005, it was emphasized that allergic diseases affect 22% of the world's population. People with asthma, allergies and other hypersensitivities are particularly vulnerable to inferior indoor environments (Leira *et al*., 2006). Compared to those who are nonallergic, a higher percentage of allergic people suffer from sick building syndrome (SBS) symptoms and complain about perceived annoyances in the indoor environment (Lundin, 1999). Allergic disease is the only group of diseases currently increasing among children in Western and developed societies. Asthma is, at present, the most common chronic disease during childhood, and in most Western countries the commonest cause of admission to hospital among children, representing up to 25% of admissions to paediatric departments in many countries (EEA, 2007). The increase of asthma and allergies in the younger population in Europe will increase the proportion of vulnerable individuals in the future work force. A 2.4-fold increased risk of suffering from asthma among adults born in 1966–1971, compared to those born in 1946–1950, was reported in studies performed in 15 industrialized countries (Sunyer *et al*., 1999). The increase occurred concurrently in most of the countries, in both males and females, and affected the onset of asthma in both childhood and adulthood. Prevalence of chronic obstructive lung diseases (bronchitis, emphysema and asthma) is increasing all over the world (WHO, 2007a), and those affected suffer more in poor indoor conditions. Good environments are particularly important for the health of such people and in preventing early retirement due to disability.

2.3 Health Effects Associated with Indoor Exposures

In recent years, there has been an accumulation of knowledge concerning the effects on health cased by exposure to agents present in indoor air, known as indoor air pollutants (WHO, 1982; 1999). Nonindustrial indoor environments are usually characterized by exposure to a complex mixture of a large number of different agents at very low exposures, mostly far below any known threshold level of biological effect. When assessing risk, it is also important to bear in mind that one effect may be caused by many different exposures, and that one exposure might cause many different effects.

2.3.1 Building-Related Illness (BRI)

Building-related illness (BRI) refers to building-related exposure to indoor contaminants. Infectious and irritative respiratory diseases, respiratory allergies (for example, to house dust mites, animal fur and dander), asthma and mucous membrane irritation are the most prevalent illnesses that have been associated with indoor exposures.

2.3.2 Respiratory Allergies and Hypersensitivity Conditions

Allergic and nonallergic asthma, rhinitis and conjunctivitis can be caused or aggravated by exposures in indoor environments. The conjunctiva of the eyes reacts in the same way as the airways, in terms of the environmental sensitivity and hypersensitivity of the conjunctiva, since symptoms can occur simultaneously in the airways and in the eyes, and can be caused by the same agents.

Respiratory effects of pollutants can, to a certain extent, be predicted by considering where the air pollutants are most likely to be deposited in the airways. Particles $<5\,\mu m$, such as allergens from house dust mites and pets, can be carried down to the peripheral part of the lung, inducing or eliciting asthma. Nanoparticles, typically formed in combustion processes, are efficiently deposited by diffusional mechanisms to all regions of the respiratory tract (Donaldson *et al*., 1998;

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Johnston *et al*., 2000; Oberdörster *et al*., 2005). Coarser particles >10 μm, such as pollen, tend to affect the eyes and upper airways, eliciting allergic rhinoconjunctivitis. Hydrophilic (water soluble) gases, such as sulphur dioxide (SO_2), ammonia (NH_3) and formaldehyde, tend to deposit in and primarily affect upper airways, not reaching the lungs, but producing powerful sensory irritation. Hydrophobic gases, such as nitrogen dioxide (NO_2), can strongly affect the lungs, while only conveying minor warnings with sensory effects to the eyes and upper airways.

2.3.3 Respiratory Infections

Modern epidemiological data on the association between dampness in buildings, indoor moulds and airway infections is limited, although the association was already well-known in the 18th and 19th century. Increased proneness to general respiratory infection is associated with poor indoor climate, particularly in association with dampness in buildings, environmental tobacco smoke (ETS) and exposure to combustion gases, but also to low ventilation rates in combination with crowding, such as in kindergartens, schools and barracks.

Legionellosis is an infection caused by the Gram-negative bacteria *Legionella*, usually by *Legionella pneumophila* (WHO, 2007b). This is a ubiquitous aquatic organism that thrives in warm environments (25–45 °C with an optimum around 37 °C) and causes over 90% of Legionnaires' disease cases. Pontiac fever is a milder respiratory illness without pneumonia caused by the same bacterium. Legionellosis usually occurs as single, isolated cases not associated with any recognized outbreak. When outbreaks do occur, they are usually recognized in the summer and early autumn, but cases may occur at any time of the year.

2.3.4 Other Respiratory Illnesses

Humidifier fever usually develops on a Monday or the first workday of the week, and has mostly been associated with humidifiers in printing offices, but has also been observed in office buildings. The hallmark of the disease is the sudden onset of fever. Other features may include muscle aches and pains and mild shortness of breath. Humidifier fever can be caused by a number of different agents including amoebae, bacteria and fungi living in the moist environment of a humidifier. Humidifier fever typically disappears once the patient is no longer exposed to the causative agent coming from the humidifier.

2.3.5 Cancer

Asbestos and benzene are known carcinogens, but exposure is now practically nonexistent. An increased risk of developing lung cancer has been linked to exposure to ETS, and to radon decay products (WHO, 2000). In areas with high radon exposure, up to 10–15% of all lung cancers occurring in the population may be attributable to indoor radon exposure (WHO, 2000). With regard to ETS, it has been estimated that nonsmokers living with smokers have about 30% increased risk of contracting lung cancer when compared to the nonexposed population. ETS has recently been almost eliminated in the working environment of most Western countries.

2.3.6 Other Illnesses

The effects of indoor air pollutants on reproduction, cardiovascular disease, as well as other systems and organs have not been well documented to date. To a certain extent, this may indicate that no serious effects occur. However, there has been very limited research to document the absence of such effects. A current issue is the use of plastic additives in the indoor environment, including flame retardants and plasticizers to which exposure has increased dramatically since World War II (Bornehag *et al*., 2004a). Animal studies have shown that some phthalates affect male reproductive development. Nanoparticle studies have shown that nanoparticles can cross cell barriers end enter into blood and lymph circulation to reach potentially sensitive target sites such as bone marrow, lymph nodes, the spleen and the heart (Oberdörster *et al*., 2005). Health effects associated with such distribution of nanoparticles are mostly unknown. Recent publications associate exposure to nanoparticles with cardiac disease (Weichenthal *et al*., 2007; Chuang *et al*., 2007), and there is an increasing number of other health-effect studies ongoing (see **Nanotoxicology-The Toxicology of Nanomaterials**).

2.3.7 Sensory Irritation, Sick Building Syndrome (SBS)

SBS consists of a group of general, mucosal and skin symptoms that are related to the time spent in particular buildings (WHO, 1982; Burge, 2004). Building occupants complain of symptoms such as irritation to eye, nose or throat, dry cough, dry or itchy skin; headache and concentration difficulties; dizziness and nausea; fatigue, as well as increased sensitivity to odours. Various factors in the indoor air environment, including dampness, temperature, thermal conditions and particle pollutants, have been suggested as causes of these symptoms. Other factors that may significantly affect the indoor environment include ventilation rates, ETS, combustion products, formaldehyde and volatile organic compounds (VOCs).

SBS is strongly related to mental stress at work (Runeson *et al*., 2004; Marmot *et al*., 2006). Mental stress has even been shown to be more important than the physical environment in explaining prevalence of SBS (Marmot *et al*., 2006). Psychosocial and personal reasons also dominated in mucus-membrane irritation symptoms and general symptoms among teachers in state schools

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when comparing 'moisture-damaged' and 'nondamaged' schools (Ebbehøj *et al*., 2005). Negative psychosocial work factors have been associated with the risk of contracting various illnesses, especially psychosomatic disorders. High demands at work together with low social job control and low job support are combinations of mental factors that may cause various negative effects on health (Theorell and Karasek, 1996).

2.3.8 Multiple Chemical Sensitivity (MCS)

Multiple chemical sensitivity (MCS) is described as a condition in which a person reports sensitivity or intolerance (as distinct from 'allergy') to a number of chemicals and other irritants at very low concentrations. There are different views among medical professionals about the existence, causes, diagnosis and treatment of this condition (EPA (Environmental Protection Agency), 2008). MCS has also been called toxic injury, chemical sensitivity, chemical injury, idiopathic environmental intolerance and toxicant-induced loss of tolerance. The cause and existence of MCS are disputed, and in particular, medical doctors disagree about whether symptoms are physiologically or psychologically generated, or both (Wikipedia, 2008). However, in view of the fact that numerous people all over the world claim that they suffer from a chronic condition characterized by several adverse and variable affects from exposure to very low levels of substances in modern human environments, it is not wise totally to disregard the existence of MCS. MCS is further discussed in **Multiple Chemical Sensitivity: Toxicological Questions and Mechanisms**.

2.4 What are the Most Important Effects Associated with Indoor Environments?

The most important effects on public health are probably allergic respiratory sensitization, aggravation of allergic diseases, increased susceptability to respiratory infection and worsening of chronic obstructive lung diseases. Respiratory diseases, asthma and allergy have mainly been associated with the indoor environment in residential buildings, while a limited number of reports are related to exposure in nonindustrial occupational environments. Reported respiratory illnesses seem mainly to be associated with exposure in damp buildings (Savilahti *et al*., 2000; Rudblad *et al*., 2004; Patovirta *et al*., 2004). Heating and cooking with gas and charcoal, ETS, and cleaning and washing products have also been associated with asthma (Viegi *et al*., 2004). Most effects of indoor environments are results of complicated interactions between several exposures, and combinations of these are difficult to study. Indications of dampness, as well as recently repainted interior surfaces, appeared to be associated with recurrent infant wheezing, with a strengthened effect due to combined indoor exposures (Emenius *et al*., 2004). Family lifestyles, according to preventive guidelines regarding breastfeeding, maternal tobacco smokers and home dampness, were synergistically associated with reductions of recurrent wheezing and asthma at two years of age (Wickman *et al*., 2003).

The most prevalent adverse effects of inferior indoor work environments are thus respiratory infectious diseases, mucosal, respiratory and skin irritation, including aggravation of allergies and other hypersensitivity symptoms, general symptoms, including headache and fatigue, as well as reduced comfort, performance and productivity (Wargocki *et al*., 2000; Wyon, 2004). Interactions and synergies among several exposures are probably important.

3 POLLUTANTS OF IMPORTANCE FOR INDOOR AIR QUALITY

The pollutants in indoor air belong to two different groups, particles and gases. These groups have different characteristics and behaviour, and partly different sources of origin. In the following the two groups of pollutants are described separately. The toxic effects of VOCs (gases and solvents) is further discussed in chapters on organic solvents (**Organic Solvents**) and the nervous system (**Neurotoxicology**).

3.1 Gases

Gases are further divided in two groups, namely the organic gases and the inorganic gases. Gas concentrations can be expressed in mass per volume of air ($\mu g\,m^{-3}$) or parts per volume, ppm (parts per million). The most typical inorganic gases in the indoor air are carbon monoxide (CO), nitrogen dioxide (NO_2), carbon dioxide (CO_2) and ozone (O_3) as shown in **Table 1**. Important organic gases are VOCs, including formaldehyde, phthalates and flame retardants as shown in **Table 2**.

3.1.1 The Combustion Gases, Carbon Monoxide and Nitrogen Dioxide

CO is a colourless, odourless and tasteless gas. $1\,ppm = 1.145\,mg\,m^{-3}$, $1\,mg\,m^{-3} = 0.875\,ppm$. CO is produced from the incomplete combustion of carbon-containing fuel. The amount of CO in the indoor air originates mainly from infiltration of CO from the outdoor air and indoor sources of CO like tobacco smoking, cooking and combustion of fossil fuels. CO is only formed during incomplete combustion, which often take place when the appliances are not well maintained, or when there is not enough fresh air supply (Lebret, 1985).

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Table 1 Sources for inorganic gases in indoor air

Chemical	Source	I/O
CO	Incomplete combustion (automobiles, cigarette smoke, heaters)	>1
CO_2	Exhaled air, combustion (cars)	≫1
NO_2	Combustion (automobiles, gas heaters, cigarette smoke)	<1
O_3	Photocopiers, laser printers	<1

I/O is the indoor to outdoor concentration ratio.

Table 2 Sources for organic compounds in indoor air

Chemical	Source	I/O
VOC	Building materials, furniture, paint, surface treatment, microbiologic sources, household and consumer products	>1
Formaldehyde	Chipboard, woodproducts, insulation foam, furniture, paint, cleaning products, cosmetics, clothing, carbonless copy paper, and tobacco smoke	>1
Phthalates	Plasticized products	>1
Flameretardants	Plastics, rubbers and household textiles, computers, TVs and other electronic equipment	>1

I/O is the indoor to outdoor concentration ratio.

NO_2 is a reddish-brown gas with a characteristic sharp, biting odour. $1\,\text{ppm} = 1.88\,\text{mg}\,\text{m}^{-3}$, $1\,\text{mg}\,\text{m}^{-3} = 0.532\,\text{ppm}$. NO_2 is one of several nitrogen oxides. The two most important nitrogen oxides are NO_2 and nitrogen monoxide (NO). These oxides are produced during combustion, especially combustion at high temperatures.

Indoor sources may be gas stoves, cigarette smoke and cooking. Elevated indoor levels of NO_2 have been associated with the use of domestic gas appliances (Dennekamp *et al*., 2001; Melia *et al*., 1990). Willers *et al*. (2006) concluded from comparison with older data that in most new homes, removal of NO_2 formed during gas cooking is more efficient than it was in home studies in the early and mid 1980s.

CO and NO_2 are, together with particles (mentioned in Section 3.3), the most important pollutants released from combustion processes. Increased risk of respiratory diseases associated with improperly vented, poorly ventilated or malfunctioning combustion appliances, occurs widely in developing countries (WHO, 1999; 2002; Viegi *et al*., 2004; Naeher *et al*., 2007). Such appliances even pose a real risk of acute poisoning by CO. Combustion products and pollution from heating systems and cooking using coal, wood, kerosene and gas have also been associated with respiratory health effects in developed countries. Pupils with asthma improved considerably when unflued gas heating was replaced by either flued gas or electric heating in a randomized controlled study (Pilotto *et al*., 2004). Use of gas stoves and wood-burning stoves/fireplaces was associated with aggravation of the disease among adult asthmatics (Ostro *et al*., 1994). Unvented gas space heaters, wood stoves and kerosene heaters were associated with respiratory symptoms among infants and women living in nonsmoking households (Thorn *et al*., 2001; Belanger *et al*., 2003; Naeher *et al*., 2007). Freestanding wood-burning stoves were associated with otitis (inflammation in the ear) among children (Daigler *et al*., 1991). The lowest risk of eczema was found in households with central heating systems and the highest where gas heaters were used (Schäfer *et al*., 1999). All indoor combustion sources must be properly vented to avoid indoor pollution from combustion gases.

Road tunnels, as well as underground and multistorey car parks are special types of indoor environments with a risk of high concentrations of CO. The ice arena is another example of a very special type of indoor environment where combustion engines are used under conditions of insufficient ventilation, with a risk of high CO levels (Hampson, 1996; Paulozzi *et al*., 1993).

Without indoor sources, the level of CO in buildings with mechanical ventilation systems depends of the location of the air intake of the ventilation system. If the air intake is placed directly against highly CO-polluted areas, for example parking places, public transport drop-off areas and locations where vehicles frequently stand at idling speed, there is increased risk of exhaust entering the air intake. Thus, this can lead to increased levels of CO in the indoor air. Another source is infiltration of CO from streets with high traffic intensity outside the building (Li *et al*., 2001).

3.1.2 Carbon dioxide

CO_2 is a colourless and odourless gas. $1\,\text{ppm} = 1.8\,\text{mg}\,\text{m}^{-3}$, $1\,\text{mg}\,\text{m}^{-3} = 0.56\,\text{ppm}$. CO_2 originates mainly indoors by exhalation air from people, but CO_2 is also produced during combustion, for example, wood-burning and smoking.

CO_2 does not affect the way people perceive the indoor air quality at the concentrations that normally occur in indoor air. The CO_2 concentration correlates, however, with emission of bioeffluents (e.g. body odour), and CO_2 measurements can be used in the indoor air context as an hygienic indicator of whether the air supply is sufficient for ventilation with regard to the number of occupants in the room.

3.1.3 Ozone

O_3 is a pale blue gas with a sharp, irritating odour. Conversion factor: $1\,\text{ppm} = 1.963\,\text{mg}\,\text{m}^{-3}$, $1\,\text{mg}\,\text{m}^{-3} = 0.509\,\text{ppm}$. O_3 in indoor air mainly originates from outdoor air.

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Several types of office equipment are known to generate O_3, including photocopiers and laser printers (Hansen and Andersen, 1986; Tuomi *et al*., 2000a). Although various photocopying processes produce O_3, most manufacturers incorporate some type of filtration system (e.g. activated carbon filters) to reduce the amount of O_3 emitted to the surrounding air. Electrostatic air filters and electrostatic precipitators can also generate O_3.

Several companies currently offer O_3 generators that claim to remove chemical pollutants from indoor air. Such devices are promoted as being safe for indoor use, which implies O_3 concentrations of 100 ppb or less (Weschler, 2000). In a review Boeniger (1995) examined the use of O_3-generating devices for the purpose of improving indoor air quality. It was concluded that O_3 is not a practicable and effective means of improving indoor air quality, especially in light of the potentially serious risk to health of O_3.

3.1.4 Volatile Organic Compounds (VOCs)

VOCs as a group have been defined as organic compounds with boiling points from about 50 °C to about 260 °C (WHO, 1989). This interval was chosen for reasons of sampling and analytical capabilities rather than from the viewpoint of health effects (Wolkoff *et al*., 1997). **Table 3** lists WHO's definitions of VOCs, more volatile (VVOCs) and less volatile volatile organic compounds (SVOCs), as well as organic matter.

A great number of VOCs occur in the indoor air. In typical indoor environments 50–300 different organic compounds can be detected with today's analytical methods. The actual compounds detected and the concentrations that can be found vary from place to place, and from time to time, dependent of the pollution sources present, and the ventilation efficiency at the actual location. **Table 4** lists VOCs in indoor air measured in European and North American field studies.

The focus on VOC in the context of indoor air quality is mainly caused by speculation about their contribution to SBS, and the general availability of methods for sampling and analysing them. The concentration of each single compound is below the level where health effects have been demonstrated, and far below the threshold limit values (TLVs). Individual VOCs have been added together to comprise an entity called total volatile organic compounds (TVOCs) in order to characterize the pollutant load in terms of VOCs. In the early 2000s a new approach called organic compounds in indoor air (OCIA) was proposed for evaluation of VOCs in the indoor air (Wolkoff and Nielsen, 2001). OCIA included organic compounds as well as organic compounds on particles, in addition to intermediary species (e.g. organic radicals) and ionic species. Compounds of microbiological origin (e.g. glucans, endotoxins) are not part of OCIA. The molecular weights of OCIA were tentatively less than 1000 Da. The idea was to use an approach that included compounds with biological relevance.

During recent years attention has moved towards identification and quantification of the individual VOCs in order to evaluate the toxicological characteristics of each compound, for example, odour thresholds, irritation thresholds, carcinogenic potential, reprotoxic or mutagenic characteristics. The importance of concepts like TVOC and OCIA has consequently decreased. Mølhave stated in 2003 that the TVOC indicator can be used in relation to exposure characterization and source characterization, but for VOCs only, not as an indicator of other pollutants and their health effects (Mølhave, 2003).

The concentration of VOCs is substantially higher indoors than outdoors. This means that the most important sources of VOCs are in the indoor environment. The sources can be divided into stationary sources, like emission from building materials, and variable sources associated with human activities.

3.1.5 Formaldehyde

Formaldehyde is a colourless gas with the formula H_2CO. $1\,\text{ppm} = 1.2\,\text{mg m}^{-3}$, $1\,\text{mg m}^{-3} = 0.833\,\text{ppm}$. Formaldehyde is widely used in indoor materials and products. Chipboard, insulation foam and paint have been major sources, but during the last decade, the emission from such products has been reduced substantially in many countries. Formaldehyde is

Table 3 Classification of organic indoor pollutants

Classification	Abbreviation	Boiling point range		Vapour pressure (kPa)
		from °C	to °C	
Very volatile organic compounds	VVOCs	<0	50–100	>10E-2
Volatile organic compounds	VOCs	50–100	240–260	>10E-2
Semi-volatile organic compounds	SVOCs	240–260	380–400	10E-2–10E-8
Organic compounds associated with particulate (organic matter)	POM	>360	—	—

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Table 4 Ubiquitous VOCs in indoor air measured in European and North American field studies in order of about 20 most abundant in descending order

Australian review[a]	European audit[b]	US review[c]	BASE study[d]	Swedish housing stock[e]	German study[f] Selected (new) VOCs
Benzene	Acetone	*o*-Xylene	Acetone	Toluene	Group 1
Tetrachloroethylene	Isoprene	Benzene	Hexane	Decane	Phenoxyethanol
p-Dichlorobenzene	2-Methylpentane	Tetrachloroethylene	Toluene	Dodecane	Butyldiglycol acetate
Ethylbenzene	Hexane	*m*,*p*,-Xylene	1,1,1,-Trichloroethane	Nonanal	Longifolene
m,*p*,-Xylene	2-Metylhexane/benzene	Ethylbenzene	Methyl chloride	Undecane	Dimethyl phtalate
1,1,1,-Trichloroethane	Heptane	Trichloroethylene	Benzene	Limonene	
o-Xylene	Toluene	Toluene	Ethanol	C_{11} alkane	Group 2
Decane	*m*,*p*,-Xylene	1,1,1,-Trichloroethane	2-Propanol	C_{12} alkane	α-Pinene
Toluene	*o*-Xylene	Dichlorobenzenes	Dichlorofluoromethane	Xylene	camphene
1,2,4-Trimetylbenzene	Decane	Styrene	*m*,*p*,-Xylene	C_{10} alkane	β-Pinene
Hexane	Trimetylbenzene	Undecane	2-Butanone	Trimethylbenzene	3-Carene
Nonane	Limonene	Dodecane	Trichlorofluoromethane	Butoxyethoxyethanol	
Limonene		Dodecane	*o*-Xylene	Butoxypropanol	Group 3
		Octane	undecane	C_7 alkane	Styrene
			Tetrachloroethylene		*o*-Xylene
			Methylene chloride		c12-alkanes
			1,2,4-Trimethylbenzene		
			Decane		Group 4
					1,2,3-Trimethylbenzene
					1,2,4-Trimethylbenzene
					Methylcyclohexane

[a]Brown, (1999).
[b]Bernhard *et al*. (1995).
[c]Holcomb and Seabrook (1995).
[d]Girman *et al*. (1999).
[e]Bornehag and Stridh (2000).
[f]Reitzig *et al*. (1998).
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also found in urea–and phenol–formaldehyde resins, which are commonly used to treat paper products to increase wet strength. Formaldehyde is also used in permanent-press clothing, carpet backings, floor coverings and adhesive binders. Other sources for formaldehyde include furniture, cosmetics, cleaning products, carbonless copy paper and tobacco smoke (Marbury and Krieger, 1991).

In the past, a high formaldehyde concentration was often considered to be the cause of increased SBS symptom prevalence. The use of proper building products (ECA, 1989) and emission control strategies have resulted in lower formaldehyde concentrations in indoor air in most of the developed countries.

3.1.6 Phthalates

Phthalates are less volatile VOCs, so-called SVOCs (WHO, 1989). Phthalates are common contaminants in the indoor environment occurring both in house dust and in indoor air. Di(2-ethylhexyl)phthalate (DEHP) is the dominant component (Fromme *et al*., 2004; Øie *et al*., 1997; Rudel *et al*., 2003).

In a Norwegian study it was shown that the risk of bronchial obstructions among children was related to the presence of polyvinyl chloride (PVC) flooring in homes (Jakkola *et al*., 1999). The plasticizer DEHP is widely used in the production of PVC and vinyl chloride resins, and accumulates to a great extent in interior surfaces of buildings. This led Øie *et al*. (1997) to propose that phthalates may cause lung inflammation through their chemical similarity to prostaglandins, naturally occurring inflammatory agents. Lagercrantz *et al*. (2005) has further shown that controlled inhalation exposures to low levels of phthalates increased levels of exhaled nitric oxide, indicating lower airway inflammation, and Bornehag *et al*. (2004a) found associations between asthma and allergic symptoms in children and phthalates in house dust. Knowledge about the exposure to phthalates and consequent health effects is still insufficient. Seeing that the use of plasticized products and consequently exposure to phthalate esters has increased dramatically since the end of World War II, and that phthalates have been demonstrated in indoor dust, more knowledge about the health risks of this exposure is required.

3.1.7 Flame Retardants

Flame retardants are widely used in plastics, rubbers and textiles to prevent or retard the initial phase of a developing fire. Organic flame retardants consist primarily of brominated compounds, halogenated and nonhalogenated phosphorous compounds, and chloroparaffins. Aluminium trihydroxide, magnesium dihydroxide and antimony trioxide (as synergistic to brominated flame retardants) are applied as inorganic flame retardants. Apart from the positive characteristic of being fire protective, several flame retardants also have negative effects on health and the environment. In 2003, the flame retardants pentabromodiphenylether (penta BDE) and octabromediphenylether (octaBDE) were banned by European Directive 2003/11/EC (European Parliament and Council, 2003).

This has lead to focus on measurements of flame retardants in products, and in the indoor environment. Brominated flame retardants, like penta and tetrabrominated diphenyl ethers, represent a new type of contaminant in indoor air (Rudel *et al*., 2003). Experimental studies have demonstrated the potential for products to emit flame retardants and degradation products (Carlsson *et al*., 1997; 2000; Salthammer *et al*., 2003). Many of these flame retardants and plasticizers have been found in indoor air (Carlsson *et al*., 1997; Rudel *et al*., 2003) and dust (Marklund *et al*., 2003; Rudel *et al*., 2003).

Knowledge about the exposure to flame retardants in indoor air and consequently knowledge of health effects is still insufficient (McDonald, 2002; Siddiqi *et al*., 2003). Seeing that the use of flame retardants has increased dramatically, and that flame retardants are known to have adverse effects on health and the environment, this may be a new area where further work is needed.

3.2 Building Materials and Surface Treatment as Indoor Sources

Building material and furniture are important sources of contamination of the indoor environment. The material, as such, can be a source for emission. However, the surface treatment of the material, as well as wear and tear, maintenance and cleaning procedures, may also contribute to emission of contaminants. The influence of material surfaces on indoor air quality can be divided into three groups: primary emission from materials, the impact of the materials on microbial flora and the ability of the materials to adsorb and desorb organic vapours on material surfaces.

Primary emission from materials can be grouped in two categories of emission sources, based on the emission decay profiles. Paints, adhesive, floor and furniture waxes, and spray products are examples of sources with a fast reduction in the emission rate. These products are used with solvents that are intended to evaporate. Evaporation and consequent drying may take anything from minutes to hours or days. However, such products may continue to release VOCs for extended periods (months or years). The second category is the sources which will emit chemicals for extended periods, for example, carpets, ceiling tiles, chipboard and other solid 'dry' materials. They may have initial emissions during the first few hours, but typically the emission rate remains high for months and even years after installation.

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Emission studies of building materials have formerly focussed on VOCs (Kelly *et al*., 1999; Wolkoff, 1995). Development of low-emitting materials has been of interest as consumers' awareness and demands have increased. Today, there are several labels for tested, low-emitting materials. Some examples are Greenguard (Greenguard, 2008), Blue Angel, the Danish Indoor Climate Labelling Scheme and The Finnish Climate Classifications (Wolkoff, 2003). Newer studies, however, focus on phthalates (Bornehag *et al*., 2005; Kolarik *et al*., 2008) and flame retardants (Carlsson *et al*., 1997; Salthammer *et al*., 2003).

Cleaning must be considered as an additional contamination source, although the intention is the opposite (Nazaroff and Weschler, 2004). Some building materials appear to increase their emission of VOCs through wet cleaning and may thus affect the indoor air quality (Wolkoff *et al*., 1998). Cleaning agents contain evaporative and nonevaporative substances, where the former have VOCs as major constituents of toxicological significance. The use of cleaning agents results in a temporal increase of the overall VOC level. The exposure levels for occupants will depend of the amount of residue, the rate of off-gassing and particle release from surfaces.

Finally, gases and particles in indoor air are able to adhere to surfaces in the indoor environment. Adsorbed organic vapours on interior surfaces can later be re-emitted, and thus lead to elevated concentrations of organic compounds in indoor environments (Jørgensen *et al*., 1999; Tichenor *et al*., 1991).

3.3 Particles

The presence of particles in the indoor air depends on particles entering from the outdoor air, the filtration efficiency of the ventilation system and particles generated within the indoor environment. Particles in the indoor air may be of a biological or nonbiological nature. This subsection will describe nonbiological particles, while biological particles are discussed in Section 3.4

3.3.1 Particle Characterization

The properties and behaviour of particles are important in order to understand their possible influence on human health. In order to characterize the particle, the following characteristics are of interest:

- Size
- Shape
- Mass
- Number
- Surface area
- Chemical composition.

3.3.1.1 Size

The size of the particle decides its fate, whether it will remain airborne or settle on surfaces. Most particles are nonspherical. They can have geometrical shapes such as cubic or cylindrical, and some have irregular shapes. The shape of the particle affects its drag force and settling velocity (Hinds, 1982). In order to describe the particle motion mathematically, the irregular particle is converted to its equivalent sphere. The aerodynamic diameter is the diameter of the unit density ($\rho = 1\ \mathrm{g\,cm^{-3}}$) sphere that has the same settling velocity as the particle. Aerodynamic diameter is the key particle property for characterizing respiratory deposition.

Particles with aerodynamic diameters less than 10 µm are of special interest since they are inhalable. Particles with aerodynamic diameter less than 5 µm are respirable (ISO (1995) use median diameter $4.25\,\mu\mathrm{m} \pm 1.5\,\mu\mathrm{m}$; in publications, both 4.5 and 5.0 µm are frequently used for respirable fraction). These particles are able to penetrate more deeply into the respiratory tree, indeed to the alveoli. In their work, Owen *et al*. (1992) have given an overview of which region of the respiratory tract particles are deposited, as a result of the particles' aerodynamic diameter. Respirable particles can be further classified. Fine particles have aerodynamic diameters less than 2.5 µm, and ultrafine particles have aerodynamic diameters less than 0.1 µm. Nanoparticles have the same size classification as ultrafine particles, at least one dimension of 100 nm or less. Some differentiate between the terms, defining nanoparticles as being engineered, but generally ultrafine particles and nanoparticles describe the same thing (Oberdörster *et al*., 2005; Donaldson *et al*., 2006). Recent publications within toxicology use the term nanoparticles.

Most of the deposited insoluble particles leave the lungs via the bronchiotracheal route by mucociliary action as free particles, or within macrophages (Im Hof and Patrick, 1994). Nanoparticles are efficiently deposited by diffusional mechanisms to all regions of the respiratory tract, and they have a greater potential to cause adverse health effects than larger particles of similar composition (Donaldson *et al*., 1998; Johnston *et al*., 2000; Oberdörster, 2001). The small size facilitates uptake into cells (Geiser *et al*., 2005) and transcytosis across epithelial and endothelial cells into blood and lymph circulation to reach potentially sensitive target sites such as bone marrow, lymph nodes, the spleen and the heart (Oberdörster *et al*., 2005).

3.3.1.2 Shape

The shape of the particle is important regarding deposition in the respiratory system and the function of the cells' defence system. A specific shape of interest is the fibre. WHO defines a fibre as a particle longer than 5 µm with a width less than 3 µm, and with a length : width ratio greater than 3 : 1 (WHO, 1997). Although fibres can be long, their shape and small width enable them

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to avoid settlement in the upper part of the respiratory system and to penetrate to the alveoli. Their ability to cause lung disease can be understood on the basis of the length of the fibres and their biopersistence in the lungs. Fibres are cleared by the mucociliary system, by being engulfed by macrophages and by dissolution (Rudd, 2004). These defence mechanisms are insufficient for long and biopersistent fibres, so that fibrosis and cancer may result.

Typical examples of fibres are asbestos and synthetic vitreous fibres (SVFs). SVFs have historically been called man-made mineral fibres (MMMFs), and are a class of inorganic fibrous materials that include glass wool, mineral wool, textile glass fibres and refractory ceramic fibres. Health effects of asbestos are well known, namely a specific fibrosis named asbestosis and cancer in lungs and airways, or in pleura and peritoneum (malignant mesothelioma). SVFs can irritate skin and mucous membrane, and some mineral wool fibres are classified as carcinogenic (Donaldson and Tran, 2004).

The presence of fibres in the indoor air originates from building materials. Lee and Van Orden (2008) have studied airborne asbestos in buildings nationwide in the USA. Their results indicated that as long as asbestos-containing materials were in place, it did not result in elevated airborne asbestos in building atmospheres approaching regulatory levels, and that it did not result in a significantly increased risk to building occupants.

3.3.1.3 Mass

Mass has historically been the main characterization aspect for particles besides size. In TLVs and guidelines, particulate matter for different size fractions has been the key description factor. PM_{10} is defined as the mass of the particles that are collected on a filter after passing a suction device with an interception efficiency of 50% at an aerodynamic diameter of 10 µm (EN, 1999). $PM_{4.5}$ and $PM_{2.5}$ are the similar fractions at aerodynamic diameters of 4.5 µm and 2.5 µm, respectively. PM_{10} has been widely use for particles in outdoor air, $PM_{4.5}$ is a fraction applied in the working environment, while $PM_{2.5}$ is applied for outdoor and indoor particles.

Several studies report good conformity between PM_{10} and respiratory effects and cardiovascular diseases (Harrison *et al*., 1999; Donaldson *et al*., 2001). The relationship between adverse health and particle mass may be the result of particle mass being a substitute for another parameter like particle number or surface area (Harrison *et al*., 1999).

3.3.1.4 Number

In terms of number, the vast majority of airborne particles are in the nanoparticle range (Morawska, 2000). The total mass of these particles is, however, often insignificant compared with the mass of a small number of large particles (Morawska, 2000). Several studies have reported that some health effects are better described by concentration given as particle number rather than by particle mass (Oberdörster *et al*., 1995; Peters *et al*., 1997; Granum *et al*., 2000; Lighty *et al*., 2000). Particle number (or surface area) correlates better than mass fractions with health effects such as inflammatory response (Oberdörster, 2001; Donaldson *et al*., 2002). Although the relevance of particle number has been well described, TLVs and standards for particles only refer to mass.

3.3.1.5 Surface Area

The surface area of the particle is essential for adsorption of chemicals, as well as for determining the potential exchange area between a particle and a body cell. Surface area together with particle number are referred to as key particle characteristics when studying adverse health effects (Tuch *et al*., 1997; Granum *et al*., 2000; Lighty *et al*., 2000). An explanation could be that the large surface area provided by nanoparticles in contact with the lung provides the opportunity for surface chemistry to have a profound effect (Donaldson *et al*., 1998). The large surface area seems to lead to oxidative stress and intracellular calcium changes that could be important in priming and activating cells for an inflammatory response (Donaldson *et al*., 2001).

3.3.1.6 Chemical Composition

Particles can cause physical irritation of the airways due to their physical characteristics (size, morphology) alone. Their chemical composition may cause additional effects due to chemical, toxic or allergic reactions. The particle can have different chemical characteristics:

- Inert particles
- Particles carrying chemical contaminants
- Toxic particles
- Allergens.

Indoor air particles will typically be inert or carry chemical contaminants present in the indoor environment. Toxic particles are more likely found in the working atmosphere. Allergens are described in Section 3.4 on biological particles.

The chemical contaminants adsorbed onto an indoor particle will depend on the chemical sources in the specific indoor environment. VOCs are frequently found adsorbed onto indoor particles (Wolkoff and Wilkins, 1994; Mølhave *et al*., 2000). Use of pesticides inside the house will result in the presence of such contaminants in indoor dust as well (Roinestad *et al*., 1993). Emissions from paint and floor coverings can also be adsorbed onto indoor particles, and thus contribute to their chemical complexity.

When a particle reaches the respiratory system, its chemical composition will influence the interaction with the cells and the mechanisms of defence. Endocytosis and biokinetics are largely dependent on the surface chemistry

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(coating) of the particle and *in vivo* surface modifications (Oberdörster *et al*., 2005).

3.3.2 Particle Sources

3.3.2.1 Tobacco Smoking

An important indoor source of particles is tobacco smoking. Tobacco smoke contains more than 4000 chemical compounds and has a significant influence on the indoor particle concentration. Several Western countries have restricted or forbidden indoor smoking in office buildings, which has limited this problem to homes. Wallace (1996) estimated the $PM_{2.5}$ concentration in homes with smokers to be in the range from 25 to 45 $\mu g\,m^{-3}$. A recent study by He *et al*. (2004) measured $PM_{2.5}$ concentration in Australian houses, and found 79 $\mu g\,m^{-3}$, which exceeds the US EPA $PM_{2.5}$ 24 hour standards of 65 $\mu g\,m^{-3}$.

3.3.2.2 Human Activities

Combustion processes are among the main indoor sources of small particles, with the majority of them being in the submicrometer range (Morawska and Zhang, 2002). Examples of significant sources are food preparation (cooking, frying, grilling), heating (kerosene heating, wood burning, electrical convection ovens), burning candles and vacuum cleaning.

In a study of Australian homes by He *et al*. (2004) very high $PM_{2.5}$ concentrations where reported for food preparation, such as cooking pizza, frying and grilling (>700 $\mu g\,m^{-3}$). They also reported that emissions varied from house to house, although they performed the same cooking procedure.

3.3.2.3 Heating

When particles are brought into contact with hot surfaces unstable components can decompose. Examples of common hot surfaces are electrical convection ovens, condensers of refrigerators and lamps. A study by Arashidani *et al*. (1996) indicated that the heating source in the air supply may generate indoor pollutants. They found that the concentration of polycyclic aromatic hydrocarbons (PAHs) increased during use of a kerosene-fuelled heater.

Pedersen *et al*. (2001) reported that the major physical change in indoor particles caused by heating was an increased number of submicron particles emitted. The particle emission started at temperatures as low as 70 °C (Pedersen *et al*., 2003). Emissions and particles can contain a range of compounds which may contribute to odour, as well as irritant effects. VOCs and SVOCs that previously were adsorbed onto particles, can, due to the heating, be desorbed and released to the environment. Hirvonen *et al*. (1994) demonstrated that organic compounds will desorb from household dust, the degree of which depends on temperature. This has been confirmed by Pedersen *et al*. (2003) showing VOC emissions from household dust started at temperatures of 150–200 °C. In a subsequent study, cell cultures where exposed to the same VOC emissions, showing that they inhibit cell proliferation and TNFα (tumour necrosis factor α) release to the growth media (Mathiesen *et al*., 2004).

3.3.2.4 Resuspension of Particles

Ventilation will cause air movement that will affect both airborne and settled particles. In dwellings the ventilation will commonly be of natural origin like opening windows and doors, and mechanic ventilation connected to the kitchen stove. Larger buildings with heating, ventilation and air conditioning (HVAC) systems, which are supposed to have a positive effect on the indoor air quality, can also be its enemy by continuously bringing particles into the breathing zone.

Resuspension caused by sweeping or vacuum cleaning brings particles into the air, which may be breathed, and at this stage the particles can have a more harmful effect than before, because of other compounds attached. Vacuum cleaning leads to extensive resuspension of the settled particles. Most vacuum cleaners do not have efficient filtration systems for small particles (<1 μm), so that these particles will pass through the filtration system and become airborne for a period, depending on their size, while the coarse fraction will to some degree remain in the filter. Appropriate cleaning methods in order to improve the indoor air quality have been studied by Franke *et al*. (1997) and Leese *et al*. (1997). When measuring the total number of particles with a laser particle counter (LPC), Leese and his group found that changing to more effective vacuum cleaners did not affect the total LPC count. This can be explained by the smaller particles remaining suspended, while only larger particles settled on surfaces and were collected by the vacuum cleaner.

3.4 Biological Particles

3.4.1 Pet Allergens

Dander (skin scales) from pets such as cats and dogs are common allergen sources in household dust (Arbes *et al*., 2004). Exposure may be different due to cultural habits, and cockroach and mouse allergens may be more important in inner cities (Matsui *et al*., 2003; Phipatanakul *et al*., 2000). The major cat allergen, Fel d I, is an acidic glycoprotein or group of proteins (Ledford, 1994). The antigen is found on the skin and fur, and is distributed by licking and grooming by the animal. The major dog allergen is Can f 1, and has been detected on the coat of the dog. Both cat and dog allergens have also been found in houses where such animals are not ordinarily kept (Almqvist *et al*., 2001; Kim *et al*., 2007a).

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Passive transport on footwear and clothes is thought to bring these allergens into the house.

3.4.2 House Dust Mites

House dust mites have been reported as frequent causes of indoor air allergy and a risk factor for development of asthma in atopic individuals (Platts Mills and Chapman, 1987). The major house dust mite is *Dermatophagoides pteronyssinus* (Der p 1) (Luczynska *et al*., 1998; Voorhorst *et al*., 1967). The main determinants for survival are high humidity, moderate temperature and an adequate food source (amply provided by human skin scales and other detritus of domestic life). Humidity is a critical parameter for establishment and survival of the mite populations. Reduction of household humidity to 45% relative humidity or less is recommended to control the growth of mites, but the most important methods for reduction are frequent cleaning and removal of breeding grounds.

3.4.3 Pollen

The proteinaceous substance in many pollen grains induces an allergic reaction commonly known as hay fever. Pollen grains are generally considered to be a risk factor for pollen allergy outdoors. Birch and grass pollen have been demonstrated in indoor air (Holmquist and Vesterberg, 1999) and settled indoor dust (Yli-Panula and Rantio-Lehtimaki, 1995). Yli-Panula and Rantio-Lehtimaki (1995) found that antigenic activity was detected two months after the peak pollen period, which indicates that indoor dust may cause pollen-allergy symptoms a long time after the season. Pollen and spores are particles too large to remain in the air for prolonged periods (Owen *et al*., 1992). Common cleaning practices such as sweeping, dusting and vacuuming will resuspend a large percentage of the particles.

3.4.4 Microbiology

Micro-organisms are a most important part of daily life, both outdoor and indoor. We breathe in considerable amounts of microbes every day, and on our skin the resident microbial flora help to keep pathogenic micro-organisms away. Exposure to microbes is therefore both a necessity and a challenge for a healthy life. However, micro-organisms are known to be important biopollutants in indoor air, giving rise not only to infections, but even more to noninfectious symptoms such as allergies and toxic syndromes. While viable microbes are a presupposition for infections, both alive, 'resting' and dead microbial cells and their metabolites are of importance for their noninfectious health implications. Among the noninfectious symptoms and clinical signs related to inhalation of microbes are symptoms and signs of allergic alveolitis, febrile reactions (organic dust toxic syndrome), irritation in mucous membranes, allergic asthma and rhinitis. The causal relation between microbes (including their metabolites) and health implications in indoor air environments are not fully understood today. A thorough introduction to the field of health implications from fungi in indoor air environments is available (Flannigan *et al*., 2001). Microbes and their metabolites have also been frequently considered as possible causative agents in connection with respiratory symptoms in indoor environments (Kim *et al*., 2007b; Meklin *et al*., 2002; Nevalainen and Seuri, 2005).

A naturally balanced microbiological flora is often attributed to normal flora. Normal flora will seldom give rise to health implications among individuals exposed on a continuous basis. Strong dominance of one species or total absence of expected groups of microbes are good markers for imbalance in the microbial flora. The microbial flora of indoor air is an important part of the total indoor climate and air quality.

The micro-organism flora will depend on a number of variables like temperature, humidity, light and nutrients. Thus, substantial differences in the flora composition will occur. Nevertheless, there are natural limitations regarding microbial composition in various environments (Dixon and Fromtling, 1995).

Certain bacteria have been specifically discussed in relation to health problems in indoor environments, for example *Legionellae*, *Pseudomonas* and *Thermoactinomycetes*. Although recently identified as a pathogen (1976), *Legionella pneumophilia* is well known as the cause of severe pneumonia-like infections in an outbreak among 200 people attending a meeting of the US American Legion, giving rise to the designation Legionnaires' disease (legionellosis) (Fraser *et al*., 1977). Legionellosis occurs after inhaling water droplets that originated from a water source contaminated with organic matter harbouring active *Legionella* bacteria, often contained in amoebae or protozoa. Potential sources of contaminated water include cooling towers used in industrial cooling water systems, as well as in large central air-conditioning systems, evaporative coolers, hot-water systems, showers, whirlpool spas, architectural fountains, room-air humidifiers, ice-making machines, mist generators and similar disseminators that use public water supplies.

The microbial flora of the indoor environment is usually determined from samples of indoor air or from settled dust samples. Apart from the normal mycoflora of indoor air, which consists mainly of outdoor air genera such as *Penicillium*, *Cladosporium* and *Aspergillus* spp., unusual genera or species may be observed, indeed, the diversity of indoor fungi is remarkable (Miller *et al*., 2000). Identification in indoor air of genera or species that typically grow on moisture-damaged building materials probably reflects the presence of excess moisture.

Certain fungi species within the genera *Aspergillus* and *Penicillium* have been shown to survive in a dried state

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for decades, while other species are shown to rapidly lose viability. A possible adaptation to increased temperatures has been reported among fungi in HVAC-treated indoor air compared to the same species in fresh air (Ahlén and Haugen, 1996; Hanssen, 2004).

Microbial toxins are produced both by fungi (Gravesen *et al*., 1994) and bacteria (Andersson *et al*., 1998; Peltola *et al*., 2001). Toxins are regularly detected in mould-contaminated building materials (Engelhart *et al*., 2002; Nielsen *et al*., 1999; Tuomi *et al*., 2000b). Thus, their occurrence may be a common phenomenon in buildings. The substrate, the material on which the organics are growing, may have a critical effect on the production of toxins and other substances with biological activity (Murtoniemi *et al*., 2001; Roponen *et al*., 2002).

Microbial volatile organic compounds (MVOCs), are volatile metabolites from micro-organisms, and are part of TVOCs in indoor air (Mølhave *et al*., 1997). Some compounds have been suggested as being of microbial origin, although few of these compounds are actually specific end products of microbial metabolism (Korpi *et al*., 1998). The toxicological relevance of MVOCs is probably negligible as they occur at very low concentrations (Korpi *et al*., 1999), but these compounds are used more and more frequently for the search for hidden mould growth (Schleibinger *et al*., 2005).

4 SOURCES OF INDOOR AIR CONTAMINATION

The main indoor environment challenges for risk management are the avoidance of dampness in buildings, the choice and maintenance of adequate ventilation rates and the elimination of pollutants from combustion sources, cooking and heating.

4.1 Sources Mainly Found Outdoors

Indoor air originates from outdoor air. Contaminants generated by outdoor sources are, for example, pollen (see Section 3.4), radon and a variety of compounds from traffic and industry.

4.1.1 Radon

Radon-222 (denoted radon) is a noble gas with a half-life of 3.8 days. It is formed from radium-226, which is the fifth decay product of uranium-238. Radon originates mainly from uranium in the earth's crust, and is especially emitted from certain granite and alum shale structures. Radon may penetrate into buildings through cracks and leakage in the base slab or walls. It is mainly a problem in the basement or ground floor in buildings without a basement. The penetration varies with the wind, temperature and pressure differences.

Radon and radon daughter elements occur normally in considerably higher concentrations indoors than outdoors. Radon in building materials normally makes only a small contribution to indoor radon (Khan, 1994), and soil gas is mainly responsible for indoor radon. Radon levels vary considerably between dwellings. Arithmetic mean concentrations in European countries range from about 20 to 100 $Bq\,m^{-3}$, with even higher levels in some regions (WHO, 2000). A North American review found analogous variations, between 25 and 131 $Bq\,m^{-3}$ (Krewski *et al*., 2006).

Radon is among the best studied of environmental carcinogens. In 1999 an extensive study by the US National Research Council estimated that 10–15% of all lung cancers in the USA were caused by radon (National Research Council, 1999). The risk for lung cancer is estimated to increase by 8.4–11% per 100 Bq m^{-3}. The dose–response relationship appeared linear and there is no indication of a threshold (Samet, 2006). In general, WHO recommends that simple remedial measures should be considered for building with radon progeny concentration of more than 100 Bq m^{-3} equilibrium equivalents of radon as an annual average, with a view to reducing such concentrations whenever possible (WHO, 2000).

4.2 The HVAC System as a Contamination Source

Pollutants generated in an HVAC system will also affect the indoor air quality. Today's indoor air, particularly that in offices, schools and similar work environments, is usually processed by HVAC systems. Unfortunately, dust from when the building was constructed often remains in the ventilation system due to lack of cleaning and quality control. Thus, these contaminants can spread to the whole area served by the HVAC system, or deposit in channels and serve as possible nutrients for micro-organisms, provided the right temperature and humidity conditions are present.

Fibreglass duct linings have been used in HVAC systems for thermal and acoustic insulation. Concerns have been expressed as to whether this use may cause glass-fibre emission into the indoor atmosphere. Great care should be taken to keep these materials intact, clean and dry throughout both the construction period and during operation of the HVAC system, otherwise fibres might be released into the air stream (Woods and Goodwin, 1997). In addition, the acoustic and thermal properties might be compromised.

Furthermore, good design and correct location of intake openings are essential for controlling moisture-related problems in HVAC systems. Prevention

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of microbiological problems depends on limiting dirt and moisture, particularly standing water, which will allow micro-organisms to proliferate. Allied to this is the avoidance of contamination by micro-organisms from outside sources, which then can colonize such systems. Thus, control involves correct design, operation, maintenance and housekeeping (ISIAQ, 1996).

Several investigations focussing on the air intake section in school and office buildings have documented that especially this part of the plant is a place where moisture and contaminants easily meet, and this may lead to microbiological growth that can result in unwanted consequences for the ventilation plant, as well as for the users. In a Norwegian study (Frydenlund *et al*., 2002), the intake sections of 30 ventilating plants were investigated. A total of nine were concluded to be plants of high potential risk because thermotolerant moulds were identified. The investigation also revealed a clear relation between microbiologic and hygienic conditions. If these findings are representative, modern ventilation systems may be potential breeding grounds for micro-organisms, which in turn may represent a health risk for the people living and working in the air-conditioned premises. A thorough introduction to successful system operation, control and prevention of potential problems is available (ECA, 2000).

4.3 Dampness in Buildings

Dampness in buildings is a risk factor for health effects among atopic (hypersensitive) and nonatopic individuals, both in domestic and in public environments (Bornehag *et al*., 2001; 2004b). It may increase the risk of health effects in the airways, such as coughing, wheezing and asthma by 40–120% or even more. Dampness is also associated with other effects such as tiredness, headache and airway infections. Remedial building measures have positive effects on health (Savilahti *et al*., 2000; Patovirta *et al*., 2004; Rudblad *et al*., 2004). The evidence for a causal association between dampness and health effects is strong, but the mechanisms are unknown (Bornehag *et al*., 2001; 2004b). Several definitions of dampness have been used in studies of these associations, and no matter how they have been formulated they all seem to be associated with health problems. The literature is not conclusive with respect to causative agents, for example mites, microbiological agents and organic chemicals from degraded building materials. Even if the mechanisms are unknown, there is sufficient evidence to take preventive measures against dampness, moisture and water damage in buildings.

If building structures are subject to more moisture and dampness than they were intended for, this may cause damage due to chemical or microbiological decomposition of the building materials. Organic dust and filth may provide nutrition for micro-organisms such as bacteria, moulds and amoebae, and insects such as mites, cockroaches and flies. Processes in built environments that are subject to more moisture than intended can therefore cause exposure to:

1. Allergens from house dust mites and other living or dead insects, germs and spores as well as from moulds and bacteria
2. MVOCs, irritating and evil-smelling vapours produced by microbiologic metabolism
3. Mycotoxins from moulds, of which many have strong biological effects; micro-organisms use toxins to suppress other organisms in their fight for survival and growth
4. Endotoxins and glucanes, which are active agents originating from bacteria
5. Chemicals, such as formaldehyde, emitted from building materials; the generation of such substances often increases due to hydrolysis and the decay of materials caused by water damage

It has also been proposed that a more efficient spreading of respiratory infection viruses in damp indoor climate can be a ‘missing link’ between allergen exposure and onset of asthma and allergic disease (Hersoug, 2005). Although many of these potential mechanisms can, theoretically, cause health effects, most exposure levels are usually much too low for health effects to actually occur. One intervention study indicated dose–response relationships between exposure to mould and health effects (Savilahti *et al*., 2000). However, as in other indoor climate cases, the measured levels were still far too low to cause health effects, even when based on current knowledge (Eduard, 2006). The association between dampness and health effects, on the other hand, is strong, although there is no reason to believe that moisture or dampness themselves are the actual causes. Obviously, possible causes must be looked for among the agents that occur due to the effects of increased humidity in buildings and other factors, such as temperature.

Dampness is sometimes associated with mite growth that can induce mite sensitization and allergic disease (Wickman *et al*., 1991). Agents other than mite allergens, which in some studies have been shown to increase the risk for symptoms and medical signs, are airborne moulds and bacteria. However, the literature is not consistent. In conclusion, it is not known which humidity-related agents in indoor air are the main causes of the health effects. Dampness and moisture phenomena in buildings, microbial and chemical exposures, and individual human responses are complex phenomena. While the causative links between exposure agents and health responses are still not well understood, the essential issue is to prevent

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problems by keeping the buildings dry and clean through good design, construction and maintenance.

5 VENTILATION

The achieved indoor air quality depends on the pollution sources in the outdoor air, the infiltration to the building from the ambient environment, the indoor sources with the influence of factors such as dampness, heating, cooling, maintenance, cleaning and finally, ventilation.

The purpose of ventilation is replacement of polluted indoor air with clean outdoor air. Although the HVAC system can be a contamination source for indoor air, it is important to keep in mind that ventilation is part of the solution to improve indoor air quality. Wargocki *et al*. (2002) made a comprehensive review and found that ventilation is strongly associated with comfort (perceived air quality) and health in terms of SBS symptoms, nasal irritative inflammation, infections, asthma, allergy and short-term sick leave. Also, an association between ventilation and productivity (performance of office work) is indicated. By applying current knowledge, indoor air quality can be improved considerably while still maintaining or even reducing ventilation rates and energy use compared to the situation today (Fanger, 2006).

6 CURRENT STATUS

When addressing indoor air quality worldwide, the focus varies between third world countries and the industrialized nations. In third world countries, the major challenges are negative health effects caused by combustion processes. Women and children are emphasized in particular, since they are close to the exposure source. In the more industrialized nations indoor pollution sources like building materials, furniture, electronic equipments, spray boxes, redecoration and so on are still important. However, focus has changed from TVOC emission and SBS only to a broader approach on the specific emission of single compounds like phthalates, flame retardants, carcinogenic compounds and other compounds with specific health effects.

Dampness in buildings is an old risk factor that has become highly important again. The evidence for a causal association between dampness and health effects is strong, but the mechanisms are unknown.

Nanoparticles are a new issue highlighted for both indoor and outdoor air. This is not a new pollution source since it has been present in both nature and in man-made combustion processes. However, engineered nanoparticles introduce new challenges to human health, due to their extremely small size and the expected huge increase in application. The number of ongoing studies related to health effects of nanoparticles and the mechanisms involved is growing rapidly, and publications of the results have just started. This can bring new knowledge into the details of how exposure to, for example, combustion particles affects human health.

It is still a major challenge to unite knowledge and practical application, in order to improve indoor air quality in future buildings. Legislation has the power to require fulfillment of regulations with respect to indoor air quality, and there is considerable potential for improvement of legislation. Most important is the practical implementation of our current knowledge in order to provide healthy indoor environments with sustainable use of energy and other resources.

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Multiple Chemical Sensitivity: Toxicological Questions and Mechanisms

Martin L. Pall

CONTENTS

1 INTRODUCTION TO MULTIPLE CHEMICAL SENSITIVITY (MCS)

Multiple chemical sensitivity (MCS) is a complex disorder with cases often apparently initiated by chemical exposure. Following initiation of illness, people with MCS report sensitivity or intolerance to low levels of a wide spectrum of chemicals. The reported symptoms of chemical exposure are diverse and variable from one patient to another, but include pain, especially headache pain, muscle and joint pain, confusion, cognitive dysfunction, asthma-type symptoms, rhinitis, sleep disturbances, fatigue and even such psychiatric symptoms as anxiety and depression and infrequently rage. In the Sorg (1999) review, a total of 41 different symptoms are listed, many of which occur only in a minority of sufferers. Among the more common symptoms following chemical exposure in MCS patients are extreme fatigue, headache, gastrointestinal problems, dizziness, anxiety, depression, upper airways irritation, muscle and joint pain, and memory and concentration difficulties (Sorg, 1999). It should be noted that six out of nine of these symptoms can probably be ascribed to central nervous system (CNS) changes. Changes in brain function have been shown in brain positron emission tomography (PET) scan studies of MCS patients (Heuser and Wu, 2001; Hillert *et al*., 2007), single photon emission computed tomography (SPECT) scan studies (Simon *et al*., 1994; Heuser *et al*., 1994; Fincher *et al*., 1997a; 1997b) and electroencephalography (EEG) studies (Bell *et al*., 1999b; Muttray *et al*., 1995; Ross *et al*., 1999; Schwartz *et al*., 1994; Fernandez *et al*., 1999; Lorig *et al*., 1991; Lorig, 1994). Miller (2001) listed 74 such symptoms that she divided into neuromuscular, head-related, musculoskeletal, gastrointestinal, cardiac, affective, airway, cognitive and other. It is likely, as is discussed below, that the profound variation in symptoms, both qualitative and quantitive among sufferers, may be due to a local mechanism whose tissue distribution may vary among different sufferers.

MCS has been given a number of different names, including chemical sensitivity, multiple chemical sensitivities, chemical intolerance and toxicant-induced loss of tolerance (TILT). The TILT name (Miller, 2001) emphasizes the observation that most cases of MCS follow exposure to one or more chemicals and the basic hypothesis that dominates much of this literature is that chemical exposure initiates cases of illness (Ashford and Miller, 1998). The Cullen case definition requires such an initiating exposure for a case to be considered to be MCS (Cullen, 1987). Furthermore, the spectrum of chemicals reported to initiate cases of MCS is similar or identical to the spectrum of chemicals to which people with MCS appear to be sensitive, suggesting that the mechanism of action of both initiating chemicals and those eliciting sensitivity responses may be similar or identical. Some researchers, mainly those who have advocated some type of psychogenic cause for MCS, have advocated calling it idiopathic environmental intolerance (IEI) and have questioned whether chemicals are in fact initiators of MCS cases.

The phenomenon of MCS has been often ignored in the toxicological literature, largely because up until recently, a series of challenging questions about MCS have been unanswered. From a toxicological perspective, the most relevant such questions include the following:

- How can such diverse chemicals be implicated in initiating cases of MCS and, having initiated sensitivity, subsequently produce responses at very low exposures?
- How can one produce high-level sensitivities to such a broad range of chemicals, with many MCS patients being estimated as being on the order of 1000-fold more sensitive than normal?
- Are there plausible physiological mechanisms that may be expected to produce the above-described pattern of sensitization?
- If so, is there any evidence supporting these mechanisms in MCS?

I will discuss each of these four questions in this review, as well as at least eight other, perhaps equally puzzling, questions about MCS.

2 DIVERSE CHEMICALS ARE REPORTED TO APPARENTLY INITIATE CASES OF MCS

There have been dozens of papers reporting a pattern of chemical exposure preceding development of cases of MCS, typically one high-level exposure or multiple

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lower-level exposures (Ashford and Miller, 1998; Sorg, 1999). Pall (2007a, Chapter 13) cited 24 distinct studies reporting chemical exposure preceding development of many cases of MCS and Miller (2000) cited 12 additional such studies and still additional studies are cited below in this section. The types of chemicals most commonly involved are the volatile organic solvents (sometimes described as volatile organic compounds (VOCs)) and pesticides, especially organophosphorus and carbamate pesticides (Ashford and Miller, 1998; Sorg, 1999; Rea, 1992; Ziem and McTamney, 1997). There are a number of additional papers reporting that exposure to organic solvent chemicals that outgas in 'sick building syndrome' situations also appear to initiate cases of MCS (Welch and Sokas, 1992; Davidoff and Keyl, 1996; Miller *et al*., 1999; Hodgson, 2000; Arnold-Llamosas *et al*., 2006; Redlich *et al*., 1997; Ross, 1997). Berglund *et al*. (1984) reported that apparently chemically sensitive individuals reacted to air piped in from such a 'sick building' in blinded fashion, but did not react to uncontaminated air, suggesting that chemicals in the 'sick building' air were causal in generating the reactions. Many of the chronic symptoms of the surviving victims of the Bhopal disaster may be ascribed to MCS (Ross, 2000; Nemery, 1996).

When Miller and Mitzel (1995) wanted to compare cases of MCS apparently initiated by two different classes of chemicals, they chose cases from recently remodelled sick buildings (volatile organic solvent exposure) and compared those with cases apparently initiated by organophosphorus pesticides. In their highly cited paper, Miller and Mitzel (1995) found these two groups of MCS patients were similar, but not identical to each other, with some differences in symptom patterns and some differences in average severity between the two groups. Because MCS cases apparently initiated in these two ways are so common, it was relatively easy for Miller and Mitzel to find substantial numbers of patients of the two types to study.

Two of the most interesting sick-building cases occurred in the then recently remodelled Environmental Protection Agency building in Washington DC, in which approximately 200 people were apparently sickened with cases of MCS (Miller, 2001) and in Brigham and Women's Hospital in Boston, part of the Harvard Medical School complex. The latter case was described in detail in a US government publication (Kawamoto *et al*., 1997), where subsequent decreases in chemical usage and increases in air flow led to substantial decreases in new cases of chemical sensitivity and related illnesses, suggesting a causal relationship between chemical exposure and illness initiation. Ashford and Miller (1998) suggested that the decreases in required air flow in buildings in the USA, as a response to the energy crises of the 1970s, led to major increases in the incidence of MCS. In an important study, occupational medicine patients differed from general patients in responses to the Toronto MCS questionnaire in much the same way that self-identified MCS patients did, albeit to a lesser extent (McKeown-Eyssen *et al*., 2001), suggesting that chemical exposure in the occupational environment may initiate substantial numbers of MCS cases. Zibrowski and Robertson (2006) reported increased prevalence of MCS-like symptoms among laboratory technicians exposed to organic solvents, as compared with similar laboratory technicians with no apparent exposure. An epidemiological study, estimating the prevalence of MCS in various occupations, including those expected to have substantial chemical exposure to classes of chemicals implicated in MCS as a consequence of the occupation, reported increased prevalence of MCS in several occupations involving such chemical exposure, again suggesting a causal role of chemical exposure (Maschewsky, 1996; 2002). Yu *et al*. (2004) found high prevalences of MCS-like symptoms among solvent-exposed printing workers, as compared with non-chemically exposed controls. There are at least a dozen studies reporting high prevalences of reactive airways disease, a common aspect of MCS, among workers occupationally exposed to organic solvents.

In addition to organic solvents and related compounds and the organophosphorus and carbamate pesticides, there are additional classes of chemicals that are reported to apparently initiate cases of MCS. These include the organochlorine pesticides chlordane, lindane, dieldrin and aldrin (Corrigan *et al*., 1994; Ziem and McTamney, 1997; Lohmann *et al*., 1996; Wallace, 1995; Pröhl *et al*., 1997) and also a variety of pyrethroid pesticides (Corrigan *et al*., 1994; Lohmann *et al*., 1996; Altenkirch, 1995; Altenkirch *et al*., 1996). Lindane has been shown to initiate animal models of MCS (Gilbert, 2001; Cloutier *et al*., 2006) as has another $GABA_A$ (γ-aminobutyric acid A receptor) antagonist (Adamec, 1994). There are reports that hydrogen sulfide exposure can initiate cases of MCS-like illnesses (Kilburn, 1997; 2003). Donnay (1999; 2000) has reviewed evidence suggesting that carbon monoxide exposure may be able to initiate cases of MCS. Furthermore, mercury and mercurial compounds are also reported to apparently initiate some cases of MCS (Eneström and Hultman, 1995; Latini *et al*., 2005; Brent, 2001; Stejskal *et al*., 1999) and dental assistants working with mercury amalgams were reported to have higher prevalences of neurological symptoms including MCS-like symptoms (Moen *et al*., 2008).

Mould exposure is also suggested to initiate cases of MCS in sick-building situations characterized by mould-infested buildings (Redlich *et al*., 1997; Claeson *et al*., 2002; Lee, 2003; Mahmoudi and Gershwin, 2000; Straus *et al*., 2003). Here, we cannot say much about what mycotoxins may be involved, although there is some evidence that *Stachybotrys* moulds may be often involved (Mahmoudi and Gershwin, 2000; Hintikka, 2004; Straus *et al*., 2003; Pestka *et al*., 2008). Hirvonen *et al*. (1999) reported that mouldy 'sick' buildings produced increases

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in nitric oxide (NO) and inflammatory cytokines in nasal passages of exposed people and similar responses were also reported in the lungs of similarly exposed people (Akpinar-Elci *et al*., 2008). NO and inflammatory cytokines are important aspects of the MCS mechanism developed in this review.

3 A COMMON RESPONSE TO INITIATING CHEMICALS: INCREASED NMDA ACTIVITY

One of the great puzzles about MCS is how can such a diverse group of chemicals produce a common biological response? In fact, one of the MCS skeptics, Ronald Gots (1996) has argued that MCS cannot possibly be a physiological response to chemicals because the diverse chemicals implicated in MCS cannot possibly produce a common response in the human body. Clearly one needs to find such a common physiological response in order to develop a compelling model of the mechanism of MCS. An important role for excessive NMDA (*N*-methyl-D-aspartate) receptor activity in MCS was first suggested by Thomas (1998) and by Dudley (1998). Pall (2002) argued that elevated NMDA[a] receptor activity is likely to have a key role in MCS and that chemicals were likely to act, in most cases indirectly, to increase such activity. There were several types of evidence reviewed in that paper suggesting a role of elevated NMDA activity:

1. MCS patients are hypersensitive to monosodium glutamate and glutamate is the common physiological agonist of the NMDA receptors.
2. In studies of the genetic polymorphism of the CCK-B gene, the allele of the gene that acts indirectly to produce higher NMDA activity was associated with increased prevalence of MCS (Binkley *et al*., 2001; see Pall, 2002 for discussion).
3. The NMDA antagonist, dextromethorphan was reported from both clinical observations and anecdotal reports to lower reactions to chemicals in MCS patients.
4. Bell and others have proposed that neural sensitization has a key role in MCS and the probable mechanism for such neural sensitization, called long-term potentiation (LTP), is known to involve increased NMDA activity.
5. Elevated NMDA activity has been shown to play an essential role in several animal models of MCS.
6. Elevated NMDA activity appears to play a role in such related illnesses as fibromyalgia (FM), chronic fatigue syndrome (CFS) and post-traumatic stress disorder (PTSD), with the most extensive evidence for such a role being found in FM (Pall, 2006; Pall, 2007a).

It should be noted that numbers 2 and 5 above suggest that chemicals initiating cases of MCS may act to increase NMDA activity and number 3 suggests that chemicals acting in those already sensitive may also act to increase NMDA activity. In fact, these two sets of chemicals are similar or identical to each other (Ashford and Miller, 1998) so it should not be surprising if they both may act via the same mechanism(s). All of these considerations raise the question about whether there are known mechanisms by which the several classes of chemicals implicated in MCS may act to increase NMDA activity?

3.1 Pesticides and NMDA Stimulation

In that Pall (2002) review, evidence was discussed showing that organophosphorus and carbamate toxicants (including pesticides) can act to produce increases in NMDA activity via the following pathway: these toxicants are acetylcholinesterase inhibitors, producing an increase in acetylcholine, which stimulates the muscarinic receptors, which produce, in turn, increased glutamate release leading to increased NMDA receptor stimulation, as well as stimulating other glutamate receptors (see diagram in **Figure 1**). There are a large number of studies showing that toxic effects of organophosphorus toxicants in mammals can be greatly lowered by using NMDA antagonists (Dekundy *et al*., 2007; Lallement *et al*., 1998; Martin and Kapur, 2008), showing that such increased NMDA activity has a substantial role in producing the response to these toxicants.

What about other pesticides and other groups of implicated chemicals? Let us take the different classes of chemicals one at a time. The organochlorine pesticides, chlordane, lindane, dieldrin and aldrin have all been shown to lower $GABA_A$ receptor activity (Gant *et al*., 1987; Corrigan *et al*., 1994; Cassidy *et al*., 1994; Brannen *et al*., 1998; Narahashi *et al*., 1995) and this, in turn is well known to produce elevated NMDA activity (Blaszczak and Turski, 1998; Watanabe *et al*., 1995; Tusell *et al*., 1992), see **Figure 1**. In fact these same citations show that seizure activity produced by these $GABA_A$ antagonists, including these pesticides, is lowered or blocked by NMDA antagonists, showing that the elevated NMDA activity produced by such toxicants has a key causal role in the mechanism of seizure generation. Because MCS involves the action of short-term stressors producing chronic illness, it may be of special interest that this pathway produces chronic changes in brain function that can be blocked by short-term interruption of the pathway (Kaindl *et al*., 2008).

Pyrethroid pesticides, which also initiate cases of MCS, act to produce long-term sodium-channel opening (Narahashi *et al*., 1995; Valentine, 1990; Wu and Liu, 2003;

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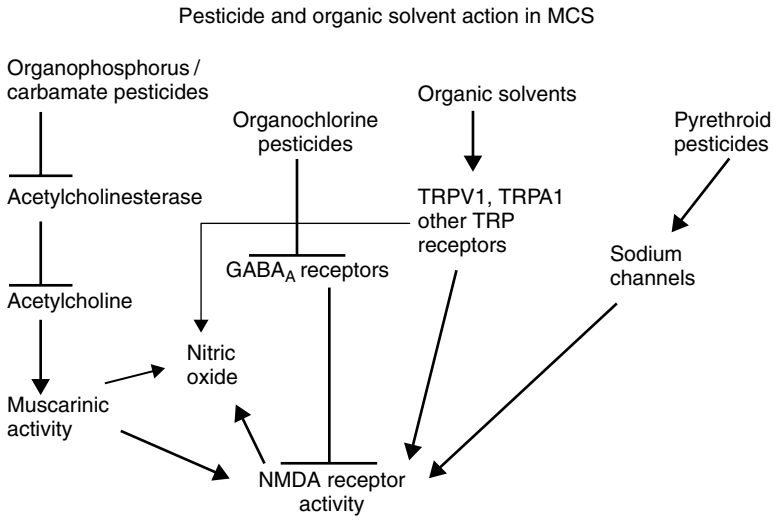

Figure 1 Pathways for action of pesticides and organic solvents. Each chemical class implicated in the initiation of cases of MCS can act along a distinct pathway to generate increases in NMDA activity, as shown in the figure. Each arrow represents a mechanism by which one parameter stimulates another. Some inhibitory (negative) interactions are also indicated. Both the organophosphorus/carbamate toxicants and the organochlorine pesticides have double-negative interactions. Such negative interactions, together with the arrows in the figure, indicate that the each of the four classes of compounds acts along one of these pathways, leading to an increase in NMDA activity.

Bradberry *et al*., 2005; Proudfoot, 2005). This in turn, produces increased NMDA stimulation (Wu and Liu, 2003; Yu, 2006; Doble, 1996), see **Figure 1**. Type II pyrethroids also act as $GABA_A$antagonists (Valentine, 1990) and may be expected, therefore, to also act along the same pathway impacted by the organochlorine pesticides, and thus lead to increased NMDA activity along that pathway as well.

3.2 Organic Solvents, TRP Receptors and NMDA Stimulation

Clearly the greatest puzzle of chemical activity in MCS is how does the huge family of organic solvents act to initiate cases of MCS or elicit sensitivity symptoms in those who have become sensitive? These chemicals are the predominant set of chemicals that trigger reactions on a day-to-day basis in MCS patients. They have also been referred to as volatile organic chemicals and yet it is clear that nonvolatile chemicals ingested or absorbed through the skin can produce reactions, so the volatility is important due to the most common mode of exposure, inhalation, rather than being an essential part of the mechanism of sensitivity. I will refer to this extremely large group of chemicals as organic solvents, even though that does not cover this entire spectrum of chemicals.

Pall and Anderson (2004) argued that the probable target for such organic solvents in MCS is the vanilloid (transfer receptor potential) TRPV1 receptor, and presented 12 distinct types of evidence arguing for such a TRPV1 role in MCS. That paper was extensively documented with 222 citations and while specific references are provided some of this discussion, for the rest the reader is referred back to that paper. One type of evidence that we presented is that some solvents well known to be involved in MCS, such as formaldehyde and other aldehydes, were quite active TRPV1 agonists, and a variety of alcohols are vanilloid agonists and may be converted into still more active aldehydes via alcohol dehydrogenases in the body. It is known that capsaicin, the classic TRPV1 agonist, requires both hydrophobic regions and a hydrogen-bonding group in order to act as an agonist, suggesting that strictly hydrophobic solvents might require cytochrome P450 metabolism in order to act as a vanilloid agonist, or might act synergistically with a solvent that does have a hydrogen-bonding group. There is evidence from animal models of MCS, which are also animal models of Gulf War illness, for such synergistic interactions of organic solvents and related compounds (Research Advisory Committee on Gulf War Veterans Illnesses., 2004): fully 28 studies of synergistically acting stressors, most, but not all, of which were organic compounds, were reviewed in that document.

Some mycotoxins are known TRPV1 agonists, so it is possible that the role of moulds in MCS may be explained through the role of the TRPV1 receptor. Chemical sensitizers, including toluene diisocyanate (TDI) and eugenol, which produce local sensitivity to a wide range of chemicals, are known TRPV1 agonists. MCS patients often report sensitivity to chlorine gas from swimming pools or from drinking water, and chlorine acts as a TRPV1 agonist *in vivo* (Morris *et al*., 2005), producing

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an irritant response. TRPV1 stimulation produces neurogenic inflammation and also reactive airways disease (Geppetti *et al*., 2008; Jia and Lee, 2007; Planells-Cases *et al*., 2005; Costa *et al*., 2008), often called reactive airways dysfunction syndrome (RADS), a form of asthma showing reaction to a spectrum of chemicals similar or identical to those involved in MCS. Both RADS and neurogenic inflammation are often aspects of MCS cases (Meggs, 1994; 1997).

Millqvist and her colleagues have published a series of papers showing that MCS patients are hypersensitive to capsaicin, the classic TRPV1 agonist, again providing support for a TRPV1 role in MCS (Johansson *et al*., 2002; Millqvist, 2000; Ternesten-Hasséus *et al*., 2002; Millqvist *et al*., 2005; 2008). Many studies have shown that capsaicin treatment leads the TRPV1-stimulated cells in several regions of the body to release glutamate neurotransmitter, leading in turn to NMDA stimulation (10 such studies are cited in Pall and Anderson, 2004). These studies provide further support for the contention that each class of chemicals involved in MCS leads to increased NMDA stimulation.

There is an additional parallel between MCS and TRPV1 stimulation. MCS patients have a phenomenon known as desensitization or masking, such that low-level chronic or repeated chemical exposure leads to decreased reactivity to chemical exposure (Ashford and Miller, 1998). This may be the basis of using low-level chemical exposure to treat MCS patients (Weaver, 1996; Rea, 1997). Low-level chronic or repeated exposure to many TRPV1 agonists leads to lowered TRPV1 activity through a complex series of changes involving increased intracellular calcium levels, complex protein phosphorylation control and probably receptor internalization (Szallasi and Blumberg, 1999; Itagaki *et al*., 2004). Thus the desensitization/masking phenomenon found in MCS may be produced, to part or in whole, by this lowered TRPV1 activity.

While there are many properties suggesting a TRPV1 role in MCS, it is clear now that some of the interpretations given by Pall and Anderson (2004) to some of the relevant data were too narrow. It was argued, for example, that TRPV1 was primarily responsible for the sensory irritation (SI) response, a response elicited by chemicals including alkanes, alkyl benzenes, halogenated benzenes, halogenated alkylbenzenes, alcohols, ketones, ethers, aldehydes, formaldehyde, isocyanates and chlorine (Nielsen, 1991; Alarie *et al*., 1998; Inoue and Bryant, 2005; Cometto-Muñiz and Abraham, 2008), a broad range of chemicals also implicated in MCS. It is now clear that this SI response involves as major players, other members of the TRP family of receptors, not just TRPV1. Specifically Bíró *et al*. (2007) discuss evidence for a role of TRPA1, TRPM8 and TRPV2, 3 and 4 receptors in this response, as well as TRPV1. Bautista *et al*. (2006) implicated specifically the TRPA1 receptor in the response to several environmental irritants. Many of the TRP receptors have roles in responding to xenobiotics (Nilius, 2007) and while our knowledge of such roles has been expanding rapidly in recent years, it is still, no doubt, incomplete. Neurogenic inflammation and reactive airways disease aspects of MCS, discussed above and below, are produced, not only through TRPV1 stimulation, but also through the action of other TRP receptors (Geppetti *et al*., 2008; Jia and Lee, 2007). Whereas some chemical sensitizers act as TRPV1 agonists, sensitizers can also act as TRPV3 agonists (Xu *et al*., 2006).

Others have argued for a central role for the SI response and the receptors involved in that response in MCS (Skov and Valbjorn, 1987; Meggs, 1993; 1997; Anderson and Anderson, 1999a; 1999b; 2003; Millqvist *et al*., 1999; Millqvist, 2000; 2008; Nordin *et al*., 2005).

In Pall and Anderson (2004), we used the desensitization response produced by low-level chronic exposure to capsaicin or other bona fide TRPV1 agonists to assess whether some solvents that had never been tested as possible TRPV1 agonists might have such activity. The reasoning was that if responses to a chemical were reported to be substantially reduced after low-level capsaicin treatment, that chemical should be labelled as a probable TRPV1 agonist, because the response to it was lowered along with TRPV1 desensitization. It is clear now that desensitization of one TRP receptor is often accompanied by desensitization of others. For example, TRPV1 and TRPA1 can undergo cross-desensitization (Rohacs *et al*., 2008; Ruparel *et al*., 2008) and TRPM8 and TRPA1 desensitization can also be produced in parallel (Zanotto *et al*., 2008). In another study, a series of TRPC receptors were desensitized together by a receptor internalization process (Itagaki *et al*., 2004). It seems likely, therefore, that some organic solvents that were argued to be probable TRPV1 agonists, as suggested earlier in this paragraph, may well be agonists of other TRP family receptors.

Of the other TRP family receptors, the one most likely to have a substantial role in MCS, based on current evidence, is TRPA1. TRPA1 is responsible for the activity of a number of different sensory irritants (Bautista *et al*., 2006; Gerhold and Bautista, 2008), with TRPV1 being responsible for others. For a number of such irritants, the chemicals react by reversible covalent modification with the TRPA1 receptor (Hinman *et al*., 2006). Among the TRPA1 agonists are certain aldehydes, including acrolein and aldehydic components of cigarette smoke (Andrè *et al*., 2008; Simon and Liedtke, 2008) and MCS patients are commonly known to be sensitive to cigarette smoke. Formaldehyde which is commonly involved in initiating cases of MCS was shown in a recent study to act via the TRPA1 receptor in a model of inflammatory pain, rather than acting via the TRPV1 receptor (McNamara *et al*., 2007).

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Activation of the TRPA1 receptor has been reported to lead to the release of the neurotransmitter glutamate, leading in turn, to increased NMDA activity (Kosugi *et al*., 2007; Ding *et al*., 2008). Given that such increased NMDA activity is also produced by TRPV1 receptor stimulation, as discussed above, it should not be surprising that organic solvent-produced changes in the nervous system can, in many cases, be blocked or lowered by using NMDA antagonists. For example, there are a number of responses to formaldehyde exposure that have been shown to be greatly lowered by NMDA antagonists (Coderre and Melzack, 1992; McMahon *et al*., 1993; Wiertelak *et al*., 1994; Wang *et al*., 1999).

In conclusion, there are compelling similarities between the diverse organic solvents and related chemicals involved in MCS and the diverse organic chemicals involved in the SI response. It seems likely that the TRP receptors are involved in both, with the two most likely members of this receptor family to be involved in chemical responses in MCS and in SI, based on current evidence, being the TRPV1 and TRPA1 receptors, both of which can produce an increase in glutamate release and consequent NMDA stimulation. These various data suggest, therefore, that the proposed pattern of chemical involvement in MCS acting through increased NMDA activity is likely to be sustained for the organic solvent group of chemicals.

Before leaving this issue of the apparent roles of TRP receptors in MCS, I need to discuss the TRPM2 receptor that may have a role in amplifying responses in MCS. The TRPM2 receptor is known to be stimulated by oxidants, including hydrogen peroxide, with much of the stimulation being produced by adenosine diphosphate (ADP)-ribose, a signalling molecule whose levels can be greatly increased by oxidants (Kühn *et al*., 2005; Fonfria *et al*., 2004; Wilkinson *et al*., 2008; Naziroglu, 2007; Buelow *et al*., 2008; Lange *et al*., 2008). The pathway of synthesis of poly(ADP)-ribose is as follows: oxidants produce nicks in DNA strands in the nucleus of cells which can lead, in turn, to a massive stimulation of poly(ADP)-ribose polymerase activity, producing poly(ADP)-ribosylation of chromosomal proteins. When this poly(ADP)-ribose becomes subsequently hydrolysed, it produces free ADP-ribose which acts as a signalling molecule. One oxidant that is very active in this process is peroxynitrite ($ONOO^-$) (Pacher and Szabo, 2008), a molecule that the author has argued (see below) has a key role in MCS and related illnesses, and whose synthesis is greatly increased by NMDA stimulation (reviewed in Pall, 2002; Moncada and Bolaños, 2006; Brown and Bal-Price, 2003). Consequently, TRPM2 activity is predicted to be elevated in MCS and to be stimulated by chemical exposure. TRPM2 may both directly and indirectly leading to increases in NO and $ONOO^-$ production, thus amplifying the already elevated levels of these compounds (see Yamamoto *et al*., 2008 for discussion). There is some evidence that another TRP receptor, TRPM7, may also have a role in this process (Miller, 2006). The role of TRPM2 and possibly 7 may be one of several interacting mechanisms that may lead to the extraordinary chemical sensitivity reported in MCS patients.

There is evidence that other TRP receptors are elevated in response to oxidants and products of oxidative stress biochemistry, including TRPV1 and TRPA1 (Taylor-Clark *et al*., 2008; Bessac *et al*., 2008; Andersson *et al*., 2008; Trevisani *et al*., 2007; Puntambekar *et al*., 2005; Schultz and Ustinova, 1998; Ustinova and Schultz, 1994), but these effects may be more modest than those on TRPM2. The effects on TRPV1 receptors makes them more susceptible to stimulation by their effectors, whereas with TRPM2, oxidative stress acts to open the receptor channel independently of any effector and so may produce a greater physiological response under many circumstances.

3.3 Other Apparent Initiators and Summary of NMDA Role

Three other apparent initiators of cases of MCS were discussed above, carbon monoxide, hydrogen sulfide and mercury. Do any of these act to increase NMDA activity?

Carbon monoxide has been reported to produce such increased NMDA activity and NMDA antagonists block or lower the toxic responses to carbon monoxide exposure (Thom *et al*., 2004; Liu and Fechter, 1995; Penney and Chen, 1996; Ishimaru *et al*., 1992). Hydrogen sulfide can also produce increased NMDA activity and again its toxic effects are lowered by NMDA antagonists (Cheung *et al*., 2007; Qu *et al*., 2008; Kamoun, 2004). Mercury, acting through its metabolic product methylmercury, also acts to produce increases in NMDA activity, and again methylmercury toxicity is lowered by NMDA antagonists (Juárez *et al*., 2005; Allen *et al*., 2002; Faro *et al*., 2002; Miyamoto *et al*., 2001; Zhang *et al*., 2003; Rossi *et al*., 1997). Methylmercury acts to produce such increased NMDA activity, at least in part, by lowering the transport of glutamate, the most important physiological NMDA agonist (Juárez *et al*., 2005; Allen *et al*., 2002).

In summary, then, we have evidence that all seven classes of compounds reported to initiate cases of MCS can each act to increase NMDA activity (**Figure 1**). At least for some members of each class under some conditions, NMDA antagonists can lower the toxic responses to each of them. While evidence linking any one of these to increased NMDA activity may be coincidental, the pattern of evidence for all seven strengthens the argument that increased NMDA activity is not likely to be coincidental. When coupled to the six types of additional evidence, discussed at the beginning of this section, on the apparent NMDA role in MCS, one can argue that there is very substantial evidence, not only that increased

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NMDA activity has a role in MCS, but also that chemicals are likely to act indirectly by increasing such NMDA activity.

There is extensive evidence that increased NMDA activity produces increases in NO and also its oxidant product $ONOO^-$ (reviewed in Pall, 2002; Moncada and Bolaños, 2006; Brown and Bal-Price, 2003), and it will be argued below that all three of these, NMDA activity, NO and $ONOO^-$, are likely to have key roles in MCS.

4 GENETIC EVIDENCE FOR CHEMICAL EXPOSURE BEING CAUSAL IN MCS

The pattern of chemical exposure preceding cases of MCS and the common mode of action of these chemicals in increasing NMDA activity strongly suggests causality of those exposures. However, one would like to have independent confirmation of causality. Such independent confirmation has come from genetic studies of susceptibility to MCS. There have been three such studies, each providing evidence that chemicals have causal roles in initiating cases of MCS (summarized in **Table 1**).

The first of these to be published was a study by Haley *et al*. (1999) on Gulf War veterans, including those suffering from what some have called Gulf War syndrome. There are several reports that the Gulf War syndrome veterans suffer from MCS or an MCS-like illness (Proctor *et al*., 2001; Reid *et al*., 2001; Miller and Prihoda, 1999; Thomas *et al*., 2006) and there is also evidence that they suffer from such related illnesses as CFS and FM (Chapter 10 in Pall, 2001a; 2007a). The Gulf War veterans were exposed to over a dozen stressors that may have had a role in initiating their illnesses (Chapter 10 in Pall, 2007a), one of which was exposure to the organophosphorus toxicants, sarin and cyclosarin, which are both potent inhibitors of acetylcholinesterases. What Haley *et al*. (1999) report is that those carrying a form of the gene for PON1 that makes them less able to metabolize these neurotoxicants, were more susceptible to developing the neurological symptoms that comprise Gulf War syndrome. This provides substantial evidence that sarin/cyclosarin had a causal role in initiating cases of Gulf War syndrome and that those less able to detoxify these toxicants were therefore more susceptible to it. Mackness *et al*. (2000) showed that British Gulf War veterans with self-reported Gulf War syndrome tended to have lowered activity for the enzyme encoded by the PON1 gene, the paraoxonase enzyme, suggesting again a link to the organophosphorus toxicants. However, in this case, the low activity was not shown to be caused by the genetic polymorphisms of the PON1 gene, so the argument for causality is weaker than in the Haley *et al*. (1999) study. Another study from the same group (Mackness *et al*., 2003), showed that among farmers using sheep dip containing an organophosphorus pesticide, farmers reporting chronic ill health tended to carry the the PON1 allele that produces lowered metabolism of that pesticide, as compared with farmers reporting good health. Unfortunately, MCS prevalence in these two groups of farmers was not studied.

Two studies somewhat similar to the Haley *et al*. (1999) study have been done, comparing a large number of civilian MCS sufferers with unaffected controls (**Table 1**). One was the Canadian study by McKeown-Eyssen *et al*. (2004) and the second, the German study by Schnakenberg *et al*. (2007). Each of these showed that three distinct polymorphic genes involved in the metabolism of chemicals otherwise implicated in initiation of MCS cases have a statistically significant influence on susceptibility (**Table 1**). In the Schnakenberg *et al*. (2007) study, there was an extremely high level of statistical significance for each of these three genes, so that the probability of getting these results by chance if there is no true correlation is less than one in 10^{11}. In total, in these three studies (Haley *et al*., 1999; McKeown-Eyssen *et al*., 2004; Schnakenberg *et al*., 2007), five genes which help determine the rate of metabolism of chemicals previously implicated in MCS have been found to have statistically significant association with the prevalence of MCS; a sixth genetic polymorphism, for the gene GSTT1 had a statistically significant effect only in conjunction with specific alleles of other implicated genes (**Table 1**). A recent similar, but much smaller study, roughly one quarter of the size of the McKeown-Eyssen *et al*. (2004) study and one ninth the size of the Schnakenberg *et al*. (2007) study, failed to find any statistically significant differences between apparent cases and controls (Wiesmüller *et al*., 2008). Of the three larger studies, we have a pattern of evidence showing that genes that metabolize chemicals otherwise implicated in MCS initiation, have substantial influence on the susceptibility to develop MCS. These results support the inference that chemicals acting as toxicants cause many cases of MCS and that those chemicals must be in their toxic form in order to so act. Therefore, alleles of polymorphic genes that either decrease or increase the metabolism of these chemicals will influence the susceptibility to MCS.

One point that should be emphasized is that genetic studies of this type may well give different results with different populations, because populations may differ in either chemical exposure or in the frequencies of the polymorphic alleles in their gene pools. The genetic roles presumably involved here are what are often described as environment X gene interactions. An apparent example of this comes from studies of autism susceptibility where the susceptibility to autism in the USA and Romania, but not in Italy was apparently influenced by the PON1 gene (Pasca *et al*., 2006; D'Amelio *et al*., 2005). The differences were ascribed to the much higher use of organophosphorus pesticides in the USA and Romania than in Italy (Deth *et al*., 2008).

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Table 1 Genetic polymorphisms influencing MCS susceptibility

Gene	Study	Function—chemical metabolism	Comments
PON1	H, M	Detoxification of organophosphorus toxicants	—
CYP2D6	M	Hydroxylation of hydrophobic compounds	Hydroxylation of compounds without hydrogen binding group may be expected to lead to greater activity as a TRPV1 agonist
NAT2	M, S	Acetylation	May produce more or less activity depending on the specific compound involved
GSTM1	S	Provide reduced glutathione for conjugation	Should increase detoxification and excretion
GSTT1	S	Glutathione conjugation	Should increase detoxification and excretion
GSTP1	S	Glutathione conjugation	Should increase detoxification and excretion; only statistically significant role was in conjunction with specific alleles of other genes

H, Haley *et al*. (1999); M, McKeown-Eyssen *et al*. (2004); S, Schnakenberg *et al*. (2007).

Are there any alternative interpretations to these genetic data, other than that the metabolism of these chemicals influences their role as toxicants in initiating cases of MCS? There is an alternative for two of the five genes, but not for the other three (**Table 1**). The gene for glutathione reductase has a very important role in the body's protective response to oxidants and oxidative stress, and the PON1 gene has a role in dealing with some of the lipid oxidation products produced by oxidative stress (Draganov and La Du, 2004), at least in lipoproteins in the blood. It follows that the roles of these two genes may be interpreted in an alternative way, but those of the other three genes cannot. The only consistent interpretation for these studies, taken as a whole, is that chemicals act as toxicants in the initiation of cases of MCS. By determining the rate of the metabolism of these chemicals, the genes help determine the incidence and prevalence of MCS.

There is strong, I would argue compelling, evidence that chemical exposure is causal in the initiation of many cases of MCS. What we need to do is to determine what physiological mechanisms are likely to be involved in such initiation. Furthermore, because low levels of similar, if not identical chemicals, trigger sensitivity responses in those already sensitive, similar pathways of action are likely to be involved in such low-level chemical responses.

5 MCS DOES NOT CENTRE ON AN OLFACTORY RESPONSE

The receptors that are implicated in the response to chemicals that are discussed above are not the olfactory receptors (Axel, 2005; Buck, 2005), and yet there have been many descriptions of MCS calling it a reaction to 'odours'. There is no evidence that the olfactory system has a central role here and there is considerable evidence against such a role. Ashford and Miller (1998) reviewed a number of studies where people with severe nasal congestion still reacted to chemical exposures. There are cases of MCS in people with no sense of smell, that is people suffering from anosmia (Doty, 1994). Many MCS patients report reacting at times when they could not smell any chemical odour. There have been three studies of patients where a nose clip was used to block off access of odourants to the nasal epithelia and those MCS patients still reacted to chemical exposure (Joffres *et al*., 2005; Millqvist and Löwhagen, 1996; Millqvist *et al*., 1999). In a recent study, regions of the brain that respond to odours were found to have lowered responses to odourants in MCS patients as compared with controls, not elevated responses (Hillert *et al*., 2007). The author is not arguing that the olfactory mechanism is never impacted in MCS cases, but rather that it does not have any essential role in the chemical sensitivity process and should not be the focus of studies, when trying to assess responses of MCS patients to chemicals. We are looking at a response to chemicals, many of which have odours, not a response to odours.

6 PREVALENCE ESTIMATES

Sorg (1999) reviewed prevalence studies of MCS by concluding that 'prevalence of severe MCS in the United States is approximately 4%'. She also concludes that those with milder chemical sensitivity are about 15–30% of the US population. Several more recent studies of MCS prevalence provide additional information on this issue (Kreutzer *et al*., 1999; Caress and Steinemann, 2003; 2004a; 2004b; 2005). Pall (2007a, Chapter 11) estimated that the prevalence of severe MCS in the USA was probably about 3.5%, with much larger numbers, perhaps 12–25% modestly affected. These

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estimates are slightly lower than the Sorg (1999) estimate. There have been few studies of MCS prevalence in other countries, but one study each from Canada (Joffres *et al*., 2001), Germany (Hausteiner *et al*., 2005), Sweden (Johansson *et al*., 2005) and Denmark (Berg *et al*., 2008) suggest prevalences of roughly 50–100% of those in the USA. All of these studies suggest that there is substantial impact of MCS on public health.

Caress and Steinemann (2003) estimated that 1.8% of the entire US population have lost their jobs due to chemical sensitivity, suggesting that many of the more severely affected may be unemployed or underemployed due to their MCS. There are no similar figures with regard to housing, but anecdotal reports suggest that the most sensitive often have great difficulty finding housing they can tolerate.

7 CASE DEFINITIONS

Probably the best review of and comparison of different case definitions for MCS was published by the Toronto group (McKeown-Eyssen *et al*., 2001). In that review, they compared seven different proposed case definitions, those of Randolph (1965), Cullen (1987), Thomson *et al*. (1985), the National Research Council, Board on Environmental Studies and Toxicology, Commission on Life Sciences (1992), Ashford and Miller (1998), Nethercott *et al*. (1993) and the 1999 Consensus (MCS Consensus Conference, 1999). These differ from each other in various ways, most notably in whether they require that the symptoms be polysystemic, associated with multiple organs, whether cases must be chronic, whether cases must be acquired as a consequence of one or more chemical exposure events and whether sensitivity responses must be produced by multiple 'unrelated' chemicals.

McKeown-Eyssen *et al*. (2001) compared various groups of patients with each other for their fit to each of these case definitions, using the University of Toronto Questionnaire. They compared the case definitions in several ways using this data, but perhaps the most crucial comparison was how well a specific case definition was able to discriminate between environmental practice patients and general practice patients. By that criterion, the Nethercott *et al*. (1993) case definition and the 1999 Consensus were the best, giving the highest odds ratio in comparing these groups of patients, with both giving odds ratios of roughly 20. The 1999 Consensus case definition (MCS Consensus Conference, 1999) is the one currently used on the Wikipedia site discussion of MCS and may be currently the most widely accepted case definition.

It should be noted that comparing occupational medicine practice patients with general practice patients also produced high odds ratios by these two case definitions, albeit lower ones than did the previously discussed comparison, suggesting that occupational chemical exposure often causes cases of MCS, as defined by these two case definitions (McKeown-Eyssen *et al*., 2001).

In contrast, the Cullen (1987) case definition only had an odds ratio of about eight, much lower than the Nethercott *et al*. (1993) or the 1999 Consensus case definition. The Cullen (1987) case definition has been criticized because of an additional, perhaps more important concern: it requires that 'no widely accepted test of physiologic function can be shown to correlate with symptoms'. However, as will be discussed below, there are a number of such tests that have been reported, tests of objectively measurable responses to low-level chemical exposure. This specific Cullen requirement may also be objected to, because it means, in effect, that we must stay perpetually ignorant of the aetiological mechanism of MCS. It should be discarded in the author's view, therefore, both for empirical and theoretical reasons.

There is one other issue that should be considered here, regarding what should and should not be part of an MCS case definition. Lacour *et al*. (2005) argued that only those patients who suffer from CNS-related complaints in response to chemical exposure should be considered to be true MCS patients. Such CNS-related symptoms include headache, fatigue, confusion and cognitive dysfunction. One possible rationale for this proposal is that Bell and others, as discussed below, have proposed a CNS mechanism for MCS involving neural sensitization in the brain, such that chemical exposure produces changes in synaptic sensitivities over substantial regions of the brain. Lacour *et al*. (2005) report that self-reported complaints of apparent MCS patients most commonly included CNS symptoms with symptoms derived from other regions of the body being less frequent. There is an argument for using a case definition for MCS that excludes patients without CNS-related symptoms.

Let us end this discussion by comparing the 1999 Consensus case definition (MCS Consensus Conference, 1999), listed immediately below with a couple of modifications that the author wishes to suggest for the reader's consideration:

1. Symptoms are reproducible with repeated (chemical) exposures.
2. The condition has persisted for a significant period of time.
3. Low levels of exposure (lower than previously or commonly tolerated) result in manifestations of the syndrome (i.e. increased sensitivity).
4. The symptoms improve, or resolve completely, when the triggering chemicals are removed.
5. Responses often occur to multiple, chemically unrelated substances.
6. Symptoms involve multiple-organ symptoms.

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7.1 Suggestion #1

The main concern here is that it is not clear what chemically unrelated means. If it means that there is no relationship among these chemicals that can be challenged, because they all may act to produce increased NMDA activity. Describing them as being chemically diverse is more accurate. This should not change how the case definition is used in practice.

1. Symptoms are reproducible with repeated (chemical) exposures.
2. The condition has persisted for a significant period of time.
3. Low levels of exposure (lower than previously or commonly tolerated) result in manifestations of the syndrome (i.e. increased sensitivity).
4. The symptoms improve, or resolve completely, when the triggering chemicals are removed.
5. Responses occur to multiple, chemically diverse substances.
6. Symptoms include those derived from multiple organs.

7.2 Suggestion #2

This suggestion includes the requirement for CNS involvement proposed by Lacour *et al*. (2005), and thus may correspond to what some consider to be the most classic aspect of MCS. I am sure that these two suggested case definitions will have much overlap in practice terms, because many will have symptoms derived from multiple organs, one of which is the brain.

1. Symptoms are reproducible with repeated (chemical) exposures.
2. The condition has persisted for a significant period of time.
3. Low levels of exposure (lower than previously or commonly tolerated) result in manifestations of the syndrome (i.e. increased sensitivity).
4. The symptoms improve, or resolve completely, when the triggering chemicals are removed.
5. Responses occur to multiple, chemically diverse substances.
6. Symptoms include those derived from apparent CNS sensitivity, such as chemically elicited headache, fatigue, depression, anxiety, memory and concentration difficulties and confusion and cognitive dysfunction.

There are two additional issues that should be considered when deciding whether a particular patient should be allowed into a study on MCS:

- There is a huge variation in severity among different MCS patients and objective changes that may be obvious in looking at more severe MCS cases may be undiscernible when looking at more modestly affected patients. There is an argument, therefore, that one should limit admission to such studies to perhaps the most affected quarter of such patients, possibly using the Miller Quick Environmental Exposure and Sensitiviy Inventory (QEESI) questionnaire (Miller and Mitzel, 1995; Miller and Prihoda, 1999) to assess severity.
- Another issue is raised by the apparent local nature of chemical reactivity in MCS. If one is, for example, looking at responses in the lungs, one should distinguish between those patients who have asthma-type symptoms from those who do not. Similar divisions should be made for those who appear to be affected in other specific regions of the body.

8 The NO/ONOO$^-$ CYCLE MECHANISM AS THE AETIOLOGICAL MECHANISM FOR MCS AND RELATED ILLNESSES

The many puzzling features of MCS are thought to require a new disease paradigm in order to explain them. This argument has been made by Bronstein (1995), Miller (1999), Rowat (1998) and Arnetz (1999). Even the MCS skeptic Gots (1996) has argued that any physiological explanation for MCS requires such a new disease paradigm. Earlier in this review, an apparently convincing argument has been made that chemicals act as toxicants in MCS, acting via different pathways, but with each producing an increase in NMDA activity. It is well established that NMDA stimulation produces increases in NO and its oxidant product ONOO$^-$ (reviewed in Pall, 2002; Moncada and Bolaños, 2006; Brown and Bal-Price, 2003), so that any or all of these may be involved in generating the properties of MCS.

There are many puzzling features of MCS, each of which must be explained by any proposed new paradigm. One of these is the relationship between MCS and several other related chronic illnesses, including CFS and FM and even PTSD. Several research groups have argued for a common aetiological mechanism for two, three or all four of these illnesses (Miller, 1999; Ziem and Donnay, 1995; Buchwald and Garrity, 1994; Clauw and Chrousos, 1997; Bell *et al*., 1998a; Wessely *et al*., 1999; Yunus, 2001; Pall, 2001a; Pall and Satterlee, 2001; Cohen *et al*., 2002; Buskila and Cohen, 2007). They are all comorbid with each other, they share a large number of symptoms and signs and they all share a common pattern of case initiation: cases of each are often initiated by a short-term stressor, exposure to which is followed by chronic illness. A fourth common

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Table 2 The stressors implicated in the initiation of these illnesses are summarized

Illness	Stressors implicated in initiation of illness
Chronic fatigue syndrome	**Viral infection, bacterial infection, organophosphorus pesticide exposure**, carbon monoxide exposure, ciguatoxin poisoning, physical trauma, severe psychological stress, toxoplasmosis (protozoan) infection, ionizing radiation exposure
Multiple chemical sensitivity	**Volatile organic solvent exposure, organophosphorus/carbamate pesticide exposure**, organochlorine pesticide exposure, pyrethroid exposure; hydrogen sulfide; carbon monoxide; mercury
Fibromyalgia	**Physical trauma (particularly head and neck trauma), viral infection**, bacterial infection, severe psychological stress, pre-existing autoimmune disease
Post-traumatic stress disorder	**Severe psychological stress**, physical (head) trauma

The stressors indicated in bold are the ones most commonly implicated for that specific disease/illness. It should be noted that the majority of such stressors are implicated in the initiation of more than one illness. Modified from the author's web site, with permission.

feature of these illnesses is that cases of each of them are stunningly variable from one patient to another, such that we need an explanation for this variability.

So what is needed, according to this point of view, is a common aetiological mechanism which explains both the similarities and the differences among cases of these illnesses. A detailed model of these four multisystem illnesses is presented below, focussing mainly on how it plays out in MCS, but also outlining how predicted variations may explain all four of these illnesses. Then and only then will the evidence be reviewed, supporting this model for MCS. Much of this discussion comes from the author's web site, with permission, and much of the evidence for it is provided in Pall (2007a) as well as other publications (Pall, 2000; 2002; Pall and Anderson, 2004).

Short-term stressors that are apparent initiators of these four illnesses are summarized in **Table 2**. You will note that each of these illnesses is initiated by multiple stressors and that these initiators include a variety of infections, physical trauma, severe psychological stress, ionizing-radiation exposure and neurotoxins such as ciguatoxin, in addition to the various chemical classes implicated in MCS initiation. These diverse stressors can all act to increase the levels of NO in the body (Pall, 2007a; 2007b; 2008; see above for MCS initiators). While each of these stressors implicated in initiation of one or more illnesses act to increase NO levels, several of these do *not* act via increased NMDA stimulation. Specifically, viral, bacterial and protozoan infections and also ionizing-radiation exposure act via induction of inducible nitric oxide synthase (iNOS) rather than acting via NMDA stimulation; NMDA receptor activation acts, in contrast, by increasing levels of intracellular calcium which stimulates, in turn, the two calcium-dependent nitric oxide synthases (NOSs), neuronal (nNOS) and endothelial (eNOS) (Pall, 2002; Moncada and Bolaños, 2006; Brown and Bal-Price, 2003). Thus it *may* be the case that MCS initiation requires increases in NMDA activity, but it is clear that CFS and FM initiation do not.

How then might short-term increases in NO produce a chronic illness? It can be argued that NO acts via its oxidant product $ONOO^-$ to initiate a complex biochemical vicious cycle that is then the cause of illness (Pall, 2000; 2001a; 2002; 2007a; 2007b), see **Figure 2**. So with each of these we have an initial cause, the short-term stressors, as well as on ongoing cause, with the ongoing cause being responsible for the properties of the chronic illness.

The vicious cycle initiated by these NO increases is shown in **Figure 2** and is centred on excessive levels of NO and its oxidant product $ONOO^-$. This vicious cycle is now being called the NO/$ONOO^-$ cycle (Pall, 2006; 2007a) (pronounced no, oh no!), based on the structures of NO and $ONOO^-$). Each of the arrows in **Figure 2** represents one or more mechanisms by which one element of the cycle acts to increase the levels of another element of the cycle. The chronic nature of these diseases is thought to be caused by the NO/$ONOO^-$ cycle, propagating itself over time through the mechanisms represented by these arrows. Most of the individual mechanisms in the cycle are based on very well-documented biochemistry (Pall, 2000; 2002; 2007a), supporting the plausibility of the cycle as a whole. Cycle elements, as shown in **Figure 2**, include not only NO and $ONOO^-$, but also superoxide, oxidative stress, the transcription factor NF-κB, the inflammatory cytokines (upper right hand corner), all three NOSs (iNOS, nNOS, eNOS), intracellular calcium levels and two types of receptors found in neuronal and non-neuronal cells, the NMDA receptor (Pall, 2007a) and the several of the TRP receptors (see above discussion; only the TRPV1 (vanilloid) receptor is shown in **Figure 2**). There are 22 distinct mechanisms that are represented by the various arrows, of which 19 are

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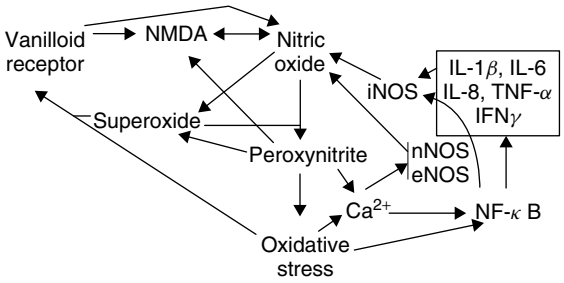

Figure 2 Vicious ($NO/ONOO^-$) cycle diagram. Each arrow represents one or more mechanisms by which the variable at the foot of the arrow can stimulate the level of the variable at the head of the arrow. It can be seen that these arrows form a series of loops that can potentially continue to stimulate each other. An example of this would be that nitric oxide can increase peroxynitrite, which can stimulate oxidative stress, which can stimulate NF-κB, which can increase the production of iNOS, which can, in turn increase nitric oxide. This loop alone constitutes a potential vicious cycle and there are a number of other loops, shown diagramatically in the figure that can collectively make up a much larger vicious cycle. The challenge in these illnesses, according to this view, is to lower this whole pattern of elevations to get back into a normal range. You will note that the cycle not only includes the compounds nitric oxide, superoxide and peroxynitrite, but a series of other elements, including the transcription factor NF-κB, oxidative stress, inflammatory cytokines (in box, upper right), the three different forms of the enzymes that make nitric oxide (the nitric oxide synthases iNOS, nNOS and eNOS), and two neurological receptors, the vanilloid (TRPV1) receptor and the NMDA receptor. (The figure and legend are taken from the author's web site with permission.)

well-established, well-accepted biochemistry and physiology (Pall, 2000; 2002; 2007a; Pall and Anderson, 2004).

Of the other three, there is substantial new evidence for each of them that was not available when that section of the Pall (2007a) book was written. The impact of NO in increasing superoxide generation from the electron-transport chain in mitochondria is now increasingly accepted (Moncada and Higgs, 2006). The effect of oxidants and oxidative stress in increasing activity of TRPV1 (vanilloid receptor) and several other the TRP receptors is also now supported by much more substantial evidence (see above discussion). And Chen *et al*. (2008) have recently provided more evidence on the impact of $ONOO^-$ on the electron-transport chain in the mitochondrion, producing increased superoxide generation. Chen *et al*. (2008) also provides important new evidence on the mechanism involved in producing this increased superoxide generation. Thus all three of the previously more weakly supported mechanisms out of the 22 are now considerably more strongly supported then they were 2.5 years ago. There is a massive amount of evidence supporting the existence of the individual mechanisms proposed to make up the $NO/ONOO^-$ cycle and the only truly original aspect to it is the simple assumption that it fits together in the way that one might assume it does, based on the individual mechanisms.

Much of the mechanism outlined in **Figure 2** is classic inflammatory biochemistry—the NF-κB actions, inflammatory cytokine induction, iNOS induction, leading to increased NO, $ONOO^-$ and oxidative stress, and consequent mitochondrial dysfunction—all of these are found in every inflammatory condition. This raises the question as to whether specific chronic inflammatory diseases, and there are dozens of them, may be $NO/ONOO^-$ cycle diseases?

There are two aspects of the $NO/ONOO^-$ cycle that are not apparent from **Figure 2**. Both add further evidence for important individual mechanisms, as well as the plausibility of the overall cycle:

1. $ONOO^-$, superoxide and NO all can act via known mechanisms to lower mitochondrial function and thus adenosine triphosphate (ATP) generation (Moncada and Bolaños, 2006; Keller *et al*., 1998). $ONOO^-$ is known to attack a number of iron–sulphur proteins, including such proteins that have important roles in both the mitochondrial electron-transport chain and in the citric-acid cycle, and also leads to mitochondrial dysfunction through protein tyrosine nitration and other mechanisms (Radi *et al*., 2002; Cassina and Radi, 1996; Keller *et al*., 1998). $ONOO^-$ is also known to produce nicks in chromosomal DNA, leading in some cases to massive stimulation of poly(ADP)-ribosylation of chromosomal proteins, and because the precursor to such poly(ADP)-ribose synthesis is NAD, this can lead to massive depletion of NAD/NADH pools and consequent lowering of mitochondrial energy metabolism (Szabo, 2003; Moncada and Bolaños, 2006). Superoxide and NO also lower energy metabolism via distinct mechanisms. They both can produce lowered activity of the aconitase enzyme (Gardner *et al*., 1997; Gardner, 1997; Castro *et al*., 1994), as can $ONOO^-$. The cardiolipin in the inner membrane of the mitochondrion is very susceptible to lipid peroxidation and superoxide generated by the electron transport chain in the mitochondrion can indirectly produce major increases in such lipid peroxidation, leading to lowered activity of complexes I, III and IV and therefore lowered ATP generation (Paradies *et al*., 2001; *et al*., 2002; Musatov, 2006). NO is a competitive inhibitor of the enzyme cytochrome oxidase (complex IV) and can therefore lower the activity of the entire mitochondrial electron transport chain by lowering its terminal oxidase activity (Cassina and Radi, 1996; Galkin *et al*., 2007). The lowered ATP generation produced by this combination of mechanisms is not only important in the generation

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of symptoms as a consequence of the NO/ONOO$^-$ cycle, but is also important as part of the proposed cycle itself; NMDA receptor activity is known to be activated by lowered availability of ATP, acting via two distinct mechanisms that are discussed below. Furthermore, the maintenance of low intracellular calcium levels involves much energy utilization via Ca^{+2}-ATPase and thus lowered ATP availability will tend to increase intracellular calcium levels, another predicted aspect of the NO/ONOO$^-$ cycle.

2. There are reciprocal interactions between ONOO$^-$ and a cofactor for the NOSs, tetrahydrobiopterin (BH4). ONOO$^-$ oxidizes BH4, leading to BH4 depletion and such depletion leads to what is called the partial uncoupling of all three NOSs (Pall, 2007b; Milstien and Katusic, 1999; Kohnen *et al*., 2001; Kuhn and Geddes, 2003). The uncoupled NOSs generate superoxide in place of NO. Thus, in tissues and regions of cells with high NOS activity, partial uncoupling leads to adjacent enzymes generating NO and superoxide, thus leading to almost instantaneous synthesis of ONOO$^-$. In this way, partially uncoupled NOS enzymes can act collectively as ONOO$^-$ synthases (Delgado-Esteban *et al*., 2002; Pall, 2007b). The ONOO$^-$ so generated will oxidize more BH4, thus leading to more partial uncoupling. This partial uncoupling may be central to the entire NO/ONOO$^-$ cycle leading to a shift in the ratio of NO to ONOO$^-$. That shift may be critical to the cycle in multiple ways, including generating increased activity of the transcription factor NF-κB; whereas ONOO$^-$ leads to activation of NF-κB, NO lowers NF-κB activity and thus the ratio of the two may be critical in determining the NF-κB regulatory response (Pall, 2007b).

Both of these aspects of the NO/ONOO$^-$ cycle are shown in **Figure 3**, a much more complete figure of the NO/ONOO$^-$ cycle. In it you will see the reciprocal relation between ONOO$^-$ (abbreviated PRN in the figure) and BH4 depletion. You will also see the role of ATP depletion inserted into the figure. One additional apparent aspect of the cycle is shown in the top left corner of **Figure 3**, indicated for the TRP receptors, specifically TRPV1, TRPA1 and TRPM2. TRPV1 and TRPA1 are both activated by the consequences of oxidative stress (Taylor-Clark *et al*., 2008; Bessac *et al*., 2008; Andersson *et al*., 2008; Trevisani *et al*., 2007; Puntambekar *et al*., 2005; Schultz and Ustinova, 1998; Ustinova and Schultz, 1994), as discussed above. The transfer receptor protein TRPM2, discussed above, is strongly activated by oxidants, presumably including ONOO$^-$, with such activation producing an influx of intracellular calcium which is predicted, in turn, to increase NO synthesis. The TRPM2 role in the NO/ONOO$^-$ cycle has not been proposed prior to this publication, but it may well be an important aspect of the cycle mechanism.

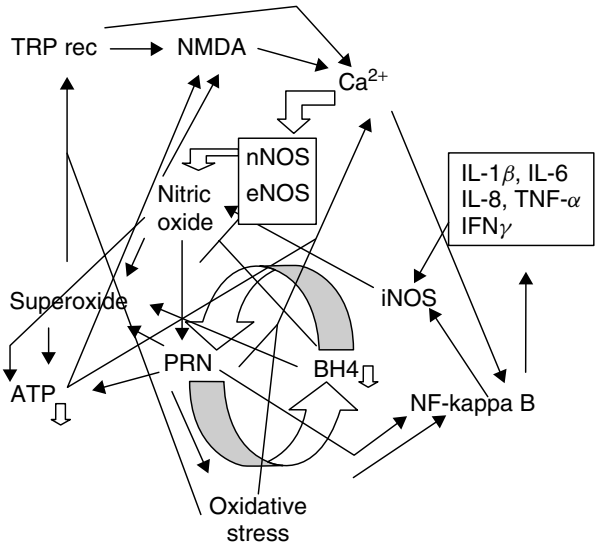

Figure 3 A more complete NO/ONOO$^-$ cycle diagram. Central to the figure are the reciprocal interactions between peroxynitrite, abbreviated as PRN and tetrahydrobiopterin (BH4) depletion. Also indicated is the ATP depletion produced by peroxynitrite, superoxide and nitric oxide. And in the upper left corner, TRP represents the three TRP receptors, TRPV1, TRPA1 and TRPM2, each of which is stimulated via distinct mechanisms by oxidative stress. Each arrow in the figure represents one or more mechanisms by which one element of the cycle stimulates another element of the cycle. (Figure and legend is taken from the author's web site with permission.)

There are three types of generic evidence that support the existence of the NO/ONOO$^-$ cycle (Pall, 2007a). By generic, I mean evidence not linked to any specific disease or illness. These are as follows:

1. Twelve studies have shown that one or both of two drugs that break down to release NO (nitroglycerine and nitroprusside) cause mammalian tissues to synthesize increased amounts of NO via all three NOSs (Chapter 1 in Pall, 2007a). These studies support the existence of a vicious cycle involving all three NOSs, as predicted by the NO/ONOO$^-$ cycle, but do not say anything about other aspects of the cycle.
2. Increased NMDA activity can increase essentially all of the NO/ONOO$^-$ cycle elements that are shown in **Figure 2** (Chapter 3 in Pall, 2007a). NMDA receptor activity directly increases intracellular calcium levels leading to increased NO levels. These studies show that most of the cycle elements can be increased simply by elevating intracellular calcium and NO, thus providing evidence for a cycle similar or identical to the NO/ONOO$^-$ cycle.
3. Hyperalgesia animal models involve all of the cycle elements shown in **Figure 2** in the generation of excessive pain in hyperalgesia (Chapter 3 in Pall,

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2007a). It is difficult to explain this involvement unless the cycle ties all of these elements together.

The NO/ONOO$^-$ cycle aetiology as an explanatory model is based on five distinct principles (Pall, 2006; 2007a; 2007b; Pall and Bedient, 2007):

1. Short-term stressors that initiate cases of multisystem illnesses act by raising NO synthesis and consequent levels of NO and/or other cycle elements.
2. Initiation is converted into a chronic illness via vicious cycle mechanisms, through which chronic elevation of NO and ONOO$^-$ and other cycle elements is produced and maintained. This principle predicts that the various elements of the NO/ONOO$^-$ cycle will be elevated in the chronic phase of illness.
3. Symptoms and signs of these illnesses are generated by elevated levels of NO and/or other important consequences of the proposed mechanism, that is, elevated levels of ONOO$^-$, NO, inflammatory cytokines, oxidative stress, elevated NMDA, TRPV1 receptor activity and/or other aspects of the cycle.
4. Because the compounds involved, NO, superoxide and ONOO$^-$ have quite limited diffusion distances in biological tissues and because the mechanisms involved in the cycle act at the level of individual cells, *the fundamental mechanisms are local*.
5. Therapy should focus on down-regulating NO/ONOO$^-$ cycle biochemistry.

Of these principles, we have discussed 1 and 2 above. Principle 3 predicts that the symptoms and signs of illness can be generated by elevation of one or more elements of the cycle. Some examples of how symptoms and signs of illness may be explained by the cycle are discussed below.

Principle 4 is so important that it takes up an entire chapter (Chapter 4) in my book (Pall, 2007a). Because NO, superoxide and ONOO$^-$, the three chemical compounds most central to the NO/ONOO$^-$ cycle, have relatively short half-lives in biological tissues, they don't diffuse very far from their site of origin in the body. NO has the longest such half-life and it only diffuses about 1 mm from its origin. Furthermore, most of the mechanisms implicated by the arrows act at the cellular level. The consequence of all of this is that the NO/ONOO$^-$ cycle may be elevated in one tissue of the body, but an adjacent tissue may show little elevation and therefore be little impacted by the cycle. This local nature of the cycle biochemistry means that we can have all kinds of variations in tissue impact from one patient to another, leading in turn to all kinds of variation in symptoms and signs from one individual to another. This striking variation in symptoms from one individual to another has been repeatedly been noted in these illnesses and has been one of the great puzzles about this group of illnesses. The variation can be easily explained by the local nature of the NO/ONOO$^-$ cycle mechanism. Principle 4 does *not* suggest that there are no systemic effects, but rather that much of the cycle effects are local.

Principle 5 states that the focus of therapy should be to down-regulate NO/ONOO$^-$ cycle biochemistry. In other words, therapy should focus on lowering the cause of illness, not just on treating symptoms. This is obviously an important principle for both patients suffering from these illnesses and for conscientious physicians trying to treat them. There is much stronger evidence for principle 5 in CFS and FM (discussed below) than in the related illness MCS.

These five principles are important as a group for three distinct but overlapping reasons:

- Taken together, they produce an essentially complete explanatory model.
- The fit to each of the five produces a very different type of evidence for the causality of the cycle. Are cases of the disease/illness started by agents predicted to initiate the cycle? Are cycle elements elevated in the chronic phase of illness? Can the symptoms and signs of illness be generated by one or more the elements of the cycle? Is there evidence for a local mechanism? Can the disease/illness be treated by agents predicted to down-regulate the cycle?
- Because the fit to each of the five gives a very different type of evidence for causality of the cycle, the fit to each of them provides a distinct criterion as to whether a particular disease/illness is a good candidate for being a NO/ONOO$^-$ cycle disease.

What the author has done, in his book and elsewhere, then, is to use these five criteria to ask whether each multisystem illness and also a number of other diseases are good candidates for inclusion under the NO/ONOO$^-$ cycle mechanism. It is the goal, then in a following section of this chapter to go through each of the criteria to see how good the fit is for MCS.

In summary, there are three distinct types of evidence that support the general notion that the NO/ONOO$^-$ cycle mechanism in an important paradigm of human disease.

1. The individual mechanisms of the cycle, represented by the arrows in **Figures 2** and **3**, are almost all well-documented biochemistry and physiology.
2. There are three generic types of evidence for the existence of the cycle, that is evidence not linked to any specific disease or illness.
3. There are a number of diseases/illnesses where one can argue based on the fit to the five principles outlined above, that they are good candidates for inclusion under the NO/ONOO$^-$ cycle paradigm.

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8.1 NO/ONOO$^-$ Cycle Mechanisms for the Generation of Shared Symptoms and Signs of Illness

It has been widely claimed that these multisystem illnesses and even their symptoms are unexplained. Clearly, for the NO/ONOO$^-$ cycle mechanism to be plausible for these multisystem illnesses, it must be possible to explain the symptoms and signs of illness as being generated by one or more elements of the cycle. Such explanations are needed for both the specific symptoms and signs and the shared ones, discussed here (**Table 3**). In Chapter 3 of Pall (2007a), evidence is provided on how these shared symptoms and signs may be generated by the NO/ONOO$^-$ cycle aetiology. The mechanisms listed in **Table 3** are *not presented as established mechanisms in these illnesses*, but they are plausible mechanisms based on substantial scientific information. Each of these only occurs in some multisystem illness sufferers, consistent with the striking variation of symptoms and signs that are a characteristic feature of these illnesses. Indeed it may be argued that the defining symptoms and signs of CFS, MCS, FM and PTSD are found in all sufferers of each of these illnesses because we required them for the diagnosis. In other words, we appear to have a very large spectrum of illness that we have more or less arbitrarily subdivided via particular symptoms.

9 FUSION OF THE NO/ONOO$^-$ CYCLE MECHANISM WITH NEURAL SENSITIZATION AND OTHER PUTATIVE MCS MECHANISMS

While what has become the NO/ONOO$^-$ cycle has produced fairly complete explanations of such illnesses as CFS and FM and also of a number of additional, well-established diseases (Pall, 2007a; Pall and Bedient, 2007), it alone did not produce a compelling explanation for the complexities of MCS (Pall and Satterlee, 2001). It was only when fused with a previous MCS model, the neural sensitization model, that a much more complete explanation became apparent.

Bell and her collaborators (Bell *et al*., 1992; 1999a; 2001a) and others (Antelman, 1994; Rossi, 1996; Friedman, 1994; Sorg and Prasad, 1997) proposed a neural sensitization model, where chemicals were proposed to act to greatly increase neural sensitization in the brain, particularly in the limbic system. The notion here is that if chemicals can act to produce such neural sensitization, greatly increasing the activity of synapses over large regions of the brain, that this could explain the basic mechanism of MCS. In this way, chemicals might generate changes in EEG activity (Lorig *et al*., 1991; Bell *et al*., 1999b; 2001b; Fernandez *et al*., 1999; Muttray *et al*., 1995) and also in brain PET scans (Heuser and Wu, 2001; Hillert *et al*., 2007) and SPECT scans (Simon *et al*., 1994; Heuser *et al*., 1994; Fincher *et al*., 1997a; 1997b) in MCS. There was a New York Academy of Sciences meeting in 2000 that focussed on the proposed neural sensitization mechanism for MCS (Sorg and Bell, 2001) and there is no question that at that time, this neural sensitization view was the most influential view of a possible physiological basis for MCS. Ashford and Miller (1998) listed 10 compelling similarities between MCS and neural sensitization, each of which may be considered to be evidence in favour of a neural sensitization model.

Nevertheless, the neural sensitization interpretation of MCS never generated explanations of how the various classes of chemicals may work nor how the roughly 1000-fold increase in chemical sensitivity that appears to occur in many MCS patients might be generated, nor the similarities to CFS and related illnesses. It did provide a framework for explaining the chronic nature of chemical sensitivity, namely long-term changes in synaptic sensitivity.

The most important mechanism of neural sensitization is that of long term potentiation (LTP), the main mechanism involved in learning and memory. The LTP mechanism is involved on a highly selective basis in strengthening synaptic interactions in the process of learning and memory, and the question raised by its possible role in MCS is what will be the consequences if chemical exposure leads to a massive activation of this process?

In the process of neural sensitization, changes in each synapse involve changes in both the presynaptic and the postsynaptic neurons. LTP is known to involve, as key elements in a complex overall mechanism activated in the postsynaptic neuron, several elements of the NO/ONOO$^-$ cycle, notably NMDA activity, NO and intracellular calcium (Albensi, 2001; Bliss and Collingridge, 1993; Bennett, 2000; Platenik *et al*., 2000; Dineley *et al*., 2001; Prast and Phillippu, 2001; Cotman *et al*., 1988). Superoxide, another cycle element also has a role, albeit a complex one (Knapp and Klann, 2002; Hu *et al*., 2007). Increased NMDA activity in the postsynaptic neuron has a role, as do the increases in intracellular calcium and NO produced by such NMDA stimulation of the postsynaptic neuron (Albensi, 2001; Bliss and Collingridge, 1993; Bennett, 2000; Platenik *et al*., 2000; Dineley *et al*., 2001; Prast and Phillippu, 2001; Cotman *et al*., 1988). NO produced in the postsynaptic neuron, acts as what is called a retrograde messenger, diffusing back to the presynaptic neuron and causing it to be more active in neurotransmitter release, including the release of glutamate, the major physiological agonist of the NMDA

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Table 3 Explanations for symptoms and signs

Symptom/sign	Explanation based on elevated nitric oxide/peroxynitrite theory
Energy metabolism/mitochondrial dysfunction	Inactivation of several proteins in the mitochondrion by peroxynitrite; inhibition of some mitochondrial enzymes by nitric oxide and superoxide; NAD/NADH depletion; cardiolipin oxidation
Oxidative stress	Peroxynitrite, superoxide and other oxidants
PET scan changes	Energy metabolism dysfunction leading to change transport of probe; changes in perfusion by nitric oxide, peroxynitrite and isoprostanes; increased neuronal activity in short-term response to chemical exposure
SPECT scan changes	Depletion of reduced glutathione by oxidative stress; perfusion changes as under PET scan changes
Low NK (natural killer) cell function	Superoxide and other oxidants acting to lower NK cell function
Other immune dysfunction	Sensitivity to oxidative stress; chronic inflammatory cytokine elevation
Elevated cytokines	NF-κB stimulating of the activity of inflammatory cytokine genes
Anxiety	Excessive NMDA activity in the amygdala
Depression	Elevated nitric oxide leading to depression; cytokines and NMDA increases acting in part or in whole via nitric oxide.
Rage	Excessive NMDA activity in the periaqueductal grey region of the mid-brain
Cognitive/learning and memory dysfunction	Lowered energy metabolism in the brain, which is very susceptible to such changes; excessive NMDA activity and nitric oxide levels and their effects of learning and memory
Multiorgan pain	All components of cycle have a role, acting in part through nitric oxide and cyclic guanosine monophosphate (cGMP) elevation
Fatigue	Energy metabolism dysfunction
Sleep disturbance	Sleep impacted by inflammatory cytokines, NF-κB activity and nitric oxide
Orthostatic intolerance	Two mechanisms: nitric oxide-mediated vasodilation leading to blood pooling in the lower body; nitric oxide-mediated sympathetic nervous system dysfunction
Irritable bowel syndrome	Sensitivity and other changes produced by excessive vanilloid and NMDA activity, increased nitric oxide
Intestinal permeabilization leading to food allergies	Permeabilization produced by excessive nitric oxide, inflammatory cytokines, NF-κB activity and peroxynitrite; peroxynitrite acts in part by stimulating poly(ADP)-ribose polymerase activity

Taken from the author's web site with permission. It should be noted that while each of these are plausible mechanisms and, in most cases well-documented mechanisms under some pathophysiological circumstances, in most cases their role in generating these symptoms in these multisystem illnesses is *not* established. The role of reduced glutathione depletion in generating SPECT scan changes is documented in Jacquier-Sarlin *et al*., 1996 and in Suess *et al*., 1991.

receptors (Zhang and Snyder, 1995; Kuriyama and Ohkuma, 1995; Williams, 1996). LTP involves not only increased glutamate release, but also changes in the post-synaptic neuron, making its synapses more sensitive to stimulation.

One point that needs to be made is that we have a striking convergence between the demonstrated role of each of the chemicals implicated in MCS, producing increased NMDA activity, and the essential role of NMDA receptors in LTP. This convergence provides, therefore, for the first time, an explanation for that pattern: only chemicals leading to increased NMDA activity may be expected to produce an up-regulation of the LTP mechanism.

Whereas the normal, highly selective role of LTP in learning and memory will not be expected to involve any substantial NO/ONOO$^-$ cycle elevation, a massive stimulation of NMDA activity over substantial regions of the brain, produced by chemical exposure, will be expected to involve substantial NO/ONOO$^-$ cycle elevation. The extraordinary chemical sensitivity seen in MCS, at least in the CNS-related symptoms, may then be generated by the following multiple mechanisms:

1. Subsequent chemical exposure will stimulate regions of the brain with already existing neural sensitization, with that neural sensitization maintained both by the standard LTP mechanism *and* by the local elevation of the NO/ONOO$^-$ cycle. This combination may be exacerbated by a series of mechanisms, each involving elements of the NO/ONOO$^-$ cycle, as follows.
2. NO acting as a retrograde messenger will act to stimulate further glutamate release by the presynaptic neurons.

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3. Energy metabolism dysfunction produced by $ONOO^-$, superoxide and NO will cause NMDA receptors to be hypersensitive to stimulation. It is known that energy-metabolism dysfunction produces a decreased membrane potential which acts, in turn, to cause the NMDA receptors in such cells to be hypersensitive to stimulation (reviewed in Novelli *et al*., 1988; Schulz *et al*., 1997; Turski and Turski, 1993; Pall, 2002).
4. Energy-metabolism dysfunction also acts on glial cells which normally rapidly lower extracellular glutamate via energy-dependent glutamate transport. Lowered energy metabolism will then lead to increased extracellular glutamate, leading in turn to increased NMDA stimulation (Gadea and Lopez-Colome, 2001; Bliss *et al*., 2004).
5. $ONOO^-$ leads to a partial breakdown of the blood–brain barrier, leading to increased chemical access to the brain (reviewed in Phares *et al*., 2007; Pall, 2002; 2003). Kuklinski *et al*. (2003) have reported blood–brain barrier breakdown in MCS patients and there is also an animal model of MCS in which similar breakdown has been observed (Abdel-Rahman *et al*., 2002; Abu-Qare and Abou-Donia, 2003; Abou-Donia *et al*., 2002b).
6. Many of the chemicals implicated in MCS are metabolized via cytochrome P450 activities and these enzymes are known to be inhibited by NO, thus possibly leading to increased accumulation of the active chemical forms (reviewed in Pall, 2002).
7. Finally TRPV1, TRPA1 and some other TRP receptors are activated through the action of oxidants, as discussed above, and organic solvents and other agents that act via these TRP receptors, such as some mould toxins, may be expected to have increased activity due to such TRP receptor activation.

This combination of multiple mechanisms, each multiplying the actions of the others, is predicted to easily produce the roughly 1000-fold increase in sensitivity that appears to occur in many MCS patients. So we have, for the first time, a hypothesis that explains the last major puzzle in MCS, how one can get this stunning increase in apparent sensitivity to such wide variety of chemicals. Having said that, while each of these mechanisms are individually well-documented and we do have aspects of some of them reported to occur in MCS, there is no currently available evidence that directly and convincingly implicates any of them in producing MCS-related sensitivity. This is not surprising, given the extraordinarily low level of research support that has been available for MCS studies.

10 PERIPHERAL SENSITIVITY MECHANISMS

MCS patients typically not only have central sensitivity symptoms that can be attributed to neural sensitization/NO/$ONOO^-$ cycle mechanisms, but also peripheral sensitivities. They often have chemical sensitivity in the upper respiratory tract, leading to rhinitis symptoms on low-level chemical exposure, they have asthma-type symptoms in response to low-level chemical exposures, they have skin sensitivities, with different patterns of skin involved in different patients, they have gastrointestinal (GI) tract sensitivities and additional organ sensitivity may be seen (Ashford and Miller, 1998). These are likely to be local sensitivity mechanisms distinct from the CNS-derived sensitivity discussed in the preceding section.

Meggs (1994; 1997), Meggs *et al*. (1996) and Bascom *et al*. (1997) and others have described the initiation of cases of RADS, where a type of asthma is initiated by chemical exposure to organic solvents and other irritants and the pattern of chemicals involved is similar or identical to those involved in MCS initiation. RADS is characterized by a wide-ranging chemical sensitivity (Meggs, (1994; 1997); Meggs *et al*., 1996; Bascom *et al*., 1997; Krishna *et al*., 1998), in addition to the more commonly studied sensitivities of asthma, those to allergens, exercise and cold. Not only are organic solvents involved, but several classes of pesticides as well (Proudfoot, 2005; Hernández *et al*., 2008; Proskocil *et al*., 2008; Fryer *et al*., 2004). Sensitization of the bronchi in response to chemical exposure, including organic solvent and pesticide exposure and also other irritants may well be commonly involved in causing occupational asthma (Jeebhay and Quirce, 2007; Gautrin *et al*., 1994). Interestingly, cases of asthma can also be apparently initiated, not only by organic solvents or pesticide chemicals, but also by exposure to mould toxins in mould-infested 'sick buildings', another similarity with MCS (Sahakian *et al*., 2008; Lee, 2003; Mahmoudi and Gershwin, 2000). Thus reactive airways disease can be seen as a common aspect of MCS, with a strikingly similar pattern of chemicals involved in the initiation process.

In addition to RADS, there is also reactive upper airways dysfunction syndrome (RUDS), in which there is chemical sensitivity initiated by previous chemical exposure, producing inflammatory responses in the upper airways, leading to rhinitis symptoms as well as ultrastructural changes (Meggs, 1994; 1997; Meggs *et al*., 1996). Similar to RADS and RUDS, there is also a reactive intestinal dysfunction syndrome (RIDS), where chemical exposure can initiate intestinal chemical sensitivity (Lieberman and Craven, 1998).

Peripheral sensitivity in the skin, lungs, upper respiratory tract, GI tract and other tissues, raises the question

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of how the mechanism of sensitivity may differ from that found in the central sensitivity discussed above? It seems likely, given the similar spectrum of chemicals involved at least in the RADS/airways response, that it also involves an NMDA stimulation pathway. There is evidence for an excessive NMDA role in asthma (Hirota and Lambert, 1996; Overstreet and Djuric, 1999; Dickman *et al*., 2004; Hoang *et al*., 2006; Said *et al*., 2001) and also for an NMDA role in skin-sensitivity responses produced by formaldehyde (Elliott *et al*., 1995; Coderre and Melzack, 1992). In MCS patients, the NMDA antagonist dextromethorphan seems to lower sensitivity responses, not only associated with central sensitivity, but also associated with peripheral sensitivity (Dudley, 1998). Glutamate ingestion of MCS patients appears to trigger symptoms associated with peripheral sensitivities, not just central (Miller and Prihoda, 1999; Ross, 1997). All of these observations suggest an NMDA mechanism in peripheral sensitivity, although the strength of the evidence on this is relatively weak. However, it seems reasonable, given the broad range of chemicals involved in these peripheral sensitivity responses, and the known action of these chemicals as producing NMDA stimulation, that NMDA receptor stimulation may well be involved in peripheral sensitivity, as it is in central sensitivity.

So what mechanisms may be likely to be involved in generating peripheral sensitivity? Clearly, of the seven mechanisms postulated for central sensitivity, one, the breakdown of the blood–brain barrier cannot be involved, and a second, the role of NO acting as a retrograde messenger is unlikely to be involved. The other five, however, may well have a role. And additional mechanisms may also be involved. Meggs has published biopsy studies of chemically sensitive peripheral tissues suggesting that neurogenic inflammation has an important role in generating the sensitivity of these peripheral tissues (Meggs, 1993; 1997; Bascom *et al*., 1997). Neurogenic inflammation may be expected to be generated by elements of the NO/$ONOO^-$ cycle, including TRPV1 activity, NF-κB activity and NO (Leffler *et al*., 2008; Kajekar *et al*., 1995; Yonehara and Yoshimura, 1999; Ruocco *et al*., 2001; Pall and Anderson, 2004; Lieb *et al*., 1997; Lin *et al*., 2007) and because of its inflammatory action, will be expected, in turn to stimulate the cycle. Mast cell activation, an aspect of neurogenic inflammation (Ruocco *et al*., 2001; Hu *et al*., 2008; Costa *et al*., 2008), has been reported to be involved in MCS (Heuser, 2000; 2001), and observations providing further support for mast-cell activation in MCS have been provided by Kimata (2004) and Elberling *et al*. (2007). Such mast-cell activation by chemical exposure may also be expected to act to exacerbate the cycle, through inflammatory cytokine elevation and other mechanisms. Mast-cell activation is reported to be stimulated by TRPV1 activation and also by NF-κB (Hu *et al*., 2008; Kempuraj *et al*., 2003; Lee *et al*., 2007), both NO/$ONOO^-$ cycle elements.

In summary, we have a number of locally acting mechanisms that are expected to act synergistically with each other to produce high levels of peripheral chemical sensitivity:

1. Chemical stimulation of regions of the body with elevated NO/$ONOO^-$ cycle activities.
2. Lowered mitochondrial function leading to increased NMDA receptor activity.
3. Lowered mitochondrial function leading to lowered local glutamate transport and therefore to increased NMDA stimulation.
4. NO inhibition of local cytochrome P450 activity and thus lowered metabolism of chemicals implicated in chemical sensitivity.
5. Local oxidative stress and $ONOO^-$ elevation, leading to increased activity of TRPV1, TRPA1, TRPM2 and possibly other TRP receptor activities, leading to both increased chemical sensitivity via these receptors and amplification of the inflammatory response by TRPM2.
6. Neurogenic inflammation produced, in part, by TRPV1 stimulation and NO, leading in turn to increased inflammation.
7. Mast-cell activation, generated in part by TRPV1 stimulation and NF-κB activity, leading in turn to increased inflammation.

It should be emphasized that while these individual mechanisms are well documented, their causal role in producing local peripheral chemical sensitivity in MCS is undocumented for most mechanisms and needs further substantial study in the others. At this point, they should be viewed as plausible predictions of the NO/$ONOO^-$ cycle fusion model which produce, in turn, plausible explanations of the peripheral sensitivities found in MCS.

11 THE NO/$ONOO^-$ CYCLE MECHANISM AS EXPLAINING PREVIOUSLY UNEXPLAINED MCS PROPERTIES

The title of the author's book *Explaining 'Unexplained Illnesses'* (Pall, 2007a) is obviously a challenge to those who have repeatedly claimed that this whole group of multisystem illnesses is unexplained, and there is no doubt that MCS has been the most challenging of this group of illnesses to explain. Kuhn, in his famous book *The Structure of Scientific Revolutions* makes clear that new scientific paradigms, developed from what he calls 'revolutionary science' (as opposed to 'normal

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science'), are judged in large measure by how well they explain previously unexplained properties of the scientific phenomena to which the paradigm may be expected to apply. That is, one does not only look at the available data and how well it supports the proposed new paradigm, but one needs to look carefully at how well it explains the many relevant, but previously unexplained properties.

Given the previous challenges in explaining MCS, one needs to ask how well the NO/ONOO$^-$ cycle fusion model for MCS explains its many previously puzzling properties. I will go through 12 of these one at a time, using a question-and-answer format. Citations are provided to document issues that were not documented above.

1. How can so many diverse chemicals produce a common response, namely initiating cases of MCS and also eliciting responses in those already chemically sensitive? By acting along different pathways to produce a series of common responses, notably increased NMDA activity, intracellular calcium, NO and ONOO$^-$.
2. Why is MCS chronic? Because the NO/ONOO$^-$ cycle propagates itself over time and probably, in addition, because of long-term changes in the synapses of the brain, leading to neural sensitization.
3. How can MCS patients be so exquisitely sensitive to low-level chemical exposure, with many estimated to be on the order to 1000 times more sensitive than normal? Possibly by a series of mechanisms in the brain predicted to lead to long-term changed neural sensitization, increased short-term sensitization, increased levels of neurotransmitter (glutamate) and increased chemical accumulation. Peripheral sensitivity may involve some of these mechanisms as well and also such mechanisms as neurogenic inflammation and mast cell activation. Two of the transient receptor potential receptors may also have roles in amplifying sensitivity responses. It is through a combination of such mechanisms, acting synergistically with each other, that such high-level sensitivity may be produced.
4. Why is MCS comorbid with such diseases/illness as CFS, FM, PTSD, tinnitus and asthma? Possibly because each of these may be NO/ONOO$^-$-cycle mechanisms and each of them certainly involves elements of the NO/ONOO$^-$ cycle in their aetiology.
5. How can diverse organic solvents be involved in MCS? Probably by stimulating, either directly or through their metabolic products, several of the TRP receptors including the TRPV1 and TRPA1 receptors. This same group of receptors is involved in the SI response to a similar or identical set of organic solvents.
6. Why are symptoms so variable from one patient to another? Because the NO/ONOO$^-$ cycle is fundamentally local, such that one can have both quantitative and qualitative variable tissue impact in different patients. This same mechanism leads to similar variability in cases of CFS, FM and PTSD.
7. Several research groups have reported apparent lowered activity of the porphyrin biosynthetic pathway, leading to accumulation of compounds derived from intermediates at multiple steps in this pathway (Downey, 2001; Matthews, 1998; Morton, 1997; see also Hahn and Bonkovsky, 1997). How can such multiple steps in the pathway be lowered? Probably because of the role of NO in regulating this pathway (Kim *et al*., 1995; Rafferty *et al*., 1996) and possibly because the last step in the pathway is an iron–sulphur protein (Dailey *et al*., 2000) and such iron–sulphur proteins are often inactivated by ONOO$^-$ or NO (Soum *et al*., 2003).
8. How can neurogenic inflammation be involved in MCS? Probably because NO/ONOO$^-$-cycle elements, including TRPV1 receptor activity and NO, can stimulate neurogenic inflammation.
9. How can mast-cell activation be involved in MCS (Pall, 2003)? Probably because both TRPV1 receptor activity and NF-κB can stimulate mast cells.
10. It has been shown that repeated or continuous low-level exposure to organic solvents can lead to desensitization/masking of the MCS response (Ashford and Miller, 1998). What mechanism is involved here? Probably by the lowering of TRPV1 and other TRP receptor activity in response to such exposure to many TRPV1 agonists (Reviewed in Pall and Anderson, 2004; Szallasi, 2002). Interestingly, the TRPA1 receptor, also suggested above to be involved in responding to organic solvents in MCS, is also reported to be down-regulated under these conditions (Akopian *et al*., 2007), consistent with a role for these receptors in masking/desensitization. The desensitization to very small amounts of xenobiotics applied as part of a therapeutic programme (Weaver, 1996; Rea, 1997) may also be produced by this same process.
11. How can moulds in 'sick-building situations' initiate cases of MCS? Probably because mycotoxins produce inflammatory responses and some mycotoxins can stimulate the TRPV1 receptor.
12. How should MCS be treated? Through chemical avoidance and the use of agents that lower aspects of the NO/ONOO$^-$ cycle, including antioxidants, agents that lower NO, ONOO$^-$ and superoxide production, agents that improve mitochondrial function, agents that lower inflammatory biochemistry, agents that lower excitotoxicity, including excessive NMDA activity and agents that help restore BH4.

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It can be seen from the above that there are reasonable explanations derived from the NO/ONOO$^-$ cycle mechanism, as it applies to MCS, for each of these puzzling questions. Previously, as best I can determine, only one of these had a good explanation: the chronic nature of MCS could be explained by the long-term synaptic changes produced by neural sensitization, but, even here, this is probably only part of the explanation and additional NO/ONOO$^-$ cycle mechanisms may be likely to be involved.

12 ANIMAL MODEL DATA ON VARIOUS ASPECTS OF THE PROPOSED NO/ONOO$^-$-CYCLE MECHANISM OF MCS

A whole series of animal models suggested as models for MCS have provided evidence for roles of various aspects of the NO/ONOO$^-$ cycle fusion model as it is proposed to apply to MCS. These include the following.

Sorg *et al*. (1998; 2001) developed a rat model showing cross-sensitization to cocaine and formaldehyde. Cocaine is known to also produce increases in NMDA activity (Laso, 2001; McGinty, 1995), as do the various initiators of cases of MCS. Her studies provide evidence for both neural sensitization and cross-sensitization. von Euler *et al*. (1994) described a similar rat model, using primarily toluene instead of formaldehyde as their main sensitizing agent, that appears to provide evidence for both neural sensitization and cross-sensitization.

Cocaine was also used in a mouse sensitization model which produced convincing evidence for cross-sensitization and increased NMDA activity, as well as an essential role of increased NO in producing the neural sensitization (Balda *et al*., 2008; Itzhak and Martin, (1999; 2000); Itzhak *et al*., 1998; Itzhak, 1995).

Gilbert (2001) reviewed an animal kindling model in response to repeated or high-level exposure to lindane and other similar pesticides, in which neural sensitization leads to overt seizure activity. The mechanism is essentially identical to the mechanism outlined earlier in this paper where pesticide produces decreased $GABA_A$ function, leading in turn to increased NMDA activity, increased subsequent intracellular calcium levels, acting in turn to produce LTP and consequent neural sensitization, leading in this situation to overt seizure activity. Cloutier *et al*. (2006) has also discussed the role of lindane in initiating an animal model for MCS and Adamec (1994) has discussed a different $GABA_A$ antagonist as such an initiator.

The mouse model of Anderson and Anderson (1999a, 1999b, 2003) of all MCS animal models is the one that has been shown to be at least superficially most similar to MCS in humans. It involves sensitization to a number of chemical mixtures implicated in MCS, cross-sensitization among different chemicals and chemical mixtures and also linkage to the SI response.

Willis (2001) described a primate model of central sensitization leading to secondary hyperalgesia and allodynia following repeated injections of capsaicin, the classic TRPV1 agonist. It provides evidence for, not only TRPV1 involvement, but also for NMDA, NO and intracellular calcium involvement, in addition, of course, to neural sensitization. Thus we have evidence of roles for five of the important elements of the model. Similar responses were reported earlier from formaldehyde injections.

Abou-Donia and his colleagues have published the most extensive studies on an animal (rat) model of MCS (Abou-Donia, 2002b). The toxicants they studied were all toxicants that the 1991 Gulf War veterans were exposed to and are therefore potentially involved in the initiation of Gulf War syndrome or illness. The Gulf War syndrome veterans suffer from MCS or an MCS-like illness (Proctor *et al*., 2001; Reid *et al*., 2001; Miller and Prihoda, 1999; Thomas *et al*., 2006), along with symptoms of other multisystem illnesses, CFS, FM and PTSD (Chapter 10, Pall, 2007a). Consequently, this rat model may be considered to be a model both for MCS and for the related Gulf War syndrome.

The specific chemicals studied by Abou-Donia and his colleagues, both individually and in combination, included the carbamate acetylcholinesterase inhibitor, pyridostigmine bromide, the insect repellant and irritant DEET (*N,N*-diethyl-*m*-toluamide) (Schoenig *et al*., 1993; Robbins and Cherniack, 1986), the pyrethroid pesticide, permethrin, depleted uranium and several organophosphorus toxicants. Of these only the depleted uranium is apparently not related to initiators of cases of MCS. In these studies, exposure to these toxicants has been found to produce chronic neurological changes, including neurobehavioural changes and sensorimotor deficits, from high-level exposures or from long-term, subclinical exposures (Abou-Donia, 2003; Abou-Donia *et al*., 2001; 2002a; 2002b; 2004; Abdel-Rahman *et al*., 2004a; 2004b). Even doses that show no signs of overt neurotoxicity produce these real, measurable and chronic neurological changes (Abdel-Rahman *et al*., 2004b).

Among the important physiological changes following chemical exposure are elevation of 3-nitrotyrosine levels, a marker of ONOO$^-$ elevation, oxidative stress as measured by elevation of 8-hydroxy-2′-deoxyguanosine levels, disruption of the blood–brain barrier and elevated NO levels (Abou-Donia *et al*., 2002b; Abu-Qare and Abou-Donia, 2001a; 2001b; 2003; Abu-Qare *et al*., 2001; Abdel-Rahman *et al*., 2002), all predicted consequences of the NO/ONOO$^-$ cycle mechanism.

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Abou-Donia and coworkers reported synergistic interactions of these chemicals (Abou-Donia *et al*., 1996; Abu-Qare and Abou-Donia, 2001a; 2003; Abdel-Rahman *et al*., 2002) and others have found such synergistic effects in animal models as well (reviewed in Research Advisory Committee on Gulf War Veterans Illnesses., 2004). They suggest at least three mechanisms for the synergistic chemical interactions: competition for a cytochrome P450 degradative enzyme (Abu-Qare and Abou-Donia, 2008); partial breakdown of the blood–brain barrier produced by one chemical, leading to increased brain sensitivity to a second chemical (Abou-Qare and Abou-Donia, 2003) and competition for cellular excretion via P-glycoprotein (El-Masry and Abou-Donia, 2006). The author suggests additional possible mechanisms for such synergism, including the synergistic action of different organic solvents, acting as TRPV1 agonists and chemical action along multiple pathways, each leading to increased NMDA activity. The synergistic interactions among chemicals produce great difficulties for toxicologists attempting to estimate the toxicity of complex mixtures of chemicals from the toxicity of the individual components.

Two chemicals and one mixture of chemicals, all implicated in cases of MCS were studied in a mouse model by Fujimaki and colleagues. They demonstrated increases in inflammatory cytokines and reactive airways disease inflammation, as well as changes in CNS neurological activity (Tin-Tin-Win-Shwe *et al*., 2007; Fujimaki *et al*., 2001; 2004; 2007). A causal role of the cytokine IL-6 in the generation of lung inflammation in response to diesel exhaust was demonstrated by comparing an IL-6 gene knockout mouse with the wild-type (Fujimaki *et al*., 2006).

Low-level exposure of several noxious chemicals, including formaldehyde, to mouse skin generated progressive sensitization, leading to both neurogenic inflammation and increased inflammatory cytokine levels (Nakano, 2007).

Fukuyama *et al*. (2008) reported on an MCS mouse model, in which repeated applications of three chemical sensitizers were used to produce sensitivity, followed by a challenge with the same sensitizer. They found that the levels of several inflammatory cytokines were elevated following sensitization and that the challenge produced a much larger cytokine elevation. Thus the pattern of exposure and the response closely parallel the pattern of chemical exposure and subsequent elicitation of sensitivity responses seen in MCS. One of the sensitizers used, TDI is known to be a TRPV1 agonist.

Plitnick *et al*. (2002) showed that the chemical sensitizers, trimellitic anhydride and dinitrochlorobenzene, known to produce airway chemical sensitivity or skin chemical sensitivity, produced increases in some inflammatory cytokines in a mouse model. Harry *et al*. (2002) also showed sensitizer induction of inflammatory cytokine mRNA in glial cells in culture.

It can be seen from the above, that a surprising number of NO/ONOO$^-$ cycle MCS fusion model elements have been found to be involved in MCS animal models. These include both neural sensitization and cross-sensitization between chemicals, as well as progressive sensitization; chemical agents that are known to act by decreasing acetylcholinesterase or $GABA_A$ activity or increasing TRPV1 or sodium channel activity; chemical linkage to the SI response; increases in NMDA activity, NO, ONOO$^-$, oxidative stress, inflammatory cytokines, intracellular calcium, neurogenic inflammation, airways sensitivity and inflammation; and breakdown of the blood–brain barrier. Most, but not all, of these have been shown to have substantial causal roles in the generation of the animal model response. Although we have evidence from these animal models for roles of many features of the NO/ONOO$^-$ cycle mechanism, as it is proposed to apply to MCS, generally, two to five of these aspects have been looked at in each animal model and it is unclear whether any single animal model will involve all of these. However, given the fact that none of these studies have been done to test the NO/ONOO$^-$ cycle mechanism, and funding for such studies has been very limited, there is a surprising amount of data supporting aspects of the cycle mechanism.

13 POSSIBLE SPECIFIC BIOMARKER TESTS? OBJECTIVELY MEASURABLE RESPONSES TO LOW-LEVEL CHEMICAL EXPOSURE

One of the obvious needs in this area of medical research, is the need for one or more specific biomarker tests that can be used to objectively confirm a diagnosis of MCS. There are similar needs for such tests for CFS and FM as well. Because the aetiological mechanism of each of these is thought to be centred on the NO/ONOO$^-$ cycle and the cycle is mostly inflammatory biochemistry, looking at whole-body markers of the consequences of such inflammatory biochemistry will not be useful as a specific biomarker test. There are many dozens of inflammatory diseases, including many chronic inflammatory diseases, so prolonged elevation of such markers will be nonspecific. Furthermore, because such chronic inflammatory diseases are so common, in most cases such markers for MCS patients will often be in the normal range, because typically abnormally elevated levels are usually defined as being two standard deviations above the norm. It is only when one compares sizable groups of MCS patients with controls that one is likely to see statistically significant differences. All of these issues create difficult challenges in trying to develop specific biomarker tests.

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Given these challenges, it may be predicted that specific biomarker tests for any NO/ONOO$^-$ cycle illness must directly or indirectly measure the impact of the cycle on whatever tissue or tissues must be involved in that specific illness. In most cases of MCS, there may be many such tissues, and the obvious way to look at the impact of the cycle on those tissues is to look at the chemical sensitivity responses in one of these tissues. We need to compare the responses of MCS patients with those of controls to low-level chemical exposure, looking at one or more objectively measurable responses. The NO/ONOO$^-$-cycle mechanism predicts that such low-level chemical exposure will produce elevated responses of NO/ONOO$^-$ cycle elements in MCS patients, but little response in normal controls. Alternatively, one might look at the consequences of NO/ONOO$^-$-cycle elevation produced by low-level chemical exposure, rather than specific cycle elements themselves. There have been quite a number of studies reporting elevated responses to low-level chemical exposure in MCS patients, as compared with controls, and this section of the chapter summarizes some of these and compares those reported responses with those predicted from the NO/ONOO$^-$-cycle mechanism of MCS. Studies of neuropsychological changes following low-level chemical exposure will not be reviewed here because the author has no competence to judge such studies.

The most extensive studies of this type are the cough responses studied by Millqvist and her colleagues in response to capsaicin challenge (Johansson *et al*., 2002; 2006; Millqvist, 2000; Ternesten-Hasséus *et al*., 2002; Millqvist *et al*., 2005; 2008). In these repeated studies, MCS patients show much elevated cough responses over normal controls in response to low-level capsaicin challenge. Capsaicin is the classic TRPV1 agonist and because TRPV1 receptor activity is thought, as argued above, to be involved in the responses to many organic solvents and related chemicals, this response appears to be quite consistent with what may be predicted by the NO/ONOO$^-$ cycle mechanism, as it is proposed to play out in MCS. Because the cough response produced by capsaicin is lowered by the use of dextromethorphan and other NMDA antagonists (Kamei *et al*., 1989; Capon *et al*., 1996; Chung, 2005), this pathway of action appears to be identical to that proposed for TRPV1 action in MCS. Millqvist *et al*. (2005) also report substantial increases in nerve growth factor (NG) activity following low-level capsaicin provocation in MCS patients, but not in controls, as predicted by two aspects of the NO/ONOO$^-$ cycle, up-regulation of TRPV1 activity and neurogenic inflammation. These responses are almost certainly local ones, as suggested by Millqvist (2000), so that the minority of MCS sufferers who do not have respiratory tract sensitivity, will not be expected to have such elevated cough responses to such capsaicin provocations.

Hillert *et al*. (2007) reported an interesting brain PET scan study, comparing MCS patients with normal controls both before and after chemical exposure. They used substantial amounts of chemicals for this study, such that both normals and MCS patients showed changes in brain PET scans after chemical exposure, but different changes. Hillert *et al*. (2007) were exploring the hypothesis that the brains of MCS patients might be particularly active in processing odour exposure information in the brain. They found that, whereas two regions of the brain have higher levels of neural activation in response to chemical exposure in MCS patients, as compared with controls, the olfactory processing regions were less responsive in MCS patients vs. controls. So the changes in olfactory processing contradicted their prediction. The two regions showing higher chemically elicited activation in MCS patients were the anterior cingulate cortex and the cuneus-precuneus. The anterior cingulate cortex is part of the limbic system, so the view presented in the current review leads us to ask whether chemical exposure might be expected to produce increased neural sensitization in this region of the brain. The TRPV1 receptor is thought, as discussed above, to often act as a receptor for various organic solvents and related chemicals in MCS, leading one to ask whether the TRPV1 receptor is located in the anterior cingulate cortex. Steenland *et al*. (2006) have found that there are quite high levels of TRPV1 activity in the anterior cingulate cortex, consistent with a local activation by chemicals in this region of the brain. While it is quite possible that this interpretation is oversimplified, it provides us with an interpretation that is compatible with the NO/ONOO$^-$-cycle–neural-sensitization model of what may be happening in the brain to generate MCS-related chemical sensitivity. In any case, the observations of Hillert *et al*. (2007) provide us with an approach to developing a specific biomarker test for MCS-related changes in the brain.

A series of EEG studies have been published in which changes of EEG patterns in MCS patients have been reported in response to low-level chemical exposure, but where normal controls show little or no similar changes (Bell *et al*., 1999b; 2001b; Schwartz *et al*., 1994; Fernandez *et al*., 1999; Lorig *et al*., 1991; Lorig, 1994). These changes, which presumably reflect changes in neural sensitization in MCS, may well provide objectively measurable changes in response to chemical exposure. My own understanding of this area is distinctly limited, so I am unable to give the reader any insights as to the pros and cons of this approach.

Joffres *et al*. (2005) reported increases in skin conductivity in MCS patients, but not in normal controls in response to low-level chemical challenge. Interestingly these skin conductivity increases were more reproducibly linked to the blinded chemical exposures in MCS patients than were there self-reported symptoms. These responses are similar to the responses measured

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in 'lie-detector tests'. The authors suggest that these responses to low-level chemical exposure may reflect a neural sensitization mechanism, indirectly influencing skin conductivity.

Kimata (2004) reported on changes in serum levels of four substances, comparing responses to low-level chemical exposures in normal controls, MCS patients and also in atopic eczema/dermatitis syndrome (AEDS) patients. The chemicals used were outgassed organic solvents in a recently painted room totalling between 3 and 3.5 $mg\,m^{-3}$. The four substances produced in response to chemical exposure were substance P (SP), vasoactive intestinal peptide (VIP), NG and histamine. The basal levels of SP, VIP and NG were elevated in MCS patients and these three, and also histamine, were elevated in the AEDS patients. These can all be viewed as inflammatory markers with SP, VIP and NG being linked to neurogenic inflammation, as suggested by Kimata (2004) and acting to increase mast-cell activation/degranulation and therefore increased histamine levels. All four of these increased in response to low-level chemical exposure in the MCS patients but *not* in either controls or in AEDS patients, although AEDS patients showed elevation of all four vs. normal controls. The increase of any of these in response to low-level chemical exposure may be useful as a possible specific biomarker test for MCS. The responses to low-level chemical exposure seem to be specific to MCS and are not produced by the inflammation seen in AEDS. Based on the data presented by Kimata, perhaps histamine may be the most interesting of these because the basal levels in MCS patients showed little, if any, elevation over normal controls, but low-level chemical exposure produced an almost doubling of these levels. These involve relatively simple serum testing, making these tests perhaps the most easily accessible in the clinical setting. One comment I have is that the data presented by Kimata (2004) show surprisingly consistent basal levels and also levels after chemical exposure from one MCS patient to another. One can't help wondering whether the patients studied here may have had MCS cases of very similar severity and it is possible that other cases with lowered severity may show lowered responsiveness.

Elberling *et al*. (2007) reported that basophils isolated from chemically sensitive patients responded to perfume exposure by releasing elevated amounts of histamine as compared with basophils isolated from normal controls. These results suggest that one can assay sensitivity even at the level of individual cells from sensitive individuals and that histamine release in response to chemical exposure may be a good assay for such sensitivity. It should be noted that the TRPV1 receptor is present on basophils (Planells-Cases *et al*., 2005), as are some other TRP receptors. It is possible, therefore, that sensitivity to chemicals mediated by these receptors might be expressed at the cellular level.

Peden (1996) reviewed studies of nasal lavage to provide objective measurement of irritant-induced nasal inflammation, including studies of multiple chemical sensitivity or sick-building syndrome. Such nasal lavage samples can be used to measure a large number of inflammatory markers, including inflammatory cytokines, NO, eicosanoid mediators, inflammatory neuropeptides and others. Some studies of this type were reported by Koren and Devlin (1992) and Koren *et al*. (1990; 1992), in which chemically sensitive people with rhinitis responses to chemicals reacted to such chemical exposure with increased measurable inflammatory markers in nasal lavage samples. These studies did not compare their results with those of normal controls without such rhinitis responses, but it would be surprising if there would be a similar inflammatory response in such people. Such controls were performed by Hirvonen *et al*. (1999), who showed that chemically sensitive people previously sensitized in a mould-infested building responded to mould exposure with increased inflammatory cytokines and increased NO production, unlike normal subjects, using nasal lavage to measure such responses. This is a good example of how nasal lavage may be used as an objective measure of sensitivity responses in 'sick-building syndrome' situations.

Interestingly, in a series of studies, Hirvonen *et al*. (1997a; 1997b) and Ruotsalainen *et al*. (1995) showed that one could show similar inflammatory responses to mould and other microbial materials in cells in culture, suggesting that such cell-culture responses could be used as a bioassay to isolate and identify materials from these organisms that produce such an inflammatory response.

In summary, these various objectively measurable responses to chemical exposure reflect three distinct predicted aspects of the $NO/ONOO^-$ cycle mechanism. The cough responses reflect a TRPV1 stimulation leading in turn to increased NMDA activity; several of the other tests presumably reflect neural sensitization responses; still others measure inflammatory responses. Many of these are likely to reflect local sensitivity, which may occur in some MCS patients, but not others. This is expected to be the case with the cough responses and nasal lavage measurements. So their possible role as specific biomarker tests may be expected to be limited to those having lung or upper respiratory tract impact, respectively.

As tests to be used in a clinical setting, perhaps the cough response to low-level capsaicin challenge, the nasal lavage tests, and the histamine and other responses studied by Kimata (2004) may be the most easily applied. One or more of these may be used, then, in a clinical context, to provide confirmation of MCS diagnoses initially based on the fit to an accepted case definition.

It is the author's opinion that the published studies suggest that we have a number of promising possible specific biomarker tests and it is essential, in my view,

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that further research be done to establish some of these as specific biomarker tests for MCS to be used for both clinical diagnostic and experimental purposes.

14 PATTERN OF EVIDENCE: FIT TO THE FIVE PRINCIPLES

The five principles underlying the NO/$ONOO^-$ cycle mechanism show how the cycle provides explanations for the wide variety of illness/disease properties. Where there is a good fit to each of the five, one can argue that a particular disease or illness is a good candidate for being caused by the NO/$ONOO^-$ cycle mechanism. In this sense, the five principles function collectively a bit like Koch's postulates. Having described much of the evidence above that is relevant to this issue of fit, it is time to summarize how good the fit is for each of the five principles in the case of MCS. I will not, in most cases, provide citations here, as they have been provided in the preceding sections of this review.

14.1 Short-term Stressors that Initiate Cases of Multisystem Illnesses Act by Raising NO Synthesis and Consequent Levels of NO and/or Other Cycle Elements

Each of the seven classes of chemicals implicated in initiating cases of MCS are known to act to increase NMDA activity and it is known that increased NMDA activity produces, in turn, increases in intracellular calcium, NO and $ONOO^-$. Elevated NMDA activity, intracellular calcium, NO and $ONOO^-$ are all elements of the cycle. It follows that there is an excellent fit to the first principle.

14.2 Initiation is Converted into a Chronic Illness through the Action of Vicious Cycle Mechanisms, through which Chronic Elevation of NO and $ONOO^-$ and Other Cycle Elements is Produced and Maintained

This principle predicts that the various elements of the NO/$ONOO^-$ cycle will be elevated in the chronic phase of illness. Here we need to go through the various elements of the cycle to determine what evidence, if any, is available for their elevation in MCS.

There are numerous types of evidence for elevation of three closely linked elements of the cycle, NO, $ONOO^-$ and oxidative stress (Pall, 2002; 2007a; and see above):

- Several organic solvents implicated in MCS have been shown to produce increases in NO.
- Organophosphorus and carbamate pesticides, through their actions as acetylcholinesterase inactivators, can lead to increased muscarinic activity, which lead in turn to increased NO synthesis.
- Neopterin, a marker of increased iNOS induction (Pall, 2000; Pall and Satterlee, 2001), has been reported to be elevated in the more severely affected MCS patients (Bell *et al*., 1998c).
- Elevated NO has been found in several animal models of MCS and in two of these, it clearly has an essential role in producing the biological response.
- Elevated levels of 3-nitrotyrosine were found in several studies of an MCS animal model and 3-nitrotyrosine is a marker of $ONOO^-$.
- MCS, and the related conditions CFS and FM, have been treated by methods that greatly elevate hydroxocobalamin levels *in vivo*, and hydroxocobalamin is a form of vitamin B_{12} that is known to be a potent NO scavenger. The across-the-board improvement in symptoms suggests that NO has a role, either directly or indirectly, in generating the symptoms of these illnesses.
- It is known that $ONOO^-$ can produce a breakdown of the blood–brain barrier and such breakdown has been reported in both MCS patients and in an animal models of MCS.
- Several types of evidence implicate elevated NMDA receptor activity in MCS and and in related illnesses, including FM. Such elevated NMDA activity is known to produce increases in NO and $ONOO^-$.
- Oxidative stress has been reported in MCS patients (Ionescu *et al*., 1999; Lu *et al*., 2007), as well as in several animal models of MCS. The notion that oxidative stress is central to the pathophysiology of MCS was first explored by Levine (1983a; 1983b) 25 years ago.

There are three types of evidence suggesting that inflammatory cytokine levels are elevated in MCS:

- Nasal lavage studies of MCS patients have reported to have elevated inflammatory cytokine levels and elevated levels of other inflammatory markers.
- Several animal models of MCS have elevated inflammatory cytokines.
- While there have not been any systemic measures of inflammatory cytokines in MCS patients, to my knowledge, there have been multiple such studies of the related illnesses CFS and FM with reported elevations.

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There are 13 distinct types of evidence implicating elevated NMDA activity in MCS; each of the seven classes of chemicals implicated in MCS can act by producing increased NMDA activity and there are also six additional types of evidence. These are all provided in Section 3 of this chapter.

Pall and Anderson (2004) listed 12 distinct types of evidence suggesting that elevated TRPV1 activity has roles in MCS. Ashford and Miller (1998) listed 10 striking similarities between MCS and neural sensitization, each of which can be viewed as evidence for neural sensitization in MCS; the animal model studies implicating neural sensitization provide an additional type of evidence. In addition, several of the putative specific biomarker tests, discussed above, provide support for a neural sensitization mechanism, providing a 12th type of such evidence.

Although there is extensive evidence for mitochondrial/energy metabolism dysfunction in CFS and FM, the only evidence for such dysfunction in MCS is from PET scan studies. Because the probe used in such PET scan studies is a glucose derivative, its transport and accumulation in the tissues is strongly impacted by mitochondrial dysfunction (Pietrini *et al*., 1998; Holthoff *et al*., 2004; Silverman *et al*., 2001).

In summary, although there have been no studies on either NF-κB elevation or BH4 depletion in MCS, to my knowledge, there are a total of 51 distinct published types of evidence supporting the role of one or more aspects of the NO/$ONOO^-$ cycle in the chronic phase of MCS. Given the paucity of research support that has been available for MCS research, that is a surprising amount of evidence!

14.3 Symptoms and Signs of these Illnesses are Generated by Elevated Levels of NO and/or Other Important Consequences of the Proposed Mechanism, that is, Elevated Levels of $ONOO^-$, NO, Inflammatory Cytokines, Oxidative Stress, Elevated NMDA, TRPV1 Receptor Activity and/or Other Aspects of the Cycle

You have seen above and elsewhere (Pall, 2007a) that we can explain a wide variety of symptoms and signs of MCS through the NO/$ONOO^-$ cycle mechanism. While these proposed explanations are based on well-established mechanisms, their roles in MCS and related illnesses should be viewed as plausible, not established.

14.4 Because the Compounds Involved, NO, Superoxide and $ONOO^-$ have Quite Limited Diffusion Distances in Biological Tissues and because the Mechanisms Involved in the Cycle Act at the Level of Individual Cells, the Fundamental Mechanisms are Local

A local mechanism is supported in MCS and related illnesses basically from two distinct types of observations: The stunning variations in symptoms and signs of illness and in overall severity going from one MCS patient to another is difficult to explain without having a local mechanism that can have variable impact among the tissues of the body. Such tissue distribution can be directly visualized in the brain PET scan and SPECT scans studies, which show striking variations from one patient to another.

14.5 Therapy Should Focus on Down-Regulating NO/$ONOO^-$-Cycle Biochemistry

There have been, unfortunately, few studies of therapy for MCS and except for one, these have been at the level of clinical observation and anecdotal reports, rather than clinical trials. The data we have available to ask for possible fit to the fifth principle are limited to the following:

- Clinical trial data on the related illnesses CFS and FM, where much more extensive data is available
- Evidence on causality from animal models of MCS
- A single clinical trial on MCS patients
- A variety of clinical observations and anecdotal reports.

The last of these is discussed in Chapter 15 of Pall (2007a) and will just be referred to here briefly.

Each of these types of observations provides evidence towards a fit to the fifth principle.

The animal model data that was discussed above provides evidence for causal roles of NO, TRPV1 activity and NMDA activity. Each of these types of studies have used agents that relatively specifically lower these activities and provide evidence, in the animal models, for what are, in effect, therapeutic effects of agents that down-regulate these specific aspects of the NO/$ONOO^-$ cycle.

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There are quite a number of clinical trials with CFS and/or FM showing apparent efficacy of agents predicted to down-regulate various aspects of the NO/$ONOO^-$ cycle (**Table 4**). The citations for these clinical trials are provided in Chapter 15, Pall (2007a), except for the more recent trials. These recent trials are for pregabalin, a drug that indirectly lowers excitotoxicity, including NMDA activity (Mease *et al*., 2008; Crofford *et al*., 2005); D-ribose (Teitelbaum *et al*., 2006; Gilula, 2007); and the antioxidant *Ecklonia cava* extract (Bierman, 2008, see also In Focus, 2007).

As can be seen from **Table 4**, of these 16 classes of agents, many have antioxidant properties, providing evidence that oxidative stress has an important causal role in generating these illnesses. Some of these agents either act as NMDA antagonists, or act indirectly to lower NMDA activity, thus providing strong evidence for a causal role of excessive NMDA activity. Carnitine/acetyl carnitine, coenzyme Q10 and possibly hyperbaric oxygen are likely to act to help improve mitochondrial function, thus providing evidence for a causal role of mitochondrial/energy metabolism dysfunction.

The potent NO scavenger, hydroxocobalamin is a form of vitamin B_{12}, but its role is much more likely to involve scavenging NO. In a clinical trial study (Ellis and Nasser, 1973), there was no correlation between initial B_{12} levels and the clinical response. Furthermore, higher doses are needed to get clinical responses here than are needed to treat a B_{12} deficiency. It seems unlikely, therefore, that hydroxocobalamin is acting to allay a B_{12} deficiency. The potent action of hydroxocobalamin as a NO scavenger is sufficiently well established that hydroxocobalamin has been used in experimental settings to establish a role for NO in biological processes (Pall, 2001b).

There is also weaker evidence for two other aspects of the NO/$ONOO^-$ cycle having a causal role. The long chain omega-3 fatty acids in fish oil are well known to have anti-inflammatory aspects, so that their reported efficacy provides some evidence for an inflammatory causal role, although an alternative interpretation to these observations is also possible. High-dose vitamin C and high-dose folate supplements help restore BH4 levels, suggesting a causal role of BH4 depletion, but again, there are other possible interpretations for their actions, so the evidence for BH4 depletion being causal must be viewed as relatively weak.

There are a number of clinical observations suggesting that these same agents are often helpful in MCS treatment, suggesting a possible similar aetiology. The various types of evidence supporting an NO/$ONOO^-$-cycle mechanism for all three of these illnesses (Pall, 2006; 2007a; 2007b) of course also suggest a common aetiological mechanism.

The only relevant clinical trial on MCS patients is that of Heuser and Vojdani (1997), which used high-dose vitamin C therapy and showed objectively measurable improvements in immune function in response to therapy.

In chapter 15, Pall (2007a), I discuss five different protocols that have used multiple agents predicted to down-regulate different aspects of the NO/$ONOO^-$ cycle. Each of these five uses at least 14 agents/classes of agents. Two of these protocols have been tested in clinical trials, one (Teitelbaum's) with both CFS and FM patients and the other (Nicolson's) with CFS-like patients. Each of the five protocols appears to produce substantially better clinical responses than do single agents. This approach may, then, be promising as a general approach to the treatment of these illnesses. Of these, only the Pall/Ziem protocol has been tried on chemically sensitive patients and the generally favourable response to this protocol is described by Dr. Grace Ziem in that chapter.

Subsequently, the author has developed a somewhat different approach to nutritional support of these patients through the Allergy Research Group, containing 22 different agents/classes of agents predicted to down-regulate different aspects of the NO/$ONOO^-$ cycle. Physicians and others using this approach report favourable responses with a large majority of patients with CFS, FM or MCS. In some cases, people who have been ill for two or more decades report rapid improvements within three or four weeks, improvements that are sustained for periods of six months or more, but do not, in general, clearly progress towards complete recovery. Clearly, the reader needs to maintain a high level of scepticism, at this point. These are unpublished observations, they do not constitute anything approaching a clinical trial and the author has a conflict of interest here, receiving some royalties from the Allergy Research Group.

In summary, there are a number of types of evidence that provide some support for the view that agents that down-regulate various aspects of the NO/$ONOO^-$ cycle produce clinical improvement in patients with MCS and in related illnesses. However, there is a great need for much more clinical study of these approaches. Clinical trial data from the related illnesses, CFS and FM, provide substantial support for the view that oxidative stress, excessive NMDA activity and NO all have causal roles; less convincing evidence suggests that inflammatory biochemistry and BH4 depletion also have causal roles in these illnesses. Various aspects of the cycle also are reported to have causal roles in MCS animal models.

15 PSYCHOGENIC CLAIMS

There have been a whole series of papers published arguing that MCS and/or the related multisystem illnesses are not physiological illnesses but are, rather, what has become known as psychogenic, having some often ill-defined psychological or psychiatric origin. These same authors have often argued that MCS should be called idiopathic environmental intolerance, a name that

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Table 4 Clinical trial studies of agents predicted to lower NO/$ONOO^-$ cycle elements in the related illnesses chronic fatigue syndrome and fibromyalgia

Agent or class	Mechanism	Comments
Vitamin C (ascorbic acid)	Chain-breaking antioxidant; lowers NF-κB activity; reported to scavenge peroxynitrite and also help restore tetrahydrobiopterin (BH4) levels by reducing an oxidized derivative of BH4	May require high doses to be effective with the latter two mechanisms; this may be the basis of so-called 'megadose therapy' for vitamin C; clinical trials on CFS and MCS used high-dose IV ascorbate
Magnesium	Lowers NMDA activity and may be useful in improving energy metabolism and ATP utilization	Magnesium is the agent that is most widely studied and found to be useful in the treatment of the multisystem illnesses
Fish oil (long chain omega-3 fatty acids)	Lowers iNOS induction; lowers production of inflammatory eicosonoids; important for brain function	Highly susceptible to lipid peroxidation and may, therefore be depleted; four studies reported improvements in clinical trials, three with CFS and one with FM
Flavonoids	Chain-breaking antioxidants; some scavenge peroxynitrite, some scavenge superoxide; some reported to induce superoxide dismutase (SOD); All three types are found in FlaviNox; some flavonoids may also act to help restore BH4 levels; lower NF-κB activity	Ginkgo extract tested in CFS; anthocyanidin flavonoids in FM; other flavonoids tested in CFS animal model
NMDA antagonists	Lower NMDA activity	Four different antagonists reported to be effective in the treatment of fibromyalgia; anecdotal reports of effectiveness for MCS
Agents that indirectly lower excitotoxicity including NMDA activity	—	Only clinical trials done with pregabalin for fibromyalgia, but other members of this class often used clinically
Acetyl L-carnitine/carnitine	Helps transport fatty acids into mitochondria; may be important here not only directly for energy metabolism but also to restore the oxidized fatty acid residues that may be produced in the cardiolipin of the inner membrane	May also help lower reductive stress; two trials in CFS
Ecklonia cava extract	Polyphenolic chain-breaking antioxidant; reported to help scavenge both peroxynitrite and superoxide; based on its reported properties, it may also help restore BH4 levels	Appears to stay in the body much longer than do the flavonoids, a useful property; reported to be helpful in a clinical trial study of fibromyalgia
Reductive stress relieving agents	These include S-adenosyl methionine (SAM or SAMe), trimethylglycine (betaine), carnitine and choline	SAM reported to be effective in multiple clinical trials with FM and CFS patients; betaine widely used clinically
Hydroxocobalamin form of vitamin B-12	Potent nitric oxide scavenger, lowers nitric oxide levels	Limited intestinal transport; often taken by intramuscular injection or as a nasal spray or inhalant; clinical trial with CFS-like illnesses; widely used for treatment of CFS, FM and MCS
Folic acid	Relatively high doses will lower the partial uncoupling of the nitric oxide synthases by helping to restore tetrahydrobiopterin (BH4)	Reacts with oxidants and therefore may be depleted due to the NO/$ONOO^-$ cycle

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Table 4 (*continued*)

Agent or class	Mechanism	Comments
Algal supplements	Probably act as antioxidants	—
Hyperbaric oxygen	May act to help restore cytochrome oxidase activity by competing with nitric oxide	My impression is that this approach needs to be used with substantial care—too high or prolonged dosage can cause damage
Trimethyl glycine (betaine), S-adenosyl methionine (SAM), choline, carnitine	Lower reductive stress; also helps with the generation of S-adenosyl methionine (SAM)	While lowering reductive stress may be the main concern, SAM generation may also be of concern; the enzyme methionine synthase is inhibited by nitric oxide and inactivated under conditions of oxidative stress, thus leading to lowered SAM and lowered methylation
Coenzyme Q10 (ubiquinone)	Important in mitochondrial function; important antioxidant, especially in mitochondrion; reported to scavenge peroxynitrite	Optimal dosage may vary considerably among different individuals; suggest taking early in day
D-ribose, RNA or inosine	Two important functions: Provides adenosine for restoring adenine nucleotide pools after energy metabolism dysfunction; when catabolized, the purine bases generate uric acid, a peroxynitrite scavenger	Each of these may act somewhat similarly; however only D-ribose has been tested in a clinical trial and reported to be effective; each of these agents has distinct drawbacks

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denies, in effect, that chemicals cause MCS or have a role in eliciting symptoms in people who suffer from MCS. It also denies that we have a mechanism that may explain the many puzzling features of MCS. The name implies that we have neither initiating causes nor ongoing causes of illness.

What this section does, is to briefly and superficially review this field, making many generalizations, some of which may not be adequately supported. To do a thorough review would take a paper considerably longer than this entire chapter, so there is not space nor time to do so. The reader is referred to Chapter 13 in Pall (2007a), which provides a more comprehensive discussion of this area, not just for MCS, but also for CFS and FM. The reader is also strongly encouraged to look at the papers advocating a psychogenic basis for MCS (**Table 5**) and the Davidoff and Fogarty (1994), the Davidoff *et al*. (2000) and the McCampbell (2001) reviews.

From a toxicological perspective, none of these psychogenic advocate papers considers the question of what chemicals are apparently involved in MCS and how they might act as toxicants in the human body. From a toxicological perspective, therefore, they all must be viewed as being flawed. This section outlines the main issues with regard to psychogenesis of MCS that were developed in Chapter 13 in Pall (2007a) and then discusses several of the reviews that have each been written from a psychogenic perspective.

There are, in the author's view (Pall, 2007a), 10 important issues that challenge the positions of psychogenic advocates of MCS and related multisystem diseases and we are considering these here one at a time.

Many such advocates argue that these multisystem illnesses are caused by 'belief' and that they are somatoform disorders generated by a mechanism called somatization. How well founded are these views? Let's consider the basis of somatoform disorders and somatization.

Somatoform disorders are defined (Smith, 1990) as a group of disorders with somatic symptoms that suggest a physical disorder, but for which no organic aetiology can be demonstrated. There is presumptive evidence of a psychological basis for the disorder.

Somatization is defined as a process whereby psychological distress is expressed in physical symptoms (Smith, 1990). So psychogenic advocates typically argue that MCS and the other multisystem illnesses are somatoform disorders generated by the process of somatization. According to its definition, it is incumbent on such psychogenic advocates to demonstrate that *no organic aetiology can be demonstrated*. That is, they not only need to show that no organic aetiology *has* been demonstrated but that none *can* be. This is a very difficult hurdle for them and none of them, to my knowledge, have even tried to jump it. They rarely, if ever, consider the detailed properties of the mechanism proposed here, or the neural sensitization interpretation or the neurogenic inflammation interpretation, nor have they developed a

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Table 5 Publications of MCS skeptics

Gots (1996)	Argues for a psychogenic 'mechanism' for MCS based mainly on dualistic reasoning
Barsky and Borus (1999)	Argues the multisystem illnesses are 'functional somatic syndromes'. Unclear whether this argues for psychogenesis, but paper is often cited by those advocating psychogenesis
Kellner (1994)	Argues that multisystem illnesses are somatoform disorders caused by somatization
Staudenmayer (1999)	Staudenmayer's book makes the longest argument for psychogenesis in MCS
Wessely *et al*. (1999)	Argues that the multisystem illnesses may not be distinct and may share an aetiology possibly centred on psychogenesis
Binder and Campbell (2004)	Similar arguments to Gots (1996), Kellner (1994) and Staudenmayer (1999); considers a broader group of illnesses
Staudenmayer *et al*. (2003a)	Goes through the Hill criteria, asking whether MCS (IEI) can be a physiological illness caused by chemical exposure.
Staudenmayer *et al*. (2003b)	Goes through the Hill criteria, asking whether MCS (IEI) can be a psychogenic illness
Wiesmüller *et al*. (2003)	Another proposal to the effect that these multisystem illnesses may be somatization disorders. While considering these illnesses from a predominantly psychiatric perspective and ignoring physiological, biochemical and animal model data, the authors are much more circumspect about their inferences than are the psychogenic advocates
Hausteiner *et al*. (2007)	A psychiatric interpretation of MCS or what they call IEI.
Eis *et al*. (2008)	Complex psychological study; argues against physiological interpretations while providing no data on them
Das-Munshi *et al*. (2006)	Review of provocation studies in MCS
Das-Munshi *et al*. (2007)	Review of MCS, from a group of psychogenic advocates from the Institute of Psychiatry, Kings College, London

compelling argument ruling out *any possible organic aetiology*.

While it may be argued that they have never even attempted to seriously fulfil this requirement, it is also the case that the very concepts of somatoform disorders and somatization have come under increasing attack (Janca, 2005; Epstein *et al*., 1999; Mayou *et al*., 2005; Dalen, 2003; Bradfield, 2006; Sykes, 2006). There are a number of reasons for this, including the issue that the concept of somatoform disorders and somatization is based on a dualistic view of human beings, where the psychological/psychiatric/mental is separate and distinct from the biological/physiological/physical. The process of somatization assumes that all of the initial causes are on one side of this dualism and somehow reach across the divide to generate physical symptoms. However this Cartesian dualism has been rejected by modern science. For example the American Psychiatric Association (1994) states that 'there is much "physical" in "mental" disorders and much "mental" in "physical" disorders'. Dualistic reasoning has been used repeatedly by advocates of psychogenesis of MCS and other multisystem illnesses and has led them astray in many circumstances. Let us consider an example: a letter published by Black (2002) on the apparent effectiveness of the drug paroxetine in the treatment of MCS. Paroxetine has been shown to lower NOS activity (reviewed in Chapter 6, Pall, 2007a) and is also a serotonin reuptake inhibitor and is a drug that has been used to treat certain psychiatric disorders. Black reports that this drug was effective in the treatment of an MCS patient and in other studies, in two other patients and concludes that, 'This case joins two others in showing *that some patients diagnosed with multiple chemical sensitivity have an underlying psychiatric disorder that, when identified, responds to medication therapy*' (italics added). Black concludes that because paroxetine has been effective in the treatment of some psychiatric diseases, it must be acting to correct a psychiatric flaw in these MCS cases. This is the same logical flaw as if one were to argue that: aspirin cures headaches; aspirin decreases blood clotting; therefore headaches cause blood clotting. The logical flaw here is obvious, but because Black is so immersed in an assumed dualism, he cannot apparently see it. I will provide some additional examples of such dualistic reasoning below.

We have discussed, thus far in this section, three weaknesses that show up in the positions of psychogenic advocates of MCS: that they base their arguments on the concepts of somatoform disorders and somatization, concepts that they have never shown to be adequately supported in MCS and concepts that have been attacked on a theoretical basis as well; that much of their position is based on a rejected dualism between the mental/psychiatric/psychological on the one hand and the physical/biological/physiological on the other; and that

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this rejected dualism has led them, in turn, to make logical flaws. These, then are three substantial flaws underlying psychogenesis—there are others.

Another important issue is that there is a long history of false psychogenic attribution in medicine. In Chapter 13 (Pall, 2007a), there is a discussion of the fact that each of the following diseases has been falsely claimed to have an aetiology that is largely or completely psychological:

1. Multiple sclerosis (MS)
2. Parkinson's disease
3. Lupus
4. Interstitial cystitis
5. Migraine
6. Rheumatoid arthritis
7. Asthma
8. Gastric and duodenal ulcers
9. Ulcerative colitis.

Each of these has been subsequently been shown to be a real physiological disease. Of that list, the psychogenic claim that has been most recently rejected by modern science is number 8, ulcers, for which two Australian physicians, Robin Warren and Barry Marshall won the 2005 Nobel prize in physiology and medicine for showing that the bacterium *Helicobacter pylori* plays a key role in the development of both types of ulcers. Ulcers are a bacterial infectious disease, with ulcers being generated when the inflammation produced by a *Helicobacter pylori* infection becomes sufficiently severe. Ulcers can be treated by a simple antibiotic regimen and this is not a psychogenic illness, as had been confidently claimed for decades.

It is essential, in the author's view, that psychogenic advocates of MCS or other multisystem illnesses show that they are not repeating the same errors that led to false psychogenic claims in the past. However, none of them has ever apparently considered this issue in their publications.

A fifth issue is the role of genetics in dealing with susceptibility to MCS or other multisystem illnesses. There is substantial published evidence for a role of genetics in determining such susceptibility, not only in MCS, but also with CFS, FM and PTSD. The role of specific genes in MCS provides strong support for the inference that chemicals are acting as toxicants in MCS and the role of the CCK-B gene also provides some evidence for a role of the NMDA receptors. Thus the genetic evidence is in very good agreement with the mechanism discussed in this review. The genetics of CFS is also consistent with a $NO/ONOO^-$ cycle mechanism (Chapter 5, Pall, 2007a). But there is a more fundamental issue with a genetic role. Genes act by influencing the structure and amounts of proteins synthesized in the body and by doing so, determine both the physical structure of the body and its biochemical activities. In a dualistic framework, they act to determine the biology and any psychological effect is indirect, produced from the biology. Staudenmayer (1999, p. 20) states that, 'The core supposition of psychogenic theory is that psychological factors are *necessary and sufficient* to account for the clinical presentations of EI [what he calls MCS] patients. Psychogenic theory emphasizes belief, somatization, psychophysiologic stress and anxiety responses, and psychogenic etiology' (italics added). Obviously if psychological factors are *necessary and sufficient*, then there is no room for a genetic role, or for any other biological role. The demonstrated genetic roles in MCS and other multisystem illnesses show that psychological factors are *not* sufficient.

A sixth issue is that psychogenic advocates rarely make clear, testable predictions. The Staudenmayer prediction discussed in the previous paragraph is a rare, perhaps unique, exception to this and as indicated immediately above, the test leads to rejection of the psychogenic hypothesis. The need to make clear, testable (and therefore potentially falsifiable) predictions is essential in science. One of the things that they do, however, is to suggest that because some (but not other) patients with multisystem illnesses clearly suffer from what are classified as psychiatric symptoms, that therefore the multisystem illnesses should be viewed as psychiatric. However, there is a large amount of literature showing that most, perhaps all, serious chronic diseases are characterized as having comorbid psychiatric symptoms, but that does not mean that these serious chronic diseases are psychiatric. The fact that cancer patients and rheumatoid arthritis patients have higher prevalences of PTSD, anxiety and depression, for example, does not make either cancer or rheumatoid arthritis a psychiatric disease.

A seventh issue is that scientists have an obligation to avoid emotion-laden rhetoric and to attempt to provide objective assessments of the scientific literature. Some examples of such emotion-laden statements from the psychogenic advocates are provided elsewhere (Chapter 13 in Pall, 2007a) and will not be repeated here. The focus here is on the need to provide an objective assessment of the literature. Let us consider some specific examples.

The Binder and Campbell (2004) review has relatively brief discussions of several illnesses, including MCS, CFS and FM with relatively few citations provided for each of them. They argue that in these illnesses, cognitive abnormalities are not caused by neurological disease, but rather are caused by 'biological and psychological factors', while concentrating their claims heavily on the psychological side. It is probably reasonable to expect that the relatively few citations on each illness will be carefully chosen to represent some relatively objective assessment of the relevant literature. Let's take a look at some of them here.

On p. 371, Binder and Campbell (2004) argue that the proposed name change from CFS to chronic fatigue and immune dysfunction syndrome was made 'despite the lack of evidence of immune dysfunction in this illness'.

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The only citation provided is that of the psychiatrist and psychogenic advocate Wessely (1997). They would apparently have us believe that the extensive evidence for immune dysfunction in CFS, reviewed, for example, by Komaroff and Buchwald (1998), by Patarca (2001) and by Klimas and Koneru (2007), does not exist because one psychogenic advocate argues that it does not.

In the MCS section of their paper, Binder and Campbell claim that the substances triggering discomfort in people with MCS are 'aromas rather than neurotoxins', citing the psychologist Bolla (2000) as their only documentation for this. They would apparently have us believe that the hundreds of citations showing that organic solvents are neurotoxicants that are cited in Kilburn (1998) or that the many citations showing that pesticides are neurotoxicants cited earlier in this chapter do not exist.

Binder and Campbell (2004) also state that sensitization 'may be initiated by aversive childhood experiences such as sexual abuse', providing Bell *et al*. (1998b) as their only documentation. What Bell *et al*. (1998b) actually report is that girls with a history of sexual abuse were at apparently greater risk for later becoming chemically sensitive, not that it directly initiated cases of MCS. But what is much more important is that they cite this one study as evidence for a possible causal role of sexual abuse in MCS, while completely ignoring the many dozens of studies showing an apparent causal role for chemical exposure in initiation of cases of MCS—and chemical exposure often leads very quickly to the development of MCS symptoms—as compared with the possible role of sexual abuse as a risk factor in the medical history of the patient. This is, unfortunately, a typical example from the psychogenic literature of only citing evidence that can be interpreted as supporting their viewpoint, while completely ignoring massive literature that contradicts it.

Binder and Campbell (2004) also dismiss a number of physiological changes found in MCS and other multisystem illnesses based on these same changes being found in what are classified as psychiatric diseases. For example they state that, 'Neuroendocrine abnormalities are associated with FM and that the illness is caused by abnormal sensory processing. *However emotional problems also are associated with neuroendocrine disorders. We know of no evidence of neuroendocrine abnormalities specific to that condition.* There was evidence of reduced cerebral blood flow in the thalamus and pontine tegmentum in patients with FM, *but similar findings are nonspecific and occur in psychiatric patients*' (italics added). It should be noted that, as discussed above, similar neuroendocrine abnormalities are also reported in FM and CFS, as well. Later in the same paper they state that, 'A fluorine [sic]-deoxyglucose PET study suggested that hypometabolism of the brain stem was found only in CFS and not in depression, *but a study using the same technique found no differences between a group with CFS and a group with somatization disorder*' (italics added). Again similar brain changes are reported in MCS and CFS, albeit with different tissue distribution. In both of these quotes, Binder and Campbell (2004) dismiss any biological significance of objectively measurable physiological changes in these multisystem illnesses, if similar changes are also reported to occur in psychiatric diseases. By their dualistic reasoning, if a physiological change occurs in a psychiatric disease it is forever dismissed as a biologically significant marker in other illnesses, based on some sort of guilt by association. The obvious inference that when these changes are seen in a psychiatric disease, they are important clues as to the pathophysiology of that disease seems to be completely lost on them.

The dualistic reasoning seen with Binder and Campbell is all too common in the psychogenic literature. The Black (2002) letter with its dualistic reasoning is discussed above. Gots' (1996) paper on MCS is essentially all based on such dualistic reasoning. In it he states, 'Stimulation of a neurotransmitter or release of a hormone occurs in response to stimulus. Evidence of response to stress or phobia, such as EEG changes or elevated cortisol levels, helps to describe part of the organic interface between stimulus and response and supplements our knowledge of how the mind produces symptoms. *These responses, however, are not indicative of organic dysfunction and do not eliminate the role of the mind in the phobic or stress response*' (italics added). The author noted (Chapter 13, Pall, 2007a) that, 'Gots would have us believe that because these are produced in response to psychological stress, cortisol or EEG changes are of no organic consequence, incapable of producing organic dysfunction. Taken to its logical conclusion, this same reasoning would have us believe that if a person responds to psychological stress by committing suicide, he or she is not "organically" dead.' Elsewhere in his paper Gots (1996) makes clear where some of his commitment to this discarded dualism comes from stating that, 'Manufacturers cannot be held responsible for responses that depend on psychological processes'. The legal issues of possible liability for the initiation of MCS cases are often discussed in the papers of psychogenic advocates and they consistently argue against any such liability. Could that be related to their roles as 'expert witnesses' in such liability trials?

In a recent MCS review, Das-Munshi *et al*. (2007), referring to a study by Baines *et al*. (2004), stated that 'a recent study suggested that people with MCS showed a nonsignificant trend towards lymphocyte depletion, but this is also known to occur in major depression, possibly as a result of hypercortisolaemia, and widespread immunological differences have also been shown in people with somatization disorders'. In that one sentence they state that the trend towards lymphocyte depletion in MCS patients was nonsignificant, whereas Baines *et al*. (2004) reported it was highly significant ($p<0.001$);

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they also discount the biological significance of this by suggesting that because similar changes occur in two apparent psychiatric diseases, major depression and somatization disorders, this aberration has no biological significance in MCS. So we see again, dualistic reasoning discounting any objective physiological changes if they occur in what are considered to be a psychiatric diseases. There is a third flaw in this sentence—that in what is *not* said. This statement, when coupled to the lack of any discussion of other objectively measurable changes in MCS, suggests that lymphocyte depletion is the only such reported change, when clearly it is not.

One of the papers that was reviewed in Chapter 13 on psychogenesis of Pall (2007a), was a paper by Staudenmayer *et al*. (2003a) raising the issue of whether chemical exposure meets the Hill (1965) criteria for initiation of cases of MCS. Hill, in his paper, stated nine criteria that were proposed to be used to help determine whether a particular environmental stressor or group of stressors might have a causal role in the initiation of some particular illness or disease. The goal here is to distinguish chance association from causation. The idea was not that all of them had to be fulfilled in order to infer probable environmental causation, but that if there was reasonably good evidence for most of them, one might infer such causation. So the question that needs to be raised in the context of MCS is whether chemical exposure is apparently causal in initiating cases of MCS, based on the Hill criteria. This seemed to be an interesting paper to analyse because Ashford and Miller (1998), themselves did an analysis of the Hill criteria as it applies to MCS (pp. 273–276), so it would be interesting to see how Staudenmayer *et al*. (2003a) might deal with these questions. Staudenmayer *et al*. (2003a) concluded (p. 244) that 'toxicogenic theory fails to meet any of the nine Hill criteria'.

The Staudenmayer *et al*. (2003a) paper is surprising in three ways: firstly they were apparently unaware of the previous Ashford and Miller (1998) treatment of this same topic in their very influential book. Secondly Staudenmayer and colleagues either did not know about or did not see the relevance of any of the cited literature that Ashford and Miller (1998) used to support their view that there was substantial evidence for fulfilling six of the nine Hill criteria with regard to chemical causation of MCS. Thirdly, in several cases, Staudenmayer failed to even ask the question that Hill requires them to ask in supposedly examining the case for the nine Hill criteria. Let's go through the first four Hill criteria one at a time to see how the Staudenmayer *et al*. (2003a) treatment compares with the scientific literature that appears to be relevant to these Hill criteria.

The first Hill criterion is strength of association. In this case, is exposure to the types of chemicals suggested to have a role in causing MCS associated with increased incidence of MCS? There are three main types of evidence suggesting such a relationship (Pall, 2007a, pp. 218–220). Firstly, there is the great increase in synthetic organic chemical production (15-fold increase from 1945 and 1980) and also a roughly similar increase in the production of pesticides, following World War II through the 1980s, paralleling the apparent incidence of MCS. One has to say apparent because we have no good epidemiological data before 1980, so we have to rely on surrogates, such as the increasing scientific and medical interest in this field around the world, as possible measures of increased MCS incidence. Secondly, we have the great increase in 'sick building syndrome' situations in the USA following the decreased requirement for indoor air flow that was put into place in 1973, after the first oil shock. By the late 1980s the US Environmental Protection Agency was reporting that fully 50% of the environmental complaints that they had to deal with were 'sick building syndrome' types of complaints (much of this information comes from Ashford and Miller, 1998 and is discussed in Pall, 2007a, pp. 218–220). So we have an apparent parallel, both with regard to increased chemical production and decreased air flow, and apparent increased MCS initiation. A third example is the genetic evidence that genes that determine the rate of metabolism of chemicals can influence the prevalence and therefore incidence of MCS. The only study that was available before Staudenmayer *et al*. (2003a) submitted their paper was the Haley *et al*. (1999) study on PON1, but there is, as discussed above, much more data available now. Staudenmayer *et al*. (2003a) state that there is no evidence for increased incidence of IEI (what they call MCS) with occupational chemical exposure; this is not accurate because Zibrowski and Robertson (2006), McKeown-Eyssen *et al*. (2001) and Maschewsky, (1996; 2002) present some data on this, as discussed above, but it is fair to state that we have very limited data. There is extensive data both on the existence of occupational asthma and the role of chemical exposure in it, and that it is part of the MCS spectrum of sensitivity, but clearly Staudenmayer *et al*. (2003a) are unable or unwilling to see that connection. Staudenmayer *et al*. (2003a) spend most of their discussion on what is supposed to be the first Hill criterion criticizing the prevalence data on MCS, rather than asking the question that must be asked for this Hill criterion—is there an association of chemical exposure with MCS incidence and prevalence, however those may be defined. In the author's judgement, the evidence for the first Hill criterion in the case of chemical causation of MCS is suggestive, but not compelling, with the exception of the more recent genetic evidence, which was not published before the Staudenmayer *et al*. (2003a) paper was submitted. However, to state, as they did, that there is no such evidence is simply incorrect.

The second Hill criterion is consistency: is there a fairly consistent illness or disease pattern that has been described in a variety of different places and circumstances? Similar observations have been made in a variety of countries around the world, including the USA, at least

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nine European countries, Canada, Australia and Japan. As stated by Miller (1997, p. 445) 'numerous investigators from different geographic regions have published strikingly similar descriptions of individuals who report disabling illnesses *after exposure to recognized environmental contaminants*' (italics added). What Staudenmayer *et al*. (2003a) discuss regarding the consistency criterion is whether or not chemical provocation studies in MCS have been properly performed, ignoring the central issue raised by the second Hill criterion.

The third Hill criterion asks whether there is some specificity to the stressors proposed to initiate a specific disease or illness. Here, Staudenmayer *et al*. (2003a) produce the strongest of their arguments with regard to any of the Hill criteria. The chemicals apparently involved have appeared to have little specificity and many of the case definitions, as seen above, discuss them as being 'unrelated' chemicals. There had been only four papers that had been published before the Staudenmayer *et al*. (2003a) paper had been submitted proposing that chemicals might act via increased NMDA activity and/or increased NO and $ONOO^-$, so perhaps it is not unreasonable that they did not consider that possibility. At this point in time, however, it should be clear that there is a substantial argument for specificity through the common response mechanism of NMDA stimulation, even though diverse chemicals are implicated in MCS initiation and in eliciting symptoms in those already sensitive.

The fourth Hill criterion, that of temporality asks, in the context of MCS, whether chemical exposure precedes or follows the initiation of illness. In Chapter 13 of Pall (2007a), the author led the reader to 30 citations that reported that chemical exposure preceded illness initiation, all apparently published before the submission of the Staudenmayer *et al*. (2003a) paper and there are a dozen additional such citations provided in Section 2 of this review; none of these 42 are cited by Staudenmayer *et al*. (2003a) in what they describe as an 'evidence-based review'. These 42 citations are not a comprehensive list of the literature and there are likely to be many other such publications as well. Among the papers ignored by Staudenmayer *et al*. (2003a) is the highly cited Miller and Mitzel (1995) paper, whose title alone implies that it is relevant to this fourth Hill criterion. How do Staudenmayer *et al*. (2003a) support their contention? They cite a single non-peer-reviewed paper by a psychogenic advocate, Terr (1993), published some 10 years earlier; the Terr paper criticizes people studying the physiological basis of MCS, based on their theoretical models and their methodology for studying the effects of chemical exposure on MCS patients. The Terr (1993) paper is, therefore, irrelevant to the issue of temporality—does chemical exposure precede or follow the initiation of illness. The Terr (1993) paper also refers to MCS as if it were an allergy, which clearly it is not.

It is difficult to see how any objective assessment of the literature can come to the conclusion that the fourth Hill criterion is not supported for MCS and the failure of Staudenmayer *et al*. (2003a) to even consider the easily accessible, extensive and obviously relevant scientific literature may be viewed as a sign of their unacceptable bias.

There is not time time nor space here to go through the other five Hill criteria as they relate to MCS, but the reader is referred to the discussion of this in Chapter 13 of Pall (2007a). The reader is also encouraged to read both the original Hill (1965) paper and also the Staudenmayer *et al*. (2003a) paper. The author's own assessment of the Hill criteria is that there is strong evidence for fulfilling six of the Hill criteria for MCS and weaker, but still suggestive, evidence for fulfilling the other three (Chapter 13, Pall, 2007a). Such evidence is not immune from criticism. It is common, as Hill (1965) suggests, that such evidence can be questioned and it is for that reason that it makes sense to weigh the evidence on nine criteria, rather than just a few, to assess the balance of evidence in the complex consideration of possible environmental causation. It is not necessary, according to Hill (1965), to find support for fulfilling all of the nine criteria in order to make a substantial case for environmental causation, but it is the author's view (Chapter 13, Pall, 2007a) that one can do just that for chemical causation of initiation of MCS cases.

Before leaving the issue of possible psychogenesis of MCS, it is essential to discuss the two masked, placebo-controlled provocation (that is controlled-exposure) studies that have been published, which together, to my knowledge, provide the only evidence that is reasonably claimed to positively argue for a psychogenic aetiology of MCS. Although there are only two such studies, given the relative paucity of direct experimental studies on MCS, it is important to look at them carefully. Both of these report on studies where they performed placebo-controlled provocation studies where the exposures were 'masked' by the presence of a presumably benign masking agent, so that the patients would be unable to tell through odour when they were exposed to the chemical. In both studies, the patients were presumably unable to distinguish the chemical exposure from the masking agent alone. One of these studies was published by Staudenmayer, Selner and Buhr (Staudenmayer *et al*., 1993) and the other was published by Smith and Sullivan (2003). Both were reviewed favourably by Das-Munshi *et al*. (2006), a group that has argued for a psychogenic mechanism of MCS and also other multisystem illnesses (Das-Munshi *et al*., 2006; 2007).

The Staudenmayer *et al*. (1993) study has been criticized for three reasons (Miller, 1997; Bell *et al*., 1997; 1999a; Joffres *et al*., 2005): the masking agent used, a heavy amount of mint, is not always benign for MCS patients (Fernandez *et al*., 1999) and therefore may not

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be the neutral masking agent that the authors claim; MCS patients can become desensitized when exposed to various chemicals and these experimenters failed to provide the patients with a substantial period away from such exposures before the provocation challenges were performed; and the patients were not chosen using a standard case definition of MCS, so that there is some question whether they were, in fact, MCS sufferers.

Somewhat surprisingly, the more recent Smith and Sullivan (2003) study may have had somewhat similar problems. Smith and Sullivan tested CFS patients, not MCS patients, and although there is a substantial comorbidity between the two, they did not use, as one would argue they should have, MCS patients who fulfilled a well-accepted case definition for MCS. They do report that their patients had self-reported food sensitivities or chemical sensitivity or both, but food sensitivity is not specific for MCS and is common among CFS patients with no apparent chemical sensitivity. Smith and Sullivan (2003) chose the chemicals to be used as follows: chemical substances chosen by an allergist based on 'clinical criteria and patients subjective responses' were previously tested on each patient until a 'reactive substance' was identified. They give trichloroethane as an example of such a reactive substance, but provide no further information on the chemicals used in this study or their frequencies of use and very little information on dosage. The masking substance used was identified as a substance to which the participants did not react—they give vanilla essence as an example, but do not provide any further information on the masking compounds used. It has been reported that vanillin, the main odourant in vanilla essence, is more of an irritant in MCS patients than in normal controls (Hillert *et al*., 2007), suggesting that it is not a neutral masking agent for MCS patients. Clearly if either the original test of the 'reactive substance' was a false positive or if the test of the possible masking compound was a false negative, the experimental test for that specific patient would have been flawed.

There is no description of any procedure being used in Smith and Sullivan (2003) to prevent desensitization of patients, caused by recent chemical exposures prior to provocation, another possible criticism. The choice of CFS patients rather than MCS patients can be criticized for an additional reason. Classical MCS patients have their symptoms resolve in the absence of chemical exposure, whereas CFS patients do not. Because they used neuropsychological tests to measure reactions here, CFS patients will have at best a low signal-to-noise ratio because of the high level of neuropsychological aberrations before any provocation exposure. Therefore, these patients were not well chosen, in my judgement, for use in such a test, even if they all did have comorbid MCS.

It should be clear that these provocation challenge experiments are complex and difficult to perform with anything approaching a bullet-proof protocol. The point here is *not* that these two experiments are flawed and that all of the experiments that support the conclusion that MCS patients react to low levels of chemicals acting as toxicants have no flaws. Rather it is that we need to maintain a high level of objectivity in analysing these complex experiments. When Das-Munshi *et al*. (2006) conclude that the Staudenmayer *et al*. (1993) and Smith and Sullivan (2003) studies have no flaws, but that all of the studies coming to the opposite conclusion have substantial flaws, their objectivity must be questioned.

16 SUMMARY OF THIS WHOLE AREA OF POSSIBLE PSYCHOGENESIS OF MCS AND OTHER MULTISYSTEM ILLNESSES

- Psychogenic advocates have failed to consider how chemicals implicated in MCS may impact the human body and specifically the human brain.
- They have failed to consider animal models of MCS and what lessons they may carry on the mechanisms of MCS.
- They have failed in most instances to provide anything resembling an objective assessment of the scientific literature about MCS. Given that most psychogenic advocates have clear conflicts of interest, either making large amounts of money testifying as 'expert witnesses' in MCS liability trials or as psychiatrists who may make substantial amounts providing psychiatric treatment for patients with multisystem illnesses, their ability or lack of same to provide an objective assessment of the literature must be subject to careful scrutiny.
- Their interpretation of MCS and other multisystem illnesses is dominated by the view that these illnesses are produced by the beliefs of the patients and that these are somatoform disorders generated by a process called somatization. However, they have failed to provide evidence that there cannot be a physiological explanation for MCS and the basic concepts of somatoform disorders and somatization have come under increasing attack.
- Their approach to MCS and other multisystem illnesses is based on the rejected dualism between the mental/psychological/psychiatric and the physical/biological/physiological.
- Belief in that dualism has apparently led them to make many logically flawed arguments.
- There is a long history of false psychogenic attribution in medicine, making it essential that psychogenic advocates show that they are not simply repeating the errors of the past. They have failed to consider this issue.
- Their argument that psychological factors are necessary and sufficient to explain MCS and other multisystem illnesses is falsified by the genetic data;

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both the specific genes implicated in MCS and their known function provide for such falsification, but also the general finding that genes have a role in determining susceptibility implicates biological factors because genes act by determining the structure and biochemical activities of the body.
- Psychogenic advocates rarely make clear and testable predictions. One of the rare exceptions to this is clearly falsified by the available data.
- Their papers are full of emotion-laden statements.

Each of these ten considerations creates, in my judgement, great challenges for psychogenic advocates of MCS. Clearly the combination of all ten create still more daunting challenges, completely apart from the main thesis of this review on the $NO/ONOO^-$ cycle and the physiological mechanism(s) of MCS.

17 SUMMARY AND AREAS OF GREATEST RESEARCH NEED

This chapter describes a detailed apparent mechanism for MCS, called the $NO/ONOO^-$ cycle, which explains, when fused with neural sensitization, neurogenic inflammation and other mechanisms, the many challenging aspects of this illness that have never been explained previously. Because new scientific paradigms are tested, often largely, by their ability to explain the many previously unexplained aspects of a scientific field, the power of the $NO/ONOO^-$ cycle as an explanatory model is of great importance. It is my view that the power of the $NO/ONOO^-$ cycle mechanism, when fused with the earlier neural sensitization mechanism as an explanatory model in MCS, and the various aspects of the model that are well supported experimentally, support the inference that the overall model is likely to be fundamentally correct. However, it could certainly be wrong in one or more details and is almost certainly incomplete.

This proposed mechanism is supported by well-established mechanisms of action of seven classes of chemicals implicated in initiating cases of MCS, all of which can act to elevate NMDA activity and produce toxic responses in the human body through such NMDA elevation. It provides mechanisms for the generation of symptoms in MCS patients, both symptoms that are shared with such related illnesses as CFS, FM and PTSD and also chemical sensitivity symptoms that are viewed as being specific for MCS. It is supported by observations implicating excessive NMDA activity, excessive NO levels and oxidative stress, neural sensitization, elevated TRP receptor activity, elevated $ONOO^-$ levels and elevated levels of intracellular calcium in people afflicted with MCS, in animal models or both. While there has been little in the way of published studies on therapy for MCS, clinical trial data on the related illnesses CFS and FM provide support for the inference that such aspects as excessive oxidative stress, NO, NMDA activity, mitochondrial dysfunction and possibly inflammation and BH4 depletion have important causal roles in the generation of this group of illnesses. We have some clinical observations suggesting that complex protocols designed to normalize these several parameters can produce substantial rapid improvement in many MCS patients also avoiding chemical exposure, even among patients who have been ill for decades.

Having said that, there are many aspects of this proposed MCS mechanism that need much study. That is not surprising, given the extraordinarily low level of funding that has been available for such studies. Pall (2002) estimated that although MCS has roughly the same prevalence as does diabetes in the USA, the funding available for research on MCS has been approximately 1/1000th of the funding for diabetes. This low level of funding is despite the fact that what little data we have on comorbid diseases for MCS (Baldwin and Bell, 1998; Bell *et al.*, 1995; Baldwin *et al.*, 1997; 1999) and the substantial impact on employment of MCS patients both suggest that the morbidity associated with MCS and its associated comorbid diseases may be comparable to that found as a consequence of diabetes.

The five areas that are in most need of further study, in my judgement, are:

1. Animal model studies testing various aspects of this mechanism that have never been adequately tested.
2. Studies to establish one or more low-level chemical exposure tests as specific biomarker tests for MCS.
3. Clinical trial studies on agents and groups of agents aimed at down-regulating various aspects of the proposed mechanism as potential therapeutic protocols for the treatment of MCS patients.
4. Studies of some of these same agents in placebo-controlled studies to determine if they can lower responses to low-level chemical exposure in MCS patients. These might be done in conjunction with the specific biomarker tests in item 2.
5. Use of bioassays described above to ascertain likely chemicals in the air of mould-infested 'sick buildings' to determine what mycotoxins are involved and what moulds produce them under what culture conditions. Promising methods have been developed for such bioassays (Hirvonen *et al.*, 1997a; 1997b; Ruotsalainen *et al.*, 1995), but we are still plagued by many examples of such 'sick buildings' due in part to our stunning ignorance about the mycotoxins involved and their mechanisms of action.

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NOTES

a. The most important physiological agonist for the NMDA receptors is L-glutamate; NMDA stands for *N*-methyl-D-aspartate, a nonphysiological agonist that is specific for these receptors, not acting as an agonist for other, non-NMDA glutamate receptors.

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Aquatic Toxicology: Concepts, Practice, New Directions

Peter G. Wells

CONTENTS

1 INTRODUCTION

Aquatic toxicology is 'the study of the effects of anthropogenic chemicals and activities, and natural chemicals, materials and activities on aquatic organisms, at various levels of biological organization, from sub-cellular through whole organisms, and to populations, communities and ecosystems' (adapted from Rand, 1995). This chapter introduces the reader to aquatic toxicology as an established subdiscipline of toxicology and environmental science.

1.1 Water Pollution and Its Effects

Aquatic ecosystems and their myriad biota and habitats are subjected to many abuses from the ever expanding human population (6.3 billion persons, circa 2008). The abuses include exposure to toxic substances in harmful amounts, physical disturbance and destruction of habitats. Since the industrial revolution, more than 200 years ago, many substances have poured into waterways causing lethal and sublethal effects to fish and other aquatic organisms, such as invertebrates and water birds, and

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generally disturbing and destroying their habitats. There is also removal of species at rates threatening their populations, for example overfishing, and the introduction of nonindigenous organisms. Massive effects, such as fish kills and water discolouration, occurred in the 1800s at locations such as eastern North America (e.g. New York and Boston Harbours) and the UK (e.g. the River Thames). Gross impacts of pollution have occurred well into the twentieth and twenty-first centuries in many countries worldwide. Owing to such impacts, notably on valuable resource species and often human health, such pollution generated concern for water quality and the condition of rivers, lakes, estuaries, coastal seas and the open ocean. This led to the preparation of chemical and biological methods for studying pollution-stressed organisms and ecosystems, and many technical engineering developments for preventing or reducing industrial discharges. Aquatic toxicology is now an established science.

1.2 History of Aquatic Toxicology: A Perspective

Water pollution has been studied and monitored over the past century. It began to attract serious regulatory attention after World War II, primarily with a focus on controlling oil spills from ships and treating the discharges of major toxic municipal and industrial effluents. In North America, the history and approaches are described by Warren (1971), Cairns and Dickson (1973), Hunn (1989) and Rand *et al*. (1995); in the UK by Lloyd (1992) and most recently by Thompson *et al*. (2005), amongst others; in Europe, by Blanck *et al*. (1978), Albaiges (1989) and Persoone *et al*. (2000); and in Australasia by R. Wu *et al*. in a series in the *Marine Pollution Bulletin* (Wu *et al*., 1999). Serious work was initiated on standardized biological test methods in the 1950s, culminating in the first general aquatic bioassay guide in *Standard Methods*, in 1961 (APHA, 1961; 1995). By the 1970s, aquatic toxicology had emerged as a new and organized subdiscipline of toxicology, with many contributors from Europe and North America. John Sprague's trilogy of papers on the measurement of pollutant toxicity to fish (Sprague, 1969; 1970; 1971) especially placed aquatic toxicology on a solid foundation in the new environmental era.

From the 1970s to the present (circa 2008), many practitioners worldwide have worked to advance the discipline and apply its methods to resolve water pollution problems. The literature is now very extensive, with between 50 000–100 000 papers and hundreds of specialized monographs and textbooks. Exposure and toxicity testing methods are now standardized, many sublethal techniques across all levels of biological organization are available (primarily as research tools), linkages to field observations and biomonitoring have been and are being established, and many special topics (see below), some of them very challenging, are being addressed. New professional meetings and societies have played a large and important role in these advances and in producing important syntheses, notably SETAC (The Society for Environmental Toxicology and Chemistry) since 1979, now in North America, Europe, Australasia, and South America, and the Canadian Annual Aquatic Toxicity Workshop series since 1974. Measurement of aquatic toxicity (acute and chronic) has been incorporated into key environmental legislation in a number of countries and is recognized by key regulatory and standards bodies (e.g. International Maritime Organization, Organization for Economic Cooperation and Development (OECD), International Standards Organization, American Society for Testing and Materials) as a key descriptor to measure, for the protection of aquatic ecosystems. Aquatic toxicity criteria are also incorporated into hazard assessment and ecological risk assessment (ERA) frameworks (Chapman and Long, 1983; Wells and Cote, 1988; EPA (Environmental Protection Agency), 1992; Wells *et al*., 1999; Suter, 2007).

Aquatic toxicology and the broader ecotoxicology, though still young fields, have made major strides towards establishing a theoretical, basic and applied foundation throughout the past 40 years (e.g. see Newman and Clements, 2008; Walker *et al*., 2006). Aquatic toxicology now is global in scope, from research to application. It is increasingly important for resolving major environmental problems, such as those in eastern European countries, with heavy industry emissions into the air and water; in the former Soviet Union (USSR) countries, with oil pipelines, various industries and nuclear wastes; in the rapidly developing, highly populated nations such as Brazil, India and China (Liu and Diamond, 2008); and in the numerous coastal states (pollution from land-based activities, oil inputs from shipping and ship-based activities, harbour and estuarine pollution, see *www.unep.org*).

1.3 Objectives and Scope of Aquatic Toxicology

Aquatic toxicology is concerned with the adverse effects of chemical substances and physical agents, alone and in combination, on aquatic organisms and their ecosystems. It started as an outgrowth of toxicology (see **Basic Elements of Toxicology**), then developed its own methods, approaches and applications. Its objectives are: (i) to understand how chemical and physical agents cause stress(es) on all phyla of aquatic organisms; this includes identifying modes of toxic action and measuring indirect effects of chemicals in and on ecosystems; (ii) to understand the implications of the effects at one level to those at other levels of biological organization; (iii) to understand the similarities and differences in response

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between different organisms in different ecosystems and geographic locations (of major importance, compared with classical toxicology); (iv) to calculate threshold concentrations for safe and unsafe biological effects, as part of the above and (v) to ensure that techniques are available for the practical roles of chemical screening, chemical and effluent regulation, and aquatic biomonitoring.

Hence the scope of the field is huge, encompassing many aquatic disciplines, environmental chemistry, basic toxicology, microbiology, ecology, statistics and law (see key references in Rand, 1995, Chapter 1). The field draws heavily upon classical toxicology, and modern biological and chemical science for its information and techniques, but it has many unique scientific features and wide management responsibilities. To practitioners, its interdisciplinarity and rate of advancement present many challenges.

1.4 Interconnections between Human and Aquatic Ecosystem Health

Making linkages between human health, and aquatic ecosystem health (AEH) and ecological integrity, as measured in aquatic toxicology, is increasingly common (Di Giulio and Monosson, 1996; Rapport *et al*., 1998; Di Giulio and Benson, 2002; Sindermann, 2006) and of undisputed importance in many locations. Contaminated and polluted waterways and coastal ecosystems can be disastrous both to people and to natural biota. One principle of aquatic toxicology is that the protection of AEH, in both surface and groundwater, lends protection to human health by ensuring good-quality drinking water, safe food and control of water-borne vectors of illness or disease. Chemicals accumulated in living aquatic species and habitats may prove toxic to people, for example, methylmercury in fish, such as at Minamata, Japan, in the 1950s; mercury in fish downstream from pulp mills in northwestern Ontario, Canada; arsenic in groundwater in Bangladesh; chlorinated solvents in drinking water; and natural toxins from microalgae in nutrient-rich waters. Other chemicals or mixtures, for example, petroleum hydrocarbons from oils, chlorinated phenols from wood, may impart off-flavours affecting taste and hence the marketability of fisheries products. Waters suffering from overnutrification and eutrophication (Schindler and Vallentyne, 2008) may harbour more algal toxin outbreaks, contaminating shellfish, such as mussels, clams and oysters, killing fish and birds, and threatening human consumers through direct ingestion or inhalation of toxic aerosols. Waters exposed to sewage and other municipal discharges may contain diseased fish and invertebrates, but also may prove threatening to swimmers, sailors and other users (GESAMP, 1990a), causing eye, skin, lung and orifice infections.

During the 1990s, phytoplankton in ship ballast water were implicated as vectors for bacteria that caused cholera in Peru and Brazil. More recently, there is concern about the security of water reservoirs from chemical agents and infectious pathogens, and the health effects of various persistent organics in seafood. Though not always shown as unambiguous cause–effect relationships, the links between water quality and human health are well recognized. In this context, aquatic toxicology contributes substantially to the cleanup, protection and security of water and its living resources.

1.5 The Practice of Aquatic Toxicology

Aquatic toxicology includes basic research with many different types of organisms (bacteria, algae and protozoa, to invertebrates, fish, amphibians and reptiles). Methods are constantly being improved to develop new understanding of how chemicals interact with aquatic species, from contact and uptake, to metabolism, excretion and recovery. For example, 43 of 58 phyla in total, including fossil phyla, are marine, according to Wilson and Peter (1988). There are 19 major groupings of freshwater organisms (World Conservation Monitoring Center (WCMC), 1998). To date, relatively few extant aquatic phyla and species have been tested in experiments for their sensitivity and responses to chemical contaminant exposures.

In addition to basic research on acute and chronic effects of chemicals, there are several practical areas of aquatic toxicology. These include chemical/substance screening and classification; regulatory testing and monitoring of industrial emissions; environmental-effects monitoring (often called biomonitoring) and water quality guidelines (WQGs) and objectives for different water uses. Aquatic toxicity criteria are used in chemical screening and hazard-evaluation systems, where chemicals or formulations are evaluated for their level of acute lethal and chronic sublethal toxicity prior to suitable classification for handling, transport and use. For example, using data provided by industry, and relevant literature, the United Nations GESAMP Working Group on the Evaluation of Harmful Substances Carried by Ships (International Maritime Organization, London, UK) uses two aquatic criteria—acute and chronic—in its hazard evaluation procedure (GESAMP, 2004); acute toxicity ratings are based on data from three types of standard tests (fish, cladoceran and microalgae) requested of industry, and the chronic rating is based on data from the standard reproductive cladoceran test, together with reliable published data on chronic sublethal effects and thresholds (Wells *et al*., 1999; GESAMP, 2004). The OECD is coordinating the harmonization of approaches and methods of aquatic toxicology used in classifying chemicals for transport, a timely example of

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the seriousness with which aquatic toxicity data are now given in scientific, industrial, transportation and legal fora.

In many countries, aquatic toxicity tests or bioassays, and appropriate end points, for example median lethal concentrations (LC_{50}s), are also incorporated into guidelines and regulations for the testing of final effluents at source (Thompson *et al*., 2005). For example, Canada initiated this approach in the early 1970s and has used it extensively to control highly toxic industrial effluents. The OECD countries evaluated and adopted the approach after an international conference in 1984 (Environment Canada, 1984; OECD, 1987). This was a landmark event, as it emphasized the importance of pollution control at source using biological tests, rather than control only after demonstrating the presence of toxic chemicals and harm in receiving waters, analogous to prevention vs. treatment in medicine. Aquatic toxicity tests and data, combined with exposure chemistry, are now used to evaluate and control the quality of industrial discharges in many countries, and the number is growing all the time (Thompson *et al*., 2005).

Aquatic toxicity measurements, largely sublethal and biochemical, also are used in biological monitoring under natural conditions. This field is also known as biomonitoring or biological-effects monitoring. Applications are effluent regulation (Mount, 1980; Servos, 1995), research looking for unexpected sublethal effects, such as using mussels and other organisms in field bags (Salazar, 1989; Salazar and Salazar, 1993), and chemical exposure and risk assessment (Chase *et al*., 2001; Suter, 2007). This is the growing field of biological indicators or bioindicators. Biological effects can be measured from field-collected organisms, from caged organisms or from *in vitro* and *in vivo* testing of environmental extracts (Hylland *et al*., 2006). Finally, aquatic toxicity data are used to establish WQGs for various uses of water and water bodies, from industrial and agricultural to conservation.

1.6 Aquatic Toxicology and the Law

Since the 1970s, a number of Western and Australasian countries have had aquatic toxicity criteria and pass–fail levels built into their regulations under their national water-pollution legislation (Thompson *et al*., 2005). For example, the USA has bioassay criteria under its NPDES (National Pollutant Discharge Elimination System) permit programme, requiring routine biomonitoring of industrial discharges (see *http://www.epa.gov/waterscience/guide/effects.html*, accessed 3 June 2008). Canada has used its Fisheries Act in this regard since 1971, with at least six industrial-sector regulations and guidelines; the rainbow trout (*Oncorhynchus mykiss,* formerly *Salmo gairdneri* Richardson) acute toxicity test has been the mainstay of the legally valid and recognized tests in Canada. Canadian courts generally recognize that a material shown in an acute aquatic toxicity test to be toxic to fish (i.e. lethal to trout) indicates a degree of harm or potential harm to aquatic systems and, by legal definition, the material is deemed to be a deleterious substance. Canada now has over 15 toxicity tests, called biological tests, developed by an expert technical group and recognized by federal and provincial regulatory authorities. It is, however, proving to be a long process to have the courts accept data other than from established fish (salmonid) and crustacean (cladoceran) tests in criminal trials, except under unusual circumstances, such as large spills of chemicals or oils. As well, litigation against polluting industries is less frequent than in the 1970s.

In Europe, the UK uses fish (salmonid) and decapod crustacean (brown shrimp, *Crangon* spp.) tests for effluent testing, sediment testing, legal purposes and the evaluation of particular substances, such as oil spill dispersants (Thompson *et al*., 2005). The PARCOM (Paris–Oslo Commission) employs several toxicity tests for controlling the disposal of harbour sediments, industrial chemicals and substances from the offshore oil sector (drilling muds, cuttings and production water). Internationally, there is an increased acceptance of toxicity test data in national legal regimes, but except for ocean dumping (disposal) under the London Convention and chemical screening under the MARPOL 73/78 Convention, there are few formal guidance frameworks for the international application of aquatic toxicity tests.

1.7 This Chapter

This chapter presents a broad overview of some of the principles, practice and new developments in aquatic toxicology. Extensive detailed treatments are found in Rand and Petrocelli (1985), Abel (1989; 1996), Munawar *et al*. (1989a), Sprague (1990), Landis and Yu (1995; 1999), Newman (1995), Rand (1995, in revision 2008), Rand *et al*. (1995), Wells *et al*. (1998), Mothersill and Austin (2003), Blaise and Ferard (2005a; 2005b), Den Besten and Munawar (2005), Mommsen and Moon (2005), Ostrander (2005), Thompson *et al*. (2005), Hylland *et al*. (2006), Sindermann (2006), Vethaak *et al*. (2006), Farris and Van Hassel (2007) and Di Giulio and Hinton (2008).

Ecotoxicology, generally defined as 'the application of toxicology to the natural environment' (Park, 2007) or the study of the effects of chemicals on natural organisms and ecosystems (M. Depledge, personal communication), encompasses aquatic toxicology and also has a growing literature. Notable recent texts include Forbes and Forbes (1994), Hoffman *et al*. (1995), Newman and Jagoe (1996), Walker *et al*. (1996; 2001), Newman (1998), Schuurmann and Markert (1998), Shaw and Chadwick (1998), Hoffman *et al*. (2003), Landis and Yu (2004),

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Helma (2005), Yu (2005), Ekins (2007) and Newman and Clements (2008), useful for both advanced students and professionals alike. New syntheses and specialized texts are being written frequently by the field's leaders, illustrating ecotoxicology's breadth, vitality, innovation and importance.

2 CONCEPTS AND PRINCIPLES

2.1 Definitions

Some key terms are essential for understanding the concepts of aquatic toxicology. The sources are GESAMP (2004), Pesch and Wells (2004) and Park (2007), with some minor changes.

Acute (aquatic) toxicity: Adverse effects that occur rapidly as a result of short-term exposure to a chemical or physical agent. In fish and other aquatic organisms, effects that occur within a few hours, days or weeks are considered acute. Generally, acute effects are severe, mortality being the most common one measured in aquatic organisms. A chemical is considered acutely toxic if by its direct action it kills 50% or more of the exposed population of test organisms in a relatively short period of time, such as 24–96 hours to 14 days.

Bioaccumulation: General term describing a process by which chemicals are taken up by aquatic organisms directly from water as well as through exposure by other routes, such as consumption of food and sediment containing the chemical(s). It results in the presence of the chemical(s) in the organism's tissue(s).

Bioconcentration: A process by which there is a net accumulation of a chemical directly from water into aquatic organisms, resulting from simultaneous uptake (e.g. by respiratory or epithelial tissue) and elimination.

Bioconcentration factor (BCF): A term describing the degree to which a chemical can be concentrated in the tissues of an organism in the aquatic environment as a result of exposure to water-borne chemical. At steady state during the uptake phase of a bioconcentration test, the BCF is a value which is equal to the concentration of a chemical in one or more tissues of the exposed aquatic organisms, divided by the average exposure water concentration of the chemical in the test.

Biomagnification: Result of the processes of bioconcentration and bioaccumulation by which tissue concentrations of bioaccumulated chemicals increase as the chemical passes up through two or more trophic levels. The term implies an efficient transfer of chemical from food to consumer, so that residue concentrations increase systematically from one trophic level to the next.

Chronic toxicity: Effects resulting from repeated or continuous exposure to a material for the lifespan of the species, or the greater part thereof.

Contaminants: Potentially harmful substances or agents in the natural environment that are present at concentrations above background natural levels, and below levels known to cause adverse effects.

Median effective concentration (EC_{50}): The concentration of a material which produces a 50% response in a defined end point in a population of organisms. The EC_{50} should be cited for a specific exposure period.

Inhibition concentration (IC): A point estimate of a chemical concentration that would cause a given percentage reduction (e.g. IC_{50}) in a nonlethal biological measurement of the test organisms, such as reproduction or growth. The IC_{50} should be cited for the specific exposure period, and for continuous rather than quantal (all-or-none) data.

Median lethal concentration (LC_{50}): The concentration in a solution which causes 50% mortality of the test species (usually for an acute exposure). It is calculated from the incidence of mortalities at various concentrations to which different groups of the test species are exposed. Since mortality will depend on the time of exposure, the LC_{50} should be cited for the specific exposure period. Previously referred to as the median tolerance limit (TL_m).

Log P_{ow}: See octanol–water partition coefficient.

Mechanism of toxicity: The way in which a chemical alters basic biological functions and structures in order to exert its toxic effect(s).

Microcosm: A small model of an ecosystem that incorporates some, but not all, of the parts and interactions of the natural system of interest.

Mesocosm: A system of large tanks, inside or outside a laboratory, or field enclosures in ponds, lakes or coastal waters, set up for the purposes of studying the fate and effects of chemicals, physical agents, natural variables or their combinations, under more natural conditions than achieved in smaller testing units.

Monitoring: Measuring the levels of substances in environmental media and living organisms and/or their effects, over time and space.

No observed effect concentration (NOEC): The highest concentration of a chemical or material in a toxicity test that has no statistically significant adverse effect on the exposed population of test organisms compared with the controls. When derived from a lifecycle or partial lifecycle test, it is numerically the same as the lower limit of the maximum acceptable toxicant concentration (MATC). Also called a no observed adverse-effect level (NOAEL) or no observed effect level (NOEL).

Octanol–water partition coefficient (K_{ow}): The ratio of a chemical's solubility in *n*-octanol and water at steady state; also expressed as P. The logarithm of P or K_{ow} (i.e. $\log P$ or K_{ow}) is used as an indication of a chemical's propensity for bioconcentration and bioaccumulation by aquatic organisms.

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Pollutant(s): Chemical or physical agents that cause adverse or harmful effects to organisms (plants and animals); to be distinguished from contaminants.
Subchronic (aquatic) toxicity: Adverse effects on aquatic organisms that occur largely from continuous long-term exposure to a chemical or other potentially toxic material or agent, alone or in combination, but where the exposure time covers only a portion of the life cycle (lifespan) of the aquatic species tested or exposed naturally. The effects may be the result of a single exposure (e.g. to a strong acid), but more often they are the consequence of repeated or continuous long-term exposures. Subchronic toxic effects may be lethal or sublethal.
Sublethal concentration: Concentrations of substances or agents above background levels that can cause sublethal (i.e. nonlethal) effects on organisms or their populations, such as delayed growth and development, impaired behaviour or abnormal embryonic development. Some sublethal effects can eventually be lethal.
Threshold level/concentration: A concentration above which some effect or response will be produced and below which it will not.
Toxic: Capable of causing adverse effects which are detrimental to the survival or normal functioning of the individual organism.
Toxic substances or chemicals: Chemicals (single or as mixtures) that are poisonous, carcinogenic or otherwise directly harmful to plants and animals at low levels.
Toxicity identification evaluation (TIE): A set of procedures, formalized by the US Environmental Protection Agency (EPA), to identify the specific chemicals or physicochemical variables responsible for the toxicity of effluents or other complex mixtures. The approach can be used to study sediment-borne chemicals.
Water-quality objectives: Numerical concentration limit or narrative statement that has been negotiated to support and protect the designated uses of water at a specified site. An objective is more specific and location-dependent than a guideline.

Also see glossaries in Klaassen (2001), Lee (2005), Yu (2005) and Rand (2009).

2.2 Approaches to Assessing Chemicals in Water Bodies

Aquatic toxicologists use a range of approaches in the assessment of the toxicity of chemicals, industrial formulations, other mixtures of chemicals and sediments. Approaches include laboratory experiments, microcosms and mesocosms, natural field situations, accidental spills and experimental field-exposure experiments (Wells, 1984b; 1989; Rand, 1995). The choice of approach and toxicity end point depend upon the questions being asked, hypotheses being formulated, and available time and resources for the assessment (Sprague and Fogels, 1977, among others). Fate-and-effects modelling supplements all of these approaches and facilitates predictions of types, magnitudes and duration of toxic effects (D. French, personal communication).

By far the most widely used aquatic toxicity tests are laboratory techniques, largely conducted with populations of single species, cultured or collected from the wild. The responses of one or more species exposed alone to chemical treatments are measured and compared, in contrast to true multispecies assays where the responses of, and the interactions between, different species are studied in the same exposure system. Single-species tests include all of the current standardized regulatory and monitoring toxicity tests used commonly in North America, Europe and Australasia (Rand, 1995; Wells *et al*., 1999; 1998; Landis and Yu, 1999; 2004; Thompson *et al*., 2005). Such tests are also used extensively in aquatic research, including species from a few other phyla or groups, for example, specific corals, polychaete worms, molluscs, crustaceans, echinoderms and teleosts. Such laboratory tests with a few key or representative species are also the backbone of research into the responses of organisms at the suborganism level, for example biochemical, cellular, tissue and organ, and the organism level (from behaviour and physiology, to reproduction, growth and development). The standard tests adopted by agencies are the simplest and most cost-effective tests to conduct, and their use has produced most of the current data and information bases on effects and threshold levels of chemicals on aquatic organisms. For example, the information bases for salmonid fish and cladocerans are huge. Ecological realism has been sacrificed for a host of practical attributes of each test method, such as control over exposure composition and time, condition and sensitivity of the test organisms and the ease of measuring and recording responses. Microscale approaches, with automation, have evolved for these reasons, and others (see below), for powerful experimental and applied toxicity testing (Wells *et al*., 1998; Persoone *et al*., 2000; Blaise and Ferard, 2005a; 2005b).

Microcosms (small volumes, in small tanks or enclosures) and mesocosms (larger volumes, in large tanks or field enclosures) permit more realistic exposure concentrations and fates, the exposure of multiple species at different trophic levels and consequently more accurate estimates of toxicity under natural conditions (see Reeve *et al*., 1977, for marine examples). Both approaches have, for example, been used extensively in the study of petroleum hydrocarbons, oil-spill control agents, metals, and herbicides and pesticides, addressing the fate of constituents and mixtures in the sediment, water and air compartments, and the effects of chronic exposures and responses of pelagic and benthic aquatic communities. They are valuable but expensive, logistically complex, research techniques. Despite hazard evaluations

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and ERAs being more realistic with these systems, their wide appeal to regulators has yet to be realized, beyond their use for assessing a few high-production pesticides, oils and constituents, and oil-spill control agents.

Studies under natural conditions have the most realistic exposure concentrations and compositions, but these are often poorly known and difficult to control and replicate, making detection and predictions of effects and clear establishment of exposure (i.e. causal) effect relationships a challenge. A recent example is the large North Sea experiment on pelagic ecosystems (Hylland *et al*., 2006); a suite of techniques showed deleterious effects of water quality near the coastline. Ultimately, such information, that is, effects of the chemicals or emissions in the receiving water bodies, is a valuable test of risk predictions and efficacy of regulatory strategies (emissions control, cleanup, recycling, replacement and conservation). Such field-oriented approaches are supplemented with valuable observations and measurements at accidental spill sites, for example, oil spills in marine waters, nuclear waste releases from sunken reactors and at experimental exposure sites, for example, oils and pesticides. However, the latter, with a few exceptions (Stokstad, 2008), have largely lost their appeal to practitioners in favour of less damaging and less expensive approaches.

2.3 Linking External Exposure, Bioavailability and Estimates of Toxicity

Whereas toxicologists dose organisms such as insects, birds and mammals directly (via oral intake, skin coatings, skin penetration or vapour inhalation), aquatic organisms receive their exposures through contact with the water column, sediment particles, ingested food (including particles) or combinations thereof. Hence, a challenge of aquatic toxicology has been to establish and control accurate chemical exposures during toxicity tests and experiments, to relate estimates of external exposure to bioavailable chemical fractions and concentrations (the so-called body residues), and to link these to indicators of toxic potency or effects. This linked sequence is necessary to establish realistic and accurate dose–response relationships and toxicity thresholds for organisms living in water (McCarty and Mackay, 1993; Beek, 2000).

Following the experiences of classical toxicology, aquatic biologists also adopted similar experimental designs and statistical treatments of exposure–response data (e.g. probit analysis, Litchfield, 1949; Litchfield and Wilcoxon, 1949; Finney, 1952; Newman, 1995). However, in so doing, they equated external water-borne and particle-borne exposures to internal doses acting at the site(s) of toxic action, assuming equivalency. This approach has at least two major limitations which have contributed to much of the variability in reported aquatic toxicity estimates for chemicals, derived using different species and water conditions. First, for many chemicals, especially those which are hydrophobic (i.e. high log P_{ow}s), external exposure (composition and concentrations) and uptake are subjected to many physicochemical and biological influences. Secondly, what is ultimately taken up by each organism more closely resembles the substance(s) causing the toxicity, providing it moves to the site(s) of toxic action rapidly and unaltered. However, this 'residue' may be subject to change through metabolism and may not resemble the substance(s) ultimately causing the effects. This situation has been long recognized, but was addressed clearly by McCarty and Mackay (1993), following studies by Abernethy *et al*. (1986; 1988). They implored toxicologists to link external exposures to body burdens and concentrations at sites of toxic action. This need is greatest for chemicals such as persistent, high-molecular-weight, organic pesticides, for example DDT (dichlorodiphenyltrichloroethane) and its residues, with low aqueous solubility, known partitioning behaviour and specific modes of toxic action.

The LC_{50}s, EC_{50}s and IC_{50}s reported in the literature are estimated based upon external concentrations causing the effects; many of these are not equivalent to LD_{50} (median lethal dose) estimates, thus complicating interpretations of precise effect concentrations and differential species sensitivities. Aquatic toxicologists generally compare the results of different sorts of tests or field exposures with different species, rather than the responses of the different species with normalized internal contaminant concentrations, a crucial distinction not yet given sufficient attention in comparative aquatic and eco-toxicology. Thus, measures of both exposure and effects provide challenges to the aquatic toxicologist.

2.4 Other Basic Concepts and Principles

Several other concepts unique to aquatic toxicology and water-pollution studies must be introduced to appreciate the complexity and uncertainties surrounding experimental estimates of toxicities of chemical substances to the many thousands of aquatic organisms living in a wide range of waters and habitats, in polar to tropical latitudes. Further detailed and fruitful discussion can be found in Forbes and Forbes (1994), Posthuma *et al*. (2002); and Newman (1995; 1998; 2001). With relatively few species and test methods in use, chosen for their high sensitivity and ease of use, aquatic toxicology attempts to estimate safe concentrations for thousands of species and hundreds of aquatic ecosystems, a daunting if not slightly overconfident task.

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2.4.1 Fitting the Test to the Task

Different tasks and questions demand different toxicity tests (Sprague and Fogels, 1977). The aim is to be cost-efficient, investigate the problem and answer the question(s) with minimal, not maximum data. For example, under the MARPOL 73/78 Convention, it was generally agreed that screening industrial chemicals and complex formulations (often with incomplete compositional data) for their aquatic toxicity required acute tests with three to seven species, and a chronic or subchronic test with one species (Wells *et al*., 1999; GESAMP, 2004), as inadequate as this may seem. In contrast, definitive data on a chemical produced, transported and applied in large volumes would be derived from a much wider range of assays, especially mesocosm and *in situ*. The screening approach is in general use in most developed countries, for example industrial effluents are controlled at source with minimal tests (in Canada, solely with the rainbow trout, *Oncorhynchus mykiss* and *Daphnia magna* Cladoceran tests).

In comparison, widely used pesticides are subjected to many research and regulatory investigations (e.g. fenitrothion in eastern Canada), and certain effluents (e.g. pulp and paper) are monitored at source and in the receiving waters with a large battery of environmental effects/toxicity tests, especially using molecular biomarkers. Other examples abound from the fields of process stream, effluent, chemical and sediment testing, for example, oil spill dispersants are generally exhaustively tested and investigated (National Research Council, 1989a; 2005); contaminated sediments are investigated with a combination of bacterial, crustacean and echinoid tests (S. Carr and M. Nipper, personal communication; Bermuda Institute of Ocean Sciences, unpublished data).

2.4.2 Choice of Species for Toxicity Tests

As stated earlier, aquatic toxicology examines the effects of chemicals and chemical mixtures on only a fraction of known aquatic organisms. The responses of many phylogenetic groups are barely understood or are unknown; for example, for coastal marine waters, the zooplanktonic organisms copepods, euphausiids (krill) and chaetognaths (arrow worms) are rarely considered (Wells, 1984b). 'Representative' organisms of only a few phyla suffice as models for other habitats, such as coastal wetlands and coral reefs. Protecting aquatic ecosystems at this time, circa early 2008, is achieved largely by using a few well-known, model bioassay organisms, deriving threshold concentrations for specific substances, setting limits for discharges and calculating objectives and standards for water and sediment quality. For screening and permitting purposes, the OECD recommends considering acute toxicity data from three groups—teleost fish (usually salmonids), crustaceans (cladocerans or shrimp) and algae (microalgae, freshwater or salt)—see *http://www.oecd.org*. For definitive marine testing, conducting additional tests with annelids (i.e. polychaetes), molluscs, echinoderms (usually echinoids) and macroalgae is also recommended. However, many aquatic phylogenetic groups and most species are omitted from regulatory consideration, opening wide the question of the adequacy of protection to most aquatic biodiversity offered by such testing. In such circumstances, field observations and measurements of specific damage are a valuable guide to further focussed toxicity testing and the selection of relevant responses or toxicity end points.

Until recently, the 'most-sensitive' species approach was axiomatic in the field and was not questioned. Over the course of several decades, it resulted in the selection of a few dozen, relatively sensitive, toxicity test organisms and the development and adoption of standard methodologies (Environment Canada, 1990a; 1990b; 1990c; 1990d; 1990e; ASTM, 1993b; 1993c; 1993d; 1993e; Thompson *et al*., 2005). However, this approach has been criticized (Cairns, 1984; Cairns and Pratt, 1985; 1989), and still is, in favour of toxicity testing with as wide an assemblage as is likely to be exposed or otherwise assaulted, within an ERA framework (EPA (Environmental Protection Agency), 1992; 1996; Suter, 1993; 2007). In practice, however, the combined approaches (limited toxicity testing, ERA and TIE) have been and are useful for protecting aquatic ecosystems. For example, much pollution control has occurred in Canada simply by using one bioassay, the rainbow trout (*Onchorhynchus mykiss*) lethal bioassay (Pessah and Cornwall, 1980; Environment Canada, 1990a). At this time, most of the assembled acute toxicity databases present data from only a few species of fish, crustaceans, molluscs, aquatic insects and microalgae; some of the available databases also routinely receive standardized data (e.g. fathead minnow; grass shrimp; fairy shrimp).

2.4.3 Minimum Time to Toxic Effect and the Toxicity Curve

Regardless of the external exposure concentration, the ultimate dose of chemical or the mode of toxic action, there is always a minimum time to death of the exposed organisms, individually and as a population. This is shown by comparing acute toxicity curves for different substances (Sprague, 1969; Newman, 1995; Rand *et al*., 1995; Abel, 1996). This information is valuable because it offers insight into the mode of toxic action of different substances, for example, water-soluble neurotoxins such as domoic acid are fast acting and cause irreversible neurological damage (Wright, 1995), compared with water-immiscible nonionic surfactants that act more slowly as nonselective toxicants and disrupt membrane integrity and function, causing reversible nervous-system impairment (Wells, 1984a; National Research Council,

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1989a). Providing that the exposures are controlled and characterized, the toxicity curves examined together allow comparisons of the toxicity of different chemicals, the sensitivity of different species (or toxicity tests) and the minimum times to toxic effects at different concentrations.

There is also considerable merit in plotting multiple concentration exposure–toxicity data as graphs of cumulative mortality vs. time, generating median effective times (ET_{50}s) and median lethal times (LT_{50}s). This is a less popular, but very informative time–response approach (Sprague, 1969; Newman and Dixon, 1996), and it requires fewer treatment concentrations to produce useful data.

2.4.4 Contributions to Measuring Environmental Quality

The conduct of aquatic toxicity tests on chemical substances and environmental samples provides vital data in the context of the broader environmental protection frameworks (for example for the marine environment, see UNEP, United Nations Environment Programme, 1985; Wells and Cote, 1988; GESAMP, 1991a; 1994; Gray *et al*., 1991) and ERA (Landis and Yu, 2004; Suter, 2007). The various frameworks guide acquisition and integration of appropriate data essential for descriptions of the condition of whole aquatic ecosystems under scrutiny; condition may also be referred to as environmental quality, ecosystem health or ecological integrity (see Wells and Rolston, 1991; Rapport *et al*., 1998; Wells, 2003; 2005, among others). Many of these data are from biological (environmental) effects-monitoring programmes, which use toxicity tests *in situ* or environmental samples in nearby laboratories. For example, with sediments, toxicity tests are applied with chemical characterization and benthic community analyses in the triad approach (Chapman and Long, 1983; Chapman *et al*., 1987a; 1987b), together with TIE analyses and preliminary estimates of risk, using ERA and a simple risk quotient method.

At present, in aquatic toxicology and, more broadly, ecotoxicology, both structural and functional measures of ecological health are measured (Munawar *et al*., 1989a; 1989b; Cairns *et al*., 1995; Di Giulio and Hinton, 2008), reflecting the current contributions of molecular biology, genetics and ecology to the methods of sublethal toxicity detection. Aquatic toxicology, through its emphasis on methods development over several decades, has contributed greatly to environmental monitoring programmes, particularly measuring status, trends and effects of chemical contamination (McIntyre and Pearce, 1980; White, 1984; Goldberg, 1986; Chase *et al*., 2001; Hylland *et al*., 2006; Sindermann, 2006) and guiding the selection of indicators of environmental quality (Bortone, 2005).

2.4.5 Predictive Toxicology

Linking simple and practical measures of toxicity of various classes of chemicals to anticipated effects of those chemicals and substances under natural conditions is a major goal of aquatic toxicology. Much effort has been in the field of QSARs (quantitative-structure–activity relationships) over the past three decades (Kaiser, 1984; Lipnick, 1995; Nendza, 1998; Ekins, 2007). The field is now called predictive toxicology (Helma, 2005). QSARs enable estimates of toxicity of chemicals in homologous series to be made from knowledge of the toxicity of a few chemicals in relation to their structures and physicochemical properties. The science is now quite well developed. It is used extensively by the regulatory community for assessing, ranking and classifying new industrial, food and cosmetic chemicals, and chemicals in transport. For example, QSARs are used in the hazard-evaluation procedure for harmful substances carried by ships (GESAMP, 1990c; 1988; Wells *et al*., 1999; GESAMP, 2004), and similar procedures through the OECD, where actual data are very limited, but the need for hazard ratings for chemicals in transit, by truck, rail and ship, is immediate.

Recently, both predictive toxicology (Helma, 2005) and computational toxicology (Ekins, 2007) have emerged from the QSAR research and applications, and utilize data from all branches of toxicology, as well as chemistry, statistics, computer science, medicine, biology and others. Predictive toxicology is the development 'of procedures or algorithms that are capable of predicting toxic effects from chemical and biological information' (Helma, 2005). Predictive models integrate information on chemical structures and properties, biological systems and toxic effects, with the use of export systems and statistics, and the aim to 'predict the toxic effects of untested compounds'. Computational toxicology, the science of applying computer methods to toxicity assessment, including environmental, has developed rapidly (Ekins, 2007). It has applications in the toxicity and risk assessment of chemical mixtures, computational risk assessment, QSARs, toxicoinformatics and regulations on the environmental side of toxicology. The impacts of these fields on aquatic toxicology and its mandate are just being felt.

3 PRACTICE OF AQUATIC TOXICOLOGY

3.1 Acute Toxicity: Measurement and Analysis

The measurement and expression of acutely toxic effects of chemicals is the backbone of aquatic toxicology. In

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this context, acute toxicity methods have been reviewed in detail by many practitioners and groups (Sprague, 1969; 1973; 1990; Buikema *et al*., 1982; Rand and Petrocelli, 1985; Cairns and Mount, 1990; Adams, 1995; Rand, 1995, 2009; Rand *et al*., 1995; Abel, 1996; Environment Canada, 1999; 2005; Newman and Clements, 2008, among others). Some of the topics important to the measurement of acute (short-term) lethal and sublethal effects of water-borne chemical contaminants are briefly introduced here. Measuring acute toxicity is particularly important to the ranking of chemicals and the quality control of industrial and municipal effluents.

Various approaches for assessing acute toxicity have been used—laboratory tests, microcosms and mesocosms (see below), *in situ* field experiments and observations at polluted field sites. Exposure (an integration of composition, concentration and time) is probably the most important variable in an aquatic toxicity test, and indeed in the aquatic environment. Exposure regimes in the laboratory can be static, static with replacement, continuous flow or intermittently flow-through, each with advantages and disadvantages to the water quality and realism of the chemical's fate (Buikema *et al*., 1982; Wells *et al*., 1984). If the organism is not exposed to toxic concentrations for a sufficiently long time, or if the chemical is in a form unlikely to enter the organism or cause sufficient external damage to sensitive tissues, for example, gills, then toxicity is unlikely to occur. External exposure in the aquatic environment differs radically from internal dosing used in mammalian toxicology. Early work relied on only nominal, external exposure concentrations of chemical(s) (i.e. those unmeasured in the test chambers), rather than on measured concentrations; hence, end points such as LC_{50}s were greatly underestimated for hydrophobic substances, that is, chemicals appeared less toxic than they were. This was especially true for complex industrial formulations and mixtures, and petroleum products, and is an ongoing problem for accurate hazard-assessment essential for regulation and safety (GESAMP, 2004).

A lot of effort has gone into developing continuous-flow devices, chemical analytical methods and the use of carrier solvents (Hutchinson *et al*., 2005) to overcome these challenges. Likewise, appropriate selection of test organisms is crucial; rationale exist and agreement has now been reached for different aquatic environments, from freshwater to marine, by many agencies and organizations, and many toxicity methods have been standardized (see recent ASTM and APHA manuals—ASTM, 1993a; 2007; APHA, 1995). As wide a range of different organisms across different phyla and life stages should be tested in single species toxicity tests as possible, and decisions made regarding hazard and risk ratings, using the lowest EC_{50}s or LC_{50}s or NOECs. A detailed literature exists for considering the various dose–response relationships and analyses of data (Sprague, 1969; Newman, 1995; Rand *et al*., 1995; Environment Canada, 2005), as well as for accounting for the many factors, physicochemical and biological, that influence exposures and expressions of toxicity (Newman, 1998; Landis and Yu, 2004).

3.2 Chronic Toxicity: Measurement and Analysis

Chronic or longer-term sublethal effects on humans from low-level exposure to trace industrial chemicals have been a concern to the medical profession for decades. The potential for such effects is very much under public scrutiny in North America and Europe. Many thousands of synthetic chemicals are introduced into our environment (air, water, soil, food) with little knowledge of their effects on reproduction, growth and development. The concern is that the exposure of humans, especially during development in the uterus, to chemicals that could act as endocrine disrupters, for example, oestrogen-mimicking compounds, such as diethylstilbestrol (DES), DDT and PCBs (polychlorinated biphenyls), may influence reproductive processes and success later in life (Rall and McLachlan, 1980). This issue has surfaced in recent years, particularly due to trace levels of persistent organic pollutants (POPs) in food, water and the workplace (Colburn *et al*., 1996; Rudel *et al*., 2003; Fitzgerald, 2007).

Likewise, many aquatic organisms and their populations, communities and habitats have been and are exposed in many locations over the longer term to low-level concentrations of persistent, bioaccumulative and potentially toxic industrial chemicals (Rand, 1995; Lotze *et al*., 2006). Concerns about chronic sublethal toxicity of chemicals to aquatic biota date back many years, for example, see reviews in Sprague (1971; 1976), Cole (1979), Waldichuk (1979; 1985), Sheehan *et al*. (1984), Rand and Petrocelli (1985), Howells *et al*. (1990), and recently Landis and Yu (2004) and Newman and Clements (2008). There is a large primary literature on sublethal effects of chemicals and mixtures on aquatic organisms, and many reviews on specific topics (e.g. endocrine disrupters—Rolland *et al*., 1997; large oil spills—Wells *et al*., 1995; Rice *et al*., 1996; metals—Langston and Bebianno, 1998), mostly addressing chronic or long(er)-term sublethal effects. In the ecotoxicology and marine-science fields, there have been 30 years of conferences, notably SETAC and PRIMA (Pollutant Responses in Marine Animals), where new research is presented on sublethal effects on many biological processes—hormonal function, immune function, physiology, behaviour, growth, development, reproduction and so on. Hundreds of responses and the concentrations causing them have been described, though many studies are unfortunately

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not designed to estimate chronic sublethal threshold concentrations.

Recently, concerns about chronic toxicity have been expressed in the context of known and suspected effects of endocrine-disrupting compounds (EDCs) on aquatic organisms, wildlife and human health (Colborn *et al*., 1996; Cadbury, 1998; Kendall *et al*., 1998; Bechmann, 1999; Hutchinson, 2002; 2006; 2007; Hutchinson *et al*., 2006), as well as in the context of the ubiquitous halogenated hydrocarbons, such as certain pesticides (e.g. mirex, toxaphenes, malathion), DDT, PCBs, dioxins and furans, and some metals (e.g. cadmium, Vetillard and Bailhache, 2005).

Chemicals (so-called industrial, priority or toxic substances) affect organisms and aquatic ecosystems at various biological levels of organization (from molecules and cells to whole organisms, to communities and ecosystems) and on a wide range of life processes, at levels often well below those killing the organism in short, constant exposures. The major types of responses are biochemical, physiological, behavioural, growth, developmental and reproductive (GESAMP, 1990b; Newman and Jagoe, 1996; Newman and Clements, 2008). Reproductive and developmental effects are most important to population survival, that is, recruitment (Newman, 2001). The important chronic experiments are those that relate the time course of chemical exposures to the generation time and lifecycle of the organism, for example 28-day flagfish experiments. Investigations over several decades have concentrated on the major types of aquatic contaminants for such effects, striving to describe NOECs and MATCs on the basis of such chronic sublethal data. The key to interpreting sublethal effects is that studies must allow some measure of lowest threshold concentrations for effects, but as stated above, such estimates are often unfortunately missing in many studies.

While sublethal effects are relatively easy to detect and measure for many substances and organisms in the laboratory (e.g. following the development of crustacean larvae or echinoid embryos for several days), finding field evidence of such effects and demonstrating the causative agent(s) proves to be difficult. However, there are examples, such as with Mirex in the Great Lakes and deformities in juvenile birds (Gilbertson *et al*., 1998); imposex in marine snails exposed to organotins (Beek, 2000; Oberdörster and McClellan-Green, 2002); reproductive failures in seabirds exposed to DDT and other insecticides (Rattner *et al*., 1984; Fry, 1995); mixed function oxygenases (MFOs) and pathologies in fish near pulp mills (Servos, 1995); various effects of POPs on Arctic species (Cone, 2005) and elevated occurrences of diseases in bottom-dwelling fish and marine mammals near urban areas (Vethaak and Rheinallt, 1992; Bernet *et al*., 1999; also see Howells *et al*., 1990; Rand, 1995; Cadbury, 1998). That such effects are occurring in organisms exposed continuously to very low levels of many chemicals over long periods of time is probably more common than currently demonstrated. However, the evidence is hard to find for some aquatic environments, such as marine (Howells *et al*., 1990; Hylland *et al*., 2006). Hence, describing the causative agents, their concentrations and the aetiologies of sublethal effects and pathologies under field conditions, and finding evidence or not of recovery remains an important challenge of aquatic toxicology.

3.3 Special Topics in Aquatic Toxicology

In recent years, ecotoxicology and aquatic toxicology have expanded quantitatively and qualitatively (note the many ASTM and SETAC publications). The standard approaches for evaluating the effects of contaminants and for measuring their presence in specific habitats and ecosystems have become more sophisticated. Several research areas have evolved substantially with new developments since the 1970s, as shown in Sections 3.3 and 3.4.

3.3.1 Techniques

3.3.1.1 Modelling

A popular approach to overcoming the difficulties associated with retrospective chemical monitoring and analysis is to forecast the behaviour and fate of chemicals with models of their environmental distribution and fate. Over the past three decades, investigators have advanced the idea that the behaviour of chemicals can be predicted on the basis of comparisons between laboratory-measured properties of the chemicals and the environmental behaviour and chemical properties of compounds for which more abundant environmental data are available. For example, Baughman and Lassiter (1978) introduced the concept of the use of hypothetical 'evaluative models', based on properties of stylized environments and contaminants; these models provided quantitative approaches to exposure estimation. Mackay (1979; 2001) followed this up with the full development of fugacity modelling. The simple exposure models used today are constructed of individual submodels of the input, transport and removal processes controlling the fate of a chemical, incorporated into a model of the environment in which the chemical ultimately will be distributed. The hypothetical model environment or environmental system is constructed by conceptualizing several environmental compartments which are considered to have distinct and uniform characteristics, such as for pH, organic matter content, salinity and temperature. The discharge, loading or emission rates of a chemical are then described by discrete models. Finally, mathematical

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relationships are developed to describe the intercompartmental transfer and removal processes that influence the dynamics of the chemical; see Mackay (2001) for a full description of the fugacity approach.

Models are being developed at three levels of increasing complexity: conceptual, comparative distribution models of a qualitative nature; quantitative, situation-specific models of a generic nature; and quantitative, site-specific models, with various applications in mind (Wrona *et al*., 2000; Koelmans *et al*., 2001; Warren and Mackay, 2005). All of them are receiving increasing attention by regulators and agencies concerned with toxic chemical management and pollution control in fresh and salt waters.

3.3.1.2 Quantitative Structure–Activity Relationships (QSARs)

Current overviews of QSARs (also called SARs or structure–activity relationships) and aquatic toxicology is found in Rand (1995), Walker (2003) and Devillers (2007), covering principles, models and uses in regulation and chemical design.

Assessing large toxicity data sets for specific chemicals provides insight into chemical structures or properties that lead to toxicity. For example, low-molecular-weight (lmw) aromatic hydrocarbons from spilled oils are biologically available and acutely toxic, the toxicity influenced by molecular weight, the presence of alkyl side chains and the species exposed; crustaceans are more sensitive than either bony fish or molluscs to lmw, readily dissolved aromatic hydrocarbons (National Research Council, 1985; 1989a; GESAMP, 1993). These relationships were developed and verified using experimental approaches and considerable knowledge of the organism's biology and physiology.

The QSAR approach proposed by Hansch (1977), in which both the physical properties and chemical structures of molecules are used to predict their biological activity, has proved highly applicable in aquatic toxicology (Kaiser, 1984; Rand, 1995). A number of experimental studies have demonstrated a high degree of toxicological predictability for aquatic organisms, with substantial agreement between expected and experimentally observed toxicity levels, within relatively homogeneous classes of organics. In some cases, the hydrophobic characteristics, expressed as the *n*-octanol/water partition coefficient (log K_{ow}), correlates highly with the activity of the molecules. Bioconcentration and bioaccumulation in some organisms can be adequately described by QSARs (Mackay, 1982), using partition coefficients such as log P (log K_{ow}), either calculated or experimentally derived. Electronic characteristics (pK_a) of the molecules have also proved useful in explaining the toxicity of several amines. A combination of hydrophobic and electronic characteristics has been shown to correlate well with the toxicities of phenols, chlorophenols and chloro-and alkylanilines. However, in many cases, log K_{ow} alone was sufficient to provide good predictive capability. The inclusion of molecular connectivity is also useful in the QSAR approach for predicting the toxicity of several other compounds.

At present, QSARs have been developed for several classes of chemicals, and allow good estimates of toxicity and mode of toxic action (Hodges *et al*., 2006; Sanderson and Thomsen, 2007). QSARs have been applied to a limited number of chemicals, necessitating expert judgement for determining the boundaries of toxicological predictability from the approach. QSARs can be applied within homologous groups of chemicals, for example, hydrocarbons, detergents and pharmaceuticals, and do offer a basis for some predictability, certainly with respect to selecting priority substances if experimental data are unavailable. QSARs have been successfully applied to the study of toxic effects of chemical mixtures and understanding some mechanisms which regulate the toxic interactions among chemicals.

To conclude, the QSAR approach is used extensively in the development of new environmentally safe products (e.g. detergents, Tsoler *et al*., 1999), in chemical ranking, classification and regulation (Walker, 2003; GESAMP, 2004) and as a component in fate modelling programmes (Mackay, 2001).

3.3.1.3 Assessing Toxicity of Chemical Mixtures

The measurement of toxic effects of chemical mixtures (Lloyd, 1987; National Research Council, 1988) remains a major topic (circa 2008) because thousands of the industrial chemicals being produced and shipped worldwide are mixtures, and most industrial and municipal emissions into air, soil, sediments and water are mixtures, not single compounds. There have been many recent studies on mixtures (e.g. Broderius, 1991; Calabrese and Baldwin, 1993, Appendix 3; Yang, 1998; Backhaus *et al*., 2000; Deneer, 2000; Parkerton *et al*., 2000; Mothersill and Austin, 2003; Lydy *et al*., 2004; Brain *et al*., 2004) and an excellent review (Lipscomb *et al*., 2007). In specific fields, such as oil pollution response, much effort has gone into determining the primary sources of toxicity in oil dispersions and mixtures of oils and dispersants (National Research Council, 1988). In some cases, for example pulp and paper effluents (Servos, 1995), and sewage, the sources of acute toxicity to fish can remain elusive due to the enormous chemical complexity of the effluents.

A number of key questions remain—can we assume that most industrial chemicals (largely organic), of similar modes of toxic action, interact additively on organisms, rather than synergistically? Is it true that 'the assessment of chemical stressors is relatively straightforward after 30 years of study', as noted by Dorward-King *et al*., in Baird and Burton (2001)? What are the best monitoring approaches to deploy near mixture outfalls? Much

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more consideration should be made of the testing, assessment and monitoring of chemical mixtures, particularly at low levels. A larger question also remains—how do we distinguish between natural and anthropogenic (chemical) factors influencing organisms and ecosystems? This distinction may be clear for some stressors, for example, DDT and thin egg shells, organophosphate pesticides and insects, organotins and molluscs, where chemicals with specific modes of toxic action were purposely synthesized, but may be very difficult to establish for other chemicals and environmental factors, for example, sediment dynamics and burdens in coastal waters. This field is called 'multiple stressors' and incorporates the concerns for assessing chemical mixtures.

3.3.1.4 Assessing Toxicity of Sediments

Many chemical contaminants become particle bound in the water column and enter bottom sediments in the aquatic environment. From there, they may reside for long periods, for example DDT, PCBs, be taken up by demersal fish, for example winter flounders, and benthic and epibenthic invertebrates, for example scallops, or flux back into the overlaying water column as shown in dynamic estuaries, for example Halifax harbour, NS. Contaminated sediments pose large problems for coastal waters in need of constant dredging; their management is controlled by the London Convention and various national legislation. The science of evaluating sediment-bound contaminants is described in Dickson *et al*. (1987), National Research Council (1989b), Baudo *et al*. (1990), Burton (1992), Ingersoll *et al*. (1997), Stolzenbach and Adams (1998) and Wenning (2005), among others. After many conferences and discussion, there is general agreement about the choice and application of the suites of bioassay tests to use, for both freshwater and marine environments. Freshwater assays include bacteria (Microtox®), annelids (oligochaetes), crustacean (amphipods) and chironomids, while marine assays include bacteria (Microtox®), annelids (polychaetes), molluscs (oyster larvae), crustaceans (amphipods, shrimp) and echinoids (sea urchins and sand dollars, young life stages).

Sediment toxicity tests are continually being re-evaluated. There has also been recent effort to evaluate the toxicity of sediment pore waters (Nipper and Carr, 2003), within the context of the TIE approach. There is much research in this field, both on basic and applied questions, with a wide range of species (Ginn and Pastorok, 1992; Power and Chapman, 1992; Meador *et al*., 1997a; 1997b; Leppänen and Kukkonen, 2000; Hartl *et al*., 2001; Leppänen and Oikari, 2001; Meador and Rice, 2001; Oikari *et al*., 2002; Burgess *et al*., 2003; Hyötyläinen and Oikari, 2004; Moore *et al*., 2005). Much of the work is conducted on sediments contaminated by oil spills, polluted harbours and pulp-mill discharges. The uptake of sediment-borne contaminants, influence on bioavailability of influencing factors, relationships between sediment contamination and toxicity and the standardization and quality control of methods have received considerable attention (Power and Chapman, 1992; Lamberson *et al*., 1992; Wenning, 2005).

3.3.1.5 Biochemical Effects and Biomarkers

An intensive effort has been underway since the 1980s to produce practical, sensitive, reproducible, low-cost and interpretable biomarker tests to detect biological effects of chemicals under natural conditions before they cause significant harm, and to monitor the presence and effects of substances in areas subjected to waste disposal (Mehrle and Mayer, 1980; McCarthy and Shugart, 1990; Gray, 1992; Rand, 1995; 2009; Butterworth *et al*., 1995; 2001; Wells *et al*., 1998; Stephensen *et al*., 2000; Denslow *et al*., 2007; Kong *et al*., 2007). Such tests can be applied at all stages of the management of chemicals and wastes that enter aquatic ecosystems. Examples of such biochemical effects procedures are MFO systems, metallothionein-complex formation (Amiard *et al*., 2005), lysosomal-membrane stability, blood-chemical assays, cytogenetic tests (including DNA-adduct analyses) and steroid-hormone assays, the latter of great recent interest due to concerns about effects of pharmaceuticals in municipal and industrial wastewaters (Sherry *et al*., 1999).

The measurement of MFOs is one example. In higher macroinvertebrates, fish and seabirds, the enzyme systems involved in detoxification are sensitive to the presence of low levels of chemicals such as PCBs, polyaromatic hydrocarbons (PAHs) and components of industrial effluents (Coakley *et al*., 2001; Reynard *et al*., 2008; R. F. Addison, personal communication). Measuring such enhanced enzyme activity demonstrates that exposure has occurred. The detection of cytogenetic patterns and responses in marine fish (eggs and larvae) and mammals again indicates that exposure has occurred and has the potential for causing fundamental toxic effects in exposed organisms. Another recent example is the measurement of DNA adducts in whale brains in contaminated estuaries (Mathieu *et al*., 1997; 1998). Biochemical and cytogenetic/cytological techniques have considerable potential for application in marine waste management. Studies are still needed to identify in detail the dose–response relationships under field conditions. The current applicability of some of these techniques is described by Giam and Ray (1987), Malins and Varanasi (1988), McCarthy and Shugart (1990), Peakall (1992) and Shugart and Theodorakis (1998).

Recent reviews and books on biochemical ecotoxicology and biomarkers proliferate. This is a growth area in aquatic toxicology due to methods flowing from molecular biology and genetics (McCarthy and Shugart, 1990; Huggett *et al*., 1992; Peakall, 1992; Walker and Livingstone, 1992; Depledge and Fossi, 1994; Fossi, 1994; Di Giulio *et al*., 1995; Ostrander, 1996; Walker

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et al., 1996; Shugart and Theodorakis, 1998; Denslow *et al*., 2007). Significant strides have been made in understanding the various modes of toxic action of diverse contaminant types—from genotoxicity (PAHs), endocrine disruption (organochlorines) and neurotoxicity (organophosphorus, carbamate and pyrethroid pesticides) to mitochondrial poisons (nitrophenols) and ATP inhibitors (dichlorodiphenyl ethylene (DDE)) (Walker *et al*., 1996), much of the evidence coming from fish and avian studies. In some cases, whole new subdisciplines of biochemical ecotoxicology have developed in recent years, such as with ecotoxicogenomics, the study of gene and protein expression in organisms exposed to chemicals (Snape *et al*., 2004), and toxicologic pathology (Gillett *et al*., 2002).

Biomarkers can be considered broadly as 'biological response(s) to a chemical or chemicals that give a measure of exposure and sometimes, also, of toxic effect' (Depledge and Fossi, 1994). Biochemical biomarkers have now been investigated extensively, with the dual roles of determining whether exposure of biota has occurred and whether or not biological damage, that is, adverse biological effects, especially cumulative, due to the exposure, has taken place under natural conditions (Depledge and Fossi, 1994; Peakall, 1994; Depledge *et al*., 1995; Karouna-Renier and Zehr, 1999; Bard, 2000; Snyder, 2000; Pretti and Cognetti-Varriale, 2001; McClain *et al*., 2002; Brown *et al*., 2003; Ferrat *et al*., 2003; Amiard *et al*., 2005; Pellacani *et al*., 2005; Valavanidis *et al*., 2005; Rose *et al*., 2006).

Biomarkers with promise for aquatic environments include metallothionein induction (metals) (Amiard *et al*., 2005), acetylcholinesterase inhibition (certain organophosphorus and carbamate pesticides) (Pretti and Cognetti-Varriale, 2001), induction of MFOs (organochlorines, PAHs) (Snyder, 2000; Reynard *et al*., 2008; Payne, J., personal communication), DNA-adduct formation (PAHs), *in vitro* genotoxic assays (Pellacani *et al*., 2005), molecular responses to oxidative stress (Brouwer *et al*., 2005; Valavanidis *et al*., 2005; Kong *et al*., 2007) and formation of stress or heat-shock proteins (metals, organochlorines, thermal stress) (Depledge and Fossi, 1994; Peakall and Walker, 1994; Branton *et al*., 1999; Karouna-Renier and Zehr, 1999). Some biomolecules, such as P-glycoproteins, provide organisms with resistance to chemical contaminants at the cellular level (Bard, 2000). Metallothioneins in invertebrates exposed to metals, despite their variability, are recommended for monitoring (Amiard *et al*., 2005). Generally, suites of biomarkers rather than one particular one, at different levels of organization, and the use of sentinel species are recommended for monitoring (Depledge *et al*., 1995; McClain *et al*., 2002; Brown *et al*., 2003; Ferrat *et al*., 2003; Pellacani *et al*., 2005; Rose *et al*., 2006).

3.3.1.6 Microscale Testing in Aquatic Toxicology

Microscale toxicity testing, for the hazard assessment of effluents, chemicals and sediments, has become an important approach over the past three decades (Persoone and Wells, 1987; Blaise *et al*., 1988; 2000; Long *et al*., 1990; Blaise, 1991; Wells *et al*., 1998; 2001; Persoone *et al*., 2000; Blaise and Ferard, 2005a; 2005b; Wells, 1999). It is also called microbiotesting, a term coined by C. Blaise, small-scale toxicity testing, or rapid-screening toxicity tests (Toussaint *et al*., 1995; Shedd *et al*., 1999). 'Small' (micron to cm) and sensitive organisms, often as embryonic or young life stages, are tested in reliable, rapid and cost-effective assays, using fundamental end points of genomic and biochemical change, physiology, growth and development, and reproduction.

Various workshops in Canada have discussed microscale methods with bacteria (Microtox®, Mutatox®); giant kelps; polychaetes; rotifers; oysters, clams and abalones; amphipods, mysids and crabs; sea urchins and smelt (Chapman *et al*., 1991; Wells, 1991; Blaise *et al*., 1996). Automated marine procedures exist, Microtox® being the best known procedure, used in >50 countries. Some assays can be conducted both in the laboratory and in the field, and some are commercially available in Toxkits (e.g. rotifers, *Artemia* shrimp, *Daphnia*) (Persoone *et al*., 2000). Microscale methods are an important source of alternative assays, hence reducing the requirement for expensively cultured or field collected macroscopic species. Regulatory agencies have now adopted such tests, in Canada, the USA and other countries, with emphasis on bacterial, microalgae, invertebrates and fish (see Thompson *et al*., 2005 for an excellent overview and Nipper and Carr, 2003, for current methods).

3.3.1.7 Microcosms and Mesocosms

The evaluation of chemicals and chemical mixtures in artificial experimental ecosystems, from simple microcosm chambers, to larger and more complex mesocosms, is a valuable technique for analysing existing problems and studying and assessing the hazards and risks of new substances. Such experimental approaches can be incorporated into tiered or sequential hazard assessment schemes for detailed evaluation of chemicals in common transport and use. The experimental and application roles were summarized by Cairns (1986) and Graney *et al*. (1995), amongst others. Through the application of microcosms and mesocosms, exposure assessment and toxicity assessment can be conducted with relatively realistic simulations of field conditions, especially incorporating multiple trophic levels, hence facilitating the measure of ecological processes. Despite the costs and logistical complexities, such testing systems are still very much in use for assaying chemicals that are in large-scale use (Caquet *et al*., 2000; Hopkins *et al*., 2004; Oikari,

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2006; Sundt *et al*., 2006; Mohr *et al*., 2007; Roussel *et al*., 2007; Sanderson *et al*., 2007).

Microcosms are a testing ground for the predictions of simple fate models, and the determination of toxic effects at simulated environmental concentrations. More complex mesocosms such as CEPEX (Controlled Ecosystem Pollution Experiment) and MERL (Marine Environmental Research Laboratory) (Grice and Reeves, 1982), very active in the 1980s, provided empirical evidence of the expected exposures and ecotoxicity under realistic conditions and at various levels of biological organization and complexity. Such work continues at the University of Guelph with mesocosms called limnocorrals, deployed in Ontario lakes to evaluate pesticide fate and effects (Solomon *et al*., 1980; 1985; 1986; Wilson *et al*., 2004; Brain *et al*., 2005; Jooste *et al*., 2005), and at the Bedford Institute in Halifax, with studies of dispersed hydrocarbons in large-wave tanks (K. Lee, personal communication). The greater cost and complexity of the microcosm and mesocosm approaches are often offset by the greater reliability, albeit greater variability, of the predictions. They are and should be used for evaluating chemicals and materials of long-term aquatic significance (e.g. pesticides, fossil fuels, effluents, dredging spoils, solid wastes, etc.) where the right choice of strategies for prevention, control, monitoring and emergency response has major economic and ecological implications.

The advantages of the above approaches are mainly the ability to conduct a priori as opposed to just a posteriori evaluations of chemical toxicity, thereby reducing uncertainties in the identification of missing data and information, improving the exploitation of existing data, and increasing the cost-effectiveness of acquiring field and laboratory exposure and toxicity data. Even more important from a scientific perspective, a far better understanding is obtained of the processes controlling the transport and interactions of chemicals in the aquatic environment.

3.3.2 Integration and Interpretation

Many areas of aquatic toxicology and its closely related fields have undergone significant advancements since the 1980s, in research approaches and applications. Some of these are briefly described below, as well as in Section 4.

3.3.2.1 Hazard Evaluation and Ecological Risk Assessment

Aquatic toxicology has contributed methods and interpretation to the hazard evaluation of chemicals and to the field of ERA. This is illustrated by the earlier classic work on chemicals and effluents (Cairns *et al*., 1978; Bergman *et al*., 1986) and more recently by work of GESAMP (Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection) on the hazard evaluation of chemicals and mixtures carried by ships, where they are evaluated for hazard based largely on their intrinsic properties (GESAMP, 1990c; 1997b; 2004; Wells *et al*., 1999). ERA has been further developed and utilized in Europe and North America, as shown by the US Environmental Protection Agency ERA framework and its many applications (EPA, 1992; 1996; Ingersoll *et al*., 1997; Newman and Strojan, 1998; Ferenc and Foran, 2000; Benjamin and Belluck, 2001; Paustenbach, 2002; Landis and Yu, 2004; Suter, 2007).

The hazard evaluation of chemicals carried by ships is conducted by assigning ratings based on seven criteria, two of which are from aquatic toxicology—bioaccumulation and aquatic toxicity (acute and chronic) (GESAMP, 1988; 2004; Wells *et al*., 1999). The criteria, the experimental methods to produce the required data, and the 'cut-off' values within each rating system have all evolved as the science of aquatic toxicology has developed over the past few decades. Bioaccumulation is assessed using the logarithm of the *n*-octanol/water partition coefficient (log P_{ow}, also often referred to as log K_{ow}), and the BCF measured using fish or molluscs as target organisms (Wells *et al*., 1999). Considerable effort went into the design of the rating scheme for bioaccumulation and the selection of the break-point of log P_{ow} 4−<5 and BCF 500−<4000 as an indication of a high potential for a substance to bioaccumulate. For toxicity, acute toxicity ratings are based on data from three sorts of standard tests—the 96 hour LC_{50} fish test, the 48–96 hour LC_{50}/EC_{50} crustacean test and the 72 or 96 hour EC_{50} microalgal growth inhibition test (see Thompson *et al*., 2005, for details of available test methods, and the different species used by various jurisdictions and sectors). LC_{50}s or EC_{50}s below 0.1–1 mg l^{-1} are considered highly, very highly or extremely toxic. For the first time in this evaluation scheme, chronic toxicity is considered formally, using NOECs from suitable tests such as the 28 day prolonged fish test, the 21 day *Daphnia* reproduction test or the early life-stage fish test, with the cut-off for high chronic toxicity being an NOEC of 0.01–0.1 mg l^{-1}.

While not without its limitations, the hazard-evaluation scheme enables identification of most substances that have high bioaccumulation and toxicity characteristics, highlighting these for the industry, shippers and regulators. Hence, hazard evaluation is precautionary and aquatic toxicology procedures are used to identify and avoid potential problems before they occur.

ERA is at the other end of the chemical-assessment spectrum. The objective is to evaluate the ecological consequences of chemical exposures in natural ecosystems, hence the probability and level of exposure is key to whether toxicity of the chemical(s) occurs. The aquatic toxicity methods involved are more complex, more expensive, field-oriented and longer-term, and they are focussed on populations and communities of organisms, rather than the organism level. One or more aquatic

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toxicity approaches, involving microcosms, mesocosms, *in situ* experimental sites and/or observations of natural systems, are deployed (Wells, 1984b; Rand *et al*., 1995; Stokstad, 2008), within the ERA framework of problem formulation, analysis (characterization of exposure, characterization of ecological effects) and risk characterization (EPA (Environmental Protection Agency), 1992; 1996; 2003). For full assessments, for example of a high volume, widely used organophosphorus pesticide such as fenetrothion, a selection of aquatic toxicity techniques might include acute tests on the active ingredient and the formulation, biochemical assays on tissue samples from exposed fish and invertebrates, residue-in-tissue monitoring using bivalve molluscs (clams, oysters or mussels) and benthic surveys for community impact analysis. Risk assessments provide the opportunity to consider all of the reliable methods in the arsenal of aquatic toxicology (Rand *et al*., 1995; Rand, 2009; EPA (Environmental Protection Agency), 2003) and use those most likely to give accurate estimates of toxicity and risk of effects under field conditions.

3.3.2.2 *Comparative Ecotoxicology or the Responses of Different Species*

There has been considerable effort to understand the relative sensitivity or responses of different species of aquatic organisms to contaminants of different types (Blanck *et al*., 1978; Kenega, 1978; Doherty, 1983; Sloof *et al*., 1983; Thurston *et al*., 1985; Blum and Speece, 1990; Posthuma *et al*., 2002). This field of research and application, traditionally called comparative ecotoxicology, now formally involves species sensitivity distributions (SSDs). The field is important from the point of view of selecting appropriate test organisms for chemical screening and ranking, and for the more difficult task of making field predictions of effects under many different conditions. Some progress has been made with selected chemical classes and compounds. For example, it is known that crustaceans, especially decapods, are more sensitive, that is, respond at lower concentrations, to petroleum hydrocarbons than fish, and that generally, across chemicals, young life stages are more sensitive than older life stages. Microtox® bacteria and sea-urchin embryos have proved to be the most sensitive assays for assessing the toxicity of sediments. The collective studies of the past 30 or so years have led regulatory agencies in many countries to select certain species for testing their chemicals and effluents/emissions with confidence. For example, Environment Canada uses fish and crustacean tests for monitoring effluents and amphipod and echinoid tests for screening sediments (see earlier sections); the OECD and the UN International Maritime Organization (IMO) recommend the joint use of fish, crustacean and algal assays; the US EPA recommends the use of fathead minnows, mummichogs, cladocerans, mysids, grass shrimp and microalgae. The selection of the phyla and particular species has been driven by our growing knowledge of interspecies sensitivity to chemicals, as well as by practical constraints of testing.

However, as introduced above, the collective knowledge of the comparative sensitivity of aquatic species has been confounded by the methods of exposure, so that in many, probably most cases, what is known is the different sensitivity of the tests employing certain species, not necessarily the differences or similarities between the species themselves. That is, different tests employ unique volumes of test solutions, temperatures, containers, lighting, duration times, and so on, all of which influence the fate of the tested substance and the contact with and uptake by the test species. Differences in exposure and partitioning, two of the 'three Ps' of aquatic toxicology (McCarty and Mackay, 1993), have compromised the actual comparison of species responses to contaminants. This problem is little appreciated in aquatic toxicology (Abernethy *et al*., 1986), somewhat surprising considering its relevance to ranking chemicals as to their toxicity, and protecting complex, multispecies, aquatic ecosystems.

The literature on comparative aquatic toxicology is large (witness the Google results for 'comparative aquatic toxicity', with >11 000 web sites, 05/08), with many recent studies across many phyla, species, life stages and chemicals and their mixtures, the effort largely focussed on understanding the sensitivities of different species, in different tests, and deriving threshold concentrations and estimates of safe environmental levels. The field needs further analysis and synthesis; infinite numbers of such studies are possible, at great cost, with little new fundamental knowledge generated unless the principles behind the different sensitivities are elucidated.

To this end, in recent years, the field of SSD-based risk assessment has developed (Posthuma *et al*., 2002), incorporating for the most part what is understood about species-specific differences in toxicant responses and thresholds, and applying the data to estimates of risk (Versteeg *et al*., 1998; Forbes *et al*., 2000; Newman *et al*., 2000; Forbes and Calow, 2002; Wheeler *et al*., 2002a; Van Straalen, 2002; Carsten von der Ohe and Liess, 2003; Duboudin *et al*., 2003; Hose and Van den Brink, 2004; Maltby *et al*., 2004). Studies are also conducted to understand the differences in sensitivity of fresh-vs. salt-water organisms (Hutchinson *et al*., 1998; Leung *et al*., 2001; Wheeler *et al*., 2002b), of polar organisms relative to temperate and tropical organisms (Perkins *et al*., 2005), of tropical vs. temperate organisms (Kwok *et al*., 2007), of various planktonic crustaceans exposed to organic pollutants (Sanchez-Bayo, 2005) and of organisms exposed under laboratory and field exposures (Seick *et al*., 2002). This field, as it attempts to uncover the basis of differential responses of organisms to persistent water pollutants, remains one of aquatic toxicology's greatest challenges.

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3.3.2.3 *Choosing Indicators of Aquatic Ecosystem Health*

Aquatic toxicology plays a key role in choosing indicators, the species and the measures, of AEH for state-of-environment (SOE) reporting, by providing the methods for short- and longer-term monitoring of water bodies. Due to the diversity of habitats and the biodiversity of aquatic ecosystems, there are a number of challenges to this role: choosing indicators that are sensitive and practical, for freshwater, estuaries and marine ecosystems; determining how they can be combined as indexes of AEH for a water body and assisting the sorting and prioritization of the key monitoring programmes and the selection of the most useful indicators.

Much has been written on the topic of aquatic indicators (e.g. see Schindler, 1996; Sindermann, 1996; Griffith, 1998; Burger and Gochfeld, 2001; Adams, 2002; Jorgensen *et al*., 2005; Wells, 2003; 2005; Bortone, 2005; Allsopp *et al*., 2007). Toxicology plays a big role in their selection—from biomarkers to disease aetiologies. Given the rate of global environmental change (loss and degradation), including climate change, the contributions of aquatic toxicology to choosing useful indicators for monitoring water, sediment and tissue quality takes on a new urgency.

4 NEW DIRECTIONS AND CHALLENGES IN AQUATIC TOXICOLOGY

4.1 Accommodating the Complexity of Issues and Ecosystems

The issues and science of aquatic toxicology are complex and interdisciplinary in nature, hence challenging for the practitioner, while offering satisfaction when solutions are found. The responsibilities are also huge. A cornerstone principle is that AEH is linked to human health in many ways! Examples abound: from the 'ocean and human health' programmes in US and Bermuda, such as Dewailly's studies on trace contaminants in Arctic peoples and linkage back to food-chain accumulations in fish and mammals (Cone, 2005); from Shaw's research on persistent chemicals and their effects in seals (Shaw *et al*., 2005); and the recent study by Kelly *et al*. on 'food-web specific biomagnifications of POPs' (Kelly *et al*., 2007). Aquatic toxicology as a discipline is highly dependent upon the sciences of limnology and oceanography, making it very different to pure toxicology itself. To quote Hartman (2007) on forestry and fresh water in British Columbia—'. . . it is unlikely anyone can accurately project the impacts of this sequence of events (the beetle infestation of BC's forests) starting with the extensive clear cutting indicted, followed by the beetle infestation and logging salvage impacts, and followed yet further with potential massive wildfire. The nature and scale of primary effects on lake and river limnology, hydrology, sediment loading, lake and stream temperature regimes and water chemistry and temperature, can only be guessed at'. Hence, the aquatic toxicologist needs a tool kit of basic toxicology, augmented by ecology and the many subdisciplines of ecotoxicology. This is illustrated further by the examples below.

4.1.1 *Influence of Climate Change*

Climate change may influence many aspects of pollutant exposure and biological effects, as changing temperatures and other variables affect migration, breeding, date of first appearance and distributional patterns (range) (Worldwatch Institute, 2007, p. 94). The Millennium Ecosystem Assessment (MEA) report of 2005 predicted that 'the impacts of climate change on biodiversity across all ecosystems will increase very rapidly' (Worldwatch Institute, 2007, p. 94–95). The most recent IPCC (Intergovernmental Panel on Climate Change) predictions are for higher air temperatures, more droughts, more storms, sea-level rise, melting of sea ice and glaciers, and so on, all of which are predicted to occur in the twenty-first century at a fast pace and to influence aquatic ecosystems profoundly. How organisms adapt, and whether aquatic ecosystems have the required resilience to adapt are critical questions (see Poff *et al*., 2002; Walker and Salt, 2006). This is where aquatic toxicology returns to its roots in environmental and comparative physiology, and ecology, and where fundamental aquatic toxicology research must be multidisciplinary and forward thinking. Chemical stressors will be interacting on biological systems and habitats in changing ecological regimes, suggesting that aquatic toxicology should be considering novel, multifactorial scenarios for chemical exposures (see Poff *et al*., 2002).

4.1.2 *Water Pollution and Invasive Species*

One of the most pervasive and serious threats to aquatic habitats and ecosystems are invasive species. Aquatic toxicologists should be more involved with *invasive species* programmes, asking questions about chemical effects in both chemically polluted and invaded (biologically changed) environments, as well as investigating further chemically based controls. Polluted or otherwise disturbed environments appear to be more susceptible to invasive species, for example, San Francisco Bay, European waters, Black Sea (GESAMP, 1997a; Sindermann, 1996; Leppakoski *et al*., 2002). What the interplay is between the presence and success of an invasive species and the level of chemical stress in an ecosystem is worthy of investigation. Many questions require answers: Can chemicals play any new roles in controlling invasives (already applied with fire ants, zebra mussels, sea

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lampreys and ballast-water organisms)? What are the implications of a continued chemical approach (often difficult and expensive, but successful) to further invasives control? Basically, do aquatic toxicologists have a new role to play with this issue?

4.2 Making Use of New Tools in Aquatic Toxicology and Ecotoxicology

DeCoen *et al*. (2005) stated the need for new tools succinctly: 'More quantitative relationships between the various levels of biological organization need to be established in the global implementation of the various ecotoxicological tools and methods. One of the main areas of uncertainty in aquatic toxicology at present is the extrapolation of toxicity data obtained from lab testing to effects in real-life ecosystems'. Ecotoxicology has six future needs—improved biomarkers, transgenic systems, novel markers at the proteome level, organism level effects—mechanisms of reproductive toxicology, realistic effect assessments by predicting effects of mixtures and ecological complexity in toxicity testing—interactions between pollutant stress and food availability. A major challenge is to optimize the knowledge related to the increased volume of toxicological data to construct molecular and genetic pathways explaining the mechanism(s) of toxic effects; increase our understanding of the consequences of lifecycle exposures to chemicals and chemical mixtures; increase genomic information for the species widely used in ecotoxicology; 'in toxicogenomics, a major application of gene expression profiling is to understand the genetic variability and susceptibility to toxic stress within ecosystems' (DeCoen *et al*., 2005).

4.2.1 Model Organisms in Aquatic Toxicology

As in traditional toxicology, model organisms are the backbone of the research, monitoring and regulatory activity, but many questions remain regarding their use: how many model organisms are enough, for the purposes of screening chemicals, and researching and monitoring their fate and effects in aquatic ecosystems? What are our primary models for understanding the effects of chemicals on aquatic organisms? What should they be? Is the concept of the model organism even valid in aquatic toxicology, given the enormous global aquatic biodiversity that requires protection based on the predictions of effects on very few organism types? Can current genomics help ecotoxicologists narrow down the list, given the many genes that organisms have in common (see Benson and Di Giulio, 2006)? There probably is not and should not be an upper limit on the number and type of 'model organisms' for aquatic research purposes, especially in basic research. But for screening chemicals for use and transport, for regulating final emissions, for monitoring and for teaching/training, there are good, mostly practical, arguments for limiting the numbers and types of model organisms to work with in such applications.

For research and regulatory applications, teleost fish have been worked with more than any other group (Cossins and Crawford, 2005; Mommsen and Moon, 2005; Vosyliene, 2007; Di Giulio and Hinton, 2008); many species are now incorporated into toxicity tests, both acute and chronic, in many countries. Standard methodologies have been and are developed under the auspices of EPA (USA), Environment Canada, ASTM, APHA, the OECD and ISO, in particular. Environment Canada has more than 10 model organisms in its regulatory ecotoxicology programmes (Inter-Governmental Ecotoxicological Technical Group (IGETG), 2004). Certain fish species are considered models—rainbow trout and other salmonids (Wells and Moyse, 1981; Environment Canada, 1990a; 1990b); zebrafish (Carvan *et al*., 2000; 2005; Bopp *et al*., 2006); American flagfish (*Jordanella floridae*); fathead minnows (*Pimephales promolus*) (Ankley and Villeneuve, 2006); killifish/mummichogs (Shedd *et al*., 1999; Weis, J., personal communication); sticklebacks (Hahlbeck *et al*., 2004a; 2004b; Ostlund-Nilsson *et al*., 2007) and Japanese medaka (Patyna *et al*., 1999; Farwell *et al*., 2006; Koyama *et al*., 2008), amongst others.

For freshwater invertebrates, for example, cladocerans (Mount and Norberg, 1984; Environment Canada, 1990c; 1990d; 1992; Anderson-Carnahan *et al*., 1995; Sarma and Nandini, 2006), rotifers (Janssen *et al*., 1994; Preston *et al*., 2000; Preston and Snell, 2001; Snell and Joaquim-Justo, 2007; McNair and Belem de Araujo, 2008), crayfish (ASTM (American Society for Testing and Materials), 1994) and bivalve molluscs (Farris and Van Hassel, 2007) have been used. Aquatic insects, such as mayflies and caddis flies, have also been utilized (see Wells *et al*., 1998; G. Persoone, personal communication).

Microalgal species are also used (Bengtson Nash *et al*., 2005), as are protozoa (Sauvant *et al*., 1999) and amphibians (e.g. frogs, toads) (ASTM (American Society for Testing and Materials), 1994). In soil ecotoxicology, nematodes and earthworms are used.

For marine invertebrates, an excellent overview was given by Persoone *et al*. (1984). Polychaetes (Pocklington and Wells, 1992), molluscs (ASTM, 2008), crustaceans (copepods, barnacles, decapods) (Persoone *et al*., 1984; Key *et al*., 2006; Raisuddin *et al*., 2007) and echinoderms (Bay *et al*., 1993; Nipper *et al*., 1993; Kobayashi, 1995) have been studied extensively. Small-scale techniques, acute and chronic, have become well used, especially for assaying contaminated sediments (Carr *et al*., 2001). In an international course in marine hazard assessment

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and ecotoxicology, *Artemia* larvae, urchin embryos and larvae, and bacteria (Microtox®) have been used for many years for assaying and evaluating the risks of contaminated inshore sediments of Bermuda (Bermuda Institute of Ocean Sciences, unpublished data).

Having the tool kit of a few reliable core tests, acute and chronic, with model species is the objective of many countries, agencies and industries. The one primary question left unanswered is: how does one define 'enough' for model organisms, given the huge biodiversity of aquatic ecosystems (e.g. 43 phyla in the oceans alone)? What can we learn from the human health and toxicology sector, dependent on only a few surrogates (e.g. rats, mice, rabbits, monkeys) for experiments and screening of chemicals? The linkage to questions of interspecies sensitivity to chemicals is also obvious. Much remains to be done before the suite of core tests, with model aquatic species, is firmly and confidently established.

4.2.2 Evaluation of Micropollutants

For many years, concerns have been expressed about the possible longer-term health and ecological effects of exposures to chemicals present in very low amounts in natural waters (Howells *et al*., 1990). Such exposures are now referred to as micropollutants—trace elements, heavy metals, POPs such as pesticides, PAHs, polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and pharmaceuticals, often present in measurable, but very low concentrations in groundwater and surface waters, particularly drinking water (Hester and Harrison, 1996; Schwarzenbach *et al*., 2006; De Verlief *et al*., 2007). Recently, concern has heightened about perchlorate found in surface and groundwater across the mid-western US states. This has implications for both ecosystem and human health, largely due to the uncertainties of the chronic effects of such trace chemicals, alone and in mixtures. The challenge of micro-pollutants was described by Schwarzenbach *et al*. (2006). They stated that 'recent research has shown that even mixtures of compounds with different modes of toxic action may cause non-negligible effects… There now are systematic investigations of mixture effects of noninteracting compounds, and countries such as Switzerland are developing water-quality criteria that include additive effects for mixtures of pesticides in surface waters'. The challenge in aquatic toxicology is to continue to conduct realistic experiments and monitoring, seeking evidence of bioaccumulation and sublethal toxicity of such trace chemicals on aquatic biota, with a special emphasis on various suspected EDCs.

4.3 Effects of Chemicals on Behaviour Such as Chemoreception

Many chemicals are pervasive and persistent in water and sediments at very low concentrations, sometimes for decadal periods, for example DDT and its residues, PCBs, PAHs. Consequently, they are of concern regarding their possible sublethal effects on basic biological processes, including the many elements of behaviour. In recent years, behavioural ecotoxicology has become an active and challenging field (Olla *et al*., 1980; Hadjinicolaou and LaRoche, 1988; Dell'Omo, 2002; Clotfelter *et al*., 2004; Kane *et al*., 2005; Gerhardt, 2007), with considerable work being conducted on the effects of pollutants (e.g. cadmium) on complex fish behaviours and their underlying physiological mechanisms (Sloman *et al*., 2003; Scott and Sloman, 2004). ASTM has issued a standard guide to the selection and application of behavioural methods for fish, amphibians and macroinvertebrates (ASTM, 2007).

Of all the behaviours, interference with chemoreception is of paramount concern. Chemoreception in fish and invertebrates plays a crucial role in food finding, reproduction, avoiding predation, schooling, finding suitable habitats, settling and metamorphosis of larvae and postlarvae (Bronmark and Hansson, 2000; Blaxter and Hallers-Tjabbes, 1992; Hebel *et al*., 1997). Some chemicals are known to interfere with chemoreception (e.g. Atema's studies with lobsters and PAHs, Atema and Stein, 1972; 1974) and many other behaviours, such as the burrowing behaviour of invertebrates in sediments (Wells and Sprague, 1976; Dell'Omo, 2002; Clotfelter *et al*., 2004). Given that organisms live in a complex chemical world, and are highly dependent upon chemical cues, subtle effects of foreign chemicals on their behaviour and chemical receptors, at very low concentrations, are not unexpected, are little understood and deserve greater attention.

Key questions that could be examined include: What are the influences of such persistent chemicals, for example the major POPs and trace pharmaceuticals in sewage effluents, on chemoreception and chemically modulated behaviour of organisms living in the water column and sediments? At what concentrations do such effects occur? What are the risks of such effects occurring in aquatic environments? Do we have sufficient techniques to permit detection of such effects, across the phyla?

4.4 Recovery and Resilience Ecotoxicology

There is a need to investigate further the potential for recovery from chemical contaminant exposures and for

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the resilience of chemically contaminated species and geographic areas. Hence, there is a developing field of aquatic toxicology called recovery and resilience ecotoxicology. Recovery has been defined as 'the process leading to partial or complete restoration of a cell, tissue, organ or organism following its damage from exposure to a harmful substance or agent' (National Library of Medicine, USA, web site, *http://www.nlm.nih.gov/*). Resilience is 'the amount of change a system can undergo (its capacity to absorb disturbance) and remain within the same regime—essentially retaining the same function, structure and feedbacks (Walker and Salt, 2006). Recovery is more organism based, resilience refers to the whole ecosystems ability to bounce back from stress. Some key questions currently are: do we conduct enough recovery and resilience aquatic toxicology? What are some good examples of the value of such research and knowledge?

Early (unpublished) work in our Bedford Institute laboratory was with sea urchins, *Strongylocentrotus droebachiensis*, that survived exposures to low concentrations of oil-spill dispersants in sea water, but later succumbed in recovery tanks (clean sea water alone)—they did not recover from exposure to the very low levels of surfactants, which in short experiments were recorded as harmless (Wells, 1982). This showed that short-term, nonlethal chemical exposures could indeed be lethal. There is a recent literature in this field (Depledge, 1998; Sánchez *et al*., 1999; Sancho *et al*., 2003; Zhao and Newman, 2006). Every effort should be made to include recovery (potential) as a dependent variable in experiments and standard assays.

4.5 Other Topics in Aquatic Toxicology

Many other topics could have been considered, briefly or in depth, in this overview of aquatic toxicology, had space permitted. They would have included:

1. Effects of chemicals on particular organisms or habitats, for example, fish, amphibians and reptiles, polar and tropical environments
2. Special topics, such as endocrine disruption, community ecotoxicology, genetics and ecotoxicology, demography and ecotoxicology, and computational toxicology
3. Biological effects monitoring (see Section 1.5 for a brief mention)
4. Coastal and marine ecotoxicology
5. Effects of multiple stressors, and complexity in aquatic ecosystems
6. The role of interfaces in aquatic toxicology
7. Bioinformatics and aquatic toxicity databases
8. Standard testing, and quality assurance/control of data
9. The role of aquatic toxicology in chemical safety assessment.

The reader is encouraged to read Rand (1995; 2009) and the other publications listed in the References and Further Reading Sections for information and guidance on these topics.

4.6 Further Directions and Challenges in Aquatic Toxicology

Aquatic toxicology is a very dynamic field at present (circa 2008), given the influence of molecular biology on its techniques, the genuine convergence of ecology with toxicology and the myriad of threats to aquatic biodiversity globally. Moore (2002) succinctly summarized some of the challenges—understanding toxic mechanisms, developing simulation models of toxic effects, making linkages across levels of biological organization and exercising precaution in the face of new industrial advances.

The field is becoming both data rich and concept rich, the latter being so important given the diversity of aquatic species and habitats under chemical exposure, and the shear impossibility of obtaining data specific to every species and habitat. Practitioners cross many disciplines in order to address the fate and effects of new chemicals on aquatic ecosystems. There is much formal contact at meetings and in new studies and their publications. The interdisciplinary nature of the field is essential (Moore, 2002); it now not only bridges the science disciplines, but also human and ecosystem health, as humans are affected by unhealthy environments, especially degraded water and food quality. Aquatic toxicology is now a mature subdiscipline of toxicology, gaining much from this affiliation in its concepts, practice and new developments, and serving the needs of both humans and the aquatic environment.

5 SUMMARY

Many threats to aquatic environments and their living resources remain, with very large economic, ecosystem and human-health consequences. Such threats are being addressed vigorously by aquatic scientists, regulatory agencies, industry and their representatives and international agencies. Methods in aquatic toxicology are being continually advanced, especially benefitting from linkages to classical toxicology, molecular biology, benthic biology and nanotechnology. Current techniques are also applied more frequently in environmental effects monitoring. Aquatic toxicology continues to be a field of much vitality, innovation and change in the new millennium,

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with new techniques, new understanding and the imperative of the vital importance of protecting the quality of aquatic ecosystems.

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Environmental Endocrine Toxicology

Poul Bjerregaard

CONTENTS

1 INTRODUCTION

The incidence of a number of adverse effects originating in the male reproductive organs increased during the last decades of the twentieth century. In humans, this was most clearly reflected in the increased prevalence of testicular cancer, but some investigations also showed a decrease in sperm-cell numbers in humans and increased frequencies of cryptorchidism and hypospadias. In nature, sexual differentiation was affected in fish downstream of discharges from sewage treatment plants and paper mills, and alligators exposed to chemicals

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after a spill showed altered sexual development. Likewise, sexual development was affected in gulls contaminated with chlorinated pesticides and in snails contaminated with tributyltin (TBT) from antifouling paints.

During the 1990s it became clear that a fairly large number of chemicals with widespread use in the industrialized world had the potential to mimic the effects of natural oestrogens in living organisms.

Based on some of these observations, Sharpe and Skakkebaek (1993) formulated the 'oestrogen hypothesis', according to which the mechanism underlying at least part of the phenomena described was the exposure of male foetuses at early stages of development to unusual amounts of the female sex hormones (oestrogens) or chemicals with oestrogenic effects.

More recently, concerns over the potential impact of chemicals with antiandrogenic effects on male development have arisen. In humans, the four phenomena mentioned above are now considered four different manifestations of the same underlying disorder—'testicular dysgenesis syndrome (TDS)'.

Disruption of endocrine systems other than the sex-hormone system has also been identified.

Table 1 Prevalence of testicular cancer (incidence per 10^5), hypospadias (%), cryptorchidism (%) and sperm cell numbers (cells ml^{-1}) in three Nordic countries

	Finland	Sweden	Denmark
Testicular cancer[a]			
1943	—	—	2.8
1960	1.0	2.6	4.6
1987	2.3	4.6	8.6
Hypospadias[b]	0.27	—	1.03
Cryptorchidism[c]			
At birth	2.4	—	9.0
At three months	1.0	—	1.9
Sperm number[d]	54	—	41

[a]Adami *et al*. (1994).
[b]Boisen *et al*. (2005).
[c]Boisen *et al*. (2004).
[d]Jorgensen *et al*. (2002).

2 TESTICULAR DYSGENESIS SYNDROME

2.1 Testicular Cancer

The frequency of testicular cancer has been increasing in most Western countries (Adami *et al*., 1994) and, in Denmark, which started systematic recording of cancer frequencies earlier than any other country, the prevalence of testicular cancer increased almost linearly from 1943 to the turn of the century (**Table 1**). Whereas Denmark, together with Norway, has the highest frequency of testicular cancer in the world, the incidence of testicular cancer is lower in Finland, although the frequency there has also increased since recording began (**Table 1**). Testicular cancer is most often diagnosed in men in their twenties and thirties, but the potential for testicular cancer is founded during early pregnancy (Skakkebaek, 1978). The division of early germinal cells is arrested and carcinoma *in situ* (CIS) cells are formed, which later in life initiate the development of testicular cancer. The precise mechanism—and the potential role of hormones or endocrine disrupters—underlying the formation of the CIS-cells is unknown.

The prevalence of testicular cancer varies between ethnic groups (Purdue *et al*., 2005) and it has been considered that genetic changes might explain the increase in the frequency of testicular cancer. However, the increase in the frequency has been so rapid that genetic changes have been rejected as a potential explanation. On the other hand, it has been strongly indicated by comprehensive Swedish investigations (Hemminki and Li, 2002) among persons migrating between the Nordic countries, that some kind of environmental impact (lifestyle factors, chemicals, etc.) seems to be a determining factor for the prevalence of testicular cancer. The Swedish frequency of testicular cancer is higher than the Finnish and lower than the Danish (**Table 1**). If Finnish or Danish couples move to Sweden, the frequency of testicular cancer in their offspring (born in Sweden) will increase (for the Finns) or decrease (for the Danes), respectively, approaching the frequency normally seen in Sweden (Hemminki and Li, 2002).

2.2 Sperm Numbers

Various investigations have shown that sperm numbers—at least in some geographical regions—have been or are decreasing. A meta-analysis of all previously published data (Carlsen *et al*., 1992) carried out in the early 1990s indicated a decrease of approximately 2% per year, and a similar trend was also seen in some (Auger *et al*., 1995)—but was not supported in other—subsequent investigations (reviewed by Safe, 2004).

Sperm numbers in humans show large individual variability (0 to approximately 500 million cells ml^{-1}). Men's fertility seems independent of sperm numbers for values higher than 35–40 million cells ml^{-1}, whereas fertility decreases linearly with sperm numbers below that threshold (Bonde *et al*., 1998). Approximately 40% of young Danish (Jorgensen *et al*., 2002) and Norwegian (Jorgensen *et al*., 2006) men have sperm numbers

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below 40 million cells ml^{-1} and this is also the case in 45% of young German men (Paasch *et al*., 2008). Sperm numbers are higher among young Finnish than Danish men (**Table 1**).

The ability of an adult man to produce sperm cells is associated with adequate masculinization of the male genital tract—including the proper development of Sertoli cells in the testes—during foetal development (Sharpe and Skakkebaek, 1993).

2.3 Cryptorchidism

Cryptorchidism is the failure of one or both testes to descend into the scrotum during pregnancy. The descent of the foetal testes from their initial position in the abdominal region is regulated by hormones and in experimental animals interference with the hormonal system may lead to undescended testes (Welsh *et al*., 2008). Some investigations have shown increased frequencies of cryptorchidism among humans, but comparison with earlier data is somewhat hampered by the previous lack of clear diagnostic criteria for this phenomenon (Toppari *et al*., 2001). Recent investigations have shown that the prevalence of cryptorchidism is approximately three times higher in Denmark than in Finland (**Table 1**).

2.4 Hypospadias

Hypospadias is an abnormality in the male genital system in which the urethra opens at a site other than the tip of the penis. This phenomenon may be induced in experimental animals by exposure to chemicals disturbing sexual development (Rider *et al*., 2008). Some investigations have shown increasing prevalence in humans but, as with cryptorchidism, comparison with earlier data are made difficult due to the previous lack of clear diagnostic criteria for this phenomenon (Toppari *et al*., 2001). However, more recent investigations show that the prevalence of hypospadias is three times higher in Denmark than in Finland (**Table 1**).

2.5 Links Between the Four Phenomena

The disturbances in human sexual development (increased frequency of testicular cancer, decreased sperm count, cryptorchidism, hypospadias) were earlier regarded as separate phenomena, but recent research shows that they should rather be seen as four separate manifestations of the same basic disturbance in sexual development, and they are now looked upon as a coherent syndrome—'TDS' (Skakkebaek *et al*., 2001).

Men who develop testicular cancer have lower fertility before the outbreak of the disease than men who do not develop testicular cancer (Moller and Skakkebaek, 1999) and boys born with hypospadias and/or cryptorchidism have an increased risk of developing testicular cancer (Toppari *et al*., 2001).

Recent investigations have shown that the frequencies of cryptorchidism as well as hypospadias among newborn boys are approximately three times higher in Denmark than in Finland, the latter also having a lower frequency of testicular cancer and higher sperm counts than Denmark (**Table 1**).

2.6 Do Endocrine-Disrupting Chemicals Play a Role in Testicular Dysgenesis Syndrome?

2.6.1 Phthalates

Exposure to certain phthalates causes reproductive disorders in rats similar to TDS symptoms (Foster, 2006), the so-called 'phthalate syndrome'. Exposure of rats to phthalates over a narrow time window during foetal development suppresses the production of foetal testosterone (a necessity for masculinization of the male genital tract) resulting in reduced Sertoli cell numbers, hypospadias, cryptorchidism and reduced anogenital distance (Howdeshell *et al*., 2008b; Scott *et al*., 2008; Welsh *et al*., 2008).

Although human exposure to phthalates has generally been assumed to be very much lower than the doses necessary to induce the 'phthalate syndrome' in rats, phthalate monoester metabolites can be demonstrated in human amniotic fluid in some women (Silva *et al*., 2004) at concentrations less than an order of magnitude lower than the concentrations found in amniotic fluid in rats (Calafat *et al*., 2006) exposed to phthalate at doses eliciting effects.

Swan *et al*. (2005) investigated pregnancy outcomes in mothers exposed to phthalates and found reduced anogenital distance in the sons of women with the highest phthalate exposures during pregnancy. Main *et al*. (2006a) found a negative association between the level of monobutyl phthalate in the breast milk of mothers and the testosterone level in their three-month-old male offspring, and a positive correlation between the monobutyl phthalate concentration and the concentration of sex-hormone-binding globulin.

Thus, it seems as if the risk cannot be neglected that exposure to phthalates or mixtures of phthalates may be one—probably among several—factor that may contribute to the development of TDS in humans (Sharpe, 2008).

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2.6.2 Pesticides

Many greenhouse workers have a higher exposure to pesticides than the general population and in a recent Danish investigation, female greenhouse workers were found to have an increased risk of delivering boys with cryptorchidism and reduced penis sizes relative to the general population (Andersen *et al*., 2008). The precise exposure regime for the female greenhouse workers could not be established, but known endocrine disrupters such as vinclozolin, fenarimol and prochloraz were among the 124 active pesticide ingredients registered as used in the greenhouses (Andersen *et al*., 2008).

2.6.3 PCBs, HCB and Chlordanes

A Swedish investigation of the concentration of chlorinated hydrocarbons in the blood of mothers whose sons developed testicular cancer (against control mothers whose sons did not develop testicular cancer) showed increased odds ratios for total polychlorinated biphenyls (PCBs), hexachlorobenzene (HCB) and chlordanes (Hardell *et al*., 2003). The concentrations of a number of individual PCB congeners were higher in the blood of the mothers of the cases; this was also true when grouped into oestrogenic and enzyme-inducing PCBs (Hardell *et al*., 2004); the PCB concentrations of the young men showed no differences.

2.6.4 Oestrogenic Chemicals

2.6.4.1 Synthetic Oestrogens

The example of diethylstilbestrol (DES) illustrates that human exposure to chemicals may affect male sexual development. During the period 1940–1970 a large number of women were treated with DES to counteract pregnancy complications. As opposed to natural oestrogen, DES is not bound to the sex-hormone-binding proteins in the blood, so the concentration of the chemical available for uptake into the cells is relatively high. Sons of these women showed an increased frequency of hypospadias, cryptorchidism and testicular cancer and low sperm count (reviewed by Sharpe and Skakkebaek, 1993).

However, in experimental animals, DES and also ethinyloestradiol (EE2) have far stronger oestrogenic effects (Howdeshell *et al*., 2008a) than any of the industrial chemicals humans are exposed to in everyday life. With the present knowledge about the potency of the different chemicals with oestrogenic effects, it is difficult to point out individual chemicals to which the general population is exposed at sufficiently high doses to cause changes in the sexual development of male foetuses (Sharpe and Skakkebaek, 2003).

2.6.4.2 The Oestrogen Exposure of the Foetus and the Lifestyle of the Mother

Various changes in lifestyle may have changed the natural oestrogen exposure of foetuses during the last half of the twentieth century, via changes in the general regulation of the blood oestrogen content of women (reviewed by Sharpe and Skakkebaek, 1993): (i) Fatty tissues metabolize oestrogen differently from other types of tissue, such that obesity leads to increased concentrations of oestrogen in the blood. (ii) A high content of fibre in food binds oestrogen excreted into the gastrointestinal tract such that enterohepatic recirculation (re-uptake of excreted oestrogen from the intestine) is reduced. (iii) A high content of phyto-oestrogens (e.g. from soya products) in food seems to increase the production of sex-hormone-binding proteins; this will lead to a decrease in the presence of free oestrogen in the blood. (iv) Mothers who smoke during pregnancy give birth to boys with lower sperm counts than nonsmoking mothers. Smoking mothers generally give birth to children with lower birth weights than nonsmoking mothers, and low birth weight by itself is associated with low sperm counts. (v) During a woman's first pregnancy, the regulation of sex-hormone-binding globulins in the blood is different from regulation during subsequent pregnancies, and this results in a higher exposure of the foetus to oestrogen. First-born boys have a higher risk of developing testicular cancer than boys born later in a group of siblings. Since women in the Western world have generally given birth to fewer children over the recent generations, this might potentially lead to an increase in the frequency of testicular cancer (but it would not explain the difference in prevalence between e.g. Denmark and Finland).

Furthermore, both underweight and overweight young men have lower sperm counts than men with a normal weight.

2.6.5 Could Mixtures Play a Role?

Common procedures for risk assessment of chemicals consider chemicals in single-compound exposure, generally ignoring the fact that both humans and organisms in the environment may simultaneously be exposed to a multitude of different chemicals; at best, it is assumed that the application or safety factors used when deriving, for example, acceptable daily intake (ADI)-values from no-effect levels identified in laboratory studies account for the potential effects of mixed exposure.

For endocrine-disrupting chemicals, a fair amount of knowledge about effects of mixtures has been gathered during recent decades. The general conclusion has been that chemicals with similar modes of action act additively and that mixture effects in such cases can be fairly accurately predicted by concentration or dose-addition models. This has been shown for oestrogenic chemicals in *in vitro* systems (Payne *et al*., 2000; Rajapakse

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et al., 2001) and *in vivo* in fish (Brian *et al*., 2007; Thorpe *et al*., 2003; 2001) and for antiandrogens (Hass *et al*., 2007), phthalates (Howdeshell *et al*., 2008b) and mixtures of different classes of antiandrogens (Rider *et al*., 2008) in rats.

The effect of mixtures with dissimilarly acting chemicals may be more complicated to predict: thus, a combination of four antiandrogens with different mechanisms of action (vinclozolin (an androgen receptor antagonist), DEHP (bis(2-ethylhexyl)phthalate, a testosterone synthesis inhibitor), finasteride (an inhibitor of steroid Type II 5-reductase) and prochloraz (multiple mechanisms)) administered to pregnant rats at doses that individually caused no or little effect caused 100% hypospadias in the male offspring (Christiansen *et al*., 2008).

Humans are known to be exposed to several endocrine-disrupting chemicals simultaneously (Main *et al*., 2006b; Silva *et al*., 2004) and the potential effect of these mixtures needs to be better understood.

3 ENDOCRINE DISRUPTION IN THE ENVIRONMENT

3.1 Feminization of Male Fish Downstream from Wastewater Discharges

The initial observations of feminization of male fish in areas affected by wastewater discharges were made in England, where early stages of eggs (ovo-testes) were found in the testes in a diclinous fish, the roach *Rutilis rutilis* (Jobling *et al*., 1998). This condition was called intersex and the degree of intersex was graded into different stages, in which stage 0 is unaffected males and stage 7 is fully feminized males (Jobling *et al*., 1998). A larger investigation was initiated in which males upstream and downstream from discharges from major sewage-treatment plants were examined. The sewage-treatment plants were chosen such that there was some kind of a physical separation of the populations upstream and downstream from the wastewater discharge. No areas were found without a certain fraction of feminized male fish and downstream from some sewage-treatment plants no unaffected males were found at all. The degree of intersex in the investigated roach populations increased with increasing contamination of the rivers with ordinary household wastewater.

The oestrogenic effect of the discharged wastewater at a number of English plants was demonstrated by the induction of vitellogenin in male trout caged in or downstream from the wastewater discharges (Harries *et al*., 1996).

Feminization of male fish in areas affected by wastewater discharges has now been found in many countries in Europe, North America, Asia and Australia, and the phenomenon has been demonstrated in approximately a dozen investigated fish species.

These effects may also extend to estuarine and coastal areas (Matthiessen *et al*., 2002).

3.1.1 The Causes of the Feminization

The oestrogenicity of the wastewater is caused particularly by the natural oestrogens 17β-oestradiol (E2) and oestrone (E1) and the synthetic oestrogen, 17α-ethynylestradiol (EE2), which is used in contraceptive pills. The three chemicals are excreted mainly from women—but for the natural oestrogens, a little also from men—in the households contributing the waste water to the sewage-treatment plants.

The oestrogens are excreted from humans mainly in the form of conjugates (especially as glucuronides and sulfates) that do not have any hormonal effects. Microbial processes in the raw sewage hydrolyse the excreted conjugates and thereby re-establish the hormonal activity (Stuer-Lauridsen *et al*., 2005). Most modern sewage-treatment plants remove the majority of the oestrogens from the wastewater, but the efficiency depends of the type of plant, and the water and sludge residence times; plants with nitrification (Stuer-Lauridsen *et al*., 2005) and high retention times are most efficient.

However, even the relatively low remaining amount of oestrogen in the purified wastewater may have the potential—at least locally—to feminize male fish in areas affected by wastewater discharges. In controlled short-term experiments in the laboratory, E2 feminizes male fish (determined by induction of vitellogenin synthesis) at concentrations between 15 and 50 $\mathrm{ng\,l^{-1}}$ (Bjerregaard *et al*., 2008); oestrone is slightly less potent (50–100 $\mathrm{ng\,l^{-1}}$), while EE2 feminizes male fish (for most of the species investigated) at concentrations between 1 and 5 $\mathrm{ng\,l^{-1}}$. Concentrations of natural and synthetic oestrogens exceeding these values have been found in many wastewater discharges (Desbrow *et al*., 1998).

E2 and E1 are degraded relatively quickly in river water ($t_{1/2} < 1$ day), but with a half life for EE2 of one to two weeks (Jurgens *et al*., 2002) this chemical has the potential to exert feminizing effects fairly far downstream the wastewater discharge.

In some cases the endocrine disruption in the freshwater environment is attributed to the discharge of oestrogenic industrial chemicals. The Aire River is situated in an area in northern England with a large woollen industry in which alkylphenolpolyethoxylates are used in large amounts to degrease raw wool. In the River Aire, concentrations of nonylphenol have been measured in the water that are almost 10 times higher than needed to

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induce vitellogenin synthesis in short-term experiments in rainbow trout (Harries *et al*., 1996).

3.1.2 The Effect of the Feminization in Populations

There is only a limited knowledge about the impact of male feminization on populations. Male roach with intersex produce lower amounts of semen than normal males (Jobling *et al*., 2002); it is, however, not known what fraction of the males must be affected (and to what degree) before it has negative consequences for the population. The timing of the readiness to spawn in the intersex males was changed relative to the females, and this lack of synchronization of spawning might be envisaged to affect the reproduction as much (or maybe even more) than the lower semen amounts of the intersex males (Jobling *et al*., 2002).

In multigeneration studies with zebra fish in the laboratory it has been found that bisphenol A and EE2 begin to reduce the reproductive success at almost the concentrations that will also induce vitellogenin production in males in short-term experiments (Segner *et al*., 2003). It is not known if the same is true for other fish species and in nature. In a Canadian field experiment, EE2 was added to a lake and maintained at approximately $5\,ng\,l^{-1}$ for a number of years. The lake's population of fathead minnow collapsed after five years (Kidd *et al*., 2007).

3.2 Masculinization of Female Fish Downstream from Paper Mills

A number of investigations in different parts of the world have documented that process water from paper production may affect the sexual development in fish. Masculinization of female fish, lower concentrations of sex hormones in the blood, reduced size of sexual organs, altered sex ratios in, for example eelpout, and several other phenomena related to normal sexual development have been observed (Larsson *et al*., 2000; Munkittrick *et al*., 1998; Tremblay and Van Der Kraak, 1999).

The exact relationships between cause and effect for these impacts have not been elucidated, but chlorinated dioxins (produced during chlorine bleaching processes) and plant sterols (present in wood) have been suspected.

3.3 The Alligators in Lake Apopka

A spill of various chlorinated chemicals in Lake Apopka, Florida, in 1980 caused a major decrease in the reproduction in the lake's population of alligators *Alligator mississippiensis* (Guillette *et al*., 1999; 1995; 1994; 1996). The male alligators developed abnormally small external sexual organs (Guillette *et al*., 1996) and the levels of sex hormones were abnormal (Guillette *et al*., 1999).

3.4 DDT-Contaminated Birds

Skewed sex ratios, feminization of male birds and reproductive problems were seen in dichlorodiphenyltrichloroethane (DDT)-contaminated populations of gulls in California and injection of relevant doses of DDT produced these effects in laboratory experiments (Fry and Toone, 1981).

3.5 Imposex and Intersex in Snails

3.5.1 Imposex

Snails belonging to the families *Buccinidae* (i.e. the dog whelk *Nucella lapillus*) and *Nassidae* are normally gonochoristic (each individual is either male or female throughout life). In the late 1960s female dog whelks were found around the Marine Biological Laboratory in Plymouth, UK, that had developed obvious male sexual characters, that is, penis and vas deferens (Blaber, 1970). An analogous phenomenon was found in the American snail species *Nassa obsoletus* (Smith, 1971; 1980; 1981), and exposure to TBT was identified as the cause of the imposed male sexual characters. In these families of snails the formation of imposed male sexual characters in females is termed *imposex* (Smith, 1971). Imposex has now been recorded in approximately 200 species of snails worldwide.

The development of imposex in females is divided into seven stages (Gibbs *et al*., 1987): Stage 0: normal female; Stage 1: formation of vas deferens initiated; Stage 2: penis development initiated; Stage 3: vas deferens 50% developed; Stage 4: vas deferens fully formed; Stage 5: vas deferens/prostate tissue beginning to block the vaginal opening; Stage 6: the capsule gland contains aborted egg capsules.

While the development of imposed male sexual characters does not seem to affect the ability to reproduce in *Nassa obsoletus* (Smith, 1980; 1981), the female dogwhelks may end up sterile in severe cases because prostate-like tissue around the vas deferens blocks the vaginal opening of the female. It is assumed that sterile females may die because they abort the eggs (that cannot be liberated via the blocked vaginal opening) into the body cavity (Gibbs *et al*., 1987).

Dog whelks are very sensitive to TBT and in adult females concentrations as low as $5\,ng\ TBT\ l^{-1}$ may

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induce growth of penis. Young and not yet sexually mature females of dog whelks are even more sensitive than the adult individuals and exposure to less than 1 ng TBT l^{-1} in nature seems to induce growth of penis and vas deferens tissue in females (Gibbs *et al*., 1987).

Imposex occurs, not only in coastal areas, but also in open marine areas with major shipping routes (Ten Hallers-Tjabbes *et al*., 1994; 2003), for example, in the Danish Belt Area and Kattegat no normal females of the whelk *Neptúnea ántiqua* were found in a recent survey (Strand and Jacobsen, 2002).

3.5.2 Intersex

In periwinkles (e.g. *Littorina littorea*) and mud snails (*Hydrobia* sp.) the masculinizing effects of TBT are exerted in a slightly different manner than in the whelks and in these species the condition is termed *intersex* (Bauer *et al*., 1995). Intersex in the periwinkles is divided into four stages. A penis is developed in the female only in Stage 4 (which is very rarely observed). The periwinkles are far less sensitive to TBT than the whelks.

3.5.3 Effects in Populations

Many of the English populations of dogwhelks declined in numbers during the 1970s and 1980s and it is assumed that population sizes start to be affected when between 20 and 40% of the females have become sterile (Gibbs *et al*., 1991; Langston *et al*., 1990; Spence *et al*., 1990).

3.6 The Effect of PCBs in Seals and Humans

The populations of Baltic seals (grey (*Halichoerus grypus*) and ringed (*Phoca hispida*)) and Wadden Sea harbour seals (*Phoca vitulina*) showed marked decreases during the 1960s and 1970s, and, especially in the Baltic seals, a number of pathological lesions were seen (Olsson *et al*., 1992).

Several lines of evidence indicated that contamination with PCBs was responsible for these effects (Kihlstrom *et al*., 1992; Reijnders, 1986). Hydroxy metabolites of certain PCB isomers have a high affinity for binding to the thyroxin-transporting protein transthyretin in the blood and in an experiment where harbour seals were fed fish from the Atlantic (low PCBs) and Wadden Sea (high PCBs) both reproductive output and thyroxin levels were reduced in the seals fed the Wadden Sea fish (Brouwer *et al*., 1989). Although the thyroxin levels were definitely affected in the seals, PCBs possess multiple of modes of action and the altered thyroxin levels may not be the only cause of the reproductive problems.

In human populations with a high intake of PCBs from contaminated fish, effects on foetal brain development have been demonstrated (Jacobson *et al*., 1990); correct levels of thyroxin are important in the final development of brain function.

4 METHODS FOR DEMONSTRATION OF ENDOCRINE EFFECTS OF CHEMICALS

The recognition that synthetic chemicals have the potential to affect endocrine regulation, that is, by interacting with hormone receptors, has necessitated the development of test systems that can reveal this type of effect.

Both *in vitro* and *in vivo* test systems for the investigation of endocrine-disrupting effects exist and, since conclusions reached after use of the two types of systems are not necessarily identical, it is necessary to stress that there does not exist one and only one well-defined and commonly accepted method to demonstrate if a specific chemical has a specific endocrine-disrupting effect or not.

4.1 *In Vitro* Methods

4.1.1 Receptor Affinity

The ability of chemicals to bind to hormone receptors can be investigated in receptor-binding assays. The principle in such an assay is relatively simple: an organ with a suitably high concentration of the relevant receptor (e.g. the oestrogen receptor) is homogenized. Radioactively labelled oestrogen is added to the homogenate, the labelled oestrogen binds to the receptor, and equilibrium between free and bound labelled oestrogen is attained. If nonlabelled oestrogen is subsequently added to the homogenate, the nonradioactive oestrogen will compete with the labelled oestrogen to bind to the receptor and a new equilibrium between free and bound labelled oestrogen will be attained. In a similar way, the ability of various chemicals to outcompete the labelled oestrogen in binding to the receptor can be investigated.

In this way, the affinity of chemicals for hormone receptors may be determined for the receptors one might have an interest in (e.g. androgen or thyroxin). The method does not, however, reveal if a chemical with a high affinity for a given receptor functions as an agonist (mimics effect of the hormone) or an antagonist (inhibits the effect of the hormone by binding to and blocking the receptor) in the system.

4.1.2 Cell Cultures

The growth of some types of breast-cancer cells is stimulated by oestrogen, and this means that the oestrogenic effect of chemicals can be investigated by evaluating

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their impact on the growth of such cell cultures. Several different cell lines have been developed for this purpose, but one of the most often utilized cell lines is the MCF-7 cell line (Soto *et al*., 1995).

The ability of chemicals to bind to and activate steroid receptors may be investigated in yeast cells into which the gene for the human oestrogen or androgen receptor has been inserted, together with a reporter gene. In the YES-assay (yeast estrogen inducible expression system) the *lac-Z* gene, coding for β-galactosidase, has been used as a reporter gene (Routledge and Sumpter, 1996). In the yeast cells used in the YAS assays, the androgen receptor has been genetically engineered into the yeast cells instead of the oestrogen receptor.

4.2 *In Vivo* Methods

4.2.1 The Uterotrophic Assay

In physiology, an oestrogen was originally defined as a compound that could cause growth of the uterus. It is therefore possible to investigate the potential oestrogenic effect of a chemical by dosing female mammals which do not themselves produce oestrogen (sexually immature animals or animals which have had their ovaries removed) with the chemical and register changes in the weight of the uterus after three days (Kanno *et al*., 2003a; 2003b).

4.2.2 The Hershberger Assay

The Hershberger assay is an androgen analogue to the uterotrophic essay. In this assay, the effect of chemicals on the weight of different organs (e.g. the prostate) associated with the sexual organs in castrated or juvenile male rats not themselves producing androgens, is determined (Owens *et al*., 2007; 2006).

4.2.3 Tests in Fish

4.2.3.1 Sexual Development

Sexual differentiation in fish is very sensitive to exposure to external sex hormones and in commonly used test species such as the zebrafish (*Danio rerio*), fathead minnow (*Pimephales promelas*) and Japanese medaka (*Oryzias latipes*), the phenotypic sex of the fish can be altered by exposure to oestrogens (feminization) and androgens (masculinization) (Holbech *et al*., 2006). Exposure to chemicals with oestrogenic, antioestrogenic, androgenic, antiandrogenic and aromatase-inhibiting effects will thus affect the sex ratio of the developing fish larvae, and this provides a sensitive system for revealing endocrine-disrupting effects which relate to the normal function of the sexual hormones.

4.2.3.2 Vitellogenin Induction

When female fish produce yolk proteins (vitellogenin) for incorporation into the eggs during preparation for reproduction, this takes place under hormonal control. Gonadotropins released from the pituitary induce the ovary to produce oestrogen, which is released into the blood. The liver responds to the oestrogen by inducing the synthesis of vitellogenin, which is released into the blood, taken up by the ovary and incorporated into the oocytes (Sumpter and Jobling, 1995).

Male and juvenile fish normally synthesize vitellogenin in very small amounts, but the synthesis can be induced by exposure to oestrogens or oestrogenic chemicals in food or water or by injection. Therefore, the concentration of vitellogenin in the blood of male or juvenile fish may be used as an excellent biomarker for oestrogenic effect, both in laboratory experiments and investigations in the field (Bjerregaard *et al*., 2008).

4.2.4 Tests for Reproductive Toxicity/Multigeneration Tests

For mammalian, avian and amphibian tests used in the risk assessment of chemicals, attempts are being made to improve and expand the scope of reproductive toxicity and multigeneration tests in order to reveal the potential endocrine-disrupting effects of chemicals. This may involve investigation of the male offspring of female rats (exposed to chemicals during pregnancy) for feminizing or demasculinizing effects. If the male rats are born with nipples or a smaller distance between the anal opening and the external genitalia (the anogenital distance) than normal, this is a sign that feminization or a demasculinization of the foetus has taken place. Thyroxin-disrupting effects of chemicals may be investigated in amphibians during metamorphosis.

4.3 *In Vivo* vs. *In Vitro* Methods

In vitro methods are generally more sensitive and less resource intensive than tests on live animals. Tests *in vivo*, however, have the advantage that the response of the intact, entire organism to the chemical is investigated.

For the inherent, endocrine-disrupting effect of a chemical to be exerted in an intact organism, the chemical must initially be taken up through the surface or via the gastrointestinal tract, be transported with the blood and thereafter reach the organ or the organs where the endocrine-disrupting effect is exerted.

In the blood, the chemicals may to a larger or smaller extent be bound to specific proteins that normally bind and transport sex hormones. If the chemicals are not bound to these proteins (e.g. in *in vitro* test systems) the amount that is available for uptake into the cells will be

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increased and thereby the potential endocrine-disrupting effect will also be increased.

Higher organisms possess relatively efficient enzyme systems that metabolize or degrade xenobiotic chemicals. There are examples where a chemical never exerts its otherwise inherent endocrine-disrupting effect, because the organism exposed to the chemical degrades or metabolizes it before it reaches the organ where the effect might have been exerted.

Some chemicals do not possess endocrine-disrupting effects in their original form, but their metabolites do.

5 ENDOCRINE-DISRUPTING CHEMICALS

5.1 Definition

There are several definitions as to what one should understand by the term 'endocrine-disrupting chemical', but one of the most often cited definitions was made by the committee—the Endocrine Disrupter Screening and Testing Advisory Committee (EDSTAC)—that advices the US Congress on test methods in this area, and it is as follows: An exogenous substance that changes endocrine function and causes adverse effects at the level of the organism, its progeny, and/or (sub)populations of organisms.

5.2 General Modes of Action

The function of endocrine systems may be disturbed at several levels, since chemicals may affect:

1. Synthesis of hormones
2. Binding to transport proteins in the blood
3. Interaction with hormone receptors in the cells
4. Metabolism or degradation of hormones.

The interaction of chemicals with hormone receptors may lead to a mimicking of the function of the natural hormone (agonism) or an inhibition of this (antagonism); the latter may take place if the chemical binds to the receptor such that the binding of the natural hormone is blocked.

5.3 Natural Oestrogens

Endogenous hormones do not have endocrine-disrupting properties in the organism where they are produced if the hormones are functioning normally. However, natural oestrogens excreted from humans may pass sewage-treatment plants and natural oestrogens have been shown to leach from agricultural areas treated with liquid manure (Kjaer *et al*., 2007) and from livestock farms (Matthiessen *et al*., 2006). Thus, natural oestrogens may be found, for example, in freshwater and coastal environments, where they may exert endocrine-disrupting effects as previously described.

5.4 Synthetic Oestrogens and Androgens

A number of synthetic oestrogens have been developed in the pharmaceutical industry and the most relevant ones in this connection are DES and 17α-EE2, the latter being used in contraceptive pills. Both DES and EE2 have shown stronger oestrogenic effect in some test systems than the two most abundant natural oestrogens 17β-oestradiol and oestrone. Like the natural oestrogens, EE2 may cause feminization in the aquatic environment upon excretion from women after use in contraconception.

Trenbolone is a very potent synthetic androgen which is used as a growth promoter in, for example, US cattle. Trenbolone has been detected in the aquatic environment in the vicinity of cattle feedlots in USA (Durhan *et al*., 2006) and it has a strong masculinizing effect in fish (Ankley *et al*., 2003).

5.5 Phyto- and Myco-Oestrogens

Certain plants and fungi produce chemicals which have endocrine disrupting—for example, oestrogenic—effects. Some of these chemicals are found in relatively high concentrations in, for example, clover and soya.

Some of these chemicals have shown oestrogenic effects in *in vitro* as well as *in vivo* test systems. In some *in vitro* test systems the most potent of these chemicals show an oestrogenic potency approaching that of 17β-oestradiol.

5.6 Alkylphenols

Alkylphenolpolyethoxylates and alkylphenols are used in a wide variety of daily products such as detergents, cosmetics, paints and as plastic additives. The global production is approximately 300 000 tonnes per year; approximately 80% are nonylphenolpolyethoxylates and octylphenolpolyethoxylates constitute the majority of the remaining 20%.

A large fraction the alkylphenolpolyethoxylates used ends in up wastewater. Nonylphenol is degraded relatively slowly in the environment. The degradation products of alkylphenolpolyethoxylates have been shown

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to be oestrogenic in *in vitro* test systems (Routledge and Sumpter, 1996; Soto *et al*., 1991) and technical nonylphenol, 4-*t*-octylphenol, 4-*t*-pentylphenol, nonylphenol carboxylic acid and nonylphenoldiethoxylate have proved to be oestrogenic in *in vivo* experiments. Alkylphenols with branched alkyl chains are generally far more oestrogenic than alkylphenols with unbranched alkyl groups; this is true in both *in vitro* (Routledge and Sumpter, 1996) and *in vivo* (Pedersen *et al*., 1999) test systems. 4-*t*-Octylphenol is the most oestrogenic of the alkylphenols and the potency is approximately 2000–10 000 times lower than that of 17β-oestradiol (Routledge and Sumpter, 1996).

In short-term experiments with adult male fish, the threshold for induction of vitellogenin by the most potent alkylphenols is in the range 5–20 $\mu g\,l^{-1}$ (Jobling *et al*., 1996). In the uterotrophic assay it takes a dose of approximately 100 $mg\,kg^{-1}\,day^{-1}$ of the most potent of the alkylphenols to elicit an oestrogenic response (Laws *et al*., 2000); vitellogenin synthesis is induced in rainbow trout with a dose of 30 $mg\,kg^{-1}$ in the food every second day (Pedersen *et al*., 2003).

Alkylphenols affect the sexual development in fish that have been exposed to the chemicals in the laboratory during the period where sexual differentiation takes place. Exposure of medaka, *Oryzias latipes*, to 100 μg nonylphenol l^{-1} from hatching until the age of three months caused a feminization of the male fish, which developed a female sex organ next to the testicles (Gray and Metcalfe, 1997). In similar experiments with carps it has, likewise, been possible to feminize genetic males by exposing them to 4-*t*-pentylphenol (Gimeno *et al*., 1996).

In certain instances nonylphenol concentrations have been measured in the aquatic environment that are higher than the concentrations needed to feminize male fish in laboratory experiments. In some rivers in England (Sheahan *et al*., 2002), the oestrogenic effect of the river water is attributed to the presence of nonylphenol concentrations as high as 180 $\mu g\,l^{-1}$.

5.7 Bisphenol A

The world production of bisphenol A ranges between 0.5 and 1 million tonnes per year. More than 99% of the production is used to manufacture polycarbonate, epoxy and fire retardants.

The oestrogenic effect of bisphenol A has been demonstrated in *in vitro* as well as *in vivo* (Dodds and Lawson, 1936) test systems.

Bisphenol A exerts approximately the same potency as 4-*t*-octylphenol in *in vitro* test systems, while the chemical appears less potent than the most active alkylphenols in *in vivo* test systems. When rainbow trout are exposed to bisphenol A in the water it takes a concentration of approximately 100 $\mu g\,l^{-1}$ before the oestrogenic response can be detected and in the uterotrophic assay it takes a dose of approximately 100 $mg\,kg^{-1}\,day^{-1}$ to provoke an oestrogenic response; when rainbow trout are fed 30 $mg\,kg^{-1}$ every second day, vitellogenin synthesis is induced.

5.7.1 Discussion of the Low Dose Effects of Bisphenol A

Whereas there is no disagreement that bisphenol A is oestrogenic, a controversy exists concerning the exposure levels required to affect sexual development in mammals (Richter *et al*., 2007) and reproduction in the freshwater snail, *Marisa cornuarietis* (Forbes *et al*., 2008; Oehlmann *et al*., 2006).

Although the details of this controversy are too comprehensive to describe in this chapter, the discussion is important because the relevance of looking for potential effects of bisphenol A in humans increases dramatically if effective doses in experimental animals decrease from the $mg\,kg^{-1}\,day^{-1}$ range to the $\mu g\,kg^{-1}\,day^{-1}$ range.

5.8 Parabens

Parabens have been used as preservatives in cosmetics and food for 75 years (Elder, 1984). The daily intake in the middle of the 1980s in an American overview was estimated to be 1–16 $mg\,kg^{-1}$ in infants and 4–6 $mg\,kg^{-1}$ in the general population (Elder, 1984).

Methyl-, ethyl-, propyl- and butylparaben all exhibit oestrogenic activity in *in vitro* test systems such that the oestrogenic effect increases with the number of carbon atoms in the alkyl chain (Routledge *et al*., 1998). Butylparaben shows oestrogenic effect in the uterotrophic assay in rats when injected subcutaneously, but not when given in the food (Routledge *et al*., 1998). Methylparaben had no effect in the uterotrophic assay and this is also true for propylparaben tested at 100 $mg\,kg^{-1}$ (Hossaini *et al*., 2000).

Propyl- and butylparaben administered orally to juvenile rats at doses close to the ADI value (10 $mg\,kg^{-1}$ per day for butylparaben) were reported to reduce semen production as well testosterone concentrations (Oishi, 2001; 2002b) and similar effects were seen for butylparaben in mice (Oishi, 2002a). None of the results on methyl and butylparaben could be confirmed in rats by Hoberman *et al*. (2008). Kang *et al*. (2002) reported that the offspring of butylparaben-exposed pregnant rats showed decreased sperm numbers and sperm motility, but a similar experiment (Taxvig *et al*., 2008) did not show clear endocrine-disrupting effects *in vivo*, although the *in vitro* results might suggest that butylparaben may inhibit steroid synthesis by inhibiting the rate-limiting transport of cholesterol across the mitochondrial membrane.

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Injected ethyl-, propyl- and butylparaben have oestrogenic effects in rainbow trout (Pedersen *et al*., 2000) and both propyl- (Bjerregaard *et al*., 2003) and butylparaben (Alslev *et al*., 2005) show oestrogenic effects after exposure via food and water; also in brown trout *Salmo trutta*, exposure to butylparaben elicits an oestrogenic response (Bjerregaard *et al*., 2008) with an EC_{50} (half maximal effective concentration) value for vitellogenin induction of $68\,\mu g\,l^{-1}$.

The parabens are assumed to be degraded relatively quickly in the skin and the gastrointestinal tract in mammals (Elder, 1984), so it has been considered unlikely that the chemicals should exert their inherent oestrogenic effect by ordinary use in food and cosmetics. In a recent experiment in which the maximally allowed amount of butylparaben was applied to the skin of volunteers, approximately 0.8% of the dose was absorbed into the blood and peak concentrations of $135\,\mu g\,l^{-1}$ were seen three hours after the administration (Janjua *et al*., 2008).

5.9 Chemical UV Filters

Compounds which absorb light in the ultraviolet region may be used as so-called chemical UV filters. These chemicals are used in sun screens and in a number of products for which an increased resistance to the effects of light is wanted, for example certain types of plastic materials.

Some UV filters have endocrine-disrupting effects (reviewed by Schlumpf *et al*., 2008). Of the UV filters tested, 3-benzylidene camphor has the strongest oestrogenic effect (Holbech *et al*., 2002).

5.10 Chlorinated Insecticides

A number of chlorinated insecticides have been suspected of exerting oestrogenic effects. Most of these insecticides were used in the Western world from around the 1950s until the use of most was prohibited during the 1960s and 1970s because of their adverse environmental effects. Some of these chemicals are, however, so persistent that this previous dispersal in nature still gives rise to exposure of humans and animals today. The continued use of some of the chemicals in developing countries likewise leads to global dispersal.

In *in vitro* test systems, *o*, *p*-DDT, an impurity in the commercial product, *p*,*p*-DDT, *o*, *p*-DDE (dichlorodiphenyl dichloroethylene), *o*, *p*-DDD (dichlorodiphenyl dichloroethane), *p*,*p*-DDD, methoxychlor, dieldrin, endosulfan, toxafene and chlordecone have shown oestrogenic effects (reviewed by Tyler *et al*., 1998).

In different types of *in vivo* test systems, *o*, *p*-DDT, *o*, *p*-DDE, methoxychlor, dieldrin, toxafene, chlordecone and γ-hexachlorcyclohexane (lindane) have shown oestrogenic effects.

Some of the examples of endocrine-disturbing effects that have been observed in nature (e.g. feminization of birds, demasculinization of alligators) are attributed to the effect of chlorinated insecticides or metabolites thereof—although without total elucidation of the mechanisms underlying the effects.

5.11 Phthalates

Phthalates are used as additives in plastics and for many other purposes and are some of the pollutants that appear in the highest concentrations in the surroundings. Of the approximately 35 phthalates that are produced commercially, nine have shown—albeit weak—oestrogenic activity in *in vitro* test systems (Harris *et al*., 1997), but the major concern is their antiandrogenic effect (Foster, 2006; Foster *et al*., 2001).

The effect doses for the antiandrogenic effects of some of the phthalates have gradually decreased over recent years and it has now been shown that the male offspring of rats treated with approximately $130\,mg\,kg^{-1}\,d^{-1}$ of the most potent phthalates during pregnancy have their sexual development affected (Howdeshell *et al*., 2008b).

Monoester metabolites of some phthalates have been demonstrated in the blood, breast milk and amniotic fluid of humans at concentrations that are not insignificant and symptoms of demasculinization (reduced anogenital distance) in newborn boys have been associated with the concentrations of phthalates in the blood of their mothers during pregnancy (Swan *et al*., 2005).

5.12 Nitrate and Nitrite

In the organism, nitrate and nitrite may be reduced to nitric oxide (NO), which acts as a signalling compound. One of the effects of NO is that it reduces the synthesis of steroids.

In rats that were exposed to nitrate and nitrite in their drinking water (at concentrations in the same range as the drinking-water limit in many countries), the testosterone concentrations in the blood decreased to approximately half of the concentration of the control group during four weeks (Panesar and Chan, 2000). In Japanese medaka, *Oryzias latipes,* the breeding success decreases when the fish are exposed to concentrations above approximately $130\,mg$ nitrate l^{-1} (Shimura *et al*., 2002).

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5.13 Polychlorinated Biphenyls (PCBs)

PCBs were produced mainly during the period from World War II until around 1975 and in a total amount of approximately 1.2 million tonnes. The chemicals were used for many purposes (in transformers, capacitors, as plasticizers, in hydraulic systems, etc.) and because of their persistence, only a minor fraction of the produced amount has been degraded.

Commercial PCB products theoretically consist of 209 different specific, chemical compounds. The individual PCB isomers have different toxicological properties and among the PCB isomers and their metabolites, oestrogenic, antioestrogenic, androgenic and antiandrogenic effects have been shown.

Hydroxylated metabolites of specific PCB isomers bind to the oestrogen receptor and treatment of tortoise eggs with 2′,4′,6′-trichloro-4-biphenylol causes complete feminization of the developing foetuses (Bergeron *et al*., 1994). Other types of hydroxy metabolites interfere with binding and transport of thyroxin in the blood (Lans *et al*., 1993).

5.14 Chlorinated Dioxins

In everyday language the term 'dioxins' covers two different groups of chlorinated chemicals with 210 specific compounds. The dioxins have never been produced for any industrial purpose, but they are formed as by-products in different processes (a number of chemical syntheses, incineration, chlorine bleaching). Certain of the dioxins have a remarkably high toxicity.

The dioxins may at very low doses (as low as $0.1\,\mu g\,kg^{-1}\ day^{-1}$) harm the reproduction in experimental animals. Male offspring of female rats treated during pregnancy show effects consistent with the oestrogen hypothesis (lower sperm counts, reduced fertility, etc.).

5.15 Tributyltin (TBT)

5.15.1 TBT as an Antifouling Agent

The use of organic tin compounds as antifouling ingredients in ship paint was initiated in the 1960s (Fent, 1996). The TBT is formulated in the ship's paint in a way such that a gradual liberation to the water phase takes place. In seawater, TBT is relatively quickly degraded by bacteria and algae to mono- and dibutyltin (Lee *et al*., 1989). The half-life for TBT in seawater is in the range of days to weeks, while the half-life in sediments may be up to six months or longer (Seligman *et al*., 1986). Molluscs, such as snails and mussels take up TBT from the water and, since they have a low capacity to metabolize TBT, they may concentrate the compound to concentration factors as high as 50 000–100 000; TBT is lost from dog whelks *Nucella lapillus* with a half-life of 50–100 days (Bryan *et al*., 1987).

Higher organisms metabolize TBT by hydroxylating and subsequently eliminating the butyl groups one by one, such that the TBT is metabolized to dibutyltin and further to monobutyltin and inorganic tin that are all less toxic than TBT (Lee, 1991).

5.15.2 The Mechanism of Action for TBT

Several mechanisms of action—that do not mutually exclude each other—have been suggested for TBT's interference with sexual development in gastropods.

It has been proposed that the mechanism of action for TBT is related to the aromatase-inhibiting effect of the chemical (Bettin *et al*., 1996). There are also indications that exposure to TBT (albeit at fairly high exposure concentrations) reduces the excretion of testosterone by inhibiting sulfate conjugation of testosterone (Ronis and Mason, 1996). Regulation of free testosterone levels may in certain invertebrates be associated with the formation of testosterone–fatty-acid esters (Gooding and LeBlanc, 2001) and TBT affects the equilibrium between free and bound testosterone (Janer *et al*., 2005; Santos *et al*., 2005). TBT accumulates in gastropods to especially high concentrations in the nervous system (Bryan *et al*., 1993) and it has been proposed that TBT exerts its effect via interaction with the formation of neuropeptides involved in regulation of steroid synthesis (Oberdörster and Clellan-Green, 2000; 2002; Oberdörster *et al*., 1998; 2005).

5.16 Presently Used Pesticides

A fairly large number of presently used pesticides show endocrine activity in *in vitro* (Andersen *et al*., 2002) and/or *in vivo* test systems and space restraints only allow the description of the most important ones.

5.16.1 Fungicides

The fungicides vinclozolin and procymidone or their metabolites show antiandrogenic effects (Gray *et al*., 1999; Nellemann *et al*., 2003) in rats and fenarimol shows antiandrogenic (Vinggaard *et al*., 2005), as well as oestrogenic effects (Andersen *et al*., 2006). The imidazol fungicide prochloraz shows endocrine-disrupting activity by several mechanisms. In male rat foetuses, testosterone production is reduced (leading to a demasculinization) (Laier *et al*., 2006), whereas the sex ratio in prochloraz-exposed zebra fish larvae is shifted towards males (Kinnberg *et al*., 2007), probably because of the aromatase-inhibiting effect of the chemical.

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5.16.2 Herbicides

Atrazine, which is widely used, especially in the USA has been reported to be an aromatase promoter that may demasculinize the clawed frog, *Xenopus laevis* (Hayes *et al*., 2002), with exposure to low concentrations, and feminize male American leopard frogs, *Rana pipiens* (Hayes *et al*., 2003). No such effects could, however, be demonstrated by Du Preez *et al*. (2008) in *X. laevis* exposed to atrazine.

The phenyl urea herbicide, linuron, shows antiandrogenic effects in rats (Gray *et al*., 1999).

5.16.3 Insecticides

A number of commonly used pyrethroids such as permethrin, fenvalerate, cypermethrin and deltamethrin show inherent oestrogenic activity *in vitro* (Andersen *et al*., 2002), and some of the pyrethroids show antiandrogenic effect *in vitro* (Tyler *et al*., 2000). Some of the pyrethroids have shown oestrogenic effects in some *in vivo* assays in rats (Kim *et al*., 2005) carried out, but not in others (Kunimatsu *et al*., 2002; Yamada *et al*., 2003).

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Assessing Impacts of Environmental Contaminants on Wildlife

Joseph P. Sullivan and Elwood F. Hill

CONTENTS

1 INTRODUCTION

Assessing the impact of contaminants on wildlife is complex because factors that influence exposure and toxicity might vary substantially among and within habitat types. Even the prescribed application of a pesticide might evoke different degrees of risk, depending on the product formulation, how, when and where it is used, and the behaviour and sensitivity of species that frequent the treated zone and its drift perimeter. Thus, evaluation of even an obvious source of exposure might be difficult unless accompanied by immediate mortality. Then, when considering the confounding influences of pesticidal interactions, uncertainty associated with mixtures of chemicals from industrial and domestic sources, and presence of natural toxics and toxins, a spectrum of studies is needed to assess impacts of contamination on wildlife.

Historically, wildlife toxicology has focussed primarily on those species, mostly birds and a few mammals, of

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aesthetic or economic interest to man. Birds have been favoured as the principal model for toxicological research because they are abundant, highly visible, have diverse behaviours and habitat associations, and many species can be studied both in the laboratory and in their natural habitats (Heinz *et al*., 1979). More recently, wildlife toxicology has expanded to include reptiles, amphibians and terrestrial invertebrates.

Wildlife toxicology has been described as the integration of three principal strategies for understanding xenobiotic effects on wildlife (Hoffman *et al*., 1990). The first strategy is chemical screening, where a variety of toxicological tests is performed in the laboratory with what are thought to be representative species. The purpose of screening is to predict likely effects in natural populations by identifying those chemicals toxic to wildlife. End points of these tests may include lethality, reproductive impairment, behavioural aberrations, developmental changes or changes in physiological indicators of health crucial to reproduction and survival.

The second strategy is the controlled field or mesocosm study. The purpose is to confine the test subjects in a simulated natural environment and expose them to real-world scenarios. The design and end points of such studies may be widely variable depending on the chemical, its uses, potential routes of exposure and the wildlife species of concern. This type of study, as well as full-scale field trials in which the subjects are free-ranging, have been occasionally required by the United States Environmental Protection Agency (US EPA) for pesticide registration, but have lost favour because of cost and limitation on extrapolation to other sites (Tiebout and Brugger, 1995; Matz *et al*., 1998; Hoffman *et al*., 2002).

The third strategy is field ecology. Natural populations are studied *in situ* in environments believed to be polluted. These studies focus on species diversity and population dynamics in critical habitats as related to residue burdens in the species of interest and various components of the ecosystem. Ecological studies are extremely complex, costly and often too specific for extensive use in risk assessment.

The above strategies provide a comprehensive evaluation of chemical risk to wildlife, but each strategy has a functional role and rarely would all three be used in a cohesive series of studies. For example, if an area is known to be contaminated, detailed residue and ecological studies (strategy 3) may be initiated to determine whether a problem exists. Carefully designed laboratory experiments would then be conducted to validate putative cause–effect relationships between the contaminant and the susceptible species or a close surrogate. Laboratory studies may borrow from standardized tests (Strategy 1) or be specially designed to address specific field observations.

In contrast to existing contamination, chemicals are constantly under review for release into the environment as pesticides or other anthropogenic by-products. Chemical screening (Strategy 1) is then initiated for protection of wildlife resources. In the USA, the US EPA provides this regulatory function. Specific tests of birds, mammals and beneficial insects and other invertebrates are required under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) and the Toxic Substance Control Act (TSCA). Most industrialized countries have similar regulations. Accepted protocols and model species have been reviewed, updated and new tests added in national and international conferences on wildlife toxicology by the US EPA, the Society of Environmental Toxicology and Chemistry (SETAC), the American Society for Testing and Materials (ASTM) and the Organisation for Economic Cooperation and Development (OECD). A framework for avian effects assessment was developed by an expert panel at a SETAC workshop (Hart *et al*., 2001).

Standardized screening tests are especially useful for regulatory purposes because they are easily interpreted and cost effective. However, other than the single-dose LD_{50} test, none of the existing protocols provide an equal basis of comparison for all chemicals and uses. In contrast to biomedical studies, where a variety of mammalian models are used to represent different physiological systems, wildlife studies may test the actual species of interest or a close surrogate. In practice, test species are usually limited to quail and ducks that can be easily reared in captivity and subjected to a wide variety of studies from single-dose through multiple-generation exposures. Impacts on wild mammals are often extrapolated from standard mammalian laboratory tests, but no standard amphibians, reptiles or passerine bird species are routinely tested.

This chapter provides an overview of wildlife toxicology and risk assessment as a component of the broader disciplines of ecotoxicology and environmental toxicology. The focus is on anthropogenic chemicals and avian wildlife with comparison with laboratory animals and other vertebrates where appropriate. Test methods and factors affecting interpretation, predictive toxicology and ecological toxicology are discussed.

2 ENVIRONMENTAL AND ECOLOGICAL TOXICOLOGY

Environmental toxicology has been comprehensively defined as the study of the effects of toxic substances occurring in both natural and man-made environments, leading to an objective assessment of risks resulting from the presence of such substances (Duffus, 1980). *The focus of this risk assessment is protection of human health.*

Ecological toxicology, or ecotoxicology, as first defined, is essentially synonymous with environmental toxicology except that *the focus is on natural systems*

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and wild animals (Truhaut, 1975). More recently, ecotoxicology has been described as the science of predicting effects of potentially toxic agents on natural ecosystems and nontarget species, but exclusive of industrial and agricultural toxicology. This distinction is losing favour because human society and its artefacts are part of natural systems, not apart from them (Hoffman *et al*., 2002).

Wildlife toxicology, as used herein, follows this more inclusive philosophy because most sources of noninfectious wildlife poisoning are of anthropogenic origin. Although crops under cultivation and point-source industries are not usually prime wildlife habitats, they may be attractive for hydration and foraging. Also, surrounding ecosystems and wildlife populations are subject to effects from chemical movement.

3 WILDLIFE SUSCEPTIBILITY TO CHEMICAL CONTAMINATION

Wildlife may be affected directly by contact with a toxic chemical or indirectly through chemical-induced changes in the invertebrate food base (insecticides, etc.) or habitat quality (e.g. industrial emissions, herbicides). Effects of chemical exposure range from acute incidents of lethal poisoning to subtle physiological dysfunctions, behavioural aberrations and reproductive impairment. Reduction of forage may induce metabolic stress that could increase sensitivity to chemical exposure, reduce reproductive success or force emigration (Grue *et al*., 1983).

Wildlife species differ remarkably in their physiology and sensitivity to xenobiotics, but their *feeding habits seem to be the overriding determinant of susceptibility to chemical hazard.* For example, the organophosphorus insecticide diazinon is extremely toxic to some birds (LD_{50}, $<10\,mg\,(kg\ body\ weight)^{-1}$) and not so toxic to mammals (LD_{50}, $>100\,mg\,(kg\ body\ weight)^{-1}$) or amphibians ($LD_{50}$, $>1000\,mg\,(kg\ body\ weight)^{-1}$) (Gaines, 1969; Hudson *et al*., 1984). When diazinon was applied, according to its label, on turf grasses, occasional incidents of mortality were reported for insectivorous birds and small mammals. Such sporadic lethality has also been observed for birds exposed to other anticholinesterase insecticides. However, when diazinon was applied to turf that was attractive to waterfowl, such as the Canada goose (*Branta canadensis*) and American widgeon (*Anas americana*), large-scale episodes of mortality were regularly documented. This unique risk to grazing waterfowl resulted in the US EPA issuing a cancellation notice for the use of diazinon products on golf courses and sod (turf) farms (US EPA, 1986). The wildlife toxicology of anticholinesterase insecticides has been reviewed extensively (Smith, 1987; Greig-Smith *et al*., 1992; Hill, 1992; 1995; 2002; Fairbrother, 1996; Eisler, 2000).

In contrast to the comparatively labile and acutely toxic poisons such as diazinon and other organophosphorus and carbamate insecticides, many chlorinated hydrocarbons such as dichlorodiphenyltrichloroethane (DDT) are stable in the natural environment and are not as acutely toxic to wildlife (Gaines, 1960; 1969; Hudson *et al*., 1984). They do, however, biomagnify in wildlife food chains and accumulate in fat stores of vertebrates. A substantial body burden of chlorinated hydrocarbon residues and metabolites may be tolerated without overt evidence of toxicity, but such residues have been shown experimentally to affect avian reproduction (Heath *et al*., 1969; Wiemeyer and Porter, 1970). The main effect was interference with calcium metabolism in the shell gland, resulting in eggshell thinning and reduced hatchability (Lundholm, 1987). Although many experiments over the past 30 years have confirmed that a variety of chlorinated hydrocarbons may thin eggshells to some degree, dichlorodiphenylethylene (DDE), a stabile metabolite of DDT, is the most potent eggshell thinning substance.

Eggshell thinning in a natural population was first detected in peregrine falcon (*Falco peregrinus*) eggs in Great Britain during the mid-1960s, and was associated with depressed reproduction and population declines in the region (Ratcliffe, 1967). Many North American species also proved to be susceptible to effects of DDE on reproduction and experienced widespread population declines (Blus, 1995; 2002). The most affected species foraged almost exclusively on fish and included the brown pelican (*Pelecanus occidentalis*), double-crested cormorant (*Phalacrocorax auritus*), osprey (*Pandion haliaetus*) and bald eagle (*Haliaeetus leucocephalus*). In contrast, other fish-eating species such as the great blue heron (*Ardea herodias*) were comparatively tolerant of chlorinated hydrocarbons.

Cancellation, in many countries, of the more persistent chlorinated hydrocarbons such as DDT, aldrin, endrin, dieldrin and heptachlor has resulted in reduction of their residues in natural systems through time and recovery of sensitive avian species (Blus, 1995; 2002). A few chlorinated hydrocarbons, such as methoxychlor and dicofol, were not included in the cancellation orders and are considered to be of low risk in the environment.

The emphasis on reproductive toxicity in birds is not meant to diminish the acute risk of some chlorinated hydrocarbons. Although only a few chlorinated hydrocarbons are extremely toxic (LD_{50}, $<10\,mg\,(kg\ body\ weight)^{-1}$) to birds and mammals, most are readily accumulated in the lipid stores of vertebrates and under conditions of energy stress (e.g. food deprivation, migration) may be translocated in lethal concentrations (LCs) to the brain. The wildlife toxicology of chlorinated hydrocarbons has been comprehensively reviewed (Blus, 1995; 1996; Keith, 1996; Eisler, 2000; Walker, 2001).

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Sensitivity of wildlife to toxic chemicals is a product of biochemical, metabolic and excretory processes. The efficiency of these processes is influenced at the organismic level by the rate, route and duration of exposure. Foremost among the physiological processes that regulate toxicity is the enzymatic mono-oxygenase system of the vertebrate liver, whose function is to increase the rate of production of water-soluble metabolites and conjugates that can be rapidly excreted. This is primarily a detoxicating system, but it can also produce metabolites that are more toxic than the parent substance. An example of mono-oxygenase toxication is the oxidative desulfuration step for maximum anticholinesterase potency of thiophosphorus insecticides such as parathion and diazinon. Mono-oxygenase metabolism of xenobiotics differs widely among species within and between taxons and generally decreases among vertebrates in the following order: mammals > birds > fish (Walker, 1978; 1980; Moriarty and Walker, 1987). Small vertebrates usually have a higher rate of mono-oxygenase metabolism than larger species, and the rate is higher in herbivores and omnivores than in carnivores (Ronis and Walker, 1989).

Other determinants of xenobiotic toxicity that vary among wildlife species include gender, age, reproductive status, genetic lineage, nutritional status and both endogenous and exogenous stressors. Except for age, the influences of these factors on wildlife responses to toxic exposure have not been thoroughly evaluated.

4 INTERSPECIES VARIABILITY

One of the challenges for assessing impacts of contaminants, particularly for site-specific assessments, is the difficulty of extrapolating among species. Often many of the species of interest on a site or of concern lack toxicity data for the contaminants of potential concern. This is particularly true for reptile species. Extrapolation among species and toxicity end points has remained one of the key areas of uncertainty in ecological risk assessment (Raimondo *et al*., 2007). A common solution when the comparative sensitivity of different species is unknown, or to protect the unknown most sensitive species, is to multiply the lowest known toxicity end point by some safety or uncertainty factor, such as a factor of 0.1 or 0.01. This approach has been criticized for introducing an unquantifiable amount of uncertainty into an assessment (Luttik *et al*., 2005). The practice of using uncertainty factors is limited by lack of consensus among scientists, regulators and practitioners about magnitudes, types and conceptual underpinnings of the uncertainty methodology (Duke and Taggart, 2000).

An important factor involved in extrapolating from one species to another is body size. Mineau *et al*. (1996) determined that a scaling factor of up to 1.55, based on body weight, was appropriate for birds when extrapolating from one species to another for a group of pesticides, mostly cholinesterase-inhibiting insecticides. Sample and Arenal (1999) found that body weight accounted for most of the variability in toxicity results among species, with scaling factors of 1.2 and 0.94 appropriate for birds and mammals, respectively, across many chemical groups.

Taxonomic relationships can be used to predict responses among Classes of vertebrate wildlife (e.g. fish, birds and mammals), but within a Class, sensitivities do not necessarily follow taxonomic line (e.g. within or across Families and Genera). Basua *et al*. (2005) investigated mercury inhibition on muscarine acetylcholine-receptor binding in the brains of five mammalian species: humans, two rodents and two mustelids. Species sensitivities, irrespective of mercury type and brain region, can be ranked from most to least sensitive: river otter (*Lutra canadensis*) > rat (*Rattus* sp.) > mink (*Mustela vison*) > mouse (*Mus* sp.) > humans.

In contrast to the taxonomic and body-weight scaling factors that have been shown to work to extrapolate acute toxicity data among species, these factors do not work well for chronic or reproductive toxicity responses (Luttik *et al*., 2005). These authors indicate the best models for extrapolation among species for chronic toxicity are those previously proposed by Luttik and Aldenberg (1995, 1997) and Luttik *et al*. (2005). The procedures proposed that safety factors of the one-sided 95% confidence limit of the hazardous dose for 5% of the species (HD_5) based on the pooled natural log (ln) standard deviations be used when only one, two and three LD_{50}s are available for a particular chemical. Otherwise, they propose to use the more conservative method (95% confidence limit) for the extrapolation, because the pooled ln standard deviation that is used is not a worst case value. Hence the probability to overpredict $\ln(HD_5)$ should be small (5%) (Luttik and Aldenberg, 1997).

Variations in diet impact how species are exposed to contaminants. Among birds, the types and numbers of seeds eaten can vary considerably (Prosser and Hart, 2005). In a study that treated seeds with the avicide 3-chloro-*p*-toluidine hydrochloride (CPTH), the model developed predicted a higher percentage of mortality for mourning doves (*Zenaida macroura*) than red-winged blackbirds (*Agelaius phoeniceus*), despite red-winged blackbirds being slightly more sensitive to CPTH. The reason more doves were predicted to be killed was because doves ate more of the treated rice than did the blackbirds (Johnston *et al*., 2005). Diet is also a driving factor in determining the normal activity levels of detoxifying enzymes. Those species that routinely eat vegetation containing natural defensive compounds tend to have greater enzyme activities than predatory species such as piscivores and most raptors (Vangilder and Drawer, 1983).

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Many insecticides inhibit cholinesterase as their mode of action against insects. This same mode of action results in the toxic impacts to wildlife species. Different species have shown varying sensitivities to cholinesterase-specific anticholinesterases. For example, fish were found to show the least sensitivity of brain cholinesterase to monocrotophos. The differences in sensitivities of brain cholinesterase to monocrotophos were thought to be the underlying cause of the differences in toxicity among rats, pigeons and fish (Qadri *et al*., 1994).

The inherent ability of different species to detoxify contaminants is related to their P450 or mono-oxygenase enzymes, among others. The specific isozymes present can enhance or diminish the ability of a species to respond to contaminants (Andersson *et al*., 2000). A species diet is involved with the suite of enzymes present capable of detoxifying or metabolizing contaminants. Fossi *et al*. (1995) looked at seven species of birds and found that the more omnivorous the diet, the greater the induction potential of their mixed function oxidases. They concluded that birds with more omnivorous diets could better survive in highly polluted environments. In situations where metabolism of a contaminant, particularly pesticides, leads to greater toxicity, such species could be at greater risk by enhancing a chemical's toxic potency through its metabolites. Such generalizations will not hold for all species for all chemicals. Hill (1971) found that blue jays (*Cyanocitta cristata*) were more sensitive to DDT than less omnivorous species.

Interspecies correlation estimation (ICE) models are log-linear least-square regression models that describe the relationship between the acute toxicity (LD_{50}; mg (kg body weight)$^{-1}$) of a range of chemicals tested in two species (Raimondo *et al*., 2007). Interspecies extrapolation models use surrogate species, often standard test organisms, to estimate toxicity of a chemical to wildlife species for which no toxicity data exists. In North America, tests are usually conducted on mallard (*Anas platyrhynchos*) and northern bobwhite (*Colinus virginianus*), whereas European countries often use Japanese quail (*Coturnix japonica*) as their standard test species. These standard test species were frequently found to be adequate surrogate species for other wildlife species. Results of these analyses provide user guidance for use and application of ICE models in wildlife risk assessment (Raimondo *et al*., 2007). A web-based ICE tool is available from the US EPA at *http://www.epa.gov/ceampubl/fchain/webice/index.htm*. Since there is an overall lack of toxicity data for reptile and amphibian species, the terrestrial ICE models are generally restricted to birds and mammals.

Shore *et al*. (2005) provide a comprehensive review of how to incorporate species-specific breeding-season information to improve the assessment of long-term exposure to pesticides. The authors focus on how to use the data routinely collected as part of the pesticide registration process to refine a deterministic risk assessment. Rather than using standardized hypothetical species, available ecological data for indicator species are incorporated into the assessment along with existing registration data to refine the deterministic assessment. This deterministic approach produces more realistic assessments of long-term risk for birds and mammals.

5 ACUTE TOXICOLOGY

The mainstay of wildlife toxicology for more than 50 years has been experimentation with relevant species in captivity. The advantage was in testing the actual species of interest or a closely related surrogate and tailoring the experiment to evaluate a specific problem. Therefore, the wildlife toxicologist tends to rely less on standardized protocols than does the biomedical toxicologist. However, wildlife toxicology has evolved several standardized protocols for chemical screening that are used in North America, Europe and elsewhere for regulation of pesticides and other toxic substances (US EPA, 1982; OECD, 1996). These protocols include avian and mammalian single-dose acute oral toxicity tests, a five-day avian dietary toxicity test and a single-generation avian reproduction test. These and similar studies have been reviewed and refined by ASTM and OECD panels. Consensus recommendations on the choices and suitability of these tests were developed by a panel of international experts (Hart *et al*., 2001).

This section is a review of acute chemical screening methods in which wildlife species are routinely used for predictive toxicology, and factors of interpretation that may influence risk assessment. The purpose of these basic tests is to protect natural populations from chemical risk of anthropogenic origin. Depending on the expected fate and toxic action of the chemical, some combination of these tests may be the only wildlife studies considered in a regulatory risk assessment. The principal end points of the basic screening tests are lethality or reproductive impairment, with ancillary observations of behaviour and gross pathology. Histological and biochemical indicators of toxicity are not usually included in chemical screening studies, but are essential for evaluation of wildlife effects from environmental pollution and incidents of mortality.

Acute toxicity tests are designed to determine exposures that cause death or other definitive end points under a prescribed protocol. Treatment levels are based on the animal's response to the test substance rather than the substance's practical environmental residues. A steep slope has the obvious risk of proportionally little chemical required to increase effect. In contrast, a shallow slope would seem inherently safer because it takes proportionally more chemical to increase effect. Generally, this is true, but it is these substances that

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may produce toxicity at very low levels, for example, below one-tenth of the LD_{50} or LC_{50}. Carbofuran is an example of a pesticide that yields a shallow slope in both acute (Hill and Camardese, 1984) and subacute (Hill and Camardese, 1986) tests with birds and has been responsible for many unexpected incidents of wildlife mortality (US EPA, 1989; Mineau, 1993).

Acute toxicity tests provide the basis for many kinds of comparisons once statistically reliable dose–response curves have been determined for a substance with several taxonomically diverse species. For example, the relative toxicity of the substance may be compared with additional species or substances by carefully designed tests of only a few animals (Link *et al*., 1996) or by use of approximate methods for determination of the median effective dose or concentration (Gad and Chengelis, 1988). When only the general order of toxicity is desired, an approximate test of lethality should be considered (Schafer *et al*., 1973; 1983; Hudson *et al*., 1984; Schafer and Bowles, 1985; Gad and Chengelis, 1988). Such abbreviated or approximate tests are cost-effective and, of more importance, designed to reduce the number of animals that need to be tested for a statistically reliable comparison.

Approximate statistical methods were used to test about 75 species of wild birds and mammals and over 1000 chemicals between the mid-1950s and early 1980s at the US Fish and Wildlife Service's Denver Wildlife Research Center (Schafer, 1972; Hudson *et al*., 1984; Schafer and Bowles, 1985). The special value of these studies is that they were all conducted by the same techniques at one laboratory. The disadvantage is that the approximate test does not provide an estimate of the dose–response curve and therefore has limited value in risk assessment. These tests were of mostly wild-captured nonbreeding adults and remain the only toxicity statistic available for many chemicals with wildlife species.

5.1 Avian Single-Dose Acute Oral Toxicity Test

Two basic protocols have been derived for wildlife from standard bioassay techniques for preliminary evaluation of the acute or short-term lethal toxicity of pesticides and other environmentally active substances. The first is the single-dose acute oral toxicity test. This test is fundamental in evaluation of virtually all substances of suspected biological activity. The primary objective is to estimate the dose–response curve and its midpoint, the median lethal dosage or LD_{50} and its error with 95% confidence limits. The test, essentially the same as for laboratory animals, is based on administration of graded dosage of chemical in relation to body mass (i.e. $mg\,kg^{-1}$). It may be adapted to virtually any terrestrial wildlife species that can be managed in captivity. Once the statistical parameters and their associated errors have been properly determined, this test provides a means of quantifying chemical potency and comparing substances of different mechanisms and sites of action (Klaassen, 1986). The acute test also provides essential information on the course and appearance of toxicity from graded exposures that is needed for comparative diagnosis of poisoning and risk assessment (Hill and Camardese, 1984; Hudson *et al*., 1984). Too often, however, comparisons and interpretation of acute tests are focussed on the LD_{50}, exclusive of its statistical reliability and without reference to the slope of the dose–response curve or other supplemental observations that provide important clues about potential risk. The LD_{50} is a convenient index of toxicity, but its indiscriminate use can be misleading (Chan and Hayes, 1989; Hill, 1994).

The acute toxicity test of wildlife can be conducted on virtually any species that can be managed in captivity. The general procedure is to dose about 10 randomly selected overnight-fasted subjects at each of five geometrically arranged dosage levels spanning the expected 10–90% mortality levels. The standard protocol uses individually caged first-year adults of both sexes that have not yet gone through a breeding cycle. A detailed protocol for acute toxicity testing of wildlife for regulatory purposes in the USA is available (Bascietto, 1985a).

Many factors may influence the reliability of an acute test of lethality even under the rigid guidelines that have evolved for testing laboratory animals. Factors may include, but are not limited to, species, strain, age, weight, gender, husbandry practises, environmental conditions, diet, nutritional status and the volume and vehicle of test substance delivery (Gad and Chengelis, 1988). With these possibilities and that many wildlife studies use wild-captured animals of unknown heritage, specific nutritional needs or previous xenobiotic exposure, results of wildlife studies must be used cautiously even though a given experiment was conducted expertly. It is also important that these tests of lethality are neither designed nor intended to imply unequivocal recovery of survivors. The amount of time it takes to recover from the initial toxicity may render an individual more vulnerable to exposure and predation.

5.2 Avian Five-Day Dietary Toxicity Test

The second test is similar to the single-dose acute oral test, except graded concentrations of chemical are presented *ad libitum* in the feed for five days. This five-day 'subacute' test is intended to augment the acute oral test by measuring response to repeated exposures from normal feeding and accumulative effects. Careful observation for changes in behaviour and rate of feeding and for onset and course of toxic signs is especially important. Most often the rate of feeding is inversely

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correlated with the graded concentration of toxic chemical provided the test subjects cannot fast for five days. This short-term feeding trial was optimized with young precocial birds, but it serves equally well for testing small mammals and other birds that can be maintained in captivity in good health (Hill, 1971; McCann *et al*., 1981; Grue, 1982; Ringer *et al*., 1991). In general, the best results are obtained for birds and mammals that weigh less than 100 g. Although birds weighing 100–200 g have been tested under this protocol, their response to graded concentrations of toxicant tended to be erratic, which resulted in artificially shallow dose–response curves and widely variable LC_{50}s (Hill *et al*., 1975; 1977; Hill, 1981; Hill and Camardese, 1981). This source of error and its implications have been evaluated elsewhere (Hill, 1971; Hill and Camardese, 1981; Grue, 1982).

The five-day test generates a dose–response curve; the mid-point of the dose–response curve is quantified as the median LC_{50}, its error with 95% confidence limits similar to the single-dose test above (Heath and Stickel, 1965). The LC_{50} may be expressed as mg a.i. (active ingredient) $(kg\ feed)^{-1}$ or as parts per million (ppm). It also provides descriptive information on response to potentially toxic food. The basic design uses the same number of animals, treatment levels and control groups as the full-scale acute test. In risk assessments, the daily consumption $(mg\ kg^{-1}\ day^{-1})$ can be used for an estimate of exposure leading to unacceptable risk. A detailed protocol for the dietary LC_{50} test of wildlife for regulatory purposes in the USA is available (Bascietto, 1985b).

5.3 Single-Dose Avian Embryotoxicity Tests

Single-dose acute tests are also conducted routinely on the avian embryo. In these tests, the embryo is exposed by topical treatment of test substance to the eggshell, injection into the air space or complete immersion of the intact egg for a prescribed duration (Hoffman and Albers, 1983). The avian egg is a relevant model for indication of environmental pollution because it has proved susceptible in nature to embryotoxicity and teratogenicity from air pollution, pesticide spray application, aquatic translocation and even from contaminated breast feathers of the adult (Hoffman, 1990; 1994). Mallard and northern bobwhite eggs are the preferred laboratory models for routine bioassays, but eggs from many species have been brought into the laboratory from the field and tested to validate putative causes of embryotoxicity.

The basic embryotoxicity test uses two approaches, although many variations of this bioassay have been reported (Hoffman, 1990; 1994). The first is to dose about 25 eggs at each of three to five levels for the purpose of generating a dose–response curve, its error and an LD_{50} or LC_{50} with 95% confidence limits. The second approach is more applied, where doses bracket actual field concentrations, thereby providing a realistic assessment of ecological risk.

The focal point of the first test is lethality, but includes stage-effect evaluation at different times of incubation, with the lethal effect including a seven-day chick survival period. Teratogenic effects are also monitored in a random subsample of eggs at various times of incubation, and often reveal effects at exposure levels well below the calculated median lethal levels. The second method is conducted the same as the first, except the objective is not to determine the median lethal dose, but to focus on teratogenic, biochemical and histological variables that are consistent with ecological observations. Another method is to use an applied bioassay, where eggs under natural incubation in the field are dosed and observed for hatchability and survival. Seminal reports on techniques and findings of embryotoxic and teratogenic studies of wildlife have been published (Hoffman, 1990; 1994; 1995; 2002; Hoffman and Albers, 1983).

5.4 Additional Acute Tests

Acute tests have also been conducted on amphibians and reptiles, but neither standardized procedures nor the preferred model species have been finalized for regulatory purposes in the USA. However, flow-through and static tank tests of bullfrog (*Rana catesbeiana*) tadpoles and oral dosing of adult bullfrogs and green anole lizards (*Anolis carolinensis*) have proved to be satisfactory techniques for comparative toxicology (Hall and Swineford, 1980; Hall and Clark, 1982; Hudson *et al*., 1984). Detailed reviews of amphibian and reptile testing procedures are available (Sparling *et al*., 2000; Linder *et al*., 2003).

The acute bioassay has been adapted to studies of inhalation and dermal toxicity to birds. Examples include a comparison of inhalation and oral toxicity of pesticides in northern bobwhite (Driver *et al*., 1991) and dermal tests where pesticides were applied to the feet of mallard (Hudson *et al*., 1979) and the breasts of passerine species (Schafer *et al*., 1973).

6 SUBLETHAL TOXICOLOGY

6.1 Special Studies

During the past 30+ years, the emphasis of wildlife toxicology has shifted from correlation of xenobiotic residues with lethality, to the development and validation of biochemical tests indicative of exposure and toxicity, behavioural toxicology, and simulated and actual field

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trials. Studies address known or theoretical questions of environmental risk and vary in complexity from routine to uniquely designed experiments for implementation in the laboratory or in nature. The studies may involve a single dose or months of continuous feeding, a spot survey or several years of field sampling. Species may include a specific strain of laboratory rodent, farm-reared ducks or quail, a wild-captured and carefully conditioned surrogate species, the actual species of interest or a natural population. The design of these studies is boundless as they usually focus on special issues of putative ecological risk; a few examples are presented.

6.1.1 Biomarkers

Biomarkers have been defined as a 'biological response to a chemical or chemicals that gives a measure of exposure and sometimes, also, of toxic effect' (Walker *et al*., 2001). A biomarker response indicates the presence of a xenobiotic, its availability and that it has reached the affected tissue or organ in sufficient amounts for a period of time long enough to elicit change. The relative value of such a response to indicate harm ranges from a simple homoeostatic response to a dose-dependent progression of responses indicating a toxic process has been initiated (Melancon, 2003). An in-depth review on biomarkers as pollution indicators in wildlife was presented by Peakall (1992).

Biomarkers of exposure may provide little information on effect, but some are fairly specific to certain classes of contaminants and are efficient substitutes for more costly and time-consuming residue analysis. Most of the techniques are based on nondestructive sampling of blood, urine or faeces and are especially useful in field monitoring. Some of the more commonly used biomarkers that have both specificity and a degree of diagnostic value have been summarized and discussed by Melancon (2003) and Walker *et al*. (2001). They include inhibition of δ-aminolaevulinic acid dehydratase (lead), inhibition of cholinesterase (organophosphorus and carbamate insecticides), anticoagulant clotting proteins (rodenticides) and induction of mono-oxygenases (chlorinated and polyaromatic hydrocarbons). Avian eggshell thinning is also classed as a biomarker that is rather specific to a few chlorinated hydrocarbon insecticides, primarily DDT and its metabolites DDE and DDD (1,1-dichloro-2,2-bis(4-chlorophenyl)ethane). Another biomarker with a high degree of specificity to a few organophosphorus compounds such as leptophos and EPN (ethyl 4-nitrophenyl phenylphosphonothioate) is neurotoxic esterase, an indicator of organophosphorus ester-induced delayed neurotoxicity. Unless a particular contaminant is suspected for which there is a highly specific biomarker, it is suggested that a suite of biomarkers be used for general monitoring of pollution (Melancon, 2003).

6.1.2 Behaviour

Many pesticides and other environmental contaminants are neurotoxic and assumed capable of affecting behaviour at clearly sublethal levels of exposure. Thus, several fairly sensitive laboratory tests and a variety of field tests have been devised for wildlife to detect chemicals that may affect natural or operant behaviour. Whereas behavioural tests seem desirable, there are two fundamental difficulties in the use of such tests in wildlife toxicology: (i) the best studied and most easily performed and quantified behaviours have the least environmental relevance and (ii) the most relevant behaviours are the most strongly conserved against change (Walker *et al*., 1996). For example, operant behaviour where animals are conditioned to respond to a cue is remote from environmental reality, but it is presumed that decreased learning ability is unfavourable. Avoidance behaviour is more directly related to survival, but difficult to quantify, as is the ability to capture prey. Nonetheless, subacute exposure of birds and mammals to pesticides and other contaminants has been shown to affect an array of behaviours such as activity level, alertness, aggression, foraging and drinking, learning and memory, navigation and reproduction (Heinz *et al*., 1979; Peakall, 1985; Grue *et al*., 1991; Bennett, 1994).

Most of the behavioural studies with wildlife are conducted under highly or quasicontrolled conditions and test response to critical survival stimuli, learning and memory competency, prey-capturing skills, and chemical discrimination and aversion. Although most of these studies were well planned and have important theoretical implications for survivability, laboratory studies are highly restrictive and their projection to natural populations is speculative (Bennett, 1994).

Response to maternal calls and fleeing from danger are vital to the survival of young animals. These variables were tested in mallard ducklings whose parents had been treated with environmental concentrations of chlorinated hydrocarbon or mercurial pesticides (Heinz, 1976; 1979). In the first test, the response of newly hatched ducklings to the maternal call was measured by the time a chick took to approach a recorded call. The other test measured the same chick's avoidance of a frightening stimulus. These tests are sensitive, but limited in their use because they were designed for precocial birds. With modification of the parental treatment regime and age of pups, the same system has been used to test avoidance of the frightening stimulus in young rodents.

Tests of learning ability based on operant conditioning appear to be a sensitive indicator of xenobiotic effect in wildlife (Heinz *et al*., 1979). These tests require few specimens, but training and testing may require months (Kreitzer, 1980). The basic test is to feed hatchlings with low dietary concentrations of chemical for several months and then train the birds by hunger motivation to peck a lighted key in the conditioning chamber for a

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food reward. After learning which lighted pattern yields a reward, the pattern is changed and the ability to adjust is measured. After the chemical has been removed, the test may be repeated to test persistence of effect. The test has been performed on northern bobwhites with realistic dietary concentrations of chlorinated hydrocarbons and organophosphorus pesticides. The domestic pigeon (*Columba livia*) has proven to be a sensitive model for operant conditioning and testing for vapour toxicity (Armstrong *et al*., 1963; Leander *et al*., 1977). Application of operant conditioning to the study of behavioural effects of pesticides has been reviewed for laboratory rats and pigeons (McMillan, 1982).

Aside from the highly controlled and extremely labour-intensive laboratory tests, comparatively few studies are expressly conducted on the effects of xenobiotics on wildlife behaviour. Instead, many reports of behavioural observations were ancillary to studies of direct toxicity. For example, in a study of secondary poisoning of Siberian ferrets (*Mustela eversmanni*) fed intact laboratory rats that had been acutely dosed with zinc phosphide, it was noted during conditioning that some of the ferrets regularly ate the entrails of the rats, whereas others did not. Once zinc phosphide-treated rats were offered, ferrets eating the poisoned rat entrails immediately showed some distress and vomited. Thereafter the ferrets avoided eating the entrails although they consumed the balance of the carcass including major organs without hesitation (Hill and Carpenter, 1982).

Avoidance behaviour is a practical protection against xenobiotic poisoning of wildlife, but it is neither well understood nor often documented in nature. This important behaviour is being carefully evaluated by the US Department of Agriculture and the chemical industry, both to protect wildlife from poisoning by agrochemical application and to protect crops from wildlife depredation (Mason, 1997). The example of the ferrets selectively avoiding the entrails of the zinc phosphide-dosed rats was unique, but potential protection against acute poisoning by emesis was previously documented for kit fox (*Vulpes macrotis*) fed zinc phosphide-dosed kangaroo rats (*Dipodomys* sp.) (Schitoskey, 1975). In this example, unabsorbed zinc phosphide is present in the stomach of the prey animal in sufficient amount to stimulate emesis and emit a strong characteristic odour. With other substances such as granular (GR) anticholinesterase insecticides, neither the physiological protective reaction of emesis nor the obvious odour are as strong, and death of predators eating poisoned prey is common (US EPA, 1991).

Xenobiotic effects on reproductive behaviour have been conducted with limited relevance to natural populations. Ringed turtle-doves (*Streptopelia risoria*) provide a satisfactory model for behavioural studies because it can be easily paired, bred and managed in small battery cage units. At least 55 distinctly different behaviours have been characterized for this species under the general topics of development (infantile and juvenile), reproductive (sexual and nonsexual), aggression, escape and a variety of miscellaneous activities (Lehrman, 1955; Miller and Miller, 1958).

Outdoor pen studies have been used to study mallard hen and brood behaviours. In one study, five-day-old ducklings were gavaged with a single low dose of methyl parathion and released with their mother and untreated siblings in pond enclosures. Many behaviours were studied, including time spent in water, time spent preening and loafing, and parental attentiveness (Fairbrother *et al*., 1988). In other studies, mallard hens were dosed with methyl parathion and their nesting, brooding and attentiveness were studied (Brewer *et al*., 1988; Bennett *et al*., 1991).

Studies have also been conducted with free-ranging birds where adults were dosed with low concentrations of chemical and their nest attentiveness evaluated (White *et al*., 1983). A different breeding behaviour was tested in free-ranging sharp-tailed grouse (*Tympanuchus phasianellus*). A single dose of malathion reduced their defence of breeding territories on leks and sometimes caused bizarre behaviour (McEwen and Brown, 1966). Field studies have also shown that species such as northern bobwhite and European starlings (*Sturnus vulgaris*) are more subject to predation after organophosphorus exposure and release (Buerger *et al*., 1991). Carbamate insecticides may also affect hen–brood interactions, as demonstrated by a pen study of operational carbofuran treatment to vegetation that was associated with aberrant approach–response behaviour in mallard ducklings (Martin *et al*., 1991). Behavioural studies of organophosphorus and carbamate insecticides are especially useful because they are applied widely during the critical reproduction cycles of wildlife and have a well-defined biochemical marker, brain and blood cholinesterase, which can be used in conjunction with behavioural changes.

Behaviours other than reproduction are also studied. For example, migratory white-throated sparrows (*Zonotrichia albicollis*) were captured and exposed to dietary acephate for 14 days to determine effects on migratory orientation (Vyas *et al*., 1995). Birds were also exposed to polarized sheets to determine the mechanism by which orientation was effected. Adult sparrows did not establish a preferred orientation, but juveniles displayed a seasonally correct southward migratory orientation.

6.1.3 Avian Nestlings

Altricial nesting birds are sensitive models for ecological risk assessment. Nestlings, like neonatal mammals, are often the most sensitive life-stage to accumulation and toxicity of many chemical substances. However, whereas mammals may move their neonates, altricial

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birds cannot. Thus, once laying commences, the eggs and nestlings might remain subject to local contamination for 5–10 weeks. During this period it is possible to evaluate natural or experimental exposure. Individual eggs, nestlings or the entire nest can be treated, and their fate monitored through observation and selective sampling. For example, specimens may be bled, biopsied or euthanized; transovarian contamination of the egg can be compared with dietary ingestion and the nestlings' oesophagus can be constricted for retrieval of food items. Some species such as American kestrels (*Falco sparverius*) and European starlings can also be attracted to nest boxes, thereby ensuring the desired distribution of nests and sample size. Similar field studies have been conducted with waterfowl, but since the precocial hatchlings do not remain at the nest site, the home area must be contained, which often restricts normal movement of the hen and brood.

Altricial nestlings can also be tested in the laboratory with species that will breed in captivity. Unfortunately, the aviculture has not been sufficiently worked out for most species and few such toxicological studies have been published. Success has been achieved with American kestrels and European starlings. The value of these species as sensitive models for toxicity testing was validated by comparison with similar studies with precocial game birds. In these experiments, hatchlings were given graded doses of chemical daily by intubation for the first 10 days posthatching. Both kestrels and starlings were consistently more sensitive to lead and a variety of herbicides and polychlorinated biphenyls than were mallards, northern bobwhites and ring-necked pheasants (*Phasianus colchicus*) (Hoffman, 1995). End points included survival, body organ and skeletal growth, and blood and organ biochemistry. The use of free-ranging and captive altricial species in contaminant toxicology has been reviewed (Hoffman, 1994; 1995).

6.1.4 Caged Field Trials

Exposure of wildlife in field enclosures to 'operational application' of pesticide was initially considered a practical approach to ecological risk assessment of agrochemical and disease-vector control products. Enclosures varied from small portable wire cages to large structures up to 30 m or more on a side. Usually one to five small passerine- to quail-sized birds were tested in the small cages for acute response. The cages were placed in the spray zone immediately prior to chemical application and removed within a few hours post-treatment. Groups of 5–20 pen-acclimatized game birds or ducks were often tested in the large pens, with observations continued for widely variable periods post-treatment. The focus of these tests was also on acute response from direct chemical application, and theoretically included effects of eating natural foods contaminated by the treatment. Such tests were also referred to as simulated field trials. The design of these studies was widely variable, but the concept was appealing and led to the US EPAs development of a standard 'pen-in-field' protocol for testing chemicals under FIFRA (US EPA, 1982). Although theoretically useful, nearly all of these tests had major flaws and the US EPA rescinded the requirement of the pen-in-field test for pesticide registration.

Although most simulated field trials have major deficiencies, there are situations where ecological risk is detectable. The best example is probably for chemicals intended for use as a foliar or systemic application to vegetation that is preferred forage for herbivorous wildlife. In this situation, comparatively small enclosures would provide ample forage for a waterfowl or small mammal species. Such a test probably would have indicated the earlier described risk to Canada geese and American widgeon from turf grass treatment with diazinon and alfalfa treatment with carbofuran. Simulated field trials would also be useful in testing the potential risk of GR formulations of pesticide. The key is to evaluate carefully how and where a chemical will be used in relation to its likely availability to wildlife before conducting such a specialized field trial.

6.2 Chronic and Reproductive Toxicity

Chronicity trials are favoured by wildlife toxicologists because they allow the study of simulated field exposure under controlled conditions with a relevant model. Most often birds are used rather than mammalian wildlife because several mammalian models are routinely tested for human health effects. Thus, there is no compelling evidence to suggest a need for additional routine chronicity testing of wild rodents and rabbits, but it has been recommended that such tests and also a carnivore such as the mink be included on a specific chemical basis (Ringer, 1988; Ringer *et al*., 1991).

6.2.1 Chronic Reproduction Test

Most avian chronicity trials are designed with reproduction as the principal end point, that is, number of young that survive to fledging or, for precocial species, the number that survive to about two weeks of age (Heinz *et al*., 1979). These tests, first designed to evaluate the reproductive effects of persistent chlorinated hydrocarbon pesticides and heavy metals, have been standardized and may be required by the regulatory agencies to support the registration of pesticides intended for use in a natural system.

The avian reproduction test provides an evaluation of a chronic dietary exposure to the chemical with the goal of determining the highest dietary concentration that produces no observed adverse effect on reproduction and the lowest concentration that produces an observed effect

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(Bennett and Ganio, 1991). The results are compared with concentrations expected in the environment. Normally, this test of avian reproduction is the only standardized test of chronic dietary exposure of wildlife and the only method used for detecting long-term effects of chemicals that are either persistent in the environment or that are applied repeatedly to the environment.

The basic protocol was designed to test farm-reared mallards and northern bobwhites, but is easily adapted to other species that can be properly managed and bred in captivity and will lay an indeterminate number of eggs over an extended period. The protocol was tailored for environmentally persistent chemicals. In contrast, test concentrations for regulatory purposes are based upon residues expected under the proposed use, and multiple levels are tested to determine effect and no-effect concentrations. Test concentrations of chemicals already present in nature are based on environmental residues. Treatment commences at least 10 weeks in advance of breeding and continues to eight weeks after about 50% of the females are in lay. Eggs are collected daily and set at weekly intervals for artificial incubation. Reproductive end points include number of eggs laid, percentage fertility, embryonic development, hatchability and chick survival. An alternative approach, of perhaps more relevance, is to allow paired birds to incubate their own eggs and rear the young, because many behavioural responses must be correct for this process to succeed (Heinz *et al*., 1979). The basic protocol and alternative procedures have been thoroughly reviewed and critiqued for regulatory purposes (Bennett and Ganio, 1991).

6.2.2 Modified Reproduction Tests

As mentioned, the chronic avian reproduction test was designed for the study of ecologically persistent and accumulative pesticides and heavy metals. Environmentally plausible dietary exposure was begun well in advance of reproduction to allow for some accumulation of residue in critical organs and systems, and continued through the reproductive cycle. This approach does not work well with more labile substances such as organophosphorus and carbamate insecticides. These chemicals do not tend to accumulate in nature, and continuous low-level exposure, if not lethal, may be accommodated physiologically without serious effect on reproduction (Grue *et al*., 1991; Hill, 1995). However, intermittent chemical exposure from repeated applications of anticholinesterase insecticides may occur throughout the reproductive cycle and may lead to different effects than for continuously exposed animals. It is also possible that the response to anticholinesterase chemicals may not be the same if the initial exposure occurs at different stages in the reproductive cycle (e.g. courtship, onset of lay, incubation, foraging for altricial young). Hence specially designed research is often necessary to evaluate properly different classes of chemicals and uses.

6.3 Terrestrial Field Studies

Field studies of contaminant effects on wildlife may include resolution of a local incident of poisoning or a designed evaluation of a chemical treatment or industrial effluent. Procedures may involve detailed examination of individual specimens for evidence of gross, enzymatic and histological pathology or complex studies of local population and ecosystem perturbation.

Field studies are necessarily complex and one model will not fit all because each environmental situation is special in some way and poses unique problems for risk assessment. However, such studies should be pursued because the final proof of true xenobiotic risk to wildlife, other than direct evidence of mortality, is demonstrated only through a comprehensive field evaluation. Even then, the results may be applicable only to the specific habitat type and species studied.

7 COMPARATIVE TOXICOLOGY

7.1 Birds and Mammals

Metabolic responses to xenobiotics are similar for birds and mammals, with any differences more quantitative than qualitative (Pan and Fouts, 1978). This conclusion was based on the evaluation of more than 300 studies of 114 chemicals and 35 species of birds. The few qualitative differences are in conjugation reactions with glucuronic acid, sulfate, glutathione ornithine, glycine, taurine, ornithine and acetate. None of the reactions are believed to be related to differential sensitivity of birds and mammals to acute toxicity of anthropogenic chemicals (Pan and Fouts, 1978; Walker, 1983).

Several quantitative enzymatic differences between birds and mammals are important to differential response to acute toxic exposure. Birds generally have lower levels of hepatic monooxygenase (HMO) and A-esterase activity than do mammals, which make birds more susceptible to many environmental contaminants (Walker, 1978; 1980; Brealey *et al*., 1980). For example, carbamate insecticides are deactivated mainly by HMOs (Kuhr and Dorough, 1976) and a variety of avian species are consistently more sensitive to acute carbamate exposure than is the laboratory rat (Smith, 1987). Organophosphorus insecticides are also deactivated by HMOs, but many are first activated to a more potent oxygen analogue by the same HMO pathway (Eto, 1974). Most active organophosphorus analogues are substrates for A-esterase hydrolysis and are rapidly

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detoxified in the liver and blood (Brealey *et al*., 1980). In a study of 14 species of birds, three laboratory mammals, domestic sheep and humans, plasma A-esterase activity was at least 13 times higher in all of the mammals than in any of the avian species (Brealey *et al*., 1980).

As a rule, birds are more sensitive to acutely toxic chemicals than are mammals, in part due to differences in HMO activity, but such generalization is not accurate for all chemicals and species. Generally, *the laboratory rat is not a good model for the prediction of acute toxicity to birds,* even for chemicals that elicit their toxicity in the same way. When chemicals of different mechanisms of toxicity and biological stability are compared, equal or larger differences between birds and rats are common (cf. Gaines, 1960; Gaines, 1969; Hudson *et al*., 1984). In contrast, *laboratory rats and mice are conservative predictors of acute toxicity to wild rodents.* In a study of the acute oral toxicity of a spectrum of pesticides to four species of voles (*Microtus orchrogastar*, *M. canicaudus*, *M. pennsylvanicus* and *M. montanus*) and laboratory rats and mice, it was determined that laboratory rodents are generally more sensitive to pesticides than the most sensitive vole, *M. canicaudus* (Cholakis *et al*., 1981). Laboratory mice were also found to be more sensitive than deer mice (*Peromyscus maniculatus*) to many pesticides and drugs, but the relationship was not consistent (Schafer and Bowles, 1985).

7.2 Factors Affecting Interpretation of Acute and Dietary Tests

7.2.1 Growth and Maturation

Acute oral LD_{50}s and five-day dietary LC_{50}s change significantly during growth and development of precocial birds. In the acute test, change is believed to be primarily influenced by developing metabolic processes that affect toxication and detoxication of xenobiotics, and an immature immune system (Hudson *et al*., 1972). The subacute test is influenced by these same processes and highly individualistic responses to dietary presentation of xenobiotic (Hill and Camardese, 1981; 1986).

Changes in avian sensitivity to acute oral dosing may follow different patterns depending on the toxic mechanism of the chemicals. For example, mallard LD_{50}s for organophosphorus compounds that require HMO activation for maximum potency (latent cholinesterase inhibitors) tend to decrease between hatch and seven days, and then increase with maturation, whereas the opposite pattern occurs for direct-acting organophosphorus and carbamate anticholinesterases. Response to central nervous system-stimulating chlorinated hydrocarbons followed the same pattern as latent cholinesterase inhibitors. In contrast, LC_{50}s typically increase with age during early growth of precocial species. The increase is probably due to larger (older) chicks eating less in proportion to body mass and being better able to reduce their food consumption (toxic exposure) during the five-day feeding trial (Hill and Camardese, 1981).

7.2.2 Physiology and Gender

Tolerance of xenobiotic exposure may be affected by many factors, such as age, sex, reproductive status, genetic lineage, nutritional status, and endogenous and exogenous stressors. Each of these variables may affect LD_{50} and LC_{50} determinations, but their importance is not well documented for wildlife. Historically, in most acute avian studies, nonbreeding subadult game birds or adult passerines of both sexes were tested. This was intended to reduce gender effects and thereby conserve the number of wild birds needed for testing general acute sensitivity to pesticides. The legitimacy of pooling sexes of reproductively quiescent birds has been validated for acute toxicity testing (Tucker and Haegele, 1971; Tucker and Leitzke, 1979). Beyond general comparisons, this narrow focus is not adequate for risk assessment, because pesticides are intensively applied during breeding seasons and knowledge of sex differences in sensitivity is essential. The importance of this variable was shown by an acute test of the organophosphorus insecticide fenthion, in which ovulating northern bobwhite were 2.3 times ($p < 0.05$) more sensitive than males (Wiemeyer and Sparling, 1991). This relationship was supported by studies with Sherman strain rats, in which females were the most sensitive sex to about 30% of the anticholinesterase insecticides tested; males were most sensitive to about 10% of the compounds (Gaines, 1960; 1969). When sex differences were detected, females were much more sensitive than males (e.g. 1.5 to fivefold); when males were most sensitive the difference was usually less than 50%. Disproportional female sensitivity is particularly important because females are more important than males for population recruitment.

7.2.3 Captivity

Adequate methods are not available to evaluate the suitability of large numbers of wild-captured animals for routine toxicity testing. Simple survival and weight maintenance for a few weeks in captivity may not reflect subtleties such as nutritional imbalance or stress response to confinement, isolation or crowding. Whether captive specimens, either wild or farm hatched and reared, truly represent their free-living counterparts is not easily determined. Limited data were provided by a series of five-day subacute feeding trials with wild blue jays, house sparrows (*Passer domesticus*), northern cardinals (*Cardinalis cardinalis*) and wild and farm-reared northern bobwhites (Hill, 1971). All wild birds were at their captive weight, in excellent plumage and believed to be

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adequately conditioned. Blue jay was always the most sensitive species and farm bobwhite was always the most tolerant. Blue jays are adaptable generalized feeders that are generally considered to be tolerant of contaminated habitats (Bernard and Wallace, 1967). However, based on LC_{50}s, blue jays were about 1.5–50 times more sensitive than the other species to DDT and several organophosphorus insecticides. Wild bobwhites had less subcutaneous and visceral fat than did farm bobwhites, weighed about 25% less and were more sensitive to all of the chemicals tested. The wild bobwhites had been in captivity for several months, appeared healthy and were from the same local population from which the farm colony had been founded about 10 generations earlier.

8 ACUTE ECOLOGICAL RISK

Prediction of acute chemical risk to wildlife may be confounded by many factors of anthropogenic and natural origin. A primary factor, and perhaps most easily addressed through limited additional testing, is the influence of pesticidal formulations on chemical availability and toxicity to wildlife. Some of the other factors that may affect acute chemical risk to wildlife to different degrees include the route, source and timing of exposure; possible interactions with other chemicals; infectious diseases; and weather stressors.

8.1 Pesticide Formulation

Pesticide regulations are usually based on tests of technical-grade or analytically pure substance (US EPA, 1982; 1996). Only rarely is technical-grade material actually applied in the field, and then only in very low volume, for example, a few fluid ounces per acre for insect vector control in populated areas. Instead, a chemical is normally applied as a formulated product that may differ substantially in its acute toxicity from the technical-grade material tested. As a general rule, it has been determined that GR formulations are most often less toxic than technical-grade materials, whereas, liquid formulations are usually more toxic than technical-grade products (Hill, 1986). This relationship was verified by a limited study of formulated diazinon and carbofuran toxicity to northern bobwhites from a single source and hatch (Hill, 1992). A single dose of insecticide was administered as technical grade (TG, 99% a.i. alone or in corn oil (CO) diluent), as granular (GR, 14–15% Al) or as emulsifiable concentrate (EC, 48% Al). The relative acute toxicity, based on statistically separable LD_{50}s ($p<0.05$), was EC>GR=TG>CO for diazinon and EC>CO=TG>GR for carbofuran.

8.2 Routes and Sources of Exposure

Wildlife is most often exposed to xenobiotics through the ingestion of contaminated food and water. Inhalation and dermal exposure also occur but are believed to be less common and less hazardous to wildlife than is oral ingestion (Driver *et al*., 1991). Accordingly, nearly all screening studies of xenobiotic toxicity to wildlife involve oral administration of chemicals, but such studies are usually based on a technical-grade chemical via gelatine capsule, gavage or dry mash.

The toxicity of most xenobiotics is primarily a function of hepatic metabolism, regardless of the route of entry (Natoff, 1967). Thus, many chemicals are potentially more potent if they can enter the circulatory system by routes other than the gastrointestinal tract, which shunts directly to the liver for first-pass metabolism and detoxication. Compounds that require HMO enhancement for maximum potency are exceptions. Usually, however, chemicals are not rapidly absorbed through skin and scales, important alternative routes to ingestion. Percutaneous risk is dependent on the rate (dose and time) at which the chemical penetrates the skin and varies widely among chemical formulations, sites of application and species (McCreesh, 1965; Vinson *et al*., 1965; Durham, 1967). An array of pesticides have been tested by application to the wrapped feet (i.e. tarsometatarsus, phalanges and webbing; about 2% of body surface) of adult mallards for 24 hour, and to 1 cm^2 of featherless skin area covering pectoralis muscles under the wing joint of small passerine birds (Schafer *et al*., 1973; Hudson *et al*., 1979). At the same time companion birds were dosed orally. Percutaneous and oral LD_{50}s were positively correlated in both studies (Mallard, $n=19$, $r=0.65$, $p<0.01$; passerine, $n=17$, $r=0.85$, $p<0.01$). The LD_{50}s were consistently highest for percutaneous routes, but the difference was larger when chemicals were applied to the feet of ducks than the breasts of sparrows.

Although potentially more toxic because most chemicals readily cross the mucus membrane of the lung, inhalation toxicity is considered minor compared with deposition of chemical on the skin (Durham, 1967; Driver *et al*., 1991). However, this concept has not been rigorously tested on wildlife species. For example, it is not known to what degree bats are at risk when returning to their roost during morning twilight, the time considered optimum for aerial application of pesticides.

Wildlife is exposed to anthropogenic xenobiotics in contaminated air, water, soil, seeds, foliage, invertebrates, vertebrates and formulated GR pesticide particles. All of these sources have killed large numbers of wildlife under varied environmental circumstances (Grue *et al*., 1983; Hardy and Stanley, 1984; US EPA, 1991; Racine *et al*., 1992; Blus, 1995). Noninfectious xenobiotics of natural origin such as mycotoxins have also killed large numbers of wildlife eating contaminated seeds and peanuts (Windingstad *et al*., 1989; O'Hara, 1996).

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8.2.1 Air

Airborne exposure of wildlife to anthropogenic contaminants is not easily distinguished from terrestrial and aquatic sources and has received comparatively little attention. Perhaps most hazardous is short-term (minutes to a few hours) exposure to pesticidal application, but as indicated previously, inhalation toxicity is minor compared with dermal and ingestion routes (Driver *et al*., 1991). Instead, the primary concern is long-term deposition of industrial effluents over space. Usually this type of contamination is not directly toxic to most animals, but alters their dependent ecosystems, for example, acid precipitation and ozone (Laurence and Andersen, 2003). US EPA has begun to incorporate inhalation exposures into ecological risk assessments through the use of its Total Risk Integrated Methodology (TRIM) for air pollutants (Efroymson and Murphy, 2001).

One group of wildlife likely to encounter contaminated air is those that burrow in contaminated soils. To assist with the assessment of whether burrow air concentrations could exceed 'safe levels', Gallegos *et al*. (2007) used Botta's pocket gopher (*Thomomys bottae*) to develop ecological screening levels of volatile organic chemicals for burrowing small mammals. Estimates of soil pore gas can be used in place of direct measurements of burrow air concentrations. Additionally, burrowing rodents have been shown to inhale soil contaminants such as manganese and cadmium. By measuring these contaminants in the olfactory bulb of California ground squirrels (*Spermophilus beecheyi*), Bench *et al*. (2001) determined that the ground squirrels not only ingested the contaminants, but a considerable portion of their body burdens resulted from inhalation. However, for most wildlife, inhalation of metals such as cadmium is minor compared to ingestion (Burger, 2008).

8.2.2 Water

Water is a common source of xenobiotic exposure to wildlife that is poorly understood because toxicity is dependent on widely variable factors of ambient water quality, movement and the solubility and stability of chemicals. The importance and details of water quality to chemical fate, toxicity and risk are discussed elsewhere in this volume (see **Soil and Fresh Water**). With regard to pesticides, water-soluble formulations usually remain available longest in natural waters (Eto, 1974; Cardarelli, 1976; Kuhr and Dorough, 1976) and tend to be the most toxic by either acute single-dose or five-day dietary exposure of birds (Hill *et al*., 1975; Hudson *et al*., 1984; Hill, 1992). How these studies relate to water-borne exposure in nature is not clear because rates of feed and water consumption vary widely among wildlife species at different ages and seasons of the year. Smaller birds and mammals have a much higher water requirement relative to body mass than do larger species under similar ambient conditions (Robbins, 1983). Even closely related birds of similar size and feeding habits vary their rates of free water consumption from about 15 to 40% of their body mass per day at an ambient temperature of 25 °C (Bartholomew and Cade, 1963).

Few studies of pesticides in water have been reported for wildlife species, but some observations are noteworthy. Black-crowned night-herons (*Nycticorax nycticorax*) were confined for 24 hours to wading pools treated with Baytex 4 (fenthion) at rates calculated to be equal to and 10 times above registered field application rates (Smith *et al*., 1986). The high treatment had about 70 mg L^{-1} of fenthion at the surface of the water at 24 hours and resulted in no overt signs of toxicity or inhibition of brain cholinesterase activity; however, blood plasma cholinesterase was inhibited an average of 32% ($p < 0.05$). Field applications of fenthion in various formulations, at 47–100 g of active ingredient per hectare, over wetlands of variable water depths killed a variety of passerine and wading birds (Seabloom *et al*., 1973; Zinkl *et al*., 1981; DeWeese *et al*., 1983). The authors concluded that contaminated insects were an important source of fenthion in the avian mortality, but the importance of contaminated water was not dismissed. Mortality of wildlife from puddling and run-off from agriculture fields has been documented for many of the more acutely toxic pesticides currently registered for use in the USA (US EPA, 1991).

8.2.3 Mining

The presence of contaminated water is especially hazardous to wildlife in arid regions where open water is widely scattered. For example, microgold mining has resulted in hundreds of cyanide-contaminated ponds across the USA. Mines are located in variable climates from the humid southeast to the Dakotas and Alaska, but most are in the Great Basin and southwestern US deserts. Open water of mine ponds is especially attractive to wildlife in desert regions and the cyanide-treated solution and tailing ponds have killed thousands of migrating birds and large numbers of many resident vertebrate species (Clark, 1991; Clark and Hothem, 1991; Henny *et al*., 1994).

In spite of industry-wide advances in the protection of wildlife from cyanide solution ponds, mines differ widely in operational practices and their potential risk to wildlife. Much research has been done to ameliorate this risk, but owing to the high toxicity of the small solution ponds that are cycled on to the ore leaching pad, the only practical resolution is total exclusion of wildlife from the leaching field, solution ponds and their connecting ditches (Henny *et al*., 1994). The toxicity of the large tailing ponds (e.g. about 50–700 ha) may be effectively reduced by various chemical treatments. Another contributor to this risk is

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that waterfowl appear to have no aversion reaction to drinking potentially LCs of cyanide (Henny *et al*., 1994).

8.2.4 Soil and Sediments

A route of exposure that needs to be included in any risk assessment is soil or sediment ingestion. A measure of sediment ingestion by waterfowl in the Coeur d'Alene River Basin in Idaho, USA. was 18% for Canada geese and tundra swans (*Cygnus columbianus*), and 12% for ducks. The 18% value corresponded to an estimated 9% sediment ingestion rate (dry weight) (Beyer *et al*., 1998). Beyer *et al*. (1994) present the soil ingestion rates of 28 additional species of wildlife. The bioavailability of contaminants in soil varies depending on the species and the type of contaminant. For metals, normalizing their concentrations in sediment to acid volatile sulfides (AVSs) has been suggested as a universal explanation of metal availability from sediments. For organics, much attention has been given to the biota-soil/sediment-accumulation-factor (BSAF), an empirical ratio defined as the chemical concentration in tissue over the chemical concentration in soil or sediment (National Academy of Sciences, 2003).

Contaminants such as lead can be absorbed by birds from ingested sediments containing environmentally relevant concentrations, and these concentrations can be toxic (Connor *et al*., 1994; Beyer *et al*., 2000; Day *et al*., 2003). In the Coeur d'Alene River Basin, waterfowl have been poisoned for many years with lead from mine tailings and smelting activities in the area. The source of the lead was ingested lake and river sediment, not the vegetation or prey items in the diet (Beyer *et al*., 1997; 2000; Sileo *et al*., 2001).

Wild mammals will also consume large amounts of contaminated soil. Mule deer (*Odocoileus hemionus*) and white-tailed deer (*Odocoileus virginianus*) near the inactive Continental Mine in northern Idaho, USA. possessed higher concentrations of lead in their pellets than was likely from incidental ingestion of lead-contaminated soil. They appeared to be intentionally ingesting contaminated soil since clumps of sand and bits of rock were found in some pellets (Beyer *et al*., 2007).

8.2.5 Soil Inclusions

Lead poisoning of waterfowl has been documented in major waterfowl concentration sites throughout much of the world (Locke and Thomas, 1996). Prior to the ban on lead shot for waterfowl hunting, it was estimated that 2–3% of the North American waterfowl population perished from ingestion of lead pellets (Bellrose, 1959). Lead poisoning of waterfowl is believed to be almost exclusively from ingestion of pellets with little evidence of poisoning from pellets embedded in muscle or visceral tissues. In contrast, avian predators and scavengers are particularly susceptible to lead poisoning in areas of waterfowl concentrations where lead shot has been used, with the source of the poisoning usually from pellets in the alimentary canal or embedded in the prey or carcass. The toxicology and diagnosis of lead poisoning in wildlife have been extensively reviewed (Eisler, 1988; Franson, 1996; Locke and Thomas, 1996; Pain, 1996).

Like lead poisoning, white phosphorus (P_4) poisoning is mainly a problem for waterfowl that pick up small particles along with other food items in sediments. The source of the contamination was detonation of smoke-producing munitions and is largely confined to a few military training facilities, primary of which is Eagle River Flats, Alaska, USA (Racine *et al*., 1992). In the atmosphere, white phosphorus autoignites and completely oxidizes, but at low temperature in anaerobic sediments it is extremely persistent (Walsh *et al*., 1996) and acutely toxic to waterfowl (mallard oral $LD_{50} = 4\,mg\,kg^{-1}$ (Roebuck *et al*., 1998)). There is also evidence of white phosphorus poisoning predators and scavengers that feed on dead waterfowl.

In contrast to lead and white phosphorus, which are believed to be ingested primarily along with other food items, some GR pesticides may be ingested selectively. When this happens, even small numbers of exposed granules of highly toxic chemicals such as carbofuran may be lethal to many animals (US EPA, 1989; 1991). Like white phosphorus and to some degree lead pellets, secondary toxicity to predators and scavengers is from feeding on granules in the alimentary canal of the carcass rather than from residual concentrations of the substance in postabsorptive tissues and organs. Secondary poisoning from these acutely toxic chemicals is usually from exposure to the unaltered parent compound rather than from residual metabolites accumulating in postabsorptive tissues and fat as described for chlorinated hydrocarbons (Blus, 1995). Regardless of the mechanism, predatory species that normally would not ingest sediments may be poisoned through an intermediate host (e.g. bald eagle, osprey and peregrine falcon).

8.2.6 Avian Food Items

Treated seeds, like GR pesticides, are an important source of chemical exposure to wildlife when the seeds are treated with acutely toxic pesticides for soil insect control, or used as baits and repellents (Stromborg, 1977; Grue *et al*., 1983; US EPA, 1991). However, in contrast to GR materials that are most often ingested haphazardly, chemically treated seeds are readily eaten by small granivorous animals in spite of being brightly coloured for safeguarding human health. Also, the risk is not limited to seeds on the surface of the soil or small animals; large-scale mortality of greylag geese (*Anser anser*) was attributed to the uprooting and ingestion of germinating organophosphorus-treated seeds (Hamilton and Stanley, 1975). It is not known whether phytometabolism of systemic organophosphorus

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insecticides has contributed to wildlife mortality, but increased risk to herbivores is plausible because sulfoxide and sulfone metabolites may be even more potent than the parent compound.

Pesticide residues in foliage, whether by topical or systemic soil application, may be extremely hazardous to wildlife in or adjacent to the treatment zone. Treatments to control insects in forests and orchards and on cultivated crops such as small grains, alfalfa and turf grasses have all resulted in mortality of wildlife over the years (Smith, 1987; US EPA, 1991). Foliar treatments may be especially hazardous because they may result in exposure by multiple routes when animals are in or possibly even near the spray zone at the time of treatment.

Contaminated arthropods have proved lethal to wildlife after application of insecticides of such diverse toxicity as monocrotophos, dimethoate and trichlorfon (DeWeese *et al*., 1979; Smith, 1987; Goldstein *et al*., 1996). Such poisonings are most likely from chemicals adsorbed on the cuticle of the arthropods. An adsorbed chemical is easily dissociated in the stomach of the consumer. The acute oral LD_{50}s for the above three substances for adult male laboratory rats are reported to be 18, 215 and 630 mg kg^{-1}, respectively, and for adult nonbreeding ring-necked pheasants they are 2.8, 20 and 96 mg kg^{-1}, respectively (Smith, 1987). Insectivorous bird such as the tree swallow (*Tachycineta bicolor*) have been exposed to detrimental levels of mercury (Brasso and Cristol, 2008) and organic contaminants, such as PCBs, (McCarty and Secord, 1999; Custer *et al*., 2003; Echols *et al*., 2004; Papp *et al*., 2007) present in aquatic sediments, by consuming the emerging insects that develop as larvae in the contaminated sediments.

Predatory and carrion-eating birds and mammals are also known to have died from eating vertebrates and carcasses contaminated with a wide spectrum of pesticides, including highly stable chlorinated hydrocarbons and mercurials (Eisler, 1987; Blus, 1995) and more labile anticholinesterases, such as aldicarb, carbofuran, monocrotophos, fenthion, mevinphos, phorate and famphur (US EPA, 1991; Hill, 1995). The chlorinated hydrocarbon and mercurial poisonings were mostly from accumulated carcass residues in prey, whereas the organophosphorus and carbamate poisonings were probably from eating unaltered chemicals in the alimentary canal of the prey, as described for white phosphorus and GR insecticides.

The potential for secondary poisoning of wildlife by anticholinesterases has also been demonstrated with bullfrog prey (Hall and Kolbe, 1980). Tadpoles exposed to as little as 1.0 mg of parathion per litre of water for 96 hours were force-fed to 14-day-old mallards at the rate of 5% of body mass. A single meal was lethal to the ducklings within 30 minutes. Because only parathion, and not its oxygen degradate, was found in the tadpoles and stomachs of the dead ducklings, it is likely that parathion concentrated in the protective mucus layer of the tadpoles and was almost immediately available on ingestion by the ducklings. The treated tadpoles appeared healthy when fed to the ducks. Another anticholinesterase, famphur, was implicated in a case of tertiary poisoning (Henny *et al*., 1985). Black-billed magpies (*Pica pica*) fed on hair and insects contaminated with Warbex that had been used as a back pour-on in cattle for warble control; the magpies either died or were incapacitated by the exposure and were eaten by red-tailed hawks (*Buteo jamaicensis*) which also died.

Soil-dwelling prey such as earthworms often provide a route for soil contaminants into the vertebrate food chain as they bioaccumulate many soil contaminants. Earthworms bioaccumulated fluorine from the soil of an industrial site in southern Germany, and once birds ate the contaminated earthworms, they spread the contaminant throughout the landscape via faeces (Breimer *et al*., 1989). In the urban environment of Montréal, Canada, earthworms bioaccumulated copper, lead and zinc from the soils, reaching an equilibrium with the soil concentrations after two to three weeks (Kennette *et al*., 2002). Earthworms are capable of developing mechanisms allowing them to survive in highly contaminated soils (Langdon *et al*., 2003) making contaminants available to vertebrate predators. Dietrich *et al*. (1995) attribute the deaths of buzzards (*Buteo buteo*) found dead in an agricultural field to consumption of earthworms contaminated with the insecticide carbofuran. Earthworms contaminated from the soils in which they live pass that contamination up the food chain, even to top vertebrate predators such as the peregrine falcon (Enderson and Berger, 1968).

8.3 Toxic Interactions: Chemical

Interaction among xenobiotics and between xenobiotics and natural stressors, such as climatic extremes and infectious diseases, has not been thoroughly studied for wildlife species. The few studies reported are based on subchronic exposure of birds to expected field concentrations of environmentally stable pesticides or contaminants followed by acute challenge with anticholinesterase insecticides, or studies of simultaneous exposure to combinations of common pesticides in the standard five-day feeding trial. The results of these studies are generally consistent with similar studies of laboratory animals, but there are some differences that may affect ecological risk assessment. For example, pretreatment of laboratory rodents with chlorinated hydrocarbon pesticides that increase HMO activity reduced the sensitivity to dicrotophos, parathion and EPN (Ball *et al*., 1954; Triolo and Coon, 1966; Menzer, 1970). In contrast, when DDE was fed to adult Japanese quail at 5–50 mg kg^{-1} in the feed for 12 weeks, the sensitivity to a single dose of parathion increased significantly (Ludke, 1977).

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However, when the quail were pretreated with chlordane, acute sensitivity to parathion was decreased (Ludke, 1977). This observation was also reported in similar studies of laboratory mice (Triolo and Coon, 1966).

Methylmercury decreases HMO in mammals (Lucier *et al*., 1972), blood plasma cholinesterase in birds and mammals (Dieter and Ludke, 1975; Hastings *et al*., 1975; Hill and Soares, 1984), and brain cholinesterase activity in birds (Hill and Soares, 1984). Acute carbofuran dosing was more toxic to Japanese quail pretreated with methylmercury (Dieter and Ludke, 1978). The same relationship was noted for laboratory rats dosed with carbaryl (Lucier *et al*., 1972). In contrast, parathion, which requires HMO activation to its more potent analogue, was also more toxic to Japanese quail pretreated with methylmercury (Dieter and Ludke, 1975). Because methylmercury depresses HMO activity, pretreatment with methylmercury should have increased the toxicity of direct cholinesterase inhibitors, as seen for carbamates and decreased the toxicity of organophosphorus compounds that require activation. It is suggested that blood plasma cholinesterase provides some degree of protection by binding a portion of absorbed anticholinesterase and rendering it unavailable for binding with cholinesterase in the central nervous system, the main target of acute anticholinesterase toxicity.

8.4 Toxic Interactions: Environmental

Temperature extremes and season of the year are natural stressors that can affect the toxicity of xenobiotics to wildlife, but neither variable has been thoroughly investigated in nature or the laboratory. Some findings and tentative conclusions follow. Continuous exposure to both high (37 °C) and low (4 °C) temperatures for 14 days increased the sensitivity of adult Japanese quail to the acute toxicity of parathion (Rattner *et al*., 1987). Low temperatures also caused birds to increase their rates of feeding per unit of body mass by an average of 67%. This combination of increased sensitivity and energy demand certainly increases the potential risk and probably contributes to the vulnerability of waterfowl and passerines to winter application of anticholinesterase-treated seed grains (Stanley and Bunyan, 1979; Stone *et al*., 1984). In contrast, the above-mentioned quail exposed to 37 °C ate about 55% less than those exposed to 4 °C. This implies some possible degree of protection from anticholinesterase poisoning, but in this study, the birds exposed to 37 °C were more sensitive to parathion than those exposed to 4 °C (Rattner *et al*., 1987). Heat stress was suggested as a contributor to dimethoate toxicity in an episode of sage grouse (*Centrocercus urophasianus*) poisoning in Idaho, USA (Blus *et al*., 1989). These and other examples of conflicting results from toxicity tests at extreme ambient temperatures simply reflect natural responses to conditions deviating markedly from thermoneutral for the species. Environmental factors affecting xenobiotic toxicity in wildlife have been reviewed (Rattner and Fairbrother, 1991; Rattner and Heath, 1995).

8.5 Diagnosis of Acute Exposure

Since the 1950s, acute toxicity of many chlorinated hydrocarbon, mercurial, organophosphorus and carbamate insecticides has resulted in wildlife mortality in association with agriculture throughout the world (Borg *et al*., 1969; Mendelssohn and Paz, 1977; Hamilton *et al*., 1981; US EPA, 1991; Goldstein *et al*., 1996). Acute lead poisoning from lead shot in major waterfowl concentration sites was also an international problem (Locke and Thomas, 1996). Though the causative agent was usually obvious in incidents of mass mortalities of mixed species, it was not so obvious in cases of individual deaths or small-scale mortality of single species, where infectious diseases or other exogenous stressors may have been present. Thus, specific diagnostic methods were needed to evaluate wildlife mortality properly.

Initially, the emphasis of diagnostic technology was on determination of chemical residue levels that were indicative of death from persistent chlorinated hydrocarbon and mercurial pesticides. As the 'DDT era' came to a close in the 1970s, attention shifted to the more environmentally labile, but acutely toxic anticholinesterase insecticides and to a myriad of agricultural and industrial effluents. With these substances chemical residues were not well correlated with effect and often could not be detected at all in key tissues and organs. Wildlife toxicologists, physiologists and chemists borrowed extensively from biomedical techniques for baseline work, but then had to adapt and validate methods for an array of species of widely variable physiology and sensitivity to xenobiotic exposure.

The basic techniques that have proved useful in the diagnosis of lethality or significant toxicity from a specific chemical include brain residues of chlorinated hydrocarbon pesticides (death; Blus *et al*., 1996), brain cholinesterase inhibition (death; Fairbrother, 1996) and red-blood-cell δ-aminolaevulinic acid dehydratase (lead exposure; Locke and Thomas, 1996). Diagnostic brain residues have been determined for DDT and its principal metabolites DDD and DDE, dieldrin, heptachlor, chlordane, oxychlordane, endrin, mirex and the polychlorinated biphenyl aroclor 1254 (Heinz *et al*., 1979; Blus, 1995). For each of these chemicals there is a general threshold level of residue associated with lethality that holds true across a wide range of birds and mammals (Heinz *et al*., 1979). Other tissues, such as fat, liver and kidney, may accumulate significant concentrations

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of lipophilic pesticides and other contaminants, but none has proved useful in conclusive diagnosis of lethality.

Brain cholinesterase inhibition has been used to diagnose death from anticholinesterase exposure, but the causative chemical is sometimes difficult to determine because neither organophosphorus nor carbamate residues tend to accumulate in postabsorptive tissues. However, a strong inferential diagnosis is possible by demonstrating depressed brain cholinesterase activity and detection of a known anticholinesterase in either ingesta or tissues (Hamilton *et al*., 1976; Hill and Fleming, 1982). A conservative threshold of about 50% depression in whole-brain cholinesterase activity is generally considered diagnostic of death from anticholinesterase poisoning (Ludke *et al*., 1975), although depression of 70–95% is commonly reported for birds killed in the field by organophosphorus insecticides (Hill and Fleming, 1982). In contrast, when birds are killed in the field by carbamates, whole brain cholinesterase activity may vary from near normal to depressions of only 60–70% (Hill and Fleming, 1982; Flickinger *et al*., 1986). Apparently, high levels of carbamate exposure kill by systemic neuromuscular blocking before significant penetration of the central nervous system has occurred (Westlake *et al*., 1981). Also, lower cholinesterase inhibition may reflect spontaneous postmortem reactivation of carbamylated enzyme (Hill and Fleming, 1982; Hill, 1989).

9 ENDOCRINE DISRUPTION

An environmental endocrine disruptor has been broadly defined as an exogenous agent that interferes with the production, release, transport, metabolism, binding, action or elimination of natural hormones in the body responsible for the maintenance of homoeostasis and the regulation of developmental processes (Kavlock *et al*., 1996). However, Guillette (2006) has recently suggested that the focus be expanded to include prostaglandin synthesis as a target for endocrine disruption, since these hormones are active in reproduction, immune responses and cardiovascular physiology.

In the early 1990s attention was drawn to impacts of endocrine-disrupting chemicals based to a great extent on studies from the Great Lakes region of the USA and Canada (Colborn *et al*., 1993). Exposure to endocrine-disrupting chemicals in the environment was believed to be associated with abnormal thyroid function in birds and fish; decreased fertility in birds, fish, shellfish and mammals; decreased hatching success in fish, birds and turtles; demasculinization and feminization of male fish, birds and mammals; defeminization and masculinization of female fish, gastropods and birds; and alteration of immune function in birds and mammals.

One of the most widely reported incidents where endocrine disruption was identified in wildlife was in alligators (*Alligator mississippiensis*) in Lake Apopka and Lake Okeechobee Florida, USA (Crain *et al*., 1998). They found that juvenile male alligators from contaminated lakes had lower levels of testosterone and increased levels of thyroxin. The reduction in testosterone levels was thought to be the cause of the highly publicized inhibition of penis growth in the juvenile males (Crain *et al*., 1997).

Various endocrine-disrupting contaminants such as some pesticides, phytoestrogens, pharmaceutical pollutants and heavy metals have the potential to alter sex determination, differentiation and maturation. Orlando and Guillette (2007) review the potential impacts that endocrine disruptors have on sex determination in different taxonomic groups. In teleost fishes, sex determination, differentiation and maturation can be affected and the genetic sex of the individual does not influence whether a response occurs or not. In frogs, crocodilians and turtles, environmental factors with oestrogenic activity can redirect sex determination, altering differentiation and maturation and producing apparently normal females or individuals with a mostly female phenotype. In birds and mammals, neither endogenous nor exogenous compounds with sex steroid activity affect sex determination—the formation of an ovary or testis is determined by the presence of specific genes.

These studies and many others led the US EPA to form the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) which published its final report in 1998. An outcome from the efforts of this committee was the development of a new series of tests for pesticides and high production volume (HPV) chemicals. The Tier 1 screening tests developed in conjunction with the OECD will include a battery of screening assays that would identify substances with the potential to interact with the oestrogen, androgen or thyroid hormone systems.

Toxicity testing required by US EPA and OECD has historically been restricted to avian tests that provide acute oral toxicity, acute dietary toxicity and reproductive toxicity. A wild mammal toxicity test protocol exists, but it is rarely performed. Once the endocrine testing has been fully implemented, the tests will be expanded to include an amphibian metamorphosis test (OECD, 2004), an amphibian two-generation test (Battelle, 2004) and an avian two-generation test (Battelle, 2005). Additional mammalian and *in vitro* tests also will have implications for impacts on wildlife.

10 PHARMACEUTICALS

Pharmacologically active compounds have been identified in wastewater treatment plant effluent (Hua *et al*., 2006) and in soils receiving treated sludge from sewage (Gielen, 2007). Pharmaceuticals are released into the environment purposefully after disposal or inadvertently

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via excretion by consumers, primarily through sewage. Numerous bioactive metabolites from any single pharmaceutical might be excreted in combination, or the parent pharmaceutical could be abiotically transformed in sewage treatment plants or by the environment. The continual release of pharmaceuticals and metabolites gives them a 'pseudo-persistent' presence in any aquatic environment that receives sewage input from humans or domestic animals (Daughton, 2003).

It is now known that a wide range of human pharmaceuticals, including analgesics, antibiotics, steroids, many cardiovascular drugs and various drugs used to treat mental illness, are all present in water treatment effluents that enter rivers (Runnalls *et al*., 2007). Little is known about the possible ecological risks of most of pharmaceutical chemicals once they become dispersed in the environment. This lack of knowledge has resulted in a substantial amount of effort to develop data and approaches for assessing the impact of pharmaceuticals in the environment (Ankley *et al*., 2007). Sanderson *et al*. (2004a) report that ecotoxicity data were available for $<1\%$ of pharmaceuticals and their adjuvants. Elsewhere, Sanderson *et al*. (2004b) suggest the greatest impact on wildlife is the potential for a pharmaceutical class of sex hormones acting as endocrine modulators in wildlife. Since pharmaceuticals most often enter the environment via wastewater, aquatic species have received more attention regarding the impacts on wildlife than have terrestrial species. Even aquatic species lack sufficient data to decide whether pharmaceuticals pose a significant threat to the environment (Crane *et al*., 2006).

A recent example of pharmaceuticals impacting wildlife is the widespread mortality of vultures on the Indian subcontinent (Green *et al*., 2004). The Oriental white-backed vulture (*Gyps bengalensis*) was once one of the most common raptors in the Indian subcontinent. A population decline of $>95\%$, began in the 1990s. The unusual pattern of mortality was first observed in early 1997, among the vultures nesting in Keoladeo National Park in northwestern India. Birds dropped from their perches to die shortly afterwards (Risebrough, 2004). Declines, also involving *Gyps indicus* and *Gyps tenuirostris*, have continued to be reported across the subcontinent. Diclofenac was identified as the cause of the renal failure that caused the deaths of both adults and subadults (Oaks *et al*., 2004; Meteyer *et al*., 2005). Diclofenac is a nonsteroidal anti-inflammatory drug used in domestic livestock, and remains available to vultures in the carcasses from recently treated animals (Taggart *et al*., 2007).

In recent reviews (Fent *et al*., 2006; Dorne *et al*., 2007), the only conclusive effects of pharmaceuticals on wildlife were the example above of the impacts of diclofenac on vultures and the impacts that synthetic steroid hormones such as 17α-ethinylestradiol (EE2) and 17β-estradiol (E2) have on fish. Until more is known about the impact that pharmaceuticals have on wildlife, the environmental matrices in addition to water likely to contain such chemicals, and the concentrations at which these chemicals have impacts, regulating them will be difficult. However, because considerable toxicological data exists for pharmaceuticals for humans and standard laboratory test species, a first step might be to extrapolate from these species to wildlife receptors (Dorne *et al*., 2007).

Most pharmaceuticals having environmental relevance share the following properties: often, but not always, HPV combined with environmental persistence and biological activity, mainly after long-term exposure (Fent *et al*., 2006). Most environmental legislation aimed at protecting wildlife from industrial chemicals sets production volumes below which either no testing is required, or the testing is tiered, becoming progressively more rigorous as production volume increases, based on the thinking that as more of a chemical is produced, the greater the concentration likely to be present in the environment, and therefore, the greater the possibility that adverse effects on organisms will occur (Ankley *et al*., 2007). Another source for exposure of wildlife to pharmaceuticals is crops that have been genetically modified to produce pharmaceuticals (Becker and Vogt, 2005). For example since 1987, the United States Department of Agriculture's Animal and Plant Health Inspection Service's Biotechnology Regulatory Services has issued 110 pharmaceutical and industrial permits in 11 crops. Since less than 350 acres have been grown since 2002 (Smith, 2005), this is currently a minor source for exposure, but that might increase.

In early 2001, a draft guideline on ecological risk assessments for non-GMO-containing drugs was published by the European Medicines Evaluation Agency (EMEA). The first tier consists of deriving a crude predicted environmental concentration (PEC) in the aquatic compartment for the active pharmaceutical ingredient (API) or its major metabolites, based on predicted amounts used and specific removal rates in sewage treatment or surface waters. If this crude PEC is $<0.01\,\mu g\,l^{-1}$ and no environmental concerns are apparent, no further assessment is deemed necessary. Otherwise, in the second tier, a crude predicted no-effect level (PNEC) for the aquatic compartment is to be extrapolated by dividing the lowest 50% effect concentration from acute ecotoxicity tests with algae, daphnia or fish (EC_{50}, LC_{50}) by an assessment factor (usually 1000). If the ratio PEC/PNEC is <1, no further assessment is deemed necessary. Lastly, in the third tier, further considerations might be needed. This may encompass refining the environmental fate information and thereby the PEC, considering further environmental compartments and their respective PECs (up to and including field studies), but also refining the PNEC (Straub, 2002). A PEC for human pharmaceuticals of $>0.1\,\mu g\,l^{-1}$ necessitates aquatic ecotoxicity testing. However, if a drug (or one of its degradation products)

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is already known, or can be reasonably expected, to affect aquatic wildlife at concentrations $<0.01\,\mu g\,l^{-1}$, then the action limit of $0.01\,\mu g\,l^{-1}$ does not apply, and further analyses must be conducted (Ankley *et al*., 2007).

11 IMMUNOTOXICOLOGY

Factors that may influence immune responses in wildlife include age, body condition, season, developmental stage of the immune system and time required for the progression of immune responses (Grasman, 2002). In birds, the thymus and bursa of Fabricius are the sites of maturation of T and B lymphocytes, respectively. Mature T and B cells and macrophages interact in the spleen during immunological responses. Hence, the mass, cellularity and histology of these organs can provide important general information on the maturation and structure of the immune system and have been assessed in several wildlife studies (**Table 1**). While not all chemicals cause atrophy and (or) histological changes in immune organs, when such alterations occur they are usually associated with altered immunological function, making these variables useful end points in studies where lethal sampling is employed (Grasman, 2002).

Laboratory assays of immunological function are highly sensitive to contaminants. Such tests include *in vivo* skin tests for T cell function, *in vivo* antibody responses, *in vitro* lymphocyte proliferation and phagocytosis assays. Other tests include the DTH (delayed-type hypersensitivity) assay which tests for T-cell-dependent inflammation. Intradermal or subdermal injections of specific antigens to which the animal has been previously immunized stimulate T cells to release cytokinins, causing a localized inflammation. The DTH test is not very practical for use in wild birds, since it often requires multiple immunizations several weeks apart. The two most commonly used *in vivo* immune function tests in birds are the phytohaemagglutinin (PHA) skin response for T cell-mediated immunity and the sheep red blood cell (SRBC) haemagglutination assay for antibody-mediated immunity (Grasman, 2002). The PHA skin response is more practical for wild birds than the DTH test since it requires only a single injection with no prior sensitization (Rollins-Smith *et al*., 2007). PCBs (polychlorobiphenyls) and other organochlorines and lead suppressed the PHA response (Rollins-Smith *et al*., 2007).

Assessment of plasma or serum protein concentrations can provide important data on health and physiological status, including immune and inflammation responses. Relative and total amounts of albumins and α-, β- and γ-globulins are affected by infections, inflammation and physiological status. Antibody responses may increase γ-globulins. Inflammation may increase positive acute phase proteins (α-globulins such as antitrypsin, α_2-macroglobulin and haptoglobin, and β-globulins such as fibrinogen, C3, C4, ferritin and amyloid A) and decrease prealbumin, albumin and transferrin (Grasman, 2002).

While measures of immunological structure assess the status of the immune system, they do not directly test immune function (i.e. how the immune system responds to specific challenges). The PHA skin response test has been used as a measure of T-lymphocyte function in laboratory experiments and human and veterinary medicine, and is one of the most common assays in wildlife studies. Common sites of injection in birds include the wing web, wattle, dewlap and interdigitary skin. Maximal inflammation occurs 12–24 hours after injection. Usually, phosphate buffered saline (PBS), the carrier for the PHA, is injected into an alternate site (e.g. other wing web or wattle). The response to PHA is calculated as the increase in skin thickness caused by PHA minus the increase caused by PBS alone (Grasman, 2002).

Like skin tests, measurement of antibody titres following immunization with an antigen integrates a large number of immunological functions and events. The tests are minimally invasive, but do require recapture of test animals approximately one week after immunization. Enzyme-linked immunosorbant assays (ELISAs) are commonly used to assess immune function in humans, rodents and domestic animals. However, ELISAs require antibodies that specifically bind to the antibodies produced by the test subjects. Such species-specific antibody reagents are not available for most wildlife species. Alternatively, the anti-SRBC haemagglutination test has been employed successfully in many species of wild birds. Blood plasma is collected from birds approximately six days after immunization with SRBC. Serial dilutions of the plasma are incubated with SRBC in microtitre plates. If sufficient antibody activity is present, the antibodies will agglutinate the SRBC, which causes a visible spreading of the SRBC on the bottom of the wells. If sufficient activity is not present, the SRBC will not spread and will form a distinct 'button' at the bottom of the V-shaped well. The higher the antibody activity or titre, the more the plasma sample can be diluted before the agglutination stops. The agglutination activity is caused by both IgM and IgG. Preincubation of the plasma with 2-mercaptoethanol dissociates IgM, and any residual agglutinating activity is attributable to IgG, which is more prevalent in memory responses (Grasman, 2002).

White blood cells are the effector cells of immune responses and have been assessed in many immunotoxicological studies of avian wildlife (**Table 1**). Lymphocytes are important for T-cell-mediated and antibody-mediated responses, as well as nonspecific natural killer cell cytotoxicity. Monocytes are important for phagocytic responses. Neutrophils or heterophils provide an important first line of defence against

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Table 1 Measures of immunological structure in toxicological studies of free-living and captive avian wildlife species

Variable	Species (captive/wild)	Contaminant(s)	Findings/comments	Reference
Immune organ mass /cellularity/ histology	Mallard (C)	Selenium	No effect on spleen mass	Fairbrother and Fowles (1990)
	Mallard (C and W)	Lead	Decreased spleen mass in females intubated with 2 #4 Pb shot	Rocke and Samuel (1991)
	European starling (C)	DMBA	Decreased spleen mass and cellularity in adults exposed subcutaneously to 125 mg (kg body mass)$^{-1}$	Trust *et al*. (1994)
			Decreased bursa mass in nestlings exposed orally to 100 mg (kg body mass)$^{-1}$	
	Tree swallow (W)	Orchard pesticides	Body and thymus masses correlated with collection date; bursa mass associated with spraying; some histological alterations associated with spraying.	Bishop *et al*. (1998)
	Herring gull (W)	Dioxin-like organochlorines	Negative association between thymus mass and liver EROD (ethoxyresorufin *O*-deethylase) activity (index of exposure to dioxin-like chemicals); no associations between thymus mass and organochlorine concentrations measured in eggs	Grasman (2002)
	Herring gull (W)	Organochlorines	Negative associations between PCBs and thymocyte numbers and between DDE and lymphoid cells in bursa in pipping herring gull embryos	Grasman unpublished data
White blood cell numbers	Japanese quail (C)	Paraquat, ethyl methanesulfonate	Increased heterophils, decreased lymphocytes and monocytes	Clark *et al*. (1988)
	Mallard (C)	Selenium	No effect on white blood cell numbers	Fairbrother and Fowles (1990)
	Mallard (C and W)	Lead	Decreased total white blood cells, heterophils, lymphocytes and monocytes in males intubated with 2 #4 Pb shot; no effect in females	Rocke and Samuel (1991)
	American avocet (W)	Selenium, arsenic, and boron	Increased heterophil/ lymphocyte ratio in one pond following *in ovo* exposure	Fairbrother *et al*. (1994)

(*continued overleaf*)

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Table 1 (*continued*)

Variable	Species (captive/wild)	Contaminant(s)	Findings/comments	Reference
	European starling (C)	DMBA	Increased heterophil/lymphocyte ratio in adults exposed subcutaneously to $\geq$50 mg (kg body mass)$^{-1}$ or orally to 300 mg (kg body mass)$^{-1}$	Trust *et al*. (1994)
	Japanese quail (C)	Lead	Marginally significant increase in heterophil/lymphocyte ratio in corn-fed quail exposed to 100 and 400 ppm lead in drinking water; no effects seen in lead-exposed quail fed commercial feed	Grasman and Scanlon (1995)
	Herring gull (W)	PCBs, DDE	No associations with white blood cell numbers	Grasman *et al*. (1996)
	Caspian tern (W)	PCBs, DDE	Positively associated with heterophil/lymphocyte ratio in one year only	Grasman *et al*. (1996)
	Mallard (C)	PCBs	No effects on white blood cell numbers	Fowles *et al*. (1997)
	Tree swallow (W)	Orchard pesticides	Heterophil numbers negatively associated with collection date; lymphocyte numbers reduced with pesticide sprays	Bishop *et al*. (1998)
	Zebra finch (C)	Oil sands tailings water	No effect on white blood cell numbers	Smits and Williams (1999)
	Herring gull (W)	PCBs, DDE, dioxin-like chemicals	In adults, total WBC and heterophil numbers positively associated with liver EROD and negatively associated with DDE, lymphocyte numbers negatively associated with PCBs, and heterophil/lymphocyte ratio negatively associated with liver EROD; in prefledglings, heterophil-lymphocyte ratio positively associated with dioxin-equivalents	Grasman *et al*. (2000b)
	Caspian tern (W)	PCBs, DDE	Plasma PCBs and DDE negatively correlated with % monocytes and positively correlated with % basophils	Grasman and Fox (2001)
Plasma proteins	Herring gull (W)	PCBs, DDE	Positive association with B_2-globulins in prefledglings	Grasman *et al*. (2000a)
	Caspian tern (W)	PCBs	Positive association with B_1-globulins and negative association with a-globulins in prefledglings	Grasman *et al*. (2000a)

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bacterial infections, while eosinophils fight parasitic infestations. Blood cell counts are minimally invasive and relatively easy to conduct. Total and differential white-blood-cell counts are commonly used as clinical indicators of immunological and infection status. Decreased numbers of specific cell types may suggest reduced functions associated with these cells, and increased numbers may suggest a response to infection. In either case, altered white blood cell counts should be followed up by tests of immune function to better characterize potential immunological effects (Grasman, 2002).

11.1 Amphibians

Amphibian metamorphosis is a complex process, during which the developing immune system is undergoing rapid development and expansion. Contaminants such as perchlorates can alter thyroid hormone activity, thus impairing the development of the immune system. Similarly, contaminants that interfere with the action of corticosteroid hormones can inhibit the naturally occurring loss of lymphocytes at metamorphosis, which could lead to the development of autoimmunity due to adult-specific lymphocytes that emerge at metamorphosis (Rollins-Smith *et al*., 2007).

Environmentally relevant concentrations of atrazine, metribuzin, aldicarb, endosulfan, lindane and dieldrin showed immunosuppression in juvenile leopard frogs (*Rana pipiens*) and African clawed frogs (*Xenopus laevis*). Spleen cell numbers were reduced and phagocytic activity was impaired. Lymphocytes from pesticide-treated leopard frogs exhibited reduced T-cell proliferation *in vitro* in response to mitogens, and the frogs developed increased parasitism when challenged with parasitic nematodes (Rollins-Smith *et al*., 2007). An impaired immune system resulting from exposure to pesticides or other contaminants also can make amphibians more susceptible to infection by trematodes. Exposure to malathion and atrazine affected the degree of infection of wood frog (*Rana sylvatica*) tadpoles by trematodes capable of inducing limb deformities. Increased malathion or atrazine caused a greater degree of parasitism and fewer circulating eosinophils (Rollins-Smith *et al*., 2007). Exposure to contaminants that alter the rate of metamorphosis could impair immune defences of amphibians in a variety of ways that are not well understood (Rollins-Smith *et al*., 2007).

Little is known regarding the impact of contaminants on immune response in amphibians in general, with even less known about the specifics in urodele amphibians (salamanders). Urodele amphibians lack some of the more evolved aspects of lymphocyte-mediated, specific immunity that are present in anurans (frogs and toads), birds and mammals (Froese *et al*., 2005).

11.2 Birds

The study of immunotoxicology in birds lags behind mammalian immunotoxicology. The structure and function of the avian immune system has often been affected by a diverse array of contaminants, including heavy metals, pesticides, petroleum hydrocarbons and organic industrial chemicals (Day *et al*., 1995; Galloway and Handy, 2003; Rollins-Smith *et al*., 2007) and possibly the explosive 2,4,6-trinitrotoluene (TNT) (Gogal *et al*., 2002).

No field studies of measured immune suppression have reported an association with an avian epizootic. It is more likely that the immune suppression in adult birds resulting from low-level chronic stress (e.g. crowding onto poor-quality habitat, food reductions or climate stress) and (or) environmental contaminants causes slow but consistent morbidity and mortality associated with multiple pathogens, rather than an acute epizootic with a single pathogen. Increased fitness costs associated with such stress may significantly alter genetic diversity and species survival over time (Fairbrother *et al*., 2004).

11.3 Terrestrial Mammals

Human and wildlife monitoring studies have identified perfluoroalkyl acids (PFAAs) and perfluorooctane sulfonate (PFOS) worldwide (Lau *et al*., 2007; Peden-Adams *et al*., 2007). There has been substantial progress in understanding additional aspects of the toxicology of these compounds, particularly related to the developmental toxicity, immunotoxicity, hepatotoxicity and the potential modes of action. Principally from studies in laboratory mice, PFAAs have led to decreased body weight, elevation of liver weight and decreases in thymus and spleen weight (both absolute and relative weights). The primary humoral response to horse red-blood cell immunization was prevented by perfluorooctanoic acid (PFOA) pretreatment while *ex vivo* spleen cell proliferation in response to both T- and B-cell activation was attenuated by the fluorochemical (Lau *et al*., 2007). Studies show that these perfluorinated compounds induce peroxisomal proliferation, induce hepatomegaly, alter steroidogenesis and decrease body weight, accompanied by a wasting syndrome; however, effects on immune function have not been addressed at length. Sulfluramid, a perfluorinated pesticide, targets T-dependent, IgM antibody production at exposure levels 10-fold less than that observed with overt toxicity (as indicated by decreases in body weight gain) (Peden-Adams *et al*., 2007).

One possible mechanism of PCB toxicity to wildlife is through immunosuppression and subsequent increase in disease rates. When white-footed mice (*Peromyscus leucopus*) were exposed to PCBs as adults via the

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diet, their offspring exhibited impacts to their immune response. The thymus was the most severely affected organ of the immune system; thymic weights did not differ between the groups, but the number of thymocytes of the treated animals was significantly lower than that of the control animals. Toxic effects, especially on the immune system, of chronic exposure to PCBs, at levels comparable to exposure found in contaminated sites in the USA, are evident in adult white-footed mice. The thymus seems to be the most persistently affected organ. However, these alterations did not translate into any evident impairment of the specific humoral immune response to the T-dependent antigen assayed (Segre *et al*., 2002).

Immune function was measured in populations of wild cotton rats (*Sigmodon hispidus*) inhabiting replicated reference and contaminated study sites at an abandoned oil refinery in Oklahoma four times from 1991 to 1992. Several measures of immunocompetence were examined including immune organ mass and cellularity, haematology, *in vivo* hypersensitivity, macrophage function, killer cell activity and lymphoproliferative responsiveness. *In vitro* proliferation of splenocytes, either spontaneous or induced with concanavalin A (Con A), was the most consistent and reliable indicator of immunotoxicity (McMurry *et al*., 1999).

11.4 Marine Mammals

Marine mammals have received attention regarding the impacts of contaminants on their immune response. Although mechanisms of action are often ill-defined in field studies, a common pattern of adverse health effects observed in contaminant-exposed individuals and populations includes developmental, immunologic and reproductive effects. Despite having been banned in North America for three decades, the highly persistent PCBs continue to present a toxic risk to wildlife and dominated the contaminant profiles in studies of British Columbia and Washington State harbour seals (*Phoca vitulina*). Gertrude Island (Puget Sound) harbour seals were particularly contaminated, having PCB concentrations that were several times higher than those sampled in the adjacent coastal waters of northern Washington State and southern and central British Columbia. The observed negative relationship between circulating thyroxin and PCB concentrations in harbour seals contributes to the notion that PCBs represent a significant health concern at the top of the food chain (Tabuchi *et al*., 2006). However, Barron *et al*. (2003) state that, based on very limited blubber data, current levels of PCBs might not pose a risk to Steller sea lions (*Eumetopias jubatus*), in comparison to immunotoxicity tissue benchmarks. They indicate that there are insufficient data to reject the hypothesis that contaminants play a role in the continued decline of Steller sea lions, and suggest that a coordinated monitoring programme be developed which can be related to key biological, ecological and laboratory toxicity data. Ross *et al*. (1995) identified immunotoxic impacts in harbour seals fed herring from the Baltic Sea, but could not determine which of the contaminants identified in the herring was responsible.

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Soil and Fresh Water

Trond Peder Flaten and Eiliv Steinnes

CONTENTS

1 INTRODUCTION

In the environmental science literature, nature is commonly divided into different spheres. In this respect, the soil is termed the *pedosphere*. The pedosphere interacts with all the other spheres; the lithosphere (the Earth's crust), the hydrosphere, the atmosphere and the biosphere. In particular, soil and water systems are intimately inter-related through a complex web of interactions. Almost all soils contain water, and all water systems contain suspended (mainly soil-derived) solid particles. Most chemical reactions in nature take place at the interface between solids and water (Stumm and Morgan, 1996). Most dissolved and suspended material in water has passed through soil and ground. Therefore, in most cases it is necessary to apply an integrated approach to the discussion of toxic substances in soil and fresh water. The prime focus in the present chapter is on soil; water will be discussed mainly as an exposure route from soil to humans.

Matter moves through the different spheres in nature in biogeochemical cycles. Transport is mostly in water via the hydrological cycle, as dissolved ions and molecules, or as colloids and suspended particles, and most substances present in fresh water will have passed through the soil. Thus, the concentrations present in water will depend on which chemical reactions these substances undergo in the soil. Also, a particular element may exist in nature as many different *species*, that is, different physicochemical forms of the same element. For example, the element iodine may exist in aqueous solution as I_2, I^-, I_3^-, HIO, IO_3^-, as ion pairs or complexes, as various organic iodo compounds or adsorbed on suspended solids (Stumm and Morgan, 1996). Properties such as mobility, toxicity and

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bioavailability of a particular element generally depend heavily on its speciation.

For a particular element, the most important variables governing speciation are: (i) particulate vs. dissolved state in aqueous solution, (ii) variations in oxidation state and (iii) ligand(s) to which the element may be chemically bound. Some general relationships between the fundamental chemical properties of different elements and their respective biological effects may be illustrated by comparing aluminium and mercury. These two metals are on the extreme ends of the scale from class A metals, which largely bind to oxygen-containing ligands (both in the environment and in biological systems), to class B metals, which seek sulphur- and nitrogen-containing ligands (Nieboer and Richardson, 1980). Mercury, being the archetypal class B metal, binds strongly to the ubiquitous sulfydryl groups on proteins and enzymes, whereas sulfydryl groups are totally irrelevant as targets for aluminium toxicity. Further, while variation in oxidation state is a major determinant of the biological properties of mercury (0 in metallic, gaseous mercury, +1 or +2 in inorganic salts and +2 in methylmercury), +3 is the only biologically relevant oxidation state for aluminium. For aluminium, on the other hand, many different potential ligands are available in the environment and in biological systems, and sorting out which ligands are relevant under different conditions has proved to be a very difficult task.

The speciation of an element may change several times from its source, through cycling in the environment and on its way through different compartments of the organism to the final target site. For example, acid precipitation (Section 5.6) leads to mobilization of aluminium from soils and bedrock (Driscoll and Postek, 1996). In the subsequent transport of dissolved aluminium through soil water, groundwater, rivers and lakes, aluminium may change ligands several times, depending on the chemistry in the different bodies of water. Relevant ligands in natural waters include OH^-, F^-, H_4SiO_4, HSO_4^- and a host of naturally occurring organic compounds. After uptake from the gastrointestinal tract, aluminium probably largely follows the iron pathway in being bound to transferrin in blood and extracellularly, while the slightly more acid intracellular environment favours binding to phosphate-containing ligands, such as inositol or membrane phosphate groups (Birchall and Chappell, 1988).

The chemical reactions taking place in water (including the aquatic phase of soils) are commonly categorized in the following types: acid–base reactions; oxidation–reduction reactions (equilibria depending largely on the availability of oxygen in the soil–water system); dissolution–precipitation reactions; complexation reactions and ion exchange–adsorption reactions (Stumm and Morgan, 1996). The overall balance of all such reactions determines how a particular substance distributes between the solid and aqueous phases in nature, and hence its mobility, and ultimately its availability and potential toxicity to humans and other organisms.

When toxic substances in soil and water are discussed, the focus is usually on anthropogenic pollutants, as in this chapter. However, one should not forget that the natural environment in many cases contains harmful concentrations of various compounds. For example, in a study conducted in pristine areas of Norway, where the mineral galena (lead sulfide) occurs in the bedrock, Låg and Bølviken (1974) reported lead concentrations (dry matter) of up to 10% in soil, 0.4% in moss and 0.04% in grass. They concluded that natural metal poisoning of soil and vegetation is more common than previously thought, and that it seems possible that in such areas the local herbivores may accumulate metals. In the Tantramar swamp in New Brunswick and Nova Scotia, Canada, Dykeman and de Sousa (1966) reported copper concentrations up to 7% dry weight in surface peat that was filtering naturally copper-rich spring water (up to 1 mg l^{-1} Cu) emerging into a marsh. These two examples concern metals, but the natural environment also contains many substances that until recently were believed to have exclusively anthropogenic sources. Notably, a large number of chlorinated and other halogenated organic compounds are produced naturally by plants, marine organisms, insects, bacteria, fungi and mammals, and by other natural processes such as volcanoes and forest fires. The number of known natural organohalogens has passed 2000 and is increasing rapidly (Gribble, 1996). Naturally produced halogenated compounds include chlorinated phenols, chloroform and even such compounds as dioxins (Ferrario *et al*., 2000) and chlorofluorocarbons (Frische *et al*., 2006). Plants and animals secrete such chemicals mainly as part of their chemical defence—as feeding deterrents, irritants or pesticides—and some of these compounds may find clinical use as antibiotics or antitumour agents.

We have chosen to devote more space to metals than to organic compounds. Among the metals, particular attention is given to cadmium, not because it is that more important than, for example, lead or mercury as an environmental health hazard, but because it serves well to illustrate different principles and mechanisms in soil- and water-related environmental toxicology.

The main emphasis in this chapter is on relevance to human health. There are numerous possible pathways of potentially toxic compounds from soil–water systems to humans. In some cases, humans are directly exposed, through airborne dust or through hand-to-mouth contact, which is particularly relevant for children. However, the transfer of toxic substances from soils to humans is usually indirect, through uptake in food plants, through further transfer up food chains (e.g. to fish and game) and

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through leaching into water and intake through drinking water.

Because it constitutes an important pathway from soil via fresh waters to humans, drinking water is treated in some detail, both under different headings in Section 5 and in Section 6 on drinking water. We have also chosen to give considerable attention to the possible human health effects of acid precipitation (Section 5.6). Ecotoxicology, that is, the branch of toxicology that studies the toxic effects of substances on nonhuman organisms that constitute the biosphere (Hoffman *et al*., 2003), is treated in other chapters in this book.

2 GENERAL DESCRIPTION OF THE NATURE AND PROPERTIES OF SOILS

The Earth's bedrock is almost universally covered by unconsolidated material known as *regolith*, the thickness of which may vary from negligible to hundreds of meters. *Soil* is usually defined as the upper layer of the regolith influenced by processes governed by climate and by living organisms. This layer, typically around 1 m thick, is where plants grow. It is therefore the basis of all higher forms of life on land, and differs from the underlying material in several respects. It is higher in organic matter, since plant residues deposited originally on the surface have been transformed by soil animals and decomposed by micro-organisms. Being near the surface, the soil has been subject to more intense weathering than the deeper layers of the regolith. The main factors determining the properties of the soil (termed *soil-forming factors*) are climate (particularly temperature and precipitation), the living organisms, the surface topography, the time period for soil formation and the properties of the mineral material (termed the *soil parent material*) where the soil formation takes place. For a standard textbook on soil and soil properties, see Brady (1990); special attention to soil chemistry is given by Sposito (1989), and McBride (1994) gives a treatment emphasizing the environmental chemical aspects of soils.

2.1 Soil Components

Soil consists of four major components: mineral material, organic matter, water and air. Water and air occupy the pore space in between solid particles of mineral and organic material. A typical volume composition of a fertile agricultural soil may be as follows: mineral, around 50%; air, 20–30%; water, 20–30% and organic, around 5%. In undisturbed natural soils, accumulation of organic matter to a much higher fraction often occurs in the surface layer, especially in temperate regions. Because the soil organic matter has a high binding capacity for many inorganic and organic chemical compounds, such *humus* layers may accumulate toxic substances added to the soil surface. The free soil water, often named *soil solution*, is very important as the medium for plant root uptake of nutrients and other substances, for the release from and fixation to the soil solid phase, for chemical reactions in soils in general, and for vertical and horizontal transport of dissolved components. This vertical transport in the soil solution is essential for the development of soil horizons and soil profiles (see below).

2.2 Soil Horizons and Soil Profiles

Soils are subject to *weathering*. Products of this weathering, especially if they have been moved vertically, give rise to characteristic layering. A vertical section of a soil, called a *profile*, consists of more or less distinct horizontal layers termed *horizons*. The properties of these horizons greatly influence the growth of higher plants. Conversely, plant growth is a main driving force behind the development of soil horizons, owing to the properties of the organic matter formed from decaying plant residues. Soil horizons with high contents of organic matter are generally dark in colour, and the organic acids formed during decomposition greatly enhance weathering of mineral matter. The different horizons in a soil profile may have very different chemical and physical properties (e.g. different pH values), so a contaminant introduced into a soil system may be strongly enriched in one of the horizons.

The soil-profile development forms the basis of soil classification. Soils are very heterogeneous, and different systems for soil classification are used in different countries and by different organizations. A detailed discussion of soil classification is beyond the scope of this chapter (see Brady, 1990).

2.3 Soil Texture

One of the factors that is important for the soil as a growth medium for plants and for the chemical reactions in the soil is the *texture* (size distribution of soil particles). The main classes are, in the direction of finer particles: gravel, sand, silt and clay. The clay fraction (particle diameter <0.002 mm) is especially important in this respect. The clay particles have a higher surface-to-mass ratio than the coarser particles, and hence a higher capacity to bind ions and molecules. Soils with high clay contents also have a greater water-holding capacity than more sandy soils, and are hence more resistant to drought.

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2.4 Mineralogical and Chemical Composition of Soils

The mineralogical composition of a newly formed soil depends on the mineral composition of the parent rocks. Young soils contain appreciable amounts of unchanged primary minerals from the parent rocks, such as various feldspars (aluminosilicates), micas (iron and aluminium silicates) and quartz (SiO_2). In the course of time the primary minerals are subject to weathering reactions, whereby they are transformed to secondary minerals (layered silicate clays) and lose most of their original content of alkali and alkaline earth elements. In very old soils, such as found in large parts of the tropical zone, the further weathering reactions have largely transformed the mineral material into oxides and hydroxides of aluminium and iron.

The elemental composition of a soil thus depends on both the character of the parent material and its weathering state. In addition, some elements may be added to the surface soil in significant amounts from the atmosphere, by natural cycling processes. In coastal areas surface soils may be enriched with elements supplied from the marine environment such as the major sea-salt cations sodium and magnesium (Låg, 1968), the halogens chlorine, bromine and iodine (Låg and Steinnes, 1976) and even the essential trace element selenium (Låg and Steinnes, 1974). This is mainly due to spray formation on the sea surface by the action of strong wind and subsequent atmospheric transfer of sea-salt aerosols to land areas. In the case of iodine (Lovelock *et al*., 1973) and selenium (Cooke and Bruland, 1987) the cycling is probably biologically driven: volatile organic compounds, for example, CH_3I and $(CH_3)_2Se$, are produced by marine organisms and released to the atmosphere, where such compounds are eventually transformed to inorganic forms by photochemical processes and transported on to land with sea-salt aerosols.

The content of a particular element in soil may vary naturally over several orders of magnitude, and may reach toxic concentrations even in cases where human influence is not involved. Thus, the concept of natural background concentrations should be used with care. All too often, the presence of high concentrations of metals or other substances in an environmental medium is interpreted as the result of anthropogenic pollution, when the explanation may be natural variations in concentrations. In **Table 1** mean concentrations in the Earth's continental crust and typical levels in soils are shown for some metals of concern in human toxicology.

Table 1 Concentration levels (mg kg^{-1}) of some metals in the Earth's continental crust (Wedepohl, 1995) and in soils (Bowen, 1979)

Metal	Mean crust	Soil	
		Median	Range
As	1.7	6	0.1–40
Cd	0.100	0.35	0.01–2
Cr	126	70	5–1500
Cu	25	30	2–250
Hg	0.040	0.06	0.01–0.5
Ni	56	50	2–750
Pb	14.8	12[a]	2–300
Zn	65	90	1–900

[a]Prior to human contamination.

2.5 Soil Organic Matter

The organic matter in soil forms an important part of the total soil system. It is formed by decomposition of plant litter and dead soil organisms, and consists of a very complex mixture of chemical compounds, from easily degradable materials such as carbohydrates and proteins to humic substances, which are secondary products very resistant to further degradation and thus form the most stable component of the soil organic matter. During the degradation, organically bound nitrogen and phosphorus are gradually transformed into inorganic forms that are reused as plant nutrients. Humic substances have a high capacity to attract cations because of the negative charges formed by dissociation of protons from acidic groups on the humus surface. These negatively charged sites may form strong, chelate-type bonds with metals such as copper, lead and mercury.

3 MATERIAL BINDING, CYCLING AND TRANSFORMATION IN SOIL

The chemical reactions and transformations that take place in soils occur in the soil solution and on the interface between the solution and soil particles. The nature and extent of these reactions depend largely on the conditions in the solution phase (pH, redox level, concentration and type of dissolved organic matter, etc.) and also on the properties of particle surfaces, which govern the distribution of ions and molecules between the solution and the solid phase. Moreover, the catalytic action of micro-organisms is essential for almost all redox reactions occurring in the soil, including the breakdown of natural and anthropogenic organic substances. Since a majority of chemical reactions occurring in soil are strongly pH dependent, the pH of the soil is a particularly important factor. The pH level is also of fundamental importance for plants and soil biota.

Chemical substances may be bound to the surface of soil particles by various mechanisms (McBride, 1994) representing a wide range of binding strengths. The types of bonds involved will largely dictate the mobility,

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bioavailability and toxicity of different substances. Ionic species in the soil solution tend to be electrostatically attached to opposite charges on the solid surface, in which case they may be easily exchanged with another ion of the same charge. In some cases stronger chemical bonds are also involved: metals such as copper, lead and mercury may be complexed by ligands on humus surfaces, in particular carboxylic acid groups. Metals bound in this way may be quite immobile in the soil, and thus hardly available for uptake into plants. The potential toxicity of the metal is therefore greatly reduced by such binding. For example, in the Tantramar copper swamp in Canada, the peaty soil, containing up to 7% copper, supports a rich growth of trees and ground-cover species typical of wet areas in the region (Dykeman and de Sousa, 1966). The reason is probably that the copper is immobilized and therefore 'detoxified' by chelation by the humic compounds in the peat. On the other hand, such strong binding may cause accumulation in the humus-rich top layers of soils to high concentrations of substances steadily supplied to the soil (see Section 5.5). Metal cations may also be complexed by humic substances dissolved in the soil solution. These low-molecular-mass compounds (fulvic acids) may thus solubilize metals from the soil solid phase. Anions such as fluoride and phosphate may be bound to mineral surfaces by so-called ligand exchange, that is, by displacement of OH^- or H_2O from coordination positions at the particle surface. Ligand exchange is favoured at low pH, whereas cation complexing is more efficient at higher pH values. This is because at low pH there are few OH^- ions in the soil solution to compete with the surface-bound anions, and at high pH there is little H^+ in the solution to compete with the metal cations. For substances that are weak bases (e.g. triazine herbicides) or weak acids, variations in binding strength as a function of pH may be difficult to predict, but binding is usually strongest at pH values in the vicinity of the pK value of the acid or base.

Organic molecules can also be sorbed to soils to a considerable degree (Nicholls, 1991). For organic ions (e.g. the cationic herbicides diquat and paraquat) the mechanisms are similar to those described above for inorganic cations and anions. However, uncharged organic molecules also show some sorption on soils, but in this case the binding mechanisms are less well characterized. In many cases, hydrogen bonds and nonspecific van der Waals bonds, which are both weak relative to ionic and covalent bonds, are probably involved. In addition, dissolution of uncharged, hydrophobic substances in water is not an energetically favourable process, because the hydrogen bonds between individual water molecules must be disrupted in the process. As a result, such substances tend to reduce contact with water and seek relatively nonpolar environments, such as soil organic matter. Hence the capacity of the soil to bind electrically neutral organic compounds increases with increasing organic matter content. In general, nonpolar substances, such as polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs), tend to be more strongly sorbed on humus than on mineral surfaces (Mackay, 2001; Ockenden *et al*., 2003).

Clay (particle diameter < 0.002 mm) is the most important size fraction for chemical reactions in soils because of its large specific surface area. The smaller the particle, the larger is its area per mass unit: The dispersed colloidal phases in natural waters may have specific surface areas as high as hundreds of $m^2\ g^{-1}$ (Stumm and Morgan, 1996). The clay fraction in soils mainly consists of three particle categories: silicate clay, oxide and humus. Clay minerals and humus generally exhibit an excess negative surface charge, which may attract cations. The surface charge of oxide minerals, on the other hand, may be predominantly positive or negative depending on pH in the soil solution. Soils thus have a high capacity to store cations, in particular if the clay content or organic matter content is high. On the other hand, sandy soils have a much smaller capacity to bind both metal cations and organic compounds.

Plant root uptake of chemical substances from the soil solution is normally restricted to ionic compounds, predominantly inorganic ions, but is not limited to ions that are essential nutrients to the plant. Some toxic metals can also be absorbed quite effectively by the plant roots and transferred into plants used for human consumption. For example, cadmium can be readily taken up from the soil by plant roots, perhaps because it is chemically similar to the essential plant nutrient zinc. Some metals may therefore be a concern as contaminants in food crops, in addition to their possible contamination of groundwater after leaching from the soil. The plant uptake of a particular element depends on its forms of occurrence (speciation) in the soil, and numerous chemical extraction procedures have been proposed to 'mimic' the fraction of one or more elements available to plant uptake (Hlavay *et al*., 2004). Extractants used are typically buffered or nonbuffered salt solutions, weak acids and solutions of strong complexing agents such as EDTA (ethylenediaminetetraacetic acid). A particularly large amount of work has been performed in relation to cadmium added to agricultural soils as an impurity in phosphate fertilizers (Singh and Steinnes, 1994). Even in this case, however, no single extraction procedure has been found that adequately predicts the plant-available fraction of cadmium in different crops and soil types. Lead and mercury, on the other hand, are in most cases rather unlikely to be taken up by plant roots and transferred to the green parts of the plant.

For elements where the uptake in plants is high relative to the amount present in the soil, a major fraction of the total amount of the element present in the soil–plant system may be continuously and rapidly circulated with uptake in plants and subsequent return to the soil surface layers when the plants die or shed their leaves, and this

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organic material is broken down and the element released to the soil solution.

Fungi possess extensive underground networks of mycelia that act as an extremely efficient system for extracting metals from the soil (Lepp, 1992; Berthelsen *et al*., 1995a). Mushrooms therefore often contain high concentrations of one or more toxic metals, but large differences exist between different species concerning which elements they seem to prefer (Lepp, 1992). Individuals with high consumption of wild mushrooms may therefore considerably increase their intake of one or more toxic elements.

Most organic soil contaminants are not likely to be much absorbed by plant roots (Ryan *et al*., 1988), so regarding human exposure, the main concern about such organic compounds is not intake via plant foods, but possible groundwater contamination. Unlike metals, they are subject to degradation in the environment. In general, the types of processes that may reduce the concentrations of organic substances in surface soils are: (i) volatilization from the soil to air; (ii) leaching from surface soil to groundwater; (iii) biodegradation; (iv) photolysis and chemical reactions; (v) physical transport processes and (vi) apparent loss through the formation of irreversibly bound, nonextractable residues (Alcock and Jones, 1996). The most persistent organic contaminants, however, such as PCBs (Meijer *et al*., 2002), dioxins (Hassanin *et al*., 2005) and PAHs (Pichler *et al*., 1996) may stay in the soil for decades, and thus represent a potential hazard, although in the case of PAHs the evidence has been subject to discussion (Koivula *et al*., 2004).

4 MATERIAL TRANSPORT BETWEEN SOIL AND WATER

When assessing the risks that toxic compounds in soil or water pose to humans, it is essential to know how the compounds are transported between soil, water and other parts of the environment, notably the atmosphere. For example, a volatile substance with low water solubility will tend to escape from water into the atmosphere. Substances with a high positive electric charge will generally bind strongly to negatively charged colloids in soils and thus be effectively immobilized.

The chemical composition of natural water is affected by several geological, atmospherical and biological factors (Bricker and Jones, 1995). Two physical factors are particularly important in determining the chemical composition of natural water; the residence time and the pathways along which the water moves in the soil–water system. The longer the residence time, the better is the opportunity for chemical reactions between the water and the materials in contact with it. The pathways determine which materials the water comes into contact with during its passage through the system; water following shallow pathways generally has a shorter residence time and comes in contact with more weathered and consequently less reactive minerals than water following deeper pathways.

Regarding trace elements in natural water, it is very important to realize that older concentration data are often erroneously high, owing to sample contamination and inadequate analytical quality control. As a consequence of clean techniques used during sampling and analysis, the concentrations of many trace elements in natural water have been found to be orders of magnitude lower than previously thought (Stumm and Morgan, 1996, Chapter 10.2).

Of the total amount of water on Earth, only about 2.5% is fresh water. Three quarters of this fresh water is locked up in ice and one quarter is groundwater, most of which is not readily available for human use. Rivers, streams and lakes together constitute less than 0.01% of the water on the planet (Berner and Berner, 1987), but this fraction is by far the most important for mankind. Water in nature is continuously in motion through the *hydrological cycle* (Berner and Berner, 1987). The cycle is driven by energy from the sun and by gravity. Only a tiny fraction of the total amount of water on Earth circulates through the cycle each year. As water moves through the hydrological cycle, constituents may be added or removed. Examples of processes adding constituents to water are mineral dissolution in soils, pollution and uptake of gases from the atmosphere. Several of the steps in the hydrological cycle may effectively remove constituents from the water, notably during the evaporation step, during percolation in soils where micro-organisms degrade organic compounds and where constituents of the water are sorbed on particles, and when water is flushed out of the system in question. Collectively, such processes are termed *self-purification* of water. The term self-purification may also be applied to soil. Self-purification of soil is generally much slower than for water systems.

5 POLLUTION OF SOIL AND WATER SYSTEMS

5.1 Introduction

For most trace elements, human activities have a major impact on both regional and global cycles. At least for arsenic, cadmium, copper, nickel, lead, vanadium and zinc, global anthropogenic emissions to the atmosphere by far exceed natural emissions (Nriagu and Pacyna, 1988). Furthermore, for most of the trace metals, the annual anthropogenic input to water exceeds the quantities emitted to the atmosphere, and direct discharges

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from human activities to soil are even higher (Nriagu and Pacyna, 1988). Thus, mankind has a major influence on the global biogeochemical cycling of many trace elements. This has resulted in considerable increases in the circulation of several toxic metals through the soil, water and air, inevitably resulting in their transfer to human food chains and increased human exposure.

The global nature of the anthropogenic influence is perhaps best seen in studies of successively dated snow and ice layers in Antarctica and Greenland, which show that, for example, lead concentrations in snowfall over Greenland increased 200-fold from pre-industrial times to the 1970s (Boutron *et al*., 1994). Of course, not only metals, but also organochlorine compounds and other anthropogenic organic contaminants, have also become widespread in the environment, sometimes on a global scale (Simonich and Hites, 1995; Gambaro *et al*., 2005).

Fortunately, however, the common view that pollution of the environment is ever on the increase is not correct in all instances. Recent decades have witnessed large efforts to reduce anthropogenic releases of many priority hazardous substances to air, water and soil. As a result, sometimes dramatic improvements of the environment have occurred, for example in some major rivers such as the Thames and the Rhine (Zehnder, 1993). In the Rhine, water concentrations of lead and cadmium have been reduced by more than 90% since the 1970s (Zehnder, 1993). In Greenland snow, the concentration of lead has decreased by a factor of 7.5 and those of cadmium and zinc by a factor of 2.5 from 1970 to 1990 (Boutron *et al*., 1991). The atmospheric deposition of lead in southern Norway, mainly by transboundary pollution from other parts of Europe, has been reduced almost 20-fold over the last 30 years (Steinnes *et al*., 2007), but most of the previously supplied lead still resides in the surface soil. For dioxins, there are clear trends in many countries and in many environmental compartments that the concentrations have been greatly reduced in recent years, resulting also in a reduced human intake of dioxin (Alcock and Jones, 1996). However, the concentrations of dioxins in soils have not declined as rapidly as in other media (Alcock and Jones, 1996), because dioxins are strongly bound and not easily degraded in soils. The bulk of the environmental burden of dioxins resides in soils (Harrad and Jones, 1992; Hassanin *et al*., 2005). Thus, although the anthropogenic release of many pollutants has been reduced in recent years, deposits of those compounds that are not easily degradable, especially in soils and sediments, will continue to affect ecosystems and humans for a considerable time.

The efforts to reduce emissions of toxic and environmentally harmful compounds are most evident in the developed countries. It is of utmost importance that economic progress in the developing world does not entail the same degree of emissions that characterized the now developed countries when they were at the same stage. Such emissions in the developing world have the potential globally to more than offset the reductions attained in the developed part of the world.

With the efforts to reduce emissions of priority hazardous substances, large point sources have become of less quantitative importance than nonpoint, diffuse sources (except in the vicinity of the point sources). Now, diffuse sources, often related to everyday use and subsequent disposal of ordinary consumer products, dominate for many pollutants delivered to the air, water and soil environment. In many cases, transport through air is very important. This transport may be effective over very long distances, in some cases even globally. The potential for environmental contamination through long-range transport may be illustrated by the concentrations of lead and cadmium in natural surface soils of southern Norway, which are 10-fold higher than the natural levels, owing to long-range atmospheric transport from other parts of Europe (Steinnes *et al*., 1989, cf. Section 5.5).

In addition to direct introduction of toxicants into the environment, human activity may mobilize naturally occurring toxic compounds, such as aluminium and arsenic. Aluminium is the most common metal in the Earth's crust, and is solubilized from soils following deposition of acid precipitation (Driscoll and Postek, 1996, cf. Section 5.6). In deep groundwater wells in Bangladesh and India, arsenic is solubilized from arsenic-containing minerals in the bedrock (Smedley and Kinniburgh, 2005, cf. Section 6.4).

Soil salinization, which is a large problem globally, also potentially increases human exposure to several elements. Soil salinization occurs largely as a result of irrigation in agriculture: as water evaporates or is taken up by plants, the salts originally present in the irrigation water may build up in the soil, and the resulting high concentrations of salts in the soil may limit crop production (Pritchard and Amthor, 2005). As the salt contents of the soil increases, certain elements may be taken up by crops and enter the food chain.

5.2 Pesticides and Fertilizers

Pesticides have received particular attention in environmental toxicology. This is not surprising in view of the fact that pesticides are introduced into the environment deliberately to eradicate or harm certain forms of life (the word 'pesticide' means a substance that can kill any organism considered by humans to be a 'pest'). If pesticides were specifically toxic to the organisms they were intended to harm, they might not represent as much of a threat. However, most pesticides are toxic to many different types of organisms, including humans, so pesticide application to soils or crops often represents a potential threat to the health of humans or to the environment. Different pesticides have very different binding

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properties and mobilities in soil–water systems. Pesticides will not be discussed further in this chapter (see **Toxicology of Pesticides**).

Fertilizers are added to soil in order to increase crop production. Whether the fertilizer is 'synthetic' (a mixture of specific chemical compounds) or 'natural' (animal manure, etc.), its application may cause contamination of soils and waters, either from the main constituents (notably phosphorus and nitrate leaching to receiving waters; health effects of nitrate in drinking water is discussed in Section 6.5), or from impurities. The most important such impurity is cadmium, which is present naturally, sometimes in high concentrations, in the sedimentary phosphate rocks which are used as raw materials for phosphate production. In addition to cadmium, arsenic, copper and zinc may be present in high enough concentrations in certain fertilizers to cause concern (Singh and Steinnes, 1994). Repeated use of high-metal fertilizers may cause accumulation of these metals in the soil, with the possibility of transfer to the human food chain. Cadmium is of particular concern in this respect, because the mean population intake is uncomfortably close to the health effect threshold (Buchet *et al*., 1990; WHO, 1992; Järup *et al*., 1998; Nordberg *et al*., 2007), because plant food is the main source of cadmium in the human diet (WHO, 1992), and because fertilizer (and sewage sludge, see below) applications in many cases have been shown to considerably increase the cadmium content of agricultural soils (Page *et al*., 1981). For example, Mulla *et al*. (1980) reported a 14-fold increase in the cadmium content of cultivated surface soil after 36 years of application of triple superphosphate.

Sewage sludge is a complex mixture of organic and inorganic compounds that are precipitated from wastewater during mechanical, biological and chemical treatment. Municipal sewage sludge is commonly used as a soil fertilizer owing to its high contents of organic matter, phosphorus and nitrogen. This use of the sludge also solves a large disposal problem. However, sewage sludge may contain a wide variety of undesired substances originating from households, from commercial buildings and from industries whose wastewater systems are connected to the municipal piping system. Repeated application of sewage sludge may cause the accumulation of such substances in the soil, uptake in food crops grown in the soil or contamination of groundwater. The most problematic chemical substances are metals, notably cadmium, but also lead, mercury, nickel, copper, zinc and chromium (Alloway and Jackson, 1991; Singh and Steinnes, 1994). There are large variations in metal concentrations in sludges between treatment plants and also between countries, probably reflecting different industrial inputs to the wastewater systems (Singh and Steinnes, 1994). In many countries, control measures have reduced the contents of cadmium and other metals in sewage sludge, and limit values have been set in the European Union (**Table 2**) and elsewhere. Thus, both phosphate fertilizers and atmospheric deposition (Section 5.5) are currently more important sources for cadmium input to agricultural soils than sewage sludge (WHO, 1992).

Various organic contaminants in sewage sludge, such as solvents, PAHs, PCBs and dioxins, may also be present in levels high enough to be of concern (Harrison *et al*., 2006). Sewage sludge may also contain pathogenic micro-organisms, which may survive in soil or water from days (bacteria) to months (viruses) to years (helminth eggs), depending on the environmental conditions (Straub *et al*., 1993). Such micro-organisms in sewage sludge thus represent a potential hazard for communicable disease transmission through contamination of groundwater.

5.3 Waste Disposal Sites

Hazardous waste disposal sites represent a large problem in the industrialized world. Soil and groundwater around such sites may become contaminated by a plethora of chemical substances, for example, metals and solvents such as di-, tri- and tetrachloroethylene and carbon tetrachloride. A related problem is leakage of petroleum components from the hundreds of thousands of underground storage tanks, notably at fuel stations. It is very difficult to ascertain the possible health effects that may result from potential human exposure to components mobilized from such waste disposal sites. Most such sites

Table 2 The European Economic Community limit values for metals when sewage sludge is used in agriculture

Metal	Cd	Cu	Hg	Ni	Pb	Zn
Soil concentrations[a]	1–3	50–140	1–1.5	30–75	50–300	150–300
Sludge concentrations[b]	20–40	1000–1750	16–25	300–400	750–1200	2500–4000
Amounts added per year[c]	0.15	12	0.1	3	15	30

[a]Limit values for concentrations of metals (mg kg^{-1} dry matter) in soils (if one or more of the metals in the soil exceeds these limit values, the use of sludge is prohibited).
[b]Limit values for concentrations of metals (mg kg^{-1} dry matter) in the sludge.
[c]Limit values for amounts of metals (kg ha^{-1} yr^{-1}) which may be added annually to agricultural land, based on a 10 year average (EEC, 2008)

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contain many different potentially toxic compounds, and producing high-quality exposure data retrospectively is usually not possible. In addition, epidemiological studies usually lack the power to definitely establish that there is no health effect, owing to the small numbers of exposed people.

The best known example of potential health problems from a waste disposal site is probably Love Canal, Niagara Falls, USA (Hoffman, 1995). Here, the Hooker Electro-Chemical Company built a residential neighbourhood on top of a site where some 22 000 tons of toxic chemical waste were buried. This waste included more than 6000 tons of the pesticide lindane, 2000 tons of chlorobenzenes and 200 tons of dioxin-contaminated trichlorophenol. Gradually, studies showed that the residential environment was heavily contaminated, and in 1978, President Jimmy Carter declared the first federal emergency for a non-natural environmental disaster in the USA. The 239 homes most seriously affected were condemned, and after nearly 10 years of very expensive clean-up efforts, the New York Department of Health issued a declaration of habitability, and a programme to redevelop the neighbourhood and move families back was started. The studies of possible health effects among the inhabitants of Love Canal were inconclusive (Hoffman, 1995).

The anaerobic waste disposal sites used so far may be replaced in the future by aerated sites, where the degradation of organic matter will go faster and leave less unhealthy products. Aeration is currently being used to stabilize existing landfills (Rich *et al*., 2008).

Technologies for cleaning contaminated soils, notably through bioremediation (Singh and Tripathi, 2007), is a rapidly developing field with some spectacular innovative efforts. Bioremediation provides technologies based on the use of living organisms, usually bacteria and fungi, to remove pollutants from soil and water, preferably *in situ*. This approach is usually more cost-effective than more traditional techniques such as incineration of soil. Bioremediation is becoming the technology of choice for the remediation of many contaminated environments, and has been shown effective for cleaning soil from a wide range of compounds including metals, petroleum hydrocarbons, PAHs, and PCBs. When using higher plants capable of hyperaccumulating metals and other substances, the term phytoremediation is often used (Audet and Charest, 2007).

5.4 Industry and Mining

A comprehensive and detailed discussion of possible impacts of mining and industrial activities on soil and water composition is beyond the scope of this chapter. Particularly, metal mining and smelting are very important sources of metal contamination of soils, both on a local and on a regional scale (Davies, 1983; Dudka and Adriano, 1997). Closed mines continue to contaminate their surroundings, mainly owing to large heaps of tailings, which are finely ground particles of ore and rock left behind as a waste product. Studies of the environmental and health impacts of historic mining sites have been conducted, especially in the UK. For example, one study shows that 100 years after the closure of two mines, large amounts of cadmium, lead, zinc and copper were still entering the surrounding soil, water and plant systems (Merrington and Alloway, 1994). Elevated concentrations of metals in plants growing on soils in the vicinity of metal smelters have been extensively documented (Davies, 1983). Thus, the potential for increased human exposure to metals through the food chain clearly exists in the vicinity of such sites.

5.4.1 Cadmium

Together with mercury and lead, cadmium has been the metal causing most concern in environmental toxicology. In contrast to mercury, where fish clearly stands out as the one main exposure source of concern, it is difficult to pinpoint even a few important sources of cadmium exposure for the general population. Cadmium came into focus as an environmental toxicant shortly after World War II, when a mysterious bone disease was found to be endemic, particularly among elderly women who had borne many children, in the basin of the Jinzu river in west-central Japan (Tsuchiya, 1978). The disease was named Itai-itai (ouch-ouch) because of the severe pain it caused in the muscles and bones of victims. After extensive epidemiological and other studies it has come to be accepted that the Itai-itai syndrome was due to long-term cadmium exposure. The cadmium was emitted into the Jinzu river from a zinc–lead–cadmium smelter upstream of rice fields, resulting in high concentrations of cadmium in rice. The characteristic skeletal changes found in the older women are not usually observed in occupational cadmium poisoning. However, Itai-itai disease was always accompanied by the classical proteinuria typical of cadmium poisoning (see below).

The Itai-itai incident is unique in that in no other nonoccupationally exposed population has the intake of cadmium been nearly as high. However, there are also indications of subtle clinical effects associated with lower-level intake in other populations after soil contamination with cadmium. The critical organ (that is, the organ where the first adverse effects occur with increasing exposure of a population) for long-term low-level cadmium toxicity is the kidney. Tubular proteinuria (increased excretion of low-molecular-mass proteins), commonly diagnosed by increased urinary β_2-microglobulin, is the earliest sign of presymptomatic cadmium-related kidney damage (WHO, 1992; Nordberg *et al*., 2007). Early renal damage seems to occur at considerably lower exposure levels than formerly

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believed (Järup *et al*., 1998; Nordberg *et al*., 2007). In the Belgian Cadmibel study (Belgium is the main producer of cadmium in Europe, with widespread environmental contamination by the metal), there was a 10% probability (in nonsmokers) of elevated urinary excretion of β_2-microglobulin, retinol-binding protein, *N*-acetyl-β-glucosaminidase, amino acids and calcium, when cadmium excretion exceeded 2–4 $\mu g\,day^{-1}$ (Buchet *et al*., 1990). The generally accepted critical level of cadmium in the renal cortex is $200\,mg\,kg^{-1}$, corresponding to a urinary excretion of about $10\,\mu g\,day^{-1}$ (WHO, 1992). However, initial signs of proteinuria seem to be present in a small percentage of people with renal cortex levels as low as $50\,mg\,kg^{-1}$ (Järup *et al*., 1998).

Soil contamination of cadmium is common around many nonferrous metal mines and smelters, particularly those processing zinc ores. Owing to the close geochemical similarity between zinc and cadmium, zinc ores always contain appreciable amounts of cadmium, and zinc mining and smelting are important sources of cadmium emissions to the environment. In the village of Shipham in the UK, which was the site of a large zinc mine between 1650 and 1850, much of the soil is severely contaminated by cadmium from old mining slag heaps. These heaps were levelled and developed for agriculture and housing, and the cadmium concentrations are some of the highest reported, up to $750\,mg\,kg^{-1}$ in surface agricultural soil, and a median value of $91\,mg\,kg^{-1}$ in surface garden soil (Thornton, 1988). Cadmium levels in locally grown vegetables in Shipham were reported to be typically 5–20 times normal values (Sherlock *et al*., 1983). For example, the mean cadmium levels (fresh weight) were $680\,\mu g\,kg^{-1}$ in lettuce and $790\,\mu g\,kg^{-1}$ in spinach. Different dietary studies showed mean daily intakes of cadmium of 29–36 $\mu g\,day^{-1}$, of which 14–17 $\mu g\,day^{-1}$ came from locally grown vegetables. Four people had cadmium intakes above $400\,\mu g\,day^{-1}$.

The intake of cadmium in Shipham was higher than average for the UK, but not much so, suggesting that the majority of the inhabitants did not consume high amounts of the heavily contaminated locally grown vegetables. The mean urinary excretion of β_2-microglobulin, an early indicator of kidney damage (see above), was higher in Shipham residents ($77\,\mu g\,day^{-1}$) than in a control population ($55\,\mu g\,day^{-1}$), but apart from this, no adverse health effects that could be attributed to cadmium were found (Strethlow and Barltrop, 1988). Furthermore, the concentrations of cadmium in soil, house dust and crops were not significantly correlated with urinary cadmium and β_2-microglobulin.

On the other hand, a study in Belgium found a strong correlation ($r = 0.76$) between soil cadmium and cadmium in residents' urine for 703 randomly selected residents in 10 districts, six of which where close to zinc smelters (Staessen *et al*., 1994). Residents in the six polluted districts had slightly higher urinary cadmium (1.18 vs. $0.89\,\mu g\,day^{-1}$), serum creatinine (100 vs. $97\,\mu mol\,l^{-1}$) and urinary excretions of β_2-microglobulin (109 vs. $95\,\mu g\,day^{-1}$), retinol-binding protein (136 vs. $118\,\mu g\,day^{-1}$) and *N*-acetyl-β-glucosaminidase (1.78 vs 1.38 U day^{-1}). Locally grown vegetables, such as celery (2.43 vs. $0.68\,mg\,kg^{-1}$) and beans (0.42 vs. $0.15\,mg\,kg^{-1}$), also contained more cadmium in the polluted districts.

Cadmium is discussed further in Section 5.6.1.

5.5 Atmospheric Deposition

It has been evident for a long time that in the immediate vicinity of a point source, severe pollution of soil and water can result from transport of contaminants through the air and subsequent ground deposition. It is only in recent decades, however, that it has been realized that even in areas very far from large sources of pollution, considerable contamination of soil and water systems may occur after long-range atmospheric transport. Even in the polar regions, studies of drilled ice cores clearly document that the deposition of metals and other compounds has increased gradually through the centuries (Boutron *et al*., 1994). Extensive studies in Norway have demonstrated the regional diffuse contamination of soil, water and vegetation resulting from long-range atmospheric transport from source regions further south in Europe, most of which are more than 1000 km away (Steinnes, 1989; Steinnes and Henriksen, 1993; Berthelsen *et al*., 1995b; Steinnes *et al*., 1997; 2007). In little-disturbed natural soils in the southernmost part of the country, the concentrations of lead, cadmium and arsenic in the humus layer are consistently many-fold higher than in corresponding soils from central and northern Norway, and the correspondence with the geographical atmospheric deposition patterns for these elements is remarkable (Steinnes *et al*., 1997, cf. **Figure 1**). The elevated concentrations were mainly found in the humus layer, that is, the uppermost few centimetres of the soil; deeper soil layers seem little affected by the atmospheric deposition. This is probably due to strong complexing of the metals by humic substances (cf. Sections 2.5 and 3). These metals may be mobilized, for example, by acid deposition (Section 5.6), but the potential for mobilization varies greatly among different metals. It is noteworthy that in large areas in southern Norway, the concentrations in the soil humus layer of cadmium are typically 1–3 $mg\,kg^{-1}$ and of lead 100–250 $mg\,kg^{-1}$, equal to the limit values for agricultural soils treated with sewage sludge in the European Union (**Table 2**). From the 1970s, however, the long-range atmospheric transport of many metals to Norway has been distinctly reduced (Amundsen *et al*., 1992, cf. **Figure 1**), owing to pollution-reducing efforts in

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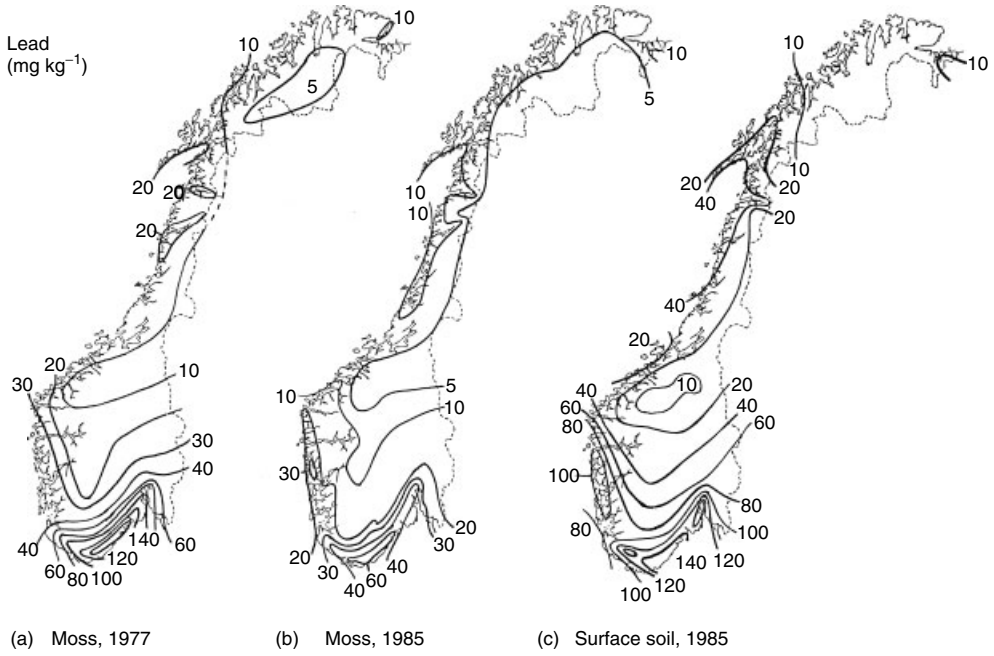

Figure 1 Concentrations of lead (mg kg^{-1} dry weight) in approximately 500 samples of terrestrial moss (*Hylocomium splendens*) sampled in 1977 (a) and in 1985 (b), and of the humus layer of natural soils in Norway (c). Because mosses lack a root system, they obtain most of their supply of chemical substances directly from atmospheric deposition. Since the carpet of living moss tissue is built up during a period of three to five years, the content of metals reflects an integrated measure of the atmospheric deposition during that period. The reduced concentrations of lead in moss from 1977 to 1985 mainly reflects reductions in the levels of lead in gasoline in Europe during this period.

most European countries. Even with fairly strong reductions in depositions, however, levels in the soil surface layers may continue to rise for elements that are very strongly complexed by humic substances.

The striking geographical patterns of metals in Norwegian surface soils (**Figure 1**) are partly reflected in local plants and further up the terrestrial food chains, indicating a potential for increased human exposure to toxic metals. For example, in a study of leaves and twigs of birch, Scots pine and cowberry, levels of cadmium were about five times higher in South Norway than in Central Norway (Steinnes, 1989). Regional studies of liver from lambs (Frøslie *et al*., 1985), moose, reindeer and red deer (Frøslie *et al*., 1984) showed similar geographical trends, most pronounced for lamb liver, where the mean value for lead was 10 times higher in the southernmost location than in the areas with lowest lead deposition (Frøslie *et al*., 1985). The levels of cadmium in kidneys of moose, reindeer and red deer also followed a similar distribution (Frøslie *et al*., 1986). For the few individuals with a high intake of organs from such game, this could represent a significant increase in the intake, especially of cadmium, but Frøslie *et al*. (1986) stated that there is no health hazard involved in the consumption of meat from wild ruminants.

When discussing the extent of soil contamination from diffuse atmospheric deposition, it is important to make a distinction between agricultural soils and natural soils. In the natural soil, the deposited metals in most cases tend to concentrate in the uppermost few centimetres of the soil (see above). In agricultural soils, on the other hand, the amount of contaminants deposited on the surface is mixed within the plough layer and therefore distributed into a larger volume of soil, so the concentrations are much lower (Singh and Steinnes, 1994). The anthropogenic contribution, however, is likely to be in a form more mobile in the soil than the natural content and hence more plant-available, in a similar way as metals added with sewage sludge or commercial fertilizers.

Persistent organic compounds, such as PAHs (Aamot *et al*., 1996), PCBs (Lead *et al*., 1997) and dioxins (Hassanin *et al*., 2005), are also supplied to soils from long-range atmospheric transport to a certain extent, in addition to contributions from local pollution sources and application of sewage sludge (Section 5.2).

5.6 Acidification and Human Health Effects

Acidification of soil and water resulting from the deposition of acid rain (or more generally acid precipitation) is a major environmental problem. The precursors of acid rain are mostly gaseous oxides of sulphur and nitrogen, introduced into the atmosphere by combustion of fossil fuels,

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smelting of metal sulfides and from natural sources. The sulphur and nitrogen oxides are oxidized in the atmosphere and react with water to form sulfuric and nitric acid. These acids are very water soluble and are deposited on the ground with precipitation. Typical pH values in precipitation over large affected regions are 4.0–4.5, but in extreme cases, pH values below 3 and even below 2 have been recorded. Much is known about effects of acidification on fish and other aquatic organisms, forests and soils (Overrein *et al*., 1980; Grennfelt *et al*., 1995), but less is known about possible human health effects (Goyer *et al*., 1985; WHO Working Group, 1986; Nordberg, 1990; Oskarsson *et al*., 1996).

Basically, two conditions have to be met for acid precipitation to cause significant problems in a particular area:

1. The rainwater has to be acidic enough. Rainwater is naturally slightly acidic because CO_2 from the atmosphere forms carbonic acid when dissolved in water; distilled water in equilibrium with the ambient atmosphere has a pH of about 5.6. In practice, rainwater pH has to be well below 5 for ecological problems to arise.
2. The buffering capacity of the soil has to be low. In most watersheds, most of the rainwater percolates through the soil before it reaches the waterways. During this percolation, more or less of the acids in the rainwater will be neutralized depending on the properties of the soil. Therefore, the ecological impact of acid precipitation in a particular area largely depends on the neutralizing capacity of its soil and bedrock. The most sensitive areas are those with shallow or no soil overlying granite or gneiss bedrock; deep, calcite-rich soils, on the other hand, have the ability to neutralize large amounts of acid, so soil and, particularly, aquatic ecosystems in such areas will be little affected.

Because it typically takes a few days for the oxides of sulphur and nitrogen to be completely oxidized in the atmosphere to sulfuric and nitric acid, serious acidification sometimes occurs in areas several hundreds and even thousands of kilometres away from the pollution sources, notably in Scandinavia as a result of transport from Continental Europe and the UK, and in Canada resulting from transport from the USA. However, in these areas, pollution-reducing efforts have resulted in reduced deposition of acids and clear tendencies for the slow recovery of acidified lakes and rivers (Wright *et al*., 2005), although the problem is far from solved. On the other hand, acidification is a large and increasing problem in China (Larssen *et al*., 2006) and other rapidly developing countries.

When assessing the possible human-health effects related to acid precipitation, it is useful to distinguish between direct effects, associated with the predepositional phase, and indirect effects, associated with the postdepositional phase (Goyer *et al*., 1985). Direct effects are those resulting from direct human exposure to acidic substances from ambient air. Such direct effects are only relevant in urban areas or close to point sources in general, and will not be discussed in this chapter (see **Air Pollution**).

The possible indirect health effects of acid precipitation are those resulting from acidification of soil and water. The major area of concern is increased mobility of toxic compounds, mainly metals (Nelson and Campbell, 1991). Since the solubility of most metals increases with decreasing pH, acid precipitation will leach soil-bound metals into the soil solution, both metals naturally present and metals deposited from the atmosphere (cf. Section 5.5). As the soil solution percolates downwards through the soil profile, the dissolved metals may precipitate when they reach a soil horizon with higher pH. With continuing supply of acid, the deeper layers of the soil may also be acidified, allowing the dissolved metals to reach the groundwater and eventually streams and lakes. Human exposure may be influenced by two main routes, namely accumulation of toxic compounds in food chains and contamination of drinking water. Increased solubility of metals in soils may lead to increased metal levels in food plants and animals, and in drinking water sources.

As a secondary effect, decreased pH in drinking water sources may lead to increased leaching of substances from corrosion in the water distribution system. Relevant substances include lead from lead pipes and solder, zinc and cadmium from galvanized pipes, faucets and solder, copper from copper pipes and asbestos from asbestos–cement pipes.

The most relevant metals for possible health effects of acid precipitation are mercury, cadmium, lead and aluminium. It is not known whether aluminium exposure constitutes a health risk to the general population. Mercury, cadmium and lead, on the other hand, are 'classical' toxic metals. All three metals tend to accumulate in the body over time, and the margins of safety between the mean population intakes and the health effect thresholds are not comfortably wide, at least not for possible susceptible subpopulations. Furthermore, exposure and body burden for these three metals, as for many other substances, follow skewed distributions in the population. This implies that a seemingly small increase in the average population intake may result in a relatively larger proportion of individuals exceeding the health effect threshold (Flaten, 1997). Therefore, every increase in population intake could have clinical relevance and should be avoided, including increases associated with acidification. Thus, although at present there is no firm evidence directly linking human health effects to soil and water acidification, the existing indications of increased

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exposure to cadmium, methylmercury and lead by itself warrant that the problem be taken seriously.

5.6.1 Cadmium

Cadmium is also discussed in Section 5.4.1. Food and tobacco smoke are the main sources of cadmium exposure for the nonoccupationally exposed population (WHO, 1992). Cereals and vegetables are the most important dietary sources. In contrast to many other metals, for example, lead and mercury, cadmium is easily taken up by plant roots, and increases in soil cadmium generally results in increased plant uptake by the metal (Page *et al*., 1981; WHO, 1992). Therefore, it is important that the cadmium concentration in agricultural soils is kept low. Cadmium, like most other metals, shows increased mobility in soil and water with decreasing pH. In addition to soil cadmium concentration, soil pH is the most important factor influencing plant cadmium accumulation. A reduced soil pH clearly increases the uptake of cadmium in various food crops, for example, rice, wheat, carrots and potatoes (Page *et al*., 1981; Öborn *et al*., 1995). For example, the average cadmium content of carrots in the study by Öborn *et al*. (1995) was about 175 μg (kg dry weight)$^{-1}$ at pH 7 and about 480 μg kg^{-1} at pH 5. Lime applications to acid soil will cause reduced cadmium levels in crops, but high liming rates may actually increase cadmium concentrations (Öborn *et al*., 1995). This puzzling effect may be due to competition between calcium and cadmium ions on soil particles, in that calcium ions from lime replace cadmium ions on soil exchange sites, thereby mobilizing cadmium and increasing its availability to the plants (Christensen, 1984).

Regarding drinking water, significant amounts of cadmium are present in some soldering materials and in parts of certain taps (faucets), but mostly in older water systems. In addition, galvanized (zinc-covered iron) water pipes always contain some cadmium. In larger waterworks, acid source water will in most cases be treated to increase pH and reduce corrosivity, but in small waterworks and single-household systems, such treatment will generally not be used. In Norway, for example, drinking water pH values in the range 4.5–5.5 are not uncommon (Flaten, 1991). Drinking water sources mostly contain less than 0.05 μg Cd l^{-1} (Méranger *et al*., 1979; Nriagu *et al*., 1996; Lydersen *et al*., 2002). This constitutes a negligible contribution to the typical daily intake of cadmium, which in most countries is around 10–30 μg (WHO, 1992). Little systematic work has been done on leaching of cadmium and other metals from plumbing systems. In Seattle, where the drinking water was quite corrosive, the median cadmium values of water samples collected in residential buildings with galvanized water pipes were 0.63 μg l^{-1} for standing and 0.25 μg l^{-1} for running water samples, and the corresponding values for buildings with copper pipes were 0.02 and 0.01 μg l^{-1} (Sharrett *et al*., 1982). Hence, although leaching of cadmium from pipes and plumbing is most probably not an important source of cadmium exposure at the population level, in certain households acid drinking water may contribute appreciable amounts of cadmium through such leaching.

In Norway, concentrations of cadmium in liver and kidneys of wild moose, reindeer and red deer are markedly higher in areas influenced by acid precipitation than in 'background' areas (Frøslie *et al*., 1986). This would represent an increased intake for individuals consuming organs from game. However, the extent to which the increased organ concentrations are due to acidification or to the concomitant atmospheric deposition of cadmium (Section 5.5) is unknown.

5.6.2 Lead

The critical effects for chronic, low-level exposure of lead are neurological and haematological; the major risk is central nervous system toxicity (Skerfving and Bergdahl, 2007). The last decades have witnessed a gradual and severalfold reduction in the threshold level for lead in blood below which no adverse effects are anticipated, and recent studies have indicated subtle cognitive and behavioural deficits in newborns and very young infants, possibly even below 100 μg l^{-1} (Schwartz, 1994; Canfield *et al*., 2003).

There are several toxicologically relevant sources of lead, notably dust, soil, paint chips, food and drinking water. Acid precipitation may contribute to increased lead intake chiefly through drinking water. Where corrosive water is supplied through lead-containing pipes and plumbing, drinking water may be the dominant source of lead (Elwood *et al*., 1984). Thus, acidification of drinking water clearly has the potential to cause increased intake of lead, where the drinking water installations contain lead. In many older urban areas, notably in the UK, lead water pipes are still in use. More commonly, the source of lead is lead-based solder joining metal pipes; lead solders have been used since Roman times. An additional source is brass, a common compound in drinking water faucets and other installations. Brass commonly contains about 2% lead to make it malleable. Raising the pH of drinking water in Glasgow and Ayr resulted in clinically relevant rapid falls in blood lead in postpartal women (Moore *et al*., 1985), demonstrating that drinking water pH really can be important for the intake and body burden of lead.

The potential of acid rain to cause increased concentrations of lead in the sources of drinking water is less than for cadmium. This is because lead is much less mobile in terrestrial ecosystems than cadmium, owing to stronger complexation by humic substances (Bergkvist *et al*., 1989). However, we do not know enough of the mechanisms involved to exclude the possibility that acidification-induced increases in the mobility of lead in

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the environment may be toxicologically relevant. The fact that a study in Norway showed the mean concentration of lead in the liver of outdoor-grazing lambs to be 10 times higher in the most acidified area than in background areas is disturbing (Frøslie *et al*., 1985). However, as mentioned in Section 5.6.1, this increased liver lead may be due to the intake of lead directly deposited from the atmosphere on plants and soil, rather than to lead mobilized by acidification.

5.6.3 Mercury

For mercury, environmental pathways, exposure routes, absorption, metabolism and toxicological properties vary widely with speciation (Berlin *et al*., 2007). Environmental transport of mercury is mainly through the atmosphere, where it exists almost exclusively in the elemental form (Hg^0), as a gas or adsorbed to the surface of small particles. This elemental mercury may then be oxidized to the more water-soluble divalent form (Hg^{2+}), dissolved in raindrops and deposited from the atmosphere. In soil and water, Hg^{2+} can be: (i) reduced to gaseous Hg^0, thereby returning mercury to the atmosphere or (ii) methylated by micro-organisms to CH_3Hg^+. The electrically neutral methylmercury species, CH_3HgCl, $(CH_3)_2Hg$ and so on are lipophilic, and can thus readily pass biological membranes and be concentrated by ecological food chains—a process known as bioamplification or biomagnification: any substance that is more soluble in the tissues of simple organisms than in the surrounding water will be a more concentrated component of the diet of the more complex species which feed on the simpler ones. Methylmercury has a very strong tendency for bioamplification. Concentrations are highest in large predatory fish such as pike; levels above $1\,mg\,kg^{-1}$ in muscle are not uncommon.

Fish and fish products are the only significant sources of human intake of methylmercury (WHO, 1990). Eating 200 g of pike containing $1\,mg\,kg^{-1}$ of methylmercury results in an intake of 200 µg, equal to the WHO recommended provisional tolerable weekly intake (WHO, 1990). In fact, northern pike is the species of fish with the highest consumption per user in the USA; the estimated number of users of northern pike is 2.5 million, with a mean consumption of $17.4\,g\,day^{-1}$ (Goyer *et al*., 1985).

The critical effects of methylmercury are neurotoxic. Extensive evidence indicates that the developing nervous system is more sensitive to methylmercury toxicity than that in adults (WHO, 1990; Berlin *et al*., 2007). Methylmercury readily crosses the placenta, so the main concern is the intake of high-methylmercury fish among women of childbearing age. The Swedish National Food Administration has since 1991 advised pregnant and lactating women, and women planning to have children in the near future, to abstain totally from eating a number of specified fresh-water fish species.

Studies in several countries show, within a given geographical area, a fairly strong negative correlation between pH values of lake water and methylmercury body burdens in fish (WHO Working Group, 1986). Although a cause-and-effect relationship has not been established, the indications that acidification increases methylmercury in fish are quite strong, and the potential increase in methylmercury intake for individuals consuming such fish is clearly toxicologically relevant.

5.6.4 Aluminium

Acidification leads to mobilization of aluminium from otherwise very poorly soluble minerals such as feldspars and kaolinite in soils and bedrock. The result is greatly increased aluminium concentrations in ground and surface water and also in drinking water (Flaten, 1991). Aluminium is the main toxicant damaging fish populations and other aquatic life in acidified areas (Driscoll and Postek, 1996). The main target organ in mature fish is the gills; death is due to disruption in iono- and osmoregulation and respiratory dysfunction.

The possible human toxicity of aluminium has been a matter of controversy for well over 100 years (Flaten *et al*., 1996; Krewski *et al*., 2007). The first human conditions generally accepted to be causally related to aluminium exposure occurred in the 1970s, shortly after the introduction of routine dialysis therapy in patients with chronic renal failure (Alfrey *et al*., 1972). These conditions are a fatal encephalopathy commonly termed dialysis dementia, fracturing osteomalacia and a microcytic hypochromic anaemia occurring despite adequate iron stores (Alfrey, 1993). The patients received aluminium into the bloodstream via high-aluminium dialysate or consumed large oral doses of aluminium-containing phosphate binders. The dialysate was prepared by diluting a concentrate with ordinary tap water. Since there is no doubt that acidification of soil and water can cause greatly increased concentrations of aluminium in fresh water, it seems highly likely that acidification has contributed to a number of cases of dialysis encephalopathy, osteomalacia and anaemia through the tap water used to prepare the dialysate. Although, of course, causal inference cannot be made for individual patients, it may be noted that the first cases of dialysis encephalopathy in Norway occurred in two hospitals situated in areas highly influenced by acid precipitation. The concentrations of aluminium at the waterworks serving the two hospitals were approximately 150 and $300\,\mu g\,l^{-1}$, respectively (Flaten, 1991). It is generally accepted that dialysate aluminium concentrations should be kept below $10\,\mu g\,l^{-1}$ to prevent aluminium accumulation and toxicity in dialysis patients. Since about 1980, hospitals have treated the water used to prepare dialysate to

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remove aluminium, mostly by reverse osmosis, and osteomalacia and especially encephalopathy are now rare in the dialysis population.

In dialysis patients, tissue accumulation of aluminium to levels high enough to cause toxicity is mainly due to a combination of: (i) very high exposure, partly directly into the bloodstream and (ii) these patients' lack of kidney function, which is the main excretion route for aluminium (Krewski *et al*., 2007). Although there are also reports of similar neuro- and osteotoxicity in certain patient groups without kidney failure (Flaten *et al*., 1996), the question whether aluminium exposure of any kind represents a health problem for the general population remains open (Krewski *et al*., 2007). The most controversial issue is whether aluminium exposure is causally related to Alzheimer's disease (Exley, 2001; Miu and Benga, 2006). Several epidemiological studies from different countries (Flaten, 2001; Krewski *et al*., 2007), have indicated a relationship between drinking-water aluminium and Alzheimer's disease. However, even at high concentrations (100–300 $\mu g\,l^{-1}$), drinking water contributes only a fraction of the total aluminium intake, which is typically in the order of a few milligrams per day (Pennington and Schoen, 1995). Persons consuming antacids may ingest more than 1 g of aluminium daily, and the handful of epidemiological studies attempting to relate antacid intake to Alzheimer's disease have been negative (Flaten, 2001; Krewski *et al*., 2007).

However, aluminium is poorly absorbed in the gastrointestinal tract; the consensus is that about 0.1–0.4% of the dietary intake is absorbed (Krewski *et al*., 2007). Hence it is possible that the aluminium present in drinking water is more bioavailable than that present in food and medications, although recent evidence seems to contradict this (Stauber *et al*., 1999; Yokel *et al*., 2001). It is also conceivable that some natural water may contain small amounts of organic aluminium complexes with properties similar to those of aluminium maltolate, stable enough to pass through the stomach and accumulate in brain and bone (van Ginkel *et al*., 1993). Hence a real association between drinking-water aluminium and Alzheimer's disease cannot be dismissed (Krewski *et al*., 2007). In any case, it is indisputable that acid precipitation has resulted in increased human exposure to aluminium through drinking water.

5.6.5 Other Substances

Copper is an essential element with a daily requirement of 1.0–1.5 $mg\,day^{-1}$ (WHO, 1996). Background concentrations of copper in fresh water rarely exceed a few $\mu g\,l^{-1}$ (Méranger *et al*., 1979; Nriagu *et al*., 1996; Lydersen *et al*., 2002), but tap water often contains much higher concentrations. More than 1 $mg\,l^{-1}$ of copper is not uncommon, because in many countries, most of the domestic water pipes are made of copper. The copper concentration increases with increasing contact time between water and piping, and flushing decreases water copper content. The time necessary to flush out the high-copper water depends on the length of the copper pipes. The corrosion rate of copper pipes is highly pH-dependent; corrosivity increases rapidly with decreasing pH. Therefore, water acidification will increase copper exposure in places where the water is not treated at the waterworks to increase pH and reduce corrosivity. Since the typical daily intake of copper is in the order of 2 mg or less (WHO, 1996), drinking water may contribute substantially to copper intake in areas with corrosive water, especially if the pipes are not thoroughly flushed before use.

For the essential trace element *selenium*, in contrast to the situation for the elements discussed above, mobility in soil and water seems to *decrease* with decreasing pH, although other variables, such as the presence of oxides in soils and the redox level may complicate the relationship (Frankenberger and Benson, 1994). Hence it is possible that human intake of selenium, which may be suboptimal for many individuals, may be reduced following acidification of the environment. A study in Sweden indicated that subjects in acidic areas had slightly lower plasma selenium levels than subjects from a reference area (medians 85 vs. 90 $\mu g\,l^{-1}$) (Svensson *et al*., 1987). In addition, there seems to be an inverse relationship between the methylmercury content of fresh-water fish and selenium contents of water. In fact, adding selenium to lakes in Sweden has resulted in severalfold reductions of the mercury level in fish (Paulsson and Lundbergh, 1989). Thus, if acidification generally reduces the availability of selenium in the environment, this may result both in reduced human intake of selenium and increased intake of methylmercury.

Exposure to *asbestos* may also increase following environmental acidification. The source is asbestos–cement, which is widely used as a material in water pipes. In addition, asbestos–cement roofing tiles have been used as catchments for household drinking-water cisterns. The calcium-carbonate matrix in the asbestos–cement dissolves in acid water, releasing asbestos fibres. Whether oral ingestion of asbestos is a significant health hazard is, however, uncertain. The data relating ingestion of asbestos to health effects, most notably colorectal cancer, are inconclusive (Homa *et al*., 1994; Browne *et al*., 2005). However, the potential for inhalation of asbestos fibres during showering may be a cause of concern.

Finally, it may be noted that acidification of soils and fresh water will also affect the bioavailability and toxicity of compounds that exist as weak acids or bases in the environment. Because essentially only the nonionized form of a lipid-soluble molecule will diffuse across biological membranes, acidification will

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result in enhanced bioavailability (and hence potential for build-up in food chains and increased exposure for humans) for weak acids and decreased bioavailability for weak bases.

6 DRINKING WATER

6.1 Introduction

Since drinking water is not specifically treated in other chapters in this book, some human-health aspects of drinking water are discussed, both in this section and in parts of Section 5. The focus is on instances where toxic substances, at least partly, have their origin in soils, and where drinking water constitutes an important exposure pathway from soils to humans.

Soils fundamentally influence the chemical composition of drinking water in that the major part of most dissolved constituents in fresh water originates from weathering of soil minerals (Stumm and Morgan, 1996). Generally speaking, the main mechanisms or processes contributing to the chemical composition of drinking water are:

- Chemical weathering of the soils, bedrock and sediments in the drainage basins
- Atmospheric transport of salt particles from the sea
- Regional and local pollution
- Corrosion of the water pipes and plumbing systems
- Water treatment
- Decomposition of organic matter
- Hydrological variables (rainfall, soil depth, terrain slope, etc.).

The number of variables regulated by drinking water standards has increased rapidly during the last decades (WHO, 2008). It is evident that under special circumstances, the concentrations of a large number of substances, both natural and anthropogenic, may reach potentially toxic levels in drinking water. On the average, however, the contribution from drinking water to the total intake is small for most drinking-water constituents. There are a few exceptions, however, the best known probably being fluoride. Furthermore, the bioavailability of certain elements is probably higher in drinking water than in most foods. This could be the case for some elements with a complex chemistry in aqueous solution and which form compounds with low solubility, such as aluminium, chromium, cobalt, iron, manganese and molybdenum, but would be less probable for the alkali and alkaline-earth elements and the halogens, for example.

6.2 Fluoride

The dominating source of fluoride in fresh water is weathering of minerals in soil and bedrock (Edmunds and Smedley, 2005; Amini *et al*., 2008). Thus, differences in the chemical composition of the local soil will determine the concentration of fluoride in drinking water, and is clearly important for dental and skeletal health. Numerous studies dating back to World War II have clearly demonstrated that the prevalence of dental caries in children is strongly related to the fluoride concentrations in drinking water (WHO, 2002). An F^- concentration around $1\,mg\,l^{-1}$ is considered optimal, and many waterworks, notably in North America, add fluoride to drinking water to achieve this concentration in the treated water. Whereas a small amount of fluoride is beneficial, higher concentrations may have deleterious effects, notably mottled teeth (dental fluorosis) and, if the concentration is high enough, skeletal deformations and malformations (skeletal fluorosis). The concentrations necessary to produce these deleterious effects depend on the water intake. In warmer climates, people consume more water, and in parts of India, Sri Lanka (Dissanayake, 2005), China and the Rift Valley area in Africa, crippling skeletal fluorosis affects millions of people (Ayoob and Gupta, 2006). High drinking-water fluoride levels originating from the local soil and bedrock is therefore a major public-health problem.

6.3 Water Hardness and Cardiovascular Disease

The 'water story', that is, the possible association between cardiovascular diseases and the hardness of drinking water, has now passed its fiftieth anniversary (Kobayashi, 1957). In more than 50 ecological (geographical correlation) studies conducted in several countries up to 1978, the mortality or incidence of cardiovascular disease was found to be typically 5–15% lower in areas with hard drinking water than in areas where the drinking water is soft, that is, has low concentrations of calcium and magnesium (NRC, 1979). The majority of epidemiological studies published after 1978 have also indicated a protective effect (Monarca *et al*., 2006). However, the ample amount of research conducted has failed to clarify a biological mechanism; possible explanations behind the 'water story' include (NRC, 1979): (i) the principal constituents of hardness (calcium or magnesium) are protective; (ii) other elements that tend to be present in hard water (e.g. selenium, chromium, silicon, lithium or vanadium) are protective; (iii) harmful elements (e.g. lead or cadmium) are present in soft water, for example as a result of the corrosive action of soft water on the

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water distribution system (cf. Section 5.6) and (iv) other (e.g. socio-economic) confounding factors are involved.

The main source of calcium and magnesium in drinking water is chemical weathering of soil minerals. Thus, differences in soil composition and soil depth dictate the concentrations of calcium and magnesium in drinking water, and would be the 'driving force' behind the 'water story'. Even at high concentrations, however, drinking water only contributes a minor fraction of the total intake of calcium and magnesium.

6.4 Arsenic

Arsenic has a complex chemistry in the environment and in biological systems. It occurs mainly in two oxidation states, +III and +V. In surface water and surface soil, most arsenic is pentavalent, while most of the arsenic introduced in the environment by human activities is trivalent. Trivalent forms are generally more toxic than pentavalent forms. Arsenic is biomethylated in nature, and may occur as many different organoarsenic species.

Arsenic exposure may cause toxic effects in many organ systems (WHO, 2001; Fowler *et al*., 2007). In Taiwan, extraordinarily high concentrations of arsenic in soil and water have been linked to an endemic peripheral vascular disease known as 'blackfoot disease', which in severe cases progresses to gangrene in the extremities (Tseng, 2005).

It is well established that ingestion of inorganic arsenic can cause skin cancer (WHO, 2001). In view of the increasing evidence that arsenic exposure may also cause other types of cancer and that its carcinogenic potency seems to be higher than previously thought (Fowler *et al*., 2007), WHO, in 1993, lowered its provisional guideline value for arsenic in drinking water to $10\,\mu g\,l^{-1}$ (WHO, 2008).

During the 1990s, endemic arsenic poisoning of enormous proportions was revealed in the Bengal alluvial delta region of Bangladesh and India (Mandal *et al*., 1996). This has been called 'the largest poisoning of a population in history' (Smith *et al*., 2000) and arsenic contamination of groundwater in general has been called 'the key environmental health problem of the twenty-first century' (Christen, 2001). In 2002, it was estimated that more than 300 000 people in the Bengal area had visible arsenic-related skin lesions, including melanosis, keratosis, nonpitting oedema, gangrene and skin cancer (Chakraborti *et al*., 2002). In the most seriously affected districts, more than 90% of the drinking water wells have arsenic concentrations above $50\,\mu g\,l^{-1}$, the highest concentrations being above $3000\,\mu g\,l^{-1}$ (Smedley and Kinniburgh, 2005). In total, an estimated 35 million people in Bangladesh and 6 million people in West Bengal are exposed to arsenic in drinking water at concentrations above $50\,\mu g\,l^{-1}$ (Smedley and Kinniburgh, 2002).

In the Bengal delta region, the traditional surface water sources for drinking were heavily contaminated with micro-organisms, causing a high burden of disease and mortality. During the 1970s and the 1980s, UNICEF worked with the local authorities to install tube-wells to provide what was presumably a safe source of drinking water for the population (Smith *et al*., 2000), and millions of wells were installed without testing the water for arsenic. The exact mechanism behind the high arsenic concentrations in the Bengal groundwater is not known, but the arsenic is generally accepted to be of natural origin. Development of strongly reducing conditions in the affected aquifers is likely the main factor controlling arsenic mobilization (Smedley and Kinniburgh, 2002). High arsenic concentrations in groundwater have later been reported in other river delta regions in Asia (Smedley and Kinniburgh, 2005), including the Red River delta in Hanoi, Vietnam (Berg *et al*., 2001) and the Mekong river floodplain in Cambodia (Buschmann *et al*., 2007). Globally, a wide variety of environmental conditions can give rise to arsenic concentrations in drinking water well above the WHO guideline of $10\,\mu g\,l^{-1}$ (Smedley and Kinniburgh, 2002).

6.5 Nitrate and Methaemoglobinaemia and Cancer

In many areas of the world, concentrations of nitrate in groundwater have been gradually increasing for several decades (Strebel *et al*., 1989; Spalding and Exner, 1993). The increase is seen mainly in agricultural areas, and is most probably due to addition of fertilizers to agricultural soils. Together with phosphorus and potassium, nitrogen is the main element in commercial fertilizers. The nitrate anion is only weakly bound in soils, and hence is fairly mobile. The mobility varies considerably with soil type and agricultural practices, and nitrate leaching from soils to groundwater may, in many cases, be efficiently controlled by agricultural practices (Strebel *et al*., 1989).

High concentrations of nitrate in drinking water was first reported to cause infant methaemoglobinaemia ('blue baby syndrome') in 1945 (Comly, 1945). Up to 1970, about 2000 cases of infant methaemoglobinaemia had been reported worldwide (Fan *et al*., 1987). This is the rationale behind drinking-water standards for nitrate, which in most countries is around $50\,mg\,l^{-1}$ of NO_3^-. Compliance with this standard seems to offer adequate protection against infant methaemoglobinaemia.

Some epidemiological studies have indicated that exposure of humans to high levels of nitrate is associated with an increased risk of several types of cancers, and later studies have indicated an increased risk for

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adverse pregnancy outcomes, but the evidence is inconclusive (Ward *et al*., 2005). The postulated mechanism is that bacteria in the alimentary tract reduce ingested nitrate to nitrite, which then reacts with nitrosatable substrates in certain foods to form carcinogenic *N*-nitroso compounds.

6.6 Chlorination By-Products and Cancer

Chlorination of drinking water, which was introduced about 100 years ago, has undoubtedly been one of the most important public health measures of the twentieth century (IARC, 1991). At present, water chlorination is still the most effective method for the control of waterborne infectious disease transmission. In 1974, it was discovered that chlorination of drinking water led to the formation of chloroform and other trihalomethanes (Rook, 1974). Since then, several hundred different halogenated organic compounds have been identified in drinking water (Richardson *et al*., 2007). However, the concentrations of even the most common chlorination by-products, chloroform and trichloroacetic acid, seldom exceed $100\,\mu g\,l^{-1}$. Most other by-products occur at levels typically below $1\,\mu g\,l^{-1}$. Several factors influence which compounds are formed, especially pH, chlorine-to-substrate ratio and the nature of the precursor compounds. The principal precursors of the chlorination by-products are humic substances, the natural products of decomposition of dead plants and animals in soil and water, which impart a characteristic yellow-brown colour to the water. Concentrations and types of humic substances vary widely in natural water, mainly as a result of local variations in soil conditions.

Among the chlorination by-products there are a number that are mutagenic and some, notably haloacetonitriles, chlorinated phenols, aldehydes and ketones, that are animal carcinogens. Practically all drinking water is mutagenic in Ames/*Salmonella* tests after chlorination, but seldom before, and mammalian cell and plant tissue assays generally give similar results (WHO, 2000; Richardson *et al*., 2007). The chlorination by-product 3-chloro-4-(dichloromethyl)-5-hydroxy-2[5 *H*]-furanone (MX) has attracted special attention, partly due to its extreme potency in the Ames mutagenic test. MX accounts for a major part of the mutagenicity in Finnish and other drinking water, although it is present in minute concentrations (below $100\,ng\,l^{-1}$) (Smeds *et al*., 1997).

More than 30 epidemiological studies of drinking water chlorination and cancer have been carried out, and although the results are not unequivocal, there is a clear tendency that cancers of the bladder, colon and rectum are associated with various measures of chlorination (Morris *et al*., 1992; Villanueva *et al*., 2004). More recently, a number of epidemiological studies have indicated that adverse pregnancy outcomes may also be associated with drinking-water chlorination (Bove *et al*., 2002; Savitz *et al*., 2006).

Although the current scientific evidence linking drinking-water chlorination to cancer and adverse pregnancy outcomes is inadequate to conclude that the relationship is causal, several waterworks have changed their treatment practices to reduce the amounts of chlorination by-products, or changed their source of raw water to one with lower concentrations of humic substances, thus reducing human exposure to chlorination by-products. One must not forget, however, that the primary public-health concern for drinking-water supplies is still waterborne disease transmission, against which chlorine provides very effective protection.

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Biological Criteria for Water Quality in Relation to Human Health

Robert A. Howd

CONTENTS

1 INTRODUCTION

Maintenance of drinking water quality is vitally important to human health, and is too often taken for granted in the modern developed world. Providing clean water has become increasingly difficult as human populations have increased, with corresponding increasing demands for high-quality water supplies. Both natural constituents and man-made chemicals can cause problems, and both surface and groundwater supplies are at risk. But it is neither useful nor cost-effective to merely remove all chemicals from our drinking water. Distilled or ultrapure water from reverse osmosis filtration does not taste good and removes desirable minerals. Therefore water providers attempt to determine what to leave in and what to remove, and the best treatments to achieve these goals that do not add other undesirable constituents or traits to the water. Increasingly sophisticated tools are available for this purpose—not only water analysis techniques, but also methods for evaluation of health risks from contaminants.

Identification of water-quality problems and attempts to solve them began over 2000 years ago (Pontius, 2003a). The most notable example of this is the construction of the Roman aqueducts, bringing water to Rome from far upriver to avoid problems caused by human sewage and other activities polluting the Tiber, which flows through the city. The same strategy was followed in other cities throughout the world where feasible, but increasingly, as populations grew, more people relied on shallow wells and urban rivers and streams for their water supply (Okun, 2003). Contamination of water by human and animal wastes led to epidemics of cholera, amoebic dysentery and other diseases—although this was not recognized to be due to micro-organisms in the water until acceptance of Pasteur's germ theory in the late nineteenth century.

Filtration of water through sand helped protect its quality by removing both sediment and bacteria. However, as medical record-keeping improved in the late nineteenth and early twentieth century, the observed variation of illness rates with water source demonstrated the inadequacy of this strategy (Frost *et al*., 2003). Disinfection of water by chlorination was begun both in Europe and the United States to avoid these problems—and was wildly successful. Incidence of water-borne diseases plummeted. Chlorination was rapidly adopted throughout the developed world (for surface water and water from shallow wells).

Regulations intended to ensure optimum chlorination to limit residual coliform bacteria began in the USA with the Public Health Standards of 1914. A few chemicals were added in 1925 (lead, copper and zinc), more in 1942, and then still more in 1962, as advances in both analytical instrumentation and epidemiology revealed the need for enhanced control of water quality. Development of appropriately health-protective water quality standards has been a long process, with gradually increasing sophistication. In the US, health risk assessment procedures for chemicals were formalized in the 1970s and early 1980s, subsequent to the establishment of the Environmental Protection Agency in 1970

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and the increased regulation of chemical contaminants in drinking water mandated by the Safe Drinking Water Act of 1974 (Howd, 2008). Risk assessment and management procedures were first comprehensively laid out in the groundbreaking publication, *Risk Assessment in the Federal Government: Managing the Process* (NAS, 1983). European Union (EU) water regulations began with the Surface Water Directive in 1975, followed by the Drinking Water Directive in 1980. The drinking water regulations were updated in 1998 with Council Directive 98/83/EC, which is still current (Hecq *et al*., 2006).

At the present time over 90 natural constituents and contaminants are regulated in the USA (US EPA, 2008a) and Canada (Health Canada, 2008a) and a comparable number are the subject of World Health Organization guidelines (WHO, 2006). The EU has promulgated standards for approximately 48 constituents or properties of drinking water in its current Directive (Hecq *et al*., 2006; EC, 2008). The UK water quality regulations are based on the 1998 European Community directive (EC, 2008), but some standards may be more stringent (DWI, 2008). Many of the standards are based on World Health Organization guidelines (WHO, 2006).

2 RISK-ASSESSMENT PROCEDURES

The policies and assumptions used in risk assessments for chemicals in drinking water have tended to converge in recent years among the USA, Canada and the EU. The US EPA guidelines and actual practice (US EPA, 2004a; 2005a; Donohue and Miller, 2008) are very similar for both carcinogens and noncarcinogens to the practices described by Health Canada (1995) and WHO (2006). The description of risk assessment below applies to all these jurisdictions except as specified.

2.1 Evaluation of Toxic Effects

For purposes of evaluating potential risks to human health associated with a chemical, several sources of data are considered, which are listed in order of general priority:

- Effects observed in humans
- Effects observed in animal studies
- Effects observed in isolated human or animal tissues
- Effects in simple cell tests such as cytogenetic assays in bacteria or yeast
- Structure–activity relationships.

2.1.1 Human Studies

Data on human effects of chemicals found in drinking water may be available from anecdotal reports (i.e. poisoning cases), from occupational epidemiology studies, from environmental epidemiology studies and, in a few cases, from purposeful administration of the chemicals to humans.

Data on the acute toxic effects of chemicals are of great interest for chemicals with prominent acute effects, such as acetylcholinesterase inhibitors (carbamate and organophosphate pesticides). Other examples include antimony and copper, for which the gastrointestinal effects (irritation, nausea and vomiting) are most obvious (Winship, 1987; Araya *et al*., 2003). Although chronic effects provide the lowest-dose responses for many chemicals, in other cases toxic effects could be most likely as a result of exposure during particularly sensitive periods and life-stages, such as pregnancy and foetal development, infancy, maturation of reproductive organs (puberty) or during senescence.

Excellent occupational studies are available for several common chemicals, including asbestos (Hodgson and Darnton, 2000), benzene (Rinsky *et al*., 1987; Lan *et al*., 2004), chromium (Park *et al*., 2004) and lead (Roels *et al*., 1994; Duydu *et al*., 2001). Good data are available for these chemicals because they have relatively specific toxic effects that can be distinguished from the general background illnesses in a study group. For example, the pulmonary mesothelioma caused by asbestos is virtually a specific diagnostic; non-asbestos-related mesothelioma is relatively rare. However, these data are not useful for risk assessment of asbestos in drinking water, because these lung effects do not occur with oral exposure to asbestos (OEHHA, 1997). Leukaemia and lymphoma caused by inhalation of benzene have been observed in several occupational studies. It is assumed for the purposes of risk assessment that the same effect will be observed after exposure to benzene in drinking water, because benzene is well-absorbed orally and this is a systemic effect. Actual concentrations of benzene found in drinking water are much lower than the concentrations in air which have been associated with leukaemia, so no confirmatory data exist.

Environmental exposures are generally to lower concentrations of chemicals than found in occupational situations. With the lower resulting doses, it is much more difficult to demonstrate specific effects of these exposures than has been the case for several occupational studies. Very large populations are required to provide statistical significance of changes in disease incidence, in the presence of many competing risk factors. Environmental epidemiology studies therefore mostly show an *association* of exposure with effects, rather than cause and effect. Even a compilation of multiple studies (often called a 'meta-analysis') may not provide compelling evidence of attributable disease. Some of the data on arsenic effects (i.e. skin keratosis) from exposure to drinking water are considered adequate to demonstrate causality because of the unique nature of the end point and the large populations studied, but data

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on most other chemicals are not as clear. In addition to the lack of such a characteristic end point and many competing risk factors, the difficulty of quantitating relative exposures among the population(s) studied is a limitation for environmental epidemiology studies.

Studies involving specific dosing of humans with chemicals of concern do not suffer the limitation of variable or unknown exposures, but are limited to low doses that do not produce frank toxicity. Examples are studies on pesticides in support of registration (US EPA, 2007), hexavalent chromium for pharmacokinetics (Finley *et al*., 1997; Kerger *et al*., 1997) and perchlorate for evaluation of hormonal effects (Greer *et al*., 2005; Braverman, 2007). This type of human study, however, introduces two other difficult issues into the data interpretation: extrapolation of short-term effect to long-term exposures and extrapolations of the observed changes at low doses to potential toxic doses and effects. In addition, concerns have been raised (Oleskey *et al*., 2004; NAS, 2004; US EPA, 2007) about the ethics of exposing people to toxic chemicals when there is no health benefit to balance the risk (as there is for drug testing). Uses of these types of data in risk assessment are discussed in more detail below.

2.1.2 Animal Studies

Data obtained in animal models are valuable because the toxic effects and their mechanisms can be studied in detail. The more information that can be obtained on what a contaminant can do, and how it does it, the more accurately the potential effects on humans can be predicted. Many different types of studies are conducted, which are discussed elsewhere in this volume, usually involving rodents. Studies in dogs, rabbits, guinea pigs, cats or a few other species may be conducted for specific purposes, such as eye irritation in rabbits. Regulatory agencies have defined good laboratory practices (GLPs) and standard operating procedures (SOPs) for purposes such as pesticide registration or drug-safety evaluation, which then may be applicable for any risk-assessment purpose (US EPA, 2007; OECD, 2008).

All toxicity data obtained in the standard test protocols are deemed relevant unless the results are shown to be inapplicable to humans by compelling evidence that biochemical pathways or physiological regulatory systems differ in humans from those in the tested species. One example of such an effect is the observation of mammary tumours in female Sprague–Dawley rats, caused by exposure to atrazine (Morseth, 1998). In this case, the US EPA has accepted the argument that the mammary tumours result from an oestrogen-stimulatory mechanism in the rats that does not occur in the human menstrual cycle (US EPA, 2006a). The effect of this decision is to greatly increase the estimated safe dose of atrazine and other triazine herbicides in humans. Toxicity test results for chemicals found in water are also frequently available for several aquatic species, including fish, amphibians and insects. These are not utilized as the basis for a human-health risk assessment due to the increased uncertainty associated with cross-species extrapolation.

In general, the lowest relevant dose effects in the most sensitive mammalian species and strain are used in the risk assessment (US EPA, 2004a). The rationale for this is that, when human data are not available, it is most prudent to assume that humans are at least as sensitive as the most sensitive mammalian species. It is also customary to assume that systemic toxic effects observed by one exposure route would be observed by all routes, so that the most sensitive effect by any exposure route can be chosen. However, there are many exceptions to this assumption based on route-specific effects. Such effects include strong point-of-entry toxicity, high pulmonary, but low oral, absorption and substantial first-pass metabolism in the liver after oral administration. Also, when results of multiple tests are available and are considered to be equally representative of a particular effect, it may be appropriate to average critical doses or cancer potency factors to estimate the toxic potency of the chemical in animals.

2.1.3 Effects in Tissues In Vitro

Effects in isolated mammalian cells and tissues can be extremely valuable in interpreting potential toxic effects. For example, evaluation of metabolism of chemicals in human liver slices and microsomes can document production of reactive metabolites that may be associated with carcinogenicity (Steensma *et al*., 1994; Born *et al*., 2000). Determination of the potency of interaction with tissue receptors can also aid in the development of sophisticated physiologically based pharmacokinetic models (Evans and Andersen, 2000). On the other hand, demonstration of an effect *in vitro* at high, unphysiological levels is not particularly useful. This is especially relevant for studies on brain tissues, because the blood–brain barrier prevents or limits access to neural tissues for many chemicals *in vivo* (Garberg *et al*., 2005). Acute *in vitro* effects (release of a transmitter in brain slices, for instance), may also be the opposite of the chronic effects (tolerance and lowered reactivity in a neural circuit).

2.1.4 Cytogenetic Assays

The standard *in vitro* mutagenicity tests, such as the Ames assay using *Salmonella typhimurium*, provide relevant supporting data for determination of the carcinogenic potential of chemicals. However, the high concentrations used can lead to false positives secondary to cytotoxicity in such tests (Kirkland *et al*., 2007). On the other hand, some solvents found in drinking water have been tested without control of evaporation or assay of

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the resulting concentrations on the test plates or culture medium, which can lead to false-negative results (Barber *et al*., 1981). The question of whether the metabolism of a chemical in an *in vitro* assay is relevant to human *in vivo* metabolism must also be addressed.

2.1.5 Structure–Activity Relationships

Literally hundreds of trace chemicals are found in drinking water, including a plethora of disinfection by-products (Zhang *et al*., 2005; Sultan and Gabryelski, 2006). No panel of toxicity test results is available for most of these chemicals (Richardson *et al*., 2007). Testing of relevant mixtures may demonstrate important adverse effects, such as mutagenicity or reproductive/developmental toxicity. Although the complexity of the mixtures may make correlations of effects with specific agents very difficult, predictions of effects and potencies through interpretations of structure–activity relationships can be quite useful. Relative toxicity values, such as for dioxins and furans, can be derived (Van den Berg *et al*., 2006; Wilkes *et al*., 2008). Thinking of such chemicals as a group may also result in methods for controlling them as a group, rather than as individual components.

2.2 Estimation of Safe Doses

Safe doses are estimated for exposure durations from acute (one exposure) up to an entire lifetime. Protection of public health requires a conservative approach, to minimize potential risk, especially for drinking water. The conventions applied to the risk assessments for individual chemicals are intended to be more likely to overestimate than underestimate potential hazards.

2.2.1 Noncancer Effects

All chemicals are evaluated for their general effects and expected toxic actions at various doses. The human and animal data are studied to determine the lowest doses associated with significant toxicity, called the lowest observed adverse effect level (LOAEL), and doses resulting in any biological effects of significance, called the lowest observed effect level (LOEL). It has been more common to derive a maximum acceptable dose using the LOAEL than the LOEL, but the public may not appreciate the distinction: they do not wish *any* unexpected effects, such as influence on thyroid hormone levels (Blount *et al*., 2006) from drinking tap water. The maximum acceptable dose is called the reference dose (RfD) by the US EPA (2004a) and tolerable daily intake (TDI) by Health Canada (1995) and WHO (2006).

Doses below the LOAEL or LOEL which result in no detectable effects are called the no observed adverse effect level (NOAEL) or no observed effect level (NOEL). From the highest NOAEL (or NOEL) associated with the lowest LOAEL (or LOEL) in a human or animal study (often called the critical dose), a virtually safe dose for the entire population is estimated by attempting to account for the uncertainty. If the critical dose level is derived from an animal study, an uncertainty factor (UF) of 1–10 is utilized to estimate what the no-effect level might be in a human. That is, assuming humans might be up to 10 times more sensitive, the animal NOAEL is divided by a number from 1–10. Ten is generally used, unless specific information is available to support a smaller number. This could include, for instance, information that the animals tested convert the chemical to a toxic metabolite much more efficiently than humans do. If the study did not identify a NOAEL, then the LOAEL may be divided by 10 to estimate the NOAEL (Alexeeff *et al*., 2002).

In addition, humans vary in many ways, including the ability to metabolize chemicals (Pelekis *et al*., 2001; Barton *et al*., 2007; Yang *et al*., 2008). For this reason, the NOAEL is divided by another UF of 1–10 to ensure that sensitive individuals and groups (such as foetuses and infants) are protected. The risk assessor would also consider the adequacy of the database, and whether the end point is a dire effect (such as death or cancer which cannot be modelled as described below), and could propose additional factors of 10 for each. These factors are multiplied together as a divisor for the NOAEL (or LOAEL). However, US EPA now limits the combined UF to 3000 for derivation of RfDs (US EPA, 2004a; Donohue and Miller, 2008). WHO designates any drinking-water value derived with a UF greater than 1000 as a provisional value, to emphasize the higher uncertainty (WHO, 2006). Guideline values with this high uncertainty are given higher priority for later re-evaluation as new information becomes available.

2.2.2 Carcinogenic Compounds

For carcinogens, a quantitative method is used to estimate human risks, which can be based on cancer incidence in either human epidemiology studies or animal experiments (Health Canada, 1995; US EPA, 2005a; WHO, 2006). Several mathematical models are available for this purpose, and can be downloaded free of charge from the US EPA web site at *http://www.epa.gov/ncea/bmds/*. The linear multistage model is most commonly used; its numerical output is cancer potency in the units of risk per $mg\,kg^{-1}\,day^{-1}$. The models calculate the maximum likelihood estimate (MLE), and upper 95th percentile confidence limit of the cancer potency slope, sometimes called the q1*. The q1* is chosen as a health-protective estimate of the cancer risk. Alternatively, the cancer model is used to calculate the lower 95% confidence

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limit on the dose associated with a 10% cancer risk. This risk level divided by dose provides a cancer potency estimate in the same mathematical form as the q1* (i.e. 0.1/dose in $mg\,kg^{-1}\,day^{-1}$ = potency in units of $(mg\,kg^{-1}\,day^{-1})^{-1}$).

The cancer risk estimation is based on the assumption that there is no practical dose threshold for production of tumours for many carcinogens, at least those which produce tumours by direct reactions with DNA. This generally means that a straight-line extrapolation through zero dose is used to estimate low-dose risk. The justification for this is discussed elsewhere in this book. However, the extrapolation to low dose, over several orders of magnitude, inherently introduces much uncertainty. Uncertainty is also introduced in the default assumption that tumour rates observed in animals are directly applicable to humans (with a dose correction for body-weight scaling). Available data on pharmacokinetics and toxicodynamics may be incorporated into the estimates if available.

The US EPA uses the cancer potency factor to calculate the lifetime risk of drinking water, based on a water consumption value of $2\,l\,day^{-1}$ for a 70 kg adult (US EPA, 2004a; Donohue and Miller, 2008). The US EPA desirable level of the carcinogen in drinking water (the maximum contaminant level goal (MCLG)) is set at zero for carcinogens, while the regulatory value (the maximum contaminant level (MCL)) has a target value of a lifetime risk in the 10^{-4}–10^{-6} risk range. Health Canada (1995) defines a maximum acceptable concentration (MAC) as being in the 10^{-5}–10^{-6} range 'whenever achievable'. WHO (2006) determines a guideline value at the 10^{-5} risk level, and acknowledges that countries may choose to set their own standards at values 'more appropriate for their circumstances', hopefully in the range of 10^{-4}–10^{-6} lifetime risk.

It is important to note that these cancer risk levels refer to each carcinogen, whereas drinking water commonly contains many chemicals with carcinogenic properties. Additive or synergistic interactions among these chemicals are largely unknown and unexplored in toxicity studies. Thus while each cancer risk estimate may be considered to be based on conservative (health-protective) assumptions, the combined risk may be greater.

Chemicals that induce cancer through nongenotoxic mechanisms may be evaluated using the noncancer methods described above. This assumes a threshold for the toxic effect and incorporates the same UFs discussed above. However, evidence for a nongenotoxic mechanism must be quite clear. Chloroform represents the most striking example of this judgement in US EPA risk assessments. This chemical, a by-product of chlorine disinfection, has been judged to produce liver and kidney tumours secondary to cytotoxicity and regenerative hyperplasia (US EPA, 2001a). Several models which incorporate this conclusion into health-risk estimates have been published (Meek *et al.*, 2002; Tan *et al.*, 2003; Liao *et al.*, 2007). However, this mode-of-action decision does not extend to the other trihalomethanes (THMs) formed along with chloroform in drinking water disinfection with chlorine. Thus, efforts to limit total THM content of drinking water continue to be based on concerns about carcinogenicity (WHO, 2005; Health Canada, 2008b).

The widely used chlorotriazine herbicides (atrazine and simazine) represent another interesting example. These chemicals and their proximate metabolites induce mammary tumours in female Sprague–Dawley rats through disruption of the oestrus cycle, leading to overstimulation of mammary cells by endogenous oestrogens (O'Connor *et al.*, 2000; Gammon *et al.*, 2005). The production of mammary tumours is considered inapplicable to humans since the menstrual cycle in humans is quite differently regulated than the rat oestrous cycle. This toxicity re-evaluation would not likely impact the EU ban on the use of these chemicals, since it is based at least in part on concerns over potential environmental effects of the chemicals.

2.3 Calculation of Health-Protective Drinking-Water Levels

2.3.1 Exposure Estimates

Once a health-protective dose is established, an appropriate maximum level in drinking water can be estimated. The calculation is based on exposure to the chemical through the water. In many cases, exposure by the dermal and inhalation routes to chemicals in the water is considered, which is related to normal activities such as showering and bathing. For noncarcinogens, exposure to the chemical from nonwater sources is also considered, because all exposure must be counted, to judge whether the dose exceeds a threshold for toxicity. This is called the relative source contribution (RSC). For carcinogen risk assessment (where no threshold is assumed), only the 'extra' risk from the water needs to be considered. Exposures to the chemical in air or food are judged not to alter the risk from exposure to the chemical in drinking water, so no RSC is incorporated into the risk calculation.

A drinking-water intake value of $2\,l\,day^{-1}$ has traditionally been used for cancer, as well as for noncancer risk assessments in the US and by WHO. This was a somewhat arbitrary value, which was later shown to represent about a 75th percentile consumption value normalized to body weight, or a 90th percentile consumption, not normalized to body weight (Ershow and Cantor, 1989). Health Canada has derived its MACs using a water

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consumption value of $1.5\,l\,day^{-1}$ (Health Canada, 1995). Values reflecting consumption by infants or children may be used when the critical end point refers to a potential effect in this group. Applicable values are $1\,l\,day^{-1}$ for a 10 kg infant or $0.75\,l\,day^{-1}$ for formula consumption for a 5 kg infant (WHO, 2006). More recently, the US EPA has considered the variability in drinking water consumption in various population groups (US EPA, 2004b; 2005b), with the intention of providing protection for a greater proportion of the population, especially infants and children. The California Office of Environmental Health Hazard Assessment (OEHHA) has incorporated the US EPA (2004b) upper 95th percentile drinking-water consumption rates into its recent risk estimates (Public Health Goals) for chemicals in drinking water for both children (OEHHA, 2008a; 2008b) and adults (OEHHA, 2009).

The additional exposures to a chemical from bathing, showering or swimming can be substantial, although dermal-uptake rates from water tend to be rather low (Thrall *et al*., 2000; Xu *et al*., 2002). Volatilization of chemicals from tap water in household uses can be significant for the small halogenated hydrocarbons like chloroform or trichloroethylene (Weisel and Jo, 1996), while inhalation of droplet mists in showering is so small that it could be relevant only for chemicals with a very high potency by inhalation compared to the oral route (Keating and McKone, 1993). US EPA (2001b) has estimated that liquid intake comprises only about 34% of the total daily dose of chloroform, with 31% from inhalation and 27% from the diet (not including tap-water uses); according to this US EPA report, Environment Canada produced similar estimates.

The US EPA guidelines for use of RSCs in their MCLG calculations specify default values in the range of 0.2–0.8 for fraction of exposure derived from water. Relatively stringent requirements for supporting data to estimate a 'true' value have been set (US EPA, 2000), and the default value of 0.2 has been most commonly used. WHO (2006) currently recommends RSC values in the range of 0.1–0.8 and also rarely uses a specific calculated value. The previous versions of the WHO guidelines recommended 0.01 for highly lipophilic chemicals, which may still be implemented in some chemical guidelines or regulations.

All of these authorities, however, make an exception in use of RSC when the data were derived from a human study of exposure to the chemical in drinking water. In this case, no RSC is used (or an RSC of 1.0), such as for barium, copper and fluoride. The rationale is that the human effects associated with consumption of a particular concentration of the chemical in drinking water were observed against a normal, usually unquantified, background exposure level, which is assumed to be about the same as current background exposures. Thus the background exposures cancel each other out.

2.3.2 Calculation Method

Using the US EPA terminology, the drinking-water guidance value for noncarcinogens is calculated as:

$$\mathrm{MCLG} = \frac{\mathrm{RfD}\times\text{body weight}\times\text{relative source contribution}}{\text{drinking water intake}} \qquad (1)$$

For carcinogens, US EPA sets the MCLG at zero as a matter of policy. However, a comparable risk-based value can be derived for carcinogens, as exemplified by the OEHHA approach, to calculate a health-protective concentration C at any particular lifetime cancer risk level, R:

$$C = \frac{R}{\mathrm{q1}^* \times \text{drinking water intake}} \qquad (2)$$

In both of these equations 'drinking-water intake' should be taken as including the dermal and inhalation exposures to chemicals derived from the tap water in normal household activities. The results (MCLG or C) are in units of $mg\,l^{-1}$.

2.3.3 Sensitive Populations

Recently increased efforts have been made to determine whether current risk assessment methods are protective of all identifiable portions of the human population, including, but not limited to, foetuses, infants and the elderly. One aspect of this question is whether the use of a 10-fold factor to account for variability among humans is adequately health-protective. For this evaluation, it is useful to separate variability in exposures from variations in susceptibility to a toxic effect.

Mean drinking-water consumption rates vary from about $13\,ml\,kg^{-1}\,day^{-1}$ in an older child (11–20 years) to $95\,ml\,kg^{-1}\,day^{-1}$ in an infant less than six months old (US EPA, 2004b), a factor of sevenfold. Other important factors, such as average breathing rate and volume vary almost as much by age (OEHHA, 2000). Acute changes associated with daily activities add variability on top of this, and long-term differences associated with lifestyle choices (physically active vs. sedentary) ensure that the mean values above underestimate the total exposure differences among a population. In addition, the effect of ambient temperature on drinking water consumption should not be overlooked. A current military operations manual notes that a person can sweat $1.5–2.5\,qt\,h^{-1}$ under desert conditions, and recommends that at least $4\,gal\,day^{-1}$ (over $15\,l\,day^{-1}$) be provided for drinking (Army Study Guide, 2005). This value is well over 10 times the average adult daily consumption of tap water documented by US EPA (2004b). For chemicals with health-protective levels estimated from relatively short-term effects, such a short-term high-level consumption could be very important, although it would be less

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relevant for a chemical with risk estimated from chronic exposures.

Variations in susceptibility to toxic effects can occur because of life-stage, genetic variability and health and nutritional influences. Foetuses are uniquely sensitive to certain toxic insults because of their rapid development. If a short-term effect on cell division occurs during the first trimester, either teratogenicity or foetal death is likely (Faustman *et al*., 2000; Thrasher and Kilburn, 2001). If the transient effect on cell division occurs at a later stage, it might delay puberty, cause short-term hair loss or go unnoticed. Risk of exposure to carcinogens also varies with age, with the very young being apparently most susceptible (Hattis *et al*., 2005; US EPA, 2005c). Many toxic effects of chemicals vary with life-stage, such as the tooth-mottling caused by fluoride. Fluoride is incorporated into tooth enamel as tooth buds develop, so the first few years of life, when permanent teeth are formed, are the most critical for development of tooth mottling.

The ideal risk assessment would include tests covering each life-stage, and would be based on the effect in the most susceptible life-stage, as described above. Thus no additional consideration of a special susceptibility would be required. This thinking was incorporated into the US Food Quality Protection Act (1996), which mandates an additional UF of 10 for setting pesticide tolerance levels in food, unless adequate pre- and post-natal test data are available. This law does not apply to determination of health-protective levels of chemicals in drinking water, but the same considerations are appropriate. The maximum concentrations of pesticides allowed in drinking water in the EU are not health based. The EU parametric values for individual pesticides ($0.1\,\mu g\,l^{-1}$) and for total pesticides ($0.5\,\mu g\,l^{-1}$) are not science based. The member states of the EU and the European Commission that adopted the Drinking Water Directive 98/83/EC are of the opinion that pesticides should not be present in drinking water. The value of $0.1\,\mu g\,l^{-1}$ is a substitute for zero, not present in water or below the detection limit (Council of the European Union, 1998).

Genetically related variations in susceptibility to toxicants are not yet well-enough documented to establish the range and distribution of human variability for most chemical classes. A few well-known variations include polymorphisms in aldehyde dehydrogenase (Isse *et al*., 2005), glucose-6-phosphate dehydrogenase deficiency (Kaplan and Hammerman, 2004; Cappellini and Fiorelli, 2008) and Wilson's disease, a disorder of copper metabolism (Mak and Lam, 2008). It is important to note that even if the variations could produce a life-threatening illness in affected individuals exposed to otherwise healthy levels of a chemical, the incidence of the condition may be too low to be a matter of concern for drinking-water suppliers. For instance, Wilson's disease, with an incidence of 1 in 10 000 to 1 in 30 000 in various populations, is ignored in setting standards for copper in water (WHO, 2004). Affected individuals, it is reasoned, should have their disease treated by a physician rather than controlled much less efficiently by changes in control of copper in water.

In the same way, differences in susceptibility to toxicants due to illnesses, special conditions and nutritional inadequacies are generally presumed to be the responsibility of the individual. Various chemicals in water, such as aluminium and chloramines, are known to affect people on dialysis for kidney failure (Arduino, 2000), but the chemicals are not regulated based on this factor. Current discussions on control of perchlorate involve, in part, the question of whether dietary deficiencies in iodine (which result in sensitivity to thyroid hormone disruption) should be considered in determination of regulatory levels of perchlorate, an iodine uptake inhibitor (Blount *et al*., 2006; FDA, 2008). A decision might revolve around the prevalence of iodine deficiency in the population, particularly in women of child-bearing age. This involves the issue of whether a practical, statistically meaningful effect on human health could be made with a drinking-water standard. The same consideration applies to cases of allergy to common chemicals. Although allergies to metals such as beryllium and mercury may provoke a very high sensitivity to these elements, the prevalence of the sensitization response is low, and therefore no attempt is made to protect against these 'idiosyncratic' susceptibilities in determination of health-protective levels of the chemicals in drinking water.

3 ESTABLISHMENT OF REGULATORY LEVELS

In the USA, the Safe Drinking Water Act mandates the development of MCLGs and MCLs for chemicals of concern in municipal drinking water supplies (Donohue and Miller, 2008), for cases in which:

- The chemical may have an adverse health impact
- The chemical is known to occur at high levels frequently enough to be of concern
- Regulation would provide a clear opportunity for significant risk reduction.

The MCLG is defined as a level 'at which no known or anticipated adverse effects on the health of persons occur and which allows an adequate margin of safety'. The actual regulatory level, the MCL, must be set 'as close as feasible' to the MCLG, considering technology available, cost and feasibility. The levels are primarily measured leaving the water treatment plant.

A few chemicals are defined in law slightly differently. Standards for lead and copper are defined as

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action levels, measured at the users' faucets. This distinction is primarily because lead and copper contamination is mostly derived from plumbing, not from the water-treatment plant. Control of their levels in drinking water may require changes in the chemistry of delivered water to alter its corrosivity in household pipes, as well as replacement of lead pipes and solder (by the user). This is regulated by 'treatment technique' (TT), which consists of a performance standard for techniques used by utilities to treat water and ensure control of contaminants. The TT regulatory mechanism also applies to acrylamide and epichlorohydrin, which are derived from materials used in water treatment (US EPA, 2008a).

The WHO (2006) Guidelines for Drinking Water Quality define a quite similar process, resulting in guideline values for contaminants. These are incorporated into specific regulations for chemicals in drinking water through European Union Drinking Water Directives. Member States of the EU 'can include additional requirements, for example, regulate additional substances that are relevant within their territory or set higher standards . . . [but] are not allowed to set lower standards' (EC, 2008).

3.1 Cost, Benefits and Feasibility

Cost, benefits and feasibility of specific regulations on drinking-water contaminants are defined in the USA through a quite lengthy process. This may involve development and laboratory validation of an improved analytical method for a chemical; conducting an extensive sampling programme, potentially in different seasons of the year; population exposure estimates; population impact assessment; evaluation of sources of the chemical (industry, agriculture, natural product, residential); investigation of methods and costs of removal of the chemical from water; and an integrated cost–benefit analysis of different control scenarios. Cost–benefit analysis is expertly described by Rauche (2003). The US EPA, as mandated by the 1996 Safe Drinking Water Amendments, has been attempting to develop an improved benefits analysis (US EPA, 2006b). Potential benefits that could be considered include: improvements in human health, enhanced aesthetic qualities, avoided costs of averting behaviour, avoided materials damages, avoided costs of market production, nonuse benefits and information benefits (US EPA, 2006b).

Feasibility of control of contaminants is a moving target, as new water-purification methods develop. Advances in ion-exchange resins, oxidation techniques and reverse-osmosis media, to list just a few, have greatly improved the ability of water suppliers to provide wholesome water supplies at moderate cost (Pontius, 2003b). One of the more exciting developments is the development of high-capacity, lower-pressure reverse-osmosis systems, which have enabled large-scale recycling and reuse of waste waters in several arid regions such as Israel and Southern California. US EPA has supported these efforts through the recent development of formal guidelines for water reuse (US EPA, 2004c). These improved technologies bring with them two further considerations: (i) are new contaminants introduced, both from new materials and from use of previously compromised water sources? (ii) as the cleanup methods improve, are cost–benefit analyses keeping pace, so that previously unattainable health goals can now be reconsidered and the higher-risk contaminant residues correspondingly reduced? Many current publications and conferences on 'emerging chemicals' address these issues, among others (Boyd and Grimm, 2001; Westerhoff *et al*., 2005; Krasner *et al*., 2006; Liebig *et al*., 2006).

3.2 Significant Impact on Public Health

The regulatory process imposes a significant burden, both in terms of the effort required to develop an appropriate regulation for a contaminant and in the monitoring and control required of water suppliers once a regulation is formalized. Therefore regulations should only be developed when the development and imposition of controls is judged to produce a significant beneficial effect. This is particularly true with regard to the WHO guidelines, since they may be applied in a wide variety of countries with greatly differing conditions (WHO, 2006).

The US EPA use of this criterion is explained in a Federal Register notice of regulatory actions for the second drinking-water contaminant candidate list, also known as CCL2 (US EPA, 2008b). US EPA did not develop new regulatory requirements for any of the 11 chemicals for which a determination was made in this particular notice, and included the justification that a regulation 'does not present a meaningful opportunity for health risk reduction', for several of the chemicals based on low incidence of detection at concentrations likely to have an impact on health. The notice acknowledges that individual states might see the situation somewhat differently (based on local problems) and that they should institute controls in their jurisdiction if needed. This does in fact occur; the state of California has developed regulatory levels (MCLs) for several chemicals that are not federally regulated, including methyl *t*-butyl ether (a gasoline additive), perchlorate (a component of rocket fuel and road flares), molinate (a rice herbicide) and others (DPH, 2008).

In this context it should also be noted that, since risk assessment is generally carried out one chemical at a time, cumulative exposures to a variety of chemicals could theoretically have a significant combined impact,

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despite lack of a documentation of harm for any of the individual compounds. This has been most thoroughly explored for disinfection by-products in drinking water, for which studies indicate a potential for increased rates of bladder cancer and reproductive effects (Nieuwenhuijsen *et al*., 2000; Villanueva *et al*., 2003), although data are insufficient for assigning blame to any individual chemical. The conventions applied to the risk assessments for individual chemicals are generally considered more likely to overestimate than underestimate potential hazards, but thinking of water as an aggregate of a great many chemicals provides a rationale for considerable caution (Richardson *et al*., 2007).

It is to be expected that techniques for risk assessments will continue to improve with increased understanding of biological mechanisms and control systems. Potential impacts of environmental contaminants on public health should be less uncertain, and UFs should be decreased. This is the driving force for advances in physiologically based pharmacokinetic modelling, as well as attempts to improve structure–activity models (Gundert-Remy and Sonich-Mullin, 2002; Aitio, 2008; Thompson *et al*., 2008). Advances in high-throughput screening methods (genomics, proteomics, metabolomics) can also be expected to enhance the science of risk assessment, although these tools have not yet been adequately correlated with traditional toxic end points and biomarkers for regulatory use (Blaauboer, 2008; Cullen *et al*., 2008).

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FURTHER READING

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Interactions between Occupational and Environmental Factors in Toxicology, Hazard Evaluation and Risk Assessment

James Gomes and B. Meek

CONTENTS

1 INTRODUCTION

Occupational toxicology examines chemical, biological or physical hazards in the work environment and evaluates their relationship with the development of adverse health effects in the worker. Environmental toxicology, on the other hand, examines the impact of the quality of the environment on human health. The health and wellbeing of an individual is certainly affected by their occupation and environment, and additionally by the interactions between occupational and environmental factors, which may be synergistic or additive in nature. This chapter examines the nature of these interactions and relevant contributing factors and the subsequent impact on hazard evaluation and risk assessment. The factors that affect health outcomes, including personal and lifestyle factors, physiological characteristics, occupational and environmental factors, and genetic and ethnic characteristics are discussed in the context of hazard evaluation, risk assessment and management.

To establish the relationship between work and illness it is necessary to first confirm the exposure at the workplace or in the environment and second, to understand the timing and the nature of exposure. Causality (i.e. understanding that a particular exposure contributes to illness) is based on consideration of factors such as the biological plausibility of the relationship and the strength of the association. It is also necessary to examine the uptake, distribution, biotransformation or excretion of the absorbed dose as affected by intensity of physical activity at work, body composition, age, gender, ethnicity, diet, smoking history, drug treatment and coexposures to ethanol and other solvents. Therefore, at the outset, a few examples of interactions between occupational and environmental factors are presented. Workers who were exposed to trichloroethylene at work and who had stopped to have beer on the way home are described by Stewart *et al*. (1974) to have developed 'degreasers flush', a condition characterized by development of blotches on their faces caused by interaction of trichloroethylene at work and alcohol consumed after work. Interaction between tobacco smoking and occupational exposure to asbestos in the causation of lung cancer has also long been described (Steenland and Thun, 1986).

Several other examples of interaction between occupational and environmental or lifestyle-related exposures have been described in the scientific literature. The interactions could occur between: (i) two industrial organic solvents, (ii) ethanol and an industrial organic solvent,

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(iii) drug and industrial organic solvent and (iv) two drugs. Exposure to toluene and xylene results in increased solvent concentration in blood and delayed excretion of hippuric acid (Tardif *et al*., 1991). A number of interactions between exposure to industrial chemicals at work and ethanol have also been described. Exposure to hepatotoxic chemicals in an industrial environment could interact additively or synergistically with alcohol resulting in liver damage (Hills and Venable 1982). Drugs may affect the toxicokinetics of organic solvents by inhibiting or stimulating the metabolism. Some of the reported effects in humans from drug–solvent interactions include impacts on blood pressure, cardiac output and hepatic function (Holtzman *et al*., 1986; Rosenberg, 1990). Juurlink *et al*. (2003) have reported that patients previously treated with clarithromycin were more likely to be admitted to hospital with digoxin toxicity because of drug interactions.

The principles of occupational and environmental toxicity, and toxic interactions between occupational and/or environmental exposures are explored in this chapter. Interactions between different agents are examined and a range of adverse health outcomes are identified. Relevant factors and those which may play a role in precipitating adverse health conditions in the exposed individual are also described. Readers are presented with a systems biology approach and encouraged to view the situation holistically. Emerging evidence of newer health outcomes and use of newer technologies, including 'omics' are explored.

2 TERMINOLOGY FOR TOXICITY AND INTERACTIONS

Toxicity is defined as the ability of a substance to cause harm or damage to an organism or substructure of an organism. The toxic effect can be at the point of contact (topical) or to the whole system of an organism (systemic) and can either affect the whole organism, or certain organs (organotoxicity) or the cellular structure (cytotoxicity) of the organism. The toxic effects can be acute effects, which occur in the first 24 hours of single exposure, or chronic effects, which occur after a latency period over a prolonged period of time. Toxicity of a substance depends upon a number of factors, such the nature of the substance, dose of exposure, individual characteristics, such as age, gender, physiological functioning and genetic makeup. Toxicity of individual chemicals has been well characterized for most of the chemicals used in industry, commerce or household products. However, toxicity of chemical mixtures is complex and involves consideration of a number of factors. Individual susceptibility to toxic interactions is dependent upon genetic, physiological and environmental factors. Among the genetic factors, individual genotypes and phenotypes play an important role in the manifestation of clinical or subclinical conditions. Among the physiological factors, age, gender, pregnancy and immune-compromised conditions are important parameters in the development of signs and symptoms (Grandjean, 1995).

The fate of a toxicant in human biological systems is described using toxicokinetics and the effect of a toxicant on the human body is described using toxicodynamics. Quantitative translation of relevant information in toxicokinetics and toxicodynamics help to describe physiologically based pharmacokinetic (PBPK) models. Absorption, distribution, metabolism and excretion (ADME) of a toxicant in human biological systems have been described by PBPK models. The ADME of a toxicant is chemical- and mode-of-action-specific, and interactions occur in human biological systems when a toxicant is not completely eliminated or when biological changes induced by a toxicant persist over a period of time. Exposures to multiple chemicals in occupational and environmental settings modify the toxicokinetics and toxicodynamics of a toxicant and, therefore, the outcome for the PBPK model of a substance (Lof and Johanson, 1998). Four possible outcomes for interactions have been described for exposures to multiple chemicals: (i) independent, (ii) additive, (iii) synergistic and (iv) antagonistic. Interaction is independent when two or more chemicals act by different modes of action, different metabolic pathways and do not compete for receptors. Additive interactions occur when two chemicals act by different modes of action, but produce the same or similar effects. Synergistic interactions produce multiplicative effects where one chemical enhances the effect of another and the combined effect is greater than the sum total of each of the interacting chemicals. Antagonistic effects occur when each of the interacting chemicals reduces the effect of another because of competing metabolic pathways.

Terminology and methodology for considering the impact of combined exposures to multiple chemicals has recently been considered under the auspices of the World Health Organization (WHO) International Programme on Chemical Safety (IPCS) project on Harmonization of Approaches to the Assessment of Risk from Exposure to Chemicals (Meek *et al*., 2009). One of the barriers to the development of harmonized methodology for assessing the effects of exposure to multiple chemicals is variation in terminology adopted in different jurisdictions and associated understanding. Also relevant to the development of a framework for risk assessment of combined exposures to multiple chemicals is a common understanding of 'mode of action'. A postulated mode of action is a biologically plausible sequence of key events leading to an observed effect, supported by robust experimental observations and mechanistic data. Mode of action contrasts with 'mechanism of action' which generally involves an

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understanding of the molecular basis for an effect so that causation can be established (Sonich-Mullin *et al*., 2001).

3 HEALTH OUTCOMES FROM INTERACTIONS

Health outcomes from interactions range from development of blotches on skin to severe adverse reactions, to developmental toxicity, to carcinogenesis. Shelley (1964) described dermatitis in a case from interaction between golf-course exposure to the fungicide thiram and alcohol consumption. A number of reports have described a range of interactions between exposure to ethanol and other industrial chemical exposures (Savolainen *et al*., 1980; Snyder *et al*., 1981; Hills and Venable, 1982). Interactions between over-the-counter drugs and alcohol have been described in animal models by Padmanabhan and Pallot (2005) as causing congenital malformations. Caffeine is also reported to produce toxic interactions with a number of drugs and in animal models teratogenic outcomes have also been reported (Beck and Urbano, 1993).

Congenital malformations and developmental effects have been observed for a number of single chemical exposures. For multiple chemical exposures, a range of effects from no interactions, to antagonistic, to additive, to synergistic, to potentiating effects have been observed (Nelson, 1994). Additive effects were observed when mean foetal weights in women were lower in those who had taken aspirin followed by alcohol (Guy and Sucheston, 1986). Wilson (1964) reported synergistic effects in animal models from coadministration of actinomycin, cyclophosphamide and fluorouracil by observing malformations in the embryos of the exposed dams. Similar results have been reported by Gomes and Lloyd (2008) when male and female mice were exposed to a mixture of organophosphorous pesticides premating and preconception. Synergistic effects were also observed in the form of increased incidence of malformations when ribavirin exposure was combined with adenine and guanine simultaneously (Willhite and Ferm, 1978). Potentiating effects are reported by Khera and Iverson (1981) when a teratogenic dose of ethylenethiourea was coadministered with *N*-methyl-2-thioimidazole.

The process of carcinogenesis is significantly affected by multiple chemical exposures and interactions between these exposures. Different exposures to a range of chemicals are reported to enhance the process of carcinogenesis through: (i) increased uptake of carcinogen, (ii) enhancement of carcinogenic activity, (iii) inhibition of DNA repair capacity and (iv) enhancement of DNA lesions (Williams, 1984). Upon exposure to several carcinogens, the observed effects are additive, and carcinogenesis could occur when exposures are sequential or concurrent (Odashima, 1959; MacDonald, 1959). On the other hand, initial exposures to agents not considered to be carcinogenic followed by subsequent exposures either sequentially or concurrently to carcinogenic agents could enhance the process of carcinogenesis (Williams and Furuya, 1984). For example, occupational exposure to ferric oxide enhances the uptake of benzo[a]pyrene, a known carcinogen, by the bronchial epithelium (Kennedy and Little, 1974) and pulmonary alveoli (Antrup *et al*., 1979). Ferric oxide also enhances the carcinogenic activity of dimethylnitrosamine (Feron *et al*., 1972). It is also reported that chemical exposures act to inhibit DNA repair in a nonspecific manner (Williams, 1984). Takahashi *et al*. (1982) reported that 3-aminobenamide acts on the DNA repair process to enhance the action of hetapcarcinogenic agents. Exposures (turpentine, chloroform) that cause hyperplasia also enhance the occurrence of tumours from exposure to methylcholantherene or tar through the process of tumour promotion (Rous and Kidd, 1941).

4 FACTORS THAT ENHANCE ADVERSE HEALTH OUTCOMES FROM INTERACTIONS

Interactions between occupational and/or environmental factors, including lifestyle exposures are adversely affected by a number of other associated factors. These factors include the following: (i) personal characteristics, such as age and gender; (ii) lifestyle characteristics, such as smoking profile, alcohol intake, drug-taking habits and so on; (iii) physiological function, including obesity, immune function, diabetes and so on; (iv) genetic make-up, including genotype and phenotype and (v) occupation or the residential environment, such as place of work, location of residence (proximity to industrial installations). These and other associated factors are discussed in this section of the chapter.

4.1 Personal Characteristics

Since enzyme function and hormone expression vary with age, manifestation of toxicity of chemicals and drugs is age dependent (Meydani, 1994). Drugs and chemicals are metabolized differently in children and adults, due to age-dependent expression of enzymes. Children are reported to more susceptible to lead exposure (Davis and Grant, 1992) then to paracetamol (acetaminophen) (Kauffman, 1992), in which case the toxicity in higher among adults. Since body fat increases during ageing, compared to lean mass, exposure to polar solvents leads to higher residual levels of polar solvents in the blood of

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the elderly, while exposure to lipophilic solvents leads to lower levels in the blood because of higher retention of such compounds in the adipose tissues (Lof and Johanson, 1998). The storage of lipophilic solvents in the adipose tissues leads to sustained release over a longer period of time. Physiological changes in adult life and during the process of ageing lead to reduced hepatic clearance of drugs and solvents in the elderly (Woodhouse and Wynne, 1992; Tam, 1993). These physiological changes include reduction of cardiac output, liver blood flow and liver weight. Also glomerular filtration and tubular clearance decreases with age, leading to reduced clearance (Cody, 1993; Rowland and Tozer, 1995). The age-related decline in hepatic clearance and renal function is also believed to reduce biotransformation and excretion of solvents and related metabolites.

The age-dependent susceptibility to toxicity has also been shown in animal models. Young experimental rats are shown to be more susceptible to *n*-propanol toxicity (Nelson *et al*., 1988) and vinyl chloride toxicity (Swenberg *et al*., 1992). Age appears to decrease Phase I metabolism, because cytochrome P450 (CYP), aniline hydroxylation and *N*-demethylation are all decreased, while age has little impact on Phase II metabolism (James *et al*., 1985; Schmucker, 1985). The severity of hepatotoxicity of allyl alcohol was higher in older rats compared to younger rats, possibly because of decreased availability of glutathione in the former (Rikans, 1984). In rats, total glutathione levels are shown to decrease with age in the liver, blood, kidneys and intestinal mucosa, and, therefore, age-dependent increased drug toxicity may be observed in the elderly (Hazelton and Lang, 1980; Stohs *et al*., 1980). Levels of hepatic glutathione are also observed to be lower in mature rats compared to their younger counterparts (Schmucker, 1985). In the elderly, the decreased elimination of solvents is probably associated with the decreased size of the liver and decreased hepatic blood flow and to a lesser extent to the changes in enzyme activity.

Women and men generally differ in average body composition in that men have leaner mass while women have higher adipose tissue. Women, therefore, tend to have a higher body burden of lipophilic xenobiotics compared to men, given that the exposures are similar. Women also tend to be exposed over a longer period of time because of sustained release of these xenobiotics from the adipose tissues (Gleiter and Gundert-Remy, 1996). Drug disposition, including toxicokinetics and toxicodynamics, in women is influenced by the level of sex hormones, menstrual cycle and oral contraceptive use (Wilson, 1984). A number of reports on gender differences and ethanol metabolism are available. Women, due to their higher body fat content, tend to have smaller volumes of ethanol available for distribution; however, there seems to be small or no differences in metabolic rates in alcohol metabolism (Pikaar *et al*., 1988; van Thiel and Gavaler, 1988). Alcohol dehydrogenase (ADH) activity is higher in men than in women and this is believed to contribute to the observed differences in blood alcohol content (Frezza *et al*., 1990; Seitz *et al*., 1993; Gentry *et al*., 1994). Women are reported to have higher peak blood alcohol levels during ovulation and immediately before menstruation than at other times (Wilson, 1984).

In animal models it has been observed that both castration and oestradiol administration suppress the hepatic microsomal ethanol oxidizing system (MEOS) activity leading to decreased hepatic biotransformation of ethanol. The suppressed hepatic MEOS activity appears to be negated by administration of testosterone (Teschke and Heymann, 1982; Lof and Johanson, 1998). Davis (1992) has reported that the chloroform-induced hepatotoxicity and nephrotoxicity in females in enhanced by dichloro- and trichloroacetic acid exposures. Sex-based differences are reported in expression of cytochrome P450 enzymes (Waxman and Chang, 1995). Hepatic clearance of solvent metabolites is expected to be higher in men than in women because of higher glomerular filtration rates in men (Lof and Johanson, 1998).

4.2 Lifestyle Characteristics

Certain lifestyle habits are believed to interact with occupational and environmental exposures, resulting in enhancement of adverse health effects. An interaction between smoking and exposure to asbestos is reported to significantly increase the incidence of lung tumours (Steenland and Thun, 1986). Caffeine is reported to alter toxicity of a number of prescription and nonprescription drugs. In animal models, synergistic effects were observed for interactions between caffeine and acetazolamide, caffeine and cyclophosphamide, caffeine and 2-ethylhexanol, and caffeine and mitomycine, and antagonistic effects for interactions between caffeine and 5-azacytidine and caffeine and propranolol (Nelson, 1994). Alcohol consumption and smoking are also believed to interact with blood lead levels and urinary *o*-cresol excretion in workers exposed to toluene (Weyermann and Brenner, 1997; Inoue *et al*., 1994).

4.3 Physiological Function

Physiological functioning including coexisting diseases, immune status and obesity can affect the toxicity of occupational and environmental exposures. In patients with liver diseases, oxidative drug metabolism is impaired, while conjugation may not be affected, but plasma-protein binding is reduced. Impaired liver function affects distribution, clearance and elimination of toxicants (Welling and Pool, 1996). When renal function is impaired because of liver disease,

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elimination of drugs such as cimetidine, antipyrine, paracetamol (acetaminophen) and theophylline is prolonged (Hoyumpa and Schenker, 1982; Welling and Pool, 1996).

Many physiological functions, including sleep–wake cycles, blood pressure, renal function and urine volume are periodic and change during the 24 hour cycle (Rowland and Tozer, 1995). Many of the hormones also undergo cyclic changes. These cyclical changes in the physiological function and hormone levels alter the pharmcokinetics of drugs, including absorption, distribution, biotransformation and elimination of xenobiotic substances (Labrecque and Belanger, 1991). Diurnal variation in alcohol concentration has been observed in blood, liver, brain and urine in animal models exposed to ethanol at different times during the day (Soliman and Walker, 1979). The circadian variations in hepatic levels of reduced glutathione is believed to be the determinant of hepatotoxicity of 1,1-dichloroethylene (Jaeger *et al*., 1973), allyl alcohol (Hanson and Anders, 1978), chloroform (Lavigne *et al*., 1983) and styrene (Decarie and Chakrabarti, 1989). Advanced insulin-dependent diabetes is associated with defective carbohydrate metabolism, hormonal perturbations and reductions in circulating testosterone, thyroid hormone and plasma growth hormone. Alterations in these hormones disrupt many liver cytochrome P450 enzymes, including induction of CYP 2A1, 2B1, 2C7, 2E1, 4A2 and 4A3 and reduction of 2A2, 2 C11 and 2 C13 (Waxman and Chang, 1995).

Induction in cytochrome P450 induces detoxification, particularly when low to moderate levels of substrates are present. Induction, therefore, is a protective mechanism and the extent of induction is inversely related to the rate of biotransformation. Enzyme induction by one chemical may also lead to increased metabolism by other compounds (Whitlock and Denison, 1995). Inhibition of cytochrome P450 can also act as a metabolic inducer (Hodgson, 1980). Inhibition involves a direct interaction with active sites on the enzymes and is generally rapid. For example, intake of ethanol increases blood concentrations of toluene in one hour. However, induction could also be a slow process; for example, intake of ethanol the evening before, followed by exposure to *m*-xylene decreases the concentration of xylene in blood. Substrates that are metabolized slowly are also potent enzyme inducers; for example, 2,3,7,8-tetracholorodibenzo-*p*-dioxin (TCDD), a potent inducer of cytochrome P450, is metabolized slowly by the enzyme (Parke *et al*., 1991).

The induction of the enzymes depends on the level of exposure to the solvent, metabolic clearance and the flow of blood to the metabolizing organs. For low-dose exposures to solvents whose intrinsic clearance prior to enzyme induction is much larger than the hepatic blood flow, the toxicokinetics do not affect metabolic induction. However, for high-dose exposures, as metabolism approaches saturation, enzyme induction is dependent upon metabolic clearance and toxicokinetic pattern (Sato, 1991). The toxicity of a solvent could be increased by the formation of reactive metabolites post Phase I enzyme induction. This toxicity can be further enhanced if the reactive metabolites are not cleared by induction of Phase II enzymes (conjugation enzymes). The toxicity and the build up of toxic metabolites of a solvent are, therefore, dependent on the balance between the induction of Phase I and Phase II enzymes (Bock *et al*., 1990; Okey, 1990). Ethanol induces CYP 2E1 by a post-translational mechanism at low ethanol concentrations in blood, and a transcriptional or messenger-RNA-stabilization mechanism at high ethanol concentrations in blood. Ethanol also induces CYP 2E1 *in vivo* by protein stabilization (Roberts *et al*., 1995).

4.3.1 Induction by Solvents

A number of solvents induce cytochrome P450 in animals and humans. Ethylbenzene, *n*-propylbenzene and *m*-xylene are effective inducers of aromatic hydroxylation of toluene in rats (Backles *et al*., 1993). Methyl *n*-alkyl ketones and *n*-alkylbenzenes induce CYP 2B1 and CYP 2B2 (Imaoka and Funae, 1991). Occupational exposures to solvents sometimes enhance biotransformation of other solvents; for example, in workers exposed to styrene, a decrease in styrene concentration in blood and an increase in metabolic clearance was observed (Dolara *et al*., 1983; Dossing, 1983). In workers exposed to petroleum, increased antipyrene clearance was observed, indicating increased metabolism of petroleum components (Harman *et al*., 1981). In experimental animals, the excretion of xylene metabolites was higher and the concentration of xylene in adipose tissues was lower following repeated exposure to xylene (Liira *et al*., 1991).

Solvents also induce Phase II enzymes. In rats exposed to methyl-*t*-butyl ether a dose-dependent increase of liver uridine diphosphate (UDP)-glucuronyl transferase activity was observed in liver and kidney microsomes (Savolainen *et al*., 1985). Repeated exposure of experimental animals to limonene increased the activity of cytochrome P450, glutathione-S-transferase (GST) and UDP-glucuronyl transferase in the liver (Maltzman *et al*., 1991; Zheng *et al*., 1992). Enhanced hepatotoxic and nephrotoxic effects were observed from exposure to organic solvents such as carbon tetrachloride, chloroform, trichloroethylene and tetrachloroethylene following an earlier exposure to ethanol, but not to simultaneous exposure to ethanol. This is a function of the induction effect of ethanol being at maximum shortly after ethanol is cleared from the body (Cornish *et al*., 1977; Sato *et al*., 1981; Zimmerman, 1986). The hepatotoxic effects of chlorinated solvents are also potentiated by aliphatic alcohols, ketones and their precursors (Traiger and Plaa, 1971; 1974; Hewitt *et al*., 1980a; 1980b; 1987).

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4.3.2 Induction by Ethanol

Chronic consumption of alcohol is shown to accelerate biotransformation of a number of organic compounds such as benzene, toluene, styrene, xylene, chloroform, trichloroethylene and carbon tetrachloride (Sato *et al*., 1980; 1981). The effects are not pronounced after 16–18 hours of consumption of alcohol and when ethanol has been cleared from the body. The effects disappear following a one day withdrawal of alcohol (Hetu *et al*., 1982). In toluene-exposed workers, it has been observed that the concentration of toluene in blood is lower with regular intake of alcohol than in nondrinkers (Waldron *et al*., 1983). However, it has been observed that drinking alcohol the evening before exposure to *m*-xylene decreases the concentration of *m*-xylene in blood and alveolar air during and after exposure (Tardiff *et al*., 1994). This effect was seen at high exposure levels only and not at low exposure levels, because biotransformation of *m*-xylene is catalysed by a high-capacity enzyme system (Lof and Johanson, 1998).

4.3.3 Induction by Drugs

Phenobarbital is reported to increase biotransformation of several solvents, including benzene, toluene, xylene, *n*-hexane, chloroform, carbon tetrachloride and trichloroethylene (Sato and Nakajima, 1985). Pretreatment of experimental animals with phenobarbital is reported to enhance the metabolism of methyl-*n*-butyl ketone (Abdel-Rahman *et al*., 1976; Couri *et al*., 1978). Although the excretion of *m*-xylene metabolites in urine increases in experimental animals following phenobarbital exposure, no increase was seen in humans from similar exposures (David *et al*., 1979).

4.3.4 Induction by Smoking

Tobacco smoke induces the cytochrome P450 system. In smokers, because of enhanced biotransformation, requirements of increased drug dosages and reduced clinical effects are observed for many pharmaceutical drugs (Miller, 1989). Tobacco smoke also enhances the metabolism of solvents. Smoking leads to a dose-dependent induction of CYP 1A2 and phenacetin transformation (Kalow and Tang, 1991; Pantuck *et al*., 1974). Decreased metabolism of toluene is reported in volunteers after three weeks of cessation of smoking (Hjelm *et al*., 1988).

4.3.5 Induction by Diet

Diet and nutritional status are believed to alter the metabolism of drugs and solvents and contribute to inter- and intra-individual variability. Diet and nutrition is reported to affect Phase I enzymes more than Phase II enzymes (Walter-Sack and Klotz, 1996). A high-protein and low-carbohydrate diet enhances metabolism and elimination of antipyrine, theophylline and phenacetin (Alvares *et al*., 1976a; Hoyumpa and Schenker, 1982). A high-protein diet is believed to increase enzyme activity (Mgbodile and Campbell, 1972; Paine and McLean, 1973). High-fat diets increase induction of CYP 2E1 (Yoo *et al*., 1990, 1991; Takahashi *et al*., 1992). High-carbohydrate and low-protein diets delay the clearance of antipyrine and theophylline (Kappas *et al*., 1976). Starvation, fasting and low-carbohydrate intake induce CYP 2E1 (Favreau *et al*., 1987; Hong *et al*., 1987). Starvation is believed to enhance the toxicity of carbon tetrachloride due to induction of metabolism (Krishnan and Stenger, 1966). Increased hepatotoxicity has been observed in fasting experimental animals exposed to halogenated hydrocarbons due to induction of CYP 2E1 (Jaeger *et al*., 1975; Diaz Gomez *et al*., 1975; Nakajima, 1997). Fasting reduces glutathione levels that increase following a high-protein or high-carbohydrate diet. However, glutathione levels continued to decrease when a higher fat diet was provided following starvation (Maruyama *et al*., 1968; Tateishi *et al*., 1974).

Diets rich in cruciferous vegetables induce CYP 1A2, leading to increased clearance of antipyrine and phenacetin in experimental animals (Loub *et al*., 1975; Vistisen *et al*., 1991). In other studies with experimental animals, cruciferous vegetables are observed to induce CYP 1A1, CYP 1A2, CYP 2B and CYP 2E1 enzymes (Vang *et al*., 1991). Charcoal-grilled meats induce CYP 1A2 and increase metabolite clearance of antipyrine, theophylline and phenacetin in humans (Kappas *et al*., 1978; Alvares *et al*., 1979). Concomitant administration of grapefruit juice is reported to increase the blood concentration of a number of drugs in humans, often resulting in adverse clinical effects. These effects are mediated by chemicals in grapefruit juice that inhibit CYP 3A4 in the gut (Bressler, 2006).

4.4 Work and/or Residential Environment

Occupational and/or environmental exposures to toxic substances cause either induction or repression of metabolizing enzymes. Toxic substances can cause inhibition of cytochrome P450 by either binding reversibly to the enzymes, or forming semi-irreversible complexes with the haeme iron atom, or by binding irreversibly to the protein or the haeme group. The toxicity of solvents from environmental and/or occupational exposures is dependent upon their interactive effects and their dosages. Metabolic interactions between solvents depend on the nature of the solvents, as well as the level of exposure and metabolic pathways through the enzyme system (Sato, 1991).

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4.4.1 Solvent–Solvent Interactions

Ethanol and methanol are both equally reactive in the peroxidative system, but ethanol is much more reactive in the ADH system. Both ethanol and 1-butanol are effective inhibitors of methanol oxidation in animal models (Makar *et al*., 1968). The accumulation of formic acid is remarkable after methanol ingestion and plays a major role in the metabolic acidosis and ocular toxicity observed in human subjects (Bergeron *et al*., 1982; Eells, 1992; Jacobsen *et al*., 1983; Medinsky *et al*., 1997). Pyrazoles (4-methyl pyrazole) also inhibit the oxidation of methanol in both animal models and humans (Jacobsen *et al*., 1996). Delayed metabolic inhibition and decreased urinary excretion of ethyl benzene and *m*-xylene metabolites have been observed following concurrent exposure to ethyl benzene and *m*-xylene (Angerer and Lehnert, 1979; Engstrom *et al*., 1984). Similarly, Shibata *et al*. (2002) and van Engelen *et al*. (1997) have reported decreased concentrations of 2,5-hexanedione in serum and urine following human exposure to *n*-hexane and methyl ethyl ketone. Concurrent exposure of methl ethyl ketone and xylene in humans is found to inhibit biotransformation of xylene by methyl ethyl ketone, but no inhibition effects have been observed following exposure to methyl ethyl ketone and toluene (Liira *et al*., 1988; Dick *et al*., 1984; Tolos *et al*., 1987). These examples show that solvent–solvent interactions generally decrease biotransformation rates, resulting in higher body burden and delayed excretion of urinary metabolites.

Competitive inhibitory effects have also been observed from exposure to two or more solvents in animal models. Sato and Nakajima (1979) have reported that toluene is an effective inhibitor of hydroxylation of benzene than benzene is of toluene side-chain hydroxylation. Another example is methyl ethyl ketone, which is a strong inhibitor of side-chain hydroxylation of *m*-xylene, but not of the benzene ring (Liira *et al*., 1991). Concurrent exposure to toluene and *n*-hexane reduces biotransformation of *n*-hexane to 2,5-hexanedione, decreases the urinary excretion of 2,5-hexanedione and decreases neurotoxicity in rats (Takeuchi *et al*., 1981; Perbellini *et al*., 1982). In a similar manner, concurrent exposure to *n*-hexane and methyl ethyl ketone causes potentiation of the neurotoxicity, although metabolites 2-hexanone and 2,5-dexanedione are reduced (Takeuchi *et al*., 1983; Shibata *et al*., 1990a; 1990b).

4.4.2 Ethanol–Solvent Interactions

Alcohol intake has a significant inhibitory effect on biotransformation of several solvents. The major (80–85%) metabolic pathway for alcohol is through ADH. The other pathways are MEOS (10–15%) and the catalase system (5–10%) (Teschke and Heymann, 1982; van Thiel and Gavaler, 1988). When blood alcohol levels are moderate, ethanol is metabolized by the ADH pathway, whereas when high blood ethanol levels are present, ethanol is metabolized by the MEOS system (Teschke and Gellert, 1986).

Ethanol–solvent interactions occur when blood alcohol levels are moderate or high. When blood ethanol levels are higher compared to the blood solvent levels, the inhibitory effects of the solvent on the biotransformation of ethanol are small, whereas when the blood levels of both ethanol and solvent are moderate or high, significant effects are observed in both animal models and humans. In animal models, high doses of ethanol and toluene inhibit biotransformation of both (Takahashi *et al*., 1987), and moderate doses of ethanol inhibit biotransformation of toluene (Dossing *et al*., 1984; Wallen *et al*., 1984). Also, moderate levels of ethanol inhibit biotransformation of *m*-xylene (Riihimaki *et al*., 1982a; 1982b), styrene (Wilson *et al*., 1983), trichloroethylene (Stewart *et al*., 1974), methyl ethyl ketone (Liira *et al*., 1990a) and 2-butoxyethanol (Dornow *et al*., 1991). Low doses of ethanol also inhibit the metabolism of toluene (Baelum *et al*., 1993). Concurrent smoking and alcohol drinking suppresses the conversion of xylene to methyl hippuric acid, as well as toluene to hippuric acid (Huang *et al*., 1994; Inoue *et al*., 1993a; Inoue *et al*., 1993b) primarily because of ethanol. Concurrent exposures to ethanol and carbon disulfide, diethyl sulphoxide, dimethyl formamide and chlorinated solvents could result in flushing, tachycardia, tachypnoea and sweat, caused by accumulation of acetyladehyde in the body (Cicero *et al*., 1980; Chadha *et al*., 1983; Kaplita and Smith, 1986).

4.4.3 Drug–Solvent Interactions

Drugs are reported to affect toxicokinetics of organic solvents through a number of pathways. Drugs unilaterally have been reported to affect hepatic blood flow (cimetidine, phenobarbital, propranolol), blood pressure (caffeine, propranolol, diuretics) and cardiac output (digitalis, propranolol) (Holtzman *et al*., 1986; Rosenberg, 1990). Drugs are also known to affect protein binding of solvent metabolites. For example, metabolites of trichloroethylene are displaced from plasma proteins by salicylates, sulfonamides and phenylbutazone (Rosenberg, 1990). Alcohol drinkers are believed to have elevated blood pressures compared to nondrinkers in a dose-dependent manner, while smoking is associated with lower blood pressure (Savdie *et al*., 1984).

Metabolic interaction of drugs and solvents occur when they are metabolized by the same enzyme. Antipyrine metabolism is delayed by the intake of cimetidine and propranolol because of competitive inhibition of cytochrome P450 (Feely *et al*., 1984). Intake of paracetamol delays the metabolism of toluene (Lof *et al*., 1990b) and acetylsalicylic acid delays the transformation of *m*-xylene to methyl hippuric acid (Campbell *et al*., 1988). Reduced biotransformation of ethanol has been

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reported from chlorzoxazone intake, because of competitive inhibition of the CYP 2E1 enzyme (Peter *et al*., 1990). Both acetone and toluene delay the urinary excretion of chlorozoxazone (Ernstgard *et al*., 1998).

4.4.4 Metal–Solvent Interactions

Metals inhibit Phase I and Phase II xenobiotic-metabolizing enzymes. In animal models, it has been shown that cadmium, cadmium chloride, mercury and methyl mercury are inhibitors of cytochrome P450 (Schnell *et al*., 1979; Alvares *et al*., 1972). Acute exposure to lead has also been reported to inhibit drug metabolism in animal models (Alvares *et al*., 1976b; 1978). Alvares *et al*. (1975) has shown that in children, antipyrine metabolism is delayed in lead poisoning. Metals such as cadmium, chromium and mercury bind to plasma proteins. Since some of the solvents also bind to plasma proteins, they compete with metals for binding sites in concurrent exposures to metals and solvents (Rosenberg, 1990). The interactions between exposure to metals and solvents depend on the levels of exposure. Exposure to mercuric chloride and zinc chloride in animal models either increase or decrease the hepatotoxicity of bromobenzene, depending on the dose (Chakrabarti and Brodeur, 1985; 1986). Aitio *et al*. (1978) have reported that cadmium iodide and mercuric acetate inhibit hydroxylation of benzo[a]pyrene, de-ethylation of ethoxycourmarin, hydration of epoxide and conjugation of glutathione.

4.5 Genetic and Ethnic Characteristics

There is a wide variation in the response of individuals to environmental and/or occupational exposures. Similar differences are observed in drug responses. One of the reasons for these differences is physiological pharmacokinetic differences and metabolic capacity (Rowland and Tozer, 1995). A number of other factors such as age, diet, lifestyle habits, and occupational and environmental exposures have also been identified (Anderson *et al*., 1986). Jang *et al*. (1997) have shown that Caucasians have higher concentrations of perchloroethylene, but lower concentrations of styrene in exhaled air compared to Orientals, following exposure to perchloroethylene and styrene respectively. Orientals were also observed to have lower concentrations of trichloroacetic acid, methyl hippuric acid and phenyl glyoxylic acid in urine compared to Caucasians following exposures to perchloroethylene, xylene and styrene, respectively.

Intra-individual differences in response to occupational and environmental exposures could possibly be due to genetic polymorphisms in xenobiotic metabolizing enzymes (Wrighton and Stevens, 1992; Daly *et al*., 1993; Idle *et al*., 1992). It is believed that poor metabolizers are homozygous for a defective gene variant, while fast or moderate metabolizers are either homozygous or heterozygous carriers of the functional gene variants. Genetic polymorphisms have been reported for a number of Phase I and Phase II enzymes, including the ones for the enzymes CYP 2D6, GST M1, GST T1 and *N*-acetyl-transferase (NAT2) (Daly *et al*., 1993; Curran *et al*., 2000). Some of these polymorphisms give rise to defective phenotypes, however, other phenotypic polymorphisms have also been reported for CYP 1A1 for polyaromatic hydrocarbons, CYP 1A2 for phenacetin and caffeine, CYP 2C19 for mephenytoin, CYP 2A6 for coumarin, CYP 2E1 for ethanol and CYP 2C9 for tolbutamin (Daly *et al*., 1993; Hassett *et al*., 1994).

ADH and aldehyde dehydrogenase (ALDH) metabolize ethanol and other hydroxylated solvents. Ethnic differences have been reported for polymorphisms in ADH2 and ADH3 loci. Variant ADH2 is reported to be present in 5–20% of Europeans and 85% of Orientals (Stamatoyannopoulos *et al*., 1975). Variant ADH3 has been detected in 25% of black Americans. Both variants are associated with increased rates of catalytic activity, which help to explain the interethnic differences in ethanol metabolism rates (Daly *et al*., 1993). Among the different variants of ALDH, Caucasians have two major variants, ALDH1 and ALDH2, while 50% of the Orientals have only one (ALDH1) (Teng, 1981; Yoshida *et al*., 1983; 1989; Mizoi *et al*., 1985). Lack of ALDH2 leads to higher acetaldehyde levels in blood post alcohol consumption (Mizoi *et al*., 1983). Higher acetaldehyde levels in the Orientals who lack ALDH2 leads to higher frequency of alcohol intoxication and facial flushing (Harada *et al*., 1985). Similar flushing has been reported among Caucasians who lack or are polymorphic in ALDH1 (Daly *et al*., 1993). Native Americans also metabolize ethanol to acetaldehyde more rapidly than Caucasians resulting in higher intoxication and flushing rates (Segal and Duffy, 1992). Polymorphisms in ALDH2 are also believed to affect the oxidation of benzylalcohol to hippuric acid. Benzylalcohol levels are lower in individuals with inactive homozygous ALDH2 genotype compared with those with heterozygous or normal homozygous genotypes (Lof and Johanson, 1993).

Ethnic differences have also been reported for biotransformation by Phase II enzymes. Fewer numbers (50%) of Europeans and Africans express NAT2 than do the Orientals (100%) and Eskimos (95%) (Daly *et al*., 1993). The GST family of enzymes catalyse the conjugation of reduced glutathione to a variety of electrophilic and hydrophilic compounds. There are four generic classes of GST enzymes: α, μ, π and θ which contribute to Phase II biotransformation of xenobiotics (Meyer *et al*., 1991; Schroder *et al*., 1992). Polymorphisms have been observed in GST T1 and GST M1 classes of GST enzymes. The Chinese and Koreans are reported to have the highest prevalence of null genotypes for GST T

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(Nelson *et al*., 1995). A number of substrates of GST T1 have been described, for example, methyl chloride, methyl bromide, methyl iodide, methylene chloride and ethylene oxide (Hallier *et al*., 1990; 1994; Fost *et al*., 1991). Lack of GST1 is reported to adversely affect solvent clearance for workers exposed to methyl bromide, for whom haemoglobin adduct levels were elevated (Iwasaki *et al*., 1989). Other studies have shown that workers exposed to methyl chloride had higher concentrations of methyl chloride in their blood and alveolar air (Nolan *et al*., 1985).

Phenotype studies have shown that 11% of the population lack conjugation activity of methyl chloride, whereas, 46 and 43% have intermediate and high activity (Warholm *et al*., 1994). In Europe, non-conjugators of methyl chloride are 11–12% in Sweden and Finland (Warholm *et al*., 1995), 30–40% in Germany (Peter *et al*., 1989) and 40% in Great Britain (Pemble *et al*., 1994). Interethnic differences in xenobiotic metabolism are supported by studies in North America in the Mexican population, where mutant allele frequency has been reported to be in the range of 30–43% (Mendonza-Cantü *et al*., 2004). Gene-dependent toxicokinetic studies have been well reported. Fast conjugators have high uptake, high metabolic clearance and lower concentration in blood, and rapid post-exposure decay, while nonconjugators show opposite effect and intermediate conjugators in between effects. Different solvent toxicokinetics have been reported for different ethnic groups.

Ethnic differences between Japanese and Chinese workers have been observed in the biotransformation of trichloroethylene (Inoue *et al*., 1989) and tetrachloroethylene (Seiji *et al*., 1989); the excretion of the metabolites in urine was lower for the Chinese workers. The concentration of hippuric acid and *o*-cresol in urine was half in Chinese and Korean workers compared to the Japanese workers, following their exposure to toluene (Inoue *et al*., 1988a; Liu *et al*., 1992). Increased susceptibility to cancer has been associated with ethnic differences for the genes NAT2, CYP 1A1, CYP 2D6, CYP 2E1, GST M1 and GST T1. Bladder cancer and gastric adenocarcinoma has been associated with genetic polymorphisms in NAT2, GST M1 and CYP 2D6 (Anwar *et al*., 1996; Brockmoller *et al*., 1996). The GST M1 null genotype is associated with increased risk of smoking-related cancers (Wolff *et al*., 1994). Polymorphisms in CYP 1A1 and CYP 2E1 are associated with lung cancers (Yu *et al*., 1995; Wu *et al*., 1997). Defective conjugation is associated with increased risk of lung cancer from cigarette smoking (Seidegarrd *et al*., 1986). The increased susceptibility to lung cancer in smokers with the GST M1 null genotype is believed to be due to nonclearance of carcinogen benzo[a]pyrene (Nakajima *et al*., 1995a).

5 RISK ASSESSMENT

In recent years, there have been advances in the development of more integrated approaches to consider the combined effects of toxins on cells, organs or organisms. This integrated approach is termed the systems-biology approach (Kitano, 2002) and allows for the examination of the modes of action, involving different targets within cells, tissues or organs. In the coming years, the systems-biology approach will be instrumental in assessing basic biology and in evaluating the conditions under which chemical stressors, either singly or in mixtures, may lead to clinically significant morbidity or toxicity in exposed population (Anderson and Denison, 2004). The systems-biology approach emphasizes the biology of organisms and dose-dependent relations for endogenous proteins and signalling molecules. The mode of action of xenobiotic chemicals is related to the endogenous perturbations of the endogenous signalling networks and manifestations of clinically relevant signs and symptoms. PBPK and physiologically based pharmacodynamic (PBPD) models of these perturbations may help to characterize the relationship between exposure and manifestation of adverse health effects.

With the development of omics (genomics, transcriptomics, proteomics and metabolomics), it has been suggested that these are potential technologies to study risk assessment of single or multiple toxicants and to predict interactions and combination effects. It is also believed that omics will provide the necessary tools needed to understand the mechanisms of action of toxicants at a molecular level (Cunningham *et al*., 2003; Bernauer *et al*., 2005). However, given the large number of chemicals in commerce and the endless combinations and complexity of interactions, a multifaceted approach may be needed, with contributions from computational technology, mechanisms of action, modes of action and mathematical modelling (El-Masri *et al*., 1995).

Current evolving frameworks for consideration of risk from combined exposures to multiple chemicals are proposed to be iterative, involving stepwise consideration of both exposure and hazard in several tiers of increasingly data-informed analyses. The approach involves decision-based analysis that takes into account relevant information at an early stage as a basis to scope the need or not for additional assessment and recommend any required data generation. Early consideration of potential for exposure (prior to any consideration of hazard) is essential in determining critical next steps, since there is no need for further assessment if there is no or minimal exposure (Meek *et al*., 2009).

The extent of assessment and nature of recommendations for generation of additional data are dependent upon the extent of the knowledge base, the magnitude of public-health concern (i.e. taking into account margins between exposure and effect) and the objective of the risk assessment (e.g. implications of potential

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risk management decisions). It is envisaged, then, that approaches will range from predictive methodologies and conservative assumptions in early tiers, to more realistic estimates of risk and rigorous descriptions of uncertainties in later tiers, based on increasingly data-informed and probabilistic approaches.

This tiered approach is considered essential to ensure that the expenditure of resources is proportional to the public-health concern and consistent with regulatory objectives. This includes the purpose of the assessment, such as prioritization for assessment, screening for additional assessment and/or risk management (including bans, controls and other options, such as education).

The proposed framework for consideration of risks from combined exposures to multiple chemicals is also hypothesis driven, involving analysis of available information followed by a conclusion in which a hypothesis is developed and refined (as necessary). This enables transparent and systematic analyses in the context of a 'weight-of-evidence' approach consistent with the IPCS Framework for Analyzing the Relevance of a Cancer Mode of Action for Humans (Boobis *et al*., 2006) and the IPCS Framework for Analyzing the Relevance of Noncancer Mode of Action for Humans (Boobis *et al*., 2008). It also enables incorporation of data from evolving technologies, including systems approaches.

6 CONCLUSIONS

Interactions occur when concomitant exposures occur to multiple chemicals from occupational and/or ambient environments, diet, drugs or lifestyle habits. The type of response in interactions can range from rash to dermatitis, to severe acute condition, to cancer. The nature of the response depends on the nature of exposure to the chemical in question and the individual that is exposed. Among the exposure- or chemical- based factors, the type of chemical, the level of exposure and the type of exposure are important and need to be considered in assessing the risk. Among the individual-related factors, age, gender, dietary habits, drugs (both recreational and therapeutic), lifestyle habits (including alcohol consumption and smoking profile), physiological characteristics (concomitant and pre-existing health conditions, chronic diseases, immune status, etc.) and genetic characteristics (including genotype, phenotype and ethnicity) are important. Many normal physiological functions, including sleep–wake cycles, blood pressure and renal function, influence the interaction response or even the response to a single chemical exposure. Altered or perturbed hormones also influence the interactions. A number of approaches to more systematically assess risk associated with combined exposures to multiple chemicals, including stepwise consideration of both exposure and hazard in several tiers of increasingly data-informed analyses, including that from evolving integrated approaches in systems biology have been proposed.

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Susceptibility of Children to Environmental Xenobiotics

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CONTENTS

1 INTRODUCTION AND HISTORICAL PERSPECTIVE

The realization that the health of a child can be drastically affected by elements in his or her environment has only been appreciated relatively recently in history. However, the growth of interest in paediatric environmental health reflects a widespread recognition that the impact of the environment on children is of major importance, not only for this generation, but also for succeeding generations in the future. Such environmental exposures often have different determinants, mechanisms and consequences for children than for adults. Whereas more than 80 000 chemicals are in widespread commercial use, most of these were synthesized within the past 50 years and few have been adequately tested for their toxic effects on humans generally, let alone newborns, infants and children (Stein *et al*., 2002).

1.1 Scope of Environmental Impacts on Children's Health

Childhood is a time of rapid growth and development, with changes in anatomy, organ-system functioning, physiology, metabolic capacity, physical size, and cognitive, developmental, psychological and sexual maturation. Children have a greater vulnerability to many environmental chemicals and other toxins than do adults, by virtue of their smaller size, increased weight-adjusted intake of some toxicants, developmental differences in body systems and functions, overall physiological and toxicodynamic differences,

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Table 1 Some determinants of child health

Social		**Health**
Family function		Physical health
Family/religious supports		Mental health
Community supports		Developmental physiology
Cultural/ethnic beliefs		Behavioural development
Cultural/ethnic practises		Cognitive development
Equity and social justice		Psychosocial development
Family stress/violence		Preventive health measures
Economic support		Physical growth
Quality of medical care		
Government	**Environment**	**Perinatal**
Health policies	Shelter	Genetics/defects
Regulations	Food and nutrition	Preconception health
Standards	Hazards/degradation	Maternal nutrition
Monitoring	Toxins/contaminants	Maternal/foetal health/exposures
Enforcement	Sanitation/hygiene	Gestational age
Health resources	Potable water	Birth weight
Labour laws	Industrial sites	Reproductive health
	Violence/aggression	Toxic waste sites
	Stress	Fertility
	Open green space/play	Labour and delivery
	Household hazards	Postnatal health
		Lactation

and differences in the environments they inhabit, the foods they eat and the behaviours they display every day. Even the prenatal environment and foetal exposures from placental transfer can determine foetal development and survival, as well as the child's postnatal health. **Table 1** illustrates some of the determinants of a child's health status that interact to contribute to a complex web of causation. The overall impact of such adverse effects, manifested by subsequent poorer health and the need for costly health careservices, is amplified by the longer lifespans of children and is associated with intergenerational transmission of harmful effects, to the detriment of mankind.

1.2 Protection of Children by Caregivers

Children are also subject to special risks by virtue of their dependencies and the social, cultural and economic circumstances of their families. A child relies on caregivers for his or her housing, water, sanitation, a healthy, nutritious diet, education, protection and nurturance. Parents must protect their children by controlling their environment: for example, by baby-proofing a home using gates and electrical outlet covers, by buckling toddlers into safety-engineered car-seats for each automobile ride and by using sun-block to protect them from overexposure to radiation from sunlight.

Caregivers can contribute to a child's risk, for example by exposing a foetus to alcohol or exposing a child to drugs of abuse or environmental tobacco smoke. Poor parenting techniques, social isolation, or aggressive and aberrant behaviours can contribute to a home atmosphere of unpredictability, disruption and violence, all of which will adversely affect the health and growth of a child. Some culturally contextual health practices may put children at risk for environmental exposures. For example, the use of tongue powders daily is promoted in certain Asian cultures to preserve infant good health. However if the tongue powder is contaminated with a heavy metal such as lead, then chronic poisoning of the infant can be significant (Woolf *et al*., 2008). Immigrant families using contaminated home remedies, herbs or even spices in cooking their food may also put their children at risk for heavy metals poisoning (Woolf and Woolf, 2005). Ayurvedic medicines, often used by people living in South Asian countries, are frequently contaminated with significant quantities of lead, arsenic, cadmium or other metals.

Disorganized families living under stress may have poor access to, or underutilize, available healthcare services, so that their children will not be afforded the preventive health programmes that contribute to healthy living. Children with underlying diseases, such as asthma or diabetes, may suffer exacerbations of their condition because of a lack of regular monitoring of their health status if families are not attentive to seeking out

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preventive-care services. For example, the prevalence of physician-undiagnosed asthma among Detroit-area children was estimated to be 14.3% (Joseph *et al*., 1996).

Workers who do not practise good industrial hygiene can inadvertently contaminate their families by bringing home occupational chemicals clinging to tools, shoes, hair, skin and clothing. Numerous studies have documented elevated blood lead levels in children whose parents are employed in industries where lead contamination is an occupational hazard. Tissue mercury levels were found to be elevated in the children of parents who worked in a thermometer-manufacturing facility (Hudson *et al*., 1987). Some children of workers employed in the manufacture of synthetic oestrogens developed gynaecomastia and breast discomfort (Budzyńska *et al*., 1967). Contact with contaminated clothing produced chloracne in children whose parents worked in industries where there was exposure to dioxin and other polycyclic halogenated compounds (Mocarelli *et al*., 1991).

1.3 Prevalence and Costs of Environment-Associated Illnesses

The prevalence of some chronic childhood conditions is inexplicably increasing everywhere. Autism rates are estimated to be as high as 1 in 150 births (Stephan, 2008). The prevalence of asthma increased by almost 40% from 3.1% of children in 1981 to 4.3% in 1988 (Weitzman *et al*., 1992). At least part of this increased prevalence is speculated to result from children's inhalation of both indoor and outdoor airborne pollutants. Childhood lead poisoning is still a threat, with more than 300 000 children in the United States suffering elevated blood lead levels (Centers for Disease Control and Prevention (CDC), 2005c). In the case of some toxins, the threat may not become manifest for years. Environmental Protection Agency data suggest that a lifetime exposure to 1 ng kg^{-1} day^{-1} of 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD) may account for 1560 additional cases of cancer per 10 000 population.

Conversely there is also great potential for averting such harmful effects by improving the quality of the environment and sparing children the consequences of exposure to harmful chemicals and toxins. One recent study estimated the economic gains and increased worker productivity to be anticipated from the reduction in environmental lead in the United States since 1976, based on population increases in cognitive abilities realized as blood lead level declines, to be in the range of $110–319 billion for each year's new cohort of two-year-old children (Grosse *et al*., 2002).

1.4 Historical Toxic Disasters: Examples Involving Children

Because of their physiological differences from adults, children are affected first and more intensely by environmental toxic spills and have higher morbidity and mortality rates than adults in such disasters. These factors are additive, marking children as the 'canaries' in much the same way that birds were lowered first into mine shafts to detect dangerous levels of toxic gases such as methane. If the birds died, than the miners would not enter the shaft. So too illness in children may be the first indication of a hazardous environment. Their casualties are disproportionately higher than those of adults because of their smaller size, their increased vulnerabilities, their dependency on adults for mature judgements and decision-making and their reduced ability to escape independently from such emergency situations.

1.4.1 Minamata Bay Mercury Poisoning

The chemical disaster that occurred at Minamata Bay in Japan in the 1950s was a watershed event in increasing the general public's awareness of the potential dangers inherent in environmental hazards having a widespread impact on children's health. Industrial discharges of very high concentrations of mercury into the bay from a vinyl-chloride-producing factory were incorporated into the flesh of fish which were subsequently caught and consumed by area residents. Neurological toxicity ensued with tremors and ataxia, paresthesias, constricted visual fields, impaired speech and hearing, and progressive neurological dysfunction. More than 3000 people were certified by the Japanese government as having 'Minamata disease' and at least 600 deaths were attributable to the contamination (Davidson *et al*., 2004). Pregnant women who ate the contaminated fish did not necessarily exhibit any symptoms themselves, but gave birth to infants with severe developmental disabilities, microcephaly, severe retardation, static encephalopathy and seizures, termed 'congenital Minamata disease'. This event alerted scientists, public-health officials and the general public to the potential greater sensitivity of the foetus to environmental chemicals conveyed via placental transfer.

1.4.2 Yusho, Japan Rice Oil PCBs Contamination

In Yusho, Japan, in 1968 it was discovered that a food staple, Kanemi rice, had been inadvertently contaminated with Kanechlor 400, a mixture of polychlorinated biphenyls (PCBs), polychlorinated dibenzofurans (PCDFs) and other dioxin-like compounds. PCBs and PCDFs comprise several hundred different chlorinated

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hydrocarbon congeners varying in halogen content, but alike in their lipophilicity and persistence in food chains and the environment. Medical effects of exposure to these chemicals include acne-like facial rashes, hyperpigmentation and eye irritation. In children, growth retardation and triglyceride, immune and endocrinological disturbances are also suspected. By 1982 more than 1788 Japanese people had been certified as having been exposed to 'Yusho poisoning'. Even as late as 30 years after the incident blood levels of total dioxins and 2,3,4,7,8 pentachlorodibenzofuran in Yusho patients were 3.4–6.8 times higher and 11.6–16.8 times higher than matched controls (Furue *et al*., 2005). Long-term effects on Yusho women included a twofold increased rate of spontaneous abortion, a fivefold increased proportion of premature births, a twofold increased rate of pregnancy loss and a fivefold increased rate of induced abortion (Tsukimori *et al*., 2008). This event revealed the potential threat for long-term harm in children occasioned by their (or their mothers') relatively short-term exposures during environmental contamination events involving persistent organic pollutants (POP).

1.4.3 Bhopal, India Methyl Isocyanate Disaster

Methyl isocyanate (MIC) is a colourless liquid with a pungent odour that reacts violently with water and is highly flammable. In an incident on 3 December 1984, at the Union Carbide chemical plant in the city of Bhopal, India, water entered a storage tank containing 40 900 kg of MIC and produced an exothermic reaction. The explosion liberated about 24 545 kg of gaseous MIC, along with 12 800 kg of by-products such as carbon monoxide, monomethylamine gases and hydrogen cyanide, over the next two hours (Sutcliffe, 1985). The health effects of MIC, previously poorly studied in humans, included dermal and ocular irritation, metabolic inhibition, acute and chronic respiratory effects, including pulmonary oedema and death. Consequently more than 1000 people living within a perimeter close to the plant died within the first two hours after the gas release, 2500 people died within the first day and over 90 000 people required emergent medical evaluations for illness. There were hundreds of delayed deaths and thousands who suffered permanent lung damage and disability. A disproportionate number of children suffered coma, seizures, ophthalmologic and pulmonary toxic effects from their exposure, and a sizable number, 199 children, died within the first 12 days postexposure (Irani and Mahashur, 1986; Bharucha and Bharucha, 1987; Mehta *et al*., 1990). There are still many lingering questions regarding the long-term residual effects of the MIC exposure on the health of the children 25 years later.

1.5 Children, Communities and Environmental Justice

Economic disparities account for racial and ethnic disparities in childhood lead poisoning, with a disproportionate number of black and Hispanic children, who are exposed to lead-containing dust in older, dilapidated housing stock.

Because of socioeconomic and racial disparities, more children live in poverty than do any other age group. Their families are more likely to live in poorer, contaminated, unhealthy communities, in close proximity to toxic waste dumps, landfills and/or 'dirty' industries where environmental regulations are nonexistent, unmonitored or unenforced. For example, people living near air-polluting electricity-generating plants have higher rates of asthma and respiratory illnesses.

Children living in urban ghettos are exposed to the hazards of dilapidated housing, substandard sanitation and insufficient 'green space' areas to play. Benzene, a contaminant of gasoline and a known carcinogen, is a problem in poverty-ridden, urban settings. Benzene levels correlate with heavy automobile traffic, and children playing in the streets in poor neighbourhoods have disproportionately high exposures (Weaver *et al*., 1996). The prevalence of asthma among children living in the Bronx, New York, was estimated to be twice the national average, with disproportionately higher rates among both Hispanic and lower-income groups (Crain *et al*., 1994). There may be racial as well as socioeconomic determinants of childhood asthma, with black children being generally more affected than whites (Weitzman *et al*., 1992; Cunningham *et al*., 1996).

New immigrants and children of migrant workers are examples of other vulnerable groups whose socioeconomic circumstances force them into substandard housing stock and whose unfamiliarity with the local healthcare system, governmental programmes, education system and other community-based helping agencies may limit their children's access to services.

2 GENE–ENVIRONMENT INTERACTIONS

The central role of environmental genomics as the mediator of risk for many chronic disease processes is well established. Gene regulators and gene cascades may differ in their roles and expression from tissue to tissue and may differ in the timing of their activity in complex ways. Genetic differences dictate the number and functionalities of molecular transporters and tissue-specific receptor sites, protein structure and function, cell-signalling processes, enzyme efficiencies and many other molecularly mediated mechanisms of subcellular, cellular and organ function. Interindividual variation in both the effectiveness of drugs and the

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susceptibility to adverse drug reactions has long been known. For example, genetic polymorphisms associated with hepatic-enzyme efficiency (i.e. 'fast' or 'slow' acetylation) not only dictate the frequency of dosing necessary to achieve a therapeutic effect of isoniazid hydrochloride, but also determine the risk for developing some adverse effects to the drug. Likewise, such genetic polymorphisms may also dictate in part the individual's susceptibility to a chemical or toxin. Genetic differences in the efficiency of the alcohol dehydrogenase enzyme system account, in part, for the susceptibility of people of Asian descent to flushing and other adverse side effects of drinking ethanol.

The effects of environmental toxins in children are similarly mediated, in part, by their genetic make-up. The existence of genetic variants and polymorphisms confers relative vulnerability or resistance to molecular perturbations and cellular dysfunctions that are manifest downstream as phenotypic variation and, in some cases, clinical toxicity. Hopkins *et al*. (2008) genotyped 422 cord-blood samples in Mexican children for mis-gene variants in haemochromatosis and transferrin genes, which in part determine gastrointestinal absorption of the cationic metals, iron and lead. These researchers found that subjects carrying missense genes had blood lead levels 11% higher than those with wild-type genes, with a 50% higher risk of having a blood lead level greater than $10\,\mu g\,dl^{-1}$. Thus children with variant iron-transport and metabolizing genes may be especially vulnerable to low environmental exposures to lead sources.

Other disorders with significant environmental contributions besides lead may also be mediated by 'environmentally responsive' genes (Herbert *et al*., 2006). Researchers showed a modest correlation between single nucleotide polymorphisms (SNPs) of the airway inflammatory proteins, glutathione-S-transferase P1 and tumour necrosis factor, interacting with geocoded ambient levels of nitrogen oxides, in a case-cohort study of 542 asthmatic Swedish children, who developed clinical indicators of allergic disease at four years of age vs. 542 controls (Melen *et al*., 2008). Variants of the chromosome 17q21 were strongly associated with risk of early-onset (aged four years or younger) asthma in 1543 subjects tested in a replication study of SNPs (Bouzigon *et al*., 2008). Significantly the same SNPs on chromosome 17q21 and the same risks were also associated with early exposure to environmental tobacco smoke. Thus allelic variability may play a complex moderator role in the ability of children to adapt to environmental xenobiotics.

Such gene–environment interactions may also be evident in disease conditions in which the environmental influence or precipitant is still unclear. Abnormal alleles in the neurexin superfamily of genes, contactin-associated protein-like 2 (CNTNAP-2), are associated with autism spectrum disorders. CNTNAP-2 proteins have shown widespread expression in the brains of animal models, including limbic circuits implicated in social interactions, and are conserved across species (Bakkaloglu *et al*., 2008).

These may be 'environmentally responsive' genes involved in synaptic membranes as molecular chaperones inembryonic neuronal migration. Rare allelic variants may disrupt protein function and predispose the child to cerebral dysplasia (Arking *et al*., 2008; Bakkaloglu *et al*., 2008; Alarcon *et al*., 2008). The resulting loss-of-function proteins may affect the attachment of axons to glia and/or the alignment of ion channels within neurons so as to affect their infrastructure and function (Stephan, 2008). Such dysplasias may also render the axonal-glial system more sensitive to the effects of neurotoxic substances such as organophosphate pesticides (Herbert *et al*., 2006).

There may also be environmental influences on epigenetic factors that contribute to the variation in the functional expression of genes. Epigenetic events can be defined as the pattern and extent of post-transcriptional DNA modulation by methylation, phosphorylation or acetylation of genes or histones. The addition of such groups can change the three-dimensional conformation of DNA which, in turn, can influence its function. Addition of methyl groups typically reduces the activity of genes, whereas acetylation is an inducer. The role of phosphorylation remains unclear. Environmental effects on epigenetic phenomena in children are areas needing further intense scientific research.

3 DEVELOPMENTAL BASIS FOR SUSCEPTIBILITY TO ENVIRONMENTAL TOXINS

The absorption, distribution, metabolism and excretion of environmental chemicals in infants and children follow different kinetics than in adults due to the developmental maturation of organ systems and physiological processes with age. Such ontogenic considerations underlie the observation that children are often more susceptible to hazards in their environment and the adverse effects of toxins to which they are exposed.

For example, young infants may have milk-based formula as a single food source (as opposed to older infants, children and adults who have multiple food sources) and, proportionate to their size, infants have a higher fluid intake per body weight. Young infants have a higher gastric pH (greater than 4) than do older children, related both to lower basal acid output and lower gastric secretions (Agunod *et al*., 1969; Rodbro *et al*., 1967). This leads to colonization of the intestines with different bacterial flora than that which colonizes older children and adults. These bacteria have a greater capacity to convert ingested nitrates to more dangerous, oxidizing nitrites. Infants less than five

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months also have reduced amounts (as little as 60% of adult amounts) of NADH-dependent methaemoglobin reductase, an enzyme responsible for reduction of potential oxidants in red blood cells. Thus if an infant's formula is made up using well water containing excess nitrates from agricultural fertilizer runoff, the infant drinking it may develop clinically significant methaemoglobinaemia (oxidized haemoglobin with impaired functional capacity to carry oxygen to cells), whereas older infants, children and adults drinking the same water experience no adverse effects (Comly, 1945).

3.1 Behavioural and Developmental Considerations

3.1.1 Pica and Oral Behaviours

Infants and young children have oral exploratory behaviours as part of their normal development, and they also put nonfood items in their mouth, defined as pica behaviours. Such hand–mouth activity of toddlers puts them at risk for ingestion of contaminant-laden household dust, paint chips, plaster, outside dirt and other nonfood items. Ingestion of outside dirt by young children from hand–mouth activity during play has been studied by several investigators. A wide range of estimates have been found: between 100 mg and 10 g of outside dirt, depending on the child's age and development (Kimbrough *et al*., 1984; Lepow *et al*., 1974; Day *et al*., 1975; Binder *et al*., 1986). Using aluminium, silicon and titanium tracers, Binder *et al*. studied 59 children one to three years old, finding a minimal estimate of 108 mg day^{-1} (maximum of 1834 mg day^{-1}) of soil intake, more than any other age group (Binder *et al*., 1986).

Such behaviours are known risk factors for oral exposures to dust-borne or paint-chip-borne toxins, such as lead, and account in large measure for the risk of childhood lead poisoning (Walter *et al*., 1980). Some groups of children are more vulnerable to toxic exposures from pica than are others. Both zinc deficiency and iron deficiency are known to be associated with increased pica behaviours in children. Children with pervasive developmental delays and autism have increased rates of pica, and may continue to display pica habits well into their school-age years (Hussain *et al*., 2007).

Children's blood lead concentrations are correlated with their intake of lead-containing dust from hand-to-mouth activities and oral exploration. This may result in appreciable absorption of lead from indoor sources, as well as from lead-contaminated soils outdoors (Walter *et al*., 1980). Arsenic contamination of residential soil in bare areas was found to correlate with urine and tissue levels of arsenic in children living near copper-smelting facilities (Binder *et al*., 1987; Hwang *et al*., 1997a; 1997b). Mean speciated arsenic concentrations in the first morning voids of 414 children less than 72 months was 9.1 $\mu g\,l^{-1}$ close to the smelter, 8.6 $\mu g\,l^{-1}$ at intermediate locations and 7.2 $\mu g\,l^{-1}$ in areas remote from the facility (Hwang *et al*., 1997b).

3.1.2 Hazards of Exploratory Play

Children generally spend more time outdoors than adults, engaged in vigorous play. This inevitably exposes them to toxins, such as chemical pollutants in the air and soil, and pesticides used on the lawn and garden. Play is the developmental 'work' of infants and young children, as the interaction with their social and physical environment teaches them, shapes their character and values, and enhances their cognitive growth through experience. However, such exploration and curiosity can also lead to injury or death from exposure to hazards. The highest death rate among children outside the newborn age period is due to injuries.

Young children play close to the ground, which places them at a high risk for exposure to pesticide applications inside the home, as well as on the lawn and garden. Exposures to household dust, which may contain hazardous particulates, biologic agents, mould spores, lead, dust mites and cockroach antigen, are more likely among children crawling on floors, pulling themselves up to windowsills and exploring indoors. Their often poor hand-washing technique exposes them to xenobiotics by ingestion.

Unintentional poisonings affect a disproportionately large number of young children. Children are developmentally at high risk for poisoning because they start walking (and running) during the second year of life, the same time at which they acquire the fine motor skills to open containers and the oral-exploratory habits of putting toys and everyday home products in their mouths. They also lack the cognitive skills and judgement to determine food vs. nonfood items, and so must rely on parents to safeguard their environment. Predictably, most childhood poisonings occur around mealtimes, when parents are preoccupied with meal preparation and distracted from the normal supervisory activities of monitoring their toddler. Of 2.4 million poison exposures recorded by poison-control centres in the United States in 2006, over 50% occurred in children aged five years and younger (Bronstein *et al*., 2007). In one analysis of costs, it was found that almost 3% of childhood hospitalizations were for the treatment of poisoning (Woolf *et al*., 1997).

The peak incidence of inadvertent poisonings is in children ages two to three years old and most such incidents occur in the child's own home. Of those poisonings that do not occur in a toddler's home, grandparents' homes are the next most common site. Thankfully most one-time potential toxic exposures in young children are medically

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trivial and can be managed at home by a period of observation for any untoward behaviours or other symptoms and signs. While exposures to plants and over-the-counter medicines, such as cough and cold remedies, are among the most frequent toddler exposures, they are rarely serious. There are notable exceptions to this rule, such as exposures to caustic compounds, volatile hydrocarbons and pesticides, some chemicals, like cyanide and arsenic, toxic alcohols, such as ethanol, ethylene glycol or methanol, and some prescription medications.

Older children have imaginary play habits, an abiding curiosity and poor judgement borne of a lack of experience, that may put them at high risk for toxic exposures or physical injury and trauma. They are more likely to explore a variety of hazardous locales, such as quarries, toxic-waste dumps, sewer drains, contaminated streams and old abandoned buildings, ignoring printed warnings and barriers to entry. Electrocution, from climbing high-tension electrical towers, drowning, from swimming in abandoned quarries, vertical falls, from climbing unguarded precipices, and death, from walking along railroad trestles, are only a few examples of the calamities that can result from playing in unsafe environments. Pedestrian injuries are a common occurrence among older children. Motor vehicle accident-related injuries and death peak in their incidence through adolescence and young adulthood.

While one would not want to change opportunities of children for constructive play, increased supervision and modifications of the environment can reduce their exposure to hazards and toxins. For example, mitigation of lead hazard in homes can lower the risk of the contamination of young children (Haynes *et al*., 2002).

3.2 Reproductive Toxicology and Foetal Exposures

Following fertilization and implantation, foetal growth and development is a remarkable symphony of cellular proliferation, differentiation and migration, orchestrated by changes in cell cycling, complex signalling and cellular interactions, downstream events and apoptosis. The human conceptus is termed an embryo until eight weeks of gestation and is characterized by several distinct periods of development: pre-implantation, blastocyst formation, implantation, gastrulation and organogenesis. Despite its extraordinary plasticity and the built-in redundancy of systems allowing foetal adaptation to changes in intrauterine life, the foetus is still vulnerable to environmental insults. All of these periods of development are characterized by rapid cell division and differentiation, and they represent critical periods of susceptibility for the embryo. The human embryo is thought to be particularly sensitive to toxic and teratogenic effects of chemicals and radiation early in embryogenesis: organogenesis occurs between 18 and 60 days of gestation (Beckman and Brent, 1984). For example, arm defects only were associated with prenatal exposure to the drug, thalidomide, in the 27–30th day of gestation, whereas if the exposure was during the 30th to 33rd days, then both arm and leg abnormalities were seen in offspring (Beckman and Brent, 1984).

Sensitivity to carcinogens is theorized to be heightened during late foetal development. The initial peak of childhood cancer by five years of age may be due to prenatal exposure to mutagens from either parent (Autrup, 1993). Foetal cells are theorized to be particularly vulnerable to oncogenic cancer-formation due to their high rate of replication, the presence of pluripotential germ cells, and the relative lack of cellular DNA repair mechanisms. Examples of such critical windows for carcinogenesis in foetal development include exposure to diethylstilbestrol and later development of vaginal clear cell adenocarcinoma in young women and the association between prenatal exposure of the mother to ionizing radiation and the occurrence of leukaemia in offspring (Olshan *et al*., 2000).

The transfer of toxic chemicals to the foetus during pregnancy has long been recognized. Spontaneous abortions or congenital malformations are potential outcomes of maternal exposure to environmental toxins during embryogenesis. Chlorinated hydrocarbon insecticides can be detected in the organs of stillborn infants and the blood of newborns (Curley *et al*., 1969; Polishuk *et al*., 1977). During the pre-implantation period, embryo exposure to dichlorodiohentltrichloroethane (DDT), nicotine and methylmethane sulfonate resulted in deficits of body and/or brain weight and embryo lethality, but not malformations (Rogers and Kavlock, 1996). Physiological changes during pregnancy can mobilize stored toxins, such as lead from bone or PCBs from fat, resulting in foetal exposure.

Healthcare providers, such as anaesthetists, dental assistants and other hospital personnel, are often exposed to potentially embryotoxic hazards, such as ethylene oxide, anaesthetic gases, solvents, mercury and antineoplastic agents (Filkins and Kerr, 1993). Studies of these groups have revealed significant risks for spontaneous abortions and congenital malformations (Ahlborg and Hemminki, 1995; Hemminki *et al*., 1982). In a study of nurses and pharmacists with occupational exposure to antineoplastic agents, exposure of the mother or handling by the mother of antineoplastic agents during pregnancy resulted in a statistically significant increased risk of spontaneous abortions and combined risk of spontaneous abortion and stillbirth (Valanis *et al*., 1999).

Toxic exposures from the mother's habits pursued during pregnancy can be conveyed directly to the foetus. Maternal alcohol ingestion can lead to foetal alcohol syndrome. Transplacental transfer of carcinogens in smoke and smoke-related, adduct-induced injury to foetal

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Table 2 Agents known or suspected to be reproductive hazards

Male reproductive toxins	Adverse pregnancy outcomes
Dibromochloropropane	Anaesthetic gases
Dinitrotoluene	Arsenic
Chloroprene	Carbon monoxide
Lead	Cytotoxic drugs
Chlordecone (Kepone)	Dibromochloropropane
Ethylene glycol ethers	Lead
Carbon disulfide	Methyl mercury
Naphthyl methylcarbamate (Carbaryl)	Organic solvents
	Ozone
Nitrous oxide	Organochlorines (DDT, DDE)
Caprolactam/Dinit	Phenoxyacetic acids (2,4,5-trichlorophenoxyacetic acid)
Alkyl mercury	
Ethylene dibromide	Polybrominated biphenyls
Toluenediamine	Polychlorinated biphenyls
Vinyl chloride	Tetrachloroethylene
	Toluene
	Vinyl chloride

Modified from Filkins and Kerr (1993).

tissues has been demonstrated (Autrup, 1993). Maternal smoking has been associated with lower birth weight and increased risk of foetal demise.

3.2.1 Fertility Issues

Parental exposure to chemicals while in the workplace or remodelling their homes, during recreational activities or while pursuing hobbies, and elsewhere poses the threat to their own fertility and can have intergenerational toxic consequences. **Table 2** presents some workplace chemicals known to be reproductive hazards. Parental exposures to chemicals may adversely affect their ability to conceive children at all. For example, endometriosis is a gynaecologic disease that often results in infertility. Dioxins have received attention as possible aetiologic agents linked to endometriosis, related to their toxicity as oestrogenic analogues (Zeyneloglu *et al*., 1997). The first study to demonstrate this possible relationship found higher levels of PCBs in 28 patients with endometriosis vs. 441 women without endometriosis in a population of women with infertility. In Belgium, the incidence of endometriosis approaches 60–80% in women with infertility and pain. Interestingly, a 1989 WHO report also found that dioxin concentrations in Belgium were among the highest in the world, providing suggestive evidence that high dioxin accumulation in Belgian women might contribute to the high incidence of endometriosis.

Male fertility can also be impaired from exposures to chemicals. One study of TCDD-exposed men with chloracne found them to have reduced libido 50% more often than either unexposed controls or exposed men without chloracne. In a longitudinal seven year follow-up study of workers exposed to the now-banned nematocide, dibromochloropropane (DBCP) in a California manufacturing plant in the 1970s, azoospermia occurred without recovery (Whorton *et al*., 1979). Differentiating spermatogonia (type B) are very radiosensitive cells, and ionizing radiation has been demonstrated to cause a significant reduction of sperm count (Giwercman and Bonde, 1998).

3.2.2 Foetal Physiology, Placental Transfer and Teratogenicity

As many as 5–8% of congenital malformations are thought to be caused by an environmental toxicant, and as many as 20% may be multifactorial, related to gene–environment interactions (Beckman and Brent, 1984). Environmental toxin exposure during the foetal period is more likely to result in impaired growth, disruption of organogenesis and poor functional maturation (Rogers and Kavlock, 1996). Thus exposure of the mother to a variety of chemicals, some of which may as yet be unrecognized as teratogens, presents, through placental transfer, an extraordinary risk to the sensitive, developing foetus. In the past the placenta has been thought to be an effective barrier to passage of toxicants, and it does have appreciable amounts of degrading enzymes such as catalase and superoxide dismutase. And yet many reactive chemicals are passed to the foetus; lipid-soluble organochlorine compounds, for example, are detectable in both mother and foetus (Polishuk *et al*., 1977). Adverse birth outcomes were correlated to maternal serum dioxin levels after the environmental disaster that released large amounts of the chemical contaminating the population in Seveso, Italy (Eskenazi *et al*., 2003). Pregnant women living in rural Sri Lanka, but not working in agricultural occupations, were studied for foetal effects of background amounts of organophosphate compounds during spray seasons (Samarawickrema *et al*., 2008). Cord blood taken from their newborn infants demonstrated inhibition of butyrylcholinesterase activity during the pesticide-spraying season; there was also evidence of associated foetal oxidative stress and DNA fragmentation.

Such environmental exposures can affect foetal outcomes at birth. For example, animal models have previously confirmed the capacity of PCB mixtures to cause reproductive dysfunction (Barsotti *et al*., 1976). PCB mixtures can induce premature uterine contractions in mice, mediated by activation of phosphorylase A2 enzymes which change intrauterine arachidonic acid levels (Bae *et al*., 1999). The metabolite of DDT, p,p′-DDE (1,1-dichloro-2,2 bis (p-chlorophenyl) ethylene), was measured in the serum of 2613 pregnant women with prospective follow-up of pregnancy outcomes in the Collaborative Perinatal Project (Longnecker *et al*., 2001). Higher DDE levels were correlated

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with in a dose–response fashion with higher rates of preterm births, as well as a higher incidence of small-for-gestational-age infants, after other potential confounders were controlled for in multivariate models. The authors speculate that the adverse reproductive effects of DDE (and DDT) may be related to its adverse effects on the binding of androgens and/or progesterone to receptor sites. They also note that a major action of DDT is its ability to impair the closing of sodium channels in neurons. Since placental tissue has a large number of sodium channels compared to other non-neuronal tissues, it may be particularly vulnerable to neurotoxins. Exposures to tobacco smoke, either because of the cigarette smoking of the pregnant woman or her exposure to environmental tobacco smoke, can result in low birth weight. Reactive species of a multitude of chemicals in tobacco smoke can pass transplacentally to the foetus, including polycylic aromatic hydrocarbons, nitrosamines, 4-aminobiphenyls and cadmium. They are found in equal levels in both maternal and cord blood and may play a role in adverse foetal outcomes (Autrup, 1993).

The physiology of the foetus makes it particularly vulnerable to xenobiotics passing through the placenta. The foetus may in fact concentrate toxins, attaining higher blood levels than the mother. For example carboxyhaemoglobin concentrations in foetal blood are approximately 10–15% higher than those in the mother. The foetus is particularly susceptible to carbon monoxide because of several physiological factors. Foetal blood has higher concentrations of foetal haemoglobin, which binds carbon monoxide more tightly than haemoglobin A or A2. Gas exchange in foetal tissues takes place on the ascending slope of the sigmoid-shaped oxygen saturation curve, at about 75–80% arterial saturation, such that minor decrements in maternal pO_2 are magnified in the inability of the foetus to perfuse its own tissues and unload enough oxygen to cells. Even small reductions in maternal oxygen tension, such as the maternal carboxyhaemoglobin levels achieved by cigarette smoking, can drop the diffusion gradient across the foetal umbilical cord to hypoxic foetal stress (Hsia, 1998).

Cryptorchidism and hypospadias are teratogenic effects linked to prenatal oestrogen exposure in animal models. A case–control study in Denmark demonstrated that an increased risk of cryptorchidism occurred in the male offspring of women gardeners. The authors suggested that the increased risk was due to the use of farming and gardening chemicals that possess oestrogenic effects (Weidner *et al*., 1998). Infants born to Taiwanese mothers with exposure to PCBs during pregnancy exhibited numerous ectodermal teratogenic defects such as hyperpigmentation, carious teeth, poor nail formation and short stature (Rogan, 1995).

Periods within foetal life may represent distinct, critical windows of susceptibility to the neurotoxic effects of environmental chemicals. Effects on postnatal cognitive development have been linked to prenatal exposures to environmental PCBs (Jacobson *et al*., 1990; Ropgan *et al*., 1986). Prenatal mercury exposures have long been associated with seizures and developmental deficits in the newborn (Koos and Longo, 1976). Pregnant women exposed to methylmercury from the contamination of fish and shellfish at Minimata Bay in Japan gave birth to children with a number of congenital malformations, cerebral palsy and mental retardation (Powell, 1991). Pregnant women can mobilize significant amounts of lead from bone, which is then conveyed to the foetus via placental transfer during critical periods of neural development, resulting in prenatal lead poisoning and postnatal neurological dysfunction. Thus current screening standards suggest testing of the blood of both pregnant women and young children for evidence of lead contamination (U.S. Preventive Services Task Force (USPSTF), 2006).

Heavy-metal intoxication in the neonate from lead (Sikorski *et al*., 1989), mercury (Sikorski *et al*., 1989) and cadmium (Alessio *et al*., 1984) can occur from prenatal placental transfer of the metals. Maternal lead exposure during pregnancy has been documented from industrial exposures (lead smelting), lead-related hobbies (jewellery and stained glass making) and construction work (renovation of old dwellings with lead-based paint). Arsenic placental transfer can occur from maternal exposures when women are exposed as agricultural workers where arsenic is used as a pesticide, when they work in copper or other smelting operations, or when arsenic is present as a natural contaminant of their drinking water. Such metals are postulated to reduce glutathione levels and increase oxidative damage to foetal organs. Animal studies of arsenic-induced damage during early organogenesis targeted brain, sensory organs, pharyngeal arches and somites with dose-, time- and valency-related injury (Zelikoff *et al*., 1995). Human exposure to environmental sources of arsenic during pregnancy has been associated with a higher risk of neural-tube defects in offspring (Shalat *et al*., 1996). After heavy-metal exposures during pregnancy, postnatal neurodevelopmental injuries have been reported in their offspring (Ghafour *et al*., 1984; Sensirivatana *et al*., 1983; Shalat *et al*., 1996). Low-level foetal lead exposure (maternal lead levels $\leq 25\,\mu g\,dl^{-1}$) has been shown to result in mild developmental delays during the first several years of life (Dietrich, 1996).

3.3 Physiological Differences in Infancy and Childhood

Table 3 illustrates some of the physiological differences between infants and adults in the absorption, distribution and elimination of xenobiotics. Children differ from adults in many aspects of their physiology and this predictably may result in different toxic responses

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Table 3 Differences in toxicokinetics between infants and adults

Absorption	Neonate compared to adult
Gastric pH	Neutral at birth to <4 by 2–4 d
Gastric acid secretion	Adult levels by 3 mo
Gastric emptying	Variable until 6–8 mo
Digestive enzymes	Lower at birth
Bacterial flora	High concentration of nitrogen-splitting bacteria
Intestinal motility	Slow and irregular in infants
Distribution	
Body water	Higher (75% body weight at birth)
Fat	Higher in infants (25% by 6–9 mo)
Muscle	Lower mass
Brain	Higher relative mass; lower myelin; higher blood flow; more porous blood-brain barrier
Plasma proteins	Reduced binding of acidic/basic compounds
Elimination	
Liver mass	Higher relative mass
Glucuronidation	Absent at birth; adult levels by 4 wk
Oxidation	Lower cytochrome P450 activity
Ester hydrolysis	Depressed levels
Renal function	Reduced glomerular filtration rate
Renal tubular secretion	Adult levels by one year of age

Modified from **Table 2** (Renwick, 1998) and **Table 1** (Scheuplein *et al.*, 2002).

to environmental chemicals than those seen in adults. For example, the reaction to organophosphate pesticides may vary with age (Pope and Liu, 1997). One study of 36 children with carbamate intoxication and 16 children with organophosphate poisoning found that their symptoms and signs of poisoning were not often the 'classic' muscarinic signs of cholinesterase inactivation typically seen in adults, but were predominately changes in consciousness and hypotonia (Lifshitz *et al.*, 1999). The authors hypothesized that blood–brain permeability in younger children might facilitate organophosphate entry into the brain to produce central nervous system (CNS) toxicity, or, alternatively, that differences in affinity binding of such pesticides to the cholinesterase enzyme in central vs. peripheral nervous system targets might account for the clinical differences in the manifestation of toxicity. Thus the toxic injury to children attributable to an environmental agent is not entirely predictable from the adverse effects to the xenobiotic demonstrable in adults.

3.3.1 Size/Weight and Body-Composition Differences

The smaller size of a child exposes them to higher doses of a toxin per unit weight than an adult. The larger extracellular and total-body water spaces, coupled with a higher water/lipid fat composition, in infants and young children alter the dispersion of chemicals. Ontologic changes in carrier proteins such as albumen and α-1-glycoprotein and changes in membrane permeability with age will affect the distribution of drugs and chemicals, both throughout the body and at target organs.

3.3.2 Gastrointestinal Absorption

Absorption of xenobiotics and toxins in the paediatric age group differs from adults with respect to several physiologic processes, such as pH-dependent diffusion, gastric emptying time and gastrointestinal peristaltic activity. In the postnatal period, the amount and rate of gastric-acid secretion does not reach the lower limits of adult secretion until three months of age. During this period, newborns are vulnerable to absorption of xenobiotics whose pK_a will favour the formation of nonionized species in this less acidic milieu. In newborns, this relatively low acidity contributed to intestinal overgrowth of bacteria that caused an outbreak of methaemoglobinaemia from well water in Iowa. This well water was contaminated by nitrates that were converted to nitrites by the bacterial overgrowth (Luyens, 1987). In the newborn period, intestinal absorption of xenobiotics is also enhanced by a gastric emptying time that lags behind adult rates for the first six to eight months of life, as well as irregular and prolonged peristalsis relative to an adult's intestinal motility.

Lead is a classic example of an environmental toxin that is known to be preferentially absorbed in the small intestine of children to a much greater extent than in adults. A one- to two-year-old child will absorb 50% of ingested lead, whereas an adult will only absorb 10% of ingested lead (Royce, 1992).

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3.3.3 Volume of Distribution

The apparent volume of distribution (V_d) for a xenobiotic is increased in the perinatal period due to several factors. Total body water (TBW) and extracellular fluid (ECF) comprise a greater percentage of body weight in the foetus and newborn vs. an adult. TBW and ECF do not decrease to adult levels until approximately one year of age. During the first year of life and *in utero*, this can result in an increase in the apparent V_d for water-soluble xenobiotics and thus potentially increase its elimination half-life. Increased apparent V_d in the foetus and newborn can also be attributable to decreased plasma protein binding of xenobiotics. Specifically, the most important proteins for binding xenobiotics in plasma (albumin, α-1-acid glycoprotein and lipoproteins) are decreased in infancy and do not reach adult levels until about one year of age. Decreased plasma protein-binding facilitates distribution of xenobiotics into tissues, where they bind to their receptors to exert their effect.

3.3.4 Respiratory and Cardiovascular Physiology

Heart and lung development begins very early in foetal life. The period of greatest risk for causing perturbations in cardiac development is during weeks 3–5 of embryogenesis, when cardiogenic cellular differentiation, neural crest cells and endocardial cushions all undergo complex and dynamic interactions resulting in organogenesis (Sadler, 2000). How environmental chemicals early in foetal life can produce congenital heart defects, or how pollutants in air, food and water can affect later cardiac function in childhood and adolescence is still largely speculative.

Children are at high risk for toxicity from inhaled toxins. The maturation of lung architecture has critical windows of vulnerability that extend from *in utero* changes, through the physiological transformation to air breathing that occurs at birth, and continues on with further development of new alveoli and greater degrees of lung volume and breathing capacity into childhood. Developmental changes in lung architecture and capacity (e.g. minute ventilation, vital capacity, respiratory rate) continue after birth and throughout childhood and alter the absorption and bioavailability of inhaled pollutants. Infants have a higher respiratory rate (40 breaths per minute in a newborn vs. 16–18 breaths per minute for an adult) and lung volume ($400\,ml\,min^{-1}\,kg^{-1}$ in a newborn vs. $150\,ml\,min^{-1}\,kg^{-1}$ in an adult), giving them higher doses of inhaled toxins relative to adults. In fact, an infant has double the amount of an inhaled chemical by body weight as compared to an adult. A higher pulse and more efficient cardiovascular system of tissue perfusion allows for more rapid dispersion of toxins absorbed across the alveoli into the blood.

Breathing zones are an important concept that can predispose a child to certain environmental toxins. Because a child's breathing zone is closer to the ground (1–3 ft compared to 4–6 ft for an adult), chemicals that are heavier than air (such as mercury, formaldehyde, pesticides, radon) will pose more of an environmental threat to them. Peak concentrations of air and surface chlorpyrifos concentrations after the pesticide's application indoors were substantially higher ($94\,\mu g\,m^{-3}$) in infant breathing zones than adult sitting zones ($63\,\mu g\,m^{-3}$), and remained higher whether or not the rooms were ventilated (Fenske *et al*., 1990). Dust also settles in infant breathing zones, exposing them to higher concentrations of a variety of biotoxins, including cockroach antigen, dust mites, other particulates and mould spores. Early exposures of crawling infants to such a variety of xenobiotics may have a role in sensitizing them, increasing the likelihood of later development of allergic reactions and reactive airway disease.

Paediatric lung development occurs in two phases: pulmonary alveoli and capillary proliferation until the age of five to eight years, followed by growth through alveolar expansion. By 12 years of age, children have nine times the number of alveoli that they had at birth. Such architectural changes of the anatomy also predispose children to anatomical damage occurring during the extended critical windows of lung maturation and development. Compared to unexposed controls, children exposed to environmental tobacco smoke experienced slower lung development and lower FEV_1 (forced expiratory volume in one second) (Tager *et al*., 1983). The combination of exposure to environmental tobacco smoke and the toxigenic mould, *Stachybotrys atra*, was possibly associated with an outbreak of acute pulmonary haemorrhage and haemosiderosis in 10 Cleveland infants in 1993–1994. Furthermore, it was felt that the rapidly growing lungs of these infants were more susceptible to the inhaled trichothecene mycotoxins produced by this mould (Committee on Environmental Health, American Academy of Pediatrics, 1998).

Bronchospasm and reactive airway constriction is more easily triggered in infants and young children compared to adults. This explains in part their propensity for wheezing as a pulmonary response to a variety of different environmental precipitants, including viruses, bacteria, dust mites, cockroach antigen and environmental tobacco smoke. Induced immunological hypersensitivity, coupled with hyper-reactivty of the airways, may explain in part the increased risk of childhood asthma (Etzel, 1995). Within the age group of children six years and younger, those with elevated blood IgE levels and a family history of allergies are an especially vulnerable group to the onset of wheezing (Martinez *et al*., 1995).

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3.3.5 Neurodevelopmental Growth and Vulnerability

The paediatric brain and CNS are particularly susceptible to injury from environmental toxins. Brain development continues after a child is born, with neuronal proliferation completed only by the age of four to five years (Rodier and Cohen, 1994). Myelination continues into adolescence (Bearer, 1995). Additionally, the blood–brain barrier (BBB) prevents access of hydrophilic chemicals to the brain, but is not fully developed at birth. The maturation of the BBB occurs at different rates in different brain regions (Rodier and Cohen, 1994; Wenzel and Felfenhauer, 1976). The concentration and functionality of subcellular molecules, such as P-glycoprotein, a member of the adenosine triphosphate (ATP)-binding transporter series, can affect the cell's ability to form an effective protective barrier by extruding xenobiotics in a variety of tissue sites (e.g. BBB, hepatocytes, renal tubular sites). Limited data suggests the level of expression of P-glycoprotein in children is lower than that in adults (Tsai *et al*., 2001). The BBB may still be relatively porous until a child is three to four years old, making them more vulnerable to the injurious effects of neurotoxicants than older children and adults.

Clinical examples of the increased susceptibility of children during central nervous system development were the infamous outbreaks of mass methylmercury poisonings in Minamata, Japan, in the 1950s; infants exposed *in utero* and in early childhood were the most severely stricken (see Section 1.4.1).

Lead is a well-researched environmental toxin, long known to be associated with cognitive impairments in children, even at what could be considered exposures to background levels and low body burdens. Preschool children absorb higher amounts of lead from the gastrointestinal tract, distribute more of the metal to water-soluble, soft tissue compartments in the body, including the central nervous system, and have increased vulnerabilities of certain tissue sites for injury from lead than do adults (Woolf *et al*., 2007). Numerous studies have shown a direct dose–response relationship between increasing body burdens of lead accumulated during early childhood and decrements in intelligence quotient (IQ) (Needleman and Bellinger, 1990).

However while lead may be the most studied neurological toxin, it is not the only neurotoxicant that children commonly encounter. Using National Health and Nutrition Examination Survey (NHANES) III data in the United States, researchers have found inverse relationships between young children's exposure to environmental tobacco smoke, as measured by plasma cotinine levels, and their IQ scores (Yolton *et al*., 2005). Other researchers have defined the substantial costs and public health implications of the IQ decrement in the US population of children attributable to their methylmercury exposures, as defined by blood mercury levels greater than $5.8\,\mu g\,l^{-1}$ (Tresande *et al*., 2005).

Noise is often an unrecognized environmental hazard for sensorineural hearing development in the foetus and infant. The human hearing apparatus completes its normal development by 24 weeks of gestation, but has been shown to be sensitive to environmental noise *in utero* and postnatally. An American Academy of Pediatrics (AAP) Policy Statement, after a review of the literature, concluded that environmental noise poses a unique threat to the foetus and newborn in several ways: (i) excessive noise exposure *in utero* can result in high-frequency sensorineural hearing loss and have an association with prematurity and intrauterine growth retardation, (ii) excessive noise exposure in the neonatal intensive care unit (NICU) can result in cochlear damage and (iii) excessive noise exposure can disrupt normal growth and development of premature infants (Committee on Environmental Health, American Academy of Pediatrics, 1997).

Many studies have demonstrated an association of maternal smoking during pregnancy with subsequent subtle behavioural and neurocognitive developmental disorders in their children. Adverse behavioural effects that have been described include increased antisocial behaviour, conduct disorder, attention deficit, hyperactivity and anxiety/depression. Adverse neurocognitive developmental disorders that have been described include poor school performance and declining performance on achievement and speech/language tests, as well as declining performance on neuropsychologic assessments such as the Wechsler Intelligence Scale for Childre—Revised. Two possible mechanisms that are theorized to produce adverse neurodevelopmental disorders are: (i) nicotine in cigarette smoke reduces blood flow to the foetus and (ii) secondary carbon-monoxide exposure leads to higher levels of carboxyhaemoglobin. The common final pathway of both mechanisms is foetal hypoxia resulting in altered brain development (Eskenazi and Castorina, 1999).

Weiss (2000) has suggested that IQ loss resulting from exposure to neurotoxins in foetal life or in infancy and childhood represents, not only a measure of cognitive injury, but also a predictor of performance later in adulthood, expressed as a loss of income and discounted future earnings. Thus the economic consequences of a neurotoxic exposure on a population's IQ can be extrapolated to societal implications. Avoidance of such environmental neurotoxic exposures in the United States alone could result in an estimated potential savings of between $5.6 and $49.4 trillion (US) dollars.

3.3.6 Developmental Immunotoxicology

The developing immune system has been clearly identified as a target that is more sensitive to the damaging effects of xenobiotic exposure than the adult immune

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system. Pluripotent stem cells are detectable early in foetal life, undergo waves of complex transformations and migrations, and are susceptible to exogenous insults. Critical stages of immune development unfold with exquisite precision to give rise to T- and B-cells, natural killer cells, macrophages and other components of the competent immune system. The neonate still has lowered amounts of complement; its neutrophils do not have effective chemotaxis and have little capacity to mount an inflammatory response.

Polycyclic halogenated hydrocarbons such as PCBs, PBBs (polybrominated biphenyls) and TCDDs, polycyclic aromatic hydrocarbons, pesticides such as DDT, chlordane and carbofuran, fungicides (hexachlorobenzene), metals (methyl mercury, lead, cadmium), hormonal substances, drugs, mycotoxins and irradiation are all environmental pollutants that can act as immunotoxicants in animal and/or human models (Holladay and Smialowicz, 2000).

TCDD is well characterized in terms of its differential, deleterious effects on the developing vs. adult immune system. TCDDs can be transferred to the foetus via the placenta. The persistent immunosuppressive effects of TCDD may be more pronounced if exposure occurs in the perinatal period vs. adulthood. Specifically, TCDD exposure during critical windows of foetal development of the immune system can result in various prolonged, postnatal impairments of cell-mediated immunity: suppression of T-cell mitogen responses, increased skin-graft rejection, increased graft-vs.-host reactivity, and increase delayed hypersensitivity.

In utero exposure to alcohol may also alter normal maturational events that occur during ontogenesis of the immune system. Specifically, children with foetal alcohol syndrome have lymphopenia, eosinophilia, decreased T-lymphocyte lymphoproliferative responses and increased vulnerability to infectious diseases (Holladay and Luster, 1999).

Immunological development continues in the newborn and throughout infancy, and competence is thought to be influenced, in part, by the timing and variety of *in utero* and neonatal exposures to environmental antigens. For example, postnatal TCDD exposure has been shown in experimental models to increase susceptibility to infectious agents.

Scientific modelling proposes that perinatal immune events involving the balanced reactions of two classes of T-helper cells (TH1 and TH2) and their cytokine milieus determine the later expression of allergen responder phenotypes. Conceptually the timing and variety of antigenic exposures in infancy, in the context of genotypic predispositions of the immune system, may determine later immune tolerance vs. responsiveness, including pathological responses manifested clinically as asthma, atopy and allergies (Boushey and Fahy, 1995; Holt, 1999). Complex interactions between cytokines, mediators of inflammation, immunoglobulins, and sensitized migratory and resident cells in the airways may predispose to the pathological responses to environmental antigens that characterize asthma in children. Different inhaled xenobiotics (e.g. air pollutants, viruses, bacterial endotoxins, moulds) may trigger varying pathogenic responses, some of which involve TH2 cells, eosinophils and IgE production, whereas others stimulate pathways involving mast cells and their cytokines. Persistent bronchial hyper-reactivity may thus be fundamentally mediated by imbalances in the cellular and subcellular mechanisms underlying the promotion and control of the inflammatory response (Boushey and Fahy, 1995).

3.3.7 Endocrine Growth and Environmental Hormonal Analogues

Foetal development is under tight hormonal regulation and various endocrine organs, such as the pancreas, hypothalamic-pituitary neuroendocrine axis, adrenal, and parathyroid and thyroid glands, have critical windows of susceptibility during prenatal organogenesis. Environmental toxins can possess the capacity to alter natural hormonal activity by acting as 'endocrine disruptors', and children have been shown to be susceptible to environmental toxins that mimic or inhibit the action of natural hormones. However, there still is little understanding of the full extent of perturbations of prenatal hormonal function possible by environmental exposures in humans.

There may be gene–environment interactions underlying the genesis of some cases of Type 1 childhood insulin-dependent diabetes. A cluster of cases of childhood diabetes was correlated with birth month in one study. Authors concluded that mothers had eaten a large amount of nitrosamine-containing cured, smoked mutton early in their pregnancies, and that this large intake of nitrosamine might have been linked to development of diabetes in their offspring (Helgason and Jonasson, 1981). They could replicate the finding in mice exposed to the same food additive (Helgason *et al*., 1982).

The foetal thyroid gland develops in the first trimester, and maternal thyroid transfer is the source of hormone through the first 12 weeks of gestation, during the critical time of cerebral neurogenesis and migration; foetal hormone thereafter regulates neuronal proliferation and differentiation (Sadler, 2000). Not only is thyroid hormone critical for CNS development, but also general foetal growth and cardiovascular and musculoskeletal development extending into the postnatal period. Elevated neonatal thyroid-stimulating hormone (TSH) levels were observed for up to three months in 200 Dutch children with a perinatal exposure to PCBs (Sauer *et al*., 1994). Another study correlated perinatal PCB exposure with elevated neonatal T4 levels (Pluim *et al*., 1993). Other environmental contaminants, including the plasticizers, bisphenol A and phthalates, have been shown in animal models to be capable of

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interfering with normal foetal thyroid development and function.

Other environmental agents, such as the DDT metabolite DDE, interfere with androgen receptor function and are believed to act as endocrine disruptors. In animal models endocrine disruptors have been associated with an increased incidence of hypospadias and other abnormalities of sexual development (Kelce and Wilson, 1997).

3.3.8 Hepatic Detoxification Systems

Both Phase 1 (acetylation, oxidation processes) and Phase II (conjugation) hepatic metabolic enzymes undergo a developmental increase in their activity postnatally. The liver itself decreases in size disproportionately to the overall growth of the child. While there is scant information available on the ontogeny of Phase II reactions, there is considerable evidence that Phase I detoxification activity occurs at reduced levels in young infants. The majority of enzymes responsible for xenobiotic metabolism or detoxification are located in the smooth endoplasmic reticulum within the microsomal fraction of the cell (Reed, 1996). The cytochrome P450 system is perhaps the best understood of these mixed-function oxidase systems.

Phase 1 hepatic enzyme activity in infants one year of age is generally about one-third that of adults. Individual enzyme systems mature at different rates. For example, CYP 3A7, a cytochrome expressed in foetal life, can detoxify such foetoactive chemicals as retinoic acid (Chen *et al*., 2000) and dehydroepiandrosterone (Kitada *et al*., 1987). Cytochromes CYP 2E1 and 2D6 increase activity shortly after birth (Vieira *et al*., 1996; Treluyer *et al*., 1991), and CYP 2C9 and CYP 2C19 become active within the first week of life (Lacroix *et al*., 1997); whereas CYP 1A2 is not expressed until one to two months of age (Sonnier and Cresteil, 1998). Many of these cytochrome mixed-function oxidases do not achieve adult levels of activity until six months of age. During this lag phase, newborns will demonstrate prolonged body elimination of xenobiotics that depend on hepatic-based metabolic pathways for their detoxification. Conversely the young infant, with incomplete cytochrome activity, may tolerate better than older children and adults other environmental chemicals that require cytochrome-mediated oxidation to create more injurious active metabolites.

3.3.9 Dermal Absorption

Percutaneous absorption constitutes a major pathway of environmental toxin exposure in the newborn and child. The skin undergoes age-dependent changes in thickness, perfusion and extent of hydration, as well as changes in the relative size and ratio of skin area relative to body weight (Rutter, 1987; Okah *et al*., 1995; Fluhr *et al*., 2000; Amato *et al*., 1992; West *et al*., 1981).

After birth, the newborn requires about five days to acquire the normal dermal keratin layer to the integument. Without this barrier, newborns are susceptible to unique poisonings from environmental toxins. In addition, a newborn's ratio of skin surface area to body weight is about 2.7 times greater than an adult's (Selevan *et al*., 2000). Consequently, for an identical percutaneous dose of an environmental toxin, a newborn's systemic absorption (bioavailability) of that toxin will exceed an adult's by a threefold margin (Reed, 1996). Thus dermal absorption of chemicals previously used to clean newborn infants resulted in hypothyroidism from iodine in betadine solutions (Clemens and Neumann, 1989) and neurotoxicity from hexachlorophene (Shuman *et al*., 1974). Hyperbilirubinaemia in newborns resulted from washing with a hospital phenolic disinfectant detergent (Wysowski *et al*., 1978).

Dermal absorption of environmental toxins is inversely proportional to the integumentary thickness of the stratum corneum (Reed, 1996). Infants have more avid uptake of chemicals through their relatively thin dermis than older children and adults. This renders them more vulnerable to topical exposures. For example, aniline dyes used in the laundering of infant diapers caused an epidemic of infantile methaemoglobinaemia because of the oxidizing effects on red blood cells of aniline absorbed through their skin (Graubarth *et al*., 1945). Topical applications of high concentrations of the insecticide, *N*-,*N*-diethyl-*m*-toluamide (DEET), can cause neurological dysfunction because of systemic absorption in infants and children. Dysarthria, confusion, ataxia and seizures have all been documented in children after application of highly concentrated DEET products (Grybosky *et al*., 1961; Zadikoff, 1979; Roland *et al*., 1985), whereas such reactions are not observed in adults. Seizures also have been temporally related to dermal absorption of other chlorinated compounds such as the antiseptic agent, hexachlorophene or the pediculoside, γ-benzene hexachloride.

3.3.10 Renal Function

Chemicals absorbed by the newborn or young infant are not eliminated through the kidneys with the same efficiency as those same chemicals in adults. Nephrogenesis begins at about the ninth week of gestation and is complete by 36 weeks gestation, with changes in both renal and intrarenal blood flow occurring after birth. For example, renal blood flow if about five times lower in neonates than it is in adults, such that toxins carried in the blood are not as readily eliminated.

Postnatal maturation of renal function extends through the first 12 months of life. The glomerular filtration rate (GFR) doubles in the first two weeks of age (Guiguard *et al*., 1975). By two months of age the mean GFR is still only about $96\,\mathrm{ml\,min^{-1}}$ $1.73\,\mathrm{m^{-2}}$ (Schwartz *et al*., 1987) and it gradually rises to adult values by 8–12 months

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of age (Kearns *et al*., 2003) Tubular function undergoes a similar postnatal maturation process. Adult levels of renal concentrating ability are not achieved until about one year of age. Thus young infants may have a longer duration of exposure to those xenobiotics (or their active metabolites) that are primarily excreted by the kidneys.

3.4 Chronic Exposures and Remote/Latent Adverse Effects

Children can have late-appearing toxic effects of their exposures to environmental agents that accumulate or leave residues stored in body tissues. Cigarette smoking in adolescence can lead to significant lung dysfunction in adulthood and, in some cases, eventual induction of lung carcinoma perhaps 30–40 years later. Chronic exposures to elevated household radon concentrations during childhood is thought to be responsible for later cases of lung cancer that only become manifest in middle age. Exposure to lead in early childhood has been linked to academic performance later on in high school and to such adult maladies as hypertension and chronic renal disease. Lead previously stored in maternal bone reservoirs may be mobilized into blood and transmitted to the foetus during pregnancy.

3.4.1 Carcinogenicity

The risk of childhood cancer peaks within the first five years of life. This disproportionate risk may be related, at least in part, to prenatal environmental exposures and induced genetic changes. The foetus is very susceptible to carcinogenic agents due to its lack of some DNA repair mechanisms. The rapidly dividing cells involved in the differentiating systems characteristic of foetal development also make the potential risk of cancer-promoting mutational changes more likely. Examples of definite human transplacental carcinogenesis are scant, although the induction of vaginal adenocarcinoma following maternal exposure to diethylstilbestrol during the first trimester is proof of principle (Herbst *et al*., 1972).

Children are more susceptible than adults to the cancer-producing effects of chemicals by virtue of their longevity. Those chemicals which promote cancer only after a lengthy latency period, such as radon or asbestos, may not express their toxicity in adults who die for other reasons before the cancer becomes manifest. Children, however, will carry the toxic agent for a longer period, with a cumulatively higher risk of the clinical expression of disease. By combining contaminant data with food consumption data, researchers estimated that the one in one million benchmark for lifetime cancer risk was exceeded for such food contaminants as arsenic, chlordane, DDT, dieldrin, dioxins and PCBs by the time a child is 12 years old (Dougherty *et al*., 2000).

Background sources of radiation illustrate the relationship of environmental exposures to a child's lifetime cancer risk. An estimated 13 000 lung cancer deaths per year in the United States are attributable to residential long-term exposures to radon daughters. Several studies have confirmed that the cancer risks associated with radon increase in relation to both cumulative and time-weighted exposures (Pershagen *et al*., 1994; Jacobi and Paretzke, 1985). The Japanese longitudinal studies of atomic bomb survivors found that persons younger than 20 years at the time of radiation exposure were more susceptible to radiation-induced cancers. Thus it is plausible that children are more susceptible to lung cancer from chronic residential radon exposure than adults (Jacobi and Paretzke, 1985; Committee on Environmental Health, American Academy of Pediatrics, 1989).

Carcinogens generally target tissues undergoing rapid growth and differentiation due to changes occurring within DNA during cell growth and a shortened period for DNA repair. This may put children at a higher risk compared to adults. For instance, an epidemic of scrotal cancer occurred among adolescent chimney sweeps in Victorian England. Although adult occupational exposure at that time was also significant for soot and other cancer-causing materials, scrotal tumours were generally seen only in the adolescent chimney sweeps. Thus, it appeared that scrotal development during adolescence was particularly susceptible to the carcinogens in soot. An increased risk of paediatric brain tumours was associated with household flea/tick pesticide use from pregnancy to diagnosis (Pogoda and Preston-Martin, 1997). Paternal occupational exposure to pesticides was also found to have a statistically significant increased risk for Wilm's tumour in their offspring (Fear *et al*., 1998).

3.5 Children's Nutrition and Toxic Exposures

Differences in children's diet and metabolism from that of adults account in part for their greater susceptibility to environmental contaminants transmitted in food and water. Infants drink twice the amount of water and eat two to three times the amount of food per body weight as adults. The average milk intake of an infant per kilogram of body weight would be the equivalent of an adult drinking 50 glasses of milk daily. The growth velocity is accelerated in childhood, such that birth weight is increased 18 times over the first 20 years of life. Moreover the types of foods eaten by children may differ from the portions eaten by adults. The one-year-old child eats two to seven times more grapes, bananas, pears and carrots. Different preferences in food choices

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may potentially lead to different risks of exposure to unacceptable daily intakes of pesticide residues and other food contaminants in children.

3.5.1 Breast Feeding, Lactation and Milk Formula

Table 4 presents some examples of environmental chemicals that can be concentrated in human breast milk. Drugs and environmental chemicals may concentrate in a mother's breast milk by many different mechanisms, including transcellular diffusion, intercellular diffusion, passive diffusion and ionophore diffusion (Berlin, 1992). Diffusion, a primary transport mechanism for toxins to enter breast milk from maternal tissues, is related in part to the lipid solubility of a chemical. The fat solubility coefficient will significantly influence the final concentration of the toxin in breast milk, as will protein-binding characteristics, low molecular weight and other factors. For example, the level of dieldrin, a widely banned pesticide, in the breast milk of a lactating woman is about six times higher than its concentration in her blood (Solomon and Weiss, 2002).

Table 4 Examples of lipophilic chemicals concentrated in human milk

Chemical	Commercial use
Aldrin	Pesticide
Benzene	Solvent, by-product
Benzene hexachloride	Pesticide
Carbon disulfide	Solvent
Carbon tetrachloride	Solvent
Chlordane	Pesticide
DDT (dichlorodiphenyl-trichloroethane)	Insecticide
Dieldrin	Pesticide
Dioxin	By-product
Furans	By-product
Heptachlor	Pesticide
Hexachlorobenzene	Pesticide, disinfectant
Hexachlorocyclohexane	Insecticide
Methylene chloride	Solvent, paint stripper
PBB (polybrominated biphenyls)	By-product
PBDE (polybrominated diphenyl ethers)	Flame retardant, manufacturing
PCB (polychlorinated biphenyls)	Flame retardant, electrical equipment
Styrene	Solvent
Trichloroethylene	Solvent
Tetrachloroethylene	Solvent
Toluene	Solvent
Xylene	Solvent

Wolff (1983); LaKind *et al*. (2001); Solomon and Weiss (2002); LaKind *et al*. (2004).

Thus, organochlorine insecticides and chlorinated hydrocarbons, such as dieldrin, heptachlor, lindane and DDT, halogenated solvents and other industrial compounds, and POPs such as PCBs, PBBs and furans concentrate in the 30% fat content of breast milk because of their lipophilicity. Women are exposed to the 17 most toxic congeners of dioxin and furan POPs (known as the 2,3,7,8 congeners) only by ingestion from dietary sources. Indeed milk expression may be the principal route of elimination of such xenobiotics from maternal tissues. Other industrial chemicals with high partition coefficients favouring breast milk vs. maternal blood include solvents such as tetrachloroethylene and carbon disulfide (Wolff, 1983).

Regional variability of exposure to POPs account in large measure for different levels of such chemicals detected in women from different countries and regions of the world (LaKind *et al*., 2001). With prohibition or declining use of a chemical in a country, a trend towards decreasing levels of the chemical in breast milk can sometimes be documented. Thus, countries such as Germany, Japan and Sweden have reported declining concentrations of hexachlorocyclohexane in human milk, and Sweden has observed declining concentrations of PCBs (LaKind *et al*., 2004). Concentrations of DDT in human milk declined from the 1970s to the 1990s in both Canada and the United States. Local use patterns of chemicals can also correlate with their concentration in milk. In Spain, a twofold difference in heptachlor epoxide levels was found in rural vs. urban women's breast milk, likely due to rural, agricultural usage patterns (Solomon and Weiss, 2002).

Rarely have environmental toxins to which an infant has been exposed been proven to have clinically important deleterious effects on the child's health directly, although any addition of xenobiotics to the background sources of overall exposure is deleterious. An association was found between transplacental and breast milk exposure to PCB and related compounds and developmental delays in young children (Gladen *et al*., 1988). And the strongest predictor of blood levels of organochlorine chemicals in a sample of 337 German seven-year-olds was whether or not they had been exclusively breast fed in infancy, with a dose-dependent relationship related to duration of lactation (Karnaus *et al*., 2000).

Lactating mothers exposed to cadmium, mercury and lead can secrete these metals into their breast milk, although since metals are not bound to proteins, this occurs only in trace amounts (Oskarsson *et al*., 1995). Breast-milk exposure of infants to methylmercury has resulted in significant neurotoxicity and has resulted in cautionary advice to lactating women against consumption of large amounts of mercury-containing fish. An epidemic of mercury poisoning occurred in Iraq in 1971–1972 after the mistaken consumption of methylmercury-contaminated grain used to make bread.

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It was subsequently confirmed that many infants developed neurotoxicity from exposure to methylmercury in breast milk (Amin-Zaki *et al*., 1976). These infants were longitudinally studied for the subsequent five years and found to develop progressively worsening hyper-reflexia, delayed motor development, mental retardation, and impaired motor, sensory and autonomic function (Amin-Zaki *et al*., 1981).

While milk formula might be considered less vulnerable to xenobiotic contamination than breast milk, this is not necessarily the case. Cows will secrete hormones, antibiotics and chemicals to which they have been exposed into their milk. During the processing of milk, contamination can occur. Milk contamination with the industrial chemical melamine in China in 2007–2008 resulted in illness in thousands of infants and death in some, due to melamine's renal toxicity. The use of contaminated water to reconstitute powdered formula is another route for the exposure of an infant to a variety of metals, biological agents and chemicals. Finally the use of lead-containing cans for milk storage or lead-containing cookware for the sterilization of milk formula is another source of potential contamination.

3.5.2 Dietary Preferences and Pesticide Residues

More than 1500 pesticides are used in the United States, and these can be combined to synthesize 50 000 commercial compounds (Needleman, 1995). It is estimated that £4.23 billion of pesticides were used in the USA in 1993 (Goldman, 1995), and worldwide the use of pesticides, including environmentally persistent, organochlorine compounds, is even higher. Such pesticide use is associated with the presence of pesticide residues detectable on many of the fruits and vegetables consumed every day by infants and children. Given the ubiquitous presence of pesticide use in agriculture, the National Resources Defence Council, Food and Drug Administration, and the US Department of Agriculture have estimated preschooler exposure to 23 different pesticides from the consumption of 27 different fruits and vegetables. Their data suggested that the pesticide residue intake of children exceeds daily allowances for a variety of organophosphates and other agricultural chemicals (Sewell and Whyatt, 1989).

Two unique features of the preschool diet also predispose them to pesticide poisoning: a greater rate of food consumption per unit weight, coupled with a dietary preference for fruits and vegetables. Due to an increased metabolic rate necessary for growth and development, infants and young children require 90–120 cal kg^{-1} daily of nutritional energy in their diets, more than twice the caloric needs for adults. Consequently preschool children generally eat three to four times as much food per pound of body weight as average American adults. Additionally, 34% of the preschooler's diet consists of fruits compared to 20% of the adult diet. The average toddler drinks 21 times more apple juice and 11 times more grape juice than adults, and eats 2–7 times more pears, bananas, grapes, carrots and broccoli (National Research Council (NRC), Committee on Pesticides in the Diet of Infants and Children, 1993). Thus children's diets differ from those of adults, exposing them to different doses of xenobiotic contaminants of food.

The combination of these two factors results in a child's daily average consumption of fruits (per kg of body weight) that is six times more than an adult woman. Consequently, children will have a proportionally greater dietary exposure to pesticides. A report from the National Research Council (NRC) concluded that consumption of pesticide residues on food products was higher in children; indeed the NRC estimated that 50% of the lifetime ingestion of pesticides occurs in the first five years of life (National Research Council (NRC), Committee on Pesticides in the Diet of Infants and Children, 1993).

3.6 Epidemiology of Special Populations of Vulnerable Children

Within the paediatric age group there may be special populations at higher risk from environmental contamination. Children with developmental delays and autism have been shown, by virtue of their continued oral exploratory behaviours, termed 'pica' for non food items, remain at risk for heavy-metal poisoning through childhood and adolescence (Hussain *et al*., 2007). Children with ataxia-telangiectasia, an inherited condition, have a defect in their DNA repair mechanisms. When exposed to conventional radiotherapy, these children may develop life-threatening complications from acute radiation poisoning (Cunlift *et al*., 1975). Children with xeroderma pigmentosa, another inherited condition, have DNA repair defects causing premature ageing, accelerated by radiation from exposure to sunlight. Children with underlying pulmonary diseases, such as bronchopulmonary dysplasia, asthma or cystic fibrosis, are more vulnerable to exacerbations of their chronic illness brought on by exposure to indoor or outdoor air pollution.

3.6.1 Example of a Special Population of Children: Childhood Asthma and Indoor Air Quality

Childhood asthma has grown at alarming rates, both in the United States and worldwide, over the last four decades, with large increases in asthma prevalence, hospitalization rates and mortality (Mannino *et al*., 1998). Allergens appear to play a critical role in the severity and chronicity of asthma, particularly in the inner city (Platts-Mills, 1994). Epidemiologic studies have established an association between elevated serum total IgE

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and asthma (Burrows *et al*., 1989), and skin-test positivity to indoor allergens, including those derived from dust mites, cockroaches, cats and dogs, is also a common finding in asthma (Eggleston *et al*., 1998).

Dust mites may be the best-studied allergen connected with asthma (Halken, 1994). From the genus *Dermatophagonides*, these mites deposit faeces onto many household places, including bedding, drapes, upholstered furniture and carpets (Lanphear, 2001). Dust mites are very potent allergens, with direct inhalation causing an immediate fall in FEV_1 and late-phase response in sensitized asthmatics (Sporik, 1990), though less prevalent in inner cities than the general population (Kattan *et al*., 1997).

Cockroach allergen is a well-described allergic trigger for asthma that is particularly important in low-income urban communities (Platts-Mills, 1994). Though many species of cockroach exist, the German cockroach (*Blattella germanica*), is the most common cockroach species infesting homes in cities in the United States and many other parts of the world, and is the best studied (Call *et al*., 1992). Children with the combination of a positive skin-test reaction to cockroach extract and a bedroom level of *Bla g* 1 exceeding 8 $U\,g^{-1}$ of dust had significantly more hospitalizations for asthma, unscheduled medical visits and parent-reported wheezing than other children (Rosenstreich *et al*., 1997). In addition to making asthma worse, recent data suggests exposure to cockroach allergen and sensitization is associated with an increased incidence of wheeze in the first year of life (Gold *et al*., 1999) and incident asthma among the siblings of the index children in a birth cohort (Litonjua *et al*., 2001).

In addition to cockroach allergen, a recent study has found connection with mouse allergen exposure (Phipatanakul *et al*., 2000a) and sensitization to severity of asthma (Phipatanakul *et al*., 2000b). There is also suggestive evidence of mouse allergen exposure as a potential contributor to the development of wheezing in the first year of life (Phipatanakul *et al*., 2005).

Mould exposure, particularly from damp housing, has been an important potential contributor to asthma as well (Strachan, 1989). Studies have suggested that exposure to dampness and mould can be a trigger for asthma symptoms (Jaakkola *et al*., 1993), as well a potential source for developing asthma in the first place (Institute of Medicine, 2004).

3.6.2 Example of a Special Environmental Toxin for Children: Bisphenol A, Phthalates

Recent concerns have been raised about the potential links between certain chemicals used in the manufacturing of plastics, and children's health.

Bis-Phenol A (BPA) is used to make polycarbonate, the plastic most commonly used for baby bottles and sippy cups. A known endocrine disruptor compound, BPA mimics oestrogen and has been linked with a range of health effects in animal and human studies (vom Saal and Hughes, 2005). It has chemical properties similar to oestradiol and can impact biological systems in very low doses (Claudio and Chace, 2006). BPA is more likely to leach from its polycarbonate matrix mainly during two instances: first, when the plastic is cracked or shows significant wear and tear; second when the contents of the actual plastic container are heated.

Phthalates represent a family of compounds with names like DEHP (diethylhexyl phthalate), DEHA (diethylhexyl adipate) and DINP (diisononyl phthalate). In 2003, the American Academy of Pediatrics in the United States came out with a consensus statement about where common sources are for children and the potential health effects (Shea, 2003). Phthalates are used to soften plastics called PVC (polyvinyl chloride), and are found in a range of products used by children, including toys. Like BPA, they are known endocrine disruptor compounds, acting as antiandrogenics and can adversely impact androgen-sensitive tissues during specific windows of development (Claudio and Chace, 2006). They have been linked with varied health effects in both animal and human studies (Sathyanarayana *et al*., 2008).

While the science regarding both BPA and phthalates is both emerging and controversial, there has been movement both in the United States and abroad to ban or limit children's exposure to both these chemicals based on the available animal and limited human epidemiologic data.

3.6.3 Child and Adolescent Workers

Over four million children and adolescents in the United States work outside the home; worldwide the figure may approach 200 million children. Entry-level, unskilled or menial jobs for which they qualify may bring them into contact with chemicals, gases and fumes, and other toxic hazards in the workplace environment. Exposure to heavy metals, pesticides, caustics and cleaning agents may be more of a risk to children working because they are not employed in regulated industries, will take greater risks to please an employer, and may not receive the appropriate training needed to work safely with dangerous chemicals. In the United States, 3% of 300 000 poison-centre occupational calls over a five year period involved adolescent workplace exposures (Woolf *et al*., 2001). The most common hazardous agents included corrosives, fumes and gases, bleaches, drugs, acids and hydrocarbons. In many cases, the exposure could have been prevented by proper training and the use of barriers, such as gloves and eyewear.

The NRC, citing the pesticide exposure risks of children working in agriculture and the risks of exposure to solvents and other chemicals to children employed in other industries, recommended in its book *Protecting Youth At Work* the development of a comprehensive plan

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for monitoring work-related injuries to children, as well as monitoring the hazards to which they are exposed (National Research Council, Committee on the Health and Safety Implications of Child Labor, Board on Children, Youth and Families, Committee on Behavioral and Social Sciences and Education, Institute of Medicine, 1998).

Families in developing countries may rely on children's workplace earnings for economic support. In such circumstances, children may work in unregulated industries, such as mining, building materials fabrication, construction, agriculture and manufacturing, with high risk of poor health due to their exposure to toxic substances, physical hazards, repetitive motion strain and injury, and physical and mental demands. Injuries account for both morbidity and mortality among working children; unhealthy work conditions have long-term implications for their future productivity. Moreover, such labour may divert them from an education and the nurturing of their youth so as to enhance their chances to become productive citizens in the future.

3.6.4 Smoking, Solvent Abuse and Problem Experimentation

Children and adolescents are at high risk for a variety of environmental toxins through their developmental vulnerability to peer pressure and experimentation. Numerous studies have shown that the age of initiation of cigarette smoking is before age 18 years, and some adolescent smokers begin to develop architectural changes in lung anatomy and measurable decrements in lung function by the time they are young adults (Woolf, 1997). As many as 30% of young adolescents experiment with volatile inhalants. Such experimentation with 'gateway' substances of abuse in early adolescence may lead to chronic use and dependence. The adult sequelae of addictions initiated in adolescence are formidable for the individual, in terms of poor health, chronic illness and shortened lifespan, as well as for the public's health, include disproportionate use of costly health services, increased frequency of social pathology and disordered relationships, and loss of future productivity.

4 LABORATORY TESTING OF SUSCEPTIBLE POPULATIONS

Both environmental testing and biological tests of biomarkers in body fluids can be helpful in determining whether or not environmental toxins are associated with specific paediatric health complaints. However such testing must always be interpreted in the context of extant clinical data. In addition the clinical applications of some laboratory analyses remain controversial; specifically both chelation challenge testing and hair analyses have been disputed as lacking in adequate scientific evidence in some of their clinical applications to the assessment of children's exposures.

4.1 Tissue Matrix Testing

Both blood and urine testing are frequently used in some environmental exposures to confirm a child's contamination. However such testing is limited by a variety of parameters, including technical (e.g. detection levels, analytical reliability), clinical (e.g. validity of normative and/or 'no-effect' levels as applied to newborns, infants and children) and circumstantial (e.g. time lag between exposure and biological test performance) issues. Blood lead levels greater than $10\,\mu g\,dl^{-1}$ have been associated with neurodevelopmental delays in children, although some research has suggested that this threshold 'no effect' level has been set too high. Red blood cell cholinesterase levels are an indirect measure of recent exposure to organophosphate pesticides, since acute exposure levels of the enzyme will show marked decrements vs. baseline concentrations. A blood mercury level equal to $5.8\,\mu g\,l^{-1}$ has been used as a 'no-effect' level used in determining when methylmercury exposures can produce cognitive decrements (Tresande *et al*., 2005). A child's serum cotinine level has been used as a biomarker of exposure to environmental tobacco smoke.

4.2 Hair Testing

Siedel *et al*. (2001) assessed the reliability of data from hair analyses performed by commercial laboratories in the United States and found that designations of normal values for different metals and minerals varied greatly between laboratories, as did sample preparation techniques and quantitative analysis methodologies. These authors submitted split hair samples to different commercial laboratories; they found that the resultant hair content analyses varied widely and were unreliable for any clinical applications. Likewise Steindel and Howanitz (2001) found that hair analyses for trace metals gave discrepant results with wide coefficients of variability and a lack of uniformity in acceptable laboratory standards in methodology. Forensic toxicology studies of cadavers have found a lack of correlation between elemental concentrations in hair and that in other human organs (e.g. cerebrum, cerebellum, kidneys, heart and liver) (Yoshinaga *et al*., 1990). Contamination of hair by external environmental sources, a lack of sample homogeneity, variable collection methods and widely variant dispersion of metals within the hair matrix confound the clinical utility of analysing this matrix. And yet in carefully

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controlled, experimental settings, using proper collection and analytic techniques, hair analysis has been found to be a valid biomarker of childhood exposures to environmental metal contaminants (Wright *et al*., 2006).

4.3 Chelation Challenge Testing

Prior to the advent of reliable blood lead testing, quantitative lead excretion in urine after an eight hour challenge with sodium calcium edetate (NaCaEDTA) was used to determine which children with suspected plumbism were candidates for five day hospital courses of parenteral chelation. With the development of very sensitive and specific whole-blood lead testing, this diagnostic test became outmoded and is no longer used clinically. The application of chelation challenge testing to other clinical uses, for example, in the determination of environmental exposures to metals of children with autism, has been disputed (Soden *et al*., 2007).

5 OTHER ENVIRONMENTS: BUILDING-RELATED ILLNESS

Children may spend much of their day out of the home, in daycare centres, nurseries or schools, where they may be exposed to indoor air and other pollutants. Building-related illness has occasionally been linked to volatile chemicals used in cleaning schools, chemicals off-gassing from newly installed floors and carpets or the routine indoor use of pesticides. Outdated and/or poorly maintained ventilation-exhaust and air-intake systems may contribute to poor air circulation and insufficient fresh air exchanges. Water damage to schools can create mould and mildew problems, old flaking ceiling tiles may expose children to asbestos and some schools are still contaminated with lead-containing paints.

Children react to indoor-air pollution from building-related causes, such as dust mites or moulds by wheezing (Etzel, 1995), or with burning eyes, headaches, sore throats and other irritant symptoms, depending on other aetiological agent(s).

6 INTERNATIONAL IMPLICATIONS

Children have long been recognized by international organizations as disproportionately affected by hazards in their environment. The World Health Organization estimates that more than 10 million children under the age of five die each year due to unhealthy environments (Gordon *et al*., 2004). More than 1.1 billion people in rural and underdeveloped areas, especially in Africa and Asia, do not have access to safe water; 2.2 billion do not have access to hygienic toilet facilities. Children are particularly susceptible to such poor sanitary conditions, which foster chronic diarrhoea, dehydration and such infections as typhoid and bacterial dysentery; more than 2 million succumb each year (Gordon *et al*., 2004). A disproportionate number of the world's children are malnourished and living in poverty, their futures prematurely shortened by the consequences of a marginal existence in a degraded environment.

Smith *et al*. (1999) employed consistent definitions and quantitative methodologies to estimate that 25–33% of all global ill health can be attributable to environmental risk factors, with children under five years of age bearing the largest environmental burden. Basic necessities, such as clean water, sanitary toilet facilities and safe food, are still out of reach in many poor, rural nations, especially in Asia and Africa. Such conditions foster childhood diseases such as diarrhoea and parasite infestations that exact a staggering toll in terms of lost lives and lost productivity. Biomass burning and indoor- and outdoor-air pollution contribute to high rates of respiratory illnesses among children living in developing countries. Globally, childhood poisonings and injuries account in large measure for high morbidity and mortality rates among infants and toddlers.

More than 200 million children worldwide between the ages of 5–14 years old forego any schooling to work outside the home, often in extremely hazardous occupations such as agriculture, construction, and mining and quarrying, to support their poverty-stricken families. Such environments expose them to physical injury, as well as to noxious chemicals, including solvents, glues, heavy metals and silica-containing dusts.

Such stark realities demand international answers addressing the pressing need for more healthful child environments.

7 RESEARCH DISPARITIES

An important context within which to consider the vulnerability of children to environmental toxins is the sparse knowledge we have of the toxicities such chemicals may produce in exposed children. Of the 3000 chemical pesticides produced in the United States at $\geq$1 million pounds annually, less than 7% have been fully studied for their effects on humans (Stein *et al*., 2002). Children are more vulnerable to xenobiotics because there is comparatively little that is known about the long-term outcomes of environmental exposures of children and the sequelae of such exposures in adult life. Much of what we know about such exposures is extrapolated from *in vitro* systems or animal studies, and such results may have errors in their extrapolation to humans, let alone to children. Often these are studies of acute

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or subacute exposures of animals to single chemicals with such gross end points as relative lethality, rather than the more typical circumstances seen in children, involving chronic, low-dose background environmental exposures with subtle adverse-effect outcomes. Pope and Liu (1997) describe the relative sensitivity of adult vs. young rats to chlorpyrifos and other organophosphate pesticides and how such age-related differences can be obscured in improperly designed experimental studies that only examine lethality, instead of neurochemical changes, as an outcome.

Many studies of the effects of chemicals in humans have involved only healthy adults exposed to higher concentrations of these chemicals found in workplace settings. Adult safety standards derived from such studies may or may not be extrapolated to the safety of children. The circumstances of such exposures frequently assume eight hour workdays and five day workweeks of exposure. Those results are then further extrapolated to daily, lower-dose background levels of repetitive exposures in children to calculate tolerable exposure levels, often utilizing 10-fold uncertainty factors to account for differences in age and 3.2-fold uncertainty factors to account for toxicokinetic and toxicodynamic variability (Renwick, 1998). However, some have questioned the adequacy of such uncertainty factors, considering the range of variability in toxicokinetic and other differences between children and adults (Weiss, 2000; Ginsberg *et al*., 2002). It may be an error to assume that children of all ages will be equally protected by modelling their theoretical risk, some researchers have suggested that the estimates are perhaps adequate for between 60 and 100% of the population over the age of six months (Dourson *et al*., 2002).

There is little research directed at questions and hypotheses specifically involving children and their environmental exposures, and only a relatively small amount of funding and research resources is dedicated to such studies. Children have been called 'research orphans' because of the lack of private and public funding and a relative dearth of both governmental and academic institutional initiatives dedicated to children and their environmental risks.

Very few studies consider an 'integrated environmental risk', that is, synergistic and/or additive interactions effected by exposures to multiple chemicals. And yet children are exposed to background levels of varying mixtures of pollutants in their environment over time. Animal models suggest that such multiple, simultaneous exposures to pollutants and xenobiotics are far more damaging, or cause damage at lower levels, than do single agent exposures in experimental settings (Carpenter, 2002; Stein *et al*., 2002).

Moreover, research studies that are done in children often consider them as a homogeneous group, rather than as heterogeneous subpopulations by age and developmental stage or by ethnic, racial and socioeconomic differences. Very few studies have addressed in children any added risk to environmental toxins engendered by an underlying chronic illness, such as childhood diabetes, seizure disorders, mental retardation or cystic fibrosis.

Thus, a new research agenda specific to paediatric environmental health concerns is of paramount importance, since the questions and challenges are so different from those of adults. There is a compelling need for better research into the health implications of children's exposures to environmental toxins, especially regarding high prevalence conditions, such as childhood cancer, asthma, endocrine and sexual disorders, and neurobehavioural toxicants (Landrigan *et al*., 1998).

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Clinical Toxicology

Alister Vale and Sally Bradberry

CONTENTS

1 INTRODUCTION

This chapter defines the specialty of clinical (or medical) toxicology and the components that should make up a comprehensive clinical toxicology service; it describes the presentation and complications of poisoning and details the general assessment and management of the poisoned patient; the chapter concludes with a discussion of the role of toxicovigilance.

2 CLINICAL TOXICOLOGY

Clinical toxicology is that discipline within toxicology which is concerned with the impact of drugs and other chemicals on humans. Some define this discipline as medical toxicology. For example, the American College of Medical Toxicology, a nonprofit association of physicians mostly from an emergency-medicine background, defines medical toxicology as a 'medical subspecialty focusing on the diagnosis, management and prevention of poisoning and other adverse health effects due to medications, occupational and environmental toxins and biological agents'. The present authors, who are physicians who have practised the discipline for several decades, believe the term clinical toxicology should be employed rather than medical toxicology to reflect the fact that clinical toxicology is both a discipline of toxicology and a medical subspecialty. This view also takes account of the fact that in North America in particular, a number of distinguished clinical toxicologists hold a PharmD and offer a professional opinion either via a poisons information centre or at the bedside. It is acknowledged, however, that in Europe most, if not all, clinical toxicologists are medically qualified.

The role of the clinical toxicologist encompasses the traditional therapeutic role, including the development of effective treatment strategies for the management of acute and chronic poisoning, the provision of expert advice via a poisons information service and the evaluation of the adverse effects of drugs and other chemicals on the body. The clinical toxicologist must be able to apply relevant kinetic and biochemical data so that treatment strategies can be developed and refined, based on the likely mechanisms of toxicity (Vale, 1992). In addition, an understanding of pathological appearances is necessary if the results of toxicity tests are to be extrapolated accurately to man and the results of forensic

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examinations are to be interpreted correctly. The clinical toxicologist will also need to be aware of the limitations of analytical methods, both as regards their sensitivity and specificity.

The increased public awareness and concern regarding the potential effects of pesticides and other chemicals means that a clinical toxicologist must be familiar with the occupational and environmental impact of a wide range of agents. A clinical toxicologist can be expected to be involved in the development of strategies for the management of major chemical disasters, including the chemical contamination of drinking water, the evaluation of antidotes used against chemical warfare agents and in the assessment of the adverse effects of pesticides, whether resulting from a single exposure or chronic low-level exposure.

Thus, clinical toxicologists will need to possess a detailed knowledge and understanding of the clinical, biochemical, kinetic, analytical, forensic, occupational and environmental aspects of toxicology if they are to play the important role that is now expected of them in today's society. They will also need to be committed to research and training.

3 A COMPREHENSIVE CLINICAL TOXICOLOGY SERVICE

It has been suggested previously that poisons centres need to offer a comprehensive clinical toxicology service if they are to survive and meet the challenges of the twenty-first century (Vale and Meredith, 1993). The combination of intensive treatment facilities for poisoned patients and substance abusers, a poisons information service and supporting laboratory is, we believe, the model that should be adopted. A comprehensive clinical toxicology service, embracing a clinical and advisory service, analytical support, research and training, with due emphasis on the occupational and environmental aspects of toxicology is needed if clinical toxicologists are to address adequately current toxicological challenges and opportunities.

3.1 A Poisons Information Service and an Expert Advisory Service to Physicians

A poisons information service must be available 24 hours a day, 365 days a year to provide expert advice to medical and paramedical staff, and, in most countries, to the public, on the toxicity of drugs and other chemicals, plants and animals. Such a service could also serve as a community resource so that health professionals and the general public can be advised both on ways to decrease the risk of poisoning and the measures to take if it occurs.

Physicians using the service must have every confidence that the advice given is evidence-based and up-to-date. To be credible such a service must include those with extensive training in clinical toxicology so that specific advice on the management of cases of poisoning can be given. It is a major advantage if these clinical toxicologists are also directly responsible for the management of patients suffering from the effects of acute or chronic poisoning. Such expertise is necessary if all the available information, circumstantial, clinical, laboratory and from other sources, is to be assessed accurately in relationship to the substances involved and the circumstances of exposure. Only in this way can a detailed plan for clinical management be developed which will maximize the chances of the patient surviving and minimize the risk of short- and long-term sequelae. This expert advice will need to be supported by a comprehensive collection of reference works and original papers, as well as by access to on-line retrieval systems.

3.2 An In-Patient Treatment Service

An in-patient service, of necessity, must be available at all times and be staffed by physicians with extensive training in clinical toxicology, who are respected for their professional competence and authority and who are capable of furnishing reliable opinions, as well as being adept at differential diagnosis. If possible, these physicians should also be responsible clinically, not only for the management of patients suffering from the effects of acute or chronic poisoning, but also for the detoxification of substance abusers. If logistically possible, it is a major advantage if the in-patient beds are in a dedicated area to act as a focus for the provision of expert medical, psychiatric and social care. It is also advantageous if the psychosocial assessments are undertaken by psychiatrists who have a specific commitment to the service.

3.3 An Out-Patient Clinical, Occupational and Environmental Toxicology Service

Many chronic medical problems are alleged to have a toxicological basis, be it occupational or environmental. Patients with such suspected toxicological problems should be assessed by an experienced clinical toxicologist to determine whether the patient's clinical features are due to a toxic exposure or not. In some countries, poisons centres are part of a much larger occupational medicine unit. Where they are not, there

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should be a close working relationship between clinical toxicologists and occupational physicians, particularly if the former are not appropriately trained in occupational medicine. Only in this way can a first-class occupational toxicology service be offered. A clinical toxicology centre should also play a major role in providing advice on environmental toxicological problems, such as on the chemical contamination of drinking water, the environmental impact of pesticides and on the management of major chemical disasters.

3.4 Advice to Government, Regulatory and International Bodies

Clinical toxicology centres will wish to play a full part in providing advice and support to Government Departments, Regulatory Authorities and Intergovernmental Bodies such as the International Programme on Chemical Safety (IPCS).

3.5 Analytical Support

Whether analytical support is provided 'in-house' or by contract with an outside laboratory is less important than the quality and speed of the service offered. In-house facilities allow a close working relationship to develop between clinical and analytical toxicologists, which will strengthen and improve the quality of the overall analytical service even further.

3.6 Research and Training in Toxicology

However outstanding its clinical service, no clinical toxicology centre can have a major impact without being closely involved in toxicological research. It is equally true that without a well-organized and relevant research programme an outstanding clinical service is unlikely to be offered. A commitment to research not only provides a stimulating environment for more senior faculty members, but also helps attract younger colleagues who are highly motivated and committed. Furthermore, a centre which is 'spearheading' clinical advances is likely to be widely consulted and have a substantial number of patient referrals.

Training programmes should ensure that those in training are able to apply relevant kinetic and biochemical data, so that treatment strategies can be developed and refined, based on the likely mechanisms of toxicity. Trainees will also need to recognize the limitations of analytical methods and be trained in the occupational and environmental aspects of toxicology.

4 POISONING

Exposure to a substance is often equated with poisoning. However, absorption is necessary for there to be a toxic effect and, even if this occurs, poisoning does not necessarily result, because the amount absorbed may be too small. In developed countries, poisoning causes approximately 10% of acute hospital medical presentations. In such cases poisoning is usually by self-administration (that is the act is deliberate) of prescribed and over-the-counter medicines, or illicit drugs. Rarely, deliberate poisoning may be the result of criminal intent (homicide). Sometimes inappropriate treatment of a patient by a doctor (iatrogenic poisoning) is responsible for the development of poisoning, for example, in the case of digoxin toxicity.

Poisoning in children aged less than six months is most commonly iatrogenic, and less frequently accidental by the parents, and involves over-treatment with, for example, paracetamol. Children between eight months and five years of age also ingest poisons accidentally, or they may be administered deliberately to cause harm, or for financial or sexual gain.

Occupational poisoning as a result of dermal or inhalational exposure to chemicals is a common occurrence in the developing world and still occurs in the developed world. As a result of changes to manufacturing processes, which may be due to criminal intent or incompetence, substantial morbidity and mortality has resulted. A famous example is the outbreak of diethylene glycol poisoning in 1937 due to the contamination of sulfanilamide (Geiling and Cannon, 1938); in recent years other outbreaks of diethylene glycol poisoning have occurred in Australia, Bangladesh, Haiti, India, Nigeria and South Africa following its inappropriate use commercially.

The type of agent taken in overdose is also heavily influenced by availability and culture. In the UK, paracetamol poisoning is responsible for approximately one third of all admissions (Bateman *et al.*, 2006), whereas in Sri Lanka, for example, the agents ingested are more often pesticides or plants such as oleander (Eddleston *et al.*, 2005). In addition, ingestion of heating fuels, antimalarials, antituberculous drugs and traditional medicines are reported frequently in the developing world.

The toxicity of a substance, and therefore the features of poisoning, can generally be predicted from its physicochemical properties, its pharmacological or toxicological actions, the route of exposure and the dose to which an individual has been exposed. The features of poisoning are classified as either local or systemic. Local toxicity is

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confined to the site of contact of the substance with body surfaces. The route of exposure (eye, skin, respiratory or gastrointestinal (GI) tract) determines the anatomical location of the interaction; the physicochemical characteristics of the substance (solubility, volatility, pH) define its nature and extent. Systemic toxicity depends on the fraction of the dose of the poison that is absorbed into the circulation; systemic toxicity is generally dose related, may be organ-specific, or may involve several organs.

While the pharmacological and toxicological effects of the poison are generally proportional to the amount that has been absorbed, the effects are modulated by variations between individuals. Some individuals react in a non-dose-dependent, idiosyncratic manner to some agents (such as metoclopramide, and some phenothiazines and butyrophenones). The speed with which features appear depends partly on the route of exposure; it is greater with inhalation and injection than with dermal exposure and ingestion.

4.1 Assessment and Management of the Poisoned Patient

The assessment and management of an acutely poisoned patient involves the following approach:

1. Taking an appropriate history
2. Assessing vital functions (the level of consciousness, ventilation, circulation and temperature) and correcting any impairment
3. Examining the patient and observing features (**Tables 1** and **2**) that help to identify the substance(s) to which the patient has been exposed
4. Identifying complications and treating them:
   (a) Fluid, acid–base and electrolyte abnormalities
   (b) Skin blisters
   (c) Convulsions
   (d) Rhabdomyolysis
   (e) Methaemoglobinaemia
   (f) Serotonin syndrome
5. Requesting appropriate investigations
6. Assessing the patient psychiatrically and offering appropriate management.

Diagnosis is based on the history, circumstantial evidence, the features present (if any) and, occasionally, on the results of haematological, biochemical and toxicological analyses.

4.2 Taking a History

About 80% of adults who have ingested an overdose are conscious on arrival at hospital and the diagnosis of self-poisoning can usually be made from the history. In unconscious patients, a history from friends or relatives is helpful, and the diagnosis can often be inferred from tablet bottles or a 'suicide note' brought by the paramedics, or made by exclusion of other causes. Self-poisoning must always be considered in the differential diagnosis in any patient with an altered consciousness level, even if relatives claim that the individual would never take an overdose.

Table 1 Features of poisoning

Features	Likely poisons
Constricted pupils (miosis)	Opioids, organophosphorus and carbamate insecticides, nerve agents
Dilated pupils (mydriasis)	Tricyclic antidepressants, amphetamines, cocaine, anticholinergic drugs
Divergent strabismus	Tricyclic antidepressants
Nystagmus	Carbamazepine, phenytoin
Loss of vision	Methanol, quinine
Papilloedema	Carbon monoxide, methanol
Convulsions	Tricyclic antidepressants, theophylline, opioids, mefenamic acid, isoniazid, amphetamines
Dystonic reactions	Metoclopramide, phenothiazines
Delirium and hallucinations	Amphetamines, anticholinergic drugs, cannabis, recovery from tricyclic antidepressant poisoning
Hypertonia and hyper-reflexia	Tricyclic antidepressants, anticholinergic drugs
Tinnitus and deafness	Salicylates, quinine
Hyperventilation	Salicylates, phenoxyacetate herbicides, theophylline
Hyperthermia	Ecstasy (MDMA), salicylates
Blisters	Usually occur in comatose patients

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Table 2 Feature clusters in acute poisoning

Feature clusters	Poisons
Coma, hypertonia, hyper-reflexia, extensor plantar responses, myoclonus, strabismus, mydriasis, sinus tachycardia, convulsions	Tricyclic antidepressants; less commonly antihistamines, orphenadrine, thioridazine
Coma, hypotonia, hyporeflexia, flexor or nonelicitable plantar responses, hypotension	Barbiturates, benzodiazepine and alcohol combinations, tricyclic antidepressants
Coma, miosis, reduced respiratory rate	Opioid analgesics
Nausea, vomiting, tinnitus, deafness, sweating, hyperventilation, vasodilation, tachycardia	Salicylates
Hyperthermia, tachycardia, delirium, agitation, mydriasis	MDMA (ecstasy) or other amphetamine
Miosis, hypersalivation, bronchorrhoea	Organophosphorus and carbamate insecticides, nerve agents

Acutely poisoned patients may be emotionally and psychiatrically distressed, and require competent, sympathetic assessment if essential information is not to be missed. It is pertinent to try to establish the nature of the substance taken, the amount involved, the route (ingestion, injection, inhalation or dermal) and the time of exposure, so that the clinical course can be anticipated and the risk assessed. However, statements about the nature and amount of what has been taken should be regarded with clinical suspicion, because these are often inconsistent with laboratory analysis of blood or urine (Mahoney *et al*., 1990; Pohjola-Sintonen *et al*., 2000). When the time of exposure is important (e.g. paracetamol poisoning), the accuracy can be improved by relating events to activities of daily life (e.g. the time of a television programme).

4.3 Assessment of Vital Functions and Their Management if Impaired

The level of consciousness, ventilation and circulation should be assessed in all patients.

4.3.1 Level of Consciousness

The Glasgow Coma Scale (GCS) is the most commonly used method to assess the degree of impairment of consciousness. The AVPU (Alert, Voice, Pain, Unresponsiveness) scale is a simplified version of the GCS (alert, responsive to verbal stimulation, responsive to painful stimulation and unresponsive) and corresponds well to GCS scores when assessing level of consciousness in the poisoned patient (Kelly *et al*., 2004). A GCS score of ≤ 8 (not obeying commands, not speaking, not eye opening) should prompt careful respiratory assessment, particularly if the laryngeal (gag) reflex is lost.

4.3.2 Ventilation

In any case of poisoning the priority is to assess and, if necessary, treat impairment of respiratory function. Food, vomit, secretions and dentures should be removed from the patient's mouth and pharynx, and the tongue prevented from falling back. The patient should be nursed with their head down in the left lateral position to minimize the risk of aspiration of the gastric contents into the lungs.

Pulse oximetry can be used to measure oxygen saturation. The displayed reading may be inaccurate when the saturation is below 70%, when peripheral perfusion is poor, and in the presence of carboxyhaemoglobin or methaemoglobin. If the patient has an arterial oxygen saturation of less than 95% (by pulse oximetry), is comatose (GCS $\leq$ 8) and/or the laryngeal (gag) reflex is absent, arterial blood gases should be measured. Only measurement of arterial blood gases indicates the presence of both hypercapnia (an increase in the arterial partial pressure of carbon dioxide) and hypoxia (a decrease in the arterial partial pressure of oxygen). The presence of ventilatory insufficiency (as determined by arterial partial pressure of oxygen ≤ 9 kPa on air and/or arterial partial pressure of carbon dioxide ≥ 6 kPa) should lead to intubation and mechanical ventilation if the central respiratory depression cannot be reversed by administration of a specific antidote, such as naloxone (see Section 4.7.2.2). Even when ventilation is satisfactory on presentation, it must be reassessed periodically

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because deterioration is well recognized (e.g. after ingestion of a sedative drug).

4.3.3 Circulation

Pulse, blood pressure and temperature (core and peripheral) should be measured to assess cardiovascular function. An electrocardiogram (ECG) should be undertaken in moderately or severely poisoned patients, particularly when a drug with a cardiotoxic action (e.g. a tricyclic antidepressant that produces QRS prolongation) has been ingested (Thanacoody and Thomas, 2005). Although hypotension (systolic blood pressure $\leq$80 mmHg) is a recognized feature of acute poisoning, the classical features of shock (tachycardia and pale, cold skin) are seen rarely, because only a minority of patients are severely poisoned.

Hypotension and shock may be caused by:

1. A direct cardiodepressant action of the poison (e.g. β-blockers [DeWitt and Waksman, 2004], calcium-channel blockers [DeWitt and Waksman, 2004], tricyclic antidepressants [Thanacoody and Thomas, 2005]);
2. Vasodilation and venous pooling in the lower limbs (e.g. angiotensin-converting enzyme (ACE) inhibitors [Lip and Ferner, 1995], phenothiazines);
3. A decrease in circulating blood volume because of GI losses (e.g. profuse vomiting in theophylline poisoning [Vale, 2007b]), increased insensible losses (e.g. salicylate poisoning [Chapman and Proudfoot, 1989]), increased renal losses (e.g. poisoning due to diuretics) and increased capillary permeability.

Hypotension may be exacerbated by coexisting hypoxia, acidosis and dysrhythmias. Young patients are generally not at risk of cerebral or renal damage unless their systolic blood pressure falls below 80 mmHg. In older patients, it is preferable to maintain systolic blood pressure above 90 mmHg. As a first step, the patient should be placed in a head-down position by elevating the foot of the bed by 15 cm. If this measure fails to produce improvement, plasma volume should be expanded by infusion of a crystalloid such as sodium chloride solution 0.9%. Invasive haemodynamic monitoring to confirm that adequate volume replacement has been administered may be appropriate. Dobutamine $2.5-10\,\mu g\,kg^{-1}\,min^{-1}$, or epinephrine $0.5-2.0\,\mu g\,kg^{-1}\,min^{-1}$, is indicated if hypotension is resistant to these measures; dopamine $2-5\,\mu g\,kg^{-1}min^{-1}$ is an alternative. A vasoconstrictor sympathomimetic drug (e.g. norepinephrine) may be necessary in severe cases, but it must be recognized that blood pressure may be raised at the expense of perfusion of vital organs, such as the kidneys.

A few drugs when taken in overdose may produce systemic hypertension. If this is mild and associated with agitation, a benzodiazepine such as diazepam may suffice. In more severe cases, for example, those due to a monoamine oxidase inhibitor, there may be a risk of arterial rupture, particularly intracranially. To prevent this, intravenous (iv) isosorbide dinitrate $2-10\,mg\,h^{-1}$ up to $20\,mg\,h^{-1}$ if necessary, or an α-adrenergic blocking agent (e.g. phentolamine, 5 mg iv every 10–15 minutes), or sodium nitroprusside $0.5-1.5\,\mu g\,kg^{-1}\,min^{-1}$ by iv infusion, may be administered until blood pressure elevation is controlled.

4.3.4 Abnormalities of Temperature

A core (rectal) temperature below 35 °C is often present in older patients who are comatose, particularly if they have been exposed to cold temperatures for several hours. Placing the patient in a room with moistened air at a temperature of 27–29 °C and covering him or her with a foil space blanket to minimize heat loss is the best way to treat hypothermia. In addition, iv and intragastric fluids at normal body temperature may be used. Local radiant heat should not be used.

Rarely, body temperature may increase to potentially fatal levels after poisoning with central nervous system (CNS) stimulants such as cocaine, amphetamines, including 3,4-methylenedioxymethamphetamine (MDMA) (ecstasy) (Freedman *et al*., 2005), monoamine oxidase inhibitors or theophylline. In such cases, muscle tone is often increased (hypertonia) and convulsions (see Section 4.5.3) and rhabdomyolysis (see Section 4.5.4) are common. Cooling measures should be instituted, sedation with diazepam should be given and, in severe cases, dantrolene 1 mg (kg body weight)$^{-1}$ should be administered intravenously.

4.4 Examination and Identification of Poison-Induced Features

Physical signs (features) are particularly important when trying to elucidate the cause of unexplained coma. A diagnosis of acute poisoning can only rarely be made on the basis of a single physical sign, but there are clusters of features that make a diagnosis of poisoning with specific drugs very likely (**Table 1**). General observations may also reveal useful information. For example, solvents or alcohol may be smelt on the breath, track marks may reveal undisclosed illicit substance abuse, atypical bruising may warn of domestic or other violence, and the signs of alcoholic liver disease may be revealed.

Inequality of the pupils is not uncommon in poisoned patients. Widely dilated pupils that react poorly to light may be caused by poisons with anticholinergic actions (e.g. tricyclic antidepressants) or sympathomimetic effects (e.g. amphetamines). Miosis (constricted pupils) is usually caused by opioid analgesics or poisons

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with cholinergic or anticholinesterase actions (e.g. organophosphorus or carbamate insecticides, nerve agents). Visual impairment (blindness) is associated most commonly with quinine (Mackie *et al*., 1997) and methanol (Barceloux *et al*., 2002) poisoning.

Strabismus (squint), internuclear ophthalmoplegia (paralysis of the muscles inside the eye that control the iris and ciliary body) and total external ophthalmoplegia (paralysis of the muscles that control eye movements) have been described in poisoning by various drugs (Hotson and Sachdev, 1982). Transient and variable strabismus (usually with the optic axes divergent in the horizontal plane) has been attributed to phenytoin, carbamazepine and tricyclic antidepressants. Although loss of oculocephalic and oculovestibular reflexes is usually regarded as evidence of severe brain-stem damage, poisoning with carbamazepine, phenytoin and tricyclic antidepressants can be associated with loss of these reflexes, but patients recover completely.

Hypertonia, hyper-reflexia and extensor plantar responses are commonly found in tricyclic antidepressant poisoning, and with other drugs with marked anticholinergic actions (e.g. the older antihistamines). However, all of these signs may be abolished in deep coma. Decerebrate and decorticate movements of the limbs often occur in unconscious poisoned patients but, in most cases, there is no irreversible brain damage and the patient recovers fully. Acute dystonic movements (muscle dysfunction characterized by spasm) are also observed in poisoning due to metoclopramide (Bateman *et al*., 1985), or less commonly haloperidol, droperidol, prochlorperazine or trifluoperazine.

4.5 Complications of Poisoning and Their Management

4.5.1 Fluid, Acid–Base and Electrolyte Imbalance

Patients who are vomiting, sweating excessively or passing large quantities of urine should be given fluids intravenously to replace GI, dermal and renal losses.

Acid–base abnormalities, particularly respiratory and metabolic acidoses, are common presentations in acute poisoning. Respiratory acidosis due to CNS depression or pulmonary toxicity, and metabolic acidosis due to lactic acidaemia or derangements of intermediary metabolism are common features of poisoning (Jones, 2007). Respiratory alkalosis is a feature of early salicylate poisoning (Chapman and Proudfoot, 1989). Some common poisons that cause metabolic acidosis are shown in **Table 3**. After correction of hypoxia and hypotension, metabolic acidosis should be treated by the administration of sodium bicarbonate 50–100 ml as a bolus dose with further boluses being given as required.

Table 3 Poisons causing metabolic acidosis

- Carbon monoxide
- Cocaine
- Cyanide
- Diethylene glycol
- Ethanol
- Ethylene glycol
- Iron
- Methanol
- Paracetamol
- Salicylates
- Tricyclic antidepressants

Information about the nature of poisons ingested can occasionally be obtained from standard haematological and biochemical investigations, and from arterial blood gas analysis (**Table 4**). Measurement of a full blood count is generally of little diagnostic value, though prolongation of the prothrombin time (international normalized ratio (INR)) may be caused by anticoagulants or by liver necrosis in paracetamol or hepatotoxic mushroom poisoning. Routine biochemistry and arterial blood-gas analysis are of value in the differential diagnosis of coma or the detection of poison-induced hypokalaemia, hyperkalaemia, hypoglycaemia, hyperglycaemia and hepatic and renal failure or of acid–base disturbances. Measurement of carboxyhaemoglobin, methaemoglobin and cholinesterase activities are of assistance in the diagnosis and management respectively of cases of poisoning due to carbon monoxide, methaemoglobin-inducing agents such as nitrites, and organophosphorus or carbamate insecticides.

Hypernatraemia can be caused by ingestion of salt and is well recognized as a nonaccidental injury in children. Urine sodium concentration is elevated in salt poisoning, but this finding does not exclude hypernatraemia caused by dehydration; measurement of the fractional excretion of sodium and water is needed to distinguish the two causes (Coulthard and Haycock, 2003). Hyponatraemia is a recognized complication of the use of MDMA ('Ecstasy'). It is caused by inappropriate secretion of antidiuretic hormone (ADH) (Henry *et al*., 1998), which impairs renal water excretion. This is often exacerbated by such patients drinking large amounts of water. It is managed by restricting the amount of fluid given to the patient.

Unless renal function is impaired or rhabdomyolysis is severe, hyperkalaemia is a relatively uncommon metabolic complication of poisoning. In contrast, marked hypokalaemia is a more common problem and may have serious sequelae. Most potassium disturbances in acute poisoning are due to disruption of extrarenal control mechanisms, notably the activity of Na^+/K^+ ATPase (adenosine triphosphatase) and potassium channels.

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Table 4 Nontoxicological investigations

- Serum sodium concentration (e.g. hyponatraemia in MDMA poisoning or hypernatraemia after salt ingestion)
- Serum potassium concentration (e.g. hypokalaemia in theophylline poisoning and hyperkalaemia in digoxin poisoning)
- Plasma creatinine concentration (e.g. renal failure in ethylene glycol poisoning)
- Prolongation of the prothrombin time (increased International Normalized Ratio—INR) (e.g. in paracetamol or anticoagulant poisoning)
- Acid–base disturbances, including metabolic acidosis and respiratory alkalosis
- Blood glucose (sugar) concentration (e.g. hypoglycaemia in insulin poisoning or hyperglycaemia in salicylate poisoning)
- Serum calcium concentration (e.g. hypocalcaemia in ethylene glycol poisoning)
- Serum phosphate concentration (e.g. hypophoshataemia in paracetamol poisoning)
- Transaminase or aminotransferase (ALT (alanine) and AST (aspartate)) activities (e.g. increased in paracetamol poisoning)
- Carboxyhaemoglobin concentration (increased in carbon monoxide poisoning)
- Methaemoglobin concentration (e.g. increased in nitrite poisoning)
- Erythrocyte cholinesterase activity (e.g. reduced in organophosphorus and carbamate insecticide and nerve agent poisoning)

Hypokalaemia occurs because of increased Na^+/K^+ ATPase activity (e.g. β_2- agonist, theophylline or insulin poisoning), competitive blockade of potassium channels (e.g. barium or chloroquine poisoning), GI losses and/or alkalosis. Hypokalaemia results in generalized muscle weakness, paralytic ileus, ECG changes (flat or inverted T waves, prominent U waves, ST segment depression) and cardiac arrhythmias (atrial tachycardia ± block, atrioventricular (AV) dissociation, ventricular tachycardia (VT), ventricular fibrillation (VF)). Severe and clinically significant hypokalaemia (e.g. that caused by theophylline (Vale, 2007b) or β_2-agonist poisoning (Vale, 2007c) should be corrected by infusing potassium to prevent arrhythmias (Bradberry and Vale, 1995).

Hyperkalaemia follows inhibition of Na^+/K^+ ATPase activity (e.g. by digoxin), increased uptake of potassium salts, disruption of intermediary metabolism (e.g. cyanide poisoning), activation of potassium channels (e.g. fluoride poisoning) and the presence of acidosis and rhabdomyolysis, particularly if the latter is complicated by renal failure. Hyperkalaemia is associated with abdominal pain, diarrhoea, muscle pain and weakness, ECG changes (tall peaked T waves, ST segment depression, prolonged PR interval, QRS prolongation) and cardiac arrhythmias (VT, VF).

Hypocalcaemia is a specific feature of ethylene glycol and hydrogen fluoride poisoning, in which calcium is deposited as insoluble oxalate and hydroxyapatite crystals, respectively.

Hypophosphataemia may contribute to morbidity and mortality in paracetamol poisoning by inducing mental confusion, irritability and coma (Jones *et al*., 1989; Schmidt and Dalhoff, 2002). Phosphaturia appears to be the principal cause of hypophosphataemia in paracetamol poisoning; it may occur in the absence of fulminant hepatic failure and indicates paracetamol-induced renal tubular damage (Jones *et al*., 1989; Florkowski *et al*., 1994). More recently, hyperphosphataemia has been shown to be a highly specific and sensitive predictor of nonsurvival in patients with severe paracetamol poisoning (Schmidt and Dalhoff, 2002). It has been suggested that hyperphosphataemia is caused by renal dysfunction in the absence of hepatic regeneration (Schmidt and Dalhoff, 2002).

Hypoglycaemia may follow an overdose of insulin, sulfonylureas or ethanol, and may occur in paracetamol-induced liver failure. It is corrected by infusing 10% dextrose, if necessary, after a bolus injection of 50% dextrose (50 ml).

4.5.2 Skin Blisters

Skin blisters may be found in poisoned patients who are, or have been, unconscious (Beveridge and Lawson, 1965; Hoffbrand and Ridley, 1972). Such lesions are not diagnostic of specific poisons, but are sufficiently

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common in poisoned patients (and sufficiently uncommon in patients unconscious from other causes) to be of diagnostic value. Unconscious patients should be turned at least every 2 hours. Bullous lesions should be left intact until they burst, to reduce the risk of infection. Deroofing should be performed when the blister bursts; a nonadhesive dressing is then applied.

4.5.3 Convulsions

These may occur, for example, in poisoning due to tricyclic antidepressants (Bateman, 2005), mefenamic acid or opioids. Usually the seizures are short-lived but, if they are prolonged, diazepam 10–20 mg iv or lorazepam 4 mg iv should be administered. Persistent fits must be controlled rapidly to prevent severe hypoxia and brain damage and should be treated with a loading dose of phenytoin 18 mg kg^{-1} administered intravenously at a rate not more than 50 mg min^{-1}, with ECG monitoring. Theoretically, phenytoin is contraindicated in poisoning due to tricyclic antidepressants as it may exacerbate sodium-channel blockade and increase the risk of cardiac arrhythmias. Rarely, muscle relaxation and mechanical ventilation are required in addition to phenytoin.

4.5.4 Rhabdomyolysis

Rhabdomyolysis is a condition in which there is dissolution of striated muscle fibres, with leakage of muscle cell contents (enzymes, myoglobin, potassium and phosphate) (Vale, 2007a). In patients who are poisoned, nontraumatic rhabdomyolysis may be caused by a direct insult to the cell membrane, affecting its ability to maintain ion gradients or be secondary to local muscle compression as a result of coma or seizures. Two clinically important complications are observed: acute renal failure (which may be nonoliguric) and peripheral nerve damage (secondary to compartment syndrome), resulting predominantly in wrist or foot drop.

Rhabdomyolysis accounts for 5–9% of all cases of acute renal failure (Grossman *et al*., 1974; Thomas and Ibels, 1985) and 5–30% of patients with rhabdomyolysis develop acute renal failure (Gabow *et al*., 1982; Ward, 1988). Three main mechanisms are involved (Holt and Moore, 2001). First, tubular necrosis occurs by free-radical-mediated lipid peroxidation. This involves redox cycling between two oxidation states of myoglobin haem: Fe^{3+} (ferric) and Fe^{4+} (ferryl) (Holt and Moore, 2000). The formation of ferryl myoglobin requires the presence of lipid hydroperoxides (LOOH). Once formed, the ferryl species reacts with lipids and LOOH to form lipid alkyl (LOO) and lipid peroxyl (L) radicals that progressively damage renal tubular membranes. Thus, ferryl myoglobin can initiate lipid peroxidation.

Secondly, renal vasoconstriction occurs due to activation of the sympathetic nervous system and the renin–angiotensin system in response to reduced effective circulating blood volume, scavenging of the vasodilator nitric oxide (NO) by myoglobin and release of isoprostanes (particularly 15-F2t and 15-E2t, which are potent vasoconstrictors), formed as a result of free-radical damage to phospholipid membranes.

Thirdly, tubular obstruction occurs due to the formation of tubular casts formed by binding of free myoglobin to Tamm–Horsfall protein (uromodulin), a renal glycoprotein (Zager, 1989), and as a result of urate crystal deposition.

Experimentally, urine alkalinization (see Section 4.7.3.2) has been shown to suppress the rate of conversion of ferryl (Fe^{4+}) myoglobin to ferric (Fe^{3+}) myoglobin. Thus, alkalinization inhibits the cyclical formation of lipid peroxide radicals and limits lipid peroxidation (Moore *et al*., 1998), so reducing tubular damage. Isoprostane release is also reduced by alkalinization, thereby lessening vasoconstriction. In addition, binding of myoglobin to Tamm–Horsfall protein is reduced under alkaline conditions, so that tubular casts are not formed (Zager, 1989). The administration of 8.4% sodium bicarbonate 225 ml should produce urine alkalinization; further boluses of sodium bicarbonate will be required to maintain the urine pH greater than 7.5. However, limited experimental and clinical data (Eneas *et al*., 1979; Homsi *et al*., 1997; Brown *et al*., 2004) suggest that early volume replacement is more important than urine alkalinization in preventing rhabdomyolysis-induced renal failure.

4.5.5 Methaemoglobinaemia

Methaemoglobin is formed when ferrous haemoglobin iron (II) is oxidized to ferric iron (III), which cannot participate in oxygen transport. Methaemoglobin concentrations are normally maintained at around 1% total haemoglobin by the action of a nicotinamide adenine dinucleotide (NADH)-dependent methaemoglobin reductase for which the physiological electron carrier is cytochrome b5 (Bunn and Forget, 1986). Excess methaemoglobin causes tissue hypoxia, not only because methaemoglobin is incapable of binding oxygen, but also because the oxidation of one or more iron atoms in the haem tetramer distorts the tetramer structure, so that the remaining nonoxidized haem subunits bind oxygen avidly, but release it less efficiently (Bunn and Forget, 1986).

Poisoning with a number of oxidizing drugs and chemicals may be complicated by methaemoglobinaemia (Bradberry *et al*., 1994a; 1994b; 2001; Bradberry, 2003). Two important chemical groups in this regard are organic nitrites (e.g. amyl and isobutyl nitrite) and amino or nitro derivatives of benzene (e.g. aniline, dapsone and lidocaine). Organic nitrites are sold as 'room odourizers'. Inhalation causes profound, though transient, vasodilatation with the intent of enhancing sexual pleasure

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or inducing a transient 'high'. Substantial absorption, which may occur after prolonged inhalation or ingestion, precipitates methaemoglobin formation. Amino and nitro derivatives of benzene are particularly potent methaemoglobin-formers because once absorbed they are activated to a metabolite that enters a cyclical process of methaemoglobin production such that even small exposures can result in clinically significant methaemoglobinaemia (Bradberry, 2007).

Even though methaemoglobin cannot bind oxygen, it is appropriate to administer high-flow oxygen to symptomatic patients with methaemoglobinaemia to maximize oxygen saturation of residual normal ferrous haemoglobin. In addition, methylthioninium chloride (methylene blue), which acts as an electron donor to reduce methaemoglobin, should be employed. In otherwise healthy individuals, methaemoglobin concentrations less than 30% usually do not require specific therapy, since such patients have only minor or no symptoms and methaemoglobin will be reduced over several hours by the intrinsic activity of methaemoglobin reductase. However, an anaemic patient may experience symptoms of hypoxia at methaemoglobin concentrations below 30%, since even in the absence of methaemoglobinaemia their overall oxygen transporting capacity is reduced. Such patients, or otherwise healthy individuals with methaemoglobin concentrations greater than 30%, warrant treatment with methylthioninium chloride $1-2\,mg\,kg^{-1}$ (the dose depending on the severity of features) intravenously over 5–10 minutes as a 1% solution. If the methaemoglobin concentration is greater than 50%, methylthioninium chloride $2\,mg\,kg^{-1}$ should be administered. Symptomatic improvement usually occurs within 30 minutes.

If there is evidence of continuing chemical absorption or prolonged methaemoglobin formation, a second dose of methylthioninium chloride $1-2\,mg\,kg^{-1}$ may be required. High doses (typically in excess of $20\,mg\,kg^{-1}$) of methylthioninium chloride can initiate severe intravascular haemolysis and doses as low as $4\,mg\,kg^{-1}$ may exacerbate the haemolytic effect of oxidizing chemicals. Severe renal impairment is an absolute contraindication to methylthioninium chloride administration, since it is eliminated predominantly renally. Methylthioninium chloride will also be less effective where nicotinamide adenine dinucleotide phosphate (NADPH) availability is reduced, as occurs in the presence of glucose-6-phosphate dehydrogenase (G-6-PD) deficiency and haemolysis, and when the chemical initiating methaemoglobin formation itself utilizes NADPH in cyclical methaemoglobin production, as occurs with, for example, dapsone and aniline.

4.5.6 Serotonin Syndrome

Serotonin toxicity results from an excess of serotonin in the CNS, which can be due to inhibition of the metabolism of serotonin (monoamine oxidase inhibitors), prevention of the reuptake of serotonin in nerve terminals (serotonin reuptake inhibitors), increased serotonin precursors (tryptophan) or increased serotonin release (serotonin-releasing agents such as amphetamines, fenfluramine, MDMA) (Isbister *et al*., 2007).

Serotonin toxicity occurs insidiously and is characterized by the development, within hours, of a triad of altered mental status (approximately 40% of patients), neuromuscular hyperactivity (approximately 50% of patients) and autonomic instability (approximately 40% of patients), though diagnosis does not depend on all parts of the triad being present (Sternbach, 1991; Isbister and Buckley, 2005; Boyer and Shannon, 2005; Thanacoody, 2007; Isbister *et al*., 2007). Features of altered mental status include agitation, confusion, delirium and hallucinations. Drowsiness and coma may occur in severe cases. Neuromuscular features include profound shivering, tremor, teeth grinding, myoclonus and hyper-reflexia. Features of autonomic instability include dilated pupils, sinus tachycardia, fever and hypertension or hypotension. Flushing, diarrhoea and vomiting are also common. In severe cases seizures, hyperthermia, rhabdomyolysis, renal failure and coagulopathies may develop.

The Hunter Serotonin Toxicity Criteria (Dunkley *et al*., 2008) have refined Sternbach's criteria and proposed seven features which are considered to be more sensitive and specific in making the diagnosis: clonus (spontaneous, inducible and ocular), agitation, diaphoresis, tremor, hyper-reflexia, hypertonicity and hyperthermia ($T^0 > 38\,^{\circ}C$). Clonus is the most important diagnostic sign.

The precipitating drug(s) should be discontinued and supportive care instituted. The control of agitation with benzodiazepines is essential. If hyperthermia supervenes, cooled iv fluids, tepid sponging and use of fans may help; paralysis and assisted ventilation should be employed if these measures do not to reduce muscle activity (Boyer and Shannon, 2005). Seizures should be controlled with diazepam 10–20 mg in an adult. Myoclonic jerks may be helped by clonazepam 1 mg intravenously over 2 minutes. The 5HT2A (5-hydroxytryptamine (serotonin) receptor 2A) antagonists, cyproheptadine 4–12 mg orally (up to 12–32 mg has been proposed during a 24 hour period (Boyer and Shannon, 2005)) and chlorpromazine 50 mg parenterally, have been used to treat serotonin syndrome following overdose, but there are no controlled trials to support the use of either agent (Isbister *et al*., 2007).

4.6 Investigations

Measurement of the concentration of a specific poison in the blood, or toxicological screening of blood or

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urine, can be used to establish a diagnosis of poisoning, particularly in severely poisoned patients in whom the cause of coma is unknown. However, although identification of a poison may reassure the clinician, this is not a good reason for the request. Before proceeding, the clinician should consider how the result of a screen will alter management. The pattern of drugs involved in poisoning in most developed countries is such that specific treatment is unlikely to be available and management will therefore be supportive. There are a small number of cases where emergency measurement of the plasma concentration will assist clinical management and/or help to determine prognosis: aspirin (salicylate), carbamazepine, digoxin, ethylene glycol, iron, lithium, methanol, paracetamol, paraquat, phenobarbital, quinine and theophylline.

Routine radiology is of little diagnostic value. It can be used to confirm ingestion of metallic objects (e.g. coins, button batteries) or injection of globules of metallic mercury. Rarely, hydrocarbon solvents may be seen as a slightly opaque layer floating on the top of the gastric contents with the patient upright, or outlining the small bowel. Some enteric coated or sustained-release drug formulations may be seen on plain abdominal radiographs, but, with the exception of iron salts, ordinary formulations are seldom seen. Ingested packets of illicit substances may be discernible on a plain radiograph, but computed tomography (CT) or magnetic resonance imaging (MRI) is more reliably able to detect such objects. Radiology may be particularly helpful in confirming some of the complications of poisoning, for example, aspiration pneumonia, noncardiogenic pulmonary oedema (salicylates), bronchiolitis obliterans (nitrogen oxides), acute respiratory distress syndrome (ARDS) and lung fibrosis (paraquat).

Performing an ECG is also of limited diagnostic value, though it should be undertaken in those ingesting potentially cardiotoxic drugs; continuous ECG monitoring may be appropriate in such patients. Sinus tachycardia with prolongation of the PR and QRS intervals in an unconscious patient should prompt consideration of tricyclic antidepressant poisoning. With increasing cardiotoxicity, VT may supervene. ECG changes in association with poison-induced potassium disturbances are discussed in Section (4.5.1). QT interval prolongation is a recognized adverse effect of several drugs in overdose (e.g. quetiapine, terfenadine and quinine) and predisposes to ventricular arrhythmias, notably *torsade de pointes*.

4.6.1 Psychiatric Assessment and Management

In adults, self-poisoning is commonly a 'cry for help'. Common precipitants include relationship problems, often in the context of depression and alcohol abuse. Those involved are most often females under the age of 35 who are in good physical health. They take an overdose in circumstances where they are likely to be found, or in the presence of others. In those older than 55 years of age, men predominate and the overdose is usually taken in the course of a depressive illness or because of poor physical health. The risks of repetition of self-harm and of suicide following self-poisoning are substantial (Hawton, 2007).

All patients require a sympathetic and caring approach, a psychiatric and social assessment and, sometimes, psychiatric treatment. Psychosocial assessment should include investigation of the events and problems preceding the act, suicidal intent and other motives for the act, psychiatric disorders, personality traits and disorders, family and personal history, psychiatric history, including of self-harm, risk of further self-harm and suicide, and coping resources and support (Hawton, 2007).

4.7 Management of the Poisoned Patient

As explained above, initial management involves the treatment of any potentially life-threatening conditions, such as airway compromise, breathing difficulties, haemodynamic instability, serious dysrhythmias and convulsions. Thereafter, fluid, acid–base and electrolyte abnormalities should be corrected and temperature disturbances treated appropriately.

Further management is aimed at:

1. Reducing absorption of the chemical(s) to which the individual has been exposed;
2. Employing a specific antidote, if appropriate;
3. Using methods to increase the elimination of the poison, if relevant.

Expert advice can always be obtained from a poisons information service.

4.7.1 Reduction of Poison Absorption

Poisons may be absorbed through the lungs, skin or from the GI tract. To reduce absorption through the lungs and skin, the exposed individual should be moved from the contaminated area, contaminated clothing should be removed and the skin thoroughly washed with soap and water.

While it appears logical to assume that removal of unabsorbed drug from the GI tract ('gut decontamination') will be beneficial, the efficacy of current methods remains unproven and efforts to remove small amounts of 'safe' drugs are clearly not worthwhile or appropriate.

4.7.1.1 Activated Charcoal

Activated charcoal adsorbs a wide variety of drugs and toxic agents; the exceptions are acids and alkalis,

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ethanol, ethylene glycol, iron, lithium and methanol. In studies in volunteers given 50 g activated charcoal, the mean reduction in absorption was 40, 16 and 21% at 60, 120 and 180 minutes, respectively, after ingestion (Chyka *et al*., 2005). Based on these studies, activated charcoal should be considered in those who have ingested a potentially toxic amount of a poison (known to be adsorbed by charcoal) up to one hour previously. There are insufficient data to support or exclude its use after one hour. There is no evidence that administration of activated charcoal improves the clinical outcome.

4.7.1.2 Gastric Lavage

Gastric-emptying studies in volunteers provide no support for the use of gastric lavage. In the single clinical study in which benefit was claimed for lavage within one hour of overdose, patients also received activated charcoal (Kulig *et al*., 1985). There was also selection bias, and hence conclusions based on these data are limited. Thus, gastric lavage should not be used routinely in the management of poisoned patients, as there is no evidence that it improves outcome, and it may cause significant morbidity (Kulig and Vale, 2004). The efficacy with which lavage removes gastric contents decreases with time. Therefore, lavage should be considered only in patients who have ingested life-threatening amounts of a toxic agent up to one hour previously.

4.7.1.3 Emesis with Syrup of Ipecacuanha

Syrup of ipecacuanha is derived from the dried roots of *Cephaelis ipecacuanha* and *C. acuminata* and contains the active alkaloids emetine and cephaeline. Emetine has a direct irritant action on the gastric mucosa, which causes vomiting within 30 minutes of administration; subsequent vomiting results from the central action of both alkaloids. Although syrup of ipecacuanha is an effective emetic, there is little evidence that its use prevents significant absorption of toxic material and, moreover, its adverse effects (e.g. persistent vomiting, diarrhoea, lethargy, drowsiness) may complicate diagnosis (Krenzelok *et al*., 2004). It should be abandoned.

4.7.1.4 Whole-Bowel Irrigation

Theoretically, the more quickly a poison passes through the gut, the less it is absorbed. Whole-bowel irrigation using polyethylene glycol electrolyte solutions does not result in absorption of fluid and electrolytes, even though large volumes are administered rapidly via a nasogastric tube. Some volunteer studies have shown substantial decreases in the bioavailability of ingested drugs, but no controlled clinical trials have been conducted and there is no evidence that whole-bowel irrigation improves outcome (Tenenbein and Lheureux, 2004). Based on volunteer studies, whole-bowel irrigation may be considered following potentially toxic ingestion of sustained-release or enteric-coated drugs and in body-packers (Hoffman *et al*., 1990).

4.7.1.5 Cathartics

Cathartics have been used alone and with activated charcoal. Two general types of osmotic cathartics have been used: saccharide cathartics (sorbitol) and saline cathartics (magnesium citrate, magnesium sulfate, sodium sulfate). Cathartics are intended to decrease the absorption of substances by accelerating the expulsion of the poison from the GI tract. Since most drug absorption occurs rapidly in the upper GI tract, the use of cathartics is most likely to benefit patients who have ingested drugs that are absorbed slowly. In volunteers, cathartics did not alter significantly the serum concentrations of lithium and salicylate when administered 30 minutes after dosing (Sørensen and Lindkær-Jensen, 1975) or change significantly the urine recovery of the metabolites of paracetamol (Galinsky and Levy, 1984) or salicylate (Mayersohn *et al*., 1977). In other studies, there was no difference in the mean area under the curve (AUC) of theophylline between the sorbitol-treated and control groups. No clinical studies have been published to investigate the ability of a cathartic, with or without activated charcoal, to reduce the bioavailability of drugs or to improve the outcome of poisoned patients. For these reasons it has been concluded that cathartics alone have no role in the management of poisoned patients. In addition, based on available data, routine use of a cathartic with activated charcoal has not been endorsed either (Barceloux *et al*., 2004).

4.7.2 Antidotes

There is no generally agreed definition of an antidote. The Shorter Oxford English Dictionary (2007) defines an antidote as a 'medicine given to counteract the action of a poison'. A WHO Working Party suggested an antidote was a 'therapeutic substance used to counteract the toxic action(s) of a specified xenobiotic' (Meredith *et al*., 1993), which could include substances such as activated charcoal. Flanagan and Jones (2001) have defined an antidote as a 'substance used to treat poisoning which has a specific action depending on the poison'. Bateman and Marrs (see **Antidotal Studies**) have adopted a similar definition.

Antidotes exert their beneficial effects by a variety of mechanisms, including forming an inert complex with the poison, accelerating detoxification of the poison, reducing the rate of conversion of the poison to a more toxic compound, competing with the poison for essential receptor sites, blocking essential receptors through which the toxic effects are mediated and bypassing the effect of the poison. These actions are described more fully in **Antidotal Studies**.

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There are only a small number of poisons for which there is a specific antidote (**Table 5**) and few antidotes are employed regularly in clinical practice. Those that are include *N*-acetylcysteine, naloxone and flumazenil, and these are described below.

4.7.2.1 Acetylcysteine

Acetylcysteine acts by replenishing cellular glutathione stores. Acetylcysteine may also repair oxidation damage caused by *N*-acetyl-*p*-benzoquinone imine (NAPQI), either directly or, more probably, through the generation of cysteine and/or glutathione, and may also act as a source of sulfate and thereby 'unsaturate' sulfate conjugation (Jones, 1998). IV therapy with acetylcysteine is preferred, if available, because in overdose paracetamol induces vomiting and oral therapy may not be absorbed. The iv regimen employed involves a 20 hour 15 min to 21 hour regimen (Prescott *et al*., 1979a; Buckley *et al*., 1999; Kerr *et al*., 2005) and involves the administration of acetylcysteine 150 mg kg^{-1} over 15–60 minutes, then 50 mg/kg over the next four hours and 100 mg kg^{-1} over the next 16 hours; (total dose, 300 mg kg^{-1} over 20 hours 15 min to 21 hours). An oral regimen (Smilkstein *et al*., 1988) used only in the USA and now largely superseded by the iv preparation involved the administration of acetylcysteine 1330 mg kg^{-1} over 72 hours. Acetylcysteine must be administered within 8–10 hours of ingestion if hepatic damage is to be minimized or prevented (Vale and Proudfoot, 1995; Kerr *et al*., 2005; Prescott, 2005).

Approximately 10% of patients treated with iv acetylcysteine (20 hour 15 min regimen) develop rash and bronchospasm within the first hour of commencing treatment; the reaction is probably the result of histamine release caused by acetylcysteine in a concentration-dependent manner (Dawson *et al*., 1989; Sandilands and Bateman 2009). These reactions are seldom serious, but infusion of acetylcysteine should be discontinued for 30–60 minutes. Antihistamines are not usually required.

4.7.2.2 Naloxone

Naloxone is a pure opioid antagonist and has been used to make a rapid clinical diagnosis of opioid poisoning in those whose clinical presentation suggests inadequate

Table 5 Antidotes of value in poisoning

Poison	Antidotes
Aluminium (aluminum)	Desferrioxamine (deferoxamine)
Arsenic	Dimercaprol, succimer (DMSA[a])
Benzodiazepines	Flumazenil
β-Adrenoceptor blocking drugs	Atropine, glucagon
Calcium channel blockers	Atropine
Carbamate insecticides	Atropine
Carbon monoxide	Oxygen
Copper	D-Penicillamine, unithiol (DMPS[b])
Cyanide	Dicobalt edetate, hydroxocobalamin, oxygen, sodium nitrite, sodium thiosulfate
Diethylene glycol	Ethanol, fomepizole
Digoxin and digitoxin	Atropine, digoxin-specific antibody fragments
Ethylene glycol	Ethanol, fomepizole
Hydrogen sulfide	Oxygen
Iron salts	Desferrioxamine (deferoxamine)
Lead (inorganic)	Sodium calcium edetate, succimer (DMSA[a])
Methaemoglobinaemia	Methylthioninium chloride (Methylene blue)
Methanol	Ethanol, fomepizole
Mercury (inorganic)	Unithiol (DMPS[b])
Nerve agents	Atropine, HI-6, obidoxime, pralidoxime,
Oleander	Digoxin-specific antibody fragments
Opioids	Naloxone
Organophosphorus insecticides	Atropine, obidoxime, pralidoxime,
Paracetamol	Acetylcysteine
Thallium	Prussian (Berlin) blue
Warfarin and other anticoagulants	Phytomenadione (Vitamin K)

[a]Dimercaptosuccinic acid.
[b]Dimercaptopropanesulfonate.

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ventilation caused by an opiate. It causes no adverse effects when given in opioid-naive individuals, though even in small doses may produce withdrawal symptoms in addicts. The dose of naloxone is titrated until the effects of the opioid have been reversed to a clinically detectable extent within 1–2 minutes. Typically, this dose of naloxone is 1.2 mg intravenously, followed by naloxone 2 mg if the response is only partial; a further 2 mg may be given 2–5 minutes later, if full resuscitation is not achieved. In patients who have not responded to naloxone 4 mg, the diagnosis of opioid poisoning should be reconsidered.

As naloxone has a half-life of some 45 and 90 minutes, which is much shorter than the half-life of opioids, repeat doses of naloxone are often required to maintain opioid reversal (Meredith *et al*., 1993). Alternatively, an infusion of naloxone may be commenced with the dose infused over one hour being 60–100% of the resuscitative dose. For slow-onset acting agents, such as methadone, the patient will need careful monitoring for several hours after the start of the infusion to ensure that appropriate levels of reversal are achieved.

Since naloxone reverses the effect of opioids on the gut there may be an increase in absorption of an orally ingested compound which leads to unexpected increases in opioid effects.

4.7.2.3 Flumazenil

Flumazenil is the specific antidote for benzodiazepines and may be given to avoid ventilation in patients with chronic obstructive pulmonary disease. Although flumazenil will reverse the respiratory and CNS depressant actions of a benzodiazepine, caution should be exercised in patients who have coingested a tricyclic antidepressant and in those known to suffer from epilepsy, because seizures and arrhythmias could be precipitated by its use. Flumazenil has a short half-life (about one hour) (Meredith *et al*., 1993). Therefore, patients with severe poisoning in whom it is indicated should be administered flumazenil 0.5 mg intravenously over one minute; flumazenil 1.0 mg should be given if there is no response or only a partial response. Once the patient has been resuscitated fully, an iv infusion ($0.5–1.0\,mg\,h^{-1}$) may be commenced, if necessary, to maintain reversal of the respiratory and CNS depressant actions of the benzodiazepine. Flumazenil is also effective, though less so, in reversing sedation in overdose from the nonbenzodiazepine hypnotics zopiclone, zolpidem and zaleplon.

4.7.3 Methods to Increase Elimination

4.7.3.1 Multiple-Dose Activated Charcoal

Multiple-dose activated charcoal is thought to produce its beneficial effect by interrupting the enteroenteric and, in some cases, the enterohepatic and the enterogastric circulation of drugs. In addition, any unabsorbed drug still present in the gut will be adsorbed onto the activated charcoal, thereby reducing drug absorption. Human volunteer studies have demonstrated that multiple-dose activated charcoal increases the elimination of many drugs, the most important clinically being carbamazepine (Neuvonen and Elonen, 1980), dapsone (Neuvonen *et al*., 1980), phenobarbital (Neuvonen and Elonen, 1980), quinine (Lockey and Bateman, 1989) and theophylline (Berlinger *et al*., 1983; Mahutte *et al*., 1983). Clinical studies have confirmed these volunteer studies and shown that the elimination of carbamazepine (Boldy *et al*., 1987; Montoya-Cabrera *et al*., 1996), dapsone (Neuvonen *et al*., 1980; 1983), phenobarbital (Pond *et al*., 1984; Boldy *et al*., 1986), quinine (Prescott *et al*., 1989) and theophylline (Mahutte *et al*., 1983; True *et al*., 1984; Amitai *et al*., 1986) is enhanced by multiple-dose activated charcoal. Based on these studies, multiple-dose activated charcoal should be considered if a patient has ingested a life-threatening amount of carbamazepine, dapsone, phenobarbital, quinine or theophylline. However, this therapy has not yet been shown in a controlled study in poisoned patients to reduce morbidity and mortality (Vale *et al*., 1999). Further studies are required to establish the role of multiple-dose activated charcoal and the optimal dosage regimen of charcoal to be administered. It is suggested that, after an initial dose of 50–100 g given to an adult, charcoal be administered hourly, every two hours or every four hours at a dose equivalent to $12.5\,g\,h^{-1}$ (Vale *et al*., 1999).

4.7.3.2 Urine Alkalinization

Urine alkalinization is a treatment regimen that increases poison elimination by the administration of iv sodium bicarbonate to produce urine with a $pH \geq 7.5$ (Proudfoot *et al*., 2003; 2004). The administration of 8.4% sodium bicarbonate 225 ml should produce alkalinization; further boluses of sodium bicarbonate will be required to maintain the urine pH greater than 7.5. The term urine alkalinization emphasizes that urine pH manipulation rather than a diuresis is the prime objective of treatment. Administration of bicarbonate to alkalinize the urine may rarely result in alkalaemia (an increase in blood pH or reduction in its hydrogen ion concentration), but there is no evidence to suggest that relatively short-duration alkalaemia (no more than a few hours) poses a risk to life in normal individuals. Hypokalaemia is the most common complication of urine alkalinization but can be corrected by giving potassium supplements. Alkalotic tetany occurs occasionally, but hypocalcaemia is rare.

Urine alkalinization increases the urine elimination of chlorpropamide, 2,4-dichlorophenoxyacetic acid, diflunisal, fluoride, mecoprop, methotrexate, phenobarbital and salicylate (Proudfoot *et al*., 2004). Based on volunteer and clinical studies, urine alkalinization should be considered as first-line treatment for patients with moderately severe salicylate poisoning (Prescott *et al*.,

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1982) who do not meet the criteria for haemodialysis. Urine alkalinization cannot be recommended as first-line treatment in cases of phenobarbital poisoning, as multiple-dose activated charcoal is superior (Ebid and Abdel-Rahman, 2001). Supportive care, including the infusion of dextrose, is invariably adequate in chlorpropamide poisoning. A substantial diuresis (approximately $600\,ml\,h^{-1}$) is required in addition to urine alkalinization if the chlorophenoxy herbicides, 2,4-dichlorophenoxyacetic acid and mecoprop, are to have their elimination enhanced in a clinically significant way (Prescott *et al*., 1979b).

4.7.3.3 Haemodialysis, Haemodialfiltration and Haemoperfusion

Haemodialysis, haemodialfiltration and haemoperfusion are of little value in patients poisoned with drugs with large volumes of distribution (e.g. tricyclic antidepressants), because the plasma contains only a small proportion of the total amount of drug in the body. These methods to increase poison elimination are indicated in patients with both severe clinical features and high plasma toxin concentrations.

Haemodialysis significantly increases elimination of ethanol, ethylene glycol, isopropanol, lithium, methanol and salicylate, and is the treatment of choice in all cases of severe poisoning with these agents (Vale, 1990). Haemodialfiltration is more widely and readily available and increases elimination of poisons such as ethylene glycol and methanol, though it is less efficient than haemodialysis. Charcoal haemoperfusion can significantly reduce the body burden of phenobarbital, carbamazepine and theophylline, but multiple-dose activated charcoal is as effective and simpler to use.

5 TOXICOVIGILANCE

The concept of toxicovigilance encompasses the active detection, validation and follow-up of clinical adverse events related to toxic exposures in human beings (Descotes and Testud, 2005). WHO has defined toxicovigilance as the 'active process of identifying and evaluating toxic risks in a community in order to reduce or remove them' (IPCS, 1997). Descotes and Testud (2005) have extended this definition and proposed that toxicovigilance 'primarily encompasses the active detection, validation and follow-up of clinical adverse events related to toxic exposures via household, occupational or environmental chemicals and products'. Drug-induced clinical adverse events are dealt with by a related process of pharmacovigilance (postmarketing drug surveillance).

Descotes and Testud (2005) believe that, 'Toxicovigilance is essentially based on the medical assessment of acute or chronic intoxications on an individual basis, which requires validated information that epidemiologists either do not look for or cannot analyse as comprehensively on a large scale'. Toxicovigilance primarily involves the collection of medically validated data from case reports, preferably using a consistent structured format to allow for the aggregation of information for global risk assessment. Thus, toxicovigilance 'generates signals that can be used to elaborate pathogenic hypotheses, and this in turn can serve as an impetus for epidemiological studies' (Descotes and Testud, 2005).

The IPCS has stated that the main goal of toxicovigilance is prevention. 'Toxicovigilance consists of the active observation and evaluation of toxic risks and phenomena in the community—an activity that should result in measures aimed to reduce or remove risks' (IPCS, 1997).

The IPCS believes that the role of poisons information centres in toxicovigilance includes (IPCS, 1997):

1. Identifying serious poisoning risks in the local community, and the substances, circumstances and population groups involved;
2. Identifying changes in the incidence of poisoning, for example, different substances of abuse, application of new pesticides and seasonal variations in the incidence of poisoning, such as carbon monoxide poisoning from heating appliances;
3. Monitoring the toxicity of commercial products, such as household, industrial and agricultural chemicals, as well as pharmaceuticals (by any route of administration), for acute, medium-term and chronic effects, with particular regard to new products and formulations (e.g. overuse of analgesics, occupational exposure to solvents);
4. Monitoring the toxic effects of drug overdosage;
5. Identifying substances that cause significant morbidity and mortality, and specific effects on organs (e.g. high incidence of renal insufficiency, foetal malformations);
6. Reporting to health authorities and other relevant bodies situations that demand preventative corrective action, and, where appropriate, calling an alert;
7. Monitoring the effectiveness of preventative measures.

Similarly, Volans *et al*. (2007) have suggested that, 'In practise, toxicovigilance is achieved through a range of activities, undertaken mainly, although not exclusively, by poisons centres. Clinical adverse events and hazards can be identified by a retrospective analysis of detailed case reports from poison centre databases to identify new patterns in cases of poisoning, or by prospective observation studies designed to answer specific questions, for example, to identify hazards associated with products and their use that can result in recommendations to improve drug safety'.

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The most well-developed national poisons surveillance system is that coordinated by the American Association of Poisons Control Centers (AAPCC). It was formerly called the Toxic Exposure Surveillance System (TESS) but is now referred to as the AAPCC National Poison Data System (NPDS). Automated, real-time toxicovigilance of the database was initiated in March 2003 as a surveillance process to identify exposures that may have public health and safety implications (Watson *et al*., 2005). The goal was to provide early identification of new product hazards, water, food or product contamination, chemical/biological terrorism incidents and emerging substances of abuse. Watson *et al*. (2005) have suggested that the methods were designed to detect:

1. Increases in total or human exposure case volume at each poison center;
2. Increases in national reporting of individual toxic (clinical) effects;
3. Cases that meet a surveillance-case definition.

These toxicovigilance tools were applied to incoming data on both real-time and near-real-time (1 to 24 hour interval) basis (Watson *et al*., 2005). The results were reviewed by clinical toxicologists with public health expertise to determine if further investigation or dissemination of findings was necessary. Clinical (toxic) effect data were collected using a list of 131 unique clinical effects, laboratory and diagnostic findings (Watson *et al*., 2005). Medical outcomes were categorized as no effect, minor effect, moderate effect, major effect and death using standardized definitions.

The medical outcomes of TESS/NDPS cases have been used to calculate a hazard factor (serious outcomes per 1000 poison exposure cases) (Watson *et al*., 2005). Hazard factor analysis provides a method for comparisons of the relative toxicity of products. An early use of hazard analysis compared the relative toxicity, defined as the rate of either major outcome or death associated with different poison exposure substance categories in children (Litovitz and Manoguerra, 1992). The hazard factor was calculated as the number of major medical outcomes and deaths per 1000 poison exposures with a documented medical outcome. This analysis showed that the three categories of substances most commonly involved in unintentional paediatric poisoning exposures, cosmetics and personal-care products, cleaning products and plants, had low hazard factors, indicating that the most common exposures were associated with minimal toxicity. Hazard-factor analysis identified a number of less frequently reported poisoning exposures that had more significant toxicity. Exposures with high hazard factors included hydrocarbons and pesticides. It was found that products containing yohimbe or ephedra were more likely to be associated with severe medical outcomes than other botanical products when risk ratios were compared (Watson *et al*., 2005).

The TESS/NDPS database has also been utilized for product safety assessment and to identify changes in substance-reporting patterns (Litovitz, 1998). Data have been used to support regulatory actions such as child-resistant closures on ethanol-containing mouthwashes, topical preparations of dibucaine and lidocaine, and acetonitrile-containing cosmetics. TESS/NDPS data have supported the reclassification of prescription medications to over-the-counter status and the cancellation of the registration of relatively more toxic pesticides, such as mevinphos and arsenical ant baits (Watson *et al*., 2005).

As Descotes and Testud (2005) have concluded, 'So far very few countries have set up structured toxicovigilance systems and it is hoped that in the future, national and international initiatives will help bridge this gap in our knowledge of the toxicity of many chemicals and commercial products to human beings'.

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Occupational Toxicology

Chuanfang Jin, Charles M. Werntz, III and Alan M. Ducatman

CONTENTS

1 INTRODUCTION

Occupational diseases are both over- and underdiagnosed. Both problems are important, but underdiagnosis in populations dwarfs the intermittent problem of occasional overdiagnosis in individuals. A Venn-diagram approach to occupational disease includes: (i) a correct clinical diagnosis (specific ICD (International Classification of Diseases) code or else a clear description of the clinical presentation), (ii) an understanding of the patient's exposure history (toxic exposures, including some sense of 'dose') and (iii) an understanding of the actual human toxicology/epidemiology of the exposure(s) in question (evidence-based exposure–outcome assessment). Incomplete understanding of the clinical presentation or failure to understand the exposure explains the large background of missed diagnoses. Fanciful attributions of exposure–outcome relationships explain intermittent overdiagnoses. Following an overview of the occupational history and laboratory testing, this chapter surveys the literature covering a broad range of toxic exposure–outcome information.

2 APPROACH TO A PATIENT WITH A CONCERN

2.1 Definitions

- Symptom—a change reported by the patient (example—pain)
- Sign—a deviation from 'normal' identified by the physician (example—a lump felt during palpation, or reddening of the skin).

2.2 Exposure History

2.2.1 Occupational History

A comprehensive occupational history starts with the first job and runs forward in time, noting approximate employment dates, job title and significant duties. Each job has a 'riskiest part', as well as most common toxic exposures or physical risks. The use and adequacy of personal protective equipment should be noted for jobs which involve toxic exposures.

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A comprehensive occupational history is a substantial effort. A single worker may be exposed to allergens, carcinogens, central or peripheral neurotoxins, and important pulmonary or dermatologic irritants, including chemicals, dusts and biohazards, in a day's work or in a working career. The pace of the modern physician's office visit makes it increasingly hard to do justice to the need for understanding patient exposures. Available office time and inadequate knowledge base are the major obstacles to good exposure histories (Harber, 2001). Sometimes, it is adequate to focus on just the aspect of a current or past job that likely relates to an abnormal pathology. (Example 1: Subacute disease. The auto body painter with asthma works with toluene diisocyanate-based paints. Example 2: Chronic disease. The retired policeman with a malignant mesothelioma helped his father, the stonemason, to mix asbestos and concrete, 60 years ago. Even these obvious, common relationships can take substantial time to elicit.) In the time-constrained environment of modern clinical practice, it is useful to consider when the patient deserves a referral to an occupational physician for purposes of comprehensive history-taking.

Electronic medical records may improve the ability to use standardized forms for exposure history-taking. Examples of job elements in a standard format would include: employer(s) listed sequentially, dates worked, hours, industry type, job duties, known health hazards, presence of protective equipment, and acute injuries/illnesses and chronic diseases attributed to work, for each job. Beyond that is a list of exposure types (e.g. acids, aerosols, biologics, zeolites) and specific toxins (e.g. asbestos, dyes, zinc chloride, smoke). There are excellent resources for finding forms and formats online. The Agency for Toxic Substances and Disease Registry (ATSDR), a part of the US Centers for Disease Control and Prevention, provides one that is attractive and has the advantage of considering home exposures and 'social' exposures, such as second-hand cigarette smoke (Centers for Disease Control, 2008). This resource is linked to a useful case study with teaching points.

While a form can adequately serve screening needs, our information technology is not yet so well developed that a formulaic approach can outperform an expert for diagnostic purposes. There are so many toxins, with so many outcomes that can be caused by toxic exposures, or by other causes. For example, in most specialty clinics, the most common cause of toxic-metabolic peripheral neuropathy referral will be diabetes, not workplace toxins. Cases of lead poisoning or other toxic causes of peripheral neuropathy can be missed easily against this background. The thought process for the occupational and environmental history resembles the thought process for creating a differential diagnosis for chronic infectious disease. What is the differential diagnosis of the symptoms and findings? Where has the patient been and what are the associated exposures? The Venn diagram of the expert thought pattern links exposure history, to clinical expert different differential diagnosis, to the epidemiology of the relationships of exposures and outcomes. Knowledge of all these characteristics is essential.

The three challenges faced by experts are these: (i) the many things we do not know about toxins; (ii) the many powerful, non-evidence-based concepts imposed on the field by (usually) well-meaning but non-evidence-based practitioners and (iii) both the patient's desire to achieve social justice and the clinician's need to perform evidence-based clinical decision-making. Patient concepts of social justice and clinician concepts of evidence are easiest to act upon when they are aligned. When they are not aligned, the clinician is obligated to the evidence. The goal of the occupational and environmental history is to link exposure evidence to clinical findings to literature evidence, or, rarely, but importantly, to propose new disease relationships in a testable fashion.

New disease relationships are rare, yet it is important to be alert that the history can uncover new aetiologic agents. Two disease relationships recently uncovered by astute observers through good history-taking include 'popcorn alveolitis' (a bronchiolitis obliterans caused by diacetyl in butter flavourings) and 'progressive inflammatory neuropathy' among slaughterhouse workers (probably caused by 'something' in aerosolized pig brains). These conditions are among the recent, new aetiologic associations whose existence was detected by astute history-taking.

Table 1 points out some common associations. It is necessarily incomplete by topic, by exposures for each topic and by outcomes for exposures. It merely demonstrates the potential role of biological, as well as chemical, agents in the workplace.

2.3 Examination

2.3.1 Confirming a Condition Suggested by the Exposure or Symptoms

The purpose of most examinations is to look for additional data to aid the physician in reaching a diagnosis. The diagnosis is an essential precursor to the further consideration of causes of disease. The occupational medicine examination is focussed upon signs of the diseases associated with the exposure, while considering the potential for new or unreported diseases, especially with novel exposures. Most physicians are adept at identifying clinical abnormalities; however, determining their environmental cause is often a less familiar activity.

A third type of occupational examination related to toxins determines fitness for specific duties, including respirator users, transporters of hazardous materials, hazardous waste operations workers and emergency

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Table 1 Exposure associations

Occupation	Exposure	Outcome
Agriculture, farm, silviculture	Pesticides, dusts, sunlight, vibration	Neurotoxic, respiratory, skin cancer, vascular
Animal handler	Pathogens, anaesthetic gases, allergens	Infections, reproductive effects, allergy
Auto body	Specialty paints and putties, isocyanates	Asthma, hypersensitivity pneumonitis, contact dermatitis
Baker	Flour, dusts	Allergy, asthma
Blaster	Nitrate explosives	Headache, sudden death
Bridge repair	Lead, silica	Neurotoxicity, pneumoconiosis
Custodian	cleaning fluids	Asthma, allergy
Degreaser, drycleaner	Solvents	Contact dermatitis, neurotoxicity, chemical hepatitis
Foundry	Heat, silica, metals	Heat stress, silicosis
Greenhouse	Mold, pesticides	Allergy, neurotoxicity
Insulator, electrician	Asbestos, manmade fibres	Pneumoconiosis, lung cancer, malignant mesothelioma
Miner	Coal dust, silica dust, radiation	Pneumoconiosis, lung cancer
Painter	Solvents, paints, lead	Central and peripheral neuropathy
Oil field or gas field	Hydrogen sulfide	'Knockdown', death
Sand-blaster	Silica	Pneumoconiosis

responders. Entry examinations for job applicants and periodic examinations for persons in these positions are intended to assure that there is not a medical condition present that would compromise critical activities or put the public at risk. In practice, incumbents who have demonstrated their ability to do the job may be less severely scrutinized.

2.3.2 Phases of an Examination

2.3.2.1 Complete History

The medical history for the occupational-exposure patient is not different from the history of other patients. There should be a chief complaint and a history of events of the present illness. Physicians who permit patients to start with exposures make the critical error of accepting a patient perspective of diagnosis and aetiologic relationships; the patient can get that without the visit. A review of systems should have a strong focus on risk factors such as tobacco and alcohol. These data are not intended to be oppositional to a patient-centred perspective; they are evidence-based.

2.3.2.2 General Examination

Vital signs, generally consisting of height, weight, pulse, respirations and blood pressure, are measured. General inspection should include abnormalities in movement, appearance, dress, speech and attention. The physician will typically assess the patient's mental function while taking their history, and may follow up on detected abnormalities during the examination. For example, if the patient provides a clear story and recalls details appropriately, then little additional testing for cognition is usually needed. On the other hand, a patient who cannot relate the story, who cannot recall details, or who reports memory problems should have further testing.

2.3.2.3 Examination of the Ears, Eyes, Nose and Throat

- Goal: ensure normal vision, including visual acuity and visual fields.
- Many chemicals can injure the eyes via desiccation or from changes in pH, especially when mixed with the tears.
- Caustics are well known to opacify the cornea, which can lead to blindness.

2.3.2.4 Inspection, Palpation and Auscultation (Listening with a Stethoscope) of the Neck

Examination of the throat and nares (nose) can reveal irritation, especially following inhalation of water-soluble chemicals or gasses, which form irritating liquids on contact with the mucosal surfaces.

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2.3.2.5 *Examination of the Chest: Heart Sounds, Lung Sounds*

Auscultation of (listening to) the heart assists the examiner to identify irregularities in rhythm, abnormal flow through the heart's valves and congenital defects. Auscultation of the lungs can find evidence of lung disease, including asthma and dust-related diseases, as well as other lung diseases. Occasionally, chest-wall percussion will reveal additional information, such as the presence of free fluid within the chest, or fluids/pus within the lung tissues.

2.3.2.6 *Inspection, Auscultation and Palpation of the Abdomen*

Although other abdominal organs are occasionally involved, the liver, with its central role in metabolism, can be affected by many toxins. Enlargement of the liver can be detected by palpation or auscultation to locate the liver edge, and can be confirmed by ultrasound. Other concerns are steatohepatitis due to abnormalities of lipid metabolism, and cirrhosis, which is the replacement of liver tissue with non-functioning scar tissue. Interestingly, this can lead to enlargement of the liver early in the disease, and then shrinking of the liver later in the disease. While hepatitis and cirrhosis are commonly associated with voluntary ingestion of ethanol, liver damage can also follow inhalation exposure to most solvents and many other chemicals.

2.3.2.7 *Neurologic*

The neurologic system consists of the brain, spinal cord and peripheral nerves that go from the spinal cord to innervate the muscles, organs and senses. The peripheral nervous system can be subdivided into the sensory-somatic nervous system, which controls the muscles and reports sensation back to the central nervous system (CNS), and the autonomic nervous system, which oversees most of the internal and basal functions of the body. Additionally, the cranial nerves exit from the brain directly, and primarily innervate the face and head, including the special senses (hearing, vision, taste and smell).

Toxins can affect all portions of the nervous system, but some toxins are specific in their actions. For example, lead prominently causes extensor muscle weakness at the wrists, as well as CNS effects, including cognitive and behavioural problems, especially following childhood exposure. Toxic peripheral neuropathies are more commonly sensory (touch, vibration).

2.3.2.7.1 Distribution of Signs/Symptoms from Neurotoxins

Most systemic toxin-related effects on the nervous system should be symmetric, meaning that they should affect the left and right sides approximately equally. Significant asymmetry should prompt consideration of nontoxic causes. However, neurotoxins often affect nerve axons based upon the length of the nerve, starting at the brain. A condition could therefore affect the feet (longest nerve pathway in the body) before affecting the hands. The common example of this is the peripheral neuropathy of diabetes, which may present with a burning sensation of the feet, then progress to affect areas closer to the brain.

A portion of the examination involves testing the patient's strength, usually by opposing the strength of the examiner. While there is some variation in strength between patients, asymmetry is an abnormal finding, either between sides (right vs. left) or between muscle groups (normal extension, then weak flexion of a muscle group).

2.3.2.7.2 Sensation (via Light Touch or Sharp Stimuli)

The skin contains many nerve endings that report stimuli to the CNS. Loss of this signal can come from impaired nerve transmission, or increase in the 'noise' from the generation of alternate signals, such as pain or 'pins and needles' sensations. Sensory loss can occur in several patterns. A dermatomal pattern follows the nerves from a specific nerve root coming out of the spine (and is usually not caused by toxins); a distal, nondermatomal pattern such as 'stocking' or 'stocking and glove' peripheral neuropathy is a common presentation in toxic exposures.

2.3.2.7.3 Reflexes

A reflex tests a complex set of functions in the nervous system. Starting with the muscle which normally pulls on the tendon relaxed, the tap from the hammer stretches the tendon, sending a signal to the spinal cord which then must be processed by the spinal cord, generating a new signal telling the muscle to contract. This tests that the sensory 'organs' are detecting the stretch, the sensory nerves between the tendon and the spinal cord, the spinal cord signal processing and then the motor nerve conduction from the spinal cord to the muscles that contract. A limited number of reflex circuits that can be tested in this way, including knee, ankle and elbow. Similar 'reflex arcs' can be tested more completely and objectively using an electrical test called an electromyelogram, typically performed by a neurologist or physiatrist.

2.3.2.7.4 Cognitive (Mini Mental-Status Exam)

Cognitive effects are associated with a variety of toxic exposures, including mood, memory (especially with solvent exposure), thought processes, reaction speed, learning and problem-solving.

The mini mental-status exam (MMSE) is a quick office test that seeks relatively profound decrements of orientation, understanding, memory registration, memory recall, calculation or spelling and the ability to follow simple directions. It will miss some subtle deficits. More detailed testing is available in formal neuropsychiatric

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testing, a test battery of several hours' duration performed by a psychologist.

Comparison of the pattern of a patient's deficits to known patterns from a variety of toxins may enable the examiner to propose a specific cause for impairments.

2.3.2.8 *Focussed Exam of the Body Part of Concern Based Upon Exposures or Symptoms*

Patient complaints about a specific body region or organ system can help the physician to provide a focus for the examination aimed at documenting findings or if there are no findings, to reassure the patient. Additional laboratory testing or imaging of the affected area may also be needed, and the examination helps guide test selection.

2.3.2.9 *Limitations*

An examination can provide only a temporal snapshot of the patient's condition. Today's normal exam inadequately predicts the presence or absence of future disease. The presence of identifiable risk factors is useful for predicting the future, but must not be overestimated. Even marked increase in risk may still describe uncommon events, if the baseline risk was already small. Patients with pleural rubs and radiographic evidence of asbestos have markedly increased risk of asbestos-related disease, yet malignant mesothelioma in any such high-risk patients is still not the most common cause of death. There are only a few exceptions, such as current urinary arsenic predicting future risk of urinary system cancers.

2.4 Laboratory Tests and Other Ancillary Tests in Occupational Medicine Practice

Laboratory tests confirm or rule out evidence of disease not detectable on examination. Tests done in connection with occupational practice are classified into several categories.

2.4.1 Tests to Assess Health Status and Potential Disease Aetiologies

Evaluation of suspected intoxication should routinely include basic laboratory tests, such as complete blood count, basic blood chemistry and urinalysis. Additional tests should depend on clinical presentation or nature of exposure. Common aetiological causes should be ruled out.

2.4.2 Nonspecific Tests of Exposure

Depending on clinical symptoms, tests are generally focussed on the nature of exposure and involved organs or systems. Examples are mean corpuscular volume; mean corpuscular haemoglobin concentration (MCHC) in case of exposure to haemotoxic agents; aspartate aminotransferase (AST-SGOT); alanine aminotransferase (ALT-SGPT) in exposure to liver toxins; erythrocyte protoporphyrin (EP), which can be measured as free erythrocyte protoporphyrin (FEP) or zinc protoporphyrin (ZPP) to help assess time course in suspected lead poisoning. Pulmonary disease might be detected by a reduction in forced expiratory volume 1 second (FEV1) for potential occupational asthma evaluation, sometimes with methacholine challenge test for recurrent asthma, or chest X-ray/computed tomography (CT)/magnetic resonance imaging (MRI) to evaluate pulmonary disorders. Neurologic tests might include electroneurodiagnostic testing for peripheral neuropathy, sensory-evoked potentials and neuropsychologic tests for central neurotoxin exposures and neuropsychologic testing for CNS damage. It should be emphasized that these tests are nonspecific for a particular exposure. An abnormal test indicates anatomical or physiologic alteration with a variety of causes, including occupational exposures.

2.4.3 Tests of Direct Biologic Indicators for a Specific Exposure

Exposure to some workplace chemical agents can be assessed through biologic testing of the exposed chemical compound or its metabolites. The chemical substance itself or a metabolite may be measured in end-exhaled air, blood or urine to detect and monitor the chemicals present in the body. Hair and nails may be useful for past exposures; these require very specific laboratory expertise. The result often represents the internal or biologic index of an exposure, referred to by many authors as internal exposure markers. For example, blood lead is elevated immediately following lead exposure, and gradually resolving evidence of elevated blood lead level can be found for many red-cell half-lives following exposure. Exposure to toluene may be detected by analysis of hippuric acid in urine, and exposure to trichloroethylene (TCE) may be ascertained by urine trichloroacetic acid or by breath analysis of the chemical compound. In contrast to lead, direct tests for solvent and pesticide exposures are valuable only for hours or days after exposure. However, abnormal tests for acetylcholinesterase inhibition following exposure to certain organophosphate pesticides may persist for a red-cell half-life (weeks or months). **Table 2** lists common tests for chemical exposures.

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Table 2 Tests for specific chemical exposures

Exposed chemicals	Test of chemicals or metabolites	Medium of testing samples	Comments
Solvents			
Styrene	Mandelic acid	Urine	Represent current exposure
Toluene	Hippuric acid	Urine	Represent current exposure
Xylene	Methylhippuric acid	Urine	Represent current exposure
Trichloroethylene	Trichloroethanol and trichloroacetic acid	Urine	Represent current exposure
1,1,1-Trichloroethane	Trichoroethanol and trichloroacetic acid	Urine	Represent current exposure
Perchloroethylene	Trichloroacetic acid	Urine	Represent current exposure
n-Hexane	2,5-Hexanedione	Urine	Represent current exposure
Benzene	Phenol	Urine	Represent current exposure
Metals			
Lead	Lead	Blood	Current exposure (screen)
	Lead	Urine	
	Zinc protoporphyrin (ZPP)	Blood	Measure body burden after EDTA (ethylenediaminete-traacetic acid) injection Elevated on lead poisoning and stayed high after cessation of exposure
Mercury (inorganic)	Mercury	Blood	Recent exposure
	Mercury	Urine	Biological monitoring
Beryllium	Lymphocyte (BeLPT (beryllium lymphocyte proliferation test))	Blood	Beryllium sensitization
	Lymphocyte (BAL (bronchoalveolar) BeLPT)	Bronchoalveolar lavage	Beryllium lung sensitization
Platinum	Pt salt (Prick test)	Skin	Pt allergic hypersensitivity
	Pt	Blood (plasma)	Monitoring treatment
Arsenic (inorganic)	Total arsenic	Urine	Affected by seafood ingestion
	Methylated derivatives	Urine	Correlate with airborne exposure
	Arsenic	Blood	Poor correlation with arsenic exposure
Cadmium	Cadmium	Blood	May reflect cadmium level in the entire body
	Cadmium	Urine	Exam kidney toxicity
	Retinol-binding proteins	Urine	Assess kidney toxicity
	β-2-microglobulin	Urine	
Gas			
Carbon monoxide	Carboxyhaemoglobin	Blood	Exposure
Chromium	Chromium (trivalent)	Urine	Biologic monitoring
	Chromium (hexavalent)	Erythrocytes	Determine body burden
Others			
Organophosphates	Cholinesterase	Plasma	Diagnosis of phosdrin poisoning
	Cholinesterase	Erythrocytes	Dx[a] of phosmet toxicity
	Cholinesterase	Plasma	Dx[a] of chlorpyrifos toxicity
	Cholinesterase	Erythrocytes	Dx[a] of dimethoate toxicity

[a]Dx: diagnosis.

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2.4.4 Tests of Genetic or Acquired Susceptibility

An increasing number of tests have been developed to establish susceptibility to a condition that may be predisposed, precipitated or aggravated by occupational exposure. For example, hereditary serum α1-antitrypsin deficiency is found to be associated with chronic obstructive pulmonary disease; glucose-6-phosphate dehydrogenase deficiency with hypersusceptibility to haemolytic agents; diaphorase deficiency with susceptibility to nitrite exposure and HLA-DPB1Glu69 is associated with beryllium sensitization, as well as development of chronic beryllium disease (McCanlies *et al*., 2004). The correct interpretation of test results requires a thorough understanding of the properties of the test. Use of the tests requires expertise in legal requirements and ethical standards in the practice setting.

Every test has a detection limit. The accuracy of a test declines as the measured value approaches the detection limit. Laboratories can report abnormal cut-offs unconventionally, including the lowest level of detection above the abnormal limit. Laboratories which report abnormals near, at, or below their detection limits are problematic.

Many occupational exposures involve essential trace elements. These substances are normally present in the body, and homeostatic mechanisms exist to regulate concentrations within a narrow range. Except in the setting of overt toxicity, measurements of elements such as zinc, copper, manganese and aluminium are of limited value in the diagnosis of occupational exposure for individual patients. (There may be population uses, a completely different topic.) In addition, many laboratories are willing to report on substances that they are ill-prepared to measure.

Many nonoccupational sources can lead to abnormal test readings of unclear significance. For example, smoking causes an increased level of cadmium, consumption of shellfish (and some shellfish-derived supplements) increases the arsenic level and some beauty products contain mercury. Some nonoccupational diseases may also affect certain test results; iron deficiency and haemoglobinopathy affect ZPP levels used to test for persistence of lead poisoning, and several heritable or disease conditions affect plasma and red-cell cholinesterase concentrations used to test for cholinesterase-inhibiting pesticide exposure.

Most tests indicate only the absorption of the substance into the body and/or its metabolism. Due to the variability of individual response, tests results do not necessarily indicate intoxication. It is important to be aware of the half-life of exposed substances, as well as natural processes that may create an appearance of exposure. Detection of chemical compounds with a short half-life suggests recent exposure, as in solvent exposure. A negative test result may be false if the sample is collected too long after exposure cessation. On the other hand, very small amounts of detected solvents may be from normal metabolic processes in the absence of exposure. The timing of sample collection for substances with a long half-life is not as critical as that of chemicals with a short half-life. Depending on toxicokinetic characteristics of exposed substances, absorption, distribution, biotransformation and elimination, these chemicals can be detected in different biological media. The half-life varies by biological medium; mercury, for example, has a 60-day half-life in urine, but approximately three days in whole blood. Marked interspecies variations in half-lives may limit the ability to generalize human outcomes from animal toxicity data.

The key to the diagnosis of occupational toxicity or toxic exposures is history. Laboratory tests and/or other tests are useful under some circumstances to confirm or exclude common nonoccupational aetiologies. Laboratory testing results should be carefully interpreted within the context of the clinical findings, including history and physical examination. Diagnoses should not be based on test results alone. When patients demand biomonitoring tests after implausibly long time periods, it is better to explain why the test is inappropriate than to try to deal with the strong likelihood of inappropriate results. Very few biosurveillance tests work well for patients outside of a workplace population context. The notable exception is blood lead, especially for children living in older homes in societies that permitted the past use of lead paints. **Table 2** lists tests for specific chemical exposures.

2.5 Common Agents by Disease or Affected Organ

2.5.1 Cancer

Cancer is a feared diagnosis, associated with a number of occupational exposures. In this section we will provide a general overview of the common toxin-associated cancers, however it is important to understand that there are many others. Excellent sources to look into these associations include the International Agency for Research on Cancer (IARC), The US Environmental Protection Agency (EPA) and the ATSDR, a part of the US Centers for Disease Control and Prevention. These agencies regularly assess the literature supporting the carcinogenic effects of various chemicals, and offer opinions both to the carcinogenic nature of the exposure, as well as the cancers that they feel support their opinion about carcinogenicity. The three sources occasionally differ in their opinions.

For any carcinogen, there may be some threshold before the exposure becomes carcinogenic. However, it is technically difficult or impossible to identify a specific level that will not lead to developing cancer.

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2.5.1.1 *Occupational Cancer*

Occupational cancer has been recognized since the eighteenth century, starting with scrotal cancer in chimney sweeps. The cause is now known to be hydrocarbons, and there are more modern jobs with similar skin cancer issues. Because of long latencies in the development of cancer, it is rare for poison centres to field calls or to feature expertise in occupational cancer. This topic is the province of the occupational physician, or less commonly, the pulmonologist, urologist or other organ-system specialist.

Table 3 links work populations with aetiologic agents and organ-system outcomes. It is intended to be illustrative, not exhaustive.

2.5.1.1.1 Lung Cancer

Lung cancer has been associated with a number of exposures, including cigarette smoke, arsenic, cadmium, silica, asbestos, radon and other uranium daughters and many more. Most of these are IARC Category I carcinogens.

Lung cancer challenges clinicians with variable treatment options, depending on the stage of disease at diagnosis. Historically, screening using chest X-ray and sputum samples has not been successful. Recent proposals to screen for lung cancer using single-breath-hold low-dose CT scans of the chest hold future promise for identifying early lung cancers (Bach *et al*., 2007). There are high costs, and yet there is still no clear survival benefit from early detection. There may be risks from the radiation needed to do the screening. At the time of writing, screening for lung cancer is not yet a recommended standard of care, not even in high-risk patients.

2.5.1.1.2 Scrotal Cancer

Scrotal cancer was identified as a hazard in chimney sweeps by Sir Percival Potts, centuries ago. This specific exposure–disease relationship is not known to occur in modern times. Requiring coke-oven workers to shower at the end of each work day has eradicated similar outcomes. The irritant properties of soot, and their carcinogenic potential, are clear.

2.5.1.1.3 Liver Cancer

There are several types of liver cancer. Angiosarcoma of the liver is associated with vinyl chloride exposure. A variety of exposures, including arsenic, are associated with hepatocellular carcinoma. Liver cancers are often asymptomatic until the patient is quite sick. While partial hepatectomy is usually survivable, many liver cancers will have spread to the point that surgical treatment is not practical when they are discovered.

2.5.1.1.4 Colon Cancer

Colon and rectal cancer are common. It is estimated that in 2008 in the US there will be 108 070 cases of colon cancer and 40 740 cases of rectal cancer diagnosed, with approximately 49 960 deaths (National Cancer Institute, 2008). Colon cancer has been associated with diet and inactivity. Some studies have associated colon cancer with chemical exposures, but these effects are often small and most of the associations are inconsistent.

2.5.1.1.5 Brain Cancer

Primary brain cancers are fairly uncommon, (as opposed to cancers from other organs that spread to the brain). There is mixed evidence for the association between brain cancers and exposures. Several associations have been proposed, including mixtures such as petrochemical exposure (Liu *et al*., 2008), but none are strongly supported. The population study of environmental mixtures as possible causes of death is important, difficult to perform or replicate, and expensive.

2.5.1.1.6 Prostate Cancer

Prostate cancer is the most common cancer in men. Many cases are indolent, some are invasive. While most prostate cancers remain confined to the prostate, the lifetime risk of developing invasive prostate cancer has increased from 7.3 to 19.6% for whites and from 8.5 to 18.6% for blacks (Merrill *et al*., 1997). A number of jobs have been associated with small increased risk of prostate cancer, but in the face of the large background rate of development of this common cancer, it is unknown whether small differences in the rate of prostate cancer are due to an exposure. The most suspect occupation is farming, and the specific concern is pesticide use, yet aetiologic causation is not established for any occupation.

2.5.1.1.7 Malignant Mesothelioma

Malignant mesothelioma is a cancer of the lining around the lungs and abdomen. It is histologically distinct from lung cancer, rarer and even more lethal. Mesothelioma was identified as an occupational disease in an asbestos-exposed cohort in the 1950s. Since that time, the connection has been repeated in many other settings. Mesothelioma, like many other cancers, develops following a period of latency, often 40 years from initial exposure. Unfortunately, mesothelioma may occur following fairly short (and often intense) asbestos exposures. It remains very difficult to treat this disease, with poor prognosis and short survivals.

2.5.1.1.8 Skin Cancer

Skin cancers have been associated with arsenic, including squamous cell (ATSDR, 2008), basal cell (ATSDR, 2008) and melanoma (Beane Freeman, 2004) types of skin cancers. For all skin cancers, early diagnosis is important, and greatly reduces the risk of spread. Some jobs may be associated with repeated episodes of sunburn, which increases the risk of skin cancer. Jobs which permit year-round tan may be less hazardous.

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Table 3 Aetiologic agents and organ-system outcomes

	Agent (where known)
Bladder cancer	
Dye workers	Arylamine
Chemical workers	β-naphthylamine
Aluminium smelters	Benzedine
	4,4′-Methylenedianiline (MDA)
	o-Toluidine
	4,4′-Methylene-bis-(2-chloraniline)
	Other aniline dyes
	3,3′-Dichlorobenzedine
	Other polyaromatic hydrocarbons
Hospitality and other	Secondhand cigarette smoke
Rubber workers	4-Aminodiphenyl
Environmental drinking water	Arsenic
Aluminium workers	Hydrocarbons
Machinists, mechanics	Hydrocarbons
Hairdressers	Hydrocarbons
Kidney Cancer	
Potroom workers, solvent exposure	Tetrachloroethylene (perc)
	trichloroethylene
Hospitality industry	Sidestream smoke
Liver Cancer	
Exposure to poor-quality foods	Aflatoxin
Exposure to animal products	Hepatitis B
	Hepatitis C
Body builders	Anabolic steroids
Plastics chemicals	Vinyl chloride (angiosarcoma)
Exposure to thorotrast (historic)	Thorium dioxide
Laryngeal Cancer	
Insulators, electricians	Asbestos
Lung Cancer	
Insulators, electricians, miners	Asbestos, and asbestos-like fibres, including some manmade fibres
Miners, sandblasters	Silica
Uranium miners, or those living in affected homes	Radon
Pleural and other mesothelial cancer (malignant mesothelioma)	
Insulators, miners, others	Asbestos
Nasal Cancer	
Forestry, wood products, textiles, nickel refining	Wood dusts, organic dusts, nickel
Skin Cancer	
Wood preservative, other	Coal tar
	Anthracene
	Coal tar and pitch
	Dibenzanthracene
	Methylcholanthracene
Chimney sweeps	Soots
Contaminated water	Arsenic
Mule spinners, oil field workers	Shale oils, crude oils, paraffins
Machinists	Mineral oils, machine oils
Fishermen, other sun-exposed	Ultraviolet light

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2.5.2 Haematopoietic Disease

Lead affects haemoglobin synthesis. The route of exposure is generally inhalation for adults, and both inhalation and ingestion for children. Children do not need to exhibit pica in order to ingest lead. When they crawl or toddle in a contaminated area, they breathe lead and they get dust on fingers, and then put fingers into mouths. Lead inhibits several enzymes along the pathway to haemoglobin synthesis. It also inhibits the synthesis of pyrimidine 5′-nucleotidase, which promotes the catabolic reuptake of RNA precursors in the immature red cell. This inhibition, combined with impairment of haeme synthesis, explains the 'basophilic stippling' seen in Wright stains of blood smears, once lead poisoning is moderately advanced. In vulnerable populations, that is, exposed children and workers, it is essential to provide surveillance in order to detect lead poisoning before it gets to this point, as multiple organ systems are affected before the classic changes of anaemia occur.

Benzene, present in gasoline and numerous other organic products, is a potent haematologic poison with demonstrable physiologic effects following respiratory exposure, even at currently permissible occupational standards. It causes epoxidization, leading to defective DNA. Exposure leads to acute bone-marrow depression, and may result in acute and chronic myelogenous leukaemia, myelodysplastic syndromes and several haematolymphopoietic cancers.

Arsine (AsH_3) is used in specialty microelectronic applications and may be generated as an undesirable by-product of soldering or galvanizing operations, or as an undesirable gas in mining. Arsine exposure induces red blood cell haemolysis. Treatment includes emergency exchange transfusions and urine alkalization to protect the kidneys from haemoglobin precipitation.

Naphthalene, an active ingredient of mothballs, causes haemolysis, which is seen most often in patients with G6PD deficiency. (Patients of African, Mediterranean and Pacific-rim ancestry are most at risk of G6PD deficiency or carrier status.) Naphthalene also causes methaemoglobinaemia (described in greater detail below). Trimellitic anhydride, a strong pulmonary irritant and allergen that is one of a variety of anhydrides used to cure resins, can cause an antibody-mediated haemolytic anaemia (associated with fever and dyspnoea). Usually, recognition and cessation of exposure is adequate to treat the condition.

Table 4 lists several toxins that can interfere with oxygen delivery into the red blood cell, or within the red blood cell, or from the red cell to tissues. (Carbon monoxide (CO) is an example of a multilevel toxin.) Of the topics addressed in the table, methaemoglobinaemia causes cyanosis and is often asymptomatic until levels are quite high. Sulfhaemoglobinaemia is uncommon in occupational settings (hydrogen sulfide usually causes other problems first). Carboxyhaemoglobinaemia is well known, as is the toxicity of cyanide; both are covered elsewhere in this text.

2.5.3 Liver Disease

Solvents have been the historic cause of occupational liver disease. Much of the evolution of industrial solvent use has been an effort to replace hepatotoxic solvents, such as carbon tetrachloride, perchloroethylene, TCE and chloroform, with lower-toxicity solvents. Nevertheless, small studies of general exposure to solvents and solvent mixtures show positive correlations of total and recent solvent exposures, serum aminotransferases and cholesterol. Of potentially great significance, the positive correlation also exists with serum glucose (Kaukiainen *et al*., 2004). In addition, liver anatomic changes can occur following solvent exposure, before there are detectable changes in 'liver enzymes' such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Brodkin *et al*., 1995).

Less commonly, settings other than solvent exposure have been associated with liver damage. Vinyl chloride, the monomer used to produce polyvinyl chloride, causes both hepatic fibrosis (Hsiao *et al*., 2004) and angiosarcoma of the liver. **Table 5** notes work settings and classes of chemicals known to be associated with hepatotoxicity. This list is meant to be instructive, not all-inclusive. Many agents can cause different types of hepatopathology at different doses, and infectious hepatitis may be associated with some occupations. It is certainly associated with the sex trade internationally.

2.5.4 Neurologic Disease

Many substances in the environment and workplace are capable of damaging the human central nervous system; a smaller number are known to damage the peripheral nervous system. Chemicals that cause peripheral damage are known to cause central damage.

Occupational toxin-induced neurologic diseases arise from exposure to metals, solvents and pesticides. A single toxin can produce a number of nonspecific syndromes and the resulting dysfunction is rarely specific to or pathognomonic of the exposure. Hence, the aetiologic diagnosis is easily overlooked, unless the clinician vigilantly pursues the role of workplace factors.

Toxic neurologic disease can be classified based on affected anatomic sites or toxic mechanisms. **Table 6** lists a classification of occupational toxic neurologic disorders.

2.5.4.1 Central Nervous System

2.5.4.1.1 Toxic Encephalopathy

2.5.4.1.2 Acute

Many toxic exposures may lead to acute or subacute onset of an altered state of consciousness, often appearing as vague malaise and eventually as a

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Table 4 Agents that interfere with red blood cell oxygen delivery

Outcome and Agent	Mechanism	Clinical
Methaemoglobinaemia[a] Aniline (percutaneous) Nitrates (including explosives) Naphthalene Dyes	Haemoglobin is oxidised by the nitrate (Fe^{++} to Fe^{+++})	Cyanosis, often asymptomatic
Sulfhaemoglobinaemia Hydrogen sulfide Pharmaceuticals	Sulphur in porphyrin ring reduces oxygen affinity	Cyanosis, often asymptomatic Loss of consciousness
Carboxyhaemoglobinaemia Carbon monoxide	Carbon monoxide binds to haemoglobin more avidly than does oxygen. 'Shifts' dissociation curves and starves tissues of oxygen.	Headache, nausea, palpitation, normal oxygen levels can be deceptive until breathing is depressed
Disrupt cellular respiration Cyanide	Disrupts electron transport	Rapid progression to death

[a]: the group of chemicals that oxidizes the iron atoms in haemoglobin from ferrous iron (Fe^{++}) to ferric iron (Fe^{+++}), rendering it unable to carry oxygen and causing metheamoglobinemia. It can lead to hypoxemia in organ tissue.

Table 5 Chemicals associated with hepatotoxicity

Chemical or class	Job type example	Outcome
Carbon disulfide	Rayon manufacture	Elevated lipids
Chlorofluorocarbons	Solvent, refrigerant	Hepatitis
Dimethyl acetamide	Industrial waste worker	Hepatitis
Hexachloroethane	Military, smoke bomb	Hepatitis
Nonhalogenated solvents; dimethyl formamide; naphthalenes	Skin exposure	Elevated enzymes, hepatitis
	Halowaxes (historic)	Cirrhosis
Paraquat	Agricultural field workers	Necrosis
Phosphorus	Matches (historic)	Osteopathy
Products of combustion	Coke oven	Elevated enzymes
Solvents (especially halogenated)	Spray paint, dry cleaning	Range: elevated enzymes to fatty liver to fulminant hepatitis
Trinitrotoluene (TNT)	Explosives	Subacute necrosis
Trivalent arsenic	Vintners	Cirrhosis
Vinyl chloride	PVC (polyvinyl chloride) manufacture	Fibrosis

confusional state. Symptoms may range from headaches, irritability, nausea, and incoordination upon mild exposure, to seizures, coma and death, with severe poisonings. Diffuse injury may affect not only the cerebral cortex (cognitive function) but also the cerebellar cortex (coordination). While symptoms are generally nonspecific, certain toxins affect specific areas of the brain. CO, manganese and carbon disulfide can generally damage the basal ganglion (Chuang *et al*., 2007; Huang *et al*., 1996; Brunssen *et al*., 2003; Prabhakaran *et al*., 2008), whereas organic mercury damages the cerebellum and occipital cortex (Chang, 1990). CO can cause diffuse frontal-lobe damage in addition to its effect on basal ganglia. Other agents, such as lead or organotins (Chang, 1990), may cause increased intracranial pressure with headaches, nausea, vomiting, seizures and papilloedema.

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Table 6 Classification of occupational toxic neurologic disorders

By affected anatomic site	By toxic mechanism
Central Nervous system	Axonopathy
Toxic encephalopathy	Myelinopathy
• Acute	Neurotransmission-associate neuropathy
• Chronic	
Movement disorders	
Peripheral Nervous system	
Toxic polyneuropathy	
Neuromuscular junction blockade	

2.5.4.1.3 Chronic

Chronic encephalopathy can take many forms. Typically, mood, memory and psychomotor function deteriorate slowly, and cognitive dysfunction becomes evident, eventually resulting in dementia.

Organic solvents commonly cause chronic encephalopathy, also known as solvent syndrome or painter's syndrome (initially reported among painters in Scandinavian literature during the 1970s). The condition comprises a constellation of CNS abnormalities, including loss of short-term memory, diminished ability to concentrate or problem-solve, change in personality, headaches, fatigue and decreased performance on neuropsychological tests.

Since the symptoms of organic solvent syndrome are nonspecific, the diagnosis depends on an accurate history of repeated exposures over the years, usually punctuated by episodes of acute intoxication characterized by transient light-headedness, headaches, a sensation of drunkenness and exclusion of other toxic-metabolic aetiologies. Standardization of a diagnostic approach to the solvent syndrome has been attempted by the World Health Organization (WHO) and summarized by Rosenberg (1995), as follows:

- Type I: symptoms only. Complaints of nonspecific symptoms; considered reversible if exposure is discontinued.
- Type 2A: Sustained personality or mood change. Complaints of nonspecific symptoms, which are not reversible.
- Type 2B: Partially irreversible impairment in intellectual function. Symptoms are accompanied by objective evidence of impairment on neuropsychological tests.
- Type 3: Dementia. Global deterioration in intellectual function; neurologic signs evident; poorly reversible, if at all. This is generally nonprogressive once exposure has ceased. However, there is emerging evidence that populations with 'mild cognitive dysfunctions' are more likely to be diagnosed with Alzheimer's disease at a later time.

Heavy metals, such as lead and mercury, may cause CNS disturbance. Relatively nonspecific clinical features, including irritability, difficulty concentrating, insomnia, inappropriate excitement, hallucinations and confusion, characterize initial presentations, depending upon the extent of poisoning. In addition, mercury poisoning (erethism) characteristically manifests as extreme timidity, shyness, embarrassment and discouragement. Other manifestations of mercury intoxication include ataxic intention tremor (also known as the 'Danbury shakes'), suggestive of a toxic mechanism beyond the cerebrum.

2.5.4.1.4 Movement Disorders

The term 'movement disorders' refers to motor dysfunction related not to weakness but to abnormalities of muscle tone, voluntary movement and posture. Movement disorders encompass involuntary movements, including tremors, dystonia, chorea, athetosis, dyskinesia, myoclonus and ballismus. The most common syndrome is parkinsonism.

Parkinson's disease, manifested with tremor, bradykinesia and rigidity caused by injury to the basal ganglia, occurs commonly in the elderly population and also, rarely, in younger patients. Occupational exposures to manganese, CO and methyl phenyl tetrahydropyridine (MPTP, a 'designer drug' contaminant not used by industry) produce syndromes that may resemble idiopathic parkinsonism, although specific differences in presentation and in anatomic variations have been described for each (Kurz *et al*., 2007). These exposures, as well as the potential role of any new or unusual compound, should be considered when evaluating patients with new-onset symptoms, especially if the patients are relatively young. At low exposure levels, manganese creates a less specific, but increasingly well-studied constellation of neuropsychologic problems, including population decrements in hand–eye coordination, hand–face arm movement, visual reaction time and tremor (Integrated Risk Information System, 2008).

2.5.4.2 Cranial Neuropathy

The cranial nerves are rarely the target of neurotoxic assault. The best-known toxic syndrome of the cranial nerves is trigeminal neuropathy related to exposure to TCE (Annau, 1981), which was once used as an anaesthetic agent. Trigeminal neuropathy is rare with current industrial practice. TCE-related trigeminal neuropathy may be caused in whole or in part by the decomposition product dichloroacethylene (Annau, 1981) since pathologic studies do not reveal such

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neuropathologic conditions after exposure to TCE alone.

2.5.4.3 Peripheral Neuropathy

Many toxins are capable of producing peripheral neuropathy. Most are primarily toxic to the axon, and the myelin sheath is affected secondarily. Presentation varies, depending on the agent and the degree of exposure. Acute polyneuropathy is rare, although it can occur with exposure to hexacarbons (such as *n*-hexane and methyl *n*-butyl ketone (MnBK)), which can be confused with acute polyradiculitis or Guillain–Barré syndrome. Repeated lower-level exposures cause polyneuropathy with mild symptoms, sometimes with only electrophysiologic abnormalities accompanied by minimal or no symptoms. Depending on whether or not the agent can cross the blood–brain barrier, the peripheral nervous system may be involved alone, or in concert with the central nervous system. **Table 7** lists toxic chemical compounds associated with peripheral neuropathies.

Table 7 Toxic chemical compounds associated with peripheral neuropathies

Chemical compounds	Clinical characteristics
Metals	
Lead	Motor deficit predominant; often asymmetric
Arsenic	Sensory, symmetric, often painful
Mercury	Often mixed neural deficits, symmetric
Thallium	Often mixed neural deficits, symmetric
Solvents	
Hexacarbons, including *n*-hexane, methyl *n*-butyl ketone (MnBK)	Often mixed neural deficits, symmetric
Trichloroethylene	Sensory deficit, featuring with trigeminal neuropathy
Carbon disulfide	Mixed neural deficits, symmetric
Styrene	Mixed neural deficits, symmetric
Gas	
Carbon monoxide	Mixed neurological deficits, may be asymmetric
Ethylene oxide	Mixed neurologic deficits, symmetric
Plastics	
Acrylamide	Mixed neurological deficits, symmetric
Pesticides	
Chlordecane	Mixed neural deficits, symmetric
Organophosphates	Delayed mixed neural deficits, symmetric

Typically, the onset of symptoms is insidious, with numbness and paresthesias of feet and hands, with progression to distal weakness. Most toxic-induced peripheral neuropathies are symmetric with distal nerve involvement. Clinical features usually present with sensory loss early on, and motor and sensory deficits as the neuropathy progresses. Sensitivity to light touch, pinprick and temperature are reduced in a stocking-and-glove distribution. Vibration and position sense are usually reduced in the distal legs prior to involvement of the arms. Cranial nerve function is usually preserved, and CNS function may or may not be clinically apparent depending on the nature of the toxic agents. Motor involvement typically shows decreased muscle tone and weakness, usually symmetric and more commonly seen in the lower extremities, such as toe extensors (extensor digitorum brevis), and foot dorsiflexors (anterior tibialis); the extensor muscles in the hands (hypothenar or dorsal interossei) (Rosenberg, 1995) may also be involved. Reflexes may be reduced or absent. Gait can be affected, likely related to neurosensory impairment, but ataxia is usually absent. Rapid alternating movement is generally intact. Clinically, large-fibre neuropathies can be distinguished from small-fibre neuropathies during neurologic testing; large fibres carry sensation for vibration and proprioception, while small fibres carry sensation for pain and temperature. Sensation for light touch is carried by both large and small nerve fibres.

Small-fibre involvement presents with painful polyneuropathy and can be associated with autonomic neuropathy. The most common example is alcoholic neuropathy (Koike and Sobue, 2006).

Although peripheral neuropathy has multiple aetiologies, the nerve has a limited number of ways to respond to injury. The damage can occur at the level of the axon (i.e. axonopathy). A disruption of the axons (e.g. trauma) results in degeneration of the axon and the myelin sheath distal to the site of the injury (i.e. Wallerian degeneration). In most toxic injuries, the most distal portion of the axons degenerates, with concomitant breakdown of the myelin sheath (known as 'dying-back', or length-dependent, neuropathy) (Edwards *et al*., 1991).

Neuronopathies occur at the level of the motor neuron or dorsal root ganglion, with subsequent degeneration of their peripheral and central processes. Because the injury is at the level of the cell body, recovery is often incomplete.

Myelinopathies occur at the level of the myelin sheath, and can be inflammatory or hereditary. In acquired demyelinating neuropathies, the injury is often patchy or segmental. Because the axons are relatively less damaged,

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recovery is often rapid (weeks to months) and complete. Hereditary abnormalities of myelin are usually diffuse, with a slowly progressive course.

Aetiologies for peripheral neuropathy are numerous; occupational toxic exposures occupy in only a small piece of a larger puzzle. Other causes of peripheral neuropathy should be ruled out, including, but not limited to, diabetes mellitus; alcohol abuse; medications in common use, such as vincristine (Citak *et al*., 2008), cisplatin (Krarup-Hansen *et al*., 2007), and podophyllin (O'Mahony *et al*., 1990), including over-the-counter agents and 'herbal remedies'; nutritional deficiency and collagen vascular diseases. Although it is important to consider other aetiologies, the presence of common risk factors such as diabetes mellitus and alcohol abuse does not exclude a sole or contributing role of workplace neurotoxins. Judgement about attribution is ultimately based on the assessment of the exposure (nature, intensity, duration), the likelihood of contribution by nonoccupational factors (for example, the nature of the presentation and the extent of alcohol use), and the nature of the process (a predominant motor neuropathy with associated new-onset joint pain in a diabetic with documented lead exposure in the workplace is likely caused by the lead). In some instances, both occupational and nonoccupational factors may be considered significant in an already susceptible patient.

2.5.4.4 Neuromuscular Junction Blockade

Certain toxins, such as pesticides or additives in plastics and petroleum products, cause blockage of the neuromuscular junction. Organophosphate pesticides inhibit acetylcholinesterase and create a cholinergic excess, preventing normal function of the neuromuscular junction. Weakness and fasciculations occur. The central and autonomic nervous systems are involved, so other manifestations include miosis, blurred vision, abdominal cramps, nausea, sweating, salivation, and confusion.

2.5.5 Respiratory

Warning properties are sensory effects of exposure; they are often unpleasant. Some chemicals, such as acid gasses, will cause burning and choking, which are very strong warning properties. These tend to be corrosives with dramatic effects. If a person is exposed to a chemical with strong warning properties, they will normally try to escape. Other chemicals, such as CO, asbestos, hydrogen cyanide and nitrogen, have essentially no odour or warning properties. These chemicals are equally dangerous, and there is even less tolerance for industrial error because there may be no human perception of exposure before harm is done.

2.5.5.1 Pneumoconiosis

Pneumoconiosis (literally 'rock lung') follows from accumulation in the lungs of one or more substances that cannot easily be removed by the immune system, either because the material cannot be dissolved by macrophages (the scavenger cells in the lungs), or the extent of the exposure is to more material than can be efficiently removed. The lung reacts with an inflammatory response, leading to fibrosis. The common pneumoconioses are asbestosis, coal worker's pneumoconiosis ('black lung') and silicosis.

The presence of pneumoconiosis can best be identified through radiographs (X-rays or high-resolution CT scans) of the lungs, whereas the extent of damage is assessed by radiographs and pulmonary function tests together. The current International Labour Organization (ILO) system for interpreting chest radiographs for the pneumoconioses is based upon the interpretation of plain posterior to anterior (PA) chest X-rays. Systems are currently being developed to provide ILO classification for digital chest X-rays and CT scans of the chest.

Unfortunately, once pneumoconiosis starts to develop, treatment is limited to preventing infections (pneumonia and flu vaccines) and controlling symptoms (for example, by providing supplemental oxygen). The disease process is not reversible; however, the rate of progression can be affected by removing the worker from further exposure. This opportunity for secondary prevention (removing a worker from exposure after they have started developing a disease, before symptoms develop) is the basis of programmes that monitor for disease development in exposed workers. The 'gold standard' of prevention programmes is to prevent disease in the first place. This goal is variably achieved.

2.5.5.2 Coal Dust (Black Lung)

Coal dust exposures include mining, both above and below ground, coal transport and coal processing. Exposure is characteristically intense at the 'face', where the coal is being cut from the earth, and is greater for underground miners. In the United States, national health surveillance programmes have found that the rate of miners with 'rapid progression', that is, >2 subcategory progression within five years, seems to be geographically based, with a 'hot spot' in central Appalachia (Antao *et al*., 2005). The current theory is that both total dust exposure and coal 'rank' (related to purity and BTUs (British thermal units)) play a role.

2.5.5.3 Asbestos (Asbestosis)

Asbestos fibres move into the distal lungs following exposure. The 'aspect ratio' of the asbestos fibres may contribute to the risk of disease development. A longer, skinny fibre may penetrate the lung tissues more deeply and may thus cause more disease than a shorter, fatter fibre. Asbestosis involves fibrosis of the lower lung parenchyma and the lung pleura. Asbestos also causes lung cancer and malignant mesothelioma. The risk of lung cancer is potentiated by concomitant cigarette smoking. Different types of asbestos fibres have

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varying capability to precipitate malignant mesothelioma. However, the concept that there is a relatively safe asbestos is a commercial concept and not well related to epidemiologic findings.

2.5.5.4 Silica (Silicosis)

Silicosis causes fibrotic changes in the lung, predominantly in lower lung fields. Although the radiologic findings within the lung tissue are similar to coal dust, there is increased frequency of calcification of the lymph nodes, with eggshell calcifications being a classic finding. Silicosis may also progress to 'complicated' disease with cascading areas of lung emphysema.

2.5.5.5 Byssinosis (Cotton Worker's Lung)

Although frequently lumped with the pneumoconioses, byssinosis is physiologically quite different. Cotton-dust exposure precipitates an asthma-like condition via immunologic response leading to constriction of the air passages, rather than the scar tissue that is a hallmark of coal, silica and asbestos exposure. The precise agent within the cotton plant that causes byssinosis is not clearly known, although a role for endotoxins is certainly indicated.

2.5.5.6 Asthma

Asthma is a manifestation of allergic disease in which the airways in the chest become reactive. Sensitization can occur to any protein and to many other smaller chemicals. Following sensitization, if there is additional exposure to the trigger, the airways are reactive to inflammation, with decreasing flow of air to the lungs. Sensitization can occur to many plant (pollen), animal (dander) and insect (scat) proteins. Additionally, some industrial exposures, such as certain sawdusts, colophony (solder flux), isocyanates and cotton dust have been associated with asthma. Irritant mixtures such as cleaning materials may also provoke reactions. Avoidance of the trigger is the most effective treatment, although there are medications available that can treat disease and even decrease event frequency.

2.5.5.7 Reactive Airway Dysfunction Syndrome (RADS)

High-dose exposure to a strong irritant can cause virtually instantaneous airway obstruction, very similar to asthma. Once reactive airway dysfunction syndrome (RADS) occurs, victims may react to a wide variety of other irritant triggers, at lower exposure levels. Patients may gradually return to normality over the course of several years. Strong irritants such as chlorine or ammonia may provoke this rapid response.

2.5.5.8 Irritants

There are several respiratory exposures which can directly irritate the respiratory tract, causing airway reactivity and lung damage. Examples include acid mists, products of combustion and any of the water-soluble acidic or basic gasses. Following exposure, responses can range from mild irritation to scar formation anywhere in the respiratory tree, including the alveoli and the lungs themselves. The only effective treatment is avoidance, either through product substitution, personnel controls or the use of personal protective equipment.

2.5.5.9 Asphyxiants

Asphyxiants block the effective use of oxygen by cells of the human body. There are four mechanisms to consider: simple, blood, cellular and central. All forms of asphyxiants can cause death. Removal to fresh air and supportive care is sufficient care for simple and central asphyxiants; additional medical care is needed for the other forms.

A simple asphyxiant merely changes the concentration of oxygen in inspired air by displacement. An example is argon gas in an enclosed space. This lethal problem is avoided by testing confined spaces for oxygen content before entry.

A 'blood' asphyxiant interferes with the transport of oxygen in the blood. An example would be CO, which preferentially binds with haemoglobin, and also interferes with the release of oxygen to tissues, thus preventing normal oxygen transport in the blood. Hyperbaric oxygen is the treatment of choice for CO poisoning.

The third mechanism is the 'cellular' asphyxiant, which uncouples oxidative phosphorylation, and thus interferes with the function of the mitochondria, blocking cells from effective utilization of oxygen. The prototype of this mechanism is cyanide. Treatment recommendations following cyanide exposure vary from supportive care only, to specific antidotes such as large doses of hydroxocobalamin to bind the cyanide.

A central asphyxiant interferes with the drive to breathe. The well-known example is hydrogen sulfide, nicknamed the 'knockdown gas' for its propensity to cause exposed oil-field workers to rapidly lose consciousness.

2.5.5.10 Bronchiolitis Obliterans

Diacetyl is used as the butter flavouring in most microwave popcorn. In 2001, bronchiolitis obliterans was noted in several workers at a plant manufacturing microwave popcorn. The greatest exposures to diacetyl and most of the disease occurred among workers in the mixing room, where large vats of heated diacetyl were added to the other ingredients. Work is ongoing to look for other exposures in the flavourings industry.

2.5.6 Skin

There are four types of exposure-related skin disease: dermatitis, allergic reaction, pigment changes and skin cancers. Each will be discussed (see above for discussion of skin cancers).

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The most common skin conditions are irritation or allergy of the skin. Both conditions are called dermatitis, although the mechanism of rash is different. Contact dermatitis can result from mechanical damage, such as exposure to acids or alkalis. Either type of dermatitis can result from a variety of exposures. Other than noting the pattern/distribution, a physician cannot identify the cause by inspection. Sometimes the pattern/distribution of a dermatitis is helpful. For example, a sharply demarcated boarder matching the edge of a pair of gloves might suggest a reaction to the gloves in one distribution, or to a chemical for which the gloves provide the barrier in another.

Allergic reactions can occur to nearly any ingredient or product. Allergic reactions can be localized (to the area of exposure) or generalized. Generalized allergic reactions may progress well beyond a skin reaction, to hives or even anaphylaxis, which can be fatal.

The third major type of skin reaction is discolouration. Populations drinking arsenic-laden water develop a dark discolouration of the feet and legs called 'blackfoot disease'. Many pigmented chemicals can leave a sometimes permanent 'tattoo' if they penetrate the skin. Other chemical exposures can lead to either darkening or lightening of the skin.

2.5.7 Renal and Urinary Tract Diseases

A wide range of occupational toxins has been implicated in causing urinary tract and kidney disease. Chemical compounds affect different sites in the urinary system. **Table 8** summarizes chemical agents that have been found to be associated with kidney diseases as well as urinary-tract disorders.

Table 8 Industrial chemical compounds associated with renal and urinary tract disorders

Condition	Offending chemical agents
Acute renal disease	
Tubulointerstitial	Halogenated hydrocarbons (carbon tetrachloride, ethylene glycol) Pesticides: Organophosphate (parathion) Organochlorine (dichlorodiphenyl-trichloroethane; DDT) Bipyridyl (paraquat) Heavy metals (arsenic, chromium, mercury, lead)
Glomerular	Hydrocarbon solvents Heavy metals
Indirect	Haemolytic agents (arsine) Rhabdomyolitic agents (ethylene glycol)
Chronic renal disease	
Tubulointerstitial	Heavy metals (cadmium, lead, beryllium)
Glomerular	Heavy metals (lead, mercury) Hydrocarbon solvents Silica and silicon compounds
Bladder dysfunction	
Bladder irritation	Pesticide (formamidine)
Sacral neuropathy	Plasticizer (DMAPN)

2.5.7.1 *Kidney Disorders*

2.5.7.1.1 Tubulointerstitial disease

The tubule is the most common site of acute nephrotoxic injury. Acute or chronic injury depends on the exposed dose and duration, with acute renal damage often occurring following high-dose, short-duration exposure. Halogenated hydrocarbons, such as carbon tetrachloride, chloroform and tri- and tetrachloroethylene, may cause acute injury to the liver and renal tubular necrosis. The primary cellular target of chloroform is the proximal tubule, with no primary damage to the glomerulus or the distal tubule. Proteinuria, glucosuria and increased blood urea nitrogen levels are all characteristic of chloroform-induced nephrotoxicity (Sinicrope *et al*., 1984). The mechanism of chloroform nephrotoxicity is related to its metabolite, trichloromethanol. This unstable, biotransformed product from chloroform releases hydrogen chloride (HCl) to form phosgene, which injures proximal tubular cells.

The effects of halogenated hydrocarbons appear to depend on dose and possibly on route of absorption. Inhalation exposures seem more likely to induce acute renal failure as the most prominent manifestation, whereas ingestion predisposes patients to hepatocellular necrosis (Lauwerys *et al*., 1985; Phillips *et al*., 1988). The difference may be explained by the first-pass effect of ingested chemical compounds cleared into the enterohepatic circulation. Solvent renal and hepatic effects are probably caused by metabolites.

Ethylene glycol is a component of commercial antifreeze. It can cause acute tubular injury following a large-dose exposure. The mechanism combines deposition of calcium oxalate crystals in renal tubular cells and lumen with direct cytotoxic effects. Glomerular proliferative changes may also be seen pathologically. The toxic metabolite of ethylene glycol, oxalic acid, is formed by alcohol dehydrogenase. Competitive inhibition of the alcohol dehydrogenase enzyme site (Guo *et al*., 2007) by (intravenous) ethanol or by 4-methylpyrazole is an effective treatment.

Heavy metals, such as inorganic mercury, arsenic, cadmium, chromium, gold, iron, silver and uranium, cause renal toxicity. Mercury is capable of inducing acute renal failure secondary to proximal tubular necrosis. The pathogenesis is partially related to mercury's affinity to membrane sulfydryl groups, resulting in alterations in cellular permeability. Damage occurs when the serum

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binding protein (metallothionein) is saturated (Barbier *et al*., 2005).

The principal target organ of chronic exposure to cadmium is the kidney. Following longstanding low-dose exposures, workers may develop proximal tubular dysfunction heralded by the excretion of β-2-microglobulin and other tubular low-molecular-weight proteinuria, as well as albuminuria. Hypercalciuria with nephrolithiasis is common. Fanconi syndrome with amino aciduria, glycosuria, phosphaturia and renal tubular acidosis can be seen in more severe cases. Osteomalacia may occur in severe cases. The changes are usually irreversible, but removal from exposure often halts disease progression.

Chronic lead exposure leads to progressive damage to the proximal tubular epithelium, followed by interstitial scarring and loss of glomeruli. Hyperuricemia, an early sign of lead nephrotoxicity, results from enhanced proximal reabsorption of urate. Subsequently, decreased glomerular clearance with hypertension may appear, usually after years of lead exposure.

Tubulointerstitial diseases can be secondary to haemolysis or rhabdomyolisis effects of primary toxins, leading to both acute and chronic forms of kidney disease.

2.5.7.2 Glomerular Diseases

Toxic insults to the glomeruli result in clinical manifestations similar to those seen in non-toxin-induced glomerulopathies, including proteinuria, reduced glomerular infiltration rate, oedema and hypertension. Organic and inorganic mercury has been found to be associated with acute glomerular injury, histologically identified as a membranous glomerulopathy.

A number of solvents used in industry, such as halogenated hydrocarbons, may increase the risk of glomerulonephritis, particularly antiglomerular basement-membrane antibody-mediated disease, resembling Goodpasture's syndrome (Churchill *et al*., 1983). A number of hydrocarbon mixtures and specific chemicals, for example, organic solvents, hairdressing solvents, gasoline, glues, trichloroethane or tetrachloroethane, have been associated with antiglomerular basement-membrane disease.

Many nephritic toxins, such as mercury and hydrocarbon solvents, are associated with chronic glomerulopathies or glomerulonephritis.

2.5.7.3 Bladder Dysfunction

2.5.7.3.1 Sacral Neuropathy

Lower urinary tract dysfunction can result from the neuropathic effects of workplace exposures. Dimethylaminopropionitrile (DMAPN), a catalyst in polyurethane foam manufacture was found to be associated with isolated urinary dysfunction. The symptoms are manifested with micturition abnormalities including retention, dysuria and delayed micturition time (Keogh, 1983). The pathogenesis is thought to be related to sacral neuropathy. DMAPN has been removed from sale and use.

2.5.7.3.2 Chemical Irritation

Pesticides of the formamidine group and toluidine are associated with bladder irritation. The exact mechanism is unclear.

2.5.8 Cardiovascular Disease

Heart diseases are leading causes of morbidity and mortality in the developed world, and they are advancing problems everywhere that 'Western' diets are adopted. Although many cardiovascular deaths may be accounted for by the known risk factors of hypertension, diabetes, cigarette smoking, hyperlipidaemia and genetic factors, many other cardiovascular deaths are not explained by these risk factors. The role of occupational risk factors and of their potential contributions, alone or in combination with other risks, to the overall burden of cardiovascular diseases, is insufficiently studied. Nevertheless, exposure to toxic chemicals in the workplace and in nonoccupational environments is clearly associated with cardiovascular disease.

Chemical compounds affect the cardiovascular system at a variety of sites. Cardiovascular toxins are generally classified by nature of abnormality or by anatomic injury location/injury mechanism (see **Table 9**).

2.5.8.1 Ischaemia

Atherosclerotic vascular disease causes cardiac ischaemia, an imbalance between the heart's demand for oxygen and the delivery of oxygen to the myocardium. Chronic exposure to carbon disulfide has been definitely linked to cardiac ischaemia (Kotseva and De Bacquer, 2000). Population investigation associates that outcome with alterations in lipid metabolism and increases in diastolic blood pressure (Kotseva and De Bacquer, 2000; Kotseva, 2001). Carbon disulfide was also found to alter thyroid function, glucose tolerance and fibrolytic activity (Cavalleri, 1975; Candura *et al*., 1979). These physiologic processes accelerate vascular atherosclerosis. Because the cardiovascular risk of carbon disulfide exposure remains after adjusting for hypertension and serum cholesterol, it is suggested that carbon disulfide may affect the cardiovascular system through other, as yet unknown, toxic mechanisms (Nurminen *et al*., 1982).

Cardiovascular diseases caused by carbon disulfide are clinically indistinguishable from nontoxic atherosclerotic vascular diseases, with one possible exception. Abnormal ocular microcirculation, characterized by microaneurysms and haemorrhages resembling those of diabetic retinopathy, is an early sign of chronic carbon disulfide poisoning (Karai *et al*., 1983a; 1983b). Laboratory tests are also generally nonspecific for individuals. There is population evidence of decreased

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Table 9 Occupational toxic cardiovascular diseases and agents

Condition	Causation		
	Definite	Probable	Possible
Cardiac ischaemia secondary to atherosclerosis	Carbon disulfide	—	Carbon monoxide, methylene chloride, nitrites, arsenic
Nonatheromatous ischaemic heart disease	organic nitrates, carbon monoxide	Methylene chloride	—
Dysrhythmias/arrhythmias	Fluorocarbons, chlorinated hydrocarbons, nitrates, organophosphates, arsenic and arsine, particulates	Antimony	—
Hypertension	—	Lead, arsenic	Cadmium, carbon disulfide
Cardiomyopathy	Arsenic, arsine, cobalt	—	Antimony, organic solvents, lead
Peripheral vascular disease		Arsine, lead, carbon disulfide	—
Alterations to lipid mechanism	Carbon disulfide, alcohol	Perfluorocarbons	—

thyroxin level and an increase in serum cholesterol level. Carbon disulfide can be measured in blood by gas chromatography-mass spectrometry; however, testing is not readily available for routine examination. A conjunction product of carbon disulfide and glutathione is excreted in urine proportionally to exposure and can be measured to monitor exposure.

Dramatically increased risk for ischaemic heart disease was also reported in large populations exposed to arsenic via contaminated well water in Taiwan (Wu *et al*., 1989; Chen *et al*., 1996). Chronic exposure to arsenic at the workplace was also found to be associated with cardiovascular disease in copper-smelter workers (Hertz-Picciotto *et al*., 2000; Axelson *et al*., 1978), including increased mortality with dose-related outcome.

Nitrate esters, of which nitroglycerine is the best known, have long been used as pharmaceuticals and as explosives. In addition to their oxidant properties and potential to induce methaemoglobinaemia, nitrates have immediate effects of variable onset and duration as arterial vasodilators. Withdrawal from nitrates will cause rebound vasospasm. Since chronic exposure to nitrates produces tolerance, acute symptoms such as severe headaches characteristically recur during withdrawal or more commonly on re-exposure after absence from work for several days (i.e. the so-called 'Monday morning headaches'). Re-exposure to nitrates after a period of nonexposure can cause paradoxic cardiac arrest. Sudden ventricular fibrillation is the likely cause (Lange *et al*., 1972; Morton, 1977).

CO causes cardiac ischaemia through several mechanisms, including avid binding to haemoglobin, with a resultant decrease in tissue oxygen delivery, as well as shifting of the haemoglobin–oxygen dissociation curve. CO exposure decreases myocardial contractility by binding to the cytochrome oxidase system in the mitochondria of cardiac muscle (Chance *et al*., 1970). Acute toxicity with high-dose exposure is common, especially in developing countries where stoves are the main means of heating in winter, or following disaster-recovery efforts with indoor use of temporary gas- or kerosene-powered generators. CO is suggested to cause ischaemic heart disease following repeated low-dose exposures (Teculescu *et al*., 2007; Koskela *et al*., 2000; 1994). Following CO exposure, workers with underlying coronary artery disease may experience exacerbation of angina and arrhythmias, even with a blood carboxyhaemoglobin of less than 10% (Sheps *et al*., 1990). The exact blood concentration of CO sufficient to produce permanent pathologic changes in humans is unknown, and likely subject to substantial variability depending on victim fitness and genetic susceptibility. At higher levels of carboxyhaemoglobin, usually above 25%, manifestations of ischaemia, dysrhythmias and electrocardiographic abnormalities are likely to be observed in otherwise healthy individuals (Prockop and Chichkova, 2007).

Methylene chloride was commonly used in paint thinner, and still persists in some products. It has cardiotoxicity, partly because of its metabolic conversion to CO. The biologic half-life of CO from this source is longer than that of inhaled CO. Carboxyhaemoglobin levels have been used to monitor methylene chloride exposure.

2.5.8.2 *Dysrhythmias*

A number of chemicals have been associated with atrial and ventricular dysrhythmias by mechanisms unrelated to ischaemia. The agents of concern include organic

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solvents, with both halogenated (particularly fluorocarbons) and nonhalogenated chemicals implicated. Cases have been reported for sudden death in solvent sniffing or inhalant abuse, especially in toluene abusers (Soden *et al*., 1996). The causes for sudden death include cardiac dysrhythmias and hypoxia-induced heart block. The toxic mechanism of sudden death is thought due to sensitization of the myocardium to circulating catecholamine. The range of solvents and solvent mixtures capable of this mischief is likely broad; an adult woman developed ventricular fibrillation and cardiopulmonary arrest after prolonged exposure to concentrated vapours from mineral spirits. Dilated cardiomyopathy and myocardial fibrosis are also reported in solvent-abuse populations.

2.5.8.3 Cardiomyopathy

Cobalt exposure has been linked directly to the development of cardiomyopathy. The association was first observed in the 1960s in drinkers of beer containing cobalt added to stabilize the foam (Bonenfant *et al*., 1967; Alexander, 1972). Occupational exposure to cobalt was observed in the production of metal alloys used especially in the manufacturing of drills and bits. A study of tungsten carbide workers exposed to cobalt-containing dust found a weak, but statistically significant, inverse correlation between resting left ventricular ejection fraction and duration of exposure (Horowitz *et al*., 1988). The findings support a possible role for cobalt in causing myocardial disease. The probable mechanism of cobalt cardiac toxicity is depression of oxygen uptake by heart mitochondria and interference with energy metabolism. It is similar to the effects of thiamine deficiency (Alexander, 1969). Antimony has also been anecdotally linked with the development of cardiomyopathy and electrocardiographic abnormalities.

Hypertension, a commonly seen chronic disease, is a major risk factor for premature atherosclerotic coronary vascular disease, cardiomyopathy and stroke. Several industrial agents can induce or aggravate hypertension. Epidemiologic studies consistently show that low-level lead exposure can chronically elevate blood pressure (Sharp *et al*., 1987; Skoczyńska *et al*., 2007). A positive relationship is reported between lead concentration and systolic blood pressure (Pirkle *et al*., 1985; Menditto *et al*., 1994). In animal studies, the mechanism of hypertension by lead is likely related to increased vascular resistance, possibly through increasing plasma renin activity, either by inhibiting angiotensin-converting enzyme or by enhancing responsiveness of vascular smooth muscle to the pressor effects of endogenous vasoconstrictors, and/or directly contracting vascular smooth muscles (Carmignani *et al*., 2000). Lead also affects calcium in tissues by inhibiting sodium-potassium-adenosine triphosphate (ATPase) and calmodulin-mediated calcium transport (Carmignani *et al*., 2000).

Other chemicals found to be associated with hypertension include carbon disulfide and organic solvents, and metals, such as cadmium and arsenic.

2.6 Other Topics

2.6.1 Indoor Air Quality

The energy crisis of the 1970s prompted efforts to increase the energy efficiency of buildings by increasing insulation and decreasing incursions of outdoor air. This trend may deepen. These laudable activities increase the risk for accumulation indoors of the products of human activity (office machines, outgassing from furniture, dust in ducts and construction materials). Decreased ventilation and increased dust are associated with a constellation of effects including nose and throat irritation, headaches, lethargy and difficulty concentrating. The exact cause of these symptoms is unclear, and removal of single constituents from the environment may not alter the effects.

Carbon dioxide is not the causative agent in indoor air problems. It is a product of human respiration that can be fairly easily monitored, and increased carbon dioxide levels in an inhabited building suggests a problem with ventilation and air quality. Thresholds ranging from 800 to 1000 ppm have been suggested. Abatement efforts typically consist of increasing the proportion of outside air that is added to the indoor environment, and retrofitting exhaust fans where they have been omitted.

2.6.2 Mould

Mould is a ubiquitous, common exposure. Human exposure has been a health concern for millennia, with instructions for removing mould from houses dating from antiquity (The Holy Bible, Revised Standard Version, 1952). Funguses do have the ability to elaborate toxins, which can be harmful to humans. For example, T-2 mycotoxin is a potential chemical weapon.

There is much lay literature focussed on the ill effects of various mould species, with most allegations of direct toxicity regarding 'black mould', especially *Stachybotrys* species. A report associated S*tachybotrys* with pulmonary haemorrhage in infants (MMWR, 1997); this report has been rescinded (MMWR, 2000). There have been no other similar reports.

Case reports following exposure to mould have also claimed a variety of direct toxic cognitive effects; these have not so far been supported by larger studies.

Allergic symptoms, including rhinitis and asthma, are mould-exposure outcomes that are clearly supported by the medical literature. People can clearly become sensitized to moulds (and to bacterial endotoxin as bacteria grow next to mould), with allergy symptoms following

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further exposure. Treatment with medications can limit these effects; however, avoidance of exposure is more effective. Removing first sources of moisture and then the mould is more effective than treating the resultant allergy. The toxicology of indoor air pollution is further discussed in **Indoor Air Quality**.

2.6.3 Nanotechology

Nanotechnology refers to materials which have a length scale between 1 and 100 nanometres (National Nanotechnology Initiative (NNI), 2008). Various geometries have been created, including single- and double-wall tubes, pyramids and fullerenes (soccer ball-like spheres, previously known as Bucky balls). There are also older products that occur in this size range, such as carbon black and titanium dioxide. There are multiple theoretical reasons for concern and, to date, scant information reporting on actual health effects associated with manufactured other geometries or materials. This is an area of active research at the US National Institute for Occupational Safety and Health. When nanomaterials are an unintended pollutant, rather than a product, they are regarded as 'ultrafine' air pollution. Ample epidemiologic evidence supports respiratory, cardiovascular and mortality outcomes. The toxicology of nanmaterials is further discussed in **Nanotoxicology-The Toxicology of Nanomaterials**.

2.7 Clinical Syndrome Presentations

Nonspecific clinical syndromes are common in the developed world. They primarily include chronic fatigue, chronic fibromyalgia and irritable bowel syndrome in the United States. Variants of these and many other syndrome presentations are found throughout the world. Less common is the nonspecific syndrome with a reference to work. Three important syndrome presentations which may be attributed to work are 'sick building', multiple chemical sensitivity (MCS), and electrical sensitivity.

'MCS' has a more than a century-old history of antecedent sociomedical history in the United States, and the recast diagnosis from the 1980s has been exported internationally. Its formal requirement, that the disorder be acquired in relationship to documented (albeit low-level) exposures, is rarely met in practice, yet such exposures are highly plausible for most of the world's population, so there is little chance to 'rule out' the diagnosis based on exclusion of minute exposures. This clinical syndrome diagnosis is therefore equally applicable across the spectrum of workers and the unemployed, in theory. In practice, the diagnosis has been historically less common in industrial-exposure groups and more common in teachers, office workers and the previously employed.

The research diagnosis involved symptoms that can be thought of as referring to more than one organ system and which recur following predictable stressors (from many unrelated chemical classes). Objective organ-system findings are not part of the syndrome and implicate other diagnoses (Cullen, 1987). Many authors have pointed out that patients who present with MCS to occupational providers typically have other practitioners who may have diagnosed 'overlap' syndromes of medically unexplained symptoms, such as chronic fatigue, chronic fibromyalgia, chronic candidiasis, chronic Epstein–Barr virus and chronic tick-borne infectious disease. The North American histories of international syndrome diagnoses, past and present, could be a book chapter in a different kind of text. Among these medically unexplained diagnoses, MCS is unique in the patient expectation that the problem is caused by other humans' behaviour with chemicals.

MCS patients present with reversible symptoms of paralyzing pain, strange skin sensations, evanescent rash, and rapid-onset and rapidly reversed episodes of confusion/memory loss, as primary examples. Triggers are odours, often starting with a single exposure and then often generalizing to a plethora of odours. Patients who perceive these triggers seek to minimize them without additional clinician guidance.

Taking an occupational-exposure history is important for all exposure patients; however, it is equally an exploration of frame of mind and historic treatment options for MCS patients. Unusual exposures from non-evidence-based remedies ranging from enemas to chelation therapy should be sought. Unusual symptoms, such as sudden paralysis or loss of consciousness are critical to understanding social risk. The patient is likely to express a goal of restored social function, and to simultaneously perceive that goal as unattainable for many details of lifestyle normalization.

In the United States, these MCS (and 'overlap') patients frequently present very unusual laboratory tests from an array of unconventional biologic media (often with brightly coloured graphics and very unconventional ways of expressing normal ranges). The quality of these tests is far from assured. Several ways to deal with these tests are to repeat the tests in a reputable lab if cost is no object, to repeat only the tests with plausible abnormalities if the usual fiscal constraints pertain to patient care or to ignore the tests if the patient is already at the point of changing belief systems. Accepting these tests at face value is the error with the largest social cost for these unfortunate patients.

It is useful to know that surveys of US populations find surprisingly high endorsement of belief systems, but actual work impairment is much rarer, and designed trials to establish aetiology either find no evidence of environmental causation, or implicate psychologic causes (Ducatman, 2007). It is nevertheless wise to remember that psychologic causes have physiologic consequences.

For the MCS patient, careful, nonjudgemental listening is far more important than therapeutic suggestion or

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further extension of already implausible test batteries. The patient may demand physiologic explanations and treatment advice, with a focus on environmental interventions. Be alert, however, that actual needs are different than those expressed. An innocent suggestion to 'avoid exposure' can further enforce an obsessive response and help this patient travel still further down the road to unconventional medical practice, social isolation and economic deprivation. And, an affirmative response to the question 'Is it all in my head?' is all it takes to send this patient back to the least evidence-based, most economically abusive provider. It is better to point out that all pain and all fatigue are experienced in our heads, and that we have not found the cause of the particular symptoms that affect our patient.

The goals of care are modest: address alarming, nonstandard lab testing as needed (and affordable); establish trust that you care; assure the patient that you do not know the physiology of the problem and create routine appointments so that medical contact can be retained in the absence of new and more alarming symptoms. Let the patient know that in this circumstance, the patient is her own best doctor, with a treatment goal to gradually improve exercise tolerance and social integration. Do not be in a hurry to make this suggestion.

Throughout Europe, MCS patients have a second manifestation of 'electrical (or electromagnetic) hypersensitivity'. The presentation is generally similar to, but less dramatic than, that of the MCS patient. Similar symptoms include headache, fatigue, weakness, lethargy and skin sensations (Lotz, 2007). The physiologic evidence basis for electrical hypersensitivity is similar to that of MCS; designed studies do not find a physiologic basis, unless it is psychologic (Ducatman, 2007; Lotz, 2007). A less challenging and more common situation is the patient who wants to be tested 'for everything' to 'prove it's safe.' In this circumstance, the goal is to be informative and believable about the limits of medical science.

2.8 Causation and Aetiology

Discussions of causation generally start with a discussion of the work of Sir Austin Bradford Hill, who proposed what are now known as 'Hill's criteria'. These points must all be considered in establishing an opinion about causation; however, it is not mandatory that all of the criteria be met to establishing causation.

2.8.1 Hill's Criteria

2.8.1.1 Temporal Relationship

The exposure must always precede the development of disease, and this must also include an appropriate latency period. While other criteria are relative, this one is absolute.

2.8.1.2 Strength

Review the epidemiological evidence, looking at the number of studies showing an association, the strength of that association and the presence or absence of studies with alternate results.

2.8.1.3 Dose–Response Relationship

This concept can have three different interpretations. The concept of dose will be reviewed, the types of dose–response relationship discussed and the application of dose–response in various settings explored.

First, consider the concept of dose. For medications, this is milligrams per tablet, times how often the tablets are taken. Applied to environmental exposures, the dose is comprised of the exposure per unit of time, times the duration of the exposure. As an example, for cigarette smokers, this would be how many packs of cigarettes a day times how many years they have smoked, yielding an exposure history expressed in pack-years. The cigarette-related diseases correlate to the pack-year total of the smoker. Expert-rated assessments start with measured or inferred contaminant levels, and then apply models to extend the measurements and estimate human exposure.

Dose–response relationships can be stochastic or deterministic. Stochastic relationships vary with the level of the exposure. An industrial example might be skin exposure to sulfuric acid, in which the amount of damage to the skin would correlate directly with the strength of the acid and the contact time. This contrasts with a deterministic dose–response relationship, in which there may be no effect at lowest doses, based upon reaching a threshold (which can vary considerably between individuals.) Most cancer outcomes are thought to be deterministic. Unfortunately, we cannot discern most workers' susceptibility to any exposure. Protective levels are necessarily set well below the level at which most individuals would develop the outcome, and still fail to be fully protective. In many cases of failure-to-protect, the problem stems from short-term excursions that are unaccounted in a larger model of total dose.

There are two types of dose–response relationship. The first of these considers the individual patient, and the second the effect on populations. In much of medicine we apply the dose–response concept to the individual patients. For example, we expect that a higher dose of a blood-pressure medicine would result in a greater lowering of blood pressure in a patient. This would apply to any stochastic toxic exposure.

Applying the dose–response concept to populations is different, and applies primarily to deterministic exposure–disease relationships. For an exposure without a threshold effect, the rates of development of disease will be highest in populations with the greatest exposure. That is not to say that all persons in the most exposed group will develop disease, just that the rate of disease in this most exposed group will be greater than in less

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exposed groups. And, as more susceptible individuals often fail to thrive as workers for health reasons, the puzzling finding of an outcome, without a strong dose–response, should not be dismissed as meaningless. While the identification of a dose–response relationship adds strength to a causation determination, this can be difficult to calculate in some exposed populations, especially if the numbers exposed are small.

2.8.1.4 Consistency

An association between an exposure and a disease should be repeatable. Multiple studies using different epidemiological methods and settings are desirable when possible. One study, even if very positive, is usually not enough to reach a conclusion. There are important exceptions for rare diseases with striking outcomes. Similarly, single negative studies should give one pause in evaluating conclusions, yet negative studies often receive less scrutiny.

2.8.1.5 Plausibility and Coherence

Plausibility is a common-sense evaluation of a scientifically reasonable association between the exposure and the disease. While current knowledge is essential to support or rule out connections between exposure and disease, one must be willing to consider novel associations that will, from time to time, change the way we think about things.

Coherence relates to findings in similar studies. This resembles thinking about drug classes that affect specific receptors. If there are similar exposures that cause similar outcomes, strength is added to the potential association. Recall that novel associations surprise us all too often, and that 'known' associations can be wrong.

2.8.1.6 Specificity

A specific exposure should cause a specific outcome. For example, reports that exposure to X causes 'cancers' or causes 'female problems' are very difficult to interpret. This is not to say that an exposure cannot cause more than one outcome; however, one should expect there to be a very short list of outcomes associated with the exposure. A problem with this criterion is that many exposures are to mixtures, so the exposure–outcome relationship becomes very complex. An inability to identify the specific component within a mixture of exposures does not mean that the exposure mixture is harmless.

2.8.1.7 Experimentation

While not one of Hill's original criteria, experimentation can offer the strongest evidence of an association. Animal testing can suggest a risk, but animal metabolic systems are somewhat different from human ones, so there may be effects from chemicals seen in animals, but not in humans, and vice versa. Recent work with perfluorocarbons illustrates the importance of choosing animals whose receptors and metabolism resemble the species of interest. Obviously, experimental exposure of humans is neither ethical nor appropriate. Commonly, we learn about human toxicity from inadvertent 'natural' experiments. There is a long industrial history of learning much from unintended consequences.

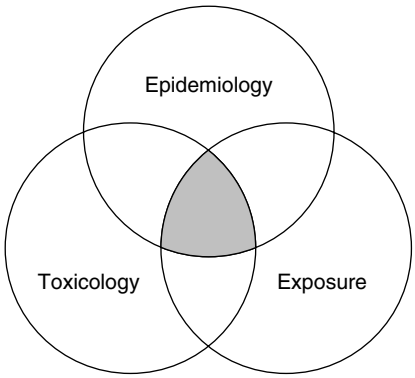

Figure 1 Discipline inputs to aetiologic decisions.

2.8.2 Consideration of Alternate Explanations

While this is really a subset of the plausibility and coherence criteria, one must always consider alternate sources of causation for the disease. If the alternate source of causation is more likely, then that should be stated. For example, a worker at a chemical plant with infrequent low-level exposure to a possible lung carcinogen who develops lung cancer may have been a two-pack-per-day smoker for the past 40 years. In this circumstance, causation is usually associated with the smoking.

2.8.3 Causation beyond Hill's Criteria

Another way to think about assessing causation is shown in the Venn diagram in **Figure 1**. Only situations where there is concurrence of exposure, toxicology and epidemiology, shown by the shading, can a causative connection be determined.

The most clinically challenging aspect of this is assuring oneself that there is a plausible exposure pathway. Mere proximity to a potential contaminant is *not* sufficient for causation; it is important to assure that the exposure pathway is completed.

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Occupational Toxicology and Occupational Hygiene within the European Union (EU) Chemicals Regulation

Steven Fairhurst and Elanor Ball

CONTENTS

1 CONTEXT

The purpose of this chapter is to discuss the interplay between the disciplines of toxicology and occupational (or industrial) hygiene in understanding and controlling the hazards and risks to health posed by chemicals, in an occupational setting.

Ten years ago, the chapter 'Industrial Toxicology and Hygiene', produced for the second edition of *General and Applied Toxicology*, offered a perspective on the main approaches, roles and responsibilities that, in a regulatory context, had held for a considerable period of time within chemical legislation. This updated chapter is written from within the European Union (EU) at a time when it is embarking on a new era of chemicals legislation that promises to change things profoundly. On 1 June 2007 the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) regulation came into force in the EU (EC, 2006). This legislation changes the nature and balance of roles between industry and regulatory

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authorities—and modifies the conventional approaches to, and interplay between, toxicology and occupational hygiene—in regulating industrial and commercial chemicals within the EU.

In its information requirements for registration purposes, REACH creates a tension between desires to improve the extent and quality of data available on chemicals, but also to minimize experimental animal testing. In relation to toxicology, this poses challenges to all concerned. REACH removes the distinction and associated data-expectation requirements between 'new' and 'existing' substances within the EU. REACH also expands and reinforces the 'customer care' principle that suppliers of chemicals take responsibility for understanding the uses to which their chemicals are to be put and specifying the risk management measures that should be followed in such uses. This will be a big challenge to the occupational hygiene profession. And all of this also comes at a time when the EU is in the process of adopting the globally harmonized system (GHS) of classification and labelling (C&L) of chemicals, which will modify the EU C&L system that has operated for the previous several decades.

So it is a time of change—and no one is quite sure how things will work out in the next 10 years. This chapter attempts to portray how these recent developments build on, or change, what has gone before, and discusses some of the key issues that are ahead for the toxicology and occupational hygiene fields, operating in this new regulatory context.

2 INTRODUCTION—BASIC PRINCIPLES

Occupational or industrial toxicology focusses on gaining an understanding of the hazardous properties of chemicals encountered at work and the risks of these properties being manifested under working conditions. Occupational or industrial hygiene, in relation to chemicals, addresses the exposure conditions created during the manufacture, formulation and use of chemicals, and the potential means of controlling and protecting against exposure. The manner in which the two disciplines interact is brought out by considering the familiar 'risk-assessment paradigm' (**Figure 1A** and **B**). Toxicology deals with the assessment of toxicological hazards and the risks of expression of such hazards under defined exposure conditions. Occupational or industrial hygiene deals with exposure assessment and the control of exposure and thereby risk. It seeks to define what exposure conditions could or will be encountered at work and addresses the technical and practical issues arising in considering and applying appropriate risk-management measures. The shared aim of both specialisms is to develop a sound scientific and technical basis for good standards of health, and effective and appropriate control measures in the workplace. There are, of course, other disciplines, such as occupational medicine and epidemiology, which make substantial contributions to achieving this goal.

Almost every chemical substance involved in modern society has an occupational exposure context and hence falls under the scrutiny of occupational toxicology and hygiene. Beyond the vast array of chemicals, such as reactants in synthetic processes, solvents, metals, oils, coatings, colourants and polymers, and the manufacturing and use situations in which they arise, conventionally thought of as 'industrial', there are other occupational exposure situations that should not be forgotten. Pharmaceuticals are made, formulated and used by medical staff in their work. Foods are processed, additives are added and cooking is done by people at work. Pesticides are made, formulated and applied by workers. Overall, there are tens of thousands of substances present in the occupational environment in most industrialized countries.

For most chemical substances the workplace is the situation where there is the potential for higher exposure than in other sectors of life. The two modes of exposure of primary interest in the workplace are inhalation of the airborne substance and surface (skin, eyes) contact with the substance. Ingestion can also be an issue, arising from contamination of material entering the mouth (food, drink, cigarettes), poor personal hygiene (hand-to-mouth transfer of chemicals) or swallowing of mucous containing inhaled substance being cleared from the respiratory tract by the mucociliary escalator).

The occupational environment holds several exposure possibilities—single, short-term exposure under normal operating conditions; single short-term exposure under uncontrolled (i.e. spillage, accident) conditions; occasional, but repeated, exposure; regular daily exposure under normal operating conditions and combinations of these. Exposure concentrations and time periods will fluctuate and exposure can also occur via more than one route. The occupational hygienist needs to give consideration to all of these aspects in making exposure assessments and contemplating control measures. The occupational toxicologist needs to consider all exposure scenarios and their potential impact on health in order to gain a full appreciation of the risks of ill-health arising from encountering a substance at work.

A particular feature of occupational toxicology is the consideration of site-of-contact effects. This can be a problem, in that much conventional toxicology has been and continues to be performed using the oral (and sometimes parenteral) route(s). Alongside the possibilities arising from the absorption, distribution around the body and metabolism (to more or less toxic derivatives) of a substance, significant occupational toxicology concern surrounds the local consequences of inhalation or skin/eye contact at and around the initial point of impact. Sensory irritation (nerve stimulation), tissue inflammation

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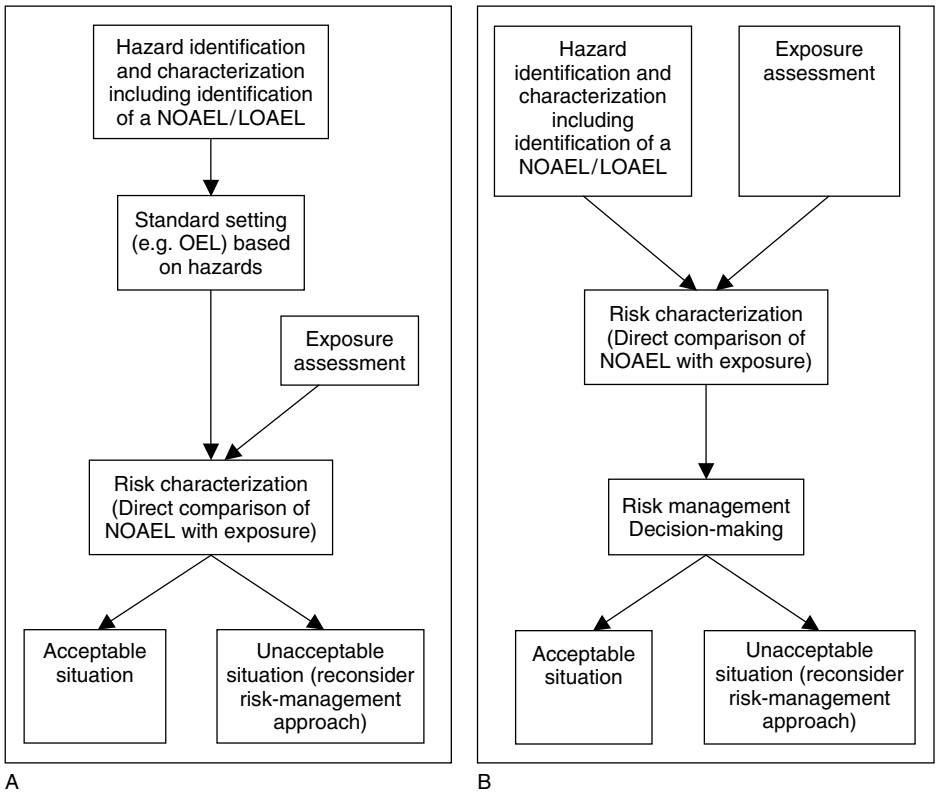

Figure 1 The interplay between hazard, exposure and risk assessment, leading to risk-management decision-making; two alternative pathways.

and damage, sensitization, mutation and cancer can arise in the lung or skin as a result of direct exposure to some reactive industrial chemicals or their locally produced metabolites. Additionally, pH effects and particle effects in the lungs (some related more to the physical than the chemical properties of a substance) are important considerations for local toxicity. The need to form a view of the potential local consequences of occasional or regular deposition of a substance in the respiratory tract and on the skin is an essential requirement in occupational toxicology.

3 HISTORICAL DEVELOPMENT

Observation of the adverse effects on health of occupational exposure to chemicals has a long history; brief allusions can be found in texts from ancient Greece and Rome. Classical figures of early toxicology and occupational medicine—Paracelsus, Ramazzini, Pott and others established their reputations from the sixteenth to the end of the eighteenth century in making the observations for which they are famed.

During the nineteenth and then the twentieth century, industrialization and the development of chemistry and the chemical industry brought about many benefits to society, but also problems, including occupational ill-health arising from exposure to industrial chemicals. The number of substances known to have produced some manifestation of occupational ill-health, be it skin inflammation, damage to internal tissues or cancer, stretches into the hundreds. It should also be acknowledged that there have been and continue to be many other, less well-substantiated claims made for evidence of manifestations of toxicity arising in the working environment.

Until well into the twentieth century there was a general acceptance or tolerance by society that where such chemical-induced ill-health at work was apparent, it was an unfortunate, but largely inescapable, feature of the world of work. Observations of affected workers were made, but there was little or no predictive toxicological testing of industrial chemicals, exposure assessment or pre-emptive action based on judgements of potential risks to health and aimed at prevention of toxicity arising from workplace exposures.

The first rudimentary toxicity testing of chemicals for the purposes of better understanding and controlling the risks to health involved in their industrial use was carried out by Lehmann in Germany in the late nineteenth century. These studies, principally on gases, involved only short-term exposure of animals and humans and were primitive in their design and conduct, but they did establish a baseline, which was important to later investigators. Similar studies were performed by others in the early part of the twentieth century, although the range

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of substances covered remained small. The findings were laid out in standard textbooks of the time (Henderson and Haggard, 1927; Flury and Zernik, 1931).

Toxicology investigations on industrial chemicals became more regular and refined from the 1940s. Nevertheless, it is generally true that the enormous and rapid expansion in the last 60 years in the number of chemical substances potentially in the occupational environment occurred largely in the absence of programmes specifically requiring the gathering and critical assessment of detailed and comprehensive toxicological data. Hence, even in the early 1970s, the American Conference of Governmental Industrial Hygienists (ACGIH) recommended a threshold limit value for vinyl chloride as high as 500 ppm (eight hours time-weighted average), commensurate with a substance being viewed as of low toxicity. Soon afterwards, vinyl chloride was clearly shown to be genotoxic and unfortunately to have already produced cancer in humans (Purchase *et al*., 1987). The situation with asbestos is even more salutary, with the tragedy still being played out of many tens of thousands of deaths from cancer, a consequence of inadequate understanding of toxicological hazard, potential exposure situations and the appropriate stringency of control necessary to secure negligible risk.

Although the field of occupational health is replete with historical accounts of industrial chemical toxicity that are both fascinating, but also tragic from a worker-health standpoint, much has been done in recent times to improve the understanding and control of risks to health posed by occupational exposures. Nevertheless, it is still true to say that the toxicology of industrial chemicals has been examined in a patchy fashion. A minority of substances have been well investigated. However, most substances of interest in relation to occupational exposure do not have extensive, detailed toxicological data on them and many have received little investigation (National Academy of Sciences, 1984; Allanou *et al*., 2000). One of the key objectives of REACH is to greatly improve this situation (see Section 7 in this chapter).

A significant consideration is the relative cost–benefit balance of pursuing better understanding of a situation as against settling immediately for tight control of that situation. Where only small quantities of a substance are being manufactured and used, and risk management to secure avoidance of ill-health is the goal, it is often more realistic to approach risk management by applying very stringent controls to achieve negligible exposure, rather than investing the time, effort and money necessary to acquire a clear understanding of the toxicology.

4 LEGISLATIVE FRAMEWORK

Since the late 1960s, a progressively increasing amount of legislation has been introduced in what is now termed the European Union, currently comprising 27 Member States; aimed at the appropriate identification and control of threats to human health (and safety, and the environment) posed by industrial chemicals. Prior to the introduction of REACH, the complex legislative framework that had developed over the last 40 years was anchored to the following general philosophy:

- *Suppliers* were deemed to be responsible for understanding the hazardous properties of the chemical(s) being supplied, toxicological information being conveyed, in a suitably interpretable manner, to recipients/users of the chemical(s). In this context *regulatory authorities* have developed classification systems that provide criteria by which to allocate substances to particular hazard categories denoted by symbols and phrases appearing on labels.
- *Users* have been held responsible for understanding the intended local conditions of use of the chemical and setting this knowledge, with its implications for levels, routes and frequencies of exposure, alongside the hazard information. In doing so, the user has been required to make a situation-specific risk assessment and draw conclusions regarding the appropriate risk management measures to apply—and then to apply them. In attempting to help the user, *regulatory authorities* have established some general risk-management measures, chiefly by extolling adherence to specified occupational exposure limits (OELs) for the airborne substance.

Although key aspects of these principles still hold now and will persist into the future, trends have been established in the EU over the last 10 or so years that have shifted things in several ways. The appearance of the new REACH legislation promises to change the picture still further, in ways that are described below. However, first we should appreciate what has been the regulatory framework for industrial chemicals up to now. This is done in the next section.

5 TOXICOLOGICAL HAZARD IDENTIFICATION: CLASSIFICATION AND LABELLING

A number of classification or categorization systems have been developed by different institutions in various parts of the world. Such systems are used to denote that a chemical belongs to a group sharing either a specified degree of toxicological hazard or a specified weight of evidence of a toxicological property (e.g. carcinogenicity) of relevance to human health. We concentrate here on the current EU system. In addition, since the mid 1990s there has been an initiative to develop a 'GHS' aimed at standardizing C&L across the world. At the time

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of writing this chapter, a new EU regulation is being negotiated that will bring the GHS into the EU (see Section 5.2).

5.1 The EU Classification and Labelling System

Since the mid 1960s, for the supply of chemicals to be used in workplaces (and elsewhere) in the EU, there has been a regulatory requirement to classify and label substances, and combinations thereof ('preparations'), in accordance with an EU-wide system described in EC Directives (and their Amendments) on the Classification and Labelling of Dangerous Substances and Preparations, together with their accompanying Annexes (EEC, 1967; EEC, 1988). This system has been implemented in each EU Member States via each country's national legislation.

The aim of this system is to harmonize understanding of the hazardous properties of any particular substance or preparation, across all of the EU Member States and across different companies with a shared interest in the same material, such that trade, and health, safety and environmental matters that might be connected with such hazardous properties are considered, based on this common currency.

This EU C&L system has required that identified hazardous toxicological properties, from whatever test situation used or observations made, are considered in terms of their relevance to human health and the C&L ultimately derived should reflect this relevance. All the data available on the chemical, including both experimental animal and human findings, needs to be taken into account. The system encompasses all toxicological end points:

- Acute toxicity arising from single exposure
- Skin, eye and respiratory tract irritancy
- Skin and respiratory sensitization
- Longer-term toxicity arising from repeated exposure
- Mutagenicity
- Carcinogenicity
- Reproductive toxicity, covering both effects on the developing foetus and newborn offspring.

Detailed criteria against which the toxicological (and physicochemical and ecotoxicological) properties of a substance are compared in order to determine its C&L are given in an annex to the directive dealing with substances. Preparations containing one or more hazardous (i.e. classified) substances are classified either by comparing the toxicological properties of the preparation against the same criteria, or by a series of rules based on the concentrations of individual substances present in the preparation.

The label that emerges from the process has three components—danger symbols, in the form of pictograms, phrases indicating hazardous properties ('R' phrases), such as 'toxic if swallowed', 'irritating to eyes' or 'may cause cancer' and phrases indicating basic advice for safe use.

The application of the C&L criteria to toxicological data available on a substance is in some instances quite straightforward. In other cases the findings of extensive and complex toxicological investigations need to be understood and considered before an appropriate judgement is reached. To take one aspect, within the current EU C&L system there are three categories of 'positively' classified carcinogen; there are detailed criteria and guidelines available to steer assessors towards the most appropriate position, within a framework in which the general meaning of the categories is as follows:

- Category 1—confirmed (i.e. directly observed) carcinogen in humans
- Category 2—presumed human carcinogen, that is, carcinogenic potential in humans judged to be likely, based on the available evidence
- Category 3—possible human carcinogen, that is, some evidence for carcinogenicity of potential relevance to humans, but surrounded by significant uncertainty.

Much toxicological endeavour and extensive and sometimes contentious regulatory debate has been expended to resolve matters of the type illustrated in **Table 1**.

Finalized positions on C&L have become ever more important, because classification now enjoys much more significance than merely information transfer. The act of classification represents a gateway beyond which there may be a large number of downstream consequences for a chemical. These are set out in legislation such as the Preparations Directive, the Carcinogens Directive, the Safety and Health at Work of Pregnant Workers Directive, the Chemical Agents Directive and the 'Seveso Directive' on control of major accidents (EC, 1999; 1998; 1996; EEC, 1990; 1992b). Thus, hazard classification can lead directly to risk management stipulations in several different spheres of chemical manufacture and use. For example, substances classified as Category 1 or 2 carcinogens, mutagens or reproductive toxicants are not allowed in consumer products, and Category 1 and 2 carcinogens and mutagens are subject to the strict risk-management specifications of the Carcinogens Directive (definitions of the categories used for classification for carcinogenicity within the EU are given above; classification for germ-cell mutagenicity and reproductive toxicity is based on similar principles).

However, the C&L scheme in itself does not specify an information requirement. Rather, it provides a set of criteria against which the observed or predicted

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Table 1 Carcinogenicity classification assigned to three substances found to produce malignant neoplasms in experimental animals

Substance	Findings in carcinogenicity studies in experimental animals	Carcinogenicity classification conclusion
Propylene oxide	Malignant respiratory tract neoplasms in rats	Category 2 carcinogen
Aniline	Malignant spleen neoplasms in rats	Category 3 carcinogen
Diethyl hexyl phthalate	Malignant liver neoplasms in rats	Not classified as a carcinogen

toxicological properties of a substance should be compared, however extensive or sparse the available data. For some substances, a regulatory C&L position has been agreed for legal implementation across the EU. However, for many others the responsibility has fallen on each individual company supplying the substance to 'self-classify' by interpreting the available data against the general criteria and guidance. This situation has (inevitably) led to inconsistency. One such example that our authority has become aware of relates to the substance 3,3′-diaminobenzidine in respect of its carcinogenicity classification.

Benzidine (**Figure 2**) is an established human (bladder) carcinogen and, as such, has a formally agreed regulatory classification in the EU as a Category 1 carcinogen.

3,3′-Diaminobenzidine (**Figure 3**) does not have a formally agreed EU classification and hence the responsibility has fallen on each supplier to self-classify. This substance has a limited toxicological database. It is genotoxic *in vitro* and no *in vivo* genotoxicity studies have been identified. In relation to carcinogenicity, no studies have been performed to current internationally accepted protocols; the results of some rather old and nonstandard studies in small groups of rats and mice do not yield a clear picture of the carcinogenic potential of 3,3′-diaminobenzidine (DFG, 1992).

Information available from a number of different suppliers indicates that 3,3′-diaminobenzidine has been marketed within the EU in recent years with self-classification verdicts for carcinogenicity of not classified, Category 3 carcinogen, or category 2 Carcinogen!

Looking ahead, one key feature of REACH, that of collaboration between companies sharing a common interest in the same substance, holds the promise of eradicating such inconsistency. This is discussed in the REACH section later in this chapter.

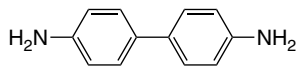

Figure 2 Chemical structure for benzidine.

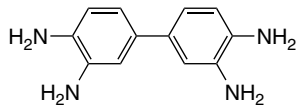

Figure 3 Chemical structure for 3,3′-diaminobenzidine.

5.2 Globally Harmonized Scheme

Stimulated by the 1992 United Nations Conference on Environment and Development (UNCED), the last 15 years have seen the development by the United Nations (UN) of a globally harmonised system (GHS) for the classification and communication of the hazards of substances and preparations (mixtures) (OECD, 2001). The model scheme for the GHS was agreed at the UN level in 2003 (UN, 2003).

The EU has recently embarked on a new regulatory initiative to bring into EU law much of the GHS, via a proposed new 'Regulation… on classification labelling and packaging of substances and mixtures…' (EC, 2007). The detail of this new regulation is under negotiation at the time of writing this chapter. It is currently envisaged that from the end of 2010 substances in the EU will be dual-classified under the stipulations of the existing EU C&L system and the new GHS rules, but labelled and packaged according to the requirements of the new legislation. From mid 2015 all substances and mixtures thereof will be classified, labelled and packaged according to the new regulation only.

5.3 (Material) Safety Data Sheets

Closely associated with C&L, the key component of information transfer from the supplier of a chemical substance or preparation to the recipient/user is the (material) safety data sheet ((M)SDS). In the EU, prior to the arrival of REACH the content of an SDS has been stipulated in the European Union Safety Data Sheets (EU SDSs) Directive (EC, 1993). The disciplines of occupational toxicology and occupational hygiene should come together to summarize the key hazard and control alerts presented in the SDS.

The REACH legislation has subsumed the EU SDS Directive and now stipulates what is required of an safety data sheet (SDS) in the EU. The degree of change in structure from what has gone before is minimal (see

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Section 8). However, experience of SDSs to date is that the quality of their content has been highly variable, with many examples of gaps, errors or ambiguities in information. Under REACH, SDSs will remain a key component of information transfer along the supply chain. It is hoped that the quality of information they contain will improve substantially in future years.

6 USER-SIDE RISK ASSESSMENT AND RISK MANAGEMENT FOR INDUSTRIAL EXPOSURE TO CHEMICALS

To reiterate points made above (see Section 4), the regulatory framework applying to workplaces in the EU has held users of chemicals responsible for understanding the intended local conditions of use of the chemical and setting this knowledge, with its implications for levels, routes and frequencies of exposure, alongside the hazard information. In doing so, the user has been required to make a situation-specific risk assessment and draw conclusions regarding the appropriate risk management measures to apply—and then to apply them. This philosophy is captured within the EU Chemical Agents Directive, implemented in each EU member state by national legislation, such as the Control of Substances Hazardous to Health (COSHH) Regulations (EC, 1998; COSHH, 2004) in the United Kingdom.

In attempting to help the user, *regulatory authorities* have specified some risk-management measures, in earlier years chiefly by extolling adherence to specified OELs for the airborne substance. This picture has started to change somewhat in recent years and the advent of REACH promises further changes. The progression to this situation is discussed below.

6.1 Occupational Exposure Limits (OELs)

6.1.1 General Principles of OELs

An OEL serves to indicate an 'appropriate' (this could be defined in a variety of ways) level for the control of exposure to an airborne substance in the workplace.

A very strong traditional representation of the process and impact of establishing OELs would run as follows. For an individual substance under consideration, the occupational toxicology profession would examine the toxicological profile of the substance to ascertain its toxicological hazards, their associated dose–response characteristics and the surrounding issues of route, frequency and duration of exposure connected with the possible appearance of the hazardous properties. Then, against a criterion for an OEL being, for example, the highest level of exposure at which no adverse effects on health would be anticipated, a toxicological judgement is made to derive the OEL value. Then the occupational hygiene profession comes in. The concept is that the OEL value is used to establish the control regime that would bring exposure to a level at or below the OEL. The associated imagery is that the occupational hygiene profession will be engaged on monitoring the airborne exposures arising in the manufacture and use of the substance, to ensure that the control of exposure to the OEL is being secured.

A variation on this process is where consideration of the practical and economic consequences of securing control to a particular airborne level is an integral part of the OEL-setting process. This approach has been used in the UK in situations where, for example, there is doubt about where a no-effect level for human exposure might lie, such as for a poorly investigated substance; or where it has not been possible to identify with confidence a no-effect level for humans, such as for a genotoxic carcinogen. In such circumstances occupational-hygiene expertise comes in earlier, to assess contemporary exposure levels and the cost and achievability of control to one or more possible lower levels of exposure, leading ultimately to determination of the eventual OEL.

These concepts and approaches have been the basis of the practice of setting OELs, an activity that has been a central plank of occupational toxicology and occupational hygiene thinking and work for more than half a century.

6.1.2 Emergence of Early OEL Lists

The first published OEL is held to be the value of 500 ppm carbon monoxide, put forward by Max Gruber in the late nineteenth century and derived from studies involving 12 rabbits, two hens and himself! (Paull, 1984). The basis was the avoidance of immediately recognizable acute effects, as it was in the first lists of harmful concentrations of substances in air that began to appear in the first quarter of the twentieth century, mainly from toxicological work performed in Germany (see Section 3).

Almost no toxicological information beyond acute toxicity and irritancy was available for substances at this time. From the 1920s, some information became available on dose–response relationships for long-term exposure to a few familiar toxicants—lead, mercury, crystalline silica, benzene—and the stated purpose of listing 'maximum allowable concentrations (MACs)' gradually changed to one of protecting the workforce against ill-health when exposure was occurring daily over a working lifetime.

Perhaps the single most significant development in this area was the establishment, in the 1940s, of the Threshold Limits Committee of what was first the

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National, and then became the American Conference of Governmental Industrial Hygienists (ACGIH), in the USA. This committee used pre-existing information and its own experience to construct and adopt its first list of MACs, comprising limit values for 160 substances, in 1946. The list of what soon became known as threshold limit values (TLVs) was first published in 1950 (ACGIH, 1950). Over the subsequent 60 or so years the TLV list has grown, as has the amount of supporting information documented in support of the rationale for the values listed.

6.1.3 Proliferation of OEL Lists

The ACGIH TLVs were extremely influential in the USA and also across the world in the second half of the twentieth century. Many countries used the values either as guidance or as the basis for national legal standards. Even now, when other systems have come and, in some cases, gone or are on the way out, listings of OELs persist in many parts of the world and contain values inherited directly from TLV lists of the past.

From about 40 years ago, institutions in various parts of the world started to establish their own systems for producing OELs, via assessment of the available toxicology and, in some cases, occupational exposure data. The first such system developed in Germany. In 1968 Germany ceased to adhere automatically to the ACGIH list and began a programme of generating its own OELs under the Maximale Arbeitskonzentrationen (MAK) Commission. Since then the MAK Commission has produced its own annual listing of limit values (DFG, 2007).

Other systems then developed, including those in the Netherlands, the UK, Nordic countries, Australia and Japan. Alongside these systems, the USSR (as was) had its own system of establishing OELs that began in the 1930s, largely based on Russian literature with findings somewhat at odds with the western world consensus at the time (Sanotsky *et al*., 1986).

From the beginning of the 1990s the EU, via one of its Directorates General (DGs) now known by the summarized title of DG Employment, developed a system for introducing EU-wide OELs (details are provided on the DG Employment web site; a link is given under Further Reading). The EU system has the potential for two types of OEL to be introduced, under the umbrella of the Chemical Agents Directive (EC, 1998). The indicative occupational exposure limit value (IOELV) is envisaged to be health-protective, in that there is confidence that adherence to it will avoid any ill-health consequences of exposure to the substances in question. The binding occupational exposure limit value (BOELV) is envisaged to reflect a balanced judgement between health, practicality and socioeconomic considerations, for substances where a health-based limit cannot be identified and/or control of such a limit cannot be achieved.

To assist it in the process of establishing EU OELs, DG Employment has its Scientific Committee on Occupational Exposure Limits (SCOEL). This committee of prominent EU experts in the field of chemicals and occupational health examines and debates the toxicological evidence and analytical methods available for a substance, and endeavours to make OEL recommendations to DG Employment. IOELVs are negotiated and implemented via EU Directives.

6.2 Effectiveness of OELs; Control-Banding Approaches

In the last 10–15 years, in the UK and some other countries, there has been some serious questioning of the reality of the above picture (see Section 95.6.1.1), and the effectiveness of tabulated lists of OELs for hundreds of individual substances. It could be argued that, to a large extent, the difference between what happens in reality and the standard representations made earlier in this section is a consequence of limitations in the availability of occupational hygiene resource, particularly at sites of receiving and using chemicals. Research conducted in the UK in the mid-1990s, in relation to the list of regulatory OELs in the UK at that time, indicated a disappointing lack of awareness and utilization of these OELs in company decision-making about chemical control (Research International, 1998). Two other key themes emerged at about this time: many smaller companies wanted to be instructed in how to secure appropriate control, rather than have to work out for themselves the control solutions; and for very many substances relatively little monitoring of exposure was occurring—and the extent of exposure monitoring seems to have been diminishing still further in recent times.

The consequence of these influences has been the development of 'control-banding' schemes. One such scheme developed in the UK is 'COSHH Essentials' which can be accessed at *www.coshh-essentials.org.uk*. Another scheme based on similar principles is the Dutch 'Stoffenmanager' scheme which can be accessed at *www.stoffenmanager.nl*.

Control-banding schemes operate on the principle that the identified toxicological hazards of a substance define its allocation to a particular category, group or band that is associated with an approximate level of occupational exposure deemed to be appropriate for substances possessing such properties. For any substance within a particular group, associated knowledge of how and in what quantities it is being used, together with its propensity to become airborne, is then used to determine the appropriate control strategy to be applied to the use of that substance in a particular industrial setting.

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The two major advantages of such schemes are that: (i) they can embrace every substance, rather than be limited to those substances for which a position (OEL) is listed and (ii) they provide users of substances with practical control advice, rather than (simply) an exposure level to be achieved.

The concept has become a gradually increasing focus of attention during the last 10 years. It has had a major influence on the concept of 'exposure scenarios' under REACH; this is discussed further in the section on REACH.

6.3 Biological Monitoring and Biological Limits

In a manner that can complement the measurement of airborne exposures and the use of OELs as control standards for airborne exposure, biological monitoring involves the measurement of a substance and/or its metabolite(s) in body fluids (usually urine or blood) or exhaled breath. A closely related concept is that of biological-effect monitoring, the measurement of a biochemical change in the body caused by exposure to a substance, the degree of change being below that associated with frank ill-health.

Biological limits are standards, based on biological monitoring or biological-effect monitoring, against which the results of biological (effect) monitoring can be compared to assess adequacy of control.

In general, biological (effect) monitoring gives a more accurate assessment of individual intake of a substance, compared with atmospheric monitoring, and is particularly useful in the following respects:

- To assess the extent of substance intake via routes other than inhalation (skin, ingestion)
- To track the potential accumulation of substances in the body resulting from long-term repeated exposure
- To examine the effectiveness of personal protective equipment (PPE) in guarding against exposure to and intake of substances.

The two most productive systems establishing biological limits are those of the ACGIH in USA and the MAK Commission in Germany, with their biological exposure indices (BEIs) and biological tolerance values (BATs), respectively. Other systems for setting biological limits exist, including the system for establishing Biological Monitoring Guidance Values (BMGVs) in the UK. The EU Chemical Agents Directive and derivative legislation also makes provision for EU biological limits, but as yet there has been little attempt to see such values introduced.

Throughout the world, the number of substances for which biological limit values exist (with associated reliable measurement methods) is less than 100. The approach is potentially very useful in some circumstances involving exposure to particular substances; for example, it has a long history of compulsory use in relation to monitoring occupational intake of lead. However, its practical use is restricted to a modest number of specified substances.

The above section has discussed the manner in which the 'user' side of industry has been required to assess and manage the risk involved in chemical exposure at its worksites. In so doing, a user company has needed to make the best of whatever information has come through the supply chain, alongside any stipulations and guidance from regulatory authorities. The next section looks at some initiatives that have been taken in the EU in the recent past to put more responsibility on to supplying companies to better assess and meet the information needs of user companies.

7 PREVIOUS (PRE-REACH) SUPPLY-SIDE DATA-GATHERING AND RISK-ASSESSMENT PROGRAMMES IN THE EU

7.1 EU 'New Substances' Legislation (1979–2008)

In its first initiative to improve the information available on industrial chemicals, the EU introduced, at the beginning of the 1980s, legislation requiring a package of data to be generated for industrial chemicals new to the EU market. The extent and quality of this information had to be deemed satisfactory by the relevant regulatory authorities before the substance could be marketed. This has been known as the 'Sixth Amendment', and later the 'Seventh Amendment' legislation, these being amendments to the original 1967 European Directive on substance C&L (EEC, 1979; 1992a). Each member state has been required to have its own national legislation and associated competent (regulatory) authority to implement the requirements of the Directive. These regulations covering *new* substances operated until the middle of 2008, whereupon they were supplanted by the advent of the REACH regulations (see Section 8).

In their first 'Sixth Amendment' form, the regulations concentrated on information generation and the communication of hazardous properties using the EU C&L system. Toxicity testing, in accordance with standard OECD test guidelines, was required, the extent being tiered according to tonnage to be placed on the EU market. Conformity with expectations was checked by Member State Competent Authorities (MSCAs). The principle was that new substances, and preparations

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containing them, being supplied to workplaces (and elsewhere) arrived for the first time with some of their toxicological (and physicochemical and ecotoxicological) properties having been explored. Where indicated by the findings of such exploration, the relevant hazardous properties would be shown on the label and in the (material) safety data sheet. Subsequently, if the quantity of the new substance on the market increased, more extensive testing was considered, embracing a wider range of toxicological end points and/or a greater depth of exploration of individual issues.

The 'Seventh Amendment' expanded the ambition of this *new substances* legislation, in that the focus became, not only hazard identification, but also risk assessment. This has required suppliers of new substances to take increased responsibility for understanding the potential exposure and therefore risk situations that could be created in the downstream use of the supplied substance, and to ensure that appropriate advice was transmitted to recipients to facilitate adequate exposure control and risk management. This development ushered in an important new feature, that of suppliers of substances taking responsibility for securing their safe use by recipients, a crucial principle that has come through into the REACH era (see later). Delivery of such a responsibility clearly requires collaboration between the disciplines of toxicology and occupational hygiene.

For *new* substances notified to the regulatory authorities at the premarketing stage, the obvious problem arises that experience has not yet been gained from which to measure directly the characteristics of exposures in use. Hence there has been a need to develop the means of predicting exposure by way of modelling. Within the EU, several approaches have been developed to meet this requirement for occupational exposure prediction, such as the knowledge-based system Estimation and Assessment of Substance Exposure (EASE) (HSE, 1995). Such models use information on the physicochemical properties of a substance and the intended characteristics of any use situation (type and scale of use, intended exposure controls, such as local exhaust ventilation) and make predictions of likely exposure either by matching such information to pre-existing knowledge of exposures created in other comparable circumstances, or by making a prediction from first principles. The occupational hygiene profession has always stressed that the predicted numerical values or ranges obtained from such models represent rough initial estimates that, as a description of any particular situation, should be considered and interpreted by an experienced occupational hygienist. The use of such exposure modelling techniques has come through into the guidance for fulfilling some responsibilities under REACH (see later).

In three decades of operation of the new substances legislation, the number of known cases of toxicity arising from exposure to a new substance within the EU is very small. This could be taken to be a strong vindication of the value and effectiveness of the legislation. However, there was always a view from industry, sometimes also voiced by others, that the legislation was too onerous—bureaucratically, in terms of the interactive process with regulatory authorities, and financially, because of the cost of commissioning toxicity (and other) testing. Associated with the latter point was a feeling that there was too much use of experimental animals to test substances that regularly showed (and, with hindsight, might have been predicted to show) little or no significant toxicological potential. Certainly the UK regulatory authority encouraged and made ever-increasing use of grouping, read-across and other structure–activity approaches, and other types of arguments, waiving the need for a test to be conducted, wherever the predictions appeared valid and beneficial in economizing on animal experimentation.

In the late 1990s the desire to change the new substances legislation grew to an extent that it has been supplanted by the REACH regulation, developed and implemented over the subsequent 10 years, in which there is no distinct identification of *new* substances. Nevertheless, many of the features of REACH are adaptations of the principles and approaches of the former new substances legislation. Importantly for toxicology, the sociopolitical pressure in the EU to minimize experimental-animal testing has continued to grow, such that REACH provides strong encouragement for imaginative alternative ways of compiling toxicological profiles of substances that are sufficiently dependable to permit appropriate regulatory judgements, without recourse to undertaking new animal experimentation.

7.2 EU Existing Substances Regulation (ESR)

In the early 1990s the EU introduced the Existing Substances Regulation (ESR) (EEC, 1993). ESR embraced similar principles to those of the 'Seventh Amendment' legislation for new substances, in that suppliers of substances (but this time substances already familiar to the EU market) were held responsible for working with regulatory authorities to address the hazardous properties of substances, and the exposure conditions and risks (to human health and the environment) that could arise in the manufacture and use of the substance. The objective was to secure appropriate exposure control and risk management of important existing industrial chemicals by instructions and measures applied through the supply chain. As for new substances legislation, ESR required each EU member state to appoint a national competent (regulatory) authority (CA).

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ESR required:

1. Collation by industry of all relevant, reliable data already available on an existing substance, including toxicological and exposure data, in a computerized proforma called a harmonized electronic dataset (HEDSET), to be sent to a central regulatory point, the European Chemicals Bureau at Ispra in Italy.
2. Storage of this information on a database called IUCLID (International Uniform Chemical Information Database) and use of the knowledge of and concerns about individual existing substances by the European Commission and EU member states' CAs to select priority substances for regulatory assessment.
3. For a selected priority substance, compilation by a *rapporteur* EU member state of an extensive document detailing the hazardous properties of the substance, its dose–response characteristics, exposure assessment and assessment of the risk judged to be involved for various sectors of the human population, including all workplace situations (and the environment) encountering the substance.
4. For each priority substance assessed, to arrive at an ultimate conclusion about the acceptability or otherwise of the risk of adverse consequences in every situation in which the substance could arise to a significant extent; wherever it was deemed that an unacceptable risk might arise, risk reduction measures to mitigate the risk were brought into play. A third, interim conclusion was also possible, that further information was required, possibly including the need for suppliers to commission additional toxicity testing in experimental animals, before a final conclusion could be reached about the risk posed in any particular situation in which the substance was present.

The features of ESR clearly indicate the need for toxicology and occupational hygiene to work in close collaboration, in addressing workplace exposure situations. It was found that occupational exposure measurements for many of the circumstances that needed to be considered were often sparse or nonexistent, necessitating the use of the predictive modelling skills of occupational hygienists to fill such gaps.

Some major difficulties arose with ESR. One was the lack of preparedness and apparent inability of industry to identify and characterize from the point of supply all of the potential workplaces, working practices and associated exposure situations that could arise with a particular substance. This led to a related, but even more general problem that the demands placed on individual ESR competent authorities to compile the extensive documents required, including the coverage of issues that supplying companies had been unable to address satisfactorily, defeated the resources available within these authorities. The rate of progress of priority substances within the ESR process and the numbers of substances that had completed their passage through the process in the 15 years of its operation were well below what was desired by the EU regulatory system when the legislation was being developed. The slow progress was identified in the late 1990s, and ESR has now been supplanted by the REACH regulation which, conceptually, aims to remedy the deficiencies that emerged under ESR.

8 REACH—A NEW ERA

REACH stands for the Registration, Evaluation, Authorization and Restriction of Chemicals. In total it replaces about 40 pieces of chemicals legislation in the EU and is intended to streamline and improve the way chemicals are regulated. It removes the arbitrary division of chemicals into 'new' and 'existing' substances and aims to place all chemicals under the same regulatory framework. REACH places important obligations on suppliers, but also imposes duties on downstream users and will have a much more direct influence on the way the risks arising from the use of chemicals are managed.

The key aims that the EU hopes to achieve with REACH are:

- To improve the protection of human health and the environment from risks posed by chemicals
- To promote alternative methods for hazard assessment
- To enhance the competitiveness of the European chemicals industry
- To ensure free circulation of substances on the internal market of the EU.

These are ambitious aims that carry within them the apparently conflicting public desires for a better understanding and control of risks arising from the use of chemicals and a move away from the use of animal models for toxicity testing. One way the European Commission hopes to achieve this is by requiring industry to share test data obtained from animal studies. In the longer term it is hoped that these aims will stimulate the development of new and better approaches to hazard evaluation.

8.1 Scope of REACH

REACH is very broad in scope. It deals with substances; substances used on their own and as they are used in preparations and in articles. REACH applies to most chemicals and it applies to most uses of chemicals. Very few chemicals are exempted from all of the provisions of

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REACH but exemptions from certain aspects are given to substances that have undergone extensive evaluations under other legislative programmes, or where limited exposure can be demonstrated; or where substances have been demonstrated to be of intrinsically low hazard. Exemptions from certain aspects of REACH are also granted for the purpose of product and process oriented research and development (PPORD) to facilitate the development of alternative substances and processes. Exemptions are described in the legislation.

8.2 Elements of REACH

There are four key elements to REACH:

Registration —is the process by which manufacturers and importers into the EU provide information on the substances that they supply to a new central coordinating body, the European Chemicals Agency (ECHA), based in Helsinki, Finland.
Evaluation —encompasses the various checks on this information from the simple completeness checks performed on all registration dossiers to the in-depth reviews that will be carried out by MSCAs on priority substances according to a rolling plan of action.
Authorization —is the mechanism by which substances with properties deemed to be of the highest concern will be phased out of use, if the risks involved in their continued use are judged to be unwarranted.
Restriction —is the mechanism by which MSCAs and the European Commission can identify and further limit the manufacture, supply and/or specific uses of substances where the risks involved in current situations are judged to merit more stringent control.

8.3 Registration and Evaluation

All manufacturers and importers of substances who supply to the EU market in quantities of 1 tonne per annum or more are required to register their substances with ECHA in order to be able to continue to supply (for substances already on the market) or in advance of supply (for substances new to the market). In order to register, manufacturers and importers need to provide a dossier of information on that substance. The amount of information that each manufacturer or importer is required to provide depends on the tonnage that the manufacturer or importer supplies, with stepwise increases in data requirements for the four tonnage bands (1–10 tonnes per annum, 10–100 tonnes per annum, 100–1000 tonnes per annum and >1000 tonnes per annum). This reflects the data-gathering process that was successfully used to gather hazard information under the previous 'new substances' legislation (see Section 7.1). There is also a requirement for suppliers to consider how their substances are being used within their own company and for all downstream uses (including consumer uses) and whether there are potential releases to the environment at different life-cycle stages. The life cycle covers all stages from manufacture to eventual disposal, including all workplace and consumer uses. If the substance is present in articles it may be necessary to consider the service life of those articles. It may also be necessary to consider the potential for human and environmental exposure during recycling. Where potential risks to human health or the environment are identified, suppliers must determine the measures necessary to adequately control those risks and must disseminate information on the operating conditions and risk management measures necessary to secure adequate control through the supply chain. This is done by means of exposure scenarios describing appropriate control strategies which are incorporated into extended safety data sheets (e-SDSs).

In the model envisaged under REACH, the onus is placed on suppliers (manufacturers and importers at the top of the supply chain) to detail the hazards and appropriate control strategies for the substances that they supply. The onus is on downstream users to apply the control strategies correctly in their respective workplaces, taking into account workplace-specific factors that might influence the efficacy of the control measures that are recommended. Downstream users are also required to communicate information on experience in use back up the supply chain to enable suppliers to optimize their risk assessments.

It is intended that the regulatory process under REACH will be transparent. To this end, ECHA will make publicly available via its web site as much information as possible from registration dossiers and the regulatory decision-making process, without compromising the need to protect confidential business information. The ECHA web site can be accessed at: *http://echa.europa.eu*.

The duty to register chemicals new to the EU market came into force on 1 June 2008. Owing to the large number of chemicals that are currently placed on the EU market in quantities of 1 tonne per annum or more (estimated to be around 30 000) the duty to register chemicals that are currently on the market is being phased in over a 10-year period. The timescales are illustrated in **Figure 4**. In order to take advantage of the phase-in deadlines, suppliers must have 'pre-registered' the substances they currently supply by 30 November 2008. The final deadline for registration of substances currently on the market is 1 June 2018, so by this time there should be core information on hazards and potential risks for every chemical that is supplied onto the EU market in quantities of 1 tonne per annum or more. Registration of chemicals new to the market is envisaged to be a continuing requirement beyond 2018.

In order to encourage harmonization of the hazard and risk management information that individual

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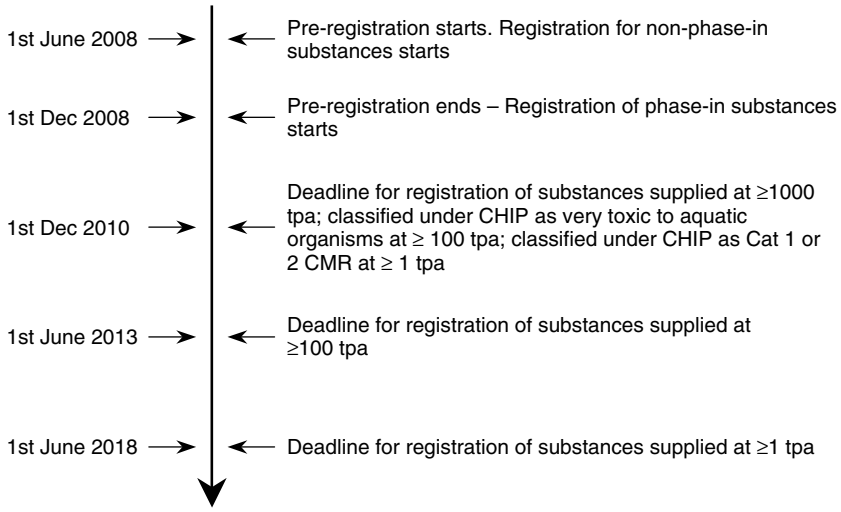

Figure 4 Timeline for implementation of registration requirements. CHIP: Chemicals (Hazard Information and Packaging for Supply) Regulations 2002; CMR: carcinogenic, mutagenic or toxic to reproduction; tpa: tonnes per annum.

suppliers disseminate through their respective supply chains, companies wishing to register a particular chemical are encouraged to 'pre-register' their interest in the substance. ECHA will then facilitate each such company joining with other companies wishing to register the same chemical in groups called Substance Information Exchange Forums (SIEFs). SIEFs enable companies to collaborate on the preparation of registration packages and share the costs. Registrants are legally obliged to share data obtained from animal test models and are encouraged to share data obtained by other types of test. In this way it is hoped that there will be a shared understanding of the hazardous properties of substances between suppliers and that this will lead to harmonization of the hazard information, C&L and recommended risk-management measures that each company provides addressing the current problems with inconsistent information between suppliers (see Sections 5.1 and 5.3).

The core data requirements for chemicals supplied in quantities of 1–10 tonnes per annum are light, at least for toxicity and ecotoxicity data. However, if it is available, companies must also submit any extra information beyond the core data requirements for their tonnage band. For example, if a carcinogenicity study had been published in the open literature this will need to be included, even if a particular registrant is only supplying at 1 tonne per annum.

The registration package for the 1–10 tonnes per annum band comprises a dossier, covering the identity of the registrant and the substance being registered plus the technical information outlined below. Registrants supplying at 10 tonnes per annum or more must also provide a chemical safety report (CSR). This documents the hazard assessment and, if required, exposure assessments and risk characterization that the supplier has performed and is the tool for identifying measures necessary to control the risks that have been identified. The chemical safety assessment (CSA) and report is discussed in more detail below.

8.3.1 1–10 Tonnes Per Annum

At the 1–10 tonnes per annum band, the minimum hazard data set includes extensive information on physicochemical properties. Toxicity data includes: information on skin and eye irritation potential based on pH or *in vitro* data; if there are no existing skin sensitization data, a local lymph node assay (LLNA) is required; registrants need to submit an *in vitro* mutagenicity test in a bacterial test system and an acute oral or acute inhalation toxicity test needs to be provided. Some ecotoxicological data are also required.

Suppliers must assign appropriate hazard C&L to their substance, based on all available data. Initially this will refer to the criteria laid out in Directive 67/548/EEC, but in the future it will refer to the criteria developed for the GHS, to be implemented within the EU as the Classification, Labelling and Packaging (CLP) Regulations, (see Section 5). It is hoped that there will be a greater degree of harmonization in the hazard C&L assigned by suppliers through collaboration in the preparation of registration dossiers. It is intended that EU regulatory committees and authorities will debate and agree harmonized C&L positions for substances only for carcinogenicity, mutagenicity and reproductive toxicity end points.

Within a registration dossier there is a requirement to provide general descriptive information on manufacture and use covering:

- Use categories for industrial, professional and/or consumer uses

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- A brief description of the manner in which it is being used, for example, in closed systems, or included into a matrix, nondispersive and dispersive uses
- An indication of significant routes of exposure for humans (i.e. oral, dermal and inhalation) and the environment (i.e. water, air, solid waste and soil)
- Patterns of exposure (i.e. will exposure be accidental or infrequent, occasional or continuous/frequent).

The supplier will also identify uses advised against. These are uses that the supplier is aware of, but is not prepared to support due to concerns for safety. The use information that is required will be sufficient to identify uses where there is the greatest potential for exposure, but will not be sufficient for the quantitative exposure assessment that is required at higher tonnages. It is anticipated that control measures will be implemented where there is a potential for exposure and limited information on potential hazards, which might well be the case for low-tonnage substances.

The technical dossier will also include guidance on safe use. This is the information that would typically appear within an SDS. SDSs are intended to provide: information on the hazards of the material being supplied; some generic advice on risk-management measures for handling, transport and storage; action to be taken in the event of a fire or spill and information on any regulations, for example, OELs that may apply to that material. Until now, information provided in SDSs has often been incomplete, risk-management advice has been generic and very basic and SDSs from different suppliers have not always been consistent (see Section 5.3). Under REACH, SDSs will continue to be a vehicle for communicating hazard information and risk-management advice along the supply chain. However, it is hoped that the need to generate core data sets and the collaborative approach encouraged for registration will improve the quality and consistency of the information included in SDS. Furthermore, there is a requirement for all SDSs to include contact information for the person responsible for its content, enabling recipients to seek clarification if required. Where exposure scenarios have been prepared these must be appended to the SDS, forming the e-SDS.

8.3.2 10–100 Tonnes Per Annum

Registrants supplying at 10 tonnes per annum or more are required to provide additional data on toxicological and ecotoxicological hazards. At the 10 tonnes per annum threshold this will include, in most cases, short-term repeated-exposure toxicity data and a screening test for reproductive/developmental toxicity. It is possible for a registrant to argue against the need to conduct repeated-exposure and reproductive toxicity testing at this level of supply, if they can confirm that there will be no human exposure arising from any of their own uses and any downstream uses. This may be the case for substances that are used in a limited number of industrial processes for which there is a high level of control and where there is no release to the environment, such as a catalyst that is consumed entirely in a chemical process.

8.3.3 100 Tonnes Per Annum and Above

Longer-term toxicity data are required once supply reaches the 100 and 1000 tonnes per annum thresholds. However, if such data are not already available, companies must not automatically conduct new experimental animal tests, but must submit testing proposals to ECHA. The testing proposal should take account of the hazardous properties that have already been identified and should focus on obtaining information necessary to clarify uncertainties in the data, rather than simply conducting tests because it is an automatic regulatory requirement. Each testing proposal submitted to ECHA will be evaluated to determine whether the tests being proposed are the most appropriate to obtain the information that is required. In evaluating the testing proposal, ECHA will consider whether sufficient information can be obtained from existing studies or whether the information could be obtained by other means, for example, *in vitro* methods, read-across to similar substances, use of structure–activity relationship information and so on. It will also be possible to use arguments based on limited or tightly controlled exposure to justify nonconduct of long-term repeated-dose and reproductive toxicity testing. In order to justify this waiver, a registrant must demonstrate that there is 'no significant' human exposure. This is done by generating exposure scenarios to allow exposures during use to be quantified, and comparing this to a level of exposure at which risks are expected to be minimal (this process is described in more detail below). For low-hazard materials, there is no need to generate any additional exposure information beyond the general descriptive information outlined above. Registrants may therefore need to weigh up the costs of conducting long-term toxicity tests against the costs of quantifying exposure during use to demonstrate that adequate control is being achieved. In the case of carcinogenicity, new testing should only be proposed for substances with widespread dispersive use, or uses that are likely to lead to long-term human exposure, and where there is evidence for mutagenic activity or effects indicating potential carcinogenic activity have been seen in repeated-exposure studies. By adopting this approach it is hoped that suppliers will be prompted to take a scientific-need-based approach to data generation and that the scientific community will be encouraged to develop alternatives to the traditional animal-based methods to complete toxicity profiles.

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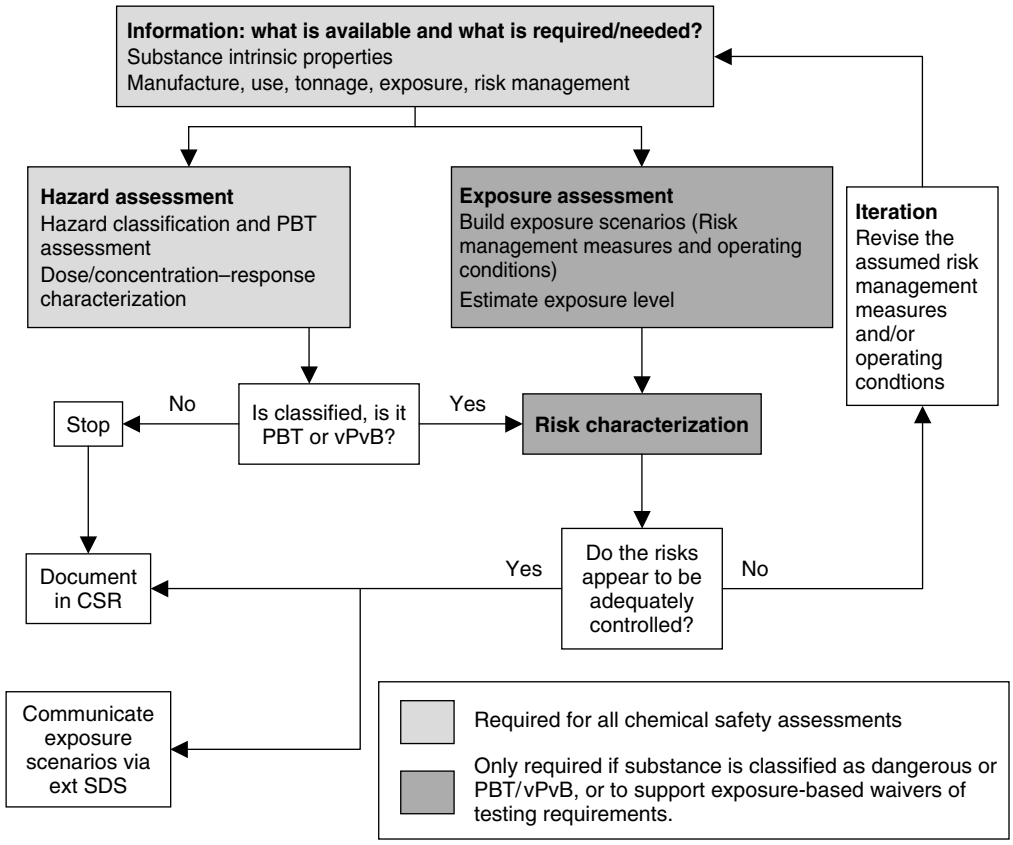

Figure 5 Illustration of the risk assessment process under REACH (based on ECHA, 2008a). PBT: persistent, bioaccumulative and toxic; vPvB: very persistant and very bioaccumulative.

8.4 Chemical Safety Assessment and Report

A CSR is required for all registrations for chemicals supplied in quantities of 10 tonnes per annum or more. It provides a written record that the registrant has undertaken a CSA and documents the outcome of the assessment. The CSA is the process by which a supplier characterizes the risks that their substance poses in any of the situations in which it will arise and determines the measures that are necessary to control those risks. An illustration of the risk-assessment process that will be followed under REACH is given in **Figure 5**.

The CSA is a two-stage process. The first stage involves an assessment of the hazardous properties of the substance and identification of the levels of human and environmental exposures that are deemed to be acceptable. If no hazards are identified there is no need to progress beyond this stage. If a substance is determined to be hazardous, that is, it meets the regulatory criteria for hazard classification for any end point, or is of concern because it is determined to be persistent, bioaccumulative and toxic (PBT) or very persistent and very bioaccumulative (vPvB) in the environment, the supplier moves to the exposure assessment and risk characterization stages. The following text focuses on the human-health risk-assessment process. A similar approach is followed for the environmental risk assessment. Detailed guidance on all aspects of the CSA process has been published on the ECHA website (ECHA, 2008b).

8.4.1 Hazard Assessment and Identification of Acceptable Levels of Exposure

Under REACH, acceptable levels of exposure are determined from data on the hazardous properties of the substance. In the case of human exposure, the acceptable levels are referred to as derived no-effect levels (DNELs). Depending on the hazards and uses for a substance, it may be necessary to derive DNELs for oral, dermal and inhalation exposure, for local and systemic effects arising from short-term/peak exposures and long-term exposures and for workers, consumers and for indirect exposures via the environment. These DNELs will then be compared with the exposures that are assessed to occur during various uses and life-cycle stages. Although the DNEL is intended to act as a benchmark against which the estimated levels of exposure will be judged, it will not have the same regulatory status as, for example, an OEL. For hazardous properties for which it is not possible to identify with confidence a no-effect level, such as is

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often the case with mutagenicity or genotoxic carcinogenicity, REACH has the concept of a derived minimal effect level (DMEL). A summary of the processes by which the DNEL and DMEL are derived is given in Box 1.

Where measured or predicted exposures are found to be below a DNEL or DMEL they will be deemed to be acceptable. If exposures are initially anticipated to be above the DNEL or DMEL then the situation appears unacceptable. However, first the registrant should look to refine their exposure and possibly also hazard assessments, in the latter case particularly if uncertainty in the hazard data has caused the registrant to introduce a number of multiplicative factors in deriving the initial DNEL value. Such a reconsideration might bring about a change in the position. The ultimate goal is to bring the situation to one where control of exposure is adequate, defined by measured or predicted exposures under the control regime advocated being below the relevant DNEL (or DMEL) value.

Box 1 Derivation of a DNEL or DMEL

- The first stage in the derivation of a DNEL is the identification of the critical health effect(s) that are relevant for the exposure pattern that is being assessed. For example, irritation may be the most relevant effect for short-term peak exposures. It is also necessary to identify whether the critical effect is mediated by a mode of action for which a threshold could be anticipated or whether the effect is a mutagenic or carcinogenic effect for which it is not possible to identify with confidence whether or not a threshold dose exists. Having identified the critical effect and mode of action it is then necessary to identify the most appropriate dose descriptor (e.g. a no (lowest) observable adverse effect level (N(L)OAEL), a benchmark dose (BMD), an LC50 or LD50, or a risk estimate obtained from an epidemiological study or some other descriptor). For threshold-based effects, for example, respiratory tract irritation, the dose descriptor is modified using assessment factors to take account of differences in the exposure patterns used for the key study and the anticipated pattern of human exposure, for example, to extrapolate from a six hour experimental exposure to an eight hour working day or to take account of differences in bioavailability if route-to-route extrapolation has been used. Additional factors are used to take account of inter- and intraspecies differences, differences in the duration of exposure (for example to extrapolate from a 90-day study to an anticipated lifetime exposure) and uncertainties in the data to arrive at the DNEL.
- In a situation where the critical effect is considered to arise through a mode of action for which it is not possible to identify with confidence whether or not a threshold dose exists, the aim is to define a level of exposure at which there is anticipated to be a minimal residual risk. This is referred to as the derived minimum effect level (DMEL) and it is determined using a semiquantitative approach. The starting point will normally be a T25 or BMD10 value. As before, it may be necessary to modify the starting point to take account of differences in exposure patterns. Two approaches may be taken to covert the modified dose descriptor to the DMEL. The first method involves linear extrapolation from the modified dose descriptor to a dose that is estimated to give a particular level of risk for the effect (e.g. 10−4, 10−5, 10−6, etc.). The actual level of risk that would be used is a matter for policy-makers to decide. Where this approach is followed, assessment factors to take account of inter- and intraspecies differences are generally not recommended because of the very conservative nature of the approach. An alternative method might involve the use of an assessment factor in the region of 10 000 to take account of inter- and intraspecies differences, uncertainties in understanding of the nature of the carcinogenic process and the fact that there is an identified risk at the starting point (i.e. the BMD10 or T25 values reflect levels of effect rather than NOAELs). Such a large assessment factor would move one to a relatively very low exposure, at which one might expect that the risk of an adverse effect occurring would also be very low.
- If it is not possible to identify a suitable dose descriptor, then a qualitative approach may be used. The qualitative approach involves selection of risk-management measures that are anticipated to provide a stringent level of control for the identified risk. Further information is available in the Chemical Safety Assessment Guidance (ECHA, 2008b)

8.4.2 Exposure Assessment, Risk Characterization and Identification of Appropriate Operating Conditions and Risk-Management Measures

The exposure assessment needs to consider each identified use and all life-cycle stages that are relevant to the identified hazards. In order to characterize exposure, detailed information needs to be obtained for each use

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and life-cycle stage on the factors that determine exposure by all relevant routes (including skin, inhalation and possibly also the oral route).

Determinants of exposure include:

- Substance-specific factors, such as volatility, particle size, chemical reactivity, and so on
- Factors relating to the operating conditions for the process, such as the type of activity, the scale, how frequently it is carried out, the process temperature, and so on
- Factors relating to any risk management measures that may be in place, for example, local exhaust ventilation (LEV) or personal protective equipment (PPE) and the effectiveness of these measures. Product packaging may also be designed to limit exposure during use and this will also need to be considered.
- Factors relating to the surroundings in which the task is performed; for example, is the task performed indoors or outdoors, in small or large spaces, what levels of natural ventilation may be present and so on.

Once assembled, the information will be used to construct exposure scenarios. The format used to describe an exposure scenario is illustrated in **Figure 6**. Exposure scenarios describe a set of process-operating conditions and risk-management measures that may be applied for a particular task and enable exposure under that particular set of circumstances to be quantified. Measured data may be available that are representative for the operating conditions and risk-management measures that are described. However, in many cases the absence of reliable exposure measurements for the situation of interest means that it will be necessary to use models.

The occupational-exposure models that are currently recommended to estimate workplace exposure are deterministic models that rely on conservative assumptions. They provide a 'reasonable worst-case' estimate of exposure. The preferred model is the targeted risk assessment (TRA) tool developed by the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) (ECETOC, 2004) which can be accessed at *www.ecetoc.org*. This is based on the EASE model (see Section 7.1) and provides estimates of inhalation and skin exposure. It does not take account of the use of PPE. If it is not possible to use this model, a German control-banding tool 'EMKG' based on the COSHH Essentials scheme developed in the UK (see Section 6.2) may be used where inhalation exposure is of concern; it can be accessed at *www.baua.de* (BAuA, 2006). These models can be used as the basis for a conservative 'Tier 1' assessment. A more sophisticated model is the updated version of 'Stoffenmanager', (version 3.5, due to be published during 2009). This will provide quantitative estimates for inhalation exposure to vapours, aerosols of low-volatility liquids and dusts, and will also provide quantitative skin-exposure estimates. At present, a complete, validated 'Tier 2' exposure assessment tool is not available, though work is underway to develop appropriate tools. The provision of better models to predict exposure is an early goal for the European Commission and the chemicals industry to facilitate the implementation of REACH.

Exposure models are also available that address exposures arising from the use of consumer products or estimate indirect human exposure via the environment.

The generation of exposure scenarios is likely to be an iterative process requiring good communication between suppliers drafting the exposure scenarios and downstream users, who have a much better understanding of the conditions within their workplace. Where an exposure scenario has been shown to produce an unacceptable level of exposure, the registrant (with input from downstream users) must rework the exposure scenario, including a more stringent set of controls. The goal is to identify the operating conditions and risk management measures that are required to achieve levels of exposure at or below the DNEL or DMEL. Looking at the process by

1	Short title of the exposure scenario
2	Process and activities covered by the exposure scenario
Operational conditions of use	
3	Duration and frequency of use
4.1	Physical form of substance or preparation; surface-volume ratio of articles
4.2	Concentration of substance in preparation of article
4.3	Amount used per time or activity
5	Other relevant operational conditions of use
Risk-management measures	
6.1	Risk-management measures related to human health (workers or consumers)
6.2	Risk-management measures related to the environment
7	Waste-management measures
Information on estimated exposure and downstream user guidance	
8	Exposure estimation and reference to its source (i.e. was the estimation based on measured or modelled data and which modelling tool was used)
9	Guidance to the downstream user to evaluate whether they work within the boundaries set by the exposure scenario

Figure 6 Information contained within an exposure scenario for a typical workplace use (ECHA, 2008b).

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which DNELs are derived and the way the DNEL is used in the risk-characterization process, it becomes clear that DNELs based on limited or uncertain data will be very conservative and it will be necessary to implement stringent control measures in order to achieve these levels of exposure. This will provide a strong impetus for industry to obtain an accurate understanding of the inherent hazards of the materials that they supply and this may, at least in the short term, prompt an increased demand for toxicity testing.

Once a set of operating conditions and risk-management measures that secure adequate control have been identified they are documented in the CSR, which forms part of the registration package. They are also appended to the SDS to form the e-SDS. When a downstream user receives an e-SDS they are obliged to check that the operating conditions and risk-management measures at their workplace comply (i.e. provide an equivalent level of control) with the measures described in the exposure scenario. To help ensure that the correct exposure scenarios are referred to, each exposure scenario is given a short title based on standardized 'use descriptors' for various tasks and processes that have been specifically developed for this purpose. In addition, standardized phrases will be used to identify process-operating conditions and risk-management measures. Further details of these phrases and use descriptors are provided in the CSR guidance. It is the duty of suppliers to ensure that the information in their SDS is provided in a form that can readily be understood by downstream users. In this way, REACH reflects the philosophy of control-banding schemes that users should be given clear and comprehensive control advice. However, downstream users still have the responsibility to ensure that the way they implement the advice within the exposure scenario is appropriate to the specific circumstances of their workplace. It is considered that the exposure scenarios provided under REACH should not obviate the need for employers to conduct the workplace risk assessments required under legislation such as the EU Chemical Agents Directive (98/24/EC) (see Section 6).

8.5 Authorization and Restriction

The remaining two elements of REACH are authorization and restriction. Chemicals that may be subject to *authorization* are referred to as substances of very high concern (SVHC). SVHC include known and probable human carcinogens, mutagens and reproductive toxicants (substances classified as Category 1 or 2 carcinogens, mutagens and reproductive toxicants under the current EU C&L scheme), substances that are PBT in the environment (i.e. those meeting the criteria for PBT or vPvB) and 'substances of equivalent concern'. Substances that are subject to the authorization regime will be listed in Annex XIV of the REACH Regulation. Each substance listed in Annex XIV will be allocated a 'sunset date'. Once this date has passed it will no longer be possible to use that substance unless a specific authorization has been granted for that use. Authorization will only be granted providing industry can demonstrate to the satisfaction of the REACH regulatory authorities that the risks are being adequately controlled or can demonstrate that the societal need for the use of a particular substance in a particular process outweighs the risks. Within the authorization process, industry will need to consider the availability of alternative substances or processes and may need to assess the risks to human health and the environment associated with those alternatives. Even where granted, authorizations will be time-limited and will impose requirements on industry to actively seek less hazardous alternatives.

The *restriction* element of REACH is designed to cater for substances that do not meet the criteria for SVHC, but on analysis are considered to pose unacceptable risks because of the way they are being used. Where these are identified, member states can propose limitations ('restrictions') on the manufacture, supply and/or specific uses for the substance. In this case, the onus is on member states to consider the availability of alternatives and to consider the effects that implementing the restriction will have on society.

9 ISSUES FOR THE FUTURE

The new EU REACH legislation will change things. The regulation is broad in scope, covering a very large number of the chemicals present in the workplace (and elsewhere). It imposes responsibilities on all actors in the supply chain for substances and preparations containing those substances—on those who make or import substances and those who receive and use them in their business. It also has ambitious objectives (see Section 8) and these objectives and some of the features of the regulation reflect political will; the practical achievability of some of the objectives has not yet been tested.

For occupational toxicology, a major challenge is posed by the objective to promote alternative (to animal experimentation) methods for the assessment of the hazards of substances. This is coupled with regular exhortations throughout the REACH text and associated guidance to minimize the extent of additional experimental-animal testing. In the field of toxicology, in 2008, we feel that it is fair to say that the balance of opinion supports a statement that our ability to predict the toxicological profile of a substance by such alternative means is limited. Hence there is an apparent tension between the desire to have a good understanding

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of the toxicological properties of a substance and the desire to minimize experimental animal testing. It has to be hoped that this tension will motivate all concerned to produce and deploy intelligent and innovative ways of making toxicological assessments. Optimistically, the next decade might see satisfactory new approaches to gaining sufficient understanding of the toxicological properties of chemicals, such that appropriate exposure control can be applied in the workplace and beyond, without recourse to extensive new animal experimentation. Pessimistically, satisfying solutions might be difficult to find.

Several features of REACH are designed to contribute to the overall objective to improve the protection of human health and the environment from the risks that can be posed by chemicals. It will be interesting to see how the following aspects work out.

Firstly, will the registration data requirements and industry collaboration mechanisms produce more complete, accurate and consistent (between different suppliers) classification and labelling of substances and preparations (mixtures) containing them? It is hoped that this will be the case, such that labels and accompanying safety data sheets contain better quality warnings about the hazardous properties of materials.

Then, alongside hazard information, REACH intends for safety data sheets to be the means by which appropriate risk-management information is conveyed from supplier to user. It will be a huge challenge to chemical manufacturers and importers to understand all of the downstream uses of their chemicals and the associated conditions of use, in order to develop and supply appropriate risk-management advice. It remains to be seen whether or not business knowledge and the occupational hygiene community can meet this challenge.

It is also anticipated that some chemicals and/or particular uses of chemicals will disappear from the scene in the EU. The extent to which this happens and the reasons for such disappearances are issues for the future. In its design, REACH has the intention of exposing to authorization decisions the uses of chemicals deemed to be 'SVHC' and the promotion of substitution of such substances with alternatives possessing milder toxicological and/or ecotoxicological hazards. This is one way in which substances and their uses might change. However, it is also possible that commercial decisions by potential registrants of substances mean that a company decides to no longer manufacture or import into the EU that substance, for purely economic reasons. What effect such decisions might have on the pattern of chemical availability and use in EU workplaces and beyond is as yet unknown.

A final point concerns the traditional roles of chemical suppliers and chemical users and the toxicological and occupational hygiene thinking and tools that each has conventionally deployed. REACH pushes suppliers to address not only hazard, but also downstream exposure, risk and risk management. To a very large extent, it is expected that under REACH chemical users should follow the instructions given to them by their supplier. This raises important issues about the future role of user-side risk assessment, and about the future of OELs and risk-management tools and guidance generated by regulatory authorities and governmental systems.

REACH is not 'big bang' legislation. Rather, the results of duty holders delivering their responsibilities will accumulate gradually over the next decade (2008–2018). It will be very interesting and very important for occupational toxicology and occupational hygiene disciplines, particularly in the EU, to monitor how things progress.

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Forensic Toxicology

Erkki Vuori and Ilkka Ojanperä

CONTENTS

1 INTRODUCTION

An extensive definition of forensic toxicology is given by the American Board of Forensic Toxicology as 'the study and practice of the application of toxicology to the purposes of law'. The definition encompasses the scope of the discipline, which involves not only science, research and theory, but also their practical application in the service of law.

By definition, forensic toxicology is always tied to the legal system of a given country. Of course, analytical methods and principles coincide internationally, and pharmacological principles apply to all human beings universally, but the legal and social contexts vary. Thus, the interpretation of the significance and the consequences of a given toxicological finding can also vary.

It is possible to divide forensic toxicology in several ways. Nevertheless, the three established specialties are postmortem toxicology serving cause-of-death investigations; clinical forensic toxicology covering drugs and driving, other 'under the influence' issues and drug-facilitated crime; and urine tests for drugs of abuse in workplaces, prisons, armed forces and so on. Additional application areas can be found within occupational, veterinary and environmental toxicology and in the investigation of terrorism. Clinical toxicology and therapeutic drug monitoring are also connected with forensic toxicology as they share similar target substances and methodology.

Forensic toxicology serves the individual, who may be a victim, an offender, a relative or a beneficiary, by providing information about accidental poisoning, malpractice, suicide and criminal offences. Revealing homicide by poisoning is the ultimate dimension of the discipline. For society as a whole, forensic toxicology provides evidence for the administration of justice, and evidence-based information for drug enforcement and other administrative purposes. Forensic

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toxicology combines the scientific principles and methods of analytical chemistry, pharmacology and epidemiology. Successful interpretation of forensic toxicological results necessitates detailed information about the case in question and a thorough understanding of the methods used to produce the results.

2 HISTORY

Poisonings, especially murder by poisoning, have troubled humanity throughout history. The toxicity of some plants and minerals was known in ancient Mesopotamia, Egypt and India. Hippocrates (460–370 BC) also mentioned poisonings by herbs and food. Plato's account of Socrates' execution by hemlock poisoning (399 BC) is a well-known example. However, it was not until the science of chemistry had developed sufficiently that the testing for toxic substances in humans could be used as evidence in court. M. J. B. Orfila (1787–1853) is often cited as 'the Father of Forensic Toxicology'. His principal work, *Traité des poisons ou Toxicologie Générale*, was published in 1814 and included a division of poisons into six categories. Orfila was the first to be able to prove the presence of a toxic substance, arsenic, in samples taken from a human body, namely that of the murdered husband in the famous Marie Lafarge case. He also established the principles for giving expert opinion in court.

Since then, many scientists have made significant contributions to the development of forensic toxicology in their respective localities and countries (Sunshine, 1998). Some chemists and forensic toxicologists of previous generations have won international recognition. Among these are J.-S. Stas (1813–1891) of Belgium and F. Otto, (1809–1870) of Germany, who developed a method for extracting alkaloids from organ samples, and E. M. P. Widmark (1889–1945) of Sweden, a pioneer in the detection and determination of alcohol in body fluids and the pharmacokinetics of ethanol. The Widmark oxidation method for determination of alcohol in capillary blood samples, although not specific to ethanol, was long a routine method for alcohol measurement in drink-driving cases in Scandinavia and Germany. He also introduced the statistical approach of triplicate measurements to confirm the final results, later expanding his method to include postmortem samples. He developed the Widmark equation (**Box 1**), which is still used to estimate the amount of alcohol consumed and the corresponding blood alcohol concentration. R. K. Bonnichsen (1913–1986) of Denmark developed the alcohol dehydrogenase enzyme method and evidential breath analyser for the determination of alcohol. A. S. Curry (1925–2007) of Britain introduced the use of ultraviolet spectroscopy, paper and thin layer chromatography (TLC) and glass column gas chromatography. His handbooks, detailing his findings, were highly influential. I. Sunshine (1916–2006) of the USA established a large international network of forensic toxicologists. He was a founding member of The International Association of Forensic Toxicologists (TIAFT) in 1963, and active in Poison Center programmes. He also made a positive impact through his lectures, workshops, scientific papers and books.

Box 1 Widmark's formula

The uncertainty associated with the use of the formula has been calculated and discussed by Alha (1951) and Gullberg (2007).

$$A = p \times r(C_t + b \times t)$$

A = amount of alcohol consumed, in grams
r = volume of distribution ($0.731\,kg^{-1}$ for males)
p = body weight in kilograms
C_t = blood alcohol concentration at time t
b = elimination rate for blood alcohol (average $0.148\ ‰\,h^{-1}$)
t = time since consumption started, in hours

3 POISONING

In forensic toxicology, a poison is defined as any substance which, when present in the organism in sufficiently high concentrations, can cause illness, be otherwise harmful, or eventually result in death by a chemical mechanism. It can exist in gaseous, liquid or solid form. Chemically, poisons range from simple inorganic compounds, such as metals and metal salts, to complex synthetic organic molecules or natural toxins.

Poisoning can occur intentionally or accidentally. Intentional poisoning can be self-inflicted or murder by poisoning. In both cases, it is possible that the original intention was not to kill but to cause harm or injure. However, dangerous mistakes are likely when administering a potentially toxic substance. Typical accidental poisonings are caused by intoxicants such as alcohol and drugs of abuse, ordinary drugs, household chemicals, pesticides, toxic plants or venoms (**Table 1**). Accidental poisonings can also be due to food, occur in pharmacotherapy or be occupational. Chemical warfare agents, although prohibited, are also potential poisons, and in countries with capital punishment, some poisons are used for the purpose of execution.

The cause of accidental poisoning is typically ignorance, carelessness or negligence. To prevent accidental poisoning in children, it is vital to provide information to parents, and that parents supervise their children closely. The means available for preventing accidental

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Table 1 Common substances constituting the most important finding in fatal poisonings

Industrialized countries
Typically found in accidental deaths
Ethanol
Opioids: heroin, morphine, codeine, propoxyphene, tramadol, fentanyls, oxycodone, buprenorphine
Cocaine
Amphetamines
Neuroleptic drugs: methotrimeprazine, promazine
Benzodiazepines: diazepam, oxazepam, alprazolam
Other analgesic drugs: paracetamol
Typically found in suicides
Antidepressants: amitriptyline, doxepin, dothiepin, mirtazapine, venlafaxine
Hypnotics: zopiclone, zolpidem, temazepam
Cardiac medicines: digoxin, propranolol, metoprolol
Insulin
Carbon monoxide: exhaust gas
Other substances: cyanide, pesticides
Developing countries
Insecticides
Rodenticides
Opiates
Benzodiazepines
Plant and animal poisons
Metals: thallium, arsenic

and suicide poisonings differ. For example, information on the risks of potentially toxic chemicals commonly found in the home may be used by unstable individuals who are considering suicide.

4 SAMPLES

Regardless of whether a sample originates from a living or deceased subject, the principles and procedures in the laboratory are identical and should conform to quality requirements. The basic procedures are the same regardless of whether the laboratory is big or small. In order to keep the quality of results reliable and to make it possible to present and defend the results in court, the *chain of custody* must be kept unbroken. This includes following written instructions for sampling, transporting, storing, analysing and calculation, and for interpreting results. In addition, careful documentation during all the phases of sample handling is vital.

The prerequisite for a forensic toxicological investigation is the justification of sampling. If the subject is a victim, the investigation is for the benefit of the individual, and it is in the subject's own interest to give the sample voluntarily. In such cases, the subject's consent should be documented. If the subject is suspected of committing a crime, sampling may be involuntary, although by law coercive measures depend on the severity of the suspected crime. The legislation in force in the country in question must be followed. Cases sometimes involve minors, and the question of the age at which a child has sovereign rights over his or her own body can be difficult to resolve. In some countries, the views of the child have to be heard if he or she is aged 12 years or older.

As to postmortem toxicology, the choice of samples is far less restricted. In addition to the usual blood and urine samples, samples are routinely taken from the stomach, liver and vitreous humour. There are several other possibilities, including hair, haematoma and bile. Not infrequently the deceased is badly injured, burned or in a state of putrefaction. In such cases, the forensic pathologist collects any sample that is available, such as 'central blood' from cavities and muscle tissue, which endures postmortem changes for a considerable time. The results in such cases must be interpreted with caution, while the condition of the samples must also be considered.

4.1 Blood

What samples are needed for forensic toxicological analysis depends on what question is to be resolved. If the objective is to establish whether a subject has been under the influence of a substance, a blood sample is obligatory. Before sampling, the skin must be cleaned, which is also the case with cadavers, but organic solvents, including alcohols, should be avoided. Although the risk is minimal, the possibility of contamination can affect the integrity of the samples (Pendlington *et al*., 2001). In living persons, blood is collected by venipuncture, usually from a cubital vein, utilizing vacuum tubes containing oxalate or EDTA (ethylenediaminetetraacetic acid) as anticoagulant, and sodium or potassium fluoride as enzyme inhibitor and to inhibit bacterial growth and activity. In a living person, all venous blood is equally acceptable. However, this is not true with blood samples from a cadaver, where the sampling site may affect concentrations. Postmortem changes may take place for a variety of reasons, such as diffusion, liposolubility, the volume of distribution of a given drug, instability and putrefaction. It is known that liver and lung tissues can have high concentrations of drugs, and of course the gastric contents can show very high amounts of substances in suicide cases involving overdoses. It is not surprising, therefore, that the concentrations in central blood can be more than 10 times higher than in corresponding femoral blood samples (Prouty and Anderson, 1990; Pounder and Jones, 1990; Rodda and Drummer, 2006). The biggest differences have been detected with tricyclic antidepressants, chloroquine,

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amphetamine, amiodarone, propoxyphene and digoxin. For this reason it is recommended that blood samples for postmortem toxicological investigations be collected from peripheral veins, for example from the femoral vein, avoiding the siphon effect which can contaminate the sample with central blood. Even if blood is correctly collected from the periphery, some redistribution is still possible, especially in the case of lipid-soluble drugs (Drummer, 2007). In order to avoid postsampling degradation, forensic samples should always be stored refrigerated or frozen (Peters, 2007). If cell-free samples are needed for biochemical investigations, for example determination of insulin and C-peptide, serum separation tubes can be used, but for optimum results the sample must be collected as soon as possible after death (Winston, 2000).

4.2 Urine

Urine samples are preferable if the question to be answered is, 'has the subject used a substance or has he/she become predisposed to a substance?' The concentrations of many drugs are high in urine and the window of detection can be long, especially in the presence of metabolites. Fast, simple immunological screening methods can therefore be used.

Free drugs that are not bound to proteins are excreted to a greater or lesser extent in urine, but only polar or ionized drugs are found in high concentrations. Most drugs are excreted as their metabolites. During biotransformation (metabolism), a drug goes through a process which exposes or adds a functional group to the parent molecule (Phase I). In Phase II the exposed functional group reacts with substances present and forms glucuronide, sulfate or glycine conjugates, which are usually more polar than the parent substance and are excreted in urine. For example, after a single dose of codeine, in Phase I the drug is *O*- and *N*-dealkylated to morphine and norcodeine, respectively. After Phase II reactions, in addition to codeine, morphine and norcodeine, the corresponding 3- and 6-glucuronides of all three substances can be found in urine (Baselt, 2004). Because some functional groups, especially phenolic and alcoholic hydroxyl groups, form conjugates, urine samples are normally treated enzymatically, for example using glucuronidase, before extraction prior to analysis.

4.3 Alternative Samples

Other common samples that can be obtained from a living subject are saliva (oral fluid), hair and gastric contents (vomit or gastric lavage).

4.3.1 Oral Fluid

The fluid present in the oral cavity has several origins. Most is secreted by the major salivary glands, but a portion is secreted by numerous accessory salivary glands and by gingival tissue. Some stems from oronasopharyngeal secretions, or possibly from food (Aps and Martens, 2005). This fluid also contains cells, bacteria and viruses, and can be contagious. Different saliva glands secrete different types of saliva: under normal conditions, submandibular saliva accounts for most of the volume, but the parotid glands when stimulated can secrete up to half of the saliva. The pH of unstimulated saliva is acidic to neutral, but stimulated saliva can be neutral to basic. The change in acidity affects the saliva/plasma ratio of those drugs that have a pK_a value close to seven.

Drugs present in the blood enter saliva by passive diffusion and are in balance with the un-ionized, unbound and thus active fraction of a given drug in blood. This is why oral fluid is the best alternative to a blood sample if 'being under the influence' has to be evaluated (Langman, 2007). However, the distribution of a particular drug between oral fluid and blood is not constant; also, many drugs may be present due to oral deposition, for example by smoking, snorting or from sublingual or buccal dosing. Hence the interpretation of the laboratory results meets numerous considerations (Cone and Huestis, 2007).

Oral fluid has several advantages over blood and urine samples in testing for drugs of abuse. Collection is noninvasive and can be performed observed but without embarrassment right away at the scene of the incident or accident. Samples can be taken without the need for medical personnel. Oral fluid has disadvantages, too. Many common drugs have an anticholinergic effect and can cause hyposalivation and dry mouth; in these cases, collection of the sample can be difficult or impossible. As with blood samples, the period during which drugs are detectable is short and the results indicate recent use only. There are several commercial devices available for collecting and analysing oral fluid samples.

4.3.2 Hair

Hair has been described as 'a recording filament' that records and preserves information about the drugs present in blood during the formation of the hair shaft in the germination centre of the hair follicle. Drugs can be incorporated via diffusion from blood, or transfer can take place via sweat, sebaceous and apocrine gland secretions (Henderson, 1993). Hair grows in cycles, and in the anagen (growing) phase, drugs are incorporated. After this active phase comes the catagen phase and then the telogen phase, when shedding occurs. In humans, each follicle has its own independent cycle of growth, and on average 85% of scalp hairs are in the growing phase

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and 15% in the resting phase at any given time (Harkey, 1993).

Hair samples are collected from the back of the head, that is, the *posterior vertex* area, where hair growth averages about 1 cm month^{-1}. In order to keep the individual hair strands synchronized, and to make segmental analysis possible, it is recommended that a bundle of hairs be tied together with a cotton thread and the sample cut close to the scalp with scissors.

Hair testing has many advantages: it increases the window of drug detection, allows resampling if the results are disputed and can discriminate a single exposure from long-term use (Kintz, 2007). External contamination of hair is always possible, and the analytical procedures used must include washing. Cosmetic treatments, such as bleaching and dyeing, can interfere by removing drugs. Hair is an excellent sample if the extent of the use of a given drug has to be evaluated in history, but has no immediate value when acute fatal poisoning is suspected. However, hair testing is also useful in clarifying a single exposure in the examination of drug-facilitated crimes. In these cases, it is recommended that samples be taken four to five weeks after the incident (Kintz, 2007).

4.3.3 Gastric Content

Analysis of gastric content or vomit shows which drugs have been last ingested, but not necessarily which drug caused the poisoning.

4.3.4 Muscle

The alcohol concentration in muscle has been shown to follow closely that in blood in cases where blood alcohol is higher than 100 mg per100 ml; at lower levels, muscle alcohol seemed to be higher (Garriott, 1991). Studies comparing drug concentrations in skeletal muscles with drug concentrations in blood have, generally speaking, produced similar findings. However, the results are not unanimous. In one study (Christensen *et al*., 1985), in which the results from skeletal muscle and venous blood from an extremity were compared, the concentrations were of the same magnitude for acidic drugs, while, with a few exceptions, good correspondence was also found for basic and amphoteric drugs. On the other hand, sometimes the reference blood is not always collected from the periphery (Garriott, 1991), which makes it difficult to assess the value of muscle as a sample. Variations have also been shown also to occur between different sampling sites of the same muscle (Christensen *et al*., 1985; Williams and Pounder, 1997). One interfering factor might be the length of survival time between dosing and death.

5 ANALYTICAL PRINCIPLES

It is convenient to divide forensic toxicological analysis according to the scope of the investigation into *targeted analysis*, including testing for drugs of abuse in various instances, and *comprehensive analysis*, which comprises postmortem toxicology and many facets of clinical forensic toxicology. Drug-testing laboratories manage with more modest equipment such as immunoassay and possibly a confirmatory technique, while expert laboratories performing comprehensive analysis and confirmation require state-of-the-art analytical instrumentation (Penders and Verstraete, 2006).

Drug testing typically involves an initial analysis (screening), and in positive cases a confirmatory analysis, of several of the following abused substances in urine: amphetamines, barbiturates, benzodiazepines, buprenorphine, cannabinoids, cocaine, ecstasy, fentanyl, LSD, methadone, methaqualone, opiates, oxycodone, phencyclidine and propoxyphene. The initial analysis is usually performed by urine drug immunoassay. Several types of immunoassay techniques are available for the substances mentioned above and for many additional drugs, such as β agonists, tramadol and tricyclic antidepressants (Verstraete and Heyden, 2005). Administrative cut-off values are used to distinguish between positive and negative results. Immunoassay seldom differentiates between individual drugs within a group, and members of the group often possess different responses to the assay in spite of the nominal cut-off limit. Homogeneous immunoassays are easy to use, while heterogeneous immunoassays offer a larger selection of tests. In positive cases, confirmation of findings in the same urine sample is usually carried out by gas chromatography-mass spectrometry (GC-MS). The procedure involves extraction and derivatization of the compounds and GC-MS analysis in the selected ion-monitoring mode, using deuterated internal standards. The cut-off limits are usually lower than those used in screening.

Comprehensive toxicological analysis is required in many areas of clinical forensic toxicology, such as in cases of drugs and driving, drug-facilitated crime and violent crime, child welfare and doping in sports. Postmortem toxicology necessitates the broadest scope of analysis, as the investigation should cover every exogenous substance potentially contributing to death. Terms like systematic toxicological analysis (STA) and general unknown screening (GUS) have been given to the organized approach of using one or several methods in search of drugs and poisons (de Zeeuw, 1998; Hartstra *et al*., 2000). A major forensic toxicology laboratory might have to have around 500 reference standards to perform its work properly. Maintaining an up-to-date reference standard collection is one of the biggest challenges facing such laboratories. **Table 2** shows the groups of drugs and poisons relevant in forensic toxicological investigations,

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Table 2 Drugs and poisons relevant in forensic toxicological investigations and the analytical techniques commonly used for their detection

Substance	Analytical technique	Approximate number of compounds
Alcohols	Head space GC	5
Volatile substances	Head space GC, head space GC-MS	50
Glycols	GC, GC-MS	5
Acidic and neutral drugs screening	TLC, GC, GC-MS, LC, LC-MS	50
Basic drugs screen	TLC, GC, GC-MS, LC, LC-MS	350
Drugs of abuse screen	Immunoassay	20
Drugs of abuse confirmation	GC-MS, LC-MS	20
Drugs related to chemical submission	GC-MS, LC-MS	50
Digoxin	Immunoassay	1
Insulin	Immunoassay	1
Salicylate	Spectrophotometry	1
Carbon monoxide	CO-oximetry, spectrophotometry	1
Cyanide	Spectrophotometry, GC, GC-MS	1
Pesticides	GC, GC-MS, LC-MS	50
Anticholinesterases	Cholinesterase activity assay	30
Metals	AAS, ICP-MS	10

GC—gas chromatography; MS—mass spectrometry; TLC—thin-layer chromatography; LC—liquid chromatography; AAS—atomic absorption spectrophotometry; ICP—inductively coupled plasma.

and the instrumental techniques commonly used for analysis.

A urine specimen is the first choice for comprehensive qualitative drug screening, as most drugs and metabolites appear in urine at higher concentrations than in blood, and as urine also possesses a longer time-window of detection (Verstraete, 2004). **Table 3** gives suggested detection times for a range of drugs of abuse in urine. Drug concentration in venous blood (plasma, serum) reflects the current level of drug action, and consequently large compilations of therapeutic, toxic and fatal concentrations in blood are available to aid interpretation (Musshoff *et al*., 2004). A good practice in forensic toxicology is to perform the analysis on two different specimens, for example urine and blood, using two independent analytical techniques. A limit of detection (LOD) and a limit of quantification (LOQ) should always be established for qualitative and quantitative analysis, respectively, and an administrative cut-off value can also be applied. Full bioanalytical method validation involves a range of additional measures, including accuracy, precision, range of measurement and uncertainty of measurement.

In recent years, much attention has been given to the development of comprehensive drug screening methods based on GC-MS and liquid chromatography-mass spectrometry (LC-MS). Large spectral libraries of organic substances are available for GC-MS, making this technique most suitable for the identification of unknown substances (Maurer, 2006). Many novel drugs, however, are polar or thermolabile in nature and therefore more amenable to LC analysis. Thus the most recent advances in GUS are related to LC-MS techniques that employ triple quadrupole or ion trap mass analysers (Mueller

Table 3 Time windows of detection for drugs of abuse in urine. The time interval depends on the cut-off level selected and also individually according to factors such as the size of the dose and the duration of the abuse[a] (Baselt, 2004; Drummer, 2001; Verstraete, 2004)

Drug	Days
Amphetamine[b]	2–9
Methamphetamine[b]	2–6
MDMA	1–2
Benzodiazepines	1–14
Cannabis[c]	
Heavy user	14–30
Daily user	5–14
Occasional user	1–5
Cocaine[c]	1–4
GHB	1
Heroin/morphine	1–2
Buprenorphine	1–6
Codeine	1–2
Methadone	2–7
Phencyclidine	5–10
LSD	0–2

[a]Reproduced from SAMHSA, 2008.
[b]Depends on pH of urine sample.
[c]Detected as metabolite.

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et al., 2005). Liquid chromatography coupled with time-of-flight mass spectrometry (LC-TOFMS), which is based on accurate mass measurement, allows tentative formula-based substance identification without reference standards. While retention times are always applied when available, target libraries containing as many as 1000 compounds can be regularly updated with fresh numerical data on new drugs, designer drugs and metabolites from the literature or other sources (Ojanperä *et al*., 2006). Quantitative analysis methods rely on the same GC- and LC-based technology, but being strictly dependent on reference standards and instrument calibration, they do not allow simultaneous quantification of more than 50–100 substances (Rasanen *et al*., 2003).

Advances in drug screening techniques have resulted in an increasing number of drugs and poisons routinely found in forensic toxicology cases. **Table 4** presents a typical postmortem case of alcohol and drug poisoning with multiple findings in urine by LC-TOFMS and subsequent quantification of the relevant findings in blood. Interpreting such findings requires both a pathologist and a toxicologist, the former being acquainted with the case history and autopsy results, and the latter being able to evaluate the drug findings against epidemiological and toxicological information.

Table 4 Example of the comprehensiveness of an initial urine analysis with a novel liquid chromatography-time-of-flight mass spectrometric method. A total of 18 drugs and metabolites, representing a vast chemical variety including basic, acidic and neutral drugs, were detected in a single run (DNo. 070764). Confirmation and quantitative analyses in blood were performed using various listed methods

Initial analysis in urine by LC-TOFMS	Blood analysis ($mg\,l^{-1}$)
Tramadol	3.8 (GC)
Nortramadol	ND (GC)
O-Desmethyltramadol	ND (GC)
Venlafaxine	1.7 (GC)
Metoclopramide	0.2 (GC)
Amitriptyline	0.2 (GC)
Carbamazepine	6.1 (GC)
Oxazepam	0.5 (GC)
Warfarin	Positive (GC-MS)
Metoprolol	0.21 (LC-MS/MS)
Oxycodone	0.16 (GC-MS)
Noroxycodone	ND (GC-MS)
Gabapentin	27 (GC-MS)
Nicotine	ND (GC)
Cotinine	ND (GC)
Hydroxycotinine	ND (GC)
Ketoprofen	ND (GC-MS)
Ranitidine	NA

ND—not detected; NA—not analysed; TOFMS—time-of-flight mass spectrometry;
MS/MS—tandem mass spectrometry.
Alcohol concentrations in blood and urine were 2.4 and 2.8‰, respectively.
Ketoprofen, nicotine, nicotine metabolites and tramadol metabolites were not detected in blood. Ranitidine was not quantified, but the substance was included in the medical history of the deceased.
The forensic pathologist classified the cause of death of the 48-year-old man as combined poisoning with alcohol and drugs, but the mode of death remained obscure.

6 INVESTIGATIONS ON LIVING PERSONS

The services of forensic toxicology are increasingly needed in today's society. This is true because, in addition to the traditional tasks (such as blood alcohol analysis in cases of drunk drivers), new duties such as workplace testing have become more frequent during the last few decades. Expertise in clinical forensic toxicology is needed for investigating the victims and offenders in cases of assault, in obstetric wards if a mother is suspected of drug abuse or a newborn baby shows withdrawal symptoms, in other child welfare cases, and in cases of drug-facilitated crimes. The contexts are often delicate and emotional, and the appropriate legislation concerning sampling and civil rights must be strictly followed.

6.1 Drunk and Drugged Drivers

Alcohol, common drugs and drugs of abuse can cause impairment of driving skills and predispose the driver to accidents. Deaths in road traffic are a serious public health problem all over the world, and driving under the influence of drink or drugs is usually penalized by law. The legally permitted limit for blood alcohol concentration varies between different countries from zero (Hungary) to 80 mg per 100 ml (USA and Canada). In most European countries and in Australia, the limit is 50 mg per 100 ml. In many countries, being under the influence of alcohol is assessed after initial breath testing with simple roadside devices or impairment tests, using evidential breath analysers. Standardized field sobriety tests typically include one-leg stand, walk and turn, and horizontal gaze nystagmus testing. If the impaired driver is not able to blow correctly, or if drugs are suspected, a blood sample is necessary. Blood alcohol concentration is usually measured using head space GC with flame ionization detection. The method is accurate, and international proficiency testing programmes typically prove a low deviation between laboratories.

Many drugs of abuse have deleterious effects on driving. For example, amphetamines and cocaine can

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cause aggressiveness and unpredictable behaviour; benzodiazepines are known to slow reaction times and cause impairment of cognitive and psychomotor functions, even at therapeutic doses (Drummer, 2001). The recognition of an impaired driver is difficult at the roadside, and new methods have therefore been developed to identify those driving under the influence of drugs. Immunoassays for testing oral fluid (saliva) are now available (Drummer *et al*., 2007), and advances in testing will produce new instruments and methods which, in turn, will increase the range of testable substances (Walsh *et al*., 2007; *www.rosita.org*). Of course, before legal penalties can be imposed, an initial positive result must be confirmed by analysis of a blood sample by confirmatory methods, preferably by MS. Some countries, such as Belgium, Germany, Finland, France, Sweden and Switzerland, apply zero tolerance in traffic, and the concentration of any drug of abuse in blood samples must be under the agreed limit. In some other countries, such as in Denmark, the UK and Norway, the simultaneous impairment of driving skills must also be proven. The legislation is exceptionally strict in Iceland, where merely a single positive urine sample will deprive the driver of his or her driving licence.

6.2 Workplace Drug Testing

Workplace drug-testing programmes have spread worldwide following drug-related accidents in transportation and aviation in the USA in the 1980s. Workplaces in transportation, the nuclear industry and aviation in particular have adopted testing programmes. In the USA, federal employees are also routinely tested. The list of tested substances varies from country to country, as do the cut-offs for positive results, but usually the Mandatory Guidelines for Federal Workplace Drug Testing Programmes of SAMHSA (Substance Abuse and Mental Health Services Administration) are followed (Federal Register, 2004; **Table 5**). Substances typically tested for in the USA include amphetamines, cannabis, cocaine, opiates and phencyclidine. In Europe, phencyclidine is not routinely tested for, but benzodiazepines are usually included. In workplace testing, adulteration of a void urine sample is always possible, and the laboratory responsible for the initial or confirmation analysis must check the samples for foul play. Normally the appearance and colour of the urine sample is checked, as well as temperature, pH, specific gravity or creatinine level, and the sample screened for

Table 5 Cut-offs for Mandatory Guidelines for Federal Workplace Drug Testing in urine, and proposed cut-offs for initial testing of alternative specimens by Substance Abuse and Mental Health Services Administration (SAMHSA), US Department of Health and Human Services (HHS) (*http://workplace.samhsa.gov*, Bush, 2008)

Initial drug-test level	Urine ($ng\,ml^{-1}$)	Hair ($pg\,mg^{-1}$)	Oral fluid ($\mu g\,l^{-1}$)	Sweat (ng $patch^{-1}$)
Marijuana metabolites	50	—	—	—
Cannabis	—	1	4	4
Cocaine metabolites	300	—	—	—
Cocaine	—	500	20	25
Opiate metabolites	2000	—	—	—
Opiates	—	200	40	25
Phencyclidine	25	300	10	20
Amphetamines	1000	500	50	25
Confirmatory drug-test level				
Marijuana metabolite[a]	15	0.05	—	—
Cannabis	—	—	2	1
Cocaine metabolite[b]	150	50	8	25
Cocaine	—	500	8	25
Opiates				
Morphine	2000	200	40	25
Codeine	2000	200	40	25
6-Acetylmorphine (6-AM)	10[c]	200	4	25
Phencyclidine	25	300	10	20
Amphetamines				
Amphetamine	500	300	50	25
Methamphetamine	500[d]	300	50[e]	25[e]

[a] Delta-9-tetrahydrocannabinol-9-carboxylic acid.
[b] Benzoylecgonine.
[c] Test for 6-AM when the morphine concentration is greater than or equal to $2000\,ng\,ml^{-1}$.
[d] Specimen must also contain amphetamine at a concentration greater than or equal to $200\,ng\,ml^{-1}$.
[e] Specimen must also contain amphetamine at a concentration greater or equal to limit of detection (LOD).

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the presence of known adulteration substances, such as nitrite or glutaraldehyde. Any final positive result must be inspected by a medical review officer (MRO), who will be able to exclude any confounding factors, such as the correct use of justified prescribed drugs or common over-the-counter drugs. Drug-testing programmes reveal many drugs of abuse, but not all. Special methods are needed to detect and determine some amphetamine derivatives, tryptamines, gammahydroxybutyrate, buprenorphine and several synthetic opioids. This is why a negative test result excludes only the substances tested for, not all possible drugs of abuse, and thus testing programmes can actually give a false feeling of safety.

6.3 Drug-Facilitated Crimes

Drug-facilitated sexual assaults often involve the use of alcohol together with short-acting benzodiazepines, hypnotics like zopiclone or zolpidem, or drugs having psychedelic or empathogenic effects (like ecstasy, cannabis or gammahydroxybutyrate). Anticholinergic drugs, like scopolamine, are associated with anterograde amnesia. Potential other substances include drugs that lower blood pressure and cause unconsciousness, like clonidine or tetryzoline, in addition to any drug affecting the central nervous system. This is why the investigation of suspected cases is always a challenge for the laboratory. No single method covers all possible substances, and a variety of analytical methods is therefore needed.

6.4 Doping

From a forensic toxicological point of view, doping means the use of banned substances to enhance performance in sports. Doping is banned for ethical, legal and health reasons. The World Anti-Doping Agency (WADA) issues annually a list of prohibited substances and methods (*www.wada-ama.org*). Some drugs are prohibited in-competition, while others are prohibited out-of-competition or in particular sports. Totally prohibited substances include anabolic agents, hormones and related substances, β_2 agonists, agents with antioestrogenic activity, diuretics and other test-masking agents. In competition, stimulants, narcotics, cannabinoids and glucocorticosteroids are also banned. In particular sports, such as shooting, alcohol and β blockers are also prohibited. The collection of urine samples for doping analysis must be supervised and follow a given protocol; for example, the tested person has the right to choose the vials for taking and storing the samples, and the original sample must be split into A and B subsamples, which are sealed and stored under the same conditions. If an A sample turns out positive, the person tested has the right to be present when the confirmation analysis of the B sample takes place. Doping analysis can be performed only in laboratories having both WADA and ISO/IEC 17025 accreditation.

7 POSTMORTEM FORENSIC TOXICOLOGY

The suspicion of poisoning normally arises from the circumstances surrounding the death. Medicines are missing or present at the scene; bottles, tablets, syringes and needles or other paraphernalia indicating abuse have been found, or the deceased is a known abuser. Sometimes the deceased has left a farewell letter with a description of the manner of intended suicide or has revealed to relatives the intention of committing suicide. Scene investigation and history do not in all cases reveal intoxicants, and routine screening for alcohol and drugs of abuse is recommended in all medicolegal cases (Gruszecki *et al.*, 2007).

An external inspection of the body can reveal stains of drugs around mouth or nose, or injection marks, while the colour or odour of the deceased can also be indicative. At autopsy, tablets or remains of drugs may be found in the gastric contents, or corrosive changes in the alimentary tract may be present. The colour of lividity is reddish in carbon monoxide and cyanide poisoning, and the blood is abnormally flowing. Congestion and oedema of the inner organs is a typical finding in cases of poisoning. However, no finding, except a recognizable odour, is fully pathognomonic; the finding may be attributable to other conditions related to sudden and unexpected death, such as asphyxia or exposure to a cold environment.

According to the classification of causes of death issued by the World Health Organization (WHO), poisoning can be an underlying cause of death (Ic), an immediate cause of death (Ia), or a contributing cause of death (II). The underlying cause of death is always the event that 'initiated the train of morbid events leading directly to death'. Opiates, cocaine, tricyclic antidepressants, neuroleptic drugs, alcohols and carbon monoxide are frequent causes of fatal poisonings in the industrialized countries, while pesticides are often involved in developing countries (**Table 1**).

Revealing a murder by poisoning is of course one of the main assignments of forensic toxicology. Toxicological information can also be decisive in solving other homicides. Drugs or alcohol may have been used to facilitate the offence, or a fire may have been started to eradicate clues or to destroy the corpse. In the latter case, no elevated carboxyhaemoglobin or cyanide will be found in the blood sample,

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which indicates that the victim had not inhaled toxic fumes.

Compared to clinical forensic toxicology, postmortem investigations suffer from several shortcomings. The samples are often far from ideal, and resampling is not usually possible. Neither the anamnesis nor the symptoms of the poisoned patient point suspicion in any particular direction, and the laboratory is left having to search for the unknown substances. On the other hand, the selection of suitable sample sites is larger than with a living person, and very sophisticated autopsy sampling is possible when needed; for example, from known sections of the brain (Stimpfl and Reichel, 2007).

It is worth remembering that the same rules do not apply to the deceased as to the living. For example, an opiate abuser may die suddenly while injecting heroin, and if the urine was originally free of opiates the abuser may have died before the opiates were secreted in the urine in detectable amounts, thus making the urine opiate concentration less than the corresponding cut-off value for the method (**Case 1**). This is why a negative urine screen in postmortem samples does not guarantee a negative blood analysis. If the background information indicates possible poisoning, a blood sample must also be screened.

Case 1 DNo. 990525

A 30-year-old man was found dead in a public toilet. At the scene on the floor, a syringe with a needle was found. The pocket of the deceased contained a small package, which was suspected to contain a single dose of an unknown drug of abuse.

An external inspection revealed two fresh injection marks. At autopsy, a fatty liver and a few minor bruises were noted. Samples of urine, blood from the femoral vein, vitreous humour, the liver, gastric contents, hair and from the injection sites were collected and sent for toxicological analysis.

No alcohol was detected in the blood or urine samples. Drug screening for common drugs was conducted on a liver sample by TLC, and temazepam was detected. The urine was screened for drugs of abuse with an immunological method. Amphetamine was positive, but the sample was negative for cannabinoids, opiates and cocaine. Quantitative analysis with a dual column gas chromatographic method revealed a blood oxazepam concentration of $0.9\,mg\,l^{-1}$ (reference value $0.1–1.4\,mg\,l^{-1}$) and a temazepam concentration of $0.4\,mg\,l^{-1}$ (reference value $0.4–0.9\,mg\,l^{-1}$). The amphetamine finding was confirmed by MS, and the amphetamine concentration in blood was found to be $0.1\,mg\,l^{-1}$ (reference value $0.03–0.6\,mg\,l^{-1}$). The blood sample was also found to contain morphine at a concentration of $0.22\,mg\,l^{-1}$ (reference value $0.08–0.12\,mg\,l^{-1}$). In urine the amphetamine concentration was $1.2\,mg\,l^{-1}$, and the sample was found to contain 6-acetylmorphine (6-AM) $0.12\,mg\,l^{-1}$ and morphine $0.07\,mg\,l^{-1}$. Amphetamine and morphine were detected at both injection sites. The hair sample represented the period of the last two months of life and was found to be positive for amphetamine and negative for cannabinoids and opiates.

Interpretation

On the basis of positive hair and urine analysis, the deceased had a history of amphetamine abuse but was naive in relation to opiates. He had obviously injected a mixture of amphetamine and heroin. He had no tolerance to opiates and had expired shortly after the last injection. Opiates can cause respiratory depression and sudden death, the risk being increased by the simultaneous presence of benzodiazepines. The combined concentration of morphine and 6-AM was less than the cut-off value for the screening method, which is $0.3\,mg\,l^{-1}$, and thus the opiates were not seen in urine with the method used for the initial analysis.

The forensic pathologist excluded other causes of death and classified the cause of death to be poisoning with heroin plus benzodiazepines, and the manner of death to be accidental.

With the standard head space GC method, it is possible to detect and quantitatively determine ethanol, isopropyl alcohol, methanol and acetone in a single run (**Figure 1**), (**Case 2**).Because of the danger of postmortem production of alcohol by microbiological activity (Zumwalt *et al*., 1982; Vuori *et al*., 1983), it is recommended to include alcohol analysis of samples other than blood (Kugelberg and Jones, 2007). Urine and vitreous humour are good alternative samples, and the results can support the blood alcohol result. The quantitative relationship between urine alcohol and blood alcohol can also be used to judge the phase of alcohol absorption at the time of death. A blood alcohol level higher than that of urine suggests incomplete absorption and recent drinking as well as the probable presence of nonabsorbed alcohol in the gastric contents. In the opposite case, the absorption and distribution of alcohol is supposed to be complete. If the deceased's body is putrefied, analysis of alcohol in the vitreous humour can lend support to the blood alcohol result. Vitreous humour alcohol correlates more closely with blood alcohol than does urine alcohol. However, in individual cases this may not be the case. If the blood sample is not available or the deceased is badly putrefied, as exhumed bodies usually are, alcohol can also be determined from a muscle tissue sample.

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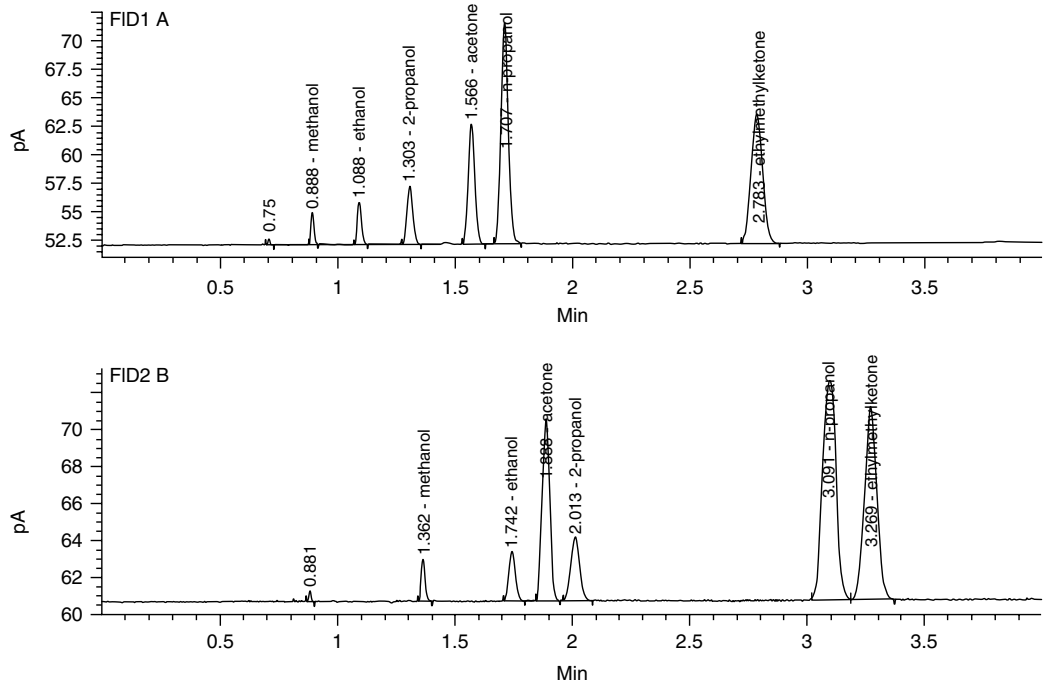

Figure 1 The detection of alcohols and some volatiles in a single run utilizing head space chromatography with flame ionization detectors and a dual column approach. Analysis of a standard mixture; the concentration of each analyte is 0.2‰, isothermic 40°C. Column A Restek Rtx-BAC1 30 m × 530 μm × 3 μm. Column B Restek Rtx-BAC2 30 m × 530 μm × 2 μm.

Volatile organic compounds (VOCs) other than ethanol are rare but important findings in postmortem toxicology (**Case 3**).Whole blood is the specimen of choice: VOC detection is as sensitive as with lung tissue and levels can be quantified. Volatile compounds usually require a dedicated head space GC or GC-MS analysis method. The terms *glue sniffing* and *solvent abuse* have now been replaced by *volatile substance abuse* (VSA). Most VSA deaths are due to gaseous fuels and aerosol propellants, especially butane. Cigarette lighter refill is a typical individual source of butane. The victims of accidental VSA death are predominantly young males. Analysis of VOCs also reveals other fuels, solvents from glues, aerosol propellants and fire extinguishers, and inhalation anaesthetics (Flanagan and Ives, 1994).

8 BIOCHEMICAL ANALYSIS

Many of the clinical chemistry investigations routinely used in clinics to diagnose disease disorders are not available postmortem. This is because postmortem blood is inevitably haemolysed and clotted, and it is impossible to recover serum or plasma. Haemolysis interferes with and precludes most investigations of blood. Hence alternative samples, such as vitreous humour and cerebrospinal fluid, which in most cases are free from contamination with blood and hence from haemolysis, have been used instead of blood. If a blood sample is to be used, the sample should be drawn very soon after death. This is often impossible, however, as the deceased may not be found for several days, weeks or even months after death. Due to postmortem changes, the opportunity to conduct several of the usual blood investigations will be lost; for example pH, oxygen tension, osmolality, sodium and potassium (Coe, 1993).

8.1 Glucose

Obtaining information on blood glucose concentrations postmortem is one of the challenges normally faced by laboratories. Hypoglycaemia can be fatal, develop rapidly and cause sudden death. The external reasons for the condition are injected insulin or excess of a given oral antidiabetic drug. Murder by poisoning with insulin is a reality, unfortunately also in hospitals and nursing institutions. So far there is no method for establishing hypoglycaemia postmortem. In addition, blood levels of insulin are normally measured using an immunological technique, and only certain laboratories can determine insulin by using chemical methods, typically LC-MS. Chemical methods can separate human, porcine and bovine insulin in postmortem blood (Darby *et al*., 2001). At present, however, recombinant human insulin or short-,

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intermediate- and long-acting human insulin analogues are used, and the old methods are no longer useful. New methods have been developed and have been validated for insulin and its degradation products in the analysis of urine and serum samples in cases of suspected doping (Thomas *et al*., 2007; Van Uytfanghe *et al*., 2007). However, these methods require ideal and nonhaemolysed samples. Immunological tests are handicapped by the fact that they merely yield the sum of all of the insulin-like active substances present: human, human analogue and antibody–insulin complexes, which may be present if external insulin has been used in treatment. One possibility is to analyse the insulin content at the alleged injection site, in which case a comparable control-site sample must be included.

Case 2 DNo. 080790

A 48-year-old man had been drinking alcohol for several days. He was brought to hospital in bad condition. The patient was restless and had a breath alcohol level of 3.78‰. He was given haloperidol to treat the restlessness. He died a few hours later.

At autopsy, no obvious cause of death could be established, and the forensic pathologist took samples for forensic toxicological investigations: blood, urine, liver, gastric contents, vitreous humour and cerebrospinal fluid.

Head space gas chromatographic analysis revealed the presence of methanol in the samples: blood 5.5‰, urine 8.4‰ and spinal fluid 7.8‰. No ethanol was detected in any of the samples. Urine was found to contain metoclopramide, the corresponding concentration in blood being $0.09\,mg\,l^{-1}$ (reference value $0.04–0.1\,mg\,l^{-1}$).

Interpretation

In methanol poisoning, symptoms arising from depression of the central nervous system are common, and the patient's restlessness could therefore have been due to methanol poisoning. Simple breath alcohol testing devices do not differentiate between methanol, ethanol and acetone, and in this case, the result of the initial test was misleading, and no other reasons for the condition were considered at hospital. The patient died shortly after administration of haloperidol and thus this drug was not detected. Metoclopramide is used in the treatment of nausea, which is also a frequent symptom of methanol poisoning. In this case, the finding has no additional forensic toxicological value.

The forensic pathologist considered accidental poisoning with methanol to be the cause of death.

Case 3 DNo. 990407

A 69-year-old man was found burned on the ice of a lake. Only a single set of footprints were noted leading to the scene. It was suspected that the man had committed suicide by burning himself with gasoline.

At autopsy, no other obvious cause of death was detected apart from burning, and the forensic pathologist took blood, urine, liver, gastric content and hair samples for forensic toxicological investigations.

No alcohol was detected in the blood or urine samples. Citalopram was detected in the liver sample by TLC. The blood citalopram concentration was found to be $0.2\,mg\,l^{-1}$ (reference value $0.04–0.4\,mg\,l^{-1}$). The blood sample also contained therapeutic, or less than therapeutic, concentrations of temazepam, oxazepam, diazepam and desmethyldiazepam plus paracetamol. The carboxyhaemoglobin content of the blood was 17%. The hair sample contained no amphetamines, cannabinoids or opiates. Purge-and-trap GC combined with Fourier transform infrared detection revealed the presence of methyl *tert*-butyl ether (MTBE) in the blood sample.

Interpretation

Citalopram is prescribed to depressed patients. Suicidal thoughts and suicide attempts are common symptoms of depression. MTBE is added to gasoline as an antiknock agent and to regulate the octane number of fuel products. It is absorbed through skin, and its presence in blood is a typical finding if gasoline has been used as burning fluid. The presence of carboxyhaemoglobin in the blood proves that, before death, the deceased had inhaled the toxic fumes produced by his burning clothes.

The forensic pathologist classified the death as suicide by burning.

On the other hand, it is possible to get information on hyperglycaemia, which is also potentially life threatening. This condition develops more gradually and typically causes ketoacidosis. In these cases, ketone bodies and acetone are usually detected in the blood or vitreous humour of the deceased. Information on the past glucose balance can be gained by determining glycosylated haemoglobin in the blood, HbA_{1c}, which remains stable postmortem and is not affected by haemolysis. The use of the combined value of glucose and lactate in the vitreous humour is a proven method for distinguishing hyperglycaemic from normal conditions postmortem (Sippel and Möttönen, 1982).

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8.2 Insulin and C-peptide

The pancreas secretes proinsulin in response to carbohydrate load. Active insulin is formed when proinsulin loses its C-peptide. Thus, in a living person, there is normally an equimolar relationship between secreted insulin and C-peptide. If someone has been exposed to external insulin, the imbalance in this ratio can be used to verify the external exposure. In postmortems, blood insulin and C-peptide are both unstable (Winston, 2000), and the ratio between them can no longer be used. The vitreous humour is not a suitable sample site since insulin and C-peptide penetrate the blood–vitreous barrier only minimally (Coe, 1993).

8.3 Potassium

The intracellular potassium concentration is higher than that of extracellular fluid. After death, cell membranes no longer maintain this difference in concentration. The serum potassium concentration therefore rises rapidly after death, making it impossible to assess the premortem potassium level (Coe, 1993). However, in the vitreous humour the rise in potassium concentration happens more slowly and is linear with respect to the time interval after death. There are several external and internal factors that need to be considered. The ambient temperature during the period postmortem has an effect, as does the age of the deceased and some metabolic conditions (Coe, 1993). Several mathematical formulas have been developed to estimate the postmortem time interval (Madea *et al*., 1990). Excluding unusual circumstances, the accuracy of the method is typically plus or minus 20 hours in the first 100 hours postmortem (Coe, 1993).

8.4 Tryptase

In an anaphylactic episode, an allergen (e.g. a medicinal substance, food or the venom of a bee or snake) releases enormous amounts of histamine and other active compounds from mast cells. The reaction is usually mediated by an interaction between an allergen-specific antibody (immunoglobulin (IgE)) and the allergen. The release and turnover of histamine are swift, and the measurement of histamine concentrations in anaphylactic deaths postmortem is not informative (Coe, 1993). It is possible to measure specific IgE allergens postmortem. A positive result means that the individual has a given allergy, but not that death is due to an allergic episode. Among many other active chemicals, the mast cells contain abundant tryptase enzyme, which is also released during an allergic episode. The release of tryptase is slower than that of histamine and peaks at 15–30 minutes survival time after exposure to the allergen. This is why, if death due to anaphylactic mechanism happens immediately after exposure, the tryptase concentration is not necessary elevated. The tryptase activity stays most stable if the sample is preserved in a freezer, but elevated levels can still be detected after several days of storage at room temperature (Yunginger *et al*., 1991). Suitable samples for the measurement of tryptase activity must be cell free. They can be obtained using serum separation tubes.

9 TERRORISM

Forensic toxicological services have also been needed for working out the possible consequences of terrorist attacks. In such cases, the use of unusual and unexpected substances has been detected, such as ricin in the Georgi Markov case in London (1978), the nerve agent sarin in Matsumoto (1994) and in the Tokyo subway (1995), a fentanyl derivative in resolving the siege by Chechen terrorists of the Dubrowka theatre in Moscow (2002), and dioxin in an attempt to murder the then presidential candidate Viktor Yushenko in Ukraine (2004).

Also exceptional was the Tylenol tampering incident, when someone replaced the original product with cyanide-containing capsules in Chicago (1982). This kind of crime is difficult to solve, and the offender was never caught.

10 ENVIRONMENTAL TOXICOLOGY

Environmental pollution can cause illnesses and can also result in death by poisoning. A typical example is air-pollution episodes, when higher than expected mortality can statistically be shown to occur. The problem in these cases is that the victims are people already suffering from cardiac and respiratory problems, primarily the elderly. However, it is impossible to prove in individual cases that the deceased is the victim of, say, a smog episode. In other environmental accidents, such as paralytic shellfish poisoning (PSP) and a disaster in a dialysis centre in Caruaru, Brazil (1996), the causative substances (algal neurotoxin in the first case and hepatotoxin in the second) have been determined. Other notorious environmental disasters have been caused by methyl mercury, other heavy metals like lead, cadmium or arsenic, polychlorinated biphenyls, dioxins and methylisocyanate.

11 NEW PERSPECTIVES

Postmortem forensic toxicology produces evidential information that is valuable in determining the cause of

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death of an individual. Together, single cases form a larger set of information, which can be useful for other purposes, for example in revealing trends in illicit drug abuse and in improving drug safety in general. To be able to utilize the information, the target population, the autopsy rate and the comprehensiveness of the data must be known as well as the analytical principles and the quality of the laboratories involved.

If the number of prescriptions or sales of drugs in an area is known, this information can be used to relate fatal poisonings to the consumption of drugs in the population. Thus, it is possible to calculate a fatal toxicity index (FTI) for a single drug or categories of drugs, and to compare the drugs with each other (Cassidy and Henry, 1987). The comparison of FTIs may reveal more dangerous drugs in a given therapeutic group of drugs. This kind of comparison has shown that, in terms of fatal poisonings, the old tricyclic antidepressant are more dangerous by far than the newer drugs, and that selective serotonin reuptake inhibitors (SSRIs) are clearly the safest of all antidepressants (Koski *et al*., 2005).

With the help of a sufficient database, the interaction between alcohol and drugs can also be established. It is known from case reports that the combination of propoxyphene and alcohol is especially fatal. With statistical methods, it has been possible to point out that the hazard is actually different with different drug–alcohol combinations (Koski *et al*., 2003). The greatest risk was identified if alcohol and promazine were present simultaneously. Amitriptyline or doxepin together with alcohol carries a similar risk to that of propoxyphene and alcohol, while the combination of alcohol and citalopram had practically no major effect.

Database studies can be extended to other combinations of drugs. It is known that there are potentially adverse drug combinations, and every effort is made to avoid these combinations in treatment. Such combinations include several serotonin-active drugs simultaneously, or a serotonin-active drug together with a monoamine oxidase inhibitor like moclobemide. These combinations are likely to cause serotonin syndrome, which can be fatal. Interaction databases are available to help avoid dangerous combinations in the treatment of patients. The most risky combinations found postmortem are medicines that together can cause serotonin syndrome, and β blockers together with verapamil or diltiazem (Launiainen *et al*., 2009). However, it is sometimes impossible to determine whether these incompatible combinations were prescribed or due to an accidental dosage by the deceased.

Traditionally, the concentrations of a particular drug found postmortem are compared with reported therapeutic, toxic and fatal concentrations, and a decision is then made on the importance of the finding. Lists have usually been collected from many sources, such as case reports over a period of several decades, and with no precise information on the site of collection, the quality of the samples or the methods of analysis. The best way so far to produce a reliable compilation is to use statistical methods for a single laboratory that has standard procedures and a large database. A Swedish group has given medians and 10–90 percentiles to several drugs found in fatal poisonings involving one drug only; to poisonings with the drug together with other drugs or alcohol; to concentrations of the drug found in fatalities other than poisonings; to concentrations detected in living drunk drivers; and to the results derived from therapeutic drug monitoring (Druid and Holmgren, 1997; Reis *et al*., 2007). In any case, a sound forensic toxicological interpretation cannot be given simply on the basis of the concentration found. Adequate background information (including possible clinical information) together with knowledge and understanding of the laboratory practices and possible analytical pitfalls are always needed (Flanagan and Connally, 2005).

Pharmacogenetics may play a role in individual cases of forensic interest. The polymorphic cytochrome P450 (CYP) enzymes, mainly CYP2D6 and CYP2C19, are involved in the metabolism of several toxicologically important drugs. Thus postmortem genotyping is recommended if statistically abnormal drug : metabolite ratios are found together with background information. As an example, a low doxepin : nordoxepin ratio has been associated with a defective CYP2D6 genotype, contributing to death from doxepin poisoning without an acute overdose (Koski *et al*., 2007).

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Veterinary Toxicology

Frederick W. Oehme and Wilson K. Rumbeiha

CONTENTS

1 INTRODUCTION

Veterinary toxicology is a diverse discipline with many subspecialities dealing with the health and care of animals, the relationship of animals in studying human disease and the concern for domestic and wild animals in the environment. As these themes cannot all be addressed adequately in the space of one chapter, the focus of this tome will be the classic role of veterinary medicine in understanding and managing chemically induced disorders of domestic animals. Animals have

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basic anatomical, physiological and biochemical differences between species and it is, therefore, not surprising that each species, including human beings, often react differently to the same toxicant. Those interspecies differences will be emphasized in this chapter.

Daily veterinary clinical practice is largely devoted to the diagnosis and treatment of poisonings in animals, although in recent years an increasing effort is devoted to zoonotic disease and public-health concerns protecting human health. The range of animals seen by practising veterinarians vary with individual specialists, but can range from small domestic animals, that is, cats and dogs, to food-producing animals (i.e. dairy and beef cattle and swine), to horses, pocket pets, pet birds, zoo animals and even wild game, such as tropical birds, caged reptiles and tropical fish. The small animals react to chemicals more or less in the same way as humans because the species are all monogastrics. The ruminants (i.e. cattle and sheep), however, react differently from the monogastrics. The ruminants have evolved a unique digestive tract structure and microbial flora which play a major role in the fermentation and digestion of the forage ingested. The ruminant's microflora is usually capable of biochemically altering foreign chemicals. As an example, cattle are more susceptible to nitrate poisoning than the horse, while dogs and cats are very resistant. Cattle are very susceptible to nitrate poisoning because the microbes in their digestive tract can convert nitrates to the proximate toxic metabolite, nitrite. Dogs, because of their relatively small gastrointestinal microbial population, are resistant to nitrate poisoning. The horse may still succumb to nitrate poisoning because of the micro-organisms in the caecum in its posterior digestive tract. However, by the time nitrate reaches the caecum, more than 70% will have been absorbed in the proximal digestive tract and little will be available for biotransformation into the toxic nitrite ion. Horses therefore require approximately threefold higher nitrate concentrations in their diet to be poisoned than do cattle.

Physiological differences among species also markedly alter the susceptibility of animals to toxicants. Birds, including pet birds, are more sensitive to toxic vapours and gases than mammals because of their respiratory-system structure and functioning. Canaries have been used in mines to test for the presence of poisonous gases because their unique respiratory system makes them succumb to lower concentrations of toxic gases than humans. Biochemical differences also contribute to differential susceptibility between and within species. Cats are more susceptible to paracetamol (acetaminophen) poisoning than are other domestic animals (Welch *et al*., 1966). The cat's ability to employ glucuronyl transferase activity for conjugating paracetamol (acetaminophen) is much lower than that of other domestic species, and feline haemoglobin is more susceptible to oxidation than that of other animals (Rumbeiha and Oehme, 1992a). Therefore, cats given what would be considered a therapeutic dose for small humans will die of methaemoglobinaemia. Biochemical differences are also found within the same species; for example, the Bedlington terrier is much more susceptible to copper poisoning than other species of dogs. These biochemical differences are of genetic origin and thus specific for animal species and breed.

Adequate comprehension of the variability in toxicity from chemicals in the domesticated species requires an understanding of the anatomy, physiology and biochemistry of the affected animals. The other general factors that affect the toxicity of chemicals must also be considered when dealing with clinical toxicities in domestic animals. These factors include the animal's age, sex, health and nutritional status, concurrent exposures to other chemicals, and the environment in which the animal lives (Osweiler *et al*., 1985). The effects of these and other factors in modifying the outcome of poisoning are of vital importance in determining the clinical outcome, and markedly affect consideration of appropriate management options. Ultimate therapy and prognostic decisions thus rely upon these overlapping insights. There is a vast literature in this area which interested readers may consult for further detailed discussions (Osweiler *et al*., 1985; Hayes, 1991; Gwaltney-Brant, 2007).

2 COMMON TOXICOSES OF DOGS AND CATS

Dogs and cats are commonly poisoned by pesticides, household products such as antifreeze and bleaches, and by human medications to which they have become accessible or with which they have mistakenly been dosed. By far the most commonly reported toxicities in these pets involve insecticides because of overzealous use of these products by owners in controlling fleas and ticks on their pets (Trammel *et al*., 1989).

2.1 Insecticides

The insecticides most commonly involved in poisoning dogs and cats are organophosphates, carbamates, pyrethrins and pyrethroids. The organophosphate and carbamate insecticides have a common mode of action in the inhibition of acetylcholinesterase (Fikes, 1990). Acetylcholinesterase is an enzyme which breaks down acetylcholine, a neurotransmitter in autonomic ganglia and at cholinergic nerve endings. The inhibition of acetylcholinesterase by organophosphate and carbamate compounds causes acetylcholine to accumulate and results in persistent firing of cholinergic nerve fibres.

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Affected animals are overexcited and show increased respiratory rates, muscle tremors and excessive salivation. Treatment of animals poisoned by organophosphate compounds involves administration of atropine and pralidoxime. Cases involving carbamates should only be treated with atropine. The organophosphate and carbamate compounds have a relatively high acute toxicity compared with chlorinated hydrocarbon (CH) insecticides, but have a lower residual activity. As such, organophosphate compounds have largely replaced the organochlorines for insecticide use because of environmental concerns.

The CHs were among the first insecticide compounds to be used, but have fallen into disfavour because of their persistence in the environment (Smith, 1991). Typical examples include dichlorodiphenyltrichloroethane (DDT) and lindane. The toxicity of these compounds in all animal species is characterized by excessive central nervous system (CNS) signs including ataxia and convulsions. Small animals are usually poisoned accidentally by being sprayed or by drinking concentrates of CH intended for spraying on crops. Although most of the organochlorines are banned or their use is highly restricted in developed countries, they are still widely used in developing countries. Therefore, cases of CH insecticide poisoning are still present in countries outside of North America.

Another class of insecticides which is increasingly commonly involved in small animal poisoning involves plant products: pyrethrins and their synthetic congeners the pyrethroids. These products are currently enjoying a resurgence because of their selective insecticidal properties and absence of environmental persistence (Valentine, 1990). These compounds are metabolized in the body mainly in the liver by glucuronidation. The cat is the most sensitive domesticated animal to pyrethrin toxicity because of the low activity of the glucuronide conjugating system in this species. Young cats, less than six weeks of age, are the most sensitive. We see frequent toxicosis in felines from pyrethroid products when they are used enthusiastically on cats for flea control or when products formulated for dogs are applied to cats or cats sleeping with treated dogs lick the pyrethroid product from their companion dog's haircoat. Pyrethroid compounds formulated with the insect repellent diethyltoluamide (DEET) were responsible for numerous deaths in cats and dogs in the early 1980s. Pyrethroids interfere with sodium channels in nerves causing them to fire repetitively (Casida *et al*., 1983). Clinical signs of pyrethroid poisoning in small animals include muscle fasciculations and tremors, ataxia and excitement. There is no antidote for pyrethrin poisoning, but treatment consists of symptomatic treatment, such as decontamination procedures and sedation (Valentine, 1990).

The more recent group of chemicals used to control ticks, fleas and other vectors on household pets are formulated with considerably reduced toxic risk and to work on insects on the pet through novel molecular or reproductive-interfering mechanisms. Fipronil is distributed in the pet's surface skin layers and is selectively neurotoxic to vertebrates and invertebrates, but less so to mammals. Imidacloprid has no systemic absorption and after topical application resides in the fatty layer of the skin. It is effective in insect control by mimicking acetylcholine and interfering with stimuli transmission in insect nervous systems. Sulfluramid is used as an ant and roach control agent, and produces potent uncoupling of insect mitochondrial respiration; the loss of ATP (adenosine triphosphate) causes insect toxicosis and death in 24–48 hours.

2.2 Rodenticides

Rodenticide poisoning is commonly encountered in small animals. They are widely used in homes and around farm houses to control rodents such as rats and mice, which destroy property and invade farm produce. Several classes of rodenticides are currently in use. These include the anticoagulant rodenticides (warfarin and the second generation rodenticides such as brodifacoum), zinc phosphide, strychnine, compound 1080 and arsenic compounds. Small animals are poisoned either by consuming baits directly or through consumption of carrion of animals which have died of rodenticide poisoning. The clinical signs will vary with the compound involved and in the majority of cases occur in dogs because of their indiscriminate eating habits. Strychnine and anticoagulant rodenticides are the most frequently reported offenders. Strychnine poisoning in dogs is a rapidly developing syndrome characterized by tonic-clonic seizures. These signs are a result of strychnine competitively blocking the inhibitory neurons in the brain (Heisser *et al*., 1992). The animals start showing signs within 20 minutes to one hour of ingesting strychnine, and if the animal has ingested a sufficient amount, death from anoxia occurs acutely. Anoxia results from paralysis of respiratory muscles. Treatment of strychnine poisoning is symptomatic and involves general decontamination procedures. Use of sedatives such as phenobarbital (phenobarbitone) and diazepam, maintenance of adequate urine output and respiratory support are supportive measures. The sedatives control the seizures and cause muscles to relax (Maron *et al*., 1971; Boyd and Spyker, 1983).

The anticoagulant rodenticides have been in use for a fairly long time. Because of the long time required to take effect, some strains of rats became genetically resistant to the so-called first-generation anticoagulant rodenticides, such as warfarin. This led to the introduction of second-generation rodenticides, such as brodifacoum. Unlike the first-generation rodenticides which took at least 24–48 hours to take effect, the second-generation

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rodenticides act fairly acutely; clinical signs can be evident within a few hours and have a long residual action. These anticoagulant rodenticides act by inhibiting vitamin K-dependent factors (VII, IX and X), decreasing prothrombin synthesis and by directly damaging blood capillaries (Coon and Wallis, 1972). Clinically, animals poisoned by anticoagulant rodenticides are weak, have swollen joints because of bleeding into the joint cavities, can show bleeding from the nostrils and may pass blood-stained faeces. Treatment of anticoagulant rodenticide poisoning involves blood transfusions if the bleeding is severe or vitamin K_3 injections. Early intervention involves general decontamination procedures to limit further absorption of toxicants, especially in the case of exposure to the potent second-generation rodenticides, followed by vitamin K_1 therapy (Pelfrene, 1991).

The toxicity of zinc phosphide is due to phosphine gas which is produced by acid hydrolysis in the stomach. Animals with partially filled stomachs are more sensitive to zinc phosphide poisoning than those with empty stomachs because of higher gastric acid secretion precipitated by the presence of food. Phosphine gas is then absorbed systemically and exerts its effects in the lungs. Poisoned animals exhibit respiratory difficulties because of the build-up of fluid in the lungs. The cause of death is respiratory failure (Stephenson, 1967). Supportive therapy, including respiratory support, is recommended in cases of zinc phosphide poisoning, but the prognosis is poor as no effective antidote is available.

Compound 1080 (sodium fluoroacetate) is a highly lethal toxicant which acts by blocking the Embden–Meyerhof pathway, thereby depriving cells of energy. *In vivo,* fluoroacetate is metabolized to fluorocitrate, which inhibits mitochondrial aconitase. This blocks ATP production (Buffa and Peters, 1950). Affected animals are initially uneasy, become excitable and will run in one direction and finally fall down in seizures and die of anoxia. There is no antidote to Compound 1080 and, invariably, poisoned animals die.

Cholecalciferol (Quintox) is a rodenticide which has been reported to be widely involved in the poisoning of dogs. The compound alters calcium homoeostasis by promoting calcium absorption from the gut and also by mobilizing calcium from bones. Consequently, poisoned animals have elevated blood calcium levels. The calcium is subsequently deposited in soft tissues, such as lung, intestinal mucosal cells, muscle, liver, heart and kidneys. Mineralization of soft tissue interferes with normal functioning of these organs. Clinically the animals do not show toxicity until 24–48 hours after ingestion of the bait. The affected animals are depressed, and have reduced urine production with low urine specific gravity. Severely poisoned animals have haematemesis, azotaemia and cardiac arrhythmias (Dorman, 1990). Animals with renal impairment are more susceptible to cholecalciferol poisoning than those with normal renal function. Cholecalciferol poisoning requires protracted treatment which may last as long as three weeks in severe intoxications (Livezey *et al.*, 1991). The treatment consists of fluid therapy to assist kidney excretion of calcium, corticosteroids to depress inflammation and calcitonin to enhance calcium resorption into bones.

Several other rodenticides can cause poisoning in small animals but do so less frequently because these rodenticides are used less often. Red squill and thallium have been used as rodenticides for a very long time. Red squill acts as a cardiotoxicant and causes death by cardiac arrest. Red squill also causes convulsions and paralysis. Thallium is a general systemic toxicant. It has a high affinity for sulfhydryl groups throughout the body. Thallium causes cracking at the corners of the lips and also hair loss. *n*-Naphthylthiourea (ANTU) causes death by inducing lung oedema, subsequently leading to anoxia. White phosphorus is a hepatorenal toxicant. Animals poisoned by white phosphorus have severe abdominal pain, hepatomegaly and signs of hepatic insufficiency, such as prolonged bleeding and hypoglycaemia.

In general, cases of rodenticide poisoning in small animals should be regarded as emergencies. General decontamination procedures such as vomiting induced with either hydrogen peroxide or apomorphine, use of activated charcoal to bind the unabsorbed toxicants or enterogastric lavage should be employed to minimize absorption of the poisons.

2.3 Household Chemicals

Antifreeze is one of the household products most commonly involved in small animal poisoning. The active ingredient in antifreeze is ethylene glycol. The characteristic sweet taste of this compound makes it very attractive to small animals. Ethylene glycol is metabolized in the liver by the alcohol dehydrogenase pathway into glycolic acid and oxalate, and the former contributes to the characteristic acidosis of ethylene glycol poisoning. The oxalate binds with calcium in the blood to produce calcium oxalate crystals, which are filtered in the renal tubules causing toxic damage to the tubular epithelium and leaving crystals that produce blockage of the renal tubules (Grauer and Thrall, 1982). Consequently, affected animals have renal failure characterized by anuria and uraemia. The binding of blood calcium to oxalate causes hypocalcaemia which, if severe, can lead to acute death. Ethylene glycol poisoning is traditionally treated by giving ethanol if the animal is presented within 4–8 hours of suspected ingestion and by giving fluids containing sodium bicarbonate to facilitate flushing the calcium oxalate crystals from the kidneys and also to correct the acid–base imbalance. Alcohol dehydrogenase, an enzyme which breaks down ethanol to acetic acid and water, prefers ethanol to ethylene glycol and in the

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presence of both substrates will selectively metabolize ethanol, leaving ethylene glycol to be excreted unchanged in the urine. 4-Methylpyrazole has more recently been confirmed to have excellent antidotal properties against ethylene glycol toxicosis in dogs (Connally *et al*., 1996). It has fewer side effects than ethanol and works by reversibly blocking the conversion of ethylene glycol to glycolic acid and oxalate by alcohol dehydrogenase. It is effective if administered up to 8–12 hours after the consumption of antifreeze, but because of species differences in enzyme specificity it is of considerably lesser benefit to cats poisoned with ethylene glycol, leaving ethanol as still the preferred treatment for ethylene glycol intoxication in cats (Dial *et al*., 1994).

Household products such as sink cleaners, dish-washing detergents and toilet cleaners are not uncommon causes of poisoning in household pets. The majority of the cleaning detergents are corrosive compounds which contain either strong alkalis, acids or phenolic compounds (Coppock *et al*., 1988). These compounds therefore act as contact poisons, causing coagulative necrosis of the tissues which they contact. Following ingestion of these products the dog or cat will vomit, have severe abdominal pain and may have diarrhoea. The vomitus and faeces may be bloody. Animals may also show other signs depending on the specific ingredients of the offending products. For example, products containing phenolic derivatives will cause acidosis and hepatotoxicty. In general, treatment following ingestion of household products is symptomatic and involves the administration of dilutants (milk or water), adsorbents, such as activated charcoal, gastrointestinal protectants such as peptobismol or egg whites, and correction of acidotic systemic disturbances which often accompany the poisoning. Animals so poisoned should be provided abundant glucose and quality protein diets.

2.4 Unusual Household Materials

The average home and household contains many materials and chemicals that could cause injury or poisoning to pets that consume them. Each year veterinarians and toxicologists are asked to deal with dogs and cats that have been exposed to one or more of such products in rooms, garages or backyards. Many induce mild to moderate effects; some are more toxic, and on being informed that illness is due to application or ingestion of the hazardous material, the animal owner usually responds with, 'I didn't know that would cause any problem!'

The materials and products that attract pets are often novel and induce the animals to play with them and in the process swallow all or a portion of them. In some instances owners offer the material to their pets believing they are harmless playtoys or snacks.

Examples of some of these products are glow jewellery, gorilla glue, super glue, homemade play dough, ice melts, fireworks and matches, mothballs, paint balls, xylitol-containing gum, macadamia nuts, grapes and raisins, hops, onions and garlic, commercial 'Greenie' treats and the numerous and varied herbal products. Each is capable of inducing unique toxic risks in pets. The extent and characteristics of each intoxication is usually a diagnostic and then management challenge that is appropriately handled by an experienced veterinarian.

2.5 Garbage Poisoning

Garbage poisoning is a frequently encountered problem in small animals. This condition is also referred to as enterotoxicosis or endotoxaemia, depending on whether poisoning is from bacterial infection or ingestion of bacterial endotoxins. Dogs that are not well fed and/or not closely supervised may seek out and eat garbage. Cats may also be affected, but only rarely because they are discriminate eaters. The bacteria most commonly involved are the coliforms, *Staphylococci*, *Salmonella* and occasionally *Clostridium botulinum*. Enterotoxaemia-affected animals develop a bacteraemia after eating infected carrion, with clinical signs normally appearing at least 24–48 hours after ingestion. The condition is characterized by severe abdominal pain, anorexia, fever, vomiting and infrequent bloody diarrhoea. In endotoxaemia the poisoning results from the bacterial endotoxins which are normally present in bacterial cell walls. The clinical signs are generally indistinguishable from those of enterotoxaemia except that with endotoxaemia there is no bacteraemia (which can be confirmed by blood culture). Although rare in occurrence, botulism is a rapidly developing fatal disease which can result from ingesting bones contaminated with *Clostridium botulinum*. In small animals the disease is characterized by an ascending paralysis. At first there is weakness and incoordination in the muscles of the hind limbs and as the paralysis progresses anteriorly there is dyspnoea and convulsions.

Garbage poisoning is rarely a severe condition in small animals because the animals invariably vomit and reduce the amount of toxicant ingested. However, in severe cases medical attention will be required. If the cat or dog is presented early after ingestion then general decontamination procedures should be instituted. Anti-inflammatory corticosteroids and antibiotics should be given, with further treatment involving supportive therapy.

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2.6 Pet Food and Dietary Hazards

2.6.1 Numerous Sources

Pets are at the mercy of their owners or care-providers for appropriate feeding and other care. Those pets that also have outdoor access may also have opportunity to browse their neighbourhood or to have material thrown into their outdoor pens if exposure to harmful substances is intended. The latter situation usually involves dogs and cats being maliciously exposed to pesticides, toxic baits or known poisons such as strychnine. The effect of these recognized toxins are discussed in separate sections.

Accidental dietary poisonings may also occur with pets gaining access to spoiled food, materials undergoing fungal growth and producing mycotoxins, and in general consuming discarded wastes that may even contain outdated drugs and discarded household materials such as coffee grounds, breads/cakes, pizza and even old chocolate pieces. As noted elsewhere in this chapter, consumption of these products is also hazardous.

Of recent additional concern has been the intentional inclusion of unidentified chemicals in commercial dietary formulations for economic gain without warning of their presence and potential toxic effects.

2.6.2 Industrially Contaminated Pet Foods

These purposely added compounds to pet diets have had confusing and largely unexpected consequences to the consuming pets. Their illegal dietary presence did not warn the veterinary and general public of potential dangers, and when clinical problems developed considerable analytical diagnostic efforts over significant timeframes impacted the ability to provide appropriate management and prevention efforts. Such was the case with the 2007 melamine contamination of dog and cat branded dietary products by the inclusion of contaminated raw ingredients from Asian suppliers.

In the Spring of 2007 observations from a small pet-food feeding study showed an incidence of adverse kidney effects. Followup investigations identified the contamination of an ingredient in the experimental diet as being melamine, cyanuric acid and related chemicals. Clinical effects were associated with the ingestion of dietary products containing melamine and cyanuric acid, producing composite crystals in the renal tubules and dose-related kidney toxicity. Both chemical contaminants were required to induce the kidney disorder.

Clinical lesions and outcomes were clearly related to the dosage of each chemical in the diet, producing an interesting classic dose–response phenomena.

2.6.3 Mixtures and Interactions Between Contaminants

Similar risks may develop by animals ingesting chemicals in mixtures or from the intentional or accidental contamination of animal foods. Owners may offer left-over table scraps to pets, not recognizing that the tasty human dietary additions may produce hazards when mixed and consumed by nondiscriminating pets. Left-over spices, onions, garlic, fruits, sweets and nuts become risky foods when eaten by animals metabolically challenged to metabolize and excrete such unaccustomed materials. The risk is increased when unknown compounds are substituted or used to replace the ingredients usually present, such as occurred in the melamine-cyanuric acid episode.

Unfortunately, testing of mixtures or unusual dietary additions is a complex effort and not on the immediate radar screen of most researchers. Industry is further compromised by not always being aware of what dietary components are most likely to be included in the mix. The result is that toxicologists and cooperating scientists are usually working with developing events as they unfold and playing 'catch-up' to identify and deal with the most prominent concerns. Identification of the issues in question is often the first priority.

2.6.4 Regulatory Actions in the Future

Since public as well as animal safety is frequently affected on the occasion of such dietary contaminant happenings, state and Federal regulatory action may result from these situations. When dietary contamination of purchased supplements is found, mandatory inspection of feed ingredients and manufacturing facilities may be an early action. Assurances from suppliers of expected quality and the meeting of ingredient standards, supported by the necessary analytical assays and attested-to documents become a part of the new manufacturing and formulating paradigm. Such regulatory requirements and oversights have intensified in response to the recent series of commercial pet food and dietary indiscretions.

2.7 Mycotoxins

Some of the mycotoxins of veterinary interest include aflatoxins, deoxynivalenol (DON). Diacetoxyscirpenol (DAS), T-2 zearalenone, ochratoxins and fumonisin B_1 (Cheeke and Shull, 1985; Keller *et al*., 1990). Mycotoxins especially are a common problem in warm climates where high temperatures and relative humidity support fungal growth and favour mycotoxin production. All animals are susceptible and clinical signs will depend on the mycotoxins involved. Rarely is only one mycotoxin

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involved because several species of fungi, for example, *Fusarium*, *Penicillium* and *Aspergillus*, coexist and often produce more than one type of mycotoxin.

The common sources of aflatoxins to pet and food-producing animals include corn and oats. When aflatoxins are ingested in parts per million quantities acute death can occur, with the affected animals showing severe gastrointestinal pain and haemorrhage. Aflatoxins are severe hepatotoxicants: therefore hepatomegaly and jaundice may be observed in severe subacute cases. Quite often, however, aflatoxin poisoning is insidious following a chronic intake of parts per billion concentrations of aflatoxin over a prolonged period of time. Clinical signs include poor weight gain, decreased milk production and poor reproductive performance, including abortions. Virtually every organ function is affected by aflatoxins. The immune system of affected animals is impaired and they succumb to infectious diseases (Pier, 1981).

Toxicity due to T-2 mycotoxins has been reported in North America and some other parts of the world, including Germany, Hungary, France and South Africa. It is less common than aflatoxin toxicity. T-2 acts by interfering with the blood-clotting mechanism. Affected animals have gastrointestinal bleeding and will pass blood-stained faeces. The animals will perform poorly, lose appetite, have low weight gain and productivity and chronic decreased food intake. T-2 is also an immunosuppressant. All animals are susceptible to T-2 mycotoxicosis. Treatment consists of withdrawal of the contaminated feed and supportive care.

Zearalenone is an oestrogenic mycotoxin which often causes toxicity in young animals, prepubertal swine being mostly affected. Consuming diets containing contaminated corn is the usual source. Affected females show swelling of the vulva and excessive straining which may cause vaginal prolapse. In male animals zearalenone will cause decreased libido. There is no effective treatment apart from withdrawing the contaminated feed.

Other mycotoxins, including deoxynivalenol (DON), diacetoxyscirpenol (DAS) and ochratoxin, are not of major economic importance, although they can be toxic and economically disruptive to food-producing animals. DAS causes necrosis and erosion of the oral mucous membranes. Consequently, affected animals may refuse to feed and have impaired growth. DON also induces vomiting and feed refusal in swine. Ochratoxins cause renal problems, including hydronephrosis.

Ergot poisoning is occasionally encountered in livestock fed diets containing grain screenings contaminated with *Claviceps purpurea*. The active ingredients are ergotoxin and ergotamine, which are vasoactive compounds. These compounds cause vasoconstriction of the peripheral vessels, especially those of the extremities, causing necrosis and gangrene of extremities. Abortions and agalactia have been reported. Therapy consists of discontinuation of the source of the toxicant and antibiotics to prevent secondary bacterial infection in necrotic tissues (Cheeke and Shull, 1985).

Fumonisin B_1 is produced by *Fusarium moniliforme*, a worldwide fungus which predominantly grows on corn. Fumonisin B_1 causes pulmonary oedema and respiratory distress in swine and other monogastrics. Deaths have been reported in swine fed contaminated corn screenings (Colvin and Harrison, 1992). This mycotoxin is also responsible for numerous sporadic outbreaks of CNS disease in horses characterized by an encephalomalacia and is epidemiologically associated with high incidences of human oesophageal cancer in cultures that have corn as a dietary staple.

2.8 Heavy Metals

Lead, zinc and arsenic are the heavy metals most frequently involved in small animal poisoning, although zinc intoxication is unique and seen in dogs with increasing frequency.

Lead poisoning is more commonly reported in the dog than the cat, but both are susceptible. The sources of lead poisoning in the dog include ingested lead objects such as lead weights and paint chips in old houses that are being renovated. The clinical signs of lead poisoning in the dog primarily involve the CNS. The dogs often present with abdominal pains, diarrhoea and CNS involvement. Lead poisoning is a chronic disease in dogs, but the overt central nervous signs may appear suddenly. Lead poisoning causes blood dyscrasia characterized by reticulocytosis and occasionally anaemia. Similar clinical signs are elicited in the cat. Treatment consists of giving chelating agents such as calcium disodium EDTA (ethylenediaminetetraacetic acid), dimercaprol (BAL), succimer (DMSA) or D-penicillamine. Identifying and removing the source in the home is important, but often is a challenge.

The playful mouthing and swallowing behaviour of dogs results in their ingesting coins that, in the acidity of the stomach, release zinc. The absorbed zinc causes chronic liver damage and red-blood-cell destruction, presenting as a haemolytic anaemia. The often puzzling clinical illness responds quickly to removal of the dissolving coins from the stomach and appropriate supportive care for several days.

Arsenic is the active ingredient in some insecticides, rodenticides and herbicides. Inorganic arsenic and the aliphatic organic arsenicals are rapidly absorbed from the gut, skin and lungs and are more toxic than cyclic organic arsenicals which are used as feed additives (Furr and Buck, 1986). Trivalent arsenic is the proximate toxicant of the pesticide arsenicals

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and it reacts with the sulfhydryl groups of proteins throughout the body. It is therefore a general poison, inhibiting all sulfhydryl-containing enzymes. The clinical signs of inorganic arsenic poisoning in dogs include severe abdominal pain, bloody diarrhoea, anorexia and hair loss, and so on, as discussed under herbicides. Treatment involves decontamination, chelation therapy with BAL and supportive therapy.

2.9 Herbicides

Herbicides are not widely involved in small animal toxicity despite their frequent use around farms. However, toxicity in dogs arising from consumption of concentrates of herbicides during mixing is occasionally reported.

The triazine herbicides act by inhibiting photosynthesis and are generally safe products. The LD_{50} of these compounds in the rat is at least 1900 mg (kg body weight)$^{-1}$. Therefore, toxicity in dogs can only occur following ingestion of large doses of the concentrated product. In experimental situations, triazine-herbicide-poisoned dogs become either excited or depressed, have motor incoordination and may show clonic-tonic spasms.

Some inorganic arsenic compounds are used as herbicides. Inorganic arsenicals are general protoplasmic poisons and therefore are hazardous to both plant and animal life. Affected dogs have severe abdominal pain, bloody diarrhoea and vomiting, and the vomitus may contain mucous shreds from erosion of the intestinal epithelium.

Paraquat, although restricted in use in most Western countries, is a very toxic herbicide which is readily available in developing tropical locales. Following intake, paraquat is rapidly metabolized in the liver and lungs with secondary oxygen-radical production. It is these secondary radicals which cause injury to tissues, especially the lungs. Poisoned animals die of acute respiratory failure.

Unlike other animals, the dog appears sensitive to chlorphenoxy herbicides such as 2,4-dichlorophenoxyacetic acid (2,4-D). The oral LD_{50} for the dog is 100 mg (kg body weight)$^{-1}$. Ventricular fibrillation is the cause of death in severely poisoned dogs. Ingestion of sublethal doses induce myotonia, stiff extremities, ataxia, paralysis, coma and subnormal temperatures (Stevens and Sumner, 1991).

Chlorate herbicides have been used on roadsides. Chlorates are rapidly metabolized in the liver to the chlorate ion, which induces methaemoglobinaemia in both cats and dogs. Cats, however, because of the greater susceptibility of their haemoglobin molecule to oxidation, are more susceptible to induced chlorate poisoning than dogs.

Organophosphate herbicides, for example, glyphosate and merphos, are weak cholinesterase inhibitors and are of moderate toxicity in dogs and cats. Carbamate herbicides are not inhibitors of acetylcholinesterase and are also moderately toxic to dogs. The LD_{50} of most of the carbaniate herbicides is at least 5000 mg (kg body weight)$^{-1}$.

2.10 Plant and Mushroom Poisonings

Although one would not expect dogs and cats to eat plants (toxic or nontoxic), plant poisoning is surprisingly often reported in these species (Fowler, 1981; ASPCA, 2008). Because of their exploratory nature, puppies and kittens are most often involved. Boredom and change of environment are some of the predisposing factors to plant ingestion in dogs and cats.

Poisonous ornamental plants, for example, *Rhododendron*, and plants used around fences such as cassia and oak are mostly involved. The subject of poisonous plants is a vast one and because the clinical signs are similar in food-producing and small animals, this subject is dealt with extensively under food-producing animal toxicoses (see **Tables 1–8**). In addition, interested readers may consult a good overview of plant poisonings in small companion animals (Fowler, 1981), as well as current online listings of common plant poisonings in pets (ASPCA, 2008).

Occasionally dogs or cats will eat or be fed poisonous mushrooms by uninformed owners. *Amanita muscaria* and *A. pantherima* are acutely toxic and induce signs within 15–30 min of ingestion. These two mushroom species cause nervous signs, which also include salivation, pupillary constriction, muscular spasms, drowsiness or excitement and eventually coma and death in severe intoxications. Ibotenic acid and muscimol are the active toxins.

However, *A. phaloides*, *A. virosa* and *A. verna* induce gastrointestinal signs which are evident 6–12 hours after ingestion. The signs include violent vomiting, diarrhoea, dehydration and muscle cramps. These mushrooms also cause hepatic insufficiency. Phalloidin and α- and β-ainanitine are the principal poisons in this group (Fowler, 1981).

3 COMMON TOXICOSES IN FOOD-PRODUCING ANIMALS

This section will address toxicoses commonly encountered in cattle, sheep, goats and swine. Swine differ from all other animals in this category in that they have a simple stomach (monogastrics), whereas the rest have a compound (four-compartment) stomach.

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Table 1 Toxic plants affecting the gastrointestinal tract of food-producing animals

Scientific name	Common name	Species commonly affected	Toxic parts and principle(s)	Clinical signs
Ricinus communis	Castor oil plant	Cattle, pigs	Seeds, leaves Ricin	Abdominal pain, vomiting, convulsions, dullness
Robinia pseudocacia	Black locust	Cattle, sheep	Bark, foliage, seed Robin, robitin, phasin	Anorexia, lassitude, posterior paralysis, cold extremities, dilated pupils
Phoradendron spp.	Mistletoe	Cattle, sheep	Berries. β-Phenylethylamine, choline, tyramine	Vomiting, diarrhoea, bradycardia, sudden death
Ranunculus spp.	Buttercup	Cattle, goats, swine	Fresh foliage Protoanemonin	Blisters on lips, salivation, diarrhoea, tucked abdomen
Phytolacca dodecandra	Pokeweed	Cattle, sheep, swine	Foliage, unripe berries. Oxalic acid, phytolaccotoxin, phytolaccin	Diarrhoea, dyspnoea, spasms, reduced milk, convulsions, ataxia
Sesbania spp.	Rattlebox	All	Seeds, foliage Sesbanine	Haemorrhagic diarrhoea, severe abdominal pain, coma, death
Agrostemma githago	Corn cockle	Cattle, swine	Seeds. Githagenin	Diarrhoea, arched back
Quercus spp.	Oak	Cattle, sheep, swine	Acorns, buds, young leaves, flowers, seeds, stem Tannic acid, gallic acid	Abdominal pain, constipation or bloody diarrhoea
Euphorbia spp.	Spurge	Cattle, sheep	Whole plant Euphoron, euphorbin, cyanide	Diarrhoea (haemorrhagic or not), blisters of skin and oral mucous membranes, salivation, abdominal pain
Xanthium spp.	Cocklebur	Pigs, cattle, sheep	Seeds, cotyledons Carboxyatractyloside	Anorexia, vomiting, tucked abdomen, depression, severe hypoglycaemia, weakness, convulsions

Most of the toxicants discussed under small animals also affect food-producing animals, but there are some toxicants which are peculiar or predominantly seen only in food-producing animals. Those frequently encountered in ruminants include nonprotein nitrogen toxicoses, copper, lead, arsenic, mycotoxicoses, nitrite poisoning, pasture-plant poisonings and algae poisoning. In swine, salt poisoning, mycotoxicoses, organic arsenic, small pasture- and feedlot-plant poisoning and gases generated in swine-confinement operations are often involved.

3.1 Poisoning by Nonprotein Nitrogen Compounds

Nonprotein nitrogenous sources in food-producing animals include urea, biuret and ammoniated feeds. These compounds are cheap sources of nitrogen, which is required by the animals for protein synthesis. Nonprotein nitrogen poisoning is a common problem and is often seen in animals that are not gradually introduced to diets containing these compounds.

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Table 2 Toxic plants primarily affecting the liver of food-producing animals

Scientific name	Common name	Species commonly affected	Toxic parts and principle(s)	Clinical signs
Senecio spp.	Groundsel	Cattle, sheep, goats	Foliage Several alkaloids, for example, jacobine, jacodine	Dullness, aimless walking, weakness, increased pulse, rapid respiration
Crotolaria sagitallis and *C. spectabilis*	Rattlebox	Cattle and swine mainly, but all species affected	Foliage and seeds Monocrotaline	Loss of appetite, weakness, emaciation incoordination, excitability, nervousness
Amsinkia intermedia	Fiddleneck	Pigs mainly but also sheep and cattle	Seeds Intermidine, lycopsamine, sincamidine	Unthrifty, icteric, haemorrhages of the gastrointestinal tract and subcutaneous tissues
Echium plantagineum	Viper's bugloss	Sheep mainly but also cattle and pigs	All parts are toxic Echiumine, echimidine	As for *Amsinkia*, contact dermatitis
Heliotropium	Heliotrope	Sheep	All parts Heliotrine, lasiocarpine, heleurine	As for *Amsinkia*; secondary photosensitization
Trichodesma europeum	—	All	Foliage Unidentified pyrolizidine alkaloids	As for *Amsinkia*
Lantana camara	—	Cattle, sheep, goats	All parts but especially foliage and berries Lantadine A and B	Severe gastroenteritis, bloody watery faeces, jaundice, secondary photosensitization
Helenium spp.	Sneezeweeds	Sheep and goats mainly but also cattle	All parts but especially leaves and flowers Helenaline, helanine, dugaldine	Severe abdominal pain, bloating, CNS involvement, for example, head pressing
Hymenoxys	Bitterweed	Sheep, cattle, goats	All parts. Hymenoxon	Unthriftiness, inappetance, salivation
Kochia scoporia	Kochia	Cattle	Foliage Unidentified alkaloids + oxalates, nitrates and a thiamine antagonist	Unthriftiness, CNS signs, bleeding disorders, photosensitization
Agave lechuguilla	Agave	Sheep, goats, but also cattle	Foliage Unidentified saponins	Listlessness, primary photosensitization
Trifolium hybridum	Alsike clover	Pigs, sheep, cattle	Foliage. Unidentified saponins	Listlessness
Lotus corniculatus	Birdsfoot trefoil	Cattle	Foliage. Unidentified principles	Listlessness, bloat

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Table 3 Toxic plants primarily causing CNS effects in food-producing animals

Scientific name	Common name	Species commonly affected	Toxic parts and principle(s)	Clinical signs
Dicentra cucullaria	Dutchman's breeches	Cattle	All parts but especially leaves and bulbs. Isoquinaline-type alkaloids, for example, apomorphine	Initially abdominal pain and diarrhoea; ataxia, trembling, respiratory distress, convulsions
Cicuta spp.	Water hemlock	Cattle, sheep, goats, pigs less often	Roots, stem base Cicutoxin, cicutol	Muscular spasms and spasmodic convulsions
Corydalis spp.	Fitweed	Sheep mostly but also cattle	Foliage Unidentified alkaloids	Clonic seizures, twitching of facial muscles
Asclepsia spp.	Milk weed	All	Foliage Cardenolides	Severe depression, ataxia, dilated pupils, laboured respiration
Gelsemium spp.	Carolina jessamine	Cattle, sheep, goats	Foliage, flowers Gelsemine and other strychnine-related alkaloids	Depression, muscle weakness, respiratory failure, convulsive movements preceding death
Calycanthus spp.	Sweet shrub	Cattle	Seeds Calycanthine	Seizures, severe tetanic spasms, muscular fasciculations
Eupatropium rugosum	White snakeroot	Young cattle, sheep	Foliage, passes in milk Tremetol	Depression, listlessness, trembling, laboured breathing
Haplopappus heterophylus	Rayless goldenrod	Cattle, sheep, goats	Foliage Tremetol	Depression, trembling, rare limb weakness
Sophora spp.	Mescal beans	Sheep mostly but also cattle and goats	All parts but seeds especially Cytisine, sophorine and nicotinic alkaloids	Nervousness, exercise-induced violent tremors, stiff gait
Xanthium spp.	Cocklebur	Pigs mostly but also cattle and sheep	Seeds, cotyledons Carboxyatractyloside	Depression, prostration, hunched posture, severe hypoglycaemia, extreme hyper sensitivity, convulsions when recumbent
Ranunculus spp.	Buttercup	Cattle mostly but also sheep, goats, pigs	Fresh foliage Protoanemonin	Irritation of oral tissues, salivation, nervousness, paralysis, depression or excitement and convulsions

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Table 4 Toxic plants affecting the autonomic nervous system of food-producing animals

Scientific name	Common name	Species commonly affected	Toxic parts and principle(s)	Clinical signs
Datura stramonium	Jimsonweed, thorn apple	Pigs mostly; sheep goats, cattle	All parts but especially seeds Atropine, scopolamine, hyoscyamine	Anticholinergic signs, for example, dilated pupils, dry mouth, muscle twitching, incoordination, paralysis
Hyoscyamus niger	Henbane	All	Seed. Hyoscyamine	As for *D. stramonium*
Solanum spp.	Nightshades	Pigs mostly; sheep, goats	Foliage, berries Solanine, dihydrosolanine, chaconine	Apathy, depression, dilated pupils, trembling, incoordination, muscular weakness, paralysis, convulsions
Physalis spp.	Groundcherry	Sheep	Tops and unripe berries. Solanine and atropine-like alkaloids	Diarrhoea, trembling, hyperthermia, weakness, paralysis
Gelsemium spp.	Carolina jessamine	Cattle, sheep, goats	(see 'plants causing CNS effects')	
Lycium spp.	Matrimony vine	Calves and sheep	Foliage Unidentified solanaceous alkaloids	Excitement, convulsion
Lobelia spp.	Wild tobacco	Sheep, cattle, goats	Foliage and green fruits Lobeline and lobelidine	Profuse salivation, dilated pupils, narcosis
Conium maculatum	Poison hemlock	Cattle, pigs	Seeds most toxic; fresh Nicotinic alkaloids, for example, conline	Muscle tremors, ataxia, muscle weakness, frequent urination and defecation, respiratory failure
Lupinus spp.	Lupine	Sheep	Pods (seeds) Nicotinic alkaloids, lupinine, lupanine	Laboured breathing, depression, salivation, ataxia, seizures
Sophora spp.	Mescal beans	(see 'plants causing CNS effects')		

It is an acute fatal condition characterized by bloating, intense abdominal pain, ammonia on the breath, frequent urination and frenzy. Often several animals will be affected at once. In ruminant animals the rumen microflora normally converts urea to ammonia, and the ammonia is rapidly utilized by the liver for protein synthesis. However, in cases of excess ammonia production, the blood ammonia concentration builds up to toxic levels very quickly and induces CNS derangement (Lloyd, 1986). Therefore, in addition to gastrointestinal signs, the animals will show fulminating central nervous effects. Treatment of the condition involves giving a weak acid, such as vinegar, and plenty of cold water orally. The rationale behind giving cold water and acetic acid is to slow the action of urease, the enzyme responsible for breaking down urea to ammonia, which requires high temperature and pH for optimal function. The cold water lowers the temperature and the acetic acid lowers the pH. Infusions of calcium and magnesium solutions should be given to alleviate tetany (Osweiler *et al*., 1985; Lloyd, 1986).

Other sources of nonprotein nitrogen (urea) poisoning in ruminants involve accidental ingestion of nitrogen-based fertilizers, such as ammonium phosphate (Gosselin *et al*., 1976. Occasionally cattle break into drums or bags of fertilizers containing these nitrogen-based compounds. Prognosis is grave in most cases if several animals are affected. In cases where only a few valuable animals are affected, a rumenotomy can be performed.

Although small ruminants have the same anatomical predisposition to suffer from nonprotein nitrogen poisoning, they are rarely involved, probably because

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Table 5 Toxic plants affecting the reproductive system of food-producing animals[a]

Scientific name	Common name	Species commonly affected	Toxic parts and principle(s)	Clinical signs
Veratrum californicum	False hellebore	Sheep	Roots and rhizomes mainly, but all parts are toxic. Jervin, veratrosin, cyclopamine	Cyclopian-type congenital malformation, anophthalmia, cleft palate
Festuca arundinacea	Tall fescue	Sheep, cattle	Foliage. Endophyte alkaloids, for example, perlolidine, perloline	Abortion, still births, agalactia
Pinus ponderosa	Ponderosa	Cattle, sheep	Pine needles Unidentified	Last trimester abortions, stillbirths, premature deliveries, retained placenta
Gutierrezia microcephala	Broomweed	All	Foliage Unidentified saponins	Abortions, premature delivery, swelling of vulva
Cupressus macrocarpa	Monterey cypress	Cattle	Foliage Unidentified	Last trimester abortions, weakness, ataxia, death
Iva augatifolia	Sumpweed	Cattle	Foliage Unidentified	Abortion in last half of gestation

[a]Other plants that cause abortion include those containing nicotinic alkaloids (such as poison hemlock and tobacco), nitrate-accumulating plants (such as locoweed), cyanogenic plants (such as sorghum) and oestrogenic plants (such as clovers and wheat germ).

they are not often fed rations containing these compounds.

3.2 Nitrate/Nitrite Poisoning

Excessive exposure of ruminants to nitrates causes nitrite toxicity, an acute, rapidly fatal disease. The commonest source of nitrates in ruminants is through consumption of forage grown on heavily fertilized fields that have accumulated a lot of nitrate compounds (Ridder and Oehme, 1974). All common animal feeds, such as sorghum, alfalfa and milo and so on, can accumulate excessive amounts of nitrates (Clay *et al*., 1976). Another common source of nitrates is contaminated drinking water. Nitrates are highly water soluble and underground water contamination can occur from heavily fertilized fields (Menzer, 1991). Run-off from fertilized fields is another source of contamination to surface pools and ponds.

Nitrates are reduced to nitrites by rumen microflora. Under normal circumstances the nitrite ion is rapidly utilized for ammonia synthesis, but in cases of excessive acute intake of nitrate and its rapid reduction, the nitrite ion is absorbed into the bloodstream where the nitrite ion reacts with haemoglobin to form methaemoglobin. Methaemoglobin is incapable of oxygen transport and the animal compensates for the anoxia by increasing its respiratory rate. Therefore, affected animals will be hyperventilating, have brownish mucous membranes, and will be weak. Chronic intake of nitrates has been reported to cause reproductive problems, such as abortion, but experimental results are inconclusive (Osweiler *et al*., 1985). Besides reacting with haemoglobin, the nitrite ion also replaces iodine in the thyroid gland, thereby interfering with the function of the thyroid hormone.

Treatment of nitrate/nitrite poisoning involves intravenous infusion of 1% methylene blue at a dose of 1.5 mg (kg body weight)$^{-1}$ and removal of the offending feed.

3.3 Copper/Molybdenum Poisoning

Sheep are more susceptible to copper poisoning than cattle, but cattle are more sensitive to molybdenum poisoning than sheep. The *in vivo* relationship between copper and molybdenum is well understood, with copper excess inducing molybdenum deficiency and copper toxicity and *vice versa.* The most frequent cause of copper poisoning in sheep is uninformed farmers, feeding them rations formulated for cattle. Copper is an essential element for cattle and is usually added to their feeds, but molybdenum is not considered highly essential and is therefore not added. Cattle feeds therefore have high copper and no molybdenum and feeding this ration

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Table 6 Toxic plants affecting the cardiovascular system of food-producing animals[a]

Scientific name	Common name	Species commonly affected	Toxic parts and principle(s)	Clinical signs
Digitalis purpurea	Foxglove	All (see 'plants causing CNS effects')		
Nerium oleander	Common pink oleander	All	Foliage and flowers Several digitoxin-like glycosides	Cardiac arrhythmias, unconsciousness, hypotension, dyspnoea
Convalaria spp.	Lily-of-the-valley	All	All parts, convallarine, convallatoxin, convallamarin	Tachycardia, diarrhoea, anorexia
Apocynum spp.	Dogbane	Cattle, sheep	All parts. Cardiac glycosides, for example, apocynamarin	Cardiac arrhythmias, fever, gastrointestinal pain, fever, gastric upset
Taxus spp.	Yew	All, but swine most sensitive	Foliage, bark, seeds Taxine	Acute heart failure (bradycardia)
Zygadenus spp.	Death camas	Sheep, cattle	All parts. Zygacine	Lowered blood pressure, salivation
Brassica spp.	Kale, rape	Cattle, sheep, goats	Foliage S-Methylcysteine sulfoxide	Haemolytic anaemia, weakness, fast respiration, haemoglobinuria, staggering, collapse
Onion	Onion	All	All parts. *n*-Propyl disulfide	As for *Brassica* spp.

[a]Several nitrate-accumulating plants, for example, *Astragalus*, sudan grass, sorghum, com and pigweed, cause methaemoglobinaemia. Some other plants, including sudan grass and sorghum, affect the cardiovascular system by virtue of their cyanogenic properties (see text).

to sheep upsets the copper/molybdenum ratio *in vivo* for sheep.

Copper toxicity in sheep is an acute condition which develops after a period of chronic copper intake. During the chronic phase copper is stored in the liver until a critical concentration is reached. Following stressful conditions such as transportation or insufficient feed or water intake, a massive hepatic release of copper may be triggered, producing a haemolytic crisis (Osweiler *et al*., 1985). Affected sheep have haemoglobinuria, are weak and death occurs acutely. The massive release of haemoglobin may induce renal failure. The prognosis is poor for animals already showing clinical signs. Chelation therapy using D-penicillamine is recommended for exposed animals not showing clinical signs. The recent availability of injectable ammonium tetrathiomolybdate effectively reduces liver copper levels and improves the prognosis of sheep accumulating low to moderately elevated concentrations of copper (Ensley, 2004).

In cattle molybdenosis is characterized by foamy diarrhoea which may be bloody; affected cattle also have depigmented hair. Molybdenosis is a subacute to chronic condition and occurs when the dietary copper:molybdenum ratio is 2 : 1 or less. The condition has a geographical distribution and occurs in areas deficient in copper or where there is excessive soil molybdenum, in parts of the USA (California, Oregon, Nevada and Florida) (Buck, 1986). Treatment of this condition calls for careful copper supplementation in the animals' feed.

3.4 Lead Poisoning

Despite an awareness of the dangers of lead poisoning in humans and domestic species, it is surprisingly the most frequently encountered heavy metal toxicity in food-producing animals. Lead poisoning is more commonly seen in cattle than in other food-producing animals.

Young animals are mostly affected because of their curiosity and indiscriminate feeding habits. There are several sources of lead for cattle, and discarded batteries and leaded water pipes are the commonest. Often uninformed owners will discard or store old batteries in farm environments and cattle will chew on them. Discarded

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Table 7 Toxic plants affecting renal function of food-producing animals

Scientific name	Common name	Species commonly affected	Toxic parts and principle(s)	Clinical signs
Beta vulgaris	Beet	Sheep and cattle	Sugar beet tops. Oxalates	Muscle tetany, renal failure
Rheum rhaponticum	Rhubarb	All	Foliage Oxalic acid, oxalates	Irritation of oral cavity and digestive tract, renal failure, death in convulsions
Halogeton spp.	Halogeton	All	Foliage and seeds Oxalates	Renal failure, dullness, weakness, slobbering
Sarobatus vermiculatus	Black grease-wood	Sheep, cattle	Foliage. Oxalates	As for *Halogeton*
Rumex spp.	Curlydock	Sheep, cattle	Foliage.Oxalic acid	As for *Halogeton*
Chenopodium spp.	Lambsquarters	All	All parts. Oxalic acid	As for *Halogeton*
Amaranthus spp.	Rough pigweed	Pigs, cattle, sheep	Foliage. Unidentified	Non-specific but related to perirenal oedema and nephrosis
Quercus spp.	Oak	Cattle, sheep	Young leaves, acorn, flowers, stem.Tannic acid, gallic acid	Abdominal pain, constipation, frequent urination, renal failure

Table 8 Toxic plants causing primary photosensitization of food-producing animals

Scientific name	Common name	Species commonly affected	Toxic parts and principle(s)	Clinical signs
Hypericum perforatum	St. John's wort	Cattle, sheep, goats	Foliage Hypericin	Acute: increased respiration and heart rate, mild dermatitis Chronic: photosensitization of unpigmented areas of the skin, photophobia
Agave lechuguilla	Agave	Sheep, goats, cattle	Foliage. Unidentified	As for *H. perforatum*
Fagopyrum esculantum	Buckwheat	All	All parts and seeds. Fagopyrin	As for *H. perforatum*
Cymopterus watsonii	Spring parsley	Sheep, cattle	Foliage. Psolalens	As for *H. perforatum*
Trifolium hybrium[a]	Alsike clover	Pigs, sheep, cattle	Foliage and seeds. Unidentified	As for *H. perforatum*

lead pipes, especially those used around oil wells, are a common source of lead poisoning (Blood and Rodostits, 1989).

Lead interferes with haem synthesis and causes prominent CNS damage. Affected animals are initially anorectic. They may become belligerent and blind at the terminal stages of the disease. Once the CNS signs have appeared the prognosis is poor, but treatment with chelating agents, for example, calcium disodium EDTA and DMPS (2,3-dimercapto-1-propane sulfonate), may be of value.

3.5 Arsenic Toxicities

Arsenic poisoning is second to lead as the most frequently reported heavy metal toxicant in food-producing animals. Arsenic is present in the environment in two forms: inorganic and organic. Inorganic arsenic is often incorporated into pesticides, which are the most common sources of arsenic poisoning in cattle. Inorganic arsenicals are also used as herbicides and cattle are sometimes exposed by eating grass clippings from recently sprayed forage. Inorganic arsenic poisoning is a rapidly developing and fatal

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disease (Radeleff, 1970). Affected animals show severe gastrointestinal abnormalities with minor CNS involvement, and have severe abdominal pain, haemorrhagic diarrhoea and depression. Usually these signs appear 24–36 hours after exposure.

Phenylarsonic arsenicals are less toxic to mammals than the inorganic arsenicals. Phenylarsonic compounds are usually incorporated into swine and poultry feed for disease-control purposes and also to improve weight gain. Examples of these compounds include arsenilic acid, 3-nitroarsenilic acid and 4-nitroarsenilic acid. Organic arsenicals are also available as trivalent and pentavalent compounds, the trivalent forms being more toxic than the pentavalent compounds. These phenylarsonic compounds are peripheral nervous system (PNS) toxicants. They cause demyeination of the peripheral nerve fibres leading to ataxia and paralysis of the hind quarters. The condition occurs frequently in swine kept on feed containing 10 000 ppm arsenic for at least 10 days or 200 ppm arsenic for 30 days or more. Therefore, unlike inorganic arsenic poisoning, which is an acute expression of the toxicosis, poisoning by phenylarsonic compounds has an insidious onset. In addition, organic arsenic is commonly involved in toxicities in swine because of its incorporation into swine feeds, whereas inorganic arsenic poisoning is more commonly seen in cattle.

Treatment of inorganic poisoning is by decontamination procedures and application of BAL chelation. Use of demulcent, to coat the gastrointestinal tract, and antibiotics is also recommended. Treatment of organic arsenic poisoning involves prompt withdrawal of the feed involved. Severely affected pigs should be culled and often cannot be used for food because of remaining arsenic residues.

3.6 Selenium Poisoning

Selenium poisoning is a regional problem in areas where the selenium content in soil is high. Selenium is absorbed and concentrated by selenium-accumulating plants such as *Astragalus*. Cattle, sheep, goats and swine consume these indicator plants; acute selenium poisoning occurs when animals consume plants containing more than 10 000 ppm. This is characterized by sudden death or the animal may have laboured breathing, abnormal movements and posture, frequent urination, diarrhoea and death. Because plants containing high selenium concentration are unpalatable, they are rarely consumed by animals, so that acute selenium poisoning is rare.

However, chronic selenium poisoning is common. Chronic consumption of plants containing as low as 50 ppm selenium can cause poisoning after weeks or months of ingesting the selenium-containing forage. Affected animals are anorectic, have impaired vision, wander, salivate excessively, are emaciated, lame and lose hair, particularly the long mane and tail hairs. Hooves are also affected resulting in overgrown hooves and difficulty walking. Removal of animals from pastures and forage that have high selenium concentrations is necessary to initiate recovery (Muth and Binns, 1964).

3.7 Blue-Green Algae

Blue-green algae poisoning occurs late in summer and early autumn when algae form a scum on pond waters. The toxicity has been prominently reported in North America and the UK. Because of husbandry practices cattle are most frequently involved. Algae of the genus *Anabaena* are commonly involved.

There are two distinct syndromes in blue-green algae poisoning: hepatotoxicity and neurotoxicity. The neurotoxic effect is peracute. Cattle that drink water containing the neurotoxic principle anatoxin-*a* can die within a few minutes and are found close to the waters with the toxin source. On the other hand, the hepatotoxic type causes a less acute poisoning characterized by lethargy and jaundice (Beasley *et al*., 1989), and death, which may occur several days or more after drinking contaminated water. Affected cattle not dying earlier become unproductive and are usually eventually culled.

Treatment involves supportive therapy for animals affected with the liver syndrome. Because of the peracute nature of the blue-green algae-induced neurological syndrome, there is hardly time for treatment, and the prognosis is poor.

3.8 Toxic Gases

Toxic gases are of primary concern in closed animal housing, especially confinement swine operations. In these intensive operations, with buildings designed to save on energy, toxic gases can accumulate causing serious health consequences in cases of ventilation failure or overstocking. These toxic gases are generated from the decomposition of urine and faeces, respiratory excretions and the operation of fuel-burning heaters. The most common and health threatening gases are ammonia, carbon monoxide, methane and hydrogen sulfide. A number of vapours from manure decomposition, such as organic acids, amines, amides, alcohols, carbonyls and sulfides, are also produced. Respirable dust particles may be loaded with endotoxins and are also a major health problem in swine and animal caretakers in confinement operations (Osweiler *et al*., 1985).

Ammonia is highly lipid soluble and will react with the mucous membranes of the eyes and respiratory passages.

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At 100 ppm or greater, ammonia toxicosis will show as excessive tearing, shallow breathing and clear or purulent nasal discharge. The irritation of the respiratory tract epithelium leads to bronchoconstriction and shallow breathing.

Hydrogen sulfide poisoning is responsible for more animal deaths than any other gas and, at 250 ppm and above, hydrogen sulfide causes irritation of the eyes and respiratory tract and pulmonary oedema. Concentrations of hydrogen sulfide above 500 ppm cause marked nervous system stimulation and acute death (O'Donaghue, 1961). To prevent hydrogen sulfide poisoning, manure pits should be agitated when pigs are not in the premises and proper ventilation should always be in place.

Carbon monoxide is produced by the incomplete combustion of hydrocarbon fuels. Poisoning by carbon monoxide is caused by operating improperly vented space heaters or furnaces in poorly ventilated buildings. Carbon monoxide binds to haemoglobin, forming carboxyhaemoglobin, thereby reducing the oxygen-carrying capacity of the blood and subsequently causing hypoxia. Concentrations of carbon monoxide greater than 250 ppm cause hyperventilation and respiratory distress; still births have been reported (Carson and Dominick, 1982).

Nitrogen dioxide is a very poisonous gas which is responsible for causing silo-fillers' disease in humans; the gas is also very toxic to animals. Nitrogen dioxide is produced during the first two weeks after silage has been cut and put in the silo. Highest concentrations of the gas are reached during the first 48 hours after filling the silo. Nitrogen dioxide dissolves in water to form nitric acid, which is very corrosive to the respiratory tract and the lungs. As low as 4–5 ppm nitrogen dioxide can cause respiratory-system disturbances (Osweiler *et al*., 1985).

Exposure to sulphur dioxide at 5 ppm or higher causes irritation and salivation in swine. The gas is soluble in water, forming the more toxic sulfuric acid, which causes eye and nasal irritation, and in severe cases haemorrhage and emphysema of the lungs (Osweiler *et al*., 1985).

The effect of these toxicants singly and in combination is a hypofunctional respiratory system, and affected animals are predisposed to respiratory tract infections. The end result is retarded performances of the affected animals. It is therefore important to ensure that animal housing is adequately ventilated to provide animals with a healthy and productive environment.

3.9 Salt Poisoning

Salt poisoning is frequently encountered in swine operations, but can also occur in feedlot cattle. The causes of this condition are twofold. Most commonly, the animals will be on a ration containing the recommended concentration of sodium chloride, but management failure can favour conditions that will cause salt poisoning to occur. These poor management conditions include the sudden absence of water, for example, by freezing in winter or breakdown of the water supply, and the possibility of accidental addition of excessive amounts of salt to the ration.

Salt poisoning has also been reported in swine operations, even when the management situation was satisfactory, the only change being that the animals were moved into a new housing facility, as occurs after weaning. Apparently, the animals were not used to the different watering facilities in the new buildings; they did not know how to obtain fresh water and went without adequate water while feeding on a normal ration. Clinically, salt poisoning is an acute neurological disorder. Affected pigs will spin on their hind limbs and fall down in convulsions. The pigs will also show a characteristic rhythmic pattern of convulsions which occur every 5–7 minutes. Several pigs are affected at the same time. The condition is corrected by providing adequate, but restricted, amounts of water for several hours, while the animals adjust to the rehydration (Dunn and Leman, 1975).

3.10 Toxic Plants

Plant poisoning is very common in areas where open grazing is practised, such as in the Great Plains of the US, the prairies of South America and the vast spaces of Africa. In North America plant poisoning is widely reported as a seasonal event during periods of drought and forage shortages.

The subject of poisonous plants is a wide-ranging one, which cannot be adequately summarized in the few pages of this chapter. The toxic potential of selected common poisonous plants is summarized in **Tables 1–8**. In these tables the plants are grouped according to the organs most prominently affected and the clinical syndromes observed. It is important to realize, however, that plants rarely affect only one biological organ, and multiorgan effects are often seen in field outbreaks of toxicity.

This presentation is an attempt to summarize the vast amount of literature on this subject. Readers interested in more extensive discussions should consult the abundant relevant literature available in numerous sources, such as the comprehensive reviews by Kingsbury (1964), Cheeke and Shull (1985), Burrows and Tyri (2001) and others.

It is important to remember that the toxicity of any given plant can vary widely depending on the prevailing natural environmental conditions of temperature, moisture, sunlight, stage of growth and nutrition. It is therefore not surprising that a given plant may be toxic under certain conditions, for example, during stressful drought

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conditions, but relatively nontoxic during other seasons and conditions.

4 COMMON TOXICOSES OF HORSES

In comparison with cats, dogs and food-producing animals, horses are less frequently poisoned. The most commonly encountered equine toxicoses involve pesticides, snake bites, arsenic, selenium, monensin, cantharidin and mycotoxins (Oehme, 1987a). Most plants discussed earlier with regard to food-producing animals are also toxic to horses, but are less frequently exposed to horses because of closer owner surveillance. However, horses are very uniquely sensitive to toxicities from monensin and cantharidin.

The pesticides most frequently encountered in equine poisoning are the organophosphate, carbamate and CH insecticides. Both the organophosphates and the carbamates are acetylcholinesterase inhibitors, and offer clinical presentations similar to those seen in food-producing animals. Affected animals salivate profusely, and develop muscle incoordination and ataxia prior to recumbency. The CHs are CNS stimulants and affected horses become excited, hyperalert and in extreme cases go into convulsions. In the vast majority of cases the exposure to pesticides is through topical application (Oehme, 1987b).

Horses are highly susceptible to monensin poisoning in comparison with other domesticated animals. Monensin and other ionophores are commonly incorporated into cattle and poultry feeds to provide improved growth efficiency by improving the movement of calcium and sodium from the gut. Horses are easily poisoned by accidentally consuming cattle or poultry feed containing amounts of monensin, recommended for those species, but detrimental for horses. Affected horses can die suddenly without any premonitory signs. Monensin affects the cardiac and skeletal muscles, and heart failure is the cause of death (Amend *et al*., 1980).

Cantharidin is the toxic agent in blister beetles. Several species of blister beetles are known and only a few contain toxic concentrations of cantharidin. Blister beetles are abundant in July–September at the time that hay is harvested in North America. Horses are poisoned by eating hay containing trapped swarms of blister beetles. Affected horses have severe colic and will kick at their belly and roll; they may die of shock. There is no effective therapy for affected horses, but treatment involves the use of pain killers such as banamine hydrochloride (Schmitz and Reagor, 1987) and intensive electrolyte therapy.

Lead poisoning in horses is characterized by neurological abnormalities. Affected horses may be either depressed or excited. Colic and diarrhoea are also observed. Horses poisoned by lead also have difficult respirations with 'roaring' because of laryngeal nerve paralysis. Abortions are also common.

Arsenic poisoning in horses is caused by consumption of foliage which has recently been sprayed with arsenic herbicides. The condition is normally acute and characterized by intense colic and haemorrhagic diarrhoea. As in food-producing animals, inorganic arsenic poisoning does not involve the nervous system, which helps differentiate this condition from organophosphate or carbamate poisoning.

4.1 Toxic Plants

Selenium is an essential element, but is toxic when excessive quantities are ingested from plants that have accumulated significant quantities from the soil. Exposure is usually through consumption of seleniferous (indicator) plants, for example *Astragalus.* Exposure to high quantities of selenium over a short period of time causes diarrhoea, which may be foul smelling and contain air bubbles. Neurological, cardiovascular and respiratory signs may also be present. Death in these animals is from respiratory failure. Chronic exposure to excessive selenium is characterized by hoof abnormalities at the coronary bands and discolouration and loss of hair. The hoof deformities and the resulting pain cause lameness (Hultine *et al*., 1979).

Other plant poisonings commonly encountered in horses are those that cause gastrointestinal problems (**Table 1**), liver damage (**Table 2**), primary or secondary nervous-system involvement (**Tables 3** and **4**) and sudden death. Plants such as castor bean, oleander and bracken fern cause colic and diarrhoea; oleander also causes heart failure.

Prolonged ingestion of some plants for several weeks can lead to liver damage and hepatic cirrhosis. Examples of commonly involved hepatotoxic plants include *Amsinckia*, *Senecio* and *Crotolaria* (Rumbeiha and Oehme, 1992b). Liver damage may compromise the ability of the horse to detoxify ammonia which accumulates *in vivo,* leading to CNS involvement.

Plants that commonly cause CNS stimulation include larkspur, locoweed, lupine, water hemlock and fitweed. Common plants that cause CNS depression include black locust, bracken fern, horsetail, milkweed and white snake root.

Like ruminants, horses will avoid eating toxic plants because they are not palatable. Therefore, consumption of poisonous plants will occur during drought conditions when the animals lack suitable pasture. Sudden death in horses can be caused by the consumption of cyanide-containing plants such as sorghum. The cyanide ion forms a complex with cytochrome oxidase, which prevents transportation of electrons and utilization of oxygen by tissues throughout the body. As a consequence, blood is well oxygenated and cherry red in colour. Treatment for this condition is an emergency and

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in the USA involves giving both sodium thiosulfate and sodium nitrite intravenously (other antidotes are used in other countries).

Horses, like other monogastrics, are more resistant to nitrate/nitrite poisoning than ruminants. However, horses can reduce nitrates to nitrites in the caecum, but require approximately three times as much nitrate to produce a similar effect as in the ruminant.

4.2 Mycotoxins

Contaminated grains are sources of mycotoxin exposure in horses. The effects are similar for the horse as for food-producing animals. The most commonly involved mycotoxins are aflatoxins, T-2 and fumonisin B_1.

Aflatoxins will cause nonspecific signs, such as not thriving, haemorrhages and abortions. T-2 is a trichothecene mycotoxin which causes prolonged bleeding time in affected animals.

A specific mycotoxin uniquely affecting horses is fumonisin B_1 which is produced by *Fusarium moniliforme*. It is responsible for the condition called equine leucoencephalomalacia. Horses are affected by the consumption of mouldy corn and become anorectic and initially depressed, but as the condition progresses animals become blind, walk aimlessly and may show head pressing. They have difficulties with swallowing and eventually die (Wilson *et al*., 1990).

5 COMMON TOXICOSES OF POULTRY

This section will mainly address toxicoses in chicken, ducks and turkeys. However, there is much interest in the toxicology of wild birds, especially those kept in zoos and wildlife parks, as well those poisonings affecting pet birds. This discussion will emphasize toxicoses encountered in poultry. Readers interested in the general subject of avian toxicology are referred to LaBonde (1991).

5.1 Chemotherapeutic Drugs

Sulfonamides have been used as coccidiostats in poultry for several decades. Although sulfonamides have inhibitory action against coccidia and other pathogenic agents, they can also be toxic to the host under certain conditions. In poultry, sulfonamide toxicity is characterized by blood dyscrasia, and renal and liver dysfunctions. Feeding chickens a mash containing only 0.2% sulfonamides for two weeks is toxic. Clinically affected birds have ruffled feathers, are depressed, pale, icteric and have poor weight gain and prolonged bleeding times. In laying birds, sulfonamides cause a marked decrease in egg production, thin, rough egg shells and depigmentation of brown eggs. The temperature of affected birds is often elevated. At post mortem, haemorrhages are found under the skin, in muscles (especially those of the thighs and breast) and in internal organs. Once these signs are noticed, the concentration of sulfonamides in the various rations should be determined and the feed involved withdrawn (Peckham, 1978).

Other chemotherapeutic agents sometimes involved in poisoning poultry include coccidiostats such as nicarbazine, zoalene (3,5-dinitro-*o*-toluamide), nitrophenide and the ionophore monensin. As little as 0.006% dietary nicarbazine causes mottled egg yolks and 0.02% produces a depressed rate of growth and depressed feeding efficiency. Feeding 0.025% nicarbazine to day-old chicks for one week results in dullness, listlessness, weakness and ataxia. Feeding zoalene at twice the recommended level of 0.025% induces nervous signs and depressed growth and feeding efficiency. The nervous signs resulting include stiff neck, and staggering and incoordination when the birds are excited. Nitrophenide possesses marked electrostatic properties and thus adheres to the sides of the feed mixer. The last portion of feed in the feed mixer will normally contain a high concentration of nitrophenide and feeding this has caused disturbances in posture and locomotion, retarded growth and significant mortality in chickens. Postural disturbances resulting include a tilted position of the head, tremors of the neck and difficulty in the righting reflex (Peckham, 1978).

In general, poultry are more resistant to nionensin toxicity than mammals. There have been reports of monensin toxicity in turkeys accidentally fed rations containing 250 ppm monensin. There is a big difference in species susceptibility among various poultry to monensin poisoning. Chickens and turkeys less than two weeks old are more resistant than older birds, but keets (young guinea fowl) seem more susceptible than adult guinea fowl and young animals of other species. For example, monensin at 200 ppm was not toxic for poults whereas 100 ppm poisoned keets.

5.2 Cresol

Cresol was previously employed as a commonly used disinfectant in poultry houses, but has been withdrawn from use and replaced by safer disinfectants. Nevertheless, cresol is still found in use in some countries. Cresol poisoning usually occurs in chickens at three to six weeks of age. Affected chicks are depressed and have a tendency to huddle. There are signs of respiratory problems, such as rales, gasping for breath and wheezing. In instances of prolonged exposure oedema of the abdomen will develop.

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5.3 Sodium Chloride

All poultry and pigeons are susceptible to salt poisoning. Young birds are more susceptible than adults. Although both acute and chronic forms of salt poisoning occur, the chronic form is more commonly encountered and results from prolonged ingestion of feed containing a high salt content. Sodium levels of 0.5% or more in drinking water or 5–10% in feed cause death in baby chicks. Signs of salt poisoning in poultry are anorexia, thirst, dyspnoea, opisthotonos, convulsions and ataxia. Increased water consumption may be the most significant early indicator of salt poisoning in poultry (Peckham, 1978).

5.4 Insecticides

Chlorinated hydrocarbon insecticides and organophosphate compounds are sometimes used inappropriately around poultry houses to control external poultry parasites (LaBonde, 1991). Commonly involved organochlorine insecticides include chlordane, dieldrin, DDT, heptachlor and lindane. Occasionally birds are also exposed by gaining access to sprayed fields, such as golf courses.

Chlordane causes chicks to chirp nervously, rest on their hocks and lie on their sides. The birds then become hyperexcitable as the condition progresses. In mildly affected adult birds there is reduced food consumption, decreased body weight and a fall in production.

Consumption of grain seeds dressed with dieldrin has been a source of exposure in wild birds. Affected birds are listless, have coordination problems while alighting, and severely poisoned birds show nervous signs characterized by lateral movements of the head and tremors of the head and neck. Birds die of violent convulsions.

DDT toxicity in chickens is characterized by hyperexcitability and fine tremors in severe cases. Moderate cases are characterized by loss of weight, moulting and reduced egg production.

Lindane dust is frequently used in chicken houses. Adult chickens poisoned by lindane become anorectic, manifest opisthotonos, flapping of the wings and clonic muscle spasms and they die in a coma (Peckham, 1978).

The organophosphate compounds commonly involved in poultry poisoning include diazinon, malathion and parathion. Diazinon is used for chicken premises, but is very toxic to ducklings; when used at rates recommended for chickens, 100% mortality occurred in one to two-week-old ducklings. Experimental studies suggest that goslings are three times more sensitive than ducks, chickens or turkeys. Poisoned birds are unable to stand, salivate profusely and manifest tremors of the head and neck. Brain cholinesterase levels in birds that die of organophosphate poisoning are on average 69% less than controls.

Other organophosphate compounds commonly used on chicken premises include dichiorvos, malathion and parathion. Birds poisoned by these compounds manifest signs similar to those produced by diazinon. Other signs that may be encountered include birds being depressed, ataxic and reluctant to move, paralysis, lachrymation, gasping for breath, diarrhoea, crop stasis and dyspnoea (Mohan, 1990). In general, ducks are more sensitive to organophosphate poisoning than chickens, and care should be exercised when using these products on premises holding ducks (Mohan, 1990).

The carbamate insecticide carbaryl is widely used as a poultry insecticide. This compound is relatively safe to use, but deaths have been reported in turkey poults kept in premises where the product has been applied at 10 times the recommended rate. The clinical signs are similar to those caused by organophosphate compounds.

5.5 Heavy Metals

Lead poisoning is not as common in poultry as in wild birds. Lead poisoning is the most common toxicity reported in the avian species (LaBonde, 1991). Lead shot has caused losses in waterfowl populations in North America. All birds are susceptible to lead poisoning, but most losses are reported in waterfowl because their feeding habits predispose them to ingestion of lead shot.

Characteristic signs of lead poisoning are those related to CNS derangement, such as ataxia, depression, paralysis of wings or convulsions. In some cases the birds present as anaemic, emaciated, regurgitating and weak. Green diarrhoea has also been reported in some birds affected with lead poisoning (LaBonde, 1991).

5.6 Rodenticides

Yellow phosphorus is a highly toxic element used as a rodenticide. Poultry and wild birds can be intoxicated by consumption of bait intended for rodents. Fragments of fireworks containing phosphorus are also a common source of poisoning in free-range birds. Affected birds are depressed, anorectic, have increased water consumption and manifest diarrhoea, ataxia, paralysis, coma and death (Peckham, 1978).

All rodenticides are potentially toxic to poultry and other birds. The clinical signs caused by rodenticides are similar to those that occur from similar compounds in small animals.

Birds occasionally consume baits containing anticoagulant rodenticides. The more potent second-generation rodenticide-containing baits, such as brodifacoum, are

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especially dangerous to birds. These coumarin-like anticoagulants act by interfering with vitamin K_1 recycling, causing bleeding because of depletion of vitamin K_1-dependent clotting factors. Poisoned birds bleed from the nares and subcutaneously, with oral petechiae. They often are weak and depressed.

Of special interest, however, is secondary intoxication from consumption by free-range birds of carrion of animals that died of rodenticide poisoning. Strychnine and sodium monofluoroacetate are compounds commonly involved because they cause acute death in primary victims and are thus present in high concentrations in carrion. Strychnine-poisoned birds manifest clinical signs within two hours of ingesting the product. The birds become apprehensive, nervous and show violent tetanic convulsions, which cause the birds to become exhausted and die of hypoxia. Sodium monofluoroacetate causes overstimulation of the CNS and also myocardial depression. Cardiac failure is the cause of death, occurring within one hour of consuming the product or portions of the contaminated carcasses (Peckham, 1978).

5.7 Mycotoxins

Mycotoxicoses are a common problem in the poultry industry in developing tropical countries. Aflatoxins are of primary concern because of the public-health risk, from consuming aflatoxin residues in eggs and meat, as well as interfering with productivity. Poultry are normally exposed by consumption of contaminated feed, especially corn. Some developing countries lack the resources to screen potentially contaminated corn adequately. Poultry feed is often made from poor quality corn rejected for human consumption; this poor quality feed may be contaminated with the aflatoxin-producing fungi, *Aspergillus flavus* and *Penicillium* spp.

Aflatoxicosis in poultry can be either acute or chronic depending on the exposure dose. Ducklings are more susceptible to aflatoxin than turkeys, pheasants or chickens (Butler, 1974). In acute cases affected birds become lethargic, their wings droop and they manifest nervous signs, such as opisthotonos. Affected birds frequently die with their legs rigidly extended backward. Chronic consumption of at least 2.5 ppm aflatoxin in the diet causes a significant drop in performance with reduced weight gain and egg production. Perhaps more important is the increased susceptibility of the affected flock to infection because chronic consumption of aflatoxins lowers the immunity of the birds. Aflatoxicosis is therefore a disease of serious economic consequences to the poultry industry in developing countries such as Uganda, both through lowered productivity and the death of affected birds.

Ergot poisoning has been reported in Europe where rye is commonly used as a feed. In acute ergot poisoning the birds' combs are cold, wilted and cyanotic. The birds are weak, thirsty and have diarrhoea. In severe cases they develop convulsions and paralysis, with death following. Ochratoxin mycotoxins have uniquely been reported to cause renal toxicity in poultry.

6 SUMMARY

In this brief chapter we have summarized the broad discipline of veterinary toxicology. We have given overviews of selected common toxicities from the various animal species so as to draw the attention of the reader to similarities and differences in their reaction to toxicants. Because some animals are more sensitive than others to the same toxicant, absolute diagnosis of some toxicoses may require the help of specialists within the veterinary profession.

This chapter is not intended as a detailed source of reference for the diagnosis and treatment of animal poisonings, nor is it meant to be all inclusive. Rather it is a summary of the commonly encountered toxicoses in the veterinary and animal health profession. Interested readers should consult the relevant references given to gain more in-depth knowledge on poisonings of interest.

From this general overview it should be clear that all animals are susceptible to some toxicants and that many toxicants are poisonous to all animals (including humans). It is therefore important to be cautious when handling chemicals around animals to avoid unnecessary risk. Animal caretakers should also provide chemically clean environments to their animals. Rations should provide well-balanced and nutritionally sound quality feed from reputable sources, and suspect feed should be avoided or atleast checked for suspected toxicants before being fed. It is also vitally important to remember that all chemicals become poisons if their exposure dose is great enough. Therefore, even some of the valuable compounds used around animals, for example, growth promoters, can be fatal if used excessively or if given to species for which they are not intended. The susceptibility of sheep to feed containing copper intended for cattle, or of poultry feed supplemented with monensin and offered to horses, are frequently tragic cases in point.

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Essentials of Epidemiology for Toxicologists

David Coggon

CONTENTS

1 INTRODUCTION

Epidemiology is concerned with the distribution and determinants of health in populations. The earliest epidemiological research provided simple descriptions of variations in mortality and in the occurrence of disease by time and place, but as its methods have evolved, epidemiology has progressed to more detailed investigation of disease causation, and extended its scope to other health outcomes, such as illness (including symptoms that occur in the absence of demonstrable underlying disease), disability and biomarkers relevant to future health. In addition, similar techniques have been used to investigate patterns of disease in animal populations (veterinary epidemiology).

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Table 1 Applications of epidemiological research

• Development of policy to maintain or improve health	This includes the identification of factors that cause disease, illness or disability, and assessment of how risks vary according to the circumstances and levels of exposure to these factors.
• Evaluation of interventions aimed at maintaining or improving health	This may be through experimental trials of preventive measures and treatments, or through observational studies.
• Characterization of prognosis and prognostic indicators	Systematic follow-up of appropriately defined populations can provide information on prognosis and its predictors. This may be useful in clinical care, and also, for example, in determining fitness for different types of work.
• Planning of health care services	Epidemiology can help in estimation of the numbers of patients who will require different treatments, and for how long.
• Determination of causal attribution for purposes of compensation	Epidemiology can help to establish whether cases of disease can reasonably be attributed to alleged causes, both in the courts and under social security regulations.

Epidemiological research has various applications (**Table 1**), but of most relevance to toxicology are its uses in the identification and characterization of toxic hazards, the assessment of risks from toxic exposures, and the evaluation of measures designed to control such risks. The contribution of epidemiology in these areas is complementary to that of experimental toxicology.

Epidemiology has the great strength that it provides direct information about determinants of health in human populations, avoiding the uncertainties that are inherent in extrapolation between species. Some toxic hazards, such as the carcinogenicity of benzene and arsenic, have been clearly apparent in epidemiological studies, but have proved difficult or impossible to demonstrate in laboratory animals. Others, such as the hepatic carcinogenicity of peroxisome proliferators in mice, appear not to extend to humans. And some health outcomes—for example, those relating to higher cerebral function and to immune sensitization—are difficult to model in the laboratory. Also, because epidemiological studies can include very large numbers of subjects (often thousands and sometimes even millions), they may be able to discern small effects from low exposures to a toxicant that could not be predicted with confidence from the laboratory. A good example is the small but important risk of cardiorespiratory disease from particulate pollutants in outdoor air.

Against this, however, epidemiology is limited by the practical and ethical constraints on research involving human subjects. Studies cannot be designed and conducted with the same rigour that is possible in the controlled environment of the laboratory, and this leads to uncertainties in interpretation.

This chapter introduces the main concepts of epidemiology, describes the most frequently employed study designs, and outlines the considerations that must be taken into account when interpreting epidemiological findings.

2 QUANTIFICATION OF HEALTH OUTCOMES

Fundamental to epidemiology is the quantification of health outcomes in populations. Various metrics are used for this purpose, but first the health outcome must be defined.

2.1 Definition of Health Outcomes

As in toxicology, measures of health outcome in epidemiology can be dichotomous, ordinal or continuous. Dichotomous outcomes usually distinguish between cases and noncases of illness or disease. For example, people might be classified according to whether or not they meet specified criteria for a diagnosis of myocardial infarction. This approach is most satisfactory for disorders that are relatively all-or-none in nature. However, it can also be applied to outcomes that occur in a spectrum of severity with no clear distinction between normality and abnormality. This requires specification of somewhat arbitrary criteria for case definition. Thus, a case of hypertension might be defined as having a systolic blood pressure >140 mm Hg and/or a diastolic blood pressure >90 mm Hg on each of three consecutive measurements over a period of three weeks.

Alternatively, an outcome such as blood pressure could be characterized by a continuous measure—for

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example, the mean systolic pressure from three consecutive readings over a three-week period. Other examples of continuous outcome measures are bone mineral density, hearing threshold on audiometry and erythrocyte acetyl cholinesterase activity.

Ordinal outcome measures are less often encountered. Examples include scales that have been devised to grade pneumoconiosis (International Labour Office, 2002) and osteoarthritis (Kellgren and Lawrence, 1957) on radiographs.

In practice, the best available measures of health outcomes are often only approximate. This may be because of inaccurate or imprecise methods of measurement (e.g. in a biochemical assay), or because indirect markers of outcome are employed—for example, in the epidemiological study that first established the human carcinogenicity of 2-naphthylamine (Case *et al*., 1954), mortality from bladder cancer as recorded on death certificates was taken as an indirect index of bladder cancer incidence. Any approximations in the assessment of health outcome must be taken into account carefully when results are interpreted. For example, when a study found elevated mortality from lobar pneumonia in occupations involving exposure to metal fume (Coggon *et al*., 1994), one possible explanation was that fume increased susceptibility to pneumonic infection. Another possibility, however, was that it increased fatality from pneumonia (i.e. the risk of death in people who have already developed pneumonia), while not influencing whether people initially acquired the disease.

Whatever the measures of health outcome that are adopted in an epidemiological study, they should be explicitly and unambiguously defined. Otherwise, results cannot be properly interpreted.

2.2 Rates of Disease[a]

As described above, most epidemiological investigations classify people dichotomously according to whether or not they are cases of a disease. In order to draw useful conclusions about the distribution and determinants of a disorder, the number of cases that occur in a population or group of people must be related to the size of the population. Several measures of disease occurrence are commonly used.

2.2.1 Incidence

The incidence of a disease is the rate at which new cases occur in a population over time. For example, the incidence of breast cancer among women aged 65–69 years in England during 2005 was 395 per 100 000 per year.

2.2.2 Mortality

A mortality or death rate is the incidence of death in a population that is attributable to one or more specified causes. For example, mortality from breast cancer among women aged 65–74 in England and Wales during 2005 was 91 per 100 000 per year.

2.2.3 Prevalence

The prevalence of a disease is the proportion of a population who are cases at a specified time or during a specified period. For example, in a survey of rural residents, the prevalence of tiredness and lack of energy during the past month among men aged 24–69 years who had at some time worked with insecticides was reported as 17.3% (Solomon, 2007), and the prevalence of Down's syndrome among babies born in 17 states of the USA during 1983–1990 was reported as 9.2 per 100 000 live-born infants (Department of Health and Human Services, 1994). The prevalence of a disorder depends both on its incidence and on the time for which it persists before recovery or death. Thus chronic diseases such as rheumatoid arthritis and multiple sclerosis have a higher prevalence relative to incidence than those that are short-lived (e.g. influenza) or rapidly fatal (e.g. pancreatic cancer).

In studies of causation, incidence is the most relevant measure of disease frequency. This is because mortality may be influenced by determinants of fatality, as well as of incidence, while prevalence depends on factors that influence disease persistence, as well as its first development. Mortality and prevalence are often used as proxy measures of incidence, but findings must then be interpreted with appropriate care.

2.2.4 Crude, Stratum-Specific and Standardized Rates

Measures of disease frequency may apply to the whole of a population (crude rates) or to specified subsets of a population defined, for example, by sex and age. Because the occurrence of most diseases varies importantly by sex and age, use of sex- and age-specific rates usually enables more meaningful comparison between populations than can be achieved with crude rates. However, the elaboration of multiple sex- and age-specific rates can sometimes be rather unwieldy, especially when data cover a large number of sex and age 'strata' (where a population is partitioned into multiple categories defined by levels of one or more variables, the categories are often termed strata of the population). In these circumstances, an alternative is to 'standardize' rates for the stratifying variables. Two methods of standardization are commonly employed.

Direct standardization entails the calculation of a weighted average of stratum-specific rates, where the

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weighting factors are derived from a defined (real or theoretical) population. Effectively, this gives the crude rate of disease that would apply in the standard population if it experienced the same stratum-specific rates that are observed in the population under study. Directly standardized rates, whether of incidence, mortality or prevalence, allow simple summary comparisons of populations that are meaningful, provided that the underlying stratum-specific rates differ consistently between the populations. However, where this condition is violated (e.g. one population has higher rates than another at older ages, but lower rates in the young), useful information will be lost. In fact, depending on the weighting factors applied, either population could be made to have the higher directly standardized rate.

In indirect standardization, the number of cases in a study population is compared with the number that would have been expected had that population experienced the same stratum-specific rates as a specified standard population (e.g. the national population of a country in a specified time period). The comparison is summarized as the ratio of observed to expected cases (often expressed as a percentage), which may be termed a standardized incidence ratio (SIR), standardized prevalence ratio or standardized mortality ratio (SMR), depending on the measure of disease frequency to which it refers. Strictly, indirect standardization is valid only as a method for comparing the study population with the chosen standard population. In most circumstances, however, comparison of standardized ratios for two or more populations (in relation to the same standard population) gives a reasonable summary of their relative frequency of disease.

2.2.5 Proportional Mortality and Morbidity

Sometimes it is not possible to derive incidence, mortality or prevalence rates for a disease because, although cases can be enumerated, the size of the population from which the cases derived is not known. For example, the number of deaths each year from sinonasal cancer among cabinet-makers in England and Wales can be established from death certificates. However, the total number of cabinet-makers nationally cannot be estimated reliably, except near the time of a national census.

In these circumstances, an alternative to the calculation of disease rates is to derive proportions. Thus, the proportion of deaths among cabinet-makers that were ascribed to sinonasal cancer might be compared with the corresponding proportion in all occupations combined, the latter information also being obtained from death certificates. Similarly, the proportions of emergency admissions that were for bleeding duodenal ulcer might be compared between two hospitals for which the exact catchment populations could not be enumerated. As for rates of disease, proportions can be directly or indirectly standardized for sex and age. For example, sex- and age-standardized proportional mortality ratios (PMRs) are often used in national or regional analyses of mortality by occupation.

Special care is needed, however, in the interpretation of proportions. This is because they depend, not only on the frequency of the disease of specific interest, but also on the overall rate of disease that provides the denominator for the proportion. For example, in England and Wales, teachers have been found to experience high proportional mortality from multiple sclerosis (Coggon *et al*., 1995). This could be because teachers are abnormally susceptible to the disease, but it is also possible that they have unusually low overall mortality, while their death rate from multiple sclerosis is unremarkable.

3 MEASUREMENT OF RISK FACTORS

As well as the measurement of health outcomes, epidemiology requires ascertainment of 'risk factors' that are known or suspected to influence or predict the occurrence of health outcomes. Risk factors for health outcomes include demographic characteristics (e.g. sex and age), genetic constitution, aspects of lifestyle, such as dietary, smoking and sexual habits, and exposure to various chemical, physical and biological agents and to psychological stressors. They may be categorical (e.g. ever or never treated with a particular drug), ordinal (e.g. level of education) or continuous measures (e.g. 24 hour urinary excretion of cadmium). Some of the methods that are commonly used to ascertain risk factors are listed in **Table 2**.

Like the assessment of health outcomes, ascertainment of risk factors is often liable to error, either because it is based on an indirect marker (e.g. job title is used as an index of exposure to a chemical in the workplace) or because of shortcomings in measurement techniques (e.g. inaccurate recall in a questionnaire). The scope for such errors can often be reduced by good study design, but the potential for residual inaccuracy must be taken into account when results are interpreted.

4 MEASURES OF ASSOCIATION BETWEEN RISK FACTORS AND HEALTH OUTCOMES

Much of epidemiology is concerned with assessment of associations between risk factors and health outcomes. Various statistics are used to summarize such associations, and their nomenclature is not yet fully standardized. The terms and definitions that follow are quite widely adopted, but not all are universally agreed.

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Table 2 Common methods for ascertainment of risk factors in epidemiological studies

• Questionnaires	Self-administered or completed at interview
• Physical examination	For example, measurement of height, weight or blood pressure
• Clinical investigation	For example, genotyping
• Measurement of biomarkers of exposure	For example, metabolites of DDT in adipose tissue, benzene in exhaled breath, or cotinine in saliva
• Personal or environmental exposure-monitoring	Using active or passive samplers

4.1 Attributable Risk

Attributable risk is the absolute difference in the risk (or probability) of a health outcome between someone who is exposed to a risk factor and someone who is unexposed or exposed at a different level. For example, a study might estimate the difference in the incidence of hepatic angiosarcoma between people who have and have not worked with vinyl chloride monomer, or the differences in the prevalence of limb reduction abnormalities between babies born to mothers who took thalidomide at different times during pregnancy. Attributable risk is the measure of association that is most relevant when making decisions for individuals. For example, when deciding whether or not to use hormone replacement therapy, a woman needs to weigh the benefits against the absolute increases in risk that she would incur for adverse effects such as breast cancer.

4.2 Relative Risk and Odds Ratio

Relative risk (RR) is the ratio of the risk of a health outcome in someone who is exposed to a risk factor to that in someone who is unexposed or exposed at a different level. For example, use of aspirin by children with acute infections has been estimated to increase the risk of Reye's syndrome by a factor of 11.5 (Halpin *et al*., 1982). Closely akin to RR is the corresponding odds ratio, defined as the ratio between the odds of the health outcome in an exposed person and the odds in someone who is unexposed or exposed at a different level (a risk of $1/N$ corresponds to odds of $1/(N-1)$). RRs lie closer to the null value of one than the corresponding odds ratios, but the difference is small for health outcomes with a risk of less than 10% in each of the exposure categories under comparison.

The relation between RR and attributable risk depends on the frequency of the health outcome. The RR of hepatic angiosarcoma from work with vinyl chloride is high (>10 000), but because the disease is extremely rare in unexposed persons, the attributable risk is modest.

4.3 Population Attributable Risk

Population attributable risk is the excess frequency of a health outcome in a population that is associated with a risk factor. In other words, it is the difference between the population's incidence, mortality or prevalence rate and that which would apply if the risk in all persons exposed to the risk factor were reduced to that of people who are unexposed (or exposed at some specified lower level). It depends on both the (individual) attributable risk and also the prevalence of exposure in the population of interest. Thus, although the individual attributable risk of mortality and serious morbidity from particulate pollution in outdoor air is small, large numbers of people are exposed, and the population attributable risk is sufficiently large to have warranted controls on sources such as motor vehicle exhaust.

4.4 Attributable Proportion

Attributable proportion (also known as population attributable fraction or aetiologic fraction) is the proportion of cases of illness or disease in a population that would be eliminated if the risk in exposed persons were reduced to that of people who were unexposed (or exposed at some specified lower level). For example, the proportion of lung cancer cases in a population that are attributable to smoking might be estimated as 80%. This would imply that if everyone in the population had the risk of a lifelong nonsmoker, 80% of lung cancer cases would be eliminated. Like population attributable risk, attributable proportion is used to inform decisions in risk management at a population level.

It should be noted that where multiple causes of a disease have been identified, their attributable proportions will often sum to more than 100%. This is because causes act in concert rather than in competition, and in persons who develop a disease following exposure to a combination of two causes, avoidance of either one of the causes would often have sufficed to prevent the disease. To give a specific example, phenylketonuria is 100% attributable to genetic constitution, but it is also 100% attributable to diet, and can be completely prevented by elimination of foods containing phenylalanine during infancy.

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4.5 Attributable Fraction in Exposed

The attributable fraction in exposed (AF_{exp}) is the proportion of cases among exposed persons that would be eliminated if their risk were reduced to that of people who were unexposed (or exposed at some specified lower level). It relates to the RR by the formula $AF_{exp} = (RR - 1)/RR$. The main application of AF_{exp} is in the determination of attribution to exposures for purposes of compensation. For example, in Britain, one justification for the classification of an occupational disease as compensatable under social-security provisions is the demonstration that a specified occupational exposure carries an AF_{exp} of at least 50%. This implies that cases of the disease in exposed persons can be ascribed to the exposure 'on the balance of probabilities'.

5 BIAS, CHANCE AND CONFOUNDING

Central to the design and interpretation of epidemiological research are the concepts of bias, chance and confounding. These terms are best understood if an epidemiological study is conceived as collecting information about a sample of people with the purpose of estimating one or more parameters in a (theoretically infinite) population from which the sample derived. For example, the odds ratio for the association between Parkinson's disease and previous work with pesticides in a general population sample might be used to estimate the odds ratio for this association more generally. Here the odds ratio in the study sample is known as a sample statistic, and serves as an estimator for the corresponding population parameter, the odds ratio in the population at large.

5.1 Bias

Bias is a systematic tendency to underestimate or overestimate a parameter of interest because of a deficiency in the design or execution of a study. Epidemiological textbooks have distinguished and named many different categories of bias, but broadly, bias can arise from two sources.

Firstly, the way in which subjects are recruited to a study may render them systematically unrepresentative of the population about which conclusions are to be drawn. The problem may arise in the specification of who is eligible for inclusion in the investigation, or from incomplete recruitment of those who are eligible for inclusion (e.g. because of missing records or unwillingness to participate).

Secondly, bias may result from inaccuracies in the information that is obtained about subjects, concerning risk factors, health outcomes or both.

Almost all epidemiological studies are liable to biases of one sort or another, and one of the arts of epidemiological research is to design and carry out studies in a way that minimizes bias, and makes it easier to gauge the potential impact of those biases that cannot be eliminated. When assessing possible effects of biases, it is important to consider in which direction they might affect parameter estimates, and to what extent. The same source of error may produce major bias in the estimation of one parameter, but have little effect on estimates of another. For example, in a survey to assess the prevalence of high alcohol consumption in a general population sample, incomplete participation might lead to serious underestimation if heavy drinkers were less willing to take part than moderate, light or nondrinkers. However, if the aim of the study were to assess the association of heavy drinking with a biomarker of liver function, this differential participation would be of less concern. Bias would only arise if the association between heavy drinking and the biomarker were systematically different in nonparticipants as compared with those who opted to take part, which seems less likely.

5.2 Chance

Even where a study sample is recruited in a way that is not systematically unrepresentative, it may still be atypical by chance. Larger samples of people are less likely to be atypical by chance than smaller samples, and the potential for chance variation in sample statistics can be quantified by methods of statistical inference.

5.2.1 Hypothesis Testing and p-values

The earliest approach to be developed was that of hypothesis testing, in which a 'null' hypothesis is assumed to apply in the theoretically infinite population from which the study sample was drawn. For example, the null hypothesis might be that in this source population there is no association between an exposure and a health outcome (or put another way, that the RR associated with the exposure is one). The probability (p-value) is then calculated of obtaining a result as or more extreme from this null hypothesis than that observed in the study sample, in a sample of the same size selected at random from the source population. If the p-value is sufficiently low, the null hypothesis may be rejected in favour of an alternative (e.g. that in the source population, the exposure and health outcome are related).

5.2.2 Statistical Power

The p-value depends on the extent to which findings in the study sample deviate from the null hypothesis, but it is also influenced by the size of the study sample. In

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a very large study sample, even a small deviation from the null hypothesis can be highly significant statistically (i.e. associated with a very small p-value). On the other hand, a large deviation from the null hypothesis could be associated with an unremarkable p-value if the study sample were small. This reflects the notion of 'statistical power'. Other things being equal, larger study samples have the capacity to discriminate smaller deviations from the null hypothesis.

When a study is being planned, the statistical power for a specified sample size can be calculated. This requires various stipulations, according to the study design. For example, in a study comparing rates of a health outcome in people with and without exposure to a risk factor, the expected rate of the outcome in people unexposed to the risk factor would be specified. In addition, the investigator would choose the lowest RR in the source population for which they wished to be confident of finding a p-value below some stated threshold (e.g. 5%). With these specifications, the probability of obtaining a p-value below the threshold could then be calculated for different sizes of study sample.

5.2.3 Confidence Intervals

In practice, decisions in the management of risks to health depend not so much on whether or not a hypothesis is true as on the estimated magnitudes of relevant parameters. For example, a decision whether or not to use a drug is determined, not simply by whether or not it is efficacious, but by the estimated extent of benefits from the treatment, and how they weigh against the costs of the drug and any risk of adverse effects. For this reason, statistical inference in epidemiology these days has largely moved away from hypothesis testing, instead focussing on the derivation of confidence intervals around estimates of parameters.

A confidence interval can be viewed as a range within which, in the absence of bias, the true value for the population parameter of interest would normally be expected to lie. Most often, 95% confidence intervals are calculated. The formula for a 95% confidence interval is specified in such a way that, on average, (in the absence of bias), 95% of such intervals will include the population parameter to which they relate. Other confidence intervals (e.g. 90% confidence intervals) are defined analogously.

Confidence intervals give an indication of the statistical uncertainty around estimates of population parameters. Other things being equal, larger samples of subjects will give tighter confidence intervals, reflecting the fact that they are less likely to be unrepresentative by chance. Once a study has been completed, the confidence interval around a parameter estimate gives the simplest and most easily interpreted index of its statistical power.

5.2.4 Statistical Inference in Context

Although p-values and confidence intervals quantify the statistical uncertainty in epidemiological findings, a final assessment of the likely contribution of chance to an observation must also take account of what is known from sources external to the study. For example, if a new epidemiological study indicated a statistically robust protective effect of smoking against lung cancer, in the absence of other explanations, one might nevertheless attribute the result to chance, given the extensive body of evidence from elsewhere that smoking is in fact a potent cause of the disease.

5.3 Confounding

Confounding is a potential source of error when conclusions about causation are inferred from statistical associations between risk factors and health outcomes. It occurs when the risk factor of interest is associated with another exposure (a confounding factor), which independently determines the risk of developing the health outcome. Depending on whether the confounding factor makes the health outcome more or less likely, confounding can cause the observed association to overestimate or underestimate the causal impact of the risk factor of primary interest. This sometimes extends to the generation of spurious associations with risk factors that in fact have no causal impact whatsoever on the health outcome. For example, several epidemiological studies have demonstrated statistical associations between higher consumption of coffee and cancer of the pancreas. However, these observations may be completely explained by a confounding effect of smoking—high consumers of coffee are more likely to be smokers and smoking is an independent cause of pancreatic cancer.

Where an exposure is recognized in advance as a potential confounding factor (e.g. because it is a known cause of the health outcome and could plausibly be associated with the risk factor of primary interest), its possible effects can be taken into account in study design. Methods include: restriction of the study to subjects with identical exposures to the confounding factor (e.g. limitation to nonsmokers), matching the groups under comparison for their exposure to the confounding factor, or measurement of the confounding factor as part of data collection and then adjusting for it in statistical analysis (by techniques such as stratification or regression modelling). However, control for identified confounding factors may be incomplete (e.g. because they are not ascertained with complete accuracy), and there is the possibility of other confounding factors of which the investigator is unaware. Therefore the potential for residual confounding is always a consideration in the interpretation of observed statistical associations.

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It should be noted, however, that in order completely to explain an observed association between a risk factor and a health outcome, a confounding factor must carry an RR for the outcome at least as high as that found for the risk factor. To this extent, higher RRs are harder to explain by unrecognized confounding.

6 STUDY DESIGNS

6.1 Descriptive Studies

Most of the earliest epidemiological research was concerned simply with summarizing disease frequency by time and place. The data generated were used, for example, to track the evolution of epidemics of infectious disease. Descriptive epidemiology of this sort remains an important source of information that is used for various purposes (**Table 3**).

Most descriptive studies use routinely collected data—for example, on death registrations, cancer registrations or hospital admissions—but more rarely, descriptive data are generated through specially designed *ad hoc* investigations. As well as analyses by time and place, the frequency of disease may be broken down according to other characteristics such as sex, age, social class, ethnic origin, place of birth and occupation.

In addition to data on health outcomes, descriptive studies may be used to quantify and monitor the occurrence of risk factors that influence health, such as smoking habits and alcohol consumption.

Table 3 Uses of descriptive epidemiology

• Planning health services	Descriptive studies can provide information about the numbers of cases of disease in a population that will require treatment.
• Surveillance for newly emerging health problems	For example, descriptive epidemiology has drawn attention to large, and as yet unexplained, increases in the incidence of testicular cancer over the past century in a number of countries.
• Monitoring the impact of preventive measures and treatment	Analysis of trends in mesothelioma in England and Wales has highlighted a failure of controls on exposure to asbestos in the 1970s.
• As a source of clues to previously unrecognized causes of disease	The discovery that dust from vegetable-tanned leather causes sino-nasal cancer arose from the observation of a cluster of cases in the English county of Northamptonshire, using routinely collected cancer registration data.
• As a source of data for use in other types of epidemiological study	For example, routinely collected data on cause-specific mortality and cancer incidence have been used as a control for comparison with rates in occupational populations exposed to known or suspected carcinogens.

6.2 Ecological Studies

Ecological studies investigate statistical associations between risk factors and health outcomes across populations—that is, with populations or groups of people as the units of analysis rather than individuals. For example, mortality from Parkinson's disease in counties of Nebraska has been correlated with rates of pesticide usage in those counties (Strickland *et al*., 1996). The populations that are compared in ecological studies may be defined in various ways, including by country or region of residence, occupation or time period.

An advantage of ecological studies is that they often exploit data that have already been collected for other purposes and therefore are relatively cheap. Against this, their capacity to adjust for potential confounding factors is usually less than that of studies focussed on individuals. Nevertheless, ecological studies have sometimes generated findings sufficiently persuasive to influence health policy. For example, a major component of the evidence underpinning national policies on particulate pollution in outdoor air has come from ecological studies that correlated daily fluctuations in mortality and hospital admissions for cardiorespiratory disease in defined urban populations with changes in airborne concentrations of particulate matter recorded at local monitoring stations.

6.3 Cohort Studies

In a cohort study, individuals who differ in their exposure to known or suspected risk factors are identified and followed up over time with systematic ascertainment

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of relevant health outcomes. Rates of health outcomes can then be compared according to levels of exposure to risk factors, allowing estimation of RR and/or attributable risk. The design is analogous to that of an animal bioassay, except that the subjects are more heterogeneous (they do not all come from the same genetic strain) and the investigator studies them as they happen to have been exposed rather than allotting the exposure that they receive (i.e. the design is observational rather than experimental). A consequence of these differences is the possibility of confounding because people with higher exposure to a risk factor of interest may be systematically different in their exposures to other determinants of the relevant health outcome. Therefore, where possible, potential confounders should be identified in advance, and taken into account by restriction, matching or statistical adjustment.

Methods of follow-up depend on the health outcomes under investigation. They include linkage with routinely collected data (e.g. on cause-specific mortality or cancer registrations), and periodic questionnaires, physical examinations (e.g. to measure blood pressure) or clinical investigations (e.g. radiography or biochemical assays). The duration of follow-up is influenced by the time period over which risk factors are expected to exert their effects (e.g. whether there is likely to be a long induction period between first exposure and eventual manifestation of a disease), and also by the incidence of health outcomes (if incidence rates are low, longer follow-up may be needed in order to have sufficient cases for meaningful statistical analysis).

Examples of cohort studies include investigations that have followed up women who differed in their use of contraceptives, to compare their incidence of diseases such as breast cancer, myocardial infarction and stroke; and studies in which serial measurements of lung function have been carried out in coal miners over a number of years and changes in their forced expiratory volumes in one second (FEV_1) have been related to their smoking habits and exposures to coal-mine dust.

Cohort studies have the advantage that they normally incorporate relatively reliable information on exposure to risk factors and potential confounders (since the assessment of exposures is the starting point for the investigation). Also, they can often be used to study multiple health outcomes simultaneously, with little additional effort. For example, studies of workers exposed to suspected carcinogens have used linkage with cancer registration records to provide information about the risk of a range of different cancers. Against this, however, in order to achieve adequate statistical power, cohort studies may need to follow up large numbers of subjects for prolonged periods, making them relatively expensive.

Sometimes this limitation can be partially countered by carrying out a cohort study retrospectively. Subjects are identified who had differing exposures to risk factors in the past, and their subsequent health outcomes over the period up to the time that the investigation is conducted are systematically ascertained and compared. For example, studies have used prescription data to identify retrospectively patients who had been treated with different nonsteroidal anti-inflammatory drugs, and then compared their subsequent incidence of myocardial infarction as ascertained from healthcare records (Garcia Rodriguez *et al*., 2004; Dick, 2000). A prerequisite for retrospective designs of this sort is that ascertainment of the study 'cohort' should not be influenced in any way by the health outcomes under study. This could be a problem, for example, if the exposure records of people who had subsequently died had been selectively deleted, leading to their exclusion from the study sample. Another requirement is that relevant health outcomes should be ascertainable retrospectively with sufficient reliability. This is most often achieved when outcomes are derived from routinely collected records (e.g. of mortality, cancer registration or hospital admission).

Another modification that may make cohort studies more efficient is to use routinely available rates of mortality or cancer incidence in the general population as a comparator for rates in an exposed cohort, thus eliminating the cost and effort of recruiting and following up internal controls. This is appropriate where exposure to the risk factor of interest in the population at large is trivial in comparison with that in the study cohort. The method has been applied, for example, to examine risks of incident (i.e. newly occurring) and fatal cancer in cohorts of workers with high occupational exposures to chemicals such as styrene (Kogevinas *et al*., 1994) and formaldehyde (Coggon *et al*., 2003). Importantly, the health outcome should be ascertained by the same method in the study cohort as in the general population. Otherwise, bias may occur. For example, if rates of cancer in the general population were derived only from cancer registrations, whereas those in the cohort took account also of cases identified from detailed review of clinical records (some of which may not have been picked up by the registration system), risk estimates could be spuriously elevated.

Where the general population provides comparison rates of mortality or cancer incidence, risk estimates are often presented in the form of an SMR or SIR. This is the ratio of the number of cases observed in the cohort during the follow-up period to the number that would have been expected had the cohort experienced the same sex-, age- and calendar period-specific rates as the general population.

6.4 Case–Control Studies

In a case–control study (also known as a case–referent study), people who have developed a health outcome of interest (cases) are identified, and their past exposure to

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known or suspected risk factors is compared with that of controls (referents) who do not have the outcome. The method is best conceived as a way of sampling efficiently from a (usually hypothetical) cohort study. In most cohort studies, information about exposure is collected for a relatively small number of people who go on to become cases and a much larger number who do not. This imbalance is statistically inefficient. In effect, a case–control study aims to characterize exposures in all the cases that occur in a cohort, but in only a representative sample of noncases. By comparing the prevalence and patterns of exposure in this way, it is possible to estimate odds ratios (usually a good proxy for RRs) and also attributable proportions. However, attributable risks cannot be estimated directly from case–control studies. Allowance for potential confounding factors is made by restriction, by matching controls to cases (either individually or in groups) according to their exposure to confounders, or by collecting information about subjects' exposures to confounders and then adjusting for them statistically. Where matching is employed, statistical analysis must be modified to take the matching into account—otherwise risk estimates may be biased.

Methods for ascertaining exposures will depend on the nature of the risk factors and potential confounders under investigation. It may be possible to determine exposures from stored records (e.g. on prescriptions or hospital treatments) or from a persistent biomarker that is not modified by the development of the health outcome (e.g. measurement of the metabolite, DDE (dichlorodiphenyldichloroethylene), in adipose tissue or blood has been used as an index of past exposure to the insecticide, DDT (dichlorodiphenyltrichloroethane) in case–control studies of breast cancer (Snedeker, 2001). Most often, however, exposures are assessed through questionnaires that are either completed at interview or self-administered.

The most challenging aspect of case–control design is usually the choice of suitable controls. The aim is that (within matching strata if there are any) controls should have exposures to risk factors and potential confounders that are representative of those in the (real or hypothetical) cohort from which the cases derived. In other words, exposures should be representative of those in people who would have been included in the study as cases, had they developed the health outcome of interest during the period of study. At the same time, it is desirable that exposures be ascertained with similar accuracy for cases and controls (ideally exposures would be established with complete accuracy in both groups, but if this is not possible, similar accuracy for cases and controls will normally ensure that any resultant bias is towards the null—that is, associations will tend to be obscured rather than exaggerated).

In practice, however, it is rarely possible to achieve both of these objectives simultaneously, especially when exposures are ascertained by questionnaire. If controls are sampled from the source population at random (or by some pseudorandom algorithm), their exposures to risk factors and confounders should be fairly representative of those in the source population. However, it is likely that they will be less motivated than cases to remember past exposures, leading to a bias that tends to inflate risk estimates. Recall bias of this sort is less likely if, for example, patients with other diseases are employed as controls. However, the exposures of such patients may not be representative of those in the source population. For example, in a case–control study of bladder cancer and smoking, controls collected from a chest clinic could give a highly unrepresentative picture of smoking patterns in the population that gave rise to the cases. Again, bias would result.

Therefore, the choice of controls in a case–control study is often a compromise. Bias can only be minimized and not totally eliminated.

Case–control studies vary in their ratio of controls to cases. If both cases and controls are readily available and can be studied at similar cost, statistical efficiency will be greatest with equal numbers of cases and controls. If, however, there is a limit to the number of cases available for study (e.g. because the disease under investigation is rare), then statistical power can be increased by recruiting more than one control per case, although the gain in power diminishes as the ratio of controls to cases increases. Therefore, unless it is very cheap and easy to enrol additional controls, it is rarely worth including more than four or five controls per case.

Case–control studies have the advantage that they are usually cheaper than cohort studies of the same statistical power, especially for the investigation of rare health outcomes. However, they are less efficient when the exposures of interest are rare in the population under study (for a dichotomous risk factor, statistical power is greatest when the prevalence of exposure in the study population is 50%, and diminishes substantially if exposure is very rare or very common). Furthermore, because of the potential for inflationary recall bias, case–control studies often give less reliable estimates of risk than cohort studies.

6.5 Cross-Sectional Studies

In a cross-sectional study, information is collected at a point in time or over a relatively short period, in a defined population, about the prevalence of current, recent or past health outcomes and/or exposure to risk factors. If only health outcomes or only risk factors are ascertained, then the study is purely descriptive (see above). However, cross-sectional studies can also be used to examine associations between risk factors and health outcomes. For example, cross-sectional

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designs have been employed to explore the relation between exposure to genotoxic chemicals and the prevalence of cytogenetic abnormalities in circulating lymphocytes.

Where cross-sectional surveys are used to investigate questions about causation, care is needed that associations are not distorted by selective exclusion of potential participants as a consequence of the study design. For example, a cross-sectional study of sheep farmers looking for a relation of previous work with organophosphate sheep dip to impaired performance on neuropsychological testing, might underestimate an adverse effect if individuals with organophosphate-related illness were more likely than others to have left farming, and therefore to be excluded from the study sample. This type of selection effect would be of less concern, however, if the study sample comprised men living in defined rural areas, who were selected solely on the basis of their age and place of residence, and not according to whether they were currently working in agriculture. Selection effects are also less likely when the health outcome of interest is asymptomatic or associated with only minor symptoms.

In addition to selection bias and possible confounding, interpretation of cross-sectional studies must sometimes give consideration to the possibility of 'reverse causation'. For example, an association between use of analgesics and dyspeptic symptoms could occur because the drugs cause gastric irritation, but might also arise because people take analgesics to relieve their dyspepsia. Reverse causation is less of a concern in cohort and case–control studies where risk factors are usually ascertained in a way that ensures they precede health outcomes in time.

6.6 Randomized Controlled Experiments

In a randomized controlled experiment (also known as a randomized controlled intervention study), eligible subjects who agree to participate are randomly assigned to receive one of two or more treatments or exposures. Subsequent health outcomes are then systematically ascertained and compared between the treatments or exposures. Studies of this sort provide the strongest evidence of causation that is possible from investigation of statistical associations in human populations. This is because if randomization is carried out on a sufficiently large scale, important confounding, even by unrecognized causes of the health outcome, becomes statistically unlikely. Randomized controlled experiments are used most commonly to assess the benefits and risks of drug treatments, but can also be applied to assess risks of minor, reversible, toxic effects of chemicals. For example, randomized controlled experiments have been used to investigate the effects of food-colouring agents on hyperactive behaviour in children (McCann *et al*., 2007).

6.7 Nonrandomized Experiments

Even where it is ethically acceptable deliberately to expose human subjects to a chemical, investigation of its effects by a randomized experiment may not be practical. For example, studies have been conducted in the past to assess the impact of fluoridating drinking water on rates of dental caries in children. However, because the intervention was at the level of the water supply systems to communities, and only a few communities could be included in a single investigation, there would have been no benefit in allocating exposures randomly. Just by chance, potentially confounding factors, such as poor dental hygiene, could have ended up being distributed unevenly between the communities assigned to fluoridation and the control communities. In this situation, a stronger design was a nonrandomized experiment, in which allocation of communities to fluoridation was done in a purposive way that balanced as far as possible the distribution of potential confounding factors between fluoridated and control communities. In addition, the design could be further strengthened by collecting information on rates of dental caries in all communities both before and after the intervention. Analysis would then compare the change in rates of dental caries following fluoridation in the communities that received the intervention with that in the control communities over the same period. Important confounding would only occur if exposure to major causes of dental caries changed differentially over the course of the study in the intervention as compared with the control communities.

The major strength of nonrandomized experiments as compared with cohort studies is that the investigator controls the exposure that is delivered through the intervention. However, like observational studies, nonrandomized experiments are liable to unrecognized confounding effects.

7 INTERPRETATION OF EPIDEMIOLOGICAL STUDIES

Unlike toxicological experiments in the laboratory, epidemiological studies, especially if observational rather than experimental, generate relatively 'dirty' data. Their findings must therefore be interpreted with care. Two studies that apparently address the same question can sometimes generate results that are quite different. In evaluating epidemiological studies and considering why findings are discrepant, six points of interpretation should be considered.

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7.1 Classification of Exposure

One reason for apparently discrepant findings may be that the exposures studied were not identical. In some cases, the differences may be clear from the way in which exposure was defined. For example, work for at least 12 months in the manufacture of boats from glass-reinforced plastics gives substantially different cumulative exposure to styrene from short-term employment in a styrene-producing chemical plant. In other cases, differences may be less obvious. Thus, two studies might both derive risk estimates for cumulative exposures to styrene in the range 10–90 ppm-years, but in one, most of the individual exposures so classified might be at the lower end of the range, whereas in the other, they were near the upper end.

7.2 Classification of Health Outcome

In the same way, two studies, although seemingly addressing the same health outcome, may differ importantly in the mix of cases investigated. For example, one case–control study of nasopharyngeal carcinoma and exposure to wood dust might include predominantly patients with squamous cell tumours, while in another, most of the cases had anaplastic cancers. If wood dust affected the risk of these histological types differentially, then the two studies could produce quite different estimates of risk.

7.3 Bias

Sources of bias and their possible impact on parameter estimates are always a major consideration in the interpretation of epidemiological data. If two studies differ importantly in their biases, this could lead to substantial differences in their results.

7.4 Chance

Chance is another possible explanation for apparently discrepant findings. The confidence intervals around parameter estimates give a good indication of their associated statistical uncertainty, and when this is taken into account, discrepancies may seem less remarkable. Sometimes formal statistical tests are carried out to explore whether heterogeneity of parameter estimates between studies is greater than might reasonably be expected by chance.

7.5 Confounding

Differences in the impact of confounding factors are another reason why studies may give different results.

7.6 Effect Modification

Effect modification occurs when the RR associated with a risk factor varies according to the presence or level of another exposure (an effect modifier). For example, the risk of bladder cancer from occupational exposure to aromatic amines has been found to vary according to genetically determined differences in activity of the enzyme, *N*-acetyl-transferase (Yuan *et al*., 2008). In other words, acetylator status modifies the effect of aromatic amines on risk of bladder cancer.

Differences in the distribution of effect modifiers are another reason why risk estimates for an association between a risk factor and health outcome can vary between studies.

If these six points of interpretation are carefully considered, it is normally possible to draw useful conclusions from epidemiological data, even when there are clear limitations in study designs. Importantly, however, results should always be viewed in context, taking into account the biological plausibility of different possible explanations for what has been found. Thus parallel toxicological findings are often a major consideration in the evaluation of epidemiological observations. Similarly, relevant epidemiology, where available, can substantially enhance the interpretation of some toxicological research.

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NOTES

[a.] Diseases are the health outcomes most commonly studied in epidemiology, and unless otherwise stated, the discussion in this section assumes that disease is the health outcome of interest. Rates of other dichotomous health outcomes such as illness and disability can be defined analogously.

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Aviation Toxicology

Thomas C. Kupiec and Vishnu Raj

CONTENTS

1 INTRODUCTION

Air travel has become an increasingly popular modality of long-distance travel for the majority of people. In recent years, a source of concern with the increase in civil and military aviation traffic is the effect of the exponential rise in the use of prescription medications. The detrimental effect of drugs and alcohol on driving performance is well documented. The complexities of aviation tasks create potentially more devastating consequences, with subtle errors by an impaired pilot. Both prescription and illicit drugs can lead to impaired performance and subsequent accidents. In addition, the medical condition for which a drug is prescribed may itself lead to accidents. Presence of drugs in biological specimens obtained from aircraft accidents may indicate exposure to drugs, as well as the possible medical conditions for which the medication might have been taken (Chaturvedi and Canfield, 1997).

The field of aviation toxicology addresses the impact of prescription and illicit drugs on pilot performance, as well as the putative role of these agents in the cause or manner of death in aviation fatalities. Applications of aviation toxicology are vast, and range from human performance to accident reconstruction efforts. Toxicological analysis of the post-mortem specimens usually includes identification of illicit drugs, controlled substances, including Schedules I–V (SI–V), prescription and nonprescription (over-the-counter (OTC)) drugs and ethanol (Chaturvedi *et al*., 2005). Examples of SI–II substances include amphetamines, cocaine and marijuana and SIII–V substances include benzodiazepines, phentermine and propoxyphene (Chaturvedi *et al*., 2005). Antidepressants, antihypertensives and anticonvulsants are example of prescription drugs, and antihistamines, antipyretics and decongestants are examples of nonprescription drugs (Chaturvedi *et al*., 2005).

In aviation toxicology, analysis of the relative levels of hydrogen cyanide and carbon monoxide (CO) among passengers and crew, may help establish fire patterns (Rainford and Gradwell, 2006). Additionally, in several situations, owing to the severity of physical trauma, toxicological examination may provide the only evidence of pathological conditions that could produce sudden incapacitation, such as hypertension and epilepsy (Rainford

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and Gradwell, 2006). Toxicological analysis of specimens from aviation accident victims necessitates a thorough investigation for the presence of prescription, OTC and illicit drugs, environmental contaminants and toxins, as well as the identification of artifacts such as post-mortem ethanol. This investigation could assist in the elucidation of human factors that contributed to the aviation accident.

Contrary to conventional forensic toxicology, drugs in aviation forensic toxicology are usually found in therapeutic to subtherapeutic levels (Chaturvedi and Canfield, 1997). Therefore, owing to their longer window of detection, the identification and determination of drug metabolites assumes a critical role in air-accident investigations. In some cases, the levels of parent drug and active metabolites may also help establish degree of intoxication.

2 ANALYTICAL TECHNIQUES

Drugs and chemicals that are commonly analysed include ethanol, stimulant and depressant drugs including prescription and illicit drugs, CO, hydrogen cyanide, solvents, fuels and insecticides. Screening for the presence of pesticide or herbicides and cholinesterase inhibition may help establish accidental exposure during crop spraying (Rainford and Gradwell, 2006). The collection of a variety of tissues and fluids is integral to successful testing. In aircraft fatalities, the availability of fluids is often impossible due to high impact forces, however; whenever possible, an adequate quantity of blood from two separate sites, sterile urine from an intact bladder and bile and vitreous humour (VH) should be obtained (Rainford and Gradwell, 2006). **Table 1** provides a listing of specimen suitability characteristics for analyses of different drugs. Samples should preferably be obtained from all accident victims. Specimens from passengers may help serve as controls for specimens obtained from flight crew, especially in instances of post-mortem ethanol production (Rainford and Gradwell, 2006).

Specimens may be subjected to various extraction techniques such as liquid–liquid and solid-phase extraction. Various techniques are available to extract drugs from tissue and these usually involve the physical breakdown of tissue with a homogenizer or blender, followed by protein precipitation, direct solvent extraction or further tissue breakdown by acid hydrolysis Mayes (1982). Osseleton introduced an extraction technique using a nonspecific proteinase to digest tissue proteins and release the drug into solution. A far higher yield was evident for the enzymatic method relative to acid hydrolysis Mayes (1982).

Headspace gas chromatography (GC) is used for the analysis of ethanol and screening for volatile compounds (Rainford and Gradwell, 2006). CO concentrations may be estimated by the spectrophotometric analysis of carboxyhaemoglobin (COHb) using a CO-oximeter (Instrumentation Laboratories (UK) Limited, Warrington, UK). However, with contaminated samples or decomposed blood, GC or derivative spectrophotometry is required. Cyanide (CN^-) is separated from acidified blood using Conway diffusion and analysed by colorimetry or GC.

Specimens are screened for drugs using immunoassay, GC and high-performance liquid chromatography (HPLC) technologies. Confirmation is performed using gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry. Mass-selective detection is essential for the identification of certain drugs (Rainford and Gradwell, 2006). Urine is preferred for the screening of prescription and OTC drugs using HPLC and GC/MS. Certain additional drugs—for example, paracetamol (acetaminophen), salicylates, quinidine, theophylline, phenytoin and propoxyphene—are also screened in urine by fluorescence polarization immunoassay (FPIA) (Chaturvedi *et al*., 2003). In the absence of urine, other specimens are screened for drugs of abuse using radioimmunoassay (RIA). The order of specimen preference for the RIA-based abused-drug screening is bile, blood, liver, kidney and other tissues (Chaturvedi *et al*., 2003).

Drugs of abuse including amphetamines, methamphetamine, phencyclidine, cocaine, opiates, cannabinoids, barbiturates and benzodiazepines are commonly screened by FPIA. The difference between the drug screen in forensic toxicology and aviation toxicology is that the forensic toxicologist is principally concerned with drugs at the overdose level (Mayes, 1982). In aviation toxicology, many drugs may impair performance at or below therapeutic levels and therefore the drug screen in aviation toxicology must be capable of detecting and identifying drugs at very low levels nanogram or picogram (Mayes, 1982).

Depending on the types and quantity of received samples, COHb, blood CN^-, alcohol/volatile, glucose and haemoglobin A1c (HbA1c) analysis and drug screening batches are prepared and submitted for analysis. Batches are prepared by transferring an aliquot of the specimens into test tubes (Chaturvedi *et al*., 2003). Batches also contain aliquots from at least two quality assurance/quality-control (QA/QC) blind samples—one negative control and one positive control (Chaturvedi *et al*., 2003). The controls are consistent with batch types, case aliquot matrices and analytes (Chaturvedi *et al*., 2003). Analysts are blinded to the location and the type or quantity of analyte(s) present in the QA/QC blind aliquots of a particular batch, and if the initial screening analysis is positive, the specimens are confirmed and quantitated (Chaturvedi *et al*., 2003).

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Table 1 Specimen characteristics for toxicological analysis (Civil Aerospace Medical Institute (CAMI))

Specimen type†	Optimal amount‡	Analysis type suitability
Blood	40 ml	Green-top tube blood for carboxyhaemoglobin (COHb), blood cyanide CN^-, haemoglobin A_1c HbA_1c) and drug analyses Gray-top tube blood for ethanol and drug analyses
Urine	100 ml	Ethanol, drug and glucose analyses
Vitreous humor	2 ml	Ethanol and glucose analyses
Spinal fluid	Available amount	Ethanol and, perhaps, drug analyses
Bile	10 ml	Abused drug radioimmunoassay (RIA) screening, when urine is not available
Gastric contents[P]	100 g	Need-based drug analysis
Liver	500 g	Ethanol and drug analyses
Muscle	300 g	Ethanol and drug analyses
Spleen	150 g	Ethanol and drug analyses
Lung	100 g	Ethanol and drug analyses
Kidney	100 g	Ethanol and drug analyses
Brain	100 g	Ethanol and drug analyses
Heart	50 g	Ethanol and drug analyses

† Drugs/substances (tablets, caplets, capsules, powder, and/or liquid) found at the accident scene are also occasionally submitted. Analyses of drugs/substances may be helpful in the investigation of accidents, particularly when they are also found in the submitted biological samples.

‡ An adequate amount considered is half of the optimal amount. Any specimen submitted in the amount less than the adequate amount is considered to be inadequate.

3 ETHANOL

Ethanol-induced impairment and the incompatibility of operating a motor vehicle under the influence of ethanol are both well recognized. The effect of ethanol may be more deleterious in aviation, where slight errors have potentially lethal consequences. In addition, effects of ethanol may also be more pronounced at higher altitudes. Alcohol use by pilots is a highly contentious matter, especially during the investigation of aviation disasters (Li *et al*., 2005). Pilot performance has been shown to be impaired at blood alcohol concentrations (BACs) as low as $0.01\,g\,dl^{-1}$, and the number and magnitude of errors increases in proportion to the BAC. **Table 2** delineates the adverse effects of alcohol on piloting performance (Cook, 1997).

A study by Canfield *et al*. (1993) reported 79 ethanol-positive ($>0.04\,g\,dl^{-1}$) specimens among 975 specimens of victims of fatal aircraft crashes, received by the US Civil Aeromedical Institute during 1989–1990 (Canfield *et al*., 1993). Based on the distribution of ethanol in urine, vitreous, blood and tissue it was determined that 21 of the positive cases could be attributed to post-mortem synthesis of ethanol, whereas 22 reflected alcohol consumption, and 36 could not be interpreted satisfactorily (Canfield *et al*., 1993). Two of the cases reported had post-mortem ethanol levels as high as $0.15\,g\,dl^{-1}$ (Canfield *et al*., 1993). Aviation fatalities present several difficulties during the recovery of the bodies and sample collection for toxicological analysis (Kugelberg and Jones, 2007). The post-mortem examination and the toxicological analyses are complicated due to extensive trauma, including stomach rupture and bursting of the bladder (Kugelberg and Jones, 2007).

Ethanol concentration measured in blood drawn from different sampling sites tends to exhibit greater variation than expected from inherent variations in the analytical methods used. It has been reported that ethanol concentrations in heart blood are generally higher than in blood from a peripheral vein, although in any individual case there are likely to be considerable variations (Kugelberg and Jones, 2007; Pelissier-Alicot *et al*., 2006; Chikasue *et al*., 1988). Femoral blood is least susceptible to post-mortem changes, and is hence the preferred specimen. The collection of additional specimens, such as urine and VH, is preferable whenever possible (**Table 1**). Blood from the intact chambers of the heart is considered suitable as a supplementary specimen to compare with femoral BAC or when the volume of the latter specimen might be limited. (Kugelberg and Jones, 2007). The worst possible specimen is a blind-stick into the chest or

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Table 2 Adverse effects of alcohol on piloting performance

Blood alcohol concentration ($mg\,dl^{-1}$)	Impaired task performance
10–30	• Terrain separation • Aircraft descent • Performance during angular acceleration ± dim lighting
30–50	• Progressive impairment of the above • Management of heavy workload conditions • Tracking radio signals • Target tracking • Airport traffic control vectoring • Flight coordination and configuration • Traffic observation and avoidance • Stimulus response tasks involving use of hand sticks and foot pedals • Complex coordination • Short-term memory • Reaction time • Performance during linear acceleration ($5\,G_2$)
50–120	• Progressively larger and more consistent impairment of all the above • Oculovestibular function • Performance during linear acceleration ($3\,G_2$)

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blood scooped from the chest cavity on opening the body (Kugelberg and Jones, 2007).

Specimen collection tubes should contain preservative such as sodium or potassium fluoride to ensure a final concentration of 1–2% w/v (Kugelberg and Jones, 2007). The fluoride ion is seemingly effective in inhibiting the activity of several kinds of enzymes, such as enolase, an enzyme important for the fermentation initiated by yeasts, fungi and so on. All containers used to collect autopsy blood specimens should be carefully labelled with the type of material, the anatomical site of origin, the date and time and important details of the case including identification of the deceased. Fluid specimens intended for determination of volatiles like ethanol should have a small air-space to minimize evaporation. Finally, the containers should be made airtight with tamper-proof seals and, if possible, transported to the laboratory refrigerated (4–8°C). Maintenance of accurate specimen chain-of-custody is integral to future legal proceedings (Kugelberg and Jones, 2007).

Alcohol is typically analysed by headspace GC which offers the benefit of protecting the chromatographic column from overloading with nonvolatile blood constituents (Kugelberg and Jones, 2007). Headspace GC entails the removal of a portion of the vapour phase in equilibrium with the biological specimen, which is kept in an air-tight glass vial at a constant temperature of 50 or 60 °C (Kugelberg and Jones, 2007). Matrix effects are minimized by diluting the blood specimen 1:5 or 1:10 using an aqueous solution of a suitable internal standard, such as *n*-propanol or *t*-butanol. Trace amounts of volatile substances may be analysed by saturating both the biological specimens and the aqueous standards with an inorganic salt such as sodium chloride or sodium sulfate. This salting-out technique raises the vapour pressure of nonelectrolytes (e.g. ethanol) in the flask and boosts the sensitivity of the headspace GC analysis. Standard addition may be performed to minimize matrix effects, when ethanol or other drugs are determined in a complex or unusual matrix (Kugelberg and Jones, 2007).

Using highly specific gas chromatographic methods of analysis, ethanol can be determined reliably even in the presence of potential interfering substances (e.g. acetaldehyde, ethyl acetate, *n*-propanol, isopropanol, *n*-butanol), that might be produced during decomposition. The biological specimens sent for analysis of ethanol should be analysed in duplicate on two different chromatographic systems, thus providing different retention times for ethanol and internal standard. The use of dual internal standards (e.g. *n*-propanol and *t*-butanol) to dilute autopsy blood specimens, has also been recommended. The tertiary alcohol (*t*-butanol) is recommended because under some circumstances small amounts of *n*-propanol might be produced during decomposition and putrefaction processes (O'Neal *et al.*, 1996).

The precision of routine blood-alcohol analysis is high and interlaboratory coefficients of variation (CVs), according to several studies, are only 3–5% compared with within laboratory CVs of less than 1% (Kugelberg and Jones, 2007). However, higher CVs are evident upon comparison of blood-ethanol concentrations from different sampling sites, and this may be partly explained by the varying fluidity of the specimens and the amounts of plasma, red cells and clots present (Kugelberg and Jones, 2007). Therefore, this site-related uncertainty needs to be considered during interpretation of post-mortem alcohol concentrations and comparison with statutory levels. The significance of a relatively low post-mortem BAC ($<0.03\,g\,dl^{-1}$) in the absence of corroborating evidence from analysis of ethanol in urine or VH, is debatable. A post-mortem BAC of less than $0.01\,g\,dl^{-1}$ should be reported as negative and is close

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to the limit of quantitation of most routine headspace GC methods.

3.1 Post-Mortem Ethanol Formation

Although the qualitative and quantitative determination of ethanol in post-mortem specimens is a relatively simple analytical procedure, the interpretation of post-mortem BAC and the consequent extrapolation of ante-mortem concentration and the individual's degree of impairment is fraught with difficulties (Kugelberg and Jones, 2007). Post-mortem diffusion of alcohol from the stomach to central blood-sampling sites is a complicating factor if a person died shortly after a period of heavy drinking (Kugelberg and Jones, 2007). In certain situations, especially involving decomposed bodies, ethanol might be produced post-mortem, due to microbial activity and fermentation of glucose. The question of post-mortem ethanol production often occurs during the investigation of mass transportation fatalities. Therefore, findings of positive BAC need to be interpreted with caution because of the heightened risk of post-mortem synthesis (Kugelberg and Jones, 2007).

Extensive trauma to a body has been known to increase the potential for spread of bacteria and heightens the risk of ethanol production after death (Kugelberg and Jones, 2007). Blood-ethanol concentrations as high as $0.19\,\mathrm{g\,dl^{-1}}$ have been reported in post-mortem blood after traumatic events such as explosions, with no evidence supporting ante-mortem ingestion of ethanol (Kugelberg and Jones, 2007). In these situations, the comparison of ethanol concentrations in blood from different sampling sites, as well as in alternative body fluids, such as urine, VH and cerebrospinal fluid (CSF) can help resolve the origin of ethanol in dead bodies (Kugelberg and Jones, 2007).

Following death, the integrity of cell membranes and tissue compartments gradually disintegrate through the action of various digestive enzymes (autolysis) (Kugelberg and Jones, 2007). During this process, bacteria from the bowel invade the surrounding tissue and vascular system. Glucose concentrations increase post-mortem, therefore providing a substrate for microbial synthesis of ethanol. Low concentrations of ethanol ($\leq 30\,\mathrm{mg\,(100\,ml)^{-1}}$) are more likely to be formed post-mortem than high concentrations (Kugelberg and Jones, 2007). Speedy recovery and refrigeration of the bodies helps to prevent microbial synthesis of ethanol.

Urine can be beneficial for alcohol analysis, owing to its high water content and a lower risk of post-mortem microbial invasion of the urinary bladder, relative to blood specimens. Additionally, the urine produced by healthy individuals does not contain significant amounts of glucose (the substrate for post-mortem ethanol synthesis). The use of urine is contraindicated if the deceased suffered from diabetes and glycosuria. An elevated urinary ethanol concentration in a specimen from a diabetic, and a negative blood ethanol concentration, is usually indicative of post-mortem urinary synthesis of ethanol due to the fermentation of glucose. If available, the determination of glucose in vitreous fluid is useful to corroborate the hypothesis of glycosuria.

Urine should be sampled directly from the intact bladder by penetrating the organ with a sterile syringe and needle and transfering to a container with a fluoride preservative present (1–2%) before shipment for analysis of ethanol (Kugelberg and Jones, 2007). Calculation of the ratio of UAC (urine-alcohol concentration)/BAC furnishes useful information about the status of alcohol absorption at the time of death. A ratio less than or close to unity suggests incomplete absorption of alcohol in all body fluids at time of death, therefore implying fairly recent drinking, with some of the ingested alcohol probably remaining unabsorbed in the stomach (Kugelberg and Jones, 2007). A ratio of 1.25 or more suggests that absorption and distribution of ethanol was complete by the time of death.

Vitreous fluid is a useful specimen for the post-mortem analysis of ethanol, drugs and endogenous biochemical constituents of the body. The main advantage of the vitreous over blood, is that anatomically it is remote from the gut and therefore less prone to bacterial contamination, especially in severe trauma and decomposition cases.

The mean vitreous humor/blood ratio of ethanol has been reported to be very close to values expected from the distribution of water in these two biological specimens, namely about 1.15–1.20:1 (Kugelberg and Jones, 2007). Correlation coefficients between VH and blood have been reported to be range between $r = 0.936$ and $r = 0.979$.

In the absence of traditional specimens, other biological specimens such as liver, brain, skeletal muscle, spleen, bone marrow, CSF and synovial fluid, as well as bile, may be used for toxicological analysis (**Table 1**) (Kugelberg and Jones, 2007). The water and lipid content of these more unusual body fluids and tissues and the stability of ethanol after sampling are important to consider, for better interpretation of the results. Organs such as liver and kidney may retain some enzymatic activity after death as the body cools, and depending on ambient temperature and availability of cofactor nicotinamide adenine dinucleotide (NAD^+) ethanol might be metabolized to some extent after death (Kugelberg and Jones, 2007). Some body organs and tissue are probably more susceptible than others to putrefaction processes, depending on their glucose and glycogen content and proximity to the bowel, thus facilitating spread of bacteria and fungal growth. In decomposed or exhumed bodies, skeletal muscle is probably the most appropriate specimen for forensic analysis of ethanol and other drugs (**Table 1**) (Kugelberg and Jones, 2007).

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3.2 Biochemical Markers

In order to distinguish between ante-mortem ethanol ingestion and the post-mortem synthesis of ethanol, biochemical indicators such as nonoxidative metabolites of ethanol have been used. Trace quantities of ethyl glucuronide (EtG), a minor metabolite of ethanol, are produced during enzymatic metabolism of ethanol (<0.1% of dose) but more importantly, EtG is evidently not produced by the action of microbes and yeasts on glucose. EtG, if present in measurable quantities, would imply that the ethanol had been metabolized during life. However, an implication of an elevated ante-mortem BAC may not be drawn from the presence of EtG, because the elimination half-life of EtG is more prolonged relative to ethanol. Analyses of EtG and ethyl sulfate are of special interest in occupational medicine for proof of abstinence among individuals performing highly skilled tasks and safety-sensitive work, for example, surgeons or airline pilots (Kugelberg and Jones, 2007).

Depending on the dose ingested, urinary EtG can disclose recent drinking for about 6–10 hours after ethanol is no longer measurable. This presence of EtG and its longer elimination time could be misinterpreted if post-mortem ethanol had been produced, and the ante-mortem BAC had reached zero due to metabolism. This scenario could probably be incorrectly interpreted to mean that the individual had alcohol in the blood at the time of death (Kugelberg and Jones, 2007). A study by Schloegl *et al*. found that EtG concentrations remained fairly constant in urine samples held in air-tight containers when these were stored at room temperature for five weeks (Schloegl *et al*., 2006). An important finding was that when EtG-free blood and liver samples were spiked with ethanol ($100\,mg\,(100\,ml)^{-1}$) there was no new *in vitro* formation of EtG during storage (Schloegl *et al*., 2006).

The urinary metabolites of serotonin, namely 5-hydroxytryptophol (5HTOL) and 5-hydroxyindoleacetic acid (5HIAA) have also been used to resolve whether a positive BAC stems from ante-mortem ingestion of ethanol or post-mortem synthesis. Finding an elevated urinary ratio of 5HTOL:5HIAA (>15) indicates that ethanol has undergone metabolism, which points to ante-mortem ingestion (Kugelberg and Jones, 2007). An article by Johnson *et al*. describes the accurate determination of the origin of ethanol origin in post-mortem urine samples, using an improved GC/MS method for simultaneous determination of 5HTOL and 5HIAA (Johnson *et al*., 2004). This approach has been used for investigating the origin of ethanol in victims of civil-aviation disasters (Johnson *et al*., 2004)

The microbial synthesis of endogenous ethanol can lead to the production of other low-molecular-weight volatiles, including higher aliphatic alcohols (isoamyl alcohol, *n*-propanol, isopropanol, *n*-butanol), acetaldehyde and propionic acid, as well as other organic acids (Kugelberg and Jones, 2007). Among these, *n*-butanol and isobutyric acid are considered to be reliable indicators of putrefaction, and their presence in blood may question the concentration of ethanol (Kugelberg and Jones, 2007; Caughlin, 1994). The use of *n*-propanol as an indicator of putrefaction, has been proposed; however, the quantitative relationship between *n*-propanol detected and the amount of ethanol produced in post-mortem blood was not strong, with the concentrations of *n*-propanol 15–20 times less than that of ethanol (Moriya and Hashimoto, 2004).

3.3 Interpretation

Traditional dose : plasma concentration relationships are not practicable for use in post-mortem toxicology owing to protein binding, drug sequestration in tissue depots and ill-defined volumes of distribution (Kugelberg and Jones, 2007). A well-recognized source of concern in post-mortem toxicology is the post-mortem redistribution of ethanol and drugs, due to passive diffusion through the stomach wall, thereby falsely increasing the concentration of these substances in surrounding tissues and blood (Kugelberg and Jones, 2007; Iwasaki *et al*., 1998). Therefore, the concentration of a drug determined during autopsy may not necessarily reflect the concentration present at the time of death (Kugelberg and Jones, 2007). Post-mortem displacement of a relatively small amount of substance from a tissue depot, for example, the liver, into the vascular system could produce a substantial change in the drug concentration determined using central blood. The passive diffusion of alcohol across a mucous membrane depends on the concentration gradient across the membrane and its permeability.

In cases of traumatic deaths, such as in an air crash, there is an increased risk of gastric alcohol contaminating the surrounding tissues. However, post-mortem diffusion and redistribution appears to be more of a concern with drugs rather than ethanol, especially those drugs with large volumes of distribution, such as tricyclic antidepressants (TCAs). Drugs that are sequestered in a particular tissue or when concentrations are sensitive to changes in pH after death are especially problematic (Kugelberg and Jones, 2007).

However, post-mortem ethanol production is of significant concern, especially in mass transportation and aviation disasters. Two methods used to establish whether measured ethanol resulted from consumption or post-mortem formation, are by the analysis of multiple specimens and the analysis for other volatile substances (Klette *et al*., 1992). Comparing the concentrations of ethanol in different specimens, such as cardiac and femoral blood, urine and VH, is integral to the rule out endogenous ethanol production. A positive blood ethanol, in combination with a negative VH or urine ethanol, two specimens resistant to the putrefaction

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process, would usually indicate that the ethanol was formed post-mortem (Klette *et al*., 1992). In addition, various biomarkers have been developed to help establish whether a positive blood ethanol arose from post-mortem synthesis or ante-mortem ingestion. Klette *et al*. in their study, reported the presence of acetaldehyde and/or *n*-propanol, two volatiles which have been observed in decomposing specimens (Klette *et al*., 1992).

4 INHALATION TOXICOLOGY

Upon exposure of aircraft cabin occupants to fire effluent, the first hazard encountered is usually smoke, containing particulates and toxic gases, causing immediate visual obscuration and painful irritation of the eyes and respiratory tract (Purser, 1996). In smouldering or small, confined, in-flight fires, where the yields of organic irritants and acid gases are likely to be high and exposure times long, the distressing effects of irritants, lung inflammation and asphyxia induced by CO are likely to be the main hazards. Types and amounts of combustion products generated depend upon the chemistry of the materials involved and the environmental conditions present during a particular fire (Chaturvedi and Sanders, 1996). Post-crash fires tend to develop rapidly into a flashover, and provide very limited time for escape before the onset of lethal conditions due to the effects of toxic smoke and heat. Combustion of cabin lining and seating materials increases the concentrations of toxic gases, especially CO and hydrogen cyanide, thereby incapacitating any remaining cabin occupants (Purser, 1996).

Besides visual obscuration, smoke components produce adverse biological effects. Exposure to CO and hydrogen cyanide, two components of smoke, may lead to incapacitation and eventual death (Chaturvedi and Sanders, 1996). CO binds haemoglobin and thus interferes with oxygen transport, whereas hydrogen cyanide inhibits oxygen utilization at the cellular level. Simultaneous exposure to both gases can produce a combined effect of severe hypoxia (Chaturvedi and Sanders, 1996). Carbon dioxide increases the respiratory rate and results in an increased uptake of the more toxic gases. The diminished oxygen at fire scenarios reduces oxygen availability and utilization (Chaturvedi and Sanders, 1996).

5 CARBON MONOXIDE AND CYANIDE

CO and CN^- are combustion products, and are often investigated in aviation accident investigations. CO in the flight crew may suggest a causal contamination possibly due to faulty heat exchangers (Rainford and Gradwell, 2006). Relative to oxygen, CO has approximately 200 times higher affinity for haemoglobin and binds it to form COHb, therefore leading to hypoxia. COHb has a half-life of 4–5 hours, and increased levels of COHb are usually attributable to smoke inhalation. CO is analysed as COHb and hydrogen cyanide as CN^- (Canfield *et al*., 2005).

Analysis of CO may help determine if the accident victim was exposed to an in-flight/post-crash fire or faulty heating/exhaust system (Lewis *et al*., 2004). Concentrations of haemoglobin in nonsmokers may be as high as 3%, while smokers may achieve levels up to 10%. Therefore, 10% saturation is a useful cut-off to separate those who have inhaled toxic atmospheres from those who have not. The fatal threshold for fire deaths has been taken as 50%, but fatalities may occur with saturations ranging from 10 to 90%. The toxic effect of high concentrations of CO in inhaled air may manifest by the inhibition of the cytochrome systems (Rainford and Gradwell, 2006).

COHb concentrations above 30% and CN^- concentrations above $1\,\mu g\,ml^{-1}$ have been reported to cause toxic effects resulting in the incapacitation of pilots (Canfield *et al*., 2005). However, the mere presence of COHb and CN^- in blood, even in potentially toxic or lethal concentrations, does not necessarily indicate that exposure to CO and hydrogen cyanide caused the death (Canfield *et al*., 2005). Elevated COHb and CN^- levels in post-mortem blood of individuals who died on impact would imply an in-flight fire, because CN^- is not produced from an exhaust leak (Canfield *et al*., 2005). An exhaust leak would be suspected in those cases with no fire, no CN^-, and blood COHb levels above 30% (Canfield *et al*., 2005).

A study on military aircraft fatalities from 1986–1990 reported greater than 10% CO saturation levels in 4% of the 535 cases where appropriate specimens were collected (Klette *et al*., 1992). These relatively low levels of COHb saturation are characteristic of most noninstantaneous deaths in post-crash fires involving military aircraft (Klette *et al*., 1992).

Canfield *et al*. investigated the presence of COHb and CN^- in 5945 aircraft fatalities, and found 223 (4%) of the cases with COHb of $\geq$10% (Canfield *et al*., 2005). Of the 223 cases, fire was reported in 201, no fire in 21, and undetermined fire status in one (Canfield *et al*., 2005). CN^- concentrations were at or above $0.25\,\mu g\,ml^{-1}$ in 103 of the 201 fire-related cases (Canfield *et al*., 2005). All nonfire cases with COHb $\geq$30% were associated with exhaust leaks (Canfield *et al*., 2005).

CO may be analysed by spectrophotometry or GC (Klette *et al*., 1992). Spectrophotometric methods of analysis are based on the comparison of COHb to reduced haemoglobin. A reducing agent is commonly employed in the spectrophotometric analysis of COHb to reduce any oxyhaemoglobin and methaemoglobin (MetHb) present in the blood, thereby eliminating their potential interference with the absorbance of COHb (Lewis *et al*., 2004). However, very low levels of haemoglobin in the fluids may increase the likelihood of an artificially

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elevated COHb. In addition, with aged or putrefied post-mortem blood, the resistance of conversion of some of the haemoglobin pigments to reduced haemoglobin, may invalidate these methods.

GC is another technique that may be used for the analysis of CO, and there are several different GC methods available for the determination of CO in blood. Although GC methods are more time-consuming than spectrophotometric procedures, their utility is unparalleled when analysing samples that are unsuitable for ultraviolet (UV) methods because of post-mortem deterioration, coagulation or the presence of a substance that obscures the UV absorption pattern of COHb (Lewis *et al*., 2004). The most common and rapid GC method determines %COHb from a ratio of the CO peak area of CO-unsaturated blood to CO-saturated blood (Lewis *et al*., 2004). While this method works very well with preserved blood samples, it does encounter limitations when analysing post-mortem blood that may have experienced some degree of deterioration (Lewis *et al*., 2004).

Post-mortem deterioration due to a lack of timely preservation, exposure to heat and as a result of the freezing of a blood sample at and above temperatures of $-30\,^{\circ}C$ can result in the oxidation of haemoglobin to MetHb (Lewis *et al*., 2004). MetHb does not bind CO, and since %COHb is calculated as a ratio of peak areas of the unsaturated and saturated samples, elevated levels of MetHb in a sample will result in a loss of CO-binding capacity, and will produce an erroneously high %COHb value (Lewis *et al*., 2004). Treating the sample with a reducing agent, such as sodium dithionite, will convert the MetHb back to haemoglobin, thus restoring the total CO binding capacity of the sample (Lewis *et al*., 2004).

6 GLUCOSE

The analysis of glucose may be a critical factor in helping establish the cause of the aviation fatality, especially among individuals with a history of hyperglycaemia. Hypoglycaemia may be a factor in aircraft accidents; however, the accurate determination of the glucose level in the blood of a fatally injured crew member may be difficult (DeHart and Davies, 1996). Depending on the location of collection, post-mortem variations in glucose levels may be evident. Glucose levels in the vitreous fluid of the eye do not change as rapidly, and therefore the vitreous is a suitable source for the estimation of post-mortem glucose (DeHart and Davies, 1996).

In addition, the VH constitutes an isolated pool of material suitable for many analyses and is far less susceptible to rapid chemical changes or contamination than the blood. Glucose concentration in VH is of particular interest when fatal hypoglycaemia or diabetes is suspected (Sippel and Möttönen, 1982). However, studies on VH have demonstrated occurrence of post-mortem glycolysis (Sippel and Möttönen, 1982).

The determination of both CSF glucose and lactic-acid values, has been proposed for the diagnosis of fatal diabetes mellitus and hypoglycaemia, since this method takes into account the breakdown of glucose to lactic acid after death (Sippel and Möttönen, 1982). The diagnosis of ante-mortem hypoglycaemia must be made with caution, owing to erratic and occasionally very marked post-mortem reduction in the vitreous glucose (Sippel and Möttönen, 1982). Sippel and Mottonen reported a rapid and irregular increase in the lactic acid content of VH after death, and therefore, the determination of vitreous glucose and lactate values may be valuable in the exclusion of hypoglycaemia as a contributory cause of death (Sippel and Möttönen, 1982). High levels of vitreous glucose and lactate are indicative of ante-mortem hyperglycaemia (Sippel and Möttönen, 1982). Relatively low glucose concentrations, on the other hand, may be suggestive of lactic acidosis. Therefore, the determination of VH glucose shortly after death (less than two hours), or the combined vitreous glucose and lactate values at one to 10 days post-mortem may be a valuable tool for diagnosing ante-mortem hyperglycaemia (Sippel and Möttönen, 1982).

Establishment of cause of death in hypoglycaemic or diabetic coma should be made in conjunction with anamnestic response, morphology, biochemical (glucose, lactate, HBA1c, ketonic bodies, insulin and C-peptide) and toxicological findings (Karlovsek, 2004). High glucose levels in VH (more than $234\,mg\,dl^{-1}$) or combined values of glucose and lactate in VH or in CSF over threshold values of 427 and $422\,mg\,dl^{-1}$ respectively, can be an indicator of the pre-mortem hyperglycaemic state with fatal outcome (Karlovsek, 2004). The determination of glycated haemoglobin, acetone and other ketone bodies improve the diagnostic values of the whole procedure (Karlovsek, 2004). Diabetic ketoacidosis (blood acetone $>0.3\,g\,l^{-1}$) is more often the cause of death of diabetic patients than the nonketotic hyperosmolal state. Hypoglycaemia is deemed fatal if the combined values are lower than $5.5\,mmol\,l^{-1}$ ($100\,mg\,dl^{-1}$) and cannot be excluded if they are lower than $8.9\,mmol\,l^{-1}$ ($160\,mg\,dl^{-1}$).

7 ANTIDEPRESSANTS

Antidepressant drugs are among the most widely used prescription medications, and they include selective serotonin reuptake inhibitors (SSRIs), monoamine oxidase inhibitors (MAOIs) and TCAs. Among these groups of antidepressants, SSRIs are the most frequently encountered. SSRIs have the potential to affect the central nervous system (CNS), and thus might impair performance and ultimately compromise aviation safety (Akin and Chaturvedi, 2003). Therefore, with the exception of a few, most of these drugs are not approved by aeromedical regulatory authorities for use by aviators,

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due to their potential for impairing performance and causing drug interactions (Akin and Chaturvedi, 2003). However, as shown in **Table 3**, SSRIs and antidepressants featured prominently among a list of medications detected in pilots following aviation accidents. As of 2003, five drugs—fluoxetine, sertraline, paroxetine, fluvoxamine and citalopram were classified as SSRIs (Akin and Chaturvedi, 2003).

Although SSRIs are relatively safe compared to other groups of antidepressants, they have the potential for drug interactions. All five SSRIs are extensively metabolized by the cytochrome P450 (CYP) enzyme system. SSRIs are known to inhibit CYP2D6-mediated metabolism with paroxetine exerting the greatest degree of inhibition and fluvoxamine the least (Mozayani and Raymon, 2004). Excluding citalopram, at higher concentrations, SSRIs have been know to inhibit their own metabolism by saturating their metabolizing CYP enzymes (Akin and Chaturvedi, 2003). Since patients with depression are frequently treated with multiple drugs, the inhibition of the metabolism of these drugs by SSRIs can lead to drug interactions, toxicity and even death (Akin and Chaturvedi, 2003). Blood concentrations of those other drugs could be higher than their typical therapeutic blood levels because of the SSRI-caused inhibition of their metabolism, and thereby their accumulation in the blood.

Fluoxetine is metabolized by CYP2D6, with CYP2C9 and CYP3A4 playing a contributory role (Hemeryck and Belpaire, 2002). Fluoxetine is a potent inhibitor of CYP2D6 and norfluoxetine has a moderate inhibitory effect on CYP3A4. Fluoxetine used concurrently with alprazolam may lead to a decrease in alprazolam clearance and concomitant increase in plasma levels plausibly due to reduction in alprazolam metabolism. Fluoxetine has also been known to inhibit the metabolism of carbamazepine by the liver, presumably due to CYP2D6 inhibition. Certain side effects of fluoxetine, including drowsiness, dizziness, abnormal vision, diarrhoea and headache, and so on, could affect pilot performance and become a factor in an aviation accident (Johnson *et al*., 2007).

A study by Johnson *et al*. determined the distribution of fluoxetine and its desmethyl metabolite, norfluoxetine, in various post-mortem tissues and fluids from 10 fatal aviation accident cases. Blood fluoxetine concentrations in these 10 cases ranged from 0.021 to 1.48 $\mu g\,ml^{-1}$ (Johnson *et al*., 2007). Akin *et al*. investigated the prevalence of SSRIs in pilot fatalities of civil-aviation accidents, using post-mortem samples from pilots involved in fatal civil-aircraft accidents (Akin and Chaturvedi, 2003). Samples were received from 4184 fatal civil-aviation accidents, and there were 61 accidents in which pilot fatalities had detectable levels of SSRIs

Table 3 Drugs detected in post-mortem specimens of pilots in aviation accidents

Psychotropic	#	Cardiovascular	#	Neurological	#
Alprazolam	8	Amlodipine	8	Carbamazepine	3
Amitriptyline	10	Atenolol	33	Phenytoin	11
Bupropion	14	Bisoprolol	4	Selegiline	1
Buspirone	1	Clonidine	1	Total	15
Chlordiazepoxide	5	Diltiazem	23		
Citalopram	13	Doxazosin	1		
Clomipramine	1	Flecainide	1		
Desalkylflurazepam	3	Labetalol	4		
Diazepam	29	Metoprolol	24		
Doxepin	2	Moricizine	1		
Fluoxetine	40	Nadolol	4		
Imipramine	10	Procainamide	3		
Lorazepam	1	Propranolol	9		
Mirtazapine	2	Verapamil	33		
Nefazodone	1	Total	149		
Nordiazepam	16				
Norfluoxetine	1				
Oxazepam	2				
Paroxetine	21				
Sertraline	26				
Temazepam	5				
Trazodone	4				
Venlafaxine	6				
Zolpidem	2				
Total	223				

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(Akin and Chaturvedi, 2003). Blood concentrations of SSRIs in the fatalities were 11–1121 ng ml^{-1}, for fluoxetine; 47–13 102 ng ml^{-1} for sertraline, 68–1441 ng ml^{-1} for paroxetine and 314–462 ng ml^{-1} for citalopram (Akin and Chaturvedi, 2003). In 39 of the 61 pilots, other drugs, such as analgesics, antihistamines, benzodiazepines, narcotic analgesics and/or sympathomimetics—and/or ethanol were also present (Akin and Chaturvedi, 2003). The authors noted that pharmacodynamic and pharmacokinetic interactions of SSRIs and their active metabolites with other drug(s) and ethanol could realistically lead to performance impairment and unpredictable pharmacological effects (Akin and Chaturvedi, 2003). Reduced oxygen availability at higher altitudes might lead to increased performance impairment and potential for adverse effects (Akin and Chaturvedi, 2003).

SSRIs have been known to inhibit their own metabolism, thereby altering the parent drug : metabolite ratios (Akin and Chaturvedi, 2003; Goeringer *et al*., 2000). These ratios are less at low SSRI doses in comparison with the ratios at high SSRI doses (Akin and Chaturvedi, 2003). Post-mortem blood concentrations of SSRIs may not necessarily reflect ante-mortem blood levels, owing to extensive post-mortem redistribution that occurs with these basic drugs (Pounder and Jones, 1990). During investigations of aviation accidents involving SSRIs, the post-mortem blood levels of SSRIs should be carefully evaluated and interpreted, particularly in the presence of other drug(s) and/or ethanol (Akin and Chaturvedi, 2003). Levels of SSRIs in other biological sample types cannot be accurately correlated with the degree of impairment or adverse effects, since such levels merely indicate the exposure of those victims to SSRIs (Akin and Chaturvedi, 2003).

8 ANTIHISTAMINES

First-generation H1-receptor antagonists are popularly used for alleviating allergy and cold symptoms, but these antihistamines cause drowsiness and sedation, thereby potentially impairing pilot performance (Sen *et al*., 2007). First generation antihistamines also depress the CNS, causing dizziness and sedation, diminished alertness, slowed reaction times and impaired performance on flight tasks, resulting in slowed reaction times, memory difficulties and impaired vigilance (Sen *et al*., 2007). Owing to these adverse effects, the first-generation antihistamines are not approved for use by civilian aviators (Silberman, 2003).

A recent study investigated the presence of the first-generation antihistamines in 5383 pilot fatalities of civil-aircraft accidents that occurred over a 16-year period. Specimens from 338 pilot fatalities were found to contain brompheniramine, chlorpheniramine, diphenhydramine, doxylamine, pheniramine, phenyltoloxamine, promethazine and triprolidine (Sen *et al*., 2007). Antihistamines alone were detected in 103 fatalities and present in combination with other drug(s) and/or ethanol, in an additional 235 fatalities. 191 fatalities was associated with diphenhydramine, followed by 112 with chlorpheniramine, and 50 with doxylamine (Sen *et al*., 2007). Antihistamines were determined to be the cause of 13 and a factor in 50 of the 338 accidents (Sen *et al*., 2007). Blood levels of the antihistamines were in the subtherapeutic to toxic range (**Table 4**). The detection of other psychotropic medications may imply concurrent use, with the potential for additive impairment of motor skills (Sen *et al*., 2007).

Interpretation of blood antihistamine concentrations must be made in conjunction with consideration of other parameters, such as the relationship between the plasma (serum) and the whole-blood drug concentrations, as well as potential for post-mortem redistribution of the antihistamines. In addition, the presence of other drugs and/or ethanol in the fatalities could also potentially affect antihistamine concentrations.

9 DRUGS OF ABUSE

Screening of blood or urine for drugs of abuse is mandatory following a fatal aviation accident. Positive

Table 4 Antihistamine concentrations found in civil aviation pilot fatalities

	Blood Concentration (ng ml^{-1})	
Antihistamines	Without other substance	With other substance(s)[a]
Brompheniramine	200 (n = 1)	5–100 (n = 7)
Chlorpheniramine	7–151 (n = 14)	4–6114 (n = 53)
Diphenhydramine	14–3800 (n = 38)	9–806 (n = 87)
Doxylamine	64–300 (n = 3)	10–1309 (n = 30)
Pheniramine	—	—
Phenyltoloxamine	—	4 (n = 1)
Promethazine	—	—
Triprolidine	—	—

[a]Includes other antihistamines, drugs and/or ethanol.

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screening results should always be confirmed and quantitated using GC/MS, to eliminate crossreactivity and false-positive results. Psychoactive drugs of abuse can be divided into:

- Narcotic analgesics such as morphine and heroin
- Stimulants such as cocaine and amphetamines
- Sedatives and hypnotics including barbiturates and benzodiazepines
- Psychedelics and hallucinogens, including cannabinoids and lysergic acid diethylamide (LSD)
- Antidepressants and antipsychotic agents.

Analysis of drugs of abuse in post-mortem cases requires certain special precautions, especially with regard to drug stability in various biological matrices.

In aviation disasters, it is highly plausible that tissues are exposed to the elements for protracted periods of time. Post-mortem chemical changes or metabolism may affect the interpretation of results (Drummer, 2004). The most common specimens used for the post-mortem analysis of drugs of abuse are blood, liver and urine. Other specimens such as VH and hair may also have important applications, whereas brain, muscle, fat, bone and pleural effusions have more specialist applications (Drummer, 2004). All drugs of abuse are detectable in bile, although buprenorphine, tramadol, other opioids, and benzodiazepines and colchicine appear to be present in higher concentrations than in blood (Drummer, 2004). **Table 5** provides a list of specimens and their relative advantages for the analysis of drugs of abuse.

Hair analysis has specialized applications for the analysis of drugs of abuse in order to establish evidence of long-term exposure. Segmental hair analyses have been used to determine degree of exposure to heroin and assess the risk of heroin use (Drummer, 2004). However, drug incorporation into hair is a complex phenomenon influenced by a number of factors, and therefore caution is advised during the interpretation of hair results (Drummer, 2004). Gastric contents may be used to establish a possible time of drug administration and to delineate oral from other routes of administration (Drummer, 2004).

Canfield *et al*. (2006), in a recent study, analysed specimens from 4143 deceased pilots who died as a result of an aviation accident. Psychotropic medications were found in 5%, cardiovascular medications found in 4% and neurological medications were found in 0.4% of the pilots in this study (**Table 3**) (Canfield *et al*., 2006). The accuracy of required reporting of medications by pilots was low. Of the 387 samples reviewed, only 8% had accurately reported the medications they were taking (Canfield *et al*., 2006). No medications were reported by 74% of the pilots found to be positive for drugs by post-mortem toxicology, and 18% had reported medications different from what was detected (Canfield *et al*., 2006). The authors also found that pilots taking psychotropic or neurological medications rarely reported the medication or medical condition on their employment medical application (Canfield *et al*., 2006).

9.1 Cocaine

Cocaine is a strong CNS stimulant that blocks the reuptake of catecholamines, particularly the excitatory neurotransmitter dopamine. Cocaine undergoes rapid absorption following smoking, snorting and intravenous administration, and is extensively metabolized to a variety of compounds, including the centrally inactive benzoylecgonine (BE), ecgonine and ecgonine methyl ester (Couper and Logan, 2004). The concurrent ingestion of cocaine and ethanol leads to the formation of cocaethylene, an

Table 5 Relative merits of post-mortem specimens for drugs of abuse

Specimen	Particular advantages
Blood/plasma/serum	Preferred specimen for most substances
Bile	Morphine, buprenorphine, tramadol, benzodiazepines, MDMA
Bone	Qualitative analysis of morphine, benzodiazepines, amphetamines
Brain	Centrally acting drugs, for example, morphine, cocaine, limited literature data
Fat	THC, and other drugs, but little literature to interpret results
Gastric contents	Orally administered drugs/poisons
Hair	All substances, particularly basic substances, and most metals
Muscle	Most drugs, however literature contains little data to interpret concentrations
Pleural effusion	Most drugs, but drugs subject to concentration changes, hence difficult to interpret
Vitreous humour	Ethanol, some biochemistries, for example, glucose, urea, creatinine

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active metabolite (Couper and Logan, 2004). Cocaine has a very short half-life of approximately 0.8 ± 0.2 hours, whereas BE has a longer half-life of six hours (Couper and Logan, 2004). Cocaine concentrations in blood are not usually associable with a degree of impairment, owing to factors such as drug tolerance and artifactual changes in cocaine upon storage (Couper and Logan, 2004).

Specimens from 3235 fatal aviation accidents over a 10 year period were analysed, among which 24 tested positive for cocaine and 17 tested positive for methamphetamine (Lewis *et al*., 2006; Botch *et al*., 2006). Among the 24 cocaine-positive fatalities, cocaine or a combination of cocaine and other drugs was found to be the probable cause of five accidents and a factor in four additional accidents (Lewis *et al*., 2006). On 12 September 1994, an airplane crashed onto the South Lawn of the White House, killing the pilot. Both cocaine and alcohol were detected in the pilot's blood (*http://www.fas.org/irp/agency/ustreas/usss/t1pubrpt.html*).

9.2 Amphetamines

Amphetamines constitute a group of sympathomimetic amines with strong stimulant properties. Stimulants drugs typically act by increasing the dopamine concentrations in the synaptic cleft. In contrast to cocaine, which blocks the reuptake of dopamine into the presynaptic neuron, the amphetamines stimulate presynaptic production and release of dopamine into the cleft (Logan, 2001). Amphetamines act both centrally and peripherally, resulting in increased pulse and blood pressure, pupillary dilation, excitation, increased alertness, anorexia, hyperactive reflexes and ataxia. Amphetamines are commonly used as recreational drugs, and chronic use results in sleep loss with associated exhaustion and sleepiness, anxiety, irritability, poorer psychomotor performance, exhaustion, depression, confusion, suspiciousness, paranoia, delusions, hallucinations, irrational behaviour and diminished attention and concentration (Logan, 2001).

Relative to other drugs, illicit amphetamine use does not appear to be as significant among pilots. Emonson and Vanderbeek (1995) studied the effects of amphetamines on pilot performance, and reported that as many as two-thirds of the pilots issued a 5 mg +-amphetamine pill every four hours to combat fatigue resulting from sustained flying operations, stated that they felt it made for safer flying operations (Emonson and Vanderbeek, 1995). Amphetamine (30 mg in divided 10 mg doses at four hour intervals) has been reported to sustain helicopter pilot performance during periods of sleep deprivation (over 48 hours without sleep) (Logan, 2002). Specimens from 3235 fatal aviation accidents over a 10 year period were analysed, among which 24 tested positive for cocaine and 17 tested positive for methamphetamine (Lewis *et al*., 2006; Botch *et al*., 2006). Fourteen of the 24 pilots tested positive for additional disqualifying substances including six for ethanol, five for methamphetamine, three for tetrahydrocannabinol (THC), two for benzodiazepines, one for methylenedioxymethamphetamine (MDMA) one for propoxyphene, two for fluoxetine and one for paroxetine (Lewis *et al*., 2006). Among the 17 methamphetamine-positive fatalities, methamphetamine or a combination was reported to be the probable cause of 12 accidents (Botch *et al*., 2006). Ten of the 17 tested positive for other disqualifying substances including seven for marijuana, five for cocaine, one each for ethanol, benzodiazepines, MDMA, fluoxetine and tramadol (Botch *et al*., 2006).

9.3 Cannabinoids

Cannabis (marijuana), a drug with psychedelic properties, is known to impair cognitive processes, divided attention and perceptual motor performance involving tasks such as fine motor performance and target tracking (Rainford and Gradwell, 2006). In a toxicological examination of 2326 occupants of aircraft mishaps, marijuana was the third most frequently detected drug as reported by DeHart and Davies, 1996. At recreational doses, effects include relaxation, euphoria, relaxed inhibitions, sense of well-being, disorientation, altered time and space perception, lack of concentration, impaired learning and memory, alterations in thought formation and expression, drowsiness, sedation, mood changes, such as panic reactions and paranoia, and a more vivid sense of taste, sight, smell and hearing (Couper and Logan, 2004). Short-term effects of marijuana use include problems with memory (**Table 6**) and learning, distorted perception, difficulty in thinking and problem-solving, and loss of coordination (Couper and Logan, 2004). Marijuana use also decreases the ability to concentrate and maintain attention, and leads to a dose-related impairment of hand–eye coordination (**Table 6**) (Couper and Logan, 2004).

A Federal Aviation Administration (FAA) report in 1973 indicted that in a two year period, 500 out of 1 000 000 applicants for a medical certificate admitted to previous marijuana use (Newman, 2004). Lewis *et al*. reported that marijuana and altitude could produce cumulative effects on some cognitive tasks, and found a reduction in work output and speed of response at altitudes of 8000 and 12 000 ft, as the dose of Δ^9-tetrahydrocannabinol (THC) increased (Newman, 2004). A study by Meacham *et al*. evaluated the performance of six pilots classed as infrequent marijuana users (Meacham *et al*., 1974). The pilots were given either a controlled dose of marijuana or a matched marijuana placebo, and each pilot operated a flight simulator sequence under both marijuana and placebo

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Table 6 Simulator studies on the effect of marijuana on pilot performance

Findings	THC levels[a, b]	Dose[a]
Impairment short term memory, attention and concentration up to 4 h	N/A	0, 0.9 mg kg^{-1} THC SM
Performance decrements up to 24 h; pilots no awareness of impairment	N/A	19 mg THC SM
Impairment with turbulent flight after high dose; impairment increased as task difficulty/complexity and pilot age increased	Serum THC 7 ng ml^{-1} at 1 h after high dose SM	0, 10, 20 mg THC SM
24 h impairment; pilots no awareness of decreased performance	N/A	0, 20 mg THC SM

[a]THC = Δ^9-Tetrahydrocannabinol.
[b]N/A = Not applicable; SM = Smoke.
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conditions (Meacham *et al*., 1974). Each pilot exhibited a significant deterioration in flying performance during the 30 minute period after taking marijuana, but not after taking placebo. The decrease in flight performance involved an increase in major errors (such as navigational errors, major altitude deviations, stalling and loss of control events) (Meacham *et al*., 1974). In a similar experiment, Janowsky *et al*. reported gross impairment of flying skills and performance, reflected in a greater number of both major and minor errors (Janowsky *et al*., 1976). Alterations in concentration, loss of orientation and temporal distortion, were noted among the affected pilots (Janowsky *et al*., 1976).

Ten experienced pilots exhibited significant impairment in number and size of aileron changes, size of elevator changes, distance off centre on landing and vertical and lateral deviation on approach to landing, 24 hours after marijuana usage (Yesavage *et al*., 1985). Leirer *et al*. studied pilots in a flight simulator, after a moderate social dose of marijuana. Pilots flew just before smoking, and 15 minutes, 4, 8, 24 and 48 hours after smoking. Marijuana impaired performance of flying tasks for up to 24 hours after smoking (Leirer *et al*., 1991). Drug dosage, time interval between drug intake and flight, pilot age and the task complexity were all reported to affect the pilot's performance (Leirer *et al*., 1991). Impairment of performance and skills appears to be at a maximum during the first four hours after taking marijuana, with some residual impairment over the subsequent 24 hours (Newman, 2004). Therefore, this combination of residual impairment at 24 hours and some other performance-reducing factors, such as increased task difficulty can result in significant deterioration of performance in pilots (Newman, 2004). Results of flight simulator studies on the effects of marijuana on piloting performance are provided in **Table 6**.

Concentrations of THC and metabolites are dependent on the pattern of use as well as dose, with THC concentrations typically peaking during smoking, while peak 11-OH-THC concentrations occur approximately 9–23 minutes after the start of smoking (Couper and Logan, 2004). Concentrations of both analytes decline rapidly and are often <5 ng ml^{-1} at three hours. Chronic users may exhibit mean plasma concentrations of THC-COOH of 45 ng ml^{-1}, 12 hours after use; corresponding THC levels are, however, less than 1 ng ml^{-1} (Couper and Logan, 2004).

One of the concerns with marijuana is the interpretation of impairment based on drug concentrations in urine or blood. Marijuana is highly lipophilic, and serum and blood concentrations are much lower relative to tissue concentrations, and therefore may not reflect degree of impairment. The time of marijuana exposure may be estimated within a 95% confidence interval using mathematical models by Huestis. (Couper and Logan, 2004). Knowing the time interval from last marijuana exposure may be used to predict impairment with concurrent cognitive and psychomotor effects based on data in the published literature (Couper and Logan, 2004). Presence of THC metabolites in urine is only indicative of prior THC exposure and not necessarily impairment. Low concentrations of THC have also been measured in OTC hemp oil products, and consumption of these products may produce positive urine cannabinoid test results (Couper and Logan, 2004).

Johnson *et al*. analysed specimens from 3235 fatal aviation accidents over a 10 year period, of which 74 tested positive for THC (Johnson *et al*., 2006). Among these 74 cases, THC was reported as a probable cause or factor in 32% of the accidents (Johnson *et al*.,

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2006). In addition to a positive THC, 24 of the 74 pilots tested positive for other disqualifying substances including seven for methamphetamine, six for SSRIs, five for ethanol, five for benzodiazepines, three for cocaine and one each for tramadol, meperidine and olanzapine (Johnson *et al*., 2006).

9.4 Opiates

Opiates comprise a group of commonly used and highly addictive drugs used for pain relief. The most commonly prescribed opiate-type compounds are hydrocodone, dihydrocodeine, codeine, hydromorphone, oxycodone and morphine (Lewis *et al*., 2005). Side effects of opioid use include drowsiness, dizziness, hypotension, unconsciousness or mental clouding, all of which can lead to significant impairment. The presence or absence of opiate compounds in post-mortem fluids and/or tissues derived from fatal civil aviation accidents may have serious medicolegal consequences and may help establish the cause of impairment and/or death (Lewis *et al*., 2005). Heroin (diacetylmorphine), is a commonly abused illicit opioid drug. Heroin is metabolized to 6-monoacetylmorphine (6-MAM), which is then metabolized to morphine. 6-MAM has not been detected following the use of morphine or other opiate compounds or the ingestion of poppy seeds and is only present following heroin use. Therefore, 6-MAM is considered a heroin-specific metabolite and is legally defensible evidence of heroin use (Lewis *et al*., 2005).

In a study by Lewis *et al*., post-mortem fluid and tissue samples obtained from eight separate civil aviation fatalities over three years that had previously been screened positive for opiates by GC/MS were re-examined. Morphine was found in three of the eight cases examined and was the only opiate detected in two of these cases (Lewis *et al*., 2005). Morphine concentrations in the various fluids and tissues ranged from 0 to 260 ng ml^{-1}, 122 to 526 ng g^{-1}, 0 to 870 ng g^{-1}, 121 to 1786 ng g^{-1} and 21 to 120 ng g^{-1} in blood, urine, liver, kidney and muscle respectively. Morphine concentrations were highest in urine and lowest in muscle.

Codeine was identified in two of the eight cases investigated at concentrations of 13 ng ml^{-1}, 78 ng g^{-1}, 68 ng g^{-1} and 19 ng g^{-1} in blood, liver, kidney and muscle, respectively; urine levels ranged from 47 to 154 ng ml^{-1}. One of the codeine-positive cases also tested positive for morphine and thebaine, while the other case tested positive for hydrocodone and dihydrocodeine. Thebaine, morphine and codeine were identified in one of the cases examined, at urinary concentrations of 22, 122 and 47 ng ml^{-1}, respectively. The relatively higher morphine, as well as the low concentration of both opiates and the presence of thebaine, was indicative of poppy seed consumption. It is important to note however, that following poppy seed ingestion, morphine and codeine may be detected in urine, without the detection of thebaine. Therefore, the absence of thebaine cannot preclude poppy seed consumption as the source of morphine and codeine (Lewis *et al*., 2005).

Hydrocodone and dihydrocodeine were detected in four of the eight cases examined at blood concentrations ranging from 18 to 102 ng ml^{-1} and 13 to 270 ng ml^{-1}, respectively. Urinary concentrations for hydrocodone and dihydrocodeine ranged from 35 to 1447 ng ml^{-1} and 78 to 2023 ng ml^{-1}, respectively. No apparent correlations were evident between hydrocodone and dihydrocodeine concentrations within specimen types.

Hydromorphone was found in one of the hydrocodone/dihydrocodeine-positive cases. Hydromorphone was below the limit of quantitation in all specimens except urine, in which it was found to be 178 ng ml^{-1}. Oxycodone was found in one of the eight cases examined with urinary concentrations being the highest, and blood concentrations the lowest (Lewis *et al*., 2005).

9.5 Post-Mortem Degradation and Redistribution

Drugs of abuse undergo post-mortem changes; however, the extent of these changes varies significantly between drugs (Drummer, 2004). Heroin and cocaine undergo rapid hydrolysis during life, as well as after death. Depending on environmental factors and the time interval between the crash and discovery of the body, varying degrees of tissue decomposition may be observed. Blood specimens containing cocaine and BE exhibited poor stability and were shown to degrade over time even when stored at ambient temperature and even when stored in fluoride/oxalate tubes (Giorgi and Meeker, 1995).

Anaerobic bacterial activity can lead to the conversion of nitrazepam, flunitrazepam and clonazepam to their respective 7-amino metabolites (Drummer, 2004). Other benzodiazepines, such as diazepam and temazepam, may also undergo degradation under putrefying conditions (Drummer, 2004). The concentration of THC in blood has also been shown to decrease with time, particularly when stored at −20 °C (Drummer, 2004). In frozen specimens, morphine is relatively stable, however, significant losses are evident when stored at 4 °C or higher for more than a few days, or in post-mortem specimens (Drummer, 2004).

Post-mortem redistribution may affect drug concentration due to drug diffusion from an area of higher concentration to an area of lower concentration. Post-mortem redistribution is a particular concern with drugs having high volumes of distribution.

Methadone, a drug with a high volume of distribution exhibits a moderate increase in post-mortem blood concentration ranging up to fourfold, although there is

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significant site-to-site variability (Drummer, 2004). In contrast, morphine, a drug with a lower volume of distribution, shows little change in post-mortem blood concentration (Drummer, 2004).

Methamphetamine has been known to exhibit a twofold variation between drug concentrations in femoral and heart blood specimens, apparently due to diffusion of drug from pulmonary circulation into the left cardiac chambers (Drummer, 2004). Although peripheral sites, such as femoral blood might show fewer changes than blood taken from the thoracic and abdominal areas, it too will show higher concentrations of drugs following a post-mortem period (Drummer, 2004). Site variability may occur due to other factors, such as differences in hematocrit, influences of other fluids and other factors affecting the quality of blood, even in the absence of significant putrefaction (Drummer, 2004). Variations in heart:femoral blood concentration ratios greater than twofold have been reported with the SSRIs fluoxetine, paroxetine, sertraline, venlafaxine and risperidone (Rodda and Drummer, 2006).

10 POST-MORTEM DNA ANALYSIS

The accuracy of analytical data on biological evidence for toxicological interpretation is imperative in order to establish the cause and manner of death.

This perspective is magnified during aircraft accident investigations, wherein multiple types of post-mortem specimens are collected for analysis from the victims, and depending upon the nature of an aircraft accident, victim bodies are frequently scattered, disintegrated, commingled, contaminated and/or putrefied (Chaturvedi *et al*., 1999). Identification of multiple specimens may be effectively resolved using deoxyribonucleic acid (DNA) profiling. However, environmental and biological factors responsible for putrefaction may also lead to degradation in the post-mortem samples that are generally obtained from aviation accident sites (Chaturvedi *et al*., 1999).

Two techniques commonly used to analyse DNA include restriction fragment length polymorphism (RFLP) and the polymerase chain reaction (PCR)-based method. Analysis of degraded DNA using the conventional RFLP technique is not as effective as the PCR-based method, which has been successfully used for the analysis of low quantities of degraded and low-molecular-weight DNA, in contaminated and putrefied samples (Chaturvedi *et al*., 1999).

Besides the identification of victims and post-mortem tissues, DNA analysis may be applied to resolving issues, such as tissue mismatching/commingling, unacceptable batch analysis and unusual analytical findings (Chaturvedi *et al*., 1999). Chaturvedi *et al*. reported the presence of atropine at unusual toxic concentrations in the blood and lung samples, but absent in the other tissue specimens, obtained from an aircraft accident victim. Three possible scenarios were postulated: intentional or accidental atropine poisoning, atropine administration by emergency healthcare providers into the thoracic cavity to modify the vasovagal activity and tissues originating from different subjects (Chaturvedi *et al*., 1999). DNA analysis performed on the blood and liver revealed tissue mismatching/commingling between the specimens, thus explaining the presence of atropine in select tissues.

11 FUTURE OPPORTUNITIES—GENOTYPING AND PHARMACOGENOMICS

In aviation fatalities, the interpretation of drug concentrations is often compounded by genetic variability in drug disposition. Pharmacogenomics is the study of the association between an individual's genotype and their response to drugs (Kupiec *et al*., 2006). Interindividual variations in drug response are often associated with polymorphisms in genes encoding for drug-metabolizing enzymes, transport proteins and drug receptors (Kupiec *et al*., 2006). The Cytochrome P450 2D6 (CYP2D6) enzyme metabolizes 25–30% of all drugs, and is also the most polymorphic CYP enzyme (Kupiec *et al*., 2006).

Based on the genetic polymorphisms of CYP2D6, three metabolizer types are recognized: ultraextensive metabolizers (UMs), extensive metabolizers (EMs) and poor metabolizers (PMs). Individuals with a UM phenotype generally have multiple copies of a gene, will metabolize drugs more rapidly, and therapeutic plasma drug levels will not be achieved at ordinary drug dosages. In contrast, individuals with a PM genotype may require substantial dose reduction to prevent toxicity. For example, in the metabolism of the tricyclic antidepressant nortriptyline, PMs have zero copies of the CYP2D6 enzyme, intermediate metabolizers have one copy, EMs have two copies and UMs have more than two copies (Kupiec *et al*., 2006). Clinical manifestations of these genetic drug metabolism profiles could range from therapeutic inefficacy to toxicity for the same dose of a drug (Kupiec *et al*., 2006).

Several commonly used prescription drugs are metabolized by CYP enzymes, for example, CYP2D6 metabolizes numerous SSRIs and opioids. SSRIs and other antidepressants have been associated with suicides and accidental deaths, and delineating a suicidal from an accidental death based on plasma drug concentrations alone might be misleading (Kupiec *et al*., 2006). An individual with a PM genotype might exhibit toxic drug concentrations at therapeutic or subtherapeutic dosages. Therefore, in aviation fatalities, caution must be exercised during interpretation of drug concentrations of drugs associated with polymorphic enzymes. Genotyping of post-mortem specimens has been proposed in order to distinguish

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drug overdose from accidental deaths due to reduced enzymatic capacity (Druid *et al*., 1999). Future research in aviation toxicology may be focussed on predictive genotyping for aviators, in order to establish optimal drug dose–response relationships, as well as identifying susceptible genotypes.

Aviation toxicology has evolved through the years into a highly specialized area of toxicology with several unique perspectives on the cause and manner of death. However, some analytical challenges, such as the paucity of samples, complexity of air-crash specimen separation and identification and decomposition issues, still remain. Toxicological interpretation is routinely complicated by putrefied or aged samples, relative to conventional forensic toxicology. Advances in related disciplines such as inhalation toxicology, pharmacogenomics and forensic toxicology have all led to a better understanding of issues in aviation toxicology. In addition, the prospect of ever-burgeoning air traffic creates an avenue for new areas of research, especially in the area of predictive genomic markers of aviation performance, as well as the potentially impairing effects of new drugs that are introduced into the market. These investigations can be expected to contribute to large-scale improvements in aviation safety with positive financial and societal ramifications.

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The Toxicologist as Expert Witness

Ira S. Richards and Marie M. Bourgeois

CONTENTS

1 INTRODUCTION

> *Science alone of all the subjects contains within itself the lesson of the danger of belief in the infallibility of the greatest teachers in the preceding generation … As a matter of fact, I can also define science another way: Science is the belief in the ignorance of experts.*
> *Richard Feynman (Root-Bernstein, 1999).*

Toxicology is the science which deals with the adverse effects of chemicals in biological systems. It has long been viewed as the first forensic science (Ramsland, 2008). Mathieu Joseph Bonaventure Orfila who published *A Treatise of General Toxicology* is widely regarded as the 'Father of Forensic Toxicology'. James Marsh in the 1830s, tested the coffee of a suspected victim of poisoning, but was unable to effectively explain to a lay jury how he had found the arsenic. This inability to effectively communicate his findings inspired him to improve his methods and make them more demonstrative for a court of law. His method became known as 'The Marsh Test' and was used extensively by contemporaries such as Orfila, to analyse both exhumed bodies and surrounding soil samples for arsenic, to assist in determining whether or not the decedent was poisoned. Dr. William Willcox in the early 1900s further developed methodologies to quantify arsenic in body tissues.

Although toxicologists initially restricted their testimony to cases of suspected poisonings, experts today offer opinions across a broad range of criminal and civil litigation (Kupferschmidt, 2004). In many ways, the science has evolved alongside the legal system and the toxicology expert witness is a commonplace legal figure today. We can define an expert witness as one who by virtue of either specialized education, experience, training, specific skills or through a combination of these, well-above those of the 'average person', may provide legal opinions, conclusions or other information that the court deems necessary to assist in clarifying the issues under specific legal consideration.

While the term forensic toxicologist may typically conjure up images of high technology laboratories and individuals gathering information from body fluids and chemical tests which will be used in a court of law, the toxicologist that serves as an expert witness, for *any* issue related to the broad discipline of the science, by definition becomes a forensic toxicologist (Kier, 1977). Today, the toxicology expert witness is involved in legal issues as diverse as driving while intoxicated, accidental or intentional poisonings, occupational toxic exposures and worker's compensation claims, product liability, regulatory proceedings, toxic torts and personal injury, environmental exposures and class-action litigation (Ellenhorn, 1978).

As science has become increasingly complex, so has the toxicologist's role as an expert witness. The ultimate

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goal is to help translate the complex scientific theories and evidence of a case to the court, so that rational decisions and recommendations can be made by a judge and jury. The problem for any expert and their legal team is that there may be, and most often are, opposing experts and their legal team who will tell a judge and jury, and often very convincingly, that your opinions are wrong. At times an expert may strongly believe that an opposing expert is embracing perhaps 'junk science' and should not have even been qualified to offer expert opinion. It would certainly not be in the best interests of an expert to tell the judge that he/she made a poor decision in qualifying that expert. Unfortunately, sometimes what makes a good expert is how convincing they are to a judge or jury. Beyond being credible in court, your testimony must always be based upon good scientific evidence. Furthermore, to be an expert witness requires your understanding and acceptance that the way the legal world determines if science is good may be different from yours (Mosteller, 2007). You may have fine credentials, are well published and even may be involved in cutting-edge research, your testimony as an expert must abide by the legal ruler for measuring good scientific evidence (Jasanoff, 2005). Remember, your expert opinions constitute an important form of evidence which can ultimately determine the outcome of a case. At times this legal ruler may seem to use a scale that is foreign to you or difficult to accept. This is especially important in courts operating under the *Daubert* standard of acceptability. The proponents of *Daubert* cited widespread scientific ignorance of the public. They maintained such ignorance had costly, sometimes catastrophic effects and led to acceptance of 'junk science' (Thigpen, 1995). A naïve jury could be swayed by anecdotal evidence (Ozonoff, 2005). This ruling established judges as 'gatekeepers', granting them sole authority over the approval of experts and their theories.

2 THE SPECTRUM OF LEGAL PROCEEDINGS

Public concern about the adverse effects from chemical exposures has risen steadily in the past few decades. This awareness can be seen on two somewhat disparate legal fronts; toxic tort litigation and regulatory proceedings. Toxicological evidence plays an integral role in both of these arenas. The courts have become burdened with cases ranging from real causation-injury to perhaps a somewhat offensive odour as responsible for producing neurologic injury. There has been a significant increase in the number of civil lawsuits and criminal prosecutions, in federal and state courts, that present evidence and theories requiring the consideration of basic and advanced science. At the same time, there has been a growing ignorance of the fundamentals of science by the public. This is not limited to advanced theoretical concepts; rather, the gap includes basic scientific principles like what constitutes anecdote and what constitutes credible evidence.

A toxic tort is the name given to a body of laws and procedures that establishes the circumstances under which individuals or companies may be held liable for the injury of a person(s) from exposures to chemicals either intentionally or accidentally and provides remedies to recover damages for those injuries (Benjamin, 1993). An important issue in tort law is to determine whether negligence was involved in the injury or not, typically what the legal profession defines as determining the 'standard of care'. The burden of proof for those who claim injury is to prove negligence sufficient to cause injury; otherwise, tort law will not compensate them.

Toxic torts typically involve pharmaceutical, consumer product, environmental or occupational exposures (Marchant, 2002). Pharmaceutical toxic tort cases are often litigated against drug manufacturers and distributors, as well as prescribing physicians. Unlike medical malpractice, pharmaceutical cases are essentially product-liability cases with the defective product being the drug. Most pharmaceutical and consumer-product toxic injury cases involve large numbers of individuals and are typically stimulated by drug and product recalls. Unlike the general population, industrial workers are often exposed to chemicals at higher concentrations and duration than the general public and therefore have a greater risk of developing disease from particular chemical exposures than the general population. Occupational exposures to chemical agents have often provided the most reliable information on chemical injury causation in humans. Probably the best example that can be provided is that of asbestos exposure. Occupational toxic tort cases differ from a worker's compensation claims which are directed against the worker's employer, while an occupational toxic tort case is usually filed with the courts against 'third parties', companies and individuals other than the employer who either manufacture or distribute chemicals and equipment for their application that can expose the workers or bystanders to these chemicals.

The role of the toxicologist is slightly different in regulatory proceedings; here, the testimony is used in support or opposition of proposed federal or state regulations with regard to a specific chemical or class of chemicals. The toxicologist uses their expertise to describe the effect of a potential exposure on a population rather than answering questions about causation.

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3 QUALIFICATIONS AND ROLE OF THE TOXICOLOGY EXPERT WITNESS

Unlike other witnesses who simply relay their version of a specific event, expert witnesses are expected to evaluate the facts of the case and render a thoughtful opinion. In the eyes of the judge and jury, credentials often equal credibility. The legal community has developed a general level of expectation outlining the professional attributes for one to qualify as an expert witness. Toxicologists base their expert opinion, in part, on a thorough review of relevant research literature. They must be capable of weighing the strengths and weaknesses of each study before they can make a determination of its value. The expert also considers fundamental precepts of toxicology before forming an opinion. This requires a thorough understanding of the actions of chemicals in biological systems. No single academic degree, research direction or career path uniquely qualifies a toxicologist as an expert.

A well-qualified toxicologist would satisfy the majority of the following:

- Possesses a graduate degree, preferably a doctorate in toxicology or a related field
- Has published papers in their discipline
- Has edited a journal or book
- Has written a book
- Has contributed chapters to a book
- Is board certified in their field
- Has practical experience in an industrial or educational environment
- Is a member of professional societies with credentialing requirements
- Has served as an advisor to a local, state or federal agency
- Has not been disqualified as an expert previously.

The role of the toxicologist in the legal arena can take many forms, including:

- Consultant
- Teacher
- Interpreter
- Investigator
- Reporter
- In-house expert
- Witness for the defence or plaintiff
- Court-appointed witness.

Toxicologists serving as legal consultants may advise attorneys regarding the development of factual evidence and the strengths or weaknesses of the opponents' case, help frame the issue, reduce wasted time and effort, guide the attorney on the relevance of documents and testing, determine cause-and-effect relationships, act as an advisor on the nature and effects of a toxicant, craft questions for an interrogatory, evaluate the theory of the case and help establish foundation (Muller, 2008).

In law, a foundation is sufficient preliminary evidence of the relevance and authenticity of evidence to admit to the court for consideration. The evidence provided by the expert can be material evidence in the form of exhibits or testimony, and its importance may greatly determine the outcome of a case (Simington, 2006). Lack of foundation by an opposing legal team is a valid objection that can be and is often used to greatly reduce the effectiveness of opposing legal counsel. Experts retained in investigatory or technical roles may conduct independent testing or research.

Consider, for example, the toxicologist called upon to offer expert opinion as to whether or not an individual was impaired through the consumption of alcohol during the operation of a motor vehicle. This witness may assume a role as an expert for either the defence of the individual or for the prosecution, by serving as a reporting witness, interpreting witness or teaching witness.

The expert may have been involved in performing or supervising the laboratory tests which were conducted to determine the blood ethanol concentration for the individual in question. The toxicologist may describe the nature of these tests to a jury, the procedures involved and the quality control standards that were in place to ensure both the accuracy and reliability of the tests which were used. The court in turn may wish to determine the qualifications of the witness to conduct such testing, which is generally satisfied by the expert qualifying by virtue of skills gained through education, experience and training. The expert must be prepared to provide convincing testimony that:

- The correct tests were done
- The equipment was in proper working order
- The sample tested was obtained through an acceptable chain of custody.

The same or another toxicologist may serve as an interpreting witness to explain the results of the tests even when a test result is self-explanatory, pass/fail or involves a statutory presumption. An opposing expert may offer opinion as well. The same or another expert witness may be called upon to offer instruction to the court on the metabolism of alcohol and its effects upon the human body and to render an opinion as to the level of impairment which would be expected at a certain blood alcohol concentration and whether or not the individual in question was impaired while operating the motor vehicle. An opposing expert may have different opinions.

Attorneys may employ in-house toxicologists as expert witnesses under certain circumstances. Such an expert has the advantage of familiarity with the product or industrial process at the heart of the lawsuit. The expert's

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employer will need to be consulted to determine the scope of allowable testimony. There may be restrictions on testimony of this sort in an effort to control the release of work-product details or detrimental information.

Occasionally, the court will appoint its own expert. The court-appointed expert is permitted under Rule 706 in the Federal Rules of Evidence. This expert can have a major impact on the outcome of the case because the jury will see him/her as an especially impartial expert. The court-appointed expert is subject to examination by the plaintiff and the defence attorneys. An expert may not be compelled to accept the request from the court to act as a court-appointed expert witness. There may be many reasons that a toxicologist may wish to decline, including commonly their time commitments and the issue of compensation, which may be problematic as the amount is fixed by the court. In civil lawsuits, attorneys from both sides are often required to contribute to their fees. This is not true in criminal cases (Federal Rules of Evidence, 2008a).

4 MARKETING YOURSELF AS AN EXPERT WITNESS

Once you have made the decision to become an expert witness, you might wonder about marketing strategies. You may wish to contact your colleagues that do expert witness consulting, as one expert may recommend another if they feel the case match to their own background and interests are weak, or they have other time commitments or potential conflicts of interest, to name a few. A strong *curriculum vitae* with an appropriate cover letter targeted to attorneys in the field of personal injury, toxic torts, worker's compensation and other areas can be extremely effective in obtaining initial assignments. You might also consider asking attorneys with whom you are acquainted to assist you in 'getting the word out' as to your areas of expertise and your interest in forensic work. The majority of attorneys find experts through colleagues, web sites and agencies which maintain databases of experts. Self-promotion, such as advertising in law journals or web sites can be counterproductive, especially if you derive most of your income as a consultant. The advertisement may appear at trial as an exhibit with the unstated implication you are a 'hired gun'.

A comprehensive *curriculum vitae* may be the most important tool at your disposal. It can be used to qualify your testimony. It will be submitted to every attorney with whom you come into contact. It can show that you constantly update your skills, detail specific accomplishments and list basic and advanced skills. It must also have a complete publication list and some references to previous testimony.

5 THE ATTORNEY–EXPERT RELATIONSHIP AND PROTECTING YOUR INTERESTS

An attorney will initially contact a potential expert witness to discuss a particular case. Most times it is relatively early in the litigation process, although there are times when trial has already been scheduled and there is a feverish rush to find and disclose an expert and complete the discovery process. Sometimes the latter is the result of poor planning by the legal firm and sometimes new information develops late and there is an urgent need for an expert witness at the eleventh hour. In either event, it is commonplace to spend some time either over the telephone or at a scheduled visit at the expert's office. Having established a collegial tone, and provided some very preliminary thoughts, the attorney may then ask if they can designate you a 'possible expert'. An important question to always ask in your initial discussion is how did your name come up as a potential expert witness? If you are an experienced witness then it might have been through a referral. Commonly, a legal assistant did a web-based search for toxicologists in the area and your name came up. In any event, a point will be reached in the discussion where you may be requested to forward a complete resume, fee schedule and perhaps the names of other attorneys with whom you have worked. An attorney should never formally disclose you as an expert without first contacting and retaining you. In some venues, misrepresenting an expert as retained to pre-empt the other side from using their services can lead to sanctions against the attorney and law firm.

You should discuss compensation as soon as feasible to avoid disagreements later. A potential expert witness should confirm whether the attorney or the client will be responsible for paying their fees and should be satisfied that the firm has adequate resources available to pay for your time, especially if it is anticipated that many hours of your time may be involved. Additionally, the expert should review any information about the attorney that he will be working with, and the legal firm, to better address concerns that they have a demonstrated record of producing the best legal efforts on behalf of their client(s) whether that will be for the defence, plaintiff or claimant (worker's compensations proceedings). It is important to provide a statement of understanding to the attorney prior to being retained. This statement of understanding should note types of billable expenses and what your retainer amount will be. Your fee structure needs to explicitly state the costs associated with research, active experimental investigation, file review and report generation, consultation and testimony at deposition or trial. Developing a fee structure can be difficult; consult with other professionals to make sure your rates are reasonable and will not price you out of the market. Higher rates are generally reserved

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for more experienced experts with a proven record of satisfaction by different legal firms. Professional societies such as the Society of Toxicology and the American Chemical Society provide legal and educational resources for their members. There are also multiple organizations of expert witnesses that can provide interested parties with stock contracts for expert witness services, consultation agreements and other necessary documents. These documents can be tailored for your individual situation; for example, an expert unwilling or unable to travel may not need an agreement that sets reimbursement rates for rental cars and hotel rates. Never accept a case with the understanding that it is on a contingency basis. You must be paid for your work whatever the final disposition of the case; win, loss, or settlement.

Once retained it is the responsibility of your attorney is to provide you with all pertinent information, including that which might be damaging to their own case. It is imperative that you have this information provided to you before you enter into deposition or trial testimony. Remember that your sworn testimony, as obtained by opposing counsel during deposition and trial, will be a meticulous and diligent probing of you as an expert to discover and emphasize your weakness to the court, including a lack of all the relevant information which may bear upon your opinions as an expert. Either your poor preparation or that of your attorney, including failure to provide all the documents needed to form your opinions, will have a negative impact upon you professionally. In either case, it may be viewed in the future as poor preparation for deposition or trial on your part.

It is your professional and sometimes personal reputation at stake; all that you say in sworn testimony is public record and available at a later time by the legal profession that may be considering retaining you on another case as their expert. It is important early on to let your attorney know that in the absence of reviewing adequate records, your full opinions cannot be formed. Sometimes following the record review, an expert may indicate to the attorney that the case is weak, and a clear basis for causality cannot be established. An excellent approach that can be taken is to let the attorney initially retain you as a consultant to evaluate the case and educate the legal team regarding strengths and weaknesses. Do not have the attorney disclose you as an expert witness until it is agreed among all concerned parties that your testimony will constitute a vital component of their legal efforts.

Expert testimony is not for the faint of heart. Your professional judgement, research skills, employment history, academic background, publications and previous testimony may all be harshly scrutinized by opposing counsel. An expert witness should be prepared to explain discrepancies between current testimony and that offered in prior depositions or trials. Meticulous recordkeeping makes this easier. It has become popular in the past few decades to accuse innovative scientific experts of practising 'junk science'. These charges can be effective because 'junk science' is a nebulous term with many possible interpretations. The adoption of *Daubert* was intended to minimize claims of this nature; however, the standard is not uniformly applied. Reliance on accepted methodology is the easiest way to avoid 'junk science' accusations.

Attacks on your credibility can be distressing, but they are rarely considered abusive. Defensiveness can be perceived by the judge and jury as an unspoken confirmation of the accusations made by opposing counsel. An expert witness should deflect hostility during questioning with a calm and professional demeanour. The expert witness should also be aware that background checks are commonplace, so potential problems should be disclosed up front. One common avenue of credibility attack is when an expert witness almost exclusively works for either the defence or the plaintiff. Your selection of which cases to consider should always be driven by your interests, experience, time commitments and other factors; however, it is best to be an expert witness who has been seated on both sides of the fence.

It should be noted that toxicologists operating in certain legal capacities may not need to be disclosed to the opposition. The scope of disclosure requirements differs greatly. Communications with experts who are not retained may be protected by work-product doctrine or disqualification. Although delaying disclosure can provide the attorney with a tactical advantage under certain conditions, there are several reasons that the toxicologist should be retained as an expert by the attorney as soon as it is feasible.

Some of the questions asked of the toxicology expert witness may centre on ethical issues related to the discipline (Elvins and Stephens, 1999). Responses should be consistent with those accepted by the profession. Toxicology, as well as other professional societies, has codes of ethics; a toxicologist interested in becoming an expert witness should be familiar with the ethical code of the societies to which they belong (Bouma, 2001). The Society of Toxicology, as an example, has such a code:

> *The Society of Toxicology is dedicated to developing and communicating knowledge to improve the health and safety of living beings and to protect the environment upon which we depend. To attain this objective, each member must maintain high ethical standards, recognize a duty to share this knowledge with the public, and be a thoughtful advocate for human, animal, and environmental health. To this purpose, this code requires a personal commitment:*
>
> - *Conduct their work with objectivity and themselves with integrity. Being honest and truthful in reporting and communicating their research.*

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- *Hold as inviolate that credible science is fundamental to all toxicological research and is the basis for communicating results.*
- *Recognize a duty to communicate information concerning health, safety, and toxicity in a timely and responsible manner, with due regard for the significance and credibility of the available data.*
- *Give due consideration to the ethical, legal, social and policy implications of their research and communications.*
- *Be a thoughtful advocate for human, animal and environmental health.*
- *Abstain from professional judgments influenced by undisclosed conflict of interest, disclose any material conflicts of interest and avoid situations that imply a conflict of interest.*
- *Observe the spirit, as well as the letter of laws, regulations, and ethical standards with regard to the conduct of human and animal research.*
- *Practice high standards of environmental and occupational health and safety for the benefit of themselves, their co-workers, their families, their communities, and society as a whole.*

Code of Ethics Adopted by the Society of Toxicology on 31 January 1985, Revised on 1 June 2005 (http://www.toxicology.org/AI/ASOT/ethics.asp).

Safeguarding your professional reputation is vital; there is no coming back once you acquire the 'hired gun' label or are viewed as an expert that is poorly prepared for sitting a deposition. An expert witness should never tailor their opinion to please the attorney. You must be capable of objectively evaluating the case and rendering an independent opinion. An expert should stress this to the attorney during their first conversation. This does not mean your judgement cannot evolve with new information.

Your attorney will also be responsible for preparing you for testimony at deposition and trial (Babitsky and Mangraviti, 1999). The testimony that you provide is a form of evidence that is obtained following the taking of a sworn oath or affirmation under penalty of perjury. It is a statement or declaration of fact, opinion, inferences or personal information that will be used in a legal action. The testimony may be oral, written or videotaped.

The rehearsals will help familiarize you with the pertinent case law, alternate theories that may be presented by the opposing counsel, and the overall process (Babitsky and Mangraviti, 1999). Depositions are almost always conducted by the opposition so they can be protracted, confrontational affairs (Hawkins, 2005). Your attorney will advise you on the nuances of testimony and perhaps even provide you with some insight about any 'tactics' likely to be used by the lawyer who will be deposing you, especially if there is familiarity from other depositions (Catto, 2008). Much advice may seem common sense:

- Know the legal issues
- Be well prepared
- Dress in a professional manner
- Answer only the question you have been asked—don't volunteer information
- Take your time
- Avoid overstatements of any kind
- Feel free to consult your notes
- Stay calm, professional and polite
- Don't be afraid to admit ignorance
- Be wary of potential traps—opposing counsel will try to damage your credibility
- Realize your testimony will be used to sway a judge or jury so use everyday language when appropriate (Gorney, 2008a)

6 TRIAL PREPARATION

According to the National Center for State Courts, 15 million civil cases are filed annually in the United States (*http://www.ncsconline.org/WC/Publications/Res_CtComm_CJRIPub.pdf*). Fewer than 5% of these will ever make it to trial. Litigation is a lengthy and complex process governed by the Rules of Civil Procedure and the Rules of Criminal Procedure (Federal Rules of Civil Procedure, 2008; Federal Rules of Criminal Procedure, 2008). An expert witness may be involved in any or all phases of the process and although there may be significant overlap, it is convenient to the litigation process in preparation for trial and the trial itself.

The expert witness is not a surprise witness who suddenly appears at trial, but rather Rule 26 of the Federal Rules of Civil Procedure outlines the details of expert testimony disclosure, and the scope of discovery and privilege (Federal Rules of Civil Procedure Rule 26, 2008). The identity of an expert who may be presenting evidence during trial must be disclosed to the opposition at least 90 days before the trial date. This is accompanied by a written report prepared by the witness. The report contains:

- A statement of opinions the expert is expected to offer
- Data used to form the aforementioned opinion
- The witness's qualifications
- A list of all cases, dating back four years, in which the expert has been deposed or testified
- A statement of compensation.

These are rules as they relate to federal court; however, they may not all be strictly applicable to state courts (e.g. there is no requirement at the state level for an expert to list all cases dating back four years for which he/she was deposed or testified), although it could be requested from

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the expert by subpoena from opposing legal counsel prior to deposition.

The pretrial is a conference held prior to trial whereby all of the interested parties may outline discovery proceedings and work with a neutral mediator in an attempt to avoid a trial, which can add significantly to overall litigation cost. Discovery is the means of obtaining information in preparation for trial (Simington, 2006). An expert witness who has been disclosed to the opposition may be required to submit copies of all of their relevant documents, reports, research and experimental data. The proposed discovery plan generated in the pretrial is submitted to the court for approval; the plan establishes the scope and limitations of the discovery process. It will also address issues of privilege or protection. Privilege refers to an immunity that allows a witness to refuse to divulge information (NOLO, 2008).

Interrogatories, depositions and production of document requests and requests for admission into evidence are tools of discovery. Interrogatories are written questions designed to elucidate the opposing party's case (Garner, 2000). Attorneys may use form interrogatories that cover typical situations, or they may write their own questions. They may also enlist the help of an expert to generate the questions. In a deposition, the opposing counsel questions the expert witness. This can be a lengthy and sometimes very heated event, which at times may be deemed by the expert as little outside of a personal attack on their professional accomplishments and opinions. All the questions are answered under oath and a transcript is recorded by the court reporter. An expert is free to consult notes or utilize PowerPoint presentations. Depositions are frequently videotaped so they may be read to or played for the jury should the witness be unavailable for trial. Deposition testimony may be used to impeach a witness during trial; witness preparation is therefore very important (Busse, 2005). Production of document requests are requests for specific documents thought to have relevance. An expert may receive a *subpoena duces tecum* from opposing counsel. This may require that the expert produce the entire file for deposition. The expert may be required to go through the file, page by page and copy documents. The time spent doing this at the request of opposing counsel should be billed to them at the normal hourly rate. Requests for admissions are statements served to get opposing counsel to stipulate to facts or documents.

7 TRIAL

A toxicologist wishing to serve as an expert witness will be required to qualify their testimony (Schroeder, 1980). *Voir dire*, from the French 'to see and speak', refers to the process used to qualify the witness. This process will differ depending on the acceptance test used by the court. Briefly, the court will apply its own standard of acceptability to the witness; it may be as simple as relevance or it might be far more stringent. The relevant statutes governing the acceptance tests are discussed elsewhere in the chapter.

Despite the differences between criminal and civil procedure, both types of trials have the following stages:

- The judge gives the jury some preliminary instructions and then the lawyers present opening statements; sometimes the opening statements on behalf of one or more parties are omitted
- Plaintiff calls witnesses and produces evidence to prove its case
- Defendant may call witnesses and produce evidence to disprove the plaintiff's case and to prove the defendant's claims
- Plaintiff may call rebuttal witnesses to disprove what was said by the defendant's witnesses
- Closing arguments are made by the lawyer on each side
- The judge instructs or charges the jury as to the law
- The jury retires to deliberate
- The jury reaches its verdict and returns to the courtroom where the verdict is announced.

(*http://www.flnd.uscourts.gov/jurors/jurySelection.cfm*)
(*www.abanet.org/publiced/practical/books/family_legal_guide/chapter_2.pdf*)

Testimony is as much an art as it is a skill. An attorney will frequently request from the expert witness a rehearsal(s) of their testimony. The direct examination should flow logically and conversationally. Your attorney may simply ask a series of open-ended questions that allow you to engage the jury. Cross examination by opposing counsel may be far more of an ordeal. The deposition should have prepared the expert for the line of questions which will be asked in court, but it can still be quite confrontational. Responses during cross examination will be much briefer than they were during direct examination. The opposing counsel will try to catch contradictory statements, impugn integrity or intellect and attempt to fluster the expert. Take time to reflect on questions before responding and do not be afraid to say 'I don't know'. Your attorney may redirect questioning following the cross examination, allowing for a further clarification of a response to opposing counsel's questions and to shore up any damage inflicted during cross examination. Opposing counsel will get one more chance to question the expert in re-cross. The expert needs to connect with the judge and jury. If the proceedings are a bench trial, that audience is the judge. Bench trials require a slightly different approach to a jury trial. The demeanour of the attorneys may change as well. Jury trials are a performance of sorts, and attorneys on both sides will play to the audience. An expert must connect

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to the audience or their testimony will not be effective.

8 RULES GOVERNING ACCEPTANCE OF EXPERT TESTIMONY

Perhaps because scientific knowledge is extremely influential in litigation, courts continue to struggle with admissibility of scientific evidence. There is still no national standard test of acceptance for the admission of expert scientific testimony (**Table 1**). In the early 1900s, courts used the commercial marketplace test. This simply held that a person capable of earning a living based on their expertise or knowledge could be considered an expert. The *Frye* test was named for the ruling in Frye vs. United States of 1923 (Frye vs. United States 293 F. 1013 (DC Cir. 1923)). This test is also referred to as the 'general acceptance rule'. The test did not specify the scope of the expert's field, nor did it welcome innovative scientific methods. Frye was the predominant rule for the United States until the early 1990s.

Table 1 State positions on *Daubert*

Adopted	Rejected	Under consideration
Alaska	Arizona	Alabama
Arkansas	California	Hawaii
Connecticut	Colorado	Illinois
Delaware	District of Columbia	Minnesota
Georgia	Florida	Missouri
Idaho	Kansas	North Carolina
Indiana	Maryland	Virginia
Iowa	Nevada	—
Kentucky	New York	—
Louisiana	North Dakota	—
Maine	Pennsylvania	—
Massachusetts	South Carolina	—
Michigan	Washington	—
Mississippi	Wisconsin	—
Montana	—	—
Nebraska	—	—
New Hampshire	—	—
New Jersey	—	—
New Mexico	—	—
Ohio	—	—
Oklahoma	—	—
Oregon	—	—
Rhode Island	—	—
South Dakota	—	—
Tennessee	—	—
Texas	—	—
Utah	—	—
Vermont	—	—
West Virginia	—	—
Wyoming	—	—

The *Daubert* standard is a legal precedent set in 1993 regarding the admissibility of expert witnesses' testimony during federal legal proceedings (Daubert vs. Merrell Dow Pharmaceuticals, 509 US 579 (1993)). The Supreme Court ruled that the 1923 Frye test was superseded by the 1975 Federal Rules of Evidence, particularly Rule 702. Rule 702 states:

> *If scientific, technical, or other specialized knowledge will assist the trier of fact to understand the evidence or determine a fact in issue, a witness qualified as an expert by knowledge, skill, experience, training, or education, may testify thereto in the form of an opinion or otherwise.*

The *Daubert* standard was further refined by two more Supreme Court rulings. These three cases are commonly referred to as the '*Daubert* trilogy'. The Supreme Court held in General Electric Co. vs. Joiner, 522 US 136 (1997) that an abuse-of-discretion standard of review was the proper standard for appellate courts when reviewing a trial court's decision on the admissibility of expert testimony (Bernstein and Jackson, 2004). This meant that appellate courts should defer to the decision of the lower-court decision. The Supreme Court held in Kumho Tire Co. vs. Carmichael, 526 US 137 (1999) that the judge's gatekeeping function identified in *Daubert* applies to all expert testimony (Nordberg, 2007). *Daubert* established that judges are the 'gatekeepers' of good science and has granted them the sole authority to either approve or disapprove the use of any particular expert and their opinions in their court.

The four tenets of the *Daubert* trilogy are:

- Has the theory been tested?
- Is there a known or potential error rate?
- Has it been subjected to peer review and published?
- Is it generally accepted within the field?

As you can see, the last tenet is the *Frye* test (Waters and Hodge, 2004). Even in states that use the *Daubert* standard, there is uneven application. Few states use the entire trilogy. The results have been mixed. Proponents of *Daubert* argued that it would reduce the acceptance of 'junk science' in courtrooms by allowing the judge to act as a 'gatekeeper' (Berger, 2005). Opponents have argued that judges are ill-prepared to act as amateur scientists. Prior to *Daubert's* adoption, judges were inclined to let the issue be sorted out during cross examination. Application of *Daubert* has led to an increase of *motions in limine* and in summary judgments; however, the overall effect appears to be changing the way trials are managed by attorneys (Haack, 2005). *Daubert* challenges to expert witnesses are used as leverage in many civil disputes (Melnick, 2005). Loss of a key expert's testimony late in the trial generally leads to a summary motion against them. Acceptance tests

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are not the last word on admissibility of expert witness testimony. The Federal Rules of Evidence, specifically Rules 602, 701–706 and 803, provide guidance for criminal and civil law (**Table 2**).

9 BURDEN OF PROOF

The burden of proof is the requirement to prove those allegations which are presented in a legal action. The toxicologist as an expert witness will be called upon to address these issues. The burden of proof rests upon the one who complains: '*maxim necessitas probandi incumbit ei qui agit*'. The burden of proof simply means responsibility. In a criminal trial, it is the state's responsibility to prove the defendant's guilt. In a civil trial claiming a personal injury from a medication that was prescribed, it is the plaintiff's responsibility to show causation (Frankel, 1986). In a toxic tort alleging that an individual user of a household cleaning product was injured during the course of its use, for example, the toxicologist may serve as a expert witness for the plaintiff who used the product in question, assisting in the legal burden of proof that would be required to provide convincing evidence to the plaintiff legal team and the court in order to prevail in a claim of personal injury.

The toxicology witness must be prepared to discuss the probability that the injury was caused from exposure to the product.

The question becomes what is the level of proof or the 'standard of proof' required to satisfy the court? Typically, most nations have two levels of proof:

- Preponderance of evidence, which is used mainly in civil trials and worker's compensation claims, represents the lowest level of proof
- Beyond a reasonable doubt, which is used in criminal trials and represents the highest level of proof.

In addition to these, the USA introduced a third standard called clear and convincing evidence. This level of proof is sometimes required in civil cases for the plaintiff to prevail. It means that the court must be persuaded that the evidence is highly probable, thus providing a heavier burden than the preponderance of the evidence standard, but less than beyond a reasonable doubt.

The preponderance of the evidence, also referred to as the balance of probabilities, is the standard required in most civil cases and is met if the conclusion is more likely to be true than not true. Essentially, the standard is satisfied if there is greater than 50% chance that the proposition is true or 'more probable than not'. Beyond a reasonable doubt means 'to the exclusion of'. That is, the state must exclude any and all reasonable doubt as to the defendant's guilt. Simply put, the phrase means that if a juror has a reasonable doubt it is their duty to return a verdict of not guilty. On the other hand, if a juror does not have a reasonable doubt then the state has met its burden of proof and it is the juror's duty to return a verdict of guilty.

Beyond a reasonable doubt is the standard required by the prosecution in most criminal cases and represents the highest level of burden of proof. This simply means that there is no 'reasonable doubt' in the mind of any reasonable person that the defendant is guilty. There can still be a doubt, but only to the extent that it would not affect a 'reasonable person's' belief that the defendant is guilty. A toxicologist may be called upon as an expert, for example, to render an opinion which may require that this standard be applied. A prosecutor may ask the toxicology expert if is it their opinion, beyond any reasonable doubt, that the decedent's death was directly caused from ingestion of this chemical. The toxicologist must recognize that the opinion is concerning the chemical and not how or who may have given the chemical to the decedent. The toxicology expert witnesses may be asked to explain the difference between scientific standards legal standards of certainty. Scientific certainty is a probability that data have sufficient statistical significance at a certain confidence level. Legal certainty merely establishes that a complaint has met the minimum amount in controversy required for a court to have jurisdiction to hear the case. Legal certainty is built on scientific certainty.

10 TRANSLATING SCIENCE IN COURT

As a part of your responsibilities as an expert witness, you will no doubt be called upon to explain to your legal team and to educate judge and jury on what you would consider to be very basic toxicological principles (Omenn, 1994). If this disturbs you in any way then perhaps you should consider not becoming an expert witness. A jury can clearly sense when an expert is trying to educate them so that they will be able to make informed decisions and when an expert is merely demonstrating their wealth of knowledge, which they may not be in a position to even actually evaluate or appreciate in the first place. Some of the concepts that the toxicology expert witness will most commonly refer to in court and those that will be directly relevant to their specific case will include:

- Dose and the dose–response relationship
- Pathways, routes, frequency and duration of exposure
- Exposure assessments and how they are determined
- Relationships between exposure and dose
- Sites of chemical action in the body
- Threshold vs. nonthreshold exposures
- What is risk?

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Table 2 Federal rules related to expert witness testimony

Rule 602. Lack of Personal Knowledge	A witness may not testify to a matter unless evidence is introduced sufficient to support a finding that the witness has personal knowledge of the matter. Evidence to prove personal knowledge may, but need not, consist of the witness' own testimony. This rule is subject to the provisions of rule 703, relating to opinion testimony by expert witnesses.
Rule 701. Opinion Testimony by Lay Witnesses	If the witness is not testifying as an expert, the witness' testimony in the form of opinions or inferences is limited to those opinions or inferences which are (a) rationally based on the perception of the witness, and (b) helpful to a clear understanding of the witness' testimony or the determination of a fact in issue, and (c) not based on scientific, technical or other specialized knowledge within the scope of Rule 702.
Rule 702. Testimony by Experts	If scientific, technical, or other specialized knowledge will assist the trier of fact to understand the evidence or to determine a fact in issue, a witness qualified as an expert by knowledge, skill, experience, training, or education, may testify thereto in the form of an opinion or otherwise, if (1) the testimony is based upon sufficient facts or data, (2) the testimony is the product of reliable principles and methods and (3) the witness has applied the principles and methods reliably to the facts of the case.
Rule 703. Bases of Opinion Testimony by Experts	The facts or data in the particular case upon which an expert bases an opinion or inference may be those perceived by or made known to the expert at or before the hearing. If of a type reasonably relied upon by experts in the particular field in forming opinions or inferences upon the subject, the facts or data need not be admissible in evidence in order for the opinion or inference to be admitted. Facts or data that are otherwise inadmissible shall not be disclosed to the jury by the proponent of the opinion or inference unless the court determines that their probative value in assisting the jury to evaluate the expert's opinion substantially outweighs their prejudicial effect.
Rule 704. Opinion on Ultimate Issue	(a) Except as provided in subdivision (b), testimony in the form of an opinion or inference otherwise admissible is not objectionable because it embraces an ultimate issue to be decided by the trier of fact. (b) No expert witness testifying with respect to the mental state or condition of a defendant in a criminal case may state an opinion or inference as to whether the defendant did or did not have the mental state or condition constituting an element of the crime charged or of a defense thereto. Such ultimate issues are matters for the trier of fact alone.
Rule 705. Disclosure of Facts or Data Underlying Expert Opinion	The expert may testify in terms of opinion or inference and give reasons therefore without first testifying to the underlying facts or data, unless the court requires otherwise. The expert may in any event be required to disclose the underlying facts or data on cross examination.
Rule 706. Court Appointed Experts	(a) Appointment. The court may on its own motion or on the motion of any party enter an order to show cause why expert witnesses should not be appointed, and may request the parties to submit nominations. The court may appoint any expert witnesses agreed upon by the parties, and may appoint expert witnesses of its own selection. An expert witness shall not be appointed by the court unless the witness consents to act. A witness so appointed shall be informed of the witness' duties by the court in writing, a copy of which shall be filed with the clerk, or at a conference in which the parties shall have opportunity to participate. A witness so appointed shall advise the parties of the witness' findings, if any; the witness' deposition may be taken by any party; and the witness may be called to testify by the court or any party. The witness shall be subject to cross examination by each party, including a party calling the witness. (b) Compensation. Expert witnesses so appointed are entitled to reasonable compensation in whatever sum the court may allow. The compensation thus fixed is payable from funds which may be provided by law in criminal cases and civil actions and proceedings involving just compensation under the Fifth Amendment. In other civil actions and proceedings the compensation shall be paid by the parties in such proportion and at such time as the court directs, and thereafter charged in like manner as other costs. (c) Disclosure of appointment. In the exercise of its discretion, the court may authorize disclosure to the jury of the fact that the court appointed the expert witness. (d) Parties' experts of own selection. Nothing in this rule limits the parties in calling expert witnesses of their own selection.

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Table 2 (*continued*)

Rule 803. Hearsay Exceptions; Availability of Declarant Immaterial	The following are not excluded by the hearsay rule, even though the declarant is available as a witness: (18) Learned treatises. To the extent called to the attention of an expert witness upon cross examination or relied upon by the expert witness in direct examination, statements contained in published treatises, periodicals, or pamphlets on a subject of history, medicine, or other science or art, established as a reliable authority by the testimony or admission of the witness or by other expert testimony or by judicial notice. If admitted, the statements may be read into evidence but may not be received as exhibits.

- Risk perception
- Toxicological causation and how it is established
- Chemical exposure and development or exacerbation of disease
- Cancer risk
- Mechanisms of chemical carcinogenesis
- The uses and limitations of animal toxicity testing
- The use of toxicological data to assess risk in individuals and populations
- The use of toxicological data to establish acceptable levels of exposure for populations
- Populations at special risk
- Regulatory guidelines as they relate to protecting individuals and populations.

11 THE EXPERT'S ROLE IN CLARIFYING THE CONCEPT OF RISK

In court, it is often necessary in many cases for the toxicologist to explain what constitutes risk to an individual of injury from a chemical exposure. This is often a challenging task, as it is necessary to clearly explain to the judge and jury the differences in risk perception and risk assessment. The concept of real risk vs. perceived risk must be emphasized when possible (Richards, 2007). Risk perception is the sensation of danger associated with an exposure. Clearly there can be no risk, whatever the toxicity of the chemical in question, if there is no opportunity for exposure.

Unlike the current Chinese infant formula crisis, where measurable amounts of melamine have been found in both cans of the powdered formula and serum samples taken from stricken children (Macartney and Yu, 2008), chemical injuries in humans rarely permit quantitative determination of exposure doses. Clinical toxicologists rely instead on case reports, case series and occasionally experimental study data to elucidate probable dose–response relationships and mechanisms of action (Chamberlain, 1996). For example, occupational and environmental exposures to polychlorinated biphenyls (PCBs) prior to usage restrictions have been assessed to determine absorption, distribution, metabolism and excretion. Human exposures occur predominantly in occupational settings, as was the case for industrial exposures to lead and asbestos (Richards, 2007). It can be difficult, if not impossible, to quantify an exposure, even one that occurs under controlled circumstances. Moreover, humans are exposed to multiple chemicals over the course of a single day. This can make it difficult to affix blame for an adverse outcome to a single specific chemical.

People may tend to overestimate or underestimate the risks of exposure for a number of different reasons. Risk assessment is a procedure used to quantify the danger of exposure. It is typically broken down into four, sequential steps:

- Hazard identification
- Dose–response assessment
- Exposure assessment
- Risk characterization.

The concepts of threshold and nonthreshold exposures are important in toxicological testing. The expert witness must be sure to differentiate between the terms; it may be simplest to define them based on carcinogenicity. Jurors should be able to understand that regulatory agencies develop guidelines based on the theory that there is no safe threshold for exposure to carcinogens. Depending on the lawsuit, the expert may then need to explain that regulatory agencies tend to exaggerate risk to keep the maximum number of people safe. Zero threshold should not be construed as unavoidably hazardous.

12 TOXICOLOGICAL CAUSATION

As an expert witness you must be prepared to render opinions as to the use of toxicological data in the assessment of individual causation. You will be required to render opinions as to:

- Whether or not the chemical(s) in question can produce or exacerbate the type of illness claimed in this case
- Whether or not an exposure to the chemical or chemicals in question had actually occurred

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- Whether or not an exposure to the chemical or chemicals in question was of a sufficient duration to produce the illness in question
- Whether or not an exposure to the chemical(s) was of a magnitude sufficient to produce the illness in question
- Whether or not medical records support the health effects of the chemical or chemicals in question
- Whether or not there are other explanations that can account for the development or exacerbation of the illness in question
- Whether or not other individuals who have been similarly exposed developed comparable illness.

In his 1965 Presidential address to the Royal Society of Medicine, Sir Austin Bradford Hill opined that:

> *All scientific work is incomplete - whether it be observational or experimental…*
>
> *(Hill, 1965)*

He went on to delineate nine criteria for assessing causation from chemical exposure:

- Strength of the association between a specific chemical exposure or process and a specific injury
- Consistency of effect among individuals similarly exposed
- Specificity of the type of injury or injuries that are produced
- Temporality between the exposure and subsequent development of an injury
- Biological gradient or dose–response should be evident from available studies
- Plausibility of the biological effect must be supported by available information
- Coherence with available experimental information should be apparent
- Experiments may support or modify current observations if available
- Analogy from other chemicals or sources of information may be enlightening.

These came to be known as Hill's Criteria of Causality and they have had a profound impact on science and law.

Interrogatories typically refer to causality criteria; an expert witness should expect a question that asks if associations are proof or are they are anecdotal events. A causal hypothesis cannot be literally proven or disproven (Cole, 1997). The role of the expert is to evaluate the facts of the case as he or she knows them and use that knowledge to generate an opinion.

Establishing causality is one of the most challenging aspects of scientific research. Toxicologists who serve as expert witnesses are frequently asked to establish or disprove causality. A controlled study is the most effective means of evaluating a causal relationship between an exposure and an outcome. '*Correlation does not imply causation*' is one of the basic tenets of statistics. Simply put, it means that the existence of a correlation between variables does not prove a causal relationship. As an expert witness, you will be called upon to provide opinions addressing a number of questions which will be asked of you. You must be prepared to answer them.

Does a temporal progression support causality? Obviously, the appearance of symptoms prior to exposure would rule out causality; however, a strong temporal relationship between exposure and symptom/sign onset is insufficient evidence of a causal relationship. In an acute injury, the time period between exposure and the appearance of symptoms is generally short and may support cause and effect, but in and of itself is not reasonable proof. Frequently though, the onset of symptoms is delayed by the number or duration of biological processes that occur as a result of the exposure. One only needs to consider a delayed inflammatory response, or acute myelogenous leukaemia as a result of benzene exposure, to support the idea of the delayed onset model. There is a latency period of approximately two years between exposure to radiation, benzene or chemotherapy drugs and the appearance of clinically recognizable acute myelogenous leukaemia. Any toxic tort claim that alleges a shorter timeframe is scientifically untenable. Asbestos is another example of a significantly delayed disease onset; symptoms of mesothelioma may not be detectable for more than 20 years following the initial exposure to asbestos fibres.

Is the medical history of the plaintiff consistent with the type of exposure alleged? A thorough medical history can be an invaluable asset in allowing you to form your opinions. Occasionally the signs and symptoms themselves are suggestive of a specific exposure. For example, acute exposure to organophosphate pesticides typically elicits complaints of headaches, nausea, dizziness, anxiety, restlessness, muscle fasciculation, diaphoresis, salivation and lacrimation.

There is currently no standardized patient information questionnaire for the toxicologist which is capable of providing all the information desired and it is often difficult to read through volumes of medical records and reports to gather the information that you deem as especially important for you to reach your conclusions. This means it is often up to the medical professional to ask the correct questions and gather the pertinent medical records. The following is a list of information that you should request from your attorney, if not provided to you:

- Past and present occupational history
- Environmental history of exposure to toxic agents
- Lifestyle characteristics (e.g. use of nicotine and alcohol)
- Family medical history (e.g. medical conditions, diseases of relatives)

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- Personal medical history (e.g. present symptoms, lab test results, past injuries, current medical conditions, diseases, surgical procedures and medical test results).

Are the complaints specific or nonspecific? Acute exposures can trigger a constellation of nonspecific symptoms, including headaches, nausea, lightheadedness and fatigue. These symptoms can be considered part of the 'human condition', something everyone has experienced at one time or another. They are triggered by a host of physical, biological and psychological factors and may be mistakenly attributed to an exposure based solely upon a temporal association. Careful medical histories require a sharp focus on patterns in the emergence of signs and symptoms. It is far easier to establish causation or lack of causation when the observed signs and symptoms that are typically seen with a specific exposure such as vinyl chloride and hepatic angiosarcoma, for example, are either present or absent respectively. Unfortunately, the vast majority of cancers and other conditions are associated with multiple causative factors.

Do laboratory tests results support exposure? Routine tests are used to detect changes in normal body status and can provide clues as to consistency with exposure to toxicants. One only needs to think of blood gases and carbon monoxide exposure as a simple example or serum cholinesterase levels for organophosphate exposure. Other routine tests, such as complete blood count, basic metabolic panels and urinalysis may be important in providing additional insight for the toxicologist. More often than not, tests to determine the specific levels of the exposure chemical(s) of concern are either unavailable or have not been performed in a timely fashion.

What other explanations could account for a given illness? Collecting a meticulous medical and occupational history is often the only way both physician and toxicologist can perform this assessment. Additionally, the medical experts may provide a differential diagnosis which either supports or fails to support that the illness in question was directly attributed to the exposure (Muller, 2008). Failure to produce such a differential diagnosis by the physician and a toxicological assessment to support this diagnosis would leave any causal connection in doubt. Careful examination of the available data, coupled with deductive reasoning, can allow the expert to determine a causal relationship between exposure and symptoms. Animal studies, pharmacological research on mechanisms of toxicity, *in vitro* studies and epidemiological data, if available, may either provide or not provide support for the toxicity of a particular compound in this case, thus influencing the expert's opinion on causality. It is considerably more difficult when the available data is scant or conflicting. In cases such as these, the expert must be prepared to explain how these results impact their reasoning.

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Regulatory Toxicology

H. Paul A. Illing and Timothy C. Marrs

CONTENTS

1 INTRODUCTION: TOXICOLOGICAL RISK ANALYSIS AND REGULATORY TOXICOLOGY

Toxicological risk analysis includes all forms of risk analysis involving effects on human health and environmental pollution. It is the process of assessing, evaluating and managing risks. Risk analysis may be conducted by the individual, the legal entity (company), national governments or supragovernmental international organizations (such as the European Union (EU) or the United Nations (UN)). This national and international toxic risk analysis is conducted on behalf of society, because decisions have been taken that the nature and type of risk management required by society is too complex to be left to the individual (*caveat emptor* cannot be adequately sustained as the buyer is so disadvantaged by a lack of relevant knowledge and experience). Furthermore, in international risk management, leaving risk management to individual governments may raise the possibility of use of spurious risk management as a nontariff barrier to trade. Legislation is introduced to regulate the ways in which these risk assessments are conducted. When toxic risk analysis is conducted by or on behalf of governments or intergovernmental organizations, the toxicology contained within the risk assessments and evaluations is referred to as 'regulatory toxicology'.

Regulatory toxicology is the process whereby information relevant to assessing and evaluating the toxicity of agents, which may be biological, chemical or physical in nature, is obtained and evaluated by or on behalf of governmental or international organizations. The aim is to protect workers, consumers, the public generally and the environment. The intention is either to provide information for the management of risks, usually in an easily understood form so that untrained individuals can manage the risks, or actively to manage the risks. It has been said that regulatory toxicology is to toxicology as military music is to music. This is because the requirements of many regulatory systems are closely defined in terms of both the studies required and the interpretations to be placed upon the results obtained, so that there is little scope for originality.

Essential to a consideration of regulatory toxicology are the concepts of risk and hazard. The latter is the inherent property of an agent or situation having the potential to cause adverse effects when an organism, system, population or subpopulation is exposed to that agent. Risk is the probability of an adverse effect in an organism, system, population or subpopulation caused under specified circumstances by exposure to that agent.

1.1 General Principles

Regulatory toxicology does not take place in a vacuum; a legislative framework is required. Society has perceived

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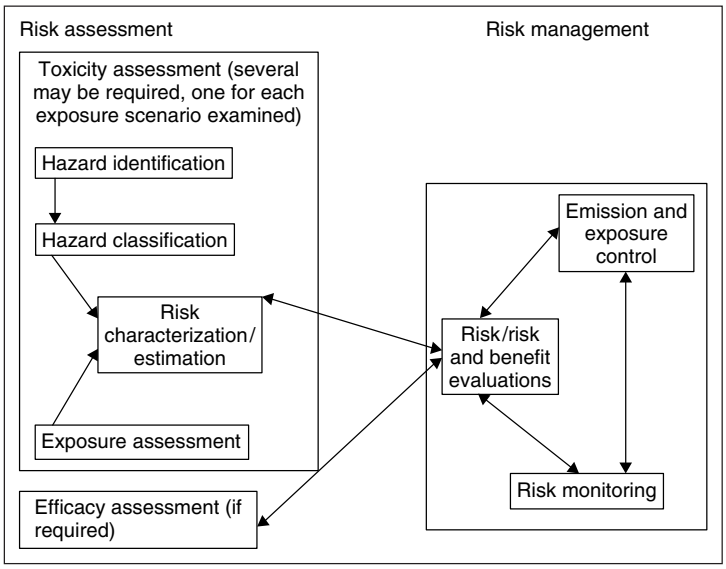

Figure 1 Risk assessment and risk management. Note: the risk analysis process is undertaken within a societally derived framework, represented by the outer box. (Adapted from Illing, 2009.)

that there is a problem and has framed a system for examining that problem. This system therefore develops from the sociological and psychological perceptions of the risks associated with the particular class of agent. Legislative frameworks have developed piecemeal, as society has seen the need to manage specific uses. The first toxicology regulation in the UK is often said to be the Alkali Act of 1863 (Alkali Act, 1863), which was intended to control pollution by heavy industry. Other early regulatory schemes were concerned with drugs and food contaminants, and regulation was later extended to pesticides and other groups of compounds.

In this chapter the regulatory systems associated with chemicals and human health will be examined, but those concerned with physical agents, such as radiation, and biological agents, such as organisms used to control pests, will not be examined further. In addition, in the main, the purely environmental risk evaluations are not included in this chapter, although risks to human health via the environment are included.

Within the legislative framework it is traditional to have separate regimes for different end uses; for example, human medicines, veterinary products, medical devices, 'cosmetics' (otherwise called personal care products), foods, food additives and contaminants, and pesticides, subdivided into plant protection products and biocides. More recently, reserve schemes have been developed for chemicals placed on the market and not included in these specific use schemes. For all of these schemes, there is a clearly identifiable entity seeking to place the chemical on the market and it is usually possible to insist that they accept the costs of premarketing and postmarketing testing and, sometimes, postmarketing monitoring and surveillance. Additional regulatory schemes deal with indoor and outdoor air quality, water quality (drinking and environmental) and soil contamination. Generally, it is difficult to identify a nongovernmental entity to underwrite testing and controlling risk when dealing with these latter schemes. Consequently, in such circumstances, the government is frequently both regulator and provider of research funds to acquire new knowledge. Another group of substances where it may be difficult to identify an entity other than the government to underwrite testing is food contaminants such as aflatoxins.

The process by which toxicological risks are assessed and managed is illustrated diagrammatically in **Figure 1**. It was first set out in two documents, one a report of a UK Royal Society Study Group (Royal Society Study Group, 1983), and the second a report of the US National Research Council (National Research Council, 1983). Many definitions for the elements of the process and of the stages in the process have recently been agreed internationally (OECD, 2003; IPCS, 2004). The definitions for several key terms associated with **Figure 1** are given in **Table 1**.

Two key terms: emission and exposure control, and risk perception, were not defined in the OECD (Organisation for Economic Co-operation and Development)/IPCS (International Programme on Chemical Safety) list of key definitions. Emission and exposure control is associated particularly with industrial hazards—the control of emissions from stacks to the atmosphere or discharges to waste water. However, it also applies to most sources of exposure to chemicals, including drugs. Drugs are controlled through designating those authorizing the obtaining of the medicine (doctors, and so on, with prescribing rights) and through designating the outlets from which they may be obtained (e.g. pharmacy only, or any supermarket). Most other regulatory schemes control emission, for example, for foods there are emission limits

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Table 1 Key definitions for use in toxicological risk analysis

Term	Definition
Exposure assessment	Evaluation of the exposure of an organism, system, population or subpopulation to an agent (and its derivatives). Exposure assessment is the third step in the process of risk assessment. (IPCS (2004) Part 2 states 'The process of estimating or measuring the magnitude, frequency and duration of exposure to an agent, along with the number and characteristics of the population exposed. Ideally it describes the source, pathways, routes and uncertainties in the assessment'.)
Hazard	The inherent property of an agent or situation having the potential to cause adverse effects when an organism, system, population or subpopulation is exposed to that agent.
Hazard characterization	The qualitative and, wherever possible, quantitative description of the inherent properties of an agent or situation having the potential to cause adverse effects. This should, where possible, include a dose–response assessment and its attendant uncertainties. Hazard characterization is the second stage in hazard assessment, and the second step in risk assessment.
Hazard identification	The identification of the type and nature of adverse effects that an agent has an inherent capacity to cause in an organism, system, population or subpopulation. Hazard identification is the first stage in hazard characterization, and the first step in risk assessment.
Risk	The probability of an adverse effect in an organism, system, population or subpopulation caused under specified circumstances by exposure to that agent.
Risk analysis	Process for controlling systems where an organism, system or population or subpopulation could be exposed to a hazard. The risk analysis process consists of three components: risk assessment, risk management and risk communication.
Risk assessment	A process intended to calculate or estimate the risk to a given target organism, system, population or subpopulation, including identification of attendant uncertainties, following exposure to a particular agent, taking into account the inherent characteristics of the agent of concern as well as the characteristics of the specific target system. The risk assessment process includes four steps: hazard identification, hazard characterization (related term: dose–response assessment), exposure assessment and risk characterization. Risk assessment is the first component in a risk analysis process.
Risk characterization	The qualitative and, wherever possible, quantitative determination, including attendant uncertainties, of the probability of occurrence of known and potential adverse effects of an agent on a given organism, system, population or subpopulation, under defined exposure conditions. Risk characterization is the fourth step in the risk assessment process.
Risk communication	Interactive exchange of information about health or environmental risks among risk assessors, managers, news media, interested groups and the general public.
Risk evaluation	Establishment of a qualitative or quantitative relationship between risks and benefits of exposure to an agent, involving the complex process of determining the significance of the identified hazards and estimated risks to the system concerned or affected by the exposure, as well as the significance of the benefits brought about by the agent. It is an element of risk management. It is synonymous with risk–benefit evaluation.[a]
Risk management	Decision-making process involving consideration of political, social, economic, and technical factors with relevant risk assessment information relating to a hazard, so as to develop, analyse and compare regulatory and nonregulatory options, and to select and implement appropriate regulatory response to that hazard. Risk management comprises three elements: risk evaluation, emission and exposure control, risk monitoring.
Risk monitoring	Process of follow-up of decisions and actions within risk management in order to ascertain that risk containment or reduction with respect to a particular hazard is assured. Risk monitoring is an element of risk management.

[a]Technically this definition confuses risk and risk–benefit. Risk alone is the criterion when dealing with an equity-based system for examining risks, risk–benefit is the criterion employed when dealing with tolerable risks in a system that can employ utility and technical-feasibility criteria when examining risks (see below).
From OECD (2003) and IPCS (2004) (Part 1) with minor textual amendments, unless otherwise noted.

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from packaging and maximum residue levels (MRLs) for additives and contaminants in foodstuffs.

Monitoring includes inspection to ensure that conditions of control are being met in the field. It also includes procedures, such as those associated with pharmacovigilance, designed to ensure that previously unidentified effects do not go unrecorded.

Risk perception was also not defined. However, the Royal Society Study Group (1983) called perceived risk the combined evaluation that is made by an individual of the likelihood of an adverse event occurring in the future and its likely consequences. In the introduction to the chapter on risk perception in the 1992 report of the Royal Society Study Group (1992), risk perception (from the perspective of the social sciences) involves peoples' beliefs, attitudes, judgements and feelings, as well as the wider social or cultural values and dispositions that people adopt towards hazards and their benefits. When aggregated, individual beliefs, attitudes, and so on, become the societal beliefs, attitudes, and so on, that both drive legislation and drive concerns about decisions taken by public bodies set up to administer and enforce the legislation.

Central to any toxicological risk analysis is the risk evaluation. There has been debate over whether it is part of risk assessment or part of risk management. It was notable that the Royal Society Study Group (1983) placed risk evaluation as part of the risk assessment, whilst the US National Research Council (1983) placed it as the first stage of risk management. In practice, risk evaluation is the key link between the two elements of risk analysis. Although in the simplest cases this risk evaluation can be conducted by individuals using guidelines on how to interpret the tests, in Europe, the more complex situations evaluations are often conducted on behalf of society by scientific committees consisting of relevant experts (and ideally some lay ('stakeholder') participation), and their recommendations implemented by the regulatory authority.

1.1.1 Basis of Risk Evaluation (Equity, Utility and Technical Feasibility Criteria)

The confusion between risk evaluation and risk/benefit evaluation in the OECD/IPCS definitions concerning risk evaluation can be examined further. It arises because of confusion about the basis of the risk evaluation. Decisions can be reached using one or more of three 'pure' criteria (HSE, 2001). These are:

- An equity-based criterion, which starts from the premise that all individuals have unconditional rights to certain levels of protection. This leads to standards, applicable to all, held to be usually acceptable in normal life. In practice this leads to fixing a limit to represent the maximum level of risk above which no individual can be exposed. If the risk characterization indicates that the risk is above this limit, the risk is held to be unacceptable—whatever the benefits.
- A utility-based criterion, which applies to the comparison between incremental benefits of measures to prevent the risk of injury or detriment, for health effects, ill health and the cost of the measures. The utility-based criterion compares the relevant benefits (e.g. statistical lives saved, life-years extended, reduced ill health and better quality of life) obtained by adoption of a particular risk prevention measure, with the net cost of introducing it, and requires that a balance be struck between the two. This balance can be deliberately skewed towards benefits by ensuring gross disproportion between costs and benefits.
- A technology-based criterion, which essentially reflects the idea that a satisfactory level of risk prevention is attained when state of the art control measures (technological, managerial, organizational) are employed to control risks, whatever the circumstances.

These criteria underlie the regulatory process first outlined by the Royal Society Study Group (1983). The scheme is based on:

- an upper limit of risk which should not be exceeded for any individual (unacceptable);
- further control, so far as is reasonably practicable, making allowances if possible for aversions to the higher levels of risk or detriment (tolerable);
- a cut-off in the deployment of resources below some level of exposure or detriment judged to be trivial (broadly acceptable).

The scheme is outlined in **Figure 2**.

In most fields of use requiring preauthorization, regulatory toxicology attempts to be protective, that is, to use a pure equity criterion and to include some disproportion in order to be conservative (Illing, 1999). This has been called gatekeeping. Thus, with pesticides it is assumed that, for most uses, no individual benefit will arise from exposure of people to pesticides. It is however realized that this is not always the case, as a benefit may arise when a pesticide is used, for example, to prevent exposure to fungal toxins in food. Risk–benefit considerations are major factors when evaluating human medicines, because of the need to balance risk of side effects against the benefit of the therapeutic effect in the patient. Risk–benefit considerations may also be appropriate when investigating occupational exposures, as it may be necessary to allow a tolerable risk to those exposed occupationally in order for society to gain great benefit from the products. In these circumstances, more explicitly considered societal judgements

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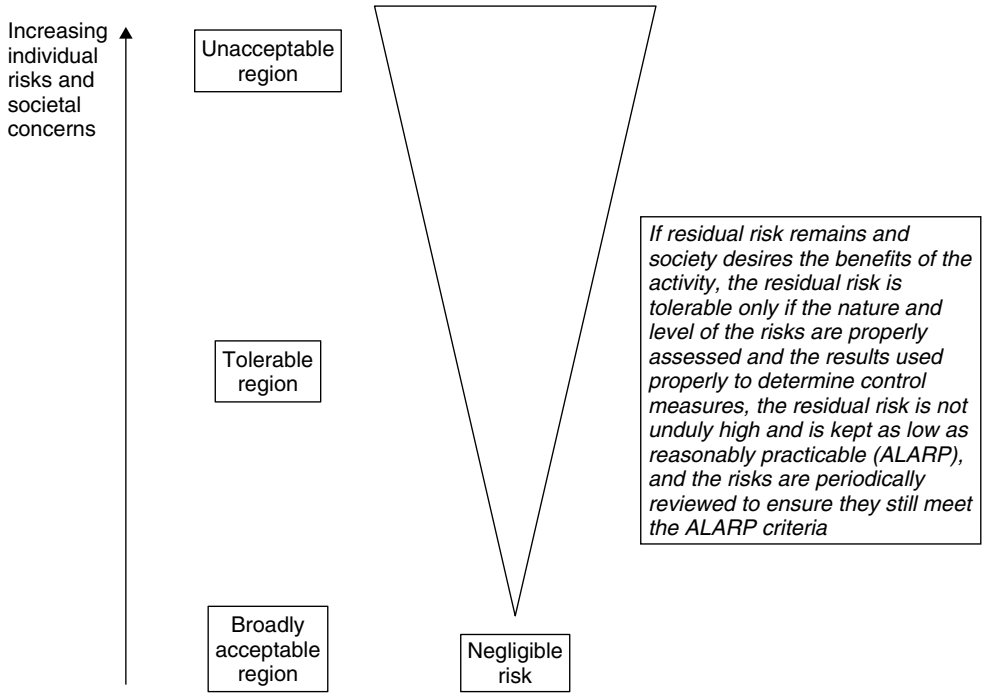

Figure 2 Outline of the Royal Society approach to risk management/tolerability of risk framework. (Based on HSE, 2001.)

concerning what constitutes 'broadly acceptable' and 'tolerable' risk criteria are required. They may also be relevant when considering the toxicity of essential nutrients, including vitamins and minerals, if the margin of exposure (MoE) between the essential minimum requirement and the level at which toxicity is seen is low. Often, air quality guidelines are set on technology-based criteria, with deadlines for requirements to meet tighter standards.

Generally, in the first instance, regulatory risk assessments are based on achieving a 'broadly acceptable' level of risk (a 'safe' level of exposure). Two processes may be used to evaluate data. These are the 'uncertainty factors (UFs)' approach and the 'margin of exposure' approach. The former mixes risk characterization with risk evaluation. In the latter, the decision concerning the acceptability is technically purer as the decision concerning the acceptability of the MoE (the risk evaluation) is independent of the evaluation of the data in the risk characterization.

1.1.2 Procedures for Regulatory Assessment and Risk Evaluation

The simpler regulatory assessments and evaluations are often conducted by an individual using guidelines. Complex regulatory evaluations may be carried out by expert committees. Before dealing with specific procedures it is necessary to set out some general principles that underlie the procedures employed.

1.1.2.1 Source–Pathway–Receptor

Many regulatory assessments have to take into account where the chemical comes from and how it is released (the source of the exposure), the passage of the chemical from the source of the exposure to the receptor (the pathway) and the receptor, at which the chemical exerts its effect, when analysing risks and setting standards (**Figure 3**). Standards may be set at various points in the transfer from source to receptor, thus risk evaluations involving toxicological input may be required at several points during this transfer. These can include limits at source such as maximum inputs into the environment from a factory, often called emission limits or discharge limits, or maximum residue levels in food. Alternatives to limits at source are intake standards measured in one or all the appropriate media (air, water, food or soil). Occasionally, as with occupational lead exposure in the EU, uptake standards may be preferred.

Modelling is required to relate input to intake and uptake. There are many types of models, usually specific to the particular exposure circumstances, and hence to the particular regulatory scheme, so they will not be considered further here.

1.1.2.2 Dose Effect and Dose Response

Most adverse effects follow a continuum of severity, from normal, through homeostatic adjustment, to compensation by input of reserve capacity with possibility of repair, to breakdown (disability and death). In the individual, severity of effect increases with increasing dose. Biological variation in the sensitivity of individuals to

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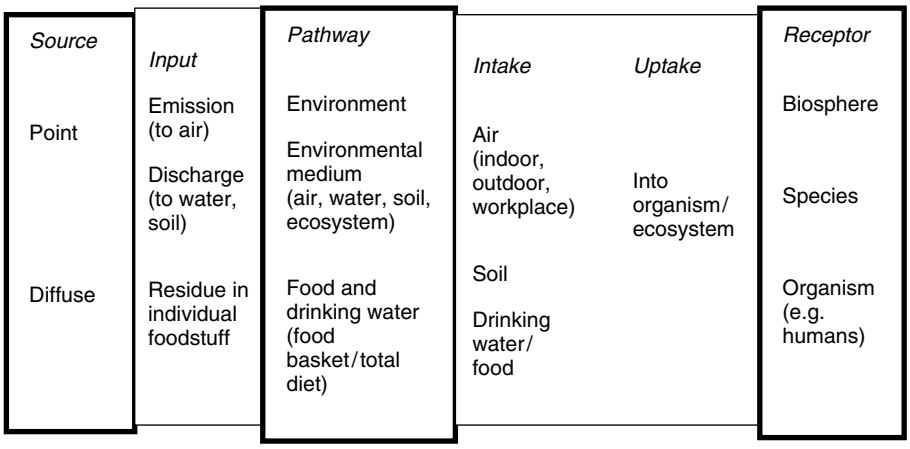

Figure 3 The passage of a chemical from source to receptor. (Reproduced with permission from Illing, 2006. © Royal Society of Chemistry.)

these effects means that the frequency of occurrence of a specific level of response can be represented by a dose–response relationship. If the individual variation in severity of effect with dose is small, a population dose–effect curve can be derived (see **Figure 4**), with its associated confidence limits, and the dose–response relationship is derivable from the statistics of the dose–effect curve.

Some substance/effect combinations (e.g. teratogenicity) are severe effects for which it is difficult to describe a dose–effect relationship, but for which there is a clear threshold. For these substance/effect combinations it is possible to set an equity-based safe limit (a broadly acceptable risk) or a utility/technological-feasibility base limit (a limit based on a tolerable risk).

Some harmful effects are quantal (all-or-nothing), appear random (stochastic) in terms of who they affect in the exposed population, and may be considered to be without threshold. Usually genotoxic substances that may cause cancer or heritable genetic damage are included in this category. Occupational asthmas are also occasionally included on grounds of very wide interindividual variations in susceptibility. Usually, the effect is considered to be 'all-or-nothing', and without a no-observed-adverse-effect level (NOAEL). A dose–effect relationship cannot be set out, but a dose–response relationship is clearly feasible and is assumed to have no threshold. In the USA, there have been attempts to describe a broadly acceptable risk for carcinogens, and methods have been developed for determining safe levels of exposure. However, these methods are not universally accepted. In other circumstances, the risk characterization, evaluation and management of these effects is based on the principle that there is no safe level of exposure; that is, that, under an equity-only-based risk management system, the substances must be banned. When present as contaminants, it may not be possible to ban the substances, and approaches based on the 'virtually safe' level of exposure have been adopted to deal with the problem. These approaches are based on utility and technological feasibility, and are therefore tolerable, rather than broadly acceptable, risks.

Approaches to toxic risk characterization and evaluation depend on whether the response is considered to have a threshold or not.

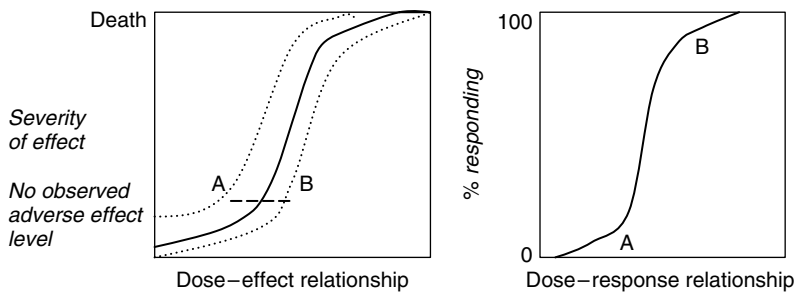

Figure 4 The relationship between dose effect and dose response. The dotted lines indicate the 95% confidence limits for the dose–effect relationship; taking the values for a fixed level of effect (AB) they can be transformed into the 5 and 95% dose values for the dose–response curve for that particular level of effect. (Reproduced with permission from Illing, 2006. © Royal Society of Chemistry.)

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1.1.2.3 Testing Strategies, Test Methods and Evaluation Procedures

From the point of view of the notifier or registrant, a premarketing regulatory scheme includes four key elements:

- when to conduct which test (testing strategies);
- the protocols considered appropriate for that test (test methods);
- an audit process (such as Good Laboratory Practice (GLP))
- the procedures for evaluating the tests and, where necessary, inserting the results into the appropriate exposure model.

Testing strategies depend on the end points of interests for the particular regulatory scheme, and this may change with intended use. Hence, for example, there are core requirements and requirements related to specified uses in the EU scheme for biocides. Testing strategies may include staged testing, and, once some data have been evaluated, in particular areas (notably human medicines and personal care products) testing in humans becomes permissible.

Test methods may cover physical chemistry and structure–activity evaluation, acute and short-term toxicity (including skin and eye irritation and skin sensitization), repeated-dose toxicity, carcinogenicity, reproductive toxicity and studies examining specific aspects of toxicity, such as neurotoxicity or immunotoxicity. Regulatory schemes tend to divide into two groups when looking at test methods. One group follows the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) route, the other the OECD route. The biggest divergence is over reproductive toxicity testing, where the protocols are incompatible with one another. However, the emergence of these two blocks has considerably reduced the need to test for the same end point using a variety of marginally different protocols to satisfy different national regulatory bodies.

The advent of GLP and Good Clinical Practice (GCP), both audit systems, has reduced substantially the amount of duplicate testing, by making a test undertaken in one country and audited in accordance with agreed internationally accepted guidelines acceptable to other countries. These audit procedures were introduced following incidents concerning the accuracy of data submitted to regulatory authorities (see **Quality Assurance in Toxicology Studies**).

The procedures for evaluation may be kept in house by a regulatory authority, or they may be carried out by an independent body or by the notifier, depending on the regulatory scheme. If they are carried out externally from the regulatory authority, then guidance is published concerning how to interpret the results.

Regulatory toxicity tests are coarse screens for toxicity. Subtle effects may not be detected. Some toxic effects are well characterized ('known knowns'), others are identified but poorly characterized ('known unknowns') and others may not be detected by regulatory testing ('unknown unknowns'). Although any regulatory scheme seeks to minimize the known unknowns and unknown unknowns, it is unlikely that they can be eliminated entirely. Thus, for the last two groups of effects it is essential to include risk monitoring and re-evaluation, either periodically or on the basis of new information/new interpretations, in any regulatory scheme.

In the case of schemes not involving prior notification (e.g. for air or drinking water quality), the chemical is already present and the risk evaluation is based on available evidence, including, significantly, often much greater availability of human epidemiological data than there would be with a new chemical entity for use as a drug or pesticide. If further studies are required, usually the regulator has to fund them.

In recent years the ability of analysts to detect extremely low levels of chemicals (usually contaminants) has considerably increased. There is a level of presence of a chemical below which it is not considered worthwhile testing. Thus the concept of a threshold of toxicological concern has been developed. However, numerical thresholds usually depend to some extent on structure–activity relationships, as they vary for different end effects. In addition, some schemes include arrangements whereby there are levels of contamination for which only reduced testing of the contaminant is required. This is a useful tool in discouraging unnecessary testing of contaminants.

1.1.2.4 Methods for Effects That Have a Threshold

1.1.2.4.1 Uncertainty-Factor Approach for Intake/Uptake

In regulatory toxicology there are two ways of conducting the risk assessment and risk evaluation. In the first way, used for standard settings, the risk characterization is combined with the risk evaluation. In one procedure, the uncertainty-factors (UFs) (formerly called safety factors) approach, the risk characterization is set against standard, predefined risk evaluation criteria, and a maximum safe (however defined) level of exposure determined (the reference dose (RfD, also called the health-based guidance value), or, for the EU REACH (Registration, Evaluation, Authorisation and restriction of CHemicals) regulation, the derived no-effect level). The definition of an uncertainty factor is:

> *A reductive factor by which an observed or estimated NOAEL is divided to arrive at a criterion or standard that is considered safe or without appreciable risk.*
> *(OECD, 2003; IPCS, 2004)*

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The UFs approach can be described by the equation:

$$\text{RfD} = \frac{\text{NOAEL (or LOAEL or BMD)}}{\text{Uncertainty and modifying factors}} \qquad (1)$$

Where LOAEL is the lowest-observed-adverse-effect level; BMD is the benchmark dose—these and NOAEL essentially are dose levels at which no or very minor adverse (and, usually reversible) effects are seen. The BMD is a statistically based procedure favoured in the USA. The critical effect is that which, under the exposure conditions, yields the NOAEL, from which the RfD is calculated. The pivotal study is the study from which the critical effect NOAEL is derived.

In this process, hazard identification, hazard characterization and risk evaluation criteria are combined to produce a maximum safe level of exposure. The hazard identification and hazard characterization includes determination of relevant physicochemical and toxicological properties through testing and, where available, appropriate human or other target-species studies (experimental studies, clinical trials and epidemiology). The specific mix of studies depends on the particular regulatory scheme. Although variants are employed by the different regulatory authorities, the actual procedure used for the combined risk characterization and evaluation is essentially that set out in EHC (environmental health criteria) 170 (IPCS, 1994), and is given in **Figure 5**.

Data packages will usually generate many NOAELs, depending on the duration, type of exposure and type of study; some studies, for example studies of developmental toxicity, will generate more than one (in this case for the dams and the offspring). Thus it may be necessary to adjust from one route to another, or from, say, a 28-day repeated-dose study to a 90-day repeated-dose study. Assumptions (usually worst-case, in the absence of information) are usually made concerning the route-to-route extrapolation. Thus, ideally, such extrapolation should be avoided. Generally NOAELs decrease with duration of exposure, so UFs may be used to adjust a NOAEL from a study of one length of duration to a NOAEL at another duration.

The original pragmatic uncertainty factor for an equity-based broadly acceptable level of risk (safe exposure) was set at 100, over 50 years ago (Lehman and Fitzhugh, 1954). It has remained at this value when a complete set of good-quality studies is available to the regulator, even though what constitutes a complete set of studies has been greatly extended. Where good experimental human data are used to derive the RfD, a UF (usually 10) is used to cover variability in the human population, and where animal data are used a further UF (often 10, making $10 \times 10 = 100$ *in toto*) is used. The 10-fold UF for extrapolation from animals to humans is used because there are often no data on the relative sensitivity of humans and animals. Both 10-fold factors have been divided, more or less arbitrarily, as being partly due to toxicokinetics and partly due to toxicodynamics (IPCS, 1994; 1999). Although detailed examinations have been conducted to validate these assumptions *post hoc*, they remain no more than pragmatic judgements based on experience. If actual data for toxicokinetics and toxicodynamics are available (e.g. from physiologically based toxicokinetic modelling or from biologically based dose–response modelling), such data can be substituted for the pragmatic assumption. A further pragmatic assumption, application of an extra factor of 10 for seriousness of effect, is often used when dealing with teratogens and nongenotoxic carcinogens; that is, when there is a severe effect for which it is difficult to describe a dose–effect relationship.

Although there have been a number of attempts to put UFs onto a more scientific basis, they remain pragmatic assumptions. This use of pragmatic assumptions means that, where the RfD is breached, it cannot be assumed that an adverse effect will occur.

One reason why pragmatism is likely to persist is that UFs may also have to take into account societal judgements (Illing, 1999). It is notable that, in the past, occupational exposures, which have to allow for utility/technical feasibility criteria as well as equity criteria, and therefore cannot afford the same level of disproportion, have tended to use smaller uncertainty UFs (Fairhurst, 1995).

1.1.2.4.2 Margin-of-Exposure Approach for Intake

The MoE approach is technically purer than the uncertainty-factors approach as it separates the risk characterization (and hence the risk assessment) from the risk evaluation.

The margin of exposure is defined as: ratio of the no-observed-adverse-effect level for the critical effects to the theoretical, predicted or estimated exposure dose or concentration (OECD, 2003; IPCS, 2004).

The margin-of-exposure approach can be described by the equation:

$$\text{MoE} = \frac{\text{NOAEL (or LOAEL or BMD)}}{\text{Predicted or estimated exposure}} \qquad (2)$$

The ideal is to use measured exposure, but predicted exposure often is the only exposure data available. Models are often used to obtain predicted exposure. These may include models for route-to-route extrapolation as the data available may be for the wrong route of exposure (e.g. oral, rather than inhalation), as well as models for predicting the frequency and dose (exposure level and duration). There are many types of models, usually specific to the particular exposure circumstances, so they will not be considered further here.

With the MoE approach, social, political and other factors affecting the risk evaluation are separated from the scientific and technical process of risk assessment.

For some experts the 'margin of safety' is an alternative term and has the same meaning as the MoE (OECD,

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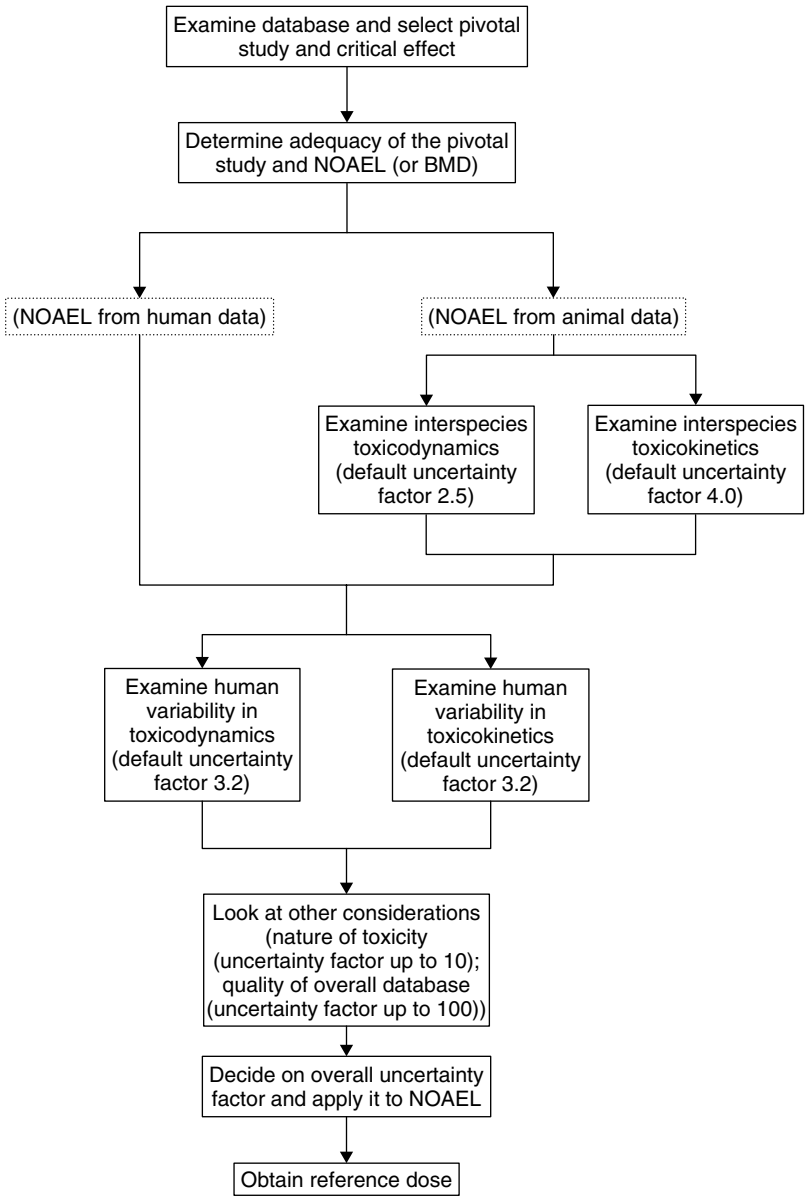

Figure 5 Procedures for the derivation of exposure standards—'uncertainty-factors' approach. NOAEL—no observed adverse effect level; BMD—benchmark dose. (Reproduced with permission from Illing, 1999. © Elsevier.)

2003). For others, the margin of safety means the margin between the RfD and the actual exposure or concentration. Thus, if the term 'margin of safety' is employed it is important to know how it has been defined.

1.1.2.5 Extrapolation for Nonthreshold Effects—Cancers and Heritable Genetic Effects

The biggest divide in toxicological risk analysis occurs in respect to attitudes to the evaluation of carcinogens. In the USA and elsewhere, extrapolation is undertaken using numerical approaches based, if necessary, on animal studies and mathematical modelling. The currently favoured model is linear extrapolation from the low 95% confidence limit for a dose associated with a 10% extra risk (LED_{10}) (IPCS, 1999). In essence the mathematical model is a device for obtaining a safe level of exposure (a broadly acceptable risk).

In the UK, regulators do not generally accept such procedures. They deem that:

- the methods are not validated;
- they are often based on incomplete or inappropriate data;
- they are derived more from mathematical assumptions than from a knowledge of biological mechanism;
- they demonstrate a disturbingly wide variation in the risk estimates, depending on the models used.

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Thus exposure is eliminated, where possible, or reduced 'as low as is reasonably practicable' (IGHRC, 2002). This approach is based, where possible, on applying an equity-based criterion for evaluation of the toxicity, and where a contaminant cannot be eliminated, applying utility and technological-feasibility criteria to establish a standard based on a risk sufficiently low as to be unquantifiable (Maynard *et al*., 1995), effectively a minimal tolerable risk.

This difference between the mathematical approach and the pragmatic approach probably arises from cultural differences associated with the acceptability of mathematical modelling, and legislative differences concerning how carcinogens should be treated.

1.1.2.6 Inputs and Input Standards

Input standards include discharge limits from factories to air or sewerage systems, and MRLs in meat or fruit and vegetables, or maximum amounts of toxic chemicals in cosmetics. Input information may also be required for prediction when dealing with the toxicity associated with major accident hazards. Modelling is required to relate input to intake. There are many types of models, usually specific to the particular exposure circumstances, so they will not be considered further here.

1.1.2.7 A Cautionary Note

In recent years the development of guideline exposure standards has become increasingly internationalized. Although international guideline standards have been set, they are usually based on first world attitudes to risk and life expectancy, thus they may be appropriate for first world nations but wholly inappropriate when dealing with other conditions. What constitutes adequate evaluation and control depends on attitudes to risk and background morbidity and mortality data. When agriculture is involved, the problems associated with the background agroecosystems also have to be taken into consideration. Hence, although the underlying hazard characterization (and toxicology) remains relevant, there is a need to adapt the risk characterizations and risk evaluations, together with the approaches to risk management, to reflect particular exposure circumstances.

1.1.2.8 Hazard Characterization

In certain regulatory schemes, notably those for chemicals based on the OECD approach, substances may be classified solely on their hazardous properties. Classifications based on simple tests, clear end points and definite criteria may be deterministic (classifiable/not classifiable) or more quantitative (grade as very toxic, toxic or harmful). When tests are complex, end points difficult to interpret and criteria difficult to define, classification is a simple deterministic statement based on quality and type of evidence. This information is then passed through the supply chain, through, for example, information sheets or safety data sheets. The various recipients conduct appropriate risk evaluations at the different stages of the supply chain. This classification is becoming more international as countries and supranational blocks, such as the EU, are implementing the UN Globally Harmonized System (GHS) for classification of the hazardous properties of a chemical (European Parliament and Council, 2008).

2 TYPES OF REGULATORY SCHEMES

Thus there are numerous regulatory systems worldwide, most concerning particular groups of compounds, for example drugs, pesticides, industrial chemicals, and originally, at least, covering one country. The tendency over the last few years has been for trading groups such as the EU and the North American Free Trade Area (NAFTA) to centralize procedures; further there have been attempts to harmonize data requirements, within and between trading blocks. The various systems fall into a few patterns (Diggle, 1999) (**Table 2**). Note that the difference between these schemes is often one of degree, and the EU schemes for pesticides and, for example, food additives are quite similar on paper, the main difference being that new pesticides are common and new food additives less common.

In the UK, pesticides were formerly regulated by a voluntary scheme (the Pesticides Safety Precaution Scheme), as were novel foods. In addition to formal regulatory mechanisms, the law of tort may apply in common law countries, if untoward effects are attributable to a substance.

Table 2 Type of regulatory framework

Type of scheme	Effect	Examples
Premarketing authorization	The marketing of the product can be authorized only after the regulatory body has examined safety and efficacy	Human and veterinary pharmaceuticals; pesticides
Allowed/proscribed lists	There are official lists of allowed and/or proscribed ingredients; individual authorization is not necessary	Cosmetics; food additives
Notification schemes	The individual substance does not need to be authorized, but its use/sale/manufacture has to be notified, together with the basic properties of the compound.	Industrial chemicals (REACH)

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It should be remembered that strict regulation is not a win–win situation in all circumstances. Thus fungal toxins are a major cause of mortality and morbidity in some countries (Wang *et al*., 1998), as are parasites in food of animal origin (Kraft, 2007); insect vectors are a major cause of disease in human populations and control is usually at least partially by chemical means. Further, large companies can deal with regulations more easily than small companies, so that regulation may inhibit competition and drive small businesses from the market (Booker, 2008).

Below are discussed some of the regulatory systems for the main groups of chemicals, using the systems in the EU and USA as exemplars.

2.1 Premarketing Authorization Systems

These systems are used most notably for human and veterinary pharmaceuticals and for pesticides. In such systems, safety and efficacy data are presented by the applicant (usually the organization wishing to market the substance) to the regulatory body, which, in the United Kingdom (UK), may rely upon the advice of an expert committee (the Committee on the Safety of Medicines, the Veterinary Products Committee and the Advisory Committee on Pesticides (ACP), respectively). In each case, national systems have largely been subsumed into an EU system. With a very few exceptions data have to be generated according to GLP.

2.1.1 Regulation of Pharmaceuticals for Use in Humans

Most countries have a system for regulating pharmaceuticals based on safety, quality and efficacy, in which the regulator has to make a judgement weighing risk against benefit for the patient.

2.1.1.1 In the European Union

In the EU, Directive 65/65 (Council of the European Economic Community, 1965) as amended, provides the legal basis for the regulation of human pharmaceuticals ('medicinal products'). Other directives established standards for testing the products. Furthermore a committee, the Committee for Proprietary Medicinal Products (CPMP), was established as well an agency, the European Medicines Evaluation Agency (EMEA) in London, UK.

The notice to applicants is a guide from the European Commission intended primarily for the pharmaceutical industry, but the notice gives a detailed overview of the system. Volume 2A covers the procedures for marketing authorization (European Commission, 2005–2007), Volume 2B covers presentation and content of the dossier (European Commission, 2006a), while Volume 2C covers the regulatory guidelines themselves (European Commission, 2003–2007). These documents are regularly updated. The format of applications for marketing authorization has been standardized throughout the EU (European Commission, 2006a).

2.1.1.1.1 Preclinical Data Requirement

The toxicological data requirements depend on a number of factors, including proposed duration of dosing and the population, for example whether the drug will be used in women of child-bearing age. Acute and repeated-dose toxicity studies are required, the latter with a duration depending on the proposed duration of human treatment. Studies of reproductive, developmental and perinatal toxicity are usually required as well as mutagenicity tests *in vitro* and *in vivo,* and carcinogenicity. Omission of particular studies needs to be justified.

2.1.1.1.2 Clinical Trials

An essential feature in bringing a new drug to market is the clinical trial. Clinical trials in the UK are regulated under the Medicines for Human Use (Clinical Trials) Regulations (2004). Studies must be in accordance with the Declaration of Helsinki (1964), which set out ethical guidelines for human volunteer studies (see also WMA, 1996). Directive 91/507/EEC (Commission of the European Communities, 1991) lays down that clinical trials should be carried out according to GCP.

2.1.1.1.3 Marketing Authorizations

To obtain a marketing authorization, there are two routes that can be followed, a centralized procedure, and the mutual recognition procedure. In the former, the application is made to the EMEA. A rapporteur, who is a CPMP member, is appointed to lead the scientific evaluation. The CPMP gives an opinion on whether a marketing authorization should be granted. This is passed to the European Commission and thence to the standing committee on medicinal products (SCMP) for human use, which comprises representatives of the member states. If the SCMP's decision is favourable, the Commission makes the final decision (or in certain circumstances the European Council). In the mutual recognition procedure, a pharmaceutical company with a marketing authorization in one member state can ask one or more other member states to recognize a marketing authorization granted by the first member state. There are procedures for objections by member states and for arbitration.

2.1.1.1.4 Pharmacovigilance

The holder of a marketing authorization has certain responsibilities after the granting of the authorization. These include gathering and acting upon information bearing on the safety of the drug, comprising, for example, suspected adverse reaction reports and

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published literature, including clinical reports and epidemiological studies.

2.1.1.2 In the USA

The basis of the regulation of pharmaceuticals in the USA is the Food, Drug and Cosmetic Act (1938), passed in the wake of a therapeutic disaster in 1937, in which a Tennessee drug company marketed a sulfonamide as an elixir for paediatric patients (Elixir Sulfanilamide). The solvent in this untested product was a highly toxic compound. Initially the requirement was for evidence of safety, but the Kefauver–Harris Amendments imposed additional requirements for evidence of efficacy. The agency regulating pharmaceuticals is the Food and Drug Administration (FDA).

2.1.1.2.1 New Drug Applications (NDAs)

The first stage after a drug development programme is the investigational new drug (IND) application. This is not an application for marketing approval. US federal law prohibits movement from one state to another of pharmaceuticals, without an approved marketing application. Because a sponsor will probably want to ship the investigational drug to clinical investigators in many states, the IND is needed for clinical trials. Approval for marketing is based on the new drug application (NDA). This now requires evidence of safety and efficacy and the information is presented in a standardized format (Applications for FDA approval to market a new drug. Content and format of an application, 1999).

Application for the IND and NDA is made to the FDA, and the toxicological data requirements are similar to those in the EU.

2.1.1.3 International Conference on Harmonisation (ICH)

The ICH brings together the regulatory authorities of Europe, Japan and the United States, together with experts from the pharmaceutical industry in the three regions, with the aim of making recommendations on ways to harmonize interpretation and application of technical guidelines and requirements. One result of this is to obviate the need for duplicating studies for guidelines that may be only slightly different, and thereby, for example, reduce experimental animal usage. The process has proceeded well, and the sixth International Conference on Harmonisation took place in Osaka, Japan, in November 2003. The main focus of the technical discussions at the conference was on the implementation of the Common Technical Document, that had been adopted at the previous conference in 2000 and which has become the required format for submissions in the EU and Japan, and the strongly recommended format in the USA.

2.1.2 Veterinary Pharmaceuticals

As with human pharmaceuticals, most counties have a system for regulating veterinary pharmaceuticals based on safety, quality and efficacy, in which the regulator has to make a judgement weighing risk against benefit for the animal. However there are two additional considerations for veterinary medicines. The first is that, in food-producing animals, human safety must be considered, in addition to that of the animals. The reason for this is that the use of veterinary medicines may give rise to residues in food. A second consideration is that, with companion animals, human exposure may be important; this is especially the case with ectoparasiticides used on cats and dogs. An additional consideration is that some veterinary medicines (e.g. sheep dips) may have a significant environmental impact.

2.1.2.1 In the European Union

In the EU, Directive 65/65 (Council of the European Economic Community, 1965), as amended, provides the legal basis for the regulation of veterinary pharmaceuticals ('veterinary medicinal products'), as with human medicines. Other directives established standards for testing the products. Furthermore a committee, the Committee for Medicinal Products for Veterinary Use (CVMP[a]), was established and this responsible for preparing the opinions of the EMEA[b] on all questions concerning veterinary medicinal products, under Regulation EC 726/2004 (European Parliament and Council, 2004a). The CVMP meets at the EMEA in London.

2.1.2.1.1 Preclinical Data Requirement

The toxicological data requirements depend on a number of factors, most importantly whether the product will be used in food-producing animals. In such animals, maximum residue limits need to be established for human safety purposes (see below). The veterinary equivalent of the clinical trial in humans is the animal trial, which may be a tolerance study (i.e. to show that the animal tolerates the drug, without unacceptable adverse effects). In addition, in food-producing animals, the safety of residues left in foods to be eaten by human consumers must be demonstrated, taking account of pharmacological, toxicological and microbiological end points, and also residue depletion studies will be required for the product. Where maximum residue levels have been set, withdrawal periods may need to be set for the product to ensure that none of the MRLs for different foods derived from the treated animal are exceeded.

2.1.2.1.2 Marketing Authorizations

As with human drugs, to obtain a marketing authorization, there are two routes that can be followed, a centralized procedure and the mutual recognition procedure.

In the former, the application is made to the EMEA and the outcome is binding on all the member states. A rapporteur, who is a CVMP member, is appointed to lead the scientific evaluation. The CVMP gives an opinion

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on whether a marketing authorization should be granted. This is passed to the regulatory committee (the standing committee on veterinary medicinal products (SCVMP)), which comprises representatives of the member states. If the SCVMP's decision is favourable, the Commission makes the final decision; the outcome, if favourable, being an EU-wide marketing authorization. In the mutual recognition procedure, a pharmaceutical company with a marketing authorization in one member state can ask one or more other member states to recognize the authorization granted by the first member state. There are procedures for objections to be made by member states and for arbitration, in which the opinion of the CVMP will be sought.

2.1.2.1.3 Maximum Residue Levels (MRLs) and Food Safety

MRLs are levels of veterinary drugs that may not be exceeded in foods of animal origin. The legal basis for pan-EU MRLs is regulation EEC 2377/90 (Council of the European Communities, 1990a). This has four annexes:

Annex I: Substances with full MRLs
Annex II: Substances which do not require MRLs
Annex III: Substances with provisional MRLs
Annex IV: Substances where MRLs cannot be established on human safety grounds.

The fundamental effect of this is that only substances on Annexes I, II and III can be used in food-producing animals. The basis for establishing the MRLs is a full toxicological data package, similar to that required for other food contaminants, including studies of acute and repeated-dose toxicity (including chronic toxicity), reproductive and developmental toxicity, genotoxicity and carcinogenicity. Additionally, pharmacokinetic and pharmacodynamic studies in laboratory animals are required as well as in the target species. The full list is given in Regulation EEC 2377/90 (Council of the European Communities, 1990a). The Commission has published a draft regulation to replace Regulation EEC 2377/90, available at *http://eurlex.europa.eu/LexUriServ/site/en/com/2007/com2007_0194en01.pdf* (see below). Risk assessment is carried out using the toxicological data. For some substances, it is also necessary to consider the pharmacodynamic effects that might occur at lower doses than those causing toxicity. The general pattern is that the appropriate NOEL, generally the lowest NOEL in the most sensitive species (known as the critical NOEL), divided by a safety factor (often 100) is used to calculate an acceptable daily intake (ADI). For microbiologically active substances, the effects on the human gut flora are also taken into account when setting the ADI. The MRLs are then based upon the fact that the ADI should not be exceeded and this must take into account distribution of the substance in the animal, the data on distribution coming from residue depletion studies in the target animal. The other data needed to elaborate MRLs from ADIs are 'food factors'; these are daily consumption quantities for animal products.

2.1.2.2 *In the USA*

In some ways, the regulation of veterinary drugs is simpler in the USA than the EU, but a major difference is that veterinary ectoparasiticides are regulated by the United States Environmental Protection Agency (US EPA) under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) (Federal Insecticide, Fungicide and Rodenticide Act, 1947; see also Protection of Environment: Part 152—Pesticide Registration and Classification Procedures, 2001), as pesticides. Vaccines are regulated by the United States Department of Agriculture (US DA). The basis of the regulation of all other veterinary pharmaceuticals in the USA is the Food, Drug and Cosmetic Act (1938), as amended (see above), and the agency responsible is the FDA, as for drugs for human use.

2.1.2.2.1 New Animal Drug Applications (NADAs)

The first stage after a veterinary drug development programme is the investigational new animal drug (INAD), which is the counterpart of the IND for drugs for use in humans. An INAD is needed for trials in animals. Approval for marketing is based on the new animal drug application (NADA). The NADA is the counterpart of the NDA.

Applications for INADs and NADAs are made to the FDA, and the toxicological data requirements are similar to those in the EU.

2.1.2.2.2 Tolerances and Food Safety

Tolerances in the USA are levels of veterinary drugs that may not be exceeded in foods of animal origin. A full toxicological database is required to assure consumer safety for veterinary products used on food-producing animals, similar to that required in the EU. Guidelines on the studies required are available and are periodically updated: these are available at the Center for Veterinary Medicine on *www.fda.gov/cvm*. Risk assessment is carried out using the toxicological data. The general pattern is that the appropriate NOEL, generally the lowest NOEL in the most sensitive species (known as the critical NOEL) is divided by a safety factor, which is often 100, but more if the database is defective or certain toxicological end points are seen. This procedure produces the ADI. A safe concentration of the drug is calculated from the ADI and a food factor (1 for muscle, higher for some offal), and a meat intake factor (500 g in an adult).

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2.1.2.3 International Activities

2.1.2.3.1 Veterinary International Cooperation on Harmonisation (VICH)

International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (Veterinary International Cooperation on Harmonisation (VICH)) is the veterinary counterpart of ICH for human pharmaceuticals and was launched in 1996. The aim of VICH is to make recommendations on ways to harmonize interpretation and application of technical guidelines and requirements. VICH is a series of meetings of officials from regulatory authorities of Europe, Japan and the United States together with experts from the pharmaceutical industry in these three regions/countries. The most recent (third) conference took place in Washington, DC, USA, 25–27 May, 2005.

2.1.2.3.2 Codex and the Joint Expert Committee on Food Additives (JECFA)

The Joint Expert Committee on Food Additives (JECFA) is an expert Committee jointly organized by the World Health Organization (WHO) and the Food and Agricultural Organization (FAO) of the United Nations. Some of JECFA's meetings are devoted to veterinary drugs and the Committee deals with residues and toxicology, producing both ADIs and MRLs. The Codex Alimentarius Commission (CAC) was created in 1963 by the FAO and WHO to develop food standards, and the MRLs and ADIs produced by JECFA are taken into the Codex Alimentarius system via the Codex Committee on Residues of Veterinary Drugs in Foods. The agreement on the application of sanitary and phytosanitary (SPS) measures (SPS agreement, WTO, 1994), achieved after tortuous negotiations in 1994, requires that, in most circumstances, CAC MRLs for *inter alia* veterinary residues be accepted for world trade purposes. This has inevitably raised the profile of the FAO/WHO expert committees such as the JECFA. Amongst the effects of the draft EU regulation to replace regulation EEC 2377/90, there would be an obligation to adapt Community legislation to include MRLs set by Codex with the support of the EU (European Commission, 2007). Thus Codex MRLs would generally be adopted by the EU.

2.1.3 Regulation of Pesticides and Biocides

2.1.3.1 In the European Union

The first European legislation to impinge on pesticides was Directive 79/117/EEC (Council of the European Communities, 1979), which forbade the marketing and use of plant protection products containing certain active substances. A comprehensive legal basis for regulation of agricultural and horticultural pesticides was commenced with Directive 91/414 (Council of the European Communities, 1991); under this directive such pesticides are referred to as plant protection products. The legal basis for regulation of nonagricultural pesticides (biocides) is Directive 98/8/EC (European Parliament and Council, 1998) (see below). Directive 91/414 is under review (European Commission, Health and Consumer Protection Directorate-General, 2004).

Before these directives came into force, the member states of the EU had their own national systems. In the UK, the first systematic controls were voluntary, through the notification of pesticides scheme, introduced in 1957, renamed the pesticides safety precautions scheme (PSPS) in 1964, following a review to ensure the scheme met the needs of the time (Miller, 1965; I. Rowland, 2008, personal communication, Pesticides Safety Directorate, York). The PSPS was a voluntary arrangement agreed between government departments and industrial associations. A major change was the introduction of the Control of Pesticides Regulations (1986) and Control of Pesticides Regulations (Northern Ireland) (1987), made under the Food and Environment Protection Act (1985), whereby the system was put on a statutory basis. The Pesticides Safety Division (later 'Pesticide Safety Directorate') of the Ministry of Agriculture, Fisheries and Food (PSD) was responsible for administering the system with regard to agricultural and horticultural pesticides and the Health and Safety Executive (HSE) was responsible for administering the system with regard to nonagricultural pesticides. In both cases, advice was given by the Advisory Committee on Pesticides (ACP), a committee of independent experts.

2.1.3.1.1 Marketing Authorizations

Directive 91/414 set out harmonized requirements for the authorization of plant protection products. The Directive provided for the compilation of three annexes.

Annex I: Active substances which may be incorporated into products intended for use.
Annex IIA: Requirements for the dossier to be submitted for the inclusion of an active substance in Annex I.
Annex IIIA: Requirements for the dossier to be submitted for the authorization of a plant protection product (Annexes IIB and IIIB deal with active substances and products consisting of micro-organisms, including viruses).

2.1.3.1.2 Data Requirements

Annex II, Part A, gives details of the toxicokinetic and toxicological data needed on the active substance (i.e. the ingredient(s) of the plant protection product with pesticidal activity); Annex II also covers environmental fate, ecotoxicology, physical and chemical properties and residues in treated crops. The toxicology data include studies of acute and repeated-dose toxicity, reproductive and developmental toxicity, genotoxicity and carcinogenicity. Any available human data as well as

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proposed medical counter-measures need to be submitted. Additionally, studies of delayed polyneuropathy may be required. Studies on the toxicity of metabolites may be required as well. Annex III lists the required toxicological studies on the product (as opposed to the active substance). These include acute oral, percutaneous and inhalation toxicity, skin and eye irritancy and skin sensitization. Any toxicological data available on nonactive substances is also requested. From these data, ADIs, acute references doses (ARfDs) acceptable operator exposure levels (AOELs) and dermal absorption values are calculated (see below). Data on operator and bystander exposure are also generally required.

2.1.3.1.3 Field Trials and MRLs

Unlike MRLs for other food contaminants, for example veterinary drugs used in food-producing animals, MRLs for pesticides are not, in themselves, safety limits, although they should be consistent with food safety. They are generated from data from field trials where the pesticide is used according to good agricultural practice (GAP), that is, the pesticide being used in the correct amounts and using the appropriate preharvest interval. One role of MRLs is to ensure that pesticides are, indeed, used according to GAP. Numerous directives have been adopted with the aim of harmonizing MRLs within the EU: recently regulation 396/2005 (European Parliament and Council, 2005) has sought to further this process. This regulation has four draft annexes:

Annex I: This includes all products for which MRLs are set, as well as the other products for which it is appropriate to apply harmonized MRLs (see also Regulation (EC) No. 178/2006 European Commission, 2006b).
Annex II: This contains MRLs already established by Directive 86/362/EEC covering MRLs on cereals (Council of the European Communities, 1986a), Directive 86/363/EEC, covering MRLs for foodstuffs of animal origin (Council of the European Communities, 1986b) and Directive 90/642/EEC MRLs for foodstuffs of plant origin (Council of the European Communities, 1990b).
Annex III, Part A: This lists all draft temporary MRLs that are thus far not harmonized at EU level.
Annex III, Part B: This lists all draft temporary MRLs of active substances harmonized at EU level in and on new commodities.
Annex IV: This lists active substances for which no MRLs are needed.

2.1.3.1.4 Obtaining Marketing Authorizations

In order to obtain a marketing authorization in the EU, an applicant must submit a dossier in line with the requirements of Annexes II and III of Directive 91/414 data. The applicant chooses a Rapporteur Member State (RMS), which will confirm if the dossier is complete, and if other member states agree, the RMS produces a monograph (draft assessment report (DAR)). Originally this was reviewed by experts at European Community Coordination (ECCO) meetings. Before the establishment of the European Food Safety Authority (EFSA), the final decision on whether to include the active substance in Annex I of Directive 91/414 was made by the European Commission after obtaining an opinion from the Scientific Committee on Plant Health (SCPH). This opinion was delivered by member states in the SCPH following consideration in full working group meetings (attended by representatives from all the member states), and was reached by qualified majority voting. Since the establishment of the EFSA, the Standing Committee on the Food Chain and Animal Health (SCFCAH) has taken over the functions of the SCPH. The current peer review process involves the circulation of the completed DAR to the EFSA and all member states for a series of written commenting rounds. There is then a discussion at an expert peer review meeting organized by the EFSA (Pesticide Risk Assessment Peer Review Unit (PRAPeR)), which agrees the toxicological end points (see *www.efsa.europa.eu/EFSA/ScientificPanels/efsa_locale-1178620753812_PRAPER.htm*). The EFSA then produces a conclusion report (see *www.efsa.europa.eu/EFSA/ScientificPanels/PRAPER/efsa_locale-1178620753812_Conclusions494.htm*), which is used by the Commission and member states to determine, in the SCFCAH, if the active substance should be listed on Annex 1 of Directive 91/414. Independent expert advice can be sought from the EFSA Panel on Plant Protection Products and their Residues (PPR—see *www.efsa.europa.eu/EFSA/ScientificPanels/efsa_locale-1178620753812_PRAPER.htm*).

2.1.3.1.5 Surveillance

Under European legislation, member states must have in place programmes for the surveillance of residues in food (Council of the European Communities, 1990b; European Commission, 2000a). There is an obligation on member states to communicate the results to the European Commission. These programmes provide checks on whether MRLs are being exceeded (i.e. GAP is being followed), that pesticide residues are not posing a risk to public health and that residues of pesticides not authorized in particular crops are not present. In the UK, pesticide residues in food and drink are monitored under an official surveillance programme conducted by the Government's Chemicals Regulation Directorate of the HSE (formerly the Pesticides Safety Directorate) and overseen by the Pesticide Residues Committee (PRC), which comprises independent experts. The UK programme is mainly directed towards foods where residues are considered likely to be present, often on the basis of previous experience, so the finding cannot be taken

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as representative of the whole UK food supply. Similar targeted approaches are used by other member states.

2.1.3.1.6 Consumer Risk Assessment

As with other food regulatory systems, the typical procedure is to use the most sensitive study in the most sensitive species (the critical study), incorporate a UF of (typically) 10 for extrapolation from animals to humans and a further UF of (typically) 10 for interindividual human variation. With all pesticides this procedure is used to generate ADIs and, with some, ARfDs. The former are used for chronic risk assessment and the latter where it is considered appropriate to undertake an acute risk assessment. Premarketing data from field trials are used for dietary risk assessment. A major difference from the USA is that nonthreshold carcinogens (genotoxic carcinogens) will not usually receive marketing authorizations.

2.1.3.1.7 Plant Metabolites

The main concern with plant metabolites is that they may represent a toxicological risk, in that, where risk assessment is based on animal studies, as it usually is, the animals may not have been exposed to such metabolites. If analysis indicates that residues in edible portions of the crop following treatment as proposed are $<0.01\,mg\,kg^{-1}$ for human food, or $<0.05\,mg\,kg^{-1}$ for animal feed, no characterization of terminal metabolites is usually required. If levels are above this, it is necessary to characterize and identify all single components of the residue which exceed $0.05\,mg\,kg^{-1}$ or 10% of the total radioactive residue and to characterize fractions or individual components of the residue which represent $0.01–0.05\,mg\,kg^{-1}$ (Directorate General for Agriculture, 1997; this document predates the establishment of the EFSA and it is likely that further guidance will be issued in the future).

2.1.3.1.8 Occupational Safety

Providing AOELs are not exceeded, it is expected that adverse effects will not be seen in those handling pesticides. In the UK, the Health and Safety Executive (HSE) is responsible for worker safety. Complaints involving pesticides are investigated by the Field Operations Directorate (FOD) of the HSE, and the Pesticide Incidents Appraisal Panel (PIAP), which comprises a mixture of government officials and nongovernment members, considers all incidents reported to FOD where there is any allegation that the use of a pesticide has caused ill health. PIAP is notified of these incidents only on completion of the inspector's investigation.

2.1.3.1.9 Review of Existing Substances

A review programme was established, under regulation 3600/92/EEC (Commission of the European Communities, 1992), for existing substances, that is, active substances on the market at the time Directive 91/414 came into force. The process for review was similar to the process for new active substances, with preparation of a DAR by a rapporteur member state (RMS), and peer review. There was considerable slippage in the first rounds of the review process. Subsequent changes to the procedures are aimed at completing the review of all existing active substances by the end of 2008.

2.1.3.1.10 Revision of Directive 91/414

This Directive is under review at the moment and important changes include the introduction of comparative assessment and (if necessary) substitution, and the removal of the option for the granting of national provisional authorizations for products containing new active substances pending Annex I inclusion. Furthermore, it is proposed that hazard triggers will take the place of much of the risk assessment presently used. The proposed revisions are subject to consultation, and industrial organizations and other interested parties have commented on the proposals (ECPA, 2005). The proposed alterations have been criticized as being not evidence based, and for having an adverse effect on food production (Trewavas, 2008; Buckenham, 2008; Titford, 2008). The effect is likely to be far ranging, with pesticides being regulated in a different way from other chemicals in the EU and from pesticides in other parts of the world (see also ADAS, 2008).

2.1.3.1.11 Pesticides in Water

The 1980 European Community Drinking Water Directive (80/778/EEC) (Council of the European Communities, 1980) set a standard for total pesticides in drinking water of $0.5\,\mu g\,l^{-1}$, and of $0.1\,\mu g\,l^{-1}$ for each individual pesticide. The latter concentration was equivalent to the detection limits of analytical methods available at the time and is not toxicologically based. These limits have been retained in subsequent EU legislation (Council of the European Union, 1998).

2.1.3.1.12 Biocides

The legal basis for the regulation of biocides in the EU is Directive 98/8/EC (European Parliament and Council, 1998). Biocides are defined so as to include disinfectants, chemicals used for preservation of products and materials (including wood), nonagricultural pesticides, products for controlling mice and rats and insects such as cockroaches and ants, and antifouling products used on hulls of boats. The Directive even covers embalming fluid!

From May 2000, new active substances have to be authorized as described below and there is provision for a 10-year review of products and active substances already on the market. There are a number of Annexes to the Directive. Of interest to the toxicologist are I, II, V and VI. Annex I is the list of permitted active substances; Annex IIA lists the core dataset required for active substances; Annex IIB lists the core dataset for products; Annex V lists the various types of biocides, and

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Annex VI lists the common principles for the evaluation of dossiers (other annexes deal with microbiological biocides). The basic principles of Directive 98/8/EC are that active substances have to be assessed and the decision on their inclusion into Annex I of the Directive made at EU level. Toxicological data requirements are similar to those required for agricultural pesticides under Directive 91/414, however the principle of comparative assessment may be used under Directive 98/8/EC, that is, an active substance may be excluded from Annex I if there are less harmful, suitable substitutes available. Member states can only authorize products which contain active substances included in Annex I, and authorization of biocidal products has to done according to Annex VI of the Directive. There is also a provision for mutual recognition of product authorizations between member states.

As Directive 98/8/EC has caused a large number of products to be removed from the market, including many disinfectants, even disinfectant soaps, it remains to be seen, in these days of meticillin-resistant *Staphylococcus aureus, Clostridium difficile* and other hospital cross-infections, whether the impact on public health will be beneficial (see Booker, 2008).

2.1.3.2 *In the USA*

The first federal pesticide legislation predates most legislation in Europe and dates from 1910. This was the Insecticide Act (1910), which was enacted to prevent the manufacture, sale or transport of impure or improperly labelled insecticides and fungicides. In 1938, the Federal Food, Drug and Cosmetic Act *inter alia* established regulatory limits for pesticides (Food, Drug and Cosmetic Act, 1938). More extensive controls on pesticides were introduced with the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), in 1947 (Federal Insecticide, Fungicide and Rodenticide Act, 1947). This has been subject to extensive amendment, including the requirement that tolerances (maximum legal residue concentrations—equivalent to European MRLs) be established for pesticide residues on food. Up until 1970, the pesticide regulatory agency had been the United States Department of Agriculture, but after that time administration of FIFRA was transferred to the US EPA (Chaffey and Dobozy, 2004). Subsequent amendments further tightened the legislation, but a major change was made with the Food Quality Protection Act (FQPA) in 1996 (Food Quality Protection Act, 1996). The safety standard was revised from 'protect public health' to 'reasonable certainty of no harm'. The FQPA required that all existing tolerances be reviewed within 10 years to the new safety standard. For the purpose of establishing tolerances, the US EPA was required to provide for the protection of infants and children, and an extra safety factor of 10× was introduced with the young in mind. The FQPA introduced two new terms; the first was 'aggregate exposure' assessment; that is, consideration of all nonoccupational sources of exposure (i.e. food, drinking water and household uses). The other term introduced was 'cumulative exposure' assessment; that is, consideration of the impact of exposure to chemicals with a common mechanism of toxicological action. Aggregate exposure assessment presents no great scientific problems. Cumulative risk assessment, however, does. The default assumptions made are that (i) no interactions are likely at residue-type doses; (ii) pesticides with qualitatively the same toxicological action will act additively (i.e. exhibit dose additivity); (iii) pesticides with qualitatively different toxicological action will act independently. This means that pesticides have to be grouped into common mechanism groups (CMGs), which is relatively easy with some groups, for example organophosphate (OP) anticholinesterases, but more difficult with others, for example azole fungicides. Calculating the acceptability of exposure to more than one pesticide in a CMG can be done in several ways (see Wilkinson *et al*., 2000), but all the methods comprise adding exposure, allowing for the toxicological potency of the individual pesticides, and comparing the sum with some level of acceptability of total exposure. Another development in the USA is the requirement for developmental neurotoxicity tests (DNTs) to be done on some pesticides, and the US EPA has issued guidelines on doing such tests (US EPA, 1998a). DNTs have now been done on many pesticides, but a full analysis of the results has not been published, although an analysis of some studies was looked at by the Joint FAO/WHO Meeting on Pesticides Residues (JMPR) (FAO/WHO, 2003). Another development has been agreement with the other countries (Canada and Mexico) of NAFTA to share work on regulation and to harmonize data requirements (NAFTA, 2003).

2.1.3.2.1 Data Requirements

Data requirements are generally similar to those in the EU, with the exception of the additional requirements discussed above.

2.1.3.2.2 Field Trials and Tolerances

Initial calculations for risk assessment are based on residues observed in crop field trials conducted at maximum application rates with the minimum preharvest intervals (i.e. according to GAP) and with the assumption that 100% of the crop is treated. These trials are used to calculate tolerance values, which as in the EU are primarily to ensure compliance with GAP; thus tolerances are not in themselves safety limits. More refined exposure assessment can be carried out using factors to allow for percentage of crop treated and other factors that will reduce calculated exposure.

2.1.3.2.3 Residues Surveillance

The FDA collects residues data on particular commodity/pesticide combinations and carries out total

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diet studies. The FDA is the agency which enforces tolerances in imported foods and in domestically produced foods shipped interstate. The US DA's Agricultural Marketing Service (AMS) conducts a residue testing programme directed primarily at raw agricultural products.

2.1.3.2.4 Consumer Risk Assessment

As in the EU, the typical procedure is to use the most sensitive study in the most sensitive species (the critical study), incorporate a UF of (typically) 10 for extrapolation from animals to humans and a further UF of (typically) 10 for interindividual human variation. With all pesticides this procedure is used to generate chronic RfDs and, with some, ARfDs. However, as discussed above, the FQPA makes a number of other demands that are not required in the EU, such as the requirement in some cases for an extra safety factor for protection of the young and for aggregate and cumulative risk assessment. Field trials are used as the source of consumption data for consumer risk assessment, and dietary surveys for food-item consumption. Where cumulative risk assessment is being undertaken, probabilistic exposure assessment is being studied as a way of avoiding compounded conservatism. Another major difference from the EU is in the treatment of nonthreshold carcinogens. Here, the cancer risk is calculated by multiplying cancer potency of the pesticide by food exposure. Dietary lifetime cancer risks of less than 10^{-6} are not generally considered to be of regulatory concern (see US EPA, 1998b; 2005; Chaffey and Dobozy, 2004).

2.1.3.2.5 Occupational Safety

Occupational safety is assured by quantifying exposure in studies undertaken during use of the pesticide. The exposure is then compared with the appropriate RfD.

2.1.3.2.6 Pesticides in Water

All sources of pesticide exposure, including exposure through drinking water, must be factored into decisions on establishing and reassessing tolerances. Intakes of pesticide via water are compared to a human health drinking water level of concern (DWLOC), which is the concentration in drinking water of a pesticide that would be an acceptable upper limit in light of exposure to that pesticide from other sources, that is, food and residential use.

2.1.3.3 International Activities

2.1.3.3.1 OECD

The Organisation for European Economic Co-operation (OEEC) came into being in 1948; the OEEC emerged as part of the reconstruction of Europe after the Second World War, in connection with the Marshall Plan. In 1961 the Organisation for Economic Co-operation and Development (OECD) superseded the OEEC. The OECD is, unlike its predecessor, a worldwide body, with its headquarters in Paris, and amongst its many activities are the generation of guidelines for the testing of chemicals, a full list of which is available at *www.oecd.org/dataoecd/9/11/33663321.pdf* (OECD, 2007). These guidelines are widely accepted internationally and are used for regulatory studies on pesticides.

2.1.3.3.2 Joint FAO/WHO Meeting on Pesticide Residues (JMPR)

The JMPR is the pesticide counterpart of JECFA. Although not officially part of the CAC structure, the JMPR provides independent scientific expert advice to the CAC and its specialist Committee on Pesticide Residues. These meetings, which occur annually, are organized by the WHO and the FAO. Some active substances that are also used as veterinary drugs are dealt with by JECFA (see Section (2.1.2.3.1)). The CAC has been discussed above: the MRLs and ADIs produced by the JMPR enter the Codex Alimentarius system via the Codex Committee on Pesticide Residues and, in most circumstances, CAC MRLs for pesticides, *inter alia,* are accepted for world trade purposes (WTO, 1994).

2.1.4 Regulation of Food Additives, Flavourings and Food Contact Materials

Food additives are substances that are added to food as preservatives and stabilizers or colours. Flavourings are used to impart odour and/or taste. Packaging materials (often plastic) are used for convenience, to prevent contamination with micro-organisms and sometimes to prevent tampering; a consideration is the toxicity of any substance that might migrate into food. The aim of regulating these materials is to ensure that additives, flavouring agents and packaging do not present a risk to human health.

2.1.4.1 In Europe

2.1.4.1.1 Food Additives and Flavourings

The legislative underpinning of European regulation of food additives is Directive 89/107 (Council of the European Communities, 1989a), and this directive has been subsequently amended on many occasions. Annex I of the Directive lists the groups of substances to which the Directive applies and it specifically excludes processing aids, substances used in the protection of plants and plant products in conformity with Community rules relating to plant health, flavourings for use in foodstuffs, falling within the scope of Council Directive 88/388/EEC (Council of the European Communities, 1988), and substances added to foodstuffs as nutrients (for example minerals, trace elements or vitamins). Substances covered fall into a number of groups including colours, preservatives, antioxidants, emulsifiers, thickeners, gelling agents, stabilizers, flavour enhancers, acids and

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acidity regulators, anticaking agents, modified starch, sweeteners and enzymes. As noted above, flavourings were already controlled by Directive 88/388/EEC (Council of the European Communities, 1988), whose annexes set maximum limits for biologically active principles and for 3,4-benzopyrene. There have been numerous amendments to Directive 89/107; for example Directive 94/35 (European Parliament and Council, 1994), which concerned sweeteners used in food. Shortly afterwards, Directive 95/2 (European Parliament and Council, 1995) carried forward the harmonization of regulation of additives other than sweeteners and colours. This Directive specified the foods in which the additives were permitted and the MRLs allowed, furthermore each additive was given a unique E number; these are number codes for food additives and are found on food labels throughout the EU. The E numbers are grouped; thus E100–E199 are colours and E200–E299 are preservatives. Harmonization, insofar as flavourings is concerned, has been slower, but the intent has been similar. According to Regulation 2232/96 (European Parliament and Council, 1996), amended by Commission Regulation 1565/2000 (European Commission, 2000b), which established an evaluation programme for flavouring substances, new numbers, so-called 'FL numbers', are to be assigned to each flavouring substance. The establishment of the EFSA brought the establishment of its scientific panel on food additives, flavourings, processing aids and materials in contact with food (AFC panel). Directive 2006/52/EC (European Parliament and Council, 2006a) amended Directives 94/35 and 95/2, making changes largely on the basis of advice from the EFSA and/or the Scientific Committee on Food (SCF). One example was change to current authorizations in order to keep the level of nitrosamines as low as possible by bringing down the levels of nitrites and nitrates added to food whilst maintaining the microbiological safety of food. Scientific advice to the EFSA is supplied by the AFC panel (see above). The panel is currently conducting a comprehensive re-evaluation of all authorized food additives in the EU in order to give scientific support to the Commission. The panel delivers its views as opinions and has both *ad hoc* and permanent working groups.

In July 2006 the Commission published a set of proposals that constitute a comprehensive review of the existing EU legislation in the area. The proposals are at second reading stage in the European Parliament. They comprise:

- a framework regulation for the control of food additives;
- a framework regulation on flavourings used in food;
- a regulation to introduce EU-wide controls on the use of enzymes in food which introduces for the first time harmonized controls across the EU on the use of enzymes in food products;
- a regulatory regulation that sets out the approval processes common to the above three regulations.

They maintain or strengthen (e.g. for enzymes) consumer protection; they consolidate the existing EU legislation which has been amended several times, and they introduce comitology for community acceptance of new additives, enzymes and flavourings.

2.1.4.1.2 Food Contact Materials

The legislative underpinning for regulation of food contact materials is Regulation 1935/2004/EC (European Parliament and Council, 2004b); this replaced the earlier Council Directive 89/109/EEC (Council of the European Communities, 1989b). Article 5 of the Regulation provides for specific measures to be put in place for the particular materials listed in Annex I, and the list includes plastics, rubber, paper, wood and cork. Most attention has been given to plastics, in particular monomers and additives such as plasticizers. Initially the evaluations were undertaken by a working group of the SCF. Since the establishment of the EFSA, the role of the SCF has been taken over by the EFSA AFC panel which has a working group on food contact materials.

2.1.4.2 In the USA

The basis of the regulation of food additives in the USA is the Food, Drug and Cosmetic Act (1938), passed, as discussed above, in the wake of a therapeutic disaster. With the exception of meat, poultry and egg products the FDA is the relevant regulatory body, while the FDA shares responsibility with the Food Safety and Inspection Service of the US DA for the safety of food additives used in meat, poultry and egg products. Regulation of food additives formally started with the Food Additives Amendment (1958) (Food Additive Amendment to the Food, Drug and Cosmetic Act, 1958), which requires manufacturers of new food additives to establish safety.

Under the US legislation, additives to food are divided into three groups, the second of which can be subdivided. They are (1) substances that are generally recognized as safe (GRAS), (2) prior-sanctioned substances; these were assumed to be safe by either the FDA or the US DA before 1958, to be used in a specific food, (3) food additives, which are subdivided into (i) direct (deliberately added to food) and (ii) indirect (food contact materials), and (4) colours.

2.1.4.2.1 GRAS Substances

These are substances that were widely used before the enactment of the Food Additives Amendment, and whose use was generally recognized as safe by a panel of experts. In 1958, the FDA published in the Federal Register the first list of substances generally recognized as safe (GRAS). The list contained nearly 200 substances. Notification of the use of GRAS substances in food is not needed. GRAS status is, of course, generally based

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upon prolonged safe usage, but if new data questions the safety of a GRAS substance, the FDA may take action to remove the substance from foods or require the manufacturer to conduct studies to meet any concerns. It is possible to get a substance affirmed as GRAS, but unless the substance was in use for a long period, this will involve submission of toxicological data.

2.1.4.2.2 Prior-Sanctioned Substances

These are similar to GRAS substances except that their use is restricted to certain foods.

2.1.4.2.3 Other Substances

Additives (including colours) other than GRAS and prior-sanctioned substances are tightly regulated and are subject to a premarketing regulatory system, based upon toxicological safety. The toxicological requirements for direct food additives and for colour additives are available in the Red Book (FDA, 2007a). A tiered approach is used whereby the minimum dataset required is determined by the level of concern assigned to the compound. The level of concern is based upon the molecular structure of the compound, and substances are assigned to categories A, B or C. Within these categories, estimated human exposure will determine the initial concern level, I being those of least concern and concern level III being those of highest concern. As concern level increases the duration of toxicological studies required also increases. The Red Book also contains guidelines for the conduct of the studies required.

2.1.4.2.4 Indirect Food Additives

These are substances that come into contact with food as part of packaging or processing. They are not intended to be added directly to, become a component of, or have a technical effect in or on the food. They include substances in food-contact articles, adhesives and components of coatings, paper and paperboard components, polymers, and adjuvants and production aids. New indirect food additives are authorized through the food contact notification programme (FDA, 2007b).

2.1.4.3 Codex and the Joint Expert Committee on Food Additives (JECFA)

JECFA, already discussed above (Section (2.1.2.3.2)), is an expert Committee jointly organized by the WHO and the FAO of the United Nations. Some of JECFA's meetings are devoted to food additives and contaminants, and the Committee deals with residues and toxicology, producing both ADIs and MRLs. ADIs and MRLs established by JECFA are taken into the Codex system via the Codex Committees. The relevant committee was the Codex Committee on Food Additives and Contaminants, but in 2006, this was split into the Codex Committee on Contaminants in Food and the Codex Committee on Food Additives. The terms of reference of the latter, which meets in China, are '(i) to establish or endorse permitted maximum levels for individual food additives; (ii) to prepare priority lists of food additives for risk assessment by the Joint FAO/WHO Expert Committee on Food Additives; (iii) to assign functional classes to individual food additives; (iv) to recommend specifications of identity and purity for food additives for adoption by the Commission; (v) to consider methods of analysis for the determination of additives in food; and (vi) to consider and elaborate standards or codes for related subjects such as the labelling of food additives when sold as such.'

2.2 Reserve Schemes for Chemicals

Reserve schemes for chemicals can be divided into 'supply-side' schemes and 'user-side' risk assessment/risk management schemes. Supply-side schemes are those requiring information, assessment and possibly further testing. Their aim is to provide information for the supply chain. The supplier classifies the substance or preparation on the basis of the toxicity found and standard criteria, and labels the packaging for the substance/preparation appropriately. The major methods for transfer of this information through the supply chain involve labelling and the preparation of safety data sheets. The headings in the safety data sheet are common internationally, although the content tends to differ slightly to accord with EU, national and state legislation. This information being transferred includes, in effect, a hazard characterization, intended to enable the user to examine the risks for their particular exposure circumstances. It also includes suggestions concerning handling and storage and the use of personal protective equipment when handling the substance/preparation. Management of the supply is achieved through different legislation.

2.2.1 Supply Side

2.2.1.1 New Chemicals — USA

The USA adopted an approach based on premanufacturing notification contained in the Toxic Substances Control Act of 1976. The new substance provisions of the Toxic Substances Control Act require that available information be sent to the EPA for evaluation prior to manufacture or import. A list of existing chemicals was drawn up. There is no legal requirement for routine testing of new substances. The decision as to whether a testing programme is required and what tests should be undertaken has to be based on available information (and any structure–activity information available concerning the new substance) and justified by the regulator, the EPA.

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2.2.1.2 New Chemicals—Excluding USA

'Supply-side' schemes for new chemicals originated in an OECD-derived approach, aimed initially at providing a minimum dataset (the minimum premarketing set of data (MPD)) for new substances, and stepped testing based on tonnages placed on the market. Most OECD adherents, including the EU, implemented this. The first step was to produce lists of existing substances—in principle substances not on these lists are 'new'. The EU scheme is typical and was originally implemented as the sixth amendment of Council Directive 67/548/EEC (Council of the European Economic Community, 1967), and reimplemented as the seventh amendment, Council Directive 92/32/EEC (Council of the European Communities, 1992). Testing is staged, based largely on tonnages placed on the market, but with some scope for varying the higher-level testing on grounds of toxicity seen in earlier studies, possible structure–activity relationships, and numbers exposed and levels of exposure seen.

2.2.1.3 Existing Chemicals/Substances

The OECD has run a risk assessment programme for high-production-volume chemicals since the late 1980s, and refocussed the programme in 1998. The chemicals chosen were those believed to be on the market at high tonnage and for which it was believed few data were available. One central requirement is a 'Screening Information Dataset'. Essentially this dataset is similar to that required under REACH Annexes VII and VIII for existing chemicals (see below), but with the omission of skin and eye irritation tests.

In general the principle has been that industry generates the data and the individual national regulator sponsors the chemical and moderates the information provided. The information is collated into a standardized format, a Screening Information Data Set (SIDS), Screening Initial Assessment Report (SIAR), and profile (summary). The moderated data is then reviewed by the OECD at a Screening Information Assessment Meeting (SIAM) and published on the IPCS web site. In the USA, the process has been conducted by the US EPA under the High Production Volume challenge, in the EU sponsorship has been by individual member states. The International Council of Chemical Associations launched a global initiative in 1998 aimed at providing data on approximately 1000 high-production-volume substances, nominally by the end of 2004, with the costs of information gathering and any testing required being borne by industry.

Within the EU evaluation of existing chemicals has been conducted using the information in an EU regulation setting out the principles for assessment (Commission Regulation (EC) No 1488/94; European Commission, 1994) and several EU regulations providing priority lists of chemicals. The drafts of the risk assessments are written by individual Member State Competent Authorities (as rapporteurs, usually in cooperation with industry), and the evaluation (including proposals for classification and labelling and areas where further work is required) is peer reviewed by the EU Scientific Committee on Toxicology, Ecotoxicology and the Environment (CSTEE). When the substance has been subject to an EU risk assessment (properly evaluation), the EU risk assessment information is accessible through the European Chemicals Bureau web site. Effectively, this programme is being subsumed into REACH.

2.2.1.4 Safety Data Sheets

Safety data sheets are required for dangerous substances and preparations. Existing substances are classified on the basis of available information. The classification of preparations may involve a calculation method for amalgamating the toxicities of the individual components given in Directive 1999/45/EEC (European Parliament and Council, 1999), or testing of the preparation. The headings and content of safety data sheets are specified in an ISO (International Organization for Standardization) standard and EU Commission Directive 2001/58/EEC (European Commission, 2001) (**Table 3**). They include the requirement to state the classification and labelling identified by toxicity testing or, in the case of a preparation, as a consequence of the application of the procedures given in Directive 1999/45/EEC.

2.2.1.5 Marketing-and-Use Directives

Unlike schemes for specified use, where the onus is on those wishing to market the chemical for that use to demonstrate safety to the regulator, here the onus is on the regulator to demonstrate that there is a need for the

Table 3 Headings for safety data sheets

Number	Title
1	Identification of the substance/preparation and company/undertaking
2	Composition/information on ingredients
3	Hazards identification
4	First aid measures
5	Fire fighting measures
6	Accidental release measures
7	Handling and storage
8	Exposure controls/personal protection
9	Physical and chemical properties
10	Stability and reactivity
11	Toxicological information
12	Ecological information
13	Disposal considerations
14	Transport information
15	Regulatory information
16	Other information

Note: the headings given in Annex II of the new REACH regulation reverse items 2 and 3.
From Directive 2001/58/EEC (European Commission, 2001).

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regulatory action proposed. This applies both in the USA (Toxic Substances Control Act) and in the EU, through the Marketing and Use Directive (Directive 76/769/EEC, Council of the European Communities, 1976). By 2008 there have been some 30 amendments to the Marketing and Use Directive seeking regulatory action on specific chemicals or groups of chemicals.

2.2.1.6 REACH and GHS

Within the European Economic Area all of these EU Directives are being replaced by the EU REACH Regulation (European Parliament and Council, 2006b), and by the introduction of the relevant parts of the United Nations Economic Commission for Europe (UN ECE) Globally Harmonised System (GHS) for Classification and Labelling of Chemicals (UN ECE, 2007). The latter is periodically updated.

The REACH regulation is being implemented over a prolonged period (2007–2022). It includes the essentials of the new substances scheme—that is, a tiered testing system based on tonnages (**Table 4**). It also includes a procedure for registering existing chemicals and ensuring that appropriate toxicity information is available, with certain high-hazard (including proven human or animal carcinogens, mutagens or reproductive toxins) and high-tonnage existing chemicals being dealt with first. The data required for the first two levels of testing must be supplied with the registration (although grouping of similar chemicals and application of structure–activity relationships and 'read across' are provided for—see **Table 4**), with proposals for appropriate further testing.

REACH requires safety data sheets and, for substances placed on the market in quantities of 10 tonnes or more, detailed chemical safety assessments for the various intended uses. Finally there is an authorization process for chemicals exhibiting specific hazardous properties. The GHS is a classification system largely intended as a means of providing harmonized hazard characterizations.

3 SCHEMES FOR AIR/WATER

3.1 Air and Water Quality

The WHO publishes air and drinking-water quality guidelines. Essentially they are drawn up and reviewed by experts after evaluating existing toxicity data, and published. National and regional governments can use these guidelines to set their own standards or develop standards themselves.

The WHO air quality guidelines global update in 2005 (WHO, 2006a) covered particulates, ozone, and nitrogen and sulfur dioxides. National or regional authorities can implement these guidelines or derive their own. In the UK, air quality objectives and standards have been set using advice from expert committees. Air quality objectives set time limits for achieving

Table 4 REACH testing proposals

Annex no.	Tonnage level	Tests proposed
VII	1 tonne or more	Skin irritation/skin corrosion (*in vitro*), eye irritation (*in vitro*), skin sensitization (mouse local lymph node assay), *in vitro* gene mutation in bacteria (Ames test), acute toxicity (oral).
VIII	10 tonnes or more	Skin irritation (*in vivo*), eye irritation (*in vivo*), *in vitro* gene mutation in mammalian cells, second acute toxicity test (dermal or inhalation, as appropriate), repeated-dose toxicity (min: 28 days), reproductive toxicity screen (OECD 421 or 422), assessment of toxicokinetics.
IX	100 tonnes or more	Additional *in vivo* genotoxicity studies, repeated-dose toxicity studies and reproductive toxicity studies, depending on the results of the earlier studies.
X	1000 tonnes or more	Additional (second) *in vivo* genotoxicity study, long-term repeated-dose toxicity study, carcinogenicity bioassay, additional repeated-dose toxicity studies.
XI	General rules for the adaptation of the standard test regime	Testing does not appear scientifically necessary: use of existing data, weight of evidence, qualitative or quantitative structure–activity relationships, *in vitro* methods, grouping of substances and read across. Testing is technically not possible (e.g. substance too volatile, too highly reactive or too unstable, or mixing substance with water causes danger of fire or explosion, or, if radiolabelling is required, the radiolabelling is unfeasible). Substance-tailored exposure-driven testing (exposure scenarios indicate that testing is unnecessary).

Note: Only the toxicity tests are included in the table. Physical chemistry and ecotoxicity tests have been omitted. Also omitted are the conditions that have to be fulfilled before conducting the tests.
(European Parliament and Council, 2004a)

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specified levels of the substance in air on the way to achieving a standard. Although the objective is an acceptable risk, the time required to achieve it is set using the technical feasibility criterion. Air quality standards are 'broadly acceptable' risks based on equity. Air quality objectives exist for benzene, 1,3-butadiene, carbon monoxide, lead and polycyclic aromatic hydrocarbons as well as particulates, ozone, and nitrogen and sulfur dioxide.

Drinking-water quality standards in the UK are derived from the WHO guidelines (WHO, 2006b) via the EU. They cover microbiological and radiation hazards as well as chemical hazards. Levels of pesticide, nitrate, lead and iron are particularly monitored. The actual standards are set out in regulations.

3.2 Occupational Exposure

3.2.1 Occupational Exposure Levels/Workplace Exposure Limits and Biological Exposure Measurements

There are two main types of standards for workplace exposure—ones based on uptake (biological exposure indices/biological monitoring guidance values) and ones based on intake (occupational exposure levels/workplace exposure limits).

These measurements and standards are intended to play a part in controlling the exposure of workers to airborne substances. Some are legally enforceable, others are for guidance. Some are influenced by what level of control is achievable with current technology without exorbitant cost, others are idealistic targets. Thus a direct comparison of numerical lists of occupational exposure limits or biological measurement values is very difficult to undertake and may not reflect the relative standards of occupational health protection in different countries. Without appropriate control regimes and means to ensure that these regimes are adhered to, these limits will be ineffective.

3.2.2 Sources of Occupational Exposure Limits

The two principal sources of occupational exposure limits in the USA are the American Association of Governmental Industrial Hygienists' 'Threshold Limit Values' and 'Biological Exposure Indices' Committee and list (revised annually), and the US Occupational Safety and Health Administration's list of Permissible Exposure Limits. The EU, through the Chemical Agents and Carcinogens Directives and the Scientific Committee on Occupational Exposure Limits, has set some exposure limits, but most limits in EU member states remain national limits. Perhaps the two most relevant airborne limits are those of the German authorities in their Technical Rules for Hazardous Substances, TRGS 900 (2006): the *Maximal Arbeitskonzentrationen* (MAK—the maximum acceptable concentration for a chemical in the workplace) and *Technisch Richtkonzentration* (TRK—technical guidance concentrations based on best available technology for carcinogens) and *Biologische Arbeitsstofftoleranzwerte* (BAT—biological tolerance value); and those in the UK, the Workplace Exposure Limits and Biological Monitoring Guidance Values (HSE, 2005). Workplace Exposure Limits are further divided into two groups, those for which adherence to the limit is adequate control and those classified in certain categories associated with carcinogenesis, heritable genetic damage and sensitization by inhalation (asthma), for which there is a duty to control exposure to as low a level below that limit as is reasonably practicable.

Biological monitoring can act as guidance or as legal limits. The chief example for biological monitoring values being used as legal limits in the EU (including the UK) is for blood lead values following exposure to inorganic lead. There are both action levels (when control measures must be reviewed) and suspension levels (where workers must be transferred away from working with lead until their levels have adequately fallen), and these levels vary according to whether the person is an adult, a woman of child-bearing age or a young person.

3.2.3 The Place of Occupational Exposure Standards in Workplace Risk Assessment/Evaluation

Occupational exposure limits are a part of any strategy concerning control of risks from chemical exposure in the workplace. They are an indication of what is an acceptable, or, at least, a tolerable risk in the workplace. Together with classification and labelling data communicated through the materials safety data sheet, occupational exposure limits should provide the underlying information for conducting risk assessments within organizations and for individual workstations. Control of the risks should be by engineering means where possible—preferably in intrinsic design of the equipment, but also by use of appropriate ventilation. If engineering means are not possible or not adequate, personal protective equipment can be used to prevent exposure. All of this has to be within management systems that encourage proper attention to worker safety and health if it is to be effective. This is the realm of the professional occupational hygienist and safety specialist and will not be examined further here.

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4 MAJOR HAZARDS

Approaches to major accident hazards toxicity are described in **Toxicology and Disasters** and will not be repeated here. The toxicology associated with these risk assessments is a very specialized area specifically concerned with predicting toxic effects following rare events. These approaches apply equally to industrial accidents, natural disasters involving chemicals and terrorist attacks involving chemical warfare agents.

5 AFTERWORD

Regulatory schemes are society's response to particular problems. They are intended to ensure that the hazards and risks associated with the chemicals to which we are exposed are adequately characterized, evaluated and controlled.

Toxicology is a major contributor to risk characterizations and evaluations, with other important contributions coming from specialists in exposure, intended effects (where relevant), structure–activity relationships, epidemiology, psychology, sociology and law, and administrators. A team effort is required if society is to be adequately satisfied with the outcomes.

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NOTES

[a.] Previously called the Committee for Veterinary Medicinal Products, but the Committee has retained the acronym CVMP.

[b.] Previously called the European Medicines Evaluation Agency. The agency has retained the acronym EMEA.

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Toxicological Considerations for Pharmaceutical Products

Lynnda Reid

CONTENTS

The contents and views expressed in this overview are my own and do not necessarily represent the views of the Food and Drug Administration (FDA), the Center for Drug Evaluation and Research (CDER) or individual divisions within CDER, or any other international regulatory body. Any questions regarding the development or safety of an investigational or marked pharmaceutical product should be directed to the relevant CDER review division or governing international agency.

1 INTRODUCTION: HISTORICAL BASES FOR NONCLINICAL REQUIREMENTS TO SUPPORT NEW PHARMACEUTICAL PRODUCTS

Early drug development was unregulated and characterized by marketplace trial and error. Therapeutic agents were often derived from poisons given in doses large

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enough to kill pathogenic organisms without ostensibly harming the host. In this sense, toxicology has always been an integral partner in the development of therapeutic agents. The systematic evaluation of the safety of medicines did not become common practice until the early 1900s. Until that time, the regulation of 'medications' was accomplished on a local/regional level, and demonstration of safety or efficacy was not required. One of the first laws regulating the marketing of drugs was the 1906 Pure Food and Drug Act which gave government authorities in the USA the power to remove drugs from sale only if they were found to be mislabelled, contaminated or adulterated. This law required that the presence and amount of selected dangerous or addictive substances, such as arsenic, alcohol and opiates, had to be labelled, and it identified the United States Pharmacopoeia and the National Formulary as official standards for drugs. However, it fell short of requiring demonstration of either safety or efficacy.

Implementation of standards requiring demonstration of safety and efficacy prior to marketing was driven primarily by two pharmaceutical disasters. In the USA, ingestion of Elixir Sulfanilamide, a cough syrup using the untested solvent diethylene glycol, was responsible for the deaths of over 100 patients, including children. In response, the 1938 Food, Drug, and Cosmetic Act was implemented, and formed the basis for regulation of modern pharmaceutical development. This act required firms to provide proof of safety prior to marketing in the USA. In Japan, government regulations requiring demonstration of safety prior to marketing were implemented in the 1950s, while in Europe, the trigger was the thalidomide tragedy of the 1960s. In the USA, the thalidomide disaster also triggered the Drug Amendments Act of 1962, which required drug firms to satisfy the Food and Drug Administration (FDA) as to both the safety and efficacy of a new drug for its intended use.

By the early 1960s and 1970s most countries had initiated some form of regional product registration controls requiring premarketing demonstration of safety and efficacy. With the globalization of markets and large multinational pharmaceutical companies it became imperative to try to harmonize requirements related to the type of data needed to demonstrate safety and efficacy. In the late 1980s, harmonization of regulatory requirements was pioneered by the European Community (now the European Union), and bilateral discussions with Japan and the USA were initiated. The joint regulatory–industry initiative on international harmonization was conceived at the World Health Organization (WHO) Conference of Drug Regulatory Authorities, in Paris, 1989. Representatives of the regulatory agencies and industry associations of Europe, Japan and the USA met in Brussels in 1990 to form the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). The ICH Steering Committee was formed to harmonize regulations regarding safety, quality and efficacy, which are the basis for approving and authorizing new medicinal products. The WHO remains active in disseminating information and providing input beyond the ICH member countries. **Table 1** contains an annotated listing of all currently available ICH guidance documents related to nonclinical testing of pharmaceutical products. **Table 2** contains an annotated listing of guidance documents generated by the US FDA and European regulatory agencies.

There are still many areas involving safety which have not been harmonized. In these instances, companies seeking marketing approval in multiple countries must meet the requirements set forth by each regulatory body as to the type, timing and duration of studies needed to support clinical development and registration.

2 CONSIDERATIONS IN THE OVERALL DESIGN OF A NONCLINICAL DEVELOPMENT PROGRAMME

The scope of nonclinical studies needed to support clinical development and approval of new pharmaceutical agents is generally dictated by the stage of development and the proposed therapeutic use of the product. Factors determining the type and duration of nonclinical studies necessary to support new drugs include the following:

- targeted population
- duration of treatment
- timing of studies in relationship to clinical development
- route of administration
- pharmaceutical class
- relevance of animal models.

2.1 Targeted Population

Initial clinical studies are performed primarily to determine a safe human dose and to collect pharmacokinetic data. Except for extremely toxic products, for example cytotoxic, genotoxic and/or chemotherapeutic products, initial clinical studies are performed in normal, healthy human volunteers who will not derive any benefit from treatment. In these cases, the medical mantra of 'do no harm' is especially important. Animal studies are usually the only basis for dose selection for 'first-in-human' studies. The minimal requirements for nonclinical studies needed to support initiation of clinical studies usually include the following:

- acute safety pharmacology studies
- *in vitro* genotoxicity studies

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Table 1 ICH guidance for industry

E8 (1997)	General Considerations for Clinical Trials	This ICH document describes the internationally accepted principles and practices both in the conduct of individual clinical trials and in overall development strategies for new medicinal products.
M3 (1997)	Nonclinical Safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals	The nonclinical safety study recommendations for the marketing approval of a pharmaceutical usually include single- and repeated-dose toxicity studies, reproduction toxicity studies, genotoxicity studies, local tolerance studies and, for drugs that have special cause for concern or are intended for a long duration of use, an assessment of carcinogenic potential. Other nonclinical studies include pharmacology studies for safety assessment (safety pharmacology) and pharmacokinetic (absorption, distribution, metabolism and excretion (ADME)) studies. These types of studies and their relation to the conduct of human clinical trials are presented in this guidance.
M3 (R1) (2000)	Maintenance of the ICH Guideline on Nonclinical Safety Studies for the Conduct of Human Clinical Trails for Pharmaceuticals	
S1A (1996)	The Need for Long-Term Rodent Carcinogenicity Studies of Pharmaceuticals	The objective of this guideline is to define the conditions under which carcinogenicity studies should be conducted, to avoid the unnecessary use of animals in testing, and to provide consistency in worldwide regulatory assessments of applications. It is expected that these studies will be performed in a manner that reflects currently accepted scientific standards. The fundamental considerations in assessing the need for carcinogenicity studies are the maximum duration of patient treatment and any perceived cause for concern arising from other investigations. Other factors may also be considered, such as the intended patient population, prior assessment of carcinogenic potential, the extent of systemic exposure, the (dis)similarity to endogenous substances, the appropriate study design, or the timing of study performance relative to clinical development.
S1B (1997)	Testing for Carcinogenicity of Pharmaceuticals	This document provides guidance on approaches for evaluating the carcinogenic potential of pharmaceuticals. These include: choice of species for a long-term carcinogenicity study and additional *in vivo* tests for carcinogenicity. Also discussed are: appropriate mechanistic studies that can be useful for the interpretation of tumour findings and their relevance to human risk assessment; general considerations in the choice of appropriate species for long-term carcinogenicity testing; and evaluation of carcinogenic potential.
S1C (1995)	Dose Selection for Carcinogenicity Studies of Pharmaceuticals	This guidance outlines four generally acceptable criteria for selection of the high dose for carcinogenicity studies of therapeutics: maximum tolerated dose, 25-fold AUC ratio (rodent : human), dose-limiting pharmacodynamic effects, saturation of absorption and maximum feasible dose. The use of other pharmacodynamic–pharmacokinetic- or toxicity-based end points in study design is considered based on scientific rationale and individual merits.
S1C(R) (1997)	Dose Selection for Carcinogenicity Studies of Pharmaceuticals: Addition of a Limit Dose and Related Notes	This guidance outlines the approaches used in determining the high dose for carcinogenicity studies. In using these approaches, it may not be necessary to exceed a dose of 1500 mg kg^{-1} day^{-1}. This limit dose applies only in cases where there is no evidence of genotoxicity and where the maximum recommended human dose does not exceed 500 mg day^{-1}.
S2A (1996)	Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals	This guidance discusses recommendations for genotoxicity testing of pharmaceuticals. Specific guidance is given for: *in vitro* tests (base set of strains used in bacterial mutation assays, and definition of the top concentration for *in vitro* tests); *in vivo* tests (acceptable bone marrow tests for the detection of clastogens *in vivo*, and use of male/female rodents in bone marrow micronucleus tests); and evaluation of *in vitro* and *in vivo* test results.

(*continued overleaf*)

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Table 1 (*continued*)

S2B (1997)	A Standard Battery for Genotoxicity Testing of Pharmaceuticals	Registration of pharmaceuticals requires a comprehensive assessment of their genotoxic potential. It is clear that no single test is capable of detecting all relevant genotoxic agents. Therefore, the usual approach should be to carry out a battery of *in vitro* and *in vivo* tests for genotoxicity. Such tests are complementary rather than representing different levels of hierarchy. This guidance recommends the following standard test battery: (i) a test for gene mutation in bacteria; (ii) an *in vitro* test with cytogenetic evaluation of chromosomal damage with mammalian cells, or an *in vitro* mouse lymphoma thymidine kinase (tk) assay; and (iii) an *in vivo* test for chromosomal damage using rodent haematopoietic cells.
S2(R1) (2008)	Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use	This draft guidance is intended to replace and combine the ICH S2A and S2B guidelines. The purpose of the revision is to optimize the standard genetic toxicology battery for prediction of potential human risks, and to provide guidance on interpretation of results, with the ultimate goal of improving risk characterization for carcinogenic effects that have their basis in changes in the genetic material. The revised guidance describes internationally agreed-upon standards for follow-up testing and interpretation of positive results *in vitro* and *in vivo* in the standard genetic toxicology battery, including assessment of nonrelevant findings.
S3A (1995)	Toxicokinetics: The Assessment of Systemic Exposure in Toxicity Studies	Toxicokinetics is defined as the generation of pharmacokinetic data, either as an integral component in the conduct of nonclinical toxicity studies or in specially designed supportive studies, in order to assess systemic exposure. The guidance highlights the need to integrate pharmacokinetics into toxicity testing, which should aid in the interpretation of the toxicology findings and promote rational study design development.
S3B (1995)	Pharmacokinetics: Guidance for Repeated Dose Tissue Distribution Studies	Tissue distribution studies are essential in providing information on distribution and accumulation of the compound and/or metabolites, especially in relation to potential sites of action. This paper provides guidance on circumstances when repeated-dose tissue-distribution studies should be considered, and on the conduct of such studies.
S4A (1999)	Duration of Chronic Toxicity Testing in Animals (Rodent and Nonrodent Toxicity Testing)	This guidance represents the current thinking on the duration of chronic toxicity testing in animals (rodent and nonrodent toxicity testing). The ICH guidance recommends nine-month chronic toxicity studies in nonrodents, although shorter studies may be equally acceptable in some circumstances, and longer studies may be more appropriate in others, as discussed in the guidance.
S5A (1994)	Detection of Toxicity to Reproduction for Medicinal Products	The aim of reproduction toxicity studies is to reveal any effect of one or more active substance(s) on mammalian reproduction. This guideline addresses the design of studies that allow exposure of mature adult animals and all other stages of development from conception to sexual maturity. These include: premating to conception; conception to implantation; implantation to closure of the hard palate; closure of the hard palate to the end of pregnancy; birth to weaning; and weaning to sexual maturity.
S5B (1996)	Detection of Toxicity to Reproduction for Medicinal Products: Addendum on Toxicity to Male Fertility	This text is an addendum to the above Guideline on Detection of Toxicity to Reproduction for Medicinal Products, and it provides amendments to the published text. The amendments are intended to provide a better description of the testing concept and recommendations, especially those addressing flexibility, premating treatment duration and observations.

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S5B(M) (2000)	Toxicity to Male Fertility, an Addendum to the ICH Tripartite Guideline on Detection of Toxicity to Reproduction for Medicinal Products	The amendments provide a better description of the testing concept and recommendations for male fertility studies, especially those addressing flexibility, premating treatment duration, and observations.
S6 (1997)	Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals	This guideline recommends a basic framework for the preclinical safety testing of biotechnology-derived pharmaceutical products. It applies to products derived from characterized cells through the use of a variety of expression systems including bacteria, yeast, insect, plant and mammalian cells.
S7A (2001)	Safety Pharmacology Studies for Human Pharmaceuticals	This guidance was developed to help protect clinical trial participants and patients receiving marketed products from potential adverse reactions to pharmaceuticals, while avoiding unnecessary use of animals and other resources. This guidance defines safety pharmacology and describes general principles and recommendations for its evaluation.
S7B (2005)	Non-clinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals	This guideline describes a nonclinical testing strategy for assessing the potential of a test substance to delay ventricular repolarization. This guideline includes information concerning nonclinical assays and an integrated risk assessment.
S8 (2006)	Immunotoxicity Studies for Human Pharmaceuticals	This ICH guidance provides: (i) recommendations on nonclinical testing approaches to identify compounds which have the potential to be immunosuppressive, and (ii) guidance on a weight-of-evidence decision-making approach for immunotoxicity testing.
Q3A (2003)	Impurities in New Drug Substances	This revised document is intended to provide guidance for registration applications on the content and qualification of impurities in new drug substances produced by chemical syntheses and not previously registered in a region or member state. It is not intended to apply to the regulation of new drug substances used during the clinical research stage of development. Biological/biotechnological, peptide, oligonucleotide, radiopharmaceutical, fermentation and semisynthetic products derived therefrom, herbal products, and crude products of animal or plant origin are not covered.
Q3B(R) (2006)	Impurities in New Drug Products	This revised document provides guidance recommendations for registration or marketing applications on the content and qualification of impurities in new drug products produced from chemically synthesized new drug substances not previously registered or approved for marketing in a region or member state. This guideline addresses only those impurities in drug products classified as degradation products of the active ingredient or reaction products of the active ingredient with an excipient and/or immediate container/closure system (collectively referred to in this guideline as degradation products). Impurities arising from excipients present in the drug product are not covered in this document.
Q3C (1997)	Residual Solvents	This guidance recommends acceptable amounts of residual solvents in pharmaceuticals for the safety of the patient, and recommends the use of less-toxic solvents in the manufacture of drug substances and dosage forms. This guidance does not apply to potential new drug substances, excipients or drug products used during the clinical research stages of development, nor does it apply to existing marketed drug products.
Q3C (2003)	Tables and Lists	This is the companion document for the ICH guidance for industry Q3C Impurities: Residual Solvents (1997), which makes recommendations as to what amounts of residual solvents are considered safe in pharmaceuticals.

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Table 2 Regional guidance documents

FDA (2005)	Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers	This guidance outlines a common process (algorithm) and vocabulary for deriving the maximum recommended starting dose for 'first in human' clinical trials of new molecular entities in normal, healthy volunteers. The goals are to: (i) establish a consistent terminology for discussion of the starting dose, (ii) provide common conversion factors for deriving a human equivalent dose, and (iii) delineate a strategy for selecting the highest recommended starting dose for normal volunteers, regardless of the projected clinical use.
FDA (1995)	Content and Format of Investigational New Drug Applications (INDs) for Phase 1 Studies of Drugs, Including Well-Characterized, Therapeutic, Biotechnology-Derived Products	This guidance clarifies requirements for data and data presentation related to the initial entry into human studies of an investigational drug, including well-characterized, therapeutic, biotechnology-derived products. The most significant clarifications are: (i) the explicit willingness to accept an integrated summary report of toxicology findings based upon the unaudited draft toxicologic reports of completed animal studies as initial support for human studies, and (ii) specific manufacturing data appropriate for a Phase 1 investigation.
FDA (1996)	Single Dose Acute Toxicity Testing for Pharmaceuticals	Acute toxicity studies in animals are usually necessary for any pharmaceutical intended for human use. The information obtained from these studies is useful in choosing doses for repeat-dose studies, providing preliminary identification of target organs of toxicity, and, occasionally, revealing delayed toxicity. Acute toxicity studies may also aid in the selection of starting doses for Phase 1 human studies, and provide information relevant to acute overdosing in humans.
CPMP (2004)	Position Paper on Non-Clinical Safety Studies to Support Clinical Trials with a Single Microdose	This position paper from the Committee for Proprietary Medicinal Products (CPMP) of the European Medicines Agency (EMEA) defines common standards of nonclinical studies needed to support human clinical trials of a single dose of a pharmacologically active compound using microdose techniques.
FAMHP (2007)	Guidance to the Conduct of Exploratory Trials in Belgium	This working document from the Belgian Federal Agency for Medicines and Health Products (FAMHP) offers a temporary framework for preparing and evaluating exploratory clinical trial applications that are outside the scope of the microdosing approach and to be conducted in Belgium. Discussed are various aspects of drug development, including types of preclinical studies needed to support exploratory clinical trials.
FDA (2004)	Botanical Drug Products (2004)	This guidance explains the circumstances in which a botanical drug may be marketed under an over-the-counter (OTC) drug monograph, and when FDA approval of a new drug application (NDA) is required for marketing. In addition, this document provides regulatory and scientific guidance to sponsors on conducting initial and expanded clinical investigations of botanical drug products, including those botanical products currently lawfully marketed as foods and dietary supplements in the USA.
CPMP (1997)	Points to Consider: The Assessment of the Potential for QT Interval Prolongation by Non-Cardiovascular Medicinal Products	This document from the European CPMP discusses considerations for assessing risk of drug-induced QT interval prolongation. Included is a discussion of the various *in vitro* and *in vivo* assays, methodological issues and their regulatory implications.
FDA (1997)	Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use (1997)	This document was developed for manufacturers of monoclonal antibody products and represents the FDA Center for Biologics Evaluation and Research (CBER)'s current (i.e. 1997) thinking on the subject. Discussed are issues relating to product manufacture, quality control, and safety testing in animals and humans, among others.

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FDA (2002)	Carcinogenicity Study Protocol Submissions	This guidance is intended to inform sponsors of the types of information the FDA Center for Drug Evaluation and Research (CDER) relies on when evaluating protocols for animal carcinogenicity studies. These include: general toxicity information, animal and human metabolic profiles, toxicokinetic data and genotoxicity.
FDA (2006)	Nonclinical Safety Evaluation of Drug or Biologic Combinations	This guidance provides recommendations on nonclinical approaches to support the clinical study and approval of fixed-dose combination (FDC) products, co-packaged products, and adjunctive therapies. The nonclinical studies that the FDA recommends sponsors use to characterize the combination will depend on the toxicologic and pharmacokinetic profiles of the individual drugs, the treatment indication or indications, and the intended population.
FDA (2002)	Drug and Biologic Drug Products: Evidence Needed to Demonstrate Effectiveness of New Drugs When Human Efficacy Studies are Not Ethical or Feasible	This final rule provides for approval of certain new drugs and biological products based on animal data when adequate and well-controlled efficacy studies in humans cannot be ethically conducted, because the studies would involve administering a potentially lethal or permanently disabling toxic substance or organism to healthy human volunteers. Although human data can be taken into account, animal studies alone can be used to demonstrate effectiveness when: (i) the pathophysiological mechanism for the indication (i.e. effects of bioterrorism) is well understood, as well as its treatment by the product; (ii) the drug effect is demonstrated in two animal species, unless one species has been well characterized; (iii) the end point in animal studies is clearly related to the human end point (e.g. survival); and (iv) PK/PD data is available to allow selection of an effective human dose.
FDA (2005)	Nonclinical Studies for the Safety Evaluation of Pharmaceutical Excipients	This document provides guidance concerning development of safety profiles to support use of new excipients as components of drug or biological products. In this document, the phrase 'new excipients' is defined to mean any ingredients that are intentionally added to therapeutic and diagnostic products but which: (i) do not directly exert therapeutic effects at the intended dosage (although they may act to improve product delivery, for example enhancing absorption or controlling release of the drug substance); and (ii) are not fully qualified by existing safety data with respect to the currently proposed level of exposure, duration of exposure or route of administration.
FDA (2006)	Exploratory IND Studies	This draft guidance describes what approaches should be considered when planning a clinical trial for a drug or biologic that occurs early in Phase 1, involves limited human exposure, and has no therapeutic intent. Such clinical studies can be helpful to: (i) gain an understanding of the product's mechanism of action, (ii) provide PK information, (iii) select a lead candidate for further development, or (iv) explore a drug's biodistribution using imaging techniques. Since such studies use a limited number of subjects with a limited dose range for a limited period of time, the preclinical requirements to support such clinical studies can be flexible, because the potential risks to human subjects are thought to be less than traditional Phase 1 studies. Examples of preclinical studies that can be used to support exploratory IND studies in humans are provided.
FDA (2006)	Recommended Approaches to Integration of Genetic Toxicology Study Results	This guidance is intended to inform industry on how CDER views positive findings in genetic toxicology assays during drug development. It provides recommendations on how to proceed with clinical studies while ensuring the safety of study participants when results in genotoxicity studies suggest a potential cancer or genetic hazard. The decision of whether to begin a clinical trial when there are positive genetic toxicity study results is based on a weight-of-evidence approach that includes consideration of the results of all genetic toxicology tests, the genotoxic mechanism of action, and the nature of the proposed clinical trial.

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Table 2 (*continued*)

CPMP (2006)	Guideline on Detection of Early Signals of Drug-Induced Hepatotoxicity in Non-Clinical Studies	This *draft* guideline provides guidance on how to identify, collect and report early nonclinical signs of medicinal product-induced hepatotoxicity in order to decrease the risk of clinical adverse liver reactions. This guideline applies mainly to new active substances. However, the principles as introduced in the present guideline would also help to identify mechanisms underlying adverse liver reactions of a medicinal product already on the market.
FDA (2002)	Immunotoxicology Evaluation of Investigational New Drugs	This guidance makes recommendations to sponsors of INDs on (i) the parameters that should be routinely assessed in toxicology studies to determine effects of a drug on immune function, (ii) when additional immunotoxicity studies should be conducted, and (iii) when additional mechanistic information could help characterize the significance of a given drug's effect on the immune system. This guidance is intended for drug products and does not apply to biological products.
FDA (1999)	ANDAs: Impurities in Drug Substances	This guidance provides recommendations for including information in abbreviated new drug applications (ANDAs) and supporting drug master files (DMFs) on the identification and qualification of impurities in drug substances. Although this guidance is similar, but not identical to ICH-Q3A, many of the recommendations found in ICH-Q3A are applicable to drug substances used in generic drug products.
FDA (1997)	FDA's Policy Statement for the Development of New Stereoisomeric Drugs	Stereoisomers are molecules that are identical in atomic constitution and bonding, but differ in the three-dimensional arrangement of the atoms. This document focusses on issues relating to the study and pharmaceutical development of individual enantiomers (individual stereoisomers) and racemates (50 : 50 proportion of enantiomers). Circumstances for additional pharmacology, ADME and/or toxicity testing of individual enantiomers are discussed.
CPMP (2002)	Note for Guidance on Specification Limits for Residues of Metal Catalysts	This draft guidance from the European CPMP recommends maximum acceptable limits on specific metal residues arising from the use of catalysts in the synthesis of pharmaceuticals. The toxicological literature was used to define the maximum safe intake limits for individual elements, or permitted daily exposure (PDE), in $\mu g\,kg^{-1}\,day^{-1}$. Included are monographs of individual metal elements.
CPMP (2006)	Position Paper on the Limits of Genotoxic Impurities	This position paper describes a general framework and practical approaches on how to deal with genotoxic impurities in new drug substances.
FDA (2008)	Safety Testing of Drug Metabolites	This guidance discusses when additional animal testing may be required for metabolites formed in humans that were not formed in the animal species used during original safety assessment studies.
FDA (1997)	Drug Metabolism/Drug Interaction Studies in the Drug Development Process: Studies *In Vitro*	This FDA guidance provides suggestions on current approaches to studies *in vitro* of drug metabolism and interactions. It describes techniques and approaches for studies conducted *in vitro* of drug metabolism and drug interactions, correlation between *in vitro* and *in vivo* studies, the timing of metabolism studies in relation to drug development, and metabolic and exposure comparisons between animals and humans.
FDA (2006)	Nonclinical Safety Evaluation of Pediatric Drug Products	This document provides guidance on the nonclinical safety evaluation of therapeutics intended for paediatric patients. Some conditions under which juvenile animals are considered meaningful predictors of toxicity in paediatric patients are discussed and recommendations on nonclinical testing are provided. It also provides guidance on the role and timing of animal studies in the safety evaluation of therapeutics intended for the treatment of paediatric patients.

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FDA (2005)	Pharmacogenomic Data Submissions	This guidance is intended to facilitate scientific progress in the field of pharmacogenomics and to facilitate the use of pharmacogenomic data in informing regulatory decisions. It discusses when pharmacogenomic data is to be submitted, the format of that data, and how the data will be used. Pharmacogenomic data must be submitted to an IND if any of the following apply: (i) the test results will be used for decision making in any clinical trial, or in an animal trial used to support safety; (ii) the sponsor is using the test results to support scientific arguments pertaining to, for example, the safety, effectiveness, dosing and pharmacology of the drug; or (iii) the test results constitute a known valid biomarker for physiologic, pathophysiologic, pharmacologic, toxicologic or clinical states or outcomes in humans, or a known valid biomarker for a safety outcome in animal studies. Data submission for NDAs is required if it is used to support scientific/clinical arguments or for labelling purposes. Otherwise, data submission is voluntary.
FDA (2007)	Pharmacogenomic Data Submissions: Companion Guidance	The recommendations made in this draft document apply to development of microarray data that might be submitted in support of INDs, NDAs and biologics licence applications (BLA)s. It covers issues relating to generation of gene expression data, genomic data and formats for data submission.
FDA (2008)	Nonclinical Safety Evaluation of Reformulated Drug Products and Products Intended for Administration by an Alternate Route	This draft guidance provides recommendations regarding the nonclinical evaluation of a new formulation containing a previously approved drug substance, and of a product proposed for use by an alternate route of administration for which the product was not previously approved.
FDA (2003)	Photosafety Testing	This guidance is intended to help applicants decide whether they should test for photosensitivity and assess potential human risk for photochemical carcinogenesis (cancer) of their drug products during the clinical development process. The guidance describes a consistent, science-based approach for testing for topically and systematically administered drug products. Basic concepts of photobiology and phototesting are described.
CPMP (2002)	Note on Guidance on Photosafety Testing	This guidance from the European CPMP defines the conditions under which photosafety evaluation of pharmaceuticals should be conducted. Also included are various test procedures, strategies and approaches to photosafety testing.
FDA (1993)	Guideline for the Study and Evaluation of Gender Differences in the Clinical Evaluation of Drugs	This guideline addresses the concern that the drug development process does not produce adequate information about the effects of drugs in women. It discusses the FDA's expectations regarding inclusion of patients of both genders in drug development, analyses of clinical data by gender, assessment of potential pharmacokinetic differences between genders and, where appropriate, assessment of pharmacodynamic differences and the conduct of specific additional studies in women. It also withdraws previous (1977) restrictions on the participation of women of childbearing potential in early clinical trials, including clinical pharmacology studies (e.g. dose tolerance, bioavailability and mechanism-of-action studies) and early therapeutic studies.
FDA (2001)	Integration of Study Results to Assess Concerns about Human Reproductive and Developmental Toxicities	This draft guidance describes a process for estimating the increase in human developmental and reproductive risks as a result of drug exposure when definitive human data are unavailable. The integration process focusses on the likelihood a drug will increase the risk of adverse human developmental or reproductive effects.

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- appropriate toxicology studies which define a no observable adverse effect level (NOAEL) as well as defining the dose-limiting toxicity and potential major toxicity targets.

For products that will first be studied in patient volunteers with serious or life-threatening conditions, in addition to the above-mentioned studies, it is also imperative that nonclinical proof of concept and/or mechanistic studies demonstrating a reasonable chance of efficacy be submitted. For most clinical studies, it is necessary to take patients off current medications; therefore, for ethical reasons, there should be some evidence that the drug will not only be safe, but may also have some benefit.

The timing of reproductive and developmental studies differs between regulatory agencies. Teratogenicity studies are generally required prior to initial studies in humans. However, some agencies may waive this requirement if initial clinical studies can be performed using reliable methods of birth control, for example physical barriers (condoms) in men and either double-barrier protection or single-barrier combined with reliable birth control methods in women, or enrolment of nonfertile female volunteers. Prior to any exposures to pregnant or potentially pregnant women, most regulatory agencies require that the entire battery of reproductive and developmental studies be completed.

2.2 Duration of Treatment

The general rule is that the nonclinical studies should be of equal or greater duration than the proposed clinical study. For registration purposes, the nonclinical studies should be at least twice the duration of the proposed labelled clinical dosing regimen up to 6 months in rodents and 6, 9 or 12 months in nonrodents. The duration of chronic treatment in nonrodents depends on regional recommendations which take into consideration the safety profile of the drug and the patient population; for example, some regulatory agencies may recommend 12-month nonrodent studies for paediatric drugs.

2.3 Timing of Studies in Relationship to Clinical Development

In recent years, the term preclinical has given away to nonclinical to better reflect the ongoing development programmes designed to keep pace with clinical development. Clinical trials are conducted in phases grouped by their purpose and objectives. ICH E8 clinical guidance has defined the phases of clinical development as follows:

Phase 1: first-in-human exposure studies generally single dose, followed by dose escalation and short-term studies to evaluate safety and determine pharmacokinetic profiles.
Phase 2: exploratory safety and efficacy studies in patients.
Phase 3: confirmatory safety and efficacy studies in patients designed to support registration and labelling.

As mentioned above, the required duration of the nonclinical studies to support clinical development is dependent on the proposed clinical duration of treatment. The timing for particular studies may be dependent on the intended patient population. For example, fertility studies need only be done in one sex if the product is intended for only male or female use, although teratogenicity studies should be done regardless of sex, since effects may be mediated by either sex. Generally prenatal and postnatal developmental studies need only be submitted to support treatment in reproductively competent female patients.

As mentioned above, *in vitro* genotoxicity studies are required prior to first time in humans, whereas the *in vivo* genotoxicity study should be submitted prior to initiation of Phase 2 of clinical development. Carcinogenicity studies for nongenotoxic products are required at the time of registration. However, for products for which the weight of evidence suggests they are genotoxic, carcinogenicity studies may be required to support chronic clinical exposures during development.

2.4 Route of Administration

Pharmacodynamics (PD) can differ significantly between species. If adequate systemic exposures cannot be achieved in animal models compared with human exposures via the clinically relevant route of exposure, it may be necessary to conduct both local and systemic toxicity studies. Local studies would be performed using the clinically relevant route of exposure, for example dermal, intrathecal, intravaginal, and so on, whereas the systemic and reproductive toxicity studies may need to be performed using an alternative route such as oral, intramuscular or subcutaneous.

2.5 Relevance of Animal Models (Species Selection)

In general, toxicology studies are required in two species, one being nonrodent. However, there are exceptions to this rule based on the relevance of the available animal models. If it can be demonstrated that only one animal model has any relevance to humans, it may be possible to justify the use of a single species. This is the case for

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many biological products which may have targets that are only expressed in primates.

Metabolic profiles are also important in determining the relevance of animal models. Metabolism is important since toxicity may be dependent on a species' ability to clear and detoxify the parent molecule or conversely to form or eliminate a toxic metabolite. If an animal model cannot be found which has a similar metabolic profile to humans, then it may be necessary to produce and test any major, uniquely human metabolites along with the parent compound.

Caution should be exercised when planning a nonclinical development plan that it does not proceed far in advance of clinical development. Human pharmacologic data are vital in determining the adequacy of the nonclinical models and studies.

3 NONCLINICAL PHARMACOLOGY

For many therapeutic products, the exact mechanism of action is difficult to determine. Most drug developers have a working hypothesis of how the drug may work prior to dedicating costly resources to development. However, proof-of-concept studies in animals are just that, and are not considered evidence of efficacy. Nonclinical proof-of-concept studies are required only for products where the risk : benefit ratio is extremely high or where ethical considerations for serious and life-threatening conditions make it unethical to remove patients from a potentially beneficial approved treatment to enroll them in a clinical trial. Another scenario which requires animal efficacy studies is for indications where it would be unethical to test products under conditions of use, such as products to prevent or treat effects resulting from exposure to chemical or biological warfare agents.

Secondary pharmacologic targets should also be explored. This should include *in vitro* receptor and/or tissue-binding profiles. Secondary pharmacologic effects may be important in explaining off-target or species-specific toxicity, or lack of efficacy.

4 SAFETY PHARMACOLOGY STUDIES

Safety pharmacology studies are usually acute studies performed at the highest tolerated doses to evaluate potential acute effects on vital functions including the cardiovascular, renal, respiratory and central nervous systems. These studies should be performed prior to first time exposure in humans. Other safety pharmacology studies include evaluation of effects on gastrointestinal (GI) motility and abuse liability, which generally should be completed to support repeat-dose clinical studies.

5 TOXICOKINETIC AND PHARMACOKINETIC STUDIES

Exposure data in nonclinical models are important not only for comparing exposures in animals to humans, but also in determining the appropriateness of the selected animal model(s). Prior to 'first-time-in-human' clinical studies, *in vitro* metabolism data should be generated using liver microsomes, comparing nonclinical species and humans to ensure qualitatively comparable metabolic profiles prior to the first exposures to humans. These data also are necessary prior to selecting the animal species for studies conducted to support clinical trials. *In vivo* data should be generated early in development to qualitatively and quantitatively compare animal and human exposures and support selection of nonclinical models for pivotal toxicology, reproductive and carcinogenicity studies.

C_{max}, T_{max}, AUC, half-life and bioavailability are important parameters in determining safe doses in humans (see **Toxicokinetics of Xenobiotic and Metabolite Distribution and Excretion**). Additionally, ADME parameters, that is, absorption, distribution, metabolism and excretion, can vary widely between species and can significantly affect the toxicokinetic profile. Bioavailability may vary widely between species and even large doses in animals may result in only limited exposures compared with humans due to differences in absorption. Differences in metabolism and elimination can also significantly affect overall exposure to either the parent or an active or toxic metabolite. Acute toxicologic effects often correlate with C_{max} and are usually associated with central nervous system (CNS), respiratory, or cardiovascular toxicity, While most chronic and tissue-related effects are related to overall exposures, that is, AUC, and drug distribution.

In order to evaluate the potential risk of products with significant adverse effects, it is necessary to compare exposures. When comparing exposures between nonclinical species and between animals and humans, the optimal parameter is either C_{max} and/or AUC, based on the type of toxicity observed. In the absence of adequate pharmacokinetic (PK) data, doses should be compared based on body surface area ($mg\,m^{-2}$), which is a more conservative estimate than using body weight ($mg\,kg^{-1}$). Conversion factors used by the FDA to convert doses to $mg\ m^{-2}$ are presented in **Table 3** (FDA Guidance for Industry: Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers).

It is also important to evaluate the effect of gender and food consumption on ADME and pharmacokinetic parameters. If the product is to be used in both paediatric and adult populations, then age-related changes in ADME and pharmacokinetic parameters should also be evaluated.

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Table 3 Conversion of animal doses to human equivalent doses (HED) based on body surface area

Species	Reference body weight (kg)	Working weight range[a] (kg)	Body surface area (m^2)	To convert mg kg^{-1} dose to dose in mg m^{-2}, multiply by *km* below:	To convert animal dose in mg kg^{-1} to HED[b] in mg kg^{-1}, either:	
					Divide animal dose by:	Multiply animal dose by:
Human:	60	—	1.62	37	—	—
Child[c]	20	—	0.80	25	—	—
Mouse	0.020	0.011–0.034	0.007	3	12.3	0.081
Hamster	0.080	0.047–0.157	0.016	5	7.4	0.135
Rat	0.150	0.080–0.270	0.025	6	6.2	0.162
Ferret	0.300	0.160–0.540	0.043	7	5.3	0.189
Guinea pig	0.400	0.208–0.700	0.05	8	4.6	0.216
Rabbit	1.8	0.9–3.0	0.15	12	3.1	0.324
Dog	10	5–17	0.50	20	1.8	0.541
Primates:						
Monkeys[d]	3	1.4–4.9	0.25	12	3.1	0.324
Marmoset	0.350	0.140–0.720	0.06	6	6.2	0.162
Squirrel monkey	0.600	0.290–0.970	0.09	7	5.3	0.189
Baboon	12	7–23	0.60	20	1.8	0.541
Micropig	20	10–33	0.74	27	1.4	0.730
Minipig	40	25–64	1.14	35	1.1	0.946

[a]For animal weights within the specified ranges, the HED for a 60 kg human calculated using the standard *km* value will not vary more than ±20% from the HED calculated using a *km* based on the exact animal weight.
[b]Assumes 60 kg human. For species not listed or for weights outside the standard ranges, human equivalent dose can be calculated from the formula: HED = animal dose in mg kg^{-1} × (animal weight in kg/human weight in kg)$^{0.33}$.
[c]The *km* is provided for reference only since healthy children will rarely be volunteers for Phase 1 trials.
[d]For example, cynomolgus, rhesus, stumptail, and so on.

6 NONCLINICAL TOXICOLOGY

In the development of pharmaceuticals, toxicology studies are used to support clinical development and to evaluate the potential risk to clinical volunteers and patients. Anyone performing toxicology studies to support submissions to regulatory agencies should consult all pertinent guidance documents. One of the most helpful and important guidances to consult when designing a nonclinical programme is ICH M3 Guidance for Industry: Nonclinical Safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals. This guidance discusses the extent and timing of nonclinical studies needed to support various stages of clinical development and an eventual submission for marketing approval.

Toxicology studies should be designed to define potential target organ toxicity, define a NOAEL and identify biomarkers that can be used to safely monitor potential toxicity in clinical trials. For new molecular entities, the toxicology studies are used to establish a safe starting dose and can influence inclusion and exclusion criteria for enrolment into clinical trials. In addition, the toxicology studies determine the scope of the initial clinical studies; for example, duration, doses, volunteer population, and so on.

6.1 Species Selection

Toxicology studies are generally conducted in two species, one rodent and one nonrodent. Although the rat and dog are the two most commonly used species, the choice of species should be based on its relevance to humans. If only one relevant animal model exits, then studies should only be conducted in that species. Whenever an alternative study design or a single or nontraditional species is used, investigators should contact the appropriate regulatory authority for concurrence.

6.2 Dose Selection

Toxicology studies are generally conducted at a minimum of three dose levels. Selection of the highest dose should

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be based on one of the following:

- Maximum tolerated dose (MTD)—the MTD is defined as the highest dose which does not result in intolerable or overt toxicity (e.g. convulsions, excessive body weight loss, necrosis, pain, paralysis, etc.) or death.
- Maximum feasible dose (MFD)—the MFD may be defined by such parameters as solubility, administration volume, dosing frequency, and so on.
- Dose-limiting pharmacodynamic effects.
- Saturation of absorption or exposure.
- Limit dose of $1000\,mg\,kg^{-1}\,day^{-1}$ (applicable for pharmaceuticals with human exposures of less than $1\,g\,day^{-1}$).

When a MTD, MFD or limit dose has been used in repeat-dose toxicology studies and the toxicity profile has been well characterized, then it may be possible in subsequent studies to use a large exposure multiple of the human exposure for the highest dose tested.

The low dose should be close to, or a low multiple of, the projected clinical dose, and ideally should identify the NOAEL. The median dose should induce mild toxicity and is used to establish the dose–response curve and help identify toxicity that may not have been evident at the high dose due to extreme toxicity or morbidity. The mid dose may also be important in establishing reversibility of findings that are lethal at higher doses. Ideally, the use of at least three dose levels allows investigators to identify target organ toxicity, potential dose-limiting effects, generate dose–response curves and identify the NOAEL. These parameters are essential for assessing risk at clinical doses and designing future nonclinical and clinical studies.

6.3 Duration

As stated earlier, the duration of the toxicology studies is dictated by the clinical development program. During development, nonclinical studies should be of equal or greater duration that the proposed clinical trial(s) that they are intended to support. For registration of a new product, the duration of the toxicology studies should be twice the duration of the proposed clinical dosing regimen up to a duration of three months. For indications that require chronic treatment greater than three months, toxicology studies should be conducted for up to six months in rodents and nine months in nonrodents (see ICH S4A Guidance for Industry: Duration of Chronic Toxicity Testing in Animals (Rodent and Nonrodent Toxicity Testing)). There are exceptions to the maximum duration of the nonrodent studies which can justify study durations of 6 or 12 months. Justifications for shorter durations may include intermittent dosing schedules, well-characterized drug classes, and/or low toxicity potential. On the other hand, 12-month studies may be requested based on use in paediatric populations, compounds with a unique mechanism of action, or late onset toxicity. For questions regarding the optimal duration for chronic nonrodent toxicology studies, one should consult the regional regulatory agency.

Use of a staged nonclinical development programme allows investigators to begin preliminary clinical investigations prior to investing large amounts of time or capital in lead compounds. Although a single-dose study in humans can be supported by single-dose studies in two animal species (one being nonrodent), two-week repeat-dose studies are preferred for a new molecular entity. Early clinical pharmacokinetic studies provide exposure data and metabolic data important for assessing risk and validating the appropriateness of the selected animal models.

All toxicology studies submitted to support clinical development or approval should be conducted in compliance with Good Laboratory Practice (GLP).

7 GENOTOXICITY

The standard battery for genotoxicity testing consists of two *in vitro* assays and one *in vivo* genotoxicity study (See ICH S2A and S2B). The *in vitro* assays should be conducted not only on the active drug substance, but also for any new excipients or impurities in the drug product. The *in vitro* genotoxicity assays should be completed and submitted to regulatory authorities to support first-time exposures in humans. The *in vivo* study should be conducted using the clinical product whenever possible and should be completed to support Phase 2 clinical studies or to support Phase 1 studies when there are positive or equivocal findings observed in the *in vitro* assays.

In general, genotoxicity is unacceptable except for drugs intended to treat life-threatening indications. Follow-up genotoxicity studies should also be performed according to the draft ICH S2(R1) Guidance for Industry: Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use. If the drug substance is determined to be genotoxic, the carcinogenicity potential of the product should be evaluated using either *in vitro* or *in vivo* carcinogenicity models prior to repeat-dose clinical studies. Single-dose pharmacokinetic studies in patients are allowed to evaluate the adequacy of the selected animal species. Interpretation of the genotoxicity risk associated with drug products should be based on a weight-of-evidence assessment and the risk/benefit profile for the particular drug and intended population.

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8 CARCINOGENICITY

Carcinogenicity studies are designed to assess tumorigenic risk associated with use of pharmaceutical products. In general, carcinogenicity studies are required for products that will be administered continuously for six months or longer, or for products that are administered intermittently with a cumulative exposure period of six months over a 10-year period (ICH S1A). For nongenotoxic products, carcinogenicity studies are generally performed during Phase 3 of clinical development, and should be filed with the supporting documents for product registration. All studies should be conducted in compliance with GLP.

Most studies are performed with food available *ad libitum*. Use of dietary restriction has been shown to decrease the number of spontaneous tumours in control animals; therefore, if this type of protocol is selected, the incidence of tumours should be compared only across historical controls from dietary-restricted studies.

For most products needing carcinogenicity assessment, two-year studies in mice and rats of both sexes are required, regardless of the intended population (i.e. male-only or female-only drugs). Six-month studies in transgenic mice may be used as an alternative for the traditional two-year mouse study. The P53 transgenic mouse model is appropriate only for genotoxic compounds, whereas the Tg.AC transgenic mouse model is appropriate only for topically applied products. Regardless of the model selected, particular attention should be made to ensure that all major human drug metabolites are represented in one or the other of the species selected. If metabolism is not qualitatively or quantitatively similar, it may be necessary to either test the metabolite alone, or spike dosing preparations with the metabolite to ensure proper assessment of carcinogenic potential.

Vehicle controls and three dose groups should generally be evaluated. Dose selection for two-year studies is most often based on 13-week toxicology studies using the same strain of animals, and studies in transgenic carcinogenicity models on one-month toxicology studies. The wild-type background strain can be used for dose selection. The high dose should be selected with care to ensure that it will not have an effect on overall survival. ICH S1C outlines five generally acceptable criteria for selection of the high dose: (i) MTD established in 13-week (or longer) toxicology studies, (ii) 25-fold rodent : human AUC ratio, (iii) dose-limiting pharmacodynamic effects, (iv) saturation of absorption or exposure, and (v) MFD. The low dose should be similar to the proposed clinical dose, and the mid dose should fall between the low and high doses, with optimally at least a three-fold difference between doses or exposures. The frequency of dosing is usually once per day, but can be varied depending on the PK parameters of the product. In general, the route of administration should be similar to the intended clinical route. An alternative route of administration may be acceptable if it can be shown to have a similar kinetic profile to the clinically relevant route. For orally administered drugs, mixing the product in the feed is acceptable as long as comparable systemic exposure levels can be achieved. In the USA, results from dose-range finding studies and carcinogenicity study protocols should be submitted prior to initiation of the studies, for concurrence on species and dose selection by the Executive Carcinogenicity Assessment Committee (see FDA Guidance for Industry: Carcinogenicity Study Protocol Submissions).

Exceptions to the requirement of carcinogenicity studies in two species are made on a case-by-case basis, and there are cases where carcinogenicity studies might we waived completely, for example an active metabolite of an already approved product, chronic tolerability issues, traditional medicines or dietary components with a long history of use, and so on. Waiver requests should be submitted to each regional authority where marketing applications will be filed.

9 REPRODUCTIVE AND DEVELOPMENTAL TOXICOLOGY STUDIES

Reproductive and developmental toxicology studies are performed to assess any effects pharmaceutical products may have on: male and female fertility; early embryo/foetal development including implantation; embryonic development through organogenesis; parturition and maternal behaviour, lactation and weaning; and postnatal development (i.e. behaviour, cognition, growth and reproductive competence). Studies can be combined into one study design or into segments: (i) fertility and early embryonic development, (ii) embryo-foetal development, and (iii) prenatal and postnatal development. Dose range-finding studies should be performed in pregnant animals to determine a MTD or MFD, and species selection should be based on applicability to humans and similar metabolic profiles. Whenever possible, the clinical route of exposure should be used; however, if this route does not provide adequate systemic exposure, an alternative route may be used. Definitive studies should be performed in accordance with GLP guidelines.

The timing of reproductive and developmental studies should coincide with the clinical development plan. Even when using reliable forms of birth control, pregnancies do occur during clinical trials. Therefore, the embryo-foetal developmental (teratogenicity) studies are generally needed to support repeat-dose clinical studies in reproductively competent women. Regional differences exist on the timing for fertility studies, but they are generally recommended prior to performing repeat-dose

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studies in reproductively competent men and women. The prenatal and postnatal reproductive and developmental studies are generally conducted during Phase 3 of development and submitted with the marketing application. For investigational products which are designed for use during pregnancy, all stages of reproduction and development, including fertility and postnatal studies, must be completed prior to dosing in pregnant women.

9.1 Fertility and Early Embryonic Development

Fertility and early embryonic development studies are generally assessed in a single species, usually the rat. Males are dosed prior to, and through, mating, while females are dosed prior to mating through implantation, that is, gestation day 6–7 of presumed pregnancy. Doses can be selected based on toxicology studies of at least two to four weeks duration. If treated animals are mated, it should be done on a 1 : 1 ratio; however, the more conservative approach is to mate treated animals with untreated animals to assess male and female parameters independently. Parameters evaluated include mating behaviour, fertility, preimplantation stages of the embryonic development, and implantation. Reversibility of any effects on fertility should be assessed.

9.2 Embryo-Foetal Development

Embryo-foetal developmental studies are assessed in two species: one rodent (generally rat) and one nonrodent (generally rabbit). Pregnant females are exposed from implantation through the period of major organogenesis and closure of the hard palate. Doses should be selected based on dose range-finding studies in pregnant animals using the same criteria used for general toxicology studies. Potential changes in pharmacokinetic parameters between pregnant and nonpregnant animals should also be assessed in the dose range-finding studies rather than in the pivotal studies since maternal stress can significantly affect reproductive outcomes.

Potential product-related effects that should be assessed include enhanced maternal toxicity relative to that in nonpregnant animals, embryo-foetal death, altered growth and teratogenicity.

Exceptions to the requirement for embryo-foetal developmental studies in two species are made on a case-by-case basis. Whenever an alternative study design or a nontraditional species is used, investigators should contact the appropriate regulatory authority for concurrence.

9.3 Prenatal and Postnatal Development

Prenatal and postnatal development studies are generally assessed in a single species, usually the rat. These studies are designed to assess effects on parturition, lactation, maternal behaviour and postnatal development. Postnatal parameters which are evaluated include survival, altered growth and development, including behaviour, learning and memory, and reproductive maturation and function. Pregnant females (F0 generation) are exposed from implantation through the end of lactation. If the active pharmaceutical substance and major metabolites are not excreted in maternal milk, it may be necessary to directly dose the first generation (F1) offspring. Following birth, litters are generally culled to allow for litter-to-litter comparisons to be made on developmental and maternal parameters. Following puberty, one male and one female from each litter are selected for assessment of reproductive function. These animals are mated to either untreated animals or to treated animals in the same dosing group to assess potential effects on fertility and early embryonic development in the next generation (F2).

10 PRODUCT-SPECIFIC CONSIDERATIONS

Certain classes of pharmaceutical products may not be amenable to the standard paradigms for toxicologic assessment, for example, two species, chronic studies including two-year carcinogenicity studies, and so on, or might not require all recommended studies. Examples of products which might fall into this category include recombinant human proteins which may elicit an immune response in all nonhuman species, botanical products which are found in either traditional medicines or in commonly consumed foods, and active metabolites of previously approved pharmaceutical substances. Consultations with the appropriate regulatory authorities are recommended when planning an alternative nonclinical development programme.

Assessment of immunogenic effects is important for all biological products (See ICH S6). The nature and extent to which antiproduct antibodies are formed in nonclinical species is important in data interpretation and study design. Antiproduct antibody can neutralize activity and affect clearance, limiting the duration of dosing and species selection. It is also important to select a species in which the biologic is active in order to assess potential exaggerated and secondary pharmacodynamic effects. If an appropriate animal model cannot be found it may be necessary to use a surrogate analogue in an appropriate species in which the analogue has similar biological function.

For many botanical products, depending on the past history of use, an abbreviated nonclinical programme

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may be recommended. If there is a long history of safe use in traditional medicine or the agent is commonly consumed at similar levels in the diet, then many of the standard studies may be waived. However, it should be noted that if the use of the product consists of purified individual components, or is administered via a route of administration different from its traditional use, then the standard recommendations for toxicology, genotoxicity, carcinogenicity, and reproductive and developmental studies will commonly be required for registration.

For pharmaceutical products that are an active metabolite, a new salt form, or a new formulation of an already approved product, then it may be possible that bridging studies can be used to bridge the safety of the new product with the already approved product. Typically, bridging studies consist of a three-month standard toxicology study. If no significant difference is observed in the toxicology profiles of the two products, no further nonclinical studies are generally required as long as the proposed clinical exposures and patient populations are similar. For sponsors with products seeking a new indication in a different patient population, for example paediatrics, opposite gender, immune deficient, and so on, specific studies may be needed to ensure the safe use in that population.

11 ROLE OF NONCLINICAL TOXICOLOGICAL SUPPORT FOR CLINICAL STUDIES AND MARKETING

Nonclinical studies play an important role in the development of pharmaceutical products. They are used as the bases for setting dosing limits and inclusion/exclusion criteria for first-time exposures in human clinical studies, to provide valuable data regarding clinical monitoring and identify relevant biomarkers, and to assess reversibility of adverse effects. Nonclinical studies, because they are done at considerably higher doses than used in clinical studies, can also be used to assess potential toxicity resulting from a medication overdose. Two areas where it is difficult if not impossible to establish safety during clinical investigation are in potential risks during pregnancy and long-term carcinogenicity potential, making these studies pivotal to evaluating the overall safety of drugs prior to marketing.

12 CLINICAL TOXICOLOGY

Monitoring for adverse effects associated with pharmaceuticals products is a continuous process. It begins in the nonclinical studies and continues in clinical trials and postapproval.

During the investigatory process and postapproval, investigators must submit annual reports detailing all reported adverse effects. The FDA requires that all product-related deaths, and unexpected life threatening adverse events requiring hospitalization, or reports of congenital malformations, must be reported to the agency within 15 days. This also applies to effects observed in animals and includes carcinogenic findings in routine toxicology studies.

In the USA, patients, investigators and physicians are informed of potential risks associated with pharmaceutical products by various means, including:

- investigator's brochures
- patient informed-consent forms
- FDA alerts
- Dear Dr. letters
- MedGuide
- Drug Safety Newsletter.

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FURTHER READING

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This publication is the result of a joint PhRMA (Pharmaceutical Research and Manufacturers of America)/FDA workshop held in November 2000. It proposes to define practical and scientific-based approaches to the use of metabolite data in the safety evaluation of drug candidates in both humans and animals. Metabolites are distinguished by: major vs. minor; pharmacologically active vs. inactive; Phase 1 (oxidative metabolites) vs. Phase II (conjugates); formation via a reactive intermediate; and possession of any structural alerts.

Chapman, K., Pullen, N., Graham, M. and Ragan, I. (2007). Preclinical safety testing of monoclonal antibodies: the significance of species relevance. *Nature Reviews—Drug Discovery*, **6**, 120–126.
This review discusses the issues in the preclinical testing of monoclonal antibodies (mAbs), with a particular focus on species relevance and primate use, and provides suggestions for how these issues might be addressed.

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Davis-Bruno, K. and Atrakchi, A. (2006). A regulatory perspective on issues and approaches in characterizing human metabolites. *Chemical Research in Toxicology*, **19**, 1561–1563.
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Combustion Toxicology and Implications for Adverse Human Health Effects

Bryan Ballantyne

CONTENTS

1 INTRODUCTION AND BACKGROUND

Combustion toxicology is essentially concerned with the nature and potential adverse health effects of products resulting from the heating or burning of materials; effects include local irritation, incapacitation, systemic toxicity and lethality. In the USA there are around 6000 deaths annually due to fires, with a very much larger number of nonfatal injuries (Alexeeff and Packham, 1984; Gad, 1990a). Annual fire- and flame-related deaths in the USA are apparently responsible for more than all natural disasters combined, and account for one of the highest fire-death rates per capita in the industrialized world (Beljan *et al*., 1987). About one-fifth of fire-related deaths occur in the workplace. Thus, the National Traumatic Occupational Fatalities (NTOF) surveillance system of the National Institute for Occupational Safety and Health (NIOSH) recorded 1518 fire- and flame-related occupational fatalities among the civilian workforce in the USA between 1980 and 1994 (Biddle and Hartley, 2000). Fires associated with tobacco use are the leading cause of residential fire deaths in the USA (Hall *et al*., 2006). Casualties that occur during or as a consequence of exposure to a fire do so for various

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reasons, of which the following are the principal causes:

1. **Direct physical trauma:** for example, resulting from structural collapse of buildings.
2. **Flame and heat:** direct thermal injuries to the skin and/or respiratory tract, or as a result of secondary complications from primary thermal injuries.
3. **Oxygen depletion:** this may be a primary factor for adverse effects and/or enhance the toxicity of respirable chemicals in the fire atmosphere (see also Section 3.2.2).
4. **Factors hindering escape:** these may increase the likelihood for further exposure to flame, heat, oxygen depletion and toxic materials in the atmosphere. Escape may be impeded by physical injury, obscuring smoke, prior use of alcoholic drinks or narcotic drugs, panic, the presence of peripheral chemosensory irritants (PCSI) or depression of central nervous system (CNS) function from specific materials in the fire atmosphere. It has been shown that alcoholic intoxication has been a significant impairing factor in some fires, and may have impeded escape. Thus, in a study of fire deaths in Maryland, USA, blood ethanol concentrations $>150\,\mathrm{mg\,ml^{-1}}$ were found in 35% of victims (Radford *et al*., 1976). In an study of 178 deaths in fires in Victoria, Australia, 53 victims had ethanol detected in blood (average concentration 0.07%), of which 42 had blood ethanol $\geq 0.01\%$ (legally intoxicated); the mean carboxyhaemoglobin (COHb) concentration was higher in those who were intoxicated (Yeoh and Braithberg, 2004). Marshall *et al*. (1998), in a series of 190 burn victims, measured ethanol in 130 adults that was found to be $\geq 0.01\%$ in 69 cases (53%). In a series reported from Scotland, Squires and Busuttil (1997) reported that in 1064 adult cases, blood ethanol could be detected in 694 cases; a mean concentration of 0.14% was reported, with a concentration of $\geq 0.01\%$ in 393 cases. The incapacitating effects of several chemicals found in fire atmospheres are appreciated, because of their effects on CNS and/or muscle function. For example, it is well known that hydrogen cyanide (HCN) and carbon monoxide (CO) can rapidly produce incapacitating effects (see Sections 5.2 and 5.3). Behavioural impairment in smoke environments has been reviewed by Purser (1996).
5. **Toxic substances in the fire atmosphere:** these may be in gaseous, vapour or particulate form, being produced by the heating or burning of materials, or released from chemical storage sites as a result of container damage. These materials may produce local (eye, skin, respiratory tract) toxicity and/or cause systemic toxicity if absorbed. Also, as, for example, with fire-fighters, in addition to paying attention to acute effects there should also be consideration of repeated exposures and the potential for cumulative and long-term effects. Immediate and long-term adverse health effects also need to be taken into consideration in developing protection factors for respiratory protective equipment (Burgess and Crutchfield, 1995).

According to the US Consumer Product Safety Committee, between 1970 and 1985 the total numbers of deaths from structural fires decreased from 5000 to 4000 per year, but the number of fatalities attributable to smoke inhalation remained constant at around 3000 per year (USCPSC, 1988). Other estimates have indicated that about 30% of major burns victims have smoke-inhalation injury, and, for fatalities, up to 80% may have had smoke inhalation (Bowes, 1976; Heimbach and Waeckerle, 1988; Haponik, 1993).

The site, structure(s) affected and related geography of fires may be major determinants of the severity and potential complications. For example, fires involving industrial complexes may present physical and toxic hazards because of the possible presence of warehouse stores of flammable and explosive materials, and of large amounts of toxic chemicals and mixtures, hence increasing the likelihood of hazards from potential physical, thermal and toxic injuries. Wildfires, which generate significant quantities of known health-damaging pollutants, including carcinogens (Naeher *et al*., 2007), present a multiplicity of problems, including the following: may affect very large areas; produce considerable heat and smoke; result in destruction of involved properties; cause the evacuation of local populations; dependent on meteorological conditions be difficult to contain and control; be a source of smoke exposure to fire-fighters and to surrounding populations, again dependant on meteorological conditions; may require sustained days of intense work by fire-fighters who are thus at increased physiological and psychological stress and result in considerable economic losses. For example, over the period 21–26 October 2007, wildfires consumed hundreds of thousands of acres and forced the evacuation of more than 300 000 people in San Diego County, California, USA. The health effects resulting from this disaster were monitored using the BioSense system of the US Centers for Disease Control (CDC), which is a national system that enables receipt, analysis and visualization of electronic health care data from emergency departments (EDs) and hospitals (CDC, 2004). For six hospitals in San Diego County, analysis of combined data for respiratory disease indicated that, compared with the previous 20 weekdays, ED visits during the five-day fire period increased from 48.6 to 72.6 $\mathrm{day^{-1}}$ for dyspnoea and from 21.7 to 40.4 $\mathrm{day^{-1}}$ for asthma (CDC, 2008).

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2 GENERATION OF FIRE ATMOSPHERES RELATED TO MORTALITY AND MORBIDITY

Smoke is a complex mixture of airborne solid and liquid particulates, vapours and gases, which are evolved when materials undergo vapourization and thermal decomposition. Thermal decomposition may conveniently be described under the following considerations:

1. **Anaerobic pyrolysis:** thermal breakdown and chemical conversion of materials in a low-oxygen environment.
2. **Oxidative pyrolysis:** thermal breakdown and chemical conversion of materials in a normal oxygen environment, but in the absence of flaming ('shouldering').
3. **Flaming combustion:** thermal breakdown and chemical conversion of materials in a normal oxygen environment in the presence of flaming.

All the above processes may be operating at the same time and at different geographical locations in a fire, and one or another may predominate. *Pyrolysis* is usually defined as the thermal degradation of a material at a temperature below the autoignition temperature. *Flaming* is the highly efficient burning of a material above the autoignition temperature in the presence of sufficient oxygen. *Thermolysis* is a generic term covering flaming, pyrolysis and shouldering.

The atmosphere from a fire is usually of extremely complex chemical composition and, because of the constantly changing conditions during the progress of a fire, the chemical composition (both the nature and the concentration of materials) varies markedly at different stages of the fire. Also, the characteristics and hazards of one fire may be entirely different to that of another. The chemical composition of the fire atmosphere and the concentrations of individual constituents depend on a large number of variable factors, of which the most important are:

1. The nature of the materials available for heating or burning
2. Phase of the combustion process
3. The potential for chemical and/or physical interactions between materials present in the fire atmosphere
4. The potential for additive or synergistic toxic effects
5. Temperature
6. Airflow and oxygen availability.

A review of European and North American literature indicates that some 50–75% of deaths that occur within a few hours of a person being involved in a fire are due to toxic effects of chemicals in the fire atmosphere. After about 12 hours, the contribution of toxic effects to mortality is considerably less. Over the past few decades the contribution of chemical toxicity to mortalities and nonfatal injuries has increased. For example, in the UK, during the period 1955–1974, the total number of fire fatalities increased by 70%; whilst those due to burns and scalds fluctuated around 400–600 annually, those attributed to the effects of smoke and gases showed a steady increase. The increasing hazard from exposure to toxic materials was also been demonstrated by consideration of the total incidence of fatal and nonfatal smoke casualties; there was a 600% increase over the 19-year period (Ballantyne, 1981). In the USA there are approximately 6000 deaths annually related to fires, with smoke inhalation being responsible for about 80% of fatalities (Alexeeff and Packham, 1984; Kaplan, 1988; Gad, 1990a). Also, there is an increased probability of death for fire victims having smoke-inhalation injury and burns (Zawacki *et al*., 1977; 1979; Shirani *et al*., 1988). There are very reasonable grounds to believe that the marked increase in smoke and gas casualties is due to the introduction of man-made materials for construction and furnishing. Thus, combustion processes involving polymers result in the generation of a variety of low-molecular-weight materials that may have significant irritant effects and acute and/or long-term systemic toxicity. The nature and relative proportions of toxic substances generated from the combustion of polymeric materials varies with both the nature of the polymer and the conditions of burning. The possible range of toxicity resulting from exposure to an atmosphere generated by combustion is reflected in the following list of products typically produced from combustion of different types of polymers: acetaldehyde, acrolein, phosgene, HCN, CO, hydrogen chloride and vinyl chloride. Mortalities resulting from the inhalation of chemicals in a fire atmosphere may be due to local chemical injury of the respiratory tract and/or systemic toxicity following absorption of inhaled materials. The latter may include disturbances of biochemical mechanisms or transport processes, or tissue injury. Nonlethal adverse health effects may be due to more restricted or less severe local respiratory-tract injury or systemic toxicity.

It should also be appreciated that inhaled smoke and fumes may contain the products of incomplete combustion, which continue to release heat following inhalation, resulting in thermal injury to the laryngotracheobronchial mucosa and, with sufficient penetration, to the alveolar epithelium. These thermal injuries will complement any chemically induced respiratory-tract injury (Zachria, 1972). Smoke inhalation may cause pulmonary oedema, in which there is increased permeability of the pulmonary microvasculature (Niemann *et al*., 1989). Studies of experimentally produced smoke injury in sheep showed that the primary, and dose-responsive, injury was acute cell-membrane damage in the trachea and bronchi leading to oedema, progressive necrotic tracheobronchitis with

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pseudomembrane formation and obstruction of airways (Hubbard *et al*., 1991). Morphological alterations occurring in the alveolar epithelium included intracellular oedema (Type II cells) and interstitial oedema. Hales *et al*. (1988; 1992) postulated that acrolein might be a major component of smoke causing pulmonary oedema. In work with anaesthetized sheep, and using smoke from burning cotton, they found an acute increase in pulmonary vascular permeability, an increase in airways resistance and decreased P_aO_2. A combined cyclo- and lipo-oxygenase inhibitor, BW-755 C, prevented all these changes, whereas indomethacin, a cyclo-oxygenase inhibitor, prevented the increase in airways resistance, but not the increase in microvascular permeability. They regarded their studies as indicating that leukotrienes may have a role in producing cotton-smoke-induced noncardiogenic pulmonary oedema.

Respiratory-tract injuries in humans resulting from smoke inhalation and causing local thermal and chemical injury may be severe and result in multiple complications, such as tracheobronchitis, pneumonia, pulmonary oedema and low ventilation/perfusion ratio. Blood creatine kinase (CK) activities are raised, possibly due to induced rhabdomyolysis, and may be prognostic of the overall severity of injury (Irrazabal *et al*., 2008). Fibre optic bronchoscopy can be useful for identifying the severity of airway injury and for performing bronchial toilet and bronchoalveolar lavage culture to permit early diagnosis of pulmonary infections. Although the role of corticosteroids in the acute management of smoke-inhalation injury is subject to controversy (Micak *et al*., 1997), others regard them as being effective for the treatment of noninfectious pulmonary complications, such a persistent acute respiratory distress syndrome (ARDS), interstitial pneumonia or bronchiolitis obliterans (Irrazabal *et al*., 2008).

Although most emphasis is usually devoted to the acute toxic effects of fire atmospheres, there is also the potential for long-term adverse health effects, particularly when exposures are prolonged and, especially, when repeated. This is a notable consideration for assessing the hazards of fire-fighting; see Section 7. The general management of casualties from fires has been discussed with reference to toxicology by Harrigan and Winograd (1994), Irrazabal *et al*. (2008) and Latenser and Iteld (2001).

Whilst most discussions on the toxicology of combustion products is of the harmful products liberated during fire situations, adverse effects also result from exposure to products resulting from the heating or burning of materials in defined occupational and domestic conditions. Two typical examples of illnesses resulting from inhalation exposure to the products resulting from heating of polymers are meat wrappers' 'allergy' and polyfume fever. Meat wrappers' allergy affects some workers exposed to fumes whilst wrapping meat in polyvinyl chloride (PVC) film. The origin of fumes comes from several sources, including cutting rolls of PVC with a hot wire (about 105°C), from heat sealing of the film ends, and from thermal fixing of an adhesive label to the wrapped product. The heating element temperature is around 200°C (Levy, 1988). The major products from hot-wire cutting of PVC film include di-2-ethylhexyl phthalate and hydrogen chloride, and those from the thermal attachment of labels include dicyclohexyl phthalate, phthalic anhydride, dicyclohexyl ether and cyclohexylbenzoate (Levy *et al*., 1978; Vandevort and Brooks, 1979). Affected meat wrappers complain of cough, wheezing, shortness of breath and chest tightness, together with symptoms and signs due to irritation of the throat and eyes. The spectrum of the illness should be interpreted as a complex response to emissions from all phases of the wrapping process (Andrasch *et al*., 1975). It is generally considered that exposure to the products resulting from the heating of PVC in the meat-wrapping environment produce effects compatible with irritation of the eyes and respiratory tract. In those with pre-existing asthma or chronic obstructive airways disease there may be an exacerbation of these conditions. The effects produced accord with respiratory-tract irritation and hyper-reactivity; the role of an immune process is debatable (Brooks, 1983). The clinical presentation is similar to that of the reactive airways dysfunction syndrome (RADS) described by Brooks *et al*. (1984). Polyfume fever is the result of exposure to the pyrolysis products of polytetrafluoroethylene (PTFE) after contamination of cigarettes with the polymer in an occupational environment. Workers who smoke PTFE-contaminated cigarettes subsequently develop an 'influenza-like syndrome', characterized by cough, chest tightness, a choking sensation and chills. There is, characteristically, a latent period of several hours between exposure and the development of symptoms; recovery is usually complete within 12–48 hours (Gantz, 1988). Long-term sequelae are unusual. The principal thermal decomposition product of PTFE up to 500°C is the tetrafluoroethylene (TFE) monomer, but also perfluoropropene and other perfluoro compounds containing four or five carbon atoms. In the temperature range 500–800°C, the major pyrolysis product is carbonyl fluoride (Hathaway *et al*., 1991).

It is relevant to note that flame retardants themselves may exert toxicity and adverse human health effects, and also lead to the release of toxic products on their combustion. Polybrominated diphenyl ether (PBDE) and polybrominated biphenyl (PBB) flame retardants are widely used in consumer products, including electronic equipments, fabrics and carpet linings (Alaee *et al*., 2003; Saito *et al*., 2007). Of the 209 PBDE congeners (Darnerud *et al*., 2001), penta-, octa- and deca-BDE have been most used commercially. PBDEs are reported to be thyroid-hormone antagonists and endocrine disrupters (Gillner and Jakobsson, 1996; Hallgren and Darnerud, 2002; Legler *et al*., 2002; Meerts

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et al., 2001; 2002; Rahman *et al*., 2001), and to be associated with neurobehavioural, reproductive and developmental toxicity (Branchi *et al*., 2003; Viberg *et al*., 2004), and possibly immunotoxic effects (Reistad *et al*., 2005). Blood levels and temporal trends of PBDEs have been reported by Schecter *et al*. (2005). The combustion products from phosphorus-based fire retardants, and their toxicological effects has been discussed in detail by Purser (1992).

3 NATURE AND TOXICITY OF FIRE ATMOSPHERES

3.1 General Considerations

The thermal decomposition of a material may produce a wide range of lower-molecular-weight species of differing irritancies and toxicities. In the atmosphere, the number, nature and relative proportions of the individual products from a fire depend on the chemical and physical nature of the material burned and the conditions of the combustion process. This may be illustrated by considering the simple burning of wood in an enclosed space (**Table 1**). Carbon, hydrogen and sulphur are available as the common combustion elements. During the early phase of burning, sulphur dioxide, carbon dioxide, CO and water are formed. As oxygen becomes depleted and burning becomes slower, more CO and sulphur dioxide are formed. With further decrease in oxygen availability, incomplete combustion occurs and hydrogen, methane, CO and free carbon are produced. In the smouldering phase, hydrogen, methane, sulphur dioxide, carbon dioxide, CO, free carbon and smoke are all generated. This simple example indicates that as a fire progresses and the temperature increases, the available oxygen decreases (especially in enclosed spaces), and toxic, irritant and flammable gases are produced, and an obscuring smoke is formed. Additionally, and also with combustion of wood, other irritant and systemically toxic materials may be produced; for example, formaldehyde, methanol, acetic acid and other organic irritants (DeKorver, 1976). Even with the apparently simple example of wood, a multiplicity of differing chemicals may be produced; for example, combustion of Douglas fir produced in excess of 75 discrete chemicals in the smoke (Packham and Hartzell, 1981). Smoke is often defined as a complex mixture of airborne solid and liquid particulates and gases produced when a material undergoes thermal decomposition (Kaplan, 1988). Smoke may be obscuring, contain smouldering particles that can produce thermal injury to the respiratory tract, and contain irritant and toxic substances in gas or vapour form, or absorbed onto the surface of particulates. As noted above in Section 2, the ubiquitous introduction of man-made polymeric materials into domestic, industrial, business and public buildings and furnishings has been associated with a wider spectrum of toxicity than that produced from natural polymers (Alarie, 1985; Ballantyne, 1981; Gad, 1990a). Products released by the combustion of synthetic polymers may have a significant contribution in morbidity and mortality in fires (Ballantyne, 1981).

Examples of the different highly toxic and irritant chemical species produced by the combustion of synthetic polymers are shown in **Table 2**. It can be seen that the nature and number of chemical species depends on the conditions of combustion for the particular specific polymer. For example, PVC yields hydrogen chloride as a principal combustion product, but, additionally, another 75 chemicals may be produced (Dyer and Esch, 1976; Wooley, 1971). Combustion of polyethylene yields 55 compounds, and polypropylene yields 56 compounds (Mitera and Michael, 1985). With polypropylene, the main thermal degradation products are formaldehyde, acetaldehyde, 2-methylacrolein, acetic aid and acetone (Frostling *et al*., 1984). N-containing polymers may yield HCN and various cyanogens.

Inhaled products of combustion consist of solid and liquid particulates, mists, vapours and gases. In general,

Table 1 Products from the burning of wood in an enclosed space, and the influence of the stage of burning

	Burning stage[a]			
Factor	Free	Slowed	Incomplete combustion	Smouldering
Oxygen (%)	20	17	15	<13
Temperature (°C)	40	205	370	550
Products	SO_2+	SO_2+	SO_2++	SO_2++
	H_2O++	H_2O++	H_2O++	H_2O++
	CO+	CO+	CO++	CO++
			H_2+	H_2+
			CH_4+	CH_4+
			Free C+	Free C+++
			Smoke+	Smoke+++

[a]The + designation shows the relative proportion of the product present.

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Table 2 Examples of toxic and irritant chemicals that may be generated by combustion of commonly occurring polymeric materials

Material	Combustion products[a]	Reference
Poly(vinyl chloride)	Hydrogen chloride Carbon monoxide	Dyer and Esch (1976). Michal (1976)
Polyethylene	Formaldehyde Acrolein	Morikawa (1976)
Polyacrylonitrile	Hydrogen cyanide	Morikawa (1978)
Polyurethane foams	Toluene di-isocyanate	Wooley (1974)
	Hydrogen cyanide Carbon monoxide	Bowes (1974)
Polytetrafluoroethylene	Hydrogen fluoride	Young *et al*. (1976)

[a]These are not necessarily the only low-molecular-weight products generated on combustion of the polymer, but are given to illustrate the more toxic materials produced. The relative proportion of the materials generated depends on the conditions of the combustion process.

the local injury from inhaled combustion products is of greater concern than thermal injury from inhaled hot smoke. The upper airways dissipate heat, and most thermal injury is limited to the supraglottic airway (Bizovi and Leikin, 1995). The site and extent of injury is determined by the nature and relative amounts of inhaled irritants and particulates. Head (1980) and Haponik *et al*. (1988) have documented the histopathology of smoke inhalation. This includes pulmonary hyperexpansion, atelectasis and congestion. Features such as focal intra-alveolar haemorrhages and pulmonary oedema may be followed by infective changes (Hill, 1996).

3.2 Factors Affecting the Generation of Combustion Products

The specific chemical species generated during combustion, and their relative proportions, are dependent on various environmental factors (**Table 3**). Additionally, these factors may quantitatively modify toxic response. Particularly relevant factors are as follows.

3.2.1 Oxygen Availability

The availability of oxygen in the in the burning area may significantly affect the generation of combustion products. For example, at a temperature in the range of 500–600°C and with low atmospheric oxygen content, polyethylene combustion gives a high acrolein yield, but with the same temperature range and a high atmospheric oxygen content the acrolein yield is low (Morikawa, 1976). As an example of the influence of atmospheric oxygen availability on toxicity, **Figure 1** shows that for any given atmospheric CO concentration, lethal toxicity increases at that concentration with decreasing oxygen content of the atmosphere. This is due, at least in part, to a proportionately lower oxygen availability to maintain vital processes where oxygen transport is already compromised by CO toxicity. This consideration is clearly highly relevant to practical fire situations, in which CO presence is ubiquitous and oxygen depletion common.

3.2.2 Temperature

The temperature in a zone of burning or smouldering may significantly influence the products released into

Table 3 Examples of the influence of various factors on the generation of thermolysis products from specific materials

Material	Variable	Observation	Reference
Polyurethane foam	Time	In the early stages, both hydrogen cyanide and carbon monoxide are produced; in the latter stages only carbon monoxide	Bowes (1974)
Polyurethane foam	Temperature	Pyrolysis at 300°C yields a polymeric smoke. At 800°C smoke decomposition to N-containing materials, notably hydrogen cyanide, acetonitrile, acrylonitrile, pyridine and benzonitrile.	Wooley (1972)
Polyethylene	Oxygen availability	Low-oxygen atmosphere (500–600°C) gives high acrolein yield high oxygen atmosphere (500–600°C) gives low acrolein yield.	Morikawa (1976)

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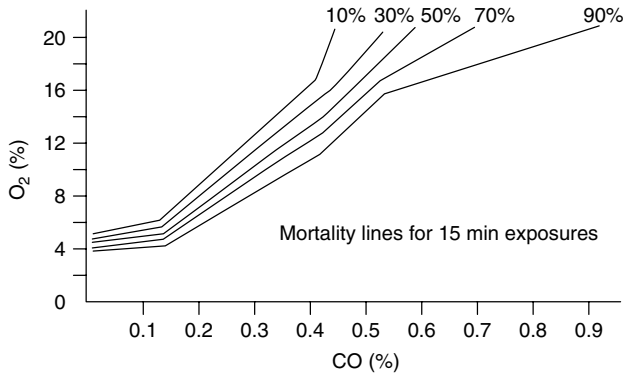

Figure 1 Mortality of male rats exposed for 15 minutes to atmospheres containing various proportions of oxygen and carbon monoxide. For any given carbon-monoxide concentration, mortality increases with decreasing oxygen content of the atmosphere. (Reproduced from Ballantyne, 1981. © Taylor & Francis Group.)

the atmosphere. For example, pyrolysis of polyurethane at 300°C yields a polymeric smoke, but at 800°C the smoke decomposes to N-containing materials such as HCN, acetonitrile, pyridine and benzonitrile (Wooley, 1972). Several investigations have demonstrated that environmental temperature may influence toxicity. For example, Nomiyama *et al*. (1980) showed an increase in acute toxicity for various organic solvents, heavy metals and agrochemicals at elevated environmental temperatures. Sanders and Endecott (1991) showed, in laboratory studies with rats, that incapacitation occurred earlier when exposure to CO was combined with elevated temperature, compared with the effects of CO or temperature alone.

4 INCAPACITATING FACTORS IN FIRES

4.1 General Considerations

In the context of a fire, incapacitating factors are those that hinder escape from the fire as a consequence of an impairment of physical and/or mental functions. Clearly, obstacles, physical injury and dense smoke are physical factors that may impair mobility and thus escape. Hypoxia (discussed in Section 4.2) may impair mental functions as a result of a wide range of effects varying from impairment of judgement to loss of consciousness. Some chemicals that may be encountered in a fire atmosphere may be absorbed from the respiratory tract and affect CNS functions, producing to a wide range of potentially incapacitating effects, including disturbance of consciousness, disturbances of coordination, weakness and decreased reaction-responsiveness times. Examples of materials that may produce such effects include volatile organic solvents, CO (Section 5.2) and HCN (Section 5.3). The above considerations of the effects of hypoxia and absorbed chemicals from the fire atmosphere on behaviour and judgement clearly apply, not only to impairment of escape from the fire environment, but also are relevant to safe and effective performance of those occupationally involved in fire-fighting operations. Impairment of behavioural reactions in smoke environments has been reviewed by Purser (1996). The growing interest and recognition of the relevance of performance deficits has lead to the introduction of neurobehavioural toxicity testing as a component of some combustion toxicology procedures (Rossi *et al*., 1996).

Several materials that are released into a fire atmosphere produce PCSI effects, and are thus capable of producing eye discomfort, excess lacrimation and blepharospasm. Such ocular effects will clearly result in a disturbance of vision and have a distracting influence, and thus cause impairment of vision, interfere with the performance of coordinated tasks and hinder escape from the area of a fire. These aspects of the PCSI response are discussed in more detail in **Peripheral Chemosensory Irritation: Fundamentals, Investigation and Applied Considerations**.

4.2 Hypoxia

4.2.1 General Considerations

Hypoxia is a broad term covering conditions in which there is a physiologically inadequate supply of oxygen to tissues or an impairment of the cellular utilization of oxygen. Hypoxaemia is a more restricted word used to describe decreased carriage of oxygen in arterial blood, as in anaemic and hypoxic hypoxia. Although tissue oxygen supply is decreased in hypoxaemia, significant damage does not occur until arterial oxygen saturation falls to about 50% and P_aO_2 falls to around 30 mmHg (Campbell *et al*., 1984). If of a sufficient degree, hypoxia may result in death. However, lesser degrees of hypoxia are also significant in fires because of the following possibilities:

1. The development, sometimes insidious, of neurological (CNS) abnormalities. These may include impaired coordination, impaired judgement, disturbance of consciousness, ranging from drowsiness to disorientation and coma (Autian, 1976; Ganong, 1977). All these clearly can produce variable degrees of mental and/or physical incapacitation.
2. Hypoxia may increase chemoreceptor activity, leading to an increase in both the rate and depth of breathing. This can result in increased minute volume and enhanced exposure to toxic materials in inspired air.

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3. Hypoxia *per se* may enhance the toxicity of some inhaled materials.

In the context of a fire, all or a combination of the following types of hypoxia may occur.

4.2.2 Hypoxic Hypoxia

This is present when there is a decrease in the arterial blood PO_2 resulting from inadequate availability of oxygen to blood in the pulmonary alveolar capillaries. There is a reduced amount of oxygen in the arterial blood, but no reduction in P_aO_2. This may be a result of depletion of atmospheric oxygen in inspired air, airways obstruction, lung injury sufficient to reduce diffusing capacity, low tidal volume and increased dead space.

4.2.3 Anaemic Hypoxia

This is present when there is a decreased oxygen transporting capacity of the blood, as, for example, with a reduced circulating erythrocytes mass. In a fire, anaemic hypoxia may occur from reduced haemoglobin oxygen-binding sites; this is often the result of the formation of COHb or of methaemoglobin (metHb).

4.2.4 Cytotoxic Hypoxia

This is an intracellular effect where there is an interference with the utilization of oxygen by cells. A common cause of this is the inhibition of cytochrome c oxidase by cyanide (see Section 5.3)

5 MATERIALS COMMONLY PRESENT IN FIRE ATMOSPHERES

5.1 General Considerations

Different fires vary with respect to the nature of materials generated and resultant hazards, mainly as a consequence of the materials available for burning, the conditions of burning, and multiple environmental factors. In these respects, each fire is unique with respect to the spectrum of toxic and irritant materials generated and the potential health problems that may ensue. A multiplicity of differing chemicals may be generated by the processes of combustion, and many are of differing chemical classes. However, a few substances are common to nearly all fire atmospheres, of which CO and HCN are examples.

5.2 Carbon Monoxide

CO is a major and ubiquitous component of fire atmospheres, and often present at potentially lethal concentrations (Jankovic *et al.*, 1991). Barnard (1979) measured concentrations of CO in 25 Los Angeles fires and found that in 12% the peak CO concentration was <100 ppm, in 40% the peak values ranged 100–500 ppm, in 25% the peak range was 501–1000 ppm, and in 23% >1000 ppm; the highest CO concentration measured was 3000 ppm. The presence of toxicologically significant amounts of CO in fire atmospheres also comes from measurements with occupationally exposed fire-fighters; see Section 7.1

A major factor in the systemic toxicity of CO is related to the high affinity of CO for haeme proteins, with haemoglobin (Hb) being most affected. Binding with Hb results in the formation of COHb; this affinity is about 200 times that for O_2 (Weaver *et al.*, 2000). A sufficient inhalation exposure to CO causes an anaemic hypoxia. An additional factor influencing the toxicity of CO is the fact that the presence of COHb causes a shift to the left in the of the O_2–Hb dissociation curve due to a modification of the Hb molecule configuration. As a consequence there is an increased affinity of Hb for O_2, and hence that at any given PO_2 the release of O_2 will be reduced compared with conditions where COHb is not present (Ayres *et al.*, 1973). Also, CO inhibits cytochrome a_3, as a function of plasma CO (Goldbaum, 1977; Goldbaum *et al.*, 1976), but binding only occurs if cytochrome c oxidase is in a reduced form and when significant hypoxia is present. Therefore tissues at greatest risk are those with the steeper intracellular O_2 gradient, including the brain and myocardium. Cytochrome c oxidase inhibition has been shown to persist for several days, and may be responsible for persistent metabolic derangements (Miro *et al.*, 1998). A further toxic mechanism of action is by brain lipid peroxidation (Thom, 1990). By competing with nitric oxide (NO) for ligand biding, CO increases the concentrations of NO in tissues, which undergoes a reaction with superoxide anion to produce peroxynitrite, which injures endothelium. There then follows adhesion of neutrophils to the injured endothelium via B_2 integrin. The proteases and reactive species released from the activated neutrophils subsequently convert endothelial xanthine dehydrogenase to xanthine oxidase (Thom, 1992; Thom and Ischiropoulos, 1999), leading to lipid peroxidation. Damage to the endothelium results in production of cerebral oedema, a common accompaniment to CO poisoning.

Interpretation of COHb values in the context of fire mortality and morbidity, particularly at low concentrations, requires to be undertaken with caution because of the influence of rural and/or urban environmental factors, and of cigarette smoking, on COHb concentrations (Ballantyne, 1981). Also, if dichloromethane is present, this may be endogenously converted to CO

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(Hathaway *et al*., 1991). Additionally, if analyses for COHb are not carried out promptly, from blood stored appropriately in containers, analytical artefacts due to losses may occur (Chance *et al*., 1986; Levin *et al*., 1990). The majority of individuals who are exposed to a fire atmosphere will have elevated concentrations of COHb, the magnitude of which depends on depends on the time of exposure and the atmospheric CO concentration. Low concentrations of COHb may be an indication that death has occurred rapidly from trauma or severe burns, and there has been insufficient time for high COHb concentrations to develop (Levin *et al*., 1990; Mayes, 1991; 1992). Thus, in fires or accidents involving fires, COHb measurements may be of value in assessing the time between physical injury and death. For example, with aircraft accidents, if there is severe impact with subsequent fire then those dying at impact will have relatively low COHb concentrations. With less severe impact situations, or if fires develop before a crash, the COHb concentration may be higher in those who survive for a period. Clearly, in such situations, the value of the COHb concentration needs to be interpreted against other forensic information; for example, inhaled smoke, pulmonary fat and bone-marrow emboli (Blackmore, 1974). However, it should be noted that even though breathing continues after exposure to a fire, tracheal debris might not be present (Rodgt and Olving, 1996). Similar considerations apply to automobile crashes when fire may be present. For example, Wirthwein and Pless (1996) reviewed 28 fatalities involving fires in automobiles, and found that COHb concentrations ranged from <10 to 92%. In 16 cases with COHb $<10\%$ a collision occurred, and in 12 of these blunt trauma was sufficient to have caused death. In seven cases with no collision, six had COHb $\geq 47\%$. From a detailed review of individual cases, the authors concluded that a COHb $>30\%$ strongly suggests that inhalation of combustion products is the main cause of death, but if COHb is $<20\%$, a search for other causes of death should be undertaken. In cases of death due solely to CO poisoning, as, for example, from coal-gas poisoning, the COHb concentration range stated to be compatible with death from acute CO is in the range 50–60%. In fire victims the COHb concentrations may show a wide range of values, some compatible with death due to CO poisoning, and others substantially lower. For example, in 90 fire deaths, Harland and Wooley (1979) found COHb concentrations $>50\%$ in half of the cases; those cases above 50% had a mean value of 67% and those below a mean value of 18%. When interpreting lower concentrations of COHb in fatal cases, it requires to be remembered that hypoxia may enhance the toxicity of CO, and that CO toxicity may be an interactive factor in the presence of other toxic substances and with physical and thermal trauma.

Maeda *et al*. (1996), in a review of cases and an evaluation of post-mortem cases with a CO-oximeter system, noted that with a COHb concentration $\geq 70\%$, acute fire deaths may be explained on the basis of the immediate effects of CO poisoning; in the range of COHb 30–70%, there is CO poisoning combined with other factor(s); below 30% COHb, other effects are the major cause of death. They noted that other oximetric findings, such as oxyHb and reduced Hb might assist in determining the final balance of blood gases in fire victims. Patterns of COHb found in differing fires may reflect the circumstances of an individual specific fire. For example, in the MGM Grand Hotel fire, 51.3% of victims had COHb $>50\%$; in this fire most of the victims were found in areas remote from the conflagration (Birky *et al*., 1985). In contrast, in the DuPont Plaza Hotel fire in Puerto Rica, 80–85% of victims had COHb $<50\%$; in this fire the majority of victims were burned and found in the area of the fire (Levin *et al*., 1990).

Exposure to sublethal concentrations of CO can result in a variety of potentially adverse health effects. Of particular practical importance are neurological and behavioural effects, which can hinder the performance of skilled tasks, or the recognition and escape from critical situations. These effects include headache, dizziness, disturbance of vision, confusion, difficulties in coordination, decreased reaction times, drowsiness, convulsions, impairment of consciousness and coma (Stewart, 1974; Zarem *et al*., 1973). Later neurological sequelae include memory loss and cognitive deficits that appear after a lucid interval of between two and 40 days (Ginsberg, 1985). Additionally, acute exposure to CO may produce cardiac arrhythmias, myocardial damage and circulatory failure (Stewart, 1974). Also, there may be aggravation of exercise-induced angina, decreased exercise tolerance, S-T segment depression in the electrocardiogram (ECG) and increased vulnerability to ventricular fibrillation (Anderson *et al*., 1973; Aronow, 1976; DeBias *et al*., 1976).

The human foetus is particularly sensitive to CO because of several differences from the adult. Thus, under steady-state conditions, foetal COHb is around 10–15% greater than the corresponding maternal blood COHb. Additionally, the partial pressure of O_2 in foetal blood is lower, at 20–30 mmHg, compared with the adult value of 100 mmHg (Longo, 1976; 1977; McDiarmid *et al*., 1991). Furthermore, the foetal oxygen–Hb dissociation curve lies to the left of the adult curve, resulting in a greater degree of hypoxia at equivalent COHb concentrations. It is also considered that the foetal half-life of elimination of CO is longer than in the mother (Margulies, 1986). Acute exposures to CO concentrations that are nonlethal to the mother have been associated with foetal loss (Goldstein, 1965; Muller and Graham, 1955), or to permanent neurological sequelae in the foetus (Cramer, 1982). These factors need to be taken into consideration if pregnant women are exposed to fire atmospheres, and also to the employment of women of child-bearing age in the fire services.

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Since CO is ubiquitous in various types of fires, all accident victims from fires should have blood COHb concentrations determined on hospital admission. Expired alveolar gas CO measurements can be undertaken to ascertain COHb concentrations, but this approach requires patient cooperation in sustained apnoea for a prolonged period (Fife *et al*., 2001). CO-oximetry measures the relative concentrations of oxygenated and deoxygenated Hb, as well as COHb and metHb, and this can be done noninvasively with sensitivity, and can identify occult CO toxicity (Suner *et al*., 2008). A portable noninvasive CO-oximeter that performs rapid COHb measurements using a fingertip probe is available for clinical use. When CO poisoning is suspected or chemically proven there should be immediate administration of 100% O_2. When available at the facility or a nearby facility, hyperbaric oxygen (HBO) therapy may be considered a therapeutic alternative, in which the patient breathes 100% O_2 at a pressure usually between 2 and 3 atm. This causes in an increase the amount of dissolved O_2 in blood plasma, according to Henry's law, which states that the amount of gas that will dissolve in a liquid at a give temperature is directly proportional to the partial pressure of the gas and the solubility factor of the gas in the liquid. HBO therapy enhances the elimination of CO; for example, a $t_{1/2}$ of 23 minutes at 3 atm compared with 60–90 minutes with 100% O_2 at 1 atm, and 240–320 minutes with room air (Reisdorff and Shah, 1993). HBO reduces tissue hypoxia by several mechanisms, which include (Domachevsky *et al*., 2005):

1. Increased pO_2 accelerates CO dissociation from Hb.
2. HBO increases the dissolved fraction of O_2 in plasma, and hence improves delivery of O_2 to injured tissues.
3. Prevention of leukocyte-mediated inflammatory changes and resultant lipid peroxidation by an inhibition of leukocyte adherence mediated by B_2 integrin, (Thom, 1993; Zamboni *et al*., 1993).
4. HBO causes vasoconstriction and reduces cerebral oedema (Nylander *et al*., 1985).

Details of the HBO management of CO intoxication have been discussed by Buckley *et al*. (2005) and Domachevsky *et al*. (2005).

There is still variable discussion on the absolute value of HBO in the management of CO poisoning, and criteria for its use (Buckley *et al*., 2005; Brent, 2005). Although coma is an undisputed indication for HBO, other circumstances suggested for the use of HBO are more debatable, and, as a result, the criteria for the use of HBO in CO poisoning have varied between different authorities (Kao and Nañagsa, 2004; Tibbles and Perrotta, 1994). Careful patient selection for HBO treatment is necessary, and involves consideration for those who have loss of consciousness, pregnancy and possibly cardiovascular (CV) dysfunction or neurological impairment (Brent, 2005). Representative examples of criteria for HBO are given in **Table 4**.

Table 4 Examples of criteria listed for the use of hyperbaric oxygen (HBO) in cases of acute CO poisoning

1. Criteria listed by Harrigan and Winograd (1994)
COHb > 25%
COHb > 15% if cardiac disease present
COHb > 10% in a child
ECG changes present
Metabolic acidosis present
P_aO_2 < 60 mmHg
Pregnancy (COHb > 15%)
2. Criteria by Myers and Thom (1994) and Ernst and Zibrak, (1998)
Coma
Any period of unconsciousness
COHb > 40%
Pregnancy with COHb > 15%
COHb > 20% with history of ischaemic cardiac disease
Current indications of cardiac arrhythmia or ischaemia
Symptoms unresolved after 4–6 h with normobaric O_2
3. Criteria by Undersea and Hyperbaric Oxygen Medical Society (Hyperbaric Oxygen Therapy, 1999)
Transient or prolonged unconsciousness
Neurological signs
Cardiovascular dysfunction
Severe metabolic acidosis
COHb > 25% (in pregnant women COHb > 10%)
4. Criteria by Bentur (2005)
Transient or prolonged unconsciousness
Neurological signs
Cardiovascular dysfunction
Severe metabolic acidosis
COHb > 25% (>10% in pregnant women)
Start treatment within 24 h of exposure
Breathing 100% O_2 at 2.8 absolute technical atmosphere (ATA) for 90 min, two sessions of 45 min each with a 5 min break in between (to achieve maximal inhibition of leukocyte adherence without oxygen toxicity)
Acute exposure (<12 h) is treated with one HBO session
Continue 'trailing treatments' as long as further improvement is noted
Discontinue treatment after two consecutive sessions if no improvement is noted
Chronic exposure (>12 h) is treated with three HBO sessions at 6–12 h intervals
If HBO is required but a contraindication for its use exists, administer normobaric O_2 for 72 h
If HBO is not indicated, administer normobaric O_2 for 24 h

5.3 Hydrogen Cyanide

Any material that contains carbon and nitrogen will liberate HCN under appropriate combustion conditions.

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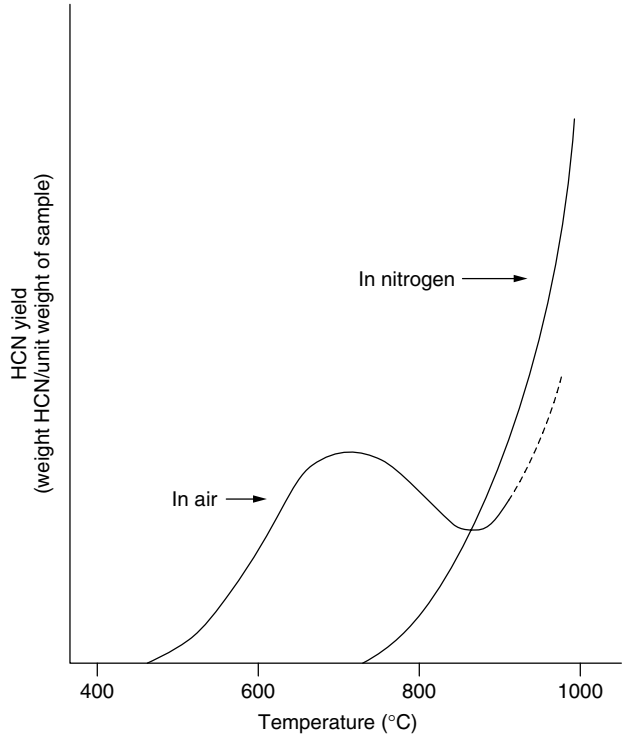

Figure 2 Graphical representation of the yields of HCN from N-containing polymers as a function of thermolysis temperature in oxidative and inert atmospheres. In air there is an initial increase in HCN yield followed by a decrease; a secondary increase in HCN yield may occur at higher temperatures.

In addition, various cyanogenic materials may be thermally generated, such as acrylonitrile, acetonitrile, adiponitrile, benzonitrile and propionitrile (Stark, 1974; Wooley, 1982). Cyanogens have also been detected in the blood of fire victims (Anderson *et al*., 1979). Polymeric materials are a particularly notable source of HCN; for example, nylon (Purser and Woolley, 1983), polyacrylonitrile (PAN) (Bertol *et al*., 1983), polyurethanes (Jellinek and Takada, 1977), urea-formaldehyde (Paabo *et al*., 1979) and melamine (Moss *et al*., 1951). Although some studies have shown that the evolution of HCN from polymers is proportional to their elemental N content (Morikawa, 1978), this is not a consistent finding. For example, Bertol *et al*. (1983) found that proportionately more HCN (1500 ppm) was evolved from PAN (19% elemental N) than from wool (200 ppm; 14.3% elemental N). Also, Urhas and Kullik (1977) found that with pyrolysis temperatures in the range 625–925°C, the yield of HCN was inversely related to the N content of three fibres. Both temperature and O_2 availability influence the yield of HCN from N-containing materials. The general effect of temperature on HCN yield from N-containing polymers in oxidative and inert atmospheres is illustrated in **Figure 2**. In oxidative atmospheres, HCN is evolved at lower temperatures, and as temperature increases so does HCN liberation, up to a maximum, and then decreases with further increase in temperature; a secondary rise in HCN liberation may occur at even higher temperatures. Polyester and polyether flexible urethane foams decompose at relatively low temperatures (200–300°C) in inert atmospheres to produce yellow smoke which is stable up to 800°C; however, over the range 800–1000°C there is decomposition yielding HCN, acetonitrile, benzonitrile and pyridine as the major N-containing products (Wooley, 1972). These and other studies indicate that the evolution of HCN varies with temperature, O_2 availability, the chemical nature of the N-containing material and burning time. Although these variables will differ at any give time, practical estimates for HCN generation have been determined. For example, Morikawa (1978) calculated that if nylon is burned at 950°C under restricted air conditions, then only 1.5 g is necessary to raise the HCN concentration to around 135 ppm in a 1 m^3 space. Bertol *et al*. (1983) calculated that a toxic concentration of HCN could be generated in an average-sized room by the burning of 2 kg of PAN.

Several laboratory-animal studies have shown that when animals are exposed to combustion products, particularly from N-containing polymers, then HCN may be generated in amounts sufficient to produce physical incapacitation or lethality. Thus, Yamamoto (1975) studied the acute inhalation toxicity of combustion products from various fibres and found that in rats exposed for up to 30 minutes, signs of incapacitation developed most rapidly (<10 minutes) following exposure to PAN. Blood removed after the development of signs showed COHb concentrations were ~10% with PAN and wool, and in the range 20–40% for silk; blood cyanide concentrations ranged from 1.5–3.0 $\mu g\,ml^{-1}$ for silk, 1.5–2.0 $\mu g\,ml^{-1}$ for PAN and ~0.5 $\mu g\,ml^{-1}$ for wool. Thus, under these conditions of exposure to the combustion products from silk and PAN, HCN was the major cause of incapacitation and blood cyanide concentrations of potentially lethal significance were measured. Purser *et al*. (1984) exposed primates to the combustion products from PAN, and a comparable group to HCN vapour. The pathophysiological effects were similar for the two groups; hyperventilation, loss of consciousness, bradycardia and cardiac arrhythmias. Following these signs, breathing was slowed and respiratory minute volume was markedly decreased. For both groups there was a relationship between chamber HCN concentration and time to incapacitation. Blood cyanide concentrations in the PAN-combustion group were similar to those in the HCN-alone group. Purser and Grimshaw (1984) exposed primates for 30 minutes to the products of pyrolysis of flexible polyurethane foam generated at 900°C or oxidative thermal decomposition of rigid polyurethane foam at 600°C. Signs included hyperventilation, followed by loss of muscle tone and limb reflexes, and then loss

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of consciousness. Venous blood COHb at the end of the exposure period ranged 17–28%, and whole blood cyanide ranged 1.9–2.3 $\mu g\,ml^{-1}$. They noted that the CNS effects could be extreme because of the cytotoxic hypoxia, circulatory failure and hyperventilation that may produce cerebral arterial constriction due to the induced hypocapnia. Thomas and O'Flaherty (1979) demonstrated that the products of pyrolysis of polyurethane foam inhibited brain and heart cytochrome oxidase activity, which was positively correlated with blood cyanide concentrations. These and other studies involving the inhalation exposure of laboratory animals to the products of combustions from various N-containing polymers have shown that significant inhalation dosage may be received, resulting in physical incapacitation, coma or lethality.

As with animal studies, there is a considerable information base suggesting that humans exposed to combustion products have absorbed cyanide, and indeed smoke inhalation is probably the major cause of acute cyanide poisoning (Eckstein and Maniscalco, 2006). Thus, the first detailed description of cyanide in the blood of fire victims was presented by Wetherell (1966), who found cyanide in the blood of 39 of 53 individuals dying in fires; the average concentration was 0.65 $\mu g\,ml^{-1}$ (range 0.17–2.20 $\mu g\,ml^{-1}$). Other representative studies include the following. Hart *et al.* (1985) described five subjects with smoke-inhalation injury who were comatose on admission to hospital; blood cyanide ranged 0.35–3.9 $\mu g\,ml^{-1}$ (avenge 1.62 $\mu g\,ml^{-1}$). The subject with the highest blood cyanide died four days after admission. In postcrash airplane fires, Mohler (1975) reported blood cyanide in victims having a range 0.01–3.9 $\mu g\,ml^{-1}$. In some cases, increased cyanide concentrations indicated death from acute cyanide poisoning. For example, Tscuhiya (1977) described two persons found dead after a fire involving a polyurethane mattress; the blood cyanide concentrations were 7.2 and 23.0 $\mu g\,ml^{-1}$. In a more extensive study, in which there were 35 deaths of convicts resulting from exposure to smoke from burning polyurethane mattresses in an insurrection in a Buenos Aires prison, Ferrari *et al.* (2001) found the average blood HCN concentration to be 3.5 $mg\,l^{-1}$ (range 2.0–7.2 $mg\,l^{-1}$); these values are compatible with death from acute cyanide poisoning. In a study of fire victims in Victoria, Australia, blood cyanide concentration measurements were conducted in 138 cases, cyanide was not detected in 52 cases, and the remaining 86 had a mean blood cyanide concentration of 1.65 $mg\,l^{-1}$, with 11 cases having a concentration $>3.0\,mg\,l^{-1}$.

Exposure to HCN released into a fire atmosphere can lead to muscle weakness, difficulty in coordination, physical incapacitation, a confusional state and partial or complete loss of consciousness. This clearly will impede escape from the area of a fire. The high concentration of cyanide measured in some fire victims has lead to suggestions for the routine use of cyanide antidotes in cases of severe smoke inhalation, either prehospital, at the scene of the fire, or on hospital admission. Clearly, for prehospital use, the chosen antidote should not cause adverse side effects and have a high benefit: risk ratio. Early suggestions included the use of stroma-free Hb solutions, but more recently evidence points to the use of hydroxocobalamin as a safe and effective antidote. Few adverse effects from hydroxocobalamin *per se* have been record, although a limited transient increase in mean arterial blood pressure has been described. For example, Uhl *et al.* (2006), in a randomized, placebo-controlled, single-dose (2.5–10.0 g) trial in healthy volunteers subjects ($n = 136$), found that mean blood-pressure changes from baseline ranged 22.6–27.0 mmHg for systolic blood pressure (SBP) and 14.3–25.4 mmHg for diastolic blood pressure (DBP), compared with placebo-group control values of 0.2–6.7 mmHg SBP and 3.0–3.8 mmHg DBP. In a pharmacokinetic study, Uhl *et al.* (2008) demonstrated that the transient increase in mean arterial pressure is closely linked to initial exposure to total and free cobalamins (III). It has been pointed out that acute cyanide poisoning is often associated with hypotension, and the hydroxocobalamin associated increase in blood pressure may be of value in improving haemodynamic stability (Baud and Borron, 2006; Fortin *et al.*, 2006). The effect of hydroxocobalamin on blood pressure is suggested, but not proven, to be due to its action as an NO scavenger, which is a free radical and an endogenous vasodilator (Gerth *et al.*, 2006). Based on eight years of experience of the Paris Fire Brigade with the prehospital use of hydroxocobalamin, Fortin *et al.* (2006) concluded that hydroxocobalamin had a risk:benefit ratio which indicated it suitable for prehospital use in the management of acute cyanide poisoning caused by smoke inhalation. It should be noted that the antidotal use of hydroxocobalamin may interfere with co-oximetry measurements (Denninghoff *et al.*, 2008; Lee *et al.*, 2007).

HCN as a product of combustion and its significance has been reviewed by Ballantyne (1987).

5.4 Interactions Between Fire Atmosphere Components

5.4.1 General Comments

The atmosphere of a fire is a continuously varying complex mixture of a multitude of materials having differing chemical structures and differing toxicities. The potential for short- and long-term adverse health effects is known for several of the individual components, but is incomplete to variable extents for many other fire-atmosphere components. Also, the likelihood for

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chemical and toxicological interactions in the changing conditions of a fire is, in general, poorly understood. This includes the formation of new compounds having differing toxicity by chemical interactive factors, and the possibility for one maternal having an influence on the quantitative toxicological expression of toxicity by another, or the potential for additional interactive toxicity between individual chemical components in an atmosphere. The potential for toxic interactive factors between a few fire-atmosphere components, particularly with binary systems, has been investigated for some materials, and estimates made of the variability in hazard according to the relative proportions of the components. Some illustrative examples are given below for three common fire-atmosphere materials, HCN, CO and carbon dioxide.

5.4.2 Binary Atmosphere of Hydrogen Cyanide and Carbon Monoxide

The investigational approaches to determining the toxic modifying interactivity between HCN and CO, a common practical combination in fires, have been variable, and include mortality studies, measurements of blood cyanide and COHb and assessment of physiological functions. Moss *et al*. (1951) found that simultaneous exposure to CO (2000 ppm) and HCN (10–20 ppm), both at individually sublethal concentrations, caused death. Smith *et al*. (1976) found that the times to death for rats exposed to an atmosphere containing 450 ppm HCN and 13 500 ppm CO (3.7 ± 0.4 SD minutes) was slightly longer than for a corresponding concentration of HCN alone (10.9 ± 2.0 SD minutes) or CO alone (5.8 ± 1.2 SD minutes). Norris *et al*. (1986) investigated the effect of a three minute inhalation exposure of mice to CO (0.3–0.66%) on the lethal toxicity of intraperitoneal (ip) potassium cyanide (KCN). A significantly lower LD_{50} for KCN was found in CO-pretreated mice (6.51 (6.04–7.00) mg kg^{-1}) than for air-alone controls (7.90 (7.36–8.45) mg kg^{-1}). In further studies they found evidence for a synergism between CO and HCN, and suggested that this may have been the result of augmentation of the inhibition of cytochrome oxidases in the CNS.

Pitt *et al*. (1979) investigated the effects of CO and HCN on cerebral circulation in the dog. When given together, CO and HCN increased cerebral blood flow in an additive manner; however, a significant decrease in cerebral oxygen consumption occurred with combined exposure to HCN and CO, neither of which alone had an effect. Ballantyne (1984; 1987) investigated the effects of differing proportions of HCN and CO in the atmosphere on lethal toxicity, and blood cyanide and COHb concentrations, and determined that the contribution of either substance to toxicity depends on their absolute and relative atmospheric concentration. Thus, when there was a marked excess of CO, the presence of HCN lowered the lethal inhalation dosage for CO by a less than additive toxicity; that is, HCN physiologically potentiated (by hyperventilation) the toxicity of CO. When there was an excess of CO with respect to HCN, but not sufficient to produce a clear biochemical evidence of death due to CO, then the blood picture indicated death not primarily due to CO or HCN. In these circumstances, because of less than additive toxicity, it is likely that both are acting at a common target site, probably cytochrome oxidase. When CO and HCN were present in equal mass proportions, biochemical evidence indicated that death was due to acute cyanide poisoning.

5.4.3 Binary Atmosphere of Carbon Monoxide and Carbon Dioxide

Nelson *et al*. (1978) found that the 30 minute lethal concentration of CO to rats was 6000 ppm, and was decreased to 2560 ppm in the presence of 1.44% CO_2. Redkey and Collison (1979) found more rapid times to death in rats exposed to 6000 ppm CO with 4.5% CO_2 (16.8 ± 0.6 minutes) compared to 6000 ppm alone. Levin *et al*. (1989), in detailed studies, found that above a certain concentration of CO (4100 ppm) some rats died, and adding CO_2 had no influence. Below 2500 ppm CO, the addition of CO_2 (up to 17.7%) was not sufficient to produce mortality. However, with a CO concentration in the range 2500–4100 ppm CO_2 (>1.5%) (which produced few mortalities *per se*) produced a high level of mortality. They noted that CO and CO_2 act together by: (i) increasing the rate of COHb formation, (ii) producing a severe acidosis which is greater than the metabolic acidosis from CO alone or respiratory acidosis, from CO_2 alone and (iii) prolonging the recovery period from acidosis.

6 INVESTIGATION OF THE TOXICOLOGICAL HAZARDS OF FIRES

6.1 General Considerations

Investigations into the toxicology of fire atmospheres and the assessment of potential adverse health effects from the products of combustion are complex and difficult because of the multiplicity of thermolysis products and the variability of factors which affect the quantitative and qualitative of the biological response to them. Therefore, from a practical point of view it is possible only to give an overview of the combustion products likely to be present under the conditions of thermolysis, and a qualitative assessment of hazards. Although detailed studies have been conducted on some binary systems, permitting quantitative assessments of interactions to be undertaken, the majority of studies have been conducted

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on combustion products. A overview of the various approaches to investigating toxicological hazards from fire is presented below. Details can be found in Gad (1990b) and Kaplan (1988).

6.2 Physiochemical Studies on Thermolysis Products

Such laboratory studies are concerned with the analytical detection, and determining the nature and relative proportions, of substances produced on thermolysis of materials under differing conditions of generation. Ideally, the analyses should be conducted under the following generation conditions; simple heating, complete combustion, oxidative pyrolysis and anaerobic pyrolysis. It is thus necessary to subject materials to a range of temperatures in atmospheres with differing oxygen contents, with the resultant effluent being analysed by appropriate instrumental procedures. In some instances highly toxic materials may be generated over a narrow temperature range, and if a differential temperature study is not carried out these materials may be missed. In addition to the influence of temperature and oxygen availability on the materials generated from combustion, it is important to study the nature of the materials generated as a function of time in the combustion phase since the pattern may change appreciably. **Table 3** gives examples of the influence of environmental variables on combustion products generated.

From a knowledge of the nature of the materials generated under different conditions of combustion, it may be possible to predict a hazard pattern for a given material, providing that there is an adequate volume of background information on the toxicology of the materials. In some cases, a major hazardous material may be identified from a large number of analytically detected substances produced by combustion of a specific material. For example, with PVC, about 75 organic products have been detected on thermal decomposition, most being aliphatic or aromatic hydrocarbons (Wooley, 1971). However, a major product that begins to be liberated at 200–300°C is hydrogen chloride, and it has been estimated that 1 kg PVC may yield about 400 g HCl on complete combustion. Hydrogen chloride causes PCSI effects of the eye and respiratory tract, and in sufficient concentrations may produce inflammatory lesions in the respiratory tract. PVC combustion is recognized as a major hazard in fires in buildings with extensive polymers in the construction and furnishings (Dyer and Esch, 1976).

Small-scale laboratory tests yield useful, though often preliminary, information on the nature of combustion products generated from specific materials under defined conditions, and allow a qualitative assessment of the hazards that may be encountered for a specific material in a fire. However, the majority of fires involve the burning of a multiplicity of materials, including structural and furnishing components. In an attempt to obtain more reliable information it may be desirable to undertake large-scale experimental fires with appropriate instrumentation. Such tests are likely to be expensive and require careful planning, specifically with respect to sampling and analysis of the atmosphere generated. Guidance on the planning of such large-scale tests will clearly be obtained from preliminary small-scale laboratory combustion-product studies.

6.3 Laboratory Exposure Studies

In a strictly physicochemical analytical approach to defining the nature and relative proportions of combustion products generated from specific materials, the likely hazards from the effluent smoke are determined by attempting to predict the probable combined toxicity of the constituents in the smoke from knowledge of their individual toxicities. Such an approach may result in misleading predictions since there is the possibility for chemical and toxicological interactions, including synergy. Attempts have therefore been made to determine the toxicity of smokes from specific materials by exposing animals to the products of thermolysis and monitoring for adverse effects by standard and special procedures. Such tests readily lend themselves to observations on irritancy and acute toxicity. The former will give an index of potentially harassing and incapacitating effects of effluent smoke from the material, and the latter can give an indication of tissue-injuring or potentially lethal adverse effects, such as lung damage, or of functional effects, such as neurobehavioural abnormalities. Such tests are frequently carried out with the smoke being generated under differing conditions of atmospheric oxygen content and thermolysis temperature. For comparative purposes, the findings from fire combustion toxicology studies are frequently referred to tests from the burning of a standard material, usually wood. Although such tests give useful information in themselves, they are particularly valuable when viewed in the light of studies on the analysis of combustion products generated under similar conditions. Thus, when interpreting laboratory data in an attempt to define possible hazards from combustion products of materials, it is highly desirable to have information on both the nature and relative proportions of combustion products, and on their effects in experimental animals exposed to combustion products generated under similar conditions. Where appropriate expertise and facilities exist, it is possible to combine analytical studies with animal-exposure tests. Also, animal exposures have been performed in large-scale fire tests.

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Problems may be encountered in defining the presence of novel or highly toxic materials for several reasons: first, because of the multiplicity of materials generated, there may be limitations on the analytical capability; second, knowledge of the toxicology of some materials generated is sparse. It therefore follows that animal studies may draw attention to the presence of highly toxic, or unsuspected, materials in a test atmosphere. This is illustrated by investigations on a fire-retarded polyurethane foam. The products from nonflaming combustion of a trimethylolpropane-based rigid urethane foam fire-retarded with an organic phosphate, *O,O*-diethyl-*N,N*-bis(2-hydroxyethyl)aminomethyl phosphonate were found to produce grand mal seizures in rats; such effects were not seen when the foam was not fire-retarded (Petajan *et al*., 1974). Subsequent chemical analyses revealed the presence of 4-ethyl-1-phospha-2,6,7-trioxabicyclo[2.2.2]octane-1-oxide in the smoke (Voorhees *et al*., 1975). This is a material of high acute toxicity (Kimmerle, 1976). Further studies demonstrated no unusual toxicity when the flame-retarded polyurethane foam was either gradually or rapidly pyrolysed at 800°C in the absence of air; however, convulsions were observed when the material was flash pyrolysed in the presence of a flow of air (Hilando and Schneider, 1977). See Purser (1992) for a discussion of caged bicyclophosphorus esters in combustion processes.

For both chemical analysis and animal-exposure studies, special equipment is usually required to permit the generation of atmospheres for the combustion of materials under differing environmental conditions. Several types of apparatus have been designed and used for combustion toxicology atmosphere generation, and have been discussed by Norris and Ballantyne (1999). The selection of such equipment will depend on the combustion conditions that are to be simulated; these may include smouldering, flaming, preflashover and postflashover.

Most recent studies on smoke-inhalation injury have called on advances in molecular toxicology. For example, Lee *et al*. (2005) developed a smoke-inhalation model and incorporated a genome-wide analysis of gene expression. Microanalysis revealed a modified brain transcriptome with changes peaking at 24 hours and subsiding seven days post smoke inhalation. Overall, smoke inhalation down-regulated genes associated with synaptic function, neurotransmission and neurotrophic support, and up-regulated genes associated with stress response, including NO synthesis, antioxidant defences, proteolysis, inflammatory response and glial activation. Thus, such investigations afford insight into the complex molecular pathophysiology of smoke inhalation in the brain. *In vitro* methods have been described for assessing toxicity of combustion products. For example, Lestari *et al*. (2006) described studies comparing three *in vitro* exposure systems coupled with cytotoxicity assays for neutral red uptake, ATP and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 H-tetrazolium (MTS).

6.4 Studies on Exposed Human Populations

Valuable and specific information may be obtained concerning the adverse effects of exposure to fire atmospheres and on the possible long-term hazards of recurrent exposures to fires, by appropriate investigations on fire-fighters and on the victims of fires. Major sources of information have been derived from post-mortem examination of fire victims; clinical, radiological and clinical-chemistry examination of nonlethal fire victims; and medical and epidemiological studies on fire-fighters. In the context of defining the requirements for respiratory and other protective equipment for fire-fighters based on the medical and epidemiological data, short-term repeated and chronic exposure situations, as well as acute exposure all require to be taken into consideration.

7 CHEMICAL HAZARDS TO FIRE-FIGHTERS

7.1 Background and General Considerations

Fire-fighters have one of the most dangerous and hazardous of occupations on essentially all occasions that they are summoned to situations, and have an associated high level of morbidity and mortality from various differing and sometimes combined causes. Overall, the most significant causes for adverse health effects are as follows:

- **Trauma:** notably from falling objects, during rescue situations, and during close-in fire-fighting operations; such trauma may result in significant physical injury and, on occasion, mortality. Also relevant is the fact that associated with the occupational noise exposure, several studies have shown that this may cause hearing loss (Kales *et al*., 1997; 2001; Tubbs, 1995).
- **Thermal:** as primary burns to the skin and respiratory tract, and heat stress. The latter is a function of environmental temperature, insulting properties of protective clothing and endogenous heat production from severe physical exertion, compounded by the additional weight of equipment such as self-contained breathing apparatus (SCBA).

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- **Ergonomic:** the high-energy costs of fire-fighting may clearly be inter-related with, and compounded by, other health concerns.
- **Psychological:** there are multiple potential causes for the development of mental trauma in fire-fighters and these include thoughts of personal security and safety, victim rescue and loss, emotional scenes and heavy social responsibility (Guidotti and Clough, 1992). All these precipitating factors occur against a background of sustained heavy workloads, physical and mental exhaustion, heat stress and the constant need for vigilance. It is therefore to be anticipated that under these very difficult conditions that postdisaster psychological reactions may develop, including post-traumatic stress disorders (Psarros *et al*., 2008).
- **Toxic chemicals:** there is sequential exposures to smoke and contained chemicals, often at high concentrations, many of which are known or suspected to produce acute and/or long-term health problems. This aspect of health concern about fire-fighting is discussed below, and by Guidotti and Clough (1992).

Fire-fighters are recurrently exposed to a multiplicity of materials that may cause acute, cumulative and/or chronic health problems; typical examples of such chemicals are CO, HCN, sulphur dioxide, hydrogen chloride, phosgene, isocyanates, oxides of nitrogen, acrolein, acetaldehyde, asbestos, polycyclic aromatic hydrocarbons (PAHs), benzene, methanol, methane, methylene chloride and toluene (Aronson *et al*., 1994; Guidotti and Clough, 1992; McDiarmid *et al*., 1991). These illustrative examples emphasize the potential for short-term, long-term and interactive toxicity from multiple exposures. That such materials may be absorbed and cause systemic toxicity has been demonstrated by several studies. For example, that fire-fighters absorb cyanide is indicated by an increased serum thiocyanate (Levine and Radford, 1978). Several studies have demonstrated that fire-fighters have an increased COHb concentrations, Thus, Sammons and Coleman (1974) found that that there was a significant difference in COHb concentrations between nonsmoking firemen (mean 5.0%; range 2.5–13.9%) and nonsmoking controls (mean 2.3%; range 1.0–11.7. Similarly, Radford and Levine (1976) found increased COHb concentrations in firemen after fighting a fire (4.53%) compared with unexposed controls (2.17%). Increased COHb concentrations were also found after fire-fighting by Loke *et al*. (1970) and Levy *et al*. (1976) (**Figure 3**). During actual fire-fighting exposures the exposure concentration and dosages received are usually unknown, and will vary between different fires and within the same fire from time-to-time, according to combustion conditions. Thus, when measurements are made, this can be helpful to assess potential adverse effects and hazards. For example, an estimate was made during a fire-fighting exercise of exposure to PAHs (Moen and Overebo, 1997).

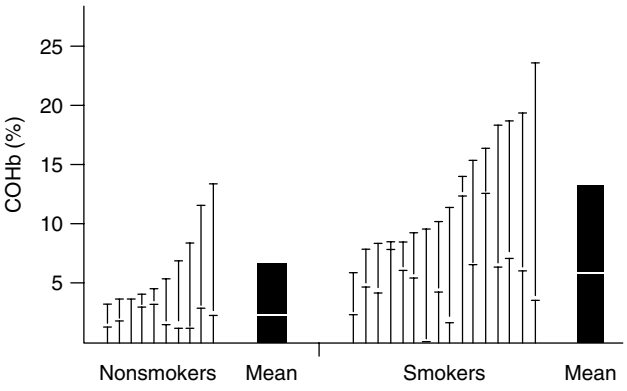

Figure 3 Increases in carboxyhaemoglobin (COHb) concentrations in fire-fighters following exposure to smoke. The lower bars represent COHb pre-exposure and the upper bars the increase in COHb postexposure. Drawn from data in Levy *et al*. (1976) and Loke *et al*. (1970).

The participants gave urine samples 6–7 hours after extinguishing burning diesel oil, and the samples were analysed for 1-hydroxypyrene by high performance liquid chromatography (HPLC). A small, but statistically significant, increase in urinary 1-hydroxypyrene was measured after the fire-fighting exercise. Because of the conditions of the exercise and the personal protective equipment used, the estimate may have represented an underestimate of actual exposure in real situations.

For a variety of reasons, fire-fighting carries a high mortality and morbidity rate. Several epidemiological studies have been undertaken with respect to general and specific causes of morbidity and mortality in fire-fighters; some of the major studies are as follows. In a study of Alberta fire-fighters, Guidotti (1993) did not find an association with cardiac or chronic pulmonary diseases, but did find a possible association with occupational exposure for renal and ureteric cancer, and limited evidence for excess urinary bladder, colon and rectal cancers. In a retrospective cohort study (1950–1989) with 5995 subjects from six fire departments in the Toronto metropolitan area (Aronson *et al*., 1994), it was found that three specific causes of death had statistically significant excesses: brain tumours (standardized mortality rate (SMR) 201; 95% confidence interval (CI) 110–337); 'other major neoplasms (SMR 238; 95% CI 145–367) and aortic aneurysm (SMR 226; 95% CI 136–354). Tornling *et al*. (1994) studied the incidences of mortality and cancer in Stockholm fire-fighters with at least one year of employment during the period 1931–1983. They found overall mortality (1951–1986, based on tracing of individuals) was lower than expected (SMR 82; 95% CI 72–91) compared with local figures. Although the overall cancer incidence was equal to that expected (SMR 100), an excess of gastric cancer was noted (18 observed vs. 9.37 expected; SMR 192). The results from various

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studies are somewhat inconsistent, as would be anticipated from the different working conditions. However, in a review analysis of major publications up to 1995, Guidotti (1995) came to the following conclusions:

- **Lung cancer:** evidence for an association, but insufficient for a general assumption of a risk.
- **Cardiovascular disease:** no evidence for increased risk overall for heart disease.
- **Aortic aneurysm:** evidence incomplete.
- **Genitourinary cancer:** evidence strong for an association and presumption of risk.
- **Lymphohaematopoietic cancers:** some evidence and a general presumption of risk, but requires a case-by-case review.
- **Rectocolonic cancer:** sufficient evidence to conclude an association, but not sufficiently strong to determine if there is a good presumption of risk.

7.2 Respiratory Disease

Smoke exposures by unprotected fire-fighters and local community members have been associated with increased ED visits and hospitalizations for chronic obstructive pulmonary disease (COPD), bronchitis, asthma and chest pain (CDC, 2008; Sutherland *et al*., 2005). Several studies have shown that exposure to a fire atmosphere produces acute changes in pulmonary function, and may be a factor in the development of chronic lung dysfunction. For example, in a study of fire-fighters from Basel, Switzerland, it was found that frequent respiratory symptoms included rhinorrhoea, cough, dyspnoea, with an odds ratio for hyper-reactivity to methacholine of 2.24 (95% CI 1.12–4.48) compared with the a general population control group (Miedinger *et al*., 2007). Musk *et al*. (1979) studied acute changes in fire-fighters during routine duties and found an average decrease in forced expiratory volume over one second (FEV_1) of 0.005 l, which was related to subjectively assessed smoke exposure; decreases in FEV_1 of 0.1 l or more were found in 30% of subjects. Brandt-Rauf *et al*. (1989) found that for fire-fighters not wearing respiratory protective equipment, there were statistically significant postfire decrements in FEV_1 and forced vital capacity (FVC). Pre- and postfire average FEV_1 values were 5.03 and 4.81 l respectively. Gu *et al*. (1996) studied acute respiratory effects in fire-fighters tackling a sustained store fire, and in whom there were irritant effects in the eyes and upper respiratory tract. Spirometry conducted two days after the fire showed that FEV_1 and FEV_1/FVC in smokers and FEV_1 in nonsmokers were significantly less than in controls; there were no significant differences in average fire-fighting times between smokers and nonsmokers. Serra *et al*. (1996) studied pulmonary function in 92 firemen and compared the findings with a group of policemen (controls). They measured FVC, FEV_1, forced expiratory flow (FEF) at 75, 50 and 25% of the FVC, total lung capacity (TLC), residual volume (RV), functional residual capacity (FRC) and CO diffusion for alveolar capillary integrity. They demonstrated the firemen to have significantly reduced FEV_1 (3.90 ± 0.50 SD vs. controls of 4.04 ± 0.44; $p < 0.05$), FEV_1/FVC (80.07 ± 5.89 vs. 83.89 ± 6.32; $p < 0.001$), FEF_{25} (1.58 ± 0.47 vs. 1.99 ± 0.69; $p < 0.001$) and RV (1.57 ± 0.28 vs. 1.76 ± 0.45; $p < 0.01$). Alveolar capillary integrity (as evidenced by CO diffusion) was not significantly different from controls. Adverse respiratory effects may also occur during the process of overhaul, which is the later stage in which fire-fighters search for and extinguish possible sources of reignition, and during which phase respiratory protective equipment may not be worn. Burgess *et al*. (2001) monitored groups of fire-fighters who either wore cartridge respirators or did not wear respiratory protective equipment during overhaul operations. Testing at baseline (pre-exposure) and at one hour after overhaul included FVC, FEV_1, serum Clara cell protein (CC16) and serum surfactant-associated protein A (SP-A). Overhaul increased CC16 in both groups, indicating increased alveolar capillary permeability. Also, SP-A increased, and FVC and FEV_1 decreased in fire-fighters wearing cartridge respirators, indicating they may not give adequate protection during overhaul. The authors concluded that SCBA should be worn during overhaul procedures to prevent the potential for lung injury.

For cumulative effects, Peabody (1977) reported a decrease in pulmonary function for San Diego fire-fighters, which was statistically significantly greater than that of the general population, and Kales *et al*. (1997) recorded a significantly decreased percentage predicted FEV_1 in hazardous materials fire-fighters, particularly in those older than 35 years of age. Over a two year period, Peters *et al*. (1974) found that in fire-fighters, the rate of decline in pulmonary function, measured by FVC and FEV_1, was twice the expected rate; these changes were significantly related to the frequency of exposure. The rate of decline in pulmonary function in fire-fighters, as assessed by FEV_1, shows variability between individuals, but in some the accelerated decline ($\geq 60\,ml\,yr^{-1}$) that has been measured (Burgess *et al*., 2004) is in excess of the average annual rate of decline in FEV_1 of around 20–30 ml in the general population (Burrows *et al*., 1986).

Wildland fire-fighters were studied preseason, postfire and postseason with respect to upper and lower respiratory effects by Gaughan *et al*. (2008). The investigation included spirometry and measurement of albumin, eosinophilic cationic protein (ECP) and myeloperoxidase (MPO) as indicators of inflammation in sputum and nasal lavage fluid (NLF). Respiratory symptom

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scores were higher postfire compared to preseason, and mean FEV_1 was lower postfire (4.35 l) compared to preseason (4.57 l; $p < 0.001$), and decreased postfire to postseason (4.54 l; $p < 0.001$). The mean postseason FEV_1 was not statistically significantly different from the preseason mean value ($p = 0.60$). There were no significant differences in FVC values preseason, postfire and postseason. Multifactor analysis of the inflammatory markers showed that, for individuals, increases in sputum and NLF, ECP and MPO from preseason to postfire were all significantly associated with postfire respiratory-symptom scores. Josyula *et al.* (2007) studied the association between cytokine genotypes and sputum concentrations on the longitudinal decline in pulmonary function in 67 fire-fighters, having a mean of 16.6 years of service (range 3–32 years). They analysed deoxyribonucleic acid (DNA) for functional polymorphisms of IL (interleukin)-1β, interleukin 1-receptor agonist (IL-1RA), IL-8, IL-10, tumour necrosis factor α (TNF-α) genes and sputum evaluated for cytokine concentration by enzyme-linked immunosorbent assay (ELISA). They determined the annual rate of FEV_1 decline was greater for fire-fighters with TT genotypes at IL-10 and with CT or TT genotypes at IL-1RA. These genotypes were not associated with concentrations of sputum cytokine, but increased IL-1RA was associated with a slower rate of FEV_1 decline, as was increased sputum macrophage count. The authors concluded that cytokine genotypes were associated with the rate of decline of FEV_1 but did not alter concentrations of sputum cytokine. They suggested that increased sputum IL-1RA may be protective.

Following the New York World Trade Center disaster on 11 September 2001, involving the deliberate crashing of civil airplanes into the Twin Towers by terrorists, concerns emerged and were stressed about the potential for acute and long-term health hazards related to exposures to particulate building material such as pulverized cement, glass, fibreglass, asbestos, silica, heavy metals and soot (Edelman *et al.*, 2003). The need for long-term medical follow-up, particularly in respect of the development and progression of respiratory diseases, was emphasized by the discovery that the US Environmental Protection Agency (EPA) misled New York citizens about the possible dangers from debris, dust and smoke in the air around the World Trade Center; this deceit was unveiled as a consequence of the US Democrats asking for hearings in respect of the allegations (Barrett, 2003). In August 2003, the internal watchdog of the EPA found that the agency, at the urging (instruction?) of Bush White House officials gave misleading assurances that there was no health risk from the dust (Ballantyne, 2005). According to the report of the inspector general, the EPA were urged to add reassuring statements and remove cautionary statements by having the National Security Council control EPA communications. This enforced misinformation was given against then existing credible published descriptions of harmful and persistent adverse effects, particularly on the respiratory tract; these included cough, bronchial hyperactivity, increased severity of asthma, bronchiolitis and spirometric changes (CDC, 2001a; 2001b; 2002; Lioy *et al.*, 2002; Prezant *et al.*, 2002; Rom *et al.*, 2002; Scanlon, 2002; Stephensen, 2002). Reports of long-term respiratory adverse health effects have continued to appear (Chen and Thurston, 2003; Gavett, 2006; Offenberg *et al.*, 2003; Payne *et al.*, 2004; Salzman *et al.*, 2004; Johnson *et al.*, 2005).

A raised risk for emphysema was found in a mortality study conducted by Demers *et al.* (1992). The importance of using respiratory protective equipment by fire-fighters was shown in a study by Tepper *et al.* (1991), who re-evaluated 632 Baltimore city firemen 6–10 years after baseline measurements, and found that those who never wore a mask had a 17.5 times greater decline than in mask wearers. NIOSH (1987) recommended a protective factor of 10 000 for the pressure-demand, SCBA used by fire-fighters.

Recently, attention has been drawn to the potential for adverse health effects following exposure to combustion-derived nanoparticles, which appear to have a common mechanism of injury through oxidative stress (Donaldson *et al.*, 2005). These materials, in addition to posing pulmonary hazards through oxidative stress, inflammation and malignancy, also have the potential to redistribute to other organs following pulmonary deposition. Nanoparticles are usually defined as primary particles with at least one diameter <100 nm, whilst ultrafine particles are defined as particles <100 nm in all directions (Lighty *et al.*, 2000).

7.3 Cardiovascular Disease

Fire-fighters are exposed to CO, CO_2, acrolein, sulphur dioxide, organic solvents, noise, heat, and physical and emotional stress. It is therefore to be anticipated that they would be at greater risk to develop CV disease. Acute myocardial incidents are reported, but rarely. For example, Jan *et al.* (2007) described a case of acute transient (four days) cardiomyopathy after smoke exposure; the patient developed acute cardiac failure, without evidence of CO poisoning. There is experimental evidence in Wistar Kyoto rats that repeated (subchronic) exposures to oil-combustion-derived combustion particles can cause inflammatory, degenerative and fibrotic myocardial injury (Kodavanti *et al.*, 2003). However, some epidemiological studies on fire-fighters do not demonstrate an excess of CV disease or mortality from CV disease (Beaumont *et al.*, 1991; Demers *et al.*, 1992; Deschamps *et al.*, 1995; Eliopulos *et al.*, 1984; Guidotti,

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1993; Sardinas *et al*., 1986). Summarizing the available evidence, Guidotti (1992) stated that population-based mortality and disability surveillance studies suggest there is a relatively small, but significant excess for disability, but not mortality, for nonmalignant CV disease for fire-fighters. More targeted cohort and case–control studies do not support such an excess, but suggest a strong healthy-worker effect (Aronson *et al*., 1994; Howe *et al*., 1988). However, some studies do suggest an excess of coronary artery disease (Musk *et al*., 1978). Holder *et al*. (2006) conducted a retrospective review of Massachusetts fire-fighters, 362 receiving Heart Presumption pensions (1997–2004) and a comparison group of 310 professionally active fire-fighters. Of the retirements, 77% were recorded as being due to coronary artery disease and 23% for other CV conditions. CV risk-factor prevalence was high among all retiree subgroups, and the authors concluded that the results support a suggestion for improved CV prevention and risk-reduction strategies among fire-fighters. Two studies have demonstrated increased SMRs for circulatory disease with increasing years of work (Demers *et al*., 1992; Vena and Fielder, 1987), and Aronson *et al*. (1994) showed an increase in aortic aneurysms.

Table 5 Site-specific neoplasms that are probably occupationally associated in fire-fighters

Neoplasm site/type	Reference
Buccal and pharyngeal	Mastromatteo (1974)
Oesophagus	Beaumont *et al*. (1991)
Colonorectal	Guidotti and Clough (1992)
Urinary tract	Deschamps *et al*. (1995), Ma *et al*. (2006)
Testicular	Ma *et al*. (2006), LeMasters *et al*. (2006)
Prostate	LeMasters *et al*. (2006)
Uterine cervix	Ma *et al*. (2006)
Lung	Heyer *et al*. (1990)
Brain	Aronson *et al*. (1994), Demers *et al*. (1992) Howe and Burch (1990)
Thyroid	Ma *et al*. (2006)
Lymphatic and leukaemia	Demers *et al*. (1992), Heyer *et al*. (1990)
Non-Hodgkin lymphoma	LeMasters *et al*. (2006)
Multiple myeloma	LeMasters *et al*. (2006)

7.4 Reproductive and Developmental Toxicity Hazards

Several chemicals that have been detected in fire atmospheres have been experimentally associated with a potential for adverse reproductive and/or developmental toxicity effects (McDiarmid *et al*., 1991). In spite of this, there is little available epidemiological evidence available on the reproductive and developmental toxicity hazards of fire-fighting. However, one study has indicated a possible excess of birth defects in the children of fire-fighters (Olshan *et al*., 1990). Peak CO concentrations measured in fires could be immediately dangerous to an unprotected female fire-fighter and her foetus (McDiarmid *et al*., 1991).

7.5 Carcinogenic Hazards

Several known, or suspect, carcinogens are present, to variable extents, in fire atmospheres; for example, PAHs, acrylonitrile, vinyl chloride, asbestos and polychlorinated biphenyls (PCBs). There are inconsistencies between some epidemiological studies on the possible excesses of cancers at various anatomical sites in fire-fighters. However, biological monitoring for genotoxic effects, including sister chromatid exchanges and PAH–DNA adducts in peripheral blood, suggests a potential for carcinogenic effects (Liou *et al*., 1989). Specific anatomical regions for neoplasms in fire-fighters that are possibly occupationally related are listed in **Table 5**. However, there is controversy for some site-specific cancers shown in **Table 5**; for example, Golka and Weistenhöfer (2008) did not confirm an increase in urothelial cancers based on analysis of mortality studies, and studies on exposure and cancer incidence. Documentation for an association between lung cancer and occupational exposure is inconsistent. For example, a Danish study (Hansen, 1990) reported an SMR of 317 for older fire-fighters, but studies from San Francisco (Beaumont *et al*., 1991) and Buffalo (Vena and Fielder, 1987) showed no excess. Cigarette smoking is a clear confounding factor (Liou *et al*., 1989), although according to one study the incidence of smoking among fire-fighters is not excessive compared with other occupations (Gerace, 1990). The excesses of certain cancers may be the result of interactions between several factors; toxic substances, alcohol and smoking (Beaumont *et al*., 1991). Ford *et al*. (1992) suggest that the immunological detection of serum β-transforming growth-factor-related proteins may be a possible biomarker for monitoring fire-fighters for potential development of cancer.

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Radiation Toxicology

Naomi H. Harley

CONTENTS

1 INTRODUCTION

Radiation and radioactivity can be both a valuable tool and a source of health effects, and thus accords with Paracelsus' dictum that everything is a poison, it is only the dose that makes it so.

There are four main sources which can be used for evaluating actual human health effects from exposure to ionizing radiation. The human risk estimates to date are derived from these studies. These are the exposures of:

1. Radium dial painters
2. Atom bomb survivors
3. Underground uranium miners exposed to radon and its decay products, together with residential radon studies
4. Those who were treated for ringworm of the scalp (tinea capitis), where X-rays were given at one time for therapy.

To date, the health effects or risk from exposure is the risk of cancer of a particular organ or tissue. The risk from ionizing radiation is that it damages DNA in specific genes.

These studies provide quantitative risk estimates for internal and external exposure. A few radionuclides are also described because they are abundant in crustal earth, although no health effects have been documented. These are uranium, thorium, lead and polonium, noteworthy as they are a significant part of the earth's background radiation and have received attention for specific reasons.

Ionizing radiation from alpha particles, beta rays or gamma ray photons are capable of causing an electron to be stripped from an atom. This results in a positive ion and an electron. DNA may no longer be capable of conducting its normal function if many such atoms are disrupted. An alpha particle has a very short range in tissue and can produce enough ionization to cause a double-strand break in DNA that is difficult to repair.

The significant DNA damage implicated from recent data is probably to checkpoint genes, such as p53, disrupting the cell cycle, allowing the cell to propagate in an uncontrolled way. The only observed health effect from high exposures and doses is cancer, except at extraordinarily high doses, when some heart disease is observed.

2 NATURAL BACKGROUND AND OTHER SOURCES OF IONIZING RADIATION

2.1 Radium

After its discovery, radium (^{226}Ra) became a popular nostrum; adverse health effects were not as yet known. It

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was approved by the American Medical Association and from 1900 to 1930 many were exposed to radium either in therapy, for example in the treatment of rheumatism, or occupationally. Radium was used in low or negligible lighting conditions in an inexpensive zinc sulfide (ZnS) phosphor to illuminate the numbers on instrument or watch dials. Luminous paint was developed before 1917 and in World War I there were several plants in New England and New Jersey where watch dials, clocks and military instruments were painted (Woodard, 1980).

The alpha particles from radium interacted with the ZnS to yield visible light photons. It was mainly women who prepared the radium-painted instrument dials. A fine tipped brush was dipped into the radium paint and applied to the dial face. The mouth was typically used to give the application brush its fine point. The dial painters developed osteogenic sarcoma (bone cancer) in a very short time after exposure, due to the ingestion of large amounts of ^{226}Ra (Martland, 1931). Radium then became the first radionuclide to show any harmful effects of radiation.

Radium behaves somewhat similarly to calcium and is a bone-seeker. The lowest bone concentrations measured that had no observed effect were determined to be 1–3 μg ^{226}Ra in the bone. At the time 1/10 of a known health risk was considered a 'safe' concentration, thus the safe concentration in bone was selected as 0.1 μg ^{226}Ra. This concept is no longer used because it is known that the probability of cancer for a given dose is a function of many biological and genetic factors. The terminology 'safe' is no longer used.

Evans (1967) studied about 500 cases of persons with radium exposure. He estimated that a measurement of 1 μCi in the skeleton several years post-exposure represented 20 μCi at the time of exposure because of its efficient removal. Using this value for a calculation of the dose to endosteal cells on bone surfaces that are targets for osteosarcoma yields a total skeletal dose of 2.9 Gy or 58 Sv (Fisenne *et al*., 1981). The estimate of bone cancer risk can be calculated as 0.35 per Gy (0.017 per Sv), with the caveat that the threshold dose is about 2.9 Gy (58 Sv).

Several thousand people were exposed to radium salts, either as part of the modish therapies using radium in the era from 1900 to 1930, or occupationally in the radium dial-painting industry around 1920. Radium therapy was accepted by the American Medical Association, and around 1915 advertisements were common for radium treatment of rheumatism and as a general tonic in the treatment of mental disorders. Solutions were available for drinking containing 2 μg, as well as vials for intravenous injection (Woodard, 1980). Luminous paint was developed before World War I, and in 1917 there were many plants in New England and New Jersey painting watch dials, clocks and military instruments (Woodard, 1980).

Radium, is first incorporated on bone surfaces then into the mineralized portion of bone. The long half-life of ^{226}Ra (1600 years) allows distribution throughout the mineral skeleton over a lifetime. However, the removal from the body is rapid and after ingestion only about 1% remains in the skeleton after one year. The target cells for osteogenic sarcoma reside in marrow on endosteal surfaces at about 10 μm from the bone surface. At long times after exposure, target cells are beyond the range of alpha particles from radium no longer on bone surfaces.

The first large studies of osteogenic sarcoma in radium-exposed people were done by Martland (1931) and Aub *et al*. (1952), who found 30 cases of bone sarcoma; Evans and associates at the Massachusetts Institute of Technology (Evans *et al*., 1969) studied 496 cases of sarcoma out of 1064 radium-exposed persons; and Rowland *et al*. (1978), studied 61 cases out of 1474 female dial painters (Woodard, 1980). **Figure 1** shows the retention of ^{226}Ra in the body from the various follow-up studies.

The loss of radium from the body by excretion was demonstrated to follow a relatively simple power function (Norris *et al*., 1958):

$$R = 0.54\, t^{-0.52} \tag{1}$$

where

R = total body retention
t = time in days.

Radium is the only radiation exposure to date that appears to have a true threshold dose before cancer is seen.

2.2 Uranium

Uranium has never been found to cause health effects in humans, even after high exposures. Uranium is of interest because of its ubiquitous use and because it supports a chain of 13 isotopes, some of which have been shown to cause cancer, including ^{226}Ra and radon (^{222}Rn). Natural uranium exists in small concentrations in all of planet earth's materials. Uranium is a very dense metal (18.7 g cm^{-3}), more so than lead (12.3 g cm^{-3}). It has been used as ballast for aircraft, as shielding for nuclear-medicine therapy sources and, as depleted uranium, as munitions and as shielding for military vehicles.

There are two long-lived uranium decay series supporting 12–13 other radioactive isotopes. One decay series is headed by ^{238}U, another by ^{235}U. The chain members include isotopes of radium, radon, lead and polonium. The parents of both decay series are very long lived, if they were not, they would not be present on the planet at this time. The ^{238}U series is shown in **Figure 2**.

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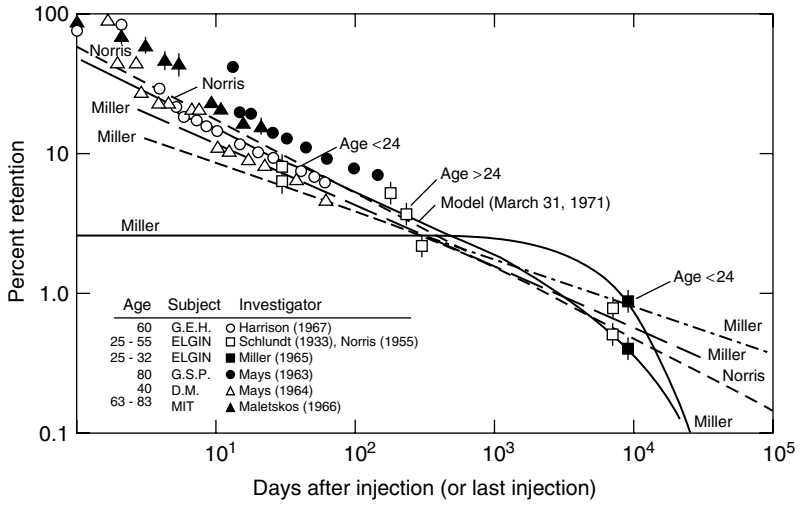

Figure 1 Whole-body radium retention in humans. Summary of all available data for adult humans. (Reproduced from Marshall *et al*, 1972 © Elsevier.)

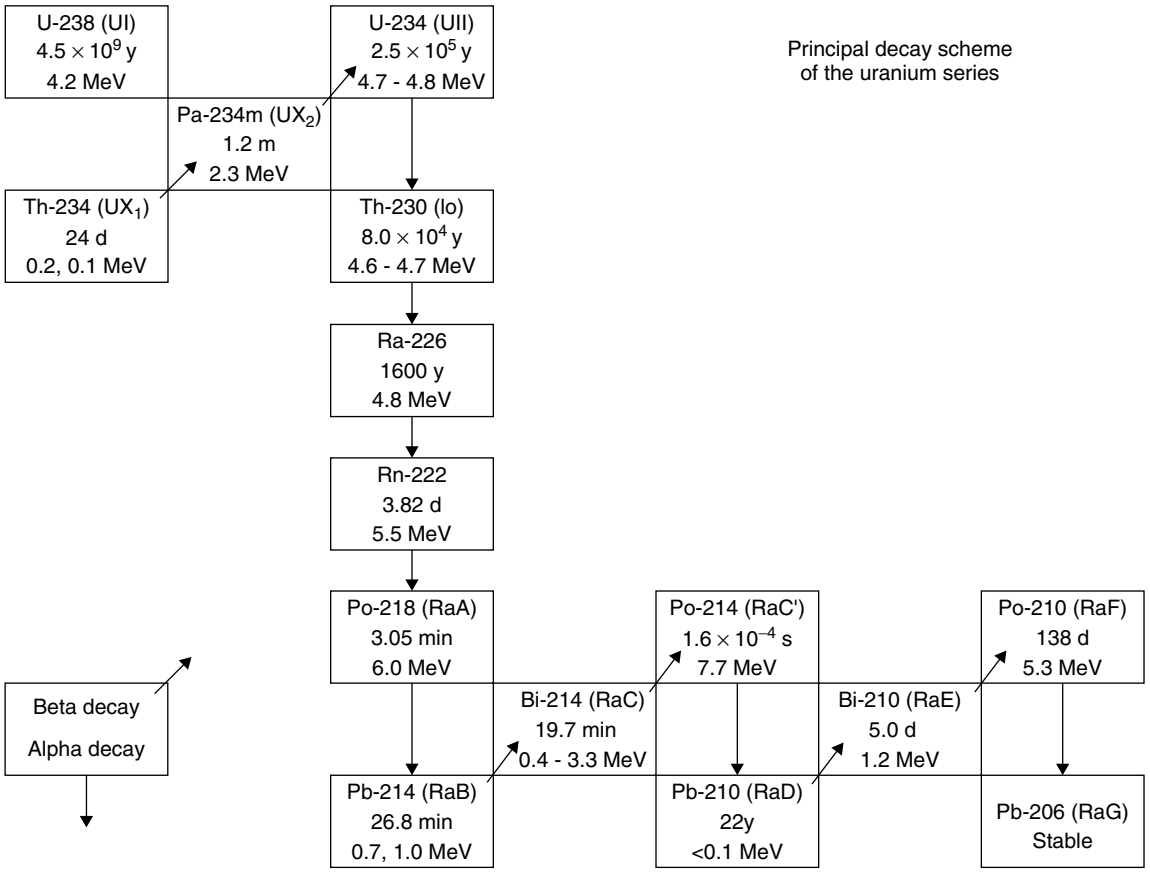

Figure 2 Uranium decay series. (Reproduced with permission from National Council on Radiation Protection and Measurements. © 1987.)

Natural ^{238}U supports its decay product ^{234}U and they are usually in radioactive equilibrium in most planetary substances. Equilibrium means that the radioactive decay rate of each is the same. In some cases, groundwater passing through soil will dissolve ^{234}U to a greater extent, and the steady-state activity in, say, drinking water is disturbed. Usually the activity ratio of $^{234}U/^{238}U$ will be from 1.0 to perhaps 1.4. The radioactivity of ^{235}U is a separate chain and it is not as radioactive as the ^{238}U series. **Table 1** shows the radioactivity of natural

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Table 1 Radiological characteristics of natural and depleted uranium

Natural uranium			
Isotope	Half-life	Isotopic %	mBq μg^{-1} natural U
^{238}U	4.468×10^9	99.2745	12.40
^{234}U	2.45×10^5	0.0055	12.40
^{235}U	7.037×10^8	0.72	0.60
Total			25.4
Depleted uranium			
Isotope	Half-life	Isotopic %	mBq μg^{-1} depleted U
^{238}U	4.468×10^9	99.800	12.40
^{234}U	2.45×10^5	0.0010	2.26
^{235}U	7.037×10^8	0.2	0.16
Total			14.8

and depleted uranium. The US Environmental Protection Agency (EPA) has set a standard of 30 μg ^{238}U l^{-1} for suppliers of drinking water to more than 25 homes. Some members of the uranium decay series are gamma-ray emitters, as well as beta-ray and alpha-particle emitters. An annual external radiation dose (about 0.5 mSv) arises from these gamma rays because the uranium series isotopes are present in all rock and soil.

Natural uranium is thus composed of three isotopes of uranium ^{238}U, ^{234}U and ^{235}U. The ^{235}U is capable of sustaining a chain fission reaction and is separated from natural uranium for use in nuclear reactors and nuclear weapons. Depleted uranium, being a very dense metal with pyrophoric properties, was used in the Gulf War in munitions and as shielding in armoured vehicles. There followed concern over health effects in veterans experiencing inhalation exposures following damage to the vehicles. Uranium is not considered a radiation hazard, rather it has the toxicological properties of a heavy metal if inhaled or ingested in very large amounts. Uranium targets the kidney and has been shown to have some short-term consequences, such as increased protein excretion, but no long-term adverse effects. Depleted uranium (DU) is less radioactive than natural uranium because most of the ^{235}U and ^{234}U have been removed (**Table 1**). In a follow-up study of over 18 000 persons employed in the atomic energy industry in the 1950s, there were no health effects from uranium, even after high, long-term, occupational exposures (Polednak and Frome, 1981). No health effects have been observed in veterans in the Gulf War exposed to friendly fire who had suffered embedded DU shrapnel (McDiarmid *et al*., 2001).

Table 1 shows the parent ^{238}U is not very radioactive because of its long half-life and thus emphasis is on its heavy-metal properties. The permitted occupational inhalation exposure to uranium is greater than that to lead, which is considered to be a more toxic heavy metal.

There are no studies to show that natural uranium or DU has caused any health effects. There is a normal dietary intake of uranium in foods each day of about 1 μg. The content in individual foodstuffs can be found in reports published by the United States National Council on Radiation Protection and Measurements (NCRP) (NCRP, 1987) and the United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR, 2000). The intake in drinking water is very variable and the range found in private wells, for example, can be over several orders of magnitude.

2.3 Thorium

Thorium is of interest only because it is one of the three primordial radioactive series supporting a chain of 13 radionuclides similar to the uranium series, and because of its universal presence in planetary materials. In soil it has a concentration of about five times that of natural uranium (10 vs. 2 ppm) (NCRP, 1987). No health effects have ever been demonstrated following thorium exposure, except in extraordinarily high exposures for medical diagnostic purposes.

Thorotrast, a thorium dioxide compound, was used from 1930 to 1960 as a contrast medium in X-ray diagnostic procedures. It was no longer used after long-term radiation effects were observed (Fisenne, 2000; van Kaick *et al*., 1991). Thorium has been used industrially for many years as mantels in incandescent kerosene (paraffin) and gas lamps, to increase their light output, as one of the rare earths used in video screens, in crucibles, in arc-welding electrodes and alloying. Thorium has been used as a fuel in breeder reactors. Thorium-232 captures a neutron, becoming ^{233}U, a fissionable material. Brazilian and Indian monazite sands are the major source of commercial thorium, and Madagascar mines thorianite

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commercially, a mineral with high concentrations of thorium (45–88%).

Fisenne (2000) prepared an extensive review of the thorium distribution in food and water, and the global environment.

2.4 Radon and Radon Decay Products

The first toxicologic report of health effects was in underground miners dying from a then unknown respiratory disease in the Erzhertzberg mountains in Bohemia (now in the the Czech Republic) (Harting and Hesse, 1879). It was not recognized until the 1950s that the responsible agent for the disease, by then established as bronchogenic carcinoma of the lung, could be the solid decay products of radon (Harley, 1953). It was realized that their deposition on the bronchial airways provided a significant alpha-particle dose to cells in the mucosa, and that radon itself gave a much lower dose to these cells, and was therefore not likely to be the carcinogen. There were other agents in mines, such as asbestos and mineral dust fumes, that could be responsible carcinogens. However, the Lubin *et al*. (1994) and NAS/NRC (1999a) studies of 11 underground mining cohorts showed a clear dose–response for lung cancer with radon exposure, and thus determined radon decay products to be a definitive carcinogen.

The lung-cancer risk was initially documented in mines and in the late 1970s, a few measurements were made in homes. It was found that radon concentrations in some homes could be as high as in mines. This caused a flurry of activity in many countries to measure residential concentrations and to set guidelines for domestic exposure. Over 89 epidemiologic studies of radon and lung cancer have been performed, with 20 being case–control studies. The case–control studies document lung-cancer cases and paired controls along with their measured residential radon concentration. To date there are four studies that have pooled the case–control studies (using the original data), and two that have performed a meta-analysis (using published data) (Darby *et al*., 2005; Krewski *et al*., 2005; Lubin *et al*., 2004; Wichmann *et al*., 2005; Lubin and Boice, 1997; Pavia *et al*., 2003).

The measured unit of exposure in mines was the working level month (WLM). The WLM is a measure of the air concentration of the actual carcinogen, that is the short-lived radon decay products, ^{218}Po and ^{214}Po. Most measurements in mines were done in the 1950s and 1960s. The WLM is a measure of the radon decay-product alpha-particle energy in 1 l of air, adjusted to the duration of exposure in work months. One work month is equal to 170 hours.

The working level (WL), was considered a 'safe' concentration in the 1950s, and was equal to 100 pCi l^{-1} (3700 Bq m^{-3}) in equilibrium with its two alpha-emitting decay products, ^{218}Po and ^{214}Po. The WL can be estimated from the radon gas concentration using an equilibrium factor, F. In homes, the equilibrium factor is 0.4, that is the decay products are 40% in equilibrium with the gas, and in mines F is about 0.3 or 30% equilibrium. The radon corrected for equilibrium with the decay products is known as the equilibrium equivalent concentration (EEC) and is then used to estimate the WL.

For a radon concentration of 10 pCi l^{-1} (370 Bq m^{-3}) in a home, the WL = (0.4)(10)/100 and the WLM = WL (hours exposed/170). Assuming 70% occupancy in a home, the exposure time is (365)(24)(0.7) = 6132 hours; WLM = WL(6132/170) = (0.04)(36) = 1.44 WLM per year.

The US EPA suggests 4 pCi l^{-1} radon gas exposure in living areas as a guide, and the occupational limit for underground miners is 4 WLM per year. The estimated excess relative risk (ERR), determined from the studies of underground uranium and other miners, averages 0.006 per WLM while the residential studies show an ERR of 1.16 for lifetime exposure to 100 Bq m^{-3}. For comparison, the residential risk for 100 Bq m^{-3} can be converted to a lifetime exposure (40 years) of about 20 WLM or an ERR of about 0.008 per WLM, in good agreement with the mining data. Over 50 countries have conducted residential radon surveys and the average global concentration is estimated to be about 40 Bq m^{-3}. The average annual dose in residences from a radon concentration of 40 Bq m^{-3} is 1.2 mGy (2 mSv).

The ERR derived from the residential studies is estimated to be the same for smokers and nonsmokers (Darby *et al*., 2005). However, the baseline risk for smokers is a factor of 12 times greater than for nonsmokers so there is a greater lung-cancer risk from radon to smokers.

Figure 3 shows the results of the lung-cancer risk from radon exposure from the pooled studies and the meta-analyses.

2.5 Lead

There are no epidemiologic studies to show health effects of ^{210}Pb. This nuclide is found in most foodstuffs and the dietary intake per day about 37 mBq (1 pCi) (NCRP, 1987). In some industrial processes both ^{210}Pb and ^{226}Ra can accumulate. Exposure rates above background radiation are seen in the scale in down-hole pipes during oil-field pumping operations (Hamilton *et al*., 2004) and are attributed mainly to ^{226}Ra. The scale must be removed with care from equipment and pipes periodically, and the material is designated as naturally occurring radioactive material (NORM).

Both ^{210}Pb and its decay product ^{210}Po are present in tobacco products and it is suggested to be one of the

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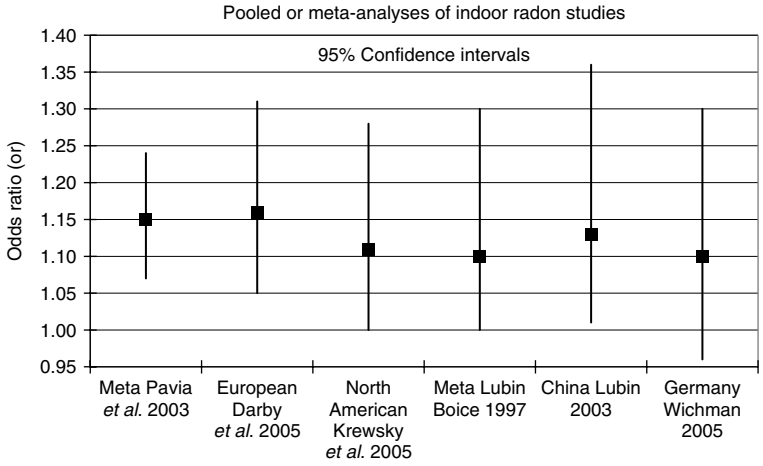

Figure 3 Pooled or meta-analysis of residential radon studies.

many carcinogens responsible for lung cancer in smokers (Cohen *et al*., 1980; NCRP, 1987).

2.6 Polonium

Polonium-210 (^{210}Po) is a decay product of ^{210}Pb in the ^{238}U series. Most polonium in the body arises from the ingestion of dietary ^{210}Pb. The fatal poisoning case of the Russian, Litvinenko, in November 2006, showed the potential for its use as an accessible poison. The use of polonium in industry is widespread, mainly for static elimination. Polonium is easily measured in urine samples as a test for dietary or other exposure. Although no human data are documented for health effects, research concerning the toxicity of polonium was conducted at the University of Rochester and is summarized by Stannard (1988).

When experimental animals (dogs, rabbits) were injected with 2.6 MBq per kg body mass, ^{210}Po lethality occurred in less than a month. Polonium distributes in the body almost uniformly and the affected organ most likely damaged enough for lethality is the bone marrow. If this is scaled to the human, 200 MBq results in a lethal internal exposure.

2.7 Potassium

Potassium is a major constituent of the human body and is a necessary requirement for human health. It is present in every functioning cell in the body. The radioactive isotope ^{40}K comprises a fraction of all natural stable potassium (^{39}K). The isotopic abundance is 0.0117% and the half-life is 1.28×10^9 years. K^{40} emits both beta and gamma rays (28 Bq beta and 3 Bq gamma per g of natural potassium). The average person contains about 120 g of potassium, thus the body content is 3.7 kBq and the annual dose from normal potassium is about 0.2 mSv. Potassium is thus the highest natural background dose from internal radioactivity, with the exception of radon.

2.8 Carbon

Naturally occurring ^{14}C is produced mainly in the stratosphere by the interaction of slow cosmic-ray neutrons with atmospheric nitrogen. It is therefore a cosmogenic radionuclide and emits a low-energy beta particle (0.16 MeV). Carbon is an important element in the human body and ^{14}C participates in body metabolism in a similar manner.

The specific activity of cosmogenic ^{14}C is 230 Bq kg^{-1} of total carbon, and the human body content is about 2700 Bq, yielding an annual effective dose of 12 μSv. The nuclear weapons tests in the 1950s and 1960s introduced about twice the cosmogenic inventory into the atmosphere. This was absorbed into the marine environment, with a half-life of six years. The current ^{14}C atmospheric residue from weapons tests is 0.05 Bq kg^{-1} carbon (UNSCEAR, 2006).

Artificially produced ^{14}C is used as an isotopic tracer to study the details of various chemical reactions involving stable carbon.

2.9 Tritium

Naturally occurring tritium (^{3}H) is produced mainly in the stratosphere by the interaction of fast cosmic-ray neutrons with atmospheric nitrogen. It is therefore a cosmogenic radionuclide and emits a low-energy beta particle (0.015 MeV). Prior to the nuclear weapons tests atmospheric ^{3}H in precipitation was about 0.6 Bq l^{-1} and the human body

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content 26 Bq (NCRP, 1987). The nuclear weapons tests increased the ^{3}H concentrations in precipitation by more than two orders of magnitude over a period of two or three years. The annual effective dose from cosmogenic ^{3}H is about 10 μSv.

Artificially produced ^{3}H has been used as an isotopic tracer in research involving chemical and hydrologic pathways. Tritium has largely replaced ^{226}Ra in luminescent signs and watch dials.

3 MEDICAL AND OTHER SOURCES OF IONIZING RADIATION

3.1 Tinea Capitis

Ringworm of the scalp (tinea capitis) was a common fungal disease before 1960. Radiation (X-ray) therapy was a common treatment modality. From 1905 to 1960, X-ray epilation in the treatment of tinea capitis in children was performed regularly. The treatment was introduced by Sabouraud in 1904 and was standardized by Kienbock (1907) and Adamson (1909). Over the 50 years it was used, perhaps 200 000 children worldwide may have been irradiated (Albert *et al*., 1986).

The first follow-up studies of the long-term effects of irradiation were performed by Albert and Omran (1968). They reported on 2200 children irradiated at the Skin and Cancer Unit of New York University Hospital (NYU) during 1940–1959. Subsequent publications studying this group have appeared at regular intervals (Shore *et al*., 1976; 1984; 1990; 2002; 2003). Subsequently, a follow-up of 11 000 children irradiated in Israel was performed (Ron *et al*., 1995).

The mean age of children irradiated in both the NYU and Israeli studies was between seven and eight years. The age range in the NYU study was 1–15 years at the time of treatment. Dose reconstruction in the NYU series was performed using a head phantom containing the skull of a seven-year-old child covered with tissue-equivalent material (Harley *et al*., 1976).

In the NYU series there were two thyroid cancers with no expected cases and 11 thyroid adenomas with one expected (Shore *et al*., 2003). In the Israeli series there were 43 thyroid cancers with 10.7 expected cases. In the NYU series there are 128 skin lesions, mostly basal cell carcinomas, with 24 expected cases. Fairness of skin is an important factor in the appearance of skin cancer (Shore *et al*., 1984; 1990; 2002).

Skin cancer was found primarily in Caucasians even though 25% of the study population consisted of blacks. As of 2002, there were 128 cases of non-melanoma skin cancer of the head and neck in the irradiated group and 21 in the control group. In the irradiated group, 125 cases were Caucasian and three were African American. This, and the fact that there appears to be a much lower dose–response on the hair-covered scalp than on the face and neck (Harley *et al*., 1983; Shore *et al*., 2002), suggest that the promotional effects of UV radiation play an important role in skin cancer.

The dose estimates for the thyroid in the Israeli study are 0.09 and 0.06 Gy in the NYU study. The skin dose estimates range from 0.1 to 0.5 Gy on the face, but 2.5 Gy near the scalp margins. The estimate for lifetime thyroid cancer risk is difficult to determine from the tinea capitis data and can be derived from a pooling of studies of thyroid irradiation (Ron *et al*., 1995); however, the estimates per Gy are arbitrary as risk depends strongly on age at exposure and gender.

The small numbers of tumours other than skin cancers in the NYU study make it of little value in estimating the lifetime risk per Gy, although an excess is appearing (Shore *et al*., 2003). There were eight leukaemia cases with one expected and six salivary gland tumours with two expected. There were 16 intracranial tumours, seven brain cancers, four meningiomas, and five acoustic neuromas (vestibular schwannomas) in the X-irradiated group, following an average brain dose of about 1.4 Gy, compared to one acoustic neuroma in the control group. The standardized incidence ratio (SIR) for brain cancer was 3.0 (95% confidence interval (CI): 1.3, 5.9).

These are important studies because children were the exposed group and because only partial body irradiation was involved. The temporal pattern of appearance of these tumours is also important. The dose was delivered over a short time interval (minutes at NYU and five days in Israel), and lifetime patterns are indicative of the underlying carcinogenic mechanisms, such as time to tumour following short-time or fractionated exposure.

Skin and thyroid cancers are of importance in documenting health effects from ionizing radiation. However, both types of cancer are rarely fatal. NCRP (1984) reported that about 10% of thyroid cancer is lethal and more recent SEER (Surveillance, Epidemiology and End Results) data indicate 3% fatality. It is estimated that the fatality rate of skin cancer is 1% (NCRP, 1990). The lifetime risk per Gy derived by NCRP for total thyroid cancer incidence (0.003 for females and 0.0014 for males for external X-ray or gamma radiation for persons under 18 years of age) is about a factor of 10 lower than that reported by Ron *et al*. (1995) in pooled data. However, the tinea irradiations were given to children with a mean age of about seven years; also in the Israeli study there is apparently an increased sensitivity resulting from ethnicity.

The effect of gender is also suggested by for thyroid cancer NCRP (1985). There is an obvious susceptibility of women for thyroid cancer and adenomas in both the NYU and Israeli tinea capitis studies. After 50 years of follow-up in the New York study of 2224 children given X-ray therapy for tinea capitis, along with a control

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Table 2 Lifetime cancer incidence per Gy from four major epidemiologic studies (in parentheses, risk per Sv for alpha emitters, $W_r = 20$, Bronchial epithelium $W_t = 0.08$)

Study, organ, type of radiation	All sites (Adults)	Leukaemia	Lung	Female breast	Bone	Thyroid	Skin
Atom bomb whole-body, gamma (ICRP, 2007)	0.041	0.0066	0.0085	0.002	0.00041	0.0008	0.0002
Uranium miner bronchial epithelium, radon decay products, alpha (UNSCEAR, 2000)	—	—	0.12 (0.08)	—	—	—	—
Tinea capitis, head X-ray (Shore *et al*., 2003; Ron *et al*., 1995)	—	—	—	—	—	0.003 female 0.0014 male	0.23
Radium ingestion, bone, alpha (^{226}Ra) (Evans *et al*., 1969)	—	—	—	—	0.35 (0.017)	—	—

group of 1380 tinea capitis patients given only topical medications, the study found a relative risk (RR) of 3.6 (95% CI, 2.3–5.9) for basal cell skin cancer (BCC) of the head and neck among irradiated Caucasians (124 irradiated cases and 21 control cases), in response to a scalp dose of about 4.8 Gy.

The base rate for the lifetime appearance of basal cell carcinoma in the US population is high, 0.3 or 30%. An estimate of the incidence per Gy is then 0.3 (3.6/4.8) or 0.23 (23%). This is shown in **Table 2**.

3.2 Atomic-Bomb Survivors

The atomic-bomb survivors are a particularly important group because the occupational radiation guidelines and some human-organ risk estimates are based on follow-up studies on these individuals. A uranium fission weapon was dropped on Hiroshima, Japan on 6 August 1945 and three days later a plutonium fission weapon was dropped on Nagasaki.

A total of 64 000 people in the two cities were killed by the blast and the thermal effects within 1 km of the explosions, and as a result of the instantaneous gamma and neutron radiation released by the weapons. Others between 1 and 2 km from the hypocentre (the point on earth directly below the detonation point in air) received radiation doses up to several Gy. Beyond 2.5 km there was no additional dose from the weapon, only natural background radiation.

A study of the mortality of A-bomb survivors was initiated by the Atomic Bomb Casualty Commission (ABCC) in 1950. This was a prospective study and is now conducted by the Radiation Effects Research Foundation (RERF). The main study, called the Life Span Study (LSS), included 120 000 people, 92 228 people within 10 km of the hypocentre and 26 850 people who were not in either city at the time of bombing (ATB). The most recent reports of the RERF (1987; 2003) are follow-ups of the cancer mortality of a subcohort over the period 1950–1997 (Preston *et al*., 2003; 2004). The cohort includes 86 611 persons with individual dose estimates. The latest updates (Preston *et al*., 2003; 2004) report that 440 (5%) of all solid cancer deaths and 250 (0.8%) of the noncancer deaths were associated with the radiation exposure.

The gamma rays account for most of the radiation doses to survivors, the neutron absorbed dose at most organ depths (and distances from the hypocentre) were about 1% of the gamma-ray absorbed dose at Hiroshima.

The occupational guidelines and risk estimates for radiation are now based on the atomic-bomb survivors. There are approximately 70 000 persons followed up since 1950. The dosimetry was re-evaluated in 2002 (*http://www.hss.energy.gov/healthsafety/IHS/ihp/japan/DS02.pdf*) to obtain a better estimate the dose received by survivors, using more modern technology than available for the first dose reconstruction in 1986 (*http://www.rerf.or.jp/shared/ds86/ds86a.html*). The risk estimates were re-evaluated by ICRP (2007) on the basis of DS02 and are shown in **Table 2**.

4 SUMMARY

The normal daily dietary intake includes naturally occurring uranium, thorium, lead-210, polonium-210, radium-226 and potassium-40. These radionuclides are present in all crustal earth. Some are gamma-ray emitters as well as alpha and beta emitters. These yield an internal body dose (about $0.2\,\mathrm{mSv\,a^{-1}}$) and an external dose from terrestrial gamma-ray radiation (about $0.5\,\mathrm{mSv\,a^{-1}}$). There are no observable health effects from this normal background radiation.

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There are four major epidemiologic studies where the exposure was to high levels or concentrations of these radionuclides: the radium dial painters, the underground uranium miners exposed to radon (also in residences with high radon concentrations) or to X- or gamma-ray radiation, the atomic-bomb survivors and the tinea capitis (ringworm) patients. These epidemiologic studies provide quantitative estimates of the cancer risk from high exposures to radiation.

5 GLOSSARY

Bequerel (Bq) = A unit of radioactivity equal to 1 disintegration per second.

Gray (Gy) = A unit of absorbed radiation dose $D = E$ (energy)/M (mass). Equal to $1\,\mathrm{J\,kg^{-1}}$.

Sievert (Sv) = A unit of radiation dose E (energy)/M (mass) equal to $1\,\mathrm{J\,kg^{-1}}$, corrected by a radiation weighting factor (W_r) to account for the type of radiation, alpha, beta or gamma rays ($W_r = 1$ for beta and gamma rays, $W_r = 20$ for alpha-particle radiation) and a tissue weighting factor (Wt) that depends on the tissue ($W_t = 0.08$ for bronchial epithelium for example). $\mathrm{Sv} = (W_r)(W_t)(D)$. See (ICRP, 2007) for detailed values of W_t.

Working level (WL) = A measure of the *potential* energy in 1 l of air from 100 pCi (3700 Bq) radon in equilibrium with its decay products for complete decay of the radon and decay products. Equal to $1.28 \times 10^5\,\mathrm{MeV\,l^{-1}}$ usually rounded to $1.3 \times 10^5\,\mathrm{Mev\,l^{-1}}$. The WL was easily measured in underground mines by a single measurement and became the exposure unit of choice because it is the decay products that deliver the bronchial dose, not the radon gas.

Working level month (WLM) = The time integral of the WL. A work month is taken as 170 hours. WLM = (WL) (hours exposed)/170.

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Risk Assessment of Chemicals

Leah D. Stuchal and Stephen M. Roberts

CONTENTS

1 INTRODUCTION

Risk assessment of chemicals is a process that describes the nature and magnitude of hazards posed by chemical exposure. The risk assessment results in a statement regarding potential adverse effects and the likelihood that an adverse effect will occur in specific exposure situations. The risk statement can be primarily qualitative (e.g. an effect is 'likely' or 'very likely') or can be quantitative in nature, with numerical expressions of risk, such as a 10^{-5} excess probability of developing cancer as a result of exposure. The risk assessment process constantly evolves as science refines estimates of exposure and toxicity. Therefore, each risk assessment reflects the current state of scientific knowledge, as well as uncertainties, that exist in human health and environmental responses to the chemical. New information on the toxicity of chemicals, as well as new methods of chemical detection and scientific approaches to detect adverse effects in their initial phases, continually change the perceived hazard of chemicals and estimates of risk.

The two main types of risk assessment are human health and ecological risk assessment. Although the approaches to both are the same at the most fundamental level, procedures for each have evolved separately to a large extent. Consequently, the specific steps involved in conducting a human health risk assessment are somewhat different from those used to produce an ecological risk assessment. Another aspect that sets the two types of risk assessment apart is the inherent degree of complexity. Human health risk assessments have the luxury of only dealing with a single species, while ecological risk assessments by necessity have to consider, not only an array of species, but also ways in which the species interact (e.g. transfer of chemicals through the food web, dependence of some species on others as food sources) in order to evaluate potential chemical effects. This chapter will cover basic aspects of both risk assessment types, with emphasis on human health.

2 RISK ASSESSMENT PARADIGM

Risk assessment of chemicals requires minimally two types of information: (i) the potency of the chemical, that is, how much is required to produce an adverse effect and (ii) measured or estimated exposure, that is, what dose of the chemical does an individual receive as a result of exposure. Information on potency is of little value without some knowledge of the dose. For example, just because the toxic potency of a certain chemical is high does not necessarily mean that exposure will produce toxicity. Even extremely potent toxicants can be used safely if the dose is small enough; for example, the injection of botulinum toxin (e.g. Botox®) into the face to remove wrinkles. Similarly, information on exposure is essentially meaningless in terms of risk if the toxic potency of the chemical is unknown.

People have been evaluating the risk from chemicals in their environment informally as long as humans have been in existence. Lessons learned the hard way about what plants can be eaten safely, for example, comprise an essential form of risk assessment information passed along through generations. Formalized processes that form the basis of contemporary risk assessment practice evolved during the latter half of the twentieth century. In 1983, the National Research Council (US National Academy of Sciences) described what are widely regarded as the four basic steps in risk assessment: hazard identification, dose–response assessment, exposure assessment and risk characterization.

- Step 1—Hazard identification: The first step in the risk assessment process is to determine if the chemical of concern is a hazard to human health. The type of adverse effect a chemical produces and its mode of action are considered. Information on health effects may be determined from case reports, clinical studies, epidemiological data or inferred from animal and laboratory studies.
- Step 2—Dose–response assessment: The dose–response assessment describes the relationship between the amount of chemical exposure (dose) and the severity of the adverse effect (response). This relationship is often determined in animals and then extrapolated across species for use in human assessments.
- Step 3—Exposure assessment: Exposure assessment is the process of estimating the magnitude, frequency and duration of exposure. This step also includes identifying the pathways through which the population of interest is exposed to the chemical (e.g. from contaminated drinking water). Exposure assessment considers multiple media (e.g. air, water, soil, sediments) and can include exposures to mixtures of chemicals. The objective of the exposure assessment is to derive estimates of the dose of chemical received from each relevant route of exposure (e.g. ingestion, dermal contact, inhalation).
- Step 4—Risk characterization: The final step uses information obtained from the first three steps and integrates them to obtain an estimate of risk. Typically, these estimates of risk are compared to risk thresholds to determine if the chemical imposes an unacceptable increase in risk to the population. Uncertainty associated with various aspects of the preceding steps of the risk assessment process is discussed to help users of the risk assessment to understand the strengths and limitations of the analysis.

Each of these steps is discussed in more detail in the following sections, as well as a note on how these

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processes will support risk management. Risk assessment is important for the development of trade and transport regulations of chemicals and this is further discussed in **Regulatory Toxicology**.

3 HAZARD IDENTIFICATION

Hazard identification is an evaluation of the intrinsic toxicity of a chemical. It asks whether the chemical is capable of producing toxicity at any dose, and if so, what effects are produced. For example, hazard identification would be used to establish whether a chemical is a carcinogen or produces reproductive toxicity. There are a variety of sources of information that can be used in hazard identification, including studies in humans (clinical studies, epidemiological studies, case reports of poisonings), studies in laboratory animals, *in vitro* tests and computer simulations. Some sources of information are more highly regarded than others, and hazard identification often includes an element of professional judgement as evidence for toxicity from different types of studies is weighed. Strengths and weaknesses of various sources of information are discussed below.

3.1 Studies in Humans

In determining what constitutes a hazard for the purposes of human health risk assessment, the most reliable source of data is from well-conducted studies of humans exposed to the chemical. For example, the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) has produced numerous reports and other toxicological evaluations regarding experimental studies on pesticides in humans. Case reports from accidental or intentional poisonings can be informative, particularly with regard to the types of effects associated with acute intoxication. Ethical issues generally preclude intentional exposure of humans to chemicals (other than pharmaceuticals) at doses producing adverse effects. However, studies of individuals exposed to chemicals as part of their normal activities are common and potentially useful for hazard identification. Often, these take the form of an epidemiological study. A very attractive feature of this type of study is that it can be used to identify adverse effects that occur under actual human exposure conditions, including chronic exposure. If the study is sufficiently large, differences in response due to gender, race or genetic susceptibility may be identified. Epidemiological studies also have several weaknesses. Unless the magnitude of exposure is high, effects produced by the chemical may be difficult to detect. For this reason, occupational cohorts, which often have higher levels of chemical exposure than the general population, are particularly useful in assessing hazard. Also, many adverse effects can have numerous causes. Unless confounding factors (i.e. alternative risk factors) are well controlled, it can be difficult or impossible to clearly associate increased disease in an exposed population with the chemical in question. The role of epidemiology in toxicology is further discussed in **Essentials of Epidemiology for Toxicologists**.

3.2 Animal Studies

For most chemicals, information from studies in humans is limited or nonexistent, and results from animal studies are used to characterize hazard. There are several advantages to using laboratory animals for hazard identification. Exposure conditions can be tightly controlled, eliminating uncertainty about doses received and the confounding influence of exposure to multiple chemicals that is typically the case in human studies. It is also possible to evaluate the health status of subjects in a much more detailed manner, including biochemical and histopathological examination of potential target tissues. This allows observations of not only overt, clinical effects, but also precursor events leading up to toxicity. The ability to detect precursor events, even at the molecular level, means that potential adverse effects can be detected with great sensitivity. It also raises the sometimes controversial question of what, exactly, constitutes a toxic response? Contact with a chemical invariably produces some kind of biological response, however subtle. Distinguishing between adaptive changes of no clinical consequence and preludes to frank toxicity can be challenging, but is critically important for hazard identification.

There are also disadvantages to the use of laboratory animals for hazard identification. Some toxic endpoints, such as subtle neurobehavioural effects, can be very difficult to detect in animal studies. And of course, there is always some degree of uncertainty as to whether the laboratory animal responds to the chemical in the same way as humans. Differences in metabolism and physiology among species can lead to different responses to chemicals. For example, a number of chemicals are carcinogenic in mice, but not rats, and *vice versa*. In this situation, what is the inference regarding the carcinogenicity of the chemical to humans? When extrapolating findings from animals to humans, it can help enormously to have information regarding the mode of action of the chemical in producing the effect. An often-cited example is increased kidney tumours in male rats produced by unleaded gasoline, 1,4-dichlorobenzene and D-limonene. Binding of metabolites of these chemicals to $\alpha_2\mu$-globulin, a protein synthesized primarily in the liver, results in its accumulation in renal tubular cells. This accumulation is associated with renal pathology,

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including tumour formation. Female rats and mice of both sexes produce much lower levels of the protein than male rats and do not develop renal tumours from exposure to these chemicals. Because humans do not synthesize $\alpha_2\mu$-globulin, this mode of action, and by extension the renal carcinogenicity of unleaded gasoline, 1,4-dichlorobenzene and D-limonene observed in male rats, are considered not relevant to human risk.

3.3 *In Vitro* Studies

Information on the toxic effects of chemicals can be obtained from *in vitro* studies. These studies typically involve cells in culture, but can also include studies using isolated tissues and organs, tissue extracts or homogenates, subcellular fractions or purified biochemical reagents (e.g. enzymes, other proteins, nucleic acids). The ability to control experimental variables is great, and experiments with *in vitro* systems can be extremely valuable in gaining an understanding of how the chemical produces its effects at the cellular, biochemical and molecular level. *In vitro* endpoints are also amenable to high-throughput screening to rapidly identify chemicals that may share a common effect or mode of action. For example, organophosphate insecticides and some chemical warfare nerve agents have in common a mode of action that involves inhibition of cholinesterase enzymes. By setting up automated assays to measure cholinesterase inhibition *in vitro,* a large library of chemicals could be screened to identify other chemicals that might have the same toxicological effect. The disadvantage of *in vitro* tests is that they are incapable of capturing the complexity of an intact human or animal, where adverse effects can result from interplay between different organs and systems in the body. Also, the relationship between biochemical and molecular changes observed in *in vitro* studies and toxicity at a clinical level is inferred. As such, *in vitro* tests can provide a basis to predict that toxicity will occur as a result of exposure, but a conclusion that a particular hazard exists (e.g. carcinogenicity) usually requires evidence from human or animal studies.

3.4 *In Silico* Studies

Structure–activity relationships (SARs) can be used to estimate the toxicity of a chemical. The high costs of animal toxicity testing, coupled with the need to evaluate, at least preliminarily, the potential adverse effects of an overwhelming number of chemicals for which no toxicity information exists, makes the use of computer modelling attractive. Commonly referred to as *in silico* testing, these models relate chemical structure to biological activity and potency. The assumption implicit in this process is that a chemical's structure determines its interaction with the biological system and reveals its toxicity. The most frequent use of this type of toxicity modelling is to predict a common mode of action for an entire class of chemicals. For example, the Ah-receptor binding ability of dioxin-like compounds has been predicted *in silico* based on SAR. It must be remembered that these results are only predictions and should be confirmed, when possible, by human or animal studies.

3.5 Weight of Evidence

Hazard identification is accomplished through a careful review of all of the available information from the various types of studies described above. This review needs to be critical, looking at the type and quality of information offered by each study. Many toxicity studies conducted before modern guidelines were in place may have some deficiencies, but still contain useful information. This is especially true when these older studies provide the only toxicity data available. The following are guidelines to consider when evaluating studies:

- Does the test measure an adverse effect or a nonadverse effect?
- Have the experiments been performed in a scientifically valid manner?
- Does the experiment contain the appropriate control groups?
- Is there proper accounting for confounding factors?
- Are observed effects statistically significant?
- Is the test species physiologically relevant to humans?
- Do the results conflict with other test species that are more physiologically relevant to humans?

In an effort to make the weighing of evidence more transparent and reproducible, formalized schemes have been proposed that involve clearly articulated quality criteria for evaluation of studies and methods for ranking them in terms of the value of information provided. Because this approach is analogous to evidence-based medicine, it has been called evidence-based toxicology.

It is important to keep in mind that hazard assessment must be viewed in the context of route and duration of exposure. Quantitative, and sometimes qualitative differences in response can occur when a chemical is administered by different routes. This is most obvious when the chemical produces its primary effects locally at the portal of entry. For example, a chemical that is an irritant or has corrosive properties would be expected to produce pulmonary effects when inhaled, gastrointestinal distress when ingested and dermal pathology when placed on the skin. Chemicals that act systemically

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can also produce route-dependent toxic effects because the concentrations of chemical and its metabolites in various tissues can differ, depending upon how the chemical enters the body. Duration of exposure is important as well. It is common for the principal adverse effects of a chemical to be different for acute vs. chronic exposure. For example, the principal concern from acute exposure to many solvents is central nervous system effects, while long-term exposure (usually at lower levels) can lead to kidney and liver toxicity. The effects of these solvents on the central nervous system are immediate, accounting for acute effects, while kidney and liver toxicity requires repeated exposure unless the exposure level is quite large.

4 DOSE–RESPONSE ASSESSMENT

Only studies in humans or animals are capable of providing dose–response information of sufficient reliability to use in risk assessment. Human studies provide this information most directly, but it is often difficult to get accurate dose estimates for exposed individuals. Further, the number of chemicals for which dose–response information is available from humans is quite limited. Consequently, as a practical matter, dose–response information used in most chemical risk assessments comes from animal studies.

Well-conducted hazard identification typically reveals that several different adverse effects are possible from exposure to a given chemical. Each effect can have its own distinct dose–response relationship, and one of the goals of the dose–response assessment is to identify the most sensitive effect of the chemical; that is, the effect that occurs at the lowest dose. From a regulatory standpoint, identifying the most sensitive effect is critical—if exposure is managed to prevent that effect, all other adverse effects will be prevented as well.

Most commonly, dose–response relationships are defined based on applied dose; that is, the dose that comes in contact with the subject, not the dose that enters the body. For example, for a study involving exposure by ingestion, the applied dose would be the amount swallowed (usually per unit time, such as milligram chemical per kilogram body weight per day, $mg\,kg^{-1}\,day^{-1}$), not the amount absorbed from the gastrointestinal tract and entering the systemic circulation (see Section 5 for a more complete discussion). Because the amount of chemical absorbed and distribution within the body can vary for different routes of exposure, dose–response relationships are route dependent. This means that dose–response relationship information is needed for each potential exposure route.

Two types of dose–response models are used in risk assessment—the threshold model and the nonthreshold model. In the threshold model, there is some dose below which no adverse effect is seen. This dose is termed the threshold dose. This model is based on the assumption that, for many chemicals, protective mechanisms exist in the body that repair or prevent the damage and stop the adverse effect from occurring. When the dose of chemical is sufficiently high, the defence mechanism is overwhelmed, resulting in an adverse effect. The threshold model is utilized whenever the mode of action is thought to involve a biological threshold. This includes some carcinogens and all effects other than cancer. The nonthreshold model, in contrast, assumes that any finite dose of the chemical poses some risk of the effect occurring, and attempts to provide an estimate of the probability of effect for a specified dose. Both models are described in the following sections.

4.1 Threshold Models

The goal of the dose–response assessment for the threshold model is to identify the threshold dose for the most sensitive effect of the chemical and use it to derive a safe dose for human exposure. Operationally, the first step in this process is to identify a dose that approximates the threshold for toxicity, usually with either the no observable adverse effect level (NOAEL)/lowest observable adverse effect level (LOAEL) or the benchmark dose (BMD) approach.

The highest dose tested for which no toxicity is observed is called the NOAEL (**Figure 1**). The lowest dose tested for which any toxicity is observed is called the LOAEL. The threshold dose is assumed to lie somewhere between these two values. So as not to overestimate the threshold dose, the NOAEL is considered to represent the threshold for toxicity from the experiment, and the safe human dose is derived from this value. In some situations, an NOAEL is not identified; all doses tested produce toxicity. Ideally, the experiment would be performed again using a lower dose range so that the NOAEL can be identified. In most such cases, additional testing is not available, and the safe human dose must be estimated from the information at hand, that is, using the LOAEL.

There are several criticisms of the NOAEL/LOAEL method. One criticism is that the NOAEL and LOAEL are determined to some extent by the doses chosen for the toxicity study. If the doses were chosen far apart, then the NOAEL may be well below the true threshold. This would lead to an overprotective safe human dose as the result of poorly chosen experimental doses. For example, suppose experimental doses of 5, 100, 500 and $1000\,mg\,kg^{-1}\,day^{-1}$ were used in a chronic oral dosing study. The $5\,mg\,kg^{-1}\,day^{-1}$ dose displayed no adverse effect and was chosen as the NOAEL. However, choosing the doses 5, 20, 50 and $75\,mg\,kg^{-1}\,day^{-1}$ may have revealed $50\,mg\,kg^{-1}\,day^{-1}$ as the NOAEL. The $5\,mg\,kg^{-1}\,day^{-1}$ NOAEL chosen based on the doses in

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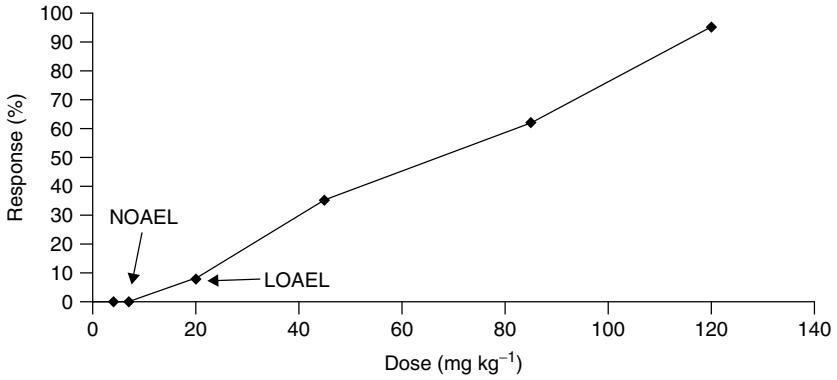

Figure 1 Dose–response relationship showing a no observable adverse effect level (NOAEL) and a lowest observable adverse effect level (LOAEL).

the first study would be at least 10 times less than the NOAEL that would arise from a different selection of doses. This would result in an overestimation of the toxicity threshold of the chemical and an unnecessarily low safe human dose. Another criticism is that the NOAEL/LOAEL method does not consider the shape of the dose–response curve or account for variability in the response. The safe human dose is derived from the NOAEL and, in essence, disregards the rest of the data points and the uncertainty surrounding them. The toxic dose of the chemical is estimated from one data point.

An alternative to the NOAEL/LOAEL approach is the BMD approach. In this approach, the shape of the dose–response curve is taken into consideration and the safe human dose is derived based on all of the response data, not just one point. The BMD approach uses mathematical models to find the curve that best fits the observed data points (**Figure 2**). This is most easily done with computer software (e.g. US EPA Benchmark Dose Software v. 2.0). Initially a benchmark response (BMR) is chosen. The BMR is the central dose estimate for a specified level of response above background. For quantal data, a BMR of 10% is usually chosen. Exceptions include a BMR of 5%, when evaluating reproductive and development studies, and a BMR of 1% for the analysis of human data. For continuous data, the BMR is defined as a 10% change in an endpoint (another term for something that is measured or assessed to show a response) that is generally considered to be biologically significant or a change in the mean value of this endpoint equal to one standard deviation from the control mean. The dose at which the BMR occurs is referred to as the effective dose (ED). For example, in a study of neurological disorders, a BMR of 10% is chosen. The dose where 10% of the study animals exhibit neurological dysfunction is referred to as the ED_{10}, or ED at an incidence of 10%. Upper and lower confidence limits are placed around the benchmark curve. For regulatory purposes, the lower confidence limit of the effective dose (LED_{10}) is chosen. The LED_{10} is known as the BMDL or the point of departure. It is used as the threshold dose below which less than 10% of the population is expected to have an adverse effect.

A BMD can be derived with three main types of data. The first is dichotomous data. These data reflect the presence or absence of an effect (e.g. an experiment in which five out of seven animals displayed kidney dysfunction). The second type of data is continuous. These data can take any value within a plausible range. Examples of endpoints yielding continuous data include body weight and sperm count. The third type of data is categorical and usually categorizes data based on the severity of the response. This is usually seen when rating histological changes (e.g. none, mild, moderate or severe tissue injury).

In order to derive an accurate estimation of the safe human dose using the BMD approach, a statistically or biologically significant dose-related trend in the data for the selected endpoint is needed. Because this method estimates the safe human dose from the dose–response curve, the software must be able to model curves that fit the data. Additionally, if there is no NOAEL in the data set, the lowest response level needs to be near the BMR. Data that have responses much greater than the BMR do not reveal the shape of the curve in the BMR region. Therefore, any shaped curve in the lower dose region would fit equally well and no additional information is gained from the software. In these cases it is more beneficial to use the NOAEL/LOAEL approach.

4.2 Application of Uncertainty Factors

Because the data available for safe human dose development usually suffer from a number of limitations, there is often considerable uncertainty whether the threshold dose approximation obtained from NOAEL/LOAEL or BMD analysis actually represents a no-effect level protective of all individuals. To address this, the approximated threshold dose may be reduced by application of one or

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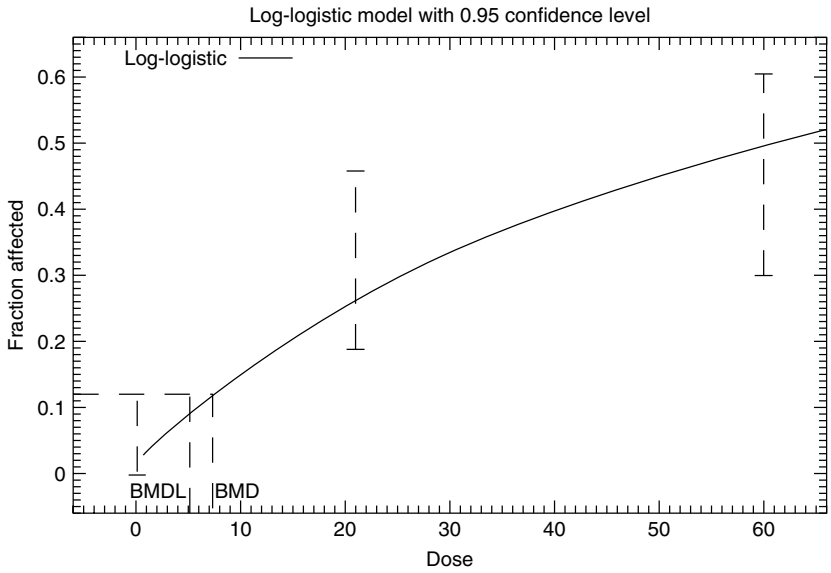

Figure 2 Benchmark dose curve displaying benchmark response. (Reproduced from U.S. Environmental Protection Agency, 2000.)

more 'uncertainty factors (UFs)'. Examples of UFs used by various agencies are shown in **Table 1**. Generally, the following types of UFs are used:

- A UF of up to 10 to extrapolate from animals to humans. This factor is used when animal studies are the source of the dose–response information and is meant to account for interspecies variability in toxicity and sensitivity to chemicals.
- A UF of up to 10 is used to account for sensitive subpopulations. The human population is full of variation, and different people may display widely different sensitivities to the same chemical. This factor is meant to insure that the safe human dose developed is protective for sensitive subgroups, such as the elderly, children and immunocompromised individuals.
- A UF of up to 10 is used if dose–response data are only available for a shorter exposure period than the one of interest; for example, if a safe human dose for chronic exposure is desired and suitable data are only available for subchronic exposure. For a number of chemicals, the threshold for toxicity decreases as the exposure duration increases. As a result, use of data from shorter-term studies could result in an overestimation of the threshold dose. This UF is intended to prevent that from occurring.
- A UF of up to 10 is used to when deriving a safe human dose from a LOAEL. Occasionally when the NOAEL/LOAEL method is used, a NOAEL is not available; that is, all of the doses tested produced toxicity. In this situation, the threshold dose must be approximated from the LOAEL. The additional UF

Table 1 Uncertainty factors

Uncertainty factors	US EPA	ATSDR	IPCS	Health Canada	Dutch RIVM
LOAEL to NOAEL	1–10	1–10	1–100	1–100	1–10
Subchronic to chronic	1–10	N/A			1–10
Insufficient database	1–10	N/A			N/A
Animal to human	1–10	1–10	1–10	1–10	1–10
Sensitive subpopulations	1–10	1–10	1–10	1–10	1–10
Modifying factors	N/A	N/A	N/A	1–5	N/A
Mutagen/teratogen	1–10	NA	1–10	1–10	N/A

US EPA—United States Environmental Protection Agency.
ATSDR—Agency for Toxic Substances and Disease Registry.
IPCS—International Programme on Chemical Safety.
RIVM—National Institute of Public Health and Environmental Protection.
NA—Not applicable.
Canadian Environmental Protection Act, 1994; IPCS, 1994; RIVM, 2001; Pohl and Abadin, 1995; US EPA, 2005.

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of up to 10 is used to reduce to LOAEL to a value closer to the no-effect level.

- A UF between 1 and 10 is used to reflect the quality of the toxicity database. This UF is applied if very few toxicity studies exist, the studies are poorly conducted, only a few animals were tested, or only a few toxic endpoints were examined. As the quality of the toxicity database decreases, the UF for this component increases. The number used for the assessment is based on professional judgement.

The UFs are multiplicative, meaning they are multiplied together to determine a final UF. For example, if the NOAEL for the most sensitive endpoint of a chemical is $5\,mg\,kg^{-1}\,day^{-1}$ and this was derived from multiple chronic animal studies, UFs for interspecies extrapolation and for sensitive human subpopulations could be applied. If UFs of 10 were chosen for both, the total UF would be 100 (10×10). The NOAEL of $5\,mg\,kg^{-1}\,day^{-1}$ is divided by the total UF (in this case 100) to arrive at the safe human dose of $0.05\,mg\,kg^{-1}\,day^{-1}$.

4.3 Expression of a Safe Dose Derived from a Threshold Model

The safe human dose is usually expressed as an amount of chemical per kilogram body weight per day ($mg\,kg^{-1}\,day^{-1}$), and is intended to represent an exposure level below which adverse health effects are not expected. The safe dose can vary depending upon the length of exposure, usually with the relationship: acute safe dose > subchronic safe dose > chronic safe dose. Acute exposures can be defined as those lasting less than two weeks, subchronic exposures as lasting between two weeks and one year and chronic exposures for time periods greater than one year. To avoid any confusion, the exposure period to which the safe dose is intended to apply should be specified. A variety of terms are used for the safe dose, depending upon the agency or organization developing and using them. Examples include 'acceptable daily intake' (World Health Organization), 'reference dose' (US Environmental Protection Agency (EPA)) and 'minimal risk level' (US Public Health Service).

Occasionally, a safe dose is not expressed as a dose at all, but as a concentration in an environmental medium such as water or air. In this case, the concentration is assumed to result in doses below those producing effects over a range of plausible exposure circumstances.

4.4 Nonthreshold Models

Nonthreshold models are generally reserved for carcinogens. In the absence of mode-of-action evidence to the contrary, risk of cancer is assumed to be a linear function of dose, with risk reduced to zero only when the dose is zero. Stated another way, any dose of a carcinogen, however small, is assumed to pose some risk of cancer, with the probability of cancer increasing as dose increases. The nonthreshold model for carcinogens originated with the study of radiation-induced cancer. It was observed that even very small doses of radiation could induce a DNA mutation and therefore had a finite risk of cancer associated with them. The assumption that any measurable dose has measurable risk has been extrapolated to all mutagenic carcinogens and to all carcinogens for which the mode of action is unknown. The absence of a threshold dose assumes that any mutation has the potential to be permanent and alter the phenotypic expression of the cell. These phenotypic changes could evolve and become cancerous.

Experimentally, it is difficult to detect reliably increased cancer rates below about 10% in laboratory-animal cancer bioassays, given the number of animals typically included in these studies and associated limitations in statistical power. Similarly, epidemiological studies have limited ability to detect low-frequency events, including cancer. As a consequence, when carcinogenicity is observed in a human or animal study, the cancer rates are substantial. For regulatory purposes, there is interest in identifying doses of chemicals that pose even small risks of cancer, below those that are seen experimentally or in human studies. This is an enormous challenge in risk assessment. When constructing a dose–response curve for cancer, the situation is one in which there is zero dose/zero risk on the left and doses producing relatively high, measurable cancer risks on the right, with only speculation as to what the shape of the dose–response curve looks like in between. There are several possibilities (**Figure 3**). The curve could bend in a supralinear manner, or be sublinear, even approaching a threshold. The effect of choice of shape of the curve in this region is profound, resulting in orders of magnitude differences in cancer risk estimates at low doses. With no way to determine the shape of the curve other than through theoretical arguments, this is one of the largest sources of uncertainty in cancer risk assessment. In the absence of a clear basis to choose one shape over another, the most common approach is to assume a linear relationship, which can involve simply drawing a straight line from the observed data points to zero.

4.5 Expression of Chemical Potency with a Nonthreshold Model

With the threshold model, a safe dose is one in which there is reasonably high confidence that adverse effects will not result. In contrast, with a nonthreshold model, all doses have some likelihood of producing adverse

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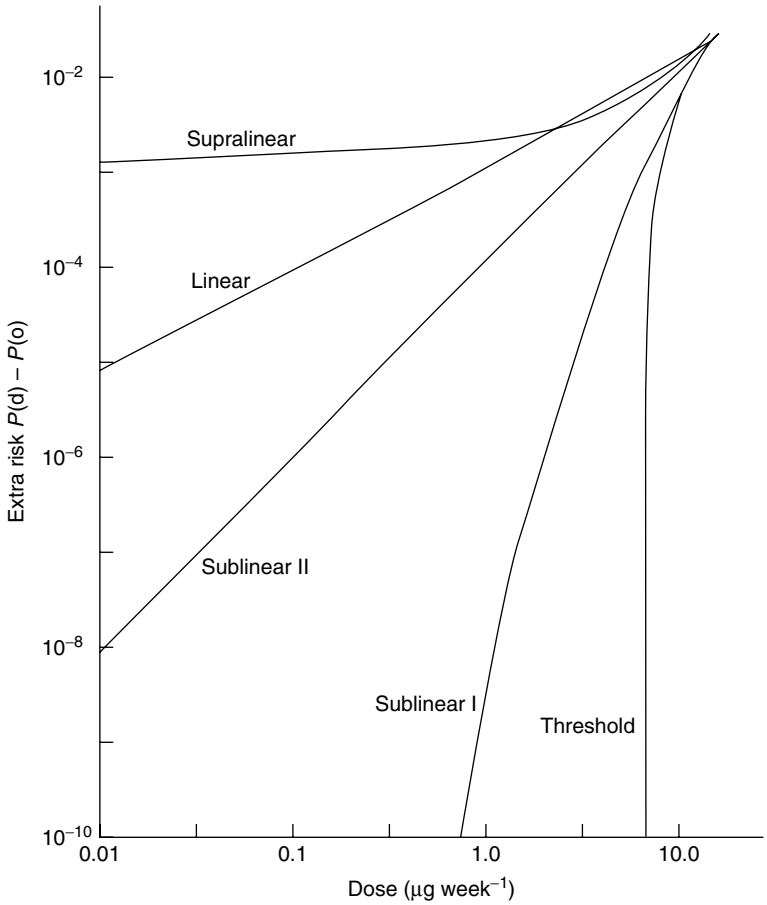

Figure 3 Results of alternative models of low-dose extrapolation of cancer risk. (From Risk Assessment in the Federal Government: Managing the Process, National Academy Press, Washington, DC, 1983.)

effects (cancer), so the concept of a safe dose must be defined differently. For the nonthreshold model, the dose–response assessment is used to determine the slope of the line that relates dose to excess probability of cancer. This value is sometimes termed the 'cancer potency factor' or 'cancer slope factor'. It is derived in such a way that the units are in reciprocal mg kg^{-1} day^{-1}. Concern for carcinogenicity is greatest when exposure is chronic. Consequently, dose–response relationships for carcinogenicity are nearly always based on long-term exposure, and in fact the cancer potency metric is generally based on lifetime exposure to the chemical. Although cancer potency could be different for shorter exposure periods, to date there has been comparatively little effort in deriving separate values for subchronic or acute exposure.

Despite the fundamental differences between toxicity values generated using threshold and nonthreshold models, there are some similarities. Toxicity values from both models (i.e. either safe doses or expressions of cancer potency) are route dependent. That means that for comprehensive evaluation of cancer risk to a chemical, separate cancer potency values are needed for each of the routes of exposure (e.g. ingestion, inhalation, dermal uptake). Also, in analysing cancer dose–response data to determine the lower end of the observation range from which extrapolation to zero should occur (termed the 'point of departure'), a process analogous to the BMD approach for threshold model data is often used. Finally, for both models, the toxicity value is sometimes expressed in concentration terms instead of an actual dose. In the case of the nonthreshold model, this takes the form of unit risk values, or concentrations in air or water corresponding to specified excess cancer risks. As with safe concentrations developed using threshold models, some assumptions regarding the extent of exposure to these concentrations, such as intake rate, must be made in order to use unit risk values.

5 EXPOSURE ASSESSMENT

Exposure assessment is an evaluation of the magnitude, frequency, duration and route of contact an individual (sometimes termed a receptor) has with the chemical. The exposure assessment is an integral part of risk assessment because, without exposure, there is no risk. For example, if soil contaminated with lead is placed

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under a maintained concrete cap, there is no contact with the soil and no chance of exposure. As long as the soil and cap remain undisturbed, there is no risk, even though high levels of lead exist in the soil. The components of an exposure assessment are discussed below.

5.1 Pathways and Media

An exposure pathway consists of four parts: (i) a source of contamination, (ii) contaminated media, (iii) contact with the contaminated media and (iv) route of exposure. Without all four of these elements, the pathway is incomplete and there is no risk to the receptor. Soil, sediment, groundwater, surface water, air and biota all function as receiving media. They become contaminated directly by a release or are indirectly contaminated through contact with other contaminated media. Relevant exposure pathways can be visualized through construction of a conceptual site model (see example in **Figure 4**). The conceptual site model conveys in concise form the source(s) of chemical release and the exposure pathways that will be considered in the risk assessment. Depending upon the level of detail, the conceptual model may also show the routes of exposure considered to be relevant. The route of exposure is the potential site of chemical entry into the body. The most common routes for environmental exposures are ingestion, inhalation and dermal absorption.

5.2 Quantifying Exposure

There are two main approaches for quantifying exposure: direct measurement and modelling. Direct measurement is usually the most accurate approach. Exposure can be quantified, for example, by analysing food intake, measuring urine output of the chemical or its metabolites or by using monitoring devices. These types of measurement are most common in controlled studies or occupational scenarios where exposure can be confined. However, in most situations, the exposure has occurred without the benefit of direct measurements. In these situations, exposure can be estimated through modelling. Fate and transport models are used to simulate the behaviour and breakdown of chemicals in the environment and estimate chemical concentrations in various media. They can estimate processes such as transport, loss, partitioning, bioaccumulation, decay and transformation. Other models or equations utilize these media concentrations to estimate intake and provide an approximate dose of chemical received during the exposure period.

Fate and transport modelling uses mathematical models to predict media concentrations, contaminant plume boundaries, effectiveness of remedial actions and time to natural attenuation. Hundreds of models exist for these purposes, including atmospheric models, surface water models, groundwater models and food-chain models. These models can be linked to integrate several media

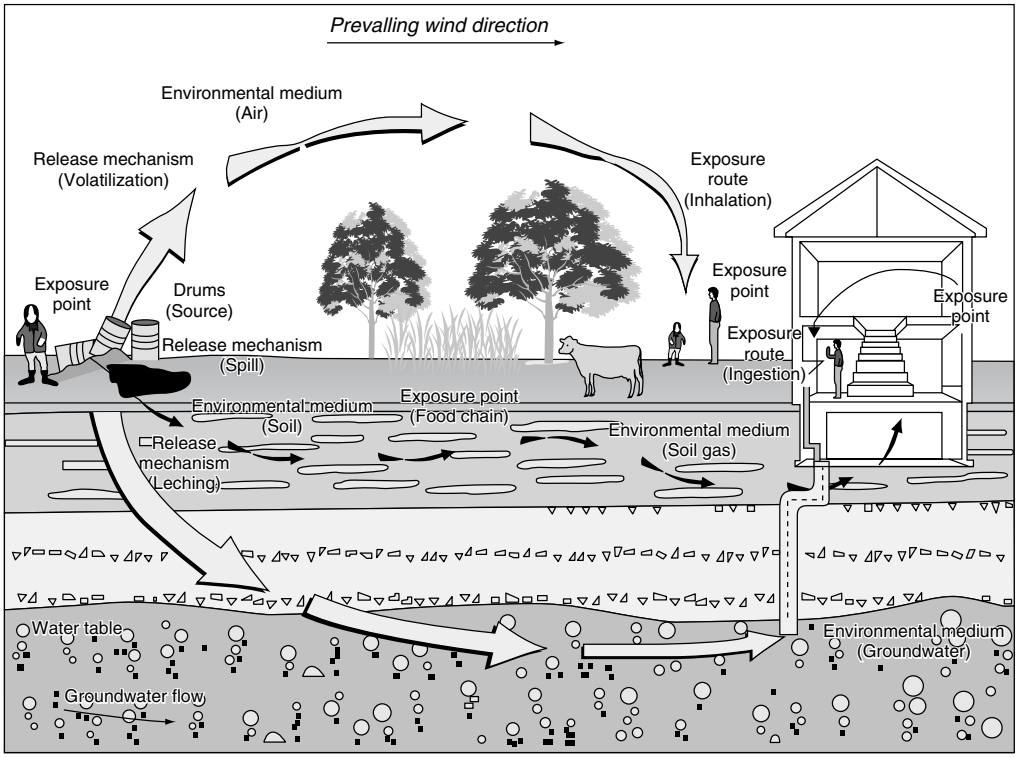

Figure 4 Example of a Conceptual Site Model. (Reproduced from U.S. Department of Health and Human Services, 1992.)

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and chemical processes together resulting in an approximation of chemical concentrations and dose received over space and time. Inputs to the model may include the concentration at the source area, chemical/physical properties of the contaminant, meteorological data, geological data and hydrological data. These models are based on assumptions and often have a large degree of uncertainty in the results. To account for this uncertainty, models tend to use parameters that may overestimate the risk. This prevents an underestimation of risk and the occurrence of adverse effects at environmental concentrations that were predicted to be without toxic effects.

5.3 Calculating Dose

Dose is the amount of chemical that is available to be absorbed into the body where it can interact with the target tissue. Dose is usually expressed per unit time, per unit body weight or both. The dose can be calculated using exposure factors. Exposure factors are variables that describe the exposed population (e.g. body weight, ingestion rate and surface area). They can be obtained from guidance such as the *Exposure Factors Handbook* (US EPA, 1997), published literature or site-specific assessments. Exposure factors will differ depending upon the population exposed (e.g. adults, children, workers) and the specific use of the contaminated area (e.g. residential or industrial). The following section provides example dose equations for some common exposure pathways.

5.3.1 Inhalation

$$\text{Dose (mg kg}^{-1}\text{d}^{-1}) = \frac{\text{CA} \times \text{IR} \times \text{EF} \times \text{ED}}{\text{BW} \times \text{AT} \times (\text{VF} \times \text{PEF})} \quad (1)$$

where

CA = chemical concentration in air (mg m^{-3})
IR = inhalation rate (m^3 day^{-1})
EF = exposure frequency (days year^{-1})
ED = exposure duration (years)
BW = body weight (kg)
AT = averaging time (days)
VF = chemical-specific volatilization factor (m^3 kg^{-1})
PEF = chemical-specific particulate emission factor (kg m^{-3})

5.3.2 Ingestion of Groundwater

$$\text{Dose (mg kg}^{-1}\text{d}^{-1}) = \frac{\text{CW} \times \text{IR} \times \text{EF} \times \text{ED}}{\text{BW} \times \text{AT}} \quad (2)$$

where

CW = chemical concentration in water (mg l^{-1})
IR = ingestion rate (l day^{-1})
EF = exposure frequency (days year^{-1})
ED = exposure duration (years)
BW = body weight (kg)
AT = averaging time (days)

5.3.3 Ingestion of Soil

$$\text{Dose (mg kg}^{-1}\text{d}^{-1}) = \frac{\text{CS} \times \text{IR} \times \text{RBA} \times \text{EF} \times \text{ED}}{\text{BW} \times \text{AT}} \quad (3)$$

where

CS = chemical concentration in soil (mg kg^{-1})
IR = ingestion rate (kg day^{-1})
RBA = chemical-specific relative bioavailability
EF = exposure frequency (days year^{-1})
ED = exposure duration (years)
BW = body weight (kg)
AT = averaging time (days)

5.3.4 Dermal Contact with Water

$$\text{Absorbed Dose (mg kg}^{-1}\text{d}^{-1}) = \frac{\text{CW} \times \text{SA} \times \text{PC} \times \text{ET} \times \text{EF} \times \text{ED} \times \text{CF}}{\text{BW} \times \text{AT}} \quad (4)$$

where

CW = chemical concentration in water (mg l^{-1})
SA = skin surface area available for contact (cm^2)
PC = chemical-specific dermal permeability coefficient (cm hour^{-1})
ET = exposure time (hours day^{-1})
EF = exposure frequency (days year^{-1})
ED = exposure duration (years)
CF = conversion factor (1 l 1000 cm^{-3})
BW = body weight (kg)
AT = averaging time (days)

5.3.5 Dermal Contact with Soil

$$\text{Absorbed Dose (mg kg}^{-1}\text{d}^{-1}) = \frac{\text{CS} \times \text{SA} \times \text{AF} \times \text{DA} \times \text{EF} \times \text{ED} \times \text{CF}}{\text{BW} \times \text{AT}} \quad (5)$$

where

CS = concentration in soil (mg kg^{-1})
SA = skin surface area available for contact (cm^2 day^{-1})
AF = adherence factor for soil (mg cm^{-2})

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DA = dermal absorption
EF = exposure frequency (days year^{-1})
ED = exposure duration (years)
CF = conversion factor (10^{-6} kg mg^{-1})
BW = body weight (kg)
AT = averaging time (days)

Dose can be summed across intake routes and media to estimate the total chemical intake per day. Time-weighted averages are commonly used when estimating dose. The time period over which the dose is averaged depends upon the length of exposure and the toxicity value that will be used to evaluate risk (i.e. acute, subchronic, etc.). For chemicals evaluated using the threshold model, the average is taken over the exposure period. For carcinogens evaluated using the nonthreshold model, cancer potency is expressed in terms of lifetime exposure (see Section 4.4). Consequently, to correspond with the toxicity value, the average dose used to estimate cancer risk should be a lifetime average. If exposure occurs for less than a lifetime, a time-weighted average that includes both exposure periods and nonexposure periods is derived. This approach basically assumes that cancer risk is a function of the cumulative cancer dose during a lifetime rather than a dosing rate during exposure (as is the case with the threshold model). With this view, for example, exposure to a chemical at a given rate for a lifetime is assumed to pose a cancer risk equal to exposure at twice that rate for half a lifetime. Experimental data to support this as a general assumption are equivocal at best, and this is another source of uncertainty in cancer risk assessment.

There are a variety of ways that dose can be expressed and it is important to understand the distinction among them. The dose metric that is likely to correlate best with response is the concentration of the chemical in the target organ or tissue, perhaps as a function of time. Measuring target organ tissue concentrations over time is difficult to do in animal studies and impossible in human studies, although it can be modelled (see Section 5.4). Provided the most relevant dose metric is known (concentration, area under concentration versus time curve, etc.) this is the optimum way of expressing dose. A more indirect way of expressing dose is the internal or delivered dose. This is the dose that is absorbed and reaches the systemic circulation. Although the precise relationship between the internal dose and target tissue concentration may be unknown, they are assumed to be proportional in some way. One of the most direct means of measuring absorbed dose is to measure the concentration of chemical in blood. This is commonly done in the clinical evaluation of potential toxicity and is used widely, for example, in evaluating risk from lead exposure in children. The internal dose can also be modelled, provided that information is available regarding the bioavailability of the chemical from environmental media for each of the routes of exposure. More indirect still is the external or applied dose. This is the dose that is ingested, inhaled or placed on the skin (as appropriate for the route of exposure). Unless toxicity is produced directly at the portal of entry into the body (e.g. irritation of the gastrointestinal tract, lung or skin), this dose is removed by at least two steps (absorption into the body and distribution to the target organ) from the target tissue concentration. The applied or external dose has one important advantage, however—it is comparatively easy to measure or estimate. Also, dose–response relationships for the vast majority of chemicals are developed using applied doses, and the dose(s) obtained from the exposure assessment need to match in order to be useful.

5.4 Physiologically-based Pharmacokinetic Models

Pharmacokinetic models (also termed toxicokinetic models when their application is in toxicology) can be used to predict chemical concentrations in target organs or tissues within the body resulting from exposure. They can serve to relate exposure measured in the form of applied doses with internal concentrations more directly related to toxicity. Further, they can be used to explore how applied dose–concentration relationships differ among species, reducing uncertainty in extrapolation of toxicity information from one species to another, including humans. They can also be used to extrapolate toxicity information obtained for one route of exposure to address exposure from other routes.

Physiologically-based pharmacokinetic (PBPK) models are mathematical models constructed to represent key anatomical compartments in the body and the flow of blood that move chemicals and their metabolites from one compartment to another. **Figure 5** shows an example of a PBPK model. The model is designed to allow input from relevant routes of exposure (in the case of **Figure 4**, from ingestion, inhalation and dermal contact) and account for elimination from the body through metabolism and excretion. Data from bioavailability studies, when available, are used to estimate the fraction of applied dose entering the systemic circulation. Flow of blood to compartments is based on physiological data. Chemical-specific data on partitioning and metabolism are used to predict movement of a chemical in and out of the compartments and conversion to metabolites. From these models, concentration of chemical and metabolite can be predicted over time as a result of specified exposures. Models are constructed for each species of interest, and model comparisons can be used to extrapolate doses across species, and as part of a determination of the appropriate interspecies extrapolation UF to use in toxicity value development.

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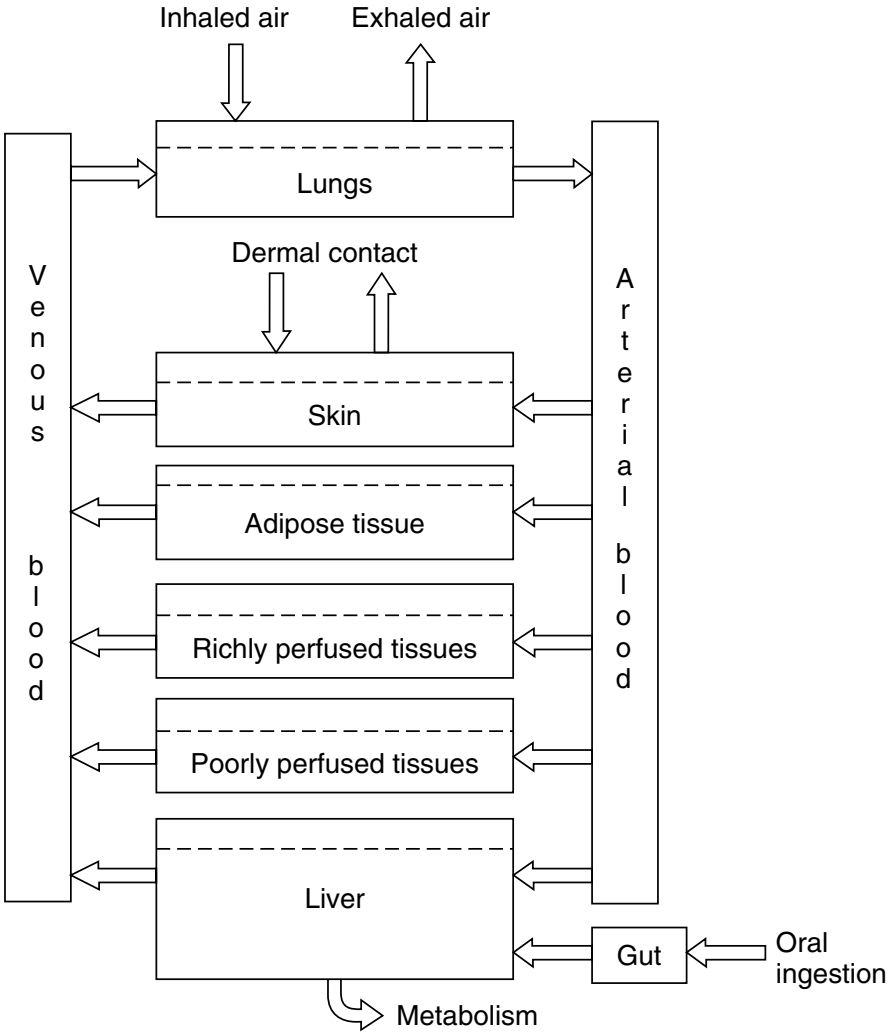

Figure 5 Example of a physiologically-based pharmacokinetic model. (Reproduced from U.S. Environmental Protection Agency, 2006.)

6 RISK CHARACTERIZATION

Risk characterization combines results from the toxicity and exposure assessments to develop a risk estimate. Risk estimates may be qualitative, quantitative or both. Qualitative risk characterization gives a general estimation as to whether the risks are high or low, but it does not provide numerical estimates. For quantitative characterization of risk, the manner of characterization depends upon the dose–response relationship thought to exist for the chemical. For threshold effects, quantitative risk characterization takes the form of a ratio involving a comparison of the estimated dose with the no-effect level or safe human dose for the chemical. When a nonthreshold approach is used, risk is expressed as a probability that the effect will occur as a result of exposure, for example, the excess cancer risk associated with exposure to a carcinogen at a specified level.

6.1 Risk Characterization for Threshold Effects

When a chemical is thought to have a toxicity threshold, one approach to characterizing risk is to calculate the ratio between the estimated dose and the safe human dose. For example, the US EPA calculates a hazard quotient as follows:

$$\mathrm{HQ} = \frac{\mathrm{D}}{\mathrm{RfD}} \tag{6}$$

where

HQ = hazard quotient
D = dose from exposure ($\mathrm{mg\,kg^{-1}\,day^{-1}}$)
RfD = reference dose ($\mathrm{mg\,kg^{-1}\,day^{-1}}$) (a safe human dose in US EPA terminology)

If the HQ is less than one, the dose received is less than the safe human dose and no adverse effects are

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expected to occur. However, if the HQ is greater than one, the possibility of adverse effects is indicated. Often, that is sufficient to stimulate regulatory action. It does not necessarily mean that toxicity is likely, however. It can only be said that the chance of toxicity increases as the HQ increases. A more precise characterization of risk in terms of probability of adverse effects is difficult because safe human doses are inexact. As discussed in the section on dose–response relationships, safe human doses are based on an observed no-effect dose and one or more UFs intended to provide a margin of safety. Where the actual threshold for toxicity lies within that margin of safety for an individual is unknown, and therefore, the extent to which the safe dose can be exceeded without producing harm is unknown. Once a quantitative determination has been made that exposure results in a HQ above one, further characterization of the risk is largely qualitative. A dose marginally above the safe human dose would not likely produce toxicity, especially if the margin of safety built into the safe human dose through application of UFs is large. On the other hand, exposure producing a dose above the safe human dose and approaching or exceeding the observed no-effect level for the chemical might be viewed as 'likely' or 'very likely' to produce toxicity.

Safe doses can vary among different routes of exposure. If an individual is exposed to a chemical by more than one route of administration, a ratio of the dose from exposure to the applicable safe human dose for each route should be calculated. For the sake of simplicity, these ratios are typically added to convey the risk to an individual from exposure to the chemical from combined routes. Following through with the example using US EPA terminology, hazard quotients from relevant routes of exposure would be summed to derive an overall ratio called a hazard index (HI):

$$HI = HQ_1 + HQ_2 + \ldots + HQ_n \tag{7}$$

where

HI = hazard index
HQ = hazard quotient

Interpretation of an HI is the same as for an HQ: if the value is equal to or less than one, exposure is unlikely to produce an adverse health effect. An HQ greater than one may trigger regulatory action, but the approach does not offer a quantitative measure of the likelihood of toxicity.

A second approach is to calculate a ratio of the no-effect level with the estimated dose from exposure. For example, the BMD (or lower confidence limit estimate of the BMD) for a sensitive effect can be used as an expression of the no-effect dose and divided by an estimate of exposure to produce what is termed the margin of exposure (MOE):

$$\text{Margin of exposure} = \frac{\text{BMD}}{\text{D}} \tag{8}$$

where

$D = \text{dose } (\text{mg kg}^{-1}\,\text{day}^{-1})$

The MOE indicates the extent to which estimated exposure is above or below the observed no-effect level. Unlike the HI, where numbers less than one indicate safety, low risk is conveyed by high MOEs. An acceptably high MOE depends in part upon confidence in the toxicity data used to derive the BMD. In general, the same kinds of considerations as in the selection of UFs for a safe human dose apply. For example, a greater MOE would be likely to be required if the BMD is derived from animal data vs. studies in humans.

6.2 Risk Characterization for Nonthreshold Effects

Because the dose–response relationship for most carcinogens is assumed to follow a linear, nonthreshold model, risk is characterized in a way fundamentally different from threshold chemicals. For these carcinogens, risk is conveyed as the lifetime excess probability of developing cancer as a result of exposure. Risk is calculated from an expression of cancer potency, such as a cancer slope factor, and an estimate of dose received from exposure:

$$\text{Risk} = \text{CSF} \times \text{D} \tag{9}$$

where

$\text{CSF} = \text{cancer slope factor } (\text{mg}^{-1}\,\text{kg day})$
$D = \text{dose } (\text{mg kg}^{-1}\,\text{day}^{-1})$

As with risks from threshold effects, cancer risks are summed for each relevant route of exposure.

6.3 Addressing Risk from Exposure to Multiple Chemicals

In animal toxicity studies, exposure usually occurs to a single chemical. Real-life chemical releases are seldom this simple and often involve simultaneous exposure to many chemicals. These chemicals may interact in a synergistic or antagonistic manner to increase or decrease the toxicity of the chemicals involved. The interactions within a chemical mixture can be even more complex, with synergistic, antagonistic and additive effects all occurring at the same time. These interactions may be chemical in nature, but can also be biological and affect

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the ability of an organism to metabolize or interact with other chemicals. Current risk assessment techniques have limited ability to account for such interactions. In cases where the interaction has been studied and is well known, it can be addressed by modifying the toxicity values. However, these interactions are usually not known and are dependent not only upon the other chemicals present, but also upon their concentrations (doses).

In the absence of both qualitative and quantitative information on the existence of an interaction among chemicals present, it is usually assumed that the effects of chemicals in combination are additive. Commonly, risk for threshold chemicals is calculated by summing the HIs for the individual chemicals. The sum of these ratios gives an indication of the overall relationship between estimated and safe levels of exposure. A more refined approach sometimes used is to combine HIs only for chemicals that produce the same type of toxicity, affect the same target organ, or have the same mode of action. The rationale for this approach is that additive effects are most likely to occur when chemicals share the same target or affect biological systems the same way. The interpretation of the HI is the same as described above. An overall HI less than one for two or more chemicals indicates that together they are unlikely to produce an adverse effect. An HI greater than one indicates that adverse effects are possible, but it does not indicate the probability of an adverse effect. For carcinogens, the total excess risk of cancer is simply the sum of the cancer risk for each chemical.

Some combinations of chemicals are found together commonly, such as chemicals present in commercial mixtures or closely related chemical structures. For these mixtures, there are three basic approaches to determining the additive effects, as described in the following sections.

6.4 Testing the Mixture of Concern

The simplest way of obtaining the toxicity of a mixture with the least amount of uncertainty is to test the mixture in an *in vivo* animal toxicity study. This direct method determines how the mixture interacts within a biological system to produce toxicity. In this approach the mixture is actually treated as a single chemical because no information is obtained on the chemical or biological interactions of the components. Unfortunately, there are thousands of mixtures and every possible combination of chemicals and their relative percentage within the mixture cannot be tested. Therefore, it is not unusual to have a mixture of concern for which no toxicity data exists. In these cases, other methods must be used to estimate the toxicity of the mixture.

If two mixtures are similar in composition, then the toxicity value for one can be used as a surrogate value for the second. There is no official guidance on how to define a similar mixture, so the use of a surrogate mixture is usually left to professional judgement and can vary among risk assessors and regulatory agencies. A surrogate mixture containing the same chemicals, but in different proportions is usually preferable to mixtures including different chemicals. The introduction of additional chemicals could change the chemical and biological interactions within the body and, in turn, alter the toxicity of the mixture.

Another method for estimating the toxicity of mixtures in the absence of *in vivo* toxicity data is the application of a PBPK model to the mixture of concern. PBPK models can utilize biological information to predict chemical interactions within the body. In these models, equations that specifically account for interactions among the chemicals within a tissue compartment are integrated into the model. The PBPK modelling predicts the magnitude and type of interactions based on individual chemical concentrations and mode of toxicity.

6.5 Dose Addition

For dose addition, the chemicals within the mixture do not require similar dose–response curves or similar toxicities. The dose addition approach is identical to the HI approach. It is the most commonly used approach in assessing the toxicity of mixtures. In this approach, the hazards for individual chemicals with the same target organ are added together. The default assumption for this method is that there is no interaction among chemicals. Consequently, this method does not take into account the synergistic or antagonistic effects that may arise from chemical interaction within the body. However, in a biological system, the absorption, distribution, metabolism and excretion of one chemical can affect the internal dose or the toxic mechanism of action for other chemicals within the mixture. The more chemicals a mixture contains, the greater the likelihood that a nonadditive interaction is possible. Therefore, the estimated toxicity for a mixture increases in uncertainty as the number of chemical components increases.

When different chemicals in the mixture have a similar mode of action, their relative potencies can be used to assess the toxicity of the mixture. An example of this method is the toxicity equivalency factor (TEF) approach utilized for chemicals with dioxin-like toxicity. In this approach, the potency of dioxins, furans and polychlorinated biphenyls (PCBs) are expressed relative to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD). The concentration of each congener is then multiplied by its relative potency factor and is given as a 2,3,7,8-TCDD equivalent concentration. The toxicity of the sum of the 2,3,7,8-TCDD equivalent concentrations is an approximation of the toxicity of the mixture. TEFs for mammals,

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Table 2 Toxicity equivalency factors (TEFs) for mammals, birds, and fish

Congener	Mammal TEFs	Bird TEFs	Fish TEFs
2,3,7,8-TCDD	1	1	1
1,2,3,7,8-PeCDD (pentachlorodibenxo-*p*-dioxin)	1	1	1
1,2,3,4,7,8-HxCDD (hexachlorodibenzo-*p*-dioxin)	0.1	0.05	0.5
1,2,3,6,7,8-HxCDD	0.1	0.01	0.01
1,2,3,7,8,9-HxCDD	0.1	0.1	0.01
1,2,3,4,6,7,8-HpCDD (heptachlorodibenzo-*p*-dioxin)	0.01	<0.001	0.001
OCDD (octachlorodibenzo-*p*-dioxin)	0.0003	0.0001	<0.0001
2,3,7,8-TCDF (tetrachlorodibenzofuran)	0.1	1	0.05
1,2,3,7,8-PeCDF (pentachlorofibenzofuran)	0.03	0.1	0.05
2,3,4,7,8-PeCDF	0.3	1	0.5
1,2,3,4,7,8-HxCDF (hexachlorodibenzofuran)	0.1	0.1	0.1
1,2,3,6,7,8-HxCDF	0.1	0.1	0.1
1,2,3,7,8,9-HxCDF	0.1	0.1	0.1
2,3,4,6,7,8-HxCDF	0.1	0.1	0.1
1,2,3,4,6,7,8-HpCDF (heptachlorodibenzofuran)	0.01	0.01	0.01
1,2,3,4,7,8,9-HpCDF	0.01	0.01	0.01
OCDF (octachlorobibenzofuran)	0.0003	0.0001	<0.0001
3,3′,4,4′-TCB (tetrachlorobenzene) (77)	0.0001	0.05	0.0001
3,4,4′,5-TCB (81)	0.0003	0.1	0.0005
3,3′,4,4′,5-PeCB (pentachlorobenzene) (126)	0.1	0.1	0.005
3,3′,4,4′,5,5′-HxCB (hexachlorobenzene) (169)	0.03	0.001	0.00005
2,3,3′,4,4′-PeCB (105)	0.00003	0.0001	<0.000005
2,3,4,4′,5-PeCB (114)	0.00003	0.0001	<0.000005
2,3′,4,4′,5-PeCB (118)	0.00003	0.0001	<0.000005
2′,3,4,4′,5-PeCB (123)	0.00003	0.0001	<0.000005
2,3,3′,4,4′,5-HxCB (156)	0.00003	0.0001	<0.000005
2,3,3′,4,4′,5′-HxCB (157)	0.00003	0.0001	<0.000005
2,3′,4,4′,5,5′-HxCB (167)	0.00003	0.0001	<0.000005
2,3,3′,4,4′,5,5′-HpCB (heptachlorobenzene) (189)	0.00003	0.0001	<0.000005

From the WHO, 2005.

birds and fish utilized by the World Health Organization are shown in **Table 2**.

6.6 Response Addition

Response addition is used when the mixture components do not necessarily have the same target organ and are believed to act through independent modes of action. In this situation, dose addition would not give an accurate estimation of the toxicity of the mixture since the different modes of action may not result in an additive toxicity. The most common use of response addition is to estimate total cancer risk from mixtures. In the response addition approach, the percentage of humans expected to develop toxicity at the exposure dose is estimated for each chemical in the mixture. These responses for each chemical are then summed to approximate the percentage of the population that can be expected to develop toxicity from exposure to the mixture at a specific concentration. This method is useful when the risks to each component are relatively small, but does not work well when one of the component risks is large. Similar to the dose addition approach, response addition does not account for synergistic or antagonistic interactions among the chemicals within the mixture. It assumes that the response to one component is the same no matter what other chemicals are present in the mixture. This may not necessarily be the case. However, interactions among chemicals in a mixture are not easy to characterize and assess. Currently, methods to routinely account for these interactive effects do not exist in risk assessment.

7 UNCERTAINTY ANALYSIS

An uncertainty analysis is a critical aspect of any risk assessment. Because of the extensive use of models and assumptions in calculating risks, the resulting risk values are inherently imprecise. In order to use these risk values effectively to assist sound risk management, it is important to describe as transparently as possible where the uncertainties lie and, if possible, the magnitude of error that may be associated with them. Ideally, an uncertainty analysis includes an exploration of alternative choices on models and assumptions, and a description of the impact that each selection had on the estimated risk

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(e.g. a statement that the choice of model X instead of model Y would result in a lower risk by 1–2 orders of magnitude). It is usually sufficient to focus the discussion of uncertainty on the model and input decisions that have the greatest effect on the risk output. A sensitivity analysis can be used to identify these key choices. In a probabilistic risk assessment (PRA), the contribution of various types of uncertainty can be addressed formally in the risk output, as described in the following section.

8 DETERMINISTIC VS. PROBABILISTIC RISK ASSESSMENTS

There is both variability and uncertainty associated with each input in the risk formulas described above. Variability describes the natural variation that occurs within a population. For example, measurement of the body weight of 100 adults might result in a range of weights between 45 and 120 kg. This range reflects the natural differences in body weight among this sample of the adult population and a range of results is expected for this parameter. Uncertainty arises from a lack of knowledge. Utilizing the same example, if body weight data were generated for this group of 100 adults by asking them their weight, some error is expected. This error could arise because they do not know their body weight with certainty, or because they might be less than candid about the value. Regardless of the cause, the possibility of error leads to uncertainty in the body weight estimates. This uncertainty could be reduced by actually weighing each individual, producing more accurate values. This is an important distinction between uncertainty and variability. Variability is an inherent property of the population while uncertainty can be reduced by additional examination, study or research.

There is uncertainty associated with both risk models and inputs into the formulas. With respect to inputs, uncertainty can arise due to measurement error, random error or systematic error. An example of measurement error is inaccuracy in laboratory measurements of chemical concentrations. Each chemical assay has limits in terms of accuracy and reproducibility that contribute to uncertainty in the chemical concentration values used in the risk assessment. Random error can occur due to limitations in the number of data points available for analysis in the risk assessment and their representativeness. Using the body weight example from the previous paragraph, body weight data from the 100 individuals could be used to estimate the distribution in body weight for the general population. However, there would be uncertainty whether this subpopulation is representative of the whole. Systematic error arises from a fundamental flaw in the data-gathering process. An example of systematic error would be chemical measurements using a faulty analytical standard.

The accuracy of the risk models is the source of several additional uncertainties. Models can be too simple and fail to capture all of the important facets of the processes they are intended to represent. They can also be conceptually incorrect, based on mistaken ideas about the relationships among component variables. Uncertainty can be introduced into the model by the use of surrogate variables. For example, estimation of cancer risk from chemical exposure in humans requires a potency value. Usually, these are not available from human studies, so a value derived from animal studies is used as a surrogate. The use of potency information derived from animals to represent cancer potency in humans contributes to uncertainty in the cancer risk the model predicts. Another source of uncertainty lies with excluded variables. These are variables that were not included in the model for the sake of simplicity. If their influence on model outcome is small, the omission of these variables can be justified. However, without including them in the risk assessment, it may be difficult to know with certainty that their contribution to risk is in fact minor. Yet another source of uncertainty in the use of models is their ability to deal with abnormal conditions. As a practical matter, it is desirable to keep models as simple as possible without sacrificing their predictive value. Although a model might seem to work well in its usual application, there can be uncertainty whether it is able to function effectively if unusual circumstances arise. A final source of uncertainty is incorrect model form; that is, knowing whether your model is structured correctly. In exposure analysis, different models can give different answers, for example in estimating the concentration of a chemical in air downwind from an emission source. In choosing output from one model over others to calculate risk, uncertainty is introduced as to whether the most accurate model was selected. In the dose–response assessment, the choice of model for extrapolation of responses in the low-dose range can have a dramatic influence on the risk estimates generated, and is an important source of uncertainty.

Risk assessments have to deal with both variability and uncertainty. Deterministic risk assessments take the simplest approach, selecting a single set of models and using a single value (sometimes called a point estimate) for each input parameter. The result is a solitary risk number for each exposure scenario considered. Given the variety of models that might be available and the range of inputs possible given both variability and uncertainty for each parameter, the choice of model and input values is critical in determining the risk output.

For any given exposure scenario (e.g. inhalation of a chemical in workplace air or contact with contaminated soil in a residential yard), differences in the extent of

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exposure among individuals is expected. As a consequence of differing exposure, risk posed by the chemical to different individuals will vary accordingly. For regulatory agencies, there will be an interest in characterizing the risk to individuals that are among the most highly exposed so that risk management strategies that are broadly protective can be developed. As a result, risk assessments performed for regulatory agencies generally include risk estimates developed using inputs that reflect the upper-end of the plausible range for a number of parameters. In order to avoid producing extreme (and unrealistic) estimates of exposure and risk, a combination of median and upper percentile values are selected for various parameters, such that together they result in a high-end, but reasonable, risk estimate.

There is a similar consideration in the choice of models used to generate risk values for regulatory purposes. If a clear choice of model cannot be made on scientific grounds, then the model that produces the highest estimates of exposure and risk will be selected. The reasoning for this is that regulatory decisions that protect public health cannot be made if risks are underestimated. To avoid underestimating risk, models producing higher risk estimates are selected. Choices in models and inputs that err on the side of overestimating risk are termed conservative.

The conservatism inherent in deterministic risk assessment is not easily quantified and this creates some controversy in the use of single point estimates for the calculation of risk. Uncertainty is also not well defined in a deterministic risk assessment. A point estimate must be chosen and the uncertainty surrounding this choice is usually reduced to a discussion in the uncertainty section of the risk assessment. Also, point estimates derived from largely uncertain values are given as much weight as those derived from well-known values.

PRA is a method for addressing some of the inherent problems with deterministic risk assessment. In the PRA, the exposure variables are defined as probability distributions. These distributions indicate the probability of each exposure value occurring within the population. The distributions can be based on variability or uncertainty. The distributions represent the range of variability or uncertainty in exposure and provide more complete information for the estimation of risk. For example, instead of using a single body weight of 70 kg in the risk calculation, a distribution of adult body weights is entered that reflects the variability within the population (**Figure 6**). The probability distribution functions (PDFs) from the exposure variables are entered into a computer and a simulation is run. In the most common type of simulation, a Monte Carlo simulation, the computer selects a variable at random from each of the PDFs and uses those chosen point estimates to calculate risk. That risk value is stored and the process is repeated a minimum of several thousand times to show how risk

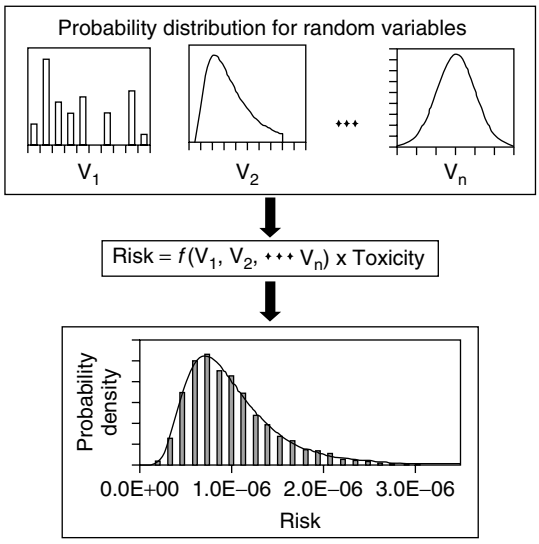

Figure 6 Probabilistic risk assessment input distributions and risk distribution output. (Reproduced from U.S. Environmental Protection Agency, 2001.)

is distributed within a population. An upper-end value (such as the 95th percentile) from this distribution is then chosen to represent the upper bound estimate of risk for the population. This number is usually compared against risk targets to determine if the exposure is of concern.

PRAs can address both variability and uncertainty simultaneously. This type of PRA is known as a two-dimensional PRA. Two-dimensional PRAs require the development of distributions for *both* variability and uncertainty for each exposure variable. While conceptually simple, separation of uncertainty from variability may not be easy in practice. The variability and uncertainty PDFs are then combined to generate a risk distribution with confidence boundaries defined by the uncertainty distributions. Another type of PRA that addresses both variability and uncertainty is called a fuzzy PRA. Fuzzy PRAs define uncertainty and variability as interval functions and then combine them in a similar manner to the two-dimensional PRA.

Variability and uncertainty exist for toxicity values as well as exposure parameters. In theory, these could be addressed in the same way, using distributions to represent variability in toxic potency among individuals within a population, and uncertainty in dose–response relationships based on model choices and source data. In practice, this has not been well accepted by regulatory agencies, usually with the explanation that methods for assigning these distributions have not been sufficiently developed.

The primary advantage of PRA is that the risk distribution includes quantitative information on variability and uncertainty. The output is not a single upper-end

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estimate of risk; rather, the result expresses a distribution of risks for the population of concern and the percentage of the population likely to exceed the target risk level. Despite these advantages, the PRA is not as widely used as deterministic risk calculations. Compared to a deterministic risk assessment, PRAs are information and labour intensive. They require the compilation of many distributions for which good data may not exist. For site-specific assessments, the distributions must be created anew and may require expensive data collection. Finally, PRAs require a different set of assumptions on acceptable risk. Because a distribution of values is used for the exposure assumptions, the computer may pick several high-end assumptions and calculate extreme estimates of risk. To avoid using these values, an upper percentile of risk (e.g. 95th percentile) is typically used to represent individuals in the general population with the greatest risk. This suggests that a small percentage of the population may exceed the target risk level, although in reality, given the overall conservativeness of the risk assessment process, there may be no one with risks over the risk target. Although the upper percentile is an acceptable estimate of the reasonable maximum exposure, communication of these concepts to the public may be difficult.

9 EVALUATING THE RISK OF LEAD EXPOSURE AS A SPECIAL CASE

The safe human dose for the majority of chemicals is determined by utilizing the applied dose, that is the dose ingested, inhaled or dermally absorbed at the point of entry into the body. An important exception is in the case of lead. The safe human dose for lead is derived using blood levels. Enough data exists on the adverse effects of lead at different concentrations in blood to directly compare blood lead levels with concentrations known to cause an adverse effect. Lead is a neurotoxicant that decreases IQ and learning ability as blood concentrations increase. It also impairs motor function and physical abilities. The most sensitive endpoint of this chemical includes its effects on the developing nervous system, which makes children a population of special concern for the toxic effects of lead. The threshold for the adverse effects of lead is not well defined because there is no apparent blood lead level below which adverse effects do not occur. Despite the lack of a threshold for effects, the current safe limit for lead in blood set by the World Health Organization in 1995 is $10\,\mu g\,dl^{-1}$. Concentrations of lead in media (e.g. water, soil, air) that result in a blood lead level above $10\,\mu g\,dl^{-1}$ are considered of concern. Because lead concentrations in environmental media cannot be directly related to blood concentrations of lead, blood lead levels are calculated using a PBPK model such as the Integrated Exposure Uptake Biokinetic Model for lead, used by the US EPA. This model predicts the likely distribution of blood lead levels in a population given the exposure at a site and determines the probability that an exposed population will exceed the target blood lead level. This information is used to determine whether the percent of exposed individuals with blood lead concentrations above the level of concern is acceptably small.

10 PLACING RISKS IN PERSPECTIVE

One of the challenges in risk assessment is to convey risk estimates in a context that gives them meaning to the public and to risk managers who use them to make decisions. One method of placing the output of a risk assessment in perspective is through comparisons with known or familiar risks. These comparisons assist risk managers by allowing them to place priorities on certain risks and allocate resources where they will be the most effective at reducing risk. By comparing the risk from exposure to a more familiar event, a non-technical comparison can be made that increases the understanding of risk by audiences outside the field of risk assessment and aids in risk communication. Several types of risk comparisons can be made. For example, risks posed by exposure to a chemical can be compared with risks experienced voluntarily in everyday life, such as driving a car or smoking. They can also be compared with risks from natural events, such as lightening strikes. **Table 3** provides examples of comparison data on the loss of life expectancy for various activities, including more familiar, voluntary and natural occurrences.

Table 3 Estimated odds of dying from various activities in the United States

Activity	Lifetime odds of dying
Driving a motor vehicle	1 in 84
Suicide	1 in 117
Falls	1 in 194
Assault	1 in 210
Ingesting narcotics	1 in 345
Drowning	1 in 1064
Fire	1 in 1192
Flying on an airplane	1 in 6460
Drinking alcohol	1 in 11 015
Animal attacks	1 in 23 672
Lightning	1 in 79 399
Earthquake	1 in 103 004
War	1 in 141 154
Flood	1 in 317 595

Based on data from the United States for 2005 taken from the National Center on Health Statistics and US Census Bureau as compiled by the National Safety Council.

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Unfortunately, comparative risk analysis is not always a straightforward process. Situations arise which complicate the process and add uncertainty into the comparison. For example, most chemicals have multiple toxic endpoints. Choosing the most sensitive endpoint to represent the chemical may be appropriate for effects screening. However, it does not reveal all the possible adverse effects that may occur from exposure to the chemical. Additionally, when chemicals produce different effects, it is difficult to compare one endpoint to another. While most people consider death a more severe effect than hypertension, it can be difficult to rank other adverse effects such as gastrointestinal effects, eye irritation and peripheral neuropathy. How, for example, should risk of kidney disease from one chemical be compared with developmental toxicity from another? It is conceivable that potential adverse effects such as these would have different relative rankings with different members of the population.

Spatial and temporal scales can also complicate the process of comparative risk analysis. It is often difficult to compare short-term adverse events such as a one-time pesticide application in a neighbourhood to a more continuous event, such as air pollution in a neighbourhood generated by a local incineration plant. The risks from the pesticide are transient, while the risks from the incineration plant may last over a person's lifetime. Differences in spatial scales can also complicate risk comparisons. Comparing the risk from a local waste site to the risk of global deposition of PCBs has little meaning.

Although some forms of comparative risk analysis can be useful in communicating risks, others can hinder that communication. The most effective forms of comparative risk analysis are those that compare risks from different chemicals released from the same source, compare risks of different chemicals to the same target organ and compare risks at different sites. Comparison of risks that are substantially different may not be effective and may even prevent comprehension. For example, comparing the risk from exposure to a chemical to the risk of being a victim of a crime may have little effectiveness since the impact of such a risk is difficult to understand. Uncertainties regarding the comparison can increase confusion and create problems in trying to communicate the risk.

Additionally, the public may interpret some types of comparative risk analysis in a negative manner. Comparison of voluntary vs. involuntary risks or natural vs. anthropogenic risks may decrease the effectiveness of the comparison. For example, comparing the risk of drinking groundwater contaminated by a local business to the risk of using a motor vehicle may not be effective. Most people understand that there is a danger in operating a motor vehicle and they willingly accept that risk every time they drive to work or a store. However, a risk from drinking water that was contaminated by someone else may be more difficult to accept, even if the actual risk is smaller. Accepted risks and imposed risks may not be different quantitatively, but they are very different qualitatively and are usually difficult for the layperson to view as equal. The same is true for comparisons of natural and anthropogenic risks. Natural occurrences (such as weather) are usually seen as beyond one's control and bear little resemblance to anthropogenic factors, which are usually seen as manageable and within the realm of control.

11 ECOLOGICAL RISK ASSESSMENT

Ecological risk assessment is a method for estimating risk to the environment as a result of physical, chemical and biological stressors. Current approaches to ecological risk assessment are utilized mainly for chemicals and focus on the risk of a chemical release into the environment. Ecological risk assessment differs from human health risk assessment in that it attempts to determine risk to a much larger entity—the environment. Human health risk assessment is focussed on one species (humans) while ecological risk assessment is concerned with the multitude of animal and plant species that inhabit an area. In addition, the ecological system as a whole and its ability to maintain current levels of diversity and types of habitat are considered. Both human health and ecological risk assessments share the same basic concepts embodied in hazard identification, dose–response assessment, exposure assessment and risk characterization. However, the way these concepts are implemented differs between the two types of risk assessment because of the greater complexity of ecological systems and somewhat different assessment goals. A summary of similarities and differences between human health and ecological risk assessment is shown in **Table 4**.

Table 4 Comparison of human and ecological risk assessment

Human	Ecological
Protects one species—humans.	Protects multiple species including plants, invertebrates and animals.
Well-established guidance and procedures.	Newer field where guidance and procedures are still evolving.
Criteria are meant to be protective of the individual, including sensitive subpopulations.	Criteria are meant to be protective of populations or habitats.
All adverse effects are considered.	Only growth, reproduction and mortality endpoints are considered.
Carcinogenic effects are considered.	Cancer is not considered an adverse effect.

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11.1 Problem Formulation

The goal of ecological risk assessment is different from that of human health risk assessment. When concerned with humans, the goal is to protect each individual, including sensitive subpopulations. With the environment, protection is usually not provided on an individual level, but on a population level. When determining the critical adverse effect, ecological risk assessment is only concerned with those toxicities that affect the fecundity of an individual. Accordingly, chemicals are assessed for toxicities that effect mortality, growth or reproduction. Other adverse effects are generally not considered. As long as the animal can survive to produce offspring, the population will be maintained and the chemical exposure is not regarded as having an adverse effect on the population. The exception to this is for species with special legal protection, such as endangered and threatened species. In these situations, protection of individual animals is required and the assessment must therefore consider adverse effects at an individual level.

The number of species present in an area of concern can be very large. Attempting to assess risks to all of them would be time consuming, expensive and full of uncertainties, because most species do not have toxicity data readily available. To streamline this process, one or more ecological entities are chosen as the focus of the ecological risk assessment. An ecological entity can be defined as one species, a representative group of species (e.g. benthic invertebrates), species from different branches of the food chain (e.g. herbivorous mammal, carnivorous mammal and insectivorous mammal) or a number of species within a habitat.

Endangered or threatened species are often the focus of assessment if they are present. Because their risks are assessed on an individual rather than population level, greater risks can occur at lower chemical concentrations. In the absence of threatened or endangered species, the ecological risk assessment usually focusses on the most sensitive species. A risk assessment that supports protection of the most sensitive species will also prevent adverse effects in species that are less sensitive, thereby ensuring the integrity of species diversity within the habitat. Although seemingly straightforward, choosing the most sensitive species is not always easy because toxicity information exists for very few species other than common laboratory animals. Instead, the most sensitive species is often inferred based on habitat and biology. For example, if a bioaccumulative chemical was released into a freshwater river, the most sensitive receptors could be piscivorous birds, piscivorous fish, piscivorous mammals and other predators at the top of the aquatic-based food chain. If a metal were released into the same river, benthic invertebrates would have a larger exposure and be more likely to experience adverse effects.

What constitutes an unacceptable chemical impact is not clearly defined for ecological risk assessment. This leaves a lot of latitude in determining which ecological entity should be protected from the release of chemical stressors in a given situation. Regulatory agencies may decide not to protect a species at all. Instead, the biodiversity of a habitat or even the type of habitat itself may be considered the ecological entity to protect. In these cases, entire populations are allowed to decline or disappear. The goal may be to maintain the same number and type of species in the affected area that are present in the reference areas. In some cases, only the total number of species is used as the endpoint. Populations are allowed to diminish as long as another species takes its place. This endpoint allows succession and the presence of invading or non-native species.

11.2 Exposure Pathways

In ecological risk assessment, different exposure routes exist for different species. Terrestrial animals may be exposed to a chemical through inhalation, ingestion or dermal absorption. Aquatic animals are exposed through ingestion and dermal absorption. Terrestrial plants are exposed through root uptake and leaf uptake of both solid and gas contaminants. Aquatic plants have direct contact with water and sediments. They may also have exposure routes similar to terrestrial plants if they are emergent. In practise, dermal contact and inhalation exposure are not usually assessed for terrestrial animals. There are two reasons for this. The first is that there is almost no data on dermal and inhalation toxicity for terrestrial animals. The second is that the largest portion of risk to the animal is obtained from ingestion of the contaminant through food sources, drinking water, and from direct ingestion of soil while feeding or grooming. Dermal absorption and inhalation do not usually contribute significantly to risk. An exception is for burrowing animals. These animals are more susceptible to direct contact from soil and inhalation of vapours because they reside within the soil. If the data are available, dermal absorption and inhalation risks should be assessed for burrowing animals. It is important to keep in mind that an exposure pathway must be complete for there to be any amount of risk. For example, a herbivorous mammal will not be affected by PCB accumulation in fish. Additionally, if a contaminant is found only in soil below the root zone, plants will not be able to absorb the chemical and would not have any risk.

11.3 Exposure Media and Biomagnification

The two major types of ecosystems in an ecological risk assessment are terrestrial and aquatic. Soil, air and sometimes sediment and surface water are the media

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of concern for terrestrial animals. Soil and air are the principal media of concern for terrestrial plants. Aquatic animals are exposed to only sediment and surface water, while aquatic plants may be exposed to sediment, surface water and air (emergent plants). Groundwater is not usually considered a medium of concern for ecological risk assessment since it is not utilized directly by most plants and animals.

An ecological risk assessment must consider biomagnification when determining exposure and identifying the most exposed species. Bioconcentration is the uptake of a constituent from water that results in an aquatic animal having a higher concentration of chemical in its body than is present in its surroundings. Bioconcentration is related to the octanol–water partition coefficient of a chemical. The more likely a chemical is to partition into the lipid phase, the more likely it will move from the water into the organism. Bioaccumulation also results in a higher concentration of chemical in the organism than is present in its surroundings. However, this term is not restricted to uptake from water. It encompasses chemical uptake through respiration, prey ingestion, dermal absorption and other means. Chemicals which bioaccumulate may result in very high body burdens for top predators. Exposure through direct ingestion alone may not accurately reflect the escalating concentrations for these species.

11.4 Exposure Parameters

Some of the exposure parameters for ecological receptors differ from those used in human health risk assessments. Ecological receptors require the consideration of additional factors when determining exposure. Some of the more common parameters are listed below.

- Area-use factor: This factor relates to the fraction of the animal's home range that is within the contaminated area. If the entire home range of the animal is within the contaminated area, then the area-use factor is one. For screening purposes, the area-use factor is assumed to be one, but may be adjusted during more detailed assessments.
- Dietary composition: The percentage of the diet that consists of each food component (e.g. 20% fruit and 80% soil invertebrates). In the absence of dietary composition information, the most conservative assumption is that 100% of the diet is composed of the most contaminated component.
- Bioaccumulation: The degree to which a contaminant biomagnifies in the food chain. The highest bioaccumulation factor reported in the literature from a well-conducted study should be used to estimate this parameter.
- Body weight
- Food ingestion rate
- Bioavailability: The bioavailability is assumed to be 100% in the absence of site-specific data.

11.5 Dose Equations

The equations used to estimate dose for ecological risk assessment also differ from their counterparts in human health risk assessment. The equations have been modified to account for different types of exposure factors. As in human health risk assessments, dose is usually expressed per unit time, per unit body weight or both. Exposure factors used in these equations can be obtained from guidance, such as the US EPA *Wildlife Exposure Factors Handbook* (US EPA, 1993), published literature or site-specific assessments. The following section provides example dose equations for some common exposure pathways.

11.5.1 Ingestion of Surface Water

$$\text{Dose (mg kg}^{-1}\text{d}^{-1}) = \frac{\text{CW} \times \text{F} \times \text{IR}}{\text{BW}} \qquad (10)$$

where

CW = chemical concentration in water (mg l^{-1})
F = fraction of water obtained from the contaminated source
IR = water ingestion rate (l day^{-1})
BW = body weight (kg)

11.5.2 Ingestion of Food

$$\text{Dose (mg kg}^{-1}\text{d}^{-1}) = \sum_{n=1}^{m}\left(\frac{\text{CF} \times \text{F} \times \text{IR}}{\text{BW}}\right) \qquad (11)$$

where

m = number of contaminated food types
CF = chemical concentration in food (mg kg^{-1})
F = fraction of diet obtained from the contaminated source
IR = ingestion rate (kg day^{-1})
BW = body weight (kg)

11.5.3 Ingestion of Soil or Sediment

$$\text{Dose (mg kg}^{-1}\text{d}^{-1}) = \frac{\text{CF} \times \text{F} \times \text{FS} \times \text{IR}}{\text{BW}} \qquad (12)$$

where

CF = chemical concentration in soil or sediment (mg kg^{-1})
F = fraction of total food intake obtained from the contaminated source

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FS = fraction of soil or sediment in the diet
IR = ingestion rate (kg day^{-1})
BW = body weight (kg)

11.6 Toxicity Values

Toxicity data are not often available for the species of interest. As with human health risk assessments, surrogate species are used to estimate toxicity. Chickens may be used to estimate toxicity to birds, ducks for aquatic waterfowl and rats for terrestrial mammals. Toxicity studies for each category of receptor (e.g. mammals, birds, plants and invertebrates) are compiled and compared to exposure levels to determine if an adverse effect is likely.

In contrast to human health risk assessments, there are no standard procedures to determine a safe dose for an ecological entity (termed the toxicity reference value (TRV)). As an initial screening step, the exposure dose could be compared with the lowest NOAEL. However, not all agencies choose to use such a conservative estimate. Since protection of ecological receptors occurs at the population level, use of the lowest NOAEL is not necessary and other estimates are often used. For example, in determining ecological soil screening levels for various constituents, the US EPA uses multiple studies to determine a NOAEL for a specific chemical and receptor group. In the derivation of their TRVs, at least three studies are required, with endpoints in the growth, reproduction or mortality effect groups. If there are at least three NOAELs available from these studies, the geometric mean of all of the NOAELs is calculated. Additional adjustments may be added to this value; however, the final value used as the TRV is rarely as conservative as the lowest NOAEL. NOAELs are used as preliminary screening values, but chemical concentrations up to and including the LOAELs may be chosen as acceptable levels for chemical constituents. Any concentration below the LOAEL is not expected to have an effect on the preservation of the population for most chemicals.

Ecological risk assessments protect entities on larger scales than human health risk assessments, so less conservatism is built into the derivation of a TRV than a safe human dose. The chemical concentrations remaining on site may result in the death of many individuals, but as long as the populations remain stable, the risks are often considered acceptable. Toxicity studies used to derive the TRV concern the fecundity of the receptor. Fecundity is affected by toxic endpoints including mortality, growth and reproduction. Cancer is not considered an endpoint for ecological receptors. The long latency period of carcinogenesis prevents this adverse effect from interfering in the preservation of the population through reproduction.

11.7 Ecological Uncertainty Factors

Similar to human health risk assessments, UFs are used to adjust the NOAEL in deriving the TRV. Some of the UFs used to adjust the NOAEL for ecological species are listed below.

- A UF of up to 10 is used to extrapolate an NOAEL from an LOAEL. This factor is used in an attempt to reduce the LOAEL to a value that is at or below the threshold dose.
- A UF of up to 10 is used to extrapolate subchronic data to chronic data. Higher exposure levels can be tolerated for shorter periods of time so the use of these data for chronic exposure may underestimate the toxicity of the dose received. Therefore, the subchronic data are divided by an UF to protect against the lower threshold dose that may come from longer exposure periods.
- A UF between 1 and 10 is used to reflect the quality of the toxicity database. This UF is applied if very few toxicity studies exist, the studies are poorly conducted or only a few animals were tested. As the quality of the toxicity database decreases, the UF for this component increases.

UFs are not used to extrapolate between species. Since toxicity data from the species of concern is rarely available, toxicity data from surrogate animals representative of the same class are usually used (e.g. quail toxicity data can be used to estimate an NOAEL for herons). This type of data is considered adequate to represent the species of concern. The ecological practice of protection at the population level negates the requirement for additional conservatism. UFs are also not used to account for sensitive individuals within the population. Since protection is on a population level, the individual is not considered.

11.8 Hazard Estimation

Because cancer is not an endpoint for ecological risk assessments, only hazard quotients are calculated. The hazard quotient for ecological risk assessments is determined in a similar manner as human health risk assessments. The estimated chemical intake is divided by the TRV to obtain the hazard quotient for ecological entities.

$$\mathrm{HQ} = \frac{\mathrm{D}}{\mathrm{TRV}} \tag{13}$$

where

HQ = hazard quotient
D = dose from exposure (mg kg^{-1} day^{-1})
TRV = toxicity reference value (mg kg^{-1} day^{-1})

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If the HQ is less than one, exposure to the chemical contaminant is unlikely to cause adverse effects on mortality, reproduction or growth. Since only population level effects were considered in the derivation of the TRV, an HQ less than one is not necessarily indicative of the absence of adverse effects. Instead, it only suggests that the population level adverse effects will not manifest at current environmental concentrations. An HQ greater than one suggests that population level adverse effects are likely. As in human health risk assessment, hazard quotients exceeding one are not a guarantee of the presence of population level effects due to the UFs included in the derivation of the TRV. However, as the HQ increases, the likelihood of population level effects increases. When there is more than one chemical present at a site, the HQs for chemicals that produce their adverse effect through the same toxic mechanism can be added, to calculate a HI.

$$HI = HQ_1 + HQ_2 + \ldots + HQ_n \qquad (14)$$

where

HI = hazard index
HQ = hazard quotient

A HI less than one indicates that a population level adverse effect is unlikely. However, the likelihood of occurrence increases with the severity of the effect and the magnitude of the HI. Uncertainty in environmental concentrations and the NOAEL used to derive the TRV can result in an inaccurate estimation of the HI and population level effects occurring at a HI less than one.

To reduce the uncertainty in whether current chemical concentrations may cause population level effects, comparisons can be made to reference areas. Reference areas are ecologically similar areas that are not impacted by the chemical(s) under investigation. Potentially impacted and reference areas are compared through the use of population metrics. Examples of these metrics include number of species, abundance of various taxonomic groups, total abundance, percentage invasive species, percentage sensitive or tolerant species, and trophic composition. Comparisons with reference areas help determine if exposure to the chemicals of concern are causing a change in the population metrics of the community. A lack of difference between site and reference areas suggests that the chemicals are not causing adverse effects on the population level even if the HI exceeds one. Bioavailability, sensitivity of the organisms to the chemical, and even the spatial and temporal distribution of the contaminants can increase or decrease the likelihood of effect on the population by altering the threshold concentration for population level effects.

12 RISK MANAGEMENT

Risk assessment alone is rarely the basis for determining whether a site requires removal of chemical contaminants. Political, technical, ethical and value-based processes all contribute to the final decision, despite the science-based risk estimates for the receptors. It is the job of the risk manager to weigh these different criteria and determine the remedial actions necessary to protect human health and the environment. The processes involved in risk management decision-making include:

- Benefit vs. risk analysis: This analysis attempts to weigh the benefits of remediation with the risks inherent in the corrective actions. Examples include release of additional product into the groundwater or destruction of valuable ecological habitat during remediation.
- Cost vs. benefit analysis: Cost effectiveness attempts to obtain the most benefit for the least cost. This can alter the type of remediation chosen (capping instead of removal) or may even end remediation when cleanup requires large costs for minimal risk reduction, that is, when the cost of additional risk reduction becomes impractical.
- Comparative analysis: If the remedial action is likely to cause additional exposure or damage the ecosystem, a comparison between the risks for contaminant removal and leaving a chemical in the environment can be performed. The goal of this analysis is to determine the action that will cause the least amount of risk to human health or the environment. This type of assessment may include relative risks, cost-benefit analysis and value judgements.
- Community acceptance: The decision-making process and final remedy should also be acceptable to the stakeholders involved. Perceptions of risk may be larger than actual risk. Obtaining stakeholder acceptance of final remedial action is essential to the decision-making process.

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Toxicogenomics in Risk Assessment

Susan L. Makris and Susan Y. Euling

CONTENTS

1 INTRODUCTION

1.1 Historical Perspective

The development and advancement of genomic technologies over the past decade has its roots in the Human Genome project that yielded a reference human genome sequence in 2000 (NRC (National Research Council), 2007a). Following this landmark event, biomolecular and informatic tools were rapidly developed to collect and analyse the global molecular response data. The potential applicability of these genomic tools to toxicology and risk assessment was readily recognized. It was anticipated that genomic information would have the potential for use in identifying and characterizing hazards, assessing individual exposures to toxicants, monitoring responses at the cellular level, characterizing mechanisms of action and predicting or characterizing individual variability in response. Ongoing efforts in the biological, medical, toxicological and regulatory communities are aimed at the organization and integration of all of the information from the many so-called -omics technologies (including genomics, proteomics, and metabolomics) to complete a picture of the genes, pathways and networks of pathways underlying regulation of the normal and perturbed states of a cell and whole organism.

The rush to utilize the new -omics technologies for broad toxicological screening is reminiscent of efforts in the 1970s to apply *in vitro* and *ex vivo* mutagenicity study data as an approach to carcinogenicity screening. Although mutagenicity data were not found to be unequivocal solitary alternatives for bioassay studies, these data are used in mode of action (MOA) assessments for carcinogenic materials and are well accepted. It is

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likely that -omics data will undergo a similar evolution in development and validation for use in human health risk assessment.

1.2 Definitions

Toxicogenomics refers to a set of technologies for assessing global molecular response after toxic agent exposure. In this chapter, the definitions used for toxicogenomics terms are consistent with the National Research Council (NRC) book entitled *Applications of Toxicogenomic Technologies to Predictive Toxicology and Risk Assessment* (NRC (National Research Council), 2007a). Genomics is the assessment of the genome, and includes technologies that assess the genome, genome sequencing and genotype analysis techniques that allow for identification of sequence variation or polymorphisms. Transcriptomics is the assessment of messenger RNA (mRNA) expression, most frequently measuring transcripts using microarrays. Proteomics is the assessment of proteins in an organism. Proteomes are complex due to the many forms of proteins (e.g. post-translational processing). Metabolomics is the assessment of low-molecular-weight (LMW) metabolic products of a biological system. Since metabolites are the most 'downstream' products of gene expression, a metabolomic profile most likely encompasses the most functional assessment of toxicity, among the -omics techniques.

Due to the near-global nature of these technologies, the generated datasets are necessarily large and complex. Consequently, methods for data analysis have been developed and continue to evolve. Bioinformatics is the application of advanced computational techniques to collect, manage and analyse quantitative biological data.

1.3 Toxicogenomics Technologies

Toxicogenomics technologies currently in use are described briefly in this section. For further details about the developing toxicogenomic technologies and associated bioinformatic tools, please refer to *Applications of Toxicogenomic Technologies to Predictive Toxicology and Risk Assessment* (NRC (National Research Council), 2007a).

Genomics technologies use genomic sequencing techniques. DNA sequencing methods have evolved rapidly since the Sanger high-throughput gene sequencing methods of the 1970s (Sanger *et al*., 1977). The new generation of automated sequencing methods includes the use of fluorescent tagging and capillary electrophoresis (Chan, 2005) and a cycle extension approach (Margulies *et al*., 2005). While new methods are being developed, the whole-genome shotgun sequencing method of Venter *et al*. (1996) is currently used the most frequently. The shotgun method breaks chromosomal DNA into fragments of ~500–1000 DNA bases that are then sequenced using automation in multiple rounds (three to seven rounds). The output sequence information from the fragments is then aligned to generate the chromosome's DNA sequence using bioinformatic analysis methods.

Sequencing of the genome allows for information about the genes that encode RNA and protein products as well as the regulatory DNA sequences. The DNA sequence of genes provides information that can be used to predict the amino acid sequences of the protein products. Comparing the predicted amino acid sequences of genes of interest across species can provide information about interspecies differences. Genomic sequencing of multiple individuals for a species can provide information about polymorphisms at a particular gene locus, giving information about intraspecies variability. Interspecies differences can potentially inform the relevance of a toxicological response in a test organism to a similar response in humans. Intraspecies information can potentially inform susceptibility to a particular toxic agent. Thus, both the interspecies and intraspecies information based on gene sequencing have the potential to be utilized in risk assessment.

Transcriptomics, or genome-wide expression profiling, provides information about gene expression at the mRNA level. Within the context of toxicogenomics, transcriptomics provides information about differentially expressed genes (DEGs) relative to the control. An understanding of the pathways perturbed by the toxic agent can be developed by performing pathway analysis on the DEG information. To date, microarrays are the most developed and utilized transciptomic technology, as well as being the most advanced regarding validation among all of the -omics technologies. The use of microarrays to study gene expression profiles from cells, tissues or organs began in 1995 (Lobenhofer *et al*., 2001). Microarrays provide a nearly global transcriptional profile of the cell, tissue or organ used to make the microarray. Microarrays are currently 'nearly global' because not all of the genes are currently annotated, for example, the rat microarrays currently allow for the simultaneous analysis of expression of ~30 000 genes.

The two most-developed types of microarrays are the complementary strand DNA (cDNA) microarray and the oligonucleotide array. The cDNA microarray is a glass slide or membrane with DNA spots from each open reading frame (ORF) from the organism of interest. These probes are used to detect cDNA: DNA synthesized in the laboratory from mature, fully spliced messenger RNA (mRNA) transcripts of the cell, tissue or organ. cDNA arrays use a radiolabelled detection system (^{32}P), which may be the most sensitive measure of gene expression

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available. One cDNA microarray is commercially available from Clontech. Some issues with cDNA microarrays have resulted in a high variability of performance and so they are no longer used in toxicogenomic studies.

The second type of microarray is the oligonucleotide array, available commercially from Affymetrix, Agilent and others. Oligonucleotides (oligos) representing the whole genome are synthesized directly onto the glass slide via a number of different methods. For example, Affymetrix probes are directly synthesized onto the arrays uses photolithography. Briefly, the slide is coated with a light-sensitive chemical compound that prevents the formation of a bond between the slide and the first nucleotide of the DNA probe being created. Arrays are synthesized and attached to the slide multiple times in order to synthesize probes that are up to 25 base residues long and produce a high density of probes on a slide. Each gene is represented by ~20 overlapping probe sequences. Paired mismatch probes are also utilized to assess nonspecific hybridization. Probe design improvements have led to less variability and increased sensitivity in microarray study results. Consequently, oligo arrays are considered the 'second generation' of microarrays and are currently used in toxicogenomic studies.

To detect gene expression either a one-dye or two-dye system is employed. For instance, Affymetrix uses a one-dye method that employs an antibody detection system with horseradish peroxidase (HRP) and streptavidin conjugates. In the case of the one-dye detection system, the absolute intensity of the individual probes of the treated and control samples are measured. These values are then used to calculate the relative gene expression change between the treated and control samples. The Agilent microarray platform uses a two-dye system (cyanine 3 (Cy3) and Cy5-labelled fluorescein dyes). For the two-dye system, the scanner detects the relative intensities of the red and green labels and gives a relative measure of the gene expression change between the control and treated samples.

Microarray analysis can be used to produce genomic signatures for toxic agents or a class of agents. Genomic signatures represent a pattern of gene expression response that may be emblematic of the single toxic agent. Genomic signatures have also been developed for a mode of action (MOA) class of chemicals by defining the common pattern of gene expression across the class (described in more detail below).

Microarrays have also been designed to detect single nucleotide polymorphisms (SNPs). Polymorphism analysis can be used to understand intraspecies differences as well as for genes involved in the metabolism of toxic agents. For example, polymorphisms in transporters and xenobiotic metabolizing enzymes can be used qualitatively and quantitatively to inform risk differences in susceptibility between groups of individuals (Guerreiro *et al*., 2003; Shastry, 2006). After DEGs have been identified from a microarray study, it is usual practice to perform some type of confirmatory or validation study. Confirmatory studies often use protein expression methods, such as immunohistochemistry and/or transcript expression methods (e.g. northern blots, reverse transcription polymerase chain reaction (RT-PCR)). The RT-PCR method involves exponentially amplifying short DNA sequences within a longer double-stranded DNA molecule using a thermo-stable DNA polymerase called Taq polymerase. Recent developments in RT-PCR technologies have led to the real-time RT-PCR (qRT-PCR) method, a kinetic approach in which the reaction is observed in the early, linear response stage. qRT-PCR, the most sensitive technique for mRNA detection and quantitation, is currently the method of choice for confirming results obtained from microarray analyses and other -omics technologies. However, differences in microarray and RT-PCR findings require further study as such an outcome does not necessarily indicate that the microarray results are incorrect.

Proteomics is the study of the collection of an organism's expressed proteins, their structural status (e.g. phosphorylated/dephosphorylated), their functional states (i.e. activity specificity and activity level) and their interactions with other cellular components (e.g. protein–protein interactions) (Pandey and Mann, 2000). Since proteins are the functional products of genes, in most cases (e.g. snRNAs (small nuclear RNAs) being another functional product), carrying out the 'work' in cells, proteomics offers advantages over transcriptomics. Toxicology and consequently risk assessment are most interested in the use of differential proteomics, or differential expression of proteins after exposure to a toxic agent (relative to a control).

The proteomic technologies include mass spectrophotometry (MS), and protein and peptide separation techniques. Other resources for protein information are gene and protein sequence databases. Bioinformatics tools for integrating the proteomic information have also been developed (for instance, see Aebersold and Mann, 2003). Gel-based and shotgun proteomics are two methods that have been developed. Gel-based methods typically use electrophoresis to separate proteins. For example, the 2D sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) technique has been frequently employed as a first step to separating proteins. Following SDS-PAGE separation, protein spot selection identifies proteins using peptide digestions, MS analysis and protein databases. Shotgun proteomic methods include digestion of proteins to peptides and liquid chromatography-coupled MS (LC-MS) to analyse the peptides (Yates, 1998). Shotgun technologies have a much better ability to analyse complex mixtures of peptides compared with gel-based methods. As with all of the -omics technologies, bioinformatics tools to organize (i.e. databases) and analyse (i.e. software) proteomic data have been developed.

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Another type of proteomics is proteomic profiling using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry to generate a spectral profile of a tissue or biological fluid (Chaurand *et al*., 1999). This technique allows for the generation of a protein pattern, similar to a genomic signature (described above). A differential proteomic signature after toxic-agent exposure may be useful for understanding the MOA for a single chemical agent or class of chemical agents. Thus, such a protein profile may be very useful for application to toxicology and risk assessment. Other proteomic methods, not based on the use of MS, are also being developed but are currently not as reproducible and sensitive as MS-based methods.

One issue in collecting proteomic information is the rapid, dynamic changes in temporal and spatial patterns of protein expression. Therefore, teasing apart variability of response vs. toxic exposure-specific changes can be difficult. Other issues include identifying low abundance proteins, identifying the number of modified forms of each protein, and the formation of intermediates. Thus, proteomic-technology-based studies cannot currently rely on the data from a single well-designed study, as is the case for microarray studies.

Metabolomics and metabonomics refer to the study of LMW molecules in response to a treatment or stimulus. The two terms are distinguished in that metabolomics refers to the study of LMW molecules within cells, whereas metabonomics refers to more systemic and complex changes in tissues and body fluids (Ekins *et al*., 2005). Metabolic intermediates reflect the most 'downstream' products of genes, in many cases, and thus these techniques are considered to be the most useful, in theory. Metabolomics and metabonomics share some of the same issues with proteomics in that metabolic products change rapidly over time and across tissues, and in response to a variety of factors (e.g. time of day, diet, stress). Information about the diversity and sensitivity of the metabolites will ultimately prove very useful. However, capturing this diversity and sensitivity is a challenge to methods development.

Methods for metabolomics and metabonomics include nuclear magnetic resonance (NMR)-based and MS-based techniques. NMR has been used most frequently for analysis of biofluids (e.g. urine: Nicholson *et al*., 2002). Some scientists have applied the ^{1}H-NMR methodology to solid tissues (Wang *et al*., 2003). MS-based methods have the advantage of detecting lower-abundance proteins. Metabolomics and metabonomics can be useful to understand toxicity in a number of ways, including defining the MOA of an environmental toxicant, biomarkers for exposure, or perhaps provide dose–response information after exposure to a toxic agent. Throughout this chapter, no attempt will be made to distinguish between metabolomics and metabonomics; the term metabolomics will be used since it is more frequently used in the literature (NRC (National Research Council), 2007a).

In order to utilize data from any or all of the -omics technologies study design is critical to generating useful and relevant data for the question at hand. For use of these kinds of data in risk assessment, some of the important study design elements are the time of exposure, organ or tissue used, time of assessment and species selected. This topic will not be further discussed herein.

2 APPLICATION TO RISK ASSESSMENT

The basic risk assessment paradigm is defined by the landmark National Academies of Science document on the topic (NRC (National Research Council), 1983). Since that time, deliberation on the implementation of the risk assessment process has recognized the importance of conducting a preliminary problem formulation effort to develop an analysis plan based upon scoping information (US EPA (Environmental Protection Agency), 2003; 2006b). The process of risk assessment is described as having four components: hazard identification, dose–response assessment, exposure assessment and risk characterization. The hazard identification and dose–response assessment comprise the overall toxicological evaluation, which characterizes the sufficiency and strength of the dataset, discusses the confidence in the data, and may include some modelling of the outcomes to better characterize dose response. Exposure assessment derives estimates of actual or potential human exposure, based upon various (e.g. environmental, occupational or residential) scenarios. The risk characterization integrates the toxicology and exposure data. It is used along with other factors (e.g. social, economic and engineering) to determine what options are available and in making a regulatory decision; this is termed the risk management process.

While the definitions and terminology have remained constant since 1983, the process of risk assessment has evolved through constant scientific research, discourse and regulatory application or precedence. In many cases, the evolution of the risk assessment process has been significantly influenced by the availability of new tools and technologies, for both the collection and analysis of data. Currently, the risk assessment process is being confronted with the new transcriptomic, proteomic and metabolomic data.

Consideration of approaches for using toxicogenomic data in a risk assessment scenario continue to be explored by many organizations and scientists (Boverhof and Zacharewski, 2006; Chan and Theilade, 2005; Cunningham *et al*., 2003; Daston, 2007; Frueh *et al*., 2004; Hackett and Lesko, 2003; NRC (National Research Council), 2007a; Oberemm *et al*., 2005). The potential

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value is widely recognized, and there have been a number of situations where these data have contributed, thus far in a qualitative manner, to some aspect of the risk assessment for an environmental agent. The following section addresses various topics that are integral to the risk assessment paradigm as it is currently applied, and discusses current and potential uses of toxicogenomic data within this context. **Figure 1** illustrates the chemical screening and risk assessment process, and the steps where toxicogenomic data may inform the process.

2.1 Exposure Assessment

Exposure is defined as the amount of a chemical or other agent presented by the immediate environment. Exposure can vary among individuals due to lifestyles, activities and life stage. In some cases it may be equivalent to the dose of the agent, although dose is often influenced by toxicokinetic (TK) factors (e.g. absorption, metabolism, excretion) and individual variability or susceptibility. The identification and quantification of sensitive predictive biomarkers to assess exposure has been and continues to be pursued for environmental agents. Traditional biomarkers of exposure have included such indicators as measurements of xenobiotic substances, metabolites, or specific molecular modifications, for example DNA adducts (Skipper *et al*., 1994). Conventional approaches used in the past have included gas chromatography and high-performance liquid chromatography.

Monitoring specific biomolecules to detect changes in expression at the individual or population level, through the use of toxicogenomic technologies, has been an important area of research. It is anticipated that the measurement of gene and protein expression, and the quantitative characterization of metabolites, will improve the sensitivity of exposure assessments.

Transcriptional profiling (i.e. mRNA), utilizing microarrays, can identify changes in most of the expressed genome that result from toxicant exposures. It has been suggested (NRC (National Research Council), 2007a) that transcriptomics could be utilized to identify

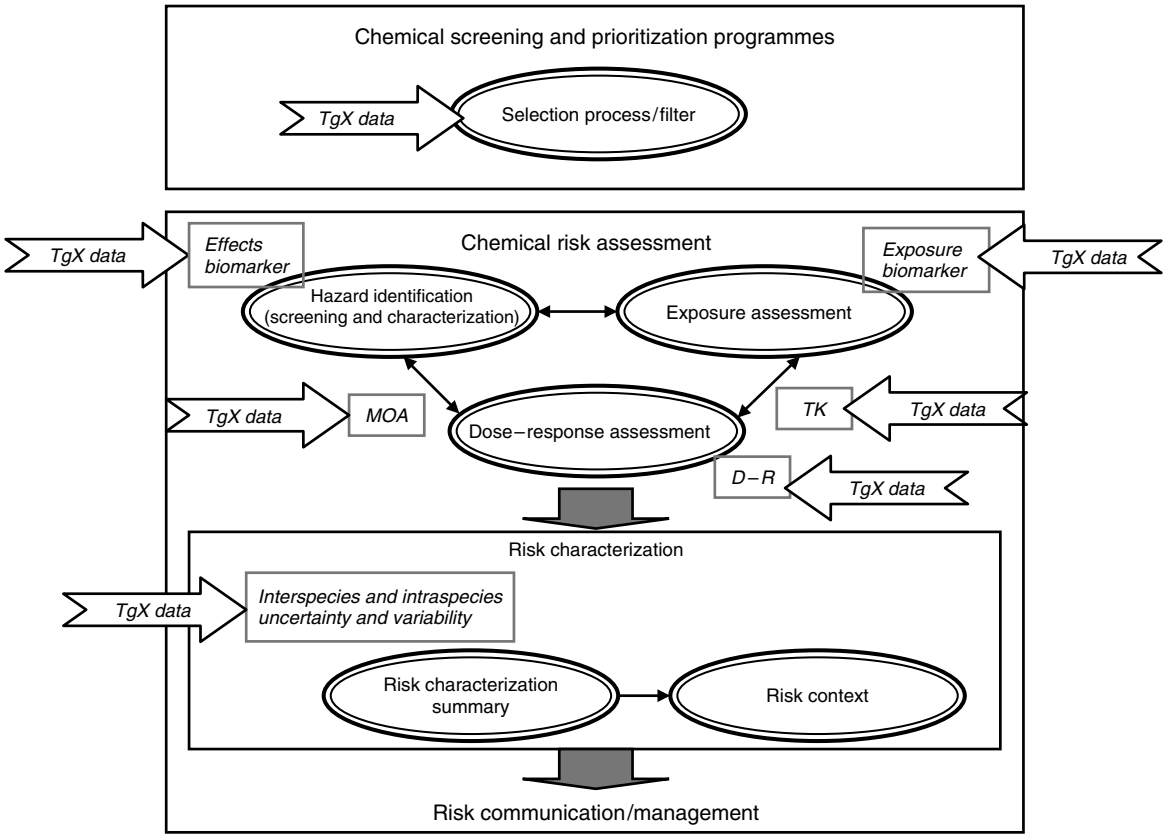

Figure 1 Chemical screening and risk assessment process: steps where toxicogenomics data may inform the process. Toxicogenomic (TgX) data, depending on the type of data and study design, have the potential to provide information to regulatory decision-making processes for chemicals. Ovals indicate the steps in screening or risk assessment. Grey boxes indicate types of information (e.g. MOA) utilized in risk assessment. Arrows with TgX data indicate places in the process where genomic data could be informative. Genomic signature data or proteomic data for oestrogen agonist chemicals, for example, could be used as a screening filter in a screening programme. Dose–response microarray data, for example, could be used in the dose–response (D–R) analysis step of risk assessment (US EPA, 2009).

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the overall pattern of gene expression, rather than just focussing on changes in just a few genes, thereby generating a more specific exposure genomic signature. The specificity gained through these techniques is an important advantage of utilizing transcriptional profiling as a biomarker for exposure assessment. While these techniques are not yet sufficiently developed for the precise characterization of human exposure, their use has been demonstrated for some micro-organisms (Begley and Samson, 2004) and cultured cell lines (van Delft *et al*., 2005) for which signatures for different toxicants could be distinguished by their transcriptional profiles. Other anticipated challenges include the use of transcriptomic data to provide an actual quantitative characterization of exposure, discerning whether changes in gene expression are the result of short-term exposures or long-term/low-dose exposures, accommodating the genetic diversity of the human population as well as epigenetic variation in exposure analyses.

Proteomic techniques (such as two-dimensional polyacrylamide gel electrophoresis or 'shotgun' proteome analysis of larger numbers of proteins) enable the assessment of changes in protein expression levels and distribution profiles of modified protein forms. While this area is very promising for exposure assessment, it has not yet been determined whether proteomic data will be able to identify changes that are toxicant specific. Other identified challenges in the use of proteomics for the assessment of human exposures include (i) the selection and accessibility of tissues or biofluids (e.g. urine, bile or bronchioalveolar lavage fluid) for biomarker analysis, and (ii) the limited sensitivity for assessing protein adducts as a post-translational consequence of exposure.

Metabolomics may provide further characterization of exposures through the identification of metabolized products of environmental chemicals and endogenous metabolites. This concept relies on the fact that alterations in gene expression may affect metabolism, and thus metabolomics techniques may reveal distinct signatures of exposure. Additionally, exposure may alter endogenous metabolites in a more persistent manner than the environmental chemicals or their metabolites, thereby allowing the detection of an exposure signature through metabolomic profiling. It is anticipated that global metabolomic profiling will be a simpler technique than transcriptomic or proteomic profiling, and for that reason may lend itself more readily to high-throughput screening. Other advantages of metabolomics are that noninvasive biofluids can be used, enabling sampling of large populations as well as sampling temporally. Also, its usefulness may be enhanced by the fact that metabolism is often conserved across species, as demonstrated by Bollard *et al*. (2005) for hydrazine treatment in mice and rats. Recommendations for standardization and reporting of metabolomic analyses have been drafted through the collaborative research efforts of the Consortium for Metabonomic Toxicology (Lindon *et al*., 2005).

2.2 Hazard Screening

Currently, most applications of toxicogenomic data to risk assessment are in the area of hazard screening. Historically, many advances in toxicogenomic technology are linked to their application within the pharmaceutical industry (Boverhof and Zacharewski, 2006). In the drug discovery process, genomic methods are often used to predict the potential for *in vivo* toxicity, thus providing a basis to determine whether to continue development and testing of a candidate chemical. Further, it is recognized that toxicogenomic data can be applied in disease diagnosis and treatment; for example, in prediction of disease predisposition, early detection of disease onset, increased accuracy of prognosis following diagnosis of disease, customization of therapy to the individual and/or to the disease, and increased accuracy in monitoring disease progression or remission during therapy (Cunningham *et al*., 2003).

In the environmental chemical context, screening of agents for toxicological outcome is primarily defined by the specific organizations in order to meet the informational (and sometimes regulatory) requirements of their mandates and goals. For example, the US Environmental Protection Agency (EPA) Office of Pollution Prevention and Toxics (OPPT) and the multinational Organisation for Economic Co-operation and Development (OECD), utilize screening-level data for industrial chemicals and pollutants in the absence of more extensive toxicological data. OECD defines this programme as the Screening Information Data Set (SIDS), and the basic tenets of this programme are also accepted and applied by OPPT for High Production Volume (HPV) chemicals. The organizing premise of this screening-level dataset is to utilize high doses, reduce the number of administered doses, assign a minimal number of animals to the test, and minimize the length of the observation period and the end points evaluated. The progression of toxicological evaluation from *in silico* methods, through *in vitro* or alternative nonmammalian techniques, and on to progressively more complex mammalian studies is utilized as much as possible. This approach is also accepted in other particular contexts within the EPA, specifically for the screening of inert substances used in pesticide formulations. Conversely, for pesticide products with food-use applications or other anticipated high-exposure potential, the Office of Pesticide Programs has regulatory requirements for a rigorous battery of toxicological tests that include more apical testing in mammalian (and other) test species. However, this approach is becoming more of an exception to standard practises, as efforts are underway in the European Union to implement a new testing paradigm based upon the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) legislation (EC (European Commission), 2006). This landmark legislation is intended to ensure the adequate toxicological screening of many thousands of industrial chemicals and

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pollutants and includes a mandate to attempt to reduce the use of test animals and replace them with validated alternative testing methodologies. This screening philosophy is consistent with a National Academies of Science report on *Toxicity Testing in the 21st Century* (NRC (National Research Council), 2007b), which includes a vision and strategy to move toxicology testing to an exclusively *in vitro*-based set of assays and other predictive tools, and with a number of other ongoing research and collaborative efforts in the US such as for endocrine disruptor screening and testing (US EPA (Environmental Protection Agency), 1998; 2007a) and for developmental neurotoxicity screening (Lein *et al*., 2007).

Toxicogenomic assays can be incorporated into screening tests, to measure changes in gene, protein or metabolite expression or response at specific doses and/or time points in the study. In a screening context, such an approach may provide sufficient additional information that might refine further testing for the chemical. Thus, while toxicogenomic studies generally require the use of animal models, they may contribute to decision-making that will ultimately reduce the number of test animals used in latter steps or tiers.

At the US EPA National Center for Computational Toxicology (NCCT), a programme entitled ToxCast™ has been implemented to develop accurate predictions of *in vivo* toxic response by analysing the combined results of hundreds of *in vitro* assays for individual chemicals (Dix *et al*., 2006). This ambitious effort directly supports the National Research Council's vision and strategy in *Toxicity Testing in the 21st Century* (NRC (National Research Council), 2007b). Genomic-based assays have the potential to be more efficient and cost-effective screening assays for several types of toxicities. Phase I of the ToxCast™ assay programme includes toxicogenomic assays as part of a large suite of assays. Similar efforts in the larger scientific community are ongoing (Mattes, 2008). The first application of ToxCast™ is to support screening and prioritization of chemicals for various EPA programmes, namely the Office of Pesticide Programs (Dix *et al*., 2006). The models were designed to predict *in vivo* toxic responses for a screening application, and thus err on the side of false positives over false negatives.

Efforts to utilize genomic signatures after toxic-agent exposure have been pursued in order to develop genomic methods for screening. For example, genomic signatures were generated from microarray data from rats exposed subchronically to individual toxic chemicals that had been tested in the National Toxicology Program (NTP) two-year rodent cancer bioassay. Algorithms were developed that demonstrated the ability of the genomic signature data to predict the lung tumour incidence based on the cancer bioassay result (Thomas *et al*., 2007). This work illustrates the utility of genomic signatures as a screening tool. One outstanding issue is that the human relevance of such predictions is not known, and this information is important for use in risk assessment.

2.3 Intraspecies Variability Including Variations in Susceptibility

The variable response of humans to environmental factors is attributable in part to interindividual genetic differences, including gene polymorphisms and epigenetic modifications. The sequencing of the human genome and the rapid advancement of genetic technologies have raised expectations that genomic information will provide a greater understanding of human susceptibilities. While this information is likely to have an important impact on clinical medicine, including personalized approaches to diagnosis and treatment of disease, the information also has the potential to influence risk assessment.

Individual variations in gene expression have been investigated by a number of researchers using toxicogenomic technologies. In some cases, human genetic variability has been shown to result in differential responses to environmental exposures. One example is the differential response to cigarette smoke and its association with lung cancer, reviewed by the NRC (National Research Council), (2007a). In other situations, differential responses to environmental insult or variability in disease outcome may be related to the modification of one gene by another, such as demonstrated in a study by McKeown-Eyssen *et al*. (2004), in which individuals with rapidly metabolizing forms of NAT2 (*N*-acetyltransferase 2) and CYP2D6 (cytochrome P450 2D6) enzymes were 18 times more likely to have chemical sensitivity than individuals with normal metabolizing forms. From a public health perspective, an increased understanding of the distribution of genetic differences in the human population is needed.

Epigenetic variability in response is associated with heritable changes in gene function that are not due to a change in the nuclear DNA sequence. The well-known example of an epigenetic mechanism is DNA methylation that alters gene expression through changes in the chemical DNA structure (Jaenisch and Bird, 2003). Epigenetic consequences of environmental exposures have been reported (Anway *et al*., 2005; Sutherland and Costa, 2003; Bombail *et al*., 2004). Although there are emerging models incorporating epigenetic and nuclear genetic information (Bjornsson *et al*., 2004), more work is needed in this area. This effort is likely to include studies on the variability in gene expression, since alterations in gene expression can be attributed to epigenetic effects, in addition to mutations of the gene, its promoter or other regulatory regions.

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The application of toxicogenomic data in the evaluation of genetic polymorphisms may quantitatively influence the risk assessment for a chemical, specifically in relation to interindividual variations in toxicokinetics, which in turn could potentially affect derivation of the intraspecies uncertainty factor applied in risk assessments. In typical risk assessment methodologies, a 10-fold range in sensitivity to xenobiotic exposures is assumed for the human population, and a 10-fold uncertainty factor is applied to account for this variability (US EPA (Environmental Protection Agency), 2002c). This encompasses both toxicokinetic and toxicodynamic aspects, thus allotting a factor of 3.16 for kinetic variability. For some chemical exposures, individual kinetic variability, particularly for neonates, may sometimes exceed 3.16-fold (Dorne *et al*., 2005). The use of toxicogenomic profiling to characterize susceptible life-stages has been demonstrated by Robinson *et al*. (2009). This study identified differences in toxicant-induced gene expression profiles between sensitive and resistant strains of mice that were associated with increased sensitivity to developmental toxicity (i.e. increased incidences of neural tube defects, embryo mortality and reduced foetal growth).

Uncertainties regarding the use of genetic mutations as a source of variability in human susceptibility to environmental toxicant exposure were identified by Haber *et al*. (2002). These uncertainties included (i) the relative contribution of multiple enzyme systems, (ii) the extent of enzyme induction and/or inhibition through coexposure, (iii) differences in mutation frequencies across ethnic groups, (iv) the lack of chemical-specific kinetic data for different genetic forms of the enzymes, (v) the large number of low-frequency mutations with significant effects, and (vi) the differences between *in vitro* and *in vivo* kinetic data. Additional data are needed to routinely apply integration of genetic information into physiologically based pharmacokinetic (PBPK) modelling in order to determine the impact on population variability.

SNPs and chromosome copy-number polymorphisms (CNPs) in genes important for the metabolism of environmental toxicants are sources of information on the impact of polymorphisms on enzyme function. They can potentially be useful in characterizing dose metrics for a specific subpopulation, or in determining how best to incorporate this information into probabilistic assessments that address population variability in enzyme function. For example, El-Masri *et al*. (1999) utilized such an approach for polymorphisms in glutathione transferase-1 following dichloromethane exposure.

2.4 MOA and Mechanistic Information

Genomics data can provide information about the mode and mechanism of action. In this regard, the genomics data can complement or provide discerning information to the MOA or mechanistic information based on all other *in vitro* and *in vivo* studies that have been conducted.

Some researchers have demonstrated common patterns of gene expression, or genomic signatures, for specific groups of chemicals. For example, Hamadeh *et al*. (2002) performed a microarray analysis on liver tissue from rats exposed to pharmaceuticals of different MOA classes. In this study, rats were exposed to either a pharmaceutical peroxisome proliferator (clofibrate Wyeth 14643, or gemfibrozil) or a CYP2B inducer (phenobarbital). Similar patterns of gene expression were observed after exposure to each of the three peroxisome proliferators, indicating that the genomic signature could provide a diagnostic for the common MOA. Additionally, the gene expression profile for phenobarbital exposure was not similar. In another laboratory, Naciff and Daston (2004) identified a set of genes that were expressed following exposure to oestrogenic compounds. Naciff *et al*. (2005) assessed the transcriptional profile in rat testis following administration of three oestrogen agonists (17α-ethynyl oestradiol, genistein, or bisphenol A), which bind to the oestrogen receptor (ER) with differing affinities. Fifty common genes were identified for the three oestrogen agonists, and gene expression was found to be altered in the same direction. Upon conduct of dose–response studies, an association of gene expression changes and dose was observed among these 50 common genes for each of the three oestrogen agonists. Additional important findings derived from these studies included that differences in gene expression patterns were dependent on the duration of exposure (Hamadeh *et al*., 2002), on the organ/tissue evaluated (Naciff *et al*., 2005; 2002), or on the life stage of the animal subject at exposure (Naciff *et al*., 2002; 2003).

In another example demonstrating the use of genomic data in elucidating mechanism of action for a toxicant, Tilton *et al*. (2008) identified an alternative mechanism for hepatic tumour promotion by perfluorooctanoic acid (PFOA) in rainbow trout. Using gene expression profiles, a novel mechanism was demonstrated that involved oestrogenic signalling underlying the tumour promotion activity of PFOA. Tumour promotion was not related to the function of PFOA as a peroxisome or PPARα (peroxisome proliferator-activated receptor alpha) agonist, but was phenotypically linked to oestrogenic gene signatures in trout liver.

Toxicogenomic data have also been found to be useful in assessing risks from exposures during development, and characterizing MOA. With the advances in human and rodent genome sequencing, there has been

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an increased understanding of development at the molecular level. Comprehensive gene expression profiling can be performed in tissues at any developmental stage, enabling analysis of and insight into disruptions in normal development by toxicant exposures. Thus far, toxicogenomic studies have focussed on known teratogens, correlating gene expression changes following exposure on a specific gestation day with defined teratogenic outcomes. Examples include studies by Hard *et al*. (2005) on alteration in brain development following ethanol exposures, studies by Kultima *et al*. (2004) on valproic acid-responsive genes that might be used as biomarkers of teratogenic outcome, and studies by Naciff *et al*. (2002) on oestrogenic chemicals (described above).

2.5 Interspecies Variability and Cross-Species Extrapolation

In a majority of risk assessments conducted for environmental pollutants, studies conducted in laboratory animal species are used to predict potential risk to human health from toxicant exposures. The scientific validity of this approach is widely accepted (NRC (National Research Council), 1983). However, it is recognized that the use of animal data in human health risk assessment relies upon the assumptions that (i) toxicological response information in animals can be used to predict adverse consequences in humans, and (ii) dose–response information from an animal bioassay is relevant for predicting risk to humans at known or expected exposure levels. Often no data are available to either support or challenge these assumptions. While information may be available on mode or mechanism of action for an animal toxicant, the relevance of these data to the human MOA may be unclear.

It is recognized that with the sequencing of the human (TIHGMC (The International Human Genome Mapping Consortium), 2001), mouse (Waterson *et al*., 2002) and rat (Gibbs *et al*., 2004) genomes, and the resulting knowledge of cross-species gene and protein homologies, studies of differential gene expression in animals may contribute to a greater understanding of human disease. The rat genome project reported that nearly all human genes that are known to be associated with disease have orthologous genes in the rat genome, and that there is approximately 90% homology among the human, mouse and rat genomes (Gibbs *et al*., 2004). Nevertheless, conservation of pathways is likely to be more important in cross-species extrapolation, since the function of a specific gene and its relationship to disease outcome may not be uniformly conserved across species (Fang *et al*., 2005).

Characterizing gene changes with toxicant exposures could also be useful in selecting the most appropriate animal model for use in toxicity testing (Aardema and MacGregor, 2002). This could contribute to increased confidence in the use of animal data to predict potential human response and to reduce uncertainties in interspecies extrapolations in risk calculations. For example, Stearman *et al*. (2005) compared gene expression data from the lungs of A/J mice treated with urethane to gene expression from human adenocarcinoma cells and found exceptional concordance, suggesting common cross-species pathobiology. Similar gene expression concordance has been found between fish and human tumours (Lam *et al*., 2006). Comparative gene expression data may also be useful in exploring the relevance for human health risk assessment of toxicological responses that are quite different across animal species.

In standard risk assessment practices, interspecies variability is addressed through the application of a 10-fold uncertainty factor (US EPA (Environmental Protection Agency), 2002c), comprised of both toxicokinetic and toxicodynamic components, and the characterization of either component could be enhanced through the use of toxicogenomics data. In an example of the use of toxicogenomic data to inform the interpretation and application of toxicokinetic data in the risk assessment, El-Masri *et al*. (1996) demonstrated changes in expression of genes or proteins associated with GSH (glutathione) depletion and resynthesis following exposure to trichloroethylene and 1,1-dichloroethylene. Modelling suggested that the consideration of GSH resynthesis was important for an adequate assessment of the toxicity of these two solvents. In a similar manner, toxicogenomic data that suggest the involvement of specific proteins in the metabolic pathway could be important in refining the parameters of dosimetry modelling. Additionally, the toxicodynamic aspects of interspecies extrapolation can be enhanced through the use of transcriptomic and proteomic data. Due to a lack of information, it is often difficult to characterize and quantify toxicodynamic differences across species; however, toxicogenomic data may be useful in qualitatively and quantitatively addressing these issues, especially in cases where clear distinctions in expression profiles are observed between species.

2.6 Dose Response

Characteristic genomic profiles of associated gene expression changes can serve as biomarkers for specific toxicity end points (Aardema and MacGregor, 2002). It is anticipated that toxicogenomic data will play an increasing role in the modelling of systems biology for use in risk assessment (Andersen *et al*., 2008; Daston, 2007). Systems biology is defined as the study of all elements in a biologic system and their interrelationships in response to exogenous perturbation (Stephens and Rung, 2006). An understanding of normal biological

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processes and compensatory mechanisms in biological systems following toxicant exposures will be needed to improve the understanding of the shape of dose–response curves at concentrations that are environmentally relevant and for adverse effects that occur at low incidences (Andersen *et al*., 2008).

Because -omics data have the potential to detect more subtle alterations at the molecular level than traditional end points collected in animal studies (Boverhof *et al*., 2004; Naciff *et al*., 2005), it is likely that these new technologies will provide greater scrutiny and insight into dose–response relationships, particularly at low doses. One difficulty in the interpretation of gene expression data over a range of doses is the ability to distinguish between homeostatic versus toxic responses. It has been proposed (Heinloth *et al*., 2004) that distinguishing toxic from nontoxic responses should take into consideration whether critical cellular systems are perturbed (e.g. stress responses, apoptosis and energy production), assessment of the magnitude of gene expression changes, and the number of genes affected as dose increases. Thus, establishing a traditional no-observed-adverse-effect level (NOAEL) may not be possible with transcriptomic data, and it may be more valid to establish a no-observed-transcriptional-effect level (NOTEL) (Lobenhofer *et al*., 2004).

Another challenge in interpreting data can result when increased dose does not just increase the magnitude of the response but also changes the response profile (i.e. affects which genes have altered expression). Such a shift in the gene expression profile at higher dose levels was observed by Sen *et al*. (2005) with dimethylarsinic acid (DMA). The nonstatic nature of gene expression data can also complicate the interpretation of dose–response relationships, as demonstrated by Boverhof *et al*. (2004), who observed time-dependent changes in hepatic gene expression in immature ovariectomized mice after ethynyl oestradiol administration.

Approaches to using microarray data in a quantitative manner in risk assessment have been developed (Yu *et al*., 2006; Thomas *et al*., 2007). Yu *et al*. (2006) utilized affected gene ontology categories as a means to quantify dose- and time-dependent effects of exposures. Thomas *et al*. (2007) developed a benchmark dose (BMD) analysis approach to identify sets of genes that were significantly altered in respiratory epithelia following acute inhalation exposure to formaldehyde. This effort demonstrated that dose–response modelling methodologies can be developed that might, with further validation, support the modelling of toxicogenomic data for chemicals with more limited *in vivo* databases. This could prove to be useful for general screening and prioritization purposes or in the future, for risk assessment. Both published approaches utilized gene ontology categorization to draw conclusions. While the use of gene ontology functional categories are useful for forming hypotheses for testing with follow up studies, some scientists consider gene ontology categories too broad to make conclusions about mechanism of action (Quackenbush, 2007).

2.7 Cumulative Risk

Cumulative risk is defined as the combined risks from aggregate exposures to multiple agents or stressors (US EPA (Environmental Protection Agency), 2003). These exposures can be accumulated over time, sources, routes or pathways. The stressors may cause the same effects or a variety of effects. When a cumulative risk assessment is conducted for multiple stressors, it may address the risks of the stressors themselves, associated health or ecological impacts one at a time, or the combined risks from some or all of the effects or impacts. This approach may need to address the potential for interactions, such as synergism or antagonism, among stressors. Considerations for cumulative risk assessment, based upon MOA or toxicological outcome, have been proposed for pesticides (US EPA (Environmental Protection Agency), 2002a) and phthalates (NRC (National Research Council), 2008). The NRC report on the cumulative assessment of phthalates cites and utilizes genomic data in the analysis of the MOA for the hazard characterization.

Typical approaches to toxicology screening and testing utilize study designs that are focussed on the effects of single chemicals. These studies, although very useful for human health risk assessment, provide no information on the potential outcomes that might result from coexposures to multiple agents. It is anticipated that gene expression data may provide a tool to help identify multisource interactions. In one example of such an application during preclinical drug development, Luyendyk *et al*. (2004) utilized toxicogenomic profiling to identify a potential pharmaceutical-induced hepatic interaction with an environmental agent (a bacterial endotoxin). Application to environmental risk assessment will certainly prove to be challenging, since coexposures are likely to be extensive, varied and poorly characterized. Utilizing toxicogenomic signatures to distinguish interactions among mixtures of toxicants, diet, pharmaceuticals, natural compounds and other environmental exposures will be a daunting task. However, the use of MOA information to define datasets and screen chemicals for similarities in gene expression may facilitate the exploration of potential pathway interactions. Such data could be utilized to test fundamental assumptions of dose or response addition that have been applied in cumulative risk assessments for environmental contaminants, for example for chemicals that activate the aryl hydrocarbon receptor (AhR) (Safe, 1990). Even for chemicals that are not structurally related, comparison of gene expression, proteomic or metabolomic profiles for individual agents could be used to identify areas in which

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individual agents overlap, determine possible agent interactions, or suggest new mechanisms of toxicity.

The potential use of toxicogenomic data to evaluate exposures with multiple components has been recognized. There are a limited number of examples of studies that utilized microarray data to assess multicomponent exposures. Bae *et al*. (2002) evaluated and compared gene expression changes in human keratinocytes that were exposed to arsenic, *N*-methyl-*N*′-nitro-*N* nitrosoguanidine, or a mixture containing arsenic, cadmium, chromium and lead. In a study by Mahadevan *et al*. (2005), the effects of a standard reference material (SRM 1649a—particulate Urban Dust) on cultured human breast carcinoma cells (MCF-7) were assessed alone or in combination with the carcinogens benzo[a]pyrene and dibenzo[a,i]pyrene; gene expression, metabolic activation and formation of DNA adducts were evaluated. These studies demonstrated the usefulness of gene expression and DNA modification data for multicomponent exposure assessment.

3 CONSIDERATIONS REGARDING THE USE OF GENOMICS DATA IN RISK ASSESSMENT

There are a number of strengths and limitations to the current use of data from -omics technologies in risk assessment. These can be separated into issues regarding (i) the technologies/assays/measures themselves, and (ii) the tools to analyse these data (**Table 1**). Broadly speaking, -omics data technologies, including microarrays, proteomics and metabolomics, are considered powerful methods because they are nearly global in scope, as discussed above. However, the technology will eventually be truly global. A method that can assess global gene expression changes is powerful for use in risk assessment because it represents a less biased approach for identifying affected pathways, and in turn, possible modes of action, for example. All of the -omics technologies can potentially identify precursor events, mechanism of action and possibly modes of action and biomarkers of effect or exposure, depending on the experiments performed and application of interest.

3.1 Variability of Response

Limitations regarding the use of -omics technology data in risk assessment include reproducibility/variability of response, a requirement for a certain level of linkage/causation between the affected gene expression changes (e.g. at the mRNA, protein or metabolic molecule level) and the outcome of interest, as well as issues related to the cost of the 'omic' technology studies. The reproducibility of microarray study results has improved with advances in gene chip technology and the MicroArray Quality Control (MAQC) project led by the Food and Drug Administration (FDA) (described below), suggesting that interlaboratory and intra-individual variability is less of an issue when provided with the same biological sample. However, variability can be fairly high even among control samples and thus, variability and study power are issues for use of these data in risk assessment. To address the issue of variability, 'omic' studies are performed with an increased number of samples, thus increasing study power for data analysis.

3.2 Experimental Design

Linkage of gene expression changes to the outcome of interest is important for using these data in risk assessment. In the ideal, data that identify changes that are measurable at each level of biological organization (i.e. protein, gene, cellular, tissue organ, whole organism) as well as a temporal sequence of changes at each of these levels would provide strong links that likely reflect a causative chain of events. However, these data are

Table 1 Current strengths and limitation of using genomic data in risk assessment

	Strengths	Limitations
Genomic technologies	• Powerful because global • Can identify: – Precursor events – Biomarker of effect – Biomarker of exposure – Mechanism/MOA	• Reproducibility • Changes in expression (in gene, protein, pathway, etc.) may not be linked to adverse effect • Studies, particularly dose-response, are costly
Data analysis tools	• Tools available for microarray studies that can identify: – Affected processes and/or pathways – Pathway networks	• Analysis of complex and large data sets • Methods continue to evolve • Current methods use statistical significance cutoffs that may not reflect biological relevance of a gene or pathway

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rarely available. Realistically defining the requirements for linkage of -omics changes of interest to the whole organism is an area that needs further exploration. Cost of the technologies is obviously an issue that limits widespread usage of -omics studies, but should decrease over time. Cost limits the use of a large number of samples, such as are needed in a multiple-dose microarray study.

Regarding the data analysis tools for -omics data, some strengths of microarray analysis methods are the ability to perform pathway analysis and gene network analysis allowing for the identification of affected processes, pathways and pathway networks. Some of these tools are publicly available (e.g. DAVID, *http://david.abcc.ncifcrf.gov/*, KEGG, *www.genome.jp/kegg/*, BioCarta, *www.biocarta.com/*), while others are proprietary (e.g. Ingenuity, GeneGo). However, a number of the analytical tools have been developed for screening purposes, such as in the development of pharmaceuticals. For analysing microarray data in the pharmaceutical industry, methods were developed for screening purposes. The purpose of screening is to exclude candidates with potential side effects and, thus, err on the side of false positives, with a low false-negative rate. Such analytical tools and cut-offs would not be as useful for application to risk assessment. There is a need for the development of methods that are tailored to the use of these data in risk assessment. A related issue is the difficulty and degree of training required to analyse these large and complex datasets. One solution to this issue, including methods development, is training of risk assessors in methods for analysing microarray data, and the formation of collaborations and information exchange between biologists/toxicologists, toxicogenomics experts and bioinformaticians.

3.3 Data Analysis and Training

Risk assessors need training in genomic data evaluation. For some, this will be the development of a basic understanding of the methods employed in order to understand a genomic study presented in the published literature. Other risk assessors will want to develop a higher-level understanding of the data in order to develop competency in the analysis of genomic data. In response to this need, the FDA Center for Drug Evaluation and Research (CDER) has developed successful genomic training courses for their scientists (*www.fda.gov/cder/genomics/Default.htm*). The EPA Risk Assessment Forum (RAF) implemented a series of internal EPA training sessions on toxicogenomics in 2007. The genomics training courses provided both an introduction to the potential applications of genomics for Agency managers, as well as intermediate technical training for genomic data review and analysis for scientists.

Finally, an issue not unique to -omics data is the use of statistical vs. biological gene expression cut-offs. When the biologically relevant level of change is not understood, as is often the case, the risk assessors and other scientists rely on the use of, at times arbitrary, statistical cut-offs. This may pertain to any precursor event such as a change in hormone levels. If the biological significance of an increment of change in hormone level is not fully understood at all levels of biological organization, then a statistical cut-off relative to control levels is utilized. Much discussion and debate about the increment of change considered to be adverse may occur. For the case of complex datasets such as -omics data, it may turn out that small changes in the expression of key genes (e.g. initiating events) could have an impact on the whole organism phenotype, even for cases when gene expression changes are not detectable using a statistical filter. However, in the absence of data supporting a causal connection between increments of gene expression change and the outcome, it is difficult to move towards the development and application of new analytical tools.

4 REGULATORY AGENCY GUIDANCE

4.1 FDA Guidance

Due to the voluntary submission of toxicogenomic data programme at the FDA, in support of pharmaceutical development, the FDA has taken a lead in the analysis and incorporation of these data into regulatory decision-making. The FDA has developed a guidance document for industry for the voluntary submission of pharmacogenomic data (US FDA (Food and Drug Administration), 2005). This guidance is intended to facilitate scientific progress in the field of pharmacogenomics and to facilitate the use of these data in informing regulatory decisions. It encourages voluntary submission of microarray data from exploratory studies, but does not include use of genetic or genomic techniques for the purposes of biological product characterization or quality control (e.g. cell bank characterization, bioassays). Data resulting from proteomic or metabolomic techniques are not addressed in this guidance.

FDA scientists also led an effort to bring researchers from government, industry and academia together to address issues of variability by contributing to the standardization of microarray procedures, the MAQC Consortium (Casciano and Woodcock, 2006; Frueh, 2006; Dix, *et al*., 2006; Ji and Davis, 2006; Canales *et al*., 2006; Shippy *et al*., 2006; Tong *et al*., 2006; Patterson *et al*., 2006; Shi *et al*., 2006; Guo *et al*., 2006). Since the

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MAQC is an ongoing consortium project, the preliminary effort described below is termed MAQC(I).

The goals of the MAQC(I) project were to identify sources of variability across microarray studies. The project evaluated the performance of microarray platforms, comparing cross-platform and interlaboratory performance of microarray technologies that were currently available. Seven microarray platforms were tested by three independent laboratories with five sample replicates. The working list of genes consisted of 12 091 reference genes. The MAQC(I) study demonstrated a high level of reproducibility within and between laboratories, and across the microarray platforms (Guo *et al*., 2006; Patterson *et al*., 2006; Shi *et al*., 2006). The performance of the microarray platforms was then compared to the results from three quantitative gene expression assays. The study found 'excellent correlation' between microarray results and quantitative gene expression results, although several sources were identified that may have resulted in the limited incongruence that was observed. These include a decreased sensitivity for low expression genes in the microarray platforms as compared to the gene expression technologies, and differences in probe location.

Subsequently, a toxicogenomic study in rats was conducted to examine the similarity of findings across microarray platforms (Guo *et al*., 2006). RNA samples were collected and processed following exposure of rats to three chemicals (aristolochic acid, riddelliine, or comfrey). Results from four microarray platforms demonstrated a high degree of similarity in gene expression findings.

To summarize, the MAQC(I) effort as well as that of Guo *et al*. (2006) found a high degree of reproducibility of findings between different microarray platforms that were tested at multiple locations. In addition, microarray results correlated well with RT-PCR findings. These studies provided important information leading to decreased variability in microarray data and the establishment of standardized quality control measures. Overall, the MAQC(I) project results provided a wealth of information that supports the incorporation of microarray data into risk assessments in the future.

4.2 EPA Policy on Use of Genomic Data

In response to the increasing availability and production of genomic data for chemicals regulated by the EPA, the Science Advisor and the Science Policy Council (SPC), which serves the Agency by addressing science policy issues that affect the EPA, released the Interim Policy on Genomics in 2002 (US EPA (Environmental Protection Agency), 2002b). This policy statement addressed how to use genomic data in decision-making. The policy encourages research in genomics, as it has been shown to provide information on MOA, biomarkers of effect, exposure and susceptibility. However, it currently restricts the use of these data in decision-making, stating that genomic data alone are not sufficient as a basis for regulatory decision-making. This has been interpreted to indicate that these data cannot currently be utilized as the critical effect in a chemical risk assessment. Recognizing the value of these data to risk assessment, it is stated that these data can be utilized in a weight-of-evidence (WOE) approach on a case-by-case basis in risk assessment. As of 2009, the interim policy is still in effect.

The SPC Genomics Task Force considered a number of the issues in using genomics in risk assessment and other regulatory decision-making. A white paper entitled *Potential Implications of Genomics for Regulatory and Risk Assessment Applications at EPA* (US EPA (Environmental Protection Agency), 2004a) was developed. This paper described the use of genomic information in regulatory and risk assessment at the EPA, and the implications of utilizing these data for programmes, policies and resources. Three areas to apply genomic data in risk assessment at the EPA, identified in the white paper, include (i) MOA analysis, (ii) susceptible population and life stage considerations, and (iii) mixtures assessments. Other activities to explore the use of genomic data in human health and ecological risk assessment at the EPA include a Colloquium on the Current Use and Future Needs of Genomics in Ecological and Human Health Risk Assessment (US EPA (Environmental Protection Agency), 2006a). Both of these activities recommended performing case studies as a means to develop approaches to using genomic data in risk assessment.

Efforts to develop guidance have been initiated at the EPA by the SPC. The EPA's SPC decided to first focus on the development of guidance for the acceptance and analysis of genomics data because this is a critical issue for use of these data in decision-making. A Genomics Workgroup focussed the guidance on microarray-based assays as this technology has been developed and refined to the greatest extent. The SPC developed an external review draft of *Interim Guidance for Microarray-Based Assays: Data Submission, Quality, Analysis, Management, and Training Considerations* (US EPA (Environmental Protection Agency), 2007b). This document provides recommendations regarding the data that should be considered for submission to the EPA for microarray studies, data quality, analysis, management and storage issues and considerations. However, guidance specifically for use of genomic data in EPA risk assessment has not yet been developed.

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4.3 Ongoing Activities to Explore and Implement the Use of Toxicogenomic Data in Risk Assessment at the EPA

The EPA is taking a leadership role in applying -omics data to human health effects research, as evidenced by the activities described in this section. There are a number of ongoing and completed activities at the EPA to address the use of genomic data in risk assessment. These activities include guidance, risk assessments, case studies, research projects (e.g. the ToxCast™ program; collaboration in the MAQC effort), and training. There are also a number of ongoing efforts to improve toxicogenomic methods and to develop computational methods that are generating toxicological information for use in the EPA in risk assessment and/or priority setting. The EPA NCCT is developing data analysis tools and databases of *in vitro* and *in vivo* assay data to support regulatory decision-making.

There are a large number of research projects to generate transcriptomic and proteomic data on chemicals regulated by the EPA. Researchers from the EPA National Health and Environmental Effects Research Laboratory (NHEERL) have published work from a number of genomic studies on (for example) conazoles, formaldehyde, triazole fungicides and perfluoroalkyl acids (reviewed in Hamernik *et al*., 2008). A great deal of their data has been incorporated into regulatory decision-making. For example, toxicogenomic studies after exposure to DMA were utilized as part of a weight-of-evidence approach in a recent EPA risk assessment for DMA. Specifically, epidemiologic studies found an association between inorganic arsenic and urinary bladder cancer. DMA is the major metabolite excreted in humans and is known to increase the risk of bladder cancer in the rat. Sen *et al*. (2005; 2007) used genomics to assess the DMA mechanism of action between humans and rats, between *in vivo* and *in vitro* rat samples, and across toxic and nontoxic doses. DMA-induced gene expression changes were reported for the *in vivo* rat (Sen *et al*., 2005). By comparing the high and low, nontoxic, dose–response findings, their study suggested that one DMA MOA for urothelium cancer was cytotoxicity. DMA-induced gene expression in human bladder cells exhibited similarities and differences from the genes affected in rat bladder cells (Sen *et al*., 2007). Twelve pathways were found to be in common between the rat *in vivo, in vitro,* as well as the human *in vitro* studies. These pathways are involved in adhesion, cellular growth and differentiation. Genes in common between the rat *in vivo* and *in vitro* models were involved in cell cycle regulation, lipid metabolism and protein degradation. The finding of common pathways affected in rats and humans suggests that urothelial cytotoxicity and regeneration, the proposed key events in bladder tumour formation in the rat, are relevant to humans. Thus, the recent EPA assessment of DMA used the genomic data to support the human relevance of the MOA (US EPA (Environmental Protection Agency), 2006c).

Some other examples of EPA risk assessments that have evaluated and utilized genomic data are the external review draft of the IRIS (Integrated Risk Information System) Toxicological Review of dibutyl phthalate (DBP) (US EPA (Environmental Protection Agency), (2006d) and the report of the Office of Pesticide Programs (OPP) Cancer Assessment Review Committee (CARC) for acetochlor (US EPA (Environmental Protection Agency), 2004b). Both of these chemical assessments used genomic data to corroborate the MOA. In addition, a vast amount of genomic data on conazoles has been generated to investigate the modes of action for this class of pesticides (reviewed in Hamernik *et al*., 2008). In summary, genomic data have been, and continue to be, used in the weight-of-evidence evaluation to inform the MOA, human relevance, and susceptibility in risk assessment as well as in screening and prioritization programmes.

Related to the risk assessment activities, a case study to assess how to utilize genomic data in EPA risk assessment has been performed for the chemical DBP (Euling *et al*., 2007; 2008; US EPA, 2009). The DBP case study was a 'paper exercise' (i.e. no additional laboratory studies were performed). The case study focussed on the male reproductive outcomes and the qualitative application of the microarray data to risk assessment because there are no available DBP dose–response microarray studies. The DBP case study addressed the use of the genomic dataset to (i) inform multiple mechanisms and/or modes of action, and (ii) interspecies differences in MOA.

There are two previously well-established modes of action for a number of the male reproductive developmental effects occurring after *in utero* exposure to DBP: (i) a decrease in foetal testicular testosterone, mediated by effects on steroidogenesis, and (ii) a decrease in insulin-like 3 (*Insl3*) gene expression. The male rodent reproductive and developmental toxicity studies were evaluated for effects on the testes after DBP exposure. Five testes effects without an established MOA were identified. Focussing on these five testes outcomes, the genomic data were evaluated for the identification of pathways affected by DBP that could explain these end points. By evaluating the DBP genomic dataset (microarray, RT-PCR and other gene expression studies), the differentially expressed genes and the direction of effect were found to be highly consistent across studies. New analyses of a DBP microarray study were performed and affected pathways were identified that include some that are related to testosterone synthesis or *Insl*3 expression and some that are unrelated. The unrelated pathways may inform the DBP modes of action for the unexplained testes outcomes.

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Interspecies differences for the steroidogenesis pathway were evaluated using three approaches. Results using all three methods found a high degree of similarity between rat and human steroidogenesis gene products. However, it is difficult to use the results to inform interspecies differences because of the difficulty in defining unequivocally a 'high' vs. 'low' degree of similarity for a given pathway.

The DBP case study draft document, provides the first systematic approach and case study example at the EPA. The approach for incorporating genomic data in risk assessment and recommendations based on the case study experience can be generalized to new risk assessments. This project also identified future research directions for integrating the genomic data evaluation within a risk assessment. One high priority research need is to perform studies to assess phenotypic linkage between the toxicogenomic results and toxicity end points of interest within a dose–response study. Specifically, one study would include a genomic assessment component (e.g. sacrifice some animals at the optimal time of assessment for use of target organs in microarray studies) and a toxicity assessment component (e.g. other animals are followed to evaluate the end points of interest). Such a study would require a large number of animals in order to identify affected *in vivo* end points at lower doses (which would occur at a low frequency). This study would be costly but beneficial to understanding the relationship between dose, genomic response and apical end-point response.

4.4 Activities of Other Organizations

A number of federal agencies (other than the FDA and EPA), nongovernmental organizations (NGOs), nonprofit organizations and industry groups have been conducting toxicogenomics studies and activities. Select examples are highlighted below. These efforts are focussed on developing standardized practices of data collection and sharing, as well as on defining and promoting research needs in the area of toxicogenomics. Additionally, the practical use of these data in multiple venues has been discussed; for example, for screening-level drug development or for human health risk assessment.

4.4.1 NIEHS Activities

The National Institute for Environmental Health Sciences (NIEHS) Division of Extramural Research and Training (DERT) issued a request for application for extramural programmes to participate in a national Toxicogenomics Research Consortium (TRC) with several goals; that is, to (i) enhance research in the broad area of environmental stress responses using microarray gene expression profiling; (ii) develop standards and practices to allow analysis of gene expression data across platforms and provide an understanding of intralaboratory and interlaboratory variation; (iii) contribute to the development of a robust relational database which combines toxicological end points with changes in gene expression profiles; and (iv) improve public health through better risk detection and earlier intervention in disease processes (NIEHS (National Institute of Environmental Health Science), 2000). The TRC was developed in 2001 and consists of six Cooperative Research Members, resource contractors and NIEHS extramural staff. This effort was followed by solicitation in Functional Proteomics in 2002 and Metabolomics Application in 2005. TRC program highlights include the conduct of ongoing standardization experiments aimed at identifying and addressing sources of technical variation in gene expression experiments across multiple technology platforms and research centres. Discourse and data sharing have been facilitated through conferences on -omics applications in the environmental health sciences (e.g. held in 2003, 2004 and 2006).

4.4.2 ILSI/HESI Activities

The International Life Sciences Institute (ILSI) Health and Environmental Sciences Institute (HESI) has been actively engaged for a number of years in the conduct of genomics studies, the analysis of genomics data and the use of these data in mechanism-based risk assessment. A standing multisector Genomics Committee was formed in 1999 to develop a collaborative scientific programme to address issues, challenges and opportunities that have emerged in the field of toxicogenomics (Pennie *et al*., 2004). The committee designed and conducted toxicogenomic studies, and analysed genomic study data. Their work was reviewed at a workshop in 2003 and resulted in a number of influential publications on the utility and conduct of DNA microarrays (Newton *et al*., 2004; Rosenzweig *et al*., 2004; Mattes, 2004); cross-laboratory analyses of gene expression changes induced by hepatotoxicants (Ulrich *et al*., 2004; Baker *et al*., 2004; Waring *et al*., 2004; Chu *et al*., 2004); the use of these data in assessing nephrotoxicity (Kramer *et al*., 2004; Amin *et al*., 2004; Thompson *et al*., 2004); and the need for qRT-PCR data (Goodsaid *et al*., 2004). Recognizing the importance of having standardized microarray data formats and public repository databases for the comparison and interpretation of these data by the scientific community, ILSI HESI has provided a publicly accessible database of the toxicogenomics work conducted from 2000 to 2004 (ILSI HESI, 2004). Additionally, ILSI HESI have continued their efforts in this area, through training courses and workshops.

4.4.3 International Activities

In 2003, the International Programme on Chemical Safety (IPCS) conducted a workshop in Berlin, which addressed

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the potential use of toxicogenomic information in various stages of the risk assessment process for the protection of human health (WHO (World Health Organization), 2003). The stated objectives of this workshop were to (i) establish a scientific forum and dialogue between relevant experts including those with expertise in molecular biochemistry, genetic toxicology, epidemiology, public health, risk assessment, computational toxicology and clinical medicine; (ii) share information about scientific-level activities involving toxicogenomics including any programmes at national, regional and international levels; (iii) discuss the potential of toxicogenomics to contribute to improvements in the risk assessment process for the protection of health from environmental exposure to chemicals, for understanding the mode of action of environmental toxicants and the relevance and scope of gene–environment interactions; (iv) identify the near-term needs and necessary steps for enhancing international cooperation to contribute to improving the scientific understanding and the potential contribution of toxicogenomic research for improving chemical safety; and (v) identify and discuss any gaps in knowledge, issues and challenges that might hinder the enhancement of awareness and use of toxicogenomics for protecting human health from environmental chemical exposures (WHO (World Health Organization), 2003). Three break-out groups addressed (i) predictive models for identifying human health hazards, (ii) human exposure and susceptibility, and (iii) risk assessment. The workshop identified key information gaps, but confirmed the view that toxicogenomics had the potential to improve the specificity and range of methods used to predict chemical hazards, to identify biomarkers of exposure, and to address uncertainties in risk assessment. The need for data quality standards, mechanisms for sharing data, and development of bioinformatics and computational capabilities was emphasized.

5 RESEARCH NEEDS AND RECOMMENDATIONS

The National Academies review of *The Application of Toxicogenomic Technologies to Predictive Toxicology and Risk Assessment* summarized a number of overarching recommendations to move the science forward (NRC (National Research Council), 2007a, pp. 201–212). These are presented in their entirety below:

1. Regulatory agencies should enhance efforts to incorporate toxicogenomic data into risk assessment.
2. NIEHS should cooperate with other stakeholders in exploring the feasibility and objectives of implementing a Human Toxicogenomics Initiative dedicated to advancing toxicogenomics.
3. Develop specialized bioinformatics, statistical and computational tools and approaches to analyse toxicogenomic data.
4. Toxicogenomic technologies should be adapted and applied for the study of exposure assessment by developing signatures of exposure to individual chemicals and perhaps to chemical mixtures.
5. Upon validation and development of adequate databases, integrate toxicogenomic screening methods into relevant current and future chemical regulatory and safety programmes.
6. Use toxicogenomic information to prospectively identify, understand the mechanisms of, and characterize the extent of genetic and epigenetic influences on variations in human susceptibility to the toxic effects of chemicals, with the goal of improving the certainty about assumptions used in the regulatory processes to address population variability.
7. Steps should be taken and tools developed to continue advancing the ability of toxicogenomics to provide useful mechanistic insight.
8. Future toxicologic assessment should incorporate dose–response and time-course analyses appropriate to risk assessment.
9. Continue to use toxicogenomics to study differences in toxicant responses between animal models and humans and continue to use genotyped and genetically altered animal model strains as experimental tools to better extrapolate results from animal tests to human health.
10. Use toxicogenomics to investigate how exposure during early development conveys susceptibility to drug and chemical toxicities.
11. Use toxicogenomic approaches to test the validity of methods for estimating potential risks associated with mixtures of environmental chemicals.
12. Actions should be taken to facilitate the technical and regulatory validation of toxicogenomics.
13. Address the ethical, legal and social issues that affect the use of toxicogenomic data and the collection of data and samples needed for toxicogenomic research.
14. Develop education and training programmes relevant to toxicogenomic applications to predictive toxicology.

As described in this chapter, many efforts have already been initiated to address each of these areas. Participants include government agencies, academia, industry and various consortia. Since the relatively recent inception of this technology, progress in the use of these data for predictive toxicology and risk assessment has been extraordinary. Ongoing and future collaborations to address the overarching issues described above are needed. Since each government agency or private sector has its own specific application needs, in response to its mission and legal mandates, projects to advance the use of genomic data for specific applications are needed.

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Thus, there are many community efforts that can work on validation and acceptance of toxicogenomic methods. Yet, individual groups will need to continue working on the use of toxicogenomic data in screening and risk assessment within their own context.

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Dose–Response Analysis in Experimental Toxicology and Risk Assessment

Robert G. Sussman, Edward V. Sargent and Todd L. Davidson

CONTENTS

1 THE CONCEPT OF DOSE–RESPONSE

The concept of dose–response dates back to the original statement by Paracelsus: 'Poison is in everything, and nothing is without poison. The dosage makes it either a poison or a remedy'. This concept led Paracelsus to use mercury as a treatment for syphilis during the 1500s (Pachter, 1961). At low doses, mercury can be considered a 'remedy' and can effectively treat the disease. However, at high doses it is considered a 'poison', as it can be toxic to the kidney and central nervous system. This was an early use of the dose–response concept which has become one of the primary underlying principles of toxicology.

When we incorporate dose–response relationships in the risk-assessment process, we are attempting to determine the point at which the response changes from an acceptable end point to an unacceptable one (e.g. a remedy to a poison, a no-effect level to an effect level, etc.). The type of response we are looking to highlight in the risk assessment depends upon the context of the assessment and the definition of what is considered 'acceptable'. A therapeutic dose of a chemotherapeutic drug may cause severe toxicity to a cancer patient taking that drug. However, the toxic effects of the treatment are considered acceptable to the patient since it may be the only way to treat their cancer. The severe toxicity that a patient considers acceptable would not be tolerated by the pharmacist or chemical operator handling these materials in an occupational setting. They will not receive the benefit of treatment as they are disease-free, but may be subjected to the same adverse effects if exposure is significant. A less obvious example would be the direct therapeutic effect of a statin, or other lipid-modifying drug. Whereas the patient taking this drug considers

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alteration of their lipid profile as acceptable, or even beneficial, alteration of a worker's lipid profile as a result of exposure to chemicals at work is considered unacceptable.

1.1 Individual Dose–Response

Individual dose–response relationships (also referred to as graded dose–response), are used to describe responses by individuals to varying amounts of chemical exposure. As the dose increases, the severity of the response increases in a continuous manner. This can be used to describe the response to a specific end point, such as an increase in liver enzymes after exposure to a solvent. As the dose increases, there is a concomitant increase in the number of damaged liver cells, resulting in progressively higher levels of liver enzymes in the blood. In addition to describing the response of a single biological end point, this terminology can be used to describe the overall response of an entire organ system. As the dose of solvent increases, the response changes from an increase in liver enzymes, to fatty infiltration of liver cells, to cirrhotic changes and eventually, to total liver failure. The concept can also be taken to the entire organism by looking at the effects on multiple organ systems. The same solvent may have central nervous system effects at low levels, liver effects at higher levels and kidney effects at even higher levels.

1.2 Population-Frequency Dose–Response

Instead of describing the effects on an individual, another way to look at dose–response relationships is to consider the number of individuals in a population that will respond with a specific end point at a given dose. This is also referred to as a quantal dose–response where an individual responds in a quantal, or 'all-or-none', manner. The population becomes divided into responders and nonresponders. As the dose increases, the number of responders generally increases as well. A quantal response is often thought of in terms of end points such as death or cancer. However, it can also be used to describe more moderate responses, such as the lowering of total cholesterol by at least 20 points. An individual whose cholesterol was reduced by 15 points would be considered a nonresponder and one whose cholesterol was reduced by 25 points would be considered a responder. The varying susceptibility to specific chemicals is a function of the biological variability within a population. It is this population-frequency or quantal dose–response that is used to define many of the parameters of toxicity we use to describe potencies of chemicals. These are the ED_{50}, LD_{50}, IC_{50} and so on that are discussed later in this chapter.

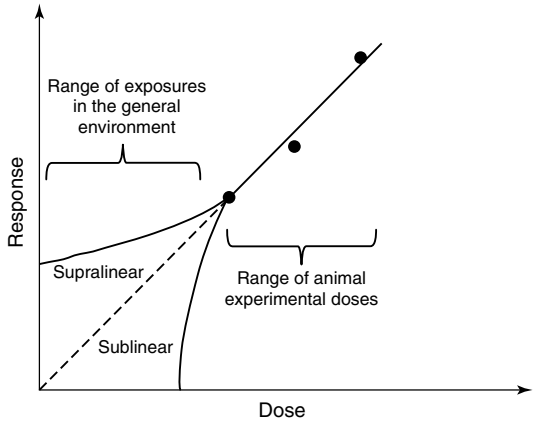

Figure 1 Hypothetical dose–response curve for an animal carcinogenicity study showing a supralinear response indicative of a background effect vs. a sublinear response indicative of a threshold effect.

1.3 Threshold Concept

If a dose–response relationship exists, we can describe that relationship graphically (**Figure 1**). Traditionally, dose is plotted on the abscissa and response on the ordinate. One of the difficulties in examining the dose–response relationship is understanding what happens when the curve approaches the origin. The data points used to plot this curve, collected from experimental animal studies, are usually orders of magnitude greater than the response rates seen in the general population. The curve may pass through the origin, but may also pass through the abscissa or ordinate. When a curve passes through the ordinate, some response is observed even at a dose equal to zero. In other words, there is a 'background' response in the population that occurs even without exposure to an exogenous chemical. However, when the curve passes through the abscissa, there is a dose below which no effect is seen. This is the concept of a threshold. The threshold is usually what we strive to identify from animal or clinical studies. As we rarely find the precise threshold dose, we use different risk-assessment methods to estimate what those thresholds may be. The assumption of the existence of a threshold implies that chemical insult at low levels will result in no damage, insufficient damage to cause a change in morphology or function, or that the body is capable of repairing itself. Noncancer or nongenotoxic end points are generally considered to be threshold responses (Renwick, 2004).

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1.4 Nonthreshold Responses

The 'one-hit' theory of carcinogenesis implies that no threshold exists and that exposure to even one molecule of a chemical can result in an effect. For a nonthreshold response, an acceptable level of risk (10^{-4} or 10^{-6}) must be selected to determine the dose that may be associated with that level of risk. Extrapolation to these low response levels is usually accomplished with biologically based extrapolation models, such as those described later and supplied by the EPA (Environmental Protection Agency) (Armitage and Doll, 1961; Altshuler, 1981; Anderson, 1989; Waddell, 2006; USEPA, 2008).

2 GUIDELINES ON OPTIMUM APPROACHES TO OBTAIN DOSE–RESPONSE INFORMATION

Risk assessors do not often have the luxury of designing an appropriate study and collecting data to be used in evaluating dose–response relationships. Often the only source of data is what already exists, either in the open literature or in proprietary studies conducted by a specific sponsor. The validity of specific studies, the relevance of their end points to humans and the applicability of the routes of exposure used in the studies to risk assessment all must be evaluated. Specific criteria should be established to determine whether or not it is appropriate to use available data in a risk assessment.

2.1 Collection and Evaluation of Relevant Data

The first step in performing a risk assessment is to assemble all available data for review and evaluation. For newly developed molecules, such as pharmaceutical products in early stages of development, a limited data set may be available for review. However, an extensive data set may exist on molecules that are either structurally or mechanistically similar to the compound in question. Data collected should include those from *in vitro* as well as *in vivo* animal toxicology and toxicokinetic studies. Data from humans, whether they are from epidemiological studies, clinical trials with drug products or incidents of accidental exposures from chemical releases or poisonings, are extremely valuable. Using human studies eliminates a source of uncertainty and obviates the need to extrapolate between species. When evaluating particular studies, emphasis should be put on whether or not these were conducted according to appropriate methodology, reported by credible sources, and that the routes of exposure and mechanisms of toxicity are relevant to humans.

The criteria used to evaluate whether or not a study is valid and should be used in a risk assessment include the following:

- There should be an adequate numbers of animals and/or test subjects per dose level
- The design of the experiment should use state-of-the-art methodology
- Practices used for animal housing/husbandry should be acceptable according to the local governmental agency
- Analytical measurements should be conducted according to an accepted methodology
- Pathological and clinical observations should be according to standard accepted criteria
- Quality assurance should be evaluated according to Good Laboratory Practice (GLP)
- Interpretation of the results should use the appropriate statistical methodology
- The results, if not proprietary, should be published in a peer-reviewed journal.

2.2 Relevance to Humans

Understanding the particular toxicokinetics of a chemical, as well as its mechanism of toxicity is fundamental to deciding whether or not a particular end point is relevant to a human-risk assessment. Several adverse effects have been reported in animal studies in recent years where those adverse effects, specifically tumours, have been found to be irrelevant in humans due to species specificity of the mechanism. Some examples include $\alpha_2\mu$-globulin nephropathy in male rats and thyroid tumours in rodents.

Certain chemicals, categorized as CIGAs (chemicals inducing $\alpha_2\mu$-globulin accumulation), cause a specific renal tubule nephropathy, leading to carcinomas, in male rats only (USEPA, 1991). The mechanism of the nephropathy has been linked to a specific metabolic pathway that causes these proteins ($\alpha_2\mu$-globulin) to accumulate in the renal tubules and cause chronic irritation leading to cell proliferation and neoplasia. These compounds are not genotoxic and the phenomenon does not occur in humans. Therefore, these tumours are not relevant to humans. In the example of thyroid tumours in rodents, microsomal induction by certain chemicals leads to the rapid metabolism of the thyroid hormone, T_4 (McClain, 1995; Capen, 1997; USEPA, 1998). This results in a continued stimulation of thyroid follicular cells to produce more T_4, resulting in hyperplasia, which leads to adenomas and carcinomas. As the induction of thyroid hormone metabolism is significantly less pronounced in humans, these tumours are not relevant.

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2.3 Establishing End Point of Concern

Once all the studies have been reviewed and evaluated, it is the responsibility of the risk assessor to select the end point of concern. Also known as the critical effect, the end point of concern is often one that occurs at the lowest levels of exposure and is relevant to humans. As it is the key end point that the risk assessment is attempting to prevent in exposed individuals, it is important that professional judgement be used in its selection.

3 DEFINITION OF DOSE–RESPONSE VALUES

Risk assessment entails characterizing and quantifying the potential adverse effects of hazards on humans or the environment (McClellan, 1999). This process involves hazard identification, exposure–response characterization, exposure assessment and risk characterization. While there are many aspects to performing a risk assessment, a consideration of the dose–response curve is one of the most fundamental elements. A variety of values are utilized when describing and evaluating dose–response relationships. This section will define some of these terms, as well as look at a few special situations where their definitions may change depending on the type of risk assessment being performed.

Several approaches have been and continue to be utilized in the field of risk assessment. These approaches vary, depending upon whether the effect has a threshold or is a nonthreshold response. As stated previously, most types of toxicological effects (e.g. target organ, reproductive/developmental, nongenotoxic carcinogenesis), are considered threshold responses (Sand *et al*., 2008). However, most risk assessments for genotoxic carcinogens make the assumption that no threshold exists and extrapolate to a *de minimus* risk level.

The traditional approach for risk assessment of nongenotoxic agents is to establish a point of departure (POD), typically a no observed adverse-effect level (NOAEL), and divide by uncertainty or safety factors to determine a health-based guidance value such as an acceptable daily intake (ADI) or a reference dose (RfD) (discussed in more detail later in this chapter). A NOAEL is the highest dose where no effect considered deleterious to the wellbeing of the test subject is observed. This differs from a no observed effect level (NOEL), which is the highest dose used in a study that causes no significant difference in any type of effect between the test subjects and the unexposed controls. In studies where a NOAEL is not established, a lowest observed adverse-effect level (LOAEL) can be used as a POD, but it must be extrapolated to a NOAEL. A LOAEL is generally defined as the lowest dose used in a study that results in the appearance of a significant adverse effect. This differs from a lowest observed effect level (LOEL), which is defined as the lowest dose used in a study that results in the appearance of some statistically significant mild biological effect (beneficial or deleterious). As mentioned earlier in this chapter, the POD may be different, depending on the type of risk assessment being performed. Ultimately, it depends upon what is considered 'adverse' and what is considered 'acceptable'.

Occasionally, neither a NOAEL nor a LOAEL can be identified. This is often the case when conducting an occupational-risk assessment for active pharmaceutical ingredients (APIs). In these cases, the POD may be a lowest therapeutic dose level (TDL) or a frank effect level (FEL), which can be higher than a LOAEL. Generally at the lowest TDL, the therapeutic benefits are expected to be more significant than any adverse effects. However, in certain cases (e.g. cytotoxic chemotherapeutic agents, steroid hormones), drugs may be administered at FELs. These dose levels result in overt, often clinically apparent, toxicity. For these compounds, data on dose levels lower than the FEL may not be available and the FEL must be used as the POD. Although this is not the preferred POD, extra uncertainty/safety factors can be added to account for the severity of effects and extrapolation to a NOAEL.

Since a number of disadvantages have been associated with using NOAELs in risk assessment, alternative models such as the benchmark dose (BMD) method (discussed in more detail later in this chapter) have been developed (Crump, 1984). In this method, the POD for extrapolation to a low-dose human exposure is obtained by fitting of a dose–response curve to experimental data. Several dose–response values that are used in the application of this method are defined below. The BMD is a dose calculated from the equation of the curve (see **Figure 2**), which corresponds to a predetermined change in the response (usually set at 1, 5 or 10%), generally referred to as the benchmark response (BMR). It is often represented by BMD_x, with x representing the percentage BMR. The one-sided lower 95% confidence limit of the BMD, the BMDL, can be used as the POD for determination of health-based guidance values. For a BMR of 10%, the BMDL is expressed as $BMDL_{10}$ and is considered a substitute for a NOAEL.

Another method used in risk assessment (typically for linear low-dose extrapolation) is the determination of a risk-specific dose (RSD). The RSD is a dose associated with a certain risk and is defined by the formula:

$$\text{RSD} = \text{Level of Risk (R)/Slope Factor (SF)}$$

R is predetermined and is the acceptable level of risk. For example, acceptable levels of risk for cancer vary by situation, but generally range from one extra

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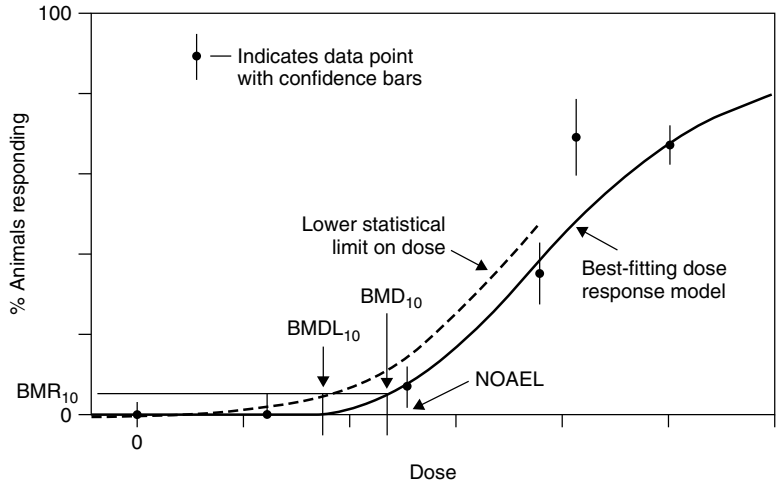

Figure 2 Graphical depiction of benchmark dose parameters.

cancer death per year per 10 000 people (10^{-4}) to one extra cancer death per million people (10^{-6}) exposed to a given contaminant over their lifetime. The SF, which is obtained from results of laboratory and/or epidemiological studies, is a numerical representation of the risk at every possible dose of a given chemical.

A number of other values are commonly used in risk assessment to describe dose–response curves. One of the most regularly used values is the median-effect dose or the ED_{50}. This dose can represent both a favourable or toxic effect of a chemical and is the dose of a chemical that causes a 50% response in a given effect. More specific ED_{50} values include the LD_{50}/LC_{50} (the dose/concentration of a substance that is lethal to 50% of the test population following a single dose), IC_{50} (the dose associated with 50% inhibition of a given biological or biochemical function), RD_{50} (exposure concentration producing a 50% respiratory rate decrease) and TD_{50} (the dose at which 50% of the population exhibit toxic effects). For drugs, the therapeutic index (TI) (also known as the therapeutic ratio) is defined as TD_{50}/ED_{50}. More specifically, it is the ratio of the median toxic dose (historically the lethal dose) to the median therapeutic dose. Drugs with a high TI have greater relative safety than drugs with a low TI. Since use of median doses in calculating the TI does not take into account the slope of the dose–response curve, another value called the margin of safety (MOS) has been used in risk assessment of drugs. The MOS is defined as TD_1/ED_{99}. It should be noted that in the risk assessment of nondrug chemicals, MOS has an alternate definition. It refers to the magnitude of the difference between an estimated exposure dose for a human population and the NOAEL or BMD from experimental animal studies. A similar term used in the risk assessment of both genotoxic carcinogens and noncarcinogenic compounds is margin of exposure (MOE). MOE refers to the ratio of a dose that causes a given effect in animals (the $BMDL_{10}$ is commonly used) to the average or predicted human exposure. The United States Environmental Protection Agency (USEPA) considers MOEs for noncarcinogens that are less than 100 to present an unacceptable risk to workers (USEPA, 2000). The European Food Safety Authority concluded that for genotoxic carcinogens, MOEs $>10\,000$ are associated with low risk and low priority for risk-management actions (EFSA, 2005).

4 FACTORS INFLUENCING DOSE–RESPONSE RELATIONSHIPS

The dose–response relationship is very complex and there are a number of modifiable (e.g. lifestyle, diet) and nonmodifiable (e.g. genetics, age) factors that can influence the shape of a dose–response curve. Several straightforward modifying factors, such as bioavailability and dosing regimen have been studied; however, this section will focus on more complex aspects that cause variability in response, such as interspecies differences, interindividual genetic variation, ecogenetics and hormesis. When all of these factors are taken into consideration, a true understanding of a dose–response curve can be attained.

Experimental dose–response data from animal studies are commonly used in risk assessments by regulatory agencies. Animal models, when properly applied, can be relevant to humans. However, differences in metabolism, pharmacokinetics and pharmacodynamics can cause vast differences in the response to any given chemical. A classic example of a species-specific metabolic difference is the hepatocarcinogenic response to the fungal toxin aflatoxin B_1. In mice, no evidence of hepatocarcinogenicity was seen at dietary doses as high as 10 000 ppb aflatoxin B_1; however, in rats, doses as low as 15 ppb caused a significant increase

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in liver tumours (Wogan *et al*., 1974). The remarkable difference between rats and mice in their susceptibility to aflatoxin B_1 has been attributed to species-specific differences in the expression of a specific form of glutathione-S-transferase that detoxifies aflatoxin B_1-8,9-epoxide, the putative ultimate carcinogen (Eaton and Gallagher, 1994). High expression of this enzyme in mice, but not rats, is responsible for resistance to the carcinogenic effects of aflatoxin B_1. In humans, a cytosolic fraction of glutathione-S-transferase isolated from liver cells was relatively ineffective at conjugating aflatoxin B_1-8,9-epoxide; glutathione-S-transferase activity towards aflatoxin B_1-8,9-epoxide was 70-, 465- and 3545-fold greater in rats, hamsters and mice, respectively, when compared to human liver cytosol preparations using microsomally generated aflatoxin B_1-8,9-epoxide (Slone *et al*., 1995). This suggests that humans may be sensitive to the carcinogenic effects of aflatoxin B_1. If dose–response data from mice or other animals resistant to the carcinogenic effects of aflatoxin B_1 were used to do a human-risk assessment, this could result in a health-based value that is too high. Therefore, it is important to understand the species-specific differences in metabolism and their effects on dose–response curves for different animals.

In addition to interspecies differences in metabolism, species-specific differences in pharmacokinetics can also affect dose–response relationships. For example, when assessing developmental toxicity, it is important to note that pharmacokinetic differences between species can affect the dose of a chemical that reaches the embryo/foetus. Factors that may differ from species to species include protein binding, half-life and maternal-conceptus pH gradient (Nau, 1986). It has also been noted that fundamental differences exist between rats, rabbits and humans in maternal-embryonic exchange during early pregnancy (Carney *et al*., 2004). It has been suggested that the varying toxic effects of thalidomide in different species may be at least partially due to pharmacokinetic (e.g. half-life, plasma-concentration–time profiles), as well as metabolic differences (Chung *et al*., 2004). Studies looking at the ability of thalidomide to cause developmental toxicity in animals treated during critical times of embryogenesis have demonstrated extreme variability in species susceptibility (Newman *et al*., 1993; TERIS, 2006). Developmental toxicity has been observed in nonhuman primates and rabbits at oral doses equal to or less than human therapeutic doses. In contrast, studies on the adverse effects of thalidomide on embryonic development in rats, mice and hamsters were generally negative or only produced positive results at higher doses. An appreciation of the species-specific pharmacokinetic factors that affect the dose–response relationship is important when conducting a human-risk assessment for thalidomide.

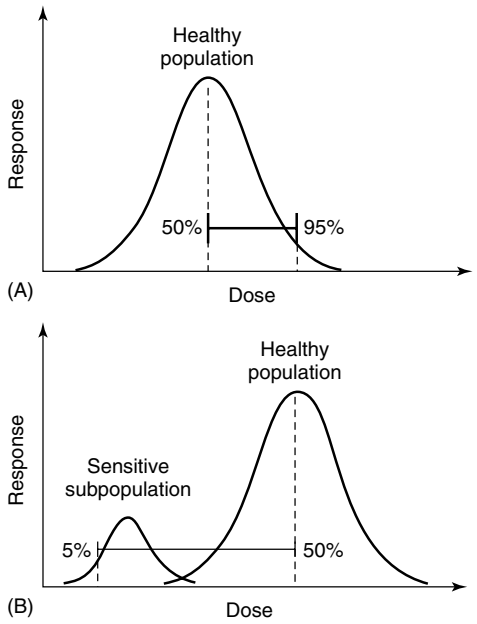

Figure 3 (a) Unimodal population showing a normal distribution for healthy individuals. (b Bimodal population showing two distinct populations, healthy individuals and a sensitive subpopulation.

Another source of variability in response is the existence of sensitive subpopulations. A healthy population can be best represented by a unimodal distribution curve demonstrating a normal distribution (**Figure 3**a). When a sensitive subpopulation is present, the population may be better represented by a bimodal distribution curve (**Figure 3**b). When a distinct sensitive subpopulation exists, the ratio of the upper tail of the most sensitive subpopulation over the mean of the healthy population provides an estimate of interindividual variability in the two populations (Naumann *et al*., 2001). It is clear from the figure that the variability in the bimodal population is greater than in the unimodal population and can be used to derive an appropriate adjustment factor in a risk assessment to account for interindividual differences.

One example of a sensitive subpopulation is the elderly. As humans age, their immune systems do not function as efficiently as younger individuals. A recent discovery in the relatively new field of immunogerontology (the study of the immune system in the elderly) was that the elderly may not develop a fever (an innate response) in response to bacterial infection and are not as efficient at producing lymphocytes as younger people. This may result in an entire subpopulation that is more susceptible to certain environmental insults than healthy individuals.

Genetic polymorphisms in critical genes can also lead to the formation of sensitive subpopulations. A genetic polymorphism exists when there are two or more clearly different phenotypes of a given gene in the population. The xeroderma pigmentosa group A

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gene (XPA) is involved in nucleotide excision repair of ultraviolet-induced DNA lesions (Miller *et al*., 2006). Xeroderma pigmentosa (XP), a rare autosomal recessive disease associated with a 1000-fold increased risk of keratinocytic cancers (including basal and squamous cell carcinomas), occurs when humans have two nonfunctional copies of an XP gene. Although it is rare to lack two functional copies of the XPA gene, polymorphisms in this gene commonly exist in the human population. The GG genotype of the A23G polymorphism has been associated with a significantly increased risk of basal cell carcinoma and squamous cell carcinoma. Genetic polymorphisms in a number of other important genes, including p53, Rb and genes involved in xenobiotic metabolism/DNA repair, have been associated with an increased risk of cancer and/or other diseases.

Several regulatory agencies (e.g. EPA, Occupational Safety and Health Administration (OSHA)) are expected to promote risk-assessment procedures that protect sensitive subpopulations or sensitive individuals. As a result, the relatively new field of ecogenetics is becoming increasingly important in assessing dose–response relationships, and is likely to play a critical role in future risk-assessment procedures. Ecogenetics is the study of how genetics alterations may represent risk factors for environmentally influenced health effects associated with exposure to toxic chemicals (Costa, 2000).

Another controversial phenomenon affecting dose–response relationships is hormesis. Hormesis is most commonly described as a dose–response relationship in which there is biological activation at low doses and an inhibition at high doses, or vice versa (Calabrese, 2002). This results in a U-, J- or inverted U-shaped dose–response curve. Under this theory, many chemicals have demonstrated beneficial, rather than harmful effects at low doses. At these low doses, the initial response may be described as an adaptive response to help protect the organism and preserve homoeostasis. A number of agents have been shown to display hormetic dose–response curves, including ionizing radiation, alcohol and essential nutrients. For example, chronic low doses of ionizing radiation are thought to stimulate cellular repair mechanisms, rather than cause DNA damage. Similar effects have also been observed with other nongenotoxic carcinogenic agents such as phenobarbital, dichlorodiphenyltrichloroethane (DDT) and ethanol (Fukushima *et al*., 2005). A better mechanistic understanding of hormesis is important since current risk-assessment models do not take into account the possibility that low doses of a chemical may be beneficial. How and if hormesis will be considered in future risk-assessment models remains to be seen.

5 DOSE–RESPONSE MODELLING IN RISK ASSESSMENT

As is stated earlier in this chapter, human-effects data or human-exposure measurements are lacking for many chemical substances. Therefore, risk assessments for many xenobiotic chemicals often use data from chronic animal bioassays. The typical chronic bioassay involves a two-year exposure of rodents to several dose levels of the test article. At least one of the dose levels is at or near a maximum tolerated dose. The primary end point of focus in the chronic bioassay is cancer, although all chronic toxic end points are examined. The ultimate goal of the study is to use it in a risk assessment to estimate human risk at very low, environmentally relevant doses. The estimation of human risk requires two extrapolations: one from animals to humans and one from high experimental doses to low environmentally relevant doses. The major issue for low-dose extrapolation is determining the shape of the dose–response curve below the experimental dose range. While linear extrapolations are most common, arguments have been made for the existence of both sublinear and supralinear dose–response (see **Figure 1**).

Low-dose extrapolation models can be classified as either dichotomous or timed-response models (Altshuler, 1981). In a dichotomous response, the outcome of the animal study is determined as the number of responders and nonresponders. Timed-response (also known as time-to-occurrence) refers to the measurement of the experimental life of the animal which is impacted by the occurrence of some chronic toxicity (i.e. cancer). The best-known dichotomous models are the log probit, the multi-hit and the multistage or linear multistage, whereas the best known timed-response models are the log-normal and multistage.

5.1 Linear Multistage Model

The multistage model was first proposed as a time-to-occurrence model by Armitage and Doll (1961) to explain epidemiological data showing tumour incidence rates increasing with time. It has been adapted for use with dichotomous or quantal data. The original model is based on six assumptions: (i) a tumour develops out of a single transformed cell, (ii) time (w) is required for the transformed cell to grow into an observable tumour, and no response is observed until time (t) is greater than time delay (w), (iii) a target cell goes through a number of discrete stages (k) in its progression to becoming cancerous, (iv) the timing of the discrete changes called transitions is governed by transition rates which do not change with time, (v) background response is governed by spontaneous transition rates and (vi) the number of dose dependent transitions (m) may be less

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than the number of transitions (k). The resulting model is expressed as a Weibull distribution:

$$-\log[1 - P(t; d)] = ad^n(t - w)^k \quad (1)$$

where $-\log[1 - P(t;d)]$ is the cumulative hazard or incidence, a is the constant of proportionality and cumulative incidence varies as the nth power of dose (d).

Crump and Howe (1984) extended the Armitage–Doll model by incorporating an assumption that the transition rate at which a cell goes through each stage is linearly related to the dose rate:

$$\gamma_{i=}\alpha_i + \beta_i d \quad (2)$$

where d is the dose rate of a continuously applied carcinogen, α_i is the background transition rate in the absence of dose and β_i is the transition rate per unit dose. The mathematical form of the linearized multistage model is:

$$P(d) = 1 - exp(-q_0 - q_1 d\_ \ldots .q_k d^k) \quad (3)$$

where q_1 is the linear term that is equal to or greater than zero, d is the lifetime average daily dose of carcinogen in mg kg $^{-1}$ day^{-1}, $P(d)$ is the lifetime probability of a cancer occurring from dose (d) and q_0 to q_k are non-negative parameters estimated from fitting the model to experimental data. Variables needed to run the linearized multistage model include dose, number of animals with a specific tumour and the number animals at risk for that specific tumour.

Due to the uncertainty in low-dose extrapolation, the upper 95% confidence limit of q_1 is used by regulatory agencies when estimating cancer risks. This new term $q_1{}^*$ is referred to as the unit cancer potency estimate and is used in the following equation to estimate risk:

$$\text{Risk} = q_1{}^*(\text{mg kg}^{-1}\text{day}^{-1})^{-1} \times d(\text{mg kg}^{-1}\text{day}^{-1}) \quad (4)$$

5.2 NOEL/LOEL Approach

Current risk assessment assumes that a threshold exists for most toxic effects other than genotoxic carcinogenicity. The traditional approach to define this threshold is to identify a NOEL or LOEL. These effect levels may be established based on statistically significant responses at the LOEL or by evidence of a continuum of response with increasing dose. LOELs are often used in conjunction with an uncertainty factor as a surrogate value for a NOEL in risk assessments (Sargent and Kirk, 1988; Naumann and Weideman, 1995). When pharmacologic effects are evaluated in conjunction with toxicologic effects, it becomes important to distinguish NOAELs and LOAELs from NOELs and LOELs. The NOEL/LOEL approach has been widely used in establishing RfDs, reference concentrations (RfCs), ADIs and occupational exposure limits (OELs) such as threshold limit values (TLVs) (ACGIH, 2008). The majority of these regulatory values have been typically derived by determining a POD based on the dose–response from the most sensitive end point(s) relative to humans.

5.3 Benchmark Dose

Since NOELs/LOELs are based on identifying statistically significant responses at specific doses or by identifying a dose-dependent response continuum, their determination is highly dependent on dose selection in the toxicity study. In addition, estimation of a NOEL or LOEL often does not explicitly utilize the entire dose–response curve and provides no extrapolation to lower risks at lower doses.

Therefore, the BMD approach was developed as an alternative to the NOEL/LOEL approach (Crump, 1984; Crump, 1995). It has gained popularity over the past 20 years, mostly in the United States, where it has been used in noncancer risk assessment to establish RfDs and OELs. The BMD approach has certain advantages over the NOEL/LOEL approach in that it is more quantitative, involves modelling the dose–response curve within the range of observable data and is not restricted to the experimental doses used in a particular study. It is not as simple as the NOEL/LOEL approach as it requires fitting a curve to a dose–response dataset using a computer model (see **Figure 2**). Also, like the NOEL/LOEL approach, a combination of end points may need to be considered in order to complete a risk assessment. The BMD is defined as an exposure due to a dose of a substance associated with a specified level of BMR, generally in the range of 1–10%, of a health effect; or the dose associated with a specified measure or change of a biological effect (CalEPA, 2004; USEPA, 2008) (see **Figure 1**). The BMD is most often based on the 95th percentile lower bound of the effective dose (LED), rather than the model's best estimate of the ED.

Guidance and software (BMDS) for deriving BMD values for use in risk assessment have been made available free of charge through the USEPA National Center for Environmental Assessment (NCEA) (USEPA, 2008). In conducting a risk assessment using a BMD approach, the usual hazard characterization is completed and end points for dose–response are selected. Generally graded monotonic responses as a function of dose and a significant dose-related trend are most useful in a BMD analysis. In addition to continuous responses, end-point data may also be quantal or dichotomous where the response

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is reported as present or absent. In either case, the latest version of the BMDS software contains different mathematical models for fitting dichotomous (gamma, Hill, logistic, multistage, probit, log-probit and Weibull) as well as continuous (linear, polynomial, power, Hill and exponential) dose–response data.

5.4 Categorical Regression

Another alternative method to the NOEL/LOEL approach for dose–response analysis of noncancer end points is categorical regression (Hertzberg and Miller, 1985; Hertzberg, 1991; Hertzberg and Dourson, 1993). Categorical regression is a metanalytical technique whereby data on how both the incidence of response and the severity of response changes as a function of dose. In this approach, severity categories are assigned to observed effects seen in a toxicity study. These categories are based on the overall toxic impact to an exposed individual. The main advantage of categorical regression is that the risk assessor is able to categorize all available data from multiple studies and is able to order those categories based on the severity of the toxic effect. A categorical regression can be run on a single end point from a single study using a generalized linear model, or it can be run on the results of different studies, each identifying a different dose–response categorical level. The different dose–response categories, including NOELs, LOELs, NOAELs, LOAELs and FELs can all be analysed and displayed as a single regression.

Evaluations using categorical regression have been conducted with a number of different chemicals (Guth *et al*., 1991; Rao *et al*., 1993). Categorical regression was used by Dourson *et al*. (1997) to evaluate risks above an RfD established by the USEPA for aldicarb. The analysis demonstrated that the combined risks of adverse effects or frank effects at 10 times the RfD are either 0.1 or 0.008% when whole-blood and red-blood-cell cholinesterase inhibition are considered as adverse effects, respectively, indicating a potential 12-fold difference in the risk estimate.

6 ISSUES WITH COLLECTION AND USE OF DOSE–RESPONSE DATA

Knowledge of dose–response relationships is not just an exercise in determining the extent of toxicity. Perhaps more important is the identification of safe and effective dosing for clinical treatments such as drug and radiation therapies. For drugs, dose–response information is needed to identify a starting dose, an appropriate means of dose adjustment, and the definition of a dose that provides the maximum benefit, while producing the least amount of unacceptable side effects (ICH, 1994). In order to select a starting dose for drug therapy, the shape of the dose–response curves for both desirable and undesirable effects must be understood. Choice of a starting dose must take into consideration interindividual differences in pharmacokinetics and pharmacodynamics, such as those arising from nonlinear kinetics, genetic polymorphism or pharmacokinetic drug–drug interactions. Utilizing dose–response data, dose adjustments can factor pharmacokinetics differences due to age, gender, race, other diseases (i.e. renal failure), diet, concurrent therapies or individual characteristics, such as body weight (Silverman *et al*., 1999).

For some drugs, safe and effective use cannot be monitored by dose–response data, but rather are followed by concentration–response information. Concentration –response data is particularly useful when pharmacokinetic differences due to diseases, like renal failure, exist or when the effects of altered pharmacokinetics of differing formulations (i.e. slow-release formulations) are assessed. In this case the concentration–response data can be better translated into dose–response information.

Dose–response data for many drugs are obtained using a dose-titration clinical study design. As a result, the tendency in interpretation of this dose–response data is to select as a recommended dose, the highest dose tested that was tolerated reasonable well. Historically, this has led to the selection of doses in excess of what was actually needed, resulting in an increased incidence of adverse effects. As an example, the initial doses of zidovudine used for the treatment of AIDS were based on the results of high-dose studies without adequate knowledge of the dose–response. When the dose–response was better characterized, it was determined that lower doses were as effective and far better tolerated in patients (ICH, 1994).

The frequency and timing of dosing also has an impact on the amount of drug administered. In a single, long dose interval, the dose–response relationships at peak and trough blood concentrations must be considered. If the dose interval is excessively long relative to the pharmacokinetic half-life of the drug, there may be a pharmacodynamic need to administer the dose at more frequent intervals.

7 APPLIED DOSE–RESPONSE IN HAZARD EVALUATION AND RISK ASSESSMENT

7.1 Establishing Acceptable Risk Values

As mentioned earlier in this chapter, the acceptability of risk is partially dependent upon the benefit being received by an individual. A patient taking a chemotherapeutic

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agent will have a higher tolerance for the risk of adverse effects from the drug than the worker (pharmacist or chemical operator) potentially exposed to that drug occupationally. The benefit to the patient is greater than to the worker. However, although the worker does not get the benefit of treatment for a disease, they are being compensated for performing their work and should be aware of the potential hazards of handling APIs. Extending this analogy one step further, an individual in the community where the manufacturing facility is located may be exposed to the pharmaceutical product in their drinking water or air. Individuals in the community have an expectation of contaminant-free air and water, and have less benefit from the potential exposure to the chemical. As a result, the generally accepted risk for community exposure scenarios is one in 1 million (10^{-6}), whereas acceptable risk for a workplace exposure is usually one in ten thousand (10^{-4}).

7.2 Establishing Acceptable Daily Intakes

One of the ultimate purposes of understanding the dose–response relationships discussed in this chapter to determine exposures that will be considered 'acceptable' to a given population of individuals. These acceptable levels or ADIs are considered the amount of a material that individuals may be safely ingest, inhale or otherwise be exposed to every day for an average lifetime. Applications of this type of risk assessment vary depending upon the type and use of the material being handled, as well as the risk–benefit profile of the group of individuals potentially exposed. Some of the ultimate uses of these assessments are enumerated below.

7.3 Product Contamination Limits

Unfortunately, it is virtually impossible to provide products for commercial use that are 100% pure and unadulterated. As analytical techniques improve, the ability to detect impurities in products also increases. Consequently, it becomes necessary to set limits on the levels of these contaminants that are safe when found in these products.

Food products, even organic ones, can be contaminated by various types of materials. These can be naturally occurring substances, direct food additives (such as food colourings or preservatives), indirect food additives (such as leachates from packaging materials) or contaminants from food processing. They can also include residuals of pesticides used to protect agricultural products from pests or antibiotics, or other drug products used to protect the health of animals used for food. The exposure of these contaminants to the general population is calculated as an estimated daily intake (EDI). When the EDI is less than the ADI, the product is considered safe.

The manufacture of pharmaceutical molecules is often comprised of multiple synthetic chemical reactions. Residual amounts of solvents or other impurities from earlier synthetic steps may be present even after the final purification step of the drug substance. The manufacturer, in filing applications for drug approval with the FDA, must set specifications on the amounts of each impurity that will exist in the final product. The rationale for these impurity and solvent-residue specifications are supported by risk assessments derived from analysis of the dose–response relationships.

Another application of product contamination assessment is used to answer the question, 'How clean is clean?' After using a specific piece of equipment to manufacture one chemical, how much residue is permitted to remain, and potentially be incorporated into the next batch of product run on the same equipment. The FDA may require dedicated equipment for chemicals with severe adverse effects profiles (cytotoxic drugs or penicillins) as they believe that no amount of residue of these compounds may be incorporated into a subsequent batch of drug product.

7.4 Occupational and Environmental Risk Values

Similar approaches can be used to establish safe limits of exposure to environmental contaminants that may occur as a result of indirect, unintentional exposure to air, water or contact surfaces. Some of these limits are described below along with the differences in exposure scenario and acceptability of risk.

For example, workers are potentially exposed to chemical compounds they may be manufacturing directly (solvents, reagents, pharmaceuticals, etc.) or using as a tool to perform some function of their job (cleaning products, paints, finishing materials, etc.). By evaluating the dose–response relationships of these substances and applying risk-assessment principles, limits can be developed to protect workers from adverse effects that may be associated with exposure to these materials. If the concern is exposure by inhalation, safe airborne limits or OELs, can be derived. OELs are defined as levels of exposure to an airborne contaminant that are considered safe to nearly all workers. Some subpopulation of workers may exist, due to individual susceptibility, which may be adversely affected at concentrations at or below the OEL. OELs are developed by selecting a NOAEL (or LOAEL if no NOAEL is available) and applying safety and uncertainty factors to develop an acceptable concentration of the contaminant in the air (Sargent and Kirk, 1988; Galer *et al.*, 1992).

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Just as limits for worker exposures can be evaluated from existing dose–response data, limits of contaminants in the ambient air can be derived to protect the general public. The EPA has established National Ambient Air Quality Standards (NAAQS) for various atmospheric pollutants such as SO_X and NO_X.

In addition to limits for acceptable levels of contaminants encountered under routine conditions, NIOSH has established short-term limits that can be tolerated during emergency situations (NIOSH, 1994). These conditions are considered immediately dangerous to life and health (IDLH) and are defined as situations 'that pose a threat of exposure to airborne contaminants when that exposure is likely to cause death or immediate or delayed permanent adverse health effects or prevent escape from such an environment'. The IDLH is established by NIOSH to 'ensure that the worker can escape from a given contaminated environment in the event of failure of the respiratory protection equipment'.

The AIHA has developed a series of similar limits known as Emergency Response Planning Guideline (ERPG) values (ERPG-Committee, 2008). They are intended to provide estimates of concentration ranges where one reasonably might anticipate observing adverse effects as described in the definitions for ERPG-1, ERPG-2 and ERPG-3 as a consequence of exposure to the specific substance.

- The ERPG-1 is the maximum airborne concentration below which it is believed that nearly all individuals could be exposed for up to one hour without experiencing other than mild, transient adverse health effects or perceiving a clearly defined, objectionable odour.
- The ERPG-2 is the maximum airborne concentration below which it is believed that nearly all individuals could be exposed for up to one hour without experiencing or developing irreversible or other serious health effects or symptoms which could impair an individual's ability to take protective action.
- The ERPG-3 is the maximum airborne concentration below which it is believed that nearly all individuals could be exposed for up to one hour without experiencing or developing life-threatening health effects.

Airborne limits are not the only concern for occupational or environmental risk values. Contamination of surfaces with chemical substances that may be absorbed through the skin in toxicologically significant amounts may be a concern to workers. Workers may be exposed to more than just airborne contaminants, however. Liquids remaining on unclean surfaces or airborne dusts settling on workplace surfaces can potentially be absorbed when a worker subsequently comes into contact with a contaminated surface. Developing acceptable surface limits (ASLs) depends upon additional data that may not have been used to calculate airborne limits. The ability of a chemical to penetrate the skin and exert its toxic effect will be determined by another set of chemical and toxicological properties, such as lipophilicity, molecular weight, percutaneous absorption data and projected surface-contact scenario.

Highly specialized exposure situations call for specific risk assessments and limits set according to those situations. Spacecraft Maximum Allowable Concentrations (SMACs) are intended to provide guidance on chemical exposures during normal operations of spacecraft as well as emergency situations (NASA, 1999). Short-term SMACs refer to concentrations of airborne substances (such as a gas, vapour or aerosol) that will not compromise the performance of specific tasks by astronauts during emergency conditions or cause serious or permanent toxic effects. Such exposures might cause reversible effects, such as mild skin or eye irritation, but they are not expected to impair judgement or interfere with proper responses to emergencies. Long-term SMACs are intended to avoid adverse health effects (either immediate or delayed) and to prevent decremental change in crew performance under continuous exposure to chemicals in the closed environment of the space station for as long as 180 days.

Similar limits have been developed for submarines (National-Research-Council, 2008). When submerged, a submarine is an enclosed and isolated environment. Its crew is potentially exposed to air contaminants in this environment 24 hours a day. Unlike workers in a typical occupational environment, submariners do not leave their work environment at the end of their shift. To protect their health, the US Navy has developed one hour and 24 hour emergency exposure guidance levels (EEGLs) and 90 day continuous exposure guidance levels (CEGLs) for a number of chemical contaminants.

Finally, environmental risk values can also be set for the contaminants in our water supply. The process for setting a safe drinking-water limit is similar to setting a safe airborne limit. The major difference between the two risk assessments is the exposure scenario. Once an ADI or RfD has been determined, the limit in the drinking water can be calculated using the default assumption of 2 l of water being ingested each day. The risk assessment can be refined by considering other sources of exposure, such as dermal absorption while swimming, bathing or showering, and inhalation of high vapour pressure compounds in the shower.

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Hormesis and Risk Assessment

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CONTENTS

1 INTRODUCTION

The hormetic dose–response model has generated considerable interest within the fields of toxicology and risk assessment (Kaiser, 2003; Renner, 2004; Calabrese and Baldwin, 2003a). Considerable efforts over the past 15 years have revealed that hormesis commonly occurs, is reproducible, has distinct quantitative features (**Figure 1**) and has a sound mechanistic foundation (Calabrese, 2005). In addition, in fair, large-scale head-to-head comparisons with the threshold dose–response model with environmental and pharmacological agents, the hormetic dose–response model was very effective in estimating below-threshold responses, whereas the threshold model performed in a consistently poor fashion (Calabrese and Baldwin, 2003c; 2001; Calabrese *et al.*, 2006). These findings raised serious questions about the scientific foundations of the threshold model and especially its capacity to offer regulatory agencies and the general public reliable toxicological predictions in the low-dose zone, the principal area of public health concern given the nature of human exposures. Despite these serious limitations of the threshold model to predict low-dose responses and the inability to validate predictions of linear-at-low-dose models that are used in genotoxic carcinogen risk assessment, these models have continued to be consistently used by federal and state regulatory agencies in the USA and all other countries. Given the serious failings of these risk assessment methodologies, and the strong performance of the hormetic dose–response model accurately to predict low-dose responses and to be validated, it is important to assess how the hormetic dose–response model may affect the risk assessment process.

2 HAZARD ASSESSMENT

2.1 Animal Model

Animal models have historically been selected for use in the chronic bioassay due to their low susceptibility to infectious disease, size, ease of handling, relatively

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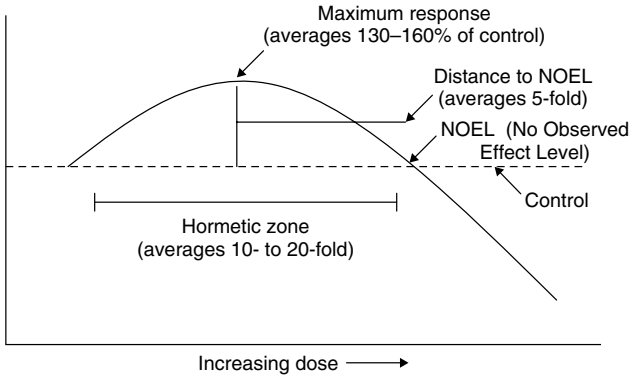

Figure 1 Dose–response curve depicting the quantitative features of hormesis.

short lifespan, fecundity, capacity to develop diseases of interest and to have a low background disease incidence in the control group. Having a high background disease incidence that is highly variable has typically been viewed as an important issue in the hazard assessment process. This issue could make it difficult to distinguish a treatment-related effect with a relatively small sample size from the control group, thus the advantage of a consistently low background disease incidence in the control.

It is not possible to assess hormetic hypotheses with animal models with very low or negligible disease incidences. In other words, it is not usually possible to practically assess whether a normally very low disease incidence may have been diminished further by the treatment, that is, demonstrating hormesis. A consideration of hormesis in animal model selection creates a conflict with traditional hazard assessment criteria that use animal models with a low background disease incidence.

A question is: is there an animal model disease-incidence optimum that could achieve an ideal compromise between being able to detect a treatment-related effect in the 'above' threshold zone (i.e. adverse treatment effect), and a treatment-related effect in the 'below' threshold zone (i.e. the hormetic effect which could be beneficial, harmful or of indeterminate medical or public-health consequence)? The answer may represent a practical balancing of research interests across a wide range of end points. To assess hormetic end points it would be of value to have a background disease incidence in the approximate range of 6–12%. A background disease incidence of less than about 6% would substantially increase treatment sample size needed to detect hormesis. Similarly, if the background disease incidence exceeded 12%, it would notably impact the sample size needed to detect a treatment effect occurring above the threshold dosage. Thus, there is a control response optimum that affects the capacity to detect below and above threshold responses. Once the hormetic concern becomes a study design priority it is necessary to base sample size calculations on balancing the goals of assessing hormetic (i.e. below threshold) and above-threshold effects. In general, there will be background disease incidence variation across end points of interest that may range from approximately 0% to greater than 20%. A key challenge for investigators is finding the optimal design for their intended purposes.

There is essentially little-to-negligible opportunity to assess disease-related hormesis hypotheses in the case of subacute toxicity studies of 13 weeks duration in which young adults are employed. An evaluation of many National Toxicology Program (NTP) subacute investigations by Calabrese and Baldwin (2003b) revealed that background disease incidences in these relatively young adults is too low to study disease-based questions related to hormesis. However, this study assessed the rate of body weight gain within a hormetic context. Other end points could be introduced if the goal was to assess hormesis within a shorter-term study. For example, numerous tumour promotion studies have been published using a single exposure protocol in which the tumour was induced within weeks to several months (Calabrese and Blain, 1999). Thus, in the case of NTP studies, hormetic hypotheses could be taken into account in the subacute and chronic bioassays but this requires proper planning.

2.2 Study Design

To study hormetic hypotheses it is necessary to reliably estimate the threshold response for the end points of interest. Since each end point would have its own threshold, the investigator needs to prioritize the end point(s) of interest or to have a sufficiently comprehensive study design for assessing multiple end points, each with a possibly different threshold. The threshold could be estimated via the traditional use of range finding tests, which identify responses of the biological model to a broad range of doses. Such an investigation provides data to guide and refine the dosing scheme for the subsequent experiment(s) to estimate the lowest dose (lowest observed adverse effect level—LOAEL) causing toxicity and an estimated threshold dose. Once the threshold and LOAEL have been estimated/determined it is possible to assess the broader dose–response continuum by explicitly including hormesis considerations into the study design.

An evaluation of more than 8000 dose responses demonstrating hormesis (Calabrese and Blain, 2005) indicates that about 80% of the examples have a hormetic stimulatory width within 1/100 of the threshold response. That is, if the threshold dose were $100\,mg\,kg^{-1}$, the hormetic zone for the strong majority of cases would be within $1\,mg\,kg^{-1}$ to less than $100\,mg\,kg^{-1}$. In general, the maximum stimulatory response has been within about

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1/5 to 1/10 of the threshold dose. In about 2% of the cases, the width of the stimulatory zone exceeded 1000-fold. Nonetheless, even in these cases the onset of the stimulatory response is very close to the threshold. Since one does not initially know what the width of the stimulatory zone may be, it reasonable to assume it could approach 1/100 of the threshold dose. Using half-log dose spacing would employ four doses (1/3, 1/10, 1/33 and 1/100 of the threshold dose) below the threshold dose. While more doses could be used, this range of four doses represents an appropriate coverage of the most likely stimulatory zone. The same study should also include a dose that is expected to approximate the threshold and at least two higher doses that assure the description of the traditional toxicological dose–response relationship. Thus, at a minimum, assessment of hormetic hypotheses would require a control group and seven treatment groups. Depending on the data obtained from this experiment, it may be necessary to alter the dose selection in a subsequent experiment.

2.3 Sample Size

Since hormetic stimulatory responses are expected to be modest, even at their maximum response, they can be difficult to distinguish from background. Thus, statistical power calculations should provide the sample sizes needed to detect statistically significant differences. This suggests that that the study designs may be unequal with respect to the number of subjects used in the treatments. For example, increasing the sample size (n) for treatments in the presumed hormetic zone at the expense of those treatments above the threshold may be an appropriate strategy.

2.4 Temporal Measures (Repeat Sampling)

Because hormesis represents an overcompensation to a disruption in homeostasis, it is best reflected in a dose–time–response relationship. This has been demonstrated in hundreds of experiments for a wide range of end points (Calabrese, 2001). Failure to recognize the compensatory response activity can result in missing the hormetic dose response. The implications of repeat measures can be different depending on the experiment. In the case of plant growth, it may represent repeated measurements of the same plants over the course of the experiment, with no loss of sample size. However, in the case of cancer bioassays the subjects are removed for sacrifice during the experiment, reducing the final sample size.

2.5 Replication

The occurrence of hormetic responses usually requires replication. The extent of the replication would depend on the strength of the original experiments, taking into consideration the study design, the statistical power and the degree that the concurrent control group shows consistency with historical control-group data, amongst other considerations.

3 EXPOSURE ASSESSMENT

The hormetic dose–response model selection would not be expected to impact the process of exposure assessment.

4 DOSE–RESPONSE

The hormetic dose–response hypothesis should be testable within standard bioassays. The data derived from the experiment should provide the basis for whether the hormesis hypothesis is supported or rejected and will provide the necessary information upon which the dose–response modelling may be constructed.

4.1 Carcinogens and Noncarcinogens

The current dominant cancer risk-assessment paradigm has assumed that genotoxic carcinogens act via processes that can best be described by linear-at-low-dose models. Essentially all other toxicological conditions have been assumed by the Environmental Protection Agency (EPA) and Federal Drug Administration (FDA) to be acting in a manner consistent with a threshold dose–response model. This dose–response dichotomy has lead to different risk assessment methodologies for the evaluation of noncarcinogens/nongenotoxic carcinogens and genotoxic carcinogens. The hormetic dose–response model, including its quantitative features, offers the distinct advantage of applying to all types of biological models, end points, chemical classes and physical agents.

5 RISK CHARACTERIZATION: DEFAULT MODEL

If hormesis were to be accepted as the default model in risk assessment, then it would be used to derive acceptable exposures for carcinogens and noncarcinogens. The hormetic dose–response model is similar to

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the threshold dose–response model at the threshold and higher doses. That is, the hormetic dose includes the existence of the threshold response, as predicted by the threshold dose–response model. Immediately below the traditional threshold, the dose enters into the hormetic zone in which the response progresses below the control value, reaching a nadir and then proceeding back towards the control values, approaching another threshold on the dose–response continuum. The quantitative features of the hormetic dose–response model could become standardized using the hormesis database. In general, with respect to carcinogens and noncarcinogens it would be expected that a threshold response would occur, below which the response decreases below the control, reaching a value of about 30–60% of the control at the nadir of the dose–response relationship. The nadir would be expected to be reached at approximately 1/10 of the threshold dose. Based on the hormesis database, the width of the hormetic zone would be approximately over a 40-fold range.

5.1 Goal of Risk Assessment: Noncarcinogens

The goal of risk assessment for noncarcinogens has been to estimate the highest dose of an agent that will not cause toxicity in the experimental model and then apply appropriate uncertainty factors (UFs), relative source contributions and adjust for gender and possibly age differences. This would ensure that the risk of experiencing toxicity from a prolonged exposure would be very low to negligible. This is based on the assumptions that the average human is 10 times more sensitive than the average animal model, that human interindividuality is 10-fold and that the experiment was relevant to human exposures including route and duration of exposure.

5.2 Carcinogens

The goal of risk assessment for carcinogens has been to estimate a dosage that will result in an acceptable level of risk. Linear-at-low-dose modelling assumes that there will be no safe level, with the risk being proportionate to dose in the low-dose domain.

5.3 How Hormesis Impacts Risk Assessment

Hormesis has the capacity to significantly affect both noncarcinogen and carcinogen risk assessment. Since hormesis provides an assessment of responses above and below the threshold it offers risk assessors more information about the agent(s) being studied. In the case of **carcinogens**, the hormetic model not only assumes that there is a threshold below which there is no adverse treatment effect, but also a response zone in which the risk decreases below that of the control and does so for normal and high-risk segments of the population. If the goal is to prevent an agent-induced cancer response, then the agent could be handled via the use of UFs, or biomathematically modelled. If traditional interspecies and interindividual UFs were selected, then it is likely that there would be little likelihood of such exposures causing cancer in the population, assuming that humans are 10 times or less more sensitive than the animal model. On the other hand, this approach ignores low-dose hormetic effects in which exposure to the agent would be expected to reduce background cancer risks. If the beneficial effects were to be assessed then decision makers could consider optimizing health within the entire population. In this case the standard may ensure that the exposure was within the hormetic zone of the normal segment of the population. This may mean that some predicted adverse effects would be expected in the high-risk segment. Alternatively, the hormetic effects could be optimized for only the high-risk segment of the population. Consequently, the hormetic dose–response model has a significant effect on how risk assessment is applied to carcinogens. It would also notably impact risk–cost–benefit assessments of carcinogen exposure standards.

In the case of **noncarcinogens and nongenotoxic carcinogens**, the hormetic model provides risk assessors with the opportunity to consider selection of dosages that would be expected to optimize the population response, rather than simply focus on the avoidance of harm for all groups within the population. As in the case of genotoxic carcinogens this would also significantly affect risk–cost–benefit evaluations.

6 RISK ASSESSMENT MODELS AND ISSUES IN PERSPECTIVE

The current models that are used as defaults in risk assessment are the threshold model with noncarcinogens and nongenotoxic carcinogens and linear-at-low-dose modelling for genotoxic carcinogens. The use of these models continues to play a dominating role in the derivation of exposure standards for environmental contaminants, food additives and chemical residues in essentially all countries. While these risk assessment models have differing historical and scientific foundations, both are now questionable.

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6.1 Threshold Model

In the case of the threshold model, it was readily adopted in the early decades of the twentieth century, institutionalized within the scientific and regulatory communities in the middle decades of the past century, never seriously questioned as judged by the published literature and, surprisingly, never explicitly tested and been attempted to be validated, until recently (Calabrese and Baldwin, 2001; 2003c; Calabrese *et al*., 2006). In two large-scale studies, designed to evaluate the capacity of the threshold model to reliably predict responses of doses below the estimated threshold, using no-adverse-effect levels (NOAELs) and various benchmark dose (BMD) values, the threshold model consistently failed to accurately predict how chemicals (from a broad range of chemical classes and causing a wide spectrum of toxic end points) acted in the low-dose zone or below the threshold. This was as surprising as it was alarming, because most regulatory actions throughout the world have been based on the assumption that the threshold model is valid and that most human exposures occur in the low-dose zone. These recent investigations have detected a serious flaw in the threshold model, a flaw that has important implications that affect its application for assessing low-dose effects. Ironically, when the hormetic dose–response model was tested it provided excellent predictive abilities, without restriction to biological model, end point or chemical class.

6.2 Linear-at-Low-Dose Modelling

In the case of the linear-at-low-dose model, it was adopted by the US EPA following the recommendation of the US National Academy of Sciences Safe Drinking Water Committee (1977), which based their acceptance on a two-decade-old decision of national and international advisory committees assessing risks from ionizing radiation. It had adopted the linear-at-low-dose model, rather than the threshold model. The linear-at-low-dose modelling was theoretically based and strongly influenced by societal fears concerning cancer.

The US government attempted experimentally to determine the shape of the dose response in the low-dose zone for genotoxic carcinogens in the late 1970s in a massive FDA-lead study, called the mega-mouse study, due to its use of over 24 000 animals. Given the significance of the study, the data underwent considerable analyses, including a detailed review by an expert committee created by the US Society of Toxicology (SOT), the findings of which were published in nearly an entire issue of the Society's journal (Bruce *et al*., 1981). The analysis indicated that the genotoxic carcinogen, 2-acetylaminofluorene (2-AAF) displayed a hormetic-like biphasic dose response with respect to bladder cancer. The findings were striking in that the hormetic response was observed in each of the six rooms that were used to house the large number of animals, in effect serving as a type of multiple internal replication of findings. While the hormetic dose–response findings were unanticipated, the major conclusions were that the study design was inadequate to assess risks below the risk of 1 cancer per 100 animals, hence it was called the ED01 study (ED—effective dose). This conclusion is particularly important because it demonstrated that linearity-at-low-zone modelling can not practically be evaluated for cancer incidence, and certainly never routinely considered. Yet the study demonstrated that the hormetic model could be evaluated and validated.

6.3 Hormetic Model

The general conclusion concerning the current risk assessment default models is that the threshold dose–response model has been demonstrated to have an inherent serious flaw, preventing it from making accurate predictions in the low-dose zone. The other modelling approach, that is, linear-at-low-dose modelling, cannot be validated at risk levels ($<1/100$) that are important to the public health. If a model's predictions can not be validated, then they are based on theoretical constructs that require 'belief' in an unverifiable model, rather than the data. Taken either separately or together, this is an untenable position for a highly industrialized and chemically dependent society.

Given these serious issues and weaknesses with respect to the current default dose–response models, it suggests the need for either model modification and/or the use of alternative models that offer the potential for improvement. The hormetic model is one such alternative since it has been observed to commonly occur, being highly generalizable according to animal model, end point and chemical class/physical stressor agent. It also has a strong mechanistic foundation. Furthermore, it has been shown to offer accurate predictions of low-dose responses in tests in which the threshold model performed poorly. It can also be tested in the 'observable' zone of experimental studies, thereby meeting a significant societal requirement that decisions should be data driven, not based on beliefs of theoretical constructs.

Practically, it may not attractive to revise the standard hazard assessment methodology to conform to the needs of the hormetic dose–response model because it affects animal model selection and study design considerations. For example, the analysis discussed indicates enhanced testing requirements for assessing hormesis, including more rigorous study designs (i.e. more doses, more subjects per dose in the below-threshold zone, and the greater need for replication of findings). However, if it were necessary to test the hormesis hypothesis,

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this task could be effectively accomplished. That is, the hormetic dose–response could be employed as a default model with validation on a case-by-case basis, as needed.

A public health advantage of the hormesis model is that it is capable of predicting and detecting possible beneficial and harmful effects below the traditional threshold dose–response model. In this respect the hormesis model provides a quantitative estimate of the dose response in the below-threshold zone that could be used by risk assessors. The magnitude of either type of response will be constrained to be usually no greater than 30–60% different from control group values. Such dose–response constraints on the quantitative features of the hormetic dose–response model offer particular value to risk assessors, as they would be able to assess the biological and population-based significance of these low-dose effects.

The hormetic dose–response model can also address the issue of mixtures more effectively than either the threshold or linear-at-low-dose models. The occurrence of chemical interactions in both of these models always assumes adverse effects, and in the case of the threshold model, effects that occur at doses greater than a threshold. In the case of hormesis, it could be similarly effective in predicting toxic interactions at the above-threshold response. However, it can also predict interactions in the below-threshold portion of the dose response. As with the case of singly tested agents, interactions could be beneficial, harmful or neutral. The magnitude of the hormetic response from chemical interactions will also be constrained by plasticity limits of the biological systems similar to that noted above for singly tested agents. Consequently, the hormetic models offer more potential value in assessing chemical interactions than the current two default models.

The issue of high-risk groups can also be effectively addressed by the hormetic dose–response methodology. Hormetic effects occur across the spectrum of susceptibilities. The quantitative features of the hormetic dose–response model are independent of the differential susceptibilities. The hormetic dose–response model creates opportunities and challenges for the risk assessor and public policy by providing the capacity for better information on the proportion of individuals who will benefit and be harmed at each level of contaminant exposure. Within this context the hormetic model takes into account harm that is expected to occur from the toxic substance as well as background disease incidence from indeterminate causes, but nonetheless real effects. This is a feature that neither of the current default models is capable of addressing and taking into account.

The hormetic model has the potential to bring various subpopulation stake holders into conflict as policy makers attempt to determine what the exposure standard may be. This is because the optimal dose for the general population may be at a dose that increases the risk of harm for a high-risk group. Since the high-risk group segment may usually be only a small fraction of the size of the general population, the conflict lies in the fact that to protect the high-risk group, a decision maker will increase the risks of the general population. These biological-based conflicts have been submerged in the present governmental risk assessment methodology because of one or more agencies' goals have been to reduce exposures to pollutant-induced risk for all groups, while completely ignoring any possible benefit that is differentially distributed across the normal and high-risk segments of society.

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Chemical Incidents—Emergency Planning, Preparedness, Response and Recovery. Community Considerations

David Russell and John Simpson

CONTENTS

1 BACKGROUND

Chemical incidents may occur at any time and at any place, reflecting a complex and well-developed chemical industry and society's needs for chemicals, whether for health, agriculture, manufacturing, food preservation, household goods and products, sanitation or leisure activities. The chemical industry, therefore, is necessarily diverse, producing 'basic' chemicals, such as plastics and additives, sealants, catalysts and coatings, soaps, detergents, bleaches, fragrances, pesticides and biotechnological products (Organisation for Economic Co-operation and Development; OECD) (2007).

The scale of this dependence on chemicals is reflected by the fact that annual sales of chemicals in the European Union amounted to €556 billion in 2003, with an estimated global market of €1736 billion in 2004 (European Chemical Industry Council (CEFIC), 2009; European Social Investment Forum (EUROSIF), 2009). Based on cross-border trade flow analyses, it is estimated that between 2002 and 2006, global export of inorganic chemicals, organic and primary plastics grew at an annual rate of approximately 17.1%, whilst future projections suggest that by 2020, the global output will be 85% higher than in 1995 (*www.global-production.com/chemicals/trendstudy/index.htm*; OECD). This increase in production not only reflects an increase in quantity of chemicals, but also the number of chemicals produced. Accordingly, within the European Union, approximately 200–300 new chemicals are produced per annum within a global market of tens of thousands of substances, some of which are produced in quantities of greater than 1 million metric tonnes (OECD).

This global market for chemicals requires both a manufacturing base and transportation of chemicals, with vast amounts of chemicals transported by road, rail, pipeline, sea and air. Therefore communities, whether rural or

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urban, are potentially at risk of exposure to industrial chemicals and subsequent health effects as a consequence of a chemical incident. The Chemical Industries Association reports that in the UK in 2004 there were 36 reported transportation accidents, equating to approximately one incident per million tonnes transported (Chemical Industries Association (CIA), 2006).

A chemical incident is defined by the World Health Organization (WHO) as being *'an uncontrolled release of a chemical from its containment that either threatens to, or does, expose people to a chemical hazard'* (International Programme on Chemical Society (IPCS), 1999). Other definitions include *'an acute event in which there is, or could be, exposure of the public to chemical substances which cause, or have the potential to cause ill health'* (The Health Protection Agency, 2007).

The potential catastrophic impact of chemical incidents upon communities and public health is graphically illustrated by the Union Carbide incident at Bhopal in 1984. Following the accidental release of approximately 2500 kg of toxic methyl isocyanate (MIC) gas, a reported 200 000 people were exposed in the nearby community and there were over 3500 deaths, with ongoing concerns regarding chronic neurological, reproductive, psychological, ocular and respiratory effects (Dhara, 1992; Dhara and Dhara, 2002). Therefore, acute exposure following a chemical incident may result in both acute and chronic health effects.

Similarly, following an explosion at a fireworks storage facility in a residential area of Enschede in the Netherlands, self reporting of somatic symptoms, sleeping disorders and anxiety and depression were at higher rates than for the general Dutch population (Van Kamp *et al*., 2006), whilst a longitudinal epidemiological study reported similar effects in both the short term and longer term (18 months) (Van Kamp *et al*., 2006; Grievink *et al*., 2007); similar results have been reported after flooding incidents.

Rural communities have also experienced chemical incidents. In 1993, following the leakage of oil from a ship (the *Braer*) off the coast of Shetland, Scotland, UK, potentially exposed individuals had significantly higher rates of headache, sore throat and itchy eyes compared to controls (Campbell *et al*., 1993; 1994). Similarly, in 1996, the *Sea Empress* grounded in Milford Sound, Pembrokeshire, Wales, UK, and resulted in the spillage of 72 000 tonnes of crude oil. A retrospective cohort study undertaken by Lyons *et al*. revealed significantly greater self reporting of physical and psychological symptoms in communities residing in the vicinity of the incident (Lyons *et al*., 1999).

Such accidental releases are not uncommon, as underlined by the fact that the Health Protection Agency reports that public-health surveillance in England and Wales demonstrates approximately 1200 chemical incidents per year, whether the result of spillage, leakage, fire or explosion in industrial complexes, recreational facilities or transportation accidents (The Health Protection Agency, 2007).

In the present climate, however, in addition to *accidental* release of chemical agents, *deliberate* release of chemicals is a possibility. The potential impact of such releases is illustrated by the release of chemicals during the First World War, with over 113 000 tonnes of chemicals utilized. Over 5000 allied troops died and 15 000 were injured. Chlorine was the first chemical to be used as a warfare agent during the campaign, having been released by German Forces in April 1915 at the Ypres Salient, resulting in many casualties and fatalities. Similarly, phosgene was used by German forces on 19 December, 1915, when 88 tonnes were released, resulting in 1069 casualties and 120 deaths (Marrs *et al*., 2007). It was subsequently utilized by the allies, and accounted for 85% of all deaths attributed to chemical warfare during this campaign (*see* **Toxicology of Chemical Warfare Agents**, **Chemical Terrorism**). Both chemicals have since developed legitimate industrial usage, with chlorine being used extensively in the production of chlorinated organic polymers, solvents and other organic chemicals, whilst phosgene is used industrially in organic synthesis, dye manufacture, in pharmaceuticals, agrochemicals, synthetic foams, resins and polymers. Therefore, chemicals with legitimate industrial usages could be utilized as agents of chemical terrorism with the potential for large numbers of casualties and fatalities ('toxic industrial chemicals').

Community chemical terrorism has already been observed, as illustrated by the release of the organophosphate chemical warfare agent sarin on the Tokyo underground in 1995, resulting in 12 deaths and approximately 5000 casualties (Okumura *et al*., 1996) (*see* **Chemical Terrorism**).

There are a vast number of globally available chemicals and an almost infinite number of scenarios for airborne, waterborne and land-borne releases. Accordingly, it is not possible to plan for all eventualities, but rather, planning should be generic, based upon a risk-prioritized strategy, following risk assessment and subsequent risk mitigation and providing an interface between occupational and community considerations.

The remainder of this chapter will therefore focus upon risk assessment and mitigation and subsequent planning and thus preparedness for a response and ultimately, recovery.

2 RISK ASSESSMENT

Before risks can be mitigated or reduced, a risk assessment procedure must be undertaken to identify and prioritize such risks. Risk assessment is the process whereby

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Table 1 The risk assessment paradigm, composed of the four steps of hazard identification, dose–response assessment, exposure assessment and risk characterization

Hazard identification—An assessment of all available evidence to explore the possibility that a chemical may cause a health effect. It is not a statement of probability, but rather of potential to cause harm.
Dose–response assessment—An assessment of available data from a number of different sources, including animal models, to assess health effects at given concentrations. This involves an examination of the relationship between dose and effect.
Exposure assessment—An estimation of population exposure to the chemical(s) of concern from all environmental sources, namely air, water and soil, according to chemical characteristics.
Risk characterization—This part of the process integrates all the information from the three steps, which, after analysis, provides a qualitative or quantitative risk, allowing subsequent decision-making by policy makers (*see* **Risk Assessment of Chemicals**).

the probability or likelihood of an event occurring is assessed (*see* **Risk Assessment of Chemicals**). In this context, it refers to the likelihood of a chemical incident occurring and its subsequent likely impact on public health, such as acute health effects, birth defects (congenital malformation) or cancer. In many instances, data are incomplete and as a consequence, the risk assessment process is an inexact science requiring utilization of limited toxicological data, extrapolation from available methodologies and expert consensus opinion. Therefore, risk assessment is more appropriately used as a policy as opposed to a scientific tool. It may be applied generically and thus be utilized to inform national policy on chemical incidents, as well as being utilized to address specific local and regional chemical industries and concerns. In this way, plans are developed that are proportional to risk and provide a basis for integration of local, regional and national chemical-incident plans (see below).

The classical risk assessment paradigm is composed of four steps (**Table 1**).

In practice, this typically entails identifying the use and storage of chemicals at industrial sites and associated transportation, the proximity and density of the local populus, together with the proximity and location of water courses, agricultural and grazing land. In addition, consideration needs to be given to all the relevant available data to enable as complete as possible a toxicological profile of the chemicals to be drawn up.

Consideration should also be given to the historical nature and frequency of chemical incidents, the accessibility of a site in the event of an incident, together with an assessment of whether the site poses a significant risk of sabotage or for chemical terrorism (Crawford *et al*., 2004a). Such an assessment may be undertaken at local, regional or national level.

Table 2 Examples of risk mitigation

Clear labelling and inventories of hazardous chemicals
Chemical substitution
Isolation and reduced storage capacity of hazardous chemicals
Bunding
Relocation of chemical industries from urban areas
Diversion of chemical transportation away from urban developments
Remediation of contaminated sites

The information from each component should be readily available and accessible and communicated in an open and transparent manner (risk communication).

Following the undertaking of the risk assessment process described above, risk deemed to be unacceptable should be reduced as far as possible by implementation of a number of practical measures (risk management or mitigation).

Examples of risk mitigation are illustrated in **Table 2**.

Such a procedure has been utilized by the US Environmental Protection Agency (EPA) for undertaking a risk assessment of hazardous waste sites. In this approach, acute hazards were identified, toxicity assessed, exposure estimated and the risk subsequently characterized. This process in turn informed risk management. (The US Environmental Protection Agency (US EPA), 2009)

In a similar fashion, major chemical installations in Europe are subject to the Seveso II Directive (1996), the principle aim of which is to prevent accidents at major installations and limit the impact of any spills on human beings and the environment. The Directive therefore covers the need for on-site—and in some instances—off-site-emergency plans, as well as the need to demonstrate adequate and appropriate safety and reliability in the design, storage and maintenance of facilities. Within this framework, industries subject to Control of Major Accident Hazard Regulations (1999) in the UK must ensure that primary, secondary and tertiary containment, pipework and pipelines, valves, control instrumentation, bunding and underground storage tanks all comply with appropriate standards, capable of being safely isolated and subject to inspection, testing and maintenance.

The risk remaining following instigation of risk mitigation is referred to as the residual risk, and forms the basis of subsequent planning and preparedness for chemical incidents and the response and recovery in their aftermath.

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3 PLANNING AND PREPAREDNESS

3.1 Planning

Emergency planning is an integrative and iterative process that collectively involves risk mitigation, planning and preparedness, response and recovery. In essence, it is the process whereby plans for incident management are constructed. Planning for major incidents and disasters (such as train and aircraft disasters, flooding and bomb explosions) has been comprehensively developed throughout much of the world. In most places, there will be a general plan covering such major incidents and disasters. In addition, there will often be a general plan covering the roles of the *emergency services* in chemical incidents. There will also usually be major incident plans in hospitals, covering most types of incidents. However, *public-health* plans to deal with *chemical incidents* historically have been less well developed; such plans need to be distinct from, but integrated with, major incident plans (Crawford *et al*., 2004b).

The purpose of the chemical-incident planning is to reduce, mitigate or prevent the public-health impact of exposure to chemicals following an incident, whether 'minor' or 'major'. The Inter Organisation Programme on the Sound Management of Chemicals (IOMC) concluded that '*Having plans ready and rehearsed has been shown to make a major difference to the management and outcome of chemical events.*' (International Programme on Chemical Society (IPCS), 1999). Therefore, a planned, prepared and practised response to incidents contributes to preparedness and serves to protect the public; preparedness refers to a state of readiness and the two aspects are closely linked (see below).

As a chemical incident is likely to involve a number of agencies and organizations, it is imperative that all are involved in the planning process (*Integrated Emergency Management),* allowing respective roles and responsibilities to be clarified, defined and mutually understood. Typical agencies and organizations likely to require participation and contribution are illustrated in **Table 3**.

This allows a multidisciplinary, multiagency, integrated approach to planning for chemical incidents, allowing an all-encompassing chemical-incident plan to be constructed. This collaborative and integrative approach can be further reinforced by developing written agreed procedures, such as memoranda of understanding, or equivalent cooperative mechanisms. It is vital that community engagement is an integral part of the process, such that risk can be put in context, and concerns and fears raised, addressed and allayed. Involvement of the community is illustrated by the activities of the petrochemical industry at Fawley Refinery, Southampton, UK, where the industry has established clear channels of communication with the community, involving written safety instructions to follow in the event of an incident, together with a dedicated contact number.

Table 3 Examples of agencies, organizations and institutions likely to be involved in emergency planning as part of integrated emergency management

Emergency services
Local government
Public-health professionals
Environmental protection agencies
Water authorities
Maritime/coastguard/port agencies
Government departments and agencies
Other non-departmental public bodies
Analytical laboratories
Academics
Voluntary agency services
Community leaders

Engaging with the media will also be an important part of planning for chemical incidents. Accordingly, media protocols and liaison panels should be in place to ensure consistency of communication messages. Any incident is likely to generate huge media interest, and thus the link with the media should be an integral part of emergency planning. The media are important for a variety of reasons, but particularly because they are one of the main channel for public communication and thus should be used for dissemination of information at each stage of the incident; local media will keep the local communities updated whilst national media reach millions and thus require timely information (International Programme on Chemical Society (IPCS), 1999).

In addition to complimentarity of major incident and chemical-incident plans, it is also necessary that plans constructed at local, regional and national level are dove-tailed. The *national* plan is strategic, reflecting government policy, identifying and leading government response, addressing national and international co-ordination arrangements, the control and command, the response of healthcare organizations and overseeing national resilience. The *regional* chemical-incident plan should address regional co-ordination of incidents, ensuring good regional communication and collaboration, but also establishing channels of communication with the public, whilst the *local* plan need to be constructed in full co-operation with local partners, local industry and communities; all plans whether national, regional or local must be fully integrated and thus provide a seamless approach to chemical-incident management.

From the above discussion regarding the vast number of chemicals and potential scenarios, chemical-incident

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plans are necessarily generic. However, they should recognize both overt and covert releases of chemicals. Overt releases of chemicals include the release of a chemical or chemicals from a fixed industrial site, whether a major industrial complex or small factory, or during transportation. Such events are typically obvious and may involve a fire or explosion or leakage/spillage of large quantities of material. The response (see below) will typically warrant a multiagency/multidisciplinary input, with first-line responders attending the scene, with clinical and public-health professionals advising, supporting and managing casualty and community concerns.

Covert releases, by contrast, are not initially detectable and will only be recognized by the presentation of unusual symptoms or symptom frequency. Such scenarios include deliberate release of chemicals, such as the poisoning of a water supply; under such circumstances detection will depend upon clinical acumen, surveillance mechanisms and alerting at points of care, such as general practitioners and emergency departments. Therefore, there is no obvious 'scene' as such, and the response therefore is in marked contrast, without the need for multiagency integrated response at a distinct location. Rather, the emphasis is on recognizing symptoms (in which Poisons Centres will have a major role to play *see* **The Role of Poison Centres in Basic and Clinical Toxicology**), identifying the source of release and the community potentially exposed, and putting in place appropriate medical, diagnostic and epidemiological measures.

3.1.1 Major Incidents

The UK Cabinet Office describes a major incident as *'... any emergency that requires the implementation of special arrangements by one or more of the emergency services..., rescue and transport of a large number of casualties; the involvement either directly or indirectly of large numbers of people, the handling of a large number of enquiries likely to be generated both from the public and the news media, usually to the police... the mobilisation and organisation of the emergency services and supporting organisations... to cater for the threat of death, serious injury or homelessness to a large number of people'* (Cabinet Office, 2003).

Major incidents therefore cannot usually be managed within the normal service arrangements and thus require the implementation of extraordinary procedures by first-line responders, healthcare professionals and local government. Such incidents have been divided into the major categories shown in **Table 4**.

In order to successfully manage such an incident, it must be underpinned by a major incident plan, the essential features of which are described by the UK's Department of Health, and highlighted by **Table 5** (Department of Health, 2007).

Table 4 Examples of types of major incidents requiring the implementation of extraordinary procedures and measures

'*Big bang*'—a major incident such as an industrial accident or transportation disaster triggers a focal, multiagency/multidisciplinary response of which the health component is likely to be a major contributor.
'*Rising tide*'—the incident is of a gradual or 'creeping' nature, such as an infectious disease outbreak or epidemic. It is only retrospectively that it becomes apparent at which point the incident became 'major'.
'*Cloud on the horizon*'—preparation is required for a threatened event which may or may not occur. Examples include a nuclear or chemical release in another country, an armed conflict or the possibility of an epidemic.
'*Headline news*'—this is a media driven headline and subsequent public concern over an actual or perceived health threat.
'*Internal incidents*'—this refers to any event, such as fire, flooding or loss of electricity supply that impairs the ability of a given service to respond to an incident.
'*Chemical, biological and nuclear materials*'—this specifically refers to the release of such materials deliberately by terrorists, disaffected individuals or dissident groups; it also includes hoaxes.

Table 5 Essential components of a major incident plan (Department of Health, 2007)

Multiagency input
Tailored to risk assessment
Addresses planning, preparedness, response and recovery
Coordinated and integrated
Establishment of appropriate command and control
Identifies and incorporates mutual aid
Regularly updated and tested
Based on best practice and information dissemination
Flexible, addressing numerous scenarios
Takes account of vulnerable populations
Incorporates business continuity management
In the public domain
Consistent with existing legislation

Reproduced from NHS, 2005.

The role of the community in emergency planning is well established, with several reports of government representatives, first-line responders and civic leaders providing input to constructing local emergency plans, raise community awareness and fully involve communities in exercises and volunteer programmes. The United Nations Environment Programme (UNEP) has developed a tool known as *the Awareness and Preparedness for Emergencies at Local Level (APELL): a Process for Responding to Technological Accidents,* which serves to mitigate the risk of exposure following the release of chemicals through the development of local plans, with particular emphasis upon community awareness of the risks and preparedness for contingencies (United Nations Environment Programme, 1998).

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3.2 Preparedness

Preparedness reflects a state of readiness and thus requires more than merely planning. Planning itself does not guarantee preparedness. Preparedness is a dynamic process and thus is ongoing (Perry and Lindell, 2003). It is composed of several components, including planning, but also comprising training, alerting and detection systems, robust communication, advance preparation of information likely to be of use during an incident, purchase and testing of necessary equipment and facilities, and ensuring access to quantitative risk-assessment tools. The whole procedure needs to be underpinned by a comprehensive exercise programme.

3.2.1 Training

The most important resource in managing acute chemical incidents is the human resource. Therefore, in order to ensure a timely, efficient, effective and appropriate public-health response to acute chemical incidents, it is necessary to identify available human resources, build capacity through training, and ensure that access to this expertise is available at all times. In the modern era, online training modules and materials have been developed, such as those developed by the Centers for Disease Control and Prevention (CDC) in the USA and the Health Protection Agency (HPA) in the UK. Examples include the Centers of Public Health Preparedness (CPHP) programme (*www.bt.cdc.gov/training/cphp/*), whilst the American Medical Association has developed web-based competencies (Weiner and Trangenstein, 2007). In addition to academic training, emergency planning also needs to address character and team building, with the virtues of '*prudence, courage, justice, stewardship, vigilance, resilience and charity'* being stated as being essential for emergency team members (Larkin and Arnold, 2003).

3.2.2 Skill Mix

The fact that chemical incidents may involve a large number of chemicals (as well the products of combustion, environmental reactions and degradation), widespread environmental contamination and the possibility of acute and chronic health effects, necessitates considerable collective expertise in a number of distinct, but related fields. These include the disciplines of environmental chemistry, environmental science, public health and epidemiology, as well as environmental and clinical toxicology. It is only through the collection, assimilation and subsequent critical analysis of available data that a risk assessment—and thus potential impact upon public health—can be undertaken. Therefore, preparedness for responding to chemical incidents requires a diverse group of skills.

The essential role of toxicologists in providing advice and support on toxi-cosurveillance, recognition of clinical syndromes, treatment and management of casualties, advising upon antidotes and decontamination procedures is well established (*see* **Clinical Toxicology**) (Schwenk *et al*., 2005). The invaluable role of toxicologists is further highlighted by the development of specific community exposure limits following the airborne release of chemicals. Community exposure limits were developed to anticipate the likely health consequences of exposure of the general public to airborne chemicals. Whilst occupational exposure limits have been developed for workers who are typically healthy adults, community exposure limits take account of susceptible populations, such as children, those with pre-existing health conditions (such as asthma or allergies, cardiovascular disease, etc.), the elderly, frail and infirm. Based upon peer review of available animal, occupational and epidemiological toxicological data and evidence, Emergency Response Planning Guidelines (ERPGs) were developed, providing quantitative data on the likely impact of exposure to a given chemical over a given period of time. For example, ERPGs predict that exposure to 1 ppm of chlorine for 1 hour would result in a noticeable odour in the majority of individuals, whereas exposure to 3 ppm of chlorine for 1 hour would probably result in sensory irritation; 20 ppm for 1 hour would result in potentially irreversible and life-threatening effects (Hansen, 1999). More recently, Acute Exposure Guideline Levels (AEGLs) have been developed by a National Advisory Committee to the US EPA, and subsequently reviewed by the National Academy of Sciences. AEGL values have been set for the time periods of 30 minutes, 1 hour, 4 hours and 8 hours, and for three levels of severity (thresholds for mild/transient effects, more significant effects including impaired ability to escape, and life-threatening effects), and have been developed for industrial chemicals and chemical warfare agents alike (see *www.epa.gov/oppt/aegl/*). Such exposure limits can be factored into emergency planning and may provide a yardstick for assessing exposure and planning for protective actions, such as evacuation or sheltering (Hansen, 1999).

3.2.3 Meteorological Data

Complementary to the need to organize beforehand is the need for rapid and prompt access to meteorological data, which provides key information on likely dispersion of the chemical(s) in question and thus populations at risk of exposure. Many chemicals are either gaseous or volatile (such as chlorine, ammonia, phosgene, the nerve agent sarin, and mustard) and therefore a release, whether deliberate or accidental, is likely to result in airborne contamination and dispersion. This information is usually requested by fire services at the scene of an incident, but is also of great value to public-health professionals. Key

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Table 6 Key components of meteorological data that may aid risk assessment following release of volatile and gaseous chemicals. Such information provides rapid access to data pertaining to potential community exposure

Wind speed and direction
Air temperature
Relative humidity
Weather conditions
Cloud cover
Air stability
Height of mixing layer
Maps of nearby communities

pieces of information that are required are illustrated by **Table 6**.

The advantage of such information is its rapid availability, whereas detailed computational modelling requires the use of complex mathematical models and the necessity of detailed environmental data.

3.2.4 Computational Modelling

Modelling of likely environmental contaminant concentrations and thus likely community exposure is time consuming and thus unlikely to be available at the beginning of an incident.

3.2.5 Datasheets

A key feature of chemical incidents is the short latency with which symptoms occur following exposure. For example, the release of ammonia at an ambient concentration of 50 ppm may induce ear, nose and throat irritation within 2 hours, whilst at 100 ppm symptoms occur rapidly; at 700 ppm symptoms occur immediately, with death likely to ensue at approximately 4500 ppm (for review see *www.hpa.org.uk*). It is therefore necessary to prepare in advance toxicological datasheets, based upon the risk assessment paradigm discussed above and addressing the key issues shown in **Table 7**.

Table 7 Key components of toxicological datasheets. The rapidity of symptom development following chemical exposure means such datasheets need to be prepared beforehand on a risk-prioritized basis

Chemical synonyms
Physicochemical properties
Acute and chronic toxicity
Environmental fate
First aid
Incident management
Decontamination (casualty and environment)

Datasheets have been developed by the Agency for Toxic Substances Disease Registry (ATSDR, *www.atsdr.cdc.gov/MHMI/mmg.html*) at the CDC in Atlanta, USA; these provide concise information on a number of industrial chemicals and chemical warfare agents. Other sources include the International Programme on Chemical Safety (IPCS) concise international chemical assessment documents (CICADs) (International Programme on Chemical Safety (IPCS), 2009). The HPA has also produced over 100 datasheets which address the commonest industrial chemicals and chemical warfare agents for the purposes of health care professionals, first-line responders and local authorities (The Health Protection Agency, 2007).

3.2.6 Communication

Communication is also a key component of preparedness, with tried and tested communication channels being an essential component of a timely, coordinated and effective response. Recently, informatics has been utilized to improve the quality and speed of information transfer. For example, cellular phone transmission of data from pre-hospital field workers to emergency departments has allowed information to be transferred on the number of casualties, clinical condition of patients, mode of transport, estimated numbers of those to be transferred and likely time of arrival. Other developments include utilizing wireless technology for secure transfer of incident and patient data between field and hospital, thereby enhancing information communication and allowing for more efficient care (Weiner and Trangenstein, 2007).

The importance of communication after an incident cannot be over emphasized, with several examples of major incidents resulting in difficulties. During the response to Hurricane Katrina, information-sharing regarding supply and demand for essentials such as bottled water was limited. Further, during the London bombings of 2005, massive demand by the public for information led to the saturation of mobile-phone networks and subsequent overload; communications involving pagers and text messaging were reported to be more resilient (Bland *et al*., 2006).

3.2.7 Alerting

As chemical incidents are by their very nature unpredictable, access to expert public-health advice will be required on a 24-hour, 365-day-per-year basis, with access provided to all agencies and organizations who may wish to provide an alert for, or seek advice on, chemical incidents. Such a system can then be utilized for accessing timely and authoritative public-health advice.

Such planning undoubtedly contributes to national preparedness. However, many incidents have the potential to be, or are, of actual concern internationally. Examples include those shown in **Table 8**.

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Table 8 Examples of incidents chemical which may be of international concern

Incidents close to national borders
Water pollution of major arterial rivers
Contamination of major food production sources
Clusters of unusual illnesses
Deliberate release of toxic chemicals
Plumes from industrial fires and explosions

Table 9 Key components of international communication

Identify key individuals
Establish rapid, robust, timely, efficient and effective channels of communication
Standardize incident classification
Promote mutual aid

Therefore, it is also necessary to open and maintain international channels of communication, which should meet the essential criteria given in **Table 9**.

In this context, the International Health Regulations (IHRs) are legally binding regulations involving 194 countries. Their principle aim is to help the global community to prevent or respond to international crises. The regulations stipulate that countries must report to the WHO outbreaks and incidents that may have international implications. Such threats include pandemic flu and Severe Acute Respiratory Syndrome (SARS), as well as chemical incidents with an international dimension (World Health Organization, 2005).

3.2.8 Surveillance

Surveillance provides invaluable information regarding the number of chemical incidents, their nature, frequency, location, type and chemicals involved, together with a range for the number of people estimated to be exposed and/or exhibiting symptoms. Therefore, the timely collection, collation and analysis of such data provides an insight into the commonest chemicals involved and the nature of incidents. For example, data collated by the HPA demonstrates that chlorine, mineral acids, carbon monoxide and mercury, are commonly released during chemical incidents; this information can be utilized to further assist risk assessment, mitigation and planning, and contribute to the development of risk-prioritized toxicological datasheets. The importance of surveillance has also been recognized in planning and preparedness for, and responding to, international chemical incidents. The World Health Assembly in 2002 agreed a resolution to strengthen surveillance systems for chemical and biological agents and nuclear/radiation events. This reflects the continuing growth of the chemicals industry as well as the fact that chemical incidents may have an impact distant from their origin (e.g. the release of cyanide into the river Tisza in Romania following a dam burst killed fish in three countries). Accordingly, the WHO has developed an international system for the verification, investigation and subsequent response to communicable disease events, chemical incidents and events of possible chemical aetiology (Global Outbreak Alert and Response Network (GOARN) (Olowokure *et al*., 2005).

3.2.9 Personal Protective Equipment

Preparedness for acute chemical incidents also requires the purchase and provision of suitable personal protective equipment (PPE) that allows first-line responders and Emergency Department staff to treat chemically contaminated casualties without becoming secondarily contaminated themselves. In essence, PPE for use in the warm zone should ensure liquid protection (including respiratory protection) against common toxic industrial chemicals and chemical warfare agents, provide good visibility and allow rapid communication. PPE, however, by its very nature is cumbersome and limits dexterity and communication. Accordingly, only limited clinical treatment can be undertaken whilst donning such clothing; it is necessary that those who use such equipment are familiar and comfortable with its use.

Complimentary to the need to utilize PPE is the need to prepare for decontaminating casualties and to be able to do so rapidly, as symptoms may occur rapidly following exposure, as discussed above. Therefore, there is a need to be able to rapidly transport and subsequently erect mobile decontamination facilities at the scene of an incident. This in itself poses a considerable challenge that is compounded by the need to be able to decontaminate a range of casualties from very few to very many (mass casualties), together with a requirement to provide copious amounts of warm water (to reduce the risk of hypothermia), basic equipment such as buckets, sponges, detergents and paper towels and replacement clothing. Further consideration needs to be given to the deployment of essential medical equipment, first aid equipment and pharmaceuticals, as highlighted by **Table 10**.

Table 10 Essential medical equipment required at the scene of a major chemical incident involving casualties

Drugs and antidotes
Intravenous lines and fluids
Resuscitation equipment (defibrillators, face masks, endotracheal tubes, etc.)
Ventilators
Oxygen
Modesty clothing
Wound dressings
Needles and syringes

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Table 11 Planning for a major chemical incident: laboratory considerations

An inventory of available and accredited laboratories and available analyses
Resilient contact information
Quality assurance procedures, including standard operating procedures for sampling, collection, transportation and analysis
Regional/local distribution of sample kits
Staff training
Data interpretation
Timely reporting

Table 12 Essential components of a successful exercise strategy

Undertaken regularly
Be well planned
Provide realistic scenarios
Involve all appropriate agencies or focus on specific practice
Test operating procedures, communications and practical issues
Tease out strengths and weaknesses
Provide timely feedback, consulting representatives of all participants
Be used for improving practice and thus preparedness

3.2.10 Sample Analysis

Provision should also be in place for environmental sampling. This requires provision for sampling at the scene of an incident and thus access to sampling equipment. Many *environmental* samples can be analysed at the scene of an incident in either a qualitative or quantitative manner. Examples include carbon monoxide, chlorine and ammonia. More complex analyses can also be undertaken at the scene, but typically are undertaken in a laboratory; examples include volatile organic compounds and heavy metals.

Biological samples are invariably sent to a laboratory for analysis (*see* **Clinical Chemistry in Toxicity Testing: Scope and Methods**). It is essential that arrangements are in place for distribution of sample collection tubes to the scene and to local emergency departments. Laboratories will advise as to appropriate sample tubes and specimens to be collected.

As part of preparedness, therefore, it is essential that the certain components of analytical sampling are in place; see **Table 11**.

As this is a complex and time-consuming process, it is vital that such procedures are in place prior to chemical incidents; establishing such channels of communication in the aftermath of an incident is too late.

Many contaminated casualties may, however flee the scene of an incident and present at hospitals. Therefore, as part of the planning and preparedness stage, it is imperative that hospitals have decontamination facilities and tried-and-tested decontamination protocols and appropriately trained staff (Dethick and Hicks, 1999).

3.2.11 Emergency Operations Centre

Major incidents will require a coordinated public-health response, collecting, collating and critically analysing information pertaining to the incident that will allow a dynamic risk assessment and the provision of advice and support to all major agencies and organizations as outlined above. This is typically achieved by planning for an Emergency Operations Centre (EOC), an essential part of preparedness. Such a centre will require robust and secure communications and access to tools such as geographical information systems, computational modelling and toxicological data. Manning of the incident over a prolonged period of time will necessitate planning for access to suitable expertise on a rota basis over days to weeks.

3.2.12 Exercises

Exercises are recognized as being a valuable tool in reducing the impact of an emergency upon communities, minimizing the interruption to businesses, increasing the chances of postincident survival and minimizing the financial, social, legal and political impact. Exercises also provide a means of measuring an organization's relative strengths and weaknesses and provide an invaluable learning tool, if well constructed (Cabinet Office, 2003). They also allow the development of interpersonal relationships and thus enhance communication. Furthermore, exercises may serve to publicize the fact that planning is under way, and signal to public and community representatives that preparedness is being attained (Perry and Lindell, 2003).

In order to test the resilience, reliability and effectiveness of the planning and preparedness discussed above, it is necessary to undertake exercises. Exercises should fulfil the criteria given in **Table 12**.

Exercises can vary in magnitude from small 'desk-top' exercises involving one aspect of the emergency planning to large multi-agency, multi-disciplinary exercises with the involvement of large numbers of 'casualties' and deployment of PPE, decontamination, ambulance, fire and hospital facilities. Some exercises also have an international dimension in order to gauge international alerting and response (see **Table 13**).

Planning and preparedness are key components and require considerable preparation and ongoing testing, with one author stating that '*you cannot be overly prepared for an emergency*' (Tumosa, 2007). They form the platform for subsequent response and recovery. The importance of this point is illustrated by a review of the Madrid bombings of 2004 which noted that there

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Table 13 Exercise 'Common Ground', an international communications exercise

Exercise COMMON GROUND was conducted as a Command Post Exercise, where organizations played into the exercise from their own emergency operations centre, over a two-day period on 23–24 November 2005. The aim of this European Commission-funded exercise was to evaluate the ability and capabilities of member states to respond to a health-related crisis, in this case an influenza pandemic. In addition to the then 25 member states, players in the exercise included the European Commission (EC), European Centre for Disease Prevention and Control, European Economic Area states, Switzerland, the World Health Organization (WHO) and other relevant European agencies and organizations. The exercise was intended, amongst other objectives, to provide the players with an opportunity to explore international coordination with the EC and was based on a model of an influenza pandemic developed by modellers at the HPA. Not surprisingly with this level of participation between national organizations, communication and coordination became a major theme of the postexercise lessons. Many areas for improvement were identified and embraced both by the European Commission and other participating organizations.
The value of exercises has clearly been recognized by the EC and it is intended that exercises become a regular feature of enhancing preparedness across Europe.

was a lack of effective emergency planning, limited interagency communication, absence of overall command and control, and no triage system (López Carresi, 2008).

4 RESPONSE

A rapid, coordinated and effective response is the best means of ensuring the reduction, minimization or prevention of the community consequences of chemical incidents and therefore of a successful public-health outcome. Its successful implementation will require multi-agency, multi-disciplinary involvement, with different skills and disciplines being required at different time points following the incident.

The response to an incident can conveniently be divided into discrete sections according to the timing of the response.

4.1 Immediate; 0–2 Hours

Following notification of a chemical incident, the first response is to assess the situation from the viewpoint of potential or actual public-health implications. It is predominantly associated with assessing emergency clinical care and will be undertaken by the emergency services in particular, targeting the areas shown in **Table 14** (Cullinan, 2002).

In the immediate aftermath of an acute incident, whether deliberate or accidental, the emergency services are responsible for managing the incident, whether a fire, explosion, leak, spillage or a toxic plume. The immediate priorities are to recognize that a chemical incident has occurred, triage and remove casualties from the immediate vicinity of the incident, decontaminate chemically contaminated casualties, provide first aid for physical injuries, such as blast injuries, wounds and trauma, and cordon the area to prevent unexposed individuals from entering the area (and to prevent contaminated and potentially contaminated individuals from leaving the scene).

Table 14 Initial assessment of a chemical incident. Immediate assessment requires a dynamic risk assessment of the actual or potential public-health implications

What, if any, are the public-health implications?
What geographical areas have been involved?
How many people are threatened, affected, injured or dead?
What are the exposure pathways?
Are critical infrastructures affected (power, water supplies, sanitation, etc.)?
What environmental media are affected?
What chemicals are involved/thought to be involved (including products of combustion/degradation)?
What other agencies and organizations are involved?
Have key personnel been notified and the emergency plan implemented?
What response actions have been taken?

4.1.1 Command and Control

A command-and-control structure will need to be rapidly established. The first act of the incident commander is to establish control of the site for the protection of attending emergency services, casualties and the wider public. This involves the establishment of an *exclusion zone* (the '*hot zone*'), where there is a risk of direct contamination from chemical exposure. It is therefore accessed only by specifically trained rescue personnel wearing *gas* tight (Type A) PPE and self-contained breathing apparatus (SCBA). In most instances, such personnel are from the fire services or 'Hazmat' teams, although considerable thought has been given to the forward deployment of medical and paramedical staff into this zone. The 'hot zone' is always established upwind of the incident, to prevent further contamination, although changes in wind direction may prove problematical and require changing the location of the zone. Casualties are brought to

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the contamination reduction zone (*'warm zone'*), where decontamination takes place. The risk in this zone is from indirect exposure to chemicals from contaminated casualties and equipment. Therefore, workers in this zone (ambulance, paramedical and medical staff) require *liquid* tight PPE (see Section 3.2). Following decontamination, casualties enter the support zone (*'cold zone'*) before transportation to hospital as appropriate.

This command-and-control structure forms part of a combined and co-ordinated response regardless of the individual role of the respective agencies and irrespective of the nature of the incident, whether chemical, biological or radiation. The overarching aims of such a joint response are to contain the emergency, save and protect lives of responders and the community alike, relieve suffering and protect property and the environment, whilst providing a channel for a unified and united channel of communication with the media and public. It also provides a mechanism for the restoration of normality and thus recovery and for identifying and implementing lessons learnt (Cabinet Office, 2003).

Within the overall command and control of the incident, the emergency services will need to instigate a mechanism for co-ordination. Typically, each service will provide strategic *command* ('gold'), *tactical* ('silver') and *operational* ('bronze') command. One or more agencies may establish a strategic command, with the express remit of establishing an overall framework, establishing clear channels of communication with tactical commanders, providing an integrated response to media enquiries, prioritizing the demands of the incident and assimilating appropriate expertise, and plan beyond the immediate aftermath of the incident, including recovery. Tactical command's primary purpose is to provide overall incident management, allocating resources according to priorities, and to ascertain when tasks will be undertaken. Operational command is established in both minor and major incidents and is active at the scene, managing front line operations (Cabinet Office, 2003). 'Big bang' incidents are likely to require invoking such a concentric command structure, whilst in others the model may vary. For example, a train crash may require several operational command sectors answering to one tactical command, whilst an underground train incident may require the establishment of one operational command centre with two tactical commands (Chemical Industries Association (CIA), 2006).

As the numbers of casualties may be large and immediate rescue and healthcare capabilities may be exceeded, it may be necessary to triage individuals.

4.1.2 Decontamination

Decontamination should always be undertaken prior to casualty treatment and medical management, thereby preventing secondary contamination of medical equipment, such as ventilators, as well as ambulances and Emergency Departments. This is of paramount importance. It means that attending staff do not have to attempt complex medical manoeuvres such as intravenous line insertion and intubation whilst donning cumbersome PPE. Decontamination is undertaken in the warm zone following removal of clothing; its purpose is to remove contamination from the body surfaces such that the toxicological consequences are minimized. It therefore needs to be undertaken rapidly. Several means of decontamination have been utilized and proposed, involving both 'dry' decontamination (using adsorbents such as fuller's earth) or 'wet' decontamination with water or weak (0.5% v/v) solutions of detergent or bleach. There is no categorical evidence regarding the best means of decontamination. However, it is widely assumed that application of water or bleach/detergent solutions through a rinse–wipe–rinse regime is the best means of removal.

Contaminated effluent, as far as possible, needs to be contained to minimize contamination of the wider environment. There is therefore a need for collection media, such as bowsers, tanks and vessels for subsequent disposal at a hazardous-waste facility. However, it is recognized that this is not always possible, and effluent may well need to be allowed to drain to waste, despite potential environmental pollution; human life should always take precedence.

4.1.3 Communications

Communications will also need to be established with key personnel, such as hospitals and general practitioners, the emergency services, laboratories, local government and other agencies and organizations. In addition, a channel of communication with the *public* will need to be established. This may involve relaying messages through the media, electing a trained spokesperson or establishing a public-health information 'hotline'.

Whatever the mechanism for dealing with the public, it is essential that communication uses appropriate, clear and concise vocabulary and is transmitted in all appropriate languages (**Table 15**).

Table 15 Risk communication with the public; key components of successful interaction. The message conveyed to the public should be:

Simple
Concise
Timely
Accurate
Relevant
Credible
Consistent

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4.2 Intermediate; 2–6 Hours

Further and ongoing assessment of the incident will be required. A risk assessment of the potential implications for the *wider community* requires knowledge of the nature of the release, the chemical(s) and quantities involved, the environmental media contaminated and the proximity of communities. Therefore, chemical incidents may result in contamination of land and crops, pollution of rivers, lakes, ground and surface water and, through the production of a plume, air pollution. It may be necessary to advise on washing or avoiding crops, the necessity for potable water supplies or for the need to shelter or evacuate.

In the great majority of incidents, *sheltering* following the production of a potentially toxic plume is the preferred option, being the most feasible—that is, staying indoors, closing all the windows and doors, and shutting down any ventilation or air-conditioning systems, until the chemical (usually in a cloud) has passed. This procedure will usually protect the community for up to 12 hours, thus providing adequate protection in the great majority of incidents. Incidents of longer duration, however, may require a different strategy, and evacuation may well be necessary. Likewise threat of exposure, such as an impending plume from the leakage of material from a transportation incident or fire at a chemical factory or storage facility (Baxter, 2005).

4.2.1 Evacuation

Evacuation may be a life-saving measure, as is illustrated by the fact that it has been reported that 34 residents were presumed to have drowned following refusal to leave a nursing home in Louisiana in the aftermath of *Hurricane Katrina* (Dosa *et al*., 2007) It entails the removal of people away from the area of (likely) contamination to a place of safety. It will often involve complex arrangements ranging from the provision of transport, to shelter, food, water and appropriate medical care (for pre-existing conditions as well as the health effects of the exposure) in the area of safety. It may also require ensuring the security of the properties left uninhabited.

In the context of a chemical incident, the major argument for evacuation is risk of explosion; sheltering in nearby accommodation during an explosion almost certainly leads to death due to the propagation of pressure waves. It may also be considered for areas that may be exposed at a later time and the likely duration of exposure is such that the protection offered by in-place sheltering will become insufficient. Risks to the evacuation process include the attendant risks of moving large numbers of people, such as falls and road traffic accidents, and disturbing the health of frail, elderly and disabled people. Generally speaking, as a rule of thumb, if the evacuation involves more than 1000 people, it is not practical or feasible to do so.

The decision to authorize return is dependent upon monitoring/modelling and multiagency experience data adequate to be able to assert that the area is safe and there is an ability to provide adequate services.

It is therefore vital that the public are informed rapidly following a chemical incident, such that they are informed and remain informed of the incident and any developments that may arise. It is therefore essential that agencies involved in an incident provide a consistent and harmonized message that can subsequently be relayed and updated through the local media; residents are often advised to '*Go in, stay in and tune in'*.

4.2.2 Environmental Sampling

Environmental sampling following a chemical incident is commonly warranted. It may involve collection of air, water and soil samples in order to assess extent and level of environmental contamination. This in turn serves as a proxy for population exposure and thus allows direction of resources towards communities or sections of communities who are potentially exposed. This allows such communities to be targeted, and clinical and epidemiological follow-up commenced, as appropriate. Initiation of sampling, following an incident, may therefore need to be commenced rapidly. Environmental Sampling should therefore be undertaken as shown in **Table 16**.

It is important to ensure that all samples taken are complimented by completion of all necessary forms (document control) and that chain of custody is maintained. These procedures allow an audit trail to be established and ensure that it may be followed at a later date. Samples will need to be kept for an agreed period of time. Further information is available from the UK Department for Food, Environment and Rural Affairs (DEFRA) (*www.defra.gov.uk/environment/chemicals/accident/sampling*) and the US Agency for Toxic

Table 16 Environmental sampling following a chemical incident. Successful and representative sampling strategies require comprehensive emergency planning:

By fully trained staff
As rapidly as possible following an incident, when safe and appropriate to do so
Utilizing standardized operating procedures
In a coordinated, systematic and representative manner
Inclusive of potentially sensitive sites, such as allotments, schools, homes and playing fields
In full consultation with all involved agencies and laboratories
Utilizing approved and, if possible, accredited methodologies
To allow rapid turnaround

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Table 17 Key characteristics of the ideal bio-marker

Be easily measurable
Be detectable rapidly and only following exposure
Decline rapidly upon cessation of exposure
Be closely correlated with environmental levels
Be closely related to acute and chronic health effects in a dose-dependent manner
Guide patient management, and community clinical and epidemiological follow-up

Disease Registry (*www.atsdr.cdc.gov/ednpha.html*). A well constructed and coordinated procedure for sampling can provide invaluable data regarding the degree and extent of contamination, and guide subsequent community biological monitoring.

4.2.3 Biological Monitoring

Biological monitoring is the process whereby a chemical or closely associated metabolite is measured in body excreta, fluid or organ, such as expired air, blood, urine or hair. The process can be subdivided into biomarkers of effect and biomarkers of exposure. The former refers to measuring the effect of a chemical on a body organ, such as the liver or kidney, whereas the latter directly measures the chemical. Ideally, a biomarker should fulfil the criteria given in **Table 17**.

Unfortunately, very few biomarkers fulfil all these criteria. In addition, the number of chemicals for which biomarkers are established is very few; the great majority do not have established biomarkers. However, collection of samples should be encouraged at every opportunity as even if the relationship between dose and health effects is poorly understood, biological monitoring may be of great value in further understanding this relationship. Examples of chemicals where biomonitoring may be helpful include carbon monoxide, lead, mercury, nerve agents and mustard.

Again it is important to liaise with laboratories prior to commencing sampling, in order to ensure appropriate specimen collection including correct timing, sample collection and transportation to the laboratory. In order to ensure appropriate sampling, the criteria given in **Table 18** need to be fulfilled.

Table 18 Pre-analytical considerations for environmental and biological sampling

Prior communication with laboratory
Sampling at appropriate times by trained staff
Correct sampling procedure (collection, appropriate media and vessels, labelling, packaging and transportation)
Laboratory accreditation
Appropriate sample turnaround and interpretation

Addressing the needs of vulnerable populations, such as children, the chronically ill (such as dialysis patients), disabled, housebound, institutionalized and transient communities should also commence, whilst continuing to communicate with, and update, the public. Comments regarding document quality assurance, document control, chain of custody and establishing an audit trail equally apply to biological specimens.

4.3 Intermediate; 6–12 Hours

In this period, the situation should continue to be frequently assessed, health response objectives updated and reviewed, requests for advice and assistance evaluated, risk communication continued, legal issues apprised, and samples collected, analysed and interpreted, whilst documenting all activities. Any available health surveillance data needs to be considered and factored into the incident management.

4.4 Extended Response; 12–24 Hours

The situation should continue to be assessed, health response objectives updated and reviewed, appropriate communications with adjacent public-health and medical structures should be initiated, requests for advice and assistance addressed, risk communication continued, legal issues apprised, and samples collected, analysed and interpreted, whilst documenting all activities. Any available health surveillance data needs to be considered and factored into the incident management.

Any voluntary aid should also be coordinated.

4.5 Longer Term; Post-24 Hours

The criteria outlined above should continue to be assessed as appropriate. In addition, however, clinical and epidemiological follow-up may be appropriate. Data obtained in this way may be invaluable in the identification of exposed individuals and subsequently may guide clinical follow up, such as clinical investigation, cancer registries and epidemiological investigation. Epidemiological investigation comes into its own in this phase, although collection of data on who may have been exposed should be implemented as soon as possible. This may well be a daunting task as those exposed may extend well into the wider community and well beyond those seeking medical care. It will be determined by the nature of the release and the environmental media contaminated. For example, an airborne release

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Table 19 Holistic overview of major components of chemical-incident management from the immediate phase through to the longer term

Detection of chemicals, both environmentally and biologically
Evaluation of the nature and magnitude of the release
Contacting specialists and reference centres
Assessment of current and delayed mass casualty needs
Risk communication
First-line responder protection
Command and control
Decontamination (rapid)
Triage implementation
First aid and medical care
Public-health protection
Collectively these procedures can be fed in to hone and improve planning and preparedness and thus mobilization of regional, national and international sources through mutual aid
Monitoring of residual hazard levels
Long-term follow up of casualties

may result in a dense plume, the footprint of which determines those potentially exposed. Enumeration therefore requires access to appropriate demographic data and identification of vulnerable/susceptible groups, whilst risk is the product of such data and expected acute and chronic health effects. All these factors should be considered before embarking upon an epidemiological investigation (Cullinan, 2002).

In summary, the response to an incident, whether accidental or deliberate should cover the systematic response steps given in **Table 19**.

5 RECOVERY

Recovery following a chemical incident encompasses the economy and the environment as well as health, which all contribute to sustainable communities.

Chemical incidents undoubtedly influence the economy, particularly in local communities. Chemical industries are major employers in developed countries, and in the aftermath of chemical incidents employment opportunities may be limited, and this together with the health consequences of incidents may result in a significant decrease in economic prosperity, wealth and standard of living. Coupled to this may be the need for clean up, remediation and restitution, which together with any ensuing litigation may be extremely costly and thus further influence economic prosperity. Alternative accommodation for residents and key facilities will need to be available if clean-up is not practical or possible, further contributing to the overall cost.

Rehabilitation following an environmental incident is a mixture of remediation and restitution of the environment, action to prevent a further occurrence, and work to improve the community's health.

5.1 Remediation

Remediation is the process of making the environment safe and clean. It may occur naturally, as with a volatile gas, or where the chemicals involved degrade rapidly. It may however, involve the physical collection of the contaminated medium and its subsequent safe removal, or it may require other measures to reduce the toxicity of the chemical. Many chemicals, such as chlorinated hydrocarbons, are environmentally persistent with long half-lives, and thus may result in ongoing contamination.

A range of expertise is needed to assess the extent of residual contamination of environmental media, such crops, soil, air and water, to devise appropriate decontamination measures where needed and to ensure that it is safe for evacuated populations to return. The extent to which contamination has contaminated environmental media, and subsequent human exposure (such as contamination of crops and livestock, with subsequent risks to the food chain) must also be appraised, and preventive or protective measures put into place. Significant contamination of the food chain is likely to require destruction of crop products, and animals grown for food. Collectively, it is a reflection of the nature and scale of the incident together with the physicochemical properties of the chemicals released and their reaction products.

Cleaning up contaminated sites or land may entail standard soil stabilization disposal technologies or biological methods of decontamination, which are developing rapidly. Material such as topsoil, equipment or crops may need to be removed for cleaning or disposal, (without endangering the new site). Rehabilitation of large bodies of waste therefore may be expensive and extremely resource intensive and time-consuming, especially when the chemical(s) have become bound to the sediment.

Individual decontamination of affected wildlife may also be thought necessary, as following a major oil spill, for example. Decontamination of the drinking water or source water may not be possible and the system may have to be flushed out, potentially causing further contamination of soil, surface and groundwater, and seawater. Alternative sources of drinking water may therefore be required.

Complete removal of the chemical or chemicals may not be possible, as it may be too difficult, hazardous or economically non-viable. If full remediation cannot be accomplished, it may be necessary to declare the area as 'limited entry' or 'limited use'. These declarations may have to be in force for many years and may seriously

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Table 20 Principle objectives of rehabilitation

Effect remediation of and restoration of the environment
Restore the health of the population at least to its state before the incident, or minimize the ill effects
Answer unanswered questions
Restore health care and community services prevailing before the incident and, whenever possible, improve these services
Provide evaluation and feedback on incident response and effect indicated changes
Monitor for unexpected effects or for potential effects when risk is uncertain
Continue risk reduction and prevention activities

disrupt the life of the community, and environmental recovery.

5.2 Restitution

Restitution is the process of returning the environment to its original state and may involve extensive remediation. Restoring the environment may involve landscaping, as well as rebuilding, replacing equipment and buildings, replanting of crops and replenishing animals and wildlife, and thus may entail considerable cost.

Addressing health concerns of communities following an incident is also of paramount importance, with a timely and effective response to community concerns vital for such community recovery. Psychological implications may well be significant even for incidents which pose no physical hazard and require few or no protective actions. Such psychological effects typically manifest as anxiety, insomnia and depression, as well as nonspecific symptoms such as nausea, vomiting and headaches. Often such stress can be resolved by good (and timely) communication of measures taken to prevent exposure whilst additional investigations to confirm a lack of health effects and environmental contamination are helpful. Involving the community in plans to reduce the risk of further incidents and to quickly alert the public-health team and the public if further incidents occur are reassuring and protective of the public's health.

The aims of rehabilitation are summarized in **Table 20**.

6 EVALUATION

At the earliest possible opportunity, the public-health response to a chemical incident should be critically evaluated. It is possible to conduct three types of review of the performance of the public-health response to the incident, but these should all be undertaken in a 'no-blame' environment. The time, effort and resources allocated to any or all of these will depend upon the nature of the incident, the complexity of the response, the time since, and the lessons learned from the last review, and the resources available. Evaluations should be set out in a methodological way, to analyse the events and to assess what impact the response made on the outcome. In particular, questions are asked and judgements made as to the difference in outcomes if there had been no public-health response, or if different responses had been given. Ideally, outside experts are brought in to add their expertise and a level of objectivity. The outcome, conclusions and recommendations should subsequently be factored into the chemical-incident planning and preparedness.

6.1 Wash-ups

Wash-ups are perhaps the simplest of the reviews, and involve a re-run of the events of the incident and the response of the public-health environmental health professionals. Errors, deviations from the plan and problems in communications are highlighted in a non-threatening way so that participants can learn as much as possible from the actual incident and the rerun, in a 'no-blame' environment. The wash-up is conducted as soon as possible after the incident, while the events are still fresh, and as soon as possible. It is usually an 'in-house' affair.

In summary, chemical-incident planning, preparedness and subsequent response is a complex process, but if undertaken in a comprehensive manner can reduce, mitigate or prevent subsequent public-health consequences.

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Biomonitoring of the Workplace and Environment

Peter J. Boogaard

CONTENTS

1 INTRODUCTION

Biomonitoring is, in its simplest definition, the measurement of a parameter in a biological specimen. This parameter is often referred to as a 'biomarker', and can be a chemical, its metabolite or an adduct to a macromolecule, or an effect, such as a change in enzymatic activity or a chromosomal aberration. In a more complex definition, a biomarker is an analytical measurement of any substance, structure or process that indicates an exposure, susceptibility or prediction of the incidence or outcome of disease (Toniolo *et al*., 1997). This chapter is restricted to human biomonitoring and aims to describe the biomonitoring of chemical exposure to humans, both in the occupational setting and through the environment. The main goal of human biomonitoring is usually to assess exposure. Biomonitoring has a number of specific characteristics that make it particularly useful for this purpose. In general, individuals may be exposed via inhalation, ingestion and uptake through the skin or mucous membranes. In contrast to most other exposure monitoring techniques, such as personal air monitoring in occupational settings or food-basket analysis in consumer settings, biomonitoring integrates all routes of exposure. In addition, biomonitoring may allow the assessment of both spatial and temporal differences in exposures between individuals. Moreover, it may also take into account differences between individuals such as variations in genetics and physiology. These advantages have been recognized for a long time, and the first human biomonitoring dates back to the beginning of last century. Amongst the first chemicals that were biomonitored were lead and benzene, substances that are still of considerable interest (Kehoe *et al*., 1933; Yant *et al*., 1936). In the 1960s and 1970s, the development of more sensitive analytical techniques not only allowed measurement of lower concentrations of chemicals in complex matrices like urine and blood, but also enabled the analysis of larger series of samples. In addition, limit values were proposed to avoid health effects of exposure (Hartogensis and Zielhuis, 1962; Zielhuis, 1959; 1971; Walkley *et al*., 1961). These developments led to an increasing importance of biomonitoring to assess occupational exposures. Historically, human biomonitoring was mainly applied in occupational settings. As

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a consequence, methodologies, techniques and interpretation of results focussed on biomonitoring of chemical exposure in the working place and during industrial applications. These principles were summarized in 'Industrial Chemical Exposure—Guidelines for Biological Monitoring' in 1983 (Lauwerys, 1983). The second edition, in 1993, had more than doubled in size (Lauwerys and Hoet, 1993), and the latest edition, again, doubled in size (Lauwerys and Hoet, 2001), reflecting the increased application of human biomonitoring in industrial settings. Similarly, in 1985 the German Research Foundation's Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area started publishing well-validated biomonitoring methods for important industrial substances under the title 'Analysis of Hazardous Substances in Biological Materials', and has over the years published 10 volumes with procedures for the biomonitoring of more than 200 substances (DFG, 2006). The World Health Organization (WHO) has also published guidance on occupational biomonitoring (IPCS, 1993; WHO, 1996a; 1996b). The importance of biomonitoring of occupational exposure is also reflected in the European Regulations, as the European Union (EU) has adopted several directives that form the base for setting biological limit values (BLVs) (Bolt and Thier, 2006).

The continuous advancements in analytical technologies and capabilities, both with respect to sensitivity and specificity, enabled the determination of increasingly lower concentrations of substances and their effects in biological media. This opened up the possibility of applying human biomonitoring to the assessment of environmental exposures. Since the capacity for testing has grown, increasingly larger numbers of individuals have themselves checked for the presence of substances in their bodies. This human biomonitoring may occur as part of national programmes, such as the German Environmental Survey (GerES) (GerES, 2009), or the USA's National Health and Nutrition Examination Survey (NHANES) (CDC, 2009) programmes, as part of scientific studies, or as a mere service. In the vast majority of cases, results from biomonitoring to assess environmental exposures show that these exposures are low. For substances for which a health-based limit value has been established, the biomonitoring values associated with environmental exposures are usually several orders of magnitude lower than the health-based limit values for occupational exposures. However, the differences in exposure patterns, potentially more susceptible subpopulations and a potential lifetime exposure make the interpretation of biomonitoring data for environmental exposures challenging. Nevertheless, in recent years a number of reports providing guidance in the interpretation of such values have been published (Boogaard *et al*., 2005; Boogaard and Money, 2008; Burke *et al*., 2006).

2 TYPES OF BIOMONITORING

Biomonitoring is a general term comprising the following subcategories:

- **Biological monitoring,** which identifies the assessment of *biomarkers of exposure*. This type of biomarker is also referred to as 'internal dose' or 'body burden'. Typical examples of biological monitoring are the determination of metals (e.g. mercury, arsenic or lead) in blood or urine, the determination of unchanged substances (e.g. dioxins, polychlorinated biphenyls (PCBs) or benzene) in adipose tissue, milk, urine, or blood, the determination of specific metabolites of a chemical (e.g. *S*-phenyl-mercapturic acid (S-PMA) or *trans,trans*-muconic acid (tt-MA) as metabolites of benzene) in urine, or volatile compounds (unchanged substances or metabolites) in exhaled breath.
- **Biochemical effect monitoring,** which identifies the assessment of *biomarkers of effective dose*, which is also referred to as 'tissue dose'. Typical examples of biochemical effect monitoring include the determination of adducts of a specific chemical (e.g. ethylene oxide or polycyclic aromatic hydrocarbons) to DNA or a protein such as albumin (Alb) or haemoglobin (Hb). It should be realized that both biological and biochemical effect monitoring solely provide evidence for exposure and cannot be interpreted in terms of health risk assessment without additional data on dose–response relationships (see also gat005). Some forms of biochemical effect monitoring, such as the determination of Hb adducts, have additional benefits since they allow time-integrated exposure monitoring over long periods of time (up to several months) at a point in time that intervention may still be possible.
- **Biological effect monitoring,** which identifies the assessment of *biomarkers of effect* and gradually flows over into the assessment of clinical parameters or *biomarkers of disease*. Typical examples of biological effect monitoring include measurements such as cholinesterase activity in blood to monitor exposure to organophosphate or carbamate pesticides; zinc protoporphyrin or δ-aminolaevulinic acid to monitor exposure to lead; sister chromatid exchanges and other chromosomal aberrations to monitor exposure to genotoxic or clastogenic substances; and several specific forms of (micro)proteinuria to detect exposure to a variety of substances.

Biological effect monitoring measures early and, in most cases, reversible biological effects, which do not necessarily lead to health effects, whereas abnormal clinical parameters are an expression of (beginning)

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disease. In general, in biological effect monitoring, natural phenomena are measured for which a 'normal' background value may be established, which may be influenced by various physiological and environmental factors that are not related to chemical exposure. This renders most methods of biological effect monitoring intrinsically nonspecific. Various parameters assessed in biological effect monitoring may also be used in clinical practice, and form a seamless continuum with clinical monitoring, as a more-or-less arbitrarily set value may demarcate the shift from a minor biological effect to an effect that is considered clinically relevant. For example, albuminuria may be used in biological effect monitoring (microalbuminuria) as a biomarker of early renal function effects, but it is also used as a clinical parameter for instance to assess renal function impairment in diabetics.

In addition to biomarkers of exposure, of effective dose, of effect, and of disease, sometimes genotyping and phenotyping are referred to as *biomarkers of susceptibility*. Biomarkers of susceptibility are indicators of an inherent or acquired ability of an organism to respond to the challenge of exposure to a specific substance. Some examples are the expression of certain isoforms of cytochrome P450, glutathione transferases or *N*-acetyltransferases. In addition, factors such as nutritional status, iron status, and so on, may also be regarded as biomarkers of susceptibility. These biomarkers differ from the other biomarkers in that they reflect potential interindividual differences in uptake and metabolism of chemicals and, consequently, potential differences in health risks. Biomarkers of susceptibility may, like lifestyle factors such as smoking and drinking behaviour, explain differences in biomarker results between individuals with identical exposure profiles (DFG, 2004).

Both biological monitoring and biochemical effect monitoring are essential methods to help better understand the complex relationships between external and internal exposure and, consequently, the potential adverse health effects that may result from exposure. Both monitoring methods should be regarded as exposure assessment with high specificity for the substance that is being measured, just like most ambient monitoring methods. In fact, in biological monitoring as well as in biochemical effect monitoring each individual serves as its own dose monitor. Although both methods measure the total, actual exposure regardless of the route of exposure, there is a fundamental difference. Biological monitoring simply reflects total internal exposure and is applicable to most substances, whereas biochemical effect monitoring reflects the effective internal exposure of a chemical, usually a potentially genotoxic substance, by measuring its reaction product with an endogenous macromolecule. Biochemical effect monitoring hence proves that the chemical was not only bioavailable but also bioactive. It not only indicates that the chemical was absorbed into the body but also that it reached the tissues (hence: tissue dose). In some cases, it is possible to measure reaction products in the ultimate target tissue and in these instances the concentration may be referred to as target tissue dose.

For genotoxic compounds, the ultimate target would theoretically be the organ in which the substance might induce cancer, and the biomarker the responsible DNA adduct. However, induction and development of cancer is a highly complex process. DNA adduct formation is considered crucial in this process, and the distribution of DNA adducts in the body usually, but not always, reflects the risk, although the concentration of adducts may be higher in nontarget tissues than target tissues (Ríos-Blanco *et al*., 2000; 2003; Potter *et al*., 1989; Otteneder *et al*., 2002; Otteneder and Lutz, 1999). This apparent lack of correlation may be due to the contribution of additional biological processes needed for the progress of an initiated cell to a malignant tumour, but it may also be due to the specific contribution of a minor DNA adduct in the development of a tumour, or a combination of both processes. For most substances, these processes are largely unknown. Moreover, since it is in the vast majority of cases not feasible to determine DNA adducts in the target tissue itself, generally DNA adducts in white blood cells are measured as a surrogate merely reflecting the effective internal exposure. In most instances protein adducts, such as Alb, and in particular Hb adducts, are better surrogate biomarkers for effective internal exposure due to their greater stability and ease of determination (Richter and Branner, 2002; Törnqvist *et al*., 2002; Boogaard, 2002; Koc and Swenberg, 2002; de Kok *et al*., 2002). Although mostly applied to the biomonitoring of genotoxic substances, it should be borne in mind that the concept of target tissue dose may also be used for other compounds with a specific target tissue, for instance cadmium in the kidney (Lauwerys *et al*., 1993; 1994; Roels *et al*., 1983).

As depicted in **Figure 1**, the accuracy of the exposure determination as well as the relevance for health increases from nonpersonal ambient monitoring (i.e. static air monitoring, monitoring of drinking water, 'food-basket' monitoring), via personal ambient monitoring (e.g. personal air monitoring, dermal exposure monitoring), to biological monitoring and biochemical effect monitoring. The relevance for health increases further with biological effect monitoring and clinical effect monitoring, but with a loss of specificity with regard to the chemical (or physical) factor associated with the health effect. In general, for health risk assessments biological monitoring and biochemical effect monitoring provide the best and most reliable information both in term of exposure and potential health effects related to certain exposures.

Biomarkers are potentially influenced by various factors, such as lifestyle and physiology, including genotype and phenotype. The influence of physiological factors may be illustrated by a number of studies where albuminuria was determined, amongst a series of other

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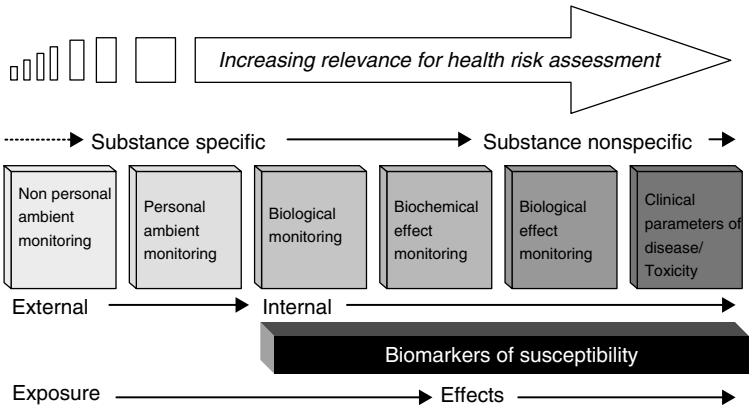

Figure 1 Monitoring techniques as part of the exposure–effect continuum in relation to risk assessment.

renal function parameters, in potentially exposed workers. In a study in 53 male oil refinery workers with potential exposure to hydrocarbons, sensitive biochemical markers of kidney function and damage were compared to those of a control group of 61 age-matched nonexposed males. No difference was found in the urinary tubular parameters β-*N*-acetyl-D-glucosaminidase (NAG), β-2-microglobulin (β2 M) and retinol-binding protein (RBP). Surprisingly, the mean albuminuria, which is considered a less sensitive parameter for renal function effects, was slightly higher ($p < 0.005$) in the refinery workers compared to the controls, although all values were in the normal range. The authors concluded that chronic low-level hydrocarbon exposure in these refinery workers did not lead to clinically significant renal abnormalities but that some findings were consistent with the possible role of hydrocarbon exposure in the induction of renal disturbances (Viau *et al*., 1987). In a study in 73 male workers of an organochlorine plant, biochemical effects on renal function were assessed by determination of urinary NAG, RBP, alanine aminopeptidase (AAP) and Alb, and compared to 35 nonexposed males. Again, albuminuria was within normal limits in both groups but slightly higher in the potentially exposed workers than the controls (Boogaard *et al*., 1993). Further investigations with additional control groups indicated that the observed effect was a physiological effect induced by shift working. In both studies with increased albuminuria, the potentially exposed operators worked in shifts or had physically strenuous workloads, whereas the controls worked only during daytime and did physically nondemanding work. As nonexposed male shift workers displayed the same small increase in albuminuria, it could be concluded that the effect was unrelated to potential chemical exposures but rather to shift work or physical work load (Boogaard and Caubo, 1994). These studies also illustrate that interpretation of biological effect monitoring data in terms of health effects in relation to chemical exposures should be undertaken with utmost caution.

In contrast, biochemical effect monitoring—due to its substance specificity—can directly be related to chemical exposures and may be highly useful in risk assessment. This is illustrated by the studies on the mycotoxin, aflatoxin B_1. From epidemiological studies the association between hepatocellular carcinoma and aflatoxin B_1 intake has been established. In large biomonitoring studies, the incidence of hepatocellular carcinoma was strongly correlated with urinary biomarkers for aflatoxin B_1 and with serum hepatitis B surface antigen, whilst there was no significant association with dietary intake of aflatoxins (Qian *et al*., 1994; Ross *et al*., 1992). The association between aflatoxin B_1 exposure and DNA-adduct levels in the liver, and with the urinary excretion of aflatoxin-*N*7-guanine was also demonstrated in animal studies (Groopman *et al*., 1992a) and confirmed in humans (Groopman *et al*., 1992). Despite the importance of hepatitis B status as an influencing factor on the development of hepatocellular carcinoma, the robust correlation of urinary aflatoxin-*N*7-guanine with biological effects strongly supports its use as biomarker in the risk assessment process (Groopman *et al*., 1993).

The characteristics of biomonitoring to evaluate internal exposure and early biochemical and biological effects make biomonitoring a potentially more accurate risk assessment tool than external monitoring such as air monitoring and surface contamination monitoring. Unfortunately, the number of substances for which a validated biomonitoring method is available is limited compared to those for which validated ambient air monitoring methods are on hand (**Tables 1** and **2**).

3 CHOICE OF SPECIMEN

Human biomonitoring is typically performed in urine or blood, but occasionally in exhaled breath, or tissues such as hair, nails or buccal cells are used. Breast milk may also be used for biomonitoring, usually to assess exposure to highly lipophilic compounds, such as chlorinated

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Table 1 Biomonitoring action levels given by different organizations

Exposure	Measured parameter	BEI® (ACGIH, 2008)	BMGV (UK HSE, 2007)	BAT or EKA (DFG, 2007)	BAL(RL) (FIOH, 2008)
Acetone	Acetone (U)	50 mg l^{-1} (T1)		80 mg l^{-1} (T1)	
Acetylcholinesterase inhibitors (organophosphates, carbamates)	Acetylcholinesterase (E)	70% of baseline (T0)		70% of baseline (T3)	30% drop (T13)
Acrylamide	*N*-(Carbonamidethyl) valine from Hb adduct (B)			15 µg l^{-1} (T2)	
Acrylonitrile	*N*-Cyanoethylvaline from Hb adduct (B)			420 µg l^{-1} (T2)[a]	
Aluminium (Al) and its inorganic compounds	Al (U) Al (S)			200 µg l^{-1} (T1, T3)	6.0 µM (T12) (0.1 µM) (T9)
4-Aminobiphenyl	4-Aminobiphenyl from Hb adducts (B)			10 ng l^{-1} (T2)	
Aniline	Aniline (U) Aniline from Hb adducts (B) *p*-Aminophenol (U)	× (T1) × (T1) 50 mg l^{-1} (T1)		1 mg l^{-1} (T1, T3) 100 µg l^{-1} (T1, T3)	10 µM (T5)
Antimony (Sb)	Sb (U)			× (T1, T5)	(9 nM) (T5)
Arsenic (As), elemental and soluble inorganic compounds	As (inorganic and methylated metabolites) (U) As (inorganic As) (U)	35 µg l^{-1} (T5)	230 µmol mol^{-1} creatinine[g]	50 µg l^{-1} (T1, T3)	70 µM (T1)
Arsenic trioxide (As_2O_3)	As (U)			50 µg l^{-1} (T1, T3)[b]	
Benzene	*S*-Phenylmercapturic acid (U) *t,t*-Muconic acid (U) Benzene (B)	25 µg g^{-1} creatinine (T1) 500 µg g^{-1} creatinine (T1)		45 µg g^{-1} creatinine (T1)[c] 2 mg l^{-1} (T1)[c] 5 µg l^{-1} (T1)[c]	14 µM (T1)
Benzidine and its salts	Benzidin (U) Benzidin-adducts (B)			× (T2) × (T1, T3)	
Beryllium and its compounds	Beryllium (U)			× (T1, T3)	(15 nM) (T5)
Bisphenol A (4,4′-isopropyli-denediphenol)	Bisphenol A (hydrolytically released) (U)			80 mg l^{-1} (T1)	

(*continued overleaf*)

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Table 1 (*continued*)

Exposure	Measured parameter	BEI® (ACGIH, 2008)	BMGV (UK HSE, 2007)	BAT or EKA (DFG, 2007)	BAL(RL) (FIOH, 2008)
1,3-Butadiene	1,2-Dihydroxy-4-(*N*-acetylcysteinyl) butane (U) *N*-(Hydroxybutenyl) valine (B)	2.5 mg l^{-1} (T1) 2.5 pmol g^{-1} Hb (T2)		4.1 mg l^{-1} (T1)[d]	
1-Butanol	1-Butanol (U)			2 mg g^{-1} creatinine (T8) 10 mg g^{-1} creatinine (T1)	
2-Butoxyethanol	Butoxyacetic acid (U)	200 mg g^{-1} creatinine (T1)	240 mmol mol^{-1} creatinine (T1)	100 mg l^{-1} (T3)	60 mmol mol^{-1} creatinine (T5)
2-Butoxyethanol acetate	Butoxyacetic acid (U)			100 mg l^{-1} (T3)	60 mmol mol^{-1} creatinine (T5)
2-(2-Butoxyethoxy) ethanol or its acetate	2-(2-Butoxyethoxy) acetic acid (U)				300 mmol mol^{-1} creatinine (T5)
p-tert-Butylphenol	*p-tert*-Butylphenol (U)			2 mg l^{-1} (T1)	
Cadmium (Cd) and its inorganic compounds	Cd (U) Cd (B) Retinol-binding protein (U)	5 µg g^{-1} creatinine (T2) 5 µg l^{-1} (T2)		7 µg l^{-1} (T2)	50 nM (T2) 50 nM (T2) (300 µg g^{-1} creatinine) (T2)
Carbon disulfide	2-Thiothiazolidine-4-carboxylic acid (U)	5 µg g^{-1} creatinine (T1)		4 mg g^{-1} creatinine (T1)	2.0 mmol mol^{-1} creatinine (T5)
Carbon monoxide	CO-Hb (B) CO (A)	3.5% (T1) 20 ppm (T1)	30 ppm (T4)	5% (T1)	5% (T1)
Carbon tetrachloride (tetrachloromethane)	Carbon tetrachloride (B)			3.5 µg l^{-1} (T3)	
Chlorobenzene	4-Chlorocatechol (U) 4-Chlorophenol (U)	100 mg g^{-1} creatinine (T5) 20 mg g^{-1} creatinine (T5)		175 mg g^{-1} creatinine (T1) 35 mg g^{-1} creatinine (T8)	
Chromium(VI), water-soluble fume (Cr)	Cr (U) Cr (E)	10 µg l^{-1} (T4) 25 µg l^{-1} (T5)	10 µmol mol^{-1} creatinine (T1)	20 µg l^{-1} (T1)[e] 17 µg l^{-1} (T3)[e]	0.1 µM (T5)[bb] 0.6 µM (T5)[cc] 0.3 µM (T5)[dd]

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Cobalt (Co) and inorganic cobalt compounds	Co (U) Co (B)	15 µg l^{-1} (T5) 1 µg l^{-1} (T5)		15 µg l^{-1} (T2)[f]	600 nM (T5)
Copper (Cu) and inorganic copper compounds	Cu (U)				(<0.50 µM) (T2) or <0.47 µmol $(24\ h)^{-1}$
Cresol (all isomers)	Total cresol (U)			200 mg l^{-1} (T1)	
Cumene (isopropylbenzene)	2-Phenyl-2-propanol (U) Cumene (B)			50 mg g^{-1} creatinine (T1) 2 mg l^{-1} (T1)	
Cyanides and hydrocyanic acid	Thiocyanate (U) Thiocyanate (S)				(85 µM[ee]; 250 µM[f,f]) (T13) (100 µM[ee]; 250 µM[f,f]) (T13)
Cyclohexane	1,2-Cyclohexanediol (U)			170 mg g^{-1} creatinine (T1, T3)	
Cyclohexanol	Cyclohexanol (U) 1,2-Cyclohexanediol (U)	× (T1) × (T1)			
Cyclohexanone	Cyclohexanol (U) 1,2-Cyclohexanediol (U)	8 mg l^{-1} (T1) 80 mg l^{-1} (T4)	2 mmol mol^{-1} creatinine (T1)	30 mg l^{-1} (T1)[g] 250 mg l^{-1} (T1)[g]	
1,2-Dichlorobenzene	1,2-Dichlorobenzene (B) 3,4- and 4,5-Dichlorocatechol (U)			140 µg l^{-1} (T1) 150 mg g^{-1} creatinine (T1)	
1,4-Dichlorobenzene	2,5-Dichlorophenol (U)			20 mg g^{-1} creatinine (T1, T3)[h]	
Dichloromethane	CO (A) Dichloromethane (U) Dichloromethane (B)	0.3 mg l^{-1} (T1)	30 ppm (T1)	0.5 mg l^{-1} (T6)[i]	5% (T1)
2,2′-Dichloro-4,4′-methylene-dianiline (MbOCA)	2,2′-Dichloro-4,4′-methylenedianiline (U)		15 µmol mol^{-1} creatinine (T1)		

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Table 1 (*continued*)

Exposure	Measured parameter	BEI® (ACGIH, 2008)	BMGV (UK HSE, 2007)	BAT or EKA (DFG, 2007)	BAL(RL) (FIOH, 2008)
N, N-Dimethylacetamide	*N*-Methylacetamide (U)	30 mg g^{-1} creatinine (T5)	100 nmol mol^{-1} creatinine (T1)	30 mg g^{-1} creatinine (T1, T5)	50 mmol mol^{-1} creatinine (T5)
N,*N*-Dimethylformamide (DMF)	*N*-Methylformamide (U) *N*-Acetyl-S-(*N*-methyl-carbamoyl) cysteine (U)	15 mg l^{-1} (T1) 40 mg l^{-1} (T9)		35 mg l^{-1} (T1)	200 µM (T1)
Dimethylsulfate	*N*-Methylvaline from Hb adduct (B)			40 µg l^{-1} (T2)[j]	
1,4-Dioxane	β-Hydroxyethoxyacetic acid (U)			× (T1)	
Diphenylmethane-4,4′-diisocyanate	4,4′-Diaminodiphenylmethane (MDA) (U)			10 µg l^{-1} (T1)	
2-Ethoxyethanol and 2-ethoxyethyl acetate	2-Ethoxyacetic acid (U)	100 mg g^{-1} creatinine (T1)		50 mg l^{-1} (T1, T3)	20 mmol mol^{-1} creatinine (T5)
2-(2-Ethoxethoxy) ethanol and its acetate	2-(2-Ethoxyethoxy)acetic acid (U)				120 mmol mol^{-1} creatinine (T5)
Ethylbenzene	Sum of mandelic acid and phenylglyoxylic acid (U) Ethylbenzene (A) Sum of 2- and 4-ethylphenol (U)	0.7 g g^{-1} creatinine (T5) × (T2)		1600 mg l^{-1} (T5)[k] 12 mg l^{-1} (T5)[k]	5.2 mM (T5)
Ethylene	Hydroxyethylvaline from Hb adduct (B)			180 µg l^{-1} (T2)[l]	
Ethylene glycol dinitrate	Ethylene glycol dinitrate (B)			0.3 µg l^{-1} (T1)	
Ethylene oxide	Hydroxyethylvaline from Hb adduct (B)			90 µg l^{-1} (T2)[m]	
Fluorides (F) (hydrogen fluoride and inorganic fluorides)	F (U)	10 mg g^{-1} creatinine (T1) 3 mg g^{-1} creatinine (T8)		7 mg g^{-1} creatinine (T1, T3) 4 mg g^{-1} creatinine (T8)	350 µM (T1) 200 µM (T12)
Formic acid and formates	Formic acid (U)				200 mmol mol^{-1} creatinine (T9)
Furfural	Furoic acid (U)	200 mg g^{-1} creatinine (T1)			
Glycerine trinitrate	Glycerine dinitrate (P/S)			× (T1)	

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Halothane (2-bromo-2-chloro-1, 1,1-trifluoroethane)	Trifluoroacetic acid (U)			2.5 mg l^{-1} (T1, T3)	
n-Heptane	*n*-Heptane (B) 2,5-Heptanedion (U)			× ×	
Hexachlorobenzene	Hexachlorobenzene (P/S)			150 µg l^{-1} (T2)	
n-Hexane	2,5-Hexanedione (U) Sum of 2,5-hexanedione and 4,5-dihydroxy-2-hexanone (U)	0.4 mg l^{-1} (T5)		5 mg l^{-1} (T1)	2 mmol mol^{-1} creatinine (T5)
Hydrazine	Hydrazine (U) Hydrazine (P)			35 µg g^{-1} creatinine (T1, T3)[n] 27 µg l^{-1} (T1, T3)[n]	
Isocyanates (MDI, TDI)	Diamine (U) Isocyanate metabolites (U)		1 µmol mol^{-1} creatinine (T7)		1 µmol mol^{-1} creatinine (T13)
Lead (Pb) inorganic	Pb (B) Pb (U)	300 µg l^{-1} (T8)[w]		400 µg l^{-1} (T2)[x]	1.4 µM (T2)
Lead (Pb) organic (tetraethyllead)	Pb (U) Diethyllead (U)			50 µg l^{-1} (T1) 25 µg l^{-1} (T1)	0.1 µM (T5)[y]
Lindane	Lindane (B) Lindane (P/S)		35 nM (T2) 70 nM (T2)	25 µg l^{-1} (T3)	
Manganese (Mn) and its inorganic compounds	Mn (B)			20 µg l^{-1} (T1, T3)	(40 nM) (T5)
Mercury (Hg)	Hg, inorganic (U) Hg, inorganic (B)	35 µg g^{-1} creatinine (T8) 15 µg l^{-1} (T5)	20 µmol mol^{-1} creatinine (T2)		250 nM (T9) 90 nM (T2)
Mercury and its inorganic salts	Hg (U)			25 µg g^{-1} creatinine or 30 µg l^{-1} (T2)	
Mercury, organic	Hg (B)			× (T2)	
Methanol	Methanol (U)	15 mg l^{-1} (T1)		30 mg l^{-1} (T1, T3)	
Methaemoglobin inducers	Methaemoglobin (B)	1.5% of Hb (T10)			
2-Methoxyethanol and 2-methoxyethyl acetate	2-Methoxyacetic acid (U)	× (T1)			

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Table 1 (*continued*)

Exposure	Measured parameter	BEI® (ACGIH, 2008)	BMGV (UK HSE, 2007)	BAT or EKA (DFG, 2007)	BAL(RL) (FIOH, 2008)
2-(2-Methoxyethoxy) ethanol and its acetate	2-(2-Methoxyethoxy) acetic acid (U)				50 mmol mol^{-1} creatinine (T5)
1-Methoxy-2-propanol and its acetate	1-Methoxy-2-propanol (U)			15 µg l^{-1} (T1)	100 µM (T5)
Methylbromide	Bromide (P, S) *S*-methylcysteinealbumin (B)			12 mg l^{-1} (T3) × (T2)	
Methyl-*t*-butyl ether (MTBE)	MTBE (B/U) *t*-Butyl alcohol (B/U)			× ×	30 µM (T11)
Methyl *n*-butyl ketone (2-hexanone)	Hexanedione + 4,5-dihydroxy hexan-2-one (U)			5 mg l^{-1} (T1)	
Methylenebis(2-chloroaniline) (MOCA)	MOCA (U)				15 µmol mol^{-1} creatinine (T5)
4,4′-Methylenedianiline (MDA)	MDA (U)		50 µmol mol^{-1} creatinine (T1 or T8)[z]	10 µg l^{-1} (T1)	50 µmol mol^{-1} creatinine[aa]
Methyleneiodide (Diiodomethane)	Carboxyhaemoglobin (B)				5% (T1)
Methylethyl ketone (2-butanone)	2-Butanone (U)	2 mg l^{-1} (T1)	70 µmol l^{-1} (T1)	5 mg l^{-1} (T1)	20 µM (T5)
Methylformiate	Methanol (U) Formic acid (U)			× ×	
Methylisobutyl ketone (4-methyl-2-pentanone)	4-Methyl-2-pentanone (U)	2 mg l^{-1}[a]		3.5 mg l^{-1}[a]	0.5 mmol mol^{-1} creatinine (T1)
N-Methyl-2-pyrrolidone	5-Hydroxy-*N*-methyl-2-pyrrolidone (U)	100 mg l^{-1} (T1)		150 µg l^{-1} (T1, T3)	
Molybdene (Mb) and its compounds except Molybdenetrioxide	Mb (U/P)			×	(1340 nM) (T5)
Naphthalene and mixtures of polycyclic aromatic hydrocarbons	Naphthol (U)				(30 nM[ee]) (T5) (300 nM[ff]) (T5)
2-Naphthylamine	2-Naphthylamine (U) 2-Naphthylamine from Hb adducts (B)			× (T1) × (T2)	

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1,5-Naphthylenediamine	1,5-Diaminonaphthaline (U)			× (T1)	
Nickel (metal, oxide, carbonate, sulfide and sulfidic ores)	Ni (U)			15 µg l^{-1} (T3)[o]	0.3 µM (T5)
Nickel, soluble compounds	Ni (U)			70 µg l^{-1} (T3)[p]	1.3 µM (T5)
Nitrobenzene	*p*-Nitrophenol (U) Methaemoglobin (B) Aniline from Hb adducts (B)	5 mg g^{-1} creatinine (T5) 1.5% of Hb (T1)		100 µg l^{-1} (T3)	
Nitroglycerine	Nitroglycols (U)		15 µmol mol^{-1} creatinine (T3)		
Nitrous oxide	Nitrous oxide (U)				700 nM (T1)
Parathion	Cholinesterase (E) *p*-Nitrophenol (U)	70% of baseline (T2) 0.5 mg g^{-1} creatinine (T1)		70% of baseline (T2) 500 µg l^{-1} (T1)	
Pentachlorophenol (PCP)	PCP (U) PCP (P)	2 mg g^{-1} creatinine (T9) 5 mg l^{-1} (T1)		600 µg l^{-1} (T2)[q] 1700 µg l^{-1} (T2)[q]	
Perfluorooctanoic acids and their inorganic salts	Perfluorooctanoic acid (S)			5 mg l^{-1} (T2)	
Phenol	Phenol (U)	250 mg g^{-1} creatinine (T1)		200 mg l^{-1} (T1)	1.3 mM (T5)
Polychlorinated biphenyls (PCBs)	PCB (S) sum of 24 isomers				(3 µg l^{-1}) (T9)[gg]
Polycyclic aromatic hydrocarbons (PAHs)	1-Hydroxypyrene (U)	× (T5)	4 µmol mol^{-1} creatinine (T1)		(3 nM) (T5)
2-Propanol	Acetone (U) Acetone (B)	40 mg l^{-1} (T5)		50 mg l^{-1} (T1) 50 mg l^{-1} (T1)	
Pyrethroids (e.g. cyfluthrin, cypermethrin, deltamethrin, permethrin, allethrin, resmethrin, phenothrin, tetramethrin)	*trans*-Chrysanthemumdi-carbonic acid, 4-fluoro-3–phenoxybenzoic acid, *cis*- and *trans*-3-(2,2-dichlo rovinyl)-2,2-dimethylcyclo propane carbonic acid or *cis*-(2,2-dibrominyl)-2,2-dimethylcyclopropane carbonic acid			× (T1)	(1 µmol mol^{-1} creatinine) (T9)

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Table 1 (*continued*)

Exposure	Measured parameter	BEI® (ACGIH, 2008)	BMGV (UK HSE, 2007)	BAT or EKA (DFG, 2007)	BAL(RL) (FIOH, 2008)
Selenium (Se)	Se (U)				(0.30 μM) (T5)
Sevoflurane	Sevoflurane (U)				30 nM (T1)
Styrene	Sum of mandelic acid and phenylglyoxylic acid (U)	400 mg g^{-1} creatinine (T1)		600 mg g^{-1} creatinine (T1, T3)	1.2 mM (T9)
	Styrene (venous B)	0.2 mg l^{-1} (T1)			
Thallium (Th)	Th (U)				(5 nM) (T5)
Tetrachloroethylene	Tetrachloroethylene (A)	5 ppm (T9)		0.6 mg l^{-1} (T11)[r]	1.2 μM (T9, T8)
	Tetrachloroethylene (B)	0.5 mg l^{-1} (T9)			
	Trichloroacetic acid (U)	3.5 mg l^{-1} (T5)			
Tetrahydrofuran (THF)	THF (U)	2 mg l^{-1} (T1)		2 mg l^{-1} (T1)	
Toluene	*o*-Cresol (U)	0.5 mg l^{-1} (T1)		3 mg l^{-1} (T1, T3)	1000 nM (T9, T8)
	Hippuric acid (U)	1.6 g g^{-1} creatinine (T1)		1 mg l^{-1} (T1)	
	Toluene (B)	0.05 mg l^{-1} (T9)			
2,4-Toluylenediamine	2,4-Toluylenediamine (U)			37 μg g^{-1} creatinine (T1)[s]	
Trichloroethylene	Trichloroacetic acid (U)	15 mg l^{-1} (T5)		20 mg l^{-1} (T1)[t]	360 μM (T5)
	Trichloroethanol (B)	0.5 mg l^{-1} (T5)			
	Trichloroethylene (B)	× (T5)			
	Trichloroethylene (A)	× (T5)			
Triethylamine	Triethylamine (U)				0.85 mM (T5)
Trimethylbenzene (all isomers)	Dimethylbenzoic acids (all isomers) (U)			600 mg g^{-1} creatinine (T1, T3)	
Vanadium (V) and its inorganic compounds (including V_2O_5)	V (U)	50 μm g^{-1} creatinine (T5)		70 μg g^{-1} creatinine (T5)[u]	600 nM (T5)
Vinyl chloride (VCM)	Thiodiglycolic acid (U)			1.8 mg $(24\ h)^{-1}$ (T5)[v]	
Xylene, *o*-, *m*-, *p*- or mixed isomers	Methylhippuric acid (U)	1.5 g g^{-1} creatinine (T1)	650 mmol mol^{-1} creatinine (T1)	2 g l^{-1} (T1)	5 mM (T5)
	Xylene (B)			1.5 mg l^{-1} (T1)	
Zinc (Zn)	Zn (U)				(<12 μM) (T5)

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The volume-based concentrations from the Finnish Institute of Occupational Health refer to values corrected to a relative density of 1.024. A—end-tidal breath; B—blood; BAL—biomonitoring action level; BAT—Biologische Arbeitsstofftoleranzwerte (biological tolerance value); BEI—biological exposure index; BMGV—biological monitoring guidance value; E—erythrocytes; EKA—Expositionsäquivalent für krebserzeugende Arbeitsstoffe (exposure-equivalent for carcinogenic substances); Hb—haemoglobin; P—plasma; RL—reference limit for nonexposed; S—serum; U—urine; ×—the marker has been listed without a reference value. Timing of sampling: T1—end-of-shift; T2—not critical; T3—in continuous exposure, after several shifts; T4—increase during the shift; T5—end-of-shift, end of working week; T6—during exposure but at least 2 h after beginning of exposure; T7—post-task; T8—prior to (next) shift; T9—prior to last shift of working week; T10—during or end of shift; T11—16 h after end of shift; T12—prior to the shift on the first day after the weekend; T13—paired samples: baseline specimen prior to shift and end-of-shift sample at end of working week or exposure period.

[a] EKA value corresponding to 7 mg m^{-3}.
[b] EKA value corresponding to 0.01 mg m^{-3}.
[c] EKA value corresponding to 3.3 mg m^{-3}.
[d] EKA value corresponding to 4.5 mg m^{-3}.
[e] EKA value corresponding to 0.05 mg m^{-3} CrO_3 in air, not valid for welding fumes.
[f] EKA value corresponding to 0.025 mg m^{-3}.
[g] EKA value corresponding to 200 mg m^{-3} cyclohexane in air.
[h] EKA value corresponding to 61 mg m^{-3} p-dichlorobenzene.
[i] EKA value corresponding to 175 mg m^{-3} dichloromethane.
[j] EKA value corresponding to 0.20 mg m^{-3} dimethylsulfate.
[k] EKA value corresponding to 440 mg m^{-3} ethylbenzene.
[l] EKA value corresponding to 117 mg m^{-3} ethylene.
[m] EKA value corresponding to 1.83 mg m^{-3} ethylene oxide.
[n] EKA value corresponding to 0.013 mg m^{-3} hydrazine.
[o] EKA value corresponding to 0.1 mg m^{-3} Ni (as metal, as nickel oxide, nickel sulfide or sulfidic ores).
[p] EKA value corresponding to 0.1 mg m^{-3} Ni (as easily soluble nickel salts like nickel acetate and comparable soluble salt, nickel chloride, nickel hydroxide, nickel sulfate).
[q] EKA value corresponding to 0.10 mg m^{-3} PCP.
[r] EKA value corresponding to 206 mg m^{-3} tetrachloroethylene.
[s] EKA value corresponding to 0.035 mg m^{-3} toluylenediamine.
[t] EKA value corresponding to 55 mg m^{-3} trichloroethylene.
[u] EKA value corresponding to 0.05 mg m^{-3} V.
[v] EKA value corresponding to 2.6 mg m^{-3} VCM.
[w] Women of child-bearing potential: 100 µg l^{-1}.
[x] Women <45 years: 100 µg l^{-1}.
[y] For foetus and pregnant women: 0.015 µM.
[z] T1 for inhalation and T8 for dermal exposure.
[aa] T1 for inhalation and T9 for dermal exposure.
[bb] For chromium platers.
[cc] For manual metal arc welders.
[dd] For shield gas welders.
[ee] Nonsmokers.
[ff] Smokers.
[gg] Fasting serum.

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Table 2 Reference values

Exposure	Measured parameter	Reference value	Reference
Aluminium (Al) and its inorganic compounds	Al (U) Al (S)	0.6 μM 0.1 μM	FIOH (2008)
Aniline	Aniline (U)	0.05 μM	FIOH (2008)
Antimony (Sb)	Sb (U)	9 nM	FIOH (2008)
Arsenic (As) and its inorganic compounds	As (U)	15 μg l^{-1}	Wilhelm *et al*. (2004)
	As (U)	30 nM	FIOH (2008)
Benzene	*t,t*-Muconic acid	2 μM	FIOH (2008)
Beryllium (Be)	Be (U)	15 nM	FIOH (2008)
2-Butoxyethanol and its acetate	Butoxyacetic acid (U)	0.5 mmol mol^{-1} creatinine	FIOH (2008)
2-(2-Butoxyethoxy) ethanol and its acetate	2-(2-Butoxyethoxy) acetic acid (U)	0.2 mmol mol^{-1} creatinine	FIOH (2008)
Cadmium (Cd)	Cd (U)	0.8 μg l^{-1}	Wilhelm *et al*. (2004)
		5 nM (nonsmokers)	FIOH (2008)
		10 nM (smokers)	FIOH (2008)
	Cd (B)	1.0 μg l^{-1}	Wilhelm *et al*. (2004)
		5 nM (non-smokers)	FIOH (2008)
		18 nM (smokers)	FIOH (2008)
	Retinol-binding protein (U)	300 μg g^{-1} creatinine	FIOH (2008)
Carbon disulfide	2-Thiothiazolidine-4-carboxylic acid (U)	0.3 mmol mol^{-1} creatinine	FIOH (2008)
Carbon monoxide	Carboxyhaemoglobin (B)	1.5%	FIOH (2008)
Chromium (Cr)	Cr (U)	0.01 μM	FIOH (2008)
Cobalt (Co) and its inorganic compounds	Co (U)	40 nM	FIOH (2008)
Copper (Cu)	Cu (U)	0.08–0.50 μM (spot)	FIOH (2008)
		0.24–0.47 μmol $(24\ h)^{-1}$	
Cyanides and hydrocyanic acid	Thiocyanate (U)	85 μM (nonsmokers)	FIOH (2008)
		250 μM (smokers)	
	Thiocyanate (S)	100 μM (nonsmokers)	
		250 μM (smokers)	
Dichloromethane	Carboxyhaemoglobin (B)	1.5%	FIOH (2008)
N,N-Dimethylacetamide	*N*-Methylacetamide (U)	0.05 mmol mol^{-1} creatinine	FIOH (2008)
N,N-Dimethylformamide	*N*-Methylformamide (U)	20 μM	FIOH (2008)
2-Ethoxyethanol and its acetate	2-Ethoxyacetic acid (U)	0.5 mmol mol^{-1} creatinine	FIOH (2008)
2-(2-Ethoxyethoxy) -ethanol and its acetate	2-(2-Ethoxyethoxy) acetic acid (U)	0.1 mmol mol^{-1} creatinine	FIOH (2008)
Ethylbenzene	Mandelic acid (U)	0.2 mM	FIOH (2008)
Fluorides, hydrofluoric acid	Fluoride (U)	100 μM	FIOH (2008)
Formic acid and formats	Formic acid (U)	70 mmol mol^{-1} creatinine	FIOH (2008)

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Table 2 (*continued*)

Exposure	Measured parameter	Reference value	Reference
α-Hexachlorocyclo-hexane	α-Hexachlorocyclo-hexane (B)	0.1 $\mu g\,l^{-1}$	Ewers *et al*. (1999)
γ-Hexachlorocyclo-hexane	γ-Hexachlorocyclo-hexane (B)	0.3 $\mu g\,l^{-1}$	Ewers *et al*. (1999)
n-Hexane	2,5-Hexanedione (U)	0.5 $mmol\,mol^{-1}$ creatinine	FIOH (2008)
Isocyanates (MDI, TDI)	Isocyanate metabolites	0.1 $\mu mol\,mol^{-1}$ creatinine	FIOH (2008)
Lead (Pb) and its inorganic compounds	Pb (B)	70 $\mu g\,l^{-1}$ (females) 90 $\mu g\,l^{-1}$ (males) 0.3 μM	Wilhelm *et al*. (2004) FIOH (2008)
Lead (Pb), organic	Pb (U)	0.015 μM	FIOH (2008)
Manganese (Mn)	Mn (U)	40 nM	FIOH (2008)
Mercury (Hg)	Hg (U) Hg (B)	1.0 $\mu g\,l^{-1}$ 50 nM 2.0 $\mu g\,l^{-1}$ 25 nM	Wilhelm *et al*. (2004) FIOH (2008) Wilhelm *et al*. (2004) FIOH (2008)
2-(2-Methoxyethoxy)–ethanol and its acetate	2-(2-Methoxyethoxy)–acetic acid (U)	0.5 $mmol\,mol^{-1}$ creatinine	FIOH (2008)
1-Methoxy-2-propanol and its acetate	1-Methoxy-2-propanol (U)	5 μM	FIOH (2008)
Methylenebis-(2-chloro-aniline)	Methylenebis-(2-chloro-aniline) (U)	0.5 $\mu mol\,mol^{-1}$ creatinine	FIOH (2008)
Methylenedianiline (MDA)	MDA (U)	0.5 $\mu mol\,mol^{-1}$ creatinine	FIOH (2008)
Methylene iodide	Carboxyhaemoglobin (B)	1.5%	FIOH (2008)
Methyl ethyl ketone (2-butanone)	Methyl ethyl ketone (U)	1.5 μM	FIOH (2008)
Methyl isobutyl ketone	Methyl isobutyl ketone (U)	0.1 $mmol\,mol^{-1}$ creatinine	FIOH (2008)
Methyl-*t*-butyl ether	*t*-Butyl alcohol (U)	1 μM	FIOH (2008)
Molybdenum (Mb)	Mb (U)	1340 nM	FIOH (2008)
Naphthalene	Naphthol (U)	30 nM (nonsmokers) 300 nM (smokers)	FIOH (2008)
Nickel (Ni) and its inorganic compounds	Ni (U)	(3.0 $\mu g\,l^{-1}$) 0.06 μM	Wilhelm *et al*. (2004) FIOH (2008)
Nitrous oxide	Nitrous oxide (U)	20 nM	FIOH (2008)
Pentachlorophenol (PCP)	PCP (S) PCP (U)	12 $\mu g\,l^{-1}$ 8 $\mu g\,l^{-1}$ or 6 $\mu g\,g^{-1}$ creatinine	Ewers *et al*. (1999)
Perfluorooctane sulfonate	Perfluorooctane sulfonate (U)	10 $\mu g\,l^{-1}$ (children) 15 $\mu g\,l^{-1}$ (adult females) 25 $\mu g\,l^{-1}$ (adult males)	Wilhelm *et al*. (2009)
Perfluorooctanoic acid	Perfluorooctanoic acid (U)	10 $\mu g\,l^{-1}$	Wilhelm *et al*. (2009)

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Table 2 (*continued*)

Exposure	Measured parameter	Reference value	Reference
Phenol	Phenol (U)	0.15 mM	FIOH (2008)
Platinum (Pt)	Pt (U)	0.01 $\mu g\,l^{-1}$	Wilhelm *et al*. (2004)
Polychlorinated biphenyls (PCBs)	PCBs (S)	3 $\mu g\,l^{-1}$ (sum of 24 PCBs)	FIOH (2008)
Polycyclic aromatic hydrocarbons	1-Hydroxypyrene (U)	0.5 $\mu mol\,mol^{-1}$ creatinine	Boogaard *et al*. (2007)
		0.5 $\mu g\,l^{-1}$	Wilhelm *et al*. (2008)
		0.3 $\mu g\,g^{-1}$ creatinine	Wilhelm *et al*. (2008)
		3 nM	FIOH (2008)
	1-Naphthol (U)	$<$30 $\mu g\,l^{-1}$	Wilhelm *et al*. (2008)
	2-Naphthol (U)	$<$20 $\mu g\,l^{-1}$	Wilhelm *et al*. (2008)
	Naphthols (U)	30 nM (nonsmokers)	FIOH (2008)
		300 nM (smokers)	
Pyrethroid insecticides	Phenoxybenzoic acid (U)	1 $\mu mol\,mol^{-1}$ creatinine	FIOH (2008)
Selenium (Se)	Se (U)	0.30 µM	FIOH (2008)
Sevoflurane	Sevoflurane (U)	1.5 nM	FIOH (2008)
Styrene	Mandelic and phenylglyoxylic acids (U)	0.2 mM	FIOH (2008)
Tetrachloroethylene (PER)	PER (B)	0.1 µM	FIOH (2008)
Thallium (Th) and its inorganic compounds	Th (U)	5 nM	FIOH (2008)
Toluene	Toluene (B)	50 nM	FIOH (2008)
Trichloroethene	Trichloroethylene (U)	50 µM	FIOH (2008)
Triethylamine	Triethylamine (U)	0.02 mM	FIOH (2008)
Vanadium (V) and its compounds	V (U)	50 nM	FIOH (2008)
Xylenes	Methylhippuric acid	0.2 Mm	FIOH (2008)
Zinc (Zn) and its inorganic compounds	Zn (U)	8–12 µM	FIOH (2008)

pesticides, dioxins and PCBs (Zietz *et al*., 2008; WHO, 2007). In principle, any bodily fluid or tissue can be used for biomonitoring. However, most tissues are not suitable for routine monitoring or for risk assessments. For routine monitoring it is essential that the body fluids or tissues are easily accessible, which rules out most tissues and fluids apart from those mentioned above. For risk assessment it is of paramount importance that the data can be compared to a reference value (see below) or at least to a dose–response curve. This is usually the most crucial factor, limiting biomonitoring to primarily urine, blood and, for some substances, hair. For some volatile substances exhaled air may be used as specimen for exposure monitoring, however the methodology, especially the sampling, is rather complex (Corradi and Mutti, 2005; Wilson and Monster, 1999). Specific advantages and disadvantages of various specimens used for biomonitoring are listed below.

3.1 Urine

Urine is in most cases the specimen of choice due to its easy collection, the large quantities and the fact that it is a noninvasive method. Many substances are excreted into the urine either as the substance itself or as water-soluble metabolites. When exposures are

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intermittent, urinary concentrations usually reflect the average exposure better than blood or exhaled air concentrations because the excretion into the urine acts as an integrator. For substances with short systemic half-lives of a few hours or less, urinary concentrations also may reflect exposures better as apparent urinary half-lives are usually considerably longer than those in blood or breath. Ideally, 24-hour urine samples are collected and analysed, but in practice spot samples are more generally used. Timing of the sample collection then becomes an important factor (see **Table 1**). Differences in hydration amongst individuals due to variations in fluid intake and perspiration may lead to significant variations in the density of the urine produced and consequently the concentration of the analyte under investigation in a spot sample. Correction of the results for the density of the urine may be essential for some substances and this can be done either by determination of the urinary density or by measuring the urinary creatinine concentration. However, whether or not correction for differences in urinary density is warranted needs to be assessed for each substance (Barr *et al*., 2005b; Alessio *et al*., 1985; Dell'Orto *et al*., 1987; Boeniger *et al*., 1993).

3.2 Blood

Blood constitutes the most important means of transport in the human body for most substances, and virtually all systemically active substances can be measured in blood. In addition, blood usually closely reflects the concentration of a substance at its place of action since blood is either itself in contact with the receptor or in equilibrium with other bodily fluids (e.g. cerebrospinal fluid), which are in contact with the receptors. As a consequence, interpretation of a biomarker measured in blood is generally more straightforward than the interpretation of a biomarker in other specimens, provided the half-life of the substance is sufficiently long. The major drawbacks of blood as a specimen are the invasive nature of sampling, and, consequently, the need for specialized personnel to collect samples, and the potential biohazards associated with blood (hepatitis, HIV), which may hamper transportation due to legal limitations. Additional practical considerations include the choice of an appropriate anticoagulant if needed, and whether the analysis is best performed on whole blood, serum, plasma or specific blood cell types (e.g. red blood cells for Hb adducts and white blood cells for DNA adducts).

3.3 Breath

For volatile substances breath may be an excellent specimen. Probably the best-known use of breath as specimen for biomonitoring is traffic alcohol controls. Alveolar air collection represents a noninvasive method, but the actual collection may be cumbersome for most substances (Wilson and Monster, 1999). Even more so than with blood or urine analysis, the timing of a breath sample collection is crucial, as most volatile substances have a relatively short half-life in alveolar air (see **Table 1**). In addition, a differentiation between 'mixed exhaled air' and 'end exhaled air' should be made in the interpretation of the results since the concentration in the latter tends to be lower during, but higher following, the exposure period.

3.4 Milk and Adipose Tissue

In environmental biomonitoring milk and adipose tissue may be suitable specimens for highly lipid-soluble substances, such as chlorinated pesticides, brominated flame retardants, and polyfluorinated compounds. Adipose samples can only be collected by (highly) invasive methodology and are therefore of limited practical use. Lipophilic compounds tend to have very long half-lives in adipose tissue and reach a steady-state level upon prolonged exposure in fat. Milk samples can be collected from lactating women and represent not only the body burden of the mother but also the exposure of the baby (Berlin *et al*., 2005; LaKind *et al*., 2005). Biomonitoring surveys using human breast milk are primarily used to assess the efficacy of policy measures to reduce exposures by measuring the trends over time in the biomarker concentration following the introduction of exposure reduction strategies (Zietz *et al*., 2008; Wang *et al*., 2005; Cerna *et al*., 2007; 2000).

3.5 Nails and Hair

Nails and hair are traditionally less commonly used for biomonitoring but provide specific opportunities for biomonitoring where other specimens are difficult to obtain, for instance in the biomonitoring of infants and small children where collection of blood or urine samples is cumbersome. More recently, methods are being developed and validated to use specimens like nails and in particular hair for biomonitoring (Bader *et al*., 1999; Legrand *et al*., 2005; Rodrigues *et al*., 2008; Schramm, 1997; 2008; Wilhelm *et al*., 2007; Bergdahl and Skerfving, 2008). Most substances have long half-lives in hair and nails. In principle, the history of exposure could be read over the length of hair.

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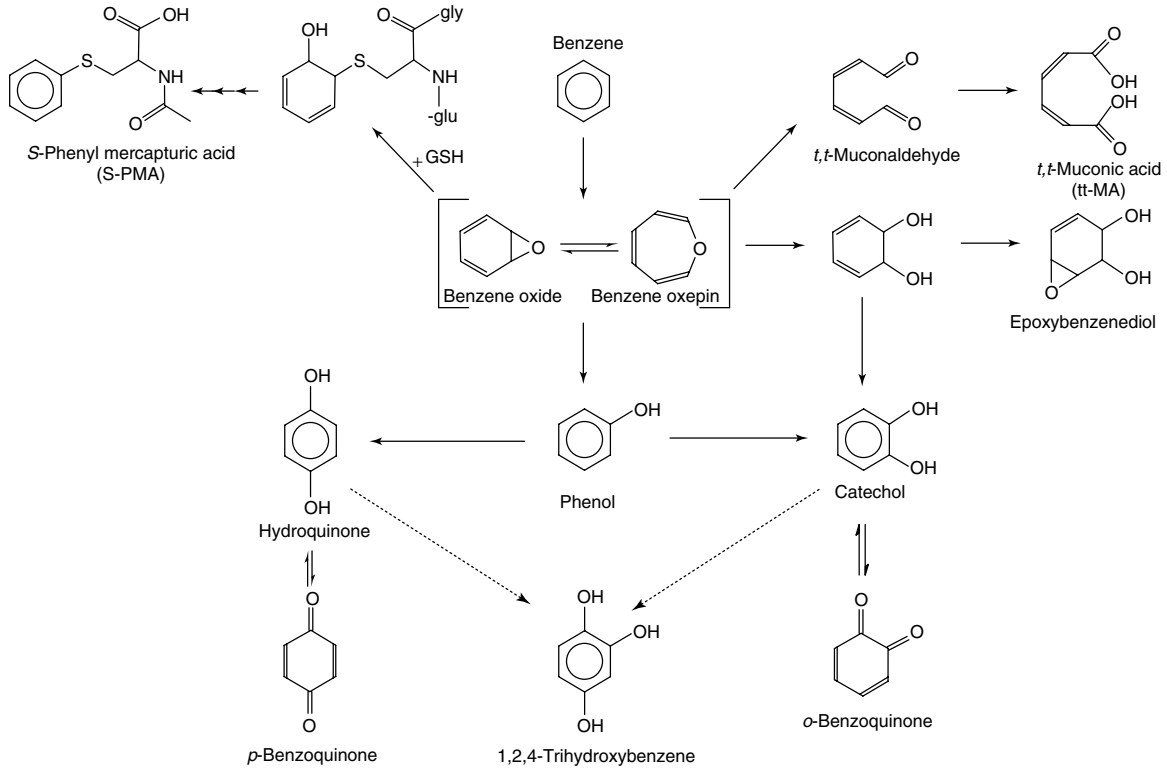

Figure 2 Simplified benzene metabolism scheme with potential urinary biomarkers for benzene exposure (GSH—glutathione).

4 CHOICE OF BIOMARKER

The choice of a biomarker for a certain substance is crucial and depends largely on the aims of the investigations and the required sensitivity and specificity. The considerations for selecting a biomarker can best be illustrated by some examples. A good example is benzene, which has been of concern for a long time and of which both metabolism and kinetics are reasonably well known. In **Figure 2** an overview of the main pathways in the metabolism of benzene is given. Its major metabolite is phenol, which is mainly excreted into the urine as sulfate or glucuronide. As mentioned in the introduction, benzene was amongst the first substances that were being biomonitored in the first half of the twentieth century, and urinary phenol was the biomarker used for this purpose. In individuals without exposure to benzene, however, background levels of urinary phenol from dietary sources may be found which correspond to airborne levels of 5–10 ppm of benzene. This background was no problem for using urinary phenol as compliance check in industrial settings when the occupational exposure limits (OELs) were in the order of 100 ppm. However, when, over time, the OELs were lowered to values below 10 ppm, phenol became useless as biomarker and other urinary metabolites, such as tt-MA and S-PMA, were investigated as biomarkers (van Sittert *et al*., 1993; Ducos *et al*., 1992). Both tt-MA and S-PMA have specific advantages and drawbacks (Boogaard and van Sittert, 1995a). As can be seen in **Figure 2**, tt-MA is part of the metabolic pathway through muconaldehyde, and more likely linked to some of the toxic effects of benzene. Theoretically tt-MA might therefore be expected to be more closely related to potential health effects of benzene. However, like phenol, tt-MA also has a background in nonexposed humans, possibly as a metabolite of sorbitol, a common food additive (Marrubini *et al*., 2002; Renner *et al*., 1999). This limits the use of tt-MA to detect exposures of up to approximately 0.5–1 ppm of benzene at the individual level. So far, no background for S-PMA has been found in nonexposed humans, which makes S-PMA suitable to assess benzene exposures also at the individual level (Boogaard and van Sittert, 1996; Aston *et al*., 2002; Pople *et al*., 2002). In addition, S-PMA has a two-times longer half-life in urine than tt-MA, which makes it a more reliable biomarker for intermittent exposures (Boogaard and van Sittert, 1995a). Several other urinary metabolites of benzene, such as 1,2,4-trihydroxybenzene, hydroquinone and catechol have been investigated as biomarkers for benzene exposure, but proved less sensitive and specific than tt-MA or S-PMA (Qu *et al*., 2000). Biochemical effect monitoring has also been developed for assessing exposures to benzene. Alb as well as Hb adducts of benzene oxide and benzoquinones have been investigated for this purpose (Waidyanatha *et al*., 1998; Yeowell-O'Connell

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et al., 1998; Lindstrom *et al*., 1998). Unlike the biological monitoring methodology using S-PMA or tt-MA, which can be applied routinely, the biochemical effect monitoring of benzene using Alb or Hb is too sophisticated at the present time for routine applications.

For many electrophilic substances, however, biochemical effect monitoring is the method of choice. This applies in particular to genotoxic substances. Although for many genotoxic substances biological monitoring methods which apply urinary metabolites are available, the use of biochemical effect monitoring has several advantages (van Welie *et al*., 1992). In particular, the use of Hb adducts to assess exposure to epoxides (e.g. ethylene oxide) or epoxide-forming substances (e.g. 1,3-butadiene) and aromatic amines (e.g. aniline) has proven very sensitive and useful (Boogaard, 2002; Sepai and Sabbioni, 1996; Sabbioni and Beyerbach, 2000; Sabbioni and Jones, 2002; van Sittert *et al*., 2000a; Törnqvist *et al*., 2002; Richter and Branner, 2002). Biochemical effect monitoring is especially useful for the biomonitoring of genotoxic substances where adducts to the Hb molecule can be used as a surrogate for the DNA adducts in the target tissue. The benefits of Hb adduct assessment as an internal dose monitor for industrial exposures were first suggested in 1976 by Ehrenberg and co-workers (Ehrenberg *et al*., 1977; Osterman-Golkar *et al*., 1976; Segerback *et al*., 1978). In industrial settings genotoxic substances are generally well contained. As a consequence the exposures are usually at low and variable levels and occur intermittently, which makes personal air monitoring very difficult. Biochemical effect monitoring may overcome these problems because it allows integrating the exposure over longer time periods. This is especially the case with Hb adducts of smaller molecules such as ethylene or propylene oxide, which have apparent half-lives of approximately four months. Over the last few decades, the methodology has improved and is now routinely applied to a large number of substances (Törnqvist *et al*., 2002; Ball *et al*., 2004; 2005). Due to the specificity and high sensitivity of Hb adducts, they can also be applied for biomonitoring of low environmental exposures. Mathematical models have been developed which allow quantitative assessments of variable external exposures based on biomarker data (Granath *et al*., 1992; Fennell *et al*., 1992).

Due to its nonspecific nature, biological effect monitoring is in general more useful for research purposes than for health risk assessments, since it is usually difficult or even impossible to relate the observed effects with certainty to a specific exposure. Biological effect monitoring can, however, be extremely useful in situations with potential exposures to a variety of substances with the same effect, as for instance may occur in the area of crop protection. Both during manufacturing and application of organophosphates and carbamates workers may potentially be exposed to a variety of products over a period of days or weeks. The mechanism of action of both organophosphates and carbamates is the inhibition of insect cholinesterases. However, human cholinesterases are also inhibited to a significant but variable extent by these products, and determination of serum cholinesterase is routinely applied to effectively monitor exposure to pesticides for decades (Aitio, 1999; Anwar, 1997; Bellino *et al*., 1978; Cocker *et al*., 2002).

Toxicogenomics, proteomics and metabolomics have opened a whole new area of biomonitoring technologies which has yet to be fully explored. Metabolomics (also called metabonomics) is basically metabolic profiling and a form of biological effect monitoring where a large range of (endogeneous) compounds is determined in a biological specimen to define the response to some external challenge. Although metabolomics has proven useful as a tool in drug development and in basic research, its application in biomonitoring is as yet very limited due to its highly unspecific nature (Griffin, 2003; Griffin and Bollard, 2004; Robertson, 2005). Toxicogenomics and toxicoproteomics, the determination of gene and protein expression in response to exposure to a toxic substance, are biological effect monitoring. Although both promising tools, they cannot be used at the present time for exposure assessment, let alone risk assessment. This is mainly due to the fact that both gene and protein expression are highly dynamic, and to the fact that one often cannot differentiate between adaptive and adverse effects with the current knowledge. Extensive studies with known toxicants using multiple doses and many time points will be required to map out the response of the genome and proteome to a toxic challenge. However, the area is highly promising since it may detect effects at concentrations lower than the lowest-observed-effect levels for more traditional end points (Andersen *et al*., 2008; Daston, 2008; Navas-Acien and Guallar, 2008; Ghosh *et al*., 2008). This may make it feasible to investigate dose–response relationships, including the shape of the curve, at the low levels of exposure which may occur in environmental settings. In addition, toxicogenomics may provide a better understanding of the effects of genetic variability on toxicity.

5 INTERPRETATION OF BIOMONITORING DATA

In general, biomonitoring data are used to assess actual exposures with the ultimate goal of making an assessment of the health risks associated with that exposure. Biomonitoring integrates all routes of exposure and can therefore be used to follow up the fate of occupational or environmental substances in the human body and may provide insight into the relative contributions of different environmental compartments (e.g. air, food, water) and emission sources to body burden, provided the external

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sources can be linked to internal exposure. In addition, biomonitoring allows the assessment of spatial and temporal differences in exposure and may therefore be used to assess the effectiveness of exposure-reduction strategies and policy decisions. Biomonitoring, especially biological effect monitoring, may provide early warning signals and be used in surveillance programmes. However, it should be born in mind that for proper interpretation of biomonitoring data, a number of factors are of paramount importance. These factors include (i) the quality of the analysis, (ii) basic understanding of the toxicokinetics of the compound under study, and (iii) basic knowledge of the dose–response curve. In addition, a weight-of-evidence approach is recommended, taking into account circumstantial evidence and additional knowledge about the compound. Only if sufficient information on these parameters is available, may quantitative reference or guidance values be derived.

5.1 Analytical Integrity

Analytical integrity of laboratory results, regardless of whether they are air measurements, food-basket analyses or biomonitoring, is crucial since they form the basis of any conclusion or action following from the results. It is beyond the scope of this chapter to address analytical integrity in detail, but like all laboratory assessments, biomonitoring analyses are potentially subject to various sources of errors. The first source relates to the preanalytical phase; that is, the phase of sample collection, sample treatment if required, sample storage and transport to the laboratory. Contamination of samples is a common problem and may be introduced at various points in the sampling procedure: from airborne dust, soiled surfaces and clothing, contaminated skin, sample vials, and so on. This is for instance illustrated by the biomonitoring of bisphenol A where contamination, resulting from leaching from plastic containers, may represent a serious problem, hampering interpretation of the biomonitoring results (Dekant and Volkel, 2008; Volkel *et al*., 2008). The contamination with metals and volatile organic solvents from collection vials is well known (Moyer *et al*., 1991; Streete and Flanagan, 1993). Usually, contamination is only a serious potential problem if the mother compound is measured, and less of a problem when a specific metabolite or adduct is measured. However, this is not always the case. A good example is the blood sampling for the determination of Hb adducts of ethylene oxide, where blood samples have to be collected in γ-radiation-sterilized tubes. Blood collected in tubes sterilized with ethylene oxide displays huge background values of Hb adducts, as traces of ethylene oxide absorbed to the glass may react after collection with the Hb in the erythrocytes (Boogaard, 2002).

For many industrial chemicals and occupational settings, optimal conditions for the preanalytical phase, such as selection of suitable specimen containers and the storage conditions under which the analyte is stable in the specimen, have been established. This information should be available as standard operating procedures at the sample collection site as well as in the analysing laboratory. For several biomarkers, standard operating procedures have been published that address the preanalytical phase (e.g. the methods series by DFG mentioned above and by the WHO (DFG, 2006; WHO, 1996b)). Other sources of error relate to the analytical phase itself. These are related to equipment, materials and training of personnel, and can be minimized by using standard operating procedures. General procedures are available for a wide variety of substances (see **Tables 1** and **2**), which can serve as the basis for standard operating procedures. As for all analytical chemistry, quality control is of utmost importance also for the analytical phase in biomonitoring, especially external quality control, which is available for a variety of substances (Schaller *et al*., 2001; 2002).

Whereas biomonitoring in occupational settings is well established, environmental biomonitoring is still developing. However, most of the lessons learned from occupational biomonitoring are also valid in the environmental field. The most important challenge is the validation of the methodologies with ever increasingly lower detection limits and concomitant greater risk of contamination.

5.2 Toxicokinetics

The main goal of biomonitoring is usually to assess exposure. In biomonitoring, individuals serve as their own dose monitors integrating all routes of exposure (oral, dermal, inhalatory), and reflecting physiological variation (including variations in workload). However, the data represent a snapshot of the presence of the substance under study in the body and, unless something is known about the toxicokinetics of this substance, the data cannot be explained in terms of exposure, let alone risk characterization. The toxicokinetics of a substance comprise its absorption, distribution, metabolism and excretion. As stated above, biomonitoring has a unique feature over other exposure monitoring techniques in that it integrates all routes of exposure, and biomonitoring accounts also for interindividual variations in toxicokinetics. In occupational settings the typical exposure routes, usually inhalation and skin contact, are often known. In environmental settings, however, the exposure routes are mostly more variable and may include, in addition to breathing and dermal contamination, also the intake via foodstuffs and drinks. In addition, dermal contact may be more extensive

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than in occupational settings (e.g. bathing and swimming). For most substances, details on absorption and distribution for the various exposure routes are not readily available. For substances with a systemic effect, this is not necessarily problematic if the aim is to characterize the overall exposure or risk. However, the impossibility of disentangling routes of exposure may hamper the evaluation of exposure sources and exposure controls.

Many substances are not biomonitored as such, but as a metabolite or reaction product with some endogenous macromolecule (e.g. protein or DNA). Using the metabolite or reaction products may increase the sensitivity and specificity. For example, 1,3-butadiene, an industrial chemical used for the production of rubbers but also a constituent of cigarette smoke, may be metabolized to several different epoxides. These epoxide metabolites form different mercapturic acids which are excreted into the urine, and also different adducts with Hb and DNA (Boogaard *et al*., 2004; Richardson *et al*., 1998; 1999; 1996; Powley *et al*., 2005). Both the urinary metabolites and the adducts can be used as biomarkers of exposure in risk assessment and were proven more reliable than personal air monitoring (Albertini *et al*., 2003; 2001; 2007; van Sittert *et al*., 2000b; Begemann *et al*., 2001; Hayes *et al*., 2001; Swenberg *et al*., 2001). On the contrary, metabolism may also pose problems if the biomarker can also be formed endogenously or from dietary components, and also when different substances can be metabolized to the same adduct or metabolite. As discussed previously, background levels due to dietary intake prevent the use of phenol and tt-MA as reliable biomarkers for benzene exposure at low exposure levels. For low-level exposures, such as might occur in environmental settings, the parent compound in urine, air or blood might therefore be a more reliable biomarker (Manini *et al*., 2008; Crebelli *et al*., 2001; Fustinoni *et al*., 1995). An other example is *N*-2-hydroxyethylvaline, one of the most commonly measured Hb adducts, and a biomarker for both ethylene oxide and ethylene (Csanády *et al*., 2000; Pauwels and Veulemans, 1998; Walker *et al*., 2000). In industrial settings, this is usually not a problem, since normally it would be known whether exposure to ethylene or to ethylene oxide could occur. In addition, the amount of *N*-2-hydroxyethylvaline formed from ethylene oxide is several orders of magnitude higher than from the same concentration of ethylene. However, in environmental settings the interpretation of *N*-2-hydroxyethylvaline levels may be less straightforward as to its source, because the adduct may be formed from ethylene oxide, for instance from cigarette smoke, or from ethylene, which is a plant hormone and present in fruits, and may also be formed by bacteria in the gut (Törnqvist *et al*., 1989; 1986). An example of interpretation problems that may arise where different compounds are metabolized to the same metabolite is that both n-hexane and 2-hexanone are excreted as 2,5-hexanedione in the urine (Prieto *et al*., 2003; Cardona *et al*., 1993). If confusion could exist about the source of the biomarker, again determination of the parent compound in exhaled air might be the preferable approach (Hamelin *et al*., 2004; Periago *et al*., 1993).

Another important toxicokinetic issue related to metabolism and excretion is the apparent half-life of a biomarker. In general terms, parent compounds in blood, urine or exhaled air have very short half-lives (minutes to hours), urinary metabolites have longer but still relatively short half-lives (hours to days), whereas DNA adducts and Alb adducts have long half-lives (days to weeks) and Hb adducts have very long half-lives (weeks to months) (Henderson *et al*., 1989). There are notable exceptions to these general rules especially after prolonged (environmental) exposures. In particular, heavy-metal biomarkers, such as lead in blood or urinary cadmium, may have very long apparent half-lives as they are stored in bone and renal tissue, respectively, from which they are only very slowly released (Philip and Gerson, 1994; Jin *et al*., 1998; Lauwerys *et al*., 1993; 1994; Sartor *et al*., 1992; Staessen *et al*., 1996). Also many highly lipophilic compounds, such as organochlorine insecticides, may have long biological half-lives since they are stored in adipose tissue and only slowly released in an equilibrium between the blood and the fatty tissues (Hunter and Robinson, 1967; Hunter *et al*., 1969). Substances may also be deposited in hair and nails and be present for measurement for long periods of time. For DNA adducts, the stability is highly dependent on the nature and the chemical structure of the adduct (Farmer and Singh, 2008). For instance *N*7-guanine adducts tend to be labile and have half-lives of only a few days due to depurination, whereas O^6-adenine adducts are more stable. Hb adducts theoretically have half-lives of about 126 days, the average lifetime of the erythrocyte before it is taken up in the spleen, and following accidental exposure of a number of individuals to high levels of ethylene oxide, indeed half-lives ranging from 120 to 130 days were found (Tates *et al*., 1995). The half-lives of Hb adducts, however, may be considerably shorter if bulky adducts or adducts with highly polar groups are formed, which is likely due to altered configuration of the Hb chains and subsequent malformation of the erythrocytes which are then more rapidly removed from the circulation (Boogaard *et al*., 1994a; 1994b).

5.3 Dose Response

Biomonitoring data indicate the mere presence of a chemical or its effect in the body. To make any attempt to characterize the health risk associated with this presence, some form of dose–response relationship is required. Usually, the first evaluations of an adverse

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health effect are based on animal data obtained from toxicological studies or from human experience resulting from accidental exposures or epidemiological evidence. An inherent problem of toxicological studies is that to observe adverse effects in experimental animals, usually very high doses are needed compared to the exposures that may occur in humans. Unfortunately, dose–response relationships at low-dose ranges relevant for the human population are generally lacking. Since at high exposure rates deviations from normal metabolism, for instance due to saturation of certain pathways, may occur, it is of paramount importance for an appropriate risk assessment that the underlying toxicological mechanisms or mode-of-action are known. Epidemiological data play a crucial role here but are often underestimated, especially when the results are negative (ECETOC, 2004).

The present analytical capabilities make it feasible to collect vast amounts of biomonitoring data in human populations (Koppen *et al*., 2007; Staessen *et al*., 2001; Denham *et al*., 2005; Schell *et al*., 2008). Although such studies may be very powerful in delineating health risks associated with (environmental) exposures, they suffer from an inherent risk of statistical chance findings. As a consequence, it is imperative that biomarker studies are hypothesis driven. The hypothesis of a causal relationship between the biomarkers and observed health effects should be based on epidemiology, animal data, or an understanding of the mode of action of a substance, and be well defined in advance, prior to the initiation of the study. Moreover, appropriate, predefined statistical methods need to be applied to the data gathered to evaluate the significance of the findings (IPCS, 2001; Boogaard and Money, 2008).

An additional problem in the interpretation of exposure data in terms of health risks, regardless of whether they are externally measured through personal air monitoring or food-basket analyses or through biomonitoring, is the interpersonal and intrapersonal variability (Symanski *et al*., 2006). This variability may hamper health risk assessment on an individual level (Cocker *et al*., 2006), but on a group level, for instance for epidemiological studies, biomarker data may provide more reliable information on exposure than air monitoring data (Lin *et al*., 2005).

5.4 Weight of Evidence

As stated above, the evaluation of causal relationships between exposures and a specific effect is highly complex. In most cases it requires the integration of data from several studies differing in terms of experimental design and parameters studied. Moreover, since often large numbers of parameters are assessed, there is a substantial risk of finding false positive associations. It is recommended to apply the criteria originally developed by Hill, and further refined for biomarker studies by Vineis and Porta, to establish whether or not an association between exposure and effect is likely to be causal (Hill, 1965; Vineis and Porta, 1996). This approach links in with the weight-of-evidence approach, which basically checks the biological plausibility and consistency for a specific hypothesis or dataset. The plausibility check may include an evaluation of the mode of action to see whether the presumed relationship can be biologically explained (Boobis *et al*., 2006; Williams, 2008). The consistency check aims to resolve contrasting findings in the literature which may be due to differences in study design, but may also be an indication that other factors than those addressed in the study have an influence on the results. If the plausibility or consistency is weak for a certain hypothesis, regardless of whether it is in favour or against, further study is required and alternative hypotheses should be evaluated.

5.5 Quantitative Interpretation

Probably the most fundamental principle of risk assessment is that adverse health effects are related to dose and, consequently, to levels and duration of exposure. In the domain of occupational biomonitoring, BLVs have been established for a number of substances. The criteria used to set BLVs may differ from one country or agency to another as well as from one substance to another (Morgan and Schaller, 1999). In many cases, these limit values correspond to an occupational limit value for airborne substances because they are based on a correlation between biomonitoring values and personal air monitoring data. These personal air monitoring data are, in turn, related to an understanding or estimation of the level of exposure associated with adverse health effects. These adverse health effects may be based either on actual data obtained from human exposure or, more and more frequently, on extrapolation from data obtained in animal experiments.

Among the most widely used BLVs are the biological tolerance values (Biologische Arbeitsstoff Toleranz-Werte, BAT values) as introduced by the Deutsche Forschungsgemeinschaft (German Research Foundation, DFG) (DFG, 2007) in 1979, and the biological exposure indices (BEI®) as published annually by the American Conference of Governmental Industrial Hygienists (ACGIH, 2008) since 1981. BAT values are by definition human-health based, and originally defined as 'the maximum permissible quantity of chemical substance, its metabolite, or any deviation from the norm of biological parameters induced by these substances in human beings' (Lehnert, 1980). The values published by the DFG since 1981 are therefore in fact ceiling values. In general, according to the scientific knowledge at present,

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exposures leading to the BAT value do not impair the health of workers, even in the case of prolonged or repeated exposures. BAT values are intended for workers, which implicitly assumes an exposure of maximally 8 hours per day or 40 hours per week for a maximum of 40 years (working life). BAT values may, however, be applied without a correction factor to other working patterns or working hours (DFG, 2007). The fact that some biological changes may be observed at exposures leading to the BAT value implies that not every variation in a biological parameter is viewed as an illness or adverse health effect. Recently, the DFG has therefore revised the concept of BAT values to describe 'the concentration of a chemical substance, of its metabolites or an effect indicator of an appropriate biological material derived by occupational medical and toxicological criteria, at which the health of an employee is usually not affected, even after repeated or long-term exposure' (Drexler *et al*., 2008). This brings the BAT values in line with the BEI® values and current scientific knowledge, assuming that changes in biological parameters are adaptive and tolerable, and that even after long-term exposure, if they are reversible, do not lead to disruption of function or the ability to compensate for effects of the exposure, do not increase the sensitivity of an individual to other external agents, and do not affect reproduction (Morgan and Schaller, 1999). Since these criteria do not apply to genotoxic substances or proven carcinogens, the DFG cannot derive BAT values for such substances. For these substances a range of values, the so-called EKA values (Exposure Equivalent for Carcinogenic Substances) are developed that reflect the biomonitoring concentration that would be observed at a corresponding range of external (airborne) exposures. In **Table 1**, EKA values are listed for concentrations that correspond closely to the threshold limit value (TLV®) of the substance where possible (the concentration chosen is listed in the footnotes).

In contrast to BAT or EKA values, BEI® values reflect the internal dose or dose surrogate arising from exposure, by inhalation only, to the TLV®. As the BEI® would of course also reflect exposure via other routes than inhalation, the underlying assumption in these cases is that any health effect is related to the internal dose irrespective of the route of exposure. In most cases this is a valid assumption. However, it should be borne in mind that, in principle, the same criteria as are used for establishing a TLV® are also applied in the setting of a BEI®. This implies that, just like for TLV® values, there is no sharp distinction between hazardous and nonhazardous exposures due to biological variabilities and, consequently, that there is not necessarily an increased health risk for an individual for whom the BEI® is exceeded. In addition, the American Conference of Governmental Industrial Hygienists (ACGIH) has recognized that for certain chemicals for which the TLV® is not based on a systemic effect but rather on local effects, such as irritation, a BEI® corresponding to such a TLV® would be less meaningful. Nevertheless, BEI® values may be derived for such chemicals to prevent systemic effects, especially when the skin is a significant route of exposure. In those cases, the BEI® does not reflect the TLV® but indicates the internal dose at which no health hazard is expected. For a limited number of substances a BEI® has been established based directly on the relationship between the internal dose and a systemic adverse health effect.

In principle, the biological limit values such as the BAT, EKA and BEI® are limited to the occupational situation. Thus far, only a limited number of health-based BLVs have been established for the general population. Some of these include the reference and guidance values for heavy metals and persistent organic pollutants established by the German Human Biomonitoring Commission (Ewers *et al*., 1999; Wilhelm *et al*., 2003; 2004). The interpretation of biomonitoring data for health risk assessment for the general population is more challenging than for occupational populations since the exposure levels are usually much lower than the exposure levels that may be present in occupational settings and usually several orders of magnitude lower than the no-adverse-effect levels (NOAELs) in animal experiments. An additional complicating factor resides in the fact that the general population is much less homogeneous than occupational populations, because it comprises individuals with greater variability in age (infants, children, elderly) and health status (Boogaard *et al*., 2005; Burke *et al*., 2006). To aid in the interpretation of human biomonitoring data, the concept of Biomonitoring Equivalent (BE) was recently developed (Hays *et al*., 2007). BE values represent quantitative benchmarks of safe or acceptable concentrations of biomarkers which are consistent with selected reference values based on the understanding of the toxicokinetics of the substance. For many substances useful reference levels have been established by a variety of regulatory bodies such as the European Food Safety Authority (EFSA), the Joint Food and Agriculture Organization (FAO)/WHO Expert Committee on Food Additives (JECFA) and the US Environmental Protection Agency (EPA). These organizations establish reference values which represent safe, acceptable or tolerable intake levels, such as reference dose (RfD) and reference concentration (RfC), acceptable daily intake (ADI) or tolerable daily intake (TDI), or minimal risk level (MRL). These values are based on epidemiology and/or animal studies and are considered to represent a safe exposure level for the general population. In essence, the BE translates such a reference value into a biomarker concentration (Hays *et al*., 2008a). This conversion can be done with greater or lesser reliability based on the available scientific information, and a communication system has been proposed to communicate the results to the general public (LaKind *et al*., 2008). BE values have been developed for a number of substances such

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as the heavy metal, cadmium (Hays *et al*., 2008b), the volatile organic solvent, toluene (Aylward *et al*., 2008a), acrylamide (Hays and Aylward, 2008), the pesticide, 2,4-dichlorophenoxyacetic acid (2,4-D) (Aylward and Hayes, 2008) and trihalomethanes (Aylward *et al*., 2008b). Applying the BE concept to large biomonitoring datasets such as obtained in the NHANES (CDC, 2009) demonstrated its usefulness in the priority setting for risk assessment follow-up.

Derivation of BE values may also be useful under REACH (Registration, Evaluation and Authorization of Chemicals), the European chemicals legislation, that was adopted at the end of 2006 (European Parliament and Council, 2006) and will gradually be applied to all chemical substances manufactured in or imported into the European Union. Under REACH, the concept of derived no-effect level (DNEL) was introduced. In principle, a DNEL needs to be derived for each relevant toxicological end point and each relevant route of exposure. The risk to human health is considered negligible if the actual exposure is less than the DNEL. Biomonitoring may be applied to assess the exposure under REACH and may have great benefits over other exposure assessment methodology to demonstrate that the actual exposure is below the DNEL. This is particularly the case since DNELs may be derived not only for workers but also for the general population, both as 'consumers' and as 'individuals indirectly exposed via the environment'. For industrial settings, OEL values—such as TLV®, permissible exposure limits (PELs) and maximum accepted concentrations (MACs)—serve as reference values. OELs may also serve as DNELs, provided they are health based and derived by recognized experts, such as the EU Scientific Committee on Occupational Standards. Applying the same principles, BEs may be derived for OELs as well, and subsequently serve as DNELs. This is very advantageous for all occupational scenarios where inhalation is not the route of exposure or not the only route of exposure. If biomonitoring results indicate that the levels of exposure are below the BE, and hence below the corresponding DNEL, it would indicate that the combined exposure via all potential exposure routes poses no human health risk. Acceptance of the use of BEs in combination with biomonitoring by regulatory authorities will not only make repetitious assessments for different exposure scenarios superfluous, but will also provide a more accurate estimate of actual exposure and hence lead to improved risk management.

6 ETHICAL ASPECTS AND CONSIDERATIONS

Biomonitoring generates data describing the exposure of an individual to certain substances. These data may be used to limit or prevent exposure in occupational settings and for health risk assessment and policy measures in environmental settings, and are consequently linked to ethical questions. It is beyond the scope of this chapter to focus on ethical considerations in detail, but a number of essential issues will be addressed here, including the nature and organization of the study, informed consent and confidentiality, and communication and interpretation of the results.

The primary ethical requirement for any biomonitoring study is a valid scientific approach. Although several issues are identical with regard to ethical aspects, it is useful to differentiate here between survey projects and research projects. Survey projects focus on periodic measurements to produce information on the prevalence of exposure to substances, whereas research projects aim at the generation or the improvement of knowledge on causal links between exposure and health effects by generation and testing of hypotheses (Knudsen, 2004). In occupational settings the vast majority of biomonitoring relates to survey projects, which includes medical health surveillance when biological effect monitoring is conducted. Occupational biomonitoring surveys are a long-standing practice, and ethical aspects are well developed. In general, a biomonitoring method must be chosen for its validity and relevance for protection of the health of the worker concerned, with due regard to its sensitivity, specificity and predictive value. As discussed, for many relevant substances biological limit values have been established that allow the proper selection of biomarkers for survey purposes. In environmental settings, where generally exposure levels are low, only a very limited number of biomonitoring methods are available that allow interpretation in terms of health risks. The classic example is lead in blood, for which guidance and limit values have been well-established and incorporated in European law (CEC, 1982).

For any study, regardless of whether it is a survey or a research project, the selection of the population under study, with well-defined inclusion and exclusion criteria, is essential, and a risk–benefit analysis (including choice of specimen) on individual and/or group level should be part of the study design. These aspects may be fairly straightforward in the case of occupational biomonitoring using well-validated biomarkers with established BLVs or reference values, as there would be no fundamental difference from personal air monitoring. However, for research studies where the validity and/or toxicological significance of the biomarkers used is less certain, ethical requirements and procedures are more stringent, and a thorough assessment by, and approval of, a competent ethical review board may be required, regardless of whether it involves an occupational setting or the general population.

Informed consent is an essential ethical requirement for research projects involving invasive procedures or not-yet-validated biomarkers. Special attention should be given to minors involved, as in mother–child studies,

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where the subjects may be not (fully) aware of the risk–benefit ratio. In all cases, clear and adequate information on the uncertainties, risks and potential benefits (either on the personal or group level) of the testing should be provided. This information should at a minimum comprise the aim of the testing and the interpretation of the possible results, including potential actions in terms of risk management, communication and publication, the sample collection procedures, the confidentiality of the data collected, and the right to withdraw at any time from the study without explanation. It is essential that informed consent is obtained by a trained professional and that it is checked whether or not the provided information is correctly understood. In general, biomonitoring data should be treated confidentially. In occupational situations the results should in principle not be treated differently from other personal occupational health data obtained (including personal air monitoring data), for which procedures are usually established. Individuals, or their legal representatives, should have the right to know their individual biomonitoring results but should also have, if they so wish, the right to not know their data. Communication of the results and their interpretation in terms of health risks is of paramount importance. The potentials and limitations of the interpretation of biomarker data, both on an individual and on a group level, are often not well understood. Moreover, the perception of the advantages and limitations of biomarker studies often differs between the investigators and the subjects as well as between health professionals (Viau, 2005). Guidance on clear communication of biomonitoring data can be found elsewhere (LaKind *et al*., 2008; Keune *et al*., 2008).

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The Role of Poison Centres in Basic and Clinical Toxicology

Alan H. Hall and Chantal Bismuth

CONTENTS

1 INTRODUCTION

1.1 Poison Centre Functions

Poisoning is a significant global health problem. For example, in 2002, an estimated 3 50 000 persons died throughout the world from unintentional poisoning. In the USA, '911' emergency telephone calls can safely and cost-effectively be diverted to Poison Centres (Anderson *et al*., 1998).

Poison Centre involvement and assistance are correlated with measurable reductions in length of hospital stays for poisoned patients, and result in substantial savings in healthcare costs and resource utilization (Vassilev and Marcus, 2007). Utilization of Poison Centres is cost effective in decreasing the number of poisoning admissions to rural hospitals (Zaloshnja *et al*., 2006).

Poison Centres are an information resource for prehospital emergency responders for chemical exposures, and participate in international alerting and surveillance for toxic terrorism (Martin-Gill *et al*., 2007; Waring *et al*., 2007). Poison Centre data can be used to develop treatment protocols and policies (Bateman, 2005a). Poison Centres have a role in protecting public health and in global risk assessment (Mathieu-Nolf, 2005), and data collected by Poison Centres can proceed from clinical practice to promoting public health (Bateman, 2005b). Poison Centres can respond to 'surges' of calls when neighbouring Centres are affected by communications failures or during widespread power outages (Vassilev *et al*., 2007; Klein *et al*., 2007) (see **Table 1**).

In the USA, non-English speakers are less likely to utilize Poison Centre services (Shepherd *et al*., 2004), although 24 hours a day, 7 days a week consultations in Spanish are available from the West Texas Poison Center in El Paso, Texas.

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Table 1 Poison Centre activities, besides providing substance and ingredient identification information and making recommendations for patient decontamination and treatment, can include:

Coordination of:
Regional antidote availability
Data Collection on/for:
Adverse events involving dietary supplements
Drug abuse trends
Hazardous materials (HAZMAT) incidents
Human experience data for risk-assessment priority setting
Infant therapeutic misadventures
International differences in toxic exposure patterns and poisoning trends over time
National epidemiological information on poisonings
Poisoning information for particularly sensitive populations, such as children and the elderly
Reflecting storm-related health hazards during hurricane seasons
Developing:
Exposure profiles for drug poisoning in children
Profiles of childhood poisoning
Structured triage protocols for paediatric poisonings
Surveillance for emerging toxic threats
Toxicological features of deliberate self-poisoning
Treatment protocols and policies
Identification of bystander pesticides exposures:
Crop dusting
Other exposure routes
Planning for:
Mass gatherings, such as the Olympic Games
Developing integrated surveillance systems for mass gatherings, such as the Olympic Games
Providing:
Drug identification services to law enforcement personnel
Environmental information
Information and consultation services during hazardous materials (HAZMAT) incidents
Information services during infectious disease outbreaks, such as the severe acute respiratory syndrome (SARS)
Poison education and Poison Centre awareness training
Public announcements on treatment errors and omissions in drug manufacturers' overdose management recommendations
Responding to:
Animal poisonings
Toxicovigilance:
Developing profiles of poisoning by country
Drug abuse/prescription-drug diversion surveillance
Early detection of poisonings with public health significance
Elucidating the varying spectrum of pesticide poisonings
Food-borne illness surveillance
Generating alerts on adverse drug events and other products of public health interest, such as contaminated foodstuffs and product recalls
Hazard identification
Longitudinal surveys of childhood poisonings
Participating in international alerting and surveillance for toxic terrorism
Pesticide-related illness and injury surveillance
Toxicant assessment in humans

(Alonzo and Laborde, 2005; Bateman, 2005a; Bentur *et al*., 2004; Bochner, 2007; Bronstein *et al*., 2007; Bryden *et al*., 2005; Burgess *et al*., 1997; Cicero *et al*., 2007; Coco *et al*., 2005; Crouch *et al*., (2004a; 2004b); Dennehy *et al*., 2005; Derby *et al*., 2005; Descotes and Testud, 2005; Dyer *et al*., 2007; Forrester, 2006; Forrester and Stanley, 2004b; Geller and Lopez, 1999; Izuora and Adeoye, 2001; Kearney *et al*., 2007; Kotwica and Czerczak, 2007; Mallows *et al*., 2005; Martin-Gill *et al*., 2007; Mazzoleni *et al*., 2006; Mehler *et al*., 2006; Mena *et al*., 2004; Mirza *et al*., 2006; MMWR, 2006; Mucci *et al*., 2006; Rutkowski, 2006; Settimi *et al*., 2007; Srivastava *et al*., 2005; Szkolnicka *et al*., 2005; von Mach *et al*., 2006; Wananukul *et al*., 2007; Waring *et al*., 2007; Wolkin *et al*., 2006; Wu and Sun, 2004).

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2 HISTORY OF THE POISON CENTRE MOVEMENT

2.1 USA

The history of poisons goes back to biblical times. Moses, in Deuteronomy 14: 9–10, describes what marine life can safely be eaten (Shannon and Haddad, 1998). Going from there to Mithridates, King of Pontus who attempted to develop antidotes, to Paracelcus in the sixteenth century who, while studying such potential therapeutic compounds as antimonials and arsenicals, noted that: 'All substances are poisons; there is none which is not a poison. The right dose differentiates a poison from a remedy.' (Shannon and Haddad, 1998).

The first meeting of the American Association of Poison Control Centers (AAPCC) was held in Chicago on 21 October, 1958, following an early 1950s study by the Accident Prevention Committee of the American Academy of Pediatrics (AAP), who found that 50% of reported childhood accidents involved some type of poisoning (Arena, 1970; Crotty and Verhulst, 1970; Shannon and Haddad, 1998). As of July, 1967, there were 550 Poison Control Centres in the USA, one or more in each of the States (Arena, 1970). In 1956, a grant was obtained from Plough, Inc. (Memphis, Tennessee) to study 14 different types of safety closures for drug containers, specifically for children's aspirin preparations, which were then responsible for almost 25% of all reported poison exposures (Arena, 1970). The results of this study were published in 1959 (Arena, 1970).

The Poison Centre movement was mainly spearheaded by paediatricians in the USA (Arena, 1970; Dart, 2004). Poison Centres were also founded in the UK, Canada, France, Germany and Italy as early as the 1960s (Dart, 2004). By 1970, there were nearly 600 Poison Centres in the USA and over 800 Centres in the USA and Canada combined (Lovejoy and Alpert, 1970). There were great variations from Centre to Centre in the size of the population served, the number of telephone calls handled, and the quality and quantity of services offered (Lovejoy and Alpert, 1970).

Prior to the 1950s, there was no formal system for poison information or treatment advice in existence in the USA (Burda and Burda, 1997). The first Poison Centre in the USA was founded in November, 1953 at Presbyterian-St. Luke's Hospital in Chicago, Illinois (Burda and Burda, 1997; Kostic *et al*., 2005). This was followed by the founding of the US Food and Drug Administration's (FDA's) National Clearinghouse for Poison Control Centers in 1957, and the AAPCC in 1958 (Burda and Burda, 1997; Cann, 1958). The National Clearinghouse contacted manufacturers and distributors for ingredient and concentration information for their products (Cann, 1958). During the 1960s to 1970s, hundreds of Poison Control Centres were created by hospitals in the USA (Kostic *et al*., 2005).

A 1998 survey of all 73 Poison Centres in the USA, which included 52 Certified Regional Poison Centres, showed that call volume had been steadily increasing, with 2 475 010 calls regarding poison exposure answered in 1997 (Youniss *et al*., 2000). The average Poison Centre utilization rate was 9.2 human poison exposures per 1000 population, and the cost was less compared with the alternative, emergency-department patient management (Youniss *et al*., 2000).

2.1.1 Poison Centres and Emergency Departments

In the USA in the 1980s, there was some debate over whether the availability of telephone poison information by Poison Centres was diverting patients away from emergency departments, or whether, in fact, this service was saving money by managing patients in the home setting who did not require evaluation in a health-care facility. In an editorial in the *Annals of Emergency Medicine*, Rumack and Rosen (1982) noted that one polarized opinion in this debate was that *all* patients exposed to a toxic substance should be evaluated by a physician. The opposing view was that 85% of children exposed to a toxic substance could be adequately managed over the telephone without being evaluated by a physician or in hospital, with subsequent cost savings.

Rumack and Rosen (1982) also noted that about 60% of telephone calls to major Poison Centres involved children of aged two years or less, and that the exposures consisted of a taste or a touch and never developed into any significant clinical condition. From one major US Poison Centre, data showed that over a 10-year period, Poison Centre referrals to emergency departments had shown a large increase. It was therefore *not* that Poison Centres' telephone advice was producing inappropriate patient management or removing patients from the emergency department. Rather, Poison Centre telephone advice was decreasing the numbers of young children unnecessarily brought for emergency department evaluation while simultaneously increasing the number of patients with potentially significant poisoning referred to emergency departments (Rumack and Rosen, 1982).

2.1.2 Institute of Medicine Report

An Institute of Medicine report commissioned by the US Health Resources and Services Administration (HRSA), published in 2004 (IOM, 2004) recommended that all Poison Centres should perform a defined set of core activities: telephone consultation; chemical and biological terrorism preparedness; capturing and reporting exposure data; training staff, physicians and pharmacists; and conducting quality-improvement activities. They should also collaborate with state and local health departments to

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develop, disseminate and evaluate public and professional educational activities.

2.2 UK and Europe

It is difficult to determine exactly when the first Poison Centre was established in Europe (Govaerts, 1970). There was a special hospital ward devoted to clinical toxicology as early as 1949 in Budapest, and a limited poisons information service was established at the General Infirmary Hospital in Leeds, UK about the same time. The UK National Poisons Information Service was founded in 1963. Gaultier's emergency service was established in Paris in 1959, and special hospital wards for the treatment of poisoning were established quite early in Budapest, Copenhagen, Edinburgh and Paris.

In the UK, a comprehensive National Poisons Information Service was inaugurated in 1963 with a principal Centre in London at Guy's Hospital and other Centres in Edinburgh, Cardiff and Belfast (Goulding, 1991; Turner, 1991). In 1967, an analytical laboratory was established at New Cross Hospital in London, and the aggregate named the Poisons Unit (Turner, 1991). Since that time, additional services have included information and treatment recommendations for the adverse effects of toxic substances in humans and animals with dedicated expertise from several disciplines, as well as planned programmes of research (Turner, 1991). Goulding (1991) also noted that in addition to acute poisonings, Poisons Units or perhaps Academic Clinical Toxicology Units, must deal with the diversity of poisoning, including chronic poisoning, occupational poisoning, forensic toxicology, chemical disasters and exotoxicology.

The European Association of Poison Control Centres was founded in Tours, France in 1964 and a European conference on poison control under the auspices of the World Health Organization's (WHO's) Regional Bureau in Europe was held in Istanbul in 1965, where several experts helped develop the concepts for organizing Poisons Centres (Govaerts, 1970). In June, 1969, the foundations for a world association for poisoning services were established in New York City (the World Federation of Associations of Poison Control Centres and Clinical Toxicology Centres) (Govaerts, 1970).

As of 1970, Govaerts (1970) noted that there were poison control programmes or clinical toxicology treatment programmes of one sort or another in 20 European countries (**Table 2**) (Govaerts, 1970).

Table 2 European countries with poison control programmes or clinical toxicology treatment programmes of one sort or another as of 1970

Belgium
Bulgaria
Denmark
France
Finland
German Democratic Republic (as it was in 1970)
German Federal Republic (as it was in 1970)
Great Britain (United Kingdom)
Holland (the Netherlands)
Hungary
Ireland
Italy
Norway
Poland
Portugal
Spain
Sweden
Switzerland
Yugoslavia (as it was in 1970)
Soviet Union (as it was in 1970)

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2.2.1 EAPCCT

It has already been noted that the European Association of Poisons Centres and Clinical Toxicologists (EAPCCT) was founded in 1964 in Tours in France (Perrson, 1992). According to its constitution, the object of the EAPCCT is 'the study, in Europe and within the context of Europe as defined by the European Office of the WHO, of problems relating to all forms of poisoning'. The association holds regular scientific congresses, some of which have been in cooperation with the International Programme on Chemical Safety (IPCS) and the Commission of the European Communities (CEC). The EAPCCT has also fostered collaboration between individual Poisons Centres by such means as regular newsletters, toxic alerts, and computer and telefax connections, recognizing that there is mutual benefit to member Centres in routine daily work (Perrson, 1992).

The EAPCCT provides advice and information to governments and government departments, as well as to industry and professional organizations, on issues involving toxicology (Perrson, 1992). The Association has promoted development of clinical toxicology as a medical discipline in its own right, and has promoted the development of technology for data storage and retrieval. It has increasingly collaborated with other international organizations such as the WHO, the IPCS, the World Federation of Clinical Toxicology Centres and Poison Control Centres, and the International Union of Toxicology (IUTOX) (Perrson, 1992).

2.3 Role of the IPCS

The International Programme on Chemical Safety (IPCS) is a cooperative programme of the International Labour

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Organization (ILO), the United Nations Environment Programme, and the WHO, established in 1980 for the purpose of responding to the needs of member states for ensuring that essential chemicals are used in such a way that they do not harm human health or the environment (Haines, 1992). IPCS activities have been undertaken jointly with the CEC and in conjunction with relevant professional associations such as the World Federation of Associations of Clinical Toxicology Centres and Poison Control Centres, the EAPCCT, the Latin American Society of Toxicology, and the International Society on Toxicology.

A meeting was held at WHO headquarters in Geneva in October 1985 and identified priority areas requiring international cooperation in the poison-control field, which included a need for provision of internationally evaluated poison information (Haines, 1992). To this end, the IPCS began preparation of a poison information package called INTOX. Poison Information Monographs (PIMs) on about 500 substances were being prepared in English, French and Spanish, beginning about 1988, and were to be supplied on CD-ROM in collaboration with the Canadian Centre for Occupational Health and Safety (CCOHS) and the Centre de Toxicologie du Québec (QTC). Some financial assistance was provided by the International Development Research Centre of Canada (IDRC). By 1992, about 80 PIMs were being put on a CD, with plans to produce another 60–80 PIMs per year over the following five years (Haines, 1992). Part of this programme will allow international data collection for validation of treatment recommendations for more rare poisonings where no one Centre or country can collect enough cases for meaningful interpretation, and for validation of chemical risk assessments based on nonhuman data (Haines, 1996).

2.4 Developing Countries

Poison Centres have multiple roles in developing countries, which include toxicology information services, training, access to laboratory services, and antidote availability (Laborde, 2004; Clarke, 2004). They may concentrate efforts on childhood and pesticide poisonings (Gupta *et al*., 2003).

2.5 Poison Centres and Standards Setting

Poison Centres organizations, such as the EAPCCT, have established activities that member Centres must carry out, and have developed minimum and maximum required standards (Della Puppa *et al*., 2006).

3 POISON CENTRE DATA COLLECTION

3.1 USA TESS/NPDS

In the USA, the name of the AAPCC national database has been changed; formerly the Toxic Exposure Surveillance System (TESS), it is now called the National Poison Data System (NPDS). The most recently published version available is for 2006 (Bronstein *et al*., 2007).

Of the 61 reporting Poison Centres, 60 now submit data electronically every 1–60 minutes, making it a real-time national surveillance system (Bronstein *et al*., 2007). In 2006, there were over 4 million calls to reporting Poison Centres recorded, including 2 403 539 human poison exposure calls. There were 1229 human poison-related fatalities reported to the NPDS in 2006. The large majority of human poison exposures were acute cases in 2006 (91.2%), and of total human poison exposures, 83.4% were accidental while 8.3% were of suicidal intent.

Since its inception in 1983, over 43 million poison exposure calls have been captured in the TESS/NPDS database. In 2006, the average daily call volume at US Poison Centres was 6937.

In 2006, the majority of cases (72.9%) were managed over the telephone and the patients were not referred to a healthcare facility/provider (Bronstein *et al*., 2007). Follow-up calls are an important part of Poison-Centre patient management. When a patient is referred to a healthcare facility/provider, telephone follow-up is done until the case has been resolved. In 2006, one follow-up call was made in 22.4% of human exposure cases, and more than one follow-up call was made in 22.0% of cases (range: 2–158).

It is noted that patients may be taken directly to a healthcare facility/provider who may not call the Poison Centre for advice or to report the case. Thus, the NPDS does not capture all the poison exposures in the USA.

It is also noted that data collection and telephone management of poison exposures and poisonings do not reflect the full extent of Poison Centre activities, which include, amongst others, poison prevention and education, and Poison Centre awareness (Bronstein *et al*., 2007). Information-only calls also comprise a major Poison-Centre activity. In 2006, such information calls involved drug identification (for the public and law enforcement agencies), drug information, environmental information, medical information, occupational information, poison information, poison prevention/safety/education, teratogenicity information, substance abuse information, administrative issues and caller referrals (Bronstein *et al*., 2007). Of drug identification calls, only 9.7% of those involved could not be identified over the telephone. Environmental calls represented 1.9% of all information calls in 2006,

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with questions relating to clean-up of mercury spills from broken thermometers and pesticides being most common.

A unique feature of NPDS is the ability to generate system alerts on adverse drug events and other products of public heath interest such as contaminated foods or product recalls (Bronstein *et al*., 2007). If a report of potential public health importance is received, more information is obtained from the reporting Poison Centre by telephone or email and then is brought to the attention of the National Center for Environmental Health at the Centers for Disease Control and Prevention (CDC). Appropriate state and local health departments may also be alerted.

In 2006, all 61 participating Poison Centres reported to the NPDS. Of these, 58 (95%) are AAPCC Certified Regional Poison Centres (Bronstein *et al*., 2007). US Poison Centres are staffed by healthcare professionals, including board-certified medical toxicologists, pharmacists, pharmacologists, registered nurses, and poison information providers (PIPs) who are allied healthcare professionals. Registered nurses and pharmacists may be either specialists in poison information (SPIs) or, after having logged a minimum of 2000 calls over a 12-month period, may take an approved examination and become certified poison information specialists (CSPIs). US Poison Centres employ (full-time equivalents) 123 PIPs, 250 SPIs and 615 CPSIs.

The NPDS verifies the difference between deaths (an observable clinical end point) and poison-related fatalities through its fatality review team (Bronstein *et al*., 2007). Of 1515 deaths reported in 2007, there were 1229 verified fatalities (Bronstein *et al*., 2007). Children 6 years of age and younger represented only 2.4% of fatalities, while 80% of fatalities occurred in patients aged 20–59 years. A single substance was implicated in 91.1% of fatalities, and 5.9% of fatality patients were exposed to two or more substances.

Poison Centre data collections, such as the AAPCC TESS data, can be 'data-mined' to understand poisoning in selected groups, such as adverse drug reactions in the elderly (Cobaugh and Krenzelok, 2006). They can also be used to show an increased relative risk of fatal outcomes in the elderly with medication poisoning. In one study using AAPCC TESS data, Rogers and Heard (2007) found that 2% of poison exposures in 1990 were in adults 60 years of age and older; this had increased to 4.7% in 2004. In addition, while persons aged 60 years and older comprise approximately 5% of poison-exposed individuals reported to Poison Centres, they make up approximately 15% of poison-related deaths (Rogers and Heard, 2007).

National data collected by Poison Centres, such as the AAPCC TESS data, have multiple uses, such as national surveillance of dietary supplement exposures (Gryzlak *et al*., 2007). Poison Centre data can be used by regulatory agencies, such as the US Environmental Protection Agency, to track pesticide poisoning incidence (Blondell, 2007) and can be used to track such exposures longitudinally (Sudakin and Power, 2007). Pesticide poisonings have been of interest to Poison Centres, particularly internationally (Kalkan *et al*., 2003), amongst both humans and animals (Motas-Guzmán *et al*., 2003). Poison Centres also identify international differences in toxic exposure patterns (Wananukul *et al*., 2007), and poisoning trends over time (Kotwica and Czerczak, 2007). They develop profiles of childhood poisonings (Szkolnicka *et al*., 2005). Food-borne illness surveillance is a potential function of Poison Centres (Derby *et al*., 2005).

One problem with Poison Centre data collections is that fatal poisoning cases that come to the attention of Medical Examiners may *not* be reported to Poison Centres (Blanc *et al*., 1995; Linakis and Frederick, 1993).

4 POISON CENTRES AND OCCUPATIONAL HEALTH

4.1 Occupational Illness Reporting

Occupational illness reporting in an important part of Poison Centre activities (Blanc *et al*., 1990; Litovitz and White, 1990). Reporting systems other than those used by Poison Centres in the USA may capture only 68–92% of occupational exposure cases, and tend to focus on clinically serious cases (Litovitz and White, 1990). Also, for each case of occupational exposure reported to a Poison Centre, there may be an average of 12 other persons exposed (Litovitz and White, 1990).

4.2 Adolescents at Work

Poison Centres provide surveillance of adolescent workplace toxic exposures (Woolf and Flynn, 2000). Each year an estimated 2 00 000 US workers between 14 and 17 years of age experience job-related injuries, and about 64 000 require emergency department treatment (Rubenstein and Bresnitz, 2001). About 70 adolescents yearly are killed in work-related incidents (Rubenstein and Bresnitz, 2001). In a study using the AAPCC TESS database (for 1997), Rubenstein and Bresnitz (2001) found 3442 cases of reported workplace poison exposures in workers aged 6–19 years, representing 7.5% of the total occupational exposure cases. The principal exposure routes in these cases were inhalation/nasal (27.2%), ocular (26%) and dermal (24.6%). The main substances involved were household cleaning substances, chemicals, and fumes/gases/vapours. In 56.6% of the cases, some symptoms ranging from minor to significant developed; there were no deaths.

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5 ADDITIONAL POISON CENTRE ACTIVITIES

5.1 Quality Improvement

Poison Centres participate in quality improvement adherence to treatment guidelines, such as those established by the American Academy of Clinical Toxicology (AACT)/European Association of Poison Control Centres and Clinical Toxicologists (EAPCCT) for gastric decontamination (Chyka and Winbery, 2006).

5.2 HAZMAT Incidents

Poison Centres provide information and consultation services during hazardous materials (HAZMAT) incidents, and collect data on such incidents (Burgess *et al*., 1997).

5.3 Sensitive Populations

Poison Centres collect information on particularly sensitive populations, such as elderly adults (Crouch *et al*., 2004a). They also follow epidemiological trends in other sensitive populations internationally, such as children under five years of age (Mena *et al*., 2004). They can define the toxicological features of deliberate self-poisoning (Bentur *et al*., 2004). Poison Centres can emphasize the dangers of maternal exposures to potential teratogens such as alcohol and anticonvulsant medications during unrecognized pregnancies (Forrester and Stanley, 2004a). Poison Centre elucidation of trends in nonprescription drug abuse in children and teenagers can assist public health officials, physicians, pharmacists and educators in attempts to curb such abuse (Crouch *et al*., 2004b). Poison Centre data can define trends in childhood poisoning (Vassilev *et al*., 2004). Poison Centres can provide information on poisoning trends and educate patients about poison prevention (McGoodwin and McKeown, 2004), as well as developing antidote stocking guidelines (Dart *et al*., 2000). Poison Centres help differentiate mass psychogenic illness from mass poisoning incidents (Doyle *et al*., 2004). They can profile iatrogenic poisonings (Ballesteros *et al*., 2003).

5.4 Treatment Recommendation Variations

Using the EAPCCT/AACT guidelines on gut decontamination for four common drug ingestions, Good *et al*. (2007) found considerable variation in recommendations amongst 11 Poison Centres in 11 different countries, although each Centre had established internal protocols. There was also a certain amount of variation regarding the 'top 20' poison exposures recorded in the various Centres, with those in developed countries tending more towards medications and household products and those in developing countries tending towards pesticides. A single Centre in Australia had a large number of envenomations by fauna (Good *et al*., 2007).

6 CONCLUSION

Poison Centres have a role in a wide variety of activities in education, research, chronic toxicity (occupational and environmental), poison prevention and public health, in addition to their primary function of providing information and treatment recommendations for patients with acute poison exposure or poisoning.

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Ethical, Legal, Social and Professional Issues in Toxicology

Steven G. Gilbert and David L. Eaton**

CONTENTS

A thing is right when it tends to preserve the integrity, stability, and beauty of the biotic community. It is wrong when it tends otherwise.

Aldo Leopold (1949)

Being mindful of suffering caused by taking poisons into our bodies and minds, we are determined to take into our bodies and minds only those things that nourish awareness, life, and love.

The Five Wonderful Precepts—Blue Iris Sangha

1 INTRODUCTION

The fewer clear facts you have in support of an opinion, the stronger your emotional attachment to that opinion.

Anonymous

Philip Handler about balancing risks and benefits: 'A sensible guide would surely be to reduce exposure to hazard whenever possible, to accept substantial hazard only for great benefit, minor hazard for modest benefit, and no hazard at all when the benefit seems relatively trivial.' (Philip Handler, former President of the National Academy of Science) (Handler, 1979).

Toxicology is the study of the health effects of chemical or physical agents. Paraphrasing Spiderman's Uncle Ben, 'With great knowledge comes great responsibility.' Toxicologists have some of the most in-depth knowledge about the adverse health effects of chemical and physical agents of any scientific discipline. Given this knowledge and the impact it has on human and environmental health, what are the toxicologists' ethical responsibilities?

Ethical issues related to the profession of toxicology have become more pronounced with the expanded knowledge about the health effects of chemicals and evolution of society. Ethical consideration in any discipline is an evolving process that reflects basic knowledge and as well the social and culture perspectives of the time. In some ways ethics is deceptively simple, for example, 'I will honour honesty and truth, I will not deceive', but there are many dimensions to ethical conduct for a toxicologist.

Ethics begins with the knowledge and perspective that we have a responsibility to consider the health and well being of other people, animals and the environment. The ethical, legal, social and professional issues that confront toxicologists have grown increasingly complex and interwoven with the demands of society. The information generated by or used by toxicologists and policy makers has enormous implications for human health and the environment, to say nothing of the financial implications. As

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our industrial, manufacturing and chemical-based society has developed, toxicological information has become an integral part of the decision-making process. We will not address issues of outright fraud or data falsification, as these actions are clearly antithetical to honesty and the scientific process (Purchase, 2004). The goal of this chapter is to present an overview of various ethical considerations that a toxicologist may need to address in the design, conduct, interpretation and assessment of toxicological studies for the greater good of the environment and human health. Our focus will be on the development of ethical concerns and decision-making process as the toxicological sciences have assumed a greater role in societal decision making. In part, this chapter provides a perspective on the interaction and evolution of toxicology and society.

The chapter is divided into five broad topic areas. *Ethical Considerations* discusses basic duties and responsibilities of toxicologists and provides a historical perspective. *Legal Considerations* explores the implications of toxicology for the regulatory system. *Social Implications* examines the broader context of toxicology in society. *Professional Considerations* addresses integrity issues that confront toxicologists and questions related to research on animals or humans. *Ethics in Education* considers how toxicologists need to be thoughtful public-health advocates while maintaining scientific integrity. While not directly discussed, several of the leading professional toxicology societies ask their members to adhere to a code of ethics. The code of ethics from the Society of Toxicology (SOT) and Society of Environmental Toxicology and Chemistry (SETAC) are included in the appendix. These serve as good examples of the issues that toxicologists need to address to demonstrate a professional and ethical approach to the issues (Saady, 2001).

2 ETHICAL CONSIDERATIONS

All human beings are born free and equal in dignity and rights. They are endowed with reason and conscience and should act towards one another in a spirit of brotherhood.

Article 1 of the United Nations Universal Declaration of Human Rights *(1948)*

We've poured our poisons into the world as though it were a bottomless pit and we go on gobbling them up. It's hard to imagine how the world could survive another century of this abuse, but nobody's really doing anything about it. It's a problem our children will have to solve, or their children.

Daniel Quinn

Human and Environmental Health: Conditions that ensure that all living things have the best opportunity to reach and maintain their full genetic potential.

Steven G. Gilbert (1999)

The emphasis on the ethical foundation of toxicology has increased in importance with the increased use of toxicological data in public health policy decisions that have broad impacts on society. Judgements and decisions based on toxicological data influence a wide range of endeavours such as drug development, manufacturing, hazardous waste site clean up, household cleaning products, and many rules and regulations governing air and water pollution, pesticide use, food safety, and consumer products, to name a few. Toxicologists must consider their duties and responsibilities to the general field of toxicology as well as the broader needs of society.

Toxicology, like all scientific disciplines, has a foundation built on ethical considerations. The Greek physician Hippocrates (460–377 BC) noted the effect of food, of occupation, and especially of climate in causation of disease. He integrated this understanding in his books, *De aëre, aquis et locis (Air, Waters and Places),* one of the earliest works on humans and the environment. He is also credited with the basic medical tenet of 'do no harm'. Bernardino Ramazzini (1633–1714), an Italian physician, identified the link between worker occupation and health, which he documented in his book *De Morbis Artificum Diatriba* (*Diseases of Workers*). He outlined the health hazards of chemicals, dust, metals and other agents encountered by workers in 52 occupations. These are but two examples, but they illustrate that knowledge was available and with that knowledge comes the ethical challenge of what to do.

Aldo Leopold, the father of 'conservation biology', and considered by many to be America's first bioethicist, summarized ethical responsibilities in a simple statement in 1949: 'A thing is right when it tends to preserve the integrity, stability and beauty of the biotic community. It is wrong when it tends otherwise.' Leopold (1949). Toxic agents can rob humans or the environment of their integrity, stability and beauty. The toxicological sciences have clearly demonstrated that some individuals, humans or animals, are more susceptible than others to the effects of a toxic agent. Susceptibility may be related to genetics, age, diet or numerous other factors. This is particularly true for children, whose rapidly developing organ systems make them more vulnerable and susceptible to the effects of many hazardous agents, and the elderly, who may have failing adaptive response systems that leave them more vulnerable, as well. Assuming that there is an obligation to protect the more vulnerable in our society, this means that children and the elderly have a right to an environment in which they can reach and maintain their full potential (Gilbert, 2005a).

More modern approaches to ethics in toxicology are grounded in biomedical ethics, which developed

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following the lessons learned from the widespread abuses of humans during the Second World War. The four principles of biomedical ethics—respect for autonomy, beneficence (do good), nonmaleficence (do no harm) and justice (be fair)—are the well defined philosophical approach to human healthcare and research (Beauchamp and Childress, 1994). These principles are also particularly applicable for toxicologists. Respect for autonomy implies that individuals have certain rights to control their bodies that must be respected by others. For example, individuals have a right to control their exposure to hazardous agents, which implies a right to know and understand what they are being exposed to. Respect for autonomy increases the importance of acknowledging and understanding individual sensitivity to agents, for example susceptibility related to age or genetics. An individual's right to know and understand what they are being exposed to also implies informed consent, which is a major part of evaluating the safety of drugs, through the process of 'clinical trials'. Beneficence and nonmaleficence implies the need to minimize the risk of a hazard, in other words, do no harm. An obligation or duty to do no harm asserts that proponents of an activity must have the information to ensure that no harm is being done to human health or the environment. Justice requires that the burdens and benefits be shared equally. In recent years this principle has been articulated as environmental justice and the unequal distribution of sources of exposure to hazardous substances or activities. More detailed discussion of these issues is available (Gilbert, 2005a; Weiss, 2001). Expanding or modifying these four principles to include sustainability and veracity has also been proposed (Gilbert, 2006).

Another important consideration involves the tension between scientific uncertainty and causation. Toxicology exemplifies science in that uncertainty is a fundamental driver of research. Uncertainty in toxicological studies or results can complicate interpretation and application to public policy related to protecting human health and/or the environment. While some level of uncertainty is inherent in almost all scientific investigations, ultimately some conclusion must be reached with regards to causation. Bradford Hill and others developed the following list of criteria to establish causation (Hill, 1965). This work was stimulated in part by the effort to assert that tobacco products caused lung cancer as well as other health effects.

1. Strength of association (relationship between independent and dependent variables).
2. Consistency of findings (replication of results by different studies).
3. Biological gradient (strength of the dose–response relationship).
4. Temporal sequence ('cause' before effect).
5. Biologic or theoretical plausibility (mechanism of action).
6. Coherence with established knowledge (consideration of alternative causes).
7. Specificity of association (cause is tightly linked to an outcome).

Hill (1965) went on to note that 'All scientific work is incomplete—whether it be observational or experimental. All scientific work is liable to be upset or modified by advancing knowledge. That does not confer upon us a freedom to ignore the knowledge we already have or postpone the action that it appears to demand at a given time.' There is thus an important responsibility to take action based on available knowledge and not use uncertainty as a rationale for inaction. This statement is also a prelude to the formulation and discussion of the precautionary principle.

In 1968, Garrett Hardin defined the 'tragedy of the Commons' and pointed out that many problems have 'no technical solution' (Hardin, 1968). The Commons was defined as a shared common area which requires management for the good of society that receives benefit from the Commons. This view of the Commons can also have implications for toxicology. For example, toxicologists have explored and characterized the foetal consequences of *in utero* alcohol exposure. The consequences of alcohol consumption during pregnancy are well established. While the way to prevent foetal alcohol exposure is obvious- provide clear advise to women to not drink alcohol during pregnancy- it also requires that society manage this issue. Society must manage this problem by investing in the education of parents and care givers, and provide support for abstinence from alcohol during pregnancy. There is a duty to use available knowledge to protect the most vulnerable.

The gradual recognition of ethical consideration in managing human and environmental health was most recently articulated as the precautionary principle, which is an interesting blending of science and philosophical considerations. The precautionary principle was first articulated in the 1992 Rio Declaration as an aid to decision making in environmental and human-health-related issues (Raffensperger and Tickner, 1999). The most widely accepted definition of the precautionary principle is from the Wingspread Conference of 1998:

> *When an activity raises threats of harm to human health or the environment, precautionary measures should be take even if some cause and effect relationships are not fully established scientifically.*
>
> Wingspread Conference *(1998) (Raffensperger and Tickner, 1999).*

The precautionary principle is not only a tool, but also provides an ethical foundation for decision making. The precautionary principle addresses the application of scientific information to protect human and environmental

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health. The implementation of the precautionary principle requires action even while acknowledging a level of uncertainty. The central elements of the precautionary principle can be summarized as follows (Gilbert, 2005b):

Central components of the precautionary principle

- establish public health goals
- taking preventive action in the face of uncertainty
- shifting the burden of responsibility (proof) to the proponents of an activity
- exploring a wide range of alternatives to possibly harmful actions
- increasing public participation in decision making.

Although controversial, the precautionary principle has been utilized as an important approach to risk management and decision making (Gilbert, 2005b; Goldstein, 2001; Grandjean *et al*., 2004; Kriebel *et al*., 2001; Myers and Raffensperger, 2006; Ter Meulen, 2005; Tickner, 2002). Opposing views have pointed to potentially unintended consequences of this approach to risk management in the face of uncertainty (Marchant, 2003).

The Earth Summit in Rio de Janeiro in 1992 also produced the foundations for an Earth Charter which was ultimately adopted by many countries, states and organizations (UNCED, 1992; Bourdeau, 2004; Strong, 1992). The Earth Charter is a broad ethical statement with the goal of establishing a global civil society. Relevant to toxicologists is one of the principles which states 'Prevent harm as the best method of environmental protection and, when knowledge is limited, apply a precautionary approach.' This clearly refers back to the precautionary principle as an important tool in decision making. The Louisville Charter for Safer Chemicals offers another statement on creating a safer and healthier environment (Geiser *et al*., 2007; Rossi, 2007).

3 LEGAL CONSIDERATIONS

Laws related to toxicology have a long history, often fraught by tragic effects of hazardous agents. One of the first laws dealing with toxicology was passed in 82 BC, by the Roman Emperor Sulla. Called *Lex Cornelia*, this law was intended to deter intentional poisoning, and came about because of the increased frequency of women poisoning men to acquire their wealth. Food adulteration in 1880 spurred Peter Collier, chief chemist, US Department of Agriculture, to recommend passage of a national food and drug law. In 1883, Germany adopted workers' insurance laws to protect workers exposed to organic chemicals, and in 1897 England adopted workers' insurance laws. These as well as other rules and regulations were adopted based on knowledge acquired from unfortunate experience with a hazardous agent.

The regulation of drugs and required testing to demonstrate efficacy and safety developed only through tragic experience. In 1937, the Elixir Sulfanilamide, containing the poisonous solvent diethylene glycol, was marketed and tragically killed 107 persons, many of whom were children. This incidence resulted in the US Congress passing, the Federal Food, Drug and Cosmetic Act of 1938, which required, among other things, drug safety testing. The next event to increase regulation on drugs occurred in 1962 with the discovery that exposure to thalidomide during a small window of susceptibility during pregnancy caused specific limb birth defects. This resulted in the passage of the Kefauver-Harris Drug Amendments which further tightened drug safety testing requirements (for a history of the US Food and Drug Administration (FDA) see *www.fda.gov/oc/history/*).

To address industrial and product-based chemicals, the US Congress passed the Toxic Substances Control Act (TSCA), which became law on 11 October 1976. TSCA was to 'prevent unreasonable risks of injury to health or the environment associated with the manufacture, processing, distribution in commerce, use, or disposal of chemical substances'. One provision of this act was that chemicals currently in use did not require additional safety assessment. TSCA Section 6 gives the US environmental protection agency (EPA) authority to control any chemical that poses an 'unreasonable risk of injury to health or the environment'. The legal, and in part, ethical question is determining, or in a court proving, what is 'unreasonable risk'. In large part TSCA failed to encourage alternatives to hazardous substances or the development and substitution of safer chemicals for industrial or commercial products. For detailed information on TSCA see the EPA web site *www.epa.gov/compliance/civil/tsca/index.html*. This situation raises the question of who is responsible for ensuring that chemicals are safe for the environment and do not harm human health.

The broad societal relevance of toxicological data to human and environmental health has resulted in a variety of laws and regulations administered by a variety of agencies. Some of these rules and regulations were derived to codify ethical conduct or as a result of unethical activities. Laws and regulations that use toxicology data have implications for occupational health and safety, hazardous waste clean up, the food supply and healthcare. In some instances the regulations are not consistent. One simple comparison is that the FDA requires that canned tuna cannot contain more than 1 ppm (1 mg/kg) of mercury, while the EPA sets a reference dose (RfD), the amount of mercury that can be safely consumed each day for a lifetime, at $0.1\,\mu g\,kg^{-1}\,day^{-1}$. Yet a 55 kg (120 lb) pregnant woman that consumed 4 ounces of tuna fish each day would receive a dose of approximately 2 µg/kg/day or 20 times the EPA's estimated 'safe' level. The EPA standard is based on an extensive risk assessment based on a solid foundation of scientific data (as well as standard assumptions and uncertainties) while the basis for the FDA standard is unclear.

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Guidelines are also established by government agencies to advise public health officials. In 1990 the Centers for Disease Control and Prevention (CDC) established a blood lead action level of $10\,\mu g\,dl^{-1}$ for children. Currently there are several studies and reports indicting that the blood lead action level should be significantly lowered (Gilbert and Weiss, 2006) to adequately protect children's health. What is the appropriate role of the toxicologist in addressing issues that combine both science and policy?

The development of the rules and regulations governing toxicological data relevant to drug development was in part implemented because of unethical conduct. Since the early 1970 s toxicological data were required to register pesticides and set exposure guidelines. During reviews of various datasets it was determined that some of the data were not accurately reported, and in some instances clearly falsified. This ultimately resulted in US and international agencies issuing Good Laboratory Practice (GLP) guidelines to ensure quality control and the ability to review and monitor all aspects of study data collection and interpretation. The US FDA specified rules for GLP in Federal Regulation 21 CFR 58. These rules are used for preclinical studies (animal-based studies) prior to clinical research in humans. Research not conducted under GLP guidance may be rejected if submitted in support of new drug applications. The Organisation for Economic Co-operation and Development (OECD) has similar rules. Similarly guidelines also govern the manufacture of drugs and collection of data from human studies. In essence these rules and regulations were developed to help ensure the trustworthy conduct of toxicological research and accurate interpretation of study data (Purchase, 2004).

4 SOCIAL IMPLICATIONS

Toxicologists must also consider the social implications of their science. For example, children's health has additional ethical and social considerations (Gilbert, 2005a). The SOT code of ethics indicates that toxicologists should be thoughtful public-health advocates (see appendix). Although not explicitly stated, codes of ethics such as that of the SOT are commonly based on the following fundamental concepts: (i) a responsibility to share and use knowledge, (ii) a duty to promote the health and wellbeing of children, and (iii) that all species have a right to reach and maintain their full potential.

The social implications of toxicology are evident in the daily media. Examples of popular reports in the media range from recalled drugs, contaminated pet food, lead-contaminated toys, cosmetics with harmful chemicals, and other chemical-laden products. These situations require the consumer to be knowledgeable about a confusing set of choices in an effort to purchase the least hazardous product. Manufacturers also struggle to produce safe and cost-effective products using the least hazardous materials. Currently many issues related to chemical exposure are addressed through the legal system. Toxicologists play an important role in addressing both sides of these issues (Eaton, 2003). The toxicologist must confront difficult issues of judgment and ethics that involve the intersection of science, legal and social concerns.

Toxicologists should be encouraged to engage with their local communities. One proposal is to actively promote the socially responsible toxicologist or citizen toxicologist (CT) (Gilbert, 2005a). The CT would be seen as a thoughtful public-heath advocate and engage in some of the following (modified from (Gilbert, 2005a)):

- Speaks to public interests.
- Recognizes the ethical, legal and social implications of toxicological research.
- Shares scientific knowledge with public.
- Volunteers time to local groups.
- Testifies before local, state and national government committees.
- Meets with local, state or national government representatives.
- Writes review papers or other papers for lay public.
- Teaches in elementary, middle and high-school classrooms.
- Encourages others to be thoughtful advocates for human and environmental health.
- Serves as a member of the board of directors or volunteering for nonprofit organizations associated that promote human and environmental health.
- Serves as a mentor for students in elementary, middle and high school or beyond.
- Lectures on or discusses science and toxicology issues with public and community groups.
- Volunteers to lecture at local community colleges or technical schools.
- Participates as a member of local groups that volunteer for public speaking.

5 PROFESSIONAL CONSIDERATIONS

5.1 Conflict of Interest/Integrity

Concern about real or perceived conflict of interest has risen in parallel with increased use of toxicological data in the decision-making process and subsequent public policies that may have a significant impact on society and financial interests (Maurissen *et al*., 2005). Essential to this discussion is a clear definition of what constitutes a conflict of interest, as opposed to a bias or opinion on the issue. Conflict of interest is often considered as some form of financial involvement related to the issue.

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Defining, and disclosure of, conflicts of interest remains an important issue despite the development of guidelines (Goozner, 2004; Krimsky and Rothenberg, 2001; NAS (National Academy of Sciences), 2003). Many professional societies, such as the SOT (see appendix below), have developed codes of ethics for members that address conflicts of interest. The SOT code of ethics specifically addresses conflict of interest, stating that members should 'abstain from professional judgments influenced by undisclosed conflict of interest, disclose any material conflicts of interest and avoid situations that imply a conflict of interest'.

In recent years charges of conflict of interest that might influence professional judgment have been directed at individuals serving on government committees. Government agencies have responded by issuing additional guidelines and requiring greater disclosure of any potential conflicts. Possible conflicts of interest have also been a concern of journal editors. Journal editors increasingly require authors to disclose any potential conflicts of interest such as financial involvement or professional relationships related to the study.

The evaluation of the toxicity surrounding the manufacture and use of vinyl chlorine (VC) is an interesting example of how issues associated with conflict of interest can effect data review (Sass *et al*., 2005). VC is used almost exclusively in the production of polyvinyl chloride (PVC). In 2002 global production of PVC was over 26 billion kg, valued at approximately US $19 billion. The article's authors charged that a 2000 review of VC toxicity was influenced by industry-funded scientists and did not fully consider all the available data (Sass *et al*., 2005).

There have also been questions related to the interpretation of the health effects of benzene (Infante, 2006). These are limited examples of the importance and implications of toxicology evaluation and the need to address conflict of interest issues. An important challenge is recognizing that conflict of interest is different from a bias, which is based on an reasoned interpretation of the available data that itself is influenced by the lifelong experiences and perceptions of the individual. Everyone has 'biases'. Thus, while it is important to acknowledge bias, it does not represent a conflict of interest, and most organizations that empanel scientists for purposes of scientific review that might impact policy clearly distinguish between conflict of interest and bias in the creation of such panels. For example, the National Academy of Sciences/National Research Council require all potential committee members to disclose all sources of both conflict of interest and bias, but only the former may be used to preclude the participation of someone on a committee. The bias information is used to ensure balance on the committee across a spectrum of perspectives, but typically will not preclude someone from serving on an NAS/NRC expert committee.

5.2 Research Considerations

The design, conduct and interpretation of results of research require that toxicologists adhere to the highest ethical standards as well as beware of appropriate rules and regulations. The experimental design must be developed to yield the best possible results with the least amount of uncertainty. It is critical to acknowledge the potential weaknesses and limitations of a study's design, or constraints due to time or material. A common problem in toxicological studies is that uncertainty can be reduced by increasing the number of subjects, but this inevitably requires more resources and time. Finding the right balance is both a scientific and ethical challenge.

5.3 Human Research Considerations

Ultimately some aspect of toxicology research will involve assessing the health effects of chemicals on humans. The principles of biomedical ethics that inform the rules and regulations that govern human subject research were largely developed out of the lessons of the Second World War and the subsequent Nuremburg trials. Another pertinent example is the Tuskegee Syphilis Study conducted from 1932 to 1972. This medical study followed black men with syphilis in Tuskegee, Alabama, that was continued even after the development of effective antibiotics for treatment (Jones, 1981; Thomas and Quinn, 1991). These experiences initiated a fundamental re-examination of the ethical implications of research on humans and ultimately lead to the development of regulations governing human-subject participation in research or data collection.

This re-examination coalesced into the four principles of biomedical ethics discussed above—respect for autonomy, beneficence (do good), nonmaleficence (do no harm) and justice (be fair) (Beauchamp and Childress, 1994). These four principles became the basis for decision making in healthcare and formed the basis for establishing Institutional Review Boards (IRBs) that oversee human subjects research. The US Federal Policy for the Protection of Human Subjects (Public Welfare: Protection of Human Participants, 2005) defines research on human subjects and addresses measures to protect human subjects. Additional information is available from the Office for Human Research Protections (OHRP) on the conduct and oversight of IRBs (OHRP (Office for Human Research Protections), 2005). The majority of US government agencies comply with the Common Rule on human subjects research (OHRP (Office for Human Research Protection), 2006).

Perspectives on human subjects research have expanded with the concept of community-based participatory research which focusses on proactive engagement of the broader community, not just the individual subjects (Arcury *et al*., 2002; O'Fallon and

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Dearry, 2002). Community-based participatory research can be summarized by the following six principles (adapted from O'Fallon and Dearry, 2002 and Gilbert, 2006):

1. Promotes active collaboration and participation at every stage of research. All parties should share ownership of the project.
2. Fosters co-learning. Researchers and participants share their knowledge and learn together.
3. Ensures projects are community driven. Projects address questions and concerns of the community, not just the researchers.
4. Disseminates results in useful terms. Project results are communicated to the participants in an appropriate and sensitive format.
5. Ensures research and intervention strategies are culturally appropriate. Investigators must be culturally sensitive to achieve the best results.
6. Defines community as a unit of identity. In collaboration with participants, consideration must be given to defining the community involved in the project.

To ensure appropriate community engagement in the research project, it has been proposed that the traditional IRBs be supplemented or even replaced by an Environmental Health and Community Review Boards (EHCRBs) (Gilbert, 2006). This approach also involves an expanded ethical construct which includes a focus on dignity, veracity and sustainability.

5.4 Animal Research Considerations

Concerns about animal welfare and discussion about the ethical use of animals in toxicology research have steadily increased over the past decades (Rollin, 2003). If animals are utilized in the research, additional ethical, animal-welfare and legal issues must be considered. In part out of ethical considerations, there has been an ongoing effort to reduce, refine the use, or seek to replace animals used in toxicity testing (Balls, 2004). The use of animals requires that study designs be reviewed by the Institutional Animal Care and Use Committee (IACUC). An important source of information about animal welfare and the use of animals in research is the American Association of Laboratory Animal Sciences (AALAS) (*www.aalas.org/index.aspx*). Organizations such as the SOT and the American College of Toxicology have policy statements on the care and use of animals in research.

6 ETHICS IN EDUCATION

Discussion of ethical issues should be incorporated into elementary, middle and high-school classrooms and continue throughout undergraduate and graduate education. Ethics primers have been developed to assist teachers in integrating ethical discussions into science classroom instruction (e.g. see Northwest Association for Biomedical Research *www.nwabr.org*). The intersection of society and science requires ongoing discussion. It is important for students and scientists to develop a basic understanding of ethics as a discipline and how to integrate ethical principles into critical and reasoned decision making. These discussions can range from the use of humans and animals in research to the use of stem cells to issues of conflicts of interest. It is very important to foster discussions and encourage critical thinking at the earliest ages in the classroom as well as within society.

7 SUMMARY

The purist idea of ethical behaviour and decision making requires the thoughtful development and articulation of fundamental principles upon which to base any action. The ethical toxicologist must consider and integrate basic ethical principles into the decision-making process. This approach moves beyond what is legally required to consider what should be done to contribute to the greater and long-term good of society. The fundamental principles that an ethical toxicologist should consider are depicted in **Figure 1**. These principles are summarized as: (i) dignity, which includes the respect for the autonomy of human and animal subjects; (ii) veracity, an adherence to transparency and presentation of all the facts so all parties can discover the truth; (iii) justice, which includes an equitable distribution of the costs, hazards and gains; (iv) integrity, an honest and forthright

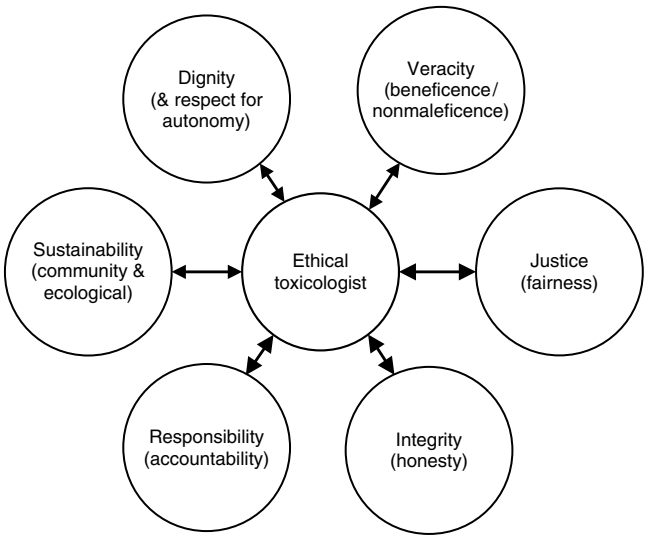

Figure 1 Key principles for consideration by the ethical toxicologist.

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approach; (v) responsibility, an acknowledgement of responsibility and accountability to all parties involved; and (vi) sustainability, consideration that actions are sustainable over a long period of time. An ethical approach requires ongoing discussion and considerations as the toxicological sciences and society evolve. Toxicologists must not only be familiar with the rules and regulations regarding the ethical conduct of research but also the underlying ethical principles. The challenge is to move beyond a purely legal adherence to the rules but towards an ethical approach grounded in carefully considered and articulated ethical principles that drive the responsible conduct of research in modern societies.

APPENDIX A

A.1 SETAC Code of Ethics

(Society of Environmental Toxicology and Chemistry—code of ethics *www.setac.org/node/18*.)

Environmental professionals are expected to exhibit the highest standards of honesty and integrity. Accordingly, our activities require honesty, impartiality, fairness and equity and we must be dedicated to the protection and welfare of environmental resources. Environmental professionals must perform under a standard of professional behaviour that requires adherence to the highest principles of ethical conduct.

Commitment to ethical professional conduct is expected of every member of the Society of Environmental Toxicology and Chemistry, and each member shall:

- Conduct themselves honourably, responsibly, ethically and lawfully, with objectivity and integrity, so as to enhance the honour and reputation of the profession.
- Avoid conflicts of interest and situations that may imply a conflict of interest.
- Give proper credit for intellectual property and honour property rights.
- Recognize and respect confidentiality, but be honest and forthcoming in all issues of public record.
- Abstain from deceptive acts such as fabricating, falsifying or suppressing results, deliberately misrepresenting research findings, or otherwise committing scientific fraud.
- Strive to accurately communicate scientific understanding and knowledge, and to avoid and discourage dissemination of erroneous, biased or exaggerated statements.
- Conduct research and related activities (i) so as to avoid or minimize adverse environmental effects of that research, and (ii) in compliance with legal requirements for protection of researchers, human subjects, or research organisms and systems.
- Abstain from discriminating against others.

A.2 SOT Code of Ethics

The Society of Toxicology is dedicated to developing and communicating knowledge to improve the health and safety of living beings and to protect the environment upon which we depend.

To attain this objective, each member must maintain high ethical standards, recognize a duty to share this knowledge with the public, and be a thoughtful advocate for human, animal and environmental health. To this purpose, this code requires a personal commitment.

Society of Toxicology members shall:

- Conduct their work with objectivity and themselves with integrity. Being honest and truthful in reporting and communicating their research.
- Hold as inviolate that credible science is fundamental to all toxicological research and is the basis for communicating results.
- Recognize a duty to communicate information concerning health, safety and toxicity in a timely and responsible manner, with due regard for the significance and credibility of the available data.
- Give due consideration to the ethical, legal, social and policy implications of their research and communications.
- Be a thoughtful advocate for human, animal and environmental health.
- Abstain from professional judgments influenced by undisclosed conflict of interest, disclose any material conflicts of interest and avoid situations that imply a conflict of interest.
- Observe the spirit, as well as the letter of laws, regulations and ethical standards with regard to the conduct of human and animal research.
- Practise high standards of environmental and occupational health and safety for the benefit of themselves, their coworkers, their families, their communities and society as a whole.

Animals in research statements:

- Position statement regarding the use of animals in toxicology (*www.toxicology.org/ai/air/air5.asp*).
- Guiding principles in the use of animals in toxicology (*www.toxicology.org/ai/air/air6.asp*).

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NOTES

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Ethical Issues Related to Animal Welfare Considerations in the Toxicology Laboratory

Robert F. Phalen

CONTENTS

1 INTRODUCTION

Ethics is a discipline that includes studying the standards of right and wrong, and analysing the values that govern or modify human behaviour. Ethics also refers to the rules that people are expected to follow. Ethical rules may vary both in time and in location. Local ethical standards, which incorporate applicable regulations and customs, are those that govern any given toxicology study. Such standards are currently evolving in the direction of increased accountability for the use of vertebrate animals.

In the context of animal toxicology, ethics involves the balancing of two contrasting 'goods': (i) the need for information for the protection of humans, animals and the environment, and (ii) the minimizing of pain, distress and death of sentient research subjects. In all cases, achieving a proper balance between these goods is a matter of judgement, good experimental design, good experimental technique and proper review by an ethics body that is not directly connected to the study. In the USA, the Institutional Animal Care and Use Committee (IACUC) reviews a proposed study, considering its importance, the necessity for use of animals, and its design. The IACUC requires modifications, or approves the study if it is appropriate, and periodically monitors all approved studies that are actually conducted. The IACUC is also required to report significant deviations from an approved protocol to the proper authorities (usually a federal agency) (NRC (National Research Council), 1996; ARENA/OLAW (Applied Research Ethics National Association/Office of Laboratory Animal Welfare), 2002; Pitts, 2002; Silverman *et al*., 2006). Similar requirements and recommendations obtain in Europe and elsewhere (Smith *et al*., 2007; Takahashi-Omoe and Omoe, 2007). Ethical review of protocols is necessary for the public acceptance of animal research.

The necessity for toxicologists to use the highest practicable ethical standards cannot be overstated. Pressure from interested public and private groups and individuals has led to the intense scrutiny of animal toxicology studies. Deviation from stringent ethical standards not only jeopardizes individual projects, but it also may

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lead to increasingly burdensome requirements, if not outright bans, on future animal studies. The primary responsibility for protecting valuable medical research falls directly on individual investigators, their personnel and their institutions. There is no adequate justification for performing poorly designed, or ill-conducted toxicology studies that involve live vertebrate animal subjects.

On the other hand, there is an ethical requirement, if not a mandate, for performing well-designed, well-conducted toxicology studies. Without such studies, new cures/treatments for diseases cannot be found, endangered species cannot be protected and other information for improving the quality and quantity of life for humans and other species will not be forthcoming. In short, a *toxicology research imperative* must be recognized, and it can be argued that it is unethical to block, impair, or otherwise impede valid institutionally approved animal studies. Nevertheless, extremists are committed to curtailing all use of animals in research, testing and teaching (Morrison, 2001; Kennedy, 2006; Young *et al*., 2006; Brumfiel, 2008).

2 PERSPECTIVE

2.1 History

Although animal studies most likely predate recorded history, Aristotle (384–322 BC) and Galen (AD 129–199) reported both dissections and demonstrations with live animals. Later, numerous reports of animal experiments, such as those by William Harvey (1578–1657), Claude Bernard (1813–1878) and Louis Pasteur (1822–1895) became commonplace (NRC (National Research Council), 1988; Bishop and Nolen, 2001). Such studies firmly established the basic principles of modern anatomy, physiology and medicine, as well as the importance of animals to medical knowledge and advancement. Toxicology has its origins in the use of animal and plant extracts as poisons and remedies over 2000 years ago (Gallo, 1996). Today, animal studies are essential to the study and understanding of carcinogens, teratogens, biological toxins, chemical toxicants, environmental pollutants, nutrients, vitamins, medications, consumer products and numerous other natural and anthropogenic substances. Although animal studies have produced unmeasurable benefits for humans and other animals, they have also generated both concern and widespread opposition (Morrison, 2002).

2.2 Regulatory Activity and Pressure

Early legislation, as in Massachusetts (1641), and England (Martin's Act of 1822), was directed against farm-animal cruelty, but antivivisectionists in England (who attempted to abolish all animal research) prompted the enactment of the 'Cruelty to Animals Act' in 1876, which required licensing of investigators who might subject vertebrate animals to pain (NRC (National Research Council), 1988; Zurlo *et al*., 1994). Since these early attempts to regulate, and even abolish, the use of animals in research, an enormous amount of public pressure, regulation and legislation has emerged (NRC (National Research Council), 1988; Sideris *et al*., 1999; VandeBerg *et al*., 1999). Today, it is clear that there is continuing pressure worldwide to restrict and regulate animal research, as well as attempts to intimidate organizations, institutions and individuals that pursue the advancement of scientific knowledge, medicine and product safety (Morrison, 2001; Kennedy, 2006; Brumfiel, 2008).

2.3 Current Regulations

The *Association for Assessment and Accreditation of Laboratory Animal Care International* (AAALAC International) (*www.aaalac.org*) publishes information on current international regulations and resources in addition to promoting 'the humane treatment of animals in science through voluntary accreditation and assessment', **Table 1** lists only a very small sample of resources on laws, guidelines and regulations for toxicologists; it demonstrates the rigorous commitment to animal welfare on the part of governments and the scientific community.

The philosophy behind current regulations relating to laboratory animals can be traced to the international guidelines for the use of human research subjects. After the Second World War, the Nuremburg Tribunal developed a list of criteria for judging whether Nazi investigators were guilty of 'crimes against humanity'; **Table 2** summarizes these criteria. By way of comparison, the US Government's 'Principles' for experimental animals (**Table 3**) mirrors the Nuremburg Code. These principles have subsequently been greatly extended and clarified as is evidenced by the most recent *Guide for the Care and Use of Laboratory Animals* (NRC (National Research Council), 1996) (the 'Guide'); **Table 4** shows the contents of the most recent 'Guide', which is widely used by Institutional Care and Use committees. Investigators should be aware that additional local regulations and guidelines are likely to apply to their specific studies. Such requirements will usually be distributed by a local institutional animal research ethics committee.

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Table 1 A small sample of sources of key international animal research regulations and guidelines

Country/region	Source of regulations/guidelines
Multinational	Council for International Organizations of Medical Sciences (CIOMS) (*www.cioms.ch/*)
	Guide for the Care and Use of Laboratory Animals (NRC (National Research Council), 1996)
	International Council for Laboratory Animal Science (ICLAS) (*www.iclas.org/*)
Asia	Japanese Association for Laboratory Animal Science (*wwwsoc.nii.ac.jp/jalas/index_e.html*)
	Korean Animal Protection Law (*www.koreananimals.org/animals/apl.htm*)
	Philippines Animal Welfare Act of 1998
	Taiwan Animal Protection Law (1998)
Australia and New Zealand	Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 7th edition, 2004.
	Australian and New Zealand Council for the Care and Use of Animals in Research and Testing (ANZCCART)
Canada	Canadian Association for Laboratory Animal Science (CALAS/ACSAL) (*www.calas-acsal.org/*)
	National Research Council, NRC-CNRC, Canada
Europe (not included are individual country resources)	Directive 86/609/EEC, (1986)
	European Biomedical Research Association (*www.ebra.org/*)
	European College of Laboratory Animal Medicine (*www.eclam.org/*)
	Federation of Laboratory Animal Science Associations (FELASA) (*www.felasa.eu/*)
India	Government of India Ministry of Environment and Forests: Animal Welfare Division
Mexico	NORMA Official Mexicana NOM-062-ZOO-1999, Especificaciones técnicas cuidado y uso de los animales de laboratorio
Russia	Russian Academy of Sciences
United States	The Animal Welfare Act, US Department of Agriculture
	Institutional Animal Care and Use Committee (*www.IACUC.org*)
	US Department of Agriculture: Animal and Plant Health Inspection Service (APHIS)
	Institute for Laboratory Animal Resources (ILAR)
	Office of Laboratory Animal Welfare, US Department of Health and Human Services (OLAW)

Adapted from *www.aaalac.org*.

Investigators are advised to begin their protocol review and approval process, which can be lengthy, well in advance of their study. Although cumbersome, the requirements for approving and performing studies must be met, as they are actually in place to protect the privilege of using animal subjects. The ultimate responsibility for compliance rests with the Principal Investigators.

2.4 Accreditation

'Accreditation', which is an institutional responsibility, is a formal acknowledgement that the institution ensures that studies are conducted under stringent requirements. Although accreditation is voluntary, many funding agencies will not support research, testing or training

Table 2 Abbreviated elements of the Nuremberg Code for human experimentation

1. 'The voluntary consent of the human subject is absolutely essential.'
2. 'The experiment should be such as to yield fruitful results for the good of society . . . '
3. 'The experiment should be so designed and based on the results of animal experimentation and a knowledge of the natural history of the disease or other problem . . . '
4. 'The experiment should be conducted as to avoid all unnecessary physical and mental suffering and injury.'
5. 'No experiment should be conducted where there is an a priori reason to believe that death or disabling injury will occur . . . '
6. 'The degree of risk to be taken should never exceed that determined by the humanitarian importance . . . '
7. 'Proper preparations should be made . . . to protect the experimental subjects against even remote possibilities of injury, disability, or death.'
8. 'The experiment should be conducted only by scientifically qualified persons.'
9. ' . . . the human subject should be at liberty to bring the experiment to an end . . . '
10. ' . . . the scientist in charge must be prepared to terminate the experiment . . . (if) . . . continuation of the experiment is likely to result in injury, disability, or death . . . '

Adapted from *http://oshr.od.nih.gov/guidelines/nuremberg*.

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Table 3 Abbreviated US government principles for vertebrate animals used in testing, research and training

1. 'The transportation, care, and use of animals should be accordance with the Animal Welfare Act . . . and other applicable Federal laws, guidelines, and policies.'
2. 'Procedures involving animals should be designed and performed with due consideration of their relevance to human or animal health, the advancement of knowledge, or the good of society.'
3. 'The animals selected for a procedure should be of an appropriate species and quality and the minimum number required to obtain valid results. Methods such as mathematical models, computer simulation, and *in vitro* biological systems should be considered.'
4. 'Proper use of animals, including the avoidance or minimization of discomfort, distress, and pain when consistent with sound scientific practices, is imperative.'
5. 'Procedures with animals that may cause more than momentary or slight pain or distress should be performed with appropriate sedation, analgesia, or anaesthesia.'
6. 'Animals that would otherwise suffer severe or chronic pain or distress that cannot be relieved should be painlessly killed at the end of the procedure or, if appropriate, during the procedure.'
7. 'The living conditions of animals should be appropriate for their species and contribute to their health and comfort.' 'In any case, veterinary care shall be provided as indicated.'
8. 'Investigators and other personnel shall be appropriately qualified and experienced for conducting procedures on living animals. Adequate arrangements shall be made for their in-service training, including the proper and humane care and use of laboratory animals.'
9. 'Where exceptions are required in relation to the provisions of these principles, the decisions should not rest with the investigators directly concerned but should be made, with due regard to Principle 2, by an appropriate review group such as an institutional animal care and use committee.'

Adapted from Appendix D, NRC (National Research Council) (1996).

activities with vertebrate animals in an institution that is not accredited by a body such as the AAALAC. Originally, the AAALAC was formed in 1965 by several scientific and educational organizations under the name *American Association for Accreditation of Laboratory Animal Care*. In 1996 the name was changed to reflect the organization's growth in other countries. AAALAC accreditation has been achieved by over 750 companies, hospitals, universities, government agencies and other institutions in 29 countries (*www.aaalac.org*). Accreditation is a clear demonstration that the institution is committed to stringent, responsible animal care and use, and that it maintains the administrative structure to accomplish that commitment.

2.5 Animal Welfare and Animal Rights

2.5.1 An Important Distinction

It can be safely assumed that the great majority of adults accept the concept of 'Animal Welfare'. For example, a recent survey of 750 New Zealanders' attitudes towards animals used for research, testing and teaching (RTT) found that just 33% were interested in the issue generally, and only 8% knew at least a 'fair amount' about legislation related to such uses of animals. The authors concluded 'the majority of New Zealanders were neither interested nor concerned about the use of animals in RTT (research, testing, and teaching)'. Also over two-thirds agreed that such animal use 'was acceptable as long as there was no unnecessary suffering by the animals' (Williams *et al*., 2007). A concern for animal welfare (and a personal attachment to animals) is also true for those who work directly with animals in the laboratory, which must be recognized and responded to by investigators (Herzog, 2002).

The elements of animal welfare according to the *American Veterinary Medical Association* (*AVMA*) (*www.avma.org*) include: a consideration for proper housing, management, nutrition, disease prevention and treatment, responsible care, humane handling and humane euthanasia. These elements are species-specific and are dealt with in laboratories under the supervision of veterinarians or other trained experts (Siglin and Rutledge, 1995). 'Animal Rights' in contrast, is a philosophy that blurs the distinction between humans and other animals, and denies the primacy of human interests over those of other species (Smith, 2007). Differential concern for, and treatment of, various animal species is often labelled as 'speciesism' by animal rightists, and even humane euthanasia in the laboratory is described as 'murder'. Yet, animal rights groups have been known to engage in euthanasia of animals left in their care (Anonymous, 2007). The animal rights philosophy is incompatible with any use of animals by humans, including for food, products, labour, exhibition and animal research and testing; it demands abolishment of such activities.

2.5.2 Activism

Although animal welfare in research, testing and teaching activities has been incrementally regulated, some individuals and groups have engaged in activism. Activism

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Table 4 Contents of the Guide for the Care and Use of Laboratory Animals

Introduction
- Regulations, polices and principles
- Evaluation criteria
- Farm animals
- Nontraditional species
- Field investigations
- Overview
- References

1. Institutional policies and responsibilities
   - Monitoring the care and use of animals
   - Veterinary care
   - Personnel qualifications and training
   - Occupational health and safety of personnel
   - References
2. Animal environment, housing and management
   - Physical environment
   - Behavioural management
   - Husbandry
   - Population management
   - References
3. Veterinary medical care
   - Animal procurement and transportation
   - Preventive medicine
   - Surgery
   - Pain, analgesia and anaesthesia
   - Euthanasia
   - References
4. Physical plant
   - Functional areas
   - Construction guidelines
   - Facilities for aseptic surgery
   - References

Appendix A: Selected bibliography

Appendix B: Selected organizations related to laboratory animal science

Appendix C: Some federal laws relevant to animal care and use

Appendix D: Public Health Service policy and government principles regarding the care and use of animals

NRC (National Research Council) (1996).

(exerting influence to further a specific goal) can be conducted legally, for example through public education, free speech and peaceful protest, but there are numerous instances of illegal activities (Kennedy, 2006; Wohlsen, 2008). Such illegal activities have included theft, vandalism, arson and even attempted murder (Borum and Tilby, 2005; Wohlsen, 2008). When individual scientists are targeted by extremist activists, a likely sequence of events has been outlined (Kennedy, 2006). These events include: photographs of apparently distressed animals being distributed to news media outlets; circulation of leaflets accusing the investigator of 'atrocities'; residential picketing; and property damage including, in the extreme, planting firebombs or other potentially lethal devices. Such activities have been officially labelled as 'terrorist acts' and they have stimulated legislation for protection of animal users, as well as widespread law enforcement responses (Borum and Tilby, 2005).

2.6 Protecting Animal Research, Testing and Teaching

It is important to realize that those who wish to abolish the use of animals for research, testing and teaching will succeed unless there are significant efforts to protect it. Several organizations (**Table 5**) are engaged in supporting animal studies by providing information resources, advice to scientists and, in some cases, education of public officials. However, they can only do so much, as the battleground is in hundreds, if not thousands, of communities and institutions. Furthermore, the high-level administrators of institutions whose scientists are attacked are often initially inclined to lay low rather than support their scientists. Therefore, it is necessary for individual scientists and teachers to become directly involved in public education.

Providing public education on how and why animals are used in research, testing and teaching can be a rewarding experience. Most people have never personally met a biomedical scientist, but they probably have an admiration of such scientists, based on their experience with medicine, dentistry, science courses, and so on. Not only are members of the general public anxious to hear the scientist's side, but they are usually completely unaware of the many protections, the approval and monitoring processes, and the efforts of investigators to protect their animals from unnecessary suffering in scientific research, testing and teaching. Science teachers in public schools, science and nonscience department chairs in colleges and universities, librarians, officers of civic groups and many others can be contacted by individual scientists to arrange visits and presentations. The audience will be especially interested in the 'big picture', that is, why the activity is important, and what the scientist's attitudes towards the use of animals are. When speaking, scientists should remember that they are, at least for that audience, representing biomedical scientists in general. It is helpful if they speak clearly, bring items of interest to display, and openly respond to all questions. Public education on the part of scientists worldwide is one of the most important aspects of protecting biomedical research and its great promise to humans and animals alike. The organizations listed in **Table 5** can offer help and teaching aids.

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Table 5 Some of the organizations that support the use of animals in research, testing and teaching

Organization	Web site
Americans for Medical Progress (AMP)	*www.amprogress.org/*
Biomedical Research Education Trust (BRET)	*www.bret.org.uk/*
Understanding Animal Research	*www.understandinganimalresearch.org.uk*
European Biomedical Research Association (EBRA)	*www.ebra.org/*
Foundation for Biomedical Research (FBR)	*www.fbresearch.org/*
National Association for Biomedical Research (NABR)	*www.nabr.org/*
Victims of Animal Rights Extremism (VARE)	*www.vare.org.uk/*

2.7 If You are Targeted by Activists

Acts of vandalism, including theft or release of animals, and public demonstrations attacking individuals, are among the acts of animal rights extremists (Morrison, 2001; Borum and Tilby, 2005; Kennedy, 2006). Although initially alarming, such acts also present the opportunity for public education (and education of family, friends, neighbours, administrators, members of the print and electronic media, the community and public officials). A scientist who is attacked will need to set aside considerable time to defend themselves and their activities. Meeting with neighbours to discuss their work is advised so that they can recognize false claims, especially if leaflets or pickets arrive. Neighbours and their children will benefit from laboratory visits. If possible, one's laboratory or other facility should be open to other legitimate visitors; members of the press and other media will insist on this, and they will usually take photographs and will film activities and interviews for broadcast. As scientists are usually not experienced in such activities, 'media training' by experts should be sought. Such training is not training to deceive; rather it is intended to prepare one for clear, effective, forthright communication. Reporters are very busy, so clear and efficient communication is required in order to help them do their jobs. They will want to see what you do, hear why you do it, and will want succinct and honest answers to their questions. It must be understood that contact with reporters presents an opportunity to educate them and the public. That opportunity can be very rewarding as well as a great service to the community. If done well, the targeted scientist will gain in stature, and even establish lasting positive relations with the public and members of the media.

3 LABORATORY ANIMAL WELFARE

3.1 General Considerations—the Three Rs

The principles listed in **Table 3** summarize the elements of humane animal use, and they must be strictly adhered to. In the toxicology laboratory, many practical issues must be considered. These include: recognition and control of pain and distress, selection and use of appropriate sedatives, anaesthetics, analgesics, tranquilizers and euthanasia agents, selection of humane end points, care in handling, use of humane food and water restriction, and attention to health and safety considerations. These and other related topics are covered in several references (Siglin and Rutledge, 1995; NRC (National Research Council), 1996; ARENA/OLAW (Applied Research Ethics National Association/Office of Laboratory Animal Welfare), 2002). In addition, the 'Three Rs', which were codified by Russell and Burch (1959), are important considerations that should be part of the planning of all research and testing procedures (Flecknell, 2002). The three Rs, 'Reduction', 'Refinement' and 'Replacement' have become mandated by legislation in the USA and Europe (Goldberg *et al.*, 1996), and they are the impetus for the increased interest for developing 'alternatives' to animals in research, testing and teaching (ARENA/OLAW (Applied Research Ethics National Association/Office of Laboratory Animal Welfare), 2002; Goldberg *et al.*, 1996), 'Reduction' usually relates to minimizing the number of animals used. Good statistical designs that insure that excess numbers of animals will not be used to test hypotheses are essential, and typically require the inclusion of an 'applied statistician' in the planning process. Good statistical planning also improves the efficiency of the study and its contribution to knowledge. Another aspect of 'Reduction' relates to only performing those animal studies that are required for answering important research questions or acquiring needed toxicity data. 'Refinement' usually relates to modifying procedures, wherever possible, to reduce and/or eliminate pain and distress. Many examples could be given, but skilful administration of sedatives and analgesics, proper handling, humane postexperimental care, use of nonlethal end points and expert humane euthanasia are some methods. 'Refinement' requires a thorough understanding of what the animals require, and what they might experience, in the study; consultation with, and participation by, an expert veterinarian is recommended. 'Replacement' commonly refers to the use of nonsentient models (e.g. cell cultures, computer models, etc.) instead of animals, or substituting lower

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Table 6 Some signs of acute and chronic pain in laboratory animals

Sign	Description
Abnormal posture	Hunching, head down, curled position, unusual gait, lameness.
Vocalization	Crying out, abnormal breathing sounds, whining, and so on.
Lethargy	Stillness, abnormal time spent lying down and poor response to stimuli.
Guarding, restlessness	Moving away, attempting to bite or scratch when approached, pacing, shifting weight, shaking or trembling.
Self mutilation	Licking, biting, scratching, rubbing, shaking.
Other behaviour	Failure to eat or drink, poor grooming, facial distortion, reluctance to move.
Clinical signs	Abnormal or absent elimination, breathing, or heart rate, lacrimation, salivation, pallor, bleeding, sweating, hypothermia, and so on.

Adapted from ARENA/OLAW (Applied Research Ethics National Association/Office of Laboratory Animal Welfare) (2002).

animals (e.g. nonvertebrates) in the procedures. Nonsentient models are usually called 'alternatives' (Zurlo *et al*., 1994). 'Replacement' is often a problem in that certain species may be required either scientifically (e.g. in exercise studies), or by regulations for testing medicines, food additives, consumer products and so on. However, many scientists are not trained in the use of alternatives, and some models, such as cell or tissue culture, require specialized facilities and training that are not always available.

3.2 Pain and Distress

Investigators must be able to identify and eliminate significant pain in laboratory animals, unless an exception is specifically obtained from the IACUC (or an equivalent review committee). Exceptions are not common, but allowed when essential to a research question that is sufficiently important in relation to the pain. 'Significant pain' may be defined as pain that is more severe than that associated with a single injection into a vein, the skin, or a muscle with a needle and syringe. The pain associated with surgery, or other significant tissue damage, must be always be anticipated and eliminated by prior administration of analgesics or other means. But pain which is more difficult to anticipate may be associated with administered pharmacological agents, toxicants, irritants, restraints, dietary manipulations, injuries, extreme environmental conditions and other intended (or unintended) factors. **Table 6** lists some of the signs of acute and chronic pain, or distress, in animals. **Table 7** describes some general methods for controlling pain or distress in animals.

It is imperative that investigators and their assistants learn to recognize the signs of pain and distress, and apply methods for eliminating these states in the specific species that they use. Siglin and Rutledge (1995) provide species-specific doses for several common species. However, it is important to consult with a qualified veterinarian who is familiar with the procedures and species involved. Prior to performing a study, it is also essential that all persons that administer pain relief be properly trained, and that the needed skills are verified.

3.3 Use of Humane End Points

The term 'end point' has two meanings that should not be confused. From the investigator's point of view it usually means the type of data that are acquired; for example blood pressure, respiration rate, tumour mass, size or number, or a variety of other responses to treatment. For the institutional review committee, inspectors, or attending animal care staff, 'end point' usually relates to how the experiment/procedure is terminated; for example, the termination of postprocedural care or intensive monitoring, return to normal housing, transfer to another study, or euthanasia. The selection of scientifically valid and humane end points is a key consideration.

Table 7 Means of eliminating and reducing pain or distress

Means	Description
Sedation	A state of decreased awareness and relaxation or sleepiness. Analgesia is not present.
Analgesia	Complete lack of pain.
Tranquilization	A state of mental calming, decreased responsiveness and relaxation. Analgesia is not present.
Anaesthesia	A complete loss of sensation in part or all of the body.

Adapted from ARENA/OLAW (Applied Research Ethics National Association/Office of Laboratory Animal Welfare) (2002).

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Survival surgeries are an example of procedures that may cause significant pain and distress in animals. Survival surgeries must be demonstrated to be essential to the study, and performed by well-trained personnel, preferably medical or veterinary surgeons, or highly skilled, experienced technicians. It is recommended that new surgical procedures be witnessed and, if necessary, supervised by a veterinarian not otherwise involved in the study. In many cases, the IACUC or other approving committee will request a report on the first new surgical procedure before permitting others. Also, the committee can require modification, or termination, of the protocol in response to the initial report. Proper presurgical preparations, including selecting a sterile suite, appropriate sedation and anaesthesia, and training of the surgical team are necessary. The means for monitoring and supporting the subject's condition (blood pressure, level of anaesthesia, and body temperature) must be available and used throughout the procedure. If complications that cannot be dealt with during the surgery are encountered, euthanasia of the fully anaesthetized subject must be performed, or at least seriously considered. Postsurgical care with initial intensive monitoring is an essential component of the procedure. Continuous monitoring is required before the animal regains consciousness. If the recovery period is lengthy, monitoring at frequent intervals for 24 hours or more may be necessary. Failure to notice and deal with postsurgical complications is considered to be serious negligence, and is likely to result in temporary or permanent cancellation of the protocol's approval.

Death by any means other than euthanasia is strongly discouraged in animal studies unless there is assurance of complete pain control. Traditional lethality studies, such as the classical LD_{50} (lethal dose for 50% of the animals during a defined postadministration period) study, have been largely replaced by studies that provide euthanasia when a premorbid state is achieved. Classical LD_{50} studies required 40 or more animals for statistical validity. Animal tests of lethality are sometimes required for toxicity testing related to chemical labelling (as for transportation), treatment of poisoning, establishing maximum dosage of therapeutics, setting dose levels for subsequent studies, and validating alternative (those not involving animals) test methods (Zurlo *et al*., 1994). Whenever possible, LD_{50} tests should be replaced with other tests. Such tests that require fewer animals have been described in a review by Rispin *et al*. (2002). As regulatory requirements for lethality testing are in a state of rapid evolution, the current regulations and literature should be searched prior to designing such studies, so that more humane procedures can be used.

Similarly, study designs involving significant food or water deprivation, noxious/irritant stimuli, cancer/tumour induction, extreme environmental conditions and other pain-producing procedures must be carefully considered. A review of toxicology testing methods edited by Derelanko and Hollinger (1995) describes traditional and available alternative testing methods. For such studies, elimination of unnecessary pain and distress, as well as minimizing the number of subjects, is necessary.

3.4 Euthanasia

Euthanasia (from the Greek, meaning 'good death') is routinely performed in toxicology studies for a variety of reasons including: to prevent suffering; to obtain tissue and other samples for analysis; to harvest organs, tissues and cells for *in vitro* preparations; and to end the lives of animals at the termination of a study. Because euthanasia itself has the potential to cause pain and distress it must be performed by trained personnel using techniques that are appropriate to the study and species used; ethical considerations should outweigh practical ones, such as cost or convenience. In recognition of the foregoing considerations, the *American Veterinary Medical Association (*AVMA*) Panel on Euthanasia* provides guidelines, which are periodically updated. The 2007 update comprises a 40-page report which is available on the AVMA web site (*www.avma.org*). The report is thorough, covering: 'general considerations'; human (scientific staff, not research subjects) and animal 'behavioural considerations'; 'inhalant agents'; 'noninhalant pharmaceutical agents'; 'physical methods'; and 'special considerations' that take into account species, age, and the reason for euthanasia (e.g. food production, or disease control). Appendices cover: 'agents and methods by species'; 'acceptable agents and methods'; 'conditionally acceptable agents and methods'; and 'unacceptable agents and methods'. The report emphasizes the responsibilities to prevent predeath anxiety, to verify death, and to insure that those who administer the euthanasia are properly trained and properly employ humane techniques; gentle handling and calming techniques are recommended. The guidelines of the AVMA Panel should be thoroughly examined and strictly followed to the extent possible in the laboratory. Scientists seeking approval for their studies can expect to be held to the AVMA Panel's guidelines by approval committees (NRC (National Research Council), 1996; ARENA/OLAW (Applied Research Ethics National Association/Office of Laboratory Animal Welfare), 2002).

4 HEALTH AND SAFETY

Investigators and their institutions are responsible for the health and safety of the animals that they acquire, transport, house and use. Discharging this responsibility requires: establishing and maintaining facilities appropriate to the species acquired; having professional (scientific and administrative) and technical staff (scientific

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Table 8 Some responsibilities that investigators have towards the health and safety of animals

Item	Comment
Diet and nutrition	Dietary modification, including food and water restriction, should not produce unrelieved pain or excess distress.
Disease	Unintended diseases should be prevented, and all diseased animals should have adequate care and treatment to the extent the study permits.
Pain and distress	Species-appropriate handling and humane treatment and procedures, to the extent permitted by the study, must be assured. This includes relief of pain and distress.
Environmental quality	Animals should not be subjected to unhealthful or stressful confinement, temperature, humidity, lighting, vibration, noise, or air quality to the extent possible in the laboratory.
Safety	To the extent possible, animals must be protected from injury in the laboratory. Attention to temporary caging, handling and laboratory equipment is required.

assistants, and vivarium personnel) that are qualified and trained; establishing protocol review and monitoring processes; providing healthful housing and caging conditions; meeting the animals' nutritional needs and environmental requirements; providing access to exercise and environmental enrichment for some species; having effective plans for protecting animals in the event of mechanical and power failures; having preparations for disasters (fires, floods, etc.); and protecting the animals from theft. These responsibilities, which fall mainly on the institution and its administration, are largely covered by regulations and by guidelines such as those used for accreditation (NRC (National Research Council), 1996).

The responsibilities of Principal Investigators and other animal users supplement those of their institutions. Some of these responsibilities are described in **Table 8**.

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Education of the Toxicologist

Robert Snyder and Jason R. Richardson

CONTENTS

1 INTRODUCTION

The idea that there can be training programmes for toxicologists bears within it the inherent concept that toxicology is a distinct scientific discipline. Among the biological sciences it has long been accepted that anatomy, biochemistry, physiology, microbiology, pathology, and even pharmacology are distinct disciplines. Although the body of scientific effort in these areas justified disciplinary designation, the requirement for learning these sciences as part of professional medical education bolstered their claim to individuality. Toxicology came late to acceptance as a unique discipline, and in some quarters that concept is not yet accepted today. Nevertheless, the knowledge that chemicals can adversely affect biological organisms has been known for many centuries. We have been familiar with the concept of poisons from the death of Socrates, through Paracelsus and Lucretia Borgia. In the nineteenth century, Orfila is credited with carrying out the first scientifically based studies of toxicity, and subsequently a number of great biologists, including Magendie, Bernard, Schmiedeberg and others performed seminal research defining the effects of chemicals on biological systems, in the study of physiology, and later, pharmacology. Today, their work could legitimately be considered mechanistic studies in toxicology.

The need to recognize a formalized profession and separate discipline of toxicology arose primarily from several distinct sources in the USA. The Food, Drug and Cosmetics Act of 1938 demanded that, prior to marketing a new drug, the US Food and Drug Administration must be supplied with data from the manufacturer showing that when taken as directed the drug is safe. While some side effects may be observed, it must be demonstrated that no serious toxicity will occur through normal use of the drug. As a result, drug companies were forced to perform toxicological studies to evaluate their new products and to present the resultant data to the agency for approval before the drug could be marketed. The second milestone in the solidification of the discipline of toxicology came when the National Academy of Sciences/National Research Council established the Committee on Toxicology in 1947. The mandate of the Committee on Toxicology extends beyond foods and now

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covers the entire range of potentially toxic industrial and environmental chemicals. A third milestone derives from the formation of the Society of Toxicology, the first professional society in toxicology, which dates from 1961–1962. Finally, the establishment of a Toxicology Training Program by the National Institute of Environmental Health Sciences (NIEHS) recognized that the discipline existed and that programmes for toxicology training would be supported by the federal government because people well prepared to study the toxicology of a large range of chemicals were essential for the health of the nation.

Although the Society of Toxicology was founded in 1961–1962, rapid expansion of training programmes in toxicology, except for a few pioneer programmes, began in the 1970s, and accelerated growth continued into the 1980s. The impetus for the rapid expansion of toxicology training came from several sources. The NIEHS, the National Institute of Occupational Safety and Health (NIOSH), the Environmental Protection Agency (EPA) and other federal agencies were established because of concern over the impact of chemicals on health and the environment. These agencies required trained personnel to carry out their functions, and created a demand for people which could only be accommodated by the development of new training programmes.

A stimulus of greater significance to the scholar resulted from a pattern of development of thought in the biological sciences which made inevitable the eventual coalescence of the discipline of toxicology. Toxicology is the study of the adverse effects of chemicals (and radiation) on biological systems. The study of adverse effects suggests that there must be a pre-existing understanding of normal biological functions before abnormal effects can be recognized and studied. The evolution of medical training in this country during the twentieth century led to the establishment of anatomy, physiology, biochemistry, pharmacology, microbiology, nutrition, immunology and pathology as the basic biomedical sciences. The accumulation of information on anatomy, which occurred over many centuries, led gradually, in the nineteenth century, to the development of physiology and then biochemistry. Pharmacology could not function as a separate discipline until the database in physiology and biochemistry, which utilized chemicals as tools to study biological functions, expanded to a critical level. Pathology was an extension of anatomy, which concentrated on a number of abnormal biological events, including effects of chemicals. Certainly, the development of new drugs required a full examination of their toxicological potential. Thus, after sufficient knowledge in the basic biomedical sciences was attained, and the practical examination of the potential toxic effects of chemicals became routine, the discipline of toxicology emerged.

Those involved in training graduate students in toxicology realize that their job is to launch the student into a lifetime of learning. The late Arnold J. Lehman, of the US Food and Drug Administration is credited with stating that 'You too can be a toxicologist in two easy lessons, each of 10 years.' (Gallo, 1996). In this discussion we will emphasize the training of graduate students and postdoctoral fellows. The average time required for formalized training is about six to eight years as a graduate student and then as a postdoctoral fellow. The bulk of the remaining 20 years of training takes place as part of career development.

2 SITES FOR TOXICOLOGY TRAINING

Historically, toxicology training programmes were housed in established pharmacology departments in medical and pharmacy schools. However, toxicology programmes are becoming increasingly more interdisciplinary and are often found as joint programmes incorporating faculty staff from a wide variety of departments in medical schools, schools of pharmacy and schools of public health. Listings of toxicology graduate programmes can be found by going to the web sites of the major toxicology societies including the Society of Toxicology (*www.toxicology.org*), the Federation of European Toxicologists & European Societies of Toxicology (*www.eurotox.com*) and the British Toxicology Society (*www.thebts.org*).

3 AIMS OF TOXICOLOGY TRAINING PROGRAMMES

The principal aim of any toxicology training programme is to offer a curriculum which will prepare the student to function at the highest possible level as a toxicologist. Although some toxicology training is available at the undergraduate level, the majority of toxicology training venues are in graduate schools. Each of the biomedical sciences can be approached at either the fundamental or the practical level, and both approaches are needed by society. If we recognize that the toxicologist is a scientist and that scientific training requires both the assimilation of known information and research to increase our knowledge, then the training of toxicologists requires a series of courses plus an opportunity for the student to learn the principles of research by performing a research project. Some aspects of the curriculum may be devoted to applied research in toxicology, but the major effort should be in basic research, preferably at the mechanistic level, which will expand our fundamental understanding of the biological responses that underlie toxicology.

Graduate training in toxicology is, of necessity, broad based. Students must be exposed to the basic biomedical sciences and chemistry because these disciplines will be the tools of their trade regardless of where

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their career paths lead them. In the course of graduate training, students will become expert in their field of study and may develop other areas of expertise as their careers develop. Although they need not be expert in the approaches of all of the biomedical disciplines, they must be sufficiently knowledgeable to know when to apply otherwise unfamiliar methodology necessary to solve specific problems. Consultation with specialists in other fields may be essential to complete the work, but the toxicologist must have sufficient knowledge to ask the proper questions, to understand the results of such studies and to involve the results in the next stage of the work.

To ensure that programmes succeed in meeting their aims there must be a faculty dedicated to graduate education. Often there is a Director for graduate education which may, or may not, be the department chair. The Director, with the help of the faculty, administers the programme by overseeing recruitment of students, admissions, record keeping, progress towards the degree, examinations, grievances and the degree-granting process. The Director is usually responsible for much of the fund-raising activities which go to support the students. Thus, obtaining teaching assistantships, externally supported fellowships, travel funds and other needs of the students falls, in large measure, on the shoulders of the Director.

Perhaps of greatest importance is the necessity for the Director to ensure that students select laboratories of mentors where they can best proceed through the process of earning the degree. Usually this requires a meeting of minds between mentor and student regarding the nature of the research project, but the issue of compatibility between the two protagonists should not be discounted. The mechanisms by which student–mentor relationships are established vary among programmes. They almost always involve a trial period in which the student spends a limited amount of time in the prospective mentor's laboratory, to permit each to determine whether a successful research partnership can be established. Once a collective decision involving student, mentor and Director is made, the student relies on the mentor for advice on courses, research, and career planning.

Most mentors follow the careers of their students and stand ready to offer assistance long after they have left to develop their own careers. Senior professors are often called upon by those seeking to fill a variety of positions in toxicology. The professor then has the opportunity to recommend former students to positions of advancement. The interplay is a two-way street; in some cases, former students having advanced in their careers may find ways to help enhance the programmes from which they graduated through teaching, providing entrees to obtaining financial assistance, offering positions to more recent graduates, and so on. The concept of the 'doctor father' in German universities, which grew out of the citing of scientific lineages, exemplifies the lasting relationships that are possible between student and mentor.

4 PREREQUISITES TO GRADUATE TRAINING IN TOXICOLOGY

Those students best prepared to study toxicology at the graduate level will have earned a bachelors degree in chemistry or biology. In addition to introductory study in chemistry, the student will benefit from courses in analytical, organic and physical chemistry. A course in biochemistry is helpful but can be taken at the graduate level. In biology, a general introductory course followed by electives in areas such as comparative anatomy, histology, cell physiology, and so on, would be helpful. Students are urged to study physics and mathematics in preparation for graduate school because of the rapid advances in application of these disciplines in modern toxicology.

5 REQUIRED GRADUATE COURSES

Although most curricula in toxicology require a basic course in general toxicology, it need not be the first course taken in graduate school. Most students require graduate-level courses in physiology, biochemistry and molecular biology. The area of biostatistics and experimental design and interpretation of results should be part of every graduate programme. Finally, the NIEHS has mandated that each programme offer a course in ethics as they apply to scientific research. Many programmes require pathology, which would be most helpful if it could precede or run in parallel with the toxicology course. Programmes associated with institutions devoted to medical training often ask students to study pharmacology because of the significant overlap between the two disciplines.[a] Other more relevant courses might be substituted for students in nonmedically related areas; for example, students directed towards a career in environmental toxicology might include courses more directly related to their interests, such as ecotoxicology. All students should be required to hone their communications skills by participation in seminar courses.[b]

The most important course offering is the first course in general toxicology, which goes by a variety of names in various institutions and is required of all students. Among the subjects that it must cover to adequately introduce students to toxicology are:

1. A brief discussion of the history and scope of toxicology.
2. A discussion of general basic principles of toxicology.

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3. Toxicodynamics; that is, what does the chemical do to the body?
4. Toxicokinetics; that is, how does the body deal with the chemical?
5. A survey of the effects of various chemicals.
6. A description of the specific toxic responses of various body organs?
7. Venues; that is, toxicity observed in the home, workplace, environment, and so on.
8. Applied toxicology: safety evaluation and risk assessment.
9. Mechanistic toxicology.

The extent to which any of these areas is covered will depend upon the time allotted to the course, the availability of advanced courses to cover any of the areas in detail, the interests of the faculty, and so on. In every course in toxicology, however, there should be sufficient emphasis given to the paracelsian admonition:

> *In all things there is a poison, and there is nothing without a poison. It depends only upon the dose whether a poison is a poison or not…*
>
> *(Jacobi, 1988)*

6 ELECTIVE COURSES

The selection of elective courses will depend upon which courses are available in any given institution, but can generally be separated into those in toxicology versus those in allied disciplines. Many students opt to take advanced courses in chemistry or the biological sciences. Advanced courses in chemistry are many and varied. Course selection is often driven by the research project in which the student is engaged. In the biological sciences, graduate students in toxicology should become familiar with microbiology, immunology and genetics.

Electives in toxicology depend upon the expertise of the faculty and may encompass such areas as neurotoxicology, pharmacogenetics/toxicogenetics, pesticide toxicology, and so on. Electives are also a mechanism for introducing students to the more applied areas of toxicology, such as safety evaluation, risk assessment, public health and the environment, and so on.

An important message for any student is that the best thing you can learn in graduate school is how to learn. Courses teach what is currently known, but cannot predict the future. Once the student has initiated an independent career, most learning will be self stimulated and will not involve attending classes. To be sure there are many opportunities for formal continuing education. Both the Society of Toxicology and the American College of Toxicology offer continuing education courses at their annual meetings. Ultimately, however, most toxicologists should keep up with recent advances through their own efforts. Therefore, the value of time spent in classes, beyond learning the basics and enough advanced information to permit the beginning of a research project, must be balanced against the value of time spent in the library and the laboratory.

7 QUALIFYING EXAMINATIONS

Advancement through graduate school is characterized by study and research on the part of the student, and evaluation of the student via a variety of metrics. Courses require that students perform well on objective exams, essay-type exams, or in the writing of research papers. Despite hurdling these barriers, in most programmes students must pass qualifying exams. The requirements for the Master of Science and the Doctor of Philosophy degrees may be dissimilar. Thus, a single examination for the MS often suffices. For the PhD, the process is usually more complex. Often there are two sets of exams: written and oral. Some programmes use a comprehensive written exam at the end of the first year of graduate school to determine whether the student should continue. Some offer the comprehensive examinations as a prelude to the oral examination to determine whether the student is prepared for the oral. Other programmes do not have written comprehensive examinations. There are a number of variations on this theme.

For many years students were required to pass one or more examinations in a foreign language, but that custom has become less popular as English has assumed a predominant position as the international language of science.

The oral examination is usually the point of decision with respect to whether or not the student becomes a candidate for the degree. There is more riding on the oral examination than on any other examination in the student's career. As a result, students study long and hard to prepare for the examination. Examination committees should inform students embarking on the candidacy process regarding faculty expectations. Toxicology is among the broadest of the biological sciences, and in each of the subdisciplines within toxicology, deals with an extensive database. Furthermore, because of the breadth, much of the information that students should know comes from other areas such as biochemistry, physiology, pathology, and so on. Thus, the oral exam may become a free-for-all, with faculty members asking questions on subjects that range from a discussion of dose–response curves to estimating the weight of Saturn (the latter as an example of how a professor once thought that a student's reasoning powers might best be measured.) A wise faculty will establish with the student the limits of required subject areas and will find a way to learn what they must from the student within the defined area.

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There are many methods for defining the range of the oral examination, but one general form which can have many benefits for the students can be addressed here. In some universities, students, with the advice of their mentors, submit to the Director, early in their graduate-school careers, a list of, perhaps, 10 hypotheses, or propositions, which together define a broad area of toxicology, but result in some delimiting of the total range of information for which the student is responsible at the time of examination. An example might be: 'The lethal effects of parathion can be explained solely on the basis of cholinesterase inhibition.' The student would then collect all of the information in the literature on this question and would defend or attack the proposition in a written paper which is presented to the members of the examination committee in advance of the examination for use as the basis of questioning. It has been demonstrated that 10 such papers prepared over a two-to-three-year period offers the committee a chance to determine how well the information has been collected, evaluated and used in creative decision making by the candidate. The proposition method permits a narrowing of the range of areas on which the student will be questioned but permits the faculty to delve as deeply into the subject as seems warranted to make candidacy decisions.

Success in the oral examination is often difficult to attain for some students because of the significance of the exam to their future careers and perceived trauma they experience during the process. Often, excellently prepared students fail to measure up to the expectations of the faculty because of nervousness, or forgetfulness induced by the pressure-packed nature of the examination. To help prevent, or to overcome, the impact of emotions on examination performance several approaches have been used. Often, graduate students within programmes offer a chance for candidates to participate in 'mock orals', in which their fellow students, or faculty members, act as the examining faculty and ask the questions they anticipate will be asked at the exam. If students appear to be in great difficulties during an exam the committee may decide to delay a decision and offer the candidate the option of completing the examination at a later date. The student is expected to complete the examination successfully at the continuation. Despite the recognized shortcomings of the oral exam system it continues to play a significant role in graduate education, both as a valuable mechanism for evaluating the qualifications of a student, and for the sense of accomplishment engendered in the successful candidate.

8 THE RESEARCH EXPERIENCE

The essence of a PhD programme in toxicology is to train people to perform scholarly research. Although the requirements of many MS programmes can be met by having the student write a library-based paper without doing a research thesis, many others require a small-scale research project leading to a written thesis. For the PhD an extensive research project is required. The subject of the research is decided upon through discussions between the student and the mentor. Some students enter graduate school with the intention of working in a specific area of toxicology, or with a specific mentor. High-quality research training can result from work in any area of toxicology. Since most students select their mentor on the basis of compatibility, they naturally work in the area of interest to the mentor, and many remain in this area long after they have completed their degrees and move on.

The components of research training include an appreciation of the concept of a hypothesis and how hypotheses are developed, selecting an experiment model, use of appropriate controls, designing experiments to test the hypothesis, data evaluation, including biostatistics, and interpretation of the data. During the training of graduate students, the mentor should provide opportunities for the student to sharpen those communication skills necessary to report the results of a research project. These would include writing of abstracts and research papers, oral presentation of results, and preparation of posters showing the work for presentation at scientific meetings. The opportunity to meet with others working in the same field should be made available by sending the student to meetings of scholarly societies.

It is during the development of the dissertation that the student should be imbued with a concept of scholarship. The importance of gaining a complete understanding of the literature in the field under study before planning a full-scale project is of the utmost importance. It is all too easy to unknowingly perform studies which have been done previously and already appear in the literature. Students tend to rely on computerized databases for literature searches. Although some online databases contain research performed prior to 1960–1970, in most cases the conscientious student has no choice but to leave the safe confines of the laboratory, venture into the challenging culture of the library, and rummage around in the stacks for what may be valuable nuggets of otherwise lost information which could be of great value in the student's research.

Hypotheses derive from creative evaluation of existing literature and seminar presentations, discussions involving the mentor, and often with other members of the research team in the laboratory, and the performance of pilot experiments to indicate whether or not there is a reasonable chance that the project will succeed. Once the hypothesis is established it is incumbent upon the student to develop an experimental design to challenge the hypothesis. Consultation with the mentor will enable the student to determine the feasibility of the planned studies. If the hypothesis is reasonable, it is necessary to ensure that it is technically possible to perform

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the experiments in the specific laboratory chosen for the research. Research costs, availability of necessary instrumentation, and, where necessary, help from other people, are issues that must be settled before a research project can begin. Assuming that these problems can be solved, the mentor must ask whether the student has selected a suitable problem. Is it sufficiently challenging to be worthy of the PhD upon completion? Can it be completed within approximately three to five years? The mentor must also consider the issues of costs, facilities and other people in the laboratory. These issues must be satisfactorily addressed to engender success in a graduate student thesis project.

In many universities the mentor–student relationship is complemented by a thesis committee, which follows the student through the process, meets with the student for regular updates, acts as a quality control mechanism, eventually decides when the project is completed, and sits as thesis defence committee. Outside reviewers are often asked to participate at some stage in the development of the thesis to act as impartial referees. An important job of the committee is to ensure that the student completes the thesis in as short a time as possible. Graduate disciplines often have cases of students who have been either improperly advised or insufficiently stimulated, who subsequently remain in graduate school for inordinately long times. The thesis committee is an important mechanism for maintaining an acceptable rate of progress towards the degree.

In toxicology the aims of the specific piece of research may vary considerably. Until the early 1980s most research in toxicology was aimed at describing the effects produced by chemicals administered via any of several routes to one or more species of animals. End points such as lethality or pathology resulting from acute or subchronic treatments were common. With the emphasis placed on cancer prevention in the USA, the National Toxicology Program was developed specifically to expose animals to chemicals for a lifetime. After the death of the animals they were examined for signs of cancer. Chronic exposure studies of this type were less commonly performed in university laboratories because of the cost and the need for specialized personnel and facilities. Nevertheless, shorter-term carcinogenicity studies were performed in a number of laboratories.

The impetus towards maturation of toxicology as a discipline came from many sources, but the emphasis by toxicologists on mechanisms of toxicity served to demonstrate that toxicology could contribute to the basic understanding of biological functions. The role of metabolism of xenobiotic chemicals, especially the formation of biologically reactive intermediates, was a significant factor in the development of mechanistic toxicology. Studies of the interactions of chemicals with enzymes or nucleic acids and the ensuing adverse effects led to a better understanding of structure–activity relationships and our ability to predict some types of toxic effects. Currently, many mechanistic studies revolve around the problems of mutagenicity, carcinogenicity, oxidative stress and mechanisms of cell death. The enrichment of mechanistic studies by the application of the principles of exposure assessment represents an ideal extension of traditional mechanistic toxicology and has been used to develop animal models of chemically induced diseases. These approaches are being used in university laboratories with the goal of understanding the underlying principles which lead to the production of adverse effects by chemicals on biological systems.

In any scholarly discipline the ultimate measure of quality research is the contribution it makes to understanding of the most fundamental issues with which the discipline is concerned. In the course of their training, graduate students play a significant role in the scientific process. They share in the development of hypotheses, perform the experiments, and focus on the results. Thoughtful interpretation of the results will lead to the emergence of new avenues of research which will benefit science, but which also enrich the training of the student and will help the student mature as a scientist.

Some students plan on careers that do not focus on laboratory research. There is a great need for toxicologists in regulatory agencies in federal and state government, in regulatory departments in industry, as consultants, in law firms, and in a number of other occupations. Nevertheless, in any work as a toxicologist it will be important to review the results of both applied and basic research. Research training is the best way to prepare a student to read and understand the results of other researchers' work. The more rigorous their research training, the better the job they can do in evaluating and criticizing research results which apply to the problems they are attempting to solve.

9 STUDENT PRESENTATIONS

At each stage in the training of a student in toxicology it is important that they learn how to communicate with their peers. Early in graduate education students should be asked to participate in exercises which will give them the opportunity to communicate is several ways. For example, the short-paper form of delivery provides an excellent training ground. Many societies offer their members the opportunity to publish an abstract of their work in advance of a meeting and then present the work in a 10–15 minute paper accompanied by a visual presentation to illustrate the data. This format makes for a good first year course for graduate students. They may work on a topic, the data for which they may find in the literature, learn how to write an abstract, prepare appropriate slides and make their presentation to other students and faculty. In the course of this activity they will gain ease in making presentations before their peers,

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learn to write concise reports and become familiar with ways of presenting their data. Learning how to make presentations in the classroom situation will help prepare them for presentations at scientific meetings.

When students go beyond the first year or two in graduate school their seminar responsibility should take on a different form. The research that they are performing for inclusion in their theses will progress from year to year, and annual oral presentations of their data in 20–30 minute reports provide a useful training tool. One approach is to have all advanced students present their progress reports in symposium format. It is an opportunity for a programme to have a day devoted to student presentations attended by the entire faculty and invited guests. The students may be asked to prepare an extended abstract to be given to the audience in advance of the presentation. The students will have benefited by having prepared short presentations earlier in their careers and can make use of that experience in the symposium format.

The first presentation by a student at a society meeting these days is most likely to be a poster presentation. Here the student presents the results of thesis research in a series of panels mounted on a board that is often 1.2×1.8 m. The poster usually contains an abstract of the work, a brief note on methodology, several panels on data and a summary statement. The poster is intended to generate discussion between the presenter and colleagues who stop to ask about the work as they stroll through the poster presentation area. Under these circumstances the student meets the questioner one-on-one, rather than as a member of a large audience. These can be much more difficult interactions since they often require detailed information at the fingertips of the student. In some cases the poster sessions are followed by a discussion period where the essential information of the posters is briefly reported by the presenters. Thus, poster sessions and poster-discussion sessions present a challenge to the student which is different from that posed by the short platform presentation or the longer symposium presentation.

The most important type of communication is the written report intended for peer review. A published report will enter the literature and become available for reference in perpetuity. As a result it is the most critically reviewed and criticized. During training the student should be prepared not only to perform the research project with excellence, but should also be trained to prepare a paper for publication at the highest possible level. The writing of the thesis, albeit a somewhat longer item of prose, should set the standard for the writing of papers which will emerge from the thesis work. Students who perform in exemplary fashion in the writing of papers may be asked to participate in the preparation of review articles. Reviews in excellent journals also undergo intensive peer review and serve as an important mechanism for deriving new ideas and research pathways based on compilation of reports from many sources. The authorship of a review article by a graduate student is unusual but can be an important step in developing a successful scientific career.

10 APPLIED TOXICOLOGY

Toxicology training as discussed above involves a series of classroom exercises and the completion of a basic research project. Toxicologists trained in this way are well adapted to careers in basic research. Many others enter careers in applied toxicology for which their university training did not prepare them directly. To be sure, learning how to understand and do science is critical for any career in toxicology. However, learning methods in areas of applied toxicology requires additional course work or on-the-job training. Historically, chemical and drug companies developed new chemicals and evaluated both their potential beneficial biological effects and their adverse effects. More recently the cost of toxicological studies has motivated companies to perform safety evaluation in laboratories whose main business is safety evaluation. Thus, the companies which develop the chemicals employ toxicologists termed ‘study directors’ to design, monitor and evaluate the toxicology studies while performed at remote laboratories. They require knowledge in the techniques of safety evaluation and should be prepared to manage and to perform these studies.

Among their responsibilities will be issues relating to the utilization of experimental animals. Animal welfare issues are of great significance. Often large numbers of animals of different species are involved; times of exposure may vary from single doses to lifetime; pathology studies, performed after the death of the animals, must be analysed; detailed reports must be prepared, often accompanied by recommendations for further action. While some of these problems may have been the subject of classroom discussion, and some similar types of studies may have been included in the thesis project, more frequently studies of these types are completely new to the recent graduate. Thus, both employee and employer must realize that advanced training, especially when the specific needs of the company must be met, is the responsibility of the company.

The processes that are termed risk assessment involve utilization of data gathered from many sources regarding the toxicology of chemicals. Risk assessment is the attempt to predict the risk to health resulting from exposure to chemicals at specific dose levels based on prior observations in animals, and when available, humans. Thus, toxicologists frequently become involved in the risk assessment process because of their training in the interpretation of the data produced in toxicology studies. The use of graphical techniques to analyse data

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leads biometricians to play an increasingly greater role in the interpretation of toxicological data. They use their understanding of mathematical functions to influence the interpretation of data. Their input is particularly critical when it is necessary to extrapolate from data observed under laboratory conditions to the real world situation in which people are exposed to chemicals. For example, given a specific response to a chemical in an animal study involving specific doses via a defined route, for various lengths of time, what might the response be in humans in a specific exposure scenario? The accuracy of these predictions depends upon the biology of the system, but the extrapolations are frequently driven by mathematics rather than by biology because we lack sufficient understanding of the underlying mechanisms. Thus, a common approach is to fall back on mathematics, making use of a variety of assumptions, rather than to do more experiments to verify the accuracy of the extrapolation. Regardless of the virtues or shortcomings of the process, toxicologists are frequently involved in risk assessment. Some may have had classes in risk assessment, but these are insufficient to prepare them for performing such an exercise and to appreciate the responsibility entailed. This is another example of where on-the-job experience is essential.

There are training locations for forensic and clinical toxicology, which are often separate from academic toxicology training programmes. Forensic laboratories are concerned with toxicological issues relating to legal matters. Their specialty is analytical chemistry. Among the most frequent calls upon these laboratories are requests to measure blood alcohol concentrations in samples from people involved in traffic accidents. Measurements of body fluids or tissues are often made in questions of poisoning. The forensic toxicologist is usually associated with a government laboratory such as a city, county or state medical examiner. The FBI has one of the largest and most complete forensic laboratories. Forensic toxicologists are frequently called upon to provide testimony in court regarding their findings. There are relatively few sites for training in this field and there has for some time been a critical shortage of personnel with sufficient expertise to work in these laboratories.

Clinical toxicology is a medical specialty concerned with the detection and treatment of poisoning in people. Victims usually arrive in the emergency ward of a hospital in a state which suggests that they have either administered a poisonous level of a chemical or drug to themselves or have been poisoned by someone else. The clinical toxicologist must decide on the basis of whatever evidence is available (i.e. interview and observe the patient, have evidence of a container in which the material was found, physical examination, laboratory analyses, etc.) and make decisions regarding treatment of the patient. The forensic laboratory plays an important role as a resource to which the clinician can turn to learn about the specific chemical, the dose, the blood level, and so on. Since this is a medical specialty, training is not usually performed in academic toxicology training centres, but as part of advanced medical training at the intern or resident level, and board certification is available to those who wish to make a career as clinical toxicologists.

11 THE POSTDOCTORAL EXPERIENCE

Toxicology training does not end with the award of the PhD. Many graduates, upon completion of the degree, take positions in companies, in government agencies, with environmental groups, and so on. In each of these venues they must undergo training to meet the specific needs of the job to meet the expectations of the employer. For those toxicologists who wish to enter a career in which research is a major component, most employers require postdoctoral training.

The postdoctoral fellowship offers the trainee an opportunity to become a full time researcher under the aegis of an experienced investigator. Postdoctoral mentors are selected by the trainee on the basis of the area of research in which they work and on the excellence of their productivity as scientists. Their laboratories may be in various departments or schools in universities, in research institutes, or in government agencies. Popular locations for postdoctoral training in the USA include those institutions awarded NIEHS training grants, or laboratories within the various National Institutes of Health. A variety of research centres, such as NIEHS Centers of Excellence offer postdoctoral training opportunities. Additional opportunities can often be found in more specialized environments such as cancer research institutes or environmental health centres. In any of these locations the postdoctoral fellowship is intended to provide the fellows with the opportunity to demonstrate their scientific creativity and productivity in an environment in which they are totally devoted to research.

The postdoctoral experience begins with the selection of a specific problem, which in most laboratories is one of a group of problems in the same general area. In an active laboratory one often finds technicians, graduate students and postdoctoral fellows. Although each works on a separate problem, proximity leads to communication which helps all in the group. Learning techniques and approaches from each other enhances the total output of the laboratory. It is clear that the success of a laboratory is related to interaction among the various participants, and the postdoctoral fellows usually take a leading role in such interactions. A frequent result of these deliberations is often to work collaboratively with one or more other members of the team. The increasing complexity of research in toxicology, and the need for the application of advanced techniques, which only some members of the team can contribute, may require the

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assembly of a group of workers each of which brings essential talents to the joint solution of the problem. Learning to operate within a collaborative framework can be an important aspect of career development for a toxicologist.

Ideally, a postdoctoral fellow should spend two to three years in training, at which point it is time to seek an independent position. Nevertheless, it has recently become more common for postdoctoral fellows to complete more than one postdoctoral training position in order to be competitive for tenure-track positions at many universities. This trend has arisen partly because of the increased complexity and breadth of science involved in the profession of toxicology, as well as the increased competition for research funds. In order to be competitive for tenure-track positions, it is advisable that postdoctoral fellows demonstrate the beginnings of independence as a scientist by successfully competing for postdoctoral research fellowship awards. There are a variety of sources for these awards from government agencies and private foundations. The experience of writing applications for postdoctoral fellowship grants is not only valuable to those with the goal of achieving a faculty position but to those that have other career plans. The ability to convey a hypothesis and an experimental plan in a written format and to then have the document undergo peer review is a valuable experience in creative thought that forces the applicant to critically evaluate the literature, identify gaps in current knowledge, and portray the importance of the research proposed as it relates to the advancement of the area proposed for study. At this point the postdoctoral fellows leave the safety, the support, the financial backing and the stipend paid by the mentor and must fend for themselves. Mentors usually help in securing positions for postdoctoral fellows, but a variety of placement services are available to assist in the job-hunting process. Clearly, those fellows who have trained in highly respected laboratories and have been productive during the training period will have the upper hand in the job search. Upon securing a position beyond the postdoctoral level the toxicologist must now direct considerable effort towards securing research funding and developing an independent laboratory. It is the most challenging time in the career of toxicologists, and success will in large measure depend upon the quality of training that they have enjoyed.

12 CONTINUING EDUCATION OF THE TOXICOLOGIST

Lehman's statement that 'You too can be a toxicologist in two easy lessons, each of 10 years' (Gallo, 1996) brings to light the necessity of continuing education, particularly for the second part of this 20 year training. Continuing education comes in a variety of forms, but is most often associated with specialized classes offered at the annual meeting of the various toxicology societies or other scientific societies to which the toxicologist belongs. However, this is not the only form of continuing education. Keeping abreast with the scientific literature, attending meetings and presenting data, networking with colleagues, and performing service to the scientific community in the form of grant and manuscript reviews all encompass aspects of continuing education. Another important form of continuing education is mentorship. For professional toxicologists in their first years of the profession, mentorship by a senior toxicologist, be it formal or informal, is an important aspect of career development that is often overlooked. These types of mentorships provide an invaluable source of advice and perspective as the toxicologist continues along their career progression. Furthermore, it lays a foundation and provides an example for the more junior toxicologist to one day become a mentor to others.

13 TRAINING IN OTHER COUNTRIES

13.1 Great Britain

An example of toxicology training in Great Britain is taken from the curriculum at the University of Surrey; here students have the opportunity to initiate their training in toxicology at the undergraduate level. Those students concentrating in biochemistry may elect to take courses in drug metabolism and pharmacokinetics, in mechanisms of toxicity, regulatory toxicology, pathology for toxicology, practical toxicopathology and forensic toxicology. Following the Bachelors degree, students may enrol in the Master of Science in Toxicology programme. Progress towards the degree requires 338 hours of lectures, 125 hours of laboratory training and 400 hours devoted to laboratory research on a dissertation project over a total of 44 weeks. The course includes tutorials, visits to industrial laboratories and utilizes a variety of assessment modes. The course work is varied, ranging from whole animal studies to molecular biology, as well as regulatory affairs and safety evaluation. The student wishing to go on to the PhD registers for the Master of Philosophy, completes the course work and writes a report on the progress in a laboratory research project, which is used to determine whether or not the student will be permitted to transfer to the doctoral programme. Upon approval of the report the student becomes a candidate for the PhD, which is awarded upon completion of the project. The total time required is usually about four years.

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13.2 Germany

Historically, toxicology in Germany has been closely associated with medical pharmacology. Toxicologists tend to be members of the German Society of Experimental and Clinical Pharmacology and Toxicology (DGPT). Indeed, a subdivision of the German Pharmacology Society is termed the German Toxicology Society, for which a separate web site has been established. In recent years the society has developed a mechanism by which individuals who hold doctorates in medicine, veterinary medicine, chemistry, biochemistry, biology, pharmacy or related disciplines can earn a certificate by which they are named Expert in Toxicology, DGPT. The certificate is awarded on the basis of an examination. However, to be eligible to take the exam the applicant must have had at least five years of experience in a toxicology laboratory, plus comprehensive knowledge in 1 area of toxicology, broad knowledge in 2 other areas of toxicology and basic knowledge in 15 areas of toxicology. They must submit three independent peer-reviewed papers or assessments of their professional skill. To meet the educational objectives of the programme, courses in specific areas of toxicology are offered in universities in Germany periodically. The courses run from three to five days. The areas covered by the courses include: animal welfare and techniques in animal experimentation; statistics and experimental design; principles of chemical and physical analysis in toxicology; pathological anatomy and histology of experimental animals; general principles of toxicology and target organ toxicology; drug metabolism and principles of toxicokinetics; principles of cellular and molecular biology and toxicology; chemical mutagenesis; chemical carcinogenesis; reproductive toxicology; immunotoxicology; epidemiology; ecotoxicology; regulatory toxicology. The German approach, unlike the American approach, embodies both training and professional certification.

More recently, the European Union has adopted a plan to ensure minimal health risks from chemicals manufactured or imported into Europe in amounts greater than a ton. The plan, which went into effect on June 1, 2007, is termed REACH (registration, evaluation, authorisation and restriction of chemical substances), and is managed by the European Chemicals Agency, under the auspices of the European Union. For each chemical a registration dossier containing the identification and the management of potential risks is prepared. This is a substantial undertaking, and as a result an increased effort at training toxicologists to help meet the requirements of REACH has begun. Training grants have been awarded at several German universities for the establishment of Masters degree programmes specifically designed to train people to meet the need of the REACH legislation. It is similar in many ways to the earlier programme cited above but is conducted within a single university and students with more diverse backgrounds may be eligible for participation.

14 BOARD CERTIFICATION

Although the formalities of earning the degree are somewhat different when comparing the American, the German and the British approach, the subject matter is largely the same. The German system carries with it the opportunity to achieve board certification. In the USA most toxicologists can apply for and earn board certification from the American Board on Toxicology by passing an examination. For more senior toxicologists who have an established career and evidence of productivity and accomplishment, the Academy of Toxicological Sciences offers the opportunity of certification on the basis of peer review.

15 SOME QUESTIONS ON RECENT DEVELOPMENTS IN TOXICOLOGY POSED TO COLLEAGUES

During the preparation of the revision of this chapter some questions arose regarding the status of toxicology as it has evolved over the past decade. Colleagues were queried with respect to their evaluation of how toxicology has fared in their countries. They were asked (i) whether there has been an increase or decrease in toxicology training programmes, (ii) what is the outlook for employment and career-building opportunities for toxicologists, and (iii) which areas appear to have the greatest need for toxicologists? Although the number of colleagues approached and the number of responses was small, the answers were informative.

It would appear that in countries where there were previously established training programmes there has been little growth, and in some cases, declines in the number of programmes. To some extent the cause is a decrease in funding and in other cases shifts in emphasis within the biological sciences. In some situations, where a single department had chairs in both pharmacology and toxicology, restructuring of resources often resulted in greater emphasis placed on pharmacology.

Despite the expected need for many toxicologists to work in the area of safety assessment, advances in biology related to genomics, proteomics, information science, and so on, have begun to play a significant role in the training of toxicologists. It was natural in the course of these communications to raise the issue, with some of the respondents, of the degree to which training programmes need to balance education in the new 'omics' with training in 'classical toxicology'. It is clear that there is a need to train students in the most modern technology

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needed for toxicological problem solving. We are still in the midst of understanding how the data obtained with these technologies can be interpreted with respect to whole-animal physiology and pathology. History has shown that the emergence of new technologies when applied to toxicology and pharmacology has ultimately led to significant advances, and there is no reason to doubt that eventually the same will be said for data derived from 'omics'.

16 THE LITERATURE OF TOXICOLOGY

Toxicology training in all countries seems to involve the classical approaches which include classroom studies highlighted by lectures and discussions, practical laboratory experience, and exercises in which students read literature and either write reports or present them orally. These are frequently termed seminars, and are an essential part of toxicology training. Although it is important that the student can write well or make oral presentations which are clear and understandable, the basis of these reports is the literature which the student has read, assimilated and is prepared to offer as evidence of having learned the material.

The literature of toxicology over time reflects a parallel growing maturity of both the biomedical sciences and toxicology. Although much has been known about the descriptive toxicology of pure chemicals and mixtures since ancient times, it is only with the past few decades that important advances in our in-depth knowledge of mechanisms of toxicity have been uncovered. Much of that information can be accessed via online databases such those of the National Library of Medicine; that is, Medline, Toxline, and so on. The explosion in online databases acted as a tool for the rapid expansion of the scope of toxicology. Formerly a literature search was conducted in the several journals with which the investigator was familiar. Asking a question to an online database often brings in references in journals heretofore thought to be outside the mainstream of toxicological publications. Although most studies published before the advent of databases must be accessed in library stacks or storage areas, there are many instances of journals and databases now scanning older documents; for example, the Journal of Pharmacology and Experimental Therapeutics has made available papers published as far back as 1909. Regardless of whether the literature can be reached online or in paper form in the library, students should trace the development of thought in the field in which they work back to its origins. Students should realize that, although technology may change with time, the thought processes behind the development of hypotheses, experimental design, and the process of gaining insight into the problem based on data from well-designed studies can be observed in the work of any era. A significant admonition worth offering to every new student is 'Science did not begin the day you walked into the laboratory'.

17 TRAINING TOXICOLOGISTS FOR THE FUTURE

Unfortunately for people who plan structured curricula, changes in the sciences occur at rates faster than they can plan. Thus, within the past few decades the basic medical sciences have largely lost their distinctive boundaries in research. The result has been mergers of departments and changing modes of teaching the basic subjects. Since toxicologists have always had to deal with approaches to problems which crossed these classical boundaries, they are prepared to take advantage of changing curricula to the advantage of their students. Furthermore, with advances in the understanding of the molecular basis of physiological function it is possible to apply the techniques generated by these exciting successes to the solution of toxicological problems. Toxicology has been called 'a borrowing science'. Scientists in any given discipline have always 'borrowed' techniques and approaches to research from each other. Borrowing of techniques from other disciplines was less common. In toxicology, however, it was the rule because there were few, if any, methods which were strictly tied to our discipline. Thus, in the current climate where the disciplines have, to a large extent, merged, the toxicologist is quite at home in 'borrowing', for example, the tools of the molecular biologist for the solution of fundamental problems in toxicology. Thus the first, and most important, message related to the training of toxicologists in the future is that we must continue to teach students broadly and to be prepared to accept new ideas in science and apply them to understand mechanisms of toxicity, as well as to apply them to the process of safety evaluation.

Toxicology, as well as its sister science, pharmacology, have been termed the study of poisons. Both toxicologists and pharmacologists are proud of that designation because the results of their studies have been a better understanding of how chemicals can cause damage to biological systems as studied by toxicologists, and the development of new and better therapeutic agents by the pharmacologists. In many cases ground breaking efforts in the nineteenth and early twentieth centuries were based on studying the effects of known poisons such as strychnine, eserine (physostigmine), nitrogen mustard, and so on. While the breakthroughs resulted in practical advances in the disciplines, they were accompanied by an increasing understanding, in these cases, of the physiology of the spinal chord, the mechanism of action of cholinesterase, and certain aspects of the biology of cancer cells. Many other examples have shown

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that as we have extended knowledge in our own discipline we have made significant contributions to the study of biology. Thus, the training of students in toxicology should include ensuring that they understand the potentially greater significance of their results that may go beyond administering a given chemical to an animal in a given protocol followed by the observance of specific responses. The student should be aware that the responses observed are reflective of the underlying physiology of the animal and may shed light on those mechanisms. In the study of cancer, early workers had no trouble identifying tumours that resulted when they painted a solution containing any of several polycyclic aromatic hydrocarbons on the backs of mice. The mouse skin assay remains one approach to determining the carcinogenicity of chemicals. However, investigations of how and why the tumours occurred, in studies where the chemical was a tool in the experiment, rather than the object of study, have led to a clearer understanding of the process of carcinogenesis.

As the biological sciences in general and toxicology in particular advance, new forms of technology are developed on a frequent basis. It is possible to gauge advances in the biological sciences associated with the development of tools such electrophysiology, the use of isotopes and their measurement, spectroscopy and chromatography in their various forms, application of immunological techniques, and now flow cytometry and imaging systems, and the computer. Toxicologists have always grasped at these innovations. In the training of toxicologists they should be made aware of advances in technology which will help them now, and to also be receptive to new technologies as they develop, since it is clear that they will develop and can represent significant improvements in our ability to find solutions to problems in toxicology. However, excellent scientific studies have been performed by toxicologists long before the advent of new high-tech laboratory equipment became available. The training of toxicologists, while making use of new technologies, should concentrate on the knowledge base of toxicology, how to solve problems using the empirical approach, and how to apply creative thought to the interpretation of data, the development of new hypotheses, and the planning of new experiments to challenge our hypotheses.

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NOTES

a. Students planning a career in the drug industry should study pharmacology.

b. Furthermore, they should be required to write papers aimed at evaluating their skills in scientific writing.

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Toxicology of Chemical Warfare Agents

Robert L. Maynard and Robert P. Chilcott

CONTENTS

1 HISTORICAL INTRODUCTION

Throughout history, humans have sought more effective means of killing and disabling their fellow men. Stones, clubs, spears, bows and arrows, gunpowder, muskets, rifles, high explosives, machineguns, tanks, warplanes, rockets and nuclear weapons comprise an apparently unending catalogue of increasing military sophistication designed for the destruction of one's enemies, while exposing one's forces to decreasing risk. Accompanying this development of military hardware, the effects of which are based on the physical disruption of men or materials, has been a very much less marked development of chemical means of attack. Some chemicals have been used as a means of killing and others as a means of incapacitating. Recently, attempts have been made to stem the development of chemical weapons, but ensuring complete destruction of such weapons is difficult (Pearson, 2007).

Chemical weapons probably began with smoke and flame, and the hurling of various concoctions of pitch and sulphur (Greek fire) dates from classical times, as recorded in the Ebers' papyrus circa 1500 BC (Bryan, 1930). Irritant smokes were described by Plutarch, hypnotic substances by the Scottish historian Buchanan, compounds allegedly capable of producing incessant diarrhoea by classical Greek authors (SIPRI, 1971) and preparations containing the saliva of rabid dogs by Leonardo da Vinci (1452–1519) (Reprint Society, 1938).

At the time these authors were writing, chemistry and chemical technology were in their infancy, and the chemical weapons developed had probably only a marginal effect on the outcome of battles. However, during the medieval period the widespread use of poisons acquired, rightly, an evil reputation and the use of such compounds came to be despised by military men. The use of poisons was seen as running counter to the tenets of chivalrous conduct; this view has persisted and lies close to the root of the objections raised during the twentieth century to the use of chemical weapons (Haldane, 1925).

At the end of the nineteenth century the first international convention to address, among other topics, chemical warfare was held. This, the First Hague Convention (1899), led to a wide-ranging prohibition of the use of chemicals in war: 'The contracting powers agree to abstain from the use of all projectiles the sole object of which is the diffusion of asphyxiating or deleterious gases' (Prentiss, 1937).

Despite this prohibition, chemical warfare was deployed on a large scale during World War I, some 113 000 tonnes of chemical weapons being used in all (Prentiss, 1937). Lefebure (1921) reported that, on 9 March 1918, German forces fired some 200 000 mustard gas shells. This resulted in many casualties, but compared with the total casualties produced during World War I, the number of casualties from chemical warfare was low. This is shown in **Table 1** (Prentiss, 1937).

From the figures given in this table a number of deductions can be made: (i) the proportion of deaths attributable to chemical warfare during World War I was

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Table 1 Chemical warfare and other casualties during World War I

Country	Battle casualties due to gas				
	Nonfatal	Deaths	Total	Total battle deaths	Total wounds including battle deaths
Russia	419 340	56 000	475 340	1 416 700	6 366 700
France	182 000	8000	190 000	1 131 500	5 397 500
British Empire	180 597	8109	188 706	585 533	2 590 509
Italy	55 373	4627	60 000	541 500	1 488 500
USA	71 345	1462	72 807	52 842	272 138
Germany	191 000	9000	200 000	1 478 000	5 694 058
Austria/Hungary	97 000	3000	100 000	1 000 000	4 620 000
Other	9000	1000	10 000	684 436	1 580 318
Total	1 205 655	91 198	1 296 853	6 890 511	28 009 723

Wounded due to chemical warfare as percentage of all wounded (including fatalities): 4.63%.
Deaths due to chemical warfare as percentage of total chemical warfare injured: 7.03%.
Deaths due to chemical warfare as percentage of all deaths: 1.32%.
UK forces deaths due to chemical warfare as percentage of total UK forces deaths: 4.3%.
After Prentiss (1937).

low; (ii) the ratio of dead to injured among those affected by chemical warfare was very low; (iii) ill-prepared forces, for example, Russian troops on the Eastern Front, suffered badly. These observations have been examined in detail elsewhere (Maynard, 1988) and have been adduced by some as evidence of the efficacy of chemical warfare, and by others as evidence of its inefficacy. Space here does not permit an examination of these interpretations.

After World War I, a widespread campaign to ban chemical warfare was mounted and the Geneva Protocol, promulgated in 1925, encapsulated widely held opinion. However, during the Italian campaign in Ethiopia (1935–1936) Italian troops used mustard gas on a large scale against unprotected native forces (SIPRI, 1971). Many casualties were produced. During the late 1930s, fear that fascist countries might use chemical warfare on a substantial scale during a future war led to the incorporation of antigas drills into Air Raid Precautions (ARPs) in the UK and the issue of gas masks to all troops and civilians (Haldane, 1938). These fears were not demonstrated to have been justified during World War II, despite the production of large quantities of chemical warfare munitions by both sides. Germany's reluctance to use chemical weapons has never been satisfactorily explained, particularly as she had what many might have considered a great advantage in having synthesized very toxic nerve agents which were unknown to the Allied Powers. The discovery of large stocks of these compounds, the acquisition of the means of production by the USSR and the worsening state of international relations, led to the expansion of research into chemical warfare in both the UK and the USA during the years following World War II.

The UK's programme of research, designed to produce chemical weapons, sometimes described as 'offensive research' (in contrast to work on antidotes and means of protection: 'defensive research'), was halted permanently in 1956, with the decommissioning of the nerve-agent production plant at the Chemical Defence Establishment out-station at Nancekuke in Cornwall, UK. Production of nerve agents continued in the USA until 1969 and began again in 1981 in order to produce the components of the so-called 'binary weapon system' (Meselson and Perry Robinson, 1980). Production in the USSR probably continued in parallel with that in the USA.

Accusations of use of chemical weapons have been common since World War II. Those of their use by Egyptian forces in the Yemen (1963–1967) seem better supported than many others. US forces used defoliants and irritants on a large scale during the war in Vietnam. That this represented a use of chemical warfare has been strenuously denied by US sources and the view that the use of CS (2-chlorobenzylidene malononitrile) tear-gas, which had been sanctioned for use in the USA against rioting civilians, could not be regarded as an exercise in chemical warfare has been voiced.

Iraq, during the Iran–Iraq war in the early and mid 1980s used chemical weapons on a large scale, mustard gas and probably the nerve agent tabun were used (United Nations, 1987). Many casualties resulted, and in one particularly distressing incident, at Halabja (Iraqi Kurdistan), some 5000 civilians were killed. Death from chemical warfare on this scale had not been known since the gas cloud attacks of 1915 and attracted international condemnation.

Among less well-known accusations are the use of 'yellow rain' in South East Asia (Seagrave, 1981) and the use of 'knock down agents' and 'black body agents' by USSR forces in Afghanistan. More recently, Russian forces are alleged to have employed an incapacitating agent (possibly a fentanyl

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analogue) against Chechen terrorists in a Moscow theatre (Wax *et al*., 2003). During 1988 reports that Libya had constructed a chemical warfare production plant appeared in the press. This report fuelled fears that terrorists might gain access to chemical weapons.

In 1988, major steps were taken by both the USA and the USSR towards a verifiable ban on chemical weapons. The chemical weapons convention came into force on 29 April 1997: this completely prohibited the use of chemical weapons (Pearson, 2007). Expansion of the capacity to wage chemical warfare has, however, continued in a number of other countries.

There is no evidence that chemical weapons were used during either Gulf War, though the production of such weapons by Iraq is not doubted. US and UK troops were provided with pyridostigmine as a pretreatment against nerve agent attack: see below.

A detailed account of the subjects considered in this chapter may be found in the recent publication: *Chemical Warfare Agents: Toxicology and Treatment* (Marrs *et al*., 2007).

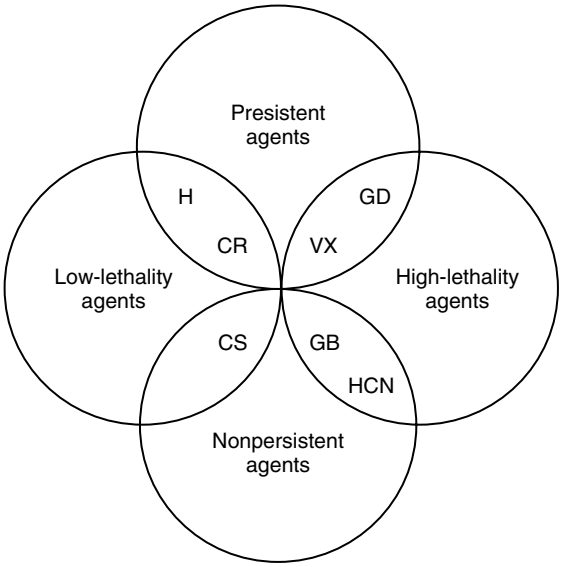

Figure 1 Categories of chemical warfare agents.

2 CONCEPTS OF USE OF CHEMICAL WEAPONS

It is often assumed that chemical weapons would be used during a war to kill as many of the opposing forces as possible. This is fallacious and misrepresents the professed purpose of modern warfare. Modern warfare is, it is generally agreed, waged to compel governments to amend their actions and not to annihilate populations (Fotion and Elfstrom, 1986). It is a fact of military experience that weapon systems which produce many casualties are likely to be more effective than systems which produce fatalities. Casualties require evacuation, nursing care and may sap morale. Because of this, chemical weapons would more likely be used to incapacitate than to kill. Some established chemical weapons, such as mustard gas, are rather ineffective as regards killing (death rate in mustard casualties during World War I was about 2%), although particularly effective as incapacitants (Haldane, 1925).

Chemical weapons can be effectively defended against by the use of protective clothing, including a respirator, gloves, boots and special overgarments. Prophylactic drugs play a much less important part in providing protection. Physical protection inevitably carries the penalty of impaired performance and commanders might be loath to institute such measures. Indeed, aggressors might consider the use of, or the expression of willingness to use, chemical warfare as successful if they force their opponents into protective clothing. Because of this, a policy of intelligent avoidance of contaminated areas would be likely to be observed. This has led to the development of the concept of use of chemical warfare agents as agents of 'ground denial' rather than as deliberate producers of casualties. Such an effect could well be exploited by an aggressor by attacks on centres of strategic and tactical importance, some 'behind the lines'. Such a use would inevitably lead to the exposure of civilians, probably less well-trained and protected than members of the armed forces, and the production of many civilian casualties. Lethal chemical warfare compounds could be used in high concentrations to permit the rapid breakthrough by well-protected troops through strongly defended points.

These concepts of use permit a number of categories of chemical warfare agent to be defined (see **Figure 1**). Only a few well-known chemical warfare agents have been included in **Figure 1**. Other compounds, such as Lewisite, could well appear in more than one category. CS has been included as a nonpersistent agent on the grounds of its rapid hydrolysis in contact with water.

It should be remembered that military concepts of use could well vary from area to area, and reports of the use of specific chemical warfare agents may prove very difficult to understand unless details of the operational scenario are available.

Much of the thinking or doctrine as regards the use of chemical warfare agents was developed during periods of East–West tension when a war between NATO (North Atlantic Treaty Organization) and Warsaw Pact forces was a possibility. Such a threat has now receded and the thinking looks dated. It is now more likely that chemicals would be used by terrorist organizations than by national forces.

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Table 2 Standard classifications of chemical warfare agents

Medical classification	Service classification
1. Agents liable to be met in warfare	
Nerve agents (G and V)	Lethal agents (nerve)
Lung-damaging agents (phosgene and chlorine)	Lethal agents (choking)
Vesicant agents (sulphur mustard, Lewisite, etc.)	Damaging agents (blister)
Psychotomimetic agents (LSD, BZ: 3-quinuclidinyl benzilate)	Incapacitating agents (mental)
Miscellaneous agents	
Cyanide	Lethal agents (blood)
Arsine	—
Herbicides	
2. Agents liable to be met in riot control and/or war	
Sensory irritants CS and CR	Riot control agents
Vomiting agents, for example, DM	Incapacitating agents (physical)

3 STANDARD CLASSIFICATION OF CHEMICAL WARFARE AGENTS

A number of different classifications of chemical warfare agents have been devised. Two broad systems, 'medical' and 'service', are in general use and are compared in **Table 2** (HMSO, 1972, 1987). Both of these systems would be regarded by many experts as outmoded. Several of the compounds listed in the table, for example, LSD (lysergic acid diethylamide), and DM (10-chloro-5,10-dihydrophenarsazine), are no longer seen as likely to be used in war, and chlorine and arsine are very unlikely to be used, though they might figure in terrorist incidents.

The possible means of production of chemical weapons have also changed in recent years, as genetic engineering and the production of complex molecules on a large scale by cultures of bacteria and yeasts have been developed. Today, chemical weapons form a spectrum ranging from the classical chemical weapons, such as nerve agents and mustard gas, to the classical biological agents, such as the anthrax bacillus or the smallpox virus. Between the two extremes are compounds originally discovered as natural products, for example, bacterial toxins, and which were classified as biological weapons, but which are, in fact, chemicals. The chemical–biological warfare spectrum is shown in **Figure 2** (Pearson, 1988). Useful though this classification is, it is likely that the simple military classification will remain in widespread use, as it lends itself to use in training and defining standard means of dealing with casualties.

4 GENERAL MANAGEMENT OF CHEMICAL WARFARE CASUALTIES

Few civilian doctors have any experience of the management of chemical warfare casualties; indeed, few military doctors in Western countries have ever

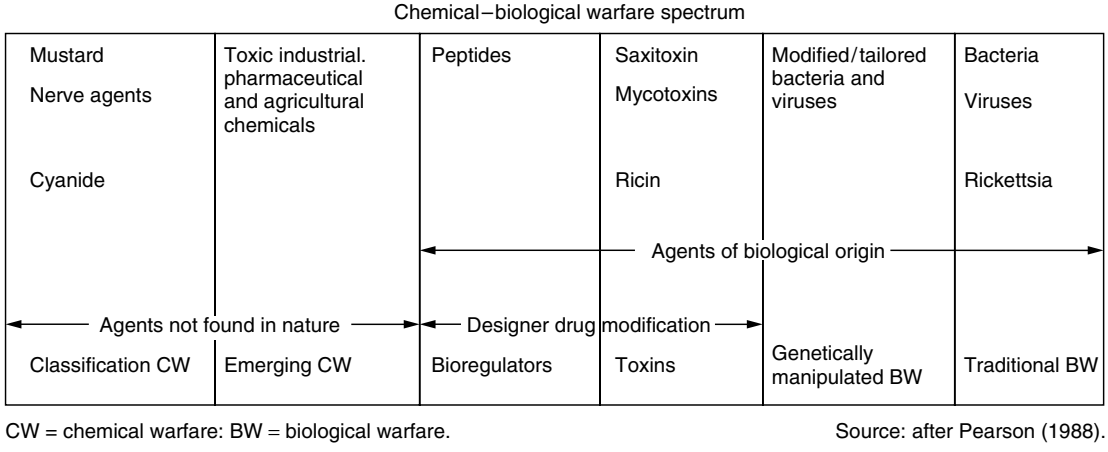

Figure 2 Chemical–biological warfare spectrum.

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seen a chemical warfare casualty. If casualties are to be well cared for and the carers are not to be placed at risk, a number of key points must be borne in mind.

1. Early treatment of casualties is essential
2. Attendants must be adequately protected from contamination
3. Disrobing and decontamination of casualties is the priority once life-saving measures, including the establishment of adequate ventilation and the administration of antidotes, have been undertaken. Fullers' earth is an excellent decontaminant for liquid agents, though it has been largely replaced by water or very dilute solutions of sodium hypochlorite (bleach). Any source of water will suffice as a means of removing liquid agents from the eyes. Care must be taken that solutions of bleach are not allowed into the casualty's eyes
4. Casualties should be moved as soon as possible after decontamination into a clean environment where clinicians may work under conditions with which they are familiar. Defence of this environment must be absolute
5. Casualties will range from the mildly affected to the moribund and the rules of triage must be rigorously applied if optimal use is to be made of clinical resources
6. Early identification of the agent responsible for the poisoning will be of great value to the clinician and every effort should be made to use such detectors and monitors as are available. Identification may, in practice, depend on the recognition of toxicological effects
7. Some casualties will require intensive nursing and should be moved along evacuation routes as quickly as possible.

5 VESICANT COMPOUNDS

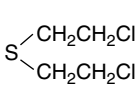
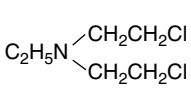
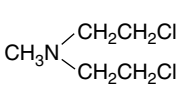
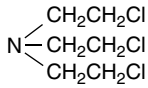
Vesicant compounds were introduced as chemical warfare agents on 12 July 1917 when German forces used sulphur mustard at Ypres (Ieper), Belgium (Prentiss, 1937). Mustard gas became the most effective chemical warfare agent used during World War I, 14 000 British casualties being produced during the first three months of its use and 120 000 by the end of the war (HMSO, 1923). This efficacy earned for mustard gas the sobriquet 'King of the Battle Gases' but, despite this, it carried only a low lethality. This low lethality, of the order of 2%, was one of the points which convinced J. B. S. Haldane (Haldane, 1925) of the desirability of chemical warfare as compared with conventional warfare.

5.1 Physicochemical Properties of Mustard Gas

'Mustard gas' is an unfortunate misnomer as the compound which at room temperature gives off vapour and smells of mustard, garlic or leeks, is a liquid with a boiling point of 217 °C. Sulphur mustard is often referred to as HS (Hun Stoff) or more commonly as HD. The nitrogen mustards are referred to as HN1, HN2 and HN3. The formulae and some of the characteristics of the mustards are shown in **Table 3**.

Sulphur mustard is poorly miscible with water, but, on mixing, hydrolysis takes place leading to the production of thiodiglycol and hydrochloric acid (**Figure 3**). The low miscibility and solubility of sulphur mustard in water leads to lengthy persistence of the compound in the field, particularly if protected from wind and rain. Sulphur mustard vapour passes quickly through clothing, although properly designed, modern military protective clothing

Table 3 Physicochemical characteristics of mustard vesicants

Characteristic	H	HN_1	HN_2	HN_3
Formula				
Appearance	Yellowish, oily liquid	←Colourless or yellowish oily liquids→		
M.P. (°C)	14	−34	−60	−4
B.P. (°C)	217	85	75	138
S.G.	1.27	1.09	1.15	1.24
V.P. (mmHg)				
10 °C	0.032	0.0773	0.130	0.00272
25 °C	0.112	0.2500	0.427	0.01090
40 °C	0.346	0.7220	1.250	0.03820
Odour	Mustard-like, garlic, leek or horseradish-like	—	Soapy or fishy	—

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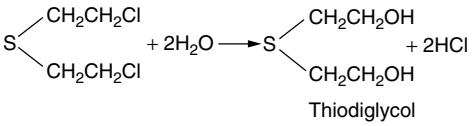

Figure 3 Hydrolysis of sulphur mustard.

provides good protection. Sulphur mustard in the liquid state passes quickly through ordinary surgical rubber gloves and heavy gloves made of butyl rubber should be worn when decontaminating casualties. The standard issue UK military respirator provides excellent protection against mustard gas vapour.

5.2 Absorption of Sulphur Mustard

Sulphur mustard, as a liquid or vapour, is lipid soluble and is absorbed across the skin. Under unoccluded conditions, ~80% of an applied dose of liquid sulphur mustard evaporates from the skin surface (Chilcott *et al*., 2000; Renshaw, 1946). Studies conducted during World War II indicated that the remaining 20% is rapidly 'fixed' in the skin. This led to the conclusion that catch-up therapies based on neutralizing the absorbed dose within the skin must 'necessarily be valueless' (Renshaw, 1946). However, other studies have demonstrated that the absorbed dose forms an appreciable reservoir of 'free' mustard which can be extracted by the topical application of appropriate solvents (Chilcott *et al*., 2000; Smith *et al*., 1919). Removal of this reservoir may represent a strategy for the future development of effective decontaminants (Chilcott, 2007; Graham *et al*., 2005).

Cameron *et al*. (1946) demonstrated, in rabbits, that some 80% of inhaled vapour was absorbed in the nose. Clinical experience suggests that the majority of inhaled sulphur mustard is absorbed in the upper airways.

5.3 Toxicity of Sulphur Mustard

The toxicity of sulphur mustard as an incapacitating agent is militarily of much greater importance than its capacity to kill, in terms of LD_{50}, and compared with the nerve agents, sulphur mustard is a comparatively nontoxic compound. Intravenous LD_{50} figures include the following: rat, 3.3 mg kg^{-1}; mice, 8.6 mg kg^{-1} (Anslow *et al*., 1948). Much more important are the effects of exposures to differing concentration/time products (*Ct* products). These are listed in **Table 4**. It should be noted that, weight for weight, sulphur mustard can be considered equipotent to nerve agents in terms of its ability to incapacitate unprotected individuals. **Figure 4** shows the interdependence of concentration and time in defining the effects of mustard gas vapour.

Table 4 Effects of sulphur mustard vapour on humans

Exposure dose (mg min m^{-3})	Effects[a]
20–50	Onset of eye effects
70	Mild reddening of the eyes—tearing
100	Partial incapacitation from eye effects
100–400	Erythema of skin
200	Total incapacitation from eye effects
200–1000	Skin burns produced
750–10 000	Severe incapacitation from skin effects

[a]The effects of sulphur mustard vapour on the skin are very dependent on ambient temperature and the range shown for skin burns encompasses the effects of decreasing ambient temperature from 80 to 50 °F.

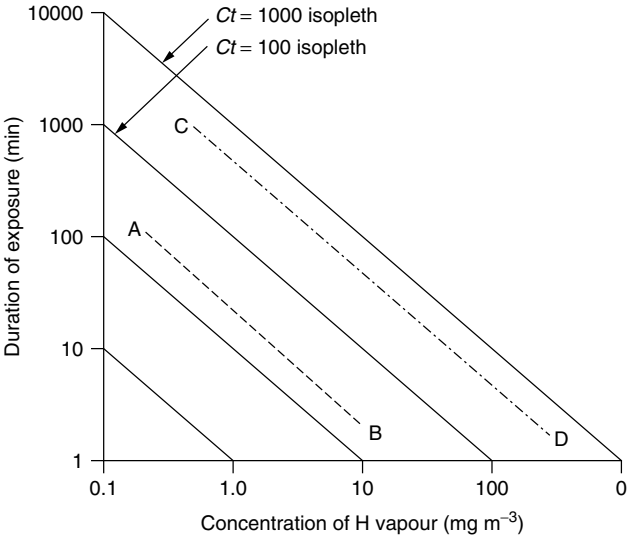

Figure 4 Effects of sulphur mustard vapour on humans. Lines AB and CD are offered as guides to early eye effects (AB: Ct = 20 mg min m^{-3}) and skin burns of significance (CD: Ct = 500 mg min m^{-3}). Because of uncertainty regarding the Ct = constant, relationship, extrapolation to extremes of time and concentration will be inaccurate.

5.4 Mechanism of Action of Sulphur Mustard

Despite nearly a century of research, the mechanism(s) through which sulphur mustard induces lesions of the epithelial cells of the skin, lung and intestinal tract remain

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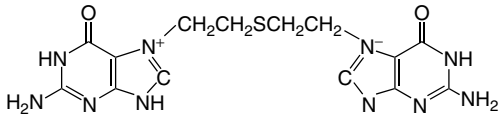

Figure 5 Crosslinking of guanine residues by sulphur mustard.

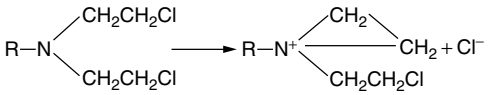

Figure 6 Formation of a quaternary ammonium compound.

to be elucidated. Research to date has identified a number of biochemical effects of sulphur mustard, although none provides an adequate link between exposure and onset of specific pathological consequences, such as skin blistering.

Sulphur mustard is an alkylating agent and a detailed account of the mode of action of such compounds can be found elsewhere (Goodman and Gilman, 1980; Fox and Scott, 1980). Both sulphur mustard and the various nitrogen mustards are bifunctional alkylating agents possessing two side chains capable of undergoing cyclization. Crosslinking of guanine in nucleic acids results from this property. Crosslinked guanine molecules are shown in **Figure 5**.

Binding of the ethylenesulfonium ion (sulphur mustard) or the ethylimmonium ion (nitrogen mustard) to DNA produces a range of effects including:

- Alkylated guanine residues tend to form base pairs with thymine rather than with cytosine, leading to coding errors and hence inaccurate protein synthesis
- Damaged guanine residues may be excised from the DNA molecule
- A pair of guanine residues may become crosslinked as shown below. This is considered by some as the most serious effect of alkylating agents on DNA (Rink and Hopkins, 1995).

Repair to DNA may take place, as long as damage is not too widespread. Juarez-Salinas *et al*. (1979) reported the polymerization of nicotinamide adenine dinucleotide (NAD^+) under the influence of the poly(ADP ribose) polymerase (PADPRP) enzyme as part of the DNA repair process. Papirmeister *et al*. (1984a; 1984b) suggested that the reduction in the cellular levels of NAD^+ following DNA repair could lead to cell death. However, there is come contradictory evidence to the PADPRP hypothesis. For example, in rat keratinocytes, NAD^+ depletion does not occur until the concentration of sulphur mustard is sufficient to cause inhibition of DNA repair (Lin *et al*., 1994); DNA repair in human keratinocytes may be accomplished within 90 minutes of exposure, whereas NAD^+ depletion is not observed until 1–3 hours postexposure (Meier *et al*., 1987) and elevation or maintenance of NAD^+ in human keratinocytes does not confer protection against the cytotoxic effects of sulphur mustard (Mol *et al*., 1989).

Early work by Dixon (1946) suggested a correlation between skin injury and inhibition of glycolysis. Although the effects of alkyating agents, such as mustard, on nucleic acids are usually stressed, it should be recalled that they also alkylate a wide range of other cellular components and enzymes. Membrane proteins at both the plasmalemma and the surfaces of intracellular organelles are liable to alkylation.

Despite this apparent understanding of how the alkylating agents act, no clear explanation of how mustard produces blisters is yet available. Miskin and Reich (1980) proposed increased protease synthesis and release after mustard-induced damage to cells. Release of such enzymes would be expected to set up an inflammatory response and would explain some of the effects of sulphur mustard. Acute inflammation does not, however, usually lead to blistering. Recent studies by Lindsay and Rice (1995; 1996) have suggested that laminin is a target for protease activation at the dermal–epidermal junction. Experiments in Yucatan minipigs showed that sulphur mustard induced microblister formation at this site. Damage to laminin was not accompanied by damage to Type IV collagen. Monteiro-Riviere and Inman (1995) have undertaken similar studies and have shown that the precise site of separation of the dermal–epidermal junction is beneath the hemidesmosomes in the upper part of the lamina lucida of the basal lamina.

The cholinomimetic effects of nitrogen mustard may be explained by cyclization leading to the formation of a quaternary ammonium compound (**Figure 6**).

5.5 Histopathology of the Skin Effects of Sulphur Mustard

Extensive experimental work was undertaken during World War I to discover the effects of sulphur mustard on human skin. This work has been reported in detail by Ireland (1926).

Volunteer studies were undertaken involving exposure of personnel to various concentrations of sulphur mustard; in some instances, biopsies of damaged skin were taken. In one study, small quantities (0.0004 ml drops) of sulphur mustard were placed on the skin. Small blisters were produced. The evolution of the lesions was followed by a timed series of biopsies.

Early vacuolation of the deeper layers of the epidermis was reported, as were nuclear changes in the stratum granulosum. Capillary dilation and leukocyte diapedesis

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Table 5 Mann's classification of mustards by effects on the eye

	'Mustard gas group'	'Nitrogen mustard group'
Compounds	Sulphur mustard and HN_3	HN_1 and HN_2
Latent period	Present, may be some hours	Absent
Limits of early damage	Cornea and conjunctiva main site of damage	Rapid penetration to anterior chamber, pupil contracts and ciliary body releases a cellular exudate within 1 h of exposure
Later effects	Petechial haemorrhages Iridocyclitis rare	Haemorrhage Iridocyclitis common Intraocular haemorrhages often seen
Resolution	Usually complete	Permanent damage likely

Modified from Mann (1948).

were also noted. These changes were established some 30 minutes after exposure. Later separation at the dermal–epidermal junction occurred with liquefaction of the epidermis near the centre of the lesion. Epithelial cells of hair follicles and sweat glands were also affected. These changes were maximally developed by 18 hours after exposure. Necrosis was followed by formation of an eschar by 72 hours, sloughing by four to six days, a pigmented scar being present by 19 days.

Histochemical studies by Vogt *et al*. (1984) have confirmed the above and, furthermore, have demonstrated two phases of increased capillary permeability. Papirmeister *et al*. (1984a; 1984b) have undertaken detailed studies using athymic human-skin-xenografted mice and have extended the above descriptions to the ultrastructural level. Their papers should be consulted for details. Damage to the extracellular matrix has been studied at the electron microscopic level by Chauhan *et al*. (1995).

5.6 Histopathology of the Effects of Sulphur and Nitrogen Mustard on the Eyes

These effects were investigated in detail by Mann (1948) who divided the compounds into two groups. These are described in **Table 5**. Note that the division does not correspond with the usual classification: sulphur mustard/nitrogen mustard.

5.7 Histopathology of the Effects of Mustard Gas on the Respiratory Tract

Several reports are available (Warthin and Weller, 1919; HMSO, 1923; Vedder, 1925; Ireland, 1926). These effects are largely confined to the conducting airways, with damage being particularly marked in the larger airways. The pseudostratified ciliated columnar epithelium becomes necrotic and sloughs; haemorrhages and inflammatory changes occur in the lamina propria and a false, diphtheritic membrane of sloughed cells, blood and exudate is formed. In severe cases, damage to the submucosa and other layers of the airway wall may occur. Surface repair is by squamous metaplasia and complete restoration, of a normal ciliated epithelium is suspected to be slow.

In the deeper lung, damage is usually less marked, although severe exposure can produce haemorrhagic pulmonary oedema. During World War I, this was clearly recorded at post mortem and found to be most marked in the alveoli adjacent to conducting airways. Repair of damage at the alveolar level involves initial organization of the exudate and its subsequent removal by macrophages. Extensive fibrotic changes following exposure to mustard gas have not been demonstrated.

The findings described above have been confirmed in rats by Anderson *et al*. (1996). In addition to the already described effects, damage to bronchus associated lymphatic tissue (BALT) and chondrocytes was reported. Examination of interalveolar septae with the electron microscope revealed perivascular oedema, though extensive pulmonary oedema was not reported.

5.8 Histopathology of the Effects of Mustard Gas on Bone Marrow

Alkylating agents in general have profound effects on rapidly dividing tissues and damage to the cells of the bone marrow produces an aplastic anaemia (Smith, 1986). The granulocyte series is first affected followed by the megakaryocytes and finally the erythropoietic series. In mustard gas casualties, the peripheral white cell count has been observed to begin to fall on about the fourth day postexposure after an initial postexposure rise.

5.9 Symptoms and Signs of Exposure to Mustard Gas

The following is based on the accounts of Vedder (1925) and Warthin and Weller (1919) and one of the author's

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Table 6 Symptoms and signs of exposure to mustard gas

Time postexposure	Symptoms and signs
20–60 min	Nausea, retching and eye smarting have all been reported. More commonly a latent period of up to an hour occurs and was clearly recorded at first hand by Adolph Hitler from experience in 1918 (Hitler, 1925)
2–6 h	Inflammation of the eyes occurs with intense and burning eye pain. Lachrymation, blepharospasm, photophobia and rhinorrhoea appear; the face becomes reddened and the voice hoarse
6–24 h	Blisters develop on skin exposed to vapour or liquid. The blisters are delicate and are often rubbed off by the patient turning in bed. Blisters are not *per se* painful, although if they occur over flexure lines at joints, pain may be produced on moving. The warm areas of the body are particularly liable to blistering with the axillae, inguinal folds, perineum and genitalia being badly affected. In contrast, the palms and soles appear to invariably escape blistering
24–48 h	Blistering becomes more marked and fresh crops of blisters appear. Coughing develops and sloughed tissue is expectorated. Intense itching of the skin may occur and frequently prevents sleep. Darkening of the skin, due to an increase of melanin in the basal layer of the epidermis, occurs and areas of dark brown or black hyperpigmentation are produced. Eye effects are maximal at this time and patients may be temporarily blinded
48 h to 6 wk	Blisters heal slowly and healing areas of skin are sensitive, and secondary blistering may be produced by friction. Peeling of areas of hyperpigmentation to leave areas of hypopigmentation occurs and a striking piebald appearance may be produced. The eye problems resolve slowly and persistent roughening of the cornea may be observed
6 wk to 6 mon	Many patients will have recovered by 6 wk although in some the process takes much longer. A state of functional neurosis was described during World War I, with depression and continuous eye problems. Persistent lachrymation and photophobia represent a particularly difficult problem

(Robert L. Maynard) observations of Iranian casualties during 1985 and 1986. The comprehensive review of Willems (1989) should be consulted for further details. The symptoms and signs are listed in **Table 6**.

5.10 Clinical Investigations

Blood should be taken for estimation of thiodiglycol. Regular chest X-rays and standard haematological investigations are clearly indicated. Pulmonary function testing should be undertaken in all whose respiratory problems appear to be resolving particularly slowly.

5.11 Management of Mustard Gas Casualties

It is the personal experience of one of the authors (Robert P. Chilcott) that human skin contaminated with liquid sulphur mustard will 'off-gas' for up to 24 hours postexposure. A similar (though less pronounced effect) has been observed with laboratory animals (Logan *et al*., 2000). Therefore, it is imperative that medical personnel treating contaminated casualties use appropriate personal protective equipment.

As already stated, casualty management divides into two parts.

5.11.1 First Aid

Casualties should be moved quickly from the source of contamination by adequately protected attendants and decontaminated thoroughly with fullers' earth. Washing of the skin with kerosene to remove liquid contamination was strongly advocated by Vedder (1925), but if undertaken should be continued for 30 minutes.

5.11.2 Medical Management

There is no specific therapy for mustard gas lesions and no antidote has been demonstrated to be clinically effective in removing mustard from the body. Despite this, considerable amelioration of the effects may be achieved and secondary infection prevented.

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5.11.2.1 Management of Skin Lesions

Large blisters should be aspirated aseptically as they are likely to be broken accidentally. Blister fluid is not harmful, despite the oft-repeated assertion that it contains free mustard and may cause damage to attendants. That blister fluid is harmless was conclusively demonstrated in volunteer studies by Sulzberger (1943). After aspiration, blisters should be covered with sterile dry dressings. Treatment by exposure seems to be as effective as by wet dressings and demands less nursing care. A variety of ointments have been used in cases of mustard gas burns, although none has been shown to enhance their rate of healing. Silver sulfadiazine cream (Flamazine) has been widely used and is useful in preventing secondary infection.

Severe itching and pain from damaged skin has been reported and in some cases narcotic analgesics have been used. This is probably unnecessary in the majority of cases and a milder analgesic combined with a sedative would be more appropriate therapy.

Most mustard gas burns are superficial or partial thickness burns. Deeper burns are occasionally seen and for these skin grafting should be considered as this may increase the rate of healing. Recent work by Rice and coworkers has demonstrated that physical removal of mustard-damaged skin by mechanical or laser debridement can significantly enhance healing rates (Rice *et al*., 2000; Evison *et al*., 2006).

5.11.2.2 Management of Eye Lesions

Early decontamination of liquid splashes in the eye is essential as a delay of more than a few minutes may make later decontamination ineffective and allow serious damage to occur (HMSO, 1972, 1987). For damaged eyes the following are recommended:

- Daily saline irrigations
- The use of petroleum jelly on the follicular margins to prevent sticking
- Chloramphenicol eye drops to prevent infection
- Mydriatics, for example, hyoscine drops, to prevent iridolenticular adhesions and to reduce pain caused by spasm of the ciliary muscle
- It has been suggested that if eye pain is particularly severe local anaesthetic drops should be used, amethocaine hydrochloride being recommended. Ophthalmological opinion should be sought before local anaesthetic or corticosteroid drops are used
- Potassium ascorbate (10%) and sodium citrate (10%) drops 'alternately, each once an hour, that is, half hourly drops, for 14 hours or the waking day', are also recommended
- Dark glasses to alleviate photophobia
- Perhaps most important of all: constant reassurance that blindness will not be produced and that recovery *will* occur, albeit slowly.

5.11.2.3 Management of Lesions of the Respiratory Tract

Antibiotic cover is recommended if the respiratory effects are more than very mild. Codeine linctus is of value in preventing coughing at night. Mucolytics, including acetylcysteine, have been used in some cases, although evidence of their efficacy is lacking. Calvet *et al*. (1996) have reported that betamethasone, given from 7 to 14 days postexposure to sulphur mustard enhanced regeneration of the airway epithelium. No clinical trials of glucocorticoids in mustard gas injuries of the respiratory tract have been reported, but this is a potentially important finding. In cases of very severe respiratory damage, a chemical pneumonitis may be produced and may demand intensive care. Respiratory physicians and anaesthetists should be consulted if evidence of deteriorating respiratory function appears.

5.11.2.4 Management of Depression of the Bone Marrow

Until recently no treatment has been available to restore the damaged bone marrow. However, recent studies have indicated that administration of granulocyte colony stimulating factor (GCSF) may be effective in reducing the duration of sulphur-mustard-induced leukopenia (Anderson *et al*., 2006).

5.12 Prognosis of Mustard Gas Casualties

Most mustard gas casualties recover fully. In a small proportion of cases, late-onset corneal problems producing blindness occur (Mann, 1948). Sulphur mustard is a recognized carcinogen and a study in Japanese mustard-gas-factory workers demonstrated an increase in the incidence of cancer (Yamada, 1963). The risk of cancer occurring as a result of a single exposure to sulphur mustard is exceedingly low.

6 LEWISITE

Lewisite, developed as a chemical warfare agent in 1918 by Lee Lewis, has never been proven to have been used in war. Despite this it has acquired a reputation as an agent of likely great effectiveness and lethality. In fact, this is unlikely to be true. In the early 1920s Lewisite was considered as a compound likely to produce more severe effects than mustard gas and was nick-named 'The Dew of Death'. It has retained its place in the standard lists of chemical warfare agents, although often as a mixture with sulphur mustard, the Lewisite lowering the freezing point of the mustard and making it more effective under cold conditions. Fears that Lewisite might

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be used during World War II prompted the work of Peters *et al*. (1945), Peters (1948) and Peters (1953) and led to the development of the chelating agent dimercaprol or British anti-Lewisite (BAL). The view that Lewisite is rather unlikely to be used in war has limited interest in the compound and only a short account of its effects and the recommended management of those effects will be provided.

6.1 Physicochemical Properties of Lewisite

These are shown in **Table 7**. Unlike sulphur mustard, Lewisite decomposes rapidly on contact with water or even in a 'damp atmosphere' (Sartori, 1939). The reaction taking place is shown in **Figure 7**.

6.2 Absorption of Lewisite

Lewisite is absorbed rapidly through the skin and mucous membranes. Its distribution in the body follows that of other arsenical compounds.

Table 7 Physicochemical characteristics of Lewisite

Formula	$ClCH{=}CHAsCl_2$
M.W.	207.32
M.P. (°C)	−13
B.P. (°C)	190
V.P. (mmHg)	
0 °C	0.087
10 °C	0.196
20 °C	0.394
40 °C	1.467
S.G.	
0 °C	1.9200
10 °C	1.9027
30 °C	1.8682
Appearance	A colourless oily liquid. Impure samples may be blue–black in colour and smell, faintly, of geraniums

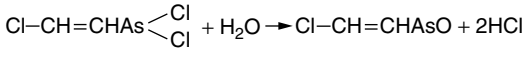

Figure 7 Hydrolysis of Lewisite.

6.3 Toxicity of Lewisite

Lewisite has a much higher systemic toxicity than sulphur mustard and 0.5 ml allowed to remain on the skin would be expected to produce severe poisoning; 2.0 ml allowed to remain in contact with the skin has been said to represent a lethal dose in humans (Vedder, 1925). Because of its systemic toxicity, few volunteers have been exposed to Lewisite and data available regarding the effects of vapour on the skin are scanty. It is the authors' impression that Lewisite vapour is more toxic to the skin and eyes than mustard gas vapour and the effects could be expected at lower concentrations.

6.4 Mechanism of Action of Lewisite

Peters *et al*. (1945) demonstrated that Lewisite attacked the pyruvate dehydrogenase system by combining with the coenzyme lipoic acid to form a cyclic compound (**Figure 8**). The essential and ubiquitous nature of lipoic acid accounts for the widespread effects of Lewisite on the body. Interestingly, as in the case of sulphur mustard, the exact link between the primary biochemical effect of Lewisite and the production of blisters remains unknown. It should be recalled that other arsenical compounds such as sodium arsenite are not vesicants.

6.5 Effects of Lewisite on Humans

Both in terms of histopathology and general patterns of symptoms and signs it is believed that Lewisite would produce effects similar to those described above for mustard gas. However, certain important differences exist and instead of repeating much of the above only these will be considered:

- Exposure of the eyes to Lewisite vapour is immediately painful and the damage produced is likely to be more severe than that produced by mustard gas vapour. Liquid contamination of the eyes is particularly painful, dangerous and demands immediate treatment
- Skin blister fluid contains Lewisite; rupture of blisters can lead to secondary blister formation if

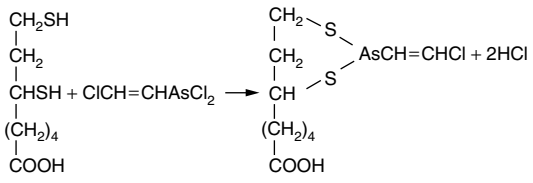

Figure 8 Reaction between Lewisite and lipoic acid.

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the fluid comes into contact with unexposed skin. Sulphur mustard-induced blisters do not contain active vesicant
- Skin blisters produced by Lewisite appear more quickly postexposure than those produced by mustard gas (Friedenwald and Hughes, 1948)
- The inflammatory response associated with Lewisite lesions is likely to be more severe than that associated with mustard lesions
- Healing of Lewisite-induced skin lesions is likely to be more rapid than of those occurring as a result of exposure to sulphur mustard. Hunter (1978) described a case of blistering following exposure of another vesicant arsenical compound, phenyl dichlorarsine, and reported that the skin lesions healed by the 10th day
- The collapse of the bone marrow seen in severe cases of mustard-gas poisoning would not be expected after exposure to Lewisite. Goyer (1986), however, has pointed out that large doses of arsenical compounds can lead to leukopenia.

6.6 Clinical Investigations

Blood and urine should be analysed for arsenic. Under military conditions, analysis should certainly be undertaken, as information regarding the identity of chemical weapons used on the battlefield might be of tactical importance.

6.7 Management of Lewisite Casualties

6.7.1 First Aid

This is as described for mustard gas.

6.7.2 Medical Treatment

Perhaps the most cheering difference between Lewisite and sulphur mustard is that specific therapy is available for the former, but not for the latter.

Dimercaprol (BAL) binds the arsenic of Lewisite and produces a harmless complex. BAL competes avidly with binding sites in the body for arsenic and removes arsenic from them. BAL is available in a form suitable for injection and is used as such in the treatment of poisoning by a range of heavy-metal compounds. Dose regimens for the management of arsenical poisoning vary from author to author, the following being given by Martindale (1989): 400–800 mg on first day of treatment, 200–400 mg on second and third days of treatment, 100–200 mg on fourth and subsequent days of treatment, all administered as divided doses.

For an alternative regimen, the HMSO publication *Medical Manual of Defence Against Chemical Agents* (HMSO, 1972, 1987) should be consulted. It is unlikely that one would need to continue treatment for more than about 14 days.

Marked reactions may be produced by the injection of BAL including tachycardia, nausea, vomiting, headache and sweating. Individual doses should not exceed $3\,mg\,kg^{-1}$ and an interval of at least four hours should separate these doses.

BAL may also be prepared as an ointment or as eye drops. Both preparations contain 5–10% dimercaprol. BAL ointment should not be used in conjunction with silver sulfadiazine (Flamazine) as chelation of silver will occur.

Recently, newer chelating agents have been proposed as replacements for BAL in the management of poisoning. The work of Graziano *et al*. (1978), Lenz *et al*. (1981), Aposhian *et al*. (1982; 1984) and Inns and Rice (1993) should be consulted for details. Despite the apparent advantages of some of the newer compounds in the treatment of systematic arsenic poisoning in experimental animals, it should be remembered that the high lipid-solubility of BAL will probably lead to the ointment retaining its place in the management of Lewisite burns.

6.8 Prognosis of Lewisite Casualties

Given that adequate, early decontamination followed by the administration of BAL is undertaken the prognosis should be good.

In war, eye splashes with liquid Lewisite would, fortunately, be expected to be rare, but if such did occur the difficulties of immediate decontamination under battlefield conditions could lead to serious eye damage.

7 NERVE AGENTS

Nerve agents, produced first in Germany during the late 1930s, are often described as second-generation chemical weapons to distinguish them from those developed during World War I. Schrader synthesized the first nerve agent, tabun, in late 1936 or 1937. Sarin followed later in 1937 and soman in 1944. Tabun was stockpiled in Germany on a large scale, 12 000 tonnes being stored by 1945. Sarin was produced on a smaller scale with some 600 tonnes being available by 1945. Tabun, sarin and soman are usually referred to by the abbreviations GA, GB and GD, respectively. Other G agents exist including GE and

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GF (cyclosarin). During the 1950s another group of nerve agents was developed and stockpiled in large quantities. These were the rather more toxic V agents including VE, VM and VX (Tammelin, 1957). Of these only VX has become well known. The history of the development of the nerve agents has been presented in some detail by Holmstedt (1963).

7.1 Physicochemical Properties of Nerve Agents

These are shown in **Table 8**. Exposure to chemical warfare agents, including nerve agents, may occur during or as a result of destruction of military stockpiles. This problem has been considered by Watson *et al*. (1992). Care should be taken in re-entering areas potentially contaminated by the nonvolatile agent VX. Nerve agents are hydrolysed by water. Khordagui (1996) has calculated that the half-life of G agents in seawater should be of the order of a few hours or less.

7.2 Military Use of Nerve Agents

Nerve agents may be disseminated on the battlefield by a variety of means and may be encountered as a vapour, liquid or artificially thickened liquid in the case of soman. Sarin is a comparatively volatile compound and presents a severe vapour hazard when encountered in the liquid state. VX on the other hand is a compound of very low volatility and presents little vapour hazard. The standard UK military respirator provides excellent protection against the effects of nerve-agent vapour.

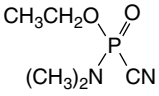
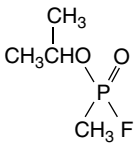
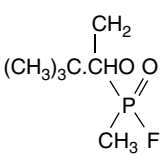
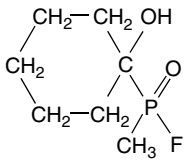
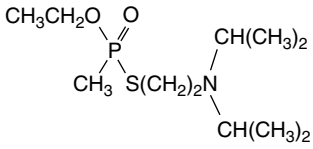
7.3 Use of Nerve Agents by Terrorists

In 1995 sarin was used in an attack on the public in a Tokyo subway: several deaths and 5000 cases of poisoning resulted (Masuda *et al*., 1995). Some 10 weeks before the sarin attack, three individuals were attacked with VX by the same terrorist group (Nozaki *et al*., 1995). The publications associated with these attacks provide details of the symptomatology of such cases of poisoning and insights into the efficacy of standard treatment. A detailed account of the medical aspects of this incident has been provided by Okumura *et al*. (2007). Interestingly, see below, the clinicians caring for the casualties found pupil diameter a reliable guide in assessing the effects of treatment with atropine.

7.4 Toxicity of Nerve Agents

Nerve agents are probably the most toxic compounds yet produced on a large scale. Many studies of their toxicity have been undertaken using animal models

Table 8 Physicochemical properties of nerve agents[a]

Characteristic	Tabun GA	Sarin GB	Soman GD	GF	VX
Formula					
M.W.	162.3	140.1	182.18	180.14	267.36
S.G. (20 °C)	1.073	1.0887	1.022	1.133	1.0083
M.P. (°C)	−49	−56	−80	−12	−20
B.P. (°C)	246	147	167	—	300
V.P. (mmHg)					
0 °C	0.004	0.52	0.044	0.006	0.00044
10 °C	0.013	1.07	0.11	0.017	
20 °C	0.036	2.10	0.27	0.044	
30 °C	0.094	3.93	0.61	0.104	
40 °C	0.23	7.1	–	0.234	
50 °C	0.56	12.3	2.60	0.501	

[a]GA, ethyl *N*-dimethylphosphorarmidocyanidate; GB, isopropyl methylphosphonofluoridate; GD, 1,2,2-trimethylpropyl methylphosphonofluoridate; GF, cyclohexyl methylphosphonofluoridate; VX, *O*-ethyl-S-[2-(diisopropylamino)ethyl]methylphosphonothioate.
From Maynard and Beswick (1992).

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Table 9 Toxicity of nerve agents[a]

Species	Route	Term	Units	GA	GB	GD	VX
Rat	Inhalation	$L(Ct)_{50}$	$mg\,m^{-3}$ per 10 min	304[b]	150[c]	—	—
	Intravenous	LD_{50}	$\mu g\,kg^{-1}$	66[b]	39[d]	44.5[e]	—
	Subcutaneous	LD_{50}	$\mu g\,kg^{-1}$	193[f]	103[g]	75[h]	12[f]
Mouse	Inhalation	$L(Ct)_{50}$	$mg\,m^{-3}$ per 30 min	15[b]	5[i]	1[i]	—
	Intravenous	LD_{50}	$\mu g\,kg^{-1}$	150[b]	113[j]	35[k]	—
	Subcutaneous	LD_{50}	$\mu g\,kg^{-1}$	250[l]	60[i]	40[i]	22[i]
Guinea pig	Inhalation	$L(Ct)_{50}$	$mg\,m^{-3}$ per 2 min	393[b]	128[m]	—	—
	Subcutaneous	LD_{50}	$\mu g\,kg^{-1}$	—	30[n]	24[o]	8.4[p]

[a]Source:
[b]Gates and Renshaw (1946);
[c]Rengstorff (1985);
[d]Fleisher (1963);
[e]Pazdernik *et al*. (1983);
[f]Jovanovic (1983);
[g]Brimblecombe *et al*. (1970);
[h]Boskovic *et al*. (1984);
[i]von Lohs (1960);
[j]Schoene and Oldiges (1973);
[k]Brezenoff *et al*. (1984);
[l]Maksimovic *et al*. (1980);
[m]Bright *et al*. (1991);
[n]Coleman *et al*. (1968);
[o]Gordon and Leadbeater (1977);
[p]Leblic *et al*. (1984).

and a very wide range of LD_{50} values are known. A small selection of these values are shown in the **Table 9**. Fortunately, the number of accidental exposures of people to nerve agents has been small and, in consequence, information on the lethal doses of the compounds in humans is scarce. Many volunteers have, however, been exposed to low concentrations of nerve agents and the effects of such exposures are well understood. Recent accounts (Maynard and Beswick, 1992; Sidell, 2007) should be consulted for details. **Table 10** gives some indication of the approximate lethal toxicity figures for nerve agents in humans.

7.5 Absorption of Nerve Agents

Nerve agents in the liquid form are absorbed across the unbroken skin and mucous membranes. There is some evidence to suggest that dermal exposure to liquid nerve agents such as VX may result in the formation of a skin depot (Chilcott *et al*., 2005). Formation of a dermal reservoir has considerable implications for the clinical management of casualties, as prolonged leaching of agent from the skin into the systemic circulation may cause the agent to outlive antidotes administered via intramuscular bolus (Chilcott, 2007). Vapour is not absorbed across the skin in significant amounts, although it is absorbed across the cornea in sufficient quantities to produce miosis as a local effect. Absorption of vapour by the lung is rapid and more than 80% of inhaled agent is absorbed. This important point should be borne in mind whenever the $L(Ct)_{50}$ figure for a volatile nerve agent is quoted. During exercise, respiratory minute volume is increased, but absorption of the high percentage of the inhaled vapour is maintained. The $L(Ct)_{50}$ for sarin in resting humans is approximately $100\,mg\,min\,m^{-3}$. On exercise, producing a fivefold increase in minute volume, this value could fall to $20\,mg\,min\,m^{-3}$. Off-gassing of vapour from contaminated clothing posed a significant hazard to medical staff dealing with the incident in Tokyo in 1995.

Table 10 Estimated values for toxicity of nerve agents in humans

Species	Route	Term	Units	GA	GB	GD	VX
Human	Inhalation	$L(Ct)_{50}$	$mg\,min\,m^{-3}$	150	70–100	40–60	—
	Intravenous	LD_{50}	$mg\,kg^{-1}$	0.08	0.01	0.025	0.007
	Percutaneous	LD_{50}	$mg\,kg^{-1}$	—	—	—	0.142

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Table 11 Ageing of organophosphorus–AChE complexes

Nerve agent	Enzyme[a]	Ageing half-life	Reference
GA	BEA	46 h	De-Jong and Wolring (1978)
GB	BEA	12 h	Benschop and Keijer (1966)
GD	BEA	4 min	De-Jong and Wolring (1980)
VX	BEA	>12 d	De-Jong, 1983, Personal communication

[a]BEA, bovine erythrocyte acetylcholinesterase.
Modified from Ligtenstein (1984).

7.6 Mechanism of Action of Nerve Agents

Nerve agents are organophosphorus anticholinesterases (anti AChEs) and exert their toxic effects by long-lasting inhibition of acetylcholinesterase (AChE) at sites of activity of acetylcholine (ACh) in the body. The action of anticholinesterase compounds, both nerve agents and pesticides, has been discussed at length by Koelle (1963; 1992), Taylor (1980), Murphy (1986) and Pope (2005) and also in **Toxicology of Pesticides**: it will not be considered here. One point should, however, be borne in mind: many organophosphorus compounds undergo a reaction generally known as 'ageing' after they have combined with AChE (Fleisher and Harris, 1965). The rate at which this reaction occurs varies with the anti-AChE and is particularly rapid in the case of GD. Ligtenstein (1984) has compiled data illustrating this point (*see* **Table 11**).

The values given in **Table 11** should not, of course, be taken as representative of the half-lives of ageing of the nerve agents at human synaptic or neuromuscular junctions, and are shown only for the purposes of comparing the rates of ageing of different agent–enzyme complexes. Differences in rates of ageing are important as regards the treatment of nerve-agent poisoning with oximes (see below).

7.7 Clinical Effects of Nerve Agents

The clinical effects of nerve agents may be deduced by recalling the sites of action of ACh in the body (Koelle, 1975). As well as acting at autonomic ganglia, peripheral parasympathetic terminals and the neuromuscular junction, it should be recalled that ACh also plays an important role in transmission within the central nervous system (CNS).

The symptoms and signs of anti-AChE poisoning are described in **Toxicology of Pesticides** and will not be described in detail here. **Table 12** shows the symptoms and signs of nerve agent poisoning in terms of short-term exposure and AChE depression.

Information from volunteer studies on the effects of nerve agents relates only to comparatively short duration exposures and, in the main, to exposures to concentration-time profiles of less than 30 mg min m^{-3}. It will be noted that considerable variations in the expected degrees of depression of AChE have been indicated in **Table 12**. This is important and all workers have stressed the considerable variation in the effects observed in different individuals at identical levels of AChE depression. This point will be returned to in the next section.

7.8 Clinical Investigations

Measurement of whole blood, erythrocyte and plasma cholinesterase activities are often undertaken in cases of organophosphorus compound poisoning. Plasma contains butyrylcholinesterase and red cells contain acetylcholinesterase. It is often assumed that a measurement of the level of active, that is, uninhibited, red-cell cholinesterase will provide an accurate reflection of levels of AChE at synaptic and neuromuscular junctions and thus reflect the severity of the poisoning which the patient has sustained. This is not so. Willems (1989) studied the patterns of cholinesterase depression in 53 cases of poisoning by organophosphorus insecticides and showed there was a poor correlation between clinical severity of poisoning and the extent of enzyme depression. It is difficult to imagine that a better relationship would exist in cases of nerve-agent poisoning.

7.9 Management of Nerve-Agent Poisoning

7.9.1 First Aid

Casualties should be removed from risk of further contamination and be decontaminated by adequately protected and trained attendants. Contaminated clothing should be removed as quickly as possible, taking care not to transfer liquid from the casualty's clothing to his skin. If protective clothing has been worn then this should be decontaminated with fullers' earth or a dilute solution of bleach before being removed. Under battlefield conditions, clean areas occupied by staff not wearing

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Table 12 Signs and symptoms of nerve agent poisoning

Short-term *Ct* (mg min m^{-3})	AChE inhibition (%) (± SD)	Symptoms and signs	
		Vapour exposure	Systemic exposure (eyes protected)
2	?	Incipient miosis, ?slight headache	Nil
5	20 ± 10	Miosis, headache, rhinorrhoea, eye pain, injection of conjunctivae, tightness of chest	?Tightness of chest
5–15	20–50 ± 10	Eye signs maximal, bronchospasm in some	Symptoms in some, ?bronchospasm
15	50 ± 10	Effects as above but more severe	Wheezing, salivation, nausea, vomiting, miosis, local sweating, muscle fasciculation in cases of skin contamination
40	80 ± 10	As above but more severe with weakness, involuntary micturition and defaecation, paralysis and convulsions	
100	100	Respiratory failure. Death	

respirators and protective clothing must be protected at all costs. Monitoring equipment should be used to confirm adequate decontamination before casualties are transferred into these clean areas. One of the most serious consequences of nerve-agent poisoning is respiratory failure produced by inhibition of the medullary respiratory centre. If respiration can be maintained while the drugs discussed below are administered, then the casualty's chances of recovery will be much enhanced. Under battlefield conditions the artificial ventilation of casualties presents formidable problems. 'Ambu'-style bags (protected by suitable rubber coverings and equipped with adequate filtres) and oropharyngeal airways can reduce these problems.

Preparations for dealing with casualties produced by the use of chemicals by terrorists and also by chemical accidents have led to the definition of three zones: (i) the hot zone, where primary contamination due to contact with disposed chemicals may occur; (ii) the warm zone where the only source of contamination is from casualties and, (iii) the cold zone. Rescue staff working in the hot zone may need closed-circuit breathing apparatus and will need impermeable protective suits. This limits the range of clinical procedures that can be undertaken in the hot zone, but the administration of antidotes to nerve-agent poisoning is possible and very important. Portable ventilators and light-weight oxygen cylinders can be deployed in both the hot and warm zones. Appropriate filters should be fitted to ventilators using ambient air.

7.9.2 Medical Treatment

Drug therapy for nerve-agent poisoning, as for poisoning by other anti-AChE compounds, divides into three: (i) cholinolytics, such as atropine, (ii) reactivators of the inhibited AChE, such as oximes and (iii) anticonvulsants, for example, diazepam or other benzodiazepines. To these should be added oxygen, which may be needed in cases of respiratory failure. The use of oxygen will not be considered further here.

In addition to the above, research has been undertaken to try to provide drugs, which if taken in advance of exposure to nerve agents, would reduce the effects of the exposure and make the treatment of poisoning resulting from that exposure the more effective. It was noted in the late 1940s that cats given the carbamate physostigmine became comparatively resistant to the effects of other anti-AChE compounds (Gilman and Cattell, 1948). It was suggested that the combination of a proportion of the available AChE with the carbamate would prevent subsequent combination of the enzyme with the organophosphorus compound and, furthermore, that the carbamate-combined enzyme would, later, spontaneously reactivate and provide the body with a supply of normal uninhibited enzyme. This hypothesis is now generally accepted. A great deal of work has been done in developing the carbamate pyridostigmine bromide for use in this way and Inns and Leadbeater (1983) summarized work on the efficacy of pretreatment, combined with the treatment detailed above, in cases of GD poisoning in guinea pigs. **Table 13** shows some of the results

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Table 13 Effectiveness of oximes and bispyridinium compounds against soman poisoning in guinea pigs receiving various supporting drug treatments. Pyridostigmine (0.32 μmol kg^{-1} im) was injected 30 minutes before challenge with soman (sc). One minute after poisoning P2S (130 μmol kg^{-1}) was injected im with atropine (50 μmol kg^{-1}). Diazepam (25 μmol kg^{-1}) was given as a separate im injection

Compound	Protective ratio (95% confidence limits)[a]			
	Atropine	Atropine, diazepam	Pyridostigmine, atropine	Pyridostigmine, atropine, diazepam
—	1.5 (1.2–1.9)	2.2 (1.8–2.7)	5.2 (4.1–6.6)	8.7 (5.7–14)
P2S	1.7 (1.5–1.9)	2.5 (1.9–3.1)	6.8 (5.4–8.5)	14 (10–19)

Modified from Inns and Leadbeater (1983).
[a]The protective ratio is defined as the ratio of the LD_{50} treated compared to the LD_{50} untreated.

obtained. This research has been most successfully prosecuted in the UK and all UK forces are issued with pyridostigmine bromide tablets (30 mg three times daily) for use, should the risk of exposure to nerve agents be deemed significant. Pyridostigmine bromide was taken by UK, US and some other forces during the Gulf War (1991). Sharabi *et al*. (1991) reported that nonspecific side effects, including dry mouth, general malaise, fatigue and weakness, were reported. Nausea, abdominal pain, increased frequency of micturition and rhinorrhoea were infrequent. Side effects tended to appear during the first few hours following each dose and then resolved. Interestingly, no correlation between AChE inhibition and symptoms was detected. Keeler *et al*. (1991) reported a study of 41 650 soldiers who took pyridostigmine bromide, in the dose described above, for between one and seven days. About half the study group reported side effects, including increased flatus, abdominal cramps, soft stools and urinary urgency. About 1% of soldiers 'believed their symptoms required medical attention', but in less than 0.1% were the side effects so severe as to require withdrawal of pyridostigmine. It was concluded that military efficiency was not impaired by taking the drug. Suggestions that the Gulf War Syndrome might have been caused by the use of pyridostigmine bromide do not appear to be well founded (Wessely and Hotopf, 2007).

7.9.2.1 Cholinolytics

Atropine is the drug of choice and should be given intramuscularly or preferably intravenously, in aliquots of 2 mg, as soon as possible after poisoning. Use of nerve agents by terrorists may lead to children being affected. For children less than eight, but more than one year of age, 600 μg of atropine should be adopted as the standard dose. For those under one year of age, 200 μg should be used. To enable servicemen to self-administer atropine and in some cases oxime, a wide variety of autoinjection devices have been developed commercially. Most contain 2 mg of atropine per injection and permit injection through clothing. Speed of administration as soon as the first signs of poisoning are detected is critical, as the progress of symptoms and signs may be very rapid. It is essential, therefore, that servicemen should be well trained in the recognition of the early signs of poisoning and in the use of the autoinjection devices. As in cases of poisoning by other organophosphorus compounds, large total doses of atropine are often said to be likely to be needed. However, in casualties surviving to reach hospital, it is unlikely that the heroic doses of atropine reported by some who were managing cases of organophosphorus pesticide poisoning would be required. In preparing supplies of atropine for treating casualties, the assumption that a casualty might need up to 30 mg of atropine would be reasonable.

The dangers of atropine overdose should always be borne in mind:

- Drying of bronchial secretions making them tenacious and difficult to remove; the careful use of suction may be required
- Large doses of atropine may induce arrhythmias particularly if the myocardium is hypoxic as a result of respiratory failure
- Bladder dysfunction may necessitate transurethral catheterization.

Atropine drops are sometimes recommended for the relief of visual impairment and eye pain caused by contraction of the iris and spasm of the ciliary muscle. Atropine drops may be expected to relieve the eye pain to some extent, but seem to do little to improve vision. The combination of mydriasis and impairment of accommodation seems to produce at least as severe an impairment of vision as the miosis produced by exposure to the nerve agent. The use of oxime, instead of atropine, eye drops has been suggested, but not widely adopted. It has already been noted that in the Tokyo subway incident, atropine eye drops were found to be helpful in those with mild poisoning (Nozaki *et al*., 1995). This may reflect a difference between military and civilian objectives as regards therapy. In the military environment further impairment of vision as a result of paralysis of accommodation might be critically disabling, whereas in a civilian context this might be acceptable, given that effective pain relief was obtained.

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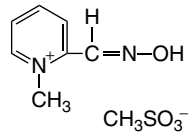

Figure 9 Pralidoxime methanesulfonate (P2S).

7.9.2.2 Oximes

The oximes, developed in the early 1950s (Wilson and Ginsburg, 1955; Wagner-Jauregg, 1956) represented a major step forward in the treatment of nerve-agent poisoning. Atropine opposes the effect of excess acetylcholine at muscarinic receptors, but has no effect at nicotinic receptors at the neuromuscular junction. Atropine cannot, therefore, relieve paralysis of respiratory muscles. Hydroxylamine was the first compound demonstrated to be capable of reactivating the AChE/nerve agent complex. More effective oximes followed and the work of Davies and Green (1956; 1959) and others led to the development of pralidoxime (PAM) methanesulfonate (pralidoxime mesilate or P2S). This compound and other PAM salts have maintained their place as the oximes of choice in the treatment of organophosphorus poisoning in many countries. P2S is included in the autoinjection device issued to the UK armed forces. A number of studies have shown that oximes block ion channels and thus work not only by reactivating inhibited acetylcholinesterase. Papers by Tattersall (1993), Alkondon *et al*. (1988) and van Helden *et al*. (1991) have stressed this mode of action. That oximes show some effectiveness in animals poisoned by soman, in which the nerve-agent–enzyme complex ages rapidly, may be explained by these direct effects.

P2S is a monopyridinium oxime; its chemical structure is shown in **Figure 9**.

A number of bispyridinium oximes have been developed, including obidoxime (Toxogonin) and those of the series of oximes referred to as the Hagedorn, or H, oximes (Oldiges and Schoene, 1970). The structures of some of the better-known compounds are shown in **Figure 10**.

If P2S is used, it should be given intramuscularly, as soon as possible after poisoning, or better by slow intravenous injection, at a dose of 30 mg kg^{-1}. The dose should be repeated at 15 minute intervals to a total dose of 2 or possibly 4 g. This dose is also regarded as appropriate in children. It should be remembered that very little experience of management of nerve-agent casualties has been accumulated. Oximes should be administered carefully and a close watch kept on the patient's condition. Side effects include headache, disturbances of vision and muscular weakness, and care should be taken to monitor the patient's condition before and after the administration of oxime to permit differentiation between the signs of deepening organophosphorus toxicity and the side effects of therapy. If given too quickly, bronchospasm and laryngospasm may occur. The use of an intravenous infusion of 2 g of P2S in 250 ml of normal (0.9%) saline delivered over 30 minutes has also been recommended. Ligtenstein (1984) has made a strong case for the continuation of the use of oximes beyond the often-suggested limit of a day or two.

The choice of oxime, if more than one is available, may be difficult. The following points should be recalled:

- PAM salts are probably still the most widely available oximes
- PAM is likely to be markedly more effective in cases of sarin and VX poisoning than in cases of tabun and soman poisoning
- Although obidoxime (Toxogonin) (3–6 mg kg^{-1} iv, four hourly) is likely to be effects in cases of tabun, sarin and VX (but not soman) poisoning, cases of liver damage following its use have been reported. A recent review has cast doubt on the likely efficacy of obidoxime in tabun poisoning (Marrs *et al*., 2006)
- The Hagedorn oximes such as HI6 and HGG12 have been shown to be effective in soman poisoning in some animal models, but are rather less effective than had been hoped in tabun poisoning (Wolthuis and Kepner, 1978; Harris *et al*., 1981; Clement, 1981; 1982a; 1982b; Clement and Lockwood, 1982). Recent studies by Kassa and Bajgar (1995) have shown that HI6 is a better antidote than obidoxime in rats poisoned with cyclosarin
- The recently developed Hagedorn oximes HLö-7 and pyrimidoxime are effective both in tabun and soman poisoning in some animal models (Eyer *et al*., 1989; 1992; Clement *et al*., 1992; Lundy *et al*., 1992)
- In general, the Hagedorn oximes are unstable in solution and demand more complex (wet–dry) autoinjection devices than the conventional oximes (Eyer *et al*., 1989). Göransson-Nyberg *et al*. (1995) reported a study of the efficacy of a new binary autoinjector containing 500 mg of HI6 and 2 mg of atropine sulfate in pigs poisoned with soman. The combination proved effective in opposing a lethal dose of soman (9 μg kg^{-1}, iv, per 20 minutes). The autoinject device contained two chambers: mixing is initiated prior to injection.

In addition to the above it should be recalled that atropine and oximes are synergistic in their effects and the administration of atropine and oxime has been shown to raise the LD_{50} of some nerve agents in animal models by a factor of more than 20 (Inns and Leadbeater, 1983). Soman remains a major problem, and the publications of Wolthuis and Kepner (1978), Wolthuis *et al*. (1981) and Clement (1981; 1982a; 1982b) should be consulted for details of the intractability of poisoning with soman, and for arguments regarding the use of H oximes. The

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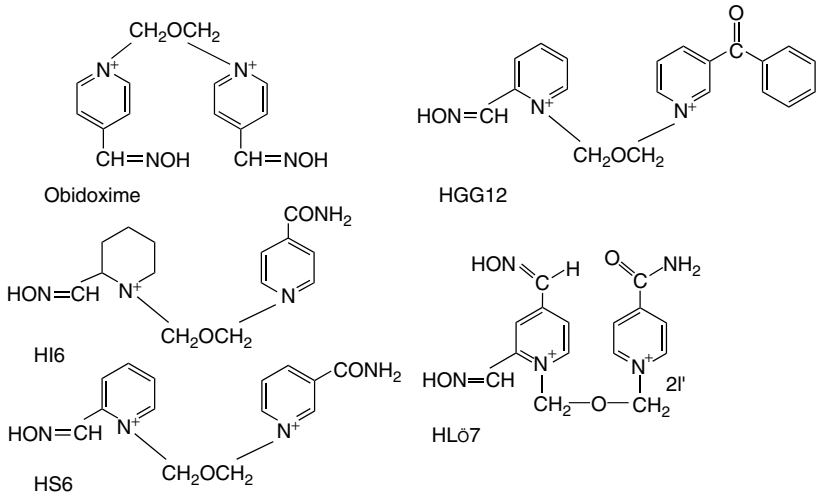

Figure 10 Structures of some of the better-known oximes.

recent account by Eyer and Worek (2007) should also be consulted.

7.9.2.3 *Anticonvulsants*

Diazepam, a benzodiazepine γ-aminobutyric acid (GABA) agonist, has come to be regarded as a valuable addition to the combination of atropine and oxime in the management of nerve-agent poisoning. Benzodiazepines, such as diazepam, do not activate the $GABA_A$ directly; rather they alter GABA binding at the $GABA_A$ receptor in an allosteric manner (Charney *et al*., 2001). Nerve-agent poisoning, and particularly poisoning with soman in monkeys, is often complicated by convulsions and the control of these convulsions with diazepam has been shown to enhance the likelihood of survival (Lipp, 1972; 1973). There is evidence from experimental nerve-agent poisoning in animals that benzodiazepines may ameliorate or prevent the development of histopathological changes in the central nervous system that are produced by organophosphates (Anderson *et al*., 1997; Hayward *et al*., 1990). A number of theories have been put forward to explain these effects, including the suggestion that diazepam may prevent the rise in cyclic GMP levels observed in the CNS of animals suffering soman-induced convulsions (Lundy and Magor, 1978): the significance of this finding is uncertain (Liu *et al*., 1988) and the phenomenon is likely to be secondary to the effect on the $GABA_A$ receptor. A number of other anticonvulsants have been studied in organophosphate poisoning and midazolam has been used clinically (see Marrs and Vale, 2005).

Diazepam should be given in a dose of 5 mg orally, intramuscularly or better intravenously. Diazepam is not suitable for combination in solution with atropine and P2S, but a recently developed lysylglycine derivative of diazepam (avisafone) is. This has been incorporated in the autoinjection devices issued to the UK armed forces.

7.10 Prognosis of Nerve-Agent Casualties

Animal studies have suggested that the combination of the pretreatment and treatment described above would probably be effective in cases of nerve-agent poisoning. It is, however, difficult to extrapolate from such work to the case of the soldier poisoned on the battlefield at a considerable distance from medical attention. For those casualties who have self-administered one or perhaps more of their autoinjection devices, but who are slipping deeper into the effects of the nerve agent, the prognosis can hardly be other than poor. Those who sustain a dose of nerve agent sufficient to produce respiratory failure are unlikely to survive. It is believed that self-administration of therapy will delay the onset of symptoms and signs of nerve-agent poisoning, and make it more likely that the poisoned person will survive and reach medical attention. The prognosis for civilian casualties exposed to significant amounts of nerve agent cannot be good: delays in administration of therapy and, of course, the absence of pretreatment will both militate against likely recovery. In those exposed to lower concentrations, survival is possible, but the need for rapid therapy remains paramount.

7.11 Long-Term Effects of Nerve-Agent Poisoning

It is well known that organophosphorus compounds can produce permanent damage to the nervous system (delayed polyneuropathy). These effects, thought to be dependent on the combination of the organophosphate with the enzyme known as neuropathy target esterase (NTE), have been studied in detail by Johnson (1992)

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and Moretto and Lotti (2005) and, in relation to insecticides, are discussed in **Toxicology of Pesticides**. Studies in animal models have demonstrated that the nerve agents are capable of producing these effects, but only when administered to animals protected by the prior administration of atropine and oxime, in doses many times in excess of their LD_{50}s. It is likely that a soldier who survives nerve-agent poisoning on the battlefield will not have been exposed to a dose of nerve agent capable of inducing delayed polyneuropathy. This also applies to civilian casualties.

Since the Gulf War, a great deal of publicity has been given to the so-called 'Gulf War syndrome'. A very wide range of psychological and physical effects have been reported in soldiers who fought in the campaign. It seems clear that veterans of the campaign have a higher self-reported prevalence of medical and psychiatric conditions than other personnel who did not see active service in the Gulf. Whether such effects are causally linked to exposure to pretreatment against nerve-agent poisoning, perhaps in combination with immunization schedules, remains unknown. The reader should consult the excellent series of papers published in 1997 (Iowa Persian Gulf Study Group, 1997; Haley and Kurt, 1997; Haley *et al*., 1997a; 1997b). Hyams *et al*. (1996) have pointed out that outbreaks of difficult-to-explain symptoms have been seen after every major war since the US Civil War and that many questions remain unanswered regarding these 'war syndromes'. The recent review by Wessely and Hotopf (2007) should also be consulted.

8 PULMONARY OEDEMA-INDUCING COMPOUNDS

During World War I, the chemical warfare compounds with the highest lethality were those which induced pulmonary oedema, and included chlorine and phosgene. Phosgene was first used by German forces on 19 December 1915 and soon acquired a reputation as a dangerous compound: 85% of deaths resulting from exposure to chemical warfare compounds during World War I were caused by phosgene (HMSO, 1972, 1987). Despite this, well-protected troops using modern respirators should not be placed at serious risk as a result of exposure to these compounds, and little development of pulmonary-oedema-producing compounds for use as chemical warfare agents has taken place since World War I. During World War I, great efforts to develop other pulmonary-damaging compounds were made and a large number of compounds were used, albeit sometimes on a comparatively small scale. Accounts dating from the 1920s should be consulted for details of these compounds. Among accounts generally available, that of Prentiss (1937) is particularly detailed and reliable. In a civilian context, chlorine and phosgene are especially worrying as they are widely used in industry, and transported by road and rail. The possibility of a terrorist organization releasing a large quantity of chlorine needs careful consideration. It will be recalled how effective chlorine released from cylinders was against unprotected troops in 1915.

During World War I, phosgene acquired a particularly evil reputation, as it was soon realized that men who had inhaled a potentially lethal dose of the compound might show few symptoms and signs during the first few hours following exposure (Vedder, 1925). Early diagnosis was therefore difficult. It was further noted that men in the symptomless latent period could collapse with florid pulmonary oedema if exposed to physical stress (HMSO, 1923). It will be appreciated that the sensible clinical advice to rest all those thought to have been exposed, for 24–48 hours, preferably in bed, was ill-received by commanders in the field. Similar problems would be faced by accident and emergency department staff (ER department staff) dealing with a large number of people all potentially exposed to phosgene as a result of a terrorist incident.

While phosgene might not present a severe toxicological risk to modern troops, it is not detected by all detectors and monitors in general military use, and troops thought to be at risk would be forced to don respirators and accept the concomitant drop in their performance.

The general effects of lung-damaging compounds have been described in **Inhalation Toxicity** and **Air Pollution**, and only those aspects of the toxicology of phosgene of importance to chemical warfare and antiterrorism are mentioned here.

8.1 Physicochemical Properties of Phosgene

These are given in **Table 14**.

Table 14 Physicochemical properties of phosgene

Formula	$COCl_2$
M.W.	99
M.P. (°C)	−118
B.P. (°C)	8.2
Vapour density	3.5
V.P. (mmHg)	
−13.7 °C	335
−10 °C	365
0 °C	555
8.2 °C	760
Odour	Stifling odour of new-mown hay

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8.2 Likely Mode of Exposure

Phosgene is rapidly dispersed by wind and is regarded as an agent of short persistence likely to be used only in surprise attacks. It may, however, linger in cellars, tunnels and hollows, as it is heavier than air. This point was discussed by Haldane (1938) who pointed out that rapid mixing with air would produce a mixture only very slightly heavier than air and that this would disperse rapidly in open air conditions. Lefebure (1921) described experiments undertaken during World War I in an attempt to convert phosgene into a more persistent compound by impregnating porous powders with the gas. These experiments do not seem to have been successful.

8.3 Absorption of Phosgene

Phosgene is not absorbed to a significant extent through the skin; it is of course absorbed by the lung. Nash and Pattle (1971) studied the reaction of phosgene with water at moist surfaces and concluded that the rate of hydrolysis would render penetration by phosgene of more than 'a few tens of microns' unlikely.

8.4 Toxicity of Phosgene

The toxicity of phosgene has been widely studied, and **Table 15** shows the range of toxicities encountered in different species.

As in the case of nerve agents, $L(Ct)_{50}$ is dependent on the respiratory state and will fall with exertion.

8.5 Mechanism of Action of Phosgene

Despite its long history, the exact mechanism of action of phosgene remains obscure. The hypothesis that phosgene acts by combining with water and forming hydrochloric acid (Winternitz, 1920; Vedder, 1925), which then produces tissue damage, was challenged by the work of Nash and Pattle (1971). These authors showed that the 'maximum concentration of acid in a blood–air barrier of thickness 1 μm, in contact with 25 ppm of phosgene is 7×10^{-10} M, which is negligible. Buffering by tissue constituents would prevent any significant change in pH'. At high concentrations it was accepted that the formation of hydrochloric acid might play a role.

Potts *et al*. (1949) proposed that phosgene combined with the amino groups of proteins to form diamides (**Figure 11**). Diller (1978) proposed a series of reactions to explain the combination of phosgene with a wider range of chemical groups (**Figure 12**). Frosolono and Pawlowski (1977) studied the biochemical changes produced by phosgene in various lung fractions prepared from rats exposed to close to an $L(Ct)_{50}$ of phosgene. A number of enzymes showed decreased activity, but the data did not allow a distinction to be drawn between reduction in enzyme activity as a result of direct inhibition and that resulting from loss from damaged cells. Recent studies shed new light on the mechanism of action of phosgene. Work by Kennedy *et al*. (1989), Guo *et al*. (1990), Sciuto *et al*., (1995; 1996; 1997; 1998; 2001; 2003) and Scuito and Hurt (2004) has led to the following conclusions:

1. Free-radical formation with associated lipoperoxidation and leukotriene formation lie at the heart of the toxicity of phosgene
2. Exposure to phosgene induces a decline in intracellular cyclic adenosine triphosphate (cAMP)

Table 15 Toxicity of phosgene

Species	Route	Term	Units	Value	Source
Rat	Inhalation	LC_{50}	mg m^{-3} per 30 min	1400	NTIS[a]
Mouse	Inhalation	LC_{50}	mg m^{-3} per 30 min	1800	NTIS
Dog	Inhalation	LC_{50}	mg m^{-3} per 20 min	4200	NTIS
Monkey	Inhalation	LC_{50}	mg m^{-3} per 1 min	600	NTIS
Rabbit	Inhalation	LC_{50}	mg m^{-3} per 30 min	1000	NTIS
Rat	Inhalation	LC_{LO}	ppm per 30 min	50	NIOSH[b]
Dog	Inhalation	LC_{LO}	ppm per 30 min	80	NIOSH
Rat	Inhalation	100% mortality	ppm per 20 min	37	

[a]NTIS, National Technical Information Service.
[b]NIOSH, National Institute for Occupational Safety and Health.
The values quoted are often described in toxicological databases as LC_{50} values, for example, the LC_{50} of phosgene for a 30 min exposure in mice is 1800 mg m^{-3}. Attempts to convert such values to $L(Ct)_{50}$ values by simple multiplication should be avoided as it is known that the $L(Ct)_{50}$ of phosgene is time dependent. It will be noted that as the duration of the exposure is increased the $L(Ct)_{50}$ value rises. This has also been noted by Ballantyne (1987) for hydrogen cyanide. A widely quoted statement of the toxicity of phosgene to humans is '50 ppm may be rapidly fatal' (for phosgene 1 ppm = 4.419 mg m^{-3} at STP). See **Table 16**.

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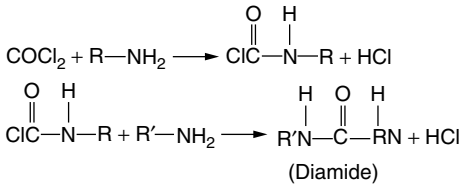

Figure 11 Formation of diamides.

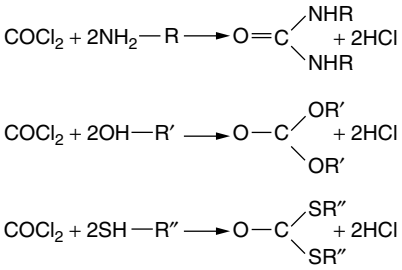

Figure 12 Reactions to explain the combination of phosgene with a wider range of chemical groups.

with an increase in capillary permeability, possibly resulting from effects on the endothelial cytoskeleton

3. Phosgene-induced decrements in tissue glutathione (GSH) levels affect regulation of intracellular calcium concentrations and this may lead to activation of transcription factors for genes controlling production of inflammatory mediators.

Jugg *et al*. (1999) reported interactions between phosgene and surfactant, and thus linked phosgene with changes in the regulation of water flux at the alveolar epithelium.

Table 16 Variations in lethal index of phosgene with duration of exposure (dogs)

Time of exposure (min)	Minimum lethal) dose mg l^{-1})	Lethal index (mg min m^{-3})
2	2.00	4000
5	1.10	5500
10	0.55	6500
15	0.46	6900
20	0.37	7400
25	0.30	7500
30	0.27	8100
45	0.20	9000
60	0.17	10 200
75	0.16	12 000

After Prentiss (1937).

In addition to the above, efforts have been made to produce a pathophysiological hypothesis to explain the production of pulmonary oedema. Phosgene clearly damages the blood–air barrier in the lung and allows the leakage of fluid from the pulmonary capillaries. At first this leak may be contained by increased flow in the pulmonary lymphatic system, but later an increase in fluid in the connective tissue spaces occurs and finally fluid spills over into the alveoli. This sequence, the standard pattern of 'permeability' as compared with 'hydrostatic' pulmonary oedema, has been most competently reviewed by Staub (1974), Teplitz (1979) and Robin (1979) and is further discussed in **Inhalation Toxicity** and **Air Pollution**. Some authors, including Everett and Overholt (1968) and Ivanhoe and Meyers (1964), have suggested that phosgene could lead to massive reflex vasoconstriction and the production of oedema as in neurogenic pulmonary oedema (NPE). In this condition a sudden redistribution of blood from the systemic to the pulmonary circulation is believed to occur, producing damage to the pulmonary capillary endothelium. The damage is believed to be such that, even when intravascular pressures have returned to normal, a leak of fluid continues. That phosgene acts by this mechanism seems unlikely, as the extraordinary systemic hypertension recorded in cases of NPE has not been observed in animal models of phosgene poisoning.

It should be understood that the exact mechanism by which phosgene causes an increase in pulmonary capillary permeability is not known. Pulmonary capillaries possess a complete endothelium, the cells being joined by tight junctions. It is often assumed that compounds such as phosgene produce some loosening of these junctions, although the changes involved at the ultrastructural level are ill understood. It would be particularly interesting to know whether phosgene produces any change in the pattern of strands of particles, revealed upon the p-face of the cell membrane by freeze-fracture techniques, which appear to be important in the structure of the tight junction.

8.6 Histopathology of Phosgene-Induced Lung Damage

The light microscopic appearances of phosgene-damaged lungs were accurately described by Winternitz in 1920. Since then many studies have been carried out, Pawlowski and Frosolono's work, 1977 at the ultrastructural level being particularly valuable. The sequence of changes described by Pawlowski is as follows:

- The epithelium of the terminal bronchiole appeared to be first affected. Intracellular vesiculation of ciliated epithelial cells and Clara cells was observed.

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These effects appeared very soon after exposure

- An increase in the amount of extracellular fluid visible in the interalveolar septae was noted
- Frank oedema of cells of the interalveolar septae was observed
- Swelling of Type II alveolar cells was noted
- Type I alveolar cells showed areas of focal disruption
- Interalveolar septae became widely distended with fluid
- Oedema fluid appeared in alveolar spaces.

Oedema fluid in phosgene-induced lung damage is protein-rich and, by light microscopy, classically eosinophilic. Damage to Type II alveolar cells is interesting, although a clear demonstration of a decline in surfactant production in phosgene-induced oedema is lacking. It is, however, known that by lowering the surface tension of the lining film of the alveoli, surfactant may reduce the forces acting to move fluid out of pulmonary capillaries (Pattle, 1965). Damage to cells which produce surfactant would then be expected to enhance the likelihood of alveolar oedema.

8.7 Symptoms and Signs of Phosgene Exposure

Because of the extensive use of phosgene during World War I, many detailed accounts of its effects on humans are available, Ireland (1926) and Vedder (1925) providing particularly valuable accounts. Accidental exposures occur and Diller (1978), Seidelim (1961), Fruhman (1974) and Bradley and Unger (1982) have also provided accounts.

Some authors describe eye irritation, coughing, lacrimation, choking and a feeling of tightness of the chest as early symptoms and signs of phosgene exposure. Doubtless these do occur in some patients, but it was clearly demonstrated during World War I that an absence of such effects did not preclude serious and sometimes lethal exposure.

The hallmark of phosgene poisoning has been recognized to be the occurrence of a latent period intervening between exposure and the onset of the symptoms and signs of pulmonary oedema. This period may range from 30 minutes to 24 hours depending, in part, on the severity of the exposure. During this period it is notoriously difficult to distinguish the mildly exposed from the severely exposed.

Once the latent period is over, dyspnoea, a painful cough and cyanosis appear rapidly. Increasing quantities of, initially whitish, but later pink, fluid are expectorated, a marked efflux of fluid, the 'champignon d'écume', sometimes appearing just before death. The cause of death is usually cardiac failure and circulatory collapse caused by hypoxia.

A World War I description of a chemist dying some hours after a brief exposure to a high concentration of phosgene during a laboratory accident gives the clinical picture:

> *His condition now (4.5 hours post exposure) rapidly deteriorated. Every fit of coughing brought up large quantities of clear, yellowish, frothy fluid of which about 80 ounces (2272 ml) were expectorated in one and a half hours. His face became of a grey, ashen colour, never purple, though the pulse remained fairly strong. He died at 6.50 pm without any great struggle for breath. The symptoms of irritation were very slight at the onset; there was then a delay of at least four hours and the final development of serious oedema up to death took little more than an hour though the patient was continually rested in bed.*
>
> (Vedder, 1925)

8.8 Management of Phosgene Poisoning

8.8.1 First Aid

Casualties should be removed from risk of further exposure by suitably protected attendants. Because phosgene would not be encountered in the liquid state, decontamination with fullers' earth or a dilute solution of bleach is unnecessary.

8.8.2 Medical Treatment

The management of phosgene poisoning is the management of permeability pulmonary oedema. No antiphosgene drug of any proven value has been discovered although hexamethylenetetramine (HMT) is discussed briefly below. Steroids, antibiotics, bronchodilators, respiratory stimulants and cardiac stimulants have all been suggested, although none has received universal support. Two measures are, however, generally agreed.

8.8.2.1 Rest

All persons thought to have been exposed to phosgene should be confined to bed. It was demonstrated repeatedly during World War I that exertion during the latent period following exposure to phosgene could precipitate acute and fatal pulmonary oedema.

8.8.2.2 Oxygen

Patients unable to maintain an adequate arterial oxygen tension when breathing air should be given supplementary oxygen. This was stressed during World War I by

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Barcroft (1920) and Haldane (1917), when experience in the management of phosgene poisoning was unrivalled.

Of measures not commanding universal support, the following should be considered:

8.8.2.3 Corticosteroids

Arguments both for and against the use of steroids in pulmonary oedema have been plentiful for some years and have been considered by Everett and Overholt (1968), Diller (1978) and Bradley and Unger (1982). Everett reported the successful use of glucocorticoids; Diller also supported their use, although Bradley found the evidence of their efficacy unconvincing. It is known that inflammatory changes are likely to occur in lung tissue damaged by phosgene and that these changes involve the release of mediators which are likely to increase capillary permeability and therefore worsen the oedema. That such release can be prevented by the prophylactic administration of corticosteroids seems likely; that such release can be reduced significantly once initiated remains doubtful. Some clinicians, perhaps the majority, have felt that the lack of serious side effects usually associated with the short-term administration of large doses of corticosteroids and the seriousness of permeability pulmonary oedema, justifies the use of these drugs. Others have felt that the lack of clear evidence of efficacy should preclude their use. In some military manuals (HMSO, 1972, 1987), the use of a large single dose of corticosteroid as soon as possible after exposure has been advocated.

8.8.2.4 Antibiotics

The provision of antibiotic cover in phosgene poisoning has been supported by both Diller (1978) and Everett and Overholt (1968). During World War I, the absence of antibiotics made pneumonia a feared complication of lung damage arising as a result of exposure to a variety of chemical warfare compounds. Selgrade *et al.* (1995) showed that exposure to phosgene impaired the capacity of mice to deal with an inhalation challenge with bacteria. This was ascribed to impairment of macrophage and natural-killer-cell activity. The choice of antibiotics is wide, penicillin G, amoxycillin and chloramphenicol all having been recommended.

Of measures not receiving wide support, only one will be considered.

8.8.2.5 Hexamethylenetetramine ('Hexamine', 'Methanamine', HMT)

Hexamine is used prophylactically as an antimicrobial drug in cases of recurrent urinary-tract infections. It has been demonstrated to be of value in animal models, if given before exposure to phosgene. Stavrakis (1971) argued that hexamine was also of value if given post-exposure in cases of phosgene poisoning and recommended the administration of 20 ml of a 20% solution, intravenously, as soon as possible after exposure. Diller (1978) reviewed the use of hexamine and concluded that there was no firm evidence to support the view that hexamine is of value if given after poisoning.

Studies by Sciuto *et al.* (1995; 1996) have investigated the use of *N*-acetylcysteine (NAC) and dibutyryl cyclic AMP (DBcAMP) in phosgene poisoning. Postexposure intratracheal administration of NAC in isolated perfused rabbit lung was shown to reduce the formation of oxidized glutathione and a number of markers of lipid peroxidation, which was shown to be induced by exposure to phosgene. Similarly, DBcAMP acted as an antioxidant. No clinical studies of the efficacy of these methods of treatment are yet available.

As in all cases of permeability pulmonary oedema, the administration of intravenous fluids should be approached with great caution.

8.9 Long-Term Effects of Phosgene Poisoning

Chronic bronchitis and emphysema have been reported as a consequence of exposure to phosgene (Cucinell, 1974). Chronic pneumonitis has been reported in rats exposed to phosgene (Gross *et al.*, 1965).

9 HYDROGEN CYANIDE

Of the poisons known to the general public, cyanide, arsenic and strychnine are perhaps the best known and it is often assumed that cyanide would be a dangerous chemical warfare agent. Despite this, hydrogen cyanide has been but little used as a chemical warfare agent and the physicochemical and toxicological characteristics of hydrogen cyanide make it unsuitable for such use on any other than a fairly small scale. It has, however, been used as a means of judicial execution and was used for the large-scale murder of prisoners in German concentration camps during World War II.

Only France used hydrogen cyanide as a chemical warfare agent during World War I, the first use of hydrogen cyanide shells being on the Somme on 1 July 1916 (Prentiss, 1937). German respirators offered poor protection against hydrogen cyanide, although this was quickly remedied and hydrogen cyanide lost most of its advantages over the alternative lethal compound, phosgene. It will be recalled that phosgene has a density equal to 3.5 times that of air, but that hydrogen cyanide is less dense than air. Rapid dispersion of hydrogen cyanide

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greatly reduced its value as a chemical warfare agent. Prentiss (1937) commented: 'Because of its extreme volatility and the fact that the vapours are lighter than air, it is almost impossible to establish a lethal concentration of hydrocyanic acid in the field and this is particularly true when the gas is put over in artillery shells.' Few authorities today believe that hydrogen cyanide would be used on a large scale as a chemical warfare agent, although it has retained its place in military chemical warfare handbooks on the grounds that successful use, at high concentration, on selected targets could probably be achieved.

In view of the above, the discussion of hydrogen cyanide as a chemical warfare agent will be limited.

9.1 Physicochemical Characteristics of Hydrogen Cyanide

These are shown in **Table 17**. Below 26 °C hydrogen cyanide occurs as a colourless to yellowish–brown liquid. In its usual slightly impure state it is unstable, although it is said to be stable when highly purified. On standing, polymerization takes place and the compound may present an explosive hazard. The risk of explosion may be much reduced by the addition of a small quantity of an acid, for example, phosphoric acid. Prentiss (1937) commented: 'Anhydrous hydrocyanic acid is extremely unstable and is quickly decomposed with the formation of a black resinous mass.' This tendency to decomposition led to difficulties with munitions and Sartori (1939) commented: 'Even in the anhydrous condition it cannot be kept long as it gradually decomposes, occasionally with explosive force. Filled in projectiles it soon becomes harmless.' Hydrogen cyanide smells of almonds, although not all individuals are able to detect the odour. The capacity to detect hydrogen cyanide rapidly wanes on exposure, owing to failure of cells of the olfactory mucosa.

Table 17 Physicochemical properties of hydrogen cyanide

Formula	HCN
M.W.	27.02
Vapour density	0.93 × that of air
B.P. (760 mmHg) (°C)	26
M.P. (°C)	−14
V.P. (mmHg)	
−10 °C	165
0 °C	256
20 °C	600
26 °C	757

Table 18 Estimated toxicity of hydrogen cyanide in humans

Duration of exposure	Concentration (C) (mg m^{-3})	L(Ct)$_{50}$ (mg min m^{-3})
15 s	2400	660
1 min	1000	1000
10 min	200	2000
15 min	133	4000

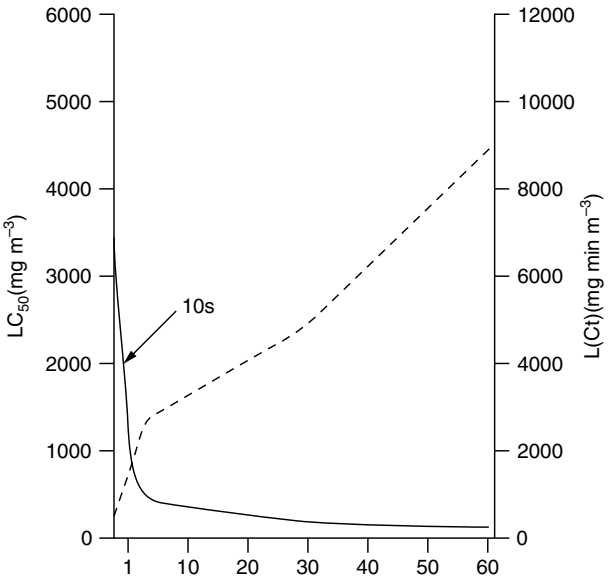

Figure 13 Acute lethal inhalation toxicity of hydrogen cyanide vapour. Solid line: LC_{50} of hydrogen cyanide (mg m^{-3}). Dashed line: corresponding L(Ct)$_{50}$ values (mg min m^{-3}). From Ballantyne and Schwabe, (1981). (Reproduced from Ballantyne and Schwabe, 1981. © Taylor & Francis Group.)

9.2 Absorption of Hydrogen Cyanide

Hydrogen cyanide vapour is readily absorbed across the lung, but only to an insignificant extent across the skin.

9.3 Toxicity of Hydrogen Cyanide

The L(Ct)$_{50}$ value for hydrogen cyanide in humans is not known with any accuracy. It is, however, known that the L(Ct)$_{50}$ value is likely to be very time dependent. This is shown by the estimates of human toxicity given in **Table 18**. The variation in L(Ct)$_{50}$ with time is believed to relate to the detoxification of cyanide by enzymatic conversion to thiocyanate. Ballantyne and

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Table 19 Estimated toxicity of hydrogen cyanide in humans

Toxic effects	Concentration (mg m^{-3})
Mild symptoms on inhalation for many hours	24–48
Maximum tolerance limit for 1 h	50–60
Hazardous to life on inhalation for 30–60 min	112–150
Death in 5–10 min	240–360
Death in 5 min	420
Death after several breaths	1000

Schwabe (1981) have shown the variation in graphical form (**Figure 13**).

Further guidance on the estimated toxicity of hydrogen cyanide may be gained from **Table 19**.

9.4 Symptoms and Signs of Hydrogen Cyanide Poisoning

It is often assumed that exposure to a given concentration of hydrogen cyanide either produces sudden death or very few ill effects. In military circles this is often summed up by the observation that the use of hydrogen cyanide leaves 'the quick and the dead', but no incapacitated or partly incapacitated individuals. This is incorrect. Work in animal models (D'Mello, 1987) has shown that exposure to sublethal doses of hydrogen cyanide can produce incapacitation. Dizziness and nausea have been recorded in people exposed to sublethal quantities of hydrogen cyanide, the effects lasting for some hours.

At lethal concentrations, collapse and death are rapid. Vedder (1925) has provided the following description:

> *In an atmosphere containing a lethal concentration the odour of bitter almonds is noticed. This is followed rapidly by a sensation of constriction of the throat, giddiness, confusion and indistinct sight. The head feels as though the temples were gripped in a vice and there may be pain at the back of the neck, pain in the chest with palpitation and laboured respiration. Unconsciousness occurs and the man drops. From this moment if the subject remains in an atmosphere of hydrocyanic acid for more than two or three minutes death almost always ensues, after a brief period of convulsions followed by failure of respiration.*

9.5 Management of Hydrogen Cyanide Poisoning

Here only a few points of relevance to battlefield casualties will be made:

- The usual points regarding the rapid removal of casualties from the risk of further contamination should be noted
- Decontamination of casualties is unnecessary unless the clothing is contaminated with liquid hydrogen cyanide. This is very unlikely
- All forms of treatment currently available demand intravenous administration. This will probably be impossible on the battlefield
- Bearing the last point in mind, it is likely that casualties who reach aid posts will be incapacitated by sublethal doses of hydrogen cyanide. Given that adequately trained medical orderlies, nursing staff or medical staff are available, antidotal therapy would be used. When deciding which therapy to recommend it should be borne in mind that the exact diagnosis of the cause of collapse may, under battlefield conditions, be difficult, and treatment known to produce severe side effects if given to casualties who have, in fact, not been exposed to hydrogen cyanide should perhaps be avoided.

9.6 Prophylaxis in Hydrogen Cyanide Poisoning

It is generally accepted that treatment of casualties suffering from hydrogen cyanide poisoning, under battlefield conditions, will be difficult and often unsuccessful. Because of this, attempts to provide a measure of pharmacological protection against the effects of hydrogen cyanide have been made. The conversion of a fraction of haemoglobin to methaemoglobin, thus providing a ready-made binding site for the cyanide, has been a favoured line of research and in 1948 Gilman and Cattell reported studies undertaken during World War II in America. *p*-Aminopropiophenone was found to be the best compound tested for the production of methaemoglobinaemia and oral doses of 2.0 mg kg^{-1} were given to volunteers. This produced a methaemoglobinaemia of some 20–30%. This was maintained by repeated dosing over seven days without ill-effects although 'at the end of this period there were

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observed the beginnings of a haemolytic anaemia'. Methaemoglobin levels of up to 15% were not found to interfere with exercise at light workloads. Animal work was reported to have shown that such levels of methaemoglobin (15%) would have provided protection against 10 lethal doses of hydrogen cyanide. On the battlefield, recalling the difficulty likely to be experienced in establishing a lethal concentration of cyanide, such a level of protection would be very valuable. Work by Bright (1987) in dogs has confirmed the above results although the issue of *p*-aminopropiophenone to troops for use in anticipation of an attack with hydrogen cyanide has, as far as is known, not been undertaken by any country.

10 LESSER CHEMICAL WARFARE AGENTS

In addition to the compounds described above, many other substances have been considered, although in the main rejected, as possible chemical warfare agents. During World War I, dozens, probably hundreds, of compounds were examined and during World War II several hundred mustard derivatives and variants were synthesized. The large number of compounds in this group which *could* be used in war makes any detailed consideration of individual compounds impossible. Instead a few comments will be made on some older compounds of interest and on the riot-control agents which could be encountered during modern warfare.

10.1 Early Irritant Compounds

Smoke from fires, used since classical times to discommode forces defending strong positions, exerted its effects by producing choking and eye irritation. In the early twentieth century, the Paris police force used grenades containing ethyl bromoacetate against rioters and it has been alleged that some of the police involved, later conscripted into the French Army, used the same grenades against German forces. The use of ethyl bromoacetate seems to have been taken up by the French and was also studied in London at Imperial College. (The military abbreviation for ethyl bromoacetate, SK, is said to stand for South Kensington, the location of Imperial College.) German workers also studied irritants and on 27 October 1914, at Neuve-Chapelle (France), high-explosive (HE) shells containing lead balls embedded in the irritant *o*-dianisidine chlorosulfonate were deployed (SIPRI, 1971). This incident was followed by the use of other compounds, including xylyl bromide, chloroacetone, benzyl bromide and iodide, ethyl and methyl chlorosulfonate, 2-chloracetophenone and bromobenzyl cyanide. Prentiss (1937) held the latter to be the most powerful irritant introduced during the war. In addition to the lacrimators, more toxic irritant smokes were developed. These included diphenylchlorarsine (DA), chlorodihydrophenarsazine (DM or Adamsite) and diphenyl cyanarsine (DC).

These compounds were described as vomiting agents and as well as producing nausea and vomiting, produced acute pain in the nose, uncontrollable sneezing, coughing, eye pain and lacrimation (HMSO, 1972, 1987). The marked sneezing led to these compounds being termed sternutators (Latin *sternuto* ... sneezing). Of these compounds only Adamsite has survived to modern use and has been used as a riot-control agent, although not in the UK.

Other arsenical compounds developed during World War I included methyl-, ethyl- and phenyldichlorarsine, collectively known as 'The Dicks' (HMSO, 1972, 1987). These were vesicant compounds of considerable toxicity and were developed in an attempt to find a 'faster-acting mustard gas' which would incapacitate casualties on contact. Attempts were also made to develop compounds combining irritancy with the lethality of hydrogen cyanide. Cyanogen bromide and chloride were produced, both effective irritants capable of killing. Despite this, neither proved a very successful chemical warfare agent (Prentiss, 1937).

10.2 Modern Riot-Control Compounds

The development of modern riot-control compounds has been dominated by the perceived desirability of developing a compound with the following characteristics:

- Rapid incapacitating effect even when used against highly motivated individuals
- Insignificant toxicity even to the very young or the very elderly bystander
- Capacity for easy dissemination
- Capacity for easy decontamination
- Long shelf-life
- Low cost.

It will be appreciated that these are difficult criteria to meet in full. At first glance, the first two criteria listed might appear likely to be mutually exclusive and yet each is met remarkably well by compounds such as CS and CR (dibenz(b.f)-1,4-oxazepine) discussed below.

A detailed review of those compounds which might be considered for use today in riot control has been provided by Ballantyne (1977) and much of the present account has been based on his work. Information on the individual compounds is given in **Table 20**.

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Table 20 Riot-control agents

Compound	Formula	Toxicometrics				V.P. 20 °C (mmHg)	Water solubility (20 °C)	Onset of effects	Recovery
		$L(Ct)_{50}$	$L(Ct)_{50}$	TC_{50}	IC_{50}				
		rat (pure compound) (mg min m^{-3})	human (estimated) (mg min m^{-3})	human eyes (mg m^{-3})	human (mg m^{-3})				
DM		3700–12 710	11 000–35 000	—	25–220	2×10^{-13}	Insoluble	Delayed for some hours	1–2 h
CN		3700–18 800	8500–25 000	0.3	20–50	5.4×10^{-3}	4.4×10^{-3}	At once	20 min
CS		88 480	25 000–150 000	4×10^{-3}	3.6	3.4×10^{-5}	2.0×10^{-4} (rapid hydrolysis)	At once	20 min; erythema for up to 24 h
CR		>425 000	>100 000	4×10^{-3}	0.7	5.9×10^{-5}	3.5×10^{-4}	At once	20 min; erythema for up to 1 h

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10.3 Dangers Associated with Exposure to Riot-Control Compounds

Despite the low toxicity of these compounds, exposure is not entirely without risk. The following points should be borne in mind.

- Exposure to levels much in excess of those anticipated, for example in a closed room, might produce significant toxic effects. CN (2-chloroacetophenone) in large doses may produce corneal damage, particularly if the compound enters the eye in the form of powder. Five deaths due to the pulmonary damage following exposure to high concentrations of CN in enclosed spaces have been reported (Gonzales *et al*, 1954; Stein and Kirwan, 1964). Exposure to high concentrations of riot-control agents can occur as a result of use of personal protection devices. These are illegal in the UK, but incidents involving their use are not uncommon. In 1995, Wheeler and Murray reported that 354 calls to the National Poisons Information Service (London) regarding CS had been received. Advice on management has been provided by Gray (1995).
- Exposure to irritants may produce transient, although significant, elevations of blood pressure. These have generally been regarded as not likely to do harm in healthy individuals, although those suffering from hypertension, aneurysms or myocardial disease might be placed at some risk (Ballantyne *et al*., 1976).
- Hysteria and panic may be produced by exposure to irritants, particularly if the means of escape from exposure are blocked. Secondary injuries may be produced by stampeding crowds.
- Each of the compounds listed in the above table, with the exception of CR, can produce contact sensitization (Holland and White, 1972; Rothberg, 1970).

10.4 Management of Casualties Exposed to Riot-Control Agents

As for all other compounds discussed in this chapter, removal of casualties from the risk of further contamination by adequately protected attendants is of first priority. Contaminated clothing should be removed and placed in polythene bags: CS and CR are notorious for spreading during decontamination of casualties. Lacrimation, blepharospasm, blepharoconjunctivitis and eye pain disappear quickly after removal from an irritant cloud. The effects on the eyes may be initially so distressing that casualties may require a great deal of reassurance that permanent eye damage has not been produced. The eyes should be kept open and those who have been regularly exposed to CS advise standing with the eyes open 'facing the wind'. Irrigation of the conjunctival sacs with 0.9% saline brings rapid, although in the authors' personal experience, sometimes temporary, relief.

Skin should be decontaminated with soap and water. In the case of CS, hydrolysis occurs quickly and decontamination is rapidly accomplished. Showering is often advised, but it should be remembered that CR, and to a lesser extent CS, may be washed out of the hair and produce secondary contamination of the eyes. Erythema generally subsides without treatment, although primary contact dermatitis may require treatment with corticosteroid ointment.

11 ALLEGED CHEMICAL WARFARE AGENTS

One of the commonest misconceptions regarding chemical warfare is represented by the view that compounds of great toxicity must, *ipso facto*, be likely effective chemical warfare agents. This view is so commonly put that it may be worth noting that in assessing the potential of a compound as a putative chemical warfare agent, a number of factors have to be considered of which acute toxicity is only one and probably not the most important. These criteria include:

- Ease of manufacture in large quantities. Chemical warfare waged on a small scale during a major war is unlikely to produce significant results. It is sometimes argued that assassinations have been carried out using small quantities of highly toxic compounds. This is true, but assassination should not be confused with chemical warfare
- The conditions of storage of the compound should not be demanding and the compound should not deteriorate on storage
- The compound should be easy to disperse using inexpensive munitions and should not be destroyed by the dispersal system. The compound should be active in the form in which it is likely to be encountered by the opposing forces: a compound only active by the intravenous route would be an unsatisfactory chemical warfare agent
- On the whole, compounds producing severe and care-demanding incapacitation are more effective chemical warfare agents than those which are inevitably lethal
- It should be possible to protect one's own forces against the effects of the chosen compound without excessive expense or loss of efficiency

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- The compound should offer advantages when compared with already available compounds which meet the above criteria.

A rigorous application of these criteria will serve to remove many substances from accounts which purport to list possible or probable chemical warfare agents. In assessing the *probability* that a compound would be used as a chemical warfare agent the question, 'Why should this compound be chosen?' should always be asked rather than the question, 'Could this compound be used as a chemical warfare agent?'

A considerable range of exotic compounds have been examined as potential chemical warfare agents: batrachotoxin, tetrodotoxin, saxitoxin, palytoxin, botulinus toxin, staphylococcal enterotoxin B, ricin and abrin have all been examined in detail. Ricin was used to assassinate G. Markow in 1978. Figures are not available for the toxicity of staphylococcal enterotoxin B when absorbed by inhalation, although it is felt the compound would be significantly more toxic when administered in this way than by ingestion (Mattix *et al*., 1995).

During the early 1980s, considerable attention was paid to toxins of fungal origin: mycotoxins. It was alleged that a group of mycotoxins known as trichothecenes had been used in Southeast Asia. The compounds on which attention centred were: T2 toxin (*Merck Index*, 11th edn, ref no. 9711) and nivalenol (*Merck Index*, 11th edn, ref no. 6581).

T2 is a caustic skin irritant which may cause dizziness, nausea, vomiting, diarrhoea and haemorrhage. The LD_{50} of T2 toxin (oral, rat) is $4.0\,mg\,kg^{-1}$. Nivalenol, also known to be capable of producing similar effects, has an LD_{50} (ip, mice) of $40\,\mu g\,kg^{-1}$. Further discussion of the toxicity of these compounds may be found in the work of Tatsuno (1968), Wade (1981), Rosen and Rosen (1982), Marasas *et al*. (1969) and Wang *et al*. (1998). Irrefutable evidence that these compounds were deliberately used as chemical warfare agents, is, however, lacking. During the Soviet occupation of Afghanistan, it was alleged that Soviet forces had used chemical warfare agents against rebel tribesmen. No clear identification of the compounds alleged to have been used has appeared and the effects reported—the induction of unconsciousness with recovery with few ill-effects an hour or so later and the production of blackened and very rapidly decaying bodies—have been hard to explain.

In examining reports of alleged uses of chemical warfare agents factors, such as the likely naivity of observers, deliberate attempts to mislead and the more common causes of death in war should be borne in mind. Before dismissing such reports, however, it should also be remembered that small-scale use of chemicals on an *ad hoc* basis might occur during a war waged by poorly disciplined forces.

All the compounds considered thus far have been characterized as likely to have effects on humans. If the definition of chemical warfare is widened a little and the attack on food production resources or woodland, which provides hiding places for troops, is included, then a number of herbicidal compounds could also be considered. Such compounds may be *per se* toxic to humans or, in the forms deployed, contain toxic contaminants. During the Vietnam War, American forces used large quantities of herbicides, including the phenoxyacetates, 2,4-D and 2,4,5-T, some preparations being contaminated with TCDD (tetrachlorodibenzodioxin). The mixture was often referred to as Agent Orange (Young *et al*., 1978). Exposure to the mixture has been alleged, though not proven, to have produced long-term effects in both Vietnamese and American veterans. Details of the toxic effects of herbicides may be found in **Toxicology of Pesticides**.

12 CONCLUSIONS

Chemical warfare has a long history, although only in the twentieth century has it been developed as a means of waging war on a large scale. In the late 1980s moves to ban the production and stockpiling of chemical weapons have been made and may yet prove completely successful between countries involved in these negotiations. It should, however, be recalled that treaties have been violated in the past and that a number of countries currently believed to have acquired or to be acquiring chemical weapons are not involved in treaty negotiations. It seems, therefore, that although the risk of chemical warfare has diminished during the past decade, such a risk still exists.

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Chemical Terrorism

Bryan Ballantyne

CONTENTS

1 BACKGROUND

Man has lost his capacity to foresee and to forestall. He will end by destroying the world.

(Albert Schweitzer)

Terrorism has a very long history. Those living in English-speaking countries will be familiar with the gunpowder plot, where a group of catholics tried to blow up parliament and King James I (James VI of Scotland) in 1605 (Fraser, 1996). In the nineteenth century, numerous assassinations were carried out by anarchists, notably that of Empress Elisabeth of Austria (Avril, 1993). It should also be remembered that states may use terror against populations, including those of neighbouring states; an example of that is the behaviour of France in the Palatinate in 1688 (Lynn, 1999). A more recent incident that could be considered terrorism against a state's own inhabitants is the use of chemical weapons in Halabja (Iraqi Kurdistan) against the Kurdish population of Iraq, by the government of Iraq in 1988 (Gosden, 1998). Nor should be forgotten the terror and the judicial murder of the King that took place during the French Revolution (Carlyle, 1906) and the terror of the Russian revolution (Pipes, 1990). Many of the actions of the Hitler government in Germany against both Germans and inhabitants of other European states could be considered as terrorism (Kohn, 1960; Lee, 1987). For a discussion of the origins and causes of terrorism see Burleigh (2008).

Richardson (2006) has defined terrorism as 'deliberately and violently targeting civilians for political purposes', with seven characteristics, as follows: (i) politically inspired, (ii) involves violence or the threat of violence, (iii) the objective is not to defeat, but to send a message, (iv) the act and the victim have symbolic significance, (v) terrorism is usually the deliberate action of substate groups, (vi) victims of the violence are usually a means for altering behaviour of a larger audience, usually a government and (vii) there is deliberate targeting of civilians.

1.1 Response to Terrorism

One of the objectives of terrorism may be to generate a disproportionate response and thereby alienate the population against the state. This creates, within organized communities, a situation of distrust, suspicion and a tendency to political dictatorship. This is a consequence

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of, and a reflection of, for example, government spying on citizens' private communications, gaining access to personal information and creating files for stated use by homeland security and government/military intelligence agencies, but clearly such information may be used for other purposes, other than those for which they were purportedly intended. Also there is a clear danger that overreaction to terrorism may damage the democracy that authorities are trying to protect.

Despite the discussion above, there are many measures that governments and other organizations may take in order to prepare for and mitigate effects of chemical terrorism and these measures are discussed below.

2 THE SPECTRUM OF TERRORIST ACTIVITIES

2.1 General Considerations

From a practical overview perspective, the term terrorist incident covers a wide spectrum of situations, the common feature of which is a desire by an individual or a group of individuals to produce an assault on organized law-abiding societies in order to attempt to draw attention to inequalities or perceived political malpractices, or to alter popular attitudes, opinions, legislation or political dictate, by the use of methods designed to make the terrorist motivation clearly and fearfully known to the appropriate members or sectors of that society. The more sophisticated terrorist will employ tactics designed to destroy the fabric of organized societies by means of occult infiltration into the social structure in order to interfere with political and governmental processes and/or financial and economic dominance. In the more usual terrorist approach, the techniques employed are designed to cause fear or panic in the targeted population, and in some cases there may be a deliberate objective to cause random or targeted deaths and mutilations. The range of weaponry potentially available to terrorists is wide and variable: weapons used may include guns or explosive devices, radioactive/nuclear sources, biological organisms and toxins (bioterrorism) or xenobiotic chemicals (chemical terrorism). It should be noted that there may be classification and use overlap between biological toxins and xenobiotics (Pearson, 2007). The use of chemicals of differing classes is generally recognized by the public as being very likely in certain situations by some terrorist groups.

At one end of the spectrum of those who might be described as terrorists are individuals, or small groups of individuals, who have, for very variable reasons, aberrant behaviour patterns against either a whole civilized society or individuals of that society. Terrorists of this type may seek vengeance or desire to frighten (terrorize) individual citizens or the population at large in order to demonstrate, and make known, the reason(s) for their antisocial and often destructive physical and amoral activities. At the other extreme of the spectrum of terrorists are larger groups or organizations, national or international, whose destructive motivation is based on cult beliefs, extreme political/ethnic persuasions, religious dogma or a combination of some or all of these factors, frequently aggravated by abnormal behavioural attributes. Furthermore, a terrorist–terrorized confrontation is frequently not a one nation vs. a different nation, but may involve one national or religious group against another within a nation or against the remainder of the same national population. This is usually the case with individuals or small-group activities.

2.2 Individual and Small-Group Terrorist Activities

An individual, or small group of individuals can draw attention to grievances or the reason(s) for such grievances, by physical or chemicals means. This type of activity is usually, though not invariably, based on circumscribed objectives and directed against a specifically targeted population. An example is the attacks by the Aum Shinrikyo cult in Japan (see below).

2.3 Major and International Terrorist Activities

Here a group of individuals will try to draw attention to grievances or the reason(s) for such grievances, by physical or chemicals means and will take on a state or a large proportion of the population therein. This type of activity is usually, although not invariably, based on circumscribed objectives and directed against a specifically targeted population. Often the number of individuals is quite small, but they may enjoy the passive support of a proportion of the population. Examples include Al Qaeda (BBC, 2008a), the Provisional Irish Republican Army (IRA) (Shanahan, 2009) and Euskadi Ta Askatasuna (ETA) (BBC, 2008b).

3 AGENTS FOR CHEMICAL TERRORIST ACTIVITIES

Until 1994, the use of chemical weaponry by terrorist groups was considered unlikely, but this all changed with

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the deployment of chemicals by a terrorist group in Japan (see below). After this, many countries started to consider the possibility that chemicals might be used elsewhere against civilian populations; indeed the United States Government organized a seminar on the consequences of chemical and biological terrorism as soon as 1995, which included British, Canadian and Japanese participants (DHHS, 1995).

3.1 Criteria for Choice of Agent

Widespread discussions and resultant recommendations have concluded that chemicals of various and different classes are likely be used by terrorist groups, depending on the depth of knowledge and degree of sophistication of the group or individual. Chemicals that might be used, and in some cases chemicals that have been used, vary widely, as do their modes of delivery. It must be appreciated that the world chemical industry is very large and that large quantities of toxic industrial chemicals (TICs) are continually being produced and transported by rail, road and ship, both within states and internationally. Whether a particular TIC could practicably be used depends on numerous factors.

On a generic basis, classes of chemicals that could be used include the following:

- Irritant and/or disorienting materials (for inducing panic)
- Nauseating materials (for inducing revulsion)
- Psychogenic materials (for inducing panic and fear)
- Severely toxic and lethal agents (for inducing panic and/or deliberate injury or murder).

It is assumed that most terrorists, or individuals using terrorist-like approaches, will choose appropriate chemicals that have the following attributes:

1. Cheap to purchase
2. Can be readily purchased or otherwise obtained, if necessary in bulk, or readily manufactured (synthesized) in 'home' laboratories; the relative ease of access to purchase and recipes using the internet requires attention and control (Yoshida, 2008)
3. Capable of causing mass incapacitation, fear, social disruption and/or mortality
4. Comparatively easy to handle and use
5. Have high biological activity and, for most circumstances, having effects that are rapid in onset
6. Can be acquired, with associated dispersal systems, without high levels of suspicion.

3.2 Possible Modes of Delivery

3.2.1 General Comments

The mode of delivery of chemicals to intended targets will depend on several factors, which include the following:

- The target population and its size, and whether individuals or random groups
- Whether the aim is deliberate killing and/or to cause fear and panic
- Whether the target is intentionally selective or random
- Whether the incident is planned and is to be conducted by individual(s) or group terrorist activity
- The planned location
- The facilities available
- The physicochemical properties of the chosen agent
- The ease of access to chosen location
- The possibility of concealment of any signs of suspicious activity
- Whether the perpetrator is prepared to die during the incident.

3.2.2 Specific Routes

Some potential scenarios are presented as follows:

1. **Inhalation exposure**: Volatile materials and substances that can be used as aerosols are likely to be used in enclosed spaces, such as rooms, indoor entertainment areas and tunnels, such as subway systems. With airborne dispersion in closed, crowded areas, the resultant panic caused by exposure to xenobiotics may lead to physical injuries and lethalities due to attempted escape to outdoor fresh air. This was seen in a Chicago nightclub as a result of a security guard discharging an irritant chemical (oleoresin capsicum) from an aerosol generator in an attempt to break up a fight; the resultant panic and stampede towards the door in a limited escape situation resulted in the deaths of 21 persons (USA Today, 2003). Lesser, but definite degrees of panic, sometimes with physical injury, have been recorded following release of irritant materials such as CS (2-chlorobenzalmalononitrile) into the atmosphere in subways (BBC, 2005) and nightclubs (BBC, 2002a).
2. **Peroral exposure**: Food and drink poisonings as terrorist-like attacks have been experienced in several countries, including Japan. Toxic substances used have included paraquat, organophosphate (OP) pesticides, cyanide and arsenate (Yoshida, 2008). Clearly there is a requirement to be aware of the potential

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for possible massive chemical poisoning exposure through locally contaminated and imported food and drink. To deliver by mouth in food there can be deliberate incorporation of the toxin randomly within shops or at distributor locations, or more specifically in foods at the locations of planned functions. For uncontrolled and widespread effects, toxin can be incorporated in basic crops at storage locations; for example, excess treatment with pesticide. For inclusion in fluids for drinking a variety of delivery modes are available depending on the planned size and geographical location of the target population. For individuals or small groups, direct precontamination of the drink may be possible, or incorporation into bottles or fluid containers. For larger-scale contamination of drinking water then it might be possible to introduce poison into fluid in storage vessels/cisterns. Cult killings, as a means of conveying a sinister message, has been effectively demonstrated by incorporating poison into drink (Thompson *et al*., 1987). With chemicals it is highly unlikely that effective intoxication would be possible by contamination of large-scale storage units, including reservoirs; the problems associated with marked dilution and nonhomogenous distribution would make this approach not feasible. It is more likely that bacterial or biotoxin contamination would be more effective in these circumstances. Other possible methods for peroral administration of potent toxins include the repacking of medicinal capsules for a specific target or, if done in pharmacies, for random 'hate' killings (see Section 3.4.2.1), although this activity has now been essentially stopped with the introduction of tamper-proof wrappings.

3. **Percutaneous absorption**: Highly potent chemicals or drugs, which are absorbed percutaneously, have been noted to be potentially a means of lethal intoxication of selected or random individuals.

3.3 Diagnosis

Preparedness for chemical incidents is described below in Section 4 and diagnostic measures for specific chemicals is discussed in Section 3.4, but it must be emphasized that determinative diagnosis should not take precedence over immediate aid to victims. Diagnosis will depend on characteristic symptomatology, possibly aided by field-deployable tests and chemical agent monitors (CAMs) (see Section 4), may be useful. There may be other clues that something untoward has happened, for example the characteristic smell or colour of a gas in the vicinity or even a labelled cylinder. Biochemical tests on blood will almost always take too long to influence management. The index of suspicion that a chemical terrorist incident has occurred will be higher with incidents where the chemical used has immediate effects and many people are involved.

3.4 Specific Chemicals

3.4.1 Organophosphate Anticholinesterases

3.4.1.1 Basis for Selection

The OP anticholinesterase (antiChE) chemical-warfare agents are known to be of high acute toxicity from studies in animals, and, to a limited extent, their use in chemical warfare and terrorism; these compounds are divided into the volatile G agents and the nonvolatile V agents (see Marrs, 2007; **Toxicology of Chemical Warfare Agents**). The related OP ester insecticides are of lower acute toxicity and in some cases of very low acute toxicity. Thus malathion has an oral rodent LD_{50} (median lethal dose) in the range of $1-5\,g\,kg^{-1}$, depending on the purity of the preparation (FAO/WHO, 1998); nevertheless some OP insecticides are of sufficiently high acute toxicity to merit consideration as possible compounds of interest to terrorists, for example mevinphos (FAO/WHO, 1997) and phorate (FAO/WHO, 1995); however these would be difficult to obtain in many countries. Details of chemistry, nomenclature and toxicology of OP pesticides are available in Ballantyne and Marrs (1992), Gupta (2006) and Marrs (2001) (see also **Toxicology of Chemical Warfare Agents**; **Toxicology of Pesticides**). OPs may be available commercially, notably as pesticides, but in developed countries, OPs of high acute toxicity are not much used. With appropriate facilities, OPs can be synthesized, although the handling of fluorine, needed to synthesize G agents other than tabun, would present challenges.

3.4.1.2 Routes of Exposure

Of the classical nerve agents, the G agents are generally considered as inhalation and skin hazards, whereas the nonvolatile V agents, for example VX, would be skin hazards, unless aerosolized (Marrs, 2007). As with V agents, OP insecticides are generally not very volatile and would not generally present an inhalation hazard unless aerosolized. OPs are generally of high vapour density and thus do not readily diffuse. In view of this and coupled, in the case of the G agents, with their volatility and the rapid onset of clinical signs and symptoms, the OPs are capable of causing large-scale mass casualties that would have significant impacts on emergency medical services (Okumura *et al*., 1998a; 1998b; Tokuda *et al*., 2006). It is generally considered that the volatile OPs would be more effective in a confined space; however, using a programme designed to model releases of environmental pollutants, Salzman *et al*. (2005) determined that the release of sarin in an open-air environment has the potential to affect more people than a similar release

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in a relatively closed space. However, it is important not to neglect routes of exposure other than inhalation and the percutaneous route. Depending on the specific OP and its physicochemical properties, OPs could be incorporated into food, drink or medicinal products for exposure by the peroral route. The toxic effectiveness with respect to contamination of foodstuffs and drink has been demonstrated by two large outbreaks of delayed-onset peripheral neuropathy from tri-*ortho*-cresyl phosphate (TOCP), namely ginger-jake paralysis in the US during 1930–1931 and in Morocco in 1959, both of which produced delayed polyneuropathy (see below).

Were a toxic OP ester to be deployed in a public space, for example a railway station, it is important to consider contamination of food and drink outlets, and, of course, a terrorist might deliberately and specifically adulterate food and water supplies with OPs.

3.4.1.3 Clinical Effects

The acute manifestations of OP intoxication are usually rapid in onset. The most widely published accounts of terrorism with OPs have been the use of sarin (*O*-isopropyl methylphosphonofluoridate; GB) in Japan by a religious cult against randomly selected human targets (Aum Shinrykio, Supreme Truth). The first incident occurred in 1994 at Matsumoto City (Nagano Prefecture, Honshu) when impure sarin was released from a truck for about 10 minutes using a heater and a fan (Morita *et al*., 1995; Okudera *et al*., 1997). About 600 people were exposed, of whom seven died and 50 were hospitalized. Secondary contamination occurred in about a third of rescuers who started relief activities within five hours of sarin release (Nakajima *et al*., 1997). A two-year follow-up revealed that of the patients who has been severely ill, some still had symptoms and clinical signs, including palpitations, dyspnoea, low-grade fever and even seizures; one victim developed sensory neuropathy seven months postexposure (Nakajima *et al*., 1999; Sekijima *et al*., 1997). The second incident involved the release of the vapour into commuter trains in the Tokyo subway (underground railway) during a Monday morning rush hour in March 1995 (Okumura *et al*., 1996; Ohbu *et al*., 1997; Okudera *et al*., 1997; Simon, 1999; Tokuda *et al*., 2006; Woodall, 1997). Five cult members released sarin vapour from plastic bags containing about 600 ml 35% diluted sarin by puncturing with the tips of umbrellas; 10 bags were distributed among five different commuter trains (Tokuda *et al*., 2006). Twelve individuals were killed, and over 5000 became symptomatic; had the sarin used been of greater purity and the delivery system more effective, casualties could have been in the thousands. Secondary contamination occurred in some physicians and nurses. See also Section 3.4.1.5 below.

3.4.1.4 Mechanisms of Toxicity

Most of the early signs and symptoms of OP antiChE intoxication are the result of inhibition of acetylcholinesterase (AChE; acetylcholine acetylhydrolase; EC 3.1.1.7) at cholinergic synapses and cholinergic effector organs, and the neuromuscular junction, as well as in the central nervous system (CNS), resulting in an accumulation of the neurotransmitter acetylcholine (ACh) and a cholinergic crisis. Cholinergic neurones release ACh which stimulates ACh receptors on the cell surface mediating the response. Cholinergic nerve fibres include preganglionic sympathetic and parasympathetic fibres, postganglionic parasympathetic fibres, somatic motor fibres and CNS ones. ACh (cholinergic) receptors are classified as nicotinic (N-cholinoceptors) and muscarinic (M-cholinoceptors) based on their response to the corresponding alkaloids. N-cholinoceptors mediate effects of ACh at the cholinergic skeletal muscle neuromuscular junction, autonomic ganglia and within the CNS. M-cholinoceptors are found at smooth muscle myoneural junctions and in the exocrine and endocrine systems. Typical acute cholinoceptor effects are as follows:

- **Muscarinic effects**: Predominant features are hyperhydrosis, hypersalivation, excess lacrimation, miosis, intestinal cramps, vomiting, diarrhoea, urinary and faecal incontinence, bronchorrhoea and bronchoconstriction
- **Nicotinic effects**: These include muscle cramps, fasciculation, weakness, paralysis and pallor.

Under normal physiological conditions, ACh at the postjunctional cholinoceptor is rapidly hydrolysed by AChE, which terminates its activity at the postjunctional membrane. The ACh molecule binds to an anionic site on the AChE molecule, and then combines with an esteratic site of the enzyme; this is followed by hydrolysis and rapid reactivation of AChE. AChE has the capacity to hydrolyse 3×10^5 ACh molecules per molecule of AChE, equivalent to a turnover of 150 µs. Inhibition of ChEs by OPs is a multistage process, with formation of a Michaelis enzyme–substrate complex and phosphorylation of a serine hydroxyl group at the esteratic site of the enzyme (see reviews by Marrs, 2001; Thompson and Richardson, 2004; Sultatos, 2006). Spontaneous reactivation of the inhibited ChE may occur, whose rate depends on factors such as species, tissue, groups (most often alkoxy ones), other than the leaving group attached to the phosphorus atom and the presence of nucleophiles that catalyse the reactivation process (see WHO, 1986; Wilson *et al*., 1992; Mason *et al*., 1993). The rate of reactivation is a major determinant of the rate of recovery from the intoxication. In some cases, a process of 'ageing' occurs, in which there is cleavage of an alkoxy–phosphorus bond with the loss

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of the alkyl group and formation of a charged monosubstituted phosphoric acid residue still attached to the enzyme protein; this structure is very stable and the aged enzyme is no longer responsive to nucleophilic reactivating agents (Wilson *et al*., 1992). Ageing is time dependant and most notably a problem with soman-inhibited AChE, where the aging $t_{1/2}$ in human erythrocytes is known to be rapid (1.3 minutes) (Harris *et al*., 1978). If the alkyl groups in the phosphorylated enzyme are methyl groups, spontaneous reactivation takes hours, if they are ethyl groups somewhat longer. Secondary or tertiary alkyl groups further enhance the stability of the phosphorylated enzyme, and the return of ChE activity may depend on new enzyme synthesis (Taylor, 1985). Tabun, an *O*-ethyl-*N*,*N*-dimethylamidophosphoro derivative, produces an inhibited enzyme that is slow to reactivate (Heilbronn, 1963), but this is not due to ageing: tabun binding of mouse AChE causes conformational changes in the enzyme that appears to stabilize the enzyme inhibitor complex (Ekstrom *et al*., 2006).

Many OP insecticides require metabolic activation mainly by mixed-function oxidases, hydrolases and transferases, For example, phosphorothioates are generally toxicologically inert until activated by conversion of the P=S to an oxon (P=O), resulting in a latent period between exposure and the appearance of cholinergic effects (see **Biotransformation of Xenobiotics**). Some lipophilic OPs are not rapidly excreted, resulting in prolonged activity or the recurrence of clinical features after treatment and apparent initial recovery.

3.4.1.5 Clinical Manifestations

3.4.1.5.1 Acute Effects

The acute central and peripheral clinical manifestations of OP antiChE intoxication are listed in **Table 1**; individual signs and symptoms appear within a few minutes to a few hours. The time to onset of symptoms and signs, and the order of their appearance varies between different OPs. Patients with severe OP poisoning typically present with pinpoint pupils, excessive sweating, reduced consciousness and difficulty with respiration. Effects on the cardiovascular (CV) system are determined by the relative predominance of M-cholinoceptor effects (bradycardia, hypotension) vs. N-cholinoceptor effects (tachycardia, hypertension). Typically there is transient sinus tachycardia followed by sinus bradycardia, A-V block, S-T and T-wave abnormalities and ventricular arrhythmias (Jorens *et al*., 2008; Karki *et al*., 2004; Kumar and Lloyd-Jones, 2007; Ludomirsky *et al*., 1982). Respiratory failure, hypoxaemia and cyanosis result from bronchospasm, bronchorrhoea, respiratory muscle paralysis and central depression of breathing; paralysis of the diaphragm has been described (Rivett and Potgieter, 1987). Respiratory effects, together with ventricular arrhythmias are the major cause of death.

In theory, the acute effects of cholinesterase inhibition are reversible, but long-term and/or irreversible neurological effects from OP antiChEs can occur. Such effects include the intermediate syndrome, delayed polyneuropathy and, probably, long-lasting central nervous system effects.

Table 1 Acute central and peripheral symptoms and signs of organophosphate anticholinesterase intoxication

Muscarinic effects[a]	
Ocular	Miosis Blurred/dim vision/ decreased visual acuity Lacrimation
Respiratory tract	Rhinorrhoea Bronchial hypersecretion Bronchoconstriction Dyspnoea
Gastrointestinal tract	Sialorrhoea Nausea Vomiting Diarrhoea and bowel incontinence Abdominal cramps
Urinary	Urinary incontinence
Cutaneous	Hyperhydrosis
Nicotinic effects[b]	
Skeletal	Twitching, fasiculations and cramping Skeletal muscle weakness and flaccid paralysis
Cardiovascular	Tachycardia Hypertension Pallor
Central nervous system effects	
Headache Irritability Ataxia Convulsions Coma Respiratory failure	

[a]Effects due to ACh accumulation at peripheral neuroeffector junctions.
[b]Effects due to ACh accumulation at skeletal myoneural junctions and autonomic ganglia.

3.4.1.5.2 Intermediate Syndrome

The intermediate syndrome which is seen after OP poisoning gains its name from the fact that signs and symptoms appear after the acute cholinergic phase, but before the anticipated time for appearance of delayed onset peripheral neuropathy. Onset is usually 24–96 hours postexposure. Clinically, the intermediate syndrome consists principally of weakness or paralysis of proximal limb muscles, neck flexors, respiratory muscles and motor cranial nerve involvement, particularly those

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innervating the extraocular muscles (Senanayake and Karalliedde, 1992). Unlike delayed polyneuropathy the intermediate syndrome is potentially lethal because of respiratory depression. If respiratory insufficiency is correctly managed, recovery occurs within 4–18 days. Using repetitive nerve stimulation techniques it was demonstrated that there was an apparent correlation between the decrement–increment pattern and the development of intermediate syndrome, and that such neurophysiological approaches may be of value in predicting the development of intermediate syndrome (Jayawardane *et al*., 2006). Karalliedde *et al*. (2006) concluded that the cause of intermediate syndrome was down-regulation of nicotinic receptors. Although intermediate syndrome has not been described with nerve agents, it has been reported with many OP insecticides, and it would be as well to be prepared for its occurrence with OP chemical-warfare agents.

3.4.1.5.3 Organophosphate-Induced Delayed Polyneuropathy

Organophosphate-induced delayed polyneuropathy (OPIDP) appears to result from covalent binding to and inhibition of a membrane-bound esterase (neuropathy target esterase (NTE)). From studies with NTE-null mice and *Drosophila*, Glynn (2006) concluded that OPs producing OPIDP caused a transient loss of NTE activity, possibly disrupting membrane phospholipid homeostasis and endoplasmic reticulum functions, such as glial–axonal interaction and axonal transport. Histopathologically, the lesion consists of axonal degeneration and demyelination of the larger diameter and longer fibres. Delay to onset of clinical signs ranges from one to several weeks. It is characterized by a distal symmetrical and sensorimotor polyneuropathy, with tingling and burning sensations, progressing to atrophy and flaccid paralysis of the lower limbs (DeJager *et al*., 1981; Lotti *et al*., 1984). The upper limbs may be affected subsequently (Cherniack, 1986). Measurement of lymphocyte NTE may predict the development of OP-induced polyneuropathy (Lotti *et al*., 1986). Most OP pesticides on the market do not give rise to OPIDP, nor is it likely to occur with chemical warfare OPs (see Marrs, 2007).

3.4.1.5.4 Long-Term Central Effects

There is evidence from laboratory studies that exposure to OP antiChEs may result in altered patterns of neurobehaviour (Chambers and Chambers, 1989; Gardner *et al*., 1984; Geller *et al*., 1987; Haggerty *et al*., 1986; Russell *et al*., 1986; Scott, 2007a; Joosen *et al*., 2009). Longer-term psychological problems that have been described following acute and chronic OP poisoning in humans include depression, schizoid reactions, impaired vigilance, memory deficit and exacerbation of pre-existing psychiatric problems (Dassanayake *et al*., 2008; Lotti, 1992; Namba *et al*., 1971; Savage *et al*., 1988). There is some evidence for sequelae, including neuropsychiatric effects, in survivors of the Japanese terrorist incidents using sarin (Yanagisawa *et al*., 2006; Okumura *et al*., 2007). The long-term and delayed effects of acute OP exposure and the effects of low-dose OP exposure have been reviewed (Institute of Environment and Health (IEH), 1998; Committee on Toxicity of Products in Food, Consumer Products and the Environment, 1999; Lader, 2001; Romano *et al*., 2001). In relation to pesticides, attention has been paid to the fact that the foetus and young children may be more sensitive to OPs than adults (Slotkin, 2005). Four pregnant women were exposed to sarin in the Tokyo exposure at 9–36 weeks' gestation (Ohbu *et al*., 1997) and all had normal offspring. Nevertheless, because of the profound effects of OPs, including chemical-warfare nerve agents, on neurotransmission, there is a strong theoretical basis for concern that effects which in adults would be reversible, would in the embryo, foetus and neonate be irreversible.

3.4.1.6 Essentials of the Management of Acute Poisoning

Detailed reviews of the management of OP antiChE intoxication are available in Marrs and Vale (2006) and Vale *et al*. (2007). The following account gives an overview of essentials, which include decontamination and protection, respiratory support and antidotes. Although severity scoring systems are described for acute OP antiChE poisoning (Johnson and Vale, 1992), in the context of chemical-terrorism incidents, because of the complexity of the situation with possible physical injuries, it is probably most appropriate to apply a general triage system.

3.4.1.6.1 Decontamination

Those responsible for management should wear appropriate protective clothing and equipment to prevent secondary contamination. If there is sufficient warning, then it may be appropriate for first responders and healthcare staff to have intramuscular prophylactic oxime (see Section 3.4.1.6.4). Decontamination of the patient should occur at the earliest stage possible and include removal of clothing and showering (Roberts and Maynard, 2007).

3.4.1.6.2 Monitoring

Activity of two enzymes are commonly measured for the diagnosis and monitoring of OP poisoning, erythrocytic AChE and butyrylcholinesterase (BChE), an enzyme that is present in the plasma and, in humans, not in the red blood cell. Erythrocyte AChE activity measurement provides a better functional index of toxicity from OPs then BChE, because of the former's identity with the enzyme at cholinergic structures, where enzyme inhibition forms the mechanistic basis for antiChE poisoning. Many OPs are more potent inhibitors of plasma BChE activity than they are of AChE activity, and thus BChE may be a sensitive index of recent exposure (Eddleston

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et al., 2005). However, BChE inhibition is not related to the clinical severity of exposure. Alkyl phosphates may be useful as markers of OP exposure, but are of little use in monitoring clinical status (Black and Noort, 2007). In addition to routine periodic clinical examination, there should be continual monitoring of heart rate, blood pressure, ECG (electrocardiography) and periodic measurement of arterial blood gases, and acid-base status.

3.4.1.6.3 Respiratory Support

Because of the potential for respiratory failure, bronchospasm and hypoxaemia, there should be provision for maintenance of the airway and the use of oxygen to combat tissue hypoxia. With severe and progressive cases of respiratory failure, intubation and mechanical ventilation may be needed, using positive end-expiratory pressure (PEEP).

3.4.1.6.4 Antidotes

Descriptions of the antidotal treatment of antiChE OP poisoning have been presented in detail elsewhere (Eddleston *et al*., 2008), and only the essentials are discussed below, with particular reference to the use of atropine, oximes and anticonvulsants.

Atropine is the mainstay of treatment as a nondepolarizing competitive blocker of the action of ACh at peripheral M-cholinoceptors (Heath and Meredith, 1992). Rapid full atropinization is required, as indicated by mydriasis, tachycardia, flushing and dry mouth. Atropine is beneficial in reducing bronchorrhoea and reversing changes in ventilation-perfusion resulting from the uneven distribution of ventilation caused by airway constriction, and atropine counteracts the convulsive effects of OPs (see McDonough and Shih, 2007).

Oximes are useful for the reactivation of inhibited AChE. The salts of pralidoxime (chloride, mesilate or methylsulfate) or obidoxime are the most commonly used. A limitation with oximes is ageing of the enzyme (see Section 3.4.1.4), and they generally become less effective with increasing time after exposure. The role of oximes is not completely clear and there are disputes about oximes in OP poisoning; for example, although oximes show value in the treatment of OP antiChE intoxication, some physicians have suggested that they are of little value in treating human OP poisoning cases. Also, there are disagreements concerning the dose and frequency of oxime administration. Oximes have been investigated extensively as agents for prophylaxis in CW situations or occupations where there is a high risk of occupational exposure to OP antiChEs, which can now include responders to antiterrorist threats where OP use is a high possibility. Clearly drugs used in prophylaxis must have a high safety ratio, and be devoid of complications that may be acceptable in antidotal usage (see reviews by Szinicz *et al*., 1996; Marrs *et al*., 2006; Eyer and Worek, 2007).

Combining atropine and oxime seems to produce antidotal synergy, and in most cases of OP antiChE intoxication, the combination is more effective than either alone. This is, in part, because separate components of the cholinergic intoxication are treated; atropine antagonizing the peripheral M-cholinoceptor effects and some central effects, and oximes producing reactivation of N-cholinoceptor AChE. Combined atropine–oxime formulations are available commercially in autoinjectors for intramuscular use.

Anticonvulsants, such as benzodiazepines, both relieve anxiety and counteract some CNS effects not alleviated by atropine (Vale and Scott, 1974). Diazepam may be of value in antiChE poisoning by reducing anxiety, reducing muscle fasciculations, arresting seizures and reducing morbidity and mortality when use in conjunction with atropine and 2-PAM (pralidoxime chloride) or P2S (pralidoxime mesilate) (Johnson and Vale, 1992 see reviews by Marrs, 2003; 2004a; Marrs and Sellström, 2007).

Pyridostigmine bromide has for many years been available to the armed forces as a pretreatment primarily to prevent soman-inhibited AChE from ageing (see reviews by Inns and Marrs, 1992; Scott, 2007b). Pyridostigmine bromide could considerably be used for high-risk civilians, for example first responders.

3.4.1.6.5 Follow-Up Care

It was the experience of the Japanese terrorist that drew attention to the high incidence of late or sustained complications that may occur with OP antiChE intoxication. For example, with the Tokyo subway incident a one year follow-up revealed that of 303 surveyed respondents, 46% still experienced physical or psychological symptoms, which included ocular effects, fatigue, headache, depression, lack of concentration, flashbacks and phobias of subways (Ohyoku *et al*., 2003; Yokoyama *et al*., 1998). There is therefore a need for provision of physical and psychological follow-up facilities for those involved in such terror incidents.

3.4.2 Cyanides

3.4.2.1 Basis for Selection and Generation

Cyanides are well known for their acute lethality, and as such make an obvious universal choice as an agent for a terrorist action intended to have acute lethal effects. The following is a brief consideration of the properties of cyanides relevant to potential use in terrorist activities. Details of the general and clinical toxicology of cyanides have been presented elsewhere (Ballantyne, 1987; Ballantyne and Salem, 2006; 2007a; Ballantyne *et al*., 2007).

Cyanides are readily available as the low boiling point and volatile hydrogen cyanide (HCN) and as soluble solid salts, generally of sodium and potassium (NaCN and KCN). They are readily available from laboratories, fumigation services and industrial organizations. Also, cyanides and cyanogens can be extracted from pips

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Table 2 Examples of information obtained about the use or potential use of cyanides (CN) in major and small group terrorist actions

Date: location: information	Reference
Major terrorist situations	
1995: Tokyo subway attack: precursors of CN found in subway bathrooms	Sauter and Kein (2001)
2002: Arrests of four Moroccans, with ties to Al Qaeda, who were plotting the use of CN to poison water supplies in areas around the American Embassy in Rome	BBC (2002b)
2002: September 18: Police raid on North London (UK) apartment: discovery of instructions for manufacturing various poisons, including cyanide from fruit pips	O'Neill (2005)
2002: December: Recovery of a store of CN in Paris: linked to three Al Qaeda operatives	Cloud (2004)
2003: Police raid on a North London (UK) apartment: crude laboratory discovered for producing CN	O'Neill (2005)
2003, May: CN 'bomb' found in the possession of white supremacists in Texas	CNN (2004)
2003: The US Central Intelligence Agency (CIA) uncovered a plot, apparently by an Al Qaeda cell, in which HCN vapour would be released into the New York Subway system, using a small device which would generate HCN vapour from NaCN and hydrochloric acid	Suskind (2006)
Small group situations	
Early 1990s and mid 1980s: Repacking of medical capsules with CN salt, either for personal individual target or for random killings by leaving bottle on pharmacy shelves	Brahams (1991) CDC (1991) Dunea (1983)

and cherry stones. Cyanides have the advantage, for the aspiring terrorist, that their toxic effects are rapid in onset. This is because the cyanides are rapidly distributed in the body and because their toxic action on cytochrome oxidase activity swiftly interrupts intracellular respiration. HCN has the advantage of having a low boiling point (26.5°C) and high volatility (vapour pressure of 600 Torr at 20°C) and thus rapidly generates a colourless vapour of high concentration (saturated vapour concentration 884 211 mg m^{-3} at 20°C). However, because of the low vapour density (0.947 at 31°5) and molecular weight of HCN (27.04), the vapour is readily diffusible, and thus its use by a terrorist would be limited to enclosed spaces, such as buildings, in-door entertainment centres, subways and small shopping centres, to be effective.

In many counties cyanides are chemicals listed as among the more likely agents to be used for chemical terror in national and international situations (Khan *et al*., 2000; NTARC, 2004; 2009). Some reported uses or potential uses of cyanides for small-scale and major terrorist uses are listed in **Table 2**. The methods used by terrorist groups and organizations for the delivery of cyanides to intended targets are likely to be variable, depending on resources available, knowledge and scientific/technical sophistication, proposed location and intent for the incident. Vapour generation could be from cylinders, or crude devices for the mixing of cyanide salts with acidic fluids. Larger-scale use could be by the adulteration of food and/or drink, as was effectively done in the Jonestown religious cult killings (Thompson *et al*., 1987). Cyanide salts could be used to contaminate food or drink. For smaller-scale incidents this could be done by incorporating cyanide into food and drinks in shops or restaurants, or into drinking-water supplies close to their entry into buildings. Larger-scale operation would involve contamination of major food and drink production, storage and transportation sites (Ballantyne *et al*., 2007). Attempted distribution by contamination of public reservoirs would not be feasible because of the massive amounts of solid cyanides that would be required to be dumped to achieve toxic concentrations in the effluent water. For use against smaller-scale or targeted groups, or against individuals, the following have been suggested/encountered; repacking of medicinal capsules (see **Table 2**), intramuscular injection from concealed syringes (Ballantyne *et al*., 1972); incorporation into cigarettes; spraying HCN in the face from a pressurized canister (Gee, 1987; Harris and Paxman, 1982; Seagrave,

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1982). Some of these incidents were really political assassinations or murder rather than terrorist incidents as previously defined.

3.4.2.2 Clinical Manifestations

Cyanides cause mental and physical incapacitation and lethality. Notable symptoms and clinical signs include dyspnoea, hyperpnoea, a cherry-red appearance (not cyanosis), anxiety, apprehension, agitation, convulsions, cardiac arrhythmias and eventually coma. A smell of bitter almonds may be detected (Hall *et al*., 1987). The occurrence of disturbances of consciousness and perception, together with muscle weakness and ataxia, will markedly impair the ability of victims to escape from an area and to undertake coordinated tasks. For humans, the concentration range required to cause lethality within 1–5 minutes is of the order of 500–4000 mg m^{-3}, and concentrations of the order of 112–224 mg m^{-3} are necessary to induce incapacitating effects (Ballantyne *et al*., 2007). A test paper (Cyantesmo®) is available for the detection of cyanide in aqueous solutions and extracts (Macherey-Nagel, 2008).

3.4.2.3 Essentials of Management

Detailed reviews of the management of cyanide poisoning are available (see Meredith *et al*., 1993; Marrs, 1998; Ballantyne *et al*., 2007). There are a number of antidotes available, including dicobalt edetate, hydroxocobalamin and the classical treatment of sodium nitrite and thiosulfate.

3.4.3 Irritant Chemicals

3.4.3.1 Basis for Selection

Under this heading fall numerous chemicals, many industrial, that can cause peripheral chemosensory irritant (PCSI) effects (Ballantyne, 2006a; see **Peripheral Chemosensory Irritation: Fundamentals, Investigation and Applied Considerations**) and, at higher concentrations, inflammatory lesions and possibly lethal respiratory tract damage or lethal systemic toxicity; some of these compounds are potentially very dangerous, for example phosgene. The PCSI effects will cause harassment and distress and could be used as warning of displeasure, or as a distraction to permit other activities. Examples of irritant chemicals include phosphine, chlorine, chloropicrin, acrolein and phosgene, which can be obtained or stolen from industrial sources. Also, terrorists may be able to obtain grenade-generated screening smokes for purposes of distraction or harassment; these include the irritant and potentially ocular- and pulmonary-injuring white and red phosphorus, titanium tetrachloride and zinc-hexachloroethane smoke compositions. Details of the toxicology, clinical effects and medical management of overexposure to such smokes have been discussed by Ballantyne and Salem (2007b). If the intent is mainly to harass without injury, then riot-control agents might be chosen for use; devices containing these agents can be purchased commercially and openly in many countries, such as the USA and Germany, their ready availability being indicated by the fact that such devices have been in the possession of some schoolchildren (Wattigney *et al*., 2008). Details of the nature, effects, toxicology and medical management of exposure to chemicals used as riot-control agents have been published by Ballantyne (2006a; 2007) and Ballantyne and Salem (2004). For maximum effects, irritant chemicals will be used by atmospheric dispersion, either as vapour or aerosol. As noted in Section 3.2.2 above, release of irritant materials into the atmospheres of enclosed spaces can result in panic, with secondary physical injuries. In addition to causing PCSI and inflammatory effects, some irritants may be absorbed and cause systemic toxicity; for example chloropicrin and phosphine may produce CV and hepatorenal effects.

3.4.3.2 Clinical Manifestations

The PCSIs effects can cause distress and distraction because of the local discomfort and associated reflexes that develops from contact with skin and exposed mucosal surfaces (Ballantyne, 2006a). For example, in the eye they produce transient discomfort or pain, excess lacrimation and blepharospasm, the last two effects producing a temporary disturbance of vision; respiratory-tract PCSI effects include discomfort in the respiratory tract, chest tightness, rhinorrhoea, cough, increased tracheobronchial secretions, bronchospasm and changes in breathing rate (see Section 3.1). At higher atmospheric concentrations than those causing PCSI effects, there is often injury to the respiratory tract; for example, both acrolein and phosgene can produce inflammatory lesions in the nasal mucosa with bronchoconstriction, tracheobronchial epithelial desquamation and inflammation, pulmonary oedema and alveolar haemorrhages (Ballantyne *et al*., 1989; Steffens, 2003). Several irritants have been used for chemical-warfare purposes with horrific consequences; for example chlorine and phosgene. For example, phosgene (carbonyl chloride) was responsible for more than 1100 casualties when 88 tonnes of the gas was used against British troops at Bieltje, Belgium on 19 December 1915 (Spiers, 1986). The use of phosgene as a chemical-warfare agent continued during World War I, being dispersed in shells, mortar bombs and projector drums, and it has been estimated that phosgene was responsible for 85% of all respiratory-related deaths attributable to chemical weapons (Karalliedde *et al*., 2000).

The severity of pulmonary damage, such as bronchiolar obstruction and/or alveolar injury, may be of a magnitude that causes hypoxic hypoxia, with resultant tissue hypoxic injury, for example renal tubular necrosis (Ballantyne and Callaway, 1972). Also, as noted above, in addition to producing PCSI and local inflammatory

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effects, some inhaled irritants may also cause systemic toxicity following absorption, and the treating physicians should be aware of this for both diagnostic and management considerations. For example, phosphine (hydrogen phosphide; PH_3), a colourless, heavier-than-air gas, is flammable and explosive, and may cause asphyxiation in enclosed, poorly ventilated spaces. It can be generated by adding water to aluminium phosphide, a rodenticide, insecticide and fumigant. NIOSH (1996) recommended that 50 ppm be considered immediately dangerous to life or health (IDLH) and the ACGIH recommendation is 0.3 ppm, as a threshold-limit-value–time-weighted average (ACGIH, 2008). It can be fatal after a 30 minute exposure to >300 ppm, and serious adverse health effects may develop after exposure to 7 ppm for several hours (Musshoff *et al*., 2008). It is a respiratory-tract irritant causing pulmonary congestion, oedema and alveolar thickening. Additionally it can cause CV, neurological and hepatorenal toxicity. CV effects include tachycardia, arrhythmia, reduced cardiac output and decreased systemic vascular resistance, with hypotension. CNS neurotoxic effects include headache, dizziness, disturbed gait, intention tremor and convulsions. Hepatotoxic effects generally develop within 48–72 hours, with the development of hepatomegaly, jaundice and elevated serum aminotransferases; additionally haematuria and proteinuria may appear. The toxicity of phosphine has been reviewed (Brautbar and Howard, 2002).

3.4.3.3 Management of Casualties

After any necessary decontamination, those having only PCSI effects usually recover following transfer to a clean-air environment and possibly flushing of the eyes with water. However, to avoid any later possible sequelae, they should be observed; those exposed to respiratory tract irritants should have an initial chest radiograph and, if considered necessary, arterial P_aO_2 testing. If inflammatory lesions have developed, they may require the use of antibiotics to prevent secondary infection and also corticosteroids, both systemic (intravenous; iv) and inhalation spray (Steffens, 2003). Symptomatic patients may require supplemental oxygen, and aerosolized bronchodilators may be considered if bronchospasm is present. Also, the development of metabolic acidosis may require the iv infusion of bicarbonate. For severe cases, mechanical ventilation may be necessary. The possibility for secondary tissue and organ injury from hypoxic hypoxia due to pulmonary damage or systemic toxicity from absorbed materials needs to be borne in mind.

3.4.4 Systemically Toxic Materials

A very large number of chemicals exist that can be absorbed into the systemic circulation, tissues and organs, and may, in sufficient dosages, be severely injurious or lethally toxic. Some of the chemicals noted above (Section 3.4.3) as having irritant properties may also exert systemic toxicity in addition to local irritation. Additionally there are a multiplicity of systemically toxic materials that can be absorbed by various routes, but causing no or little local irritant warning effects, many of which are readily obtainable through commercial or industrial sources. OP antiChEs and cyanides have been considered above, and a few additional illustrative examples are noted below that have toxicity, making them likely as candidate agents for use in urban terrorist incidents.

3.4.4.1 Arsine

Arsine is a gas of vapour density 2.7 which is readily absorbed by inhalation exposure, and causes malaise, fever, nausea, vomiting, paresthesia, haemolysis, haematuria, rhabdomyolysis, acute renal failure and anuria. Treatment of severe poisoning may require DMSA (dimercaptosuccinic acid) chelation, erythrocyte exchange, plasmapheresis and haemodialysis (O'Connor *et al*., 2005; see review by Pakulska and Czerczak, 2006).

3.4.4.2 Paraquat

Paraquat is a bipyridylium herbicide (1,1-dimethyl-4,4-bipyridinium), whose toxicology has been discussed in detail elsewhere (Bismuth and Hall, 1995; FAO/WHO, 2004; Marrs, 2004b, see also **Toxicology of Pesticides**).There have been deaths due to paraquat poisoning, some accidental and others in suicidal attempts, most cases having resulted from drinking agricultural concentrates, rather than home-garden preparations (Carson and Carson, 1976; Fletcher, 1974). Agricultural paraquat formulations are mostly sold as solutions, and because of its toxicity and low volatility it is likely that any attempt to use paraquat in terrorist situations would likely involve contamination of food or drink. The estimated human acute peroral LD_{50} for paraquat is of the order of 30 mg kg^{-1} (Fletcher, 1974), and it causes local and systemic toxicity, the former being concentration dependent and the latter dose dependant (Proudfoot, 1999). After swallowing paraquat, local effects occur (see below), but except with very large doses, there is commonly a latent period of many hours or days before the clinical symptoms and signs of systemic pulmonary toxicity occur; the effect on the lung is usually the most serious of the toxic injuries. This latency may present a diagnostic and medical management problem. Toxicity also affects the liver, kidneys and heart. Thus, swallowing paraquat causes widespread organ injury, but with the most severe and lethal action on the lung. Pulmonary toxicity is a consequence of the active uptake of the material by the lung by a saturable process in Type II alveolar cells (Chen *et al*., 1992; Smith, 1982; Smith *et al*., 1990). After swallowing paraquat, there is irritation of the mouth, throat, oesophagus and gastrointestinal tract, resulting in dysphagia, epigastric pain, nausea,

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vomiting and diarrhoea. The initial signs of systemic toxicity are usually of renal tubular injury starting at one to three days, with increased blood urea, reduced creatinine clearance and diminished urine output (Gardiner, 1972). Renal dysfunction may be a combined result of nephrotoxicity coupled with hypovolaemia. Hepatotoxicity is common, with centrilobular necrosis (Bullivant, 1966). Respiratory effects include pulmonary oedema and haemorrhage followed by marked fibrosis, leading to severe respiratory distress. Although it may not be clinically apparent until later, pulmonary injury may start early in paraquat poisoning, particularly if large amounts of paraquat are swallowed (>6 g). For example, Yoneyama *et al*. (1969) described death with pulmonary haemorrhage one day after swallowing a large dose of paraquat, and von der Hardt and Cardesa (1971) found pulmonary haemorrhage and loss of alveolar and bronchiolar epithelium two days after swallowing paraquat. Death from pulmonary injury secondary to swallowing of smaller doses (>5 g) is frequently delayed for several weeks (Fletcher, 1974). Autopsy findings in the lung following lethal paraquat poisoning include pleural effusion, pulmonary and subpleural haemorrhages and pulmonary oedema. Histopathologically there is a destructive alveolitis, with oedema and initial involvement of Type I cells and subsequently of Type II alveolar and Clara cells. This is followed by fibroplastic proliferation in the alveolar walls with infiltration by mononuclear cells, polymorphonuclear cells, macrophages and eosinophils (FAO/WHO, 2004; Marrs, 2004b; Marrs and Proudfoot, 2003). The longer the survival time, the greater is the degree of proliferation of alveolar epithelium and fibroblasts (Carson and Carson, 1976). Plasma paraquat concentrations measured within 24 hours of exposure are predictive of outcome in around 90% of cases (Proudfoot, 1995). In spite of the large number of substances that have been tried experimentally and clinically for the treatment of paraquat poisoning (Fletcher, 1974), currently there is no specific antidotes, and management is directed mainly at restriction of absorption and increased elimination, and symptomatic treatment (Bismuth and Hall, 1995; Marrs, 2004b). It has been noted that when pulmonary effects occur, the use of oxygen should be postponed for as long as possible, since it may exacerbate pulmonary fibrosis (Bismuth *et al*., 1982).

3.4.4.3 Thallium

Thallium is a soft, grey malleable metal, used and available industrially, which produces systemic multiple organ toxicity. Thallium has no smell, It has been used for intentional criminal poisonings (Rusyniak *et al*., 2002; Saddique and Peterson, 1983) and maliciously contaminating food (Meggs *et al*., 1994). There is a report of food being intentionally contaminated with thallium in Iraq and causing acute poisoning with delayed mortalities in two families in Baghdad during January 2008 (CDC, 2008a). By acute peroral poisoning, thallium causes abdominal pain, nausea and vomiting within a few hours of ingestion, that could be misdiagnosed as being due to other causes. However, within a few days, and even with acute poisoning, there is hair loss and a painful ascending peripheral neuropathy of the lower limbs, with paresthesia and muscle weakness. Hypertension and tachycardia may develop. Diagnosis can be confirmed by measurement of thallium in blood and urine. Treatment includes the use of ferric hexacyanoferrate (FHF: Prussian blue) as a sequestering agent (Rusyniak *et al*., 2003; Yang *et al*., 2008). The physical properties of the material and its acute and delayed onset effects make this a candidate agent for listing as a potential agent for incorporation in foods for a localized or generalized terrorist threat. In such circumstances, the sudden appearance of gastrointestinal symptoms followed in a few days by signs of alopecia and peripheral neuropathy should prompt clinical consideration of thallium poisoning. Thought should be given to stockpiling of FHF in a few selected centres for distribution when and where necessary.

3.4.4.4 Fentanyls

Fentanyls are synthetic opiates having short-acting and highly potent narcotic, analgesic, anaesthetic and immobilizing properties (Salem *et al*., 2005). Fentanyl (*N*-phenyl-*N*-[1-(2-phenylethyl)-4-piperidinyl]propanamide) is a potent narcotic analgesic also used for the induction and maintenance of general anaesthesia. Several analogues of fentanyl have been synthesized for use in anaesthesia. All are synthetic opiates that produce short-acting and potent analgesic, anaesthetic and immobilizing effects (Hess and Knakel, 1985; Janssen, 1984; Salem *et al*., 2006). Because of their high lipid solubility, the fentanyls penetrate the blood–brain barrier rapidly, which accounts for their quick onset of action. They all have the pharmacological characteristics of opium, including analgesia, euphoria, miosis and respiratory depression. The respiratory depression has been shown to be the result of direct rhythm-generating respiratory neurones in the pre-Boetzinger complex of the brain stem (Manzke *et al*., 2003). 5-Hydroxytryptamine (5-HT) 4(a) receptors are strongly expressed in these neurones, and their selective activation protects spontaneous respiratory activity. Rats dosed with a 5-HT 4 receptor-specific agonist overcame fentanyl-induced respiratory depression and re-established stable breathing rhythm without loss of analgesic activity. Several studies have been conducted on dissociating respiratory depressant effects from opiate-induced sedative activity of fentanyls (Brown and Pleuvry, 1992; Mioduszewski, 1994; Mioduszewski and Reutter, 1991). Adverse effects of opiates include bradypnoea, respiratory depression and hypoxic hypoxia. Narcotic antagonists are effective to various extents when given simultaneously with fentanyl/analogues (receptor

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competitive exclusion) or when given postfentanyl for reversal of effects (receptor displacement) (Langguth *et al*., 1990). Some of these investigations were carried out to improve safety in patients receiving fentanyl, and other studies were probably conducted in government laboratories to produce a formulation that could be used in antiriot/antihostage situations.

One incident where fentanyl/fentanyl analogues were used on a large scale, producing mortalities was by Russian authorities in 2002. Many innocent citizens were killed in an antihostage situation in Moscow on 23 October, the incident involving a Chechnian terrorist group holding about 800 hostages in the Moscow Dubrovka Theatre Centre. Government authorities incapacitated the hostage-takers possibly by pumping fentanyl into the building (Couzin, 2003), although some believe that a mixture of fentanyl and halothane was used (Salem *et al*., 2006; Zilker *et al*., 2003). The result was at least 129 of the hostages died. It is possible that remifentanil might have been used because of its extreme potency coupled with relatively fast onset of action, short duration of action and its rapid metabolism (Salem *et al*., 2006). It has further been suggested that massive doses of the potent opiate carfentanil were used to saturate the theatre and obtain a maximum effect by inhalation (Salem *et al*., 2006), or a mixture of carfentanil and halothane (Wax *et al*., 2003) There is reason to believe that the authorities did not inform local hospitals and physicians of the agent used (Glasser and Baker, 2002). It has been argued that since fentanyl is not listed in any of the Schedules of the Chemical Warfare Convention and because of its rapid-onset–short-duration analgesic effects, it can be legally considered a riot-control agent and not covered by international conventions banning chemical-warfare agents (see Pearson, 2007). It is to be hoped that the tragic consequences of the Russian incident will be used as a lesson against such a conclusion or perhaps that the conventions need to be modified.

Several fentanyl analogues have been illegally synthesized for sale on the illicit drug market as Persian white, China white, Mexican brown and synthetic heroin (CDC, 2008b; Kram *et al*., 1981; Poklis, 1995; Salem *et al*., 2006). Nonpharmaceutical fentanyl is apparently easy to synthesize, recipes being available on the Internet (CDC, 2008b). The Drug Enforcement Agency (DEA) on 23 April 2007 began regulating access to *N*-phenylethyl-4-piperidone, a chemical used in the manufacture of illicit fentanyl. Abusers use these substances by iv injection, snorting or smoking, and these practices can lead to addiction or habituation. Additionally, fentanyl is available in transdermal patches, which have been increasingly abused by extracting fentanyl gel from them with a syringe (Arvanitis and Satonik, 2002; Reeves and Ginifer, 2002; Shoemaker *et al*., 2006). Many epidemics related to the illicit abuse of fentanyl have been reported, particularly in the USA (Hibbs *et al*., 1991; Martin *et al*., 1991; Schumann *et al*., 2008; Smialek *et al*., 1994). Fentanyl abuse has been common amongst healthcare workers (Silsby *et al*., 1984). The serious health consequences in outbreaks of fentanyl or analogue distribution by drug dealers is demonstrated by mortality statistics; for example, in a Chicago April 2005 to December 2006 epidemic, there were 342 fentanyl-related deaths (Schumann *et al*., 2008); in Detroit during 2006 illicit fentanyl was the cause of death in 178 cases (Boddiger, 2006; Goodman, 2007); terrorists could obtain fentanyls from illicit drug producers or by stealing from healthcare institutions, for use in operations where it is desired to induce a narcotic and immobilizing state. Because of the lack of a morphine-derivative metabolite, most conventional urine drug screens cannot detect fentanyl, and there is thus a need in such circumstances for a sensitive and specific method to be developed for diagnosis.

The management of opioid intoxication includes airway support and the use of narcotic antagonists such as naloxone; large doses of naloxone may be required for reversal (Marquardt and Tharratt, 1994; Poklis, 1995; Schumann *et al*., 2008).

3.4.4.5 *Osmium Tetroxide*

This is included to demonstrate that some chemicals selected as being potentially toxic by terrorists, may not be suitable because of technical problems with generation and dispersion. British antiterrorist police conducted raids at 24 locations throughout London on 30 March 2004 following the tracking of groups of terrorist suspects and after the British electronic eavesdropping agency, the Government Communications Headquarters, Cheltenham (usually known as GCHQ), intercepted some interesting telephone calls. Suspected British terrorists of Pakistani origin were arrested for allegedly discussing the use of osmium tetroxide in a chemical bomb in crowded civilian locations in London (Norton-Taylor and Cowan, 2004). Exposure to the vapour causes severe irritant effects on the eye, skin and respiratory tract, with delayed onset pulmonary oedema. Another latent effect from vapour is 'blacking' of the cornea. Although the plot did not go beyond the planning stage it is highly unlikely that the use of osmium tetroxide by dispersal from an explosive device would be successful because of its ready thermal decomposition (Baker and Kosai, 2004), although the possibility for dispersion as a vapour by other means requires that this material should be kept on the list of suspect potential terrorist threat chemicals that could be used in small-scale attacks (Makarovsky *et al*., 2007).

3.4.4.6 *Miscellaneous*

The above examples of systemically active chemicals indicate the wide range of chemical classes that are likely to be encountered in chemical terror incidents, the choice of which depends on a multiplicity of factors. The list could be extended significantly, depending on whether

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the intention of the terrorist/organization is to be deliberately focal or randomly to kill or to engender fear and in this way influence society. The antiterrorist authorities, who presumably are maintaining preparedness by keeping records of chemicals and drugs likely to be used by terrorists, need to consider the wide choice of alternatives available and how these can be obtained. Additional substances likely to be employed include hallucinogenic drugs (e.g. indoleamines, phenylalkylamines, anticholinergics and cannabis).

3.4.5 Biotoxins

The bacterial toxin *Clostridium botulinum* neurotoxin (Tendler and O'Neill, 2005; **Toxicology of Chemical Warfare Agents**) and the plant toxin, ricin (see **Ricin: Chemistry, Sources, Exposures, Toxicology and Medical Aspects**) are of extreme toxicity and have been acknowledged as possible agents for use in terrorist operations.

3.4.5.1 Ricin

Ricin, a highly potent glycoprotein toxin derived from the castor bean plant, *Ricinus communis (Euphorbiaceae)*, and is proscribed in the Biological and Toxin Weapons Convention and listed in Schedule 1 of the Chemical Weapons Convention (Pearson, 2007). Ricin has been used for political assassination purposes in public places (Simon, 1999), including assassination on Waterloo Bridge, in London, by agents of the Bulgarian secret police assisted by the Komitjet Gosudarstvjennoj Bjezopasnosti (KGB) (the secret service of the Union of Soviet Socialist Republics), in 1978 of the Bulgarian defector Georgi Markov. Mr Markov, who had sought refuge in the United Kingdom, was injected in the thigh with a small ricin-containing metal pellet using a specially devised umbrella (Crompton and Gall, 1980; Knight, 1979).

Ricin has been discovered with production instructions during security force raids on apartments housing terrorists in London (Craven, 2005; O'Neill, 2005). It had, apparently, been planned to smear ricin on door handles in London, and to contaminate toiletries, including face creams, mouthwashes and toothbrushes. Other apparently unconnected discoveries have been made suggesting terrorist intent in various countries (Mirarchi and Allswede, 2008). Ricin has been considered by terrorists because it is highly toxic, noninfective, easy to extract, relatively stable and a solid. It is likely that the major threat from ricin is powdered material that could be dispersed in the atmosphere, although food and drink contamination is a possibility. An electrochemoluminescence immunoassay has been developed to screen samples of food and drink for ricin (Garber and O'Brien, 2008). The estimated human lethal dose is $1\,\mu g\,kg^{-1}$ (Wannemacher and Anderson, 2006).

The toxin consists of two dissimilar polypeptide chains joined by a disulfide bond, which links cysteinyl residues near the carboxy terminus of the A-chain and the amino terminus of the B-chain. The A-chain polypeptide chain is a potent inhibitor of protein synthesis, and the B-chain is a galactose- or an *N*-acetylgalactosamine-binding lectin (Lin *et al*., 1971). The toxicity of ricin is attributed to A-chain biological activity, and the B-chain function is to bind toxin to cell-surface receptors (Wannemacher and Anderson, 2006). By inhalation, ricin aerosols cause acute-onset alveolar oedema, alveolitis, peribronchovasculitis and acute bronchitis (Hewetson *et al*., 1996; Fritz *et al*., 1993; Griffiths *et al*., 1995). With mice, and depending on the strain, the whole body aerosol LD_{50} in the range of $2.8–11.2\,\mu g\,kg^{-1}$ (Hewetson *et al*., 1996; Wilhelmson, 1997). Parenteral dosing of experimental animals with ricin causes lymphoid tissue necrosis, liver injury, adrenal gland haemorrhagic necrosis, myocardial degenerative lesions, renal tubular hyaline changes and gastrointestinal haemorrhages (Bingen *et al*., 1987; Griffiths *et al*., 1987). There is currently no effective antidote for ricin intoxication, and the major treatment is supportive for respiratory distress. The Department of Health and Human Services (DHSS) has published a response guideline for ricin (DHHS, 2006). See also **Ricin: Chemistry, Sources, Exposures, Toxicology and Medical Aspects**.

3.4.5.2 Botulinum Neurotoxin

During and following World War II, botulinum neurotoxins were developed as biological weapons because of their ability to cause a high incidence of morbidly and mortality (Middlebrook and Franz, 1997). Several countries have developed programmes to produce and stockpile botulinum toxin as an effective bioweapon. If employed by terrorists, botulinum neurotoxin would be capable of causing massive loss of life (Arnon *et al*., 2001). There have been attempts to use botulinum toxin as a terrorist weapon, notably by the Japanese extremist group Aum Shinrikyo, who spread botulinum toxin over Tokyo three times between 1990 and 1995 (Arnon *et al*., 2001). These attempts failed because of poor microbiological techniques, deficient aerosol-generating equipment and internal sabotage, The *Clostridium botulinum* neurotoxins act by impairing the release of acetylcholine (ACh) at cholinergic neuromuscular and neuroeffector junctions (Simpson, 1981). Eight immunologically distinct botulinum neurotoxins exist (A, B, C1, C2, D, E, F and G). Neurotoxins A, B, E and F are responsible for human poisoning. Botulinum neurotoxins are anaerobically secreted as inactive 150 kDa single-chain protoxins, surrounded by a complex of several associated proteins. The protoxin is subsequently cleaved to form the active disulfide-linked dichain neurotoxin consisting of a 100 kDa heavy chain (HC) and a 50 kDa light chain (LC) (DasGupta and Sugiyama, 1972). The amino-terminal region of the HC promotes translocation of the LC into the cytosol (Koriazova and Montal, 2003). The LC is a

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Zn-containing endoprotease that blocks ACh-containing synaptic vesicles for from fusing with the subsynaptic membrane at cholinergic neuroeffector and neuromuscular junctions. With peroral poisoning, the earliest symptoms are generally observed 12–36 hours postexposure and include visual disturbances including diplopia, dysphasia, dysphonia and dysarthria, reflecting the high susceptibility of cranial-nerve efferent terminals (Brin, 1997). Gastrointestinal effects sometimes occur, including nausea and diarrhoea, which may be of parasympathetic origin (Ambache, 1951). A progressive descending symmetrical skeletal muscle paralysis occurs from upper to lower limbs, and involving the diaphragm and intercostal muscles, which can progress to respiratory failure (Robinson and Nahata, 2003), but without sensory or CNS involvement (Brin, 1997; Simpson, 2004). Management includes the use of respiratory support, but further work is still required in the development of therapeutic inhibitors of LC protoxins (Adler *et al*., 2001). It is likely that use in terrorist incidents would involve contamination of food with toxin spores, and possibly by aerosol generation, both requiring specialist knowledge (Adler, 2006). Botulism is discussed in detail in **Toxicology of Chemical Warfare Agents**.

4 PREPAREDNESS FOR CHEMICAL TERROR INCIDENTS

4.1 General Considerations

In general with major terrorist incidents or suspected incidents, preparations for should be made as for any large industrial or transportation chemical incident, but it should be remembered that there may be secondary devices or other further action by the terrorist. The security forces and first responders should be prepared for this. In general, incidents may be of two general types: (i) those involving an obvious abrupt local incident, such as the abrupt release of a chemical into the environment and (ii) those involving the covert use of a toxic substance so that there is likely to be widespread, and possibly scattered, outbreaks of intoxication, which may at first not be obvious. For example, contamination of food might be mistaken for food poisoning of microbiological origin, and symptoms and signs might not occur for some hours. Were food outlets at transport hubs involved, the victims might be far away before adverse effects became apparent.

In the case of an abrupt use of a toxic chemical, the fact that something untoward has happened will be apparent immediately, but the incident may not be ascribed to terrorism. The necessary preplanned readiness actions can be put into operation immediately the cause is known. However, in many cases there may be a relatively slow appreciation of the possibility of terrorist activity, particularly with occult use of a chemical.

Detailed reports on how to plan for, and cope with, terrorist incidents have been published by national and local organizations (see Khan *et al*., 2000; Roberts and Maynard, 2007; Ballantyne, 2006b; Baker, 2007), and the following is a résumé of the more important overview considerations.

1. There should be frequent liaison between military, civil and intelligence agencies (and the governments) in order that all may be kept informed on the likelihood, nature, possible geographical location(s) and timing of possible incidents. This was stressed by one of the unfortunate lessons of the 11 September 1991 New York City World Trade Center incident, in which informed discussion and exchange of information between government agencies was far from optimum. Subsequent incidents suggest that there is still work to be done in respect of information and communication needs at both major and individual incidents, as exemplified by the police killing of an innocent Brazilian in the UK.
2. Continual updating of lists of the materials likely to be used in major and restricted terrorist situations is necessary, together with details on methods of obtaining, dispersal and likely locations of use. There needs to be continual updating and maintenance of manuals and checklists for of what is needed for management of incidents; for example, medical and rescue equipments, protective and precautionary measures, antidotes.
3. Frequent collaborative training sessions for dealing with terrorist actions in all likely scenarios, and for dealing with situations on discovery, before actual planned incidents, are necessary.
4. An essential element of preparedness is to ensure that adequate supplies of protective equipment and clothing are available; planning should provide for the needs not only of the on-site situation, but also of healthcare institutions where there may be secondary contamination as casualties are brought for medical and/or surgical management. This need should cover, at least:
   (a) Respiratory protective equipment. At the active on-site area, and, because initially the agent may be unknown, positive-pressure, self-contained breathing apparatus (SCBA) will be needed.
   (b) Whole-body protective clothing and gloves should be available.
5. Essential for on-site identification of agents used is the need for constant updating of analytical methods for specific threat chemicals; the specificity and sensitivity should be noted. Ideally, methods should be available for the on-site sampling analysis, preferably by portable instant readout devices for quantitative evaluation; such devices include the Improved

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Chemical Agent Monitor (DOD, 1999; Smiths Detection, 2009; Ram and Bhethanabotler, 2009).

4.2 On-Site Provisions

With abrupt-onset terrorist situations, at the actual site of an incident there may a multiplicity of initial complex considerations and uncertainties, such as the nature of the agent(s) used and the extent and severity of the possible degree of contaminations. Therefore, from the outset it is essential that the most rapid and efficient responses must be obtained by the interactive cooperation between official and trained first responders and appropriately qualified experts in chemical terrorism. In this manner the most appropriate resources can be deployed in a correct manner. The division of the site into a hot zone, with an inner cordon separating it from the warm zone, with triage facilities between the two, is advised. There should be decontamination facilities before casualties are moved into the cold zone, and an outer cordon should be established (see ASA, 2004; Home Office, 2004). In nearly all incidents, the most important immediate measure will be to remove casualties from further exposure.

4.3 Role of Healthcare Establishments and Professional Healthcare Providers

Hospitals dealing with the general public have major incident plans. However, a lesson from the Tokyo use of sarin was that people tended to leave the scene of the incident by all means available and many made their way to hospital or to general practitioners by means other than ambulance. Moreover, many individuals went home when they felt better as, even after getting to healthcare facilities, they did not feel inclined to wait. There was some evidence of secondary contamination of hospital staff in Tokyo (see Vale *et al*., 2007).

5 CONCLUSION

Terrorism has a very long history and it is unwise to assume it will go away soon. There are obvious attractions for the terrorist in the use of chemical substances, and it is wise for governments to prepare for the use of chemicals, as previous uses in incidents (e.g. in Japan) have shown that facilities in unprepared countries can be quickly overwhelmed.

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Evaluating the Long-term Health Consequences of United States Combat Deployments: Toxicology and the Law

Mark Brown and Kenneth Hyams

CONTENTS

1 INTRODUCTION

For as long as there have been wars, combat veterans and their families have expressed concerns about potential long-term health consequences from exposure to deployment-related hazards. One of the oldest stories ever written describes how Gilgamesh, the King of Uruk—who coincidentally lived about 2700 BC in what is today Iraq—goes off to war in the 'Great Cedar Forest'. This ancient story of a foreign military campaign includes a vivid account of the fears and worries of deployed warriors and their families, both before and after the battle. The equally vivid description of the serious readjustment issues for Gilgamesh and other survivors following their successful war is perhaps the first description of combat-related post-traumatic stress disorder (PTSD) (Mitchell, 2004).

More recent conflicts involving US forces and troops from other Western countries are no exception to this history of war-related health concerns. Particularly since the Vietnam War, US veterans and their supporters have spent a great deal of effort trying to identify and understand possible long-term health consequences associated with deployment-related exposures to occupational and environmental hazards. For the Vietnam War, military herbicides contaminated with dioxin (like Agent Orange) became the focus for a wide range of health concerns among combat veterans.

US veterans of the 1991 Gulf War have raised similar concerns but over a much broader array of potential deployment-related health hazards, including vaccines and prophylactic drugs, pesticides, chemical warfare agents, depleted uranium (DU) munitions, oil-well fire smoke, and many other potential exposure risks (Joseph *et al.*, 1998). For the 1991 Gulf War, all of these potential health hazards have become contentious issues as well in the United Kingdom (UK), Canada and Australia, because these countries deployed substantial numbers of troops to this conflict.

In contrast to the first Gulf War, health concerns for the current conflicts in Iraq and Afghanistan have so far focussed primarily on the long-term mental health impacts of deployment-related stress and various degrees of traumatic brain injury (TBI) from encounters with

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explosive weapons. Deployment-related environmental health concerns are still unfolding for the present conflict. Recently, some veterans relate apprehension over additional environmental and occupational exposures, which will probably increase in the coming years if the Vietnam and Gulf War experience is any guide.

Once the fighting stops in Iraq and Afghanistan, the focus may shift from acute physical and psychological trauma to long-term health problems, like cancer, which will inevitably be attributed to environmental exposures. A survey of new combat veterans coming to the Department of Veterans Affairs (VA) War-Related Illness and Injury Study Center (WRIISC) programme between June 2004 and January 2006, found that most veterans (55%) had mental-health concerns, but exposure concerns were commonly reported for DU, multiple vaccinations and poor air quality related to smoke from burning trash (Helmer *et al*., 2007).

The VA in the USA is responsible for providing a wide range of federal benefits to the nearly 24 million military veterans alive today and to their dependents, including nationwide programmes offering healthcare, financial assistance, education and training, home loans, pensions, rehabilitation, vocational training, burial and survivor benefits, and other assistance for veterans and their families. The broad scope of this mission is reflected in its budget and staff, making VA the second largest Cabinet level department in the Executive Branch of the US government.

The USA does not have a national healthcare system that serves all citizens. However, VA is the largest US healthcare provider, offering a full spectrum of medical, surgical and rehabilitative care to 5.5 million unique veteran patients at VA medical facilities in 2007, up from 3.8 million in 2000. Counting repeat appointments, in 2007, VA outpatient clinics accommodated 55.7 million visits. To handle this patient load, VA operates more than 1000 sites of care, including 153 hospitals, 731 community-based outpatient clinics, 135 nursing homes, 47 domiciliaries for veterans needing living quarters adjacent to VA healthcare facilities and 207 Veterans Centers for readjustment counselling.

The VA generally does not provide healthcare to US troops currently serving on active military duty. And, not all US military veterans are eligible for VA healthcare, although the VA serves a large number of military veterans. Veterans who do not have a health problem related to military service and veterans in higher income brackets may not be able to enrol for VA healthcare or may have to pay for a portion of their healthcare.

VA's disability and pension benefits are important for the support of veterans, just as healthcare is. VA disability assistance affects millions of Americans by providing a form of worker's compensation for former military personnel, in the form of monetary support paid to veterans who are disabled by injuries or illnesses they suffered or which were aggravated during their active military service. These include health problems thought to be due to deployment-related environmental and occupation hazards. Other veteran benefits include education assistance, home-loan guarantees, life insurance and survivor benefits.

The VA in the USA has learned one fundamental lesson from responding to the diverse needs of new combat veterans since the Vietnam War: for each combat deployment, critical stakeholders—veterans and their families, the general public, government leaders and the media—all demand to know how the health of new combat veterans has been affected and how best to care for these veterans. Along with the US Department of Defense (DoD), the VA will inevitably be asked to respond to the following key health questions following each wartime deployment:

1. Are combat veterans experiencing greater health problems than their nondeployed military counterparts?
2. Do returning veterans suffer from greater rates of specific diseases, like cancer, lung disease and endocrine disorders, and are they experiencing higher rates of mortality?
3. What is the impact of combat deployment upon veterans' families and upon their reproductive health?
4. Which specific environmental and occupational hazards encountered during deployment may be causing health problems among returning veterans?

Veterans' supporters often focus on the immediate healthcare and disability needs of recent combat veterans. They generally have limited patience for governmental statements that answering essential questions about the health status of combat veterans takes time in order to conduct epidemiological studies or overcome technical hurdles, like the absence of reliable exposure data in war zones during active hostilities. In the absence of immediate information, and pressed with the real concerns of ill veterans, supporters of veterans' interest may push the VA, DoD and other responsible government agencies into short-term solutions that do not adequately address veterans' needs. Although the best guidance on veteran health issues comes from well-designed, longitudinal mortality and morbidity studies that focus on veterans from a specific deployment, the government has to respond to veterans' health concerns long before this process can be implemented.

Well-conducted research studies take time to design, to assemble a population-based cohort, to locate a comparable control group, and finally to generate long-term data. Because years and even decades of research may be needed, exclusive use of a scientific and research approach by the government is often viewed as nonresponsive or even as governmental stonewalling.

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The VA's more immediate mission, to provide healthcare to veterans and to compensate them for disabilities caused by injuries received during military service, can be accomplished without time-consuming research, although occasionally these responsibilities distract from opportunities for careful scientific inquiry. Furthermore, because of the paucity of useful exposure data, particularly in combat zones, epidemiological studies have often been inconclusive in tying specific illnesses to a specific deployment-related environmental hazard.

Demonstrating a plausible link between specific exposures and unique illnesses among veterans has frequently been achieved only via the use of all available science related to a potential health hazard. Studies of veterans themselves have often not been sufficient for this purpose because of the noted limitations of these studies. However, for conclusions based on broad reviews of mainstream science to gain acceptance from veterans and their supporters, an independent assessment from a scientifically credible group is often needed.

In addition to research studies, much more immediate but less informative clinical data can be obtained from special health examination programmes; but specialized clinical 'registries' often suffer from being unrepresentative of a deployment cohort. Recent combat veterans can also be surveyed rapidly for self-reported health problems and exposures via telephone contacts and mass mailings. However, the data from these surveys generally cannot be confirmed. Reporting bias is a major problem in populations sensitized to potential health hazards by inaccurate and sensational media reports.

Although clinical registries and self-reported surveys fall far short of well-designed epidemiological studies, they can answer some basic questions about the types of illnesses and concerns exhibited by recent combat veterans. Consequently, they are viewed as a highly responsive approach by the government to immediate health questions from stakeholders.

As will be more thoroughly discussed, the VA in the US has utilized a number of approaches to evaluate the health consequences of military deployments and to address veterans' health concerns. As one of two US federal agencies directly responsible for military and veterans' health, the VA has tried the following:

1. establishment of deployment-unique clinical registries
2. epidemiological studies of veterans' health status
3. independent reviews of scientific literature relevant to specific deployment-related occupational and environmental hazards
4. recent use of 'combat veteran rosters', which are employed to provide comprehensive data on healthcare utilization based on VA and DoD electronic records.

2 HEALTHCARE REGISTRIES

For every US combat deployment, Congress and the general public rally in support of the troops. One way to show support is to demand solid answers to questions about the impact of combat deployment on veterans' health, and then to make sure troops receive optimal healthcare. The DoD is responsible for the health of US troops before they leave the military and become veterans eligible for VA benefits. After these veterans leave active military duty, the VA has the lead for veterans' health needs, including the tracking and monitoring of health status.

Historically, the quickest answers VA can supply about the health of recent combat veterans have come from review of the clinical-care needs of these veterans, often via establishment of special, voluntary 'clinical registries'. To obtain comparable clinical information, these registries entail a systematic examination with uniform requirements and reporting for the medical history, clinical examination and laboratory testing. Of course, registry participants are also clinically evaluated for outstanding health problems that are not specifically addressed by a registry protocol.

Once begun, these registries generally are not stopped even long after initial health questions are answered. The VA has ongoing special health-examination registries for veterans of the Vietnam War and the 1991 Gulf War—the 'Agent Orange' and 'Gulf War' registries. As of December 2007, the VA had provided 443 767 'Agent Orange' registry examinations for Vietnam War veterans and 102 535 Gulf War registry examinations for veterans from that conflict. Recently, the 'Agent Orange' registry has been opened to non-Vietnam War veterans who may have been exposed to herbicides contaminated with dioxin during military service outside Vietnam, and the Gulf War registry has been opened to veterans who served in the current conflict in Iraq.

Despite the focus on specific groups of veterans, special clinical registries have in general not been very useful in answering basic questions about the overall health impacts of a specific combat deployment or exposure (Institute of Medicine, 1998; Murphy *et al*., 1999). This fact should come as no surprise because participation in a special clinical registry is of necessity voluntary; with most veterans electing not to undergo additional examination, registries are not population based, and focus on healthcare-seeking patients; registries generally are a one-time clinical examination and lack any plausible control group, and clinical registries produce only limited data on combat-related hazardous exposures, usually based solely upon self-reports (Gray *et al*., 1998; Kroenke *et al*., 1998).

These limitations together render clinical registries ineffective for establishing scientifically defendable conclusions about the health of combat veterans as a whole or the causes of their health problems. The

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primary advantage of clinical registries is how quickly they can generate information on veterans' health problems, regardless of their limitations. Inevitably, clinical registries are no substitute for the data supplied by well-designed, population-based epidemiological studies.

2.1 Findings of the VA's Gulf War I Registry Examination

Officially authorized by Congress and the President in November 1992, all veterans of the 1991 Gulf War became eligible for participation in the VA's Gulf War registry. Despite the limitations of registries in general, the large number of veterans who have been examined and the systematic examination process used in the VA's Gulf War registry programme has produced important clinical insight into the variety of illnesses suffered by Gulf War veterans. This registry also has served as a source of hypotheses for further research. Moreover, any widespread serious physiologic disease should have been detected in this very large case series. Of note, the US DoD, the Ministry of Defence in the UK and Canada, and the Australian government all established similar clinical registries after the first Gulf War (Lee *et al*., 2002; Goss Gilroy, Inc., 1998; Kelsall *et al*., 2004).

Review of the diagnoses among over 100 000 1991 Gulf War veterans who participated in the VA's clinical registry indicates that these veterans presented with a wide range of common illnesses, which would be expected among any group of adult veterans (Department of Veterans Affairs, Veterans Health Administration, 2002). Interestingly, approximately 12% of participants in this registry had no outstanding health problem, presumably participating out of concern or interest to be registered. Although no unique illness stands out in this predominantly outpatient population, the self-selected nature of the group, lack of controls and absence of good exposure data make detailed interpretation problematic.

Unfortunately, the media have misinterpreted routine outpatient health problems as indicating substantial and serious illness among all Gulf War veterans. One misinterpretation of the data is that the >100 000 Gulf War veterans volunteering for an outpatient examination out of a total of 700 000 Gulf War veterans serving in that conflict means that one out of seven Gulf War veterans had a serious mystery illness or 'Gulf War Syndrome' (Department of Veterans Affairs, Veterans Health Administration, 2002).

In actuality, these veterans were diagnosed as having a wide range of health problems expected in an adult population of veterans. Importantly, a new or unique syndrome was not identified by systematic clinical examinations of over 100 000 Gulf War veterans. Similar common health problems without evidence of a new syndrome were also found in the British, Canadian and Australian Gulf War registries (Lee *et al*., 2002; Goss Gilroy, Inc., 1998; Kelsall *et al*., 2004).

Review of 1999 VA revised registry data indicates that many participants were 'well' when they were evaluated. While 74% of evaluated veterans report their health status as 'all right', 'good' or 'very good', the remaining 26% report 'poor' or 'very poor' health. **Table 1** shows the most common symptoms reported by Gulf War veterans in decreasing frequency: (i) loss of memory, (ii) headache, (iii) fatigue, (iv) skin rash, (v) muscle/joint pain, (vi) sleep disturbances, (vii) diarrhoea and other gastrointestinal symptoms, and (viii) shortness of breath.

These are common symptoms frequently reported in outpatient populations. During the Gulf War Syndrome controversy, statistics on symptoms were

Table 1 Most frequent complaints among 19 721 veterans in the VA's revised Persian Gulf registry[a]

Complaints	Frequency	Percentage[b]
Loss of memory and other general symptoms	5794	29.4
Headache	5204	26.4
Fatigue	4639	23.5
Skin rash	4574	23.2
Muscle/joint pain	4096	20.8
Sleep disturbances	2553	12.9
Diarrhoea and other gastrointestinal symptoms	2352	11.9
Shortness of breath	2050	10.4
Chest pain	1041	5.3
Choking sensitivity	939	4.8
Abdominal pain	916	4.6
Other symptoms involving skin and integumentary tissue	692	3.5
Cough	676	3.4
No complaint	1527	7.7

[a]Data as of February 1999, prepared by the VA Environmental Epidemiology Service.
[b]Percentage of 19 721 veterans.

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often misreported without noting whether the symptoms were inconsequential and transient or whether they were associated with well-known health problems. This type of symptom reporting led to substantial confusion about a potential mystery illness. As will be noted, subsequent improvements in VA's electronic medical record allowed for a much more rapid and comprehensive compilation of actual, physician-derived diagnoses rather than just symptoms, which obviated much of the speculation about a new symptom-defined syndrome among veterans following the invasion of Afghanistan and Iraq.

Table 2 shows that the most common primary diagnoses among veterans of the 1991 Gulf War are diverse and related to: (i) musculoskeletal and connective tissue conditions, (ii) mental disorders, (iii) respiratory conditions, (iv) skin and subcutaneous conditions, (v) digestive conditions, and (vi) nervous system conditions. As found here among Gulf War registry volunteers, musculoskeletal problems are consistently identified to be the most common medical problems in young populations of military veterans. Although it is difficult to identify control patients for comparison purposes in clinical case series, the VA's clinical registry data were very similar to the diagnoses found among British, Canadian and Australian veterans in their clinical registry programmes.

The VA registry provided limited information about the relative severity of diagnosed illnesses among Gulf War veterans. However, some information about severity is found in disability statistics for veterans awarded financial compensation (Department of Veterans Affairs, Veterans Benefits Administration, 2007). Of 621 230 Gulf War veterans separated from military service and eligible for VA programmes, 160 461 have had illnesses or injuries connected to military service with a disability rated to a level of 10% or more, which is compensable. A 1999 review found that the most common claim was for impairment of the knee, followed by skeletal system disability, back strain, arthritis due to trauma, scars and hearing loss (Department of Veterans Affairs, 1999).

There were 3280 veterans granted claims for disabling symptoms due to undiagnosed illnesses—a new compensation claim category, established by Congress in 1994, to assist veterans debilitated from unexplained symptoms. By 1999, there were 3077 claims granted for disability caused by undiagnosed illnesses. Granted claims represented a wide range of conditions, with the most common relating to: (i) musculoskeletal disease, followed by (ii) miscellaneous neurological conditions, (iii) systemic disease, (iv) lower digestive system, (v) skin, and (vi) skull and ribs (Department of Veterans Affairs, 1999). For determination of the number of veterans who may suffer from the putative 'Gulf War Syndrome', this group of veterans, who received compensation for undiagnosed illnesses, is probably the most representative because their unexplained symptoms were the cause of demonstrable disability.

2.2 Value of Registries

Ultimately, clinical registry programmes for Vietnam and Gulf War veterans fulfilled an initial short-term role as a rough and quick assessment of the health of combat veterans. For example, at the end of the 1991 Gulf War, VA healthcare experts, who saw televised images of enormous dark clouds of smoke from burning oil wells in Kuwait, became concerned about potential long-term respiratory problems among returning veterans, and in response established the initial Gulf War clinical registry programme. Early analysis of data from the programme served to dispel concerns about widespread respiratory illnesses in this combat cohort. The value today of all registry programmes may ultimately lie in the area of outreach and education. The registry examinations offer a unique opportunity for veterans to discuss their

Table 2 Distribution of diagnoses for the 19 721 veterans in the VA's revised Persian Gulf registry[a]

Diagnosis	Number	Percentage
Musculoskeletal and connective tissue	7286	36.9
Mental disorders	6887	34.9
Skin and subcutaneous tissue	3813	19.3
Respiratory system	3626	18.4
Digestive system	3451	17.5
Nervous system	3441	17.4
Circulatory system	2083	10.6
Injury and poisoning	2020	10.2
Infectious diseases	1785	9.1
Genitourinary system	1126	5.7
Neoplasm (malignant)	149	0.8
No medical diagnosis	4664	23.6

[a]Data as of February 1999, prepared by the VA Environmental Epidemiology Service.

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deployment-related health concerns with an informed VA healthcare provider.

The noted limitations of clinical registries linked to participation in particular combat deployments do not necessarily apply to registries based on specific diseases. These types of registries have a long history of usefulness. Clinical registries of specific diseases, like cancer or neurological disease, can be very helpful for research purposes and for outreach efforts to patients with unique health problems and health risks.

3 EPIDEMIOLOGICAL STUDIES OF VETERANS

Military veterans and their supporters, and many medical researchers have advocated epidemiological studies as the gold standard to assess the health consequences of military service. Properly designed epidemiological studies of combat veterans' health offer advantages over specialized clinical registries for evaluating the overall health of new combat veterans and for studying potential risk factors of illness. In particular, epidemiological studies can provide information on a population as a whole, rather than just sick and healthcare-seeking groups.

Note that epidemiological studies are not as useful in identifying a new disease because the most critical initial requirement in this process is to find unique clinical characteristics among affected patients; that is, identification of pathognomonic symptoms or signs. Clinical case–control studies are often a better approach for identifying new diseases than epidemiological studies. Epidemiological research is therefore not the first step when predominantly clinical questions, like the possible existence of a new or unique syndrome, need to be answered.

In recognition that properly designed epidemiological studies are critical in answering general questions about long-term deployment health effects, the US federal government has to date committed more than $274 million on Gulf War veteran health research, which includes a significant number of epidemiological studies (Department of Veterans Affairs, 2004; Department of Veterans Affairs, Deployment Health Working Group Research Subcommittee, 2006). A number of excellent reviews and summaries of the remarkably large number of studies of Gulf War veterans have been published (Institute of Medicine, 2006; Wessely, 2006).

The 2006 report *Gulf War & Health Vol. 4: Health Effects of Serving in the Gulf War*, which was produced by an independent committee of the National Academies of Sciences Institute of Medicine (IOM), represents a particularly deliberate and thorough review of all published scientific literature on veterans of the 1991 Gulf War (Institute of Medicine, 2006). For this IOM study, which was requested by the VA, the expert committee identified over 4000 potentially relevant scientific reports and focussed on 850 epidemiological studies. They concluded that the 'VA and DoD have expended enormous effort and resources in attempts to address the numerous health issues related to the Gulf War veterans. The information obtained from those efforts, however, has not been sufficient to determine conclusively the origins, extent, and potential long-term implications of health problems potentially associated with veterans' participation in the Gulf War'.

Tellingly, the IOM committee also identified serious limitations in existing epidemiological studies of Gulf War veterans, resulting largely from the lack of reliable exposure data. However, they did 'not recommend that more such studies be undertaken for the Gulf War veterans'. Rather, they recommended that there is 'value in continuing to monitor' Gulf War veterans for a few illnesses that they may be at greater risk for; specifically, brain and testicular cancer, amyotrophic lateral sclerosis (ALS), birth defects and postdeployment psychiatric conditions.

The IOM committee further noted that 'every study reviewed by this committee found that veterans of the Gulf War report higher rates of nearly all symptoms examined than their nondeployed counterparts.' Given this finding, not surprisingly the IOM committee concluded that symptom-defined illnesses, consistent with chronic fatigue syndrome, fibromyalgia, irritable bowel syndrome and multiple chemical sensitivity, were the most common health problem reported in these studies. They also found that 'Gulf War veterans consistently have been found to suffer from a variety of psychiatric conditions', including PTSD, anxiety, depression and substance abuse.

The IOM also reported that studies have 'not demonstrated differences in cognitive and motor measures' in deployed versus nondeployed veterans, and show no apparent increase in risk of peripheral neuropathy, cardiovascular disease or diabetes. They reported finding no consistent pattern of birth defects among offspring of male or female Gulf War veterans, but that congenital urinary tract abnormalities were found to be increased in 'more than one well-designed study'. They also found little data supporting objective respiratory illnesses among Gulf War veterans.

Finally, the IOM concluded that 'the results of that research indicate that although deployed veterans report more symptoms and more severe symptoms than their nondeployed counterparts, there is not a unique symptom complex (or syndrome) in deployed Gulf war veterans'. This report underlined the important fact that after more than one-quarter of a billion dollars of research, a unique 'Gulf War Syndrome' has not been confirmed.

For future deployments, the IOM committee recommended better use of predeployment and postdeployment health screening, better exposure assessments and continued surveillance among Gulf War veterans for

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cancer, ALS, birth defects, adverse pregnancy outcomes, postdeployment psychiatric problems and mortality. As recognized by the IOM, longitudinal mortality and morbidity studies have provided the most reliable data on the general health status of Gulf War I veterans. For example, according to a VA study of mortality among all Gulf War veterans, mortality risk for Gulf War veterans was essentially identical compared to their nondeployed military peers (except for a small increased risk for accidental death in the first five years postdeployment). Interestingly, the mortality of US veterans of the 1991 Gulf War was less than one-half the mortality of a demographically similar US civilian population, which is probably a reflection of the healthy worker or warrior effect (Kang and Bullman, 2001).

Mortality studies are useful because relatively objective and accurate mortality data is available for the entire cohort of US war veterans. Consequently, mortality studies have already been started by the VA for the current cohort of combat veterans who served in Iraq and Afghanistan.

Because many health questions remain unanswered, substantial government funding continues for studies of Gulf War veterans more than 17 years after the war (Department of Veterans Affairs, 2004). Most research has focussed on the possibility of a neurological abnormality as the cause of chronic unexplained symptoms among Gulf War veterans. Evidence has been found in studies conducted at Southwestern Medical School in Texas that some Gulf War veterans may be suffering from a previously uncharacterized neurological disease, possibly due to exposure to pesticides or organophosphate chemical agents during the Gulf War (Haley *et al*., 1997).

4 INDEPENDENT REVIEWS OF ALL RELEVANT SCIENTIFIC LITERATURE—CURRENTLY THE MOST ACCEPTABLE APPROACH

A key weakness of epidemiologic studies of combat veterans has been their frequent inability to find evidence of a link between specific health outcomes and exposures during combat deployment. This problem results from the nearly complete absence of useable environmental and occupational exposure data among combat troops deployed to a war zone. The difficulty of accurately recording the location of individual troops during hectic wartime deployments adds to this problem.

4.1 Impact of Poor Exposure Data

Lack of exposure and troop location data have seriously handicapped governmental efforts to craft fair and equitable policies for linking specific deployment hazards to specific injuries and disabilities among returning veterans, and to provide appropriate compensation. A critical problem for policy makers and veterans alike has been how to document exposure to wartime health hazards, including even basic information about where deployed troops were located. From a preventive medicine and research study standpoint, the well-understood model of work-place occupational exposures monitoring cannot be translated to the battlefield.

In general, occupational monitoring has not proven practical during active engagement in war situations. Combat deployments are not large epidemiological studies. In fact, attempts to collect extensive exposure information could hinder war-fighting capabilities and endanger combat troops. Accurate documentation of troop locations is similarly difficult and hazardous during chaotic wartime events.

These serious limitations in obtaining exposure data have generally frustrated attempts to evaluate long-term health consequences of combat-related exposures and to develop sound policies to respond to affected veterans. Similarly, disentangling military deployment-related from civilian, non-military-related risks has been problematic. In general, military environmental and occupational health risks are comparable or even identical to risks experienced by civilians with similar occupations. Consequently, it is hard to make distinctions between risks accrued during military service and risks encountered during civilian life.

A good illustration of these problems is the federal government's response to health concerns related to Vietnam War veterans and their potential exposure to herbicides contaminated with dioxin. Many returning veterans developed health problems that have been attributed to exposure to Agent Orange and related herbicides used in Vietnam, and these concerns extended to birth defects among their children. Congress held their first of many hearings on herbicide use in Vietnam in 1970, and Congressional interest in this topic remains high to this day.

Initially, the US government had difficulty in responding to Agent Orange health concerns. In part this was because at the beginning of the controversy scientists were only just beginning to understand the various health effects that could be caused by exposure to dioxins. Similarly, the VA had few well-developed policies and procedures in place for evaluating and compensating illnesses potentially related to military environmental and occupation hazards in cases that were not clearly linked to military service.

Those early concerns initiated a long struggle by the government to fairly and equitably evaluate possible long-term health effects among Vietnam veterans exposed to herbicides. Many observers at the time—back in the late 1970s—considered that the obvious approach would be to study Vietnam veterans themselves. That

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is, the ideal way to evaluate potential health effects from Agent Orange exposure would be a well-designed epidemiological study of Vietnam War veterans. Congress supported this approach in 1979, by passing Public Law 96–151, which directed the VA to 'conduct an epidemiological study of the long-term health effects on individuals from exposure to dioxins (including 'Agent Orange') used during the Vietnam conflict'.

Conduct of an epidemiological study was the research approach that the VA was directed by law to embark upon in 1979. Four years later, in 1983, VA representatives were forced to announce that they had been unable to develop a feasible study, primarily because of inadequate herbicide exposure data (Institute of Medicine, 1994). In frustration, Congress then urged the VA to transfer the study to the CDC (Centers for Disease Control) (United States Government Accountability Office, 1990). In response, the VA and CDC signed an interagency agreement transferring funds ($70.4 million dollars in 1980s-appropriated VA dollars), personnel positions, prior work, and the authority for the CDC to conduct the study (United States Government Accountability Office, 1990). Several years and tens of millions of dollars later, the CDC also concluded that available military records were not adequate for establishing meaningful herbicide exposure data among Vietnam veterans, and that an adequate Agent Orange epidemiological study was not feasible.

In retrospect, this approach was fated to be disappointing because we now appreciate that trying to conduct an occupational-health-type study on combat-deployed troops is extremely challenging, due primarily to the lack of usable exposure and troop location data. The basic problem is that this information is generally not available. Similarly, more than a decade later following the 1991 Gulf War, essentially the same high initial expectation arose over the value of epidemiological studies in understanding battlefield health hazards. A similar history could unfold in the aftermath of the current conflicts in Iraq and Afghanistan (Helmer *et al*., 2007).

The inability of epidemiologic studies of Vietnam War veterans to prove a link between their health problems and herbicide exposure because of a lack of basic information—reliable exposure and troop location data—was difficult for nonscientists to understand. Efforts are still underway to develop reliable exposure models more than 30 years after the last American soldier left Vietnam (Institute of Medicine, 2008b).

Given the lack of reliable exposure data, Congress and the President bypassed this problem for policy development purposes in 1991 by enacting Public Law 102-4, the 'Agent Orange Act of 1991', which stipulates that all Vietnam veterans—any veteran who spent even one day 'in-country'—be considered as exposed to Agent Orange. Further, this law directed the VA to develop a new approach for incorporating scientific data into Vietnam veteran disability compensation policies relative to Agent Orange exposure.

Instead of relying solely on studies of Vietnam veterans themselves, the mandated approach was based upon regular, biennial reviews by the independent and prestigious National Academies of Science Institute of Medicine of the entire scientific literature on health effects from herbicides and related dioxin in any exposed population. Veterans would thus potentially benefit from scientific findings in any exposed human population and not just research on veterans who had participated in hectic and confusing combat operations.

This new approach, still used today, requires the VA to use the IOM reports to help determine whether an automatic link should be recognized between a specific illness and Vietnam service. In bureaucratic terms, that is whether a 'presumption' of service connection for disability compensation is warranted by virtue of a 'positive association' between herbicide exposure and disease as identified by the IOM. The law defines a 'positive association' as existing when the credible scientific evidence for the association is equal to or outweighs the credible evidence against the association. In making the determination, the VA is given latitude to consider all other credible medical and scientific information, in addition to the IOM reports.

In evaluating IOM reports for this purpose, Public Law 102-4 directs the VA to consider whether the findings of an association are statistically significant, are capable of replication, and withstand peer review. VA is required then to announce any new policies for compensating Vietnam veterans for injuries related to herbicide exposure, or to publish an explanation of the scientific basis for why not. Thus, a standard was set for disability determination that does not rely on establishing the dose of dioxin exposure or the probable health effects of that exposure on any individual veteran.

The first IOM report, 'Veterans and Agent Orange', published in 1994, set the pattern for all future reports as a *tour de force*, exhaustive review of the entire published scientific and medical literature on health effects from exposure to herbicides used in Vietnam. Since then, the 'Agent Orange Act' has required that the IOM report be updated every two years, taking into account new scientific and medical publications that may affect the results of previous IOM reviews.

Based upon findings contained in that initial 1994 and subsequent biennial IOM updates through 'Update 2006', (Institute of Medicine, 2008b), the VA has established new presumptions of service connection for Vietnam-era veterans who served in Vietnam and are diagnosed with:

1. soft tissue sarcoma
2. non-Hodgkin's lymphoma
3. chronic lymphocytic leukaemia
4. Hodgkin's disease

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5. chloracne
6. respiratory cancers
7. prostate cancer
8. multiple myeloma
9. early-onset transient peripheral neuropathy
10. porphyria cutanea tarda
11. Type 2 diabetes
12. spina bifida among the children of Vietnam War veterans.

Even before this formal review process was set in motion, the VA had previously on its own authority recognized chloracne, non-Hodgkin's lymphoma and soft tissue sarcoma as related to herbicide exposure for Vietnam veterans. The IOM review process confirmed the validity of these earlier decisions and has subsequently added many additional diseases to this list.

In effect, IOM is used as a scientific arbiter so that Vietnam veterans benefit from service connection for any health effect positively associated in studies of populations exposed at any level to Agent Orange or other herbicides used in Vietnam. Most of the hundreds or thousands of studies reviewed by the IOM are in fact of non-Vietnam veterans, and include civilian workers exposed on the job and civilians exposed during industrial accidents. Studies based upon nonmilitary veteran subjects have been quite helpful because they almost always involve much better-characterized exposure data with correspondingly substantially larger and longer exposures, compared to that experienced by the average Vietnam veteran. Veterans gain the advantage of disability compensation policies based upon studies that provide more reliable information than studies of potentially affected veterans themselves.

This part-legislative, part-scientific and part-administrative approach to determining eligibility for disability is still used today. It is generally accepted as having worked to the benefit of potentially affected veterans by all stakeholders, because it is viewed as giving the benefit of the doubt to veterans. Congress has found this approach acceptable and has renewed the VA's authority to use it through 2015.

4.2 Limitations in Evaluating Commonplace or Well-Characterized Health Effects

More recent attempts to extend this approach of using the IOM to help link disability to military service among other groups of veterans have been more difficult and have produced few new benefits. For example, in response to concerns of veterans of the 1991 Gulf War and their families that their health may have been affected by exposure to a wide variety of environmental and occupational hazards during that conflict, two federal laws, Public Laws 105–277 and 105–368, were enacted. Drawn directly from the Agent Orange Act of 1991, these two statutes invoked the now-familiar formal IOM committee review process, including regular and thorough reviews of all the scientific and medical literature relevant to health and Gulf War exposures.

Several problems have emerged from using this approach with more recent war veterans. The environmental and occupational exposures experienced by Gulf War veterans were quite different compared to the Vietnam War. In contrast to the relatively narrow range of herbicides used in Vietnam and their dioxin contaminant, hazardous exposures potentially related to the 1991 Gulf War involved many dozens of different and unrelated agents (Joseph *et al*., 1998). The two laws actually specified at least 33 different Gulf War-related environmental and occupational hazards, all to be evaluated through this formal process (**Table 3**).

In addition to these differences, when the Agent Orange Act was enacted in 1991 there was a relatively small number of relevant scientific publications on herbicides and dioxin health effects for IOM committees to evaluate. In contrast, many of the Gulf War environmental and occupational hazards are well studied, with an abundant health-effects literature, much of which suggests a small health risk from low-level exposure. Consequently, the six major and a couple of minor IOM committee reviews produced as of 2007 have generated few new or original insights into the health effects of exposure relative to these agents. In fact, the IOM reports tend more to mirror summaries of human health effects found in standard occupational health and toxicology textbooks.

In 2000, the first IOM committee report in this series on 1991 Gulf War health effects reviewed relevant scientific literature on sarin, cyclosarin, pyridostigmine bromide (a chemical warfare pretreatment used by the US and UK military), DU and anthrax botulinum toxoid vaccines (Institute of Medicine, 2000). The second (2003) IOM study reviewed the potential long-term health effects from insecticides and solvents used in the Gulf War (Institute of Medicine, 2003). The third IOM study (Institute of Medicine, 2005) examined health effects from Gulf War fuels, combustion products and rocket propellants. The fourth study (2006) reviewed all scientific studies on health effects of serving in the Gulf War (Institute of Medicine, 2006). Potential infectious disease risks were reviewed in 2007 (Institute of Medicine, 2007a). The sixth study (2008) reviewed health effects associated with deployment-related stress (Institute of Medicine, 2008a). Finally, the potential health effects of sarin exposure was re-reviewed in 2004 because of new research findings (Institute of Medicine, 2004).

In addition to this legally mandated series of studies, since the 1991 Gulf War the IOM has produced

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Table 3 Statutory list of potentially hazardous exposures during the 1991 Gulf War to be evaluated by the IOM

The OP pesticides chlorpyrifos, diazinon, dichlorvos and malathion	The methyl carbamate pesticides proxpur, carbaryl and methomyl
Pyridostigmine bromide	Mustard agents 'at levels below those which cause immediate blistering'
Nerve agents 'at exposure levels below those which produce immediately apparent incapacitating symptoms' including sarin and tabun	Other pesticides and repellents including lindane, pyrethrins, permethrins, rodenticides and DEET[a]
Volatile organic compounds	Hydrazine
Red fuming nitric acid	Solvents
Uranium and depleted uranium	Microwave radiation
Radio frequency radiation	Hydrogen sulfide
Oil fire by-products	Diesel heater fumes
Sand 'microparticles'	
Time compressed administration of multiple live, 'attenuated' and toxoid vaccines	Diseases endemic to the region including leishmaniasis, sandfly fever, pathogenic *Escherichia coli* and shigellosis

[a]N,N-diethyl-m-Toluamide

nearly 20 other, separate studies for the VA and DoD on a wide range of Gulf War veteran health issues. (Joellenbeck and Hernandez, 2002). The breadth and thoroughness of the IOM scientific review process and their reputation for independence and scientific excellence make them a highly credible source for what often are contentious questions surrounding health and possible deployment-related hazardous exposures. Taken together, these IOM committee studies on potential health effects from participation in the 1991 Gulf War have produced somewhat predictable findings on well-understood environmental exposures.

As with the Agent Orange Act of 1991, the new statutes covering Gulf War veterans give the IOM responsibility for evaluating the underlying science, but the VA retains responsibility for translating their conclusions into relevant healthcare and disability policy, as appropriate to its mission. The end result of this process has not led to many new presumptions for disability compensation. Nevertheless, the IOM's largely negative findings about serious health consequences from participation in the 1991 Gulf War have been generally acceptable to all stakeholders because of the IOM's reputation for competence and fairness.

4.3 Toxicology versus the Law

The VA has had difficulty applying IOM committee findings to 1991 Gulf War veterans precisely because many of the proposed Gulf War-related hazards represent well-characterized occupational exposures that are commonly experienced by US citizens (Brown, 2005). For example, the pesticides, solvents and air pollutants reviewed by IOM committees represent virtually the identical agents that US civilians are frequently exposed to domestically. Similar low-level exposures in both the civilian and military setting are no accident because the DoD generally adheres to civilian guidelines established by the US Environmental Protection Agency and others when selecting pesticides or other agents for use in combat deployment situations.

Even the seemingly more unusual exposures such as to sarin (a chemical warfare nerve agent) or DU (used in special armour-piercing munitions) still have a substantial health effects literature derived from exposed workers and other populations (Brown and Brix, 1998). Many people have been involved in the manufacturing of chemical warfare agents and DU and other uranium components, and general populations have been exposed to terrorist incidents, like the 1994 Tokyo, Japan, incident involving thousands of civilian exposures and some deaths (Morita *et al*., 1995). There is also extensive clinical experience in the use of pyridostigmine bromide for the treatment of myasthenia gravis.

Because of the relatively short-term (a few months) and low-level exposures experienced by most 1991 Gulf War veterans to hazardous agents associated with that deployment, the VA has had particular difficulty applying the IOM's conclusions based upon studies of involving long-term (years or decades) occupational exposures among civilian workers or short-term but relatively high-dose exposures in industrial accidents. To complicate policy development, the laws governing this process, like those for Agent Orange, do not take into account exposure magnitude or duration, leading to the somewhat scientifically implausible result of treating any exposure level as equally likely to lead to a specific long-term health effect, no matter how universal or trivial

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the exposure magnitude may have been for most Gulf War veterans.

As an example of the difficulty in developing fair disability policies, the 2003 IOM committee report on long-term health effects from insecticides and solvents documents a slight increase in leukaemia risk among chemical industry workers with large and prolonged occupational exposure to benzene (Institute of Medicine, 2003). But such findings do little to inform us about the potential leukaemia risk for the typical Gulf War soldier experiencing an unremarkable benzene exposure during a few months of wartime deployment.

On the other hand, there are certainly examples of 1991 Gulf War veterans who experienced more than just everyday or commonplace benzene exposure, for example, veterans who regularly worked on vehicle maintenance. Such cases may involve benzene exposure at levels more comparable to the typical civilian occupational exposures that formed the bases of the studies reviewed by the IOM. Importantly, the applicable laws do little to distinguish between these two exposure extremes. However, individual veterans in the USA can still provide evidence of significant hazardous exposure to any known health threat and receive compensation based on their distinctive circumstances.

In a similar vein, many IOM committee findings about long-term health effects arise only in cases involving an unusually large exposure sufficient to cause immediate (acute) and serious health effects at the time of exposure. For example, certain well-documented long-term health effects from organophosphorus (OP) pesticides are found only as the result of severe and immediate initial poisoning, typically via an occupational exposure, and usually leading rapidly to hospitalization (Brown and Brix, 1998). Lesser exposures not leading immediately to serious clinical signs and symptoms have generally not been associated with long-term effects. For the 1991 Gulf War, all potential exposures to sarin and related OPs are thought to have been subclinical because there were no documented instances of acute OP-type toxicity reported among deployed coalition troops from any country (Institute of Medicine, 2000).

From a policy standpoint, how should the VA apply such findings by the IOM to the vast majority of veterans who experienced only unexceptional, asymptomatic exposures to these agents? All Americans experience small, perhaps continuous exposures to common OP pesticides (available at gardening stores everywhere) or benzene (a component of gasoline) during their lifetimes. However, such exposures are generally considered to have negligible health risks, given their small magnitude.

For these reasons, the VA has been hindered in developing new presumptive service-connected disability policies in response to IOM committee findings on the health effects posed by the wide range of common but potentially hazardous occupational and environmental exposures associated with the 1991 Gulf War. However, the IOM findings have generally been considered credible and the VA's response to these findings appropriate.

As noted, a presumption of service-connection allows the VA to award disability compensation without the individual veteran having to provide evidence that they were harmed due to their military service. As previously noted, it is important to understand, however, that even without a presumptive service connection, veterans can, on an individual basis, provide evidence that they were exposed to a toxic agent and suffered harm and then receive compensation. Disability compensation is usually awarded through this direct method in the VA. As a result, IOM findings have not precluded veterans from obtaining medical and financial assistance for health problems caused by hazardous exposures during military service.

5 LESSON LEARNED—BETTER WAYS TO ASSESS VETERANS' HEALTH THROUGH ELECTRONIC 'COMBAT VETERAN ROSTERS'

The type of voluntary clinical registry used by the VA for Vietnam and Gulf War veterans has become obsolete. The VA's recent advances in developing a comprehensive electronic patient record and the development by the DoD of an accurate roster of Iraq and Afghanistan veterans has led to enormous improvements in the VA's ability to track and evaluate the healthcare needs of new combat veterans. The VA's recent experiences tracking VA healthcare utilization for specific groups of veterans using electronic 'combat veteran rosters' demonstrates that this approach has many advantages over special clinical registries. The approach also provides very rapid results, which have been well received by all stakeholders concerned about the health of new combat veterans.

Key to this improved approach has been the VA's electronic outpatient and inpatient clinic records, which include diagnostic and demographic data for all veterans who receive VA healthcare, including International Classification of Disease (ICD-9) codes, for every clinic visit and hospitalization at a VA medical facility. The recorded data is mainly administrative data that reflects clinical impressions at the time of the clinical encounter, which means that not all diagnoses are confirmed when recorded. Nevertheless, each recorded diagnosis requires clinical follow-up and evaluation, and so accurately reflects healthcare needs. All inpatient and outpatient encounters are now captured by the VA's electronic health record, whereas only inpatient data was computerized just after the 1991 Gulf War. By matching this electronic database with the DoD's roster of recently deployed combat troops, the VA can identify all combat veterans accessing VA healthcare, and track their healthcare needs.

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The capturing of both inpatient and outpatient visits in an electronic health record is a major advance in VA healthcare that has occurred since the first Gulf War. The use of VA electronic healthcare records to monitor veterans' health needs first took place in early 2000, in an evaluation of recent VA healthcare utilization among Project Shipboard Hazard and Defense (SHAD) veterans who may have been exposed to biological and chemical agents in military tests conducted during the Cold War. Potential SHAD exposures include infectious agents, possible carcinogens, and chemical warfare nerve agents.

Although not a new combat deployment, nevertheless Project SHAD veterans and their supporters in Congress and the media had similar questions about potential long-term health effects from involvement in these tests as has been asked previously for recent combat troops. Using its electronic health record, the VA was able to quickly supply some answers about possible health problems among this veteran cohort of about 5000 identified individuals.

Although this clinical information is not research data and did not represent a formal epidemiological study where rates of various diseases could be compared, it nevertheless provided a rapid assessment of the healthcare needs of this cohort. No unusual health problems were identified in this aging group of veterans.

Perhaps the most useful conclusion from this summary of clinical care was that since no particular health problem stood out among Project SHAD veterans, no recommendations for specific testing or evaluation of this cohort were feasible. SHAD veterans therefore should be assessed individually on the basis of their clinical presentation to receive optimal healthcare.

Although this electronic health record approach generates rapid results, it is limited in that it only contains health information for those SHAD veterans electing to seek healthcare from the VA, and there is no comparison group to assess rates. Thus, clinical findings cannot be generalized to the overall health status of SHAD veterans. More detailed analysis clearly requires a properly designed population-based epidemiological study. Consequently, in 2002 the VA contracted with the IOM for a study of potential long-term health effects among SHAD veterans in comparison to suitable controls.

Because the IOM study of SHAD veterans required more than four years to complete, the initial analysis of their clinical care using the VA's electronic health record was very helpful in understanding their healthcare needs and answering veterans' questions. Much later, the formal IOM epidemiological study was unable to definitely identify any long-term health effects among SHAD veterans that occurred at greater rates compared with controls (Institute of Medicine, 2007b).

5.1 'Operations Iraqi Freedom/Enduring Freedom' Combat Veteran Roster

The electronic Combat Veteran Roster approach has also proven remarkably successful in meeting the short-term requirements for information on new veterans returning from the current conflicts in Iraq and Afghanistan. The VA produces healthcare utilization reports with summary statistics for these combat veterans on a quarterly basis. The January 2008 report includes information on healthcare needs among 799 791 Operation Iraqi Freedom/Operation Enduring Freedom (OIF/OEF) veterans (both former active duty and Reserves/National Guard) who left active duty and became eligible for VA healthcare since fiscal year (FY) 2002 ('Analysis of VA Health Care Utilization Among US Global War on Terrorism (GWOT) Veterans, Operation Enduring Freedom Operation Iraqi Freedom', Veteran Health Administration Office of Public Health and Environmental Hazards, January 2008).

Among all 799 791 separated OEF/OIF veterans, a cumulative total of 299 585 (37%) received VA healthcare since FY 2002, with most (96%) seen as outpatients only. Their diagnosed health problems covered more than 8000 discrete ICD-9 diagnostic codes, with the three most common problems being musculoskeletal ailments (principally joint and back disorders), mental disorders, and 'Symptoms, Signs and Ill-Defined Conditions' (a frequent initial diagnosis in outpatient populations that includes 160 subcategories of symptoms and clinical findings not coded elsewhere in the ICD-9).

This clinical information demonstrates that new Iraq and Afghan veterans are presenting to the VA with a wide range of medical and psychological health problems. As with SHAD veterans, no unexpected conditions stand out, and therefore these new combat veterans are being assessed as individuals to identify all outstanding health problems.

5.2 Combat Veteran Roster Approach—Advantages over Conventional Registries

Although not a research study with confirmed diagnoses, the electronic Combat Veteran Roster approach continues to be useful in meeting immediate information needs on the health of new combat veterans. This nonresearch approach to evaluating healthcare needs—matching the DoD-provided roster of new combat veterans with VA electronic patient care data—clearly offers significant advantages compared to earlier special clinical registries such as the Vietnam War and Gulf War health examination registries. It is faster to implement, popular with stakeholders, generates a snapshot of major diagnoses and

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clinical care requirements, and encompasses every single veteran who uses VA healthcare—not just veterans who elect to volunteer for a special health registry programme.

As an example of its advantages, in contrast to the approximately 14% (103 000) of Gulf War I veterans who underwent a special Gulf War registry examination during the last 17 years, already the healthcare needs of more than 37% of OIF/OEF veterans are being followed in the new electronic database maintained by the VA. Moreover, the Combat Veteran Roster approach documents every time OIF/OEF veterans obtain VA care, not just on the one occasion that they elect to have a registry examination.

The evaluation of all healthcare encounters provides a much more in-depth and longer-term assessment of the healthcare utilization of these veterans because many of them return frequently for VA healthcare, and because veterans are often seen in different clinics or even different parts of the country for specialized healthcare. Lastly, this approach gives a more accurate assessment of veterans' health problems because it relies on physician-diagnosed health problems and not self-reported symptoms, which may not be significant because they are transient or nondebilitating.

Although medical research, particularly epidemiological studies, is still necessary to answer questions about the long-term health of combat veterans and to investigate potential hazardous exposures and other risk factors (Kang *et al*., 2000; Cowan *et al*., 1997) the VA's OIF/OEF Combat Veteran Rosters have proven to be enormously popular with veterans and their supporters, who deserve rapid answers to questions about their healthcare needs. Consequently, the VA has committed to continue to monitor the healthcare utilization of recent OIF and OEF veterans using updated deployment rosters provided by the DoD to ensure that the VA tailors its healthcare and disability programmes to meet the needs of this newest generation of war veterans.

6 CONCLUSIONS

The VA is repeatedly called upon by critical stakeholders—veterans and their families, the US public, government leaders and the media—for data on the health consequences of military deployments. The VA therefore must be ready to rapidly address many difficult-to-answer questions each time troops are sent into combat. Are combat veterans experiencing more health problems than they should be? Have specific environmental hazards encountered during deployment caused health problems among returning veterans? Do returning veterans suffer from greater rates of specific diseases including cancers or mortality? And, what is the impact of combat deployment upon veterans' family and upon their reproductive health? Everyone of course wants immediate answers to these questions.

Although the best data on deployment-related health problems eventually comes from well-designed, long-term mortality and morbidity studies, the VA has developed a range of more rapid responses, including voluntary health examination registries, which have had many limitations, and more recently, electronic 'Combat Veteran Rosters' based on VA universal electronic healthcare records, which have been quite successful.

A unique clinical programme has also been developed within the VA to provide in-depth clinical assessments and healthcare to US veterans with health problems that are difficult to diagnose and treat. These health problems, which are often characterized as unique war syndromes, have been encountered after every major modern war (Hyams *et al*., 1996). There are now three WRIISCs in Washington, DC, New Jersey and Palo Alto, CA that provide for this specialized healthcare (Department of Veterans Affairs, 2008). Besides these clinic-based efforts, a concerted outreach and risk-communication programme is maintained by both the VA and DoD to inform veterans and their families about deployment-related health hazards and availability of healthcare and compensation (Brown *et al*., 2002).

Well-designed epidemiology studies are still needed after wartime deployments because they provide the most reliable information about the risks to veterans' health and because they are fixed in the public mind as the gold standard of research. However, they are generally not satisfactory for linking specific deployment-related exposure to specific occupational or environmental hazards with any specific health outcome, primarily because of the lack of useful exposure and troop location data during chaotic wartime conflicts.

Without good data linking hazardous military exposures to specific illnesses, policy development in the USA to assist military veterans often has had to rely on systematic reviews of all relevant medical literature for various exposed groups, whether military or civilian, by an independent scientific group such as the National Academies of Sciences Institute of Medicine. The development of fair and helpful policy for the healthcare and assistance of combat veterans potentially exposed to environmental hazards is not totally reliant on good science and frequently cannot be based on research studies of the exposed veterans themselves.

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Biological Toxins in Warfare and Terrorism

R. Gregory Evans

CONTENTS

1 INTRODUCTION

Toxins are harmful biological agents produced by bacteria, plants and animals. They differ from chemical agents in that they are naturally occurring, are nonvolatile and, except for mycotoxins, are not dermally active. Toxins can be much more toxic than chemical agents (US Army Medical Research Institute of Infectious Diseases, 2005). They are also not persistent in the environment and are not transmittable from person to person. Toxins that are stable in the environment, highly toxic and easy to produce are candidates for use as biological weapons. Some toxins, such as mycotoxins and ricin, require much larger quantities than toxins such as botulinum to be effective in an open-air aerosol exposure; however, they can be effectively used in an indoor release. In this chapter, we will discuss the four toxins that are considered most likely to be used as biological weapons either by state entities or terrorist organizations (Franz *et al*., 1997). The list includes one highly lethal toxin, botulinum, two fairly lethal toxins, ricin and mycotoxins, and one that is considered primarily an incapacitating agent, staphylococcal enterotoxin B (SEB). Botulinum is a Center for Disease Control and Prevention (CDC) Category A agent, while the other toxins are CDC Category B agents. The likely route of intoxication for outdoor and indoor release of these toxins is aerosol; however, gastrointestinal intoxication can occur through poisoning of food. Aerosol release might be in a building, subway or tunnel. Water supplies might also be contaminated; however this is less likely because of chlorination and dilution. It is most likely that these toxins will be delivered by aerosol release because their lethality is much greater when inhaled than when ingested (Franz *et al*., 1997).

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2 BOTULINUM TOXINS

2.1 Introduction

The botulinum toxins are a group of seven neurotoxins (types A through G) produced by bacillus *Clostridium botulinum* (*C. botulinum*) and two other *Clostridium* species. All seven types of neurotoxins act by inhibition of presynaptic acetylcholine release. Humans are naturally exposed to the toxins through food poisoning and through rare incidents of wound botulism and infant botulism (Tacket *et al*., 1989). *C. botulinum* toxin is a likely agent for use in biological warfare or as a terrorist agent because of its toxicity, stability, ease of production, and because humans are generally not immunized against it (Villar *et al*., 2006). Also, large quantities can be produced through fermentation. The toxin can be delivered by aerosol or can be used to contaminate food. One gram of crystalline toxin, evenly dispersed and inhaled, could kill more than 1 million people (Arnon *et al*., 2001). If the toxin is released outdoors, it would be widely dispersed and contaminate far fewer people than if the same amount were released indoors. Botulinum neurotoxin (BoNT) is easily denatured by environmental conditions that detoxify it within 12 hours in air and 1–3 hours in sunlight (US Army Medical Research Institute of Infectious Diseases, 2005). It is also inactivated by 20 minutes exposure to 3 mg l^{-1} free available chlorine.

Botulinum spores are found worldwide in soil and can germinate into vegetative bacteria that produce toxins during anaerobic incubation (Popoff, 1995). Naturally occurring animal and human disease arises from exposure to spores that germinate and produce BoNT *in vivo,* or from ingestion of preformed BoNT in edible or potable materials. Botulinum is the first biological toxin to be licensed for treatment of human disease such as cervical torticollis, strabismus, blepharospasm associated with dystonia, and cosmetically to treat wrinkles. Medical uses do not pose a bioterrorist threat because the amounts dispensed are far below a lethal dose.

Botulinum toxin was one of the first agents to be weaponized by the USA in the now-defunct offensive bioweapons programme begun during the Second World War and ending in 1970 (Arnon *et al*., 2001). Since then several countries and terrorist groups have used the toxin as a weapon. Evidence from the United Nations indicates that in 1995, Iraq had filled and deployed over 100 bombs with approximately 10 000 l of botulinum toxin (United Nations Security Council, 1995), although these weapons were destroyed prior to the US invasion of Iraq. The Japanese cult Aum Shinrikyo manufactured and attempted to disseminate botulinum toxin obtained from soil in Tokyo prior to their 1995 sarin nerve gas attack on the Tokyo subway (Tucker, 2000). The attacks failed because of faulty microbiological technique and inadequate aerosol-generating equipment (Arnon *et al*., 2001).

2.2 Description

C. botulinum produces neurotoxins that are the most toxic substances, per weight of agent, known to man, requiring only one nanogram per kilogram of body weight to kill 50% of the animals exposed. Botulinum toxin type A is 15 000 times more toxic by weight than the well-known organophosphate nerve agent VX, and 100 000 times more toxic than sarin (US Army Medical Research Institute of Infectious Diseases, 2005). BoNT is a protein with a molecular mass of approximately 150 kDa. The incubation period and severity of botulism depend on the rate and amount of toxin absorbed. Symptoms of food-borne disease may begin between 2 hours to 8 days after exposure (typically 12–72 hours) (Koenig *et al*., 1967). Timing of the onset of symptoms in inhalational exposure is difficult to estimate because of inadequate data; however, monkeys showed signs of botulism 12–80 hours after exposure (Franz *et al*., 1993). The incubation period might be considerably shorter after an aerosolized release of large amounts of BoNT.

C. botulinum is a classification that is applied to four diverse groups of organisms that produce similar neurotoxins (Aureli *et al*., 1986). They are anaerobic, motile by peritrichous flagella and are initially Gram positive but are often Gram negative or Gram variable after longer growth. When cultured anaerobically on blood agar, they are greyish-white translucent, round colonies with irregular borders that are encircled by a thin zone of partial haemolysis (Popoff, 1995). The four groups of *C. botulinum* are based on phenotypic physiologic characteristics (Hatheway and Bartlett, 2004; Popoff, 1995), and all are proteolytic. Group I organisms are proteolytic, producing BoNT/A and some produce BoNT/B or BoNT/F. Group II organisms are nonproteolytic and have a lower optimal growth temperature and lower maximum temperature of spore resistance than other groups. All BoNT/E producing strains are in Group II along with some that produce BoNT/B and BoNT/F. Group III organisms produce BoNT/C and BoNT/D but are distinguished from Groups I and II by their production of propionic acid as a product of fermentation. Group IV organisms lack any sugar fermentation or lipase production, but are proteolytic. BoNT/G is produced only by group IV (Greenfield *et al*., 2005).

Botulism, the disease caused by the BoNT has historically been a by-product of unsuccessful food preservation. *C. botulinum* grows and produces the neurotoxin in the anaerobic conditions encountered in home canning and other forms of food preservation such as smoking. The term *botulism* derives from the Latin for sausage, *botulus,* because frequent outbreaks in Germany resulted from making sausage by stuffing pig's stomach and then smoking it (Erbguth and Naumann, 2000). The spores can only be inactivated by heating to at least 85°C for 5 minutes.

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Botulinum toxin is a simple dichain polypeptide made up of a 100 kDa 'heavy' chain joined by a single disulfide bond to a 50 kDa 'light' chain (Lacy *et al*., 1998). The toxin's light chain is a Zn^{2+}-containing endopeptidase that blocks acetylcholine-containing vesicles from fusing with the terminal membrane of the motor neuron, resulting in flaccid muscle paralysis (Montecucco, 1995). The mechanism of action is described by Arnon *et al*. (2001) in a consensus paper.

Release of acetylcholine at the neuromuscular junction is mediated by the assembly of a synaptic fusion complex that allows the membrane of the synaptic vesicle containing acetylcholine to fuse with the neuronal cell membrane. The synaptic fusion complex is a set of SNARE proteins, which include synaptobrevin, SNAP-25 and syntaxin. After membrane fusion, acetylcholine is released into the synaptic cleft and then bound by receptors on the muscle cell. Botulinum toxin binds to the neuronal cell membrane at the nerve terminus and enters the neuron by endocytosis. The light chain of botulinum toxin cleaves specific sites on the SNARE proteins, preventing complete assembly of the synaptic fusion complex and thereby blocking acetylcholine release. Botulinum toxins types B, D, F and G cleave synaptobrevin; types A, C and E cleave SNAP-25; and type C cleaves syntaxin. Without acetylcholine release, the muscle is unable to contract. SNARE stands for 'soluble NSF-attachment protein receptor'; NSF for '*N*-ethylmaleimide-sensitive fusion protein'; and SNAP-25, 'synaptosomal-associated protein of 25 kDa'.

2.3 Clinical Disease

Symptoms of botulism include difficulty seeing, speaking and/or swallowing. Neurological findings include ptosis, diplopia, blurred vision, often enlarged or sluggishly reactive pupils, dysarthria, dysphonia and dysphagia (Arnon *et al*., 2001; Franz *et al*., 1997; Shapiro *et al*., 1998; US Army Medical Research Institute of Infectious Diseases, 2005). Because of peripheral parasympathetic cholinergic blockage, the mouth may be dry and the pharynx injected. There are few sensory changes except infrequent circumoral and peripheral paraesthesias from hyperventilation resulting from fright. Extension of paralysis beyond the bulbar musculature results in loss of head control, hypotonia and generalized weakness (Arnon *et al*., 2001). Gastrointestinal symptoms of nausea, vomiting, diarrhoea or constipation and abdominal cramps may be present with food-borne exposure, but occur in only a little over half of the patients observed (Arnon *et al*., 2001). In the absence of secondary infection, fever is not present (Arnon *et al*., 2001; Bleck, 2000). Patients do not exhibit confusion or obtundation because BoNT does not pass the blood–brain barrier. The lethal dose of botulinum toxin in humans has been estimated at 0.09–0.15 μg intravenously, 70 μg orally and 0.70–0.90 μg inhalationally (Schantz and Johnson, 1992). In untreated patients death results from airway obstruction and inadequate tidal volume.

Some patients may be mildly affected and others so paralysed that they appear comatose and require months of ventilatory support. Severity and time of onset depend on dose. Recovery results from new motor axon twigs that sprout to reinnervate paralysed muscle fibres (Duchen, 1972; Mann *et al*., 1981).

A mouse bioassay that demonstrates BoNT in blood or stool neutralized by antisera is used for confirmatory diagnosis; however the test takes several days to complete (Middlebrook and Franz, 1997).

Mortality from food-borne botulism is approximately 6% (CDC, 1998). In surviving patients, paralysis requiring fluid and nutritional support, assisted ventilation and treatment of complications can last for weeks to months (Arnon *et al*., 2001). Therapy consists of supportive care and passive immunization with equine antitoxin. If passive neutralizing antibodies are administered early, nerve damage and severity of disease will be minimized but existent paralysis will not be reversed (Tacket *et al*., 1984). A pentavalent toxoid of *C. botulinum* toxin types A, B, C, D and E is available as an IND (investigational new drug) for pre-exposure prophylaxis, but is not widely available.

3 RICIN TOXIN

3.1 Introduction

Ricin toxin is derived from the bean of the castor oil plant *Ricinus communis*. It is one of the most toxic plant toxins, classified as a Category B bioterrorism agent and a Schedule Number 1 chemical warfare agent. Ricin is a likely agent for use in biological warfare or as a terrorist agent because of its toxicity, heat stability, relative ease in acquiring the castor bean, and the fact that the toxin can be extracted with little difficulty through a salting-out process (Wannemacher *et al*., 1992), and large quantities can be produced with low-technology equipment (US Army Medical Research Institute of Infectious Diseases, 2005). The toxin can be delivered by aerosol, ingestion and injection. Although the median lethal dose for ingestion in mice is $30\,mg\,kg^{-1}$, it is only $3–5\,\mu g\,kg^{-1}$ for inhalation, a 1000-fold difference (Audi *et al*., 2005). As little as $20\,\mu g\,kg^{-1}$ was a lethal inhalation dose in mice (Wong *et al*., 2007). If ricin was released outdoors, it would require a quantity that would be difficult for a terrorist to amass; however, an indoor release would require a much smaller quantity. It is stable under ambient conditions, can be detoxified

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after 10 minutes at 80°C, inactivated by 100 mg l^{-1} free available chlorine, and decontaminated with soap and water (US Army Medical Research Institute of Infectious Diseases, 2005).

Over 1 000 000 tonnes of castor beans are processed annually. The waste mash is 3–5% ricin by weight. Humans can be naturally exposed to the toxin through the consumption of the castor bean. The US Chemical Warfare Service began studying the weaponization of ricin near the end of the First World War and this continued during the Second World War (Cookson and Nottingham, 1969). It was only in the 1990s after ricin was believed to be a potential terrorist weapon that work began on therapies and prophylactic measures (Franz and Jaax, 1997). Recently, ricin has been used by several extremist groups (Sharn, 1995; Goodman, 1996), and was mailed to a South Carolina postal facility (Schier *et al*., 2007).

3.2 Description

Ricin is a globular protein of 66 kDa that when purified is a water-soluble white powder (Parker *et al*., 1996). The toxic heterodimer consists of a 32 kDa A-chain that is bonded to a 32 kDa B-chain (Robertus, 1988). Both chains must be associated for toxicity. Both the A- and B-chains are globular proteins, with the A-chain tucked into a gap between two domains of the B-chain (Franz and Jaax, 1997). It is in a family of compounds that includes diphtheria, cholera, shiga and pseudomonas toxins (Kotb and Bronze, 2005). The toxicity of ricin varies between different animal models and route of exposure (Balint, 1974). Oral toxicity is 1000 times less than inhalational toxicity because of poor absorption of the toxin from the gastrointestinal tract (Franz and Jaax, 1997). Ricin's toxicity requires an association between both its catalytic A subunit and its B subunit. The ricin A-chain is a *N*-glycosidase that depurinates adenine, making the depurinated RNA susceptible to hydrolysis. The modified ribosomes are then unable to support protein synthesis, resulting in the toxicity.

The B-chain of ricin can bind to galactosides of cell-surface carbohydrates, while the A part is enzymatically active. The B-chain facilitates the transport of the A-chain into the cytosol (Youle and Neville, 1982). After entering the cytoplasm, the A-chain enzymatically attacks the 28S ribosomal subunit, causing cell death in 8–24 hours *in vivo* (Franz and Jaax, 1997). The endocytotic uptake of the protein is initiated by the binding of the B-chain to glycoside residue on the carbohydrate-containing protein and glycolipids (Franz and Jaax, 1997). The Golgi apparatus is involved in the pathogenesis of ricin intoxication (Gonatas *et al*., 1998).

3.3 Clinical Disease

The clinical signs and symptoms of ricin toxicity vary with the dose and route of exposure (Olsnes and Pihl, 1982); however ricin is deadly by all routes of exposure (Kotb and Bronze, 2005). Most of the information that we have on signs and symptoms comes from animal studies; there are limited data from human poisonings except from assassination attempts (Crompton and Gall, 1980) using injected ricin, and from children and adults ingesting castor beans (Rauber and Heard, 1985). Inhaled ricin will probably result in respiratory distress and airway and pulmonary lesions within 8 hours of exposure (Franz and Jaax, 1997). Symptoms might include cough, dyspnoea, arthralgias and fever (Audi *et al*., 2005). From 12 to 30 hours after exposure, inflammatory cells and total protein counts increase. Ricin binds to ciliated bronchiolar epithelial cells, alveolar lining cells and alveolar macrophages (Franz and Jaax, 1997). Toxicity increases with dose, and death occurs from severe hypoxaemia resulting from pulmonary oedema and alveolar flooding (Kotb and Bronze, 2005).

As indicated above, ricin is much less toxic by oral ingestion; however, eating 3–20 beans can be fatal (Bradberry *et al*., 2003; Franz and Jaax, 1997). Ingestion of sufficient quantities of ricin usually results in abdominal pain, nausea, vomiting, diarrhoea, dehydration, decreased urine production, gastrointestinal haemorrhage, hypotension, haemolysis, and necrosis of the liver, spleen and kidneys, vascular collapse and shock (Bradberry *et al*., 2003; Franz and Jaax, 1997; Kotb and Bronze, 2005). Other symptoms may include fever, sore throat, thirst, headache, confusion, convulsions and papillary dilation (Bradberry *et al*., 2003). Death in fatal cases is the result of multiorgan failure.

Information on injected ricin is quite limited, but in these limited studies, injections of high doses result in local lymphoid necrosis, liver necrosis, gastrointestinal haemorrhage, diffuse nephritis, diffuse splenitis (Franz and Jaax, 1997), nausea, vomiting, fever, chest and abdominal pain, myalgias and headache (Crompton and Gall, 1980; Fine *et al*., 1992; Targosz *et al*., 2002). Death is due to multiorgan failure.

Treatment for ricin poisoning by all routes is primarily supportive. It includes intravenous fluid and vasopressors for hypotension (Audi *et al*., 2005).

4 TRICHOTHECENE MYCOTOXINS

4.1 Introduction

Mycotoxins are a group of compounds that are products of fungal metabolism. They have been associated with disease in humans and animals that have

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eaten fungus-infected agricultural products (Ciegler and Bennett, 1980; Sudakin, 2003). Mycotoxins are usually associated with terrestrial filamentous fungi commonly called moulds (Moss, 1989; Bennett and Klich, 2003). Mycotoxins that are of primary concern for causing disease are aflatoxins, rubratoxins, ochratoxins, fumonisins and trichothecenes (Wannemacher and Wiener, 1997; Ciegler and Bennett, 1980). They have acute, chronic, carcinogenic and teratogenic toxicity. This toxic effect is called mycotoxicosis (Peraica *et al*., 1999; Pitt, 2000).

The trichothecene mycotoxins are a group of 40 compounds that are the most likely mycotoxins to be used as a bioweapon against humans (Etzel, 2002; Zapor and Fishbain, 2004; Madsen, 2001; Holstege *et al*., 2007); therefore, we will limit further discussion to this group of toxins. They are of small molecular weight and are stable in the environment (US Army Medical Research Institute of Infectious Diseases, 2005), are active in small amounts, and can be delivered as dusts, droplets and aerosol from a variety of dispersal devices (Kotb and Bronze, 2005; Wannemacher and Wiener, 1997). They are the only biologically active toxins that are effective through dermal exposure. They are, however, maximally effective through the respiratory and gastrointestinal portals. There is evidence that mycotoxins were used as a bioweapon by the former Soviet Union in aerial attacks in Laos, Kampuchea and Afghanistan in the 1970s, and much of our information on human exposure derives from these attacks that were described as 'yellow rain'. There is still some controversy about this contention; however, there is substantial epidemiological and intelligence information to support the claim of use of trichothecenes in Southeast Asia and Afghanistan (Ember, 1984; Wannemacher and Wiener, 1997). This evidence indicates that trichothecenes are an effective bioweapon because they can be delivered by aerosol, can be produced in large quantities, and in powder and liquid forms that are stable when exposed to air, light and UV radiation (Committee on Protection Against Mycotoxins, Board on Toxicology and Environmental Health Hazards, Commission on Life Sciences, National Research Council, 1983). They also require high heats, over 580°C for 10 minutes or 260°C for 30 minutes for complete inactivation (Wannemacher and Wiener, 1997). Although past use has been outdoors, they can also serve as an effective terrorist agent that can be used in smaller quantities indoors.

4.2 Description

Trichothecenes are nonvolatile proteins of low molecular weight, between 250 and 550 Da (Cole and Cox, 1981), that can be absorbed slowly through skin but rapidly from the gastrointestinal tract and pulmonary mucosal surface. The major organ for metabolism is the liver (Matsumoto *et al*., 1978); however other tissues are capable of metabolizing the toxin (Wannemacher and Wiener, 1997). Carboxylesterase is important for detoxifying trichothecenes (Johnsen *et al*., 1986). The metabolized toxin is excreted in the urine and faeces but very little of the parent trichothecenes is excreted intact; they are eliminated via detoxification by biotransformation (Wannemacher and Wiener, 1997).

Trichothecenes have low solubility in water but are highly soluble in organic solvents (Cole and Cox, 1981). When extracted, they form a yellow-brown liquid that forms a greasy, yellow crystalline product upon evaporation. They rapidly inhibit protein synthesis (Zapor and Fishbain, 2004), and are toxic to eukaryotic cells (Wannemacher and Wiener, 1997). Trichothecenes act by inhibiting the initiation or the elongation process of translation and possibly by interfering with peptidyltransferase reactions (Wannemacher and Wiener, 1997). The primary toxic effects of the trichothecenes are caused by their ability to inhibit protein synthesis and the activation of the stress response that synergistically induces apoptosis and cell death (Pestka and Bondy, 1994). They are particularly cytotoxic to rapidly dividing cells such as in the bone marrow, gastrointestinal tract, skin and germ cells. These cytotoxic effects are referred to as radiomimetic because they imitate the haematopoietic and lymphoid effects of radiation sickness. They also have an immunosuppressive effect that increases susceptibility to infections (Kotb and Bronze, 2005), and do not appear to require metabolic activation prior to exerting their biologic effect (Busby and Wogan, 1981).

4.3 Clinical Disease

Unlike many toxic materials, a number of toxic responses are similar for all routes of exposure to trichothecenes, but can vary depending on whether the exposure is acute or chronic (Wannemacher and Wiener, 1997). The most likely mycotoxins to cause human disease are the T-2 mycotoxins (Kotb and Bronze, 2005). Acute exposure is most likely with warfare or a terrorist attack, and symptoms can begin within minutes to hours (Henghold, 2004). In these situations, T-2 can penetrate the skin, be inhaled, or be ingested, causing a multitude of symptoms (Nelson *et al*., 1994; Peraica *et al*., 1999; Hussein and Brasel, 2001; Etzel, 2002; Sudakin, 2003).

All routes of exposure produce gastric and intestinal lesions. Haematopoietic and immunosuppressive effects are radiomimetic (Wannemacher and Wiener, 1997). Central nervous system toxicity results in anorexia, lassitude, nausea and vascular effects resulting in hypotension and shock. In addition to these common symptoms,

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there are specific symptoms for dermal, oral, gastrointestinal and ocular exposure. Dermal symptoms such as burning pain, redness, tenderness, blistering and skin necrosis frequently begin within minutes of exposure (Henghold, 2004). Exposure to the mouth and throat causes pain and bleeding and, if swallowed, gastrointestinal toxicity resulting in abdominal pain, nausea, vomiting and bloody diarrhoea. Symptoms of ocular exposure include pain, burning, conjunctivitis, tearing and impaired vision. Inhalation results in nasal pain, itching and rhinorrhoea; the throat will be painful with possible voice change; bloody cough, low-grade fever, dyspnoea, wheezing and chest pain are frequent (Wannemacher and Wiener, 1997; Etzel, 2002). Systemic toxicity occurs via all routes of exposure. Symptoms include dizziness, loss of coordination, weakness and prostration. Death can occur in minutes, hours or days and is frequently accompanied by hypothermia, hypotension and tachycardia.

All therapy for mycotoxin toxicity is supportive, including respiratory support. There is no specific antidote or therapeutic regimen available. If ingested, standard poison therapy includes use of superactivated charcoal to absorb the toxin.

5 STAPHYLOCOCCAL ENTEROTOXIN B

5.1 Introduction

Staphylococcal Enterotoxin B (SEB) is produced by the *Staphylococcus aureus* bacterium (Jay, 1992). SEB is an exotoxin because it is excreted by the organism. It normally affects the intestines and therefore is referred to as an enterotoxin (Le Loir *et al*., 2003). SEB is one of the pyrogenic toxins that are produced by unrefrigerated meats, dairy and bakery products. It is the second most common source of food-borne outbreaks. Inhalation of SEB does not occur naturally and should be considered an act of terrorism. Most likely, an aerosol attack would occur in a closed environment such as a theatre, sports arena, conference centre or subway. Although an attack would not likely produce significant mortality, it could render 80% or more of exposed people incapacitated for one to two weeks (US Army Medical Research Institute of Infectious Diseases, 2005; Henghold, 2004). Symptoms from inhalation appear at doses 100 times less than the lethal dose (US Army Medical Research Institute of Infectious Diseases, 2005).

SEB was one of the biological agents stockpiled by the USA until 1969 (Ulrich *et al*., 1997). Because it could produce significant incapacitation after inhalation, SEB was considered valuable for military uses (Franz *et al*., 1997). It is also a potential terrorist agent if used in large-enough quantities to cause death.

5.2 Description

SEB is one of seven enterotoxins produced by strains of the *S. aureus* bacteria. It is a clump-forming, Gram-positive cocci that consists of 239 amino acid residues and has a molecular mass of 28 kDa (Ulrich *et al*., 1997; Johns and Khan, 1998). SEB is a relatively stable protein that is soluble in water. SEB production can be inhibited by sodium chloride and acetic acid; and alkaline conditions can also decrease production of SEB (Le Loir *et al*., 2003). It can withstand temperatures of up to 100°C. The effective dose (ED_{50}) for an aerosol release is $0.0004\,\mu g\,kg^{-1}$, and the lethal dose (LD_{50}) is $0.02\,\mu g\,kg^{-1}$ (Henghold, 2004).

The primary effects of SEB are mediated stimulation of T lymphocytes. SEB binds to the major histocompatibility complex (MHC) class II proteins, resulting in the stimulation of the proliferation of large numbers of T lymphocytes. It belongs to a class of immune stimulants referred to as 'superantigen's. They can bridge the MHC II on the antigen-presenting cells, and the T cell receptors on the CD4- and CD8-T cells (Kotb, 1998). This bridging results in a cascade of pro-inflammatory cytokines (interleukin-6, tumour necrosis factor-alpha and interferons) responsible for the systemic effects of the toxin (Proft and Fraser, 2003; Stiles *et al*., 1993). The gastrointestinal disease is, however, associated with histamine and leukotriene release from mast cells (Scheuber *et al*., 1987). Lethality of the pyrogenic toxins may be enhanced by endotoxin from Gram-negative bacteria (Stiles *et al*., 1993).

The cytokines cause a recruitment of additional immune effector cells, and the counter-regulatory feedback loops become poorly activated. Therefore, it is the body's own inflammatory response that mediates many of the toxic effects of SEB. The pulmonary oedema sometimes reported with aerosol exposure is more than likely secondary to T cell proliferation within the respiratory mucosa (Ulrich *et al*., 1997; Stiles *et al*., 1993).

5.3 Clinical Disease

There are markedly different clinical signs and symptoms when SEB is inhaled compared to when ingested. Gastrointestinal SEB toxicity is generally debilitating but can be fatal (Schlievert *et al*., 2000). The incubation period for ingestion is 4–6 hours (Bergdoll, 1985). Symptom onset is usually abrupt and includes intense nausea, vomiting, acute salivation, malaise, abdominal pain and diarrhoea. Physical examination may show hypotension, hyperperistalsis, tachycardia and diffuse nonlocalizing abdominal pain. Fever is generally present in gastrointestinal SEB toxicity. Most cases resolve in 8–24 hours.

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There is little data available on the morbidity and mortality of inhalational SEB. Most of our information comes from studies on rhesus monkeys, other animals, and a small number of accidentally exposed laboratory workers (Ulrich *et al*., 1997; Rusnak, 2004). Symptoms associated with inhalation would most likely appear within 3–12 hours and include anorexia, emesis, diarrhoea and mastication. After 48 hours, symptoms can progress to lethargy, dyspnoea and facial pallor (Ulrich *et al*., 1997). Gastrointestinal symptoms may accompany inhalation due to swallowing of the toxin after mucocilliary clearance. On physical examination, patients are acutely short of breath and have substernal severe chest pain. Unlike gastrointestinal toxicity, patients may have a fever up to 41°C. Disease may progress to respiratory failure from pulmonary oedema caused by systemic inflammation that might lead to death. In general, evidence indicates that SEB is a potent immune stimulant and that the intense stimulation of T cells plays an important role in mediating the pathology of the disease (Ulrich *et al*., 1997).

Treatment is limited to supportive care that might include artificial ventilation and pain control.

6 SUMMARY

Protecting people from biological toxins that might be used by states or terrorist groups requires more research on rapid diagnostic methodologies to improve our ability to rapidly treat victims of toxin release. It is equally important that we develop improved vaccines, antitoxins and therapies to provide prophylactic and treatment alternatives if intelligence indicates an increased likelihood of the use of a specific toxin (Middlebrook, 2005). Although the toxins discussed in this chapter are the ones that current knowledge indicates are the most likely to be used, we must also keep our minds open to the possibility that there are other toxins that might also be used as bioweapons. Finally, we must be aware that innocuous microorganisms can be genetically altered to produce a toxin that is even more lethal than those naturally available.

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Radiation Terrorism

Robin Beverly McFee

CONTENTS

1 INTRODUCTION

Hospitals and healthcare professionals (HCPs) are symbolic of public preparedness, healthcare leadership and safety within a community. The public hold healthcare facilities (HCFs) in esteem and hold high expectations that when a crisis occurs, hospitals and clinicians will be ready to respond and deliver appropriate care. Yet, the medical response to radiation—whether the result of radiological warfare, terrorist deployment of improvised radiation dispersal weapons, political assassination, occupational or industrial accidents, or the medically radiated patient—remains one of the least taught among all disciplines within medical education and least emphasized in non-nuclear designated facilities. This is in spite of a generation raised on the Cuban Missile Crisis, Three Mile Island and Chernobyl!

Research continues to demonstrate that many HCFs lack even the most basic and necessary antidotes suggested by the toxicology community (McFee *et al*., 2005; Geller *et al*., 2003; Waselenko *et al*., 2004; Leikin *et al*., 2003). If the most commonly used antidotes are inadequately stocked, what can be said about the supply of medications critical to treat WMD, especially radiation toxicity?

Going one step further, even within professional preparedness circles and in the aftermath of 9/11, when government initiatives focussed on medical vulnerabilities to toxicant threats, of all the categories of weapons of mass destruction (WMD)—whether using the CBRNE (chemical, biological, radiological, nuclear, explosive) or NBC (nuclear, biological, chemical) acronym—radiation is the least taught in professional schools, responder cultures or civil preparedness organizations. Yet a radiological event remains considered to be one of the most significant preparedness vulnerabilities, especially in terms of knowledge about and responding to such an emergency. To date, few HCPs—prehospital (emergency medical services (EMSs)) or hospital-based—possess the fundamental knowledge or skills to identify, let alone treat, a radiation victim; this vulnerability is made even more obvious in the aftermath of the high-profile assassination of former Russian agent Alexander Litvinenko. He was poisoned with polonium-210 (Harrison *et al*., 2007; McFee and Leikin, 2009; 2008; Stather 2007; International Atomic Energy Association (IAEA), 2009a).

Moreover, most HCPs and first responders consider themselves inadequately prepared for radiation events. Consider a recent online poll conducted by the *Journal of Emergency Services* (*JEMS*). The question was posed 'do you feel prepared to handle victims of a dirty (radioactive material) bomb?' (McFee and Leikin, 2008). Of the 246 respondents, 82% replied 'NO!' Compound this with the fact that most HCFs remain underprepared—from training to detection equipment to appropriate management materials. Since clinicians at HCFs are often insulated from the environmental circumstances or actual event, EMS can offer important insights; better training can enhance their ability to assist HCPs, especially emergency department (ED) personnel if a radiological event occurs.

Clearly, not all radiological events will be obvious. Nuclear materials or radiation-based weapons, without an explosion, may not be readily considered. Radiation is odourless, tasteless. An index of suspicion in the appropriate context will enhance the likelihood of considering and ultimately properly responding to such an exigency.

Mass or individual exposures to radiation present unique challenges to the entire response continuum from law enforcement, first responders and emergency medical care (Leikin *et al*., 2003).

For the prehospital responder, who often is the only medical provider with access to or first-hand experience of the event, recognition of signs and symptoms of exposure, focussing on the appropriate decontamination of patients and observing the entry/no-entry rules concerning hot, warm and cold zones is essential. Enhanced training should be initiated in the diagnosis, management and follow up of radiation among prehospital and emergency department professionals (Leikin *et al*., 2003; Flynn and Goans, 2006; Walter *et al*., 2000; 2003; NCRP, 2001; Thomas and Walter, 2008; National Institute of Occupational Safety and Health, 2009). As such, it is critically important for the first responder to convey as much information as possible to the receiving HCF. Self-protection of rescue personnel and reducing the risk to bystanders, as well as patients, is of paramount concern.

Most major metropolitan areas have designated nuclear-response hospitals, which are staffed and equipped to handle such an event. However, a radiation incident may occur closer to a rural or community hospital rather than a tertiary care or designated specialty facility. Consider the transit routes that radioactive wastes and medical radioactive materials traverse nationwide, and it is readily apparent that an incident en route could occur in any type of area—isolated or urban.

Given that radioactive substances are ubiquitous—from university, government and industrial research facilities to energy plants and military installations—it is likely that additional resources may be regionalized. However, in the immediate aftermath of an event—intentional or accidental—first response may be inadequately equipped or trained before transport is possible to advanced care. With radiation sources being in or transported through virtually every region nationwide, it is essential to increase preparedness, even among community and rural HCFs.

Managing radiation injuries effectively requires access to specialized equipment and expertise (Leikin *et al*., 2007; McFee and Leikin, 2005; 2008; 2009; Stather 2007; International Atomic Energy Association (IAEA),

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2009a; Jarrett, 1999; Christensen *et al*., 2008a; 2008b; Berger *et al*., 2006; Goans and Waselenko, 2005; Goans, 2007; Koenig *et al*., 2005; Ricks and Fry, 1990; Ricks *et al*., 1990; 2002; Schleipman *et al*., 2004; NCRP, 1979; REAC/TS, 2009; Fliedner *et al*., 2001; Gusev *et al*., 2002). Once at the HCF, clinicians must realize patients with radiation injuries can suffer from complex patterns of injury, reflective of the tissue and organs damaged, as well as trauma. Radiation sickness is progressive and may require acute, critical and long-term care throughout the course of illness.

Regardless of the source, preparedness rests upon acknowledging a threat exists and dedicating the resources to address the risks, including the enhancement of training and equipment. Accepting a threat exists suggests we go beyond thinking in terms of a nuclear holocaust or Chernobyl-like meltdown, to include dirty bombs or industrial incidents.

In this chapter you'll be introduced to the critical information you need to prepare for, diagnose and manage victims of radiation/nuclear events, assist your facility in identifying vulnerabilities, including training gaps, as well as opportunities to enhance awareness about the security of radioactive materials that might be easily stolen. Various threat scenarios, as well as well-documented events that have involved radiation will be discussed. Additionally, the type of equipment, agencies, experts and resources available to assist you in radiation response and other key considerations necessary to enhance your ability to respond and manage these patients will be covered in this chapter.

2 THE GLOBAL NUCLEAR THREAT

The chilling reality is that nuclear materials and technologies are more accessible now than at any other time in history.

Source: Former Director—US Central Intelligence Agency, John Deutch

Few threats evoke images of horror and raise as much concern as nuclear weapons or radiation events. The proliferation of nuclear and radiological materials globally, and the technology to create radiation-based weapons is evolving and spreading. Consider the growing group of nations in possession of nuclear weapons and others pursuing dual-use programmes—for energy and weapons. Numerous suitcase-sized one kilotonne thermonuclear weapons reportedly are missing and unaccounted for from the former Soviet Union. Concerns persist that they will appear on the black market and in the hands of terrorists or rogue nations (Graham and Talent, 2008). Terrorist groups, including Aum Shinrikyo—the cult that already demonstrated the willingness *and* ability to harm people in a mass casualty incident in Tokyo using the nerve agent sarin, resulting in more than 5500 people requiring medical treatment, and several deaths—Al-Qaeda and other terrorist groups, as well as Chechnyan extremists, have expressed interest in obtaining, have tested and attempted to deploy various types of radiological weapons.

The devastation from a nuclear device was clearly demonstrated when, in 1945, a 15 kilotonne weapon (by today's standards an improvised nuclear device (IND)) detonated over Hiroshima, Japan. In the blink of an eye there were an estimated 150 000+ casualties and 75 000 fatalities, thus illustrating the power resulting from unleashing nuclear energy weapons (Graham and Talent, 2008; International Atomic Energy Association (IAEA), 2009a; Jarrett, 1999).

Although the possession of nuclear weapons, even improvised ones, can dramatically alter the balance of power in a region (think North Korea, or India and Pakistan, or the potential for Iran to become a nuclear weapons nation) one of the primary reasons terrorists may elect to use radiological weapons is not necessarily to cause immense physical damage or loss of life, but to create widespread panic, psychological fear and undermine civilian authorities (Graham and Talent, 2008). The mere mention of a potential radiological release could cause significant and immediate public concern. Consider the number of inquiries the British Authorities received in the aftermath of the Litvinenko event (Harrison *et al*., 2007; McFee and Leikin, 2008; 2009; Stather 2007; International Atomic Energy Association (IAEA), 2009a).

In the December 2008 report to the United States President and Congress *World At Risk* by the Commission on the Prevention of Weapons of Mass Destruction Proliferation and Terrorism, 'unless the world community acts decisively and with great urgency, it is more likely than not that a weapon of mass destruction will be used in a terrorist attack somewhere in the world by the end of 2013'. The Commission further believes that terrorists are more likely to be able to obtain and use a biological weapon than a nuclear weapon. But they hasten to caution, nuclear materials are increasingly unaccounted for worldwide; worrisome since certain terror groups have expressed interest in deploying a radiological or nuclear weapon (Graham and Talent, 2008).

According to the US Secretary of Defense Robert Gates, 'every senior leader, when you're asked what keeps you awake at night, it's the thought of a terrorist ending up with a weapon of mass destruction, especially nuclear'.

The WAR report suggests unsecured materials, weapons trafficking and the ability for 'dual use' of nuclear materials—items that can be used in civilian energy or weapons - is an increasing threat worldwide.

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On 28 October 2008, Dr Mohamed El Baradei, the Director General of the International Atomic Energy Agency (IAEA), stood at the rostrum of the United Nations General Assembly and warned the world about nuclear terror (International Atomic Energy Association (IAEA), 2009b). 'The possibility of terrorists obtaining nuclear or other radioactive material remains a grave threat,' said Dr El Baradei. 'The number of incidents reported to the Agency involving the theft or loss of nuclear or radioactive material is disturbingly high,' he said. 'Equally troubling is the fact that much of this material is not subsequently recovered. Sometimes material is found which had not been reported missing'.

In every terrorist strike anywhere in the world, to every innocent life lost must be added thousands more who were just hours away from having been at that ground zero—in those moments of danger, we are all, first and foremost, citizens of a WAR, with the common cause of protecting the innocent and preserving our way of life.

According to National Intelligence Estimates by the US, as well as security evaluations from Interpol and other global watch-dog agencies, there is high likelihood of increasing numbers of nuclear nations, including North Korea and probably Iran in the not distant future, as well as increased trafficking of radiological materials—for legal and peacetime uses and black-market enterprises.

The future stability of Pakistan is a significant source of concern as a nuclear nation, that is, increasing its arsenal; their ability to secure those weapons in an unstable and dangerous region remains unknown. In a 2007 poll sponsored by the well-respected magazine *Foreign Policy*, among 117 terrorism experts queried about nuclear threats, 74% considered Pakistan the country most likely to transfer nuclear technology to terrorists in the next three to five years.

According to the National Nuclear Security Administration, Russia is a great concern as a source for radioactive materials. Then President, Vladimir Putin, admitted there were several suitcase thermonuclear weapons unaccounted for after the fall of the Soviet Union and international inspectors raise concern that some fissile and radioactive materials remain poorly secured in certain regions of Russia.

The availability of radioactive and nuclear materials to terrorist organizations is widely considered both a grave international threat and realistic exigency. It is well recognized that instructions on how to make a crude radiological or nuclear weapon are available on the Internet. The expertise is also available. The 'x' factor—and final, lethal step to successfully developing a viable weapon, is acquiring the appropriate radioactive materials.

Though beyond the scope of this text, while numerous agencies operating out of the United Nations and individual nations are tasked with controlling the spread of nuclear materials, it is important to realize that radioactive sources are in HCFs and universities, largely poorly secured, and provide an opportunity for theft and crude weapons development.

From a security, military perspective, **Table 1** lists the high-value radioactive materials (Jarrett, 1999).

Over the years there have been numerous industrial and criminal events, as well as terrorist actions and foiled attempts to disperse radioactive materials (**Table 2**).

2.1 Nuclear Terrorism: Radiation Threat Vulnerabilities

Nuclear terrorism is defined as the use of radioactive material in various forms to produce maximum disruption, panic, injury and fear in the general population (McFee *et al.*, 2004; Timins and Lipoti, 2003; Jarrett, 1999; NTI and The Center for Nonproliferation Studies at the Monterey Institute of International Studies, 2004).

Table 1 Threat-potential radioactive materials (Jarrett, 1999; Christensen *et al.*, 2008b)

Radioactive material	Radionuclides	Type of ionizing radiation
Americium	Am^{241}	Alpha, detectable by FIDLER from gamma. Heavy metal toxicant
Caesium	Cs^{137}	Beta and gamma
Cobalt	Co^{60}	Beta and gamma
Depleted uranium	DU	Alpha, beta and gamma (an inhalation and wound threat more than an emission threat. Absorption based upon chemical state of uranium)
Iodine	$I^{131,132,134,135}$	Beta and gamma
Phosphorus	P^{32}	Beta
Plutonium	$Pu^{238,239}$	In pure form alpha; when contaminated with americium it will have a detectable X-ray with the use of thin walled gamma probe.
Tritium	H^3	Beta emitter
Uranium	$U^{238,235,239}$	Alpha, beta and gamma

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Table 2 Worldwide events associated with radiological/nuclear materials (McFee and Leikin, 2005; ED Management, 2002; Timins and Lipoti, 2003; Jarrett, 1999; NTI and The Center for Nonproliferation Studies at the Monterey Institute of International Studies, 2004)

Date	Location	Event
1987	Iraq	Allegedly tests radiological weapon. Does not generate high radiation levels; considered a failure.
	Brazil	The worst radiation incident in the Western Hemisphere. A source containing 1375 curies of ^{137}Cs is removed from a medical unit and its shield by scavengers. Numerous people are exposed. More than 100 000 individuals are screened, 249 are contaminated and four deaths and numerous hospitalizations result.
1991	Russia	Authorities are warned of a planned Chechnyan attack on a nuclear power plant.
1993	Russia	The Russian Mafia allegedly places gamma-emitting pellets in the office of a Moscow businessman, who subsequently dies.
1995	Russia	Chechnyan rebels bury a container with ^{137}Cs in a Moscow park.
1996	United States	Three individuals are arrested after discovery of their plot to place radium in the food, cars and toothpaste of Long Island politicians.
	Russia	Authorities are warned of a planned Chechnyan attack on a nuclear power plant (Balakovo).
1998	United States	Nineteen tubes of caesium used for treating cancer are reported missing from a North Carolina hospital. They are not recovered.
	Chechnya	Chechnyan Security Service discovers/defuses an RDD booby trap near a railway.
1999	Chechnya	In September, unidentified thieves attempt to steal a container housing 200 g of radioactive material from a chemical factory in Grozny. One of the thieves dies half an hour after exposure to the container; the other is hospitalized in critical condition. (Each carried the container for only a few minutes.)
	United States	The government issues a high alert during a search for six men who allegedly have photos and details of a US nuclear power plant.
	Russia	In December, two people scavenging for lead in a lighthouse on the northern coast are hospitalized after exposure to the powerful radioactive strontium source used to power the lighthouse. (In January 2003, the US government announces a plan to aid Russia in safely replacing the energy sources of all of its nuclear lighthouses.)
	Republic of Georgia	In December, heat-emanating containers, each housing 40 000 curies of strontium, are found by woodcutters in Lilo. Within hours, the men become ill with nausea, vomiting, and dizziness, and later develop severe radiation burns on their backs. The International Atomic Energy Agency (IAEA), on recovering the containers, finds that the amount of radiation of each is equivalent to the amount of radioactive strontium released immediately after the Chernobyl accident of 1986.
2002	United States	In June, the FBI arrests Jose Padilla, a US citizen also known as Abdullah al-Mujahir, at Chicago's O'Hare airport. Padilla is known to have ties to al-Qaeda and is suspected of researching dirty bombs with the intention of detonating one inside the country's borders.
2003	Afghanistan	Evidence uncovered in Herat leads British intelligence agents and weapons experts to conclude that al-Qaeda has succeeded in constructing a small dirty bomb, although the device has not been found. They believe the terrorist organization received radioactive sources used in medical devices from the Taliban regime.
	Iraq	In April–May, after the fall of Saddam Hussein's regime, a US military team finds that extensive looting has taken place at nuclear facilities. An IAEA team is allowed to inspect the looted facilities in June. It finds radioactive materials lying on the floor.
	Republic of Georgia	In May, Georgian police conducting a routine search of a taxi find two boxes of ^{137}Cs and ^{90}Sr, and another box containing nerve gas concentrate. The materials appear to have been headed to Turkey for resale. This event is one in a series of recoveries of radioactive materials in Georgia.
	Thailand	Thai police arrest a public school teacher in Bangkok after he attempts to sell a container filled with ^{137}Cs (believed to be of Russian origin) for $240 000.
	Great Britain	In July, a van containing 30 lbs of depleted uranium is stolen outside a British radioactive waste-processing firm.
	China	A Chinese nuclear medicine expert buys ^{192}Ir. He then places the pellets in his colleague's ceiling as a form of revenge. Soon after, the poisoned colleague begins complaining of memory loss, fatigue, loss of appetite, headaches, vomiting and bleeding gums. Before the radioactive pellets are uncovered, another 74 hospital staff members are found to have similar symptoms.

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There are several forms of threat that could result in a radiological event (McFee and Leikin, 2005; Jarrett, 1999; Leikin *et al*., 2003; Walter *et al*., 2000; Christensen *et al*., 2008b; Schleipman *et al*., 2004; NTI and The Center for Nonproliferation Studies at the Monterey Institute of International Studies, 2004).

Five categories of terrorist threat can be considered:

- Simple radiological device (SRD) sometimes referred to as a simple dispersal device (SDD)
- Radiological dispersal device (RDD)
- Nuclear reactor sabotage
- Improvised nuclear device
- Nuclear weapon.

The most plausible threat would be an SRD or RDD, because hundreds of thousands of radioactive sources are available in industry and medicine around the world.

2.1.1 Simple Radiological Devices (SDRs); Simple Dispersal Device (SDDs); Radiation Exposure Device (REDs)

Use of an SRD, SDD or radiation exposure device (RED) is the deliberate act of placing a high-energy source or spreading radioactive material in a highly populated area, such as an airport, train station, port or sports venue, to expose people to various levels of radiation (McFee and Leikin, 2005; Jarrett, 1999; Christensen *et al*., 2008b).

SDRs/SDDs or REDs have been deployed and remain a threat that can be utilized by terrorists (**Table 2**). A psychological weapon, this form of radiation threat is more suited to cause panic than widespread injury. The mere mention of radiation causes concern and can create a panic, overwhelming HCFs with worried well, in addition to actual victims. Consider the 2006 murder of Alexander Litvinenko in London by polonium-210. Although widespread panic did not occur, nevertheless, the British National Health Service was deluged with calls and visits by people who feared they were exposed; albeit most were the worried well, and this was a selective target of one person, nevertheless, when over 1000 are concerned, imagine the impact, had the entire community or a section of London been targeted. However, the toxicity of such a device should not be taken lightly, especially given the radioactive materials suitable for such a weapon are ubiquitous and widely available sources are found in radiation oncology, industrial radiography and any number of other applications in medicine.

Caesium (Cs) is one of the most likely radioactive materials to be used in an SRD/SDD or RDD. It is widely available and utilized industrially. Other radionuclides include americium, which can be found in smoke detectors, and iridium.

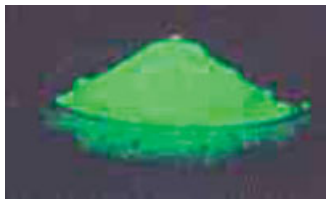

Figure 1 Caesium (McFee and Leikin, 2005). (Reproduced with permission from Radiation Emergency Assistance Center/Training Site (REAC TS).)

Especially problematic is the stealth with which an SDD could be deployed. If not announced as a weapon, no one would know about the device until patients presented with a variety of symptoms. Unless the clinician considered radiation—and this is unlikely in the early stages of illness based upon the level of radiation knowledge and preparedness among healthcare workers, and the results of the Litvinenko case where he was initially misdiagnosed—appropriate management would be delayed (Harrison *et al*., 2007; McFee and Leikin, 2008; 2009; Stather 2007; International Atomic Energy Association (IAEA), 2009a).

Though not an intentional act of terrorism, the events of 1987 in Goiânia, Brazil demonstrate the impact upon a community that 'innocently' spread radioactive materials can have and underscore our vulnerabilities (McFee and Leikin, 2005; Oliveira *et al*., 1991a; 1991b). Two thieves stole a hospital therapy device, and sold it for scrap metal. Unbeknownst to them it contained caesium-137 (^{137}Cs) in its shielding. The source consisted of 1375 Ci of ^{137}Cs. The source was broken up and shared among various individuals. At the time, these persons were not aware they had a radioactive source, and the incident was not detected for 15 days. When the container was opened, the contents, characteristically gave off a slight iridescence (**Figure 1**). Children and others started handling the caesium and subsequently started to develop a variety of symptoms. The medical response and cleanup phase took several months to complete. In this situation there was both exposure and contamination. When the government became involved, it developed into a significant public-health concern. The regional authorities required outside logistical support. The overall findings were as follows: 112 800 persons were surveyed for contamination, which involved providing a urine sample. The Brazilian government utilized a local sports venue to aggregate the population.

Victims included were as follows:

- 249 persons were contaminated
- 120 persons were externally contaminated on clothes and shoes
- 129 persons were both externally and internally contaminated

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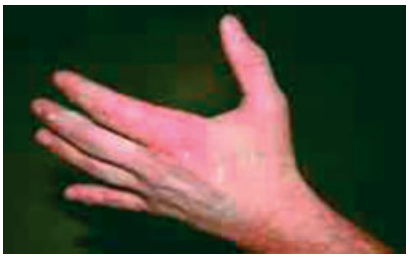

Figure 2 The early phase of acute local radiation injury is marked by atypical erythema extending roughly beyond the irradiated areas. Changes of sensitivity, burning pain, intense itching and tingling may occur (McFee and Leikin, 2005). (Reproduced with permission from Radiation Emergency Assistance Center/Training Site (REAC TS).)

- 20 persons required specific hospital treatment
- 14 persons had subsequent bone-marrow depression
- Eight persons received treatment with granulocyte-macrophage colony-stimulating factor (GM-CSF)
- Four persons died as a result of haemorrhage and infection.

A child ended up having an amputation. One deceased child at autopsy emitted several Gy.

^{137}Cs is ubiquitous as a medical and industrial radiation source. A common industrial usage is in the construction of highways, in which it is used in devices that measure density of asphalt. During the last 36 months, several such devices in the southeastern United States have been stolen or are missing; their whereabouts remain unknown. Sixteen brachiotherapy sources of ^{137}Cs were stolen from a hospital in North Carolina and, in Florida, an industrial radiography source of iridium-192 (^{192}Ir) was stolen. These sources have not been found. In addition to radiation risk from ^{137}Cs, this substance is highly toxic. There are numerous forms of Caesium. Some are explosive when exposed to air and water, some must be stored in special containment and, as a β-emitter, can cause significant dermal injury on direct contact. The initial burn can look like a thermal or sunburn (**Figure 2**).

Hospitals, industry and scientific research facilities, as opposed to military installations, are especially vulnerable to theft of radioactive materials. Industries that use such materials must implement safeguards and surveillance systems to ensure that these radiation sources remain fully accounted for.

2.1.2 Radiological Dispersal Devices (RDDs)

In an RDD, explosives are attached to a radioactive isotope and detonated. This can result in large areas of local environmental contamination, as well as patients being contaminated. RDD, though not posing the risk of devastation associated with thermonuclear devices, can nevertheless spread radioactive materials. The materials integrated in an RDD could be in several forms: gas, aerosol, solid, particulate, liquid or other. RDDs use conventional explosives, for example, trinitrotoluene (TNT), dynamite, plastic explosives or others, to pulverize or vaporize, then spread radioactive materials. This is commonly called a 'dirty bomb'. Radioactive materials that could be used in such devices are the same as those that could be used in SDDs above. However, RDDs as terror weapons would be highly effective; the explosion causing serious physical injuries from blasts, shrapnel/flying debris and thermal injuriess in addition to the threat of radiation toxicity. Moreover the psychological impact of 'radiation' in addition to the terror associated with a bombing would be highly effective (McFee and Leikin, 2005; Jarrett, 1999; Christensen *et al*., 2008b).

^{137}Cs has been identified as a potential radiation source for such a 'dirty' bomb or RDD. In this era of terrorism, responders must be aware of the secondary risk in responding to an explosion threat (McFee and Leikin, 2005; Jarrett, 1999; NTI and The Center for Nonproliferation Studies at the Monterey Institute of International Studies, 2004; US Environmental Protection Agency, 2009). The targeting of first responders using secondary devices, usually explosives such as the IED (improvised explosive device) is well known. The additional threat of toxic substances—radioactive or not—enhance the risk. Therefore, the risk of a potential secondary device as well as a 'dirty' primary device must always be considered. The wide distribution of lower-level radioactive hazards that are frequently found in academia, medicine and industry are a persistent vulnerability, given they are relatively unsecured or located in moderately controlled settings.

2.1.3 Nuclear Reactor

In the Western world, the probability of a terrorist attack on a nuclear reactor is low, but recent events highlight the possibility. The low probability is due to the high security surrounding a nuclear reactor and the safety systems incorporated into it. There is extensive shielding around a reactor; therefore, very significant amounts of explosive would be required to breach the reactor core. Terrorist events could include use of very large amounts of explosives, but it would be extremely difficult for terrorists to breach the security cordon. It is possible that a jumbo jet could crash into a reactor or a nuclear pond of used reactor cores; given the events of 9/11, not as far fetched an exigency as previously considered (McFee *et al*., 2004; McFee and Leikin, 2005; Christensen *et al*., 2008b; NTI and The Center for Nonproliferation Studies at the Monterey Institute of International Studies, 2004).

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Recently published results of computer and engineering studies suggest that the construction of most reactors would sustain a direct hit from a commercial aircraft flying into a reactor at less than 300 mph. However, some scientists question these findings. Most people are aware of the reactor accidents at Three Mile Island in Pennsylvania and the deadly explosion in 1986 at Reactor 4 of the nuclear power plant of Chernobyl, in the Ukraine, which at the time was a satellite member of the now former Soviet Union. The amount of radiation released from Three Mile Island was small indeed, and no radiation injuries resulted.

The most common type of incident of concern involving a nuclear reaction would be the disruption of the cooling system for the reactor core of nuclear fuel. Loss-of-coolant accidents (LOCAs) can result in failure to cool a reactor core, allowing it to reach temperatures at which the nuclear fuel and associated assembly melts (hence the term 'meltdown'). The cause may be a pipe break, valve failure or pump failure that interrupts cooling. Reactors generally are now designed to avoid an LOCA by automatic insertion of control rods to shut down the reactor before criticality occurs and sets the stage for a 'meltdown'. LOCAs may result in the release of radioisotopes such as srontium-90 (^{90}Sr), ^{137}Cs and iodine-131 (^{131}I), to name a few (Jarrett, 1999; Christensen *et al*., 2008b; NTI and The Center for Nonproliferation Studies at the Monterey Institute of International Studies, 2004). Medical management of workers or other individuals near the site of a reactor incident with release of radioactive materials may require immediate care for medical conditions, surgical conditions and thermal burns. If a plume were allowed to escape the reactor facility and containment structure, it would drift with the current wind conditions direction. Airborne, ground, environmental, animal and human contamination could occur in the plume's path, as with a nuclear weapon detonation. The impact from Chernobyl as a result of the plume effect reached into Scandinavia and beyond.

The Chernobyl reactor incident resulted because several safety systems were bypassed, while a set of experiments with the reactor were performed. There were two explosions, with fires and meltdown of the reactor core, leading to serious widespread contamination of the environment.

The early phase of the accident had the following results (Jarrett, 1999; International Atomic Energy Association (IAEA), 2009b; The Chernobyl Forum 2003–2005, 2009; World Health Organization, 2009):

- 237 persons were hospitalized
- 134 persons had acute radiation syndrome (ARS)
- 28 persons died within the first three months
- Two persons were killed in the initial explosion
- One person died of heart failure
- 250 000 persons were permanently evacuated from the area.

The primary radioactive isotopes responsible for health problems were ^{137}Cs and ^{131}I. Considering the gravity of the accident, it is commendable that the response-team actions led to low overall mortality.

2.1.4 Improvised Nuclear Device

An IND, if successful, could produce a nuclear yield similar to that of Hiroshima and Nagasaki, with release of radiation, blast and thermal pulses, together with significant radioactive fallout. In reality, a terrorist organization might be able to produce a partial yield, producing less effect. The conventional explosive would detonate and blow the device apart, resulting in environmental contamination with weapons material, such as plutonium or uranium (McFee and Leikin, 2005; Jarrett, 1999; Leikin *et al*., 2003; Walter *et al*., 2000; Christensen *et al*., 2008b; NTI and The Center for Nonproliferation Studies at the Monterey Institute of International Studies, 2004).

A review of the data suggests that the mean lethal dose (LD) of whole-body radiation necessary to kill by 60 days 50% of people exposed (LD50/60) is between 3.25 and 4 Gy, without supportive medical care. Survivors of Hiroshima had estimated doses of less than 3 Gy.

Construction heretofore of such a device has been considered highly difficult because of the sophisticated engineering and expertise required. More recent studies conducted by Harvard University and projects assessing the threat of nuclear terrorism have come to the conclusion that a capable, well funded and organized group could make, deliver and detonate at least a crude nuclear bomb capable of incinerating the centre of any major city in the world, *if* the necessary fissile materials were obtained.

For a crude 10 kilotonne weapon (two thirds that of the Hiroshima bomb) detonated at Grand Central Station—a major transportation hub located in Manhattan, New York City—it is estimated over 500 000 would be killed immediately and hundreds of thousands more would be injured. Depending upon wind direction, Manhattan would have to be evacuated—the mass exodus creating more injuries. Much of the island would be destroyed. Environmental impact and direct costs would exceed $1 trillion. The psychological impact would be incalculable (Kristoff, 2004).

2.1.5 Nuclear Weapons

Today's nuclear weapons are far more sophisticated than the kilotonne bombs detonated over Hiroshima and Nagasaki, with destructive power in the megatonne or higher category (McFee and Leikin, 2005; Jarrett, 1999; Leikin *et al*., 2003; Walter *et al*., 2000; Christensen *et al*., 2008b; NTI and The Center for Nonproliferation

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Studies at the Monterey Institute of International Studies, 2004).

Nevertheless, detonation of such devices in populated areas could result in tens, even hundreds of thousands of deaths. Physical trauma and thermal burns are the immediate hazards to life and limb. *Immediate deaths would not be the result of exposure to ionizing radiation* (*IR*). Patients not immediately near the epicentre who might survive may require treatment for physical injuries, thermal injuries, as well as radiation-induced injuries and illnesses (Christensen *et al*., 2008a; 2008b).

Recognize that a nuclear weapon, as with all explosions, creates enormous blast forces, which are the primary destructive force, along with thermal energy and radiation energy.

There are three forms of blast injury: primary, secondary and tertiary (Basic Disaster Life Support (BDLS) Course, 2004).

- Primary blast injuries involve the direct blast waves through overpressure forces, which are especially dangerous to gas-filled organs such as ears, lungs and the gastrointestinal (GI) tract. The brain can also be affected, and concussions can occur with seemingly no external injury. Bear in mind, if a victim has suffered from a tympanic membrane rupture, communications will be compromised, confounding the medical response. Also pneumothorax, pneumomediastinum, 'shock lung' and other air-membrane interfaces can be affected, some of which can result in significant medical/surgical attention, if not death.
- Secondary blast injuries result from flying debris that is now travelling at projectile velocity, resulting in blunt and penetrating injuries.
- Tertiary blast injuries result in the patient becoming the projectile. Numerous injuries are possible.

In addition to the blast effect would be significant thermal injuries. These burns, immediate from the explosion and some evolving from the radiation effect, require highly specialized burn and radiation infection-control measures.

Medical management of such a scenario would require extensive radiological and medical triage of patients to ensure appropriate and efficient utilization of public health and emergency medical resources.

While the probability of stealing a sophisticated nuclear weapon in the Western world is considered by many to still be a remote exigency because of high security, others are less optimistic (Graham and Talent, 2008; Christensen *et al*., 2008b; NTI and The Center for Nonproliferation Studies at the Monterey Institute of International Studies, 2004; Basic Disaster Life Support (BDLS) Course, 2004).

However, there are two situations warranting concern. First, among the nuclear nations, at least two are politically and/or militarily unstable and pose a security risk in the region, and perhaps globally if their governments fail to control their weapons—North Korea and Pakistan. Second, and widely supported as worrisome, both Vladimir Putin and members of the Russian general staff, as well as security officers who have defected to the West, have stated publicly that several (estimates range from a handful to 50) suitcase nuclear weapons with a one kilotonne rating are unaccounted for in the former Soviet Union. These weapons are potential 'suitcase bombs', and represent an increasing threat, especially on the black market (McFee *et al*., 2004; Graham and Talent, 2008; Christensen *et al*., 2008b; International Atomic Energy Association (IAEA), 2009b; NTI and The Center for Nonproliferation Studies at the Monterey Institute of International Studies, 2004; Basic Disaster Life Support (BDLS) Course, 2004).

The consequences within the first minute of detonation of a one kilotonne nuclear weapon are as follows (Jarrett, 1999; Nuclear Threat Initiative (NTI), 2009; Samuel and Dolan, 1977):

- Blast range would reach 400–500 yd
- Thermal radiation would reach approximately the same distance as the blast
- Nuclear radiation, that is, gamma particles and neutrons, would reach half a mile
- Radioactive fallout could produce high exposure rates up to half a mile.

Regardless of the nature of the threat, clearly the most serious scenario results in patients exposed to high level, high dose, acute whole-body or even partial-body gamma irradiation. As evident in **Table 1**, numerous radionuclides considered a military/security threat are gamma emitters. As will be discussed in the next section, gamma rays require special shielding, are high energy and potentially lethal.

Whether an IND, nuclear accident or nuclear weapon, widespread human, animal and agricultural impact from airborne, and environmental contamination are likely (Christensen *et al*., 2008b).

Since radioactive iodine (^{131}I) will be a likely threat, prophylactic thyroid-blocking therapy with potassium iodide (KI) will need to be initiated. KI supplies are stockpiled within a 10 mile radius of nuclear power plants. This will be discussed in Section 5.4 on Antidotes (International Atomic Energy Association (IAEA), 2009a; Christensen *et al*., (2008a; 2008b); Basic Disaster Life Support (BDLS) Course, 2004).

The National Council on Radiation Protection and Measurements (NCRP) Report Number 65 also contains an in-depth overview for dosages and methods of delivery (NCRP, 1979). When considering upgrading facility preparedness, it is important to become familiar with NCRP reports (Christensen *et al*., 2008b). Identification and quantification of internal contamination with various radionuclides is needed to guide chelation and/or other

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decorporation therapies. Recognize there will be disruption of public infrastructure that will require mobilization of federal resources, such as the Federal Radiological Monitoring and Assessment Center (FRMAC), a multi-agency response contingency managed by the National Nuclear Security Administration (NNSA) of the US. For preparedness purposes, The Radiation Emergency Assistance Center/Training Site (REAC/TS, 2009) is a valuable consulting resource. If you are not located in the United States, identify the national and regional authorities in your country that would provide similar medical, security, environmental and resource services.

3 RADIOACTIVITY AND IONIZING RADIATION: A PRIMER

3.1 Radiation Chemistry

The periodic table of elements contains over 100; these are the building blocks for all substances on earth. Each element has an atomic structure consisting of a nucleus, electrons, neutrons and protons. Elements may occur in several forms called isotopes that differ in their nuclear properties (McFee and Leikin, 2005; Timins and Lipoti, 2003; United Nations, 2000; International Atomic Energy Association (IAEA), 2009a; Jarrett, 1999; Walter *et al*., 2000; Christensen *et al*., 2008a; 2008b; World Health Organization, 2009).

Isotopes may be either stable or unstable; the latter are referred to as radioisotopes because of the radioactive decay occurring in the basic structure which underpins the instability. There are literally thousands of radioactive sources.

Radioactive chemicals or isotopes within the element can also demonstrate toxic properties beyond radioactivity. There are forms of caesium and compounds including that element that can cause burns, or explode on contact with water (McFee and Leikin, 2005; US Environmental Protection Agency, 2009). Of note, unlike traditional chemical toxicants, radiation cannot be diluted with water to neutralize the toxicity. Water may create distance or pose limited shielding for relatively weak particles, but it cannot detoxify radiation. It is effective, especially with soap, in decontamination procedures to wash off particles from patients (Jarrett, 1999; Leikin *et al*., 2003; Christensen *et al*., 2008a; 2008b).

Knowing the decay rate, energy, amount and type of radiation associated with a particular isotope (radioisotope) is necessary to characterize the level of risk that a weapon or source hazard poses to human health.

Decay rate is usually expressed as the *half-life,* which is the amount of time it takes for half of the radioactive sample to decay. In notation it would appear as $t_{1/2}$. For example, after one half-life, 50% of the material is gone; after two half-lives 25% of the material remains, and so forth with each subsequent half-life. The range of possible $t_{1/2}$s for radioactive sources varies from seconds to thousands of years! The latter clearly has the potential to pose an environmental challenge (Leikin *et al*., 2007; McFee and Leikin, 2005; Timins and Lipoti, 2003; United Nations, 2000; Jarrett, 1999; Christensen *et al*., 2008a; 2008b).

Energy emission is isotope specific and measured in mega electron volts. In notation it would appear as MeV. The higher the MeV, the deeper the penetration into tissue, for example (McFee and Leikin, 2005; Timins and Lipoti, 2003; United Nations, 2000; International Atomic Energy Association (IAEA), 2009a; Christensen *et al*., 2008a; 2008b).

Amount of radiation is characterized by the number of nuclear transformations (disintegrations) per time period (usually a second), referred to by the conventional (US) unit of measure the curie (Ci) named after the discovering chemist and pioneer in radiation studies, Marie Curie, or the Système International (SI) term becquerel (Bq). One Bq = 1 disintegration per second; one Ci = 3.7×10^{10} Bq or disintegrations per second. In practical terms, what is the significance of the Ci for human health? Consider the banana—considered a good source of potassium in the diet. Each banana contains the radioisotope potassium-40 (^{40}K), making bananas a radiation emitter! However each banana contains 10^{-11} Ci. Compared to a radioactive cobalt pellet (^{60}Co) found in radiotherapy units to treat certain forms of cancer, which is 2 Ci; the banana is one billionth the emitter of Co^{60} (McFee and Leikin, 2005; Timins and Lipoti, 2003; United Nations, 2000; Christensen *et al*., (2008a; 2008b); Goans, 2007).

Either term (Bq or Ci) can be used to refer to the amount of radioactive material released into the environment. It is estimated that 81 million Ci of radioactive caesium was released into the atmosphere during the Chernobyl disaster.

Radiation is the emission and propagation of energy through a medium or space in the form of waves or particles; there are a variety of types of radiation within the electromagnetic spectrum, most of which do not have the energy to cause ionization. Radioactivity occurs because the nucleus of the atom is unstable, resulting in emission of energy (Leikin *et al*., 2007; McFee and Leikin, 2005; Timins and Lipoti, 2003; United Nations, 2000; International Atomic Energy Association (IAEA), 2009a; 2009b; NTI and The Center for Nonproliferation Studies at the Monterey Institute of International Studies, 2004; Basic Disaster Life Support (BDLS) Course, 2004; World Health Organization, 2009).

Radiation can be measured in terms of emission from a radioactive source, absorption by a person or risk that a person might suffer health effects—the biological risks (Leikin *et al*., 2003; 2007; McFee and Leikin, 2005; Timins and Lipoti, 2003; United Nations, 2000; Thomas and Walter, 2008; Christensen *et al*., 2008a; Goans, 2007;

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Ricks *et al*., 1990; REAC/TS, 2009; Basic Disaster Life Support (BDLS) Course, 2004; World Health Organization, 2009). Depending on the aspect of radiation under discussion, different units of measurement are used. A release in the form of radiation is in proportion to the number of disintegrations of radioactive atoms in a radioactive material over a period of time. Radioactivity is a naturally occurring phenomenon; we are exposed to small amounts on a daily basis (Leikin *et al*., 2003; 2007; McFee and Leikin, 2005; Timins and Lipoti, 2003; United Nations, 2000; Thomas and Walter, 2008; Christensen *et al*., 2008a; Goans, 2007; Ricks *et al*., 1990; REAC/TS, 2009; Basic Disaster Life Support (BDLS) Course, 2004; World Health Organization, 2009). When a person is exposed to radiation, energy is deposited in the body. Radiation dose refers to the absorption of radiation energy per unit mass of absorber. The amount deposited per unit of weight, using the US conventional term is called the rad or radiation absorbed dose; the basic unit of measuring exposure dose is defined as the deposition of 0.01 J of energy into one kilogram of tissue. The Système International (SI) unit of measure is the gray (Gy); 1 Gy = 100 rad. The number of rads a patient has been subjected to allows clinicians to anticipate the potential severity of ARS and plan clinical management accordingly (Leikin *et al*., 2007; McFee and Leikin, 2005; 2009; United Nations, 2000; Harrison *et al*., 2007; International Atomic Energy Association (IAEA), 2009a; Jarrett, 1999; Waselenko *et al*., 2004; Flynn and Goans, 2006; Walter *et al*., 2000; NCRP, 2001; Christensen *et al*., 2008b; Berger *et al*., 2006; Goans and Waselenko, 2005; Goans, 2007; Koenig *et al*., 2005; Ricks and Fry, 1990; Ricks *et al*., 1990; 2002; NCRP, 1979; REAC/TS, 2009; Fliedner *et al*., 2001; Gusev *et al*., 2002; Basic Disaster Life Support (BDLS) Course, 2004).

The risk that a person will suffer adverse health effects from radiation is measured using the unit rem (roentgen equivalent in man). The rem quantifies the amount of damage suspected from a radiation exposure. The SI unit is the Sievert (Sv); 1 Sv = 100 rem. The rem is adjusted to reflect the type of radiation absorbed and the likely damage produced (Leikin *et al*., 2007; McFee and Leikin, 2005; 2009; United Nations, 2000; Harrison *et al*., 2007; International Atomic Energy Association (IAEA), 2009a; Jarrett, 1999; Waselenko *et al*., 2004; Flynn and Goans, 2006; Walter *et al*., 2000; NCRP, 2001; Christensen *et al*., 2008b; Berger *et al*., 2006; Goans and Waselenko, 2005; Goans, 2007; Koenig *et al*., 2005; Ricks and Fry, 1990; Ricks *et al*., (1990; 2002); NCRP, 1979; REAC/TS, 2009; Fliedner *et al*., 2001; Gusev *et al*., 2002; Basic Disaster Life Support (BDLS) Course, 2004).

Key Facts: For the intent and purpose of acute care, roentgens are equivalent to rads which are equivalent to rems.

Type of radiation refers to whether a source is IR or nonionizing radiation (Leikin *et al*., 2003; 2007; McFee and Leikin, 2005; Jarrett, 1999; Christensen *et al*., 2008a; Ricks and Fry, 1990; Schleipman *et al*., 2004; NCRP, 1979; REAC/TS, 2009; NTI and The Center for Nonproliferation Studies at the Monterey Institute of International Studies, 2004; World Health Organization, 2009).

3.2 Nonionizing Radiation

Nonionizing forms of energy radiation include microwaves, as well as light—visible, ultraviolet and infrared. These forms of non-IR can still cause tissue damage by creation of heat (Leikin *et al*., 2007; 2003; Christensen *et al*., 2008a; World Health Organization, 2009).

3.3 Ionizing Radiation (IR)

Ionizing radiation is a form of energy that has the potential to remove electrons from other atoms. For example, in IR, by stripping a negative electron from an atom, an 'ion pair' is created—an electron and a positively charged atomic remnant. This process of 'ionization' creates instability in molecules, the most important of which, from a medical perspective, is cellular genetic material—deoxyribonucleic acid (DNA). Ionizing radiation can also damage cells by the hydrolysis of water to form highly reactive 'free radicals' which can propagate tissue destruction by chemical reactions (Leikin *et al*., 2003; 2007; Jarrett, 1999; Walter *et al*., 2000; Christensen *et al*., 2008a; World Health Organization, 2009).

Not all IR is the same. Within this category are several types of IR; the differences among types of ionizing radiation relate to their energy and charge. Their respective properties determine tissue toxicity: deeply ionizing radiation can penetrate cells, tissues or organs as they deposit energy along their tracks.

While the type of IR and resulting range of penetration vary, they also determine the nature of the absorber or barrier necessary to block their respective energy. This is the basis for shielding.

Penetration is determined by the circumstance—tissue/host type, distance from source and shielding, which have implications in terms of the health threat, type of personal protective equipment (PPE) required and prognosis. IR comprises alpha and beta particles, and gamma and X-rays (Leikin *et al*., 2003; 2007; Jarrett, 1999; Christensen *et al*., 2008a; World Health Organization, 2009).

Various technologies are used to characterize, interpret and even identify IR. 'Ionization' is the primary basis for locating radioactivity and radioactive materials. 'Spectrometry' is used to identify the energy of the radiation, allowing it to be compared to a known spectrum,

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and is utilized to identify specific radionuclides. Each radionuclide has a unique energy spectrum much like every human has unique fingerprints.

3.3.1 Types of Ionizing Radiation

3.3.1.1 Alpha Particles

Alpha particles are large, charged particles (Leikin *et al*., 2003; 2007; McFee and Leikin, 2005; 2008; 2009; Harrison *et al*., 2007; Stather 2007; Jarrett, 1999; Christensen *et al*., 2008a; Ricks and Fry, 1990; Schleipman *et al*., 2004; NCRP, 1979; REAC/TS, 2009; NTI and The Center for Nonproliferation Studies at the Monterey Institute of International Studies, 2004; World Health Organization, 2009). They are essentially helium nuclei consisting of two protons and two neutrons. They have significant mass and kinetic energy that can cause direct ionization of other atoms or molecules. Because of their size-to-speed characteristics and 2+ charge, they have little penetrating power. Intact skin, clothing, even paper, can stop alpha particles and are effective barriers to penetration. Examples of alpha emitters include americium, plutonium and polonium, which will be discussed later in greater detail. If the radiation source is strictly an alpha emitter, universal precautions are sufficient protection from external danger. However, it is important to recognize alpha emitters are significantly dangerous once internalized—through ingestion, inhalation or percutaneously via insertion/injection or contamination of wounds. IEDs that integrate toxicants, including radioactive materials, can internalize alpha particles into wounds. Intentional poisoning, as with the Litvinenko assassination, demonstrated the deadly nature of internalized alpha emitters (Harrison *et al*., 2007; Stather 2007; McFee and Leikin, 2008; Jarrett, 1999; Leikin *et al*., 2003; Flynn and Goans, 2006; Christensen *et al*., 2008a; Ricks and Fry, 1990; Ricks *et al*., (1990; 2002); REAC/TS, 2009). Once internalized, alpha emitters can cause ARS, and wide range of tissue effects, symptoms and organ damage. These particles pose a contamination risk and must be removed from victims (Leikin *et al*., 2003; 2007; McFee and Leikin, 2005; United Nations, 2000; Jarrett, 1999; Christensen *et al*., 2008a; Ricks and Fry, 1990; Schleipman *et al*., 2004; NCRP, 1979; REAC/TS, 2009; NTI and The Center for Nonproliferation Studies at the Monterey Institute of International Studies, 2004; World Health Organization, 2009).

It is important to make certain *before an event* that radiation detectors can identify alpha particles. Responders should be familiar with the capability and limitations of the detectors. Usually special wands or sensor attachments are necessary and applied in specific ways.

3.3.1.1.1 The Alexander Litvinenko Case: Death by Polonium 210

On 1 November 2006, a previously healthy, athletic, 43-year-old Russian male, who had emigrated to London five years earlier, presented to a North London hospital with acute, severe, progressive GI symptoms (Harrison *et al*., 2007; McFee and Leikin, 2008; 2008; 2009; Stather 2007). As is now well known, he was a former Russian agent before becoming a writer in the UK. Whether Litvinenko intentionally withheld his prior occupational history or it was not initially pursued by clinicians remains unclear. Litvinenko's health rapidly deteriorated: his hair fell out and he developed pancytopenia. His symptoms were consistent with radiation toxicity; physicians obtained urine and blood samples and subjected them to gamma spectrometry. Not surprisingly, the tests came back negative, given that these were inadequate to the task (see following section on testing/management). Subsequently clinicians began searching for other causes of illness, including exotic toxins, biologicals and poisons; the patient's condition steadily declined. Because Litvinenko was not responding to treatment, his urine was sent for more advanced laboratory analysis to Britain's Atomic Weapons Establishment (BAWE), where tests revealed significant amounts of alpha-particle radiation (McFee and *et al*., 2008).

On November 23, Litvinenko died of internal contamination from Polonium-210 (^{210}Po). Twenty-one weeks elapsed between the poisoning and realization that radioactive materials were used, and as of January 2007, at least 12 people have tested positive for contamination. The incidental exposure of these individuals did not appear to pose a risk. However, fears of ^{210}Po contamination led thousands of people to contact the National Health Service's direct helpline, established in the aftermath of the assassination.

3.3.1.1.2 Polonium

^{210}Po is considered one of the most hazardous radioactive materials, but like other alpha emitters, it must be internalized to pose a toxic threat. Patients affected by ^{210}Po do *not* pose a health risk to responders. Intact skin is a good barrier. However, inhalation and ingestion are access points (Harrison *et al*., 2007; McFee and Leikin, 2008; 2008; 2009; Stather 2007).

Polonium was discovered in 1897 by Marie and Pierre Curie; Madame Curie named it after her native Poland. There are more than 20 isotopes of Po; ^{210}Po, the most stable form, is an alpha-particle (5.3 MeV)-emitting radionuclide with a radio-decay half-life ($t_{1/2}$) of 138 days. Unlike other radioactive elements, ^{210}Po is relatively safe to transport. However, if these high-energy particles are inhaled, ingested or inserted into abraded skins or wounds, they can damage tissue.

In the case of alpha emitters, such as ^{210}Po, identifying the source's nature as a contaminant, on the victim or in the area, requires equipment capable of detecting the full range of ionizing radiation types (Christensen *et al*., 2008a). Also, in the case of alpha emitters as toxicants, once inside the patient, especially if the victim

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is not externally contaminated, further investigation to determine the aetiology is recommended on the basis of the clinical picture, because external detection methods are unlikely to be of value for an internal toxicant. Advanced laboratory testing is required to evaluate biological samples and conduct blood tests.

Few human data are available on the health effects of ^{210}Po, but the toxicity of orally administered ^{210}Po appears to be consistent with the amount reaching the blood (bioavailability). Damage to the gut mucosa is a probable contributory cause of death after oral administration. In a Russian case of interest, a male worker accidentally inhaled an aerosol of ^{210}Po. Nine deaths occurred after 13 days (Harrison *et al*., 2007; Scott, 2007). Vomiting was severe at the time of admittance to a clinic, two to three days after the intake. A high fever was reported, but there was no diarrhoea. Thrombocyte counts were 150×10^9 l^{-1} on day 6 and 80×10^9 l^{-1} on day 8. Within minutes of ingesting ^{210}Po, the cells lining the victim's GI tract would begin to die and slough off, which would cause nausea, pain and severe GI bleeding. Other systems would be affected by ^{210}Po, and, unless early decorporative (chelation) treatment is initiated to lower the body burden and other medical interventions were provided, significant morbidity and ultimately death would be anticipated.

The earlier the initial symptoms of radiation poisoning (nausea and vomiting) occur, the more likely it is that it is a severe exposure (Jarrett, 1999; Christensen *et al*., 2008b). Although profound fear response and/or psychological exposure without actual radiation exposure can also cause nausea and vomiting, these don't tend to cause rapid hair loss and disruption of blood counts. In the presence of an actual exposure, persistent GI symptoms within the first two hours of exposure portends a poor prognosis and potentially fatal outcome.

There are other important alpha emitters of concern (**Table 1**) (Jarrett, 1999). These include americium, which can be found in smoke detectors, and plutonium, which is a highly controlled commodity produced from uranium (^{239,238}U) in reactors, often contaminated with americium and used in thermonuclear devices.

3.3.1.1.3 Lessons Learned from the Litvinenko Case

Although Litvinenko's symptoms were consistent with radiation illness, as a result of the initial hospital assessment, which apparently focussed on gamma radiation, radiation was abandoned and other diagnoses were sought. Of note, the Centers for Disease Control and Prevention (CDC) recommend a 24 hour urine collection to assay for the presence of ^{210}Po when suspected; levels in excess of background are suggestive of internal contamination (CDC, 2006). The essential issue is suspecting radiation toxicity early on and employing a full range of testing until it can be ruled out (Harrison *et al*., 2007; McFee and Leikin, 2008; 2008; 2009; Stather 2007; Scott, 2007; CDC, 2006).

3.3.2 Beta Particles

Beta particles (**Table 1**) are identical to electrons, have a negative charge, are very low in mass (1/2000 that of protons or neutrons) and possess a spectrum of energies. High-energy beta emitters can penetrate a few meters through air and a few millimetres into tissue. Like alpha emitters, beta emitters pose an internal hazard. However, beta emitters also pose an external hazard risk (McFee and Leikin, 2005; United Nations, 2000; Jarrett, 1999; Leikin *et al*., 2003; Flynn and Goans, 2006; Berger *et al*., 2006; Goans and Waselenko, 2005; REAC/TS, 2009; International Atomic Energy Association (IAEA), 2009b; NTI and The Center for Nonproliferation Studies at the Monterey Institute of International Studies, 2004; Oliveira *et al*., 1991a; 1991b; US Environmental Protection Agency, 2009; Basic Disaster Life Support (BDLS) Course, 2004; World Health Organization, 2009; Hall and Giaccia, 2005).

Caesium-137 (^{137}Cs) is a ubiquitous beta emitter that has demonstrated an ability to cause human illness and has been utilized in SDDs (McFee and Leikin, 2005; Jarrett, 1999; Christensen *et al*., 2008a; Goans, 2007; Oliveira *et al*., 1991a; 1991b; US Environmental Protection Agency, 2009).

Recall the concept of MeV (McFee and Leikin, 2005; Christensen *et al*., 2008a). ^{137}Cs is a beta emitter (McFee and Leikin, 2005; Jarrett, 1999; Christensen *et al*., 2008a; Oliveira *et al*., (1991a; 1991b); US Environmental Protection Agency, 2009; World Health Organization, 2009). Beta emitters with just 0.1 MeV of energy emission can penetrate 0.15 cm into human tissue. Severe beta particle burns can occur on skin or the eye with protracted contact (**Figure 2**). Aluminium, tin foil or thick plastic protective clothing is required as an effective barrier. Caesium is a common source for beta particles. Like alpha particles, beta particles pose a significant internal hazard. These particles pose a contamination risk and must be removed from victims.

It is important to make certain *before an event* that radiation detectors can identify beta particles. Responders should be familiar with the capability and limitations of the detectors.

3.3.3 Gamma Rays

Gamma rays, also called 'photons' consist of electromagnetic energy or electromagnetic radiation. These high-energy uncharged particles are similar to X-rays and can readily penetrate most materials and matter, including skin and clothing, depending upon their energy. Gamma rays result in whole-body radiation and can damage multiple organ systems, albeit different tissues exhibit varying degrees of radiosensitivity; at

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large enough exposures virtually all systems will be affected. Gamma emitters pose both an internal and external hazard (McFee and Leikin, 2005; Jarrett, 1999; Leikin *et al*., 2003; Christensen *et al*., 2008a; Goans, 2007; World Health Organization, 2009).

High-atomic-weight elements are used for shielding—heavy metals such as lead or depleted uranium. Concrete barriers can be used to shield against gamma radiation, usually in concert with heavy metals; in an emergency, setting up makeshift barriers, establishing perimeters that place space between people and gamma sources, and limiting time of exposure in the danger zone may be the most immediate, if imperfect, safety option. Doubling the distance from a source reduces the threat by 75%.

3.3.4 X-rays

X-rays are similar to gamma rays; high energy and able to readily penetrate skin, clothing and most matter. X-rays have longer wavelengths than gamma rays. Again, like gamma rays, special shielding is necessary (McFee and Leikin, 2005; Jarrett, 1999; Leikin *et al*., 2003; Christensen *et al*., 2008a; Goans, 2007; World Health Organization, 2009).

3.4 Radiation: 'Exposed' (Irradiation) and 'Contaminated'

Understanding the difference between these terms is critical to medical management (Leikin *et al*., 2003; 2007; McFee and Leikin, 2005; 2008; Timins and Lipoti, 2003; United Nations, 2000; Jarrett, 1999; Flynn and Goans, 2006; NCRP, 2001; Berger *et al*., 2006; Goans and Waselenko, 2005; Goans, 2007; Ricks and Fry, 1990; Ricks *et al*., 1990; 2002; Schleipman *et al*., 2004; NCRP, 1979; Gusev *et al*., 2002; World Health Organization, 2009; Nuclear Threat Initiative (NTI), 2009; Hall and Giaccia, 2005). The 'irradiated' patient is one who has been 'exposed' to ionizing radiation (International Atomic Energy Association (IAEA), 2009a; Christensen *et al*., 2008a; NCRP, 1979; Mettler and Upton, 1995). One can consider it similar to patients who have had diagnostic X-rays. Just like the patient you have sent for an X-ray, purely irradiated or radiation exposed patients are *not* radioactive and pose no risk to others.

On the other hand, patients who are 'contaminated' have radioactive material on them (external contamination) or in them (internal contamination). This would be likely after exploring an SDD, stolen materials, as in the Goiana, Brazil event involving ^{137}Cs, or in the aftermath of a 'dirty' bomb or RDD (ED Management, 2002; Harrison *et al*., 2007; McFee and Leikin, 2008; 2009; Stather 2007; Jarrett, 1999; Thomas and Walter, 2008; Christensen *et al*., 2008a; Berger *et al*., 2006; Goans and Waselenko, 2005; Goans, 2007; Koenig *et al*., 2005; NCRP, 1979; REAC/TS, 2009; NTI and The Center for Nonproliferation Studies at the Monterey Institute of International Studies, 2004). Patients are thus likely to have external contamination with radioactive materials on clothing or skin. These patients may pose a risk to others, depending upon the type of IR and quantity present. The proper use of PPE can significantly reduce this risk. Another important strategy: *removal of clothing* in such cases can reduce external contaminants by ~90% (McFee and Leikin, 2005; Jarrett, 1999; Leikin *et al*., 2003; Christensen *et al*., 2008a).

Internal contamination can occur with radioactive materials being inhaled, ingested, as occurred in the Alexander Litvinenko murder using ^{210}Po, penetration or percutaneous absorption (McFee and Leikin, 2005; 2008; 2009; Harrison *et al*., 2007; Stather 2007; Jarrett, 1999; Christensen *et al*., 2008a; 2008b; Goans, 2007; NTI and The Center for Nonproliferation Studies at the Monterey Institute of International Studies, 2004). In the event of a radiation-associated explosion, wounds may become contaminated with radioactive materials, which may become embedded in tissue.

Key Facts: The radiation-poisoned patient poses little risk to healthcare workers, especially if proper precautions are taken. Do not delay life-saving care because the patient is a 'radiation victim'. Hospital personnel safely handle patients on a daily basis who have had nuclear medicine scans and who are internally contaminated with injections of radioactive materials.

Enlist the assistance of a radiation health physicist (HP) early, as s/he will be well schooled in the critical science and convergence points of radiation and human health.

3.4.1 Radiation Damage: 'Deterministic' and 'Stochastic' Effects

The amount of damage to an organism is related to the dose, which, in terms of radiation, is the total amount of energy deposited. Traditional tenets of managing the poisoned patient are built upon the notion that the dose determines the difference between harmless and harmful—a 'deterministic' effect. These have a threshold at which an expected effect will appear. Above that threshold, as radiation dose increases, the effects become worse, as will be discussed in Section 4 on Acute Radiation Syndrome (McFee and Leikin, 2005; Christensen *et al*., 2008a; Goans, 2007).

An important linked consideration is the dose rate—the time over which ionizing radiation energy is deposited. The shorter the time period during which the energy is delivered, the greater the effects.

The impact of the dose is also to some degree influenced by the volume of cells or tissues absorbing the dose—the larger the volume of tissue irradiated, the greater the effects.

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The type and function of the cells receiving the dose also influences the outcome; some tissues are more radiosensitive at certain doses than others.

Of note is that the schedule of dose delivery has an influence on outcomes as well. If a dose is delivered in smaller divided doses or is *fractionated*, compared to giving the entire dose at one time, more significant effects will be seen with the single acute dose. Fractionation allows some repair of damage between doses.

As with other toxicants, the type and quality of radiation is important.

Host considerations play a role. The young and old are more susceptible to radiation injury and illness than more robust adults. Patients suffering from other injury or illness, for example, combined injuries, defined as radiation injury with trauma and/or thermal burns have increased morbidity and mortality. There are, as can be seen in more traditional illnesses, individual variations in susceptibility. No two persons are alike in their ability to withstand any kind of insult, whether it is a disease or toxic exposure involving radiation, chemicals, biological toxins, trauma or thermal injuries.

'Stochastic effects' have 'no lower threshold' (NLT) at which the effects are seen (Christensen *et al*., 2008a). They are random or probabilistic 'events' rather than 'effects'. In the crudest terms, radiation exposure imposes greater than a zero threat. Put another way, the number of cancers generated, or cancer incidence, increases with increasing radiation dose to a population, but the various types of cancer that develop do not become 'worse'. For purposes of emergency care, a thorough understanding of stochastic effects is not necessary, but will be necessary for patient counselling about the potential for various cancers after an incident.

3.4.2 Tissue Sensitivity

Cells that are less differentiated are generally more radiosensitive than more differentiated cells. Stem cells or precursor cells are generally more sensitive to radiation than more differentiated cells, for example, red blood cells (RBCs), sperm, striated muscle cells or neurons, to name a few. However, at high enough doses, these, too become susceptible to the effects of radiation.

Cells that are actively dividing are generally more radiosensitive than those that are not actively dividing. Haematopoietic cells in bone marrow actively divide and are much more sensitive compared to nervous system tissues that do not rapidly divide (Jarrett, 1999; Christensen *et al*., 2008a; Goans, 2007).

Sensitivity is also determined by life stage. Embryos and the foetus are more sensitive than later development *in utero*. Children are more radiosensitive than adults. Some organs are more radioresistant than others, for example, the thyroid gland is more radiosensitive than the liver.

3.5 Protection Against Radiation

The three primary ways to protect against radiation are time, distance and shielding (Leikin *et al*., 2007; McFee and Leikin, 2005; Jarrett, 1999; Thomas and Walter, 2008; Christensen *et al*., 2008a).

- Time: Exposure and absorption of radiation are dose and time dependent. By limiting the time in a radiation environment, the exposure is limited—one of the bases for rotating medical and response teams, and keeping individual doses to a minimum during victim rescue. No treatment should occur in the exposure or hot zone.
- Distance: Distance reduces the exposure. Keeping the maximum distance possible between yourself and the source is an essential consideration in response, and benefits both the rescuer and the victim. The inverse square law applies: if you double the distance between yourself and the source, the exposure will be reduced to one fourth of its original value. The reduction of exposure is exponential.
- Shielding: Different types of shielding strategies are required, based upon the various ways the different forms of IR behave (Leikin *et al*., 2003; 2007; McFee and Leikin, 2005; Jarrett, 1999; Christensen *et al*., 2008a). Paper or hospital protective clothing, such as universal precautions will stop alpha particles and external alpha contamination. Beta particles require stronger shielding, which includes aluminium or thick plastic. Universal or standard protective clothing will provide only partial protection. Gamma and X-rays can be attenuated, but not fully stopped, by lead, thick concrete or depleted uranium. Buildings, vehicles or makeshift barriers with heavy materials may provide some shelter in an emergency; limiting exposure time may be the only option.

Recognize that contaminated materials or clothing can pose a threat to the ambulance and crew—ideally removing such materials at the incident site and appropriate storage will enhance responder and patient safety.

3.6 Biological Effects of Radiation

Radioactivity is a naturally occurring phenomenon; we are exposed to trace amounts on a daily basis. Whenever a person is exposed to radiation, energy is deposited in the body. (Jarrett, 1999; Christensen *et al*., 2008a; Goans, 2007; World Health Organization, 2009; Leikin *et al*., 2007; McFee and Leikin, 2005; International Atomic Energy Association (IAEA), 2009a; Hall and Giaccia, 2005).

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3.6.1 Survival Probable

Patients with exposures less than 100 rad should have very few signs and symptoms if any, although nausea and vomiting are possible, which generally subside in a few hours (Christensen *et al*., 2008a; 2008b).

3.6.2 Survival Possible

Patients exposed to 200–800 rad present with nausea and vomiting that may last 24–48 hours. Reverse isolation, haematology, infectious disease and immunology management are critical. Patients will develop bone-marrow and haematopoietic suppression. Death can occur without strict attention to infection control, the use of blood replacement products and colony-stimulating factors (CSFs). Even antiviral and antifungal agents may become necessary (Jarrett, 1999; Christensen *et al*., 2008a; 2008b).

3.6.3 Survival Improbable

Patients who have received whole-body exposures in excess of 800 rad (some authorities suggest 1000 or above) face a poor prognosis. Even with short-term survival, long-term outcomes are poor. While there have been successfully treated patients who received 800–1200 rad, they often develop severe lung damage a few months later and die (Jarrett, 1999; Christensen *et al*., 2008a; 2008b).

If a mass casualty incident has occurred, patients suspected of receiving very high doses are to be made as comfortable as possible and assigned a non-clinical companion for compassionate care, because survival is unlikely, and resources and clinical manpower limited.

Depending upon the dose, patients are more likely to survive in this era with the development of more effective chemoprophylaxis, infection control, antidotes and intensive care than in prior times.

4 ACUTE RADIATION SYNDROME (ARS)

4.1 Overview

ARS describes a serious pattern of injury that occurs when the body receives a high dose of penetrating radiation over a short period of time (Leikin *et al*., 2003; 2007; McFee and Leikin, 2005; 2009; Harrison *et al*., 2007; International Atomic Energy Association (IAEA), 2009a; Jarrett, 1999; Waselenko *et al*., 2004; Flynn and Goans, 2006; Christensen *et al*., 2008a; 2008b; Berger *et al*., 2006; Goans and Waselenko, 2005; Goans, 2007; Koenig *et al*., 2005; Ricks and Fry, 1990; REAC/TS, 2009; Fliedner *et al*., 2001; NTI and The Center for Nonproliferation Studies at the Monterey Institute of International Studies, 2004; Basic Disaster Life Support (BDLS) Course, 2004; World Health Organization, 2009; Hall and Giaccia, 2005; Mettler and Upton, 1995). The illness follows a predictable clinical course proportionate to the significance of exposure over a few hours to several weeks. The time interval from exposure to symptoms depends upon dose; the higher the dose the shorter the time interval (Leikin *et al*., 2003; 2007; McFee and Leikin, 2009; 2005; Harrison *et al*., 2007; International Atomic Energy Association (IAEA), 2009a; Jarrett, 1999; Waselenko *et al*., 2004; Flynn and Goans, 2006; Christensen *et al*., 2008a; 2008b; Berger *et al*., 2006; Goans and Waselenko, 2005; Goans, 2007; Koenig *et al*., 2005; Ricks and Fry, 1990; REAC/TS, 2009; Fliedner *et al*., 2001; NTI and The Center for Nonproliferation Studies at the Monterey Institute of International Studies, 2004; Basic Disaster Life Support (BDLS) Course, 2004; World Health Organization, 2009; Hall and Giaccia, 2005; Mettler and Upton, 1995).

High dose, high dose rate, penetrating or whole-body exposure are likely to produce severe, possibly fatal ARS. Host factors can play a part in the illness. Age, pre-existing medical conditions, especially chronic, debilitating disease and genetic considerations are also factors. In terms of clinical considerations, the source of radiation, once ARS occurs, becomes irrelevant. Whether a weapon of mass destruction, an industrial accident or a nuclear reactor, if the dose is high enough, it will produce the same ARS effects.

ARS represents clinical manifestations—a spectrum of radiation-induced illnesses whereby the signs and symptoms associated with a specific range of exposure dosages will appear relatively predictably over time.

For example, at low, survivable doses there may be a delay between time of exposure and time of symptoms for days or weeks and, in some cases, months. ARS, which is divided into subsyndromes—haematological (haematopoietic), cutaneous, GI and cardiovascular/neurovascular syndromes—is characterized by the development of groups of symptoms and signs that are manifestations of acute irradiation of the whole body or significant portions of it.

Living cells are differentially sensitive to radiation, referred to as their 'radiosensitivity' or 'radioresistance' (Christensen *et al*., 2008a). This variable sensitivity is the basis for the constellation of symptoms and signs that will appear as the dose of acute whole-body irradiation (WBI) increases. The reactions of various tissues to radiation exposure depend upon a number of factors, including, but not limited to, dose, dose rate, volume of tissue irradiated, schedule of dose delivery, type and quality of radiation, and host factors such as age, other medical conditions and individual variations, among others.

It is important to recognize that *the time to onset of symptoms and signs becomes shorter as the radiation dose increases* (Leikin *et al*., 2003; 2007; McFee and

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Leikin, 2005; Jarrett, 1999; Christensen *et al*., 2008b). The duration of clinical signs and laboratory findings also becomes shorter, that is, the injury or illness progresses more quickly as the dose increases.

Circulating peripheral lymphocytes are among the most radiosensitive. Lymphocytes are highly differentiated (International Atomic Energy Association (IAEA), 2009a; Christensen *et al*., 2008b; Goans, 2007; Basic Disaster Life Support (BDLS) Course, 2004; World Health Organization, 2009; Hall and Giaccia, 2005; Mettler and Upton, 1995; Daniak *et al*., 2003). As they do not undergo mitosis they can nevertheless be killed by radiation during interphase. Many other cells are more sensitive at mitosis. Because of the sensitivity of lymphocytes to radiation injury, loss of these cells can occur relatively quickly—within hours to days. This feature allows clinicians to use lymphocyte depletion kinetics as a biodosimeter.

Stem cells or precursor cells to sperm and blood cells (spermatogonia and myeloblasts) are radiosensitive (Christensen *et al*., 2008a; 2008b; Berger *et al*., 2006; Goans and Waselenko, 2005; Goans, 2007; Koenig *et al*., 2005; Ricks and Fry, 1990; Ricks *et al*., 1990; 2002; REAC/TS, 2009; Fliedner *et al*., 2001; Gusev *et al*., 2002; International Atomic Energy Association (IAEA), 2009b; Basic Disaster Life Support (BDLS) Course, 2004; Hall and Giaccia, 2005; Mettler and Upton, 1995; Daniak *et al*., 2003). Damage to these cells will not become manifest for some days to weeks after radiation injury. Tissues that are considered 'body barriers', such as skin, mucosal linings of the GI system and respiratory tract, and small blood vessels, are less radiosensitive, especially at low-dose short exposure. Significant damage to these cells appears much later, on the order of weeks to months, and sooner at higher doses. Osteocytes, spermatocytes, mature sperm, mature white blood cells, mature RBCs, fibrocytes, chondrocytes, muscle cells and nerve cells are progressively more radioresistant (Christensen *et al*., 2008a; 2008b; Hall and Giaccia, 2005; Mettler and Upton, 1995; Daniak *et al*., 2003). The appearance of damage to these cells occurs over weeks, months or years, and at even higher doses.

It is important to remember two distinct, but important, scenarios:

1. If a known event involving radiation has occurred, practitioners can be expectant of certain systemic illnesses related to radiation exposure
2. If patients present to hospital without a known history of radiation exposure, it is important in the appropriate context of a constellation of symptoms that may not initially seem the result of a 'unifying' infection or pathology, to keep radiation and toxicity within the differential diagnosis. Clearly in the early onset of exposure, especially a low-dose exposure, such patient presentations can be confusing to the unwary clinician. Depending upon the nature and timing of exposure(s), patients may present with mixed symptoms and several signs of ARS.

Key Facts: The notification of a radiation exposure can be worrisome for both the patient and clinician. It is important to remember the following: ionizing radiation at *survivable* doses does not cause the following:

- *Sudden* death
- Incapacitation.

Fairly sudden loss of consciousness, seizures, hypotension and other signs can occur with acute ionizing radiation exposure, but would indicate an LD if the exposure was acute.

Ordinarily, especially in low or survivable doses, loss of consciousness, seizures, hypotension—such severe symptoms and signs will have another aetiology. Even after lethal doses of ionizing radiation, death should not occur for many hours to days, sometimes even months.

Remember—very early, 'non specific' symptoms such as anorexia, nausea, vomiting and low-grade fever may appear with significant *survivable* doses of ionizing radiation AND these are usually *transient*. They are merely a prelude or a prodrome to the phases of ARS to follow.

4.2 Phases of Acute Radiation Syndrome

The evolution of ARS and its various subsyndromes may roughly be divided into four phases: the prodrome, the latent phase, the period of manifest illness and, lastly, the period of recovery or death. The time to onset of each phase and the duration of symptoms and signs is determined by a large number of factors, as discussed above (Leikin *et al*., 2003; 2007; McFee and Leikin, 2005; 2009; Harrison *et al*., 2007; International Atomic Energy Association (IAEA), 2009a; 2009b; Jarrett, 1999; Waselenko *et al*., 2004; Flynn and Goans, 2006; Christensen *et al*., 2008a; 2008b; Berger *et al*., 2006; Goans and Waselenko, 2005; Goans, 2007; Koenig *et al*., 2005; Ricks and Fry, 1990; Ricks *et al*., 1990; 2002; REAC/TS, 2009; Fliedner *et al*., 2001; Gusev *et al*., 2002; NTI and The Center for Nonproliferation Studies at the Monterey Institute of International Studies, 2004; Basic Disaster Life Support (BDLS) Course, 2004; World Health Organization, 2009; Hall and Giaccia, 2005; Mettler and Upton, 1995; Daniak *et al*., 2003).

- Prodromal phase: In the prodrome, any signs and symptoms are generally considered 'non-specific' such anorexia, nausea, vomiting, fatigue and perhaps low-grade fever (Leikin *et al*., 2007; McFee and Leikin, 2005; Jarrett, 1999; Christensen *et al*., 2008a; 2008b; Goans, 2007; World Health Organization,

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2009; Hall and Giaccia, 2005; Mettler and Upton, 1995; Daniak *et al*., 2003).

- Latent phase: No clinical signs are apparent at all. Significant doses will result in a more-or-less slow progression of cellular damage that will not be evident until the manifest illness phase (Leikin *et al*., 2007; McFee and Leikin, 2005; Jarrett, 1999; Christensen *et al*., 2008a; 2008b; Goans, 2007; World Health Organization, 2009; Hall and Giaccia, 2005; Mettler and Upton, 1995; Daniak *et al*., 2003).
- Manifest illness phase: Symptoms and signs of damage to specific cells and tissues will begin to appear, based upon their radiosensitivity (Leikin *et al*., 2007; McFee and Leikin, 2005; Jarrett, 1999; Christensen *et al*., 2008a; 2008b; Goans, 2007; World Health Organization, 2009; Hall and Giaccia, 2005; Mettler and Upton, 1995; Daniak *et al*., 2003).
- Period of recovery or death: If the dose absorbed is not too high, recovery can be complete or can be incomplete, with persistent or long-term sequelae (Leikin *et al*., 2007; McFee and Leikin, 2005; Jarrett, 1999; Christensen *et al*., 2008a; 2008b; Goans, 2007; World Health Organization, 2009; Hall and Giaccia, 2005; Mettler and Upton, 1995; Daniak *et al*., 2003).

It is important to recognize that, like other clinical conundrums, the greatest challenge to the clinician is to make a diagnosis of acute or chronic radiation-induced injury or illness in the absence of an event history. This difficulty arises because the various constellations of symptoms and signs of radiation illness, especially in the early stages, look much like more common injuries and illnesses.

4.3 Syndromes of Acute Radiation

While the clinical team will be called upon to address radiation injury, it is important to remember these patients may suffer from combined injuries, that is, radiation injury or illness, plus trauma or thermal burns (**Table 3**). Combined injuries significantly increase morbidity and mortality, that is, they significantly lower the radiation LD50/60. The key to treating these patients is understanding the likely progression of physiological damage associated with exposure, and planning treatment rapidly and accordingly (Jarrett, 1999; Christensen *et al*., 2008b; Goans, 2007).

4.3.1 Radiation Lethal Dose 50/60 (LD50/60)

In humans, the LD50/60 is approximately 3.5 Gy (350 rads) for acute WBI without treatment. Of a population uniformly exposed to such a dose of ionizing radiation, 50% will die within 60 days if not aggressively treated. The goal is to push the LD50/60 to a higher dose.

Table 3 Acute radiation syndromes and the exposure range associated with them (Jarrett, 1999; Christensen *et al*., 2008b; Goans, 2007)

Acute radiation subsyndromes	Exposure range
Subclinical	<0.75–1 Gy (75–100 rad)
Haematopoietic	>1–2 Gy (>100–200 rad)
Cutaneous	>3 Gy (>300 rad)
Gastrointestinal	>6 Gy (600 rad)
Respiratory	>6 Gy (600 rad)
Cerebrovascular/Central Nervous System (CV/CNS)	>30 Gy (3000 rad)
Neurovascular	

Gy = 100 rad.

4.3.2 Subclinical

Most patients who receive below 100 rads (<1 Gy) are unlikely to have significant illness resulting from their exposure. About 10% of patients exposed at this range may still vomit. Nevertheless, the prognosis is good (International Atomic Energy Association (IAEA), 2009a; Jarrett, 1999; Waselenko *et al*., 2004; Christensen *et al*., 2008b; Berger *et al*., 2006; Goans and Waselenko, 2005; Goans, 2007; Koenig *et al*., 2005; Ricks and Fry, 1990; Ricks *et al*., 1990; Fliedner *et al*., 2001).

The complete blood count (CBC) may be useful in determining the effect of the radiation on the haematopoietic system. The dicentric assay from chromosomal aberration analysis may be necessary to determine doses below 100 rads and guide management.

4.3.3 Haematopoietic Syndrome

The haematopoietic system exhibits the earliest indications of the severity of radiation exposure manifest by the rapid decline of the lymphocyte cell line. This decline leads to immune system dysfunction, increased risk for infectious complications, anaemia, delayed and impaired wound healing and haemorrhage. A whole-body exposure of as little as 100–800 rad will produce the haematopoietic syndrome (Leikin *et al*., 2007; International Atomic Energy Association (IAEA), 2009a; Jarrett, 1999; Waselenko *et al*., 2004; Walter *et al*., 2000; Christensen *et al*., 2008b; Berger *et al*., 2006; Goans and Waselenko, 2005; Goans, 2007; Koenig *et al*., 2005; Ricks and Fry, 1990; Ricks *et al*., 1990; Fliedner *et al*., 2001; Daniak *et al*., 2003).

Infection remains a leading cause of death among radiation-exposed victims resulting from immune-system

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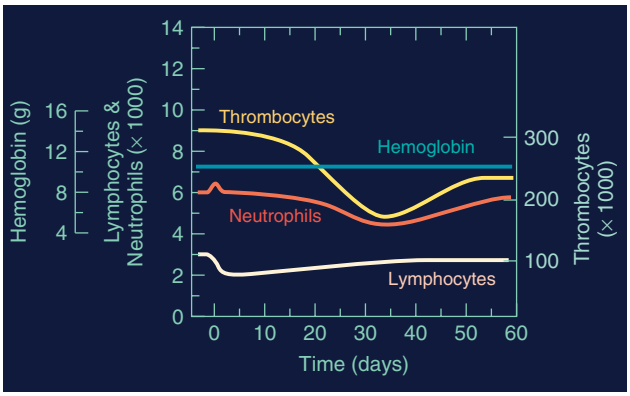

Figure 3 Haematological response to whole-body ionizing radiation at 1 Gy. (Reproduced with permission from International Atomic Energy Agency (IAEA).)

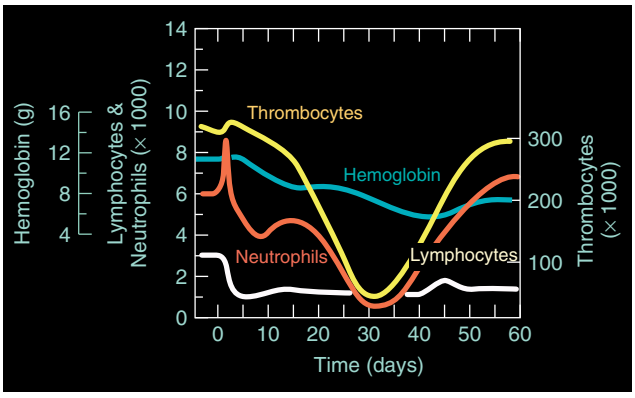

Figure 4 Haematological response to whole-body ionizing radiation at 3 Gy. (Reproduced with permission from International Atomic Energy Agency (IAEA).)

dysfunction. Strict attention to infection control, promotion of host defences through CSFs, nutritional support and chemoprophylaxis is essential.

Acute radiation, or WBI with doses from 1 to 2 Gy (100–200 rad) can cause progressive reduction in cells within bone marrow and peripheral blood as the dose increases (**Figures 3** and **4**; (International Atomic Energy Association (IAEA), 2009a; 2009b).

Stem cells can become depleted. Leucopenia and sometimes pancytopenia may result (International Atomic Energy Association (IAEA), 2009a; 2009b; Jarrett, 1999; Christensen *et al*., 2008b; Goans, 2007; Koenig *et al*., 2005; Ricks and Fry, 1990; Ricks *et al*., 1990; Basic Disaster Life Support (BDLS) Course, 2004; World Health Organization, 2009; Hall and Giaccia, 2005; Mettler and Upton, 1995; Daniak *et al*., 2003). Not unexpectedly, the risk of infections in the radiated patient is expected. Other complications include haemorrhage, anaemia and impaired wound healing. The latter requires early surgical consultation in the

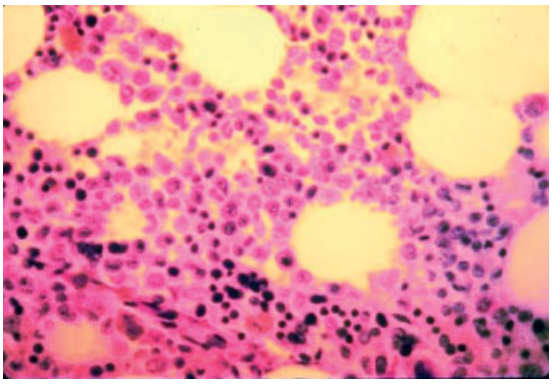

Figure 5 Normal bone marrow. (Reproduced with permission from International Atomic Energy Agency (IAEA).)

event of trauma or injury, since critical decisions when to perform surgery and wound closure must be made based upon the likely and predictable progression of haematological and immune effects associated with radiation illness.

Patients exposed to radiation in the potentially low- to mid-lethal range (2–6 Gy) will experience depression of bone-marrow function with cessation of blood-cell production that will lead to pancytopenia (International Atomic Energy Association (IAEA), 2009a; 2009b; Jarrett, 1999; Christensen *et al*., 2008b; Goans, 2007; World Health Organization, 2009; Hall and Giaccia, 2005; Mettler and Upton, 1995; Daniak *et al*., 2003).

Recall that bone marrow *contains the renewal systems for three haematopoietic cell systems, each with different responses* in terms of their evolutionary/developmental cycles, cellular distribution patterns and post-irradiation responses (**Figure 5**) (International Atomic Energy Association (IAEA), 2009a; 2009b; Jarrett, 1999; Christensen *et al*., 2008b; Goans, 2007; World Health Organization, 2009; Hall and Giaccia, 2005; Mettler and Upton, 1995; Daniak *et al*., 2003). These three lines include erythropoietic (red cell), myelopoietic (white cell) and thrombopoietic (platelet) and evolve from the pluripotential stem cells. The bone marrow houses a complex interplay of compartments; each cell-renewal system consists of and depends upon a stem-cell compartment for the production of erythrocytes, leucocytes (lymphocytes, granulocytes, monocytes, etc.) or platelets, a dividing and differentiating compartment, a maturing (nondividing) compartment and a compartment containing mature functional cells.

Because tissue with rapid cell turnover tends to be the most sensitive to radiation exposure, the stem-cell lines of the bone marrow are among the most radiosensitive tissue of the body owing to their high mitotic rate and their high nuclear-to-cytoplasmic ratio (**Figure 6**) (International Atomic Energy Association (IAEA), 2009a; 2009b).

Of note, and important in determining appropriate medical care, is that with an exposure to ionizing radiation, even in the dose range of 2.5 Gy and higher,

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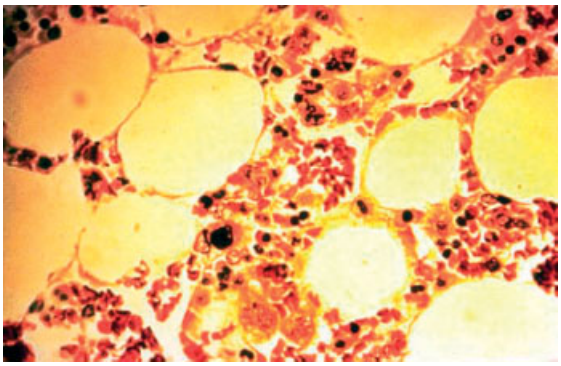

Figure 6 Abnormal/radiated bone marrow. (Reproduced with permission from International Atomic Energy Agency (IAEA).)

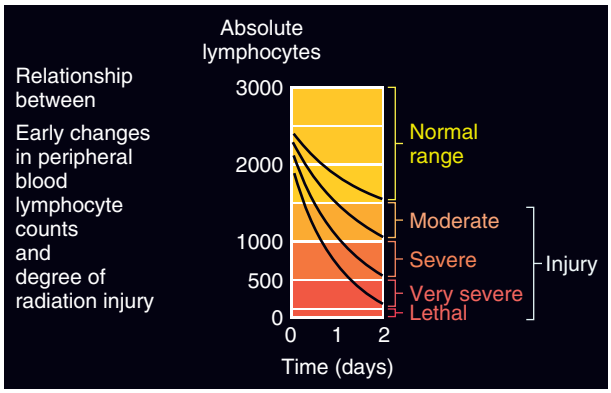

Figure 7 Effect on lymphocyte count after exposure to radiation. (Reproduced with permission from International Atomic Energy Agency (IAEA) and Jacocks, 2003.)

it is likely that a small percentage of the stem-cell population will survive and be capable of continuously expanding progeny (International Atomic Energy Association (IAEA), 2009a; 2009b; Jarrett, 1999; Leikin *et al*., 2003; Christensen *et al*., 2008b; Goans, 2007; Koenig *et al*., 2005; Ricks and Fry, 1990; Ricks *et al*., 1990; Hall and Giaccia, 2005; Mettler and Upton, 1995; Daniak *et al*., 2003). Of concern—the surviving stem cells will be tasked with replenishing their own populations—are the committed stem cells and also the vital differentiated precursor blood-line cells. Given the impact on the bone marrow, patients must be supported as the counts of the shorter-lived cell populations drop and it will take time for recovery, assuming the patient survives. The likelihood of cell replenishment and ultimately survival is dependent upon the dose of radiation, host factors, timeliness and aggressiveness of medical care.

Patients exposed at a high enough level to suffer from the haematopoietic syndrome will undergo the various stages of ARS.

Prodromal symptoms include nausea and vomiting, which, compared with those for the other more advanced syndromes (GI and neurovascular), are less severe, of shorter duration (hours) and easier to control. Time of symptom onset from exposure can range from an hour to about 24 hours.

Central nervous system syndrome signs are unexpected, unless a high-dose exposure occurred. One can expect slight if any change in blood pressure, and only mild watery diarrhoea. Fatigue, malaise, anorexia and drowsiness may be observed, but are not consistently correlated to dose received, as are the onset and severity of nausea and vomiting.

The next, or latent phase may last up to a month. During this period, mild symptoms may occur but these usually only include general malaise, tiredness and weakness.

Subsequently the patient will be in the manifest illness phase, which is characterized by neutropenia, fevers, systemic and localized infections, sepsis and haemorrhage. Patients are highly vulnerable during this phase.

4.3.4 Specific Blood Lines

4.3.4.1 Red Blood Cells

After exposure to ionizing radiation, there is usually no depression or only a very mild depression of RBCs, unless there is concomitant haemorrhage (International Atomic Energy Association (IAEA), 2009a; 2009b; Jarrett, 1999; Christensen *et al*., 2008b; Goans, 2007; World Health Organization, 2009; Hall and Giaccia, 2005; Mettler and Upton, 1995; Daniak *et al*., 2003).

4.3.4.2 Granulocytes

Lymphocyte counts are usually the first blood counts to drop after exposure to ionizing radiation. A drop in lymphocytes occurs 24–48 hours after the injury (**Figure 7**). The speed and extent of the lymphocyte drop is linearly proportional to the severity of the dose to the bone marrow (International Atomic Energy Association (IAEA), 2009a; 2009b; Jarrett, 1999; Christensen *et al*., 2008b; Goans, 2007; Koenig *et al*., 2005; Ricks and Fry, 1990; Ricks *et al*., 1990; World Health Organization, 2009; Hall and Giaccia, 2005; Mettler and Upton, 1995; Daniak *et al*., 2003).

A minor drop is noted after doses of 0.25–1 Gy. At about 1.5 Gy, the drop is around 50%. At 3 Gy, the count drops to 1000 mm^{-3}; at 4–5 Gy, it drops to less than 500 mm^{-3}. A drop to zero implies a dose of greater than 6 Gy.

The drop in lymphocyte count is a crude, but sensitive, important estimation of severity of injury within 48 hours of exposure. For example, a patient whose lymphocyte count stays above 1500 mm^{-3} after 48 hours may have received a clinically significant dose, but the overall prognosis is quite good. On the other hand, a patient whose count drops to less than 500 mm^{-3} in 24 hours demonstrates a profound life-threatening injury.

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Exposure doses >2 Gy can cause an initial paradoxical rise in lymphocyte counts; but this lasts only hours or days and will be followed by a significant drop. The transient rise is caused by demargination of white cells into the circulation. Take caution in interpreting early laboratory results—this is why sequential monitoring is critical. Any CBC taken when the paradoxical rise occurs may be misinterpreted as evidence of infection or even worse, of a good immune system! Patients exposed to moderate doses of radiation are at risk for infection and should be treated within radiation protocols, accordingly.

Doses of 2–5 Gy can cause a second transient rise, which interrupts the initial dramatic drop in counts for several days or a week. This second rise is caused by the products of final differentiation and entry into circulation of marrow polymorphic nucleated cell (PMN) precursor cells, which do not need to undergo further mitotic divisions. The extent and duration of this second rise is variable. Historically it can last for a week and be associated with a rise from about 50% to about 75% of normal. But then, near day 25–35 post-exposure, the neutrophil count continues dropping to a nadir—with levels near 0–20% of normal.

Injury to bone marrow and nucleated blood cells can occur at IR doses as low as 0.25 Gy, but clinical depression of cell counts is usually not noted until a dose of 0.5 Gy.

Key Facts: The lymphocyte count and neutrophil count as biodosimetric tools can be useful for the uncomplicated patient without other injury. With a radiation patient who is also a victim of trauma or has burns—these can cause changes in blood cell counts that will confound interpretation of expected radiation-induced blood-cell kinetics.

4.3.4.3 Platelets

After exposure to ionizing radiation, platelet counts generally drop along with declining neutrophil counts. Unlike with granulocytes, a transient rise in platelet count is not typically expected. Platelet counts drop to zero or near zero with doses that exceed 5 Gy (International Atomic Energy Association (IAEA), 2009a; 2009b; Jarrett, 1999; Christensen *et al*., 2008b; Goans, 2007; World Health Organization, 2009; Hall and Giaccia, 2005; Mettler and Upton, 1995; Daniak *et al*., 2003).

Predictably, the damage to the immune and haematostatic systems emperil the patient. Not only are the number of cells reduced, but the function of those remaining may be altered and thus not fully capable of protecting the patient. Among the few neutrophils and platelets remaining, they are old (senescent) or damaged.

Decrements in RBC and platelet counts occur much later than the decrements of lymphocytes and other leucocytes. Bleeding and anaemia may be significant at higher survivable doses, but do not occur for several weeks after irradiation.

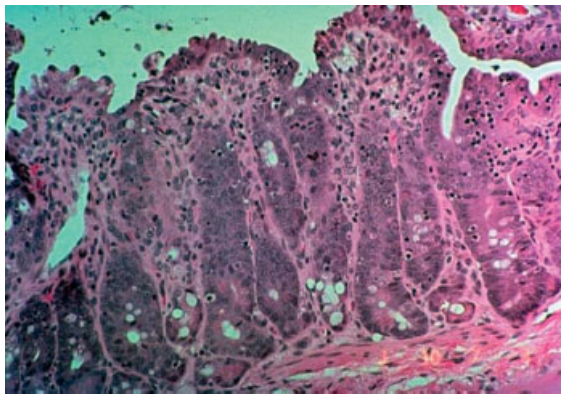

Figure 8 Irradiated gastrointestinal mucosa. (Reproduced with permission from International Atomic Energy Agency (IAEA).)

If, during the 15 day recovery period from the neutropenia, the patient survives neutropenic fever, complications of sepsis and uncontrollable haemorrhage, recovery following the nadir period can be expected.

The goal of clinical management is to lessen the severity of thrombocytopenia and neutropenia, while preventing/minimizing and/or treating infections.

4.3.5 Gastrointestinal Syndrome

The GI tract, like other tissue exhibiting high cell turnover, is very radiosensitive. High-dose exposure can lead to loss of integrity of the cells lining the large intestine (**Figure 8**). Doses of 600–3000 rad (6–30 Gy) will produce this syndrome. The classic symptoms are nausea, vomiting and diarrhoea, as seen in the Litvinenko case (Leikin *et al*., 2007; McFee and Leikin, 2008; 2005; 2009; Harrison *et al*., 2007; Stather 2007; International Atomic Energy Association (IAEA), 2009a; 2009b; Jarrett, 1999; Christensen *et al*., 2008b; Goans, 2007; World Health Organization, 2009; Hall and Giaccia, 2005; Mettler and Upton, 1995; Daniak *et al*., 2003).

It is worth noting that these symptoms can also occur as a psychological response to a terrorism event. The time from exposure to onset of vomiting and diarrhoea provides an important insight into the potential severity of injury; patients who vomit in the first 2–6 hours likely obtained a very high dose. These symptoms result in significant loss of fluids and can set the stage for opportunistic infections that can lead to sepsis. Persistent high fever and bloody diarrhoea are serious. Death can occur within one to two weeks.

Gastrointestinal injury can occur and become evident after an exposure dose of 6–8 Gy (600–800 rad) WBI. As expected in radiation-related injuries, the signs and symptoms of IR, especially as they pertain to the GI syndrome, can be relatively immediate in the aftermath of significantly high doses or take upwards of two weeks

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at survivable doses of WBI. Severe anorexia, nausea, vomiting are the initial GI symptoms.

Generally, exposure to a dose range of 8–30 Gy is fatal, since that dose range causes reproductive death of the mucosal crypt stem cell. Loss of these cells eliminates the bowel's ability to replenish the short-lived mucosal cells that normally slough off during routine GI function.

Since the rate of cell turnover is highest in the small intestine, radiation injury is usually most severe in that section of the GI tract. Disruption of neural control of the gut, abnormal release of intestinal neurohormonal peptides, submucosal oedema and engorgement or stasis of the submucosal blood vessels can be expected, especially in greater than low-dose exposures. As a result, dysfunctional bowel motility, resulting in severe bloody diarrhoea, anaemia, ileus, severe electrolyte disturbances and malnutrition.

Denuding of sections of bowel can be problematic and allow the invasion of bacteria normally relegated to the intestinal lumen into the circulation. Loss of fluid and electrolytes, diminished absorptive capability are likely to accompany this level of damage. Gastrointestinal haemorrhage can occur. Clearly disruption of the GI tract will compromise nutrition.

Like the other types of ARS, the GI syndrome involves three phases: prodromal, latent and manifest periods.

- Prodromal period: Within 30 minutes to two hours of exposure, patients who have received radiation doses of 8–30 Gy may experience severe anorexia, nausea, vomiting and occasionally watery diarrhoea with abdominal cramps, which increase in intensity for 4–8 hours and may be difficult to control with antiemetic medications. These symptoms are usually accompanied by drowsiness, malaise and fatigue. Occasionally, the parotid gland is involved and will be painful. Patients have also complained of a metallic taste sensation. During this phase, depending upon the dose and clinical picture, mild hypotension and tachycardia may be present. The prodromal symptoms continue, but diminish over the first 48 hours after exposure.
- Latent (subacute) phase: The patient's symptoms may subside. This relative asymptomatic period may last for hours or days, depending upon the exposure. Nevertheless, the patient will be very tired and weak.
- Manifest phase: This phase sees the return of severe GI symptoms, including vomiting and diarrhoea that will go through stages: diarrhoea with fever, bloody diarrhoea, then possibly shock and death. Aggressive medical intervention may be able to save some patients.

The pathophysiology of the GI syndrome involves the depletion of the epithelial cells lining the lumen of GI tract, which allows the intestinal bacteria to gain free access to the body. As the GI tract is progressively damaged, it will necrose. Haemorrhage through denuded areas will lead to hypotension and shock. If the dose is not sufficient to cause massive disruption, there will, nevertheless, be a loss of absorptive capacity.

Management of the GI syndrome will need to take into account the haematological syndrome as well.

Key Facts: Instrumentation of the GI tract, such as endoscopy is relatively contraindicated, given the potential for perforation.

NB: At about 6 Gy (600 rad) and higher WBI, multi-organ system damage may become evident. Damage to the endothelium of smaller blood vessels is a basis for multiple organ damage or failure. At 6 Gy or higher, these vessels can be expected to occlude. Tissue/organ ischaemia, necrosis and death can occur.

4.3.6 Pulmonary Effects

Radiation doses in the 8–30 Gy range usually cause death from the GI syndrome. However, such doses also produce potentially life-threatening pulmonary effects of respiratory insufficiency, acute respiratory distress syndrome (ARDS) and pneumonitis (International Atomic Energy Association (IAEA), 2009a; 2009b; Jarrett, 1999; Christensen *et al*., 2008b; Goans, 2007; World Health Organization, 2009).

Pneumonitis is likely to be caused by a complex of factors, including breakdown of vascular permeability, fluid imbalance, free-radical tissue interactions, infectious agents, biological and chemical toxin damage, and inhalation injury from heat, smoke and fumes.

The patient injured by fatal whole-body radiation may die of the GI syndrome before the pulmonary effects become manifest. However, partial-body shielding or other situations may set the stage for pulmonary effects.

4.3.7 Cerebrovascular/Central Nervous System Syndrome

Neuronal tissue is not as radiosensitive as other tissue. Doses of 3000 rad (30 Gy) and above produce this syndrome, which may cause microvascular leaks within the brain. Doses in this range cause significant direct effects, as well as free-radical overload of the cells and basement membranes of the microcirculation system. This leads to, among other damage, massive loss of serum and electrolytes through leakage into the extravascular space, circulatory collapse, oedema, increased intracranial pressure and cerebral anoxia. Acute radiation doses of 30 Gy and above cause death within 72 hours and usually between 24 and 48 hours (Jarrett, 1999; Christensen *et al*., 2008b; Goans, 2007).

Early vomiting and increased cerebral pressure occur within minutes. Patients tend to become confused, disoriented. Convulsions, coma and severe hyperpyrexia result; death is within a couple days. Death is imminent and no recovery from this is likely. Comfort measures and compassionate care should be initiated.

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- Prodrome: In less than an hour and possibly within minutes of exposure, patients receiving these doses begin experiencing prodromal symptoms: a burning sensation within minutes, and severe nausea and usually projectile vomiting within an hour. The symptoms, which are severe and may last more than 24 hours, also include diarrhoea that is occasionally bloody, cutaneous oedema and erythema, hypotension, hyperpyrexia, disorientation, prostration, loss of co-ordination and possibly seizures.
- Latent: Pain may subside. Following the prodromal phase, there may be a brief latent phase of apparent clinical improvement; but this will last for only hours, perhaps a day or two.
- Manifestation: Finally, the victim will succumb to a complex of gross central nervous system dysfunction and total cardiovascular collapse, leading to a relatively prompt and inevitable death.

4.3.8 Cutaneous Syndrome

Radiation injury to the skin, like the other ARS syndromes, has a clinical course with three phases—prodrome, latent, manifest periods (Leikin *et al*., 2007; McFee and Leikin, 2005; 2009; Harrison *et al*., 2007; Stather 2007; McFee and Leikin, 2008; International Atomic Energy Association (IAEA), 2009a; 2009b; Jarrett, 1999; Christensen *et al*., 2008b; Berger *et al*., 2006; Goans, 2007; Koenig *et al*., 2005; Ricks and Fry, 1990; Ricks *et al*., 1990; 2002; Basic Disaster Life Support (BDLS) Course, 2004; World Health Organization, 2009).

- Prodrome: There may be early erythema (**Figure 2**) that may be accompanied by dysesthesias, vague and uncomfortable sensations of heat or pinching.
- Latent: This period, like that of other ARSs, is characterized by the appearance of no clinical change.
- Manifest: The skin injury may ensue with progression of skin damage. Sloughing can occur. Depending upon the extent, it is important to address proper burn management and wound care.

4.3.9 Acute Local Injury

Injuries to smaller or circumscribed areas may result initially or fully as skin damage only. However, it is also possible for more severe and deeper damage, that can include the dermal structures and vasculature, as well as subcutaneous regions. Depending upon the level of exposure, the damage can be profound, requiring aggressive surgical management, even amputation. Surgeons with experience treating immune-compromised patients (cancer patients) or radiation experience are preferred (Leikin *et al*., 2007; McFee and Leikin, 2008; 2005; 2009; Harrison *et al*., 2007; Stather 2007; International Atomic Energy Association (IAEA), 2009a; Jarrett, 1999; Christensen *et al*., 2008b; Goans, 2007; Fliedner *et al*., 2001).

Key Facts:

1. ARS can accompany acute local injury. Clinicians should monitor these patients as with other radiation exposed.
2. Estimating the dose exposure associated with an acute local injury is a complex process; consultation with an HP and/or REAC/TS or other radiation expertise is advised. The delay in appearance of ionizing radiation injury accounts for the difficulty identifying radiation injury and managing these patients.

5 MEDICAL MANAGEMENT OF THE RADIATION-EXPOSED PATIENT

5.1 Initial Response

It is well accepted that a radiation incident could cause a surge of potentially contaminated patients to the ED. Given most HCFs have limited surge capacity and limited experience of handling a hazardous material incident (HAZMAT), let alone a radiation event, one can appreciate the challenge to protecting the staff, HCF and providing rapid, appropriate care (Leikin *et al*., 2003; 2007; McFee and Leikin, 2005; International Atomic Energy Association (IAEA), 2009a; 2009b; Jarrett, 1999; NCRP, 2001; Christensen *et al*., 2008b; Goans, 2007; Koenig *et al*., 2005; Ricks and Fry, 1990; Ricks *et al*., 1990; 2002; Fliedner *et al*., 2001; Basic Disaster Life Support (BDLS) Course, 2004; World Health Organization, 2009).

When responding to a radiation victim, the precise source is not critical to initial care, but is important in selecting the most appropriate protective strategies. It is important to recognize historically the provision of healthcare to radiologically inured or ill patients is a low risk activity; no known history of significant exposure to IR has caused serious injury to healthcare providers.

It is important to acknowledge that the presentation of the patient may not be initially recognized as a radiation victim. However, when radiation is suspected, the use of PPE, at a minimum, and disrobing the patient may reduce the risk posed by a potentially contaminated patient. HAZMAT and radiation experts should be consulted immediately upon even suspecting a radiation victim. Most HCFs have a radiation safety officer or HP—a valuable resource in pre-event preparedness planning, as well as in the initial approach to the patient. They should be well known to the ED, prehospital responders

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and others likely to interact with the radiation patient. They have the expertise, equipment and resources to assist the clinician. Moreover, their knowledge should go beyond direct patient care to assisting with environmental, public-health and community-infrastructure concerns.

If the patient is to have a chance of surviving, this is the overview of requisite care:

- Rapid diagnosis
- Address life-threatening injuries
- Administer specific antidotes as soon as possible
- Decorporative (chelators) and other treatments for internal contamination need to be considered early in the clinical course in order to minimize morbidity and mortality
- Standard toxicological treatment
- Symptomatic and supportive care
- Address each of the ARS syndromes rapidly
- Haematological
- Aggressive supportive care
- Antibiotic prophylaxis
- Treatment of bleeding disorders and anaemia
- Cytokines therapy
- Stem-cell transplants must begin early
- Gastrointestinal
- Protect alimentary function
- Prevent infection
- Provide low-microbial nutrition.

5.2 Medical Management of Radiation Patients

The appropriate medical management of the radiation-exposed patient or group of radiation-terrorism victims is predicated upon the following (Leikin *et al*., 2003; 2007; McFee and Leikin, 2005; International Atomic Energy Association (IAEA), 2009a; 2009b; Jarrett, 1999; NCRP, 2001; Christensen *et al*., 2008b; Goans, 2007; Koenig *et al*., 2005; Ricks and Fry, 1990; Ricks *et al*., 1990; 2002; Fliedner *et al*., 2001; Basic Disaster Life Support (BDLS) Course, 2004; World Health Organization, 2009; Hughes *et al*., 2002):

- Trained clinicians
- Availability of an HP
- A well-developed and practised response plan
- Key personnel are identified and have worked together on drills
- Well-posted information on REAC/TS and other appropriate resources
- Functional and calibrated radiation-detection technology
- Readily available
- Team is practised on the use
- Capability and limitations of technology are well known
- Availability of radiation-specific
- Equipment
- Disposal materials
- Antidotes.

Considering radiation in the differential diagnosis is essential.

The medical management of radiation/combined injuries can be divided into three stages (International Atomic Energy Association (IAEA), 2009a; Jarrett, 1999; Leikin *et al*., 2003; Flynn and Goans, 2006; Walter *et al*., 2000; NCRP, 2001; Christensen *et al*., 2008b; Goans, 2007; Schleipman *et al*., 2004):

1. Triage
2. Initial emergency care
3. Definitive care.

Effective, quality care can be provided, both when there are few casualties and a well-equipped facility and when there are many casualties and a functioning worldwide evacuation system. The therapeutic modalities vary according to current medical knowledge and experience. Precisely implemented modalities will depend on the number of casualties, available medical facilities and resources. Recommendations for the treatment of a few casualties may not apply to the treatment of mass casualties because of limited resources.

It is essential to implement good infection-control measures as soon as radiation. Clearly that should be the dictum in all healthcare, given the rising rate of hospital-acquired infections, but no more critically than in an expected immune-compromised patient.

The management of a radiation casualty involves three critically important and inter-related issues (Leikin *et al*., 2003; 2007; McFee and Leikin, 2005; International Atomic Energy Association (IAEA), 2009a; 2009b; Jarrett, 1999; Waselenko *et al*., 2004; Flynn and Goans, 2006; Walter *et al*., 2000; NCRP, 2001; Goans, 2007; Koenig *et al*., 2005; Ricks and Fry, 1990; Ricks *et al*., 1990; 2002; Schleipman *et al*., 2004; REAC/TS, 2009; Fliedner *et al*., 2001; Gusev *et al*., 2002; NTI and The Center for Nonproliferation Studies at the Monterey Institute of International Studies, 2004; Hall and Giaccia, 2005; Mettler and Upton, 1995; Daniak *et al*., 2003; Hughes *et al*., 2002):

1. Managing severe immune-system compromise
2. Managing/preventing the resulting infectious complications
3. Preventing haemorrhage and severe anaemia due to thrombocytopenia.

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Dose exposures in the haematopoietic syndrome range are survivable. The therapeutic goal is to lessen the severity of neutropenia and thrombocytopenia, while minimizing the likelihood of and/or aggressively treating infection.

Look for and treat immediately life-threatening injuries. Recognize that, in addition to clothes, body fluids can be contaminants, as well as necessary to submit for sampling and diagnostic purposes. Recall that removal of clothes has the potential to reduce contamination by upwards of 90%.

Different types of radiation accidents require different resources and management strategies. These range from simple medical attention to highly sophisticated care at specialty HCFs. Although the radiation exposure may pose a serious health risk to the patient, irradiation or exposure is not necessarily a medical emergency or immediately life-threatening. Moreover, the severity or extent of the radiation injury may not be immediately obvious.

The first priority for the radiation victim is consistent with good medical practice—stabilize the patient and attend to life-threatening injuries first. Attend to the 'ABC' basics—Airway, Breathing and Circulation. Airway protection and fluid replacement may be required. Pain management and control of vomiting may also be necessary.

Triage will be based on associated injuries, presenting signs and symptoms. Remember, radiation is rarely *immediately* life threatening. Other injuries—burns and trauma—should be attended to. If radiation is suspected, rendering surgical care and other interventions must be done in consultation with radiation-health expertise, and infectious disease, surgical and haematology specialists. Once the patient is stabilized and decontaminated, more definitive care can be provided.

5.2.1 Patient Triage

If a multiple or mass casualty event has occurred, especially one that may involve radiation, it is important to implement triage—the sorting of patients into classes of injury/disease for the purpose of expediting clinical care and maximizing the available clinical services and facilities (Jarrett, 1999; Flynn and Goans, 2006; Walter *et al*., 2000; NCRP, 2001; Christensen *et al*., 2008b; Goans, 2007; Koenig *et al*., 2005; Ricks and Fry, 1990; Ricks *et al*., 1990; 2002; Schleipman *et al*., 2004; Liu and Rodgers, 2008; Kennedy *et al*., 1996; Lai *et al*., 2003; Lanoix *et al*., 2002).

One of the main tasks is to determine the level of emergency care. If the accident produces only a small number of casualties, medical management should not cause major problems in most countries. An accident involving tens or hundreds of individuals exposed, or suspected of exposure, would cause great difficulties, especially in hospitalization.

Preplanning is clearly important, and should be adapted to the system of medical care contemplated for catastrophic event situations.

In a radiation patient, the early clinical symptoms serve for the basis of sorting persons exposed to radiation and deciding upon proper medical care at an individual level. Proper planning, teamwork and the availability of detection technology, expertise and critical resources, including antidotes, will determine the success of response. In subsequent sections detection technology will be discussed.

As will be discussed momentarily in the medical and physiological sections, the most important early clinical signs are onset of nausea, vomiting and diarrhoea. The time from exposure to appearance of symptoms is quite telling in terms of potential severity of illness. Keep in mind the psychological impact of an event; this may also cause mild GI symptoms.

Estimating the degree of radiation exposure and attendant damage is challenging. Ongoing diagnosis and reassessment is required throughout the patient's clinical course (Jarrett, 1999; Christensen *et al*., 2008a; 2008b; Goans, 2007; Koenig *et al*., 2005; Ricks and Fry, 1990; Ricks *et al*., 1990; 2002; Schleipman *et al*., 2004; International Atomic Energy Association (IAEA), 2009b).

Recall there is the prodrome, when symptoms begin within hours of exposure, characterized by GI signs and symptoms, including nausea, vomiting, diarrhoea, fatigue, weakness, fever and headache. These symptoms generally do not last longer than 24–48 hours after exposure, but a vague weakness can persist for an undetermined length of time.

Note that the time of onset, severity and duration of these signs are dose dependent and dose-rate dependent and should be used in conjunction with early biological parameters, such as granulocyte and lymphocyte levels, to determine the presence and severity of the ARS.

Given radiation will affect blood cells, in a dose-dependent fashion, an initial baseline sample should be obtained as early as possible after irradiation. Frequent sampling will increase the reliability of dose estimates. If lymphocytes have decreased by 50% and are less than $1000\,\mu l^{-1}$ within 24–48 hours, the patient has received at least a moderate dose of radiation (International Atomic Energy Association (IAEA), 2009a; Christensen *et al*., 2008b).

At the initial stage of patient evaluation, take into account the physiology of radiation illness, the relationship between exposure dose and symptoms, and have ready the appropriate surveillance equipment, as well as expertise.

From the moment of exposure, the clock is ticking. A likely fatal exposure may experience the following:

Vomiting within the first hour, especially if accompanied by significant, possibly haemorrhagic diarrhoea, is associated with fatal doses. Vomiting, even within the

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first 2–3 hours, is usually associated with a severe radiation dose. Due to the transient nature of these symptoms, it is possible that the patient will have already passed through the initial phase of GI distress before being seen. However, the severity of these symptoms is usually memorable by the patient and should be inquired about.

Casualties who have received a potentially lethal radiation injury show a significant rise in body temperature within the first few hours postexposure. The occurrence of a fever and chills within the first day postexposure is an ominous sign, often associated with a severe and life-threatening radiation dose. Hyperthermia may occur in patients who receive lower, but still serious radiation doses (2 Gy or more).

A person who received a WBI dose of more than 10 Gy, or comparable doses to local body regions, is likely to develop erythema within the first day postexposure, albeit the erythema should be restricted to the affected area. With whole-body doses, lower, but still in the potentially fatal range, erythema is less frequently seen (Jarrett, 1999; Christensen *et al*., 2008b; Goans, 2007; Koenig *et al*., 2005; Ricks and Fry, 1990; Ricks *et al*., 1990; 2002; Schleipman *et al*., 2004; World Health Organization, 2009; Hall and Giaccia, 2005; Mettler and Upton, 1995; Daniak *et al*., 2003).

A noticeable drop in systemic blood pressure has been reported among victims who received a lethal dose of WBI. Severe hypotension after irradiation is associated with a poor prognosis.

Neurological tissue requires a large dose to develop damage. Virtually all persons who demonstrate clear signs of damage to the central nervous system within the first hour postexposure have received an LD. Symptoms include mental confusion, convulsion and coma. Intractable hypotension as the cardiovascular system becomes involved, along with neuro effects, will probably accompany these symptoms. Despite vascular support, death usually occurs within 48 hours.

In the triage of multiple patients, identifying patients likely to have been exposed is essential to managing patients effectively. The following quick thoughts are likely to assist.

- Unlikely exposure: The patient with unlikely radiation injury demonstrates no more than episodes of nausea and vomiting and usually retains lymphocyte counts above 1500 mm^{-3} in 48 hours.
- Probable exposure: The patient with probable radiation injury has prodromal symptoms that include more pronounced or severe and sustained nausea and vomiting that began within one hour to one day of exposure and last for hours. The lymphocyte count will drop to around 1000–1500 lymphocytes mm^{-3}, but there should be no vascular or central nervous system symptoms.
- Likely severe or fatal exposure: The patient who has received a severe, potentially fatal radiation injury (who may become categorized as 'expectant') in a triage situation presents with uncontrollable nausea and vomiting within one hour after exposure. Those symptoms are frequently associated with explosive, bloody diarrhoea, hypotension, a burning skin sensation and obvious CNS signs.

5.3 General Surgical Considerations

Radiation can complicate wound healing. Therefore surgical procedures should be performed early in the course of a radiological exposure, preferably within 24–36 hours. Surgical procedures performed after 36–48 hours from the time of the radiation injury may be complicated by failure to heal and a variety of infections. Tissue oedema, leakage of fluids and vascular damage make an ideal medium for growth of micro-organisms. The immune compromise associated with radiation-induced haematopoietic damage can also complicate management. Skin grafts, if performed too early in the course of a radiation injury, may not take, especially if there is significant injury to the microvasculature supporting the skin. As in other clinical situations, the risk–benefit ratio must be considered; emergency surgery must be performed in life-threatening situations, taking into account the ongoing evolution of immune and haematology damage. Precautions, including anticipating unexpectedly active bleeding should be part of preoperative planning for the radiation victim (International Atomic Energy Association (IAEA), 2009a; 2009b; Jarrett, 1999; Leikin *et al*., 2003; Christensen *et al*., 2008b; Goans, 2007; Koenig *et al*., 2005; Ricks and Fry, 1990; Ricks *et al*., 1990; 2002; REAC/TS, 2009; Fliedner *et al*., 2001; Gusev *et al*., 2002; World Health Organization, 2009; Hall and Giaccia, 2005).

5.4 Antidotes

The medical management of acute radiation patients, especially in a high-dose exposure is complex. The use of immune stimulation, infection control/prophylactic chemotherapy and antidotes may all be required (McFee *et al*., 2004; Leikin *et al*., 2003; 2007; United Nations, 2000; International Atomic Energy Association (IAEA), 2009a; 2009b; Jarrett, 1999; Waselenko *et al*., 2004; Flynn and Goans, 2006; NCRP, 2001; Christensen *et al*., 2008a; 2008b; Berger *et al*., 2006; Goans and Waselenko, 2005; Goans, 2007; Koenig *et al*., 2005; Ricks and Fry, 1990; Ricks *et al*., 1990; 2002; Fliedner *et al*., 2001; Gusev *et al*., 2002; Oliveira *et al*., 1991b; Basic Disaster Life Support (BDLS) Course, 2004; CDC, 2006).

There are several antidote classes available: these include decorporative (separate the patient from the

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poison), mechanistic (interfere with the process of toxicity) or other mechanisms (McFee and Leikin, 2005; Jarrett, 1999; Christensen *et al*., 2008b; Goans, 2007).

Selecting which approach to take requires consultation with toxicologists and radiation experts, as well as haematologists and infectious disease experts (Christensen *et al*., 2008b; Goans, 2007; Daniak *et al*., 2003).

The uptake and/or incorporation of radioactive materials is relatively fast for some of the security-threat or nuclear-accident radionuclides. These include radioiodines and radiocaesium. Recalling the toxicologists axiom 'time is tissue', treatment should begin very quickly—ideally within hours.

'Antidotes' may not be curative (Christensen *et al*., 2008b). Nevertheless, they can often 'buy time' for the body to heal or regroup. Antidotal therapies are generally categorized into the following broad categories:

- Saturation and blocking, for example the use of stable KI to prevent thyroid uptake and incorporation
- Ion binding, for example the use of Prussian blue (ferric ferricyanate or ferric hexacyanoferrate) for radiocaesium as well as both radio- and nonradioactive thallium
- Competitive displacement, for example the use of calcium to compete with radioactive strontium
- Chelation, for example, the use of diethylenetriamine pentaacetate (DTPA or pentetate) for the transuranic radionuclides: americium, curium and plutonium
- Isotopic dilution, for example the use of high intakes of water for tritium that has ^{3}H or is radioactive. Recall the hydrogen in water (H_20) can compete with the 3H in tritium. Caveat—be cautious of fluid status.

5.4.1 Antidotes for Specific Situations

5.4.1.1 Caesium (^{137}Cs)

^{137}Cs is often found in medical radiotherapy and industrial instruments. It was used in a Chechen threat as a component of an SDD deployed in Moscow. It emits both beta and gamma, is completely absorbed by the lungs, from the GI tract and from wounds. Soluble in most forms, metabolically it is a potassium analogue. Following intake, caesium redistributes much like potassium. Radioactive caesium is internalized, transported, metabolized and excreted exactly like nonradioactive caesium. The pathology from internalization of ^{137}Cs is related to its radiological toxicity (McFee and Leikin, 2005; Jarrett, 1999; Christensen *et al*., 2008b; Goans, 2007; Oliveira *et al*., 1991a; 1991b; US Environmental Protection Agency, 2009; Basic Disaster Life Support (BDLS) Course, 2004).

Primary toxicity with ^{137}Cs like other radiation risks is by WBI (Jarrett, 1999; Christensen *et al*., 2008b; Goans, 2007; Oliveira *et al*., 1991b; US Environmental Protection Agency, 2009; Heyltex Industries, 2009).

Treatment consists of including the full range of clinical care—from dose estimation, to addressing the impact and likelihood of ARS, to providing early administration of Prussian Blue (Radiogardase®) or ferric hexacyanoferrate, which binds radioactive and nonradioactive caesium and thallium toxicants in the GI tract by ion exchange, adsorption and mechanical trapping within the structure; it is used for decorporation of such toxicants (Jarrett, 1999; Christensen *et al*., 2008b; Heyltex Industries, 2009; US Department of Health and Human Services, 2009). This ion exchange binds and helps to prevent the absorption of the toxicant, reducing the biological half-life of radioactive caesium to approximately one-third of normal, which follows an enteroenteric cycle (Christensen *et al*., 2008b; Heyltex Industries, 2009; US Department of Health and Human Services, 2009).

Prussian Blue is available in 500 mg capsules that can be safely opened and mixed with food or beverages to ease administration (CDC, 2009; Heyltex Industries, 2009; US Department of Health and Human Services, 2009). The usual dose for adults, breastfeeding women and children older than two years of age is 3 g, three times a day for at least 30 days. Possible adverse effects include nausea, constipation and bluish teeth or faeces (opened capsules).

5.4.1.2 Cobalt (^{60}Co)

Cobalt is widely used in medical radiotherapy devices, as well as in food irradiators. Most likely this threat will occur from theft or improper disposal of medical/industrial equipment, similar to the Goiano, Brazil event (only this involved caesium). However, with the ready availability in often undersecured locations, Co may be used in an SDD or RDD (Jarrett, 1999; Christensen *et al*., 2008a; 2008b; Goans, 2007; World Health Organization, 2009).

It is rapidly absorbed from the lung; less than 5% is expected to be absorbed from the GI tract. Absorption from wounds is largely understudied or uncharacterized. The primary toxicity is expected from a WBI resulting in ARS.

Although some suggest gastric lavage or other GI decontamination, there are insufficient data to commend this practice without consultation on a patient-by-patient basis with toxicologists and HPs. The US Military suggest the use of penicillamine chelation for extreme cases.

5.4.1.3 Americium (^{241}Am)

Americium is a decay 'daughter' of plutonium, and is an alpha emitter. Found in smoke detectors, as well as industrial instruments, it is easy to acquire. Fallout from the detonation of a nuclear weapon will result in ^{241}Am. It is a heavy-metal poison, but in large amounts can cause WBI. Emission of gamma can be detected through the use of a FIDLER (field instrument for the detection of

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low-energy radiation) detector (McFee and Leikin, 2005; Jarrett, 1999; Christensen *et al*., 2008b; World Health Organization, 2009).

GI absorption is minimal, but inhalation results in 75% absorption, with 10% retained. It may be readily and rapidly absorbed through wounds. Elimination is through urinary and hepatic excretion.

Treatment (see Section 5.4.1.5 on Transuranic Elements) includes chelation with zinc diethylenetriamine pentaacetate (Zn DTPA) and calcium diethylenetriamine pentaacetate (Ca DTPA) with in the first 24–48 hours following pulmonary exposure. EDTA (ethylenediaminetetraacetic acid) chelation can be used until DTPA is available. Consultation with medical toxicologists and HPs is a critical early strategy.

5.4.1.4 Iodine ($^{131-135}I$)

Radioactive iodine will occur after nuclear reactor accidents or their destruction by terrorism. It is a normal fission product in fuel rods. The primary toxicity is via beta rays, with some gamma. The thyroid gland is the most vulnerable organ; uptake of radioactive iodine resembles therapeutic thyroid ablation, especially untreated. Following the Chernobyl disaster, there was a high incidence of childhood thyroid cancer in the region (McFee *et al*., 2004; Leikin *et al*., 2007; McFee and Leikin, 2005; International Atomic Energy Association (IAEA), 2009a; Jarrett, 1999; Flynn and Goans, 2006; Walter *et al*., 2000; NCRP, 2001; National Institute of Occupational Safety and Health, 2009; Christensen *et al*., 2008a; 2008b; Schleipman *et al*., 2004; Basic Disaster Life Support (BDLS) Course, 2004; The Chernobyl Forum 2003–2005, 2009; World Health Organization, 2009; Kristoff, 2004; Nuclear Threat Initiative (NTI), 2009; Samuel and Dolan, 1977).

Administration of KI, ideally within six hours of exposure is essential for those near the site, or downwind of a plume of debris. Adults should receive the 130 mg tablet, while children 3–18 can receive 65 mg, children one month to three years should receive 32 mg and infants up to one month can receive 16 mg daily until the environmental threat subsides. Pregnant women and their infants can take normal doses. Children who weigh 150 lbs (~70 kg) can take the adult dose. Adverse affects may include fever, rash, abdominal pain, nausea and a metallic taste.

Oral propylthiouracil has been suggested, but this should be done in careful consultation with radiation-health experts and endocrinologists.

5.4.1.5 Transuranic Elements: Plutonium, Americium, Californium, Curium

The FDA has approved the use of two heavy-metal chelators, Ca-DTPA and Zn-DTPA for the treatment of internal contamination by plutonium, americium, californium and curium transuranic soluble salts (Food and Drug Administration (FDA), 2009; International Atomic Energy Association (IAEA), 2009a; Jarrett, 1999; Christensen *et al*., 2008b; Goans, 2007; World Health Organization, 2009; US Department of Health and Human Services, 2009).

DTPA is a chelating agent which exchanges either calcium or zinc for another metal with greater binding ability, carrying it to the kidneys where it is excreted in the urine. The chelating efficacy is greatest immediately, or within one hour, following uptake, when the radionuclide is circulating in or available to tissue fluids and plasma.

Ca-DTPA is approximately 10 times more effective within the first 24 hours than Zn-DTPA for chelation of transuranics and therefore should be used whenever larger body burdens are expected. However, after 24 hours, Zn-DTPA is as effective as Ca-DTPA and should be used for protracted therapy because of its lesser toxicity. If Zn-DTPA is not available, or you are treating a pregnant patient, Ca-DTPA is acceptable for the duration of therapy. Monitor for toxicity.

Zn-DTPA is recommended for sensitive populations such as children, pregnant women and patients with known kidney disease or bone-marrow suppression.

The dose of either Ca-DTPA or Zn-DTPA for adults is 1000 mg and for children under 12 years of age 14 mg kg^{-1}, not to exceed 1 g. It should be administered by a 3–4 minute slow intravenous push or dilute infusion in 100–250 ml of dextrose 5%, or 0.9% sodium chloride, or lactated Ringer's solution over 30 minutes. If inhalation was the route of exposure, consider nebulizing either Ca- or Zn-DTPA in a 1:1 mixture with saline or sterile water via a nebulizer. Caution and extra monitoring with asthma patients is recommended, as underlying pulmonary illness can be exacerbated (Christensen *et al*., 2008b; US Department of Health and Human Services, 2009; Food and Drug Administration (FDA), 2009).

Adverse events can include headache, lightheadedness, nausea, metallic taste, pruritis, injection-site discomfort, dermatitis, chest pain, nausea and diarrhoea.

Key Facts: Chelation therapy can have a deleterious impact upon electrolytes. DTPA agents can deplete zinc, magnesium and manganese. Regular monitoring and replenishing electrolytes is suggested.

Follow-up and additional therapy may be continued. DTPA chelating agents are manufactured by Hamelin Pharmaceuticals in Germany.

DTPA should not be used for uranium or neptunium internal uptake.

5.4.1.6 Strontium (^{90}Sr)

Strontium-90 (^{90}Sr) is a fission product and radioactive 'daughter' of uranium. Sr^{90} and its daughters emit beta and gamma rays, as well as pose an external hazard in large quantity. Strontium biologically follows calcium in the body. It is readily absorbed by both the respiratory and GI routes (Jarrett, 1999; Goans, 2007).

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Immediately after ingestion, the use of over-the-counter (OTC) antacids such as aluminium phosphate can reduce GI absorption of strontium. Aluminium-containing antacids are the most effective, reducing strontium uptake by 50–85%. In the acute aftermath of radioactive strontium exposure, calcium gluconate 2 g mixed into 500 ml solution can be given intravenously. The use of barium sulfate (300 g po), in the immediate postaccident or exposure setting may block intestinal absorption. Large doses of calcium and acidification can increase excretion.

5.4.1.7 Tritium (^{3}H)

Tritium is a hydrogen with a nucleus composed of two neutrons and one proton. Used in nuclear weapons and luminescent gun sights, it is unlikely to pose a significant threat unless in a closed space, as tritium gas rapidly diffuses. Tritium is a beta emitter. Though not a high-threat irradiation hazard, nevertheless, it equilibrates with body water rapidly and is excreted in urine; samples will be positive within one hour of a significant exposure. Biological $t_{1/2}$ is 10–12 days. Oral hydration should help reduce body burden, but caution should be exercised to not fluid-overload the patient (Jarrett, 1999).

5.4.1.8 Uranium ($^{238,235,239}U$)

Uranium is found in depleted uranium, naturally occurring uranium, fuel rods and as nuclear-weapon-grade material (Jarrett, 1999; Christensen *et al*., 2008b; Goans, 2007).

Uranium and its radioactive 'daughters' emit alpha, beta and gamma radiation. Like other toxicants, absorption is determined by the chemical state. Soluble salts, as expected, would be readily absorbed, while the metal isn't. Inhaled uranium compounds can be metabolized and excreted in the urine. Following acute exposure, urinary levels of 100 $\mu g\,dl^{-1}$ may cause renal failure.

Generally, uranium presents a greater chemical than radiological hazard. Alkalize the patient with sodium bicarbonate in order to promote excretion. Alkaline urine forms a nontoxic uranium carbonate complex that is promptly excreted through the kidneys.

Important factors in determining the effectiveness of this method include the chemical form and particle size of the inhaled uranium. Sodium bicarbonate should be administered either orally or intravenously.

Tubular diuretics may be beneficial. Regularly monitor these patients. In addition to the 'standard' radiation-related lab tests, perform a urinalysis, 24 hour urine uranium bioassay, serum BUN (blood urea nitrogen) and creatinine, β2-microglobulin, creatinine clearance and liver studies. Involve a nephrologist, especially one familiar with radiation effects, early in the management of such patients (**Table 3**).

5.5 Haematological Support

5.5.1 Cytokines

Growth factors or CSFs are critical to management of the haematopoietic syndrome. Granulocyte colony stimulating factor (G-CSF), GM-CSF and the pegylated form of GCSF are three cytokines used to treat cytopenias in oncology patients. The rationale for their use in the medical management of patients from radiation incidents is based upon that experience. Cytokines cause proliferation and differentiation of stem cells that lead to increased numbers of circulating blood cells. For optimal efficacy, they need to be administered early, preferably as soon as it is determined that there may have been an acute WBI of >1.5–2 Gy (150–200 rad) (Leikin *et al*., 2007; McFee and Leikin, 2005; 2009; Harrison *et al*., 2007; International Atomic Energy Association (IAEA), 2009a; 2009b; Jarrett, 1999; Flynn and Goans, 2006; Christensen *et al*., 2008a; 2008b; Berger *et al*., 2006; Goans and Waselenko, 2005; Goans, 2007; Oliveira *et al*., 1991b; Basic Disaster Life Support (BDLS) Course, 2004; World Health Organization, 2009; Scott, 2007; CDC, 2006; Hall and Giaccia, 2005; Mettler and Upton, 1995; Daniak *et al*., 2003; Christensen *et al*., 2008b; Goans, 2007; Scott, 2007; CDC, 2006; Hall and Giaccia, 2005; Mettler and Upton, 1995; Daniak *et al*., 2003).

Cytokines stimulate proliferation and differentiation of haematopoietic stem cells. The following are recombinant forms of stem cells:

- G-CSF (Neupogen, Amgen Inc.)
- GM-CSF (Leukine, Berlex)
- Pegylated form of G-CSF (Neulasta, Amgen Inc.).

Cytokines include GM-CSF, macrophage colony-stimulating factor (M-CSF), G-CSF, termDefstem cell factor (SCF) and the interleukin series (IL-1 to IL-16).

GM-CSF and G-CSF have been available for the treatment of radiation myelosuppression. G-CSF and GM-CSF are currently in widespread clinical use for the treatment of acute neutropenic conditions, and, in turn, the management of infections, following radiochemotherapy of cancer patients.

G-CSF and GM-CSF are valuable tools used that act upon uncommitted stem-cell populations within the bone marrow to increase the mitotic rate and accelerate repopulation, differentiate daughter cells to become committed stem cells, speed the maturation process and improve the function of existing granulocytes. Given that it is likely after a radiation exposure that some stem cells remain, even in damaged bone marrow, these factors can improve the immune function of existing cells, while speeding the recovery of stem-cell populations and reducing the time of immune system compromise. Studies of neutropenic

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cancer patients reveal that use of these cytokines reduces infection rates, admissions and days hospitalized.

GM-CSF and G-CSF confer an obvious advantage: their effect on granulocytes and myeloid stem-cell lines do not come at the expense of other marrow cell lines.

5.5.1.1 Dosing

G-CSF, for example Filgastim (Neupogen/AMGEN) is given at 100–200 mcg m^{-2} day^{-1}. GM-CSF, for example Sargramostim (Leukine/Immunex) is given at 200–400 mcg m^{-2} day^{-1}. Both are given intravenously or subcutaneously and should be initiated promptly upon diagnosis of significant bone-marrow damage and continued until recovery of neutrophil counts is sustained above 800 mm^{-3}.

Key Facts:

- G-CSF and GM-CSF increase rate of haemopoietic recovery in patients after radiation exposure and may obviate need for a bone marrow transplant (BMT), when stem cells are still viable. ILs (IL-1 and IL-3) act in synergism with GM-CSF
- Cytokines were successfully used for radiation victims after Goiânia, San Salvador, Israel, Belarus and Istanbul accidents
- A benchmark absolute lymphocyte count (ALC) of less than 500 μl^{-1} may be considered a threshold for beginning cytokine therapy in the first two days. The therapeutic goal of cytokine application is to minimize the extent and duration of radiation-induced neutropenia, and hence minimize the subsequent risk of infection.
- In order to achieve maximum clinical response, G-CSF or GM-CSF should be started 24–72 hours after exposure. This provides the opportunity for maximum recovery. Cytokine administration should continue, with daily consecutive injections, to reach the desired effect of an absolute neutrophil count (ANC) of 1000 μl^{-1} after the ANC bottoms.

A side effect associated with G-CSF (filgrastim) is medullary bone pain, which may be observed shortly after initiation of G-CSF treatment and again just before onset of neutrophil recovery. G-CSF may exacerbate pre-existing inflammatory conditions.

Side effects from GM-CSF (sargramostim) are fever, nausea, fatigue, headache, bone pain and myalgia. It is not clear whether side effects of G-CSF or GM-CSF differ markedly when conventional doses are administered.

5.5.2 Blood Products

Trauma or thermal burns may require the early use of blood products. If the emergency situation permits, these blood products should be leukocyte-reduced and irradiated to 25 Gy to prevent transfusion-associated graft vs. host disease. Do not withhold life-saving blood products. Packed RBCs and platelets for haematopoietic damage will not be required for several weeks at survivable acute WBI doses (Christensen *et al*., 2008b).

5.5.3 Stem-Cell Transplants

Pancytopenia and depletion of stem cells from higher radiation doses may eventually require stem-cell transplants. There are a number of methods for stem-cell transplantation, however this is not a matter for concern in the ED early in the clinical course. Blood for tissue typing should be drawn in the ED if possible, however. Later consultation with a specialist in haematology/oncology may be required to evaluate the necessity of using the valuable resource (International Atomic Energy Association (IAEA), 2009a; 2009b; Jarrett, 1999; Goans, 2007; Koenig *et al*., 2005; Ricks and Fry, 1990; Ricks *et al*., 1990; 2002; Schleipman *et al*., 2004; Scott, 2007; Hall and Giaccia, 2005; Mettler and Upton, 1995; Daniak *et al*., 2003).

5.5.4 Immunoglobulin Administration

Immunoglobulins have not been shown to be beneficial for radiation casualties on a general basis. However, their use may be beneficial in specific diseases (International Atomic Energy Association (IAEA), 2009a).

Passive intravenous administration of immunoglobulin (IgG) following acute radiation injury is a therapeutic measure employed to counter the suppressive effects of irradiation on the immune system and to diminish susceptibility to infection or its pathological consequences. The therapeutic strategies for using IgG treatment fall into two, quite distinct, camps. The first, more commonly applied strategy (e.g. in WBI patients receiving bone-marrow transplants) involves periodic blood infusions in an attempt to bolster the diminished IgG blood plasma levels that are critical in combating a variety of infections. The second strategy, which is more experimental in nature and less certain in terms of efficacy, is designed to selectively control the pathogenic responses related to septic shock and associated overexpression of inflammatory cytokines.

5.6 Preventing Infections

Nosocomial infections can be a disaster in radiation casualty cases. The loss of a functional immune system predisposes these patients to severe, potentially fatal infections (International Atomic Energy Association (IAEA), 2009a; Jarrett, 1999; Christensen *et al*., 2008b; Goans, 2007; Koenig *et al*., 2005; Ricks and Fry, 1990; Ricks *et al*., 1990; 2002; Basic Disaster Life Support (BDLS) Course, 2004; Hall and Giaccia, 2005; Mettler and Upton, 1995; Daniak *et al*., 2003).

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Once the granulocyte count is affected, reverse isolation should be initiated. Nursing and infection control personnel should be forewarned of the need for rigorous environmental and visitor control.

Overall recommendations for management of infection are:

- Initial care of medical casualties with moderate and severe radiation exposure should include early institution of measures to reduce pathogen acquisition, with emphasis on low-microbial-content food, acceptable water supplies, frequent hand washing (or wearing of gloves) and air filtration.
- Early collaboration with infectious-disease and HP expertise.
- A standardized plan for the management of febrile, neutropenic patients must be devised.
- Empiric regimens must contain antibiotics broadly active against gram-negative bacteria, but antibiotics directed against gram-positive bacteria need be included only in institutions where these infections are prevalent.
- No single antimicrobial regimen can be recommended above all others, as pathogens and susceptibility vary with time.
- If infection is documented by cultures, the empiric regimen may require adjustment to provide appropriate coverage for the isolation. This should not narrow the antibiotic spectrum.

5.7 Fever

While the irradiated patient is at severe risk of sepsis, any neutropenic fever should be aggressively evaluated and, unless a specific pathogen is identified, empiric triple antibiotic therapy should be initiated (International Atomic Energy Association (IAEA), 2009a; Jarrett, 1999; Waselenko *et al*., 2004; Christensen *et al*., 2008b; Goans, 2007; Koenig *et al*., 2005; Ricks and Fry, 1990; Ricks *et al*., 1990; 2002; REAC/TS, 2009; Hall and Giaccia, 2005; Mettler and Upton, 1995; Daniak *et al*., 2003).

Empirical/specific therapy should be maintained until granulocytes exceed $800\,mm^{-3}$. If antibiotics fail to control the fever, or if specific fungal or viral pathogens are suspected on clinical grounds, aggressive use of antifungal and antiviral agents should be considered.

This 'empirical' regimen of antibiotics should be selected, based on the pattern of bacterial susceptibility and nosocomial infections in the particular institution and the degree of neutropenia. Broad spectrum empirical therapy with high doses of one or more antibiotics, avoiding aminoglycosides whenever feasible due to associated toxicities, should be used. Broad-spectrum antibiotic coverage includes double β-lactam antibiotics such as a third-generation cephalosporin and ureidopenicillin, monotherapy with imipenem or a third-generation cephalosporin, and vancomycin plus a third-generation cephalosporin.

Neutropenic fever therapy:

- Survey for possible source, pancultures
- Administer antibiotics for ANC $<500\,mm^{-3}$
- Use-broad spectrum antibiotic coverage
- Add amphotericin for prolonged fever lasting five to seven days after starting standard antibiotics
- Continue antibiotics for duration of ANC $<1000\,mm^{-3}$.

If there is evidence of resistant Gram-positive infection, vancomycin should be added.

Although antibiotics and antibiotic-associated diarrhoea is a well-described, as well as an emerging, hospital-acquired pattern of infection, often caused by *Clostridium difficile*, nonetheless, stool cultures should be examined for *Salmonella*, *Shigella*, *Campylobacter* and *Yersinia* as well as *Clostridium difficile*.

Oral and pharyngeal mucositis and esophagitis suggest herpes simplex infection or candidiasis. Empiric antiviral and/or antifungal therapy should be considered.

Surveillance cultures may be useful for monitoring acquisition of resistant bacteria during prophylaxis and emergence of fungi. A once- or twice-weekly sampling of surveillance cultures from natural orifices and skin folds (e.g. axillae, groin) would be reasonable, but should be modified according to the institutional patterns of nosocomial infections. A chest radiograph should be considered at initiation of empirical therapy. This may aid in definitive diagnosis of a new pulmonary infiltrate obtained during the course of neutropenia. Several principles have been stated that are generally applicable to the febrile neutropenic patient and provide a foundation upon which a specific initial regimen may be selected.

Antifungal coverage with amphotericin B should be added, if indicated, for patients who remain persistently febrile for seven days or more on antibiotic therapy, in association with clinical evidence of infection, or if they have new fever on or after day 7 of treatment with antibiotics.

Loss of peripheral circulating lymphocytes and leukocytes, as well as decrements in marrow stem cells result in an immunologically compromised patient who will be susceptible to an array of infectious events. This phase of the haematopoietic syndrome is almost identical to the clinical course of human immunodeficiency virus (HIV) infection. Infection control is a fairly complicated matter. Haematology/oncology and infectious disease expertise will be needed.

Those patients with doses of $>2\,Gy$ (200 rad) acute WBI should be placed in reverse isolation in a general

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hospital. Patients with doses of 5–6 Gy (500–600 rad) and those with combined injuries will probably require treatment at a specialty hospital or medical centre.

Antibiotic prophylaxis and specific antidotes will be required. Antibiotic use should be directed towards specific foci of infection in non-neutropenic patients. Control of infections in the neutropenic phase of the haematopoietic syndrome is critical, is the most difficult to manage and is the major limiting factor for a successful outcome in otherwise salvageable patients. Neutropenic patients with an ANC of $<0.500 \times 10^9$ cells l^{-1} should be given a broad-spectrum fluoroquinolone or a fluoroquinolone with coverage for *Streptococci*. A febrile event in the neutropenic patient who has been given prophylaxis, but without other focal evidence of infection should be interpreted as the beginning of Gram-negative sepsis that can be rapidly fatal. Fluoroquinolones should be stopped and treatment of sepsis should begin. This treatment needs to include coverage for *Pseudomonas aeruginosa*, in particular, as well as for organisms known to be opportunists in the particular hospital.

5.8 GI Considerations

The radiation-injured patient endures significant nutritional and metabolic stress. Maintaining the integrity of the GI tract, while attempting to reduce pathogen risk and mechanical injury to the bowel mucosa are challenging. Collaboration with HP and GI disease specialists, especially experienced in radiation-related illness, and nutritional experts are critical, as a health team is necessary to aid the patient to recovery. Some suggest the use of an 'elemental' diet which delivers nutrients directly to surviving mucosal cells, limits mechanical stress to the small intestine, preserves physiological functioning, limits growth of abnormal bowel flora and limits injury of the bowel from pancreatic enzymes and bile acids. Some research has suggested that an increased concentration of glutamine in the diet may help to speed regeneration of crypt-cell populations, but data are lacking to make strong recommendations; a case-by-case approach is warranted (Harrison *et al*., 2007; McFee and Leikin, 2009; 2008; Stather 2007; International Atomic Energy Association (IAEA), 2009a; Jarrett, 1999; Waselenko *et al*., 2004; Leikin *et al*., 2003; NCRP, 2001; Goans, 2007; Koenig *et al*., 2005; Ricks and Fry, 1990; Ricks *et al*., 1990; 2002; REAC/TS, 2009).

5.9 GI Infection

In a situation of severe immunocompromise, as with ARS, a significant source of infection may be pathogenic bacteria that normally inhabit the human colon. The pathophysiological changes that occur during radiation injury, combined with use of antibiotic therapy, significantly alter the normal gut flora and cause many GI pathogens to gain access to the patient's circulation.

Historically and now a subject of some debate in combating this problem is the early selective decontamination of the gut using poorly absorbable oral antibacterial drugs. Quinolones eliminate potentially pathogenic aerobic gram-negative bacilli, prevent colonization and subsequent infection. Preserving anaerobic bacteria, they assist in maintaining digestion of overexposed persons developing the GI syndrome. Other attractive candidates for this purpose are, in order, ciprofloxacin, polymyxin B and trimethoprim-sulfamethoxizole or other sulphur-based oral medications. Some experts advocate performing a sensitivity screen of bowel flora prior to the period of manifest illness to better define a panel of antibiotics to be used if needed, but the possibility of an unscreened pathogen causing illness should still be kept in mind. Antiviral drugs (e.g. acyclovir) should be guided by a positive anti-HSV (herpes simplex virus) antibody test or empirically, if a test is not available.

Other authorities consider gut decontamination no longer beneficial except for patients with abdominal wounds or infection such as *Clostridium difficile* enterocolitis. Neutropenia and immunocompromise will allow mucosal overgrowth of *Candida* species, which may be partially controlled with fluconazole, 400 mg daily. Some *Candida* species, moulds and *Aspergillus* are resistant to fluconazole. The presence of these organisms requires special considerations beyond the scope of this work. Disruption of cutaneous and mucosal barriers also help create a favourable environment for opportunistic infections.

Remember, managing infectious complications of ARS will require specialty consultation. Radiation-induced cellular damage creates an ideal medium for reactivation and growth of quiescent infectious agents such as HSV and cytomegalo virus (CMV). Shortly after the nadir of lymphopenia, HSV reactivation may occur in those patients who have previously been infected. Prophylaxis with acyclovir or one of its congeners will be required. Serologies for HSV are recommended; however, in the presence of mucositis, empirical treatment may be started. Stomatitis from these infections can look like radiation-related stomatitis. Similarly, the presence of *Pneumocystis carinii* pneumonia requires special considerations.

Maintenance of gastric acidity (avoidance of antacids and H2 blockers) may prevent bacteria from colonizing and invading the gastric mucosa and may reduce the frequency of nosocomial pneumonia due to aspiration of these organisms. The use of sucralfate or prostaglandin analogues may prevent gastric haemorrhage without decreasing gastric activity. When possible, an early oral

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immuno-incompetent diet is preferred to intravenous feeding to maintain the immunological and physiological integrity of the gut.

5.10 Nutritional Support

Surgical implantation of a subcutaneously tunnelled central venous catheter can be considered to allow frequent venous access, but meticulous attention to proper care is necessary to prevent disastrous catheter-associated infections (International Atomic Energy Association (IAEA), 2009a; Christensen *et al*., 2008b; Goans, 2007; Koenig *et al*., 2005; Ricks and Fry, 1990; Ricks *et al*., 1990; 2002).

An area of debate, not just for radiation victims, but in other clinical situations, involves whether and when to use total parenteral nutrition (TPN) vs. an elemental diet delivered orally and/or through a feeding tube. TPN has the advantage of delivering large amounts of carefully and regularly adjusted nutrients to a patient whose GI function may be compromised. Furthermore, if the damage to the GI tract is especially severe or in the case of severe vomiting, ileus or obstruction, TPN is the only option available.

5.11 Psychosocial Effects and Support

Psychosocial concerns, for example widespread panic, may interfere with medical management of patients involved in an radiological/nuclear (R/N) incident. The general lack of knowledge about radiation and radioactive materials, coupled with the generally high level of anxiety, even fear, about all things radiological may result in exaggerated responses (McFee *et al*., 2004; Jarrett, 1999; Christensen *et al*., 2008b).

Psychosocial support professionals need to be identified *prior to* an R/N incident and engaged early following an incident.

Support personnel should include psychologists, pastoral care and social workers, public information officers and others.

Many patients will present to healthcare facilities, who only have the *perception* of significant exposure or contamination and who are really without the need for medical care—their needs will be primarily for reassurance and psychological support.

The real issue for first responders and first receivers is to determine who really has significant irradiation and thus the potential for significant radiation-induced injuries and illnesses.

Planning and preparation for R/N terrorist incidents must take these factors into account in order to communicate with and control patients effectively after an incident.

Key Facts: For healthcare professionals, responding to a mass-casualty incident, with or without radiation, as well as responding to a radiological terrorism event will be stressful even to the most seasoned clinician. Provide appropriate psychological care in the immediate aftermath of the response to staff *and* their families, who may be concerned about radiation threats at home. While that exigency is unlikely, remember information is the best antidote for fear.

6 PREPAREDNESS CONSIDERATIONS AND REQUIRED EQUIPMENT

6.1 Radiation/Nuclear Preparedness

Among the WMD, radiological threat response is rarely emphasized (McFee *et al*., 2004; Leikin *et al*., 2003; 2007; McFee and Leikin, 2005; ED Management, 2002; Jarrett, 1999; Waselenko *et al*., 2004; Walter *et al*., 2000; Christensen *et al*., 2008a; Schleipman *et al*., 2004; REAC/TS, 2009; NTI and the Center for Nonproliferation Studies at the Monterey Institute of International Studies, 2004; Basic Disaster Life Support (BDLS) Course, 2004; Liu and Rodgers, 2008; Kennedy *et al*., 1996; Lai *et al*., 2003).

The deadly explosion in 1986 at Reactor 4 of the nuclear power plant of Chernobyl in the former Soviet Union demonstrated the impact on human health, responders and the environment that radiation events can have. Although rare, this high-profile event demonstrated the potential for multiple casualties and long-term illness. Moreover the threat to those outside the immediate region—as weather patterns spread some of the toxicity as far away as Northern Europe illustrate the danger of radiation when safeguards are bypassed or inadequate. The health effects are still being observed 20 years later.

6.2 Healthcare Facility Preparedness

First and foremost is training (Leikin *et al*., 2003; Walter *et al*., 2000; Christensen *et al*., 2008a; Liu and Rodgers, 2008; Kennedy *et al*., 1996; Lai *et al*., 2003). Having the appropriate medical interventions including antidotes to the radionuclides identified as potentially the most problematic is essential (**Table 4**). Now is a good time to develop or ensure collaborations with regional planners and establish memoranda of understanding with other HCF to optimize the availability of critical medications.

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Table 4 Radioactive material and the treatments associated with them

Radionuclide	Antidote
Caesium-137	Prussian blue (ferric hexacyanoferrate), which adsorbs caesium in the GI tract. It may enhance elimination.
Iodine-131	KI blocks thyroid uptake.
Plutonium-239	DTPA can be used as a chelator and for wounds. EDTA is also a suitable chelator. Aluminium hydroxide antacids may bind plutonium in the GI tract
Radium-226	Immediate lavage with 10% magnesium sulfate followed by saline and magnesium purgatives. Ammonium chloride may increase faecal elimination.
Strontium-90	Aluminium hydroxide antacids may bind strontium in the GI tract. Aluminium phosphate can decrease absorption by 85%. Ammonium chloride can acidify the urine and enhance excretion. Barium sulfate may reduce strontium absorption. Stable strontium competitively can inhibit the metabolism and increase excretion of radiostrontium.
Tritium	Oral fluids reduce the biological half-life from 12 d to approximately 6 d. Do not overhydrate patients.
Uranium	Sodium bicarbonate renders the uranyl ion less nephrotoxic. Diuretics

6.3 Preparedness Considerations: Equipment

A mass casualty incident can lead to numerous, even contaminated patients presenting to a HCF, especially if it involves chemicals or radioactive materials. Of paramount consideration is the protection of the HCF and personnel. Keeping potentially toxic substances and people contaminated with such materials out of the HCF protects other patients and critically vital staff from becoming ill. Essential to protecting the HCF is ongoing and real- or near-realtime communications between the incident zone and the receiving institutions. Otherwise a facility can be overrun by the sick, injured or worried well—any of which can be contaminated. An index of suspicion is critical, especially if patients self transport from a scene in advance of first responders arriving at the incident, as happened during the sarin nerve agent of the Tokyo subway in 1995. During that time 23% of hospital staff involved suffered acute poisoning symptoms from secondary exposure from nerve agents. Nearly 10% of emergency responders were also affected (Jarrett, 1999; Christensen *et al*., 2008a; Liu and Rodgers, 2008; Kennedy *et al*., 1996; Lai *et al*., 2003).

In addition to an index of suspicion, technology plays an important, but yet incompletely appreciated or defined, role in early detection. Detector technology includes the traditional hand-held Geiger–Muller counter (GC), the radiation detection portal (RDP). In limited situation studies, both technologies were useful, *if* they were used appropriately, calibrated and capable of detecting the potential threat. Not all detectors, especially older models, are able to detect the full range of IR. Often special attachments, such as wands, are needed to allow the GC to detect alpha emissions.

The ability to detect, locate and quantify radioactive materials is a major advantage not universally afforded by other hazards like biological or chemical agents. Biological agent releases are usually surreptitious; unless the event is announced, detecting the pathogen at the point of dispersal or those exposed is unlikely. Bear in mind that there is an incubation period, which is the lag period between exposure and illness. Although a variety of detection technologies are deployed or under development, realtime alert capacity remains largely unrealized. Identification of a biological event, alas, occurs as patients present. Chemical agents, even unannounced incidents, yield symptoms much closer temporally than biological weapons. Moreover, there is likely to be a group of individuals simultaneously exposed, albeit they may present to different facilities. There are numerous detectors that can identify various chemical agents, although false positives remain a challenge. Radiation is a distinctive threat in terms of detection capability. The main challenge for the clinician or responder is considering radiation, not detecting it—with a caveat that all detectors do not necessarily detect all types of radiation.

6.4 Types of Radiation Detectors

- **Geiger–Muller counters (GMC)** (**Figure 9**) or so-called 'Geiger counters' are used to detect ionizations. There are a number of other detectors available, but GMC are the most common and easiest to use.
- **Dose-rate meters** (**Figure 10**) are used to *quantify* radiation levels. They are scaled to read in 'roentgens per hour' or 'milliroentgens per hour' or 'microroentgens per hour'.
- **'Survey meters'** (**Figure 11**) detect radiation; they are used to count ionizations in counts per minutes (cpm).

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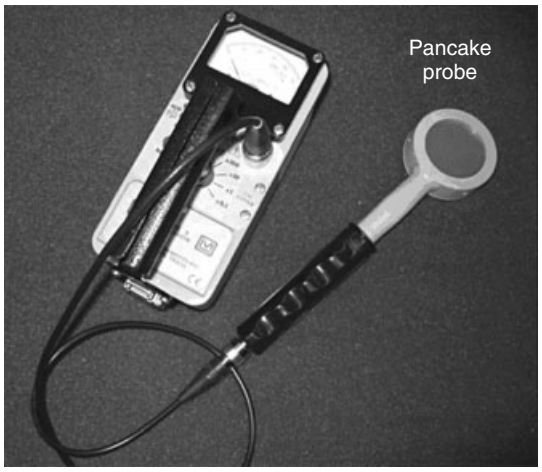

Figure 9 Geiger–Muller counter with pancake probe. (Reproduced from Christensen 2008a; 2008b, with permission from Radiation Emergency Assistance Center/Training Site (REAC TS).)

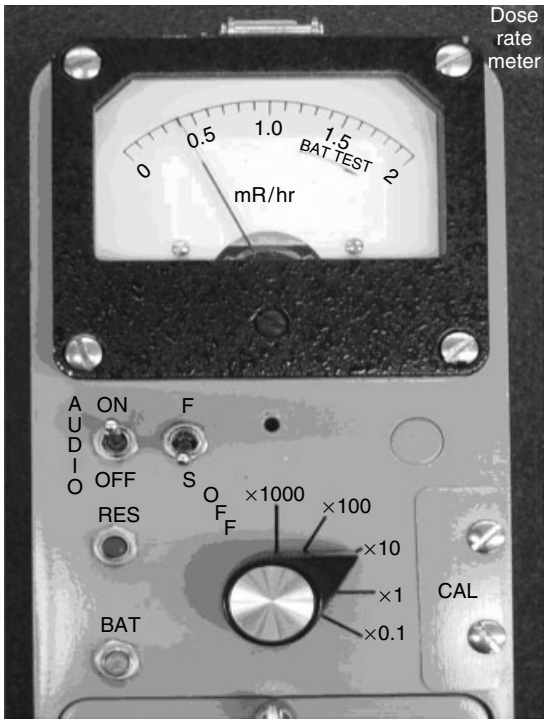

Figure 10 Dose-rate meter. (Reproduced from Christensen 2008a; 2008b, with permission from Radiation Emergency Assistance Center/Training Site (REAC TS).)

Prior to use, remember to check the level of background radiation in the area where patients from a suspected radiological incident will be evaluated and treated (Jarrett, 1999; Christensen *et al*., 2008a; Ricks and Fry, 1990). Background levels vary with geographical area. For example, the background levels in Oak Ridge, TN are in the range of 20–40 cpm. Bear in mind that higher levels may generally be found in areas at higher elevation; however, other factors may impact background levels.

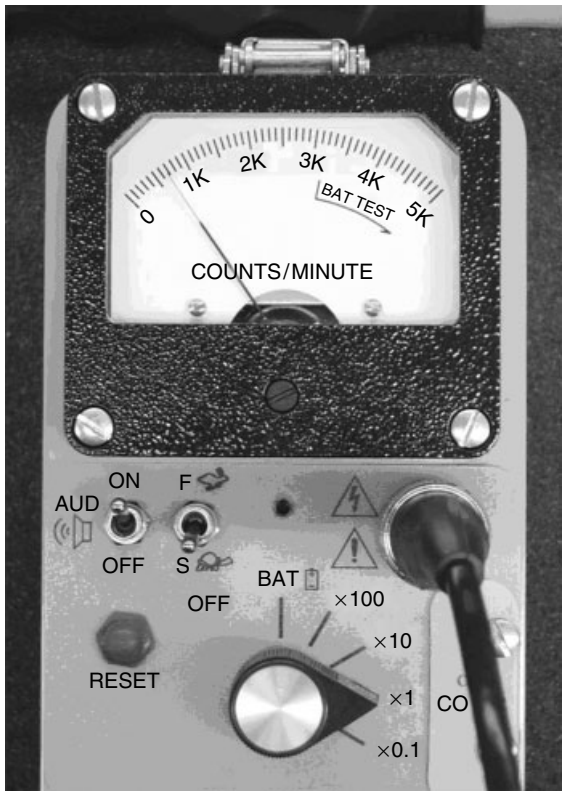

Figure 11 Survey meter. (Reproduced from Christensen 2008a; 2008b, with permission from Radiation Emergency Assistance Center/Training Site (REAC TS).)

First responders in the EMS system and public safety (fire, police) as well as first receivers may unknowingly be exposed to radiation or contaminated with radioactive materials. While increasingly the EMS has a variety of detectors in the field, radiation remains an underpractised area.

The following is a description of the equipment necessary to have ready in advance of anticipated radiation victims. The objective is to facilitate decontamination, safe collection and containment of potentially contaminated clothes and fluids (decontamination run-off and body fluids), create, as necessary, barriers along corridors and floors, staff/medical teams wearing appropriate barrier clothing and utilizing detection technologies to identify the threat.

6.5 Identifying Radioactive Materials

GMC or Geiger Counters (**Figure 9**) can be used to detect and locate radioactive materials, not to *identify* radiation and radioactive sources. Pancake probes can be used to distinguish between alpha, beta and gamma radiation in the appropriate setting and by a trained professional. Identifying the radiation type(s) involved is highly

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Figure 12 Direct-reading dosimeters. At the top are 'pen' or 'pencil' or 'pocket' dosimeters from which photon doses can be read directly by looking through the device to see a reading of dose to the dosimeter. At the bottom is a digital direct-reading dosimeter. (Reproduced from Christensen 2008a; 2008b, with permission from Radiation Emergency Assistance Center/Training Site (REAC TS).)

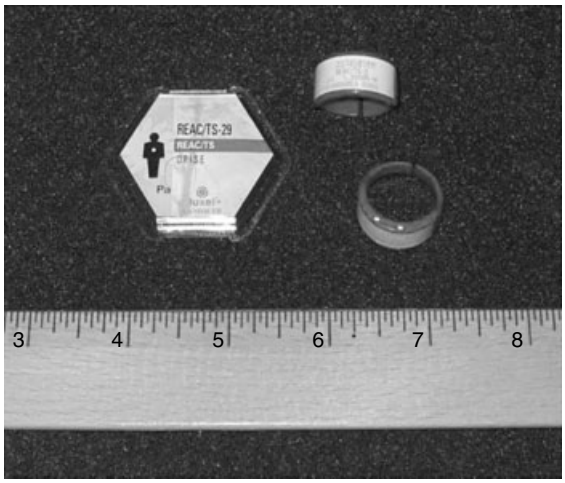

Figure 13 On the left is an optically stimulated luminescence dosimeter (OSLD) usually attached to clothing between the shoulders and the waist. On the right are two views of ring dosimeters. Both types must be 'developed' by a special process to determine dose absorbed in the area where the dosimeter is worn. (Reproduced from Christensen 2008a; 2008b, with permission from Radiation Emergency Assistance Center/Training Site (REAC TS).)

useful in planning medical management (Jarrett, 1999; Christensen *et al*., 2008a; Ricks and Fry, 1990; Salen *et al*., 2007; Gollnick, 2000; Thermo Scientific, 2009).

Identification of radioactive material can be accomplished with a gamma spectrometer. This is a relatively sophisticated piece of equipment that may be located only at fairly specialized research, response or designated radiation response HCFs. However, to effectively prepare for a radiation event, such facilities that can provide critical identification services need to be identified, agreements developed with and then included in the ED response plan *before* an incident. Of note, there are portable spectrometers available on the market, but they may require the expertise of an HP.

Figure 14 Electronic personal dosimeter from Thermo Scientific (NB: this is not an endorsement of any specific product or manufacturer; for illustrative purposes only!). (Reproduced with permission from Thermo Scientific 2009.)

6.6 Dosimeters

Unless an incident has occurred in a known radiation-controlled environment, such as radiological or nuclear industries or laboratories, most victims will come in equipped with 'dosimeters' (**Figures 12–14**). A 'physical' dosimeter is a device that can be used to determine how much radiation is absorbed at the location on the body where the device is worn. These devices are designed to detect some, but not necessarily all, radiations, and they must be properly selected in advance according to likely scenarios and within their performance windows. HPs need to be consulted for decisions about devices to be used in EDs in advance of an incident. In the event the victims are wearing a dosimeter, an HP should be consulted to assist in accurate interpretation (Jarrett, 1999; Christensen *et al*., 2008a; Ricks and Fry, 1990; Salen *et al*., 2007; Gollnick, 2000).

There are a variety of dosimeters available. These include electronic personal dosimeters (EPDs) that are worn much like pagers on waist belts, have audible alarms and can be preset to a variety of alert settings (**Figure 14**). Some electronic devices that will directly report dose rates or radiation doses absorbed by the devices in measurable quantities in realtime.

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Others include thermoluminescence dosimeters (TLDs), worn by radiology department personnel, or optically stimulated luminescence dosimeters (OSLDs), which must be developed after the fact by a special process. Dosimeters that must be 'developed' in this way will be of no immediate help with dose assessment. 'Ring' dosimeters may be worn by personnel handling radiation sources with their hands, such as during extraction of a piece of shrapnel or other surgical procedures. Direct-reading 'pen', 'pencil' or 'pocket' dosimeters may be worn by healthcare personnel during management of patients (**Figure 12**). These devices respond to photons, not alphas or betas. They can be read directly after or during patient management. These dosimeters must be 'zeroed' or the reading noted prior to use.

6.7 Hospital Facility Detection

Protecting a facility from surreptitious radiation exposures—hidden SDDs or preventing thefts of radioactive materials—has long been a security vulnerability. A wide range of approaches have been undertaken to develop 'arms-length' detection technology. These include large portal detectors strategically placed at ED entry points, small sensors placed strategically thoughout a facility and linked to integrated computer programs and other products. To date, no universally accepted product has been in widespread use. However, in November 2008, Washington Hospital Center in Washington, DC installed a radiation detection system as part of a pilot programme. The technology places a wide array of sensors that are integrated into an interpretation centre; the information is disseminated in realtime or near-realtime to key personnel. It is designed to identify gamma radiation from dirty bombs (RDD) as well as the theft of medical radiological material, contamination of patients and so on. Whether it has false alarms or the ability to detect during real events, or discriminate in a useful manner other forms of IR, remains to be seen. The 'holy grail' of detection technology that will identify a surreptitious release in the facility, or persons contaminated with or smuggling out (or in) radioactive materials, in realtime, remains to be proven cost-effective and practical for the average HCF.

6.8 Radiation Protection and Contamination Control

Protection from radioactive materials and radioactivity is not much different to protection from biological or chemical agents. It is relatively easier to provide protection from radiological materials because of the ability to detect them. Contamination control is the most important way to prevent exposures. A very basic level of understanding of physics, how radioactive materials behave and how to control radioactive contamination is all that is needed by healthcare providers.

The equipment necessary can be found in **Table 5**. Personnel can help protect themselves by using:

- Universal precautions
- Sterile technique.

6.9 Decontamination

Contamination should be considered as nothing more than dirt with radioactive atoms mixed in, so remove the dirt and the radioactive materials go with it. Decontamination of the potentially radiation-contaminated patient is similar to that for chemically exposed patients, with a few caveats.

First, soap and water is adequate. There is no need for harsh chemicals, shampoos, shaving cream, industrial nonsolvent grease removers and any number of other types of cleansers.

Warning: Degreasers or other solvents should not be used because defatting of the skin may damage it enough to allow percutaneous absorption of radioactive and other potentially toxic materials (Christensen *et al*., 2008a).

Second, be thorough—hair, under the nails, orifices, intertriginous areas—but avoid abrading the skin, which can increase the risk of absorption.

Priorities for decontamination should be given to open wounds first, then body orifices, in particular the nose and mouth, or any other area that may be a portal for radioactive materials to enter the body. A superb reference for decontaminating the potentially contaminated patient with radioactive materials is *http://orise.orau.gov/reacts/guide/procedures.htm*.

Total-body decontamination, like showering, may be necessary, even with the presence of radioactive materials. In many cases, biological or chemical hazards may be more hazardous than radiological materials, therefore management of those contaminants will dictate the best method of decontamination.

Body hair, including beards and eyebrows, should not be shaved, because microabrasions may allow absorption and internalization. Given the deleterious effects of radiation on hair follicles, shaved eyebrows may not regrow.

Decontamination of wounds with intravenous (iv) fluids and a syringe is a very useful technique for wound management. Wound care, irrigation, debridement and repair can generally proceed as usual, but, given the effect on the immune system and healing, consultation with surgeons knowledgeable in treating such patients, along with an HP can greatly enhance medical outcomes. Bear

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Table 5 Decontamination and radiation response equipment (Leikin *et al*., 2003; 2007; Jarrett, 1999; Christensen *et al*., 2008a; Ricks and Fry, 1990; Liu *et al*., 2008; Kennedy *et al*., 1996; Lai *et al*., 2003; Salen *et al*., 2007; Gollnick, 2000; Thermo Scientific, 2009)

Universal PPE	Hospital greens and surgical gown (waterproof, any openings taped shut)
	Surgical trousers taped to shoes
	Caps (water resistant/water proof)
	Face shield (water resistant/water proof)
	Booties (water resistant/water proof)
	Double glove: inner layer taped to surgical gown
Plastic apron	—
Dosimeters	Pencil dosimeters
	Thermoluminescent dosimeters (TLD)
	Finger TLD
	Survey meters
	QFD + charger
	Electronic readout dosimeter/electronic personal dosimeter (EPD)
	Personal air sampler
Plastic sheeting (polyethylene)	Wall and floor
	Drapes
Ventilation filter paper	—
Plastic bags and large garbage cans	—
Butcher paper	—
Radiation signs and tape	—
Decontamination stretcher	Stretcher should have drainage capability and storage container tank
Lead storage containers	—
Respiratory masks—n95 or greater	—
Radiation detector	Capable of detecting alpha, beta, gamma, X-ray.
Advanced level PPE	Level A, B or C depending upon response role
Neoprene industrial gloves	—

in mind, if a life-saving medical intervention would be necessary on this patient in the absence of radioactive materials, then the intervention can usually be done in the presence of radioactive materials.

6.10 Self-Protection

Key points:

- Minimize *time* spent around or near sources of ionizing radiation
- Maximize *distance* from sources. The inverse square law defines the effect of distance upon dose. If the distance is doubled, the dose will be quartered. If the distance is halved, the dose will be four times higher
- Maximize *shielding* from sources
- Minimize *amounts* of radioactivity.

7 CONCLUSION

The threat of radiation—whether from an industrial or nuclear reactor accident or the intentional use of a weapon, is increasing. With widespread proliferation of radioactive materials, securing these is a daunting task, as is preparing an HCF to address radiation victims, especially in a world of competing demands and financial pressures. Nevertheless, it is the HCP the public looks to as the definitive protector in the event of a radiological event. Given most HCFs, as well as EMSs and clinicians have not seen, let alone treated a radiation victim, greater training is warranted. While most departments would fail to certify a clinician to perform intubation after only practising once a year or less, is it wise to 'certify' or consider a HCF competent to respond to a radiation event when plans are old or nonexistent, drills rarely performed and training has been cut back?

The management of the patient acutely poisoned by radiation is complex and requires a team of experts drawn from a variety of medical and health disciplines. Working together to develop familiarity prior to an event will go far to enhance capabilities when the real thing occurs.

Medical management hinges upon early recognition, which will require awareness, detection technology, treating life-threatening injuries and anticipating the long-term effects of ARS. Such patients will require long-term care and monitoring. Infection control, nutritional and psychosocial care will be necessary.

Given most regions of the industrialized world have nuclear reactors, radiation research and industrial sources of radioactive material, preparedness is worth the effort. A rapid and well-performed response will save lives.

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8 QUICK REFERENCE: RESPONDING TO A RADIATION POISONED PATIENT

The following is a checklist when radiation/nuclear patients may present. Consider the ability to conduct each section an ersatz list of competencies to which HCPs should be held.

8.1 Essential Tasks

ED/receiving medical personnel should be able to perform initial surveys for radiation and radioactive contamination.

Key Facts: The history (suspected/actual) of a radiological or nuclear incident is as essential as the medical history to the medical diagnosis and management of patients:

- History of the incident—document
- Radiation—yes or possible?
- History of mechanism of injuries—document.
- Radiation injury alone
- Combination trauma/burn/radiation
- History and times of onset and duration of symptoms—document.
- Call for health physics support
- Radiological and healthcare personnel should be identified, trained and integrated into ED processes *prior* to an incident.
- Patient radiation surveys
- Incident history and reconstruction
- Identification of radioactive materials
- Radiation dose estimates—help guide medical management.
- The process of incident history and dose estimation need to be repeated.
- These patients require ongoing re-evaluation to guide medical management.
- Conduct a complete physical examination and document including:
- Vital signs
- Temperature
- Weight
- Skin appearance
- Involve consultation with key specialists early
- Infectious disease
- Haematology
- Surgery
- Call for psychosocial, pastoral and other support
- Psychosocial support personnel need to be identified, trained and integrated into ED processes *prior* to an incident.

8.2 Laboratory Studies

Key Facts: Laboratories and the necessary sampling equipment required to identify and quantify radioactive materials on clothing, dressings or in-body fluids (bioassays) need to be identified *prior* to an incident:

- Save and double-bag contaminated clothing and dressings as samples for further radiological analyses
- Have appropriate secure/safe location for potentially radiation-contaminated materials
- Suspicious incidents are likely to be considered part of a crime scene; the issues associated with chain of evidence can complicate the medical response.

Preparedness planning and practice in advance of an actual radiological or terrorist criminal event will allow greater interoperability and smoother working relationships with law enforcement, preventing the potential for obstructive territoriality. It will also enhance the collection of forensic samples and the need for heightened attention to proper collection, containment and identification with patient name, patient identifier, date and time of collection and name of collector.

- Laboratories that can perform cytogenetic biodosimetry need to be identified *prior* to an incident
- Cytogenetic biodosimetry
- Usually, only one lithium-heparin tube is needed
- Special handling and packaging are required
- Cytogenetic laboratories
- Most of those that perform chromosomal analyses do so for genetic disease diagnosis and counselling rather than dose assessments
- They are *not* typically set up to perform chromosomal analyses for radiation biodosimetry
- Two federally funded cytogenetic biodosimetry laboratories to date are available in the United States
- The Armed Forces Radiobiology Research Institute (AFRRI) in Bethesda, MD
- The Radiation Emergency Assistance Center/Training Site (REAC/TS) at the US Department of Energy's Oak Ridge Institute for Science and Education (ORISE) in Oak Ridge, TN
- Draw all laboratory specimens and perform diagnostic testing as required for other injuries and illnesses
- Laboratory specimens and special studies that may be required for evaluation of radiological injuries:
- Complete blood count (CBC)
- Absolute lymphocyte counts (ALCs) every six hours for the first 24 hours than every 12 hours for two days, then daily
- Absolute neutrophils counts (ANCs) every six hours for the first 24 hours than every 12 hours for two days, then daily

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- Check initial urine specimen for radioactivity with a Geiger counter—this is referred to as 'rad urine'on the Radiation Patient Treatment decision support algorithm in **Figure A.1**
- Some high-energy photons from ^{137}Cs might be detected in urine depending upon how soon after an event the initial spot urine is checked.

A negative initial spot urine radiation survey is *meaningless*—only positives that indicate internal contamination are significant.

- Begin 24-hour urine and faecal collections for 'radiobioassays'.
- Such laboratories need to be identified *prior* to an incident.
- Obtain a baseline chest X-ray
- Perform other special studies as needed
- Obtain a serum amylase initially, then at 24 hours
- Significant irradiation of the salivary glands can result in high amylase levels.
- Interpret high amylase levels with caution if the patient has suffered from trauma affecting the face, head, neck, chest or abdomen.
- Obtain blood for typing
- Cross match only if needed for other medical or surgical conditions.
- Obtain blood for HLA tissue typing
- Draw one lithium-heparin tube for cytogenetic biodosimetry
- Culture wounds, blood or other body fluids as indicated.

In severely neutropenic patients, particularly those who are also anaemic, they may not mount a normal inflammatory response. Examples include:

- A urinary tract infection without pyuria
- Pneumonia without chest X-ray infiltrate
- Skin infection without typical cellulitis.

Radiation/nuclear events can cause anxiety and fear. This may result in significantly more patients, families—the worried well—disproportionate to the event. This exaggerated concern response can include healthcare providers. Information is the best cure for fear.

- Expect larger numbers of patients with the *perception* of radiation injury or illness than those who actually may have *real* significant radiation exposures or contamination.

9 APPENDIX A: FAST FACTS

The following 'fast facts' can be photocopied and place strategically in your department to guide your preparedness and response to a potential radiation or nuclear event.

9.1 Enhancing Your Preparedness

First and foremost, every department that will be called upon to respond to a radiation or nuclear event should have the following:

- An updated RAD/nuclear response plan that is practised regularly
- Team members who have received training in RAD/nuclear patient care
- Readily available and key personnel are practised in the use of updated detection and surveying technology
- Have on display *validated algorithms* such as those from REAC/TS (**Figure A.1**).

9.1.1 Basic Considerations

- Most radioactive materials (and radioactivity) are relatively easy to detect with appropriate instruments:
  - If well maintained
  - If they cover the full range of ionizing radiation (IR)—alpha, beta, gamma/X-Ray)
- Once identified, the patient can be managed effectively employing simple and readily applied practices:
  - Universal precautions with appropriate personal protective equipment (PPE)
  - Integrate health physicists early in the process
  - Remember the immediate needs of the patient:
    - Trauma/life threatening illnesses
    - Time is tissue—radiation damage is progressive, but may not immediately be apparent
    - Antidotes and chemoprophylaxis must be initiated early—example of key antidotes and the respective radionuclide can be found in **Table 6**
    - Surgery and other aggressive/advanced interventions must be decided in concert with experts in radiation response, taking into account the potential weakening of immune response and healing response associated with radiation
    - These patients, especially once disrobed and decontaminated (soap and water) do not pose a threat to the HCF, personnel or other patients and thus should not be denied access to conventional diagnostic or other services
- Historically, nonterrorist radiation events have been classified into five categories:
  - *High-level external* radiation—external exposure

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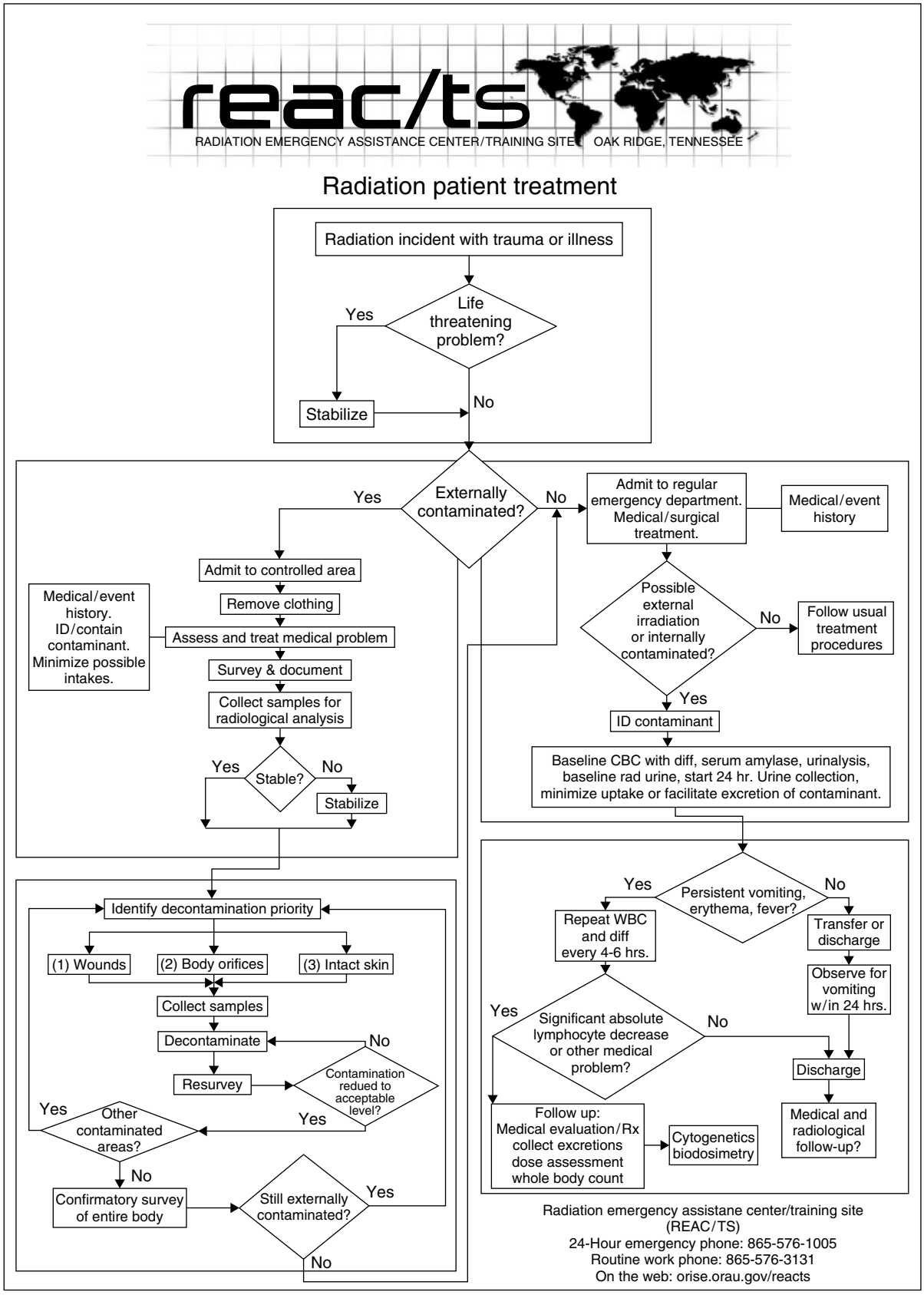

Figure A.1 REACTS algorithm REAC TS/ORAU/Oak Ridge. (Reproduced with permission from Radiation Emergency Assistance Center/Training Site (REAC TS).)

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Table 6 Radionuclide and suggested antidote/therapy

Radionuclide	*Antidote*
Cesium 137	Prussian blue (ferric hexacyanoferrate), which adsorbs cesium in the GIT. It may enhance elimination.
Iodine 131	KI blocks thyroid uptake.
Plutonium 239	DTPA can be used as a chelator and or wounds. EDTA is also a suitable chelator. Aluminum hydroxide antacids may bind plutonium in the GIT
Radium 226	Immediate lavage with 10% magnesium sulfate followed by saline and magnesium purgatives. Ammonium chloride may increase fecal elimination.
Strontium 90	Aluminum hydroxide antacids may bind strontium in the GIT. Aluminum phosphate can decrease absorption by 85%. Ammonium chloride can acidify the urine and enhance excretion. Barium sulfate may reduce strontium absorption. Stable strontium competitively can inhibit the metabolism and increase excretion of radiostrontium.
Tritium	Oral fluids reduce the biologic half-life from 12 days to approximately 6 days. Do not overhydrate patients.
Uranium	Sodium bicarbonate renders the uranyl ion less nephrotoxic. Diuretics

- Late systemic symptoms (two to three weeks) including neutropenia and sepsis
- Similar industrial and accidental events have occurred with, but not limited to:
- Caesium-137
- Cobalt-60
- Iridium-192

- *Low-level external* radiation—external exposure
  - Normal clinical examination without physical damage expected
- *Local injury*
  - Usually to an extremity or specific area of the body
  - Systemic symptoms unlikely unless unrecognized/untreated high level of local radiation resulting in infection, necrosis
- *Internal—inhalation or ingestion*
  - Depending upon the dose internalized these exposures can range from nontoxic to fatal
  - The initial physical examination may be normal
  - Subsequently the patient may develop symptoms referable to radiation exposure, depending upon the dose
- *Iatrogenic/hospital/treatment* associated
  - Cancer and other radiation treatment
  - Brachytherapy
  - Nuclear medicine studies
  - *Caveat:* Radiation pericarditis is often overlooked

- Healthcare professionals (HCPs) can decrease their risk by:
  - Disrobing the patient (removes ~90% of contamination)
  - Decontaminating patient (soap and water)
  - Realizing patients do not pose a significant risk to HCPs.

9.1.2 Radiation-Related Terrorism

- May or may not be announced or heralded by explosion
- If associated with explosion it is important to recognize there may be four types of injuries:
  - Blast
    - Patients may have serious internal damage without obvious external signs
    - Tympanic membranes, lungs and hollow organs are highly sensitive to blast injuries
  - Burn
  - Radiation injury
  - Psychological trauma
- Clinical acumen and index of suspicion will be critical to assess the various clinical clues; include radiation in the differential diagnosis. Time is tissue; delayed diagnosis can be costly
- Victims of a radiation terrorism event involve three critical considerations:
  - Surgical care
    - Radiation can confound wound healing—surgical evaluation in consultation with infectious disease, haematology and radiation specialists is critical to determine the optimal time to consider surgery
  - Medical care
    - Radiation terrorist events may result in traumatic injuries, as well as exposure to radioactive materials
    - Always treat life-threatening injuries first
  - Radiological care
    - Radiation-related effects may not manifest for hours if the exposure is significant; most likely it will take days to weeks before clinical evidence of radiation sickness occurs

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- While treating trauma, surgical and other medical issues it is important to determine the estimated dose each patient received
  - Collaborating with a health physicist or other radiation expertise is essential to determine the exposure as the course of acute radiation syndrome is largely based upon the dose received and the underlying health and clinical situation of the patient.
  - The lethal dose for 50% of persons receiving whole-body irradiation or exposure to die in 60 days (LD50/60) is ~3.5–4.0 Gy (350–400 rad) with supportive care only. Some texts use the LD 50/30 signifying survival to 30 days
  - The LD 50/60 with aggressive expert medical care may be upwards of 6 Gy. Some suggest 8 Gy is survivable under these conditions. Greater than 10 Gy exposures carry a poor prognosis
- Once the exposure dose is estimated, the patient can be triaged into the categories of acute radiation syndrome (ARS) given in **Table 3**
- Acute radiation syndrome (ARS)
  - Symptoms vary by
    - Type of radiation
    - Individual radiation sensitivity
    - Amount absorbed
  - ARS is a time-phased illness
    - Prodromal phase—relatively rapid symptoms
    - Latent phase—dose dependent
    - Radiation illness
  - ARS radiation illness
    - Haematopoietic
    - Gastrointestinal
    - Neurovascular
- Initiate appropriate management of radiation illness, including:
  - Antidotes
  - Colony-stimulating factors
  - Infection prophylaxis
  - Other interventions

9.1.3 Challenges to Effective Response

- Lack of training persists among all healthcare professions concerning radiation
- Least emphasized of all preparedness threats—natural, industrial or intentional
- Radiation may not be considered at the first-responder level
- There may be a delay between recognizing that an event occurred and the patients who present—radiation events may be surreptitious or lack warning signs typical of other threats, such as explosives or chemicals.
- Radiation is rarely considered in the differential diagnosis
- Lack of familiarity with radiation detection equipment
- Lack of familiarity with critical resources including expertise, such as
  - Radiation Emergency Assistance Center/Training Site (REAC/TS) in Oak Ridge Tennessee (Emergency phone ask for REAC/TS: **865.576.3131** or **865.576.1005** or online at *http://orise.orau.gov/reacts/* (accessed 29 April 2009)).
  - Armed Forces Radiobiology Research Institute (AFRRI) in Bethesda, Maryland
  - Local HAZMAT
  - Healthcare facility radiation physicist
  - Regional poison information centre.

9.1.4 Opportunities to Enhance Preparedness

- Enlist expertise to assist your healthcare facility
- Collaborate *before* an event with local and regional responder groups to become accustomed to working with different performance cultures
- Conduct regularly scheduled training and update sessions
- Test your plans with drills, scenarios and table-top exercises
- Check your radiation response equipment regularly
- Ensure your facility has the appropriate countermeasures
  - Collaborate with regional preparedness, especially for chemotherapeutics
    - Antidotes—certain radiation appropriate medications may need to be acquired from the government
  - Personal protective equipment
  - Decontamination equipment
  - External resources—mutual aid agreements
  - Other medical interventions.

9.2 Radiation Terrorism Response: Fast Medical Facts

Make certain your department has an updated response plan and everyone on the team is aware of it, has practised it and has critiqued it.

9.2.1 Arrival

- The management of patients who are *irradiated only* and who are *not contaminated* requires no special area within the treatment facility.

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- Removing the victim's clothes is likely to eliminate ~90% of the external contamination threat to clinicians and responders
  - Using appropriate hazardous waste disposal procedures is critical
- Prepare a special area for receipt, evaluation and treatment of contaminated patients.
- *Priorities for decontamination*
  - *Open wounds*
  - *Body orifices*
  - *Intact skin*
- Decontamination must be accomplished to prevent/minimize internal contamination
- As soon as possible after medical and surgical conditions have been stabilized survey and collect using swabs or other testing materials as suggested by your radiation health physicists the following areas:
  - Face
  - Mouth
  - Nose
  - Open wounds
  - Don't forget intertrigonous areas.

9.2.2 Treatment

- Perform a patient assessment, keeping in mind the possibility of radiation
  - The initial survey should include appropriate detection technology
- Use a Radiation Patient Treatment algorithm
- *Treat trauma first: surgical and medical emergencies are a priority*
  - Remember to look for burns and blast injury
  - Combined injuries are defined as radiation injuries/illnesses *plus* trauma and/or thermal burns
    - Combined injuries significantly increase morbidity and mortality
  - Radiation-related illness may take hours, days or weeks to manifest

NB: Morbidity and mortality from radiation injuries and illnesses will usually be delayed, unlike some traumatic and thermal injuries

- A good history is essential; especially if there was no explosion and the patient may be the index case
  - Determining if radiation is a potential cause, especially in an unannounced event is a significant challenge for the clinician
  - Taking into account the patient is a good biological dosimeter by virtue of the relative radiosensitivity of tissue types can guide the clinician in determining if ARS is present and to what extent
- If radiation is considered, enlist the assistance of radiation HPs *early* in the management of the patient
  - *Estimation of dose will be essential to guide medical management*
  - Begin specific antidotes for some radioactive materials.

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Depleted Uranium: Toxicology and Health Consequences

Alexandra C. Miller

CONTENTS

1 INTRODUCTION

Advances in metallurgy and weapons design in the past several decades have led to new munitions whose effectiveness has provided tactical advantages on the battlefield and consequently, saved the lives of military personnel. Depleted uranium (DU) kinetic energy penetrators are perhaps the best-known example of these advanced munitions, primarily because of their outstanding, well-publicized performance against enemy armour in the 1991 Persian Gulf War. DU munitions were again used in NATO military actions in Bosnia-Herzegovina (BiH) (1995) and Kosovo (1999), and, more recently, coalition actions in Iraq.

DU munitions were initially used only by Coalition forces during the 1991 Gulf War, but that use led to DU fragment injuries among Coalition forces as a result of friendly fire incidents. Other personnel may have been potentially exposed via inhalation/ingestion after working around vehicles struck by DU munitions. Such exposures were not considered especially dangerous at the time, because numerous epidemiological studies of uranium miners and millers working with natural uranium had shown few concrete health effects from exposure and DU is chemically the same as natural uranium, with the difference being the uranium isotopic concentrations. However, the exposure of wounded personnel to uranium as embedded fragments had no medical precedent, so the earlier studies, dealing primarily with inhalation or ingestion exposures in miners, were of uncertain utility. As a result, questions were soon raised as to whether it was wise to leave in place fragments possessing the unique radiological and toxicological properties of DU, especially when considering that exposures might extend as long as the 40–50 years remaining in the individuals' lives. As these treatment questions were being addressed,

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Table 1 Transformation of human osteoblast cells: comparison of DU, tungsten alloy, nickel and beryllium

	Transformation: biological properties of transformed HOS cells					
	Untreated	DU (soluble)	DU (insoluble)	rWNiCo (insoluble)	Beryllium (insoluble)	Nickel (insoluble)
Transformation frequency (per survivor $\times 10^{-4}$)	4.4 ± 1.1	49.6 ± 4.8	71.2 ± 4.8	37.6 ± 5.1	31.4 ± 4.4	39.5 ± 3.1
Morphology	Flat	Transformed	Transformed	Transformed	Transformed	Transformed
Saturation density ($\times 10^5$ cells)	2.6	6.9	6.6	6.1	6.3	7.1
Soft-agar colony formation (plating efficiency %)	3	47	56	34	31	28
Tumorigenicity (mice tumours/mice inoculated)	0/82	9/20	14/20	8/20	7/20	6/20
Kras expression (densitometric analysis; relative to actin)	0.5	8.1	7.3	6.9	7.0	5.7
pRb phosphorylation (densitometric analysis)	6.1	1.0	1.2	4.0	4.6	1.1

Cells were exposed to DU, tungsten alloy, nickel or beryllium and assessed for neoplastic transformation. The transformation frequency was determined. A comparison of cell morphology, transformation frequency, saturation density, soft-agar colony formation, tumorigenicity, *Kras* expression, pRb phosphorylation was done.

a growing public concern about the long-term health and environmental impact of using a radioactive metal like DU on the battlefield fuelled forceful national and international efforts to ban the use of DU in munitions. The impact of DU on civilian populations and the environment was unknown as well.

This chapter aims to summarize the current status of knowledge about the potential health effects of DU, based on cellular, animal and limited human studies. Toxicological assessments have been conducted using cultured cells and animal models and have attempted to answer questions relating to toxicity, carcinogenicity and the involvement of radioactivity. Additional information and research is still needed to provide a more complete understanding of DU human health effects.

2 BACKGROUND

2.1 Uranium

An understanding of DU requires a review of the element uranium. Uranium was discovered in the mineral pitchblende in 1789 by the German chemist Martin Heinrich Klaproth. Uranium possesses a unique role in radiation science, since a sample of uranium was used by the French physicist Henri Becquerel in his discovery and formulation of the concept of radioactivity in 1896. Uranium does not exist in pure metallic form in nature because it is quickly oxidized in air and occurs most commonly as U_3O_8, uranium oxide, in ores such as pitchblende. Refined uranium metal used in reactors is in the form of UO_2, uranium dioxide.

Natural uranium has three predominant natural isotopes, ^{234}U, ^{235}U and ^{238}U, all of which are radioactive; other uranium isotopes such as ^{236}U can be produced artificially in a reactor. **Table 1** shows the relative contributions of the uranium isotopes to DU and natural uranium. Uranium isotopes are alpha-particle emitters; alpha particles are positively charged ions composed of two protons and two neutrons. Due to physical characteristics, alpha particles lose their kinetic energy quickly and have little penetrating power because of their charge and relatively large size. The range of an alpha particle is approximately 4 cm in air and considerably less (20–32 μm) in tissue (ATSDR, 1999a). Therefore, uranium is primarily an internal radiation hazard, since an alpha particle will only penetrate the outermost layer of skin. Thus, alpha particles represent little hazard when on the surface of the skin, but are potentially a significant hazard if inhaled or ingested, whereupon they come into close contact with sensitive tissues (Hartmann *et al*., 2000). In the decay process, beta and gamma radiation are emitted, radiations which are significantly more penetrating than alpha particles requiring the necessity of providing shielding for DU when it is not being used (ATSDR, 1999b). Uranium isotopes decay to other radioactive elements that eventually decay to stable isotopes of lead (ATSDR, 1999b). The half lives of the isotopes are: 2.44×10^5 years for ^{234}U, 7.10×10^8 years for ^{235}U and 4.5×10^9 years for ^{238}U, and their composition in natural

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Comparison of the relative contribution of Uranium isotopes[a] (Natural and Depleted)

Isotope	Specific activity (μCi g^{-1})	DU SA by WT% (μCi g^{-1})	Natural uranium SA by WT% (μCi g^{-1})
^{238}U	0.333	0.332	0.331
^{236}U (not naturally occurring)	63.6	0.0001	0
^{235}U	2.2	0.0044	0.051
^{234}U	6200	0.093	0.310
Total		0.4295	0.692

[a]Contribution of daughter products is not include.

Figure 1 Comparison of relative contributions of isotopes of uranium (natural and depleted). The specific activities by weight of natural and depleted uranium are shown and the contribution of each uranium isotope is shown.

uranium by mass is: 0.005% ^{234}U, 0.711% ^{235}U and 99.284% ^{238}U. The isotopes of uranium have the same chemical properties because they all have the same number of protons, 92. Like all other elements, variation in the number of neutrons gives the isotopes different radiological properties. The radioactivity of isotopes can be compared using specific activity, a measurement of the number of nuclear transformations (disintegrations) per second per unit mass. The most abundant naturally occurring uranium isotope, ^{238}U, has the lowest specific activity (0.33 μCi g^{-1}) (Squibb *et al.*, 2005). The high specific activity of ^{234}U (6200 mCi g^{-1}) contributes to more than half of the radioactivity of natural uranium, even though by weight its percentage is extremely small. Enriched uranium is quantified by its percentage of ^{235}U (specific activity 2.2 μCi g^{-1}) which can range from 2% to more than 90% (US AEPI, 1995). A comparison of DU to natural uranium is shown in **Figure 1**. This chart quite simply shows that DU has an approximately 50% lower specific activity than natural uranium.

Considering the isotope half-lives and their mass percentages, it can be calculated that 48.9% of the radioactivity of natural uranium is derived from the isotope ^{234}U, 2.2% from ^{235}U and 48.9% from ^{238}U (ATSDR, 1999a). Thus, ^{234}U contributes as much to the radioactivity of natural uranium as does ^{238}U, despite the fact it is 20 000 times less abundant. Natural uranium has a low specific activity of about 0.68 μCi g^{-1} or 1.8 $\times$ 10^7 Bq, which means natural uranium is considered only a weakly radioactive element. However, chemically, uranium is categorized as a heavy metal (i.e. any metal with a specific gravity of 5.0 or greater) and like other heavy metals, that is, nickel and arsenic, has a significant chemical toxicity. Similar to those heavy metals, the chemical toxicity of a uranium compound varies depending on the nature of the compound, its solubility, and its route of exposure, different to that of natural uranium.

2.2 'Depleted Uranium'

The use of uranium as nuclear fuel or in nuclear weapons requires enrichment of the fissionable isotope ^{235}U. The enrichment process concentrates ^{235}U in the metal to specific activities required to sustain nuclear reactions. The by-product of enrichment is uranium with reduced levels of the ^{235}U isotope, or 'depleted' uranium. The Nuclear Regulatory Commission considers the specific activity of DU to be no more than 0.36 μCi g^{-1}, but more aggressive enrichment processes can drive this value slightly lower ($\sim$0.33 μCi g^{-1}) (ATSDR, 1999b). This means DU has roughly 50% of the radioactivity of natural uranium. Even though DU has less specific activity than natural uranium, it retains all of its chemical properties. The large-scale production of enriched uranium for nuclear weapons and fuel over the decades has resulted in an abundance of cheap DU, a factor that has played a role in its use in a wide variety of applications (e.g. radiation shielding, compact counterweights, armour, kinetic-energy weapons). The properties of DU that make it useful as an armour-penetrating munition are its density (1.68 times that of lead) and the ability to engineer into it a molecular structure that facilitates entry into a hardened target by 'shedding' outer layers of the metal during penetration. The chemical and physical properties of DU are ideal for many military and commercial uses. It is 65% more dense than lead (with a density of 18.9 g cm^{-3}), has a high melting point (2070°F, 1132°C), is highly pyrophoric (it ignites when it fragments), has a tensile strength comparable to most steels and is chemically highly reactive (Kirk and Machalowski 1982).

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2.3 Depleted Uranium Exposures

Beginning with the 1991 Persian Gulf War, US weapons systems have utilized DU (frequently alloyed with 0.75% titanium by weight to reduce oxidation) for offensive and defensive purposes (Parkhurst *et al.*, 1995; OSAGWI, 1998). Heavy armour tanks have a layer of DU armour to increase protection. Offensively, DU increases the penetration effectiveness of the kinetic-energy cartridges and ammunition rounds used by the Army (105 and 120 mm tank ammunition), Air Force (armour-piercing munitions for the Gatling gun mounted on the A-10 aircraft), Marine Corps (Harrier aircraft and tank munitions) and Navy (rounds for the Phalanx Close-in Weapon System) (OSAGWI, 1998). The Army used an estimated 9500 DU tank rounds during the 1991 Gulf War, for both training and practice purposes (OSAGWI, 1998). The use of DU munitions in further military operations includes NATO military actions in Bosnia-Herzegovina (1995) and Kosovo (1999), and, more recently, coalition actions in Iraq.

Known exposure of US personnel to DU during the 1991 Gulf War occurred as the result of friendly fire incidents, cleanup operations and accidents (including fires). DU-containing projectiles struck 21 US Army combat vehicles (15 Bradley Fighting Vehicles and six Abrams tanks) (US AEPI, 1995). After the war, assessment teams and cleanup and recovery personnel (including explosive ordnance disposal, battle damage assessment and radiation control teams and salvage personnel) may have had contact with DU-contaminated vehicles or DU munitions. In July 1991, a large fire occurred in Camp Doha near Kuwait City. This site housed a number of combat-ready vehicles, and the series of blasts and fires damaged or destroyed vehicles and munitions including M1A1 tanks and DU munitions. Troops at the scene and those involved in cleanup efforts may have been exposed to DU residue. Other troops may have been exposed through contact with vehicles or inhalation of DU-containing dust. It is difficult to assess the exposure of Iraqi soldiers and other nonmilitary personnel during military operations.

A range of DU concentrations have been measured within struck vehicles in test firings of large-calibre DU rounds (US Department of Defense, 2004), and inhalation intakes of DU oxides for surviving crew have been estimated to be about 10–20% greater in an Abrams tank with DU armour, compared to one with conventional armour, and about three times greater in a struck tank than in a less heavily armoured Bradley Fighting Vehicle. In one test firing the activation of the tank's ventilation system reduced the concentration and hence the estimated inhalation intake of DU by about 90% (Capstone Report, 2004), but in friendly fire incidents in the Gulf War it is not clear whether ventilation systems were active, and assessment of risks in most studies are based on the cautious assumption of no ventilation. Those surviving within, or close to, a tank struck with a DU round will inhale DU oxides in the impact aerosols, and may also receive DU shrapnel wounds, and are therefore believed to be at highest risk from DU, although a broader view of the risks to soldiers on the battlefield is required (Miller, 2006; Spratt, 2006). Radiation risks to military personnel of handling DU rounds, and to tank crews from the DU rounds stored within tanks, are considered to be very low, as exposure levels can easily be monitored and managed. A discussion of civilian populations and the environment with regard to DU is found later in this chapter.

Most studies of the hazards of DU munitions have focussed on battlefield exposures, but there are also concerns about longer-term exposures to local populations who live in, or return to, areas where DU munitions were deployed. In the Gulf War, the conflicts in the Balkans and in Iraq, the great majority of the total mass of DU that was deployed was small calibre DU rounds (30 mm; ~300 g of DU), fired in strafing runs by attack aircraft, with only a small proportion being large-calibre tank rounds (mostly 120 mm; ~4.5 kg of DU). Most of the DU penetrators in strafing runs miss their intended targets and end up embedded several feet in the ground, where they corrode (UNEP, 2002). In the years following a conflict, the local population may also be exposed to DU by inhalation of resuspended soil containing DU oxides, unless careful clean-up procedures are used (UNEP, 2002).

Understanding the exposure pathways of most significance for soldiers and local populations, and the resulting risks to health, requires estimates of intakes of DU for a number of different scenarios. Exposures on the battlefield have usually been considered in one of three categories (US Department of Defense, 2004), an approach that provides a useful framework for considering the potential intakes and risks, and which was used in the Royal Society, Capstone and Sandia reports.

- Level I includes soldiers surviving in a vehicle struck by a DU penetrator(s), or first responders entering the struck vehicle to rescue the occupants. Intakes are likely to be dominated by inhalation of DU particulates and, for surviving crew, by DU shrapnel wounds.
- Level II includes those who work within struck vehicles for substantial periods, at some time after the impact, and who may be exposed to inhalation of DU oxides resuspended within the contaminated vehicle.
- Level III includes all others on the battlefield who may be exposed from being downwind of struck vehicles or from briefly entering struck vehicles.

Identification and health monitoring of soldiers who are believed to have received Level I and II exposures has been the main priority for veterans of the Gulf War and subsequent conflicts. The number of soldiers

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who are known to have received Level I exposures is relatively small. For example, after the 1991 Gulf War, approximately 100 United States soldiers known to have been involved in friendly fire incidents were assessed as being in this category (McDiarmid *et al*., 2006 and 14 United Kingdom soldiers were assessed as having received Level I exposures in the Iraq conflict during 2003 (UK Ministry of Defence, personal communication; (Spratt, 2006). However, there are likely to be larger numbers of Iraqi soldiers from the 1991 Gulf War who had Level I exposures, but no data appear to be available. Exposures for soldiers categorized as Level III are likely to be much lower than for those in Level I or II, but, as this group is potentially very much larger, estimates are required of the range of exposures (and health risks) for soldiers and support personnel who were on or near the battlefield where DU munitions were deployed.

3 URANIUM ABSORPTION

Previous studies on uranium absorption, retention and excretion have been done almost exclusively following an acute uranium exposure and are very different from the chronic DU exposure scenarios that were discussed above. The amount of uranium that the body absorbs depends largely on the route of exposure and the solubility of the uranium compounds to which the individual is exposed. Absorption of uranium can occur in three ways, through inhalation, ingestion and wounding.

Following inhalation of uranium, insoluble uranium compounds may remain within the pulmonary tissues, especially the pulmonary lymph nodes, for a long time and thus constitute a localized radiological hazard. In contrast, uranium is less readily absorbed from the intestinal tract than from the respiratory tract, resulting in lower doses per unit intake. Chemical toxicity, characterized predominantly by renal dysfunction as a consequence of acute exposure to soluble uranium, and lung injury potentially caused by the ionizing radiation from uranium decay isotopes are the best-characterized consequences of acute exposure to uranium compounds. However, the chemical and radiological properties of uranium could act cooperatively to cause tissue damage, and therefore, it cannot be assumed that excess cancers would be due solely to the radiological effects of uranium or that organ damage is exclusively due to its heavy-metal properties.

4 URANIUM TRANSPORT, RETENTION, EXCRETION

Following acute uranium exposure, absorbed uranium forms soluble complexes with bicarbonate, citrate or proteins in the plasma (Pellmar, 1999a; Stevens *et al*., 1980; Cooper *et al*., 1982). Estimates show that approximately 47% of blood uranium forms a complex with bicarbonate in plasma, 32% of uranium binds to plasma proteins and 20% binds to erythrocytes (Chevari and Likhner, 1968). The retention time and excretion of the bicarbonate complex of uranium depends on the pH of the solution and differs in various bodily compartments (Berlin and Rudell, 1986). The low-molecular-weight bicarbonate complex passes through the renal glomerulus and is excreted in the urine at a rate that depends on urinary pH. At high pH, small amounts of uranium are retained within the walls of the tubular lumen of the kidney. At low pH, bicarbonate–uranyl (and citrate–uranyl) complexes dissociate. The uranyl ion forms complexes with proteins on the surface of cells lining the tubule. In contrast, protein-bound uranium is more likely to remain in blood since little protein passes through the glomerulus.

Based primarily on animal studies, the rate of deposition and clearance of inhaled uranium particles from the lung depends on their chemical form and particle size. Larger particles are transported by mucociliary action from the respiratory system to the pharynx, where they are swallowed and then eliminated in the faeces. The clearance of the smaller particles that are deposited in the lungs depends on the solubility of the compounds. Particles that contain the more soluble forms of uranium are more rapidly absorbed into the bloodstream and excreted in urine within one to five days (Damon *et al*., 1984), while smaller particles of less soluble forms can be retained for up 30 months (Leach *et al*., 1973). It is again important to consider the differences between acute and chronic uranium exposure when considering uranium kinetics. An example of the differences between acute and chronic uranium exposure and uranium retention can be observed by examining the contrasting studies of Lang and Raunemaa (1991) and Pellmar *et al*. (1999a). After administering high dose UO_2 to rats by an intragastric tube, no significant uranium was detected in liver, kidney, muscle, bone, brain, blood and urine within 48 hours post ingestion. In contrast, Pellmar *et al*., using embedded DU pellets (chronic exposure 1–18 months) in rodents, demonstrated that bone and kidney were the primary reservoir for the uranium that had dissolved from embedded DU fragments. Uranium also localized within the central nervous system (CNS), lymph nodes, testes and spleen (Pellmar *et al*., 1999a). Low levels of uranium were noted in the serum at all time points.

5 DU TOXICITY: *IN VITRO* RESEARCH APPROACHES

The earliest studies on DU effects involved using cellular model systems and were initiated and conducted

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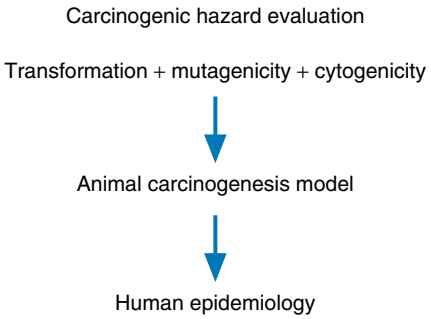

Figure 2 The process of carcinogenic hazard evaluation. The standard approach to assess carcinogenicity is to use cellular end points initially, followed by animal carcinogen studies. Human epidemiology studies contribute to the evaluation of carcinogenesis, if available.

at the US Armed Forces Radiobiology Research Institute, beginning in the mid 1990s. A strategic research approach, involving the progression from cellular studies to animal models and finally to human epidemiology considerations, was used by Dr. Miller and colleagues (**Figure 2**). Using this multistep approach, an immortalized human cell line was used to examine multiple end points, including neoplastic transformation, genomic instability, genotoxicity and radiation-induced damage. First the carcinogenic potential of soluble and insoluble forms of DU was evaluated using the neoplastic transformation model approach (**Figure 3**). In this approach, human cells were exposed to the test material, processed and analysed for cell colony formation. An evaluation of the colony morphology is used to define the state of transformation of the exposed cells. These were the first studies to demonstrate that DU could transform human cells into the malignant phenotype. A comparison to other heavy metals, like nickel and tungsten alloy, was also done. Neoplastic transformation of human osteoblast (HOS) cells to a tumorigenic phenotype after exposure to both uranyl chloride, a soluble DU compound, and uranium dioxide, an insoluble DU compound, was observed (Miller *et al.*, 1998b). These malignant (DU-treated) cells also demonstrated anchorage-independent growth, increased levels of the *k*-ras oncogene and decreased levels of the Rb tumour-suppressor protein. The latter changes are associated with the malignant phenotype in other heavy-metal-exposed cells. A comparison to insoluble DU (uranium dioxide), and other nonradioactive heavy metals was done to enable a comparison of DU-induced effects to those of better-known carcinogenic heavy metals, like nickel. Transformation rates of DU-exposed cells were 9.6 times those of untreated controls, and transformed cells formed tumours in nude mice. The results from all these transformation studies are compiled in **Table 1**. A comparison of the transformation values indicates that DU can neoplastically transform human cells similar to other better-known heavy metals and at a similar magnitude. These results were the first indication that DU could be carcinogenic; however, as is the case for any determination of carcinogenicity, *in vivo* studies would be necessary to more fully understand DU's potential as a carcinogen. Not only was the human-cell model excellent as a means to study DU, but the same model was later used to evaluate additional carcinogenic, mutagenic and genotoxic end points, providing a continuity in assessing the results.

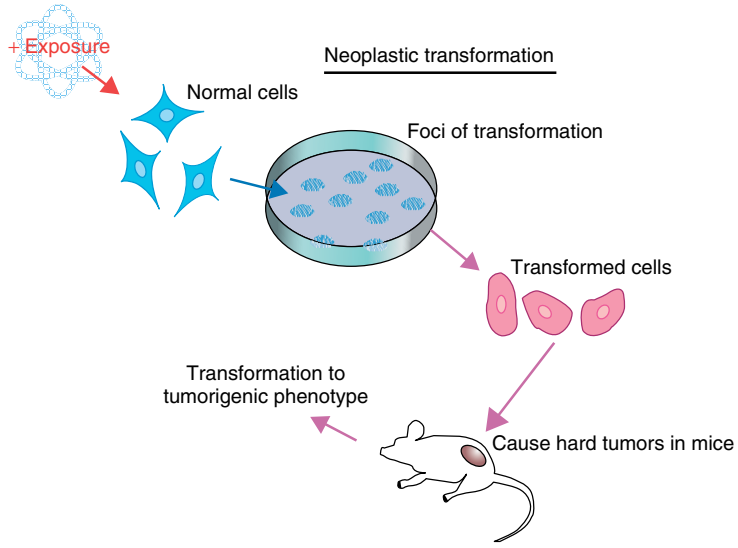

Figure 3 Neoplastic transformation assay. The neoplastic transformation assay is an *in vitro* assay used to assess the phenotype of cells following exposure to the suspected carcinogen. Cells are plated onto petri dishes, incubated for cell growth and assessed after the appropriate growth time for colony formation. Colonies are counted and individual colonies are expanded to a cell line. Cells are then injected into immunocompromised mice to evaluate tumorigenicity.

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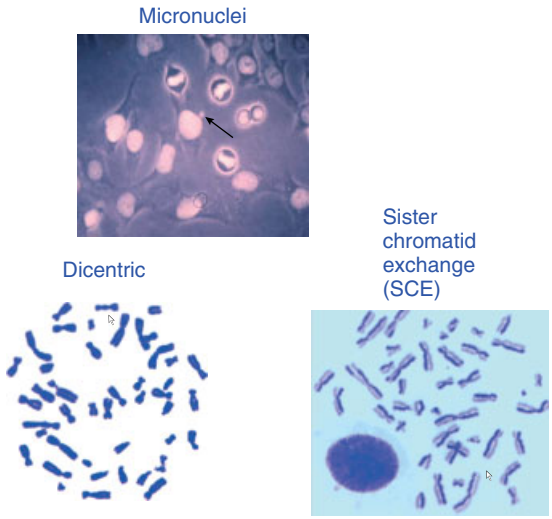

Figure 4 Examples of genotoxic damage. Chromosomal measures of genotoxic damage include micronuclei, dicentric chromosomes and sister chromatid exchanges.

In vitro studies demonstrated that DU was genotoxic by measuring the induction of sister chromatid exchanges, micronuclei and dicentric chromosomes in the same human-cell model used to study neoplastic transformation. Three examples of chromosomal damage measured in these studies are shown in **Figure 4**, including micronuclei, dicentric chromosomes and sister chromatid exchanges. Studies examining the effect of DU exposure on the induction of these chromosomal changes revealed that DU (in both soluble and insoluble forms) could induce an increase in genotoxic damage in comparison to control levels (**Table 2**). Experiments with cultured rodent cells have also demonstrated the capacity of DU to induce genotoxic changes. Lin (1993) showed that uranyl nitrate increased frequencies of micronuclei, sister chromatid exchange and chromosomal aberrations in Chinese Hamster Ovary (CHO) cells. The results describing the development of dicentric chromosome damage were the first studies to demonstrate that DU could induce a radiation-specific marker, a dicentric chromosome. The nonradioactive heavy metals, like nickel, which were genotoxic, causing sister chromatid exchanges and micronuclei formation, did not induce dicentric chromosomes; therefore, these data also suggest that DU causes genotoxic damage via a radioactive mechanism. This finding led to additional studies addressing the potential role of alpha-particle radiation in damage caused by DU exposure.

Inhalation studies in a rodent model showed that exposure to DU by inhalation resulted in DNA strand breaks in bronchoalveolar lavage (BAL) cells and in an increase of inflammatory cytokine expression and production of hydroperoxides in lung tissue, suggesting that the DNA damage was in part a consequence of the inflammatory processes and oxidative stress (Monleau *et al*., 2006a). The observed effects, which seemed to be linked to the DU doses, were independent of the solubility of DU compounds and correlated with the type of inhalation. Repeated inhalations resulted in a potentiation of the effect in both BAL and kidney cells. Measurement of DNA damage by the Comet assay in neutral conditions revealed that DNA damage in BAL cells was composed partly by double-strand breaks. Since double-strand DNA breaks are associated with radiation-induced, but not chemically induced damage, the results suggest that radiation could have contributed to the DU genotoxic effects observed *in vivo*. In contrast, the formation of uranium–DNA adducts and mutations in mammalian cells after *in vitro* exposure to another DU compound, uranyl acetate, were not associated with the generation of free radicals. These results suggest that chemical effects played a significant role in the DU-induced DNA damage (Stearns *et al*., 2005; Yazzie *et al*., 2005).

To more definitively address the question of the role of alpha-particle radiation and DU, Miller *et al*. (2002a) showed that incubating HOS cells with uranyl nitrate solutions at a fixed uranium concentration, but increasing specific activity, resulted in increasing transformation rates (**Figure 5**). These results demonstrated that uranium toxicity can result from both chemical and radiological toxicity. Previously it was thought that DU caused its effects through chemical effects alone, since the radioactive contribution was calculated to be low. The results from the uranium-isotope study have indicated that the role of radiation in DU's cellular effects cannot be discounted (Miller *et al*., 2002b). Further support of DU's radiation effects includes a

Table 2 Heavy-metal genotoxicity of human osteoblast cells. Cells were exposed to DU, tungsten alloy, nickel, or beryllium and assessed for genotoxic damage. Genotoxic measures used were micronuclei, dicentric chromosomes, sister chromatid exchanges and DNA double-strand breaks

	DU (soluble)	DU (insoluble)	rWNiCo	Be	Ni
Micronuclei induction	↑	↑	↑	↑	↑
Sister chromatid exchange	↑	↑	↑	↑	↑
DNA filter elution (DNA strand break)	↑	↑	↑	↑	↑
Dicentric formation	↑	↑	No change	ND	No change

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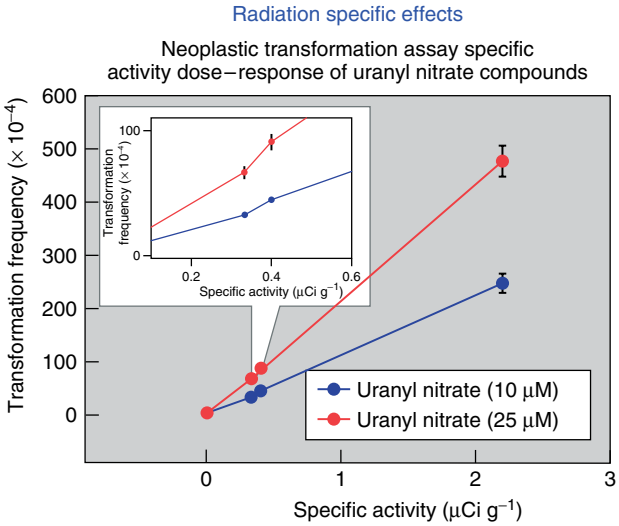

Figure 5 Radiation specific effects. To determine if DU induces radiation-specific damage, human osteoblast cells were exposed to equal concentrations of uranyl nitrate compounds (DU, specific activity (SA) 0.43 μCi g^{-1}; 238U, SA 0.33 μCi g^{-1}) that had different specific activities. Neoplastic transformation frequency was assessed. (Reproduced from Miller *et al.*, *Radiation Protection Dosimetry*, **90**, 4, 2002.)

recent mutagenicity study. Mutagenicity at the hypoxanthine (guanine) phosphoribosyltransferase (*hprt*) locus was measured by selection with 6-thioguanine. There was a dose-dependent increase in mutagenic response following DU exposure (10.50 μm); the average increase in mutagenicity above background ranged from 2.54 ± 1.19 to 8.75 ± 1.8 ($p < 0.05$). Using the same concentration (25 μM) of two uranyl nitrate compounds that have different uranium isotopic concentrations and, therefore, different specific activities, the effect on *hprt* mutant frequency *in vitro* was examined. Results showed that at equal uranium concentration, a 1.33-fold increase in specific activity resulted in a 1.27 ± 0.11-fold ($p < 0.05$) increase in *hprt* mutant frequency. Taken together, these data support earlier results showing that radiation can play a role in DU-associated mutagenicity *in vitro*.

Since evidence has shown that radiation can induce a transmissible persistent destabilization of the genome, studies were done to evaluate the involvement of genomic instability in the mechanism of DU-induced effects (Miller *et al.*, 2003). This means that damage to cells that are irradiated is transmitted over time to offspring cells. A variety of end points can be used to assess genomic instability, including cell survival, neoplastic transformation and genotoxic damage. One study has demonstrated the yields of cell lethality and DNA damage measured immediately, or at delayed times after DU, Ni and gamma-radiation exposure in a human cell model. Micronuclei yields were also measured in clonal progeny of surviving DU-exposed cells. Following the initial concentration-dependent acute response for both end points, data demonstrated that there was *de novo* genomic instability in the surviving progeny. Delayed reproductive death was observed for many generations (36 days, 30 population doublings) following cellular exposure to DU, Ni or gamma radiation. Delayed production of micronuclei was observed at times up to 36 days post DU exposure. There was also a persistent increase in micronuclei in all clones isolated from individual cells which had been exposed to nontoxic concentrations of DU. Cell clones isolated from gamma-irradiated individual cells (at doses equitoxic to the DU exposure) and Ni-exposed cells, did exhibit an increase in micronuclei frequency. From this investigation, it appears that DU exposure *in vitro* resulted in genomic instability manifested as delayed reproductive death and micronucleus formation. These studies confirm previous results demonstrating that heavy metals, like cadmium and Ni, could induce genomic instability *in vitro* similar to that observed with radiation (Coen and Mothersill, 2001; Mothersill and Seymour, 1998). Since there are also extensive data showing that alpha particles can induce a persistent instability in the genome of progeny of irradiated cells, it is difficult to determine whether the alpha particle or metal component of DU is responsible for the induced genomic instability. There have been no published studies determining whether the mechanism of genomic instability associated with DU exposure involves radiation, is exclusively chemical or involves both effects.

In conclusion, the *in vitro* studies examining cellular effects of DU compounds demonstrate that the DU compounds tested are neoplastically transforming, genotoxic, mutagenic and can induce genomic instability. Cellular damage following DU exposure is caused in part by radiation effects, but the contribution of chemical vs. radiation effects is still not known. These findings strongly support the hypothesis that DU is carcinogenic. In the next section of this chapter we will review what the limited carcinogenesis *in vivo* studies have shown regarding the question of DU carcinogenicity in animal models.

6 DU TOXICITY: *IN VIVO* RESEARCH APPROACHES

6.1 Solid Tumours in Laboratory Animals

Experiments with laboratory animals have expanded our understanding of the carcinogenic potential of

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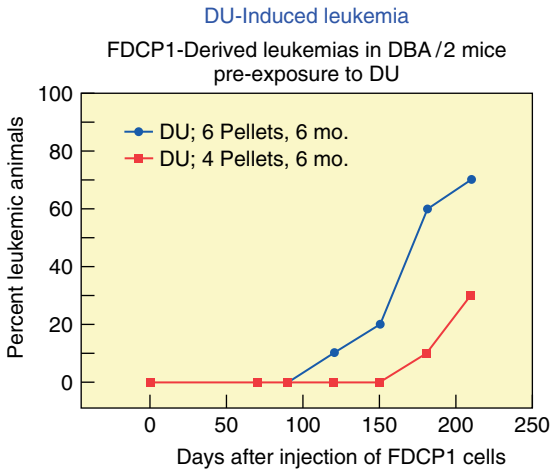

Figure 6 DU-induced leukaemia *in vivo*. Rodents were chronically exposed to implanted DU pellets for three months. The animals were then injected with haematopoietic progenitor cells and monitored for leukaemia development for 225 days. Leukaemia was determined by white blood counts and verified by necropsy.

uranium and DU. Not long after the 1991 Gulf War, in an effort to understand more about the potential health effects in personnel wounded in that conflict by DU shrapnel, Pellmar *et al.* (1999a) carried out a toxicological investigation using Sprague–Dawley rats implanted with various numbers of DU pellets (cylinders 1 mm in diameter and 2 mm long) to mimic shrapnel injuries in humans. Although cancer was not specifically designed as an end point in these studies, necropsies of subject rats showed no increased number of tumours in DU-implanted rats compared to tantalum-pellet-implanted controls. The interpretation of that data was confounded by the high levels of spontaneous tumour development typical of Sprague–Dawley rats and the lack of a statistically relevant number of rats. In similarly designed studies with appropriate numbers of rodents, Hahn *et al.* (2002) implanted male Wistar rats with either pellets or thin foils (1 × 2 or 2 × 5 mm) of DU in their hind limbs. This study showed that DU pellets of sufficient size were associated with soft-tissue tumours; the types of tumours included malignant fibrous histiocytoma, fibrosarcoma and osteosarcoma (Hahn *et al.*, 2002). All tumours were in the soft tissues of the hind legs directly associated with the implanted DU. In some tumours, black shards, particles of implanted fragments could be seen scattered through most of the tumour tissues. These histologic findings lend further credence to the association of the implants with the tumours. An increased tumour incidence was observed in the rats with the largest DU implants when compared with the sham or foreign-body (Ta) controls. In addition, increased tumour incidence was related to increased fragment size in the DU-treated rats. The response could not be explained by physical surface area alone because the tumour incidence with the Ta implants of similar size was much lower. There was a correlation with the initial alpha-particle radioactivity on the pellet surface. However, radiography demonstrated that the physical shape of the DU fragments changed prior to tumour development and therefore the surface alpha radioactivity changed with time as the shape of the implants changed, making it difficult to determine to what extent radioactivity was involved in the development of DU-associated tumours. In comparison, in experiments assessing the carcinogenic potential of DU and a tungsten alloy proposed as a surrogate for DU in armour-penetrating munitions, Kalinich *et al.* (2005) demonstrated DU pellets (1 × 2 mm cylinders) implanted into the leg muscles of Fisher 344 rats for 18 months caused no tumour development. These conflicting results have not provided a clear answer as to whether chronic exposure to embedded DU causes solid tumours, but highlight the usual confounding problems in heavy-metal carcinogenesis research. These confounding factors include the relevance of size-dependent tumour formation, heavy-metal solubility and species/strain selection.

6.2 Leukaemia in Laboratory Animals

While studies using rat models showed that DU caused a solid-state induction of solid tumours, other results indicated that DU implants could induce leukaemia in a mouse model. Recent studies from Miller's laboratory demonstrated that internalized DU could be a carcinogenic risk by causing leukaemia in laboratory mice (Miller, 2005). To better assess this risk, they developed an *in vivo* leukaemogenesis model using murine haematopoietic cells (FDC-P1) that are dependent on stimulation by granulocyte-macrophage colony-stimulating factor. Although immortalized, these cells are not tumorigenic on subcutaneous inoculation. As shown in **Figure 6**, intravenous injection of FDC-P1 cells into DBA/2 mice was followed by the development of leukaemias in 76% of all mice implanted with DU pellets In contrast, only 10% of control mice developed leukaemia. Karyotypic analysis confirmed that the leukaemias originated from FDC-P1 cells. The full impact of these results is not understood, since human studies have not shown the development of leukaemia in DU-exposed individuals; however, it appears that a DU-altered *in vivo* environment may be involved in the pathogenesis of a DU-induced leukaemia in an animal model. Further studies are necessary to determine how the DU-altered environment enhanced the development of leukaemia in mice.

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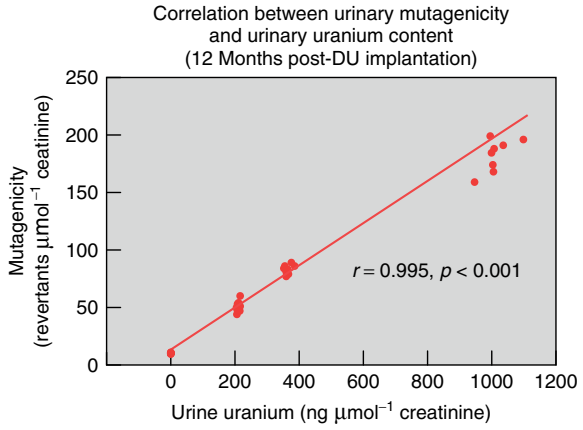

Figure 7 DU-induced mutagenicity *in vivo*. Rodents were chronically exposed to implanted DU pellets for 3–18 months. Urine was collected periodically and assayed for mutagenicity using the Ames test. (Reproduced from Miller *et al.*, *Mutagenesis*, **13**, 643–648, 1998.)

6.3 Uranium Genotoxicity/Mutagenicity

There have been very few studies evaluating uranium exposure in humans with genotoxic end points. Such studies are relevant because destabilization of the genome can indicate an increased susceptibility to cancer development. Martin *et al.* (1991) reported that levels of chromosomal aberration, sister chromatid exchange and dicentrics measured in nuclear-fuel workers increase proportionally with uranium exposure. McDiarmid *et al.* (2004), in their 10 year follow-up of 39 veterans exposed to DU in friendly fire incidents during the 1991 Gulf War, reported that study participants exposed to the highest levels of DU showed a statistically significant increase in chromosomal aberrations compared to the low-exposure groups. HPRT mutation frequencies were also significantly higher in the high-DU groups, but sister chromatid exchanges were not.

The consistent observation of uranium-induced genetic changes remains a cause for concern since they are known to precede cancer development. Hu and Zhu (1990) injected uranyl fluoride into the testes of mice and showed that chromosomal aberrations in spermatogonia and primary spermatocytes are dependent on the amount of injected uranium. In experiments in which rats were implanted with pellets of DU and/or the biologically inert metal tantalum, urine mutagenicity levels increased in a DU dose-dependent manner (Miller *et al.*, 1998a). **Figure 7** demonstrates that DU internalization results in the excretion of a mutagen which is assumed to be uranium.

6.4 Nephrotoxicity

The review by Hodge (1973) of uranium toxicity prior to the Manhattan Project (1942) shows that it has long been known that uranium is toxic to humans, animals and other living things. Kidney toxicity of uranium was first recognized in animals around the middle of the nineteenth century, and kidney toxicity remains the primary basis for the regulation of uranium exposure. Limits for inhalation and ingestion of uranium are aimed at not allowing uranium content in the kidney to exceed a set value, which for most countries is set at a maximum of 3 µg of uranium per gram of kidney tissue; effects caused by exposure of the kidneys at these levels are considered to be minor and transient.

The pharmacokinetics and pharmacodynamics of uranium and, therefore, DU are well established (Wrenn *et al.*, 1985; Leggett, 1994; Taylor and Taylor, 1997; ICRP, 1995; Leggett and Pellmar, 2003). There have been many studies that have investigated the results of uranium exposure in laboratory animals (Morrow *et al.*, 1982; La Touche *et al.*, 1987; Ortega *et al.*, 1989; Wrenn *et al.*, 1989). Once absorbed it circulates in the blood, primarily as the stable uranyl ion UO_2^{2+} bound to bicarbonate, albumin or proteins (Diamond, 1989; Kocher, 1989; Leggett, 1989). In the kidney, uranium is filtered through the glomerulus and most is excreted within 24 hours. Renal kidney toxicity occurs when residual uranium is subsequently taken up by the proximal tubules and causes damage by forming complexes with phosphate ligands and proteins in the tubular walls, thereby impairing kidney function (Blantz, 1975).

Pellmar *et al.* (1999a) showed that DU from pellets implanted in muscle of rats can be measured in their urine within one day after pellet implantation. Over the course of the 18 month experiment, kidney uranium content reached levels well above 5 µg g^{-1} of kidney tissue, a concentration that, if reached in an acute exposure, would normally prove lethal to the animal. The findings suggested that the kidney adapted to the high levels during the chronic exposure. This aspect of DU exposure has not been fully examined and the potential for adaptive responses to DU remains an intriguing possibility.

6.5 Bone Effects

Bone is a major site of uranium deposition. Neuman and colleagues, in a series of early articles designed to understand how uranium interacts with normal bone metabolism, published the first observation demonstrating that bone has a high affinity for uranium. Of a toxic dose, 20–30% of intravenous uranium could be found in the bones of male rats within 2.5 hours after administration, and 90% of the uranium retained by the body

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after 40 days was in bone (Neuman *et al*., 1948a). They showed that young growing rats or rats deficient in dietary calcium incorporated greater amounts of uranium than controls (Neuman *et al*., 1948b). They also showed that uranium is preferentially incorporated in areas of active calcification and becomes more refractory to resorption as new calcification covers areas of uranium deposition (Neuman and Neuman, 1948).

Uranium incorporates itself into the bone matrix by displacing calcium to form complexes with phosphate groups in the matrix (Domingo *et al*., 1992; Guglielmotti *et al*., 1989). Bone-bound uranium establishes equilibrium with uranium in the blood, and as the circulating uranium is excreted by the kidneys, bone-bound uranium slowly returns it to the circulation over time (Wrenn *et al*., 1985). Pellmar *et al*. (1999a) demonstrated that DU from implanted pellets rapidly distributes throughout the body and accumulates at high levels in the bone, though histological examination showed no bone lesions as a result.

6.6 Neurological/Behavioural Effects

The neurophysiological effects of uranium exposure have been investigated for many decades. Among the early findings was the observation that uranyl ions potentiate the twitch response of frog sartorious muscles by prolonging the active state of contraction. The fact this effect was reversed by administration of phosphate ions suggested that uranium prolongs the action potential (Sandow and Isaacson, 1996). In a study of uranium workers, Kathren and Moore (1986) showed individuals excreting up to 200 µg U per l urine manifested abnormal mental function. High doses of oral (210 mg kg^{-1}) or subcutaneous (10 mg kg^{-1}) uranyl acetate caused tremors in rats (Domingo *et al*., 1987). It was also shown that uranium applied at high concentrations to the ileal longitudinal muscle of guinea-pig (Fu and Lin-Shiau, 1985) and mouse phrenic nerve–diaphragm preparation (Lin *et al*., 1988) enhanced muscle contraction.

In a study investigating the toxicology of embedded pellets of DU in rats to mimic shrapnel wounds in wounded 1991 Gulf War veterans, Pellmar *et al*. (1999b) demonstrated that DU crosses the blood–brain barrier and accumulates in the hippocampus, where electrophysiological changes were observed. Briner and Murray (2005) tested behavioural effects and brain lipid peroxidation in rats exposed to various concentrations of uranyl acetate in drinking water for two weeks or six months. Open-field behaviour was altered in male rats receiving the highest dose of DU after two weeks of exposure; female rats demonstrated behavioural changes after six months of exposure. Lipid peroxidation levels increased in the brain and correlated with some of the behavioural changes, but the correlation was ambiguous. Barber *et al*. (2005) sought to determine the kinetics of uranium content in the brains of rats following a single intraperitoneal injection of uranyl acetate (1 mg kg^{-1}). They found that uranium content in all areas of the brain tested increased rapidly after injection and remained elevated for seven days post-injection. Interestingly, rats stressed by daily forced swimming before uranium injection accumulated less uranium in their brains and had lower levels than unstressed animals, seven days after exposure.

Additional models have been used to evaluate neurotoxicity. The neurotoxicity of DU-uranyl acetate in *C. elegans*, using various neuronal green-fluorescent-protein reporter strains to visualize neurodegeneration, was studied (Jiang and Aschner, 2007). Results demonstrated that DU-uranyl acetate had low cytotoxic potential, did not result in significant changes in cellular energy metabolism, thiol metabolite oxidation or lipid peroxidation. Furthermore, the *C. elegans* studies did not show any significant neurodegeneration following DU-uranyl acetate exposure. Together, these studies suggest that DU, in the form of uranyl acetate, has low neurotoxic potential.

Studies employing enriched uranium have also been conducted to evaluate the functional neurological effects of accumulation of uranium in the brain, Houpert *et al*. examined the effects of chronic exposure to enriched uranium in the drinking water of rodents (Houpert *et al*., 2007). Concomitant with significant accumulation of uranium in the hippocampus, enriched uranium exposure altered the spatial working memory capacities of rodents and this effect was correlated with previously described accumulation of uranium in the hippocampus, which is one of the cerebral areas involved in this memory system.

Limited studies have been done to assess behavioural effects of DU exposure and the results have been conflicting. An evaluation of the effect of implanted DU in rats indicated that DU did not have a significant negative impact on their general health or neurobehavioural capacities, when assessed after long-term DU exposure (Arfsten *et al*., 2005). In contrast, repeated inhalation of DU resulted in uranium uptake in several brain tissues and an associated decrease in spontaneous locomotion activity and spatial working memory in exposed rodents (Monleau *et al*., 2006b).

6.7 Reproductive/Developmental Effects

Despite nearly a century of studies of uranium toxicity, there were few detailed studies of uranium reproductive and developmental toxicity until the late 1980s (Domingo, 1995). In most exposure scenarios, the chemical toxic effects from uranium compounds appear to occur at lower exposure levels than radiological toxicity

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(Hartmann *et al*., 2000), and this is thought to be the case for reproductive effects as well (Domingo, 1995). In the early 1980s, Domingo and his colleagues began extensive investigations of uranium reproductive toxicity, and they have provided most of our current knowledge on the subject to date. These studies, however, involved acute high-dose exposures to uranium and are very different from the chronic exposures examined in recent DU investigations. There are a few recent studies investigating the reproductive and developmental health effects of DU specifically. It is beneficial, however, to review the information regarding acute high-dose uranium exposures for comparison.

Early studies (Maynard and Hodge, 1949) identified uranium as a possible reproductive toxicant in rats. Male and female rats fed diets containing 2% uranyl nitrate hexahydrate for seven months, followed by normal diets for five months produced fewer litters, with fewer pups per litter, than control rats (Maynard and Hodge, 1949). However, it was difficult to determine in these experiments whether uranium toxicity or nutritional effects arising from retarded weight gain in the uranium-fed rats caused the decreased reproductive success. In follow-up studies, rats fed diets containing 2% uranyl nitrate hexahydrate for a single 24 hour period after weaning also produced fewer litters with fewer pups per litter than control rats, with no signs of maternal toxicity (Maynard *et al*., 1953), an observation that strengthened the connection between uranium exposure and reproductive toxicity.

Llobet *et al*. (1991) showed that male mice continuously receiving water containing uranyl acetate dihydrate and mated with untreated females resulted in a significantly decreased, but dose-unrelated, pregnancy rate, but testicular function and spermatogenesis were unaffected. Domingo *et al*. (1989) showed that pregnant female mice given uranyl acetate dihydrate ($0.05–50\,mg\,kg^{-1}\,day^{-1}$) by oral gavage from gestational day (GD) 13 through postnatal day (PND) 21 demonstrated no significant decrease in litter frequency, litter size and pup viability except at the highest dose ($50\,mg\,kg^{-1}$; about one-fifth of the oral LD_{50}). On the other hand, injection of 1/40 to 1/10 of the subcutaneous LD_{50} dose ($20\,mg\,kg^{-1}$) into pregnant female mice between gestational days 6–15 produced both maternal and foetal toxicity (Bosque *et al*., 1993). Some of the malformations noted in pups could have occurred as a result of maternal toxicity, but defects such as cleft palate and certain other variations are not known to be associated with maternal toxicity and were interpreted to be the result of uranium developmental toxicity (Domingo, 2001).

Recent studies have been conducted to examine the potential reproductive effects of chronic DU exposure. Using a Sprague–Dawley rat model, Arfsten and colleagues showed that internalized exposure to DU pellets (150 days total exposure), did not significantly affect the concentration, motion and velocity of sperm isolated from DU-implanted animals, in comparison to sham surgery controls (Arfsten *et al*., 2006). Reports of the health status of military veterans of the 1991 Gulf War have provided certain insights into the possible reproductive health effects of DU as well. A follow-up examination of DU-exposed individuals (via embedded DU fragments and/or inhaled DU dust) showed that there were no significant differences in semen and sperm characteristics among veterans with high- or low-DU urine concentrations (McDiarmid *et al*., 2000; 2004). Data from animals chronically exposed to DU pellets (seven months), however, have demonstrated a DU-dose dependence on the induction of sperm DNA damage measured by the Comet assay (Merlot and Miller, 2007) suggesting that DU exposure could have a deleterious reproductive effect in exposed males. Limited studies examining the reproductive effects on female rodents exposed to DU have been done as well. Oestrogen receptor-dependent responses in female mice were observed following long-term exposure (>120 days) to DU in drinking water (Raymond-Whish, 2007). These oestrogenic responses included selective reduction of primary follicles, increased uterine weight, greater uterine luminal epithelial cell height, accelerated vaginal opening and persistent presence of cornified vaginal cells. Indicators from this study that DU is an endocrine-disrupting heavy metal may have implications for reproductive health in females exposed to DU, but there are no data on human females and DU exposure to answer this question.

The impact of diminished reproductive parental health on offspring following parental DU exposures has been addressed in limited studies and, in general, the results demonstrate a DU dose- and time-dependent effect. Investigations on the involvement of DU exposure and offspring effects have been conducted using several model systems including Sprague–Dawley rats and C57/BL Big Blue transgenic mice. Short-term exposure (90 days) of Sprague–Dawley rats to embedded DU pellets did not have a negative impact on P1 reproductive success, survival, or body-weight gain (Arfsten, 2005). Furthermore, there were no statistically significant differences in F1 birth weight, survival and litter size at PND 0, 5 and 20 and no gross physical abnormalities were identified in the offspring (Arfsten, 2005). In contrast, long-term, high-dose DU-pellet exposure (seven months; eight pellets) in a 'Big Blue' transgenic mouse model, caused a significant reduction in F1 birth weight ($>12.3 \pm 1.1\%$) and litter size ($>22.4 \pm 2.0\%$) (Miller and McClain, 2007). Previous studies have demonstrated that paternal preconceptional exposure to radiation or heavy metals like cadmium can induce cancer in unexposed offspring (Lord, 1999; Dubrova, 2003; Nomura, 2004). The paternal transmission of genetic damage in offspring of male rodents carrying embedded DU pellets, using a transgenic mouse model, was examined. In this system λlacI gene mutation frequency in multiple tissues obtained from progeny of exposed

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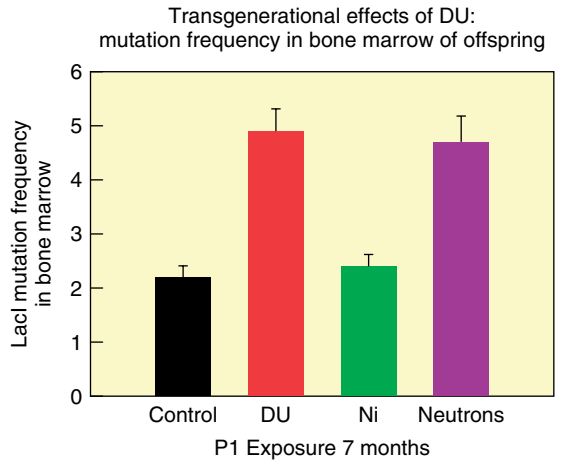

Figure 8 Transgenerational effects of DU: mutation frequency in bone marrow of offspring. An evaluation of preconceptional paternal exposure to DU was done. Male 'Big Blue' C57BL/6 mice which are genetically engineered to carry the *LacI* gene were chronically exposed to embedded DU for seven months. They were mated with nontransgenic unimplanted female C57Bl mice. The offspring were tested for *LacI* inheritance and offspring that carried the *LacI* gene were further tested for *LacI* mutagenicity (bone marrow). A comparison to implanted nickel and neutrons (1 Gy; 0.55 Gy min^{-1}) was done.

fathers is assessed by employing a λ*lac*I shuttle vector carried by cells of a transgenic mouse (Big Blue: strain C57BL/6 hemizygous mice containing 40 copies of a λ*lac*I shuttle vector per cell). Male rodents were internally exposed to implanted DU (high and low dose) and the mutation frequencies in testes and bone marrow were then measured. Offspring from exposed male parents were genotyped and those positive for the λ*lac*I gene were assessed for transmission of genetic damage in bone-marrow tissue. Data demonstrate that offspring from DU-exposed (high-dose) male parents exhibited a significant increase in the mutation frequency of the λ*lac*I gene in their bone marrow (2.4-fold elevation) in comparison to offspring from control or nickel-exposed fathers (**Figure 8**). The *LacI* mutation frequency in progeny from neutron-irradiated fathers was also significantly increased above control levels. The results from this study indicate the possibility of transmission of genomic instability from male parents carrying embedded DU to the somatic cells of their offspring. Paternal occupational exposures, including metals, (Tomatis, 1994) have been associated with an increased risk for a variety of childhood malignancies and transgenerational carcinogenesis has been shown in several rodent models with a spectrum of exposures, including chemical, metal and radiation (Cheng, 2004). Therefore, the possibility that DU exposure may be relevant to offspring health needs to be considered. This conclusion is further supported by results demonstrating that parental exposure to enriched uranium induced a delayed hyperactivity in rat offspring, suggesting that offspring studies should not be restricted to effects observed in young rodents (Houpert *et al.*, 2007) and that offspring effects are not related to carcinogenic possibilities, but include behavioural modification as well.

As of 1999, 50 of the Gulf War veterans in the McDiarmid studies had fathered 35 children since the conflict, and none had birth defects (McDiarmid *et al.*, 2001). The relatively small number of individuals involved in these studies and the end points that were possible limit their contribution towards fully understanding reproductive health effects and the studies were unable to address late effects in offspring.

7 EPIDEMIOLOGICAL STUDIES—DU EXPOSURE

A series of significant epidemiological studies of nuclear-industry workers and uranium miners and millers carried out since the mid-1960s have added a wealth of data to the uranium health-effects database. Several investigations of uranium millers (Wagoner *et al.*, 1965; Archer *et al.*, 1973; Waxweiler *et al.*, 1983), workers whose occupation exposes them to uranium dust inhalation in the workplace, used death certificates and, in some cases, health records to investigate cancers and other diseases (e.g. renal) as a cause of death. These studies failed to clearly identify a link between uranium exposure and any specific health effects, including cancer. Studies have also been carried out on workers at the Y-12 nuclear processing plant in Oak Ridge, Tennessee (Dupree *et al.*, 1995; Loomis and Wolf, 1996). The studies, which often included controls for age, race, gender, radiation dose, other chemical exposures and medical history (when available) showed no association between cancer and occupational exposure to radiation from external and internal sources. The relatively small sizes of these epidemiological studies, uncertainties about the amount of uranium workers were exposed to, and the impact of confounding factors, such as parallel exposures to agents such as radon, silicates and other toxic metals (e.g. arsenic), lead to large statistical errors in the results, so caution should be exercised in overinterpreting the results of such studies.

As discussed earlier, in the 1991 Gulf War, an unknown number of personnel were exposed to DU aerosols (primarily uranium oxides) after being in vehicles that were struck by DU munitions, rescuing personnel in struck vehicles, reclaiming or investigating struck vehicles or moving through areas where DU dust was left in the environment. Even though satisfactory exposure models exist for such personnel, it is generally considered that the brief exposures to DU dust experienced by personnel would have been far below

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exposures experienced by uranium miners and millers in earlier studies, so no cancers would be expected by any route of exposure. McDiarmid *et al*. (2004) calculated radiation dose estimates for personnel carrying DU shrapnel in their bodies as a result of fragment injuries. Whole-body radiation counting, using the International Commission on Radiological Protection (ICRP) 30 biokinetic model for uranium, yielded an upper dose limit of 0.1 rem per year, a dose is not considered particularly dangerous.

The fact that human uranium epidemiology studies continue to remain controversial as to whether uranium exposure by any route is associated with cancer (IOM, 2008; ATSDR, 1999a) the *in vivo* results from the DU studies are significant, and only serve to strengthen the relevance of *in vitro* experiments showing genetic changes consistent with cancer development. The BEIR IV report (BEIR IV, 1988) on radon and other alpha-particle emitters states that large statistical uncertainties in most of the epidemiological studies looking for cancer in uranium workers may be hiding small populations of adversely affected individuals; it cautions against minimizing the risk until more studies are available. The same is probably true for DU exposure.

8 DU ASSESSMENTS IN THE BALKANS

8.1 Background

Since DU has been used by the US military and Coalition forces in Kuwait (1991) and Iraq (2003–present), and by NATO in military operations in the Balkans (1995, 1999), there has been concern among the civilian populations in those regions as to their potential exposure to DU. To evaluate potential environmental contamination by DU in the individual countries where it was used, the United Nations Environmental Programme (UNEP) and the French Defense Radiological Protective Service (Service de Protection Radiologique des Armées (SPRA)) have conducted studies. The UNEP has conducted studies: (i) in Kosovo in 1999; (ii) in Serbia and Montenegro in 2001; (iii) in BiH in 2002 and (iv) in Kuwait, with the International Agency on Atomic Energy (IAEA) in 2003; (v) the French SPRA conducted a radiological assessment in Kosovo in December 1999 to evaluate DU exposure in the environment as well. To assist UNEP, in 2000, the North Atlantic Treaty Organization (NATO) provided UNEP with new information concerning the use of DU during the Kosovo conflict. This information included maps, amount of DU ammunition used and coordinates of the targeted areas. It enabled UNEP to conduct one of the first-ever international assessments on the environmental behaviour of DU following its use in a real conflict situation. The French government was concerned as well, since French soldiers had been stationed very near to specific DU-targeted sites in Kosovo, so they commissioned their own radiological assessment. Since a small number of sites outside Kosovo, in Serbia and Montenegro, had also been targeted with ordnance containing DU during the Kosovo conflict, UNEP decided that they should also be assessed. In 2002, the Council of Ministers of BiH requested UNEP to conduct a similar assessment in BiH related to the use of DU ordnance during the war 1994–1995. UNEP assisted the IAEA in conducting a study in Kuwait in 2002 as well. In the Balkans and during the IAEA Kuwait mission, mainly air-to-ground ammunition of the types 20/25 mm penetrators (Kuwait) and 30 mm penetrators (Balkans) were studied.

As indicated previously in this chapter, natural uranium is found in trace amounts in all rocks and soil, in water and air, and in materials made from natural substances. Therefore, each individual is exposed to a 'background' level of uranium in the environment. Uranium is commonly found in air, soil, drinking water, leafy vegetables, root vegetables and milk products. Natural uranium is incorporated into the human body, mainly through the ingestion of food and water, and the inhalation of air. The United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR) has estimated that the average person ingests 1.3 μg of uranium per day, corresponding to an annual intake of 0.46 mg or 11.6 Bq, primarily through the consumption of drinking water. Typically, the average person receives an annual dose of less than 116 μSv from exposure to natural uranium via ingestion and inhalation. Most of the uranium ingested is excreted in faeces within a few days and never reaches the bloodstream. The remaining fraction will be transferred into the bloodstream and excreted in urine within a few days, but a small fraction remains in the kidneys and other soft tissue, as well as in bones. All totalled you will probably receive a whole-body internal dose of approximately 116 μSv of natural uranium in a year.

Uranium is not generally transferred effectively along food chains, so in environmental assessments, inhalation is usually the exposure pathway that merits primary attention. Processes, such as migration through the soil, deposition of resuspended material onto crops and transfer to groundwater, might be of greater interest in the longer term. In a combat situation, the main radiological hazard associated with DU munitions is the inhalation of the aerosols created when DU munitions hit an armoured target. Studies carried out at test ranges show that most of the DU aerosols created by the impact of penetrators against an armoured target settle within a short time of the impact and in close proximity to the site, although smaller particles may be carried a distance of several 100 m by the wind. A possible exposure pathway for those visiting or living in DU-affected areas after the aerosols have settled

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is the inhalation of the DU particles that are resuspended through the action of the wind or human activities. One possible pathway of exposure that merits consideration is the inadvertent or deliberate ingestion of soil, although doses from this exposure pathway were, however, found to be much lower than doses associated with other pathways. Furthermore, exposure of individuals who find and handle unexploded DU munitions via external irradiation, due to the beta particles and gamma rays emitted by the DU, could occur. However, the dose received would be significant *only if* a person were in contact with DU projectiles over a considerable period of time, since the contact dose to the skin from DU is about $2.3\,\mathrm{mSv\,h^{-1}}$. It is therefore unlikely that even prolonged contact with DU would lead to skin burns or any other acute radiation effect. For perspective, the worldwide average annual effective dose from natural background radiation is 2.4 mSv, with a typical range of 1–20 mSv. The most significant contribution to the worldwide average annual effective dose comes from exposure to radon and its decay products (1.15 mSv); exposure to terrestrial gamma rays and cosmic rays accounts for 0.48 and 0.38 mSv, respectively. It is important to keep in perspective, the amount of natural radiation exposure an individual is exposed to each year.

8.2 Results from UNEP Assessments of DU in the Balkans

Results were obtained by the UNEP on the DU assessments that took place in the Balkans and the joint IAEA-UNEP mission to Kuwait. It should be noted that the time between the military conflict and the corresponding assessment in Kosovo was 1–2 years, in Serbia-Montenegro 2–3 years, in BiH 7–8 and 13 years in Kuwait. Since UNEP did not have its own laboratory capacity, it relied on the assistance of several international laboratories to analyse the samples and complete the results. The laboratories involved were the Swedish Radiation Protection Institute (SSI), the Swiss SPIEZ laboratory, the Italian Environmental Protection Agency and Technical Services (APAT) and the University of Bristol, UK.

At the sites investigated by the UNEP DU assessments, where a total of approximately 10 000 penetrators were fired, more than 200 penetrators, fragments and jackets (casings) were found on surfaces, and more than 500 penetrators were discovered buried in the ground. UNEP divided its penetrator assessment analysis into several areas including isotopic concentration, actinides/transuranic contamination and corrosion.

UNEP found that the composition of the DU used for 30 mm ammunition in the Balkans had been constant: ^{235}U ($0.200 \pm 0.005\%$ by mass); ^{234}U ($0.00055 \pm 0.005\%$) and ^{238}U ($0.790 \pm 0.006\%$). A similar value was found on Type 20/25 mm penetrators, analysed by the IAEA from the joint IAEA mission to Kuwait. The presence of ^{236}U, a resulting product from uranium enrichment was confirmed ($0.0028 \pm 0.0002\%$ by mass). UNEP also confirmed the presence of plutonium in penetrators used in the Balkans, but the concentrations were found to be extremely low, ranging from less than 0.8 to $88\,\mathrm{Bq\,kg^{-1}}$ for the sum of ^{239}Pu and ^{240}Pu. From the IAEA Kuwait mission $^{239+240}$Pu was found to be in the range $0.6–6.2\,\mathrm{Bq\,kg^{-1}}$ in the DU penetrators. The values found by UNEP and IAEA were lower compared to the values published by the US Army in January 2000 on DU used as tank armour, ranging from 85 to $130\,\mathrm{Bq\,kg^{-1}}$.

After lying in the ground for over seven years in Bosnia, the penetrators were heavily corroded and intensive pitting (corrosion attack producing small holes) of the DU surface had taken place. Based upon information provided by the US military, it was estimated that the penetrators had lost 34% of their weight due to corrosion (24) and penetrators laying on the ground surface were much less corroded than those buried below the ground surface. While unconfirmed, evidence was observed during the IAEA Kuwait mission that penetrators and fragments showed a similar corrosion pattern under desert conditions as in the Balkans.

Multiple contamination places and types were examined including more than 500 points of contamination by DU, including contamination of ground, buildings, vehicles, air and water. A contamination of the ground surface may be either *localized* or *widespread* over a large area, depending on the properties of the aerosols and the prevailing meteorological conditions. Widespread contamination exists in cases where the contamination can be found over a couple of 100 m from the source of contamination resulting in an large affected area. In general, widespread contamination occurs as a result of initial dispersion in air during the use of DU penetrators and then by dispersion/resuspension of DU by wind. UNEP determined that there were multiple sites of localized ground contamination, but found no evidence of widespread ground contamination by DU.

Contamination of vehicles was not observed by UNEP, since any previously hit vehicles had been removed prior to their assessment. A very comprehensive study on the effects of DU and vehicle contamination has been published by the US Army Center for Health Promotion and Preventive Medicine (2004).

The DU assessments in Kosovo, and Serbia and Montenegro did not find any DU-contaminated buildings. In contrast, fragments and DU dust were found in two buildings which were still in use by the local population or the army in BiH at two sites which reportedly were targeted by A10 aircraft penetrators. Both scratch and smear samples were collected, demonstrating a primary deposition of debris and dust from the initial impact of

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DU penetrators on the concrete floor inside the buildings. DU contamination inside the buildings ranged between 11 and 1070 mg m^{-2}. Smear samples taken from surfaces of artillery guns and grenade boxes, considered to be secondary contamination, showed a surface contamination of 59–270 μg m^{-2}. The resuspension factor for this building was calculated and found to be 5.2 10 9 m^{-1}. UNEP, using ICRP models, calculated that this contamination constituted a minor radiological risk. Therefore, UNEP recommended several decontamination measures that were to be completed by the local authorities.

Water sampling and analysis in Kosovo, Serbia and Montenegro, and Kuwait showed no contamination of water by DU. However in BiH, DU contamination of groundwater was found at one of the 11 sites investigated. Uranium isotopic analysis of all 11 water samples demonstrated that DU was present in only one source. The concentration of DU in that source was considered low, however, and measures were taken to prevent future water use. Water from that source had been used in farming, but no further action was taken.

As with water samples, measurements on possible contamination of air by resuspended DU from contaminated ground were carried out in Serbia and Montenegro, Kuwait and in BiH. The levels in air in Serbia and Montenegro and Kuwait were insignificant. In the Balkans investigations, several sampling sites demonstrated an increase in uranium concentrations in the air at two sites that sustained more DU munitions usage. Using ICRP models, UNEP calculated that the concentration of DU in air was in the nanograms per cubic metre, 0.05–6.0 ng m^{-3}, range and was classified as low. Normal uranium concentration in the air was also in the nanograms per cubic metre range, 0.08–0.80 ng m^{-3} (radiation dose: 1 μBq m^{-3}). From the radiation-dose viewpoint, UNEP concluded that effective doses from airborne uranium are very small, even if the concentration is several hundred times higher than normal.

In terms of botanical materials, at all sites UNEP primarily sampled lichen. In Bosnia, some lichen showed traces of DU that suggested earlier or ongoing contamination, possibly via resuspension through the air from contaminated ground. No vegetables tested were found to be contaminated by DU. Several farming areas in Kuwait were investigated in detail in the IAEA Kuwait assessment. There was no evidence of the presence of DU in any of the soil samples taken in the farms. However, in some crop samples in Kuwait low concentrations of DU were found; these results are confusing since neither the water taken from the site nor the soil, nor any other vegetables demonstrated any DU contamination. UNEP concluded that a possible cross-contamination had occurred at the time of packaging the samples at the end of the assessment.

Based on UNEP's work to date, they have concluded that the residue of DU munitions does not present a significant risk to the health of the general population in the countries they assessed. On a site-specific basis, the main risks are still considered to be toxicological, based on exposure to a heavy metal. The radiological risks are very small and less than or equal to background natural radiation. However, based on the precautionary principle and on existing scientific uncertainty regarding the environmental behaviour of DU, risk reduction measures, such as access restrictions and clean-up, should be adopted. In addition, UNEP recommends that long-term monitoring of groundwater should be employed and that the authorities should also be active by informing the local residents and workers at such sites of the hazards associated with collecting DU residues and other possible actions.

9 SUMMARY AND CONCLUSIONS ON DEPLETED URANIUM HEALTH EFFECTS

Since internalization of uranium in any form will result in a combined chemical and radiation exposure, there exists the potential for subtle differences in health effects between DU and uranium. Recent developments in cell-biology technology and understanding are providing more sensitive approaches towards understanding those differences (Miller *et al*., 2007; 2001). Furthermore, since there are conflicting results regarding the carcinogenicity of chronic DU exposure, (negative solid tumour results vs. positive leukaemia results), the question still remains whether chronic DU exposure is a significant toxicological threat.

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Toxicology and Disasters

H. Paul A. Illing

CONTENTS

1 INTRODUCTION

Disasters ('great or sudden misfortunes'—Concise Oxford Dictionary, 1990) occur from time to time. Because they are portrayed and analysed extensively in the news media and subjected to careful examination in subsequent public enquiries, they become entrenched in everyone's mind. Unfortunately, the ideas on how to handle or prevent potential disaster situations occurring have often been developed from the lessons learnt through analysing previous disasters. In many cases, disasters can be avoided and the effects of accidents minimized by careful planning. In addition, by examining how to handle the consequences of an accident once it has occurred, it should be possible to mitigate the effects. It is these thoughts that have led to legislation aimed at considering the safety aspects of certain hazardous situations at an early stage in order to minimize the likelihood of their becoming disasters.

Disasters may be naturally occurring, as with earthquakes and volcanoes. They may include the incidental results of human activity, as with incidents involving food supplies or industrial plant. Although fortunately rare, they may also be the intentional results of human activity, as with terrorist incidents. Some disasters are the consequences of a toxicant entering a biological system and creating a damaging perturbation to that system. These effects may be largely environmental (e.g. the consequences of an oil ranker spill in coastal waters) or they may affect human health, either directly (through ingestion of contaminated drinking water or inhalation of a toxicant as it is dispersed in air) or indirectly (e.g. via uptake into food species). Hence, a knowledge of the effects of toxicants can be important when examining disasters and potential disaster situations.

In this chapter, concepts involved in planning to prevent disasters occurring are discussed first. This is followed by an evaluation of different types of disaster or serious incidents involving toxicants. Although purely environmental effects should not be ignored, the examples chosen are all associated with human health effects. Subsequently, approaches to the planning associated with presenting disasters due to toxicants, and mitigating their effects, are discussed.

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2 THEORETICAL CONSIDERATIONS

Many of the concepts used in analysing major hazards and minimizing their potential for causing disasters have their origin in engineering concepts associated with the design of military equipment, aircraft and nuclear plant. As a consequence, different national and international organizations have developed definitions for aspects of this work, and these definitions need examining, especially as difficulties can ensue if toxicologists and engineering-based risk assessors employ different interpretations of the same words. Although 'hazard' and 'risk' are interchangeable terms to the general public, they have separate meanings in the context of risk assessment, so their definitions will be examined carefully.

2.1 Hazard

Hazard is an intrinsic property of a substance or situation. The Royal Society Study Group (1992) defined hazard as 'a situation that could occur during the lifetime of a product, system or plant that has the potential for human injury, damage to property, damage to the environment or economic loss', and the Institution of Chemical Engineers called it 'a physical situation with a potential for human injury, damage to property, damage to the environment or some combination of these' (Jones, 1992). The most recent toxicological definition from the joint Organisation for Economic Co-operation and Development (OECD)/International Programme on Chemical Safety (IPCS) project on the harmonization of hazard/risk assessment terminology (OECD, 2003) calls hazard 'the inherent property of an agent or situation having the potential to cause adverse effects when an organism, system or (sub) population is exposed to that agent'. A chemical hazard was further defined by the Institution of Chemical Engineers (Jones, 1992) as 'a hazard involving chemicals or processes which may release its potential through fire, explosion, toxic or corrosion effects'. A major hazard is an imprecise term: the Royal Society Study Group called it 'a large scale hazard which may have severe consequences'. The Institution of Chemical Engineers calls a major hazard 'an imprecise term for a large-scale chemical hazard, especially one which may be released through an acute event'. Given that a major accident is 'an occurrence (including, in particular, a major emission, fire or explosion) resulting from uncontrolled developments in the course of the operation of any establishment and leading to serious danger to human health or the environment, immediate or delayed, inside or outside the establishments, and involving one or more dangerous substances' (The Control of Major Accident Hazards, 1999), it can be concluded that a major hazard is a hazard having the potential that, if realized, could constitute a major accident. Both of these latter definitions use a narrower remit to excluded natural hazards, hazards from the use of nuclear power and terrorist hazards.

For chemicals or radiation to represent toxic hazards, they must be present in sufficient quantities to exert toxic effects on the individual. If they are to be major hazards, they must be present in quantities which, if released or dispersed, could result in effects being seen in many people.

2.2 Risk

Risk differs from hazard, as it involves a consideration of the probability or likelihood of a consequence occurring as well as what the consequence might be. The Royal Society Study Group (1983) defined risk as 'the probability that a particular adverse event occurs during a stated period of time or results from a particular challenge'. The Institution of Chemical Engineers Working Party (1985) and Jones (1992) extended the statement to 'the likelihood of a specific undesired effect occurring within a specified time or in specified circumstances'. They go on to say that 'it may be either a frequency (the number of events occurring in unit time) or a probability (the probability of a specified event following a prior event), depending on circumstances'. The OECD (2003) definition is 'the probability of an adverse effect in an organism, system or (sub) populations caused under specified circumstances by exposure to an agent'. If risk is quantified, it is a statistically based parameter.

2.3 Relationship between Hazard and Risk

In engineering terms, the risk to the individual is obtained by identifying possible events associated with the release and dispersion of significant amounts of a toxic substance, and/or by analysing potential failure mechanisms which would allow the release to take place (Health and Safety Executive, 1989; 1992). Likely frequencies and sizes of releases can then be calculated. The hazard is a quantitative statement of the exposure conditions associated with a specified level of harm ('harm criterion').

For major hazards, risk associated with a failure includes identification of a geographic area for which, following dispersion, exposure matches or exceeds the harm criterion; that is, the risk is risk of the initiating event. In contrast, occupational and public health toxicologists and epidemiologists often think of risk in terms of likelihood of end effect occurring for a given level of exposure (the risk of end effect arising at the exposure level defined as the harm criterion). To the engineering risk assessor, this is 'uncertainty in defining the hazard'.

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For the purposes of this chapter risk will be taken as risk associated with the initiating event and dispersion pattern.

2.4 Individual and Societal (Population) Risk

There are two principal types of risk which can arise from major hazards: individual risk and societal (or population) risk. The Health and Safety Executive (1988a; 1992) has called the individual risk associated with major industrial hazards 'the risk to any particular individual, either a worker or a member of the public'. A member of the public is considered to be 'either anybody living within a defined radius from the establishment or somebody following a particular pattern of life'. Societal risk (or 'risk giving rise to societal concerns'; Health and Safety Executive, 2001) was 'the risk to society as a whole, as represented, for example, by the chance of a large accident causing a defined number of deaths and injuries'. It is expressed numerically as a frequency (F) that there will be a disaster harming more than a particular number of people (N), and the criteria for acceptability of societal risk can be aggregated in the form of an F–N curve (**Figure 1**). Although the individual risk remains the same for each person, the societal risk is governed also by the number of people likely to be affected. The individual risk of living 1 km from a major hazard (industrial or natural) remains the same irrespective of whether the hazard is located in an unpopulated area or in it city, but the societal risk is very different! The term population risk is used for environmental purposes, where usually it is the risk to the species or habitat, rather than to the individual organism, that matters.

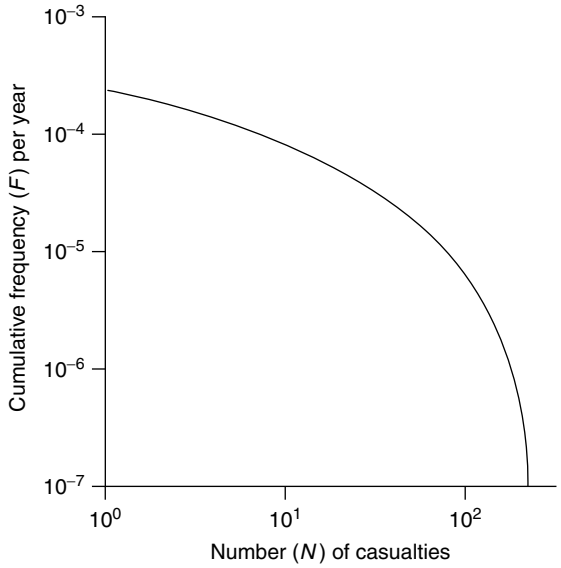

Figure 1 An F–N curve for societal risk. In assessing societal risk, a particular actuarial or estimated curve must be compared with a criterion curve. Actuarial curves (based on historic data) are usually only available for high-frequency—low-number events. Curves for low-frequency—high-number events are normally estimated.

2.5 Risk Assessment, Management and Perception

The concepts of hazard and risk are fundamental to analysing the causes of disasters and to preventing their recurrence. However, these concepts must be contained within a framework of analysis, management and communication if they are to be applied usefully to a given situation. In practice there is a multistage process involved in handling any hazard (**Figure 2**), and most of the people analysing and managing hazards from industrial plants have engineering or chemistry backgrounds. Their specialisms cover plant design and failure rates, event and fault tree analyses, dispersion modelling for releases of clouds of substances and rates of combustion and so on, for explosions and burning gasses (Wells, 1997; Mannan, 2005). Geologists are often the principal people interested in the causes of natural disasters involving toxicants. These are 'overt' disasters where a clear point source can be identified readily.

Only when the substance released is a toxicant or is transformed into a toxicant will any toxicological input become important. This input will largely be in defining the hazard; it will be concerned with identifying whether the agents present are toxic, and defining the combination of exposure size and duration likely to produce a given toxic effect. Clinical toxicologists are also able to advise on the treatment of victims following an incident; that is, they can have a role in the event of an incident occurring.

'Disseminated' disasters are those which only become apparent because of evidence of effect. Identifying the cause when the effect is ill health may require persistent, painstaking research. If the suspected cause is noninfective, the investigation team will need the assistance of toxicologists in identifying the agent responsible.

Often risk can be managed in more than one way. The aim of risk management is to reduce the risk, both in terms of the frequency of an event and in terms of the nature of the potential consequences, to an acceptable or tolerable level (see **Table 1** for definitions of acceptable and tolerable risk). This involves choices as to what chemical physical agents are usable by society and in what circumstances, and on selecting which processes to employ when manufacturing, using or disposing of these agents and their waste products (general risk management). It also involves choices on where to site the major hazards (including waste sites)

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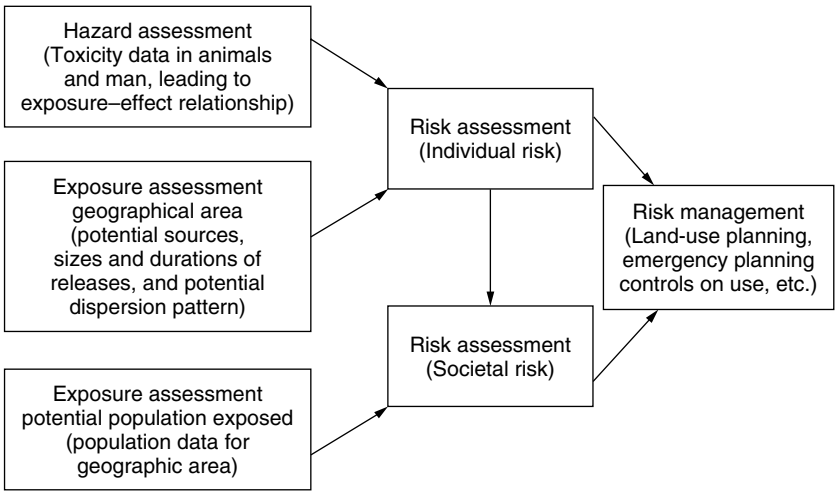

Figure 2 Block diagram illustrating the processes involved in risk assessment and risk management.

Table 1 Various aspects of risk

Objective, actual or statistical risk	A statistically calculated risk evaluation
Perceived risk	The combined evaluation that is made by an individual of the likelihood of an adverse event occurring in the future, and its consequences
Acceptable risk	An acceptable risk is one which, on objective criteria, should generally be regarded as not worth worrying about by those exposed to it
Tolerable risk	A tolerable risk is one which society is prepared to live with in order to have certain benefits
Accepted risk	People tend to accept the risks which they have experienced, but are less prepared to accept new risks, even though, on 'objective' criteria, the risks are similarly acceptable. The new risk is regarded as worse
Voluntary and involuntary risk	Certain risks are accepted willingly by choice (e.g. in sport) by individuals but are not accepted (even by the same individuals) if they are unable to choose to accept the risk
Risk and benefit	Costs occur in reducing risks, either directly or indirectly. For example, many drugs used to treat cancers carry a significant risk that they will cause cancer in that patient at some future time; whooping cough vaccine may prevent severe illness and possible death in most recipients but can cause severe damage in a very few recipients. Risks to nonbeneficiaries are regarded as worse than risks to beneficiaries

Based on Royal Society Study Group (1983), Lovell (1986) and Health and Safety Executive (1988a; 1988b).

and whether to permit housing and other developments around major hazards (land use planning). In addition, risk management is concerned with examining how to handle the consequences of an untoward event at a particular site (emergency planning).

How risks are perceived is also important. The Royal Society Study Group (1983) called perceived risk the combined evaluation that is made by an individual of the likelihood of an adverse event occurring in the future and its likely consequences. In the introduction to the chapter on risk perception in the 1992 report of the Royal Society Study Group, the study group wrote that 'risk perception (from the perspective of the social sciences) involves peoples' beliefs, attitudes, judgements and feelings, as well as the wider social or cultural values and dispositions that people adopt towards hazards and their benefits'. Ultimately, Government (on behalf of society) decides whether a societally regulated risk is generally acceptable or tolerable. What is an acceptable, or at least a tolerable, risk is a problem which depends on a number of factors, some of which are listed in **Table 1**. Individuals or groups of individuals may attempt to vary the decision as it relates to their specific circumstances and perceptions. For this reason it is essential that those evaluating risks and benefits seek to maintain the confidence ('trust') of the general public in their decisions. This includes communicating the issues involved in risk assessment and management and the basis of the evaluations as well as the outcomes in a manner that can be understood by society as a whole. Clear, effective and consistent communication between all parties ('stakeholders') is seen more and more as essential if risks are to be managed satisfactorily. This is a multidirectional process, and requires the

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technical specialist to understand the views of individuals and society as well as explaining his or her assessment to the general public. In the final analysis, risk acceptability revolves around personal and political decisions.

3 DISASTERS AND SERIOUS INCIDENTS INVOLVING TOXICANTS

Many disasters are the result of major hazards fulfilling their potential for harm. They are part of a continuous spectrum of possible consequences, which can arise from the accidental release of chemicals at a point source. These consequences range from minor difficulties through serious incidents until, in the worst cases, they become disasters. Most of these disasters are not due to toxicants. Their primary effects are physical in nature, and include crush injuries and burns. Other disasters may be due to the ordinary exposure associated with covenanted releases of potential toxicants. This may be the result of failure to set and meet adequate exposure standards. Failure to set appropriate standards may be because the appropriate knowledge was not available or the criterion against which the knowledge was set was unsuitable. In either case the resulting standard can be inadequate. Alternatively, it may be because of some accidental or, as with terrorist attacks, intentional deviation from the standard. Classification of disasters is given in **Table 2**. This classification is based on the type of event that caused the disaster or serious incident.

Health effects due to toxicants may result from inhalation or from absorption through skin or gastrointestinal tract. Inhaled toxicants may be gases, liquids (aerosol mists) or solids (dusts, fumes). Particle size is important when particulate material is inhaled since, if inhaled, a particle may be deposited in the lung alveoli or—as a result of the 'tracheobronchial escalator'—it may be swallowed. An inhaled toxicant may cause injury to the lung, it may restrict the transfer of oxygen (asphyxiation), or it may act systemically in a particular organ (including skin). Skin and eye effects are often phenomena of surface contamination.

Gastrointestinal absorption may contribute to the overall toxicity of inhaled material. However, it is the main route of entry for those toxicants which are transmitted to humans in food or drinking water.

Examples of different types of disaster or serious incident with the potential for disaster now follow.

3.1 Natural Disasters

Disasters due to natural causes include phenomena caused by the movement of the earth's crust and also the consequences of abnormal weather. In general, these types of disasters cause injuries due to physical effects, or disease due to infective organisms. Toxicants are rarely involved, and then usually secondarily (e.g. due to water contamination), except in the cases of volcanoes, and gas emission from lakes. Asphyxiation can be caused as a consequence of an emitted gas or irritant dust causing respiratory dysfunction, bronchial obstruction and pulmonary oedema.

3.1.1 Volcanoes

Volcanic eruption can be divided into two types: explosive and effusive. Each type may present different health hazards. In a volcanic eruption, magma (molten rock and associated dissolved gas below the earth's surface) is extruded to the surface. When it reaches the surface it may appear as liquid (lava), fragments (pyroclastic debris) and exsolved gasses. In effusive eruptions these flows are usually slow moving. Gas releases are steady and most of the limited amount of dust produced is nonrespirable. Explosive eruptions tend to be more dangerous, the principal toxic hazards being hot ash release and gas emission. Volcanoes may change their nature from one type to the other.

Table 2 Classification of types of disasters involving toxic agents

Cause of disaster	Type	Example
Natural	Volcanic	Vesuvius, AD 79; Mount St. Helens, 1980
	Nonvolcanic	Lake Nyos, 1986
Human-made	Plant failures	Seveso, 1976; Bhopal, 1984; Chernobyl, 1986
	Fire	Manchester Airport, 1985; Kings Cross, 1987
	Food and drink adulteration	Ginger paralysis, 1930; toxic oil, 1981
	Accidental contamination	North Cornwall, 1988; Epping jaundice, 1966
	Environmental contamination	Minamata and Niigata, 1951–1974
Interaction of humans and nature	Shipping	MV Braer, 1993
	Mines and tunnels	Tokyo subway, 1995
	Waste disposal	Love Canal, 1960s; Minamata and Niigata, 1951–1974

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The gas emitted by volcanoes is principally steam, but includes carbon dioxide, carbon monoxide, hydrogen sulfide, sulfur dioxide, hydrogen chloride and hydrogen. Plumes normally disperse by dilution in the atmosphere and are carried on the wind above human settlement. However, sulfur dioxide, hydrogen chloride or hydrogen fluoride may occasionally be present in sufficient quantities to contaminate air within settlements, water supplies and animal feedstuffs. Denser-than-air gases, principally carbon dioxide and hydrogen sulfide, can flow into valleys and low-lying basins, and displace oxygen, giving rise to asphyxiation. A nuée ardente (a cinder cloud carrying trapped gases) can flow rapidly down slopes and may also endanger life.

Pyroclastic debris (tephra) and ash products vary in size. Blocks and bombs are large (over 64 mm), lapilli vary between 2 and 64 mm, and cinders and ashes are smaller particles. Lapilli and ash, when released to the atmosphere, rise in a hot convection cloud which may be transmitted widely (several hundred kilometres) downwind. Eventually they fall to earth and blanket large areas. Finer particles are deposited further away. The particles have the ability to cause darkness during daylight hours. Ash products include respirable particles containing significant levels of crystalline silica (quartz and cristobolite). Volcanic ash can affect the respiratory tract and eyes. Severe tracheal injury, pulmonary oedema and bronchial obstruction can occur, leading to death from pulmonary injury or suffocation. Ash may also act as a respiratory tract and eye irritant. Irritation and inflammation of the upper and lower respiratory tract may persist if low-level chronic exposure occurs.

Lava is molten rock, the liquid product from the volcano. Lava is derived from the molten magma, but differs from it because the dissolved gases in the magma escape with the reduction of pressure that occurs as the material approaches the surface.

Debris flows occur when loose rock mixes with surface water or groundwater and flows as a mass of rock, mud and water.

This description of volcanoes is inevitably very short and much simplified. More detailed information can be found in Sheets and Grayson (1979) and Newhall and Fruchter (1986).

3.1.1.1 Vesuvius, Italy, AD 79

Perhaps one of the best-known historic volcanic eruptions is that of Vesuvius in Italy in AD 79. A contemporary description of the eruption and its effects on one victim was provided by Pliny the Younger in two 'letters' to Tacitus (Radice, 1969). More detailed information on the eruption and its consequences has been obtained during archaeological investigation of the sites at Pompeii and Herculaneum, both of which were buried in the eruption (Jashemski, 1979; Sigurdsson *et al*., 1985).

Vesuvius had been quiescent for many centuries before the eruption of AD 79. The first sign of its reawakening was an earthquake in AD 62 which damaged almost all the buildings in Pompeii. Then came the eruption of AD 79. There were three phases to this eruption. The first stage was expulsion of the vent plug on 24 August. This was followed by expulsion of ashes. There were six surges and pyroclastic flows during the last eruption.

Both Plinys were at Misenum when Vesuvius erupted, together with Pliny the Elder's sister (Pliny the Younger's mother). Pliny the Elder, who was in command of the Roman fleet at Misenum, gave instructions that a ship should be made ready so that he could investigate the phenomenon. However, by the time the ship got under way with the elder Pliny on board, he changed the mission to one of attempting to rescue the people living along the shore of the bay at the foot of Vesuvius (see **Figure 3**). Pompeii and Herculaneum were probably being buried at this time, the former from deposition of

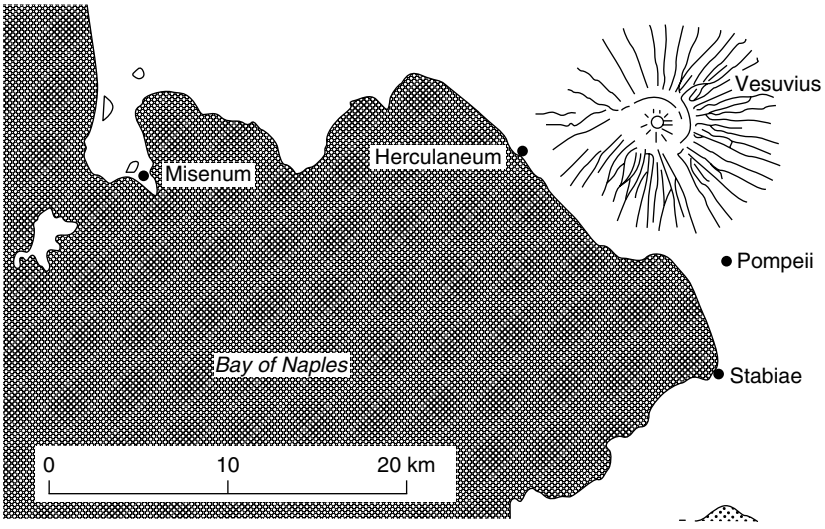

Figure 3 Sketch map of the Bay of Naples. (Based on Jashemski, 1979.)

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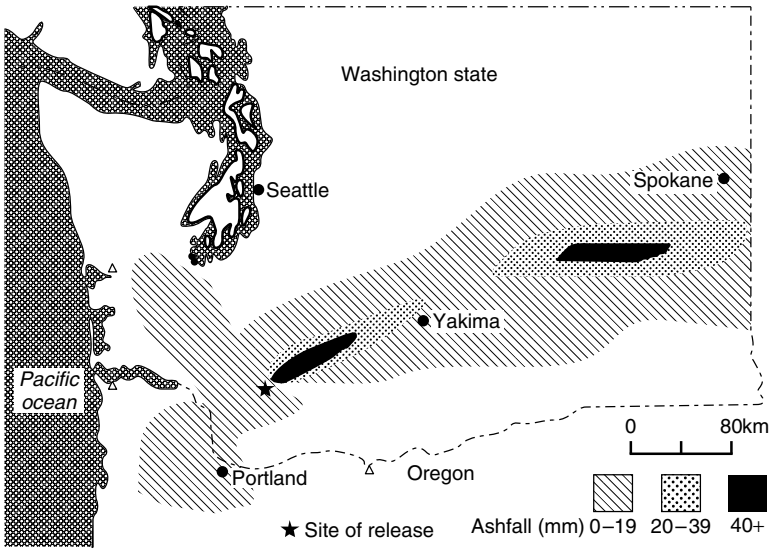

Figure 4 Sketch map of Washington State, showing deposition patterns of ash for the first three eruptions of Mount St. Helens in 1980. The star indicates the position of the volcano. (Based on Baxter *et al*., 1981.)

lapilli and ashes and the latter from a mud and tephra flow. Pliny found his mission impossible because of the falling debris near the shore, and eventually made port at Stabiae, where he stayed the night. During the night the courtyard of the house in which he was staying filled with ashes and debris. On the morning of 25 August it was still dark at Stabiae after dawn. Pliny the Elder went to the shore to investigate the possibility of escape by sea, and died. Pliny the Younger described the death as because 'the dense fumes choked his breathing by blocking his windpipe, which was constitutionally weak and narrow and often inflamed'. In modern terms this might he described as asphyxiation.

The crew of the ship later successfully got away, and the younger Pliny and his mother were evacuated from Misenum on 25 August as ashes started falling there. Both survived. Although the 'letters' only describe ash and lapilli, there was also a lava flow on the north side of the volcano. The results of excavations at Pompeii suggest that at least 2000 people died. Presumably many more deaths went unrecorded. Most deaths in Pompeii and Herculaneum were probably due to asphyxiation caused by inhalation of the hot ash material in the first surge of the ash eruption (Sigurdsson *et al*., 1985). Recent studies on 80 skeletons unearthed of a group of 300 people who had taken shelter in the boat chambers at Herculaneum indicate that they probably died from thermal shock, not suffocation (Mastrolorenzo *et al*., 2001).

3.1.1.2 Mount St. Helens, USA, 1980

A much more recent volcanic eruption was that of Mount St. Helens in the Cascade Range in the west of North America. Premonitory earthquakes started on 20 March and, towards the end of April, a bulge developed in an area to the north of the summit (Buist and Bernstein, 1986). On 18 May a major earthquake occurred, the roof of the bulge slid downhill and an explosive blast took place. Large quantities of ash, super-heated steam and gas were released. There were five additional explosions over the following five months, and ash falls accompanied four of these eruptions. The dispersion pattern is shown in **Figure 4**. The main land areas covered by the vented material lay in a northeasterly direction from the volcano.

There were 35 known deaths and at least 23 people missing without trace following the eruption (Baxter *et al*., 1981; 1983; Buist and Bernstein, 1986). Asphyxiation was the cause of death in 18 of the 23 victims autopsied. Ash probably acted as an irritant to the respiratory tract and eye, causing tracheal injury, pulmonary oedema and bronchial obstruction in those dying. The irritation of the respiratory tract continued as the result of chronic low-level exposure to ash, but there appeared to be few long-term sequelae. However any potential pneumoconiotic effect from the single massive exposure to silica could not be detected because of the short time span since the eruption. Interview studies of patients with pre-existing chronic lung disease showed that the ashfall exacerbated the condition in these patients. There were dose-related increases in the prevalence of three psychiatric syndromes associated with disaster stress and generalized anxiety, major depression and post-traumatic stress (Shore *et al*., 1986). The duration of effect was related to the level of disaster stress suffered by the subject.

The toxic effects seen in the victims of the Mount St. Helens eruption were largely those due to the nature of the ash deposited. Fortunately, the toxic gases that were emitted were vented to the atmosphere and diluted to nontoxic levels through dispersion.

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3.1.2 Other Natural Disasters

3.1.2.1 Lake Nyos, Cameroon, West Africa, 1986

Volcanoes are not the only natural phenomenon which can cause major disasters due to toxic substances. On 21 August 1986 there was a catastrophic release of gas from Lake Nyos, Cameroon (Freeth and Kay, 1987; Kling *et al*., 1987; Baxter *et al*., 1989b). The cloud of gas was lethal at distances up to 10 km from the source. About 1700 people, 3000 cattle and many other animals died, mostly from asphyxiation. An earlier, smaller release from Lake Monoun, also in Cameroon, had resulted in 37 deaths, presumably from similar causes.

The generally accepted cause of the disaster is that because of the geochemical and geophysical characteristics of the Cameroon rocks and the geological conditions in the Lake Nyos area, waters rich in carbon dioxide developed. The gas accumulated in the lake to near-saturating conditions. Although the trigger mechanism for the release is unknown, a small disturbance would have been sufficient to cause degassing in the form of a large release of the carbon dioxide. The gas cloud produced was denser than air and dispersed through the river valleys. Simultaneously, a water surge resulted in the loss of about 200 000 tonnes of water from the lake. That release was heard as a series of 'rumbling sounds' lasting 15–20 seconds, and one observer reported seeing a white cloud rise from the lake.

Many people lost consciousness rapidly, and survivors woke 6–36 hours after the event, weak and confused. Cutaneous erythema and bullae were present in about 19% of survivors treated in hospital. Very limited pathological investigations on those dying suggested that carbon dioxide was the toxicant, as it appeared that the potential toxicants, carbon monoxide, cyanide or hydrogen sulfide were not relevant to the cause of death. Reports of the odour of sulphur compounds were probably a result of the sensory hallucination due to exposure to high levels of carbon dioxide.

Respiratory symptoms and peak expiratory flow in survivors have been compared with nonexposed subjects living in the same area (Afane Ze *et al*., 1996). There were no differences in the frequency of dyspnoea, cough, sputum production and peak expiratory flow at 55 months postincident, suggesting that there were no long-term respiratory consequences among survivors.

This disaster was a consequence of the special geology of the area. Therefore, although a rare event, it does illustrate that natural disasters involving toxicants are not confined to volcanic releases.

3.2 Human-Made Disasters

3.2.1 Plant Failures

Plant failures are a well-known cause of major disasters. Those involving the release of toxic chemicals or radioactivity are relevant to toxicologists, and some examples are examined here.

3.2.1.1 Seveso, Italy, 1976

An escape of toxic substances occurred at an industrial plant at Seveso, Italy, in 1976. The circumstances surrounding the escape and the potential health effects caused by the escape were investigated by a Parliamentary Commission of Enquiry (Orsini, 1977). Both the engineering and the chemical aspects of the incident have been reviewed (Marshall, 1987; Skene *et al*., 1989). Recent work has been published on potential long-term and reproductive effects. The incident was important because of its influence on European Community legislation (the 'Seveso' Directives) concerned with major industrial chemical hazards.

The plant produced trichlorophenol by reaction of tetrachlorobenzene with sodium hydroxide (**Figure 5**). Following the reaction, the solvent (ethylene glycol–xylene) was partially vacuum-distilled off by the end of shift, at which time the heating and agitation were switched off. Some 7.5 hours later a safety plate on the reactor vessel burst and there was a consequent venting of the reaction mixture, including approximately 2–3 kg of the impurity dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) to the atmosphere.

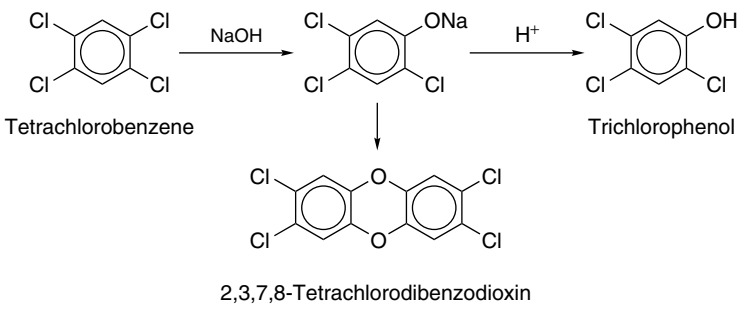

Figure 5 Reaction scheme for the formation of dioxin from tetrachlorobenzene. The condensation to form dioxin is a minor reaction compared with that of synthesizing trichlorophenol.

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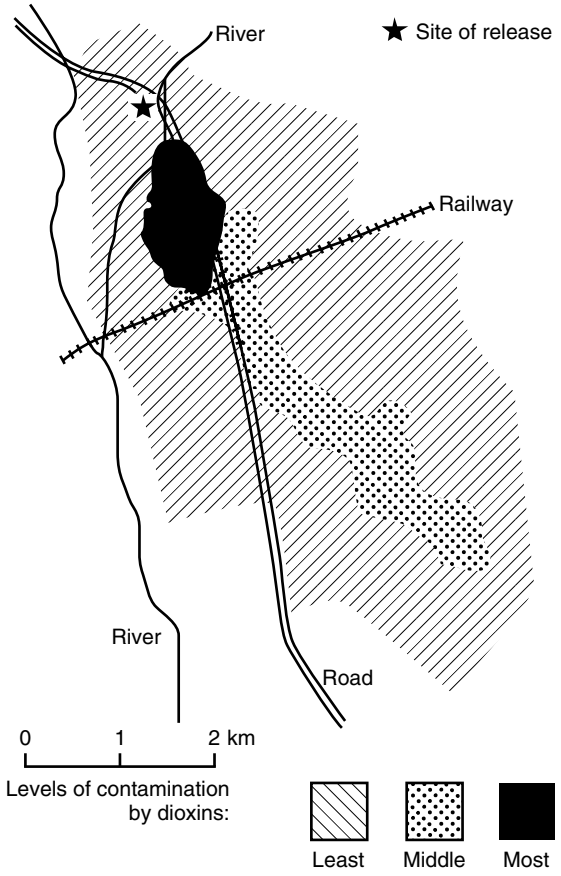

Figure 6 Sketch map of Seveso, showing areas of most, middle and least contamination by dioxin. (Based on Marshall, 1987.)

Once in the atmosphere, the dioxin was spread over a wide area downwind of the plant and settled on fields and houses. Three major zones were identified according to the levels of dioxins present in the vegetation and soil (**Figure 6**). The resident populations in the zones were 733, 4800 and 22 000 people in the most contaminated, middle and least contaminated areas, respectively. A medical surveillance programme was undertaken on these people.

Apart from burns arising directly from contact with the caustic reaction products, the other major effect was chloracne. This was reported some six weeks after the accident, with a frequency correlating approximately to the levels of dioxins in the soil. By the end of 1978 the chloracne had disappeared. Repeated-dose animal studies suggested that dioxin could cause porphyria and was hepatotoxic. In animal studies on reproductive effects, dioxin was a potent foetotoxin and teratogen. Hepatocarcinogenicity has also been established in animal studies. Therefore, these effects were examined in the follow-up to the single acute exposure at Seveso.

Studies on liver effects, including porphyria, in 700 children, failed to identify significant illness, although two indicators of liver dysfunction, γ-glutamyl transferase and alanine aminotransferase, were slightly elevated in boys from the most contaminated zone.

A birth defects register was set up after the disaster. There were no birth defects that could be unequivocally linked to dioxin exposure among the limited number of births to residents of the high-exposure zone. In addition, although there were wide variations in the spontaneous abortion rate between zones, these could not be ascribed to dioxin. Examination of chromosomes in aborted tissue following artificially induced abortions suggested that there might have been a higher frequency of chromosomal aberrations in foetuses from mothers potentially exposed to dioxins, but it was not possible to establish whether these aberrations would have led to adverse reproductive outcomes.

The influence of dioxin exposure from Seveso on endocrine hormone disruption and semen quality has been examined in men 22 years postincident (Mocarelli *et al*., 2008). The study concluded that exposure to dioxin in infancy resulted in permanently reduced sperm concentration and motility, with an opposite effect being seen when exposure occurred during puberty. For both exposures, permanent reductions in oestradiol and increases in follicle stimulating hormone were claimed. These effects were claimed to occur at concentrations of less than 68 ppt (parts per trillion). The biological importance of these findings is, at present, unclear. Examination of women aged 20–40 and not using oral contraceptives at follow-up (20 years postincident) gave no clear indication of ovarian dysfunction in those exposed to high levels of dioxin in the most contaminated and middle zones (Warner *et al*., 2007).

Perhaps the greatest long-term worry from the Seveso incident was cancer. Mortality has now been followed up for 25 years (Consonni *et al*., 2008). Risks of deaths from certain individual cancers and from circulatory disease were elevated. There are excesses of lymphatic and haematopoietic tissue neoplasms in the most exposed zone (six deaths, rate ratio 2.23; 95% confidence intervals 1.00–4.97) and intermediate zone (28 deaths, rate ratio 1.59; 95% confidence limits 1.09–2.33). The small increases in numbers of premature deaths due to certain specific causes seem to suggest that there have not been the feared large-scale increases in the overall numbers of deaths from cancer.

The half-life of dioxin has been determined in blood samples collected up to 16 years postincident for 20 male and 25 female Seveso children (those less than18 years in July 1976) from the high-exposure zone (Kerger *et al*., 2006). The study demonstrated that children and adolescents have shorter dioxin half-lives than adults and this effect is augmented at higher body burden.

The Seveso incident illustrates how an accidental release of a chemical may be perceived by the general public as a major disaster. So far, relatively few human deaths have resulted from the single-dose exposure to dioxin. Those suffering chloracne or burns recovered.

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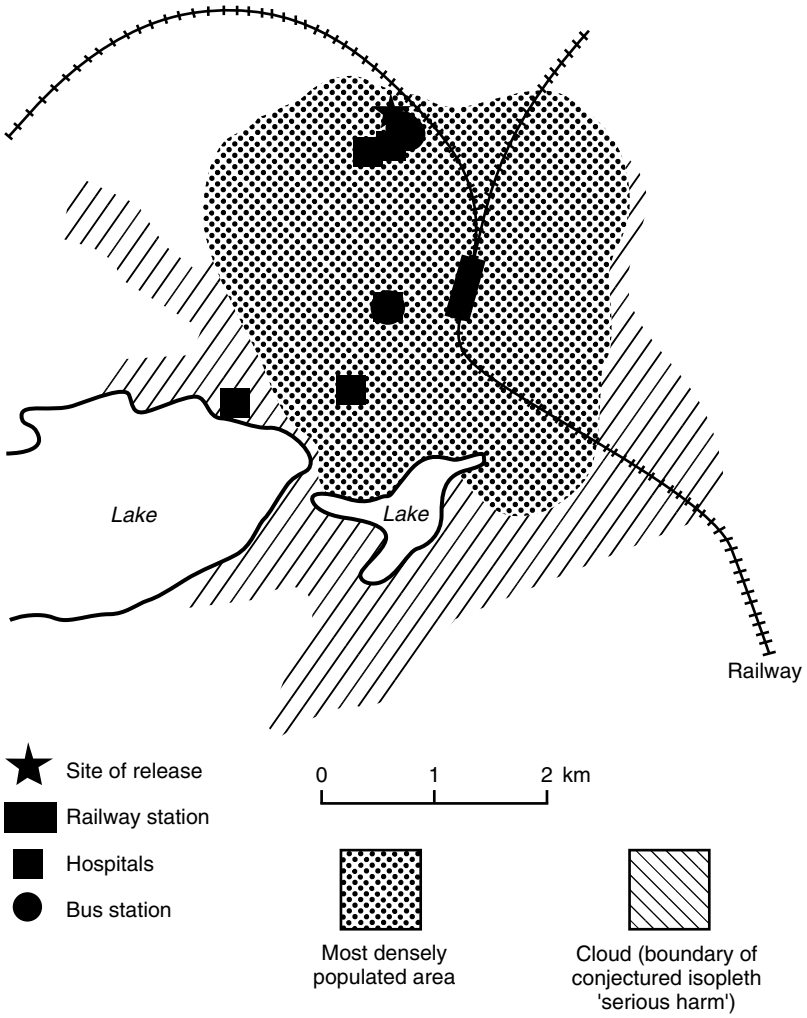

Figure 7 Sketch map of Bhopal, showing overlap between populated area and the approximate boundary for receiving serious harm from the vapour cloud. (Based on Marshall, 1987.)

Fears of large numbers of people being severely affected by potential long-term and reproductive effects have not been confirmed. Nevertheless, the Seveso incident is important, as it raised the general awareness of the potential that for ill health following major plant failure, and was a major factor leading to the European Union (EU) legislation concerning the control of major industrial accident hazards.

3.2.1.2 Bhopal, India, 1984

Methyl isocyanate is a toxic substance that was responsible for a major disaster at Bhopal, India in December 1984, at a factory manufacturing the pesticide carbaryl. The cause of the accident was the introduction of water into a storage tank containing methyl isocyanate. This resulted in the production of carbon dioxide:

$$CH_3NCO + H_20 \longrightarrow CH_3NH_2 + CO_2 \qquad (1)$$

The combination of rising temperature, due to a runaway exothermic reaction, coupled with gas evolution led to a build-up of pressure which caused about 27 tonnes of methyl isocyanate to be vented to the atmosphere in a 1–2 hour period (Dhara and Dhara, 2002). The venting occurred during the night and the cloud dispersed over a densely populated area (**Figure 7**).

In 1994 an International Medical Commission was set up with the aim of assessing respiratory, neurological and other health effects attributable to exposure to the vapour released at Bhopal. Until then investigations had been relatively piece-meal. Many of the studies suffered from limitations associated with study design, bias and exposure classification.

Initial estimates of the number of deaths which resulted front the release vary between 1700 and 55 000, with up to 60 000 people being seriously injured (Bucher, 1987; Andersson *et al*., 1988). Dhara and Dhara (2002) indicate that, although some uncertainty exists in the number of disaster-related deaths, independent agencies had estimated that by 2002 the estimate was between 15 000 and 20 000. Survivors reported that the vapour cloud gave off considerable heat and had a pungent odour.

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Acute irritant effects on eye and respiratory system were the major findings following this incident (Dhara and Dhara, 2002). Initial lung effects (oedema, focal atelectasis) were probable causes of death and led, in survivors, to more persistent changes in lung function, and possible fibrosis and inflammation. Persistent small-airway obstruction, attributed to exposure to the vapour, was still present in survivors 10 years after the incident (Cullinan *et al*., 1997). Although severe eye effects, including temporary blindness, were seen in many survivors, they did not persist (Andersson *et al*., 1988). However, there was evidence of persistent eye watering in some cases and tear secretion deficiency in others, suggesting that the exposure had led to a chronic inflammatory process which might have been exacerbated by the very poor housing conditions in Bhopal slum areas (Dhara and Dhara, 2002).

Epidemiological surveys suggested that miscarriages and infant mortality were high in women living near the plant at the time of the release (Varma, 1987; Dhara and Dhara, 2002). Similar foetotoxic effects were seen in mice treated with methyl isocyanate (Schwetz *et al*., 1987; Varma, 1987).

Psychological and neurobehavioural effects were also manifest (Dhara and Dhara, 2002). These included post-traumatic stress syndrome, pathological grief reactions, emotional reactions to physical problems and exacerbation of pre-existing psychiatric problems.

The Bhopal incident illustrates many important points. The reasons for the water entering the methyl isocyanate tank were never clearly identified. However, the consequences were made substantially worse than they need have been because several of the design safety features had been rendered unusable and because there were substantial numbers of shanty houses right up to the factory fence. Also, there was a paucity of toxicological data on methyl isocyanate prior to the incident, which has now been partly rectified by undertaking substantial studies in animals as well as by following up the victims (Bucher, 1987). At the time of the incident there was only one substantial published report on the toxicity of methyl isocyanate, and that was restricted to an animal study on the acute effects following a single exposure (Kimmerle and Eben, 1964).

3.2.1.3 Chernobyl, Former USSR, 1986

Chernobyl (Chornobyl when transliterated from the Ukrainian), 80 km north of Kiev, Ukraine was the site of probably the worst accident to have occurred at a nuclear plant. In April 1986, a test was being conducted on a reactor during shut-down for routine maintenance (Henderson, 1987; Bertazzi, 1989; Wells, 1997). However, the planning of the test was poor and safety devices were deliberately switched off to allow the test to proceed. By the time it was realized that something was wrong, uranium oxide fuel elements in the upper part of the core had probably started to disintegrate because of the high temperatures. Explosions followed, which released considerable quantities of radioactivity to the atmosphere, much of which was dispersed over the former Soviet Union and western and northern Europe.

After the accident, the people in the area around Chernobyl were evacuated. They have not been allowed to return and the area was converted to a national park where human activity, including farming, was banned.

By one year after the event only 31 people had died as a result of it; they died in the immediate aftermath of the accident, from acute radiation sickness.

The Chernobyl Forum (the International Atomic Energy Agency (IAEA), the World Health Organization (WHO), the United Nations Development Programme (UNDP), the Food and Agriculture Organization of the United Nations (FAO), the United Nations Environment Programme (UNEP), the United Nations Office for the Coordination of Humanitarian Affairs (UNOCHA), the United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR), the World Bank Group, Belarus, Russian Federation and Ukraine) second report (Chernobyl Forum, 2006) includes information on 20 years of follow-up from the accident. Apart from a small number of emergency and recovery workers, exposures of emergency and recovery workers and of residents in areas designated as 'contaminated' were to relatively low whole-body doses of radiation, not much higher than those due to natural radiation.

Although not apparent for the first three years after the accident, by the end of 1994 a clear increase in childhood thyroid cancer had been seen in children from the surrounding areas in Belarus and Ukraine (Likhtarev *et al*., 1995; Stsjazhko *et al*., 1995). The Chernobyl Forum reports that more than 4000 cases of thyroid cancer were diagnosed between 1992 and 2002 in children and adolescents (0–18 at the time of the accident). A cohort study conducted on childhood thyroid cancers in Ukraine concluded that exposure to radioactive iodine was strongly associated with increased risk of thyroid cancer among those exposed as children and adolescents (Tronko *et al*., 2006). It should be pointed out that this is a treatable cancer.

An International collaborative population-based case–control study on childhood leukaemia in Belarus, Russia and the Ukraine indicated that there might be a significant increase in leukaemia risk associated with increasing radiation dose to the bone marrow (Davies *et al*., 2006). There was a lack of significant dose-responses in Belarus and Russia. The large and statistically significant dose-response in the Ukraine might be accounted for, at least in part, by an overestimate of risk. However, it is unclear as to whether the results are due to a true radiation-related excess, a sampling-derived bias in the Ukraine, or some combination of the two. The authors conclude that the study provides no convincing evidence of an increased

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risk of childhood leukaemia as a result of exposure to Chernobyl radiation.

The Chernobyl Forum 2006 also indicated that there was an increase in psychological problems among the affected population, compounded by insufficient communication about radiation effects and by the social disruption and economic depression that followed the break-up of the Soviet Union. The Forum also suggests that radioactivity levels in the area surrounding the reactor have declined several hundredfold since the incident because of natural processes and countermeasures. The majority of the 'contaminated' territory is now safe for settlement and economic activities, but restrictions on land use in the Chernobyl exclusion zone and some other limited areas will need to be retained for decades to come.

The radioactivity from the accident was washed from the skies and entered food chains, notably in areas of high rainfall. In the UK this led to the banning of the sale for human consumption of sheepmeat from the badly affected areas for over one year.

This disaster is one in which future illness and premature deaths are the primary effects on humans. It is this fear of the future effects of radiation which has made the nuclear industry so heavily regulated in comparison with other industries.

3.2.2 Fire

Thermal injuries, heat stress and physical trauma from collapsing structures are obvious problems in major fires. However, fire statistics indicate that deaths consequent to being overcome by smoke are the most common type of death (Committee on Fire Toxicology, 1986). Such deaths and incapacitations are due to the evolution of toxic combustion products. As toxicants are likely to be funnelled upwards and diluted to nontoxic levels in unconfined fires, major fire disasters usually occur in confined spaces when it is not possible to escape from the effects of the toxic combustion products.

Smoke includes all airborne products from the pyrolysis and combustion of materials (Committee on Fire Toxicology, 1986). Full oxidation of substances present in fires would result in products such as carbon dioxide, water (steam), nitrogen dioxide, sulfur dioxide and chlorine. However, complete combustion rarely occurs and other products such as carbon monoxide, soot (particles), hydrogen cyanide, hydrogen chloride, hydrogen fluoride, acrolein and other organic materials are often present. The toxicity of fires therefore arises from the evolution of smoke (including gases, dust/fume and aerosol) containing irritants and asphyxiants and, potentially, carcinogens. Those most at risk will normally be the fire-fighters in close proximity to the fire, and thus near enough to inhale undispersed toxic smoke and gasses. Potentially, risks from acute health effects of dispersed combustion products may occur in warehouse fires such as that at Brightside, Sheffield (Health and Safety Executive, 1985) or large oil terminal fires such as that at Buncefield, Hertfordshire, UK in December, 2005 (Aus *et al*., 2006). At Brightside, onlookers who were in close proximity to the fire may have acquired sore throats and chest symptoms due to effects from the fire. However, potential combustion-product toxicants were dispersed to levels thought too dilute to pose a risk to the general population within very short distances. Asbestos roofing materials were dispersed widely in this fire, but the longer-term health effects for people exposed to single doses of dispersed fire products were considered to be minimal. At Buncefield, some effects were reported by police officers at the front line, but evacuation of damaged houses and workplaces and advice to 'go in, stay in' to those under the plume of smoke ensured that there were no serious health problems for the general public (Aus *et al*., 2006). Two examples of disasters in which the evolution of toxic combustion products from fires contributed significantly are given below. In both examples most of those who died were incapacitated by the effects of inhaling toxic smoke and gases in relatively confined spaces, and thus became unable to move to less polluted atmospheres.

3.2.2.1 Manchester Airport Crash, UK, 1985

One area where there is a potential for disasters due to toxic hazards is aircraft fires. An example of such a catastrophe was the Manchester (UK) Airport crash of 1985 (Air Accidents Investigation Branch, Department of Transport, 1989).

On 22 August 1985 a British Airtours Boeing 737 aircraft hound for Corfu (Greece) was taking off from Manchester International Airport when the left engine suffered an uncontained failure which punctured a wing fuel tank access panel. The leaking fuel ignited and burnt as a large plume of fire trailing directly behind the engine. The crew abandoned take-off and cleared the runway by turning on to a taxiway as they stopped. A light wind carried the fire on to and around the rear fuselage (**Figure 8**). After the aircraft stopped, the hull was penetrated rapidly and smoke and possibly flames entered through one of the cabin aft-doors, which had just been opened. Fire subsequently developed inside the cabin and generated a dense black toxic/irritant smoke. Despite prompt attendance by the airport fire services the aircraft was destroyed and 55 people (53 passengers and 2 crew) died, one after six days in hospital. All those who died on board the aircraft had general congestion and oedema of the lungs with carbon particles in the air passages, consistent with inhalation of smoke. The cause of death was inhalation of smoke for 48 people and direct thermal injury (burns) for only 6 passengers.

Of those engulfed by smoke, only 38 (47%) survived. The survivors reported that a single breath of the cabin atmosphere was burning and painful, immediately

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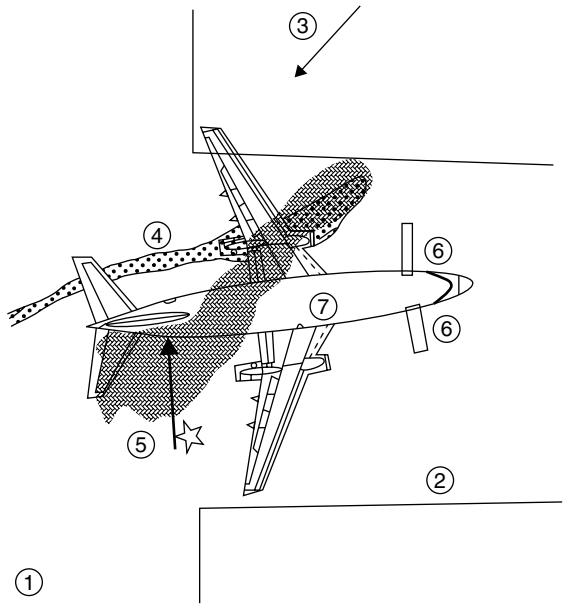

Figure 8 Sketch map to illustrate the position of the stopped plane in the Manchester Airport crash, 1985. 1—Main runway; 2—taxiway; 3—wind direction (wind speed 6 knots); 4—pool of jet fuel from punctured tank; 5—plume of fire/smoke from ignited fuel; 6—forward emergency doors (used to escape). Star indicates rear emergency door through which flames/smoke may have penetrated cabin. (Based on data in Air Accidents Investigation Branch, Department of Transport, 1989.)

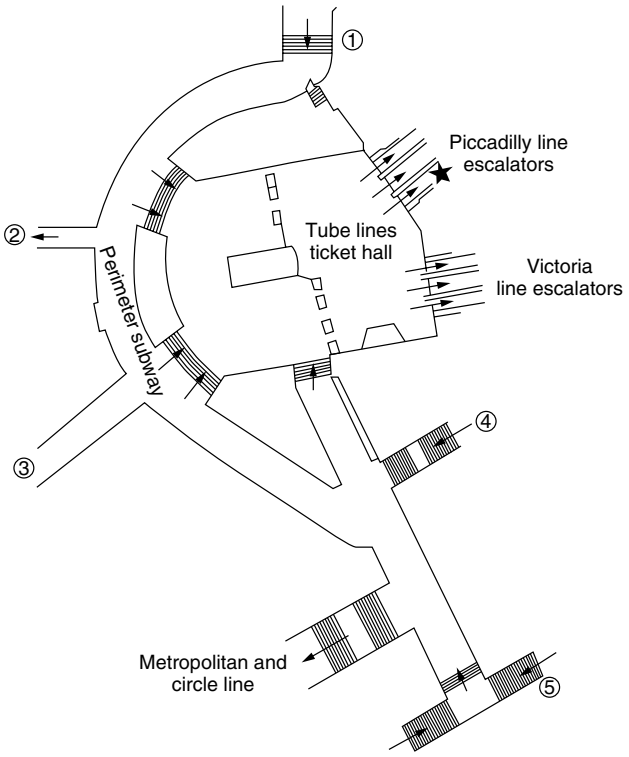

Figure 9 Sketch map of the layout of the subsurface ticket hall and subways at Kings Cross Underground station. 1—Entrance from Kings Cross mainline station; 2—entrance from St. Pancras mainline station; 3—entrance from Pancras Road; 4 and 5—entrances from Euston Road. Star indicates escalator in which fire started. (Based on Fennel, 1988.)

causing choking. They experienced drowsiness and disorientation. Eight survivors actually collapsed, but recovered sufficiently to get out from the plane. Most of the deaths due to incapacitation might have been prevented if the people concerned had been protected from smoke or if external assistance had been more quickly available. All except one of the survivors of the immediate accident made their exit within 7 minutes of the aircraft stopping. The only survivor recovered by firemen was taken out after 33 minutes. He died six days later from severe pulmonary damage and associated pneumonia.

The thermal decomposition products of cabin materials included toxic or irritant gases, such as carbon monoxide, hydrogen chloride and hydrogen cyanide. Some fluorinated materials (used as decorative films) yielded hydrogen fluoride. It was probably the combined effects of toxic combustion products that caused death. Elevated levels of carbon monoxide and cyanide and the metabolic product of cyanide, thiocyanate, were found in all except 6 of those dying on board the plane. Individually lethal levels of carbon monoxide were found in 13, and of cyanide in 21 of these people (9 had levels of both substances, either of which could have been lethal); for the remainder, the sum effects from the total amounts of toxic materials present probably caused death.

This aircraft accident is an example of a disaster where many of those who died might, in other circumstances, have been rescuable. One element that played a part in the disaster was disablement by the smoke from a fire in a confined space before exit from that space could be achieved. This has encouraged investigations into the potential for placing smoke-hoods of suitable design on aircraft for passenger use in this type of emergency, the investigation into possible 'in-cabin' fire-fighting systems and provision of low-level lighting systems to indicate the route to fire exits. So far, only the last of these has been implemented.

3.2.2.2 *Kings Cross (London) Underground Station, UK, 1987*

Thirty-one people died and many more were injured in an escalator fire at Kings Cross Underground Station. The fire took place in the evening at an extremely busy Underground station where four lines cross (**Figures 9** and **10**). Access to the three deep lines (Piccadilly, Victoria and Northern) is from the ticket hall below the mainline station forecourt. The ticket hall is approached by subways from Kings Cross and St. Pancras mainline stations and from street level. According to the Inquiry

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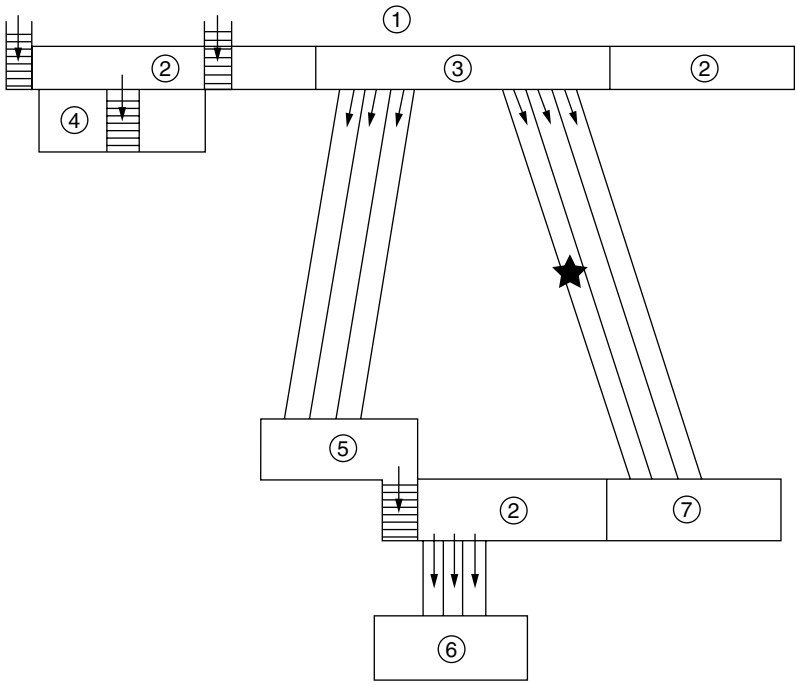

Figure 10 Sketch map of the levels of the components of Kings Cross Underground station. 1—Surface level (Kings Cross mainline station forecourt); 2—subways; 3—ticket hall; 4—Metropolitan and Circle Lines; 5—Victoria Line; 6—Northern Line; 7—Piccadilly Line. Star indicates escalator on which fire started.

Report (Fennel, 1988), the fire started in the Piccadilly line escalator, among an accumulation of grease and detritus (dust, fibre and debris) on the running tracks, possibly as a result of a lighted match passing through the skirting board. This fire preheated the balustrades and decking, which were wooden. As a consequence of a 'trench' effect, the fire initially burnt cleanly and then produced dense, black smoke. 'Flashover' occurred as the fire erupted into the ticket hall. The deaths all occurred among people in the ticket hall at around the time 'flashover' occurred.

The inquiry did not pursue in detail the question of cause of death for the 31 people who died, but concluded that many deaths were due to the toxic effects of the smoke rather than to burns. In the report the Inspector says:

After hearing expert evidence about the role of toxic gases in the fire and the findings of pathologists on post-mortem tests, I determined that the cause of death in individual cases could not be pursued any further in this investigation. On the evidence available to me no reliable assessment could be made of the relative importance of various materials present in the station to the production of toxic fire fumes or to the sources of toxic materials found in the bodies. Although separate statutory Coroner's Inquests were held, the Coroner decided not to take the matter further.

A major part of the inquiry focussed on the procedures by which London Underground dealt with escalator fires. It was clear that, at the time, the Underground lacked an adequate approach to safety in terms of their attitudes to safety matters, their attention to staff training in safety and their equipment, and procedures to be used in emergencies. The lack of preparedness led to an emergency becoming a disaster. Regulations made subsequent to the report also led to the replacement of wooden escalators in subsurface Underground stations with metal ones, mandatory installation of automatic fire sprinklers and heat detectors in escalators, and fire safety training.

3.2.3 Food and Drink

Mass poisonings due to contamination of food or drink may be considered disasters. Many episodes of this type of mass poisoning are the consequences of bacterial or fungal contamination of foods consumed by the victims. If the causative agent is pathogenic, then health can suffer. However, a portion of such poisonings is due to the introduction of a chemical toxicant into the consumed material, either directly or via a food chain.

Because of the indirect way in which the contamination affects humans, it can be difficult to demonstrate cause and effect. Ill health may occur indirectly, in a species (humans) remote from that (e.g. wheat) to which the toxicant was administered, possibly after transmission through a food chain (fed to farm animals, and so on) or directly (in bread). It may be some time before the toxicant accumulates sufficiently for the victim to exhibit symptoms of ill health, or for the ill health to be

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manifest following ingestion. Consequently, considerable detective work may be needed in order to identify the cause of ill health, and on occasion it may never be properly characterized.

A series of examples of incidents involving contamination of food or water follow. Further examples can be found in Aldridge (1987).

3.2.3.1 *Adulteration of Food or Drink*

Adulteration (debasing by adding other or inferior substances: Concise Oxford Dictionary, 1990) of foodstuffs and deliberate poisonings by admixture in food have gone on from time immemorial. Adulteration for commercial reasons was rife in the eighteenth and nineteenth centuries (see Smullen, 1989). Bread from bakers often contained chalk, lime, lead salts or even bone to make it look white. Leaves of hawthorn, sloe or ash were used to dilute tea. With hindsight, several of these adulterants were toxic chemicals and may have had disastrous consequences for the recipients. Deliberate adulteration, when it occurs, is still capable of causing major disasters.

3.2.3.1.1 Ginger Jake Paralysis, Midwest and Southwest USA, 1930

During prohibition in the USA, the Prohibition Bureau ruled that the US Pharmacopeia (USP) fluid 'extract of ginger' was a nonpotable beverage, an alcoholic patent medicine, and its sale was not restricted (Baum, 2003). This beverage was an alcoholic extract of material from the ginger plant (obtained from Jamaica—hence the 'Jake') and was freely drunk. An adulterated 'fluid extract of ginger' entered circulation through dealers (nonpharmacists), mainly in Ohio and Tennessee early in 1930s. Cases of paralysis started to occur in mid February. Adult men (typically those living alone in cheap rooms, unemployed or employed on a menial job) were the principal victims, and those showing symptoms seemed to do so some 10 days to three weeks after drinking the suspect ginger extract (Smith and Elvove, 1930). Ultimately some 50 000 people were affected (Morgan, 1982).

The paralysis first appeared as soreness of the muscles of the arms and legs, with occasional numbness in the fingers and toes. Foot and wrist drop and weakness of the fingers developed. The symptoms were found in distal parts of the limbs and were more marked in the feet and legs. This neuropathy was a primary axonopathy caused by demyelination and dying back from the distal end of the long nerves. In some cases recovery was very limited (Aldridge, 1987).

In a very detailed piece of work, the causative agent was identified chemically as tri-*o*-cresyl phosphate (**Figure 11**) (Smith, 1930; Smith and Elvove, 1930). Both the adulterated ginger extract and the presumed adulterant, tri-*o*-cresyl phosphate, were found to cause the symptoms of neuropathy in calves, chickens and, to a much less marked degree, rabbits, but had little effect on monkeys or dogs following oral ingestion.

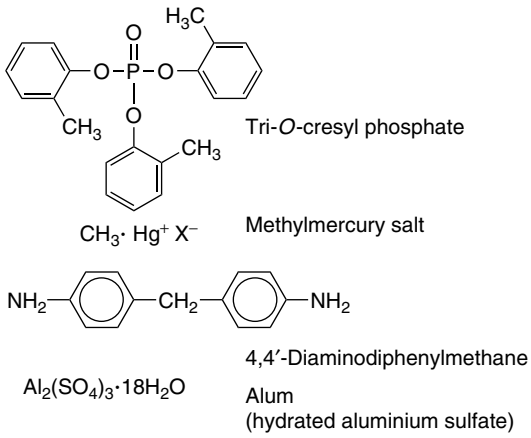

Figure 11 Structures of chemicals implicated in disasters mediated through food or water supplies.

3.2.3.1.2 Toxic Oil Syndrome, Spain, 1981

Toxic oil syndrome was a previously unknown disease syndrome which appeared in Spain in May 1981, principally in Madrid and the northwest provinces (World Health Organization, 1984; Aldridge, 1985; 1987; Gelpi *et al*., 2002). The epidemic was at its peak in mid June and faded away thereafter. By March 1983, 340 deaths had occurred and over 20 000 cases had been recorded. To the end of 1997 there has been no subsequent excess of deaths in those exposed to toxic oil.

The disease developed in two phases. In the acute phase, a pleuropneumonia sufficient to cause respiratory distress and death in severe cases. This pleuropneumonia did not respond to antibiotic treatment and about 20% of survivors did not recover completely. A chronic phase of the disease, a sensorimotor peripheral neuropathy of variable appearance, developed together with sclerodermal-like skin changes. There was little evidence of central nervous system involvement. Although initially thought to be due to an infective agent, the syndrome was rapidly associated with the consumption of an oil sold for food use in 51 cans by itinerant salesmen. The oil was rapeseed oil, denatured with aniline, intended for industrial use. In most cases the oil had been re-refined, mixed with other seed oils, animal fats and poor-quality olive oil or chlorophyll, but in the cases of a small number of victims in the Seville area (well away from the main outbreak) the re-refined oil had not been further processed. Recent studies have suggested that the disease is strongly associated with the consumption of oils containing the fatty acid esters of 3-(*N*-phenylamino)-1,2-propanediol (Gelpi *et al*., 2002). Whether these esters are simply markers for the toxicity of the oils or have the capability to induce the disease has not been determined, in part because of a lack of suitable animal models for the syndrome.

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3.2.3.2 *Accidental Contamination*

As opposed to deliberate addition of materials to foodstuffs or drinks, accidental addition of toxicants can also occur. If the toxicant is sufficiently effective and affects a large number of people, a major disaster could result.

3.2.3.2.1 Pollution of Drinking Water in North Cornwall, UK, 1988

Mass intoxications, in theory at least, could occur due to contamination of drinking-water supplies. That this is not such a remote possibility was demonstrated in 1988, when the South West Water Authority found that a truck load (20 tonnes) of alum (aluminium sulfate; **Figure 11**) had accidentally been released into the drinking-water supply to Camelford, a small town in Cornwall, and the surrounding district (Lowermoor Incident Health Advisory Group, 1989; Lowermoor Incident Health Advisory Group, 1991; COT, 2005). The material was delivered into the treated-water reservoir at Lowermoor Treatment Works. The pH of the water dropped below 5 and aluminium levels were raised to over $10\,mg\,l^{-1}$, considerably above the $0.2\,mg\,l^{-1}$ set on aesthetic grounds (potential discolouration) in the European Community Drinking Water Directive. Although initial advice from local sources suggested that little ill health would occur, newspapers reported considerable acute health symptoms and speculated that there were potential long-term effects. The expert assessment was that the early symptoms of gastrointestinal disturbances, rashes and mouth ulcers were probably due to the incident, but these would be short-lived. Up to 400 (out of a population of about 12 000) attributed continuing effects to the incident. Later complaints included joint and muscle pain, malaise, fatigue and memory problems. The 1991 report ascribed these symptoms to a heightened awareness provoked by the incident and subsequent events. Media coverage ascribed further symptoms, including shedding of toenails, to the incident, although these symptoms could not be confirmed from reliable sources. In the 2005 report, additional concerns were voiced concerning cancer and thyroid disease. The Lowermoor Incident Advisory Group concluded that there was no reason to predict any late consequences, but it could not categorically exclude them. Its recommendations included a follow-up study monitoring hospital discharges. This study of hospital-discharge rates up to the fifth anniversary of the incident indicated a continuing significantly higher level of hospital admissions arising from the Camelford area. However, despite examining diagnoses by cause, the authors were unable to identify a statistical excess likely to be linked to aluminium exposure (Owen and Miles, 1995). Compared with deaths in Cornwall over the period 1988–1997, mortality in the area of water pollution was lower than expected (standardized mortality ratio 81.6), and compared with England and Wales it was still lower (77.7) (Owen *et al*., 2002).

There was a higher level of talipes in foetuses from mothers exposed to the aluminium sulfate (four cases, one control, $p = 0.01$), but, because of the low numbers involved (88 exposed mothers) and the likely general under-reporting of positional talipes, the importance of this finding is unclear. The only conclusion drawn was that there was no evidence from the study of major problems apparent at birth (Lowermoor Incident Health Advisory Group, 1991; Golding *et al*., 1991).

The incident is a sufficient reminder of the possibility of a disaster occurring as a result of contaminated water supplies. It also illustrates the difficulties which occur in allaying fears when the affected population receives initial advice which was, to quote the conclusions in the first report of the Advisory Group, 'contradictory, confusing and sometimes inappropriate', and when media reports claiming further health effects continue. The 1991 report suggests that many of the longer-lasting symptoms and complaints arising from the Lowermoor incident would be expected as a consequence of psychological reactions. They cite evidence that, even when the victims concerned have suffered no direct physical damage, then worry, anxiety, depression and 'post-traumatic stress syndrome' may follow a major accident or disaster and can produce real mental and physical symptoms. The Committee on Toxicology report of 2005 also identified that many of the claims associating possible longer-term effects with the incident were for effects occurring at similar levels to those in the unexposed population, although in some cases, they recommended that further studies be undertaken.

3.2.3.2.2 Contamination of Food during Storage—Epping Jaundice, UK, 1965

Epping jaundice was an outbreak of jaundice which affected at least 84 people in the Epping area of London during February, 1965 (Kopelman *et al*., 1966a; 1966b). It was traced to ingestion of wholemeal bread made from flour contaminated with 4,4′-diaminodiphenylmethane (**Figure 11**). The chemical had spilled from a container on to the floor of a van, which was carrying flour as well as chemicals.

In most of the cases, severe, intermittent pains in the upper abdomen and lower chest areas of the body preceded jaundice and liver enlargement. Normally, these pains were of acute onset (50 patients), but sometimes onset was insidious (27 patients). Of the 57 patients further investigated, most had raised serum bilirubin, alkaline phosphate and aspartate aminotransferase levels. Needle biopsies were performed on four patients within three weeks of the onset of symptoms; all showed considerable evidence of portal inflammation and bile duct cholestasis, and evidence of hepatocyte damage. The lesion was reproducible in mice given 4,4′-diaminodiphenylmethane (Schoental, 1968). The patients slowly recovered over succeeding weeks.

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One feared outcome has been cancer. Of the 83% of the group that have been followed up over 38 years postincident, 37 deaths were recorded (Nichols, 2004). Mortality from all causes was close to expectation (observed 25, expected 26.7, standardized mortality ratio (SMR) 82) in females, and below (observed 12, expected 26.7, SMR 45) in males. There were no observed deaths from cancer of the liver or from nonmalignant liver disease.

The jaundice was the result of an accidental, undetected (until too late) contamination of a foodstuff because it was stored during delivery adjacent to chemicals which were insufficiently securely contained within the packaging. The chemicals were absorbed by the flour through the sacking and, following baking, were present in the bread.

3.2.3.3 Poisoning due to Consumption of Foodstuff Not Intended for Human Consumption

3.2.3.3.1 Methylmercury Poisoning in Iraq 1971–1972

An outbreak of organomercurial poisoning due to the consumption of treated grain by farmers and their families occurred in Iraq in 1971–1972 (World Health Organization, 1976). There were 459 deaths and 6000 further cases admitted to hospital.

Poisoning cases started to appear in hospitals in late December. Farming families only were affected, and the cause of the poisoning was identified as consumption of home-made bread, an important element of their diet, made from wheat treated with seed dressing. There was a latent period of up to 60 days from first consumption to the appearance of signs and symptoms of poisoning, and in many cases consumption of contaminated grain ceased before the symptoms occurred.

Symptoms included speech disturbances, abnormal behaviour, loss of auditory and visual acuity, and ataxia. The severity varied from minimal effects to severe disability and death. Most of those showing only mild or moderate symptoms were symptom-free two years later, although symptoms were still present in severe cases. Mercury levels in hair were found to be good indicators of the dose of mercury received.

Organomercurials, such as the methylmercury (**Figure 1**) involved in this episode are fungicides, the methylmercury being used as a seed dressing to prevent wheat bunt and other crop diseases. Grain dressed with methylmercury was distributed to farmers between mid September and early December, 1971. Although much of the wheat had been consumed before a cause–effect relationship had been established, surplus treated grain was withdrawn to storehouses once the cause of the outbreak was known. The problem arose because wheat intended as seed for next year's crop was eaten by the farmers and their families rather than used for its intended function.

3.2.3.3.2 Environmental Pollution—Minamata and Niigata, Japan, 1951–1974

The examples of major disasters arising from toxic substances so far discussed arise from contamination of the foodstuff. It is also possible for contamination to arise indirectly as a result of an environmental pollutant entering a food chain. Two examples of this occurred in Japan, at Minamata and Niigata (Tsubaki and Irukayama, 1977).

Over the period 1951–1974 there were over 700 recognized cases and 80 deaths due to 'Minamata disease', and over 2000 other people had applied for recognition as Minamata disease patients. The principal geographical areas affected were in two prefectures, Kumamoto and Kagoshima, which border Minamata Bay. The disease occurred mainly in fishermen and their families who consumed large quantities of locally caught fish containing high concentrations of mercury. The patients' nervous systems were affected, with symptoms of sensory, motor and visual involvement. These neurological signs have continued for at least 30 years following cessation of exposure (Ekino *et al*., 2007). Domestic cats (presumably also largely fed fish) exhibited similar clinical signs, and abnormal behaviour occurred among crows in the affected areas. Congenital effects also occurred.

The outbreak of poisoning at Niigata was first identified in 1965, with over 520 patients being identified by the end of 1974. The epidemic was also apparent in the domestic animal population.

In the Minamata outbreak, the effects were due to organically bound mercury present in sludges from industrial plant. The mercury was used as a catalyst. Mercury from the sludge or from the waste-water outlet entered food chains and was bioconcentrated in both shellfish and fish. Humans eventually consumed these foodstuffs. At Niigata, river fish from the lower reaches of the Agano River were the main foodstuff consumed which contained organic mercurial compounds. The source of the mercury was an industrial plant waste-water discharge containing low concentrations of mercury (**Figure 11**). Methylation took place in sediments, and considerable bioconcentration occurred in the fish, as evidenced by the much higher levels of mercury in the fish when compared with the river water.

At both sites, consumption of contaminated fish had gone on for a considerable time before the causes of the disease were identified. This points to the great difficulties involved in deriving a cause–effect relationship when the effect is remote from the source of the causative agent.

Although the particular examples chosen to illustrate this effect related to human health, another species may be the final consumer in a food chain. The story of the consequences of spraying persistent organochlorine insecticides, their bioconcentration in the food chain and

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their disastrous effects on raptor populations because the concentrations reached were sufficient to cause eggshell thinning and failure to reproduce effectively, summarized by Smith (1986), is well known.

3.3 Interactions of Humans and Nature

This section includes a miscellaneous collection of disasters in which human activity and natural events have interacted. It includes disasters involving tide, current and weather, in addition to those due to human activity on (in) the land.

3.3.1 Shipping Disasters

The classic shipping disaster involving toxic effects is the spilling of oil from wrecked oil tankers. Most of the consequences are environmental, but there is also potential for indirect or direct effects on the human population.

3.3.1.1 Wreck of the MV Braer, Shetland Isles, Scotland, 1993

On 5 January 1993, the oil tanker MV Braer grounded in the Shetland Isles (off the north coast of Scotland) in bad weather (Ritchie and O'Sullivan, 1994; Topping *et al*., 1997; Whittle *et al*., 1997). The cargo of 8500 tonnes of a biodegradable light crude oil was released over the next seven days. This oil was dispersed by the severe weather; dispersants were not used because of these weather conditions.

Human health effects from direct (airborne) exposure were minimal and were confined to headache, throat irritation and itchy eyes (Campbell *et al*., 1993). There was some evidence for self-reported somatic symptoms, anxiety and insomnia, based on a general health questionnaire (Campbell *et al*., 1994).

Although the principal toxic effects were environmental, there was a potential human health problem arising from the food chain (Topping *et al*., 1997; Whittle *et al*., 1997). The area contaminated was a fishing ground and it included several salmon farms. Because of potential human health problems, an exclusion order under the Food and Environment Protection Act 1985, banning the harvesting for market of fish, shellfish and farmed salmon from within a designated zone around the wreck was made on 8 January. On 27 January, that zone was extended in order to ensure that all areas where contamination took place were included. **Figure 12** shows the approximate area covered by the zone. The order was lifted for fish on 23 April 1993, on crustaceans on 30 September 1994 and on molluscs in February 1995. The farmed salmon usually remain in sea cages for two winters. Both the 1991 and 1992 fish (date is year of transfer of the salmon smolts (young fish) to sea cages) were destroyed, but smolts transferred in early spring 1993 were marketed. The basis of the decision to allow marketing depended on analysis of polycyclic aromatic hydrocarbons present in the fish and taint analysis. Because no formal limits for acceptable levels of these contaminants in foodstuffs were available, chosen were:

1. Fish and shellfish caught within the zone should not contain taint associated with crude oils and petroleum fractions.
2. Concentrations of aliphatic and polycyclic aromatic hydrocarbons should fall within the background range of values for fish and shellfish from outside the exclusion tone.

This approach to the potential problem of contamination of the food chain was successful, in that it upheld the reputation of the Shetland Isles seafood industry, prevented entry of contaminated food into the chain and provided public reassurance concerning the wholesomeness of marketed fish and seafood. However, it did little to alleviate the direct environmental effects of the release of oil.

3.3.2 Mines and Tunnels

People have died as a result of lung cancer due to exposure to radon in mines or pneumoconiosis caused by exposure to coal dust or silica, but the exposure to these agents was a consequence of inadequate working conditions rather than specific accidental exposures. Fire is a potential problem in mines and tunnels. For this reason, when there was an underground coal mining industry in the UK, very strict precautions were developed to prevent fires starting underground. These included testing and certifying machinery for use underground and strict prevention of miners taking potential ignition sources underground.

In general, toxicants are unlikely to be a primary cause of a specific mining disaster, as the conditions causing ill health are unlikely to develop suddenly. Historically, particularly before the use of forced ventilation, disasters due to asphyxiation or the release of toxic gases did occur. Canaries were taken underground to act as fail-safe biological monitors for these effects ('when the singing ceased ...'). Disasters can also occur in tunnels used by the public as a result of the deliberate release of toxic agents. The Kings Cross disaster, already examined, is a demonstration of the potential for disaster that can result from fire occurring in a confined space.

3.3.2.1 Terrorist Attack on the Tokyo Subway, 1995

The dramatic effects of deliberately releasing a toxic agent in a confined space were demonstrated in a terrorist attack in the Tokyo subway in 1995 (Masuda

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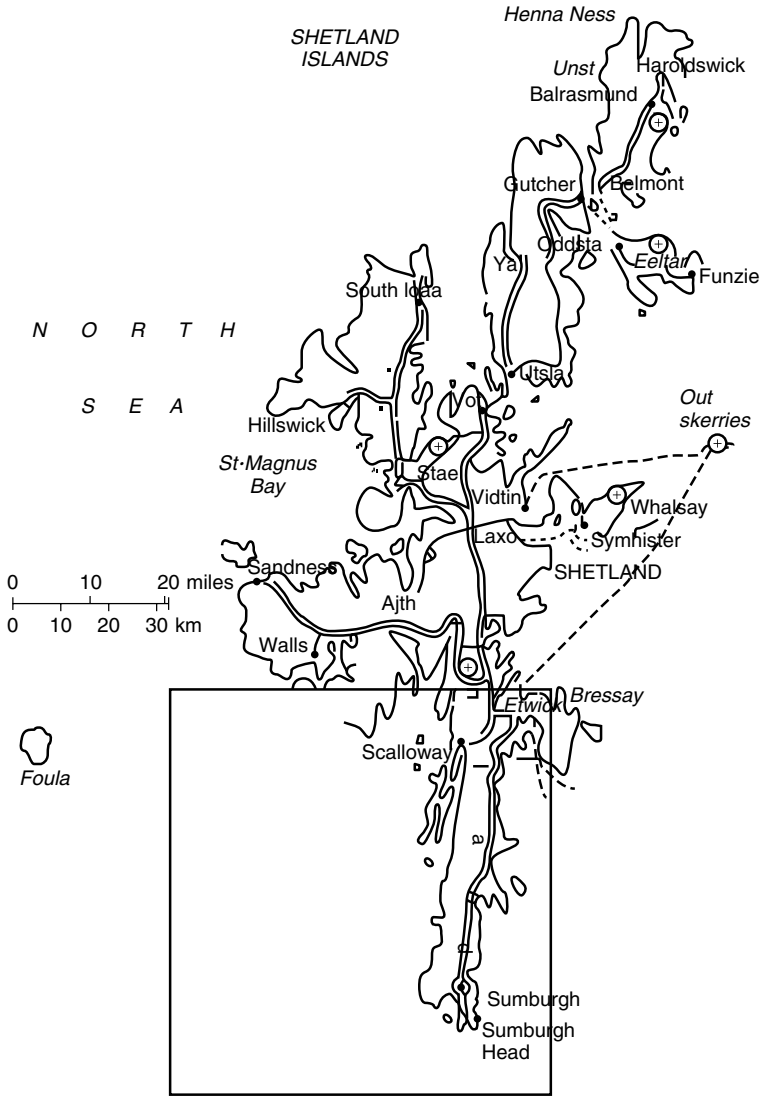

Figure 12 Map of Shetland Isles showing exclusion zone.

et al., 1995; Nozaki and Aikawa, 1995; Nozaki *et al*., 1995; Suzuki *et al*., 1995; Miyaki *et al*., 2005). Sarin, an organophosphorus nerve agent, was released. Twelve people died and over 5000 were poisoned by the attack. Classical symptoms of organophosphorus poisoning were exhibited. Atropine and pralidoxime iodide administration were the principal treatments employed. However, it was claimed that the atropine was of limited value as it is effective against muscarinic dominant responses, and sarin was thought to have produced principally nicotinic dominant responses. It was reported that, as a group, those exposed exhibited a chronic decline of psychomotor function and memory function seven years post incident (Miyaki *et al*., 2005).

3.3.3 Waste Disposal

Inadequately thought-out or poorly controlled disposal of wastes may cause disasters. Love Canal was one such incident.

3.3.3.1 Love Canal, USA

Love Canal was a waste disposal site, which contained municipal and chemical waste disposed of over a period of 30 years up to 1953. Homes were built on the site during the 1960s. Leaching became a problem in the late 1960s, when chemical odours were detected in basements. These were followed by fears of potential ill health which led to considerable psychological stress. Dibenzofurans and dioxins were identified in the organic phases of leachates and were presumably derived from the disposal of waste products of the manufacture of chlorinated hydrocarbons. Animal studies conducted on the organic phase of the leachate indicated that there could be risks of immunotoxic, carcinogenic and teratogenic effects (Silkworth *et al*., 1984; 1986; 1989a; 1989b). Low birth weights have been found in the offspring of residents of Love Canal, together with a higher proportion of female offspring and higher rates of congenital malformations (Vianna and Polan, 1984; New York State

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Department of Health, 2008). No causal link has been established for exposure to chemicals and cancer in humans (Janerich *et al*., 1981; New York State Department of Health, 2008).

Serious social and psychological consequences have resulted from the use of the site for houses and the initial lack of understanding of the fears of residents concerning the toxic properties of the chemicals dumped at the site (Holden, 1980).

Love Canal is important because it was a key incident leading to the US 'Comprehensive Environmental Response Compensation and Liability Act' (CERCLA) of 1980, and its amendment, the Superfund Amendments and Reauthorization Act (SARA) of 1986, commonly known as 'Superfund'. These provided broad US Federal authority to respond to releases or threatened releases of hazardous substances that may endanger public health or the environment. They also created a tax on the chemical and petroleum industries (which continued to 1995), which went into a trust fund for cleaning up abandoned or uncontrolled hazardous waste sites when no responsible party could be identified. The trust funds ran out in 2003, since when such clean-ups have been paid for from general taxation.

3.3.3.2 *Minamata and Niigata*

The mercury poisonings at Minamata and Niigata, already described, were caused by the disposal of industrial wastes in such a manner that the toxicant was concentrated to dangerous level, in a food chain in the aqueous environment.

3.4 Conclusions

Toxicants, whether derived from nature or the chemical plant, can cause disasters. Identifying the cause is relatively easy when the toxicant is airborne and the ill health occurs during or shortly after exposure. It is usually more difficult if the effect is mediated via the food or water supply and/or if the effect is not immediately apparent. The former have been called 'overt' disasters and the latter 'dilute' disasters (Bertazzi, 1989). As 'dilute' implies some diminution of effect, it might be better to refer to the latter type of disaster as a 'disseminated' disaster.

In overt disasters due to airborne toxicants, there is usually a primary event such as volcanic eruption or a plant failure. This is followed by dispersal of the toxicant, which depends on the buoyancy of the material released and the meteorological conditions at the time of release. Any immediate dose received by humans will depend on the level and duration of the exposure. Only very simple post-event preventative measures can be used to minimize the dose received. Although usually ill health is immediate, long-term effects such as carcinogenicity can occur. However, they are difficult to link to a primary event. Toxic material can be deposited on to surfaces, and ill health due to the consequent continuing lower-level exposures may be prevented by segregation (e.g. collection and containment of the contaminated material and/or banning public access to it).

Most known airborne toxicants involved in major disasters affect the respiratory tract. Irritation, asphyxiation (chemically induced by binding of toxicant, or physically induced by blockage of the respiratory tract) and pulmonary oedema are common (Schwartz, 1987). Cancers are possible effects, although they have been better characterized for radiation, and only recently has it been possible to detect cancers following chemical exposures epidemiologically. Other toxic effects may occur in organs away from the respiratory system, although this appears to be rare. When airborne, the toxicant reaches the lung and respiratory system first: consequently, these appear to be the most frequently affected organ systems.

When transport and dispersal of a toxicant are in water, then buoyancy, flow rates and ability to be taken up and released by sediment or biota become important determinants of the concentration present. Transport of toxic substances deposited on land depends, in large part, on the weather. In dry conditions, air dispersal of the material absorbed on particles is possible; in wet weather, dispersal will be along the same routes as those followed by the rainwater. Soil adsorption and uptake by plants and animals may also occur. Waterborne (or soil-borne) toxic substances may affect human health as a result of direct intake as drinking water or via foodstuffs contaminated with the toxic substances. This type of dispersal may result in 'overt' disasters when the source is readily apparent, or in 'disseminated' disasters when the source of the toxic substance is not obvious.

The causes of 'disseminated' disasters are frequently more difficult to identify and these disasters are often identified initially by end effect. Tracing the cause may take a considerable time, particularly as there may be a significant time delay between exposure occurring and ill health taking effect, and transfer between environmental compartments may have occurred. In consequence, withdrawal of the food, and so on, containing the toxicant, although desirable, may not be possible until it is too late to be an effective post-event preventative measure.

One consistent complicating factor in major accidents and disasters is the possibility of psychological reactions being considered as physiological responses to exposure to a particular agent. Long-lasting consequences, including severe worry, anxiety, depression and 'post-traumatic syndrome', were identified following the Mount St. Helens eruption, Bhopal, the Lowermoor and Love Canal incidents. Although not toxicological responses *per se*, these effects can be real, distressing and disabling. In the absence of sufficient authoritative and accurate information, popular speculation generally favours the worst possible outcomes,

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however improbable. This is likely to worsen psychiatric consequences of the event and to complicate the emergency response. The provision of timely, accurate advice in an understandable form is one need that must be considered when examining how to handle the consequences of a serious incident.

4 DISASTER PREVENTION AND MITIGATION

One role of industry, individual governments and international organizations is to develop and implement procedures for preventing or minimizing the effects of potential or actual disasters. In most countries governments develop a series of legislative requirements in order to provide a framework of regulation within which to work. Rather than compare different national frameworks, this section will concentrate on conceptual approaches to handling the risks arising from major hazards.

Assessment of potential hazards and assessment and management of the likely consequences are the key elements in any process for dealing with major hazards. In the case of toxic hazards, one aspect of the hazard evaluation is an examination of the likely toxicity of the materials involved in the potentially disastrous situation, including the prediction of the amounts of toxic materials likely to cause these effects. Procedures can then be adopted for the assessment of the risks associated with the various uses. These procedures of risk management differ fundamentally according to the type ('overt' or 'disseminated') of potential disaster envisaged.

4.1 Judging Risk

'Is it a risk?' is usually the first question asked when a potential hazard is being examined. Once the concept of risk has been explained, two questions will follow: 'What is the risk?' and 'Is it acceptable/tolerable?' (see **Table 1** for definitions). This involves trying to define general criteria for acceptable or tolerable risk. These criteria should be based on societal judgements, but judgements are often made by the technical expert on behalf of society as a whole. Once criteria for acceptability (tolerability) have been defined, a method is needed in order to compare the risk for the particular problem under study with the relevant criterion for acceptable risk. That method may be qualitative, of the form 'acceptable'/'unacceptable', based on a judgement of the data available against broadly defined criteria. However, for many purposes, including much engineering-based risk assessment for an initiating event, quantitative, numerical approaches are adopted. These numerical approaches are called quantified risk analyses. These quantitative analyses render decision-making easier, especially when choosing between options, as they give a clearer indication of the magnitude of a risk or of the relative risks for different options. However, because these decisions depend, in the final analysis, on societal judgements concerning acceptability, they may be unacceptable to certain individuals or groups within that society. Major reports on risk management and standard setting have emphasized that both communication between technical experts and key 'stakeholders' (acting on behalf of society) and transparency of decision taking process are essential if decisions taken on behalf of society are to be accepted by society (Presidential/Congressional Commission on Risk Assessment and Risk Management, 1997; Royal Commission on Environmental Pollution, 1998). Public (or 'stakeholder') involvement should be included in strategy formulation rather than solely in consultation on already drafted proposals, and may be accomplished using 'focus groups', citizen juries, consensus conferences and deliberative polls as well as by more traditional means.

When considering the risk of an event leading to an end effect, a surrogate for that end effect, a 'dose' or exposure–time combination (a 'harm criterion') is often used. Uncertainty in the relationship between this surrogate and the end effect (normally considered 'risk' by toxicologists and epidemiologists) is regarded, in this context, as uncertainty in defining the hazard.

Judging the acceptability of a human health harm criterion depends on three factors: the frequency level (the frequency with which the end effect is likely to occur), the definition of the biological effect (death, serious injury, etc.) and the status of the receiving individual (a 'normal' or a 'vulnerable' individual).

What constitutes a major accident to the environment is more difficult to define and the definition may involve subjective judgements. Generally loss of populations or loss of habitats, rather than loss of individuals is required when considering nonhuman species. The more extensive the areas of natural or seminatural resources damaged, the longer the effects are likely to last. And the more intense or severe these effects are, then the more likely it is that the event will be regarded as a major accident to the environment. Moreover, if the land is designated for nature conservation purposes (and especially Natura 2000 or Ramsar sites (Ramsar sites are named after the place in Iran where the Convention on Wetlands was signed in 1971)), then the event is likely to be regarded as a major accident at lower thresholds than those that apply to other designated areas, amenity areas, the wider countryside or the more common types of agricultural land. One set of definitions for the types of effects for a major accident to the environment is that the incident:

- Poses knock-out threats to human health by contamination of food or drinking water or impact on sewage treatment works.

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- Affects large areas of land designated for conservation, amenity or planning purposes. (Note that this may include extensive agglomerations of fragmented habitats.)
- Results in long-term or persistent damage and/or inhibits natural processes of regeneration.
- Results in significant, permanent or long-term damage to the ecosystem (direct, indirect or knock-on), such as reducing breeding success of protected species, or reducing biodiversity of protected habitats (including local or national extinctions of protected species), or destroying/reducing the quality of a significant portion of a rare habitat.

(Department of the Environment, Transport and the Regions, 1999).

4.2 Framework for Risk Assessment

There are several levels of risk (either of initiating event or of end effect) that can be elaborated. At one level there is the 'trivial', 'negligible' or 'completely acceptable' risk. At the other extreme there is the 'intolerable' or 'unacceptable' risk. In between lies a range of risks which are tolerable under certain circumstances and/or provided they are minimized (**Figure 13**). In between the upper and lower bounds of acceptability lies a region where risks from known hazards should be reduced as far as is reasonably practicable. This means that the cost of reducing the risk should not be disproportionate to the level of risk encountered. A small reduction in an already low risk may not be justified if it is very expensive.

4.2.1 Harm Criteria for 'Acceptable Risk' (Human Health)

When comparing risks, the risk of end effect, and not of initiating event, is being examined. It is necessary to ensure that the particular end effect (e.g. 'death'; in reality foreshortening of life, often to the extent that death occurs during or shortly after the event occurring) for which the likelihood is being calculated is the same for all the likelihoods being examined, in terms of both the biological event and the type of recipient (usually a 'normal' individual). Criteria for assessing this 'risk of death' to the individual have been obtained by comparisons with everyday risks associated with various activities (Royal Society Study Group, 1983; Health and Safety Executive, 1988a; Health and Safety Executive, 1988b; Health and Safety Executive, 1989). For major hazards risk, the acceptable 'risk of death' for exposure to the given agent has to be converted to a 'harm criterion' (exposure concentration–time relationship) for inclusion in the calculation of the overall risk arising from the initiating event.

A likelihood of about 1 in 10^6 per year that the exposure will result in the individual concerned dying from a given cause appears to be generally regarded in the UK as the boundary between tolerable and acceptable (Royal Society Study Group, 1983; Health and Safety

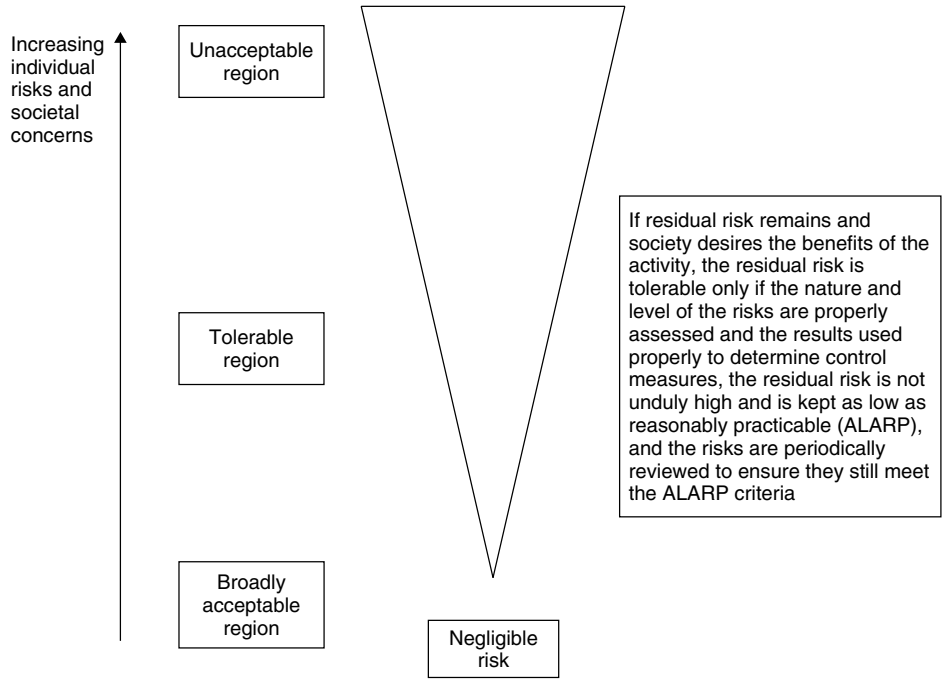

Figure 13 Outline of the Royal Society approach to risk management/tolerability of risk framework. (Based on Health and Safety Executive, 2001.)

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Table 3 Comparisons of risk of death from various causes

Cause of death	Risk (as annual experience) (per year)	Year of statistic	Risk (as annual experience) (per year)	Year of statistic
Cancer	2.8×10^{-3}	1985	2.58×10^{-3}	1999 (England and Wales)
Deep sea fishing	8.8×10^{-4}	1984		
All accidents and external causes			2.46×10^{-4}	1999 (UK)
Quarrying	3.0×10^{-4}	1985		
Coal extraction, manufacture of solid fuels	1.1×10^{-4}	1986–1987		
Mining and quarrying (energy producing material)			1.09×10^{-4}	1996–1997 to 2000–2001 (GB)
Agriculture	8.7×10^{-5}	1986–1987	5.81×10^{-5}	1996–1997 to 2000–2001 (GB)
Construction	9.2×10^{-5}	1986–1987	5.88×10^{-5}	1996–1997 to 2000–2001 (GB)
Road accident	1.0×10^{-4}	1985	5.95×10^{-5}	1999 (UK)
Gas incidents	1.8×10^{-6}	1981–1985	6.62×10^{-7}	1996–1997 to 2000-2001 (GB)
Lightning	1.0×10^{-7}	Average over several years	5.35×10^{-8}	1995–1999 (England and Wales)

Data on industrial accidents are based on accidents to the employee; data on deaths from other causes are averaged over the general population.
Compiled from Health and Safety Executive (1989; 2001). The data from the 1980s were used by the Health and Safety Executive when defining the criteria of 'risk of death'. Note how the data underlying the criteria have improved over time.

Executive, 2001). This was justified by considering the death rates for different activities considered 'safe' (see **Table 3** for some comparative 'risks of death'). The 'risk of death' for workers in recognized dangerous occupations in the 1980s was of the order of 1 death per 10^3 per year; this is considered the highest bound of 'tolerable' risk for a lifetime risk. Any higher level is considered intolerable and unacceptable at all times. Because working in a 'risky' industry is, at least to some extent, voluntary, the individual risk just tolerable for a member of the general public living in the neighbourhood of a hazard is considered to be 10-fold less (1 death per 10^4 per year) (Health and Safety Executive, 1989; 2001). This is approximately the risk from death in a road traffic accident and less than the risk of developing any form of cancer. All of these likelihood criteria relate to individual risk of end effect for the average individual. As risk levels associated with industrial accidents decrease, it could be argued that the criterion value for tolerable/unacceptable risk for workers should be adjusted to reflect more recent statistics.

The type of population being considered may differ according to circumstances. Rather than examining the frequency of achieving a given biological effect in terms of the event occurring in an average individual in a 'normal' population, it may be that the consequence should be considered for an individual from a population containing a large number of particularly vulnerable (or susceptible) individuals. A frequency of 3.3 in 10^7 per year for when there is a high proportion of 'highly susceptible' people receiving a 'dangerous dose' has been proposed in place of the 1 in 10^6 per year for an individual from a population containing a normal balance of 'highly susceptible' individuals (Health and Safety Executive, 1989). The identity of the 'highly susceptible' people will depend on the effects seen, and the proportion will differ according to effect. Homes for the elderly, caring institutions and long-stay hospitals are considered to contain a high proportion of 'highly susceptible' people. However, the choice of the ratio of 3.3 : 1 (a half-order of magnitude) is a pragmatic decision.

A more conservative approach has been elaborated in the Netherlands (Versteeg, 1988). It depends on the frequency of deaths from natural causes and is based on the idea that industrial activity should not increase the background mortality by more than 1%. An upper bound of 10^6 deaths per year has been derived with a lower bound of 10^8 per year being considered trivial. If the biological effect being looked at changes, and/or the type of individual being examined is different, then the numerical value of any harm criterion ought also to be altered. When quoting a harm criterion it is therefore necessary also to mention the effect and the type of

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recipient. Although these types of numerical comparisons have been made for individual likelihoods associated with severe end effects, such as death, other end effects may be more important for evaluating the potential toxic effects of chemicals for disaster prevention and mitigation. Risks for events leading to more minor ill health may be the evaluation required, and the risk may be societal (or population) risk. Although attempts have been made to consider what frequencies of end effect should be applied for many of these circumstances (e.g. Illing, 1991; Calman, 1996), consensus is absent.

Although descriptive criteria as to what constitutes a major disaster to the environment have been developed for environmental incidents (Department of the Environment, Transport and the Regions, 1999), these descriptions have not been related to predictive information. Thus judgement has to be used.

4.2.1.1 'Overt' Disasters

Risk of the initiating event is the risk being examined. The aim of the risk assessment is therefore to examine the likelihood of events resulting in a release of a chemical or chemicals of a size and type that results in the harm criterion being exceeded in a given geographic location. Potential 'overt' disasters can be averted or minimized by a combination of land-use planning and emergency planning. Good land-use planning can minimize the potential of a release (a catastrophic failure of plant, etc.) leading to a disaster by restricting the size of any interaction of event and consequence. A hazardous process or a large store of hazardous material can be sited well away from large numbers of people (or, for environmental effects, away from important buildings or sensitive wildlife habitats). Potential developments involving significant numbers of members of the public can be sited away from natural or human-made major hazards.

There will remain a residue of risk after appropriate land-use planning has been achieved. Occasionally there will be the situation where inappropriate combinations of people and major hazards occurred prior to the time when the need for appropriate land-use planning became apparent. In addition, other considerations, such as a need for local employment, may mean that other factors were decisive when the planning decision was made. The consequences, should the event occur, can still be substantially reduced, provided that appropriate emergency planning has been undertaken. This includes both enabling potential victims to survive better prior to treatment, and making available appropriate treatment sufficiently rapidly. It also involves protecting water and food supplies intended for human consumption.

Generally, owners (or prospective owners) of potential major hazard installations will prepare 'safety cases' or 'safety assessments' for those sites. These often include assessments of the risks and consideration of the administrative and other arrangements for minimizing risks and for handling emergencies.

4.2.1.1.1 Land-Use Planning

For land-use planning in the UK, the 'Dangerous Toxic Load' (DTL) describes the exposure conditions, in terms of airborne concentration and duration of exposure, that would produce a particular level of toxicity in the general population (Health and Safety Executive, 2008).

The 'Dangerous Dose' is a harm criterion, a description of the exposure conditions producing a specified level of toxicity (SLOT) where (Health and Safety Executive, 1989; Fairhurst and Turner, 1993):

1. Severe distress will be caused to almost everyone in the area.
2. A substantial fraction of the exposed population will require medical attention.
3. Some people are seriously injured and require prolonged treatment.
4. Any highly susceptible people may be killed.

This type of criterion reflects that there is a range of individual ill-health effects and imprecision in the estimate of overall effect seen in the population.

Another DTL is that for the significant likelihood of death (SLOD; a DTL relating to 50% mortality in a normal exposed population) (Health and Safety Executive, 2008).

Some ratios have been enunciated for the relationships between the harm criterion for exposure conditions leading to 'death' (SLOD) and 'dangerous dose' (SLOT; taken as 1% mortality in Health and Safety Executive, 2008) in the same population. Such a value will depend on the LC_{50} (lethal concentration expected to cause 50% mortality) for different durations of exposure and the slopes of the dose–response lines for both the SLOD and the SLOT. Comparisons for chlorine, ammonia, hydrogen fluoride and sulfur dioxide give the relationship:

$$\text{Risk of } LD_{50} = \frac{(\text{Risk of Dangerous Dose})^{1.12}}{2.97} \qquad (2)$$

(Franks *et al*., 1996).

In this exercise, LD_{50} (LD—'lethal dose') is an amount of a substance, a combination of concentration and time. When there are insufficient data to derive a relationship between SLOD and SLOT (or LD_{50} and Dangerous Dose), the Health and Safety Executive (HSE) takes a default factor of 4 and divides the LC_{50} (for a given exposure time) by this (Health and Safety Executive, 2008).

4.2.1.1.2 Emergency Planning

Emergency planning is essentially concerned with mitigating and recovering from the consequences after an

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event has occurred. This has very wide application, and includes preparedness for chemical, biological, radiological or nuclear hazards, including those arising from terrorist activity. In the UK, emergency preparedness for non-'Control of Major Accident Hazards Regulations' (COMAH) sites is covered by the Civil Contingencies Act 2004, and guidance has been published (HM Government, 2005). In the USA it is dealt with by the Department of Homeland Security. For certain industrial hazards, the COMAH Regulations (The Control of Major Accident Hazards, 1999) apply. There is a need to cover both 'on-site' and 'off-site' planning, including immediate ('emergency') shutdown, responses of emergency services, the medical management of the immediate and long-term health effects, and the management of food and drinking-water supplies and potential environmental effects (OECD, 1994). Such planning is concerned with a much wider range of biological effects than death. For immediate effects on human health, these include the 'severe health effect' (disability, requiring hospital treatment), and 'mild health effect' (discomfort or distress), as well as the 'detectable but not a true health effect' (nuisance) (Baxter *et al*., 1989a; Illing, 1989). Both the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), through their 'emergency exposure indices' and the US National Research Council, through their 'acute exposure guideline levels' (AEGLs) have defined three harm criteria for airborne concentrations for exposures lasting up to a specified time (ECETOC, 1991; National Research Council, 2001). These are criteria above which direct toxic effects are likely to lead to one of the outcomes: death/permanent incapacity, disability or discomfort. Although hospital treatment may be essential for recovery from the severe health effects, it will usually have little influence in the case of milder effects (discomfort). It may also be necessary to consider longer-term health effects, protection of food and drinking-water supplies (by withdrawing affected foods and water from the supply chain), and possibly protection (if feasible) of the environment. There is also a need to plan for an appropriately sized event. One proposal categorizes three types of release: small but likely accidents, severe but reasonably foreseeable events, and large, unlikely events (Baxter *et al*., 1989a). If the severe, reasonably foreseeable event is chosen for planning purposes, then it should be possible to scale up or down the response for an individual event.

4.2.1.2 'Disseminated' Disasters

Essentially, 'disseminated' disasters should only occur as a result of failure of a regulatory system resulting in exposure to a toxicant. The risk should be that of the initiating event, the failure of the regulatory system. That failure may be because a new effect was uncovered by the disaster; alternatively, a known effect might have occurred because of nonadherence to existing regulatory requirements. The primary means of control has to be preventative, ensuring that, under normal conditions of exposure, the frequency of even minor ill health is acceptably low. A sufficiently rigorous enforcement system is needed in order to ensure that failures of control which result in higher frequencies of ill health or more severe ill-health effects do not occur. This type of approach is based on the conventional no-effect level and safety factors, leading to such concepts as maximum 'acceptable daily intake' and 'occupational exposure limits' (Royal Society Study Group, 1983). Where carcinogenic or other stochastic effects are concerned, the approach will be to minimize exposure as far as is reasonably practicable.

4.2.2 Determining Risk Levels

Determination of the risk levels associated with likely release sizes and dispersion patterns is required for airborne overt hazards. There are essentially similar processes for determining risk levels for land-use planning and emergency planning (Health and Safety Executive, 1989; ECETOC, 1991). The process can be divided into seven steps (Health and Safety Executive, 1989):

1. Identification of possible hazardous release events.
2. Identification and analysis of the failure mechanisms which would allow a release.
3. Estimation of rates and durations of the release.
4. Estimation of the frequencies of releases using the analysis of failure mechanisms.
5. Estimation of the injury consequences of releases, taking account of mitigating factors.
6. Combination of the frequencies and consequences to determine the overall risk levels.
7. Judgement of the significance of the risk levels (by comparison with appropriate criteria).

Although this process was described for land-use planning in the vicinity of major industrial hazards in the UK, it can also be used more generally. Essentially, it can be described as three elements—a description of a source, a model for looking at the dispersion of the substance, and a means of entering into the dispersion model parameters describing the conditions which will give a biological effect.

Models have been developed for airborne dispersion. Models are also available for dispersion through surface and groundwaters and soils and sediments, and for dispersion through food chains.

The source term for inputting into the dispersion model can be obtained by 'fault tree' analysis of the frequency of events (failure rates), by 'event tree analysis' leading to an estimate of failure rates, or by engineering judgement based on historical event frequency. The actual method(s) chosen will depend on the type of release being

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studied and the available data. The sizes and durations of the postulated/actual releases are also examined (Health and Safety Executive, 1989). For land-use planning there is a continuum of event frequency and size of release. Usually, sets of release sizes and durations are selected from the continuum and assigned appropriate frequencies. However, for emergency planning the process can be simplified by using the 'severe, but reasonably foreseeable event' as the appropriate basis on which to plan (Baxter *et al*., 1989a). This event was exemplified as a large hole in, or fracture of, a liquid chlorine pipe, or of a road tanker delivery coupling at a chlorine installation. Scaling a response up or down should then be possible when an event actually occurs if the plan is sufficiently flexible. The modelling then required is concerned with the dispersion of the toxic cloud in air or the toxic material through surface or groundwaters or in soils and sediments. Several models for airborne dispersion have been described (see McQuaid, 1989; Brighton *et al*., 1994). In general, these models require a knowledge of the buoyant density of the cloud under different weather conditions. The dispersal conditions need to be combined with a function describing the combination of exposure concentration and time, resulting in a defined frequency of a specified effect. For example, one concentration–time combination may be described as the 'dangerous dose' for land-use planning, while a different combination could be used for the outer boundary for 'discomfort' for emergency planning. When combined with the information on the source, the model is then used to calculate 'isopleths'. These are boundaries of areas within which, at a point in time, the concentration–time combination of exposure to the toxic substance would exceed that expected to give the identified biological effect. An overall isopleth envelope can then be calculated for a given set of release conditions by combining the individual isopleths for each time point post-event. Similar approaches can be used for dispersion in river systems and for groundwater dispersion.

The information on the various isopleth envelopes for different types of release and meteorological conditions, when combined using knowledge of the frequencies with which these conditions occur, gives a generalized 'contour' for all source terms and dispersal conditions. This contour is a risk statement for individual risk for a person within the specified boundary. An estimate of societal risk can then be obtained if data on the distribution of people within the geographic area are input. In view of the complexity of these systems, computer programs have been developed to carry out the calculations associated with the models. The aim of the toxicologist is to find appropriate information on which to base a function describing the concentration–time relationship for a particular biological effect or combination of effects.

4.2.2.1 Contribution of Human Health Toxicology to Hazard and Risk Assessment

The most likely direct hazards arising from a major accidental release of a toxic substance are the biological consequences of short-term exposure during dispersal of the toxicant. Longer-term or delayed effects should be considered in addition to those more immediately apparent. Indirect consequences due to contamination of food or water supplies or to inhalation of dusts following settlement and subsequent disturbance are potentially important, but less immediate problems, and should be amenable to post-event measures aimed at preventing significant exposure.

4.2.2.1.1 The Data Available

In theory, at least, the ideal assessment of toxicity of a potential major hazard substance or agent would be based on accurate observations of the appropriate effect in humans. Reliable reports on severe effects in humans are, fortunately, few, and mainly as a result of accidental exposure or exposure in wartime. Controversy often surrounds the exposure levels associated with such studies (Withers and Lees, 1987; Marshall, 1989) or the sources of the information (MHAP Toxicology Working Party, 1993). Results from studies on sublethal effects in humans may be more plentiful. Nevertheless, the data on most substances are restricted to accidental exposures, often affecting only one or two people and rarely containing accurate exposure information. Except for radioactivity, human evidence concerning long-term effects of single exposures to most agents and substances is virtually nonexistent. Further studies which require deliberate exposure of men (or women) to dangerous levels of a substance or agent are unethical.

As a consequence of the limited human data, heavy reliance has to be placed on animal and other experiments in attempting to predict the adverse effects in the human population. Often these animal data are confined to short-term effects (sometimes only lethality) following single exposures. For example, prior to Bhopal, the information available on the toxicity of methyl isocyanate was confined to a single published paper (Kimmerle and Eben, 1964) on short-term effects following acute exposure (Bucher, 1987). Also, many animal studies on the types of substances which might constitute major hazards were performed a long time ago and the data are, by modern standards, of poor quality. In view of the very variable nature of the toxicity information, it is essential to evaluate this information critically and to understand the uncertainties introduced as a consequence of the nature and quality of the data (Illing, 1989; Marshall, 1989; ECETOC, 1991; Fairhurst and Turner, 1993). This means that assessments will normally be conducted using the original reports or published papers.

As the information may be required for quantitative risk analysis associated with an initiating event, where

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possible it will need to be described in numerical terms. Uncertainty has to be handled by sensitivity analysis. By inputting various potential values for particular effects into the risk assessments, it is possible to discover the differences that variation of the input parameter will have in terms of outcome, the areas of map covered by the contour envelope related to a particular risk value. This sensitivity analysis is an essential part of the overall risk assessment.

Usually there are few, if any, data on the immunological, carcinogenic or reproductive effects likely after single exposures to major hazard substances/agents. Indirect effects mediated through food chain uptake and bioconcentration, or disturbing settled material also need to be considered. If data are available, they should be evaluated. However, that evaluation will normally be descriptive as, except for the effects of radioactivity, there is currently no satisfactory way of obtaining the data in a form suitable for more quantitative approaches.

4.2.2.1.2 Problems of Extrapolation

The difficulties encountered in extrapolating from data in animals in evaluating the hazard to humans are similar whatever the nature of the hazard being examined. Four principal problems—extrapolation for different exposure times, interspecies variation, population heterogeneity (interindividual variation) and route-to-route extrapolation—are particularly relevant when data for major hazard substances are examined.

Extrapolation for different exposure times is considered under 'modelling the toxicity information'.

Interspecies variation in biological effects is an important area of uncertainty in the evaluation. In the absence of suitable alternatives, the 'default option' is to use the most sensitive relevant species, that is, after excluding those species for which mode of action or other data clearly suggest they are inappropriate models for humans. Toxicokinetic and toxicodynamic relationships between species, together with information on the relevant biochemical parameters in humans, can lead to a better understanding of the relevance of results in a particular species for humans, but are rarely available.

Extrapolation between species is usually based on allometric scaling. As lung surface area (for absorption) and body weight or body surface area (for effect) can both be scaled allometrically, such scaling is not usually relevant to inhalation studies.

In addition to interspecies variation, it is necessary to consider interindividual variation (or population heterogeneity) and the nature of the population being studied. This is considered further below under 'modelling the toxicity'.

The UK tended to avoid the use of uncertainty factors for interspecies variation (Fairhurst and Turner, 1993). The factor of 4 in moving from SLOD to SLOT (see below) should include an element associated with interindividual variation as well as a component associated with the move from LC_{50} to a low level of deaths. The US National Research Council (2001) recommended use of uncertainty factors of 1–10 to cover both interspecies and interindividual variation, but more recent analysis suggests that these factors may be inappropriate (Bogen, 2005).

Occasionally, toxicity data may be used which were obtained for a different route of exposure from that for which they are being assessed (Pepelco and Withey, 1985; Pepelco, 1987). For example, in the absence of sufficient inhalation data it may be possible, in some circumstances, to make use of oral data and to relate them to equivalent inhalation exposures. However, comparisons cannot be made if there are substantial differences in the toxicokinetics for the two routes. Often, effects on the lung are critical. These can be considered local effects, in which case it is not appropriate to extrapolate from oral data. Great caution is required when extrapolating data from studies using different routes of exposure. Extrapolation of laboratory animal data to other, nonhuman species, although possible, is only done when there is a specific requirement.

4.2.2.2 Modelling the Toxicity Information

The best way of relating exposure level, duration and effect for quantitative risk assessment is probably based on toxicokinetic and toxicodynamic modelling, similar to that used for workplace exposure to inhaled gases and vapours. This has been used for developing AEGL values for trichloroethylene (Boyes *et al*., 2005). However, appropriate data are not usually available, so other, more pragmatic approaches are adopted.

A generally available pragmatic approach is based on the need to obtain (i) a concentration–time relationship, usually based on the midpoint (LC_{50} or EC_{50} (EC—'effective concentration')) of the effect(s) being examined and, if required, (ii) an appropriate set of exposure conditions that are taken to result in a particular level of effect in the population (ECETOC, 1991; Fairhurst and Turner, 1993; Franks *et al*., 1996; Health and Safety Executive, 2008).

As the concentration–time (ct) relationships often have to be based on LC_{50} or EC_{50} data for various times (they are frequently the only data available), they normally refer to midpoint concentration, that is, where the effect is seen in 50% of the population. In general, the values have to be adjusted to yield the boundary between effects (disability with few immediate deaths for 'dangerous dose'; discomfort with few disabled in the case of defining zones where people needing hospital treatment are likely to be located). The dose response may also need to be adjusted to account for differences in the make-up of the populations being examined. It must be emphasized

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that any boundary will be approximate because of the nature of the data and the models being used.

In the early years of this century, studies on the acute inhalation toxicity for a limited number of gases yielded mortality data suggesting the following relationship:

$$ct = \text{constant} \quad (3)$$

where $c =$ concentration and $t =$ time.

(Haber, 1924). This is usually known as the Haber rule. More recent observations have suggested a more general relationship:

$$c^n t = \text{constant} \quad (4)$$

(ten Berg *et al*., 1985; Klimisch *et al*., 1987; Marshall, 1989; Miller *et al*., 2000; National Research Council, 2001). An alternative form of this equation, which can be used to plot data, is

$$n\log c = \log t + \text{constant} \quad (5)$$

(the constant here is the logarithmic value of the constant in Equation 4.)

Although a wide range of values for n have been cited, usually it appears to be close to the integer value of 1 or 2. Where it is possible, the relationship should be derived from experimental data, ideally from a single series of experiments in the same laboratory and using the same strain of animal. If data are combined from different laboratories and different strains or species, the relationship generated may represent interspecies (or interstrain) variation, rather than a genuine concentration–time relationship.

Occasionally, as with data for rats exposed to sarin, a more complex relationship, including an interaction term is required (Mioduszewski *et al*., 2002). This may reflect the toxicokinetic and toxicodynamic influences associated with substances that cause their effects systemically, rather than at the site of administration.

Frequently there are insufficient data to verify the concentration–time relationship, and the 'default' value of $n = 1$ is used. It is probably adequate when extrapolating to longer times where this figure usually overestimates the consequences. However, the Haber rule could seriously underestimate the exposure if data are not available for shorter periods and extrapolation from longer exposure periods is undertaken (Illing, 1989; Boyes *et al*., 2000). Thus the UK Health and Safety Executive works from single, short-term exposures (up to 4 hours duration) and uses a default value of 1 (Health and Safety Executive, 2008). Also, the US National Research Council recommends using a default value of 1 when extrapolating from shorter to longer time points and a value of 3 when extrapolating from longer to shorter times for AEGLs (National Research Council, 2001). Because of underlying assumptions about steady-state toxicokinetics inherent in any of these relationships, they are all likely to be overestimates at, say, less than 5 minutes, while equilibration to some form of pseudo-steady state is taking place.

Usually it is also necessary to define the concentration–time combination for a boundary condition rather than for the midpoint value for an effect (**Figure 14**). Ideally this should be obtained from experimental data. Often it has to be derived from the slope of the dose–response curve. If data on the slope are not available, it may be possible to use Equation 2 (see above), provided that the effects seen and the populations for which the predictions are required are reasonably similar to those used in deriving the equation. The slope of the dose–response curve depends on the type of population being studied (see **Figure 15**). In transferring from LC_{50} to an LC representing the boundary death–disability, it is necessary to consider population heterogeneity. Animal experiments, particularly those performed in recent years, have been conducted using animals specially bred to limit variations in response, and are usually young, healthy individuals. This implies a relatively steep dose–response curve. In many older studies, much less closely defined animals were used. The general human population is more heterogeneous still, containing groups of individuals who must be considered 'highly susceptible' because of age, genetic constitution or disease state. Under these circumstances the dose–response relationship will be much shallower. A default of 4 has been suggested when moving from LC_{50} (the SLOD) to very low mortality (suggested as 1%) for the SLOT (Health and Safety Executive, 2008). This is probably a reasonable overestimate of the allowance for the movement from a tightly defined population variation due to the use of inbred strains to a more heterogeneous population typical of the normal human populace.

As an alternative to the approach to concentration–time relationships outlined above, it is sometimes possible to describe mathematically a line for the boundary conditions of the concentration–time relationship using scatter diagrams, showing all concentrations and times at which the effect occurred (**Figure 16**) (ECETOC, 1991). This approach normally requires access to more detailed data, including, ideally, the results for individual animals. It also can suffer from problems relating to population heterogeneity and interspecies and interlaboratory variation.

4.2.2.3 Additional Points

Whatever information emerges from animal toxicity data must always be compared with any data that are available in the target species (humans). Those from the most sensitive species are normally chosen as representing a conservative approach.

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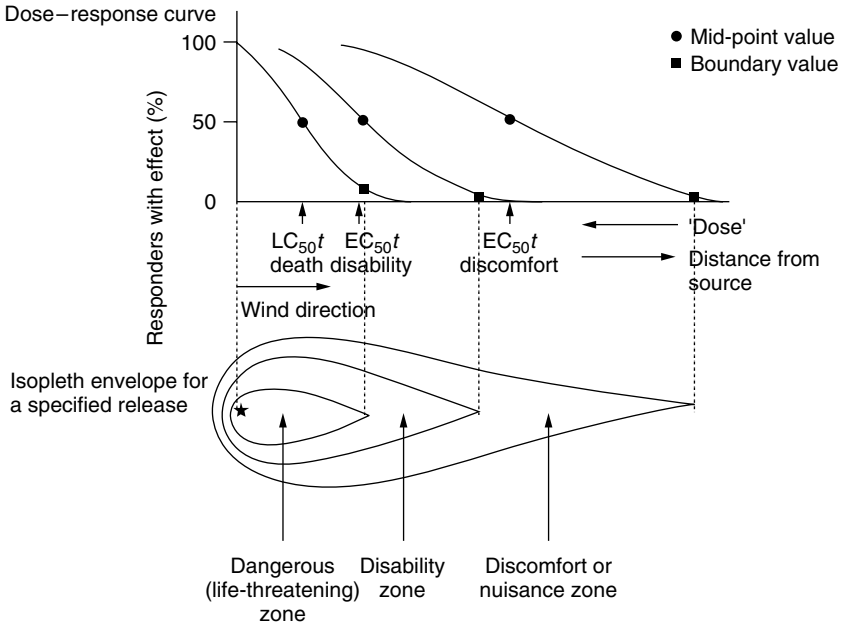

Figure 14 Diagram to illustrate the correlation between response and isopleth envelope. 'Dose' is the combined exposure concentration and duration (time) function for the particular effect. Time after initial event is given as *t*. The isopleth envelope described is that bounding the overall combination of concentrations and durations of exposure for a release, and can be built up from a series of isopleths describing the dispersion at different intervals after the start of the release. A combination of isopleth envelopes becomes a risk estimate when it incorporates the frequencies for different source and dispersion conditions.

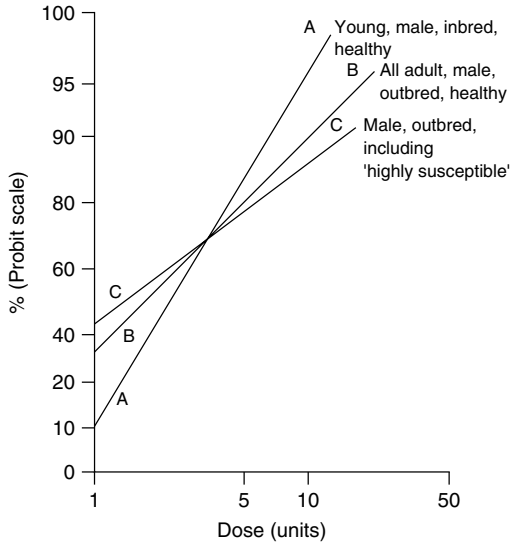

Figure 15 Diagram to illustrate hypothetical dose–response lines for different populations.

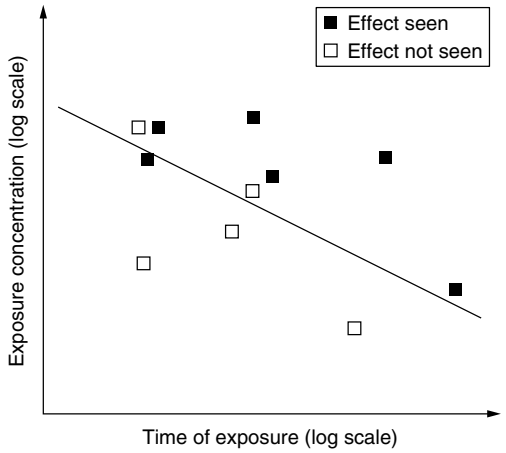

Figure 16 Idealised scatter diagram of the hypothetical relationship for an exposure-time–concentration relationship for an effect. By plotting on logarithmic axes the slope of the boundary line can be used to derive *n*. In the example the slope is $-n$, and $n = 2$.

The data are generally used predictively in order to describe the geographic areas where effects may occur or where the risk levels are associated with particular consequences. Best estimates are therefore usually considered preferable to more 'conservative' values based on the more severe confidence limit for the data. How closely defined the geographic areas affected by the risk analysis are when the toxicity data are limited in quality and quantity needs to be checked by uncertainty analysis in which the more extreme toxicity interpretations can be compared with the best estimate. Great accuracy is not required in view of the approximate nature of the overall evaluation.

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4.2.3 Outcomes

The requirements from the overall assessment of the toxic risks are different for land-use planning and for emergency planning.

4.2.3.1 Land-Use Planning

The outcome from a quantitative risk analysis is a risk statement based on a series of isopleth envelopes. This statement can be judged against predetermined criteria to decide whether a risk is acceptable in predetermined 'objective' terms. The risk assessor can recommend that no objection be raised to the proposed development or that the development should not go ahead. Acceptance by the general population may depend on different, often subjective, criteria. There is a political process associated with land-use planning (in the UK, based on the local authority, with access to a planning enquiry on behalf of central government) in order to allow expression of the objective and subjective opinions on the development. A decision has to be taken by the local authority councillors or a government minister on behalf of society as a whole.

4.2.3.2 Emergency Planning

In emergency planning, the aims include delineation of appropriate zones over which to provide public information on the type of hazard and on the protective measures that can be taken to ameliorate the likely damage. They also include delineating ways of tackling the problems likely to emerge post-event, guiding general emergency planning, search and rescue, planning for the medical needs of casualties, including setting permissible exposures for the people involved, planning for the protection of food and drinking-water supplies and planning (when possible) for protection of the environment. Emergency planning and management is an important topic in its own right, and the ways in which emergencies are tackled can vary with the circumstances surrounding the site. For example, the management of an emergency on an oil rig will differ from that for a land-based chemical plant or nuclear reactor. The type of medical planning is also important and is discussed in greater detail elsewhere (Baxter *et al*., 1989a; OECD, 1994; Heptonstall and Gent, 2006).

5 CONCLUSION

Disasters can be caused when large amounts of toxicants interact with a (human) population and cause deleterious effects on that population. They can occur in many forms; some result from natural causes and many from human efforts, accidental or intentional. Describing and understanding past disasters can be the first stage to preventing or ameliorating the effects of potential future disasters.

In general, sites which contain toxicants causing them to be classified as major hazards are so classified because of the amount of toxicant present, and also because of the toxicity of the substance. Both the evidence concerning the toxicity of many of these toxicants and the risk management procedures for managing the hazards and risks they pose have been greatly enhanced in recent years. In addition, there has been recognition of the need to respond to the emergencies that can be caused by terrorist attacks using chemicals, and this has also driven approaches to major incidents involving chemicals. When the first edition of 'General and Applied Toxicology' was published it was easy to bewail the lack of knowledge and procedures. The situation, though not yet ideal, has improved very substantially.

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Toxicology of Food Additives

Susan M. Barlow

CONTENTS

1 INTRODUCTION

Evaluation of food additives for safety in use has been continuing for over 50 years, both at the international level and by national regulatory and advisory bodies. In most countries, food additives cannot be marketed unless they have undergone a full safety evaluation. Nevertheless, consumers are often sceptical about the adequacy of toxicological testing of food additives. For example, in a survey conducted in 2005 of over 24 000 consumers from 25 European countries (Eurobarometer, 2006), when asked an open-ended question on what came into their mind when thinking about possible problems or risks associated with food, 7% of those surveyed cited food additives as a problem. It was the sixth most frequently cited issue, with food poisoning being the most frequently mentioned (by 16%) and chemicals, pesticides and toxic substances the second most frequently mentioned (by 14%). When specifically asked about additives like colours, preservatives or flavourings used in food and drinks, 22% said they were very worried, 39% fairly worried, 27% not very worried and 11% not worried at all, a level of concern that was very similar to that expressed by the survey respondents about genetically modified products in food and drinks.

This chapter will consider the history of food additives and discuss the approaches used to test for toxicity of food additives and the way in which such data are used to evaluate safety in use. Examples of how emerging science has affected the regulatory status of some additives that were previously considered to be safe will also be discussed.

2 ORIGIN OF FOOD ADDITIVES

Chemicals have been used to preserve food and to influence the colour and taste of food for centuries. Methods of preservation, such as smoking, salting, pickling, fumigation with sulphur dioxide, and the use of honey and sugar, have long been utilized to extend the wholesomeness of foods gathered in season or in times of plenty, for later use. The use of saltpetre (potassium or sodium nitrate) to preserve meat and fish can be traced back to the Middle Ages. Similarly there is a long history of use of salt (sodium chloride) and vinegar (acetic acid) to preserve fruits and vegetables. When the modern era of processed food manufacture emerged in the twentieth century, the numbers of food additives increased, reaching over 400 by the beginning of the twenty-first century.

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Food additives include, not only chemicals that can be synthesized in the laboratory, but also substances that occur naturally, or are synthesized in the laboratory to be nature-identical. In the functional class of colours, for example, there are both synthetic and natural colours. Examples of synthetic colours include quinoline yellow, tartrazine and amaranth. A number of synthetic food colours are azo dyes and were originally (but no longer) derived from coal tar. The azo group (–N=N–) confers intense colouring properties, the hue varying depending on the other structural components of the molecule. Other food colours are derived from natural sources, such as plants, animals and micro-organisms, but are not necessarily from sources that are also used as human food. Examples of natural colours include, beet red from beetroot, lycopene from tomatoes or micro-organisms, anthocyanins from grape skins and other fruits and vegetables, cochineal from insects and chlorophyll from grass, lucerne or nettles.

Examples of other types of food additive that are derived from plant and animal sources are lecithins from soya beans or chicken eggs, which function as both antioxidants and emulsifiers, beeswax from honeycomb produced by bees, which acts as a glazing agent to impart surface shine and prevent food items sticking together, pectins derived from fruit, which act as gelling and thickening agents, and a number of gums and waxes, such as gum arabic, karaya gum and carnauba wax derived from plants, which have a variety of technological actions. Examples of additives derived solely from micro-organisms are gellan gum and xanthan gum. Additives such as ascorbic acid (vitamin C) and citric acid (from lemon juice) can be derived from natural sources, but nowadays may more often be made synthetically.

3 DEFINITIONS OF FOOD ADDITIVES

Various definitions of food additives can be found in advisory and legal texts. Working definitions differ somewhat by region or country, the principle differences being whether food additives are defined solely as those chemicals that are deliberately added to food to exert a technical function, whether certain categories of technical function are excluded from the definition (e.g. the exclusion of flavourings and enzymes under European Union (EU) legislation), and whether substances migrating from food contact materials are regarded as (indirect) food additives, as they under United States (US) legislation, but not under EU legislation.

The Food and Agricultural Organization of the United Nations (FAO) and the World Health Organization (WHO) have played a key role in evaluation of the safety of food additives. The first Joint FAO/WHO Conference on Food Additives proposed the following definition of food additives (FAO/WHO, 1956):

> *Non-nutritive substances added intentionally to food, generally in small quantities, to improve its appearance, flavour, texture, or storage properties.*

Later, this definition was expanded (CCFA, 1965):

> *The term 'food additive' means any substance, not being a food per se, the intended use of which results, or may reasonably be expected to result, directly or indirectly, in it or its by-products becoming a component of, or otherwise affecting the characteristics of a food. The term includes any substance intended for use in producing, manufacturing, packing, processing, preparing, treating, packaging, transporting, or holding food; and includes any source of radiation intended for any such use.*

In the European Community legislation, a food additive is defined as (European Commission, 1989):

> *Any substance not normally consumed as a food in itself and not normally used as a characteristic ingredient of food whether or not it has nutritive value, the intentional addition of which to food for a technological purpose in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food results, or may be reasonably expected to result, in it or its by-products becoming directly or indirectly a component of such foods.*

In the USA, the Code of Federal Regulations defines food additives as follows (FDA, 2008):

> *Food additives includes all substances not exempted by section 201(s) of the act, the intended use of which results or may reasonably be expected to result, directly or indirectly, either in their becoming a component of food or otherwise affecting the characteristics of food. A material used in the production of containers and packages is subject to the definition if it may reasonably be expected to become a component, or to affect the characteristics, directly or indirectly, of food packed in the container. 'Affecting the characteristics of food' does not include such physical effects, as protecting contents of packages, preserving shape, and preventing moisture loss. If there is no migration of a packaging component from the package to the food, it does not become a component of the food and thus is not a food additive. A substance that does not become a component of food, but that is used, for example, in*

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preparing an ingredient of the food to give a different flavour, texture, or other characteristic in the food, may be a food additive.

4 RANGE AND IDENTIFICATION OF FOOD ADDITIVES

4.1 Functional Classes

Food additives perform a variety of technical functions in food (see **Table 1**). They include additives with important functions, such as preservatives, which maintain food in a safe condition free from microbial and fungal contamination. Antioxidants similarly serve an important function to retard or prevent oxidative deterioration of nutritional ingredients such as fats and vitamins. Other additives are essential for some of the technical processes that are used to make processed foods; without them, it would not be possible to manufacture certain foods. They include, for example, emulsifiers that allow the mixing of fat and water in foods such as margarine, sauces and dressings and stabilizers that maintain the stability of such mixtures. Some additives are used to improve the keeping quality of the foods, for example, humectants that maintain moisture levels and softness in foods such as dried fruits and cakes. Other additives are used to affect appearance, taste or texture, such as colours, glazing agents, flavour enhancers, sweeteners and thickeners.

4.2 International Numbering System

Food additives are identified by their technical function or class name, as shown in **Table 1**, and by their individual name and a code number. Code numbering is based on the internationally agreed system adopted by the Codex Alimentarius Commission (CAC) in 1989 and since updated (CAC, 2007). Its purpose is to ensure that there is no confusion between additives with similar names. It also allows additives to be listed on food packaging labels by numbers rather than names so that less space is occupied. In the EU, permitted additives are numbered according to the international coding system, prefixed by the letter 'E', signifying that it has been approved as safe for food use in Europe and is included in positive lists in the relevant legal directives. Each food additive has a separate specification which lays down purity criteria for the additive (FAO, 2006). Labels on processed foods may list additives either by their international code number (or in the EU by the E number) and/or by their common name.

5 EVALUATION OF THE SAFETY IN USE OF FOOD ADDITIVES

5.1 Food Additive Evaluation at the International Level

Evaluation of the safety in use of food additives was initiated at an international level following a joint FAO/WHO Conference on Food Additives, held in Switzerland in 1955 (FAO/WHO, 1956). The conference recommended that an expert committee be convened to address the technical and administrative aspects of chemical additives and their safety in food. In 1956, the first meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) was held and has been convened regularly ever since. The JECFA serves as an independent scientific committee which performs risk assessments and provides advice to FAO, WHO and the member countries of both organizations, as well as to the CAC. While the CAC does not have the same force of law as an EU directive or national legislation, Codex limits are used as a reference point for countries that are looking at revising or creating legislation on food. The requests for scientific advice are, for the main part, channelled through the subsidiary bodies of the CAC in their work to develop international food standards and guidelines under the Joint FAO/WHO Food Standards Programme. The advice to the CAC on food additives is normally provided by the Codex Committee on Food Additives (CCFA), formerly known, between 1971 and 2006, as the Codex Committee on Food Additives and Contaminants (CCFAC) (IPCS, 2008).

5.2 The Principle of the Acceptable Daily Intake

The term 'acceptable daily intake' (ADI) for a food additive was first used by the JECFA. The development of the principles behind the ADI concept began at the first meeting of the JECFA in 1957 (FAO/WHO, 1957) and the first ADIs for additives were elaborated at its sixth meeting (FAO/WHO, 1962). The concept is based on the premise that organisms have some capacity to cope with chemical challenge without the development of adverse effects and only when that threshold capacity is exceeded will toxic effects be manifested. The purpose of the ADI was to act as an acceptable reference value, derived from the available toxicity database, which would be substantially below any level that could be harmful to consumers. It acts as a guide to enable regulatory authorities, where necessary, to set maximum limits for the concentrations of additives in foods and to compare

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Table 1 Functional categories of food additives according to the CAC (2007)

Functional classes (for labelling purposes)	Definition	Subclasses (technological functions)
1. Acid	Increases the acidity and/or imparts a sour taste to a food.	Acidifier
2. Acidity regulator	Alters or controls the acidity or alkalinity of a food.	Acid, alkali, base, buffer, buffering agent, pH adjusting agent
3. Anticaking agent	Reduces the tendency of particles of food to adhere to one another.	Anticaking agent, antistick agent, drying agent, dusting powder, release agent
4. Antifoaming agent	Prevents or reduces foaming.	Antifoaming agent
5. Antioxidant	Prolongs the shelf-life of foods by protecting against deterioration caused by oxidation, such as fat rancidity and colour changes.	Antioxidant, antioxidant synergist, sequestrant
6. Bulking agent	A substance, other than air or water, which contributes to the bulk of a food without contributing significantly to its available energy value.	Bulking agent, filler
7. Colour	Adds or restores colour in a food.	Colour
8. Colour retention agent	Stabilizes, retains or intensifies the colour of a food.	Colour fixative, colour stabilizer
9. Emulsifier	Forms or maintains a uniform mixture of two or more immiscible phases such surface as oil and water in a food.	Emulsifier, plasticizer, dispersing agent, surface active agent, surfactant, wetting agent
10. Emulsifying salt	Rearranges cheese proteins in the manufacture of processed cheese, in order to prevent fat separation.	Melding salt, sequestrant
11. Firming agent	Makes or keeps tissues of fruit or vegetables firm and crisp, or interacts with gelling agents to produce or strengthen a gel.	Firming agent
12. Flavour enhancer	Enhances the existing taste and/or odour of a food.	Flavour enhancer, flavour modifier, tenderizer
13. Flour treatment agent	A substance added to flour to improve its baking quality or colour.	Bleaching agent, dough improver, flour improver
14. Foaming agent	Makes it possible to form or maintain a uniform dispersion of a gaseous phase in a liquid or solid food.	Whipping agent, aerating agent
15. Gelling agent	Gives a food texture through formation of a gel.	Gelling agent
16. Glazing agent	A substance which, when applied to the external surface of a food, imparts a shiny appearance or provides a protective coating.	Coating, sealing agent, polish
17. Humectant	Prevents food from drying out by counteracting the effect of an wetting agent atmosphere having a low degree of humidity.	Moisture/water retention agent, wetting agent
18. Preservative	Prolongs the shelf-life of a food by protecting against deterioration caused by micro-organisms.	Antimicrobial preservative, antimycotic agent, bacteriophage control agent, chemosterilant/wine maturing agent, disinfection agent
19. Propellant	A gas, other than air, which expels a food from a container.	Propellant

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Table 1 (*continued*)

Functional classes (for labelling purposes)	Definition	Subclasses (technological functions)
20. Raising agent	A substance or combination of substances which liberate gas and thereby increase the volume of a dough.	Leavening agent, raising agent
21. Stabilizer	Makes it possible to maintain a uniform dispersion of two or more immiscible substances in a food.	Binder, firming agent moisture/water retention agent, foam stabilizer
22. Sweetener	A nonsugar substance which imparts a sweet taste to a food.	Sweetener, artificial sweetener, nutritive sweetener
23. Thickener	Increases the viscosity of a food.	Thickening agent, texturizer, bodying agent

assessments of actual exposure to food additives to their respective ADIs.

The ADI is defined as:

> *An estimate of the amount of a food additive, expressed on a body weight basis, that can be ingested daily over a lifetime without appreciable health risk*
>
> (WHO, 1987).

For the purposes of the definition, a standard body weight of 60 kg is assumed. ADIs are expressed in milligrams per kilogram of body weight, as a range from zero to an upper limit, which the JECFA considered to be the zone of acceptability of the substance. Expressing the ADI as a range was designed to encourage the lowest levels of use of an additive that would be technologically feasible.

From its use of the words 'estimate' and 'appreciable risk', it can be seen that the JECFA considered that absolute safety could not be guaranteed. However, by building a suitable margin of safety into the numerical value of the ADI, the committee aimed 'to allow for any species difference in susceptibility, the numerical differences between the test animals and the human population exposed to the hazard, the greater variety of complicating disease processes in the human population, the difficulty of estimating the human intake, and the possibility of synergistic action among food additives' (FAO/WHO, 1958). The derivation of a numerical ADI from the available toxicological studies on a food additive is described later in Section 5.5.

5.3 Current Methods of Safety Testing

5.3.1 Rationale

The purpose of toxicological testing of substances proposed as food additives is to determine whether the substance, when used in the manner and in the quantities proposed, would pose any appreciable risk to the health of consumers. Such testing should provide, not only information relevant to the average consumer, but also relevant to those population groups whose pattern of food consumption, physiological or health status may make them vulnerable, for example, young age, pregnancy, diabetes and so on. Testing of proposed food additives is designed to establish their general toxicological profile, to identify any target organs for toxicity, and to provide a basis for establishing an ADI.

In general, the safety of food additives has been more intensively studied than that of major food components, or natural and environmental contaminants of food (excluding pesticides and veterinary drug residues). Such studies have shown that most food additives have low inherent toxicity since they are designed to perform a technical function in food rather than to have an effect on biological organisms. Toxicity studies are usually carried out in animals or in *in vitro* models, serving as surrogates to be used in risk assessment for humans. Human studies are rarely required by regulatory authorities for approval of food additives, but studies at the preapproval stage are sometimes carried out in human volunteers, for example, to establish gastrointestinal tolerance to bulk sweeteners or to assess the effects of artificial sweeteners on biochemical parameters in humans with diabetes. Similarly, experimental and epidemiological studies may be conducted in humans after a food additive has been marketed.

5.3.2 Range of Recommended Toxicity Tests

Recommendations have been published for the types of toxicity studies that are usually necessary to establish the safety in use of a food additive (see, for example, WHO, 1987; FDA, 2000; SCF, 2001; Barlow *et al*., 2002). A comprehensive range of toxicity studies is usually carried out, including short-term/subchronic repeated-dose tests,

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long-term chronic toxicity/carcinogenicity tests, genotoxicity tests, reproduction and developmental toxicity tests. In addition to toxicity studies, toxicokinetic (TK) studies of absorption, distribution, metabolism and excretion (ADME) of the additive are also often conducted.

In some cases, a lesser range of toxicity studies may be sufficient to establish safety. Considerations for fewer toxicity studies for a proposed food additive might include one or several of the following:

- If it will be consumed in small amounts
- If it is a substance that is a normal component of the diet
- If it has a structure considered to be of low toxicological potential
- If there is likely to be negligible systemic absorption because of high molecular weight and resistance to breakdown in the gut
- If it is readily metabolized into known innocuous substances or into substances known to be endogenously present in the body.

The US Food and Drug Administration (FDA) has for many years recommended a tiered approach to testing, based on the assumption that the degree of effort expended to reduce uncertainty about the safety of an additive used in food should relate to the likelihood that the additive could pose a risk to human health. This likelihood is based two considerations, the chemical structure of the additive and the predicted daily dietary exposure to the additive. Under this tiered approach, an additive is assigned to one of three levels of concern, low (I), intermediate (II) or high (III), based on information on the toxicological potential of the additive predicted from its chemical structure and an estimate of cumulative human exposure (FDA, 1993) (see **Figure 1**). The minimum set of recommended toxicity tests is related to the Concern Level (I, II or III), to which the additive is assigned (FDA, 2006), as shown in **Table 2**.

In the EU, no fixed programme of testing is laid down, but a general framework describing core tests and other tests is recommended (SCF, 2001). Food additive petitioners are asked to submit the range of studies recommended as a core set and to consider whether any other types of study might also be appropriate. If a study of a type that might be expected is not submitted, then the scientific reasons for that should be provided. The reasons for carrying out any unusual studies should also be explained. The core set of toxicity tests is shown in **Table 3**.

Guidelines and protocols for the conduct of toxicity tests for regulatory submissions have been published by various international and national bodies. The majority of national regulatory systems for food additive approval will accept studies conducted according to the internationally accepted Organization for Economic Cooperation and Development (OECD) guidelines for the testing of chemical health effects (OECD, 2008). The FDA has its own study guidelines in the FDA Redbook (FDA, 2000). OECD and FDA guidelines differ on some points of detail (e.g. in the recommended numbers of animals/sex/dose to be treated), but generally follow closely similar lines on study designs, observations to be made and reporting of results. Studies conducted for regulatory submissions should normally be conducted according to the principles of Good Laboratory Practice (GLP) (OECD, 1998). However, for many food additives already on the market, some for as long as 50 years, toxicity testing may have been conducted at a time when GLP was not a standard requirement.

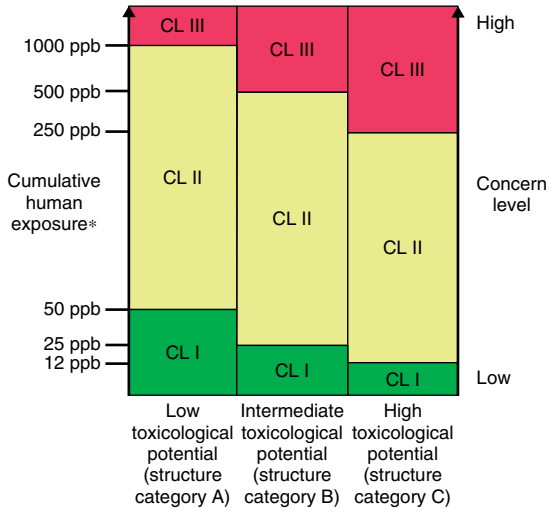

Figure 1 The additive is placed in one of three broad structural categories by comparison with the structures of known toxic substances: Category A is for low toxicological potential, Category B is for intermediate toxicological potential and Category C for high toxicological potential. Within each structure category (A, B and C), estimated human exposure will determine the initial Concern Level to which the additive is assigned. *Cumulative human exposure is expressed as parts per billion (ppb, equivalent to microgram per kg diet) of daily dietary consumption of additives. For conversion of ppb to microgram per kilogram body weight per day, divide by 20, assuming a daily diet of 3 kg. (Reproduced from the FDA, 2006.)

5.3.3 Routes of Administration of Test Substances

The most appropriate route of administration of test substances in laboratory animal studies designed to assess the safety of food additives is the oral route. Administration in the diet or the drinking water most closely simulates the usual conditions of human ingestion of additives in food and beverages. Additives with strong tastes, such as intense sweeteners, may give rise to palatability problems which can limit the maximum amount that may be

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Table 2 Summary of toxicity testing recommended by the US FDA for food additives

Toxicity tests	Concern levels		
	Low (I)	Intermediate (II)	High (III)
Genetic toxicity tests	X	X	X
Short-term toxicity studies with rodents	X[c]	X[a,c]	X[a,c]
Subchronic toxicity studies with rodents	—	X[c]	X[a,c]
Subchronic toxicity studies with nonrodents	—	X[c]	X[a,c]
One-year toxicity studies with nonrodents	—	—	X[c]
Chronic toxicity or combined chronic toxicity/carcinogenicity studies with rodents	—	—	X[c]
Carcinogenicity studies with rodents including *in utero* exposure phase	—	—	X
Reproduction studies	—	X[c]	X[c]
Developmental toxicity studies	—	X[b,c]	X[b,c]
Metabolism and pharmacokinetic studies	—	X[b]	X[b]
Human studies including epidemiology studies	—	—	X[b]

[a]If needed as preliminary to further study.
[b]If indicated by available data or information.
[c]Including screens for neurotoxicity and immunotoxicity.
Adapted from FDA, 2006.

Table 3 Core set of toxicity tests recommended by the EU (SCF, 2001)

Toxicity test	Type/species
Metabolism/toxicokinetics studies	Single and repeat-dose, usually in rat
Subchronic toxicity studies	90 day studies in a rodent and a nonrodent
Genotoxicity studies	*In vitro* studies. Positive results *in vitro* should be followed up with *in vivo* studies
Chronic toxicity and carcinogenicity studies	Usually rat (24 months) and mouse (18 or 24 months)
Reproductive toxicity study	Usually rat, two-generation, one litter per generation
Developmental toxicity studies	Rodent and nonrodent, usually rat and rabbit

administered via the diet or drinking water. The solubility of the substance may also limit the amounts that can be administered via drinking water. In the case of reduced palatability of the diet, pair-fed controls can be incorporated into study designs to enable any effects of reduced food intake to be separated from those of any induced toxicity.

Administration by oral gavage is also used to overcome palatability problems and can enable higher amounts of test substance to be administered (e.g. it is often favoured for developmental toxicity studies). Oral gavage may also be an appropriate route for administration of additives that may be consumed in a short timeframe, such as an additive for use in soft drinks, or for additives that are consumed in a single daily ingestion, such as an additive proposed for use in food supplements taken in the form of tablets or capsules. When substances are administered by oral gavage, as a so-called 'bolus' dose, peak concentrations reached in blood may considerably exceed the concentrations that would be reached if the substance were normally ingested via the diet. At high doses, gavage administration may also overwhelm the metabolic capacity to detoxify a substance. These aspects need to be taken into consideration in the interpretation of the likely risks for human health of any observed toxicity.

For a few food additives, such as propellant gases, toxicity studies may only be available using the inhalational route. These studies can be used in risk assessment, provided any necessary adjustments are made for route-to-route extrapolation (see, for example, IGHRC, 2006).

In subchronic and chronic toxicity studies, the test substance is usually given via the diet for practical reasons. The majority of food additives are substances with relatively low molecular weights and thus suitable for testing using protocols such as those developed by OECD for low-molecular-weight substances. They are also usually added to foods in relatively low amounts. However, some types of additive, such as modified starches or celluloses, are high-molecular-weight substances and may be added to foods in higher amounts, for example, as bulking agents. In tests on laboratory animals, it may not be possible to administer these via the diet in high amounts on a milligram per kilogram body-weight basis, because their inclusion may

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impact on the nutritional value of the diet. It is generally recommended that the concentration of a test substance that is not a nutrient should not exceed 5% of the diet. This may mean that the highest dose tested may not be much above the likely human intake, expressed on a body-weight basis.

Monitoring of food (and/or drinking water) consumption at regular intervals is an important parameter in all repeat-dose studies on food additives, both as an indicator of palatability or toxicity problems and in order to generate an accurate measure of the amount of the additive that has been ingested on a daily basis.

5.3.4 ADME Studies

ADME studies can give useful information about a proposed food additive, including whether it is broken down in the gastrointestinal tract, the proportion of the administered parent substance (and/or of its gut metabolites) that is absorbed, its metabolic fate following systemic absorption (e.g. whether it may be metabolized by the liver to innocuous products), and how quickly it is cleared from the body by excretion in urine and faeces, and/or expiration via the lungs. ADME studies can thus help, not only in the selection of appropriate doses for subsequent toxicity studies, but also for the interpretation of results of the toxicity studies and for human-risk assessment.

Besides providing information on gastrointestinal absorption and overall elimination rates, they can also reveal any changes in the kinetic behaviour of the substance with repeated administration (e.g. more rapid elimination due to enzyme induction, or increasing body burdens due to accumulation in tissues such as fat). If comparative ADME studies are available, particularly from the laboratory testing species and from humans, they can help in the evaluation of any species differences. They may also provide information on other aspects relevant to toxicity, such as placental transfer, induction or inhibition of metabolizing enzyme systems and reversible or irreversible binding to tissue sites and carrier proteins. *In vitro* studies, employing enzymes, sub-cellular organelles, cell cultures and perfused organs, can also contribute useful information in the investigation of metabolic pathways, mechanisms of toxicity and effects on enzymes.

5.3.5 Acute Toxicity Tests

Acute toxicity tests, utilizing single-dose administration and generally followed by up to 14 days of observation, are considered not essential for the evaluation of the safety in use of food additives. The reason for this is because such tests generally use high doses that are irrelevant to consumer exposure to food additives, which is usually characterized by much lower exposures and ingestion over long periods of time. However, acute studies, if available for other purposes, can aid in the design of subsequent repeated-dose toxicity studies, by providing information on target organs for toxicity that should be particularly scrutinized and information on dose selection for subsequent studies.

The FDA previously only recommended acute oral toxicity studies if they were considered necessary as preliminary tests to further studies (FDA, 1993; 2000). Since 2006, the FDA no longer recommends acute oral toxicity studies for food additives (FDA, 2006). The guidance on food additive submissions for the EU (SCF, 2001) states that acute toxicity studies are not required for evaluation of the safety of food additives, but notes that they may have been conducted for other purposes, such as worker safety, and if such studies are available, they should be submitted, as they may provide useful background information.

5.3.6 Short-Term Toxicity Tests

Short-term toxicity tests are repeat-dose studies generally not exceeding two to four weeks in duration. They are recommended by the FDA for all additives of low concern (FDA, 2006). Such tests, together with genotoxicity tests, may be all that is needed to establish safety in use at Concern Level I (see **Table 2**). At Concern Levels II and III, the FDA only recommends short-term tests if they are needed as a preliminary to further studies.

The EU guidelines do not recommend the use of any short-term, repeat-dose toxicity tests, advising that they are not normally sufficient, by themselves, for evaluation of potential subchronic toxicity. The EU does, however, request that any short-term range-finding studies that have been conducted should be submitted.

5.3.7 Subchronic Toxicity Tests

Subchronic toxicity tests are repeat-dose tests in which the period of administration is around 10% of the animal's normal lifespan. The major objective of subchronic toxicity studies is to determine the general toxicological profile of the test substance following repeated administration. Such studies provide information on the target organs/tissues for toxicity, on the nature and severity of any effects, and on dose–response relationships. They normally include investigations of haematology and clinical chemistry of blood and urine, as well as histopathology of a comprehensive set of tissues. Such studies should allow determination, not only of any adverse effects, but also of the dose at which adverse effects are no longer observed, that is, the no-observed-adverse-effect level (NOAEL). If subchronic studies are conducted according to current protocols (e.g. OECD), they will include parameters that provide an apical screen for neurotoxicity, immunotoxicity and some aspects of

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reproductive toxicity. Thus they may provide triggers for additional studies on these effects. Subchronic studies may also be helpful for estimating appropriate dose levels for the conduct of chronic toxicity studies.

The FDA recommends subchronic tests in a rodent and a nonrodent for all additives assigned to Concern Level II. For additives assigned to Concern Level III, they are recommended if they are needed as a preliminary to further studies of longer duration (FDA, 2006). EU guidelines recommend that any new additive should normally be tested in subchronic oral toxicity studies in two laboratory species, usually a rodent and a nonrodent, such as a rat and a dog, for a period of at least 90 days, but such studies may be waived if there are longer duration studies available on the same species, with full recording of haematological, clinical chemical and urinalysis parameters, alongside the usual histopathological examinations.

5.3.8 One-Year Toxicity Studies

A one-year toxicity study in a non-rodent and a study in a rodent of a minimum of 12 months are recommended by the FDA for any additive assigned to Concern Level III (FDA, 2006). Such studies are not recommended in EU guidelines.

5.3.9 Chronic Toxicity and Carcinogenicity Studies

Carcinogenicity studies in two species (rat and mouse) are recommended by the FDA for any additive assigned to Concern Level III (FDA, 2006). They further recommend that an *in utero* exposure phase be included in the rat study. The FDA may accept data from a study using a combined chronic toxicity/carcinogenicity protocol, but point out that, because of the *in utero* dosing phase, there are problems in administering appropriate dose levels when the two types of study are combined. Studies in transgenic mice may be accepted as supplemental data, but are not considered by the FDA to be substitutes for two-year carcinogenicity bioassays.

For the EU, chronic toxicity and carcinogenicity studies (using separate or combined protocols) in two species are recommended, with no specific recommendation to include an *in utero* dosing phase. The recommended species (and durations) are rat (24 months) and mouse (18 or 24 months).

5.3.10 Genotoxicity

Genotoxicity studies are recommended by the FDA for all substances proposed as food additives, irrespective of the Concern Level (I, II or III), unless the cumulative dietary exposure is below $1.5\,\mu g\,day^{-1}$, corresponding to a concentration of 0.5 ppb in the daily diet. The recommended battery of tests is: (i) a test for gene mutations in bacteria, (ii) either an *in vitro* test for cytogenetic damage in mammalian cells, or an *in vitro* mouse lymphoma *tk* gene mutation assay and (iii) and *in vivo* test for chromosome damage in mammalian haematopoietic cells. If exposure is below a concentration of 50 ppb in the daily diet, only (i) and (ii) are recommended.

For the EU, any new additive should normally be tested for genotoxicity in order to assess its mutagenic and carcinogenic potential. The recommended battery of tests is: (i) a test for gene mutations in bacteria, (ii) an *in vitro* test for gene mutations in mammalian cells (preferably the mouse lymphoma *tk* assay) and (iii) an *in vitro* test for chromosomal aberrations in mammalian cells. Positive results in any of these *in vitro* tests will normally require further testing for genotoxicity *in vivo*.

5.3.11 Reproductive and Developmental Toxicity Studies

The FDA recommends reproduction and developmental toxicity studies for all Concern Level II and III substances. The recommended reproduction study is a two-generation study, one litter per generation, in which animals in the second (F2) generation are sacrificed at weaning. The study guideline contains optional procedures for including additional litters, additional generations and a test for teratology and developmental toxicity. Developmental toxicity studies are also required on two species, the rat and the rabbit, unless there is information to indicate a different species may be more relevant. They can be conducted either as stand-alone studies or as part of the rat two-generation study.

For the EU, a new additive should normally be tested in reproduction and developmental toxicity studies. A multigeneration reproduction study should be conducted in one laboratory species, usually rat, and comprise at least two generations and one litter per generation. It should include assessment of end points relevant to endocrine disrupter potential. Developmental toxicity studies should be conducted in two laboratory species, usually a rodent and a nonrodent, such as rat or mouse and rabbit. Administration of the test substance should cover, not only the period of embryogenesis, but continue to the end of gestation in order to ensure detection of, for example, endocrine disrupter potential. In order to ensure that a new additive does not affect postnatal development and function, including neurological function and behaviour, the physical, functional and behavioural development of animals exposed from at least the beginning of embryogenesis through to weaning should be studied. This can be done as a separate study or as part of a multigeneration and/or developmental toxicity study.

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5.4 Interpretation of ADME and Toxicity Tests

Extensive guidance on the interpretation of toxicity tests on food additives in order to characterize any risk have been published elsewhere (WHO, 1987; Moch *et al*., 1997; Renwick *et al*., 2003). Some key considerations are outlined below.

If ADME studies indicate that a proposed food additive shows evidence of accumulation in the body (for example if it has a long half-life) then it may be considered unacceptable as a food additive or it may be advised that its use should be severely restricted. One such example is the food colour canthaxanthin, which has been shown to accumulate in the eye in humans (SCF, 1989; WHO, 1996). This discovery came, not from its use as a food additive, but from the marketing of canthaxanthin as an orally ingested cosmetic for 'bronzing' the skin. Consequently, it has a very restricted use as a food additive in the EU (permitted only in one type of sausage), but is also permitted as a feed additive for chickens to colour egg yolks and for farmed fish such as salmon and trout.

Whilst the toxicity of food additives is generally low, the effects most commonly observed are effects on growth and body weight, which may often provide the earliest indication of toxicity, or effects on the liver and kidney which, being the major organs of metabolism and elimination, are generally the tissues exposed to the highest concentrations of an additive. Deciding which treatment-related effects are adverse, which are probably not, or which effects are not relevant for human-risk assessment, is a matter for expert judgement and is done on a case-by-case basis, taking into account all the available information. For example, certain hydrocarbon-based compounds induce kidney damage, eventually resulting in tumours in male rats via the accumulation of α-2-microglobulin protein droplets, an effect which is unique to the male rat kidney. Thus if kidney damage can be shown to be due to this mechanism it is not regarded as relevant for human risk assessment (IARC, 1998). However, in the absence of clear knowledge or information to demonstrate that an effect is not adverse or is not relevant for humans, the default assumption for food additives is that it should be taken into account in the risk assessment.

In tests on food additives, the following effects are not uncommon and are usually regarded as toxicologically insignificant, minor or non-dose-related: changes in biochemical parameters without any other indications of toxicity and without evidence of more severe damage to the indicated organ at higher doses; changes in the gut due to administration of high levels of nondigestible substances; adaptive changes, such as liver hypertrophy with enzyme induction due to metabolic overload; staining of tissues with food colours. Occasionally, however, such effects may have human-health implications, as is the case with bulk sweeteners which are not absorbed and can have laxative effects on the gut, sometimes experienced by high consumers.

There are two particular types of toxicological effect which are of special significance in safety evaluation of food additives. These are carcinogenicity and mutagenicity. If a substance is shown to possess genotoxic potential which is expressed *in vivo*, then it will not be approved as a food additive since such effects may be without a threshold and some degree of risk could occur at any level of exposure. If a substance is shown to be a carcinogen in a long-term test then it may not be approved unless it can be clearly demonstrated that the induction of tumours is by a nongenotoxic mechanism, with a threshold at a dose well above the highest likely level of human exposure.

An example of an approved food additive which has a carcinogenic action by a thresholded mechanism is the artificial sweetener saccharin. It causes tumours in the male rat bladder, due to the particular conditions in the bladder of the male rats of high concentrations of sodium ions and elevated urinary pH, which encourages tissue irritation and crystal formation (SCF, 1997). The relevance of such tumours for humans has been questioned, and indeed the JECFA did not base its ADI on that effect (WHO, 1993), although the European Commission's Scientific Committee for Food (SCF) did take it into account (SCF, 1997). Another example is the antioxidant, butylated hydroxyanisole (BHA), which causes forestomach tumours in rodents triggered by early onset hyperplasia and hyperkeratosis. In rodents, food is held for some time in the forestomach, but in species such as humans and other primates there is no forestomach and food passes relatively quickly through the stomach. There is also an indication that at very high doses it causes slight hyperplasia in the oesophagus of animals without a forestomach, such as monkey and pig (Barlow, 1990). BHA is not genotoxic and, although the relevance of forestomach tumours for humans is questionable, an oral dose has been established in rodents at which there is no increase in cell proliferation, as measured by cellular uptake of radiolabelled thymidine. The dose at which no effect was seen on cell proliferation was used to establish the ADI for BHA (WHO, 1989).

Other toxic effects, including teratogenicity, also have demonstrable thresholds, that is, for a given substance there will always be a dose below which no effects occur. Thus, in the absence of genotoxic effects, the various toxicological studies may be assessed to determine an overall no-effect level. The no-effect level is usually termed the 'no-observed-adverse-effect level'. Sometimes the term 'no-observed-effect level' (NOEL) is used to denote that there is an effect observed at a higher dose, but it is not considered adverse, such as induction of a liver enzyme that is indicative of an adaptive effect.

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5.5 Derivation of the ADI

Any adverse effects observed in the range of toxicity tests conducted are identified, together with the doses at which they occur (dose–response assessment). All effects that are regarded as adverse and potentially relevant to humans should be considered. When results from two or more laboratory animal species are available, the ADI is based on the most sensitive species, that is, the species that displayed a toxic effect at the lowest dose, unless pharmacokinetic, metabolic or mechanistic data are available to establish that the most sensitive species is not appropriate for human risk assessment. The toxic effect seen to occur at the lowest dose across the range of studies is generally taken to be the critical toxicological effect. Provided the dose at which it occurs does not coincide with the lowest dose utilized in the particular toxicity test under consideration, an overall NOAEL for the additive can be identified.

Long-term toxicity tests, in which the additive is administered for most of the lifespan of the animal, or multigeneration studies generally (though not always) yield the most sensitive effect at the lowest dose in all the tests conducted. Thus the majority of ADIs for food additives are set on the basis of these repeat-dose studies. This is due to the long periods of administration, which often drive the effect and no-effect levels downwards compared with shorter durations of exposure, and the fact that such studies cover a number of critical periods in the lifetime (e.g. pregnancy, neonatal and juvenile periods, ageing).

If there is a comprehensive database, the overall NOAEL is used to derive an ADI. The NOAEL, in milligram per kilogram body weight per day, is divided by a suitable safety factor (see below) to calculate an upper limit (X) for the ADI, which is then expressed as a range ($0–X$ mg (kg body weight)$^{-1}$). Risk managers can then compare estimates of intake of the additive with the ADI to ensure intake is below the ADI for all exposed population groups.

It should be noted that whilst the ADI is generally considered to be applicable to children, it is not applicable to infants under the age of 12 weeks (Walker, 1998). This is because the tissues and organs of young infants may be more biologically sensitive to a chemical on a body weight basis than older children or adults, and because of physiological and metabolic immaturity (Scheuplein *et al*., 2002). Moreover, the usual range of toxicity studies do not adequately mimic the special situation of a baby receiving infant formula (in which some additives are present) as its only source of food in the first weeks of life.

To determine the ADI, a default safety factor (sometimes termed 'uncertainty factor') of 100 is usually applied to the overall NOAEL to provide a conservative margin of safety that takes account of the inherent uncertainties in extrapolating from animal toxicity data to potential effects in humans and variation within the human species. The use of a default factor of 100 is arbitrary, but was first proposed in 1954 by two officials working in the FDA (Lehman and Fitzhugh, 1954). The same default factor of 100 (10 for interspecies differences multiplied by 10 for intraspecies variation) was adopted by the JECFA soon after, which considered it would provide an adequate margin of safety for the consumer, assuming that humans might be up to 10 times more sensitive than the most sensitive laboratory test species and that there might be an additional 10-fold difference in sensitivity range among the human population (WHO, 1987).

Subsequently, studies have been done to investigate whether the use of these arbitrary 10-fold safety factors can be underpinned by supporting scientific data (Renwick and Lazarus, 1998; Renwick, 1998; Walton *et al*., 2001a; 2001b; Dorne *et al*., 2001a; 2001b; 2004; 2005; Dourson *et al*., 2002; Dorne and Renwick, 2005; Dorne, 2007). Renwick (1993) first proposed the use of data-derived safety factors in a scheme, based on comparative information from studies on animals and humans, which allows subdivision of each of the 10-fold components of the 100-fold default safety or uncertainty factor into separate factors for toxicokinetics (TK—fate of the chemical in the body) and toxicodynamics (TD—actions of the chemical in the body). The usual 10-fold factor for interspecies differences is subdivided into a factor of 2.5 for TD and a factor of 4.0 for TK ($2.4 \times 4.0 = 10$), reflecting the fact that there is a greater potential for TK differences than for TD differences between humans and test species. For intraspecies differences, the potential for variability between humans for TK and TD is considered to be similar, and so values of 3.2 are used for both ($3.2 \times 3.2 = 10$). Where appropriate TK or TD data are available for a particular chemical from humans and/or test species, the subdivisions of the default uncertainty values can be replaced with 'correction' factors based on actual data, and these are termed chemical-specific adjustment factors (CSAFs) (WHO, 1994; 2001).

Similarly, if, for example, the critical study for an additive is one involving human subjects, then a reduced default safety factor of perhaps only 10 may be applied, since interspecies differences are no longer relevant. Such is the case, for example, with the food colour erythrosine. This affects the human and rat thyroid, ultimately causing tumours in the rat due to excessive production of thyroid stimulating hormone (TSH). Its mechanism of action is well understood and an NOAEL for increases in thyroid hormone levels in humans has been established and used, in conjunction with a 10-fold rather than a 100-fold safety factor to set the ADI (SCF, 1989).

If the toxicology database is considered complete, then a full ADI will be assigned. Occasionally 'temporary' ADIs may be set when it is reasonable to conclude from the available data that it is safe to consume the

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additive for the short period of time required to generate some further data which are necessary to complete the safety evaluation. A previously assigned ADI may also be changed to a temporary ADI when new data emerge that appear to call into question the safety of the additive or the assigned value of the ADI. When setting a temporary ADI, a higher safety factor is often used. If new data are provided that show the additive is no longer acceptable for use in food, then the ADI is withdrawn (see Section 5.6 for some examples).

A numerical ADI can be established solely on the basis of toxicological information, without the need for data on intended or actual use(s) or for estimates of dietary exposure. However, in setting ADIs, it may be necessary to know whether particular subpopulations are exposed, since the ADI should be applicable to the whole population. Therefore, general information about exposure patterns should be known at the time of the safety assessment. For example, if a food additive is to be used in foods for infants and young children, then estimates of their daily exposure are essential as they may well be higher than that of adults on a body-weight basis, not because of a higher absolute intake in milligrams per day, but because of their much lower body weights (Ostergaard and Knudsen, 1999).

Occasionally, the toxicity database may not indicate a clear NOAEL in cases where an effect is still seen at the lowest dose tested. If this is the critical effect for the whole database, then a decision has to be made as to whether a change observed can be considered as minor (low incidence, low severity). If so, the dose at which it occurs is called a lowest-observed-adverse-effect level (LOAEL). An LOAEL may be used to derive an ADI, usually incorporating an additional safety factor of 2–3, to allow for the fact that it is not a NOAEL.

If the database cannot be used to derive a numerical ADI, for example, if there is no toxicity observed, even at the highest doses tested, or it is not considered necessary to conduct a full range of toxicity tests (see Section (5.3.2) for circumstances where this may be the case), then the ADI may be expressed as an ADI 'not specified', an approach that has been used by the JECFA. The JECFA defines this term to mean that, on the basis of available data (chemical, biochemical, toxicological and other), the total daily intake of the substance, arising from its use at the levels necessary to achieve the desired effect and from its acceptable background in food, does not, in the opinion of the Committee, represent a hazard to health (WHO, 1987). Alternatively, if a numerical ADI cannot be established, but use of the proposed additive is considered acceptable, then the overall NOAEL for the additive can be compared with the estimates of intake to see whether there is a sufficient margin of safety. This latter approach has been used in the past by the European Commission's Scientific Committee on Food (SCF) and is used now by the European Food Safety Authority (EFSA), when a numerical ADI cannot be derived.

5.6 Removal of Additives with Safety Problems from Approved Lists

Occasionally, new data emerge some time after the approval of a food additive that cast doubt on its safety. When the occurrence of a newly discovered adverse effect is relatively clear, there are two options, either to lower the ADI or, in cases where the continued use of the additive is considered unacceptable, to withdraw the ADI. If lists of approved food additives are consulted according to the international numbering system (CAC, 1989) or European E numbers (FSA, 2007), it is apparent that some numbers in the sequential numbering system are missing. This is because the additives have either been withdrawn by industry or have been removed from the approved list by the regulatory authorities because the ADI has been withdrawn or, in cases where an ADI has not been set, they are considered unacceptable for use.

Some colours, such as butter yellow (toxic to the liver) and Sudan 1 (toxic and later found to be carcinogenic) were banned for food use almost 100 years ago. A number of other colours were banned for food use in the 1950–1970s when they were found to be toxic. Examples include certain colours such as (E) 103, 105 107 and 111 (Alkanet, Fast Yellow AB, Yellow 2G and Orange GGN, respectively), that were used in food many years ago, but have now been withdrawn or banned for such use. In some European countries that are or were outside the EU, many of the azo-containing colours are or were banned.

In the EU, the food colour, Red 2G has been removed from the approved list (European Commission, 2007), following withdrawal of its ADI by the European Food Safety Authority (EFSA, 2007). The information that prompted the withdrawal of the ADI was that Red 2G had been shown to be metabolized into aniline, which is regarded as an animal carcinogen and for which a genotoxic mechanism of action cannot be excluded. EFSA therefore concluded that Red 2G should be regarded as being of safety concern and withdrew the previously established ADI of 0–0.1 mg (kg body weight)$^{-1}$. Up to then, its uses in the EU had been restricted to breakfast sausages and hamburger meat with minimum cereal contents of 6 and 4%, respectively (European Commission, 2007). At the time it was first approved for use in the EU (SCF, 1975), it was not known that it could be extensively metabolized to aniline and it is only relatively recently that a consensus view of the carcinogenicity of aniline has been reached in the EU (EU, 2004).

Another example concerns one of the parabens, propyl paraben. Parabens are antimicrobial preservatives allowed for use in foods, drugs, cosmetics and toiletries. The European Commission's former SCF evaluated the parabens in 1994 and allocated a temporary group

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ADI of 0–10 mg (kg body weight)$^{-1}$, for the sum of methyl, ethyl and propyl *p*-hydroxybenzoic acid esters and their sodium salts (SCF, 1996). Following a request from the European Commission, the EFSA Scientific Panel dealing with food additives re-evaluated the toxicology of these substances, including findings from recent studies and the safety of their usage in foods. These findings included the demonstration that several of the parabens had oestrogenic activity *in vitro* and that propyl paraben affected the male reproductive system *in vivo*. The Panel concluded that a group ADI of 0–10 mg (kg body weight)$^{-1}$ could be established for the methyl and ethyl parabens and their sodium salts. However, the Panel considered that propyl paraben should not be included in this group ADI because propyl paraben, unlike the methyl and ethyl forms, showed adverse effects on sperm production at a relatively low dose in juvenile male rats. The Panel was unable to recommend an ADI for propyl paraben because of the lack of a clear NOAEL for this effect (EFSA, 2004).

5.7 Food Colours and Hyperactivity

The question of whether consumption of food additives may be associated with behavioural effects, especially in children, has been a subject of research and debate for many years. Feingold (1975) was the first to suggest that exposure to synthetic food colours and other food additives may be linked to hyperactivity in children. Since then there have been more than 20 human studies that have addressed the 'Feingold hypothesis'. The majority of these have been elimination trials conducted in children considered to be hyperactive or having a diagnosis of attention deficit hyperactivity disorder (ADHD). ADHD is characterized by increased activity, impulsive and inattentive behaviour and it can impact adversely on social interaction and learning. Two more recent studies, both conducted by a research group at the University of Southampton, UK, have differed from previous studies in that the subjects were children selected from the general population and excluded children on medication for ADHD (Bateman *et al*., 2004; McCann *et al*., 2007).

Many of the earlier studies have been reviewed in two meta-analyses (Kavale and Forness, 1983; Schab and Trinh, 2004) and discussed in an opinion of the European Food Safety Authority (EFSA, 2008). Early studies investigated the effects on behaviour of administering a 'Feingold diet' (a diet without synthetic colours and flavours) under double-blind conditions to relatively small groups of hyperactive children. In some studies children identified as possible responders were challenged with food additives in double-blind studies. Several authors reported improved behavioural characteristics (or deterioration on challenge with additives) in some, but not all, children in their study population, while other studies did not provide support for the Feingold hypothesis. The study subjects were generally hyperactive children or children considered to be responsive to food additives, often only small numbers of subjects were studied and parent or teacher ratings of behaviour were used. Mostly, the studies were not designed to address dose–response dependency and did not link the adverse behavioural effect to a specific food additive. The most recent meta-analysis of 15 double-blind, placebo-controlled trials that met the authors' inclusion criteria concluded that, despite the limitations of many of the studies, the results strongly suggested an association between ingestion of artificial food colours and hyperactivity (Schab and Trinh, 2004).

The two more recent UK studies used much larger numbers of subjects than earlier studies (nearly 300 in each study) and administered mixtures of food colours (carmoisine, ponceau 4 R, sunset yellow, tartrazine) and a preservative (sodium benzoate) in drinks in a double-blind, placebo-controlled, crossover design. In the first study on three-year-old children, the authors concluded that there was a general adverse effect of the mixture of food colours and preservative on the behaviour of the children, which was detectable by the parents, but not by clinic assessment (Bateman *et al*., 2004). A detailed review of the study results by a UK government advisory committee, the Committee on Toxicity (COT), noted that the reported effects were small and there were limitations in the study design, concluding that it was not possible to reach firm conclusions about the clinical significance of the observed effects (COT, 2002a). In the subsequent UK study, in which sodium benzoate and the same four colours plus allura red AC were given as two different mixtures to both three- and eight- to nine-year-old children, the authors concluded that artificial colours plus the preservative resulted in increased hyperactivity in both age groups (McCann *et al*., 2007). Behaviour was scored separately by parents, teachers and independent classroom observers. This study has also been reviewed in detail by the COT, which concluded that the study provided supporting evidence suggesting that certain mixtures of artificial food colours, together with the preservative sodium benzoate are associated with an increase in hyperactivity in children from the general population, but noted that the increases in mean levels of hyperactivity observed were small relative to normal interindividual variation, and that changes in behaviour were not evident in all children in any one group and were not consistent across age groups or across the different mixtures used in the study (COT, 2007). The EFSA similarly concluded that this second study provided limited evidence that the two different mixtures of synthetic colours and sodium benzoate tested had a small and statistically significant effect on activity and

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attention in children selected from the general population, although the effects were not statistically significant for the two mixtures in both age groups and that the clinical significance of the observed effects remains unclear (EFSA, 2008). EFSA also noted that it was largely the parental scores that accounted for the statistical significance of the findings and concluded that, in the context of the overall weight of evidence and in view of the considerable uncertainties in the study, the findings could not be used as a basis for altering the ADI of the tested food colours or sodium benzoate. Subsequently, EU legislators, following the recommendations of the European Parliament, have decided to include warnings in the labelling of products containing any of the five colours tested to the effect that the colours may have an adverse effect on activity and attention in children.

It is interesting to note that none of the studies conducted since the Feingold hypothesis was first proposed have yet provided answers to key questions, some of which were first raised in the 1970s (Lipton *et al*., 1979), concerning the duration and clinical significance of the observed effects, whether the effects can be confirmed by objective laboratory tests, whether specific subpopulations may be affected and whether specific colours are involved. Whatever the role of colours, specialists in the field of ADHD have pointed out that it is a complex, multifactorial condition and that other important risk factors for development of ADHD include genetic factors, maternal smoking during pregnancy, premature birth and institutionalized upbringing (COT, 2007).

5.8 Safety of Additives in Combination

Concern is sometimes expressed about the possible synergistic effects of consuming foods containing several additives, often referred to as the so-called 'cocktail effect'. The testing of all possible combinations of additives that can occur in food for risk characterization is, in practice, impossible, given the large numbers of different combinations that may be present in foods. However, the issue of combined action is amenable to generic research approaches.

In recent years there have been major advances in understanding mechanisms of combination toxicology and a significant theoretical and experimental database has been developed (Ito *et al*., 1995; Jonker *et al*., 1996; 2004; Groten *et al*., 2000; Feron and Groten, 2002; Feron *et al*., 2002). Dose-addition occurs when substances produce toxicity via the same mechanism of action, whereas response-addition occurs when the substances act via different mechanisms. Synergism and antagonism may arise from either TK or TD reasons, but usually requires that each substance is present at active doses or concentrations.

One of the major lessons learned from this research is that exposure to mixtures of chemicals at levels that are nontoxic for each individual chemical generally will not result in a health risk. An important exception to this is when dose addition occurs for a mixture of chemicals with a common mode of action because the summing of a number of essentially inactive doses can give sufficient activity to produce an effect (see, for example COT, 2002b; DVFA, 2003; Kortenkamp, 2007; 2008). Although synergistic interactions can never be fully excluded on theoretical grounds, they are likely to occur only when there is a biologically active dose level of at least one of the chemicals involved.

On food additives specifically, the possibility that structurally unrelated food additives could show either joint actions or interactions has been assessed based on their potential to share common sites and mechanisms of action or common pathways of elimination (Groten *et al*., 2000). All food additives approved in the EU and allocated a numerical ADI were studied, using the data contained in the relevant JECFA reports. In all but a very few cases, the possibility of joint actions or interactions could be excluded on scientific grounds. The exceptions were on the liver (curcumin, thiabendazole, propyl gallate and BHT), the kidney (diphenyl, *o*-phenylphenol and ferrocyanide salts), the blood (azorubine and propyl gallate) and the thyroid (erythrosine, thiabendazole and nitrate). The authors concluded that many of those additives which could not be excluded from showing joint actions or interactions would have low intakes and that joint actions or interactions between additives do not represent a significant health concern.

There are examples in the food additives area of structurally or metabolically related compounds which display similar toxic effects, and for these group ADIs are established that include all members of the group of compounds, in order to limit their cumulative intake. For this procedure to be feasible, each of the substances must of similar toxic potency so that summation of the intakes of the different substances does not have to include a correction for potency. In cases where different substances are converted essentially quantitatively to a common the metabolite, the Group ADI may be expressed on the basis of the common metabolite; a recent example is the ADI for the sweetener steviol and related glycosides, which is expressed as steviol equivalents (WHO, 2005).

The use of a group ADI is also appropriate for compounds that cause additive physiological or toxic effects, even if they are not closely related chemically. For example, it may be appropriate to establish a group ADI for additives such as bulk sweeteners that are poorly absorbed and cause laxation by a common osmotic

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mechanism, to ensure that cumulative intake does not exceed the human threshold for undesirable laxation effects.

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Safety Assessment of Foods Obtained from Crops Developed Using Biotechnology

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CONTENTS

1 INTRODUCTION

1.1 What Are Foods Obtained from GE Crops?

This chapter will describe the methods used to assess the safety of foods obtained from genetically engineered (GE) crops; therefore it is appropriate that the chapter begin by defining just what is meant when these terms are used. For the purposes of this chapter, 'foods obtained from GE crops' refers to products obtained from field crops that are or could routinely be consumed as part of the human or livestock diet. For example, whole maize grain and soya beans may not be considered staples of the human diet, however, corn and soya beans currently constitute the most widely cultivated GE crops. Accordingly, there is a substantial body of literature that has assessed the safety and nutritional quality of milled maize grain from GE corn and processed feed fractions obtained from GE soya beans (e.g. soya-bean meal). Within the context of the current chapter, these substances would be considered as foods obtained from GE crops.

1.2 What is Biotechnology?

Within the context of agriculture, the term biotechnology refers to the modification of field crops for desired traits using methods other than traditional breeding. Biotechnology has also been described as genetic modification or genetic engineering. The process of developing a field crop using biotechnology begins with identification of the desired trait. For the purposes of this chapter, the word 'trait' refers to the genetic factors responsible for a defined biochemical or agronomic property that is not otherwise present in the target crop, though it could also refer to crops developed by selectively increasing or decreasing the expression of endogenous plant molecules using biotechnology. Most currently marketed GE crops are agronomic in nature in that they were developed to increase the yield of field crops by introduction of traits intended to counteract two stressors that consistently result in yield loss from field crops: (i) competition with weeds for environmental resources; (ii) infestation with insect pests.

1.2.1 Herbicide Tolerance

To address the issues of competition with weeds, biotechnology has been used to develop GE crops that are tolerant to application of nonspecific herbicides. The first

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example of this is RoundUp Ready™ soya beans that were developed by the Monsanto Company. RoundUp Ready™ soya beans were developed following identification of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene from *Agrobacterium* strain CP4 (Stallings *et al*., 1991). The nonselective herbicide glyphosate inhibits endogenous plant EPSPS proteins and causes toxicity by inhibiting the synthesis of aromatic amino acids. In contrast, the CP4 EPSPS protein is not inhibited by glyphosate, so that plants in which it is expressed are tolerant to treatment with glyphosate (Padgette *et al*., 1991; 1995). GE crops in which the transgenic CP4 EPSPS protein is expressed have an obvious advantage over crops in which this protein is not expressed, in that they tolerate field application of glyphosate, whereas the weeds competing with the crop do not. The tools of biotechnology have also been used to express the CP4 EPSPS protein in other field crops including corn, cotton and canola.

An alternative method for conferring tolerance to glyphosate in field crops was developed by isolation of the gene for the glyphosate acetyltransferase (GAT) protein found in *Bacillus licheniformis*. This gene was modified using gene shuffling techniques designed to screen for increased activity towards acetylating (and thereby inactivating) glyphosate (Castle *et al*., 2004; Siehl *et al*., 2005). The DuPont Company has developed field crops that are tolerant to glyphosate by expression of the *gat* gene in soya beans and corn under the market name of Optimum® GAT®.

Biotechnology has also been used to produce field crops that are tolerant to the nonspecific herbicidal active ingredient glufosinate ammonium by the Bayer Company. The phosphinothricin acetyltransferase (PAT) protein that was originally isolated from *Streptomyces viridochromogenes* metabolically inactivates glufosinate ammonium by acetylation of phosphinothricin. The gene for this protein has been expressed in field crops under the market name Liberty Link™.

1.2.2 Insect Resistance

Insect pests are also responsible for loss of crop yield, though they may be controlled by numerous classes of chemical pesticides including organophosphates, carbamates and pyrethroids. However, the use of chemical pesticides can result in effects on nontarget insects, development of resistance in target insects, and possible adverse effects from human exposure to residues. The development of insecticidal protein traits in plants, as discussed further below have minimized these effects by reduction of chemical pesticide use.

Farmers of organic foods have used the ubiquitous soil bacteria *Bacillus thuringiensis* (Bt) for insect control for more than 40 years. Biochemical studies have demonstrated that selective pesticidal activity is attributable to the crystalline (Cry) proteins expressed by these bacteria (Höfte and Whiteley, 1989). Biotechnology has been used to transfer individual *cry* or *vip* genes from different strains of Bt into field crops for the purpose of insect control. The particular Cry proteins used in these crops were chosen based on their selective toxicity towards target insect species. For example, the Cry1F and Cry1Ab proteins display a selective toxicity towards European corn borer and other lepidopteran larvae, whereas the Cry3Bb1 and Cry34Ab1/Cry35Ab1 proteins display selective toxicity towards corn rootworm and other coleopteran larvae. The use of GE corn in which Bt Cry proteins are expressed has also been reported to result in decreased concentrations of mycotoxins; an effect that is believed to be attributable to improved health of the plant (Hammond *et al*., 2004a; Wu, 2006).

1.3 Crops Produced Using Conventional Breeding Methods vs. Biotechnology

Traditional or conventional crop breeding has been used for millennia to improve the yield and hardiness of domesticated plants. Selecting and saving seed from the 'best' performers to replant the following season, or crossing (hand-pollinating) a reliable producer with a plant of the same species expressing a new, desirable phenotype are examples of traditional breeding. More recently in the past century, new crop varieties have been created using tools for genetic modification, including forced recombination of entire genomes or chromosomes across species or even genus boundaries (wide-cross hybridization), and the creation of large numbers of random, widespread gene disruptions, by exposing plants to ionizing radiation or mutagenic chemicals (induced-mutation breeding) (National Academy of Sciences (NAS), 2004; Harlander, 2002).

For the past 20 years, plant molecular biologists have been introducing new functionality into cultivated crops using gene isolation and DNA-transfer techniques of modern biotechnology. Recombinant DNA technology, the combining of genes and elements some of which may originate from different organisms, has been used to create new traits of benefit to agriculture. Insect-resistant and herbicide-tolerant corn, soya beans and cotton are some of the first products commercialized using recombinant DNA technology, in which entire coding sequences (genes) with well-characterized functions were stably integrated into the genome of the recipient plant. For these crops, expression of the encoded protein or proteins *in planta* confers the anticipated phenotype. It is also possible to utilize recombinant DNA technology to modulate expression of endogenous plant genes (double-stranded (ds) RNA-induced gene silencing; Kusaba, 2004) or entire biochemical pathways

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(altered transcription factors; Broun, 2004) for alternative crop phenotypes (e.g. drought tolerance, nitrogen retention). In fact, it is now known that major phenotypic advances in plant domestication using traditional breeding methods were the result of selection for naturally occurring specific transcription factor variants or mutations in cis-regulatory genes (Doebley, 2006; Li *et al*., 2006; Wang *et al*., 2005).

The obvious similarity between recombinant DNA technology and conventional breeding is that the phenotype of the new crop variety is conferred by altering DNA sequence or expression. However, there are two important differences. The first is that in conventional breeding and selection methods, only a proportion of the progeny will express the phenotype of interest. Secondly, most of the progeny will contain additional genetic traits in addition to the intended trait—potentially undesirable qualities that must be bred out by repetitive backcrossing to the parental line. Recombinant DNA technology is more directed than other genetic modification techniques in that only the intended DNA sequence(s) is transferred. It has been documented that considerably greater heterogeneity exists within the genomes, proteomes and metabolomes of conventional varieties of a crop than between a new GE crop and its conventional progenitor (Catchpole *et al*., 2005; Shewry *et al*., 2007; Cheng *et al*., 2008; Ruebelt *et al*., 2006). Within the context of safety assessment, this is curious because regulatory agencies in many countries require safety assessment of crops produced using biotechnology, but little or no safety characterization of crops produced using conventional breeding methods (Harlander, 2002; National Academy of Sciences (NAS), 2004; Parrott, 2006).

The second difference between these methodologies is that recombinant DNA technology eliminates the species barrier across which DNA can be transferred. While the traits imparted into crops from recombinant technology are mediated by genetic modification, just as they are with conventional breeding practices, it is possible that public concern about the safety of GE crops is at least partly attributable to the introduction of foreign genes into a species that would not otherwise have expressed them, since within conventional breeding, forced crosses above the genus level are seldom successful.

2 PRODUCT DEVELOPMENT

Conceptually, the process of product development using biotechnology is relatively straightforward following the identification of the genetic elements responsible for the desired trait. It involves engineering the trait DNA into an expression vector, which is then transferred into the seed germline of the selected crop. At a minimum, the vectors contain DNA responsible for: (i) conferring the desired trait or traits; (ii) the transgenic elements required for their expression and (iii) a selectable marker required to identify transformed plant tissues. The ensuing inheritance and expression of the inserted DNA fragment confers the desired trait to the progeny of the transformed plant. The actual process by which genetically modified (GM) crops are developed is considerably more complicated.

While not typically discussed in the safety assessment of GM crops, it should be noted that the transformation process and selection of efficacious events represent two critical steps in identifying viable candidate plants for product development. Following construction of a transgenic vector, individual transformants are produced by the insertion of the transgenic DNA into the seed germlines. A variety of methods have been used to produce candidates, including particle-based methods (e.g. gene gun) and transfer using nonpathogenic bacteria (Taylor and Fauquet, 2002). The processes used to insert the transgenic DNA into seed germlines are not 100% efficient and do not always result in transfer of functional transgenic DNA. The screening of individual transformants for viability and efficacy is one of the most important steps in product development of GM crops. For example, approximately 10 000 individual transformants were created during the process of development of the corn-rootworm-resistant maize line DAS-59122-7, most of which did not survive the transformation process or produce efficacious events, and only one of which was commercialized (unpublished data).

3 SAFETY ASSESSMENT OF GE CROPS

Assessment of the safety of foods or food components obtained from GE crops is a process that considers numerous factors, some of which relate to the transgenic proteins or other transgenic elements expressed in GE crops, while others focus on assessment of the safety of whole foods obtained from GE crops. The overall goal of the safety-assessment process for any individual GE crop is to determine whether it is *as safe as* its appropriate conventional non-GE comparator, which presumably has a history of safe use. Accordingly, the safety assessment is conducted to determine if the production of the particular GE crop resulted in unintended effects (i.e. pleiotropism) and, if observed, whether the changes are adverse.

The current chapter focusses on assessment of human-health risk from foods obtained from GE crops. A comprehensive safety assessment that would be conducted for a regulatory dossier would also consider a number of additional factors, including environmental effects, such as stability of the trait, possible effects on nontarget insects and, depending on the nature of

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the transgenic event, the potential impact on insect resistance.

3.1 Safety Assessment of Transgenic Proteins

Most dietary proteins are metabolized into individual amino acids and small peptides that are absorbed for nutritive purposes. However, a small number of proteins have been reported to cause two adverse effects: allergenicity and toxicity. The following sections discuss the processes used to assess transgenic proteins for potential to cause allergenic or generalized toxicity.

3.1.1 Allergenicity

Allergenic proteins have been identified in a number of foods and all known allergenic substances identified in foods are, in fact, proteins. Therefore, one of the potential risks that has been identified with the expression of transgenic proteins in GE crops and foods obtained from them is that the proteins could be allergenic, however, this concern appears to be based solely on the fact that they are proteins. During the research phase of one project, a methionine-rich protein from Brazil nuts was transferred into the soya bean seed germline to increase the concentration of sulphur in soya beans for animal nutritional purposes. The safety assessment of the transgenic product conducted early in development determined that the protein that was selected from the Brazil nut was allergenic (Nordlee *et al*., 1996). The product was not commercialized, but it did highlight the need for the assessment of the potential allergenicity of transgenic proteins.

While toxicologists often evaluate the safety of various substances, including proteins, using animal models, there are currently no validated animal models to assess the potential allergenicity of candidate transgenic proteins, even though a number of models have been investigated (McClain and Bannon, 2006; Ladics *et al*., 2003; Helm, 2002; Goodman *et al*., 2008). Nevertheless, guidelines have been developed to assess the potential allergenicity of transgenic proteins by a number of scientific and regulatory authorities (Metcalfe *et al*., 1996; FAO/WHO, 2001; Codex, 2003a). Current guidelines call for a weight-of-evidence approach that considers a number of end points and properties of the transgenic protein (Codex, 2003a).

Assessment of the potential allergenicity of transgenic proteins begins with consideration of the source from which the DNA encoding the transgenic protein was obtained. Sources with a documented history of causing allergic reactions in individuals are considered more likely to contain allergenic proteins than those without a documented history of causing allergic reactions. Many foods have been documented to cause allergic reactions in humans (Hefle *et al*., 1996), but the majority of allergic reactions from foods are attributable to a relatively small number of foods (approximately 200 foods or food groups) including peanuts, tree nuts, seafood, shellfish, egg, milk, soya and wheat. Therefore, mining the sequences of these particular foods for proteins that could be useful in GE crops is typically avoided. However, the known allergenic proteins contained in such foods represent a very small number in the total proteome. Consequently, it should be noted that the proteins or other transgenic elements derived from such commonly allergenic food sources should not necessarily imply that they are allergenic.

Another component in the weight-of-evidence approach for assessing the potential allergenicity of transgenic proteins involves using computer-based bioinformatics tools to determine the similarity of the particular amino acid sequence of the transgenic protein expressed in a GE crop to other known allergenic proteins contained in a database (i.e. AllergenOnline; *www.allergenonline.com*). The AllergenOnline Database is a publically available, peer-reviewed, and curated database that is updated on an annual basis. A variety of bioinformatics tools are available for this application, including FASTA (Ladics *et al*., 2007). According to current guidelines, bioinformatics analyses are conducted to determine if there are any eight contiguous identical amino-acid matches representative of 'theoretical' IgE binding epitopes or an overall sequence identity of greater than 35% over 80 or greater amino acids between the transgenic protein and known allergenic proteins (Codex, 2003a; Silvanovich *et al*., 2006).

Studies have reported that nonallergenic proteins are degraded following *in vitro* exposure to digestive enzymes (Astwood *et al*., 1996). Accordingly, the allergenicity assessment of transgenic proteins also includes an evaluation of their resistance to degradation by digestive enzymes, including pepsin (in simulated gastric fluid) and pancreatin (in simulated intestinal fluid) *in vitro*. The transgenic Bt and herbicide tolerance proteins used in currently marketed GE crops are degraded in these conditions (Herman *et al*., 2003).

It has since been reported that the relationship between digestibility and allergenicity is not absolute, as a number of allergenic proteins were found to be degraded under these conditions and nonallergenic proteins were found to resist digestion under these *in vitro* conditions (Fu *et al*., 2002). Nevertheless, assessment of the *in vitro* digestion of heterologously produced transgenic proteins remains a common component of the protein allergenicity assessment of practically all guidelines.

One example of a transgenic protein that was resistant to digestion under *in vitro* conditions is Cry9C (i.e. Starlink) that was expressed in corn intended to be used for livestock applications. This protein was modified by a one-amino-acid change that made it resistant to digestion

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and then expressed in corn intended for use in livestock applications. This corn was unfortunately commingled into the human food supply and resulted in a recall of products containing Cry9c from the market (Bucchini and Goldman, 2002). Although the Cry9 C protein was resistant to degradation by digestive enzymes under *in vitro* conditions, a property of some allergenic proteins, there was no indication that exposure to the Cry9 C protein actually resulted in any adverse effects, allergenic or other, in the human population.

In some cases, heat lability of transgenic proteins is also assessed and may be required by some regulatory agencies. This is based on the concept that allergenic proteins would be more likely to remain structurally intact following exposure to heat; however, this relationship has not been clearly demonstrated. Additionally, it should be noted that there are no clear guidelines describing how this particular assessment should be conducted. In cases where this property has been investigated, the data are usually expressed as an activity curve that reports the enzymatic activity at different elevated temperatures (up to 100 °C) relative to that observed at cooler temperatures (for example see Delaney *et al*., 2008c).

When considering the potential for a transgenic protein to cause allergic reactions, the individual components discussed above are considered in a weight-of-evidence approach rather than a check-the-box or flow-chart approach. In other words, there is not always a clear yes–no approach in the assessment for any particular transgenic protein. Rather the individual components discussed above, when considered collectively, give a good indication of whether or not the protein is likely to be allergenic.

3.1.2 Toxicity

A small number of proteins have also been reported to cause mammalian toxicity that is unrelated to allergenicity. The majority of these proteins are bacterial in origin, including shiga toxin, though a number of plant-derived protein toxins are also known to exist, including lectins and antinutrients (e.g. trypsin inhibitor). Because the traits expressed in currently marketed GE crops are proteins, recommendations to determine whether individual transgenic proteins are likely to be toxic have recently been published by the International Food Biotechnology Committee (IFBiC) of the International Life Sciences Institute (ILSI; Delaney *et al*., 2008a). They bear a number of similarities to those used to assess the allergenicity, but are structured in a two-tiered approach rather than a weight-of-evidence approach.

The first tier (potential hazard identification) includes assessing information about the source of the transgenic DNA and whether that source has been documented to cause toxicity in mammalian species. The reasoning for including this particular information is that it is considered that species not known to cause toxicity are less likely to contain proteins that are toxic, than those known to produce protein toxins. This is the reason why transgenic DNA used in GE crops has been obtained from innocuous species such as *B. thuringiensis* rather than from pathogenic bacteria or from plants known to produce protein toxins.

Another piece of information in the first tier is a bioinformatics comparison of the amino-acid sequence of the transgenic protein with that of known protein toxins. In contrast to the bioinformatics assessment conducted for allergenicity, there are currently no internationally accepted standards that constitute a significant sequence or structural match between transgenic proteins and toxic proteins. Further, there are currently no databases where the sequences of toxic proteins are actively maintained. However, these analyses are typically conducted using the same types of bioinformatics tools.

An additional factor to consider in the safety assessment of transgenic proteins is information about their mechanism of action. The more information that is known about the mechanism by which a particular protein acts will aid substantially in the assessment of the safety of that protein. For example, if a transgenic Cry protein obtained from a particular *B. thuringiensis* species interacts with a specific receptor that is only expressed within the target insect, it would be conceptually easier to determine that the protein represents little risk to any other species of insects.

The assessment of transgenic protein resistance to degradation by digestive enzymes conducted under the rubric of allergenicity assessment is also a component of the toxicity assessment. To date, there has been no systematic assessment of whether toxic proteins are more resistant to degradation by digestive enzymes than nontoxic proteins. Nevertheless, the data obtained from the allergenicity assessment is applicable to the toxicity assessment of these proteins, because toxic proteins must remain intact to exert evidence of toxicity. Therefore it is expected that proteins that are degraded by digestive enzymes are less likely to cause toxicity to an organism for the simple reason that they have little or no opportunity to be absorbed intact (reviewed in Delaney *et al*., 2008a).

In many instances, the fundamental elements addressed to assess the toxicity of transgenic proteins in the first tier of the ILSI/IFBiC recommendations alone are adequate to assess the potential for a particular transgenic protein to exhibit toxicity. Nevertheless, a second tier (hazard characterization) was recommended to be considered in the case where the evidence from the first tier is not conclusive. The elements contained within the second tier include considerations for additional general toxicity studies to assess the general safety of the transgenic protein (acute and repeated-dose rodent studies) and, in some cases, hypothesis-based testing.

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It has been reported that even though most proteins are not toxic, those that are toxic appear to act through acute mechanisms of action (Sjoblad *et al*., 1992). Accordingly, an assessment of the acute toxicity of transgenic proteins is often conducted, though different methods have been used to assess the acute toxicity of transgenic proteins.

In most cases, transgenic proteins were administered to mice rather than rats for practical reasons because the amount of test substance required for mouse studies is substantially lower than that required for rat studies. In some cases the test substance has been administered to mice via oral gavage in accordance with OECD (Organization for Economiic Cooperation and Development) limit dose guidelines (i.e. 2000 mg (kg body weight)$^{-1}$ OECD, 2001b) to assess the potential for adverse effects following oral exposure—the presumed route of exposure to most proteins in foods from crops developed using biotechnology. In other cases, the test substance was administered to mice via alternative routes, including intraperitoneal and intravenous exposure routes. Despite the differences in the methods used to assess the acute toxicity of transgenic proteins, to date there is no evidence that any transgenic proteins used in GE crops exhibit any evidence of acute toxicity (reviewed in Delaney *et al*., 2008a).

Repeated dose (28 day) toxicology studies have been recommended by some regulatory agencies to assess the safety of transgenic proteins used in GE crops. While few studies have been conducted to date, the studies that have been conducted reported no evidence of adverse effects (Delaney *et al*., 2008b; Cerven *et al*., 2008a; 2008b; Mathesius *et al*., 2009; Juberg *et al*., 2009).

The final element of toxicity testing recommended by ILSI/IFBiC is hypothesis-based studies. Conceptually, these types of studies would only be necessary when other studies or information indicated that they would be necessary. One example that has been discussed is that of antifungal protein (AFP; Delaney *et al*., 2008a). Bioinformatics analysis of this particular protein reported sequence similarity between this protein and scorpion toxin. In that case, AFP was isolated and compared to scorpion toxin using electrophysiology methods to demonstrate that it did not possess the same biochemical properties as scorpion toxin, though products containing AFP have not been commercialized to date.

3.1.3 Challenges

One of the problems with conducting allergenicity and toxicity testing with transgenic proteins expressed in GE crops is that of obtaining sufficient quantities of the pure proteins required to conduct these studies. In most cases, the transgenic proteins in GE crops are expressed at low concentrations, making it impractical to isolate them from the crops in which they are expressed to conduct *in vitro* or *in vivo* toxicology studies. Therefore, these studies are typically conducted using heterologously expressed proteins, which necessitate biochemical and analytical studies to demonstrate that, in fact, any studies conducted with heterologously expressed proteins are truly representative of the proteins when expressed *in planta*.

3.2 Safety Assessment of Foods from GE Crops

The fundamental concept for the safety assessment of GE crops is called 'substantial equivalence' and was first proposed by the World Health Organization (WHO) and the OECD (WHO, 1991; OECD, 1993; reviewed in Kok and Kuiper, 2003). This concept was developed because it is not possible to demonstrate that any whole food is absolutely safe, even though countless foods have a long history of safe consumption. Since the original publication, numerous variations have been published by other scientific and regulatory authorities (Codex, 2003b; 2007; EC, 1997; 2003a; 2003b; 2004; EFSA, 2006; 2008; FAO, 1996; FAO/WHO, 2000; ILSI, 1997; 2003b; 2004; OECD, 1997; 2003; WHO, 1995). While there are differences among these different sets of recommendations, in essence, they all recommend using scientific evidence to demonstrate that the only difference between the GE crop and the non-GE counterpart crop is the presence of the transgenic DNA and associated expressed protein or phenotypic trait.

There is controversy regarding DNA and RNA in foodstuffs, but they are both normal components of the human diet and people have been estimated to consume up to 1.0 g day^{-1} of RNA and DNA from the diet (Carver and Allan Walker, 1995; Jonas *et al*., 2001). The DNA present in genetic inserts in foods obtained from GE crops does not differ structurally or in other biochemical properties from the DNA already present in food and represents a very small fraction of the total DNA present within the foods (Parrott *et al*., 2008). Though consumption of both DNA and RNA do not cause adverse effects, it has been recommended that the intake should not exceed 2 g day^{-1}, at least in populations that are sensitive to elevated concentrations of uric acid, such as those with gout (Clifford and Story, 1976). The presence of additional DNA or RNA in the foods obtained from GE crops relative to the DNA present in foods obtained from non-GE foods contributes virtually nothing to the typical daily dietary exposure to either substance.

3.2.1 Characterization of the Genetic Insert

Characterization of the transgenic insertion is normally conducted to determine copy number, integrity, orientation, number of insertion loci, whether the process of

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transformation resulted in insertion of backbone DNA from the vector, and whether the transgenic DNA was inserted into an endogenous gene. The methods used to conduct these types of analyses have been discussed elsewhere and are beyond the scope of this paper.

3.2.2 Compositional Analysis

In practice, the principle of substantial equivalence has been applied in the comparison of the composition of GM crops with the composition of non-GM crops of closely related genetic lineage (near isogenic) from field trials in which they were both grown under similar conditions. These studies are analytical in nature and are extensive in the number of ingredients that are analysed, including primary nutrients (nutritional proximates), amino acids, fatty acids, vitamins, minerals and antinutrients on a crop-specific basis with known biological importance, either to the plant itself or to their nutritional importance (ILSI, 2003a; OECD, 2001a). Numerous such comparative trials have been published for field crops, including soya beans (Taylor *et al*., 1999; McCann *et al*., 2005), corn (Sidhu *et al*., 2000; Ridley *et al*., 2002; George *et al*., 2004; Herman *et al*., 2003; 2007), wheat (Obert *et al*., 2004) and rice (Oberdoerfer *et al*., 2005). Collectively, these studies have determined that the composition of the GM crops is comparable to the composition of their conventional crop counterparts.

3.2.3 Nutritional Equivalence Studies with Foods Obtained from GE Crops in Livestock Species

In most cases, the information necessary to determine that the foods obtained from a particular GE crop are substantially equivalent to their non-GE comparator can be obtained from characterization of the insert, composition testing and agronomic field studies. However, feeding studies have also been conducted with processed grains and feed fractions from a number of GE crops. To be clear, these are not toxicology studies; however they can provide valuable information about the nutritional quality of the foods obtained from individual GE crops to determine whether adverse pleiotropic effects could have been introduced or expressed from the process of genetic modification that may not have been detected from compositional testing.

Because early generation GE crops were soya beans and corn and a large proportion of the grains obtained from these crops are used as livestock feed, these feeding studies have been conducted in livestock species. These feeding studies have been referred to colloquially as nutritional equivalence studies. Of particular relevance for these types of studies are the feeding studies that have been conducted in broiler chickens. Broiler chickens represent a commercially important commodity that grows rapidly. These animals grow from approximately 40–50 to 2000 g over a 42 day feeding interval, which is the standard lifespan for broiler chickens used by commercial poultry farmers, and nutritional comparison studies conducted in broiler chickens are considered to be very sensitive to nutritionally imbalanced diets (ILSI, 2003c; 2007).

The basic design of these studies is to compare nutritional performance metrics from groups of broiler chickens consuming diets produced with processed (i.e. milled) grains and feed fractions from GE crops (the experimental group) to those observed in groups of broiler chickens consuming diets formulated with corresponding processed grains and feed fractions from non-GE crops. Additional groups are fed diets produced from unrelated commercially available non-GE crop varieties to provide within-study control values for the response variables that are assessed. The response variable typically assessed in these types of studies included body-weight gain, feed consumption, feed-conversion efficiency, and an assessment of commercially relevant carcass traits, including yield of breast, wings and thighs. Numerous feeding studies with processed grains and feed fractions obtained from GE soya beans and/or whole grain from individual and stacked trait GE maize plants have been published though no adverse nutritional effects have been reported to date (Taylor *et al*., 2003a; 2003b; 2003c; 2003d; McNaughton *et al*., 2007a; 2007b).

3.2.4 Subchronic Toxicology Studies with Foods Obtained from GE Crops in Rodents

Subchronic (90 day) rodent toxicology studies have also been conducted with the processed grains and feed fractions obtained from GE crops. These studies were conducted to determine whether the process of engineering the particular crop resulted in adverse pleiotropic effects that were not detected during compositional analyses. These studies were originally designed to assess the health effects of long-term exposure to 'pure' chemicals, using a comprehensive overall health screen in rats, as described in OECD 408 guidelines (OECD, 1998). Subchronic rodent studies conducted with foods from GE crops are similar to subchronic rodent toxicology studies conducted with chemicals or food additives, in that they are the same duration and assess the same growth performance (body weights, feed consumption, etc.) and clinical pathology response variables (serum chemistry, haematology, coagulation, organ weights and organ histopathology).

However, there are differences between the design of subchronic rodent toxicity studies conducted with foods from GE crops and those conducted with food ingredients. The most obvious of these is the dose–response curve, in which the test substance is administered to rodents at tens, hundreds or thousands of times in excess

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of anticipated human exposure for purposes of quantitative risk assessment. When conducted with whole grains or processed feed fractions from GE crops, these studies represent what is essentially a qualitative safety assessment because they have been designed and conducted to compare the effects of two substances (food from GE crops vs. food from non-GE crops). A variety of study designs have been employed to assess these differences, but it should be noted that the studies themselves are as rigorous as the toxicology studies conducted for quantitative risk assessment of food ingredients (Delaney *et al*., 2008a). When conducting feeding studies with whole foods, whether derived from GE or non-GE crops, it is usually not possible to administer the test substance at levels that are tens of times greater than estimated human exposure. Even if it were physically possible to formulate diets that met these criteria, it would likely result in formulation of nutritionally imbalanced diets that would result in corresponding differences in the response variables evaluated in these studies that are not related to exposure to the particular test substance.

Subchronic rodent feeding studies with processed grains and feeding fractions from GE crops have been conducted by formulating rodent diets according to the specifications of commercially available rodent feed. The experimental diets in these studies typically contain processed grains or feed fractions from GE crops, whereas the control diets are formulated with the corresponding fractions from the nearest isogenic comparator (i.e. near isoline). For example, one commonly used commercial diet in the United States is Purina Mills Certified LabDiet 5002 which contains 33–38% whole maize grain. Subchronic studies with whole corn from GE crops have therefore been conducted by formulating rodent grain-based diets with ~35% GE maize grain in the experimental group and ~35% near-isoline maize grain in the control group.

These studies have essentially borrowed the elements of study design from nutritional comparison studies as conducted in livestock species. They usually compare nutritional performance (body weights, feed consumption) and a more extensive battery of toxicology response variables (haematology, coagulation, clinical chemistry, organ weights and histopathology) between rats consuming diets formulated with the foods obtained from the GE crop and the non-GE control group. The combination of the large number of response variables that are evaluated in these studies and the fact that the primary statistical comparison is between two groups results in a high probability that some statistical differences will be observed between the control and experimental groups. By default, it could be assumed that statistical differences between the two groups are attributed to consumption of the experimental diet. However, it is equally likely that statistical differences could be attributed to deviations in the values observed in the control group. That is, if statistical differences are observed between the control and the experimental group and it can be demonstrated that the values observed in the experimental group fall within the range of anticipated values when the values observed in the control group do not, it is unlikely that the statistical difference represents a biologically significant difference. For that reason, it is common to include additional 'reference' control groups in subchronic rodent feeding studies. The reference groups are fed diets containing the same concentrations of processed grains or feed fractions from genetically unrelated non-GE crops to provide within-study ranges for the response variables typically evaluated in subchronic rodent toxicology studies. It is also common to refer to the values observed in historical control groups to determine whether statistical differences observed between the response variables evaluated in control and experimental groups are within the anticipated ranges to determine whether they are biologically relevant.

In most cases published to date, these types of studies have also included at least three additional groups of rats that were fed diets produced in accordance with commercial standards using commercially available, but unrelated, varieties of non-GE grain fractions (i.e. reference groups). The inclusion of the reference groups has proven useful for statistical analysis of the values in what is essentially a two-group comparison study. Because statistical significance in these studies is typically set at a default of $p < 0.05$ and there are a large number of response variables, it would be anticipated that each independent study would contain a number of statistical differences between the control and experimental groups. In the absence of a true multiple exposure dose–response design it can be difficult to determine whether the statistical differences are biologically meaningful or not. In fact, practically every subchronic rodent feeding study conducted with foods from GE crops to date has reported statistically significant differences between the control and experimental groups for at least one response variable. The data obtained from the concomitant reference groups has therefore been particularly useful in establishing within study ranges for the response variables. Data from historical control groups is also useful for this purpose.

Numerous studies of this design have been reported for corn (MacKenzie *et al*., 2007; Malley *et al*., 2007) and soya beans (Appenzeller *et al*., 2008; Delaney *et al*., 2008b) from GE crops. Some subchronic rodent studies with foods obtained from GE crops have also been conducted using an additional dose group that contains lower concentrations of GE maize grain to construct a dose–response curve, at least with relation to exposure to the test substance (Hammond *et al*., 2004b; 2006a; 2006b; Healy *et al*., 2008). However, it is unclear whether the dose–response design utilized in those studies was useful for quantitative risk assessment, since no differences have been reported among the treatment groups consuming

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different concentrations of the whole foods obtained from GE crops.

Different methods of conducting subchronic feeding studies have also been reported. In some studies, different concentrations of maize grain from GE corn (50 and 70%) were incorporated into experimental diets and fed to rats for 90 days. Control groups were fed diets containing the same concentrations of non-GE, near-isogenic maize grain. The diets in these studies were formulated to meet the guidelines of AIN-93G standards (Reeves *et al*., 1993). Additional control groups were fed commercially sourced AIN-93G diets. The statistical analysis of data obtained from studies of this design have been difficult to interpret, but, to date, studies of this design have not reported biologically significant differences between rats consuming control and experimental diets (He *et al*., 2008).

Subchronic rodent toxicity studies have also been conducted with GE rice. In those studies ground flour from GE rice expressing the Cry1Ab protein or the parental wild-type rice were administered to rats at a dietary concentration of 60% and fed for 90 days. In that study, a number of statistically significant differences were observed between the toxicology response variables of rats consuming the control and experimental diets, but the authors concluded that the differences were not adverse (Schrøder *et al*., 2007). Similar observations were made from a subchronic rodent feeding study conducted with GE rice expressing the insecticidal snowdrop lectin (GNA (*Galanthis nivalis* agglutinin); Poulsen *et al*., 2007a).

In another variation, a recent subchronic rodent toxicity study was conducted, in which rats were fed control (non-GE) or rice that was produced experimentally to express the toxic kidney bean lectin (PHA-E (phytohemagglutinin-E). In this study a third group was included that was fed the PHA-E-expressing rice grain and an additional dietary concentration of 0.1% pure PHA-E (Poulsen *et al*., 2007b). The authors indicated that the intent of designing the study that way was to increase the sensitivity and specificity of the study to detect possible adverse effects. For practical purposes, studies of this design would be difficult to reproduce in many instances because the transgenic proteins and other elements expressed in foods from GE crops are expressed at very low concentrations and it may not be physically possible to obtain the amount of purified test substance that would be necessary to conduct a study according to that type of design.

3.2.5 Other Toxicology Studies with Foods Obtained from GE Crops in Rodents

Toxicology studies other than subchronic have also been conducted using foods obtained from GE crops. For example, multigenerational reproductive studies have been conducted in mice using GE soya beans, maize grain and potatoes, though no evidence of adverse effects were observed (Brake and Evenson, 2004; Brake *et al*., 2004; Rhee *et al*., 2005). Additionally, no adverse effects on the immune system were observed in rats or mice following consumption of GE soya beans (Teshima *et al*., 2000).

4 QUALITATIVE SAFETY ASSESSMENT

The previous sections of this chapter describe the types of studies that are often conducted when assessing the safety of foods or food components from GE crops. What needs to be kept in mind is the overall goal of these studies—to determine if the foods obtained from GE crops are *as safe as* those obtained from non-GE crops (i.e. substantial equivalence). Some typical toxicology studies are included in the assessment, but numerous additional *in vitro, in silico* and analytical studies are also included.

The process of safety assessment of foods from GE crops can therefore be considered as a qualitative safety assessment in that the overall goal is comparative in nature; however, the process itself is certainly no less rigorous than the data considered in the quantitative safety assessment of dietary ingredients (Delaney, 2007).

5 SAFETY ASSESSMENT OF FUTURE GE FOODS

The majority of currently marketed GE crops are relatively simple in nature, in that traits that were engineered into them are mediated primarily by expression of exogenous proteins. The future or GE crops will include new traits intended to confer additional agronomic benefits, such as drought tolerance and nitrogen-use efficiency, and consumer benefits, including modified fatty-acid profiles and altered nutritional properties. It is likely that these effects will not be mediated by expression of exogenous proteins, but rather by the modification of endogenous biochemical pathways of field crops, using such techniques as gene silencing and altered expression of endogenous proteins using transcription factors. Introduction of new traits may also involve more complicated proteins, such as transmembrane proteins that, at least using currently available technology, cannot be isolated in the quantities necessary to conduct safety and toxicology studies such as those described above.

As new molecular and biochemical technologies are developed through scientific research, it will be increasingly important to carefully evaluate these techniques for their utility in safety assessments of GE crops. It has been suggested that 'omics' profiling studies

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(e.g. metabolomics) may be useful in assessing the safety of the next generation of GE crops (Chassy *et al*., 2004; Kok *et al*., 2003; Kuiper *et al*., 2003; Shewry *et al*., 2007). 'Omics' studies provide a large number of data points about expressed genes, metabolites or proteins of GE crops well beyond the analysis of current studies. However, it should be kept in mind that producing additional data alone will not provide greater information about the safety of future GE crops without an understanding of the biological significance of this data and a better understanding of the normal range of variation for the individual components in non-GE crops. For example, many of the components evaluated in current composition analysis studies are based on those for which biological and nutritional properties are known and for which established ranges in non-GE crops have been determined. It is critical to evaluate new molecular and biochemical technologies such that a complete understanding of the biological and safety significance of this data is established. Without this understanding, such techniques have limited utility in the safety assessments of GE crops.

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Toxicology of Pesticides

Ian C. Dewhurst and Timothy C. Marrs

CONTENTS

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1 INTRODUCTION

Pesticides are a group of substances with heterogeneous toxicity, whose desired activity is the killing of unwanted living organisms, in a more or less specific manner. In order to review the toxicity of pesticides, some method of classification is needed. In this chapter, they are divided by class of action, into insecticides, fungicides and herbicides, molluscicides and rodenticides, these groups being subdivided by chemical group. The terms acaricide or miticide are used for substances that kill mites: most are also insecticides. Some chemical groups have more than one type of pesticidal activity, and therefore appear in more than one category, for example, the carbamates and organophosphates (OPs) which include insecticides, herbicides and fungicides and the phosphides used as both fumigants and rodenticides.

The very definition of pesticides may vary, particularly in relation to regulatory affairs. Some veterinary products, for example, compounds used as ectoparasiticides on cattle, sheep and domestic pets, are regulated in many countries under different legislation than when the same active ingredients are used on arable commodities. Moreover, pesticide legislation often controls substances such as plant growth promoters and defoliants: some of these will be briefly discussed here.

There are a number of possible ways in which humans can be exposed to pesticides, thus the toxic effects of pesticides may have consequences for consumers of food, as well as farmers and other applicators. Moreover, pesticides used domestically in wood preservation or as household insecticides may be important sources of exposure of the general public. Furthermore, pesticides may get into the water supply. These various routes of exposure may be isolated or may need to be considered in combination when assessing potential effects in humans. It should also be remembered that the more acutely toxic pesticides have been used for suicide and murder.

The effects of pesticide residues in food and water probably cause the greatest public concern. However, reports of clinical poisoning by residues seem to be extremely rare, certainly by comparison with occupational intoxication and deliberate and accidental poisonings. The reasons for this are complex, but may reflect a true rarity of poisoning or the fact that occupational poisonings are far more easily identified. Occupational intoxications would be expected to be more severe than food-borne poisonings, and the proximity of cause and effect in occupational poisoning certainly makes diagnosis easier. Food-borne pesticide intoxication, especially where clinical signs are nonspecific or trivial, would probably pass unnoticed or may not be attributed to pesticides. This would be particularly likely where the signs and symptoms could be ascribed to a microbiological cause. Acute poisoning, where pesticides had been used in accordance with regulations, seems likely to be a very uncommon occurrence, if it occurs at all, at least in developed countries. The problem of consumers who eat high levels of some foodstuffs is taken into account when residue levels are pronounced to be toxicologically acceptable by comparison with the acceptable daily intake (ADI) or the more recently developed concept of the 'acute reference dose (ARfD)'. Bearing in mind that, as a minimum, a 100-fold safety factor is used in calculating ADIs and ARfDs from animal studies (WHO, 1990a), residues of many multiples of the maximum residue limit (MRL) would probably be necessary to produce acute poisoning. Thus it would seem highly unlikely that extreme consumption, by itself, could give rise to pesticide poisoning from approved uses. In fact, analysis of reported consumer poisonings by pesticides shows that most reported instances occur from:

1. Spillage of pesticides on to food during storage or transport
2. Eating grain or seed potatoes treated with pesticides, where the food article was not intended for human consumption
3. Improper application of pesticides or failure to observe pre-harvest intervals.

The pesticides responsible have often been ones with low LD_{50}s (<20 mg (kg body weight)$^{-1}$), such as the insecticides endrin, parathion or aldicarb or the rodenticides such as thallium sulfate or sodium fluoride. Other pesticides that have produced morbidity by ingestion with food include organic mercury fungicides (Ferrer and Cabral, 1991).

Weighed against the disadvantages of pesticides that accrue from their toxic effects is the fact that insects and fungi are important sources of agricultural loss and give rise to much damage to buildings, particularly in those countries such as the USA and Canada, where construction is often of wood. Furthermore, many insects carry diseases such as malaria and sleeping sickness, which in the absence of control measures may render land uninhabitable or agriculturally unusable; despite control measures these diseases continue to be major sources of morbidity and mortality. In addition, fungal contamination of agricultural produce can give rise to such conditions as ergotism and aflatoxicosis.

The key to a successful pesticide is selective toxicity. An ideal pesticide will interfere with a biological system in the pest which has no counterpart in nontarget species; this is the advantage of the juvenile-hormone-analogue insecticides. In the case of agricultural insecticides, the pesticide should be toxic to insects, but less toxic to plants, to humans and to other nontarget organisms. Thus, the neuroactive insecticides exploit the relative accessibility of the insect nervous system to xenobiotics,

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when compared to humans, together with the lack of a nervous system in plants.

In this text it is not possible to give more than an outline on the toxicology of the main groups of pesticides, pointing out major problems which occur in regulation of the use of pesticides and allowable residues in food and the treatment of poisoning.

1.1 Nomenclature of Pesticides

Pesticides have, as well as chemical names, national (for example British Standards Institute (BSI)) common names, as well as International Organization for Standardization (ISO) common names (ISO, 1965; ISO, 1981). ISO names are allocated in the English and French languages and the English language ISO name is not always the same as the BSI or US common name. A few pesticides are used as drugs, either in human or veterinary medicine. As such they have international nonproprietary names (INNs). In some cases these differ from the ISO pesticide names; thus trichlorfon, a pesticide, is the same as metrifonate, a pharmaceutical used in human medicine. INNs are bestowed by the World Health Organization (see WHO, 2008). Pesticides are commonly sold under trade names, given by their manufacturers.

2 INSECTICIDES

Many insecticides affect the nervous system of insects and, since many have some activity against the mammalian nervous system, in man, the neurotoxic effects of insecticides are often prominent. The main groups of insecticides are listed in **Table 1**.

2.1 Organochlorines

This group was formerly of great importance and includes dichlorodiphenyltrichloroethane (DDT), hexachlorocyclohexane (HCH), the cyclodienes, dieldrin, endrin, heptachlor and toxaphene. However, the use of organochlorines (OCs) has, in recent years, been severely restricted in most countries. This has mainly been due to the persistence of OCs in the environment and wildlife, as well as in humans, rather than to their toxicity to mammals. Despite the fact that OCs are less used than formerly, their toxicology is still important because they continue to be present in the environment and because, in foodstuffs on sale in, or exported from, developing countries, residues, sometimes above international maximum residue limits (MRLs), continue to arise (Working Party on Pesticide Residues, 1997). OCs are excreted in breast milk (Siddiqui and Saxena, 1985) and their persistence is shown by the continuing presence of OCs in human milk, though usually at declining levels (Working Party on Pesticide Residues, 1982). The most recently used OC in agriculture was lindane, the γ isomer of HCH, but DDT is still used in vector control for malaria (see Rogan and Chen, 2005; Ross, 2005; WHO, 2007).

The most prominent effects of the OCs are those referable to the nervous system, where OCs have an effect on Na^+ channels. DDT produces tremor and incoordination in lower doses and convulsions in high doses, with a clear incremental dose response. By contrast, HCH and the cyclodienes may produce convulsions as the first sign of intoxication, as well as fever, by a central effect possibly linked to disturbances in γ-aminobutyric acid (GABA)-mediated inhibitory neurotransmission (Cole and Casida, 1986). OCs have been associated with a chronic toxicity syndrome, which includes apathy, headache, emotional lability, depression, confusion and irritability (Proudfoot, 1996).

OC poisoning is treated symptomatically and diazepam is usually used to deal with the convulsions.

2.1.1 Carcinogenic Effects of OCs

Chlorinated hydrocarbons produce microsomal enzyme induction and characteristic histopathological changes in the livers of experimental animals (hepatocellular hypertrophy with accumulation of lipid). If administration is prolonged and at high enough doses, liver nodules may appear and tumours are seen in rodents. These tumours do not appear to be indicative of genotoxic carcinogenicity. In human populations OCs have been linked with non-Hodgkin's lymphoma (Axelson, 1987; Cantor *et al*., 1992) but where a link between non-Hodgkin's lymphoma and OC exposure has been observed, the association has been weak, and some studies, for example, Rothman *et al*. (1997) have shown no such association. Some OCs have weak oestrogenic properties. The suggestion has been made that exposure to OCs is related to increased susceptibility to breast cancer. Some epidemiology studies showing a link between high fat levels of DDT/DDE (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene) and breast cancer and there is a similar link between β-HCH and breast cancer. The studies linking DDT/DDE and breast cancer were summarized by Key and Reeves (1994), who concluded that it was unlikely that DDT increased the risk of breast cancer. Suspicion has also fallen upon dieldrin, where Høyer *et al*. (1998) showed a doubling in the risk of developing breast cancer in women with the highest serum diedrin concentration compared to those with the lowest; moreover, Høyer *et al*. (2000) found that high serum dieldrin was associated with an adverse effect on survival and breast-cancer-specific survival. A review of the postulated association between OCs and cancer found

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Table 1 The main groups of insecticides

Group	Subgroups	Examples
Organochlorines (OCs)	—	DDT Endrin Aldrin Dieldrin Endosulfan γ-Hexachlorocyclohexane (lindane)
Anticholinesterases	Organophosphates (OPs)	Malathion Fenitrothion Dichlorvos Diazinon
	Carbamates	Carbaryl Aldicarb
Cholinergic group	Nicotine and the neonicotinoids	Nicotine Imidacloprid
Pyrethrins and synthetic pyrethroids	—	Pyrethrum Permethrin Cypermethrin Flumethrin
Natural compounds, other than pyrethrins and nicotine	—	Abamectin Ivermectin Rotenone
Substances which interfere with systems specific to insects	Juvenile hormone analogues Chitin synthesis inhibitors Ecdysone agonists	Cyromazine Diflubenzuron Tebufenozide
Miscellaneous synthetic insecticides	Formamidine $GABA_A$-blocker	Amitraz Fipronil

no convincing evidence of an association (Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment, 2004).

2.1.2 Specific OCs

2.1.2.1 Lindane (γ-HCH)

Lindane is an OC insecticide, used as a wood preserver, in arable agriculture and in human and veterinary medicine as an ectoparasiticide. It is the γ isomer of 1,2,3,4,5,6-hexachlorocyclohexane, which is sometimes known as benzene hexachloride. 1,2,3,4,5,6-hexachlorocyclohexane has eight stereoisomers of which the α, β and γ are the most important. Of these isomers, only lindane (>99% γ isomer), is still approved in the West, but in some countries preparations of lesser purity continue to be available and these contain other isomers. Lindane is less biologically persistent than DDT and, in rodents, the α and β isomers of HCH accumulate more readily than does lindane (Eichler *et al*., 1983). As well as differences in pharmacokinetics between the isomers, there are qualitative differences in toxicology: thus the effect of lindane on the central nervous system (CNS) is stimulatory, causing convulsions, whereas the α and β isomers have a depressant effect (Coper *et al*., 1951; van Asperen, 1954).

As with other OCs, lindane produces histopathological changes in the livers of experimental animals. The mutagenicity of lindane *in vitro* and *in vivo* has been reviewed and the preponderance of evidence is that the compound is not mutagenic (FAO/WHO, 1990). Over the years its usage on arable crops and in veterinary medicine has become more and more restricted, but its uses have long outlived those of DDT.

The European Union decided to withdraw lindane in 2000 (European Commission, 2000), while the United States Environmental Protection Agency (USEPA) cancelled all remaining registrations for lindane in 2006 (USEPA, 2006).

2.2 Anticholinesterases

Two groups of anticholinesterases, the OPs and the carbamates, are widely used as agricultural insecticides and veterinary medicines (Marrs, 1996); in addition

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some anticholinesterases are used as human drugs, for example, malathion in the treatment of headlice and metrifonate in schistosomiasis (Aden-Abdi *et al*., 1990). Several carbamates are used in human medicine, for example, pyridostigmine in myasthenia gravis. The anticholinesterases are often more acutely toxic than the OCs, although some OPs, especially the P=S phosphorothioates (see below), are of low acute mammalian toxicity. The action is respectively to phosphorylate or carbamylate esterases, particularly the enzyme acetylcholinesterase, causing accumulation of the neurotransmitter, acetylcholine. A variety of cholinergic symptoms and clinical signs occurs at parasympathetic effector sites, including bronchorrhoea, salivation, constriction of the pupil of the eye and abdominal colic. Sympathetic effects can also ensue, together with signs of central nervous system involvement, such as confusion and apprehension. Convulsions will occur in severe poisoning. Actions at the neuromuscular junction result in muscle fasciculation and later paralysis. The terminal event in fatal poisonings seems to be respiratory paralysis, which may be of central or peripheral origin, although with pesticidal OPs both will usually contribute. It is generally believed that, provided the patient survives (without suffering cerebral anoxia), the symptoms and clinical signs of the cholinergic syndrome of anticholinesterase poisoning are reversible. Moreover, histopathological changes from the anticholinesterase effects of OPs and carbamates are *per se* exiguous and, in acute lethal poisoning, specific pathological changes are usually not noteworthy. However, there is recognition that survival after high, perilethal doses of OPs and perhaps also lower doses and after overt carbamate intoxication may result in long-term clinical and electrophysiological changes in the central nervous system.

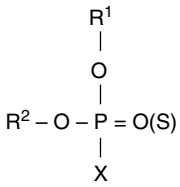
It is often stated that the main difference between the OPs and the carbamates is that the former produce irreversible inhibition of acetylcholinesterase, whilst the latter produce reversible inhibition: this is only true in a relative sense. With certain exceptions (see below), reactivation of the dialkylphosphoryl–enzyme complexes produced by OP-induced inhibition of cholinesterases is produced by hydrolysis, but the stability of the phosphorylated enzyme is generally greater than that of the carbamylated one, so that carbamate poisoning is less long-lasting.

The numerous cholinesterases in the body show different intensities of sensitivity to inhibitors. Although by no means always the case, plasma cholinesterase is usually the cholinesterase most open to inhibition and it reactivates slowly (Skrinjarič-Spoljar *et al*., 1973), so that this enzyme is a useful marker of exposure. However, correlation with cholinergic symptoms is poor, so that plasma cholinesterase inhibition can be taken as a marker of exposure and no more. Erythrocyte acetyl cholinesterase inhibition often correlates better with cholinergic symptomatology, but reactivation can take place sufficiently quickly to interfere with the validity of blood tests, both in clinical and experimental situations, unless care is taken (Mason *et al*., 1993; FAO/WHO, 1993), reactivation occurring *ex vivo* in blood samples. The great sensitivity to inhibition and poor correlation with clinical cholinergic effects of inhibition of erythrocyte cholinesterase and, even more so, plasma cholinesterase have caused the Joint Meeting on Pesticide Residues (JMPR) to prefer inhibition of brain cholinesterase in the derivation of ADIs for OPs (WHO, 1990a). It is uncertain if such an approach will cover the implications of peripherally mediated intoxication (Ligtenstein, 1984) and the policy has since been modified (Herrman, 1998).

2.2.1 Organophosphates

OP anticholinesterases are esters of phosphoric, phosphonic or phosphorothioic or related acids.

Their general formula is:



The R groups in pesticides are generally either both methyl groups or both ethyl groups. The X or leaving group can be any one of large variety of moieties. The OP pesticides have some similarities with the chemical warfare nerve agents, but these are often phosphonofluoridates. Many pesticidal OPs are phosphorothioates and those containing P=S groups, such as malathion tend to be of lower acute mammalian toxicity than their corresponding phosphates and phosphonates. Thus, paraoxon is much more toxic than parathion, as is malaoxon compared to malathion. The reason for this is that the P=S phosphorothioates are inactive as anticholinesterases *in vitro* and only acquire toxicity after conversion of the P=S moiety to a P=O moiety, forming the oxon (WHO, 1986). Although OPs share their cholinergic symptomatology with the carbamates, the toxicology of OPs has certain features which do not resemble the effects of carbamates. These features are organophosphate-induced delayed polyneuropathy (OPIDP) and the so-called intermediate syndrome. Since the OP pesticides all have the same qualitative anticholinesterase action, and this property is responsible for their acute lethal toxicity, quantitative differences in toxicity are partly due to differences in absorption, distribution and metabolism. However, the rates of formation of the OP–acetylcholinesterase complex, of hydrolysis of this complex and of the ageing reaction (see below) must also be considered.

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2.2.1.1 Anticholinesterase Activity

Inactivation of cholinesterases by OPs involves a reaction in which the leaving group (X) is lost, typically producing a dialkylphosphoryl enzyme. Since most insecticides contain two methyl or two ethyl R groups, inactivation of cholinesterase produces a dimethoxyphosphorylated enzyme or a diethoxyphosphorylated enzyme and the kinetics of reactivation are the same for each derivative regardless of the structure of the leaving group of the OP. Reactivation of the dimethoxy phosphorylated enzyme will occur within a few hours and is considerably quicker than the diethoxy equivalent, while phosphorylated complexes containing one alkylthio group and one alkoxy group reactivate faster than those containing two alkoxy groups. By contrast, spontaneous reactivation of complexes containing larger R groups, for example isopropoxy and di-*sec*-butoxy is slow or nonexistent (WHO, 1986; Wilson *et al*., 1992; Mason *et al*., 1993), although large halogen-containing groups are an exception and seem to reactivate. With soman (pinacolyl methylphosphonofluoridate), a phosphonofluoridate nerve agent that has as one of its R groups a pinacoloxy moiety, reactivation fails to occur, but the mechanism is somewhat different: a further reaction takes place after dialkylphosphonylation of the enzyme in which the pinacolyl group is lost to leave a monoalkylphosphonyl enzyme. Such derivatives are refractory to oxime-induced reactivation and do not undergo spontaneous reactivation. With soman-inhibited enzyme the half-time for ageing is a few minutes. No pesticidal OP gives rise to a complex that ages at such a rate, but therapeutic failure with oximes has been attributed to ageing with certain pesticides (Glickman *et al*., 1984; Gyrd-Hansen and Kraul, 1984), and ageing half-lives for dimethoxyphosphorylated cholinesterases are in the region of 2–9 hours (Wilson *et al*., 1992).

2.2.1.2 Intermediate Syndrome

Senanayake and Karalliedde (1987) reported a syndrome that followed therapy for and resolution of the cholinergic effects of OP intoxication. As the syndrome developed before the late effects of OPs, the authors called this syndrome the intermediate syndrome. Since 1987, other examples have been recorded, for instance that by Karademir *et al*. (1990), and this phenomenon is probably the same as the Type II syndrome described by Wadia *et al*. (1987). The syndrome comprises a proximal limb paralysis starting one to four days after poisoning. The progression is not altered by atropine or oximes and, as the respiratory muscles are affected, respiratory support is necessary. A myopathy has been described *post mortem* in cases of human poisoning (de Rueck and Willems, 1975) and in experimental animals (Preusser, 1967; Wecker *et al*., 1978), but Karalliedde *et al*. (2006) concluded that intermediate syndrome was probably caused by down-regulation of nicotinic acetylcholine receptors at the neuromuscular junction as a consequence of acetylcholine accumulation. The myopathy appears to be initiated by calcium accumulation in the region of the motor endplate (Inns *et al*., 1990) again as a consequence of acetylcholine accumulation. So that rather than being causally related, intermediate syndrome and myopathy have a common origin in acetylcholine accumulation.

2.2.1.3 Organophosphate-Induced Delayed Polyneuropathy (OPIDP)

OPIDP is a symmetrical sensory-motor axonopathy, tending to be most severe in the long axons, occurring 7–14 days after exposure. It is a polyneuropathy in that there are central and peripheral components. There is degeneration of axons and Schwann-cell proliferation in the peripheral nervous system (Bouldin and Cavanagh, 1979a; 1979b; Cavanagh, 1982), and also changes in the spinal cord and medulla oblongata (Barrett *et al*., 1985). Clinically, the most disabling feature is the paralysis of the legs which may result. Less severe cases exhibit a characteristic high-stepping gait, and some recovery may occur, but there is no specific treatment (Barrett *et al*., 1985). The initial event in the pathogenesis of the syndrome appears to be inhibition of neuropathy target esterase (NTE). This is followed by an ageing reaction similar to that described for soman with acetylcholinesterase above (Johnson, 1975). On the basis of studies with NTE-null mice and *Drosophila*, Glynn (2006) hypothesized that OPs producing OPIDP caused a transient loss of NTE activity, disrupting membrane phospholipid homeostasis and endoplasmic reticulum functions, such as glial–axonal interaction and axonal transport; the distal parts of long axons would be particularly susceptible to loss of these functions. It should be noted that the structural requirements for inhibition of acetylcholinesterase and NTE are different as shown by the fact that many OPs with powerful anticholinesterase properties are devoid of the ability to produce OPIDP. It should be noted that the nerve agents, which are powerful anticholinesterases, have little propensity to cause OPIDP (Gordon *et al*., 1983), while tri-*o*-cresyl phosphate, which has little anticholinesterase activity, is powerfully neuropathic.

Regulatory authorities typically demand the use of tests to detect propensity for the development of OPIDP and this has resulted in the disappearance of most OPs that are capable of producing OPIDP from the market. The usual test that is carried out is one using hens, the reason for the use of hens being that these creatures are very susceptible to the syndrome. However, mice and rats also develop OPIDP (Veronesi *et al*., 1991) and the reported resistance of rodents to the development of OPIDP has been attributed to the use of young animals in studies (Moretto *et al*., 1992). Several OPs not in current use as pesticides produce OPIDP, including mipafox and diisopropyl phosphorofluoridate, while leptophos, also not currently used in most countries, has been consistently reported

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to produce OPIDP. There is some limited experimental and clinical evidence that a few OPs currently used in some countries can produced OPIDP, for example, *O*-ethyl *O*-(4-nitrophenyl) phenylphosphonothioate (EPN), methamidophos, cyanofenphos and trichloronat (El-Sebae *et al*., 1981; Gallo and Lawryk, 1991). There is some evidence that the hen test may give misleading results with chiral OPs, which are usually used as insecticides as racemic mixtures (Battershill *et al*., 2004); the vast majority of insecticidal OPs are not optically active, but a few, for example methamidophos, are.

2.2.1.4 Other Delayed Effects of OPs on the Nervous System

The behavioural neuropsychiatric toxicity of anticholinesterases, *inter alia* OPs, has been reviewed (D'Mello, 1993; Lader, 2001). Since acute intoxication with OPs can cause major effects, such as convulsions, respiratory failure and cardiac arrhythmias, all of which can result in cerebral anoxia, it is hardly surprising that major intoxication is sometimes associated with long term CNS changes (Tabershaw and Cooper, 1966; Bartels and Friedel, 1979; Korshak and Sato, 1977; Hirshberg and Lerman, 1984; Holmes and Gaon, 1956; Burchfiel *et al*., 1976; Duffy *et al*., 1979; Durham *et al*., 1965; Savage *et al*., 1988). More information has come available in studies following up survivors of the use of sarin, an OP ester, in Tokyo (see Okumura *et al*., 2007). More debatable is whether long-term low-dose exposure produces delayed or chronic effects. Such an outcome is biologically less plausable than delayed effects of acute exposure, but the two problems have frequently been conflated. Studies pertaining to long-term low-dose exposure, such as those by Stephens *et al*. (1995), Beach *et al*. (1996), Ames *et al*. (1995) and Fiedler *et al*. (1997) have been reviewed (Eyer, 1995; Steenland, 1996; IEH, 1998; Committee on Toxicity of Products in Food, Consumer Products and the Environment, 1999). Steenland (1996) concluded that studies had shown chronic subclinical effects in the central and peripheral nervous system in individuals previously poisoned by OPs, but that the outcome after long-term low-level exposure was less consistent, although some studies had shown effects. Further, the effects of low-level exposure to OP pesticides on foetal and childhood health have also been reviewed (IEH, 2002). A number of OP pesticides (and other compounds) have been tested in the USEPA's developmental toxicity study in the rat (Makris, 2006) and the results of these studies are starting to become available (FAO/WHO, 2003).

2.2.1.5 Other Effects of OPs

It should never be forgotten that OPs may have properties, some of which may be entirely independent of their anticholinesterase effects including mutagenicity and carcinogenicity as well as organ specific toxicity to the heart and kidney and other organs (Singer *et al*., 1987; Baskin and Whitmer, 1992; Pimentel and Carrington da Costa, 1992; Wedin, 1992). Pancreatitis has frequently been reported as a complication of acute OP poisoning (Dagli and Shaikh, 1983).

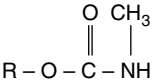
2.2.1.6 Treatment of OP Poisoning

The treatment of OP poisoning involves symptomatic treatment and the use of antidotes. Atropine, an anticholinergic compound, and an oxime enzyme reactivator such as pralidoxime chloride (2-PAM) or obidoxime is used (Bismuth *et al*., 1992; Szinicz *et al*., 1996), while convulsions and muscle fasciculation respond to diazepam (Johnson and Vale, 1992). The antidotal treatment is only effective against acute poisoning and specific treatment is not available for OPIDP or other long-term effects (see reviews by Marrs and Vale, 2005; Roberts and Aaron, 2007).

2.2.2 Carbamates

Carbamates are used as insecticides, herbicides and fungicides, but only the first group has marked anticholinesterase activity. Methiocarb (section 6), an anticholinesterase, is used as a molluscicide.

The anticholinesterase carbamate pesticides are closely related to human drugs such as physostigmine and pyridostigmine. The general structure of most carbamate insecticides is as follows:



In most cases, the R group is a substituted phenol or heterocycle, notable exceptions being aldicarb, which has an *N*-alkyl R group, and pirimicarb, which has two *N*-methyl groups.

In general, the carbamates produce toxicity similar to that of OPs, but less severe. A major difference is that carbamate-inhibited cholinesterases reactivate more rapidly than enzyme-inhibited by OPs, with the result that the effects do not last as long in carbamate poisoning. Indeed reactivation of carbamylated enzyme may be quick enough to render detection of cholinesterase depression difficult in both experimental animals and clinical situations. However, an insecticidal carbamate, aldicarb, is one of the few pesticides that has given rise to poisoning in consumers of treated food. Residues have occurred in cucumbers, watermelons, squashes and similar products, sufficient to cause illness, in some cases severe. Outbreaks have occurred in the USA and Canada (Hall and Rumack, 1992) and in the Irish Republic (Department of Agriculture and Food, 1992). The reason for these problems is that aldicarb, in other respects a satisfactory pesticide, has a high mammalian toxicity, with an acute oral LD_{50} of about 1 mg (kg body weight)$^{-1}$. Atropine is effective in carbamate poisoning, but it is unclear whether oximes are, or are not, beneficial

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(Mercurio-Zappala *et al*., 2007). Due to the rapid reactivation of carbamoylated acetylcholinesterase, the value of oximes is questionable from a theoretical point of view.

2.3 Nicotine and the Neonicotinoids

2.3.1 Nicotine Sulfate

Nicotine is extracted from tobacco (*Nicotiana tabacum*) or other *Nicotiana* species and is one of the oldest botanical insecticides. Nicotine is very toxic to mammals by mouth (oral rat LD_{50} 52–56 mg (kg body weight)$^{-1}$ Lazutka *et al*., 1969), and it is readily absorbed through the skin (rabbit dermal LD_{50} 50 mg kg^{-1}, Negherbon, 1959). When nicotine was widely used as an insecticide it was responsible for many suicides and accidents (see review by Ujváry, 2001). In some parts of the world nicotine is permitted in organic agriculture.

2.3.2 Neonicotinoids

The neonicotinoid group of insecticides comprise what is regarded by some as the most important new class of synthetic insecticides of recent times, and the history of this group of compounds has been reviewed (Tomizawa and Casida, 2003). These insecticides are structurally related to nicotine and the first to be commercialized was imidacloprid. As with nicotine, this group of compounds acts as agonists at the cholinergic nicotinic receptor, but the neonicotinoids are safer for humans than nicotine. This reduced toxicity in mammals is possibly associated with the different receptor binding characteristics (see below). The cholinergic nicotinic receptor has many subtypes in organisms, including mammals, and these receptors are part of a superfamily of ligand-gated ion channels with γ-aminobutyric acid A ($GABA_A$) receptors and glycine receptors (Ortells and Lunt, 1995). Each receptor comprises five subunits arranged around a channel that passes through the cell membrane (for reviews see Kaminski and Ruff, 1999; Kalamida *et al*., 2007). There is evidence that the toxicity of neonicotinoid insecticides may involve action at multiple subtypes and that minor changes in molecular structure may confer selectivity towards insects (Tomizawa and Casida, 1999). An extensive quantitative structure–activity study was undertaken by Debnath *et al*. (2003) using electrotopological state atom indices. This study showed that nitroimines, nitromethylenes and cyanoimines exhibited selectivity towards the *Drosophyla malanogaster* nicotinic receptor and predicted that such structures would be relatively safe for mammals, whereas *N*-substituted imines had greater affinity for an important subtype ($\alpha_4\beta_2$) of mammalian nicotinic receptor. It has also been hypothesized that the ionized nicotine binds at the anionic subsite in the mammalian nicotinic receptor, while the negatively charged neonicotinoids bind at a cationic subsite on the insect nicotinic receptor (Zhang *et al*., 2002).

2.3.2.1 Imidocloprid

Commercially, the most important of the neonicotinoids is imidacloprid. The selective toxicity of imidacloprid to insects and not to mammals is partly attributable to differences in the binding affinity or potency at the nicotinic acetylcholine receptor (Chao and Casida, 1997; Tomizawa and Casida, 2005; see also Ohno *et al*. 2009). Also, imidacloprid is poorly ionized in neutral media, in which respect it differs from nicotine, and thus passes easily through insect lipophilic barriers (Sone *et al*., 1994; Roe *et al*., 1999). The toxicology of imidacloprid has been reviewed (Sheets, 2001; FAO/WHO, 2002). Imidacloprid is of moderate acute toxicity in rats (LD_{50}, 380–650 mg (kg body weight)$^{-1}$) and mice (LD_{50}, 130–170 mg (kg body weight)$^{-1}$) (FAO/WHO, 2002). In repeated-dose studies, reductions in body weight gain were seen in mice, rats, rabbits and dogs. The other changes observed were in the liver, where induction of hepatic microsomal enzymes, disturbance of hepatic function and/or histological changes, such as mild hepatocellular hypertrophy and necrosis, cell nuclear swelling and round-cell infiltration were seen. Increased liver weight was also apparent. Imidacloprid was neither carcinogenic nor teratogenic, nor was it a reproductive toxin. In the full range of genotoxicity studies required premarketing, imidacloprid was not genotoxic. The JMPR concluded that imidacloprid was unlikely to be genotoxic because an adequate range of assays for genotoxicity *in vitro* and *in vivo* gave negative results. Weak induction of sister chromatid exchange was found in one test with Chinese hamster ovary cells *in vitro,* but not *in vivo* (FAO/WHO, 2002). Karabay and Oguz (2005) reported that imidacloprid and an imidacloprid/methamidophos mixture were mutagenic in *Salmonella typhimurium* strain TA 98, with or without S9; furthermore imidacloprid and the mixture both induced micronuclei in rat erythrocytes and chromosomal aberrations in rat bone-marrow cells, both after 90 days dietary administration. In a single-dose rat neurotoxicity study, clinical signs of cholinergic toxicity were seen. These included tremor and uncoordinated gait; nasal staining was also observed. A few cases of human poisoning with imidacloprid have been reported in the literature, including three fatal cases (Proença *et al*., 2005; Huang *et al*., 2006; Agarwal and Srinivas, 2007; David *et al*., 2007). In the case reported by David *et al*. (2007), where poisoning was caused by intentional ingestion and which was nonfatal, the main findings were drowsiness, tachycardia (followed by bradycardia) and vomiting. In the other nonfatal case, that described by Agarwal and Srinivas (2007), there were severe psychiatric manifestations, which the authors ascribed to central

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nicotinic stimulation; the patient also had rhabdomyolisis, which the authors suggested could have been caused by nicotinic actions at the neuromuscular junction or by fever. Exposure in this case was by inhalation. The two cases described by Proença *et al*. (2005) were both found dead, hence there were no descriptions of clinical signs and symptoms; however, the presence of imidacloprid in body fluids and tissues was established and there was circumstantial evidence that they had ingested imidacloprid. The case described by Huang *et al*. (2006) was a woman who drank about 200 ml of a 9% solution of imidacloprid. The patient had hypertension for six years; in addition, eight months before the poisoning, she had a stroke. On admission she was disorientated and vomiting. She developed intermittent ventricular fibrillation and died 12 hours after admission.

Both the data from experimental animals and from human poisonings suggest that imidacloprid is capable of producing cholinergic signs, so that specificity towards insects is clearly not complete. However, imidacloprid has achieved widespread usage and is clearly a comparatively safe compound under approved conditions of use.

2.3.2.2 Other Neonicotinoids

A number of other neonicotinoids have been developed. These include acetamiprid, nitenpyram, thiacloprid, chlothianidin, nithiazine and thiamethoxam. Kayser *et al*. (2004) found that while acetamiprid, nitenpyram, thiacloprid, chlothianidin and nithiazine displaced ^{3}H-imidacloprid competitively from specific binding sites on membranes from the aphids *Mysus persica* and *Aphis craccivora*, thiamethoxam did so noncompetitively, suggesting that the last-named compound bound at a different site or in a different way to the aphids' nicotinic receptor. Thiamethoxam has been observed to cause liver tumours in long-term studies in mice, but not rats (Green *et al*., 2005a). It has been hypothesized that this effect is produced by a metabolite and is exacerbated by a second metabolite, which is an inhibitor of nitric oxide synthase; blood concentrations of these metabolites were much lower in rats than in mice in metabolic studies (Green *et al*., 2005b).

2.4 Pyrethroids (and Pyrethrins)

Pyrethrins are natural insecticides produced from *inter alia* pyrethrum, a plant of the Compositae group, and are esters of pyrethric or chrysanthemic acids. Pyrethrins are broken down and deactivated very readily in the environment, particularly by sunlight. The synthetic pyrethroids are structurally similar compounds rendered photostable by various substituent groups, such as chlorine, bromine or cyanide. Some of the newer ones bear a more distant structural relationship to the pyrethrins. Because of their low mammalian toxicity, high insecticidal potency and lack of persistence in the environment, the synthetic pyrethroids have achieved widespread usage in agriculture, as household insecticides and in wood preservation. Synthetic pyrethroids are also used in mosquito control (White, 1993). They have also been widely used on humans to treat such conditions as scabies, and as veterinary ectoparasiticides. However they are very toxic to aquatic organisms (Zitko *et al*., 1979) and their lack of persistence can be a problem when used as wood preservatives. The advantageous properties of the synthetic pyrethroids result from the fact that they hydrolyse relatively easily, both in the mammalian body and in the environment. Consequently, bioaccumulation does not occur to a significant extent, nor do they persist in soils.

In general, by the oral route, the synthetic pyrethroids are of low acute toxicity, but this is not the case when they are administered to mammals parenterally when they are neurotoxic by virtue of their action upon voltage-dependent sodium channels (Vijverberg and van den Bercken, 1990; Vijverberg, 1994). As a result, there is often a large difference between the oral LD_{50} and LD_{50}s by parenteral routes. Despite the neurotoxicity of this group of compounds, underlying pathological changes in the nervous systems of animals exposed to pyrethroids are slight (Aldridge, 1990). Pyrethroids can be separated into two classes on the basis of the central neurotoxic syndrome that they produce by parenteral routes in experimental animals (Vijverberg, 1994). Type I synthetic pyrethroids, which include permethrin and resmethrin as well as the components of natural pyrethrum, lack an α-cyano group and give rise to T-syndrome. The T-syndrome is characterized by fine tremor, hypersensitivity to stimuli and aggressive sparring, progressing to coarse whole-body tremor; this syndrome is rather similar to that produced by some of the OCs. Type II compounds, which include deltamethrin, flumethrin, cyfluthrin and γ-cypermethrin have an α-cyano group and give rise to CS syndrome. The CS syndrome consists of initial pawing and burrowing and later marked choreoathetosis, salivation, course tremor and convulsions (Aldridge, 1990; Joy, 1994). As well as these central effects in experimental animals, pyrethroids cause peripheral nerve damage (in rats) with functional impairment when administered repeatedly at near-lethal dose levels. (Rose and Dewar, 1983). Axonal degeneration has been described, but generally at near lethal doses and there is no evidence that pyrethroids can produce delayed neuropathy of the OP type (Aldridge, 1990).

Despite the findings in the central and peripheral nervous systems of animals, in humans the most prominent effect of the pyrethroids is to cause transient paraesthesia, mainly in the face, and there is little evidence of any permanent effects in man. Pruritis with blotch erythema, itching, rhinorrhoea and lachrymation have also been described (Aldridge, 1990). Deltamethrin seems more potent and permethrin less so, in producing these effects. Additionally, the pyrethroids are potent allergens

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and allergic rhinitis, asthma and extrinsic allergic alveolitis have been reported (Bismuth *et al*., 1987).

Specific treatment of the effects of synthetic pyrethroids is rarely necessary, as systemic effects are very unusual in humans and where reported these are transient.

Table 2 Insecticides of natural origin

Group	Active ingredients
Avermectin group	Abamectin
	Ivermectin
Spinosins	Spinosad
Derris and similar insecticides	Rotenone
Cholinergic group	Nicotine
Pyrethrum	Pyrethrins

2.5 Insecticides of Biological Origin Other than Pyrethrum and Nicotine

A number of insecticides are available that are of biological origin. The pyrethrins, discussed above with the synthetic pyrethroids, form one group. Of the others, only the avermectins, spinosad and derris are at all widely used (see **Table 2**).

2.5.1 Avermectins

Abamectin is the common name for a mixture of avermectin B1a and B1b, macrocyclic lactone disaccharide antibiotics from *Streptomyces avermitilis*. Abamectin is an insecticide and acaricide used in crop protection, while, in veterinary medicine, it is used as an anthelmintic. The dihydro derivative, ivermectin, is used to control nematodes and arthropods, in animals, and, in man, for the control of onchocerciasis (Wright, 1986) (see below). The insecticidal activity is based on action upon GABAergic nerve transmission; because mammals only have GABAergic synapses in the CNS, the mammalian blood–brain barrier ensures a degree of specificity. A notable feature of this group of compounds is their low LD_{50}s; thus, the oral LD_{50} for abamectin in mice is $14–24\,mg\,kg^{-1}$ and for ivermectin is $25–40\,mg\,kg^{-1}$ (Lankas and Gordon, 1989). The acute oral toxicity of abamectin was shown to be directly related to the expression of P-glycoprotein genes; homozygous negative CF-1 mice were over an order of magnitude more sensitive than heterozygous and homozygous positive CF-1 mice (WHO, 1998). Abamectin and ivermectin are neurotoxic, producing tremors, weakness and incoordination, and can induce malformations in offspring in teratological studies in CF-1 mice and in cultured embryos (Lankas and Gordon, 1989; FAO/WHO, 1993; Chaconas and Smoak, 1997). Ivermectin, used in human infestations with parasites, rarely gives rise to effects related to outcomes observed in animal toxicology studies; thus, reactions (encephalopathy) reported to the use of ivermectin in infestation with *Onchocerca volvulus* were correlated to the load of microfilaria of *Loa loa*, and were probably not a direct toxic effect of ivermectin (Gardon *et al*., 1997). Both abamectin and ivermectin are nongenotoxic and almost certainly not carcinogenic (WHO, 1991; 1996; FAO/WHO, 1993).

2.5.2 Spinosad

Spinosad is the ISO name for a mixture of compounds from fermentation carried out by a soil organism *Saccharopolyspora spinosa*. It causes rapid excitation of the insect nervous system. Spinosad is of low acute toxicity by mouth, administered dermally or by inhalation in experimental animals. In repeated-dose studies, spinosad caused increased liver weights in rodents. In dogs, increased thyroid weights and histopathological changes in the lymphatic system (vacuolation of cells in the spleen and lymph nodes) were seen. Also in dogs, in the liver there were vacuolated hepatocytes and Kupffer-cell proliferation and in the pancreas, vacuolated acinar cells. Vacuolation was seen in other organs. Spinosad gave negative results in an adequate range of assays for genotoxicity *in vivo* and *in vitro*. The JMPR concluded that spinosad is not genotoxic and unlikely to pose a carcinogenic risk to humans. Spinosad was only foetotoxic at maternally toxic doses and was not embryotoxic or foetotoxicity in a multigeneration study (FAO/WHO, 2002; see review by Huang *et al*., 2009).

2.5.3 Derris and Similar Insecticides

The other insecticide of biological origin that is very well known is rotenone, under the name derris. It has acquired this name as the garden insecticide marketed under this name is derived from two Asiatic plants, *Derris eliptica* and *Derris mallaccensis*. Another plant *Lonchocarpus utilis*, from South America, is a further source of an insecticide containing rotenone. Rotenone blocks mitochondrial electron transport at Complex I and this is responsible for the toxic effects in mammals, including humans. It can also cause dermatitis. In severe human poisoning, the main effects that have been observed have been vomiting, depressed respiration and eventually apnea (DeWilde *et al*., 1986). Intravenous infusion of rotenone into rats at doses of $1–12\,mg$ (kg body weight)$^{-1}$ day^{-1} was reported to produce cardiovascular toxicity. Lower doses produced nigrostriatal dopaminergic lesions in the CNS. The

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Table 3 Insecticides that interfere with insect growth and development

Group	Examples
Insect growth regulators (juvenile hormone analogues)	Methoprene Hydroprene Fenoxycarb Cyromazine Dicyclanil
Chitin-synthesis inhibitors	Diflubenzuron
Ecdysone agonists	Tebufenozide

relevance of this observation to human illnesses such as Parkinson's disease is dubious in the light of the dose and route of administration (Betarbet *et al*., 2000).

2.6 Juvenile Hormone Analogues, Chitin Synthesis Inhibitors and Ecdysone Agonists

The attraction of these groups of insecticides is that they have no direct target organ or system in mammals analogous to those that the insecticides target in insects. The toxicity is therefore generally nonspecific and requires relatively high exposures. Examples are given in **Table 3**.

2.6.1 Insect Growth Regulators

The insect growth regulators (juvenile hormone analogues) are esters of long-chain fatty acids, in the case of hydroprene and methoprene, or trazines in the case of cyromazine, while dicyclanil is a cyclopropylaminopyrimidine. Fenoxycarb is a carbamate. They prevent metamorphosis of insect larvae into viable adults, when applied to the larvae. They are generally of low acute mammalian toxicity (oral LD_{50} about 5 g (kg body weight)$^{-1}$. They are usually nonteratogenic and nongenotoxic, and are without endocrine activity in mammals (FAO/WHO, 1985; Ray, 1991). Methoprene caused changes such as bile duct proliferation in the livers of rats during a two year study at doses of about 220 mg (kg body weight)$^{-1}$ day^{-1} (FAO/WHO, 1985). Cyromazine (Vetrazine®), which is used as a sheep ectoparasiticide, is of low acute toxicity. In short-term studies in rats and dogs and in long-term studies in mice and rats, effects on body weight were seen. Red blood cell counts and haemoglobin levels were reduced in dogs at high dietary concentrations. In a rat multigeneration study, cyromazine did not affect fertility, but there was increased perinatal pup mortality and reduced pup weight, at maternally toxic doses. Abnormalities, including cyclopia with multiple head anomalies in two foetuses, were observed in a rabbit study seen by the JMPR, but there was no clear dose response and JMPR did not consider cyromazine to be teratogenic, nor was it genotoxic (FAO/WHO, 1991). Dicyclanil is also used on sheep. Dicyclanil is slightly hazardous by mouth (LD50 >2000 mg (kg body weight)$^{-1}$). In a three month study in dogs, neurotoxity (ataxia and tremor), decreased food consumption and body-weight gain, and changes in clinical chemistry and erythrocyte parameters (reduced haemoglobin and haematocrit with microcytosis and erythrocytic hypochromasia) at the high doses, while severe liver toxicity was seen in a one year dog study. The Joint Expert Committee on Food Additives (JECFA), considered that dicyclanil was not genotoxic nor did it pose a carcinogenic risk to humans. Dicylcanil was not a reproductive toxin, while the NOAEL (no observable adverse effect level) for pup toxicity (reduced body weight gain) was slightly below the NOAEL for parental toxicity. Dicyclanil was not teratogenic and was only foetotoxic at dose above those that were maternally toxic. The carbamate, fenoxycarb, exerts most of its insecticidal activity as a juvenile hormone analogue and is of low mammalian toxicity (Evans, 1993; Tomlin, 2006).

2.6.2 Chitin Synthesis Inhibitors

Chitin synthesis inhibitors, such as diflubenzuron, are also generally of low toxicity. These insecticides, which are benzoylphenylureas, act by interfering with the formation of the insect cuticle and the action is very specific, in that the compounds do not appear to inhibit hexosamine transferases, which are responsible for connective tissue glycosaminoglycan formation in mammals (FAO/WHO, 1982; Tomlin, 2006). A notable toxicological effect of diflubenzuron in experimental animals is to cause changes in haematological parameters, such as methaemoglobinaemia and sulfhaemoglobinemia, together with a fall in the red blood cell count and increased Heinz-body formation. This probably results from the metabolism of the compound to *inter alia* 4-chloroaniline (FAO/WHO, 1982).

2.6.3 Ecdysone Agonists

Tebufenozide mimics the action of ecdysone by binding to its receptor and bringing about a lethal unsuccessful moult. It is of low toxicity to rodents, but in experimental studies in laboratory animals it causes mild haemolytic anaemia. Methaemoglobinemia is seen in mice. The compound is neither carcinogenic nor genotoxic (FAO/WHO, 1997; 2004).

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2.7 Miscellaneous Synthetic Insecticides

2.7.1 Amitraz

Amitraz is a formamidine compound, has resulted in a number of intoxications (Aydin *et al*., 1997). The main feature is central nervous system depression, which is accompanied by hypotension, hypothermia and bradycardia. Hyperglycaemia also occurs.

2.7.2 Fipronil

Fipronil is an insecticide with many uses. It is used as a malarial control agent and to control fleas on cats and dogs. Fipronil has a novel mode of action, acting on the $GABA_A$ receptor (FAO/WHO, 1998). At high doses in experimental animals, neurotoxicity is observed. Mohamed *et al*. (2004), who reported a series of poisonings from Sri Lanka, found that fipronil poisoning was characterized by vomiting, agitation and convulsions; in most cases the outcome was favorable.

3 FUNGICIDES

The fungicides are a heterogeneous group of chemicals that defy convenient chemical classification; their action is often upon the cytoskeleton and therefore relevant to both fungi and mammals. The main classes of fungicides are given in **Table 4**.

3.1 Metals

3.1.1 Inorganic Metallic Fungicides

Some of the first fungicides used in agriculture were neutralized copper sulfate preparations such as Bordeaux mixture, which was introduced in the nineteenth century and consists of lime and copper sulfate. Compounds of mercury, both mercurous chloride (calomel) and mercuric chloride have long been used as fungicides, but have now been withdrawn in many countries due to their toxicity and environmental concerns.

3.1.2 Organometals

The organometals are in general fairly toxic compounds, many being neurotoxic. Trimethyltin and triethyltin are highly neurotoxic, but not used as pesticides. Tributyltin oxide (TBTO) was, for a time, extensively used in wood preservatives and in antifouling paints on boats, but use has been considerably restricted some countries, for example, the UK (Advisory Committee on Pesticides, 1990; Cavanagh and Nolan, 1994). Substances such as fentin (triphenyltin) and fenbutatin oxide, which are used in agriculture and horticulture, are poorly absorbed by mouth, and are of low oral toxicity (FAO/WHO, 1992; 1993), and any neurotoxicity they possess is mild and nonspecific (Manzo *et al*., 1981; Bock, 1981). Organotins are immunotoxic; thus, fentin causes lymphopenia and lymphocyte depletion of the spleen and thymus in experimental animals (FAO/WHO, 1992). The immunotoxicity of TBTO was reviewed extensively by WHO (1990b).

Organic compounds of copper and zinc are also used as fungicides, especially as wood preservers.

3.2 Carbamate Fungicides and Thiabendazole

A number of carbamates, predominantly the dithiocarbamates and benzimidazole derivatives, possess fungicidal activity.

3.2.1 Dithiocarbamates

The dithiocarbamates include three main groups of fungicides, methyl dithiocarbamates, metam, dimethyl dithiocarbamates, ferbam, thiram and ziram, and ethylenebis(dithiocarbamate)s (EBDCs), maneb, zineb and mancozeb, the last group being degraded to ethylene thiourea. Propineb is closely related to the last group and is polymeric zinc propylenebis(dithiocarbamate). All dithiocarbamates seem to have the potential to affect the thyroid, decreasing plasma thyroxine levels and increasing TSH levels; the active compounds being the alkyl-thiourea metabolites.

The dimethyldithiocarbamates, ferbam, thiram and ziram, contain the same core molecule, together with iron, in the case of ferbam, and zinc in the case of ziram. Amongst their metabolites is carbon disulfide, which may account for hepatotoxicity. The dithiocarbamates are generally not highly toxic, but they interact with alcohol in humans. In this context, it should be noted that the drug disulfiram (Antabuse), used to treat alcoholism, is the ethyl analogue of thiram (tetramethylthiuram disulfide). Both thiram and ziram, but, on the basis of limited studies, apparently not ferbam, have shown mutagenic potential in bacteria. Thiram and ziram are considered not to be mutagenic in mammalian systems *in vivo* (FAO/WHO, 1993; 1997).

Maneb is polymeric manganese EBDC, zineb is the same compound with zinc replacing the manganese, while mancozeb is a complex of the two. A notable feature of the EBDCs is that one of their metabolites is ethylene thiourea and this substance forms a major part of the residues, to which consumers are exposed. The EBDCs reduce iodine uptake by the thyroid

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Table 4 Main classes of fungicides with example compounds

Group	Subgroup	Example	
Metals	Inorganic	Copper sulfate (Bordeaux mixture) Mercurous chloride	
	Organic	Organomercurials	Methyl mercury sulfate
		Organotins	Tributyltin oxide Fentin
Carbamates	Dithiocarbamates	Methyl dithiocarbamates	Metam
		Dimethyl dithiocarbamates	Ferbam Ziram Thiram
		Ethylenebis dithiocarbamates	Maneb Zineb Mancozeb
	Benzimidazole carbamates and similar compounds	Benomyl Carbendazim Thiophanate Thiophanate-methyl Thiabendazole	
	Other compounds similar to carbamates, for example, dicarboximides	Iprodione	
Chloroalkyl thio compounds	—	Captan Folpet Captafol	
Phenols and derivative	—	Dinocap Pentachlorophenol Dichlorophen 2-Phenylphenol	
Hydantoins	—	Vinclozalin	
Organophosphates	—	Pyrazophos Tolclofos-methyl	
Azoles	—	Hexaconazole Penconazole Tebuconazole Cyproconazole Fenbuconazole Imazalil	
Morpholines	—	Dodemorph Fenpropimorph Tridemorph	
Miscellaneous fungicides	—	Quintozene Chlorothalonil Creosote Boron compounds	

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and repeated administration produces thyroid hyperplasia that is initially reversible and is associated with decreased plasma thyroxine and triiodothyronine levels; these effects are also observed in studies of ethylene thiourea (FAO/WHO, 1994). Prolonged dosage produces thyroid tumours in animals (FAO/WHO, 1994). Propineb is similarly metabolized in plants and animals to propylene thiourea, which is goitrogenic and interferes with thyroid function in the same was as ethylene thiourea (FAO/WHO, 2000). Maneb is one of a number of pesticides where an association with exposure and development of Parkinson's disease has been suggested (see review by Barlow *et al*., 2007; IEH, 2005). Manganese is itself associated with Parkinsonism (Perl and Olanow, 2007).

3.2.2 Benzimidazole Carbamates

The benzimidazole carbamates include benomyl and its hydrolysis product carbendazim, as well as thiophanate and thiophanate-methyl. Thiabendazole, although not strictly a carbamate, shares many of the properties of the benzimidazole carbamate fungicides. The acute toxicity of carbamate fungicides of this type is usually low. Thus thiophanate has an oral LD_{50} of more than 5 g (kg body weight)$^{-1}$ in rodents (Hashimoto *et al*., 1970). Carbendazim and benomyl have detrimental effects on fertility of male rats (Carter and Laskey, 1982; Carter *et al*., 1987) and these two fungicides have been shown to be embryotoxic and teratogenic, under experimental conditions; thus, in rats, benomyl produces craniocerebral and ocular abnormalities (Kavlock *et al*., 1982; Janardhan *et al*., 1984; Cummings *et al*., 1990; Hoogenboom *et al*., 1991; FAO/WHO, 1996). Both carbendazim and benomyl have produced microphthalmia in certain experimental studies using high dose levels (FAO/WHO, 1996). With benomyl and carbendazim, a major area of concern has been the possibility of these two pesticides being spindle poisons and both pesticides cause numerical chromosomal changes *in vitro* and *in vivo* (Georgieva *et al*., 1990; Pandita, 1988; FAO/WHO, 1996). Some of these effects may be related to the effects of benomyl and carbendazim upon microtubules and thus to the fungicidal effects, which are mediated through an effect in binding to fungal tubulin and preventing polymerization.

3.2.3 Other Compounds Similar to Carbamates

Iprodione is a dicarboximide, of low acute toxicity. It is not genotoxic, but in long-term studies, high doses produced skin lesions in dogs, and increased liver and testicular tumours in rodents. In dogs, a reduced red blood cell count was seen, with, in some studies, Heinz-body formation (FAO/WHO, 1993; 1996). There are some reports that iprodione may disrupt androgen functions.

3.3 Chloroalkylthio-Containing Fungicides

This group consists of captan, folpet and captofol. They are of low acute mammalian toxicity. However, because of the similarity of their molecules to thalidomide, there was considerable concern over their effect on reproductive and foetal development. The negative results of reproductive studies into the chloralkylthio fungicides at doses not producing maternal toxicity, suggest that another part of the thalidomide molecule is responsible for the teratogenicity of that drug (Edwards *et al*., 1991). There also have been concerns over the ability of some fungicides of this class to induce gastrointestinal tumours in experimental animals, and captan and folpet are mutagenic *in vitro,* but not *in vivo* (FAO/WHO, 1991; 1996). Indications are that the gastrointestinal tumours are related to the irritant effects of the thiophosgene moiety (FAO/WHO, 1996).

3.4 Phenols

One of the most widely used wood preservers has been pentachlorophenol, which is a potent fungicide. Due to concerns over its persistence and content of chlorinated dibenzo-*p*-dioxins, pentachlorophenol is now severely restricted in some parts of the world and banned in others. Other phenolic derivatives with fungicidal properties include dinocap, which is an ester of an alkyl-substituted dinitrophenol and dinitro-*o*-cresol. The main mammalian toxic action of this group of compounds is to cause uncoupling of oxidative phosphorylation and hyperthermia, with collapse and death at high dose. Liver and kidney involvement occurs in fatal human poisoning, while in experimental animals the onset of *rigor mortis* is notably fast after death. Some compounds of the phenol group of fungicides induce cataracts in experimental animals, for example dinocap (FAO/WHO, 1990).

3.5 Hydantoins

This group includes vinclozolin, which has been a cause of concern because of its reproductive toxicity (Advisory Committee on Pesticides, 1992). In a long-term study in rats, there was atrophy of the accessory sex glands and an increased incidence of Leydig cell tumours, while in multigeneration studies in the same species, there was infertility in the males associated with feminization of the external genitalia. Changes have been observed in other species and it is likely that vinclozolin acts as an antiandrogen (FAO/WHO, 1996).

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3.6 OPs

Pyrazophos, although an anticholinesterase OP, is used as a fungicide. It has anticholinesterase properties and, insofar as mammalian toxicity is concerned, has many features in common with OP insecticides (FAO/WHO, 1993). Tolclofos-methyl is an OP fungicide, which has limited anticholinesterase properties and low mammalian toxicity (PSD, 1993).

3.7 Azoles

Fungicides of this group (the 'conazoles') inhibit sterol synthesis in fungi and they have some activity on steroid metabolism in animals. The toxicology of all members is rather similar, effects on the liver (hypertrophy and enzyme induction) being noted in subacute and long-term studies and, with some compounds, effects on the reproductive system, such as delayed parturition, which are mild and do not appear adversely to affect reproductive performance. Anaemia is seen, for example, with hexaconazole. The compounds are mostly not carcinogenic, although Leydig cell tumours are sometimes observed and fenbuconazole produces effects on the thyroid, including tumours via effects on thyroxine metabolism (FAO/WHO, 1998). Foetotoxicity is seen at maternally toxic doses and, in some cases, for example, tebuconazole and diniconazole, teratogenicity, although again generally only at maternally toxic doses (Advisory Committee on Pesticides, 1990; 1993).

Hexaconazole, a fairly typical triazole, produces effects on the female and male reproductive systems. From studies *in vitro,* it is believed that hexaconazole inhibits testosterone production. Nevertheless, reproductive parameters are not affected in multigeneration studies. This compound is not genotoxic and probably not carcinogenic, although the frequency of Leydig cell tumours appeared to be treatment related in rats (FAO/WHO, 1991). The compound is foetotoxic at maternally toxic doses, but it is not teratogenic, although in a rabbit study there was reported to be some delayed ossification in rabbit foetuses. The toxicology of penconazole is similar; with this compound, delayed ossification was observed more consistently than with hexaconazole (FAO/WHO, 1993). Cataracts were observed in the eyes of dogs fed tebuconazole (Advisory Committee on Pesticides, 1993). Imazalil was reported to have effects on the liver in a long-term rat study; furthermore there was a decreased number of live births at the top dose in a multigeneration study (FAO/WHO, 1978; 1985). This fungicide is been used in humans and in veterinary medicine under the name enilconazole (INN). It appears to have been well tolerated (Edwards *et al*., 1991).

3.8 Morpholines

This group of fungicides has a similar action in fungi to the azoles. Dodemorph, fenpropimorph and tridemorph all inhibit the synthesis of ergosterol. They are of low acute toxicity, although tridemorph is reported to be teratogenic and foetotoxic in rodents (Barbiere and Ferioli, 1994).

3.9 Miscellaneous Fungicides

3.9.1 Chlorothalonil

Chlorothalonil is a nitrile of low acute toxicity but severe irritant potential. There has been concern about findings from long-term rat and mice studies. In rats, there were gastric and renal changes, including renal epithelial hyperplasia, adenomas and carcinomas, forestomach hyperplasia and hyperkeratosis, and gastric papillomas (FAO/WHO, 1991; 1993).

3.9.2 Creosote

Coal-tar creosote, a widely used wood preserver in English-speaking countries, is a complex mixture, which contains quantities of various carcinogens and phenols. The amount of benz(a)pyrene in some preparations has caused some concern and is subject to limits in a number of countries: the use of creosote has been banned in the European Union. However, it is likely that the irritancy reduces the potential of creosote for causing skin cancer and it probably presents little hazard to the public.

4 HERBICIDES

Herbicides are substances that kill plants, and are of variable degrees of specificity. The main groups of herbicides are given in **Table 5**.

4.1 Inorganic Herbicides

Substances such as common salt (sodium chloride) have, for many years, been used as nonselective herbicides and sodium chlorate, continues to be used in this way. If ingested by humans, sodium chlorate produces immediate vomiting, abdominal pain and diarrohea. A noteworthy feature is that acute poisoning with sodium chlorate produces methaemoglobinaemia and intravascular haemolysis may also occur (Proudfoot, 1996).

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Table 5 Main groups of herbicides, with examples

Group		Examples
Inorganic		Sodium chlorate
Bipyridylium		Paraquat Diquat
Organic acid	Phenoxy	2,4-D 2,4,5-T Mecoprop Fenoprop Haloxyfop
	Other organic acids	Dicamba
Substituted anilines		Alachlor Propachlor Propanil
Ureas and thioureas		Diuron Linuron Monolinuron
Nitriles		Ioxynil Bromoxynil
Triazines and triazoles	Triazines	Atrazine Simazine
	Triazoles	Amitrole
Organophosphate group	Phosphonic acid derivatives	Glyphosate
	Phosphinic acid derivatives	Glufosinate

4.2 Bipyridylium Herbicides

The two well-known pesticides in this group are paraquat and diquat. The mechanism of action involves cyclic reduction–oxidation reactions producing reactive oxygen species and depletion of NADPH.

4.2.1 Paraquat

Paraquat is selectively toxic to the lungs, producing an acute alveolitis followed by fibrosis. Both Type I and Type II alveolar cells, as well as the Clara cells are destroyed. Associated with this process, it has been noted the lung tissue accumulates paraquat by a saturable uptake process (Rose *et al*., 1974; 1976; Smith, 1982; Smith *et al*., 1990). In experimental animals, the main result of paraquat toxicity is histologically a proliferative pneumonitis with fibroblasts, alveolar oedema, perivascular and peribronchial oedema and accumulation of neutrophils and macrophages (Schoenberger *et al*., 1984). The precise histological changes observed in rats depend upon the dose of paraquat and time to death. In those dying early, haemorrhage and oedema is very prominent, while those dying later show greater evidence of fibrosis. These changes appear to occur largely independently of the route of administration of the paraquat and it should be noted that, in mice, similar histopathological findings have been observed after exposure to paraquat aerosol (Popenoe, 1979).

Many cases of paraquat poisoning, mostly due to deliberate ingestion of the agricultural concentrate, have been reported since this herbicide was introduced (FAO/WHO, 2004). Poisoning with paraquat in man initially produces damage to the gastrointestinal tract, including the mouth and pharynx, and to the liver and kidneys. Often partial recovery occurs and then from 10 days onwards, clinical signs and symptoms referable to the respiratory tract develop (Higenbottam *et al*., 1979; Schuster *et al*., 1981). Death occurs normally from respiratory failure. There is no effective treatment for paraquat-induced lung damage and the only effective measures involve efforts aimed at the prevention of absorption of the herbicide from the gastrointestinal tract.

Paraquat is one of a number of pesticides where an association with exposure and development of Parkinson's disease has been suggested (see reviews by Barlow *et al*., 2007; IEH, 2005)

4.2.2 Diquat

Diquat poisoning differs from paraquat poisoning in that renal effects are more prominent and lung changes do not generally occur, because diquat does not have the nitrogen atoms in the correct spatial positions to permit active uptake by the lung. Cataractogenesis has been observed in long-term experimental animal studies, but not in humans (FAO/WHO, 1994). In man, death in overdose is caused by renal failure (Vanholder *et al*., 1981).

4.3 Organic Acid Herbicides

4.3.1 Phenoxy Herbicides

The phenoxy herbicides, which are widely used to destroy broad-leaved weeds, are chemical analogues of plant growth hormones (auxins) of the indole acetic-acid type, and are used for the selective control of broad-leaved plants in monocotyledenous crops. Herbicides of this type have no hormonal action in animals. The phenoxy herbicides include 2, 4-dichlorophenoxyacetic acid (2,4-D), 2, 4, 5-trichlorophenoxyacetic acid (2,4,5-T), mecoprop and fenoprop. Similar compounds are used as rooting powders (i.e. they stimulate rooting of cuttings). One herbicide of the group, namely 2,4,5-T, has come under a cloud as result of contamination with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, levels of which are now subject to strict limits. The whole group has attracted some suspicion, because of certain epidemiological studies linking non-Hodgkin's

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lymphoma and soft-tissue sarcoma with herbicide manufacture or application. 2,4,5-T is rarely used nowadays, but 2,4-D is extensively used as a selective pesticide on lawns and on monocotyledonous crops. The mammalian toxicology of all the members of the group is quite similar and appears to mediated by several mechanisms, including cell membrane damage, uncoupling of oxidative phosphorylation and disruption of acetylcoenzyme A metabolism (see reviews by Bradberry *et al*., 2004a; Marrs, 2004).

4.3.1.1 2,4-D

2,4-D is a moderately toxic compound with LD_{50}s >400 mg (kg body weight)$^{-1}$. It may be used as the acid (2,4-dichlorophenoxyacetic acid) or as esters, amine salts or the sodium salt. In subacute and chronic studies in experimental animals, changes were found in the kidneys and sometimes the liver. Mutagenicity tests on 2,4-D have produced somewhat contradictory results (Pilinskaya, 1974), so that IARC (1977) concluded that results on mutagenicity and carcinogenicity were inadequate for a proper evaluation. However, it seems unlikely that pure 2,4-D is mutagenic. The JMPR (FAO/WHO, 1997) observed that mutagenicity test results indicated that 2,4-D was not genotoxic, although conflicting results had been obtained for mutation in *Drosophila melanogaster*. In man, large doses are necessary to produce major toxic effects, which include alterations in consciousness, muscle fasciculation, vomiting and convulsions. Gross overdose produces stupor, muscle hypotonia and coma, which may be prolonged, metabolic acidosis, hypotension and pulmonary oedema may occur (Ecobichon, 1994; Proudfoot, 1996). There is some evidence that alkaline diuresis is beneficial in 2,4-D poisoning and also with mecoprop.

4.3.1.2 2,4,5-T

2,4,5-T is not much used in Western countries any more. The toxicology is similar to that of 2,4-D, however human case reports must be studied in the context of the content of TCDD in the material. At high dose levels, 2,4,5-T is reported to be teratogenic to rats and mice, but not rabbits, monkeys and sheep (FAO/WHO, 1980).

4.3.1.3 Other Phenoxy Herbicides

The main target organs for fenoprop in experimental animals are the liver and kidneys (USEPA, 1988), and the material is not carcinogenic. By contrast, the toxicology of haloxyfop is notable for the production of liver tumours; these are observed in mice and are associated with peroxisome proliferation (FAO/WHO, 1996). The animal toxicology of mecoprop is very similar to 2,4-D (PSD, 1994). A case of combined 2,4-D and mecoprop poisoning was reported by Kerr *et al*. (1997). The main features were gastrointestinal effects, hypotension, coma, muscle weakness, hyperthermia and tachycardia.

4.3.2 Other Organic Acid Herbicides

Dicamba is a herbicide of low acute toxicity, and the few poisoning cases observed in humans have been complicated by ingestion of phenoxy herbicides at the same time. In animals, muscular spasms and dyspnea occur in acute studies, whilst in long-term studies, organ-specific effects are few (Stevens and Sumner, 1991). Dicamba is reported to cause peroxisome proliferation in experimental animals (Espandiari *et al*., 1998). In a prospective questionnaire-based cohort study, although associations between exposure and lung and colon cancer were observed, Samanic *et al*. (2006) did not find clear evidence of an association between cancer risk and dicamba exposure.

4.4 Substituted Anilines

These are used as herbicides and include alachlor, propachlor and propanil. Some herbicides of this group have the general property of causing methaemoglobinaemia, as do many other aniline derivatives (Kiese, 1970). The probable mechanism of the methaemoglobinaemia is *N*-hydroxylation to the corresponding hydroxylamine, which then takes part in an intraerythrocytic cycle with the corresponding nitroso derivative, at the same time generating methaemogloblin. If this is the mechanism, for propanil the proximate methaemoglobin former would be *N*-hydroxy-3,4-dichloroaniline (McMillan *et al*., 1990). In the case of propachlor, which is a tertiary amine, it would seem unlikely that the above mechanism would operate, and rat data suggest that this is indeed the case (Panshina, 1973). However, it must be remembered that the rat is not a good experimental animal for demonstrating methaemoglobinemia (Calabrese, 1983), with the dog considered a more appropriate model for man. As well as causing methaemoglobinaemia, propanil causes reduced red-cell survival (McMillan *et al*., 1991).

4.4.1 Alachlor

Alachlor is, like other substituted aniline herbicides, not a substance of high acute toxicity (Tomlin, 2006). The substance is carcinogenic in rodents, producing posterior nasal and stomach tumours, possibly by a nongenotoxic mechanism (Berry, 1988).

4.5 Ureas and Thioureas

The herbicidal ureas, such as diuron, linuron and monolinuron, are of low acute toxicity. They appear to interfere with photosynthesis in plants. In man, they cause methaemoglobinemia, intravascular haemolysis and haemoglobinuria (Proudfoot, 1996).

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4.6 Nitriles

The nitrile herbicides include ioxynil and bromoxynil and they may act as toxicants partly by uncoupling oxidative phosphorylation and as inhibitors of oxidative phosphorylation (Stevens and Sumner, 1991; Proudfoot, 1996).

4.7 Triazines and Triazoles

Atrazine and simazine are triazines, while amitrole is a closely-related triazole; they are a very widely used group of herbicides, which inhibit photosynthesis. These compounds are of low toxicity and the effects of ingestion by humans are nonspecific. In rats, mice and sheep, amitrole has effects upon the thyroid (hyperplastic changes) (FAO/WHO, 1994).

4.8 OP Herbicides

Two OPs, both of which have low or nonexistent ability to produce cholinesterase depression, are used as herbicides. Glyphosate (*N*-phosphonomethyl glycine) is an inhibitor of amino-acid synthesis in plants. In mammals, it appears to be very nontoxic, with LD_{50}s in the 5 g (kg body weight)$^{-1}$ range (Atkinson, 1985; FAO/WHO, 1987). El-Demerdash *et al*. (2001) reported that glyphosate, at high concentrations *in vitro* (IC_{50} about 700 mM) could inhibit 'serum acetylcholinesterase', however there is no indication that significant cholinesterase inhibition occurs in mammals *in vivo*. Indeed, structure–activity consideration would not lead to the expectation that glyphosate would inhibit cholinesterases. In repeated-dose studies in experimental animals, the toxicity tends to be rather nonspecific, failure to gain weight being the most frequent observation. As very high dietary concentrations were used in some of these studies, this effect may be due to unpalatability of the diet and caloric dilution. There is no evidence of carcinogenic potential in long-term studies, nor of teratogenic potential. There is little evidence of genotoxicity in a variety of *in vitro* and *in vivo* tests (Williams *et al*., 2000). Dallegrave *et al*. (2007) found that exposure to glyphosate induced adverse effects on the reproductive systems of rats at puberty and during adulthood, these rats having been exposed to glyphosate *in utero* and during lactation. Glyphosate is of low acute toxicity in humans, and most case reports and series have shown that high doses are necessary to produce fatality (see review by Bradberry *et al*., 2004b). However, the lethal dose in humans seems somewhat variable, some patients surviving doses which were fatal in others. Massive overdose of glyphosate produces gastric irritation, hypotension and pulmonary insufficiency, for which constituents of the formulation other than glyphosate may be to blame (Talbot *et al*., 1991). Damage to the larynx can occur following respiratory aspiration (Hung *et al*., 1997). It has been suggested that the trimethylsulfonium salt is more toxic than the other salts in humans (Sørensen and Gregersen, 1999). Glufosinate ammonium is a nonselective phosphinic acid herbicide. It is a substance of natural origin from the tripeptide L-phosphinothricyl-L-alanyl-L-alanine, which was first isolated from *Streptomyces viridichromogenes* or *Streptomyces hygroscopicus* (Hoerlein, 1994). Glufosinate ammonium acts as an inhibitor of glutamine synthetase in plants. It has some inhibitory action on glutamine synthetase in experimental animals, especially in the kidney (FAO/WHO, 1992). On the basis of studies in mice, Matsumura *et al*. (2001) suggested that the convulsions caused by glufosinate ammonium were mediated through *N*-methyl-D-aspartate (NMDA) receptors. Serious acute poisonings in humans do not appear to be particularly common. Watanabe and Sano (1998) reported a case of a 64-year-old man who ingested glufosinate with suicidal intent. Initially there were mental disturbances and haematological changes, together with gastrointestinal effects and he later developed generalized convulsions, impaired respiration and circulatory failure. During recovery he exhibited loss of short-term memory. A range of genetically modified crops with resistance to glyphosate and glufosinate ammonium are being developed.

5 DEFOLIANTS AND DESSICANTS

A number of substances are used as defoliants and dessicants in agriculture. Thus, sulfuric acid is widely used to destroy potato haulms. *S,S,S*-tributyl phosphorotrithioate (DEF) and *S,S,S*-tributyl phosphorotrithioite (merphos) are OPs used as cotton defoliants, since they produce leaf abscission. A notable feature of the toxicity of DEF and merphos is that in hens they produce OPIDP (Baron and Johnson, 1964).

6 MOLLUSCICIDES

Certain pesticides are used to kill slugs and snails. One of the more noteworthy is metaldehyde. This quite commonly causes mild poisoning when ingested by children and sometimes more severe poisoning in pet animals. Severe intoxication with metaldehyde is primarily due to its breakdown to acetaldehyde and is characterized by convulsions, hyperpyrexia and metabolic acidosis (Proudfoot, 1996). Poisoning is not infrequently reported in pet animals, especially dogs. Thus Yas-Natan *et al*. (2007) reported a case series of

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14 dogs from Israel. Clinical signs observed included convulsions, hyperthermia, tachycardia and muscle tremor. Serum biochemistry abnormalities including acidaemia were seen. Methiocarb is a molluscicide with cholinesterase-inhibiting properties and relatively high acute oral toxicity (LD_{50}s 20–300 mg (kg body weight)$^{-1}$, FAO/WHO, 1999).

7 RODENTICIDES

7.1 Anticoagulant Rodenticides

Many rodenticides are anticoagulants and include warfarin, as well as newer substances, such as brodifacoum, bromadialone and chlorophacinone. The main difference between warfarin and the newer compounds is the increased single-dose toxicity and persistence of anticoagulant action. The mechanism of action is inhibition of the synthesis of blood-clotting factors VII, IX and X, the vitamin-K-dependent ones. Poisoning in humans is similar to that seen in the rodents and usually follows ingestion. Spontaneous haemorrhages occur from the nose, into the skin and into internal organs. The prothrombin time is prolonged. Oral vitamin K_1 (phytomenadione) can be used as an antidote or alternatively, in extreme cases, transfusion of fresh frozen plasma or blood may be indicated.

7.2 Nonanticoagulant Rodenticides

7.2.1 α-Chloralose

α-Chloralose causes hypersalivation, increased muscle tone, hyper-reflexia, opisthotonus and convulsions. Rhabdomyolysis is a possible complication and coma may occur.

7.2.2 Phosphides

These are used as fumigants in grain stores, and so on, and as rat poisons. Zinc and aluminium phosphides both give rise to phosphine on combination with stomach acid or moist air. Phosphine is very toxic by inhalation, producing local irritation to mucous membranes, together with hepatic effects and reductions in the red blood cell count. By the oral route the phosphides affect the liver and cause convulsions in experimental animals. In humans, exposure to phosphine causes coughing, dyspnoea, tight chest, headache, giddiness and retrosternal pain; pulmonary oedema, with resultant cyanosis may be seen. Ingestion of metallic phosphides causes nausea, vomiting, hypotension and shock (Proudfoot, 1996). The mechanism of the poisoning is unclear. There is some evidence for inhibition of cholinesterases in cases of human poisoning; thus, Rastogi *et al*. (1990) found that plasma cholinesterase was decreased in patients poisoned by aluminium phosphide by the oral route. Brain cholinesterase was not affected. Plasma cholinesterase inhibition did not appear to influence prognosis and aminotransferase estimation did not suggest liver disease, so that a hepatic origin for the decreased cholinesterase activity seems unlikely. Some epidemiological work has reported an increased incidence of chromosomal aberrations following occupational exposure to phosphides or phosphine gas (Garry *et al*., 1989; 1992) although other work has not confirmed this finding (Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment, 2001).

8 TOXICITY OF COMBINATIONS OF PESTICIDES

There has been some concern as to the possibility of deleterious effects from multiple pesticides exposure, either as residues in food or at the workplace. The former seems unlikely to be much of a problem, as clinical effects from exposure to pesticide residues in food are exceedingly uncommon, although it has been suggested that adverse effects of long-term exposure to multiple residues, even at low doses, may be a problem. The workplace is more of a potential concern, raising the question of the safety of tank-mixing of pesticides and other sources of combined exposure. Frequently, data on a particular combination are scanty and general principles are necessary for predictive purposes. The possible types of toxicological combined action with pesticides are: (i) additivity, (ii) potentiation (synergy) and (iii) antagonism. Often, compounds with the same toxic effects act additively and the observed effect is dose additivity. If toxic effects were only determined by actions at receptors, this would probably always be the case. However, one has also to consider alteration by one pesticide of the pharmacokinetics or metabolism of the other, and as a result interactions may be exceedingly complex. However, at doses below NOAELs, combined effects other than dose addition are unlikely to occur. Thus, in combination, the acute toxicity of OCs at moderate dose levels is usually additive, although potentiation and antagonism can occur. With pairs consisting of one OP and one OC, the same is true (Keplinger and Deichmann, 1967). It has been shown that some pairs of OPs exhibit greater than additive toxic effects when administered together (Dubois, 1961). Because of the predictive difficulty, experimental approaches have been proposed (GIFAP, 1988). A major problem in this area is the poor design of many studies in the literature (Borgert *et al*., 2001).

The problem of exposure to mixtures of pesticides was first systematically addressed in practical terms in

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the USA. The Food Quality Protection Act (FQPA) of 1996 (USA, 1996) mandated the consideration of all sources of pesticide exposure when carrying out risk assessments, and also the consideration of the effects of combined exposure to different pesticides. The act also introduced the terms aggregate exposure assessment (consideration of all sources) and cumulative exposure assessment (consideration of combined exposure to different pesticides); both terms have been much criticized, especially the latter, but they have both passed into general use. The USA has made considerable progress in delineating groups of pesticides with common mechanisms of action (common mechanism groups (CMGs)), where dose additivity might be expected (USEPA, 1998; 1999; 2000a; 2000b; 2001). The first group to be tackled was that comprising OP compounds (USEPA, 2002a), and more recently the *N*-methyl carbamate compounds were evaluated (USEPA, 2007). Much less has been done in the European Union. The UK Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (2003) prepared a report on the subject and this was followed by a rather impractical action plan (FSA, 2007). Germany (Federal Institute for Risk Assessment, 2005) and other member states of the EU are also addressing the problem in an uncoordinated way, for example the Netherlands (Boon and van Klaveren, 2003) and Denmark (Danish Veterinary and Food Administration, 2003). Even when the CMGs have been defined, there remains the question of how to do cumulative risk assessment. Available methodology has been reviewed (Wilkinson *et al*., 2000). The European Food Safety Authority is taking steps to provide a European approach to combined assessments following a symposium held in 2006 (EFSA, 2007) and the setting up of a working group. To date, none of the combined assessments performed has identified significant risks from combined exposures that merited immediate risk-management action.

9 MICROBIAL PEST CONTROL AGENTS

Microbial pest control agents (MPCAs) are plant protection products that have a micro-organism as the active substance. The micro-organism can be a bacterium, fungus, virus, protozoan, microscopic nematode or microsporidium. An extensive compilation of basic information on biopesticides can be found in Copping (1998).

The first MPCAs are reported to have been introduced commercially in the 1930s (Jarvis, 2001). The majority of MPCAs have a specific action against a limited range of pests, resulting in limited use, often in niche markets. However, due to the loss of older pesticides following review programmes and public pressure against traditional pesticides, interest in biological pesticides has been increasing. Currently, the only products with significant usage are those based on *Bacillus thuringiensis* (Bt) representing approximately 88% w/w of the MPCAs applied in California (Rubin, 2001). Possibly because of the limited use, and the fact that to gain approval they should be benign, there is little information in the public domain on the toxicity, infectivity or pathogenicity to mammals of most MPCAs, the exception being Bt.

Generic concerns relating to microbial pesticides include: production of antibiotics/toxins, ability to infect mammals, sensitizing potential due to the presence of antigenic macromolecules and contamination with pathogenic organisms.

9.1 Toxicity of Particular Organisms

9.1.1 *Bacillus Thuringiensis*

Bt is a spore-forming, aerobic, gram positive bacillus closely related to *B. cereus,* an organism which has been associated with human infections. Bt produces crystalline protein inclusion in its spores that is absent in *B. cereus;* in other respects Bt and *B. cereus* are closely related and Giffel *et al*. (1997) showed that six out of 20 isolates previously identified as *B. cereus* by classical biochemical techniques were more correctly Bt. There is thus a degree of uncertainty about whether Bt can produce infections in humans. The properties of Bt strains differ and it is important to relate data to specific strains of Bt wherever possible.

Protein crystals produced by Bt spores are in the form of a protoxin that becomes active only after it is ingested, solubilized by the alkaline conditions, and activated by enzymes, in the insect gut. The C-terminal of the protein binds to receptors on the gut epithelial cells, is variable and provides species specificity in terms of pesticidal activity. The N-terminal is conserved between strains of Bt and produces a pore in the gut epithelial cells, leading to cell lysis and death (IPCS, 1999).

Some strains of Bt also produce other toxins. The 'β-exotoxin' is a heat stable nucleotide that inhibits RNA polymerases and is toxic to most animal phyla, including insects and mammals. An enterotoxin, similar to that produced by *B. cereus* is thought to be responsible for producing diarrhoea and vomiting in mammals. Several other poorly characterized toxins have also been reported (IPCS, 1999). The Bt strains supplied for insecticidal use are reported to be 'β-exotoxin' free.

In addition to the animal studies on toxicity, pathogenicity and infectivity, a range of studies have been performed on Bt strains used as MPCAs. These indicate that use of Bt as a pesticide is without significant risk to human health, though some preparations

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have been found to possess irritant properties (IPCS, 1999; McClintock *et al*., 1995). Green *et al*. (1990) investigated reports of infection over a two year period of Bt spraying. Out of a total of 95 bacillus-positive cultures, 55 contained Bt. Of these, 52 were considered to have been associated with contamination of skin, tissue, surfaces or plates. In the three remaining cases where Bt involvement could not be entirely discounted, the medical history indicated the immune system may have been compromised. Noble *et al*. (1992) performed an epidemiological investigation to monitor any health effects relating to the widespread use of Bt *kurstaki* to eradicate the Asian Gypsy moth in British Columbia. The authors concluded that though there was clear evidence of exposure to Bt *kurstaki* and occasional reports of irritation, in both workers and the general population, there was no case indicating pathogenicity or infection. Damgaard *et al*. (1997) reported the presence of four strains of Bt in infections in burn wounds and from fluid used to treat the burns. None of the strains had insecticidal activity. It is not clear whether these strains of Bt were involved in producing the infection or were merely present as contaminants.

A number of adverse findings relating to Bt have been reported, however, it is not always clear how these reports relate to the actual use of Bt strains as MPCAs. A French soldier injured by a land mine was found to have Bt serotype H34 in necrotic soft tissue from the site of injury. Initial studies with this strain in immunosuppressed mice showed it to induce myonecrosis following cutaneous administration (Hernandez *et al*., 1998). Four strains of Bt related to those used in pesticide formulations were found to give positive results in tests for *B. cereus* enterotoxin. (Carlson *et al*., 1994). Investigations into the levels of *Bacillus* diarrhoeal enterotoxin in one strain of *B. cereus* (positive control) and 10 strains of Bt (one pure strain HD-1; an isolate from a case of bovine mastitis; and eight commercial preparations were performed). The enterotoxin titres in Bt strains varied between 15 and 242, with the *B. cereus* strain having a titre of 1629 (see review by Rosas-Garcia, 2009).

9.1.2 Beauveria Bassiana

Beauveria bassiana ATCC 74040 has activity against a wide range of insect pests, possibly involving the production of beauvericin, a proteinaceous toxin that enhances membrane permeability. Intraperitoneal, oral, dermal or intratracheal administration at up to 2.5×10^9 CFU per animal did not produce any indications of infectivity. An inflammatory response was noted in the lungs of animals dosed intratracheally, but this is considered to be a normal reaction to the presence of large titres of foreign material. A formulation based on ATCC 74040 was found to be a skin sensitizer, although no hypersensitivity incidents have been reported (USEPA, 2000c).

9.1.3 Granulosis Viruses and Nuclear Polyhedrosis Viruses

Granulosis viruses and nuclear polyhedrosis viruses are members of the family of baculoviruses, complex viruses that are protected by a protein overcoat and forming an occlusion body. A number of baculoviruses have insecticidal uses, primarily against moths. Susceptible insect larvae ingest the virus, which becomes active only after ingestion of the occlusion bodies. In the larval gut, the protein overcoat quickly disintegrates, and the viral particles proceed to infect digestive cells and interfere with food absorption. Larvae die after a few days. These viruses occur naturally and are considered to present no known risks to humans or other mammals, as they are unable to jump between phyla (USEPA, 2002b).

9.1.4 Pseudomonas Chloraphis

Pseudomonas chloraphis MA342 has activity against fungi that attack crop roots and can be used to protect cereal seed. The precise mechanism of action has not been identified, but is believed to involve the secretion of an iron-chelating protein and the production of 2,3-deepoxy-2,3-dihydrorhizoxin (DDR). MA342 is not toxic or pathogenic to rats via the oral or inhalation routes and is not closely related to any organisms considered to be pathogenic to humans. The metabolite DDR has been found to be a potent *in vitro* aneugen, inducing a significant increase in c-mitotic cells at concentrations of >25 pM in Chinese hamster V79 cell cultures. This aneugenicity was reproduced *in vivo* with oral doses of 2 and 18.6 mg (kg body weight)$^{-1}$, producing increases in micronucleated polychromatic erythrocytes in NMRI mice. However, DDR is only produced in contact with the target fungi and hence the use of *Pseudomonas chloraphis* MA342 as a seed treatment results in negligible human exposure to DDR (SCP, 2002).

The views expressed in this chapter are those of the authors and do not necessarily reflect the views of any UK Government Department or agency.

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NOTES

[a.] Maximum residue level and maximum residue limit exist for the same concept, but in this chapter we have standardized on limit.

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Toxicology of Biocides

Bryan Ballantyne

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1 BACKGROUND

The definition of biocides, sometimes referred to as antimicrobials, can be confusing because of the regulatory use and misuse of this term and what materials are included in approved listings. Generally, biocides are defined as chemicals or biological agents that are capable of destroying, preventing or rendering harmless pathogenic organisms. They were originally defined in relation to antimicrobial activity, but in regulatory language the spectrum of activity and the types of organisms affected has widened considerably, to the extent that biocides can be interpreted as nonagricultural pesticides. Thus, the Biocidal Products Directive (BPD) of the European Union (EU) defines biocidal products as 'active substances or preparations containing one or more active substances—intended to destroy, deter, render harmless, prevent the action of, or otherwise exert a controlling effect on any harmful organism by chemical or biological means'.

In the USA, industrial biocides require approval by the Environmental Protection Agency (EPA) for use, are regulated by the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), and require extensive chemical, efficacy and toxicological testing before approval. In Europe, biocides are regulated through the EU by the BPD, 98/8/EC. This covers a multitude of different products in four main groupings: disinfectants, preservatives, pest control agents and 'other biocidal products' (see **Table 1**). Authorization by the BPD is a two-step process; active substances are evaluated and listed under the aegis of the EU commission, and products are authorized at a national level with individual member states. For a product to be authorized for marketing it requires to be proven that it is sufficiently effective against the intended target organisms, imposes no unacceptable effects on nontarget organisms, exhibits no unacceptable effects on human or animal health, and does not impose unacceptable effects on the environment. Within this process are a multitude of legal and administrative contortions that make the actual scientific and public-health consequences pale into insignificance. The required data for registration of chemical biocides generally includes, but is not necessarily limited to, physicochemical properties, relevant human toxicology, ecotoxicity, environmental fate and behaviour, results from exposure modelling studies and biocidal efficacy studies.

2 USE PATTERNS

Many industrial processes, such as pulp and paper manufacturing, use large amounts of recirculated water that, if left untreated, will become contaminated, leading to odours, equipment fouling, and failure and contamination of the finished product. Aside from general contamination, bacteria in untreated systems can attach to equipment surfaces. The initial interaction of the bacteria with the surface is reversible, but eventually

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Table 1 Main grouping of biocidal products covered by the European Union Biocidal Products Directive 98/8/EC

Main group 1: *Disinfectants and general biocidal products*
1. Human hygiene biocidal products
2. Private area and public-health area disinfectants and other biocidal products
3. Veterinary hygiene biocidal products
4. Food and feed area disinfectants
5. Drinking water disinfectants
Main group 2: *Preservatives*
6. In-can preservatives
7. Film preservatives
8. Wood preservatives
9. Fibre, leather, rubber and polymerized materials preservatives
10. Masonry preservatives
11. Preservatives for liquid-cooling and processing systems
12. Slimicides
13. Metalworking-fluid preservatives
Main group 3: *Pest control*
14. Rodenticides
15. Avicides
16. Molluscicides
17. Insecticides, acaricides and products to control other arthropods
19. Repellents and attractants
Main group 4: *Other biocidal products*
20. Preservatives for food or feedstocks
21. Antifouling products
22. Embalming and taxidermist fluids
23. Control of other vertebrates

becomes irreversible due to adhesion of exopolysaccharide polymers (Costerton *et al*., 1987). The attached bacteria form a biofilm on the surface that may cause major problems in a given system, such as clogging of equipment, breaking off to move into downstream systems, adding pressure to the system and slowing down the process. In addition, there can be costly down time associated with cleaning out systems plagued by biofilm. Also, biofilms may tend to harbour anaerobic bacteria under the biofilm layer. These sulfate-reducing bacteria (SRB) cause additional corrosion problems in tanks and pipes, leading to process leaks and potentially dangerous situations. In many cases, the problems created by biofilms (sessile bacteria) are much more serious and detrimental than those caused by free-floating (planktonic) bacteria. Sessile and SRB, however, are typically more difficult to kill than planktonic bacteria. This is due, in part, to transport limitations into the biofilm and potentially phenotypic changes in bacteria, and making the bacteria more resistant to certain biocides (Grobe and Stewart, 2000; Costerton *et al*., 1987). Accordingly, industrial biocides vary in their ability to control these bacteria.

Industries that use biocides include oil-field water flooding, pulp and paper mills, cooling towers, retort processing systems, wastewater treatment and textile processing. These systems are dynamic in nature with periodic turnover of the treated water that requires a sump-side addition of the biocide. Biocides used in these systems do need to sterilize the systems, but must be able to reduce micro-organism load and maintain low enough levels to help prevent biofilm formation. Some biocides are added continuously at low doses to maintain control, while others are added as slug doses (higher concentrations added quickly with repeated additions over time). The type of addition will depend on the system being treated, as well as the biocide.

Aside from treating dynamic systems, biocides are also used to preserve formulations used for personal care products (e.g. shampoos and cosmetics), detergents (e.g. fabric softeners) and industrial applications (e.g. concrete admixtures, paints, adhesives and lubrication fluids). When used as a preservative, a biocide must have long-term activity, as it is added only once to the product and is intended for long-term storage and repeated insult. Preservatives used in these applications include isothiazolones, glutaraldehyde (GA), 2-bromo-2-nitropropane-1,3-diol (BNP), *o*-phenylphenol, formaldehyde and formaldehyde-releasing compounds, carbamates and parabens.

Sanitizers, disinfectants and sterilants represent another class of biocides. Many countries have specific criteria that must be met to qualify for these labels. The level and type of organism reduction is important. In general, sterilants are required to completely kill bacterial spores, which, aside from prions, are considered amongst the most difficult of organisms to kill, followed by mycobacterium, nonlipid or small viruses, fungi, vegetative bacteria, and lipid or medium-sized viruses. The Center for Disease Control and Prevention (CDC, Atlanta, Georgia, US) classifies sterilization and disinfection processes using liquid chemicals as follows:

- Sterilization: sporicidal chemical, prolonged contact (e.g. GA, chlorine dioxide, hydrogen peroxide, peracetic acid (PAA)).
- High-level disinfection: sporicidal chemical, short contact time (e.g. GA, chlorine dioxide, hydrogen peroxide, PAA).
- Intermediate-level disinfection: some tuberculocidal activity (e.g. phenolics, iodophors or chlorine compounds).
- Low-level disinfection: No tuberculocidal activity, kills most bacteria and viruses quickly, can include sanitizers (e.g. quarternary ammonium compounds, some iodophors and some phenolics).

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For sterilization, autoclaving is typically the method of choice, unless the instrument is heat sensitive. In that case ethylene oxide (EO), as gas plasma sterilization, is used. The low-temperature liquid chemical sterilants referred to here (GA and PAA), are typically used for high-level disinfection rather than full sterilization, even though both chemicals are sporicidal under certain conditions. High-level disinfectants are used to disinfect semicritical devices which enter the body and may come into contact with mucosal surfaces, but do not, in general, break through these membranes and enter the normally sterile areas of the body. In the USA, high-level disinfectants must be able to kill spores under some conditions, and must be able to kill some spores and all mycobacteria under the label conditions.

3 CHEMISTRY OF BIOCIDES

Biocides are typically separated into two main classes; oxidizers such as halogens, and nonoxidizers such as isothiazolones, GA, 2,2-dibromo-3-nitrilopropionamide (DBNPA), quarternary ammonium compounds, tetra-(hydroxymethyl)-phosphonium sulfate (THPS), BNP and methylenebisthiocyanate (MBT). Industrial biocides are chemically diverse compounds and include many classes of chemicals such as phenolics, surface-active agents, aldehydes, halogens, heavy metals and many other miscellaneous compounds. They can be nucleophiles or electrophiles, oxidizing agents or reducing agents. Because of this diversity, there is no general mechanism that can be used to describe the mode of action of these chemicals (Denzer, 1995). While individual metabolic processes may be affected by biocides, the broad spectrum of lethality exhibited by many of the most successful biocides suggests a certain lack of specificity. For example, GA is known to nonspecifically react with amine and thiol groups of cellular and membrane proteins and enzymes. Phenolics interact with the cell wall, interfere with membrane potential, and react with thiols and amines. Surface-acting agents, such as quarternary ammonium compounds, affect general membrane permeability (Denzer, 1995; Favero, 1994).

Because of the nonspecific nature of the mechanisms of action of the many industrial biocides, micro-organisms do not develop genetic resistance to biocides, as do organisms to antibiotics and other chemotherapeutic agents. There are, however, many instances of organisms developing some degree of resistance or tolerance to particular biocides (Chapman, 1998; Chapman *et al.*, 1998; Grobe and Stewart, 2000). The mechanisms for this resistance presumably differs with each biocide. In some cases it may be opportunistic growth of a tolerant subclass, in others the stress induced by the biocide or inclusion in a biofilm may trigger phenotypic changes in the organism making it more difficult to kill. In any case, the biocide industry has developed strategies to deal with these issues, such as alternating biocides to prevent development of tolerance and using synergistic blends.

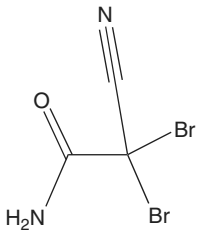
Since biocides have many differing mechanisms of action, they also have differing degrees of activity against the organisms of interest. Therefore, not every biocide is suitable for each application, and in some cases biocides are used together or in succession in order to obtain optimum biocidal activity. Some biocides are primarily used for long-term preservation, whilst others are used for quick kill and short half-life. Some of the more important and widely used biocides are discussed below.

4 INDIVIDUAL BIOCIDES

4.1 2,2-Dibromo-3-nitrilopropionamide (DBNPA)

4.1.1 Identities and Physicochemical Properties

Structural formula:



Molecular formula: $C_3H_2Br_2N_2O$
CAS No: 10222-01-2
Chemical family: Halogenated cyanoacetamide
Synonyms: 2,2-Dibromo-2-cyanoacetamide, Dibromo-2-carbamoylacetonitrile, DBNPA
Molecular weight: 241.86
Melting point: 123–125 °C
Thermal decomposition: > about 60 °C
Vapour pressure: 8.25×10^{-4} mm Hg (25 °C)
Specific gravity: 2.375 (21 °C)
Appearance: White crystalline powder
Odour: Mild antiseptic
Solubility: acetone: 35 g $100\,g^{-1}$; ethanol: 25 g $100\,g^{-1}$; dimethylformamide: 120 g $100\,g^{-1}$; polyethylene glycol 200: 120 g $100\,g^{-1}$; soluble in water to $17\,g\,l^{-1}$ (25 °C)
K_{ow}: 6.3
DBNPA is available commercially as a powder, or 5–20% solutions containing 40–60% glycol.

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Sources of information: Clearon Corporation (2000), (Paulus (1993), pp. 241–249) and Wuzhou International Company (2002).

4.1.2 Mechanism of Action

DBNPA has been demonstrated to bind to living and dead cells, suggesting it is not transported into cells, but binds to the surface membrane. DBNPA reacts with thiols, and most likely inhibits proteins and enzymes on the cell surface by reacting with key groups within these enzymes. In particular, DBNPA has been shown to interfere with the transport of *o*-nitrophenyl-β-D-glycosidase and ^{14}C-glucose into bacteria (Friend and White Kettle, 1980).

4.1.3 Stability and Decomposition

DBNPA is stable at acidic pH, but begins to degrade quickly as the pH is raised ($t_{1/2}$ at pH 6.0 = 155 hours; $t_{1/2}$ at pH 9.0 = 0.3 hours) (Friend and White Kettle, 1980). DBNPA is not very stable in the presence of organics, nucleophiles or UV light (Blanchard *et al*., 1986), and is particularly sensitive to thiols and sulfides. DBNPA degrades to dibromoacetamide and ultimately to CO_2, NH_3 and Br. In a secondary pathway, DBNPA is debrominated by UV light to cyanoacetamide (Blanchard *et al*., 1986; Exner *et al*., 1973).

4.1.4 Biocidal Uses

DBNPA has a broad spectrum of biocidal activity against Gram-negative and Gram-positive bacteria, yeast and moulds. Because of its relatively short half-life in neutral to alkaline formulations, DBNPA is typically used in industrial applications, such as cooling towers, pulp and paper processing, water recirculating systems and RO membranes. DBNPA can be added at a low concentration continuous dose (0.5–5 ppm) or at a higher slug dose (5–20 ppm), added periodically, as needed for control. Laboratory studies have shown that DBNPA is effective in destroying *Legionella pneumophilia* at concentrations of 10^5–10^6 viable cells ml^{-1} (Anonymous, 1979; Skaliy *et al*., 1980).

4.1.5 Toxicology

4.1.5.1 Acute Toxicity

Peroral: DBNPA is of moderate acute peroral toxicity, with an LD_{50} of 308 mg kg^{-1} in the rat. Upper alimentary tract corrosive effects may develop.
Percutaneous: By 24 hour occluded contact, DBNPA is of slight acute percutaneous toxicity, with an LD_{50} of >2000 mg kg^{-1} in the rat.
Vapour exposure: A low acute toxicity is reported in the rat with a 4 hour LC_{50} of 0.32 mg m^{-3} (Clearon Corporation, 2000).

4.1.5.2 Primary Irritation

Skin: Undiluted DBNPA is reported as being moderately irritating to the skin (Clearon Corporation, 2000).
Eye: The undiluted material is reported as corrosive to the rabbit eye (EPA, 1994).

4.1.5.3 Subchronic Repeated Peroral Toxicity

An NOEL (no observed effect level) of 15 mg kg^{-1} day^{-1} was reported for a 13 week peroral study in the rat (Clearon Corporation, 2000).

4.1.5.4 Chronic Toxicity

No information available.

4.1.5.5 Oncogenicity

No information.

4.1.5.6 Genetic Toxicology

Not mutagenic in an Ames test. Not clastogenic in a chromosomal aberration test with Chinese hamster ovary (CHO) cells or a chromosomal aberration test with human lymphocytes. Did not induce unscheduled DNA repair *in vitro* with rat hepatocytes (Clearon Corporation, 2000).

4.1.5.7 Developmental Toxicology

Reported as being not teratogenic in rabbits, in which test an NOAEL (no observed adverse-effect level) of 10 mg kg^{-1} day^{-1} was reported for foetotoxicity (Clearon Corporation, 2000).

4.1.5.8 Reproductive Toxicology

In a two-generation study with rats, a NOEL for reproductive effects was cited as $\geq$30 mg kg^{-1} day^{-1} (Clearon Corporation, 2000).

4.1.5.9 Human Toxicology

By local contamination, DBNPA will produce moderate to severe skin irritation and severe (corrosive) lesions to the eye. Those handling the material should wear appropriate protective clothing and eye goggles. Because of its corrosive properties, swallowing of DBNPA should be regarded as an emergency medical situation, since complications of upper alimentary corrosive lesions may develop and there is a potential aspiration hazard. Because of these considerations, vomiting should not be induced in cases of ingested DBNPA.

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No American Conference of Government Industrial Hygienists ACGIH threshold limit value (TLV) is available.

US Department of Transportation (DOT) Packing Group II, Class 6.1

4.1.6 Ecotoxicology

Values for aquatic toxicity are as follows:

Rainbow trout: 96 hour $LC_{50} = 2.3\,mg\,l^{-1}$
Sheepshead minnow: 96 hour $LC_{50} = 3.4\,mg\,l^{-1}$
Bluegill sunfish: 96 hour $LC_{50} = 0.72\,mg\,l^{-1}$
Mysid shrimp: 96 hour $LC_{50} = 0.72\,mg\,l^{-1}$
Eastern oyster: 96 hour $LC_{50} = 0.37\,mg\,l^{-1}$
Daphnia magna: 48 hour $EC_{50} = 0.86\,mg\,l^{-1}$

Values for avian toxicity are as follows:

Bobwhite quail, acute oral $LD_{50} = 354\,mg\,kg^{-1}$
Mallard duck, dietary $LC_{50} > 5620$ ppm
Bobwhite quail, dietary $LC_{50} > 5620$ ppm.

4.2 Methylenebisthiocyanate (MBT)

4.2.1 Identities and Physicochemical Properties

Structural formula: NCS-CH_2-SCN
CAS No: 6317-18-6
Molecular weight: 130.13
Melting point: 101–105 °C
Appearance: Yellow powder
Solubility: Water—poorly
Sources of information: Paulus (1993, pp. 421–422).

4.2.2 Mechanism of Action

MBT interferes with cell metabolism by complexing with iron in enzymes such as cytochromes and disrupts energy transfer reactions that rely on iron-containing enzymes.

4.2.3 Stability and Decomposition

MBT is commercially available as a dilute liquid or in a dry powder form (95% active) (Friend and White Kettle, 1980; Hugo and Russell, 1982). MBT is more stable at acidic pH than at alkaline pH ($t_{1/2}$ at pH 6 = 288 hours; $t_{1/2}$ at pH 9 = 1 hour), rapidly hydrolyzing at alkaline pH to cyanide and thiocyanate (Friend and White Kettle, 1980).

4.2.4 Biocidal Uses

MBT has been used as a biocide for over 30 years. It has a broad spectrum of activity against bacteria, yeast, moulds, algae and anaerobic SRB (Friend and White Kettle, 1980; Wehner and Hinz, 1971). MBT is usually formulated as a liquid in organic solvent or aqueous suspension. It is primarily used in slug doses of 2–10 ppm in cooling towers, pulp and paper processing and leather applications. Because of its antifungal activity, it is also used, at higher concentrations, as an antisapstain wood preservative for short-term preservation of freshly sawn wood.

4.2.5 Toxicology

4.2.5.1 Sensitizing Potential

Skin: Shown to have a strong sensitizing potential in a guinea-pig maximization test (Andersen and Hamann, 1983). Human cases of possible allergic contact dermatitis have been described (Jappinen and Eskelinen, 1987).

4.2.5.2 Subchronic Toxicity

Peroral: Male and female Fischer 344 rats and $B6C3F_1$ mice were given MBT by gavage at dosages of 1, 2, 4, 8 or 16 $mg\,kg^{-1}$ daily for five days per week for 13 weeks. Mortalities occurred at 2, 4, 8 and 16 $mg\,kg^{-1}$ with rats and 8 and 16 $mg\,kg^{-1}$ with mice. Histology demonstrated squamous mucosal hyperplasia and hyperkeratosis in the forestomach, with an NOEL of 4 $mg\,kg^{-1}$ for male rats and 2 $mg\,kg^{-1}$ for female rats, and male and female mice (NTP, 1993a).

4.3 Quarternary Ammonium Compounds (Quats)

There are many different biocidally active quarternary ammonium compounds in use. Each may differ in some properties, such as specific efficacy, sensitivity to organic load or salt, and degree of foaming. Quats are available commercially as clear to yellow liquids and are very soluble in water (Paulus, 1993, pp. 375–387).

4.3.1 Mechanism of Action

Quats are cationic compounds containing a nitrogen bonded to four alkyl or heterocyclic groups and a small counterion. In order to have antibacterial efficacy, at least one group must be C_8-C_{18} in length

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(Hugo and Russell, 1982). Variation in chain length and type can significantly affect biological activity. Quats are considered surface-active agents. The hydrophobic chains are able to orientate themselves in the cell wall with the hydrophilic portion remaining outside the cell. In this manner, quats disrupt cytoplasmic membranes, releasing K^+ ions and other constituents (Merianos, 2001). Quats also dissociate conjugated proteins in the membrane, and in this manner alter membrane permeability.

4.3.2 Stability and Decomposition

Quats have varying sensitivities to hard water (salt) and organic load. The extent of inactivation varies with the chain length or substitution on the benzyl ring of *N*-alkyl-*N*,*N*-dimethylammonium chloride (ADAC) (Merianos, 2001). They tend to be more active under alkaline conditions, and less soluble as the hydrophobic chain increases in length. They tend to be inactivated and potentially precipitate in the presence of anionic surfactants (Hugo and Russell, 1982).

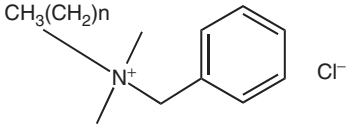
4.3.3 Biocidal Uses

Quats are more active against Gram-positive bacteria (minimum inhibitory concentration (MIC) > 50 ppm). Quats are used as hard surface disinfectants in household products, food processing, hospitals and animal housing. Quats are not normally used as high-level disinfectants or instrument sterilants since they lack mycobacterial and sporicidal activity (Hugo and Russell, 1982; Merianos, 2001; Paulus, 1993). Quats are active against lipophilic viruses and fungi, and are used in pools against algae. In addition to disinfection, quats are used as preservatives in personal care products (e.g. shampoos) as well as household products, and in water recirculating processes and cooling towers. Because quats have a tendency to foam, they require care when applied in large systems. Quats are often used in combination with other biocides for synergistic effects, For instance a GA/quarternary ammonium formulation is used in cooling towers and pulp and paper processing. Two representative quats are discussed below.

4.3.4 N-Alkyl-N,N-Dimethyl Ammonium Chloride (ADAC)

4.3.4.1 Identities and Physicochemical Properties

Structural formula:

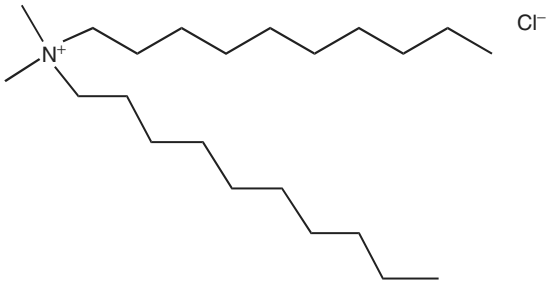


$n = 8–16$
CAS No: 68391-01-5
Molecular weight: 354
Freezing point = +5 to −12 °C
Boiling point: 80 °C (50%)
Flash point: 34 °C
Density: 0.95 g cm^{-3}
Viscosity: 860 mPa

4.3.5 Di-N-Decyl-Dimethyl Ammonium Chloride (DDAC)

4.3.5.1 Identities and Physicochemical Properties

Structural formula:



CAS No: 7173-51-5
Molecular weight: 362.09
Freezing point: −20 °C
Flash point: 29 °C
Density: 0.91 g ml^{-1}
Viscosity: 52 mPa

4.3.5.2 Toxicology

Sensitizing potential

Skin: A few cases of occupational allergic contact dermatitis have been described (Dejobert *et al*., 1997).

4.3.5.3 Ecotoxicology

The following acute toxicity values have been obtained for aquatic species (Farrell *et al*., 1998):

Acipenser tyransmontanus: 24 hour $LC_{50} = 1–10$ ppm
Pimephales promelas: 96 hour $LC_{50} = 0.39$ ppm
Platichthys stellatus: 96 hour $LC_{50} = 2.0$ ppm
Daphnia magna: 48 hour $LC_{50} = 0.037$ ppm
Mysidopsis bahia: 48 hour $LC_{50} = 0.039$ ppm
Hyalella azteca: 48 hour $LC_{50} = 0.106$ ppm
Neomysis mercedis: 48 hour $LC_{50} = 0.947$ ppm

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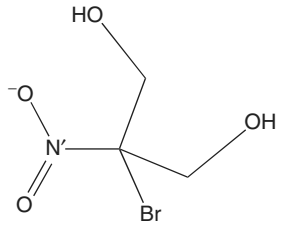
4.4 2-Bromo-2-nitropropane-1,3-diol (Bronopol; BNP)

4.4.1 Identities and Physicochemical Properties

Structural formula:



Molecular formula: $C_3H_8BrO_2N$
Molecular weight: 200
Melting point: 130–133 °C
Appearance: White crystalline solid
Aqueous solutions of BNP release 30% formaldehyde over time.
Sources of information: (Paulus, 1993, pp. 55–57, 70–72)

4.4.2 Mechanism of Action

Formaldehyde released from BNP is not the active species that kills the micro-organisms. BNP is electrophilic in nature, and its activity is due primarily to oxidation of thiols in proteins to form disulfides. BNP also causes membrane damage (Stretton and Manson, 1973). Because BNP activity is seen mainly with dividing cells, the primary effect likely involves metabolic processes. Specifically, BNP has been shown to inhibit the dehydrogenases involved in glucose metabolism located in cell membranes and the thiol-acetylation of CoA (Stretton and Manson, 1973). BNP is active against Gram-positive and Gram-negative bacteria, as well as yeast and fungi. The effect is primarily biostatic instead of biocidal, but a change in mechanism may occur. Cell death occurs as the oxidized thiols produce peroxide and superoxide radicals. Cidal concentrations of BNP are typically 10 times those of biostatic (preservative) concentrations (Gutherie, 1997; Shepherd *et al*., 1988). The activity increases with increasing pH, from 5.5 to 8.0.

4.4.3 Stability and Decomposition

Aqueous solutions of BNP are more stable at an acidic than an alkaline pH. As pH increases (>9.0), BNP decomposes, releasing formaldehyde to form bromonitroethanol and, eventually, Br, nitrite and nitroalcohols (Bryce *et al*., 1978; Challis and Yousaf, 1991; Paulus, 1993, pp. 55–57, 70–72). BNP also loses activity in the presence of thiols. When thiols are added to a formulation containing BNP, they can reverse the effects of BNP on the enzymes (Bryce *et al*., 1978; Stretton and Manson, 1973).

4.4.4 Biocidal Uses

Because of its biostatic properties, BNP is typically used as a preservative in slightly acidic formulations, which include cosmetics, toiletries and household products, as well as paints and adhesive (Bryce *et al*., 1978). BNP is particularly effective against Gram-negative bacteria such as *Pseudomonas aeruginosa* (MIC ≈ 50 ppm). It is used at 50–500 ppm against bacteria and 200–2000 ppm against fungi (Paulus, 1993, pp. 55–57, 70–72).

4.4.5 Toxicology

4.4.5.1 Acute Toxicity

Peroral: BNP has a moderate peroral toxicity in the rat, with LD_{50} values in the range 254–342 mg kg^{-1} (Eriksson *et al*., 1995).
Percutaneous: In aqueous solution, BNP is of low acute percutaneous toxicity in the rat, having an LD_{50} of >1600 mg kg^{-1} (Eriksson *et al*., 1995).
Subcutaneous: By subcutaneous doing BNP is of moderate toxicity, with an LD_{50} of 200 mg kg^{-1}.

4.4.5.2 Primary Irritation

Skin: At 2% and above BNP is a severe skin irritant.
Eye: Causes severe eye irritation at 5% and above.

4.4.5.3 Sensitization

Skin: Animal studies indicate that BNP has a skin-sensitizing potential. Controlled human studies and cases of occupational exposure show that BNP can cause allergic contact dermatitis. Although some studies suggest that BNP is a common cause of skin sensitization (Peters *et al*., 1983; Storrs *et al*., 1989), others show a low incidence of sensitization, For example, in a study of 8149 patients in Europe, patch testing showed an incidence of only 0.21% reaction to BNP (Frosch *et al*., 1990). A similar low reactivity rate of 0.46% in 2152 patients for patch testing to BNP was found in the United Kingdom (Shaw, 1997).

4.4.5.4 Toxicokinetics and Metabolism

In vivo studies in the rat using ^{14}C-BNP showed that about 40% of an epicutaneously applied dose was absorbed through the skin in 24 hours. Of the applied

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radioactivity, about 19% was excreted in urine, faeces and expired air. The 24 hour recoveries of ^{14}C in urine and expired air were 15 and 2%, respectively, after the cutaneous application, compared with 74 and 9% for ^{14}C-BNP given intravenously. Urine thin layer chromatography (TLC) showed three metabolites, but no unchanged ^{14}C-BNP, following both epicutaneous and intravenous dosing (Buttar and Downie, 1980).

4.4.6 Ecotoxicology

BNP has a low volatility (vapour pressure 1.6×10^{-3} Pa). A logP_{ow} of 0.18 indicates a low bioaccumulation potential. BNP is hydrostable at pH 4 and 6. At pH 8 the $t_{1/2}$ is two months (Eriksson *et al*., 1995). Photolysis occurs at a high rate, with a $t_{1/2}$ of about 35 hours. Biodegradation of 1.0 mg l^{-1} of ^{14}C-BNP at 22 °C resulted in rapid transformation (78%) after three days. Cited acute aquatic toxicity values are as follows:

Scenedesmus subspicatus: 72 hour-$EC_{50} = 0.02$ mg l^{-1}; No observed effect concentration (NOEC) = 0.01 mg l^{-1}
Daphnia magna: 48 hour $LC_{50} = 1.4$ mg l^{-1}
Misido bahia: 96 hour $LC_{50} = 5.9$ mg l^{-1}
Brown shrimp: 96 hour-$LC_{50} = 121$ mg l^{-1}
Oyster larvae: 48 hour-$EC_{50} = 0.69$ mg l^{-1} (abnormal larvae); 48 hour $LC_{50} = 1.7$ mg l^{-1}

A reproduction study with *Daphia magna* gave a 21-day NOEC of 0.27 mg l^{-1}. The Swedish National Chemicals Inspectorate has calculated a predicted no-effect concentration (PNEC) for aquatic ecosystems of 0.2 μg l^{-1} based on the lowest NOEC for algae.

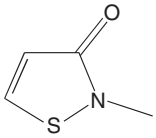
4.5 Isothiazolones

Most commercially available isothiazolone biocides contain both 2-methyl-4-isothiazolin-3-one (MIT) and 5-chloro-2-methyl-4-isothiazolin-3one (CMIT). Most tests for efficacy, mammalian toxicology and ecotoxicology have been conducted using formulations containing both molecular species.

4.5.1 Identities and Physicochemical Properties

MIT

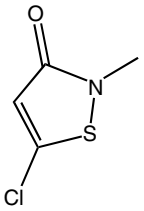
Structural formula:



CAS No: 2682-20-4
Molecular weight: 115.16
Melting point: 47–50 °C
Boiling point: 93 °C

CMIT

Structural formula:



CAS No: 26172-55-4
Molecular weight: 149.60
Melting point: 52–55 °C
Sources of information: (CIREP (Cosmetic Ingredients Review Expert Panel), 1992; Lewis *et al*., 1991).

4.5.2 Mechanism of Action

Isothiazolones are immediately bacteriostatic, but take several hours to achieve biocidal activity. The sulphur atom of N-substituted isothiazolones is electrophilic and reacts with nucleophiles (Crow and Gorman, 1969). These electrophilic molecules target dehydrogenase enzymes in the Krebs cycle, affecting cellular metabolism (Chapman and Diehl, 1995). Cell damage can be measured by the loss of protein thiols. The following mechanism has been proposed:

- Covalent modification via direct electrophilic attack of thiols
- Generation of a secondary electrophile by disulfide exchange and tautomerization to a thioacyl chloride
- Intracellular generation of free radicals, stressing free-radical defence mechanisms.

Bacteria act as a sink for CMIT, concentrating 1.0 ppm as much as 400-fold, and leading to strong association of CMIT within the cell. As the concentration of CMIT increases, the rate of association and the rate of cidal effects increases (Diehl and Chapman, 1999).

4.5.3 Stability and Decomposition

CMIT and MIT are less stable at alkaline pH, with hydrolysis beginning at pH 8. MIT is more stable at alkaline pH than CMIT. Cu^{2+} can be added as a stabilizer for CMIT, protecting it from nucleophilic attack. CMIT and MIT are also sensitive to thiols, reversing biostatic activity and preventing cell death (Chapman and Diehl, 1995).

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4.5.4 Biocidal Uses

MIT/CMIT has broad spectrum antimicrobial activity. The MIC for *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* have been determined to be 30 $\mu g\,cm^{-3}$ (Zeelie and McCarthy, 1983). Commercially available isothiazolones usually contain both CMIT and MIT in the ratio of 2.7:1 respectively. CMIT is the more effective biocide, being 100–1000-fold more active than MIT. The CMIT is very water soluble and the mixture is commercially available in 1.5 to ≈14% active solutions (Paulus, 1993, pp. 321–326). Other isothiazolones containing different alkyl chains or other modifications are available for particular applications.

Isothiazolones are used as preservatives in cosmetics and personal care. They are supplied to cosmetic manufacturers in the form of a mixture containing 0.35% MIT and 1.15% CMIT (1.5 MIT/CMIT; CIREP (Cosmetic Ingredients Review Expert Panel), 1992). They are also used in paints, slurries, adhesives and other industrial applications at 5–15 ppm active. In pulp and paper applications, cooling towers and recirculating systems, 0.5–1 ppm isothiazolones are added either continuously or as a fast-feed slug dose. Isothiazolones are sometimes added in combination with, or alternated with, GA or DBNPA. To help prevent the development of bacterial tolerance. at least one preservative formulation is available in combination with BNP. Isothiazolones are also used to prevent marine biofouling of piers and boat hulls, and certain formulations are used in wood preservation. Details of the uses of MIT/CMIT in cosmetic and noncosmetic applications are given in CIREP (Cosmetic Ingredients Review Expert Panel) (1992).

4.5.5 Toxicology

4.5.5.1 Acute Toxicology

Peroral: MIT/CMIT mixtures are moderately toxic to rats, with LD_{50} values in the range of 40–79 mg ai (active ingredient) kg^{-1}. A 1.5% MIT/CMIT mixture had an LD_{50} of 3350 $mg\,kg^{-1}$. Signs of toxicity included those of severe gastric irritation, lethargy and ataxia (CIREP (Cosmetic Ingredients Review Expert Panel), 1992). A 1.5% solution of MIT/CMIT dosed in methylcellosolve to rabbits had an oral LD_{50} of 30 $mg\,kg^{-1}$. Signs of toxicity were decreased motor activity, decreased breathing and those of gastric irritation.

Intraperitoneal: In aqueous solution, acute intraperitoneal LD_{50} values to Wistar rats were 4.6 $mg\,kg^{-1}$ (males) and 4.3 $mg\,kg^{-1}$ (females). The major sign was decreased motor activity and the principal lesion was peritonitis.

Percutaneous: Several MIT/CMIT mixtures were tested, with resultant LD_{50} values determined to be >75, 87, 94, 112 and 130 mg ai kg^{-1}.

4.5.5.2 Primary Irritation

Skin: In New Zealand white rabbits and using standard occluded skin-irritation tests with concentrations in the range 1.1–13.7%, there was severe irritation. Aqueous dilutions of MIT/CMIT were tested on rabbit skin and 0.056% ai was found to be nonirritating, 0.28% ai was moderately irritating, 0.56% ai was severely irritating, and 5.6% ai was corrosive. In humans, the irritation potential of MIT/CMIT is concentration-dependant, with 400–800 ppm being strongly irritating, 200 ppm being slightly irritating, and 100 ppm essentially nonirritating (CIREP (Cosmetic Ingredients Review Expert Panel), 1992).

Eye: In rabbit eye irritation tests, mixtures of MIT/CMIT in the concentration range 1.1–14.0% produced severe irritation, 0.56–1.7% was moderately to severely irritating, 0.28% slightly irritating and 0.056% essentially nonirritating (CIREP (Cosmetic Ingredients Review Expert Panel), 1992). In a rabbit cumulative eye irritation test, 0.1 ml of 0.0056% MIT/CMIT was instilled into the conjunctival sac every 15 minutes for two hours, and this procedure repeated daily for five consecutive days. Mild intermittent conjunctivitis, comparable to tap-water controls, was seen. In a chorioallantoic membrane (CAM) test, 0.03% MIT/CMIT was nonirritating, but higher concentrations produced hyperemia, haemorrhage and coagulation (CIREP (Cosmetic Ingredients Review Expert Panel), 1992).

4.5.5.3 Sensitizing Potential

Skin: With guinea pigs receiving up to nine epicutaneous induction dosages given over three weeks and challenge doses two weeks later, it was found that delayed contact dermatitis developed, the incidence of which was dependant on both the induction and challenge concentrations. In one study, the EC_{50} (half maximal effective concentration) for guinea pigs challenged with 1000 ppm MIT/CMIT was 88 ppm (Chan *et al.*, 1983). The sensitizing potential of MIT/CMIT has been investigated extensively in humans (CIREP (Cosmetic Ingredients Review Expert Panel), 1992). There is general agreement that the MIT/CMIT complex has sensitizing potential, although the potency of CMIT in this respect may be greater than that of MIT. However there is some discrepancy in the cited eliciting concentration. Based on an extensive review of the extensive data, the Cosmetic Ingredients Review Expert Panel (CIREP) has concluded that for cosmetic preparations, MIT/CMIT may be safely used in 'rinse-off' products at a concentration not exceeding 15 ppm, and in 'leave-on' products at a concentration not exceeding 7.5 ppm; this conclusion refers to a mixture containing 23.3% MIT and 76.7%

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CMIT (CIREP (Cosmetic Ingredients Review Expert Panel), 1992).

4.5.5.4 Short-Term Repeated and Subchronic Toxicology

Inhalation: Male rats were exposed for six hours a day, five days a week for two weeks to an aerosolized aqueous solution of MIT/CMIT at 0, 0.03, 0.07 and 0.13 mg ai l^{-1}. Mid- and high-dose rats had decreased body-weight gain. There were a few deaths amongst low and high exposure-concentration rats. Lesions included pulmonary haemorrhages and enlarged livers. Thus, under the conditions of this study the NOEC was <0.03 mg l^{-1}.

Peroral: MIT/CMIT was included in the diets of rats for three months to give average daily dosages of 0, 3, 10 and 30 mg kg^{-1}. No mortalities occurred. There was a dosage-related increase in absolute and relative adrenal gland weights in females, and high-dose males had slight, but statistically significant increases in serum glutaric-oxaloacetic transaminase. There were no histopathological lesions. Beagle dogs had average daily dietary dosages of 0, 3, 10 and 30 mg kg^{-1} for three months. This resulted in no abnormal effects with respect to signs, chemical pathology or histology. In another study rats received MIT/CMIT in the drinking water at average daily dosages of 0, 3, 8 and 20 mg kg^{-1} for 13 weeks. There was no mortality, but water consumption was decreased in all MIT/CMIT-treated rats. A significant decrease in serum globulin concentration with increased A/G (albumin/globulin) ratio occurred in the high-dosage females. Relative liver and kidney weights were increased in males and females of the high-dosage group. Slight gastric irritation was seen in the high-dose males and females. The NOEL was 8 mg kg^{-1} day^{-1}, and the minimal effects dosage was 20 mg kg^{-1} day^{-1}.

Cutaneous: Male and female New Zealand white rabbits had cutaneous applications of 0, 0.1, 0.2 and 0.4 mg kg^{-1} daily, five days a week, for 13 weeks to both intact and abraded skin. Deaths, attributed to endemic respiratory disease, occurred in all treatment groups; 3/12, 5/12 and 4/12 respectively for the low-, mid- and high-dosage groups. There was dosage-related slight to severe local erythema and slight oedema, but no treatment-related findings at necropsy or histology.

4.5.5.5 Chronic Toxicology and Oncogenicity

A chronic skin-painting study was conducted in which 25 µl per rat of a distilled water solution containing 400 ppm MIT/CMIT was applied daily, three times a week, for 30 months to the dorsal skin of male Charles River CD-1 mice. A positive control group received 1000 ppm 3-methylcholanthrene in acetone, and a negative control received tap water. In the MIT/CMIT group there was no indication of an increase in local or systemic neoplasms, but all animals in the 3-methylcholanthrene group had local squamous cell carcinomas within six months of the start of treatment.

4.5.5.6 Genetic Toxicology

MIT/CMIT was mutagenic in *Salmonella typhimurium* strain TA 100 with and without metabolic activation, but not with strains TA98, TA1535, TA1537 or TA1538 (Wright *et al*., 1983). When tested separately, CMIT, but not MIT, was mutagenic in TA100 (Scribner *et al*., 1983). MIT/CMIT was also mutagenic, without metabolic activation, in *Escherichia coli* WP2uvrA(p) (Wright *et al*., 1983) and in mouse lymphoma L5178Y cells (CIREP (Cosmetic Ingredients Review Expert Panel), 1992). MIT/CMIT was not genotoxic in the following tests; unscheduled DNA synthesis using rat hepatocytes (Scribner *et al*., 1983); *in vitro* chromosomal aberration test with Chinese Hamster lung fibroblasts; DNA binding *in vitro* with mouse lymphoma cell line; gender-linked recessive lethal test with *Drosophila melanogaster* (Scribner *et al*., 1983); mouse *in vivo* cytogenetics assay; *in vivo* DNA binding with rat testis; and detection of induced cell transformation in mouse embryo fibroblast cell line C3H 10 T1/2 (Scribner *et al*., 1983; CIREP (Cosmetic Ingredients Review Expert Panel), 1992).

4.5.5.7 Developmental Toxicity

When given by gavage to Dutch belted rabbits over gestational days (GD) 6–18 at dosages of 0, 1.5, 4.4 and 13.3 mg ai kg^{-1} day^{-1}, MIT/CMIT was maternally toxic with dose-related mortalities and signs of toxicity (ataxia, diarrhoea and gastric irritation). There were decreases in the number of live foetuses, increased resorption sites and increased postimplantation losses, but no malformations. Thus, in this test, MIT/CMIT was embryofoetotoxic but not teratogenic at maternally toxic dosages (CIREP (Cosmetic Ingredients Review Expert Panel), 1992). When given by gavage to Sprague-Dawley rats over GD5–15 at dosages of 0, 1.5, 4.5 and 15 mg kg^{-1} day^{-1}, there was a low incidence of dosage-related mortalities and signs of toxicity, but no signs of embryofoetotoxicity or teratogenic effects. Male and female Charles River rats were given MIT/CMIT in drinking water at daily average dosages of 0, 3, 8 and 20 mg kg^{-1} for 15 weeks. Rats from the same dosage groups were then mated. There were no adverse effects on fertility, reproduction, foetal survival or foetal health (CIREP (Cosmetic Ingredients Review Expert Panel), 1992).

4.5.5.8 Toxicokinetics and Metabolism

With the Sprague-Dawley rat, ^{14}C-MIT/CMIT was rapidly distributed following intravenous dosing with a total recovery of 94–111%. A rapid elimination of ^{14}C from plasma with sustained maintenance of whole blood ^{14}C (3 µg g^{-1}) at 6–96 hours postdosing suggested ^{14}C to be erythrocyte-bound. In support of this was slow elimination of ^{14}C from the spleen

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and liver. Tissue elimination was biphasic, with a $t_{1/2}$ of >4 hours. By 96 hours faeces, urine and $^{14}CO_2$ accounted for 35, 31 and 4% of the administered dose, respectively. Following epicutaneous application, cutaneous recovery of ^{14}C-CMIT ranged 89–94% over a concentration range of 0.05–0.4%, and was 13% greater than ^{14}C-MIT at 0.2%. Approximately 50% of the radioactivity was associated with the treated skin. Elimination of ^{14}C from the application site had a $t_{1/2}$ of 13.1 days, suggesting a potential for local accumulation by repeated topical application. As the applied concentration was increased, the proportion of ^{14}C in skin decreased and that in excreta increased (Debethizy *et al*., 1986). Other comparative studies in the rat indicate that ^{14}C-MIT and ^{14}C-CMIT were similar in the degree of absorption, binding and excretory patterns following intravenous, peroral and cutaneous dosing. However, MIT produced higher blood concentrations after oral and cutaneous dosing, and a 45% greater relative absorption after peroral dosing than CMIT. Both dose-dependant and saturable processes governed absorption, distribution and elimination of MIT/CMIT in the rat (CIREP (Cosmetic Ingredients Review Expert Panel), 1992).

4.5.6 Ecotoxicology

MIT/CMIT is completely soluble in water, and has relatively high vapour pressure at 8.3 Pa for MIT and 2.4 Pa for CMIT. Henry's constant is calculated to be 9.56×10^{-7} Pa m^3 mol^{-1} for MIT and 3.59×10^{-7} Pa m^3 mol^{-1} for CMIT, indicating low volatility. At pH 5 and 7 CMIT is hydrolytically stable, but at pH 9 hydrolysis occurs with a $t_{1/2}$ of 22 days. MIT is hydrolytically stable at pH 5, 7 and 9. An aerobic degradation study in river water using ^{14}C-CMIT resulted in conversion of 25% to $^{14}CO_2$ after 29 days. Another aerobic degradation study with sediment/water at 25 °C and ^{14}C-CMIT at a concentration of 1.0 mg ai l^{-1} resulted in rapid transformation with a DT_{50} (degradation time with a 50% activity reduction) of 17.3 hours. After seven days, ≈2.8% of radioactivity had been evolved as $^{14}CO_2$ and 57% was bound to sediment. An aerobic degradation study with ^{14}C-MIT in water/sediment at 25 °C, and a test concentration of 1.0 mg ai l^{-1} gave a DT_{50} of 9.1 hours. At seven days ≈9% of radioactivity evolved as $14CO_2$ and 68% was bound to sediment. These studies indicate that MIT/CMIT is inherently, but not readily, biodegradable. MIT/CMIT has high water solubility and respective logP_{ow} values of −0.5 and 0.4, indicating a low potential for bioaccumulation. However, a bioaccumulation study in bluegill sunfish resulted in bioconcentration factors (BCF) of 102, 114 and 67 for CMIT at respective doses of 0.02, 0.12 and 0.8 mg l^{-1}, suggesting that CMIT bioaccumulates. The BCF for MIT is 2.3 at a dose of 0.12 mg l^{-1}, indicating the absence of bioaccumulation. Acute toxicity values for MIT/CMIT are as follows:

Skeletonema costatum: 72 hour $EC_{50} = 0.01$ mg l^{-1} with NOEC < 0.01 mg l^{-1}
Daphnia magna: 48 hour $EC_{50} = 0.18$ mg l^{-1}
Brachydanio rerio: 96 hour $LC_{50} < 0.27$ mg l^{-1} with NOEC = 0.084 mg l^{-1}
Cyprinodon variegatus: 96 hour $LC_{50} = 0.3$ mg l^{-1} with NOEC = 0.18 mg l^{-1}

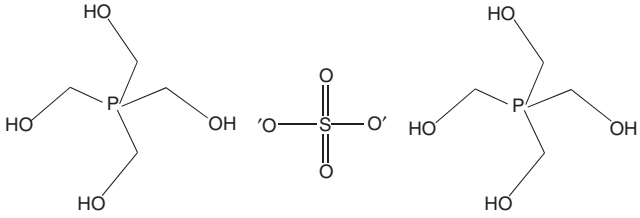
Prolonged testing indicates high toxicity:

Oncorhyncus mykiss: 24 hour $LC_{50} = 0.14$ mg l^{-1}; 14 day $LC_{50} = 0.07$ mg l^{-1}; NOEC = 0.05 mg l^{-1}
Pimephales promelas: Four day $LC_{100} = 0.23$ mg l^{-1}; NOEC = 0.02 mg l^{-1}
A reproduction study with *Daphnia magna* resulted in a NOEC (21 days) of 0.1 mg l^{-1}.

4.6 Tetra-(Hydroxymethyl)-Phosphonium Sulfate (THPS)

4.6.1 Identities and Physicochemical Properties

Structural formula:



CAS No: 55566-30-8
Molecular weight: 406.49
Boiling point: 108.5 °C (75%)
Density: 1.3 g ml^{-1} (75%)
Appearance: Clear straw coloured liquid
Solubility: Miscible with water
Sources of information: (Paulus, 1993, pp. 55–57, 137–138).

4.6.2 Mechanism of Action

Although THPS is a quarternary phosphonium compound, its mode of action differs from that of other quarternary ammonium compounds. THPS has an acidic β-proton that is cleaved by base to form

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tris(hydroxymethyl)phosphine (THP) and formaldehyde (Hellman and Schumacher, 1960; Kirby and Warren, 1967). The phosphine is the biologically active species. It is known that Ph_3P reduces disulfides to thiols. THPS presumably acts as a reducing agent in a similar manner, disrupting the disulfide bonds in proteins and enzymes (Burns *et al*., 1991; March, 1992; Overman and Petty, 1975; Overman *et al*., 1974). After reaction with disulfide, THPO is formed, which is no longer active (Overman and Petty, 1975).

4.6.3 Stability and Deactivation

THPS is stable at acidic pH, but at alkaline pH it releases formaldehyde to form the active THP species. THP is unstable in the presence of disulfides, acting as a reducing agent to reduce disulfides and degrading to THPO. THPS is also unstable in the presence of oxidizers such as chlorine and peroxide. Both the oxidizer and THPS are consumed in these reactions. THPS is inactivated by reducing agents such as bisulfite. If used in the presence of the oxygen scavenger ammonium bisulfite, THPS has been known to react with the bisulfite in water resulting in an increase in the dissolved oxygen content.

4.6.4 Biocidal Uses

THPS is commercially available as an aqueous solution up to 75% active. Some formulations contain surfactants. THPS is a fast-acting biocide with activity against Gram-positive and Gram-negative bacteria, fungi and algae, as well as SRB. THPS can be added continuously or as a slug dose. It is used as an oil-field biocide at 10–300 ppm in slug doses. It is also used in pulp and paper manufacturing (15–350 ppm), although not in conjunction with oxidizing biocides. THPS has some nonbiocidal uses, such as leather tanning and as an FeS scavenger.

4.6.5 Toxicology

4.6.5.1 Subchronic Toxicity

In a 13 week gavage study F344/N male and female rats received doses of THPS ranging 5–60 mg kg^{-1} and B6C3F1 male and female mice received doses in the range 2–180 mg kg^{-1}. Mortality and reduced body-weight gain occurred in both sexes and both species. Vacuolar degeneration of hepatocytes and hepatocellular necrosis was a common histopathological finding (NTP, 1987).

4.6.5.2 Chronic Toxicity and Oncogenesis

F344/N rats and $B6C3F_1$ mice received 0, 5 or 10 mg THPS kg^{-1} in distilled water by gavage daily for five days a week for 103 or 104 weeks. Mean body weights of rats and mice were comparable to those of controls and no signs of toxicity were seen. There was a dosage-related increase in the incidence of cytoplasm vacuolization of liver cells in rats, of focal hyperplasia of the adrenal medulla in high-dose male mice, and of thyroid gland follicular cell hyperplasia in high-dosage female mice. There was no evidence for carcinogenicity of THPS in either sex of rats or mice (NTP, 1987).

4.6.5.3 Genetic Toxicology

THPS was not active in a *Salmonella typhimurim* reverse mutation test with and without metabolic activation (Ulsamer *et al*., 1980). THPS induced forward mutations in mouse lymphoma L5178Y cells (NTP, 1987). In a micronucleus test, THPS produced small increases in micronuclei at 700 and 1000 mg kg^{-1}, and in a dominant lethal assay using ICR mice, THPS produced a significant decrease in the number of pregnant females and an increase in the number of mortalities at 1000 mg kg^{-1}. There was no increase in the average number of implants per pregnancy (Ulsamer *et al*., 1980).

4.7 Peracetic Acid (PAA)

4.7.1 Identities and Physicochemical Properties

Molecular formula: CH_3-CO-O-OH
CAS No: 79-21-0
RETECS No: SD8750000
Synonyms: Peroxyacetic acid; acetyl hydroperoxide; ethaneperoxoic acid; PAA
Molecular weight: 76.05
Specific gravity: 1.15 (20 °C)
Viscosity: 3.280 cP
Freezing point: −30 °C
Boiling point: 105 °C (40% solution)
Vapour pressure: 2.6 kPa (20 °C)
Flash point: 40.5 °C
pK_a: 8.20
Appearance: Clear colourless liquid
Odour: Acrid type
Sources of information: (ICSC, 2000; Lewis, 1993; NAS, 1995; Paulus, 1993).

4.7.2 Mechanism of Action

PAA acts as a protein denaturant and oxidatively disrupts sulfhydryl and sulphur bonds within essential metabolic enzymes (Lefevre *et al*., 1992). In addition, PAA disrupts membrane transport by rupturing or dislocating cell membranes, and thereby altering their function (Fraser, 1986). The mechanism for this disruption is likely by radical formation.

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4.7.3 Stability and Decomposition

Lower concentrations of PAA are in equilibrium with acetic acid, peroxide and sulfuric acid. The sulfuric acid is added to favour an equilibrium towards PAA. Eventually the PAA decomposes to acetic acid and water (Block, 2001; Fraser, 1986). Concentrated PAA is not very stable unless stabilizers are added to prevent decomposition to acetic acid and water. PAA is less active in the presence of organics, and most stable at acidic pH. It is most active at pH 2.5–4.0, and inactivated by thiols and other reducing agents (Block, 2001; Paulus, 1993, pp. 430–432). Concentrated PAA requires handling with care because of the potential to be explosive at relatively low temperatures (Paulus, 1993, pp. 430–432).

4.7.4 Biocidal Uses

PAA is commercially available, for use as a general biocide, as an equilibrium mixture with acetic acid, hydrogen peroxide and sulfuric acid in water at about 50% PAA. It is also available as an ≈35% concentrate for instrument sterilization. PAA is regarded as a highly effective disinfectant, but experience with the substance is limited and it is suggested that it should be used in sealed or exhaust-ventilated facilities (Working Party (Report of a Working Party of the British Society of Gastroenterology Endoscopy Committee), 1998). It is more expensive than GA, less stable and larger volumes have to be stored. In a comparative study with chlorine dioxide, PAA was found to produce a high abatement of micro-organisms and considered to be a valuable alternative in the disinfection of wastewater (Stampi *et al*., 2001). Disinfection of wastewater with PAA has been reviewed by Kitis (2004).

PAA is considered more potent than peroxide and better able to control bacteria and spores at safe levels (Block, 2001). PAA has broad-spectrum activity. Aside from being active against bacteria, fungi and viruses, it is also mycobacteriocidal and sporicidal. Therefore, PAA is one of the few biocides capable of low-temperature-liquid high-level disinfection and sterilization (0.1–0.5% active). In addition, PAA vapour can also be used as a sterilant/high-level disinfectant. One commercially available sterilant uses concentrated PAA that is diluted to ≈0.2% active at 55 °C in a specially designed reprocessor (Schneider, 1994). This is a single-use process, requiring fresh PAA for each cycle. Another manufacturer makes a sterilant/ high-level disinfectant at a much lower concentration (0.08%). This formulation also contains 1% hydrogen peroxide and is reusable up to 14 days. Since PAA degrades into acetic acid and water, it is regarded as easy to use and to dispose of. It is, however, somewhat corrosive and repeated use as a sterilant requires the use of a corrosion inhibitor to preserve the integrity of the instruments (Schneider, 1994). PAA is used to disinfect food-processing equipment, including in meat processing, dairies and breweries. Because of the fast decomposition of PAA, these surfaces do not usually require rinsing after use. The low-concentration equilibrium formulations are used to treat cooling towers and water-recirculating systems in slug doses of 1–10 ppm. PAA is also one of the few biocides used to reprocess renal dialysers and to control mould growth on fruits and vegetables. It has also been convenient and effective as a sterilant for apparatus and equipment used to maintain gnotobiotic animals (Fordham, 1978).

4.7.5 Toxicology

4.7.5.1 Acute Toxicity

Peroral: Rat peroral LD_{50} is 1540 mg kg^{-1} for a 40% solution (Anonymous, 1982; NAS, 1995).
Percutaneous: LD_{50} for the rabbit is 1410 mg kg^{-1} (NAS, 1995).

4.7.5.2 Primary Irritancy

Skin: A 40% solution is corrosive (NAS, 1995).
Eye: At 0.1% in water, PAA is not irritating to the rabbit eye. A 10% solution causes ulceration and perforation of the cornea, and formation of synblepharon (Grant and Schuman, 1993). A 40% solution can cause severe eye burns (NAS, 1995).

4.7.5.3 Peripheral Sensory Irritation

PAA is a relatively potent peripheral sensory irritant. In mice, a one hour RD_{50} of 5.4 ppm has been determined (Gagnaire *et al*., 2002). The authors suggest that this value indicates a TLV-STEL (short-term exposure limit) of 0.5 ppm (0.1 RD_{50}) and a TLV-TWA (time-weighted average) of 0.2 ppm (0.03 RD_{50} (exposure concentration producing a 50% respiratory rate decrease)).

4.7.5.4 Genetic Toxicology

When given by intraperitoneal injection at a dosage of 25 mg kg^{-1} day^{-1} for 35 days, PAA produced a slight increase in the rate of mouse sperm-head abnormalities (Koch *et al*., 1989). PAA did not induce DNA-repair synthesis (Coppinger *et al*., 1983).

4.7.6 Occupational Medical Aspects

Skin or eye contact with a 40% solution will cause serious burns. Inhalation of high vapour concentrations or mists will lead to burning sensations in the nose, throat and chest, and coughing, wheezing and shortness of breath (NAS, 1995). These clinical findings indicate that local exhaust ventilation is required, and protective clothing, gloves and eye protection are required when working with PAA (ICSC, 2000).

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4.8 Glutaraldehyde (GA)

4.8.1 Identities and Physicochemical Properties

Molecular formula: OHC-CH_2-CH_2-CH_2-CHO
CAS No: 111-30-8
Synonyms: Glutaral; Glutaric dialdehyde
Molecular weight: 100.13
Freezing point: −21 °C (50%)
Boiling point: 100.5 °C (50%) (760 mm Hg)
Density (20 °C): 50% = 1.129 g ml^{-1}; 25% = 1.064 g ml^{-1}; 10% = 1.026 g ml^{-1}; 2% = 1.006 g ml^{-1}
Vapour pressure (25 °C): 50% = 0.116 mm Hg; 15% = 0.041 mm Hg; 2% = 0.0051 mm Hg
Vapour concentration (25 °C): 50% = 207 ppm; 15% = 53 ppm; 2% = 7 ppm; 0.1% = 0.3 ppm; 0.01% = 0.03 ppm
Henry's Law constant: 0.0000331 atm mol^{-1}
Odour: Apple-like
Odour threshold: 0.04 ppm
Solubility (20 °C): Miscible with water, acetone and isopropanol; methylene chloride = 36 g l^{-1}; ethyl acetate = 30 g l^{-1}; toluene = 4.4 g l^{-1}; *n*-hexane = 0.96 g l^{-1}
$P_{ow} = 0.98$
Sources of information: (BIBRA, 1991; CIREP (Cosmetic Ingredients Review Expert Panel), 1996; Olson, 1998)

4.8.2 Mechanism of Action

GA is an aliphatic dialdehyde that is capable of reacting with amines and thiols. When GA reacts with amines, it forms a stable six-membered cyclic compound that can be further reacted with another GA molecule or nucleophile (Banin *et al*., 1992; Berrien *et al*., 1994; Francois *et al*., 1998). GA has been used to synthesize cyclic organic compounds in this manner (Banin *et al*., 1992; Berrien *et al*., 1994). GA reacts with amino acids (such as the free amine of lysine) in proteins in a similar manner, producing a crosslinked cell. The formation of a stable six-membered ring is likely to be the reason GA is a potent biochemical dialdehyde. In addition to crosslinking, there are indications that GA inhibits protein synthesis precursor uptake and some cellular enzymes (Scott and Gorman, 2001). The nonspecific nature of the mechanism of action of GA leads to broad-spectrum activity. GA biocidal activity increases at alkaline pH, presumably because, as the pH reaches the pK_a of the available amines, the unprotonated amines become more susceptible to reaction with GA.

4.8.3 Stability and Decomposition

GA is stable at pH 3.0–4.5. As the pH increases (>10), GA forms both soluble and insoluble polymers through aldol condensation. GA is inactivated by bisulfite and primary amines. Complex formation with bisulfite is reversible at elevated temperature (>55 °C). GA is manufactured commercially as a 50% aqueous solution because 100% GA is very unstable and forms clear glass polymers in a relatively short time.

4.8.4 Biocidal Uses

GA is commercially available in aqueous solutions (≈20–50%) (Paulus, 1993, pp. 37–38, 45–47). There are some formulations with ADAC quats at a ratio of 7:1 GA:ADAC. Instrument sterilant formulations are typically 2–4% active GA, some containing surfactants.

GA is used as a general biocide at 25–200 ppm active in pulp and paper processing, cooling water towers, water-recirculating systems, and oil-field recovery (Paulus, 1993, pp. 37–38, 45–47; Scott and Gorman, 2001). GA is also used in some industrial preservative applications at 100–1000 ppm active, and as an animal-housing sanitizer (1000 ppm). It also finds use in reprocessing renal dialysers. GA is sometimes used in conjunction with other biocides, such as quarternary ammonium compounds. GA is a fast-acting biocide, effective against Gram-positive and Gram-negative bacteria, yeast and fungi (>500 ppm) and SRB. GA has been shown to be effective against biofilm. Any reduced activity is linked to transport limitation rather than phenotypic change in the biofilm (Grobe and Stewart, 2000). Since GA is effective against mycobacterial spores, it is one of the few biocides available that can be used as a cold high-level disinfectant or sterilant for endoscopes and other thermosensitive devices (Scott and Gorman, 2001). When used as a sterilant, most acidic solutions of GA (≈2.5% active) are activated by a buffer system to produce an alkaline solution (Ballantyne *et al*., 1997). These sterilants are reusable for up to 28 days. They can be used for manual disinfection, as well as in automatic reprocessors. The instruments require at least a 20 minute soak in the sterilant at 20 °C and then copious rinsing to remove residual GA from the endoscope (Ballantyne and Jordan, 2001). Unlike many other biocides, GA also has many nonbiocidal uses, including leather tanning, tissue fixation, enzyme immobilization, embalming and crosslinking bioprostheses such as heart valves.

4.8.5 Toxicology

4.8.5.1 Acute Toxicity

4.8.5.1.1 Peroral

Aqueous solutions of 5% and higher are of moderate acute peroral toxicity, and those of 2% and lower are

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Table 2 Acute lethal peroral toxicity of aqueous solutions of various concentrations of glutaraldehyde for male rats and mice[a]

Species	GA (% w/w)	LD_{50} (95% confidence limits)	
		ml kg^{-1}[b]	mg GA kg^{-1}[c]
Rat	50	1.30 (0.27–1.94)	733 (452–1095)
	45	1.19 (0.84–1.69)	538 (422–849)
	25	1.54 (1.14–2.08)	410 (303–553)
	15	1.17 (0.72–1.88)	183 (112–293)
	10	1.62 (1.01–2.62)	166 (104–269)
	5	3.25 (2.40–4.39)	170 (122–222)
	2	3.34 (2.53–4.43)	67 (51–89)
	1	12.30 (9.13–16.70)	123 (92–168)
Mouse	50	0.27 (0.18–0.40)	152 (102–225)
	25	0.65 (0.32–1.30)	173 (85–346)
	5	0.62 (0.36–1.04)	31 (18–53)
	1	3.36 (1.35–8.41)	34 (14–84)

[a]Data from Ballantyne and Myers (2001).
[b]As volume of glutaraldehyde solution dosed.
[c]As contained glutaraldehyde dosed.

of slight toxicity, with the mouse being more sensitive than the rat (Ballantyne and Myers, 2001) Typical LD_{50} values are given in **Table 2**. Expressed as the volume of solution dose (ml kg^{-1}), the LD_{50} values increase with dilution, but when expressed as the amount of GA given (mg GA kg^{-1}) the values decrease with increasing dilution. This may have implications for the first-aid management of swallowed GA solutions. Mortality usually occurs within one to three days. Dilute solutions produce only minor irritation of the upper alimentary tract, but more concentrated solutions produce severe irritation and corrosive lesions of the upper alimentary tract. Signs of toxicity include piloerection, sluggishness, rapid breathing and diarrhoea. Survivors recover from these effects by one to five days postdosing, and generally do not show any gross pathology. Decedents usually show congestion and haemorrhage of the gastric mucosa with thickening of the pyloric area, and congestion of the small intestinal mucosa.

4.8.5.1.2 Percutaneous

Aqueous solutions of 45% and higher are of moderate acute percutaneous toxicity, those of 25% of slight toxicity, and 15% solutions do not produce systemic toxicity (**Table 3**; Ballantyne and Myers, 2001).

Table 3 Acute percutaneous lethal toxicity of various concentrations of aqueous glutaraldehyde for male rabbits[a]

GA (% w/w)	LD_{50} (95% confidence limits)	
	ml kg^{-1}[b]	mg GA kg^{-1}[c]
50	2.54 (1.46–4.41)	1434 (824–2489)
46	2.00 (1.18–3.39)	1004 (593–1703)
25	8.80 (1.91–33.5)	2341 (508–8911)
15	Killed 0/5	

[a]24 hour occluded contact and 14 day observation period. Data from Ballantyne and Myers (2001).
[b]As volume of glutaraldehyde solution dosed.
[c]As contained glutaraldehyde dosed.

4.8.5.1.3 Vapour Exposure

Exposure to vapour generated dynamically or statically at ambient temperature does not produce mortalities, and signs are limited to those of respiratory tract and ocular irritation (blepharospasm, lacrimation, nasal discharge, abdominal and mouth breathing). Typical exposure situations for ambient generation conditions are shown in **Table 4**. Definitive toxicity is seen only in animals acutely exposed to GA vapour generated at high temperature (Ballantyne and Myers, 2001). For example, when rats were exposed to GA vapour generated at 65 °C, they showed an exposure-concentration-related mortality pattern, allowing the calculation of four hour LC_{50} values of 23.5 ppm for males and 40.1 ppm for females. A further study compared the acute vapour exposure toxicity to rats of GA vapour generated at ambient (24 °C) vs. elevated (60 °C) temperature (Ballantyne and Myers, 2001). Under elevated temperature conditions there were four hour LC_{50} values of 36.9 ppm for males and 44.3 ppm for females. Signs included blepharospasm, lacrimation, mouth and audible breathing, and hypoactivity. In contrast, with ambient temperature conditions and GA vapour concentrations of 27.1 and 27.4 ppm, there were no mortalities. The marked differences between the elevated and ambient temperature generation findings may, in part, be due to the ability to generate higher vapour concentrations at the elevated temperature, but the possibility that a toxic

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Table 4 Acute vapour exposure studies conducted at ambient temperature with rats, and vapour generated to give saturated vapour conditions[a]

Generation	GA vapour (ppm)[b]	Exposure time (h)	Mortality[c]	Signs
Static	48.1 ± 6.77	6	0/6	Lacrimation Perinasal discharge
	4.9 ± 3.4	6	0/6	Periocular wetness Laboured breathing
Dynamic	8.1 ± 2.2	4	0/10	Blepharospasm Rhinorrhea
	16.3 ± 15.0	4	0/10	Blepharospasm Audible breathing
	14.5 ± 2.1	4	0/10	Blepharospasm Periocular wetness

[a]Data from Ballantyne and Myers (2001).
[b]As mean ± SD.
[c]As number dying/number exposed.

species is produced during the heating of GA solutions cannot be excluded. For example, in a four hour saturated vapour study (23 °C), in which the GA vapour concentration was 22.2 ppm, there were no mortalities. In contrast, in a study with vapour generation at 65 °C and a vapour concentration of 23 ppm 2/6 males and 2/6 females died. These findings, at comparable GA vapour concentrations, suggest the generation of a toxic species under the elevated temperature conditions.

4.8.5.2 Primary Irritation

4.8.5.2.1 Skin

Standard rabbit primary skin-irritation studies (four hours occluded contact with 0.5 ml) have shown concentration-related effects (**Table 5**). At 45–50%, aqueous solutions of GA produce severe local inflammation (erythema, oedema, desquamation) and corrosion; at 25% there is moderate inflammation, at 5–10% minor to moderate inflammation and 1% is threshold. Alkalinization of 2.2% aqueous GA solutions has no significant effect on skin-irritating potential (Ballantyne *et al*., 1997). In a controlled study with human volunteer subjects it was found that 0.5% aqueous GA solution was slightly irritant (erythema), 0.2% was marginal and 0.1% was not irritant (Ballantyne and Berman, 1984). Regional variations in cutaneous irritant effects occur, which are primarily related to the thickness of the skin (Juhlin and Hannson, 1968; Prigant *et al*., 1996; Reifenrath *et al*., 1985).

4.8.5.2.2 Eye

Rabbit eye irritation tests (Ballantyne and Myers, 2001) have shown the lowest concentration of GA to produce corneal injury is 1%, and the no-effect concentration is 0.5%. Corneal *injury* is mild at 2% GA, but severe at 5%. For conjunctival irritation, the threshold is 0.2% GA and the no-effects concentration is 0.1%. At 1–2%, conjunctival hyperemia and chemosis are marked. Alkalinization of 2.2% GA solution with buffer resulted in corneal injury and conjunctival inflammation being greater and persisting longer than with acidic solutions (Ballantyne *et al*., 1997). Only a few case reports of human eye injury from GA have been published (Dailey *et al*., 1993; Murray and Ruddy, 1985).

Table 5 Primary irritant effects of 0.5 ml of various concentrations of glutaraldehyde in water applied under occluded conditions to shaven dorsal trunk skin of rabbits[a]

GA (% w/w)	Contact time	Observations
50	4 h	Necrosis
50	1 h	Erythema, oedema, desquamation, alopecia, necrosis
50	3 min	Minor transient erythema
45	4 h	Moderate erythema, mild oedema, punctatenecrosis
25	4 h	Moderate erythema, mild oedema, punctatenecrosis
10	4 h	Moderate erythema, mild oedema, punctatenecrosis
5	4 h	Minor erythema and oedema
2	4 h	Minor erythema
1	4 h	None

[a]From Ballantyne and Myers (2001).

4.8.5.3 Sensitizing Potential

4.8.5.3.1 Skin Sensitization

Evidence for a low incidence of skin sensitization comes from laboratory animal studies, controlled human volunteer studies, and clinical and occupational case reports. A contact hypersensitivity-type reaction has been demonstrated with the mouse ear swelling test (MEST) (Descotes, 1988; Gad *et al*., 1986; Sailstad *et al*., 1993). Mouse local lymph node proliferate assays (LLNAs) have show concentration-related stimulation of nodal activity, indicating a skin-sensitizing potential for GA (Ballantyne and Jordan, 2001; Dearman *et al*., 1999; Hilton *et al*.,

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1998a). A dosage-related hypersensitivity response was obtained in the guinea pig and mouse that was statistically significant at 0.3% in mice and 3% in both species (Stein *et al*., 1987). Unbuffered 2.2% GA had a greater skin-sensitizing potential than buffered GA (pH 7.8) in a guinea-pig maximization study (Ballantyne *et al*., 1997). Using an LLNA procedure, Hilton *et al*. (1998b) found GA had a greater potential to induce skin sensitization than did formaldehyde.

In a repeated-insult patch test with human volunteer subjects (Ballantyne and Berman, 1984) it was found that 0.1 and 0.2% GA did not cause a sensitizing reaction, but with 0.5% GA induction there was a challenge reaction in one of 109 subjects. There are several reports of allergic contact dermatitis from occupational exposure to GA and also cases of skin sensitizing reactions to GA when used in the treatment of various dermatological diseases (reviewed by Ballantyne and Jordan, 2001). There is some evidence for regional variations in susceptibility to skin sensitization with GA, and which appear to be related principally to differences in skin thickness (Maibach and Prystowsky, 1997). Cross sensitization to formaldehyde does not occur (Gordon, 1983; Lyon, 1971; Maibach, 1975), but cross sensitization to other dialdehydes is possible (Zemtsov, 1992). The available evidence indicates that GA has a potential to cause skin sensitization, with an eliciting threshold concentration of 0.5%. Alkalinization of GA solutions may reduce the incidence of skin-sensitizing reactions (Ballantyne *et al*., 1997). Rarely, allergic contact dermatitis has resulted from exposure to GA vapour (Fowler, 1989). GA does not produce phototoxic or photoallergic reactions (Ballantyne and Jordan, 2001).

4.8.5.3.2 Respiratory Sensitizing Potential

A guinea-pig bronchial hypersensitivity study did not show any change in breathing rate or respiratory waveform after GA vapour challenge exposures (Werley *et al*., 1995). However, a murine IgE induction study produced a concentration-related increase in serum IgE that was significant for 25 and 10% GA, but not 5% GA (Ballantyne and Jordan, 2001). Also, in a cytokine secretion profile study in BALB/c mice (Dearman *et al*., 1999), high levels of interleukins-4 and -10, but low levels of interferon-γ were produced, which is typical of a Th2 (T-helper cell 2) response. Several publications have drawn attention to the possibility that asthmatic-like symptoms may occur following exposure to GA vapour in the occupational environment (summarized by Ballantyne and Jordan, 2001). Some patients have been investigated with respect to the possible role of the immunological system (Curran *et al*., 1996; DiStefano *et al*., 1998; 1999). The correlation between specific IgE antibodies and clinical symptoms is poor, and the role of IgE in the pathogenesis of GA occupational asthma is currently unclear. In most cases the clinical findings are similar to those of reactive airways dysfunction syndrome (RADS), and result from overexposure to GA vapour. In situations where there are good industrial-hygiene controls on potential exposure, then occupational asthma does not occur. Thus, in an epidemiological study involving 218 workers assigned to GA production or drumming units, there was no indication of skin or respiratory sensitizations related to GA exposure (Teta *et al*., 1995). A cross-sectional study of 135 nurses using GA solutions for instrument cold sterilization was conducted in 26 hospitals in Australia (Pisannello *et al*., 1997). There was no evidence for an increased incidence of respiratory effects, including asthmatic symptoms, in the nurses exposed to GA. A large cross-sectional survey of endoscopy nurses was conducted in the UK, using 348 currently employed nurses (Vyas *et al*., 2000). Whilst nurses exhibited irritant symptoms, there were neither clinical nor investigational (respiratory function tests, specific IgE antibody studies) indications of asthma and no evidence that GA is a respiratory sensitizer. Paiczyński *et al*. (2001) evaluated changes in the cytogram, protein content, eosinophil cation protein and mast-cell tryptase concentrations in nasal lavage fluid (NLF) after GA challenge of patients with a positive history of GA-induced asthma. There was a significant increase in eosinophil absolute number and percentage, and in albumin eosinophil cation protein and tryptase concentrations in the NLF from the patients with occupational asthma and rhinitis. The authors concluded that the findings indicate an immunological mechanism of GA-induced asthma and the applicability of NLF analysis.

4.8.5.4 Peripheral Sensory Irritation

GA is a typical peripheral chemosensory sensory irritant (PCSI) material, and can interact with sensory nerve receptors in the skin and exposed mucosal surfaces, resulting in local sensations and related reflexes (see also Ballantyne 1999; **Peripheral Chemosensory Irritation: Fundamentals, Investigation and Applied Considerations**). Exposure to suprathreshold concentrations of GA vapour may result in eye discomfort, excess lacrimation, discomfort in the nose and chest, rhinorrhea, cough and sneezing. The PCSI effects of GA have been investigated by an animal model and by studies with human volunteer subjects. Measurement of the depression of respiratory rate in ND4 Swiss Webster mice gave a calculated RD_{50} of 13.86 ppm (Werley *et al*., 1995). Using a correlative correction factor of 0.03 (Alarie, 1984), it was calculated that 0.2 ppm is a value at which sensory irritation would be anticipated to be negligible in humans. A lower RD_{50} value of 2.6 ppm has been reported for Swiss OF1 mice (Zissu *et al*., 1994). One human study demonstrated a sensory irritant threshold for GA vapour of 0.24–0.26 ppm (Whitmore, 1976), another study gave a human irritation threshold of 0.3 ppm (Colwell, 1976) and Cain *et al*. (2007) obtained a 50% detection (which they called 'feel') in the eye and

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nose at 0.39 ppm and 0.47 ppm respectively in young adult females. Cain and Schmidt (2002) investigated the sensory detection of GA in drinking water, and reported that persons with normal sensitivity detected GA above chance at 6.25 ppm.

4.8.5.5 Subchronic Repeated Exposures

4.8.5.5.1 Peroral

Because the irritant effects of GA may limit dosing by the epicutaneous and inhalation routes of exposure, studies have been conducted by the peroral route to permit maximum dosage, and hence allow the greatest potential for the expression of toxicity. Drinking-water studies have been conducted in the rat, mouse and dog, with dosing up to three months and full monitoring for signs, body weight, food and water consumption, haematology, clinical chemistry, urinalysis, necropsy, organ weights and histology. Dosages were up to 1000 ppm for rats and mice and 250 ppm for dogs. The major, and only, findings in all species were decreased food and water consumption (probably related to an aversion to the taste and/or irritancy of GA), decreased body weight, and decreased urine volume with increased specific gravity. In no species was there any clinical, haematological, biochemical or morphological evidence for target organ or tissue systemic toxicity. All the findings are compatible with decreased urine production secondary to decreased water consumption resulting from an aversion to GA in the drinking water (Hermansky *et al*., 1995a).

4.8.5.5.2 Epicutaneous

Fischer 344 rats had 20 epicutaneous occluded applications (six hours a day) of aqueous GA solutions resulting in dosages of 50, 100 and 250 $mg\,kg^{-1}\,day^{-1}$ (Werley *et al*., 1996). There were no treatment-related mortalities or signs of systemic toxicity. Local skin irritation was minimal (minor erythema and slight oedema). There were slight decreases in body weight (at 250 $mg\,kg^{-1}\,day1^{-1}$), reduced body-weight gain (100 and 250 $mg\,kg^{-1}\,day^{-1}$), and slightly reduced food and water consumption (250 $mg\,kg^{-1}\,day^{-1}$). The only haematological findings (females only) were increased platelet and reticulocyte counts. Clinical-chemistry results showed a slight increase in blood urea nitrogen. Urinalysis was normal. Adrenal gland weights were slightly increased for the 100 and 150 $mg\,kg^{1}\,day^{-1}$ females. Histopathological findings were only seen in the dosed skin area, and consisted of acanthosis, hyperkeratosis, parakeratosis, dermatitis, epidermitis and dermal fibrosis. The minor effects noted are common findings in rodents receiving cutaneous applications of irritant materials (Hermansky *et al*., 1995b). There was no evidence for systemic target organ or tissue toxicity.

4.8.5.5.3 Vapour Exposure

Several short-term (9–12 days) repeated vapour-exposure studies have been conducted in rats and mice as preliminary investigations to definitive subchronic studies. These have been reviewed by Ballantyne and Jordan (2001). Although conducted under differing conditions of vapour generation, vapour analysis and monitoring, the following are common findings. For vapour concentrations of 2 ppm and greater, mortality was exposure-concentration related. The lowest concentration of GA vapour causing mortality was 0.63 ppm (1/20 rats), and all studies showed a steep slope on the vapour-concentration–mortality data. The available evidence suggests a greater degree of lethality for vapour generated at elevated temperatures (about 50 °C) than for vapour generated at ambient temperature. All studies showed effects on the respiratory tract typical of those for an irritant material. At $\geq$5 ppm, respiratory tract histopathology was extensive, but at $\leq$1.6 ppm, effects were generally restricted to the nasal mucosa. Threshold concentrations for effects in the nasal mucosa were 0.3 ppm (rats) and 0.5 ppm (mice), and the no-effect concentration was 0.16 ppm for both species. A detailed subchronic vapour exposure study was conducted with Fischer 344 rats at concentrations of 20, 50 and 200 ppb, with exposures for six hours a day, five days a week, for a total of 14 weeks. There were minimal signs of respiratory tract and ocular irritation, and slightly decreased body weight, at 50 and 200 ppb, but no evidence for respiratory-tract inflammation or systemic toxicity (Greenspan *et al*., 1985). In another study, $B6C3F_1$ mice were exposed to 100 ppb GA vapour for six hours a day, five days a week for 52 or 78 weeks. Lowered body-weight gain was measured for female mice, but males gained weight. Histopathological effects were seen in female, but not male, mice and were restricted to the nasal vestibule. The findings included hyperplasia of the squamous epithelium lining the dorsal wall and lateral aspect of the atrioturbinate, and was associated with intraepithelial and subepithelial cellular infiltration (granulocytes and lymphocytes). This lesion was significantly increased ($p < 0.05$) in female mice of the 52 week (15/48 vs. 2/49 controls) and 78 week (14/28 vs. 6/28 controls) exposure groups. Additionally, epidermal ulceration and erosions were seen with squamous cell and inflammatory exfoliation in the nasal cavity, but there was no squamous metaplasia. Also, there was no histological evidence for systemic toxicity (Zissu *et al*., 1998). In a more extensive study conducted under the US National Toxicology Program, Fischer 344 rats and $B6C3F_1$ mice were exposed for 6.5 hours a day, five days week, for 13 weeks, to GA vapour concentrations of 0, 62.5, 125, 250, 500 and 1000 ppb. Monitors for toxicity included signs, body-weight, haematology, clinical chemistry, sperm morphology, vaginal cytology, and gross and microscopic pathology. The only sign with rats was difficulty with breathing (1000 ppb). Mice were more susceptible, with 10% mortality at 500 ppb and all animals dying at 1000 ppb. Histopathology was restricted to the nasal mucosa and (mice only) larynx, and consisted of inflammation, hyperplasia and squamous

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metaplasia. Effects were moderate at 1000 ppm and mild at ≤500 ppb. A no-effect concentration for nasal mucosal effects from GA vapour was established at 62.5 ppb for the male rat. Minimal inflammation was present in half of the female mice at 62.5 ppb (NTP, 1993b).

4.8.5.6 Chronic Toxicity and Oncogenicity

Studies have been conducted by the peroral and inhalation routes. The peroral study was conducted by lifetime (two-year) exposure of Fischer 344 rats to GA in the drinking water at concentrations of 50, 250 and 1000 ppm, resulting in respective average daily dosages of 3.6, 17.1 and 63.9 mg kg^{-1} for male rats and 5.5, 25.1 and 85.9 mg kg^{-1} for females (Van Miller *et al.*, 2002). Interim sacrifices were conducted at 12 and 18 months after the start of dosing. No dosage-related mortality occurred. Absolute body weights and body-weight gains of the 250 and 1000 ppm males and females were reduced over the study in a dose-related manner. Food and water consumption by the 250 and 1000 ppm groups were decreased in a statistically significant dose-related manner over the study, and mean water consumption by the 50 ppm rats was slightly reduced, but not with statistical significance. The 250 and 1000 ppm groups had a dosage-related decrease in urine volume with increased osmolality and slightly reduced pH. Absolute kidney weights were increased in the 250 and 1000 ppm groups at the 12 and 18 months sacrifices, and decreased at the two-year sacrifice. Relative kidney weights were increased at all sacrifice times for the 1000 ppm group, at 52 weeks for the 250 ppm group and at 72 weeks for the 50 ppm group. The urinalysis and renal weight changes are compatible with a physiological compensatory adaptation to reduced water consumption. Gross and histological evidence of gastric irritation was observed, principally in the 1000 ppm rats euthanized at two years and in animals dying during the study. Bone marrow hyperplasia and renal tubular pigmentation, seen in rats that died and in at the two year sacrifice, may have been secondary to a low-grade haemolytic anaemia in animals with large granular lymphocytic leukaemia (LGLL); (Stromberg *et al.*, 1983). The only neoplasm that showed a statistically significant increase was LGLL, which occurred at a high incidence in both sexes and in all groups, including controls, for both animals that died and at the two year sacrifice. The overall incidences for LGLL in the spleen of the 0, 50, 250 and 1000 ppm groups were, respectively, 43, 51, 40 and 46% for males, and 24, 41, 41 and 53% for females. In assessing the significance of the increased LGLL it needs to be noted that the increased incidence occurred only in females, that LGLL is a spontaneously occurring neoplasm in the Fischer 344 rat (Losco and Ward, 1984; Hermansky *et al.*, 1992) and that the incidence of LGLL in control Fischer 344 rats shows temporal intra-and interlaboratory variations (Hermansky *et al.*, 1992; Stefanski *et al.*, 1990). It is possible that the low incidence of LGLL in the control female rats was a consequence of random biological variation. However, even if the increase in LGLL was treatment-related, it is unlikely to have been the result of a genotoxic carcinogenesis. No clear dosage–response relationship was apparent and there was no increased incidence in males. Also, genetic toxicology studies have failed to demonstrate a mutagenic or clastogenic effect in the intact organism (see below). There is the possibility that the increased incidence of LGLL in female Fischer 344 rats was a consequence of the chronic dosing with GA having a modifying effect on one or more of the factors influencing the expression of this spontaneously occurring neoplasm the female rat.

A two year chronic toxicity and oncogenicity study was conducted with GA vapour by the inhalation route with Fischer 344 rats and $B6C3F_1$ mice (Van Birgulen *et al.*, 2000). For rats, groups of 50 males and 50 females rats were exposed to 0, 250 and 750 ppb GA vapour for 6 hours 25 minutes a day, five days a week for 104 weeks. The two year survivals for the 0, 250, 500 and 750 ppb groups were, respectively, 12/50, 14/50, 6/50 and 6/50 for males and 26/50, 3/50, 15/50 and 14/50 for females. Mean body weights of all males, and the 500 and 750 ppb females, were generally slightly reduced compared with the 0 ppb controls. Histopathological effects were mainly limited to the nasal mucosa and seen as exposure-concentration-related squamous epithelial hyperplasia, squamous metaplasia, inflammation, goblet cell hyperplasia and olfactory epithelial hyaline degeneration. Inflammation consisted of infiltrates of neutrophils, lymphocytes and plasma cells. Squamous epithelial hyperplasia and inflammation were present at 250 ppb with statistical significance. Similar sized groups of mice were exposed to 0, 62.5, 125 and 250 ppb GA vapour. The respective two year survival rates were 31/50, 27/50, 40/50 and 38/50 for males and 34/50, 37/50, 35/50 and 32/50 for females. Major findings were nasal histopathological effects, which were qualitatively similar to those for the rats. The incidence of respiratory epithelial squamous metaplasia was increased in the 250 ppb males and females and the 125 ppb females. Respiratory epithelial hyaline degeneration was increased significantly in all groups of GA-exposed females, but not in an exposure-related fashion. Nasal inflammation was increased in 250 ppb females. Turbinate necrosis was seen in 2/50 of the 125 ppb males and in all groups of exposed females. For rats and mice there was no other non-neoplastic histopathology. Under the conditions of this study, there was no evidence for an oncogenic potential with GA and no evidence for systemic toxicity.

4.8.5.7 Genetic Toxicology

Numerous *in vitro* and *in vivo* genetic toxicology studies have been conducted, and are summarized by Ballantyne and Jordan (2001) and Zeiger *et al.* (2005). Mutagenic activity, generally weak, has been demonstrated in *Salmonella typhimurium* strains TA100, TA102, TA104,

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TA1535/PSK1002, BA9 and BA13 and in *Escherichia coli* strain WP2uvrA. Results with *in vitro* CHO cells depend on the gene locus investigated, with no activity being seen at the hypoxanthine-guanine-phosphoribosyl transferase (HGPRT) locus and weak activity at the thymidine kinase (TK) locus. *In vitro* increases in CHO sister chromatid exchanges generally do not occur and the results from chromosomal aberration tests vary from no to weak activity. DNA damage and repair tests have shown variable results. *In vivo* studies have generally shown no evidence for genotoxic activity (micronucleus, chromosomal aberration, dominant lethal and *Drosophila* tests; Vergnes and Ballantyne, 2002). The absence of genotoxic effects *in vivo* may, in part, be related to the rapid metabolism and protein-binding characteristics of GA, and the fact that ^{14}C-GA cannot be detected in nuclei (Ranly *et al*., 1990). Speit *et al*.(2008) characterized the genotoxic potential of GA using an alkaline comet assay using V79 cells. They demonstrated the induction of DNA–protein crosslinking at a concentration of 10 μM and above. Crosslinking concentrations of GA were also cytotoxic; that is, inhibited cell growth of V79 cultures. They also showed a small, but statistically significant, increase in sister chromatid exchange and micronuclei.

4.8.5.8 Developmental and Reproductive Toxicity

Several developmental toxicology studies have been conducted in various species. Mice given GA by gavage on GD6–15 over a dosage range of 16–100 mg kg^{-1} day^{-1} showed maternal toxicity (mortality and decreased body weight) at 50 and 100 mg kg^{-1} day^{-1} with some indication of foetotoxicity (foetal body weight). However, foetotoxicity and teratogenic effects were not seen at maternally nontoxic doses (Marks *et al*., 1980). With rats given GA by gavage at daily doses of 25, 50 or 100 mg kg^{-1} over GD6–15, maternal mortality occurred at 50 and 100 mg kg^{-1} and body-weight gain was reduced at 100 mg kg^{-1}. There were no effects on implantations, resorptions or number of live foetuses. Foetal body weight was reduced at 100 mg kg^{-1}, but malformations were not seen (Ema *et al*., 1992). In another rat peroral developmental toxicity study, pregnant Wistar rats were given GA in drinking water at average daily dosages of 5.2, 25.7 and 68.0 mg kg^{-1} over GD6–16. The only effect noted was a reduction in drinking water consumption at the mid and high dosages (Ballantyne and Jordan, 2001). In a rabbit study, GA was given by gavage at daily dosages of 5, 15 or 45 mg kg^{-1} over GD7–19. At the highest dosage there were signs of maternal and embryofoetal toxicity, but no malformations. Neither maternal nor developmental toxicity was present at the mid and low dosages (Ballantyne and Jordan, 2001). With respect to reproductive toxicity, several subchronic repeated-exposure toxicity studies have not shown any toxicity to the male or female reproductive tract. Also, a dominant lethal assay in mice by gavage showed no evidence for reduced fertility and no effect on embryofoetal viability (Tamada *et al*., 1978). In a definitive reproduction study, rats received GA in drinking water over two generations at 50, 250 or 500 ppm. This resulted only in a dosage-related decrease in parental water consumption and body weight, with no effects on reproductive performance (Neeper-Bradley and Ballantyne, 2000). Also, two epidemiological studies in hospital workers showed that exposure to GA was not associated with a risk of spontaneous abortion or other risks to reproductive performance (Hemminki *et al*., 1982; 1985).

4.8.5.9 Toxicokinetics and Metabolism

An *in vivo* comparative intravenous and epicutaneous study was carried out using rats and rabbits (McKelvey *et al*., 1992). By cutaneous application, a high proportion of ^{14}C-GA was recovered from the skin (45–61% in the rat, 31–45% in the rabbit). Autoradiography showed activity over the stratum corneum, hair shafts and at foci of dermal necrosis. The proportion of GA absorbed percutaneously in the rat was calculated at 0.3–2.1%, and in the rabbit at 7.5–24.9%. Toxicokinetic profiles showed a bi-exponential form, suggesting a two-compartment model. The absorption rate constants were low in both species (range 0.2–2.0 hour^{-1}). That the rat is a more appropriate model for the human, and shows low skin penetration, as strongly suggested by comparative *in vitro* studies. With isolated human skin it was found that GA did not penetrate isolated thick stratum corneum (sole of foot), whereas 2.8–4.4% of GA penetrated isolated abdominal skin epidermis and 3.3–13.8% penetrated isolated thin stratum corneum from chest and abdominal skin (Reifenrath *et al*., 1985). A detailed *in vitro* species comparison (rat, mouse, guinea pig, rabbit and human) was undertaken with 1,5-^{14}C-GA (Frantz *et al*., 1993). Highest recovery was with the skin of male mice (1.73% with 0.75% GA) and lowest with rat (0.05–0.06% for females and males). Human female skin was low; 0.2% recovery with 7.5% GA and 0.16% with 0.75% GA. GA is metabolized extensively to CO_2, probably through a series of oxidation, decarboxylation and hydrolysis reactions. The major route of metabolism is probably by oxidation by kidney and liver to glutaric-γ-semialdehyde, and then to glutaric acid, which is used to synthesize glutaryl CoA, with further metabolism to glutaconyl CoA, crotinyl CoA, β-hydroxybutyryl CoA and acetyl CoA, and finally CO_2 (Beauchamp *et al*., 1992; Hjelle and Peterson, 1983; Karp *et al*., 1987; Myers *et al*., 1986; Ranly *et al*., 1990).

4.8.5.10 Human Occupational Medical Features

The use patterns of GA imply a potential for human contact with the liquid on skin and eye, as well as

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exposure to vapour. Concentration-related skin and eye irritation, and allergic contact dermatitis, are well-known overexposure effects and are discussed earlier in the chapter. Sensory irritation and other symptoms have been documented extensively from exposure to the vapour (Binding and Witting, 1990; Guthua *et al*., 2001; Jachuck *et al*., 1989; Nayebzadeh, 2007; Norback, 1988). Typical are eye discomfort, excess lacrimation, blepharospasm, blepharitis, rhinorrhoea, irritation and dryness of the throat, sneezing, coughing, slight breathing difficulties, headache, nausea and fatigue. These effects are reversible within a few minutes to a few hours of the cessation of exposure. Epistaxis has been reported following vapour exposure (Wiggins *et al*., 1989) and allergic contact dermatitis from vapour contact with skin (Fowler, 1989). The occurrence of asthmatic symptoms has been discussed previously. Limited epidemiology studies on GA-exposed workers have not indicated an increase in malignant tumour incidence (Collins *et al*., 2006; Teta *et al*., 1995). Additionally, inadequately decontaminated endoscopes and medical instruments may be a source of exposure for patients, and lead to inflammatory complications (reviewed by Ballantyne and Jordan, 2001). This has been noted especially for colitis and proctocolitis (Burtin *et al*., 1993; Durante *et al*., 1992; McHanson *et al*., 1998; Stein *et al*., 2001; Zissin *et al*., 1999). Also, a potential has been noted for synovitis (Harner *et al*., 1989), oesophageal injury and dysphagia (Isserow *et al*., 1998; Leudtke *et al*., 2003), bowel injury following peritoneal contamination from a GA-contaminated laparoscope (Karpelowsky *et al*., 2006), laryngotracheitis (Belani and Priedkains, 1977) and ocular toxic anterior segment syndrome (Mamalis *et al*., 2006; Unal *et al*., 2006).

The TLV for GA (activated or unactivated) is 0.05 ppm ($0.2\,mg\,m^{-3}$) as a ceiling value, with irritation and sensitization notations, and an A4 notation (not classifiable as a human carcinogen) (ACGIH, 2007; Anonymous, 2001). The UK Health and Safety Executive has established a maximum exposure limit (MEL) of 0.05 ppm (Evans *et al*., 1997). Inasmuch as the odour threshold (usually cited as 0.04 ppm) is significantly lower than these values, it is likely that GA vapour will be detected by smell before reaching an overexposure concentration. Cain *et al*. (2007) found a greater sensitivity (higher odour potency) for GA vapour in young adult females with a 50% threshold for odour at 0.3 ppb.

4.8.6 Ecotoxicology

GA is hydrolyzable with a DT_{50} of 508 days at pH 5.0, 102 days at pH 7.0 and 46 days at pH 9.0. Photolysis in water at pH 5.0 shows a DT_{50} of 196 days (Eriksson *et al*., 1995). With a P_{ow} of 0.98, the potential for bioaccumulation is low. An aerobic metabolism study (water/sediment) indicated GA ($10\,mg\,l^{-1}$) to be oxidized to glutaric acid. The *t* 1/2 was 10.6 hours, indicating ready biodegradability. Toxicity to aquatic organisms has been determined as follows:

Scenedesmus subspicatus: 72 hour $EC_{50} = 0.85\,mg\,l^{-1}$; NOEL $= 0.31\,mg\,l^{-1}$
Daphnia magna: 48 hour $EC_{50} = 16\,mg\,l^{-1}$
Leptomis maccochirus: 96 hour-$LC_{50} = 12\,mg\,l^{-1}$
Oncorhynchus mykiss: 96 hour $LC_{50} = 12\,mg\,l^{-1}$
Cyprinodon variegatus: 96 hour $LC_{50} = 31\,mg\,l^{-1}$ (flow-through)
Crassostrea virginica: 48 hour $LC_{50} = 0.55\,mg\,l^{-1}$; 96 hour EC50 (shell growth) $= 0.87\,mg\,l^{-1}$ with NOEL $= 0.16\,mg\,l^{-1}$
Carcinus maenus: $LC_{50} = 465\,mg\,l^{-1}$
Palaemonetas bugio: $LC_{50} = 41\,mg\,l^{-1}$

A reproductive study with *Daphnis magna* showed moderate toxicity with a 21 day NOEL of $2.1\,mg\,l^{-1}$.

A PNEC of $6.2\,\mu g\,l^{-1}$ for GA to aquatic ecosystems has been suggested by the Swedish National Chemicals Inspectorate (Eriksson *et al*., 1995). This is based on the lowest NOEL for the most sensitive strain (algae).

Extensive summaries of the toxicology, occupational medical and industrial hygiene aspects of GA can be found in the following—Ballantyne and Jordan, 2001; CIREP (Cosmetic Ingredients Review Expert Panel), 1996; Evans *et al*., 1997; Jordan, 1995.

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Dermal Toxicology of Cosmetics and Body-Care Products

David Basketter and Linda Lea

CONTENTS

1 GENERAL INTRODUCTION

There is a tendency in both regulatory thinking and in public perception towards the view that cosmetics are in some way different from other products. The reality of course is that they are formed from a combination of chemical ingredients, raw materials which, in fact, can be found in a very wide range of products. Consequently, the toxicology considerations which apply to other products also apply to cosmetics and body-care products. Application of appropriate toxicological review of product ingredients and of the final formulation is key to meeting the requirement that such products do not cause harm to the consumer. Failure to undertake such a review to an adequate standard gives rise to a risk of adverse reactions (reviewed in Modjtahedi *et al*., 2008). In this chapter, we set out some general thoughts on how a review of product safety generally should be approached for these types of product. Whilst details of its execution inevitably will reflect our own biases, we hope that it will serve to illustrate the basic concepts of what is necessary.

2 CHEMICAL PROPERTIES AND SPECIFICATION

In common with any toxicological review, the starting point is to have a clear chemical definition of the ingredient(s) in terms of chemical composition, chemical structure, purity and potential impurities. When evaluating toxicological data on existing chemicals, the toxicologist should consider the specification of the ingredient tested, as even small levels of impurities can effect the toxicity of the ingredient being tested. This is especially important when toxicological studies have been carried out at different time points by different investigators. Similarly, where new toxicological testing is being planned, the ingredient should be fully characterized chemically and all testing carried out on the same specification and, where possible, on the same manufacturing batch. This should also be representative of the ingredient to be used in the marketplace.

Physical chemical properties such as solubility, partition coefficient and so on can also be valuable to any toxicological evaluation as they can be predictive of some toxicological properties, for example small-molecular-weight-compounds are more likely

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to penetrate the skin than are hydrophobic chemicals. Therefore, a good understanding of the chemical properties of an ingredient is required before the toxicological evaluation can begin.

3 ACUTE TOXICITY

Many ingredients used in cosmetic products have already undergone registration as new chemicals, as many are also used in other products. Where this is the case there will usually be acute toxicity data available and it is unusual for there to be a need to generate acute toxicity data. Quite often the data may be old and not meet current testing standards and/or be compliant with Good Laboratory Practice (GLP) standards. However, the data may still be of value in assessing acute toxicity and should be considered before embarking on new acute toxicity tests. The data from acute toxicity tests should be reviewed to identify any ingredients with low LD_{50} (median lethal dose) values against likely exposure levels. Given the generally low exposure to individual ingredients in a cosmetic product, acute toxicity is rarely a limiting factor in a safety evaluation of cosmetic ingredients.

If new data need to be generated then one of the three test methods now adopted by the Organization for Economic Cooperation and Development (OECD) for assessment of acute oral toxicity would have to be used. The tests are OECD 420: fixed dose method (OECD, 2001a), OECD 423: acute toxic class method (OECD, 2001b) and OECD 425: up and down procedure (OECD, 2008). All are considered to refinements to the traditional LD_{50} test.

4 DERMAL IRRITATION

Skin irritation is a complex matter (Chew and Maibach, 2003; Weltfriend and Maibach, 2008; Fluhr *et al*., 2008; Modjtahedi *et al*., 2008). Probably the overwhelming adverse reaction experienced in relation to cosmetics and body-care products comes under this all-encompassing banner. Skin irritation in fact represents a broad family of (unrelated) dermal reactions to direct chemical insult. What is measured in regulatory toxicology is the acute irritant response to a single contact with a high concentration of an individual substance. The contribution that such data makes to the assessment of risk is minimal. The types of adverse reaction experienced by consumers ranges from mild sensory effects (tightness, itching, burning, stinging), to urticaria (sometimes called immediate contact reactions), through to cumulative irritant responses (dryness, redness) and ultimately to severe irritation, even skin corrosion, the gross skin damage that can result, for example, from overexposure to strong alkalis present in hair-relaxing formulations. These cannot be addressed in a single assay and certainly not by the now largely redundant Draize rabbit skin irritation assay (Draize *et al*., 1944).

So, how does the toxicologist proceed? In the first place, although of limited utility, there is some merit in having information on the acute irritant potential of substances. *In vitro* methods for the identification of corrosive materials and those of stronger irritant potential are now well described and are generally accepted in regulatory toxicology (Basketter and Jones, 2008; Jones, 2008). These techniques permit the predictive identification of substances, which if used as ingredients at high concentration might well deliver significant irritant effects into the final formulation. However, in our experience, this is not a common issue for this type of product. As mentioned earlier, the acute irritant response is uncommon, even in occupational irritant contact dermatitis (Malten, 1981; Chew and Maibach, 2003). In reality, frank skin irritation arises as a consequence of repeated exposures, hence 'cumulative' irritant contact dermatitis is the more appropriate terminology. This skin reaction, characterized in its mild form as dryness and redness (erythema) is most commonly associated with repeated exposures to surfactants. So, surfactant-based products, such as liquid soaps, shampoos, shower gels and so on, can (and do) produce this reaction if exposure is sufficient. Much less commonly, and notably only in very susceptible individuals, nonsurfactant-based products with high levels of skin exposure can also produce irritation. The topic of individual susceptibility will be discussed later, but first, how can cumulative irritancy be assessed?

In the past, *in vivo* models of cumulative skin irritancy were developed, but they have no place in twenty-first century toxicology and will not be discussed further. Where inspection of a new product formulation gives rise to any concern (for example, the introduction of a new surfactant type or mixture), then the proper recourse for the toxicologist is to set up an appropriate human study. Clearly, such a course of action demands its own safety assessment, independent ethical review and medical oversight, but for cosmetics and body-care products it represents a safe and wholly appropriate strategy (Walker *et al*., 1996; 1997; Basketter and Reynolds, 1997). Detailed protocols are beyond the scope of this review chapter, not least since the products under question represent a very diverse community. However, a number of key principles can be mentioned. The aim of this type of clinical study typically will be to assess whether a formulation has an unexpected capacity to produce cumulative irritancy. Hence, the test must involve repeated exposures. Then, since the test aims to predict what may occur in a large exposed population by use of small group of volunteers, the exposures may need to be exaggerated compared to normal product use. Thirdly, to ensure that the test is scientifically robust, a

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positive control material will need to be incorporated. Typically, this might be a currently marketed product from the same category where there exists a substantial history of safe use. For example, if the new product is a liquid soap formulation, it could readily be compared to another marketed liquid soap which has an acceptable market history. Exaggerated repeated exposures to these formulations, for example, by hand immersion, would permit their relative irritancy to be compared simply as a function of the number of exposures necessary to produce a slight reaction. Such a type of protocol ensures volunteer safety by limiting the exposures to the point where no more than a slight reaction occurs, but nevertheless provides real data on the end point of concern in the species of concern. It is worth noting here that acute irritancy, such as that measured by conduct of a patch test in humans does not predict cumulative irritancy at all well (Hannuksela and Hannuksela, 1996). In addition, where products are already very mild in respect of effects on the skin, then it has to be recognized that this in itself can present a substantial challenge in terms of the design of a sufficiently sensitive and robust protocol (Farage, 2008).

Nonimmune immediate contact reactions (urticaria) represent another potential adverse skin reactions, typically appearing as redness and swelling (oedema) within minutes of contact and subsiding within an hour (Lahti and Basketter, 2006; Amin *et al*., 2008). There is little report that this is a significant adverse effect seen with the products covered in this chapter, although to an extent this may represent an absence of evidence rather than vice versa. To assess the end point, only a human study will suffice, as there are no animal models. However, as the effect arises from a single contact, is rapid and transient, and with no lasting effect, then a simple open-exposure protocol in a suitable panel of volunteers is sufficient for the assessment of urticarial potential (Basketter and Wilhelm, 1996; Amin *et al*., 2008). It is worth noting here that contact urticaria involves a number of mechanisms, many poorly characterized, with the consequence that individuals sensitive to one standard urticant material (e.g. ethyl nicotinate, cinnamal, benzoic acid) may not be particularly reactive to another. Thus, the identification of a 'sensitive panel' for this end point is complex (Amin *et al*., 2008; Coverly *et al*., 1998).

An important set of skin reactions, though, comprise sensory effects, the most commonly referred to of which is stinging, but which also include itching, burning, tingling and so forth (Coverly *et al*., 1998; Marriott *et al*., 2003; 2005; Peters *et al*., 2006; Modjtahedi *et al*., 2008). A common error is that these end points are all related, such that susceptibility to one will predict reactivity to another (Coverly *et al*., 1998; Marriott *et al*., 2003; 2005). Unfortunately, this is not the case, rendering the clinical evaluation more complex than some have supposed, particularly where there is a wish to use so-called 'sensitive panels'. The reality is that each of the end points being separate, the construction of such a panel requires a great deal more than the identification of those who are sensitive to lactic acid stinging in the nasolabial fold (Marriott *et al*., 2003; Peters *et al*., 2006).

5 DERMAL SENSITIZATION

Whereas irritation arises from a direct local response to skin insult, sensitization requires the systemic involvement of the immune system. So, chemicals which can penetrate into the viable epidermis, react with skin protein, cause local trauma and so be perceived by the immune system as foreign are termed skin sensitizers. Such chemicals have the capacity to induce delayed contact hypersensitivity (contact allergy), a T-cell-mediated response. With further skin contact, the same or a closely similar substance can elicit the local inflammation characteristic of this type of allergy, commonly termed allergic contact dermatitis (reviewed in Rustemeyer *et al*., 2006).

Extensive reviews have been written concerning the guinea pig as the *in vivo* model for the identification of skin sensitization hazards, that is of those substances which possess the intrinsic property that they can cause skin sensitization (Andersen and Maibach, 1985). The guinea-pig maximization test (Magnusson and Kligman, 1970) and the Buehler occluded patch test (Buehler, 1965) are the main tests, and they share certain features in common, in that there is a set of procedures with test substances to attempt to induce sensitization, then the extent, if any, of that sensitization is revealed by challenge testing (application of an occluded patch to the skin) and the reading of any reactions during the next 24–72 hours. Details of the regulatory protocols are in the relevant OECD Guidelines (OECD, 1992). Extensive lists of chemicals tested using these procedures have been published (Wahlberg and Boman, 1985; Cronin and Basketter, 1994).

Towards the end of the twentieth century, a mouse test, the local lymph node assay (LLNA) was developed (Kimber and Basketter, 1992; Kimber *et al*., 1994). The animal-welfare advantages, the objective and quantitative nature of the end point, the simple prediction of skin-sensitization hazards and the full independent validation of the assay (NIH, 1999; Gerberick *et al*., 2000; Balls and Hellsten, 2000; Dean *et al*., 2001) led to this method having its own OECD Guideline (OECD, 2002). Most recently, it has become the preferred assay under REACH (Registration, Evaluation, Authorisation and restriction of Chemicals) and in globally harmonized guidelines for the assessment of skin sensitizers (Commission of the European Communities, 2006). Lists of chemicals tested using the LLNA have been published (Gerberick *et al*., 2004; ICCVAM, 2008).

The identification of chemicals with skin-sensitization hazard may satisfy regulatory toxicology, but is rarely

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adequate for the proper protection of human health, for example for those chemicals used in cosmetics and personal-care products. Rather, it is simply an essential prerequisite on which to build further analysis, particularly the measurement of the relative potency of a skin sensitizer. In combination with potency data, information on exposure permits a risk assessment to be undertaken. The measurement of the relative potency of skin-sensitizing chemicals has been reviewed recently (Kimber *et al*., 2001; Basketter *et al*., 2005a; van Loveren *et al*., 2008). Guinea-pig tests were not designed for potency assessment and have not been proven to be particularly useful in this respect. In contrast, the LLNA has dose–response assessment and the method used for potency measurement has been to estimate the concentration necessary to cause a threshold effect. In the LLNA, this is termed the EC3 value, the concentration necessary to cause a threefold stimulation compared to concurrent vehicle-treated controls (Basketter *et al*., 1999). What is more important is that the EC3 values correlate well with human predictive test thresholds (Basketter *et al*., 2000; 2005a; 2008b; Ryan *et al*., 2000; Gerberick *et al*., 2001b; Griem *et al*., 2003; Schneider and Akkan, 2004). It is critical to note that this correlation uses the available threshold data from published human experiments, and does not relate to either induction or elicitation thresholds associated with consumer and occupational exposure safe limits—for that, exposure information is required.

Of course, an EC3 value, whilst not an absolute measure, is quantitative and has proven to be very reproducible over time and between laboratories (Dearman *et al*., 1998; Warbrick *et al*., 1999; Basketter *et al*., 2007).

Owing to the lack of definition of a measure of a no observed-effect level (NOEL) within guinea-pig testing strategies, the assessment of skin sensitization risk generally was achieved by application of comparative toxicology and use of complementary processes such as the human repeated-insult patch test (HRIPT). These techniques are detailed elsewhere (Basketter *et al*., 1996). With the advent of the LLNA EC3 value, which is able to predict the HRIPT induction threshold (Safford, 2008), then it has been possible to devise an approach to risk assessment similar to that used in many others of repeat-dose toxicity. Quantitative risk assessment (QRA) for skin sensitization now uses the LLNA EC3 value (together with any other available information) to predict what is effectively an NOEL in the HRIPT. This value is then adjusted by a number of uncertainty (i.e. safety) factors to determine a maximum acceptable exposure level (Gerberick *et al*., 2001a; Felter *et al*., 2002; 2003). There are several important issues that must be noted. Firstly, exposure is measured in terms of dose per unit area, this being the key measure for skin sensitization (Kimber *et al*., 2008). Then, the maximum exposure level is calculated for specific product types since the uncertainty factors incorporate elements of the nature of the exposure which may occur (e.g. rinse off vs. leave on), as well as of the vehicle matrix in which exposure occurs. An outline of the QRA process is presented in **Figure 1**. This process has been evaluated for a range of types of allergen, including fragrances (Gerberick *et al*., 2001a; Api *et al*., 2008), transition metals (Basketter *et al*., 2003) and preservatives (Basketter *et al*., 2008a; Basketter, 2009). It has also been adjusted to take into account exposure at mucosal surfaces (Farage *et al*., 2003). Furthermore, by combining the daily doses from multiple (product) exposures, it represents a transparent tool for the completion of a more comprehensive strategy for the establishment of safe exposure levels than has heretofore been available. Ultimately of course, as in all areas of toxicology, this risk assessment contains assumptions and requires elements of expert judgement and thus is most appropriately used to guide a final safety decision rather than being regarded as a precision tool.

The elimination of animals from this area of predictive toxicology seems a more realistic goal than even just a few years ago and has been the subject of recent reviews which discuss the approaches being currently explored (Casati *et al*., 2005; Basketter and Maxwell, 2007). The prospect of predictions on the basis of chemical structure continue to offer limited utility (Patlewicz *et al*., 2007). Nevertheless, these approaches do offer some very specific benefits, not least that it is not necessary to synthesize the chemical in order to test its sensitizing potential. However, the estimation of chemical reactivity by evaluation of peptide binding (Gerberick *et al*., 2004; 2007; Natsch *et al*., 2007), particularly if combined with other information (Natsch *et al*., 2009), does provide confidence that current efforts are proceeding in a potentially fruitful direction. Cell-based assays have also shown some promise, particularly those utilizing dendritic-like cell lines (Ashikaga *et al*., 2002; 2006; Yoshida *et al*., 2003; Sakaguchi *et al*., 2006; 2007; Python *et al*., 2007). These assays have generally been the subject of some degree of interlaboratory evaluation, but much of the data remains unpublished. All of these and other methods suffer from one defining limitation, that they provide data only on one aspect of the sensitization induction mechanism. For example, peptide-binding assays inform on the potential for chemical reactivity, but do not tell us about epidermal bioavailability, the capacity to produce danger signals or the intrinsic antigenicity of the hapten-modified self-protein structure. It seems probable therefore that data from these various elements of the induction process will have to be combined in order to deliver a nonanimal equivalent to the LLNA or guinea-pig methods. An outline of the strategic approach to this has been presented already (Jowsey *et al*., 2006; Basketter and Kimber, 2009). A first attempt to translate this theory into practice has recently been published and already shows considerable promise, with a good degree of accuracy (approximately 85%) in terms of both the identification and potency characterization of skin-sensitizing chemicals (Natsch *et al*., 2008).

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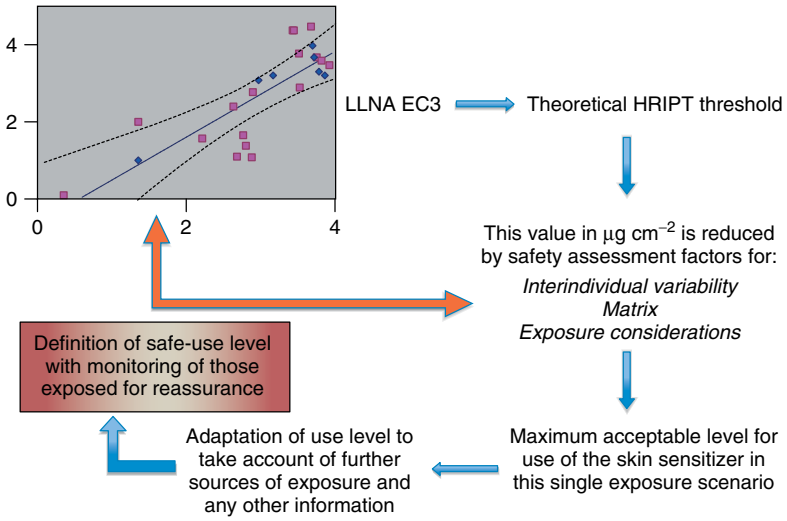

Figure 1 Outline of skin sensitization quantitative risk-assessment process. For a new substance, the definition of the LLNA EC3 value (or its estimation using *in vitro* techniques) permits the prediction of the threshold for the induction of skin sensitization in the human repeated-insult patch test. This latter threshold is then adjusted by the consideration of three safety-assessment factors, each from 1 to 10× (normally, human variability is always taken as 10×, complementing the assumed 100 subject variation in the HRIPT). The calculation yields a maximum acceptable exposure level, expressed in $\mu g\,cm^{-2}$ per day. However, this is associated with the individual sensitizer and exposure scenario under consideration, so the safety assessor must also take account of other potential exposures to the particular substance. Finally, the ultimate test, safety in use, should be followed and any clinical feedback on the success or failure of the safety assessment can be used to refine the critical variables and assumptions implicit in the QRA process.

6 GENOTOXICITY

An assessment of genotoxicity is required for all cosmetic ingredient products with the three main end points under evaluation being:

- Mutagenicity at the gene level
- Clastogenicity (chromosome damage and/or rearrangements)
- Aneugenicity (numerical chromosome aberrations).

Currently there is no single test method that is capable of detecting all these end points and it is usual for a battery of tests to be employed to assess genotoxicity. Previously, it was considered sufficient for a battery of at least two *in vitro* tests, normally a bacteria gene-mutation test (OECD, 1997a) and an *in vitro* mammalian cell chromosome-aberration test (OECD, 1997b) to be carried out (SCCNFP, 2003) and indeed this type of assessment has been widely used for many years. It has now been suggested that the *in vitro* micronucleus test (OECD, 2004a) should be used for the detection of chromosomal effects as it is able to detect both clastogens and aneugens (Kirsch-Volders *et al.*, 1997; 2004). Moreover, in the sixth revision of the notes of guidance for the testing of cosmetic ingredients, the Scientific Committee on Consumer Products (SCCP) now recommends that the first stage of genotoxicity testing should comprise two gene-mutation tests, a bacterial test and a mammalian cell test (OECD, 1997c), along with an *in vitro* micronucleus test to assess clastogenicity and aneugenicity (SCCP, 2006). Other genotoxicity-testing strategies for chemicals have been proposed (Cimino, 2006; WHO, 2008; Pfuhler *et al.*, 2007), as well as that defined in the European Union (EU) Technical Guidance Document (Technical Guidance Document, 2003). This is an area of ongoing discussion and the reader is referred to other chapters (see **Mutagenesis**; **Cytogenetics**; **Genetic Toxicology Testing and its Relevance to Human Risk and Safety Evaluation**) in this book for a more detailed discussion of this subject. In practice, any *in vitro* testing strategy undertaken for a new ingredient should be justified scientifically.

As part of an initial screen, a (Q)SAR ((quantitative) structure–activity relationship) evaluation of new chemicals is now common practice before embarking on any *in vitro* testing. There are a number of commercial and noncommercial software packages available for the prediction of genotoxicity end points, for example TOPKAT, DEREK, MCASE, OECD Toolbox. Whilst a (Q)SAR is unlikely to replace the need for *in vitro* testing, it can provide a useful adjunct to *in vitro* tests.

If the battery of *in vitro* screening tests and any associated SARs analysis are negative then the ingredient can be considered as acceptable for use as a cosmetic ingredient. However, if any of the tests give a positive result, further investigations are required. The nature and extent of the additional testing will be dependant on

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the positive results in the *in vitro* assays so needs to be considered on a case-by-case basis. It may involve additional *in vitro* tests or there may be a need for *in vivo* tests to be carried out, but a thorough review of all the available toxicology data on the ingredient and, if available, data on structurally related materials should be carried before defining any *in vivo* testing strategy. The most common *in vivo* assays used are the *in vivo* micronucleus and/or the unscheduled DNA synthesis (UDS) assay (OECD, 1997d; 1997e), but before undertaking any testing it needs to be established that the there is adequate exposure to the target tissue to ensure the validity of a negative *in vivo* result.

One of the main issues with current *in vitro* testing batteries is the number of false positives generated. Kirkland *et al*. (2005) evaluated the ability of a battery of three *in vitro* genotoxicity tests to discriminate rodent carcinogens and noncarcinogens and found that the specificity of the mammalian cell tests was low (45%). This can present a problem for evaluating new cosmetic ingredients as a positive result in an *in vitro* mammalian cell test may well define the need for *in vivo* testing, which, under the provisions on the Seventh Amendment to the Cosmetics Directive 76/768/EEC (2003/15/EC), may not be possible. There is also the question as to whether it is economically viable to pursue testing of an ingredient that has given a positive result *in vitro*, as the testing requirements to negate the positive test can be significant and also there is no guarantee that the outcome will be favourable.

For existing chemicals being considered for use as cosmetic ingredients, genotoxicity data should already be available. However, many different test methods both *in vitro* and *in vivo* have been used over the years to make this assessment, not all complying with current testing guidelines and the toxicologist can sometimes find themselves presented with a large number of conflicting results for evaluation. In such cases each study should be evaluated carefully in terms of its quality compared to standard testing guidelines and GLP compliance, as well as scientifically. A weight-of-evidence approach should then be taken to make an assessment of the overall genotoxic potential of the ingredient. Genotoxicity and/or carcinogenicity data from structurally related materials can also be relevant to this evaluation, as well as (Q)SAR predictions.

7 SYSTEMIC TOXICITY

It is the responsibility of the manufacturer of cosmetic products to carry out an appropriate risk assessment to ensure that the products they place on the market will not cause harm to human health, and this includes an assessment of systemic toxicity. This is normally considered in terms of the individual components contained in the product and not on the finished formulation.

The general principles of the risk-assessment process have been well documented by others (WHO/UNEP/ILO, 2001; European Commission, DG Health and Consumer Protection, 2000). The safety assessment process as applied to chemicals used as cosmetic ingredients is the same as for any other chemical. This can be summarized as follows:

- Hazard identification: this is the identification of the intrinsic toxicological properties of the chemical as determined by *in vitro* or *in vivo* toxicological tests. It may also include quantitative structure–activity relationship (QSAR) evaluations.
- Dose–response assessment: this establishes the relationship between the observed toxic effect and the dose. For effects with thresholds it is usually defined as a no observed adverse-effect level (NOAEL) or in cases where no such level can be established a lowest observed adverse-effect level (LOAEL) is sometimes used.
- Exposure assessment: this defines the expected human exposure to the chemical from its intended use.
- Risk characterization: this calculates the likelihood that the chemical could cause damage to human health. For an effect with a threshold, the margin of safety (MOS) is calculated, the MOS being defined as the NOAEL/exposure. For systemic toxicity, an MOS greater than 100 is usually considered acceptable. For other end points, a different MOS may be appropriate. For nonthresholded carcinogens, a slightly different approach is applied, but these chemicals should not be used in cosmetic products, so will not be considered further.

The final stages in the process are risk management and risk communication, which are the responsibility of the regulatory authorities and not considered further here.

7.1 Hazard Assessment/Dose–Response Assessment

The most appropriate hazard study to assess systemic toxicity is a repeat-dose (28 or 90 day) dermal toxicity study. This design of this study closely follows that of a repeat-dose oral toxicity study in that three dose groups and a control group of a minimum of five male and five female rats are used. The maximum dose level is normally $1000\,\mathrm{mg\,(kg\,bw)^{-1}\,day^{-1}}$. The dose is applied to an area of clipped and shaved skin, which should not be less than 10% of the body surface area of the animal, daily for a period of 28 days. The treated area is covered with a fixed dressing to ensure that the animal does not ingest any of the test chemical. Clinical observations are made daily to check for signs of toxicity, and body

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weights and food intakes recorded throughout the study. At the end of the study blood is taken for the measurement of a range of biochemical and haematological parameters. At termination, selected organs are weighed and any gross pathological changes recorded. Histopathological examination of a range of selected tissues is also carried out. After evaluation of all the recorded data an NO(A)EL, expressed in $\mathrm{mg\,(kg\,bw)^{-1}\,day^{-1}}$, is established, which is the maximum dose used in the test producing no adverse effect. If adverse effects are recorded at all dose levels then an LOAEL is established. Full details of the test methodologies can be found in the relevant testing guidelines (OECD, 1981a; 1981b).

In reality, most chemicals are evaluated for the purposes of general chemical use and not specifically for dermal application and in many cases a repeat-dose dermal toxicity study may not be available. However, a repeat-dose oral toxicity study may have been conducted on the chemical and an NOAEL derived from oral exposure is often used to calculate the MOS for dermal exposure.

For some chemicals, particularly chemicals that have been used for many years, more than one repeat-dose study may be available. Some studies may have been conducted more than 30 years ago and may not have evaluated the same end points as more recent studies. The dose levels, species and strains of animals, animal numbers, duration of study and route of administration may differ between the studies. This leaves the safety evaluator with the dilemma of which study to use to calculate the MOS as NOAELs/NOELs can vary between studies.

A more recent study conducted to current testing guidelines that is compliant with GLP standards will normally take precedence over an older, nonstandard, non-GLP study. However, it is fair to say that 20 or 30 years ago many chemical and end-product manufacturers had their own in-house animal testing facilities and so had the opportunity to conduct investigative studies for their own purposes; these studies are often now in the public domain. These studies, whilst not complying with current testing guidelines, can sometimes provide valuable information. There was also less concern over the number of animals used for safety assessments. This is in contrast to the current situation, where many manufactures now contract out their toxicity testing, which, combined with the increasing pressure to reduce animal testing, means that studies on newer chemicals tend to be the more standard toxicity tests required for chemical registration.

There are no hard and fast rules on which study to select for calculating the MOS and each study should be evaluated on its merit. It may be the case that a repeat-dose oral toxicity study is chosen over a repeat-dose dermal toxicity study if the former is considered to be more scientifically valid. The NOAEL could also from a longer-term chronic toxicity test, carcinogenicity study or reproduction toxicity test. It is usual practice to take the lowest NOAEL from the scientifically valid studies reviewed to the safety evaluation. Whichever study is chosen as the pivotal study for the safety evaluation, a justification for the selection should be documented.

Given that oral toxicity studies are often used for the safety evaluation of products applied dermally, there is a need to understand the percutaneous absorption of the chemical across the skin, so an accurate estimate of the systemic exposure can be made. Taking a pragmatic approach before any testing is carried out, it is worth considering calculating the MOS assuming 100% dermal absorption (DA), that is, systemic exposure is equal to dermal exposure. If taking this worst-case approach there is an adequate MOS, then testing may not be necessary. If however, the MOS is not acceptable, then there is a need to generate specific data on DA. This can be determined through either *in vivo* or *in vitro* DA assays such as those outlined in OECD Guidelines 427 and 428 (OECD, 2004b; 2004c). Given the restrictions outlined for animal testing in the Seventh Amendment to the Cosmetics Directive 76/768/EEC (2003/15/EC), in Europe the *in vivo* test option will no longer be available after March 2009 for the assessment of new cosmetic ingredients.

The *in vitro* skin absorption test is described fully in OECD 428 (OECD, 2004b), but in summary consists of applying the chemical to excised skin in a specially developed diffusion cell, which consists of a donor chamber and a receptor chamber between which the skin sample is positioned. The amount applied should be representative of the human exposure expected, typically in the range $1-5\,\mathrm{mg\,cm^{-2}}$ for solids and up to $10\,\mu\mathrm{l\,cm^{-2}}$ for liquids. Although human skin is the obvious choice, availability can sometimes be a problem, in which case pig is the preferred alternative, as it has similar characteristics to human skin. Rodent skin is not normally used, as it is not representative of human skin (ECETOC, 1993). The skin integrity should be verified before the start of the study and afterwards a full mass balance should be carried out.

DA can be affected by a number of factors, such as the physical and chemical properties of the skin, the composition of the formulation, the concentration in the formulation, the site of exposure and whether occlusion occurs (Schaffer and Redelmeier, 1996; ECETOC, 1993). Some cosmetic products are formulated specifically to enhance penetration of other ingredients through the skin. For this reason the full formulation of the cosmetic product needs to be assessed and where such penetration enhancers are present it should be assumed that the systemic exposure is 100% of the dermal exposure. Similarly for ingredients used in cosmetic products intended to come into contact with mucous membranes, the systemic exposure should be considered to be equivalent to the dermal exposure.

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7.2 Exposure Assessments

It is standard risk-assessment practice to consider exposure scenarios resulting from intended use or foreseeable misuse of the product, but not abuse. For cosmetic products, exposure is primarily via the skin, although for some lip and dental-care products, ingestion should also be considered; this is outside the scope of this chapter, but in general the same principles apply as for other ingested products. The main considerations for exposure to cosmetic products are as follows:

- Frequency of exposure. The product may be intended for multiple applications daily, such as a hand cream, daily use, such as a shower gel, or intermittent use, such as hair colouring products.
- Duration of exposure. Products such as skin creams are intended to be left on the skin, whereas products such as shampoo or shower gel are rinsed off the skin.
- The concentration of the ingredient in the finished product. Some ingredients, such as the 'actives', preservatives, colours and fragrance ingredients, are used at low levels in the formulation, whereas others that form the base of the formulation can be present as high concentrations. Possible cumulative exposure should also be taken into consideration, as many ingredients are used in many different product types.
- The area of skin exposed. In some cases, such as a body lotion, a large surface area is exposed, whereas for a make-up product only the face will be exposed.
- The quantity of product used at each application. Again this will vary considerably according to product type. Also, some products, such as soaps, are applied in a fairly dilute solution, which will affect the overall exposure.
- Cumulative exposure. Many ingredients are common to different types of cosmetic products, so an estimation of the cumulative exposure from a range of products should also be considered.
- Other factors. There may be a number of other special considerations that can affect exposure. These include: skin integrity—some products are specifically designed for damaged skin and here the skin barrier may be compromised increasing skin penetration; products intended for babies and small children, as these have a high surface area to body weight ratio; oxidative hair dyes, where the ingredients react on the hair and exposure to the reaction products not present in the formulation occurs; products intended to come into contact with mucous membranes where the thin epithelial skin layer is minimal.

This list is not meant to be exhaustive, but gives an indication of the factors to be considered for the exposure assessment. Each new product needs to be considered on a case-by-case basis to ensure all aspects have been covered.

Many companies have their own habits and data practices for particular product types. But there are also a number of published sources of typical exposure data for a large number of cosmetic products, such as those from the Cosmetics, Toiletry and Fragrance Association (CTFA) and the SCCP notes of guidance for the testing of cosmetic ingredients and their safety evaluation (Loretz *et al.*, 2005; 2006; Hall *et al.*, 2007; SCCP, 2006). Using the above, exposure scenarios can be developed that reflect the use of the substance in various applications and can be used to calculate the systemic exposure dosage (SED) in $\mathrm{mg\,(kg\,bw)^{-1}\,day^{-1}}$. For example:

$$\mathrm{SED} = \frac{\mathrm{DA}\left(\frac{\mu g}{cm^2}\right) \times 10^{-3}\frac{\mathrm{mg}}{\mu g} \times \mathrm{SSA(cm^2)} \times F(\mathrm{day^{-1}}) \times R}{60\mathrm{kg}} \quad (1)$$

where

DA—dermal absorption in $\mu g\,cm^{-2}$
SSA—skin surface area in cm^2
F—frequency of application of finished product per day
R—retention factor, for example, 100% for leave on products to 1% for rinse off products
60 kg—typical default human body weight.

There will be considerable variation in the exposure between individuals, so in many instances it may not be possible to measure the exposure accurately and a conservative approach is taken to the exposure assessment. The gathering of exposure data is an ongoing activity and as more data becomes available, the default figures may be refined and updated. It should also be noted that the use of cosmetic products can be very susceptible to changes in fashion and trends in the marketplace.

7.3 Risk Characterization

The final stage in the safety assessment of a cosmetic ingredient is the calculation of the MOS. The MOS is the NOAEL divided by the SED. For a cosmetic ingredient to be considered safe for use, a default value of 100 for the MOS is considered acceptable (SCCP, 2006). It allows a factor of 10 for the interspecies differences between experimental animals and man, and a factor of 10 for human variability. This value is used generally in toxicological risk assessment and is not specific to cosmetic ingredients. An MOS of 100 is not an absolute cut-off and each MOS is considered on a case-by-case basis, as in some circumstances, additional data, for example toxicokinetic, may be available

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which suggests that a higher or lower figure may be acceptable.

7.4 Alternative Methodologies for Assessing Systemic Toxicity

Within the EU there is a requirement to develop and validate alternative methods to animal testing to meet the scientific objectives of the 3 R strategy of refinement, reduction and replacement of animal testing. In the Seventh Amendment of the Cosmetics Directive (2003/15/EC) the marketing of finished products containing an ingredient that has been subjected to any animals testing after 2013 is prohibited. At the moment there are no alternative *in vitro* methods or even relevant proposals for alternative methods to evaluate systemic toxicity. This is not surprising given the complexity of evaluating the effects on the whole organism. However, one area that is active is that of developing alternative methodology for carrying out safety evaluations, such as the application of the threshold of toxicological concern (TTC) concept to cosmetic ingredients.

7.5 Use of the TTC

The TTC concept is not new; it is based on a generic exposure threshold that originally formed the basis of the US Food and Drug Administrations Threshold of Regulation for Indirect Food Additives (Food and Drug Administration, 1983; 1993; Threshold of Regulation for Substances used in Food Contact, 1995). Since then, the concept has been continuously developed and undergone many refinements (Munro *et al*., 1996; 1999; Kroes *et al*., 2000; 2004) to its current application in the area of risk assessment (Kroes *et al*., 2005). The basic principle is that a threshold of human exposure can be established below which there is a very low probability of adverse effects on human health. The threshold level is dependant on chemical structure and the known toxicity of a large number of existing chemicals that share similar structural characteristics. Application of the TTC requires the user to work through a decision tree comprising a number of steps which require a yes/no response to progress to the next step. The outcome is that the threshold value is compared to the estimated exposure; where the exposure is lower the chemical is predicted not to be a health concern and where the exposure is higher than a further risk assessment is necessary using compound-specific toxicity data. There is insufficient space here to describe the full history and the methodology, but this can be found in Kroes *et al*. (2005; 2007).

The TTC concept has its origins in food safety and this is where it has been most widely used, particularly in relation to food contact materials and food flavourings. It is also used as a tool in the safety assessment of contaminants in pharmaceutical products (Delaney, 2007). Kroes *et al*. (2005) first suggested the idea that the concept may be suitable for application to nonfood chemicals and many toxicologists have been interested in evaluating whether this approach could be widened to the risk assessment of consumer products. This led to the European Cosmetic Toiletry and Perfumery Association (COLIPA) sponsoring a group of experts to evaluate potential use in the safety evaluation of cosmetics ingredients. The focus of the Expert Group was to evaluate a number of factors related to the use of chemicals as cosmetic ingredients and their exposure. In particular, the similarity of cosmetic ingredients to the chemicals used to establish the thresholds of concern (Cramer classes); differences in metabolism between the oral and dermal route of exposure; default adjustment figures for percutaneous penetration to adjust for systemic exposure via the skin; consideration of the use of adjustment factors to assess the exposure for rinse-off products and products used intermittently; and total exposure to the ingredient. The outcome of this detailed evaluation by the Expert Group has been published by Kroes *et al*. (2007), with an overall conclusion that the TTC approach is suitable for evaluating cosmetic ingredients. Their proposal is for the application of the TTC for cosmetic ingredients to consist of the following steps:

1. Define product type, its intended use and related skin surface area involved
2. Define concentration of ingredient in the product
3. Estimate external exposure per day
4. Estimate skin absorption of the ingredients based on its physical and chemical characteristics
5. If it is a rinse-off product apply retention factor
6. Establish use pattern: for example, daily or intermittent use, if the latter is the case apply the default factor related to the use interval
7. Calculate adjusted internal exposure per person per day
8. Where relevant, calculate total (aggregate) exposure when several cosmetic products contain this target ingredient
9. Use this average aggregate internal dosage in the TTC decision tree.

Using the TTC approach, if the systemic dosage is below the threshold level for the chemical class for the given chemical under consideration, then it is considered not to present a human-health concern and is acceptable for use in cosmetic products. If the level is higher, then the TTC approach cannot be used and ingredient-specific toxicity data is needed for the safety evaluation.

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If one compares the steps outlined above with the standard methodology for safety evaluation there is commonality in the process. In particular, both methodologies require a rigorous estimate of exposure, as this is key to providing a reliable safety evaluation. The Expert Group state that the use-related adjustment factors should be regarded as provisional and may need refinement as more data becomes available (Kroes *et al*., 2007). This is also true for any exposure assessment.

Clearly, for some macroconstituents of cosmetic products, a TTC approach is not appropriate, as the exposure will always be above any threshold of no concern. Substances, such as heavy metals, proteins and those with specific structural alerts, that were excluded in the original decision tree developed by Kroes *et al*. (2004) are excluded from inclusion in the TTC approach, as are new chemical ingredients that are suspected to have pharmacological activity (Kroes *et al*., 2007). Furthermore, plant extracts could not be evaluated by the TTC as these are usually complex mixtures of chemicals and the starting point for a TTC evaluation is having a known chemical structure (Kroes *et al*., 2007).

The SCCP was requested to review the proposal put forward by the Expert Group and also address more generally the use of the TTC concept in consumer products and others, where a significant exposure to chemicals is likely to occur through normal-use situations. They also considered whether there was a distinction between intentionally added ingredients and those chemicals that are present as inadvertent contaminants. A draft opinion was published in 2008 (SCCP/1171/08, 19.11.08). Whilst broadly supportive of the TTC approach, the opinion was that the current database of toxicity data used to establish the threshold exposure levels is inadequate for the assessment of chemicals used as cosmetic ingredients. The rationale being that after comparison of the 15 000 cosmetic ingredients listed in the EU database (CosIng) with the chemicals listed in the Munro database (Munro *et al*., 1996), cosmetic ingredients were only covered to a limited extent. A similar conclusion was found for impurities present in cosmetic ingredients. However, at the time of writing, this draft opinion had only just been published and whilst disappointing, the recommendation from the SCCP that the toxicity database needs to be validated and extended before a TTC approach becomes more generally acceptable is one that is likely to be addressed in the near future. The application of the TTC concept in the risk assessment of cosmetic ingredients is still in its infancy and no doubt this is an area where there will be significant focus in the future, as it provides significant opportunity for advancement of alternative methodologies for safety evaluation. As discussed here, the TTC is only being used to consider systemic toxicity, and local end points require separate evaluation, although a proposal for a TTC approach to sensitization risk assessment has already been discussed elsewhere in this chapter.

8 OTHER TOXICOLOGICAL END POINTS

In this review we have focussed on the end points which we consider to be the essential elements of a review of the dermal toxicology of cosmetic and beauty-care products. However, in some instances, further information may be required, such as phototoxicity data, where the cosmetic product is intended for sun-exposed skin (see, for example, Basketter and Jones, 2008). Also, carcinogenicity and/or reproductive toxicity data may be required, due to potential adverse findings in the studies conducted, for example evidence of toxicity to the reproductive system in a repeat-dose toxicity study, or the expected use levels and skin penetration may be high. This has to be evaluated on a case-by-case basis. For high production volume, existing chemical reproductive toxicity and/or carcinogenicity data may already be available, and in such cases any existing data should be taken into account. If there is a need to generate new data this must to be considered carefully, as there will be a requirement for significant animal use with considerable costs and in many cases, unless the ingredient brings significant benefit to the end product, the additional testing requirements may not be justified.

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Organic Solvents

Håkon L. Leira, Frode Fonnum and Tore Syversen

CONTENTS

1 GENERAL PRINCIPLES

Organic solvents are a chemically heterogenous group of small lipophilic compounds defined on the basis of their common function, namely the ability to dissolve and disperse solids not soluble in water. Solvents are fluid at room temperature and many of them are highly volatile. These common properties make the number of potential solvents quite large, and several hundred different compounds are in use. However, only a minority of these are used on a regular basis in industry and/or in consumer products. Organic solvents belong to several chemical classes, for example, aliphatic and aromatic hydrocarbons, as well as chlorinated and oxygenated compounds such as alcohols, ketones, aldehydes, esters, glycols and glycol ethers, see **Table 1**.

Solvents are mainly used for metal degreasing and as intermediate chemicals in industry. They are important ingredients in varnishes, paints, lacquers, dyes and adhesives, as well as liquid fuels (gasoline, diesel, jet fuel) and fuel additives (benzene, methyl-*t*-butyl ether). Solvents are also found in the general environment and contamination of drinking water is a growing problem in many parts of the world. Chlorinated solvents, in common with many other halogenated compounds, may deplete stratospheric ozone, thus increasing the amount of incoming solar radiation.

Although exposure to solvents is ubiquitous, the main health challenge results from occupational exposure. The doses encountered in industry may be orders of magnitude greater than in other settings, with one exception. Due to their inebriating properties, various solvents are extensively abused ('glue-sniffing'), particularly among disadvantaged children and adolescents in many countries throughout the world. Part of our knowledge of the potential health effects in humans of organic solvents originate from studies among such abusers.

In industry, single compounds may be used, but exposure to solvent mixtures is the general rule. The literature on health effects of single compounds is extensive, but our understanding of the health effects of solvent mixtures is limited. Thus, toxicological risk evaluations in this field are challenging. In addition to the problem of mixtures, the question of exposed groups further complicates the risk assessment. It is well established that the risk profile of solvent exposure can differ substantially between, for example, neonates, children, pregnant women, diseased people and the elderly, as well as by gender.

In addition to the neurotoxic effects, some solvents have been classified as carcinogens or mutagens. In some cases also the immunological system is affected (Dreiem *et al.*, 2003; Harris *et al.*, 1997). However, except for turpentine, organic solvents are not allergens.

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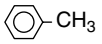
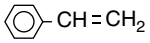
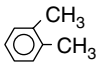
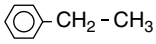
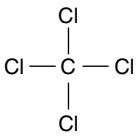
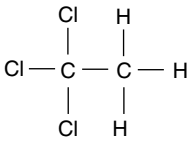
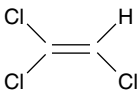
Table 1 The structures, molecular weights and oil/water partition coefficients of selected solvents

Solvent		MW[a]	p.c.[b]
Benzene		78	177
Toluene		92	659
Styrene		104	1168
o-Xylene		106	1318
Ethylbenzene		106	2243
Cyclohexane		84	2754
n-Hexane		86	8710
Carbon tetrachloride		154	631
1,1,1-Trichloroethane		133	309
Trichloroethylene		131	263

[a]Molecular weight.
[b]Oil–water partition coefficients according to Leo *et al*. (1971).

1.1 Exposure and Absorption

Occupational exposure affects a large number of workers in most countries of the world. It is estimated that 10 million workers are exposed in the USA (OSHA (Occupational Safety and Health Administration), 2006). In a representative survey in 1997 by the national bureau of statistics in Norway, 11% of male workers and 7% of female workers stated that they were occupationally exposed to solvents on a daily basis. In many industrialized countries, exposure to organic solvents in industry has decreased over the last decades (Caldwell *et al*., 2000; Burstyn and Kromhut, 2002).

Exposure is particularly common in painters, printers, fibreglass laminators and floor-layers, as well as in petrochemical and refinery workers. Volatility and evaporation surface area are the main determinants of solvent concentrations in air. Spraying procedures lead to high concentrations of solvent vapour as the aerosols represent a vast surface from which the solvents can evaporate. Industrial spray painters are thus among the occupational groups most at risk.

Inhalation and skin absorption represent the most significant routes of solvent uptake. Uptake by inhalation is a simple physical process: the molecules diffuse from the alveolar space into the blood where they dissolve. The solvent molecules will partition between air and blood during the absorptive phase and between blood and other tissues during the distribution phase. The more water soluble a vapour is, the more will be absorbed into the blood during each respiratory cycle. The amount of vapour taken up by various tissues depends on the affinity of the organic solvent for each tissue. Thus, the rate of absorption of organic solvents in the lungs and the distribution to tissues varies considerably and depends on the blood/gas and fat/blood partition coefficients, respectively (Fiserova-Bergerova, 1985; Sato and Nakajima, 1987). Hydrophobic solvents have low blood–gas partition coefficients and their fat–blood partition coefficients are much larger than one. Hydrophilic solvents have blood–gas partition coefficients larger than 200 and fat–blood partition coefficients much smaller than one (Fiserova-Bergerova, 1985). Differences in the uptake and distribution of various organic solvents have been shown predominantly to result from differences in such solubility factors. The pulmonary uptake of many organic

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solvents in physically active individuals is much larger than in resting individuals, provided that the diffusion of the solvent from the alveolar space into the blood is not rate-limiting (Åstrand, 1983; Fiserova-Bergerova, 1985).

Percutaneous absorption of solvents is generally considered to be of minor importance compared to pulmonary uptake. However, several solvents can be absorbed through the skin, depending on their physical and chemical properties (Nakaaki *et al*., 1980; Kezic *et al*., 1997; Brooke *et al*., 1998). The degree of uptake also depends upon exposure conditions and is proportional to exposure time and the amount of skin surface exposed. Hydrophobic solvents of low molecular weight permeate the skin better than those with high molecular weights or hydrophilic solvents. Solvents that damage the skin will affect the skin integrity and this may enhance the percutaneous absorption of the agent. The glycol ethers 2-methoxyethanol (2-ME) and 2-ethoxyethanol (2-EE) are readily absorbed through the skin, and skin contact of both forearms and hands for 15 minutes may cause a body uptake exceeding inhalation for eight hours at the occupational exposure limit value (Kezic *et al*., 1997). Toluene, xylene, tetrachloroethylene, 1-propanol, methanol, 2-hexanone (methyl butyl ketone (MBK)), 1,4-dioxane and 2-butoxyethanol are examples of other solvents that easily penetrate the skin.

1.2 Metabolism

The metabolism of solvents can be divided into two phases. Phase I is characterized by oxidation, reduction and hydrolysis rendering the molecule more water soluble, while phase II reactions conjugate the metabolite, rendering a product having a molecular weight which is more suited for excretion (through bile or urine). The products of phase I reactions are often more reactive and toxic than the parent compound (metabolic activation), while phase II reactions usually represent a true detoxification reaction. Metabolizing enzymes are widely distributed throughout the body, although the liver is the major metabolic organ.

Several solvents are hepatotoxic due to their conversion to toxic intermediates or metabolites in the liver (Sipes and Gandolfi, 1982). Many of the haloalkanes and haloalkenes appear to have similar toxic effects in liver and kidney (Kitchin and Brown, 1989; Dekant *et al*., 1989). The hepatotoxic and carcinogenic effects of haloalkanes and haloalkenes are generally thought to be due to reactive metabolites rather than the parent molecule. Free-radical intermediates are involved in the toxicity through mechanisms including, for example, lipid peroxidation, covalent binding and cofactor depletion (Cheeseman *et al*., 1985). Ethylene glycol, for example, is metabolized by alcohol dehydrogenase (ADH) to glycolaldehyde and further to glycolic acid. Glycolic acid is oxidized to oxalic acid by glycolic acid oxidase (von Wartburg *et al*., 1964; Liao and Richardson, 1973; Gessner *et al*., 1960). The oxalate may cause acute renal tubular necrosis through the formation of insoluble oxalate crystals in the kidney (Gabow *et al*., 1986; Turk *et al*., 1986). The lethal dose of ethylene glycol is reported to be approximately 100 ml in an adult, but only 5–10 ml in children. ADH catalyses ethanol oxidation, and ethanol can be used as an antidote in ethylene glycol intoxication, as it will reduce the production rate of oxalic acid (Jacobsen and McMartin, 1986). Specific toxic effects of several other solvents such as, for example, benzene, styrene, *n*-hexane, glycol ethers and methanol are also due to production of toxic metabolites, and are discussed elsewhere in this chapter.

1.2.1 Metabolic Interactions of Solvent Mixtures

A thorough review on the toxicology of exposure to mixtures is given in **Toxicology of Chemical Mixtures**. Organic solvents are more commonly used as mixtures than as individual chemicals. Thus the metabolism, and hence the toxicity, of one solvent may be modified by other solvents in the mixture.

The effect of ethanol on metabolism of organic solvents has been shown to be rather complex (Sato *et al*., 1981). At high concentrations, ethanol may act as an inhibitor of solvent metabolism. Thus, concurrent exposure to ethanol and solvents may reduce the clearance of other solvents and the result is increased and sustained internal dose of the solvent (Cherry, 1993). However, an acute dose of ethanol will increase the metabolism of toluene and trichloroethylene (TRI) in rat liver, and ethanol may enhance the hepatic metabolism of these solvents (Sato *et al*., 1981). Ethanol may impair the metabolic clearance of *m*-xylene, causing raised xylene blood concentration and a decrease in the excretion of methylhippuric acid (Riihimäki *et al*., 1982). Some people appear to be more susceptible to combined ethanol–xylene exposure than others, and may develop nausea and dermal flush (Riihimäki *et al*., 1982).

Coexposure of hexane and toluene decreases hexane neurotoxicity and urinary excretion of hexane metabolites in rats (Takeuchi *et al*., 1993). Methyl ethyl ketone inhibits the *in vitro* metabolism of *n*-hexane in a noncompetitive pattern and decreases the levels of the toxic metabolite 2,5-hexanedione (Mortensen *et al*., 1998). Concomitant treatment of rats with a nonhepatotoxic dose of dichloromethane potentiates the hepatotoxicity of carbon tetrachloride (Kim, 1997). Several investigators have demonstrated that coadministration of toluene with benzene protects animals from benzene-induced haematotoxicity (Ikeda *et al*., 1972; Andrews *et al*., 1977; Sato and Nakajima, 1979; Tunek *et al*., 1982). However, such interactions are exhibited only at high concentrations.

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Several kinetic studies conducted with volunteers have demonstrated similar metabolic interactions in humans (Engström *et al*., 1984; Wallen *et al*., 1985; Liira *et al*., 1988). In male workers exposed to benzene and toluene, the metabolism of both solvents was depressed, indicating a mutual suppression of metabolism between benzene and toluene (Inoue *et al*., 1988). Coexposure to xylene (100 ppm) and methyl ethyl ketone (200 ppm) in humans has resulted in reduced xylene metabolism (Liira *et al*., 1988). However, the combined exposure did not cause any change in methyl ethyl ketone metabolism. In humans, metabolic interaction took place when the subjects were exposed to a combination of 95 ppm toluene and 80 ppm xylene, whereas no interaction was detected after exposure to a combination of 50 ppm toluene and 40 ppm xylene (Tardif *et al*., 1991). Benzene and toluene competitively inhibit each other's metabolism (Sato, 1993). Male workers exposed to a mixture of solvents, including toluene (18 ppm), methyl ethyl ketone (16 ppm), isopropyl alcohol (7 ppm) and ethyl acetate (9 ppm) (Ukai *et al*., 1994) showed no signs or symptoms that suggested anything other than irritation effects due to toluene exposure. There was no evidence to suggest any modifications of toluene toxicity or metabolism due to coexposure. The results indicate that metabolic interaction depends on the solvents and the level of exposure.

Gasoline (petrol) is a complex mixture of aromatic and aliphatic chemicals. The same enzyme responsible for the metabolism of benzene metabolizes many components of gasoline. Studies at the Chemical Industry Institute of Toxicology (CIIT) have shown that the interactive effect of a complex mixture, such as benzene in gasoline, may only occur at saturating concentrations of these chemical mixtures. At lower and environmentally relevant concentrations, however, these interactions become negligible (Bond *et al*., 1997). 1,2,4-Trimethylbenzene (1,2,4-TMB) occurs mainly in petroleum products, and in man it has been shown that metabolism of 1,2,4-TMB is inhibited after exposure to white spirit compared to exposure to 1,2,4-TMB alone (Järnberg *et al*., 1997). It appears that components in white spirit inhibit the metabolism of 1,2,4-TMB.

1.3 Enzyme Induction

A number of solvents may induce xenobiotic metabolizing enzymes, for example, CYP 2E1 (Conney, 1968; Nebert *et al*., 1978). This enzyme catalyses the metabolism of a large number of solvents, including ethanol. As a socially accepted toxin, ethanol is consumed in large quantities compared to other solvents. Ethanol is known to inhibit drug metabolism acutely, and to stimulate xenobiotic metabolism through enzyme induction when administered repeatedly. Thus the effects of ethanol on the metabolism of other solvents are complex, depending on the timing and frequency of ethanol intake. It has been shown that chronic ethanol administration induces CYP 2E1. Thus, in regular drinkers, elimination of solvent metabolites appears to be faster compared to nondrinkers, presumably caused by enzyme induction (Cherry, 1993). In addition to a faster elimination of solvents, chronic ethanol intake may lead to a faster and increased formation of metabolites, and therefore increased risk of adverse effects due to production of toxic metabolites. It has been shown that alcoholics have an increased susceptibility to solvents such as carbon tetrachloride and benzene (Morimoto *et al*., 1993; Ronis *et al*., 1996; Lindros, 1997). While extinguishing a fire, seven workers were exposed to high concentrations of carbon tetrachloride. However, only the two workers with a high ethanol intake sustained hepatotoxic and nephrotoxic effects (Manno *et al*., 1996).

It has been shown that even a single dose of benzene, toluene or *m*-xylene may induce the activity of CYP 2E1 significantly, and thereby enhance metabolism of other solvents (Kim and Kim, 1996). Methyl ethyl ketone pretreatment induced rat liver cytochrome P450 and increased the formation of the toxic metabolite 2,5-hexanedione in rat liver (Mortensen *et al*., 1998). Exposure to small aromatic hydrocarbons shows that the addition of methyl groups to the aromatic ring affects the inductive capacity of different CYP isoenzymes (Pathiratne *et al*., 1986; Backes *et al*., 1993; Gut *et al*., 1993; Yuan *et al*., 1995; Wang *et al*., 1996). Sex differences in enzyme induction have been observed. Ethylbenzene treatment induced CYP 2B1 and CYP 2B2 to a greater extent in male rats than in females, and CYP 2E1 only in female rats (Sequeira *et al*., 1992). Several studies have indicated no induction of hepatic ADH following treatment with ethanol (Gillion *et al*., 1985; Guthrie *et al*., 1990; Singh and Pandey, 1991; Aasmoe and Aarbakke, 1999). The glycol ethers 2-ME and 2-EE induced liver ADH in male rats only, while no induction was observed in female rats (Aasmoe and Aarbakke, 1999).

However, induction is not always reflected in increased *in vivo* metabolism. If the hepatic blood flow rather than the enzyme activity becomes rate limiting to the metabolism of a solvent, then enzyme induction will not affect the solvent toxicokinetics. In such cases, the blood flow limits the extent of their metabolism and enzyme induction has little effect on metabolism when exposure concentration is low. Examples of such solvents are TRI and xylene. In humans, enzyme induction resulted in increased metabolism of TRI only at high exposure concentrations (Sato, 1993). Ethanol consumption increased the metabolism of xylene about fivefold in rats only at high exposure concentrations (Kaneko *et al*., 1993).

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1.4 Toxic Effects of Organic Solvents

The common effects of most solvents include anaesthetic effects on the central nervous system (CNS) and irritation of mucous membranes and tissues. These are the dominant signs of short-term exposure at a high concentration. Distinct from general effects are the specific toxic effects of individual solvents. They are often related to the metabolism of the solvent. The specific toxic effects are often not caused by the parent compound, but rather by the metabolites created by this metabolic activation.

1.4.1 Irritation of Mucous Membranes and Tissues

Irritation is a physiological response to chemical or physical stimuli. Depending on dose, most organic molecules have some irritant properties, such as stinging, burning, prickling or tingling (Cometto-Muñiz and Cain, 1992). Such irritation may damage the skin, lungs or eyes. If certain functional groups are added to an organic molecule, the irritant properties of the chemical will increase. Examples are amines and acids, which will add corrosive properties to the molecule. Aldehydes and ketones can produce narcosis, but this effect is usually avoided because the vapours are irritating to the eyes and respiratory tract at low concentrations (James, 1985). It has been shown that the irritation thresholds decrease logarithmically with carbon-chain length for a homologous series of ketones, secondary and tertiary alcohols, acetates, aldehydes and alkylbenzenes (Cometto-Muñiz and Cain, 1991; 1994; Cometto-Muñiz *et al*., 1998). Eye irritation thresholds and nasal pungency thresholds were well below the odour threshold (Cometto-Muñiz and Cain, 1995). However, irrespective of molecular size or chemical functional group, threshold for nasal pungency is observed at a fairly constant percentage of vapour saturation, which implies an important role for a physical rather than chemical interaction with the nasal mucosa (Cometto-Muñiz and Cain, 1993). Thus, irritation of mucous membranes is probably related to the size as well as to the structure of the molecule.

1.4.2 Clinical Effects on the Central Nervous System

Cross-sectional epidemiological studies have supported the hypothesis that occupational low-level, long-term exposure to solvent mixtures may cause adverse CNS effects (Tähti, 1984; Bleecker *et al*., 1991; Triebig *et al*., 1992; Hakkola, 1994; Hogstedt, 1994). They have demonstrated significant dose-response relations between exposure, mental symptoms and neurobehavioural performance. Occupational solvent exposure may be the cause of mental and cognitive impairment that can become permanent. In complex exposures, the adverse CNS effects seem to occur at exposure levels well below the accepted threshold limit values (Mikkelsen, 1997). Such values are, in most cases, set according to mucus membrane irritation, and in no case according to the risk of chronic CNS effects.

At occupational exposure levels, organic solvents can cause various symptoms and signs of CNS origin: fatigue, anxiety, irritability, headache, weakness, depression, neurobehavioural changes and impairment of cognitive performance (Milanovic *et al*., 1990; Evans and Balster, 1991; Hakkola, 1994; Kishi *et al*., 1995; Mikkelsen, 1997).

Long-term occupational exposure to solvents may cause a neurotoxic syndrome commonly known as chronic toxic encephalopathy. Symptoms and clinical signs typical of chronic toxic encephalopathy are impairment in memory and learning skills, increased irritability, fatigue, sleeping problems and changes in personality (Ng *et al*., 1990; Hein *et al*., 1990; Mikkelsen, 1997). Epidemiological studies on occupational solvent exposures have shown impairment in short-term memory and in psychomotor function (Baker, 1994). In cross-sectional studies, differences between exposed and unexposed workers have been most commonly seen in performance tests of memory function and particularly in short-term memory ability (Moen *et al*., 1990; Bleecker *et al*., 1991; Hänninen *et al*., 1991). Notably the performance in associated learning tasks and in memory span has been reduced in solvent-exposed workers. In many epidemiological studies, a dose–response relationship has been demonstrated. In heavily exposed workers the neurobehavioural deficits are similar to those seen in persons with pronounced neurobehavioural dysfunction due to solvent abuse (Baker, 1994). Follow-up studies have shown that after removal from solvent exposure, persistent deficits can be observed in persons with severe initial impairment, but no progression is seen once exposure is stopped (Gregersen, 1988; Edling *et al*., 1990). Of particular interest is a Swedish follow-up study of floor layers who, as a group, did not show neurobehavioural deterioration compared to controls (carpenters) at evaluations after 5 and 20 years of exposure. During the following 18 years, exposure was low, but the floor layers most heavily exposed during the first 20 years of exposure showed a significant deterioration in visual memory and perceptual speed, relative to the carpenters and to the less-exposed floor layers. The results are consistent with the view that the negative effects of exposure to solvents may interact with the normal ageing process, primarily at heavy exposure (Nilson *et al*., 2002).

Intentional inhalation of volatile organic compounds (VOCs) is well known and the abuse of volatile substances (glue sniffing, inhalant abuse, solvent abuse) in order to achieve intoxication has been reported from most parts of the world (Giovacchini, 1985; Flanagan and Ives, 1994). Small doses can lead to euphoria,

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delusions and hallucinations. Higher doses may produce life-threatening effects, such as convulsions and coma. Death may ensue indirectly after aspiration of vomit or from direct cardiac or CNS toxicity. Chronic abuse of toluene-containing products and of chlorinated solvents such as 1,1,1-trichloroethane can produce severe organ damage, especially in the liver, kidneys and brain. Prolonged abuse may be fatal.

The question of solvent-induced toxic encephalopathy is controversial (Fiedler and Lerman, 2007), although the condition is accepted as an occupational disease in many European countries, including the European Union. In the Nordic countries it was recognized as an occupational disease in the 1970 and 1980s, almost reaching epidemic proportions. In parallel with the decline of exposure in industry, there has been a decline in the number of new cases. Thus, the condition is no longer considered to be a major problem in these countries.

In electrophysiological studies on solvent-exposed workers, some changes in electroencephalography consistent with diffuse cortical dysfunction have been found (Orbaek *et al*., 1988). Impairments in posture-control mechanisms (Niklasson *et al*., 1997), colour vision (Mergler and Blain, 1987) and olfactory functions (Schwartz *et al*., 1990) have also been associated with the neurotoxic syndrome caused by chronic exposure to organic solvents. Also, temporal epileptic seizures have been found as a result of occupational exposure to solvents (Jacobsen *et al*., 1994).

1.4.3 Effects on the Peripheral Nervous System (PNS)

Most organic solvents will have a nonspecific effect on the CNS, some leaving chronic effects after high or prolonged exposure. Peripheral polyneuropathy, on the other hand, results from exposure only to certain specific compounds, namely *n*-hexane, *n*-MBK and carbon disulfide (CS_2). Among these, *n*-hexane has been the most studied.

There are various industrial applications of *n*-hexane, including use in solvents and thinners in rubber, food and the pharmaceutical industry. Other uses are in adhesive tape and in glue in shoes, as well in cleaning agents. This hydrocarbon is also used in the production of polypropylene and polyethylene. In most circumstances, *n*-hexane is mixed with other aliphatic hydrocarbons and toluene for its primary industrial uses as a solvent.

About 15% of inhaled *n*-hexane vapour is absorbed. Large quantities (50–60%) of *n*-hexane are expired by the lungs after vapour exposure. The remaining *n*-hexane is biotransformed by the cytochrome P450 mixed-function oxidase system to 2-hexanol. 2-Hexanol is subsequently oxidized to 2,5-hexanediol, 2-hydroxy-5-hexanone, 2-hexanone, 2,5-hexanediol and 2,5-hexanedione (see **Figure 1**). The metabolites

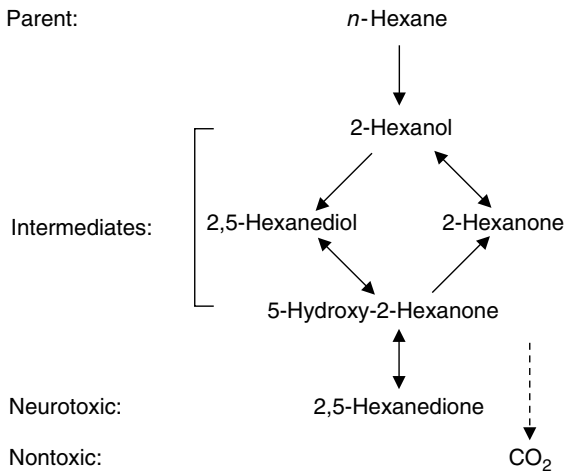

Figure 1 The biotransformation of *n*-hexane. Formation of the neurotoxic metabolite 2,5 hexanedione.

of *n*-hexane are more neurotoxic than the parent compound. The neurotoxic potency of *n*-hexane and some of its metabolites are (in ascending order): *n*-hexane < 2-hexanone < 2,5-hexanediol < 2,5-hexanedione. *n*-Hexane is known to cause peripheral neuropathies following long-term occupational exposures (Scaumburg *et al*., 1983).

Short-term, high-dose *n*-hexane exposure can cause narcotic effects, with headache and nausea at 1500 ppm, and confusion and dizziness at 5000 ppm within some minutes (Jørgensen and Cohr, 1981).

Long-term exposure to *n*-hexane and other liquid aliphatic hydrocarbons that undergo biotransformation to γ-diketone can cause peripheral axonal damage (peripheral axonopathy) (Ruff *et al*., 1981; Cavanagh, 1982; Bachmann *et al*., 1993; Graham *et al*., 1995). This toxic effect of *n*-hexane is based on the biotransformation product 2,5-hexanedione (**Figure 1**). The toxic mechanism appears to be that 2,5-hexanedione can bind and subsequently crosslink neurofilaments (Abou-Donia and Lapadula, 1988; Graham *et al*., 1995). It has been proposed that the γ-diketone, 2,5-hexanedione, reacts with lysine amino groups of neurofilaments to form pyrrole rings, which can interact with other proteins (DeCaprio, 1987). Neurofilaments accumulate above the nodes of Ranvier and form giant axonal swellings characteristic of γ-diketone-induced neurotoxicity (see **Figure 2**). Neurofilament crosslinking has been demonstrated both *in vitro* and *in vivo*. Although more than one mechanism may be operating, the following cascade of events seems to be supported by present knowledge: *n*-hexane is metabolized to 2,5-hexanedione, which causes decreased kinase-mediated phosphorylation of neurofilament proteins. This leads to a breakdown of the cytoskeletal matrix and dissociation of neurofilament proteins. Accumulated neurofilaments can then

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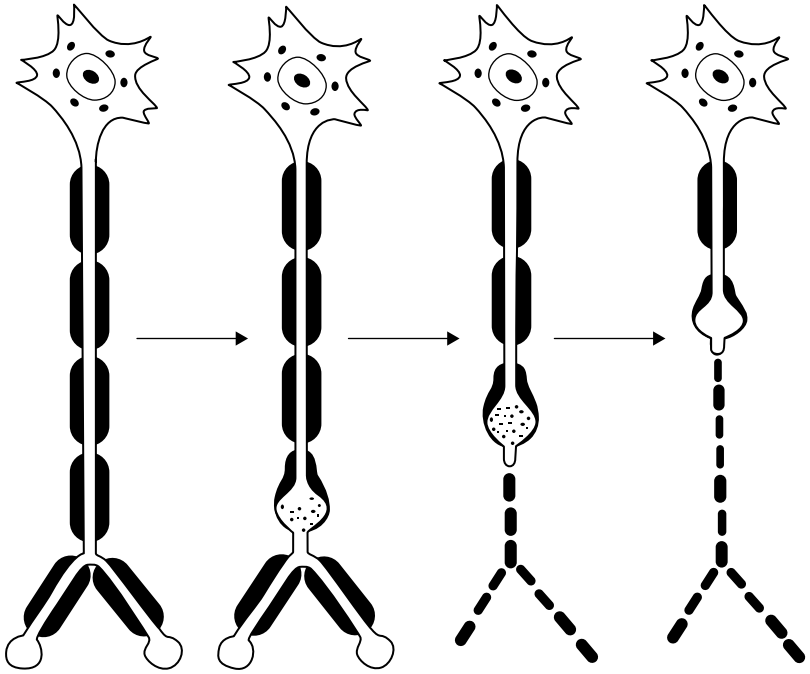

Figure 2 The mechanism of *n*-hexane-induced peripheral neurotoxicity. The axonal swellings are caused by the metabolite 2,5-hexanedione. Because of the axonal swelling there is no transport from the neuronal cell body to the peripheral part behind the swelling, and this part degenerates.

react with 2,5-hexanedione to form crosslinked neurofilaments, which results in the giant axonal swellings and distal axonopathy (**Figure 2**.). Based on studies with specific marker proteins for both the peripheral nervous systems (PNSs) and CNS, long-term *n*-hexane exposure in rats has shown changes in the PNS, but no significant changes in the CNS (Huang *et al*., 1992; 1993). The PNS seems to be the most sensitive target for *n*-hexane, while toluene mainly causes CNS toxicity (Tähti *et al*., 1997). Toluene has been shown to inhibit hexane metabolism and decrease its neurotoxicity (Takeuchi *et al*., 1993).

Although chemically similar to *n*-hexane, *n*-pentane, *n*-heptane and the alkenes, hexene and heptene, do not produce similar toxic metabolites to those of *n*-hexane and they have not been shown to cause peripheral axonopathy.

The polyneuropathy of MBK resembles that of *n*-hexane and is caused by the same toxic metobolite, 2,5-hexanedione. MBK is roughly 12 times more potent than *n*-hexane (Bos *et al*., 1991). In MBK neuropathy, swellings have also been found in the cerebellar white matter in the CNS and in the distal optic nerve, causing spastic gait and impairments of vision and memory (Bos *et al*., 1991). The long ascending and descending tracts of the spinal cord are also particularly vulnerable.

Carbon disulfide also causes polyneuropathy by neurofilament-filled swellings of the distal axons, similar to that produced by the metabolite of *n*-hexane (Graham *et al*., 1995).

1.4.4 Mechanisms of Neurotoxicity

The mechanisms of the CNS toxicity of solvents are not fully understood. Generally it has not been possible to identify specific sites of toxic effects in the CNS. Chronic high exposures to, for example, toluene cause multifocal disorders, including cerebral, cerebellar and brain-stem atrophy (Ikeda and Tsukagoshi, 1990). When inhaled at high concentrations, organic solvents produce CNS depression. The effect is related to the concentration of the solvent in the brain (Papper and Kitz, 1963; Lowe, 1972; Eger, 1974; Tichy, 1983). There is no simple relationship between the partition coefficient and the chemical structure or molecular weight which would help predict the uptake or the effects of the solvent in the nervous system (Sato and Nakajima, 1987). The length of the narcosis induced by organic solvents correlates well with their octanol–water partition coefficients (Wasserkort and Koller, 1997). A linear relationship between the 50% effective dose (ED_{50}) for CNS depression and the olive-oil–water partition coefficients for 20 different narcotics have been reported (Miller *et al*., 1972). As a broad generalization, the CNS depressant activity of an organic compound correlates with the length of its carbon chains and thus its lipid solubility.

Aliphatic, alicyclic and aromatic hydrocarbons have in common the ability to interact with biological membranes (Engelke *et al*., 1996). This interaction has been thought to cause changes in neural membranes, which then

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cause the CNS-depressant effect. According to the 'lipid theory', the anaesthetic potency of inhalation aesthetics and the CNS-depressant effects of organic solvents are related to their lipophilicity and to their effects on the lipid bilayer (Seeman, 1972). Organic solvents readily pass the blood–brain barrier and distribute into the lipid membranes of the neural cells, where they can affect neural membrane target proteins (Franks and Lieb, 1978; Korpela and Tähti, 1988; Edelfors and Ravn-Jonsen, 1992; Tähti *et al*., 1992; Naskali *et al*., 1993). The CNS depressant effect is greatly enhanced by halogenation, and to a lesser extent by alcoholic functional groups (James, 1985; Sato and Nakajima, 1987). Many organic solvents have effects similar to anaesthetics, and several halogenated solvents are the systemic anaesthetic agents of choice in surgery. In the past, TRI, diethyl ether and chloroform were used in clinical anaesthesia. Today, halogenated solvents such as sevoflurane, isoflurane and halothane are commonly used as anaesthetics.

Solvents with an aromatic structure are thought to localize in hydrophobic pockets in the protein or simply to be concentrated at the protein–lipid interface. The accumulation of organic solvents in neural cell membranes produces bilayer disorder (Engelke *et al*., 1992; 1996). The disorder in the phospholipid bilayer can indirectly affect the function of membrane enzymes (Edelfors and Ravn-Jonsen, 1992; Engelke *et al*., 1992). The physical/chemical activity relationship has been found important for the effects of organic solvents on synaptosomal membrane Na+/K+-ATPase activity and membrane fluidity (Naskali *et al*., 1993; Tanii *et al*., 1994). Both groups reported that partitioning of solvents into the lipid bilayer changes the membrane fluidity and leads to the inhibition of membrane-bound enzymes.

An effect which is seen with many lipophilic substances, such as polychlorinated biphenyls and brominated flame retardants is oxidative stress, or the production of free radicals (Mariussen and Fonnum, 2006). Intraperitoneal injection of toluene caused a significant elevation of the rate of production of reactive oxygen species (ROS) in the mitochondrial fraction of the liver and the synaptosomal fraction of the brain (Mattia *et al*., 1993a). The ROS was assayed with 2,7-dichlorodihydrofluorescein, which can detect hydrogen peroxide alone and in the presence of peroxidase, nitric oxide, peroxynitrite and hydroxyl radical (Myhre *et al*., 2003). Mattia *et al*. (1993a) confirmed the oxidative stress by showing a fall in the antioxidant glutathione. By employing a spin trap they were able to show that the hydroxyl radical was produced. Using the same technique they also showed that injection of toluene (0.5, 1 and 1.5 $mg\,kg^{-1}$) was accompanied by ROS production in the mitochondrial fraction from lung, kidney and liver, and in the synaptosome fraction from cerebellum and hippocampus (Mattia *et al*., 1993b). It was subsequently shown that exposure both to aromatic and dearomatized white spirit (0, 400 and 800 ppm, six hours a day, seven days a week, for three weeks) produced definite oxidative stress in subcellular fractions of brain, kidney and liver tissue of rats. Both a reduction in glutamine synthetase, which is an enzyme marker for oxidative stress, and a decrease in glutathione was found (Lam *et al*., 1994; Bondy *et al*., 1995). After exposure of white spirit to rats at days 7–20 during pregnancy, the rats were killed at day 35 and synaptosomes prepared. The synaptosome fraction from exposed animals showed an increase in calcium concentration (Edelfors *et al*., 1999). Also, synaptosomes exposed *in vitro* to white spirit showed an increase in calcium (Edelfors *et al*., 1999; von Euler *et al*., 1990). This can also be related to oxidative stress.

The studies on oxidative stress have been expanded to compare the effect of C6–C10 aliphatic, naphthenic and aromatic hydrocarbons on synaptosomes and on cerebellar granule cells. The results show that the alicyclic solvents were the most active, followed by the aliphatic hydrocarbons. Since the general feeling was that the aromatic hydrocarbons were the most toxic, it was a surprise to find that aromatic solvents were the least active (Myhre and Fonnum, 2001; Dreiem *et al*., 2002). The alicyclic and the naphthenic hydrocarbons were active in the range 100–800 μM, whereas the aromatic hydrocarbons were active at much higher concentrations (2–6 mM). The most active hydrocarbons in each group were *n*-octane, trimethylcyclohexane and *n*-butylbenzene respectively. When the granule cells were exposed to 200 μM *t*-butylcyclohexane, 1000 μM *n*-decane and 800 μM *n*-butylbenzene, cell death was encountered with the naphthenic and the aromatic compounds, but not with the aliphatic compounds. Both vitamin E and glutathione decreased the amount of cell death (Dreiem *et al*., 2005). Mechanistic studies of ROS production in synaptosomes showed the involvement of the neuronal nitric oxide synthetase, phospholipase A2 and mitochondria (Myhre and Fonnum, 2001). Similar studies were carried out with hydrocarbons on human granulocytes and gave similar results as with granule cells (Dreiem *et al*., 2003) The effect on granulocytes indicates that the immune system may be affected by exposure to hydrocarbon solvents.

As hydrocarbons interact with membranes, one would expect an effect on transporter molecules and receptors. Chronic exposure to styrene did not increase the binding of the dopamine agonist radopride to D2 dopamine receptors, but induced a persistent decrease in affinity of the neostriatal D2-agonist binding sites, possibly mediated by membrane perturbations (von Euler and Bjørnaes, 1990). Further experiments in the rat indicated that subchronic exposure to toluene at low concentrations caused a slight, but persistent deficit in spatial learning and memory, a persistent increase in dopamine-mediated locomotor activity due to an increase in the number of dopamine D2 receptors (von Euler *et al*., 1993). Exposure of rats to white spirit was also accompanied by

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an effect on the serotonin transporter and receptor (Lam *et al*., 2001). In conclusion, the lipophilic hydrocarbons affected both ROS formation and neurotransmitter transporters and receptors. Neuronal death is probably in some way linked to ROS formation, although it is surprising that aliphatic hydrocarbons with high ROS-producing activity did not produce cell death *in vitro*. The effects on biogenic amines could be involved in some of the neurobehavioural changes that are observed.

2 TOXICOLOGY AND EFFECTS OF INDIVIDUAL SOLVENTS

2.1 Aliphatic Solvents

Aliphatic hydrocarbons are open-chain carbon-containing chemicals derived from crude oil. The group can be divided into three subgroups. Alkanes or paraffins (C_nH_{2n+2}) are saturated hydrocarbons. Alkenes or olefins (C_nH_{2n}) contain one or more double bonds. Alkynes or acetylenes (C_nH_{2n-2}) contain one or more triple bonds.

Aliphatic hydrocarbons containing four or less carbon atoms are gases (e.g. methane, ethane, propane, butane, 1,3-butadiene and acetylene). Compounds with a 16-carbon atom chain or longer are solids at room temperature. Aliphatic hydrocarbons containing 5–16 carbon atoms are liquids. C_5-C_8 compounds are very volatile solvents.

Aliphatic hydrocarbons are found in fuels (natural gas, gasoline, kerosine), propellants, solvents, dyes, inks, plastics and coatings, dry-cleaning agents and chemical intermediates. Aliphatic volatile alkanes (e.g. pentane, hexane, heptane, octane and nonane) are CNS depressants in high concentrations. They may also irritate mucous membranes, as well as cause defatting of the skin that may develop into dermatitis. *n*-Hexane may cause peripheral polyneuropathy (see Section 1.4.3).

Alkenes, the unsaturated aliphatic solvents, are toxicologically very similar to alkanes. Acetylenes, the aliphatic solvents with triple bonds, are toxicologically inert, but may displace oxygen in confined spaces and thus act as asphyxiants.

2.2 Chlorinated Aliphatic Solvents

Chlorinated aliphatic hydrocarbons have been widely used as solvents, dry-cleaning agents, aerosol propellants and as raw materials in the chemical industry. They may be found as by-products of drinking-water chlorination. Exposure to chlorinated aliphatic solvents can cause CNS depression, and, after chronic exposure, permanent damage such as impaired memory, concentration difficulties and personality changes (Stevens and Forster, 1993). Chlorinated solvents have also been shown to cause CNS depression in experimental animals (Honma, 1990), and chloroform and TRI have been used as inhalation anaesthetics.

TRI and tetrachloroethylene are classified as probable carcinogens (Group 2A) by International Agency for Research on Cancer (IARC) while carbon tetrachloride, chloroform, 1,2-dichloroethane and dichloromethane are classified as possible carcinogens (Group 2B). Most of the chlorinated solvents will sensitize the myocardium to endogenous catecholamines, so acute high exposure may lead to ventricular fibrillation and death. The chlorinated solvents are nonflammable, but upon heating they disintegrate partly into chlorine-containing compounds, which are very irritating to the bronchial and lung mucosa. Among these is phosgene, which may cause lung oedema and death, and was therefore used as a war gas during World War I.

2.2.1 Carbon Tetrachloride (Tetrachloromethane/ Perchloromethane)

Carbon tetrachloride (CCl_4) is a well-known hepatotoxic compound (Kefalas and Stacey, 1991). Its use has been declining as it has been replaced by less toxic chlorinated solvents such as TRI, perchloroethylene (tetrachloroethylene) and methylchloroform (1,1,1-trichloroethane) (see **Table 1**.). However, it is still used, mainly in the synthesis of fluorocarbon refrigerants, solvents and aerosol propellants.

Carbon tetrachloride is well absorbed by the lungs and gastrointestinal tract and percutaneous absorption can also occur. CCl_4 is concentrated in fatty tissues, while the main excretion route (50–80%) is as the unchanged compound via the lungs. Dechlorination occurs in the liver microsomal cytochrome P450 system, and the formation of free radicals may cause lipid peroxidation and subsequent hepatocellular damage. A small part of the dose is excreted as carbon dioxide via the lungs or kidney.

Acute exposure to CCl_4 causes CNS depression followed by hepatic and renal dysfunction. The mechanisms of the CNS effects are not clear. However, it has been demonstrated that acute exposure to high doses of CCl_4 leads to the same kind of CNS effects as acute exposure to other organic solvents, for example headache, dizziness, blurred vision and constricted visual fields (Stevens and Forster, 1993).

The cerebellum is particularly sensitive to the actions of CCl_4. Bilateral visual field constriction, optic atrophy and amblyopia (reduced vision) characterize the effects of CCl_4 on the optic system. Alcoholics are especially sensitive to CNS effects. Death results from respiratory depression or from dysrhythmias (Hyatt and Salmons, 1952).

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The liver toxicity is based on the formation of free radicals from the biotransformation reactions. The dechlorination of CCl_4 produces phosgene and the trichloromethyl free radical, which is subsequently metabolized to chloroform and carbon dioxide, as well as hexachloroethane. Free radicals can attack proteins and destroy membranes by affecting membrane lipids and producing lipid peroxidation (Sáez *et al*., 1987). Hepatotoxicity caused by CCl_4 is characterized by acute fatty degeneration of the liver, leading to hepatic necrosis. There are also theories that toxic liver cell death might be associated with alterations of Ca^{2+} homeostasis due to CCl_4 (Rechnagel, 1983). CCl_4 inhibits the ability of microsomes to sequester Ca^{2+}, but does not prevent the influx of extracellular Ca^{2+}. The release of Ca^{2+} into the cytosol will result in a number of regulatory alterations (Rechnagel, 1983), which can lead to triglyceride accumulation in the cell. Calcium-channel blockers have been shown to protect against CCl_4-induced liver toxicity (Romero *et al*., 1994). CCl_4 has been considered a potential carcinogen. It has produced hepatocellular carcinoma in hamsters and hepatomas in mice and rats. The data on humans are inadequate to link CCl_4 to liver cancer. CCl_4 can also produce renal damage via acute tubular necrosis.

2.2.2 Chloroform (Trichloromethane/Methyl Trichloride)

Chloroform ($CHCl_3$) is now restricted to industrial uses as a solvent or a chemical intermediate. In 1847 it was introduced as a general anaesthetic because it was less volatile and not as flammable as ether. The use as an anaesthetic was banned early in the twentieth century. The industrial use of chloroform is also restricted, because it can cause liver and kidney cancer in experimental animals. Chloroform is a potent CNS depressant that produces a variety of acute symptoms, for example, nausea, headache and coma (Schroeder, 1965).

Chloroform is rapidly absorbed via the lungs and gastrointestinal tract, the peak blood concentration is reached in one hour. A considerable amount (17–67%) is expired by the lungs. In the liver, cytochrome P450 enzymes dechlorinate chloroform by oxidation to trichloromethanol, which spontaneously dehydrochlorinates to phosgene. Phosgene can react with water to form carbon dioxide, or bind covalently to cellular macromolecules. It produces hepatotoxicity characterized by fatty infiltration and necrosis (Larson *et al*., 1993).

Chronic oral exposure to chloroform has caused hepatocellular tumours in mice and kidney tumours in rats (Byron *et al*., 1994). However, Jorgenson and coworkers could not find any tumours in the livers of mice after administration of chloroform in drinking water (Jorgenson *et al*., 1985). The studies of Larson and colleagues have shown that chloroform administered in corn oil by gavage causes hyperplasia in mouse liver and in rat kidney (Larson *et al*., 1993). Chloroform-induced mouse liver cancer may be secondary to events associated with induced cytolethality and cell proliferation (Butterworth *et al*., 1998). Chloroform is an example of a nongenotoxic cytotoxic carcinogen. Chloroform-induced cytotoxic effects (necrosis, inflammation and regenerative cell proliferation) can start the carcinogenic process and enhance endogenous and exogenous mutagenic activity (Butterworth *et al*., 1998). The possible carcinogenicity of chloroform has been of particular concern as drinking-water purification using chlorination results in the formation of trace amounts of disinfection by-products such as chloroform. Chloroform is often the most prevalent by-product in the chlorination process.

Chloroform may cause renal toxicity 24–48 hours after exposure characterized by proteinuria. Like most chlorinated hydrocarbons, chloroform sensitizes the myocardium to endogenous catecholamines.

2.2.3 Trichloroethylene

TRI ($CHCl{=}CCl_2$) has been widely used in metal degreasing, dry cleaning and chemical synthesis and as a component in household cleaning agents. It was also used as a nonflammable narcotic during the early 1900s. It occasionally produces euphoria, and consequently it has been abused for that purpose. In cases of sniffing, TRI has caused fatal accidents, perhaps through the induction of ventricular fibrillation.

TRI is readily absorbed into the body through the lung and gastrointestinal mucosa. Elimination involves two major processes: pulmonary excretion of unchanged TRI and relatively rapid hepatic biotransformation to urinary metabolites. The major metabolites of TRI are trichloroethanol, trichloroethanol-glucuronide and trichloroacetic acid. Reactive intermediates can be produced in the biotransformation process: TRI-epoxide, dichloroacetic acid, dichlorovinyl-cysteine, dichloroacetyl chloride and chloroform. The liver toxicity, as studied in animal models, is caused by the metabolism of TRI to trichloroacetic acid and dichloroacetic acid, although a possible role of TRI-epoxide cannot be ruled out.

The acute toxic effects of TRI are CNS depression, visual disturbances, mental confusion, fatigue and nausea. In experimental studies in developing rats, effects on myelin formation in the hippocampus were found (Isaacson and Taylor, 1989). TRI can cause changes in the proportion of the long-chain polyunsaturated fatty acids in rat brain after subchronic and chronic exposure (Kyrklund *et al*., 1986). The changes in membrane fatty acid composition might alter brain function. A reduction in myelin could, in part, be responsible for the behavioural effects observed in TRI exposure (Isaacson and Taylor, 1989). TRI can also cause arrhythmia through sensitizing the heart to catecholamines and also pulmonary oedema if the exposure is severe. Compared

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with carbon tetrachloride and chloroform, TRI is a weak hepatotoxin. Hepatocellular carcinoma has been found in mice after high doses of TRI (Kimbrough *et al*., 1985). Epidemiological studies have presented conflicting evidence for the carcinogenicity of TRI in humans (Kaneko *et al*., 1997). A cohort study of 2050 male and 1924 female workers indicated excess of cancers in the CNS and increased risk of multiple myeloma among TRI-exposed workers. However, the overall cancer incidence within the cohort was similar to that of the Finnish population as a whole (Anttila *et al*., 1995). A review from 2000 found the strongest evidence for a causal relationship with cancer of the kidney, liver and non-Hodgkin's lymphoma (Wartenberg *et al*., 2000). The IARC has characterized TRI as a probable human carcinogen causing non-Hodgkin lymphoma and cancer of renal cells, and of the liver and bile ducts (IARC, 1995).

2.2.4 Tetrachloroethylene (Perchloroethylene)

Tetrachloroethylene ($CCl_2{=}CCl_2$) is used particularly in dry cleaning and degreasing. The toxicity of tetrachloroethylene resembles that of TRI. Tetrachloroethylene has caused loss of myelin-enriched lipids, which might indicate a persisting loss of myelin membranes (Kyrklund *et al*., 1990). According to studies with experimental animals, tetrachloroethylene may cause liver cancer (Kyrklund *et al*., 1990). A cohort study of dry-cleaning workers exposed mainly to tetrachloroethylene has shown an increased risk of oesophageal, intestinal, pancreatic and bladder cancers (Ruder *et al*., 1994). IARC has characterized tetrachloroethylene as a probable human carcinogen causing non-Hodgkin's lymphoma.

2.2.5 1,1,1-Trichloroethane (Methylchloroform)

1,1,1-Trichloroethane (CH_3CCl_3) has many industrial and household applications. The relatively low acute toxicity of trichloroethane is mainly a consequence of the low blood–gas partition coefficient compared to other chlorinated solvents. Due to the low blood solubility, the uptake of trichloroethane is low. The principal effect is CNS depression, and high doses can sensitize the heart to the effects of catecholamines and thus cause arrhythmia. In high concentrations, trichloroethane can cause changes in the fatty acid pattern in rat brain (Kyrklund and Haglid, 1991).

2.3 Alicyclic Hydrocarbons

These colourless liquid solvents are saturated or unsaturated hydrocarbons in which three or more carbon atoms join to form rings. They include cycloalkanes, cycloparaffins and naphthenes. They are present in petroleum solvents and are used in the manufacture of organic chemicals. Alicyclic hydrocarbons have similar toxic effects to aliphatic hydrocarbons, but have a greater depressive or anaesthetic effect on the CNS. Alicyclic solvents with small molecular weight, such as cyclopropane, have been used as anaesthetics. The higher-molecular-weight compounds, for example, cyclohexane, cannot be used as narcotics because the safety margin between the narcotic dose and the lethal dose is narrow. Cyclohexane does not change the neural membrane fluidity to the same extent as aromatic hydrocarbons (Engelke *et al*., 1996).

2.4 Aromatic Hydrocarbons

2.4.1 Benzene

Benzene has mainly been used as a raw material in chemical processes and as a solvent in rubbers and glues. It is also used in the synthesis of ethylbenzene, styrene, cumene, phenolic resins, ketones and various dyes. However, in most industrial products, benzene has been substituted by other organic solvents because of its myelotoxicity and since it can cause aplastic anaemia and leukaemia (Vigliani and Forni, 1976; Infante *et al*., 1977). Benzene is a natural constituent of gasoline and can be added as an anti-knocking agent at concentrations of up to 5% and thus is a constituent of gasoline fumes and automobile exhaust. Tobacco smoke also contains benzene. Benzene is one of the VOCs in the city air, and benzene concentrations are monitored in many cities. In industrialized countries workplace exposures to benzene are quite limited, but in the developing world exposure is widespread, especially in artisan work, shoe manufacture and small chemical enterprises. Some of these workplaces may include children in the labour force (Hricko, 1994; Tompa *et al*., 1994; Vermeulen *et al*., 2004).

Benzene is metabolized in the liver to phenols by the cytochrome P450 mixed-function oxidase system (**Figure 3**). Benzene epoxide is the most toxic metabolite of benzene and may be responsible for benzene-induced haematological abnormalities. The conjugation products with glutathione form phenyl mercapturic acid, which is excreted in the urine (Schlosser *et al*., 1998).

Acute exposure to benzene results in CNS depression characterized by euphoria, headache, nausea and ataxia. These symptoms may progress to a change in gait, convulsions and coma. Acute exposure to benzene may

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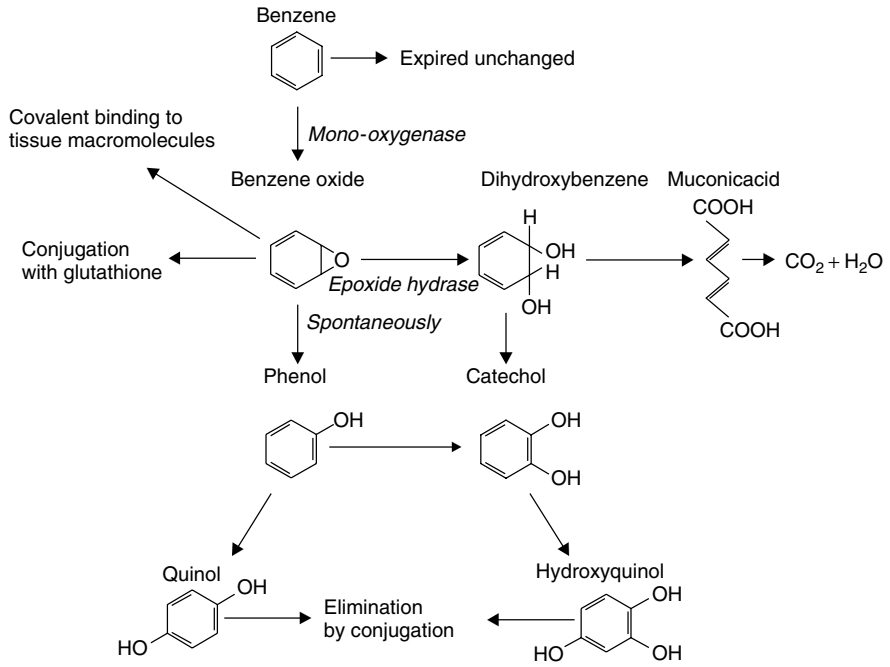

Figure 3 The biotransformation of benzene. The intermediate metabolite benzene oxide (epoxide) is highly reactive and glutathione conjugation removes this metabolite via formation of phenylmercapturic acid.

cause arrhythmia due to myocardial sensitization to endogenous catecholamines (Snyder and Kocsis, 1975).

Chronic occupational exposure of humans to benzene can lead to bone-marrow damage, which may be manifested as anaemia, leukopenia and/or thrombocytopenia. Benzene has been found to induce leukaemia in humans (Snyder and Kalf, 1994). The fact that benzene is carcinogenic is supported by the chromosomal aberrations found in the peripheral blood lymphocytes of exposed workers (Tompa *et al*., 1994). Benzene does not induce leukaemia in rats and mice, although benzene oxide has been shown to induce oxidative microsomal metabolism of benzene in mice and rats, as well as humans (Schlosser *et al*., 1998). Benzene oxide may be an important link between benzene and leukaemia (Snyder *et al*., 1978), but even though it has been shown that metabolism plays a role in benzene toxicity, and that benzene can interfere with DNA, the exact mechanism of benzene-induced leukaemia is not known (Snyder, 2004).

2.4.2 Toluene

Toluene (methylbenzene) is used as a solvent for paints, lacquers, thinners, coatings, glues and cleaning agents. Toluene is also used in the production of other chemicals and in pharmaceutical industries. The important exposure route is by inhalation, but toluene is also absorbed through the skin.

The biological half-life of toluene ranges from several hours for white adipose tissue to a few minutes in highly perfused organs (Pyykkö *et al*., 1977). After inhalation exposure of rats to toluene, the distribution to various tissues is rapid. Toluene accumulates in white adipose tissue, from which the elimination is slow, compared to other tissues (Pyykkö *et al*., 1977). Approximately 18% of the absorbed toluene is expired unchanged via the lungs, and less than 0.1% is eliminated unchanged in the urine. Toluene is metabolized via cytochrome P450 to benzyl alcohol, which is further oxidized via ADH and aldehyde dehydrogenase to benzaldehyde and benzoic acid, respectively (Pyykkö, 1983). Benzoic acid conjugates with glycine to form hippuric acid, which is excreted in the urine (**Figure 4**). Some other solvents (e.g. *n*-hexane, benzene, styrene, xylene and TRI) can inhibit toluene biotransformation (Sato and Nakajima, 1979; Tähti, 1984; Liira *et al*., 1988). Competitive metabolic inhibition is the plausible mechanism of this toxicokinetic interaction between the individual solvents. Toluene causes induction of microsomal liver enzymes at relatively low concentrations (Pyykkö, 1983). Toluene has neither carcinogenic nor mutagenic potency. Kidney damage has been reported in sniffers of glues that may have contained toluene and other solvents.

The CNS is sensitive to the effects of toluene. Acute exposure to toluene causes CNS depression, including the following symptoms: drowsiness, tiredness, headache, dizziness and nausea. The anaesthetic effect of toluene, as with other CNS-depressing solvents, is thought to be based on the ability of toluene to disturb the lipid–protein interaction in neural membranes (Engelke *et al*., 1992; 1996). Due to their lipid solubility, solvents are readily incorporated into neural membranes (**Figure 5**). Solvent molecules can bind to the hydrophobic parts of membrane

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integral proteins, and disturb the ion balance in the membrane (Naskali *et al*., 1993). Exposure for a short time to very high concentrations of toluene (10 000–30 000 ppm) produces unconsciousness and anaesthesia. Long-term inhalation of toluene by abusers is known to cause a multifocal neurological disorder called toluene leukoencephalopathy which is characterized by ataxia, tremors and emotional lability as well as cerebral, cerebellar and brain stem atrophy (King *et al*., 1985; Ikeda and Tsukagoshi, 1990; Filley *et al*., 2004). The acute effect of toluene has been described in controlled conditions in exposure chambers at dose levels which are consistent with those seen in the work places. According to such studies, toluene can cause impairment of visual vigilance (Dick *et al*., 1984) and vestibular function (Hydén *et al*., 1983).

Experimental studies have given some evidence that toluene can cause irreversible effects on noradrenaline, dopamine and 5-hydroxytryptamine mechanisms in various brain regions in rats (Ladefoged *et al*., 1991) and in peripheral sympathetic ganglia (Alho *et al*., 1986). Several case histories describe a consistent pattern of neurological damage in individuals repeatedly inhaling toluene at high concentrations because of its euphoric properties (Fornazzari *et al*., 1983; Spencer and Schaumburg, 1985; Rosenberg *et al*., 1988). There is evidence for an increased risk of neuropsychiatric disorders and behavioural disturbances after chronic toluene intoxications (Kishi *et al*., 1995).

2.4.3 Xylene

There are three isomers (*ortho*-, *meta*- and *para*-) of xylene (dimethylbenzene) in the commercial product, *meta*- being the major isomer. Xylene is one of the most common solvents in paints and varnishes, and it commonly also occurs in glues and printing inks. Furthermore, xylene is used as a solvent in the rubber and leather industries and in pathology laboratories. Xylene is also used in the chemical manufacture of synthetic fibres, plastics, and enamel, as well as the formulation of

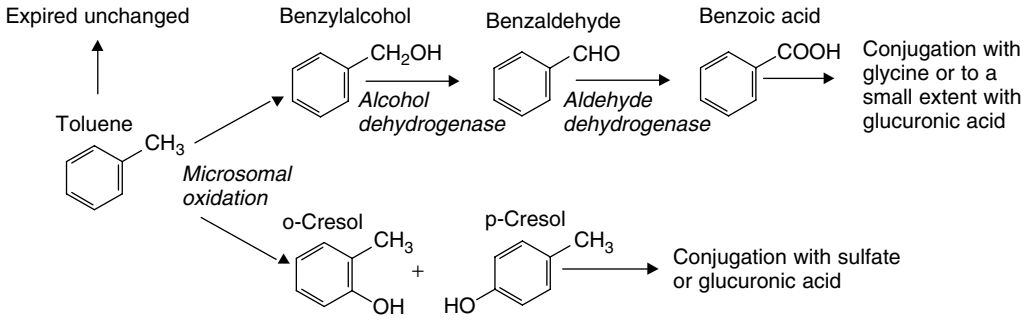

Figure 4 The biotransformation of toluene. The main metabolite in the urine is hippuric acid.

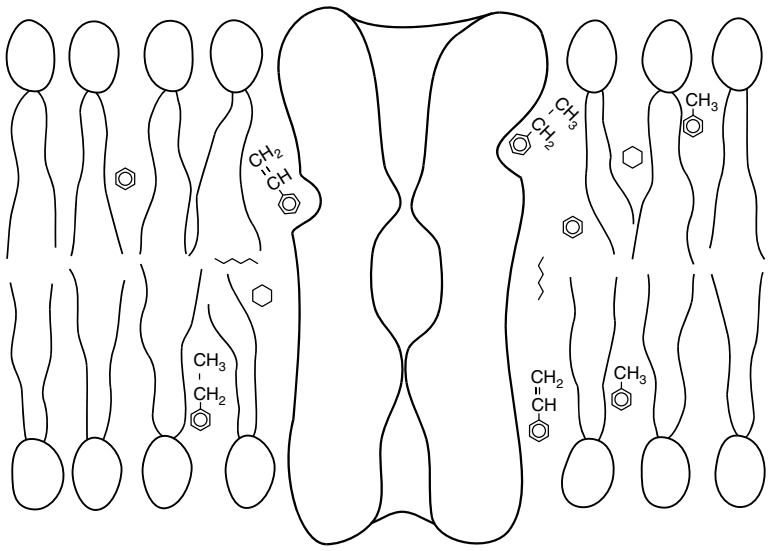

Figure 5 A schematic representation of the incorporation and location of solvent molecules in the neural membrane. Solvent molecules are drawn approximately at the same scale as the membrane molecules. Solvent molecules can bind in the hydrophobic pockets in the integral proteins (enzymes, e.g. Na^+, K^+-ATPase) and disturb the ion balance across the membrane.

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insecticides. Xylenes are often used to replace benzene as a solvent.

Xylene is rapidly absorbed through the lungs and approximately 65% of the inhaled dose is retained by the lungs. Xylene is also absorbed through the gastrointestinal tract and the skin. Only 3–6% of the absorbed xylene is exhaled via the lungs. Most of the remaining xylene is oxidized to methylbenzoic acid, which is conjugated with glycine to form methylhippuric acid. Only 2% of the absorbed xylene is excreted in urine as xylenols (Riihimäki *et al*., 1979).

The acute toxicity of xylene is greater than that of toluene, although the symptoms are quite similar. The major effect of xylene is CNS depression, resulting, at low doses, in lightheadedness, nausea, headache and ataxia. High dose exposure to xylene produces confusion, respiratory depression and coma. Xylene causes conjunctivitis, nasal irritation, sore throat and respiratory irritation. Xylenes have been shown to induce microsomal enzymes in the liver, although their hepatotoxicity is considered to be low. Concentrations as high as 10 000 ppm produce a reversible and minor increase in hepatic aminotransferase activity, and reversible renal failure. Like other aromatic solvents, xylenes may also cause skin and eye irritation.

2.4.4 Styrene (Vinyl Benzene)

More than 50% of the world's production of benzene is used in the manufacture of styrene. Styrene materials account for 20% of all plastic production, for example, polystyrene, resins and polyesters.

Styrene is readily absorbed via the lungs and gastrointestinal tract and also to some extent via the skin. Styrene is metabolized to mandelic acid (70%) and phenylglyoxylic acid (30%), both of which are excreted in the urine. Styrene is also metabolized to styrene oxide, which is more toxic than the parent compound (Mendrala *et al*., 1993). Hippuric acid is also excreted in the urine following exposure to styrene and this metabolite has been used for biological monitoring of occupational exposure.

The major effect of styrene is on the CNS (Pahwa and Kaira, 1993; Naskali *et al*., 1994; Pierce *et al*., 1998). In addition to CNS depression, styrene causes mucosal irritation. Symptoms and clinical signs include those of eye irritation, as well as nasal and respiratory tract irritation.

Styrene is an indirect genotoxic agent, which requires metabolic activation to an epoxide in order to bind covalently to DNA. Styrene 7,8-oxide, the active metabolite of styrene, is a carcinogen in rodents and has been shown to be genotoxic in most *in vitro* test systems. Styrene has been found to be weakly genotoxic in the sister chromatide exchange assay, especially in mice. Cytogenetic damage has been reported in many studies of workers, mainly from the reinforced plastics industry, where ambient concentrations of styrene have been high (Norppa and Sorsa, 1993). Styrene has been characterized as possibly carcinogenic to humans (IARC, 2002)

2.5 Alcohols

2.5.1 Methanol

Methanol (CH_3OH) is used as a solvent, an antifreeze agent and an ingredient of car-windscreen washing fluid. Methanol is also used in the production of stains, enamels and photographic films.

Plans to use methanol as a fuel or as a component in gasoline may widen the probability of exposures to methanol. Inhalation and skin absorption are the most important entry routes of methanol in occupational exposure. Ingested methanol is absorbed from the gastrointestinal tract almost completely (Liesivuori and Savolainen, 1991). The first step in the metabolic pathway of methanol is oxidation to formaldehyde. In humans, ADH is the enzyme that catalyses oxidation. The resultant formaldehyde is spontaneously hydrated to methanediol, which is further metabolized by ADH to formic acid (Liesivuori and Savolainen, 1991). Urinary formic acid can be used for biological monitoring of methanol exposure in the working place (Liesivuori and Savolainen, 1987). The symptoms caused by methanol exposure are dizziness, nausea and vomiting, various types of visual disturbances, headache and metabolic acidosis. At high doses, methanol can cause permanent blindness resulting from retinal damage (Røe, 1946; Liesivuori and Savolainen, 1991; Eells *et al*., 1996). Methanol poisoning is characterized by severe metabolic acidosis which can inhibit cellular respiration and hasten cellular failure. Progressive acidosis will also induce circulatory and respiratory failure. This leads to tissue hypoxia and lactic acid production, both of which further increase the acid load (Jacobsen and McMartin, 1986). The toxic effects of formic acid are due to inhibition of the cytochrome oxidase complex at the terminal end of the respiratory chain in the mitochondria, leading to tissue hypoxia. In addition to the optic nerve and retina, other organs (e.g. brain, heart, kidney) with a high rate of oxygen consumption are possible targets. Oral ingestion of methanol as a single moderate to large dose ($0.4–1\ g\ kg^{-1}$) may cause blindness. Other common clinical manifestations of methanol poisoning in humans include CNS depression, weakness, headache and vomiting (McMartin *et al*., 1980). The symptoms and signs of methanol poisoning may be delayed for up to 24 hours due to the time taken for methanol oxidation to formic acid. The elimination of formic acid from the body is slow. In human methanol poisonings, the half-life of formic acid is about 20 hours (Shahangian

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et al., 1984; Shahangian and Ash, 1986). The treatment of methanol poisoning includes administration of ethanol (Røe, 1946) and folic acid. The ethanol will compete for ADH and thus reduce formate production, while the folic acid will enhance formate oxidation to carbon dioxide (Becker, 1983). Dialysis and alkalinization of urine have been used to increase elimination of formic acid (Liesivuori and Savolainen, 1991). In recent experiments with methanol-intoxicated rats, a significant recovery of rod- and cone-mediated function after exposure to light in the red and near-infrared range has been documented. Such exposure also protects the retina from histopathological changes induced by methanol-derived formate (Eells *et al*., 2003)

2.5.2 Ethanol

Ethanol (CH_3CH_2OH) is extensively used in the chemical industry and in the production of cosmetics and drugs. Exposure to low doses of ethanol by inhalation is not uncommon in industry. Chronic alcohol consumption causes significant CNS toxicity characterized by cerebellar atrophy and dilation of cerebral ventricles. Harper and Kril (1990) have proposed a model for alcohol-induced brain damage. The model includes loss of dendritic arbour and shrinkage of the neuronal body. These changes are potentially reversible and occur in many brain regions. The second phase is neuronal death, which is irreversible and has been demonstrated primarily in the frontal cortex.

A serious consequence of ethanol consumption is its multiple effects on the developing embryo and foetus (Pratt, 1982). Foetal alcohol syndrome occurs in the children of approximately 10% of the women drinking heavily during pregnancy (Sokol *et al*., 1986). Recent studies have indicated that a possible explanation for the toxic effects is the enhanced production of acetaldehyde in the placenta and foetus due to induction of CYP 2E1 (Boutelet-Bochan *et al*., 1997; Carpenter *et al*., 1996; 1997; Rasheed *et al*., 1997). One mechanism for the interindividual variations is that induction varies among heavy drinkers, and may be genetically controlled (Rasheed *et al*., 1997).

Ethanol is oxidized to acetaldehyde and on to acetate, mainly through the action of ADH and aldehyde dehydrogenase (ALDH), primarily in the liver, but ADH is found also in the stomach, lungs and eye. Minor amounts are metabolized by catalase and CYP 2E1. Due to its high affinity to ADH, ethanol is effective in the treatment of methanol intoxications (Røe, 1946). It can also be used in the treatment of ethylene glycol and diethylene glycol poisoning. CYP 2E1 can be induced by ethanol consumption, leading to increased toxic effects of other solvents and some drugs that are metabolized by this enzyme.

On average, an adult will metabolize 16 mg $100\,ml^{-1}\,hour^{-1}$, but there are important genetic polymorphisms concerning the ethanol-metabolizing enzymes. Some Asiatic populations have a high frequency of a rapidly acting form of ADH and of a slow acting form of ALDH. After ethanol consumption, such individuals may experience acetaldehyde intoxication with effects such as nausea, flushing and hyperventilation. Disulfiram, which is an ALDH inhibitor used in the treatment of chronic alcoholism, will produce a similar syndrome. Women metabolize ethanol less effectively than men. This may, in part, be due to a less pronounced activity of ADH in the stomach mucosa of females and to a smaller distribution volume, thus increasing blood concentrations of ethanol, making women more prone to negative health effects.

2.5.3 Isopropanol

Isopropanol (isopropyl alcohol, $CH_3CH(OH)CH_3$) is used in industry as a solvent and disinfectant. Isopropanol is metabolized via ADH to acetone, which is eliminated through the kidney and the lung. Acetone is further oxidized to acetic acid, formic acid and CO_2 (Daniel *et al*., 1981).

Exposure to high concentrations of isopropanol causes CNS and cardiovascular depression. Lower concentrations can cause mild irritation of eyes. Isopropanol exerts its primary effect on the CNS in both humans and laboratory animals, showing a more potent narcotic effect than ethanol (Daniel *et al*., 1981). It is the second most ingested alcohol after ethanol (Litovitz *et al*., 1985) and has caused poisonings in connection with heavy drinking (Rich *et al*., 1990)

2.6 Glycol Ethers

The acute toxicity of the glycol ethers is low. Until the mid-1980s, the focus regarding these solvents was on the toxicity towards the CNS and haematopoietic systems, blood, liver, and kidneys. In later years, attention has also been focussed on the effects on reproduction and foetal development. Several animal studies have shown that 2-methoxyethanol (2-ME) and 2-ethoxyethanol (2-EE) have teratogenic and spermatotoxic effects (Lamb *et al*., 1985; Nagano *et al*., 1981). Workers exposed to 2-ME and 2-EE were found to have lower sperm counts compared to nonexposed workers (Ratcliffe *et al*., 1989; Welch *et al*., 1988). A case–control study among patients at a clinic for reproductive disorders showed an association between a diagnosis of impaired fertility and exposure to glycol ethers (Veulemans *et al*., 1993). Several studies of female workers have shown an increased risk of spontaneous abortions, subfertility and congenital malformations due to glycol ether exposure (Beaumont *et al*., 1995; Cordier *et al*., 1997; Correa *et al*., 1996; Swan *et al*., 1995).

The metabolism of glycol ethers is a key factor in understanding the biological interactions and

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structure–activity relationships of these compounds. The adverse effects have been attributed to the corresponding alkoxyacetic acid metabolites of the glycol ethers, methoxyacetic acid (MAA) and ethoxyacetic acid, respectively (Miller *et al*., 1982; Foster *et al*., 1983; Brown *et al*., 1984; Moss *et al*., 1985). These acids arise after oxidation with ADH and ADHL. A competing metabolic pathway is *O*-dealkylation to ethylene glycol and alkyl aldehyde.

Longer-alkyl-chain glycol ethers are more haematotoxic than those having a shorter chain, while shorter-alkyl-chain glycol ethers are more embryotoxic than those having a longer chain (Nelson *et al*., 1984). Alkoxyacetic acids added to postimplantation rat embryo cultures increase the incidence of abnormalities in a dose- and structure-related manner, MAA being more effective than ethoxyacetic acid (Rawlings *et al*., 1985). Ethoxyacetic acid has been shown to produce less severe testicular toxicity, *in vivo* as well as *in vitro*, in the rat, compared to MAA (Foster *et al*., 1987). On a molar basis, 2-EE produces less severe toxic effects than 2-ME (Wess, 1992).

Little is known about the biochemical mechanisms that induce the embryotoxicity and testicular toxicity of 2-ME and 2-EE, and the possible contribution from the alkoxyacetic acid metabolites. Several studies in rats and mice indicate that MAA undergoes further metabolism, and that these metabolites could be responsible for developmental and testicular toxicity (Welsch *et al*., 1987; Mebus and Welsch, 1989; Mebus *et al*., 1989; 1992; Sumner and Fennell, 1993). Developing embryo and late-stage spermatocytes both undergo rapid nucleic-acid biosynthesis. MAA or a product of its metabolism can influence common biochemical pathways by affecting the availability of one-carbon units, thus causing depression of purine and pyrimidine biosynthesis (Mebus and Welsch, 1989; Mebus *et al*., 1992; Sumner *et al*., 1992). Alterations in the availability of these precursors might be expected to affect DNA and/or RNA synthesis and thereby influence normal cellular proliferation and differentiation. Some studies indicate that 2-ME induces spermatocyte apoptosis in rats and guinea pigs (Brinkworth *et al*., 1995; Ku *et al*., 1995; Wine *et al*., 1997). It has been shown that MAA induces cell death of spermatocytes in both rat and human seminiferous tubules in the same dose ranges (Li *et al*., 1996). MAA has been shown to increase progesterone production in rat and human luteinized granulosa cells, and this would probably result in reduced fertility due to a disturbed menstrual cycle (Almekinder *et al*., 1997).

3 FUTURE CHALLENGES

The wide use of organic solvents in industry will in the future also present an important occupational health risk. In particular, prospective epidemiological studies will be needed to study the effects of long-term exposure to low levels of organic solvents. At least one such study has been underway (Williamson, 1996).

A particular problem that has not been well addressed is the question of exposure assessment. Workers are typically exposed to solvent mixtures and the doses may vary considerably during exposure periods. The usual proxy for exposure, namely the number of years employed in the trade, may lead to serious misclassifications.

The classification of effects is also challenging. Experimental techniques have been developed for studies of CNS functions in experimental animal models (Vorhees, 1987; 1991; Stanton and Freeman, 1994). However, extrapolation of behavioural data from animals to humans may be difficult and possibly misleading. An overview of available test batteries has been presented by Anger who offers a summary of their features, benefits and limitations (Anger, 2003).

Genetic polymorphism may be of importance. It has been shown that polymorphisms of glutathione-S-transferase may influence the risk of solvent-induced toxic encephalopathy (Söderkvist *et al*., 1996; Kezic *et al*., 2006).

Epidemiological studies have shown that long-term exposures to specific solvents, for example, benzene, TRI, tetrachloroethylene and carbon tetrachloride, may increase the cancer risks. Occupational exposure to mixtures of solvents in paints has consistently been associated with a 40% increased risk of lung cancer (Lynge *et al*., 1997). However, addressing these questions is becoming ever more demanding, since most long-term exposures are to mixtures rather than to single chemicals at any one time.

Early health effects of organic solvents may be difficult to detect and in many cases it may be hard to know even what kind of effect to look for.

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Toxicology and Biological Monitoring of Metals

Monica Nordberg and Gunnar F. Nordberg

CONTENTS

1 INTRODUCTION

In total, 111 elements have been identified, 90 occur naturally on earth and 67 are metals. All metals but one of these, that is Hg, are solid. The chemistry of metals represents a major part of inorganic chemistry. Understanding of the toxicology of metal species has advanced substantially during recent decades, thanks to the considerable contributions of bioinorganic chemistry, that is the discovery that some metal species undergo biomethylation and thus can form organometallic compounds. This discipline goes hand in hand with chemical speciation, which is instrumental in understanding metal toxicology and related adverse health effects.

It was pointed out by Duffus (2002) that the oldest scientific use of the term 'heavy metals' to be found in the English literature is cited in Bjerrum's *Inorganic Chemistry* published in 1936. The Bjerrum definition is based upon density of the elemental form of the metal, and he classifies heavy metals as those metals with elemental densities above $7\,g\,cm^{-3}$. Over the years, this definition has been modified by various authors, and there is no consistency—the term is not recommended (Cornelis and Nordberg, 2007). The term 'heavy metals' has sometimes been used to describe metals or metalloids that can give rise to toxicity. Such use should be discouraged. The Scientific Committee on Toxicology of Metals (SCTM) under the International Commission on Occupational Health (ICOH) had already concluded in the 1970s that the term should not be used (Task Group on Metal Accumulation, 1973; Nordberg, 1976).

2 DEFINITION OF METALS AND SOME GENERAL ASPECTS OF METAL TOXICOLOGY

2.1 Definition of Metals and Metalloids

Metals are usually defined on the basis of their physical properties in the solid state. The physical properties of great technological significance are those such as high reflectivity which is responsible for the characteristic metallic luster, high electrical conductivity, decreasing with increasing temperature, high thermal conductivity and mechanical properties, such as strength and ductility.

Metals in the solid state are also characterized by their crystal structure, by the specific chemical bond in which electrons are delocalized and mobile, and by their magnetic properties. These physical properties have only limited value for understanding the systemic toxic effects, although some may be important in understanding the local effects of metal aerosols.

A more useful definition of metals, in order to make it possible to explain the toxic effects, is based on their properties in aqueous solutions. This definition is: 'a metal is an element which, under biological conditions, may react by losing one or more electrons to form a cation'. In the following text the discussion will be based on the behaviour of metals/metal ions in solution and, where applicable, in biological media.

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The distinction in metal toxicology between metals and nonmetals, whether based on their physical or on their chemical properties is not sharp. In metal toxicology, some metalloids are included, since they produce adverse health effects in humans, either by themselves or by interaction. They exhibit certain properties that are typical of metals, while other properties make them similar to nonmetals. In general, in some groups of the periodic system, there occurs a gradual transition of properties from nonmetals to metals when descending from the lighter to the heavier elements, for example, C, Si, Ge, Sn, Pb in Group 14. Borderline elements such as As, Ge, Sb, Se and Te are sometimes called metalloids. Metals are persistent and naturally occurring in the environment and show reactivity. Some have long biological half-lives.

2.2 General Aspects of Metal Toxicology and Biological Monitoring

Metals may bioaccumulate and some are toxic at low-level exposure. Even essential elements may cause toxicity at high exposures.

A number of factors influence the toxicity of metals. Such toxicity depends on general factors, such as dose, exposure conditions, bioavailability and species. Essential metals important for life might cause adverse health effects if exposure is excessive or if the chemical species or exposure route is not the physiological one. The toxicology of metals vary with nutritional factors like iron status, intake of protein, calcium and zinc. Elemental speciation is of fundamental importance in human-health risk assessment (WHO/IPCS, 2006a) where 'chemical species' is defined as a 'specific form of an element defined as to isotopic composition, electronic or oxidation state, and/or complex or molecular structure'. In metal toxicology the elements are often classified as essential or toxic metals, and both categories include metals and metalloids. Metalloids are sometimes referred to as semimetals (WHO/IPCS, 2006a).

Most metals display systemic toxicity, which means that the toxic effect is on an organ other than where the metal is taken up. After absorption, transport, metabolism and kinetics, the metal compound under consideration gives rise to an adverse effect in the critical organ, as defined for each metal compound and exposure condition. Methylation of metals generally increases their toxicity by rendering them more lipid soluble and facilitating their crossing lipid barriers, such as the cell membrane or a blood–tissue (e.g. blood–brain) barrier. However, the opposite is seen for the metalloids arsenic and selenium, where methylation can serve as a means of detoxification (WHO/IPCS, 2006a). Some metals may give rise to cancer. The International Agency for Research on Cancer (IARC) is a part of the World Health Organization (WHO) and is located in Lyon, France. This organization has classified a number of metals and their compounds with respect to their carcinogenicity (IARC, 2008). Either a chemical compound or a specified exposure circumstance is classified by its carcinogenicity. IARC have classified beryllium, cadmium, chromium [VI] and nickel compounds, for example, as carcinogenic to humans (Group 1) and a number of other metals and their compounds as probable or possible carcinogens to humans (Group 2A and 2B) (**Table 1**).

Metals can be organized into toxic metals, essential metals and nonessential metals of major concern in metal toxicology. Metals can be listed by increasing number in the periodic system (**Table 2**). However, in this chapter the metals are listed in alphabetical order.

Table 1 Cancer Classification by International Agency for Research on Cancer (IARC, 2008)

Group 1 Carcinogenic to humans	Group 2A Probably carcinogenic to humans	Group 2B Possibly carcinogenic to humans	Group 3 Not classifiable as to carcinogenicity to humans
Beryllium	Cisplatin[a]	Cobalt and cobalt compounds[d]	Mercury and inorganic mercury compounds
Cadmium	Indium phosphide[b]	Cobalt sulfate and other soluble cobalt(II) salts	Selenium and Selenium compounds
Chromium [VI]	Lead compounds, inorganic	Titanium oxide	—
Nickel compounds	Exposure circumstances: cobalt metal with tungsten carbide[c]	Nickel metal and alloys	—
		Methyl mercury	

[a]Overall evaluation upgraded from 2B to 2A with supporting evidence from other relevant data
[b]Overall evaluation upgraded from 2B to 2A
[c]for exposure circumstances cobalt metal with tungsten carbide
[d]Evaluated as a group

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Table 2 Some elements with more than one biologically relevant valence (in order of atomic number)

Atomic number	Name[a]	Symbol	Speciation
23	Vanadium*	V	IV/V
24	Chromium*	Cr	III/VI
25	Manganese*	Mn	II/III/IV
26	Iron*	Fe	0/II/III
27	Cobalt*	Co	II/III
28	Nickel*	Ni	II/IV
29	Copper*	Cu	0/I/II
30	Zinc*	Zn	0/II
33	Arsenic*	As	III/V
34	Selenium*	Se	II/IV/VI
42	Molybdenum*	Mo	II/III/IV/VI
46	Palladium	Pd	II/IV
47	Silver*	Ag	0/I/II
50	Tin*	Sn	II/IV
51	Antimony*	Sb	III/V
52	Tellurium*	Te	0/II/IV/VI
78	Platinum	Pt	II/IV
80	Mercury*	Hg	0/I/II
81	Thallium*	Tl	I/III
82	Lead	Pb	II/IV
92	Uranium*	U	III/VI
94	Plutonium*	Pu	III/IV/V/VI

[a]Elements marked with an asterisk are taken from Yokel *et al*. (2006). From WHO/IPCS (2006a).

Biological monitoring is defined as continuous or repeated measurement of potentially toxic substances or their metabolites or biochemical effects in tissues, secreta, excreta, expired air, or any combination of these in order to evaluate occupational or environmental exposure and health risk, by comparison with appropriate reference values based on knowledge of the probable relationship between ambient exposure and resultant adverse (health) effects. Biological assessment of exposure is used as a synonym (Nordberg *et al*., 2004).

The most commonly used media in biological monitoring for assessing exposure and risk assessment are blood, urine and hair. Which media is sampled depends on the kinetics of the respective metal and which biomarkers are most feasible (Aitio *et al*., 2007).

3 ALUMINIUM (Al)

Alum was used by the Greeks and Romans as an astringent in medicine. The element was named aluminium, derived from the Latin words *alumen* or *alum*. Aluminium is widely used in modern society for aeroplanes, in building materials, electrical equipment and so on.

The toxicology and biological monitoring of aluminium has been reviewed by WHO/IPCS (1997) and more recently by Sjögren *et al*. (2007).

3.1 Uptake, Metabolism and Excretion

Aluminium is only absorbed to a limited extent in the gastrointestinal tract, and in the lungs to a variable extent, depending on solubility of the Al compound inhaled and its particle size. Excretion is mainly by the kidneys, probably as aluminium citrate.

3.2 Toxic Effects and Dose–Response Relationships

Aluminium is a well-known neurotoxicant. Accumulation in the human body has been related to the presence of aluminium in dialysis fluids and the concomitant intake of aluminium-containing drugs. This accumulation has resulted in dialysis encephalopathy that is often fatal. The occurrence of this disease has decreased dramatically due to the control of aluminium levels in dialysis fluid (see Section 3.5). Neurotoxic effects have been observed in welders with aluminium in urine $>100\,\mu g\,l^{-1}$. The upper reference limit among nonexposed individuals is $16\,\mu g\,l^{-1}$. Aluminium has been suggested to be one of several factors that might contribute to Alzheimer's disease, although this has not been satisfactorily demonstrated. Occupational exposure to aluminium powder has resulted in pulmonary fibrosis. Asthma has been associated with the inhalation of aluminium sulfate, aluminium fluoride, potassium aluminium tetrafluoride and the complex environment in pot rooms during aluminium production (Sjögren *et al*., 2007).

3.3 Carcinogenicity and Genotoxicity

Cancer and coronary heart disease have been observed among aluminium production workers. However, it is unlikely that aluminium *per se* is responsible for these diseases. Simultaneous exposure to other compounds, such as polyaromatic hydrocarbons, is considered important. Aluminium production is classified as a human carcinogen (IARC, 1987).

3.4 Biological Monitoring

Levels of aluminium in biological fluids that are related to adverse health outcomes are given in Section 3.2 and **Table 3**.

3.5 Recommendations

Aluminium is used as a coagulant in water treatment. WHO has set a practical level in drinking-water plants using aluminium-based coagulants, of $0.1\,mg\,l^{-1}$ or less

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Table 3 Concentration of aluminium in biological fluids and organs

Exposures based on urine levels in different occupations
High aluminium exposure (urine concentrations often $>100\,\mu g\,l^{-1}$)
Intermediate aluminium exposure (urine concentrations sometimes $>100\,\mu g\,l^{-1}$)
Low aluminium exposure (urine concentrations seldom $>100\,\mu g\,l^{-1}$)

From Sjögren *et al*., 2007.

in large water treatment facilities, and $0.2\,mg\,l^{-1}$ or less in small facilities (WHO, 2004). Similarly, Health Canada established an operational guidance value of $<0.1\,mg\,l^{-1}$ for drinking water from treatment plants using aluminium-based coagulants and $<0.2\,mg\,l^{-1}$ for other types of treatment systems.

The occupational exposure limits for aluminium have decreased during the last decade in many countries. Occupational biological exposure limits have been established in some countries. Finland has a urine biological action level (BAL) of $160\,\mu g\,l^{-1}$ ($6\,\mu mol\,l^{-1}$) (Kallio *et al*., 1999). Germany has a biological tolerance value for occupational exposure of $200\,\mu g\,Al\,l^{-1}$ of postshift urine (Deutsche Forschungsgemeinschaft, 2004). Physicians determine dialysate aluminium levels twice a year. It has been recommended that dialysate aluminium should be $<10\,\mu g\,l^{-1}$ and stated that it must be $<20\,\mu g\,l^{-1}$ (Rob *et al*., 2001).

The amount of aluminium in vaccines in the United States is limited to 0.85 mg per dose if the level is assayed or 1.14 mg if determined by calculation on the basis of the amount of aluminium compound added. The daily intravenous intake of more than $4–5\,\mu g\,Al\,kg^{-1}$ from total parenteral nutrition solutions can produce aluminium accumulation and central nervous system (CNS) and bone toxicity. To avoid this problem, the US FDA (Food and Drug Administration) established a labelling requirement that large- and small-volume additives used in parenteral nutrition must state the maximum aluminium concentration (US FDA 2002; Health Canada, 2009) see also **Toxicities Associated with Parenteral Nutrition**.

4 ANTIMONY (Sb)

The use of Sb as the standard chemical symbol for antimony is due to the abbreviation of the name *stibium*. Antimony is a silvery-white brittle metal of medium hardness and exists in four valence states: 0, −3, +3 and +5. The toxicology and biological monitoring of antimony was recently reviewed by Tylenda and Fowler (2007).

4.1 Uptake, Metabolism and Excretion

Most absorbed antimony is excreted rapidly through urine and faeces. Elimination and route of excretion depend on the type of antimony compound. Urinary excretion is higher for pentavalent than for trivalent antimony compounds, whereas the gastrointestinal excretion is higher for trivalent than for pentavalent antimony. Some data on humans, as well as on animals, indicate that a small part of absorbed and retained antimony may have a long biological half-life of years, especially in the lung (Leffler *et al*., 1984). After acute or chronic oral or parenteral exposure to antimony, the highest concentrations are found in the thyroid, adrenals, liver and kidney.

4.2 Toxic Effects and Dose–Response Relationships

Industrial exposure may give rise to symptoms of irritation in the respiratory tract. Pneumoconiosis, occasionally in combination with obstructive lung changes, has been reported after long-term exposure in humans. Focal fibrosis of the lung has been seen in animal trials. Effects on the heart, even fatal, have been related to long-term industrial exposure to antimony trioxide. Secondary cardiovascular effects as a result of treatment of parasitic disease with antimony compounds have also been reported. In addition, cardiovascular effects have been observed in animal experiments.

4.3 Carcinogenicity and Genotoxicity

Rats exposed to antimony trioxide through inhalation for long periods of time showed a high frequency of lung tumours. IARC (1989) classified antimony trioxide a Group 2B and antimony trisulfide a Group 3 carcinogen.

4.4 Biological Monitoring

Antimony levels in human tissues have been summarized by Tylenda and Fowler (2007). Among members of the general population in various countries, levels are $30–100\,\mu g\,g^{-1}$ in lung tissue and $10–30\,\mu g\,g^{-1}$ in liver, wet weight. Blood and serum concentrations of antimony in normal subjects have been reported to be around 3 and $1\,\mu g\,l^{-1}$, respectively. The 24 hour urinary excretion of 16 subjects ranged from 0.5 to 2.6 µg (Wester, 1973). Smith and Griffiths (1982) found a median antimony concentration of less than $1\,\mu g\,l^{-1}$ for persons not occupationally exposed to antimony and $54\,\mu g\,l^{-1}$ for occupationally exposed workers. Determination of urinary antimony is

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considered useful for biological monitoring of exposure. Significant correlation was found between the concentration of antimony in air, and in the blood and urine of workers in a lead battery factory (Kentner *et al*., 1995). Because quality control programmes were not reported in any of the studies cited, the results on normal levels of antimony in biological materials, especially in urine and blood, must be regarded as tentative only. A study of workers in a textile factory that uses antimony-containing flame retardants compared urinary antimony levels in two groups of workers and controls. The 'high' exposure group had a urinary antimony level of $0.39 \pm 0.26\,\mu g\,l^{-1}$ compared with $0.10 \pm 0.06\,\mu g\,l^{-1}$ in the controls (Iavicoli *et al*., 2002). However, increased urinary antimony levels are seen in cases of exposure to even low levels of antimony in air and may be useful for biological monitoring.

5 ARSENIC (As)

The name arsenic is derived from the Latin word *arsenicum* and the Greek word *arsenikon* (yellow orpiment). Arsenic in the environment occurs in both organic and inorganic compounds in its trivalent or pentavalent state. Certain fish and crustaceans contain very high levels of organic arsenic, often as arsenobetaine. In most other foodstuffs, levels of arsenic are low and the form is not known. The total daily intake of arsenic in the general population is reported to be approximately a few tenths of a milligram, but varies to a great extent, depending on the amount of fish consumed.

The literature on toxicology and biological monitoring has been reviewed by Fowler *et al*. (2007), WHO/IPCS (1981b; 2001).

5.1 Uptake, Metabolism and Excretion

Both organic arsenic in seafood and inorganic arsenic in water, beverages and drugs are readily absorbed (70–90%) by the gastrointestinal tract (Fowler *et al*., 2007; WHO/IPCS, 2001). Some reports also indicate a fairly high degree of absorption after inhalation of arsenic. Absorbed arsenic, irrespective of form, is widely distributed in the body. After exposure to inorganic arsenic, clearance of arsenic from the skin, upper gastrointestinal tract, epididymis, thyroid and skeleton is slower than from other organs. The highest levels of arsenic in humans are normally found in hair, nails and skin. The main route of excretion is through the kidneys. After ingestion of arsenite or arsenate, approximately 35% of the dose is excreted within two days. From animal experiments it seems that insoluble inorganic arsenic inhaled through the airway is deposited and retained in lung tissue for a relatively long time. Animal data indicate accumulation of arsenobetaine in cartilage, testes, epididymis and muscle. Of ingested arsenobetaine, 50–80% is excreted by the urine within two days. Biotransformation of inorganic arsenic has been shown to occur in both animals and humans. Methylated compounds, such as monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA), have been detected in urine after ingestion or inhalation of inorganic arsenic. Reduction of arsenate and oxidation of arsenite *in vivo* have been demonstrated in experimental animals. Recently, a human arsenic methyl transferase has been identified. Medications, contaminated food, beverages, and drinking water have given rise to a number of episodes of arsenic poisoning.

Most arsenic that is absorbed from the lungs or the gastrointestinal tract is excreted in the urine within one to two days. Inorganic arsenic can easily pass through the placenta.

5.2 Toxic Effects and Dose–Response Relationships

Inorganic arsenic-induced skin lesions such, as dermatoses, which may include eruption, pigmentation or leukodermal hyperkeratosis, may ultimately lead to the development of skin cancer and Bowen's disease. Effects on the nervous system (e.g. peripheral nervous disturbance), as well as on the heart and circulatory system (e.g. abnormal electrocardiograms, and peripheral vascular disturbances with gangrene of the extremities), have also been reported after chronic exposure to inorganic arsenic. Haematological changes after inorganic arsenic exposure are characterized by anaemia and leukopenia.

Arsenic poisoning among industrial workers is characterized by perforation of the nasal septum, skin changes and peripheral neuritis. Arsine gas is a powerful haemolytic poison encountered under some industrial conditions. Arsine poisoning is characterized by nausea, vomiting, headache, shortness of breath and haemoglobinuria (WHO/IPCS, 2001).

5.3 Carcinogenicity and Genotoxicity

Arsenic is a human carcinogen (IARC, 1987) Group 1. That evaluation applies to the group of compounds as a whole. IARC (2004) classified arsenic in drinking-water as Group 1. They summarized data on cancer in specific organs, that is, skin, lung, liver, bladder and kidney. There is substantial epidemiological evidence of an excessive risk of lung cancer among workers exposed to arsenic

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and experimental data in animals also gives support to carcinogenicity (Pershagen *et al*., 1984).

5.4 Mechanism of Action

Inorganic arsenic undergoes methylation. Monomethylarsonic acid (MMA) and DMA are thus formed and are excreted in the urine. Methylated forms of arsenic undergo demethylation. Methylation of As has previously been regarded a detoxifying process, but has been shown to induce reactive oxygen species (ROS), playing important roles in cellular toxicity.

5.5 Biological Monitoring

Fowler *et al*. 2007 reviewed available information on As in human organs. The highest levels have usually been found in liver, followed by kidney and the other organs.

It is not known to what extent concentrations in human organs represent inorganic or organic arsenic. Concentrations of 0.014–0.21 mg As kg^{-1} wet weight have been observed in the lungs of smelter workers who had retired 2–19 years before death, whereas unexposed controls had 0.001–0.018 mg kg^{-1}, indicating a long retention time for arsenic in the lungs after inhalation, which may occur in certain occupations (Brune *et al*., 1980).

Arsenic was detected in human breast milk at concentrations of 0.13–0.82 µg l^{-1} wet weight (Somogyi and Beck, 1993). In a population of Andean women exposed to high concentrations (approximately 200 µg l^{-1}) of inorganic arsenic in drinking water, concentrations of arsenic in breast milk ranged from about 0.8–8 µg kg^{-1} wet weight (Concha *et al*., 1998).

5.5.1 Urine

Measurement of urinary arsenic levels is generally accepted as the most reliable indicator of recent arsenic exposure. Normal levels of arsenic in the urine of people with no known high exposure are apparently in the range of 5–50 µg As l^{-1} (Buchet *et al*., 1980). Ingestion of seafood may, however, increase the concentrations to more than 1 mg As l^{-1} (review Fowler *et al*., 2007).

Arsenic concentration in urine may be used as an index of exposure, but a number of factors, such as a diet containing mainly seafood and the time between exposure and urine sampling have to be considered. Because 'fish arsenic' arsenobetaine is essentially nontoxic, total urinary arsenic content may overestimate exposures to arsenic species that are of health concerns. Analytical differentiation of the forms of arsenic in the urine is an appropriate way of determining how much and what form of arsenic has been absorbed (Buchet *et al*., 1980). The sum of urinary excretion of inorganic arsenic and its monomethylated and dimethylated metabolites is a good indicator of exposure to inorganic arsenic when there is some fish consumption. Buchet *et al*. (2003) determined such values by means of hydride generation combined with atomic absorption spectroscopy (AAS) or flame atomic absorption spectroscopy (AAF) and reported a mean value of approximately 7 µg g^{-1} creatinine and a 95% cut off of 18 µg g^{-1} creatinine, in a group from the general population of Belgium. In a population group from an area near the sea in China not known to be exposed to inorganic arsenic, there was a mean of approximately 25 µg g^{-1} creatinine and a 95% cut off of 50 µg g^{-1} creatinine (Buchet *et al*., 2003).

5.5.2 Blood

By use of neutron activation analysis (NAA), Brune *et al*. (1966) found a mean concentration of 0.004 mg kg^{-1} in whole blood of normal people. In studies of As-exposed populations, Concha *et al*. (1998) average blood total As levels of 8–10 µg l^{-1} have been reported. The range of blood As concentrations in an unexposed population was 1–2 µg l^{-1}. By use ofhigh-performance liquid chromatography/inductively coupled mass spectrometry (HPLC-ICP-MS), Mandal *et al*. (2004) were able to separate and quantify the As species in the blood of an As-exposed population, with a total As of 10.1 µg l^{-1}. They found arsenobetaine, DMA, MMA, and inorganic As.

The clearance of arsenic from blood is very rapid. The time lapse between exposure and sampling will, therefore, be of importance if the blood levels are being related to the exposure. Furthermore, intake of seafood may greatly influence the (total) blood arsenic levels. For these reasons, blood arsenic cannot be regarded as a useful indicator of exposure.

5.5.3 Hair

Available information about levels of As in hair, as a biomarker of exposure and the internal dose of As has been reviewed by Fowler *et al*. (2007). In the past, somewhat higher levels in hair were reported, for example, an average of 0.81 mg kg^{-1} in the 1960s. More recently, hair As from an unexposed population in the USA was 0.17 µg g^{-1}, with a range of 0.06–0.33 µg g^{-1} (10–90 percentiles), (Pellizzari and Clayton, 2006).

Speciation of hair by HPLC-ICP-MS showed the presence of As(III), As(V), MMA(V), and DMA(V) in hair, but no arsenobetaine; therefore it does not accumulate in hair. The mean concentration of arsenic in hair has been found to reflect the degree of arsenic pollution in community air in areas polluted by airborne As from coal

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burning (Bencko *et al*., 1971). Among persons with high water exposures to As, Hinwood *et al*. (2003) found a geometric mean of 5.5 $\mu g\,g^{-1}$ As in the hair with a range of 1.2–20 $\mu g\,g^{-1}$.

There are several caveats related to the analysis of hair As that must be taken into consideration when evaluating an individual or population suspected to have chronic or acute As exposure or poisoning. It may not be possible to distinguish between arsenic adsorbed onto hair from external contamination and arsenic incorporated into hair from the internal body burden.

6 BARIUM (Ba)

Barium stems from Greek *barys*, meaning 'heavy'. The toxicology and biological monitoring of barium was reviewed by WHO/IPCS (1990c), Dallas and Williams (2001) and more recently by Oskarsson and Reeves (2007).

6.1 Uptake, Metabolism and Excretion

The free ion is readily absorbed in the lung or gastrointestinal tract, but barium sulfate remains essentially unabsorbed. After absorption, barium accumulates in the skeleton; an accumulation also takes place in the pigmented parts of the eye.

6.2 Toxic Effects and Dose–Response Relationships

The toxicity of barium compounds depends on their solubility. Acute or chronic exposure to barium salts results in a number of disorders, including renal intoxication, hypertension and cardiac malfunction in experimental animals. A higher incidence of hypertension has been observed after occupational barium exposure, but no association was found between blood pressure and barium in drinking water, up to 10 $mg\,l^{-1}$, corresponding to approximately 0.21 mg Ba kg^{-1} day^{-1}. The kidney seems to be the most sensitive target organ in rats and mice exposed repeatedly to barium chloride in drinking water.

Poisoning with soluble barium compounds has resulted from accidental or suicidal ingestion or when barium sulfate, used as an opaque contrast medium for X-ray studies of the gastrointestinal tract, has been contaminated with soluble barium. The Ba^{2+} ion is a muscle poison, causing gastrointestinal, cardiac and skeletomuscular stimulation, followed by paralysis. In the Szechuan province of China, a subacute form of barium poisoning (pa-ping) was endemic because of use of contaminated table salt (Oskarsson and Reeves, 2007).

6.3 Mechanism of Action

Barium seems to act as a potassium antagonist and calcium agonist. The mechanism of action is partly due to blocking of the K^+ channels of the Na–K pump in cell membranes, increasing the active inward transport and inhibiting the passive outward transport of potassium. Cases of barium poisoning are accompanied by severe hypokalemia, and potassium infusion is an effective antidote. If administered promptly, soluble sulfates are also an effective antidote, because they form a precipitate with barium that is not readily absorbed from the gastrointestinal tract or lung. Inhalation of barium sulfate dust causes a benign pneumoconiosis ('baritosis') with conspicuous radiographic manifestations, but no impairment of pulmonary function. The condition has been reproduced in rats.

6.4 Biological Monitoring

Most or all barium in blood is in the plasma fraction. Serum reference values of barium have been reported, varying from 1 (Cornelis *et al*., 1994) to 60 $\mu g\,l^{-1}$ and higher with increasing age, especially in women (Rahil-Khazen *et al*., 2000). A temporary increase occurs after barium absorption, but the excess disappears from the bloodstream practically completely 24 hours after cessation of absorption. Exposure to soluble barium compounds during welding resulted in median plasma levels up to 24.7 $\mu g\,l^{-1}$ and median urinary levels up to 101.7 $\mu g\,l^{-1}$ (Zschiesche *et al*., 1992). Urinary levels of barium were determined by ICP-MS in 1437 samples collected from US patients (Komaromy-Hiller *et al*., 2000). The mean level was 3.5 $\mu g\,l^{-1}$ (standard deviation (SD), 2.2) and the calculated representative ranges 1.0–7.0 $\mu g\,l^{-1}$ (95% confidence interval after exclusion of the top 10% of the results), which was somewhat higher than the reference values, 0.25–5.7 $\mu g\,l^{-1}$ from a control population, as reported by Minoia *et al*. (1990). Rosborg *et al*. (2003) determined 34 elements in hair in females from acid and alkaline regions in southern Sweden. The median hair concentration of barium was 1057 $\mu g\,kg^{-1}$ (range, 10–7150). The concentrations of barium were significantly higher in hair collected in acid compared with alkaline regions ($p < 0.001$). No correlation was found between barium levels in drinking water and in hair.

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7 BERYLLIUM (Be)

The name of the element can be derived from the Greek words *beryllos* and *beryl.* Aquamarine and emerald are precious forms of the mineral beryl. Beryllium is a strategic and critical material for many industries. It is widely used, in spite of its relatively high cost. Three primary forms of beryllium are produced. Copper beryllium alloy is the largest, followed by pure beryllium metal and beryllium oxide ceramics. As a result of the increasing industrial use of beryllium, occupational exposure to the metal may be an important issue. At present, the levels in the occupational environment are below the occupational exposure limits of $2\,\mu g\,m^{-3}$, but this exposure level is not protective of pulmonary disease among sensitive persons in a worker population. The toxicology and biological monitoring of beryllium was reviewed by WHO/IPCS (1990b) and more recently by Jakubowski and Palczynski (2007).

7.1 Uptake, Metabolism and Excretion

Beryllium and its compounds are poorly absorbed in the gastrointestinal tract. In general, inhalation exposure to beryllium compounds results in long-term storage of appreciable amounts of beryllium in lung tissue, particularly in pulmonary lymph nodes, and in the skeleton, which is the ultimate site of beryllium storage.

7.2 Toxic Effects and Dose–Response Relationships

Exposure to beryllium compounds has caused dermatitis, acute pulmonary inflammation and chronic beryllium disease (CBD). Exposure to soluble beryllium salts may cause skin reactions such as oedematous, erythematous and papulovesicular dermatitis. Those changes usually disappear after cessation of exposure. Granulomatous necrotic changes and ulcerations due to skin penetration by insoluble beryllium salts were also observed. These pathological changes are based on delayed allergic hypersensitivity. Acute toxicity of beryllium at concentrations usually above $25\,\mu g\,m^{-3}$ is manifested by skin, eye, nose and throat irritation, followed by upper- and lower-airway inflammation, pulmonary oedema and (above $100\,\mu g\,m^{-3}$) chemical pneumonitis.

CBD (chronic pulmonary granulomatosis, berylliosis) is the most common health problem caused by exposure to beryllium. The beryllium blood lymphocyte proliferation test (BLPT) is used as a medical surveillance tool for assessment of persons at risk for developing clinical and subclinical CBD. The LOAEL (lowest observed adverse-effect level) for beryllium sensitization and CBD progression was suggested to be $0.55\,\mu g$ Be m^{-3}. However, recent reports suggest that sensitization and CBD were associated with beryllium air TWA (time-weighted average) levels exceeding $0.2\,\mu g\,m^{-3}$. These results suggest that to avoid sensitization and CBD, the occupational exposure limits should be lower than $0.2\,\mu g\,m^{-3}$.

7.3 Carcinogenicity and Genotoxicity

An excess of lung cancer occurs in persons occupationally exposed to beryllium and its compounds. IARC (1993) have classified beryllium as a Group 1 carcinogen (sufficient evidence for carcinogenicity in humans). In general, it seems that the lung cancer observations related to occupational exposure to beryllium are linked to higher exposure levels, which were associated with acute beryllium pneumonitis and predominated prior to 1950s.

7.4 Mechanism of Action

CBD is a T-cell-mediated disorder. Beryllium, acting as a hapten, interacts with the antigen-presenting cells in the lungs.

7.5 Biological Monitoring

According to Apostoli and Schaller (2001), the 'normal' urinary Be concentrations reported earlier in the literature were too high, mainly as a result of the poor specificity and sensitivity of the adopted analytical methods. In the control groups investigated by Apostoli and Schaller (2001), all results of determinations were below the detection limits of $0.03\,\mu g\,l^{-1}$.

The data for biological monitoring of exposure to beryllium are not available because of the small number of occupational activities involving exposure to this metal and the lack of appropriate analytical methods in the past.

Apostoli and Schaller (2001) examined 65 metallurgic workers in two electric steel plants and two copper alloy foundries. Beryllium concentrations in urine varied from <0.03–$0.54\,\mu g\,l^{-1}$. A significant correlation was found for the relationship between external and internal exposure. Urinary Be levels were in the range 0.12–$0.15\,\mu g\,l^{-1}$ in workers exposed to Be concentrations in respirable dust; below the recommended threshold limit value (TLV) TWA of $0.2\,\mu g\,m^{-3}$.

8 BISMUTH (Bi)

The name originates from the German *Weisse Masse*, (white mass), later transformed to *Wismuth* and *Bisemutum.* There are no reports on occupational exposures.

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For the general population, the total daily intake via food is about 5–20 μg, with much smaller amounts contributed by air and water. An important source of exposure for specific segments of the population in the past was the therapeutic use of bismuth compounds. The cosmetic use of bismuth compounds still continues to be fairly widespread. The toxicology and biological monitoring of bismuth was recently reviewed by Fowler and Sexton (2007a).

8.1 Uptake, Metabolism and Excretion

Bismuth compounds are considered to be poorly to moderately absorbed following inhalation or ingestion, but there are no quantitative data. Absorbed bismuth is distributed throughout the soft tissues and bone, the highest concentrations being found in the kidney and liver. Absorbed bismuth is excreted primarily via the urine. The biological half-life for whole-body retention is about five days, but intranuclear inclusions containing bismuth seem to remain for years in the kidneys of patients treated with bismuth compounds.

8.2 Toxic Effects and Dose–Response Relationships

High-level exposure causes renal failure associated with degeneration and necrosis of the epithelium of the renal proximal tubules, fatty changes and necrosis of the liver, reversible dysfunction of the nervous system, skin eruptions and pigmentation of the gums and intestine. For information on neurotoxic effects in persons taking Bi medicine see Section 8.3, Biological Monitoring.

Bismuth subnitrate has been used to induce metallothionein (MT) in heart tissue and kidneys, and to attenuate the toxicity of adriamycin and cisplatin compounds, respectively.

8.3 Biological Monitoring

Studies in Belgium (Vanhoe *et al*., 1993) on measured bismuth in human serum by ICP-MS showed a range of $<0.007–0.067\,\mu g\,l^{-1}$ in 19 healthy adults. Slikkerveer and de Wolff (1989) reported that the normal concentration of bismuth in blood is in the range of $1–15\,\mu g\,l^{-1}$. Serum concentrations of bismuth of $242\,\mu g\,l^{-1}$ have been reported in patients with bismuth encephalopathy originally diagnosed with Alzheimer's disease (Summers, 1998). Even higher levels are found in those with renal dysfunction.

9 CADMIUM (Cd)

The Latin word *cadmia* and the Greek word *kadmeia* are ancient names for calamine (zinc carbonate) and the element was discovered from an impurity in zinc carbonate. Cadmium is chemically similar to zinc; it occurs naturally with zinc and lead in sulfide ores. Elevated concentrations in air, water and soil may occur close to industrial emission sources, particularly those of nonferrous mining and metal-refining industries.

Cadmium metal has been used as an anticorrosive, electroplated onto steel, and Cd compounds are used as pigments, often in plastics. Cadmium and its compounds are also used in electric batteries, electronic components and nuclear reactors. Because some of the applications of Cd can be performed by other less-toxic materials, the use of Cd has, therefore, been restricted by law in some countries. The toxicology and biological monitoring of cadmium was reviewed by WHO/IPCS (1992), Satarug and Moore (2004) and recently by Nordberg *et al*. (2007a).

9.1 Uptake, Metabolism and Excretion

Absorption of Cd compounds through the skin is negligible. Inhaled Cd will be absorbed, to between 10 and 50% depending on particle size. The absorption is greater for smaller particles and fumes than for larger dust particles. Humans, absorb 5–10% of ingested Cd. A low intake of calcium, zinc or iron increases the degree of absorption; for example, in iron-deficient individuals, the gastrointestinal absorption rate may be as high as 20% (WHO/IPCS, 1992). Experimental studies have indicated that cadmium, following absorption, is bound to albumin and other high-molecular-weight proteins in the blood plasma. Most cadmium in blood is, however, found within the red blood cells. In the liver, cadmium binds to MT, a low-molecular-weight protein (6.5–7.0 kDa) that is induced by cadmium and zinc. Each MT molecule can bind seven atoms of cadmium. Cadmium is transported in plasma when bound to MT and/or to certain high-molecular-weight proteins (Nordberg and Nordberg, 2000; Nordberg and Nordberg, 2009). Cadmium is accumulated in most organs and the half-lives of 10–30 years are reported for humans in muscle, kidney and liver tissue.

9.2 Toxic Effects and Dose–Response Relationships

The average amount of Cd ingested in most European and North American countries is about $10–20\,\mu g\,day^{-1}$.

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The corresponding average urinary excretion is about 0.5–1.0 μg day^{-1}. Most of the Cd in blood is located in the cells. The average blood concentration is about 0.5–1.0 μg l^{-1} in nonsmokers; it is twice as high in smokers because of Cd absorption from cigarette smoke. Concentrations of 10–20 μg kg^{-1} are usually found in the kidney cortex of nonsmokers in European countries. Although the intake of Cd through food has been higher in Japan than in Europe, and the reported tissue levels are correspondingly higher, the food intake of Cd has decreased in Japan during the last few decades.

Ingestion of highly contaminated food or drink results in acute gastrointestinal effects with concomitant diarrhoea and vomiting. Acute inhalation of Cd in air—for example, from soldering or welding fumes—may lead to severe chemical pneumonitis. Long-term exposure to low air levels may lead to chronic obstructive lung disease and lung cancer. Long-term excessive exposure from the air or food leads to renal tubular dysfunction. The first sign of damage is a low-molecular-weight proteinuria. This condition is the critical effect of such exposure to Cd and is used in quantitative risk assessment.

Long-term exposure from food, often combined with other means of delivery, may also lead to disturbance of calcium metabolism, osteoporosis and osteomalacia, mainly among postmenopausal women. A disease exhibiting these features—called *Itai-itai* disease—occurred in the 1950s in Cd-polluted areas of Japan; 124 cases were diagnosed up to 1970 and decreasing numbers of clinical cases have been diagnosed later with 66 cases during the period between 1970 and 2006 (WHO/IPCS, 1992; Nordberg *et al*., 2007a).

9.2.1 Exposure–Response Relationships: Treatment of Poisoning

Exposure to Cd in the air at concentrations of 5–10 μg m^{-3} during a working life of 45 years may give rise to renal tubular dysfunction in a small proportion of exposed workers. At about 100 μg m^{-3}, signs of chronic obstructive lung disease may develop, even after exposure for a shorter duration. After a lifetime of exposure from food at an average intake of about 200 μg day^{-1}, adverse effects on the kidney with low-molecular-weight proteinuria have been observed at age 50. There is considerable individual variation in the sensitivity to development of these renal effects. It has been suggested that such effects can be avoided if renal cortex levels are kept below 50 μg kg^{-1} and urine levels below about 2 μg g^{-1} creatinine. Recent reports of low, but statistically significant, increases at even lower levels of urinary Cd are, however, noteworthy. Such increases are observed in the general population, particularly among people with diabetes.

There is no specific treatment for Cd poisoning. When there are signs of osteomalacia, large doses of vitamin D should be given. Because of the long half-life of Cd in the kidneys, which are the critical organs, and the irreversibility of the effect, primary prevention is essential. Prevention can be assisted through environmental and biological monitoring.

9.3 Carcinogenicity and Genotoxicity

In animals exposed to Cd through injection, inhalation or oral exposure, cancer may develop at the injection site, in the lungs and prostate, or in other organs. Although some epidemiological studies have found an increase in the rates of cancer of the lungs and prostate, other studies have not demonstrated such effects. Since 1993, cadmium has been classified as a human carcinogen (Group 1) by the IARC (1993).

9.4 Mechanism of Action

Cadmium stimulates MT production in the same manner as other bivalent metals, such as zinc, copper and mercury. MT-bound Cd in plasma is filtered through the renal glomeruli and reabsorbed in the tubuli, where the metal ion is released following lysosomal degradation of the protein. The unbound Cd stimulates the production of new MT, which binds the Cd in the renal tubular cells. There is a continuous accumulation of Cd in the kidney tubules in long-term cadmium exposures. When not all of this accumulated Cd is bound, toxic effects occur, possibly because of the interference of Cd with zinc-dependent enzymes and/or membrane function.

9.5 Biological Monitoring

9.5.1 Biomarkers of Exposure

The biomarkers of exposure include biomarkers of external and internal doses and accumulation in critical organs. The utility of using the Cd content in blood and urine to indicate these three dose measurements is discussed in the following text.

9.5.1.1 Cd in Blood

Cadmium in blood occurs mainly in the blood cells. Whole-blood levels in nonsmokers from the general population, in areas without Cd pollution, are below 1 μg l^{-1} in most countries. Smokers have higher values. There is a fast and a slow component in the elimination of Cd from blood. The half-life in the fast component is approximately 100 days on average; the slow component

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is 7–16 years. The 'fast' component may reflect the turnover of cells in the blood.

The Cd concentration in blood may be a useful indicator of the degree of exposure in recent months. After long-term Cd exposure, however, an increasing proportion of blood Cd will be related to the body burden, and blood Cd is a good indicator of internal dose and accumulation in the kidney in long-term orally exposed population groups (Nordberg *et al*., 2002). These considerations are in concordance with the general understanding of Cd kinetics and the toxicokinetic multicompartment model for Cd. Because of the 'fast' compartment of blood Cd (Järup *et al*., 1983), high blood Cd values may be found before critical levels are reached in the kidneys. The level of blood Cd is used for occupational biomonitoring (e.g. in Sweden, a level of 50 nmol l^{-1} (5.5 µg l^{-1})) is used as an action level.

9.5.1.2 Cd in Urine

Urine levels of Cd often are adjusted for variations in volume that occur as a result of variable fluid intake. The most common adjustments are for creatinine or specific gravity, the latter usually being preferred to Cd, but the following discussion will focus on creatinine (CR)-adjusted values, because such values are those available in the published literature.

In urine, 'normal' or 'reference' levels will also vary with age, area and smoking habits, but they are generally $<1\,\mu g\,g^{-1}$ CR (or $1\,\mu g\,l^{-1}$ adjusted to specific gravity 1.024), although levels of $1–4\,\mu g\,g^{-1}$ CR have been reported from some nonpolluted areas in Japan (Ezaki *et al*., 2003; Nordberg *et al*., 2007a). Variation with age is seen in Sweden.

The Cd content in urine increases with the body burden of Cd and also after renal damage has occurred and is usually not related to recent exposure.

It was demonstrated in the 1970s (Nordberg, 1972), from animal experiments, that during the early phase of exposure, before renal tubular impairment has occurred, a correlation exists on a group basis between the body burden and the urinary concentration of Cd. Such information has also been reported in humans in several epidemiological studies. Urinary Cd is presently widely accepted as an indicator of body burden and kidney accumulation of Cd (Järup *et al*., 1998; Nordberg *et al*., 2002). Estimated relationships in the general population between the levels of urinary and kidney cortex Cd and the risk for developing tubular dysfunction are given in Section (9.2.1).

9.5.1.3 Cd in Placenta

Cadmium accumulates in the human placenta, and placental samples can be used as an indicator of Cd exposure during pregnancy. Lagerkvist *et al*. (1996) found levels of 2.6 and $5.0\,ng\,g^{-1}$ wet weight in Swedish nonsmokers and smokers, respectively. Placental levels were four times higher than those in the maternal blood.

9.5.1.4 Cd in Hair, Faeces and Other Biological Materials

For people exposed to Cd almost exclusively from food, the average daily Cd content in faeces is a good indicator of the daily intake, because 90% or more of the ingested Cd passes through the gastrointestinal tract unabsorbed and reaches the faeces. The average daily faecal Cd varies with age in a manner similar to the average daily energy intake. Faecal Cd has been used in several studies to estimate the average daily intake from food in Cd-polluted areas and in populations exposed to background levels of Cd.

Cadmium in hair may be used as an indicator of exposure and of the internal dose in oral Cd exposure (Nordberg and Nordberg, 1988). Because Cd levels in hair are fairly low, there is a risk of external contamination; thus, Cd in hair has not been used significantly for biological monitoring.

9.5.1.5 Cd in Kidney and Liver, Measured In vivo, Body Burden

Among adults in Sweden, USA and Germany, concentrations of Cd in the liver is approximately $1–3\,mg\,kg^{-1}$ and in the kidney cortex $15–50\,mg\,kg^{-1}$ (corresponding to approximately $10–30\,mg\,kg^{-1}$ calculated for a whole kidney). Higher values are found among smokers than among nonsmokers.

In vivo NAA or *in vivo* X-ray fluorescence measurements of liver and kidney Cd has made it possible to measure the correlation between the levels of Cd in the indicator media and in the critical organ, the kidney cortex. Roels *et al*. (1981) found no increase in the blood Cd with increased body burden. The generally high blood levels of Cd (5–30 µg l^{-1}) among the workers studied reflected their high daily exposures. The levels of urinary Cd, on the other hand, correlated with the body burden in workers not exhibiting renal damage. Ellis *et al*. (1984) calculated the relationship between the Cd levels in the kidney or liver measured with *in vivo* NAA and the prevalence of renal tubular dysfunction. Other studies have not succeeded in establishing these relationships because of the lower levels measured and the related larger proportion of values falling below the detection limit (for a review, see Järup *et al*., 1998).

10 CHROMIUM (Cr)

Chromium takes its name from the Greek word for colour, *chroma*. Chromium (Cr) is found in nature primarily as chromite ore with Cr in the trivalent (Cr(III)) form. This ore is used for manufacturing monochromates, dichromates, chromic acid and Cr pigments, as well as Cr metal. Chromium chemicals are of great significance to

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the health of workers. Chromium is an important component of stainless steel and is widely used in the rust protection of iron.

The toxicology and biological monitoring of chromium was reviewed by WHO/IPCS (1988) and recently Langard and Costa (2007) have reviewed the toxicology and biological monitoring of chromium.

10.1 Uptake, Metabolism and Excretion

The uptake of Cr through the airways and the digestive tract is much quicker in the hexavalent Cr(VI) than in the Cr(III) state. Chromium is found in all human organs of adults and newborns. The Cr concentrations are generally highest in lung tissue, where the concentration tends to increase with age, most likely resulting from inhalation and retention of Cr compounds with low water solubility. Chromium is excreted through the urine and faeces, predominantly through the urine. Chromium excretion in rats has been reported to take place exponentially, with half-lives of 1.5, 5.9 and 83.4 days.

Trivalent Cr seems to be a nutritional supplement for humans and animals and may play a role in the in metabolism of glucose. However, the mechanism still remains to be further explained. The daily diet is the main source of Cr in humans, and minute amounts of the intake come from drinking water and inhalation, but any daily requirement is still not fully defined.

10.2 Toxic Effects and Dose–Response Relationships

Exposure to Cr(VI) compounds is the main source of acute and long-term untoward effects of Cr. Allergic reaction of the skin is the adverse effect for Cr most frequently observed, and Cr(VI)-induced ulcerations of the mucosa of the nasal septum and ulcers of the skin are still seen frequently in developing countries. Long-term inhalation exposure to various Cr(VI) compounds has been shown to result in a high risk of carcinomas of the respiratory organs. Some of the Cr(VI) compounds, when inhaled seem to be among the most potent human carcinogens. Many animal experiments with instillation and injection of different Cr compounds, have resulted in local cancers in the skin and muscle tissue.

The most significant exposure to Cr(III) compounds in the general population is through food, but no untoward effects of Cr have been reported on this basis.

10.3 Carcinogenicity and Genotoxicity

Manufacture, exposure and analyses of Cr compounds have been reviewed by IARC (IARC, 1990) and chromium (VI) has been classified as carcinogenic to humans (Group 1), while chromium (III) compounds and metallic chromium are Group 3.

10.4 Mechanism of Action

Compounds of Cr(VI) are shown to induce mutations, chromosomal aberrations, DNA damage in the form of single-strand breaks, and DNA–protein and DNA–DNA crosslinks. The end reductant product of Cr(VI) in the cell is Cr(III), which is thought to be responsible for much of the DNA damage and mutations induced by Cr(VI).

10.5 Biological Monitoring

The highest concentration of Cr in humans is found in hair, with values from 200 to 2000 $\mu g\,kg^{-1}$ reported (Mertz, 1969). Levels of Cr in various human organs obtained at autopsy from persons in the general population of various countries show the highest levels in lung and lower levels in other tissues (review by Langard and Costa, 2007).

The Cr concentration in all tissues decreases from the moment of birth up to the age of approximately 10. After this time, there is a slight increase in lung concentration, but a continuing fall in all other organs (Schroeder *et al*., 1962). This indicates that Cr in the lungs is a result of deposition from inhaled air, whereas Cr in food is the main source of Cr in other organs.

Previously reported normal levels of Cr in blood up to 3–30 $\mu g\,l^{-1}$ were probably too high. Later the concentration of Cr in plasma was reported to be 0.14 $\mu g\,l^{-1}$ (Veillon *et al*., 1979). Urinary Cr has been used for exposure biomonitoring in welders with levels of 16–58 $\mu g\,g^{-1}$ creatinine in the 1970s and 1980s (Aitio, 1988).

11 COBALT (Co)

Cobalt was isolated by the Swedish chemist Georg Brandt in the middle of the eighteenth century, although compounds containing cobalt were already used in ancient Egypt. *Kobold* was a name applied during the sixteenth century to ores eventually found to be toxic, arsenic-bearing cobalt ores.

Cobalt is a relatively rare element in the earth's crust, which is essential to mammals in the form of cobalamin (vitamin B_{12}). The adult human body contains about 1 mg of cobalt, 85% of which is in the form of vitamin B_{12}. Human dietary intake of cobalt varies between 5 and 50 $\mu g\,day^{-1}$ and most of the cobalt ingested by humans is

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inorganic, vitamin B_{12} representing only a small fraction. The oral bioavailability of inorganic cobalt varies with the solubility of the cobalt compound (5–45%). In occupational settings, workers are exposed to cobalt compounds by inhalation of dust. The main industrial use of cobalt is for the manufacture of alloys and hard metals. The toxicology and biological monitoring of cobalt was recently reviewed by WHO/IPCS (2006b) and Lison (2007).

11.1 Uptake, Metabolism and Excretion

The absorption rate of inhaled cobalt also varies with the species considered. Cobalt does not accumulate in the organism and is rapidly excreted in urine. The concentration of cobalt in urine or in blood may be used as a biomarker of recent exposure to soluble cobalt species (WHO/IPCS, 2006b).

11.2 Toxic Effects and Dose–Response Relationships

The respiratory system is the main target organ (asthma, fibrosing alveolitis, lung cancer) upon inhalation exposure to cobalt, with a higher risk of fibrosing alveolitis (hard-metal disease) and lung cancer in the hard-metal industry, where workers are exposed to cobalt metal mixed with tungsten carbide particles. A physicochemical interaction leading to the formation of ROS may account for the increased toxicity of this mixture of particles. Other target organs include the haematopoietic system, the myocardium, the thyroid gland and possibly the reproductive system.

11.3 Carcinogenicity and Genotoxicity

Cobalt and cobalt compounds (evaluated as a group) were classified by IARC as Group 2B (1991). In 2006, cobalt metal without tungsten carbide was classified as Group 2B and cobalt metal with tungsten carbide as Group 2A; cobalt sulfate and other soluble cobalt(II) salts are Group 2B agents (IARC, 2006a).

11.4 Biological Monitoring

In nonoccupationally exposed subjects, a wide range of cobalt concentrations in urine and blood have been reported, and normal values are probably $<2\,\mu g\,l^{-1}$ (or $\mu g\,g^{-1}$ creatinine) for urine and $0.5\,\mu g\,l^{-1}$ for blood (Lauwerys and Hoet, 2001). Increased urinary cobalt concentrations may be observed in individuals taking multivitamins and patients with a Co-containing prosthesis (Schaffer *et al*., 1999).

Lison (2007) reviewed available data on the relationship between urinary cobalt and exposure in the hard-metal industry. Several studies have demonstrated a good correlation between cumulative exposure via air during the workweek and Co in urine samples on Friday afternoon.

On the basis of the correlation analyses (Ichikawa *et al*., 1985) reported that an airborne exposure to $100\,\mu g\,m^{-3}$ was associated with a cobalt concentration of $5.7–7.9\,\mu g\,l^{-1}$ in blood and $59–78\,\mu g\,l^{-1}$ in urine (95% confidence intervals). The importance of the solubility of cobalt compounds to which workers are exposed is evident from studies by Christensen and Poulsen (1994), who observed increased concentrations of cobalt in blood ($0.2–24\,\mu g\,l^{-1}$) and urine ($0.4–848\,\mu g\,l^{-1}$) of pottery-plate painters who used a soluble cobalt pigment, whereas only slightly increased values were measured in those using an insoluble cobalt pigment (0.05–0.6 and $0.05–7.7\,\mu g\,l^{-1}$ in blood and urine, respectively).

Biological monitoring of workers (Lison *et al*., 1994) exposed to cobalt oxides revealed increased blood and urine levels compared with nonexposed subjects, but parameters poorly reflected recent exposure levels. In contrast, when exposure was to soluble cobalt compounds, urine and/or blood cobalt at the end of the workweek would be recommended for monitoring. It was calculated that an eight hour exposure to 20 or $50\,\mu g\,m^{-3}$ of a soluble form of cobalt would lead to average urinary concentrations of 18.2 and 32.4 μg Co g^{-1} creatinine, respectively (postshift urine sample collected at the end of the workweek). In workers from the hard-metal industry, cobalt in urine rapidly increased in the hours immediately after exposure, with a peak approximately 2–4 hours after exposure, and a subsequent decrease (more rapid for the first 24 hours) in the following days (Apostoli *et al*., 1994).

There is no biological marker of effect that would be specific for cobalt or hard-metal exposure. The determination of the HLA-DP genotype may have some relevance in terms of individual susceptibility to hard-metal disease.

12 COPPER (Cu)

The element was discovered in prehistoric times and takes its name from the island of Cyprus (Latin: *cuprum*). The toxicology and biological monitoring of copper was recently reviewed by Tapiero *et al*. (2003), and Ellingsen *et al*. (2007). Copper is an essential trace element and

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is a vital part of several enzymes (e.g. ferroxidases, cytochrome c oxidase, superoxide dismutase (SOD), tyrosinase, lysyl oxidase and dopamine β-hydroxylase).

In the general environment, humans are exposed to copper via food and drinking water which are the most important sources of copper exposure in humans. It is widely used in cooking utensils and water distribution systems as well as in fungicides. Major sources for copper distribution in the environment are mining operations, agriculture, solid waste, and sludge from treatment factories.

12.1 Uptake, Metabolism and Excretion

Absorption depends on the amount ingested, its chemical form and the composition of other dietary components, such as zinc. Drinking water may contribute significantly to the daily copper intake because of the widespread use of copper pipes. Absorption is regulated by homeostatic mechanisms in the liver, and biliary excretion increases when copper is in excess. Copper is found in all organs. The highest concentrations, both in adults and in the newborn, are found in the liver; the brain is also rich in copper.

Excretion is mainly through the bile, and only a small percentage of absorbed copper is found in urine. The total-body biological half-life in groups of healthy humans after oral administration has been calculated in the range of 2–5 weeks.

In people with Wilson's disease, an inherited deficiency of ATP7B, biliary excretion of Cu is impaired and, as a consequence, Cu is accumulated in the liver and other organs. In Menkes disease, there is an uptake of Cu in some organs, and related severe, often fatal, intracellular tissue deficiency due to deficiency in ATP7A.

12.2 Toxic Effects and Dose–Response Relationships

Ingestion of a large amount of copper salts causes gastrointestinal disturbances. The first symptom to occur is nausea, with increased reporting starting at approximately $4\,\mathrm{mg\,l^{-1}}$ of copper in drinking water.

In severe cases, systemic effects, especially haemolysis, and liver and kidney damage, can occur. Indian childhood cirrhosis is a rare liver cirrhosis with increased liver Cu levels. The disease is of unclear aetiology, affecting children from one to six years of age. In Wilson's disease, the impaired excretion of Cu gives rise to tissue accumulation with increasing damage to the liver and brain. In contrast to data obtained after ingestion, comparatively little is known about health effects related to the inhalation of copper and copper fumes in an industrial setting. Copper may cause irritation in the respiratory tract and metal fume fever. Lung changes in vineyard workers have been attributed to copper sulfate, but the role of copper has not been fully explained.

12.3 Mechanism of Action

Crucial for appropriate biliary excretion of copper is the presence of the copper exporter ATP7B. Mutations in the ATP7B gene are responsible for the genetic disorder Wilson's disease.

12.4 Biological Monitoring

Several markers of exposure have been assessed for the purpose of biological monitoring, but none of them have shown clear associations with excessive exposure. Because copper is a highly reactive metal and thus harmful to cells if present as free ions, intracellular copper levels are strictly controlled by a number of integral transmembrane transporters, metallobuffers and metallochaperones.

Ellingsen *et al*. (2007) reviewed data on tissue levels of Cu in humans. In normal humans the highest levels are found in the foetus, decreasing after birth to about $5\,\mu\mathrm{g\,kg^{-1}}$ in liver. In patients with Wilson's disease and in children with Indian childhood cirrhosis considerably higher levels occur.

In the blood, copper is distributed into a plasma pool associated with large proteins, an exchangeable fraction of low-molecular-weight copper complexes and a red cell pool that is partly nonexchangeable. Most of the copper in blood plasma is bound to ceruloplasmin, approximately 15% to albumin, 10% to transcuprein while small amounts are bound to small peptides and amino acids (see review by Ellingsen *et al*., 2007). Transcuprein and albumin represent the bulk of the exchangeable copper pool in plasma. The percentage of plasma copper bound to ceruloplasmin is approximately 60–95%. The copper concentration in red blood cells is about the same as in plasma. Copper is also found in leukocytes. In erythrocytes, approximately 60% of the copper is bound to Cu–Zn–SOD. The normal concentration of copper in plasma is approximately $1\,\mathrm{mg\,l^{-1}}$ ranging up to approximately $1.5\,\mathrm{mg\,l^{-1}}$. Several factors, related to changes in ceruloplasmin levels, make plasma copper an unreliable marker of copper status, except in more severe deficiency states. Increased serum copper levels have been measured in clinically severe cases of copper-sulfate poisoning. The increased concentrations of copper in serum measured in various liver diseases,

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carcinomas, acute and chronic infections, rheumatoid arthritis and other conditions may be related to the increased synthesis of ceruloplasmin as an acute-phase reactant, and thus not related to copper status as such. The half-life of apoceruloplasmin (five hours) is much shorter than the half-life of holoceruloplasmin (5.5 days). Women have generally higher plasma copper levels than men (Milne, 1998). Higher levels have also been measured in women taking oral contraceptives and post-menopausal women receiving oestrogen. Serum copper and serum ceruloplasmin concentration increase during pregnancy.

In healthy adults, the urinary excretion of copper is generally between 30 and 60 μg Cu day^{-1}. An oral intake severalfold higher than the daily recommended intake does not increase the urinary copper excretion over a three week time period. Thus, urinary copper is unsuitable for biological monitoring at moderately elevated oral intakes. However, whether this is true for the occupational setting where exposure occurs by inhalation, has hardly been studied. An increased urinary excretion >100 μg Cu day^{-1} is often observed in patients with Wilson's disease.

13 GALLIUM AND SEMICONDUCTOR COMPOUNDS (Ga)

The name gallium stems from the Latin word *Gallia,* the name for Roman provinces roughly coterminous with modern France. Semiconductors may be elemental materials such as silicon and germanium, or compound semiconductors such as gallium arsenide and indium phosphide, or alloys such as silicon germanium or aluminium gallium arsenide.

Gallium is a member of Group III of the Periodic Table of Elements, and new uses have been found for different chemical forms of this metal over the past two decades in the fields of semiconductor materials, lasers, cancer and malaria chemotherapy, and dental materials. The production of gallium for these uses has increased greatly during this period. Analytical methods for gallium include atomic absorption/emission spectrometry, X-ray fluorescence and, more recently, inductively coupled plasma spectrometry. The toxicology and biological monitoring of gallium and semiconductors was recently reviewed by Fowler and Sexton (2007b).

13.1 Uptake, Metabolism and Excretion

Gallium absorption is independent of exposure route and very limited. After injection it is taken up by bone, kidney and specifically by neoplastic tissue. It is excreted in urine and faeces (Nordberg and Nordberg, 1996).

13.2 Toxic Effects and Dose–Response Relationships

The main health concerns for gallium have been centered on workers in the semiconductor industry engaged in the production of gallium-arsenide-based devices. Toxicity studies in experimental animal systems have shown gallium nitrate and gallium arsenide to produce toxicity to the lungs, immune system, kidneys and haematopoietic systems. Injected gallium compounds may give rise to kidney damage and neuromuscular toxicity.

13.3 Carcinogenicity and Genotoxicity

In 2006, the IARC classified gallium arsenide as a human carcinogen (Group 1). Studies of semiconductor workers are limited, but clinical trials in humans treated with gallium nitrate for various cancers have demonstrated renal toxicity as the primary side effect.

13.4 Mechanism of Action

Gallium is transported in the circulation, bound to transferrin, and competition with iron for transferrin binding seems to be one mechanism by which it may act as an anticancer agent.

13.5 Biological Monitoring

Liao *et al*. (2004) reported gallium concentrations in blood and urine from workers in Taiwan engaged in the production of III–V semiconductors. The gallium blood levels were approximately 0.5 μg l^{-1} for workers in various occupations and office workers (controls). Gallium concentrations in urine were 0.2–0.3 μg l^{-1} among workers and 0.15 μg l^{-1} in controls. Although blood levels were not consistently higher in 'exposed' workers than office workers, urinary gallium was significantly higher ($p < 0.05$) in all other workers compared with the officer workers (controls).

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14 GERMANIUM (Ge)

In 1871, Mendeleev predicted the existance of germanium (Latin *Germania* for Germany), as a missing analogue of the silicon group, and called it 'ekasilicon'). Tao and Bolger (1997) reviewed the adverse effects of Ge and more recently Faroon *et al*. (2007) summarized available evidence on toxicity and biological monitoring of Ge.

14.1 Uptake, Metabolism and Excretion

Animal experimental data show that germanium compounds, both inorganic and organic, are rapidly and almost completely absorbed from the lungs and the gastrointestinal tract. The distribution among the organs and tissues is fairly uniform, and there is no evidence of preferential uptake or accumulation. The systemic toxicity is low. Absorbed germanium is rapidly excreted, mainly in urine. Data on biological half-lives are inadequate, but for the rat the whole-body retention half-life has been estimated at about 1.5 days.

14.2 Toxic Effects and Dose–Response Relationships

Germanium tetrachloride is a strong irritant of the respiratory system, the skin and the eye, possibly because it is easily hydrolyzed, producing hydrogen chloride; in mice high-level inhalation exposures caused necrosis of the tracheal mucosa, bronchitis and interstitial pneumonia. Systemic toxicity of germanium compounds is comparatively low. The specific target or critical organs cannot be identified, but nephropathy, neuropathy and hepatotoxicity are usually observed. Trialkylgermanium compounds are less toxic than the corresponding lead or tin alkyls. Germanium compounds do not seem to be carcinogenic. Dimethylgermanium oxide is teratogenic in chickens, but sodium germanate has not produced malformations in hamsters.

There is little information on the toxicity of inorganic germanium compounds to man, except that germanium tetrachloride may produce skin irritation. In clinical trials, spirogermanium, an organogermanium antitumour agent, has been shown to be neurotoxic. Recently, germanium was reported as having anticancer effects. A patient with $175\,\mu g\,g^{-1}$ of germanium in their hair showed a variation in the fibre sizes of the skeletal muscles, with dense cytoplasmic bodies, reduction of cytochrome c oxidase activity, mitochondrial myopathies and vacuolar degeneration; similar findings were observed in rats treated with germanium oxide.

High doses of germanium compounds (taken as supplements) induced remarkable lactic acidosis, hydropic degeneration of the proximal convoluted tubules, with presence of inclusion bodies, and some cellular necrosis and subsequent renal failure; however, the renal glomeruli and the renal interstitial tissue appeared normal. Neurological effects involved negative deep-tendon reflexes in the lower extremities and a persistent tingling sensation in the palms and soles. In addition to severe cardiac dilation, vacuolar degeneration of myocardial cells and interstitial oedema were observed.

Inhalation is the main route of exposure under occupational conditions; the main source of germanium for the general population is food.

14.3 Biological Monitoring

Faroon *et al*. (2007) have recently summarized available data on tissue levels in humans and biological monitoring of Ge. Urinary Ge concentrations can be used for biological monitoring of occupational Ge exposure. Swennen *et al*. (2000) reported urinary concentrations of $<0.25\,\mu g$ Ge g^{-1} creatinine in control workers. In exposed workers, they reported geometric mean urinary concentration ranges from 1.36 (Monday) to 3.6 (Friday) μg Ge g^{-1} creatinine before the working shift to 4.22 (Monday) to 4.34 (Friday) μg Ge g^{-1} creatinine after the working shift. Ranges of individual values before the shift were $0.23–70\,\mu g\,g^{-1}$ creatinine and after the shift $0.16–195\,\mu g\,g^{-1}$ creatinine.

15 INDIUM (In)

The name of the element alludes to the brilliant indigo line in its spectrum. One unusual property of indium is that its most common isotope (^{115}In) is slightly radioactive; it very slowly decays by β-emission to tin. Indium compounds of toxicological interest include indium trichloride, indium oxide, indium sulfate, indium sulfide, indium sesquioxide and colloidal indium hydroxide. Indium also forms organometallic compounds such as trimethylindium, a polymeric solid. Indium phosphide and indium arsenide are used in the manufacture of III–V semiconductors, and indium phosphide is used in the production of nanoparticles, nanowires and dots. Available evidence regarding indium toxicology has been reviewed by Fowler and Sexton (2002) and more recently by Fowler (2007).

15.1 Uptake, Metabolism and Excretion

Indium (In) compounds are poorly absorbed when ingested and moderately so when inhaled. The most

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common routes of exposure for the general population are inhalation and ingestion; for occupationally exposed persons it is inhalation. The use of In for nanotechnology may increase the possibility of dermal absorption in work environments.

Muscle, skin and bone constitute the main storage sites, but the excretion routes vary depending upon the form. Ionic indium is excreted primarily in urine; faecal elimination is the predominant route for removal of colloidal indium. A biphasic pattern of excretion and a whole-body biological half-life on the order of two weeks have been reported for both forms of indium.

15.2 Toxic Effects and Dose–Response Relationships

Ionic indium is concentrated in the kidneys, producing renal failure; colloidal indium is taken up by the reticuloendothelial system, causing damage to the liver and spleen. Intravenous administration of ionic indium to pregnant hamsters has been reported to produce malformations of the foetal digits.

15.3 Carcinogenicity and Genotoxicity

In 2006, indium phosphide was upgraded from Group 2B to 2A (IARC, 2006a).

15.4 Mechanism of Action

Ionic indium has been shown to produce marked ultrastructural damage in the endoplasmic reticulum of both hepatocytes and renal proximal tubule cells with associated disruption of haem metabolism and haemoprotein function.

15.5 Biological Monitoring

Smith *et al*. (1978) have extensively reviewed the literature concerning reported tissue concentrations of indium in animal and human tissues, and most values have been found to be $<0.05\,\mu g\,g^{-1}$.

16 IRON (Fe)

The name stems from the Latin word *ferrum*. Iron has been known since prehistoric times. It is the fourth most abundant metal in the earth's crust and the most abundant transition metal. Iron can easily change valence and form complexes with oxygen, and iron-mediated reactions support the respiration of nearly all aerobic organisms. An adequate oral intake of iron is thus essential to human life. However, unless appropriately shielded, iron catalyses the formation of radicals that can damage biological molecules, cells, tissues and entire organisms. A review of iron biochemistry and toxicology has recently been presented by Ponka *et al*. (2007).

16.1 Uptake, Metabolism and Excretion

Exposure to excess iron—typically from multiple blood transfusions over many years—can lead to numerous pathological consequences. In contrast, severe iron deficiency may also have serious health consequences. Because of the inherent danger of iron, specialized molecules for the acquisition, transport and storage of iron (ferritin) in a soluble nontoxic form have evolved.

16.2 Toxic Effects and Dose–Response Relationships

Despite these homeostatic mechanisms, organisms can face the threat of either iron deficiency or iron overload. Acute iron poisoning occurs in children as a result of accidental ingestion of medical iron preparations. Such ingestion gives rise to gastrointestinal symptoms with blood vomiting and acidosis; subsequently hepatic and renal damage may develop. Hereditary haemochromatosis is an inherited inability to downregulate intestinal iron absorption. The disease gives rise to gradually increasing excessive iron level with related liver disease. Other forms of haemochromatosis give rise to damage to the heart. In addition, occupational inhalation exposures to iron may give rise to siderosis, a benign condition easily diagnosed by chest X-ray. Emerging knowledge of iron metabolism increasingly emphasizes the simultaneously vital and threatening nature of this most important and abundant metal.

16.3 Carcinogenicity and Genotoxicity

IARC (1987) classified iron-related industrial activities and iron compounds as to their carcinogenicity. Iron and steel founding are Group 1, that is, carcinogenic to humans, iron–dextran complex is Group 2B, while iron–dextrin complex and iron-sorbitol–citric-acid complex is Group 3. It should, however, be noted that

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the carcinogenic agent in the occupational environment is most probably not iron, but other compounds generated in the process, such as polyaromatic hydrocarbons.

16.4 Mechanism of Action

Delivery of iron to most cells occurs after the binding of transferrin to transferrin receptors on the cell membrane. The transferrin–receptor complexes are then internalized by endocytosis, and iron is released from transferrin by a process involving endosomal acidification and reduction. Iron is then transported through the endosomal membrane by the Fe(II) transporter DMT1/Nramp2. Importantly, this same transporter is involved in the absorption of inorganic iron in the duodenum, a process facilitated by the ferric reductase, Dcytb, which presumably provides Fe(II) for DMT1/Nramp2. Organisms and cells possess limited ability to excrete excess iron, and only some specialized cells have active mechanisms to export iron. Iron release from these 'donor cells' (primarily enterocytes and macrophages that recycle haemoglobin iron) is mediated by ferroportin 1. The ferroxidase activity of copper-containing proteins, hephaestin and ceruloplasmin, facilitates the movement of iron across the membranes of enterocytes and macrophages, respectively. Cells are also equipped with a regulatory system that controls iron levels in the labile pool. Levels of iron modulate the capacity of iron regulatory proteins (IRPs) to bind to the iron-responsive elements (IREs) present in the untranslated regions of messenger RNAs (mRNAs) for several proteins involved in iron metabolism (e.g. ferritin, transferrin receptor, DMT1); these associations, or lack of them, in turn control the expression of these proteins. In fact, important information about regulation of iron metabolism came from studies of proteins (e.g. HFE (gene encoding for haemoachromatosis), ferroportin and hepcidin) coded by gene mutations that cause different types of hereditary haemochromatoses.

16.5 Biological Monitoring

Iron status can be monitored by measurements of serum iron, transferrin and ferritin. Acute iron poisoning is monitored by measurements of serum iron. Haemochromatosis is evaluated based on serum ferritin levels.

17 LEAD (Pb)

The chemical symbol Pb is derived from the Latin word for lead, *plumbum*. Inorganic lead is certainly the most extensively studied of all toxic agents. Occupational exposure occurs in a wide variety of settings. There is also widespread exposure in the general environment. However, after the ban of lead addition to petrol, the exposure has decreased dramatically in several parts of the world. The toxicology of lead and its compounds has been reviewed by WHO/IPCS (1995), ATSDR (Agency for Toxic Substances and Disease Registry) (2005) and more recently by Skerfving and Bergdahl (2007).

17.1 Uptake, Metabolism and Excretion

Uptake of lead from the gastrointestinal tract is variable: 4–70% may be taken up depending on conditions of ingestion and dietary composition. Absorption via inhalation is dependent on particle size and solubility of the lead compound being inhaled. Inhalation of small particles of water soluble Pb compounds gives higher uptake, up to 60%.

After uptake into the blood, Pb is distributed to blood cells. A major proportion, approximately 99% is in blood cells and only 1% in plasma. From plasma, Pb distributes to other soft tissues and is gradually incorporated into the mineral part of the bone, where it has a long biological half-life, from two years to several decades, related to bone structure. Excretion is mainly via urine and faeces.

17.2 Toxic Effects and Dose–Response Relationships

Heavy exposure to lead causes anaemia and symptoms of the nervous system, involving both the brain and the peripheral nerves with paralysis. Toxic effects of somewhat lower exposure to inorganic lead may occur in the central and peripheral nervous systems, blood (including inhibition of haem synthesis, which also affects other cells), kidney, and cardiovascular, endocrine and immune systems, gastrointestinal tract and male reproduction (sperm quality). Lead causes increase in blood pressure; several studies have reported slight effects, increase by 1 mm Hg, in adults with a mean blood Pb (PbB) of $0.4\,\mu mol\,l^{-1}$ when PbB was doubled. Furthermore, lead passes through the placenta and may cause effects on the nervous system of the foetus. Lead in the skeleton is mobilized during pregnancy and lactation, and is transferred to both the foetus and the lactating infant. Slight (but adverse) effects on the mental development of infants and children in terms of performance in neurobehavioural tests have repeatedly been reported at a mean PbB of $0.5\,\mu mol\,l^{-1}$, about $100\,\mu g\,l^{-1}$ or even less, in the pregnant woman. The most important organolead compounds are tetraethyl and tetramethyl lead, which have been used in enormous quantities in leaded petrol. They are easily

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absorbed through inhalation and through the skin and may cause acute encephalopathia.

17.3 Carcinogenicity and Genotoxicity

Lead is carcinogenic in animal experiments, but there is only limited evidence for carcinogenicity in humans. Inorganic lead compounds are classified as Group 2A (IARC, 2006b) and organic lead compounds as Group 3 (IARC, 2006b).

17.4 Mechanisms of Action

In the adult, the early effects of lead exposure are effects on the synthesis of haemoglobin. In children a neurotoxic effect is the critical effect. The mechanism for this is not quite fully understood. It is, however, suggested that impairment of haem metabolism might cause interference with cellular energy metabolism.

Lead inhibits enzymes in the haem synthesis pathway, particularly (aminolevulinic acid dehydratase (ALAD) and ferrochelatase. The latter effect means that iron cannot be incorporated into haem and an erroneous metabolite zincprotoporphyrin (ZPP) is formed. These effects are sometimes used for biological monitoring (see Section 17.5). Anaemia in lead-exposed persons may also be partly related to tubular toxicity in the kidney, with decreased production of erythropoietin.

17.5 Biological Monitoring

Most of our knowledge about dose–response relationships of Pb comes from studies using biological monitoring. There are well-established biomarkers of lead exposure and also biomarkers of some of the adverse effects. The level of lead in whole blood (PbB) is the most commonly used biomarker of exposure and internal dose. The PbB reflects both recent exposure and long-term accumulation of Pb. There is a curvilinear relationship between PbB and lead uptake. At low uptakes there is a steady increase of PbB, but at high uptakes the PbB level flattens out. Recently plasma and serum levels of Pb have also been reported. Determination of these levels, which are considerably lower than those in whole blood is now possible as a result of improved chemical analyses. However, experience is still limited from studies using this, possibly improved, biomarker of exposure and internal dose (Bergdahl *et al*., 1997).

Existing levels of PbB vary among populations and are related to the use of lead in petrol. Unleaded petrol is presently used in most countries. For example, in the Swedish town of Trelleborg, PbB levels in children have decreased since the 1990s when there was a switch from leaded to unleaded petrol. The mean level in 2003–2005 was 16–12 $\mu g\,l^{-1}$. Gellein *et al*. (2007) reported Pb levels of 1.32 and 0.2 $\mu g\,l^{-1}$ (arithmetic average and standard error of the mean (SEM)) in the CSF of adults in Norway by high resolution (HR)-ICP-MS. Comparable values for the serum analyses for Pb was below detection limits. In 33 patients with Parkinson's disease, serum concentration, expressed as $\mu g\,l^{-1}$, for Pb, average and SD, was 0.29 and 0.43, respectively (Gellein *et al*. 2008c). Dose–response relationships for PbB and adverse health effects are given in Section 17.2. Urinary lead levels are also used in biological monitoring, particularly as a test of 'chelate lead'. In order to measure 'chelate lead', either ethylenediamine tetraacetic acid (EDTA) or dimercaptosuccinic acid is administered to the patient and subsequently urine is collected and the urinary Pb level determined. It is believed that 'chelate lead' should reflect the total body burden of lead, but several studies have shown that it reflects mainly soft tissue levels of lead (Gerhardsson *et al*., 1998).

Lead interferes with haem synthesis (Section 17.4) and ZPP accumulates in blood with increasing PbB, starting at 1–2 $\mu mol\,l^{-1}$ of PbB, differing among individuals. Increased levels of ALAD in red blood cells are related to PbB levels above 30 $\mu g\,l^{-1}$ (0.15 $\mu mol\,l^{-1}$) and thus ALAD is a very sensitive biomarker of lead effects. Other metabolites in the haem synthesis pathway have also been used as biomarkers for lead, for example aminolevulinic acid (ALA) in blood plasma or urine. There are differences in the effect on ALA depending on the ALAD genotype (Schwartz *et al*., 1997). Lead accumulates in teeth and in the skeleton. Pb in deciduous teeth is a useful biomarker of cumulative exposure in children. Pb in bone may be determined by *in vivo* methods, which reflect long-term uptake.

18 MANGANESE (Mn)

The name manganese stems from two black minerals from Magnesia, in Greece. *Magnes* was later called *magnesia and* in modern times manganese dioxide. The metal isolated from magnesia negra, *Manganesa* was recognized as manganese, from the German, Mangan.

Manganese is ubiquitous in the environment. It occurs in soil, air, water and food. Its concentration in food varies markedly, but on the whole, food is the major source of manganese intake for humans. The toxicology and biological monitoring of Mn has been reviewed by WHO/IPCS (1981a), WHO (2004), ATSDR (2000) and Sarić and Lucchini (2007).

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18.1 Uptake, Metabolism and Excretion

Manganese absorption is by inhalation, ingestion and through the olfactory pathway. The absorption and homeostasis of ingested manganese is regulated by absorption mechanisms, but manganese homeostasis is primarily achieved by excretion. Absorbed manganese is rapidly eliminated from the blood and first concentrates in the liver, where it conjugates with the bile and is excreted into the intestines. Reaching the intestines, most of the element is excreted in the faeces. Urinary excretion is only approximately 0.01% per day of the body burden. The biological half-life in humans is two to five weeks, depending on body stores. Manganese crosses the blood–brain barrier and accumulates in the brain, where the half-life is much longer. Manganese is an essential element for humans and animals. Although many people are likely to be below the estimated safe and adequate daily manganese intake, no large-scale deficiency has been reported.

18.2 Toxic Effects and Dose–Response Relationships

Long-term exposure to increased concentrations of manganese, associated primarily with inhalation in occupational settings, may result in neurological and neurobehavioural effects. On the basis of the cumulative mechanism of neurotoxicity, concerns have been raised regarding the possible long-term effects on the CNS, represented by parkinsonian disturbances. Manganism, a manganese-induced brain disease, is a progressive irreversible impairment. Positron emission tomography (PET) with ^{18}F-dopa or single-photon emission tomography (SPET) afford a differentiation between manganism and parkinsonism, which is marked by neurodegradation in the dopaminergic nigrostriatal pathway, whereas manganism-induced damage occurs postsynaptic to the nigrostriatal system (Racette *et al*., 2005). Lungs are the second target organs affected by exposure to manganese. Inhalation exposure to high concentrations of manganese dioxide (or tetroxide) can cause chemical pneumonitis, an inflammatory response in the lung. Moreover, even at rather low exposure levels, increased incidence of acute respiratory diseases—bronchitis and pneumonia—have been reported, indicating impaired resistance to respiratory infections. There is also some limited evidence of adverse manganese effects on the reproductive, cardiovascular, haematological, endocrine and immunological systems.

18.3 Carcinogenicity and Genotoxicity

Although manganese causes mutations, it is not carcinogenic. As part of an alloy with chromium, increased incidence of tumours has been seen in experimental studies.

18.4 Mechanism of Action

The principal mechanism of manganese neurotoxicity has not yet been found. Manganese may enhance the auto-oxidation or turnover of various intracellular catecholamines, such as dopamine, leading to an increased production of free radicals, ROS and other cytotoxic metabolites. It may also disturb γ-aminobutyric acid (GABA) regulation, as well as glutamatergic transmission. It also seems to impair the cellular antioxidant defence mechanism.

18.5 Biological Monitoring

Manganese can be measured with good sensitivity in biological fluids and tissues, and levels in blood, urine, faeces and hair have been investigated as possible biomarkers of exposure (Sarić and Lucchini 2007).

The average normal level in whole blood is $7–12\,\mu g\,l^{-1}$. Approximately 85% of blood manganese is in the erythrocytes. In serum and plasma, average manganese concentrations are reported to be 0.6–4.3 $\mu g\,l^{-1}$.

The reported normal concentrations of manganese in urine vary to a large extent, but normally less than $1\,\mu g\,l^{-1}$ is found. As a group, workers exposed to a mean concentration of $1\,mg\,Mn\,m^{-3}$ had higher levels of manganese in the blood and urine than unexposed controls (Roels *et al*., 1987). The group average levels in blood seem to be related to manganese body burden, whereas average urinary excretion levels are judged to be most indicative of recent exposures. However, the individual measurements do not correspond to individual exposures.

In a study by Lucchini *et al*. (1995), blood and urine levels were correlated with exposure levels on an individual basis. In this study, a correlation was observed between a cumulative exposure index (CEI) and manganese in blood (MnB), and both MnB and CEI were associated with neurobehavioural outcomes. Workers had been tested during a month of layoff, and all the correlations increased proportionally to the latency from the exposure cessation. Thus the influence of current exposure was decreased. A factor that limits the usefulness of measuring current MnB and urine as a measure of excess manganese exposure is the relatively

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rapid rate of manganese clearance from the body, with very little excretion in urine.

In a Canadian study by Baldwin *et al*. (1999) on the relationship between the levels of manganese in air and blood manganese, significantly higher levels of blood manganese were correlated with high levels of airborne manganese. Data obtained in this study combined with the occupational studies indicated that there might be a plateau level of homeostatic control of manganese.

At low levels, blood manganese concentrations are related to food, water and air levels of manganese, and large differences in individual blood levels are observed. At high exposure levels, such as in occupational environments, this plateau may be reached or exceeded.

Manganese-dependent superoxide dismutase activity in lymphocytes increases with increased manganese uptake. It has been suggested that this could be used, in conjunction with serum manganese levels, to assess low and moderate levels of exposure to manganese.

Magnetic resonance imaging (MRI) can be useful to track manganese distribution in the brain among industrial workers exposed to airborne manganese, and to identify accumulation of manganese in children exposed to excess manganese.

The use of hair as a possible biomarker of manganese exposure is problematic. In hair, manganese concentrations are reported to be normally $<4\,\mathrm{mg\,kg^{-1}}$. There is no consensus concerning the validity of scalp hair analysis as a biomarker of exposure.

Ethylene thiourea is readily detected in the urine of humans (and animals) exposed to maneb and mancozeb, but there is no clear relationship between dose and urinary levels (Kurttio and Savolainen, 1990; Kurttio *et al*., 1990).

Clara cell protein CC16 in blood serum or urine is a potential biomarker for pulmonary effects from exposure to MMT (Halatek *et al*., 1998). Clara cells are found in the bronchioles. Damage to these cells causes a significant change in the levels of CC16.

Serum PRL (prolactin) has been shown to be a possible biomarker of manganese effects on dopamine neurotransmission. Namely, the tuberoinfundibular dopaminergic system, which exerts tonic inhibition of PRL secretion, is affected by manganese action (Smargiassi and Mutti, 1999). Still, it is not clear whether PRL levels indicate recent or cumulative exposure. It has to be mentioned, also, that in a study by Roels *et al*. (1992) and more recently in a large South African study on ferroalloy workers (Myers *et al*., 2003), serum PRL levels were not increased in workers chronically exposed to airborne manganese.

19 MERCURY (Hg)

Hg, the chemical symbol for mercury, stems from the name of the planet Mercury and is derived from the Latininized Greek word *hydrargyrum* ('liquid silver') 'water' and 'silver'. Mercury occurs as elemental mercury and as inorganic and organic compounds (mercury vapour, mercury liquid, mercury salts, short-chain alkylmercury compounds, alkoxyalkylmercury compounds and phenylmercury compounds), all having different toxicological properties.

Mercury is circulated naturally in the biosphere, with 30 000–50 000 tons being released into the atmosphere by degassing from the earth's crust and the oceans. In addition, 20 000 tons of mercury are released into the environment each year by human activities, such as combustion of fossil fuels and other industrial release. Annually, approximately 2000 tons of mercury is produced for industrial use, a small part of which is used for synthesizing organic mercury compounds. The world production of mercury for commercial uses has been slowly declining over the past 20 years. In nature, methylmercury (MeHg) is produced from inorganic mercury as a consequence of microbial activity. In mammals, oxidative demethylation occurs *in vivo* to produce inorganic mercury. In fish, the major amount of mercury is MeHg. Factors determining the MeHg concentration in fish are the mercury content in the water and bottom sediments, the pH, the redox potential of water, and the species, age, and size of the fish.

The toxicology and biological monitoring of Hg and its compounds has been reviewed by WHO/IPCS (1990a; 1991b) and more recently by Clarkson and Magos (2006) and by Berlin *et al*. (2007).

19.1 Uptake, Metabolism and Excretion

There are considerable differences in toxicokinetics among various mercury compounds. Mercury vapour is efficiently taken up (about 80%) by inhalation. Metallic mercury, if swallowed, passes the gastrointestinal tract almost without uptake. After uptake of mercury vapour, it is initially transported in blood, bound to erythrocytes, but is gradually oxidized to divalent mercury. Because of its lipid solubility, mercury vapour in blood is efficiently taken up by the brain. Higher brain levels and more toxicity to the brain thus occurs from exposure to mercury vapour compared to exposure to other inorganic forms of Hg. Divalent mercury compounds, if inhaled, are taken up to a considerable extent, but there is no specific information on this. When ingested, approximately 2% of these compounds are taken up in adults and probably a larger proportion in infants and children, as indicated by animal experiments. Divalent mercury, regardless of whether it is derived from mercury vapour or directly absorbed, is efficiently taken up in the proximal tubules. The hazards resulting from long-term intake of food containing MeHg and in occupational exposure to

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MeHg are due to the efficient absorption (90%) of MeHg in man and the long retention time (mean half-life of 65 days but up to 190 days in about 10% of a human population). With continuous long-term exposure there is an accumulation of MeHg in the brain. MeHg also crosses the placental barrier.

19.2 Toxic Effects and Dose–Response Relationships

The toxic properties of mercury vapour are due to mercury accumulation in the brain causing neurological signs, involving an unspecific psychoasthenic and vegetative syndrome (micromercurialism). At high exposure levels, mercurial tremor is seen, accompanied by severe behavioural and personality changes, increased excitability, loss of memory and insomnia. Mercurials may also affect other organ systems, such as the kidney. On a group basis, exposure levels are likely to be reflected in mercury concentrations in blood and urine. Occupational exposure to mercury concentrations in air $>0.1\,mg\,m^{-3}$ may produce mercurialism. Micromercurialism has not been reported at concentrations $<0.01\,mg\,m^{-3}$. Exposure to mercury vapour inhibits foetal brain development in primates. The exact dose–response relationship in humans is not known. Inorganic, but not MeHg, has been found to induce and bind to the low-molecular-weight metal-binding protein, MT. The acute and long-term actions of mercuric salts, phenylmercury compounds, and alkoxyalkylmercury compounds are likely to be gastrointestinal disturbance and renal damage, appearing as a tubular dysfunction progressing to tubular necrosis in severe cases. The lethal dose in man is approximately 1 g of mercuric salt. The mercury load on the kidney is best determined by analysis of renal biopsy. Mercury concentrations in the kidney between 10 and $70\,mg\,kg^{-1}$ have been reported in poison cases with renal injury. Levels $<3\,mg\,kg^{-1}$ may be found in normal cases. Occasionally, mercuric compounds may cause idiosyncratic skin symptoms, which may develop into severe exfoliative dermatitis or may cause glomerular nephritis. Animal and clinical observations have shown that mercuric mercury stimulates, and MeHg inhibits, the immune system, in a specific form of idiosyncrasy, called acrodynia or pink disease, seen in children. Most cases are associated with mercury exposure where increased levels of mercury in urine are observed.

Chronic poisoning by MeHg results in degeneration and atrophy of the sensory cerebral cortex, paraesthesia, ataxia, hearing and visual impairment. Prenatal exposure causes cerebral palsy and, in less severe cases, psychomotor retardation. MeHg concentration in blood and hair reflects the body burden and the MeHg concentration in the brain. Intake resulting in body burdens of $<0.5\,mg\,kg^{-1}$ body weight (bw) is not likely to give rise to detectable neurological signs in adults. This intake corresponds to blood values of $<200\,\mu g\,l^{-1}$ and mercury levels in hair of $<50\,mg\,kg^{-1}$. However, this level of MeHg exposure in pregnant women may result in inhibited brain development of the foetus with psychomotor retardation of the child. The highest level of MeHg load in pregnant women, not associated with inhibition of foetal brain development, is not known.

19.3 Carcinogenicity and Genotoxicity

IARC (1993) classified mercury and inorganic mercury compounds as Group 3 and MeHg compounds (evaluated as a group) as Group 2B carcinogens.

19.4 Mechanism of Action

Glomerular injury of the kidney subsequent to inorganic mercury exposure is due to an autoimmune reaction, with formation of an antigen against the glomerular membrane. Uptake of divalent Hg into the proximal kidney tubule takes place via the pathway of the amino acid cystine, because gluthathione complexes with mercury (II) are connected to cysteine complexes that mimic the amino acid cystine. Such uptake leads to tubular necrosis at higher levels of accumulation in the tubules (see Section 19.2). MeHg toxicity to the brain is considered to be related to the inhibition of tubulin polymerization in naive cells.

19.5 Biological Monitoring

Biological monitoring of mercury exposure is possibly by means of determination of Hg levels in blood, urine or hair. For exposures to mercury vapour, urine or whole-blood levels have been used. Relationships between urine Hg and neurological signs, that is, tremor have been described (Chaffin *et al.*, 1973) at urine levels of $10\,\mu g\,l^{-1}$. Urine levels of Hg usually are below $5\,\mu g\,l^{-1}$ among adults without exposure. For exposure to inorganic mercury compounds, urine Hg levels are also used. Dose–response relationships are, however, not well established for this less common exposure.

When there is exposure to MeHg, whole-blood erythrocytes or hair are the most useful indicators, because the highest levels of this mercury compound occur in these media, while serum or plasma and urine levels are low. There is a well-established relationship of 1/250

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between MeHg levels in human blood and hair. Levels of Hg in hair exceeding 10 mg kg^{-1} in pregnant women have resulted in adverse effects on the foetal brain, with increased occurrence of neurodevelopment deficits in their children (Debes *et al*., 2006).

Gellein *et al*. (2007) found Hg concentrations of 1.87 µg l^{-1} in whole blood and 0.5 µg l^{-1} in plasma and 2.53 µg g^{-1} (Gellein *et al*., 2008b) in hair among persons without excessive exposure, in Norway.

20 MOLYBDENUM (Mo)

Molybdenum comes from the Greek and means 'lead-like'. Molybdenum is an essential element for humans and dietary recommendations have been established. Dietary intakes in humans are usually within the range of the recommendations. The toxicology and biological monitoring were reviewed by Turnlund (2002) and by Turnlund and Friberg (2007).

20.1 Uptake, Metabolism and Excretion

Soluble molybdenum compounds are readily absorbed when ingested. The highest molybdenum concentrations are found in the kidneys, liver and bone. Excretion, primarily via the urine, is rapid. The biological half-life ranges from a few hours to a few days. Turnover is much more rapid when intake is high than when intake is low. The metabolism of molybdenum is affected by copper and sulphur intake in some species. In ruminants, copper generally has a beneficial effect on the symptoms caused by excessive molybdenum, and thiomolybdates increase the excretion of copper. Both positive and negative effects of the interaction between these three elements have been reported. The effects and their magnitude vary between animal species.

20.2 Toxic Effects and Dose–Response Relationships

In livestock, chronic molybdenum poisoning known as 'teart disease' is caused by a diet high in molybdenum and low in copper. Symptoms include anaemia, gastrointestinal disturbances, bone disorders and growth retardation. In laboratory animals, excessive molybdenum may give rise to morphological and functional changes in the liver, kidneys and spleen. It has a growth-depressing action and deformities of bone may occur.

A few cases of pneumoconiosis have been reported among workers exposed to metallic molybdenum and molybdenum trioxide. Increased blood uric acid values and gout-like symptoms have been reported among workers exposed to molybdenum in a copper-molybdenum plant, as well as among the general population living in an area with high molybdenum and low copper contents in soil and vegetables.

20.3 Mechanism of Action

Molybdenum is important for enzymes such as the mammalian molybdoenzymes, which are involved with the catabolism of sulphur amino acids, purines and pyrimidines; defects in these enzymes result in changes in metabolites. *S*-sulfocysteine, taurine, xanthine and hypoxanthine increase and uric acid is low (Turnlund, 2002).

20.4 Biological Monitoring

So far, no biochemical marker for molybdenum status has been identified. Levels in many tissues and excretion reflect dietary intake in animals and humans. However, none of these has yet been directly related to molybdenum status (Turnlund and Friberg, 2007). In normal adults, median values for the liver, kidneys and adrenal glands have been found to be approximately 1, 0.3 and 0.7 mg kg^{-1} wet weight, respectively. Data on daily urinary excretion of molybdenum in 'normal' human subjects have been presented in groups varying between 49 and 71 µg day^{-1}. Urinary excretion is directly related to recent dietary intake (Turnlund *et al*., 1995). However, the values do not necessarily reflect molybdenum status. A wide range of plasma molybdenum concentrations were reported before 1973; however, more recent estimates on the basis of NAA and ICP-MS resulted in concentrations of from 0.3 to 1.1 µg l^{-1} (Versieck and Cornelis, 1980). As with urinary excretion, blood plasma concentrations also reflect recent dietary intake, but not molybdenum status. They are considerably higher, from 2 to 4 µg l^{-1}, when intake is unusually high, from 500 to 1500 µg day^{-1} (Turnlund and Keyes, 2004).

21 NICKEL (Ni)

The German word *Kupfernickel* meaning 'Old Nick's (Satan's) copper' is considered as the origin of the name, nickel. Nickel is widely used in steel and alloy production, electroplating, in coins, household items and so on. It is an essential metal for the growth of bacteria and for lipid metabolism in rats, but its essentiality for humans has not been finally established. Its toxicology and biological monitoring was reviewed by Lu *et al*. (2005) and by Klein and Costa (2007).

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21.1 Uptake, Metabolism and Excretion

Ni is absorbed to a very limited extent from food, approximately 1%, but much more from drinking water. It is taken up via inhalation to an extent of approximately 20%. Ni uptake through the membranes is facilitated by transport proteins like DMT1. It binds to transferrin and competes with iron. Ni is efficiently excreted via urine and is poorly retained in tissues, with a biological half-life of approximately one day. The highest levels are found in the kidneys.

21.2 Toxic Effects and Dose–Response Relationships

Acute nickel poisoning can occur as a result of inhalation of nickelcarbonyl ($NiCO_4$). Initial symptoms are modest, but after two to three days progressive cough, dyspnoea, pneumonia and respiratory distress are seen. There may also be myalgias, delirium and convulsions.

Less severe respiratory symptoms related to long-term inhalation exposures to Ni dust have been reported in workers. Long-term exposures may give rise to pulmonary fibrosis and pneumoconiosis. Perforation of the nasal septum is common in Ni workers. Dermal Ni exposure may give rise to contact allergy. Ni allergy is a very common disorder affecting as much as 30% of the population in industrialized countries, particularly among women who have previously had skin piercing. Nickel crosses the placental barrier and is teratogenic in animals. Limited human evidence indicates an increased rate of spontaneous abortions among women Ni workers.

21.3 Carcinogenicity and Genotoxicity

The IARC concluded, in 1990, that metallic nickel and alloys, were human carcinogens Group 2B, and the majority of animal data *in vivo* and genetic toxicology data *in vitro* prior to that time suggested that the insoluble particulate nickel species were the most carcinogenic; however, more recent human epidemiology and experimental data is pointing to the water-soluble nickel compounds as perhaps of equal hazard. Accumulating molecular and genetic toxicology data show that soluble nickel chloride ($NiCl_2$) can in fact, be mutagenic and genotoxic, especially when cells are chronically exposed for long durations up to several weeks to allow sufficient nickel uptake. Upon reconsideration of the previous experimental studies with shorter 24–48 hour acute exposures to the insoluble nickel compounds *in vitro*, it is likely that the actual nickel exposures were in fact of much longer duration than previously thought, owing to the insufficient removal of adherent nickel particles from the cell surfaces.

21.4 Mechanism of Action

Nickel (Ni(II)) can generate ROS and activates a number of cellular signal-transduction pathways. The newest information on the molecular mechanisms of nickel's action in biological systems has been summarized by Davidson *et al.* (2007).

21.5 Biological Monitoring

Both serum and urinary nickel measurements can be useful monitors of environmental or occupational exposures. Precautions have to be taken in blood sampling to avoid contamination from stainless-steel needles. Normal levels of nickel in unexposed adults are reported to be in the range of 0.05–1.1 μg Ni l^{-1} serum and a mean value of 1.5 μg g^{-1} creatinine in urine (WHO/IPCS, 1991a). In a study of Finnish factory workers (Kiilunen *et al.*, 1997), after-shift urinary concentrations of nickel were 0.1–2 μmol l^{-1} and were still elevated after a two to four week vacation. However, concentrations of nickel in urine in this study showed no correlation with nickel concentrations in the air. In a Norwegian study of potential nickel exposure from nearby Russian refineries, nickel levels in the urine of residents living near the refineries were significantly lower than for urban residents living away from the refineries in areas of high vehicular traffic (Smith-Sivertsen *et al.*, 1997). In flux-core-wire (FCW) stainless-steel welders exposed to workplace air levels of nickel of 50.4 μg m^{-3} (ranging from <2 to 416.7 μg m^{-3}), whole blood and plasma levels were found to be low at 0.84 μg l^{-1} and 0.57 μg Ni l^{-1} and urine levels were also very low at 2.5 μg g^{-1} creatinine (Stridsklev *et al.*, 2004). These values were all near the current detection limit ranges for Ni in whole blood (0.48–1.5 μg Ni l^{-1} whole blood), plasma (0.3–0.9 μg Ni l^{-1}) and urine (0.3 μg g^{-1} creatinine). Although there were no associations between the Ni levels in air and in biological fluids; statistically significant associations between Ni levels in whole blood and plasma before and after work were found. Compared with manual metal arc welding (Stridsklev *et al.*, 1993), the levels of nickel in biological fluids were the same or slightly higher for FCW welders. When measuring blood, plasma and serum levels of nickel, investigators must consider the use of non-stainless-steel needles, because of the potential for unintended Ni contamination of the samples. Nickel measurements in smokers' blood range from 0.01 to 0.42 μg Ni l^{-1}, not much higher than

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in nonsmokers' blood (0.01–0.26 μg Ni l^{-1}), whereas nickel in the urine of smokers (<0.01–8.20 μg l^{-1}, median) is significantly higher than nonsmokers (<0.01–4.60 μg Ni l^{-1}) (Stojanović *et al*., 2004).

22 PALLADIUM (Pd)

Palladium is named after the asteroid Pallas, which had been discovered at the beginning of the 1800s, just before Pd. Palladium is one of the six platinum group metals and possesses strong catalytic activity. Demand for palladium is high for its uses in electrical equipment, dental materials and automobile catalysts. Environmental levels of palladium in water, soil and ambient air are not high, and environmental exposure and intake from food are not significant. However, palladium concentrations are increasing in the general environment because of its increased use in automobile catalysts. Workers in palladium mines and refineries are possibly exposed to palladium, as are dental personnel during the processing of dental alloys containing palladium. The toxicology and biological monitoring were reviewed by WHO/IPCS (2002) and more recently by Satoh (2007).

22.1 Uptake, Metabolism and Excretion

The general population may be exposed to palladium through inhalation of ambient respirable particles from automobile catalytic converters that incorporate palladium, but relevant data are rare. Among the general population, skin exposure may occur through contact with jewellery containing palladium.

Uptake of palladium ions via the gastrointestinal tract ranges from 0.5–4% in animal experiments. Aqueous aerosols of ionic palladium are taken up to a greater extent. After uptake, Pd is distributed to the kidneys, liver, lymph nodes and spleen. Placental transfer is limited. Excretion is via urine to a considerable extent.

22.2 Toxic Effects and Dose–Response Relationships

The oral toxicity of palladium is believed to be low, although it does depend on the water solubility of the palladium compounds. Therefore, similar intravenous LD_{50} values have been reported for several palladium compounds: from 3 to 6.4 mg kg^{-1} bw. From a 28 day toxicity study of tetraamine palladium hydrogen carbonate in rats, performed through gavage, the no observed adverse-effect level (NOAEL) was 1.5 mg (kg bw)$^{-1}$ day^{-1}. Many case reports exist describing palladium sensitivity and recovery after removal of dental restorations. The symptoms that have been observed include swelling of the lips and cheeks, stomatitis, oral lichen planus, itching, dizziness, asthma and chronic urticaria. ^{103}Pd has been used as a radioactive source implanted directly into a malignant tumour; no palladium-related complications have been reported. Although sensitization effects, such as bronchial asthma, can occur among workers exposed to palladium and its compounds, its incidence is extremely low.

22.3 Biological Monitoring

Studies in Germany gave levels of 0.03–0.2 (mean, 0.1) μg l^{-1} for 24 hour urine samples from 14 nonsmokers (Schramel *et al*., 1997), 0.02–0.08 μg l^{-1} (mean, 0.04) for 24 hour urine samples (Begerow *et al*., 1997c), 0.03–0.08 μg l^{-1} (mean, 0.05) for whole blood (Begerow *et al*., 1997a), 0.03–0.22 μg l^{-1} (mean, 0.14) for 24 hour urine samples (Begerow *et al*., 1997b) and slightly lower values of 0.013–0.048 μg l^{-1} (mean, 0.031) for morning urine. Urine levels among groups living in areas having different automobile traffic densities did not display a definite difference.

23 PLATINUM (Pt)

The word *platinum* comes from the Spanish word *platina*, meaning 'little silver'. Commercial sources of platinum are sulfide and arsenide minerals, and, increasingly, recycled platinum. The main source of platinum in the environment is release from vehicle catalysts, and platinum concentrations near highways and urban areas have gradually increased. In occupational exposure, the form of platinum is mainly coordination complexes, most often with chlorine as a ligand, whereas the platinum released from automotive catalysts is metallic or oxidic. Kiilunen and Aitio (2007) have recently reviewed the toxicology and biological monitoring of Pt.

23.1 Uptake, Metabolism and Excretion

Animal experiments with intratracheal instillation of platinum-coated aluminium particles less than 5 μm (a model aerosol for automobile exhaust) showed that 97% of the dose was excreted in faeces the first two days. After day 5 there was also some urinary excretion. Oral dosing of platinum(IV) chloride in rats showed a

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limited systemic uptake (2%). After intravenous administration or administration by other routes, Pt mainly accumulates in the kidneys. Systemically absorbed Pt is excreted in urine and faeces. Distribution and excretion of *cis*-platinum used in chemotherapy is different, with faster initial clearance from blood plasma, but *cis*-platinum also accumulates in kidneys.

23.2 Toxic Effects and Dose–Response Relationships

The acute toxicity of platinum compounds depends mainly on their solubility, soluble platinum salts being more toxic than compounds with lower solubility, such as oxides. Chloroplatinates are irritating to the eye and skin. The main health effect of platinum compounds is sensitization; the symptoms of platinum-salt sensitivity include irritation of the eyes and upper respiratory tract, and asthma. The allergenic potency of platinum compounds seems to be limited to coordination complexes containing halogen ligands as leaving groups, hexa- and tetrachloroplatinates being the most potent sensitizers. Neutral coordination complexes such as diamminedichloroplatinum do not seem to be allergenic. Bromine and iodine analogues of platinates may cause sensitization, but seem to be less potent.

23.3 Carcinogenicity and Genotoxicity

With the exception of platinum-containing chemotherapeutic drugs such as cisplatin (*cis*-diamminedichloroplatinum), no relevant human or experimental data are available on the potential carcinogenicity or teratogenicity of platinum compounds. Cisplatin and other platinum-containing chemotherapeutic drugs are genotoxic in a variety of test systems, and other platinum compounds have induced mutations *in vitro* (but not *in vivo*).

Platinum compounds used as anticancer drugs, such as cisplatin and its analogues, are not covered in detail in this review. However, cisplatin was classified as a Group 2A carcinogen (IARC, 1981; 1987) and in combination with certain agents as a Group 1 carcinogen, according to IARC (2000).

23.4 Mechanism of Action

The mechanism of platinum-salt allergy is likely to be Type 1 (i.e. involves IgE). Atopic constitution is not associated with susceptibility to platinum sensitization. Smokers have a higher risk of platinum sensitization. No health effects from environmental exposure to platinum have been reported.

23.5 Biological Monitoring

Previous studies have reported high concentrations for platinum in biological fluids, but are not considered reliable. Dental gold alloys were found to increase urinary and saliva platinum concentrations (mean age, 35.9 years, 326 urine samples, median 9.3 ng g^{-1} creatinine with dental gold inlays and 5.3 ng g^{-1} creatinine without dental inlays, $p < 0.001$, and 5–2770 pg g^{-1} saliva, median 160 pg g^{-1} and 4.8–26 pg g^{-1} saliva, median 8.1 pg g^{-1} saliva, $p < 0.001$) (Schierl, 2001). Also, the German Environmental Survey (GerES III) from 1998 (Becker *et al*., 2003) found a positive relation between the platinum levels in urine and teeth with dental inlays, crowns and bridge elements, whereas road traffic was related to urinary platinum concentrations. The geometric mean value for all occupationally unexposed persons (age, 18–69 years, $n = 1080$) was 2.18 ng l^{-1} (1–75 ng g^{-1} creatinine) and the 95% CI from the log-normal distribution was 2.01–2.36 ng l^{-1}; (1.60–1.90 ng g^{-1} creatinine). Those with no dental inlays ($n = 509$) had a geometric mean platinum concentration in urine of 1.32 ng l^{-1} (0.99 ng g^{-1} creatinine) with 95% CI of 1.1–1.47 ng l^{-1} (0.89–1.11 ng g^{-1} creatinine). From Italy, Iavicoli *et al*. (2004) reported a mean urinary platinum concentration of 4.56 ng l^{-1} in 58 people. They found a statistical difference between females and males (4.09 vs. 5.77 ng l^{-1}, $p = 0.004$). The subjects older than 40 years of age ($n = 28$) showed statistically significantly higher mean platinum concentrations in urine than those of younger colleagues ($n = 21$, $p = 0.03$). In children, the Pt concentrations were 0.9 ± 1.1 ng g^{-1} creatinine, and they were not associated with traffic density in the area of residence (Caroli *et al*., 2001).

In Budapest and Vienna, platinum concentrations in the urine of adult inhabitants without occupational exposure were on average 10.1 ± 8.8 and 3.7 ± 4.3 ng g^{-1} creatinine ($n = 100$), respectively (Záray *et al*., 2004).

The serum platinum concentration in Germany in nonexposed persons was 6.35 ng l^{-1} (median; range, <5.0–42.1 ng l^{-1}, number of subjects not given) (Merget *et al*., 1999). In Italy, the blood platinum level was 10 ± 2 ng l^{-1}, in urine the level was 5 ± 0.1 ng l^{-1} and in hair 50 ± 8 ng kg^{-1} ($n = 25$) (Petrucci *et al*., 2005).

24 SELENIUM (Se)

The name is derived from the word *selene*, the Greek name for moon. The element was associated with

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tellurium, named after *Tellus*, the Earth. Selenium is an essential trace element and has been shown to be a natural component in the enzymes glutathione peroxidases (GSH-Pxs 1–5), iodothyrodine deiodinases, thioredoxin reductases, selenoprotein P and other proteins, 25 altogether. The toxicology and biological monitoring of selenium has recently been reviewed by Högberg and Alexander (2007).

24.1 Uptake, Metabolism and Excretion

Most selenium inorganic and low-molecular-weight compounds are water soluble and can efficiently be taken up in the intestine. Soluble, as well as nonsoluble, compounds can be taken up by the lungs. Selenium occurs as cysteine in selenoproteins, whereas selenomethionine can non-specifically substitute for methionine in proteins. The selenium requirement to compensate minimal losses in man is 50–70 μg day^{-1}.

When given in excess, selenium compounds are rapidly distributed to major organs of the body. In the liver, many selenium compounds are biotransformed to excretable metabolites. Identified metabolites include selenosugars in urine and dimethylselenide in breath. Biotransformation seems to be a major mechanism by which selenium homeostasis is maintained during excessive exposure, and there is a rapid phase and a slow phase of elimination. The half-life of the rapid phase is one to three days, depending on the compound ingested, and for the slow phase it is 30–110 days. Blood levels are approximately 0.027 μg ml^{-1} in geographical areas with low selenium intake, and an intake of about 90 μg day^{-1} would correspond to approximately 0.11 μg ml^{-1}.

24.2 Toxic Effects and Dose–Response Relationships

Toxic effects have been seen at blood levels ranging from 0.179 to 7.5 μg ml^{-1}. In most parts of the world, normal urine levels are 0.03 mg l^{-1}. Occupationally exposed workers usually excrete <0.1 μg ml^{-1}.

The LD_{50} ranges between 1.5 and 6 mg (kg bw)$^{-1}$ for many selenium compounds and animal species. The CNS seems to be the target organ at these dose levels, but the liver, heart and lungs may also be affected. Cases of acute selenium poisoning in humans have been described, a few with fatal outcomes. These cases occurred either after consumption of selenium or after exposure through inhalation. Gastrointestinal and neurological symptoms predominated.

Chronic poisoning caused by long-term exposure has been reported in livestock and humans from geographical areas where soil contains high levels of selenium. In rodents, liver cirrhosis is a common effect, whereas typical effects in domestic animals are emaciation, deformation of hooves, loss of hair and joint erosions. In humans consuming 1–5 mg Se day^{-1}, hair and nail problems have been reported, and are very common at 5 mg Se day^{-1}. An intake of approximately 1200 μg day^{-1} is the LOAEL for clinical selenosis, and 850 μg day^{-1} can be taken as a NOAEL for clinical selenosis. However, a clinically insignificant rise of serum alanine aminotransferase (ALAT) has been reported at slightly lower doses. Other adverse effects among a few individuals in larger study cohorts have also been reported at lower doses. Skin lesions and depigmentation are also common signs of intoxication. In more severe cases, neurological and gastrointestinal symptoms predominate. Death, albeit rarely, has been associated with chronic selenium poisoning.

Teratogenicity has been observed, particularly in avian species and fish. Selenite in hamsters and mice and selenomethionine in macaques were essentially negative. Selenium may prevent or alleviate toxic effects of arsenic, cadmium, mercury, platinum and silver. Conversely, some of these metals protect against selenium toxicity.

24.3 Carcinogenicity and Genotoxicity

Selenium and selenium compounds are classified as Group 3 carcinogens (IARC, 1987). Although several selenium compounds have been tested for possible carcinogenic potential, only selenium sulfide in large oral doses has shown convincing carcinogenic effects. Conversely, some of these compounds have been shown to prevent the development of cancer. Some selenium compounds can cause DNA damage, probably by inducing oxidative stress.

24.4 Biological Monitoring

The reference urinary level should not exceed 0.03 μg ml^{-1} (Robberecht and Deelstra, 1984). It is recommended that selenium in urine is measured in 24 hour samples or is related to the creatinine level. This is also a biomarker of exposure.

Urinary levels in workers exposed to 0.2–0.4 mg Se m^{-3} were usually <0.1 mg l^{-1}. At air concentrations as high as 3.6 mg m^{-3} times the urinary level averaged 0.25–0.45 mg l^{-1}. A garlic odour on the breath usually accompanied these higher levels (Glover, 1967).

Toxic effects of selenium are reflected by increased blood, urine and hair levels. In China, people consuming selenium-rich food and exhibiting signs of selenosis had

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mean levels in blood, urine and hair of 3.2 μg ml^{-1}, 2.7 μg ml^{-1} and 32.2 μg g^{-1}, respectively. The corresponding values in people from a high selenium area without selenosis were 0.44 μg ml^{-1}, 0.14 μg ml^{-1} and 3.7 μg g^{-1} in blood, urine and hair, respectively. In selenium-adequate areas, the levels were 0.095 μg ml^{-1}, 0.026 μg ml^{-1} and 0.36 μg g^{-1} in blood, urine and hair, respectively (Yang *et al*., 1983).

Supplementation of the diet with yeast or wheat, so that 200 μg Se day^{-1} was consumed, resulted in an increased plasma level from 0.07 to 0.17 μg ml^{-1} within 11 weeks (Levander *et al*., 1983). Supplementation with selenate was less efficient. On a low-selenium diet (33 μg day^{-1}), the level in breast milk was 5.8 μg l^{-1}. At a higher dietary level (50 μg day^{-1}), the milk level was 10.0 μg l^{-1} (Kumpulainen *et al*., 1984). Many supplementation studies have been carried out since then.

The highest levels are often achieved on supplementation with selenomethionine, a common form in plants, which is nonspecifically incorporated into proteins. The efficiency of selenium compounds to raise plasma selenium levels also depends on the selenium status (Alexander and Meltzer, 1995; Rayman, 2004). In deficiency, selenium in blood or plasma is decreased.

25 SILVER (Ag)

Silver has been known since ancient times. The symbol 'Ag' is from the Latin for 'silver', *argentum.*

The toxicology and biological monitoring has recently been reviewed by Holler *et al*. (2007).

25.1 Uptake, Metabolism and Excretion

Silver compounds may be absorbed to varying degrees through inhalation. Silver salts are absorbed by up to 10–20% after ingestion. The highest concentrations of silver are usually found in the liver and spleen, and to some extent in the muscles, skin and brain, after ingestion. The biological half-life for silver ranges from a few days for animals up to approximately 50 days for the human liver; it is possible that skin deposits have an even longer half-life, but there are no quantitative data on this for man. Silver binds to high-molecular-weight proteins and MT in tissue cytosol fractions. Excretion of silver from the body is primarily biliary.

25.2 Toxic Effects and Dose–Response Relationships

Water-soluble silver compounds, such as the nitrate have a local corrosive effect and may cause fatal poisoning if swallowed accidentally. Chronic exposure of humans leads to argyria, a clinical entity characterized by grey-blue pigmentation of the skin and other body viscera. Repeated exposure of animals to silver may produce anaemia, cardiac enlargement, growth retardation and degenerative changes in the liver.

25.3 Biological Monitoring

Plasma samples from an unexposed population were all <1 μg l^{-1} (Wan *et al*., 1991). Tipton *et al*. (1966) found between 0.006 and 0.015 mg day^{-1} in urine and between 0.02 and 0.11 mg day^{-1} in faeces. Wester (1971), by use of NAA, found 0.9–1 μg day^{-1} in urine and 0.9–97 μg day^{-1} in faeces of two patients with pancreatic insufficiency. In urine, Bostrom and Wester (1968) found 1 μg day^{-1} in normal persons and 0.7–7.5 μg day^{-1} in patients with untreated hyperthyroidism. Wan *et al*. (1991) observed urinary excretion levels in a control group generally to be <2 μg day^{-1}.

26 TELLURIUM (Te)

Tellurium stems from Latin *tellus* meaning 'earth'. The main source of exposure in the general population is food, for example, meat, dairy products and cereals. The toxicology and biological monitoring was recently reviewed by Gerhardsson (2007).

26.1 Uptake, Metabolism and Excretion

In the work environment, inhalational exposure predominates. Small amounts of organic tellurium compounds can also be absorbed through the skin. There are no quantitative data on absorption of tellurium or tellurium compounds in humans. In animals, the intestinal absorption has been estimated to range between 10 and 25%. The highest tissue concentrations have been observed in the kidneys. Increased levels have also been noted in blood, heart, lungs, liver, spleen, muscle and bone. The main accumulation is in bone, which harbours >90% of the total body burden. Tellurium can pass both the placenta and the blood–brain barrier. Parenterally administered tellurium is predominantly excreted in the urine, whereas orally ingested tellurium salts through biliary secretion are mainly excreted in the faeces. Small amounts, probably approximately 0.1%, of absorbed tellurium are exhaled, presumably as dimethyltelluride. In rat experiments, biological half-lives ranging from nine days in blood to 23 days in kidney have been reported.

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The whole-body retention model for man estimates a biological half-life of approximately three weeks. The elimination from bone is slow, with an estimated half-life of approximately 600 days.

26.2 Toxic Effects and Dose–Response Relationships

Acute systemic effects of tellurium toxicity in rats include listlessness, decreased locomotor activity, somnolence, anorexia, weight loss, gastrointestinal disturbances, changes in fur, and, occasionally, epilation and hind-leg paralysis. Long-term studies of chronic effects are sparse. Dominant and critical effects have been reported from the nervous system, including peripheral neuropathy, characterized by segmental demyelination and minor axonal degeneration. In the brain, black changes caused by dark tellurium particles that are localized in lipofuscin granules in neuron cytoplasm have been observed. Other effects have been reported from the liver (fatty degeneration and necrosis), kidney (proximal tubular lesions, oliguria, or anuria) and heart (cell necrosis, oedema and congestion). Reproductive effects, including, for example, hydrocephalus, oedema, exophthalmia and ocular haemorrhage have been described.

Acute exposure to tellurium in occupational settings may cause acute respiratory irritation followed by the development of a garlicky odour on the breath and sweat, drowsiness, headache, malaise, lassitude, weakness and dizziness. Gastrointestinal symptoms such as anorexia, nausea, vomiting, metallic taste, dry mouth and constipation may appear. Dermatitis and blue-black discoloration of the skin may follow from exposure to tellurium hexafluoride. Severe intoxication may lead to depression of the respiratory system and circulatory collapse. No specific antidote for tellurium poisoning has been found.

26.3 Biological Monitoring

Blood and urinary analyses may be used for biological monitoring as biomarkers of exposure, but data are very scanty. Tellurium values for normal subjects have been reported for blood $0.15–0.3\,\mu g\,l^{-1}$ (van Montfort *et al.*, 1979) and urine $<0.1–10\,\mu g\,l^{-1}$ (Fodor and Barnes, 1983; Kobayashi and Imaizumi, 1991). Only few and incomplete data are, however, available from the working environment, regarding the relationship between tellurium in air, blood and urine. Quantitative data on the relationship between internal dose and adverse health effects are still lacking and, thus, risk assessment cannot be carried out. An early and classical sign is the garlicky odour on the breath, which may appear after ingestion of $40\,\mu g$ of soluble tellurium.

27 THALLIUM (Tl)

Thallium originates from Greek, *thallos*, and means a green shoot or twig. The name comes from thallium's bright green spectral emission lines. The toxicology and biological monitoring have been reviewed by WHO/IPCS (1996) and more recently by Kazantzis (2007).

27.1 Uptake, Metabolism and Excretion

After rapid, almost total, absorption from the gastrointestinal tract, soluble thallium compounds are widely distributed in the body, the highest concentration being accumulated initially in the kidneys. Excretion occurs through urine and faeces, the disappearance of thallium from the tissues following first-order kinetics. The biological half-life of ^{204}Tl in the rat has been calculated at three to four days. In man, increased concentrations of thallium may be found in urine and faeces for several weeks after absorption. Excretion also occurs through hair, which, in unexposed subjects, has been shown to contain the highest concentration of thallium in any tissue. In the rat, 21 days after dosing by parenteral or oral routes, up to 60% of the remaining body burden was found in the hair.

27.2 Toxic Effects and Dose–Response Relationships

Thallium has caused acute and often fatal poisoning as a result of unintentional, criminal or suicidal ingestion. Fatalities have also occurred after the now obsolete therapeutic administration of thallium. Gastroenteritis, collapse and later peripheral neuropathy are the principal features in acute poisoning, but, with longer survival, alopecia becomes a characteristic feature after two to three weeks. In chronic poisoning, the main features are vague ill health, paraesthesias and, in some cases, loss of hair; this pattern is seen after low-level occupational exposure. The widespread use of thallium as a rodenticide has been responsible in some countries for the death of domestic and wild animals and their natural predators. Dose–response relationships for symptoms of adverse health effects from thallium exposure in the general environment are discussed in Section 27.4.

27.3 Mechanism of Action

There are similarities between the ionic transport of thallium and of potassium through cell membranes, but

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once intracellular, thallium is less rapidly released than potassium.

27.4 Biological Monitoring

Thallium intake in polluted areas may exceed the normal dietary intake in the general population, estimated to be $<5\,\mu g\ day^{-1}$, mostly from foodstuffs, which does not constitute a threat to health. Where environmental monitoring shows thallium levels significantly above background, biomonitoring of the population should be carried out and where this reveals evidence of excessive exposure, emissions should be reduced.

Weinig and Zink (1967) determined the thallium concentration in early-morning urine samples in nine subjects to range from 0.13 to $1.69\,\mu g\,l^{-1}$. In three carefully controlled population-based studies involving a total of 686 unexposed subjects, the range of urinary thallium concentrations was $0.06–1.2\,\mu g\,l^{-1}$, with a mean thallium urinary concentration of $0.3–0.4\,\mu g\,l^{-1}$. With a short biological half-life and an assumed steady state in such population-based samples, the urinary excretion value can be considered as an indicator of total dose after inhalation and total dietary intake (WHO/IPCS, 1996). The concentration of thallium in whole blood in a population of 320 children in New Jersey was measured by AAS. Values ranged between nondetectable ($<5\,\mu g\,l^{-1}$) and $80\,\mu g\,l^{-1}$, with a mean concentration of $3.0\,\mu g\,l^{-1}$. Approximately 80% of the children showed no detectable thallium in the blood, whereas 17.5% had between 5 and $20\,\mu g\,l^{-1}$. Five children (1.6% of the total group) had blood thallium levels between 40 and $80\,\mu g\,l^{-1}$, but all were without evidence of thallium toxicity (Singh *et al*., 1975).

In a population sample living in the vicinity of thallium emission into the atmosphere, the mean urinary thallium concentration was $5.2 \pm 8.3\,\mu g\,l^{-1}$, with a range of $0.1–76.5\,\mu g\,l^{-1}$. From a questionnaire, a clear dose–response relationship was identified between thallium concentrations in urine and the prevalence of tiredness, weakness, sleep disorders, headache, nervousness, paraesthesia, and muscle and joint pain, with a similar dose–response relationship when thallium in hair was taken as an indicator of exposure (WHO/IPCS, 1996).

From these limited studies, it has been suggested that an approximately 15-fold increase in urinary thallium excretion above the mean unexposed level of $0.3–0.4\,\mu g\,l^{-1}$ may be related to subjective symptoms that could possibly be considered as early adverse effects. The WHO Task Group (WHO/IPCS, 1996) considered that exposures with urinary thallium concentrations $<5\,\mu g\,l^{-1}$ are unlikely to cause adverse health effects. In the range of $5–500\,\mu g\,l^{-1}$, the magnitude of risk and severity of adverse effects are uncertain, whereas exposures with urinary thallium concentrations $>500\,\mu g\,l^{-1}$ have been associated with clinical poisoning.

Extensive data on thallium levels in persons from the general population is available from the NHANES studies in the United States. Thallium levels in urine, blood and hair are useful biomarkers of exposure to thallium.

28 TIN (Sn)

Tin stems from the Latin *stannum* and is one of the earliest metals known and used as a component of bronze, as early as 3500 BC, even if not used until about 600 BC as just tin. Tin is not an essential metal and is widely used in industry. In nature, it occurs both in inorganic and organic forms. The toxicology and biological monitoring was reviewed by WHO/IPCS (1980; 1990d) and more recently Ostrakhovitch and Cherian (2007).

28.1 Uptake, Metabolism and Excretion

The gastrointestinal absorption of soluble tin salts is only a few % of the ingested dose. In chronic exposures, bone is the major storage organ for tin, and its biological half-life in bone is approximately 100 days. The excretory routes of tin compounds may vary, depending on the type of tin compounds and the mode of exposure.

28.2 Toxic Effects and Dose–Response Relationships

Most of the inorganic tin compounds are nontoxic because of their low solubility and absorption. In workers who inhale tin oxide, it accumulates in the lung and causes ‘stannosis’, a benign pneumoconiosis without any tissue reaction or pulmonary dysfunction. Although insoluble tin compounds are nontoxic, soluble tin compounds can be toxic. Oral administration of $45\,mg\,kg^{-1}$ of stannous chloride can induce vomiting and diarrhoea in cats. In human, high intakes of inorganic tin compounds can cause abdominal pain and anaemia. Inhaled tin hydride gas can cause damage to nerves. Epidemiological study shows a positive relationship of increased tin intake to a decrease in copper status, changes in cholesterol metabolism and ischaemic heart disease. Tin can be converted by chemical and biological reactions in the environment to form more toxic organotin compounds. Short-chain alkyltin compounds, such as trimethyl and triethyl tins are well absorbed from the gastrointestinal tract, whereas long-chain alkyltin compounds are not much absorbed. The toxicity of organotin compounds differs, depending on their physicochemical properties. Alkyl and aromatic tin compounds are neurotoxicants. Triethyltin can cause encephalopathy and cerebral

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oedema. Severe dermatitis has been described in workers exposed to tributyltin and painters applying formulations. Tributyltin is widely used as an antifoulant in marine paints to prevent any growth on ocean vessels. However, the use of tin-containing paints for smaller marine vessels is restricted in many countries.

28.3 Carcinogenicity and Genotoxicity

Tin as tannic acid and tannins are classified as Group 3 agents by IARC (1987). Organotin compounds can penetrate cell membranes because of their lipophilicity and cause damage to cell membranes, interrupt oxidative phosphorylation and damage mitochondria. They can inhibit synthesis of haem oxygenase and can be immunotoxic and genotoxic. There is no definite evidence that inorganic or organic tin compounds can cause carcinogenic or teratogenic effects, although in a few studies, effects have been reported. Tributyltin and triphenyltin are implicated in endocrine disruptions, and hence cause reproductive failure in marine organisms.

28.4 Biological Monitoring

The average concentration of tin in urine is approximately 4.2–42.2 nmol l^{-1} and in hair is approximately 0.42–3.37 nmol g^{-1}. The average concentration of tin in blood of normal subjects is 0.14 mg l^{-1} and is found mainly in the erythrocytes (Baselt and Cravey, 1989). Tissue analysis has shown that tin is present in lungs, adrenal glands and liver at concentrations of 37, 23 and 23 mg kg^{-1}, respectively. The highest amount of tin (in dry ash) has been found in the cecum (130 mg kg^{-1}), ileum (79 mg kg^{-1}), rectum (57 mg kg^{-1}) and sigmoid colon (45 mg kg^{-1}) (Schroeder *et al*., 1964). Inorganic tin does not accumulate in soft tissues with increasing age (Baselt and Cravey, 1989). Concentrations of organotin compounds in organisms are very high near sources such as ports, pleasure-boat marinas, shipyards and much-travelled shipping routes. The bioconcentration factors for triphenyltin have been found to be 2090 in carp kidney and in the range of 80 000–440 000 in the crab hepatopancreas (Kannan *et al*., 1995b; Tsuda *et al*., 1987). The levels of triphenyltin in mussels and samples of sea birds from coastal areas of Japan are approximately 0.45 and 0.05 μg g^{-1}, respectively. In fish from the same coastal areas, the maximum concentration of triphenyltin has been reported to be 2.6 μg g^{-1} in 1989 and 0.25 μg g^{-1} in 1995. Butyltin compounds in the tissue of mussels from Egremont are in the range 0.217–0.508 mg kg^{-1} dry wt, slightly higher than the range of 0.11–0.201 mg kg dry wt at New Brighton (Harino *et al*., 2005). The concentration of tributyltin reached 1000 ng kg^{-1} in bream muscles from the Elbe estuary (Shawky and Emons, 1998). The concentration of trisubstituted organotins is higher in digestive glands than in other tissues.

Tributyltin concentration in digestive tissue and the gills has been reported to be twice as high as in mantle and muscle (Laughlin *et al*., 1986; Shim *et al*., 1998). The average estimated level of triphenyltins in fish and other marine products ranges from 0.03 to 1.3 mg kg^{-1} (Ishizaka *et al*., 1989). In fish from sampling sites of the North Sea, concentrations of tributyltin range from 27–202 ng g^{-1} in muscles and 54–223 ng g^{-1} in liver (Shawky and Emons, 1998). Concentrations of tributyltin in the muscle of fish from the Gulf of Mexico were from 0.1 to 0.196 mg kg^{-1}, whereas in fish from Switzerland, the concentration reported is 0.17 mg kg^{-1} (Fent and Hunn, 1991). A recent study from Denmark determined the levels of total butyltin in shellfish and fish, and they were approximately 10–440 ng g^{-1} (Nielsen and Strand, 2002). It was shown that concentrations of butyltin compounds in the liver of dolphins from the Italian coast of Mediterranean Sea were in the range of 1.2–2.2 μg g^{-1} (Kannan *et al*., 1995a).

The total butyl content in the liver of mice exposed to tributyltin is approximately 1 mg kg^{-1} and remains unchanged 24 hours after administration. The contents of tin in the liver of rats and guinea pigs are lower at three hours and higher at 24 hours than those of mice (Ueno *et al*., 2003). The main form of butyltin in the liver of rats treated with tributyltin was dibutyltin. After a single oral dose of tributyltin, high levels of tributyltin were seen in the frontal and temporal lobes and in the cerebellum of rabbits. Despite the possibility of human exposure to butyltins, only a few studies have examined butyltins in human tissues. The concentrations of butyltins in the liver of Japanese subjects ranged from 59 to 96 ng g^{-1} wet wt, whereas monobutyltin and dibutyltin, degradation products of tributyltin, were predominant (Takahashi *et al*., 1999). The hepatic deposition of butyltin in a group of Danish men was from 1 to 33 ng g^{-1}, whereas total butyltin concentrations in human liver samples collected from Poland were in the range 2.4–11 ng g^{-1} (Nielsen and Strand, 2002). The variation in concentrations of butyltin among individuals from different countries may probably reflects the differences in dietary intake of fish (Nielsen and Strand, 2002). The concentration of butyltin in blood collected from 32 volunteers during a blood drive organized by the American Red Cross in central Michigan in 1998 ranged from 0 to 101 ng ml^{-1} (Kannan *et al*., 1999). After removing the highest concentration from the data, the average concentration was approximately 4.5 ng ml^{-1}. The occurrence of butyl compounds in human blood suggests the widespread exposure to these compounds from a variety of sources. When tributyltin in a mixture

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of 3 ml cherry brandy and 7 ml ethanol was given orally to a volunteer, only 5.1–5.4% of the dose was excreted in the urine, mainly as dibutyltin metabolites (Uhl, 1986). In 1999, more than 1000 people were exposed to methyltin contained in cooking oil in Southeast China. The concentration of trimethyltin in blood samples from these people after the incident was approximately 70 ng g^{-1}, whereas the levels of di- and trimethyltin in urine were approximately 80 ng ml^{-1} (Jiang *et al.*, 2000). Analysis of tin in organs showed high concentrations of methyltin in liver (1.93 µg g^{-1} dimethyltin and 1.42 µg g^{-1} trimethyltin), kidney (1.05 µg g^{-1} dimethyltin and 0.47 µg g^{-1} trimethyltin) and heart (0.1 µg g^{-1} dimethyltin and 1.48 µg g^{-1} trimethyltin).

29 TITANIUM (Ti)

Titanium was named after the Titans of Greek mythology. There is no evidence indicating that titanium is an essential element for humans or other animals. Titanium belongs to the first transition group; its chemical behaviour is similar to that of silicon and zirconium. The toxicology and biological monitoring was recently reviewed by Jin and Berlin (2007).

29.1 Uptake, Metabolism and Excretion

Although titanium compounds are, in general, absorbed poorly through ingestion and inhalation, titanium still can be detected in the blood, brain and parenchymatous organs of individuals in the general population, with the highest concentrations being found in the hilar lymph nodes and the lungs. Titanium is excreted in urine; information on other routes of excretion is lacking.

29.2 Toxic Effects and Dose–Response Relationships

Studies on titanium alloys used in implants and titanium compounds—such as its salicylate, oxide and tannate derivatives—used in cosmetics and pharmaceuticals, have not indicated any significant local effects on tissues, but, under certain circumstances, cases of inflammatory reactions and systemic effects have been observed. Thus, titanium cannot always be considered as being inert and biocompatible, as was once believed. Titanium tetrachloride, a strong irritant to mucous membranes and the eyes, can cause skin burns through accidental exposure. A soluble titanate given to rats in their drinking water was found to disturb their reproduction in a three-generation study on rats.

29.3 Carcinogenicity and Genotoxicity

Several epidemiological studies have suggested that TiO_2 dust has no carcinogenic effect on human lungs. IARC has listed titanium dioxide within Group 2B (IARC, 2006c). There is sufficient evidence from experimental animal studies for the carcinogenicity of TiO_2, but no adequate evidence exists for the carcinogenicity of titanium oxide in humans. Intramuscular injections of powdered titanium metal have induced fibrosarcomas and lymphosarcomas in rats; similarly, the organotitanium compound titanocene has induced fibrosarcomas in rats.

29.4 Mechanism of Action

Bronchoalveolar lavage cells from rats instilled intratracheally with TiO_2 particles induce *hrpt* gene mutations in rat alveolar cells *in vitro*. Experimental animal studies, clinical studies and some epidemiological surveys have indicated that titanium dioxide is generally biologically inert, but cases of adverse reactions have been reported. In experimental studies on animals, titanium carbide, hydride, nitride and boride have exhibited slight fibrogenic activity. Artificial fibres—such as potassium octatitanate and titanium phosphate fibres—possess fibrogenic properties.

29.5 Biological Monitoring

It has been reported that the blood concentration of titanium ranges from 0.03 to 0.15 mg kg^{-1} (Hamilton *et al.*, 1973). Titanium concentrations of 0.8 mg kg^{-1} wet weight in the brain, 1.3 mg kg^{-1} in the kidney cortex, 1.3 mg kg^{-1} in the liver, 3.7 mg kg^{-1} in the lungs and 0.2 mg kg^{-1} in muscle have been reported (Hamilton *et al.*, 1973). Titanium tends to accumulate with age in lung tissue, but not in other organs (Schroeder *et al.*, 1964). Titanium concentrations of 33 mg kg^{-1} dry weight in the lungs and 150 mg kg^{-1} in hilar lymph nodes have been reported (Teraoka, 1981). No analytical methods have been described for the measurement of titanium dioxide in urine. It has been observed that serum titanium concentrations were approximately 50 times greater, because of abrasion and wear of the titanium base material, in patients with failed patellar components of titanium-based joint implants (average concentration of serum titanium, 135.57 ng ml^{-1}) compared with those of a control group. There were, however, no significant

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differences in the levels of urinary titanium. Elevated serum titanium may, therefore, serve as a marker of failed patellar components in subjects who have undergone total knee replacements with titanium alloy bearings (Jacobs *et al.*, 1999).

30 TUNGSTEN (W)

Tungsten is also known as wolfram. Apart from occupational sources, small quantities of tungsten are present in food and water; trace quantities, related to industrial emissions, have been found in the general atmosphere. Tungsten is also present in trace amounts in human serum and in urine and faeces, elimination approximately balancing the intake of the metal in the few nonindustrially exposed subjects studied. Industrial, medical and military uses of tungsten have been expanding rapidly, as has the potential for the spread of tungsten to the environment (Leggett, 1997). The toxicology and biological monitoring of tungsten was recently reviewed by Kazantzis and Leffler (2007).

30.1 Uptake, Metabolism and Excretion

Almost one-half of an ingested dose of tungsten in the form of a soluble salt, and one-third of a deposited inhaled aerosol of tungstic oxide, were rapidly absorbed in those animal models which have been studied. Most of the absorbed tungsten was rapidly excreted in the urine. The small amount of tungsten retained was transferred in part from plasma to red blood cells and distributed mainly to spleen, kidney and bone. About three months after ingestion and six months following inhalation, the largest proportion of the body burden of tungsten was found in bone, although this was only a very small part of the administered dose. Sodium tungstate resembles molybdate in chemical properties and antagonizes the normal action of molybdate in its role as metal carrier for certain enzymes.

30.2 Toxic Effects and Dose–Response Relationships

Little is known about the toxicity of tungsten compounds, although the LD50 of soluble salts in the rat is relatively high. Following occupational exposure to tungsten carbide dust by inhalation, cases of pulmonary fibrosis have been reported, but this 'hard-metal disease', as it is often called, is more likely to be caused by cobalt, with which tungsten carbide is fused.

30.3 Carcinogenicity and Genotoxicity

Recent studies have shown a risk of cancer induction following exposure to toxic-metal tungsten alloys, which requires further investigation.

30.4 Biological Monitoring

In man, the mean serum concentration of tungsten, estimated by NAA, was found to be 5.8 ng ml^{-1} in eight healthy subjects, with an SD of 3.5. The urinary excretion of tungsten, again measured by NAA, ranged from 2.0 to 13.0 µg per 24 hours in four subjects in eight estimations. Faecal elimination in the same subjects ranged from 1.6 to 5.7 µg W per 24 hours (Wester, 1973).

31 ZINC (Zn)

Zinc was used in alloys centuries before it was identified as an element. Metallic zinc was produced in thirteenth-century India. The pure zinc metal was rediscovered in the eighteenth century. The toxicology and biological monitoring of zinc was recently reviewed by Sandstead and Au (2007).

Zinc (Zn) is environmentally ubiquitous and essential for life. However, Zn serves as a nutrient and as a toxic metal. There is a risk of deficiency or excess. Human Zn deficiency is common throughout the life cycle, especially in populations with diets that infrequently include animal flesh, and thus are limited primarily to plant products, many of which are rich in indigestible Zn-binding ligands. One estimate suggests that 20% of the world's population is at risk of Zn deficiency. The link between Zn, geology and health effects is presented in Nordberg and Cherian (2005).

31.1 Uptake, Metabolism and Excretion

Zn abundance in humans is approximately half that of Fe. A 70 kg adult male contains approximately 2–3 g, and women contain less. Approximately 60% is in muscle, 30% in bone, 8% in skin and hair, 5% in the liver, 3% in the gastrointestinal tract and pancreas, and other organs contain <1% (Wastney *et al.*, 1986). Soft tissue Zn

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($\mu g\,g^{-1}$ fresh tissue) ranges from approximately 12 in the adrenal glands to 102 in the prostate (Tipton and Cook, 1963). The body has no readily available Zn store similar to the store of Fe. However, Zn in bone is available to other tissues when bone turns over. When bone turnover is increased, much of the released Zn is excreted (Fell *et al*., 1973), although some is reused (Masters *et al*., 1986).

31.2 Toxic Effects and Dose–Response Relationships

Manifestation in animals given high doses include abortion, teratology and neonatal death. Human Zn toxicity is uncommon. Inhalation of Zn oxide fumes generated by welding and certain other industrial processes causes metal-fume fever, a self-limited flulike illness. In contrast, inhalation of zinc chloride, from smoke bombs, causes severe pulmonary injury and may be fatal. Ingestion of food or drink contaminated by exposure to galvanized surfaces can cause resulting nausea, vomiting, cramps and diarrhoea that is seldom fatal. Uncontrolled consumption of dietary supplements rich in Zn represents a hazard; intestinal absorption of Cu is suppressed, and Cu deficiency can occur. A similar phenomenon can occur when oral intakes of Zn are physiological or near physiological, and intakes are low. Present data suggest the risk of Cu deficiency is increased when the oral intake Zn/Cu molar ratio is >18; however, the critical level is unknown.

31.3 Biological Monitoring

The zinc content of serum/plasma is normally approximately $1\,mg\,l^{-1}$. The lower limit of 'normal' for morning fasting plasma Zn is approximately $700\,\mu g\,l^{-1}$ (Yokoi *et al*., 2003). Plasma Zn is higher in the morning and decreases after meals. Approximately 60–80% of plasma Zn is bound to albumin, and most of the remainder is bound to transferrin and an α2-macroglobulin (Wastney *et al*., 1986). Plasma Zn concentration is relatively insensitive to changes in Zn status; concentrations may be maintained within the accepted normal range for several weeks to months when subjects are fed 2.6–3.6 mg day^{-1} (40–55.5 µmol day^{-1}) (Milne *et al*., 1983; 1987), amounts of Zn that are inadequate for neuropsychological function (Penland, 1991). In addition, plasma Zn may not be reflective of foetal growth and maternal muscle Zn, although maternal leukocyte Zn is closely related to maternal muscle Zn and foetal growth (Meadows *et al*., 1981).

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Toxicology of Substances that Affect Performance and Behaviour

John P. Thompson

CONTENTS

1 INTRODUCTION

The use of drugs to enhance performance has become a matter of increasing concern in recent years. The use of foods containing active substances, for example caffeine, which may be taken to improve perceived levels of concentration, is well known. However, with the advent of a greater range of pharmacologically active substances, and the possibility of psychological or financial reward, the temptation to use drugs in the pursuit of enhanced sporting performance has increased.

Within different sports, drugs are taken for a variety of reasons. Some drugs are taken in the expectation that they will alter physical characteristics which will improve performance. Not only are drugs used to enhance performance in sports, studies have shown their use to improve the perception of body image and their use is prevalent in some sports facilities and gymnasia.

Performance-enhancing drugs of abuse may, or may not, be required to be taken at the time of competition. This has implications for those programmes which aim to detect the unfair use of drugs in sport.

For drugs that have to be present at the time of competition it is a relatively simple process to introduce testing programmes designed to detect banned substances. However, drugs which are intended to increase muscle mass may be used 'out of season' to develop increased muscle mass, which is then maintained by regular exercise and training throughout a competitive season. Since these drugs may be completely metabolized by the time of competition, detecting unfair practice may be much harder and require the use of random testing

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throughout the whole year. Similarly, the abuse of substances which are present naturally within the body presents a particular problem for regulatory authorities, as demonstrating that they are present in an unusual amount may be difficult.

Drugs of abuse are not only taken by athletes seeking to improve their performance. They may also be taken to improve the perception of body image, and by those not participating in competitive sport. The prevalence can be difficult to ascertain as studies often rely on self-reporting or the results from screening programmes. A study of people attending fitness centres, using both voluntary questionnaires and direct interviews suggested a high prevalence of doping, 12.5%, and also that use of other illegal substances was common in this group (Simon *et al*., 2006).

2 EPIDEMIOLOGY

In many sports, the absolute limits of performance have been increasing regularly. High-profile cases of very high-performing athletes who have subsequently been found guilty of using performance-enhancing drugs have raised the question as to whether such performances would have been possible without the misuse of drugs. It has also been suggested, that for some sports, the ever-increasing levels of performance are starting to plateau, since the introduction of more vigorous testing regimes.

2.1 Rationale for Banning Particular Substances in Sport

There is a moral and ethical debate to be had about the use of drugs in sport. Arguably, a variety of techniques are used by athletes to improve their performance. Society judges some of these, for example training, to be acceptable, whilst others, including the use of drugs, are largely considered unacceptable. However, there are additional reasons, other than performance enhancement, which should also be considered in determining what is acceptable to society and to those authorities which regulate sport. The use of drugs in sport not only offers the opportunity to obtain better performance, but may also put the individual at increased risk of an adverse event, which may be life-threatening.

Banned substances therefore not only include those which may improve performance, but also include those which may put the athlete or others, at risk, for example alcohol in motor sport.

International regulation of drug abuse for many sports is undertaken by the World Anti Doping Agency (WADA). Not only are particular individually named substances banned under the WADA regulations, but the WADA code of practice also proscribes generic approaches to unfair enhancement of performance (The World Anti-Doping Code, 2009). It is hoped that this will mitigate against the use of small molecular alterations to develop 'designer drugs', which do not appear on a particular static list, with the regulatory authorities always playing 'catch-up'.

Such a generic approach to regulation can also be used against techniques designed to improve oxygen delivery to the tissues. Various approaches to this are possible. Allegations of blood-doping have been made historically. In these techniques, an athlete could, for example, undergo a blood transfusion prior to competition which would transiently increase the oxygen-carrying capacity of the blood. A different approach to increasing oxygen delivery, also banned, is the use of erythropoietin (EPO) to stimulate increased red-cell production.

Categories of drugs and methods prohibited by WADA include (The World Anti-Doping Code, 2009):

- Anabolic agents
  - Anabolic androgenic steroids
  - Other anabolic agents
- Hormones and related substances
- Beta-2 agonists
- Hormone antagonists and modulators
- Diuretics and other masking agents
- WADA-prohibited methods:
  - Enhancement of oxygen transfer
  - Chemical and physical manipulation
  - Gene doping.

Athletes abusing drugs may attempt to hide their use from the regulatory authorities. Numerous techniques have been attempted for this, including substitution of test fluids (e.g. urine) from a nonexposed individual, to the inclusion of substances which would not of themselves enhance performance, but might make the analytical detection of a banned substance difficult or impossible.

2.2 Prevalence

Historically, there have been allegations of organized misuse of drugs in sport. It is reported that following the collapse of the German Democratic Republic, evidence became available that hundreds of physicians and scientists performed doping research and administered prescription drugs, as well as experimental drug preparations. In addition they contributed to the development of methods of drug administration that would evade detection by international doping controls (Franke and Berendonk, 1997). There are numerous examples of high-profile international athletes

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who have been stripped of medals in international competition. Use outside competitive sport also appears prevalent.

A survey of 12th grade male students in 46 American high schools reported that 6.6% had used anabolic steroids and that over two thirds of the group had initiated their use when aged less than 16 years old; 21% reported that a health professional was their primary source (Buckley *et al*., 1988). In 2003, a study of Indiana high school students suggested that 6.3% had used androgenic anabolic steroids (AASs). The average age of first use was 14, and 15% began taking them before the age of 10. Other athletes, physicians and coaches were listed as sources of AASs (Stiger and Yesalis, 1999). In Sweden, a study of over 5000 males aged 16- or 17-years-old reported that 3.6 and 2.8%, respectively, had misused AASs. Among female adolescents there was no reported use of these drugs (Nilsson *et al*., 2001). This may be due to the side-effect profile of these drugs. It has been suggested that use of anabolic steroids is prevalent more commonly in body-builders and weight-lifters than in other populations (Eklo *et al*., 2003; Thiblin and Petersson, 2005). It is suggested that the use of AASs by females is very much less than in males, but, calls to an antidoping telephone hotline suggest doping may be increasing (Doping Jouren, 2009).

2.3 Prohibited Substances

The WADA produces, each year, a list of substances which are prohibited in particular sports. It also provides useful advice on its web site for athletes and those involved in their training. The particular substances which are prohibited may be specific for particular sports. Similarly, some substances may be banned only at the time of competition, but may be permissible at other times.

2.4 Therapeutic Use Exemption

The use of prescription medicines by athletes poses particular problems. An athlete may have a legitimate medical need for a drug which would otherwise be banned. For example, it would clearly be wrong to ban an asthmatic athlete from using beta-2 agonists, whilst their use by a nonasthmatic competitor for the sole purpose of obtaining an unfair advantage would not be desirable. This issue is addressed by the use of exemptions for therapeutic use.

The principles of therapeutic-use exemptions are defined by the WADA. The antidoping guidelines are updated regularly and copies of current guidelines are held on the WADA web site (*www.wada-ama.org*). General principles for the therapeutic use of medicines are:

- An application for therapeutic-use exemption should be made in advance
- There would be a significant health impairment if the substance were withheld
- No additional performance-enhancement should occur, other than that which might be anticipated by a return to a state of normal health following the treatment of a legitimate medical condition
- Increasing 'low-normal' levels of any endogenous hormone is an unacceptable therapeutic intervention
- No reasonable alternative therapeutic intervention is available (WADA, 2009).

Some of the drugs which appear on lists of prohibited substances have legitimate medical uses. It is accepted that there are occasions when it would be appropriate for an athlete to be taking these and that this should not be a reason to be barred from competing. However the therapeutic use of these substances should not confer an unfair advantage upon an athlete. Generally the use of such drugs should be agreed prospectively, although some athletics associations may agree to *post hoc* agreement. There is a need to demonstrate that the medication is necessary for treatment of a medical condition. In some cases, for example the appropriate use of insulin in an insulin-dependent diabetic, this will be easy to verify and monitor. However, the situation is not always so clear. For example, the use of stimulants in psychiatric conditions may be harder to justify. In any case, the use should confer an improvement in the medical condition, rather than an unfair advantage in performance over an athlete not taking the medicine. With this principle in mind, alternative therapies for the particular condition should be considered which might not confer a performance advantage before other drugs are considered. For illnesses with limited treatment options the case is fairly clear cut, but for others, the situation may be much more complicated. Finally, the therapeutic-use exclusions do not allow the use of an otherwise prohibited substance to treat the consequences of prior misuse of substances on the prohibited list.

3 PERFORMANCE-ENHANCING EFFECTS OF PARTICULAR DRUGS

Whilst it would be unlikely that athletes would use drugs of abuse unless they had a beneficial effect, objective evidence for their efficacy is harder to find. The possible beneficial and adverse effects may be considered by looking at the following illustrative examples.

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3.1 Androgenic Anabolic Steroids

AASs are said to have three beneficial effects for an athlete (Calfee and Fedale, 2006). Binding to androgen receptors produces an anabolic state by causing nitrogen to accumulate in muscles, although whether this produces a major effect in males has been questioned. In males, most of the available androgen receptors are already saturated by endogenous testosterone. It is thought that in overtrained individuals, there is an excess of glucocorticoids and that these increase the breakdown of muscle glucogen to release energy. The effect of exogenous steroids in an individual whose androgenic receptors are already saturated is to inhibit, competitively, the binding of other steroids, including those which would have catabolic effects. Another effect of anabolic steroids is that on emotion. In particular it is claimed that they increase aggression. This may be of benefit during a competitive performance, or may allow athletes to trainer harder of for longer (American Academy of paediatrics Committee on Sports Medicine and Fitness, 1997). The use of anabolic steroids increases muscle mass. This is due to both muscle fibre hypertrophy and the formation of new muscle fibres (Kadi *et al*., 1999). Volunteer studies have also shown an increase in muscle pennation following 12 weeks exposure to testosterone (Blazevich and Giorgi, 2001). The increase in muscle mass is associated with an increase in strength. Increases in performance have been demonstrated following abuse of anabolic steroids. In a randomized controlled trial, subjects treated with 3.5 mg kg^{-1} testosterone ethanate and undergoing resistance training were assessed. The ability to perform a one-repetition maximum bench press was increased (Blazevich and Giorgi, 2001). An increase in strength is not, however, necessarily associated with an increase in the aerobic work rate (Haupt and Rovere, 1984).

3.1.1 Adverse Effects of Anabolic Steroids

Anabolic steroids have adverse effects upon the cardiovascular system via several different mechanisms. A study of body-builders using, or not using, AASs, and of matched controls showed that those body-builders using AASs had a greater left ventricular mass, a larger right ventricular diameter and depressed diastolic function of both ventricles than those in other groups (Kasikcioglu *et al*., 2009). Similarly, post-mortem findings have suggested hypertrophy, ventricular dilatation and premature atherosclerosis (Madea and Grellner, 1998). The effects on left ventricular mass may be greater when anabolic steroids are used in association with growth hormone (Karila *et al*., 2003). Male subjects taking 3.5 mg kg^{-1} $week^{-1}$ intramuscular testosterone developed an increase in systolic blood pressure of 10 mmHg (Giorgi *et al*., 1999).

Androgenic steroids have a deleterious effect on lipid profiles. A study of men taking a combination of methandrostenolone, testosterone and nandrolone showed a severe depression of high density lipoprotein cholesterol (HDLC) and an associated rise in low density lipoprotein cholesterol (LDLC) (Webb *et al*., 1984). An eight week course of androgens in power-lifters was associated with a 54% decrease in HDLC (Alen and Rahkila, 1984). Similarly, endogenous testosterone has a strong negative association with HDLC concentrations (Semmens *et al*., 1983).

Numerous hepatic side effects have been reported in association with the use of ASSs. In particular, cholestasis and the production of hepatic tumours are of concern. Although most of the tumours are benign, and may regress once the steroid use is terminated, there is an associated risk of life-threatening haemorrhage (Patil *et al*., 2007) and of malignant transformation (Socas *et al*., 2005).

Numerous reports have suggested that the use of anabolic steroids is associated with aggression. Results of epidemiological studies may be difficult to interpret as use of AASs may be associated with misuse of other drugs. However, a study in Sweden showed that weapons offences were almost twice as common amongst those who tested positive for AASs than in those who did not. However, there were no statistically significant differences between users and nonusers with respect to violence, either in the incidence of convictions or the proportion of individuals convicted (Klotz *et al*., 2006).

3.2 Creatine

Creatine is formed from arginine, methionine and glycine. Once formed, 95% is stored in the muscles. Supplementation with dietary creatine, often using a loading regimen followed by maintenance dosing has been shown to increase muscle creatine and phosphocreatine. Creatine is converted to phosphocreatine by the enzyme creatine kinase. Phosphocreatine, in turn, is a source of energy for the muscle (Calfee and Fedale, 2006). It has been suggested that phosphocreatine is a major source of early energy in anaerobic metabolism, after free adenosine triphosphate is consumed during the first few seconds of activity. Creatine supplementation has been shown to increase the ability to perform anaerobic exercise (Balsom *et al*., 1993) and this ability to increase early exercise performance has been suggested to be due to increased turnover of adenosine triphosphate (ATP) during muscle contraction (Birch *et al*., 1994).

Creatine powder has been shown to improve sprint cycling performance when compared to creatine serum in volunteers supplemented with either 20 g per day of creatine powder or 5 ml per day of serum creatine. The

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total work (9.6%) and peak power (3.4%) were both increased (Blazevich *et al*., 2004).

3.3 Beta-2 Agonists

Although the acute inhalation of beta-2 agonists, for example salbutamol, has not been demonstrated to have substantial effects on respiratory function in normal subjects, ingestion of beta-2 agonists such as clenbuterol and salbutamol may increase muscle mass. Clenbuterol has been used in animals as a growth enhancer. It is sometimes taken by body-builders or athletes for its lipolytic and anabolic effects. However it may have serious cardiac side effects. A case report describes a myocardial infarction in a 17-year-old body-builder (Kierzkowska *et al*., 2005).

3.4 Beta Blockers

Beta-blockers reduce tremor and heart rate. These may therefore be beneficial in sports which require accuracy, for example target shooting.

3.5 Peptide Hormones

A number of peptide hormones occur in the WADA list of prohibited substances. These include EPO, growth hormone, insulin-like growth factors, insulin, gonadotrophins and corticotrophins. EPO, a glycoprotein hormone, is discussed in Section 4.3

3.5.1 Gonadotrophins

3.5.1.1 Human Chorionic Gonadotrophin (HCG)

Human chorionic gonadotrophin (HCG) has been used for doping and causes an increase in testosterone levels. This increase in testosterone is associated with an increase in epitestosterone, thereby mitigating the risk of being detected, by calculation of a testosterone to epitestosterone ratio (Barroso *et al*., 2008).

3.5.1.2 Luteinizing Hormone (LH)

In men, luteinizing hormone (LH) is said to increase production of testosterone from the testes, whereas in women it produces changes in progesterone and oestrogen. Detection relies on either the use of immune assays, or on sensitive assays using tandem mass spectrometry. Given that these hormones are present in varying amounts in healthy females, the interpretation of results to determine whether they have been administered exogenously may be challenging.

3.5.2 Human Growth Hormone (HGH)

Historically human growth hormone (GH) was formerly extracted from the pituitary gland. With concern over Creutzfeld–Jacob disease, recombinant growth hormone (rGH) was developed. The degree of similarity between the endogenous and the recombinant forms of growth hormone, its pulsatile secretion and marked interindividual variability makes detection of doping difficult. Similarly, levels of growth hormone increase substantially after periods of exercise, hypoglycaemia and stress, potentially confounding tests for misuse.

The use of growth hormone in sport is said to depend, not only on its anabolic properties, but also on its effect on carbohydrate and fat metabolism (Saugy *et al*., 2006).

That GH can be used as a doping agent is not disputed. GH increases circulating levels of insulin-like growth hormone I (IGF-I), primarily of hepatic origin (Velloso, 2008). However, objective evidence for its effect on strength, rather than on changes in lean body mass is not so clear. Clinically, an excess of endogenous growth hormone in adults results in acromegaly and is associated with raised blood pressure, diabetes and increased mortality.

Detection of doping with growth hormone presents many difficulties. Urine samples contain concentrations of growth hormone that are several orders of magnitude lower than blood samples. rGH consists of a 22 kDa isoform, whereas endogenous GH is present in the blood in several forms. The ratio of rGH to other isoforms will vary when rGH is administered acutely. With chronic use, endogenous secretion of the 22 kDa form may be suppressed, perturbing the ratios. Two approaches have been investigated to detect GH abuse, one detecting different pituitary isoforms and another, the measurement of GH-dependent markers (Holt and Sonksen, 2008).

3.6 Diuretics

Diuretics produce a decrease in body water. Acutely, this is associated with a decrease in weight and therefore may confer advantages in sports which are categorized in terms of weight. Use of a diuretic may allow an athlete the ability to compete in a weight category in which they would otherwise not be able to compete.

3.7 Cofounders and Masking Agents

The use of substances which serve to make the detection or interpretation of the presence of a banned substance is forbidden.

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4 ENHANCED OXYGEN DELIVERY

Whilst drugs such as anabolic androgenic steroids may increase power or strength, they would not be expected to enhance aerobic performance. Numerous techniques have been attempted to improve oxygen delivery to the tissues. These include high-altitude living, 'living high'–'training low', experimental hypobaric hypoxia, transfusion of red blood cells or other substances to increase the oxygen-carrying capacity of the blood and administration of EPO.

4.1 Oxygen Deprivation

'Altitude therapy' may be effective. For example, a study of athletes who lived at 2500 m for 27 days and trained at 1250 m, showed that circulating EPO levels 20 hours after ascent were nearly double their initial value at sea level. Haemoglobin increased 1 g dl^{-1} over the course of the trial, maximum oxygen intake increased and one third of athletes achieved personal-best times (Stray-Gundersen *et al*., 2001).

Intermittent hypobaric oxygen has been studied to determine its effects on EPO, red-cell volume and haemoglobin mass. Four weeks of intermittent exposure, three hours per day, five days per week, resulted in an increase in EPO, but not in red-cell volume or haemoglobin mass (Gore *et al*., 2006).

4.2 Blood Doping

The use of blood transfusions to improve sporting performance was suggested decades ago and allowed an increase in haemoglobin greater than that found from altitude training. However, the introduction of synthetic EPO enhanced the opportunity to manipulate the oxygen-carrying capacity of the blood. The ability to synthesize recombinant human EPO as a medicine has enabled its illicit use in sport.

4.3 Erythropoietin

EPO is a 30.4 kDa glycoprotein hormone that is the main synthetic control for red blood cell formation. It consists of isoforms that differ in their patterns of glycosylation and biological activity (Skibeli *et al*., 2001; Sherwood *et al*., 1988). Naturally, it is synthesized largely in the kidneys, but also from other tissues, including liver and brain.

Administration of exogenous EPO results in an increased red-cell mass and haematocrit. A study of exogenous EPO demonstrated an increase in haemoglobin concentration and an associated increase in maximal aerobic power (VO_2 max). The increase in VO_2 max per g dl^{-1} increase of haemoglobin was no different from that which was seen following transfusion (Ekblom and Berglund, 1991).

In otherwise healthy individuals, there is some evidence to suggest that an increased haematocrit is associated with increased cardiovascular risk. Use of EPO is associated with headaches, hypertension and increased risk of thrombotic events, in addition to other effects not directly related to an increase in haemoglobin concentration (Lappin *et al*., 2002). Due to the abuse of several different agents, it is sometimes difficult to ascribe an adverse effect to a particular agent, for example as was the case with a 26-year-old professional cyclist who developed a sagital sinus thrombosis, with a history of using EPO, growth hormone and vitamin A and E supplements (Lage *et al*., 2002).

Suggestions that EPO may be misused in sport date from the late 1980s. As a test that could distinguish recombinant human EPO (rHuEPO) from endogenous EPO was not initially available and early indicators of misuse were indirect.

Indirect evidence for the abuse of Epos may come from comparison of haemoglobin concentrations in athletes competing in different years, which suggested that these were increasing. Analysis of blood samples taken from elite cross-country skiers in World ski championships suggested that half the medal winners and one third of those finishing in 4th–10th place had highly abnormal haematological profiles (Stray-Gundersen *et al*., 2003). Similarly, haemoglobin concentrations were found to rise as time went on, with concentrations in ski teams increasing between 1989 and 1994 and again in 1996. Following the introduction of a rule limiting maximum values, a fall of 1.5 g dl^{-1} in males and 4.2 g dl^{-1} in female skiers was noted (Videman *et al*.). Riders have admitted using EPO in the 1996 Tour de France (Elliott, 2008).

5 DETECTION

5.1 Testing Programmes

Testing programmes are potentially fraught with difficulties. Physicians involved in an athlete's training may also be involved in testing programmes and the prescription of therapeutic drugs to the athlete. They may, in some cases, also be responsible for the supply of illicit substances. This may produce conflicts of interest.

A unified approach to the control of substances is fostered by the WADA, which produces annually a list of prohibited substances. In order for a drug-testing programme to have credibility it must fulfil a number

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of different criteria. Firstly, it must be possible to obtain samples at a time when they might be being used and have a chance of being detected. As some drugs of abuse are used outside the competition season, for example to produce increased muscle bulk, which is subsequently maintained by training, it is essential that athletes must be prepared to undergo random testing, including testing which occurs outside the competitive season. Similarly, there must be confidence that the sample to be tested comes from the specified individual. Once a sample has been obtained, a chain of custody must be demonstrable throughout the analytical procedure.

For convenience, a noninvasive screening programme, for example using urine samples, would be preferable. However, to detect some proscribed activities, for example those used in blood doping, it may be necessary to take more invasive samples.

Once a sample has been obtained, there should be a robust analytical process available to detect a proscribed substance, or reliable markers to show that it has been present. Once detected, it must be able to be demonstrated that the substance has not occurred naturally, or that if it might be detected as a natural phenomenon, it is present in abnormal quantities. Defining what is 'normal' and what is 'abnormal' may be quite difficult. For some compounds, the decision-making process is relatively easy, as no compound should be detectable at all. For other substances, the process is more complex and a decision-making 'cut-off' concentration or ratio has to be determined.

5.2 Collection

Following the selection of the individual to be tested, and the timing of the test, collection is the first step in the analytical process. This will involve the witnessed collection of the relevant sample from an unambiguously identified athlete. The athlete may be allowed to select the sample container into which the sample is taken. Once obtained, specimen validity checks may be commenced, for example density and pH measurements for urine samples. The sample will be labelled in front of, or by the athlete, and the whole process documented carefully, with signatures being obtained from both the tester and the athlete. Once obtained, the sample will generally be split into two separate sealed samples, one of which is available to be retested in case of an appeal (Green, 2006).

5.3 Analysis

For competition at Olympic level, samples must be analysed by a laboratory which is accredited by WADA. If the sample is negative, the process is terminated. In the event of a positive test, a further sample from the same sample will be tested. If positive, the result may then be reported, with the second sample bottle available for further testing.

It should be remembered that the presence of a negative test does not exclude the use of banned substances, merely that the test did not detect them. This may be due to the presence of a novel compound for which current analytical techniques are inadequate, or, more likely, that the substance is not there. Even the lack of a banned substance for which there are robust analytical techniques does not mean that it has not been abused; merely that it was not detected. For example, AASs used out of season would not be expected to be detected during an in-season test.

For some substances, trace amounts may appear in the urine for several months following a positive test. A repeat positive test does not necessarily mean that further abuse has occurred. If an athlete does not receive a permanent ban having tested positive, it may be required that a negative sample is obtained before return to competition.

Problems associated with testing for drugs of abuse may occur when the prohibited substance occurs as a natural hormone.

5.3.1 Testing for Androgenic Anabolic Steroids (AAS)

Anabolic steroids may occur naturally within the body or may be administered exogenously. Similarly, exogenous steroids may have the same chemical structure as endogenous hormones, or may be artificially synthesized with subtle differences in structure. Recently, 'designer' anabolic steroids have been manufactured and detected, perhaps in the hope that they would not be identified.

Generally, urine testing for anabolic steroids will be undertaken using gas chromatography–mass spectrometry analysis (GC-MS), relying upon the retention time and spectrometry of both parent compounds and metabolites. If a screening test is positive, further tests to identify the specific compound will be undertaken.

Testing for anabolic steroids illustrates some of the generic difficulties in testing for prohibited substances to identify positively which substance may have been abused. For example, when testing for 19-nortestosterone (nandrolone), a 'positive' result may come from the detection of its metabolites, 19-norandrosterone (19-NA) and 19-noretiocholanolone (19-NE), rather than 19-nortestosterone itself. However, these two metabolites may also be formed from the metabolism of 19-norandrostenedione and 19-norandrostenediol. The presence, *per se* of 19-NA does not of itself indicate conclusively that 19-nortestosterone has been abused. However, as these compounds are now also considered as prohibited substances this may be of

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limited consequence. It is also accepted that small amounts of 19-NA may occur in pregnant women and following the ingestion of norethesterone-containing oral contraceptives. In this case, the detection of other metabolites of norethesterone may clarify the picture. It is also accepted that some males may excrete small amounts of 19-NA. A threshold value is therefore used, albeit with very low concentrations set (Green, 2006).

With improved analytical techniques, it has become possible to detect exogenous and 'designer' steroids more readily and to show that they would not be expected to be found naturally. This has made the use of naturally occurring substances more attractive for abusers, as the defence that they have occurred naturally may then become possible. It becomes necessary to determine whether they are present in unusual concentrations, or in an unusual ratio when compared to other endogenous compounds.

Naturally occurring substances, for example testosterone, administered endogenously will produce the same chromatograph. However, naturally occurring testosterone is found in approximately equal concentrations as epitestosterone, although there can be considerable interindividual variation. It therefore becomes possible to use the ratio of testosterone to epitestosterone to provide a threshold suggestive of exogenous administration. However two potential problems may arise. Firstly, some individuals have a naturally high ratio. However, as the intraindividual ratio is relatively constant, serial measurements may be used to determine what is usual in a particular individual. The second problem is that manipulation of the ratio of testosterone to epitestosterone is possible by abusing epitestosterone to manipulate the ratio. For this reason, both the ratio of testosterone to epitestosterone and the concentration of epitestosterone must be considered. In difficult cases, the use of a ketaconazole challenge (which changes the ratio of testosterone to epitestosterone in different directions, depending upon whether the testosterone is endogenous or exogenous) may be considered. An alternative approach is to use isotope ratio mass spectrometry (IRMS) (Green, 2006).

IRMS is able to compare the prevalence of carbon isotopes ^{12}C and ^{13}C. Different plant groups fix different proportions of the two isotopes of carbon during photosynthesis. As exogenous steroids may be manufactured from plant substrates, the ratio of carbon isotopes from exogenous anabolic steroids may be different from the ratio found in steroids synthesized endogenously by an athlete (Green, 2006).

5.3.2 Detection of Erythropoietin

Direct tests for EPO are available, indicating the presence of exogenous EPO isoforms. Indirect tests are also available, indicating haematological changes induced by exogenous EPO administration, measuring, for example haematocrit, reticulocyte haematocrit, serum soluble transferring receptors and the proportion of macrocytic red cells (Wilber, 2002).

As early as 2000, a technique was reported for distinguishing between EPO forms. Using this technique, 102 frozen urine samples from the 1998 Tour de France cycling competition were analysed. Twenty-eight samples had EPO levels above the normal range. The 14 samples with the highest levels underwent further analysis and gave rise to banding patterns typical of recombinant hormone (Lasne and de Ceaurriz, 2000).

The tests used include those based on a combination of isoelectric focussing and double immunoblotting and distinguish between endogenous and exogenous EPO. However, concerns over interpretation of results have been expressed due to the use of a primary antibody which is not monospecific, although careful pre-analytical care may improve the reliability (Delanghe *et al*., 2008). Some have suggested that false positives may occur occasionally in post-exercise protein-rich urine (Beullens *et al*., 2006), although this view has been challenged (Catlin *et al*., 2006; Lasne, 2006).

6 CONCLUSION

It is clear that xenobiotics are abused, both by individuals seeking to improve their body image and by those who are seeking to gain an improvement in their performance in competitive sports. There is objective evidence that a variety of substances are capable of improving performance and that drugs have been abused, even by those competing at the highest levels, where testing is known to occur. This alteration in performance, however, may be achieved to the detriment of the health of the individual.

It is a matter of concern that pressure, perhaps from peers or coaches, may be brought to bear upon young athletes who may be placing their health and even their lives at risk. It is a societal decision to determine which techniques to improve performance (e.g. diet or training) are acceptable within competitive sport and which are not (e.g. drug abuse). The simplest and most attractive toxicological approach is to ban the use of drugs taken with the intent of improving performance, rather than to treat an underlying medical condition. The concept of a toxicological 'level playing field' is very attractive.

Achieving the level playing field is becoming increasingly difficult, but perhaps more successful. There are huge rewards available to successful sportsmen and women. These rewards provide the temptation to cheat, and the possibility that it might be worth 'investing' heavily to achieve them. This 'investment' could include the development of new or modified xenobiotics, in an attempt either to gain further improvements in performance, or to avoid detection. In order to combat this trend, drug-abuse control schemes have had to develop, both organizationally and analytically.

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The introduction of out-of-season and random testing is a prerequisite for a successful antidoping scheme. Merely testing during competition will not catch all those who abuse drugs. For example, drugs may be used out-of-season to gain strength, with muscle bulk retained due to subsequent training. This may also be an issue if a decision is made to allow a banned athlete to return subsequently to competitive sport. Just how long do the effects last?

Those involved in the analytical detection of banned substances need to be constantly vigilant if new substances are to be detected. Considerable advances have been made in recent years. Once a substance has been detected, a decision then has to be made to determine what is 'normal' or 'acceptable' and what is not. The development of synthetic human hormones has been a particular challenge for regulatory authorities. Increasingly, it may become necessary to determine an individual's personal 'profile' to inform the decision-making process. With future developments, increasingly invasive sampling may become necessary, as may diagnostic challenges with test agents, to determine whether a particular biochemical or haematological profile, which represents only a static picture, is due to natural homeostasis or the exogenous administration of banned substances.

The abuse of drugs in sport remains an area which will challenge physiologists, and clinical and analytical toxicologists.

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Poisons of Animal Origin

Gregory P. Wedin, Daniel E. Keyler and Elisabeth F. Bilden

CONTENTS

1 INTRODUCTION

Poisons of animal origin is a broad topic that covers a great variety of toxic substances found in thousands of animal species. The scope of the problem that these animals pose throughout the world is presented here through a discussion of representative species whose poisons are significant hazards to humans.

It is important to first distinguish between animals that are poisonous and those that are venomous. Venomous animals, as the name implies, produce venom in specialized glands or cells that can be administered in some way to its enemy or prey. Poisonous animals, on the other hand, possess a toxin(s) within their tissue that can have deleterious effects on those who eat them.

The venom apparatus, mode of envenomation and constituents of venom vary considerably throughout the animal kingdom. Venomous animals can either bite or sting their victims and some are capable of squirting or spitting venom. The venom of most species is a unique, but complex proteinaceous mixture. The biochemical and pharmacologic properties of most venoms are incompletely understood due to their complexity. Interspecies differences, as well as difficulties in obtaining sufficient venom and in extracting individual components further complicate this issue.

An animal may use its venom offensively or defensively. For example, it may be used to subdue or kill its prey and aid in digestion. It also may be used to ward off predators. Most venoms are multipurpose and cannot be narrowly classified. Obviously, the toxic effects that result from envenomation are greatly influenced by these functional properties and the corresponding venom constituents.

Most poisonous animals accumulate toxin through the marine food chain. The most common initial source is unicellular sea algae (dinoflagellates). A number of these dinoflagellates are responsible for the often-publicized 'red tides' which are linked to poisonous shellfish.

Although people who live in regions inhabited by venomous or poisonous animals are at greatest risk for toxic exposure, such encounters can occur virtually anywhere. Venomous animals, for example, are imported by hobbyists and have been shipped inadvertently in produce or other goods. Likewise, poisonous seafood may be shipped to distant markets. This may present a therapeutic dilemma to a practitioner who is unfamiliar with such exposures. Poison Information Centres are staffed with specially trained professionals who can provide expert information and advice on managing such cases. These centres also have access to the Antivenom Index, which was developed to assist with locating the appropriate antivenom for exotic bites and stings. The Index is a joint effort of the American Association of Zoological Parks and Aquariums and the American Association of Poison Control Centers.

2 VENOMOUS SNAKES

Venomous snakes have fascinated mankind for millennia, and continue to be of significant importance, not only to native peoples in the vanishing natural world, but also to scientists, religious groups, and in recent decades, amateur herpetologists (Stocker, 1990; Bates *et al*., 1988; Ivanyi and Altimari, 2004). The encroachment of human beings into once remote habitats is rapidly changing the natural landscape of our world and the ability for venomous species to survive has become more complex and difficult. As a consequence, many venomous snake species exist in quite precarious environments (O'Shea, 2005).

Human encounters with snakes occur across large geographic regions, and are a frequent part of everyday living in many third-world countries and rural areas of more developed countries, with the consequence of snakebite having a significant impact on public health (**Table 1**). All too frequently it is children and the elderly who suffer the greatest mortality (Russell *et al*., 1997). In stark contrast has been the increasing number of exotic snakebites that have occurred to amateur collectors due to a booming interest in the herpetoculture of exotic venomous snakes. Species from nearly every genus listed in **Table 1** are bred in captivity, and available online via the Internet (Trestrail, 1982; Pearn *et al*., 1994; Keyler, 2006). Additionally, with the drive to study snake venoms in biomedical research, even professionals are

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Table 1 Venomous snakes frequently responsible for envenomation to humans in nature: taxonomy, geographic distribution and venom effects

Family/*Genus*	Common names	Native country	Chief venom effects
Atractaspididae			
Atractaspis	Burrowing asps	Africa	Vasoconstriction, myocardial effects
Colubridae			
Dispholidus	Boomslang	Africa	Coagulopathy, haemorrhage
Philodryas	Cobra-verde	C, S America	Coagulopathy, haemorrhage
Rhabdophis	Keelbacks	Asia	Coagulopathy, haemorrhage
Elapida			
Acanthophis	Death adders	Australia	Paralysis
Bungarus	Kraits	SE Asia	Paralysis
Dendroaspis	Black/green mamba	Africa	Paralysis
Hemachatus	Rinkhals	Africa	Paralysis, local necrosis
Hoplocephalus	Broad-headed snakes	Australia	Coagulopathy
Micropechis	Small-eyed snake	New Guinea	Paralysis, coagulopathy, myolysis
Micrurus	Coral snakes	N, C, S America	Paralysis
Naja	Cobras/spitting cobra	Africa/Asia	Paralysis/corneal ulceration
Notechis	Tiger snakes	Australia	Paralysis, coagulopathy, myolysis
Ophiophagus	King cobra	Asia	Paralysis
Oxyuranus	Taipan	Australia	Paralysis, coagulopathy, myolysis
Pseudechis	Mulga/black snakes	Australia	Coagulopathy, myolysis
Pseudonaja	Brown snakes	Australia	Coagulopathy, paralysis
Tropidechis	Rough scaled snake	Australia	Coagulopathy, paralysis, myolysis
Hydrophiidae			
Astrotia *and many other genera*	Sea snakes	Indo-Pacific Oceans	Paralysis, myolysis
Viperidae			
Crotalinae (pit vipers)			
Agkistrodon	Cantils, copperheads, moccasins	N, C, S America	Coagulopathy, necrosis
Bothrops	Lanceheads	C, S America	Coagulopathy, necrosis
Calloselasma	Malayan pit viper	Asia	Coagulopathy, necrosis
Crotalus	NA rattlesnakes, tropical rattlesnakes	N America C, S America	Coagulopathy, necrosis Paralysis, myolysis
Hypnale	Hump-nosed vipers	Asia	Local necrosis
Lachesis	Bushmaster	C, S America	Coagulopathy, necrosis
Sistrurus	Massasauga, pigmy rattlesnake	N America	Haemorrhage, local necrosis
Trimeresurus	Bamboo vipers	SE Asia	Coagulopathy, necrosis
Viperinae (true vipers)			
Bitis	Gaboon/puff adder	Africa	Cardiovascular, coagulopathy
Causus	Night adders	Africa	Local necrosis
Cerastes	Horned vipers	Africa/Asia	Coagulopathy, necrosis
Daboia	Russell's vipers	SE Asia	Coagulopathy, myolysis, renal
Echis	Carpet, saw-scaled vipers	N Africa/Asia	Coagulopathy, necrosis
Vipera	Adders, asps, European vipers	Eurasia	Cardiovascular, coagulopathy, necrosis, paralysis

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not immune to the consequences of envenomation (Ivanyi and Altimari, 2004; Keyler, 2008).

Globally, venomous snakebite has been reported to cause 3 million envenomations annually, resulting in 150 000 fatalities with the greatest morbidity and mortality occurring in tropical regions (White, 2000). In North America, rattlesnakes have been responsible for the greatest number of venomous snakebites, and the greatest number of fatalities (Russell, 1990). In the United States, 2001 data from the Toxic Exposure Surveillance System revealed that 2450 venomous snakebites had occurred, with 47% caused by rattlesnakes, and 5% caused by exotic species. Interestingly, the percentage of bites by exotic snakes was greater than the percentage (3.5%) caused by the native coral snake (*Micrurus fulvius*). Only two fatalities were documented, and both were from rattlesnake bites (Watson *et al*., 2002). However, according to data from 1979 to 1998 in the National Vital Statistics System, 97 deaths ($\approx$5 year^{-1}) were documented by medical examiners. Young white males aged 25–34 years were the most common victims, with the greatest number of bites occurring in Texas, Florida and Georgia, respectively (Morgan *et al*., 2004). Alcohol involvement is not uncommon, and bites frequently result from poor judgment and bare-handling (Gold *et al*., 2002).

Adders, bamboo vipers, cobras, mambas and rattlesnakes are all familiar names, but may leave the medical clinician at a loss with respect to their venom effects, consequences of their envenomation to humans, and what constitutes appropriate medical management. Thus, regardless of where medical professionals practise, it is important to have some basic knowledge of snakebite medical management if a favourable outcome is to be achieved.

2.1 Venomous Snakes, Venoms and Envenomation

2.1.1 Venomous Snakes

The taxonomy of venomous snakes was traditionally based on hierarchal biological classifications, but has been a constantly evolving process in recent years, due to the increasing flow of DNA analysis data used in constructing snake phylogenies (Slowinski and Lawson, 2002). Currently, there are approximately 420 different snake genera representing 2900 species that range geographically over six continents and the Indo-Pacific oceans (Greene, 1997). The venomous snakes that are frequently involved in envenomation to humans are represented in five families, with the family Viperidae divided into two important subfamilies, the true vipers (viperinae) and the pit vipers (crotalinae), which possess infrared heat-sensing pits between the eye and nasal opening (**Table 1**).

Venomous snake species of the different genera are all equipped with fangs, but not all fang structures are the same, as there are evolutionary differences in dentition type for venom delivery (Kardong, 1979). Sea snakes have conical fangs with no grooves (aglyphous), boomslangs and many other colubrids have enlarged rear fangs with a ventral groove (opisthoglyphous), cobras and most elapids have large anterior, fixed-position fangs with deep frontal grooves (proteroglyphous), and vipers and pit vipers, such as rattlesnakes, have the most sophisticated snake dentition with large tubular canaliculated fangs with hinge-like action (solenoglyphus) (Greene, 1997). The larger the fang structure, the greater the ability to penetrate tissues with a bite, and the potential for deep venom-dose delivery. Spitting cobra fangs are unique and capable of ejecting venom for distances of several feet due to a forward-angled venom-exit-orifice modification in their fangs that allows the venom to be sprayed towards their victim.

Venom is delivered to the fangs either by a duct from a venom gland, as found in vipers, pit vipers, most elapids and sea snakes, or from a modified salivary structure of secretory cells that allow the toxins to be elaborated onto the fangs or to grooves in the fangs, such as the case with venomous colubrid snakes. Rear-fanged snakes (i.e. boomslang, *Dispholidus typus*) and many short fixed-fanged elapids (i.e. sea snakes, *Hydrophiidae* spp.) will usually grab their prey, or human victim, and repeatedly chew, while most vipers (i.e. Gaboon viper, *Bitis gabonicas*; Russell's viper, *Daboia russelli*) and pit vipers (i.e. rattlesnakes, *Crotalus* spp.; Fer-de-Lance, *Bothrops lanceolatus*) will rapidly bite and release. Either method can effectively deliver venom in quantities sufficient to subdue prey, or cause envenomation to humans. Interestingly, the fangs of dead snakes are not without potential effect as envenomation has been documented to result from the accidental penetration of fangs into the digits (Griffen and Donovan, 1986; Keyler and Schwitzer, 1987).

2.1.2 Snake Venoms

Venoms are basically biochemical cocktails of greater than 20 different components that are primarily enzymatic and nonenzymatic proteins, such as hyaluronidase, phospholipases, hydrolases and polypeptide toxins (Stocker, 1990; Iyaniwura, 1991). Venom composition has undergone divergent evolution and varies considerably among different snake families and species (Ivanov and Ivanov, 1979). Further complicating our understanding of venom effects associated with a specific species are the findings of intraspecific, subspecific and geographic variations in venom and, consequently, different toxicity profiles (Glenn *et al*., 1994; Chippaux and Goyffon, 1998; Keyler and Van De Voort, 1999). A few venom components,

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like the polypeptide α-neurotoxins, may be quite selective and act like a pharmacological bullet for a specific target (i.e. neuromuscular junction receptors), a feature common to many elapid venoms. In vipers and pit vipers, multiple venom components may function collectively and in concert or synergistically, to produce a staged progression for achieving a net toxic effect or effects on a physiological system or systems. Certain venom components have even been shown to induce apoptosis, a form of programmed cell death (Suhr and Kim, 1996).

The important concept about different snake venoms is that they possess their own library of different venom components, yet quite similar toxic effects may be induced via a multitude of different pharmacologic mechanisms. These venom actions and their associated pathophysiologic effects can be simplified and generalized in order to provide a better basic understanding of venomous snakebite and responsible species (**Table 2**). However, there are exceptions, despite the generalities. For example, several species of spitting cobras (*Naja kaouthia*, *Naja mossambica* and *Naha nigiricollis*), whose venoms contain neurotoxins, are also capable of causing significant localized necrosis, and potentially lethal cardiotoxic events (Blaylock, 1982; Kunkel *et al*., 1983–1984; Minton, 1990). Similarly, the majority of rattlesnake envenomations generally cause local tissue damage and coagulopathies, but rarely true neurotoxicity. However, the bite of the Mojave rattlesnake (*Crotalus scutulatus*) produces less local swelling and pain, and victims can exhibit profound neurotoxic effects (Russell *et al*., 1975; Kunkel *et al*., 1983–1984; Jansen *et al*., 1992).

2.1.3 Envenomation Effects

The effects of envenomation are initially dependent on the pharmacokinetics of the venom, the dose delivered and its route of delivery. The dose of venom a snake delivers can vary widely, especially in cases of human envenomation. Pit vipers will use their heat-sensing pits to size up their prey prior to biting, and then when delivering their bite will meter the dose to conserve venom, as there is no need to use excessive amounts

Table 2 Snake venom toxins: generalized actions, pathophysiology, clinical symptoms and representative species

Toxin actions/pathophysiology	Clinical symptoms	Representative species
Cardiotoxic		
Coronary vasoconstriction/vasodilation, direct myocardial effects	Hypotension, arrhythmias	Mole/stiletto vipers
Cytotoxic/vasculotoxic		
Cell membrane destruction, Inflammation	Ocular ulceration,[a] blebs, erythema, oedema, necrosis, fluid shifts, hypotension, pain	Spitting cobras,[a] N, S American and Asian pit vipers, African vipers
Haemotoxic		
Anticoagulant/procoagulant, fibrinolysis/haemolysis, direct haemorrhagic effects, microangiopathy, platelet aggregation/inhibition	Haemorrhage, thrombi formation, ecchymosis, haematuria, thrombocytopenia	Boomslang, green pit vipers, N, S American pit viper spp., Asian viper spp., African and Asian pit viper spp.
Myotoxic		
Rhabdomyolysis, selective ion channel blockade	Myoglobinaemia, myoglobinuria, pain, renal failure	Sea snakes, tiger snakes, taipans
Necrotoxic		
Direct tissue destruction	Local necrosis, pain	N, S, American and Asian pit vipers, African and Asian vipers, spitting cobras
Nephrotoxic		
Direct nephrotic damage, acute tubular necrosis, renal cortical necrosis	Acute renal failure, anuria/oliguria, haematuria, haemaglobinuria, myoglobinuria	Russell's vipers, N, S, American and Asian pit vipers, African and European vipers
Neurotoxic		
Pre/postsynaptic blockade, neurotransmitter destruction	Flaccid paralysis, respiratory arrest, fasciculations, ptosis, dysphagia	Cobras, coral snakes, kraits, mambas, sea snakes, Mojave and tropical rattlesnakes

[a]Ocular ulceration most commonly associated with spitting cobras.

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of venom to subdue a small prey animal. When a human–snake encounter occurs, the snake is biting out of a fear response and may expel venom amounts ranging from none (dry bites) to 90% of its venom gland reserves (Russell, 1990). In fact, 'dry bites' may occur in up to 30% of Crotalidae bites, 50% of Elapidae bites and 75% of Hydrophiidae bites (Kunkel *et al*., 1983–1984). However, vipers or pit vipers may inject potentially large venom doses, due to their sizeable venom gland and fang structure. This is exemplified with the bite of the terciopelo (*Bothrops asper*) as approximately 3 ml of venom can be injected in a single bite (Avila-Aguero *et al*., 2001). This sizeable venom dose can lead to the rapid development of both local and systemic symptoms. At the other extreme is the microlitre quantities injected with sea-snake bites (*Hydrophiidae* spp.); however, even these minute doses have been fatal (Heatwole, 1999). Whatever the dose, it may be delivered subcutaneously, intramuscularly or intravenously. The route of delivery influences the absorption kinetics of the venom and the resulting onset and severity of symptoms. Many bites to humans are superficial and absorption may be incomplete, resulting in only minor medical consequences (Russell, 1990). In contrast, intravenous envenomation, although uncommon, results in instantaneous systemic uptake of the entire venom dose and leads to more rapidly progressing systemic toxicity, causing profound morbidity or death (Davidson, 1988). The anatomical location of the bite may be important. It has been noted for pit-viper envenomation that distal appendage bites are more likely to result in less severe envenomation, but more proximal appendage bites lead to more severe envenomation and complications (Moss *et al*., 1997). Bites to the eye, face and tongue have occurred, and resulted in severe complications due to oedema-related airway obstruction (Gupta *et al*., 1995; Lewis and Portera, 1994; Gerkin *et al*., 1987). Severe bites have also occurred to unusual anatomical sites such as the penis, further complicating treatment (Crane and Irwin, 1985). Additional factors that can affect the venom pharmacokinetics include: the chemical make-up of the venom; the species of snake; the age, size and health of the victim; and an individual's susceptibility to the venom (Gold *et al*., 2002).

A final feature influencing both toxicokinetic (venom distribution and time to target tissues) and toxicodynamic (magnitude of clinical and systemic symptoms) effects from envenomation is the interval between the time of the bite and the time to medical treatment. Increased time to adequate medical care, including antivenom administration, correlates with increased morbidity and mortality from envenomations (Nishioka and Silveira, 1992; Russell *et al*., 1997).

2.2 Venomous Snakebite: Characteristics and Toxic Manifestations

The potential pathophysiological effects to humans having sustained venomous snakebites are wide-ranging, as venoms can affect nearly every tissue and organ system, depending on the species of snake involved. Although most snake venoms have multiple venom-toxin actions, they usually induce one or two predominant clinical effects (**Table 2**). A few generalized broad categories are of major clinical importance: (i) cardiotoxicity; (ii) coagulopathy; (iii) flaccid paralysis; (iv) haemorrhage; (v) renal injury and (vi) localized tissue injury and destruction (White, 2005).

2.2.1 The Snakebite

The bite of a venomous snake can easily be confused with that of a nonvenomous one initially, or in cases of a dry bite. If skin where the bite occurred is not broken there is usually very little risk of toxicity. If the skin is broken then the history of how the bite occurred becomes important. Bites can have the appearance of scratches or defined puncture wounds, and in cases involving front-fanged snakes, the classic two-fang punctures are often seen, but not always.

When an amateur herpetologist tells emergency department staff that a black mamba bit them, then probably they have been bitten by a black mamba! Usually the amateur knows exactly what they were bitten by, though they may know little else. The difficulty in confirming venomous snakebite usually involves individuals bitten in a natural setting, when the offending snake was not clearly seen. Rattlesnakes are usually identified with some reliability, but it is important to confirm that rattlesnakes are indigenous to the geographic locality where the bite occurred. Many nonvenomous snakes are frequently misidentified as venomous, therefore, the resource and consultation of a knowledgeable herpetologist is helpful. If there is any question about the species involved, it is best to keep the patient for observation for a minimum of six hours or longer, especially in paediatric cases (Guisto, 1995; Swindle *et al*., 1992). This leaves the clinician to rely on the observation of objective signs and symptoms of envenomation for the diagnostic confirmation of a venomous snakebite (**Table 2**).

2.2.2 Local Toxic Effects

Envenomation by pit vipers and vipers typically results in significant localized pain, swelling and oedema within 30–60 minutes, and in cases of severe envenomation these intensify and progress rapidly (Russell, 1991; Gold *et al*., 2002). Ecchymosis may develop and spread following the path of lymphatic drainage (Roberts and

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Greenberg, 1997). Vesicles or blebs may also appear, which are usually filled with clear fluid or blood in severe cases. Local tissue necrosis also may follow due to local ischemia and direct action of venom enzymes. Pain and swelling are generally more severe following bites by the larger rattlesnake species such as eastern and western diamondbacks, while bites by small *Sistrurus* rattlesnake species (Massasauga and pygmy rattlesnakes) tend to be less locally severe (Russell *et al*., 1975). However, even copperheads can cause significant local pain and tissue destruction (Thorson *et al*., 2003). Pit-viper envenomation-associated pain may persist for days, and there may be long-term sequalae associated with recurrent oedema due to limb activity that lasts for weeks, and poorly healing wounds can fester for months (Spiller and Bosse, 2003). Similar local wound complications have been reported with the bites of African vipers and spitting cobras (Bey *et al*., 1997; Spawls and Branch, 1995).

2.2.3 Coagulopathies

A wide range of coagulopathies, which can be a complex consequence of snakebite, can be difficult to manage, and often cause significant morbidity and mortality (Markland, 1998; White, 2005). A variety of snake species may produce haemostatic disorders including members of the Colubridae, Elapidae and Viperidae (pit viper and vipers) families (White, 2005). Venom constituents will interact at various points of the coagulation cascade, depending on the species involved.

Principal coagulopathic effects are due to procoagulant (Factors V, IX, and X activation, prothrombin activation and fibrinogen clotting) and anticoagulant (protein C activation, Factors IX and X activating protein, thrombin inhibitor and phospholipase A_2) actions, fibrinolytic activity (fibrin degradation and plasminogen activation), platelet activity (platelet aggregation inducers and inhibitors), plasma protein activation and vessel wall disruptors (haemorrhagins) (Hutton and Warrell, 1993; Markland, 1998). Disseminated intravascular coagulation (DIC) may complicate severe cases, and frequently renal failure ensues, as the kidneys are excessively burdened with degradation products (Curry and Kunkel, 1985; White, 2005).

Procoagulant effects result in consumption of coagulation factors. Laboratory data can suggest the potential for profound clinical bleeding, but this may or may not occur, and the resolution of complications may be highly variable. Anticoagulant venoms contain toxins that are indirect or direct in their ability to inhibit clotting mechanisms. The end clinical picture of anticoagulant effect is similar to the end effect of the consumptive route observed with procoagulant venoms, although the risk of pathologic bleeding is less. Haemorrhagic toxins are usually metalloproteinases that disrupt the integrity of the vascular endothelium and are present in numerous viperid snake species (Markland, 1998). Platelet function is compromised by intravascular clotting and consumption of platelets, sequestration of platelets at the site of envenomation or direct destruction of platelets by the venom (Riffer *et al*., 1987). The degree of thrombocytopenia may directly correlate with the severity of envenomation; however, the extent of thrombocytopenia and increased risk of bleeding in cases of snakebite is less clear (La Grange and Russell, 1970; White, 2005).

2.2.4 Cardiotoxic and Haemodynamic Effects

Cardiovascular shock leads to systemic complications and is a common cause of death in humans due to crotalid envenomation (Hardy, 1986). Reduced cardiac output secondary to venom-induced cardiac changes, as well as the release of mediators such as bradykinins, histamine and serotonin may also contribute to haemodynamic compromise (Curry and Kunkel, 1985; Christopher and Rodning, 1986). Electrocardiographic abnormalities, including T-wave inversion and heart block have also occurred following envenomation by a number of snake species (Lalloo *et al*., 1997) (**Table 2**). Tropical rattlesnake (*Croatlus durrissus terrificus*) envenomation has been implicated to cause direct venom-induced changes in myocardial enzymes (Cupo *et al*., 1990). Viper and pit-viper envenomation frequently cause haemodynamic changes that alter membrane permeability of the pulmonary blood vessels, resulting in pulmonary oedema and blood pooling in the vasculature, but almost all organ systems can be affected (Russell, 1991). This sets the stage for shock as a result of third-spacing of fluids and intravascular volume depletion (Schaeffer *et al*., 1979).

2.2.5 Neurotoxicity

Neurotoxicity results from envenomation by elapids such as coral snakes, cobras, mambas and kraits; the hydrophid sea snakes; the South American tropical rattlesnake (*Crotalus durissus terrificus*) and the North American Mojave rattlesnake (*Crotalus scutulatus*) (Ekenback *et al*., 1985; Cupo *et al*., 1988; Jansen *et al*., 1992). Sea snakes have potent venom compared to many other snakes, and their neurotoxin binds postsynaptically on acetylcholine receptors (Tu and Fulde, 1987). The venom of kraits acts presynaptically inhibiting the release of acetylcholine at the myoneural junction (Minton, 1990). Neurotoxic signs and symptoms in general progress as a descending paralysis with ptosis, dysphagia and blurred vision being early signs of envenomation, and respiratory paralysis the most common cause of death (Kitchens and Van Mierop, 1987). The onset of symptoms may occur in minutes or be delayed hours, and symptoms may persist for days (Minton, 1990). Crotalid envenomations from timber rattlesnakes and western diamondback rattlesnakes

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have also been reported to cause paraesthesias, muscle fasciculations, myokymia (including lingual myokymia) and weakness (Russell, 1980; Brick *et al*., 1987; Clark *et al*., 1997).

2.2.6 Ocular Effects of Venom

Several species of African and Asian cobras are capable of spraying or 'spitting' venom defensively into the eyes of a predator. The venom of these snakes can produce a spectrum of corneal lesions, such as intense conjunctivitis, pain, tearing, corneal oedema, de-epithelialization and opacification (Ismail *et al*., 1993a; Minton, 1990). Corneal opacification syndrome has been attributed to the cardiotoxins in their venom (Ismail *et al*., 1993b). Ocular exposure to the venom of several species of pit vipers (rattlesnakes and cottonmouths) has been reported, but reactions of minimal irritation and burning resolved rapidly (Troutman and Wilson, 1989).

2.2.7 Other Toxic Manifestations

Many potential systemic toxic effects may result from direct actions of venom or as secondary complications of cardiovascular, neuromuscular or coagulation disorders. Renal failure is a frequent complication associated with direct venom toxicity, or haemostatic complications from envenomations by crotalidae, hydrophidae, viperidae and some elapid envenomations, and may be further complicated by DIC or cardiovascular shock (George *et al*., 1987; Nelson, 1989; Vijeth *et al*., 1997). Adult respiratory distress syndrome may develop in severe cases. The aetiology is unclear but shock, DIC, multiple blood-component transfusions and the venom itself are all postulated (Curry and Kunkel, 1985). Pancreatitis and hepatocellular necrosis have also been reported following snakebite, the aetiology of which is unclear (Kjellstrom, 1989; de Silva *et al*., 1992; Narvencar, 2006). Hepatic necrosis has been reported in cases of envenomation by *Crotalus* spp. in South America (Barraviera *et al*., 1989).

2.3 Out-of-Hospital Management

The actions taken prior to a venomous snakebite victim's arrival at a medical facility can have a major influence on the course of envenomation. Initially, providing reassurance to the victim followed by removal of bracelets, watches, rings, or any constrictive jewellery or clothing, including shoes, are important first steps. However, the use of arterial tourniquets, cryotherapy, electric shock, excision and incision have all resulted in various forms of increased morbidity, and in cases of severe envenomation may exacerbate venom-induced toxicity and mortality (Dart and Gustafson, 1991; McKinney, 2001). Negative pressure suction remains controversial, and if used should not involve incision or other invasive techniques (Bush *et al*., 2000; Alberts *et al*., 2004). If the use of a compression bandage or the pressure immobilization technique has been implemented, they are best left in place until arrival at a hospital and comprehensive evaluation (Watt *et al*., 1988; Hardy and Bush, 1998).

It is also beneficial to know if the victim has a prior history of venomous snakebite or treatment with antivenom as both can influence the potential for allergic or anaphylactic complications. If the victim is being transported via emergency medical services (EMS) and venomous snakebite has been confirmed, baseline vital signs should be taken, cardiac monitoring should be initiated, the progression of signs and symptoms should be documented, intravenous access should be established and supportive measures should be performed as needed.

2.4 Medical Management

Effective management of venomous snakebite patients requires an understanding of snakes and their behaviour, envenomation pathophysiology, proper clinical assessment, knowledge of what not to do and the timely implementation of appropriate pharmacotherapies (**Table 2** and **Table 3**). The scope of discussion here will not be to provide specific treatment protocols, but to give a general overview of the important concepts involved in the medical management of the snakebite patient.

2.4.1 Initial Measures

In Australia and some Asian countries, the immunodiagnosis of snakebite can be made using enzyme-linked immunoassay technology (ELISA), which can serve as an aid in the prognosis of symptoms and selection of appropriate antivenoms. However, economic factors have hindered development and use in countries outside of Australia (Ratanabanangkoon *et al*., 1987; Hawdon and Winkel, 1997; Rodriguez-Acosta *et al*., 1998). In addition to confirming the venomous snake involved in the bite history, the patient's medical history should be documented (particularly relating to previous snakebite or antivenom exposure), evaluation of the bite wound performed and tetanus vaccination status determined (Russell, 1991). The bitten limb should be maintained in an immobilized functional position, and blood/urine taken for laboratory analysis. In cases of pit-viper and viper envenomation, circumferential measurements at a point above and below the bite site should be documented, and repeat measurements taken at 15–20 minute intervals (Gold *et al*., 2002). The accepted period of patient observation in a medical facility following snakebite in the United States has been six hours with crotalid envenomation, but caution should be taken, as even crotalid bite victims have had severe symptoms delayed

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Table 3 Clinical therapeutic intervention options used in the treatment of snakebite patients

Physiologic system	Pharmacotherapeutic agents	Supportive measures
General	Tetanus Analgesics Antibiotics (aerobic/anaerobic gram negative bacilli primarily)[a] Antivenom?	Reassurance
Cardiocirculatory	Antivenom Epinephrine	Blood replacement products Fluid volume replacement
Dermal tissues	Antibiotics	Wound debridement Wound soaks/baths
Gastrointestinal	H_1, H_2 antihistamines	Fluid
Musculoskeletal	Antivenom	Limb elevation
Nervous (CNS and autonomic)	Antivenom Anticholinesterases	—
Ocular	Antibiotic eye drops Heparin solution Epinephrine drops	Voluminous irrigation
Pulmonary	Antivenom Epinephrine (anaphylaxis cases)	Endotracheal intubation Mechanical ventilation
Renal	Antivenom	Fluids—maintain urine flow Haemodialysis

[a](Jorge *et al*., 1998).

up to 12 hours (Swindle *et al*., 1992; Guisto, 1995). Victims of coral-snake envenomation have been reported to have a delayed development of symptoms, up to 12 hours in some cases (Gold *et al*., 2002). Accordingly, antivenom therapy may be a consideration for all victims of eastern coral snake bite and other neurotoxic snake bites when there is evidence of skin penetration by fangs (Otten, 1983). In general, if there is any question of envenomation and an apparent bite is evident (especially in small children), there is no harm in admitting the patient for 24 hours of close monitoring. Although medical personnel are inclined to administer antibiotics prophylactically, their use in snake-venom poisoning is rarely needed (Talan *et al*., 1991; Clark *et al*., 1993; Kerrigan *et al*., 1997). In some instances this may be due to the antibacterial activity of snake venom itself (Talan *et al*., 1991). Exceptions requiring the use of antibiotics usually result from an extensive delay to medical treatment (common in rural areas), or the use of inappropriate first aid measures, such as tourniquets (Jorge *et al*., 1998).

2.4.2 Laboratory

Laboratory data are of great utility for assessing the severity of toxicity, directing the course of treatment and monitoring the effectiveness of treatment. Initial baseline laboratory values, drawn as soon as possible following the bite for coagulation profile (prothrombin time (PT), partial thromboplastin time (PTT), fibrinogen, fibrin split products) and haematology, should be determined in all cases of confirmed or questionable envenomation, regardless of the species involved. Complete blood count (CBC), creatine kinase and lactate dehydrogenase levels, along with urinalysis (haematuria, haemaglobinuria, myoglobinuria), renal function and hepatic function tests are useful. The typing and crossmatching of patient blood should also be done prior to antivenom administration to avoid potential immuno-induced haemolytic complications (Miwa and Fujii, 1996). Tests should be repeated at regular intervals during the course of antivenom therapy, and particularly following antivenom infusion (Gold *et al*., 2002). Values may not reverse readily, but when adequate doses of antivenom have been administered and venom is neutralized the progressive coagulopathic deterioration should stop.

2.4.3 Antivenom Therapy

Definitive treatment of confirmed envenomation is ideally with species-specific antivenom. Venom differences across a genus may exist, and intraspecific and geographic venom variability occurs, yet some degree of paraspecific coverage is frequently afforded by many polyvalent antivenoms (Minton, 1967; 1990; Chippaux and Goyffon, 1998). However, despite the crossreactivity of snake venom components, a monovalent or monospecific antivenom developed

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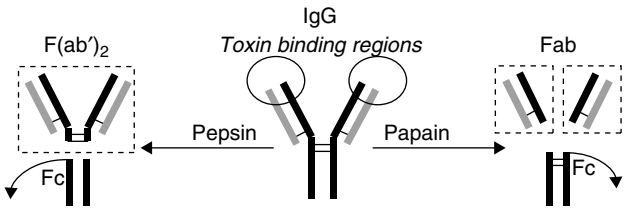

Antibody structure	$F(ab')_2$	IgG	Fab
Molecular Wt.	102 kDa	150 kDa	50 kDa
Immunizing snake venoms	Tropical rattlesnake common lancehead	Death adder	Eastern diamondback Western diamondback Mojave rattlesnake Water moccasin
Immunized host animal	Horse	Horse	Sheep
Specficity Valency	Polyspecific Polyvalent	Monospecific	Polyspecific Polyvalent
Country of production	Mexico	Australia	United States

Figure 1 Molecular properties and immunological characteristics of three forms of antivenom produced in three different countries. Venom from a single snake species or venoms from several species may be used to immunize a host animal. Antibodies formed against venoms are harvested from sera of host animals and purified to make antivenom. Whole antibody, immunoglobulin G (IgG), is the most common antivenom form. Each mole of IgG has two binding regions, and each binds or neutralizes 1 mol of venom toxin. IgG digestion with pepsin yields a single $F(ab')_2$, two-thirds the size of IgG, that can bind two moles of venom toxins. IgG digestion with papain yields two smaller Fab subunits, each one-third the size of IgG, that can bind a single mole of venom toxin. Each form has its own pharmacokinetic and therapeutic advantages/disadvantages.

for treatment of envenomation from a single species may be ineffective or have limited effectiveness for treating envenomation by related subspecies, or even the same species from a different geographic region (Berger and Bhatti, 1989; Gillesen *et al*., 1994; Chippaux and Goyffon, 1998). As a result, the tendency has been towards the development of numerous polyvalent or polyspecific antivenoms that provide a broader spectrum of coverage (Laloo and Theakston, 2003).

Snake antivenoms are prepared by harvesting antibodies from the sera of horses, sheep or goats that have been hyperimmunized with the venom of a single species of snake, or the venom of multiple snake genera and species, or by pooling venoms for immunization (**Figure 1**). Antibodies are proteins that are usually affinity purified, and may be used as whole immunoglobulin G (IgG), or enzymatically digested into an $F(ab')_2$ subunit that is two thirds, or digested to two subunits that are one-third the parent IgG size, respectively (**Figure 1**). Efforts to make even smaller humanized single-chain antibodies with predefined binding specificities for specific target toxins of South American rattlesnake (*Crotalus durissus terrificus*) venom has been accomplished using phage technology, which may open new avenues for passive immunotherapy in snakebite (Lafaye *et al*., 1997).

The variant antibody forms have different chemical properties, pharmacokinetics and immunogenic characteristics (**Figure 1**). The renal elimination of Fab is possible because of its small molecular size, but IgG and $F(ab')_2$ antivenoms, being larger, have prolonged elimination (Ismail *et al*., 1998). Thus, Fab in excess of the quantity needed to bind and neutralize all the venom toxins, is readily eliminated (Meyer *et al*., 1997; Seifert and Boyer, 2001). This feature is good in one way, but is also a limitation. The larger $F(ab')_2$ and IgG antibody forms remain in the systemic circulation longer due to slower elimination, and, as such, remain available to bind persistent venom toxins that slowly release from tissue depots to the circulation (Seifert and Boyer, 2001; Quesada *et al*., 2006). One must remember that once venom toxins are complexed to antivenom, not only do the pharmacokinetics of the toxin change, but the antivenom pharmacokinetics may change as well (Laloo and Theakston, 2003).

Another feature of different antibody forms stems from the antigenicity they exhibit based on their differing molecular sizes. The small Fab form is less antigenic than IgG, and consequently possesses less risk of allergic side effects following its administration. Both the Fab and $F(ab')_2$ forms have had the Fc region (compliment binding region) digested away, and this also reduces antigenicity (Seifert and Boyer, 2001) (**Figure 1**). Since the antibodies that constitute the make-up of any given

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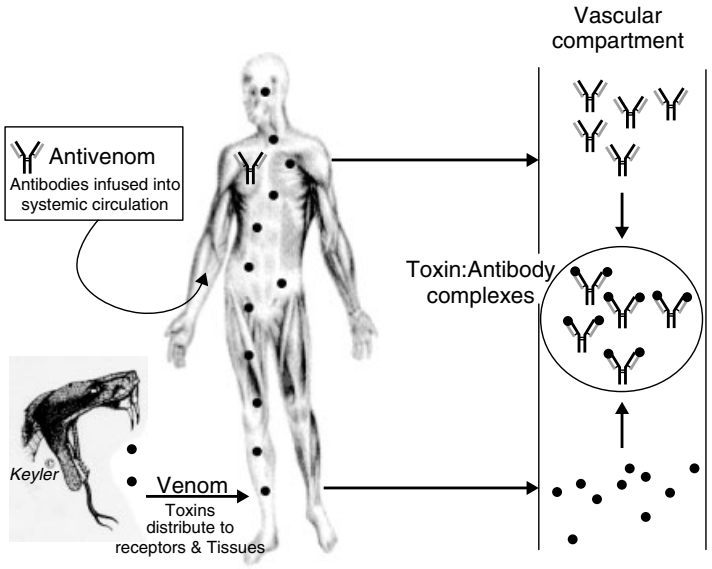

Figure 2 Snakebite can deliver venom subcutaneously, intramuscularly or intravenously. Venom is taken up by the systemic circulation and toxins distributed to target tissues and receptors resulting in toxic effects. Antivenom infused into the vascular compartment results in antibody recognition and binding of venom toxins, forming toxin:antibody complexes that essentially render the toxins pharmacologically inactive. Ideally, antivenom doses should be escalated in increments to achieve a total dose that is sufficient to neutralize all toxins.

antivenom are proteins, derived from other mammalian species, the lack of purity in their pharmaceutical preparation can also contribute to antivenom antigenicity. However, high-quality purified IgG antivenoms can be administered safely to humans (Chippaux and Goyffon, 1998).

Antivenom should be administered intravenously. Antivenom cannot alter the venom dose injected, but does alter distribution and elimination pharmacokinetics of venom and venom components by action of the antibodies binding to venom. Venom or venom components, bound to antibody (toxin:antibody complexes) become inaccessible to target tissues and receptors and are essentially neutralized and rendered pharmacologically inactive (**Figure 2**). Therefore, the total dose of antivenom should be great enough to theoretically bind/neutralize all the venom dose injected. Ideally, antivenom dosing would be based on actual stoichiometric venom:antibody relationships in order to effectively neutralize all venom components. Since the quantity of venom injected with a bite is unknown; clinical signs, symptoms and laboratory analyses are used to guide treatment, and the antivenom dose is empirical. Basically, the larger the quantity of venom injected, the greater the dose of antivenom required. Antivenom administration, preparation, dosing guidelines and other useful information are normally clearly printed and provided in the manufacturer's package insert. Dosing is generally done using a given number of vials or units at intervals and, as such, the total effective dose is accomplished via gradual escalation. On occasion, venom-induced coagulopathy may recur after an initial response to treatment with Fab, $F(ab')_2$ or IgG antivenom, even after lengthy periods, which may require repeated antivenom dosing. This is a result of the pharmacokinetic mismatch between venom components and the antibody make-up of the antivenom, with the most pronounced mismatch occurring with Fab antivenom (Seifert and Boyer, 2001).

The selection of appropriate antivenom will depend on the species of snake, and in many cases this leaves attending medical personnel at a loss as to sources of antivenom and the need for information concerning the management of venomous snakebite, especially in cases of exotic envenomation. Contacting a regional poison centre can be most useful to obtain snakebite toxicology consultation, and to identify sources of antivenom. In the United States there are two antivenoms that are FDA (Food and Drug Administration) approved for use in treating venomous snakebite victims: Crotalidae Polyvalent Immune Fab (Ovine) is available for treating envenomation by all species of North American pit vipers (**Figure 1**) and; coral snake antivenom that is an equine IgG preparation is available for two subspecies of eastern coral snake (*Micrurus fulvius fulvius/tenere*). However, other foreign-produced antivenoms have also been shown to provide significant protection, in animal models, against the effects of envenomation from North American coral snakes, and may become available for human use in the future (Wisniewski *et al*., 2003; de Roodt *et al*., 2004; Sanchez *et al*., 2008).

The rationale for the administration of antivenom early-on following envenomation is based on the pharmacokinetic relationships between venom dose and

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antivenom (antibody) dose, with the timely coordination of these leading to the neutralization of venom toxins and the halting of the progression of venom-induced pathophysiologies and symptoms. However, there may be limitations as to the effectiveness of antivenom in preventing the worsening of local tissue damage. Regional administration of antivenom intramuscularly has been demonstrated to be of no benefit in limiting necrosis following envenomation in animal models (Norris *et al*., 2003). Other studies with pit-viper venoms (i.e. Jararaca, *Bothrops jararaca*) revealed that venom in the microcirculatory complex at the site of fang punctures can significantly alter the microcirculation, resulting in reduced antivenom absorption kinetics, and impaired antivenom-venom neutralization, with any neutralization that does occur being delayed (Battellino *et al*., 2003).

In general, the sustained plateau of laboratory test values, their gradual recovery towards normal and the general clinical stabilization of the patient suggests adequate neutralization of venom by antivenom has been achieved. On occasion, continued absorption of venom from a depot at the bite site may prolong the clinical course. As a result, antivenom may need to be administered over a period of days. Importantly, antivenom therapy should not be ruled out, even if the patient presents for treatment after significant delay, if toxic effects of venom are evident (Rosen *et al*., 2000; Bebarta and Dart, 2004).

In the event of an allergic response, the potential risks and benefits of therapy must be weighed carefully, but if life or limb is at stake, antivenom therapy should commence after taking appropriate precautions. Patients who are treated with antivenom also are likely to develop a delayed immune complex reaction commonly known as serum sickness. This is an IgG- and IgM-mediated process, which results in the formation of antigen–antibody complexes, and the subsequent activation of the complement system (Otten and McKimm, 1983). Lastly, the use of antivenom does not preclude the use of symptomatic and supportive management, as it is the combination of these that will optimize the potential for reducing morbidity and mortality.

2.4.4 Supportive Treatment and Other Therapeutic Measures

Since intravascular volume depletion is a primary cause of cardiovascular shock, adequate fluid replenishment is essential. Oxygen should be administered and appropriate respiratory support provided as needed. Whole blood or blood products may be needed to treat acute blood loss and specific coagulopathies, but should be used judiciously (Burgess and Dart, 1991). Paralysis from elapid venom-induced neurotoxicity has been successfully treated with anticholinesterase agents such as edrophonium and neostigmine (Watt *et al*., 1986; Gold, 1996; Lalloo *et al*., 1996). In the absence of pharmacotherapeutic agents in cases of neurotoxic envenoming, respiratory support has proven life-saving (Deer, 1997; Pochanugool *et al*., 1997).

2.5 Ocular Venom Exposures

Snake venom can be ejected into the eyes of a human by spitting cobras, and resulting complications can be significant (Ismail *et al*., 1993a; Ismail *et al*., 1993b; Cham *et al*., 2006). This risk is obvious to those who live in spitting cobra habitats of various countries, but amateur and professional herpetologists are also at risk when manipulating these snakes. If at all possible, immediate irrigation of affected eyes should be performed prior to reaching medical care, and can be accomplished with water or normal saline solution. Irrigation with an aqueous heparin solution (2500 U ml^{-1} in saline) or tetracycline (1% solution) were effective in limiting the corneal opacification syndrome induced by several African cobras such as Rinkhals (*Hemachatus haemachatus*), forest cobra (*Naja melanoleuca*), black-necked spitting cobra (*Naja nigricollis*) and the Cape cobra (*Naja nivea*) (Ismail *et al*., 1993a). Recent studies of venom-induced epithelial damage, inflammation and scarring effects from the black spitting cobra (*Naja sumatrana*), native to Malaya, Borneo and Sumatra, revealed that topical application of heparin (5000 U ml^{-1}), or topical application of reconstituted antivenom, significantly reduced corneal damage (Cham *et al*., 2006).

2.6 Pregnancy and Snakebite

The pregnant snakebite patient presents a serious and perplexing situation, as treatment must be rendered to two patients concurrently. Depending on the species of snake, the severity of envenomation and time interval to medical care, there can be foetal and maternal mortality in the absence of treatment (Dunnihoo *et al*., 1992; Pantanowitz and Guidozzi, 1996). Data are limited, and although it has been suggested that envenomation in early gestation may have a less favourable prognosis, the outcome is highly variable (Parrish and Khan, 1966; Seneviratne *et al*., 2002). In two large case-series reviews of 30 and 39 cases, a 43% foetal mortality (20% spontaneous abortions), 10% maternal mortality and 30% abortion rate were reported, respectively (Dunnihoo *et al*., 1992; Seneviratne *et al*., 2002). Venom may cross the placenta resulting in systemic foetal poisoning, in the absence of maternal symptoms (James, 1985). The best way to provide any chance of foetal survival is to insure maternal survival. Both foetal and maternal monitoring is essential.

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If coagulopathy is evident, the use of blood-component replacement alone will be insufficient, and the use of antivenom should be considered (Pantanowitz and Guidozzi, 1996). Crotalidae Polyvalent Immune Fab (Ovine) antivenom, five vials, has been successfully used in treating a 32-year-old patient (28 weeks gestation) following copperhead envenomation (Kravitz and Gerardo, 2006).

2.7 Surgery and Snakebite

Surgical management of venomous snakebite has been performed for decades, and in recent times has been carefully scrutinized (Glass, 1991; Hall, 2001; Dart, 2004). Surgical procedures, such as incision of the bite site as a first-aid measure, and excision of the bite to remove enough venom in order to avoid the need for antivenom were frequently used interventions (Glass, 1976; Huang *et al*., 1974). These are no longer recommended procedures (Hall, 2001). The main issue prompting surgical intervention is the possibility of compartment syndrome that can cause severe muscle necrosis and permanent nerve damage, with loss of functionality (Dart, 2004). When this process occurs in a digit the possibility of dermotomy is raised, and when it occurs in the larger limbs the question of fasciotomy arises. Justification to perform either should be cautiously approached, as compartment syndrome from snakebite results from a toxin-induced compartment syndrome rather than that caused by physical trauma. Collectively, the data from multiple research studies suggest that surgery does not improve the outcome of venom-induced compartment syndrome; however, it may be considered in carefully selected cases (Hall, 2001; Dart, 2004). Only two out 1257 cases, in case series collectively reported, required fasciotomy (Hall, 2001). Technology now provides for the measurement and monitoring of compartment pressures, which should be used to confirm the diagnosis of true compartment syndrome (Mars *et al*., 1991; Seiler *et al*., 1994). Clinical evaluation is an important adjunct to compartment pressure measurements and is critical in the decision-making process for surgical intervention.

2.8 Allergies, Anaphylaxis, Antivenom and Venom

Acute allergic reactions to snake venom from multiple species from all geographic regions of the world have been documented to occur following repeated venomous bites (Schmutz and Stahel, 1985; Wadee and Rabson, 1987; Domingos *et al*., 1990; Bush and Jansen, 1995; Alonso *et al*., 1995). In regions of the world where native populations encounter indigenous species of snakes on a regular basis, there is evidence of active immunization, with some protection afforded (Theakston *et al*., 1981; 1983). However, this is the exception, as amateur herpetologists have tried self-immunization, resulting in unfavourable effects that further contributed to the complications of their envenomation (Keyler, 1991). Thus, individuals who have routine contact with snakes, and persons who have repeat bites, can present with complex clinical findings of both allergic and toxic manifestations. Active immunization against snake venoms has been formally attempted in man, but the antibody concentrations achieved following immunization have been short-lived, and protection limited and too brief to be practical (Theakston, 1989).

Antivenom therapy has also been frequently associated with systemic allergic reactions, both from single and repeat treatment exposures (Malasit *et al*., 1986; Pugh and Theakston, 1987; Jurkovitch *et al*., 1988; Premawardena *et al*., 1999; Chen *et al*., 2000; LoVecchio *et al*., 2003; Fan *et al*., 2006). In cases of IgE-mediated allergic reactions to snake venom or antivenoms the patient is at immediate increased risk for potential respiratory and shock complications requiring pharmacologic and supportive interventions (Moran *et al*., 1998; Chen *et al*., 2000; Fan *et al*., 2006). Thus, there should be close observation of the patient, both following the actual snakebite and during administration of antivenom, with epinephrine, antihistamines and life support at the ready.

Two useful manoeuvres can be taken to reduce the likelihood of adverse reactions with antivneom administration in patients, with or without allergy history. First, adequate dilution of the antivenom is important. Dilution should be based on manufacturers' guidelines. Second, the rate of antivenom administration can be controlled, and in cases of reaction, the rate can be reduced, or even stopped, and then gradually restarted at a slower rate (Russell, 1983).

3 GILA MONSTER/BEADED LIZARD

The genus *Heloderma* contains the only two species of lizards known to be venomous to humans. The Gila monster (*Heloderma suspectum*) and the beaded lizard (*Heloderma horridum*) are further classified to five subspecies. The banded Gila monster (*Heloderma suspectum cinctum*) is the only subspecies with part of its geographical range in the southwestern United States. The beaded lizard ranges from southern Mexico on down the Pacific coast to Guatemala (Campbell and Lamar, 2004). Because of their unique features and colour patterns, and the fact that they are venomous, they have become quite popular with amateur collectors. Thus, they can be present in virtually any state or country far from their native range.

Both species have a broad head, solid bodies and legs with thick tails, but the forked tongue of the Gila

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monster is black while that of the beaded lizard is pink (Beck and Lowe, 1991). Their short curved teeth are peg-like and grooved on both the anterior and posterior surfaces. A venom duct opens at the base of the teeth, and when the lizard bites, the muscle contraction of the jaw causes the chanelling of venom along the tooth grooves bidirectionally via capillary action.

3.1 Heloderma Venom

Venoms of helodermids are quite similar between both species, and possess numerous enzymatic properties, including a phospholipase A_2 and kallikrein-like enzymes such as, 'helodermatine', along with an acidic neurotoxin, 'gilatoxin' (Alagon *et al*., 1982; 1986; Mochca-Morales *et al*., 1990). In animal toxicity studies these toxins have induced lethargy, partial paralysis and hypothermia (Mochca-Morales *et al*., 1990). Although Gila monster venom lacks apparent haemolytic or haemorrhagic activity, it does contain vasoactive peptides capable of inducing vasodilation, and a Type III phospholipase A_2 that blocks platelet aggregation (Hendon and Tu, 1981; Huang and Chiang, 1994). These may produce profound systemic effects, such as hypotension, coagulation disorders and a rapid loss of consciousness (Fry *et al*., 2005).

3.2 Heloderma Bites

Heloderma bites that occur naturally are very rare (Hooker and Caravati, 1994). Neither species of lizard attacks humans, despite the stories that are rumoured. It takes considerable provoking to get them to bite, and although they appear sluggish, they can rapidly flex their stumpy body, deliver a substantial clamping bite and they are notorious for remaining attached to their victim. When victims present to the emergency department for treatment the circumstances leading to the visit usually involve prodding and bare handling of the lizards. Their bite can result in considerable mechanical trauma in addition to venom toxicity.

3.3 Toxic Manifestations of Heloderma Envenomation

Reports of human envenomation by the Gila monster and beaded lizard have been sporadic, frequently exaggerated, and documentation is rare (Russell, 1983; Strimple *et al*., 1997; Cantrell, 2003). Although fatalities have been reported, their legitimacy is highly questionable (Russell, 1983). Bites should be treated, however, as medical emergencies, with appropriate evaluation and care.

3.3.1 Local Toxic Effects

Puncture wounds usually result, due to their powerful bite, and occasionally teeth become lodged in the tissue and even break off when trying to disengage the lizard. Bluish discoloration around the bite may be noted, but tissue necrosis is unlikely. Injected venom causes pain that can be intense and may radiate throughout the extremity. Oedema generally develops more slowly and is less severe than that which occurs with snakebite, but in some cases it can become quite marked and tense (Russell and Bogert, 1981; Roller, 1976; Hooker and Caravati, 1994). This may contribute to the pain of envenomation. Lymphadenopathy and lymphadenitis have also been reported (Russell and Bogert, 1981; Bou-Abboud and Kardassakis, 1988).

3.3.2 Systemic Effects

Systemic manifestations include generalized weakness, faintness or dizziness, diaphoresis, nausea and vomiting. Hypotension and tachycardia are complications of significant concern (Hooker and Caravati, 1994; Cantrell, 2003). Profound hypotension has been reported in several cases (Streiffer, 1986; Piacentine *et al*., 1986; Heitschel, 1986; Bou-Abboud and Kardassakis, 1988; Preston, 1989). Additionally, cardiovascular effects have been described, including nonspecific electrocardiographic changes, ventricular arrhythmias and myocardial infarction (Roller, 1976; Streiffer, 1986; Bou-Abboud and Kardassakis, 1988; Preston, 1989). Impaired renal function has also been reported as a possible result of prolonged hypotension (Preston, 1989).

Swelling of the lips and tongue have been noted in two separate reports, as anaphylaxis from Gila monster envenomation, in two patients without a prior bite history (Piacentine *et al*., 1986; Caravati *et al*., 1999). Marked swelling of the tongue and lips has also been reported in a case of beaded lizard envenomation in a patient without previous exposure (Cantrell, 2003). Given these three similar profiles, it may be that symptoms of swelling of the lips and tongue are potentially direct venom effects.

3.3.3 Laboratory

Laboratory abnormalities may include hypokalemia and leukocytosis. Thrombocytopenia has been rarely reported (Russell and Bogert, 1981; Bou-Abboud and Kardassakis, 1988; Preston, 1989). Thrombocytopenia associated with reduced fibrinogen and increased PT, PTT and fibrin split products indicated a consumptive coagulopathy in two cases (Bou-Abboud and Kardassakis, 1988; Preston, 1989).

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3.4 Management of Heloderma Envenomation

There is no antivenom; therefore the management of toxic manifestations is limited to supportive and symptomatic care. Removal of the offending lizard is commonly a first-aid measure required, and is important, because the longer the lizard is attached the greater the potential envenomation. Approaches that have been tried with mixed success include: (i) prying the jaws apart with a stick; (ii) submersion of the extremity and attached lizard under water or (iii) lighting and holding a match under the Gila monster's jaw. The bite site should be rinsed off to remove venom on the skin and further reduce venom absorption. Keep the patient warm, immobilize the extremity and transport the patient to an appropriate healthcare facility.

The site of the bite should be thoroughly cleansed and examined for remaining teeth. Careful exploration of the wound should be performed and soft-tissue radiography of the site may be helpful (Cantrell, 2003). Elevation of the limb may reduce swelling. Tetanus immunization should be updated, and follow-up wound care is essential. Prophylactic antibiotics are not necessary (Strimple *et al*., 1997). Vital signs, including pulse oximetry, and laboratory values should be regularly monitored. Pain management with opiates is usually beneficial, but should be used with caution in patients experiencing hypotension. Observe for electrocardiogram abnormalities, as transient T-wave anomalies and conduction abnormalities may occur (Bou-Abboud and Kardassakis, 1988). Volume resuscitation usually corrects the hypotension, but vasopressors may be necessary (Strimple *et al*., 1997). Systemic symptoms usually resolve within one to two days with adequate supportive care, depending on the degree of envenomation (Cantrell, 2003).

4 HYMENOPTERA

Flying insects of the order Hymenoptera can be found throughout the world. Only a few of the thousands of known species are significantly hazardous to man. These include honeybees, bumblebees, wasps, hornets and yellow jackets. Honeybees and bumblebees belong to the family Apidae while the others belong to the family Vespidae.

Honeybees, *Apis mellifera*, account for most apidae stings. People are most likely to be stung by honeybees when around flowering plants or if they disturb the bees' colony. The introduction of an African race of honeybee, *Apis mellifera scutellata* or *adansoni*, to South, Central and North America raises some additional concern. Most of the time, their behaviour is the same as other honeybees. However, if disturbed, these hybrid bees attack more quickly, attack in greater numbers, pursue for longer distances and sting continually over longer distances (Kim, 1999).

The vespids are generally more aggressive and attack vigorously if disturbed. Yellow Jackets, *Vespula maculiforma*, are the most common source of Hymenoptera stings (Kemp, 1998; Sigford, 2000). Yellow jackets tend to nest on the ground or in decaying wood. They will scavenge for food and may be pests at picnics or around garbage cans. Hornets build nests in trees or shrubs, while wasps often nest under eaves of buildings. Venom-sensitive patients may be more likely to be stung than nonsensitive individuals. It is postulated that intrinsic attractants may account for this phenomenon (Stone *et al*., 1992).

Their stinging apparatus consists of a modified ovipositor that is connected to a venom sac. They grasp the victim's skin with their claws and then pierce the skin with their stinger. The stinger of honeybees is barbed, so it and the venom sack remain attached to the victim's skin when the bee flies away. This results in the honeybee's demise. Vespids and bumblebees are generally able to withdraw their stingers and are capable of stinging again. Some yellow jackets, however, also may lose their stinger. Hymenoptera venom is composed of various proteins and peptides, some of which can cause vasoactive responses.

4.1 Toxic Manifestations

Stings from these insects may result in manifestations ranging from minor local pain and swelling to life-threatening respiratory and cardiovascular compromise (Ceyhan *et al*., 2001; Wagdi *et al*., 1994). The toxic effects may result from direct local and systemic effects of the venom, as well as anaphylactic reactions to venom proteins.

Up to 50 µl of venom may be injected with each sting. This dose is insufficient to produce systemic toxicity; however, vasoactive constituents of venom will produce local irritation with redness, swelling, tenderness and pain at the site of the sting. If a person is stung repeatedly on one occasion, then sufficient venom may be injected to produce systemic toxicity. Systemic toxicity due to multiple bee stings typically resembles an allergic reaction because of the histamine effects of the large dose of venom. Acute renal tubular necrosis resulting from multiple bee or wasp stings has been reported (Beccari *et al*., 1992; Kim *et al*., 2003). Systemic toxicity and death from a single bee sting is usually due to an anaphylactic reaction in those individuals who have developed specific IgE antibodies from prior stings (Kemp, 1998; Freeman, 2004).

Toxicity due to vespid stings may include an acute allergic-like response, followed by delayed effects such as

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haemolysis, rhabdomyolysis and renal failure (Bousquet *et al*., 1984).

The normal physiologic response to a hymenoptera sting consists of a small, painful, urticarial lesion that lasts only a few hours. Approximately 10–17% of people may develop a large local reaction that includes swelling and erythema greater than 5 cm in diameter, which may last more than 24 hours (Maguire and Geha, 1986; Reisman, 1989; Lin, 2003; Freeman, 2004). This large local reaction likely represents a cell-mediated, Type IV hypersensitivity reaction.

4.2 Allergic Manifestations

The allergic reactions to hymenoptera stings deserve detailed discussion. The allergic response may range from cutaneous effects of pruritis, urticaria and angioedema, to broader effects including nausea, vomiting, diarrhoea, abdominal cramps, uterine cramps, light-headedness and dizziness. The reactions with greatest risk include dyspnoea, bronchospasm, hypotension, dysrhythmias and cardiovascular collapse (Freeman, 2004). It is estimated that hymenoptera stings account for at least 40–50 deaths annually in the United States, more deaths than result from all other venomous animals (Golden, 1989). The systemic allergic response to hymenoptera stings is an IgE-mediated, Type I hypersensitivity reaction. Indirect complement activation may also play a role in insect sting anaphylaxis (Van Der Linden *et al*., 1990).

Unusual serum-sickness-like reactions have also been reported (Reisman and Livingston, 1989). There is considerable crossreactivity among the various vespids but cross-reactivity between vespid and bee venoms is relatively uncommon (Wright and Lockey, 1990).

The incidence of systemic allergic reactions may be as low as 0.4–0.8% in children, but as high as 4.0% in adults (Settipane *et al*., 1972; Golden *et al*., 1982). Up to 10–15% of the population may be sensitized to hymenoptera venom, but have not had an allergic response (Golden *et al*., 1982; 1997).

There is limited data to characterize which patients may develop none, some or all of these manifestations. Important factors include the insect type, age of the patient, underlying mastocytosis, underlying cardiovascular disease, treatment with β-adrenergic blockers, history of prior stings, time interval between stings, number of stings, venom sensitization, as documented by skin test or RAST (radioallergosorbent tests) and severity of the previous response to hymenoptera stings. Approximately 75% of the population is not sensitized to hymenoptera venom and will develop a normal local response and less than 1% will develop a systemic allergic reaction (Golden, 1989). Those who have previously had normal or large local reactions, but have a positive skin test or RAST have a 10–20% risk of developing a systemic reaction (Golden, 1989). Patients with a previous history of a systemic reaction who have a positive skin test or RAST have a 50% chance of developing another systemic reaction (Golden, 1989; Golden *et al*., 1997).

Individuals with bee-venom allergies are at a greater risk of systemic reactions on subsequent stings than are those with vespid-venom allergies (Bilo *et al*., 2005). It is commonly thought that patients develop more severe reactions with subsequent stings. While it is true that patients who receive repeat stings within a relatively short period (weeks to months) are more likely to develop a systemic reaction, most patients develop more mild or similar reactions with subsequent stings (Golden, 1989; Valentine *et al*., 1990; Bilo *et al*., 2005). Insect-sting allergy, in fact, is a self-limited disease for most people (Reisman, 1989). In other words, the more time between stings, the less likely a person is to have a serious systemic reaction. However, tolerance sometimes develops with very frequent stings, as observed with beekeepers (Bilo *et al*., 2005). Patients with mastocytosis, cardiovascular diseases or who are being treated with β-blocking drugs are associated with more severe sting reactions (Bilo *et al*., 2005).

The typical allergic response in children is very different to that in adults. Children are more likely to have cutaneous manifestations, but less likely to develop hypotension (Golden, 1989). Also, children much less frequently develop recurrent systemic manifestations. In fact, children who have previously had non-life-threatening allergic reactions are unlikely to subsequently develop a life-threatening reaction (Schuberth *et al*., 1983; Valentine *et al*., 1990).

Most fatalities occur within one hour of envenomation and are usually due to airway obstruction, hypotension or both. Almost all fatalities that occur do so within five hours of envenomation (Erickson, 2007).

4.3 Management

First-aid treatment for hymenoptera stings includes removing the stinger, if left behind, as rapidly as possible, by any method. Quick timing of stinger removal seems to be more important than potentially delaying removal by scraping across the site with a blunt edged object, as previously recommended (Visscher *et al*., 1996). The area should be washed well with soap and running water. Local reactions can be managed by applying ice to reduce swelling, oedema and pruritus. An antihistamine such as diphenhydramine may also help relieve pruritus. Large local reactions may improve with elevation of the extremity and administration of an analgesic, as well as a corticosteroid such as prednisone. The combined use of H_1- and H_2-antagonists has

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been suggested as a means to decrease the severity of late-phase cutaneous reactions (de Shazo *et al*., 1984).

Epinephrine (adrenaline) is the drug of choice for systemic sting reactions. Intramuscular administration is adequate in mild to moderate cases. In the event of hypotension, epinephrine may be administered intravenously. Vasopressin has also been used successfully to treat severe anaphylactic shock due to hymenoptera envenomation (Kill *et al*., 2003). Intravenous fluids and aggressive cardiopulmonary resuscitation should be provided if epinephrine therapy alone is not adequate. Intravenous antihistamines and corticosteroids should be used in addition to epinephrine for systemic reactions. Inhaled β_2-agonists may help relieve bronchospasm.

Patients who are at risk of developing systemic allergic reactions should be prescribed an emergency kit that contains epinephrine for subcutaneous injection. This includes those patients with a previous history of systemic allergic effects, and those who have had large local reactions and have a positive skin test or RAST.

Venom immunotherapy (VIT) may be useful in selected cases. It is presently indicated for those patients who have previously had life-threatening systemic reactions and have a positive skin test or RAST (Maguire and Geha, 1986). Adults are more likely candidates than children, since adults are at greater risk for developing a repeat systemic reaction. However, immunotherapy should be considered in children who have systemic allergic reactions, therefore following the same indications as with adults (Gruchalla, 2004; Golden *et al*., 2004). Other patients with less severe systemic reactions who have positive skin tests or RAST may benefit from VIT, but the cost of therapy and other factors, such as their age, medical history, occupation, outdoor activities or hobbies must be considered.

Conventional VIT consists of administering a gradually increasing dose of venom over 4–12 weeks to a maintenance dose of 50–100 µg. Rush protocols, in which venom doses are administered while the patient is hospitalized, achieve maintenance doses within only four to five days. The incidence of adverse reactions varies greatly among studies, but it seems that 50% or more of patients develop at least mild systemic effects. The variability reported may result from differences in patient populations, dosing regimens and the species of venom used for VIT. Bee venom, for example, is not as well tolerated as vespid venoms (Muller *et al*., 1992; Youlten *et al*., 1995). Both conventional and rush VIT protocols have produced protection rates up to 97% (Golden and Valentine, 1984; Reisman and Livingston, 1992). The protection rate for honeybee venoms, however, is only about 80%.

The issue of when to discontinue VIT is still controversial. Recent evidence, however, suggests that VIT can be safely discontinued after five years in most patients (Graft and Schoenwetter, 1994; Golden *et al*., 1996).

In an animal model, an antivenom has been effectively administered to treat mass bee envenomation. This has not been used in humans and is not commercially available (Jones *et al*., 1999).

5 ANTS

Ants belong to the family Formicidae and comprise the third group of venomous Hymenoptera. Thousands of ant species are found throughout the world, some of which can inflict painful venomous stings. Local or systemic allergic reactions can also occur. Not all venomous ants sting. Some, such as the carpenter ant, *Camponotus atriceps* and weaver ants, genus *Oecophylla*, of the subfamily Formacinae, bite their prey and then spray venom into the wound. Formic acid, which is a potent cytotoxin, is the primary constituent of their venom (Rhoades *et al*., 1977).

Fire ants (*Solenopsis* spp.) are native to both North and South America. Fire-ant envenomation is a significant health hazard in the southern United States (Blum, 1984; Stafford *et al*., 1989a; Tracy *et al*., 1995). In fact, up to 60% of the population in an infested area are stung each year (de Shazo *et al*., 1990). The red imported fire ant (*Solenopsis invicta*) has rapidly spread throughout the southern United States and has overtaken the less aggressive native species (*S. xyloni*), as well as the black imported fire ant (*S. richteri*). The imported fire ants of the United States are so named because they are thought to have been introduced via produce shipped from Brazil to Mobile, Alabama in 1918 (Hung and Vinson, 1978).

Ants sting to subdue their prey and as a means of defense. A fire ant grasps its victim with its mandibles then, using its head as a pivot, it swings its abdominal stinger to inflict multiple stings (Diaz *et al*., 1989). Unlike other Hymenoptera, fire ants sting slowly and may inject venom for seconds to minutes (Stafford *et al*., 1989b). With each sting the ant injects from 0.04 to 0.11 µl of venom (de Shazo *et al*., 1990).

The venom of most ants and other Hymenoptera consists primarily of protein (Blum, 1984). Imported fire-ant venom, on the other hand, is 90–95% piperidine alkaloids, which cause histamine release and skin necrosis. This venom contains only 0.1% protein (Hoffman *et al*., 1988a; Bilo *et al*., 2005). Four proteins have been identified, namely: Sol i I, Sol i II, Sol i III and Sol i IV (Hoffman *et al*., 1988a; Bilo *et al*., 2005). All these proteins are significant allergens.

5.1 Toxic Manifestations

Toxicity associated with fire-ant envenomation is normally limited to the site of the sting. *In vitro* studies

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indicate that the venom has haemolytic, cytotoxic, bactericidal and insecticidal properties (Rhoades *et al*., 1977; Adrouny *et al*., 1959). Multiple stings (approximately 10 000), however, have not resulted in systemic toxicity (Diaz *et al*., 1989).

The local response to envenomation includes instant pain, an initial wheal and a flare reaction. Superficial vesicles with clear fluid develop at the site of the sting within four hours. This fluid is lost and replaced within 8–10 hours by cloudy fluid, which becomes purulent. A pustule develops within 24 hours, which may be surrounded by a red halo (Car *et al*., 1957). This lesion is pathognomonic in the United States for fire-ant stings (Lockey, 1990). An important complication of fire-ant stings is secondary infection (Parrin *et al*., 1981).

Large local reactions may also occur, which may be immunologically mediated (Diaz *et al*., 1989). These reactions are characteristic of 'late-phase reactions' that occur secondary to ragweed (de Shazo *et al*., 1984). Systemic allergic reactions characteristic of those caused by other Hymenoptera also may occur. The natural history of such responses has not been well studied, but is probably comparable to that for other Hymenoptera. Anaphylaxis may result in up to 1% of stings (de Shazo *et al*., 1990). Anaphylactic deaths due to fire-ant envenomation have occurred (Prahlow and Barnard, 1998).

The prevalence of asymptomatic sensitized people to fire-ant venom is comparable to that which exists for other Hymenoptera, approximately 16% (Hoffman *et al*., 1988b). Crossreactivity may exist between bee or wasp venom and fire-ant venom. Sol i II has been identified as the crossreactive protein (Hoffman *et al*., 1988b; Bilo *et al*., 2005).

Neurologic sequelae, including seizures and mononeuropathy have also been reported (Fox *et al*., 1982; Candiotti and Lamas, 1993). The aetiology of such reactions is not known.

Envenomations by the samsum ant, *Pachycondyla sennaarensis* found in the African tropics and Arabian Peninsula, and Australian bull ants (*Myrmecia* spp.) have caused anaphylactic reactions.

Giant black ants, genus *Paraponera* found from Nicaragua to the Amazon basin cause severe pain and fever, and lymphangitis has also been reported (Dib *et al*., 1995; Erickson, 2007).

5.2 Management

First aid for fire-ant envenomation consists primarily of thorough washing with soap and water. Although a number of therapeutic measures have been evaluated as a means to alter the development of pustules, neither topical nor parenteral therapies had any effect on developing lesions (Parrin *et al*., 1981). A cold compress may help relieve some swelling and discomfort. The pustules should be bandaged to prevent excoriation. Large local reactions and systemic allergic reactions due to fire ant and other ant envenomations should be managed as previously described for other Hymenoptera.

As with other Hymenoptera, the indications for immunotherapy for fire-ant envenomation are not clear. The relatively high risk for stings in sensitized people, however, causes many to undergo such therapy (Stafford *et al*., 1989c). To further confound the issue, only whole-body extracts are available for therapy, which contain variable quantities of venom. Some evidence of its effectiveness, however, has been presented (Hylander *et al*., 1989; Brown *et al*., 2003).

VIT for the Australian bull ant using purified *Mymecia* ant venom has been shown to be effective in individuals with known allergy to these ants (Brown *et al*., 2003).

6 SPIDERS

Spiders are arthropods of the order Arachnidae. Most spiders are venomous; in fact, all but two families of spiders have venom glands. Thousands of venomous spiders exist throughout the world, however, only a few are of significant medical importance. Most notable are spiders of the genus *Latrodectus* and *Loxosceles*. Other spiders of clinical significance include the *Tegenaria*, *Hadronyche*, *Phoneutria* and various genera of tarantulas.

6.1 Widow Spiders

The true black widow spider (*Latrodectus mactans*) is found in temperate zones of North America, including all of the United States except Alaska. Other *Latrodectus* species are found throughout the world (Rauber, 1983–1984) including Australia, Europe, South America, Asia and the Middle East. The major differences between these species are in their body markings and habitats. *L. mactans* has a characteristic shiny black coloration with a red hourglass-shaped marking on its abdomen. Generally these spiders are nonaggressive and bite defensively when threatened or disturbed. They are typically found in undisturbed, protected areas such as storage buildings, wood piles and trash heaps. Female spiders make irregular funnel shaped webs in which to trap prey and suspend their egg sacs.

Only the females of this species are hazardous to man. The male is too small to cause significant envenomation. The black widow has claw-like hollow fangs that are connected to two venom glands in its cephalothorax. The venom glands have striated musculature that controls the injection of venom. While there are some interspecies

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differences in venom constituents, the toxic fraction affecting vertebrate nerve endings, α-latrotoxin, appears to be the same (Rauber, 1983–1984; Müller, 1993).

The venom of *Latrodectus* spiders, which is one of the most potent of all animal venoms, is primarily neurotoxic. The venom gland of the black widow spider contains just less than 0.2 mg of venom (Binder, 1989). The mean lethal dose ranges from 0.005 to 1.0 mg kg^{-1} in various animal species (Edlich *et al*., 1985). The venom acts at the neuromuscular synaptic junction, causing the release of acetylcholine and norepinephrine from presynaptic vesicles (Rauber, 1983–1984; Binder, 1989). This results in excessive neuromuscular stimulation and, as expected, other cholinergic and adrenergic signs and symptoms. The venom causes local muscle pain initially, which then generalizes to involve primarily large muscle groups (Kobernick, 1984).

6.1.1 Toxic Manifestations

Most bites in humans occur above the waist on the forearm or torso (Moss and Binder, 1987). Bites are more prevalent during late summer and early fall, when there are increased numbers of both young and mature spiders (Moss and Binder, 1987). Although the bite itself is often not initially painful and may go unnoticed, pain at the site is the most common early symptom of envenomation (Moss and Binder, 1987). Other common symptoms include severe abdominal pain and cramping, as well as lower extremity pain and weakness. Hypertension, tachycardia, fever, leukocytosis, vomiting, restlessness, mental status changes, headache, rash, priapism, paraesthesias, albuminuria, ptosis and periorbital oedema also have been reported. Facies latrodectismica, with spasm of facial muscles, blepharitis, lacrimation and rhinitis, has been described (Maretić, 1983). In severe cases shock, coma, respiratory failure and pulmonary oedema may occur (Binder, 1989; LaGrange, 1990). In most cases, resolution of symptoms usually occurs within a few days, however pain may last for over a week. The mortality rate from black widow envenomation is probably less than 1% (Binder, 1989).

6.1.2 Management

There are no specific first-aid measures for black-widow envenomation. The bite site should be thoroughly cleansed and tetanus immunization should be updated if necessary. Medical management is primarily directed at relieving muscle spasms and pain. Opioid analgesics and benzodiazepines appear to be the most effective treatment for mild to moderate envenomations (Clark *et al*., 1992). Calcium gluconate has been used, however, it does not appear to be routinely effective (Clark *et al*., 1992). In addition, centrally acting muscle relaxants, such as methocarbamol, do not appear to be effective (Key, 1981).

Lactrodectus antivenom is available in Argentina, Australia, Mexico, South Africa and the United States. These are all equine-derived antivenoms. Some of these are whole IgG and some are $F(ab')_2$ IgG fragments, the latter having reduced antigenicity. The use of antivenom should be weighed against the potential risk of acute hypersensitivity and delayed serum sickness. In general, antivenom should be reserved for cases of severe *Lactrodectus* envenomation, including those with systemic involvement or severe local symptoms refractory to other treatments. It also can be used in life-threatening situations and in those patients at high risk for severe morbidity, such as the very young or old, and in those with underlying hypertension, cardiac or cerebrovascular disease.

6.2 Brown Spiders

Bites of some spiders result primarily in local tissue destruction. This condition has been referred to as necrotic arachnidism. The most notorious of these spiders are those of the genus *Loxosceles*. Spiders of this genus are found throughout South America, southern North America, Africa, Australia and southern Russia, as well as the Mediterranean and the Orient (Gertsch and Ennik, 1983; Gendron, 1990). *Loxosceles* spiders are fawn to dark brown in colour with relatively long skinny legs and have a characteristic violin-shaped marking on their dorsal carapace (Binder, 1989). The terms brown, violin or fiddleback spider are used to describe these species. The brown recluse spider, *Loxosceles reclusa*, is the most important species in the United States, as it is responsible for the majority of envenomations. It has been extensively studied and exemplifies the toxicity associated with these spiders.

The diagnosis of *Loxosceles* envenomation and its actual incidence are difficult to establish because of other potential causes of necrotic wounds, including bites of other spiders and confusing medical conditions, such as infections or toxic epidermal necrolysis (Russell and Gertsch, 1983). Diagnostic difficulties also have hampered attempts to study various treatment modalities. For example, in one series of 95 cases of presumed brown-recluse-spider bite, only 17 cases could be confirmed and ultimately studied (Rees *et al*., 1987). A venom-specific immunoassay has been used to identify *Loxosceles* envenomation (Berger *et al*., 1973a). Also, a highly specific passive haemagglutin inhibition test has been used for determining entry into controlled treatment trials for brown-recluse-spider bites (Barrett *et al*., 1993). In addition, a *Loxosceles* species venom ELISA has been developed for detection of *Loxosceles* species spider venom (Gomez *et al*., 2002). However, no diagnostic test is readily available to confirm *Loxosceles* venom exposure in a clinical setting.

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The brown recluse spider is found primarily in the south-central United States (Majeski and Durst, 1976). Most reported bites have occurred in Arkansas, Kansas, Missouri and Oklahoma. These spiders inhabit primarily warm, dry, secluded places and can be found both indoors and outdoors. The household closet is the most frequently reported site of discovery (Rees and Campbell, 1989). Other potential sites include wood piles, storage buildings, stored clothing, attics, basements and other quiet locations. The brown recluse is nocturnal and, as a result, most bites occur during the night (Rees and Campbell, 1989). These spiders are most active in summer months and hibernate during the winter.

6.2.1 Toxic Manifestations

The venom of *Loxosceles* spiders is both cytotoxic and haemolytic. At least nine proteins have been identified, most notably a hyaluronidase, which accounts for the spreading of injected venom, and sphingomyelinase D, which likely contributes to its haemolytic and cytotoxic properties (Rees and Campbell, 1989; Wasserman, 1988; Hobbs and Harrell, 1989). Sphingomyelinase D activity has been found in a worldwide sampling of *Loxosceles* (Binford and Wells, 2003). It is this component to which dermonecrosis is attributed. The quantity of injected venom is relatively small and by itself is unlikely to cause significant injury. The destructive nature of the venom is apparently facilitated by complement activation and subsequent inflammatory response (Hobbs and Harrell, 1989; Rees *et al*., 1983). This results in endothelial cell damage, haemorrhage, infiltration of polymorphonuclear leukocytes and thrombosis of venules and arterioles, causing necrosis (Berger *et al*., 1973b).

The bite of a brown recluse spider results in little more than a stinging or prick sensation and may go unnoticed by the victim. In contrast to this, the bite of the South American *Loxosceles laeta* can cause severe pain and oedema, but less tissue necrosis. The clinical manifestations following envenomation depends on the amount of venom injected and the site of envenomation, as well as the age, underlying health and immune status of the victim (Wasserman and Anderson, 1983–1984; Majeski and Durst, 1976). Not all patients develop the characteristic necrotic lesion or potentially severe systemic effects. It may be that many bites go unrecognized because only minimal envenomation occurs and victims experience only mild discomfort that resolves uneventfully within a few days (Berger, 1973).

Most patients, even following significant envenomation, do not present for treatment until many hours after the bite, once the initial signs of a necrotic lesion become evident (Gendron, 1990). The most common presenting signs and symptoms include erythema, cellulitis, generalized rash, blister, pain, pruritus, malaise, chills and sweats (Rees *et al*., 1987). The characteristic lesion begins as a blister with surrounding ischaemic discolouration (Wasserman and Anderson, 1983–1984; Hobbs and Harrell, 1989). An erythematous ring may surround this area giving a characteristic 'bulls eye' or 'halo' appearance (Wasserman and Anderson, 1983–1984). The blister subsequently becomes a bluish macule, the centre of which generally sinks below surrounding tissue (Hobbs and Harrell, 1989). Over several days the necrotic lesion may progress, resulting in an area of eschar that sloughs off after 7–14 days. This leaves an area of ulceration from 1 to 30 cm in diameter (Wasserman and Anderson, 1983–1984; Hobbs and Harrell, 1989). Bites in fatty areas of the body tend to become more extensive (Wasserman and Anderson, 1983–1984). It may take weeks to months for this area to heal by secondary intention. A small percentage of patients may develop persistent lesions, which could subsequently progress to the development of pyoderma gangrenosum and pseudoepitheliomatus hyperplasia (Rees *et al*., 1987; Hoover *et al*., 1990). The extent of local bite findings may not correlate with the presence or severity of systemic findings.

Systemic toxicity occurs less commonly and may not develop for 24–72 hours after the bite. Systemic toxic effects may include fever, malaise, arthralgias, myalgias, rash, convulsions, haemolysis, thrombocytopenia, anaemia and DIC (Wasserman and Anderson, 1983–1984; Williams *et al*., 1995). Nephrotoxicity may result as a complication of haemolysis and subsequent haemoglobinuria. Shock without the presence of DIC, possibly due to an endotoxemic-like effect of *Loxosceles* venom, has been reported (Walter *et al*., 1999). Death due to the systemic effects of *Loxosceles* envenomation has occurred.

6.2.2 Management

The treatment of necrotic arachnidism, regardless of the spider involved, should consist of sound local-wound management. This should include thorough cleansing, tetanus prophylaxis as necessary, immobilization, elevation and rest (Wasserman, 1988). Cool compresses may help relieve inflammation and pain (Gendron, 1990). Prophylactic antibiotics generally are not indicated and steroid therapy has not been found to be effective (Wasserman, 1988; Cole *et al*., 1995). Symptomatic relief can be provided with the use of antipruritic, analgesic and antianxiety agents. Other treatments including dapsone, glucocorticoids, diphenhydramine, dermal nitroglycerine and hyperbaric oxygen have been described, but efficacy has not been established (Burton, 1995; King and Rees, 1983; Elston *et al*., 2005; Phillips *et al*., 1995).

Surgical debridement may be needed, but should be delayed until progression of erythema has stopped and eschar margins are defined, usually occurring about one to two weeks after the bite. In cases of systemic involvement, therapy should be directed at specific complications. Platelets and packed red-blood cells may

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be indicated in the presence of thrombocytopenia or anaemia, respectively. Good hydration should be maintained and renal function monitored. Alkalinization of the urine may be considered in the presence of haemoglobinuria or haematuria.

Brown-recluse-spider antivenom has been prepared and has been shown *in vitro* to abolish the dermonecrotic activity of brown-recluse venom (Rees *et al*., 1984). Equine-derived *Loxosceles* antivenom, is available in endemic areas of South America, including Brazil and Peru, for severe dermal and systemic effects of *Loxosceles* envenomation. Results are often disappointing given the delayed presentation and therefore delayed treatment (White *et al*., 1995).

6.3 Hobo Spiders

Hobo spiders, *Tegenaria agrestis*, build their funnel-shaped webs in undisturbed areas. They are found in Europe, west central Asia, British Columbia and areas of the United States, including the Pacific Northwest and Utah (Boyer *et al*., 2007). The components of their venom are not well identified and no necrotic component has been found. The venoms of the European spiders and those in the United States appear to be similar, but no medical problems have been reported following envenomation by the European *T. agrestis* (Binford, 2001; Boyer *et al*., 2007).

6.3.1 Toxic Manifestations

Necrotic arachnidism has been attributed to the Hobo spiders of the Pacific Northwest but often the spider is not identified and further study has been suggested (Vest, 1996; Vetter and Isbister, 2004). Systemic effects are also reported and these include headache, visual changes, hallucinations, weakness and lethargy (Vest, 1996).

6.3.2 Management

Treatment is supportive and includes local wound care with cleaning and debridement as needed and tetanus prophylaxis when indicated.

6.4 Funnel Web Spiders

The clinically significant species *Hadronyche* (or *Atrax*) are found in southeastern Australia, Tasmania, Papua New Guinea and the Solomon Islands (Boyer *et al*., 2007). They live in protected spaces such as silk-lined burrows in the ground or they create shallow, funnel shaped burrows with silk under objects such as rocks or thick vegetation. The venom of these spiders is complex with components that include both high and low-molecular-weight proteins and various acids. The Sydney funnel web spider, *Atrax rorobustus*, is an aggressive spider, which causes widespread neurotransmitter release (Mylecharane *et al*., 1989).

6.4.1 Toxic Manifestations

The clinical result of envenomation includes severe local pain followed by muscle fasciculations, diaphoresis, tachycardia, hypertension, hypotension, agitation and pulmonary oedema (Mylecharane *et al*., 1989; Isbister *et al*., 2005). Although uncommon, death has occurred due to respiratory failure, hypotension or cardiac arrest (Isbister *et al*., 2005).

6.4.2 Management

In addition to local-wound care and tetanus prophylaxis if indicated, treatment is similar to that for Australian snakebite. A compression wrap is placed on the affected extremity and the extremity immobilized. This dressing should only be removed after antivenom administration is started (Isbister, 2006). Antivenom, an IgG product, is produced against *Atrax robustus* and is also effective against the *Hadronyche* spp. It should be reserved for and appears effective in the treatment of severe funnel-web-spider envenomation. The risks of immediate allergic reaction and delayed serum sickness exist but appear to be infrequent (Isbister *et al*., 2005).

6.5 Banana Spiders

Phoneutria spiders of South America are large, nocturnal spiders. *Phoneutria nigriventer* is found in southern Brazil, Argentina and Uruguay. Other lesser-known species are found in Bolivia and Columbia (Boyer *et al*., 2007). Spiders of this genus are aggressive hunters. Their venom contains a mixture of histamine, serotonin, amino acids, hyaluronidase and other peptides (Costa *et al*., 2003). The venom effect is on both the central and peripheral nervous systems (Costa *et al*., 2003).

6.5.1 Toxic Manifestations

Most envenomations by *Phoneutria* are not significant. However, in some cases, severe local pain that radiates proximately occurs. Following this, diaphoresis, salivation, vomiting, priapism, hypothermia, hypertension, tachycardia and death, usually due to respiratory muscle paralysis, may occur (Lucas, 1988).

6.5.2 Management

Little information is available about the treatment of *Phoneutria* envenomation. Cleansing the wound and tetanus prophylaxis, when indicated, should be

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performed. When pain is present, injecting a local anaesthetic near the bite site often provides relief (Lucas, 1988; White *et al*., 1995). The use of opioid analgesics may potentiate the venom-induced respiratory compromise and suggestions against their use, in the setting of systemic toxicity, have been made (Boyer *et al*., 2007).

Monovalent and polyvalent antivenoms, active against *Phoneutria* species, are available in Brazil. Given the risks of immediate and delayed hypersensitivity reactions, their use should be reserved for cases of severe envenomation.

An ELISA has been developed for rapid detection of venom antigens, but is not readily available (Chávez-Olórtegui *et al*., 2001).

6.6 Tarantulas

Tarantulas belong to the Theraphosidae family and suborder Mygalomorphae. About 800 species have been described worldwide on all continents, with the greatest number in tropical regions. They live in burrows with trip-line threads. The venoms' multiple constituents vary and include hyaluronidase, nucleotides, polyamines and proteins, depending on species. Some genera, including *Haplopelma*, *Lasiodora*, *Grammostola*, *Acanthoscurria* and *Brachypelma*, located in the western hemisphere, are unique in that they have urticating hairs. When threatened, these spiders send these specialized hairs, located on their tarsi (feet), towards a victim by rubbing the hind legs across the dorsal abdomen.

6.6.1 Toxic Manifestations

Most tarantula bites cause mild local symptoms such as immediate pain and redness. Some cause more severe pain, swelling, numbness, lymphangitis, weakness and emesis. No deaths have been reported due to envenomation. The urticating hairs irritate skin and mucous membranes. These barbed hairs can penetrate human skin causing oedematous, pruritic papules; itching may persist for weeks. Ophthalmic complications such as keratoconjunctivitis, and ophthalmia nodosa have been reported (Belyea *et al*., 1998).

6.6.2 Management

Standard wound care, oral analgesics, and, when indicated, tetanus prophylaxis is the treatment for tarantula envenomation. Removal of urticating hairs can be achieved by repeated use of sticky tape and irrigation with water. Oral antihistamines and oral corticosteroids may be of benefit.

7 SCORPIONS

True scorpions are arachnids of the order Scorpionida. It is estimated that up to 1400 species are distributed worldwide, only a few of which are of significant medical importance (Hutt and Houghton, 1998; Lucas and Meier, 1995). Most of these are in the Buthidae family. One, however, belongs to the Scorpionidae family (Radmanesh, 1990; Bush, 2007). Scorpions primarily inhabit deserts and semi-arid regions. While scorpions are native to certain locales, it is important to recognize that they may be inadvertently transported in luggage or other goods to distant areas (Trestrail, 1981).

In the United States, the most significant of all the scorpions is *Centruroides sculpturatus* (bark scorpion), which is found primarily in Arizona, but also inhabits areas within New Mexico, California, Texas and Nevada. *Centruroides exilicauda* is found in Mexico (Likes *et al*., 1984; Curry *et al*., 1983–1984; Bush, 2007). A number of other scorpions that can produce significant envenomation may be found in Central America, South America, the Caribbean, Asia, Africa, India and the Middle East (Banner, 1989; Bush, 2007; Lucas and Meier, 1995).

Scorpions are nocturnal and take shelter during the day under rocks, piles of debris or may hide inside houses in clothing or shoes. The bark scorpion notoriously shelters under the loose bark of trees, and in crevices of dead trees or logs (Likes *et al*., 1984). Scorpions feed primarily on insects, spiders and occasionally on other scorpions (Banner, 1989).

Scorpions have a hard exoskeleton and three primary body parts: the cephalothorax, to which are attached a pair of pincers; the abdomen, which has four pairs of legs; and the tail, which is segmented and ends in a telson which contains the stinging apparatus. The telson contains two venom glands that lead via independent ducts to the stinger. The scorpion uses its pincers to grab its prey and then arches its tail over its body and head to inject venom. Likewise, the scorpion may grab the skin of humans and sting, sometimes repeatedly, in self-defense.

7.1 Toxic Manifestations

The venom of scorpions is primarily neurotoxic. This property appears to result from its effects on voltage-dependent ion channels. The best studied of these are sodium channels. The activation and inactivation of sodium channels ultimately results in the release of catecholamines and acetylcholine (Wang and Strichartz, 1983; Banner, 1989). Other venom fractions exhibit enzymatic, anticholinesterase, coagulopathic, haemolytic, cardiotoxic and pancreatotoxic properties (Banner, 1989). The venom of one genus, *Hemiscorpion lepturus*, belonging to the Scorpionidae family, also has cytotoxic properties (Bush, 2007; Radmanesh, 1990).

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Most envenomations occur on the extremities. Adults are most commonly stung, however, children are more likely to develop serious toxicity (Curry *et al*., 1983–1984; Likes *et al*., 1984; Berg and Tarantino, 1991). The sting of *Centruroides* causes local pain, numbness, hyperaesthesia, salivation, agitation, wheezing, tachycardia, hypertension and muscle spasms (Likes *et al*., 1984). Usually, there are no visible findings at the site of envenomation. In severe cases, cranial nerve and somatic motor abnormalities, such as eye movement disorders, blurred vision, tongue fasciculations, difficulty swallowing and jerking of the extremities, may develop (Curry *et al*., 1983–1984). Respiratory failure, pulmonary oedema, multiorgan failure and rhabdomyolysis have been described in severely envenomated children (Berg and Tarantino, 1991).

Tityus scorpions are of significant importance in South America, Central America and the Caribbean. Clinical manifestations are similar to those produced by other scorpions and result from excess neurotransmitter release from the sympathetic and parasympathetic nerve endings and adrenal medulla. Severe envenomation may result in cardiocirculatory failure and pulmonary oedema (Hering *et al*., 1993). *Tityus* scorpion envenomation may also produce significant local pain and erythema. The *Tityus trinitatis* species has also been reported to produce pancreatitis (Bartholomew, 1970).

In South Africa the most important genera of scorpions include *Parabuthus* and *Bothotus.* These scorpions most commonly sting their victims, however, certain species of *Parabuthus* are also capable of squirting venom for up to 1 m (Newlands, 1978). If the venom enters the eye or an open wound it can cause toxicity comparable to that of the spitting cobra. Local effects following envenomation by these South African scorpions includes local burning and possibly swelling. Systemic toxicity may include muscle contractions, convulsions, perspiration, salivation, tachycardia, arrhythmias and irregular respirations (Newlands, 1978). Death may result from respiratory or cardiac failure.

Red scorpion (*Mesobuthus tamulus*) envenomation in India can cause severe toxicity and death. Excessive release of catecholamines can result in myocardial damage, arrhythmias, cardiac failure and pulmonary oedema (Alagesan *et al*., 1977; Rajarajeswari *et al*., 1979; Bawaskar, 1982). Other reported manifestations in both fatal and nonfatal cases include profuse sweating, mydriasis, vomiting and priapism (Bawaskar, 1982).

The yellow scorpion (*Leiurus quinquestriatus*), as well as *Androctonus* and *Buthus* species, inhabit the Middle East and northern Africa. The yellow scorpion also causes excessive release of catecholamines, which can result in myocardial damage and congestive heart failure (Barzilay *et al*., 1982). Arrhythmias and pulmonary oedema have also been reported (Alagesan *et al*., 1977; Rahav and Weiss, 1990). Pancreatitis is a common complication of envenomation by the yellow scorpion and may explain the abdominal pain and vomiting commonly seen following envenomation (Sofer *et al*., 1991).

Hemiscorpion lepturus inhabits southwest Iran, Iraq, Pakistan and Yemen. The cytoxic venom can cause significant tissue destruction. Usually, a 3–4 mm dark blue macule develops and is surrounded by a red halo on the skin. In some cases, this area becomes indurated and inflamed and eventually skin sloughing may occur leaving a disfiguring wound. In severe cases, central nervous system and cardiovascular involvement may occur (Radmanesh, 1990).

7.2 Management

First aid for scorpion envenomation consists of good local-wound care, including thorough cleansing and tetanus prophylaxis, if necessary. Cold compresses can be applied to help relieve pain and inflammation, if present. Opioid analgesics may be needed to treat severe pain, which can sometimes occur. Systemic manifestations of envenomation can generally be managed conservatively with traditional supportive measures. Atropine sulfate may be indicated to control excessive parasympathetic manifestations, however, this is often not necessary (Banner, 1989). Clinical reports describe successful management of the cardiovascular manifestations of scorpion envenomation with β-blockers, calcium-channel blockers, vasodilators, such as hydralazine, and angiotensin-converting enzyme inhibitors (Gueron *et al*., 1992). The selection of specific therapy should be based on the clinical findings and manifestations of the patient. Prazocin is first-line treatment in India for hypertension and pulmonary oedema caused by the red scorpion, *Mesobuthus tamulus* (Bawaskar and Bawaskar, 1997). Sodium nitroprusside is used for severe cases of pulmonary oedema. However, these treatments have not been rigorously studied. For severe symptoms due to *C. sculpturatus* envenomation, a continuous midazolam infusion, administered in an intensive care unit, has been retrospectively evaluated and felt to be safe for the treatment of these symptoms (Gibly *et al*., 1999). This provided an alternative to previously available antivenom.

Antivenom therapy has an important role in therapy for scorpion envenomations in some parts of the world. Twenty-two types of scorpion antivenom are listed in the American Zoo and Aquarium Association Antivenom Index. Scorpion antivenom is available in Brazil, India, Israel, South Africa, North Africa and Mexico (Banner, 1989; Bush, 2007). In the United States, an IgG antivenom for *C. sculpturatus* had been derived from goat serum, but it was not approved by the Food and Drug Administration and is no longer available (Rachesky *et al*., 1984). It had been used

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safely and successfully to relieve severe signs and symptoms of envenomation (Gateau *et al*., 1994). An $F(ab')_2$ *Centruroides* antivenom, manufactured by Instituto Bioclon in Mexico, is undergoing clinical trials in the United States. One advantage of this antivenom is less antigenicity when compared with the previous whole IgG antivenom. Experience with antivenom in other parts of the world is also limited and issues relative to safety, efficacy and specificity remain to be resolved (Banner, 1989). Given the risks, which include immediate and delayed hypersensitivity reactions, the use of antivenom should be reserved for symptoms consistent with severe scorpion envenomation.

8 JELLYFISH

The phylum Cnidaria includes the class Scyphozoa, the true jellyfish. The Portuguese man-o'-war, although generally considered a jellyfish is actually a Cnidarian of the class Hydrozoa. Because of marked similarities, however, the Portuguese man-o'-war will be discussed here with the true jellyfish.

The most notable of these species are: the box jellyfish (*Chironex fleckeri*), which inhabits the coastal waters of Australia and the Indo-Pacific region; the Irukandji jellyfish (*Carukia barnesi*), found in the northern coastal waters of Australia; the Portuguese man-o'-war (*Physalia physalis*), located in the more tropical waters of the Atlantic; the sea nettle (*Chrysaora quinquecirrha*), which is endemic to the Chesapeake Bay and the mid-Atlantic coastal waters of the United States; and the Pacific Portuguese man-o'-war (*Physalia utriculus*), which is often responsible for stings off the Hawaiian coast. Multitudes of other jellyfish species are also found in these and other waters throughout the world.

The hanging tentacles of jellyfish contain thousands of stinging organelles known as nematocysts. Within the nematocyst is a coiled thread-like structure coated with venom. In response to pressure or changes in osmolarity, the nematocyst fires its thread, which can penetrate the skin to cause envenomation.

The venom of jellyfish contains various polypeptides and enzymes. It is both toxic and allergenic. In animal studies, the venom has been shown to produce dermonecrosis, vasopermeability, haemolysis, cardiotoxicity, neurotoxicity, musculotoxicity and cytotoxicity (Burnett and Calton, 1987a). A kinin-like fraction has also been identified which is believed to account for pain (Burnett and Calton, 1987a). The exact mechanisms by which most jellyfish produce their toxic syndromes remain to be elucidated.

8.1 Toxic Manifestations

The most common manifestations from Cnidaria envenomation are local toxic effects. Severe systemic and allergic reactions also may occur. The severity of envenomation is dependent on a number of factors, including: the species of jellyfish; the extent and duration of contact with tentacles; the resultant number of fired nematocysts; the amount of venom available in the nematocyst at the time of firing; the thickness of the skin at the site of the sting; and the size, age and underlying health of the victim (Burnett *et al*., 1987a; Lumley *et al*., 1988).

Dermal contact with jellyfish tentacles typically results in linear, urticarial and painful eruptions, which result from the direct toxic effects of the venom (Burnett and Calton, 1987a). The sting of the Portuguese man-o'-war is generally considered more painful than that of the sea nettle and the local pain from envenomation by the box jellyfish can be excruciating (Burnett *et al*., 1987a). The resultant lesions produced by jellyfish envenomation may be vesicular, haemorrhagic, necrotizing or ulcerative (Burnett *et al*., 1987a). Subacute or chronic reactions may include localized hyperhidrosis, desquamation, lymphadenopathy, angioedema, urticaria, keloid formation, hyper- or hypopigmentation, local fat atrophy, contractions, vasospasm, gangrene and nerve damage (Burnett *et al*., 1987a; Burnett and Calton, 1987b). Envenomation by the small carybdeid jellyfish *Carukia barnesi*, on the other hand, may result in a relatively minor initial sting followed by the delayed onset of a potentially severe systemic reaction (Little *et al*., 2003).

An allergic response may contribute to the local effects (Burnett *et al*., 1983). In fact, a large local reaction comparable to that seen with hymenoptera stings has been described (Burnett and Calton, 1987a). Delayed, persistent or recurrent eruptions at the site of the initial sting and at distant sites have also been reported (Burnett *et al*., 1983; 1987b; O'Donnell and Tan, 1993). It has been postulated that an antigen depot must exist for this to occur. There is also evidence that individuals may crossreact with different animals of this phylum (Burnett *et al*., 1987b). The potential for an anaphylactic reaction must be considered.

Jellyfish stings to the cornea cause intense pain, tearing and photophobia. The injuries are typically self-limited without long-term sequelae. Unusually severe reactions consisting of iritis, mydriasis, loss of accommodation and increased intraocular pressure have been reported (Glasser *et al*., 1992).

Jellyfish envenomation may result in severe systemic toxic reactions and even death. The most common systemic toxic effects include headache, nausea, vomiting, malaise, weakness, perspiration and lacrimation (Burnett *et al*., 1987a). More severe manifestations include hypotension, cardiac conduction disturbances, arrhythmias, respiratory depression, pulmonary oedema and cardiovascular collapse. Irukandji syndrome,

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resulting from the stings of the Irukandji jellyfish (*Carukia barnesi)* may include the delayed onset (30 minutes) of severe backache, muscle pains, chest and abdominal pain, nausea and vomiting, headache and sweating (Little *et al*., 2003). Patients are often hypertensive on presentation. Pulmonary oedema is also a potential complication.

Death from jellyfish envenomation may result from either allergic or toxic mechanisms. The box jellyfish is the most toxic of all marine animals, and has been implicated in most jellyfish-related fatalities. It is not clear whether death results from cardiotoxicity, respiratory failure or both (Lumley *et al*., 1988; Stein *et al*., 1989). The precise aetiology, in fact, may be dose dependent (Lumley *et al*., 1988).

8.2 Management

Victims of jellyfish envenomation should be kept quiet and the affected limb should be immobilized, because muscle activity may increase the firing of nematocysts. Compression bandages, which are used to impede lymphatic flow following snakebite, have been shown to cause the mechanical expression of additional venom from discharged nematocysts and should be avoided (Pereira *et al*., 2000). The exposed area should be rinsed with seawater. Fresh water is contraindicated, as this too will increase nematocyst firing due to the osmotic change.

The next step is to inactivate the nematocysts. For most species this can be accomplished by flooding the area with household vinegar (5% acetic acid). Vinegar should be applied to a small test area first, since this may aggravate some jellyfish stings by causing nematocysts to fire. Nematocysts can then be removed from the skin by scraping with a blunt edged object such as a seashell, credit card or by shaving with a razor.

Application of a cold pack has traditionally been recommended to help relieve mild to moderate pain (Exton *et al*., 1989). However, hot water (45 °C for 20 minutes) immersion of the affected part has recently been shown to be significantly more effective than ice packs following Portuguese man-o'-war stings (Loten *et al*., 2006). In addition, the pain did not recur, suggesting that hot water immersion deactivates the venom. This theory is supported by data showing that the lethality of *Chironex fleckeri* venom is reduced by exposing the venom to temperatures above 39 °C (Carrette *et al*., 2002). The extent to which this treatment will prove effective for other species of jellyfish remains to be determined. Topical anaesthetics or steroid creams, as well as oral analgesics may benefit some patients. Hyperpigmentation can be treated with a bleaching agent such as topical hydroquinone (Kokolu and Burnett, 1990).

Topical corticosteroids and cycloplegics are recommended as initial treatment for corneal jellyfish stings (Glasser *et al*., 1992). Topical or systemic therapy for increased intraocular pressure should be administered as needed.

Systemic manifestations are managed with traditional supportive measures. The definitive treatment of box jellyfish envenomation is a specific antivenom from the Commonwealth Serum Laboratory in Australia (Holmes, 1996). The calcium-channel antagonist verapamil hydrochloride has been shown to inhibit the action of box jellyfish cardiotoxin and prolong survival in mice (Burnett and Calton, 1983). However, this mode of therapy has become controversial, as more recent data from an investigation using mechanically ventilated piglets demonstrated that verapamil actually exacerbated cardiovascular collapse and increased mortality from box jellyfish venom (Tibballs *et al*., 1998).

Opiate analgesics such as morphine are generally required to treat the intense pain associated with Irukandji syndrome. Early experience with parenteral magnesium sulfate for the management of pain and hypertension associated with Irukandji syndrome is favourable, however, further study is needed before it can be recommended (Corkeron *et al*., 2004).

Antihistamines may be useful if there is evidence of an allergic response to the venom. Anaphylactic reactions should be managed accordingly. In the event of recurrent dermal eruptions, a tapering dose of a corticosteroid may be employed.

In addition to the Portuguese man-o'-war, another important hydrozoan is fire coral. The fire coral, while not a true coral, is so named because of its marked resemblance to these species. Polyps, which contain nematocysts, protrude through pores of its calcareous skeleton. It typically produces only mild dermatitis and burning discomfort (Kizer, 1983–1984). The class Anthozoa includes the sea anemones, which also contain modified nematocysts capable of inflicting stings and local effects as described for other cnidarians.

9 STINGRAYS

Another important costal hazard is the stingray. Approximately 50 marine species and 20 freshwater species have been described (Barbaro *et al*., 2007; Haddad *et al*., 2004). These cartilaginous fish are often found partially buried in the sand and are a significant hazard to beachcombers and to those who swim or play in shallow water. Although normally very docile, if they are stepped on or are otherwise abruptly disturbed, stingrays will lash their tails forward and sting with a spine located near the base of the tail. Some species of stingray contain more than one spine and are capable of inflicting multiple simultaneous stings (Grainger, 1987).

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A venom-containing integumentary sheath covers the stinging spine(s) of stingrays. As the spine enters the victim this sheath may rupture, resulting in the release of venom into the wound. The spines of stingrays vary by species and may range from 2.5 to 12 cm in length (Grainger, 1985). The spine is curved and serrated, enabling it to inflict significant trauma in addition to envenoming the victim. Lethal injury can result if the stinging spine penetrates a vital organ.

9.1 Toxic Manifestations

Most stings are to the lower extremities (Russell *et al*., 1958; Clark *et al*., 2007). The upper extremities, abdomen or thorax may be involved due to careless handling or under extraordinary circumstances. The stingray's venom consists of a heat-labile protein that causes intense pain at the site of the sting, which is out of proportion to the physical trauma. In the event that the wound is not characteristically painful and other toxic manifestations do not occur, it is likely that the integumentary sheath had already been lost or it was not disrupted during the sting.

The venom can produce local tissue necrosis, which complicates the healing process (Fenner *et al*., 1989). Necrosis and inflammation is much more prominent with injuries produced by freshwater stingrays (Barbaro *et al*., 2007; Haddad *et al*., 2004). Freshwater species have been found to have a larger number of secretory cells spread over the entire stinger epidermis, whereas the protein secretory cells in marine species are found only around or inside the ventrolateral grooves (Pedroso *et al*., 2007). The presence of hyaluronidase and other enzymes in the venom of freshwater stingrays may also explain the more severe tissue necrosis caused by these species (Haddad *et al*., 2004; Barbaro *et al*., 2007).

Stingray venom has also been shown in animal models to possess both cardiotoxic and neurotoxic properties (Russell *et al*., 1958). Secondary infection is also possible (Clark *et al*., 2007).

9.2 Management

First aid for stingray envenomation consists of washing the wound with seawater and as soon as possible soaking the site in water which is as hot as the patient can tolerate for 30–90 minutes. This is done to denature the thermolabile venom (Fenner *et al*., 1989; Russell *et al*., 1958). The wound should then be surgically explored to remove any remaining fragments of the sheath or spine. Necrotic tissue should be debrided and an antiseptic should be used to cleanse the wound, which should be left open to heal by natural intention. Tetanus immunization should be updated, if necessary, and prophylactic antibiotics should generally be administered (Fenner *et al*., 1989; Clark *et al*., 2007). Narcotic analgesics may be necessary to control pain. No antivenom is available and systemic manifestations should be managed with traditional symptomatic and supportive care.

10 STINGING FISHES

Other venomous underwater creatures include fresh- and salt-water fishes. Common examples include the lionfish, scorpion fish, stonefish, catfish and weever fish. All these fish sting with venomous spines on their fins. The sting of these fish is much less traumatic than that associated with the stingray, but in other respects their stings are very similar.

Fish stings may result when handling venomous species and by chance encounters in shallow waters. The lionfish is popular with marine aquarists and has been responsible for many stings to the hand and fingers (Kizer *et al*., 1985; Trestrail and Al-Mahasneh, 1989).

10.1 Toxic Manifestations

Fish stings result in an initial sharp stabbing pain when the spine penetrates the skin. In some cases the spine may break free and remain lodged in the wound. Severe pain may ensue, which can radiate to involve the entire extremity. The pain may become excruciating and incapacitating in some cases. Pronounced local swelling occurs commonly, and vesicles may form at the puncture sites (Auerbach *et al*., 1987). The vesicular fluid can itself be harmful and prompt drainage of the fluid is recommended. Delayed onset of Raynaud's phenomenon following a weever fish sting to the finger has been reported (Carducci *et al*., 1996).

Systemic manifestations may occur with significant envenomation resulting from multiple stings, or single stings from certain species such as the stonefish. These toxic effects may include nausea, vomiting, diaphoresis, bradycardia or tachycardia, conduction disturbances, hypotension, myocardial ischemia, respiratory distress, pulmonary oedema, muscle tremor, weakness, delirium, convulsions and death (Kizer *et al*., 1985; Ell and Yates, 1989; Lehmann and Hardy, 1993). Delayed onset of systemic manifestations has been reported following lionfish envenomation (Bangh, 1997).

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10.2 Management

As with the stingray, the venom of these fish consists primarily of heat-labile proteins. Accordingly, first-aid treatment should include immersion of the affected site in water as hot as the patient can tolerate for 30–90 minutes. Afterwards the site should be thoroughly cleaned and in some cases the wound may need to be surgically explored to remove any remaining spine. Tetanus prophylaxis should be updated as necessary. Prophylactic administration of antibiotics is not routinely necessary. Catfish stings may pose a greater risk of infection and should be managed accordingly (Baack *et al*., 1991).

Failure to promptly treat these inflictions may result in significant tissue necrosis, permanent scaring and physical impairment (Kasdan *et al*., 1987; Mann and Werntz, 1991). Systemic manifestations should be managed with traditional supportive and symptomatic measures. An antivenom is available for the management of stonefish envenomation from CSL Ltd. (Parkville, Victoria, Australia). Stonefish antivenom may be useful in treating severe evenomation by other fish species, based on similarities of symptoms resulting from envenomation by the various fish species, similar pharmacological activity between fish venoms and the observed crossreactivity of stonefish venom with the venom of other species (Church and Hodgson, 2003).

11 POISONOUS FISH AND SHELLFISH

In addition to those animals that cause human poisoning by envenomation, a number of toxic syndromes may result from ingestion of various fish and shellfish. In some cases these animals excrete the toxin, but most concentrate toxins that are produced by dinoflagellates or bacteria. Fish can be categorized based on the tissue that contains the toxin (Halstead, 1964). Ichthyosarcotoxic fish, which cause most poisonings, have toxin in muscle, viscera, skin or mucus. Ichthyootoxic fish produce toxin that is concentrated in the gonads. Ichthyohemotoxic fish, which rarely produce poisoning, have toxin in their blood. There are many common characteristics between these syndromes and some distinct differences (**Table 4**). Diagnosis is primarily based on signs and symptoms and a history of ingesting fish or shellfish.

11.1 Ciguatera Fish Poisoning

Ciguatera intoxication is the most common type of ichthyosarcotoxic fish poisoning. The primary responsible toxins, ciguatoxins, are produced by the dinoflagellate *Gambierdiscus toxicus* (Eastaugh and Shepherd, 1989). Ciguatoxins activate voltage-sensitive sodium channels at nanomolar and picamolar concentrations, resulting in spontaneous firing of a variety of nerve types, giving rise to the neurologic signs and symptoms of intoxication (Lewis, 2006). Ciguatoxin is a heat-stable, lipid-soluble compound that is resistant to gastric acid (Eastaugh and Shepherd, 1989).

Ciguatoxins are concentrated up the food chain; as a result, large fish are most likely to cause human poisoning. Hundreds of fish species have been reported to harbour ciguatoxins; common examples include the barracuda, grouper, snapper, amberjack and sea bass (Halstead, 1964).

Most outbreaks of ciguatera intoxication occur in the Caribbean, South Pacific and Indian Oceans. In the United States, most cases have been reported in Hawaii and Florida (Hughes and Merson, 1976). Ciguatera poisoning, however, has also been associated with ingestion of fish caught from the southeastern United States coastal waters as far north as North Carolina (Morris *et al*., 1990). The ability to readily transport fresh fish great distances may result in ciguatera poisoning in virtually any geographic region.

11.1.1 Toxic Manifestations

Ciguatera poisoning is characterized by gastrointestinal, neurological and cardiovascular disturbances. Although signs and symptoms usually develop within six hours, there is considerable variability. Common gastrointestinal effects include diarrhoea, vomiting and abdominal pain (Morris *et al*., 1982). These symptoms generally occur early and resolve within 24 hours. Other initial symptoms may include malaise, and pain and weakness in the lower extremities, as well as pruritus and paraesthesias around the mouth and of the extremities (Hughes and Merson, 1976; Morris *et al*., 1982). A characteristic finding of cold allodynia (dysesthesia when touching cold water or objects) is nearly pathognomic of ciguatera poisoning. This is commonly referred to incorrectly as ‘temperature reversal’ (Isbister and Kiernan, 2005). Neurologic manifestations may persist for weeks or months. Other common findings include rash, dry mouth, metallic taste, myalgias, arthralgia, visual disturbances and a sensation of loose teeth. Bradycardia, hypotension and respiratory paralysis may occur in severe cases (Hughes and Merson, 1976).

11.1.2 Management

Treatment is primarily symptomatic and supportive care. Fluid and electrolyte balance should be maintained. Atropine and intravenous fluids have been effective in the treatment of bradycardia and hypotension. Mannitol was found to dramatically improve neurologic and gastrointestinal toxicity in a group of 24 patients (Palafox *et al*., 1988). Mannitol has also been shown to benefit patients with chronic symptoms, even with delayed administration (Blythe *et al*., 1992; Eastaugh, 1996). It is postulated

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Table 4 Characteristics of poisoning by ingestion of marine toxins

Poisoning syndrome	Characteristic symptoms	Toxin	Mechanism of action
Ciguatera fish poisoning	Diarrhoea, abdominal pain, cold allodynia, numbness, paraesthesia, myalgia, arthralgia, headache, pruritis, dizziness, asthenia	Ciguatoxins	Activate sodium channels
Paralytic shellfish poisoning	Paraesthesias, headache, dizziness, nausea, hypersalivation, diaphoresis, weakness, muscle paralysis, respiratory paralysis	Saxitoxins	Block sodium channels
Neurotoxic shellfish poisoning	Nearly identical to ciguatera poisoning, but less severe	Brevitoxins	Activate sodium channels
Amnesic shellfish poisoning	Vomiting, abdominal cramps, diarrhoea, headache, confusion, memory loss	Domoic acid	Excitatory neurotransmitter
Tetrodotoxic fish poisoning	Paraesthesias, nausea, numbness, weakness, ascending paralysis, respiratory impairment, hypotension, bradycardia, (consciousness may be maintained)	Tetrodotoxin	Block sodium channels
Histamine fish poisoning	Urticaria, flushing, hypotension, headache, abdominal cramps, diarrhoea, vomiting, itching	Histamine	Histaminic action
Diarrhetic shellfish poisoning	Nausea, vomiting, diarrhoea, abdominal cramps	Okadaic acid and its analogues	Phosphorylase phosphatase inhibitors

that mannitol may have inactivated the toxin or competitively inhibited its action on the sodium channel (Palafox *et al*., 1988). The possibility that ciguatera may cause oedema in the nervous tissue, which mannitol could reverse, has also been hypothesized (Palafox, 1992). In a recent double-blind randomized trial of mannitol therapy for ciguatera poisoning, however, there was no significant difference between mannitol and normal saline in relieving signs and symptoms (Schnorf *et al*., 2002). The authors postulated that dilution of the toxin or restoration of a volume deficit with normal saline provided therapeutic benefit. The use of mannitol is still a generally accepted practice, but further study is warranted. Gabapentin, an anticonvulsant that has been used successfully to treat neuropathic pain, has recently been shown to also relieve symptoms of ciguatera poisoning (Perez *et al*., 2001).

11.2 Paralytic Shellfish Poisoning

Paralytic shellfish poisoning results from ingestion of contaminated bivalve molluscs, such as clams and oysters. These molluscs concentrate neurotoxins known as saxitoxins, which are produced by a number of dinoflagellates, including those of the *Alexandrium* spp., *Gymnodinium catenatum* and *Pyrodinium bahamense* (Lehane, 2001). The toxin is a water-soluble, heat and acid-stable compound that cannot be destroyed by ordinary cooking (Auerbach and Halstead, 1989). The toxin acts by blocking sodium channels, thereby inhibiting neuromuscular transmission.

11.2.1 Toxic Manifestations

As the name implies, paralytic shellfish poisoning affects primarily the nervous system. The onset of symptoms occurs within 30 minutes of shellfish ingestion (Eastaugh and Shepherd, 1989). Prominent toxic effects include paresthesias of the lips, face and extremities, headache, weakness, dizziness, vertigo and difficulty walking (Hughes and Merson, 1976; Eastaugh and Shepherd, 1989). A sensation of floating has also been described (McCollum *et al*., 1968). In severe cases, muscle paralysis may occur. Death may result due to respiratory arrest if adequate life-support cannot be provided. Some neurologic symptoms such as headaches, memory loss and fatigue may persist for up to two weeks (Rodrigue *et al*., 1990).

11.2.2 Management

The mainstay of therapy is symptomatic and supportive care. Respiratory function should be monitored closely with ventilatory assistance provided as needed.

11.3 Neurotoxic Shellfish Poisoning

A milder intoxication, known as neurotoxic shellfish poisoning, results from ingestion of shellfish contaminated with brevitoxins, produced by the dinoflagellate *Gymnodinium brevis* (Isbister and Kiernan, 2005). The costal waters of western Florida, Texas, North Carolina and New Zealand have been affected by *G. brevis* (Sakamoto *et al*., 1987; Morris *et al*., 1991; Ishida *et al*., 1996). These toxins activate sodium channels, producing

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a clinical syndrome nearly identical to that produced by ciguatera, but less severe (Daranas *et al*., 2001). Toxic manifestations include nausea, vomiting, diarrhoea and paresthesias. As with ciguatera poisoning, patients may experience cold allodynia or 'temperature reversal' (Sims, 1987; Hughes and Merson, 1976; Isbister and Kiernan, 2005).

11.4 Amnesic Shellfish Poisoning

A unique toxic syndrome has been described that resulted from ingestion of contaminated mussels from Prince Edward Island in Canada (Perl *et al*., 1990). Domoic acid was implicated as the responsible toxin, which was apparently produced by the marine algae *Pseudo-Nitzschia* spp. Domoic acid is an excitatory neurotransmitter structurally similar to glutamic acid and kainic acid (Teitelbaum *et al*., 1990).

The most common acute symptoms of intoxication included nausea, vomiting, abdominal cramps, diarrhoea, headache and memory loss (Perl *et al*., 1990). In severe cases altered mental status, seizures, myoclonus and cardiovascular instability resulted. Death was reported in four cases. The initial widespread neurotoxicity and subsequent chronic residual memory impairment differentiates this syndrome from either paralytic or neurotoxic shellfish poisoning.

Following this outbreak of amnesic shellfish poisoning (ASP) in Canada, an action limit of 20 μg of domoic acid per gram of shellfish was introduced in Canada and subsequently other parts of the world. The thorough study and documentation of this outbreak, and these subsequent action levels, appears to have prevented further large-scale outbreaks of ASP (Jeffery *et al*., 2004).

11.5 Tetrodotoxic Fish Poisoning

Tetrodotoxication is primarily associated with ingestion of the puffer fish and related fish of the order Tetraodontiformes. Other animals may also contain this neurotoxin, for example the Californian newt (*Taricha torosa*), Pacific goby (*Gobius criniger*) and Costa Rican frog (Tibballs, 1988). Two species of gastropod molluscs collected off the southern Taiwan coast have also been implicated in an outbreak of tetrodotoxin poisoning (Yang *et al*., 1995). The bite of the blue-ringed octopus also causes tetrodotoxication due to toxin in the salivary glands (Hwang and Noguchi, 2007).

Tetrodotoxin acts similar to saxitoxin in that it blocks neurotransmission by action on sodium channels (Narahashi, 1972). This results in motor, autonomic and sensory-nerve impairment (Tibballs, 1988). It also has direct action on the medulla, stimulating the chemoreceptor trigger zone and depressing the respiratory centre (Eastaugh and Shepherd, 1989). The type, severity, onset and range of symptoms depend on the amount of toxin ingested (Hwang and Noguchi, 2007).

11.5.1 Toxic Manifestations

Prominent signs and symptoms of intoxication include persistent vomiting, paresthesias, weakness, respiratory impairment, hypotension and bradycardia (Tibballs, 1988; Sims and Ostman, 1986). Hypertension has been reported in patients with pre-existing hypertensive disease (Deng *et al*., 1991; Yang *et al*., 1995). The precise mechanism of the hypertensive response is not understood. Other manifestations may include headache, dilated pupils, salivation, diaphoresis, myalgias, dysarthria, ataxia, muscle fasciculations and seizures (Sims and Ostman, 1986). Severe poisoning rapidly progresses to flaccid paralysis, but consciousness is typically maintained, except in the most severe cases (Isbister and Kiernan, 2005). Death may result in severe cases due to respiratory failure or cardiovascular collapse.

11.5.2 Management

Treatment primarily consists of symptomatic and supportive care. Patients should be admitted for observation until peak effects have occurred and improvement is noted. Paralysed patients should be sedated to minimize psychological stress. Ventilatory assistance may be required in severe cases. Hypotension and bradycardia should be managed with atropine and fluid therapy. Vasopressors, such as dopamine hydrochloride may be required.

11.6 Histamine Fish Poisoning

Histamine fish poisoning (HFP), often referred to as scombroid fish poisoning, is a toxic syndrome that resembles an acute allergic reaction. HFP results from ingestion of spoiled fish that is contaminated with histamine and possibly other toxins. Histamine is formed as a result of bacteria that cause enzymatic decarboxylation of histidine, which is normally present in the flesh of certain fish species (Lerke *et al*., 1978). Fish most commonly involved are those of the families Scombroidae, such as the tuna and mackerel, and Scomberesocidae, such as the saury. Other nonscombroid marine fish such as bluefish, sardines, anchovies and mahi-mahi have also been implicated in outbreaks of HFP (Lehane and Olley, 2000).

Although histamine is an important factor in the pathogenesis of scombroid poisoning, the exact mechanism is still unclear. Histamine has limited activity

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when administered orally, due to rapid metabolism and elimination in the urine (Garrison, 1990). However, markedly elevated urinary histamine concentrations have been measured in symptomatic patients after ingestion of scombrotoxic fish (Morrow *et al*., 1991). This evidence and the effectiveness of antihistamines to relieve symptoms of scombroid intoxication implicate histamine as the causative toxin.

A variety of hypotheses have been put forward to explain the pivotal role of histamine in the pathogenesis of scombroid intoxication, despite the lack of a straightforward dose–response relationship. These include: the toxicity of other biogenic amines, the inhibition of histamine detoxification by histamine potentiators, disruption of the intestinal barrier or the release of endogenous histamine from mast cells by sombroid toxins (Lehane and Olley, 2000). None of these hypotheses has proven totally satisfactory. This is an area that warrants further investigation.

11.6.1 Toxic Manifestations

HFP usually results in a relatively mild, self-limited syndrome, which typically begins within one hour of ingestion and lasts for eight hours or less (Merson *et al*., 1974; Hughes and Merson, 1976). Typical manifestations include nausea, diarrhoea, abdominal cramps, vomiting, throbbing headache, oral blistering or burning sensation, flushing, burning sensation of the skin and urticaria. Tachycardia, palpitations, bronchospasm and respiratory distress may also occur (Merson *et al*., 1974; Hughes and Merson, 1976; Blakesley, 1983).

11.6.2 Management

Treatment is primarily symptomatic and supportive care. Antihistamines, such as diphenhydramine, are the mainstay of therapy. The use of cimetidine in a few cases has been shown to dramatically relieve the signs and symptoms of HFP (Blakesley, 1983; Auerbach, 1990). Bronchodilators may be needed in the event of bronchospasm.

It is important to differentiate between HFP and fish allergy. An incorrect diagnosis of fish allergy will unnecessarily limit the diet of the patient. Considerations include the patient's prior response to ingestion of the implicated fish species and the response in others who consumed the same meal (Taylor *et al*., 1989). The food can be analysed for the presence of histamine, but at this time there are no diagnostic tests that can be performed on the patient (Lerke *et al*., 1978; Taylor *et al*., 1989).

11.7 Diarrhetic Shellfish Poisoning

Several species of the dinoflagellate *Dinophysis* produce okadaic acid and its analogues, dinophysistoxin-1 and dinophysistoxin-2, the primary toxins responsible for diarrhetic shellfish poisoning (DSP) (Daranas *et al*., 2001). DSP is a worldwide public-health problem with documented outbreaks in Japan, Europe, Chile, Thailand, Nova Scotia and New Zealand (Tester, 1994). DSP toxins produce gastroenteritis within 30 minutes to two hours, resulting in diarrhoea, nausea, vomiting and abdominal cramps. These symptoms are self-limiting with recovery occurring within hours to a few days. Treatment should be directed at maintaining fluid and electrolyte balance.

Okadaic acid and some dinophysistoxins are potent protein phosphorylase phosphatase Type 1 and 2A inhibitors, which cause phosphorylation of numerous proteins, resulting in sodium release from intestinal mucosal cells and causing diarrhoea (Sobel and Painter, 2005). These compounds and have also been shown to be potent tumour promoters (Daranas *et al*., 2001). Recently, it has been postulated that the ingestion of shellfish with low levels of DSP toxins is an important risk factor for colorectal cancer (CRC) and is increasing the incidence of CRC in the European Union (Manerio *et al*., 2008).

Other DSP toxins, the yessotoxins and pectenotoxins, do not cause diarrhoea, but more importantly, are hepatotoxic (Daranas *et al*., 2001). The extent to which these or yet other unidentified toxins produce other toxic syndromes remains to be determined.

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Poisons of Plant Origin

Deon van der Merwe

CONTENTS

1 INTRODUCTION

We share our world with a vast number of plant species. Species of seed plants alone number around 400 000 (Govaerts, 2001). Plants are adapted to many different environments and growing conditions that, in turn, are related to the production and/or accumulation of a multitude of biologically active compounds, also referred to as phytochemicals, that make it possible for plants to thrive under different environmental and competitive conditions. Examples include compounds produced to deter herbivores of various kinds, from large mammals to insects, compounds to defend against infection by bacteria, fungi and parasites, as well as compounds that increase success in competing for resources against other plants. Some biologically active compounds are accumulated to provide a store of nutrients or to facilitate specialized physiological processes, or may simply reflect the relative abundance of chemicals in the plant's environment. The diversity of biologically active phytochemicals is a valuable resource, both as nutrients and as compounds that have health benefits when consumed in moderate quantities. Many important classes of pharmaceuticals were originally derived from plants, or are extracted directly from plants. Traditional herbal medicines, derived directly from plant tissues or crude extracts, are the mainstay of healthcare in many parts of the world, especially where access to modern healthcare is limited (Kofi, 2005), while persisting to varying degrees in societies with the most advanced healthcare systems (Bent and Ko, 2004).

Since biologically active compounds are so commonly encountered in plants, it is not surprising that some plants, under conditions of sufficient exposure and phytochemical absorption, have the ability to cause toxicity. As with most other toxicants, the dose required to produce toxicity varies tremendously between compounds, routes of exposure and with the susceptibility of the individual.

The relative importance of plant toxicities is illustrated by the fact that human exposures to plants that are perceived as poisonous, and reported to Poison Control Centers in the United States, comprised 2.7% of total reported exposures to toxicants in 2006. This represents over 64 000 incidents (Bronstein *et al*., 2007). The 25 most frequently reported poisonous-plant exposures in the United States from 1987 to 2006 are listed in **Figure 1**. These reports indicate the relative importance of popular, decorative garden and house plants in a predominantly urban, Western society, like the United States. Due to cultivation in nurseries and wide distribution to gardens and homes, where growing conditions can be controlled, exposure to these plants is no longer correlated with their original distributions under natural conditions, and exposures can be expected in almost any urban environment. In societies where naturally occurring, local vegetation is commonly used in traditional medicine and as a food source, the most frequent poisonous-plant exposures are likely to reflect local abundance and use of plants. An ominous development in recent years has been the speed and efficiency of distribution of potentially hazardous food ingredients, which may be contaminated with poisonous plant material (Prakash *et al*., 1999). A recent example is an Australian recall of

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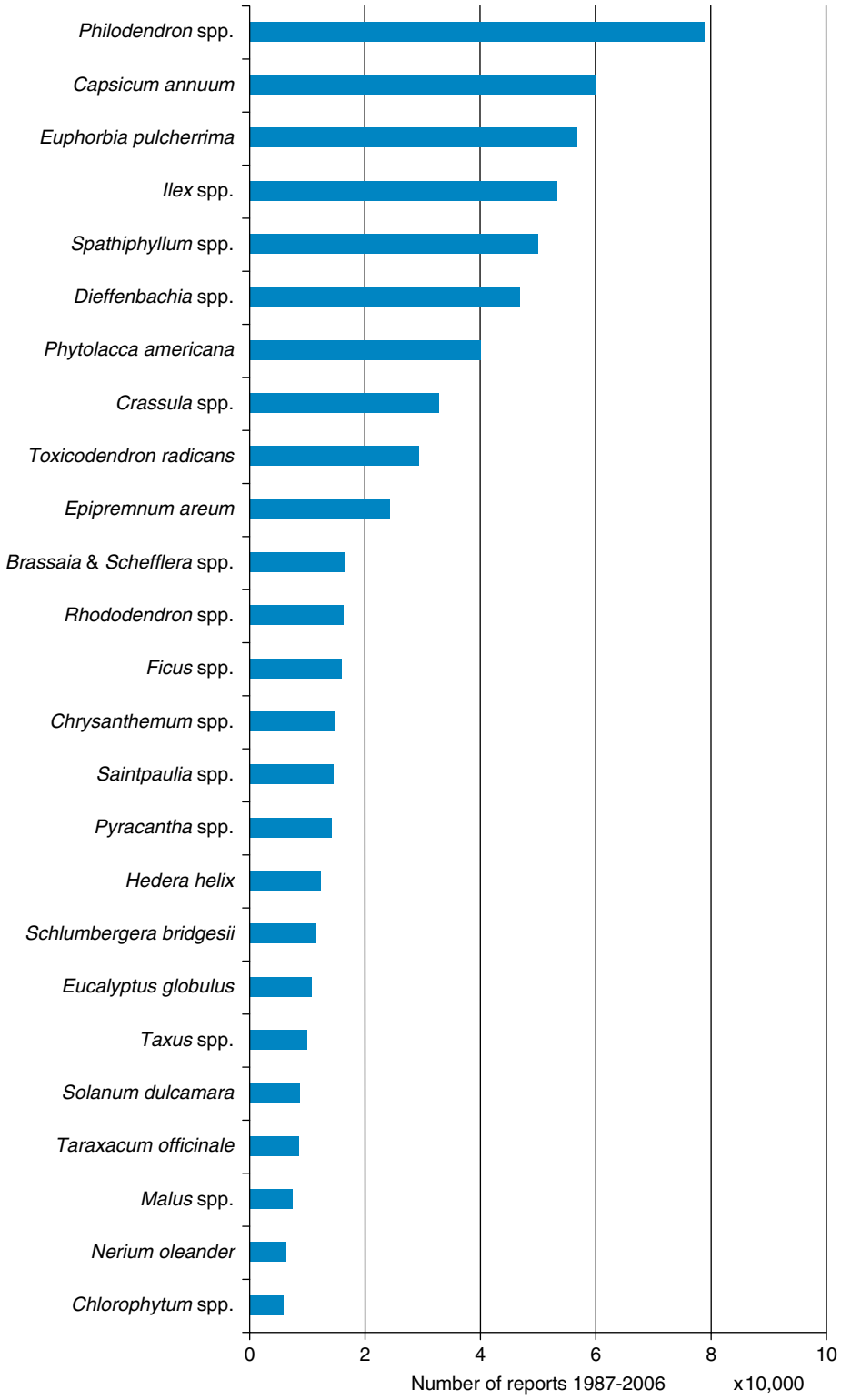

Figure 1 The 25 most frequent poisonous plant exposures reported to United States Poison Control Centers from 1987 to 2006. (Bronstein *et al.*, 2007; Lai *et al.*, 2006; Litovitz *et al.*, 1988; 1989; 1990; 1991; 1992; 1993; 1994; 1995; 1996; 1997; 1998; 1999; 2000; 2001; 2002; Watson *et al.*, 2003; 2004; 2005.)

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vegetarian crackers in January 2008 that were manufactured using cyanogenic glycoside-containing cassava as an ingredient (SADH, 2008). Rapid and massive distribution of processed and poorly recognizable hazardous food ingredients of plant origin presents a difficult challenge.

Fortunately, the majority of poisonous plant exposures do not result in toxicity. Most plant toxins, with some notable exceptions, produce severe toxicity or lethal effects only at relatively high doses, and exposures to highly toxic doses are uncommon under normal conditions. Our interactions with plants are therefore mostly harmless, even when we are exposed to potentially poisonous plants. It is only when exposed to plant species of exceptional toxic potential, or when the route of exposure and dose leads to ample absorption of toxic compounds, that we see toxic effects. This represents a problem in its own right, however, because it may lull us into disregarding as trivial those rare exposures that may lead to severe toxicity or death (Nelson *et al*., 2006). For this reason, it is important that healthcare professionals have a basic knowledge of the most important and harmful poisonous plants.

Correct identification of a poisonous plant may be crucial in the treatment of patients where significant exposure took place and/or the patient is experiencing symptoms of poisoning. One of the common pitfalls when dealing with plant exposures is the incorrect identification of the plant, based on common names and other assumptions made by nonexperts. The identity of a plant should not be based only on a common name, but should at least be consistent with detailed descriptions of the physical characteristics of the plant. Ideally, the plant should be identified by experts, such as botanists familiar with the plant, based on a properly collected and prepared physical example of the plant, or by direct comparison with herbarium specimens. Where timely submission of plant samples to an expert or herbarium is impractical, plant material should be preserved in a plant press or a substitute, such as a large, heavy book. Whenever possible, reproductive parts, including flowers, fruits and seeds should be included, as well as detailed descriptions of the plant's location and growth form. When rapid identification is needed, references on poisonous plants containing detailed descriptions and pictures are available (Frohne and Pfänder, 2005; Nelson *et al*., 2006; Gloster, 2004), and should be consulted when necessary in the absence of expert opinion. Although online sources should be interpreted with caution, searches for plant images using services such as Google Image Search™ often produce multiple images of the most common and important poisonous plants that can be helpful in attempts to rapidly obtain a probable identification based on prominent morphological characteristics. Comprehensive, reliable online sources are available that can be consulted for additional information that are useful for identification. Some sources are more relevant to specific parts of the world, such as the United States Department of Agriculture (USDA) Plants Database in North America (USDA, 2008), while others have a global coverage, such as the electronic Plant Information Centre (Royal Botanic Gardens, Kew, 2008). Poison centres and other poisoning information services are readily available in many parts of the world, and are often able to give sound advice on plant identification, plant poisoning diagnoses and case management.

The information that follows below is mostly limited to poisons of plant origin that are considered to be important because they are associated with common or serious toxicity. However, discussions of some plants are included that are only mildly toxic and very rarely cause poisoning, but which are often perceived as harmful by the general public and result in frequent enquiries to Poison Control Centers and other healthcare professionals. Plants are discussed as groups that share specific or closely related toxic compounds or that share specific toxicity syndromes. Descriptions include the most prominent families and/or genera associated with poisoning, the toxic agents and their mechanisms of action, risk factors, toxicity syndrome descriptions and suggested management strategies. Where available, frequently used common names for families and genera are included, but it should be noted that common names are often regional, the same name may be used to describe more than one plant and a specific plant may be known by various names.

Finally, plant poisoning is a dynamic field. The distribution of potentially poisonous plants and plant products change over time as people make use of distant sources of food ingredients, which may contain unfamiliar contaminants, as new species and varieties become popular as decorative plants, and as biologically active plants and plant products, used as traditional medicines or mind-altering drugs in one part of the world, become known and used/abused in other parts of the world where the traditional knowledge of preparation methods, dosages and risks are unknown. New information on active constituents and mechanisms of action often change how we approach diagnoses and treatment. Healthcare professionals need to keep abreast of new plant-poisoning hazards and developments in treatment to be in the best possible position to give sound advice and intervene successfully in cases of plant poisoning.

2 ANTICHOLINERGIC ALKALOIDS

2.1 Plants

Anticholinergic alkaloids are mostly associated with members of the Solanaceae (potato) family, including

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Atropa spp. (belladonna), *Brugmansia* spp., *Datura* spp. (Jimsonweed), *Hyoscyamus* spp. (henbane), *Solandra* spp. (chalice vine) and *Solanum* spp. (nightshade). These plants are typically forbs and small shrubs found in most habitats around the world. They are also commonly cultivated for use in gardens.

2.2 Toxic Agent and Mechanism

Anticholinergic alkaloids, also known as tropane alkaloids, include atropine, hyoscyamine, hyoscine and scopolamine. All plant parts are toxic, but alkaloid concentrations are especially high in green plant parts and unripe fruit (Knight and Walter, 2001). Alkaloids are resistant to breakdown and may persist as contaminants in foods, even following extensive processing into secondary products (Perharic, 2005; Lee, 2007). The tropane alkaloids competitively antagonize the effects of acetylcholine on muscarinic receptors in the central and parasympathetic nervous systems.

2.3 Risk Factors

One of the most common sources of anticholinergic alkaloid poisoning is the abuse of *Datura* spp. (Jimsonweed) seeds as mind-altering drugs, either by ingestion or inhalation (Guharoy and Barajas, 1991; Soneral and Connor, 2005). Variations in the alkaloid levels of individual plants make its use as a mind-altering drug hazardous, even for experienced users. Poisoning may also result from mistaken identification when gathering 'edible' wild leafy greens and berries. This is of particular concern where wild leafy greens are common in the diet. Indiscriminate harvesting and consumption of apparently edible berries, such as those on *Atropa belladonna* is associated with poisoning, mostly in children, where these plants are cultivated as garden subjects. Contaminated flour has been associated with mass outbreaks of poisoning (Perharic, 2005). *Atropa belladonna* poisoning is one of only a handful of plant poisonings where secondary poisoning has been described from eating the meat of animals that grazed on the plant, or from ingesting contaminated honey (Lee, 2007).

2.4 Toxicity Syndrome

Suppression of the parasympathetic nervous system leads to an apparent overexpression of the sympathetic system. Classically, the clinical syndrome has been described as 'blind as a bat', due to ciliary body and iris muscle paralysis that leads to mydriasis and cycloplegia, 'dry as a bone', due to suppression of salivation resulting in a profound dry mouth sensation, 'red as a beet', due to flushing of the skin, 'hot as a hare', due to effects on temperature regulation leading to hyperthermia, and 'mad as a hen', due to central nervous system effects that include visual hallucinations, agitation, impaired judgment and risk-taking behaviour, delirium, seizures and coma (Spina and Taddei, 2007; Lee, 2007). Death may result from respiratory or cardiac arrest. Signs appear from 30 minutes to four hours following ingestion and may last from several hours to several days.

2.5 Management

Initiate supportive and symptomatic treatment. Sedation with a benzodiazepine may be of benefit. Physostigmine by slow intravenous injection ($1–2\,mg\,kg^{-1}$ in adults; $0.02\,mg\,kg^{-1}$ in children) is an antidote. Note that the duration of alkaloid activity can be longer than the antidote activity and may require repeated administration of the antidote (Nelson *et al*., 2006).

3 CARDIAC GLYCOSIDES

3.1 Plants

Cardiac glycosides are produced by a wide range of flowering plants including both monocotyledons and dicotyledons, with growth forms ranging from bulbs to shrubs and small trees. Plants include members of the Apocynaceae (dogbane) family, including *Acokanthera spp.* (poison arrow plant), *Adenium* spp. (desert rose), *Nerium oleander* (oleander), *Pentalinon luteum* (hammock viper's tail), *Strophanthus* spp. and *Thevetia* spp.; the Asclepiadaceae (milkweed) family, including *Asclepias* spp. (milkweed), *Calotropis* spp. and *Cryptostegia* spp. (rubbervine); the Crassulaceae (orpine) family, including *Cotyledon* spp. (pig's ear), *Kalanchoe* spp. (neverdie) and *Tylecodon* spp.; the Hyacinthaceae (hyacinth) family, including *Bowiea volubilis* (climbing onion), *Urginea/Drimia* spp. (snake's head) and *Ornithogalum* spp. (star of Bethlehem); the Iridaceae (iris) family, including *Homeria* spp. and *Moraea* spp. (tulip); the Liliaceae (lily) family, including *Convallaria* spp. (lily of the valley) and *Scilla/Merwilla* spp. (squill); the Melianthaceae (melianthus) family, including *Melianthus* spp. (honey flower); the Ranunculaceae (buttercup) family, including *Adonis* spp. (pheasant's eye) and *Helleborus* spp. (hellebore); the Santalaceae (sandalwood) family, including *Thesium* spp. (flaxleaf); and the Scrophulariaceae (figwort)

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family, including *Digitalis* spp. (foxglove). Although most of these species have specific habitat requirements that restrict their distribution under natural conditions, many produce attractive flowers and foliage that make them popular as garden subjects and contribute to their availability all over the world. Examples of commonly cultivated plants grown for their showy flowers include *Digitalis purpurea* (foxglove) and *Ornithogalum umbellatum* (grass lily). *Nerium oleander* (oleander) and *Thevetia peruviana* (yellow oleander) are popular in many parts of the world as a hardy hedge plants.

3.2 Toxic Agent and Mechanism

Glycosides consist of two parts: sugars, referred to as the glycone portion of the molecules, are bonded via glycosidic bonds to nonsugar moieties, referred to as the aglycone portion. The aglycones of cardiac glycosides are steroid derivatives and are responsible for the toxic effects (Kellerman *et al*., 2005). Generally, all plant parts are considered toxic.

Cardiac glycosides inhibit Na^+/K^+-ATPase, which causes intracellular Na^+ accumulation, followed by Ca^{2+} accumulation (McDonough *et al*., 2002). These changes result in increased cardiac muscle inotropy and bradycardia. Increased vagal tone contributes to a slowing of the heart rate. As the dose increases, the heart muscle becomes more excitable and the heart becomes susceptible to dysrhythmias of various types, including frequent premature ventricular beats, bradycardia, paroxysmal atrial tachycardia with block, junctional tachycardia and bidirectional ventricular tachycardia (Ma *et al*., 2001). Na^+/K^+-ATPase inhibition also affects other smooth muscle tissue, such as the vascular system, where vasoconstriction occurs in the atrial and venous systems, and in the digestive tract smooth muscle, where excessive smooth muscle contraction is induced.

3.3 Risk Factors

Cardiac-glycoside-containing plants and extracts are commonly prescribed in traditional medicine for a variety of indications and as tonics, but in particular for the treatment of heart failure. Toxicity often results from the uncontrolled use of these plants as medicines, especially when the users have limited experience and training in the safe use of these potent medicines (McVann *et al*., 1992; Tracqui *et al*., 1997). The common use of cardiac-glycoside-containing plants as garden subjects places people at risk (Cheung *et al*., 1989). Cardiac-glycoside-containing plants are occasionally used to commit murder and suicide (Tracqui *et al*., 1997; Driggers *et al*., 1989).

3.4 Toxicity Syndrome

Plant-associated cardiac-glycoside poisoning closely resembles poisoning due to digitalis overdose (Tracqui *et al*., 1997). The most obvious initial clinical signs of cardiac-glycoside toxicity are abdominal pain, nausea and vomiting. Headache, dizziness, blurred vision and dyschromatopsia (disturbance of colour vision) may also occur. After an initial bradycardia, the pulse becomes rapid and weak. Various heart dysrhythmias are discernable by electrocardiography. Hyperkalaemia may be seen in severely affected patients. Other typical signs associated with severe toxicity include weakness, muscle tremors, paresis, dyspnoea, cardiogenic shock, convulsions and coma.

3.5 Management

Consider consulting a Poison Control Center. Prompt administration of activated charcoal is of benefit, but standard decontamination and supportive treatment on its own is often not adequate in severe cases involving life-threatening dysrhythmias, cardiogenic shock and hyperkalaemia (Eddleston, 2003). Digoxin-specific antibody fragments (Digibind™) are available as an antidote. Although it was developed specifically for digoxin, there is sufficient crossreaction with other cardiac glycosides for the drug to be useful in the treatment of cardiac-glycoside poisoning in general (McMillin *et al*., 2002). The initial dose in adults and children should be 400 mg intravenously. Additional doses may be needed, depending on the response to treatment.

4 CYANOGENIC GLYCOSIDES

4.1 Plants

Cyanogenic glycosides are produced by a wide variety of flowering plants as a means of defence against herbivores (Gleadow and Woodrow, 2002). Toxic species include members of the Caprifoliaceae (honeysuckle) family, including *Sambucus* spp. (elderberry); the Caricaceae (papaya) family, including *Carica papaya* (papaya); the Euphorbiaceae (spurge) family, including *Manihot esculenta* (cassava); the Fabaceae (legumes), including *Lotus* spp. (trefoil), *Phaseolus* spp. (tepary bean) and *Trifolium* spp. (clover); the Hydrangeaceae (hydrangea) family, including *Hydrangea* spp.; the Juncaginaceae

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(arrow-grass) family, including *Triglochin* spp. (arrow-grass); the Poaceae (grasses), including *Sorghum* spp. and *Zea mays* (maize/corn) and the Rosaceae (rose) family, including *Eriobotrya japonica* (loquat), *Malus* spp. (crab apple) and *Prunus* spp. (plums, prunes, cherries, peaches, apricots and almonds).

4.2 Toxic Agent and Mechanism

Cyanogenic glycosides may be found in most plant parts in affected plant species, but concentrations tend to be highest in the seeds of the Rosaceae, while high concentrations in the grasses are mostly found in young or resprouting plants. Intact cyanogenic glycosides are not toxic. Toxicity occurs when cyanide in the form of HCN (hydrocyanic acid), also referred to as prussic acid, is released from its glycoside form by a process of hydrolyses. Enzymes that are able to hydrolyse cyanogenic glycosides are often present in plant tissues, but are sequestered in different cell compartments and only cause HCN release when plant tissues are damaged. HCN release can also be facilitated by digestive-tract enzymes (Knight and Walter, 2001). This may be a rapid or slow process depending on conditions in the digestive tract. After release, HCN is readily absorbed. It inhibits cytochrome oxidase, which blocks cellular respiration and causes tissue anoxia. Rapid death may result from the failure of organs that are dependent on continuous and relatively efficient oxygen-dependent energy production, such as the brain and the heart. Exposure to toxic doses during pregnancy may lead to teratogenic effects (Frakes *et al*., 1985).

4.3 Risk Factors

The cyanogenic glycoside concentrations in certain plant species, especially the grasses such as *Sorghum halepense* (Johnsongrass) and other sorghum species vary depending on their growth stage. Grazing animals are often at risk if the plants are consumed during their early growth phase, or as plants regrow after a severe frost or mowing, when cyanogenic glycoside concentrations are high (Knight and Walter, 2001). High soil nitrogen content tends to promote cyanogenic glycoside accumulation in plant tissues. The flesh of ripe fruits often does not contain cyanogenic glycosides, even if concentrations in the seeds or leaves are hazardous. Human exposure to toxic levels of cyanogenic glycosides is comparatively rare because people typically do not consume large quantities of those plant parts that are hazardous. Food preparation, such as cooking, further reduces the risk. An exception to this general rule is poisoning by varieties of *Manihot esculenta* (cassava), which accumulate high cyanogenic glycoside concentrations. Cassava poisoning is a common occurrence in regions where home-prepared cassava roots and leaves are a staple food, such as rural areas of central Africa. The cyanogenic glycoside content of cassava can be reduced to safe levels by adequate preparation, which should include extended soaking in water and thorough cooking. When these steps are omitted or deficient, poisoning may occur (Ngudi *et al*., 2003; Tylleskar *et al*., 1992). Another occasional source of human poisoning is the ingestion of large quantities of raw apple seeds or pit kernels from *Prunus* species such as *Prunus armeniaca* (apricot). Note that the amount of seeds or kernels that people consume as part of a normal diet is usually not hazardous. Poisoning typically occurs only when unusually large amounts are ingested.

4.4 Toxicity Syndrome

Cyanogenic glycoside toxicity is often delayed for a variable length of time, up to several hours, because of the time required for glycoside breakdown and cyanide release in the digestive tract. Typical early signs of acute toxicity are nausea, vomiting and abdominal pain. The patient may sweat and complain of headache, disorientation, vertigo, faintness or a burning sensation in the mouth and throat. Signs of hypoxia appear and may include exercise intolerance, tachypnoea and tachycardia. The lack of oxygen uptake in tissues leads to oxygen buildup in venous blood, causing venous blood to become highly oxygenated, similar to arterial blood, and bright red in colour. Compensatory anaerobic energy production leads to lactic acidosis. Signs of hypoxia become progressively worse. Terminal signs may include convulsions, respiratory depression, bradycardia, cardiogenic shock, lung oedema, paralysis and coma (Yen *et al*., 1995; Holland and Kozlowski, 1986).

4.5 Management

When poisoning is suspected, the rapid progression of cyanide poisoning makes it imperative that treatment with an appropriate antidote should be given promptly, even when confirmatory tests are unavailable, as antidote therapy is often essential to survival. The antidote of choice in human poisoning is hydrocobalamin, which is typically packaged in concentrated, stable lyophilized powder form and must be reconstituted before use using suitable diluents such as 0.9% NaCl, lactated Ringer's solution or 5% dextrose solution. The adult dose is 5 g hydrocobalamin by slow intravenous injection (over 15 minutes). If needed, the dose may be repeated for a total of 10 g. Alternative treatments include breaking

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a nitrite pearl and holding it under the patient's nose for 30 seconds each minute. Sodium nitrite may be administered intravenously (the adult dose is 10 ml of a 3% solution). This should be followed by intravenous administration of sodium thiosulfate (the adult dose is 50 ml of a 25% solution). Although it has been used successfully (Mannaioni *et al*., 2002), the safety of combining hydrocobalamin with other treatments has not been established. Antidote therapy should be combined with appropriate supportive care.

5 PLANT-INDUCED DERMATITIS

5.1 Plants

Skin effects due to contact with plants are commonly reported to poison-control centers (Nelson *et al*., 2006). Among the 25 most reported toxic-plant exposures in the United States (**Figure 1**), eight are associated with dermal toxicity including *Crassula* spp. (jade plant), *Toxicodendron* spp. (poison oak/poison ivy), *Schefflera* spp. (umbrella tree), *Ficus* spp. (fig), *Chrysanthemum morifolium* (florist's daisy), *Hedera helix* (English ivy), *Eucalyptus* spp. (blue gum) and *Taraxacum officinale* (dandelion). Many, if not most, plants have the potential to induce allergic contact dermatitis, usually following repeated exposure. A listing of all the plants that have been associated with contact dermatitis is, therefore, impractical and it is prudent to assume that any plant has the potential to induce allergic contact dermatitis in susceptible individuals.

5.2 Toxic Agent and Mechanism

Phytotoxins may affect the skin through a variety of mechanisms, including physicochemical disruption of specific cellular processes, mechanical and/or chemical irritation, allergies and phototoxicities. Although the aetiologies differ, the skin's response to acute insult from plant toxins or mechanical injury from plant structures is most often an inflammatory response of varying severity and extent. Reactions to allergens, such as urushiol found in *Toxicodendron* spp. and a wide variety of macromolecules found on or in the tissues of most plants, are variable, depending on the degree, frequency and duration of exposure, and on the allergenic potential of the molecule(s) involved. Persistence of the allergen in the skin may also be an important factor. Type IV hypersensitivity, also referred to as allergic contact dermatitis, is the most common allergic reaction in the skin, but other types of hypersensitivities are possible (Nelson *et al*., 2006). Exposure to light is a prerequisite for the development of dermatitis associated with phototoxicity (Greeson *et al*., 2001), because it depends on the absorption of photons by phototoxic compounds, and the release of the absorbed energy to surrounding tissues in the form of higher-energy particles. Mechanical damage to skin resulting from barbs, thorns, calcium oxalate raphides, commonly found in species of the Araceae family, or stinging trichomes, such as those found in nettles (*Urtica* spp.), is often accompanied by the deposition of irritant compounds that may intensify pain and induce pruritis (Nelson *et al*., 2006).

5.3 Risk Factors

Most cases of plant-associated dermatitis are due to allergic reactions and require prior sensitization in susceptible individuals. Plants, however, vary widely in their potential to induce allergic reactions, as well as the severity and duration of reactions. *Toxicodendron* spp., for example, have a relatively high potential for inducing severe reactions with 50–70% of the general population being susceptible (Tanner, 2000), while reactions to other commonly implicated plants, such as species of *Taraxacum, Eucalyptus* and *Hedera* are usually less severe. There is tremendous variability between individuals' general susceptibility to plant allergies and the plants to which they react, which is likely to be influenced by inheritable traits. Minor exposures, such as lightly brushing against leaves, may induce a severe reaction to *Toxicodendron* spp., while repeated, intimate exposure is often required for reactions to be induced by other species, such as *Chrysanthemum morifolium*, which typically induce allergies in nursery workers who handle the plant. Even with intimate, repeated exposure to unprotected skin, however, the incidence of sensitivity is much lower compared to Toxicodendron sensitivity and appears to be variable between different population groups, being less common in Asian populations (Tanner, 2000). The part of the plant and its cooked or raw status can make a difference. Some individuals, for example, experience dermatitis if they handle uncooked, peeled potatoes, while cooked potatoes or other potato plant parts do not induce dermatitis. The importance of repeated exposure is also demonstrated by the increased incidence of sensitivity to onions in some subpopulations. Onions more commonly cause chronic hand eczema in older, frequently exposed women who handle onions during food preparation, compared to other sections of the population (Cabanillas *et al*., 2006). Phototoxic effects may occur with repeated, high-dose use of herbal remedies containing St. John's wort (*Hypericum perforatum*) (Jacobson *et al*., 2001). Ironically, though, topical St. John's wort treatment has also been shown to be of benefit in the treatment of atopic dermatitis (Schempp *et al*., 2003).

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5.4 Toxicity Syndrome

The symptoms resulting from different aetiologies most commonly presents as an inflammatory response of varying severity. The overlap between the symptoms associated with various aetiologies makes the differentiation between specific aetiologies, based on lesions and symptoms, challenging. Allergic reactions to plants that result in dermal inflammation is arguably the most common aetiology of plant-associated contact dermatitis, because many different plant species have the potential to induce allergies in susceptible individuals; and because some of the plants that have a high potential for inducing contact allergies, such as *Toxicodendron* spp. are widespread and hard to avoid when entering habitats where the plants are common. Exposures in sensitive individuals typically presents as a rash or eczema on exposed skin, which may include redness, itching, swelling, blistering and pain. The development of lesions related to Type IV hypersensitivity may take hours to days to fully develop, and symptoms may persist for several days up to two weeks. Secondary infection may result in the formation of pustules and purulent exudates. The severity of lesions associated with allergic contact dermatitis is often not proportional to the level of exposure, but rather depends on the level of sensitization in the individual. Pain and dermatitis due to plant-derived chemical and/or mechanical irritants, from exposures to plants such as stinging nettles and capsicum preparations, do not require prior exposure and sensitization. Reactions to these plants and plant-derived products are rapid and the severity is proportional to the level of exposure. Since exposure to light is a prerequisite for phototoxic effects, only sunlight-exposed skin is typically affected. Lesions tend to be more severe on skin surfaces that receive more light exposure, such as the face and hands, and light-skinned individuals are more susceptible. In some instances, photosensitivity may be linked to plant-associated allergic contact dermatitis, a situation that is more commonly encountered in older individuals (Frain-Bell and Johnson, 1979).

5.5 Management

Avoidance is the simplest and most effective approach to allergic dermatitis-inducing plants. Species that are commonly implicated in serious contact dermatitis, such as *Toxicodendron* spp., should be eradicated from areas where contact is expected to be frequent and difficult to avoid. Effective herbicides, registered for this purpose, are available. Hand-pulling by nonsusceptible individuals and repeated grazing by goats and sheep may also be effective. Burning is not an effective control method and urushiol may be carried in smoke, potentially leading to skin and respiratory reactions in smoke-exposed individuals (Nelson, 2000). If avoidance or eradication is not practical, physical protection of exposed skin using clothing or barrier creams is necessary. Barrier creams containing 5% quaternium-18 bentonite are effective in preventing contact dermatitis if properly applied (Scott *et al*., 2002). Sensitive individuals should also be alerted to the fact that second-hand exposure via pets or clothing may be hazardous. Some individuals are allergic to multiple plant species, making identification of particular species that must be avoided challenging. In such cases, attempts should be made to associate the appearance of symptoms with visits to specific areas or habitats. If associations can be made, identified areas or habitats should be avoided or appropriate precautions taken.

Early skin decontamination may be effective in preventing or reducing the symptoms of exposure to plant-associated contact allergens. This can be achieved by gently rinsing the skin with copious amounts of water. The addition of soap or the use of mild solvents, such as isopropyl alcohol, may help in allergen removal from the skin, but care should be taken not to damage the skin surface. Treatment is aimed at symptom alleviation and may involve the use of corticosteroids, analgesics, anti-inflammatory drugs or antihistamines. An important aim in treatment is to prevent itching and scratching, which leads to secondary skin damage and infections. Corticosteroids are the mainstay of the treatment of severe allergic contact dermatitis because they counter lymphocyte proliferation and cytokine production. Topical medications, such as medicated creams and ointments, are preferred if they are effective. Systemic corticosteroids may be needed if topical treatment is ineffective, usually with good results (Mark and Slavin, 2006). Lesions may become infected with bacteria, requiring systemic antibiotic treatment. Pain and dermatitis associated with direct irritants can be controlled with analgesics and anti-inflammatory drugs. If done shortly after exposure, gentle washing with copious amounts of warm, soapy water may be effective in removing irritating substances from the skin. Avoid deep scrubbing that may cause damage to the stratum corneum. Avoidance of sunlight is essential to control symptoms associated with phototoxicity. Additional alleviation of symptoms may be obtained by using analgesics and anti-inflammatory drugs as needed.

6 GASTROINTESTINAL IRRITANTS/STIMULANTS

6.1 Plants

A wide variety of plants affect the gastrointestinal tract (GIT). Commonly implicated genera and their principle toxins include: *Euphorbia* (spurges), containing diterpene esters; *Alocasia*, *Arum*, *Colocasia*, *Philodendron*, *Dieffenbachia*, *Schlefflera*, *Spathiphyllum* and related

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Araceae genera, as well as *Brassaia* (umbrella tree), *Caryota* (fishtail palm) and *Parthenocissus* (creeper) containing calcium oxalate raphides; *Daphne* (paradise plant) *Senna* and *Wisteria*, containing glycosides; *Phytolacca* (pokeweed), containing triterpenes; and *Ranunculus* (buttercup), containing protoanemonin. Some plants may be associated with GIT upset only when ingested in high quantities or at specific growth stages, while being safe under other conditions. Examples include the ingestion of greening or sprouting potatoes, unripe fruit or large quantities of dried fruit.

6.2 Toxic Agent and Mechanism

The mechanisms of action of most plants that affect the GIT have not been well defined, but can be classified into two broad categories: nonspecific irritant effects and specific receptor activity leading to GIT effects. Irritants are found in many plants and may be associated with specific growth phases or plant parts, such as unripe fruits. Irritants stimulate GIT smooth-muscle contraction directly and/or induce an acute inflammatory response, typically associated with increased fluid secretion into the GIT lumen. Severe irritation may lead to ulceration. calcium oxalate raphides leads to local tissue damage in the upper GIT associated with severe inflammation, enhanced by proinflammatory compounds associated with the raphides. Plants have evolved a variety of specialized strategies for effectively delivering raphides, including the formation of needle-shaped crystals, and pressurized crystal idioblasts that forcibly expel raphides into tissues upon contact (Franceschi and Nakata, 2005). Stimulation of cholinergic receptors appears to be a major specific mechanism of inducing increased GIT smooth-muscle contraction due to plant toxins (Nelson *et al*., 2006). GIT peristaltic and secretory control, however, depends on a complex system of balanced responses and multiple receptor ligands (Grider, 2004), offering many potential toxicity targets.

6.3 Risk Factors

Due to the relatively common occurrence of irritants in plants, indiscriminate ingestion or chewing on unfamiliar plants often leads to GIT upset. This is especially relevant to young children who often chew on indoor plants. Gathering and eating wild plants is a risk when the collectors are unfamiliar with the plants they collect, particularly when plants are gathered in early growth stages before the development of flowers and fruits. Poorly controlled production and use of herbal remedies intended for the treatment of constipation can be hazardous due to lack of consistency in active compound concentrations and dose formulations. Even when herbal remedies are appropriately formulated and produced, popular perceptions of herbal medicines as 'natural, safer' alternatives to other chemical laxatives, occasionally lead to ingestion of large doses that cause severe, long-lasting diarrhoea.

6.4 Toxicity Syndrome

The first signs of GIT irritant exposure are generally nausea and vomiting. Other typical irritant effects include hypersecretion and smooth-muscle contraction, which leads to diarrhoea, and abdominal pain associated with cramping. Severe gastric irritation or ulceration may lead to blood products appearing in the vomitus, which may have a 'coffee-granule' appearance. Diarrhoea can range widely in severity and appearance. Young children are particularly vulnerable to electrolyte loss and dehydration.

6.5 Management

The management of plant poisoning involving gastrointestinal symptoms is nonspecific, and aimed at symptom alleviation and countering the consequences of fluid and electrolyte loss. Vomiting can often be controlled with drugs such as metoclopramide. The treatment of diarrhoea depends on severity and should be aimed at the control of water and electrolyte loss and its replacement. The use of antidiarrhoeal drugs, such as loperamide, is generally not recommended (Nelson *et al*., 2006). Rehydration and electrolyte replacement therapy may include oral rehydration or intravenous fluids, depending on the need. Since GIT upset is often one of the first effects seen in poisoning cases that eventually include systemic toxicity, the potential for the gastrointestinal effects being part of a more widespread, systemic toxicity should always be considered (Nelson *et al*., 2006).

7 ION-CHANNEL ACTIVATORS/INHIBITORS

7.1 Plants

Many members of genera in the Ericaceae (heath) family have effects on ion channels, including *Rhododendron* spp. (azalea), *Kalmia* spp. (laurel), *Lyonia* spp. (staggerbush), *Leucothoe* spp. (doghobble) and *Pieris* spp. (fetterbush). Other affected families are the Ranunculaceae (buttercup) family, including *Aconitum* spp. (monkshood); the Liliaceae (lily) family, including *Zigadenus* spp. (deathcamus), *Schoenocaulon* spp. (feathershank) and *Veratrum* spp. (false hellebore); and the Taxaceae (yew) family, including *Taxus* spp. (yew).

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7.2 Toxic Agent and Mechanism

Depending on the plant and predominant active compound, various ion channels may be activated or inhibited. One of the most common mechanisms for toxicity is sodium-channel activation, typical of plants in the heath family, containing grayanotoxins. Excessive influx of sodium causes persistent depolarization of neurons and other conductive tissues such as cardiac muscle cells (Nelson *et al*., 2006). Taxine-derived alkaloids, of which several have been identified, are found in *Taxus* spp. (yew) (Wilson *et al*., 2001). Taxines are calcium- and sodium-channel antagonists. Indirect evidence suggests that taxine toxicity is largely attributable to its calcium-channel blocking effects (Wilson *et al*., 2007). Taxine-B induces increased AV (atrioventricular) conduction time and QRS duration in cardiac muscle. Intestinal effects observed in experimental animals included contraction of the duodenum and ileum and inhibition of peristalsis (Wilson *et al*., 2007).

7.3 Risk Factors

Consumption of honey derived from bees harvesting nectar from *Rhododendron* species, sometimes referred to as 'mad honey' is an important risk factor because grayanotoxins are present in nectar and remain active after nectar processing into honey (Koca and Koca, 2007). Consumption of Rhododendron flowers or nectar in other forms, such as wine, juice or as an ingredient in cakes may also be associated with poisoning (Lee *et al*., 2007).

Yew trees and shrubs are hardy, evergreen and produce attractive, bright red fruit. Their widespread and popular use as garden subjects makes them easily accessible for most people. Eating the flesh of the fruit, a bright red aril with a pleasant, sweet taste is, fortunately, not associated with poisoning (Nelson *et al*., 2006; Pietsch *et al*., 2007). Indiscriminate ingestion of seeds with the fruit's flesh, or chewing on twigs and leaves can, however, be hazardous. Yews are also commonly implicated in animal poisoning when animals gain access to garden waste (Wilson *et al*., 2007). A number of web sites dealing with suicides mention yew as a classical method for committing suicide (Pietsch *et al*., 2007). This may partly explain its relatively common use in suicide.

7.4 Toxicity Syndrome

Excessive sodium-channel activation on neurons leads to a variety of effects associated with the central nervous system, including nausea and vomiting, disorientation and seizures. Peripheral effects include paresthesia, excessive perspiration, hypersalivation and muscle weakness, fasciculation and paralysis. Cardiac effects may include hypotension and dysrhythmias ranging from mild bradycardia to complete heart blocks (Gunduz *et al*., 2008; Koca and Koca, 2007; Nelson *et al*., 2006; Lee *et al*., 2007). Taxus poisoning is associated with initial dizziness, dry mouth and mydriasis, followed by nausea and vomiting, abdominal cramping and pain, tachycardia followed by bradycardia, hyperkalaemia, convulsions and respiratory paralysis (Pietsch *et al*., 2007; Nelson *et al*., 2006).

7.5 Management

Life-threatening cardiac effects associated with sodium-channel activation can be countered with rapid saline infusion and atropine (Gunduz *et al*., 2008). Severe hypotension can be counteracted with norepinephrine (noradrenaline). The use of sodium-channel blocking agents, such as lidocaine and amiodarone, makes sense in terms of their mechanism of action, but superior outcomes using these drugs have not been proven (Nelson *et al*., 2006). Specific antidotes for taxus poisoning are not available. Treatment is based on decontamination, such as gastric lavage and activated charcoal, and symptomatic treatment to counteract cardiac dysrhythmias.

8 MICROTUBULE POLYMERIZATION INHIBITORS

8.1 Plants

Plants that inhibit microtubule polymerization include members of the Apocynaceae (dogbane) family, including *Catharanthus* spp. (periwinkle); the Liliaceae (lily) family, including *Colchicum* spp. (crocus) and *Gloriosa* spp. (flame lily); and the Berberidaceae (barberry) family, including *Podophyllum* spp. (mayapple).

8.2 Toxic Agent and Mechanism

Toxic alkaloids that inhibit microtubule polymerization include some of the most well-known antitumour drugs, such as vincristine and related dimeric alkaloids derived from *Catharanthus roseus* (Madagascar periwinkle) (Barnett *et al*., 1978). These compounds destabilize microtubules, which leads to mitotic arrest and, at sufficient exposure levels, cell death (Groth-Pedersen *et al*., 2007). The effects are more pronounced in rapidly dividing cells, which makes it useful for the treatment of tumours, but also affects rapidly dividing normal cells,

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such as bone marrow, intestinal epithelium and hair follicles. At high doses or following prolonged exposures, organs with relatively rapid cell turnover rates, such as the liver and pancreas can be affected (Brvar *et al*., 2004). It also affects peripheral nerve function and reduces the number of intraepidermal nerve fibres (Siau *et al*., 2006).

8.3 Risk Factors

The leaves of some toxic members of the Liliaceae, such as *Colchicum autumnale* (autumn crocus), may be confused with that of *Allium ursium* (wild garlic) and is a cause of poisoning in individuals that gather wild herbs for use in food (Hermanns-Clausen, 2005).

8.4 Toxicity Syndrome

Initial signs of acute poisoning usually involve the GIT and include nausea, vomiting and diarrhoea (Brvar *et al*., 2004). Ulcerations in the mouth and gastrointestinal necrosis are also possible (Nelson *et al*., 2006). Subsequent effects potentially include failure of various organ systems, including the heart, liver and pancreas. Bone-marrow suppression leads to pancytopenia (Brvar *et al*., 2004). Acute clinical signs may include colic, hepatosplenomegaly and jaundice, while more chronic exposure may lead to cirrhosis or ascitis following hepatic venous occlusion (Nelson *et al*., 2006).

8.5 Management

There are no specific treatments available. Supportive treatments for liver failure, immune suppression and other manifestations of poisoning should be instituted to alleviate symptoms and promote tissue repair. Severe liver failure may be irreversible and successful treatment could require liver transplant (Nelson *et al*., 2006).

9 NICOTINIC ALKALOIDS

9.1 Plants

Nicotinic alkaloids are found in a wide range of plants, including members of the Apiaceae (carrot) family, including *Conium maculatum* (poison hemlock); Berberidaceae (barberry family), including *Caulophyllum* spp. (cohosh); Campanulaceae family, including *Hippobroma* spp. and *Lobelia* spp.; Fabaceae (pea) family, including *Baptisia* spp. (wild indigo), *Gymnocladus* spp. (coffee tree), *Laburnum* spp. and *Sophora* spp. (necklacepod); Ranunculaceae (buttercup family), including *Delphinium* spp. (larkspur), and Solanaceae (potato family), including *Nicotiana* spp. (tobacco).

9.2 Toxic Agent and Mechanism

Nicotinic alkaloids can be found in a variety of alkaloid groups, such as diterpenoids (found in *Delphinium* spp.), piperidines (found in *Conium* spp.) and pyridines (found in *Nicotiana* spp.) (Pfister *et al*., 2001). Nicotinic receptors are pentameric ligand-gated cation channels, with many subtypes, that are activated by acetylcholine (Schmitt, 2000). They are found in the central nervous system, parasympathetic and sympathetic peripheral nervous systems and at neuromuscular junctions. Depending on the ligand-binding characteristics and the specific binding conditions, nicotinic receptors can transition between closed, open and desensitized states, leading to suppression, excitation or nonreactivity of affected central and peripheral neurological or neuromuscular pathways and functions (Giniatullin *et al*., 2005). Nicotine is the classic nicotinic alkaloid. It binds to nicotinic receptor subtypes relatively indiscriminately and tends to induce initial stimulation followed by nonreactivity in affected pathways. Many nicotinic alkaloids derived from plants share this characteristic to some extent, but different nicotinic alkaloids differ in receptor binding characteristics (Dobelis *et al*., 1999).

9.3 Risk Factors

The most problematic and widespread risk factor associated with nicotinic alkaloids is chronic exposure to tobacco smoke and other tobacco products, which is generally considered to be one of the most important preventable health risks in modern society. This is due, in part, to the tremendous addictive potential of nicotine (Bierut *et al*., 2008). Acute toxicity is, however, much less common and is usually associated with accidental exposure following misidentification of nicotinic-alkaloid-containing plants (Durand *et al*., 2008). The infamy of some plants, such as *Conium maculatum* (poison hemlock) that was used in the execution of Socrates, unfortunately contributes to their use in criminal poisoning (Trestrail, 2007).

9.4 Toxicity Syndrome

The acute effects of nicotinic alkaloids are characterized by initial stimulation, followed by loss of reactivity and

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function in nicotinic pathways in the central and peripheral nervous systems, and at neuromuscular junctions. Typical symptoms include nausea and vomiting, salivation, diaphoresis, trembling, dyskinesia, weakness, bradycardia followed by tachycardia, hypertension, increased respiration rate, urination, convulsions, paralysis, coma and death (Nelson *et al*., 2006; Vetter, 2004).

9.5 Management

Early decontamination, including stomach lavage and activated charcoal, may be effective in reducing toxic effects (Vetter, 2004). Treatment is symptomatic and should be targeted at correcting serious vital sign abnormalities, such as countering hypertension with diltiazem or nitroprusside. Seizures can be treated with intravenous benzodiazepines. Ventilatory support may be necessary in cases of respiratory muscle weakness or paralysis (Nelson *et al*., 2006).

10 PYRROLIZIDINE ALKALOIDS

10.1 Plants

Pyrrolizidine alkaloid toxicity is associated with a wide variety of plants including members of the Asteraceae (aster) family, including *Callilepis* spp. (ox-eye daisy) and *Packera* spp. (ragwort; formerly known as *Senecio* spp.); Boraginaceae (borage) family, including *Echium* spp. (viper's bugloss), *Heliotropium* spp. (heliotrope) and *Symphytum* spp. (comfrey); Fabaceae (bean) family, including *Crotalaria* spp. (rattlebox), *Sesbania* spp. (riverhemp); Lamiaceae (mint) family, including *Teucrium* spp. (germander); and Zygophylaceae (creosote-bush) family, including *Larrea* spp. (creosote bush). Many other plant families also have members that produce pyrrolizidine alkaloids and, although they are not as often associated with poisoning, can be potentially toxic, including the Apocynaceae (dogbane) family, Celastraceae (bittersweet) family, Convolvulaceae (morning-glory) family, Proteaceae (protea) family, Orchidaceae (orchid) family and Ranunculaceae (buttercup) family.

10.2 Toxic Agent and Mechanism

Pyrrolizidine alkaloids and related N-oxides are not directly toxic, but are metabolized by microsomal enzymes to pyrroles, which are dehydro- forms of the alkaloid, in a process that occurs mostly in the liver. Pyrroles are powerful alkylating agents leading to the formation of DNA adducts and crosslinking of proteins, amino acids and glutathione. Exposure is associated with various genotoxic effects including tumorogenicity, mutagenicity and teratogenicity (Fu *et al*., 2004), but a clear link between human cancer and pyrrolizidine alkaloid exposure has not been made (Prakash *et al*., 1999). The most prominent acute lesions occur in the liver, where high doses can cause centrilobular necrosis. Acute endothelial injury can also occur in the pulmonary vasculature in animals associated with the ingestion of *Crotalaria* spp. (Knight and Walter, 2001), but similar lesions have not been described in human pyrrolizidine alkaloid poisoning (Nelson *et al*., 2006). Chronic exposure leads to nonthrombotic veno-occlusive liver disease and, eventually, liver cirrhosis (Stickel and Seitz, 2000).

10.3 Risk Factors

The use of pyrrolizidine-alkaloid-containing plants in herbal medicines and dietary supplements is common in many parts of the world. It is one of the most important health risks associated with the unregulated use of herbal products (Stickel *et al*., 2000; Sheikh *et al*., 1997; Seeff, 2007). Inclusion of pyrrolizidine-alkaloid-containing plants as contaminants in foodstuffs, such as flour, is a potentially massive source of exposure (Prakash *et al*., 1999). Pyrrolizidine alkaloids are excreted through milk (Panter and James, 1990), which may be a potential source of exposure when milk-producing animals consume pyrrolizidine-alkaloid-containing plants and infants may be exposed through mother's milk. Lactation may partially protect lactating mothers, while exposing vulnerable infants (Schoental, 1968). Pyrrolizidine alkaloid exposure in pregnant women may lead to foetal poisoning (Rasenack *et al*., 2003). A particularly tragic example of the risks associated with inappropriate use of pyrrolizidine-alkaloid-containing herbal medicines is the lethal poisoning of young children due to toxic *Packera* spp. in South Africa (Steenkamp *et al*., 2000). The collection of plant material by nonexperts for sale in markets, and the difficulty of differentiating between toxic and nontoxic *Packera* spp., contribute to the problem.

10.4 Toxicity Syndrome

The major target organ in pyrrolizidine alkaloid poisoning is the liver. Hepatic degeneration and necrosis is mostly centrilobular, but panlobular necrosis is possible at massive exposure levels. Chronic liver lesions include fibrotic and cirrhotic changes. The clinical presentation depends on the level and duration of exposure. Massive, acute exposure leads to gastrointestinal

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symptoms, abdominal pain, hepatosplenomegaly and icterus (Nelson *et al*., 2006). Biochemical indicators such as aspartate ammotransferase (AST) and bilirubin levels are consistent with liver failure (Nelson *et al*., 2006). Chronic exposure can lead to cumulative effects, including cirrhosis, ascites and, potentially, liver carcinoma (Nelson *et al*., 2006; Prakash *et al*., 1999).

10.5 Management

There are no specific treatments available. Spontaneous repair of liver damage may occur, depending on the level of injury. Severe liver damage or cirrhosis may require liver transplant (Nelson *et al*., 2006).

11 TOXALBUMINS

11.1 Plants

Toxalbumin-containing plants include species of the Cucurbitaceae family (*Momordica*), the Fabaceae family (*Abrus*, *Robinia*), the Euphorbiaceae family (*Hura*, *Jatropha*, *Ricinus*) and the Visacaceae family (*Phoradendron*). The most infamous example of a toxalbumin-producing plant is *Ricinus communis* (castorbean), because it is the source of one of the most potent toxins known, ricin, which has been used for assassination and is of concern as a potential weapon of terrorism. The number of species containing similar toxalbumins shows, however, that toxalbumins are produced by a fairly large number of plant species.

11.2 Toxic Agent and Mechanism

The toxalbumins in plants that cause most concern are members of a large group of glycoproteins, called lectins, that have the ability to specifically bind or crosslink carbohydrates. They may be loosely classified into high-potency lectins, such as ricin (produced by *Ricinus communis*), abrin (produced by *Abrus precatorius*) and curcin (produced by *Jatropha curcas*), and low-potency lectins (present in most undercooked beans). The toxalbumins are typically water soluble and not present in oil extracts, such as castor oil, in significant concentrations. They denature under high temperature and are therefore wet-heat labile. Due to their large size and polar characteristics, the toxalbumins are poorly absorbed from the GIT and the parenteral lethal dose is typically much smaller than the oral lethal dose. The toxalbumins consists of two distinct chains, called the A-chain and the B-chain. The toxic effects are due to the inhibition of protein synthesis through irreversible inactivation of ribosomal subunits by the A-chain, while the B-chain is necessary for toxalbumin binding to glycolipids and glycoproteins on the cell surface, followed by entry into the cell by endocytosis (Doan, 2004). Human poisoning with low-potency lectins has not been well described, but it has been suggested that acute gastrointestinal distress following the ingestion of inadequately cooked legumes is due to inhibition of plasma-membrane repair (Miyake *et al*., 2007).

11.3 Risk Factors

Ricinus communis (castorbean) is a common weed in many parts of the world. Some varieties are also occasionally used as decorative garden plants. The seeds are quite attractive and may be of interest to children coming in contact with the plant. Although the seeds contain hazardous concentrations of ricin, its release requires mastication or maceration. All plant parts, including leaves and roots, may create a hazard when used in traditional medicines (Audi *et al*., 2005). An increase in the production of *Jatropha curcas* (physic nut) for use as a biofuel may create increased risk of poisoning, especially in children who are often interested in the attractive seeds and occasionally ingest seeds out of curiosity (Kulkarni *et al*., 2005; Menezes *et al*., 2006). A similar risk occurs due to the use of seeds from *Abrus precatorius* (precatory bean), which are particularly attractive (bright red with a black patch on one end), to make jewellery.

11.4 Toxicity Syndrome

Oral exposure is the most relevant route of exposure for unintentional poisoning by toxalbumins. Signs typically appear 2–4 hours, and occasionally as much as 10 hours, after ingestion and may include nausea, abdominal cramps, heartburn, vomiting, diarrhoea, hypotension, dehydration and hypovolemic shock. Haematemesis and melaena are possible in severe cases. Hepatotoxic and renal effects have been recorded, but are not typical. Flulike symptoms, including fatigue, headache and muscle pain may also occur. Laboratory findings may include leukocytosis, elevated transaminases and creatinine kinase, hyperbilirubinemia, renal insufficiency and anaemia (Audi *et al*., 2005; Kulkarni *et al*., 2005). Although possible, lethal poisoning with oral toxalbumins is uncommon and appears to follow severe damage to the gastrointestinal mucosa that allows increased toxalbumin absorption followed by effects on the liver, pancreas, muscle and other tissues. Acute exposure to high concentrations of low-potency lectins, such as those found in

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undercooked beans, is associated with nausea, vomiting and diarrhoea (Miyake *et al*., 2007).

11.5 Management

Treatment is mainly supportive and symptomatic and could include intravenous fluids, electrolyte correction and vasopressive treatment for hypotension. Gastric evacuation is considered to be of limited value, but can be considered if ingestion occurred within an hour or less. Activated charcoal may be of benefit if the patient is not vomiting (Audi *et al*., 2005).

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Mycotoxins with a Special Focus on Aflatoxins, Ochratoxins and Fumonisins

Pieter S. Steyn, Wentzel C.A. Gelderblom, Gordon S. Shephard and Fanie R. van Heerden

CONTENTS

1 INTRODUCTION

Mycotoxins comprise a structurally diverse group of mostly small-molecular-weight compounds, derived from the secondary metabolism of filamentous fungi. Several of the toxins are important environmental and carcinogenic agents and are ubiquitous in a broad range of commodities, causing toxic responses when ingested by mammals (such as man and higher animals), poultry and fish. Initially, mycotoxin production was mainly linked to the so-called storage fungi, growing saprophytically (post-harvest) on stored grains and nuts; however, fungi growing parasitically (preharvest) on plants also have the propensity to produce mycotoxins.

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Not all fungal growth on plants and plant products cause mycotoxin production, therefore detection of fungi, even toxigenic fungi, on foods and feeds does not necessarily imply the presence of mycotoxins. Worldwide, an exponential growth in all aspects related to mycotoxin research is occurring. This chapter is directed at updating the review of Steyn and Stander (1999) with a focus on recent findings.

These toxic secondary metabolites are produced by a number of fungi, including members of the *Aspergillus*, *Penicillium*, *Fusarium*, *Claviceps* and *Alternaria* genera. Several explanations may be given for their production, however, it is likely that mycotoxins could play a role in facilitating competition with other micro-organisms for nutrients and space, and the generation of favourable germination conditions for fungal spores (O'Brien and Dietrich, 2005). Bennett and Arnold (2001) reviewed the genomics of fungi and focussed on the *Aspergillus flavus* complex that encompasses a cluster of anamorphic, haploid species involved in *A. flavus* and *A. parasiticus*, the mycotoxin producers, and the koji moulds, *A. oryzae* and *A. sojae*. Several leading research groups are making considerable progress on the genomics of aflatoxigenic species, as well as on ochratoxigenic and other mycotoxigenic species, and it is envisaged that a better understanding of the genomics of fungi could enhance methods of mycotoxin control. The seminal findings of O'Callaghan and Dobson (2006a; 2006b), O'Callaghan *et al*. (2006c), Geisen *et al*. (2006) and Atoui *et al*. (2006) contributed significantly to a better understanding of the polyketide synthase genes from *Aspergillus ochraceus* and the molecular characterization of ochratoxin A (OTA) biosynthesis.

The epoch-making discovery of the aflatoxins in the 1960s, the causal agents of turkey X-disease in the UK (Lancaster *et al*., 1961; Sargeant *et al*., 1961), subsequently led to their classification as human carcinogens and linked them to primary liver cancer (PLC) in humans. This introduced an unabated worldwide interest in mycotoxins and led to the discovery of the ochratoxins, fumonisins, phomopsins and the tremorgenic mycotoxins. (See the reviews by Murphy *et al*. 2006, and the monograph on *Mycotoxins in Food*, edited by Magan and Olsen, 2004). The aflatoxins, such as aflatoxin B_1 (AFB_1), are transformed in mammals, for example, humans, into reactive mutagenic electrophiles through metabolizing processes. These electrophiles react readily with DNA and are thus considered to be the ultimate carcinogens.

In structural complexity, mycotoxins vary from simple C4-compounds, for example moniliformin, produced by *Fusarium moniliforme*, to complex substances such as the phomopsins, fumonisins, sporidesmin and the tremorgenic mycotoxins. See **Figure 1** for the structures of some representative mycotoxins which are associated with specific human and animal mycotoxicoses. Although there are hundreds of mycotoxins, few of these are considered responsible for large-scale losses associated with contaminated agricultural products. There can be significant year-to-year fluctuations in the levels of mycotoxins in foods, due to many factors, such as adverse climatic conditions that favour fungal invasion, growth and mycotoxin formation. Plant toxins cause local problems, mainly affecting livestock; however, mycotoxins put the world food supply under threat due to the worldwide distribution of toxigenic fungi, and the international trade in foods and feeds. Post-harvest losses in the developing world are particularly severe owing to the quality of the produce and inadequate storage facilities. It is claimed that approximately 60% of Africa's grain supplies are at risk, owing to fungal contamination and mycotoxin formation.

The global threat to mankind is based on well-documented human mycotoxicoses, such as ergotism (St Anthony's fire) which occurred frequently in the middle Ages in Europe, alimentary toxic aleukia (ATA) in Russia, acute aflatoxicoses in South and East Asia, and human PLC in Africa and South East Asia. OTA is suspected of playing a role in Balkan endemic nephropathy (BEN) amongst the population living in the former Yugoslavia (Mantle, 2002) and chronic interstitial nephropathy (CIN) in North Africa. The fumonisins are implicated in the aetiology of the high incidence of oesophageal cancer among the inhabitants of the Transkei region of South Africa. Although the role of mycotoxins in diseases among domestic animals is better established, diagnosis of the mycotoxicosis is extremely difficult owing to the numerous pharmacological effects of the causative toxins, for example, aflatoxins (Turkey-X disease), fumonisins (leukoencephalomalacia in horses and pulmonary oedema in swine (Bezuidenhout *et al*., 1988; Gelderblom *et al*., 1988)), ochratoxins (nephropathy in swine (Danish porcine nephropathy (DPN)) (Krogh, 1976; Stoev *et al*., 2002)), phomopsin A (lupinosis in sheep (Culvenor *et al*., 1989)), sporidesmin A (facial eczema in sheep (Mortimer *et al*., 1978)) and zearalenone (hyperoestrogenism, vulvovaginitis and abortion in swine). Outbreaks of diplodiosis amongst farm animals are linked to feeds contaminated with *Diplodia maydis*.

Mycotoxins induce powerful and dissimilar biological effects. Some are carcinogenic (aflatoxins, ochratoxins and fumonisins), mutagenic (aflatoxins and sterigmatocystin), teratogenic (ochratoxins), oestrogenic (zearalenone), haemorrhagic (trichothecenes), immunotoxic (aflatoxins and ochratoxins), nephrotoxic (ochratoxins), hepatotoxic (aflatoxins, ochratoxins and phomopsins), dermotoxic (trichothecenes) and neurotoxic (ergotoxins, penitrems, lolitrems and paxilline), whereas others display antitumour, cytotoxic and antimicrobial properties. The human ingestion of mycotoxins is due to the consumption of the mycotoxins in plant-based foods, such as grains, like maize, barley and rice, coffee, nuts and their residues, and metabolites in

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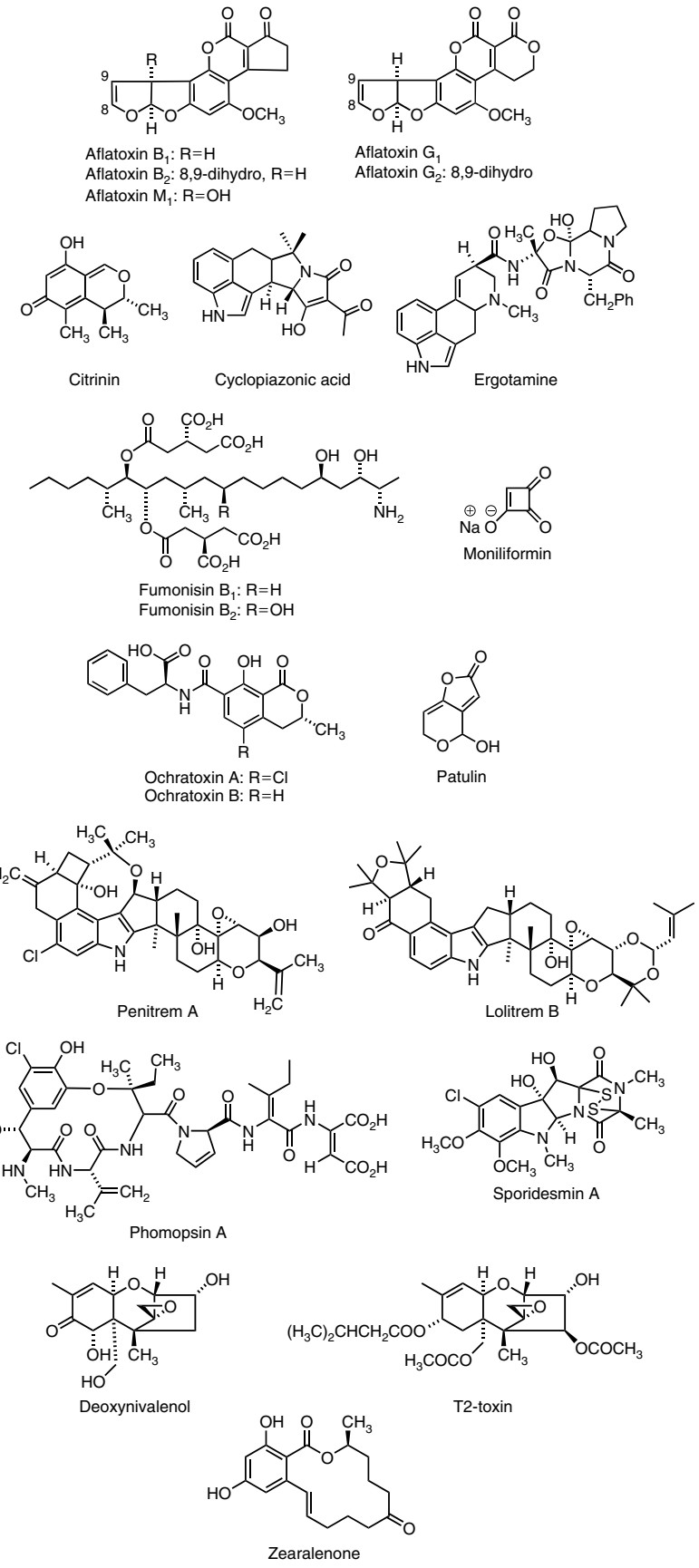

Figure 1 Some representative mycotoxins associated with human and animal mycotoxicoses.

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animal-derived foods, for example aflatoxin M_1 (AFM_1) in milk and meat products. In addition, mycotoxins may occur in beer and wine; worldwide, OTA is a frequent contaminant of wines. Some of the toxic effects of mycotoxins observed in experimental animals are consistent with the characteristic symptoms seen in a number of human and animal diseases.

The impact of mycotoxins on health depends on the amount of the mycotoxin consumed, the toxicity of the compound, for example, acute or chronic (e.g. carcinogenic) effects, the body weight of the individual, the presence of other mycotoxins (synergistic effects) and other dietary effects. Hsieh (1990) maintained that all of the following criteria have to be satisfied to link a mycotoxin to a specific human disease: occurrence of the mycotoxin in food supplies; human exposure to the mycotoxin; correlation between exposure and incidence; reproducibility of the characteristic symptoms in experimental animals; similar mode of action in human and animal models.

In nature, most cereal grains, oil seeds, tree nuts, fruits and dehydrated fruits are susceptible to fungus contamination and mycotoxin formation. Under laboratory conditions, at least 300 mycotoxins have been produced by pure cultures of fungi and chemically characterized. Fortunately, only about 20 mycotoxins are known to occur in foodstuffs at significant levels and frequency to be of food-safety concern. These toxins are mainly produced by five genera of fungi: *Aspergillus, Penicillium, Fusarium, Alternaria* and *Claviceps* (O'Brien and Dietrich, 2005). The mycotoxins produced by these fungi are: *Aspergillus* toxins, aflatoxin B_1, G_1, M_1, OTA, sterigmatocystin and cyclopiazonic acid; *Penicillium* toxins, patulin (PAT), OTA, citrinin, penitrem A (PA) and cyclopiazonic acid; *Fusarium* toxins, deoxynivalenol, nivalenol, zearalenone, T-2 toxin, diacetoxyscirpenol, fumonisins and moniliformin; *Alternaria* toxins, tenuazonic acid, alternariol and alternariol methyl ether; *Claviceps* toxins, ergot alkaloids.

The Council for Agricultural Science and Technology (CAST) considered aflatoxins, trichothecenes, fumonisins, zearalenone, OTA and ergot alkaloids as most relevant for human health (see Serra *et al*., 2005), although the extent of harm caused by each toxin is highly species dependant. This chapter mainly focusses on the mycotoxins aflatoxins, OTA and fumonisins, owing to their high priority for control purposes and their frequent and worldwide distribution in agricultural products. Many mycotoxins are highly resistant, and survive food processing to enter the food chain and provide a threat to human health. Reliable and accurate analysis of mycotoxins at the ppb (parts per billion) and ppt (parts per trillion) levels in the various commodities is essential for protecting human and animal health, as well as for conforming to the standards set for national and international trade. The newer methods which involve antibody-based mycotoxin screening techniques, high-performance liquid chromatography (HPLC) and tandem mass spectroscopy impacted positively on several of the techniques. These procedures are described in more detail in later subsections.

Detoxifying contaminated foodstuffs and feedstuffs is of prime importance in protecting consumer health; and each of the subsections contains a part dedicated to this matter. The decontamination processes aim to destroy, inactivate or remove the mycotoxin, and not to produce or leave toxic or carcinogenic/mutagenic residues in the final products or in the food products obtained from animals fed decontaminated feeds. Regulations for the control of mycotoxins in various commodities have received much international attention, and are contained as a subsection of this chapter. In addition, risk assessment is generic to all the mycotoxins, therefore, a subsection is devoted to this matter as well.

2 AFLATOXIN

The aflatoxins comprise a group of hepatocarcinogenic mycotoxins, mainly produced by the filamentous fungi *Aspergillus flavus* and *Aspergillus parasiticus*. Most foods and feeds, particularly high-energy nuts, are at risk of contamination by these fungi and by the notorious aflatoxins. A cause–effect relationship between dietary aflatoxin intake and human hepatocellular carcinoma (HCC) has become internationally recognized, and their role in human and animal mycotoxicoses firmly established. AFM_1 is found in milk of animals that have consumed feeds contaminated with aflatoxins. Aflatoxins B_1, G_1 and M_1 have been recognized to be possibly carcinogenic to humans (Group 2B) by the International Agency for Research on Cancer (2002), and a plausible mechanism for their mode of action has become generally accepted. As a result, acceptable levels of aflatoxins in foods, feeds and milk have been established and enforced worldwide. The importance of aflatoxin is emphasized by a recent outbreak of aflatoxicosis in Kenya in April 2004 in which 125 people died (Lewis *et al*., 2005).

2.1 Producing Organisms for Aflatoxins

The most important aflatoxin-producing organisms in terms of agricultural products remain *Aspergillus flavus* and *A. parasiticus* (Steyn and Stander, 1999). Although *A. flavus* is mostly associated with oilseed crops (maize, peanuts and cottonseed), the fungus is an extremely common soil fungus, and under improper storage conditions aflatoxin production may be a problem with many other crops (Klich, 2007). Other aflatoxin-producing

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species include *A. flavus* var. *parvisclerotigenus*, *A. toxicarius*, *A. nomius*, *A. pseudotamarii*, *A. zhaoqingensis* and *A. bombycis* (all from *Aspergillus* section *Flavi*), *A. ochraceoroseus* and *A. ambellii* (*Aspergillus* section *Ochraceorosei)* and *Emericella astellata* and *E. venezuelensis* (*Aspergillus* section *Nidulantes)* (Frisvad *et al*., 2005; Cary *et al*., 2005).

The B-type aflatoxins (AFB_1 and AFB_2) are produced by all the organisms whereas the G-type (AFG_1 and AFG_2) aflatoxins are produced by only some of the species in *Aspergillus* section *Flavi.* Species in this subgroup that produce B-type aflatoxins, but not G-type aflatoxins, often produce cyclopiazonic acid. Production of both the G-type aflatoxins and cyclopiazonic acid by a single strain has not yet been observed (Frisvad *et al*., 2005).

Substantial progress has been made on the sequencing of the genome of *A. flavus* and closely related fungi (Machida *et al*., 2005). Information on the genome sequence of aflatoxin-producing organisms will assist in understanding the pathogenicity and regulatory aspects of aflatoxin biosynthesis (Bhatnagar *et al*., 2003; Chang *et al*., 2007; Kim *et al*., 2005; Yu *et al*., 2007). It has been shown that the genes encoding the biosynthesis pathway of aflatoxins are in a 70 kb cluster of the chromosome (Yu *et al*., 2004). To investigate aflatoxin production, gene expression profiles in aflatoxin-supportive media vs. non-aflatoxin-supportive media were evaluated in *A. flavus* and *A. parasiticus* and genes consistently expressed in several aflatoxin-supportive media were identified (Yu *et al*., 2007).

2.2 Analytical Methodology for Aflatoxins

Since their discovery in the early 1960s, numerous analytical methods have been applied to their analysis in a range of contaminated commodities. These have been highlighted in a previous review (Steyn and Stander, 1999). Analytical methods for aflatoxins have advanced along with general advances in analytical chemistry methods and instrumentation. These improvements have involved changes in sample extract purification with the widespread introduction of immunoaffinity columns (IACs), the increasing use of mass spectrometry (MS) as a HPLC detection tool and the further development of antibody-based rapid methods.

2.2.1 Chromatographic Methods for Aflatoxins

Thin-layer chromatography (TLC) remains widely in routine use in many laboratories. To eliminate the need for chlorinated solvents, IAC clean-up was linked with one-dimensional TLC for analysis of various food matrices at levels significantly lower than European Union (EU) limits (Stroka *et al*., 2000a). As a technique related to TLC, overpressured layer chromatography has been used to achieve reproducible determination of aflatoxins in samples of red paprika (Moricz *et al*., 2007).

A number of papers published in the last five years described the optimization of existing methods to achieve specific goals. Vega (2005) described a method to reduce solvent consumption in the extraction of aflatoxins from peanut butter, by reducing sample size and extraction-solvent volume. Möller and Nyberg (2004) investigated the extraction of aflatoxins from defatted peanut-meal reference samples using four different extraction solvent mixtures, and concluded that the widely used methanol–water $(80 + 20)$ may not always be the best for all matrices, as it achieved a result considerably below the certified value of the test material. Post-column derivatization with iodine or bromine is a widely used method to enhance the fluorescence of AFB_1 and AFG_1 in reversed-phase chromatography. A collaborative study of a post-column photochemical derivatization using a reaction coil wound around an ultraviolet (UV) lamp has been successfully performed and accepted by AOAC International (Waltking and Wilson, 2006). Extraction of high-pigment samples, such as chilli powder, green bean and black sesame was achieved by matrix solid-phase dispersion using neutral alumina, followed by elution of aflatoxins through a bed of graphitic carbon black to remove pigments that interfered with the HPLC analysis (Hu *et al*., 2006). The problem of low recoveries on extracts of herbal samples of high acidity was solved by using 0.1 M phosphate buffer for dilution and neutralization (Ip and Che, 2006). For the purpose of analysing very low levels of mycotoxins in a duplicate diet study, a method for AFB_1, AFM_1 and OTA was developed, which involved chloroform extraction and liquid–liquid extraction prior to IAC clean-up and HPLC determination (Sizoo and Van Egmond, 2005). The limits of quantitation for these mycotoxins were 5, 24 and 16 $ng\,kg^{-1}$ (ppt), respectively.

The introduction of lower EU regulatory limits at 2 $\mu g\,kg^{-1}$ for AFB_1 and 4 $\mu g\,kg^{-1}$ total aflatoxins required the validation of a new official method for aflatoxins in peanut butter, pistachio paste, fig paste and paprika powder which involved IAC clean-up of extract and reversed-phase HPLC with fluorescence detection following post-column bromination (Stroka *et al*., 2000b). Similarly, a collaborative study validated an analytical method for AFM_1 in milk at levels above 0.02 $ng\,ml^{-1}$, which was based on IAC clean-up of a centrifuged milk sample followed by reversed-phase HPLC (Dragacci *et al*., 2001). This methodology, involving extraction with nonchlorinated solvent mixtures, dilution of the filtered extract, clean-up on IAC and reversed-phase HPLC quantification with post-column derivatization has been extended

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to a number of commodities. Thus, methods have been developed for the determination of aflatoxins in medicinal herbs (Ali *et al*., 2005; Arranz *et al*., 2006a; Braga *et al*., 2005; Gomez-Catalan *et al*., 2005), in tiger-nut-based soft drinks (Arranz *et al*., 2006b), in maize (Brera *et al*., 2007), in hazelnut paste (Senyuva and Gilbert, 2005) and in pig liver (Chiavaro *et al*., 2005). The commercial availability of IACs containing antibodies specific to more than one mycotoxin has resulted in the development of methods for aflatoxins and OTA in foods (Chan *et al*., 2004) and in botanical roots (Trucksess *et al*., 2006; Trucksess *et al*., 2007), and for aflatoxins, OTA and zearalenone in rice, rye and pig feed (Göbel and Lusky, 2004).

MS as an HPLC detection system has made rapid advances in the last decade. Methods have been developed in which the conventional fluorescence detectors used for aflatoxins have been replaced by bench-top mass spectrometers operating with various atmospheric pressure ionization (API) sources. The application of these new techniques to a range of mycotoxins has been comprehensively reviewed (Zöllner and Mayer-Helm, 2006). The ability to combine quantification with confirmation from specific fragmentation patterns has distinct advantages. Although some authors have used atmospheric pressure chemical ionization (APCI) (Abbas *et al*., 2006; Liau *et al*., 2007), the majority of methods developed employ electrospray ionization (ESI) in the positive ion mode to yield protonated molecular ions (Cavaliere *et al*., 2007a; 2007b; Lattanzio *et al*., 2007; Spanjer *et al*., 2008; Sulyok *et al*., 2006; Ventura *et al*., 2004). The use of atmospheric pressure photoionization (APPI) has found limited application, although it has been shown to reduce chemical noise (Cavaliere *et al*., 2006; Takino *et al*., 2004). Confirmation of analysis can be achieved by use of time-of-flight mass spectrometry (TOF-MS) (Tanaka *et al*., 2006) or the use of multiple reaction monitoring (MRM), in which one fragment ion is used for quantification and a second for confirmation purposes (Spanjer *et al*., 2008). In many cases, the MS detector has been confined to the detection of the four main aflatoxin analogues. For example, methods have been developed for the determination of aflatoxins in maize (Abbas *et al*., 2006; Cavaliere *et al*., 2007a), olive oil (Cavaliere *et al*., 2007b), hazelnuts (Bacaloni *et al*., 2008), medicinal herbs (Ventura *et al*., 2004) and traditional medicines (Liau *et al*., 2007). In contrast to the above, a few groups have exploited the versatility of MS to develop multimycotoxin methods. This approach can be restricted to mycotoxins for which multitoxin immunoaffinity clean-up columns are available (aflatoxins, OTA, fumonisins, deoxynivalenol, zearalenone, T-2 toxin and HT-2 toxin)(Lattanzio *et al*., 2007) or it can encompass a much larger number of toxins (Delmulle *et al*., 2006; Spanjer *et al*., 2008; Sulyok *et al*., 2006; Tanaka *et al*., 2006). A recent publication allows detection of 90 mycotoxins, secondary fungal metabolites and plant-derived metabolites of mycotoxins (Berthiller *et al*., 2007). Due to the multiplicity of toxin chemistries, no clean-up is available for such mixtures. In these cases, the extracts are diluted and then injected. Matrix effects are monitored and for quantitation, matrix-matched standards are used (Sulyok *et al*., 2007).

2.2.2 Immunological Methods for Aflatoxins

Immunological methods in a number of different formats continue to be developed for aflatoxins. Lateral flow devices have been prepared which incorporate colloidal gold particles to produce the visible end point, the design of the assay being such as to provide a visible line at aflatoxin levels below a designated level. This level is set by adjusting the amounts of reagents used in the assay. The presence of a control line ensures the good performance of the kit in that it indicates that antibodies have migrated past the test line. Such kits have been developed for rapid detection of aflatoxins in pig feed (Delmulle *et al*., 2005), grain and animal feed (Shim *et al*., 2007), and rice, maize and wheat flours (Xiulan *et al*., 2006). In its most popular format, the assay provides a single visible cut-off level, although it can be combined with a photometric strip reader for semiquantitative determinations over a limited range (Xiulan *et al*., 2006). Although the majority of immunological methods utilize a colour reaction (generated by enzyme action or by the use of colloidal gold particles) for the end point, some researchers have developed electrochemical detection systems. These are based on enzyme-linked immunosorbent assay (ELISA) principles using disposable screen-printed electrodes on which the relevant antibodies are coated and the end point is measured by a suitable voltametric or amperometric electrochemical method (Ammida *et al*., 2004). Immunological methods such as ELISA have generally been confined to determination of single mycotoxins. However, a few methods have emerged which allow the simultaneous determination of both aflatoxin and OTA. Using a membrane-based method involving the separate spotting of specific antibodies, good agreement was found with individual ELISA measurements for the toxins in chilli samples (Saha *et al*., 2007). A method for the detection of both AFB_1 and OTA with specific cut-off levels was incorporated in a sorbent tube, which was packed with a clean-up layer at the base and separate detection immunolayers for the two mycotoxins above (Goryacheva *et al*., 2007a). Extracts of spice samples were drawn through the tube from the base, allowing on-tube clean-up prior to the extract solution passing through the immunolayers containing mycotoxin antibodies. The flow through the tube was reversed, and after washing and developing the colour, the presence

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or absence of the individual mycotoxins determined by the absence or presence of colour, respectively.

2.3 Occurrence of Aflatoxins

Contamination of groundnuts in the UK by aflatoxins was first reported by Lancaster *et al*. (1961) and Sargeant *et al*. (1961) as a result of the death of turkey poults that were fed contaminated groundnut meal. The highly carcinogenic toxic compounds became known as aflatoxins B_1, B_2, G_1 and G_2. Aflatoxins have been found in a variety of agricultural commodities of which the most important are groundnuts, tree nuts, maize and other oil seeds, such as cottonseed.

The levels of aflatoxins occurring in groundnuts, grown in tropical climatic conditions are generally high, as found in Brazil, Cambodia, India, Jordan, Malaya, Kenya, Nigeria, Uganda, and warm regions of the USA and Australia (Strange, 1991). The EU has established the maximum tolerated level (MTL) of AFB_1 in groundnuts for direct consumption as $2\,\mu g\,kg^{-1}$. Many reports of AFB_1 in ground nuts have appeared over the years. For example, Dos Santos *et al*. (2001) reported contamination of 28 out of 77 samples of raw peanuts with AFB_1 and AFG_1 in Brazil 24 samples had contamination $>30\,\mu g\,kg^{-1}$. A total of 145 raw shelled peanut samples from Malaysia were analysed for the contamination of AFB_1, AFB_2, AFG_1 and AFG_2 (Sulaiman *et al*., 2007). The percentage, along with the range of incidence of the AFB_1 (50%, $0.85–548\,\mu g\,kg^{-1}$) and AFG_1 (37%, $1.37–376\,\mu g\,kg^{-1}$) in raw shelled peanut samples were very high, whereas AFB_2 and AFG_2 were not detected in the samples. In a survey of the occurrence of aflatoxins, OTA and fumonisins in retail foods in Japan, aflatoxin contamination was determined in 10 of 21 peanut-butter samples, while aflatoxins could not be quantified in other foods. The average concentrations for AFB_1, AFB_2, AFG_1 and AFG_2 were 1.07, 0.27, 0.40 and $0.21\,\mu g\,kg^{-1}$, respectively (Sugita-Konishi *et al*., 2006b). In a survey that was aimed at the occurrence of mycotoxins relevant to the feed industry in various parts of the world, eight peanut-meal samples out of nine from Asia and Oceania were contaminated with AFB_1, for which an average concentration of $184\,\mu g\,kg^{-1}$ was calculated (Binder *et al*., 2007). The EU maximum limit of AFB_1 in all feed materials for cattle, sheep, goats, pigs and poultry is $20\,\mu g\,kg^{-1}$.

Levels of aflatoxin contamination of maize is a worldwide problem. It may vary from region to region within a country due to environmental factors, high temperature and humidity that favour its production. For example, the problem of contaminated maize in the southeast of the USA is far greater than in other parts of the USA (Shotwell, 1991). Aflatoxin formation in Australian maize is uncommon (Pitt and Hocking, 2004). Due to maize processing, the consumer is often not exposed directly to mycotoxins in raw maize, but rather in derived products of maize. Thus, the amounts of mycotoxins in raw maize are unlikely to reflect the concentrations in the finished product. Maize is often processed with other feedstuffs for animal feed. A three-year surveillance programme assessed the extent of mycotoxin contamination of key foods and feeds grown in Bangladesh (Dawlatana *et al*., 2002). Of the foods and feeds tested, the levels of contamination of maize, roasted and raw groundnuts, and poultry feed were highest, with average total AFB_1 contents of 33, 13, 65 and $7\,\mu g\,kg^{-1}$, respectively. In Morocco, 58 samples of cereals for human consumption (20 corn flour, 17 wheat flour) and poultry feeds (21) purchased from local markets were analysed (Zinedine *et al*., 2007a). The incidence of aflatoxin in corn flour, wheat flour and poultry feeds was about 80, 17.6 and 66.6%, respectively. Levels of contamination of analysed samples with AFB_1 ranged from 0.23 to 11.2, 0.03 to 0.15 and 0.05 to $5.38\,ng\,g^{-1}$ for corn flour, wheat flour and poultry feeds, respectively. Results showed also that the contamination of 10% of samples of corn flour was higher than the limit set by EU regulations for AFB_1 and total aflatoxins. In Brazil, aflatoxins were found to co-occur with fumonisins on freshly harvested maize kernels (Ono *et al*., 2001). Aflatoxins were detected in 11.3% of the total samples and all the aflatoxin-positive samples (range $38.0–460.0\,ng\,g^{-1}$) were cocontaminated with fumonisins. The co-occurrence of AFB_1 and fumonisins was previously reported in Indian maize and poultry feeds by Shetty and Bhat (1997). Co-occurrence of aflatoxin and OTA seems to be rarer. Maize and barley samples intended for animal feed in northern Iran were analysed for aflatoxin and OTA. AFB_1 and AFB_2 were detected in 88.8 and 66.6% of the corn samples, at a mean level of 15.83 and $2.99\,\mu g\,kg^{-1}$, respectively (Yazdanpanah *et al*., 2001). Only one of the AF-contaminated maize samples was cocontaminated with OTA at a concentration of $0.35\,\mu g\,kg^{-1}$. Neither aflatoxin nor OTA was found in any of the barley samples. In India (Thirumala-Devi *et al*., 2002) ingredients intended for incorporation into chicken feed, which included groundnut cake, maize, millet, rice bran, sorghum, soybean, sunflower and mixed feeds, were assayed for aflatoxins and OTA contamination. Of the samples, 38% were contaminated with aflatoxins and 6% with OTA. AFB_1 has been reported in conserved hay and in maize silage used as animal feed (Lacey, 1991).

Although reported in more than 20 countries, the incidence and levels of aflatoxins in small grains such as wheat, barley, rye, oats, sorghum, millet and rice are relatively low (Yoshisawa, 1991). Ayalew *et al*. (2006) recently reported the occurrence of mycotoxins in the Ethiopian staple cereals, barley, sorghum, teff (*Eragrostis tef*) and wheat. AFB_1 and OTA were detected in samples of all the four crops. AFB_1 was detected in 8.8%

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of the 352 samples analysed at concentrations ranging from trace amounts to concentrations of $26\,\mu g\,kg^{-1}$. The results indicated higher mycotoxin contamination in sorghum, which could be related to the widespread storage of sorghum grain in underground pits. AFB_1 was found in 58.3% of *menu* (crushed fermented soybean cake) samples in Korea, with an average concentration of $7.3\,ng\,g^{-1}$ (Kim *et al*., 2001).

The occurrence of aflatoxin in cottonseed is of great concern in the USA due to its wide usage in feedstuffs. The oil from cotton kernels is used for manufacturing of margarine and soap, and the remaining press cake is used for animal feed. The occurrence of aflatoxin in cottonseed is uncommon in Australia (Pitt and Hocking, 2004). The occurrence of AFB_1 in other oil seeds such as soya has received attention as a potential health risk. A total of 694 food samples collected from six different regions of South Korea were analysed for their AFB_1 content (Ok *et al*., 2007). Thirty-two samples, including three soybean products, were found to be contaminated with AFB_1 up to $48.6\,\mu g\,kg^{-1}$. The level of AFB_1 contamination in 28 of the 32 food products was below $10\,\mu g\,kg^{-1}$, which is the legal tolerance limit in Korea. The major contributors to the dietary intake of AFB_1 were soybean paste and soy sauce, which composed 91% of the total exposure to AFB_1. Sunflower is an important ingredient of animal feedstuff and is a source of concern. For example, in the study of Thirumala-Devi *et al*. (2002) (see above), the following incidence scores of aflatoxin contamination in excess of $10\,\mu g\,kg^{-1}$ were found in poultry feed: 41 of 95 for maize, 18 of 30 for mixed feeds, 10 of 37 for groundnut, 6 of 29 for sorghum, 5 of 10 for sunflower, 3 of 14 for rice bran and one of eight for millet.

Zinedine *et al*. (2006) reported the analysis of 55 samples of spices (14 of paprika, 12 of ginger, 14 of cumin and 15 of pepper) purchased from markets in Morocco. Spice samples were analysed for aflatoxins and the average contamination found for AFB_1 was 0.09, 0.63, 2.88 and $0.03\,\mu g\,kg^{-1}$ for black pepper, ginger, red paprika and cumin, respectively.

AFM_1 and AFM_2, the hydroxylated metabolites of AFB_1 and AFB_2 (Holzapfel *et al*., 1966), are found in milk products obtained from livestock that have ingested contaminated feed, such as peanut, maize and cottonseed meal. About 1–2% of AFB_1 in animal feed is transformed to AFM_1 in milk (Van Egmond, 1989). The European Commission has set a maximum tolerated level of $0.05\,\mu g\,kg^{-1}$ for AFM_1 in milk, whereas the maximum level set by Codex Alimentarius is $0.5\,\mu g\,kg^{-1}$. In a study in Egypt, Salem (2002) reported that all feedstuff samples from six farms and two from feedstuff factories were found to contain total aflatoxin levels ranging between 2 and $60\,\mu g\,kg^{-1}$, with four samples exceeding the Egyptian maximum levels ($20\,\mu g\,kg^{-1}$). AFM_1 was found in 58.8% of the investigated milk samples with concentrations not reaching the MTL of the EU countries. Kim *et al*. (2000) showed that, the incidence of AFM_1 in milk was 76%, with a mean concentration of $18\,pg\,g^{-1}$ in Korea. Rastogi *et al*. (2004) showed that the incidence of contamination of AFM_1 in milk was 33%, with a concentration range of $28–164\,ng\,l^{-1}$ in Indian markets. In Brazil, Sassahara *et al*. (2005) analysed 42 samples of milk: 10 (24%) were contaminated by AFM_1 and three (7%) were above $0.5\,\mu g\,l^{-1}$. Aflatoxin contamination was investigated in 48 raw milk and 48 feed samples in Turkey (Ayar *et al*., 2007). Altogether, 20 raw milk samples (41.67%) and 15 feed samples (31.25%) exceeded the relevant legal limits established by the Turkish Food Codex (TFC) and the EU. Similarly, Unusan (2006) reported a mean value of $108.17\,ng\,l^{-1}$ for AFM_1 in 129 samples of commercial UHT (ultra-high temperature) whole milk from Turkey and an incidence rate of 58%. Of the contaminated samples, 47% were well above the limit permitted by the EU. Kamkar (2005) found that in 76.6% of samples from Iran the presence of AFM_1 was detected in a range between 0.015 and $0.28\,\mu g\,l^{-1}$. Of positive samples, 40% exceeded the concentration limit permitted by the EU. Similarly, 64% of 186 milk samples from different dairy farms in Iran were contaminated (Ghiasian *et al*., 2007), having a mean concentration of AFM_1 in contaminated samples of $43.4\,ng\,l^{-1}$. Fourteen (11.76%) contaminated samples had AFM_1 in concentrations in excess of the maximum levels specified in EU regulations. In a study on Feta cheese Kamkar (2006) reported an 82.5% incidence of AFM_1 in the range $0.15–2.41\,\mu g\,l^{-1}$. In Italy, a total of 332 samples were collected from dairy farms delivering milk to factories for Parmigiano Reggiano cheese production over a six year period (Pietri *et al*., 2003). While AFM_1 was present in 95.5% of the samples, with 8.4% exceeding the limit set by the EU, it was reported that in later years there was a general trend towards lower AFM_1 levels in dairy products. In a study in Brazil, Iha *et al*. (2007) found that 18% of UHT and pasteurized frozen goat-milk samples contained between 20 and $98\,ng\,l^{-1}$ AFM_1. However, Oliveira and Ferraz (2007) found the incidence in Brazil to be as high as 69% with mean concentrations of 0.58, 0.56 and $0.72\,\mu g\,l^{-1}$ for UHT, powder and pasteurized goat milk, respectively. Virdis *et al*. (2006) found 17.3% of bulk goat milk at a cheese factory in Sardinia, Italy, to contain between 5 and $40\,ng\,l^{-1}$ AFM_1, whereas ripened tank cheese had an incidence of 9.8%, with concentration values between 79 and $389\,ng\,kg^{-1}$.

The staple food in many African countries is frequently contaminated by aflatoxins. Prenatal exposure of the foetus to AFB_1 in Ghana, Kenya and Nigeria was revealed by analysis of cord blood (Shephard, 2004). Exposure of infants to aflatoxins in human breast milk has been reported in Egypt (El-Sayed *et al*., 2000), Gambia, Ghana, Kenya, Sierra Leone, Sudan and Zimbabwe (Shephard, 2004). Aflatoxins are common contaminants of staple foods in sub-Saharan Africa and are associated with increased risk of liver cancer and impaired

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growth in young children (Wild, 2007). In an intervention study of exposure to infected groundnuts from 20 African farms, the concentrations of blood aflatoxin–albumin adducts from 600 people immediately after harvest and at three and five months post-harvest were monitored. In control villages mean aflatoxin–albumin concentration increased from 5.5 pg mg^{-1}, immediately after harvest, to 18.7 pg mg^{-1} five months later (Turner *et al*., 2005).

2.4 Biological Activity of Aflatoxins

As mentioned by Steyn and Stander (1999), the aflatoxins, particularly AFB_1, can be regarded as potent toxins, carcinogens, teratogens and mutagens. Of the four naturally occurring aflatoxins (B_1, B_2, G_1 and G_2), AFB_1 is the most toxic and carcinogenic. AFM_1, a metabolite of AFB_1 present in milk, is considered to be as toxic, but less carcinogenic than AFB_1 (Bailey *et al*., 1998; Wogan and Paglialunga, 1974). Different animal species have different oral LD_{50} values for AFB_1, as is quoted by Leung *et al*. (2006): rabbits (0.3 mg (kg body weight)$^{-1}$), mink (0.5–0.6 mg (kg body weight)$^{-1}$), cats (0.55 mg (kg body weight)$^{-1}$), dogs (1.0 mg (kg body weight)$^{-1}$) and guinea pigs (1.4–2.0 mg (kg body weight)$^{-1}$), being more sensitive than mice (9.0 mg (kg body weight)$^{-1}$) or hamsters (10.2 mg (kg body weight)$^{-1}$).

AFB_1, the most potent natural carcinogen (Bennett and Klich, 2003), is converted in human liver to the 8,9-epoxide, which can bind to both DNA and proteins (**Scheme 1**). Although Essigmann *et al*. (1977) were the first to postulate that the AFB_1 epoxide was the active intermediate in the hepatocarcinogenicity of AFB_1, it was only after the availability of the epoxide by chemical synthesis (Baertschi *et al*., 1988) that the mechanism was unravelled. In the human liver, AFB_1 is metabolized by cytochrome P450 (Guengerich, 2003) to yield the highly reactive 8,9-epoxyaflatoxin B_1 (Kelly *et al*., 1997). In chemical synthesis (Baertschi *et al*., 1988) and enzymatic reactions (Ueng *et al*., 1995), both the *exo*- and *endo*- epoxides are formed in varying ratios. However, it has been proven that the *exo*-stereoisomer is the carcinogenic metabolite and that the *endo*-epoxide is 1000-fold less active than the *exo*-isomer. It is postulated that furocoumarin intercalates between the bases of DNA and that with the *exo*-epoxide, an S_N2 reaction between the N^7 of guanine and the epoxide is possible, whereas with the *endo*-epoxide, attack is blocked by the epoxide group (Iyer *et al*., 1994). However, Bren *et al*. (2007) have proved by calculations that the higher activity of the *exo*-epoxide already exists in aqueous solution and that it is only further enhanced by DNA intercalation.

AFB_1 epoxide undergoes hydrolysis to form a diol, which under slightly basic conditions, is transformed into a dialdehyde. The dialdehyde can form a Schiff base with the primary amine group of lysine to form protein adducts (**Scheme 1**). AFB_1 *exo*-epoxide also reacts with DNA after intercalation to form a *trans*-8,9-dihydro-8-(N^7-guanyl)-9-hydroxyaflatoxin adduct (Croy *et al*., 1978; Brown *et al*., 2006; Johnson and Guengerich, 1997). The cationic DNA adduct promotes depurination, resulting in an apurinic site or, under basic conditions, the imidazole ring opens to form the stable AFB_1 formamidopyrimidine. The initial cationic DNA adduct and the two secondary DNA lesions promote GC → TA transversions (Bailey *et al*., 1996). A characteristic G to T mutation is observed at the third position of codon 249 of the p53 tumour suppressor gene in more than 50% of human HCC cases associated with AFB_1 exposure (Smela *et al*., 2002).

In the human liver, at least four different cytochrome P450 enzymes can metabolize AFB_1 to the more carcinogenic epoxide, but Kamdem *et al*. (2006) have shown that cytochrome P450 3A4 plays the most important role and should be targeted for the development of inhibitors. Although AFB_1 is mostly associated with liver cancer, there is also the risk of lung cancer, either by inhalation of grain dust or by dietary exposure (Kelly *et al*., 1997; Van Vleet *et al*., 2002). He *et al*. (2006) have shown that AFB_1 can also be activated by cytochrome P450 2A13, an enzyme predominantly expressed in the human respiratory tract (Zhu *et al*., 2006).

PLC is the fifth most common cancer in the world and the third most common cause of cancer-related deaths (Lau and Lai, 2008). The incidence of liver cancer is much higher in developing countries than in developed countries, with a very high incidence in sub-Saharan Africa and Asia. The risk factors for liver cancer include exposure to hepatitis B or C and aflatoxin-contaminated food (Lau and Lai, 2008; Henry *et al*., 1999; Wang and Tang, 2004). An extensive study by Qian *et al*. (1994) on human HCC patients has shown that although aflatoxin exposure as detected by urinary aflatoxin (relative risk = 3.4), and hepatitis B infection as detected by hepatitis B surface antigen (relative risk = 7.3), play a role in the development of HCC, a significant increase in the relative risk was observed for people who tested positive for both (relative risk = 59).

Poultry, and especially turkeys, are extremely sensitive towards the toxic effects of AFB_1 and small amounts of AFB_1 cause reduction in growth rate, feed efficiency, hatchability and increased susceptibility to disease. Klein *et al*. (2000) have shown that turkey livers are more efficient in the epoxidation of AFB_1 than rodent livers and that turkeys are deficient in the glutathione S-transferase (GST) isoform that efficiently detoxifies AFB_1. This information was crucial in the development of dietary

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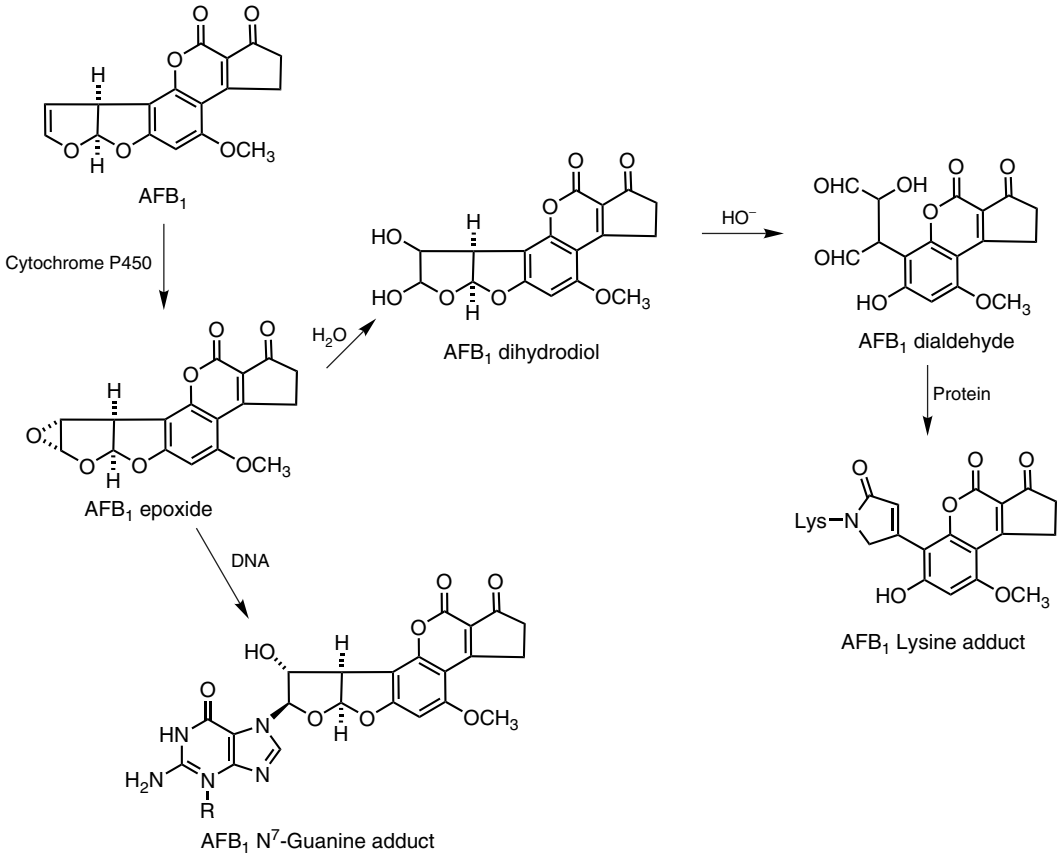

Scheme 1 Metabolism of aflatoxin B_1 in humans. (Based on Guengerich, 2008; Bedard and Massey, 2006.)

antioxidants for the protection of turkeys against AFB_1 toxicosis (Coulombe *et al*., 2005). Yip and Coulombe (2006) have cloned and expressed a cytochrome P450 from turkey livers which can transform AFB_1 to the AFB_1-8,9-oxide and AFM_1.

2.5 Control and Decontamination of Aflatoxins

Approaches to decontaminate aflatoxin-containing food or feedstuffs, or to minimize the effect thereof, include treatment with ammonia or ozone and addition of a sorbent (e.g. activated charcoal, montmorillonite or bentonite clay), microbial degradation, or the addition of antioxidant dietary supplements to the food or feedstuff.

Treatment of plant-derived material with ammonia in the gaseous phase or in solution is a well-known process and has been discussed by Piva *et al*. (1995) and Steyn and Stander (1999). The efficacy and safety of any decontamination process need constant scrutiny. Allameh *et al*. (2005) have done a comparative study on one-day-old broiler chickens fed on uncontaminated, ammonia-treated uncontaminated, aflatoxin-contaminated and ammonia-treated aflatoxin-contaminated maize and found that aflatoxin-contaminated maize led to a suppression of dietary intake, body-weight gain and feed conversion ratio in chickens, but that ammonia-treated maize (both with or without aflatoxin) did not affect any of these parameters and that treatment of aflatoxin-containing maize with ammonia can significantly suppress aflatoxicosis. Hoogenboom *et al*. (2001) investigated the absorption, distribution and excretion of aflatoxin-derived ammoniation products in lactating cows by feeding the cows naturally contaminated peanut-meal spiked with radiolabelled aflatoxin. It was observed that the reduction of aflatoxin levels by ammoniation of contaminated peanut meal resulted in a strong reduction of aflatoxin-related residues in milk and meat of cows.

Treatment with ozone has also been investigated as a method for the decontamination of aflatoxin. McKenzie *et al*. (1998) have shown that turkey poults fed on aflatoxin-contaminated whole-kernel corn treated with ozone did not exhibit any of the negative symptoms of aflatoxicosis. Inan *et al*. (2007) found that the AFB_1 content of red pepper (*Capsicum annuum*) could be reduced by 80 and 93% by exposing it to 33 and $66\,mg\,l^{-1}$ ozone, respectively, for 60 minutes. Akbas and Ozdemir (2006) investigated the effect of ozone on aflatoxin-containing pistachio kernels and ground pistachios, and found that with pistachio kernels the

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AFB_1 levels could be reduced by 23% (9 mg l^{-1} ozone, 420 minutes, 20 °C), whereas only a 5% reduction was observed for ground pistachios under the same conditions. Furthermore, although no change in the fatty acid composition of both the kernels and ground pistachios was observed, significant changes in the organoleptic properties of the ground pistachios were detected. Proctor *et al*. (2004) observed degradation of aflatoxins in peanut kernels and flour by gaseous ozonation and mild heat treatment. In a study on the safety and efficacy of ozonation by Prudente and King (2002), it was found that although ozonation (10–12 wt%) reduced aflatoxin levels by 92% and had a minimal effect on the fatty acids of the uncontaminated corn, it had an effect on the fatty acids of the contaminated corn and that extracts of the treated contaminated corn showed mutagenic effects in the Ames assay. It is clear that the safety of ozonation as a decontamination method for aflatoxins needs further investigation.

Clay additives are used to pelletize animal feeds and as an anticaking agents to improve the flow characteristics thereof. It was also found that certain clays can reduce the toxic effects of certain mycotoxins, especially the aflatoxins. The clays are added to dry feeds and, therefore, absorption of the mycotoxins must occur after ingestion. Several studies have shown the benefits of different clays against aflatoxicosis (Piva *et al*., 1995). For instance, Bailey *et al*. (2006) have investigated the effect of montmorillonite clay (NovaSil PLUS) on the performance of full-term broiler chickens fed on a diet containing 4000 μg kg^{-1} aflatoxin. The birds fed with the clay received a considerable protection against aflatoxicosis for most parameters measured (tissue analyses of blood serum, relative liver, spleen and kidney weight, and histological examination of sampled organs). However, it was clear that at exposure of this high level of aflatoxin, full protection was not received, since the performance of the chickens was not as good as the control group. In a short-term study, Wang *et al*. (2005) investigated the safety of this montmorillonite clay (NovaSil) on humans. Upon ingesting either 1.5 or 3.0 g per day for 14 days, no negative symptoms were observed. In a study among residents of Qidong, People's Republic of China, who are at high risk of developing of PLC, partly from aflatoxin ingestion, treatment with chlorophyllin, water-soluble derivatives of chlorophyll (100 mg per day, three times per day for four months) reduced the level of biomarkers associated with aflatoxin-induced liver cancer by 55% (Egner *et al*., 2001).

Extensive research has also been done on compounds/extracts that will inhibit aflatoxin biosynthesis. Many of the active inhibitors are plant-derived compounds. A review article by Holmes *et al*. (2008) summarizes a large number of such products.

Teniola *et al*. (2005) studied the biological degradation of AFB_1 by *Rhodococcus erythropolis* in liquid cultures and in cell-free extracts. A dramatic reduction of AFB was observed, and after 72 hours only 3–6% of residual AFB_1 remained. Similar results were obtained by employing *Mycobacterium fluoranthenivorans*. The high degradation rate and wide temperature range for degradation by *R. erythropolis* and *M. fluoranthenivorans* indicated the potential for application in the food and feed industries.

3 OCHRATOXIN

The ochratoxins, metabolites of several *Aspergillus* and *Penicillium* species, described by Van der Merwe *et al*. (1965), comprised the first major group of mycotoxins discovered subsequent to the aflatoxins. The ubiquitous ochratoxins formed the substance of many reviews (Steyn, 1993; Steyn and Stander, 1999; Blank *et al*., 1999; Gareis, 2000; Varga *et al*., 2001; Frank, 2001; Mantle, 2002; Belli *et al*., 2002; Aish *et al*., 2004; Huijbregts *et al*., 2005; Ringot *et al*., 2006; Clark and Snedeker, 2006; Pardo *et al*., 2006; Murphy *et al*., 2006; Pfohl-Leszkowicz and Manderville, 2007). The production of OTA by the black *Aspergilli* on grape berries, leading to the contamination of wines, established OTA as the most important mycotoxin in wine (Serra *et al*., 2005). These findings led to a renewed interest in ochratoxigenic micro-organisms, and the toxicology of OTA (Battilani *et al*., 2006; Leong *et al*., 2006a).

OTA comprises a pentaketide-derived (R)-3,4-dihydro-3-methylisocoumarin moiety linked via its 12-carboxy group to L-β-phenylalanine, as verified by X-ray crystallography (Bredenkamp *et al*., 1989) (See **Figure 1**). De Jesus *et al*. (1980), employing stable isotopes, verified the biosynthetic origin of OTA, its 12-carboxy group being derived from a C1 unit, such as methyl-methionine. The physical data for OTA and ochratoxin B (OTB) were described by Van der Merwe *et al*. (1965) and Bredenkamp *et al*. (1989). OTB, the dechloro derivative of OTA is essentially non-toxic.

3.1 Ochratoxin A-Producing Micro-Organisms

A. ochraceus, *A. carbonarius* and *Penicillium verrucosum* are the three major ochratoxigenic fungi. These fungi grow in atmospheres with a relatively high moisture content. Therefore, harvesting crops at high moisture contents and storage of contaminated commodities under damp conditions favour the production of OTA (Birzele *et al*., 2000; Esteban *et al*., 2006a; 2006b; Leong *et al*., 2006b; 2006c). However, many fungal species have the propensity to produce OTA. Frisvad *et al*. (2004) described new species of *Aspergillus* section *Circumdati*, namely *A. cretensis*, *A. flocculosus*, *A. neobridgeri*,

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A. pseudoelegans, *A. roseoglobulosus*, *A. steynii* and *A. westerdijkiae*. Except for *A. neobridgeri*, all of these species are producers of OTA. *A. ochraceus, A. westerdijkiae* and *A. steynii* were reported by Frisvad *et al*. (2004) as the most important species regarding OTA production in coffee, rice, beverages and feedstuffs. Vega *et al*. (2006) described four potent *Penicillium* species as OTA producers, namely *P. brevicompactum*, *P. crustosum*, *P. olsonii* and *P. oxalicum*. Rizzo *et al*. (2002) reported that *Aspergillus* and *Penicillium* fungi contaminate cereals and foodstuffs, and found five new isolates of *Aspergillus* which produce OTA namely: *A. albertensis*, *A. auricomus*, *A. wentii*, *A. fumigatus* and *A. versicolor*. Al-Julaifi (2003) found that *Eurotium amstelodami*, *E. herbariorum* and *Euortium* spp. isolated from barley all produced OTA.

3.1.1 Producers of OTA from Grapes

Although OTA was first detected in wine during 1996, the role of the black *Aspergilli* in OTA production was only discovered in 1999. The black *Aspergilli*, namely *Aspergillus* section *Nigri* are the producers of OTA on grape berries/vines with *A. carbonarius* as the main producer (Battilani *et al*., 2003; 2004; 2006; Serra *et al*., 2003; 2005; Bau *et al*., 2005; Medina *et al*., 2005; Belli *et al*., 2006; 2007; Leong *et al*., 2006a; Chulze *et al*., 2006; Bejaoui *et al*., 2006b; Gomez *et al*., 2006; Martinez-Culebras and Ramon, 2007; Ponsone *et al*., 2007; Hocking *et al*., 2007; 2008). Esteban *et al*. (2006b) observed that *A. carbonarius* is able to grow and produce OTA in a wide range of water activities (a_w) and temperatures. Leong *et al*. (2006b) investigated the effects of a_w and temperature on the survival of *A. carbonarius* spores *in vitro* and concluded that increased incidence of black *Aspergillus* spp. in dry soils and from grapes in dry conditions may result partly from prolonged survival of spores. In fact, *A. carbonarius* grows optimally at 32–35 °C and is resistant to sunlight. In a related study, employing a simulated grape-juice medium, Leong *et al*. (2006b) observed that maximum growth for *A. carbonarius* occurred at about 0.965 a_w and 30 °C, and for *A. niger*, at about 0.98 a_w and 35 °C. In Spain, Belli *et al*. (2004a) found that the fungal infection in grape berries increased with time, reaching a maximum at harvest. A total of 386 isolates of *Aspergillus* section *Nigri* and 10 of *Aspergillus* section *Circumdati* were isolated and tested for their ability to produce OTA. Twenty-one strains produced OTA (18 *Aspergillus* section *Nigri* and three *Aspergillus* section *Circumdati).*

To monitor and quantify *A. carbonarius,* a specific primer pair Ac12RL_OTAF/Ac12RL_OTAR, was designed from the acyltransferase (AT) domain of the polyketide synthase sequence Ac12RL3 to amplify 141 bp by polymerase chain reaction (PCR) (Atoui *et al*., 2007). Among the toxigenic fungi tested, only *A. carbonarius* gave a positive result. This specific primer pair was successfully employed in real-time PCR conjugated with SYBR Green I dye for the direct quantification of this fungus in grape samples. A positive correlation was found between *A. carbonarius* DNA content and OTA concentration in 72 grape samples, allowing for the estimation of the potential risk from OTA contamination.

Bau *et al*. (2006) developed RFLP (restriction fragment length polymorphism) characterization of *A. niger* aggregate species on grapes from Europe and Israel. All of the strains belonging to the *A. niger* aggregate were classified into the two RFLP types previously defined: type N (43%) and type T (57%). Out of the 173 strains of *A. niger* aggregate, 20 produced OTA ($0.1–10.5\,\mu g\,g^{-1}$). All the OTA-producing species belonged to the N-RFLP type.

3.1.2 Producers of OTA from Cereal Grain

OTA was initially obtained from cultures of *A. ochraceus*, isolated from grain sorghum (Van der Merwe *et al*., 1965). In Poland, Czerwiecki *et al*. (2002a; 2002b) studied 200 samples of cereal grain for the presence of OTA and contamination by microscopic fungi. These fungi were classified as *P. cyclopium*, *P. viridicatum*, *A. ochraceus* group, *A. glaucus* and *A. versicolor*. OTA-producing *Penicillium* strains were isolated from 71% of the samples; in 28% of samples, only *Aspergillus* strains (species known to be OTA producers) were noted.

Czaban *et al*. (2006) studied the production of OTA by *P. verrucosum* on wheat grain and wheat-grain barley, respectively. *P. verrucosum* occurs in the cool and temperate climates of Northern Europe and Canada, and is the cause of the formation of OTA in cereal products. Gareis and Gareis (2007) reported that toxigenic *P. nordicum* and *P. verrucosum* produced guttation droplets containing high amounts of OTA and OTB when grown on Czapek yeast extract.

Bogs *et al*. (2006) developed a PCR method for differentiation and detection of the two known OTA-producing species, *P. verrucosum* and *P. nordicum.* It is based upon two genes of the OTA biosynthesis, namely the OTA polyketide synthase gene (otapksPN) and a nonribosomal peptide synthetase gene (otanpsPN) from *P. nordicum.* Both OTA-producing *Penicillia* differ characteristically in the PCR result, making a taxonomic differentiation possible.

3.1.3 Producers of OTA from Coffee

Frank (2001) found *A. niger, A. carbonarius* and *A. ochraceus* (and other *Circumdati* species) on coffee. *A. ochraceus* is the source of OTA contamination in coffee, whereas the commonly occurring *A. niger*, with a low capacity for OTA production, is an important quality

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degrader. Related findings were reported by Bucheli *et al*. (2001), Urbano *et al*. (2001), Suarez-Quiroz *et al*. (2004), Pardo *et al*. (2004) and Kouadio *et al*. (2007a). Recently *Aspergillus westerdijkiae* was also described as a potent OTA producer in coffee beans (Mata *et al*., 2007; Morello *et al*., 2007).

Leong *et al*. (2007) studied the biota of green Vietnamese coffee beans: *A. niger* infected 89% of Robusta beans, whereas *A. carbonarius* and yellow *Aspergilli* each infected 12–14% of beans. OTA was not produced by *A. niger* or *A. ochraceus*, but was detected in 110 of 113 isolates of *A. carbonarius*, 10 isolates of *A. westerdijkiae* and one isolate of *A. steynii*.

Sartori *et al*. (2006) developed a multiplex-PCR for detection of *A. carbonarius, A. niger* and *A. ochraceus* in coffee-bean samples, and succeeded in developing specific primers for *A. niger* detection. The primer designed (OPX7372) provided an amplicon of 372 pb in all *A. niger stricto sensu* isolates. Subsequently a multiplex-PCR method for detection of *A. carbonarius, A. niger* and *A. ochraceus* species in coffee beans was developed.

3.1.4 Producers of OTA from Nuts and Olives

In studies on OTA and the occurrence of ochratoxigenic black *Aspergilli* in stored peanut seeds from Argentina, 43 isolates (27%) of *Aspergillus* section *Nigri,* were found to be ochratoxigenic. The highest percentage of ochratoxigenic strains (57%) was found within the *A. carbonarius* spp. (Magnoli *et al*., 2006). Moroccan olives and olive cake contained strains of *A. niger* which produced OTA (Roussos *et al*., 2006).

3.2 Analytical Methodology for Ochratoxin A

3.2.1 Chromatographic Methods for Ochratoxin A

The present trend in the analysis of mycotoxins is to use IAC as a clean-up and enrichment technique. The OTA in the resulting purified extract is determined by HPLC linked to fluorescence detection or, more recently, to mass spectrometric detection. This methodology has been used for establishing analytical methods for OTA in various matrices at levels consistent with intended EU regulatory limits. Interlaboratory collaborative studies have successfully been completed for OTA in baby food (Burdaspal *et al*., 2001), OTA in currants, raisins, sultanas, mixed dried fruit and dried figs (MacDonald *et al*., 2003) and OTA in green coffee (Vargas *et al*., 2005). Determination of OTA in roasted coffee was achieved with a sequential clean-up of the extract, using first a phenyl silane solid-phase extraction cartridge and then an IAC specific for OTA (Entwisle *et al*., 2001). In a study of three wine samples distributed to 24 laboratories from 17 countries in five continents, 75% of the laboratories used IAC clean-up and HPLC with fluorescence detection (Pascale *et al*., 2000). Only three outliers were found over all the analyses performed, demonstrating the reproducibility of methods for OTA determination in wine. IAC clean-up with HPLC determination has also been used for the determination of OTA in physiological samples, such as urine and blood serum (Yordanova *et al*., 2005). All the above methods have used fluorescence detection, although the purified extracts can also be quantitated by liquid chromatography-mass spectrometry (LC-MS). Chung and Kwong (2007) reported the determination of OTA at ppt levels in cereal products by IAC clean-up and LC-MS. OTA was extracted from cereal products with acetonitrile–water, the extract was diluted with a buffer and cleaned up on an IAC before LC-MS analysis. Two multiple-reaction monitoring (MRM) transitions were used, one for quantification of OTA and one for confirmation of identity. A recent report described the simultaneous determination of OTA, together with aflatoxins, deoxynivalenol, zearalenone, and T-2 and HT-2 toxins in maize by LC-MS/MS after multitoxin IAC clean-up with a new multitoxin IAC containing antibodies for all these mycotoxins (Lattanzio *et al*., 2007). Detection and quantification of the 11 mycotoxins were performed by reversed-phase liquid chromatography coupled with electrospray ionization triple quadrupole mass spectrometry (LC-ESI-MS/MS) using, as chromatographic mobile phase, a linear gradient of methanol/water containing 0.5% acetic acid and 1 mM ammonium acetate. Method performances were quite satisfactory for all tested mycotoxins at contamination levels close to or below the relevant EU maximum permitted or recommended levels. Trucksess *et al*. (2006; 2007) used an IAC with antibodies against OTA and aflatoxin to determine these toxins in ginseng, ginger and other botanical roots, by fluorescence detection with LC-MS confirmation.

Apart from the IAC clean-up methods reviewed above, other extract purification methods are still being investigated. Sugita-Konishi *et al*. (2006a) compared two clean-up procedures, namely a multifunctional column and an IAC, for HPLC determination of OTA in cereals, raisins and green coffee beans. The results suggest that a multifunctional column could be used to detect OTA in wheat and corn grits at a concentration as low as $0.5\,\mu g\,kg^{-1}$; however, it was difficult to detect OTA in green coffee beans and raisins at such a low level. Although an IAC could be used for all the test samples in this study, from a low level to a high level, the recovery rates were lower than with a multifunctional column. In a related study, employing ultra-performance liquid chromatography-MS/MS (UPLC-MS/MS), Ren *et al*. (2007) described the simultaneous determination of multicomponent mycotoxin contaminants in foods

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and feeds. The treatment of the homogenized samples involved extraction, followed by clean-up with multifunctional cartridges, filtration, concentration and secondary filtration. The limit of quatitation (LOQ) of selected analytes ranged from 0.01 to 0.70 $\mu g\,kg^{-1}$. Reinsch *et al*. (2005) reported the use of anion exchange/reversed-phase clean-up for the determination of OTA in mulled and red wine. The substances were analysed by LC-MS/MS. The method was validated with naturally contaminated and spiked wine samples with OTA contents from 1.34 to 3.48 $\mu g\,kg^{-1}$. A clean-up method for beer, which involved zinc acetate and silica solid-phase extraction cartridges, was developed (Medina *et al*., 2006). Another method developed for beer involved clean-up on a polymeric sorbent and determination of both aflatoxins and OTA by UPLC-MS/MS (Aresta *et al*., 2006).

Apart from the above methods, which all involve sample extract clean-up prior to chromatography, certain laboratories have developed methods which utilize the sensitivity of MS detection to avoid clean-up altogether. Timperio *et al*. (2006) developed a rapid and highly sensitive method for detection of OTA in grapes by using nano-reversed-phase high-performance liquid chromatography with electrospray ionization mass spectrometry (nano-RP-HPLC-ESI-MS). This method gives a detection limit in the fg ml^{-1} range, without resorting to IAC clean-up or concentration. The advantage of coupling a direct-injection technique with LC-MS analysis lies in its ability to undertake multitoxin determination of a wide range of toxins. This has been achieved for OTA and other toxins in maize and wheat matrices (Sulyok *et al*., 2006; 2007; Spanjer *et al*., 2008).

3.2.2 Capillary Electrophoresis Methods for Ochratoxin A

Apart from the analytical separation achieved by chromatography, a few papers have described the application of capillary electrophoresis (CE) to the determination of OTA. Koeller *et al*. (2006) compared ELISA and CE with laser-induced fluorescence (LIF) detection in the analysis of OTA in low volumes of human blood serum. Both methods showed an LOD of 0.5 ng ml^{-1}. However, comparing the precisions of both methods, the data show that the quantified concentrations in ELISA are higher than the corresponding concentrations in the CE-LIF method. Hong and Chen (2007) achieved the selective enrichment of OTA using human serum albumin (HSA)-bound magnetic beads as the concentrating probes for OTA in aqueous solutions or in wine. Sensitive detection was achieved using CE coupled with electrospray ionization mass spectrometric (ESI-MS) analysis. This method features speed and cost-effectiveness, making it suitable for the purpose of rapid screening. The lowest detection limit for OTA was approximately 4 $\mu g\,l^{-1}$.

3.2.3 Immunological Methods for Ochratoxin A

Apart from conventional commercial ELISA methods, a number advances have been made in the area of antibody-based rapid techniques for OTA. Sibanda *et al*. (2001) developed a flow-through enzyme immunoassay for the fast screening of green coffee for OTA contamination. Saha *et al*. (2006) applied membrane-based dot immunoassays to mycotoxin analyses and developed a method for homogeneous spotting of antibodies on membranes. This method gave twofold increased sensitivity in a competitive assay of the toxin compared to conventional spotting methods. The detection limit of OTA obtained in wine (1 $\mu g\,l^{-1}$) and coffee (2.5 $\mu g\,kg^{-1}$) is superior to values reported recently. De Saeger *et al*. (2006) developed a rapid antibody-based mycotoxin screening technique incorporating microporous membranes for use at the place of sampling. When using this clean-up tandem-assay column for the detection of OTA in roasted coffee, a cut-off value of 6 $\mu g\,kg^{-1}$ was reached. No false positive results were obtained, however the false negative rate was 8%. An immunosensor based on an optical waveguide lightmode spectroscopy (OWLS) technique was developed for the measurement of AFB_1 and OTA in barley and wheat flour, and the results were in good correlation with those measured by ELISA (Adanyi *et al*., 2007).

A novel quartz crystal microbalance (QCM)-based immunosensor was developed by immobilizing anti-OTA antibodies onto the surface of the 16-mercaptohexadecanoic acid (MHDA)-modified electrode, and allowing competition between free OTA and that conjugated with BSA to occur (Tsai and Hsieh, 2007). The OTA-specific assay exhibited a working range of 50–1000 ng ml^{-1} and a detection limit of 16.1 ng ml^{-1}. Goryacheva *et al*. (2007b) used a rapid all-in-one three-step immunoassay for noninstrumental detection of OTA in high-coloured herbs and spices. The same research group developed a simultaneous detection of AFB_1 and OTA using a clean-up tandem immunoassay column (Goryacheva *et al*., 2007a). The described method was applied to the simultaneous detection of AFB_1 and OTA in spices with cut-off levels at 5 and 10 $\mu g\,kg^{-1}$, respectively. Results were confirmed by LC-MS/MS with IAC clean-up.

3.3 Occurrence of Ochratoxins

Although OTA was discovered in 1964, it was only found in nature in the late 1960s by Shotwell *et al*.

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(1969). OTA is regarded as one of the most commonly occurring mycotoxins, found, not only in cereals (Czerwiecki *et al*., 2002a; Beretta *et al*., 2002), grapes, raisins and wines (Zimmerli and Dick, 1996), in nuts (Magnoli *et al*., 2006; 2007), in beers (Mateo *et al*., 2007), in coffee (Jørgensen, 1998; Pardo *et al*., 2004), in cocoa (Bonvehi, 2004), in spices (Aziz *et al*., 1998), in dried figs (Senyuva *et al*., 2005), in mouldy fruit (Engelhardt *et al*., 1999) and in dairy and meat products of animals (Aish *et al*., 2004; Jørgensen, 1998), but also in human blood and milk. Cereals are considered the major source of human OTA intake, with wine as the second most important source (Miraglia and Brera, 2002). The main regions of concern are Europe and, for some foods, Africa. Occurrence data may vary in a region due to year-to-year variation in climatic conditions, and variation in agricultural and processing practices. A plethora of papers and reviews have appeared on the occurrence of OTA. A selection of the most recent findings will be discussed in this section.

3.3.1 OTA in Human Food

Wheat, barley, oats, maize and rye are highly susceptible to fungal infection and mycotoxin contamination. The EU laid down an MTL of $5.0\,\mu g\,kg^{-1}$ for raw cereals (European Commission, 2006; Food and Agriculture Organization, 2004). In the BEN areas, investigations revealed the mean concentration in maize of positive samples to be $73.4\,\mu g\,kg^{-1}$ (50% incidence) in 1997 (Jurjevic *et al*., 1999). In Spain and Portugal (83 samples including rice, wheat, barley, rye, oats and maize) OTA was detected in 22% of cereal samples, with mean concentrations of 0.93 and $0.64\,\mu g\,kg^{-1}$, respectively, for each country (Juan *et al*., 2008). In Poland, rye from the conventional farms showed a 5.8% OTA contamination and a mean concentration of $1.38\,\mu g\,kg^{-1}$, while rye from ecological farms was contaminated over six times as frequently (37.5%), with a mean concentration of $3.17\,\mu g\,kg^{-1}$ (Czerwiecki *et al*., 2002a). Yet, a study by Biffi *et al*. (2004) of commercial cereal products in the Italian market revealed that all products contained OTA in concentrations below the Italian legal limit of $3\,\mu g\,kg^{-1}$, except four samples of baby food that exceeded the Italian permitted level of $0.5\,\mu g\,kg^{-1}$. Analyses of 338 samples of baby food indicated that 16.8% contained detectable quantities of OTA and 3.4% contained OTA above the Italian permitted value (Beretta *et al*., 2002). OTA was present in 60% of semolina-based baby foods ($0.14–0.65\,\mu g\,kg^{-1}$) and in 80% of multicereal formulas (up to $0.4\,\mu g\,kg^{-1}$).

A survey of the occurrence of aflatoxins, OTA and fumonisins in retail foods in Japan revealed that OTA was present in low levels ($<0.8\,\mu g\,kg^{-1}$) in oatmeal, wheat-flour, rye, buckwheat flour, green and roasted coffee beans, raisins, beer and wine, but not in rice or corn products (Sugita-Konishi *et al*., 2006b). Similarly in Brazil, no OTA could be detected in rice (68 samples analysed) (Simionato *et al*., 2003). However, the incidence of occurrence of OTA in rice was found to be 90%, with an average value of $4.15\,\mu g\,kg^{-1}$ in Morocco (Zinedine *et al*., 2007b). In Korea, where rice is the major contributor (>90%) to the Korean dietary intake, it was found to contain $2.1–6.0\,\mu g\,kg^{-1}$, presenting no considerable risk to the consumer (Park *et al*., 2005).

OTA in beer has its origin mainly from malting barley. OTA occurs in beer with high frequency, but in low levels all over the world (Scott and Kanhere, 1995). In a study in Denmark, a 100% incidence was achieved for 21 samples of beer, with an average concentration of $0.049\,\mu g\,l^{-1}$ (Jørgensen, 1998). Similar results of high incidence, but low levels of OTA were obtained for Belgian beers (Tangni *et al*., 2002). This was again confirmed by a recent survey in which more than 50% of analysed samples do not show detectable levels, that is, $<0.2\,\mu g\,l^{-1}$ by Mateo *et al*. (2007). Yet, in the case of some traditionally brewed beers in South Africa, 34% of traditional beers prepared from the raw ingredients maize and sorghum, contained OTA concentrations of $1.5–2340\,\mu g\,l^{-1}$ (Odhav and Naicker, 2002).

OTA in red wine was first reported in 1995 (Zimmerli and Dick, 1996), followed by numerous surveys throughout the world (Belli *et al*., 2002; Battilani *et al*., 2006; Serra *et al*., 2006). The occurrence of OTA in grapes is normally higher than in wine, since some OTA is removed with the removal of spent fractions during the winemaking (Fernandes *et al*., 2007). Generally there is an increase in occurrence for wines from warmer climates. Levels as high as $15.6\,\mu g\,l^{-1}$ have been reported in Southern Europe (Miraglia and Brera, 2002). Yet, El Khoury *et al*. (2006) reported low values (maximum $0.126\,\mu g\,l^{-1}$) for higher-lying areas along the Mediterranean, such as the Bekaa valley in the Lebanon. Stander and Steyn (2002) reported on a comprehensive study on the occurrence of OTA in South African wines. The levels of OTA of most red and white wines were substantially lower than the permissible levels, however, high levels of OTA were detected in a few noble late harvest wines. For the USA, no quantifiable levels of OTA in wine were reported in some investigations (Ng *et al*., 2004); in general North American wines have lower levels of OTA contamination than European wines (Otteneder and Majerus, 2000). Likewise, in Brazil, a mean concentration of $0.0344\,\mu g\,l^{-1}$ was detected in 28.75% of positive samples from 80 samples analysed (Rosa *et al*., 2004) and in Argentina and Chile no OTA could be detected on the 2002 and 2003 markets (Pacin *et al*., 2005). OTA levels generally decrease in the order red wine, rosé and white wine, and usually dessert wines have higher levels than dry wines (Shephard *et al*., 2003; Finoli *et al*., 2004; Rosa *et al*., 2004). OTA has also been detected in low levels in Australian wines (concentration range $<0.05–0.62\,\mu g\,l^{-1}$), but here no significant difference was observed between red and

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white wines (Hocking *et al*., 2003). Similarly Stefanaki *et al*. (2003) found that the OTA concentration in red dry wines did not differ significantly from that found in white and rosé wines in Greece. A survey of the presence of OTA in sweet wines produced in Spain and other countries showed the highest levels for Spain, France and Greece with mean concentrations of 0.645, 0.212 and 0.833 $\mu g\,l^{-1}$, respectively (Burdaspal and Legarda, 2007). The highest OTA concentration reported for dessert wine was 15.25 $\mu g\,l^{-1}$ (Belli *et al*., 2004b). In a study of wine and grape juice on the Polish market, it was found that 92% of the red wine samples contained 0.0022–6.710 $\mu g\,l^{-1}$ OTA, while 100% of the juice samples contained 0.0016–0.0647 $\mu g\,l^{-1}$ (Czerwiecki *et al*., 2005). The EU laid down an MTL of 2.0 $\mu g\,l^{-1}$ for wine, but no levels are set for wines with alcohol strength of more than 15% vol.

Dried fruit is a further source of OTA in human diets (Engelhardt *et al*., 1999; MacDonald *et al*., 2003). OTA has been found in much higher concentrations in Europe (50–70 $\mu g\,kg^{-1}$) in dried vine fruit than in wine or grape juice (Battilani *et al*., 2006; Miraglia and Brera, 2002). Yet, the average OTA contamination of dried vine fruits in the USA during 1998–1999 was reported as 1.27 $\mu g\,kg^{-1}$. It was reported that 79% of samples of raisins and 59% of sultanas from the Canadian retail market had OTA levels above the limit of quantification (Lombaert *et al*., 2004), which was comparable with incidence rates reported in a Greek survey (Stefanaki *et al*., 2003). The incidence of OTA on unprocessed sultanas from Turkey was reported as 68% with a mean concentration of 3.4 $\mu g\,kg^{-1}$ (Meyvaci *et al*., 2005), while an incidence of 90.7% and a mean concentration of 1.36 $\mu g\,kg^{-1}$ were reported for processed sultanas in Turkey (Aksoy *et al*., 2007). In Morocco, the incidence of occurrence of OTA in raisins and dried figs was found to be 30% and 65% with average values for positive samples of 0.96 and 0.33 $\mu g\,kg^{-1}$, respectively (Zinedine *et al*., 2007b). In a study over two years an incidence of 14–15% (>0.5 $\mu g\,kg^{-1}$) of occurrence of OTA was found in dried figs in Turkey (Senyuva *et al*., 2005). The incidence of contamination of dried fruit is influenced by factors such as the suitability of the fruit as a substrate and the possibility of fungal infection of the fruit, as well as the harvesting and drying conditions (Drusch and Ragab, 2003).

OTA contamination of green and processed coffee has been documented since the 1970s (Levi *et al*., 1974). Romani *et al*. (2000) showed that 65% of samples of green coffee beans from various countries tested positive for OTA, with concentrations up to 48 $\mu g\,kg^{-1}$. In a recent study, coffee and corresponding byproducts from seven different geographic regions were investigated (Napolitano *et al*., 2007). Costa Rica and Indian green coffees were the most contaminated samples, with mean concentrations of 13 and 11 $\mu g\,kg^{-1}$, respectively, while Ethiopian coffee was the least contaminated, with 3.8 $\mu g\,kg^{-1}$ of OTA. No significant differences in OTA occurrence between the two main varieties Arabica and Robusta could be observed. The hypothesis is that roasting and processing of coffee reduce the levels of OTA in the final product (Bayman and Baker, 2006).

Only a few studies have evaluated OTA in cocoa products. The highest levels of OTA in 170 samples from different geographical origins were detected in roasted cocoa shell, raw cocoa shell, cocoa cake and cocoa powder with mean concentrations of 11, 2.79, 2.60 and 2.41 $\mu g\,kg^{-1}$, respectively (Bonvehi, 2004). The occurrence of OTA in 18 pure cocoa powder samples purchased from supermarkets, organic product shops and retail shops in Italy was investigated (Tafuri *et al*., 2004). Nine samples were positive and had concentrations ranging between 0.22 and 0.77 $\mu g\,kg^{-1}$ with a mean of 0.43 $\mu g\,kg^{-1}$. Of 14 conventional samples analysed, four samples were above the suggested legal limit (0.5 $\mu g\,kg^{-1}$); this provided a cause for concern, as cocoa is widely used as an ingredient in children's snack foods.

Few surveys of the natural occurrence of OTA in stored peanuts have been reported. In a recent study in Argentina, OTA was found in 60% of the peanut samples, with mean levels ranging from 5.6 to 130 $\mu g\,kg^{-1}$ (Magnoli *et al*., 2006; 2007). In Morocco, the incidence of occurrence of OTA in walnuts and peanuts was found to be 35% and 25%, respectively, with average values for positive samples of 0.11 and 0.68 $\mu g\,kg^{-1}$ (Zinedine *et al*., 2007b).

The occurrence of OTA in olive oil has drawn attention due to its importance as a fat source in the Mediterranean diet. Preliminary data on the presence of OTA in black table olives from Moroccan origin revealed a mean of 0.60 $\mu g\,kg^{-1}$ for seven contaminated olive samples from 10 samples taken from supermarkets and retailers (El Adlouni *et al*., 2006). It was claimed that contaminated black olives could contribute significantly to the total daily intake of OTA. In another study, the occurrence of aflatoxins and OTA was determined in 28 samples of Sicilian extra virgin olive oil produced from olives from traditional and biological agriculture, as well as in 45 samples of green and black olives. (Finoli *et al*., 2005). The OTA levels were 0.052–0.244 $\mu g\,kg^{-1}$ for oil and 0.101–8.391 $\mu g\,kg^{-1}$ for olives. Ferracane *et al*. (2007) found labelled olive oil to be less contaminated by OTA than unlabelled olive oil (mean values of 0.66 $\mu g\,kg^{-1}$ and 2.47 $\mu g\,kg^{-1}$, respectively).

OTA is a frequent contaminant in spices, as reported by Scheuer and Gareis (2002) in a survey of 681 samples of 50 different spice commodities from the German retail market. On comparing the regions of origin of these spices, higher levels of contamination were found in spices from Southeast Asia than from America, Africa, Europe and Near Asia. The highest frequency of occurrence was found for chilli (100%) and pepper (23%) from Southeast Asia and India, and for paprika (23%) from Israel.

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3.3.2 OTA in Animal Feed and Animal Products

The presence of OTA in animal feeds raised concern for the livestock industry. The occurrence of mycotoxins in animal feeds and feed ingredients in relation to the regions of their sourcing has been reported by Binder *et al*. (2007). Of the 1507 samples from European and Mediterranean markets and 1291 samples from the Asia-Pacific region, 52% and 30%, respectively, tested positive for *Fusarium* mycotoxins and the non-*Fusarium* toxins, OTA and AFB_1. OTA was found to be a minor contaminant when compared with deoxynivalenol, zearalenone, T-2 toxin, fumonisins and aflatoxins.

OTA is found in dairy products and the meat of animals consuming OTA-contaminated grains, that is, cow's milk, pork and poultry (Boudra *et al*., 2007). In a survey in Denmark of pork and poultry meat and liver, the mean values ranged from 0.02 to 0.11 $\mu g\,kg^{-1}$ (Jørgensen, 1998). Compared to cereal and cereal products the contribution from pork and poultry by the Danish population is considered of less importance. Samples of organic cow's milk, conventional cow's milk and cow's milk-based infant formulas were analysed for the occurrence of OTA (Skaug, 1999). OTA was detected in 6 of 40 conventional cow's milk samples (11–58 $ng\,l^{-1}$), and in 5 out of 47 organic milk samples (15–28 $ng\,l^{-1}$). No OTA was detected in any of the 20 infant formula samples. The OTA levels in cow's milk found in this investigation are sufficient to cause a higher intake of OTA than the suggested tolerable daily intake (TDI).

3.3.3 OTA in Human Blood and Milk

The possibility for maternal milk to be contaminated exists when mothers are exposed to OTA in food. A study was carried out on lactating women in Northern Italy in which OTA was detected in 85.7% of the milk samples with a mean value of 6.01 $ng\,l^{-1}$ (Turconi *et al*., 2004). The results of this work indicated that a high percentage of babies on maternal milk were found to be exposed to mycotoxin levels greater than the TDI value of 0.2 ng (kg body weight)$^{-1}$. Elevated concentrations of OTA are often found in the sera of people exposed to this mycotoxin. In a study of patients affected with renal diseases Grosso *et al*. (2003) found that the average OTA concentration for patients with urinary tract disease (excluding cancer patients) was $0.99 \pm 1.28\,\mu g\,l^{-1}$, while that for the non-nephropathic patients was $0.53 \pm 1.00\,\mu g\,l^{-1}$.

3.4 Biological Activity of Ochratoxins

OTA exhibits many adverse toxicological effects, including renal and hepatic toxicity, mutagenicity, carcinogenicity, teratogenicity, immunotoxicity and neurotoxicity. It is linked to the aetiology of DPN and poultry mycotoxicoses. OTA is relevant to human health and implied in BEN and urinary system tumours in North Africa (Golian, 2000; Mantle, 2002; Gekle *et al*., 2002; Fuchs and Peraica, 2005; O'Brien and Dietrich, 2005; Castegnaro *et al*., 2006a; Ringot *et al*., 2006; Tanaka and Yoshizawa, 2006; Voice *et al*., 2006; Pfohl-Leszkowicz and Manderville, 2007; Pfohl-Leszkowicz *et al*., 2007). Based on the ground-breaking research of Krogh and coworkers, OTA became accepted as the cause of DPN (Krogh, 1978). These findings strongly influenced the association of OTA with BEN.

In a study of German food and its exposure to consumers, OTA was found in 57.2% of the food samples and in 98% of the blood samples of consumers (Gareis *et al*., 2001). Contamination of food by OTA occurs primarily during preharvest periods (Al-Anati and Petzinger, 2006). (see Section 3.3 for the worldwide occurrence of OTA in commodities). Studies on the effect of OTA in human proximal tubule cells and human fibroblasts in primary culture (Schwerdt *et al*., 2007) suggested that consumption of OTA is almost unavoidable, leading to the detection of low blood concentrations, previously considered harmless, which impacted on the reassessment of the risk of OTA consumption. OTA has a half-life of about 35.5 days in humans, 21 days in monkeys, 72–120 hours in pigs and 40 hours in mice (O'Brien and Dietrich, 2005). Mantle (2008) interpreted the pharmacokinetics of OTA in blood plasma of rats, during and after acute or chronic ingestion. Based on animal and epidemiological studies, OTA has been classified by the International Agency for Research on Cancer (2002) as a Group 2 B carcinogen (possibly carcinogenic to humans). Maximum permissible OTA concentrations of 5 and 3 $\mu g\,kg^{-1}$ in raw cereals and processed cereal products, respectively, were reported by O'Brien and Dietrich (2005). Although carcinogenic effects of OTA have been demonstrated only in laboratory animals until now, several European countries have enacted or proposed regulations for levels of OTA in food. This has prompted adoption of regulatory limits in several countries (Monaci and Palmisano, 2004).

3.4.1 Toxicological Effects

In monogastric species, OTA is highly bioavailable with 40–60% of an oral dose being absorbed from the gastrointestinal tract. Due to extraordinary high plasma-protein binding, elimination of OTA is a rather slow process and high concentrations are found in blood, kidneys and the liver. Different from monogastric species, ruminants may tolerate larger amounts of OTA due to hydrolysis to the less toxic metabolite ochratoxin α by micro-organisms within the forestomach contents (Blank *et al*., 1999).

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Several mechanisms have been proposed to be involved in the toxicity of OTA: inhibition of protein synthesis, promotion of membrane peroxidation, disruption of calcium homeostasis, inhibition of mitochondrial respiration, apoptosis and DNA damage (O'Brien and Dietrich, 2005; Ringot *et al*., 2006). The contribution of metabolites of OTA in its genotoxicity and carcinogenicity is still unclear (See Section 3.4.4). More recent studies are focussed on the OTA ability to disrupt cellular signalling pathways thereby influencing cell viability and cell growth parameters. The effects of OTA in the nanomolar range and its interactions with cellular signalling networks in different renal cells proposing OTA to act as a signal modulator have been recently reviewed (Gekle *et al*., 2005). Several nephrotoxicants and renal carcinogens have been shown to alter cell–cell signalling by interference with gap junction intercellular communication (GJIC) and/or cell adhesion. It was therefore important to investigate if the disruption of cell–cell interactions occurs in kidney epithelial cells in response to OTA treatment. A study in kidney epithelial cells (MDCK) utilizing the scrape-load/dye-transfer assay suggested that altered cell adhesion and GJIC and cell–cell signalling may contribute to OTA toxicity and carcinogenicity (Mally *et al*., 2006).

When exposed to different concentrations of OTA for 24 hours, apoptotic and necrotic renal cell death was induced in MDCK, porcine renal epithelial cells (LLC-PK1) and RK-13 renal cells in a cell type- and concentration-dependent manner (Petrik *et al*., 2005). Apparently, MDCK and LLC-PK1 cells are more sensitive to OTA than RK-13 cells. Repeated exposure of renal epithelial cells to OTA or OTB provided a subpopulation of cells with reduced ochratoxin-sensitivity and alterations in growth characteristics (Heussner *et al*., 2007).

Investigations of the biotransformation and nephrotoxicity of OTB in rats showed that, in contrast to OTA, no tissue-specific retention of OTB was evident after single and repeated administration (Mally *et al*., 2005). These data suggest that OTA and OTB have a similar potential to induce cytotoxicity *in vitro*, but large differences occur in their potential to induce nephrotoxicity in rodents. OTB is also more extensively metabolized and more rapidly eliminated than OTA, which could explain differences in toxicokinetics and lower nephrotoxicity. The synergistic effects of OTA, OTB, citrinin (CIT) and PAT were investigated *in vitro*, using the well-established porcine renal cell line LLC-PK1 and the MTT reduction test as a cytotoxicity end point (Heussner *et al*., 2006). The results confirmed a potential for interactive effects of CIT and OTA and possibly other mycotoxins in cells of renal origin. The combined treatment with fumonisin B_1 (FB_1), beauvericin and OTA resulted mostly in additive effects, especially after a 24-hour exposure, although synergistic, as well as antagonistic interactions, could not be excluded, depending on toxin concentrations and time of exposure (Klaric *et al*., 2007).

The immunomodulatory effects of OTA and OTC on the human monocyte/macrophage line THP-1 were investigated (Muller *et al*., 2003). Metabolic activity, cell proliferation, cell membrane integrity, cell differentiation, phagocytic behaviour, nitrogen-oxide synthesis and cell surface markers were largely suppressed by OTA and OTC at concentrations ranging between 10 and 1000 ng ml^{-1}.

3.4.2 OTA as a Liver Toxin

Although no hepatic disease is correlated with OTA consumption, the adverse toxic effects in the liver of experimental animals are highly relevant. The overall early effects of chronic OTA treatment on rat liver, analysing different aspects related to: (i) fibrosis; (ii) oxidative stress and stress response and (iii) a possible tumour-promoting effect have been investigated (Gagliano *et al*., 2006). These *in vivo* results showed that OTA-induced liver injury involved a reduction in the ability to counterbalance oxidative stress, presumably leading to altered GJIC and loss of cell adhesion and polarity. These findings suggest that mild oxidative damage might be a key factor, in combination with other cytotoxic effects, in triggering the promotion of liver tumours after exposure to OTA.

The underlying mechanisms of OTA-induced cellular toxicity in liver (HepG2) cells were investigated by determining the impact of serum concentration, dietary antioxidants and glutathione-modulating compounds (Bosch-Saadatmandi *et al*., 2006). It has been suggested that oxidative damage contributes to its cytotoxic effects, therefore, dietary antioxidants, such as vitamin E and polyphenols, may counteract OTA-induced cell death. The effects of serum concentrations, as well as different dietary antioxidants on the viability of OTA-exposed liver (HepG2) cells showed that the cytotoxicity was strongly dependent on the protein concentration in the cell culture medium and was not prevented by preincubation with dietary antioxidants (Bosch-Saadatmandi *et al*., 2006). Although cellular GSH levels are influenced by OTA incubation, mechanisms other than oxidative stress are likely to be involved in OTA-induced cell death in HepG2 cells.

3.4.3 OTA as a Teratogenic Toxin

OTA is teratogenic in the rat, mouse, hamster and chick, with reduced birth weight and craniofacial abnormalities being the most commonly observed malformations (O'Brien *et al*., 2005). Neither the potential of OTA to cause malformations in humans nor its teratogenic mode of action is known. Using the standard American Society for Testing and Materials (ASTM) 96 h exposure protocol and the FETAX (Frog Embryo Teratogenesis Assay Xenopus) system, an embryotoxicity assay

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system, with a high correlation to animal models and epidemiological data, the effect of OTA and OTB on the *in vitro* development of *Xenopus laevis* embryos was assessed. Both OTA and OTB caused craniofacial malformations, while OTA also caused reduced embryo growth. As expected, OTA was far more potent in inducing these effects than OTB, presumably, as discussed above, owing to greater accumulation of OTA. The critical gestation period for inducing teratogenicity in pregnant Wistar rats is between six and seven days and the minimum single oral dose of OTA was 2.75 mg (kg body weight)$^{-1}$ (Patil *et al*., 2006).

The teratogenic effects of the simultaneous prenatal oral exposure of Wistar rats on days 6–15 of gestation to OTA and AFB_1 were investigated (Wangikar *et al*., 2004). OTA and AFB_1 alone and in combination caused various gross skeletal and visceral anomalies. The occurrence was considerably less pronounced in foetuses of AFB_1 and combination groups as compared with those of OTA group foetuses. However, new manifestations, such as gastroschisis and syndactyly were observed and the incidence of cardiac defects was increased in foetuses due to the combined treatment. The results indicated reduced teratogenic activity of OTA in the presence of AFB_1.

3.4.4 OTA Nephrotoxicity and Its Role in Balkan Endemic Nephropathy

OTA is nephrotoxic to all animal species tested, therefore, it is not surprising that the kidneys are most susceptible to OTA-induced toxicity. The renal lesions associated with the diseases include degeneration of the proximal tubules, interstitial fibrosis in the renal cortex, hyalinization of the glomeruli and atrophy in the tubular epithelium (Steyn and Stander, 1999).

In 1964, the WHO provided a description of Balkan endemic nephropathy (BEN), and the endemic kidney disease became recognized as a new nosological entity. BEN is geographically linked to rural villages in Bosnia, Bulgaria, Croatia, Romania and Serbia, and is considered by several authors to be associated with OTA, the environmental mycotoxin. The clinical features and pathomorphological changes of BEN have been described (Pfohl-Leszkowicz and Manderville, 2007 and references cited therein). Early histopathological changes of BEN are characterized by tubular and degenerative and regenerative processes of epithelial cells, without reduction in organ size. In chronic cases, the size of the kidneys is considerably reduced and diffuse interstitial fibrosis without inflammatory cells is the most prominent finding. The three affected countries are Bulgaria, Croatia and Romania and the association between BEN and urinary tract tumours (UTT) has been highlighted. The clustering of BEN to specific families living in these rural areas suggests that a genetic predisposition may be involved. Such a genetic factor for BEN could be due to genes located in the region between 3q25 and 3q26 (Toncheva *et al*., 1998).

In countries such as Bulgaria, a much higher prevalence of OTA, exceeding 2 μg l^{-1} in the blood of affected population, was observed (Castegnaro *et al*., 2006a). To quantify human exposure to OTA in the Vratza district, Bulgaria, OTA intake was monitored for one month by examining the OTA in blood and urine from healthy volunteers, from two villages with high risk for BEN (Pfohl-Leszkowicz *et al*., 2007). Food samples were collected daily, blood and urine at the beginning of each week. The average weekly intake of OTA varied from 1.9 to 206 ng (kg body weight)$^{-1}$, twice the tolerable weekly intake recommended by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), with OTA blood concentrations reaching 10 μg l^{-1}. Biomarkers of biological effects such as DNA adducts were detected in patients affected by UTT, similarly detected in rat studies. It was concluded that all these data support a causative role of OTA in BEN and UTT. DNA adduct formation and persistence of OTA/CIT and aristolochic acids (AA) adducts in human kidney cell culture were compared to DNA adducts in several tumours from human kidney suspected to be exposed to either OTA and CIT, or AA. In kidney cell cultures, the formation of specific OTA–DNA and AA–DNA adducts was detected in the same range (around 10 adducts/109 nucleotides) and were time- and dose-dependent. DNA adducts related to the mycotoxins, OTA and CIT were found in human kidney tissues from the Balkans, France and Belgium, whereas no DNA adducts related to AA could be found in any tumours of BEN patients from Croatia, Bulgaria or Serbia. OTA and CIT were more frequently found in rural areas, whereas AA was never detected. The findings of this study were in support of those reported previously (Castegnaro *et al*., 2006a). Providing further evidence for the involvement of mycotoxins, especially OTA, in BEN and UTT.

However, it was recently reported that the pathologic lesions in kidneys of rats treated with OTA appear rather differently from the clinical and pathologic characteristics of endemic nephropathy (Mally *et al*., 2007). Moreover, increasing evidence suggests that OTA does not bind to DNA, but induces tumours by an epigenetic route, implying a threshold mechanism by which doses below a certain level will induce no adverse health effects. Based on food consumption data and OTA serum concentrations, it appears that human exposure, even in areas with relatively high dietary exposure to OTA, is several orders of magnitude below doses known to cause nephrotoxicity and tumour formation in laboratory animals. These observations suggest that OTA is not likely to be an aetiologic factor involved in BEN and indicate a need to search for new clues towards the aetiology of this endemic kidney disease.

Worldwide, several studies have been conducted to assess the potential role of OTA in the aetiology of

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BEN and CIN. The occurrence of OTA in human sera from patients in Tunisia was reported (Grosso *et al*., 2003). In cases where the high incidence of CIN of unknown aetiology were pointed out, the involvement of OTA was suspected, but findings on the rather low degree of exposure failed to provide sufficient proof for its causative role. In Portugal, the levels of OTA in haemodialysis patients in Coimbra were higher than in Aveiro (Dinis *et al*., 2007). In the Pomeranian region in Poland, OTA was determined in human serum and kidney samples from a healthy group and from patients after nephrectomy. The mean concentration of OTA in the healthy group was $0.37\,\mathrm{ng\,ml^{-1}}$. In patients subjected to nephrectomy it reached $1.06\,\mathrm{ng\,ml^{-1}}$ in men and $0.94\,\mathrm{ng\,ml^{-1}}$ in women. These results support the role of OTA in kidney diseases (Grajewski *et al*., 2007).

3.4.5 Mechanisms in OTA Genotoxicity and Carcinogenicity

The genotoxic status of OTA is still controversial because contradictory results were obtained in various microbial and mammalian tests, notably regarding the formation of DNA adducts. It is of importance to note that the genotoxicity of OTA was originally established (Knasmueller *et al*., 2004), whereas under identical conditions OTB was devoid of genotoxic activity, although it caused pronounced inhibition of cell division, even at doses lower than OTA. Evidence of OTA genotoxicity was derived from the sensitive ^{32}P-labelling assay. OTA facilitates guanine-specific DNA adducts *in vitro*, and in rat and pig kidney when dosed orally. One adduct comigrates with a synthetic C-bonded C8-dG OTA-adduct standard. Like other chlorinated phenols, OTA undergoes an oxidative dechlorination process to generate a quinone (OTQ)/hydroquinone (OTHQ) redox couple that may play a role in OTA-mediated genotoxicity. To determine whether the OTQ/OTHQ redox couple of OTA contributes to genotoxicity, the DNA adduction properties of OTHQ were compared to OTA in the absence and presence of metabolic activation (pig kidney microsomes) utilizing human bronchial epithelial (WI26) and human kidney (HK2) cells (Tozlovanu *et al*., 2006). OTHQ generates DNA-adduct spots in the absence of metabolic activation. These results establish structure–activity relationships for OTA-mediated DNA adduction and provide new evidence for the potential role of the OTQ/OTHQ redox couple in OTA-induced genotoxicity.

The genotoxicity of OTA was investigated in primary human urothelial cells by means of alkaline single-cell gel electrophoresis (Comet assay), indicating great interindividual differences (Lebrun *et al*., 2006). To evaluate these differences, the influence of the genotype of the isoenzymes of GST, namely GSTT1, GSTM1 and GSTP1 on the genotoxic potential of OTA was examined. It was concluded that genetic predisposition has the potential to influence OTA genotoxicity. The genotoxic potential of OTA in the kidney of adult female Wistar rats was studied using the Comet assay, monitoring the tail length, tail intensity and tail moment. Changes in the kidney tissue in all the OTA treated groups were significantly higher than in controls ($p < 0.05$) thereby confirming the genotoxic potential of OTA (Zeljezic *et al*., 2006). The severity of DNA lesions in kidneys also correlated with OTA concentration. These results were in agreement with the findings on the genotoxicity of OTA in primary human urothelial cells (Lebrun *et al*., 2006). In the leading review, the current understanding of OTA toxicity and carcinogenicity (Pfohl-Leszkowicz *et al*., 2007) is debated. The genotoxic activity could play a central role in OTA-mediated carcinogenesis and may be divided into direct (covalent DNA adduction) and indirect (oxidative DNA damage) mechanisms of action. The available evidence suggests that OTA is a genotoxic carcinogen by induction of oxidative DNA lesions coupled with direct DNA adducts via OTQ formation. In an earlier paper the molecular details of the binding of OTA to HSA, and the affect on the transport across epithelial cell membranes by organic anion transport proteins, the oxidative activation of OTA and the formation of OTA adducts with biological molecules were investigated (Dai *et al*., 2004). These studies contributed to a better understanding of a detailed chemical model for the transport, accumulation, and genotoxic and carcinogenic effects of OTA.

A recent study showed that OTA inhibits cell proliferation, strongly reduces protein synthesis and induces a decrease of GSH in a concentration-dependent manner in both HepG2 and Vero cells (Hassen *et al*., 2007). In a subsequent study, the reduction of Nrf2-regulated gene expression resulting in oxidative DNA damage was evidenced by formation of abasic sites *in vitro* and confirmed in kidney *in vivo* (Cavin *et al*., 2007). All OTA-mediated effects observed were prevented by pretreatment of cell cultures with inducers of Nrf2 activity. These data suggest that reduction of cellular defence against oxidative stress by Nrf2 inhibition may be a plausible mechanism of OTA nephrotoxicity and carcinogenicity.

Although the mechanisms of the genotoxicity and carcinogenicity of OTA have been thoroughly studied, many controversial findings have been reported. The ability of OTA to produce single-strand DNA breaks and oxidative DNA damage in the human renal proximal tubular epithelial cell line (HK2) was evaluated (Arbillaga *et al*., 2007). The authors also attempted to determine if biotransformation processes mediate OTA genotoxicity. Single-cell gel electrophoresis assays were performed after three and six hour treatments using different OTA concentrations, both cytotoxic and noncytotoxic doses, in order to distinguish the apparent genotoxic effect from an indirect effect derived from its general cellular toxicity. No effect was shown in the

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absence of cytotoxicity, both in the presence and in the absence of metabolic activation, suggesting that OTA is not acting as a direct genotoxic carcinogen and that oxidative stress is likely to be responsible for the genotoxicity and cytotoxicity observed in human renal cells. A similar study involving single-cell gel electrophoresis investigated the genotoxic potency of OTA and its metabolites in NIH/3 T3 cells stably expressing the human cytochrome P450 isoenzymes CYP 2C9 and CYP 3A4 (Simarro *et al*., 2006). Their results confirmed the hypothesis that biotransformation processes mediated OTA toxicity and affected the genotoxicity. Measurement of the production of reactive oxygen species (ROS) showed that the OTA-induced ROS production corresponded to the OTA-induced genotoxicity.

The DNA ploidy distribution, measured in experimental renal tumours, that occurred in 12 ageing male Fischer rats in response to chronic dietary OTA exposure, was diploid in all renal adenomas and aneuploid in all carcinomas, correlating with their typical organized and disorganized histopathology, respectively (Brown *et al*., 2007). Thus, an experimental rat renal carcinoma could arise within an adenoma directly from certain persistent karyomegalic tubular epithelial cells, long after their particular genetic damage has been caused during a protracted period of OTA insult. A marked change in DNA ploidy over the adenoma–carcinoma continuum in OTA-generated rat renal tumours was observed. No case of testicular cancer occurred in over 250 male rats given dietary OTA at renal tumourigenic doses; these findings refuted the suggestion by (Schwartz, 2002) that OTA could be the cause of testicular cancer in young men.

A study using a toxicogenomics approach to identify new plausible epigenetic mechanisms of OTA carcinogenicity was conducted in male Fischer rats for two years (Marin-Kuan *et al*., 2007). Renal tumours were discovered during the last six months of the study, and the total tumour incidence reached 25% at the end of the study while gene expression profile was analysed in groups of animals taken in intervals from 7 days to 12 months. Tissue-specific responses were observed in kidney versus liver. In the kidney, several genes known as markers of kidney injury and cell regeneration were significantly modulated by OTA. The expression of genes known to be involved in DNA synthesis and repair, or genes induced as a result of DNA damage, was only marginally modulated. Another study found very little or no effect amongst genes associated with apoptosis (Marin-Kuan *et al*., 2007). The depletion of these genes is likely to impair the defence potential of the cells, resulting in chronic elevation of oxidative stress in the kidney. The inhibition of the defence mechanism appears to be a highly plausible new mechanism which could contribute to OTA carcinogenicity. OTA-induced mutagenesis was also investigated in mammalian cells and mutations characterized as point mutations (48%) and a lack of a detectable reverse-transcription polymerase chain reaction product (52%) (Palma *et al*., 2007). The pattern of OTA-induced point mutations was similar to that of spontaneous mutants, suggesting that OTA induced an increase of the endogenous oxidative metabolites, but not covalent DNA adducts. These data support a model where OTA is mutagenic via oxidative DNA damage induction.

3.5 Decontamination of Ochratoxins

OTA, the dangerous and ubiquitous mycotoxin, is frequently found in different foods, as well as in beverages such as grape juices, and wines; see Section 3.3 in this chapter. The inactivation of mycotoxins by enzymes or by selected micro-organisms is an attractive strategy for the decontamination of agricultural commodities without significant losses in nutritive value and the palatability of the detoxified feed or food (Bata and Lasztity, 1999). Several of these approaches are described in this section.

The ochratoxins contain an amide bond, therefore, it should be prone to hydrolysis by hydrolases to its nontoxic components OTα and β-phenylalanine. Stander *et al*. (2000) screened 23 lipases and esterases for their ability to hydrolyse OTA and found that only a lipase preparation from *A. niger* degraded OTA. It is very likely that proteases or amidases are responsible for the hydrolytic activity observed with the crude lipase preparation. Stander *et al*. (2001) subsequently undertook a kinetic study into the hydrolysis of the ochratoxins and their analogues by carboxypeptidase and found that the halogen-containing toxins were much slower to hydrolyse than the analogues without the halogen group, such as OTB. Abrunhosa *et al*. (2006) reported the ability of several commercial proteases to hydrolyse OTA into OTα. Upon an incubation period of 25 hours at pH 7.5, significant hydrolytic activities for protease A (87.3%) and for pancreatin (43.4%) were detected.

Bejaoui *et al*. (2006a) studied the biodegradation of OTA by *A*. section *nigri* species isolated from French grapes in order to reduce OTA in grape juices and musts. Forty isolates of the *Aspergilli* species from French grapes, namely, *A. carbonarius, A. niger aggregate* and *A. japonicus* were assessed for their capacity to degrade OTA in Czapek yeast extract broth (CYB) and in a synthetic grape juice medium (SGM) contaminated with OTA at $2\,mg\,l^{-1}$ (5 µM). In both media these fungi had the ability to degrade OTA to OTα. In SGM and CYB, 77 and 45% of the isolates, respectively, were able to degrade more than 80% of the OTA. *A. niger* was regarded as the most effective species for OTA detoxification processes, followed by *A. japonicus*. These results are consistent with the findings of Stander *et al*. (2000). Abrunhosa and Venancio (2007) isolated and

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purified a metalloenzyme hydrolysing OTA from *A. niger* leading to OTα and L-β-phenylalanine, based on the known capacity of *A. niger* to hydrolyse OTA. The OTA hydrolytic enzyme presented a V_{max} of 0.44 μM min^{-1} and a K_m of 0.5 mM when the reaction was carried out at pH 7.5 and 37 °C. The 33.9 megabase genome of *A. niger* CBS 513.88, the ancestor of currently used enzyme production strains was recently reported in *Nature* by Pel *et al*. (2007). Noteworthy are the large number of major facilitator superfamily transporters and fungal zinc binuclear cluster transcription factors, and the presence of putative gene clusters for fumonisin and OTA synthesis. A reconstructed metabolic network comprising 1069 unique reactions illustrates the versatile metabolism of *A. niger* (Pel *et al*., 2007).

Varga *et al*. (2005) examined several filamentous fungi representing the genera Rhizopus and Mucor for their ability to degrade OTA, AFB1, zearalenone and PAT in a liquid medium. OTA was successfully degraded by *Rhizopus stolonifer*, *R. microsporus*, *R. homothallicus* and two *R. oryzae* isolates, and by four unidentified *Rhizopus* isolates. The *Rhizopus* isolates were able to degrade more than 95% of OTA within 16 days, furthermore an isolate of *Rhizopus stolonifer* effectively decomposed OTA on moistened wheat.

In their studies on the fate of OTA during vinification of grapes, Leong *et al*. (2006d) observed a reduction in the amount of OTA in juice and wine at every solid–liquid separation stage. The OTA concentration (μg kg^{-1}) in white and red wine after racking was 4 and 9%, respectively, of that in crushed grapes. The OTA content was divided between solid and liquid phases at each stage of vinification. OTA did not appear to be transformed either chemically or biologically by yeast during fermentation. However, it was discarded with the marc, juice lees and gross lees. Fernandes *et al*. (2007) similarly investigated changes in OTA levels during the making of the Portuguese wine, Vinho Verde. It was found that vinification consistently reduced the levels of OTA, independent of the initial OTA concentrations in the grapes used. The reduction in OTA was associated with the removal of spent fractions during the wine making rather than degradation by yeasts or bacteria. The findings of Fernandes *et al*. (2007) were confirmed by Del Prete *et al*. (2007) who observed that OTA was not degraded by cell-free extracts of wine lactic acid bacteria (LAB). In fact, no degradation products of OTA were detected in the bacterial pellets, therefore the removal of OTA by wine LAB was a cell-binding phenomenon. Shetty and Jespersen (2006) reviewed the mycotoxin-binding capacity of *S. cerevisiae* and LAB, and concluded that their strains have prospects as mycotoxin-binding organisms. More importantly, *S. cerevisiae* and LAB are the major micro-organisms involved in food fermentations in tropical countries with high levels of mycotoxin contamination.

Angioni *et al*. (2007) reported the *in vitro* interaction during alcoholic fermentation between OTA and 20 different strains of *S. cerevisiae* and *Kloeckera apiculata*. The yeast cells do not adsorb OTA and, for all yeasts, OTA levels did not affect the alcoholic fermentation. Only some yeast strains reduced the levels of OTA, thereby demonstrating that the reduction of OTA levels to genus or species characteristic, but a strain trait. Cecchini *et al*. (2006) studied the influence of the yeast strains on OTA content during the fermentation of red and white wines. Depending on the yeast strain involved in the fermentation, there was a difference in the OTA levels in the wines. The absence of degradation products indicated an adsorption mechanism.

Bejaoui *et al*. (2004) assessed the efficiency of removing OTA from yeast peptone glucose (YPG), SGM and natural grape juice by viable and dead (heat and acid-treated) oenological *Saccharomyces* strains (five *S. cerevisiae* and one *S. bayanus*) compared with a commercial yeast-cell-wall additive. A significant decrease of OTA levels in YPG medium and SGM was observed for many of the growing strains reaching a maximum of 45%, but no degradation products were detected. With both heat- and acid-pretreated yeasts, OTA removal was enhanced, indicating that adsorption, not catabolism, is the mechanism to reduce OTA concentrations. Approximately 90% of OTA was bound rapidly within five minutes and up to 72 hours of incubation with heat-treated cells of either *S. cerevisiae* or *S. bayanus.*

Steyn *et al*. (2000) reported that certain yeasts, such as *Rhodotorula*, *Cryptococcus* and *Pichia* species have the ability to hydrolyse OTA to OTα and phenylalanine. Peteri *et al*. (2007) observed OTA degradation and adsorption by astaxanthin-producing yeasts, namely *Phaffia rhodozyma*. This yeast is able to convert OTA to OTα, by a conversion possibly mediated by carboxypeptidases. In addition, viable and dead *P. rhodozyma* cells have the ability to absorb significant amounts of OTA. Schatzmayr *et al*. (2006) isolated a novel yeast strain, *Trichosporon mycotoxinivorans*, from bovine rumen fluid capable of degrading OTA and zearalenone.

OTA is mainly produced by the black *Aspergilli*, namely *A. carbonarius and A. niger.* Therefore, Bleve *et al*. (2006) isolated a total of 144 antagonistic yeasts from the epiphytic flora associated with grape berries and tested these isolates for a killer activity against the two black *Aspergilli*. Of 28 yeast isolates selected, two isolates of *Issatchenkia orientalis* and one each of *Metschnikowia pulcherrima*, *Kluyveromyces thermotolerans*, *Issatchenkia terricola* and *Candida incommunis* were finally selected and screened on wounded grape berries for their ability to inhibit infection by ochratoxigenic moulds. The best antagonistic activity was shown by the two *I. orientalis* isolates. Results suggest that antagonist yeasts with the potential to control *A. carbonarius* and *A. niger* on grapes can be found among the microflora associated with the berries.

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Valero *et al*. (2006) observed that maintaining the temperature at or above 30 °C during dehydration of grapes may provide some control against OTA accumulation by *A. carbonarius.* Savino *et al*. (2007) reduced OTA contamination in red wines by adding oak-wood fragments. The effectiveness of the treatment depended upon the quantity of the oak fragments used; the best results were obtained by using the oak powder. During the roasting of Arabica coffee at 190 °C (mild roast) and 210 °C (dark roast) 63 and 82%, respectively, of the OTA was degraded (Santos *et al*., 2006).

4 FUMONISINS

Fumonisins are mycotoxins mainly produced by fungi of the genus *Fusarium* and predominantly associated with maize. The high concentrations found on maize worldwide and particularly on maize produced by subsistence farmers are of great concern. FB_1 is the major structural analogue produced on maize, whereas FB_2 and FB_3 are produced to a lesser extent. The toxicological profiles of the different analogues are similar and have been reviewed in detail; they provide the scientific base of certain regulations and guidelines (WHO, 2001). Fumonisins are regarded to be possibly carcinogenic to humans (Group 2B) by the International Agency for Research on Cancer (2002).

4.1 Fumonisin-Producing Organisms

Owing to the widespread occurrence of the fumonisins in feed and foods, numerous studies focussed on the ability of different fungal spieces to produce these mycotoxins. High levels of fumonisins are generally produced in hot and dry weather followed by periods of high humidity. Environmental factors, such as temperature, humidity, drought stress, insect damage and rainfall during preharvest and harvest periods could result in the formation of extremely high levels of the fumonisins. Although 28 analogues of fumonisin have been characterized, which include the fumonisin A, B, C and P series, specific studies reported on the production of FB_1, FB_2 and FB_3, of which FB_1 predominates as the main fumonisin produced in nature. Fifteen *Fusarium* species are known to produce fumonisins, which belong to sections *Liseola*, *Dlamini*, *Elegans* and *Arthroporiella* (Rheeder *et al*., 2002), although the verification of fumonisin production of certain strains needs confirmation. The two main fumonisins producing strains of *Fusarium,* occurring on maize worldwide, are *F. verticillioides* and *F. proliferatum*. Two other fungal species known to produce the fumonisins are *Alternaria alternata* (Fr.) Keissler f. sp. *lycopersici* (Mirocha *et al*., 1996) and *Aspergillus niger* (Frisvad *et al*., 2007).

Maize and maize products are known to contain high levels of the fumonisins, which pose a health risk to humans and animals. Other agricultural important crops such as sorghum and millet, colonized by certain *Fusarium* spp. are also contaminated with the fumonisins (Marasas *et al*., 2001; Nirenberg and O'Donnel, 1998). Certain traditional herbal medicines that enter the human food chain in developing countries via muthi markets have been shown to contain the fumonisins, although the specific fungal species responsible for the production are not known (Sewram *et al*., 2006). The relative stability and accumulation of the fumonisins in the soil (Williams *et al*., 2006) could also be responsible for its widespread contamination of plant products utilized for human consumption.

4.2 Analysis of Fumonisins

Since the discovery of FB_1 and FB_2 in 1988 (Gelderblom *et al*., 1988), 28 individual analogues have been reported (Rheeder *et al*., 2002), as well as a number of reaction products with sugars (Poling *et al*., 2002). In addition, the fumonisin chemical structure containing two ester functional moieties implies the potential occurrence of partially and fully hydrolysed forms of each analogue. Despite this multiplicity of forms, the majority of analytical methods have focussed on the determination of FB_1, FB_2 and FB_3, which are the most abundant naturally occurring members of this group of mycotoxins (Shephard *et al*., 1996). A wide range of analytical techniques have been applied to the fumonisins. The majority of the literature on natural occurrence of fumonisins is based on their determination by HPLC with fluorescence detection of appropriate derivatives. More recently, the coupling of HPLC with mass spectrometry (LC-MS) via ESI has allowed both sensitive quantification and confirmation of fumonisins. Apart from these techniques, both TLC and ELISA methods are available. Other methods that have been used to a limited extent for the determination of fumonisins include gas chromatography (GC) (Plattner *et al*., 1990), CE (Maragos, 1998), fluorescence polarization (FP) (Maragos *et al*., 2001) and biosensor techniques (Mullett *et al*., 1998). The fumonisins occur primarily in maize and hence the analytical methods have been predominantly developed for their determination in maize and its food and feed products. For this reason, the following subheadings relate mainly to analytical techniques.

4.2.1 *Thin-Layer Chromatographic Methods for Fumonisins*

TLC has found limited application in fumonisin analysis. A method with a detection limit of 1 mg kg^{-1} was developed, which included reversed-phase and strong anion exchange clean-up of the extract. The reversed-phase

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TLC plate was developed with ethanol–water–acetic acid (65:35:1) and FB_1 was visualized by a fluorescamine spray technique (Schaafsma *et al*., 1998). Comparison with HPLC results over a range of FB_1 contamination levels up to 250 mg kg^{-1} demonstrated a significant positive correlation. Further improvements in sample extract purification can be achieved by using IACs. These have been combined with reversed-phase TLC separation, followed by fluorescamine spray and fluorodensitometry for quantification of FB_1 in corn with a published detection limit of 0.1 mg kg^{-1} (Preis and Vargas, 2000). In order to separate FB_1 from a cochromatographing impurity, Shephard and Sewram (2004) reacted a cleaned-up maize extract with fluorescamine and separated the FB_1 derivative by reversed-phase TLC, using a developing solution of methanol–aqueous 4% potassium chloride (70:30). Based on visual comparison, the quantification limit of the method in maize was estimated to be 0.5 mg kg^{-1}.

4.2.2 High-Performance Liquid Chromatographic Methods for Fumonisins

Fumonisins are widely determined by HPLC and methods for their determination in maize have been validated by international collaborative study (Sydenham *et al*., 1996; Visconti *et al*., 2001). Typically, these methods or their modifications involve extraction of the sample with methanol–water or methanol–acetonitrile–water mixtures by shaking or homogenization, and clean-up on reversed-phase (C18) or strong anion-exchange solid-phase extraction cartridges or on IACs containing specific fumonisin antibodies, prior to derivatization and subsequent separation on reversed-phase HPLC with fluorescence detection. Nevertheless, a number of issues continue to be studied and reported. Extraction of fumonisins from processed foods, such as maize-based infant foods, was investigated and the optimum extraction solvent found to be acidified 70% aqueous methanol (Sewram *et al*., 2003). However, recovery studies still showed that some products, especially those containing a high proportion of maize starch, gave poor recoveries of spiked standards. Similar problems with extraction recoveries from spiked samples of rice flour have been reported, in that recovery of the spike can decrease over time (Kim *et al*., 2002a). IACs have been used extensively in mycotoxin analysis and a study has reported the possible regeneration and reuse of these columns for fumonisin analysis (Fazekas *et al*., 1999), whereas other authors have highlighted the problems that can arise if extracted impurities interfere with the antibodies, leading to under-reporting of the analyte levels (Castegnaro *et al*., 2006b). Existing methods for fumonisins in maize have been adapted to determine fumonisins in corn silage and swine liver. In the former, fumonisins were extracted with 0.1 M EDTA (ethylenediaminetetraacetic acid), cleaned-up on IAC and finally determined by HPLC with fluorescence detection after derivatization with naphthalene-2,3-dicarboxaldehyde (Kim *et al*., 2004). The method for swine liver involved clean-up on a hydrophilic–lipophilic balance cartridge, which retained both the FB_1 and its fully hydrolysed analogue (Pagliuca *et al*., 2005). Thereafter, the extract was derivatized with *o*-phthaldialdehyde and determined by HPLC on a phenylhexyl column with fluorescence detection.

A large number of authors have described the use of LC-MS or LC-MS/MS, mostly with ESI in the positive ion mode, for the detection, quantitation and confirmation of fumonisins in a number of matrices. These methods have been the subject of reviews (Musser *et al*., 2002; Zöllner and Mayer-Helm, 2006). Confirmation of fumonisins is readily achieved in MS/MS mode as, apart from loss of water during fragmentation, the fumonisin molecule readily fragments with the loss of one or both of the tricarballylic acid side chains, leading to a characteristic pattern of product ions (Musser *et al*., 2002). As in the case of aflatoxins, some authors have confined their work to fumonisin analogues (Faberi *et al*., 2005; Seefelder *et al*., 2003a; Sewram *et al*., 2001; Paepens *et al*., 2005a), whereas others have used the versatility of MS to develop multimycotoxin methods. These latter methods utilize extract dilution and direct injection (Spanjer *et al*., 2008; Sulyok *et al*., 2006; 2007) or extract clean-up with multimycotoxin IAC (Lattanzio *et al*., 2007) or with conventional solid-phase extraction cartridges (Cavaliere *et al*., 2005; Royer *et al*., 2004). A LC-MS/MS method for the determination of fumonisins in maize-based foods, which involved reversed-phase and graphitized carbon solid-phase clean-up, achieved detection limits for fumonisins of the order 1–2 μg kg^{-1} (Faberi *et al*., 2005). A method for fumonisins in cornflakes, which involved IAC clean-up of an acidified methanol–water extraction solution, was successfully validated in-house (Paepens *et al*., 2005b). Many of the developed methods use matrix-matched standards to overcome potential matrix effects in the ionization process (Sulyok *et al*., 2007). Apart from its use to determine fumonisins in food, Sewram *et al*. (2001) used LC-MS/MS to measure fumonisins in hair as a suitable biomarker of exposure. Following methanol extraction, the sample extract was cleaned-up on strong anion exchange (for fumonisin analogues) and on reversed-phase C18 (for hydrolysed analogues) solid-phase extraction cartridges prior to injection on the LC-MS/MS. Quantitation limits were of the order of 60–80 pg on column.

4.2.3 Immunological Methods for Fumonisins

In common with the other major mycotoxins, a number of immunological methods have been developed for

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the determination of fumonisins. A commercial competitive direct ELISA with colorimetric end point was studied collaboratively for determination of total fumonisins in maize and achieved AOAC official status (Bird *et al*., 2002). Greater sensitivity and shorter assay times were obtained in a direct competitive ELISA developed with polyclonal antibodies and enhanced chemiluminescence detection for FB_1 (Quan *et al*., 2006). Although IACs are widely used for clean-up of sample extracts prior to further chromatographic separation and analysis, under suitable conditions and with suitable matrices, the resulting purified extract has itself been used, after derivatization, for quantitation of total fumonisins by direct fluorescence measurement (Duncan *et al*., 1998). However, such methods must be carefully validated for the analytical matrix, as incorrect reports of fumonisin contamination are possible if used outside their area of validation (Shephard *et al*., 2005a).

For rapid screening of samples at a set level of contamination, lateral flow and flow-through enzyme immunoassays have been introduced. Wang *et al*. (2006) developed both flow-through and lateral flow formats which utilized colloidal gold as the visual component in test and control lines. Paepens *et al*. (2004) developed a flow-through immunoassay with a colorimetric end point. The method was successfully applied to maize, maize flour, maize grits and to an evaluation of fumonisin levels in cornflakes on the Belgian market (Paepens *et al*., 2005b).

4.3 Occurrence of Fumonisins

The main producing fungi of fumonisins are *Fusarium verticillioides* (Sacc.) Nirenberg (formerly *F. moniliforme* Sheldon) and *F. proliferatum* (Matsushima) Nirenberg, which are associated with maize in most areas of the world (Marasas, 1996). As a consequence, fumonisins are ubiquitously found in maize and the vast majority of publications on natural occurrence of fumonisins deal with either maize or maize products. The first comprehensive survey of fumonisin occurrence was published by Shephard *et al*. (1996), and this was followed by a comprehensive compilation of published data undertaken by the JECFA (Bolger *et al*., 2001). Subsequently, other reviews of fumonisin occurrence have also been published (Soriano and Dragacci, 2004; Weidenbörner, 2001).

The natural occurrence of fumonisins in unprocessed maize has been investigated by numerous researchers in many countries of the world. The contamination levels found vary greatly and are influenced by a number of factors, such as weather, temperature and plant stress induced by drought or insect herbivory (Miller, 2001). Consequently, contamination frequency and levels can vary greatly between geographic areas, seasons, years and climate zones. Typically, contamination levels vary from detection limits of $10–20\,\mu g\,kg^{-1}$ up to a level of some several thousands or tens of thousands of $\mu g\,kg^{-1}$. Studies in four regions of Iran have shown how levels can vary in different climate zones in that maize harvested in Mazandaran had mean total fumonisin levels of $10674\,\mu g\,kg^{-1}$, whereas samples from Fars, Kermanshah and Khuzestan had mean levels of 215, 71 and $174\,\mu g\,kg^{-1}$, respectively (Ghiasian *et al*., 2006). Relative to the other growing areas, Mazandaran is an area of relatively high rainfall, higher relatively humidity and the lowest minimum altitude. Another study in Mazandaran demonstrated how contamination levels can vary between harvest seasons in that mean FB_1 levels were shown to vary from 2.27 to 3.18 to $6.14\,mg\,kg^{-1}$ over three consecutive years (Yazdanpanah *et al*., 2006). Depending on the region, maize can be infected with more than one fungal species and the resulting crop contaminated with multiple mycotoxins. Of particular concern is the co-occurrence with fumonisin of another carcinogenic mycotoxin, aflatoxin. This situation has been studied in various countries, such as Brazil, where analysis of 214 unprocessed maize samples showed 212 fumonisin-contaminated samples (mean FB_1 of $2200\,\mu g\,kg^{-1}$), of which 82 samples were co-contaminated with a mean aflatoxin level of $9.4\,\mu g\,kg^{-1}$ and a maximum level of $129\,\mu g\,kg^{-1}$ (Vargas *et al*., 2001). A multiyear study in Arkansas, USA, showed that cocontamination is common among a large number of commercial hybrids, that it varies from year to year and that natural infection with *Fusarium* spp. does not protect against aflatoxin production (Abbas *et al*., 2006). The association of fumonisins with high incidence of oesophageal cancer has increased interest in studying contamination of maize grown in these specific areas. In the affected areas of Asia, studies in Mazandaran have shown high levels of fumonisin in maize (Ghiasian *et al*., 2006). A comparison of maize samples from three areas of China (one of high oesophageal cancer, one of high liver cancer and one of low incidence of both cancers) showed a significantly higher fumonisin contamination in the high cancer-incidence areas (mean FB_1 of 2.84 and $1.27\,mg\,kg^{-1}$ vs. mean FB_1 of $0.65\,mg\,kg^{-1}$, respectively) (Sun *et al*., 2007). Results of an extensive field study comparing maize kernels from Bt maize hybrids (containing the Cry1Ab protein from *Bacillus thuringiensis* (Bt) that controls European stalk borer infestation) with near isogenic controls confirmed that in a significant number of locations, kernels of the Bt maize varieties showed considerably lower fumonisin contamination than their conventional controls due to reduced insect herbivory (Hammond *et al*., 2004). A comparison carried out in Spain between maize samples collected from conventional and nearby organic farms showed very low levels of fumonisin (mean $FB_1 < 50\,\mu g\,kg^{-1}$) in both sets of samples, with no significant difference between the two farming systems (Arino *et al*., 2007).

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Industrially processed maize products intended for human consumption and derived from contaminated unprocessed maize will generally retain, at a lower level, some fumonisin contamination. Industrial milling processes, which generate a number of maize milling fractions, result in the fumonisin being reduced in fractions intended for human consumption (grits, flour, maize meal, polenta and starch) and to be concentrated in fractions intended for animal feed (bran and germ fractions) (Brera *et al*., 2004; Saunders *et al*., 2001). Cooking of maize either as home porridge or polenta, or industrial canning and baking produce zero to low levels of fumonisin reduction, whereas the higher temperatures and pressures associated with extrusion products can result in higher losses depending on the processing time and temperature (Brera *et al*., 2004; Bullerman *et al*., 2002; Shephard *et al*., 2002). Market surveys of commercial maize-based foods confirm the widespread presence of fumonisins. Corn flour, corn snacks and corn flakes collected in Spain had mean levels of FB_1 of 363, 46 and 17 $\mu g\,kg^{-1}$, respectively (Velluti *et al*., 2001). Analysis of breakfast cereals in France showed that 94% of samples containing maize, oats or rice contained FB_1 at levels between 1 and 1110 $\mu g\,kg^{-1}$ (Molinie *et al*., 2005). A survey of 196 samples of infant food in Campinas, Brazil showed fumonisin contamination depended on the ingredients (De Castro *et al*., 2004). Infant cereals containing corn starch or wheat as main ingredients were free of fumonisins, whereas those containing corn flour or corn meal contained mean total fumonisins of 664 and 2242 $\mu g\,kg^{-1}$, respectively. Other surveys in countries such Canada (infant cereal foods), Japan (frozen corn, corn flakes and corn grits) and Korea (various corn products) have all shown that fumonisin contamination of maize-based foods at levels ranging up to several 100 $\mu g\,kg^{-1}$ is a universal occurrence (Kim *et al*., 2002b; Lombaert *et al*., 2003; Sugita-Konishi *et al*., 2006b). The possible binding of fumonisins to the food matrix during processing has been investigated. For a range of Canadian samples, including corn flakes, maize-based breakfast cereals, tortilla chips and corn chips, it was shown that there was an average of about 30% more FB_1 in a bound form than was determined by conventional extraction techniques (Park *et al*., 2004).

The process of nixtamalization, in which maize is treated with lime as the first stage of tortilla production by Hispanic groups in America, is known to reduce fumonisin levels. Reductions of over 80% were measured in a study of the operation of four small tortilla plants in southern Texas (De la Campa *et al*., 2004). A laboratory study in which contaminated maize was processed into tortilla chips also achieved a measured FB_1 reduction of over 80% and also determined a similar decrease in biological activity measured by *in vitro* inhibition of ceramide synthase (Voss *et al*., 2006a). The presence of fumonisins has been demonstrated in a number of traditional African foods. Of 75 samples of kenkey (a Ghanaian fermented maize product), 55 were found to contain total fumonisins ranging from 15 to 1035 $\mu g\,kg^{-1}$ (Kpodo *et al*., 2006). Eighteen maize beer samples collected in the former Transkei region of South Africa were all positive for FB_1, with a mean level of 281 $\mu g\,l^{-1}$ and a range of 38–1066 $\mu g\,l^{-1}$ (Shephard *et al*., 2005b).

Apart from their ubiquitous presence in human food, fumonisins are widely found in a variety of animal feeds, frequently in conjunction with other mycotoxins. Poultry feed in Kuwait was found by ELISA to be contaminated with fumonisin levels between 1.4 and 3.2 $mg\,kg^{-1}$, in addition to aflatoxin (maximum 3.26 $\mu g\,kg^{-1}$), deoxynivalenol (maximum 0.29 $mg\,kg^{-1}$), OTA (maximum 9.6 $\mu g\,kg^{-1}$) and zearalenone (maximum 67.6 $\mu g\,kg^{-1}$) (Beg *et al*., 2006). Poultry feeds in Rio de Janeiro State, Brazil contained up to 5.5 $mg\,kg^{-1}$ (Oliveira *et al*., 2006), whereas a survey in Portugal reported much lower levels of fumonisin contamination in mixed-feed for laying hens (FB_1 maximum level 110 $\mu g\,kg^{-1}$) (Martins *et al*., 2006). A major survey of animal feed and feed ingredients was performed with samples drawn from Europe, Asia and Oceania (Binder *et al*., 2007). Fumonisins were frequent contaminants in Asia and southern Europe and the Mediterranean. Mean and maximum total fumonisins were 432 and 530 $\mu g\,kg^{-1}$ in northern Europe, 757 and 3120 $\mu g\,kg^{-1}$ in southern Europe, 612 and 1090 $\mu g\,kg^{-1}$ in Oceania, 1011 and 14 714 $\mu g\,kg^{-1}$ in northern Asia, 491 and 4866 $\mu g\,kg^{-1}$ southeast Asia, and 397 and 2217 $\mu g\,kg^{-1}$ in southern Asia. In addition to the above studies, fumonisins have also been determined in corn silage samples collected in the midwestern USA at contamination frequencies of 97% and with mean FB_1 levels of 615 $\mu g\,kg^{-1}$ (maximum 1824 $\mu g\,kg^{-1}$) (Kim *et al*., 2004).

Apart from their association as a universal contaminant of maize and maize-based food and feed, fumonisins have been found in a number of other commodities. Low levels of FB_1 (up to 70 $\mu g\,kg^{-1}$) have been reported in stored einkorn, emmer and spelt wheat (collectively referred to as farro in Italy) (Castoria *et al*., 2005) and pearl millet in Georgia, USA (mean 121 $\mu g\,kg^{-1}$ in one cultivar) (Wilson *et al*., 2006). Limited data is available on the occurrence of fumonisins in sorghum. Of 39 sorghum grain samples from Ethiopia, analysed by ELISA, three showed fumonisin levels ranging from 1370 to 2117 $\mu g\,kg^{-1}$ (Ayalew *et al*., 2006). Three of 46 sorghum malt samples collected in Botswana had FB_1 levels ranging from 47 to 1316 $\mu g\,kg^{-1}$, but no fumonisin was detected in corresponding sorghum wort or beer samples (Nkwe *et al*., 2005). Of 30 samples of healthy asparagus spears collected in Shandong province, China, 24 were shown by LC-MS to contain FB_1 and FB_2 at levels ranging from 24 to 670 $\mu g\,kg^{-1}$ (mean 123 $\mu g\,kg^{-1}$) for FB_1 and 17 to 138 $\mu g\,kg^{-1}$ (mean 35 $\mu g\,kg^{-1}$) for FB_2 (Liu *et al*., 2005). Also, analysis of asparagus spears from Germany, naturally infected with *F. proliferatum*, showed nine of ten samples to contain FB_1 at levels between 36

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and 4514 $\mu g\,kg^{-1}$ (Seefelder *et al*., 2004). Lower levels of FB_2 and FB_3 were also detected. Investigations have also revealed that FB_1 can occur in tea and medicinal plants. Of 87 samples of black teas and medicinal plants collected in Lisbon, Portugal, 55 contained FB_1 at levels as high as 700 $\mu g\,kg^{-1}$ (Martins *et al*., 2001). A low incidence of FB_1 contamination of medicinal plants collected in Istanbul, Turkey (Omurtag and Yazicioglu, 2004) and in the Eastern Province of South Africa (Sewram *et al*., 2006) was reported, although individual levels could be as high as 1487 $\mu g\,kg^{-1}$ in Turkey (two samples of 115 were contaminated) and 1553 $\mu g\,kg^{-1}$ in South Africa (eight samples of 49 were contaminated). Four samples of South African cowpeas were found to contain FB_1 at levels between 0.12 and 0.61 $mg\,kg^{-1}$ (Kritzinger *et al*., 2003).

4.4 Toxicology of Fumonisins

4.4.1 Studies in Domestic Animals

4.4.1.1 Equine Leukoencephalomalcia (ELEM)

Natural outbreaks of leukoencephalomalcia (LEM) in donkeys and horses have been recorded in the USA since the early 1900s, and sporadically occurred during the 1930s, 1980s, specifically 1989 and during mid 1990s (Marasas, 1995; Voss *et al*., 2007). Horses are the most sensitive species to fumonisin toxicity, which is associated with prominent brain lesions associated with liquefactive necrosis of the white matter, oedema and haemorrhage. Clinical signs associated with these changes are decreased feed intake, depression, ataxia, blindness and hysteria. Anorexia occurs as a result of glossopharyngeal paralysis associated with the loss of ability to grasp and chew feed. High exposure, however, is normally associated with hepatotoxicity with an increase of serum bilirubin and liver enzyme activities with lower doses favouring the neurotoxic syndrome. The main liver lesions following high-dose exposure include centrilobular necrosis and perilobular fibrosis. These two forms of the disease may develop concurrently with the one that dominates depending on different factors including the dosage used, length of exposure and individual susceptibility. The first reports on the causative role of FB_1 in LEM resulted from intravenous application by Marasas *et al*. (1988) and Laurent *et al*. (1989), and oral dosing by Kellerman *et al*. (1990). A recent study showed that the clinical signs for the neurological disease are induced after intravenous application (Foreman *et al*., 2004). Cardiovascular effects were also present during the development of the neurological disease, which provide interesting mechanistic perspectives regarding the kinetics of the disease (Smith *et al*., 2002).

4.4.1.2 Pulmonary Oedema Syndrome (PES) in Pigs

Following the first report of the causative role of FB_1 in pulmonary oedema syndrome (PES) by Harrison *et al*. (1990), several studies were conducted to investigate the progression of the disease to elucidate relevant mechanisms. In general, respiratory distress and acute cyanosis, occur in the four to seven days preceding the acute pulmonary oedema and hydrothorax. Perivascular oedema and dilatation of lymphatics occur in the interstitium in the lung, and are regarded as early features of the disease. A reduced cardiac contractility and output, with a concomitant increase in pulmonary artery pressure were observed, implying acute left-side heart failure (Smith *et al*., 2000; Hasheck *et al*., 2001; Constable *et al*., 2000; 2003). Inhibition of L-type calcium channels of cardiac myocytes and the decrease in Ca^{2+} release and resultant cardiac contractility are proposed as a possible mechanism. Hepatic injury is always present, and depending on the dose of exposure, varies from mild to acute effects, including hepatocellular necrosis and apoptosis, with a resultant increase in cell proliferation A dose–response study showed that a FB_1 dosage of 10 ppm in the diet revealed two mild cases of pulmonary oedema which were associated with an increase in serum aspartate aminotransferase activity, indicating pathological changes in the liver (Zomborszky *et al*., 2002). Exposure to even lower dietary levels, up to 1 ppm FB_1, revealed pathological alterations in the lung associated with proliferation of connective tissue fibres in the subpleural and interlobular connective tissue (Zomborszky-Kovacs *et al*., 2002). A study in three-week-old weaned pigs showed that an average dose of 0.5 mg $FB_1\,kg^{-1}$ for six days significantly increased the colonization of the small and large intestine with *Escherichia coli*, suggesting a predisposition to infectious diseases (Oswald *et al*., 2003). A gavage treatment of piglets with a crude extract of FB_1 (0.5 mg kg body weight)$^{-1}$ also predisposes pigs to lung inflammation caused by *Pasteurella multocida*, which was associated with an increased expression of tumour necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-18 (Halloy *et al*., 2005). A study in male weanling pigs fed a diet of 8 mg $FB_1\,kg^{-1}$ fed for 28 days led to an immunosuppressive effect after immunization with *Mycoplasma agalactiae*. The specific antibody levels, and the expression of Type 2 T-helper cell (Th2) cytokines, IL-10, IL-4 and IL-6 were decreased (Marin *et al*., 2006). Female weanlings were more resistant.

4.4.2 Studies in Ruminants

FB_1 induces hepatotoxic and nephrotoxic effects in calves after an intravenous dose of 1 mg (kg body weight)$^{-1}$ for 7–14 days. Hepatic lesions consisted of disorganized hepatic cords associated with apoptosis, hepatocyte and bile ductular proliferation. Renal lesions include

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apoptosis, karyomegaly and proliferation of proximal renal tubular cells (Mathur *et al*., 2001). Feeding studies in lambs and goats also showed renal and mild hepatic injury (See review by Voss *et al*., 2007).

4.4.3 Miscellaneous Studies

FB_1 was also found to be hepato- and nephrotoxic to rabbits after receiving multiple intravenous doses for four to five days ranging from 0.15 to 1 mg (kg body weight)$^{-1}$ (Bucci *et al*., 1998). Studies in vervet monkeys showed that single dosages of FB_1 and FB_2, as well as repeated dosages of FB_1 increased the levels of the liver function enzymes in a reversible time-dependent manner (Van der Westhuizen *et al*., 2001a; 2001b). No effects were noticed when considering the serum markers for urinary functions in any of the studies. Serum parameters and liver pathological alterations in the liver were also reported in a 13-year study of culture material of *F. verticillioides* in vervet monkeys (Gelderblom *et al*., 2001d). Studies in ducks showed that FB_1 and/or crude preparations containing FB_1, FB_2 and FB_3 failed to induced necrotic lesions in the liver and kidneys while the serum enzyme markers showed an increase at the highest dose level of 128 mg FB_1 kg^{-1} feed (Bailly *et al*., 2001; Tran *et al*., 2005; Tradieu *et al*., 2006). Apoptosis, a key toxic parameter induced by the fumonisins also seems not to be important in the liver. The mechanisms involved in the resistance of ducks to the toxic effects of the fumonisins are not known at present. FB_1 increased the activities of drug-metabolizing enzymes in duck liver, of which the Phase 1 enzyme activities are the most sensitive while the Phase 2 enzymes are weakly affected (Raynal *et al*., 2001). The activities of different isozymes of cytochrome P450 are increased, this reaction may affect the metabolism of other carcinogens, steroids, numerous drugs and chemicals. Chickens and minks were also found to be relatively resistant to FB-induced toxic effects (see review by Voss *et al*., 2007). Hepatic necrosis and biliary hyperplasia were noticed in chicks with 200 to 300 ppm, while hepatic enzymes were elevated with a dietary dose of 80 mg FB_1 kg^{-1}. A study in mink showed no clinical evidence of disease at dietary levels between 118 and 254 ppm. Quail chicks showed an increased susceptibility to *Salmonella gallinarum* infection after feeding culture material containing 150 mg FB_1 kg^{-1} (Deshmukh *et al*., 2005). Consumption of pellets to obtain a dosage of 0.5 and 5 mg FB_1 (kg body weight)$^{-1}$ increased liver and kidney function enzymes of one-year old carp fish after 42 days (Pepeljnjak *et al*., 2003).

4.4.4 Studies in Rats and Mice

The effects of the fumonisins in rats and mice have been reviewed extensively (Bolger *et al*., 2001; Voss *et al*., 2001; 2007).

4.4.4.1 Toxicological Effects

Dose-dependent effects in the liver include the appearance of apoptotic and necrotic hepatocytes resulting in mitosis as initial events. Hepatotoxic effects are associated with an increase in serum chemical indicators for liver injury, including alanine and aspartate transaminases, alkaline phosphatase and lactate dehydrogenase. Other serum parameters included an increase in cholesterol and triglyceride levels. Advance liver lesions following long-term chronic exposure include a toxic hepatitis that eventually developed into cirrhosis, cholangiomatous lesions, fibrosis and eventually in nodular regeneration and cirrhosis. This was also associated with bile duct and oval cell proliferation resulting in the formation of cholangiofibromas (Lemmer *et al*., 1999a; 2004). Kidney lesions include apoptosis in the proximal tubules as an initial event, characterized by sloughing of the cells into the lumen, resulting in an increased number of mitoses and foci of tubular hyperplasia, and interstitial fibrosis and inflammation during severe injury. These changes are associated with an increased serum creatinine levels. Although the liver and kidneys are the main target organs in rats, strain and sex differences exist, as the kidney is the most sensitive organ in Sprague-Dawley and Fischer 344 rats, while in BD IX rats, the liver is the main target organ (Voss *et al*., 2007). The liver was also found to be the most sensitive organ in mice, whereas the kidneys were more resistant, even when mice were exposed to dietary levels of 484 ppm FB_1 for 28 days (Voss *et al*., 2001).

4.4.4.2 Reproductive Toxicology

Foetotoxicity has been reported in rats which also exhibited maternal toxicity, with no evidence that FB_1 crosses the placenta (See review by Voss *et al*., 2007; International Agency for Research on Cancer, 2002). The first evidence that FB_1 crossed the placenta was obtained in LM/Bc and CD 1 mice, showing teratogenic effects by inducing neural-tube defects (Gelineau-van Waes *et al*., 2005; Voss *et al*., 2006a). The FB1-induced effects were reduced by folate and the sphingolipid GM_1, colocalized with the folate-binding protein in the mouse yolk-sac membrane.

4.4.4.3 Carcinogenesis Studies

Hepatocarcinogenicity of naturally infected maize and *F. verticillioides* culture material has been demonstrated in Fischer 344 and BD IX male rats, respectively (Wilson *et al*., 1985; Marasas *et al*., 1984). Retrospective analyses of the total fumonisin content of the samples showed that a daily total fumonisin intake of between 2.3 and 3.2 mg affects a carcinogenic response in male Fischer 344 rats (Gelderblom *et al*., 2004). In male BD IX rats, a daily exposure of 3.2 and 6.9 mg FB resulted in 66% of the rats developing liver cancer (Gelderblom *et al*., 2001a; 2004). Varying doses of between 0.4 and 1.3 mg

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FB_1 (kg body weight)$^{-1}$ per day yielded a far lower carcinogenic response in the liver (Jaskiewicz *et al*., 1987). A no observed effect level (NOEL) of 0.8 and $<$0.3 mg (kg body weight)$^{-1}$ per day was determined using pure FB_1 for the induction of liver cancer and hepatocyte nodules in male BD IX rats, respectively (Gelderblom *et al*., 1996; 2001b). These studies are in contrast with the two year study in male Fischer 344 rats, showing that an exposure of between 2.2 and 6.6 mg (kg body weight)$^{-1}$ did not cause any pathological lesions in the liver (Howard *et al*., 2001a). As different diets were used in the studies, the role of certain dietary constituents, such as the protein content and deficiencies in certain micronutrients could be responsible for differences in the organ-specific responses. A long-term study in Fischer 344 rats showed that female rats are more resistant than male rats in developing a rare variant of kidney tumours that locally invade and metastasize to the lungs (Howard *et al*., 2001a; Hard *et al*., 2001). In B6C3F1 mice FB_1 increased the development of adenomas, and carcinomas developed spontaneously in the liver of female mice, whereas no effect was noticed in males (Howard *et al*., 2001a).

4.5 Synergistic Interactions

The interaction between different mycotoxins that simultaneously occur in feed and food is of importance in order to elucidate possible additive and/or synergistic effects. This synergy is of relevance, as different mycotoxins have been shown to co-occur, which could increase their individual risks in the development of certain human and animal diseases. The studies, which mainly focussed on the interactions of FB_1 and AFB_1 utilized different experimental models including rats (Gelderblom *et al*., 2002; Theumer *et al*., 2003; 2008), weaned piglets (Dilkin *et al*., 2003), broiler chickens (Bianchi *et al*., 2005), male Fischer 344 rats (McKean *et al*., 2006), rabbits (Orsi *et al*., 2007) and rainbow trout (Carlson *et al*., 2001). Sequential treatment of rats with AFB_1 and FB_1 synergistically increased the hepatotoxic effects in the liver and also rendered the liver more susceptible to the cancer-promoting and initiating properties of FB_1 (Gelderblom *et al*., 2002). Coexposure of FB_1-containing maize culture material and AFB_1 enhanced the decrease in body-weight gain while the coexposure increased the toxic effects in the liver and kidney (Theumer *et al*., 2008). A synergistic increase in the Sa/So (sphinganine to sphingosine) ratio in the kidneys was noticed with the combined treatment. A study in weaned piglets showed that feed consumption and feed conversion were the only parameters affected, due to the joint effects of the toxins. Although the highest dose of FB_1 (30 mg (kg feed)$^{-1}$) induced the typical signs of pulmonary oedema, no combined and/or additive toxicological effects were noticed in the lung, liver and kidneys (Dilkin *et al*., 2003). In broiler chicks, the combined effects of FB_1 and AFB_1 did not alter the haematological and serological parameters, although certain changes in the liver and kidneys may be of relevance (Bianchi *et al*., 2005). Coadministration of AFB_1 and FB_1 in male Fischer rats showed that the latter additively increased the mortality caused by the acute toxicity of AFB_1 (McKean *et al*., 2006). Combined OTA and FB_1 treatment of male Wistar rats showed a significant increase in the tail intensity in kidney cells using the Comet assay (Domijan *et al*., 2006). A study in New Zealand using white rabbits showed synergistic liver and kidney toxicological effects when considering the serum biochemical and histology analyses after coadministration of AFB_1 and FB_1 (Orsi *et al*., 2007).

4.6 Fumonisin Biomarker Studies

Studies in animals showed that the disruption of sphingolipid biosynthesis via inhibition of ceramide synthase with the resultant accumulation of Sa, and the Sa/So ratio could be utilized as a biomarker for fumonisin exposure. These studies included investigations in horses (Wang *et al*., 1992), pigs (Riley *et al*., 1993), ducks (Tran *et al*., 2006) and rodents (Wang *et al*., 1999; Enongene *et al*., 2002). The validation of fumonisin biomarkers, including free FB_1 excretion in the faeces, Sa/So and SaP/SoP (sphinganine phosphate to sphingosine phosphate) alteration in the plasma and urine was recently investigated in male mice (Kim *et al*., 2006) and male Fischer rats (Garren *et al*., 2001; Cai *et al*., 2007). Treatment of ICR mice with FB_1 (10 mg kg^{-1}, intraperitoneal (ip)) for five days indicated that the SaP/SoP ratio in the serum was the most sensitive marker of exposure. A time-course study in the Fischer rats over a period of five weeks showed that the urinary SaP/SoP ratio was also the most sensitive biomarker for FB_1 exposure. Free faecal and urine FB_1 levels were strongly correlated with urinary sphingolipid metabolites and also were suggested as possible biomarkers for exposure in humans.

4.7 Studies in Humans

The impact of fumonisin exposure to humans and its involvement in certain human diseases are not known at present. A detailed review of the possible involvement of fumonisins, other fungal toxins and risk factors in human diseases, including oesophageal and liver cancer, and neural-tube defects has been conducted (Bolger *et al*., 2001). Most of these associations have been linked to poor socioeconomic populations using maize and/or maize products as a staple diet. More recent studies focussed on the development of valid biomarkers for fumonisin exposure in human populations consuming

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contaminated maize. A study in three different regions, including the former Transkei region of the Eastern Cape, the Madadeni distract in Kwazulu-Natal in South Africa and the Bomet district in western Kenia showed that that no differences exist in the Sa/So ratios in the plasma and urine between males and females, between the different regions or as a function of the home-grown maize collected (Van der Westhuizen *et al*., 1999). These areas are known to have high prevalence of oesophageal cancer, with the populations utilizing maize as the main dietary staple. It was suggested that the Sa/So biomarker was not sensitive enough to monitor fumonisin exposure in humans. Two independent studies conducted in China showed contradictory findings regarding the use of the Sa/So ratio as a biomarker for oesophageal cancer and FB exposure. Randomly selected cases from a prospective control study in Linxian showed no association between the serum Sa/So ratio and the development of oesophageal squamous cell carcinoma (Abnet *et al*., 2001). In another study, the urine Sa/So ratio was suggested to be useful marker to assess FB_1 exposure in northern China (Qiu and Liu, 2001). A study conducted in high maize-consuming populations in northern Argentina and southern Brazil showed that, despite similar FB_1 intake profiles, the urinary Sa/So ratio was significantly higher in the residents from northern Argentina (Solfrizzo *et al*., 2004). Although a significant increase in the ratio was noticed when compared to residents in central Argentina and southern Italy, with low or no maize consumption, changes in the Sa/So values could not be associated with high fumonisin exposure. It would appear that the specific study design and the biomarker used are important when validating the sphingolipid biomarker in humans. A recent study showed that urinary FB_1 appear to be a more sensitive marker for fumonisin exposure and was correlated with tortilla consumption in a Mexican population (Gong *et al*., 2008).

Studies related to the possible role of fumonisins in the development of neural-tube defects (NTD) have been reviewed by Marasas *et al*. (2004). A recent study showed that the consumption of tortillas during the first trimester was associated with an increased risk of developing neural-tube defects (Missmer *et al*., 2006). Based on the Sa/So ratio in postpartum blood, increasing levels of fumonisins exposure were associated with increasing odds ratios for NTD incidence. Estimated fumonisin exposure in Guatemala, based on the consumption of nixtamalized maize products in the form of tortillas, was found to exceed the recommended provisional maximal TDI of 2 μg (kg body weight)$^{-1}$ per day, especially in the rural areas (Torres *et al*., 2007). The association of the high FB exposure and the development of NTD in this region are not known at present.

4.8 Biochemical Mechanisms of Action

4.8.1 Studies Utilizing Cell Cultures

Details of the FB_1-induced cytotoxic effects and possible mechanisms involved in different cell culture systems have been reviewed by Bolger *et al*. (2001). Some of the most recent studies, addressing important aspects regarding the mechanisms involved in FB_1-induced biological effects in different cell culture systems are summarized in **Table 1**. The studies utilized different cell culture systems including kidney, immune and inflammatory cells, cardiomyocytes, intestinal epithelial cells, oesophageal cells and gliomal cells to name a few. Most of the studies implied the disruption of sphingolipid metabolism and the accumulation of sphingoid bases and their phosphate metabolites in the modulation of cytotoxicity, inhibition of cell proliferation and induction of apoptosis. Another mechanism has been proposed implying mitochondrial damage and induction of lipid peroxidation. Irrespective of the mechanism involved, the fumonisins influenced a variety of cells and apart from selective cytotoxicity and the modulation of cell growth parameters, a number of immune cells are also affected, indicating potential immunosuppressive effects.

The comparative and interactive effects of FB_1 and other mycotoxins, including AFB_1, the tricothecenes, nivalenol and deoxynivalenol, and zearalenone have been conducted *in vitro* utilizing different cell lines (**Table 1**). Although differences exist in the specific mechanisms involved, most of the studies showed synergistic and/or additive effects when considering different toxic parameters.

4.8.2 Toxicological Effects in Experimental Animals

Initial studies on the possible mechanisms involved in fumonisin-induced toxic effects were based on the finding that the fumonisins disrupt the *de novo* biosynthesis of sphingolipids by inhibiting ceramide synthase (reviews by Merrill *et al*., 2001; Voss *et al*., 2001). The immediate response of ceramide synthase inhibition is the accumulation of sphinganine and, to a lesser extent, sphingosine, resulting in an increased Sa/So ratio in the blood, urine, kidney and liver, while the more complex sphingolipids are depleted. The sphingoid bases are catabolized by phosphorylation and lytic cleavage resulting in increased levels of sphingoid base 1-phosphate and ethanolamine phosphate, which are incorporated in the phospholipid phosphatidylethanolamine, affecting membrane integrity and fluidity. The sphingoid bases are also substrates for the *N*-acetyltransferase, resulting in the accumulation of *N*-acetylsphinganine metabolites. Apart from the accumulation of the sphingoid bases, the synthesis

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Table 1 Some mechanisms involved in FB_1-induced biological effects in different cell culture systems

Model	Biological effects/end points	Biochemical alterations	Reference
Porcine renal epithelial (LLC-PK_1)	Cytotoxicity and apoptosis	Increased caspase-3 activity; elevation of sphinganine levels; resistance of CHO cells related to lower sphinganine accumulation.	Yu *et al*. (2001)
Chinese hamster ovary (CHO)	—	—	—
LLC-PK_1	Apoptosis	Accumulation of sphinganine; blocking of sphinganine accumulation prevents apoptosis; specific role of calmodulin in FB_1-induced apoptosis.	Kim *et al*. (2001)
LLC-PK_1	Cell viability (MTT assay) and cytotoxicity (lactate dehydrogenase) release	Inhibition of sphinganine accumulation protects against FB_1-induced cytotoxicity; modulation of TNFα expression prevents toxicity independent from sphinganine pathway.	He *et al*. (2002)
LLC-PK_1	Apoptosis	Repression of membranous PKC; inhibition of cytosol to nuclear translocation of NFkB and expression of TNFα; Increased caspase-3 activity.	Gopee and Sharma (2003a)
LLC-PK_1	Sphingoid base modulation of PKC activation	Activation of PKCα due to the accumulation of Sa-1-phosphate; repression of PKC isoforms due to accumulation of Sa or its metabolites.	Gopee *et al*. (2003b)
LLC-PK_1	Sphingolipid accumulation	Decrease in pERK2 not related to accumulation of sphingoid bases or reduction of ceramide levels; related to inhibition of glycosphingolipid biosynthesis.	Rentz *et al*. (2005)
COS-7 and human embryonic kidney cells (HEK-293T)	Ceramide synthesis	Transfected cells with uog-1 confer FB_1 resistance and continue to produce ceramide, which was channelled into neutral glycosphingolipids, containing primarily stearic acid.	Venkataraman *et al*. (2002)
African green monkey kidney cells (CV-1 and COS-7)	Cell arrest and apoptosis	Alteration of expression of specific genes; induction of TRAP2 and binding to TNFR-1 suggest a TNF-induced death pathway; resistance of COS-7 cells related to lack of expression of certain genes	Zhang *et al*. (2001)
CV-1 cells Mouse embryo fibroblast (MEF)	Apoptosis	Supports involvement of the TNF pathway by inactivation of caspase-8; p53 not required for FB_1-induced apoptosis; Bcl-2 not effective inhibitor of FB_1-induced apoptosis.	Jones *et al*. (2001)

(*continued overleaf*)

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Table 1 (*continued*)

Model	Biological effects/end points	Biochemical alterations	Reference
Human SCLC cells (Ms-1)	Regulation of apoptosis	FB_1 inhibits inostamycin-induced apoptosis by preventing cytochrome c release and caspase-3 (like) activation. Apoptosis mediated by PKC-regulated ceramide generation	Kawatani *et al*. (2000)
Human embryonic kidney (HEK-293)	Resistant to cytotoxic effects	Resistance related to the rapid formation of Sa-1-phosphate via sphingosine kinase.	Sharma *et al*. (2004a)
Human proximal tubule-derived cells (IHEK)	Ceramide synthesis, apoptosis.	Fumonisin derivatives increase sphinganine. Only FB_1-induced apoptosis.	Seefelder *et al*. (2003b)
Porcine intestinal epithelial cells (IEPC-1) LLC-PK_1	Cell proliferation	FB_1 blocks proliferation of IPEC-1 and LLC-PK_1 cells in G0/G1 phase. Barrier function of intestinal cells was decreased.	Bouhet *et al*. (2004)
Intestinal cells (Caco-2)	Absorption and toxicity	HFB_1 is more bioavailable than FB_1 and the partial hydrolized FB_1.	Caloni *et al*. (2002)
Caco-2 cells	Absorption of FB_1 and HFB_1 into cells	FB_1 not absorbed by Caco cells. HFB_1 absorption is limited by a P-glycoprotein efflux mechanism	Caloni *et al*. (2005)
NIH 3T3 fibroblasts	Glycosphingolipid synthesis	Ceramide inhibition results in the up-regulation of the activities of enzymes in the synthesis of the neutral glycosphingolipids (Gb_3)	Meivar-Levy and Futerman (1999)
Rat splenic macrophage and lymphocytes cells	Nitric oxide (NO) production; T-cell activation	FB_1 stimulates and potentiates TNF-γ-induced NO production in macrophages. FB_1 potentiates proliferation of splenic cells in the presence of a NO synthase inhibitor.	Dombrink-Kurtzman *et al*. (2000)
Murine macrophages (J774A.1)	NO and PGE_2 production	Increased NO and prostaglandin E_2 production related to increased iNOS and COX-2 expression	Meli *et al*. (2000)
J774A.1	Cytotoxicity; lipid peroxidation	FB_1 enhances susceptibility to oxidative damage; increased membrane microviscosity; enhanced horseradish peroxidase endocytosis, which is reverted by IFN-γ	Ferrante *et al*. (2002)
S-59 $\gamma\delta$ T lymphocytes	Growth arrest in IL-2-dependent proliferation	WCI-induced growth arrest via the accumulation of ceramide. FB_1 blocked WCI-induced growth arrest.	Kirkham *et al*. (2000)
Mouse cytotoxic T-cells (CTLL-2)	Regulation of CD95-induced apoptosis	FB_1 does not block activation-induced cell death (AICD); myriocin, inhibitor of serine palmitoyl-CoA transferase, blocks AICD; sphinganine counteracts the inhibition of myriocin.	Solomon *et al*. (2003)

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Table 1 (*continued*)

Model	Biological effects/end points	Biochemical alterations	Reference
Avian immune cells peripheral blood lymphocytes (PBL), splenocytes and thymocytes)	Cell viability and proliferative response after mitogen stimulation	FB_1 lacks any effect on PBL; increase number of viable thymic cells with no effect on mitogenic response; secreased spleen viability and mitogenic response	Keck and Bodine (2006)
Swine peripheral blood mononuclear cells	Lymphocytes proliferation and IL-2 production	FB_1 decreases cell proliferation in G0/G1 phase. IL-2 production is decreased by FB_1 in ConA-stimulated cells.	Marin *et al*. (2007)
Murine macrophages (J774A.1) cocultured with liver epithelial cells (NMuLi)	Cytotoxicity measurements and cytokine production	Cocultures exhibit cytotoxic effects against FB_1 while separate cultures show no effect; sphinganine accumulates to a similar extent, while cocultures express TNFα and IL-12; media of FB_1-treated macrophages induce IFNγ expression in NMuLi cells. Cytokine expression potentiated FB_1-induced cytotoxic effects	Sharma *et al*. (2004b)
Neonatal mouse cardiomyocytes	Cell viability (MTT assay)	FB_1 prevented nitrosoglutathione-induced cell death via the reduction of ceramide accumulation	Rabkin (2002)
Rat brain glioma cells (line C6)	Cell viability; cell cycle arrest	FB_1 arrest cells in G2/M phase; induced hypermethylation of DNA; inhibition of protein synthesis	Mobio *et al*. (2000)
Astrocytes and oligodendrocytes of rat cerebrum	Sphingosine accumulation; proliferation	Modify proliferation and differentiation of glial cells	Kwon *et al*. (2000)
C6 gliomal and mouse embryonic fibroblasts (MEF)	Cytotoxicity, protein and DNA synthesis	Induction of lipid peroxidation that was inhibited with antioxidants; oxidative DNA damage in both cell lines; gliomal cells undergo apoptosis while embryonic cells were resistant; MEF cells lost protective effects mediated by p53.	Mobio *et al*. (2003)
Glioblastoma cells (U-118 MG)	Toxicity and oxidative damage	FB_1 increase lipid peroxidation and production of ROS; depletion of GSH associated with decreased cell viability; increased caspase-3 and DNA fragmentation; oxidative stress and apoptosis key determinants for neurotoxicity	Stockmann-Juvala *et al*. (2004)
Oesophageal cancer cells (SNO)	Cytotoxicity	FB_1 predisposes cells for cytotoxicity of diethylnitrosamine. Inhibited cytotoxicity induced by dietary catechol, a constituent of cigarette smoke	Myburg *et al*. (2002)
Comparative and interactive effects between different mycotoxins in cell culture models			
Swine alveolar macrophages	Cytotoxicity and DNA fragmentation	FB_1 and AFB_1 immunotoxic to swine via independent mechanisms; FB_1 decreases the expression of IL-1β and TNF-α; FB_1 induces apoptosis and AFB_1 not; both induce the expression of apoptosis-related heat shock protein.	Liu *et al*. (2002)

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Table 1 (*continued*)

Model	Biological effects/end points	Biochemical alterations	Reference
Insect pupal ovary cells (SF-9)	Cytotoxicity and apoptosis	Nivalenol (NIV) and deoxynivalenol (DON) more cytotoxic than FB_1; cell arrest in G0/G1 phase; mycotoxins induce apoptosis	Fornelli *et al*. (2004)
Human erythroleukaemia cells (K562)	Cytotoxicity, proliferation and apoptosis	NIV and DON more cytotoxic than FB_1; cytotoxicity not related to membrane damage. NIV and DON induce more late stage apoptotic events.	Minervini *et al*. (2004)
Rat brain glioma (C6); human colon cancer (Caco-2); green monkey kidney (Vero) cells	Cytotoxicity	Synergistic effect of FB_1 and ochratoxin A	Creppy *et al*. (2004)
Human colon cancer (Caco-2)	Cell viability; DNA and protein synthesis; lipid peroxidation	FB_1 more cytotoxic when using the MTT assay than DON and zearalenone (ZEN); toxicity related to disruption of the mitochondria; DON more effective in the inhibition of protein and DNA synthesis; FB_1 more effective in inducing lipid peroxidation; DON and ZEN more effective in disruption of lysosomes.	Kouadio *et al*. (2005)
Human hepatoma cells (HepG2); human bronchial epithelial cells (BEAS-2B)	Cell cytotoxicty	IC_{50} values show that AFB_1 is more cytotoxic; combined effects showed an antagonistic effect in HepG2 cells and an additive effect in BEAS-2B.	McKean *et al*. (2006)
Human lymphoblastoid cells (Jurkat T)	Cytotoxicity, cell proliferation and apoptosis	ZEN reduces cytokines IL-2 and IFNγ and not FB_1; FB_1 increases cell proliferation, while ZEN shows inhibition; combined effects enhance ZEN inhibitory effects indicating interactive effects.	Luongo *et al*. (2006)
Porcine kidney epithelial cells (PK15)	Cell viability, GSH and thiobarbituric acid reactive substances (TBARS) levels	FB_1, ochratoxin A, beauvericin, increased TBARS, and decrease GSH. Combined effects is additive. Synergistic and antagonistic effects depending on time of exposure and dose.	Klarić *et al*. (2007)
Caco-2	Lipid peroxidation, protein and DNA synthesis, DNA fragmentation and methylation	ZEN, DON and FB_1 additive effects on DNA damage. Increase methylation of DNA with no additive effects. Less than additive effects on DNA synthesis. Synergistic effects on lipid peroxidation.	Kouadio *et al*. (2007b)

of complex sphingolipids from ceramide is reduced. These sphingolipids include sphingomyelin and gangliosides, which are important structural components of the cell membrane and growth-factor receptors, respectively. Ceramide synthase disruption appears to be a critical step as it normally precedes FB-induced toxic effects in different organs of many animal species. The disruption of this pathway and the effect on TNF-α and Fas ligand-induced apoptosis still need to be elucidated. The binding of TNF-a to Fas activates sphingomyelinase, yielding ceramide from sphingomyelin, may be linked to the biological action of IFN-γ and arachidonic acid release (Soriano *et al*., 2005). Modulation of protein kinase Cα, an internal acceptor for tumour promoters, may be related to FB_1 involvement during multistage carcinogenesis. Studies in TNFα (TNFR1 and TNFR2) knockout mice showed reduced hepatotoxic effects associated with the reduction in sphinganine accumulation (Voss *et al*., 2002; Sharma *et al*., 2001; 2002).

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However, double knockout mouse showed increased apoptosis compared to the wild-type, indicating the complexity of the mechanism involved in FB_1 toxicity (Sharma *et al*., 2002). FB_1 caused the expression of oncogenic transcription factors and apoptotic genes, as well as activation of the cytokine network in the liver of mice, which was suggested to play a role in cancer promotion (Bhandari and Sharma, 2002; Sharma *et al*., 2003). A different cytotoxic pathway, associated with sphingolipid deregulation seem to exist when considering hepatotoxic effects in mice (Sharma *et al*., 2006). A recent study showed that the induction of nitric oxide (iNOS) gene also plays an important role in sensitivity to the hepatotoxic effect of FB_1 via the regulation of sphingosine kinase and the generation of sphingosine 1-phosphate (Suzuki *et al*., 2007). Changes in the disruption of signalling pathways related to sphingolipid metabolism and subsequent defects in the apoptotic and cytokine expression have been associated with the resistant of hepatocytes in a specific mice strain (Sharma *et al*., 2005). Depletion of the T cells by antibodies against mature peripheral T lymphocytes abolished FB_1-induced hepatotoxic effects in mice by inhibiting the formation of proinflammatory cytokines (Sharma *et al*., 2006). Upregulation of proinflammatory cytokines was also associated with neurodegeneration in murine brain after intraventricular infusion with FB_1 (Osuchowski *et al*., 2005). The hepatotoxic effects were also shown to be independent of the peroxisome proliferator-activated receptor (Voss *et al*., 2006b).

The relative sensitivity of different organs such as rat liver and kidneys was suggested due to differences in fumonisin accumulation and clearance, as well as sphingolipid metabolism into their phosphate metabolites (Soriano *et al*., 2005; Riley and Voss, 2006). The rapid turnover of sphinganine in the liver and intestine and the persistent accumulation in the kidneys is likely to explain the sensitivity of the latter to FB_1-induced toxic effects. Differences, however, exist in mice where the liver appears to be the more sensitive organ, as compare to the kidneys. The accumulation of sphingoid base 1-phosphate due to the induction of sphingosine kinase and inhibition of sphingosine 1-phosphate lyase are key determinants in FB_1-induced apoptosis (Kim *et al*., 2007). The exact role of the sphingolipid mediators in fumonisin toxicity is, however, not known at present and other mechanisms also should be considered.

The induction of oxidative stress and the resulting oxidative damage to membranal and subcellular organelles and macromolecules, such as DNA, should also be considered (Abel and Gelderblom, 1998). Recent reviews have indicated the involvement of sphingolipid turnover in regulating the cellular redox status (Won and Singh, 2006; Kitatani *et al*., 2008). The role of sphingomyelinase activation and cellular glutathione in the regulation of reactive oxygen and nitrogen species has been suggested. Downstream effects of ceramide are also important in the regulation of NADPH oxidase, mitochondrial integrity and antioxidant enzymes. TNF-α, an inducer of oxidative stress and stimulated by FB_1, enhances the generation of long-chain ceramide through a sphingosine recycling pathway, while other studies have reported on the ability of TNF-α to stimulate ceramide formation through sphingomyelinase. It was shown that FB_1 alters cellular redox balance in the liver and kidneys, leading to changes in the glutathione cycle and the activation and expression of redox-sensitive signalling molecules such as mitogen-activated protein kinases (MAPKs) (ERK and p38) and heat-shock proteins (Rumora *et al*., 2007). The interaction of changes in sphingolipid metabolism and the cellular redox balance could be important in clarifying the cytotoxic effects of the fumonisins.

4.8.3 Carcinogenic Effect in Rats and Mice

The fumonisins are regarded as nongenotoxic carcinogens as they lack mutagenic and genotoxic effects in many *in vitro* assays. Although characterized as liver-cancer promoters, they also exhibit cancer-initiating properties in the liver. The *in vivo* genotoxic effects were therefore the topic of many investigations, with the induction of oxidative cell damage as one of the possibilities of being important in the genesis of DNA damage.

4.8.3.1 Genotoxicity of Fumonisins

In vitro mutagenesis assays and *in vivo* DNA repair assays failed to indicate any direct interaction of the fumonisins with DNA. Indirect evidence of interaction with DNA resulted from studies conducted under conditions where toxic effects prevail, implying the interaction of reactive oxygen species with macromolecules, including DNA (see Bolger *et al*., 2001 for review). A study in C6 glioma and mouse embryonic fibroblast (MEC) cells showed that FB_1 induced lipid peroxidation, which was associated with a dose-related increase in 8-OH-deoxyguanine and DNA fragmentation (Mobio *et al*., 2000). However, studies in human fibroblasts and rat astrocytes showed that apoptotic-type DNA damage occurred without increased ROS production (Galvano *et al*., 2002a; 2002b), indicating that lipid peroxidation is an end result rather than a cause of FB_1-induced injury, as suggested previously (Abel and Gelderblom, 1998; Lemmer *et al*., 1999b). In human-derived hepatoma cells, FB_1 affected clastogenic changes, suggested that the mycotoxins may act as a genotoxic carcinogen in humans (Ehrlich *et al*., 2002). The genotoxic effects are exerted in the absence of cell cytotoxicity and induced early by modulating the expression of some genes (Galvano *et al*., 2002a). An *in vivo* study showed that FB_1 induced DNA damage in the kidney and liver prior to inducing oxidative damage, suggesting that changes in sphingolipid metabolism may play a role in DNA damage (Domijan *et al*., 2007). In the liver, however, the oxidative markers

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and changes to the sphingolipid levels coincided with DNA damage.

4.8.3.2 Cancer Initiation

Studies in rat liver showed that FB_1 exhibited cancer-initiating activity (Gelderblom *et al*., 1991; 1994; 1996; 2001b; 2008a). Based on the role of cell proliferation and the irreversible nature thereof, cancer initiation of FB_1 mimics that of genotoxic carcinogens, although the kinetics differ depending on the dose and time of exposure. The fact that different cancer-promoting stimuli selectively promote the development of FB_1-induced initiated hepatocytes into nodules further emphasizes the cancer-initiating potency. A hepatotoxic threshold exists for cancer initiation in the liver, with high doses fed over a short-time period of exposure. The irreversible nature of the cancer-initiating effect was demonstrated by a five-week exposure to FB_1 that resulted in the development of adenomas after one year (Lemmer *et al*., 2004). A long-term study, feeding low dose levels also initiated cancer, which emphasized the complexicity of FB_1–tissue interactions. A specific role for spontaneously initiated cells in the development of FB_1-induced liver carcinogenesis could not be demonstrated in older rats when utilizing a short-term cancer initiating/promoting model (Gelderblom *et al*., 2008a). Although the exact nature of the initiating step is not known, a possible role of oxidative DNA damage has been suggested (Abel and Gelderblom, 1998; Mobio *et al*., 2003).

4.8.3.3 Cancer Promotion

Two major mechanisms of FB_1-induced carcinogenesis, both involving lipid metabolism, have been proposed: (i) the role of FB_1-induced apoptosis due to the disruption of ceramide synthase in the development of hepato- and nephrocarcinogenesis in rats and mice and (ii) the disruption of phospholipid and, mainly, fatty-acid metabolic pathways as important events in the development of hepatocarcinogenesis in rats (Burger *et al*., 2007; Gelderblom *et al*., 2001c; 2008b).

4.8.3.3.1 FB_1 Apoptosis Hypothesis

The induction of apoptosis and necrosis, resulting in a compensatory regenerative response could theoretically increase the likelihood of cancer development (Dragan *et al*., 2001; Howard *et al*., 2001b; Riley *et al*., 2001; Voss *et al*., 2002). In rat kidneys, cell death is exclusively of an apoptotic nature followed by regeneration of the renal tubule epithelial cells. Another possibility is the development of a subpopulation of cells that are resistant to FB_1-induced apoptosis accumulating heritable genetic changes during their increased lifespan. The balance between effectors, which either induce apoptosis (increased ceramide or free sphingoid bases) or stimulate proliferation (decreased ceramide and increased sphingoid 1-phosphate) could eventually determine the outcome regarding the tumourigenic response (Riley *et al*., 2001). Cells sensitive to the proliferative effect of decreased ceramide and increased sphingoid 1-phosphates will survive, thereby creating a proliferative or mitogenic response, eventually leading to cancer. On the other hand, cells sensitive to sphingoid-base-induced growth arrest, due to the accumulation of free sphingoid bases, will die, while the resistant cells will survive, thereby increasing the risk of developing cancer specifically if they are genetically altered. At present the phases of cancer development have not been characterized in the kidneys. Possible precursor lesions were the atypical tubule regenerative lesions which were noticed in the proximal tubules 24 months after chronic treatment with FB_1 (Hard *et al*., 2001). No information regarding the kinetics of lesion development or information regarding the disruption of sphingoid metabolism and the levels of sphingoid metabolites in these and/or the cancerous lesions has been reported in an attempt to clarify the role of sphingolipids.

4.8.3.3.2 Resistant Hepatocyte Hypothesis

Several studies have shown that FB_1 affects both cancer initiation and promotion in the liver and as such mimics genotoxic carcinogens. The mechanism involved during the induction of resistant hepatocytes is not known at present, but a chronic toxic effect resulting in oxidative stress is likely to be involved. The genesis of the resistant hepatocytes induced by FB_1 has been characterized and found to be similar to that induced by genotoxic hepatocarcinogens with respect to its dependence on hepatocyte proliferation of the regenerative type. Proliferation of the mitogenic type failed to support initiation by FB_1 (Gelderblom *et al*., 2001b). A recent study showed that the initiated hepatocytes were selectively stimulated to grow by phenobarbital, a known cancer promoter (Gelderblom *et al*., 2008a). It was also shown that spontaneous initiated hepatocytes, normally present in the liver of older animals, could not explain the cancer-initiating properties of FB_1 in rat liver. The mechanism of promotion by FB_1 was based on the principle of differential inhibition, whereby FB_1 inhibits the proliferation of normal cells, while the resistant hepatocytes are allowed to proliferate. The apparent mechanism involved the inhibition of δ-6 desaturase, a key enzyme in the fatty acid metabolic pathway, resulting in the reduction in the long-chain polyunsaturated fatty content. In hepatocyte nodules, the reduction in long-chain polyunsaturated fatty acids (LCPUFAs) rendered them more resistant to oxidative stress, while normal cells will undergo apoptosis (Abel *et al*., 2001). In addition, the increased levels of arachidonic acid due to an enhanced level of phosphatydylethanolamine provide the nodules with a selective growth advantage due to an increased formation of prostaglandin E2. Interaction of AA with ceramide via sphingomylinase also

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links the fatty-acid metabolic pathway with the sphingolipid pathway affecting different cell-survival parameters. These interactive pathways and secondary responses, involving different cell-cycle signalling pathways have been reviewed in detail (Gelderblom *et al*., 2001c; 2008b; Van der Westhuizen *et al*., 2004; Burger *et al*., 2007).

4.9 Reduction, Decontamination and Inactivation Procedures

Several detoxification processes of fumonisins have been investigated. Ammoniation, effectively used in the decontamination of aflatoxins, destroyed only 50% of the FB_1, when applying similar conditions (Chourasia, 2001). As the primary amino group in FB_1 is a prerequisite for cytotoxicity, many attempts have been focussed on either blocking it, thorough diazotation (Lemke *et al*., 2001), or removing this moiety by reaction with glucose (Liu *et al*., 2001; Fernandez-Surumay *et al*., 2004; 2005). These studies showed that the modification of the primary amine significantly reduced the toxic effects of the fumonisins. Other treatments included the use of bentonite clay, fermentative bacteria, clinoptilolite-rich zeolite tuff containing octadecyldimethylbenzyl ammonium ions at different pH values (Miazzo *et al*., 2005; Dakovic *et al*., 2007; Niderkorn *et al*., 2007) and activated carbon (Piva *et al*., 2005). Bentonite and activated carbon failed to reduce the toxic effects of FB_1, while *Lactobacillus* and the organozeolites significantly bound the fumonisins in a pH-dependent manner. Conflicting findings were reported on the effect of gamma irradiation. Application of a radiation dose of 5 kGy completely destroyed FB_1 in wheat, maize and barley (Aziz *et al*., 2007). The viability of *Fusarium* in seeds also decreased with increased radiation dose levels. Conversely, a study in maize showed that a radiation dose of 30–100 kGy totally eliminated *Fusarium* spp. in ground and whole maize (D'Ovidio *et al*., 2007). No reduction, however, was noticed in the level of FB_1 in corn samples irradiated under similar conditions. Nixtamlization of maize and the reduction of the fumonisins in maize have been extensively reviewed (Bolger *et al*., 2001). FB_1 and hydrolysed FB_1 (HFB_1) were found to account for 50% in tortillas prepared in a traditional way (Palencia *et al*., 2003). The effect of shelling and heating were also investigated recently. Mechanical dehulling of maize reduced the fumonisin levels by approximately 60%, depending on the method used (Fandohan *et al*., 2006). Temperatures above 150–200 °C reduced the fumonisin content of different products; the reduction depended on many factors, including the type of sugar present, cooking time, pH, moisture content and the specific recipe used (Humpf and Voss, 2004). Traditional African cooking of maize reduces the FB_1 levels by 23% (Shephard *et al*., 2002).

Reduction of fumonisin contamination via genetic modification or transgenic approaches, resulted in the development of *Bacillus thuringiensis* (Bt) maize (Duvick, 2001). Field studies using Bt maize have been widely applied and showed a significant reduction in fumonisin contamination. Alternative detoxification and/or inhibitory effects on fumonisin production were investigated *in vitro* by utilizing essential oils, cyclic terpenes and naturally occurring phenols. Although certain oils inhibited the growth of *F. verticillioides*, and therefore fumonisin production, they adversely affected kernel germination (Fandohan *et al*., 2004). In contrast, oil from neem seeds accelerated the growth of the fungus. Of the cyclic terpenes, limonene and thymol showed the highest inhibitory effect on FB_1 production (Dambolena *et al*., 2008). Of the plant phenols, chlorophorin was the most active phenol in reducing FB_1 production in culture, followed by caffeic acid and ferulic acid (Beekrum *et al*., 2003).

5 RISK ASSESSMENT OF AFLATOXINS, OCHRATOXINS AND FUMONISINS

5.1 Aflatoxins

When considering the epidemiological data, studies in animals, and different metabolic studies *in vivo* and *in vitro*, aflatoxin is treated as a human carcinogen (Group I). In such cases, the exposure must be kept as low as reasonably achievable (WHO, 2008a; International Agency for Research on Cancer, 2002), and a hypothetical standard ($20\,\mu g\,kg^{-1}$) was therefore set for aflatoxin exposure by the WHO (2008a). However, the risk of liver cancer attributable to aflatoxin and other factors is up to 30-fold higher in populations chronically infected with the hepatitis B and hepatitis C viruses. It was concluded that for populations from western Europe and the USA with a low hepatitis B surface antigen (HbsAg)-positive prevalence, aflatoxin levels below $20\,\mu g\,kg^{-1}$ in maize would be unlikely to decrease the incidence of liver cancer. Similar results were obtained for aflatoxin in groundnuts. Reducing the hypothetical standard from 20 to $10\,\mu g\,kg^{-1}$ would reduce the estimated population risk of liver cancer to two additional cancer cases per year per 10^9 people, as compared to an estimated 300 cases of cancer in people that are carriers of hepatitis B. Populations with a high HBsAg-positive prevalence and a high aflatoxin intake would thus benefit from such reductions, except in cases where there is increased risk of malnutrition or starvation. However, if the EU regulation of $4\,\mu g\,kg^{-1}$ of aflatoxins is adopted, this would encourage the export of the better-quality crops, with

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the poor quality crops being left for domestic consumption, which in turn could increase the risk of liver cancer among hepatitis-infected populations (Wu, 2004).

As a result of consecutive outbreaks of acute aflatoxicosis in Kenya in 2004 and 2005, which resulted in more than 150 deaths, a workgroup of international experts and health officials was convened by the Centers for Disease Control and Prevention and the World Health Organization. They identified gaps in current knowledge about acute and chronic human-health effects of aflatoxins, surveillance and food monitoring, analytic methods and the efficacy of intervention strategies (Strosnider *et al*., 2006).

5.2 Ochratoxins

Both genotoxic and nongenotoxic mechanisms have been suggested to play a role in the development of renal tumours by OTA, although the presence of DNA adducts containing OTA has not yet been confirmed. Consequently, different approaches to the risk assessment of OTA have been adopted by regulatory bodies in different countries, depending on whether the carcinogenicity arises through a threshold or a nonthreshold mechanism. Currently, risk assessment of increased tumour formation induced by OTA is based on a nongenotoxic mode of action exhibiting a threshold. Evaluation by JECFA, both in 2001 and 2007, retained the previously established provisional tolerable weekly intake (PTWI) of 100 ng (kg body weight)$^{-1}$ per week (WHO, 2001; 2008b). Current dietary exposure levels of humans to OTA of 8–17 (kg body weight)$^{-1}$ per week is well below the PTWI, particularly when one considers the consumption of processed cereals in Europe. As contamination levels of raw cereals are below 5 μg kg^{-1}, with only a small number of samples reaching 20 μg kg^{-1}, MTLs of this order will have little impact on dietary exposure to OTA. The situation in developing countries, however, is not clear, as very little data is available on contamination levels and dietary exposure.

5.3 Fumonisins

The NOEL for cancer induction in the liver has been estimated to be in the order of 0.8 mg FB_1 (kg body weight)$^{-1}$ per day, which represents a dietary level of 25 mg FB_1 kg^{-1} over a period of two years (Gelderblom *et al*., 2001a). When using the safety factor approach, a TDI of 0.8 μg (kg body weight)$^{-1}$ per day was calculated for humans, using a safety factor of 1000 (Gelderblom *et al*., 1996). The probable daily intake (PDI) levels for humans will depend on the level of fumonisin contamination and the maize intake profiles, which differ between countries and specific population groups within the same country. In South Africa, urban blacks consume less maize than rural blacks (250 vs. 500 g per person per day, respectively) while the European community consumes extremely low levels of maize (approximately 5 g per person per day). The health risk as a result of fumonisin exposure will therefore differ.

When using the NOEL for kidney toxicity, a provisional maximum tolerable daily intake (PMTDI) of 2 μg (kg body weight)$^{-1}$ per day was calculated, using a safety factor of 100 (WHO, 2002). The MTL established using the exposure and hazard assessments are levels of about 2 and 4 mg kg^{-1} for maize products and whole maize kernels, respectively (FDA, 2001). However, using the risk parameters developed from the NOEL for liver-cancer development, Marasas (1997), proposed levels 10–20 times lower, between 0.1 and 0.2 mg kg^{-1}. These MTL levels however, would seriously affect the food supply and the maize chain between producer and consumer (Viljoen and Marasas, 2003). In developed countries, the PDI levels are well below the PMTDI level proposed by JECFA (WHO, 2002), due to the consumption of small amounts of maize with the lowest contamination levels. However, certain subpopulations in developed countries that consume high quantities are still at risk (De Nijs *et al*., 1997). With respect to subsistence farmers in developing countries, it was suggested that large amounts of maize are consumed, often contaminated with high levels of fumonisins, which provide PDI levels between 10 and 50 times higher that the PMTDI. Another subpopulation that could be adversely affected, when using maize as a monocereal diet in rural areas, is children. As control of fumonisin levels in rural areas is critical, importation of maize with fairly high contamination levels from developed countries could also pose a problem, and specific control measures need to be implemented. However, details of these concerns have already been raised and were recently debated (Shephard *et al*., 2007; Marasas *et al*., 2008; Gelderblom *et al*., 2008b).

6 REGULATIONS FOR AFLATOXINS, OCHRATOXINS AND FUMONISINS

Since the discovery of aflatoxins in the 1960s, an increasing number of countries have legislated MTLs for an increasing number of mycotoxins with the aim of protecting both human and animal populations from the harmful effects of mycotoxin exposure. The most recent international effort to collate these regulations was undertaken under a Food and Agriculture Organization (FAO) project during 2002 and 2003, and a comprehensive compilation has been published as an FAO Food and Nutrition paper (Food and Agriculture Organization,

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2004). These have also been reviewed in the literature (Van Egmond *et al*., 2007).

Aflatoxin remains the most widely legislated mycotoxin. At least 99 countries have regulatory limits for AFB_1 or the sum of AFB_1, AFB_2, AFG_1 and AFG_2 in food and/or feed. The MTLs themselves and the details of the regulations vary widely between countries. In general, limits for AFB_1 in food in different countries range from 1 to 20 $\mu g\,kg^{-1}$, with the limits in the EU for AFB_1 and for total aflatoxins now set at 2 and 4 $\mu g\,kg^{-1}$, respectively, for human food. These MTLs legislated in the EU have elicited considerable international comment concerning the potential effect they may have on exports from developing countries, especially those in Africa. Based on economic modelling of the exportation of edible groundnuts from nine African countries, it was postulated that these MTLs would lead to a 36% reduction in trade and a loss of exports of US$670 million (Otsuki *et al*., 2001). A subsequent World Bank Policy Research Working Paper put the actual loss at hundreds of thousands, rather than hundreds of millions (Jaffee and Henson, 2004). An empirical economic model was developed to further quantify expected export losses for a range of groundnut-exporting countries, including Argentina, China, USA and Africa, if the EU model was internationally adopted. This model predicted total losses would be of the order of US$446 million (Wu, 2006).

OTA is regulated in cereals and/or cereal products in over 30 countries, mainly in Europe. MTLs generally fall in the range 3–50 $\mu g\,kg^{-1}$. For example, within the EU, two limits have been set, namely 5 $\mu g\,kg^{-1}$ for raw cereals and 3 $\mu g\,kg^{-1}$ for processed cereal products intended for direct human consumption. In some countries, other sources of OTA may also be regulated. In the EU, dried vine fruit (raisins, sultanas and currants) have a MTL for OTA of 10 $\mu g\,kg^{-1}$, roasted coffee beans 5.0 $\mu g\,kg^{-1}$, instant coffee 10.0 $\mu g\,kg^{-1}$, wine and grape juice 2.0 $\mu g\,kg^{-1}$, and foods for infants and young children 0.50 $\mu g\,kg^{-1}$ (European Commission, 2006; Food and Agriculture Organization, 2004).

As a relatively recently discovered mycotoxin, only six countries in the FAO survey reported regulations for fumonisin ranging between 1000 and 3000 $\mu g\,kg^{-1}$ (Food and Agriculture Organization, 2004). However, with new EU regulations coming into force, this number has increased significantly. These regulations specify regulatory levels between 4000 $\mu g\,kg^{-1}$ for unprocessed maize (unless intended for wet milling), to 1000 $\mu g\,kg^{-1}$ for maize intended for direct human consumption and down to 200 $\mu g\,kg^{-1}$ for baby foods for infants and young children (European Commission, 2007).

7 CONCLUSION

Although the discovery of the aflatoxins as the causal agents of turkey X-disease in the UK happened 50 years ago, there is still an unabated worldwide concern about mycotoxins, as evidenced by the explosive growth in mycotoxin literature, particularly on the fumonisins and ochratoxins. Sterling progress on the genetics and genome sequence of the some of the filamentous fungi has been achieved, as well as a molecular understanding of the genomics of the toxicity of mycotoxins. Advances in immuno-based and physicochemical techniques have enabled the detection of mycotoxins at ppb levels in food and feeds. Based on these considerations, several countries and international organizations have introduced legislation to control mycotoxin contamination. The prevention of mycotoxin contamination of commodities remains a daunting challenge. In future, the focus should be on better agricultural practice, breeding of mycotoxin-resistant cereal and nut-producing cultivars, storage of crops under hygienic conditions and inactivation of mycotoxins by employing enzymes and micro-organisms.

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Botulism

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CONTENTS

1 INTRODUCTION

Botulinum neurotoxins (BoNTs) produced by *Clostridia* species are among the most potent identified toxins. These toxins cause the potentially fatal paralysis known as 'botulism'; this serious intoxication can be a medical and public health emergency for which prompt diagnosis and early treatment are essential. Five well-defined forms of botulism are identified in relation to the route of exposure: foodborne, infant and adult intestinal, wound, iatrogenic and inhalation botulism; some authors add a sixth form as botulism of unknown source (Johnson and Montecucco, 2008). All these forms are finally characterized by the same clinical syndrome, which is caused by toxin-induced neuromuscular paralysis. Among the seven known neurotoxins (types A–G), the types A, B and E (rarely F) are toxic in humans, whereas the other mainly cause disease in animals.

1.1 Definitions

In all its forms, botulism is a poisoning related to the toxic effects of specific xenobiotics, the BoNTs. The

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disease in not contagious and patients do not require isolation: only a hypothetical exposure originating from an aerial dispersion (e.g. terrorist attack) could potentially contaminate the nonexposed rescuers. The following 'clinical description, laboratory criteria for diagnosis and case classification' refer to the USA CDC (Centers for Disease Control and Prevention), (1997) and EU European Union (EU) (2002).

1.1.1 Foodborne Botulism

Foodborne botulism results from consumption of preformed BoNT complexes in food:

1. **Clinical description**: Ingestion of BoNTs results in an illness of variable severity. Common symptoms are diplopia, blurred vision and bulbar weakness. Symmetrical paralysis (descending) may progress rapidly
2. **Laboratory criteria for diagnosis**: Detection of BoNTs in serum, stool or patient's food or isolation of *Clostridium botulinum* from stools
3. **Case classification**:
   - **Confirmed**: A clinically compatible case that is laboratory confirmed or that occurs among persons who ate the same food as persons who have laboratory-confirmed botulism
   - **Probable**: A clinically compatible case with an epidemiological link (e.g. ingestion of home-canned or other potentially suspect products within the previous 48 hours).

1.1.2 Wound Botulism

Wound botulism, as with tetanus, occurs when spores of *Clostridium* sp. contaminate and colonize a wound in anaerobic conditions with a subsequent *in situ* multiplication of the organism and production of BoNTs that finally enter into the bloodstream. However, the frequency of wound botulism is much lower than tetanus, despite the fact that the population is not immunized against botulism, whereas many Western populations are immunized against tetanus:

1. **Clinical description**: An illness resulting from toxin produced by *C. botulinum* that has infected a wound. Common symptoms are diplopia, blurred vision and bulbar weakness. Symmetric paralysis may progress rapidly
2. **Laboratory criteria for diagnosis**: Detection of botulinum toxin in serum or isolation of *C. botulinum* from wound
3. **Case classification**:
   - **Confirmed**: A clinically compatible case that is laboratory confirmed in a patient who has no suspected exposure to contaminated food (i.e. has been ruled out as a food-borne botulism case) and who has a history of a fresh, contaminated wound during the two weeks before onset of symptoms.

1.1.3 Intestinal Toxaemia (Adult Intestinal and Infant Botulism)

Botulism can occur as a result of *Clostridium* sp. colonization of the intestinal lumen with subsequently production *in vivo* of BoNTs causing the form of botulism poisoning defined as 'intestinal toxaemia' (Arnon, 1995). The colonization of the intestinal tract by *Clostridium* sp. in an infant under one year of age is defined as 'infant botulism', while in children older than one year or adults cases are defined as 'adult intestinal botulism' (also referred as 'hidden botulism' or 'other botulism').

1.1.3.1 Adult Intestinal Botulism

Adult intestinal botulism is rare and resembles infant botulism in its pathogenesis and clinical status. People with altered intestinal flora are particularly vulnerable; also anatomical or functional bowel abnormalities may permit neurotoxigenic Clostridia colonization:

1. **Case classification**:
   - **Confirmed**: A clinically compatible case that is laboratory confirmed in a patient aged greater than or equal to one year who has no history of ingestion of suspect food and has no wounds.

1.1.3.2 Infant Botulism

Infant botulism results from absorption of BoNTs produced *in situ* by neurotoxigenic Clostridia colonizing the intestinal lumen (large intestine) in infants aged less than one year, who have ingested the spores.

Infant botulism is a disease that affects infants between one and 52 weeks of age, and not later. Infant botulism, first recognized in 1976 (Midura and Arnon, 1976), is the most common form of human botulism in the USA, where it is already included in the National Organization for Rare Disorders:

1. **Clinical description**: An illness of infants, characterized by constipation, poor feeding and 'failure to thrive' that may be followed by progressive weakness, impaired respiration and death
2. **Laboratory criteria for diagnosis**: Detection of BoNTs in stool or serum or isolation of *C. botulinum* from stools
3. **Case classification**:
   - **Confirmed**: A clinically compatible case that is laboratory confirmed, occurring in a child aged less one year.

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1.1.4 Iatrogenic Botulism

Iatrogenic botulism, sometimes referred as 'inadvertent botulism' is caused by injection of BoNTs for cosmetic or therapeutic purposes.

1.1.5 Inhalation Botulism

Inhalational botulism, a disease resulting from aerosolized toxin, has been produced experimentally in primates (Franz *et al*., 1993), has been attempted by terrorists (Zilinskas, 1997; WuDunn *et al*., 1998; Tucker, 2000; Arnon *et al*., 2001) and has occurred accidentally in humans (Holzer, 1962).

1.1.6 Botulism from Unknown Source

This form should be considered in cases one year of age or older in whom no plausible food vehicle, or intestinal or wound colonization can be demonstrated:

1. **Clinical description**: See foodborne botulism
2. **Laboratory criteria for diagnosis**: Detection of BoNTs or isolation of *Clostridium botulinum* from clinical specimens
3. **Case classification**:
   - **Confirmed**. A clinically compatible case that is laboratory confirmed in a patient aged greater than or equal to one year who has no history of ingestion of suspect food and has no wounds.

1.2 History

The recorded history of botulism begins in 1735, when minor outbreaks were described. Later, the first well-recognized outbreak occurred in Wildbad in the state of Baden-Württemberg, Germany in 1793, when six people over 13 died after eating a popular blutwurst (blood sausage, black pudding). Following this, the number of reported cases of sausage poisoning rapidly increased, prompting a study of the disease in southern Germany by Kerner, who described 230 cases, as well as the clinical syndrome (Kerner, 1817). He also performed animal and clinical experiments, and hypothesized the possible therapeutic use of botulinum toxin. At approximately the same time, some Russian physicians identified a disease with similar clinical manifestations termed 'fish poisoning' (Young, 1976). In 1870, the German physician Muller derived the name botulism from the Latin term *botulus*, which means 'sausage' (Torrens, 1998). The isolation of the clostridial organism responsible for botulism was made in the nineteenth century by the microbiologist Emilie Pierre Van Ermengem, who conducted a meticulous investigation as a response to an extraordinary outbreak in Ellezelles (Belgium) in December 1895, involving 34 musicians who had consumed raw-salted ham after a funeral meal. During the next two days, 23 of the musicians became ill with increasing neuromuscular paralysis (mydriasis, diplopia, dysphagia, dysartria and progressive muscle paralysis): three of them died. In the investigation of this outbreak, Van Ermengem established that botulism was an intoxication, not an infection, and that the toxin was produced by a spore-forming obligate anaerobic bacterium, called '*Bacillus botulinus*' (Van Ermengen, 1897). The pathogen was later renamed *Clostridium botulinum* to acknowledge both its spindle shape (*kloster* is Greek for spindle) and its anaerobic metabolism (Erbguth, 2008).

A later outbreak in Darmstadt (in the German state of Hesse) associated with canned white beans showed a second type of botulism: the new strain was Type A, while the Van Ermengem strain probably was Type B. In 1922, Type C botulism was identified as causing disease in chickens in USA by Bengston and in Australian cattle by Seddon. Meyer and Gunisson identified the type-D in the USA, and Bier the type-E in Ukraine. Type-F was first described by Moller and Scheibel from a Danish outbreak of botulism involving homemade liver pâté. Finally, type-G botulism was identified in soil from cornfields in Argentina by Giménez and Ciccarelli (Gunn, 1979; Kriek and Odendaal, 1994; Geiges, 2002; Erbguth, 2008).

2 CLOSTRIDIA PRODUCING BOTULINUM NEUROTOXINS

BoNT-producing Clostridia display heterogeneous bacteriological characters and are divided into several species and groups. The taxonomic position of the *C. botulinum* species was originally based on the production of a BoNT, and nontoxic variant strains, although genetically related to *C. botulinum,* were assigned to different species. Moreover, some of the seven different serologically distinct BoNTs (termed with letters from A to G) can be further divided into subserotypes; for example, there are four subserotypes of BoNT/A (termed A1–A4), which are approximately 90% identical in their amino-acid sequences (Arndt *et al*., 2006).

BoNT can be produced by other *Clostridium* species such as *C. butyricum* and *C. baratii*, which have been implicated in human botulism (Hall *et al*., 1985). The toxinogenic property of the *Clostridium* strains remain an important characteristic, mainly in clinical aspects, but this single property is not sufficient to define a bacterial species. Moreover, toxigenicity is unstable, and it appears to be transferable to a wide variety of *Clostridium* species.

C. botulinum strains are divided into four physiological groups:

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- Group I includes *C. botulinum* A, and proteolytic stains of Types B and F, and it is characterized by proteolytic activity and by the nonacidification of carbohydrates. The optimal temperature of growth is 37 °C. The thermoresistance of the spores is usually high (above 120 °C).
- Group II (*C. botulinum* E, and glucidolytic strains of types B and F) acidifies many carbohydrates and produce mainly butyric and acetic acids from trypticase–yeast-extract–glucose broth. They hydrolyse gelatin, but they are nonproteolytic. They have a lower optimal temperature of growth (around 25–30 °C), and they can grow and produce toxins at very low temperature. Spores are only moderately resistant to heat, not withstanding 10 minutes at 90 °C.
- Group III (*C. botulinum* C and D) is poorly proteolytic, and is rather heterogeneous with respect to sugar fermentation and production of indol and hydrogen sulfide. These bacteria grow at higher temperature than the other *C. botulinum* strains (30–37 °C optimal range), with most strains growing well up to 45 °C. Heat resistance of spores is intermediate.
- Group IV (*C. argentinense*). This group includes *C. botulinum* G strains, which have been assigned to a different species termed *C. argentinense*. These bacteria are proteolytic, hydrolyse gelatin and do not ferment any of the usual carbohydrates. Contrary to the other strains of the *C. botulinum* groups, *C. argentinense* strains do not produce a lipase. The optimum temperature of growth is 30–37 °C.

C. butyricum toxigenic strains are nonproteolytic, do not hydrolyse gelatin and acidify strongly various carbohydrates, but they do not produce lipase or lecithinase. Toxigenic and nontoxigenic *C. baratii* strains display similar morphological and biochemical characteristics and are phenotypically related to *C. perfringens*. They produce a lecithinase, but not a lipase.

BoNT-producing *Clostridia* are usually straight to slightly curved rods, 0.6–2 µm wide and 2–22 µm long. They are usually motile and peritrichous, except *C. baratii*, which is nonmotile. Spores are oval and swell one terminus of the cell. These bacteria grow well in usual anaerobic liquid media, with production of gas. Surface colonies can be grown on blood agar plates, incubated under anaerobic conditions.

All the BoNTs producing *Clostridium* belong to Group I of 23 S rRNA homology, which also includes *C. butyricum.* The genome sequence of *C. botulinum* A strain Hall has been completed (Sebaihia *et al*., 2007). It consists of a chromosome (3 886 916 bp) and a plasmid (16 344 bp), which carry 3650 and 19 predicted genes, respectively.

The genes encoding for the different types of BoNT are present on different genetic elements, including phages, plasmids or chromosomes, depending on the species and strain of Clostridia (Minton, 1995). These genes are associated with genetically mobile elements allowing for a loss or acquisition of neurotoxigenicity. In *C. argentinense,* the neurotoxin gene is present within a large plasmid, and in *C. botulinum* A, B, E and F, the toxin genes are located on the chromosome. BoNT/C and BoNT/D are present within bacteriophages and their genes can be transferred in different *Clostridium* host strains, determining their toxinotypes; indeed, transposable elements have been identified in *C. botulinum* C and D (Marvaud *et al*., 2000).

2.1 Ecology of Botulinum-Neurotoxin-Producing Clostridia

C. botulinum is widespread in soils and in sediments of lakes and seas in most parts of the world, and quantitative information on the presence of spores was summarized by Dodds (1994). BoNT-producing Clostridia, as the other Clostridia, are present in the environment, mainly in the form of spores, which are able to survive for very long periods under extreme conditions (including heat, dryness, radiation, chemicals and oxygen). Spore germination and cell division occur only under anaerobic conditions and in the presence of the appropriate nutritional requirements. This restricts the habitat of the *Clostridium* to anaerobic (low oxygen) milieux containing adequate amounts of organic materials. Based on their physiological properties (tolerance or extreme sensitivity to oxygen, requirement of particular pH, temperature and substrate for growth or spore germination), the distribution of the different *Clostridium* species in nature is not uniform. Saccharolytic *Clostridia* such as *C. butyricum* are able to grow on carbohydrates and are mainly found in decomposing vegetables and fruits. Proteolytic and gelatinolytic *C. botulinum* are preferentially associated with animal cadavers, and soils or sediments rich in organic material. They can also be found in the digestive tract of healthy humans and animals, and, after death, they participate in the process of cadaver decomposition.

3 TOXIN STRUCTURE AND MODE OF ACTION OF BOTULINUM NEUROTOXINS

BoNTs bind to and enter inside peripheral cholinergic terminals and cause a sustained block of acetylcholine release, with ensuing flaccid paralysis and loss of function of autonomic glands. These neurotoxins are metalloproteases which exert their enzymatic action in the cell cytosol on selected proteins, which form the core of

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the neuroexocytosis machinery. As a net result of such double specificity of binding (presynaptic membrane and substrate) and of their catalytic activity, these neurotoxins are the most potent toxins known, with a mouse LD_{50} (median lethal dose) between 0.4 and 1.2 ng ip of toxin per kilogram of body weight (Gill, 1982). Such extremely low values are expected to be even lower in the wild, where even a very small deficit in mobility may be sufficient to impair the survival of intoxicated animals.

3.1 Toxin Structure

BoNTs are synthesized in the bacterial cytosol as a 150 kDa polypeptide chain without a leader sequence, and are released in the culture medium after bacterial autolysis as a noncovalent assembly with a set of nontoxic proteins (Minton, 1995; Sakaguchi, 1982). The neurotoxin moiety interacts with a large nontoxic, nonhaemagglutinin protein (NTNH) via its C-terminal domain. This dimeric complex (12 S, MW 300 kDa) serves as scaffold for the assembly of larger complexes formed by the incorporation of three types of additional proteins with haemagglutinin activity: HA of 17 kDa (HA17), HA of 34 kDa (HA34) and HA of 70 kDa (HA70).These additional forms are the large (16 S, MW 500 kDa) and the extra-large (19 S, MW 900 kDa) progenitor toxins. Complexed BoNTs are more stable than isolated BoNTs to proteolysis and denaturation induced by temperature, solvent removal or acid pH (Sakaguchi, 1982). It has been found that the HA component binds to the microvilli of the small intestine through interactions with cell-surface glycoconjugates (Nakamura *et al*., 2008). Whether accessory proteins do promote the toxin uptake from the intestinal lumen into general body-fluid circulations, however, is not known. *In vitro,* small amounts of purified BoNT in the absence of HA proteins can pass through polarized epithelial monolayers by trancytosis (Maksymowych and Simpson, 1998). It has been recently reported that the HA of a 16 S BoNT/B complex was able to disrupt the intercellular junctions of the intestinal epithelium so as to allow passive protein transport via a paracellular pathway (Matsumura *et al*., 2008). The BoNTs transcytose from the apical to the basolateral side of intestinal epithelial cells (Maksymowych and Simpson, 1998) and the toxin form appearing in the lymph is the 150 kDa 'naked' protein (Heckly *et al*., 1960). Once in the tissue fluids, they then reach their specific targets, which are the peripheral cholinergic nerve terminals (Rossetto *et al*., 2006; Montecucco *et al*., 2004). The single-chain 150 kDa neurotoxins are inactive and are activated by specific proteolysis within a surface-exposed loop subtended by a highly conserved disulfide bridge. Several bacterial and tissue proteinases are able to generate the active dichain neurotoxin. The heavy chain (H, 100 kDa) and the light chain (L, 50 kDa) remain associated via noncovalent interactions and via the conserved interchain S–S bond, whose integrity is essential for neurotoxicity (Schiavo *et al*., 1990).

The lengths of the polypeptide chains of BoNTs vary from the 1251 amino-acid residues of *C. butyricum* BoNT/E to the 1297 residues of BoNT/G. Since the first molecular glimpse of BoNT/A in 1998 (Lacy *et al*., 1998), there has been a profusion of X-ray crystal structures, including the BoNT/A and BoNT/B holotoxins, the BoNT/B binding domain and the LC structures of BoNT/A–/G (structures are available at the protein data bank: *http://www.rcsb.org/pdb/*). BoNTs are organized in three distinct 50 kDa domains: an N-terminal metalloprotease domain (light chain, LC), disulfide linked to the first half of the heavy chain (H_N), which is essential for the entry into the cell and the C-terminal domain H_C responsible for the neurospecific binding (Lacy *et al*., 1998; Swaminathan and Eswaramoorthy, 2000).

The H_C binding domain consists of two functionally distinct subdomains of ~25 kDa each, the N-terminal half (H_CN) and the C-terminal half (H_CC), with little protein–protein contacts among them. H_CN is similar to some legume lectins, which are carbohydrate-binding proteins. The amino-acid sequence of this subdomain is highly conserved among BoNTs, suggesting a closely similar folding. On the other hand, the sequence of the H_CC subdomain is overall poorly conserved, but folds similarly to protein of the Kunitz-type trypsin inhibitor family. H_CC harbours one binding site for the oligosaccharide portion of polysialogangliosides in BoNT/A and /B (Rummel *et al*., 2004a). The crystalline structures of the complex between BoNT/B and the luminal domain of synaptotagmin II show that H_CC of BoNT/B also harbours a cleft which fits in segment 44–60 of this synaptic vesicle protein (Jin *et al*., 2006; Chai *et al*., 2006). The translocation domain H_N features a cylindrical shape determined by the presence of a pair of unusually long and twisted 10 nm long antiparallel α-helices (Lacy *et al*., 1998; Swaminathan and Eswaramoorthy, 2000). The overall structure of H_N resembles that of membrane-interacting viral colicin and influenza haemagglutinin proteins, which undergo an acid-driven conformational change.

The metalloprotease domain LC is disulfide bridged to the H_N and has little similarity with related enzymes of known structure, apart from the α-helix segment, including the zinc-binding motif. The active site zinc atom is coordinated by the imidazole rings of the two histidines of the motif, a water molecule bound to the glutamic acid of the motif and a distant glutamic residue (Glu262 in BoNT/A), which is conserved among clostridial neurotoxins. The Glu residue of the motif is particularly important because it coordinates the water molecule directly implicated in the hydrolytic reaction

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of proteolysis. The loss of the Glu residue by mutation leads to complete inactivation of these neurotoxins (Li *et al*., 2000). The active site is similar to that of thermolysin and identifies a primary sphere of residues essential to the catalytic function, which coincides with the zinc-coordinating residues. In addition, there is a secondary layer of residues, further from the zinc centre, including Arg362 and Tyr365 (numbering of BoNT/A), which appear to be directly involved in catalysis by providing transition-state stabilization (Rigoni *et al*., 2001; Binz *et al*., 2002). The active site of BoNTs is long and deep, and shielded by the H chain; it becomes accessible to the substrate upon reduction of the interchain disulfide bridge and it appears to be capable of accommodating a 16 residue-long segment of the substrate.

Such structural organization is functionally related to the fact that BoNTs intoxicate neurons via a four-step mechanism consisting of: (i) binding, (ii) internalization, (iii) membrane translocation and (iv) enzymatic target modification (Rossetto and Montecucco, 2008).

3.2 Mode of Action of Clostridial Neurotoxins

3.2.1 Neurospecific Binding

From the site of production or adsorption, BoNTs diffuse in the body fluids and eventually bind with high affinity and specificity to the presynaptic membrane of skeletal and autonomic cholinergic nerve terminals. The H_C domain plays the major role in the neurospecific binding (Jin *et al*., 2006; Chai *et al*., 2006). Polysialogangliosides are involved in BoNT binding (Schiavo *et al*., 2000; Rossetto and Montecucco, 2008). In fact, the H_CC subdomain of BoNT bind polysialogangliosides, particularly GD1a, GT1b and GQ1b, via the conserved peptide motif H . . . SXWY . . . G (Rummel *et al*., 2004a). More recently, polysialogangliosides GD1b and GT1b and phosphatidylethanolamine were reported to be the functional receptors of BoNT/C and BoNT/D, respectively (Tsukamoto *et al*., 2005). However, a double receptor model resulted from the existence of two classes of binding sites distinguished by different affinities and from the discovery of protease-sensitive binding to neurons (Montecucco, 1986). Indeed, the identification of synaptic vesicle proteins synaptotagmin I and II as protein receptors for BoNT/B and /G (Rummel *et al*., 2004b; Nishiki *et al*., 1996; Dong *et al*., 2003) and of SV2 for BoNT/A (Mahrhold *et al*., 2006; Dong *et al*., 2006) confirmed this model. The crystal structures of the complex between BoNT/B and the luminal domain of synaptotagmin II show that H_CC of BoNT/B also harbours a cleft which fits in segment 44–60 of this synaptic vesicle protein (Jin *et al*., 2006; Chai *et al*., 2006). The synaptotagmin-binding cleft and polysialoganglioside-binding pocket are located in the H_CC subdomain and double mutants at the two binding sites abolish neurotoxicity. Therefore, this opens the question of the role of the H_CN subdomain, which still remains unknown (Rossetto and Montecucco, 2007).

3.2.2 Internalization

BoNT binding is followed by endocytosis via a temperature- and energy-dependent process (Dolly *et al*., 1984). The binding of BoNT/B to the luminal portion of synaptotagmin I and II and of BoNT/A to the luminal domain of SV2 suggest that they are endocytosed within synaptic vesicles. This would provide a simple explanation for the accelerated intoxication brought about by increasing the nerve activity (Hughes and Whaler, 1962), because there is a tight coupling between neuroexocytosis and synaptic vesicle retrieval (Cremona and De Camilli, 1997). The protein receptors only become exposed to the external medium for a short time, between the times when the vesicle fuses with the presynaptic membrane and the times when the fission event allows vesicle endocytosis and recycling. Therefore the synaptic vesicle cycling is the cellular event that brings the toxin inside the nerve terminal; synaptic vesicles, hence, act as neuronal Trojan horses for BoNTs. BoNTs are known to act within the peripheric nerve terminal. On the other hand, a closely related clostridial neurotoxin, the tetanus toxin is retrogradely transported and acts at the central nervous system. It is not known if, and to what extent, BoNTs migrate retroaxonally. Direct measurements of retroaxonal transport indicate that 125I-BoNT/A does not reach the central nervous system (Habermann and Dreyer, 1986). However, it was recently reported that BoNT/A was retrogradely transported by central neurons and motoneurons in mice and was then transcytosed to afferent synapses, in which it cleaved SNAP-25 (synaptosome-associated protein of 25 kDa) (Antonucci *et al*., 2008).

3.2.3 Membrane Translocation

To reach the cytosol, the L chain must cross the hydrophobic barrier of the vesicle membrane, and the acidity of the lumen is essential for such a movement. BoNTs have to go through a low-pH step for nerve intoxication to occur (Schiavo *et al*., 2000). This causes a conformational change from a water-soluble 'neutral' structure to an 'acid' structure, with the surface exposure of hydrophobic patches, which mediate the interaction of the H and L chains with the hydrocarbon core of the lipid bilayer (Montecucco *et al*., 1989). Following this low-pH-induced membrane insertion, BoNTs form transmembrane ion channels in planar lipid bilayers (Koriazova and Montal, 2003) and in cell membranes (Sheridan, 1998). Membrane translocation of BoNTs is

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the least understood step of their mechanism of action. For BoNT/A, the following translocation mechanism has been envisioned: an acid-pH-induced membrane insertion step is coupled to L-chain unfolding and entry into the H-chain chaperone/channel; then the L chain is conducted through the H-chain channel in an N- to C-terminal orientation, is subsequently released from the chaperone by reduction of the disulfide bridge and is concomitantly refolded at the cytosol (Koriazova and Montal, 2003; Brunger *et al*., 2007).

3.2.4 Proteolysis of SNARE Proteins

Once in the cytosol, BoNT L chains display their catalytic activity. BoNTs are remarkably specific proteases that recognize and cleave only three proteins: VAMP (vesicle-associated membrane protein), SNAP-25 and syntaxin, which form the core of the neuroexocytosis machinery (Schiavo *et al*., 2000; Rossetto and Montecucco, 2008). VAMP, SNAP-25 and syntaxin form a heterotrimeric coiled-coil complex which juxtaposes the vesicle to the target membrane and is involved in their fusion (Chen and Scheller, 2001). VAMP is a synaptic vesicle transmembrane protein. Syntaxin is anchored to target membranes via a C-terminal hydrophobic tail and SNAP-25 is a 25 kDa protein bound to the cytosolic face of the presynaptic membrane.

BoNT/B, /D, /F and /G cleave the VAMP at different single peptide bonds; BoNT/C cleaves both syntaxin and SNAP-25, two proteins of the presynaptic membrane, whilst BoNT/A and /E cleave SNAP-25 at different sites within the COOH terminus (Schiavo *et al*., 2000). Proteolysis of one SNARE protein prevents the fusion complex formation or it may result in a nonfunctional complex (Humeau *et al*., 2000).

The biochemical basis of the BoNT specificity for the three SNAREs is only partially known. Experimental evidence clearly indicates that they recognize a nine-residue-long motif characterized by the presence of three carboxylate residues alternated with hydrophobic and hydrophilic residues (Rossetto *et al*., 1994; Pellizzari *et al*., 1996; Evans *et al*., 2005). A recent cocrystal structure of LC/A and SNAP-25 (146–204) defined additional regions of interaction external to the cleavage site and to the motif (Breidenbach and Brunger, 2004; Breidenbach and Brunger, 2005). More recently, the fitting of an extended region of the substrate (residues 189–203) within the long active-site cleft was defined, following extensive mutagenesis of LC/A and SNAP-25 (Chen *et al*., 2007).

The cleavage of the SNARE proteins by the BoNTs within the nerve cells does not cause cell death, as with other bacterial toxins (diphtheria toxin, shiga toxins, etc.). Within a time period that varies with the type of nerve, the animal species and the type of BoNT, the toxin is inactivated within the nerve and the new SNARE protein is synthesized, leading to full functional recovery.

Such recovery is the end result of a series of events, which include, not only the lifetime of the L chain, but also the replacement rate of the nonfunctional SNARE protein, which depends on several factors. This replenishment tends to be rapid, if the SNARE protein is nonfunctional, but it is slower in the case of the limited modification caused by BoNT/A and /C because these truncated SNAP-25 molecules are still capable of forming SNARE complexes (Meunier *et al*., 2003). Experimental evidence supports the proposal that an important role is also played by the dominant negative effect on neurotransmission exerted by the BoNT/A- or /C-truncated SNAP-25 (Rossetto *et al*., 2006; Montecucco *et al*., 2005). The time required for a full recovery of nerve function varies with the amount and type of L chain, the type of nerve and muscle involved, the animal species and other factors. In general, recovery from BoNT/A and /C intoxication is slower (two to four months in humans), in agreement with the slower recovery from botulism caused by these neurotoxins, whilst recovery from BoNT/B, /E and /F is shorter (one to two months in humans) (Montecucco and Molgó, 2005; Eleopra *et al*., 2006). The recovery from BoNT intoxication in mice is much shorter than in humans for all BoNTs (Morbiato *et al*., 2007).

4 CLINICAL USE OF BOTULINUM NEUROTOXINS

The finding that BoNTs cause only a transient impairment of neuroexocytosis with complete functional recovery provides the scientific basis of the booming growth of the use of BoNTs in the therapy of a variety of human diseases caused by hyperfunction of cholinergic terminals (Scott *et al*., 1989; Jankovic, 2006). Injection of BoNT is currently recognized as the best available treatment for dystonias and for certain types of strabismus, and new uses are continually being found (Montecucco and Molgó, 2005). In addition, BoNT/A inhibits acetylcholine (ACh) release at autonomic nerve terminals, which innervate the glands and smooth muscle, and it is currently used to treat diseases such as hypersalivation and hypersudoration (Naumann and Jost, 2004). Injections of minute amounts of BoNT into the muscle(s) or the gland to be paralysed lead to a depression of the symptoms, lasting months. The duration of the beneficial effect varies according to the type of toxin used and to the type of cholinergic terminals. Autonomic terminals are affected for many months, in certain cases for more than one year.

The use of BoNT/A has been increasingly reported in many conditions of pathological pain, including migraine and other headache disorders, musculoskeletal pain, such as myofascial pain, low back pain and other chronic

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pain syndromes (Jeynes and Gauci, 2008). On the basis of the differential effect of BoNT on glutamatergic and γ-aminobutyric acid (GABA)-ergic nerve terminals in hippocampal neurons (Verderio *et al*., 2004), BoNT/E was tested as a potential therapeutic for epilepsy, and it was found to effectively decrease the symptoms induced in mice by the epileptogenic, kainic acid (Costantin *et al*., 2005).

There are comparatively few side effects of the treatment with BoNT/A, the most important one is the production of antibodies, which make the patient resistant to the toxin. In general, this occurs with the large doses of toxin necessary for the treatment of spasmodic torticollis; here the aim is to weaken certain powerful muscles in areas rich in lymph nodes, primarily in the neck. If there are neutralizing anti-BoNT/A antibodies, one has to switch to a different type. BoNT/B has been introduced for the treatment of cervical dystonias, and other types of BoNT are undergoing clinical tests, with very encouraging results having been obtained with BoNT/C (Morbiato *et al*., 2007; Eleopra *et al*., 2006).

5 OUTLINE OF EPIDEMIOLOGY OF HUMAN BOTULISM

The worldwide incidence of botulism is still unknown, especially in developing countries. Moreover, some differences regarding the commonest botulism form are present; infant botulism is the prevalent form in USA, whereas foodborne botulism is more common in the EU. Botulism remains underdiagnosed because clinicians are unfamiliar with the signs of presentation, and the first symptoms can be mistaken for several more common clinical entities. In recent years, increased surveillance by *ad hoc* surveillance systems established by health authorities in several countries has contributed to an increase in the number of cases identified.

5.1 Foodborne Botulism

Epidemiological studies have indicated that the home-prepared foods causing the highest incidence of botulism included home-canned vegetables and meat, and garlic, mushrooms and other vegetables packed in oil or brine not suitable because it is not salty enough. Since the beginning of industrial canning (1920s), commercial products have generally had a good safety profile. Few outbreaks due to commercial foods are reported worldwide, and some of them are shown in **Table 1**.

The majority of incidents of foodborne botulism have been caused by Clostridia producing BoNT/A, BoNT/B and BoNT/E. Type A is predominant in the western USA, Argentina and China, most frequently related to vegetables and fruits. Type B is prevalent in Europe (excluding northern countries) and meat has been recognized as the main involved source; only in Italy and Spain are vegetables more frequently incriminated. Type E botulism is found mainly in the colder regions of the northern hemisphere (Alaska, Canada, Greenland, Scandinavia, Japan). Type E due to neurotoxigenic *C. butyricum,* has been reported only in China, India and Italy (Anniballi *et al*., 2002). Type F foodborne botulism is very rare, and only a few poisonings have been

Table 1 Foodborne botulism cases/outbreaks due to commercial food occurred worldwide in the last 25 years

Year	Country	Incriminated food	BoNT type	No. of cases
1983	Canada	Garlic in oil	B	36
1986	Taiwan	Unsalted peanuts in water	A	9
1987	USA and Israel	Uneviscerated salted airdried fish (‘kapchunka’)	E	8
1989	United Kingdom	Hazelnut yoghurt	B	27
1989	Italy	Black olives	B	5
1991	Egypt	Uneviscerated salted fish (‘faseikh’)	E	91
1992	US—Alaska	Uneviscerated salted fish (‘moloha’)	E	8
1993	US—Georgia	Cheese sauce	A	8
1993	Italy	Roasted eggplant in oil	B	7
1996	Italy	Mascarpone cheese	A	8
1997	Germany	Hot-smoked vacuum packed fish	E	2
1998	Iran	Cheese in oil	A	27
2001	USA—Texas	Chili sauce	A	16
2006	USA—Georgia and Florida	Carrot juice	A	4
2007	USA—Texas and Indiana	Canned chili sauce	A	2

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recognized; they were in Langeland Island (Denmark), California, Norway and Italy.

The first European Communicable Disease Epidemiological Report (European Centre for Disease prevention and Control, 2007), included data reported from 20 EU member States and Norway from 1995 to 2004. A total of 2388 human cases of botulism were included; Poland reported the highest number (850 cases), accounting for 35% of the total. In 2005, a total of 152 cases were reported by 22 EU countries, with an overall estimated incidence of 0.03 per 100 000.

In the UK, only 37 cases of foodborne botulism, confirmed in the laboratory, were reported to the Health Protection Agency Centre for Infections between 1980 and 2006. In France, a total of 56 incidents (96 patients) of botulism have been notified from 2003 to 2006; 50 of them, involving 64 patients, were laboratory confirmed. In Italy, a total of 283 cases (177 incidents) were confirmed between 1984 and 2007 by the National Reference Centre for Botulism, with an annual incidence of 0.02 per 100 000. The most frequent confirmed vehicle of intoxication (76.3% of confirmed) was home-preserved food, whereas commercially canned foods more rarely caused foodborne botulism. No restaurant-prepared foods were involved. Only two cases of BoNT/F were observed, and *C. butyricum* Type E was isolated from a patient who had consumed a noncanned homemade pasta. The republic of Georgia has the highest rate of foodborne botulism in the world, with 879 cases from 1980 to 2002 (80% attributed to BoNT/B).

In USA, 2757 cases of foodborne botulism were reported by Centers for Disease Control and Prevention (CDC) from 1899 to 2006. Until the 1950s, nearly all outbreaks of botulism were caused by BoNT/A or /B. From 1950 to 1996, 289 outbreaks (65.1%) have been traced to home-processed food and 31 (7%) to commercially processed foods, including food served in restaurants. Type E botulism associated with Alaskan native food has increased in recent decades because traditional practices have been altered in an unsafe manner. In Canada, about 90% of incidents are associated with marine products and due to BoNT/E. Native people are mostly involved, primarily Inuits for whom the risk of botulism is over 1000 greater than for other Canadians.

Argentina reported the highest number of cases in South America. From 1922 to 2006, 695 cases (114 deaths) of foodborne botulism were registered. Type A botulism occurred in 71.6%, while Type B in 9.0%, Type E in 3.0%, Type F in 1.5% and Type Af in 1.5%. In Brazil, 15 cases of confirmed foodborne botulism (BoNT/A identified in 10 cases) were reported in 1997–2007.

Since 1989, 2861 cases of botulism have been reported in China, while in Thailand, an isolated outbreak in 2006 caused 203 cases. From 1955 to 1998, 351 cases (68 fatal) were reported in Japan (mostly BoNT/E).

5.2 Infant Botulism

Several neurotoxigenic Clostridia can cause infant botulism: BoNT/A/B/E and /F can be involved. Most cases are due to BoNT/A or proteolytic BoNT/B produced by *C. botulinum.* A BoNT/F case, produced by *C. baratii,* was first reported in New Mexico in 1979, whereas the first two cases of Type E occurred in Italy in 1984.

Since its first recognition in 1976, infant botulism has become the most common form of human botulism diagnosed in the Western Countries. A total of more than 3100 cases of infant botulism were globally identified from 1976 to 2007 (**Table 2**): the highest incidence of cases is reported in the USA, followed by Argentina, Australia, Canada and Italy. In the USA, approximately 80–110 cases are annually recorded, mostly recognized in California. In Europe, a total of 67 cases were diagnosed in 14 countries from 1978 to 2007.

5.3 Adult Intestinal Colonization

Few cases have been reported worldwide: adult intestinal botulism was reported only in the USA (15 cases of BoNT/A or /F from 1981 to 2003), Italy (three cases of BoNT/E and /A from 1994 to 1997) and Japan. Cases have been related to *C. botulinum* Types A, B, Ab and F. *C. baratii* Type F and *C. butyricum* Type E have also been involved (Fenicia *et al.*, 1999; Gupta *et al.*, 2005).

5.4 Wound Botulism

First recognized in 1943, until 1985, 33 cases were reported in the USA. Among the 25 laboratory-confirmed cases, 17 were due to BoNT/A, seven to BoNT/B and one to BoNT/AB (CDC (Centers for Disease Control and Prevention), 1998). The most affected population is composed of young men, almost exclusively with traumatic or surgical wounds.

From 1979, only five cases of wound botulism were diagnosed in Italy. Four of these were laboratory-confirmed and BoNT/B or *C. botulinum* Type B was detected in serum or tissue samples. Two cases of BoNT/A were reported in 2001 in Ecuador.

Since 1982, there has been a change in the epidemiology of wound botulism: the number of cases has increased dramatically due to a growth in the number of users of injectable drugs, where wound botulism is associated with needle-puncture sites, and, less frequently, in chronic cocaine sniffing via nasal/sinus lesions. About 1000 cases have been reported worldwide so far.

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Table 2 Worldwide infant botulism cases, from 1976 to 2007

Country	Time period	No. of cases	BoNT/A	BoNT/B	BoNT/E	Other BoNTs	BoNTs unknown
China	1986–1989	2	—	1	—	—	1
Japan	1988–2008	18	15	1	1	1 (C)	—
Taiwan	1987	1	—	1	—	—	—
Australia	1978–2006	32	12	15	1	—	4
Israel	1994–2006	2	—	2	—	—	—
Kuwait	2005	1	—	—	—	—	1
Yemen	1989	1	—	1	—	—	—
Canada	1979–2006	27	22	5	—	—	—
Mexico	2001	1	1	—	—	—	—
USA	1976–2006	2419	1079	1310	2	29	1
Argentina	1982–2006	410	409	1	—	—	—
Chile	1984–1995	3	2	—	—	—	1
Venezuela	2000	1	—	1	—	—	—
Italy	1984–2007	28	4	19	4	1 (Ab)	—
Spain	1985–2007	11	2	3	—	—	6
UK	1978–2007	8	2	5	—	1 (Bf)	—
Norway	1997–1999	4	4	—	—	—	—
Germany	1981–2000	4	1	1	—	—	2
Sweden	1985–2006	3	2	1	—	—	—
Netherlands	2000–2005	3	1	2	—	—	—
France	1987–2007	3	—	2	—	—	1
Denmark	1995–2000	2	—	—	—	1	1
Hungary	1995–2002	2	—	—	—	—	—
Czech Republic	1979	1	—	1	—	—	—
Switzerland	1987	1	1	—	—	—	—
Finland	2002	1	—	1	—	—	—
Greece	2007	1	1	—	—	—	—

Seventy-eight cases were reported in drug users (mostly using injectable drugs) in the USA between 1986 and 1996, and 178 cases (mostly BoNT/A, two fatal) from 2001 to 2006 (Werner *et al*., 2000).

The first European case of wound botulism in illicit drugs users was reported in Norway in 1997, followed by at least three further cases. Subsequently, another nine cases have been identified in Switzerland and one in the Netherlands. From 2000 to 2007, a total of 136 wound botulism cases were reported in UK in those using injectable drugs. In 2007, the first wound case in a user of injectable drugs was reported in Sweden, and in 2008 an outbreak of six patients was reported in Ireland. Simultaneous wound botulism in two brothers addicted to cocaine was reported in Spain in 2007.

6 CLINICAL SYNDROME OF HUMAN BOTULISM AND PATHOPHYSIOLOGY OF THE DIFFERENT FORMS

Human botulism is a 'typical' toxicological syndrome characterized by neurological symptoms and signs resulting from a toxin-induced blockade of the voluntary motor and autonomic cholinergic junctions; it is quite similar for each type of BoNT and route of exposure (foodborne, wound, intestinal colonization—infant or adult, iatrogenic or other less frequent forms).

The route of exposure influences the delay from exposure to the appearance of the typical syndrome. Depending on the route of exposure, the causal 'agent' entering the body may be the *Clostridium* organism itself (e.g. intestinal, wound) or the preformed BoNT (foodborne), whilst in all cases the clinical syndrome of intoxication is due to the preformed neurotoxins or those produced *in situ.* Consequently, the specific treatment is aimed at preventing the binding between BoNTs and the neuromuscular junctions, although in some forms (wound and intestinal forms), additional treatment modalities can be added to prevent or limit the *in situ* production of toxins.

The rapidity of onset and the severity of botulism depend on the rate and amount of toxin absorbed. Generally, BoNT/A is more severe than the other BoNTs; however, patients with Type E botulism have the shortest incubation periods, while patients with Type B botulism show the longest incubation periods. Cases of Type A botulism will most likely require intubation (Woodruff *et al*., 1992).

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The typical clinical syndrome of botulism described in medical literature refers to the foodborne form, which historically has been the commonest. This is characterized by an acute or subacute afebrile, symmetric descending flaccid paralysis that always begins in the bulbar musculature, which is the most densely innervated and well supplied with blood (IX, X, XI and XII cranial nerves). Related main symptoms are dysphagia, dysarthria and dysphonia, frequently associated with autonomic dysfunction (i.e. dry or sore mouth and throat) and preceded by gastrointestinal discomfort (such as diarrhoea followed by constipation) in patients with normal mental status, sensation and reflexes (Gomez, 2005; Goldfrank and Flomenbaum, 2006; Schwartz and Morgan, 2007). These complaints are often underestimated, while the first signs observed on clinical examination are those involving cranial abducens (VI) or oculomotor (III) nerves, with diplopia, blurred vision, mydriasis (often fixed), bilateral ptosis, lateral rectus palsy, external ophthalmoplegia. Pupillary reactions may remain abnormal for months after motor recovery (Friedman *et al*., 1990). Nystagmus is only occasionally noted. The clinical picture is completed by progressive respiratory weakness progressing to respiratory failure, as well as urinary retention (Simcock *et al*., 1994; Gomez, 2005).

A neurological syndrome due to cholinergic blockade may be present at first evaluation; visual disturbances, dysarthria, dysphagia and dry or sore mouth and throat are the four most specific neurological symptoms (Hughes *et al*., 1981; CDC (Centers for Disease Control and Prevention), 1998). If the disease is mild, no other symptoms develop and the initial picture will gradually resolve.

Early gastrointestinal signs and symptoms consist of nausea, vomiting, abdominal discomfort, pain and diarrhoea. The presence and ingestion of other bacteria or their toxins in improperly preserved food, or changes in bowel motility are linked to gastrointestinal symptoms, such as pain, nausea, vomiting and diarrhoea that often precede or accompany the typical neurological syndrome; constipation occurs in subsequent days in about 70% of patients (Schwartz and Morgan, 2007). Although gastrointestinal disturbances are common, about 30% of patients may not present any abdominal/gastrointestinal signs or symptoms (CDC (Centers for Disease Control and Prevention), 1998).

The clinical syndrome of botulism usually presents within 12 to 36–48 hours after ingestion of contaminated food (Hughes *et al*., 1981); in some cases, however, onset of initial symptoms can be delayed as long as 10–15 days after ingestion. Variability in the time of onset of symptoms depends on patient, type and quantity of toxin ingested, route of exposure and may make the diagnosis difficult. Generally, Type A botulism is significantly associated with neurological signs and symptoms while Type B seems to produce more autonomic dysfunctions and is characterized by a late and slow progression; Type E is related more to gastrointestinal symptoms than neurological signs (St Louis *et al*., 1988). In these last cases, the suspicion of botulism poisoning is more difficult. Nonspecific neurological symptoms such as malaise, weakness, headache, dizziness and paresthesias may be present, but rarely (CDC (Centers for Disease Control and Prevention), 1998).

In severe cases, the prolonged extensive flaccid paralysis causes life-threatening complications; respiratory dysfunction may result from either upper airway obstruction (the weakened glottis tending to close during attempted inspiration) or diaphragmatic weakness. The most common complication in the first period of botulism poisoning is aspiration pneumonia (Gomez, 2005).

The period requiring mechanical ventilatory support ranges from one to eight weeks, but patients can remain ventilator-dependent for seven to eight months; the mean period for ventilatory support is 58 days for toxin Type A and 26 days for Type B (Hughes *et al*., 1981). After this, most patients have residual symptoms, including easy fatigability and exertional dyspnoea at one year follow-up (Schmidt-Nowara *et al*., 1983), loss of responsiveness to postural change (orthostatic hypotension), hypothermia, alterations in the resting heart rate and urinary retention (Vita *et al*., 1987). Secondary medical complications from prolonged ventilator use, such as nosocomial infection can occur. Recovery results from new motor axon twigs that sprout and reinnervate paralysed muscle tissue in a process that may take months to complete (Duchen, 1972).

The case:fatality ratio was high (approximately 60%) in the fist half of the twentieth century, and has gradually decreased in the second half to 15.5%; at present the mortality rate of foodborne botulism is around 5–15 % of cases (Hughes, *et al*., 1981; Shapiro *et al*., 1998). Early death results from a failure to identify the severity of the disease, or from acute pulmonary or infectious complications. The decline in mortality rate (for all toxin types) is due primarily to improvement in supportive and respiratory intensive care and perhaps to prompt administration of antitoxin.

6.1 Wound Botulism

The clinical syndrome is similar to other forms of botulism, except for the lack of gastrointestinal symptoms and the possible presence of fever. The incubation period may be longer (from 7 to 14 days) than with foodborne botulism (Merson and Dowell, 1973). In nearly 30–50% of cases, toxins are not detected in sera and *Clostridium* sp. are not isolated from the wound.

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6.2 Intestinal Toxaemia (Adult Intestinal Botulism and Infant Botulism)

6.2.1 Adult Intestinal Botulism

Rarely, cases of adult botulism have been reported with no known contaminated food, no wound present and no history of drug abuse, and, for these reasons, such cases were defined as 'hidden botulism'. Most of these patients represent adult variations of infant botulism and often have a history of abdominal surgery, gastrointestinal tract abnormalities following inflammatory intestinal disease, recent use of antibiotics that may have altered the natural gastrointestinal flora (which acts as barrier to intestinal colonization), and surgically or pharmacologically induced achlorhydria (Goldfrank and Flomenbaum, 2006; Fenicia *et al*., 2007a).

Cases due to colonization of *C. botulinum* producing toxin Type A and B, *C. baratii* producing toxin Type F and *C. butyricum* producing toxin Type E have been described (McCroskey *et al*., 1991; Fenicia *et al*., 1999). In some cases, a relationship with Meckel's diverticula and *C. butyricum* producing toxin Type E has been hypothesized (Fenicia *et al*., 1999). McCroskey and Hatheway (1988) documented the detection of the toxin in serum 47 days after ingestion of a suspected food, which was contaminated with organisms, but did not test positive for preformed toxin, suggesting continued production of toxin by organisms colonizing the gut. In other cases, organisms were cultured from the stool 119 days after the onset of illness. The production of endogenous antibody to botulinum toxin has been described in a single case report (Griffin *et al*., 1997). The finding of *C. botulinum* in faeces of adult patients is almost always associated with clinical botulism (Dowell *et al*., 1977). The clinical picture is similar to that of foodborne botulism, but the onset is generally gradual and less dramatic.

6.2.2 Infant Botulism

The onset of infant botulism can only be detected by careful observation; in mild cases or in the early stages of illness, the physical signs may be subtle and easily overlooked. On initial presentation, the typical patient has some or all of the following findings: lethargy, listlessness, ptosis, weak cry, diminished suck and gag, drooling and/or pooling of saliva, dysphagia, dilated and/or sluggishly reactive pupils, disconjugate gaze, blunted facial expression, poor head control, decreased anal sphincter tone, hypotonia and generalized weakness, and difficulty in breathing (Arnon *et al*., 2006). Infant botulism is also considered a possible occult cause of the sudden infant death syndrome (Bartram and Singer, 2004). However, in the absence of complications, patients recover completely from the disease.

Certain factors can predispose to infant botulism. Botulism spores are unable to germinate in the presence of high acidity, a mature gastrointestinal immune system or in the copresence of aerobic and anaerobic bacteria. The gastrointestinal tract of infants (up to four to six months) does meet these conditions; in fact the infantile gut is characterized by a more alkaline gastric environment, a paucity of normal flora and a lack of mature mucosal immunological defences including lysozyme, complement and secretory IgA (Arnon, 2004; Istre *et al*., 1986). The difference in pH of faeces may play a role in multiplication of *C. botulinum* and in the production of toxins (Brook, 2007).

Honey (Fenicia *et al*., 1993; Aureli *et al*., 2002), household dust (Nevas *et al*., 2005) and chamomile (Bianco *et al*., 2008) may be considered as known sources of neurotoxigenic *Clostridia* spores, but in about 85% of patients the source remains unknown (Brook, 2007). Another predisposing factor for infant botulism seems to be concomitant enteroviral infection, which may facilitate the colonization with *C. botulinum* (Fenicia *et al*., 2004).

7 DIAGNOSIS AND DIFFERENTIAL DIAGNOSIS

Diagnosis of botulism is essentially clinical in the first stage: the role of the laboratory is mandatory to confirm the clinical diagnosis, to identify the different BoNTs involved and the source of poisoning.

Foodborne botulism poisoning is often underdiagnosed or misdiagnosed because physicians rarely encounter this disease; the initial symptoms can be confused with more common clinical conditions, such as stroke or the autoimmune acute demyelinating polyneuropathy known as Guillain–Barré syndrome (Miller–Fisher variant) (Shapiro *et al*., 1998). Some cases are undoubtedly entirely unrecognized (CDC (Centers for Disease Control and Prevention), 1998). When the gastrointestinal syndrome is prevalent, such as with BoNT/E-related poisoning, the suspicion of botulism is more difficult. The diagnosis can be easy when the index of suspicion is high, as in the setting of a large outbreak, but often cases occur singly and in most of them patients are referred first to an oculist, otolaryngologist or neurologist and only subsequently to an emergency department because of worsening of clinical features. Indeed, the results from many outbreaks have suggested that early cases are commonly misdiagnosed; in other situations an outbreak was recognized only after a second cluster of cases was associated with the same food vehicle (St Louis *et al*., 1988). In some cases the diagnosis has been made retrospectively (after death),

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Table 3 Key signs and symptoms for the diagnosis of foodborne botulism

Recent medical history	Negative for infectious diseases
First evaluation	Dry mouth/sore throat and/or dysphagia
	Anxiety
	Normal mental status
	Afebrile
	Absence of cardiac dysfunction (rarely bradycardia)
	Gastrointestinal discomfort
	Weak
Physical examination	Symmetric oculobulbar symptoms
	Symmetric descending neurological abnormalities

when a cluster of cases finally alerted the public-health authorities to an outbreak of botulism. Therefore, correct diagnosis of the first case of poisoning is very important to ensure the planning of appropriate clinical management of subsequent cases and particularly to avoid serious delay.

Various constellations of features have been suggested to consider diagnosis of botulism early in its presentation (**Table 3**) (CDC (Centers for Disease Control and Prevention), 1998). Botulism must be suspected whenever a patient presents with any kind of weakness: generalized, ocular or oropharyngeal, sometimes associated with a history of acute onset of gastrointestinal dysfunction (Schwartz and Morgan, 2007). Bilateral cranial nerve abnormalities and progression of descending paralyses should raise the suspicion of botulism. Epidemiological criteria may be useful in formulating a correct diagnosis in some cases.

The differential diagnosis of human botulism includes myasthenia gravis, stroke, Guillain–Barré syndrome, bacterial and chemical food poisoning, paralysic tick diseases, chemical intoxication (e.g. methyl alcohol, atropine, magnesium), adverse drug reactions, poliomyelitis, diphtheria and psychiatric illness. About 10.5% of patients with suspected botulism reported to the CDC are finally diagnosed as having Guillain–Barré syndrome, and 3.4, 3.2 and 3% as having carbon monoxide poisoning, food poisoning of unknown aetiology and staphylococcal food poisoning, respectively (CDC (Centers for Disease Control and Prevention), 1998).

Routine laboratory tests are not useful in confirming the clinical suspicion of botulism because they will all be normal, if secondary complications are absent. Some laboratory studies are helpful to differentiate botulism from other diseases; thus, normal cerebrospinal fluid (CSF) may discriminate botulism from Guillain–Barré syndrome, although a slightly elevated CSF protein level is occasionally observed with botulism, and the protein level may be initially normal in Guillain–Barré syndrome (Hughes *et al*., 1981). A negative tensilon test helps to differentiate botulism from myasthenia gravis; also neuroradiological studies may be useful to rule out stroke (St Louis *et al*., 1988).

7.1 Diagnosis and Differential Diagnosis of Infant Botulism

The diagnosis of infant botulism should be based on clinical symptoms; assays for toxin in the serum rarely yield positive results, and a definitive diagnosis may be confirmed by isolation of toxin or organism from the stool (CDC (Centers for Disease Control and Prevention), 1998). Because of constipation, an enema with sterile, nonbacteriostatic water (not saline) commonly is needed to obtain a faecal specimen for diagnostic examination. Electromyography (EMG) may play an important role in instrumental confirmation of the diagnosis, even if normal findings do not rule out the disease. The differential diagnosis of infant botulism includes: sepsis, myopathies, Guillain–Barré syndrome, familial infantile myasthenia gravis, spinal muscular atrophy and poliomyelitis. Approximately the 5% of the 681 patients who were treated (antidote or placebo) in a randomized, controlled clinical trial were not affected by infant botulism; in most of these cases an alternative diagnosis was established as spinal muscular atrophy Type I, metabolic disorders, infectious diseases, miscellaneous and probable infant botulism (Francisco and Arnon, 2007).

7.1.1 Electrophysiological Studies

In all forms of botulism, the EMG pattern is characterized by low amplitude, short-duration and abundant motor-unit action potentials (BSAPs) (Engel, 1975). The most consistent electrophysiological abnormality is a small evoked muscle action potential (MAP) in response to a single supramaximal nerve stimulus in a clinically affected muscle (Cherington, 1998). The EMG findings alone should never be used to differentiate other diseases from botulism. In fact, as many as 15% of patient with botulism may have normal findings on EMG, improvement with edrophonium may be noted in 25% of patients with proven cases of botulism and low-frequency findings supporting myasthenia gravis may be observed in 20% of patients who suffer from botulism (St Louis *et al*., 1988). Anticholinesterase drugs, such as edrophonium, have no effects on the mechanism of action of the botulism toxin; in rare cases, in the first stage of the disease, limited improvement in strength can occur, but is not comparable with the rapid response in patients with myasthenia gravis (Pickett, 1988).

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7.1.2 Laboratory Methods to Detect Clostridium Botulinum and Botulinum Toxins

The laboratory diagnosis of botulism is based on the detection of BoNTs in clinical or food samples and the isolation of BoNT-producing Clostridia from stools (CDC (Centers for Disease Control and Prevention), 1997). The most direct way to confirm the diagnosis is to demonstrate the BoNTs in the patient's serum or stools by injecting serum or stools into mice and looking for signs of botulism. The bacteria can also be isolated from the stools of subjects with foodborne and infant botulism. Since other Clostridia can produce human cases of botulism, the presence of other BoNT-producing Clostridia, such as *C. butyricum* and *C. baratii,* will have to be included in the criteria for laboratory diagnosis (Fenicia *et al*., 2007a).

7.1.2.1 Laboratory Safety

The United States Centers for Disease Control and Prevention include BoNTs in the Category A infectious agents. Botulinum toxins may be present in food, serum, gastric contents, faeces and environmental samples. Absorption is possible by accidental ingestion and through damaged skin and mucous membranes, including the respiratory tract. Appropriate Biosafety Level 2 containment facilities, including gloves and a face shield, and trained personnel are therefore the minimum required. A Biosafety Level 3 is required when manipulations producing aerosols or droplet formation from toxic materials are expected. Sentinel Laboratories (Level A) should collect appropriate specimens and, after consultation, ship the specimens to a Reference Laboratory. Restricted entry to the laboratory dealing with the manipulation of presumptive BoNT-contaminated materials has to be secured.

7.1.2.2 Detection and Typing of BoNTs: Mouse Lethality Bioassay

The mouse bioassay (intraperitoneal injections of white mice unprotected and protected with polyvalent botulinum antitoxin) is currently the only accepted method to confirm the presence of BoNTs. Mouse type-specific botulinum antitoxin is used to define the type (typing) of the toxins produced from different strains. Typical botulism symptoms in mice sequentially consist of ruffled fur, 'wasp-like' aspect, hind-limb paralysis, laboured breathing and, finally, death due to respiratory failure.

Mouse bioassay is the only standard validated method for the detection of BoNT in food: the method shows a high degree of repeatability and reproducibility (Kautter and Solomon, 1977), with one intraperitoneal mouse LD_{50} dose corresponding to 5–10 pg and the detection limit being 0.01 ng ml^{-1} of sample. False positive results due to *in vivo* toxin formation from a high number of *C. botulinum* spores, endotoxins from Gram-negative bacteria and tetanus toxins have been reported.

7.1.2.3 Detection and Typing of Neurotoxigenic Clostridia

7.1.2.3.1 Culture Methods

Six phenotypically distinct groups of Clostridia are known to be capable of producing BoNTs. Conventional detection and isolation of *C. botulinum* are based on culturing in liquid medium and subsequent detection of BoNTs in the culture supernatant by the mouse bioassay. Positive samples are streaked on solid media, and the toxin formation by colonies is traditionally further confirmed by the mouse test. Clinical samples, such as serum and faeces, can be cultivated directly or pretreated with ethanol to eliminate vegetative bacteria while recovering bacterial spores. Alternatively, heating may be used to eliminate non-spore-formers, but care should be taken in selecting the temperature; 80 °C for 10 minutes for Group I spores and at 60 °C for 10–20 minutes for Group II spores has been recommended. An enrichment step in a non-selective medium trypticase peptone glucose yeast extract (TPGY) broth followed to isolation in egg yolk agar is used for detecting the suspected BoNT-producing strains of Clostridia. In the isolation step of the neurotoxigenic strains on egg yolk gar, lipase-negative colonies (*C. butyricum* and *C. baratii*), as well as lipase-positive colonies have to be examined (Fenicia *et al*., 2007a). Mouse bioassay is used to confirm BoNT production in subcultured colonies.

7.1.2.3.2 Molecular Method

Nucleic-acid techniques, especially the polymerase chain reaction (PCR), have emerged rapidly as method of choice allowing rapid, specific and sensitive detection of pathogen in clinical, food and environmental samples. In the case of *C. botulinum,* as well as for other toxigenic bacteria, PCR-based methods detect merely the presence of botulinum DNA, but they do not detect the activity of the different DNA genes, nor the presence of the toxins. PCR is a suitable tool for confirming the presence of BoNT-producing Clostridia in enrichment broth and in isolated strains, or for typing isolated strains. A different conventional (gel-based electrophoresis) PCR method has been proposed, based on single PCR reactions for specific detection of the different toxin genes. The use of a general primer pair able to amplify the four toxin genes, while specific internal probes are then used to identify the toxin types of strains is also suggested. An improvement has been the development of a multiplex PCR assay that enables the detection of BoNT/A, BoNT/B, BoNT/E and BoNT/F genes simultaneously (Lindstrom *et al*., 2001).

More recently, realtime PCR has been used to detect pathogenic micro-organisms in food, environmental and clinical samples. Several fluorescent-probe-based real-time PCR studies have been carried out to detect BoNTs using TaqMan probes (Akbulut *et al*., 2004; Yoon *et al*.,

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2005) and the simple and less expensive double-stranded DNA-binding dye SYBR green (Fenicia *et al*., 2007b). While the majority of the PCR methods are based on the specific detection of BoNT genes, the use of the NTNH gene as a target for PCR has been successfully proposed as a single target to identify human and animal BoNT-producing Clostridia (Raphael and Andreadis, 2007). The use of PCR could reduce dramatically the use of animals in laboratories dealing with surveillance and research into botulism (De Medici *et al*., 2006).

7.1.3 Role of the Laboratory Tests in the Diagnosis and Management of Poisoned Patients

BoNTs and/or *Clostridium* sp. toxigenic strains may be detected in patients' specimens (serum, gastric contents, stool and wound) and in suspected foods. Early sample collection (stools and/or gastric content) before antitoxin administration may increase the likelihood of obtaining positive results. In the cases of foodborne botulism reported in the USA from 1975 to 1988, the toxin was present in 37% of sera (126/240 cases), 23% of stools (65/288) and 5% of gastric aspirate (3/63) (Woodruff *et al*., 1992). Tests in sera were positive in more than 60% of specimens, if collected within two days, in 44% of specimens collected within three days after toxin ingestion and in only 23% of that obtained by day 4 or later. The positivity of toxin assays of sera was much higher for BoNT/A compared to Types B and E. Stools were positive in 50% of specimens collected within one day following the ingestion, in 39% collected within three days and 20% of specimens obtained at day 5 or later. Cultures of stools and gastric aspirates for *Clostridium* sp. from cases of foodborne botulism showed the presence of toxins in 45% of gastric aspirate and 80% of stools at day 2 after ingestion, 40% of specimens remaining positive seven to nine days after ingestion. However, laboratory confirmation of botulism poisoning by either toxin assay or culture could not be obtained in 32% of cases, primarily due to reduced sensitivity of diagnostic testing if undertaken more than three days after toxin ingestion. False-negative results are common when sample collection is made late in the course of the poisoning. Post-mortem diagnosis may be made by identification of toxins or organisms in autopsy specimens of the small or large intestines, the or liver (Dodds, 1994). Laboratory confirmation of botulism may require days before results are available. Since antidotal treatment with botulinum antitoxin is thought to be more effective when given early after poisoning, usually the treatment is applied before the analytical confirmation. BoNTs detection and *Clostridium* sp. isolation from the sera and wounds confirmed most cases of wound botulism (Werner *et al*., 2000).

In cases of botulism due to inhalation of the toxin, the identification of the toxin in typical human specimens, such as serum, stools and gastric contents, may be difficult (Franz *et al*., 1997; Arnon *et al*., 2001). Toxin may be detectable in the nares for up to 24 hours after the contact and a nasal mucosal swab may be useful to help the diagnosis, using an enzyme-linked immunosorbent assay or PCR techniques that are in the course of validation (Chao *et al*., 2004; Akbulut *et al*., 2005; Dembek *et al*., 2007).

8 TREATMENT

The treatment of botulism includes procedures for decontamination, administration of antidotes and, when required, support of respiratory function; only few differences are related to the route of exposure.

8.1 Foodborne Botulism

8.1.1 Gastrointestinal Decontamination

All cases of foodborne botulism, if no contraindications are present, should be evaluated for gastrointestinal decontamination, with the aim of removing the spores and toxin from the gut. Gastric lavage or induced emesis is indicated only in asymptomatic patients that have very recently ingested a possible contaminated food.

Most of the patients present symptoms some days after ingestion; in these cases gastrointestinal decontamination must be applied because of the permanence of contaminated food (and spores and toxins) in the gastrointestinal tract due to the anticholinergic effects. Then, upper and lower decontamination with an orogastric tube and cathartics, or whole-bowel irrigation could be performed, even if the efficacy in suspected and confirmed cases of botulism poisoning are not assessed. Sorbitol is better than magnesium salts to obtain cathartic effects because the latter may exacerbate neuromuscular blockade. Effective whole-bowel irrigation may be difficult in the presence of drug- or toxin-induced ileus; in some cases neostigmine, which inhibits the enzymatic degradation of acetylcholine, appears to be useful in reversing ileus (Isbister *et al*., 2001). Activated charcoal administration must be considered in poisoned patients because it absorbs BoNT/A *in vitro* (Gomez *et al*., 1995).

8.1.2 Antidotes

The aim of the antidotal treatment consists in neutralizing the circulating toxin molecules still unbound to the nerve endings. This mechanism of action was shown to be effective many years ago in experimental studies (Oberst *et al*., 1968). But the specific treatment with

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antitoxin limits the involvement of new nerve endings, but cannot reverse the paralysis nor neutralize the toxins already bound to the nerve receptors. Symptoms often progress for up to 12 hours after antitoxin administration before an effect is observed (Dembek *et al*., 2007). The type-specific antitoxins are ineffective against any other antigen. Botulism antitoxin should be administered as soon as botulism poisoning is suspected. The antitoxin is most often used for foodborne botulism, although it would also be an effective antidote in the inhalation form. Animal studies showed that early administration, after an aerosolized release of lethal concentration of botulinum toxin, may be effective (Arnon *et al*., 2001).

Equine-derived antitoxin, available since 1970, is the unique and specific antidotal treatment available for botulism poisoning. Before 1950, the mortality rate for botulism was approximately 60%, even when equine antitoxin was administered in heroic doses (Shapiro *et al*., 1998). During the late 1950s the fatality rate decreased precipitously until it reached the current rate of 3–5%, due, mainly, to the improvement of intensive-care support, quality of respiratory assistance (including the application of tracheotomy) and the introduction of procedures for the prevention of secondary infection (Sobel, 2005).

Although there is evidence of efficacy in experimental studies in animals, no controlled studies have been done in humans to evaluate the usefulness of antitoxin therapy: the clinical evidence for the efficacy of antitoxin use in humans is today supported only by case reports and retrospective analyses of small numbers of patients. Morbidity and mortality studies are difficult to perform because of the rarity of the poisoning and the usual delay in diagnosis, which means that the toxin is already permanently fixed to the neuromuscular junction.

Tacket *et al*. (1984) analysed 132 cases of BoNT/A foodborne botulism reported to the CDC from 1973 to 1980, to evaluate primarily the effect of the trivalent equine antitoxin therapy on the outcome of patients. Patients who had received antitoxin within 24 hours after the onset of symptoms had a lower fatality rate (10%) than those who had received antitoxin after more than 24 hours (15%) and than those who did not receive antitoxin (46%). In addition, survivors that received the antitoxin early had a median hospital stay of only 10 days, compared with 41 days for those who received antitoxin late and 56 days for those who did not receive antitoxin.

In the USA more than 80% of patients with botulism poisoning are treated with antitoxin. In the remaining cases, the diagnosis was delayed, the treatment was considered to be of no benefit and, therefore, antitoxin was not administered (Shapiro *et al*., 1998).

Some evidence shows that the early administration of antitoxin, especially within 24 hours, is more effective in preventing the progression of paralysis and in shortening the duration of mechanical ventilation and intensive-care stay (Tacket *et al*., 1984; Chang and Ganguly, 2003). Nonetheless, respiratory support may be necessary for long time, usually two to six weeks and in some cases may be longer: 58 days for botulism due to BoNT/A and 26 days for BoNT/ B (Hughes *et al*., 1981).

At the present time, only limited data are available on the relationship between dose (intravenous/intramuscular administration) and the amount of circulating antitoxin found in treated patients, their half-life and the toxin-neutralizing capacity. The half-life of circulating antitoxin was evaluated at 6.5, 7.6 and 5.3 days for antitoxin Types A, B and E, respectively. Peak serum concentrations of antitoxin are 10–1000 times higher than the concentrations estimated to be necessary to achieve toxin neutralization (Hatheway *et al*., 1984). Furthermore, 90% of the activity of the antitoxin administered was detected when all the circulating toxin was neutralized (Metzger and Lewis, 1979). The half-life of five to eight days and the exceedingly small quantities of toxin measured explain the limited decrease in antitoxin titres following toxin–antitoxin binding (Goldfrank and Flomenbaum, 2006). Published and unpublished clinical data support, as a correct approach in the USA, the administration of one vial of botulism antitoxin, which produces a high level of toxin type-specific antibodies (100-fold greater than that needed to neutralize the largest amount of circulating toxin ever measured at the CDC) (Hatheway *et al*., 1984).

As with other heterologous proteins, administration of horse-serum-derived preparations can result in adverse effects. During 1967–1977, the overall rate of adverse effects (including hypersensitivity and serum sickness) was 9–17% with an incidence of anaphylaxis of 1.9% (Black and Gunn, 1980). However, in previous decades, the recommended dose was two- to fourfold higher than those currently used in the USA. After changing to single-vial dosing, the incidence of hypersensitivity may be smaller than previously reported; at present, the risk of serum sickness may be approximately 1–4% (Sobel, 2005).

Throughout the world different formulations containing different quantities and combinations of specific types of antitoxins are available.

Currently there is only one product available in the USA, obtainable through the CDC, licensed and FDA-approved for adults (Type-AB-bivalent antitoxin), and containing in each 10 ml vial, 7500 and 5500 IU of Type A and B antitoxins, respectively. The recommended dose is one vial intravenously. Also, a monovalent antitoxin Type E (containing 5000 IU) is available in USA through an investigational new drug (IND) protocol: one vial of antitoxin Type E should be administered consecutively with bivalent (Types A and B) antitoxin if the type of botulism is not known or suspected to be E. Trivalent equine antitoxin (containing in each vial 7500 IU Type A, 5500 IU Type B, 8500 IU Type E of antitoxin), is no

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longer available from the CDC because of its declining antitoxin titres to antitoxin Type E (Dembek *et al*., 2007).

In addition, two other equine antitoxin preparations (investigational use) against all the seven toxins serotypes have been developed by the US Army Medical Research Institute of Infectious Diseases (USAMRIID) for treating botulism: botulism antitoxin, heptavalent, equine types A, B, C, D, E, F, G, named HE-BAT and botulism antitoxin $F(ab')_2$ heptavalent, equine toxin-neutralizating activity types A, B, C, D, E, F, G, named Hfab-BAT. The purification of the product resulting in Fab fragments potentially reduces the risk for serum sickness and hypersensitivity reactions. Both investigational products are available for botulism due to terrorism events, which may involve the use of any of the seven toxin serotypes (Dembek *et al*., 2007).

In Europe, the formulations currently available are trivalent and contain a different amount of total antitoxin (**Table 4**). The quantity of antitoxin per ml of antidote is quite similar in all the EU and USA formulations, while an important difference is related to the total amount of antitoxin that must be administered following the registered indications. The EU product marketed in Western Europe is recommended by the producer at a dose approximately 50-fold greater than the US formulation. Considering that acute or delayed hypersensitivity reactions have been reported significantly more frequently in patients treated with more than four USA vials of antitoxin, suggesting a dose-related phenomenon (Black and Gunn, 1980), a critical analysis of the minimal optimal dose to administer is needed. It must be considered that, generally, products derived from horse globulin may contain an extra amount of 10–20% of bulk antitoxin.

8.1.3 Supportive Airway Treatment

The mainstay of treatment for severe cases of botulism poisonings is early and advanced medical and nursing supportive care, with particular attention to respiratory status. Because of the high risk of rapid respiratory failure and because respiratory compromise is the usual cause of early death from botulism, close monitoring of respiration is needed throughout the disease.

The patient with suspected botulism poisoning not yet ventilated should be put in the reverse Trendelenburg position at 20–25° with cervical support; this seems to be useful to enhance diaphragmatic function, decreasing the pressure of abdominal viscera and to reduce the risk of aspiration, but the application in moderate to severe cases has not been validated (Arnon *et al*., 2001).

8.1.4 Antibiotic Therapy

Antibiotic therapy is ineffective on the toxin mechanism of action, even if it can be useful when secondary infections are present. Also, in wound botulism, the antibiotic therapy alone is inadequate. In any case, aminoglycoside antibiotics and clyndamicin should not be used because they may exacerbate the neuromuscular blockade (Santos *et al*., 1981).

8.1.5 Experimental Treatments

Guanidine has been used in the past to promote acetylcholine release, but its use, also evaluated in placebo-controlled studies, failed to ameliorate the clinical course of the disease (Roblot *et al*., 1994). Other studies including 4-aminopyridine, a potassium channel-blocking agent, which increases the release

Table 4 Comparison among principal marketed botulinum antitoxin products

Antitoxin product	Available from	Formulation	Total amount of antitoxin (IU)		Antitoxin per ml (IU)	Recommended dosage
Bivalent (A/B) equine	Aventis Pasteur CDC	Vial 10 ml	Type-A	7500	750	1 vial
			Type-B	5500	550	
Monovalent (E) equine	Aventis Pasteur CDC	Vial 10 ml	Type-E	5000	500	1 vial
Trivalent (A/B/E) equine	Biomed (Poland)	Vial 10 ml	Type-A	5000	500	1–5 vials
			Type-B	5000	500	
			Type-E	1000	100	
Trivalent (A/B/E) equine	Behring (Germany)	Bottle 250 ml	Type-A	187 500	750	2 bottles
			Type-B	125 000	500	
			Type-E	12 500	50	
Tetravalent (A/B/E/F) equine	Chiba Serum Institute (Japan)	Vial 20 ml	Type-A	10 000	500	1–3 vials
			Type-B	10 000	500	
			Type-E	10 000	500	
			Type-F	4000	200	
Trivalent (A/B/E) equine	Instituto Butantan (Brazil)	Vial 20 ml	Type-A	7500	375	1 vial
			Type-B	5500	275	

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of acetylcholine in the nerve terminations have been proposed (Qiao *et al*., 1997). 3,4-Diaminopyridine does not cross the blood–brian barrier to a substantial extent, and this limits central nervous system adverse reactions, such as seizures, potentially induced by 4-aminopyridine (Goldfrank and Flomenbaum, 2006). However, although some improvement has been reported in moderate cases, its efficacy has not been established (Dock *et al*., 2002). The only benefit observed regards an improvement in ocular and limb muscle strength in some patients, but there has been no benefit on respiratory paralysis (Neal and Dunbar, 1990). Steroids, immunoglobulins, chloroquine and plasmapheresis, have been tried in a few patients, with dubious benefits.

8.2 Wound Botulism

Treatment of wound botulism requires surgical debridement with extensive wound irrigation, preferably, if possible, after administration of the antitoxin and antibiotic therapy. Aerobic and anaerobic cultures of the wound should be performed. In some cases, aggressive surgical wound debridement is needed to remove the source of the toxin (Werner *et al*., 2000). The use of antibiotics is controversial, most likely because of the minimal delivery of drug that reaches the hypoperfused anaerobic wound site where *Clostridium* sp. proliferates. Penicillin G seems to be the first-line antibiotic, even if its clinical efficacy has not been firmly established; high-dose penicillin with other broad-spectrum antibiotics is appropriate, however, until a definitive diagnosis can be made (Mechem and Walter, 1994).

8.3 Intestinal Toxaemia: Infant Botulism

No specific indications are available regarding the treatment of adult intestinal botulism. Conversely, several studies support some different approaches to infant botulism. Supportive care, including respiratory assistance and nutritional support through parenteral feeding, is the mainstay of therapy for infant botulism.

Clostridiocidal antibiotics, such as penicillin, have not been demonstrated to be useful and should be administered with care, according with the clinical conditions, because of the possibility of rupture of the cells of the botulinal agent, causing additional toxin to be released; the use of nalidixic acid or trimethoprim-sulfamethoxazole antibiotics, to which *C. botulinum* is resistant, should be avoided (Swenson *et al*., 1980).

In infant botulism, circulating toxins are believed to be present at very low concentrations, and antitoxin has no effect on toxin-producing organisms in the gut. Therefore, antitoxin is not expected to halt syndrome progression (Goldfrank and Flomenbaum, 2006).

Equine antitoxin used for adult patients, theoretically has a limited role, mainly because of the short half-life (five to seven days) and the known side effects.

The Infant Botulism Treatment and Prevention Program in California, in collaboration with the US Food and Drug Administration and the CDC, produces and distributes the orphan drug botulism immune globulin intravenous (Human) (BIG-IV), a human-derived botulism antitoxin that neutralizes botulinum toxin (Arnon *et al*., 2006). The purified immunoglobulin is derived from pooled adult plasma from persons immunized with pentavalent botulinum toxoid, who were selected for their high titers of neutralizing antibody against BoNT/A and /B.

The treatment of patients with infant botulism Type A or Type B with BIG-IV reduced the mean length of the hospital stay for all patients to 3.1 weeks, and the mean length of stay in the intensive-care unit, the mean duration of mechanical ventilation, the mean duration of tube or intravenous feeding, and the mean hospital charges per patient were also significantly reduced. The only adverse effect perhaps related to treatment with BIG-IV was a transient, blush-like erythematous rash (Arnon *et al*., 2006).

9 INHALATION BOTULISM

Primate experiments designed to study treatment for inhaled botulism toxin showed that doses of 5–10 LD_{50} cause death in 24–96 hours in animals neither immunized nor treated with antitoxin (Franz *et al*., 1993). The syndrome in humans was first described in three German veterinary workers after conducting post-mortem examinations on laboratory animals. With a delay of three to four days, the patients presented with sore throat, dysphagia, mydriasis, mental numbness and extreme weakness; they were treated with antitoxin on the fourth to fifth day after the incident and fully recovered in two weeks. BoNT/A was detected in a serum sample from all three affected patients (Holzer, 1962). Monkeys showed signs of botulism 12–80 hours after aerosol exposure to four to seven multiples of the monkey median lethal dose. The time to onset of inhalational botulism cannot be stated with certainty because so few cases are known. Any outbreak of a large number of simultaneous cases with a common geographic factor, but without a common dietary exposure and, possibly, with an unusual botulinum toxin type (e.g. Types C, D, F, G) may suggest a deliberate release of botulinum toxin as an aerosol attack (Arnon *et al*., 2001). Botulism is at least 5000 times more lethal than sarin and 1800 times more lethal than VX nerve agent.

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Botulinum toxin was developed as a biological weapon at least 60 years ago, especially during World War II, by state-sponsored programmes in the USA, Soviet Union, Japan, Germany and Iraq. Although the 1972 Biological and Toxin Weapons Convention prohibited offensive research and production of biological weapons, some signatories, such as Iraq and Soviet Union, continued to produce botulinum toxin to use as a weapon. During the last decade, countries suspected to sponsor terrorism are believed to be still developing botulinum toxin as a weapon. Botulinum toxin has already been used as a biological weapon: aerosols were used in at least three occasions between 1990 and 1995 in Japan by the Japanese Cult Aum Shinrikyo, but these attacks failed.

A deliberate release of botulism either by aerosol or contaminated food would be able to cause a large number of casualties in a civilian population. Predicting the consequences of environmental dissemination is difficult and problematic, since data about the stability of the toxin in water or sunlight are lacking; it has been estimated that an aerosol release of toxin could intoxicate 10% of people within 500 m. The decay rate of the toxin in air is estimated at 1–4% per minute and models of aerosol exposure show that inactivation may take up to two days, and would be accelerated in extreme conditions of temperature and humidity (Arnon *et al*., 2001).

A mass casualty attack with botulinum toxin could likely overwhelm the healthcare system and drastically involve the medical service and response to victims; the medical support required for poisoned patients includes mechanical ventilation as well as urgent attendant medical care, together with the activation of hospital emergency plans, including actions for additional respiratory and intensive-care unit support for mass poisoning. Furthermore, when an outbreak happens, involving a large number of patients, there may be a problem to have an adequate quantity of antitoxin to administer. A recent experience of a massive outbreak of botulism in northern Thailand tested the international capacity to respond to a public-health emergency. An outbreak due to contaminated home-canned bamboo shoots has caused illness in 209 patients, of whom 134 were hospitalized and 42 required mechanical ventilation (Ungchusak *et al*., 2007). A global search for antitoxin has been applied, involving international agencies, embassies, national laboratories, airlines and commercial organizations in seven countries. Sufficient antitoxin was obtained from four sources for treatment of 90 patients, but with delays in treatment of five to nine days from time of exposure. This suggests that a stronger commitment through formal international agreements is urgently needed, including creation of global and regional stockpiles of critical medicines and supplies. Modelling for bioterrorism incidents have been used and analysed in order to plan all the action to counteract the phenomenon.

10 IATROGENIC BOTULISM

The most recent form of botulism described is iatrogenic botulism, which can result mostly from erroneous injection of botulinum toxin (toxin Types A and B) for cosmetic or therapeutic purposes. A distant effect from the site of injection of toxin is also hypothesized: experimentally, the catalytically active BoNT/A is transported in a retrograde manner along microtubules, transcytosed and taken up by afferent terminals (Antonucci *et al*., 2008).

The dose usually administered to obtain local muscle paralysis is not, theoretically, able to cause severe systemic effects. A typical clinical application varies from 4 to 20 units for cosmetic purposes up to 300 units for therapeutic use (Chertow *et al*., 2006). The human lethal dose of crystalline BoNT/A is unknown: data on primates suggests an approximate intravenous or intramuscular human lethal dose of 40 U kg^{-1} (Scott and Suzuki, 1988), each unit being the estimated LD_{50} for mice (CDC (Centers for Disease Control and Prevention), 1998). When used in therapeutic doses, BoNT/A is considered to be safe and effective, and only systemic minor adverse effects are described, such as flu-like symptoms, transient fatigue, nausea, ptosis and generalized weakness. Subclinical effects of the toxin on neuromuscular transmission distant from the site of injection have also been reported, even for doses of 23 U kg^{-1} of BoNT/A (Olney *et al*., 1988; Goldstein, 2006). The analysis of all adverse events suggests that patients receiving more than 1000 U of BoNT/A were more likely to have adverse effects (Crowner *et al*., 2007).

Nonetheless, the reports of adverse effects or electrophysiological abnormalities following therapeutic and erroneous cosmetic use of toxin Type A have increased in recent years.

The first laboratory-confirmed cases of iatrogenic botulism were a cluster of four adult patients who may have each been injected with a highly concentrated (2857 times the estimated human lethal dose by injection), unlicensed preparation of botulinum toxin Type A, for cosmetic treatment (Chertow *et al*., 2006). The serum toxin levels (collected before antitoxin treatment) were equivalent to 21–43 times the estimated human lethal dose. The typical syndrome of botulism poisoning (extreme weakness, vertigo and initial cranial neuropathies) presented two to three days after injection; subsequently the patients presented with dry mouth, diplopia, blurred vision, ptosis, mydriasis, dysphagia, dysarthria, dyspnoea, tachypnoea and respiratory failure. All patients received bivalent anti-AB equine antitoxin; three patients received antidote on day 4 and one patient on day 10. After prolonged mechanical ventilation (from 36 to 171 days), physical rehabilitation and hospital stay, all patients were discharged from the hospital.

The EU has posted a series of warnings concerning botulinum toxin on its web site, the latest in March

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2007, alerting physicians in its 27 member states to the need to monitor for signs of botulinum toxin adverse events (European Medicines Agency (EMEA), 2007). Contemporarily, the Danish Medicines Agency published an analysis of adverse reactions from the four products marketed in the EU. The results showed that, since the entry of the product on the market, more than 600 reports of adverse events related to botulinum toxin have been registered, and approximately half of all serious adverse reactions were caused by spread of toxin. These adverse events are substantially under-reported and connected with cosmetic, as well as therapeutic, uses (The Danish Medicines Agency, 2007). Recently, serious adverse effects arising from approved medications (botulinum toxin Types A and B) have been reported by US nonprofit public organizations and also by the FDA, and warnings have been diffused (Med Watch, 2008).

11 NOTIFICATION OF THE DISEASE

As foodborne botulism results from ingestion of preformed BoNTs in foods that still may be available to cause illness in others, a single case of foodborne botulism represents a public-health emergency that might herald a larger outbreak (Shapiro *et al*., 1998). Prompt diagnosis and epidemiological investigation for rapid indentification of the food vehicle is essential, especially for commercial foods. For this purpose, the prompt activation of the Rapid Alert System for Food and Feed, and the withdrawal of goods from the commercial network, could prevent the onset of international outbreaks.

Botulism is a statutory disease and it is mandatory to notify the State Health Department/Officers of cases of suspected poisoning. Serum and stool samples should be obtained (if possible, always before antitoxin administration), and a call to a Poison Control Centre or a National/Regional Reference Centre may be helpful for appropriate clinical management and possible laboratory support.

12 BOTULISM AND PREGNANCY

Clinical experience and experimental data confirm that the large molecular weight of the toxin (150 kDa) blocks passive diffusion through the placenta (Polo *et al*., 1996; Magri *et al*. 2006); no cases of clinical or serum evidence of botulism in infants born from mothers poisoned were observed. Adequate care and preparation for maternal complications of delivery seems to offer the best potential outcome for a normal infant. The use of botulinum toxin Type A in pregnant women appears to be relatively safe for both the expectant mother and the foetus (de Oliveira Monteiro, 2006; Morgan *et al*., 2006). Pregnancy is not considered a contraindication to antitoxin administration, which has been used successfully (Robin *et al*., 1996).

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Ricin: Chemistry, Sources, Exposures, Toxicology and Medical Aspects

J. Michael Lord and Gareth D. Griffiths

CONTENTS

1 INTRODUCTION

Ricin is a potently toxic protein that occurs in the seeds of the castor oil plant, *Ricinus communis*. This plant thrives in tropical and subtropical regions of the world, but also grows well in Europe including the United Kingdom. It is extensively cultivated in warmer climates for the extraction of castor oil. Approximately 1.3 million metric tonnes of castor oil seeds, grown mainly in India, but also Brazil and China, are processed annually to extract castor oil. This product is used widely in the manufacture of many commodities, including lubricants (oils and greases), polishes, paints and varnishes plasticizers and cosmetics. Ricinus plants exist in a many different forms known as cultivars and, broadly speaking, have green leaves and stems, or red leaves and stems, and the seeds may also vary considerably in size and colour, but the outer husk always bears a characteristic lighter pattern on a darker background (**Figure 1**).

Ricin toxin (also referred to as RCA_{60}, *Ricinus communis* agglutinin with molecular weight of 60 kDa) is abundantly present in the seeds of all cultivars where, together with the closely related lectin (RCA_{120}, *Ricinus communis* agglutinin with molecular weight of 120 kDa), it can account for up to 5% of the total seed protein. Ricin is synthesized during seed development, and is stably stored in mature seeds in an organelle termed the vacuole, together with several other abundant seed proteins. Collectively these proteins are called storage proteins: they are rapidly hydrolysed during the early stages of seed germination to provide a source of amino acids for the synthesis of proteins encoded by genes that are expressed at this stage of development. After a few days of post-germinative growth, the storage proteins, including ricin, have disappeared entirely as

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Figure 1 Examples of *Ricinus* seeds from various cultivars of *Ricinus communis.* (A) *Ricinus yunnan* castor (China); (B) *Ricinus shangdong* 108 (China); (C) *Ricinus zanzibariensis* (Africa); (D) *Ricinus dehradun* (India); (E) *Ricinus sanguineus* (Africa).

the developing seedling is increasingly able to synthesize the amino acids it needs via photosynthesis. The biological function of ricin, therefore, seems to be as a seed-storage protein, with the added advantage that its potent toxicity deters herbivores from eating the seeds.

Although the toxicity of *Ricinus* seeds had been known from antiquity, the active principle was first described as a toxic lectin in 1888, in the inaugural thesis of Hermann Stillmark. Stillmark worked at the University of Dorpat in what was then Estonia, as a student of Robert Kobert. In one of his *in vitro* experiments, he mixed an extract of the seeds with blood and observed that the erythrocytes rapidly became agglutinated. He found that the agent responsible was a protein which he termed 'ricin', the first lectin to be identified (Stillmark, 1888). Lectins were initially designated as 'haemagglutinins', or 'phytohaemagglutinins' because the first examples of lectins came from plants (Etzler, 1985). We now know that the haemagglutination observed by Stillmark was largely due to RCA_{120} rather than ricin, but in 1888 he reasonably concluded that the toxicity of *Ricinus* seeds was caused by their ability to agglutinate red blood cells. Shortly afterwards, another student of Kobert's, H. Hellin, found that the toxic extracts of the jequirity bean (*Abrus precatorius*) also caused red blood cells to agglutinate, and the second haemagglutinin to be described was called 'abrin'.

Ricin gained considerable notoriety as the likely poison in the small pellet used to kill the Bulgarian journalist Georgi Markov in the so-called 'umbrella-tip' assassination in London in 1978 (Knight, 1979). A more recent concern focusses on the potential use of ricin as a weapon of bioterrorism. *Ricinus* seeds are readily available from the extensive cultivation of the plant for commercial and now, horticultural purposes around Asia, Europe and the Americas. Ricin can be easily isolated from the seeds using uncomplicated and low-cost procedures that require neither complex laboratory facilities nor scientific expertise, thereby making the toxin a desirable biological agent for terrorist organizations. Indeed, descriptions on how to isolate this toxin in an active state have been found in several terrorist manuals. As such, both the UK and US Governments list ricin as a category B agent for bioterrorism.

2 STRUCTURE AND BIOGENESIS

Ricin is the best-characterized member of a family of plant proteins collectively known as ribosome-inactivating proteins (RIPs) (Lord *et al*., 1994). It is a heterodimeric protein (**Figure 2A**) composed of a ribosome-inactivating enzyme (32 kDa, designated the A chain or RTA) linked to a galactose/*N*-acetyl-galactosamine-binding lectin (34 kDa, the B chain or RTB; Figure **2A**) (Lord *et al*., 2004). RCA_{120}, in contrast, is tetrameric, composed of two ricin-like heterodimers (**Figure 2B**), each of which contains an A chain (32 kDa) and a galactose-binding B chain (34 kDa). In addition to their structural differences, these two proteins also differ in their biological properties. Ricin is a potent cytotoxin, but a weak haemagglutinin, RCA_{120} is only weakly toxic to intact cells, but is a strong haemagglutinin. However, although RCA_{120} is only weakly toxic to intact cells *in vivo,* its isolated A chain is an RIP of comparable activity to the ricin A chain in terms of its ability to modify ribosomes *in vitro* (O'Hare *et al*., 1992). The sugar-binding specificities are also similar, but not identical. Overall, however, ricin and RCA_{120} are closely related proteins, and antisera raised against individual ricin A or B chains cross-react strongly with the corresponding RCA_{120} subunits and vice versa. The complete primary structures of the ricin and RCA_{120} subunits are known. Overall the A chains differ in 18 of their 267 residues and are thus 93% homologous at the amino-acid level, whereas the B chains differ in 41 of 262 residues, giving 84% homology (Roberts *et al*., 1985).

The ricin gene encodes a preproprotein containing both the RTA and RTB sequences. Preproricin consists of 576 amino acid residues, the first 35 of which include a 26 residue N-terminal signal sequence and a nine residue propeptide, followed by the mature A chain sequence (267 residues) joined by a 12 residue linker peptide to the mature B chain sequence (262 residues). The 12 residue linker sequence contains a targeting signal that directs the ricin precursor to the vacuole (Frigerio *et al*., 2001).

During ricin biosynthesis, the N-terminal signal sequence directs the transport of the nascent polypeptide across the endoplasmic reticulum (ER) membrane

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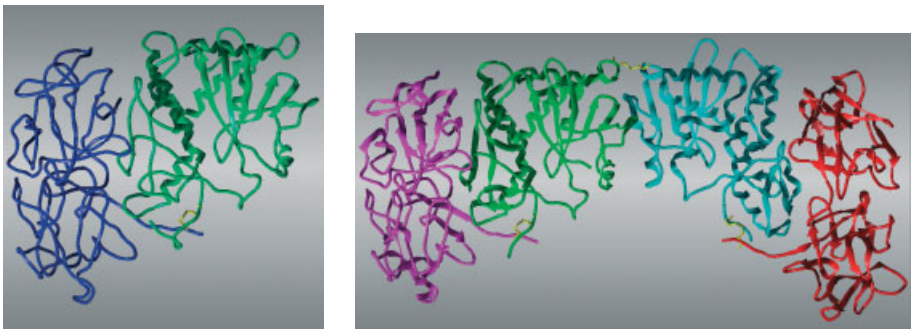

Figure 2 (A,B) Representations of the RCA_{60} and RCA_{120} structures, respectively. (A) Ricin (RCA_{60}) is a heterodimer in which the catalytically active A chain (green) is covalently linked to the galactose-binding B chain (blue) by a single disulfide bond (yellow). (B) *Ricinus communis* agglutinin (RCA_{120}) is a tetramer containing two A chains (green and light blue) and two B chains (pink and red), which are highly homologous to their ricin counterparts. The RCA_{120} A chain contains an extra cysteine residue, not present in the ricin A chain, which forms a disulfide bond linking the A chains of two ricin-like heterodimers (yellow) to give the tetramer.

of the seed endosperm cells and into the ER lumen. Translocation across the ER membrane is accompanied by three major modifications (Lord and Roberts, 1993). First, the N-terminal signal peptide is cleaved by ER luminal signal peptidase. Second, proricin is N-glycosylated as it enters the ER lumen. Proricin contains four N-glycosylation sites, two within the A chain sequence and two within the B chain. Third, protein disulfide isomerize catalyses the formation of five disulfide bonds in the folding proricin. In mature ricin, four of these disulfide bonds are in the B chain, while the fifth one is the disulfide bond linking RTA and RTB. Proricin is transported from the ER, via the Golgi complex, to the vacuole. The attached glycans are modified during intracellular transport (Lord, 1985) and, upon reaching the vacuole, proricin is processed by an endopeptidase that removes the 12 residue linker sequence between RTA and RTB, and the nine residue N-terminal propeptide. The two subunits are still covalently joined by a disulfide bond because cleavage of the linker occurs within a disulfide loop connecting the A and the B chain sequences (Lord and Harley, 1985).

The three-dimensional structure of ricin has been solved by X-ray crystallography (Montfort *et al*., 1987). RTA is a globular protein with a pronounced active site cleft, while RTB is a dumbbell shaped, two-domain protein, the two domains apparently the result of a gene duplication event (Villafranca and Robertus, 1981).

3 RIBOSOME MODIFICATION BY THE RICIN A CHAIN

RTA, in common with other RIPs, is an enzyme that removes a specific adenine residue from the ribosomal 28 S RNA of the large subunit of eukaryotic ribosomes (Endo *et al*., 1987a). The adenine removed, adenine 4324 in the case of rat liver 28 S ribosomal RNA (rRNA), is located in a region of the rRNA that contains one of the longest universally conserved rRNA sequences. This highly conserved region of rRNA functions in the ribosomal elongation cycle, and the adenine residue removed as a result of RTA-catalysed depurination is the binding site for elongation factors 1 and 2. Since RTA-modified ribosomes are rendered unable to bind elongation factors, they are unable to achieve protein synthesis, leading eventually to cell death. Because it is an enzyme, a very small amount of RTA inside a cell can inactivate numerous ribosomes, making ricin one of the most cytotoxic compounds in nature.

4 INTOXICATION OF INTACT CELLS BY RICIN

Because RTA's ribosomal substrate is in the cell cytosol, RTA must enter this compartment in an active conformation in order to intoxicate the cell. In common with other members of the so-called A-B toxin superfamily, ricin's bipartite structure is crucial in the regard. Ricin binds, via its B chain lectin activity, to any cell surface glycoprotein or glycolipid having a terminal β-1-4-linked galactose residues. Such potential ricin binding sites are plentiful on mammalian cells: each HeLa cell, for example, can bind 3×10^7 molecules of ricin (Sandvig *et al*., 1976). Surface-bound ricin enters the cell by endocytic uptake, and is initially delivered into early endosomes. Much of the ricin that enters the cell is returned to the cell surface in recycling endosomes, or is transported via late endosomes to the lysosomes, where it is destroyed. Some of

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the ricin in early endosomes follows an intracellular transport route that is productive for cellular intoxication. This intracellular trafficking entails retrograde transport to the ER via the Golgi complex (Spooner *et al*., 2006), the ricin holotoxin reaches the lumen of the ER, from where RTA crosses the ER membrane to reach the cytosol. The molecular details of this retrotranslocation step from the ER lumen to the cytosol are not yet completely established, but it is thought that RTA and other toxin subunits do so by exploiting the quality-control system known as endoplasmic reticulum-associated protein degradation (ERAD) (Lord *et al*., 2006). The ER lumen is a major protein-folding compartment. Newly synthesized proteins in the ER that fail to fold or assemble correctly are disposed of by ERAD. This entails their retrotranslocation from the ER to the cytosol, where they are degraded by the proteasome, facilitated normally by polyubiquitination (Romisch, 2005). The emerging explanation for RTA retrotranslocation is as follows. After its arrival in the ER lumen, ricin is reductively separated into the A and B subunits by the action of ER protein disulfide isomerase (Spooner *et al*., 2004). Free RTA contains a sequence of hydrophobic amino acids that is covered by RTB in the ricin holotoxin. This exposed hydrophobic stretch inserts into the luminal surface of the ER membrane, which triggers the partial unfolding of RTA (Day *et al*., 2002). RTA is perceived by the ER quality-control system as a misfolded membrane protein and therefore a substrate for ERAD. This results in RTA retrotranslocation, but in order to intoxicate the cell, at least some of the retrotranslocated RTA avoids the normal fate of ERAD substrates, namely proteasomal degradation. The low lysine content of RTA and other protein toxins that retrotranslocate from the ER facilitates this uncoupling from a degradative fate (Deeks *et al*., 2002; Rodighiero *et al*., 2002). The fate of RTA in the cytosol is influenced by molecular chaperones and cochaperones, which assist in regaining the native conformation (Spooner *et al*., 2008).

Earlier studies *in vitro* (Hughes *et al*., 1996) demonstrated that ricin induced apoptosis in bovine pulmonary endothelial cells. Subsequently, ricin-induced apoptosis has been demonstrated in many other cell lines, and studies have been undertaken to further elucidate the mechanism of this form of cell death (e.g. MDCK cells, Oda *et al*., 1999; HeLa cells, Gan *et al*., 2000; U937 cells, Kageyama *et al*., 2002; RAW 264.7 cells, Higuchi *et al*., 2003; human cervical cancer cells, Rao *et al*., 2005; primary human macrophages, Korcheva *et al*., 2007).

5 EXPOSURES TO RICIN

Considerable information has been published on the intoxication of various cell types by ricin. As touched upon above, this *in vitro* approach offers great potential for understanding the mechanism of action of toxins like ricin, but the likely consequences of poisoning in man, and the development of medical countermeasures and therapies, our ultimate concern, can only fully be understood and developed through the use of animal models.

Ricin has been of concern as a possible warfare weapon for some time, but is now also considered a feasible terrorist weapon because of the abundance of the castor oil seeds and the relative ease with which a crude ricin toxin may be extracted. This is further supported by the internet publications describing simple extraction and preparation of extracts from *Ricinus* seeds using low technology.

From a military perspective, the primary route of concern for intoxication by ricin is by inhalation, although poisoning is also possible by other routes, including access to the bloodstream across broken skin or by ingestion. Clearly, these potential routes of intoxication could also be relevant to civilian casualties in a terrorist situation. The inhalation toxicology of ricin will be examined first, before going on to consider the other possible routes of intoxication.

6 INHALATION TOXICOLOGY OF RICIN

There is probably more toxicological information available from laboratory studies of experimental ricin poisoning by inhalation than for the other possible routes of intoxication. The inhalation toxicology of ricin in rodents, both mice and rats, has been examined in the UK at the Defence Science and Technology Laboratory (Dstl), while the US Army Medical Research Institute of Infectious Diseases has examined this route of ricin exposure in rodents and nonhuman primate species (Wannemacher and Anderson, 2006). A number of factors must be considered when designing inhalation toxicological experiments, including, not least, the choice of animal model, its airway physiology and anatomy, the respiratory properties of the species and the size of the aerosol particles produced by the aerosol generator.

6.1 Aerosol Generation

Ricin and other protein toxins could theoretically be produced as aerosols from liquid solutions or from powdered material, using a variety of aerosol generators. In studying inhalation toxicology, key issues to be satisfied include the delivery of sufficient material of the appropriate particle size, its mass median aerodynamic diameter (MMAD), to reach the desired target site.

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Inhalation toxicological studies with ricin at Dstl have been conducted using solutions of ricin, generated as respirable aerosols for the rodent models (rats and mice), using a Liu–Lee nebulizer (Liu and Lee, 1975), with modifications to enable a constant output (Leong *et al*., 1982). The ricin solutions were made in phosphate-buffered saline, which was tagged with sodium fluorescein, to enable an estimate to be made of aerosol concentrations achieved during each exposure. The generator produced aerosol particles which, when dry, had a MMAD of between 0.89 and 0.91 μm, with a geometric standard deviation of 1.6. This means that the range of particle sizes produced was very small and largely homodisperse. Rodents were exposed to aerosol by nose only, rather than whole body, being held in metal plethysmography tubes sealed at the 'tail end' with a rubber bung and around the neck of each animal using a latex collar. This enabled respiratory parameters to be measured during the experimental period (online pneumotachography), which, in conjunction with calculated aerosol concentration (from fluorescein tagging) allowed for more accurate determination of the quantities of toxin inhaled.

The inhaled quantities of a challenge agent are usually expressed in terms of aerosol concentration (C; mg m^{-3}) and the time (t) for which exposure was maintained (minutes) giving a composite value (Ct) of mg min m^{-3}. While the likely quantity of toxin inhaled over a period of time can be derived from the information gathered during the experiment, it is far more difficult to estimate the actual quantity of the toxin retained in the respiratory tract. An approximation of retained material is usually made on the basis of the aerosol inhaled during the exposure time and may be expressed as μg or mg per kg bodyweight. Although LD_{50} (median lethal dose) or LCt_{50} (median lethal concentration and time) are normally quoted as single values, there is usually a fairly wide margin of error. In the case of inhalation toxicology, this variation may be between 10 and 20% either side of the quoted mean value (95% confidence limits). There may be several reasons for such variation, one of which is that the lethal Ct is usually determined from relatively few animals, each of which may have different susceptibilities or resistance to the challenge agent.

6.2 Inhalation Toxicity of Ricin

Following observation of the health status and survival of the experimental rodents, it was possible to express aerosol toxicity in terms of lethal Ct_{50} (LCt_{50}), the product of aerosol concentration and the exposure time producing death of 50% of the recipient animals. Alternatively, information may estimate an approximate lethal dose 50 (LD_{50}).

Solutions of different concentrations of pure ricin were made for aerosolization from two different seed cultivars of *Ricinus communis,* Hale Queen (a commercial preparation from Sigma) and *Ricinus zanzibariensis*. Adult rats (approx. 250 g bodyweight) were exposed to aerosols of either material for 10 minute time periods and, following a period of observation lasting 14 days, lethality data were derived. It was interesting to find that the LCt_{50} values determined for the toxicity of the two preparations were different, being between 4.54 and 5.96 mg min m^{-3} (estimated LD_{50} 3.7 μg kg^{-1}) for Hale Queen and 12.7 mg min m^{-3} (estimated LD_{50} 9.8 μg kg^{-1}) for *Ricinus zanzibariensis.* Times to death were related to dose received, varying from about 36 hours at higher Cts to between 66 and 138 hours at lower Cts. Details of these studies have been published (Griffiths *et al*., 1994). The inhalation toxicology of pure ricin extracted from *Ricinus zanzibariensis* in the Balb/c mouse was also examined in our laboratory using the same exposure system, and the LCt_{50} was found to be 6.52 mg min m^{-3} (95% confidence limits: lower 6.153, upper 7.038). This approximated to an LD_{50} of 6 μg kg^{-1}.

Inhalation toxicity studies of ricin have been undertaken by other laboratories using the mouse as the small-animal model. For example, Balb/c mice were challenged by inhalation of pure ricin using a whole-body exposure method (Wannemacher and Anderson, 2006) and an LD_{50} of 11.2 μg kg^{-1} was determined. Differential susceptibilities to inhaled ricin were also mentioned in relation to mouse strain and the LD_{50} for inhaled ricin in the most sensitive strain, BXSB, was approximately 2.8 μg kg^{-1}. It was also intimated by these authors that the toxicities of ricin prepared from different *Ricinus* plant cultivars can vary, an observation supported by our own studies on inhaled ricin purified from two cultivars of the plant.

6.3 Histopathology and Symptoms of Inhalation Intoxication

The pathogenesis of damage resulting from the inhalation of pure ricin aerosol particles (from *R. zanzibariensis*) in the rat, at an approximate LCt_{30} challenge dose (i.e. a quantity of ricin which would kill approximately 30% of recipients) was found to be confined to the lungs (Griffiths *et al*., 1994). Using light microscopy it was evident that ricin had reached the deep lung, since, by 24 hours, lung tissue sections showed acute inflammatory cell infiltration into the alveolar walls with frequent flooding of air spaces by protein-rich oedema (Oe) fluid. By 48 hours (**Figures 3B** and **C**) lung sections clearly illustrated florid intra-alveolar Oe and large numbers of darkly stained migratory inflammatory cells within the alveolar septae. This damage was limited to the lung and other organs examined remained normal in appearance. Using

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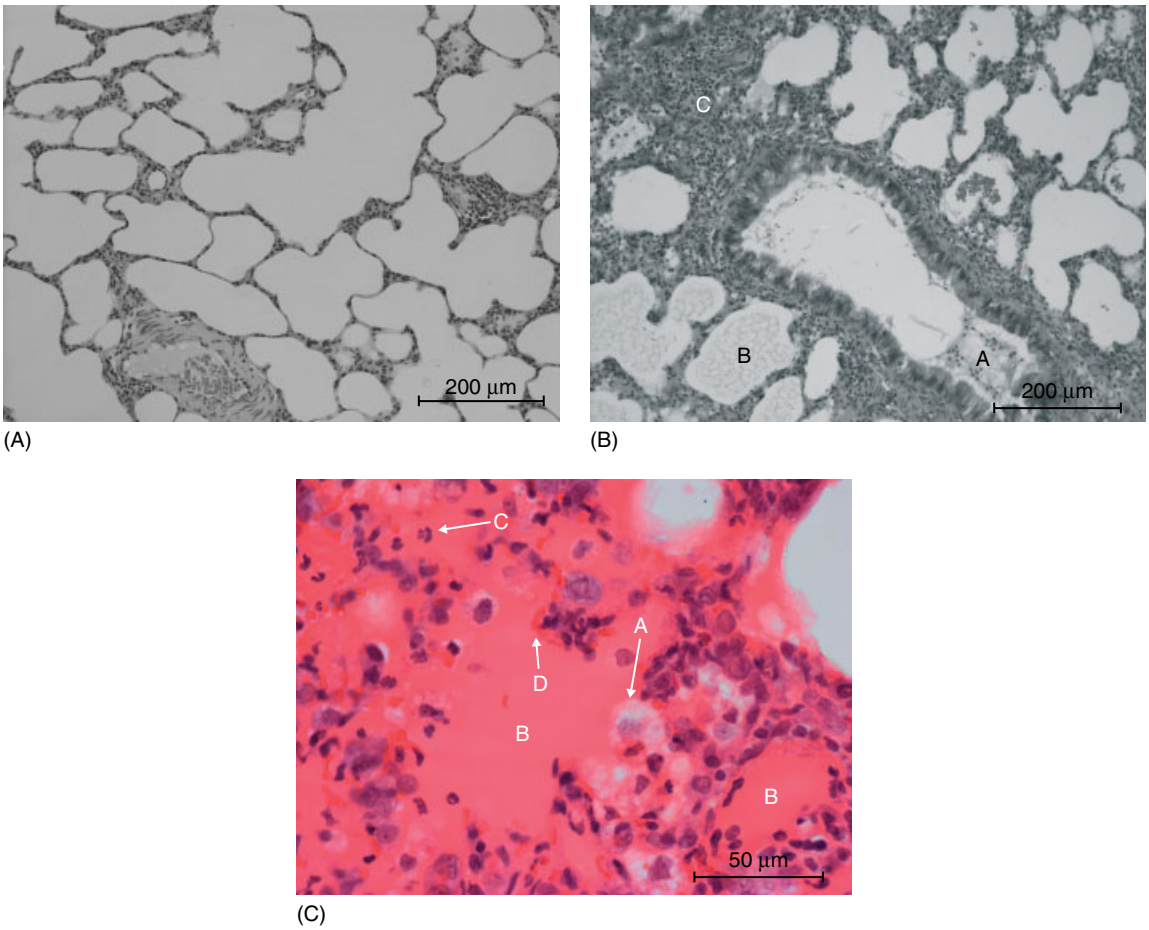

Figure 3 (A–C) Sections from lungs of rats, focusing on the alveolar region. (A) Normal rat lung, showing open, clear alveolar spaces bordered by thin epithelial membranes. (B) Section through alveolar region of lung of rat taken 48 hours after inhalation exposure to ricin toxin (LCt_{30}). The picture shows: A, residual proteinaceous oedema fluid containing inflammatory cells within the lumen of a bronchiole; B, residual proteinaceous oedema fluid within the airspace of an alveolus and C, congestion of the interstitial tissue, between alveolae, with inflammatory cells. (C) A section through the alveolar region of the lung of a rat taken 48 hours after inhalation exposure to ricin toxin (LCt_{30}). The picture shows: A, an example of macrophage next to other cells around what appears to be an alveolus (B); other macrophages may be seen throughout the section. The alveolar epithelial boundary is no longer clear, as a result of damage. An alveolus with epithelium partially intact features on the bottom right of this figure. In another alveolar region, a neutrophil is indicated (C); further neutrophils are evident elsewhere in this section. Red blood cells can be seen (stained bright red) within the alveolar spaces and also amongst interstitial cells as a result of haemorrhage through damage to capillary endothelia. An example is indicated (D).

the higher resolution afforded by electron microscopy, Brown and White (1997) observed the initiation of Oe formation, initially perivascular, from 12 hours following inhaled ricin aerosol (LCt_{30}). The situation progressed from 15–36 hours to include interstitial and intra-alveolar regions. Additionally, inflammatory cell infiltration was evident from 12 hours post challenge and the greatest numbers of macrophages and polymorphonuclear leucocytes were noted between 24 and 48 hours.

Aerosolized ricin particles penetrating the deep lung of the rats induced tissue pathology and the epithelia of both the lower respiratory tract and the alveoli were subject to destructive inflammatory damage, with evidence of necrotic and apparently apoptotic processes. Apoptosis was identified purely on cell morphology, including rounding up, cytoplasmic contraction and frequent appearance of discrete particles of apparently nuclear material. The same picture was maintained at 96 hours when signs of repair were also noted in the form of hyperplasia of Type II epithelial cells. Beyond this time, a gradual clearing of Oe fluid was observed although complete resolution of damage was not achieved until 14 days post exposure. This progressive pathogenesis was also reproduced in studies conducted in mice in our laboratory.

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The major problem caused by the inhalation of ricin seems to be pulmonary flooding of the lungs resulting from primary damage to epithelia and presumably underlying blood-vessel endothelia. Damage is probably exacerbated by the massive inflammatory response mounted by the host to the presence of the toxin and the primary damage caused. Together, these events lead to respiratory insufficiency which, in severe cases, leads to death.

There is some debate as to whether inhaled ricin gains access to tissues beyond the lung. In our studies at Dstl we have not found evidence of inhaled ricin causing damage to other tissues. In an earlier inhalation study using ^{125}I-labelled ricin (Doebler *et al*., 1995), mice were exposed nose-only to the aerosol and radiolabel was found in the lungs, but also in the gastrointestinal tract and minimally in the circulation. Some inhaled aerosol is likely to be swallowed, although it is unlikely that it would contribute significantly to the lethality of the total dose received, since the oral toxicity of ricin is orders of magnitude lower than that of the inhaled toxin. It is also difficult, when tracing molecules carrying a radioactive label, to be clear whether one is measuring the intact molecule or a fragment of the molecule. In a more recent study the intrapulmonary instillation of a lethal dose of ricin was found to cause an accumulation of proinflammatory messenger RNA (mRNA) transcripts, primarily in the lungs, but also in other body tissues including the liver, spleen and blood, as well as elevated levels of proinflammatory cytokines. This challenge dose also produced pathology in the kidney, including tubular fibrosis and impaired renal function. The LD_{50} quoted by the authors (Wong *et al*., 2007) for the ricin administered by this method was 10 µg (100 g bodyweight)$^{-1}$ which is equivalent to 100 µg kg^{-1}. In our experience in the rat, the LD_{50} of ricin by instillation was very close to the inhaled LD_{50} and within the range of 1–10 µg kg^{-1} bodyweight. If this is true for the mouse, the lethal dose derived in this study was very high. This might suggest that a significant quantity of the ricin instilled was being delivered to the stomach and intestine, although the lungs were also clearly receiving damaging amounts of the toxin.

7 OTHER ROUTES OF INTOXICATION AND TOXICOLOGY

7.1 Ingestion of Ricin

The toxicity of ricin when delivered by the oral route is much lower than by inhalation and parenteral delivery routes. In the mouse, the LD_{50} for orally administered ricin is quoted by Franz and Jaax (1997) as 20 mg kg^{-1} bodyweight with a time to death of approximately 85 hours. The lower toxicity by this route is probably a reflection of the poor absorption of ricin from the intestine. Another review by Audi *et al*. (2005) reported the LD_{50} of ricin by the oral route to be 30 mg kg^{-1} and a human LD_{50} estimate was given as 1–20 mg ricin kg^{-1} bodyweight. A probable human oral lethal dose given in the TOXNET Hazardous Substances Database for ricin is less than 5 mg kg^{-1}.

While there have been many reported suicide attempts through the ingestion of castor oil seeds, there is great difficulty in the interpretation of the toxic dose because of variables, including differences in ricin content of seed types (e.g. geographic region of plant growth, time of harvesting seeds, degree of hydration of seeds) size and weight of seeds ingested, as well as the degree of mastication to release the seed contents, including the water-soluble ricin.

7.2 Histopathology and Symptoms of Oral Intoxication

Victims of oral ricin poisoning feature prominent gastrointestinal symptoms, which may include colicky abdominal pain, vomiting, diarrhoea, heartburn and oropharyngeal pain. In addition, haematemesis and melena are less commonly-reported symptoms. Fluid loss may result in electrolyte imbalance, dehydration, hypotension and circulatory collapse (Audi *et al*., 2005), and associated symptoms may include tachycardia, tachypnoea, sweating and peripheral cyanosis (Bradberry *et al*., 2003). Histopathology of lethal cases of oral ricin intoxication includes multifocal, often severe, ulceration and haemorrhage of the gastric and small intestinal mucosa. Also necrosis of the draining mesenteric lymph nodes, gut associated lymphoid tissue and spleen. Necrosis is observed in liver tissue, especially the reticulendothelial Kupffer cells and diffuse inflammation of renal tubules (nephritis) and the spleen (Franz and Jaax, 1997). Apoptotic cell death is also cited in the histopathology of ricin toxicity by ingestion (Audi *et al*., 2005).

At Dstl we have limited experience of oral ingestion of ricin in Balb/c mice. Three mice were administered (by gavage) 10 or 20 mg kg^{-1} of ricin purified from *Ricinus zanzibariensis* seeds. Animals appeared normal over a seven day period following oral dosing with 10 mg kg^{-1}. Forty-eight hours after dosing with 20 mg kg^{-1} ricin, however, all mice were relatively inactive, they had lost the inquisitive habit typical of rodents and their fur was piloerected. One animal died on the fourth day and a photograph (**Figure 4**) of the stomach and small intestine shows evidence of inflammatory reddening of the small intestinal wall, close to its point of exit from the stomach. From this very limited study it appears that in

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Figure 4 Inflammation of the duodenum proximal to the stomach in the gastrointestinal tract removed from a female Balb/c mouse, four days after oral dosing with ricin (20 mg kg^{-1}).

this species, the LD_{50} of pure ricin (from *Ricinus zanzibariensis*) probably lies around 20 mg kg^{-1} (unreported observations). This concurs well with other reported data (Franz and Jaax, 1997).

7.3 Parenteral Intoxication with Ricin

Routes of intoxication under the 'parenteral' category include intravenous (iv), intraperitoneal (ip) and intramuscular (im). Gill (1982) gives a figure (LD_{50}) for ricin by the iv route in the mouse of 2.7 μg kg^{-1}. This compares fairly closely with the value quoted by Franz and Jaax (1997) of 5 μg kg^{-1} (90 hours to death) for the iv route in the mouse, but 22 μg kg^{-1} (100 hours to death) if ricin is administered by the ip route. The im route is not given by these authors, but they do quote a value for a subcutaneous route of 24 μg kg^{-1} (100 hours to death).

7.4 Histopathology and Symptoms of Parenteral Intoxication

Studies on im dosing of rodents with ricin have used immunocytochemical methods to demonstrate the presence of the toxin in a lymph node draining the injection site (Griffiths *et al*., 1989b). Closer examination of the draining process suggested that ricin reached the lymph node an hour or so after injection and was bound to different cell types, including lymphocytes, by a mechanism which could be inhibited, *in vitro,* by lactose (Griffiths *et al*., 1989a). Damage to the small intestine was a feature following im poisoning with ricin, with the infiltration of plasma cells and activated macrophages into the lamina propria and apoptotic deletion of these and structural cells in the ileum (Leek *et al*., 1989). The most notorious case of alleged ricin poisoning by im injection was that of the murder of Georgi Markov in London in 1978, in which approximately 0.5 mg of ricin may have been delivered in a hollow pellet (Crompton and Gall, 1980; Knight, 1979).

Symptoms included pain at the injection site of the pellet followed by nausea, vomiting, fatigue and fever developing over the 24 hours after injection. Gastrointestinal haemorrhage, hypovolaemic shock and renal failure developed subsequently and Markov died three days after being 'injected' with the pellet. Histopathology subsequently revealed the wide distribution of ricin around the body, including the presence of pulmonary oedema, haemorrhagic necrosis of the small intestine and damage to the lymph nodes draining the original injection site, but also to the myocardium, pancreas and testes.

The fate of iv administered ^{125}I-labelled ricin was examined in the rat (Ramsden *et al*., 1989) and indicated that some 46% of the injected dose reached the liver within 30 minutes of administration, followed by the spleen (9.9%) and muscle (13%). Greatest tissue concentration of labelled ricin was in the spleen (33% of original dose g^{-1} of tissue) while some entered the intestine (10–12% at between 3–12 hours and <5% at 24 hours).

There is little information on ricin administered by the ip route, although the LD_{50} for this route is given as 22 μg kg^{-1} with death at around 100 hours later (Franz and Jaax, 1997).

8 A CONSIDERATION OF ANIMAL MODELS OF HUMAN TOXICITY, FOCUSSING ON INHALATIONAL TOXICITY

Toxicological studies are ultimately concerned with the implications of observations gained in animal species to humans. In many situations there may be human exposure data to help corroborate or refute animal-derived data, but such information is unavailable for inhaled ricin. It is extremely important that the limitations of animal models and the experimental conditions used must be understood for such extrapolations to be appropriately made, even more so when there are no human data to compare.

Modelling inhalation toxicity requires special consideration because, in addition to species' relative sensitivity to the toxin, which affects all routes of intoxication, several other factors must be considered which could lead to misinterpretation of data.

In studies of inhalation of aerosol particles, the aerosol particle size (MMAD) is most important in governing the penetration of the material through the respiratory airways into the deep lung. Other key factors influencing depth of aerosol penetration are the airway diameter and breathing patterns of the animal species studied. Generally, the smaller the species, the lower the MMAD

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required for deep-lung penetration, especially if the animals are obligate nasal breathers and have a highly efficient nasal filtration system.

Humans may breathe both nasally or orally and the nose and upper airways (laryngeal, in particular) quite efficiently adsorb aerosol particles larger than about 2 μm aerodynamic diameter, In nasal breathing, particles larger than approximately 0.5 μm MMAD are effectively removed by inertial impaction in the nose, but also the larynx. Smaller particles (e.g. ~1 μm) will reach the deep lung, but may not deposit there, while larger particles (e.g. between 1 and 5 μm), which actually reach the alveoli, stand a better chance of depositing there (Heyder *et al*., 1986). In rats and mice, species commonly employed in inhalation toxicology, the smaller dimensions of the airways and the fact that these species are obligate nasal breathers, requires a lower MMAD in the region of ideally 1–2 μm for penetration of particles through the nasal turbinates and airways down to the deep lung. It has been shown experimentally that in small species, particles of aerodynamic diameter less than 3 μm are required (Raabe *et al*., 1988) in order to study deep-lung penetration. Roy *et al*. (2003) examined the comparative distribution of inhaled aerosolized ricin particles of different size ranges of 1 μm and 5–12 μm MMAD. In general terms, this study showed that, in mice exposed nose-only to aerosol, approximately 60% of the 1 μm ricin particles were deposited in the lungs, followed by the trachea, with very small amounts being measured (using enzyme-linked immunosorbent assay (ELISA)) in the nares and stomach (~1–2%). In the case of the 5–12 μm particles, most ricin was found to be in the trachea, with less in the lungs and very little in the nares and stomach. No animal deaths were noted following the exposure to the 5–12 μm particles at a Ct which, for 1 μm particles would have been $4LD_{50}$.

In terms of species' relative sensitivity to ricin, it was established during World War II that a range of susceptibilities to ricin by inhalational exposure was apparent between animal species (Cope *et al*., 1946). Using an impure sample of ricin, animals were exposed by inhalation to atomized aqueous samples of crude ricin droplets (MMAD 1.4 μm), and the order of sensitivity was established (based on derived LCt_{50}s). In decreasing sensitivity species were: mouse, rabbit, guinea pig, cat, rat and monkey. It has since been shown that such differences may be found, even between strains of the same species, by comparative toxicology studies undertaken in the mouse (Wannemacher and Anderson, 2006).

Thus, care must be taken in the selection of animal models for such studies and in the interpretation of information gained with reference to man, as discussed by Paddle (2003).

9 MEDICAL COUNTERMEASURES

There are two potential approaches to the development of medical countermeasures for ricin. A pretreatment strategy could be adopted, involving vaccination or, alternatively, a post-exposure therapeutic strategy might be considered. Here, for example, a passive immune approach through the timely administration of preformed anti-ricin antibodies (antitoxin) might be employed or, alternatively, the administration of inhibitors of the *N*-glycosidase enzyme activity of ricin or of compounds to prevent ricin attachment to tissues might be considered. The potential for both pre- and postexposure strategies has been explored by several laboratories in the UK and in the USA and key points arising from these studies will be identified below.

9.1 Pretreatment Approaches: Vaccines

When pretreating against poisoning by protein toxins, the key feature is to engender sufficient quantities of neutralizing antibodies, which are available in the right place at the critical time. The vaccine antigens must be as closely preserved as possible to the native protein, in this case, ricin, in order to ensure that antibodies having the appropriate qualities are produced in the vaccinee. Early studies in our laboratory investigated the efficacy of the long-standing toxoiding method, where the protein is incubated with formaldehyde, to produce a nontoxic vaccine candidate. This approach has been used for the preparation of vaccines currently in use, including diphtheria/tetanus toxoids with adsorbed acellular pertussis, as produced by Wyerth–Ayerst and GlaxoSmithKline (Informed Choice, 2009).

At Dstl we examined the efficacy of a formaldehyde-treated ricin (toxoid) vaccine in Porton Wistar rats. Animals were vaccinated with toxoid by three subcutaneous injections at three-week intervals, in the absence of an adjuvant, before challenge by inhalation to two LCt_{50}s of ricin. All vaccinated animals survived aerosol challenge and had mounted strong ricin-specific antibody responses, which, in a blood sample taken just before challenge, included predominantly IgG but also IgA and IgM isotypes with ricin specificity. Although the rats had been protected against toxin challenge, their lungs still showed abnormal histopathology consistent with ricin poisoning, but the study indicated that the toxoid showed promise as a basis for pretreatment against mortality from inhaled ricin toxin (Griffiths *et al*., 1995).

In order to attempt to achieve better protection of the lungs, ricin toxoid was liposomally encapsulated and administered by intratracheal (it) instillation to rats. After challenge ($3LD_{50}$ ricin, it) animals were found to be well

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protected, with mild signs of intoxication, producing high titres of ricin-specific antibody with IgA isotype in serum and lung washes. One cause for concern, however, was that ricin-specific IgE was measured in both sera and in lung fluid washes (Griffiths *et al*., 1997). An alternative RTA-based vaccine candidate was compared in rats with ricin toxoid, and each antigen was administered it in liposomal formulations. Protection is believed to depend upon the production of protective, ricin-neutralizing antibodies. One week after vaccine boosts, ricin-specific antibody titres were similar with each antigen, but antibody levels in lung fluid post challenge ($3LD_{50}$, it) with ricin were lower in the RTA-vaccinated group. Once again, ricin-specific IgE levels were measured in rats which had been vaccinated with the toxoid preparation (Griffiths *et al*., 1998).

This quality of protection offered by it vaccination with liposomal ricin toxoid or RTA was compared by analysis of markers of inflammation in lung-fluid protein over several days following challenge with ricin ($3LD_{50}$, it). Rats which had been protected with toxoid had very low levels of protein or inflammatory neutrophils compared with the RTA group. When liposomal RTA had been administered subcutaneously instead of it, lower levels of inflammatory markers were noted after ricin challenge (Griffiths *et al*., 1999).

Overall, therefore, studies indicated that ricin holotoxoid vaccine produced a better quality of protection than the A-chain-based vaccines. One potential problem over formaldehyde-toxoided ricin was that the preparation could revert to active toxin. However, investigation of established formaldehyde-toxoid vaccines in clinical use revealed that residual formaldehyde is included in the formulation in order to prevent the reversion of toxic activity.

Other laboratories have explored the utility of a formalin-inactivated ricin toxoid as a vaccine candidate. Yan *et al*. (1996) demonstrated protective immunity against a supralethal challenge of ricin aerosol, one year after intranasal (in) immunization of mice using ricin toxoid microencapsulated in poly(lactide-coglycolide) (PLG) or polylactide (PLA) microspheres. Kende *et al*. (2002) demonstrated the production of serum antibodies (IgG2a and IgA classes) following oral administration of PLG-encapsulated ricin toxoid in mice. Seven administrations of 25 μg toxoid in PLG over a period of 30 days protected 50% of the immunized mice. For direct comparison, a single subcutaneous dose of microencapsulated ricin toxoid was fully protective. The protection of mice produced against ricin has also been examined by in administration of deglycosylated ricin A chain (Kende *et al*., 2006) or recombinant A chain (Kende *et al*., 2007) in the presence of mucosal adjuvants prepared by mutations of *Escherichia coli* heat-labile enterotoxin. The adjuvants were shown to substantially improve ricin-specific antibody titres in serum and lung lavage. Full protection against death was produced following subsequent inhalation challenge with supralethal amounts of ricin toxin aerosol, although lung damage was still evident.

Growing interest in the use of RTA-chain vaccine candidates is evident over the past 10–15 years. The A chain of ricin and other RIPs, have been used for a long time to produce immunotoxins, by coupling them with antibodies against tumour cell surface-specific antigens (Stirpe and Battelli, 2006). In this connection, it was found that the A chains had adverse effects on human umbilical vein endothelial cells and could cause vascular leak syndrome in Phase I/II clinical trials, so potentially limiting the quantity of immunotoxin which may be administered to patients (Soler-Rodriguez *et al*., 1993). This property would also be undesirable in an RTA-chain-based vaccine.

Further studies by Baluna *et al*. (1999) identified a tripeptide sequence in the A chain of two-chain or single-chain RIPs, with a common aspartic acid residue, flanked by several variable residues, which for ricin were leucine and valine (leucine, aspartic acid, valine tripeptide). This tripeptide enabled binding to and damage of endothelial cells. Engineered recombinant ricin A-chain molecules with mutations in the sequence, or in flanking regions, produced an RTA which behaved like the ricin A chain as an immunotoxin, but did not cause vascular leak in mice (Smallshaw *et al*., 2003). It is important to note that for use as an immunotoxin, the toxic activity of the A chain should be preserved, while the ability to cause vascular leak should ideally be removed. For RTA-based vaccines, however, it is desirable to engineer out the sequence causing vascular leak, but also achieve inhibition of toxic *N*-glycosidase activity.

Mutant recombinant ricin A chains prepared by Smallshaw *et al*. (2002), having a single mutations in the enzyme active site and another in the vascular leak tripeptide, were made. These mutants did not produce pulmonary vascular leak in mice, were immunogenic and protected the animals from an ip challenge with a supralethal quantity of ricin. One successful mutant vaccine candidate named RiVax, has been taken forward as a potential human ricin vaccine candidate. It has been shown to be safe, immunogenic and protective in mice and rabbits (Smallshaw *et al*., 2005). RiVax has passed human clinical trials involving three im dose levels, where antibody titres with neutralizing antibody levels were detected (Vitetta *et al*., 2006).

The vaccine has been shown to prevent lethality of mice when challenged orally (gavage) or by inhalation with ricin (10 LD_{50}), when administered at doses of 1, 3.3 and 10 μg, although minimal pulmonary damage was present even in the highest vaccine dose tested (Smallshaw *et al*., 2007). Future studies will investigate whether higher vaccine doses or the inclusion of an adjuvant will induce more robust, longer-duration immunity with even less lung pathology. The efficacy of this vaccine is currently being evaluated in nonhuman primates (Rhesus

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macaque) at Tulane National Primate Research Center in Louisiana (Dor BioPharma, 2008).

Another mutant recombinant RTA vaccine candidate, RTA1-33/44-198, lacks hydrophobic residues implicated in the aggregation and precipitation of recombinant A chain molecules (Olson *et al*., 2004). The molecule is very stable to freezing and thawing, has substantially reduced toxicity compared with the wild-type molecule and has a higher thermal stability. Although the vascular leak tripeptide was not modified, animal studies did not reveal signs of this problem and the candidate protected mice against 5–10 lethal doses of ricin by inhalation.

A novel approach to the preparation of a recombinant ricin A-chain candidate (Marsden *et al*., 2004) involved the incorporation of a natural inhibitory 25 residue peptide from an inactive maize RIP into ricin A chain to produce a recombinant protein, RTA-MPP (maize propeptide). The construct had approximately 300-fold reduced *N*-glycosidase activity, compared with wild-type A chain. RTA-MPP was re-associated with ricin B chain to produce ricin-MPP. This reconstituted mutant holotoxoid was nontoxic to cells in culture. When given to rats with adjuvant it protected them against $5LD_{50}$ of ricin when administered by it instillation. This might be a viable candidate ricin vaccine, but further work would be required to completely eliminate *N*-glycosidase activity.

In summary, studies on vaccinations to protect against intoxication by inhaled ricin indicated that a holotoxoid approach gave a better quality of protection than the subunit approach, although both types of candidate could prevent lethality (Griffiths *et al*., 1998; 1999).

9.2 Postexposure Approaches: Ricin Antitoxin

There are few reports in the open literature on the therapy of ricin intoxication (systemic or inhaled) in animal models. A rabbit affinity-purified polyclonal IgG raised against ricin formaldehyde toxoid (Foxwell *et al*., 1985), and a monoclonal antibody (IgG) raised against the B-chain of ricin (Guo *et al*., 2005) have been shown to be effective when administered 40 and 20 minutes, respectively, following systemic ricin challenge.

Protection against inhaled ricin by prophylactic and postexposure use of an affinity-purified mouse antiricin IgG has been reported in a review article of otherwise unpublished results (Wannemacher and Anderson, 2006). Aerosolized antibodies have also been reported as effective when administered prior to an aerosolized ricin challenge (Poli *et al*., 1996). Instilled antibody was not effective, indicating that pulmonary deposition patterns are important for the toxicity and therapy of inhaled ricin.

The production of antitoxins suitable for use in humans exposed to ricin by inhalation are being developed at Dstl. Antibodies raised in sheep against RTA-chain, RTB-chain and the formaldehyde toxoid of whole ricin (using Alhydrogel adjuvant) have been examined for the postexposure therapy of ricin poisoning in mice. IgG raised against the formaldehyde ricin holotoxoid gave the best protection in mice (ip ricin challenge) when antitoxin was administered intravenously, 60 minutes following challenge (Poole *et al*., personal communication; Holley *et al*. 2006). While the time to death was delayed, actual survival was not achieved. Subsequent studies to prepare formaldehyde ricin holotoxoid in sheep used Freund's incomplete adjuvant. This IgG antitoxin produced was more effective achieving 100% survival when administered two hours after an ip challenge with ricin in mice. Further investigation of this antitoxin to develop a treatment for humans exposed to ricin has involved the production of antibody ($F(ab')_2$ and Fab') fragments. The rationale was that the removal of the reactogenic Fc portion of the antibody should make these fragments more suitable for eventual human use. The antitoxin IgG was marginally more effective than $F(ab')_2$ for the postexposure therapy of a systemic ricin challenge in mice, where the latter complete protection was accompanied by some weight loss, while there was no weight loss in the IgG treated mice. The smaller Fab' fragment was unable to prevent death. Of particular significance was the observation that IgG and $F(ab')_2$ were both able to protect mice against a lethal inhaled challenge ($3LCt_{50}$ ricin) when administered two hours after the challenge. Again the quality of protection was slightly better for IgG than the $F(ab')_2$. It is believed that good protection requires the maintenance of a critical level of antitoxin in the blood to access and neutralize the ricin. For equivalent doses of IgG and $F(ab')_2$ our pharmacokinetic studies have shown that plasma levels of IgG are higher than those for $F(ab')_2$, following iv injection of the antitoxins in mice. Continuing studies show greater promise, with a longer therapeutic window to administer antitoxin against inhaled ricin.

Other interest in an antitoxin approach has been reported, involving a novel single domain antibody to the A-chain of ricin. This was developed using a computer-guided molecular design approach and has demonstrated a remarkable ability to neutralize the *in vitro* toxicity of ricin (Wang *et al*., 2006). This highlights the potential of human V_H (the hypervariable region of the immunoglobulin molecule which recognizes antigen epitopes) to display biostructure and biofunction of peptides designed on the ricin A-chain functional domain and could be useful in developing new antidotes to ricin.

Both vaccination (prophylaxis) and antitoxin (therapeutic) approaches bring positive and negative aspects in terms of giving protection against intoxication by ricin. It is unlikely that a vaccination strategy would be adopted for the population of a whole country, when the likelihood of mass exposure to ricin toxin is improbable. On the other hand, it would be reasonable to vaccinate

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the Armed Forces and key personnel in a vulnerable organization. It is likely that policy would dictate that a therapeutic approach would be adopted on a needs basis. Such an approach would ideally require robust indication that ricin had been deployed, but also reliable and timely identification of victims of ricin exposure. Since the administration of large quantities of foreign protein (antitoxin molecules) may well not be without side effects, the antitoxin would not be given unless necessary.

9.3 Other Possible Postexposure Approaches

A number of lines of research have recently been explored, which aimed to interfere with the process of intoxication by ricin using a number of approaches. Studies have attempted to identify strategies which may interfere with the binding of toxic lectins, including ricin, with cell-surface glycoconjugates, or have looked at molecules interfering with the cellular uptake of ricin or through the production of inhibitors of the *N*-glycosidase activity of RTA. Examples of these approaches will be examined in more detail.

One novel approach for the potential therapy of ricin intoxication was the investigation of mono- or polyvalent carbohydrate inhibitors (galactose and lactose analogues) to inhibit ricin binding to cell surface receptors. These molecules were less effective than galactose and lactose themselves. However, promising results were obtained for a self-assembled lyotropic mesophase gel containing novel synthetic galactose-based surfactants, which sequestered ricin from aqueous solution (Dawson *et al*., 2006). Another group investigated the potential for synthetic glycoclusters based on calixarenes with thiourea-linked galactose or lactose moieties to bind the ricin-related ribotoxin viscumin. Calyx[6,8]arenes were reported to be very effective in this respect (André *et al*., 2008). Potential inhibitors of the *N*-glycosidase enzyme, which is the toxic activity attributed to the RTA chain, have been explored by Sturm *et al*. (2007). Synthetic circular DNA or DNA/RNA hybrid nucleotide sequences were characterized as substrates for RTA *N*-glycosidase and as potential inhibitor scaffolds. Small circular DNA and DNA/RNA tetramers were found to be promising scaffolds for RTA inhibitor design. A nanomolar inhibition constant was determined for the circular oligonucleotide containing the RTA transition state analogue diazabutadiene methyl (DADMe)-*N*-benzyl (*N*-Bn). The best inhibitor was cyclic G(*N*-Bn)GA with a K_i of 70 nM, making it the lowest-molecular-weight nanomolar inhibitor of RTA. Various modifications to the structures of potential inhibitor sequences may improve performance, but these compounds hold promise as potential inhibitors of RTA.

A further research study has targeted the retrograde transfer of ribotoxin A chain through the trans-Golgi network from the ER to the cytosol, where they gain access to their ribosomal RNA substrate. Two novel compounds which interfere with this process and prevent shiga toxin and ricin-mediated inhibition of protein synthesis have been identified (Saenz *et al*., 2007).

In summary, a vaccination approach is very feasible against fatal intoxication by ricin, but development and licensing is time-consuming and expensive. A therapeutic approach using, for example, antitoxin, is also feasible and cheaper, but adds the requirement for specific and timely knowledge of exposure in order to implement treatment. Several novel lines of research are currently investigating the potential to protect against ricin poisoning through interfering with toxin binding, toxin *N*-glycosidase activity or the retrospective transfer of ricin A chain to the cytosol via the trans-Golgi network. Such inhibitor studies are only at the developmental stage and have yet to be tested *in vivo.*

Research using cultured primary epithelial cells has demonstrated that the addition of ricin induces them to express multiple proinflammatory molecules through the activation of stress-activated protein kinases (SAPKs) and nuclear factor (NF)-$\kappa\beta$ (Wong *et al*., 2007). Studies *in vivo,* covered above, demonstrate that ricin administration leads to tissue inflammation. Using a chemical inhibitor or short, interfering RNA, the inhibition of p38 MAPK or NF-$\kappa\beta$, respectively, reduced the expression of proinflammatory genes. The authors have speculated that these would be good targets to consider for the modulation of ricin-induced inflammation to ameliorate the consequences following a ricin poisoning.

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Occupation, Toxic Exposures and Health Effects

Hilton C. Lewinsohn

CONTENTS

1 INTRODUCTION

It is impossible to discuss a topic such as 'Occupation, toxic exposures and health effects' without referring to Bernardino Ramazzini's unique treatise *De morbitus artificum* ('Diseases of Workers') first published in Modena in 1700 and rewritten in 1713 (Wright, 1993). Nobody before this had written a comprehensive treatise on occupational diseases and their possible prevention. Ramazzini stated that 'Various and manifold is the harvest of diseases reaped by certain workers from the crafts and trades that they pursue'. His major literary contribution to the study of occupational and environmental illness is in these two treatises, and the importance of a physician's medical history is outlined by him as follows:

> *When you come to a patient's house, you should ask him what sort of pains he has, what caused them, how many days he has been ill, whether the bowels are working and what sort of food he eats. So says Hippocrates in his work Affections. I may venture to add one more question: What occupation does he follow?*

The recognition of clinical findings and disease, related to occupation and industry, resulted in great strides occurring towards the development of a systematic classification of this subject. The recognition of occupational medicine can be attributed to Hippocrates and his followers and he advised them 'to observe the environment of their patients' (Ellenbog, 1524). As a result of this practical approach, the basis of curative occupational medicine was developed. In mining towns silicosis was diagnosed, as were lead poisoning and other lung diseases (Ellenbog, 1524).

Another important physician, Georgius Agricola (1494–1555) observed the mining of gold and silver.

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In *De Re Metallica* he described 'consumption', which caused early deaths among miners, and which today would probably be considered to be due to silicosis or some other pneumoconiosis (Hunter, 1955).

Paracelsus (1493–1541) studied the diseases of metallurgists and wrote about 'acute and chronic forms of mercurialism'. He also described diseases affecting miners (Hunter, 1955). But, it was obviously Ramazzini who added the significant question regarding occupation, and then went on to describe diseases which he attributed to the various work types involved. Hunter (1955) states that

> *In the course of carrying out his resolve Ramazzini inquired into the conditions of work and the occupational diseases of the following types of workers: miners of metals, healers by inunction, chemists, potters, tinsmiths, glass workers and mirror makers, painters, sulphur workers, blacksmiths, workers with gypsum and lime, apothecaries, cleaners of privies and cesspits, fullers, oil pressers, tanners, cheese makers and other workers at dirty trades, tobacco workers, corpse carriers, midwives, wetnurses, vintners and brewers, bakers and millers, starch makers, sifters and measurers of grain, stone cutters, laundresses, workers who handle flux, hemp and silk, bathmen, salt makers, workers who stand, sedentary workers, runners, grooms, porters, athletes, those who strain their eyes over fine work, voice trainers, singers, farmers, fishermen, soldiers, learned men, nuns, printers, scribes and notaries, confectioners, weavers, coppersmiths, carpenters, grinders of razors and lancets, brick makers, well diggers, sailors and rowers, hunters and soap makers.*

Ulrich Ellenbog (1440–1499) wrote the first work on an occupational disease in 1473, but it was not published until 1524. This pamphlet described the miners' diseases and is said to be the first known work on industrial hygiene and toxicology (Ellenbog, 1524). The study of occupational hazards apparently lay dormant with regard to making specific medical investigations of diseases they caused. The physicians following Ramazzini did not concentrate on industrial illnesses as a whole, but described individual cases which they attributed to isolated tasks.

Hunter (1978) points out that the changes which took place in England between the years 1760 and 1830 are comparable to a revolution in industry, and the term *die industrielle Revolution* was used as early as 1845 by Friedrich Engels in his *Die Lage der arbeitenden Klasse in England.* Hunter states that the credit for originating the comparison is generally ascribed to Arnold Toynbee, whose book, unfinished when he died, was published in 1884 under the title *Lectures on the Industrial Revolution in England.*

As a result there were no significant advances in the study of occupational medicine until Sir Thomas Legge (1863–1932) began to study public-health matters in Europe (Harrington, 1998). Based in the United Kingdom, while serving as medical inspector, he promoted the investigation of occupational diseases and measures to be taken to prevent them. Among the subjects he studied were lead poisoning, anthrax, toxic jaundice and brass-founders' ague. He visited workplaces, examined workers, undertook statistical analysis, risk assessments and, finally, proposed preventive measures. He pointed out that there was widespread ignorance of occupational diseases in the medical profession and recommended that a detailed curriculum on occupational medicine be developed for medical students.

> *As a result of his close contact with the worker he was able to show that lead poisoning resulted from the inhalation of lead and he could then propose preventive measures to decrease the incidence of cases.*
>
> (Harrington, 1998)

The subject of occupation, toxic exposure and health effects, dealt with in this chapter, will review processes involving chemicals, used in various forms, in the manufacture of products in common use by the populations of industrialized countries. It has to be understood that the time lapsed since reports on occupational toxicology in the past century, and the present requirements for the manufacture and modern day usage of industrial chemicals, the hazards and environments presently described are very different from those studied by Legge and others. In this chapter, an attempt will be made to discuss the toxicity and health problems associated with the use of chemicals in the workplace in the twenty-first century. The workplace includes, not only the factory using the raw chemicals and producing finished products, but the source of origin of the materials, the uses of the manufactured products and the disposal of waste generated. The entire course of manufacture and usage will be discussed. Industry standards and government regulations educate workers and their employers in the safe handling of toxic materials, and industrial hygiene standards, which establish exposure levels and regulations for their control. The composition of factory work populations and the life standards they now enjoy, will be taken into consideration when examining the toxicity of the chemicals with which they are in contact.

2 TOXICOLOGY

Toxicology is the science which studies toxic substances, how to detect them and their chemical actions in exposed

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individuals. The development of control standards, exposure monitoring and the treatment of toxic health effects is the practical outcome of such findings.

Peter S. Thorne has stated that:

> *Occupational toxicology is the application of the principles and methodology of toxicology toward chemical and biological hazards encountered at work.* (Thorne, 2001)

In order to understand occupational toxic hazards likely to influence the twenty-first century work environment, it is necessary to study modern processes and compare them to those originally responsible for occupational toxicological illnesses. The study of occupational toxicology requires that a systematic approach is used to identify workplaces and the types found in the global environment. Employment, which is characteristic of the twenty-first century, requires documentation of materials in use, their handling and the work safety procedures in use. The introduction of modern safety and health standards is developed from knowledge of the exposure quantities involved, confirmatory data derived from human, experimental animal and cell studies, and research presenting evidence of engineering controls eliminating harmful effects.

A leading proponent of change in approach to toxicity definition and toxicity testing is the National Research Council (NRC) of the National Academies, and this is dealt with in the recent publication *Toxicity Testing in the 21st Century –A Vision and a Strategy* (NRC, 2007). The National Academies are advisers to the US Government on science, engineering and medicine, and the four academies are listed in the abovementioned publication (*http://www.national-academies.org/*). They consist of the National Academy of Sciences, The National Academy of Engineering, The Institute of Medicine and the NRC. Three committees work as agents of the groups. They are the Committee on Toxicity Testing and Assessment of Environmental Agents, the Board on Environmental Studies and Toxicology and the Institute for Laboratory Animal Research Council. Other reports of the Board on Environmental Studies and Toxicology, and the Institute for Laboratory Animal Research publications are listed in that publication (*see also* **Regulatory Toxicology**).

This chapter's focus is on occupational toxicity, on better understanding of the health effects and their prevention, by modification of hazardous existing standards. It is important to understand why an individual or group of workers develops disease when employed in a specific occupational circumstance. To do so, the work environment, and the materials handled in the various processes in manufacture of a product, must be studied. In addition, disease processes studied must be considered in the light of exposure types, and manufacturing techniques encountered. The nature of hazardous materials must be characterized and defined, depending on quantitative exposure measurements, types of materials used and human body defences studied.

Natural body defences will be dependent upon the type of material in use and the dose of exposure encountered. It is important to recognize the various portals of bodily entry, and the human tissues in particular, and organ systems in general, which react to the toxic substances encountered. Studies of the materials involved have to be conducted to determine the physical and chemical agents which are likely to cause specific effects. Occupational toxicology is concerned with the workplace environment, and occupational physicians are uniquely placed to study the effects of industrial toxins on the persons in it. Occupational toxicity is studied for the influence of societal factors, such as the general availability of healthcare, the level of cultural and technologic development of the population, and the degree of government and industry regulatory control exercised. In the USA, the National Toxicology Programme (NTP), which is in the Department of Health and Human Services (DHHS), relies on its Office of Chemical Nomination and Selection for public comments on toxicological studies to be considered by the NTP. The agency which is involved is the National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health (NIH). The requirements are published by NIEHS in the *Federal Register* (FR) specifying how NTP solicits and accepts nominations for toxicological studies to be undertaken by the programme. Nominations of substances of potential human-health concern are received from federal agencies, the public and other interested parties. These nominations are subject to several levels of review before selections for testing are made and toxicological studies are designed and implemented. Brief background information and preliminary study recommendations regarding nominations for study by the NTP are provided, public comment and study recommendations on nominations are solicited, and the submission of additional relevant information for consideration by the NTP in its continued review of these nominations is requested.

The NTP actively seeks to identify and select for study chemicals and other substances for which sufficient information is not available to adequately evaluate potential human-health hazards. The NTP accomplishes this goal through a formal open nomination and selection process.

Our environment, in which we work and live, must be kept under surveillance for illnesses, to detect hazardous chemicals, and other workplace factors, which could account for them. For example, cancer research scientists have shown in many studies of our work environment, and our living circumstances, how they contribute to the development of cancer.

> *The scope of toxicology is enormous, since virtually every known chemical, if present in sufficient amount,*

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has the potential to produce injury or death. In our modern society, toxicologic tests are used to evaluate the potential health hazards that may result from exposure to environmental pollutants, food additives, pharmacologic agents, and occupational exposures among others.

(Logan, 1994)

3 WORKPLACE HAZARD RECOGNITION

Recognition of workplace hazards has been discussed in many different publications in the occupational health literature since at least 1700 AD. In the opening remarks in this chapter this has been pointed out already. Over time, the science of toxicology has evolved and the study of industrial chemical and physical hazards has been developed. The recognition of workplace toxic hazards is now an important factor in the development of safety and health-control-management processes in the work environment. The toxic effects threat is not only confined to the chemical manufacturing industry, but general industry must also be aware of it, since the chemicals will be used in many processes. In addition, it must be recognized that in some instances the hazard spreads beyond the factory fence and exposes the local population to its ill effects. The general industrial population may be exposed in many processes, such as painting, packaging products, cleaning plant machinery, using adhesives for purposes of assembly and many other similar tasks. Modern industrial processes have changed extensively, and, in the twenty-first century, potential exposures are now covered by the term 'occupational and environmental health'. Modern production processes have dramatically changed and are now responsible for 'atmospheric pollution, carcinogenesis, biological monitoring, ergonomics, epidemiology, product safety and health promotion' (Groneberg and Fischer, 2006). The Occupational Safety and Health Act (OSH Act) of the United States Department of Labor Occupational Safety and Health Administration (OSHA) has defined toxic industrial chemicals (TICs). The OSH Act defines toxic industrial TICs as 'industrial chemicals that are manufactured, stored, transported and used throughout the world'. TICs may be gases, liquids or in the solid state. Chemical hazards may be carcinogenic, reproductive toxins, corrosives or pulmonary or haematotoxic hazards. Physical agents may be flammable, combustible, explosive or reactive. **Table 1** is published by the OSHA and lists the most common TICs by their hazard index. It is interesting to note in the twenty-first century, that the OSHA feels that, because of terrorist events, concern has been expressed by some about the possibility of an attack using TICs or toxic industrial materials (TIMs). The OSHA notes that these agents can be highly toxic and are produced in large quantities. It is necessary for industrial workers to understand what TICs are and how they may affect their health. The occupational physician has to be aware of the toxic effects attributable to materials and products present in the workplace, and the possibility of extending their exposure to the outside world. The physician has to be able to determine the risk to factory workers, and to users of the finished products, as well as the general population exposure to waste dusts, gasses and other environmental contaminants. The magnitude of the risk will determine how to safely control the environment for the populations potentially exposed. The physician and other occupational-health workers, such as engineers and industrial hygienists, must maintain records to enable them to eliminate risks. It is important to correctly identify suspected hazardous materials. One example of such a procedure is for an occupational hygienist, personally protected by suitable safety equipment, to collect samples of suspected harmful materials and have them analysed by a laboratory specializing in such work. In the USA, users of known hazardous materials must maintain material safety data sheets (MSDSs) prepared by the manufacturer. These sheets are found to give reasonable information on safety hazards, but describe incompletely toxic effects of compounds.

Toxic dose is measured in milligrams per kilogram body weight ($mg\,kg^{-1}$) or, in the case of fluids, milligrams per litre ($mg\,l^{-1}$). The amount of chemical present is expressed in milligrams (mg); the weight of the exposed subject (human or animal) is expressed in kilograms (kg) and l stands for litre of air. The lethal toxicity of a substance may be expressed according to the dose that causes death in half the test animals exposed and is called an LD_{50} (lethal dose) or LC_{50} (lethal concentration). Tests are usually performed using rats or mice. The higher the LD_{50} or LC_{50}, the lower the toxic fatal dose, therefore indicating that more chemical exposure is needed to result in death. The opposite finding, of a low LD_{50} or LC_{50}, means that there is a higher toxicity, and a lesser amount of the chemical causes death in 50% of the population being tested. These results are due to acute exposure effects, but cannot provide data to allow determination of chronic health effects.

In product manufacture, in the twenty-first century, exposure of factory workers is probably negligible, as processes are largely carried out in enclosed equipment, at least in well-developed countries. Mixing and formulation may not be as easily controlled and packaging, warehousing and distribution are still likely sources of exposure to workers. Workers who repair, maintain and clean factory production equipment in which the products are manufactured are still at risk of being exposed. Potential for exposure exists in handling the raw materials which may be delivered by pipeline, rail, ship or barge, or in tank-trucks.

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Table 1 Toxic industrial chemicals (TICs) listed by hazard index

High	Medium	Low
Ammonia (CAS# 7664-417)	Acetone cyanohydrins (CAS# 75-86-5)	Allyl isothiocyanate (CAS# 57-06-7)
Arsine (CAS# 7784-42-1)	Acrolein (CAS# 107-02-8)	Arsenic trichloride (CAS# 7784-34-1)
Boron trifluoride (CAS# 10294-34-5)	Acrylonitrile (CAS# 107-13-1)	Bromine (CAS# 7726-95-6)
Boron trifluoride (CAS 7637-07-2)	Allyl alcohol (CAS# 107-18-6)	Bromine chloride (CAS# 13863-41-7)
Carbon disulfide (CAS# 75-15-0)	Allylamine (CAS# 107-11-9)	Bromine pentafluoride (CAS# 7789-30-2)
Chlorine (CAS# 7782-50-5)	Allyl chlorocarbonate (CAS# 2937-50-0)	Bromine trifluoride (CAS# 7787-71-5)
Diborane (CAS# 19287-45-7)	Boron tribromide (CAS# 10294-33-4)	Carbonyl fluoride (CAS# 353-50-4)
Ethylene oxide (CAS# 75-21-8)	Carbon monoxide (CAS# 630-08-0)	Chlorine pentafluoride (CAS# 13637-63-3)
Fluorine (CAS# 7782-41-4)	Carbon sulfide (CAS# 463-58-1)	Chlorine trifluoride (CAS# 7790-91-2)
Formaldehyde (CAS# 50-00-0)	Chloroacetone (CAS# 78-95-5)	Chloroacetaldehyde (CAS# 107-20-0)
Hydrogen bromide (CAS# 10035-10-6)	Chloroacetonitrile (CAS# 7790-84-5)	Chloroacetyl chloride (CAS# 79-04-9)
Hydrogen chloride (CAS# 7647-01-0)	Chlorosulfonic acid (CAS# 7790-94-5)	Crotonaldehyde (CAS# 123-73-9)
Hydrogen cyanide (CAS# 74-90-8)	Diketene (CAS# 674-82-8)	Cyanogen chloride (CAS# 506-77-4)
Hydrogen fluoride (CAS# 7664-39-3)	1-2-Dimethylhydrazine (CAS# 540-73-8)	Dimethyl sulfate (CAS# 77-78-1)
Hydrogen sulfide (CAS# 7783-0604)	Ethylene dibromide (CAS# 106-93-4)	Diphenylmethane-4.4′-diisocyannate (CAS# 101-68-8
Nitric acid, fuming (CAS# 7697-37-2)	Hydrogen selenide (CAS# 7783-07-5)	Ethyl chloroformate (CAS# 101-68-8)
Phosgene (CAS# 75-44-5)	Methanesulfonyl chloride (CAS# 124-63-0)	Ethyl chlorothioformate (CAS# 2941-64-2)
Phosphorus trichloride (CAS# 7719-12-2)	Methyl bromide (CAS# 74-83-9)	Ethyl phosphonothioic dichloride (CAS# 993-43-1
Sulphur dioxide (CAS# 7446-09-5)	Methyl chloroformate (CAS# 79-22-1)	Ethyl phosphonic dichloride (CAS# 1066-50-8)
Sulfuric acid (CAS# 7664-93-9)	Methyl chlorosilane (CAS# 993-00-0)	Ethyleneimine (CAS# 151-56-4)
Tungsten hexafluoride CAS# 7783-82-6)	Methyl hydrazine (CAS# 60-34-4)	Hexachlorocyclopentadiene (CAS# 77-47-4)
	Methyl isocyanate (CAS# 624-83-9)	Hydrogen iodide (CAS# 10034-85-2)
	Methyl mercaptan (CAS# 74-93-1)	Iron pentacarbonyl (CAS# 13463-40-6)
	Nitrogen dioxide (CAS# 10102-44-0)	Isobutyl chloroformate (CAS# 543-27-1)
	Phosphine (CAS# 7803-51-2)	Isopropyl chloroformate (CAS# 108-23-6)
	Phosphorus oxychloride (CAS# 1795-48-8)	Isopropyl isocyanate (CAS# 1795-48-8)
	Phosphorus pentafluoride (CAS# 7647-19-0)	n-Butyl chloroformate (CAS# 592-34-7)
	Selenium hexafluoride (CAS# 7783-79-1)	n-Butyl isocyanate (CAS# 111-36-4)
	Silicon tetrafluoride (CAS# 7783-16-1)	Nitric oxide (CAS# 10102-43-9)
	Stibine (CAS# 7803-52-3)	n-Propyl chloroformate (CAS# 109-61-5)
	Sulphur trioxide (CAS# 7446-11-9)	Parathion (CAS# 56-38-2)
	Sulfuryl fluoride (CAS# 2699-79-8)	Perchloromethyl mercaptan (CAS# 594-42-3)
	Tellurium hexafluoride (CAS# 7783-80-4)	sec-Butyl chloroformate (CAS# 17462-58-7)
	n-Octyl mercaptan (CAS# 111-88-6)	tert-Butyl isocyanate (CAS# 1609-86-5)
	Titanium tetrachloride (CAS# 7550-45-0	Tetraethyl lead (CAS# 78-00-2)
	Trichloroacetyl chloride (CAS# 76-02-8)	Tetraethyl pyrophosphate (CAS# 107-49-3)
	Trifluoroacetyl chloride (CAS# 354-32-5)	Tetramethyl lead (CAS# 75-74-1)
		Toluene 2,4-diisocyanate (CAS# 584-84-9)
		Toluene 2,6-diisocyanate (CAS# 91-08-7)

The table, prepared by OSHA, lists the most common TICs by their hazard index.
Reproduced from National Institue of Justice, 2001.

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Methyl isocyanate (MIC), used in the manufacture of aldicarb, is a hazardous material and its effect on the general population of Bhopal in India is an example of one of the worst industrial accidents known, resulting in the loss of thousands of lives due to exposure to it.

There is now a great need for the redevelopment of the technology used in estimating toxic hazards in exposed humans, either occupational or environmental, because there is a great difference in the types of hazards in the workplace from those first recognized. The potentially toxic materials now in use are found in industrial situations which vary and in concentrations which differ greatly. It is necessary, therefore, to recognize, not only the *presence of exposure*, but also the variations in *amounts (dose)*.

> *Exposure is the amount of a given agent presented by the immediate environment, whereas dose is the amount of an agent absorbed by the individual. Thus, dose has an implicit time component, which depends on the duration of exposure. Individuals in the same environment for the same amount of time may experience different doses of a given compound because of differences in absorption or metabolism.*

4 IDENTIFIED ROUTES OF EXPOSURE AND POSSIBLE TARGET ORGANS

Occupational health depends on avoiding exposure to toxic substances present in the workplace and to products from which they may escape into the general environment.

Table 2 is an example of the incidence of occupational illnesses and injuries, and in this instance, by using an example from the Bureau of Labor Statistics (BLS) statistics published for the United States; the characteristics can be compared for a particular industry, for example chemical manufacturing, with those for private industry in general. It is important to determine how often exposure occurs, the number of times an individual or group of individuals is exposed and the timeframe of exposure occurrence. Exposure may be acute, when the actual time of exposure may be seconds or minutes and may occur at frequent or long intervals, possibly even up to a week. Chronic exposure occurs over many years or may even be regarded as lifetime in some instances. Chronic exposure is thought to be determined as spanning at least 10% of a lifetime and represents seven or more years in humans. Exposure which is between acute and chronic, as described above, and extends from more than a week to less than seven years, is labelled subchronic exposure.

5 POTENTIAL HEALTH EFFECTS IN TARGET ORGANS AND ON GENERAL BODY FUNCTION

> *Toxicity testing… is poised to take advantage of the revolutions in biology and biotechnology. Advances in toxicogenomics, bioinformatics, systems biology, epigenetics, and computational toxicology could transform toxicity testing from a system based on whole animal testing to one founded primarily on in vitro methods that evaluate changes in biologic processes using cells, cell lines, or cellular components, preferably of human origin. Anticipating the impact of recent scientific advances, the U.S. Environmental Protection Agency (EPA) asked the National Research Council (NRC) to develop a long-range vision for toxicity testing and a strategic plan for implementing the vision.*
>
> (NRC, 2007)

The occupational health hazards encountered in this early part of the twenty-first century are beginning to reflect the need for more accurate methods of detection and product composition in the light of modern engineering controls. Future progress in this field may depend upon the ability to use the knowledge and science developed by the Human Genome Project, one of the greatest biological research efforts ever made (*see also* **Genetic Toxicology Testing and its Relevance to Human Risk and Safety Evaluation**).

The project began to be considered in the late 1980s and, because of the efforts exerted, it fostered the development of rapid DNA sequencing technologies. The genomic sequence of the bacterium *Haemophilus influenzae* was determined in 1995, since when genomes of dozens of organisms have been defined and, above all, the human genome sequence became known by the year 2000. Scientists, clinicians, engineers and information scientists have developed new molecular and bioinformatic tools that make it possible to collect and analyse biologic datasets of unprecedented magnitude and detail. The new analytical techniques are called genomic technologies that allow sequence analyses on a genome-wide scale. The NIEHS of the US DHHS is collaborating with the National Academies' investigative arm, the NRC, to examine the toxicogenomic technologies on predictive toxicology.

The NRC's panel has clarified its task by defining the terms 'toxicogenomics' and 'predictive toxicology' as follows:

- **Toxicogenomics** is defined as the application of genomic technologies (e.g. genetics, genome sequence analysis, gene expression profiling, proteomics, metabolomics and related approaches) to study the adverse effects of environmental and

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Table 2 Number and rate[a] of nonfatal occupational injuries and illnesses by selected industry, all USA, private industry, 2006 (Numbers in thousands)

Characteristic	Private industry[b,c,d]		Chemical manufacturing	
	Number	Rate	Number	Rate
Injuries and illnesses				
Total cases	4085.4	4.4	25.6	2.9
Cases with days away from work, job transfer or restriction	2114.6	2.3	15.3	1.7
Cases with days away from work[e]	1183.5	1.3	6.6	0.8
Cases with job transfer or restriction	932.1	1.0	8.7	1.0
Other recordable cases	—	2.1	10.3	1.2
Injuries				
Total cases	3857.4	4.2	22.6	2.6
Illnesses				
Total cases	228.0	24.6	3.1	35.0
Illness categories				
Skin disorders	41.4	4.5	0.7	7.4
Respiratory conditions	17.7	1.9	0.2	2.3
Poisoning	3.4	0.4	—	—
Hearing loss	24.4	2.6	0.4	4.2
All other illness cases	141.1	15.2	1.7	19.6

[a]Incidence rates represent the number of injuries and illnesses per 100 full-time workers (10 000 full-time workers for illness rates) and were calculated as: (N/EH)×200 000 (20 000 000 for illness rates) where N is the number of injuries and illnesses; EH is the total hours worked by all employees during the calendar year; 200 000, base for 100 full-time equivalent workers (working 40 hours per week, 50 weeks per year); 20 000 000, base for 10 000 full-time equivalent workers (working 40 hours per week, 50 weeks per year).
[b]Excludes farms with fewer than 11 employees.
[c]Data for Mining (Sector 21 in the North American Industry Classification System—United States, 2002) include establishments not governed by the Mine Safety and Health Administration (MSHA) rules and reporting, such as those in oil and gas extraction and related support activities. Data for mining operators in coal, metal and nonmetal mining are provided to BLS by the Mine Safety and Health Administration, US Department of Labor. Independent mining contractors are excluded from the coal, metal and nonmetal mining industries. These data do not reflect the changes the Occupational Safety and Health Administration made to its recordkeeping requirements effective 1 January 2002; therefore estimates for these industries are not comparable to estimates in other industries.
[d]Data for employees in railroad transportation are provided to BLS by the Federal Railroad Administration, US Department of Transportation. These data do not reflect the changes the Occupational Safety and Health Administration made to its recordkeeping requirements 1 January 2002; therefore estimates for these industries are not comparable with estimates for other industries.
[e]Days-away-from-work cases include those that result in days away from work with or without job transfer or restriction.
Note: Dashes indicate data that do not meet publication guidelines.
Reproduced from U.S. Department of Labor, 2008.

pharmaceutical chemicals on human health and the environment. Toxicogenomics combines toxicology with information-dense genomic technologies to integrate toxicant specific alterations in gene, protein and metabolite expression patterns with phenotypic responses of cells, tissues and organisms. Toxicogenomics can provide insight into gene–environment interactions and the response of biologic pathways and networks to perturbations. Toxicogenomics may lead to information that is more discriminating, predictive and sensitive than that currently used to evaluate exposures to toxicants or to predict effects on human health.

- **Predictive toxicology** is used (in this report) to describe the study of how toxic effects observed in model systems or humans can be used to predict pathogenesis, assess risk and prevent human disease.

At present, applying toxicogenomic technologies to risk assessment and regulatory decision-making are still mainly exploratory. The ability to enhance the measurements of risk assessments, by introducing toxicogenomic techniques, is not yet available. Toxicogenomics is not practical at present to replace existing industrial hygiene testing abilities needed for determining regulatory toxicology needs or developing practical risk assessments.

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This subject is progressing rapidly, however, and should be carefully considered when health hazards occur in the workplace and they need to be studied by toxicological and toxicogenomic means.

Occupational toxicology studies the harmful health effects which workers may develop from employment in their industrial environment, and also includes the study of effects which can be seen in experimental animals and other researches being conducted to determine the mechanism of action of specific agents. The composition of workforces will differ according to industry, geographic location of manufacturing sites and government regulations in effect.

Industrial hygiene is the science of anticipating, recognizing, evaluating and controlling workplace conditions that may cause workers' injury or illness. Industrial hygienists have to have training in production methods, company management, toxicology, occupational medicine, epidemiology, government regulations and safety standards (legal and corporate). Industrial hygiene includes knowledge of harmful noise levels, effects of radiation, control of hazardous heat levels and heat stress, and increasingly the presence of biohazards in the workplace and its surroundings (*see also* **Occupational Toxicology and Occupational Hygiene within the European Union (EU) Chemicals Regulation**).

The information collected in the workplace and its environment will determine the programme to be developed in order to ensure the health and safety of workers and, if necessary, the population living in the neighbourhood of the manufacturing facility. In this chapter, the general workplace health programme will be discussed as determined by the industrial findings and the government-regulated materials and processes in use. The effects of chemicals may vary in different organs or body tissue exposed to them. The responses may be designated as *target organ toxicity* or *systemic toxicity*. These effects must be distinguished from *local toxicity*.

The importance of determining in-plant exposures and the various groups who handle materials as they pass through stages of manufacture, packaging and waste disposal, is necessary in subsequently implementing health controls. It is important to consider that an indirect toxic risk may be present for the population residing in the neighbourhood of a plant which contaminates their environment. The target-organ toxicity, systemic toxicity and local toxicity of chemicals used in the plant will develop the methods used for medical surveillance of exposed workers and other populations. The health and biological effects to be kept under surveillance are usually determined from either experimental animal or other studies.

The study of the toxic effects of various materials to which the population is exposed is directly related to the means of being exposed and the organs involved. The routes of exposure determine the nature of the harmful effects from the materials encountered. Toxic responses may affect target organs or biological systems (systemic toxicity). Local toxicity directly affects the site of exposure or immediate site contact, such as may occur due to acidic or corrosive action on exposed skin, inhalation damage or exposed eye surface.

Systemic toxicity requires the absorption of the harmful agent which may result in immediate damage to the organ involved, as well as expression of the harmful effect at a distant site. The organ directly affected by the harmful agent is known as the primary target organ and those less directly or seriously affected are referred to as secondary target organs.

> *The scope of toxicology is enormous, since virtually every known chemical, if present in sufficient amount, has the potential to produce injury or death. In our modern society, toxicological tests are used to evaluate the potential health hazards that may result from exposure to environmental pollutants, food additives, pharmacologic agents, and occupational exposures among others.*
>
> (Logan, 1994)

It is well recognized that chemicals in the workplace may readily affect the skin surface if it is not protected. Many chemicals occur in the nature of fine dusts which become airborne and can enter the respiratory tract, where they produce harmful diseases, or as chemicals in a gaseous state which is readily inhalable and damaging to the respiratory system organs.

Toxic effects from exposure to various chemicals may affect:

1. The liver
2. The kidney
3. The nervous system
4. The haematopoietic system
5. The immune system
6. The cardiovascular system
7. The gastrointestinal (GI) system
8. The respiratory system.

Chemicals may be present in the workplace, with reproductive and developmental toxicity. Developmental toxicity leads to foetal death (foetotoxicity) or to birth defects (teratogenicity). Exposure to chemicals and dust products may be responsible for exerting carcinogenic effects in the human body. These effects include:

1. Lung cancer
2. Skin cancer
3. Leukaemia
4. Breast cancer
5. Prostate cancer.

The following organs are known to have been studied epidemiologically and related to occupational exposures.

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Table 3 Histological classification of liver injury

Lesion type	Examples
Necrosis (type of cell death)	Carbon tetrachloride Beryllium
Steatosis (intracellular fat accumulation)	Carbon tetrachloride Ethanol
Cholestasis (cessation/flowing of bile flow)	Organic arsenicals
Fibrosis/cirrhosis (end-result of injury)	Carbon tetrachloride Trinitrotoluene
Neoplasia (development of cancer)	Carbon tetrachloride Vinyl chloride

5.1 The Liver

The liver is an important organ, and chemicals which are absorbed by the digestive tract can reach it directly via the portal blood circulation. Toxic materials may also reach the liver via the hepatic artery or portal vein, even when they arise from other routes of exposure and can be concentrated, biotransformed and excreted. Employees in the workplace may suffer liver injury following exposure to a number of materials they may encounter, which can cause either acute, possibly reversible effects or chronic cell damage leading to such conditions as cirrhosis or carcinoma. **Table 3** is an outline of the histological classification of liver injury due to exposure to toxic materials (*see also* **Hepatotoxicity**).

Liver injuries, including cancer, have been described in many workers and attributed to chemicals found in their workplace or present in the environment where their factory is located. Modern methods of workplace improvement and exposure control are reducing the occurrence of liver disease and carcinoma in employees, and also in persons exposed to environmental hazards in the atmosphere in the neighbourhood. Hepatic lesions have been attributed to workplace exposure to a number of chemicals by various patterns of exposure, ranging from acute (various organic solvents) to short-term (tetrachloroethane and trinitrotoluene) and chronic exposure (arsenicals, vinyl chloride and some organic solvents) (Stacey *et al*., 1993).

5.2 The Kidney

Occupational exposure to chemicals, such as lead and cadmium, may be involved in causing renal failure (Bernard and Lauwerys, 1992). Coke-oven workers, exposed to emissions from this process, are at risk of developing malignant neoplasm of the kidney, and unspecified urinary organs (Goldham, 1994). Occupational exposure in the work environment that may be associated with renal disease include metals (e.g. mercury, lead, cadmium, etc.), organic solvents (e.g. carbon tetrachloride, trichloroethylene), glycols, chemicals involved in plastics manufacture and resins (acrylonitrile, styrene, etc.) and herbicides such as diquat and paraquat (Haschek, 1993a) (*see also* **Responses of the Kidney to Toxic Compounds**).

5.3 The Nervous System

> *… no precise figures are available on the total number of chemicals in existence that are potentially neurotoxic to humans.*
>
> (US Congress, 1990)

The nervous system consists of the brain, spinal cord and many nerves that control most important body functions. Neurological disorders may develop in workers in various occupations as the result of exposure to specific chemicals, which result in pathological lesions. Nerve cells affected by specific toxic substances are usually unable to recover, with the exception of the peripheral nervous system. Effects of neurotoxicity may be classified as follows:

- Immediate: Almost instant effect which may continue for several hours
  - *Examples*:
    - Antiseizure preventive agents
    - Alcoholic beverages
    - Fumes from a can of paint
- Repeated: Over weeks or years
  - *Examples*:
    - Regularly breathing solvent fumes in the workplace
    - Eating food or drinking water contaminated with lead.

Some chemicals have the ability to permanently damage the nervous system after only one exposure. It should also be recognized that substances, which individually are not toxic, may become hazardous if combined with certain other chemicals. It must also be recognized that some chemicals may be regarded as safe at one concentration, but then become neurotoxic in larger doses. It is not possible to present an exhaustive section of this chapter to deal with the nervous system and neurotoxicity. In an attempt to describe neurotoxicity briefly and include this in the overall subject heading for this chapter, 'Occupation, toxic exposure and health effects', tables from literature on occupational toxicology and texts from occupational and environmental medicine have been abstracted and are presented (**Tables 4**, **5** and **6**).

It is important to recognize that the diagnosis of neurotoxicity depends on demonstrating suspected substances in the workplace or general environment to which the work population or neighbourhood population has been exposed. The effects of such exposure may be associated

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Table 4 Occupational diseases: brain and spinal cord

Neurological effect	Agent(s)
Toxic encephalopathy	
Acute	Organochlorine pesticides
	Arsenic (and arsine)
Damage to basal ganglion	Carbon monoxide
	Manganese
	Carbon disulfide
Damage to cerebellum	Organic mercury
Damage to occipital cortex	Organic mercury
Intracranial pressure	Lead
	Organotins
Chronic	Carbon disulfide
	Organic solvents
	Lead
	Inorganic mercury
	Arsenic
	Manganese
Encephalic syndromes	
Parkinsonism movement disorders	Carbon monoxide
	Manganese
	Carbon disulfide
	Methylphenyltetrahydropyridine
Dyskinesia and tremors	Methyl bromide
Toxic tremors	Mercury
	Organophosphorus compounds
	Tetrachloroethane
	Hexachlorocyclohexane
Isolated convulsive crises	Organochlorine pesticides
	Carbon tetrachloride
	Nitrophenols
	Nitrocresols
	Lead
Bulbar (brain stem) syndromes	Hydrocyanic acid
Spinal cord syndromes	
Polyneuritis	Thallium; triorthocresol phosphate

Adapted from Chapter 3, 'Systemic Toxicology', Occupational Toxicology pages 51–52, Edited by Neill H. Stacey, published by Taylor & Francis, 1993.

with findings demonstrated by various examination techniques during clinical examination and the use of supplemental tests such as electroencephalography and electromyography (*see also* **Neurotoxicology**; **The Role of Behavioural Toxicity in Risk Assessment**).

More than 65 000 chemicals are in the US Environmental Protection Agency's (EPA's) inventory of toxic chemicals, and each year the EPA receives approximately 1500 notices of intent to manufacture new substances (NAS, 1984). Greater exposure to some toxic substances can affect workers in industry and agriculture than occurs in the general population. The US National Institute for Occupational Safety and Health (NIOSH) has recognized neurotoxicity as one of the United Nations' 10 leading causes of work-related disease and injury. Neurotoxic substances include metals, such as lead and mercury, organic solvents, pesticides, such as organophosphates, thallium and chlorinated phenol derivatives, gases, such as methyl bromide and carbon dioxide, and other organic compounds.

It has been noted that an increase in some brain cancers has been reported in a number of occupations involving exposure to chemicals. Ionizing radiation is an established environmental cause of brain tumours (Preston-Martin, 1996). Occupational exposure to radiation has been consistently linked to adult brain tumours (Alexander and DiMarco, 2001) and has also been linked to childhood brain tumours in the offspring of people exposed to radiation at work. It has been suggested that there is an increased risk of brain cancer in farmers than in the general population (Blair *et al*., 1985) which may be due to pesticide exposure (Viel *et al*., 1998). Solomon (2003) summarizes this problem as follows:

> *... brain tumors may be caused, at least partially, by environmental factors. Although living on a farm*

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Table 5 Agents causing occupational neuropathy

Metals:
Arsenic
Lead
Mercury (organic)
Organic solvents:
Carbon disulfide
n-Hexane
Methanol
Methyl-*n*-butyl ketone
Trichloroethylene
Pesticides:
Lead arsenate
Organophosphorus compounds
Organotin compounds
Thallium
Chlorinated phenol derivatives
Chlorodecane
Gases:
Methyl bromide
Carbon dioxide
Other organic compounds:
Acrylamide
o-Arylphosphates (such as TOCP)
Dimethylaminopropionitrile
Styrene
TCDD

DDT, dichlorodiphenyltrichloroethane; PBBs, polybrominated biphenyls; PCBs, polychlorinated biphenyls; TCDD, 2,3,7,8-tetrachlorodibenzine-*p*-dioxin; TOCP, tri-*ortho*-cresyl phosphate; 2,4D, 2,4-dichlorophenoxyacetic acid.
(Derived from Chapter 3, 'Systemic Toxicology', Occupational Toxicology page 56) lists recognized neurotoxins. (Winder, 1993).

Table 6 Occupations associated with an excess of brain cancer

Aluminium workers	Oil refinery workers
Chemists	Petrochemical workers
Lead smelter workers	Pharmaceutical workers
Machinists	Rubber workers
Medical personnel	Veterinarians
Dentists	Vinyl chloride workers

Reproduced from Winder, 1993. © Taylor and Francis Group.

is clearly associated with an increased risk of this disease, it is not clear whether the causal factor is related to pesticides, microorganisms, petroleum products, or other farm exposures.

She goes on to state:

The data on other factors, such as solvents, industrial chemicals, EMF (electromagnetic field), and cell phones are conflicting, and it is not clear to what degree these links are important in the disease.

5.4 The Haematopoietic System

The haematopoietic system has two components, blood and bone marrow. Blood cells are produced in the bone marrow, which keeps their content at a normal level in the circulating system and allows them to function in the body. Bone-marrow function and development may be suppressed or bone-marrow carcinogenesis may result from toxic exposures. When the red-cell development is suppressed it will be manifested as anaemia and examples are those caused by overexposure to lead or arsenic. Cytopenia may result in some instances, and, depending on the nature of exposure, all cell types may be affected. Pancytopenia and aplastic anaemia are two examples of this being attributable to such chemical exposures as benzene or trinitrotoluene. Bone marrow may also be harmed by chemical therapeutic drugs or radiation. Benzene exposure has also been associated with leukaemia.

Direct toxicity can be found to lead to red cells or white cells which have already entered the blood stream being reduced in number. Arsine may cause harmful toxicity to red cells and produce hemolytic anaemia. Chemicals which are toxic to white cells in the circulation cause cytopenia. Certain chemicals may cause methaemoglobinaemia (e.g. aniline, nitrobenzene) or carboxyhaemoglobinaemia due to exposure to carbon monoxide. Platelets may aggregate when exposed to toxins and white cells may be phagocytosed. In view of the possible effect of chemicals on the haematopoietic system it is important to maintain a medical surveillance programme for workers where exposure to such substances may occur. It is advised that a routine blood count be done, as well as a haematological examination. Workers should also be subjected to a periodic clinical medical examination with particular emphasis on the haematopoietic system. If any abnormal findings are present, the worker affected should be kept under surveillance after removal from exposure, and appropriate treatment performed. As an example of a substance affecting a worker over time, lead exposure may induce chronic effects, of which anaemia is one. These ill effects usually resolve on removal from exposure. The reader is advised to study the literature and be aware of other chemicals or industrial circumstances which may be toxic to the haematopoietic system.

5.5 The Immune System

Immunotoxicology is the study of toxic effects of xenobiotics on the cellular and humoral components

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Table 7 Cellular components of the immune system

Cell type	Function
Macrophage/monocyte	Phagocytose foreign material
Polymorphonuclear cells or granulocytes	Phagocytose foreign material
Lymphocytes	
T	Cell-mediated cytotoxicity (MHCr)[a]
TH or Ts	Helper or suppressor
B	Antibody production
Natural killer (NK)	Cell-mediated cytotoxicity (non-MHC)

[a]MHCr, major histocompatability complex, restricted.
Reproduced from Stacey *et al.*, 1993. © Taylor & Francis Group.

> *of the immune system. The multiplicity of chemical and biologic agents in the home and workplace and their complex interactions within the immune system make this field confusing and difficult to assess.*
>
> (Donald and Faenham, 1994)

The immune system includes a circulatory component, as well as a presence in other tissues. T-lymphocytes arise in the neonatal thymus, while others are processed into B-lymphocytes in the bone marrow, lymph nodes, appendix and other GI-tract-associated lymphoid tissues. Some lymphoid stem cells become natural killer (NK) cells without direct involvement of the thymus and bursal tissue. The major cell types and their functions are shown in **Table 7** published by N.H. Stacey in Chapter 3, Systemic Toxicology, page 62 (*Occupational Toxicology,* Edited by Neill H. Stacey, published by Taylor & Francis, 1993).

Peter S. Thorne states:

> *Injury affecting the immune system may arise from the immune-suppressive effects of chemicals such as dioxins or toxic metals. Many occupational diseases of the immune system occur due to hypersensitivity leading to respiratory or dermal allergy or systemic hypersensitivity reactions. Autoimmune syndromes have been associated with occupational exposures to crystalline silica and vinyl chloride.*
>
> (Thorne, 2001)

John E. Salvaggio writes:

> *Symptoms allegedly resulting from chronic exposure to low levels of synthetic and natural organic and inorganic environmental chemicals have led to the coining of terms such as 'environmental illness', 'ecologic illness', 'twentieth century disease', 'multiple chemical hypersensitivity syndrome' and 'chemical AIDS.*
>
> (Salvaggio, 1994)

Salvaggio goes on to indicate that these effects are from exposure to many industrial and domestic chemicals, atmospheric pollutants, exhaust fumes, insecticides, preservatives, cigarette smoke and related environmental contaminants (Salvaggio, 1994).

It is now recognized that the immune system may be affected by toxicity as a result of exposure to industrial hazards, environmental pollutants from chemical manufacture and from the manufacture of pharmaceutical products. Immunotoxicity of materials to which humans may be exposed is not well defined as yet, but is now becoming a subject of evaluation and concern. Three subsections of immunotoxicity have been identified (Luster, 1994), namely:

- Direct immunotoxicity
- Hypersensitivity
- Autoimmunity.

Jacques Descotes *et al*. state that 'little is actually known of the immunotoxic effects of chemicals in humans' (Descotes *et al*., 1996).

The US Congress, Office of Technology Assessment has published *Identifying & Controlling Immunotoxic Substances-Background Paper, OTA-BP-BA-75* (Washington, DC: US Government Printing Office, April 1991), which is a valuable resource and 'examines a field of study rife with jargon' (OTA, 1991). In this paper, 'immunotoxicity, or an immunotoxic effect, is defined as an adverse or inappropriate change in the structure or function of the immune system after exposure to a foreign substance'.

The study examines industrial, transportation, agricultural and household chemicals, as well as drugs and food additives. Antigens are substances that provoke an immune response. The reader is referred to the International Programme on Chemical Safety (IPCS), which is a joint venture of the United Nations Environmental Programme, the International Labor Organization and the World Health Organization (WHO). The document is found in the WHO Library Cataloguing-in-Publication Data (WHO, 1996). The subject of the immune system, its functions and testing workers for the effects of exposure to various agents, is a matter for this chapter to discuss in detail. **Table 8** is equivalent to US Congress Office of Technology Assessment (Table 1-1) referred to previously. When copied into this section of this Chapter it is valuable in studying the common immunotoxicological tests which can be used (*see also* **Toxicology of the Endocrine System**).

5.6 The Cardiovascular System

Occupational hazards in the workplace which cause cardiovascular injuries or disease are difficult to recognize and are relatively uncommon. Many chemicals

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Table 8 Common immunotoxicological tests

Category	Test
Pathology	Haematology
	Organ weights
	Histology
	Cellularity
Humoral immunity	Antibody plaque forming cell (PFC) response
	B cell litogen response
	Immunoglobulin levels in serum
	Quantitation of splenic and/or peripheral blood T cells
Cell-mediated immunity	T-cell mitogen response
	Cytotoxic T lymphocyte (CTL) cytolysis
	Delayed hypersensitivity response
	Mixed leucocyte response (MLR)
	Quantitation of splenic and/or peripheral blood T cells
Nonspecific immunity	Natural killer cell activity
	Macrophage counts
	Neutrophil counts
Host-resistance	Syngeneic tumour cells:
	PYB6 sarcoma (tumour incidence)
	B16F10 melanoma (lung burden)
	Bacterial models:
	Listeria monocytogenes (mortality)
	Streptococcus species (mortality)
	Viral models:
	Influenza (mortality)
	Mouse cytomegalovirus (mortality)
	Parasite models:
	Plasmodium yoelii (parasitaemia)
Hypersensitivity	Draize test
	Open epicutaneous test
	Buehler test
	Freund's complete adjuvant test
	Optimization test
	Split adjuvant test
	Guinea pig maximization test
	Mouse ear swelling test
	Respiratory rate measurement
	Serum IgE measurement
	Local lymphnode proliferation assay

Note: Immunotoxicologists seldom perform each test or type of test for a particular substance. They often divide the tests into tiers, using tests that indicate immune system damage at a fairly gross level to screen for potential immunotoxicants, and applying more sensitive and specific tests only to those substances that indicate possible toxicity in the primary screen. These tests are described in Chapter 1 of the Background Paper 'Identifying and controlling Immunotoxic Substances'.
Reproduced from Congress of the United States, Office of Technology Assessment, 1991.

studied experimentally in animals may be found to be cardiotoxins, but in low levels in the workplace, their ability to produce cardiovascular lesions in workers has not been proven. W. M. Haschek has tabulated some of the many ways of classifying types of cardiovascular injury with illustrative examples in **Table 9** (Haschek, 1993b).

There are many sources in industry for exposure to carbon monoxide, such as furnaces and exhaust fumes from in-plant automotive trolleys. The symptoms from exposure to carbon monoxide may be headaches, dizziness and chest pain from myocardial ischaemia. On electrocardiography (ECG), where overexposure to carbon monoxide has occurred, abnormalities may be seen, such as extrasystoles or atrial fibrillation.

Other toxic materials which may be encountered by workers are those used as solvent and cleaning materials. Materials which can contain such agents are paint thinners and degreasing products, such as trichloroethylene or methylene chloride. Again, arrhythmias may be

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Table 9 Types of cardiovascular injury, with examples

Heart
1. Disturbances in electrical impulse formation or conduction (leading to arrhythmia), for example halogenated hydrocarbons, organophosphates, arsine.
2. Direct myocardial injury, for example arsenic, arsine.
3. Ischaemia due to coronary vasospasm, for example organic nitrates
4. Asphyxia, for example carbon monoxide, cyanide, hydrogen sulfide
Vessels
1. Vasoconstriction (functional injury leading to hypertension), for example lead, carbon disulfide
2. Atherosclerosis/arteriosclerosis, for example carbon disulfide
3. Vasculitis, for example arsenic, gold. Many drugs

Table 10 Agents associated with work-related cardiovascular disease

Antimony	Fluorocarbons[a]
Arsenic	Heat
Carbon disulfide[a]	Hydrocarbons (solvents)
Carbon monoxide[a]	Methylene chloride[a]
Cobalt	Nitrates[a]
Cold	Heavy metals (lead, cadmium)

[a]Well-supported medical evidence.
Reproduced with permission from McCunney and Schmitz, 1994. © Little, Brown and Company.

caused by these solvents and sudden death may occur in some cases. According to Haschek (1993c), 'Many chemicals are known to be potentially cardiotoxic; however few have been shown to be of unequivocal importance in the workplace'. He goes on to state: 'Those workplace chemicals which have been identified as significant include: substituted aliphatic hydrocarbons, such as chloroform, fluorocarbons and propylene glycol; heavy metals such as cadmium, lead and nickel and carbon monoxide and carbon disulphide'.

Table 10 (published as Table 14.6) lists agents associated with work-related cardiovascular disease (McCunney and Schmitz, 1994) (*see also* **Cardiac Toxicology**).

5.7 The Gastrointestinal System

> *... the most important examples of occupational gastrointestinal disease, in terms of numbers and impact, are toxic hepatitis and liver and gastric cancers.*
>
> (Flemming and Beckett, 1992)

This statement indicates that persons occupationally exposed can develop gastric and liver disease as a result.

> *Toxicity is the degree to which something is able to produce illness or damage to an exposed organism. Toxicity can refer to the effect on a whole organism, such as a human or a bacterium or a plant, or to a substructure, such as a cell (cytotoxicity) or an organ (organotoxicity) such as the liver (hepatotoxicity).*
>
> (Wikipedia, 2008)

This section will summarize GI occupational diseases, and effects on the liver, from exposure to toxic chemicals encountered by persons in the industrial workplace. It is important that manufacturers and importers of chemicals identify the risks involved and keep exposed workers under medical surveillance. There are numerous chemicals used in industry, and not all have been fully classified or studied as yet. It is noted in this chapter that there may be three types of toxic entities, namely chemicals, biological toxins and physically toxic entities. The scope of this chapter is further decreased and only devoted to brief discussions of specific substances already well studied and evaluated.

The GI tract can be directly exposed to a toxic material after eating or drinking it in the workplace or it may be exposed by oral contact with a hand which handled a toxic chemical (Kemple, 2002). The following are examples of toxic effects of industrial materials to which the GI tract, or the hepatic system, are exposed and will be used for the purposes of this chapter.

5.7.1 Inorganic Lead

The history of toxicity of lead has been known for more than 2000 years. In the twenty-first century, lead toxicity is still occurring and being described, and research is being carried out on it throughout the world. Lead overexposure is still widely occurring in industry and causing illnesses. Overexposure results in impairment and disease of various forms, and may occur after varying timeframes ranging from days (acute exposure) to, in some cases, years of exposure (chronic exposure). Lead is used in some storage batteries, may be found in paint, can be incorporated into ceramic materials and can be found in explosives and bullets. Lead is of no physiological significance in the human body, but if exposure to it and absorption of it occur, it can mimic the effects of metals such as calcium, iron and zinc (see *http://en.wikipedia.org/wiki/Lead_poisoning*). There may be a history of severe GI problems such as constipation, diarrhoea, vomiting, poor appetite and weight loss associated with acute poisoning. Long-term lead exposure or overexposure to its inorganic compounds can result in severe GI disturbances and anaemia.

It is not intended to enter into prolonged discussion on lead poisoning, but merely to bring the GI system's involvement in lead toxicity to the reader's notice. There is extensive literature available on lead and its effects. It is interesting to note that most cases of lead

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poisoning probably involve young children and are from ingestion of lead dust or flakes from deteriorating lead-based paints. It must now be remembered that fuel used in automobiles does not contain lead any longer, thus removing an environmental exposure.

The US OSHA has responsibility for the OSHA Lead Standard which 'specifies the permissible exposure limit (PEL) of lead in the workplace, the frequency and extent of medical monitoring, and other responsibilities of the employer. OSHA has set a PEL (enforceable) of lead in workplace air at 50 μ/mg^3 averaged over an 8-hour workday for workers in general industry' (*see also* **Toxicology and Biological Monitoring of Metals**).

5.7.2 Vinyl Chloride Monomer

This chemical is included here as it has been shown to be a human carcinogen which causes occupational cancer of the liver when exposed to it. A study of the mortality of vinyl chloride monomer workers employed in the United Kingdom in 1940–1974 was published and discussed this problem (Jones *et al*., 1988). These workers found that in a cohort of 5498 male workers employed for at least one year during 1940–1974 in the vinyl chloride industry in the United Kingdom and followed to December 1984, there was a significant excess of nonsecondary liver tumours with 11 deaths, of which seven were angiosarcomas. The authors found that all these deaths occurred in autoclave workers with a median latency of 25 years from the date of first exposure. In 2003, mortality from neoplasms in 10 109 men employed in a job exposed to vinyl chloride for at least one year between 1942 and 1972 at any of 37 North American factories was updated and assessed (Mundt *et al*., 2000). The authors state that previous analyses indicated associations between employment in vinyl production and increased mortality risk of cancers of the liver and biliary tract, due to increased mortality from angiosarcoma of the liver and brain cancer. Their study concluded that excess mortality from cancer of the liver and biliary tract, largely due to angiosarcoma, continued. The risk of mortality from brain cancer has attenuated, 'but its relation with exposure to vinyl chloride remains unclear'.

The above two examples of GI and liver disease, due to occupational exposure to workplace agents, are a reminder of the importance of keeping employees under surveillance and investigating any unusual health issues noted (*see also* **Organic Solvents**).

5.8 Respiratory System Toxicology

The respiratory system consists of:

- The nasal pharyngeal compartment (nares to vocal cords)
- The tracheobronchial compartment (vocal cords to respiratory bronchioles)
- The parenchymal compartment (respiratory bronchioles to alveoli)
- The pleural space.

Each component of the respiratory system has specific functions to perform and the reader should study the anatomy and known functions thereof in the abovementioned compartments. In brief, workers in industry should be protected from harmful materials which they can inspire, and in designing pulmonary defences, the type of exposure should be studied, so that inhaled toxicants are avoided, the toxicants are deactivated if possible, the employees are encouraged to clear toxicants by means of coughing and their lungs remain able to discharge toxins up the mucociliary pulmonary escalator, while also using phagocytosis (Baker, 1993).

Some diagnostic features involved in the study of respiratory toxicology will now be mentioned. Exposure to inhalable agents determines the type of effect seen, based on the anatomic areas involved or the protective systems which are affected. Exposure to respirable toxicants may produce pulmonary fibrosis, lung cancer and such conditions as occupational asthma. Occupational lung diseases are well documented and there is an extensive scientific literature database for the interested reader to study. Specific conditions which have been described and discussed over many years include coal workers' pneumoconiosis, silicosis, asbestosis, byssinosis and occupational asthma. To illustrate the means which should be utilized to prevent occupational respiratory illness, an initial medical examination should be performed and then followed on a regular basis with a periodic evaluation. The interval between initial and periodic tests, and physical exams will depend on the symptoms and signs which develop. In addition, an industrial hygiene programme should be developed to coordinate medical examination findings with exposure measurements and develop safe working conditions based on these findings. **Table 11**, published by Baker (1993) summarizes methods for detecting respiratory disease (*see also* **Inhalation Toxicity**).

6 CONTROL OF HAZARDS IN INDUSTRY

6.1 Occupational and Environmental Hazards

In the United States of America, and other countries in the industrialized world, organizations exist to regulate and control hazards in the workplace, and those which may spread in to the general environment. Illnesses, which have been discussed briefly in preceding sections of this

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Table 11 Methods for detecting respiratory disease

Method	Compartment			
	Naso-pharyngeal	Tracheobronchial	Parenchyma	Pleura
Questionnaire	+ + +	+ +	+	—
Spirometry	—	+ + +	+ +	+
Exercise	—	+ +	+ + +	+
Chest radiograph	—	+	+ +	+ + +

The clinical methods for measuring respiratory pathophysiology have different usefulness in the different respiratory compartments.
+, some; ++, reasonable; + + +, most.
Reproduced from Baker, 1993. © Taylor & Francis Group.

chapter are, in addition to providing care to workers, studied by various experts for developing safety standards and methods of controlling such hazards. Workplaces are sampled for hazardous materials, which have previously been studied and shown to cause occupational disease, and industrial hygienists run programmes which measure the levels of exposure in the work environment. Epidemiological studies are of value in developing preventive industrial hygiene standards, based upon the exposure levels in the workplace, and the medical findings associated with them. Engineers are trained in the design and development of workplace machinery which control air contaminants or processes which may cause direct contact with solids or fluids. It is important to develop an overall occupational programme, which includes occupational medical examinations, industrial hygiene monitoring, chemical-control engineering and routinely calculated epidemiological results, to study deviation from normal, thus indicating development of health dangers (*see also* **Occupational Toxicology and Occupational Hygiene within the European Union (EU) Chemicals Regulation**).

6.2 Basic Factors in Industrial Exposure Standards

It is the main task of the industrial hygiene specialist to develop a programme which will recognize and assess exposure levels to chemicals, physical agents or noise in the work environment. Another issue to be taken into account may be biological abnormalities caused by exposure to a harmful agent in the workplace. It is not a subject for discussion in this chapter, but ergonomic factors in industry fall within the field of industrial hygiene.

In modern industrial plants, because of strict standards imposed, most chemicals encountered at work are not hazardous at the levels found there. In some chemical manufacturing plants, exposure levels may be significantly increased, and the need arises for industrial hygiene monitoring programmes there and constant surveillance of the health of exposed employees. If the worksite is not completely isolatable, exposed workers will need to be protected by specifically designed clothes, by skin protection and by respiratory protection using appropriate breathing apparatus. It goes without saying, that a medical surveillance programme must be in effect to follow the health of such workers, and if indicated remove them from any exposure likely to be toxic to them. Employees must be aware of the hazards in the workplace by reading warning labels on materials used by them, by being warned by supervisors to read MSDSs, and by being thoroughly familiar with the Occupational Safety and Health Act requirements for preventing exposures above the OSHA Standard set, and equivalent regulations in other countries. Chemical suppliers are required by the OSHA to provide users with an MSDS for chemicals they use (known as the 'Hazard Communications Standard'). As mentioned previously, workplaces must be checked for physical hazards, biological hazards and ergonomic hazards.

6.3 Recognition and Control of Hazardous Substances

'Industrial hygiene is the science of anticipating, recognizing, evaluating and controlling workplace conditions that may cause workers injury or illness. Industrial hygienists use environmental monitoring and analytical methods to detect the extent of worker exposure and employ engineering, work place controls and other methods to control potential health hazards' (OSHA, 1998). It is stated that material contained in this publication is in the public domain and may be reproduced, fully or partially, without permission of the Federal Government.

Developing and setting occupational health standards requires determination of employee exposures to hazards and deciding how to control them. Training provided to industrial hygienists lets them anticipate, recognize, evaluate and recommend controls of the hazards detected. Their goal is to protect the health of workers and thereby prevent the contamination of their homes and communities.

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Their method of achieving their task is to study the workplace for contamination and evaluate the possible routes of exposure which can be studied and controlled.

The main routes of exposure are inhalation, ingestion, skin contact and accidental injection. The role of an industrial hygienist is to evaluate the magnitude of exposure. This may be done by measuring air levels or levels in other media in use in the factory. Exposure may be calculated in parts per million (ppm), milligrams per metre cubed ($mg\,m^{-3}$) or fibres per cubic centimetre ($f\,cm^{-3}$). It is important to know how long such exposure occurs, ranging from minutes to a lifetime. The frequency with which exposure occurs may range from hours to days to longer, such as weekly, and may take place over a working lifetime.

It is not the subject of this chapter to go into great depth regarding industrial hygiene, but it is important to know that engineering, work practices and administrative controls are the primary means of reducing exposure to industrial health hazards.

- Engineering controls:
  - Replacing known toxic materials with nonhazardous ones
  - Enclosing work processes
  - Confining operations
  - Installing general and local ventilation systems
- Work-practice controls:
  - Follow required procedures to minimize exposure while operating production and control equipment
  - Regular inspection and maintenance of equipment
  - Implementing good housekeeping
  - Good supervision
  - Prohibition of eating, drinking, smoking, chewing tobacco or gum and applying cosmetics in regulated areas
- Administrative controls:
  - Schedule production and workers' tasks to minimize exposure levels.

When all the above controls cannot be implemented then it may be necessary to provide exposed workers with appropriate personal protective equipment, such as safety goggles, gloves, shoes and protective clothing, as well as appropriate respiratory equipment.

7 MEDICAL EVALUATION OF HEALTH IN INDUSTRY

7.1 Principles of Medical Surveillance and Hazardous Substances

The recognition that hazardous materials may be found in industry, and the finding of potential ill-health of workers exposed to them, has resulted in the development of surveillance programmes which follow up both the exposure findings and the health of the exposed population. The implementation of such a programme requires intimate collaboration between company management, industrial hygienists, employees and the corporate medical services. The management involved will observe employees throughout their period of work in an environment suspected of being associated with an occupational hazard. A surveillance programme notes any medical abnormality occurring in individuals in the workplace, and develops an industrial hygiene surveillance programme which will allow potential hazards to be documented. A work programme has to be developed which will intervene and lead to disease reversal or prevention. A programme established to follow the health of employees is known as medical surveillance.

7.2 Medical Surveillance

7.2.1 Work History

The new employee provides an occupational history. Details of potential exposures encountered in previous occupations will be recorded. Attention will be paid to the answers in case the content of the medical examination indicated, and special tests required, need to be modified.

7.2.2 Medical Examination

The medical history is relevant to the industry involved and known hazards in the particular workplace to which the individual is being assigned. There are two types of examination involved, namely the initial medical examination and the periodic medical examination.

7.2.2.1 Initial Medical Examination

It is important to record the applicant's past medical history and establish what previous toxic materials may have been encountered elsewhere. This may influence the initial examination and tests required for potential disease which may already be established; an example would be exposure to asbestos, which does not necessarily exhibit findings for many years.

A complete medical examination will consist of:

- Medical history
- Occupational history
- Physical examination
- Special examination such as, for example:
  - Radiology, for example chest X-rays
  - Haematology, for example blood lead counts
  - Urine analysis, for example proteins and so on
  - Pulmonary function tests, for example spirometry.

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In asbestos workers, the medical surveillance programme data records must be kept for at least 30 years and for the employee's lifetime, if possible. Similarly, such records should also be retained for 30 years in silica-exposed workers. These requirements are laid down in the USA by the OSHA Standard on Access to Employee Exposure and Medical Records (29 CFR 1910.1020).

7.2.2.2 Periodic Medical Examination

The object of the periodic medical examination is to re-evaluate the medical history and the findings which have been specifically designed for the workplace hazard involved. The periodicity of the examination will depend upon the type of hazard occurring and the timeframe for development of the effects. It may be necessary to examine the workers involved frequently, such as a matter of months, annually or even at an interval of five years. It may be decided to reduce the time between such periodic examinations when it has been determined that the longer the exposure, or time since first exposure, the more ill-effects will become recognizable. For example, since the ill-effects from exposure to asbestos dust may not manifest themselves for 15–20 years (or longer!) the periodic examination of workers exposed will be initially at five yearly instances and then reduced to annually later.

The medical examination will essentially use the same history and physical findings to determine whether the worker can continue the job, be temporarily removed from it and then reinstated on return to physical normality, or be permanently removed from further exposure. The medical examination results must be part of the overall study of the workplace, in conjunction with employers, industrial hygienists and workers.

In summary therefore, a baseline evaluation should be done for each worker potentially exposed to a workplace hazard, the workers should be followed-up at an appropriate interval, and when leaving the job should have an exit evaluation. The periodicity of the examination depends upon the exposure and the epidemiological history findings, which will suggest the time between initial and periodic examinations.

8 SUMMARY

A brief presentation of the history and evaluation of occupation, toxic exposures and health effects has been attempted in this chapter. The history of occupational medicine, as it applies to evaluation of the materials used in the manufacture and use of chemicals in various sectors of industry, is briefly outlined and famous scientists over time are mentioned.

Toxicology is the science which studies toxic substances. This chapter briefly presents the findings of studies which are involved in methods of detecting chemical actions in manufacturing units affecting exposed individuals. Occupational toxicology can be studied by occupational physicians, chemical engineers, industrial hygienists and workplace management, allowing protective controls to be developed. Toxic effects from exposure to various toxic chemicals is briefly reviewed. The review studies the effect of hazardous chemicals and presents a brief outline section for each. Included in the chapter for study of their effects are the liver, the kidney, the nervous system, the haematopoietic system, the immune system, the cardiovascular system, the GI system and the respiratory system. The control of occupational and environmental hazards is briefly discussed. The object is to mention that an overall programme must include occupational medical examinations, industrial hygiene monitoring, chemical-control engineering and routinely calculated epidemiological results. Studying the deviation from normal will allow evaluation of the results the health dangers present.

A brief outline is presented to allow familiarity with engineering controls, work practice controls and administrative controls.

Medical surveillance is briefly discussed and outlines the content of initial and periodic medical examinations.

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Biotechnology: Safety Assessment of Biotherapeutics and Agribiotechnology-Derived Foods

John A. Thomas

CONTENTS

1 INTRODUCTION

1.1 History

Biotechnology has been used for centuries, but only recently has it taken on more molecular complexities in terms of the genetic manipulation of both plant and animal cells. Classically, biotechnology refers to any technique that uses living organisms (or components of these organisms) to modify or create, to improve plants or animals or to develop micro-organisms for specific uses. Biotechnology has been used for centuries in making wine, cheese, yogurt, bread and in the selective crossbreeding of both animals and plants, all leading to an enhancement of specific desirable traits. The term 'biotechnology' was first coined by a Hungarian, Karl Ereky, towards the end of WorldWar I. Ereky purportedly used the term to refer to intensive agricultural methods (Thomas, 1998). Unfortunately, the term genetically modified organisms (i.e. GMOs) is a somewhat erroneous term with regard to issues of safety or risk pertaining to foods. GMO is an old term that was coined nearly two decades ago, and by those seeking to give it a negative connotation. In retrospect, many other terms could have been adopted, included that of selective crossbreeding. Genetic engineering involves the ability to manipulate, modify or otherwise 'engineer' genetic material to produce desired characteristics. This has been the integral part of biotechnology (Liberman *et al*., 1991). Genetic engineering also encompasses recombinant DNA technology. The contemporary era of biotechnology began about three decades ago with the discovery of endonucleases or 'restriction enzymes', and their ability to selectively cut-and-paste segments of DNA. Such enzymes act as highly specific chemical scalpels and can be used to obtain specific sequences within genetic material. Thus, restriction enzymes recognize and cut DNA at precise locations. Recombinant DNA and similar genetic engineering procedures led to the transfer of genetic material across species barriers. Actually, cell biology has become another overlapping core discipline of modern

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biology, along with genetics and biochemistry. Of course the sequencing of the human genome will undoubtedly lead to significant advances in other areas of biotechnology and to a better understanding of genetic-related diseases.

There are many uses of these new biotechnologies, such as hormone substitutes, nutritional supplements and improved crop yields, but a few examples of how this new biotechnology will impact on the quality of life. The biotechnology industry encompasses several segments, including therapeutics, vaccines, diagnostics (e.g. monoclonal antibodies (mAb)), single nucleotide polymorphisms (SNPs), agriculture and the environment. There is the potential to discover many new drug entities for the treatment of various diseases.

1.2 Transgene Technology

The introduction of transgene technologies involving the transfer of a gene from one species to another species. Genetic manipulation of laboratory mouse models for biomedical research has progressed rapidly, with so-called 'designer mice' (i.e. transgenics) being used to study immune deficiencies, cancer and developmental biology (Grosveld and Kollias, 1992; Goodnow, 1992; Thomas, 1997) (**Table 1**). Over a decade has elapsed since the development of a transgenic mouse model and its introduction into biomedical research. Transgenic animals are produced by inserting a foreign gene into an embryo, with this foreign gene subsequently becoming an integral part of the host animal's genetic material. Increasingly, there have been improvements in the techniques used for gene introduction into animals. There are several techniques that have been used, including microinjections of DNA into the pronucleus, retrovirus infection and embryonic stem cells, which can be grown *in vitro* from explanted blastocysts. All of these techniques have been employed to create chimeric animals. Transgenic animal models are only one type of at least four other classes of models that can be used to study specific disease states (Thomas, 1997).

Generally speaking, progress has been slower in developing transgene species in the plant cell than in the animal cell. Plant breeding techniques have evolved over the millennium with the purpose of improving crops. Artificially induced mutations and selection have hastened the improvements in major cereal crops. Initially, improvements in plant biotechnology have been directed at enhancing agronomic traits, including pest resistance, herbicide tolerance and disease resistance (e.g. viruses, fungi). Significant progress has been made in improving the nutritional value of many cereal grains.

Table 1 Transgenic mouse models simulating human diseases[a]

AIDS	Inflammatory diseases
Demyelinating diseases	Neonatal hepatitis
Diabetes mellitus	Oncogenesis
Glomerulosclerosis	Osteogenesis imperfecta
Hepatocarcinogenesis	Sickle-cell anaemia
Hypertension	

[a]Modified from Goodnow, 1992.

1.3 Regulatory Considerations

The regulatory approval of genetically engineered products has varied widely from country to country and from product to product. The safety and regulatory-approval process for therapeutic proteins has generally proceeded without any major impediments. The approval processes for what are usually considered as 'biologics' have long been established. Alternate sources of insulin for the treatment of diabetes mellitus and of growth hormone for treating children with deficiencies have not been controversial and posed no significant risk. Other more novel therapeutic proteins have undergone more rigorous testing to insure safety and efficacy prior to any regulatory approval. Agribiotechnology involving improvements in food quality, however, has not been universally accepted by the public nor in every instance by regulatory agencies. Generally, the somewhat slower progress in the regulatory approval of genetically modified (GM) foods has not been an issue of human safety, but of environmental impact on wildlife and nontarget species.

Several countries have well-established regulatory committees or groups for biotechnology-derived drugs or biopharmaceuticals (**Table 2**). In the United States, the Food and Drug Administration (FDA), through the Center for Biologic Evaluation and Research (CBER), has oversight responsibilities for insuring safety and efficacy of biopharmaceuticals.

Regulatory responsibility for biotechnology-derived foods may involve several US agencies, including the United States Department of Agriculture (USDA), the Environmental Protection Agency (EPA) and the FDA. The FDA again has oversight on food safety (like drug safety), as it related to new plant varieties (e.g. dairy products, food-processing aids, etc.). The USDA regulates poultry products and meats and has the oversight of field tests for genetically modified crop plants. The EPA, through its regulatory responsibility for pesticides, oversees aspects of genetically altered plants with traits such as herbicide tolerance, pest resistant and potential effects on nontarget wildlife species.

Globalization of commerce has seen many agencies worldwide become involved in the regulation of GMOs (**Table 3**). GMOs involve both plant and animal biotechnologies. Several of these organizations dealing

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Table 2 Regulatory aspects of biotechnology[a]

Country(s)	Regulatory committee/agency
Europe (EU)	Committee Proprietary Medicinal Products (CPMP) Safety Working Party (SWP) Biotechnology Quality Working Party (BQWP) Operations Working Party (OWP) Council Directive 87/22/EEC Concertation Procedure Notes for Guidance, e.g. preclinical safety (1987)
Japan (Ministry of Health, Labour and Welfare (MHW))	Pharmaceutical Affairs Bureau (PAB) Central Pharmaceutical Affairs Council (CPAC) Committee on Drugs Committee on Antibiotic Drugs Committee on Blood Products National Institute of Hygienic Sciences Notification No. 243 (1984) Notification No. 10 (1988)
United States (FDA)	Center for Drug Evaluation and Research (CDER) Center for Biologic Evaluation and Research (CBER) Public Health Services Act Food, Drug and Cosmetic Act Federal Register: biotechnology notice (1984) Federal Register: regulation of biotechnology Product (class) oriented 'Points to Consider' (PTC)

[a]From: Bass *et al*., 1992; Thomas, 1995.

Table 3 Some global organizations involved in plant and animal biotechnology

ACNFP	Advisory Committee on Novel Foods and Processes (UK)
BQWP	Biotechnology Quality Work Party (EU)
CBD	Convention on Biological Diversity
CBER	Center for Biological Evaluation and Research (US)
CDER	Center for Drug Evaluation—Research (US)
CPMP	Committee for Proprietary Medicinal Products (EU)
GIBiP	Green Industry Biotechnology Platform
ICH	International Conference on Harmonisation
NIBSC	National Institute Biological Standards and Control (UK)
OECD	Organization for Economic Cooperative and Development
UNCED	UN Conference in Environment and Development
UNEP	U.N. Environmental Program
UNIDO	U.N. Industrial Development Organization

with GMOs have been identified (**Table 3**). Some of these organizations have no regulatory authority, yet have proposed various guidelines for the use of GMOs. Harmonization and standardization of safety protocols for both biotherapeutic agents and agribiotechnology products has become well established.

Regulatory guidelines offer both advantages and disadvantages to regulatory guidelines (**Table 4**). Importantly, the advantages outweigh the disadvantages. Harmonization or adoption of consistent review protocols is imperative and will lead to better overall assessment of safety and efficacy of new biotechnology-derived products. Importantly, the review must be science-based, and it continues to evolve and keep abreast of the latest breakthroughs in technologies and food processing.

2 BIOTHERAPEUTICS

2.1 Introduction

The development of recombinant DNA technologies has enabled the pharmaceutical-related industries to manufacture large quantities of macromolecules in relatively pure forms. This has led to the production of such therapeutic proteins as human insulin, human growth hormone (hGH), human recombinant plasminogen activator (rtPA) and human erythropoietin (EPO) (Thomas, 1998). While hormone substitutes (e.g. insulin, hGH, etc.) represented an early entry in biopharmaceuticals, many other classes of therapeutic agents have been produced and are undergoing development or clinical trials. Most of the early biopharmaceuticals were represented by simple hormone replacement therapies (e.g. insulin), they did not undergo the same extensive preclinical safety and efficacy studies, such as those that would be required for

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Table 4 Advantages and disadvantages of establishing regulatory guidelines[a]

Advantages

- Formalization of a consensus scientific opinion
- Promotion of consistency of review
- Improvement of quality of studies performed
- Assistance to industry

Disadvantages

- Nonuniformity in study designs
- Retreat to 'check-the-box' approach due to uncertainties
- Disincentive to industry to be creative
- Failure to appropriately incorporate new and evolving technologies

[a]Reproduced from Henck *et al*., 1996. © John Wiley & Sons Ltd.

interferon(s) (Vial and Descotes, 1994) and other drugs. Biopharmaceutical products have been approved for a variety of clinical indications (**Table 5**). New clinical indications have been approved for the interferon(s) and for other immunomodulating agents. Blood factor(s) deficiencies or disorders have benefited from these biotechnological advances as have blood-clot dissolution proteins. There has also been an increasing number of regulatory approvals for mAb. It is noteworthy that the regulatory approval of rhGH (recombinant hGH) was expedited by the discovery that some cadaveric hGH was possibly contaminated with latent neural viruses responsible for Creutzfeldt–Jacob disease.

Uses of SNPs can yield information about promising drug targets for a host of diseases. Gene-based medicine, known also as functional genomics, may hold the key to understanding and treating various diseases (e.g. Alzheimer's, diabetes mellitus, etc.).

The preclinical safety assessment of new biotherapeutic agents for human medical therapies has highlighted the issue of immunogenicity between species (Cavagnaro, 1997; Thomas, 1999). Macromolecules derived through biotechnology expectedly will challenge the immune system of the laboratory animal used for toxicological testing protocols. Despite some of these unique and early concerns, guidelines for the preclinical testing of large-molecular-weight biopharmaceuticals has proceeded, and has generally been adopted by regulatory agencies. Macromolecules represent a challenge to the design of toxicological protocols, and have also led to challenges in the formulation and delivery of biopharmaceuticals (Panchagnula and Thomas, 2000). Macromolecules also represent a challenge in noninvasive drug-delivery systems, and the use of supercritical fluid technology may produce dry powder formulations suitable for inhalation or needle-free administrations (Tservistas *et al*., 2001).

2.2 The New Biologies

The scientific disciplines of genetics, immunology and molecular biology have provided a strong impetus for advances in biotechnology, and particularly in biopharmaceuticals (Tomlinson, 1992). Certainly, biotechnology-derived products could not have progressed to their current state had it not been for the coincidental and independently developed technology of monoclonal antibody production. Certainly, recombinant DNA technologies and monoclonal antibody production are two areas that are inseparably important to the development of new biopharmaceuticals. mAb are essential in the identification, extraction and purification of most macromolecules. The term molecular biology has come to signify the biochemical study of nucleic acids, advanced by the discovery of a series of enzymes (e.g. restriction enzymes or endonucleases) that allows for specific manipulation of RNA and DNA. There are several techniques or approaches used in molecular biology (**Table 6**) (Ausubel *et al*., 1995). These technologies are advancing at a very rapid pace. The ability to manipulate the genetic material of a cell in order to modify gene expression has utilized a variety of new techniques, thus facilitating the discovery of new biopharmaceuticals. Using the appropriate mammalian expression system for the development of biopharmaceuticals is very important (Werner *et al*., 1998). New advances in the development of highly sensitive and highly specific analytical methods (e.g. high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS-MS)) and caco-2 cell techniques will facilitate the entry of new biopharmaceuticals into the market (Panchagnula and Thomas, 2000).

2.3 Therapeutic Proteins

Developments in recombinant DNA technologies have focussed upon mammalian macromolecules, often peptides, for discovering therapeutically active entities (Kelley, 1996). Mammalian biotechnology has advanced more rapidly than agribiotechnology, although the latter field promises to be very fast moving. Early successes witnessed the use of prokaryotic cell systems (e.g. *E. coli*) capable of producing rather complex mammalian proteins. Subsequently, and with more advances in techniques employed in molecular biology (**Table 7**), prokaryotic cell systems were found to be able to secrete even more complex proteins and glycoproteins. Improved methods of molecular characterization made it possible to create more complex and more highly purified therapeutic entities. Mammalian organisms possess a plethora of proteins that modulate intricate physiological systems and, through biotechnology, some of these will be discovered for their therapeutic usefulness.

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Table 5 Selected list of biopharmaceuticals

Generic name	Trade name	Classification	Indication(s)
Alteplase (rTPA)	Activase	Recombinant protein	Acute myocardial infarction
Tenectaplase	TNKase	Recombinant protein	Pulmonary embolism Acute mycardial infarction
Epoetin alfa (EPO)	Epogen Procrit	Recombinant protein	Anaemia of chronic renal failure Anaemia secondary to zidovudine treatment in HIV-infected patients
Antihaemophilic factor	Recombinate, Humate-P, Refacto	Recombinant protein	Haemophilia A
Factor VIII	KoGENAteFS, Helix-ate FS	Recombinant protein	Haemophilia A
Factor VIIIc	Monoclate-P	Recombinant protein	Haemophilia A
Factor IX	L-Nine SC	Recombinant protein	Haemophilia B
Tumour necrosis factor	Embrel	Recombinant protein	Active rheumatoid arthritis
Filgrastim (G-CSF)	Neupogen	Recombinant protein	Neutropenia following chemotherapy
Sargramostim	Leukine	Recombinant protein	Myeloid reconstitution after bone marrow transplantation
Interferon-α-2a	Roferon A	Recombinant protein	Hairy cell leukaemia AIDS-related Kaposi's sarcoma
Interferon-α-2b	Intron A	Recombinant protein	Hairy cell leukaemia AIDS-related Kaposi's sarcoma Hepatitis, non-A, non-B/C Condylomata acuminata
Interferon-alpha	Wellferon	Recombinant protein	Chronic hepatitis C
Interferon-α-n3	Alferon N	Human	Genital warts
Interferon-β-1a	Avonex	Recombinant protein	Multiple sclerosis
Interferon-β-1b	Betaseron	Recombinant protein	Multiple sclerosis
Interferon-γ-1b	Actimmune	Recombinant protein	Chronic granulomatous disease
I-IGF-1	Myotropin	Recombinant protein	Amyotrophic lateral sclerosis (ALS)
Aldesleukin (IL-2)	Proleukin	Recombinant protein	Kidney cancer
Dornase-α	Pulmozyme	Recombinant protein	Cystic fibrosis
Human insulin	Humulin	Recombinant protein	Diabetes mellitus
Somatrem (recombinant methionyl-hGH)	Protropin	Recombinant protein	Growth hormone deficiency
Somatropin (rhGH)	Humatrope Saizen Nutrotropin	Recombinant protein	Growth hormone deficiency
Follicle stimulating hormone	Puregon	Recombinant protein	Induce ovulation
Somatotropin (recombinant bovine GH)[a]	Posilac	Recombinant protein	Increases bovine lactation
Pegaspargase (polyethylene glycol-1-asparaginase)	Oncaspar	Polyethylene glycol modified protein	Acute lymphocytic leukaemia
Haemophilus B conjugate vaccine	HibTITER	Recombinant protein	Prophylaxis against haemophilus influenza
Hepatitis B vaccine	Engeridx-B Recombivax HB	Recombinant protein	Prophylaxis against hepatitis B infection
Satumomab	OncoScint CR103	Monoclonal antibody	Colorectal cancer imaging
Satumomab	OncoScint OV103	Monoclonal antibody	Ovarian cancer imaging
Muromonab CD3	Orthoceone (OKT3)	Monoclonal antibody	Acute allograft rejection in renal
Alemtuzumab	Compath	Monoclonal antibody	Chronic lymphocytic leukaemia

[a]Veterinary product.

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Table 6 Some molecular cell biology tools[a]

Recombinant DNA techniques
■ Site-directed mutagenesis
■ Single-strand conformational polymorphism (SSCP)
■ Ligated gene fusions
Hybridoma technology
■ For production of antibodies
Carbohydrate engineering
Novel instrumental techniques
■ Polymerase chain reaction (PCR) and reverse transcription polymerase chain reaction (RT-PCR)
■ Subtractive hybridization differential display
■ Immunoblotting
■ Confocal laser microscopy
■ Fluorescence-activated cell sorting (FACS)
■ Scanning tunnelling electron microscopy
Pulsed field electrophoresis
Northern blotting and solution hybridization
In situ hybridization
Southern blotting: gene deletions

[a]From Demain, 1991; Davis, 1996.

Modern molecular biology, particularly through the use of polymerase chain reactions (PCRs), has become exceedingly important in the discovery and production of recombinant proteins. Acting as a molecular 'photocopier', PCR has been employed extensively to amplify DNA sequences. Subsequently, the design of new biopharmaceuticals has proceeded with sufficient quantities of purified material in order to fulfill the amounts required to undertake both preclinical and clinical studies. Nowadays, robot gene sequencers (e.g. ABI Prism 3700) and high-speed computers have greatly facilitated the discoveries associated with the human-genome project.

The majority of new biopharmaceuticals usually must be administered parenterally. Proteins or large peptides are vulnerable to gastric degradation and experience a loss of biological activity. There is considerable interest and need to develop drug-delivery systems for these macromolecules (cf. Tservistas *et al*., 2001). Selective drug targeting is a characteristic that is highly desirable. Progress has been made with some biotherapeutic agents by modifying their biologic dispersion characteristics (**Table 8**). Such approaches have included site-directed mutagenesis, hybrid site-specific proteins and supercritical fluid technologies. In addition, significant progress has been made towards the development of site-specific drug-delivery systems for protein and peptide biopharmaceuticals (Pettit and Gombotz, 1998)

2.4 Safety Assessment

2.4.1 General Considerations

Assessing the 'safety' of drugs produced by biotechnology resembles the assessment of conventional new chemical entities (NCE), but with certain major differences in testing protocols (Zbinden, 1990; Dayan, 1995; Cavagnaro, 1997). Several safety considerations must be incorporated into the preclinical testing protocol (**Table 9**) (Tomlinson, 1992). The quality of each biotechnology-derived product necessitates careful control because of the concern about immunogenic proteins or peptides, endotoxins released by the harvesting of prokaryotic cell systems that secrete the product, and other possible chemical contaminants emanating from processing procedures. There are at least three areas of concern regarding biotechnology-derived products: (i) toxicology issues pertaining to differences in pharmacodynamic properties, (ii) 'intrinsic' toxicity, that is, adverse effects due to the molecule itself and not directly related to pharmacological actions and

Table 7 Methodological advances in the characterization of biological products[a]

Method	Examples
In vitro bioassays	Cell culture techniques for hormones and cytokines
Gene analysis	PCR analysis (viral contaminations of blood products)
Immunological tests	Enzyme-linked immunosorbent assay (ELISA)
	Epitope mapping by panels of monoclonal antibodies
	Immunoblotting of gels (western blots)
	Testing for process-related impurities
Advanced chromatographic techniques	Size-exclusion and reversed-phase HPLC
	Capillary zone electrophoresis
Analysis of 3-D structure	Circular dichroism
	Nuclear magnetic resonance
Protein analysis by mass spectrometry	Fast atom bombardment (Fab)-mass spectrometry (antigen binding)
	Electrospray mass spectrometry
Analysis of glycosylation isoforms	Detailed structure–function studies required

[a]Reproduced from Tomlinson, 1992. © Nature Publishing Group.

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(iii) 'biological' toxicity, responses resulting from the activation of a physiological mechanism (e.g. antigen–antibody reactions) (Zbinden, 1990). There are safety issues that form the basis for toxicity testing. There are safety issues pertaining to the intrinsic actions of the product itself, and there are safety issues pertaining to the cellular systems or processes that lead to the production or secretion of the macromolecule (Thomas and Thomas, 1993; Dayan, 1995; Thomas, 1995; Cavagnaro, 1997) (**Table 10**). Product-related impurities or variants might include mutants, aggregated forms and aberrant glycosylation. Process-related impurities might include pyrogens, host-cell-derived proteins, viruses and possibly prions (Jeffcoate, 1992). Reliable cell systems, expressing the correct gene and insuring its proper insertion along with any promoter is of fundamental importance. The secretion or presence of other bioactive moieties must always be considered, and needs to be addressed through proper extraction and purification processes. Protein or peptide contaminants can produce both unwanted immunologic and nonimmunologic actions. Clinical or conventional toxicologic protocols may not be relevant in attempting to characterize the phrmacodynamics of a macromolecule. Nonimmunologic testing is dependent upon the product's potential therapeutic use, and, at a minimum testing, should include biodistribution, developmental and reproductive and mutagenic/oncogenicity assessments. Protocols that define the kinetics and safety of infused recombinant proteins include bolus delivery, continuous infusion and other physiological parameters (Yaksh *et al*., 1997). It is very important that a panel for detecting possible immune alterations be an integral component of the toxicology testing protocol (**Table 11**). The first tier consists of a screening panel that evaluates immunopathology, cell-mediated immunity and humoral immunity. Tier 1 should also identify any agents that may elicit an immune response. Those agents testing positive in Tier 1 must undergo further evaluation. Tier 2 seeks to evaluate the presence of any immunopathological effects.

Table 8 Approaches for modifying the biological dispersion of therapeutic proteins[a]

Approach	Therapeutic protein
Site-directed mutagenesis	α_1-Antitrypsin
Hybrid site-specific proteins	Immunoglobulin and toxin
Linked synthetically	Fragments
Fused-gene products	Growth factors
Protectants	
Polyethylene glycols	Interleukins
Administration	
Frequency and rate	Growth hormone (effectiveness altered)
Route	Insulin (site of injection)
Staging	Combinations of interferon and tumour necrosis factor

[a]From Demain, 1991.

Table 9 Some safety considerations of biotechnologically derived products[a]

Product-related impurities or variants
Genetic variants and mutants
Aggregated forms
Glycosylation patterns
Process-related impurities
Pyrogens
DNA and potential oncogenicity
Host-cell-derived proteins
Viruses (human, simian, murine, bovine)

[a]Reproduced from Tomlinson, 1992. © Nature Publishing Group.

2.4.2 Antisense Drugs

The use of antisense oligonucleotides is probably the most mature in the development of macromolecular drugs (cf. Crooke, 1992; Juliano *et al*., 2001). Base pairing between relatively short oligonucleotides (12–18 residues) and complementary sequences in messenger RNA (mRNA) provide highly selective recognition that can affect gene expression.

The completion of the sequencing of the human genome was one of the early applications of antisense technology in which the primary sequencing information can be used to design short oligonucleotides to hybridize to a specific mRNA (Bennett *et al*., 1998). Once bound to the targeted mRNA in a cell, the antisense oligonucleotide prevents expression of the protein product encoded by the targeted RNA. Antisense oligonucleotides can interrupt gene expression. Antisense molecules are highly selective, and when inserted into a cell can bind particular mRNA molecules and prevent them from being translated into proteins.

Antisense drug therapy may affect pathophysiological processes at the genetic level (Harvey, 1998). Such therapies involve using modified oligonucleotides that bind specifically to target sequences of RNA to affect translational arrest or to sections of DNA to block transcription. It is noteworthy that there are naturally occurring antisense RNAs (Dolnick, 1997). Naturally occurring antisense RNAs may act as mediators of alterations in gene expression.

Some technical issues need to be overcome in order to fully exploit antisense drug development. Such issues include the size of the molecule in order to insure specificity, the chemical stability often needs to be enhanced and control of the agent's uptake and tissue distribution. Unmodified oligonucleotides are negatively charged and do not readily enter cells. However, modification of the internucleoside phosphate linkages to uncharged

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Table 10 Selected guidelines for evaluating biopharmaceutics[a]

Toxicological issues arising from the producing system
■ Prokaryotic production system (recombinant DNA) Correct gene and promoter, stable expression, contaminating toxins, and so on.
■ Eukaryotic production system (recombinant DNA) Correct gene and promoter; stable expression, presence of antigens, other bioactive peptides, and so on.
■ Other Chemical modifications, infection-free animal vectors, and so on.
Toxicological issues arising from the production process
■ De- and renaturation of protein(s)
■ Presence of other bioactive molecules
■ Presence of chemical residues
■ Microbial contamination (e.g. endotoxins)
Toxicological issues arising from biopharmaceutics
■ Pharmacodynamics
■ Pharmacological and toxicological actions
■ Immunological
■ Other (e.g. live or attenuated vaccine)

[a]Reproduced from Dayan, 1995. © Elsevier.

Table 11 Panel for detecting immune alterations[a]

Tier 1
Haematology (e.g. leucocyte counts)
Weights—body, spleen, thymus, kidney, liver
Cellularity—spleen, bone marrow
Histology of lymphoid organ
IgM antibody plaque-forming cells (PFCs)
Lymphocyte blastogenesis
T-cell mitogens (PHA, Con A)
T-cell (mixed leucocyte response MLR)
β-cell (lipopolysaccharide, LPS)
Natural killer (NK) cell activity
Tier 2
Quantitation of splenic B and T Lymphocytes (surface markers)
Enumeration of IgG antibody PFC response
Cytotoxic T lymphocyte (CTL)
Cytolysis or delayed hypersensitivity response (DHR)
Host resistance
Syngeneic tumour cells
PYB6 sarcoma (tumour incidence)
B16F10 melanoma (lung burden)
Bacterial models
Listeria monocytogenes (morbidity)
Streptococcus species (morbidity)
Viral models
Influenza (morbidity)
Parasite models
Plasmodium yoelii (parasitemia)

[a]Condensed from Luster *et al.*, 1992.

methylphosphonates enables the oligonucleotides to gain access to the cell's interior (cf. Persaud and Jones, 1994).

Oligonucleotides may inhibit target gene expression by several mechanisms (cf. Gibson, 1994; Bennett *et al.*, 1998; Toulme, 2001). Oligonucleotides can induce degradation of the target DNA by the cellular enzyme RNase. Other mechanisms proposed to explain how protein synthesis might be inhibited include the steric blocking of ribosome assembly and splicing, and capping—all events involved in mRNA production. Also suggested is the maturation of mRNA or its translation into protein.

First-generation oligonucleotides have met with limited success due primarily to the rapid degradation of natural DNA by serum and cellular nucleases. Chemical modifications, including oligonucleotides that are resistant to nuclease degradation, have had some limited therapeutic effectiveness. The most commonly used modified oligonucleotides are the phosphorothioate oligonucleotides, in which one of the nonbridging oxygen atoms in the phosphate backbone is replaced with sulphur (cf. Bennett *et al.*, 1998).

Modified oligonucleotides have half-lives that range from 30 to 60 minutes following intravenous administration. Oral bioavailability is low due principally to nucleases present in the gastrointestinal (GI) tract. Biodistribution reveals relatively high concentrations in the liver and kidney.

The pharmacologic implications for antisense therapy may encompass virology, oncology and the cardiovascular system. Pulmonary, immuno- and neuropharmacology are also potential targets. Cancer-related targets such as Bcl-2, Raf-1, the protein kinase C (PKC) isoenzymes, and BCR-Abl have attracted considerable attention. Members of the Bcl-2 family are important regulators of apoptosis and represent attractive targets for the development of anticancer drugs. Targets involved in inflammation (e.g. ICAM-1) and viral diseases are also being developed (Juliano *et al.*, 2001). Most emphasis has been placed on the development of antiviral therapy, cancer therapy and anti-inflammatory therapy. Forevirsen (ISIS 2922) is an agent indicated for cytomegalovirus (CMV) retinitis; vitravene is an antisense agent used for the topical treatment of CMV retinitis in AIDS patients.

Antisense phosphorothioate nucleotides exhibit four distinct types of toxicity (Bennett *et al.*, 1998):

1. Sequence-specific toxicity due to exaggerated pharmacological actions
2. Sequence-specific toxicity due to serendipitous hybridization to nontarget RNA
3. Sequence-specific toxicity due to nonantisense effects
4. Sequence-independent toxicity; nonantisense effects.

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The major toxicological concerns associated with antisense drugs are related to their interaction with non-nucleic-acid targets. Expectedly, species differences play a role in the toxicity of the antisense drugs. In rodents, immune-cell activation appears to be a dose-limiting toxicity. In primates, acute effects on the complement system and anticoagulation effects have been observed. Transient fluctuations in blood pressure occurring during preclinical toxicology assessment may be due to the activation of the complement system.

Emerging technologies will allow the effective intracellular delivery of large molecules including antisense oligonucleotides. Despite early failures, antisense technology will likely play an important role in the development of new biotherapeutics.

2.4.3 Gene Products

Gene therapy has been defined as the transfer of exogenous genes to somatic cells of a patient in an effort to correct an inherited or acquired gene defect or to introduce a new function or characteristic. Gene-therapy products can be considered biotherapeutic agents. Human gene-therapy products include naked DNA, and viral and nonviral vectors containing nucleic acids (cf. Verdier and Descotes, 1999). Notwithstanding some disappointing preliminary clinical results, gene therapy has become an established concept in medicine. The future success of gene therapy depends upon further development of the basic sciences in the discovery of new disease-related genes, as well as identifying suitable animal models (Barzon *et al*., 2000). It has been estimated that nearly 400 gene-therapy clinical trials are ongoing.

By the insertion of plasmid DNA into target cells it may be possible to rectify genetic disorders, and to produce therapeutic agents in the form of peptides and proteins or antigens *in situ* to stimulate the immune system (cf. Davis, 1997). Advances in gene therapy may also allow for controlling pain, and could even be used to block the production of pronociceptive molecules (Yang and Wu, 2001). Other attractive targets for gene therapy include the epithelial surfaces of the lungs and GI tract, endothelial cells lining the blood vessels, muscles, myoblasts and skin fibroblasts. Three principle means of gene-delivery systems include:

1. Viral vectors
2. Nonviral vectors (e.g. particles, polymers)
3. Direct injection (e.g. ‘gene guns’).

Generally, nonviral-vector systems have been favoured, but they too possess certain disadvantages. In order for nonviral vectors to be effective it is essential that the negatively charged plasmid DNA be condensed into a nanoparticulate structure. This can be accomplished by the use of cationic lipids and cationic polymers, in which the interaction between the selected cationic materials and the anionic DNA results in a condensed/compacted structure. Such complexes provide increased stability of the genetic material as well as improved uptake into target cells (cf. Davis, 1997). Safety evaluation of gene therapy products entails a number of toxicological issues (cf. Harris and Dayan, 1998; Verdier and Descotes, 1999). While some of these issues are common to all biological products, there are others that are specific to gene-therapy products. Specific preclinical safety issues for gene-therapy products are depicted on **Table 12**. Of course, all test materials must be in compliance with current good manufacturing practices (cGMPs). Both *in vivo* and *in vitro* assays for adventitious viral contaminants must be undertaken. Human cells employed in the production of adenovirus vectors may be assayed for tumorigenicity in nude mice. Establishing the absence of replication-competent viruses is essential. Modification of the gene-expression construct may potentially alter the product’s biodistribution and/or gene expression, thus necessitating additional toxicologic testing.

Table 12 Pre-clinical safety issues for gene therapy[a]

Characteristics of gene-therapy product
Quality assurance of gene-therapy product
Safety evaluation of the vector
Toxicity of expressed protein (s)
Selection of animal species
Dose selection
Selection of route of administration

[a]Reproduced from Verdier and Descotes, 1999. © Oxford University Press.

No general test is available to predict the virulence or pathogenicity of recombinant viral vectors (cf. Smith *et al*., 1996). Some viral vectors may induce toxic shock and possess other specific cytotoxic properties.

In considering the toxicity of the expressed protein(s), the amount of protein equivalent to the expected total gene expression is compared to the dose of protein used in the safety evaluation (cf. Verdier and Descotes, 1999). The selection of the animal species is critical, and generally only nonhuman primates provide the most relevant model. Dose selection is often based on preliminary dose-ranging studies in animals, but should take into consideration the proposed dose used in human clinical trials. Similarly, the selection of the route of administration should be the same in both the preclinical and clinical studies.

2.4.4 Monoclonal Antibodies (Mab)

Monoclonal antibody technology has revealed that immunoglobulins (Igs) exhibit a wide range of biological activities—they are very specific. Antigen-combining sites on antibody molecules have great potential for developing bioactive peptides. Earlier, technical problems arose in Mab production because

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Table 13 Applications of monoclonal antibodies[a]

Disease	Humanized antibody	Antigen
Coronary artery dsease	C7E3Fab	Platelet glycoprotein 11b/111a receptor
Mycosis fungoides	Anti-CD4	CD4
Non-Hodgkin's B cell lymphoma	CAMPATH-IH	CAMPATH-I;Lymphoid/monocytes
Systemic vasculitis	CAMPATH-IH	CAMPATH-I
Refractory rheumatoid arthritis	CAMPATH-IH	CAMPATH-I
Generalized pustular psoriasis	Anti-CD4	CD4
Severe psoriasis	Anti-CD4	CD4
Cardiac transplant	Anti-Tac-H	CD4
Metastatic colorectal C/A	IgGI antibody 17-IA	Glycoprotein antigen
Rheumatoid arthritis	CD4 (CM-T412)	CD4
Septic shock	HA-IA	Lipid A region-Gram(-)

[a]Modified and condensed from Vaswani and Hamilton, 1998.

of host-anti-antibody immune responses. Subsequently, 'humanized' antibodies were developed by inserting the rodent hypervariable regions into human framework regions. In addition, Mab have been obtained by selecting antibodies from recombinatorial-phage-display libraries (cf. Dougall *et al*., 1994). Mab have been used for both diagnostic and therapeutic indications. There are a number of clinically approved Mab (see **Table 13**). Mab have been used diagnostically in colorectal cancer and ovarian cancer imaging. Antibodies (e.g. hMN-14) are under development for the treatment of ovarian, breast and medullary thyroid cancers. Still other antibody-targeted chemotherapeutic agents represent a new class of anticancer therapies (e.g. CMA-676-Mylotarg). This recombinant humanized antibody is linked with a cytotoxic antitumour antibiotic (e.g. calicheamicin), and may be indicated in the treatment of acute myelogenous leukaemia (AML).

2.5 Regulatory Considerations

Pharmaceutical companies and regulatory agencies responsible for public health must establish relevant and meaningful guidelines for the preclinical and clinical evaluation of new biopharmaceutical agents. There have been some difficulties in conceiving and applying guidelines for the safety evaluation of biotechnology-derived products (Claude, 1992; Cohen-Haguenauer, 1996; Cavagnaro, 1997). Safety is highly dependent upon the particular industrial process and its quality control. When possible, there should be efforts to compare the new biopharmaceutical to a natural biologic entity. Study designs often use a case-by-case approach (Cavagnaro, 1997). Regulatory guidelines must be mindful of the importance of selecting the appropriate animal model.

Good manufacturing practice (GMP) for biopharmaceuticals is an integral part of the safety and regulatory process (Jeffcoate, 1992; Federici, 1994). Other steps in the manufacturing process that convert the new biologies into biopharmaceuticals require due diligence throughout the entire process (**Table 14**).

Table 14 Aspects of regulatory procedures manufacturing procedures

Source materials:
Genetically engineered micro-organisms
Transformed mammalian cell lines
Hybridoma technology
Manufacture:
Clear production strategy and in-process controls
Validation of virus inactivation and removal
Purification of final product:
Fraction and chromatographic procedures
Affinity purification (e.g. Mab)
Pasteurization
Lyophilization
End-product quality:
Accurate and precise methods
Rigorous specifications

[a]Modified from Jeffcoate, 1992.

Biopharmaceuticals are generally classified as 'biologicals' by regulatory agencies. This classification would ordinarily include 'any virus, therapeutic serum, toxin, antitoxin or analogous product applicable to the prevention, treatment or cure of diseases or injuries to man'. The quality-control process of biopharmaceuticals engenders essentially the same as those applied to the analysis of conventional or low-molecular-weight pharmaceutical products or drugs.

3 AGRIBIOTECHNOLOGY

3.1 Genetically Modified Foods

Plants are one of many novel hosts that can be used, not only in the production of more nutritious foods, but also in the production of biopharmaceuticals. Since

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Table 15 Biotechnology offers many advantages for crop and food production

Insect resistance
Bacterial and fungal resistance
Viral resistance
Herbicide resistance
Stress tolerance
Extended shelf life
Nutrient modification

the early discoveries by Mendel, geneticists have been interested in the prospects of directed genetic change. Genetic modification of crop plants, in order to improve many of their qualities or traits, has led to a proliferation of recombinant products.

Modern-day biotechnology techniques are more precise and more rapid. Agribiotechnology has many potential benefits, including increased crop yields and livestock productivity, enhanced micronutrient composition and improved pest control through the development of herbicide-resistant crops. This technology may also improve food processing and even provide diagnostic tools for detecting plant pathogens. Still another important dimension of transgenic plants is the development and production of edible vaccines and other biotherapeutic agents. Genetic engineering of plants is not unlike the types of modifications that have been used by previous generations of plant breeders, but the techniques are more complex and diverse.

Several agronomic traits can be enhanced through agribiotechnology (Stark *et al*., 1993) (**Table 15**). Transgenically-introduced traits which have received considerable attention include herbicide tolerance (e.g. glyphosate), virus resistance and insect resistance. Other traits include resistance to fungal and bacterial infections, and tolerance to different stresses such as extremes of salt concentrations, heavy metals, temperature, nitrogen levels and phytohormones.

Some agribiotechnology-derived products have been introduced as having altered traits, including both food and vaccines (**Table 16**). Canola can be modified using an acyl carrier-protein thioesterase gene. A glufosinate-tolerant canola can be made using a phosphinothricin acetyltransferase gene obtained from *Streptomyces viridochromogenes*. A glyphosate-tolerant cotton can be made using the enolpyruvylshikimate-3-phosphate gene from *Agrobacterium* sp. strain CP4. Likewise, canola and soybeans can be rendered glyphosate tolerant. Insect-protected potatoes and corn can be made using the CryIIIA gene from *Bacillus thuringiensis*. Squash can be rendered virus resistant by using coat-protein genes from the watermelon mosaic viruses or the zucchini yellow mosaic virus. Viral resistant GM papayas and viral resistant sweet potatoes have flourished locally, but regulatory and cultural issues from other countries have precluded their usefulness and distribution. A modified fruit-ripening tomato has been achieved using several different gene-modification approaches. The insertion of several genes into a plant, often referred to as 'gene stacking' can lead to several new species with enhanced levels of various micronutrients. For example, rice can be nutritional enhanced, with both increased levels of iron and β-carotene (i.e. vitamin A). Genes from the daffodil (a yellow flower containing high levels of endogenous β-carotene) can be transfected into rice to, not only give rise to a 'golden' colour, but, more importantly, a crop plant with enhanced vitamin A. It should be noted that there are several areas involved in food biotechnology, and not just the alteration of plant traits and other forms of resistance. Actually, food biotechnology constitutes several areas:

- Processing aids (e.g. enzymes) and additives produced by fermentation with GM micro-organisms
- GM microbial starter cultures for the production of fermented foods (e.g. dairy products, cereals, vegetables, etc.)
- GM crops for fresh and processed food
- GM animals (e.g. husbandry of lean or less fatty animals).

All these areas of food technology are important advancements, but the public focus has been on GM-crop alterations that relate to human safety and perceptions of environmental risk.

Methods to establish food safety of GM foods (or components) are to determine whether or not it is 'substantially equivalent' to a conventional food product. Equivalency takes into account the biochemical and

Table 16 Selected biotechnology products[a]

Product	Altered trait	Source of gene(s)
Canola	High lauric acid	Turnip, oilseed rape, and so on.
Cotton	Herbicide resistance	Bacteria, virus
Potato	Resistance to beetles	Bacteria
Soybean	Herbicide resistance	Bacteria, virus
Squash	Viral resistance	Virus
Tomato	Delayed ripening	Tomato, bacteria, virus
Vaccinia virus	Vaccine	Rabies virus

[a]From Paoletti and Pimentel, 1996.

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chemical composition, the nutritional value comparisons and several other factors (e.g. antinutritional components). In order to demonstrate 'substantial equivalence', several criteria need to be considered:

- The composition and characteristics of the conventional food to which the GM food is being compared
- A knowledge of any new components (i.e. introduced genes) either expressed or otherwise present in the GM food.

Additionally, various food-processing techniques can affect a new GM food. During the entire process of ensuring similar composition and characteristics, it is necessary to establish its safety (e.g. allergenicity). Establishing the concept of 'substantial equivalency' between a conventional food and a GM food has provided the basis for regulatory approval in the US and other countries (Constable *et al*., 2007).

3.2 Altering Genetic Traits

3.2.1 Herbicide Tolerance

A herbicide is a chemical agent that kills or significantly interrupts plant growth and development. Every year, US growers use an estimated 971 million pounds of pesticides, mostly to kill insects, weeds and fungi (Brown, 2001). These chemicals interfere with metabolic processes that are essential for plant growth and vitality. Their phytotoxicity is due to their adverse actions on normal enzyme activity. Crops and weeds are naturally resistant to many herbicides. This resistance can be due to metabolic detoxification, prevention of the herbicide from reaching its site of action, or resistance at its site of action. Glyphosate-based weed-control products are among the most widely used broad-spectrum herbicides in the world (Giesy *et al*., 1996). The herbicidal properties of glyphosate were discovered over three decades ago. Glyphosate-based herbicides are used in agriculture, in industry, in ornamental gardening and in residential weed management. In agriculture, they are used in applications involving genetically modified plant varieties selected for their capacity to withstand glyphosate treatment.

Glyphosate (e.g. Roundup™) is a nonselective broad-spectrum herbicide that ordinarily cannot be used on crops without severe plant injury. Once glyphosate enters the plant, it inhibits the enzyme, 5-enolpyruvolylshikimate-3-phosphate synthase (EPSPS), thereby preventing the plant from producing aromatic amino acids essential for protein synthesis. EPSPS is the only physiological target of glyphosate.

Glyphosate-resistant (glyphosate-tolerant) crops employ a target-site modification wherein a glyphosate-resistant EPSPS is introduced into the plant by genetic engineering. Upon exposure to glyphosate, the plant is now unaffected, because the continued action of the glyphosate-resistant EPSPS enzyme provides for the plant's needs for amino-acid synthesis. An adjacent weed, for example, would not be protected from the glyphosate and hence would die because of the inhibition of amino-acid synthesis.

A number of herbicide-tolerant crops now available commercially (e.g. glufosinate/bialaphos, bromoxynil, etc.), but glyphosate was one of the first to be marketed. It has also undergone extensive safety testing and therefore is a representative prototype. The development of glyphosate-resistant (glyphosate-tolerant) crops has withstood two decades of safety testing and field trials.

Glyphosphate's mechanism of action is through its binding and blockade of EPSPS, an important enzyme necessary in the synthesis of aromatic amino acids both in crops and in weeds. Glyphosate's inhibition of EPSPS prevents a plant's ability to produce aromatic amino acids that are essential for protein synthesis. EPSPS can be found in all plants, in bacteria and in fungi. EPSPS is not found in animals, and therefore aromatic amino acids must be present in the diet.

Herbicide-tolerant crops are more environmentally friendly since they effectively lower the usage of conventional chemical herbicides. Glyphosate is readily degraded to metabolites that are nontoxic and nonresidual. Glyphosate in the environment tends to bind tightly to soil and to particulate matter. It is essentially not available to plants and other soil organisms (Giesy *et al*., 1996).

3.2.2 Bt (Bacillus Thuringiensis) Crops

The gene for the Bt toxin (*Bacillus thuringiensis*) was discovered over a 100 years ago. This soil bacterium has been used extensively in so-called organic farming. Since 1991, the Bt gene has been inserted into more than 50 plant crops (cf. Paoletti and Pimentel, 1996). There have been some concerns about the development of resistance to Bt toxin both in field and laboratory tests (Lambert and Peferoen, 1992; Stone *et al*., 1991; Tabashnik *et al*., 1992). Despite some of these early concerns, and now with the benefit of another decade of evaluation, Bt crops have been approved by US regulatory agencies and other agencies throughout the world. The use of Bt crops requires less chemical pesticide to be sprayed on the crops. Insect resistance can be provided by a gene from the soil bacterium Bt. This gene (*crylAB*) directs cells to synthesize a crystalline protein that is toxic to selected insects, particularly caterpillars and beetles that destroy food crops. The toxin gene in various strains of Bt can affect different insects, and hence a seed can be tailor-made to provide the best protection.

Bt-protected plants provide a safe and highly effective method of insect control (Betz *et al*., 2000). Bt-protected

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corn, cotton and potatoes were introduced into US agriculture nearly 10 years ago. Not only do they produce higher yields to the farmer, but they also reduce levels of fungal toxins (e.g. fumonisin in corn). Non-Bt corn and other species do not take up the toxin released into the soil in root exudates of Bt-corn. The persistence of the toxin in soil for up to 180 days after its release indicates that the pesticide remains bound to surface-active particles which protect the toxin from biodegradation.

Safety-evaluation tests, including acute, subacute and chronic exposures, have been undertaken with Bt-protected plants. All have been fully approved by US regulatory agencies, including the FDA, EPA and the USDA. These Bt-protected crops have been determined to be 'substantially equivalent' in both food and animal feeds derived from non-GM or from conventional foods.

3.3 Safety Assessment

Safety issues concerning GM plants have involved both foods for human consumption and animal feeds. Toxicological studies have been aimed at ensuring their safety and to establish compositional equivalency. 'Substantial equivalence' establishes important safety criteria for GM foods and animal feeds (Delaney, 2007).

Safety concerns also involve wildlife species and possible adverse effects upon the environment and ecosystems. Concerns for nontarget species (e.g. monarch butterflies) have been raised by environmentalists. Will innocent nontarget species be affected by insecticides that are incorporated into GM crops? The risk appears to be very small, yet continued surveillance and additional research is necessary. Thus far, no 'superweeds' arising from genes inserted into crops have migrated or jumped to nonGM plants.

3.3.1 Allergenicity and Digestible Proteins

In order to assess allergy-inducing potential, it is necessary to examine the chemical composition of each novel protein produced by the GM plant against a known allergen. It is estimated that there are 500 or more known allergens. Several food-allergen sequences can be retrieved from public-domain databases for proteins and nucleic acids (cf. Metcalfe *et al*., 1996).

There are thousands of different proteins in the body. Only a few proteins are capable of causing food allergies. Because genetically engineered crop plants introduce new proteins, there is always the possibility that the protein encoded into this new genetic material might produce an allergic response. Many foods, not genetically modified, including milk, eggs, tree nuts, shellfish and certain legumes (e.g. peanuts, soybeans) are commonly allergenic. Food allergies/intolerances differ significantly across multiple countries (Woods *et al*., 2001). A food crop (e.g. legume) that is genetically modified is apt to be just as allergenic in a sensitive person as in the sensitive person consuming the same legume from a non-GM version of the same crop.

There is no evidence that recombinant proteins in newly developed foods are more allergenic than naturally occurring proteins. In fact, evidence suggests that the vast majority of these proteins are safe to consume (Lehrer *et al*., 1996). The stability (or lack of) of food allergens to gastric digestion can determine their potential immunogenicity (Astwood *et al*., 1996). Moreover, often overlooked are the temperature extremes that might be used in the processing of the final food product and the effect it may have on allergenicity. A decision tree for evaluating recombinant food proteins for immunogenicity can be very useful (Lehrer *et al*., 1996; see also **Table 11**). Recombinant proteins from known allergens can be readily tested using a tier of *in vitro* immunochemical assays. Many biochemical and immunotoxicity tests can be used to evaluate allergenicity. Unfortunately, recombinant proteins from unknown allergenic sources are not easily recognized nor necessarily anticipated. Hence, it may be difficult to assay recombinant proteins from sources with undetermined allergenic activity. Importantly, there is no evidence that recombinant proteins are more allergenic than naturally occurring proteins. In fact, the vast majority of proteins present in new food products are safe for consumption. Nevertheless, it is important that new food products, regardless of source, be prudently developed and tested for any potential immunogenicity.

Food allergens that cross the mucosal membranes of the GI tract are generally a prerequisite for allergenicity. High temperatures associated with food processing and the acidity of the stomach do not necessarily result in their being digested. A protein that is stable under such environmental conditions (i.e. proteolysis) has an increased probability of reaching the intestinal mucosa intact. Many allergens exhibit stability despite such conditions (cf. Metcalfe *et al*., 1996). It is possible to compare the relative stability of proteins engineered into plants with a number of commonly known allergenic food proteins. There are several major classes of food allergens, including egg-white allergens, milk allergens, soybean allergens, peanut allergens and mustard allergens. The toxicologic assessment of many of these allergens can be carried out using simulated gastric and digestive models of mammalian digestion, as described in the US Pharmacopeia. It is possible to compare the relative stability of proteins engineered into plants (e.g. Bt insecticidal protein, CP4 EPSPS synthase, etc.) with a number of commonly known allergenic food proteins. The *in vitro* digestibility of Bt Cry proteins in simulated gastric fluid ranges from 30 to 60 seconds (Betz *et al*., 2000). These *in vitro* models can be used to evaluate the digestibility of both plant and animal proteins, as

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well as certain food additives. Not surprisingly, many of the known food allergens are stable to digestion in these simulated GI digestive models. The allergen (or a protein fragment) may be stable for at least two minutes; major allergens are typically stable for as long as one hour. Common food proteins with no history of allergenicity undergo rapid degradation in these simulated gastric and intestinal models. Thus, it is possible to add this *in vitro* battery to safety testing for evaluating new GM foods.

3.3.2 Feeding Studies

Feeding studies are an integral part of the overall safety assessment of GM foods and/or animal feeds. Animal feeding studies, whether using laboratory animals or domestic animals, provide insight into the nutritional status of the new GM product. It also allows for the monitoring of growth rates of the test animals. Daily dietary regimens using several different intake levels and durations (e.g. 4–10 weeks depending upon the species) aid in establishing normal growth patterns and nutritional balance studies. From such studies, it may be possible to set safety margins. Often, several different species can be used to determine nutritional parameters, and might include laboratory rats, as well as domestic animals commonly found in the human food chain. Feeding studies involving invertebrates (e.g. catfish) may also be undertaken, not only for assessing another food-chain component, but also for any potential ecological effect(s).

The chicken is an excellent animal feeding model since it can be easily studied through its lifespan. It also undergoes a severalfold increase in body weight in just over six weeks, and provides additional information about the nutrient value of the new GM food/feed. To accommodate such a rapid body-weight increase during this time period, the chicken's protein requirements are significant, and hence it is a very sensitive model for assessing nutritional equivalency.

3.3.3 Nontarget Species

In the overall safety assessment of GM foods and the safety of animal feeds that are derived from GM plants, is necessity to evaluate the effects of such crops on nontarget species (e.g. monarch butterflies, swallowtail butterfly larvae, honeybees, etc.). Field studies that include any potential harmful effect on nontarget species should be conducted as a routine evaluation of a GM food/feed. If studies are conducted in a laboratory setting, they must closely approximate those conditions found in the natural setting. The environmental risks that GM crops might pose to nontarget species is not completely known, but experiments to date have not revealed any adverse effects. Further, the cultivation of GM food crops (e.g. Bt-protected plants) may result in fewer adverse impacts on nontarget organisms than would be incurred from the use of chemical pesticides. Glyphosate formulations do not affect honeybees nor do they affect nontarget terrestrial plants (Giesy *et al*., 1996). Glyphosate does not bioaccumulate in fish or other animals. There must be continued vigilance to determine any possible ecological consequences arising that might harm nontarget species of wildlife. The weight of evidence indicates no unreasonable adverse effects of Bt Cry proteins expressed into plants on nontarget wildlife or beneficial invertebrates (e.g. earthworms, soil microbials or flora).

3.4 Nutritional Improvement

The nutritional health and wellbeing of humans is dependent on plant foods that are either directly or indirectly consumed by certain domestic animals. Crop foods provide most essential vitamins and minerals along with a host of other nutritionally related phytochemicals. Oftentimes, micronutrients are found only in low concentrations in staple food crops. Essential micronutrients in the human diet consist of about 17 minerals and 13 vitamins. Unfortunately, there are many developing or underdeveloped countries in the world that are unable to provide proper or adequate nutrition. GM foods, particularly those that increase yield and productivity, may allay malnutrition. It has been estimated that about 800 million people, mostly children, have some degree of malnourishment.

Modern agriculture, particularly in the last few decades, has witnessed advances in crop breeding leading to increases in productivity and yields. While efforts have been focussed upon attaining caloric needs, genetically engineered crops now afford the opportunity to enhance the micronutrient composition of crop foods (cf. Comaci, 1993). Through nutritional genomics, it is now possible to increase plant micronutrients leading to an improvement in human health (DellaPenna, 1999). By inserting foreign genes into various crop foods, it is possible to achieve nutritional enhancement of certain micronutrients. Initial efforts to attain GM foods with traits that endow them with herbicidal resistance, ordinarily involve the insertion of one or two foreign genes. Multiple gene insertions, sometimes referred to as 'gene stacking', may involve over a half dozen gene insertions and utilize more than one of the plant's metabolic pathways.

Nutritional genomics using multiple gene insertions is exemplified by rice (e.g. 'golden rice'). Millions of people depend upon rice as a staple food. Through nutritional genomics it is possible to enhance rice with increased levels of micronutrients, namely, β-carotene (vitamin A) and iron. 'Golden mustard oil', much like 'golden rice' can be made with high levels of β-carotene, a precursor to vitamin A. Four encoded enzymes that provide rice with the ability to synthesize β-carotene, and three other encoded enzymes, allow the kernals to accumulate additional iron. Both vitamin A deficiency

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Table 17 Production of biopharmaceuticals in transgenic plants[a]

Potential application/indication	Plant	Protein
Anticoagulants:		
Protein C pathway	Tobacco	Human protein C (serum)
Indirect thrombin inhibitors	Tobacco, oilseed Ethiopian mustard	Human hirudin variant 2
Recombinant hormones/proteins:		
Neutropenia	Tobacco	Human granulocyte-CSF
Anaemia	Tobacco	Human erythropoietin
Antihyperanalgesic (opiate)	Thale cress, oilseed	Human enkephalins
Wound repair	Tobacco	Human epidermal growth factor (EGF)
Hepatitis C and B	Rice, turnip	Human interferon-α
Liver cirrhosis	Potato, tobacco	Human serum albumin
Blood substitute	Tobacco	Human haemoglobin
Collagen	Tobacco	Human collagen
Protein/peptide inhibitors:		
Cystic fibrosis	Tobacco	Human α-l-antitrypsin
Trypsin inhibitor	Maize	Human aprotinin
Hypertension	Tobacco, tomato	Angiotensin-converting enzyme (ACE) Inhibitors
HIV	*Nicotiana bethamiana*	α-trichosanthin (TMV-UI)
Recombinant enzymes:		
Gaucher's disease	Tobacco	Glucocerebrosidases

[a]Modified and condensed from Giddings *et al*., 1996, see also Goldstein and Thomas, 2004.

and iron deficiency, particularly in children, can potentially benefit from this novel form of GM rice. Efforts are currently underway to even further increase the levels of these micronutrients in this GM rice, as well as in other food crops. By enhancing the levels of carotenoids and the glucosinolates in GM foods, it might be possible to reduce the risk of certain forms of cancer. Likewise, the enhancement of phyto-oestrogens, as well as selected phytophenolic compounds might reduce the risk of other diseases, including cardiovascular diseases, osteoporosis and certain hormone-related cancers. Aqua farming using GM salmon may prove to be an important alternate source of protein. Commercial 'fish farms' growing GM salmon transfected with a growth hormone from other fish species', that have a four times greater growth than the salmon have been produced and will soon be reviewed for regulatory approval. By inserting the growth hormone gene into the fish's egg, the gene will be present in every cell in the fish's body. Fish with this integrated gene can then be used to create a breeding stock of a newer and faster-growing variety.

3.5 Agribiotechnology and Medicines

Biotherapeutics, which involves the genetic manipulation of mammalian cells, is more focussed upon the discovery of new medicines and other therapeutic products for the treatment of various diseases. Early successes in developing alternate sources of often scarce or animal-origin hormones through recombinant technologies led to recombinant human insulin and human growth hormone. These were indeed scientific breakthroughs enjoyed by the then new biotechnologies. Progress in developing new medicinal therapies through agribiotechnology has not been as rapid nor successful, yet there are many potential indications (Giddings 1996) (**Table 17**). Many of the expression systems used in the production of biopharmaceuticals have employed agrobacterium mediated transformations (AMTs). Finally, the production of nutraceuticals using potato or rice plants may be useful in the eventual treatment of provitamin A deficiencies and amino-acid deficiencies (Giddings *et al*., 1996). Improved crop traits have produced healthier foods such as 'trans'-free margarines, and improved protein and vitamin levels. Agribiotechnology can use GM crop foods as a vector for the delivery of edible vaccines (Richter, 1997; Richter and Kipp, 1999) (**Table 18**). When plants genetically engineered to produce bacterial or viral proteins are consumed as food, they can trigger an immune response (Arntzen, 1998). Oral immunizations using transgenic crops could provide a low-cost approach to protecting against several different diseases. Cholera vaccines, using the potato as a vehicle, could provide

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Table 18 Production of vaccines from plants[a]

Potential application/indication	Plant	Protein
Hepatitis B	Tobacco	rHBsAg (recombinant hepatitis B surface antigen)
	Tobacco	Marine hepatitis epitope
Dental caries	Tobacco	*Streptococcus mutans*
Autoimmune diabetes	Potato	*Vibrio cholerae* toxin B
	Potato	Glutamic acid decarboxylase
Cholera and E. coli Diarrhoea	Tobacco, potato	*E. coli* enterotoxin LT-B
Oral vaccine (cholera)	Potato	*V. cholerae* toxin CtoxA/Ctox-B
Mucosal vaccines	Cow-pea	D2 peptide (fibronectin-B) *Staph. aureus*
Diarrhoea (Norwalk virus)	Tobacco, potato	Coat protein (Norwalk virus)
Rabies	Tobacco, spinach	Rabies virus glycoprotein
HIV	Tobacco, blackeyed pea	HIV epitope (gp 120)
	Cow-pea	HIV epitope (gp 41)
Rhinovirus	Blackeyed pea	Human rhinovirus epitope (HR14)
Foot and mouth	Blackeyed pea	Foot and mouth virus epitope (VPl)
Malaria	Tobacco	Malarial B-cell epitope
Influenza	Tobacco	Haemagglutinin

[a]Modified and condensed from Giddings *et al.*, 1996.

immunity to this disease. Likewise, a GM potato could be used as a vehicle for developing vaccines against enterogenic *E. coli* (ETEC) infections. Transgenic bananas, since they can be eaten raw and where cooking might destroy a vaccine, might provide the necessary vehicle for a hepatitis B vaccine. In an effort to control dosage, the transgenic banana could be homogenized into a measured baby food to ensure the effectiveness of the vaccine. Thus, pharmaceutical foodstuffs through oral immunization using a transgenic plant may provide the basis for new immunomodulating drugs and vaccines.

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Laboratory Recognition of Potential Xenobiotic Respiratory Sensitizers

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CONTENTS

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1 INTRODUCTION

The immune system functions to establish and maintain homeostasis by distinguishing endogenous ('self') from exogenous ('nonself') components, thus protecting the body from infectious agents (bacteria, viruses, fungi, parasites) and certain tumours. Perturbation of this homeostasis by interaction with some xenobiotics can result in adverse events. These include immune responses to otherwise innocuous nonself agents (generally proteins) leading to hypersensitivity development. Although most proteins are capable of eliciting immune responses, not all proteins are allergens (Viana *et al.*, 2002). Hypersensitivity reactions can significantly impact patient quality of life (discomfort, lack of activity participation, inability to concentrate at work or school) and produce economic difficulties (medical costs, lost productivity).

Most hypersensitivity responses develop in genetically predisposed individuals. Generally, no symptoms are experienced during the sensitization phase (induction). Instead, disease symptoms appear upon subsequent re-exposure/challenge (elicitation) in previously sensitized individuals.

Hypersensitivity reactions are divided into four types, as originally proposed by Gell and Coombs (**Table 1**). Although these divisions are based upon differences in the underlying immune mechanisms, the four groups are

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Table 1 Gell and Coombs classification of hypersensitivity reactions

Hypersensitivity	Mechanism	Examples
Type I Immediate type hypersensitivity	Th2-mediated induction of cytophilic antibody (IgE)	Allergic rhinitis, asthma, systemic anaphylaxis
	Cross-linking of mast cell receptor bound IgE results in mediator release (e.g. histamine)	—
Type II	IgM, IgG antibodies (Ab) react with cell- or matrix-associated antigen (Ag) results in cell cytolysis	Some drug allergies, for example, penicillin
	IgG reacts with cell-surface receptors alters signalling	Chronic urticaria (Ab to FcεR1α)
Type III	IgM, IgG complexed with Ag (immune complexes) activates complement and phagocytes leading to inflammation	Serum sickness, Arthus reaction
Type IV delayed-type hypersensitivity (DTH)	Th1 cells react with soluble Ag resulting in macrophage activation	Contact dermatitis, tuberculin Rx
	Th2 cells react with soluble Ag resulting in eosinophil activation	Chronic asthma, chronic allergic rhinitis
	Cytotoxic T cells react with cell-associated Ag resulting cytotoxicity	Contact dermatitis

not mutually exclusive and hypersensitivity responses are often mixed. In some cases the pathology appears to be similar, suggesting some overlap. Mechanistically, hypersensitivity Types I–III are antibody-mediated and Type IV is cell-mediated.

The incidence of Type I hypersensitivities (atopy or immediate-type hypersensitivity), has increased dramatically over the last several decades in the USA and other industrialized nations, particularly for allergic asthma. This trend has been noted in both the general population and in occupational settings (Petsonk, 2002). It is assumed that changes in environment, lifestyle and/or medical practices have contributed to the increase, as the change is more dramatic than would be expected for a simple population genetic shift (Mannino *et al*., 1998; Pew, 2000).

During the induction of Type I hypersensitivity, cytokines produced by T helper 2 (Th2) subset CD4+ T cells induce B cells to undergo immunoglobulin isotype switching and produce antigen-specific cytophilic IgE or IgG1 antibodies. Subsequent re-exposure induces crosslinking of antigen-specific mast cell/basophile-bound antibodies. This crosslinking induces the immediate release of preformed mediators (including histamine and prostaglandins), which produce bronchoconstriction, increased vascular permeability and inflammation. The resulting allergic reactions may have a variety of clinical manifestations, ranging from skin rashes and rhinitis to life-threatening asthmatic and anaphylactic reactions. In the case of asthma, a late-phase response may also be seen between 2 and 12 hours after challenge. This prolonged response is driven by leukotrienes, chemokines and cytokines synthesized by activated mast cells and CD4+ Th2 lymphocyte cells. It is characterized by mucus hypersecretion, bronchoconstriction, airway hyper-responsiveness (AHR) to nonspecific stimuli (e.g. histamine, methacholine (Mch)) and eosinophilic airway inflammation. The early and late phases of allergic asthma are predominantly a Type I hypersensitivity. However, some late phase and chronic components are Type IV.

Type IV or delayed-type hypersensitivity (DTH) reactions are mediated by CD4+ Th1 and cytolytic CD8+ T cells, not antibodies. Activated T cells may induce cell damage and death directly (CD8+ cells) or indirectly, by recruiting and activating macrophages and other T cells (Th1 cells). An example of a Type IV response is the classic tuberculin reaction, in which antigen-specific Th1 cells induce local accumulation of inflammatory cells and vascular dilation and leakage. An example of Type IV pulmonary hypersensitivity is hypersensitivity pneumonitis (HP; extrinsic allergic alveolitis). This inflammatory lung disease, a mix of Type IV and Type III (circulating antigen–antibody complexes) responses, results from the inhalation of organic dusts, including bioaerosols (containing fungi/bacteria), food ingredients (baker's lung) and certain metals (e.g. beryllium). Although HP is usually induced by occupational exposure, residential mould exposures have recently also been associated with the syndrome. HP is characterized by a predominantly mononuclear inflammation, as well as granuloma formation. In addition to hypersensitivity, development of chronic HP may also require immunomodulatory factors such as viral infections and/or microbial components (endotoxin, fungal glucans).

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Human studies of hypersensitivity are frequently impossible for ethical or logistical reasons. Therefore, alternate methods are required to help elucidate mechanisms of pathology. This chapter focuses on the laboratory methods used in hazard identification and the assessment of the health risks associated with xenobiotic exposures that might induce respiratory hypersensitivities.

2 ANIMAL MODELS

Animal models have been developed to assess the risk of hypersensitivity induction and/or elicitation associated with inhalation exposure. These models have been used to identify causal agents (generally proteins) and to assess the relative allergenic potency of various proteins/extracts such as dust mites, moulds and cockroaches in residential environments and detergent enzymes, biotechnology products and latex in occupational settings. These models have also been used to assess the adjuvant activity of common air pollutants (ozone, diesel, NO_2) in respiratory hypersensitivity (Selgrade *et al*., 2000), and to elucidate the mechanism(s) involved in disease pathology.

A good animal model should mimic all or most of the responses characteristic of the human condition under study. Samples and assessments can be tailored to evaluate end points relevant to the hypersensitivity disorder under investigation. For Type I hypersensitivities, these end points include the identification of Th2 cytokines and cytophilic antibodies (IgE and IgG1) in serum and bronchoalveolar lavage fluid (BALF), BALF inflammatory cells (particularly eosinophils), and the induction of antigen-specific lymphocyte proliferation. Whole-body plethysmography can be used to assess both early- and late-phase airway responses.

Unfortunately, animal models are less successful in mimicking the inflammatory changes characteristic of chronic asthma (such as airway remodelling) or HP. Early HP models used rabbits and required extensive exposure protocols. More recent HP mouse models utilize both aerosolized antigen administration and adjuvant co-stimulation to produce lung pathology. End points of interest include demonstration of the presence of Th1 cytokines (interferon γ (IFNγ), interleukin-12 (IL-12)), and BALF inflammatory cells (neutrophils, lymphocytes).

Animals used in hypersensitivity models are primarily mice, rats or guinea pigs, although rabbits, monkeys and dogs are occasionally used. The predominance of murine models largely stems from the availability of inbred and transgenic mouse strains, providing opportunities to address research questions regarding disease susceptibility in sensitive subpopulations. The popularity of these models is further enhanced by the wide spectrum of commercially available murine-specific immunological reagents. Mice and humans share similar mechanisms of sensitization and recognize many of the same allergenic epitopes. Mouse strains relevant to the study of respiratory hypersensitivity include the Th2-biased high IgE responders BALB/c, DBA/2 and A/J mice, and the Th1-biased C57BL/6, B10.D2 and SJL/J mice.

In addition to choosing a model that displays relevant disease characteristics, the experimental design must also allow sufficient time for the adaptive response involved in respiratory sensitization to develop. Furthermore, the experimental design must include controls that distinguish adaptive immune responses from those induced by the antigen delivery vehicle, adjuvants or nonspecific inflammatory or irritant responses. Methods for exposures, sample collection and subsequent analysis are provided below.

2.1 Exposure Methods

Sensitization can be initiated by systemic or local exposures, or by cell transfer. Systemic exposure can be accomplished by intraperitoneal (IP) injection, generally in the presence of an aluminium-based adjuvant, by subcutaneous injection or by dermal exposure. Following systemic sensitization, one or more respiratory challenges are required for airway responses to become evident (Saloga *et al*., 1994).

Respiratory sensitization and challenges can be accomplished using local exposure techniques (inhalation, intranasal, intratracheal (IT) and IT aspiration). Inhalation exposures using whole-body or nose-only systems expose the entire respiratory tract to the aerosolized test agent. However, determination of total inhaled dose in whole-body exposures can be complicated by concurrent dermal, ingestion (grooming), and mucosal exposures, as well as by individual variability in respiration rates. Nose-only exposures limit exposure by nonrespiratory routes, but are more stressful to the animals. Animal stress can be reduced by chamber acclimatization prior to study initiation.

Intranasal exposures may be performed with or without short-term anaesthesia (e.g. isoflurane). The animal is restrained in a supine position with nose pointed upward. The agent is placed at the nostril opening and inhaled by the animal. This method exposes the upper airways, while the amount of agent reaching the lower airways is directly proportional to the volume instilled. However, there is the potential for ingestion of the agent.

IT exposure can be performed under general anaesthesia using a tracheal cannula. This method ensures precise dose delivery to the lower respiratory tract, but requires considerable skill to perform and may produce trauma to the animal.

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Another method of lower airway exposure is aspiration, sometimes called intratracheal aspiration (IA) or oropharyngeal aspiration. Aspiration requires a short period of anaesthesia (~one minute isoflurane). The anaesthetized animal is vertically suspended by the incisors. The oesophagus is occluded and swallowing prevented by gently pulling the tongue out of the mouth in an upward direction. The agent is then placed in the oropharynx while the nose is briefly occluded, inducing aspiration of the agent.

3 SAMPLE COLLECTION

Samples may be collected at various times during the sensitization and challenge process to evaluate end points characteristic of allergic asthma or other respiratory hypersensitivities. Samples include blood, BALF and tissues such as lungs, lymph nodes and spleen. Most of these samples can only be acquired during terminal procedures, although some blood sampling and respiratory physiology assessments can be made in intact animals.

3.1 Blood Collection

To determine if the exposure regime is producing an IgE or IgG1 response (or other end point), it may be useful to periodically collect small blood samples (100–200 µl). Blood collection is survivable if the volume collected is <10% of blood volume (~8% body weight). Some techniques permit repeated sampling of individual subjects. One of these methods is blood collection via the lateral tail vein either by using a needle and syringe (easier in rats than mice) or by nicking the vein.

Small blood samples may be obtained from the retro-orbital plexus/sinus in both mice and rats, although this is not the method of choice for rats. This method requires the use of an ophthalmic anaesthetic or general anaesthesia and a skilled technician to minimize pain and distress. Briefly, the tip of a glass capillary tube is placed at the medial canthus and rotated until the sinus is penetrated and blood is collected. Care should be taken to ensure haemostasis following the procedure. Each orbit should not be sampled more than once every two to three weeks.

Blood may be obtained from the saphenous vein in both rats and mice. Mice are restrained by placing them head-first into a 50 ml conical centrifuge tube (perforated to permit air flow). The mouse is gently held in the tube with the rear legs extended. The fur on the side(s) of one rear leg can then be shaved. Coating the skin surface with silicone grease prior to puncturing the saphenous vein with a needle (e.g. 23 g) optimizes blood flow by minimizing blood loss onto the surrounding skin. Following blood sample collection (~100 µl total), gentle pressure is applied to the area to stop bleeding. This procedure requires more skill and time than either tail bleeding or retro-orbital sampling, but repeated samples can be obtained by removing the scab at the venipuncture site.

A minimally stressful blood sampling method is submandibular venous puncture. The mice are restrained by grasping the dorsal neck skin (the 'scruff') and cradling the mouse's body in the palm of the hand. The submandibular venous sinus can then be punctured using a 5 mm lancet. A maximum of eight drops of blood (~25 µl each; total 200 µl) can be collected at each sampling. Following the sampling, gentle pressure should be applied to ensure that bleeding has stopped.

Collection methods for larger blood volumes are nonsurvival procedures, and require lethal or surgical planes of anaesthesia. Blood collection by intracardiac bleeding is appropriate for both mice and rats (~1 ml from an adult mouse; 4–8 ml from an adult rat) while abdominal aorta puncture can be performed in rats. Abdominal aorta puncture is performed by opening the abdominal cavity, exposing the descending aorta (located on the animal's left side), separating the mesentery covering the vessel and withdrawing blood using a Vacutainer or syringe.

Blood samples are usually processed to provide plasma or serum and possibly a blood smear (see below). Blood collected in the presence of an anticoagulant (e.g. heparin, Alsever's solution) may be centrifuged to separate blood cells from the fluid portion (plasma). Allowing blood to clot also removes cells (and certain other factors) from the fluid portion (now called serum).

3.2 Bronchoalveolar Lavage (BAL)

Bronchoalveolar lavage (BAL) allows assessment of pulmonary inflammation. BAL fluid (BALF) contains cells, cytokines and antibodies that can be assayed to determine an animal's allergic status. It is accomplished in lethally anaesthetized animals or following sacrifice by cannulating the trachea and washing the airway one or more times with a saline solution such as Hank's balanced salt solution (HBSS) or phosphate-buffered saline. Lavage volumes may be determined based on animal body weight or a fixed volume may be used (0.75–1 ml for mice). This volume may be adjusted if some lung tissue will be removed before lavage.

3.3 Organ Tissue

Organs such as lung, lymph nodes, spleen and liver can be collected at necropsy. Differences in tissue

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wet weights between treated and control animals may suggest responses such as oedema, cellular influx or cell proliferation. Harvested organs may be frozen or fixed in formaldehyde for histopathologic analysis. Tissues may also be snap-frozen or placed in RNase-inhibitor for RNA isolation and gene expression analysis. Alternately, single cell suspensions may be prepared from the tissue and cultured to provide insight into the disease status or mechanism (see Section 5 on Cell Culture).

3.4 Airway Reactivity Assessment

An important feature of allergic asthma is airway reactivity, which includes both antigen-specific immediate airway responses and antigen nonspecific AHR after experimental exposure to cholinergic agents (e.g. Mch) or histamine. Commonly used methods for AHR assessment include pulmonary function monitoring and whole-body plethysmography.

Pulmonary function monitoring allows measurement of airway responses in anaesthetized animals, but is a terminal procedure. Systems may be built in-house or commercially available monitoring systems may be used. Generally, heart rate and waveform are monitored throughout the experiment. Flow and pressure measurements for each breath are averaged to calculate airway compliance and resistance. A baseline is recorded prior to challenge with increasing doses of Mch, each delivered over one second via a jugular cannula and electronic syringe pump. A brief recovery period is allowed between doses. Airway opening pressure is monitored directly, while total respiratory system resistance and compliance are calculated using software such as that described by Gavett *et al*. (1999). Data may be expressed as the effective dose of Mch causing a percentage increase in airway pressure and airway resistance over baseline values, or a percentage decrease in compliance.

Whole-body plethysmography is a nonterminal method of assessing immediate responses to agent challenge or AHR. Airway responses are measured in unrestrained mice or rats with or without anaesthesia. The results are expressed as enhanced pause (PenH), a unitless parameter derived from whole body flow measurements (respiratory rate, tidal volume, inspiratory and expiratory times, peak inspiratory and expiratory flows and relaxation time). Although PenH has been correlated with airway resistance (Hamelmann *et al*., 1997), some authors (Adler *et al*., 2004; Bates *et al*., 2004) have criticized its use because it is partially based on measurements not related to airway resistance. However, this parameter does reflect changes in airflow, as illustrated in **Figure 1**.

Our laboratory has used plethysmography to follow the progression of immediate airway responses to allergen sensitization/challenge. Baseline measurements were recorded for each animal. Subsequently, mice were administered fungal extracts or HBSS control by aspiration and placed back in the plethysmograph chambers. PenH measurements were collected and averaged over a one hour postinstillation period (Viana *et al*., 2002; Chung *et al*., 2005). A similar approach has been used to assess immediate responses to house dust mite (HDM) in rats (Lambert *et al*., 2001).

An *in vitro* method for assessing airway responses is to measure tracheal tissue smooth-muscle contractions

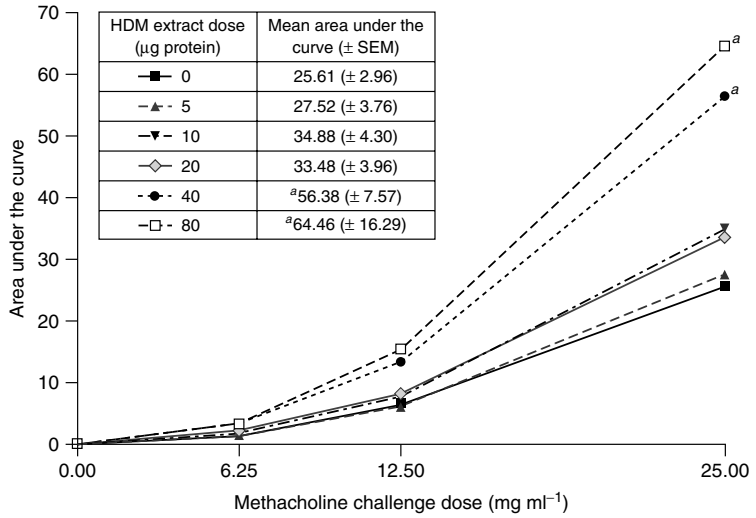

Figure 1 Airway hyper-responsiveness. BALB/c mice were exposed four times over four weeks to house dust mite (HDM) extract at doses ranging from 0 to 80 µg protein. Three days after the final HDM exposure the mice were placed in a whole-body plethysmograph and challenged with doubling doses of Mch (6.25–25.00 mg ml^{-1}). Mice that had received multiple 40 or 80 µg HDM exposures had significantly higher responses to Mch challenge at $p < 0.05$, indicated by *a*. The responses are presented as area under the curve. $n = 5–10$.

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in response to cholinergic stimuli (Mch, carbachol) (Garssen *et al*., 1990). At selected time points following allergen challenge, the trachea is resected, cleaned of extraneous tissue and sectioned. The tracheal sections are fixed to a strain gauge in an organ bath and then suspended in a temperature-controlled chamber filled with Krebs–Ringer solution. After equilibration, cholinergic-induced contractility is recorded with a force (isometric) transducer. Typically, cholinergic stimulant dose–response curves are generated from both allergen-challenged and naive/control animal tracheas for comparison.

4 SAMPLE ANALYSIS/ASSESSMENT

4.1 Blood-Smear Analysis

Blood smears can be used to evaluate circulating blood-cell populations. A small drop of fresh blood is placed near the end of a slide. After bringing the edge of a second slide (at 30–40°) into contact with the blood, the drop is allowed to bank evenly along the slide edge and push the 'spreader' in a smooth, quick motion. The blood cells will be distributed in a thickness gradient and somewhere along that gradient should be a satisfactory distribution of cells. If the blood drop is too large, the red blood cells may obscure the leukocytes. The smear is air-dried and stained, then examined under a microscope for cell evaluation.

4.2 BALF Analysis

BALF can provide information regarding the quantity and quality of the pulmonary cellular influx following allergen sensitization and challenge. Following centrifugation, the supernatant may be submitted for biochemical (e.g. total protein (oedema), lactate dehydrogenase activity (cell damage)) and immunologic (e.g. antibodies, cytokines) analysis. The cell pellet is resuspended in an isotonic solution. The total cell concentration of the suspension can be determined by microscopic examination using a haemocytometer or by counting cell nuclei spectrophotometrically using instruments such as a Coulter Counter (Coulter Corp., Miami, FL). Cell viability can be determined with trypan blue exclusion staining using the microscopic, but not the spectrophotometric, method.

For differential cell counts, another portion of the cell suspension, usually 100–150 µl, is adhered to glass slides by centrifugation. Wright–Giemsa staining allows the cell populations to be identified by differential staining and morphological discrimination. **Figure 2A** shows an example of BALF total and differential cell counts from a HDM dose–response study conducted in our laboratory.

4.3 Lung Histopathology

Although BALF assessments provide some insight into lung inflammation, fixed lung samples provide direct evidence of lung pathology. For our laboratory, lungs are sectioned along the mainstem bronchus and embedded in paraffin. Sections are stained with haematoxylin–eosin solution prior to histopathologic examination. The severity of inflammatory, degenerative and proliferative changes can be graded on a numeric scale. Pathology scores may be expressed as *incidence* (the number of mice in a group exhibiting a particular pathology) and *severity* (the average of the pathology scores for a particular lesion per treatment group).

Fixed lung sections can also be stained immunohistochemically to identify the presence, source and location of molecules of interest. A study in our laboratory demonstrated that certain neurotrophins (NGF (nerve growth factor), NT-3, NT-4) are elevated in the BALF of mould extract-exposed mice, as is seen in human asthmatics. Subsequent immunohistochemical analysis identified mononuclear and epithelial cells as the source of NGF (Chung *et al*., 2007).

4.4 Immunologic Assays

The serum, plasma or BALF from experimental animals can be assayed for proteins of interest in allergy models. These proteins include cytophilic antibodies, such as IgE (mice and rats) and IgG1 (guinea pigs and mice), which arm mast cells/basophiles for allergic responsiveness. Both antigen-specific and total antibody isotype concentrations can be evaluated. Other proteins of interest in allergy models include Th2-relevant and inflammatory cytokines (IL-4, IL-13, IL-5, IL-10, TNFγ) and chemokines (Eotaxin, RANTES, IL-8).

4.4.1 Enzyme-Linked Immunosorbant Assay (ELISA)

The enzyme-linked immunosorbant assay (ELISA) is a method for the quantification of proteins such as antibodies, cytokines or chemokines. Two common assay designs are the antibody-capture ELISA and the two-antibody sandwich ELISA. In the antibody-capture ELISA, the test agent is immobilized on a solid matrix (microtitre plate). Agent-specific antibodies in the sample (serum, BALF) are then allowed to bind to the immobilized agent. Bound sample antibodies may be detected either directly (labelled primary antibody) or

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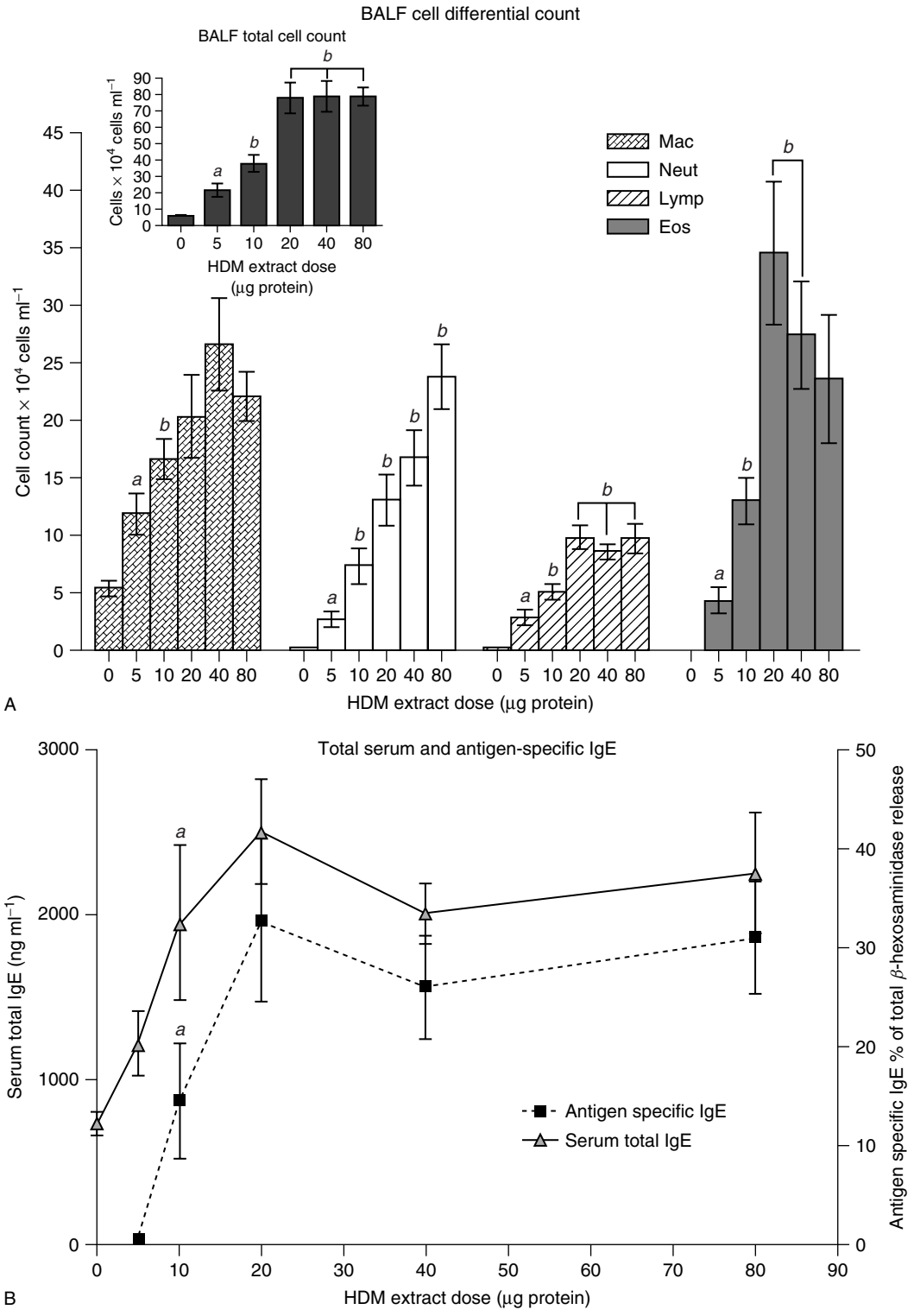

Figure 2 Three days after the fourth HDM (0–80 μg protein) exposure BALF and blood were collected from BALB/c mice. (A) BALF total and differential cell counts are shown. (B) Serum total IgE (ng ml^{-1}) and antigen-specific IgE (% of total mast cell mediator release) are shown. *a* indicates lowest significant dose compared to 0 μg dose; *b* significantly higher than lower doses at $p < 0.05$. $n = 5–10$.

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indirectly (labelled secondary antibody, e.g. biotinylated anti-IgE). In the two-antibody sandwich ELISA, an antigen-specific antibody is bound to the matrix. These antibodies then bind the target antigen (e.g. serum IgE, cytokines) in the test sample. Subsequently, a second antigen-specific antibody is allowed to bind. The two antibodies are often referred to as paired antibodies because both bind the target antigen but do not hinder the binding of each other. Many kits and paired-antibody sets are commercially available, particularly for mouse samples. **Figure 2B** shows serum total IgE ELISA data from the HDM dose–response.

4.4.2 Measurements of Functional IgE

Other methods may be used to evaluate the functional status of sample IgE. The passive cutaneous anaphylaxis (PCA) assay may be used for detection and quantification of even small quantities of cytophilic antibodies. PCA assays may be homologous (serum donor and recipient animals are the same species) or heterologous (donor and recipient animals are different species). In either case, serum from an allergen-sensitized animal (guinea pig, rat or mouse) is serially diluted and injected intradermally into a nonsensitized animal. Subsequently the test allergen, mixed with Evans blue dye, is injected intravenously. Allergen-specific antibodies from the donor serum bind the allergen and induce mast-cell degranulation, producing a localized cutaneous reaction (wheal and flare) called the Prausnitz-Kustner reaction. This appears as a dark blue area of dye extravasation due to local capillary dilation and leakage. The inverse of the highest dilution factor at which the dye is visible determines the IgE (IgG1) antibody titre.

An alternate measure of functional antigen-specific IgE is the rat basophile leukaemia (RBL) assay (Hoffmann *et al*., 1997). RBL cells (RBL-2H3) are passively sensitized with rat or mouse serum samples. Subsequently, an empirically derived amount of antigen sufficient to provide IgE crosslinking is added, resulting in mediator release (histamine, ß-hexosaminidase or serotonin). Mediator release measurement data are expressed as percent of total mediator release, thus providing an indirect measurement of antigen-specific IgE. In our laboratory, we measure ß-hexosaminidase release (Chung *et al*., 2005). In addition to spontaneous (no serum added) and total release (cells lysed with Triton X100), we measure an adjusted spontaneous release by treating cells with antigen alone to identify any direct effects of the extract. **Figure 2B** shows antigen-specific IgE data from the HDM dose–response study conducted in our laboratory.

4.5 Protein-Expression Analysis

In addition to traditional methods for protein(s) detection, multiplexing techniques have been developed to permit the simultaneous characterization and quantification of multiple proteins. Generally, these multiplexing platforms are variations of either sandwich or antibody-capture ELISAs. With a relatively small sample volume, these techniques can assay for the presence of cytokines, chemokines, growth factors, other plasma proteins and antigen-specific antibodies.

4.5.1 Multiplex Fluorescent Bead Assays

In fluorescent bead assays, polystyrene beads are loaded with a mixture of red and infrared fluorophores in defined proportions. Each mixture generates a unique fluorescent signature, which is reserved for a single target analyte. The fluorescent beads are then bound to agent-specific capture antibodies. Following incubation with the sample and an orange-fluorescing detection antibody, the beads are drawn past two lasers to identify (red laser) and quantify (green laser (orange fluorescence)) the analytes of interest. Advantages of this technology include the wide variety of sample types (including BALF, serum and tissue or cell culture lysates) that can be analysed, and the ability to assay multiple analytes in a small sample volume (Farraj *et al*., 2007). In some cases, this technology can be used to discriminate between protein variants, such as total and phosphorylated forms of cell signalling factors (Rhyne *et al*., 2003). In addition to bead sets that detect preselected protein targets (cytokines, soluble receptors, etc.) custom bead sets can be produced in-house or purchased.

However, many bead-based assays are intolerant of even minor changes in assay conditions, resulting in a tendency for bead ‘clumping’, thus producing a greater degree of variability than that seen with plate-based ELISAs. As a result, at this time, these assays may be better suited for demonstrating qualitative, rather than quantitative differences in protein expression.

4.5.2 Protein Microarrays

Protein microarray platforms employ a solid substrate, such as a glass slide coated with nitrocellulose. This substrate can then be dotted with a pre-defined arrangement of antibodies specific for the proteins of interest. Sample protein binding to these antibodies can be detected and quantified using fluorescently-labelled detection antibodies. Alternately, array substrates can be dotted with sample proteins for subsequent detection using labelled antibodies of known specificities or modified to facilitate the study of protein–protein interactions. Additionally, proteins of known identity can also be spotted on to slides and used to probe for the presence of antigen-specific antibodies in the sample. A brief overview of this technology has recently been published in the journal *Nature* (Eisenstein, 2006).

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4.6 Gene-Expression Analysis

Gene-expression analysis can identify differentially up-regulated or down-regulated genes following exposure to the test agent compared to control samples. This technology is very useful when direct detection of target proteins is not possible or when the proteins of interest are unknown. Additionally, the data can be used to identify biomarkers of sensitization or elicitation thus suggesting immune mechanisms as well as targets for disease intervention. Several analytical options are available, from whole-genome-transcript evaluation to more targeted assays evaluating the expression of one to a few dozen genes. Further information on the use of gene-expression analyses in toxicology screening may be found elsewhere in this text (see **Toxicogenomics and the Evolution of Systems Toxicology**).

Gene-expression analysis avoids some of the technical difficulties associated with protein detection (e.g. the need for species-specific reagents), but it must be remembered that the presence of a messenger RNA (mRNA) transcript does not necessarily mean that the protein product is also present. Furthermore, mRNA transcripts provide no information regarding the post-translation processing or activation state of the protein product.

4.6.1 Microarray Gene-Expression Analysis

Microarray (or 'gene chip') analyses allow simultaneous identification and relative quantification of thousands of mRNA transcripts on a single sample. They can provide excellent whole-genome screening for differentially expressed genes or gene families. Depending upon the study goals, data derived from microarray experiments can be used to identify differentially affected pathways for later study, to identify targets/actions for new drugs, or for discovery of 'biomarkers' of sensitization, exposure or overt toxicity.

Gene-expression microarray assays are similar in design to other nucleotide hybridization-based techniques. Briefly, DNA oligonucleotides are arranged (by spotting or synthesis *in situ*) at specific locations on a solid surface, such as a glass slide. Fluorescently labelled RNA fragments from the test sample are added and allowed to hybridize to complementary DNA sequences. Subsequent evaluation of the treated chip allows determination of the presence or absence of specific RNA transcripts, with fluorescence intensity at each site proportional to the quantity of sample RNA bound there.

Array analyses can be performed on RNA obtained from whole-tissue homogenates, from cell culture lysates, or from cell populations separated *ex vivo* by centrifugation or laser capture microdissection. Whole-genome analysis microarrays are available for humans, mice, rats, zebrafish, *Xenopus*, *Caenorhabditis elegans* and other species.

A major limitation to the use of microarrays is their expense. Ideally, only one subject sample is used per chip. However, studies designed in this manner become costly if dose–response evaluations or large numbers of replicates are required. Some researchers economize by pooling RNA samples to allow the use of fewer chips. Unfortunately, expression results derived from pooled samples cannot be correlated with other biological parameters for individual animals, making phenotypic validation of array results difficult or impossible (Jolly *et al*., 2005).

Another constraint in microarray analysis is the requirement for extremely high quality RNA, since the amplification and hybridization steps are very sensitive to RNA degradation and genomic DNA contamination (Medeiros *et al*., 2007). Finally, compared to polymerase chain reaction (PCR)-based methods (see below), microarrays suffer from a lack of detection sensitivity for low abundance RNA transcripts, as well as from limited quantification abilities. For these reasons, it is recommended that 'key' gene targets obtained by microarray analysis be confirmed using an alternate method, such as the quantitative real-time polymerase chain reaction (qPCR). In addition, demonstration of the presence of the protein products of gene targets identified by expression analyses should be performed whenever possible.

The volume of data generated during a microarray experiment can make interpretation of the results very difficult. Whole-genome transcript analyses require the use of powerful computer programmes and statistical tools to make valid determinations of differential gene expression profiles between individuals and groups (**Table 2**). Differentially expressed gene sets may then be superimposed upon known biological pathways or networks ('pathway analysis') to determine relevant relationships, as well as in the context of annotated gene databases (**Table 2**).

The annotated gene databases include detailed information on known biological functions, tissue expression and species-specific homologous gene orthologues. Several of these programmes include their own literature-based, independently curated annotation databases. Other programmes use 'meta-search' type algorithms to compile and cross-reference information from other databases, including pathway databases, tissue expression databases, protein-interaction databases, and disease-association databases.

4.6.2 Real-Time PCR-based Assays

Although gene-expression arrays offer an unmatched ability to rapidly 'screen' for genes or pathways of interest, they do have significant limitations, as mentioned above. Since the mid 1990s qPCR has been used to evaluate gene expression. This technology provides amplification and quantification of mRNA

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Table 2 Selected resources for analysis of microarray data (programme name: source; major features and programme type)

Program Name	Source	Major Features	Program Type
Rosetta Resolver	Rosetta Biosoftware, Seattle, WA, USA; *www.rosettabio.com*	Processing of microarray raw data, comparison and statistical analysis of intensity data; data clustering	Desktop
Ingenuity Pathway Analysis	Ingenuity Systems, Redwood City, CA, USA; *www.ingenuity.com*	Biological, metabolic and toxicologic pathway analysis, biomarker discovery, molecular network analysis	Web-based
GeneGo	GeneGo Inc., St. Joseph, MI, USA; *www.genego.com*	Biological and metabolic pathway analysis, protein interactions, network analysis, disease associations	Web-based
Panther classification system	Applied Biosystems, Foster City, CA, USA; *www.pantherdb.org*	Functional analysis of gene or protein lists, pathway analysis, SNP functional analysis	Web-based and Desktop
DAVID (database for annotation, visualization and integrated discovery)	National Institute of Allergy and Infectious Diseases, NIH, USA; *http://david.abcc.ncifcrf.gov/home.jsp*	Compilation and crossreference of other databases, including pathway analysis, tissue expression, protein interactions and disease associations	Web-based and Desktop
GSEA (gene sequence expression analysis)	Broad institute, Massachusetts Institute of Technology, Cambridge, MA, USA; *www.broad.mit.edu/gsea/index.jsp*	Statistical comparison of gene sets from different phenotypes/treatment groups	Web-based and Desktop
Gene expression omnibus	National Center for Biotechnology Information, NIH, USA; *www.ncbi.nlm.nih.gov/geo/*	Depository for gene-set data and analyses	Web-based

transcript concentrations over time, thus permitting more accurate quantitation of gene expression than is possible with a microarray. Additionally, because qPCR produces exponential amplification of transcript numbers, it is better suited for the detection of low-abundance transcripts than microarray analyses. Although the sensitivity and precision of qPCR technology makes it well-suited for detailed evaluation of a small number of gene transcripts, multiple targets in a large sample size can quickly consume valuable samples.

Recently PCR technology has expanded with the development of qRT-PCR-based 'arrays', such as the TaqMan Gene Signature Array (Applied Biosystems). In these arrays, individual wells of a 384 well microfluidic card are preloaded with gene-specific primers and probes. Sample and reaction master mix is added to the card through 'ports' and distributed to the wells by centrifugation, after which the plate is processed in the usual manner. Different plate configurations allow for simultaneous evaluation of up to 384 analytes per sample, or up to 24 analytes in eight samples.

5 CELL CULTURE

Animal studies provide logistical challenges. Animals must be bred, housed, exposed and sacrificed under controlled conditions to minimize confounding factors. Due to the complexity of sensitization development requiring tightly regulated interactions between multiple cell types, including lymphoid cells, innate immune system cells (e.g. dendritic cells, mast cells) and epithelial cells (e.g. keratinocytes, alveolar epithelial cells), animal studies have been considered essential for hypersensitivity hazard screening and understanding disease pathogenesis. Although *in vitro* cell culture techniques have been adapted for overt cytotoxicity screening, adaptive immune responses are difficult to model *in vitro*

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and their use thus far has been limited. However, *in vitro* cell culture can provide a homogenous system to dissect out pathways and mechanisms, leading to a better understanding of disease pathology and possibly targets for disease intervention. It is currently feasible to model some early cellular responses in culture systems. Therefore, *in vitro* methods for hazard screening are increasingly being employed in support of or as alternatives to traditional *in vivo* assessments.

Despite the limitations, there are several important reasons to include selected *in vitro* techniques in the sensitization screening process. Cell-culture systems may be developed to evaluate several agents at multiple concentrations simultaneously for known or putative biomarkers. Also, the validity of nonhuman test-subject data used in interspecies extrapolation can be partially addressed by human-derived cell-assay systems.

Culture supernatants or lysates can be harvested following exposure and evaluated for protein or gene expression using methods such as ELISA, and protein- or gene-expression microarrays. Alternately, putative sensitizers could be evaluated for their effects on surface phenotype, cell function or cellular proliferation.

5.1 Primary Cell Cultures

Culture systems involving *ex vivo* exposure of target cells to potential sensitizers avoid many of the difficulties associated with immortalized cell lines. In general, these cells exhibit minimal changes in phenotype and function when compared to the same cell types *in situ*, but have a limited lifespan. Historically, acquisition of sufficient quantities of the desired cell type could be difficult, especially for human-derived cells. However, several commercial sources for both animal- and human-derived primary cells now exist.

Primary cell cultures may display a significant donor-related variability in function. These differences may be exploited in studies comparing cellular responses to an agent in cells from allergic and nonallergic subjects. Both airway epithelial cells and peripheral blood-derived dendritic cells have been demonstrated to behave differently, depending upon the atopic status of the donors (Lordan *et al*., 2002; De Smedt *et al*., 2002). The most frequently used *ex vivo* cell types in exposure studies include exfoliated tracheobronchial epithelial cells and bone marrow or peripheral blood monocyte-derived dendritic cells. Some commercial sources of primary bronchial epithelial cells offer cells categorized by donor type, including nonallergics, asthmatics and smokers.

Ex vivo cultures of dendritic cells may be obtained by selective culture of either $CD34^+$ bone-marrow-derived dendritic cells (BMDCs) or peripheral blood monocytes (monocyte-derived dendritic cells; MoDC). BMDCs are more difficult and costly to obtain than MoDC. However, donor-related differences in response may be greater in MoDC (De Smedt *et al*., 2002).

5.2 Cell Lines

Immortalized cell cultures from a variety of species have been used to study biological phenomena such as cell signalling and apoptosis, and to investigate cytotoxicity. Established culture techniques and the high degree of phenotypic stability demonstrated by many of these cell lines permits some flexibility in protocol optimization and exposure synchronization. However, phenotypic stability, particularly in prolonged culture, does not necessarily imply that the responses are predictive of 'normal' cell behaviour. Additionally, immortalized cell lines may lack features considered characteristic of the parent cell type (e.g. lack of IgE receptor on the human mast cell line HMC-1 (Love *et al*., 1996)). Furthermore, well-established lines may develop sudden alterations in phenotype or function (e.g. prolonged culture of the canine C2 mast cell line results in the loss of IgE receptor function (Brazis *et al*., 2002)). It is also important to remember that there is significant variability in response between cell lines, even those derived from the same species and parent cell type.

Epithelial-derived cells are frequently used in the modelling of respiratory-tract responses. Alveolar epithelial cells represent the largest respiratory-tract cell population and are responsible for many innate defence responses against inhaled xenobiotics, such as surfactant production. One widely used respiratory epithelial cell line is the human alveolar epithelial cell line A549, which displays characteristics analogous to Type II alveolar cells. Other frequently used cell lines are derived from tracheobronchial epithelium and include the human-derived BEAS-2B, 16HBE14o- and Calu-3 cell lines. These lines form polarized cell layers with intercellular tight junctions (physical barrier (Wan *et al*., 2000)), and may retain some secretory properties (e.g. epithelial-derived mucous). Both characteristics are critical components of the airway barrier *in vivo*. Therefore, these cell culture systems can provide insight into agents or other substances that are transported across or cause damage to the airway epithelium.

Dendritic cells represent a far smaller cell population than epithelial cells, but are known to play a vital role in the development of both allergic and nonallergic airway inflammation. Immortalized 'dendritic cell' lines are frequently myeloid-derived cells that have been cultured under conditions that induce the phenotypic development of mature or immature dendritic cells. Commonly used lines include the human KG-1, THP-1 and MUTZ-3 cell lines, as well as the mouse XS52 cell line.

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5.3 Three-Dimensional/Coculture Systems

Recent advances in cell-culture techniques have enabled the development of three-dimensional and coculture models. Although first developed as models for screening of contact irritants and sensitizers, modifications of these culture systems permit the study of respiratory immunotoxicity and sensitization.

Cocultures provide a simulated airway environment to evaluate interactions between epithelial and immune-system cells. Commonly, epithelial cells or fibroblasts are cultured on a porous surface, such as a synthetic or organic transwell membrane. This facilitates the development of a polarized monolayer, and permits the subsequent removal and histological/immunochemical examination of the layer *in toto* (Blank *et al*., 2006). Once the monolayer is established, other cell types or agents can be added to the culture. These systems are especially well suited for direct or indirect measurements of epithelial permeability, such as transepithelial resistance or mannitol permeability. Although increased epithelial permeability is not necessarily predictive of sensitization potential, the induction of significant epithelial disruption may increase the systemic availability of other substances, potentially facilitating sensitization (Wan *et al*., 2001)

Coculture experiments can be performed using primary cells, established cell lines or a mixture of both. Bronchial or alveolar epithelial cells are frequently cultured with antigen-presenting cells such as *ex-vivo*-derived macrophages or dendritic cells. One particularly detailed triple coculture includes all three cell types, with peripheral-blood-derived macrophages and dendritic cells seeded apically and basally to A549 epithelial cells (Rothen-Rutishauser *et al*., 2005). Alternately, epithelial cells may be cultured with endothelial cells or fibroblasts to model epithelial–stromal interactions.

This type of culture system is labour intensive and requires a high degree of technical proficiency. In addition to the coordination of the epithelial monolayer culture with the preparation of another cell type for addition to the system, sufficient time must be allotted for the monolayer development and acclimation of the additional cell types before the test compound is added. Despite the technical complexities of these multicell systems they can provide insight into intercellular interactions.

6 SUMMARY

Allergic diseases, particularly asthma, have been increasing in prevalence in Western societies over the past several decades (Pew, 2000). One possible explanation for this phenomenon is increased exposure to environmental sensitizers. Recent advances in industry and biotechnology have produced an unprecedented expansion of novel items, including nanoparticles and bioengineered foods. The sensitization risk represented by these items remains to be determined. This chapter provides an overview of selected laboratory methods used in hazard identification and health-risk assessment of potential respiratory sensitizers.

Animal models of hypersensitivity have been used to elucidate disease pathogenesis, for hazard identification and for the determination of occupational exposure limits. Our laboratory has not only used BALB/c mice to evaluate the allergic potential of several mould extracts (Ward *et al*., 2000; Viana *et al*., 2002; Chung *et al*., 2005), but also to identify IgE-inducing mould proteins (Ward *et al*., in press) and to demonstrate that not all proteins can induce allergic responses (Viana *et al*., 2002). Additionally, the dose–response induced by exposure to mould extract (Chung *et al*., 2005) suggests that this model has utility in assessing the relative allergenicity of various indoor allergens. Other rodent models of allergy have been used to elucidate mechanisms associated with HDM hypersensitivity (Lambert *et al*., 2001) and to demonstrate adjuvant effects of air pollutants, such as NO_2 (Gilmour *et al*., 1996), residual oil fly ash and its metal components (Lambert *et al*., 1999; 2000). Additionally, mouse models have demonstrated that HP severity is modulated by viral infections (Gudmundsson *et al*., 1999) and cytokines (Gudmundsson *et al*., 1998a; 1998b).

Of particular relevance, the detergent industry has demonstrated that animal models can be used to set safe human exposure levels for detergent enzymes (Sarlo *et al*., 1997; Robinson *et al*., 1998). Following the establishment of occupational exposure guidelines for the detergent enzyme, *Bacillus subtilis* subtilisin A (Alcalase), <20% of the manufacturing population has been sensitized (developed IgE) to enzymes in a 10 year period, with no new cases of occupational asthma since 1994 (Sarlo, 2003). Determination of the relative allergenicity of new detergent enzymes compared to Alcalase has been predictive of human health outcomes and has provided a method to ensure the protection of these industrial workers.

In addition to traditional *in vivo* models of hazard screening, promising *in vitro* screening techniques are being developed, enabled by recent advances in cell-culture technology. Multiple-cell-type cocultures allow the evaluation of interactions between airway epithelial cells and extracellular stroma, endothelium and/or cells of the immune system, and provide an approximation of the complex airway environment. The use of cell cultures in toxicity screening has also been facilitated by the increased commercial availability of both established cell lines and primary cells from a variety of species. These techniques are particularly

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well-suited for high-throughput screening assays, and may eventually be used for first-tier new product evaluation.

In conclusion, several models have been developed for the screening of potential respiratory sensitizing agents. Early identification of potential allergens can facilitate reduction or elimination of environmental exposure and may reduce the risk of asthma development. The methods and models discussed in this chapter have been used in our laboratory and others as a means of determining the risks associated with bioaerosol exposure. However, they could be applied to other putative sensitizing agents and could be used to address a number of issues related to environmental exposures and respiratory hypersensitivity diseases.

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National and International Aspects on Bioterrorism and Biosecurity

Philippe Bossi

CONTENTS

1 INTRODUCTION

Bioterrorism is defined as the intentional use or threatened use of micro-organisms or toxins derived from living organisms to cause death or diseases in humans, animals or plants on which we depend. The other major point is to generate fear in the general population.

The deliberate use of micro-organisms as weapons has been attempted throughout history. One of the first major biological warfare attacks was reported during the fourteenth century, in 1346, during the siege of Kaffa, with *Yersinia pestis* The attacking Tatar force catapulted the bodies of their plague-infected soldiers over the city walls. This attack initiated the second major outbreak of plague, the Black Death that decimated one third of people living in Europa, and probably millions of people in Asia. The last biological warfare attack was the deliberate release of *Bacillus anthracis* in the United States shortly after the terrorist attacks of 11 September 2001 and this terrorist act, due to an unknown criminal group, caused 22 cases of anthrax, including five deaths.

More than 150 pathogens have been reported to be potential agents for bioterrorism (**Table 1**). These agents are classified by the Centers for Disease Control and Prevention (CDC) into three different categories, according to the infectiousness, virulence, public perception, impact, cost and sophistication in fighting against the agents. Category A includes agents that can be easily disseminated or transmitted person-to-person, cause high mortality, with potential for major public-health impact, might cause public panic and social disruption, and require special action for public-health preparedness. Category B includes agents that are moderately easy to disseminate, cause moderate morbidity and low mortality, and require specific enhancements of diagnostic capacity and enhanced disease surveillance. Category C includes emerging infectious agents that could be engineered for mass dissemination in the future because of availability and because of ease of production and dissemination, and potential for high morbidity and mortality and major health impact. In biological warfare, these agents would most likely be disseminated by aerosols, which are invisible, silent, odourless, tasteless and, theoretically, easily dispersed without detection. Infective doses for agents commonly considered to be potential biological warfare agents are reported in **Table 2**. In 1970 the World Health Organization (WHO) predicted that a city of 500 000 people would be devastated following an aerosol release of 50 kg of biological warfare agent, if deployed under ideal meteorological conditions; for example, an aerosol of anthrax spores could be associated with 125 000 incapacitated, including 95 000 deaths, and an aerosol of plague with 85 000 incapacitated, including 19 000 deaths (WHO, 1970).

In 1973, the Convention on the Prohibition of the Development, Production and Stockpiling of Biological and Toxin Weapons and on their Destruction, also known as the Biological and Toxin Weapons Convention or BWC, prohibited the development, production, stockpiling and acquisition of these weapons. Despite the international agreements to ban such weapons, there is no effective international mechanism for controlling either the development of biological weapons or their use. It has been reported that the number of countries

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Table 1 Biological terrorism agents

Category	Bacteria	Viruses/toxins
A	*Bacillus anthracis* (anthrax) *Yersinia pestis* (plague) *Francisella tularensis* (tularemia)	Variola major (smallpox) Haemorrhagic fever viruses (Ebola, Marburg, Lassa viruses . . .) *Clostridium botulinum* toxin
B	*Burkholderia mallei* (glanders) *Brucella* species (brucellosis) *Coxiella burnetii* (Q fever) Food and waterborne pathogens	Encephalomyelitis viruses (Venezuelan equine encephalitis (VEE), extracellular enveloped vaccinia (EEV), western encephalomyelitis virus (WEV)) *Ricinus communis* toxin (ricin)
C	Mutidrug-resistant *M. tuberculosis*, etc.	Encephalomyelitis viruses (tick-borne encephalitis (TBE), etc.)

Table 2 Infective aerosolized doses for common biological agents (3)

Agents	Infective dose
Bacillus anthracis	8000–50 000 spores
Brucella spp.	10–100 bacteria
Burkholderia mallei	Low
Yersinia pestis	100–500
Francisella tularensis	10–50
Coxiella burnetii	1–10
Encephalitis viruses	10–100 viruses
Haemorrhagic fever viruses	1–10
Smallpox	10–100
Clostridium botulinum toxin	0.001 μg kg^{-1}

known or suspected of having biological-weapons capability has doubled since the convention went into force in 1975. From 1980 to 2000, it has been estimated that 40 deliberate uses of micro-organisms as weapons have been perpetrated throughout the world. In the United States alone, during the last decade of the last century (1988–1999), intentional use of infectious agents was considered in six cases.

2 NATIONAL PLANS

The last deliberate release of anthrax in the United States in October 2001 brought about a radical change in people's perception of the risk of bioterrorism. The impact was not limited to the United States. These bioterrorist events, unlike others before, had a worldwide impact, not only in respect of security and public health, but also in other sectors. Before that time, public-health preparedness for bioterrorism was not a political priority in most countries.

In many countries, civil protection, security and law enforcement services were put on alert, and public-health systems had to manage numerous events relating to letters suspected to be contaminated with anthrax. The disturbance and psychological effects of these incidents became of great concern to society and decision-makers alike. The need for bioterrorism preparedness and planning for response at multiple levels has been recognized in many countries. Throughout the world, these countries set up new administrative and operational structures and adapted their preparedness and response plans in order to deal with the new kind of threat. On the wider international level, a concerted global-health security action to strengthen the public-health response to the threat of international biological, chemical and radionuclear terrorism has been also initiated. Governments and international entities with responsibilities related to maintenance of peace, security, safety and health protection reviewed urgently their political, economic, diplomatic, military and legal means to face up to such attacks and embarked upon major efforts to increase their preparedness.

Most of the countries involved against bioterrorism, such as European countries, the United States or Canada, have developed plans to prepare a programme to improve national and international cooperation on the evaluation of risks, alerts and intervention, the storage of such means, and in the field of research. Countries re-examined their preparedness plans and adapted them to these new threats, and legislation needed to implement countermeasures was reviewed. The national authorities also discussed the necessity of stockpiling medicines and other medical supplies and most upgraded their stocks of vaccines and antibiotics, especially smallpox vaccine.

To improve the ability to recognize a deliberate release, countries developed local, national and international training programmes for different kinds of first responders. These programmes also cover the detection and identification of infectious and toxic agents, as well as the prevention and treatment of chemical and biological attacks.

Early diagnosis and prompt institution of effective control measures are critical determinants of the eventual impact of any infectious-disease emergency. All clinicians must remain open to the possibility that they

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may be the first person to recognize a deliberate release and must be prepared to consult urgently with their local infectious-disease specialist, clinical microbiologist and public-health department on suspicion alone, without waiting for a definitive diagnosis. That is why numerous guidelines have been written, especially concerning the principal agents that could be used as biological weapons. Most clinicians do not know these infectious agents. Clinical aspects, diagnosis and treatments have been detailed in these guidelines.

In a few countries, the establishment of a preoutbreak national team that could investigate and manage initial suspected or confirmed cases of unusual agents has been performed. Special teams that could manage initial cases of smallpox have be vaccinated or revaccinated against smallpox. Most of the volunteers of these teams have been revaccinated using first-generation vaccine. In some countries, second generation has been used. Development of a third generation is ongoing and will be studied soon.

Specific departments of infectious diseases or emergency have been designated for receiving the first patients suspected, or actually infected with a biological agent. These departments include identifying a space (ideally one or several negative-pressure rooms) suitable for airborne infection isolation and respiratory isolation, and ensuring that staff understand when and how it should be used; review of available personal protective equipment (PPE: gloves, gowns, face and eye protection, surgical masks or other respiratory protection e.g. N95-type respirators), hand-cleaning facilities, and sharps safety and disposal arrangements; assessment of staff competency in choosing, and safely using, removing and disposing of the PPE available, ensuring that, if N95-type respirators are to be used, staff have been fit-tested and know how and when to perform fit-checks; reinforcement of the importance of hand hygiene; arrangements for cleaning and ensuring environmental hygiene; and setting triggers for notifying the infection-control team, public-health department and seeking further expert advice.

National plans for hospital preparedness have been written and tested. The overall aim of hospital preparedness for a bioterrorism attack is to be able to provide adequate medical care to those affected, whilst at the same time continuing to provide essential medical services to the community. Effective preparedness planning requires multidisciplinary involvement, involving emergency planners, clinical practitioners, laboratorians, managers and administrators, emergency responders, pharmacists, voluntary agencies, mental-health and occupational-health services, religious and spiritual advisors, and support staff, including catering, housekeeping, security, medical records, communications, information technology and transport/courier services. Clear, pre-event, designation of roles and responsibilities, clear chains of command, control and communication, and regular testing and evaluation of 'major incident' or emergency operations' plans by drills and exercises are needed.

The laboratory capacity required to diagnose most of the infectious agents that might be used in case of biological attack (biosafety level (BSL)-4) has been found in most countries. Laboratories and laboratorians have been involved in planning, and writing protocols for the safe collection, transport and external referral of clinical specimens.

Local, national and international exercises have been performed in order to test emergency teams, and effective communication. Effective communication appears crucial to management of infectious-disease emergencies, and a communication plan is an integral part of any emergency-management plan. Communication is a broad term that encompasses provision of accurate, timely, complete, easily understood information to the community about what the emergency is, what is being done to control it and what people can do to make themselves safer; provision of information to healthcare professionals about diagnosis, investigation and pre- or postexposure interventions; communication with families and others close to those affected by the emergency; communication with the media; and communication within and between all levels of those involved in managing the emergency.

Most of these countries involved against bioterrorism have compiled and planned national stockpiles of vaccines, antibiotics, antivirals and antitoxins. Most of the smallpox vaccines available have been in storage since the 1970–1980s. New vaccines were developed and studied at the end of 2002.

For smallpox vaccines, stockpiles of first generation and production capabilities for the new generations vaccines are probably sufficient in case of a massive bioterrorist attack using smallpox as an agent. For antibiotics, production capabilities are also sufficient to meet demand in the event of an emergency. A limiting factor in both cases is the lead time to increase production to meet increased demand.

Advice, algorithms and response protocols for investigation and management of possible cases or case clusters have been published, and give details of reporting mechanisms, diagnostic specimens, infection-control measures and other containment responses.

Bioterrorism has also initiated a discussion on the need to improve preparedness through reinforcing existing public-health structures that are in charge of monitoring and controlling diseases.

3 INTERNATIONAL PLANS

In parallel with these developments at national levels, many well-focussed international activities were also carried out.

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On the wider international level, health ministers of the G 7+ (US, Canada, Japan, France, Italy, UK, Germany and Mexico) agreed at a meeting in Ottawa in November 2001 to initiate concerted global-health security action to strengthen the public-health response to the threat of international biological, chemical and radionuclear terrorism.

In the European Commission (EC), in response to the increased threat of bioterrorism, a task force on health security was established in 2002. Task-force members address a broad range of issues related to preparedness for and response to bioterrorist events and seek to bring about a greater collaboration between the European Union (EU) member states.

On 19 October 2001, at the European Council in Ghent, Belgium, the Council of Ministers of the EU and the EC were asked by the heads of state and government to prepare a programme to improve the cooperation between member states for the evaluation of risks, alerts and intervention, the storage of the necessary means and collaboration in the field of research. The programme had to cover the detection and identification of infectious and toxic agents, as well as the prevention and management of chemical and biological attacks. Shortly afterwards, the EU Council of Ministers requested the Commission to develop an action programme of cooperation on preparedness and response to biological- and chemical-agent threats. In response, the Commission launched a series of coordinated actions across the civil protection, health, enterprise, research, nuclear, transport and energy fields. On the initiative of the European Commissioner for Health and Consumer Protection, the Health Security Committee was set up with representatives of the ministries of health from each member state. In December 2001, the Health Security Committee agreed on a programme for cooperation on preparedness and response to biological- and chemical-agent attacks. The overall goal was to improve cooperation between the member states, with the aid of the commission, and to facilitate collaboration between the different national authorities involved in public-health preparedness for bioterrorism. In May 2002, a task force was set up by the Directorate-General of Health and Consumer Protection. The task force's main objective was to implement the health-security programme. One of the initial priority activities was the setting up of a mechanism for rapid information exchange, consultation and coordination on all aspects dealing with bioterrorism. All designated authorities of the EU member states have been connected through this network by means of e-mail, fax and telephone. This tool makes it possible for the task force and the member states to disseminate information rapidly about suspected or confirmed incidents and for national authorities to evaluate measures planned with their implications for EU and for other member states. The task force is available 24 hours a day, seven days a week to facilitate the process.

The system has been used in EU-wide exercises and for alerts about suspicious events communicated by the member states. The early detection of a deliberate release or bioterrorist attack is an essential part of a programme of preparedness.

When the task force was established, several lists of agents that could be involved in a biological attack and considered a major bioterrorist threat had been established and published. Against this background, an approach more suitable to the demands for focussed action in Europe was developed though a matrix model designed to provide an evaluation of the public health impact of any given agent that could be used in bioterrorism. The clinical management, including recognition, of many of the diseases caused by the bioterrorist agents is likely to be difficult, since they are, to a large extent, unknown to the average clinician in Europe today. To provide clinicians with guidance and a source of generally agreed-upon information, several clinical guidelines have been compiled and published in a European journal.

The laboratory capacity required to diagnose most of the potentially toxic and infectious agents that might be used in cases of deliberate release is believed to exist within the EU as a whole; however, no member state has the complete spectrum of diagnostic capabilities needed. To improve the collective preparedness and to make more efficient use of the available resources, the laboratory capacities already in place were studied. Additionally, concrete collaborations between laboratories were initiated to share techniques and participate in common quality assurance schemes. Assisted by the task force, the BSL-4 laboratories in the EU are currently forming a network that will enhance their research activities through common projects. They have been asked to develop procedures to make BSL-4 diagnostics available to all member states and to handle, under high safety-level requirements, a substantial workload of analysis of environmental samples that might arise following a series of bioterrorist incidents or threats.

The European Agency for the Evaluation of Medicinal Products (EMEA) has developed guidelines on the use of authorized pharmaceuticals in case of deliberate release of biological and chemical agents. This includes guidelines for treatment and prophylaxis of the agents on the so-called CDC A-list of the most important threats as bioterrorist agents.

Stockpiling of medicines and vaccines (in particular, smallpox vaccines) has been extensively discussed between the different member states and the EC. Smallpox has become the model for much of the work performed in the area of preparedness for bioterrorist incidents. Vaccination has been performed in several European countries, mainly in volunteers that have been vaccinated during the past. Severe side effects have not been reported in these European-specific groups.

Preparedness also includes improved coordination between the Commission and international

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organizations working in this area such as the WHO and the parties of the Global Health Security Action Initiative (Canada, France, Germany, Italy, Japan, Mexico, the United States, the United Kingdom and the EC, with WHO participating as a resource). Multiple initiatives regarding coordination of actions in this area were pursued by the European Centre for Disease Prevention and Control in Sweden.

4 BIOLOGICAL AGENTS

Many guidelines have been published for treatments and prophylaxis of agents used as biological weapons. Prompt recognition of biological-warfare-associated diseases can lead to a more rapid mobilization of public-health and medical assets. We report examples of biological agents that could be used as biological weapons.

4.1 Anthrax and Bioterrorism

Anthrax is seen as one of the most likely biological agents for use as a weapon. *B. anthracis* spores can be transmitted by aerosolization. Inhalation anthrax has a high mortality rate and the organism's spores, compared with other potential biological warfare agents, are quite stable in the environment (Inglesby *et al*., 2002; Bossi *et al*., 2004a; Centers for Disease Control and Prevention, 2001a). It has been estimated that 50 kg of *B. anthracis* spores released over an urban population of 5 million would sicken 250 000 and kill 100 000 (Health Aspects of Chemical and Biological Weapons, 1970). The use of anthrax in warfare has been recorded throughout history. During World War I, Germany used *B. anthracis* to infect livestock during trans-shipment. In December 1941, the British Government began testing the effect of anthrax on sheep on the Scottish island of Gruinard. Due to the durability of anthrax spores, decontamination was unsuccessful, and the island was quarantined until 1986, when a determined effort was made to decontaminate the island using formaldehyde. It was finally declared safe in 1990 (Inglesby *et al*., 2002). By 1945 the Japanese programme (Unit 731) had stockpiled 400 kg of anthrax spores to be used in bombs. The United States has weaponized anthrax spores, as did other countries in the 1950s and 1960s. This was evidenced by the accidental aerosol release of *B. anthracis* spores from a Soviet military microbiology facility in Sverdlovsk in the former Soviet Union in April 1979 (Meselson *et al*., 1994). This was the largest known outbreak of inhalational anthrax in the twentieth century: 68 of the 79 patients with inhalational anthrax died (Meselson *et al*., 1994). The first victim died after four days; the last one died six weeks later. Cases have also been reported in animals located more than 50 km from the site. Initially, the Soviet Government claimed the deaths were caused by intestinal anthrax from tainted meat. It wasn't until 13 years later, in 1992, that President Boris Yeltsin admitted that the anthrax outbreak was the result of military activity at the facility.

In October 2001, 22 cases of bioterrorism-related anthrax were reported in the United States: 11 confirmed inhalational, and seven confirmed and four suspected cutaneous cases (Jernigan *et al*., 2001; Centers for Disease Control and Prevention, 2001b). It seems that the cult of Aum Shinrikyo in Japan tried on several occasions to disperse anthrax unsuccessfully in Tokyo before the sarin attack (Inglesby *et al*., 2002), but they used an attenuated vaccine strain. No other bioterrorism-related outbreak or deliberate release has been reported in the literature.

Many guidelines have been published for treatments and prophylaxis of anthrax (Inglesby *et al*., 2002; Bossi *et al*., 2004a; Dixon *et al*., 1999; Centers for Disease Control and Prevention, 2001c; EMEA, 2002b). Private room placement for patients with inhalational anthrax is not necessary.

For inhalational anthrax, the duration of treatment is eight weeks. Duration of treatment for cutaneous anthrax is shorter; 7–10 days. The same antibiotics are recommended for postexposure prophylaxis. Oral ciprofloxacin is also recommended as first choice for prophylaxis for persons at risk of inhalational anthrax and must be taken for at least 60 days, unless exposure has been excluded. It has been demonstrated that treatment with antibiotics beginning one day after exposure to a lethal aerosol can provide significant protection against death. Much stockpiling of that antibiotic has been performed in countries involved against bioterrorism: this antibiotic could also be used for prophylaxis and treatment of others agents such as *Y. pestis* or *Francisella tularensis*.

4.2 Smallpox and Bioterrorism

Smallpox is a viral infection caused by the variola virus which belongs to the family of Poxviridae, subfamily Chordopoxvirinae and genus *Orthopoxvirus*, which includes monkeypox virus, smallpox vaccine and cowpox virus (Breman and Henderson, 2002). It was declared eradicated worldwide by the World Health Organization in 1979 following a smallpox-eradication campaign begun in 1958 and intensified in 1967, and the last case of endemic smallpox occurred in Somalia in 1977 (Arita, 1979). The last fatal case was due to a laboratory-acquired infection in the United Kingdom in 1978 (Shooter, 1980).

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Smallpox is seen as one of the most likely viruses to be used as a biological weapon. Using this virus in warfare is an old concept: it was used with contaminated clothing in the eighteenth century by the British to create epidemics among the native American tribes. More recently, during World War II, the Japanese military explored the weaponization of smallpox in Mongolia and China. The variola virus exists legitimately only in two laboratories in the world: one in the Centers for Disease Control and Prevention in Atlanta, Georgia, USA and the other in the State Research Centre of Virology and Biotechnology in Novosibirsk, Russia (Henderson *et al*., 1999). There is no documentation of clandestine stock of the virus. Any new case of smallpox would have to be the result of human accidental or deliberate release. The aerosol infectivity, high mortality and stability of the variola virus make it a potential and dangerous threat in biological warfare (Franz *et al*., 1997; Bossi *et al*., 2004b). In addition, animalpox viruses such as monkeypox or a recombinant variant of smallpox could be developed as biological weapons. However, for the monkeypox virus, data indicate that it has limited potential for person-to-person transmission and furthermore, is not able to sustain an epidemic indefinitely in a community by human transmission only (Huhn *et al*., 2005).

Patients with smallpox must be isolated and managed, if possible, in a negative-pressure room until death, or until all scabs have been shed (about three weeks) (The European Agency for the Evaluation of Medicinal Products, 2002; Breman and Henderson, 2002; Henderson *et al*., 1999; Franz *et al*., 1997). There is no established antiviral treatment for smallpox. Cidofovir, an antiviral drug, is active *in vitro* on isolates of variola virus (Bray *et al*., 2002). Obviously, no data are available for humans. The most effective prevention is vaccination before exposure (Frey *et al*., 2002a; 2002b). The frequency of complications is low, but is higher than the most commonly used vaccines. Vaccination can modify the course of the disease and reduce mortality if given immediately after exposure (mortality can be reduced by up to 100%) and up to four days after (mortality can be reduced by up to 50%).

The vaccine used to eradicate smallpox was highly efficacious; it was prepared using live animal skin as a substrate. During the WHO smallpox eradication programme (1967–1978) many countries produced and used such 'first-generation' products despite their known reactogenicity, as it was accepted that the benefits outweighed the risks. Nowadays, quality requirements for vaccines have become much more stringent, leading to the development and testing of second-generation vaccines. These are produced using the same vaccine strains as the first-generation vaccines on tissue-culture substrate, allowing better production consistency and quality-control testing. For Europe, the EMEA has developed guidelines for development and production of second-generation vaccines (EMEA, 2002a). In 2002, many countries implemented a preparedness programme in which smallpox vaccine was administered to volunteers.

Throughout the world, vaccination against smallpox has been discontinued since about 1982. In France, for example, routine childhood immunization using the Lister strain ceased in 1979, and reimmunization of healthcare workers in 1984. This means that a large population is susceptible and any new case of smallpox would have to be the result of human accidental or deliberate release, could be associated with a major epidemic. In March 2003, the French Ministry of Health implemented a programme on preparedness and response to a biological attack using smallpox as weapon. This programme included the establishment of a preoutbreak national team that could be revaccinated against smallpox and called upon to investigate and manage initial suspected or confirmed cases of smallpox in France. People selected to this team were eligible volunteers, had received at least one dose of smallpox vaccine in the past and had no contraindications to a reimmunization.

In the United States, 665 000 people were vaccinated against smallpox during the 2002–2004 smallpox vaccination campaign (Casey *et al*., 2005; Sejvar *et al*., 2005). Serious adverse events such as neurologic disorders, myocarditis and/or pericarditis with generalized vaccinia were infrequent (Casey *et al*., 2005; Sejvar *et al*., 2005). There is a need therefore to develop a third generation of smallpox vaccines, with an acceptable safety profile (Bozzette *et al*., 2003). This could be achieved by attenuation or genetic engineering of vaccinia vaccine strains, while retaining their immunizing properties.

After the events of September and October, 2001, many governments took actions to improve their level of preparedness against smallpox—included updating and releasing a smallpox response plan. Right now, in many countries stockpiling of smallpox vaccine to vaccinate every person in the country in the case of a smallpox emergency seems to be enough. Anyone who has had contact with a smallpox patient will be offered smallpox vaccination as soon as possible. Then, the people who have had contact with those individuals will also be vaccinated (ring vaccination). Ring vaccination of direct contacts of infected persons seems sufficient to contain an epidemic of smallpox if infectious cases are rapidly diagnosed. National and international plans (different levels) have been written to vaccinate all the population, in the case of a massive biological attack using smallpox, in a limited time.

4.3 Plague and Bioterrorism

Y. pestis appears to be a good candidate agent for a bioterrorist attack. The use of an aerosolized form of this agent could cause with an explosive outbreak of primary plague

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pneumonia in the exposed population, or alternatively the bacteria could be used to infect the rodent population and precipitate a secondary outbreak in humans living in poor conditions (Inglesby *et al*., 2001; Levison, 2000). Intentional aerosol release should be suspected in patients presenting with plague pneumonia in nonendemic areas or in patients without risk factors. *Y. pestis* is a relatively fragile organism that remains viable for only an hour after an aerosol release. Nevertheless, as few as 1–10 bacteria are sufficient to infect rodents via the oral, intradermal, subcutaneous or intravenous routes (Brubaker, 1991). Estimates of human infectivity by the respiratory route vary from 100 to 20 000 organisms (Inglesby *et al*., 2000; McGovern and Friedlander, 1997). The Tatars were the first to use plague as a biological weapon, in 1347, during the siege of the Genoese-controlled Black Sea port of Kaffa; they hurled the bodies of their plague victims over the city walls. It has been reported that the Japanese army dropped plague-infected fleas in China during World War II; as many as 15 million fleas were used per attack to infect the population with plague (Inglesby *et al*., 2000). It has been also reported that cases of plague occurred among the Japanese soldiers shortly after a biological attack on Changteh in 1941; 1700 deaths were reported in the Japanese army. In 1970, it was reported that if 50 kg of *Y. pestis* were released over a city of 5 million, plague pneumonia could occur in as many as 150 000 people, including 36 000 deaths (Health Aspects of Chemical and Biological Weapons, 1970). In May 2000, during the virtual exercise TOPOFF (for 'top officials') in the United States, simulation of a deliberate release of an aerosol of *Y. pestis* at a performing-arts centre estimated that 3700–4000 cases of plague pneumonia and between 950 and over 2000 deaths might have resulted (Inglesby *et al*., 2001).

A live attenuated vaccine is available in the USA, but it retains some virulence and is therefore not considered suitable for human use in most countries (Tiball and Williamson, 2001). Recent vaccine research in Europe is focussed on the development of a subunit vaccine containing F1 antigens and recombinant V antigens, which proved to be efficacious against pneumonic plague in mice (Tiball and Williamson, 2001; Du *et al*., 2002).

4.4 Other Biological Agents

Many others bacteria (*Francisella tularensis, Clostridium botulinum, Brucella* spp., *Coxiella burnetii, Burkholderia mallei* and *Burkholderia pseudomallei*, etc.) have been reported to be potential agents for bioterrorism (Franz *et al*., 1997; Bossi *et al*., 2004c; 2004d; 2004e). Many of such agents are zoonotic and could have a considerable impact on agriculture, as well as on human health. Interest in such agents as a biological weapon stems from the fact that airborne transmission of these agents is possible. For example, *Brucella* spp. is highly contagious, as it can enter through mucous membranes such as the conjunctiva, oropharynx, respiratory tract and skin abrasions. It has been estimated that only 10–100 organisms are needed to constitute an infectious aerosol dose for humans (Hoover and Friedlander, 1997). For *C. burnetii*, an infectious dose of very few organisms is required to cause infection. It has been estimated that as few as 1–10 organisms could cause disease. *B. mallei* and *B. pseudomallei* infection may be acquired through direct skin contact with contaminated soil or water (abraded or lacerated skin) or through mucosal surfaces of the eyes and nose. Ingestion of such contaminated water or dust is another route of transmission. These agents have been studied for weaponization in several countries in the past. They have been used during both the First and Second World Wars (Byrne, 1997). However, all these agents might be used more as an incapacitating agent, as the disease is associated with a high morbidity combining a protracted illness.

Many other viruses are also good candidates for bioterrorist agents: encephalitis-associated viruses and haemorrhagic fever viruses could be used by aerosolization during a bioterrorist attack. Most of these viruses have been weaponized (Bossi *et al*., 2004f; 2004g).

5 CONCLUSION

At the beginning of the twenty-first century, similarly to what has been observed throughout history, among the risks of nonconventional weapons known as nuclear, radiological, biological and chemical weapons, bioterrorism remains a potentially important risk, even if terrorist acts using chemical or radiological weapons or bombs appear to be more plausible.

Technology moves with time. It thus appears prudent to consider the use of modified infectious agents by not very scrupulous spirits. Infectious agents are more virulent, have a shorter incubation, are associated with more serious clinical signs, are more easily transmitted human-to-human, are more difficult to diagnose and would be more resistant to standard treatments. Due to this significant risk, our governments must remain vigilant and, as best as possible, prepare to face such a situation. The preparation for clinicians, general practitioners and specialists consists of a better knowledge of the little taught, not-well-known clinical signs generated by infectious agents, which are rarely observed in the majority of our countries. Let us be vigilant, let us be ready, without pouring in an exacerbated alarmism.

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Mycotoxins and Mycotoxicoses: Significance, Occurrence and Mitigation in the Food Chain

Wayne L. Bryden

CONTENTS

1 INTRODUCTION

Invasion of human food and animal feed supply chains by filamentous fungi or moulds can result in contamination by secondary fungal metabolites. Many of these metabolites are toxic and are called mycotoxins. Mycotoxicoses are the diseases resulting from ingestion of these compounds. There are some 100 000 species of mould, and though not all are toxigenic, thousands of secondary metabolites have been isolated in the search for antibiotics and mycotoxins and many are toxic to man and animals (Cole *et al*., 2003). Historically, contamination of foodstuffs has been of concern because of the economic cost of spoilage rather than the potential hazard to animal and human health. Nevertheless, as mycotoxins are associated with field crops and stored plant produce, it is likely that they have affected man since the beginning of agriculture. It has long been recognized that mouldy food can be toxic, with reports from China some 5000 years ago and a suggestion of a biblical role for mycotoxins (Schoental, 1980; 1994), including the 10 plagues of Egypt in the Old Testament (Marr and Malloy, 1996). Ergotism has been known since the Middle Ages (Matossian, 1989) and the association of mouldy food with disease has been recognized in Japan, Russia and the USA for over a century (Joffe, 1986; Matossian, 1989; Richard, 2008). The neglect of the disease aspect of mouldy foods and feeds was altered dramatically by outbreaks of 'Turkey X' disease in England in the 1960s (Allcroft *et al*., 1961; Blount, 1961) and the concurrent report of an epizootic in trout from the USA (Halver, 1965). In both instances the causal agent was shown to be aflatoxins, which is a group of highly toxic metabolites produced by some species of the fungal genus *Aspergillus* (Austwick and Ayerst, 1963). Subsequent research has shown that aflatoxin B_1 is an extremely potent carcinogen (Wogan and Newberne, 1967). This finding highlights the potential health hazard of these natural chemical environmental contaminants and has prompted worldwide interest in toxigenic fungi.

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Since the discovery of aflatoxins, research has continued apace on these mycotoxins (Richard, 2008). It is generally accepted that aflatoxin is the causative agent of 'Turkey X' disease, but Cole (1986b) has presented a persuasive case for the involvement of cyclopiazonic acid in this syndrome. The neurological signs (including opisthotonus) and enteritis observed in 'Turkey X' disease are not associated with aflatoxicosis of turkeys and he suggested that cyclopiazonic acid, which is a tremorgenic mycotoxin (Bryden, 2008) and produced by *A. flavus*, may have been a co-contaminant and have given rise to the clinical neurological signs exhibited by the turkeys. Subsequently, Bradburn *et al.* (1994) found cyclopiazonic acid in a sample of peanut meal from the original 'Turkey X' disease outbreak during the intervening period much has been accomplished in delineating the toxicology of the other major mycotoxins; the trichothecenes, ochratoxin, ergot alkaloids, zearalenone and fumonisins (see **Mycotoxins with a Special Focus on Aflatoxins, Ochratoxins and Fumonisins**). However, the mycotoxins likely to be encountered by human and animal populations differ between countries. This reflects different crops, agronomic practices and climatic conditions, which dictate the fungi that are present in a farming system. Mycotoxins may also be produced during storage and transport of food commodities. The temperature and humidity of the storage/transport environment are major factors determining fungal growth and toxin production. These aspects of the food chain, which mediate the level of food contamination, coupled with local socioeconomic factors and food cultural practices determine exposure to mycotoxins (Wild and Hall, 1996; Miller, 1998; Bryden, 2007; Wild, 2007; Shephard, 2008).

Globally, mycotoxins have significant human health, economic and international trade implications, which are considered in the opening section of this chapter. The subsequent sections are concerned with the ecology of toxigenic fungi and the elaboration of mycotoxins and strategies that have been developed to control and manage the occurrence of these naturally occurring compounds in the food chain. Emphasis, in these sections, is given to cereal crops as the most important source of mycotoxins. The prevention of mould growth and subsequent mycotoxin formation in crops and in other components of the food chain requires an integrated understanding of crop biology, agronomy, fungal ecology, harvesting methods, storage conditions and food processing. It is beyond the scope of this chapter comprehensively to describe all of these aspects and the interested reader is referred to texts (including Abbas, 2005; Barug *et al.*, 2004; 2006; Beasley, 1989; CAST, 1989; 2003; Chelkowski, 1991; Cole, 1986a; Cole *et al.*, 2003; De Vries *et al.*, 2002; Desjardins, 2006; Eaton and Groopman, 1994; Jackson *et al.*, 1996; JEFCA, 2001; Joffe, 1986; Logrieco and Visconti, 2004; Magan and Olsen, 2004; Matossian, 1989; Miller and Trenholm, 1994; Oswald and Taranu, 2008; Richard and Thurston, 1986; Sinha and Bhatnagar, 1998; Smith and Henderson, 1991; Summerell *et al.*, 2001) that detail mycotoxin occurrence, toxicology and control in the food chain.

2 GLOBAL SIGNIFICANCE OF MYCOTOXINS

It has been estimated that some 25% of the world's grain crops are effected annually by fungal invasion and mycotoxin contamination (Mannon and Johnson, 1985). It is interesting to speculate that with global warming, the threat from fungal invasion of crops will increase (Garrett *et al.*, 2006). It is therefore imperative that all measures be taken to reduce the occurrence of these compounds in the food supply chain. The complexities of this task can be gauged from **Figure 1**. Once formed either pre- or postharvest, mycotoxins, most of which are chemically stable, remain intact during food processing.

All food commodities can be contaminated with mycotoxins (**Table 1**) and prominent foodstuffs include: barley, cheese, coffee, cottonseed, grapes, maize (corn), milk, peanuts (groundnuts), rice, sorghum, tree nuts and wheat, especially maize and peanuts. The level of contamination found in these commodities varies with location and reflects climatic conditions and the invading fungal species. Globally, the most important genera of mycotoxigenic fungi are *Aspergillus*, *Fusarium* and *Penicillium* (Miller, 1998). The major toxins produced by these genera include: aflatoxins, ochratoxins, trichothecenes, fumonisins and zearalenone (Miller, 1998). The ergot alkaloids produced by *Claviceps* (Bryden, 1994) and toxins produced by *Alternaria* (Ostry, 2008) also cause disease-related problems from time to time. Mycotoxins produced by *Fusarium* sp. are invariably found in cereals and include trichothecenes (Beasley, 1989), fumonisins (Marasas *et al.*, 2001) and zearalenone (Hagler *et al.*, 2001). Aflatoxin and ochratoxin can be found in a wider range of food commodities preharvest. Significant quantities of aflatoxin can be found in maize, peanuts and treenuts. Wheat, rye, barley, grapes and coffee can be contaminated with ochratoxin.

2.1 Developed and Developing Countries

Mycotoxin contamination can occur throughout all phases of the food supply chain. However, there are differences between developed and developing countries (Wild, 2007; Shephard, 2008). In developed countries, most mycotoxin contamination occurs preharvest, in contrast to developing countries, where contamination occurs both pre- and postharvest. The differences reflect agronomic practices in the different regions and also the storage facilities available in developed compared to developing countries. As will be discussed later in

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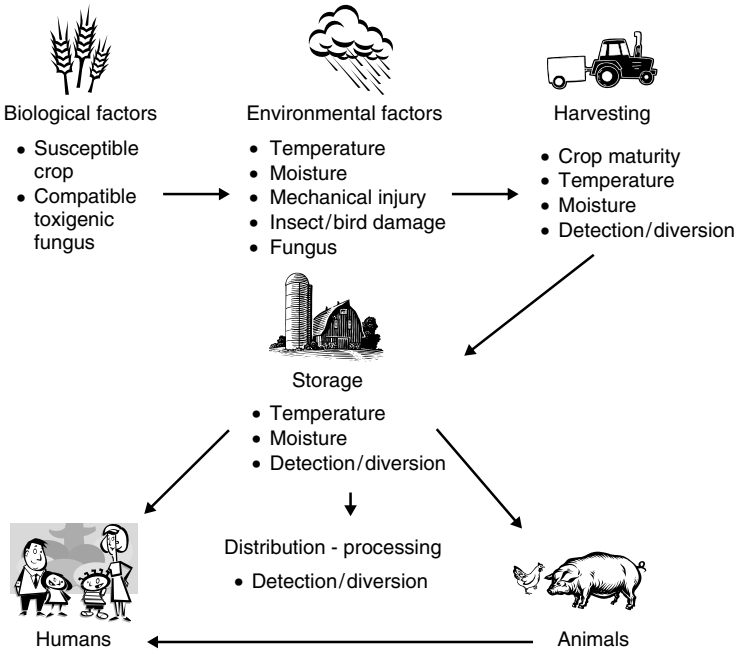

Figure 1 Factors affecting mycotoxin occurrence in the human food and animal feed chains. (Adapted from Pestka and Casale, 1990.)

Table 1 Food commodities and major associated fungi and mycotoxins

Commodity	Fungal species	Mycotoxins
Peanuts; maize; dried fruits	*Aspergillus flavus; A. parasiticus*	Aflatoxins
Peanuts	*A. flavus*	Cyclopiazonic acid
Cereal grains; coffee	*Penicillium verrucosum; A. ochraceus*	Ochratoxin A
Grapes	*A. carbonarius*	Ochratoxin A
Cereal grains	*P. citrinum; P. expansum*	Citrinin
Apple products	*P. expansum*	Patulin
Cereal grains	*Fusarium sporotrichioides; F. poae*	T-2 toxin
Cereal grains	*F. sporotrichioides; F. poae*	Diacetoxyscirpenol
Cereal grains	*F. culmorum; F. graminearum*	Deoxynivalenol
Cereal grains	*F. culmorum; F. graminearum*	Zearalenone
Maize	*F. verticillioides; F. proliferatum*	Fumonisins
Fruit; vegetables; cereal grains	*Alternaria alternata*	Tenuazonic acid
Rice	*P. citreoviride*	Citreoviridin
Cereal grains	*Claviceps purpurea*	Ergot alkaloids

the chapter, conditions of moisture and temperature, especially during storage, have a major impact on fungal growth and mycotoxin production. The well constructed and maintained storage facilities in developed countries largely mitigate against fungal growth. In developing countries, where storage facilities are often rudimentary, it is not unusual for grain to become moulded during storage. The human populations in developing countries will therefore have greater exposure to mycotoxins than their counterparts in developed countries. The major concern of mycotoxin contamination in developing countries is its impact on human health (Miller, 1998; Shier *et al*., 2005; Wild, 2007). In developed countries, mycotoxin contamination in the food and feed chains is tightly regulated to reduce human and animal exposure. The additional costs to the producer and/or the consumer to meet the economic burden of regulating the food and feed supply is the major mycotoxin concern in developed economies (Shier *et al*., 2005). The second major concern in both developed and developing countries is the impact of mycotoxins on animal health and production (Shier *et al*., 2005).

2.2 Mycotoxin Exposure and Disease

Mycotoxins pose a significant risk to the health and wellbeing of humans and animals and are a significant

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food safety issue. Regulations and limits to the levels of mycotoxins that can be permitted in foods require that Governments and international agencies have a transparent process for arriving at acceptable levels (Moy, 1998). The process of risk assessment, which is overseen by a number of different international agencies, including the World Health Organization (WHO) and the Food and Agricultural Organization of the United Nations (FAO), is the paradigm used to determine the probability of risk posed by mycotoxins (FAO/WHO, 1995). It involves a number of different steps including hazard identification, hazard characterization, exposure assessments and, finally, risk characterization and evaluation, culminating with the communication of risk (Kuiper-Goodman, 1998; 2004). Each step in this process involves the collection and analysis of data based on scientific principles. It is obvious from the discussion in this chapter that the hazards from different mycotoxins have been identified in both man and animals. However, in many instances it is difficult to determine actual mycotoxin exposure and toxicity in human populations (Kuiper-Goodman, 1990). These important aspects of risk assessment are explored below. Risk assessments have been prepared for aflatoxin B_1, aflatoxin M_1, deoxynivalenol (DON or vomitoxin), fumonisins, ochratoxin A (OTA) and zearalenone (see Kuiper-Goodman, 2004; JEFCA, 2001). It is necessary to revise risk assessments as more data becomes available.

2.2.1 Exposure

Human populations are predominantly exposed to mycotoxins through plant-derived foods (Miller, 2008) and on occasion can be subjected to mycotoxins in the air, where dust or airborne spores are the vehicle, and occasionally dermally (Jarvis, 2002). Only trace levels of mycotoxins or their metabolites are likely in animal products (Pestka, 1995), with the possible exception of milk (van Egmond, 1989). When acute intoxications occur, it is possible to determine the source of the toxin, but in situations where mycotoxin exposure occurs at low levels for a prolonged period, the source and degree of exposure is more difficult to delineate. Diagnosis of a mycotoxicosis has largely depended upon the absence of other readily diagnosed diseases and the finding of a mycotoxin in the suspect food (Richard and Thurston, 1986). In making a diagnosis, not only must mycotoxins be considered for their unique effects, but they must be evaluated for possible interactions with other toxins, infectious agents and environmental stressors commonly encountered (see **Figure 2**) which can determine the severity of an intoxication.

Analysis of food commodities for mycotoxins, which is discussed below, is not always the appropriate approach when attempting to delineate human mycotoxin exposure. In situations where the subject may have been subjected to low-level, long-term exposure to heterogeneous distributions of mycotoxins in food, the time lag between mycotoxin intake and the development of chronic disease and the inaccuracy associated with food-intake questionnaires suggest that biomarkers are the most robust approach when attempting to delineate the epidemiology of human exposure and the aetiology of diseases associated with mycotoxins (Wild, 2007; Groopman *et al*., 2008).

A detailed understanding of the toxicology of aflatoxin has allowed the development of a biomarker (aflatoxin albumen adduct in serum) which is reliable in assessing aflatoxin intake (Wild and Turner, 2002). Its application has allowed the definition of the relationships between aflatoxin intake and immunosuppression, impaired child growth and development (Gong *et al*., 2002; Williams *et al*., 2004), and the role of aflatoxin in the aetiology of hepatocellular carcinoma. The demonstration of aflatoxin-induced mutations in the p53 tumour suppressor gene, *TP53*, has contributed significantly to the confirmation of aflatoxins as human carcinogens (Groopman and Kensler, 2005). Biomarkers have not been as well defined for other mycotoxins. Nevertheless, the use of clinical measures and mycotoxin levels in foodstuffs and body fluids has allowed an estimation of population exposure to mycotoxins. Using these approaches it has been shown, both in developed and developing countries, that exposure to mycotoxins, including fumonisins (Shephard, 2008) and DON (Turner *et al*., 2008a; 2008b), may exceed the recommended maximum tolerable daily intake.

2.2.2 Mycotoxicoses

A mycotoxicosis is the final stage in a series of events and the interaction of many factors that commenced with the growth of a mycotoxigenic fungus. The diseases arising from ingestion of one or a combination of mycotoxins may affect every system of the body resulting in various toxicity lesions which have been reviewed by Haschek *et al*. (2002). The adverse effects of mycotoxins may result in acute, overt disease or chronic, insidious conditions. Fortunately, contamination levels in food are usually not high enough to cause an overt toxicosis. Low levels of toxins in foods are likely to result in an array of metabolic disturbances (Riley, 1998), which may or may not be accompanied by pathological changes (Haschek *et al*., 2002). The effects will be unpredictable, as toxicity will depend on the toxin(s) present, dosage, duration of exposure and a variety of other factors, including age, nutrition and concurrent disease (Bryden, 2007; Wild, 2007). The gut microflora may also modify mycotoxin toxicity (Swanson *et al*., 1988; Annison and Bryden, 1998; Eriksen *et al*., 2002).

Mycotoxins have been associated with a number of human diseases, some acute and others chronic (**Table 2**), but only rarely has a direct involvement of a mycotoxin been established in the aetiology of the disease (Beardall

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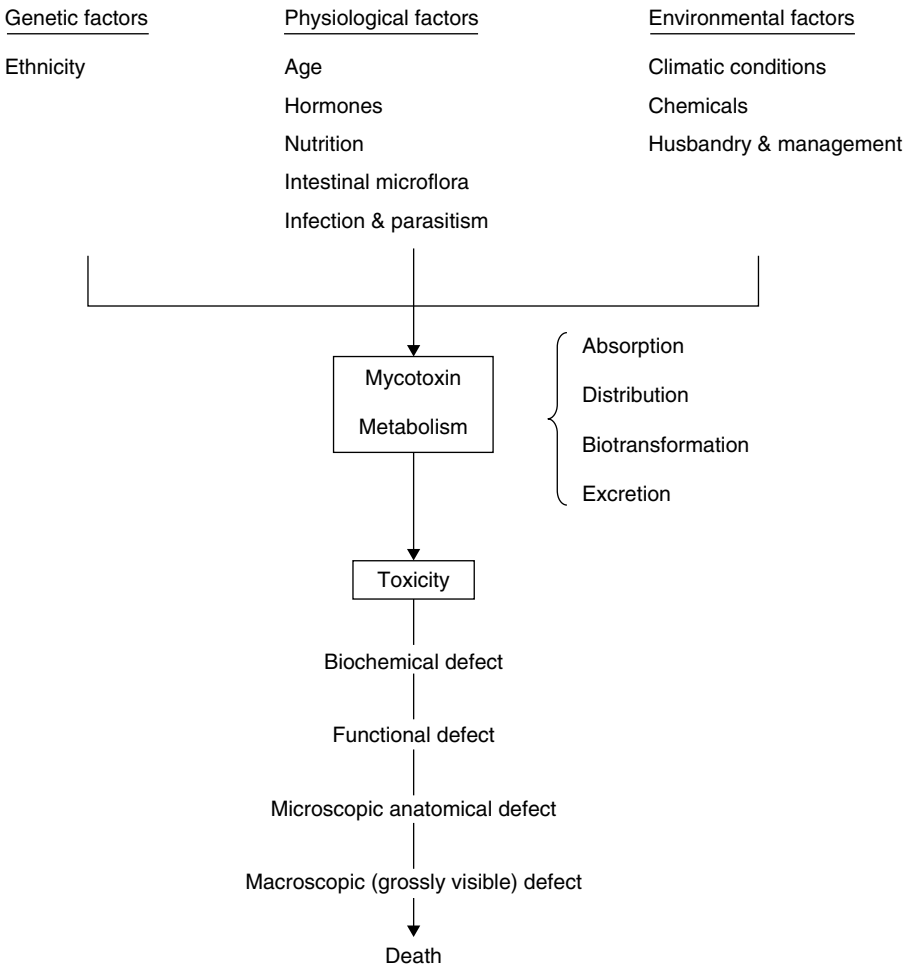

Figure 2 A simplified representation of some general relationships in a mycotoxicosis. (Adapted from Bryden, 1982.)

and Miller, 1994). Although rare, acute human intoxication with mycotoxins does occur, with the most recent case of aflatoxicosis occurring in Kenya in 2004 (Lewis *et al*., 2005). There are other recent recorded cases of intoxications with DON (Bhat *et al*., 1989) and fumonisins (Bhat *et al*., 1997) in India. The last major outbreak of ergotism occurred in Ethiopia (King, 1979). In developing countries, the vast majority of the population have limited food choices and rely on major dietary staples. If these staples are contaminated with mycotoxins, the human population will be exposed, in most instances, to low-level mycotoxin intake. If exposure is prolonged, the population is likely to suffer from a number of associated chronic diseases. It has now been demonstrated unequivocally that low-level aflatoxin intake in conjunction with hepatitis infection are major aetiological factors in the development of hepatocellular carcinoma worldwide (Henry *et al*., 1999; Groopman and Kensler, 2005). There is increasing evidence from both Africa and China that children may be exposed to aflatoxin *in utero* and this increases substantially after weaning. The result is childhood stunting and reduced growth rate, and suppressed immune function (Williams *et al*., 2004; Groopman *et al*., 2008). When this occurs, other infections and vaccination failures are likely to be the sequel (Bondy and Pestka, 2000; Oswald *et al*., 2005). During infection, the body will repartition nutrients away from growth and development to the immune system (Elsasser *et al*., 2000) and this phenomenon may contribute to the growth depression noted in children exposed to aflatoxin. Aflatoxin exposure has also been implicated in the aetiology of kwashiorkor (Hendrickse, 1991).

There is increasing evidence that other populations may also be exposed to low levels of mycotoxins. The data are indicative of a much wider global public-health problem. OTA has been associated with Balkan endemic nephropathy (BEN) and chronic interstitial nephropathy in northern Africa, and kidney tumours in humans (Bayman and Baker, 2006). There is epidemiological evidence to suggest that the fumonisins which cause equine leukoencephalomalacia and porcine pulmonary oedema (Haschek *et al*., 2002), and are carcinogenic in rats (Howard *et al*., 2001), may be a major aetiological factor in the incidence of human oesophageal cancer in southern Africa and China (Marasas *et al*., 2001). Recent epidemiological evidence demonstrate a higher

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Table 2 Some human diseases in which mycotoxins have been implicated

Disease	Mycotoxin source	Fungus
Akakabio-byo	Wheat, barley, oats, rice	*Fusarium* spp.
Alimentary toxic aleukia	Cereal grains (toxic bread)	*Fusarium* spp.
Balkan nephropathy	Cereal grains	*Penicillium* spp.
Cardiac beriberi	Rice	*Aspergillus* spp., *Penicillium* spp.
Celery harvester's disease	Celery (Pink rot)	*Sclerontinia*
Ergotism	Rye, cereal grains	*Claviceps purpurea*
Hepatocarcinoma	Cereal grains, peanuts	*Aspergillus flavus, A. parasiticus*
Kwashiorkor	Cereal grains	*Aspergillus flavus, A. parasiticus*
Neural-tube defects	Maize	*Fusarium verticillioides, F. proliferatum*
Oesophageal tumours	Maize	*Fusarium verticilloides, F. proliferatum*
Onyalai	Millet	*Phoma sorghina*
Reye's syndrome	Cereal grains	*Aspergillus*
Stachybotryotoxicosis	Cereal grains (grain dust)	*Stachybotrys atra*

incidence of neural tube defects (NTDs) in babies from Texas, China, Guatemala and southern Africa, where the populations rely on foods prepared from maize (Marasas *et al*., 2004). International surveys of maize for fumonisin contamination has found that there are very few instances where maize can be shown to be totally free of this mycotoxin (Shephard *et al*., 1996) and this reflects the endophytic relationship of the fungus with maize (Bacon and Hinton, 1996). An association between NTD and fumonisins is likely (Voss *et al*., 2006), as these mycotoxins induce folate deficiency, which is a known cause of NTDs in humans (Stevens and Tang, 1997). Recent studies in mice, both *in vivo* and *in vitro* have demonstrated that fumonisins may cause NTDs (Gelineau-van Waes *et al*., 2005). The teratogenicity of other mycotoxins has been demonstrated for some 40 compounds which have been shown to be teratogenic and/or embryotoxic (Cawdell-Smith *et al*., 2007b) and is another potential consequence of low-level mycotoxin ingestion (see **Developmental Toxicology**).

The accepted paradigm is that the chronic effects of mycotoxins occur in populations in developing countries that rely heavily on a limited number of food staples. However, recent research in the United Kingdom demonstrates that low-level mycotoxin intake can occur in highly industrialized populations. In this study, Wild and his colleagues (Turner *et al*., 2008a; 2008b) developed an analytical procedure for the quantification of DON in human urine. They subsequently carried out a survey where they calculated cereal intake from the UK Adult National Diet and Nutrition Survey and compared it to 24 hours urinary DON excretion. The results demonstrated that the majority of adults in the United Kingdom were exposed to DON on the basis of urinary excretion of the toxin (Turner *et al*., 2008b). It was estimated that some individuals may exceed the European Union (EU) recommended maximum tolerable daily intake of 1000 ng DON (kg bodyweight)$^{-1}$ (Turner *et al*., 2008b). In a follow-up study it was demonstrated that removal of wheat-based foods from the diet reduced DON urinary excretion rates 11-fold (Turner *et al*., 2008a). These are very interesting and very disturbing results, as they are the first indication that populations in developed countries could be subjected to ongoing low-level mycotoxin ingestion. Implications of continued low-level DON ingestion by human populations has been reviewed (Pestka and Smolinski, 2005). In animal studies it has been demonstrated that chronic dietary exposure to DON causes impaired weight gain, anorexia, decreased nutritional efficiency and immune dysregulation (Trenholm *et al*., 1984; Rotter *et al*., 1996; Haschek *et al*., 2002). Trichothecenes, including DON, are immunostimulatory or immunosuppressing depending on dose, and frequency and duration of exposure (Pestka, 2008). The implications of these findings are further complicated by a recent Canadian survey of breakfast cereals which found frequent low-level multiple contamination with DON, fumonisins, OTA and zearalenone (Roscoe *et al*., 2008).

The scenarios following low-level mycotoxin ingestion are similar to what has been observed in different intensive animal production systems (cattle, pigs and poultry), which rely on grain-based diets. In these systems it has been shown that low-level mycotoxin intake can result in reduced feed intake, poor growth rate, lower egg production, changes in carcass composition, reduced fertility and hatchability of eggs, and immunosuppression (Bryden, 1982; Hamilton, 1982; Smith and Henderson, 1991; Dersjant-Li *et al*., 2003; Oswald and Taranu, 2008). However, the most insidious effects of low-level mycotoxin ingestion result from impairment of native and acquired resistance to disease. These effects are often difficult to recognize because signs of the disease are associated with infection rather than with the toxin that predisposed the animal to infection (Oswald *et al*., 2005). This is because the immunological effect of mycotoxins usually occurs at much lower levels of intake than do toxin effects on production parameters, such as growth rate or egg production (Bryden, 1982). Changes in the composition of grain following fungal

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growth and alterations in nutrient intake and utilization that accompany mycotoxin ingestion further complicate diagnosis (Bryden, 1982; Hamilton *et al*., 1988; Dersjant-Li *et al*., 2003). Grazing animals may also consume mycotoxins if pastures are infected with fungi, especially endophytes (Bryden, 1998). The endophytic fungal genera *Neotyphodium* colonizes pasture grasses (tall fescue and perennial ryegrass) and produces a series of ergot alkaloids and related compounds (Bryden, 1994; Haschek *et al*., 2002; Cawdell-Smith *et al*., 2007a). The severity of any of these effects in different animal-product systems will depend on the level of mycotoxin present in the feed supply chain, the duration of exposure, the physiological status of the animal, and other environmental and disease factors that impact on the uptake, biotransformation, deposition and excretion of these toxins.

2.3 Economic Impact of Mycotoxins

A number of authors (Charmley *et al*., 1994; Lubulwa and Davis, 1995; Lamb and Sternitzke, 2001; Vardon *et al*., 2003; Robens and Cardwell, 2003) have attempted to quantify the potential costs of mycotoxins to society, but have found it difficult because of the uncertainty of the level and variability of contamination; variable price of affected commodities, and the costs associated with attempts to mitigate contamination. Nevertheless, the annual cost of mycotoxin contamination of food and feed in the United States is of the order US$1–2 billion (Robens and Cardwell, 2003). Moreover, the insidious nature of many mycotoxicoses, in both humans and animals, make it difficult to estimate their incidence and economic impact. If the various aspects of possible loss during a mycotoxicosis are considered, a multitude of possibilities exist. Obviously, they are complex and deal with many aspects of crop and animal production, storage, transport and processing of food commodities and implementation of food-safety regulations. Many factors would have to be considered in attempting to determine economic loss: reduced crop yield and quality; mycotoxin analysis; epidemiological surveys, including clinical and laboratory procedures; impaired human health and associated losses in productivity; reduced animal productivity, increased mortality, and morbidity and predilection of animals and birds, ingesting mycotoxins, to infection (Charmley *et al*., 1994). There is also a considerable cost to the industry as a whole, in terms of research, monitoring and extension; extra handling and distribution costs; increased processing costs and loss of consumer confidence in the safety of food products (Robens and Cardwell, 2003). Development and use of simulation models will greatly assist in the quantification of losses from mycotoxin contamination (Vardon *et al*., 2003).

Table 3 National maximum tolerated levels for aflatoxins in human food (Food and Agriculture Organization, 2004)

Nation	Total aflatoxin standard in human food ($\mu g\,kg^{-1}$)
Australia	5
China	20
European Union (EU), harmonized	4
Guatemala	20
India	30
Ireland	30
Kenya	20
Taiwan	50

With the establishment of the high toxicity and carcinogenicity of some mycotoxins, international regulations that limit levels in food and feedstuffs for both man and animals have been imposed (Food and Agriculture Organization, 2004; van Egmond and Jonker, 2004). Maximum tolerated levels of aflatoxins in human food for selected countries appear in **Table 3**. These limits have implications for international trade in grain crops and in some instances can result in a barrier for the export or import of commodities from different parts of the world. Interestingly, the outbreak of 'Turkey X' disease that occurred in the United Kingdom in 1960s was the result of the importation of peanut meal from Brazil, which was subsequently incorporated into turkey diets and resulted in the death of many thousands of poults (Richard, 2008). This is an example of how the international grain trade can result in the movement of mycotoxins between continents. Moreover, it should be remembered that fungi do not recognize international boundaries, as fungal invasion and toxic production is associated with seasonal conditions and particular crops.

Wu (2004) has completed a comprehensive risk and economic analysis of lowering acceptable levels of fumonisins and aflatoxin in world trade. In that study she demonstrated that the United States would experience significant economic losses from tighter controls. The developing countries China and Argentina were more likely to experience greater economic losses than sub-Saharan Africa. The disturbing outcome of this detailed analysis was that tighter controls were, overall, unlikely to decrease health risks and may have the opposite effect (Wu, 2004). Otsuki *et al*. (2001) estimated health risks would be reduced by 2.3 deaths per million people per year in the EU if a lower aflatoxin standard was implemented. In other words, very stringent international trade regulations could lead to the situation where exporting countries, especially developing countries, would retain higher risk commodities, which would subsequently be available for their own populations, communities which are already exposed to higher level of mycotoxins than consumers in developed countries (Wu, 2008).

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3 OCCURRENCE OF TOXIGENIC FUNGI AND MYCOTOXINS

Fungi are a normal part of the microflora of standing crops and stored foods, but the production of mycotoxins depends upon the fungi present, agronomic practices, the composition of the commodity and the conditions of harvesting, handling and storage.

3.1 Biology and Ecology of Mycotoxigenic Fungi

Many studies have examined the fungal flora of food commodities from all climatic zones of the world. The species most often encountered belong primarily to five genera: *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium* and *Penicillium*. Other genera, including *Chaetomium*, *Claviceps*, *Diplodia*, *Myrothecium*, *Phoma*, *Phomopsis*, *Pithomyces* and *Stachybotrys*, also contain mycotoxic fungi (Moss, 1991). These moulds produce many different toxic compounds, but not all isolates of the same species produce toxins. The genera of most concern globally are *Aspergillus, Fusarium* and *Penicillium* (Miller, 1998), which are predominantly soil borne. An understanding of fungal biology and ecology, especially the manner in which fungi interact with their plant hosts, gives a good indication of the likely occurrence of mycotoxins, that is, where mycotoxins will be found, and the conditions that promote their synthesis and production by toxigenic fungi. Moreover, toxin production is often unrelated to total fungal biomass and the ecological requirements for growth and mycotoxin production may differ considerably between fungal species (Magan, 2006).

3.1.1 Conditions Favouring Mycotoxin Production

All food commodities serve as a suitable substrate for mould growth and mycotoxin formation. Toxigenic fungi can demonstrate variable toxin production depending on physical factors (moisture, relative humidity (RH), temperature and mechanical damage), chemical factors (carbon dioxide, oxygen, composition of substrate, pesticide and fungicides, etc.) and biological factors (plant variety, stress, insects, spore load, etc.) (Frisvad, 1995; Wicklow, 1995). Moisture and temperature are two factors that have a crucial effect on mould growth and mycotoxin production and are so intimately linked that it is difficult to discuss one independently from the other. Moisture is expressed in terms of RH and water activity (a_w). a_w has taken the place of moisture percentage or content as the most useful expression of the availability of water for micro-organism growth (Pitt and Hocking, 1997). The a_w is the ratio of the vapour pressure of the water in the substrate or feedstuff to that of pure water at the same temperature and pressure. The lower the a_w, the less available water is for micro-organisms. All micro-organisms, including toxigenic fungi have a minimum, optimum and maximum a_w for growth. Minimum water requirements for growth are different as temperature and substrate changes. The minimum a_w for growth can be lowest at the optimum temperature for growth and highest near the minimum and maximum growth temperatures. Also, the better the substrate for growth, the lower will be the limiting a_w. In general, the higher the a_w, the lower the temperature at which moulds will grow and produce mycotoxins (Pitt and Hocking, 1997).

Although a_w is the best measure of the availability of water in a commodity to fungi it is often more convenient to measure and think in terms of the moisture content (Wicklow, 1995) and the relationships for different cereals are shown in **Table 4**. Pathogenic fungi that invade crops prior to harvest usually require high moisture levels (20–25%) for infection compared to fungi that can proliferate during storage and grow at moisture levels in the range of 13–18%. Hence, most foods with moisture contents above 13% are susceptible to growth of toxic moulds and mycotoxin formation; although some commodities that have a high oil content (i.e. oilseeds) may be susceptible to mould growth at moisture contents below 13% (CAST, 1989; Wicklow, 1995).

3.1.2 Crops and Field Conditions

The accumulation of *Fusarium* toxins in cereal grains occurs during high moisture conditions around harvest (Sutton, 1982) (**Figure 3**), whereas preharvest aflatoxin contamination of crops, including peanuts (Diener *et al*., 1987) and maize, is associated with high temperatures, insect damage and prolonged drought conditions (Payne, 1998). Moreover, because *Aspergillus* can tolerate lower a_w than *Fusarium,* it is more likely to contaminate commodities both preharvest and postharvest, whereas *Fusarium* is more likely to be found as a contaminant preharvest (Abramson, 1998). Likewise, *Penicillium* usually invades plant produce postharvest (Leistner, 1984). These examples demonstrate that although it may be convenient to describe fungi as pre- and postharvest contaminants, the actual colonization and proliferation of fungi is not clear cut, but depends on the environmental and ecological circumstances, and the contaminating toxins will differ accordingly (**Table 5**).

Fusarium plant pathogens can cause major economic losses to crop production as they may cause seedling blights, root rots, crown rots, leaf blights, head blights and head and cob rots. These different plant pathological conditions result from infection by different *Fusarium* species and occur on different crops (Summerell *et al*.,

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Table 4 Relationship between moisture content (wet weight basis, %) and water activity (a_w) for some key cereals at 25 °C

	Moisture content (%)				
a_w	Maize	Wheat	Sorghum	Rice	Peanuts
0.98	30–32	30–34	31–32	26–28	16–17
0.95	26–27	26–28	26–27	23–24	14.5–15
0.90	23–24	21–22	22.5–23	20–21	12.5–13.5
0.80	16–17	16–17	18–19	17–18	9–10
0.70	15–16	14–14.5	16–17	14–14.5	7–8

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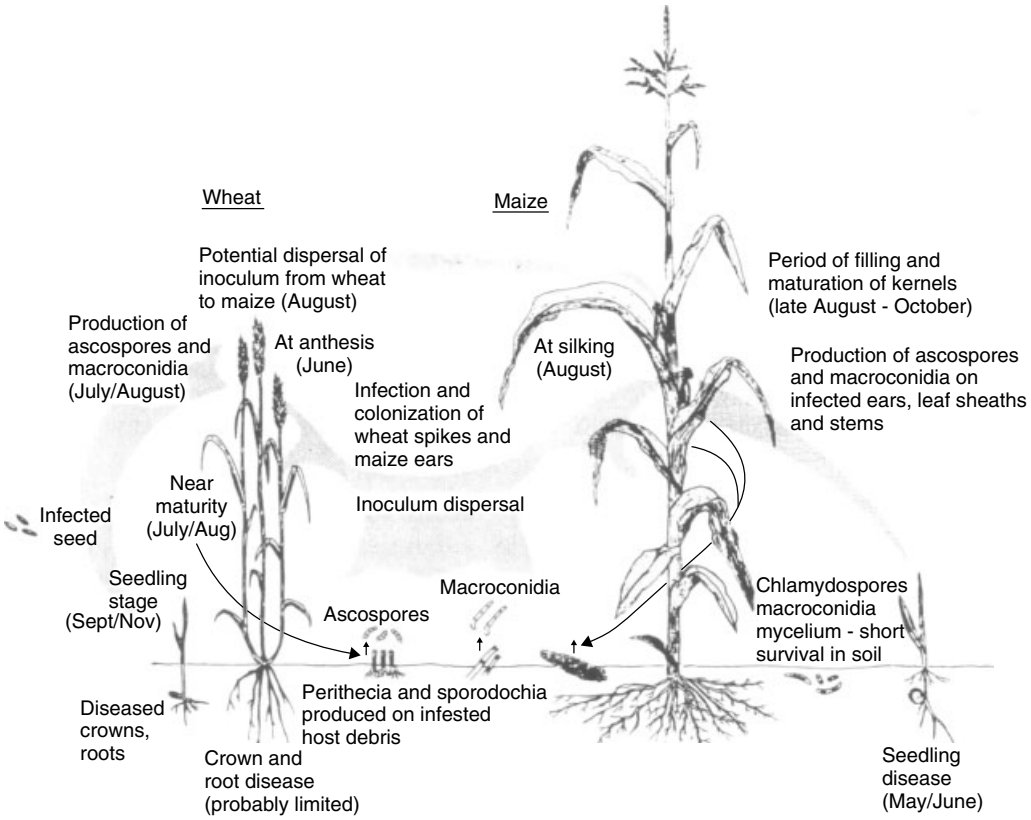

Figure 3 Disease cycle of *Fusarium graminearum* in wheat and maize. (Reproduced with permission from Sutton, 1982. © Canadian Phytopathological Society.)

2001). Glenn (2007) and Miller (2008) have recently reviewed the role of *Fusarium* species in mycotoxin contamination of small grains and maize. The most prominent DON-producing *Fusarium* species are *F. graminearum* and *F. culmorun*. These fungi are closely related and cause *Fusarium* head blight in small grains and *Gibberella* ear rot in maize. These infections occur more frequently when rainfall is high during flowering. Which of the two species will dominate depends on the temperature, with *F. graminearum* being associated with cereal grain in warmer areas and *F. culmorun* in cooler areas. *F. verticillioides* and *F. proliferatum* are associated with maize and produce fumonisins. These fungi cause *Fusarium* kernel rot, which occurs in warm, dry years and often in insect-damaged plants. Descriptions of the role of *Fusarium* species in plant disease can be found in a number of books, including Miller and Trenholm (1994), Leonard and Bushnell (2003) and Summerell *et al*. (2001).

Mycotoxin production by *Fusarium* species can be confusing for those who are new to mycotoxicology. In the past, *Fusarium* species were sometimes misidentified and recent changes to fungal taxonomy has resulted in the changing of the names of a number of species. In the last 20 years, the number of mycotoxigenic *Fusarium* species has increased from 13 to 42 (Desjardins, 2006). Marasas *et al*. (1984) were the first to attempt to reconcile *Fusarium* taxonomy and mycotoxin production. This was an excellent beginning and it has since been followed by Burgess *et al*. (1994), Leslie and Summerell (2005)

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Table 5 Pre- and postharvest contamination of cereals with their most commonly associated mycotoxins

Cereals	Preharvest	Post-harvest
Barley	DON, NIV, Zea, HT-2, T-2	OTA, Afla, Cit
Maize	DON, Fum, Zea,	Zea, Afla,
Oats	DON, NIV, HT-2, T-2	OTA, Cit
Rice	—	Afla, Sterig, OTA
Rye	Ergot	OTA
Sorghum	—	Afla
Wheat	DON, NIV, Zea, ergot	OTA, Afla, Cit

Note: Afla, aflatoxins; Cit, citrinin; DON, deoxynivalenol; Ergot, ergotamine; HT-2, HT-2 toxin; NIV, nivalenol; OTA, ochratoxin A; Sterig, sterigmatocystin; T-2, T-2 toxin; Zea, zearalenone.
Adapted from Petterrson, 2004.

Table 6 Some mycotoxigenic *Fusarium* species and the mycotoxins they produce[a]

Species	Mycotoxin
F. acuminatum	Acuminatum, aurofusarin, beauvericin, chlamydosporol, enniatins, fusarins, moniliformin, trichothecenes
F. armeniacum	Beauvericin, fusarins, trichothecenes
F. crookwellense	Aurofusarin, butenolide, culmorin, cyclonerodiol, fusaric acid, fusarins, trichothecenes, zearalenone
F. culmorum	Aurofusarin, butenolide, chlamydosporol, culmorin, cyclonerodiol, cyclonerotriol, fusarins, moniliformin, trichothecenes, zearalenone
F. equiseti	Beauvericin, equisetin, fusarochromanone, moniliformin, trichothecenes, zearalenone
F. graminearum	Aurofusarin, butenolide, chlamydosporol, culmorin, cyclonerodiol, fusarins, trichothecenes, zearalenone
F. proliferatum	Beauvericin, enniatins, fumonisins, fusaric acid, fusaproliferin, moniliformin
F. pseudograminearum	Trichothecenes, zearalenone
F. sporotrichioides	Aurofusarin, beauvericin, butenolide, culmorin, enniatins, fusarins, moniliformin, trichothecenes
F. verticillioides	Fumonisins, fusaric acid, fusarins, naphthoquinones

[a]Adapted from Desjardins, 2006.

and most recently Desjardins (2006). These books trace the evolution of *Fusarium* taxonomy and show quite graphically the number of metabolites that different *Fusarium* species can produce.

Historically, *Fusarium* classification relied on morphological characteristics of the fungus, but with recent advances, molecular techniques have been used to confirm previous classifications (Benyon *et al*., 2000; Desjardins, 2006). However, as emphasized by a number of authors, in particular Burgess and his colleagues (Burgess *et al*., 1994), it is critical when classifying fungi that a large number of isolates of the fungus in question are adequately assessed before making any definitive conclusions on the status of a species. The following examples give an indication of the classification difficulties that have occurred. *Fusarium moniliforme* was the species from which fumonisins were isolated and it has been reclassified as *Fusarium verticillioides*. This does create some confusion in the literature as neither fumonisins nor moniliformin, which were named after *F. moniliforme* (a species name which no longers exists), from which they were isolated, now relate to the fungus in question (Desjardins, 2006). *Fusarium graminearum*, which was described as two populations designated Group 1 and Group 2 (Burgess *et al*., 1994) has now been classified as *Fusarium pseudograminearum* and *Fusarium graminearum,* respectively (Desjardins, 2006). This has significant ramifications, as different crops are affected by these two species. *Fusarium* species may produce very different mycotoxin profiles, as shown in **Table 6**. Moreover, it can be seen from **Table 6** that many of the *Fusarium* produce a range of mycotoxins, many of which are structurally distinct (Cole *et al*., 2003) and give rise to different clinical signs and pathology in man and animals.

Table 1 shows the array of mycotoxins that can be produced by fungi and **Table 6** indicates that some fungi produce many different toxins. As noted above, it is considered globally that there are five major toxins that

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may cause significant food losses, and also impair human and animal health. In addition to these there are a number of other toxins, especially produced by *Fusarium* that could have significant and profound effects on man and animals. Interestingly, some of these toxins are produced by many species, whereas other toxins are produced by relatively few (Bryden *et al*., 2001; Desjardins, 2006; Jestoi, 2008). In most instances there have not been systematic surveys of these toxins because of the lack of routine analytical methods, so there is no appreciation of the extent of their occurrence in nature. Moreover, the possible interactions between toxins in disease aetiology and pathogenesis requires further study (CAST, 2003; Speijers and Speijers, 2004). In many instances intoxication is likely to result from the ingestion of low concentrations of a number or 'cocktail' of mycotoxins. The role that mycotoxins play for fungi in nature is a question that creates much speculation (Desjardins, 2006).

Many of the initial studies that examined the infection of agricultural commodities by *Aspergillus* species concentrated on stored commodities. However, it was eventually shown that aflatoxins could also be formed in plant products prior to harvest, especially in peanuts and corn (Payne, 1998). The *Aspergillus* sp. that produce aflatoxins are *A. flavus*, *A. parasiticus* and *A. nomii* (Dorner, 2008). The factors that contribute to *Aspergillus* invasion of peanuts are shown in **Figure 4**. In overview, as conditions of water and heat stress affect plants, especially late in the growing season, this gives the fungus a competitive advantage over other fungi, and, at the same time, places the plant, whether it be a peanut or a maize plant, under considerable stress. The initial isolation of aflatoxin was made from groundnuts. In this crop, plant varieties with a high incidence of pod splitting, drought conditions and soils with a low water-holding potential interact to increase fungal invasion and aflatoxin production before harvest (Diener *et al*., 1987; Dorner *et al*., 1989). Interestingly, very high kernel moistures may prevent aflatoxin formation, presumably by facilitating growth of other micro-organisms, or it may be a reflection of active host cells that can resist invasion. In some field situations, especially in peanuts, where *A. flavus* tends to be the dominant species, cyclopiazonic acid can also be formed. Cyclopiazonic acid, like ochratoxin, can be formed by both *Aspergillus* and *Penicillium* species and be a co-contaminant with aflatoxin (Bryden *et al*., 2004) in cereal grains. The contamination of grapes and wine with ochratoxin following field invasion of the crops with *Aspergillus* species, especially *A. carbonarius* is of growing concern internationally (Visconti *et al*., 2008). The rheological behaviour of starch is influenced by fungal growth in, and DON contamination of, the wheat from which it was derived (Lancova *et al*., 2008a).

Aflatoxin production in field maize (Payne, 1998; Munkvold, 2003) is facilitated by drought stress and other stress conditions that predispose the plant to attack by a number of pests, including toxin-producing fungi. Fungal attack is facilitated by a damaged seed. Insects cause the type of damage generally associated with fungal infection and probably serve as vectors of the initial fungal inoculum (Dowd, 1998). Developing kernels are most susceptible to infection during the late milk-dough stage. Damaged kernels are usually associated with mycotoxin contamination, but intact, undamaged kernels have been reported to contain aflatoxin, suggesting fungal invasion via the silk (Payne, 1998). These inter-relationships are shown graphically in **Figure 5**.

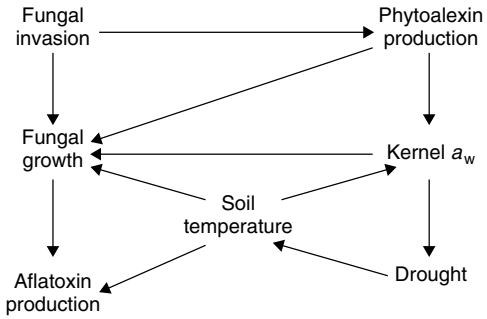

Figure 4 The inter-relationship of several factors involved in preharvest aflatoxin contamination of peanuts. Arrows indicate an influence of one factor upon another. (Reproduced from Dorner *et al*., 1989. © Springer.)

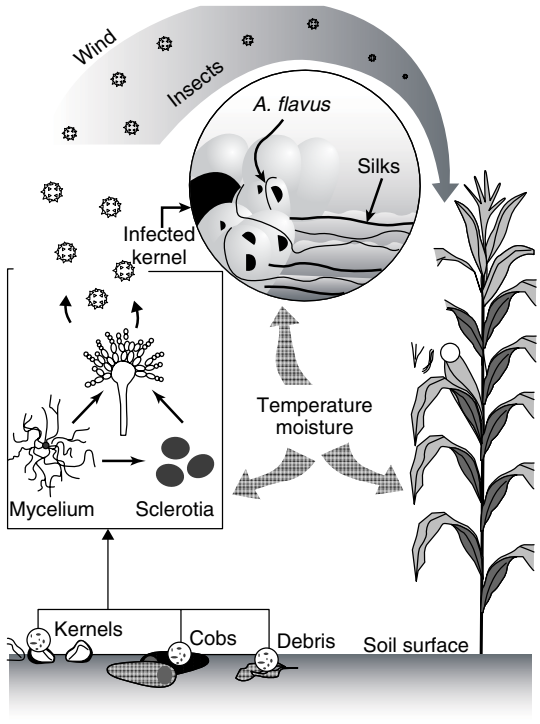

Figure 5 Schematic diagram showing possible sources of inoculum of *Aspergillus flavus* and the routes of colonization of the fungus on maize. (Reproduced from Payne, 1998. © Springer.)

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3.1.3 Storage Conditions

Stored grain is not static; it is in a dynamic state and may be contaminated both externally and internally by fungi and insects. These interrelationships are affected by climatic factors such as temperature and humidity, by geographical location, by the type of storage container and by the mode of grain handling and transport (Christensen, 1974; Chelkowski, 1991; Jayas *et al*., 1995). Moisture depends mostly on water content at harvest, the amount of drying, aerating and turning of the grain before or during storage as well as the respiration of insects and microorganisms. Provided grain is dry when placed in storage, moisture can only rise from leaks or condensation. Grain may go into storage at a uniform temperature but over a period the grain mass will cool at a different rate in the centre than at the periphery. As a result of temperature differentials moisture migrates through the storage bin, resulting in condensation and the provision of ideal conditions for mould growth or the development of 'hot spots' in localized areas (Wicklow, 1995). If grain of widely different moisture contents is blended to give a presumably safe average moisture content, there is a great danger of development of self-propagating 'hot spots'. Similarly, microbial and insect growth in stored grain also results in moisture condensation and the potential development of 'hot spots'. The minimum critical levels for the growth of fungi are 7–15% moisture (depending on commodity) and 80–85% RH. Temperatures at which toxin production can take place vary from 0 to 35 °C, depending on the fungal species concerned (Wicklow, 1995; Frisvad, 1995).

3.2 Mycotoxins

Mycotoxins vary greatly in their structure and chemical composition (Cole *et al*., 2003) which has significant ramifications for analysis, metabolism and toxicity.

3.2.1 Mycotoxin Analysis

Mycotoxin analysis is a highly developed field that continues to expand, and the interested reader is referred to the following reviews, which detail the latest developments in mycotoxin analysis (Gilbert and Anklam, 2002; Logrieco *et al*., 2005; Maragos, 2004; Krska *et al*., 2008). Sampling is the greatest source of error in quantifying mycotoxin contamination (Whitaker *et al*., 1995; Whitaker, 2004) because of the difficulty of obtaining food samples representative of that which may have caused a mycotoxicosis or for regulatory purposes from large grain consignments. These difficulties arise because of the existence of 'hot spots' of fungal proliferation and the resulting uneven distribution of toxin within a commodity and the low levels, ranging from microgram per gram to milligram per gram, at which mycotoxins occur (CAST, 1989). Other major difficulties for the analyst are the vast array of chemical compounds that constitute mycotoxins and the different food matrices in which they occur. This is further complicated by the occurrence of conjugated mycotoxins in foodstuffs. These so-called 'masked' mycotoxins can occur following plant metabolism (Gareis *et al*., 1990; Berthiller *et al*., 2007) and are not detected with conventional analytical procedures. For example, zearalenone-4-glucoside, a conjugate of zearalenone, and DON-3-glucoside, a conjugate of DON, can constitute up to 20% of the total content of the precursor mycotoxin in the foodstuffs (Berthiller *et al*., 2005; 2006; Lancova *et al*., 2008b). It is likely that these conjugates will be hydrolysed following ingestion, thus increasing exposure to the precursor toxin. There is also evidence that OTA and fumonisins are conjugated by plants (Berthiller *et al*., 2007) and fumonisins may also be conjugated during food processing (Humpf and Voss, 2004). Recently, Krska and his colleagues have developed chromatographic techniques that allow the simultaneous determination of mycotoxins and their conjugates (Berthiller *et al*., 2007; Krska *et al*., 2005; 2008).

The analytical procedures for mycotoxins can be summarized as follows (Cole, 1986a). After a sample has been obtained, it is ground and subsampled prior to analysis. Mycotoxins are usually extracted from food or agricultural products using organic solvents, and after extraction filtration it is often necessary to remove unwanted solids. Various clean-up techniques have been developed to remove substances that interfere with accurate quantification of the mycotoxins during analysis. A variety of solid-phase extraction columns or immunoaffinity columns are used extensively in the clean-up of samples for mycotoxin analysis. Aflatoxin, zearalenone, patulin and ochratoxin contain a chromaphore and can therefore be quantified using thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC). Mycotoxins that do not contain a distinctive chromaphore, for example, fumonisins and some of the trichothecenes, can be derivatized and analysed by HPLC or gas chromatography (GC). Alternatively, these compounds can be analysed directly by GC/mass spectrometry (GC/MS) or LC/MS.

Application of immunological methods to mycotoxin analysis is an exciting advance in mycotoxin detection (Pestka, 1994). Immunoassays appear to offer the best opportunity for the development of rapid, repeatable and sensitive assays. These techniques are based on the highly specific reaction between an antibody and an antigen and require the development of an antibody against the mycotoxin that is to be assayed. Mycotoxins are nonantigenic, but are able to illicit an antibody response after conjugation to a protein or polypeptide carrier. The availability of antibodies to a number of mycotoxins has allowed the development of radioimmunoassays (RIAs)

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and enzyme-linked immunosorbent assays (ELISAs) for the detection of toxins in feedstuffs and residual mycotoxins or metabolites in body fluids (Krska *et al*., 2005). The ELISA technique offers advantages over RIA in that radioisotopes and appropriate monitoring equipment are not required. The ELISA is also more adaptable to field use. Commercial ELISA kits have become available for aflatoxins, zearalenone, DON, ochratoxins and fumonisins.

3.2.2 Mycotoxin Occurrence

Fungi are able to grow on all kinds of food, including cereals, fruit, vegetables, nuts and the products produced from these different commodities. Extensive surveys have been published on the occurrence of mycotoxins, including those by Scott (1978), van Egmond and Speijers (1994), Filtenborg *et al*. (1996), Meister and Springer (2004), Drusch and Aumann (2005), Binder *et al*. (2007), Roscoe *et al*. (2008) and Trucksess and Scott (2008). Some mycotoxins remain within the fungal mycelium or within spores, but most are excreted by the fungus into the food. In grains and solid foods like cheeses, bread and apples, the diffusion of the toxin from the fungus is usually only in the immediate area surrounding the fungus, leaving much of the product uncontaminated. For liquid foods, and fruits like peaches and tomatoes, diffusion of the mycotoxins is throughout the food product. However, most mycotoxins are very stable and once in a food product are resistant to further food processing. The corollary to this is that the use of mouldy material during food processing may result in the product being contaminated with the mycotoxin. This has been demonstrated with the transfer of DON from barley into beer (Lancova *et al*., 2008b) and ochratoxin from grapes into wine (Visconti *et al*., 2008).

As indicated above, there are five major classes of mycotoxins that are considered to be the most significant threat to food safety globally. The fungi that produce these toxins also produce other toxins (**Table 1**) and in field situations co-contamination is likely to occur. Binder *et al*. (2007) have completed a global survey of the major mycotoxins and found low-level contamination throughout the world, with significant regional differences, especially between tropical and temperate areas. Most food products, because of their chemical composition and the conditions under which they are produced and/or processed, have only a limited number of toxigenic fungi associated with them and it has been estimated that there is usually only 2–10 species that will contaminate any given food commodity (Filtenborg *et al*., 1996).

In many situations, mycotoxin-contaminated cereal grains are diverted to manufacture feed for livestock. This poses the question, is there likely to be significant contamination of animal products with mycotoxins or their metabolic products that will be subsequently consumed by the human population? The major area of concern is the transfer of mycotoxins into milk (Shreeve *et al*., 1979) especially aflatoxin (van Egmond, 1989; Fink-Gremmels, 2008). The hydroxylated metabolite of aflatoxin B_1, aflatoxin M_1, is excreted into milk from 1 to 6% of dietary intake (Petterrson, 2004; Fink-Gremmels, 2008). This large variation in the carry-over of aflatoxin M_1 into milk may reflect the studies undertaken, including feeding practices, breed and productivity of cows, toxin excretion rates and/or analytical procedures used. Nevertheless, it is recognized that milk can be an important vehicle for aflatoxin exposure, not only in dairy animals, but also in human breast milk (El Nezami *et al*., 1995).

OTA is rapidly metabolized in the rumen to ochratoxin α and phenylalanine, but low levels may escape rumen metabolism and low quantities have been detected in cow's milk. OTA has been found in tissues of pigs and poultry that had been fed OTA-contaminated feed. There are significant differences amongst studies and this is presumably due to differences in absorption and metabolism of the toxin; it has been shown that the half-life in pigs and chickens is 180–140 hours and approximately four hours, respectively (Petterrson, 2004). Residues of other mycotoxins, including zearalenone, trichothecenes and fumonisins are not considered to be of public-health importance, as only very low levels of the toxins have been found in the tissues of animals that had been fed very high levels of the toxins in experimental situations (Pestka, 1995; Petterrson, 2004). Cyclopiazonic acid, which can occur as a co-contaminant with aflatoxin, has been shown experimentally to accumulate in meat, milk and eggs of animals and poultry fed this toxin (Dorner *et al*., 1994). Pestka (1995) conducted an extensive review on the possibility of residues occurring in animal products and concluded that trace levels of mycotoxins and their metabolites may carry over into edible tissues of meat-producing animals, but the evidence suggests that the levels transmitted do not pose a public-health risk.

4 STRATEGIES FOR MYCOTOXIN MITIGATION

Conditions favouring the development of mycotoxins in food commodities before and after harvest have been discussed in preceding sections. There are many factors that affect mycotoxin formation and any control or management programme that seeks to reduce contamination by these compounds must take into account the complex interaction between these different factors. Overall, there are a number of approaches that can be taken to minimize mycotoxin contamination in the food chain, and these involve prevention of fungal growth and therefore mycotoxin formation, strategies to reduce or

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eliminate mycotoxins from contaminated commodities or diverting contaminated products to low-risk uses.

4.1 Genetic Modification of Fungi and Crops

Fungal geneticists have unravelled the pathways and the genes responsible for the synthesis and regulation of the production of the major mycotoxins, especially aflatoxin and the trichothecenes (Yu and Keller, 2005; Bhatnagar *et al*., 2008). In the future this may allow the development of approaches that will reduce or eliminate mycotoxin production by the fungus, including the production of inhibitors which could be incorporated into plants and thus reduce the synthesis of the toxin and/or increase the tolerance of the plant to the toxin (Holmes *et al*., 2008). There has been much interest in developing crop varieties that are resistant to toxigenic fungi, but studies in maize (Duvick, 2001; Munkvold, 2003) and peanuts have shown that it is difficult to achieve a high level of genetic resistance to toxigenic fungi. Moreover, in many field situations, the extremes of climate and changes in agronomic practices may render a crop variety vulnerable to fungal infection and effectively eliminate any advantages made in genetic progress.

There has been increasing interest in the application of transgenic resistance against toxigenic fungi and mycotoxins (Bhatnagar *et al*., 2008). There are three basic strategies that can be used; (i) reducing infection by the pathogen, (ii) inserting genes capable of degrading the toxin or (iii) reducing mycotoxin accumulation by interfering with the biosynthetic pathway. Theoretically, these techniques have great promise for the reduction of mycotoxin accumulation in crops, but are only in their infancy, and numerous technical, economic and social aspects will need to be overcome before there will be widespread acceptance of these approaches (Bhatnagar *et al*., 2008; Wu, 2008). Moreover, the difficulty with any of these transgenic approaches is that they are likely only to be effective against one toxin or perhaps a fungal genera and this then leaves the crop vulnerable to the accumulation of other toxins. In some respects, this is demonstrated by the success of the Bt maize hybrids, which have been developed as a means of transgenic insect protection. The transgenic Bt corn or maize contains a gene from the soil bacterium *Bacillus thuringiensis* which encodes for a protein that is toxic to common lepidopteran maize pests. These hybrids offer a new tool for mycotoxin management because insect damage is often a major aetiological factor in facilitating toxigenic fungal infection of crops (Wicklow, 1995; Dowd, 1998).

Bt maize is effective in reducing the incidence of fumonisin contamination, but less effective in reducing DON contamination. This reflects different disease patterns and pathogens as DON is associated with *Gibberella* ear rot, whereas fumonisin production is associated with *Fusarium* ear rot and the occurrence of *Gibberella* ear rot is not as strongly influenced by insect damage as is fumonisin accumulation. Aflatoxin contamination of maize has not been consistently reduced in Bt hybrids compared with non-Bt hybrids (Munkvold, 2003). Aflatoxin accumulation is influenced by insect injury to the plant, but this is probably overridden by drought and high temperatures, which explains the inconsistent results that have been found with aflatoxin accumulation and the use of Bt maize hybrids.

4.2 Agronomic and Biological Control Measures

There are a number of agronomic factors that need to be considered if fungal infection and mycotoxin production is to be prevented. Essentially these techniques involve reducing crop stress or changing the ecological niche of the fungus in such a manner that the spore load of the fungus is reduced and the probability of fungal infection of the crop correspondingly decreased. Good Agricultural Practice (GAP) would indicate that crop rotations, tillage and ploughing practices, soil fertilizer regimens and planting date all have a significant impact on the viability of a crop and whether it can withstand fungal attack (Munkvold, 2003; Jouany, 2007). Other agronomic aspects, including insect control, weed control and also tillage practices have a major impact on the spore load. Weeds are often natural reservoirs for many fungi, especially *Fusarium* (Burgess *et al*., 1975). Depending on crop rotations and the depth to which the remnants of the previous crop is ploughed into the ground, the debris is often a major source of subsequent fungal infection, as all of these fungi are soil organisms (Dill-Macky and Jones, 2000). The complexity of fungal infection in the field, especially with *Fusarium* sp., make it difficult to control these diseases with fungicides; application rate and application timing are important for optimum efficacy (Jennings, 2004). Suboptimal application rates of some fungicides can result in the stimulation of mycotoxin production (Magan, 2006).

An aspect of the ecology of the fungus that has been recently exploited by a number of groups is using the concept of 'competitive exclusion', in which a nontoxigenic fungus is used to displace the toxigenic fungus in the field. Studies have been conducted with peanuts, cotton and maize. In the studies with peanuts (Dorner and Horn, 2007; Dorner, 2008) it has been shown that using a nontoxigenic strain of *A. flavus* over a couple of growing seasons considerably reduced the incidence of aflatoxin contamination of field peanuts. It appears

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that *A. flavus* strains are more effective than *A. parasiticus* in displacing toxigenic strains in the environment (Dorner and Horn, 2007). The other aspect of the application of nontoxigenic strains to field situations is that great care must be taken to ensure that the fungus does not produce other toxins; for example, in the work carried out with peanuts, only *A. flavus* strains that did not produce cyclopiazonic acid were used (Dorner, 2008). If this approach is applied to *Fusarium* it may be more difficult to find strains that are nontoxigenic, because as noted above (**Table 6**), many *Fusarium* species produce a number of different toxins.

4.3 Climate Modelling to Predict Mycotoxin Risk

Climate conditions during fruiting or seed setting and at harvest can play a significant role in toxin accumulation. If, for example, peanuts are harvested and left in wind rows without adequate drying, significant accumulation of aflatoxin can occur. With maize, the stage of the kernel at harvest can have significant ramifications for contamination by fumonisins and other *Fusarium* toxins, and infection by *Aspergillus* at this time can lead to greater build up of aflatoxins.

Examination of the climatic conditions under which crops are grown gives a good indication of the possible threat of fungal invasion and mycotoxin accumulation prior to harvest. With this understanding a number of groups have used climatic conditions to model or predict preharvest toxin production and accumulation (Schaafsma and Hooker, 2007). Models for aflatoxin contamination of peanuts (Wright *et al*., 2005; Dorner, 2008) and maize (Chauhan *et al*., 2008) have been developed. A model has also been developed to minimize DON production in wheat. The model developed in Australia and called AFLOMAN by Wright *et al*. (2005) allows farmers to evaluate the risk of aflatoxin contamination of their peanuts. They may decide to harvest earlier than normal to minimize aflatoxin contamination and therefore avoid the financial penalties that occur when this mycotoxin contaminates their crop. The Canadian model DONcast (Hooker *et al*., 2002) for the prediction of DON in wheat has been used successfully commercially and adapted for use in the United States, Uruguay and France (Schaafsma and Hooker, 2007; Miller, 2008). Greater difficulty has been experienced in developing models to predict aflatoxin, DON and fumonisin accumulation in maize, due to the more complex fungal, insect/crop interaction compared with wheat (Schaafsma and Hooker, 2007). Nevertheless, it is possible to develop models to predict mycotoxin risk in crops and use that information to change management practices on a season-by-season basis. At this time, this approach is only appropriate for developed countries with significant infrastructure that allows online monitoring of meteorological data. In the future, as remote sensing technology becomes more sophisticated and available, it should be possible to apply modelling technology in less developed economies.

4.4 Storage Management

Moisture and temperature are two factors that have a crucial effect on fungal proliferation and toxin elaboration during storage, as noted above. Moisture depends mostly on water content at harvest, and the amount of drying, aerating and turning of the grain before or during storage, as well as the respiration of insects and micro-organisms (Christensen, 1974). Rewetting may occur by leaks or flooding into storage units, but by far the most common cause is condensation, which can give rise to the development of 'hot spots'. Condensation often results from moisture migration in bulk stores under the influence of temperature differentials within the grain that are mediated by fluctuations in ambient temperature (CAST, 1989). These problems are minimized if storage time is kept to a minimum. As described above, with knowledge of the composition of the food commodity and storage conditions it should be possible to predict the likely fungal spoilage organisms and develop control programmes that target those fungi and prevent their growth and the production of mycotoxins in the food commodity (Filtenborg *et al*., 1996).

Prevention of mycotoxin formation in stored feeds is a major goal in controlling mycotoxicoses in livestock and poultry (Smith and Henderson, 1991). Good feed management practices (for example, systematic clean-up of feedlines and augers; feed grain storage at proper moisture content, that is, below 13%; inspection of grain regularly for temperature, insects and wet spots) will limit the possibility of fungal development in feedstuffs and complete feeds. The risk of feed contamination will be reduced in units with rapid turnover of feed because there will be less time for fungal growth and toxin production. Apart from methods that modify the fungal environment, many compounds are available that will inhibit mould growth in feedstuffs. Organic acids, especially propionic acid, form the basis of many commercial antifungal agents used in the stockfeed industry and give excellent protection (Hamilton, 1985). An alternative approach is the application of essential oils, such as cinnamon and clover-leaf oil, which have the capacity to control fungal growth (Magan, 2006).

4.5 Food Processing

Many studies have been completed that have followed the levels of mycotoxins in food commodities as they have been processed (Jackson and Bullerman, 1999;

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Humpf and Voss, 2004; Murphy *et al*., 2006). Mycotoxins may appear to be destroyed, but in many instances the mycotoxin is not destroyed, but accumulates in one fraction of the commodity during the process. As is shown in **Table 7**, the processing of peanuts through colour sorting, blanching and so on can virtually remove all the aflatoxin from the finished product (Dorner, 2008). In instances like this, all of the contaminated peanuts are removed and those remaining are essentially toxin free. The oil is subsequently removed from the contaminated nuts and the resulting meal is often diverted to animal feed, but only if below regulatory levels.

In instances where there is only surface colonization of the grain, removal of bran fractions will remove most of the toxin, and these fractions can then be diverted to animal feeds (Meister and Springer, 2004). In many situations, removing screenings from maize will remove the greatest portion of the grain that is contaminated with fumonisins. However, as this can be a substantial fraction of the total amount of grain, this can be uneconomical in many circumstances. Wet milling of maize results in starch almost free of zearalenone, fumonisins, DON and nivalenol (NIV). DON and NIV are recovered in steep water, whereas fumonisins and zearalenone are found mainly in the gluten fraction. In dry-milled food products, the distribution of mycotoxins among the fractions depends on the fungal penetration of the endosperm (Meister and Springer, 2004; Castells *et al*., 2005). Scudmore *et al*. (2008a; 2008b) have shown that the relationship between the concentration of *Fusarium* mycotoxins in cereal grains and finished products is toxin and process dependent.

The primary amine group in fumonisins is key to its chromotagraphic detection and can be derivatized with acids and/or react with reducing sugars (Humpf and Voss, 2004). The products formed in these processes are essentially nontoxic. Alkaline processing of maize contaminated with fumonisins releases the tricarballylic groups and hydrolysed fumonisins have reduced toxicity. It appears that the cooking, steeping and rinsing processes involved in the production of tortillas (Mexican Style) is effective in reducing the fumonisin content (Murphy *et al*., 2006), but baking *per se* has little effect on the fumonisin content in the final product (Humpf and Voss, 2004). The difficulty with many studies that have attempted to quantify the toxicity of products after processing is that the biological activity of fumonisin breakdown products is unknown. Moreover, these products may bind to the food matrix and become 'hidden', but may become reactivated following ingestion. Ammoniation has also been shown to reduce fumonisin concentrations in maize (Norred *et al*., 1991).

It has been shown that roasting peanuts resulted in greater reductions in chemically detectable aflatoxins than boiling (Dorner, 2008). There are other processes that involve alkali treatment and boiling of aflatoxin-containing commodities and these will reduce aflatoxin to very low levels (Murphy *et al*., 2006). Alkali processing, or nixtamalization, has been shown to significantly reduce aflatoxin levels in maize-based products. There are also reports that acidification of a product following nixtamalization can reconstitute the aflatoxin postprocess (Murphy *et al*., 2006). Perhaps this has implications for gastrointestinal kinetics of aflatoxin following these processes.

4.6 Detoxification

Approaches to detoxification of mycotoxin-contaminated grain and other food commodities have included physical, chemical and biological treatments (Cole, 1989; Trenholm *et al*., 1989; CAST, 2003; Kabak *et al*., 2006; Jouany, 2007). In cases of light to moderate mycotoxin contamination, physical methods for cleaning the kernel surface, and hence removing the more heavily contaminated particulate matter, have proven effective in reducing mycotoxin concentrations as noted above. A range of chemical treatments have been applied to contaminated products and have been found to vary in their effectiveness at reducing mycotoxin concentrations in contaminated grain or feed (Galvano *et al*., 2001). Ammoniation has been used widely to reduce aflatoxin levels and there have been some commercial plants developed for this process (Park and Price, 2001). However,

Table 7 Reduction of aflatoxins postharvest[a,b]

Process	Aflatoxin level ($\mu g\,kg^{-1}$)	Reduction (%)	Cumulative reduction (%)
Farmer's stock	217	—	—
Belt separator	140	35	35
Shelling plant	100	29	54
Colour sorting	30	70	86
Gravity table	25	16	88
Blanching/colour sorting	2.2	91	99.0
Re-colour sorting	1.6	27	99.3

[a]Results were obtained from processing of a 40 000 kg lot of contaminated peanuts.
[b]From Cole *et al.*, 1995.

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because of expense this process is not widely adopted. All processes that seek to decontaminate infected grain, especially where they involve rigorous chemical or heat treatments, must be cost effective and must not reduce the nutritional content of the grain or food if they are to be accepted generally.

A diverse variety of substances have been investigated as potential mycotoxin-binding agents (Galvano *et al*., 2001; Huwig *et al*., 2001), including lucerne, synthetic cation- or anion-exchange zeolites, bentonite, spent canola oil, bleaching clays and hydrated sodium calcium aluminosilicate (HSCAS). Several studies have examined the efficacy of HSCAS to reduce the toxicity of a variety of mycotoxins in several animal species. HSCAS is a high-affinity absorbent for aflatoxins, capable of forming a very stable complex with the toxin and hence reducing its bioavailability (Phillips, 1999). Other approaches have been used to bind mycotoxins (Jouany, 2007), in particular a yeast cell-wall-derived glucomanin prepared from *Saccharomyces cerevisiae* has been shown to efficiently adsorb aflatoxin, zearalenone and fumonisins (Dawson *et al*., 2001). A feed additive that is a new stabilized bacterial species can detoxify trichothecenes by removal of the epoxide group *in vivo* (Binder *et al*., 2001; Fuchs *et al*., 2002) and is a novel approach to mycotoxin decontamination (Molnar *et al*., 2004).

Many approaches have been used to reduce the toxicity of mycotoxin-contaminated commodities. However, most methods have been tested on a limited number of specific toxins. Since contaminated grain may contain a broad range of toxins of differing chemical characteristics, including heat stability, solubility and adsorbent affinity, a detoxification procedure that works well for individual toxins may not be effective for the diverse mycotoxin combinations that may occur naturally. Moreover, no single treatment has proved completely successful in degrading or removing toxins and retaining the nutritional and functional qualities of the treated commodity.

4.7 Integrated Mycotoxin Management

An integrated approach to mycotoxin control is the application of the Hazard Analysis and Critical Control Points (HACCP). This system is used extensively in the food industry to control bacterial contamination (Ropkins *et al*., 2003) and there has been much interest in its application to the control of mycotoxins throughout the food chain (Lopez-Garcia, 2001). Aldred and Magan (2004) have reviewed the application of this approach to mycotoxins and, as shown in the **Table 8**, there are seven principles that define a HACCP programme. The successful application of this approach should reduce mycotoxin contamination in all sectors of the food chain and have the added advantage of increasing production efficiency, which is an important consideration given the increasing need for food production globally. The development of a HACCP programme should evolve in conjunction with other complimentary approaches to agricultural production, namely GAP, Good Manufacturing Practice (GMP), Good Hygiene Practice (GHP) and Good Storage Practice (GSP) (Aldred and Magan, 2004).

Table 8 The HACCP principles

Principle 1: Identification of hazards; assessment of risk; description of control measures.
A hazard is something which can have a negative effect on health.
Risk is the probability of an adverse effect resulting from a hazard.
Principle 2: Identification of the critical control points (CCPs).
A critical control point is a step in a process where control is possible, and loss of control may lead to an unacceptable health hazard. A CCP may be a raw material, a location, a practise, a procedure or a process stage, but it must be specific.
Principle 3: Establishment of critical limits
Critical limits are the tolerances applied to CCPs which maintain a safe condition.
Principle 4: Establishment of procedures for monitoring of CCPs and
Principle 5: Establishment of corrective actions
Principle 6: Establishment of verification procedures
Principle 7: Documentation and record keeping

Adapted from Aldred and Magan, 2004.

A number of HACCP programmes (Aldred and Magan, 2004; Lopez-Garcia *et al*., 2008) have been developed for aflatoxin in maize, animal feeds, copra and coconut, groundnuts and pistachio nuts, and also for OTA in coffee. The success of these programmes will require the development of rapid diagnostic tools to monitor the occurrence of mycotoxigenic fungi using molecular technology (Niessen, 2008) and the application of molecular imprinted polymers and lateral flow devices for quantification of mycotoxins (Magan, 2006). The difficulty of applying this approach, especially in high-risk areas in developing countries, is that these processes may not be economically feasible or practical, and therefore the other approaches are required to minimize mycotoxin contamination.

4.8 Human Intervention

In the preceding sections, the emphasis has been on techniques that reduce exposure to mycotoxins, but complete elimination of mycotoxins from the food chain is extremely difficult, if not impossible. Therefore interventions that modify the metabolism and deposition of these toxins have been explored in animal models.

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In the animal feed industry, for example, it has been shown that feed additives that adsorb or degrade mycotoxins are effective in significantly reducing risk. In the developing world, education programmes that describe the appropriate storage of food commodities is an important strategy to reduce mycotoxin contamination (Turner *et al*., 2005). Nevertheless, in many high-risk regions, it will be essential to include additional intervention strategies. In this regard HSCAS-marketed NovaSil is being evaluated in human clinical trials as a means of adsorbing or trapping aflatoxin and preventing its absorption from the gastrointestinal tract (Phillips *et al*., 2008), as discussed above.

Another interesting compound with anticarcinogenic properties is chlorophyllin, a water-soluble derivative of chlorophyll, which is able to sequest aflatoxin and also appears to induce enzymes that metabolize the toxin (Fahey *et al*., 2005). It has been shown to protect against aflatoxin carcinogenesis in trout (Dashwood *et al*., 1998) and rats (Simonich *et al*., 2007). It has been demonstrated in animal and human studies that oltipraz, a dithiolethione, is an effective agent in blocking aflatoxin DNA-adduct formation, which primarily reflects the induction of aflatoxin-detoxifying enzymes and increased conjugation of aflatoxin with glutathione (Kensler *et al*., 1999). However, the multiphase and long-term development of hepatocellular carcinoma may limit the effectiveness of chemotherapeutic agents, along with the cost and possible adverse effects of long-term drug intake (Kensler *et al*., 2004). It is the considered the view of the WHO that, because of the interaction of aflatoxin and the hepatitis B virus, the most cost-effective intervention to reduce the incidence of hepatocellular carcinoma is a vaccination against viral infection (Henry *et al*., 1999). Nevertheless, to break the cycle that begins with infection at birth, universal vaccination must be carried out for at least two generations (Kensler *et al*., 1999).

Groopman *et al*. (2008) have reviewed the interventions that can be applied to reduce aflatoxin-induced carcinogenesis, especially in developing countries. In addition to the possible interventions already noted, other possibilities include green tea polyphenols (Moyers and Kumar, 2004), which inhibit various chemically induced cancers in experimental animals, and sulforaphane (Talalay and Fahey, 2001). Glucosinolates, which are found in many plants (Cheeke, 1998), are a precursor of sulforaphane and therefore a potentially affordable chemopreventive agent. These examples and the lessons that have been learnt from aflatoxin will, in the future, no doubt be applied to other mycotoxins. A reoccurring point made by Groopman *et al*. (2008) is the importance of having suitable biomarkers, so that reliable data on mycotoxin exposure and dose can be determined. Biomarkers are also necessary to monitor the efficacy of intervention programmes.

5 REFLECTION

The link between human health and agriculture is often ignored by historians, but any consideration of population demographics demonstrates the importance of agricultural production and public health (Scholthof, 2003). In her fascinating book *Poisons of the Past: Mould, Epidemics and History,* Mary Matossian (1989) contends that mycotoxins have probably been responsible for more loss and suffering in animals and man than any other class of food toxin. In particular, she makes the following observation:

> *It may seem irregular to use plant health and climatic indicators of plant health as an index of human health, but the two are intimately related. If plants that people eat are toxic or diseased, people will either be diseased or simply not as healthy and fertile as they would be if they were properly nourished. The population in the area identified more or less, in the heart of western civilization, did not begin to thrive until the sixteenth century (and then only slowly until about 1750) and they grew at different rates in different regions. These regional differences in fact highlight the effects of plant health on human health because of the varied conditions of plant growth in different environments.*

She further explains that epidemics, outbursts of bizarre behaviour and low fertility and high death rates from the fourteenth to the eighteenth century may have been caused by food poisoning from mycotoxins, especially ergot alkaloids, in bread, the staple food in Europe and America during this period (Matossian, 1989).

Mycotoxins, which are natural environmental compounds, will continue to contaminate the food chain and remain a threat to global food security (Strange and Scott, 2005). In times of drought, flooding and other natural disasters, mycotoxins are likely to be more prevalent (Bryden, 2007). Food commodities that are subject to fungal attack, especially cereal grains, will continue to be important economically and nutritionally. There now are a number of strategies that include agronomic practices, plant breeding and transgenics, biotechnology, toxin binding/trapping food additives and education that are available to reduce the risk of mycotoxin exposure. Any realistic assessment of the present-day risk to human health posed by mycotoxins will show that the risk is very dependent on the country and the area in which the individual is living. The risk is greatest in developing countries (Wild, 2007), and acute mycotoxicoses still occur periodically in these populations.

However, it is the chronic effects of mycotoxins on human populations through modulation of the immune system and increased cancer risk that are of major concern to national and international regulatory agencies.

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The ubiquitous nature of these natural contaminants in the food chain make it imperative that every effort be made to ensure that exposure is minimal, as a means to improve environmental, animal and human health.

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Basic Toxicological Issues in Product-Safety Evaluations

David W. Hobson

CONTENTS

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1 INTRODUCTION

Toxicology is an essential part of safety evaluation for a wide variety of products, including pharmaceuticals, medical devices, cosmetics, industrial chemicals and materials, and a wide variety of different types of consumer products. Historically, the introduction of formal toxicology studies for use in the evaluation of product safety is a relatively recent event, but the concept of recognizing and making note of the potentially poisonous properties of manmade products or formulations has been occurring for millennia. So the concept of 'safety by design' that sometimes seems to be of modern origin, when espoused by modern-day toxicologists, chemists, engineers and even consumer groups, most likely had its conceptual origin somewhere in antiquity with the routine formulation of medicinal treatments and the design of potentially dangerous mechanical devices, along with the practical necessity of food tasters to ensure the safety of food products for the aristocracy.

Today, 'safety by design' means little without careful application of modern toxicology for many products. Some of these products require toxicological evaluation prior to entry into their respective markets, due to formal regulatory requirements (pharmaceuticals, pesticides, food additives, medical devices, etc.), whereas others (cosmetics, paints, textiles, foods, art materials, etc.) may not have formal guidance and specified requirements, but many of these products are often evaluated toxicologically in order to determine if some types of precautionary labelling are required, and to help assure product safety and quality for both the manufacturer and the consumer.

Product-safety data is used in many ways by a wide variety of industries. Chemical manufacturers, for example, need to know the toxicologic characteristics of their raw materials, as well as finished products in order to properly design manufacturing processes and select personal protective equipment (PPE) to protect their workers and to develop warning labels, as well as material safety data sheets (MSDS). Pharmaceutical companies need to have toxicologic data for the screening and selection of lead compounds for further development into pharmaceutical products, and the pharmaceutical products themselves require toxicologic evaluation to establish safe levels for administration to animal and human subjects in studies required for approval and registration of the pharmaceutical by regulatory bodies, such as the US Food Administration. Insurers and company risk managers also need to know about product safety in order to establish risk for marketed products. Products, such as pharmaceuticals, cosmetics, coatings, dyes, colourants, food additives and so on, may be evaluated for risk in a company's insurance portfolio, and toxicological data, combined with the potential for exposure, can be useful in obtaining rational estimates for a product's risk. Virtually any industry that one can think of requires some sort of use of toxicological data to establish product safety for the myriad of products that are manufactured or are in use all over the world every day.

2 THE DESIGN AND DEVELOPMENT OF SAFE PRODUCTS

Safe products don't 'just happen' as a result of the design and development of products that serve the purpose for which they are intended. Often, product design is so heavily focussed on function or aesthetics, such as appearance, fragrance, sound, feel and so on, that it is easy for product designers or innovators to put safety considerations aside, while the essential elements of design to meet market needs or intellectual property requirements are satisfied. Under these conditions, product safety obviously becomes more an afterthought of the design process or may be thought to be a necessary 'evil' or 'hurdle' that a newly developed product prototype must overcome. A typical product-development scheme of this nature is depicted in **Figure 1**. Industries, and the companies within them, that are in highly competitive markets often approach the 'normal' course of discovery and development of new products in such a manner that safety or toxicologic considerations are included 'along the way' in their product-development programmes. In companies of this nature, project-management schemes are typically focussed toward the earliest possible release into the market and they 'hope' that any required toxicological testing does not result in any 'snags' on the way to market. Such development programmes typically can be identified, not only by the position that the toxicology evaluation process occupies in their project management plans, but also often in the minimalism and frugality of their conception and implementation to do nothing more than 'satisfy' the most essential regulatory premarket requirements. Companies operating in this manner usually like to describe themselves as being 'market driven' and typically pride themselves on the number of new product launches achieved per year and the speed at which they are able to move products from concept to launch through their pipelines, using efficient and often aggressive, time and cost-efficient techniques. This aggressive road to market is considered necessary and simply 'good business' practice by companies competing for their market shares.

Naturally, most toxicologists are interested in doing their best to assist in the development of safe and effective or useful products that improve the quality of life. However, sometimes being able to do this to the best of their abilities and knowledge becomes a challenge, given the aggressive business practices in some of the companies they serve, and the never-ending need to put new products into their markets, to stay competitive and be responsive to customer needs.

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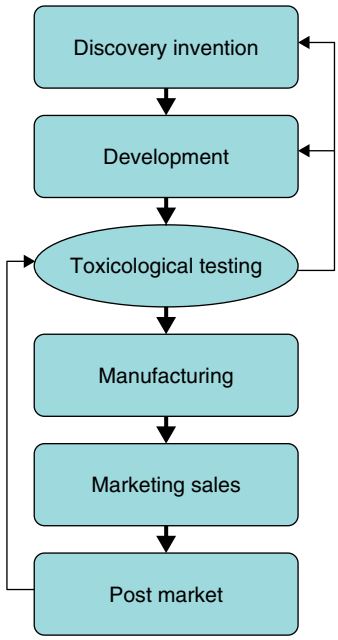

Figure 1 Typified product-development scheme for companies in highly competitive markets, showing the 'in-process' position of the toxicological evaluatory programme.

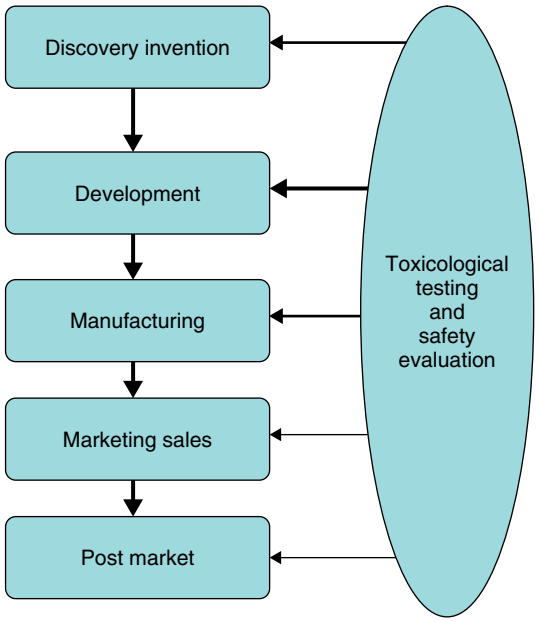

Figure 2 A product development scheme for companies operating with a high degree of product-safety awareness showing the 'continuous' nature of the toxicological evaluatory programme.

Under these conditions, product safety becomes less a toxicologically driven concern and more an issue of product liability (PL), regulatory compliance and market acceptance. Toxicologists working under these conditions often must continuously look for means and methods for improving the rate of 'throughput' and lowering the cost of their evaluatory programmes.

Thus, increasing throughput and lowering cost can become basic driving issues of product-safety programmes that may become a toxicological issue by way of creating a need for more short-term, lower-cost alternatives to currently accepted and established tests and evaluatory procedures.

Companies operating with a high degree of safety awareness or need, independently or within industries that demand a primary focus on product safety (e.g. pharmaceutical, medical device, pesticide, some truly 'green' companies, etc.), typically understand that toxicologic considerations need to be an integral part of their entire discovery, design and manufacturing processes. **Figure 2** shows how such a product development process might typically operate.

Not surprisingly, companies operating with a high safety need also need more short-term, lower-cost alternatives to currently accepted toxicologic procedures, not just because of a desire to improve throughput in their product lines and reduce the cost of testing, but also because there is a lot more for them to do early in the discovery process to select raw materials and develop prototypes with a high potential for proving themselves safe. The basic toxicologic issue for product safety in these development programmes becomes efficiency and accuracy of the tests and their results, such that toxicology evaluation in the early phases of discovery and development does not become a rate-limiting factor or 'bottleneck', where there are potentially many selections to be made based on safety data. Thus, no matter what drives the company with respect to the need for toxicologic evaluation, there is always a demand for improved testing procedures and evaluatory processes. Indeed, many of the 'emerging' methods and procedures presented in various chapters in this book have as their genesis some aspect of responding to this ongoing need. The purpose of the remainder of this chapter will be to define and describe how toxicologists arrive at concluding that a given product is 'safe' for its intended use, basic toxicologic requirements, recommendations and issues for product safety within different industries and basic product-safety testing and evaluation issues currently engaging the toxicologic community.

3 DEFINITIONS, CONCEPTS AND EXPRESSION OF PRODUCT SAFETY

'Safety' is defined as being the condition of being safe from undergoing or causing hurt, injury or loss (Merriam-Webster Online Dictionary, 2009). When used in context with the development and use of any of products of any sort, it is clear that a safe product would be an item 'produced' in some fashion (grown,

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hand-made, developed, manufactured, etc.) that somehow achieves or otherwise demonstrates itself capable of achieving the condition of being safe from undergoing or causing hurt, injury or loss of some significance.

Thus, a general perception would be that implementing product safety in any fashion somehow involves the prevention and/or protection of humans, animals, plants and the environment from the potentially harmful or damaging effects (including hurt, injury or loss) of a given product. Obviously, it is not surprising that the implementation of product safety for any product requires the collection of specific information, including the composition and physical nature of the product and its conditions of use, as well as its potential to cause physical and/or toxic damaging or harmful effects.

Since toxicology is the area of science primarily concerned with determining the adverse effects of substances and materials on biological systems, or more simply whether or not something is toxic to people, animals, plants or the environment, the public, as well as the scientific community at large looks to this discipline to produce data and arrive at sound, scientific findings and conclusions regarding the safety of the products that we discover, develop, manufacture and use. Because toxicologists, in the course of their comprehensive scientific investigations of product safety, also must learn something about the nature and potential for exposure to the substances and compositions of the materials and products that they investigate, they also begin to understand something of the nature and potential for these substances and their various combinations and forms to be hazardous to people, animals, plants and/or the environment. As in all areas of science, how much we know is dependent on the type, amount and quality of the toxicologic data we have available.

Thus, knowledge about the toxicity that a given product might produce, as well as the nature and magnitude of any exposures with respect to human, animal, plant or environmental systems, allows us to define and understand the hazard that the product could represent. This concept is generally expressed toxicologically with the following relationship:

$$\text{Hazard} = \text{Toxicity} \times \text{Exposure} \qquad (1)$$

where 'Hazard' refers to the extent to which a particular adverse effect or event might occur in an exposed population due to a specific type of toxicity (response) and exposure (dose) for a given substance or product.

Evaluation of the toxicity or toxicologic characterization of raw materials, product prototypes, formulations, finished goods and by-products from product degradation and disposal throughout a product's life cycle can all be issues that often must be addressed in product safety evaluations. Indeed, product 'life cycle' analyses to include such toxicologic assessments have become an integral part of current movement toward the development and use of 'green' or 'environmentally friendly' products.

How a toxicologist arrives at a sound science-based conclusion, judgement or finding on a product's 'safety' is not guesswork, as some might tend to believe. A conclusion of safety, or lack thereof, is rather the end stage of a process of investigation that requires the highest state-of-the-art skill and knowledge that toxicologists have toward collecting, analyzing and evaluating as much evidence as is possible regarding dose–response effects and the estimation of toxicologic risk for the product under investigation.

'Dose–response assessment' is the process that toxicologists typically use to quantitatively evaluate the toxicity of a given chemical agent or formulated product as a function of exposure (human, animal or plant) to that agent or product. The relationship between the dose of the agent or product administered or received into the human, animal or plant test system, and the incidence of adverse biologic effects in the exposed test population, forms the basis for the 'quantitative dose–response relationship'. From these relationships, toxicity values (e.g. reference doses and slope factors) are derived that can be used to estimate the incidence or potential for adverse effects (hazard) might occur in an exposed population.

'Hazard identification' is the process of determining whether exposure to a chemical agent or formulated product might cause an increase in the incidence of a particular adverse health effect (e.g. death, cancer, birth defects) and whether the adverse health effect is likely to occur in humans, animals or plants. This process examines the available scientific data for a given chemical or product and develops a data package called termed 'weight of evidence' to determine and characterize any links between the potential for adverse or negative effects, and exposure to the agent or product.

'Toxicologic risk' generally refers to the potential or likelihood that adverse health effects may be induced by exposure to a chemical toxicant, biological toxin or some types of physical agents (e.g. radiation).

'Risk Assessment' is a process that aims to understand, describe and, in some cases, quantify the likelihood that potentially adverse health effects may be induced by exposure to chemical, biological and/or physical agents.

'Risk', in toxicologic terms, refers to the probability or likelihood that a toxicologically significant exposure might occur for a given population. Often this is given as incidence of occurrence of a toxicologically significant event (e.g. death, organ damage, cancer) per some number of individuals in the population (e.g. per 1000 animals or people). Sometimes risk is expressed in terms of a reference or control population in the form of an 'excess likelihood' of an adverse event (e.g. cancer) occurring, relative to a 'baseline' or 'background' incidence of the same adverse event in a nonexposed reference population. For example, when estimating risk

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for low levels of exposure to relatively low levels of carcinogens in drinking, the US Environmental Protection Agency (EPA) has used the available data and a theoretical 'model' of how chemicals cause cancer to estimate the carcinogenic potency of a given chemical. This potency is considered an 'upper-bound estimate' such that the true risk is not likely to be higher and may be lower. The estimated lifetime cancer risk for exposure to the chemical is the upper-bound estimate of the increase in one's probability of contracting cancer as a result of exposure for a lifetime. The 'unit cancer risk' is the EPA's estimate of risk per some unit of exposure such as one's increased risk from drinking 1 g of the chemical per litre of drinking water for a lifetime. The higher the unit risk, the greater is the estimated carcinogenic potency of the chemical (US EPA, 1997). Risk can usually be estimated in some manner for all types of exposures to chemicals, as well as toxicological effects if the exposure or dose can be determined in a reasonable manner and there is a quantifiable relationship between dose and the effect.

'Risk characterization' is the estimation of the incidence and severity of the adverse effects likely to occur in a human or animal population due to actual or predicted exposure to a substance. This process usually involves comparing the exposure (measured or estimated) with the hazard.

'Relative risk' (RR) is the risk of a toxic adverse event (or of toxic injury) relative to exposure. RR is a ratio of the probability of the event occurring in the exposed group vs. that of a nonexposed group.

$$\mathrm{RR} = \frac{P_{\text{exposed}}}{P_{\text{non-exposed}}} \tag{2}$$

For example, if the probability of developing lung cancer among smokers was 30% and among nonsmokers 1%, then the RR of cancer associated with smoking would be 30. Smokers would be 30 times as likely as nonsmokers to develop lung cancer.

Another term for the RR is the 'risk ratio' because it is the ratio of the risk in the exposed divided by the risk in the unexposed.

Taking everything above into consideration, it should be clear that 'safety' is a concept or an expression that attempts to describe the condition of being relatively low or free from toxicologically significant risk. In practice, safety is often expressed in toxicologic studies as an observed incidence of adverse outcomes (or lack thereof) as being no different (or sometimes even less) than a nonexposed control or reference group or population sample. This same concept is very often applied in preclinical studies, epidemiology studies, clinical trials and environmental field studies, wherever a suitable control or reference population can be identified and included in the study design. Thus, a finding of 'safety' based on this type of data is predicated on having a suitable control or reference group included in the study design and selection of appropriate endpoints for toxicologic investigation. Since these studies often involve animals or human subjects, they must be very carefully planned, and executed with the highest degree of scientific integrity and data quality possible in order to obtain results that are most likely to be reproducible.

Another means of expressing safety might be use of an 'odds ratio (OR)' approach. This can sometimes be of value in expressing and communicating toxicologic findings to the general public. The OR is a measure of effect size in a population group exposed to a toxicant relative to effect size in a nonexposed or control population group. It can also be used to compare effect size in one exposed population group to that of another exposed population group (e.g. differences between men and women, one product vs. another product, etc.). An OR is defined as the ratio of the odds of an event occurring in one group to the odds of it occurring in another group, or to a sample-based estimate of that ratio. These groups might be males and females, an experimental group and a control group, or any other dichotomous classification. If the incidence of a potentially adverse event (e.g. skin damage) in each of two product-formulation-exposed groups are 'Formulation A' and 'Formulation B' then the results matrix for a population sample of 1000 subjects to which both formulations were applied prior to a similar regimen of sunlight exposure might be expressed as follows:

	No skin damage	Skin damage
Formulation A	884	116
Formulation B	147	853

and the OR comparing Formulation A with Formulation B relative to 'skin damage' is:

$$\mathrm{OR} = \frac{884 \times 853}{147 \times 116} = \frac{754052}{17052} = 44.2 \tag{3}$$

This indicates that Formulation-A-exposed treatment areas were substantially more likely to be free of skin damage than Formulation-B areas.

An OR of one would indicate that the condition or event under study is equally likely in both groups, which could denote a condition of 'safety' between a product treatment group and an appropriate control or vehicle treatment group. An OR greater than one indicates that the condition or event is more likely in the first group and an OR less than one indicates that the condition or event is less likely in the first group. The OR must be greater than or equal to zero. As the odds of the first

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group approaches zero, the OR approaches zero. As the odds of the second group approaches zero, the OR approaches positive infinity.

For example, suppose that in a sample of 100 men, 90 have developed headaches while taking a product over the past month, while in a sample of 100 women taking the same product only 20 developed headaches in the same period. The odds of a man developing a headache are 90 : 10 or 9 : 1, while the odds of a woman developing a headache are only 20 : 80 or $1 : 4 = 0.25 : 1$. The OR is thus 9/0.25 or 36, showing that men taking the product are much more likely to develop a headache than women.

The above example also shows how ORs are sometimes sensitive in stating relative positions: in this sample men are $90/20 = 4.5$ times more likely to develop a headache than women, but have 36 times the odds.

'Safety factors' are also a historical means of approximating safe levels of exposure to toxic materials or products. Using this approach, 'safety factors' are added to experimental toxicity data to account for uncertainties in data and evaluation processes. For example, if a toxicant dose is observed to be safe for a laboratory rodent at a particular 'no-effect' dose level, one might assume that one hundredth of that dose would be safe for a human, allowing a safety factor of 100 to allow for interspecies differences between two different mammals; if the toxicant exhibits reproductive or other adverse effects at a high dose level, then the safety factor applied to the rodent no-effect dose level might be 1000. On the other hand, if toxicant exposure data is available from two substantially dissimilar mammalian species (e.g. laboratory rodent and miniature pig) and no substantial adverse effects are observed at the highest dose levels in either species, application of a safety factor of 10 to the 'no-effect' level for the species most similar in body mass, anatomy and biochemistry to the human (i.e. the miniature pig) might be applied to estimate a reasonably safe exposure level. An increased protection factor may also be used for individuals believed to be more susceptible to toxic effects, such as in pregnancy or with certain diseases; or, a newly synthesized and previously unstudied chemical that is believed to be very similar in effect to another compound could be assigned an additional protection factor of 10 to account for possible differences in effects that are probably much smaller. Obviously, this approach is very approximate, but such protection factors are deliberately very conservative and the method has been found to be useful in a wide variety of applications.

The magnitude of the RR or safety for the use of a given product is determined by some function related to the size of the population that would be affected, and any additional events or circumstances that might differentially affect the probability of exposure or toxicologic effect for different segments of the population (age, gender, genetics, health status, environmental conditions, etc.).

In reality, differing perceptions and definitions of 'safety' for different types of products occur and this results in concepts of 'product safety' being viewed differently by various industries and often even between different product lines within an industry. In actuality, there is no such thing as a 'completely safe' product because, no matter how small, there is always some amount of risk associated with the use of any product. So, for this reason, many find the concept of 'safe for the intended use' to be the practical concept for product safety to which formal risk assessment practices may be applied in order to arrive at some level of 'acceptable risk', and a determination of product safety that can be supported with data and communicated for a given product to the community in which its perception of safety is operating.

A conclusion of 'safety' or lack thereof, from a toxicologic perspective, is therefore best arrived at following a careful evaluation of dose–response relationships involving potential toxicologic endpoints that are relevant to the product under review, followed by the application of some form of rational risk assessment directed toward the known or anticipated product-use conditions. This is not something that is easily mastered and there are many potential pitfalls and opportunities for making mistakes. Product-safety conclusions should always be performed with the cognizance that they will be scrutinized by other experienced toxicologists (and sometimes the public) that will likely have the advantage of time in the intended market and outcomes from actual use to draw from. Therefore, it is highly recommended and indeed imperative that any product-safety finding be carefully documented or 'qualified' with a succinct, but comprehensive disclosure of the information that was available and the procedures used to arrive at the conclusion.

Products found to be safe and placed in their respective markets should always be followed or 'tracked' in some fashion by the manufacturer, distributor or market-responsible party to ensure that any possible adverse effects experienced by product handlers or users are collected and periodically evaluated. Assisting companies with the development of effective and manageable product-safety tracking systems often can be an issue that is addressed most effectively and timely with expert toxicologic assistance, as a lack of proper vigilance in this regard for some products can result in substantial problems for the ongoing success of a marketed product such as PL litigation, loss of product acceptance and declining brand recognition.

4 STRATEGIES AND PROCEDURES FOR SAFETY ASSESSMENT

Modern strategies for the assessment of product safety using toxicological data vary with the type of product

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being evaluated, as well as the elements of applying sound, evidence-based, risk assessment and toxicological principles to arrive at acceptable communications of product safety and risk. Product-safety evaluations are, therefore, typically done on a individualized product and intended-use basis, with data packages to support the assessment of risk and a finding of acceptance or rejection of 'safety for intended use' that is soundly supported by scientific evidence. When provided this type of data package and a clear, unbiased assessment, appropriately experienced toxicologists are able to evaluate the type, nature and quality of toxicological information presented and will arrive at reasonably similar conclusions.

Figure 3 shows a basic strategy for product-safety assessment leading to the filing of an investigational new drug (IND) application to the US Food and Drug Administration (FDA). A similar process is followed when preparing formal safety assessments based on toxicologic data for pesticide applications submitted to the US EPA and to other international regulatory bodies concerned with pharmaceutical or pesticide regulation and registration. This type of product-safety assessment is 'regulatory driven' because the entire process is always focussed on completion of a safety assessment that meets the criteria for acceptability, both in content and quality, for the planned regulatory submission. The information collected and studies performed in support of the safety assessment for a pharmaceutical product are directed specifically towards the clinical indications for the product, anticipated therapeutic dose level or dose range and the desired route of administration. Similarly, information required and studies performed in support of a pesticide application are focussed on the determination of any potential toxicity to humans, animals or the environment, as well as determination of no-observed-effect exposure levels that can be used to evaluate product safety.

Not all product-safety assessments are regulatory driven. Many are performed to identify any potential safety concerns and to establish that a product is safe for its intended use, or to determine the magnitude to which people need to be protected from exposure to the material due to the potential for harm. These types of assessments can also be of substantial value to insurers for accurately determining PL. Some insurance companies require this information as part of their PL determinations. **Figure 4** shows a strategy to conduct a product-safety assessment that is driven by primarily by practical needs and concerns only rather than specific regulatory requirements. Although the overall objective is similar to the regulatory-driven assessment, the process differs in that it lacks the strict regulatory guidance, data quality requirements and agency interactions necessary for regulatory safety evaluation and is, therefore, often less costly and also may include tests, such as *in vitro* alternatives and so on, that are not yet accepted for regulatory-driven evaluations. This type of safety evaluation is conducted largely because it is simply good business practice to have this type of information in a product portfolio to satisfy internal concerns regarding the safety of a company's products for employees (management, safety personnel, R&D, manufacturing, packaging, warehouse, etc.), as well as to be able to provide scientifically sound information to distributors, transportation workers, customers, insurers, for MSDS preparation and so on, when needed.

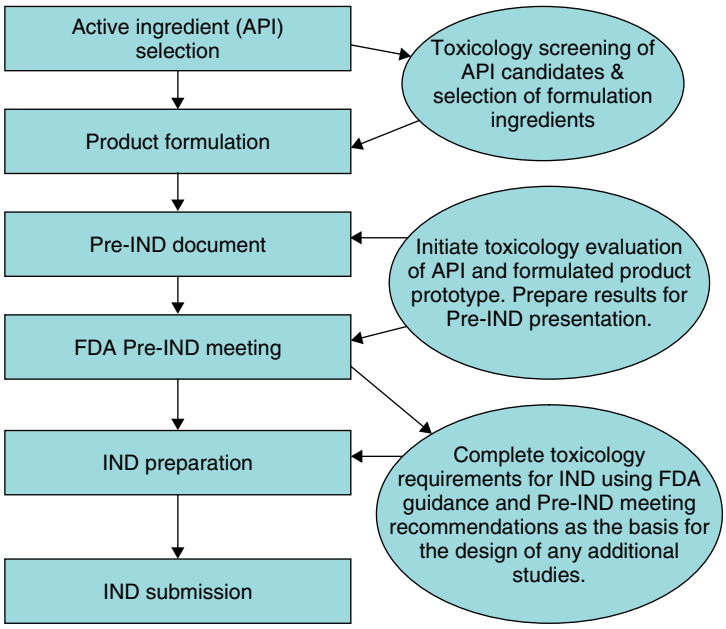

Figure 3 A basic strategy for product-safety assessment leading to the filing of an investigational new drug (IND) application to the US Food and Drug Administration (FDA).

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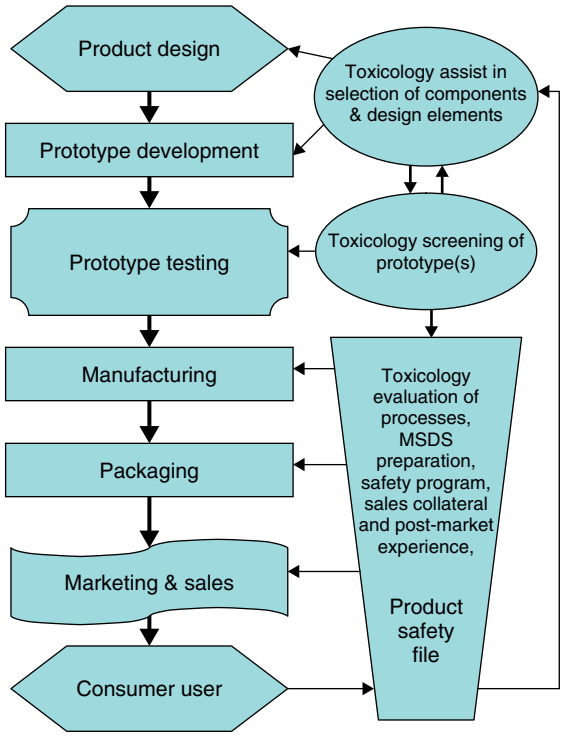

Figure 4 A strategy for toxicological involvement in product-safety assessment, monitoring and improvement that is not regulatory driven.

Whether formal and regulatory driven or business driven, the conduct of basic, unbiased, toxicological assessments of product safety with a high degree of consensus requires: (i) the involvement of a toxicologist with training, certification and experience to be competent to perform risk and product-safety assessments and (ii) the development and implementation of a sound product-safety programme that is focussed on quality and is supported at the highest levels of a company or organization that understands and expects integrity, quality and clarity in its product-safety assessments.

5 WHO SHOULD PERFORM PRODUCT-SAFETY EVALUATIONS?

As indicated above, training and certification of toxicologists to perform risk and product-safety assessments in a comprehensive and competent manner is an issue that gets the attention of experienced toxicologists, product developers and regulatory officials. Although chemistry, physiology, biochemistry, pathology and even pharmacology are disciplines integral to training in toxicology, a chemist is not necessarily a toxicologist, nor is a biochemist, pathologist, pharmacologist or pharmacist, unless they have had formal training in the subject adequate to the toxicologic issues they are attempting to address. Even though there are many academic programmes offering toxicology training, sometimes leading to a formal degree in toxicology, there are no commonly accepted requirements that a graduating toxicologist demonstrate competence in being able to complete a basic risk assessment and product-safety evaluation prior to successful graduation from these training programmes. This creates a situation where some training programmes are stronger than others with respect to this practical necessity of the commercial market for toxicologists. This has been a topic of discussion for some professional toxicology organizations such as the American College of Toxicology (*www.act.org*) and the Society of Toxicology (*www.toxicology.org*) that occasionally offer continuing education courses and workshops that attempt to address this need. Bodies that certify toxicologists by education, experience and examination, such as the American Board of Toxicology Inc. (*www.abtox.org*) make an effort to evaluate whether or not a practising toxicologist that is applying for certification demonstrate, by examination, that they have some degree of basic competence with toxicologic risk assessment and product-safety testing and evaluation. Thus, not all 'toxicologists' are created equal with respect to this practical and industr- important capability, which is why the internet, news media and forums not subject to peer review by competent and experienced toxicologists are so full of product-safety claims and concerns that have little or no basis in actual scientific fact. Therefore, at present, the best means of establishing that a toxicologist is competent to complete an acceptable risk assessment and product-safety evaluation appears to be a demonstrated history of doing so in a successful manner, combined with a degree in toxicology from an accredited institution of higher learning known for the quality of its toxicology training programme, and board certification from an internationally recognized certifying organization that requires demonstrating risk assessment and product-safety evaluation ability as one of the competencies that it evaluates as a requisite for certification.

Computational methods for evaluating toxicological information are commonly used today and are generally accepted as tools for making some types of predictions and as aids in experimental design (Lyons *et al*., 2008; Rabinowitz *et al*., 2008; Bruce *et al*., 2008; Clark *et al*., 2004; Blaauboer, 2003). However, computational methods are also used for toxicological risk assessment and it is generally understood by the toxicologists that use these methods that they have limitations with respect to the quantity and quality of useful data that are used to make risk predictions for given chemical substance or product formulation. These models have been found to be useful in the evaluation of environmental risk from complex mixtures, and may also

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be useful for making safety predictions for formulations of some complex products (e.g. nanoproducts) in the very near future (Liao *et al*., 2002; Ahlers *et al*., 2008) The development of these computational tools for use by experienced toxicologists who understand their limitations now appears to be entering a new phase in response to an increasing demand for rapid or 'instant' assessment of toxicologic risk for chemical substances by nontoxicologists for a variety of purposes, including queries regarding potentially safe levels of toxic substances in commerce or in the environment, as well as making predictions with respect to formulated products for public 'right-to-know' regulations and chemical registration (Worth *et al*., 2007; Grindon *et al*., 2008; Combes *et al*., 2008; Lilienblum *et al*., 2008). This use of computational toxicology and expert systems may prove useful in the protection of people, animals or the environment from potential harm, as well as aid nontoxicologists in the regulation of toxic substances and products that contain them. Chemists, engineers and lawmakers may find such systems valuable in making rapid, rudimentary determinations of potential risk for chemical substances that have been entered into computational databases due to the need for vigilance and concern. Some of these systems look up information in data tables and apply this information to conditions that are input by the user regarding the conditions of exposure, and others have as their basis for estimation the use of quantitative structure–activity relationships (QSARs) that attempt to relate chemicals or substances of interest on the basis of the chemical and/or molecular structural similarities or dissimilarities to known toxicants. While these systems may be useful for determining whether there is a potential concern sufficient to involve a toxicologist or not, such methodologies must be rigorously tested and validated before they are used to arrive at any risk assessment or product-safety evaluation that would be likely to be acceptable for establishing, with respect to current standards of practice, any sort of risk estimate adequate for product-safety evaluation. Nevertheless, it is conceivable that, in time, expert computational systems will emerge that can access more information more quickly than even the best toxicologist, and even compile and evaluate content substantially for use in performing preliminary risk assessments that, with appropriate involvement of an experienced toxicologist, could be used as the basis for product-safety evaluations in the future.

However, we are all aware that it is always the actual experience with the product in use over time that determines whether or not a product is truly safe for its intended use or not, so there is always a need for formal, ongoing vigilance practices with respect to continuous monitoring of the use history, with respect to ensuring the that the products we develop and use are safe.

6 APPLICATION OF TOXICOLOGY PRINCIPLES TO PRODUCT SAFETY

The foundation of a sound product-safety programme for products where toxicity is a potential concern always involves the collection of some sort of toxicologic data relevant to evaluation of the potential for adverse effects to occur under controlled experimental conditions that relate to anticipated use exposures and/or 'worst case' in-use (e.g. accidental) exposure. It is for this reason that the proper application of toxicology to product-safety evaluation requires a substantial knowledge of the applied and well-validated aspects of the discipline, including the proper selection and design of toxicologic evaluation programmes, as well as individual study design. This generally requires a reasonable understanding of any regulations and guidance that are applicable to the product type being evaluated. There are many sources of regulations and guidance internationally that provide the toxicologist with useful perspectives on toxicology programmes and study-design considerations, as well as quality, content and format requirements for the successful collection of toxicologic data and preparation of product-safety evaluations (US FDA, 1994; 1995; 1996; 1997; 2008; US EPA, 1998a; 1998b; 1998c; 1998d; OECD, 1981; 1992; 1995; 1998a; 1998b). There are also valuable published resources for the design of product-safety programmes, as well as the design of toxicologic studies to support product-safety evaluations (Gad, 1999; Derelanko and Hollinger, 2002; Ross, 2003). It is also important to acquire and have available computational capabilities sufficient to manage and analyze the data necessary to perform scientifically rational safety evaluations for the specific product types of interest, for the preparation of reports of product-safety findings and for archiving.

A current awareness of emerging techniques and new or changing regulations is also necessary to ensure that product-safety evaluations are conducted in accordance with toxicologic formats that meet or exceed the data content and quality required for the type of product being evaluated. There are many sources of regulations and guidance for the evaluation of different types of products. Some examples of different types of product categories and United States, European Union (EU) or other international agencies or bodies regulating and/or providing product-safety guidance for them, are shown in **Table 1**.

Navigating the data and study-design requirements to arrive at the completion of a toxicology testing portfolio that may be considered 'state of the art' for the evaluation of safety for a given product is not a trivial process. Without appropriate training and practical experience, it is not uncommon to have just enough knowledge to get started doing relatively straightforward, short-term toxicity studies, without adequate planning for repeat-dose studies, that are generally done using two

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Table 1 Examples of various sources of product-safety regulatory requirements and/or guidelines for different types of products

Organization	Product responsibility	Purpose
US Food and Drug Administration (FDA)	Pharmaceuticals (human and animals), biologics, medical devices, food including food additives	Regulatory oversight for the United States. Provides guidance for safety evaluation by documentation or consultation. *http://www.fda.gov*
US Consumer Product Safety Commission (CPSC)	Consumer products of all types, except food, over the counter drugs, pesticides and so on	Regulatory oversight of consumer product safety. Guidance on product safety. *http://www.cpsc.gov*
US Environmental Protection Agency	Pesticides, environmental (air, water, soil) effects of all types of products mined, manufactured, waste and so on. Registration of chemicals under the Toxic Substances Control Act (TOSCA)	Regulatory oversight of pesticide products. Provides guidance for registration of pesticides and for environmental impact assessment. *http://www.epa.gov*
US Department of Transportation (DOT)	Packaging and labelling for transportation on US roadways, railways, waterways or airways.	Regulatory oversight of packaging and labelling of consumer products shipped in the USA *http://www.dot.gov*
Organization for Economic Cooperation and Development (OECD)	Serves member nations and convenes expert panels to develop recommendations and methods with member acceptance.	Produces standard toxicological testing methods for various endpoints and test materials.
European Chemicals Agency (ECHA)	European Union (EU) Agency responsible for administration of the REACH	Reference and repository of REACH information and records. Chemical regulation is by EU member states.
European Medicines Agency (EMEA)	European Union Agency responsible for administration of medicinal products (human and veterinary)	Scientific advice and assessments for medicinal products. Regulation is by EU member states.
European Food Safety Authority (EFSA)	European Union Agency responsible for risk assessment regarding food and feed safety.	EFSA produces scientific opinions and advice to provide a sound foundation for European policies and legislation. Regulation is by EU member states. *http://ww.efsa.eu.int*
European Environment Agency (EEA)	European Union Agency responsible for assessments and reports regarding environmental issues.	EEA Produces reports to support environmental policies and legislation. Regulation is by EU member states. *http://www.eea.europa.eu/*

different species. Experienced contract research organizations (CROs) sometimes make study-design errors that are even more likely when working with assumptions or incomplete or confused information from a sponsor that is pushing to get studies completed as quickly as possible. This practice often results in the sponsor jumping into costly and time-consuming studies that may not provide the data necessary for a reasonable determination of safety necessary to satisfy most regulatory bodies.

Effective and efficient product-safety evaluations begin at the product-design or product-formulation stage. By having the toxicologist evaluate the raw materials that are going to be used in formulating or manufacturing the product, as well as having input into the design and manufacturing process, product development can be adjusted in ways that are insignificant to the overall outcome, but can eliminate problems that might cause toxicologic problems when finished products are evaluated for safety. An example of this is the elimination of sources of latex or lanolin containing raw materials and elements of the manufacturing process that come into contact with the formulated product in the manufacture of dermatologic products. Other examples are the careful selection of preservatives, surfactants and other ingredients used in the manufacture of ophthalmic products and the prequalification of new cosmetic raw materials or new raw material sources for cosmetics using *in vitro* testing to help eliminate skin and ocular irritation.

Early-stage involvement by the toxicologist in the product development and manufacturing process can

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also be of substantial value to the toxicologist in the design of a comprehensive product-safety plan for the new product. Having a complete understanding of the formulation essentials, as well as its manufacturing process allows the toxicologist to be more effective in making constructive and workable suggestions to help eliminate product-safety issues, should they arise. This knowledge is also valuable, should the toxicologist be called upon to provide expert testimony in cases involving frivolous claims of PL or negligence.

7 ESSENTIAL TOXICOLOGY CONSIDERATIONS FOR PRODUCT-SAFETY EVALUATIONS

Initiation of a successful product-safety evaluation requires some planning by the toxicologist. The process of developing a product-safety data portfolio that is adequate to cover potential hazards associated with the development, manufacture, sale and use of the product requires some forethought and design.

Beginning with a complete understanding of the product itself, as well as any exposures that may be toxicologically significant from development and throughout the product life cycle, a determination of the information needed to support safety assessments is made. Some of the informational needs can be obtained from interviews and literature information that is obtained and evaluated as a preliminary to determining what types of studies will be needed to complete the data required to prepare the evaluation. Some of these studies may be of a chemical or physical characterization nature and others will be toxicology studies. In some cases, environmental monitoring of the workplace and/or effluents and disposal sites may also be necessary.

Some basic informational considerations for effective product-safety evaluations that should be collected into a comprehensive product development file for each new product include the following.

7.1 Product Concept

A clear and concise product concept should be developed that answers essential questions for the toxicologist, such as how the product is going to be used, who will use the product and where the product will be used. Any constraints on the formulation or manufacturing process that are required for manufacturing or by the intended market should be provided (e.g. colour, no colour, fragrance, no fragrance, etc.). Any constraints on the safety-assessment process, such as the desire for the product to not be tested using animals, should also be included in the product concept.

7.2 Claims

A draft of the desired or anticipated use claims to include claims of efficacy and safety for pharmaceuticals, medical devices, cosmetics, food additives and so on, should be developed for the product with the toxicologist participating if possible. Pharmaceuticals and medical devices should also provide their desired therapeutic claims or indications, as well as list the type and nature of any active ingredients and their modes of action, if known.

7.3 Composition or Formulation

Formulated products should begin by listing any and all active ingredients, as well as all known formulation components and their amounts (preferably as percentage weight/weight values and Chemical Abstracts Service reference numbers (CAS number). If there are any known hazardous materials used in the manufacture or formulation of the product, MSDSs should be obtained and placed into the product information file. Similar considerations and information should be obtained for the composition of nonformulated products that also includes what is known about any components that might be released, off-gas or leach from the finished product.

7.4 Exposure

The type and nature of any human, animal, plant or environmental exposure to the product should be determined and listed in order of maximum likelihood of occurrence. If the product is a pharmaceutical, the anticipated product dose should be provided. If it is a cosmetic, the location and amount of intended application should be determined. Other manufactured products, including medical devices, should note the area of the body that is most likely to come into contact with the item and in what form the product might be when coming into contact with biological tissues (e.g. surface material, off-gassed chemicals or particulate emissions, liquid release or transfer, etc.). All potential routes of exposure should be considered and listed, if applicable, for each product.

7.5 Life cycle

Product life-cycle analysis is fast becoming a fixture in the development of products, especially products that wish to demonstrate themselves to minimally impact the environment, for example ‘green’ products. Sourcing and disposal of raw materials to avoid excess or waste entering the environment, as well as streamlining the

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manufacturing process to reduce waste, including the use of excess energy and methods for the final disposal of the used product, including any packaging, are all considerations that a toxicologist should be aware of and allowed to make contributions to.

7.6 Required Labelling and Warnings

Any regulatory requirements for labelling or product-safety warnings should be identified as well as the agencies, domestic and international, that will regulate the products. In most large companies this aspect of the product-development portfolio can be provided by the regulatory affairs section or departments. In some cases, this is the responsibility of the legal department. In smaller companies, this may be the responsibility of the toxicologist or some other department that has knowledge of product regulatory and labelling requirements.

7.7 Alternatives or Competing Products

Information regarding composition, claims, safe-use history and so on, should be collected and maintained on an ongoing basis in the product file. This is not for marketing purposes, but rather allows the toxicologist to consider the prior history of product use in the market and make any necessary or desirable changes or alterations in the emerging new product that improve product safety. This offers, not only a potential competitive claim, but it also takes advantage of 'lessons learned' to develop better products for everyone.

After collecting the initial information, the product-safety evaluation programme is designed and the required toxicology studies are identified and designed appropriately. Basic considerations for the design of product-safety studies are presented below.

8 STUDY DESIGNS FOR PRODUCT-SAFETY EVALUATION

Once the objectives and requirements of the product-safety evaluation are understood, the selection of appropriate toxicological studies and their design can commence. During this process, certain types of information are required, whether the toxicologist is going to conduct the studies in-house or work with a CRO. A practical means of initiating this sort of study planning is to prepare an outline of all the studies required for completion of the dataset needed for product-safety evaluation. Having this outline is helpful in determining what studies need to be completed sequentially and what studies can be performed within the same timeframe. The basic information necessary for the design of initial studies includes the following:

- Chemical composition of the test substance or material (test article)
- Physical characteristics of the test article
- Route, or routes, of administration or exposure to the test article
- Expected dose or exposure level, or range, to the test article
- Duration of administration or exposure to the test article
- How and where dose preparation is to be done
- Toxicological endpoints to be evaluated for safety
- The data quality requirements (GLP or non-GLP)
- Source for dose confirmation analysis
- Source for bioanalytical analysis
- Selection of an experienced study director, toxicologist and pathologist.

The route and duration of exposure combined with the overall objective of the product-safety evaluation studies is then used to establish what studies should be included in the product-safety data portfolio. The exposure duration is determined relative to the anticipated longest duration of exposure to the product that is expected in actual use. If the product exposure will be accidental then an acute or single-dose study at a very high dose level, using the anticipated exposure might be adequate. The exposure route should be that which will be used or encountered in actual administration or exposure to the product and may include other routes such as intravenous administration as necessary to maximize the systemic dose or to evaluate 'worst case' exposure conditions.

The order in which toxicological studies are performed to complete the portfolio is also important, and generally starts with short-duration or 'acute' studies and progresses to longer-duration or 'chronic' studies, as necessary. This is both practical and necessary because acute studies often provide information essential to the estimation of doses to be used in longer-term, repeat-dose studies, and they are also generally less expensive and allow the toxicologist and the company an opportunity to stop and re-evaluate further development of a product, should initial results indicate that is has undesirable toxicologic concerns. Studies typical of this initial phase of product-safety evaluation include the following:

- Mutagenicity: evaluates potential to cause genotoxic effects
- Skin corrosivity: evaluates the potential for direct contact to cause skin corrosion

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- Primary dermal irritation: evaluates the potential for a single dose to cause skin irritation
- Primary ocular irritation: evaluates the potential for a single dose to cause eye irritation
- Dermal sensitization: evaluates the potential for delayed-contact hypersensitivity
- Phototoxicity: evaluates the potential for sunlight photoactivation to cause toxicity
- Photosensitization: evaluates the potential to cause photoactivated delayed-contact hypersensitivity.
- Lethality: determines the potential for a single dose to be lethal.

Not all of the studies above are required and the selection of the studies to be included depends on the route of exposure, prior information known about the test article and objectives of the safety-evaluation programme. For example, lethality studies may not be required if there is sufficient evidence that the test material has been tested previously or historically to the extent that there is essentially no need to conduct these studies. In some cases, structure–activity data may be used to estimate the potential for lethality sufficient to reduce or eliminate this requirement.

Acute studies with different routes may be necessary if more than one type of accidental exposure is possible and there is a need to evaluate different endpoints for each route. Typical routes used in acute study designs are oral, dermal, intravenous, subcutaneous, intraperitoneal, ocular and inhalation.

Following completion of acute studies, longer-duration studies can be designed and initiated as necessary. Longer-duration studies are needed for product-safety evaluation if exposure is expected to be repeated over the course of a period of time necessary to treat a disease condition, or if the product is only going be in contact with the body for some duration of time (i.e. treatment of a skin rash, short-duration pain, healing wounds, etc.). The common durations of these studies, as well as the general purposes for each duration is provided in **Table 2**. In these studies, doses are administered at intervals, or continuously, in such a manner that the route and frequency of exposure in actual use is simulated as closely as experimentally possible.

Special studies, such as reproductive outcome, multigenerational, teratological, behavioural and so on, may be required if the potentially exposed population has special considerations, such as being of reproductive age, or if the product has characteristics that indicate the need to evaluate special endpoints (behavioural toxicity, renal toxicity, neurotoxicity, immunotoxicity, etc.).

9 SCHEDULING AND PLACEMENT OF TOXICOLOGY STUDIES

Planning and networking are keys to successful scheduling, placement and completion of toxicology studies in a timely and cost-effective manner. Since a product-safety evaluation for a new product will likely require that at least a few studies be conducted with the product, some whose design requirements are dependent on the outcome of others, it is obvious that the order of completion can be important. The longest-duration studies do need to get started as soon as possible, but not before preliminary studies that yield essential information regarding critical design parameters are completed. For example, toxicokinetic information essential for determining the dose levels and dosing intervals may be necessary before designing longer-term

Table 2 Durations and general purposes of repeat dose or continuous exposure toxicology studies

Study objectives	2 wk	4 wk	13 wk	'Lifetime chronic'
Establish dose levels or exposures for longer studies	X	X	X	—
Evaluate organ toxicity	X	X	X	—
Identify highly toxic test articles doses or exposures	X	X	X	—
Estimate lethality potential	X	X	—	—
Evaluate potential for cumulative effects	X	X	X	—
Evaluate toxicokinetics (blood, urine, tissues)	X	X	X	—
Evaluate effects on weight gain and food consumption	X	X	X	X
Investigate pharmacokinetic characteristics	—	X	X	—
Evaluate physiological changes (ECG, ocular tonometry, blood pressure, core temperature, etc.)	—	X	X	—
Investigate the nature of target organ toxicity observed in earlier studies	—	X	X	X
Evaluate reversibility of toxic effects	—	—	X	—
Identify 'specialized' systemic effects (endocrine, immunological, tissue repair, neurological, etc.)	—	X	X	—
Identify potential carcinogens	—	—	—	X

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studies to ensure that high dose levels are at a tolerance level acceptable to support the study requirements. Studies that are independent of the outcome of others may be completed whenever it is desirable within the programme to do so, as long as they are completed and reported well before completion of the longest-duration study that will be required.

Often the scheduling and placement of toxicology studies is driven by budgetary constraints, as well as time-to-market concerns. While these factors should always be a consideration, quality in the performance of the study and preparation of the study report can outweigh concerns related to financial performance when it comes to studies that are critical for the establishment of product safety. Therefore it is essential that the toxicologist concerned with documentation of product safety carefully select sources with proven performance records in completion of studies on time, with the highest standards of data and report quality.

A toxicologist experienced in product-safety testing is valuable in all aspects of the safety-evaluation process. However, virtually anyone can begin contracting for product-safety studies with some testing facilities. This approach may work satisfactorily for tests with simple, straightforward designs that require little in the way of data interpretation, but it doesn't take much deviation from the expected state of affairs, with respect to test outcomes, to get the novice into trouble. Further testing, if done without experience, can simply create more problems of interpretation and indeed confound the product data set to the point that, as one attorney who attempted this once said, 'this product is beginning to smell', indicating that the safety-data package was in such poor shape that the product (which actually was quite safe) had safety portfolio issues that could be legally challenging under certain circumstances. Product-safety portfolios must be objective, coherent and as comprehensive as possible. They must be up to date with current regulatory requirements, as well as test methods, without creating conflicting findings due to poor study design or management. This is why corporate regulatory affairs professionals and toxicologists will often engage the services of an experienced consulting toxicologist to provide advice and assistance with product-safety programme design and the conduct of safety testing, particularly with products of extreme importance to the company. Here are some reasons why a consulting toxicologist can add significant value to a product-safety evaluation programme:

- Experience with all phases of the product-safety evaluation process, including insurance and litigation, in some cases, that is of value in designing and establishing appropriate testing strategies to ensure safe products for current and future markets.
- Contacts and experience working with many more CROs and the ability to select sources for testing, based on current knowledge that often allows rapid identification of the study locations based on appropriate experience, timing and pricing.
- Experience working with regulatory agencies and up-to-date knowledge of critical regulations.
- Broad product experience across several companies and markets.
- Experience in the preparation of study protocols with many different CROs.

10 THE PRODUCT-SAFETY FILE

All products placed into a marketplace where exposure to humans, animals or the environment is possible, should have some sort of product-safety file. Pharmaceutical and medical device product-safety files are typically quite comprehensive and contain much of the information mentioned above for the product-development file, plus all information collected from the published literature, as well as all study data and reports of product testing that relate in any fashion to the safety and efficacy of the product.

Product-safety files are often opened and maintained by the research and development department, initially, and then are turned over to the regulatory-affairs department upon completion of a new drug or medical device. In other industries, this file can be maintained by a safety, engineering or legal department, or the concern that has been designated to respond to any inquiries regarding product safety.

Having product-safety information readily available and properly summarized is necessary to answer any regulatory inquiries for highly regulated products such as pharmaceuticals, medical devices (which also require a comprehensive design history to be included) and pesticides. This is also extremely valuable in other industries for working most cost-effectively with insurers, responding to product-safety inquiries, concerns, claims or litigation. It should not come as any surprise what can happen to companies not having the ability to rapidly demonstrate an appropriate amount of concern for product safety with products in markets where there is a reasonable possibility of human, animal or environmental exposure.

A product-safety file should contain data sufficient to support a reasonable, up-to-date risk assessment for the intended use of the product, as well as a product-safety assessment if the product is to have significant contact with humans, animals or the environment at any stage of its life cycle, and data on the market history of the product, to include any and all complaints and their

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responses that have any bearing whatsoever on product safety.

Product-safety files should be reviewed regularly (e.g. annually) for any update needs, including product-safety testing and should be maintained actively throughout the market life of the product and then archived for the life of the company.

11 INDUSTRY AND PRODUCT-SPECIFIC SAFETY-EVALUATION ISSUES

11.1 Bulk Chemicals and Raw Materials

The chemical industry includes a wide variety of concerns and is generally concerned with product safety, from raw-material mining through to finished goods production, use and disposal. Worldwide, this industry is regulated heavily in developed countries, with registration requirements under programmes such as the Toxic Substances Control Act (TOSCA), requirement for chemical registration in the US and the EUs new regulation concerning the registration, evaluation, authorization and restriction of chemicals (REACH). Product-safety evaluations that include toxicological testing requirements for registration, inclusion in MSDS documents, environmental-impact assessment and life-cycle analysis are all areas of potential toxicological issues that may arise. At present, the need to perform safety testing using a variety of new methodologies developed for use with REACH is an issue of note for chemical companies doing business in the EU (Combes *et al*., 2008; Ahlers *et al*., 2008). The European Chemicals Agency (ECHA), in Helsinki, Finland is responsible for administration of the REACH program. The ECHA began taking applications for REACH chemicals on 1 June 2008. REACH requires manufacturers and importers to generate data on the substances they manufacture or import, to use these data to assess the risks related to these substances and to develop and recommend appropriate risk-management measures. REACH registration provisions require the submission of a 'registration dossier' containing information in accordance with the guidance provisions of the regulation (e.g. Article 10). More information regarding the registration process and guidance on REACH requirements can be found at the ECHA web site (*http://echa.europa.eu/reachen.asp*). The REACH paradigm attempts to move away from extensive standard testing to a more intelligent, substance-tailored approach (see **Regulatory Toxicology**; **Risk Assessment of Chemicals**).

11.2 Nanomaterials

Nanotechnology is an emerging science involving manipulation of matter at the nanometre scale. Nanomaterials have characteristic morphological features smaller than a one tenth of a micrometre in at least one dimension Engineered nanomaterials, nanoparticles are 'nanoproducts' that represent emerging new segments of the chemical and manufactured products industries, that are gaining support and are increasing in usage internationally in a relatively quiet fashion, despite various concerns from some toxicologists and other scientists and engineers that their safety is relatively unknown and that use of these materials should be slowed or even stopped until safety assessments can be performed. The issue for these materials and products is that scientists, as well as regulatory agencies, are not decided on what sort of testing is of value and should be required despite the availability of proceedings from many meetings and conferences over the past several years that have considered this problem and made many recommendations. The problem seems to be that there is indifference to safety testing in some industries that are essentially self-regulated, whereas in highly regulated industries, nanoproducts have already been or are already being evaluated for safety on a case-by-case basis, which seems the most rational and prudent course to take, at least until there is sufficient data available to indicate otherwise.

It has been established in many toxicologic studies that nanomaterials behave differently to larger particles or chemical substances. Therefore, due to handling and dose-preparation issues alone, it is necessary to develop specialized approaches to testing and monitoring their effects on human and animal health and on the environment (Stern and McNeil, 2008).

Due to concerns over nanomaterial risks, there has been an increase in safety research with nanomaterials of various types. The toxicology community is currently working to identify areas of agreement and discordance with regard to: (i) the potential for nanomaterial exposure, (ii) the relative hazard nanomaterials pose to humans and the environment and (iii) the present deficits in our understanding of risk. Nevertheless, product-safety studies have been performed with nanomaterials, and nanoparticulate pharmaceutical forms have been approved and currently have a safe-use history in their respective markets. In designing product-safety evaluations involving nanomaterials or nanoparticles, special attention must be paid to study design and methodologies. The particle size, surface area, shape, surface charge, chemical composition and aggregation state are variables that can impact the outcomes of some toxicologic tests. These are practical complexities encountered with nanomaterial safety assessment atypical of other product forms. Recent data demonstrate the impact of surface characteristics on nanomaterial biocompatibility and point to the inadequacy of the current

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size-dependent mechanistic paradigms, with nanoscale materials lacking unique or characteristic toxicity profiles (Stern and McNeil, 2008).

The lung, gastrointestinal tract and skin appear to act as significant barriers to the systemic exposure of many nanomaterials and the acute systemic toxicity of many nanomaterials appears to be low. In contrast, the potential pulmonary toxicity of certain nanomaterials (e.g. carbon nanotubes) appears to be of greater concern, and more comprehensive studies are needed to adequately support risk assessment. Many scientists, including some toxicologists, feel that nanomaterial safety data are limited substantially, to the point that until such time as exposures, hazards and the environmental life cycles of nanomaterials have been more clearly defined, cautious development and implementation of nanotechnology is the best course.

The Organization for Economic Cooperation and Development (OECD) Chemicals Committee has established the Working Party on Manufactured Nanomaterials to address this issue and to study the practices of OECD member countries with regards to nanomaterial safety. OECD is currently developing methods for the testing of nanomaterials for the purpose of safety evaluation and has initiated the evaluation of safety for a few of the more common nanomaterials.

Nanomaterials and nanotechnologies are expected to yield important health and healthcare advances, such as more targeted methods of delivering drugs, new cancer therapies and methods of early detection of diseases. Engineered or manufactured nanoparticles have increased surface-area-to-volume ratios and their greater specific surface area (surface area per unit weight) is thought to potentially lead to increased rates of absorption through the skin, lungs or digestive tract; however, the main problem with nanoparticles may actually be recognition by the immune system, which may cause some unwanted localized effects, whether substantially absorbed or not.

There is concern over the use of engineered nanomaterials in food products to enhance aesthetic characteristics, improve nutritional content or reduce caloric levels. In September 2008, the US FDA convened hearings to begin to address concerns regarding the regulation of nanotechnology products in food, drugs and cosmetics. This is an emerging area of toxicologic safety assessment that will, most likely, result in a number of new regulatory guidance documents that could have some impact on the way safety-assessment studies are designed and conducted with nanomaterials (see **Nanotoxicology-The Toxicology of Nanomaterials**).

11.3 Alternative Energy Products and Fuels

There is an increasing interest in the USA and EU in the development and use of alternative fuels for use primarily in transportation, heating and power stations. Safety evaluations of alternative fuels and their associated manufacturing, use and life-cycle properties are the responsibility of the US Department of Energy (DOE) in the USA. The DOE has been evaluating the safety of a flexible-fuel transportation system in the USA since 1991 that could easily switch between petroleum and another fuel, depending on price and availability. Safety evaluations for some fuels such as gasohol have been reported (US DOE, 1991).

11.4 Pesticides

Except for antimicrobial agents intended for use as disinfectants in medicine, the US EPA is responsible for the oversight of pesticide registration and use in the USA. This includes fungicides, insecticides and rodenticides, under the Federal Insecticide, Fungicide and Rodenticide Act, which was first passed in 1947 and then underwent a major revision in 1972. The EPA has different review processes for three categories of pesticides: antimicrobials, biopesticides and conventional pesticides. The three categories have a similar application process, but have different data requirements and review policies. Depending on the category of pesticide, the review process can take several years. The application of nanotechnology to the formulation of pesticides is one of the most significant emerging issues for the EPA, which recently issued a white paper stating its current position on how it intends to establish risk and conduct safety assessments for these emerging products (US EPA, 2007).

11.5 Toxic Toys

Safety evaluation of toys and other consumer products that might pose a significant toxicologic risk is the responsibility of the Consumer Product Safety Commission (CPSC) via the Federal Hazardous Substances Act (FHSA). The FHSA requires precautionary labelling on the immediate container of hazardous household products to help consumers safely store and use those products and to give them information about immediate first aid steps to take if an accident happens. The act also allows the CPSC to ban certain products that are so dangerous or the nature of the hazard is such that the labelling the act requires is not adequate to protect consumers. An example of how the CPSC acts occurred in late 2007, when China confirmed US findings that exports of toys were contaminated with a toxic chemical. US safety officials voluntarily recalled about 4.2 million of these Chinese-made toys. In late 2007, a highly popular toy was found to contain a butylene glycol adhesive solvent that, once metabolized, converts into the toxic 'date rape' drug γ-hydroxy butyrate (GHB).

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The CPSC warned that children who swallow the beads can become comatose, develop respiratory depression or have seizures. This incident serves as an example of the product alerts that the CPSC issues each year. Obviously, it is the responsibility of the manufacturer and distributor to ensure product safety. A careful review of the raw materials and manufacturing process (including solvents used) by an experienced toxicologist might have eliminated the problem in favour of an alternate, less toxic, solvent system. Based on a significant number of recent product alerts in the US regarding potential toxicity from exposures to imported products from certain countries, it would seem prudent for importing companies to perform product-safety evaluations to include toxicity hazard assessment for these products prior to export, if possible.

11.6 Pharmaceuticals

Pharmaceutical products are of note as a basic toxicologic issue for product safety because they are subject to continuous scrutiny and monitoring for safety by the pharmaceutical companies, the FDA, international pharmaceutical regulatory agencies and clinicians using them. In some cases, a pharmaceutical with a tainted safety record may attract the attention of the legal profession. In any case, toxicologists engaged in the development of pharmaceutical products must realize this and be continuously vigilant with respect to any potential for a product-safety concern through all aspects of development, for the pharmaceuticals for which they are responsible. This vigilance extends beyond product launch and includes monitoring for any reports of adverse effects (especially for patterns of effects that may indicate toxicity beyond what was known at NDA (US FDA New Drug Application) submission). Toxicologists should review any manufacturing changes, including site changes that may introduce a component or contaminant that was not evaluated in studies submitted for registration and perform any required additional evaluation or testing as necessary to ensure that the product remains safe for its intended use (see **Toxicological Considerations for Pharmaceutical Products**).

11.7 Medical Devices

Some 'medical devices' consist entirely of a chemical formulation that is intended to produce its desired effect by purely physical means, with little or no substantial absorption and biochemical interaction with the patient. Toxicologists need to be aware of this because product safety is usually determined by evaluating the nature and extent of systemic and local exposure to the device or its constituent chemicals by methods that often require some degree of oversight and data review. 'Combination' medical devices are devices designed to deliver a drug substance in some controlled and/or special fashion. These so-called 'drug-device' combinations are typically evaluated for safety on the basis of: (i) the nature of the effects of the device itself on the systemic and/or local areas that it is in contact with or may affect by direct physical contact or physical emanation, such as electrical, heat, light, sound and so on, and; (ii) the amount and quality of the dose of drug released by the device to the patient. The design and interpretation of safety-assessment studies with such combination devices can be challenging and create issues beyond those encountered for drug substances or medical devices alone. An example of such a device is a 'microneedle' transdermal drug-delivery system. These systems are exciting new collaborations merging engineering with pharmaceutical technology. By painlessly punching arrays of microscopic holes in the stratum corneum, microneedles appear to have the potential to expand the range of drugs and vaccines that can be delivered transdermally (Bal *et al*., 2008; Coulman *et al*., 2008; Donnelly *et al*., 2009; Vandervoort and Ludwig, 2008). Microneedles alone can cause differential effects in the skin due to design factors such as needle length and construction. Safety evaluation for this type of device must necessarily focus on both the effects of the physical damage and the drug being delivered, such that all questions regarding potential risks from the use of this device are investigated appropriately in preclinical studies are addressed in the product-safety evaluation (see **Toxicological Considerations in Relation to the Regulatory Safety Evaluation of Medical Devices**).

11.8 Food Products

Toxicologists must be involved in the evaluation of product safety for food additives. Involvement in the safety review and submission of food-additive petitions to ensure the safety of food products is the responsibility of experienced toxicologists working for the food industry. US FDA regulations require the 'views of experts qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food'. For example, the US FDA makes formal determinations of 'generally recognized as safe' (GRAS) food ingredients according to the following provisions of Section 170 of Title 21 of the US Code of Federal Regulations.

> *Sec. 170.30 Eligibility for classification as generally recognized as safe (GRAS).*
> *(a) General recognition of safety may be based only on the views of experts qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food. The basis of such views may be either (1) scientific procedures or (2) in*

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the case of a substance used in food prior to January 1, 1958, through experience based on common use in food. General recognition of safety requires common knowledge about the substance throughout the scientific community knowledgeable about the safety of substances directly or indirectly added to food.
(b) General recognition of safety based upon scientific procedures shall require the same quantity and quality of scientific evidence as is required to obtain approval of a food additive regulation for the ingredient. General recognition of safety through scientific procedures shall ordinarily be based upon published studies which may be corroborated by unpublished studies and other data and information.
(c) (1) General recognition of safety through experience based on common use in food prior to January 1, 1958, may be determined without the quantity or quality of scientific procedures required for approval of a food additive regulation. General recognition of safety through experience based on common use in food prior to January 1, 1958, shall be based solely on food use of the substance prior to January 1, 1958, and shall ordinarily be based upon generally available data and information. An ingredient not in common use in food prior to January 1, 1958, may achieve general recognition of safety only through scientific procedures.
(2) A substance used in food prior to January 1, 1958, may be generally recognized as safe through experience based on its common use in food when that use occurred exclusively or primarily outside of the United States if the information about the experience establishes that the use of the substance is safe within the meaning of the act (see Sec. 170.3(i)). Common use in food prior to January 1, 1958, that occurred outside of the United States shall be documented by published or other information and shall be corroborated by information from a second, independent source that confirms the history and circumstances of use of the substance. The information used to document and to corroborate the history and circumstances of use of the substance must be generally available; that is, it must be widely available in the country in which the history of use has occurred and readily available to interested qualified experts in this country. Persons claiming GRAS status for a substance based on its common use in food outside of the United States should obtain FDA concurrence that the use of the substance is GRAS.

The introduction of nanotechnology-derived materials into foods is an emerging product-safety assessment issue for food toxicologists that presents challenges in dose preparation and the design of toxicology studies, as well as the selection of appropriate methods to evaluate the distribution and metabolism. This was the topic of a public meeting held in September 2008 by the US FDA, seeking information and input for consideration of the need to provide guidance to industry for use of nanomaterials in food products (US FDA, 2007).

The US EPA has also recently issued a position paper on nanotechnology products. (US EPA, 2007).

11.9 Dietary Supplements

Many people regularly take some type of dietary supplement, and there is some evidence that these vitamins and minerals and other products could offer significant health benefits. In the USA, the Dietary Supplement Health and Education Act (DSHEA) of 1994 amended the Federal Food, Drug and Cosmetic (FD&C) Act to establish a regulatory framework for these products. DSHEA is intended to balance between providing consumers access to safe dietary supplements they choose to improve their health, and giving the FDA, or the Agency, regulatory authority to take action against supplements and supplement ingredients that present safety problems, have false or misleading claims, or are otherwise adulterated or misbranded. DSHEA establishes special requirements for dietary supplements that differ in some respects from those covering 'conventional' foods. DSHEA defines the term 'dietary supplement' as a product that is intended for ingestion, is intended to supplement the diet, is labelled as a dietary supplement, is not represented as a conventional food or as a sole item of a meal or diet, and contains one or more 'dietary ingredients'. 'Dietary ingredients' are defined as vitamins, minerals, amino acids, herbs or other botanicals, dietary substances (such as enzymes) and concentrates, metabolites, constituents, extracts or combinations of the preceding types of ingredients. Dietary supplements may be found in many forms, such as tablets, capsules, powder, liquids or bars. The need to evaluate the safety of dietary supplement products is thus an issue for toxicologists because these items, while not being closely regulated by the FDA, nevertheless have a regulatory authority to act if product-safety concerns become an issue.

There is no requirement for manufacturers of most dietary supplements to provide evidence of product safety to the FDA prior to marketing. The FDA regulates the safety of dietary supplements primarily through a postmarket evaluation of whether the product is adulterated under one of the provisions of the FD&C Act. The burden of proving lack of safety rests with the Federal Government. There is a 75-day premarket notification requirement for dietary supplements that contain certain dietary ingredients that were not marketed in the United States before 15 October 1994, or 'new dietary ingredients'. The manufacturer or distributor of a supplement that contains one or more new dietary

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ingredients must submit a premarket notification to the FDA, unless all new dietary ingredients in the product have been present without chemical alteration in the food supplement as articles used for food. In this notification, the manufacturer or distributor of the supplement must submit information, including citations to published scientific articles that form the basis for the firm's conclusion that the dietary supplement containing the new dietary ingredient will reasonably be expected to be safe. This information is best prepared and submitted with the conclusions of a trained and experienced toxicologist. The supplement will be deemed adulterated by the FDA, unless there is a history of use or other evidence of safety establishing that the new dietary ingredient will reasonably be expected to be safe when used as recommended or suggested in the labelling.

11.10 Cosmetics

Cosmetics, like dietary supplements, are not actively regulated by the FDA like pharmaceuticals or medical devices (except sunscreens, which are typically regulated by the FDA as over-the-counter (OTC) products). The FDA, however, may act to remove a cosmetic product from the market, if the agency has sufficient evidence to show that the product is unsafe. Therefore, it is incumbent on cosmetic companies to evaluate their products for safety prior to market and to monitor the consumer experience with the products in such a manner that any adverse effects are noted and potentially harmful products are reformulated or are removed from the market. Since cosmetics also don't require premarket approval, a basic toxicologic issue for product-safety evaluation with this class of products is what form, if any, should toxicology testing take? Cosmetic ingredients are reviewed for safety by the Cosmetic Ingredient Review (CIR) panel of expert toxicologists, and their reviews are regularly published in the International Journal of Toxicology on an annual basis. Since many cosmetic consumers and consumer groups don't feel that cosmetics should be tested using animals, the evaluation of cosmetics for safety has been at the forefront of attempting to identify alternative tests that are usually conducted using *in vitro* procedures. Historical data from studies performed with animals on cosmetic ingredients can be used in the safety assessment process, even though no animals are used in testing the formulated product. Toxicologists involved in safety assessments for cosmetic products are generally aware that the initial *in vivo* experience with the product will most likely be human exposure in an exposure study involving human subjects, or in premarket tests with small numbers of consumers and monitor for any indication of an adverse reaction to the new product (see **Dermal Toxicology of Cosmetics and Body-Care Products**).

12 CURRENT TOXICOLOGY ISSUES FOR PRODUCT-SAFETY EVALUATION

12.1 Changing Markets with Expanding International Participation

Products that require safety testing to protect workers, consumers and the environment are developed in an expanding international marketplace. Product-safety testing has been expanding internationally to include new operations in China and India, as well as the many established operations in the USA, EU and Japan. Similarly, product-safety evaluations are becoming necessary to meet the requirements of registration programmes that are growing worldwide. The acceptance and success of many products, as well as their sustainability in many markets (foods, drugs, cosmetics, nutritional supplements, toys, etc.) is becoming as dependent on product safety as it is performance.

12.2 New Materials and Methods of Manufacturing or Formulation

New materials for use in the fabrication and manufacturing of new products are increasing the need for evaluations of materials as well as product safety. Nanomaterials are currently the best example of new materials that have raised much concern over the need for re-evaluation of product-safety evaluations to ensure that they meet the needs for determining the safety of emerging products that contain nanomaterials. Much thought has been given to the need for the testing of new nanomaterials for safety, and as a result there have been many recommendations as to approaches that might be taken (NIST, 2008; NIOSH, 2006; European Commission, 2008). Regulatory agencies may eventually make some recommendations and provide guidance on the safety evaluation of products that contain nanomaterials, however, in actuality, the necessity for testing these materials is new and companies, as well as their insurers and consumers are required to perform product-safety evaluations as a matter of 'best practise' in a world that requires knowledge of product safety as a prerequisite to market acceptance and product sustainability.

12.3 Consumer 'Right-to-Know' Regulations

In some parts of the world, there is consumer interest in having product labelling regulations that include affixing product labelling informing consumers of ingredients

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or components that have individually shown to have significant adverse effects (carcinogenicity, reproductive effects, teratogenicity, etc.). California Proposition 65 is one such regulation (State of California, 1986). Toxicologists involved in product-safety testing of products that will be marketed in these regions need to be aware of their requirements and perform product-safety evaluations that are capable of detecting any potential for adverse effects of concern.

12.4 New Methods for Safety Evaluation

There is increasing interest in the use of computational, QSAR and *in vitro* methods for use in product-safety evaluation, as the recent literature and adoption of some of these techniques for routine use bears out (Benfenati, 2007; Bruce *et al*., 2008). These methods have the attraction of being less costly and time consuming than traditional testing procedures, while reducing animal requirements. However, in order for a new method to be accepted by toxicologists as a complete replacement, these new 'alternative' methods have to be rigorously validated against those traditional methods they seek to replace. Whether or not an emerging new method is capable of complete replacement of a traditional method at times may not be the issue, if the new method can be used as a first tier test to screen out and reduce potentially unsafe materials or formulations from a larger number of alternatives that may then be tested using more costly traditional methods.

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) is comprised of representatives from 15 US Federal regulatory and research agencies that use, generate, or disseminate toxicological information. ICCVAM promotes the scientific validation and regulatory acceptance of toxicological test methods that more accurately assess the safety or hazards of chemicals and products and that reduce, refine (decrease or eliminate pain and distress) and/or replace animal use. The activities of ICCVAM are well worth monitoring for any developments in toxicologic testing alternatives of value for product-safety assessments (*http://iccvam.niehs.nih.gov/*).

In October 2007, ICCVAM forwarded recommendations on two *in vitro* test methods to identify ocular corrosives or severe irritants to Federal agencies for their consideration of regulatory acceptance as required by the ICCVAM Authorization Act of 2000 (42 USC. 285 l-3). These are the first alternative nonanimal test methods for ocular safety testing reviewed by ICCVAM and transmitted to agencies. ICCVAM recommended that the bovine corneal opacity and permeability (BCOP) test method and the isolated chicken eye (ICE) test method be used in a tiered testing strategy to determine ocular hazards, with specific limitations for certain chemical classes and/or physical properties. Substances that test positive in these assays can be classified as ocular corrosives or severe irritants without further testing in animals. The report also recommends that these *in vitro* test methods should be considered before using animals for ocular testing and used when determined appropriate. These recommendations, which have been accepted by Federal agencies, will result in a reduction of the number of animals used for safety testing to determine permanent or temporary damage to the eye. ICCVAM estimates that the use of these two alternative test methods will likely reduce the use of live animals for eye safety testing by 10% or more and that the use of these tests will eliminate eye safety testing in animals of most substances likely to cause the most severe pain and discomfort.

On 28 February 2008, ICCVAM forwarded recommendations on the use of *in vitro* test methods for estimating starting doses for acute oral systemic toxicity tests. ICCVAM recommended that the *in vitro* basal cytotoxicity test methods tested in the joint ICCVAM/ECVAM (European Centre for the Validation of Alternative Methods) validation study should be considered before using animals for acute oral toxicity testing, and that the methods should be used where determined appropriate. Two cytotoxicity test methods were recommended, the BALB/c 3T3 NRU method and the NHK NRU method. Details for the conduct and validation of each method can be found in a report issued by the National Institutes of Health (2006). ICCVAM recommends that data from the test methods should be used in a weight-of-evidence approach for determining starting doses for *in vivo* studies. Using these *in vitro* methods, where appropriate, is expected to reduce the number of animals required for each toxicity test. ICCVAM concluded that the *in vitro* test methods are not sufficiently accurate to replace animals for regulatory hazard classification purposes (see **Alternatives to In vivo Studies in Toxicology**).

12.4.1 Local Lymph Node Assay

The murine local lymph node assay (LLNA) is a method for assessing the potential of a test substance to induce allergic contact dermatitis. In January 2007, the US CPSC requested that the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and ICCVAM assess the validation status of: (i) the LLNA as a stand-alone assay for potency determination for classification purposes; (ii) modified LLNA protocols; (iii) the LLNA limit test; (iv) the use of LLNA to test mixtures, aqueous solutions and metals and (v) the applicability domain for LLNA. In response to comments received, ICCVAM endorsed these activities as high priorities. NICEATM and ICCVAM prepared drafts for each topic, which were peer-reviewed in a public meeting in March 2008. The

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peer-review panel's initial LLNA report is now available on the ICCVAM website (*http://iccvam.niehs.nih.gov/*) and another panel meeting to review additional uses of the LLNA is scheduled for April 28–29, 2009, to evaluate three nonradioactive modified versions and new applications for the murine LLNA. Updates and final reporting of this activity and all LLNA-related documentation may be found at the ICCVAM website by searching for the keyword 'LLNA'. The ICCVAM has been receptive to receiving alternative methods for consideration and further evaluation as warranted. Alternative methods for dermal corrosion testing, as well as proposed methods for endocrine disrupter and teratogenesis are currently under consideration.

In the EU, the REACH program has established as its goal for its Optimized Strategies for Risk Assessment of Industrial Chemicals through Integration of Non-Test and Test Information (OSIRIS) project to develop integrated testing strategies (ITS) fit for REACH that enable the programme to significantly increase the use of nontesting information for regulatory decision-making, and thus to minimize the need for animal testing (reducing costs and time) without sacrificing accuracy, validity and reliability of the results. In this programme, operational procedures are developed, tested and disseminated that guide a transparent and scientifically sound evaluation of chemical substances in a risk-driven, context-specific and substance-tailored manner.

OSIRIS periodically conducts workshops to provide overviews of the preliminary results of OSIRIS and its contributions to the REACH characterization and risk-assessment process. These workshops involve experts from industry, academia and government that present the available testing methods and explain how they can be used in REACH. These workshops also allow for an open scientific discussion on the pros and cons of the approaches taken by REACH.

Under the OSIRIS program, a decision-theory framework is envisaged, including alternative methods, such as chemical and biological read-across, *in vitro* results, *in vivo* information on analogues, qualitative and quantitative SAR, thresholds of toxicological concern and exposure-based waiving. OSIRIS also allows for cost-benefit analyses, and it recognizes the gap between risk and societal risk perception.

12.5 Computational Risk-Assessment Modelling

There has long been an interest in using computational tools and toxicology-oriented databases to predict potential safety issues for various materials and products (Holmes, 1976; Jaworska *et al*., 2005; Cronin *et al*., 2003; Patlewicz *et al*., 2008). Initially, the power and speed, as well as the data storage and archiving capabilities of computational systems created practical limitations; however, this is no longer a significant issue and there is more interest in these methods than ever. The purposes of these efforts has been varied, but most often includes a desire to reduce animal requirements, reduce the cost of toxicologic testing, screen raw materials more rapidly and to shorten the time required to complete product-safety risk assessments. A recent project, OSIRIS has as its goal to develop ITS fit for REACH that enable a significant increase in the use of nontesting information for regulatory decision-making, and thus to minimize the need for animal testing. To this end, operational procedures are developed, tested and disseminated that guide a transparent and scientifically sound evaluation of chemical substances in a risk-driven, context-specific and substance-tailored manner.

OSIRIS takes into account cost–benefit analyses, and it aims to close the gap between risk and societal risk perception. It is based on the new REACH paradigm to move away from extensive standard testing to a more intelligent, substance-tailored approach.

Europe's REACH regulations have become the subject of much debate among toxicologists, as well as industry officials. A general consensus has emerged that full implementation of REACH would require testing of around 30 000 existing substances, cost more than 2 billion Euros and could require the use of 10–22 million test animals, if no alternatives to animal testing were available. Simultaneously with the implementation of REACH, the EU desires to develop a policy of replacing, reducing, and refining (the 3 Rs) the use of animals in safety tests. QSARs, have emerged as a valuable means by which to determine and express mathematical relationships between the biological activity of a series of chemical compounds and their physicochemical properties. Until recently, QSARs were unlikely to be acceptable in a regulatory context for a number of reasons. QSARs were developed for a narrow range of closely related molecules, and were unlikely to be relevant for the wide diversity of chemicals falling under REACH. Also, QSARs were generally not developed for toxicological endpoints required under REACH. Furthermore, for all QSARs, validation is not a trivial problem. Despite considerable knowledge of computational methods, recent toxicology graduates may lack the experience with animal models to develop and validate QSARs. So the best approach requires a team of scientists to develop the models and then validation by comprehensive review from an independent team or multiple teams. This process takes time, despite the attractiveness of QSARs, and sometimes results in translational problems between *in vivo* experimental results and *in silico* QSAR assumptions or constraints. However, due to the substantial increases in computational power in recent years, coupled with the availability of good-quality toxicological datasets, the construction of comprehensive

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QSARs is much more amenable and, with the use of extensive data mining and machine learning capabilities, the possibilities for developing QSARs that can operate using more heterogeneous data sets is improving.

The OECD has recently developed comprehensive guidance on what is considered acceptable for a QSAR to be used in a regulatory context with regard to validation requirements (OECD, 2008). To be useful for regulatory purposes, the OECD guidelines suggest that QSARs should have:

1. A defined endpoint; the QSAR must relate to a toxicological parameter used in REACH as an indicator of safety.
2. An unambiguous algorithm; the model must have been generated by a specific definable and repeatable mathematical process.
3. A defined domain of applicability; types of chemicals for which the model is valid must be stated.
4. Appropriate measures of goodness-of-fit, robustness and predictability; in addition to statistical tests for validation, it is now required that an external test set be applied and the resulting accuracy of prediction specified.
5. A mechanistic interpretation, if possible; an understanding of how the model works at the biological level is useful in establishing its validity.

Computer-Assisted Evaluation of Industrial Chemical Substances According to Regulations (CAESAR) is a current project to develop robust, validated QSARs specifically for REACH, and to provide the models free of charge on the internet. CAESAR emerged from an earlier EU project, DEMETRA (Development of Environmental Modules for Evaluation of Toxicity of Pesticide Residues in Agriculture), which developed QSARs for pesticides (Benfenati, 2007) for use by those involved in the pesticide industry or its regulation. This approach seemed ideal for REACH chemicals, and so CAESAR was born. An important lesson learned in DEMETRA and applied in CAESAR was that quality assurance at all stages of the QSAR process is vital. Built into the CAESAR project plan is a series of steps involving checking and cross checking of data by two or more partners with expertise in biochemistry, toxicology, computational chemistry and data mining. A further major step in the early part of the CAESAR project was the quality assurance of the structural data. In cross-checking the many thousands of structures, using desktop molecular modelling software, many errors were found in the published data files, as well as, occasionally, in proprietary 'chemical-finder' web sites. Datasets had to be 'cleaned' of all incorrect structures, ambiguous or mixed structures and those containing inorganic elements, cross-checked by at least two partners and then submitted to partners for descriptor calculation and mathematical modelling. A range of mathematical techniques are used, including linear regression, neural networks, self-organizing maps, genetic algorithms and machine learning.

The availability of CAESAR project output is anticipated in mid-2009. The project team anticipates that a set of models for developmental toxicity, mutagenicity, skin sensitization, bioaccumulation and carcinogenicity, valid for a wide range of chemical types, will be submitted to the European Chemicals Bureau for validation for use within REACH.

In addition to CAESAR and DEMETRA, a wide variety of publicly available and commercial computational tools have been developed, or are under development, that are suitable for the development and application of (Q)SARs. These tools include methods for a range of QSAR development-related tasks, including data management and data mining, descriptor generation, molecular similarity analysis, analogue searching and hazard assessment. All of these computational tools are built upon some experimental toxicity data with rules derived from the data (Matthews and Contrera, 1998; 2007; Kruhlak *et al*., 2007; Contrera *et al*., 2007; Jacobson-Kram and Contrera, 2007). Examples of QSAR rule-based systems include TOPKAT and MCASE. Knowledge-based systems include Derek, OncoLogic® and HazardExpert, whereas other systems, such as TIMES and ECOSAR, are hybrids.

Although QSAR is not likely to provide the complete product-safety assessment, it will undoubtedly be used as part of the 'body of evidence' for a wide range of chemicals, especially in the early years of REACH, when the completion of submissions may have to rely heavily on nonanimal data.

13 RECENT DEVELOPMENTS IN INTERNATIONAL CONSUMER PRODUCT-SAFETY LEGISLATION

Some recent developments in consumer product-safety reporting and legislation of interest to toxicologists include the following, by country or region.

13.1 United States

The Consumer Product Safety Improvement Act of 2008, HR 4040, became Public Law No 110–314 on 14 August 2008 (US Government, 2008). This act treats as a banned hazardous substance under the FHSA, any children's product (which is defined as a consumer product designed or intended primarily for children 12 years of age or younger) containing more than specified amounts of lead. If this is not technologically feasible, alternate limits are allowed which require periodic review and, when

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technologically feasible, more stringent limits. The limits are inapplicable to any component that is not accessible to a child through use and abuse. Specific products or materials may be excluded if the products or materials will neither: (i) result in human lead absorption, taking into account reasonably foreseeable use and abuse by a child, nor (ii) have any other adverse impact on public health or safety. It prohibits considering paint, coatings or electroplating to be a barrier rendering substrate lead inaccessible to a child, or to prevent human absorption of lead. The act also provides for special regulations related to lead exposure for certain electronic devices such as batteries and products that contain them.

This act makes it unlawful to manufacture, sell, distribute or import a children's toy or childcare article containing specified concentrations of particular phthalates. It requires the CPSC to begin appointing a Chronic Hazard Advisory Panel to study the effects on children's health of all phthalates and phthalate alternatives, as used in children's toys and child care articles.

The act requires a manufacturer of a children's product, before importing any children's product that is subject to a safety rule, to have the product tested by an accredited third party for compliance with such rule and to certify that the product complies. It also requires the CPSC to: (i) publish accreditation requirements and a list of accredited entities; (ii) initiate a programme by which a manufacturer may label a product as complying with the certification requirements and (iii) establish protocols and standards relating to the testing of children's products.

Specific CPSC reforms are provided under this act, including enhanced enforcement authorities.

13.2 European Union

Directive 2001/95/EC, enacted by the European Parliament, is now in force, as of 15 January 2004 (European Parliament, 2001). This directive, also known as the General Product Safety Directive (GPSD), is designed as an EU-wide effort to improve consumer health and safety, and the availability of quality products. Originally created 3 December 2001, the directive sets safety requirements for consumer products ranging from sports equipment and household products, to medical-device equipment. The directive empowers member states to establish requirements, depending on the risk and hazard posed by individual products either being introduced within the EU or already in circulation.

This directive empowers member states to take any necessary action to remove any 'serious risk requiring rapid action'. These actions include, but are not limited to:

- An entire ban and recall where a 'serious risk requiring rapid action' is identified
- Information regarding such actions being available to the general public
- A ban on exporting the product from the EU to other countries.

Work is underway in the EU to establish a list of potentially dangerous products of EU-wide concern, as well as establishing within the member states means of monitoring and testing these products. The GPSD is a 'per-product' format directive, meaning that the requirements and actions required vary across products and are evaluated on a product-by-product basis.

On 13 March 2007 the Commission adopted a 'Consumer Policy Strategy' (European Commission, 2007). The strategy sets out the challenges, role, priorities and actions of EU consumer policy. The priorities of the strategy are to:

- Increase consumer confidence in the internal market—which contributes to the improvement of business competitiveness—by establishing a uniform regulatory environment that is equally enforced across the European market and which effectively protects consumers.
- Strengthen consumers' position in the marketplace by developing consumer-education tools, the active support of EU consumer organizations and their involvement in policy-making.
- Ensure that consumer concerns are taken into account in all EU policies.
- Complement member states' consumer policies.
- Collect consumer-related data to support the development of legislative proposals and other initiatives.

'RAPEX' is the recently introduced (EU) rapid alert system for dangerous consumer products (food, pharmaceutical and medical devices are covered by other reporting means), that facilitates the rapid exchange of information between member states and the Commission on measures taken to prevent or restrict the marketing or use of products posing a serious risk to the health and safety of consumers (European Commission, 2004). Measures ordered by national authorities and measures taken voluntarily by producers and distributors are reported by RAPEX:

- Every Friday, the Commission publishes a weekly overview of the dangerous products reported by the national authorities (the RAPEX notifications).
- When a product (e.g. a toy, a childcare article or a household appliance) is found to be dangerous, the competent national authority takes appropriate action to eliminate the risk. It can withdraw the product from the market; recall it from consumers or issue warnings. The National Contact Point then informs the European Commission (Directorate-General for Health and Consumer Protection) about the product,

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the risks it poses to consumers and the measures taken by the authority to prevent risks and accidents.
- The European Commission disseminates the information that it receives to the National Contact Points of all other EU countries. It publishes weekly overviews of dangerous products and the measures taken to eliminate the risks on the internet.
- The National Contact Points in each EU country ensure that the authorities responsible check whether the newly notified dangerous product is present on the market. If so, the authorities take measures to eliminate the risk, either by requiring that the product be withdrawn from the market, by recalling it from consumers or by issuing warnings.

13.3 United Kingdom

The General Product Safety Regulations 2005 (GPSR) is a statutory enactment of the parliament of the United Kingdom that demands that, 'No producer shall (supply or) place a (consumer) product on the market unless the product is a safe product', and provides broad enforcement powers. The regulations implement EU directive 2001/95/EC.

Unless the product is a safe product, the GPSR regulations require that no producer shall:

- Place a product on the market
- Offer or agree to place a product on the market, or expose or possess a product for placing on the market
- Offer or agree to supply a product or expose or possess a product for supply
- Supply a product.

A producer must provide appropriate information to consumers. Either a producer or distributor must inform an enforcement authority if they become aware that they have supplied a dangerous product (regulation 9). The authority must inform the Secretary of State, as of 2008 the Secretary of State for Business, Enterprise and Regulatory Reform, who must inform the European Commission.

A distributor must exercise due care in helping to ensure safety by:

- Not selling dangerous products
- Providing information to purchasers
- Maintaining traceability
- Cooperating with enforcement authorities.

13.4 Japan

Japan's national legislature, the Diet, enacted the country's first law specific to products liability: the PL Law (Law No. 85, 1994) (PL Law) on 1 July 1994. The law, which introduced the concept of strict liability and expanded the definition of a liable manufacturer, went into effect one year later, on 1 July 1995.

Prior to the PL Law, no specific cause of action for strict PL existed in Japanese jurisprudence. Individual plaintiffs were forced to bring claims under Japan's Civil Code using a theory of contractual liability or negligence. These theories afforded little relief for injured parties—and left individual plaintiffs at a particular disadvantage. The number of PL lawsuits filed in Japan has increased since the PL Law went into effect. Many of these PL lawsuits are being brought by individuals or small groups for relatively small claims. Even more importantly, individual plaintiffs are now prevailing in their lawsuits and obtaining judgments against manufacturer defendants for claims that a few short years ago would have been unactionable.

14 THE ROLE OF TOXICOLOGY IN PRODUCT-SAFETY VIGILANCE

The maintenance of successful products in the market requires continual vigilance on many fronts, including performance, satisfaction, quality, cost and so on, but for many reasons, the importance and significance of product safety cannot and must not be overlooked. A long history of product safety can, in fact, be of great benefit to product longevity and marketing. However, if overlooked, consumer and regulatory concerns can rapidly result in significant losses to a product's market share, as well as affect a company's brand recognition. In fact, even a well-established product can rapidly and completely lose market share if a problem in manufacturing or distribution occurs due to a cause that is not rapidly identified and promptly addressed.

Toxicologic problems with products create substantial concern with consumers and can even become exaggerated if not rapidly addressed and resolved. Toxicologists can be of assistance in such circumstances, but not without appropriate data. Therefore, it is important that product-safety evaluation be an essential part of the development process for all products, most especially consumer products that enter a highly diverse international marketplace where there is a high likelihood of having unexpected things happen. So, the design of toxicological safety assessment programmes must learn to anticipate and 'expect the unexpected' to the greatest degree possible. The establishment of an effective post-market vigilance programme can do a lot to provide the information needed for continuous review and improvement of a product's safety file, including any additional testing, as indicated by market experience. Without such vigilance, the product is left in the hands of the consumer

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to do with what they will and this may create unexpected liability issues for the producer and distributor, including some of a toxicologic nature.

Products that are currently being marketed should always be monitored for product safety and all substantive issues or complaints addressed effectively and promptly. An experienced toxicologist can be of substantial value in establishing and maintaining an effective product-safety programme for any product involving consumer contact or exposure. Even if the product is only used within a particular industry, where worker protection is in place to help document and ensure that such protections are adequate to protect against any potential hazards. If the product-safety file is lacking with respect to information on an important issue, effort should be expended to obtain this information in an expeditious manner to protect the consumer, as well as the producer and distributor.

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Cellular Tight Junctions as Mediators of Adverse Effects

Yula Sambuy

CONTENTS

1 INTRODUCTION

Epithelial and endothelial cells form diffusion barriers that generate and maintain the distinct composition of the different body compartments by selective exchange functions. Epithelial cells lining the surfaces of the body that are exposed to the external environment provide a selective barrier to allow the physiological exchange of beneficial molecules and the exclusion or removal of potentially toxic compounds. Endothelial cells lining the blood and lymphoid vessels express various degrees of selectivity in their barrier function, depending on the vascular district, and play important roles in the maintenance of tissue integrity, vascular permeability, leukocyte extravasation and angiogenesis. To perform these functions, epithelial and endothelial cells are equipped with highly specialized junctional complexes (tight and adherens junctions) that join cells to each other at the apical end of the lateral membrane (Schneeberger and Lynch, 2004). Tight junctions serve both as 'gates' that seal the paracellular space and as diffusion 'fences', to maintain the apical/basolateral polarity that is essential to perform asymmetrical exchanges. Tight junctions maintain epithelial cell polarity by preventing intermixing of plasma membrane proteins and restricting diffusion of lipids in the exoplasmic membrane leaflet (Cereijido *et al*., 2008). Epithelial cells, in their position at the border between the external and the internal environment, are exposed to all kinds of injuries, which are dealt with by frequent cellular replacing and by specific repair mechanisms. For these dynamic processes to occur, tight junctions need to be plastic structures, rapidly responding to extracellular stimuli to maintain the integrity of the epithelial cell monolayer. This occurs both in adult life (i.e. in the small intestinal mucosa, epithelial cells proliferate in the crypts, migrate towards the apex of the villi and are finally shed in the lumen), but is particularly important in tissue remodelling during embryogenesis (Cereijido *et al*., 2007).

In recent years, changes in the permeability of epithelial and endothelial barriers have increasingly been reported to represent early indicators of adverse effects from exposure to chemical or infective agents, but also

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to be involved in the development of morbidity and in the progression of various diseases (Forster, 2008; Mullin *et al*., 2005; Prozialeck and Edwards, 2007). The aim of this chapter is to briefly review structure and regulation of epithelial tight and adherens junctions and to report some examples of the mechanisms involved in alterations of tight junction permeability during toxic exposure and in pathological states.

2 TIGHT AND ADHERENS JUNCTIONS

In early ultrastructural studies, epithelial junctional complexes were described as tripartite structures between adjoining cell membranes formed by tight junction (*zonula occludens),* intermediate junction (*zonula adhaerens)* and desmosome (*macula adhaerens),* in which the tight junctions were characterized by fusion of the adjacent cell membranes (Farquhar and Palade, 1963). Later on, employing freeze-fracture electron microscopy, a technique that allows visualization of the hydrophobic membrane surfaces, the tight junctions appeared as a circumferential network of anastomizing strands that are generally assumed to represent polymers of interacting transmembrane components. The barrier is formed where strands of adhesive transmembrane proteins contact across the paracellular space, and it behaves as if perforated by pores possessing size and charge selectivity, the exact molecular nature of which is still unknown despite the identification of several tight junction proteins (Van Itallie *et al*., 2008b). In the tight junctions, transmembrane proteins (occludin, claudins and JAMs (junctional adhesion molecules)) mediate cell–cell adhesion and form the paracellular diffusion barrier, and bind to a group of cytoplasmic plaque proteins that function as cytoskeletal linkers, scaffolds, and regulators of junction assembly and function (**Figure 1**). Most of the transmembrane proteins of the tight junction contain special sequences on their cytoplasmic tail, called PDZ-binding domains, that recruit and bind proteins containing PDZ (PSD95/DlgA/ZO-1 homology) motifs, including the peripheral proteins of the cytoplasmic plaque, the zonula occludens proteins (ZO-1, ZO-2, ZO-3) and other regulatory proteins (Schneeberger and Lynch, 2004). In addition to proteins exclusively associated with the tight junctions, a recently described class of proteins appear to shuffle between the membrane and the nucleus, the *N*uclear *A*dhesion *Co*mplexes (NACo) proteins, where they are involved in the regulation of transcription (Matter and Balda, 2007).

2.1 Adherens Junctions

Adherens junctions are structures that link membrane and cytoskeletal components at discrete contact regions situated just below the most apically located tight junctions (**Figure 1**). Adherens junctions consist of two basic adhesive units: the cadherin/catenin and nectin/afadin complexes. Classical cadherins, such as

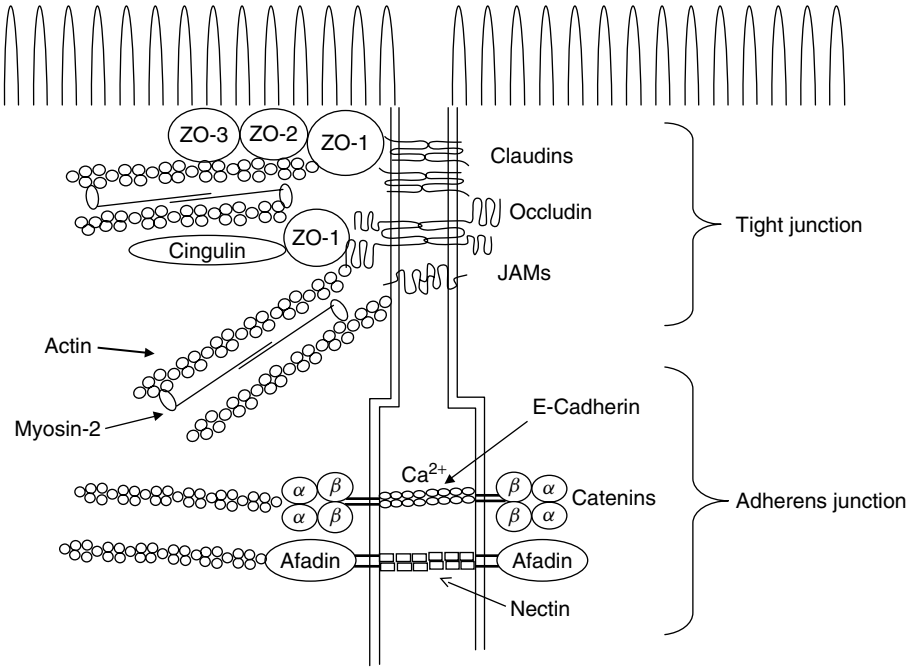

Figure 1 Tight and adherens junction composition. A schematic drawing of the composition of tight and adherens junctions in which only the major proteins and their interactions are shown. Several regulatory proteins participating in the control of junction assembly and function have been omitted for the sake of clarity.

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E-cadherin, are single-pass transmembrane glycoproteins that promote adhesion between homotypic opposing cells by a Ca^{2+}-dependent mechanism, but also bind directly and indirectly to many cytoplasmic proteins, particularly members of the catenin family (i.e. p120-catenin, α-catenin and β-catenin), which locally regulate the organization of the actin cytoskeleton, cadherin stability and intracellular signalling pathways involved in the control of gene transcription. Nectin, a member of the IgG (immunoglobulin G) superfamily of Ca^{2+}-independent adhesion molecules, links through its C-terminal PDZ-binding domain to the actin-binding protein afadin, which also interacts with the Ras/Rap family of GTPases as well as with other actin-binding proteins such as ZO-1 and α-catenin (Niessen and Gottardi, 2008). Adherens junctions play important roles in stabilizing the epithelium by promoting tight junction formation and epithelial cell polarity. When junctions are formed *de novo* or following epithelial injury, adherens junctions assemble first, and this leads to formation of tight junctions (Schneeberger and Lynch, 2004).

In recent years the great molecular complexity of the organization and regulation of epithelial apical junctions (tight and adherens junctions) is fully emerging, and some excellent reviews provide ample details and updated references on the subject (Anderson and Van Itallie, 2008; Ebnet, 2008; Hartsock and Nelson, 2008; Miyoshi and Takai, 2008; Niessen and Gottardi, 2008). Although the adherens junctions will also be mentioned in the chapter, major attention will be placed on the involvement of tight junctions in toxic and pathological events.

2.2 Integral Membrane Proteins of the Tight Junction

2.2.1 Occludin

Occludin, the first transmembrane protein identified in tight junctions, is a tetraspan membrane protein with two extracellular loops, a short intracellular turn, and N- and C-terminal cytoplasmic domains. Its C-terminal tail binds actin and ZO-1, which in turn associates with actin and other proteins of the cytoplasmic plaque (Paris *et al*., 2008). Occludin appears also to interact, directly or indirectly, with claudins, and is recruited into the strands formed by co-transfection of claudins 1 and 2 into fibroblasts (Furuse *et al*., 1998a). When first discovered, occludin was believed to be responsible for tight junction barrier properties, although a single protein could hardly account for the wide range of permeabilities encountered in different epithelial and endothelial tight junctions. Transfection and transgenic mice experiments later showed that occludin expression was not essential to the formation of structurally and functionally mature tight junctions, although occludin-null mice showed postnatal growth retardation and complex phenotypes in various organs. The C-terminal tail of occludin has several residues that can be phosphorylated and dephosphorylated by various protein kinases and phosphatases, providing means of rapid regulation during tight junction assembly and disassembly (reviewed in Chiba *et al*., 2008).

2.2.2 Claudins

Claudins constitute a large family of proteins that share the tetraspan transmembrane topology with occludin, tricellulin and connexins (gap junction proteins), without considerable sequence homology (Krause *et al*., 2008). Claudin expression is fundamental for strand structure since their expression in nonepithelial fibroblasts was shown to be sufficient to recapitulate the strand morphology of tight junctions (Furuse *et al*., 1998b). Claudins can form homotypic or heterotypic interactions among different members of the family. The interactions of claudins of neighbouring cells occur between the two extracellular loops, but it is the first loop, which varies widely among claudins in the position and number of charged amino acids, that is believed to be responsible for the characteristics of the paracellular pores for cations or anions (Van Itallie and Anderson, 2006). Permeability of small uncharged solutes appears to be proportional to the pore number, but small electrolytes may be subject to further selectivity by the specific profile of claudins expressed, thus explaining the frequently reported dissociation between the permeability to small uncharged solutes and the electrical resistance (Van Itallie *et al*., 2008b).

The 24 claudins that have so far been identified are expressed in specific combinations, conferring tissue-specific barrier properties that can range from almost complete tightness of the paracellular route for solutes, as observed in the bladder, to the formation of paracellular pores for specific charged ions (cations or anions), as observed in different segments of the renal tubules (recently reviewed in Chiba *et al*., 2008; Krause *et al*., 2008). Claudin 16/paracellin 1, for example, is primarily expressed in the thick ascending loop of Henle, where it is specifically involved in Mg^{2+} resorption (Simon *et al*., 1999). At least seven claudins (claudins 2, 3, 4, 7, 8, 12 and 15) are abundantly expressed along the intestinal tract with remarkable variations in the level of expression both throughout the intestinal segments and along the crypt-to-villus axis (reviewed in Chiba *et al*., 2008). Although results of transfections or other experiments in single tissues or cell types have ascribed specific functions to single claudins, the distinct tightness properties of a given tissue seem to be largely dependent on the combination of the claudins that are expressed and on the manner

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in which they co-polymerize. Through a PDZ-binding motif at their C-terminus, most claudins directly bind to PDZ domain-containing peripheral membrane proteins of the cytoplasmic plaque (i.e. ZO proteins); this binding appears to be essential for the spatial organization of claudin-based membrane strands in epithelial cells, and to the transmission of signals to the regulatory proteins (Guillemot *et al*., 2008). The dynamic nature of tight junctions appears to be conferred, at least in part, by the fast turnover of claudins (with half-lives much shorter than other transmembrane proteins) and by their many phosphorylations, indicating the existence of short-term regulatory mechanisms. Phosphorylation by different protein kinases of various claudin isoforms can result in rapid changes in tight junction permeability, frequently accompanied by movement of the claudins away from the membrane and dissociation from other junction proteins (Gonzalez-Mariscal *et al*., 2008; Krause *et al*., 2008).

2.2.3 Tricellulin

Tricellulin has been identified as a four-pass transmembrane protein that is concentrated at the vertically oriented tight junction strands of tricellular contacts of epithelial cell sheets, and is required for the maintenance of the transepithelial barrier (Ikenouchi *et al*., 2005). It shows a 30% homology in the C-terminal tail with occludin, and recent data indicate that occludin is required for tricellulin correct localization to the tight junction (Ikenouchi *et al*., 2008). Down-regulation of tricellulin by RNA interference in cultured epithelial cells produced disorganization of both bicellular and tricellular tight junctions (Ikenouchi *et al*., 2005). Tricellulin is ubiquitously expressed in epithelial junctions throughout the body, and different splice variants in the tricellulin gene have been described in humans affected by autosomal recessive hearing impairment (Chishti *et al*., 2008; Riazuddin *et al*., 2006). The mechanisms for such a critical role of tricellulin in the inner ear have not yet been fully elucidated, and it has been suggested that at least one underlying cause of cellular dysfunction in the cochlea may be the inability to incorporate these mutant tricellulin proteins into the cytosolic scaffold formed by ZO proteins (Riazuddin *et al*., 2006).

2.2.4 Junctional Adhesion Molecules

Junctional adhesion molecules (JAMs) are glycosylated transmembrane proteins that belong to the immunoglobulin (Ig) superfamily, and consist of two extracellular Ig-like domains, a single transmembrane region and a C-terminal cytoplasmic domain with a PDZ-binding domain. Based on their PDZ-binding domain they can be distinguished and assigned to two groups, JAM-A, JAM-B, JAM-C, and JAM-4, CAR, CLMP and ESAM (Ebnet, 2008). They show homophilic and heterophilic interactions through their extracellular domains among distinct cell types (i.e. leukocytes, platelets and/or endothelial cells), and also bind to various regulatory proteins of the cytoplasmic plaque (Chiba *et al*., 2008). Except for JAM-A, there is only little information on the role of these proteins in the development or the function of tight junctions. All but CLMP interact with tight junction-associated scaffolding proteins of the cytoplasmic plaque like ZO-1, PAR-3 and MAGI-1. For JAM-A there is good evidence for its involvement in the regulation of epithelial and endothelial tight junctions and in the development of apical/basal cell polarity in epithelial cells by recruiting multiprotein signalling complexes crucial for apical domain formation (Ebnet, 2008). In addition, certain JAMs have been implicated in the regulation of leukocyte extravasation through endothelial cells (Cook-Mills and Deem, 2005).

2.2.5 Cytoplasmic Plaque Proteins

The cytoplasmic plaque is composed of a series of proteins (ZO-1, ZO-2, ZO-3) belonging to the membrane-associated guanylate kinase (MAGUK) superfamily, and of an array of other molecules, including cingulin; symplekin; the Ras target, leukaemia fusion-6 (AF-6); the multi-PDZ protein, MUPP-1 (multi-PDZ domain protein 1); the membrane-associated guanylate kinase inverted proteins (MAGIs); and proteins involved in the establishment of epithelial cell polarity (PAR-3, PAR-6, PALS-1 and PATJ). Many of the scaffolding proteins contain multiple PDZ domains and other protein–protein interaction domains, with which they bind to and regulate different types of signalling proteins, ranging from small and heterotrimeric GTP-binding proteins and guanine nucleotide exchange factors for Rho-family GTPases, to protein phosphatases and kinases (for reviews, see Aijaz *et al*., 2006; Ebnet, 2008; Guillemot *et al*., 2008; Schneeberger and Lynch, 2004). More than 30 proteins have been described to be associated with the cytoplasmic aspect of the tight junctions (Schneeberger and Lynch, 2004), playing a scaffolding role to couple the transmembrane proteins to the actin cytoskeleton and to other regulatory proteins in the cytoplasm, as well as a coordinating role for the complex signalling pathways involved in the regulation of epithelial junction assembly and permeability.

2.2.5.1 NACo Proteins (Nuclear Adhesion Complexes)

Recent evidence suggests that tight junctions also participate in signal transduction mechanisms that regulate epithelial cell proliferation, gene expression, differentiation and morphogenesis. One important class of tight junction-associated signal transduction mechanisms is based on dual localization of certain proteins both at junctions and in the nucleus (Matter and Balda, 2007).

At the level of the adherens junction the best and most intensively studied example is the β-catenin, an adherens

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junction-associated NACo that binds to cadherins and plays crucial roles in the Wnt/Wingless signalling pathway, important in development and cancer. Once stimulated, β-catenin accumulates in the nucleus, owing to decreased ubiquitin–proteasome-mediated degradation, and binds to and activates the transcription factor LEF/TCF (lymphoid enhancer factor/T cell factor), leading to transcription of genes involved in proliferation and epithelial–mesenchymal transition (Cadigan, 2008).

In tight junctions, the Y-box transcription factor ZONAB (*ZO*-1-associated *N*ucleic *A*cid *B*inding protein), the canine homologue to human DbpA, was described in postproliferative confluent epithelial cells to be transcriptionally inactive and to reside exclusively in the cytoplasm, bound to ZO-1, and in proliferating cells to move away from the junction to the nucleus to initiate transcription (Balda and Matter, 2000). ZONAB has been shown to affect G1/S-phase transition by regulating the nuclear accumulation of the cell division kinase CDK4 (cyclin-dependent kinase 4) and the expression of genes involved in cell cycle regulation (Balda and Matter, 2003; Sourisseau *et al*., 2006). Interaction of the heat shock protein, Apg-2, with ZO-1 was found be stimulated by heat shock and to result in nuclear translocation and transcriptional activation of ZONAB, presumably by displacing it from its junctional adaptor (Tsapara *et al*., 2006). The ZO-1–ZONAB pathway is therefore emerging not only as a mediator of proliferation, but also in cellular responses to certain stresses. Although it is currently not known which types of stresses may affect the transcriptional activity of ZONAB, it is possible that conditions that interfere with junctional integrity (i.e. oxidative stress or energy depletion) also induce ZONAB activation, as recently shown for the ethanol metabolite phosphatidylethanol (see below).

3 ENDOTHELIAL JUNCTIONS

The endothelium forms a semipermeable barrier lining the vascular vessels that regulates fluid and solute exchange between the blood and the interstitial fluid. The junctional structures joining together endothelial cells are related to those found in epithelia, as they contain tight junctions and adherens junctions (**Figure 1**); however, their organization is more variable, and in most vascular beds their topology is less restricted than in epithelial cells. In pathological conditions, endothelial barrier dysfunction can lead to protein-rich tissue oedema, as observed in atherosclerosis and diabetes-associated vascular disease (reviewed in Bakker *et al*., 2008; Hadi *et al*., 2005).

Adherens junctions represent the majority of junctions comprising the endothelial barrier, in contrast to the epithelium where tight junctions predominate, and are composed of vascular endothelial (VE)-cadherin consisting of five cadherin-like repeats that associate homotypically with VE-cadherin on the adjacent cell in a Ca^{2+}-dependent manner. The juxtamembrane domain of VE-cadherin binds p120-catenin, whereas the C-terminal domain binds with β-catenin and α-catenin, which in turn link to the actin cytoskeleton. Although adhesion between cadherin and catenins is required for maintaining adherens junction integrity, p120 binding to cadherins is perhaps the most important determinant of their stability. The scaffold function of p120 serves to regulate the interactions between cadherins, kinases, phosphatases and Rho GTPases, which in turn control the phosphorylation state and stability of cadherin interaction with one another, as well as with catenins (reviewed in Vandenbroucke *et al*., 2008).

Endothelial tight junctions are much less well known than their epithelial counterparts. They contain occludin, ZO-1, ZO-2 and ZO-3 that associate to cortical actin through cingulin. Only claudin 5 is expressed in endothelial tight junctions. In the different segments of the vascular tree the tight junctions show considerable variability in strand complexity and composition with strong impact on vascular permeability and leukocyte extravasation (the process by which at inflammatory sites, mostly in postcapillary venules, leukocytes cross endothelial cells either through transcellular or paracellular routes) (Vestweber, 2007; Wallez and Huber, 2008). Large artery endothelial cells, which are exposed to high flow rates, display a well-developed system of tight junctions. Within the microvasculature, tight junctions are less complex in capillaries than in arterioles, and even less in venules. The blood–brain barrier (BBB) and the blood–retinal barrier are particularly rich in tight junctions whose barrier properties are believed to be determined by interactions with the perivascular astrocytes and the closely associated pericytes. Both cells produce factors, including angiopoietin-1, TGFβ (transforming growth factor β) and probably other uncharacterized molecules, that are required to induce the barrier phenotype (reviewed in Wallez and Huber, 2008).

Various evidence supports a different mode of tight junction regulation in epithelial and endothelial cells, and in the following paragraphs the focus will be primarily on epithelial tight junction regulation, because of the still limited information available on the endothelium (Liebner *et al*., 2006).

4 REGULATORY PATHWAYS INVOLVED IN CONTROL OF EPITHELIAL TIGHT JUNCTION PERMEABILITY

Several regulatory pathways are involved in the control of tight junction assembly and maintenance during embryogenesis, tissue remodelling, normal physiological

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turnover and in response to toxic or pathological signals. This highly dynamic structure, whose degree of sealing is controlled by external stimuli, requires a complex crosstalk between distinct regulatory pathways relying on activation of signalling cascades for rapid modulation of protein phosphorylation and on the production of intracellular second messengers leading to mobilization of specific transcription factors. For an extensive review of the subject refer to Gonzalez-Mariscal *et al*. (2008). Briefly, the state of phosphorylation of tight junction proteins is regulated by an array of kinases and phosphatases. Among these are the phosphokinase C (PKC) family, 12 known Ser/Thr-specific isozymes that differ in their mechanism of action, subcellular distribution, substrate type and expression; the phosphokinase A (PKA), regulated by the rise in intracellular cAMP, and the phosphokinase G (PKG) whose activation by cGMP in brain endothelial cells exerts different effects depending on the state of the tissue. While Ser/Thr phosphorylation of several tight junction proteins promotes the barrier function of tight junctions, several protein phosphatases, PP1, PP2A and PP2B, exert opposite effects on the barrier function of tight junctions depending on the role of the kinase isoform they are inhibiting. The Rho family of small GTPases includes RhoA, Rac and Cdc-42, which regulate distinct actin structures in response to diverse signals, and are therefore involved in the maintenance of the stability of the scaffold between the junction proteins and the cytoskeleton, thus affecting junction permeability. Another important pathway involved in regulation of tight junction permeability by external stress or pro-inflammatory cytokines is the Myosin Light Chain Kinase (MLCK) pathway that Ser-phosphorylates the Myosin 2 Light Chain (MLC), leading to contraction of the perijunctional actomyosin ring and opening of the paracellular space. In certain systems a crosstalk between PKC and MLCK has been proposed where MLCK is itself phosphorylated by activated PKC. Finally, another important pathway in tight junction regulation is that of the mitogen-activated protein kinases (MAPKs). This signalling pathway has a membranous and a cytoplasmic phase, the first in close proximity to the growth factor receptors, involving the activation of small GTP binding proteins (i.e. Ras), that initiates a cascade of sequential activations of several cytoplasmic kinases, MAP3K, MAP2K and finally MAPK, all phosphorylated on Ser/Thr residues. MAPKs can then phosphorylate a large number of proteins that are located both in the cytoplasm and the nucleus, including transcription factors that regulate the expression of diverse genes. The different MAPKs expressed in mammalian tissues are the extracellular signal-regulated kinases (ERKs), also known as classical MAP kinases that have been found to interact with occludin, the c-Jun N-terminal kinases (JNKs) and p38 isoforms activated by stress stimuli and involved in cell differentiation and apoptosis, and ERK5, participating in cell proliferation and activated by both growth factors and stress stimuli.

In conclusion, given the potential for feedback and crosstalk linking all the regulatory pathways involved in the control of tight junction permeability, it is unlikely that simple linear pathways will adequately explain the complex relationship between external toxic or pathological stimuli, intracellular signalling and junction responses. It is therefore important to abandon the view that single kinases, phosphatases or GTPases will mediate a single cellular or tissue outcome (Braga and Yap, 2005).

5 EXPERIMENTAL MODELS AND ASSESSMENT OF TIGHT JUNCTION PERMEABILITY

In clinical practice, changes in intestinal permeability as indicators of several pathological conditions of the gut, including inflammatory bowel disease (IBD) and coeliac disease, are measured by the lactulose/mannitol test, and in the renal disease, kidney creatinine or inulin clearance are employed. However, an increasing number of pathological conditions are found to be associated with changes in permeability of the epithelial or the endothelial barrier, and simple clinical tests are not available for all areas. For the study of the molecular mechanisms involved in pathological changes to epithelial and endothelial barriers, both animal disease models and excised mucosal tissue mounted in Ussing chambers have been employed (Li *et al*., 2004). The use of knockout mice for genes coding for tight junction protein, and the positional cloning of gene mutations in several hereditary diseases involving tight junction proteins, are two different experimental approaches that have recently contributed to further understanding of tight junction function and regulation *in vivo* (Furuse, 2008).

But in many cases, detailed dissection of the molecular composition and regulation of tight junctions during assembly or disassembly, in response to a wide array of different physiological and pathological stimuli, has required the use of cell-culture models of well-differentiated epithelial and endothelial cells from various organs. These *in vitro* models also allow expression of exogenous or silencing of endogenous genes, as well as expression of chimaeric proteins, and all these techniques have been extensively utilized to dissect the roles of single components within the junctional complexes. Although cell culture models cannot account for immune-mediated or neural-controlled stimuli, they have been essential to single-out and unravel molecular mechanisms of tight junction regulation.

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Among the most widely used models for the study of epithelial tight junctions are the Madin Darby canine kidney (MDCK) cell lines and the human intestinal Caco-2 cell lines. In MDCK cell lines, differences in transepithelial electrical resistance (TER), which distinguish the high-resistance MDCK Type I line from the low-resistance MDCK Type II line, have been attributed to changes in the expression of the pore-forming claudin 2 and in the basal activity of the regulatory kinases ERK 1/2 (Lipschutz *et al*., 2005), while strand structure and ZO-1 content were identical in the two lines. Caco-2 cells, originally obtained from a human colon adenocarcinoma, when grown on permeable filter supports, differentiate over a period of two to three weeks in a postmitotic monolayer of well-polarized cells coupled by functional tight junctions and expressing many characteristics of mature small intestinal enterocytes (Delie and Rubas, 1997). Both MDCK and Caco-2 cell lines have proved very useful for investigation of epithelial junction permeability, although care should be taken in the comparison of results from different laboratories since extensive cell- and culture-related variability has been reported with these culture models (Sambuy *et al*., 2005; Volpe, 2008). Other well-differentiated cell culture models useful to study tight junction regulation in different tissues are the renal porcine proximal tubules line LLC-PK1 (Gopalakrishnan *et al*., 2003), the human pancreatic cell line HPAF-II (Rajasekaran *et al*., 2004), as well as primary or established cell lines of the human airways epithelium (Bao and Knoell, 2006a; Verstraelen *et al*., 2008).

The BBB is a highly regulated barrier of brain capillaries that segregate the central nervous system (CNS) from systemic blood circulation and maintain a delicate homeostasis of the CNS environment. In the BBB, glial astrocytes surround endothelial cells, providing them with a biochemical support. Culture models of endothelial brain cells have a low TER and form tight junction networks of low complexity. However, when they are maintained in co-culture with astrocytes or in astrocyte-conditioned media the TER doubles, tight junctions more closely resemble those of brain endothelium *in vivo* and the cells express specific BBB markers. An established co-culture method of bovine brain capillary endothelial cells (BBCECs) with rat glial cells (Cecchelli *et al*., 1999) has recently been optimized by replacing the co-culture with a differentiation medium containing glial-conditioned medium and by shortening the time required to obtain well-differentiated endothelial cells resembling those of the BBB (Culot *et al*., 2008). Another cell culture model is the immortalized human brain microvascular endothelial cell line hCMEC/D3 that retains the morphological characteristics of primary brain endothelial cells and expresses specific brain endothelial markers and cell surface adhesion molecules (Weksler *et al*., 2005). It was recently shown that these cells, when cultured on hollow microporous fibres under physiological flow conditions, maintain *in vitro* the physiological properties of the BBB even in the absence of abluminal astrocytes, and respond to inflammatory stimuli with barrier failure and extravasation of leukocytes (Cucullo *et al*., 2008).

Properties of the epithelial paracellular barrier include electrical resistance and solute permeability, which can be independently assessed by measuring the TER, the transepithelial ionic diffusion potentials determined by short circuit currents (I_{sc}), or the diffusion of labelled charged or uncharged tracer molecules of various sizes (Rajasekaran *et al*., 2008). TER is the most sensitive measure of mucosal barrier function since it allows for fast and continuous assessment of changes in the paracellular conductance. In relatively leaky epithelia such as that of the small intestine, TER essentially reflects the paracellular resistance, and alterations of the apical membrane conductance by activation of ionic channels have little effect on total resistance. However, in tight epithelia (i.e. in the colon or in renal proximal tubules), activation of ionic membrane channels may lead to large changes in TER, and appropriate control of selective ionic permeability by I_{sc} measurement must be undertaken to distinguish between transcellular and paracellular contributions to TER (Madara, 1998). Measurements of transepithelial ionic diffusion potentials can also assess the ionic selectivity of the tight junction pathway, while measurements of diffusion of tracer molecules have the advantage of being size selective. This is particularly relevant in conditions of toxic or pathological changes where both transcellular permeability (largely controlled by ionic channel activity) and paracellular permeability (tight junctions) can contribute to different extents to the observed changes.

Paracellular solute permeability can be measured with molecules that are excluded by the cells and detected by colour (phenol red), fluorescence (Lucifer yellow or FITC-dextran) or radioactive label (^{3}H- or ^{14}C-labelled inulin or mannitol). The size of the tight junction pores can be evaluated employing paracellular tracers of different sizes as recently shown in different epithelial cell lines and in porcine ileum, with a series of noncharged polyethylene glycols (PEGs) detected by high-performance liquid chromatography (HPLC) after fluorescent derivatization (Van Itallie *et al*., 2008b). In experimental practice a widely shared opinion is that TER and noncharged tracer diffusion studies are complementary, but not mutually exclusive methods to measure paracellular permeability.

In addition, when using whole-tissue mounts in Ussing chambers (i.e. intestinal mucosal sheets) some investigators have employed methods of impedance analysis that allow one to differentiate the epithelial and the subepithelial portion of transmural resistance, resulting in a more accurate assessment of the electrical pathways contributing to the overall TER (Gitter *et al*., 1998).

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Although these methods are not very commonly utilized, as they require more sophisticated instrumentation and mathematical modelling, it is important to recognize the possible contribution of the various constituents of TER in the evaluation of the physiological significance of recorded changes in tight junction permeability in the different experimental models.

Since tight junctions are highly dynamic structures that rapidly respond to various physiological and pathological stimuli, appropriate experimental approaches are necessary to correctly interpret the mechanisms involved. Until recently, for example, it was difficult to analyse the mechanisms by which actin depolymerization led to tight junction disruption, as studies of fixed tissues showed morphological changes only after loss of barrier function. The development of well-validated fluorescent fusion constructs of representative tight junction proteins have allowed continuous real-time assessment of tight junction structure and function in live epithelial monolayers, demonstrating that during exposure to drugs depolymerizing actin, occludin was removed from the junction by caveolae-mediated endocytosis at the precise time that barrier function was disrupted, while claudin 1 and ZO-1 delocalization occurred at a later stage (Turner, 2006).

6 AGENTS CAUSING INCREASED TIGHT JUNCTION PERMEABILITY

First studies reporting alterations to tight and adherens junction permeability were conducted on cultured epithelial cells treated with pharmacological agents causing either actin depolymerization (Madara, 1987) or calcium chelation (Cereijido *et al*., 2000). Several different stimuli leading to changes in tight junction permeability were found to be mediated by the actin cytoskeleton, suggesting a physical link between the cytoskeleton and the junction proteins. This physical link was later identified in the scaffolding role of cytoplasmic plaque proteins (i.e. ZO-1) linking the apical ring of filamentous actin to the adhesive transmembrane proteins of the tight and adherens junctions (reviewed in Miyoshi and Takai, 2005). Calcium mediates the adhesion between extracellular domains of E-cadherin in adherens junctions, and calcium depletion causes endocytosis of adherens junction (E-cadherin) and tight junction (claudins, occludin) proteins (Hartsock and Nelson, 2008) The rapid disassembly/reassembly of junctions in response to changes in extracellular calcium have been exploited in the 'calcium-switch' model that is frequently utilized to follow and characterize the molecular mechanisms involved in modulation of the apical junction (Cereijido *et al*., 2000). Since the early studies, this field has grown enormously both in the number of proteins and regulatory pathways identified and in the complexity of their interactions. Over the years, an increasing number of noxious stimuli have been reported to affect the structure and permeability of epithelial and endothelial tight junctions, and these early toxic effects have frequently been shown to precede further damage to the cells, leading to cell death by apoptosis or necrosis. Examples of chemical noxious stimuli to epithelia and endothelia that will be discussed here are ethanol, oxidative stress and metals (in particular cadmium, iron and zinc).

6.1 Ethanol

Ethanol is one of the numerous exogenous compounds thought to have harmful effects on the gastrointestinal mucosa, in particular due to the regular and repeated contact with ethanol of the gastrointestinal mucosa of chronic alcohol consumers. Chronic ethanol consumption is associated with an increased risk of developing cancers of the digestive tract, and in particular colorectal carcinomas, and strong proliferative response and mucosal hyperplasia in colon and rectum have been reported following alcohol abuse (Boffetta and Hashibe, 2006). In addition, high ethanol concentrations are known to produce significant mucosal damage, allowing the paracellular passage of luminal substances that may favour cytotoxicity and inflammation. Ethanol effects are not restricted to the gastrointestinal tract since alcohol consumption is also a major risk factor in other pathological conditions such as neurobehavioural diseases, inflammation disorders in different organs and enhanced susceptibility to bacterial infection, frequently involving changes in junction permeability of epithelial and endothelial cells, and accompanied by a strong inflammatory response.

Ethanol treatment of cultured intestinal epithelial cells was first shown to activate MLCK, leading to disorganization of the perijunctional actomyosin ring and increase in paracellular permeability (Ma *et al*., 1999). Regulation of tight junction permeability by MLCK phosphorylation of MLC and of tight junction proteins is a mechanism common to different stimuli, including oxidative stress and pro-inflammatory cytokines, that has been described in several epithelial and endothelial cells both *in vivo* and *in vitro* (Eutamene *et al*., 2005; Haorah *et al*., 2005b; Ma *et al*., 2005). The toxic effects of ethanol are generally attributed to the production of acetaldehyde by specific isoforms of alcohol dehydrogenase (ADH) in epithelial and endothelial cells and in the intestinal lumen by the action of enteric microbial fermentation. Acetaldehyde is a highly mutagenic and carcinogenic molecule, also reported to generate a number of reactive oxygen metabolites. At the level of the intestinal epithelium, acetaldehyde has been shown to disrupt tight junctions and

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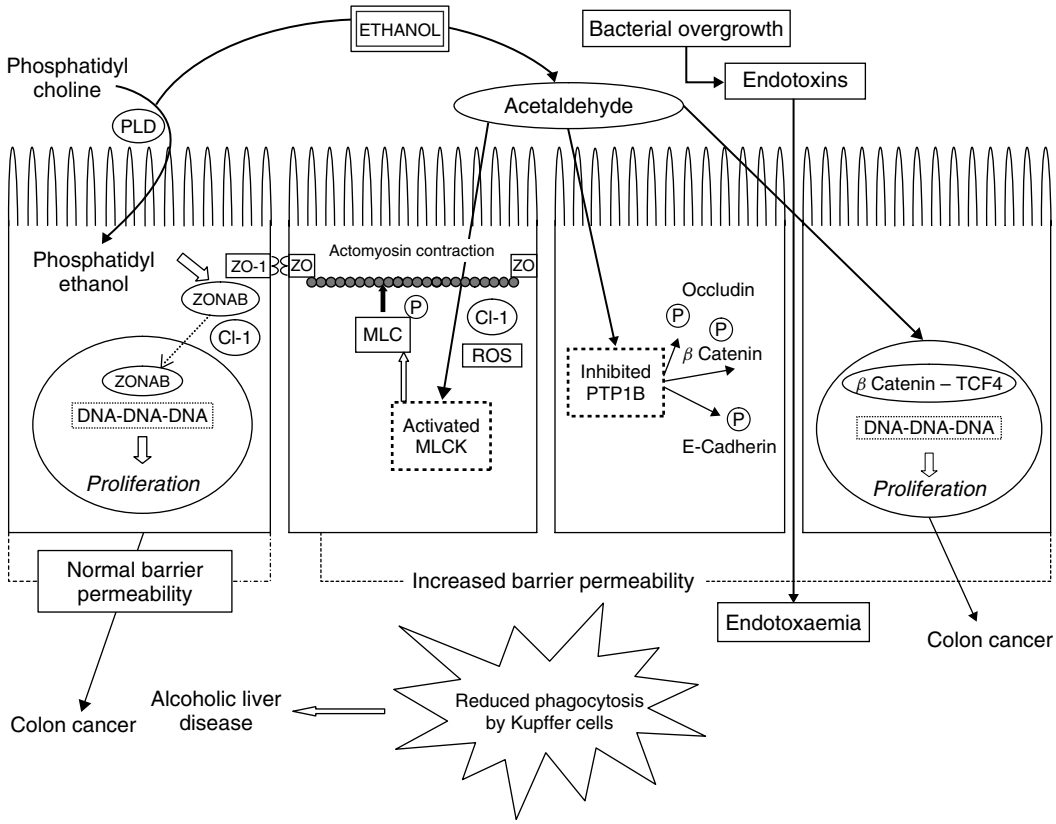

Figure 2 Multiple pathways of ethanol toxicity in the intestinal epithelium leading to diverse pathological effects. Formation of phosphatidylethanol from ethanol and phosphatidylcholine by the membrane enzyme phospholipase D (PLD) can cause dissociation of the transcription factor ZONAB from ZO-1 and its translocation to the nucleus, leading to the activation of genes involved in reinitiation of proliferation. These effects occur in the absence of changes in intestinal barrier function. Acetaldehyde, produced in intestinal cells by the enzyme alcohol dehydrogenase, can activate phosphorylation (P) of myosin light chain kinase (MLCK) either directly or through the formation of reactive oxygen species (ROS), which in turn phosphorylates myosin 2 light chain (MLC) and causes the contraction of the perijunctional actomyosin ring and increased epithelial barrier permeability, with claudin 1 (Cl-1) internalization. Acetaldehyde can also inhibit the protein tyrosine phosphatase 1B (PTP1B), blocking occludin, β-catenin and E-cadherin in their phosphorylated state. Phosphorylated occludin is internalized and contributes to barrier dysfunction. Phosphorylated β-catenin dissociates from E-cadherin and migrates to the nucleus where, with the transcription factor TCF4, it activates genes involved in proliferation. Chronic ethanol consumption can enhance intestinal bacterial overgrowth with increased production of endotoxins that can freely migrate across the permeabilized intestinal mucosa, leading to endotoxaemia. In the liver, acetaldehyde reduces endotoxin clearance by Kupffer cells and can contribute to alcoholic liver disease. Activation of proliferative events by different ethanol metabolites can contribute to the development of colon carcinogenesis.

increase paracellular permeability by different mechanisms (**Figure 2**). In the human intestinal Caco-2 cell line that lacks ADH activity, acetaldehyde treatment induced inhibition and dissociation of Protein Tyrosine Phosphatase 1 B (PTP1B) from the E-cadherin–β-catenin complex of the adherens junction, resulting in sustained tyrosine phosphorylation of β-catenin that led to its dissociation from E-cadherin and loss of homophilic interaction between extracellular domains of E-cadherin (Sheth *et al*., 2007). Inhibition of PTP1B was also shown to maintain occludin phosphorylation, causing its redistribution to the cytoplasm together with ZO-1 (Basuroy *et al*., 2005). The combined effects of acetaldehyde on the phosphorylated state of proteins of tight and adherens junctions resulted in increased epithelial barrier permeability. Such effects could be prevented by treatment with EGF or L-glutamine, a protection that was shown to be mediated by ERK activity (Basuroy *et al*., 2005; Seth *et al*., 2004).

However, acetaldehyde is not the only toxic mediator of ethanol effects (**Figure 2**). Recently, chronic low-dose ethanol exposure was shown both *in vivo* and *in vitro* to be associated with increased production of phosphatidylethanol in intestinal epithelial cells, resulting in changes in the localization of claudin 1 and ZO-1 and its associated transcription factor ZONAB, in the absence of changes to tight junction permeability (Pannequin *et al*., 2007). Detachment of

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ZONAB from the tight junction protein ZO-1 and its migration to the nucleus resulted in transcriptional activation of genes involved in cell proliferation. Therefore, the disruption of ZONAB-mediated physiologic signals sent by the tight junction complexes to stop the proliferation of confluent epithelial cells might, in addition, facilitate the stimulatory role of other ethanol metabolites, such as acetaldehyde, on the proliferation of cells within intestinal crypts, thereby contributing to the well-established co-carcinogenic role of alcohol consumption in the colon (Pannequin *et al*., 2007).

Elevated intestinal permeability appears to be one of the major factors in the mechanism of alcohol-induced endotoxaemia, a crucial event in the pathogenesis of alcoholic liver disease (Nagata *et al*., 2007). Bacteria of the intestinal flora are the source of endotoxins (lipopolysaccharides (LPS), derived from the intestinal wall of Gram-negative bacteria) that normally penetrate the intestinal mucosa only in trace amounts. In chronic ethanol consumption, microbial proliferation is increased and the acetaldehyde-mediated opening of intestinal tight junctions can significantly enhance the passage of endotoxins into the circulation. Plasma endotoxin levels are higher in patients with alcoholic liver disease (ALD) than in normal subjects or in patients with nonalcoholic cirrhosis. In addition, alcohol metabolites appear to reduce the phagocytic activity of Kupffer cells, while endotoxins activate cytokine production, further contributing to the development of liver disease. The role of intestinal permeability and endotoxaemia in alcoholic liver disease has been reviewed by (Rao *et al*., 2004).

In cultured brain microvascular endothelial cells (BMVECs), an *in vitro* model of the BBB, ethanol activated MLCK, leading to phosphorylation of serine residues of claudin 5 and occludin that resulted in BBB impairment (Haorah *et al*., 2005a). In addition, ethanol exposure significantly increased the levels and activity of ADH, producing toxic acetaldehyde, and of the ethanol-metabolizing enzyme, cytochrome P450-2E1 (CYP2E1), that enhances reactive oxygen species (ROS) generation, implicating oxidative damage in ethanol-induced BBB damage (Haorah *et al*., 2005b). The *in vivo* effects of chronic ethanol drinking on the BBB were studied in rat brain endothelial cells and neurons challenged with the bacterial endotoxins LPS. Increase in endothelial tight junction permeability correlated with decreased mRNA expression and increased phosphorylation of junction proteins, associated with an increase in ERK, p38 MAPK, JNK, and activation of RelA-p50 and p50-p50 (inducers of pro-inflammatory signals). Thus, chronic alcohol drinking may cause higher predisposition to infection and inflammation-related diseases possibly by augmenting the effects of pathogens on the BBB (Singh *et al*., 2007).

6.2 Oxidative Stress

ROS and reactive nitrogen species (RNS, e.g. nitric oxide and its radical, $NO^{\bullet}$) are well known to play a dual role within the cell, both as deleterious toxic agents and as key second messengers in numerous signalling pathways involved in transcriptional regulation, differentiation, proliferation and programmed cell death (apoptosis). The complexity of the involvement of free radicals and antioxidants in normal physiological functions and human disease have recently been extensively reviewed (see Valko *et al*., 2007 and **Nitric Oxide Pathways in Toxic Responses**; **Reactive Oxygen Species in the Induction of Toxicity**). In addition, ROS and RNS have frequently been implicated in changes to the permeability of epithelial (Basuroy *et al*., 2006; Han *et al*., 2004; Hashimoto *et al*., 2008; Holgate, 2007; Oshima *et al*., 2007) and endothelial (Usatyuk *et al*., 2006; Vandenbroucke *et al*., 2008) tight junctions. Pathways frequently involved in ROS-mediated alteration of tight junction permeability in different epithelia are those involving the MAPKs. Generation of ROS by direct means (i.e. metals or other redox-active substances), or via cytokines and growth factors binding to specific receptors, can transmit signals by activating MAPKs signalling pathways (reviewed in Gonzalez-Mariscal *et al*., 2008; Valko *et al*., 2007). MAPKs (ERKs, JNKs and p38 isoforms) have been involved in tight junction protein phosphorylation in both epithelia and endothelia exposed to oxidative stress, resulting in delocalization of the proteins from the junctions and increased permeability, although their exact role in barrier function appears to vary, possibly due to cell type or stimulant specificity (Gonzalez-Mariscal *et al*., 2008). In human intestinal Caco-2 cells, EGF treatment prevented the oxidative-stress-induced Tyr phosphorylation and Thr dephosphorylation and redistribution of occludin and ZO-1 from the junctions, and disruption of the actin cytoskeletal architecture by a MAPK-dependent mechanism involving direct ERK interaction with occludin (Basuroy *et al*., 2006). In a different study performed on the Caco-2 cell line, hydrogen peroxide treatment increased tight junction permeability by a delocalization of claudin 4 from the membrane, accompanied by phosphorylation of p38 MAPK. This effect could be reversed by a specific inhibitor of p38 MAPK and by exogenous expression of the protein phosphatase Wip1, a member of the serine/threonine-specific protein phosphatase type 2C (PP2C) family, that selectively dephosphorylates and inactivates p38 MAPK, but not JNK or ERK (Oshima *et al*., 2007). Recently, two secretory proteins, p40 and p75, isolated from the probiotic strain *Lactobacillus rhamnosus GG* have been reported to significantly diminish the disruption of barrier function induced by hydrogen peroxide treatment of Caco-2 cells, attenuating the delocalization of tight junction (occludin and ZO-1) and adherens junction (β-catenin

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and E-cadherin) proteins from the membrane. The probiotic proteins induced the membrane translocation of two PKC isoforms (β1 and ε) and activated ERK1/2, thus blocking both PKC- and MAPK-dependent alterations to the tight junctions (Seth *et al*., 2008).

The vascular endothelium, which regulates the passage of macromolecules and circulating cells from blood to tissues, is a major target of oxidative stress, playing a critical role in the pathophysiology of several vascular diseases and disorders. Specifically, oxidative stress increases VE permeability and promotes leukocyte adhesion, which is coupled with alterations in endothelial signal transduction and redox-regulated transcription factors (Lum and Roebuck, 2001). In bovine lung microvasculature endothelial cells (BLMVECs), treatment with 4-hydroxy-2-nonenal (4-HNE), one of the major aldehydes formed during inflammation and oxidative stress, led to protein Tyr phosphorylation and actin cytoskeletal rearrangements, and activation of ERK 1/2, p38 MAPK and JNK, resulting in increased junction permeability (Usatyuk *et al*., 2006) Using two different models of the BBB, it was shown that ROS led to endothelial barrier dysfunction by cytoskeletal rearrangement, redistribution and disappearance of occludin and claudin 5, mediated by RhoA activation of phosphatidyl inositol 3 (PI3) kinase and its downstream target, PKB (Schreibelt *et al*., 2007). Overall, oxidative stress is certainly involved in the regulation of tight junction permeability, although often it is not easy to determine whether it is a primary signal or secondary to inflammatory mediators.

6.3 Metals

The study of the toxicity of metals on epithelial and endothelial barriers is relevant not only for the toxic metals (i.e. cadmium, lead and mercury) but also for those metals (i.e. iron, zinc and copper) that are essential micronutrients and play important roles in cell physiology but, at high doses, can exert toxic effects (see **Toxicology and Biological Monitoring of Metals**). A correct supply of essential metal micronutrients from the diet is therefore of primary importance, especially in certain population groups, such as children, pregnant women and the elderly, in which suboptimal metals micronutrient status can have adverse health effects. In some cases, however, supplementation not warranted by a verified deficiency can cause excess metals accumulation with possible noxious effects. This is an important point in the light of the increasing use of nonprescribed micronutrient supplements whose allegedly health-promoting effects are widely advertised. Some examples of the effects of metals on the maintenance of epithelial and endothelial barrier function will be discussed.

Iron uptake in mammals is exclusively regulated at the level of the intestinal mucosa as there is no pathway for iron excretion. Iron is an essential micronutrient but, when present in excess, it can generate oxidative stress and can be toxic (Puntarulo, 2005). In cultured human intestinal Caco-2 cells, Fe(II) treatment was reported to exert a two-phase effect: a rapid increase in tight junction permeability, associated with claudin 4 delocalization and F-actin disorganization was followed, after iron removal, by a late-phase onset of apoptosis and necrosis that was ascribed to the generation of oxidative stress (Ferruzza *et al*., 2002; 2003). *In vivo* iron overload is well known to induce toxic effects in various organs (Puntarulo, 2005), but also iron supplementation could cause damage to the intestinal mucosa, as shown by the increased intestinal permeability found in African schoolchildren who had received iron supplements (Nchito *et al*., 2006).

Zinc is an essential micronutrient that plays fundamental housekeeping roles in physiology, cellular metabolism and gene expression. Depletion of intracellular zinc in airways epithelial cells was shown to increase cytokine-induced cell barrier dysfunction (Bao and Knoell, 2006a) and to enhance apoptosis (Bao and Knoell, 2006b). In intestinal epithelial cells, zinc deficiency was found to interfere with tight and adherens junction formation by delocalization of ZO-1, occludin and β-catenin, and changes to junction protein phosphorylation, and to promote chemokines secretion and transepithelial neutrophil migration (Finamore *et al*., 2008). A specific protective effect of zinc administration on cadmium toxicity to the kidney was observed *in vivo* in rats chronically intoxicated with repeated cadmium injections. Among the toxic effects of cadmium on ionic handling, apoptosis and necrosis in several renal areas, a specific effect on tight junction organization was observed, with disorganization of claudins 2 and 3 in proximal and distal epithelium and of claudin 5 in the endothelium, while co-treatment of zinc and cadmium completely reversed these effects and reduced apoptosis (Jacquillet *et al*., 2006). The protective role of zinc against several toxic or stress stimuli has been reported in various organs (Stefanidou *et al*., 2006), although a direct role for zinc on intercellular junctions has not been described.

Human exposure to the toxic metals cadmium, lead and arsenic can result in damage to a variety of organ systems, although the mechanisms of this toxicity have yet to be fully elucidated. Recently, the results from studies on the toxicity of these metals in the kidney and the vascular epithelium have been reviewed (Prozialeck and Edwards, 2007; Prozialeck *et al*., 2008). An interesting conclusion of both reviews was that, in most cases, a primary target of metal toxicity were the molecules responsible for cell–cell adhesion in epithelia and endothelia of the various organs, and changes in adhesion properties among cells often

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occurred well before cellular death. Although some evidence exists for adverse effects of lead and mercury on brain barriers function, the mechanisms involved at the level of the endothelial junctions are still largely unknown. In a previous review of the toxic effects of metals on the blood brain barriers, the need for studies on the susceptibility of brain endothelial tight junctions to metal toxicants was emphasized (Zheng *et al*., 2003).

7 BARRIER FUNCTION AND DISEASE

The effect of disease processes on barrier functions of epithelia and endothelia almost invariably results in an increase in leakiness, often reflecting changes to the permeability of tight junctions. Pathogenetic or toxic mechanisms associated with alterations in tight junction permeability in different tissues and organs include inflammation, immune-mediated or direct response to pathogens and their toxins, response to oxidative stress, and specific response to toxic substances directly or indirectly altering tight junction structure. These mechanisms are involved to a different extent in a highly heterogeneous group of diseases that are still difficult to classify but that share early impairment of epithelial or endothelial tight junction permeability.

7.1 Genetic Diseases

A special case is represented by diseases caused by single mutations in genes coding for proteins of the tight junction or other genetic defects. Examples of these have recently emerged along with better molecular characterization of junction components. A human renal disease known as familial hypomagnesaemia, characterized by renal wasting of Mg^{2+} and Ca^{2+}, is caused by a dysfunction of claudin 16, paracellin, that concentrates at the tight junction of the thick ascending limb of Henle and functions as a paracellular pore for Mg^{2+} (Simon *et al*., 1999). Another example is congenital deafness due to mutations of the gene encoding claudin 14, which normally acts as a cation-restrictive pore in the tight junctions of reticular lamina in the organ of Corti of the inner ear. In the presence of mutated claudin 14, the tight junctions are unable to maintain the proper ionic composition of the fluid surrounding the basolateral surface of outer hair cells due to incorrect recruitment of the mutated protein to the tight junction (Wattenhofer *et al*., 2005). For further details on diseases caused by genetic defects the reader is referred to some recent reviews (Cereijido *et al*., 2007; Chiba *et al*., 2008; Forster, 2008; Lai-Cheong *et al*., 2007).

7.2 Cancers

Cancers are typically multifactorial diseases, but a common trait of all tumoural cells is their ability to spread metastatically, a property directly related to their loss or down-regulation of cell–cell adhesion that, for epithelial cell-derived tumours, is initiated by loss of tight junction barrier function. This break-down of tissue organization allows entry of single cells into the blood vessels and also facilitates nutrient uptake from luminal fluids (Mullin *et al*., 2000). Several alterations of tight junction structure (i.e. reduced number of strands and structural disorganization) as well as changes in the expression of tight and adherens junction proteins have been described in different epithelial tumours (reviewed in Forster, 2008; Gloushankova, 2008; Mullin *et al*., 2000). Unlike other junction molecules, expression of claudins is often increased in various tumours. For example, claudins 3, 4 and 7 are frequently up-regulated in a variety of cancers such as those originating from the ovary, fallopian tubes, breast, uterus, prostate, thyroid, pancreas, stomach, colon and bladder (reviewed in Chiba *et al*., 2008; Morin, 2007). Although the exact roles of over-expressed claudins in cancer are unclear, expression of some claudins in epithelial cells promotes cell invasion, motility and/or survival, suggesting potential functions of these proteins in tumorigenesis and metastasis. (Hewitt *et al*., 2006). In addition, the recent characterization of the role of ZO-1-associated ZONAB transcription factor in the control of genes involved in cellular proliferation suggests a possible novel connection between the loss of tight junction barrier and the pathogenesis of some epithelial cancers (Balda and Matter, 2003; Sourisseau *et al*., 2006).

In summary, tight junctions are typically disorganized, down-regulated or simply absent from epithelial neoplasia, and these changes occur very early in the progression of the tumour. However, when dealing with specific tight junction proteins there is no general rule on whether they are down-regulated or up-regulated in the disease. But given the importance of homotypic and heterotypic interactions between tight junction components, alterations in the proportion of specific molecules can disrupt the balance on which the junction is maintained, often resulting in altered junction permeability.

7.3 Pathological Micro-organisms

One of the challenging tasks of the gastrointestinal tract is to mount an aggressive response against enteric microbes while maintaining tolerance to commensal bacteria and to food antigens. Similarly, other epithelial and endothelial barriers delimiting the internal from the external environments need to tightly control 'what to let in and what to leave out'. On the other hand, pathogens have evolved

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ways to get across these obstacles and often utilize apical junctional complexes as sites of entry into the body, even exploiting the same mechanisms of physiological junction regulation to overcome epithelial and endothelial barriers (Vogelmann *et al*., 2004). In most cases, bacterial pathogen effects on epithelial and endothelial barriers result in inflammatory reactions that can lead to barrier dysfunction. In this section, the discussion will be restricted to some examples of pathogens that affect barrier permeability by directly interacting with structural proteins of the junction or the cytoskeleton, or by targeting regulatory elements from inside or outside the cells (**Figure 3**).

Clostridium perfringens is a common cause of food-borne and antibiotic-associated diarrhoea. *C. perfringens* enteroxin (CPE) cytotoxicity is a multistep process that initiates with the binding of CPE to an extracellular loop on claudins 3 and 4, followed by formation of SDS-resistant complexes that contain claudins and occludin (McClane, 2001; Smedley *et al*., 2007). These large complexes are thought to represent the cytotoxic pores, which create a hole in the plasma membrane, allowing Ca^{2+} influx and ultimately leading to cell death. Toxin-induced cytolytic pore formation requires residues in the NH_2-terminal half, whereas residues near the COOH-terminus are required for binding to claudins. Although the isolated claudin-binding domain, whose structure has recently been elucidated (Van Itallie *et al*., 2008a), is not cytotoxic to cultured epithelial cells, its binding increased paracellular permeability accompanied by disappearance of claudin 4, indicating a direct effect on tight junctions (Kondoh *et al*., 2005). However, the increased tight junction permeability and removal of claudins from the junctions does not appear to be linked to CPE toxicity, thus the involvement of tight junction components in the toxicity of CPE may be restricted to its role as membrane receptor for the toxin.

Bacteroides fragilis, which accounts for only 0.5% of the human colonic flora, is the most commonly isolated anaerobic pathogen, due, in part, to its potent virulence factors. Some strains produce a toxin, a zinc-metalloproteinase called fragilysin or *B. fragilis* toxin (BFT), implicated as a cause of diarrhoeal disease in farm animals and humans. The pathogenic mechanisms of *B. fragilis* in the intestine have recently been reviewed (Holton, 2008). Briefly, fragilysin binding to a still-unknown receptor on intestinal epithelial cells requires its own protease action and leads, directly or indirectly, to the hydrolysis of the extracellular domain of E-cadherin, disrupting intercellular adhesion

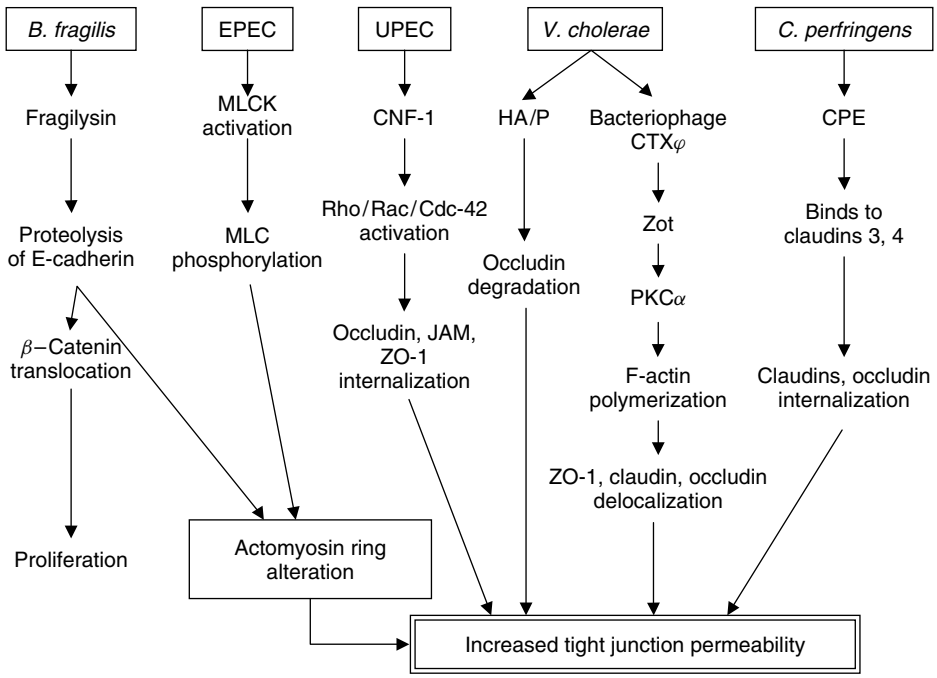

Figure 3 Epithelial tight junctions can be altered by various pathogens and their toxins. The disruption of tight junction barrier function induced by several pathogens can result from direct modification of tight junction proteins (claudins, occludin, ZO-1, JAM) or by alterations of the perijunctional actomyosin ring through activation of myosin light chain kinase (MLCK) phosphorylation of myosin 2 light chain (MLC). In addition, the proteolysis by the bacterial toxin fragilysin of the adherens junction protein E-cadherin, leading to actomyosin disruption and to proliferation, is also indicated. The regulatory pathways involved, when known, are indicated. Refer to the text for details. EPEC—enteropathogenic *E. coli*; UPEC—uropathogenic *E. coli*; PKCα—phosphokinase C member α; CPE—*C. perfringens* enterotoxin; ZOT—zonula occludens toxin.

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and thus increasing the permeability of the epithelium. The cleavage of E-cadherin releases β-catenin into the cytoplasm, and is accompanied by morphological changes and redistribution of F-actin. β-Catenin translocates to the nucleus, stimulates *MYC* transcription and ultimately cell proliferation. In addition, when exposed to fragilysin, colonocytes secrete cytokines (i.e. interleukin IL-8, epithelial neutrophil-activating peptide-78, and growth-regulated oncogene-α) under the control of the transcription factor NFκB and the MAPK pathways, and this leads to the induction of an inflammatory response.

Pathogenic *Escherichia coli* strains producing cytotoxic necrotizing factor 1 (CNF-1), such as the Uropathogenic *E. coli* (UPEC), have been associated with human disease including enteritis, urinary tract infections and prostatitis. The effect of CNF-1 targets the Rho family of GTPases to disrupt epithelial tight junctions. CNF-1 applied to polarized cells activates Rho/Rac-1 and Cdc-42 and blocks GTPase activity (i.e. preventing the hydrolysis of GTP to GDP), locking the Rho proteins in their GTP-bound activated state, and resulting in permanent activation of the proteins. This results in massive internalization of tight junction proteins (occludin, JAM and ZO-1) while adherens junctions remain grossly intact (Fasano and Nataro, 2004; Hopkins *et al*., 2003; Vogelmann *et al*., 2004). It has recently been shown that both pathogenic *E. coli* producing CNF-1 and CNF-2 and *Yersinia pseudotuberculosis*, producing CNF(Y), activate, by deamidation, the same Rho GTPases, although they bind to different surface receptors for internalization in host cells (Blumenthal *et al*., 2007). Others, such as the enteropathogenic *E. coli* (EPEC) have evolved two different strategies: they tightly adhere to the cell surface and modify the actin cytoskeleton beneath the attachment site, and through the secretion apparatus inject into the host cell effector molecules altering electrolyte secretion, tight junction permeability and inflammatory response. An increase in Ca^{2+} influx is associated to activation of the MLCK cascade, leading to perijunctional actomyosin ring contraction and loosening of tight junctions.

The discovery of zonula occludens toxin (Zot), an enterotoxin expressed by *Vibrio cholerae* that reversibly opens tight junctions, has greatly contributed to the understanding of some mechanisms of regulation of the intestinal paracellular pathway. Zot is a single polypeptide chain encoded by the cholera toxin bacteriophage (CTXϕ) present in the toxigenic strain of *V. cholerae*. Zot action is mediated through a cascade of events that lead to PKCα-dependent polymerization of actin microfilaments strategically localized to regulate the paracellular pathway. Zot, which is a homologue of the human intestinal zonulin, binds to the zonulin surface receptor, whose distribution along the intestine (expressed in the jejunum and distal ileum but not in the colon) coincides with the regional effect of Zot on intestinal permeability and with its preferential effect on the differentiated cells of the villi. Following binding to the zonulin receptor, Zot induces actin polymerization, followed by ZO-1–occludin and ZO-1–claudin detachment and by a down-regulation of occludin gene expression occurring only when cells are chronically exposed to the toxin (Fasano, 2008; Fasano and Nataro, 2004). *V. cholerae* also produces a haemagglutinin protease (HA/P) toxin that represents a typical example of direct effects of a bacterial protease on tight junction proteins. Epithelial cell monolayers exposed to HA/P showed decreased TER, disruption of ZO-1 and of the actin cytoskeleton, in a dose-dependent manner, as well as occludin degradation, indicating a specific effect on intestinal permeability (Wu *et al*., 2000).

7.4 Inflammatory Conditions

A central role of epithelial and endothelial barriers in the modulation and progression of several disease states has emerged for conditions ranging from IBD (Weber and Turner, 2007) to airway inflammation in asthma (Holgate, 2007) and cystic fibrosis (Coyne *et al*., 2002), coeliac disease (Fasano, 2008; Schulzke *et al*., 1998), and neuroinflammatory conditions such as multiple sclerosis, meningitis and encephalitis (Forster, 2008; Persidsky *et al*., 2006). In all these conditions, diverse and not always defined stimuli such as bacterial toxins, toxic chemicals, food allergens, and so on, can initiate an inflammatory response, leading to the secretion of pro-inflammatory cytokines that alter tight junction permeability and recruit neutrophils, macrophages and other inflammatory mediators, further contributing to tissue damage (see **Xenobiotic-Induced Inflammation: Pathogenesis and Mediators**; **Food Allergy and Intolerance**). In mucosal tissues, the inflammatory response is central to effective host defence against invading pathogens, yet must be tightly regulated to prevent abnormal responses to innocuous environmental antigens and commensal organisms which result in allergy or chronic inflammatory diseases (Kelsall, 2008).

A wide array of cytokines including tumour necrosis factor alpha (TNF-α), interferon-gamma (IFN-γ) and several interleukins, have been reported to perturb epithelial and endothelial barrier function by influencing the structure and function of the tight junction (reviewed in Capaldo and Nusrat, 2008). Although it is often difficult to determine whether the initial alteration of tight junction permeability precedes or is a consequence of early inflammatory response, tight junction dysfunction and inflammation are almost invariably associated. A vicious cycle is therefore created in which barrier dysfunction allows further leakage of contents from the extracellular milieu, thereby triggering an immune response that in turn promotes leakiness and further damage to the cells.

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Impairment of the intestinal barrier was originally described *in vivo* in patients with small intestinal Crohn's disease (Hollander, 1988), but was later observed in patients or experimental models of a variety of inflammatory, immune-mediated and infectious intestinal diseases (Clayburgh *et al*., 2004). For Crohn's disease, intestinal barrier dysfunction may not only be a consequence of the inflammatory conditions but may also contribute to the activation of the disease. This early pathogenetic role of barrier dysfunction in the disease was suggested by the increased intestinal permeability in first-degree relatives of Crohn's disease patients in the absence of clinical symptoms (Weber and Turner, 2007) and by the higher risk of relapse in patients with clinically inactive disease presenting altered intestinal permeability (D'Inca *et al*., 1999; Wyatt *et al*., 1993). Recent studies have shown that in several forms of IBD (e.g. Crohn's disease, collagenous colitis, ulcerative colitis) the composition and organization of intestinal epithelial tight junctions is severely affected. A common finding in all these conditions was an elevated expression of claudin 2, the cation-pore forming claudin, a reduced number of strands and meshwork depth, and increased strand discontinuities in the tight junctions. The severity of these changes was correlated with the degree of inflammation of the tissue (Mankertz and Schulzke, 2007). High levels of the TNF-α are consistently observed in IBD, and anti-TNF-α antibodies have been shown to counteract the progression of inflammation. TNF-α has been shown to directly impair tight junction function in a number of epithelial and endothelial cell lines (Capaldo and Nusrat, 2008). However, conflicting responses to TNF-α have been reported in several systems, which may reflect cell-type-specific responses as well as experimental differences. Treatment of epithelial cells in culture with physiological concentrations of TNF-α caused increased tight junction permeability which was found to be mediated by the activation of the transcription factor NFκB, increased MLCK expression and phosphorylation, leading to contraction of the actomyosin ring (Turner, 2006; Ye *et al*., 2006). In intestinal cells, TNF-α was also reported to activate MLCK by transcriptional stimulation under the control of NFκB (Graham *et al*., 2006). The *in vitro* results on the effects of TNF-α on intestinal epithelial barrier function were also confirmed in a mouse *in vivo* model of acute immune-mediated diarrhoeal disease where epithelial barrier impairment, defined as both increased paracellular protein flux and tight junction disruption, was mediated by MLCK-dependent MLC phosphorylation. Such barrier dysfunction was required for diarrhoea to occur after T cell activation, but could be prevented by MLCK inhibition (Clayburgh *et al*., 2005). Under inflammatory conditions, target cells are simultaneously exposed to a variety of cytokines, and *in vitro* epithelial and endothelial cell models exposed simultaneously to IFN-γ and TNF-α showed increased paracellular permeability with several changes to the actomyosin ring, and localization of tight junction protein (reviewed in Capaldo and Nusrat, 2008). An interesting finding was that in intestinal monolayers the two cytokines acted synergistically towards transcriptional up-regulation of MLCK (Wang *et al*., 2005), and that IFN-γ priming of the cells activated the cell surface TNF-α receptor (Wang *et al*., 2006). Other pro-inflammatory cytokines that have been shown to alter tight junction permeability are the interleukins. IL-1β in Caco-2 cells activated MLCK by an NFκB-dependent pathway (Al-Sadi *et al*., 2008). Many other interleukins (i.e. IL-4, IL-6 and IL-13) were shown to act as modulators of tight junction components, controlling occludin, claudin and ZO-1 protein levels, leading to the formation of tight junctions with altered permeability characteristics. Conversely, IL-2 and IL-10 could antagonize the action of pro-inflammatory cytokines on tight junction permeability, although the mechanisms involved are poorly understood (Capaldo and Nusrat, 2008). The aberrant expression of claudins 2, 5 and 8 that was observed in Crohn's disease patients in inflamed intestinal segments was different to the claudins expression in noninflamed segments or in periods of remission of the same patients, suggesting that cytokines can induce tight junction remodelling and changes in claudin expression (Zeissig *et al*., 2007).

In endothelial cells, MLC phosphorylation and RhoA activation are early events after TNF-α treatment, and correlated with increased permeability (McKenzie and Ridley, 2007; Wadgaonkar *et al*., 2005). In addition, long-term effects of TNF-α treatment on endothelial cells were shown to involve occludin down-regulation and delocalization of claudin 5 and JAM-1 (McKenzie and Ridley, 2007). Disruption of the endothelial BBB has been described as a crucial step of neuroinflammatory conditions including brain tumours, cerebral ischaemia, meningitis, encephalitis and multiple sclerosis. Under normal conditions, the brain microvasculature endothelial cells form a tight barrier and limit extravasation of cells of the immune system (monocytes, lymphocytes and other leukocytes). In multiple sclerosis, an autoimmune condition characterized by myelin sheath destruction by reactive T lymphocytes, increased leukocyte extravasation was associated with endothelial tight junction disorganization (Forster, 2008). Reactive T cells interact with brain macrophages and microglial cells serving as antigen-presenting cells (Grigoriadis *et al*., 2006). Activated macrophages synthesize and produce cytokines (IFN-γ, TNF-α), matrix metalloproteinases (MMPs) and NO (Raivich and Banati, 2004). Cytokine-dependent opening of tight junctions in the BBB was shown to occur by degradation and decreased synthesis of junctional proteins, especially occludin and ZO-1 (Forster, 2008). Serum from multiple sclerosis patients induced down-regulation of occludin and VE-cadherin in cultured endothelial

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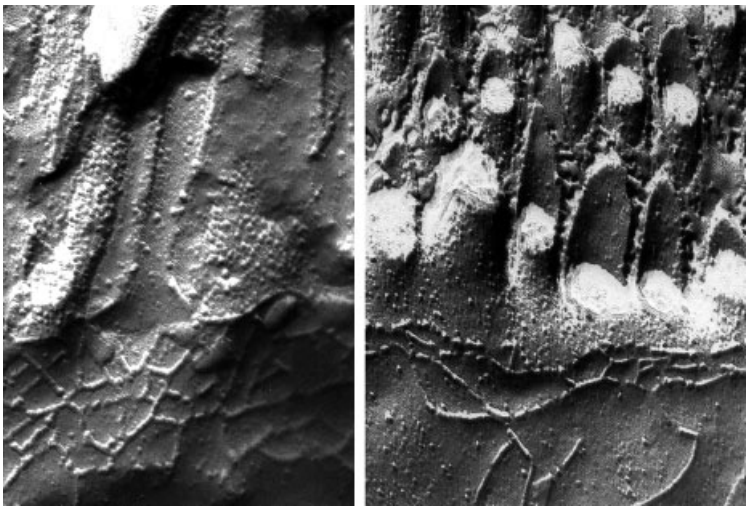

Figure 4 Freeze fracture replicas from human surface duodenal epithelium of normal (A) and of acute coeliac disease patient (B). The freeze fracture electron micrographs show the bottom part of the apical brush border and the intramembranous particle strands of the tight junctions. The mean strand count was reduced from about 5.3 in control tissue to 3.0 in the inflamed tissue from the coeliac patient (B), who also presented villus atrophy and diarrhoea. (Kindly provided by Prof. J.-D Schulzke, Dept. of Gastroenterology, Charité Berlin, Berlin, Germany.)

cells, and these effects appeared to precede massive infiltration of T lymphocytes that attack myelin sheath, forming demyelinating plaques (Minagar and Alexander, 2003).

7.5 Autoimmune Disease: Coeliac Disease

Coeliac disease is an immune-mediated enteropathy triggered by gliadin, a component of the grain protein, gluten. Gliadin consumption by affected patients leads to increased intestinal permeability, a postulated early event in the pathogenesis of coeliac disease. Mucosa of coeliac patients is characterized by a decrease in tight junction strand number in all regions of the jejunal surface–crypt axis (Schulzke *et al.*, 1998) (**Figure 4**), an indication of reduced tight junction complexity often associated with chronic inflammatory reactions, including IBD (Mankertz and Schulzke, 2007). The gliadin stimulus has been shown to activate the zonulin pathway. It has recently been shown that gliadin binds to the CXC chemokine receptor 3 (CXCR-3), and leads to MyD88-dependent zonulin release and increased intestinal permeability in cultured intestinal cells and human biopsy specimens, thus enabling paracellular translocation of gliadin and its subsequent interaction with macrophages in the submucosa. Zonulin is the mammalian homologue of Zot, that when secreted by the intestinal cells binds to its own receptor and initiates a cascade of reactions involving activation of phospholipase C, release of inositol 1,4,5-tris phosphate and diacylglycerol, activation of PKCα, phosphorylation of target proteins leading to F-actin polymerization and tight junction proteins delocalization and increased barrier permeability (Fasano, 2008). In addition, mucosal expression of CXCR-3 was found to be elevated in active coeliac disease but returned to baseline levels following implementation of a gluten-free diet (Lammers *et al.*, 2008).

8 CONCLUSIONS

Tight junctions are highly dynamic structures, undergoing rapid reorganization in response to many physiological stimuli. In the presence of toxic/pathological events the response is just as rapid and is often contrasted by the body/tissue/cellular stress responses, repair mechanisms or immune-mediated reactions. However, if the tissue is unable to rapidly restore barrier function or if the immune response is excessively or continuously activated, disruption of tight junctions can lead to uncontrolled passage of potentially harmful molecules that can then reach internal organs and elicit further toxic events. The complexity and diversity of the mechanisms involved in tight junction dysfunction in response to different toxic stimuli is often difficult to interpret. However, one simple unifying concept is that several different stimuli in epithelial and endothelial cells from different organs, through either distinct or interacting regulatory pathways, almost invariably lead to the same end point, an increase in tight junction permeability, opening the way to several local and systemic adverse

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effects. Thus, in endothelia and epithelia the initial toxic effect of several substances appears to be targeted to the molecules governing cell–cell interactions, well before the onset of additional cytotoxic mechanisms leading to cell death by apoptosis or necrosis.

The importance of investigating these changes by appropriate experimental approaches is particularly relevant, as the complexity of the pathways involved and the rapidity of tight junction modulation may lead to an overestimation of final end points, while missing the initial events causing alteration of barrier properties. In addition, the use of *in vitro* cell culture models has proved very useful to dissect molecular responses to different toxic stimuli, although *in vivo* or *ex vivo* studies are required to recreate the complex interactions between epithelia and other cell types to gain insight into the development of actual disease symptoms. Results need therefore to be interpreted within the perspective of the experimental model utilized, especially in this highly dynamic and critical cell function that can be influenced by very diverse stimuli from the internal and the external environments.

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Chemokines as Mediators of Toxicant-Induced Inflammation and Fibrosis

François Huaux, Sandra Lo Re and Dominique Lison

CONTENTS

1 INTRODUCTION

1.1 The Chemokine Network

Chemokines represent a large superfamily of 8–15 kDa structurally related proteins and are usually secreted upon cell stimulation. Defined by a tetra-cysteine motif, these small proteins are subdivided into four distinct families according to the configurations of the cysteine residues at their amino terminus. There are two large subfamilies: CCL1 through CCL28 and CXCL1 through CXCL16 (CXCL—cysteine X cysteine chemokine ligand), as well as two small subfamilies: XCL1 through XCL2 and CX_3CL1 (**Table 1**) (Coelho *et al*., 2005; Schuh *et al*., 2003; Panina-Bordignon and D'Ambrosio, 2003). With nanomolar affinities, all chemokines bind to seven transmembrane spanning Type V G-protein couple receptors, which are also distributed in two large subfamilies: CCR1 through CCR10 and CXCR1 through CXCR6 (CXCR—cysteine X cysteine chemokine receptor), as well as two small subfamilies: XCR1 through XCL2 and CX_3R1 (**Table 1**) (Coelho *et al*., 2005; Schuh *et al*., 2003; Panina-Bordignon and D'Ambrosio, 2003). To date, almost 50 chemokines have been identified, which exert their effects through over 20 distinct receptors. A certain degree of promiscuity exists among chemokines, with the possibility for multiple ligands to be associated with a single receptor and vice versa. Indeed, most chemokine receptors can bind multiple ligands, for example, CCR3 can bind CCL5, CCL7, CCL8, CCL11, CCL13, CCL24 and CCL26. Many chemokines are capable of binding to more than one receptor; for example, CCL5 binds to CCR1, CCR3 and CCR5. A few chemokines and chemokine receptors demonstrate exclusive binding, such as the specific CCL25/CCR9 and CXCL12/CXCR4 pairs. Recent reports show that chemokines can also act as antagonists for chemokine receptors. For example, CXCL10 is a natural antagonist of CCR3 (Coelho *et al*., 2005; Palmqvist *et al*., 2007).

Chemokines can be further separated into constitutive and inducible, depending on their mode of expression. Constitutive chemokines are expressed primarily in secondary lymphoid organs, where they play a role in leukocyte homing. Inducible chemokines are involved mainly in the recruitment of cells to inflammatory sites and comprise most of the chemokine family members. These inflammatory chemokines are generally induced to high levels of expression by inflammatory stimuli such as lipopolysaccharide (LPS), interleukin (IL)-1 or tumour necrosis factor (TNF)-α (Coelho *et al*., 2005; Schuh *et al*., 2003).

1.2 Chemokines Orchestrate Leukocyte Recruitment

Chemokines play pivotal roles in coordinating tissue leukocyte navigation. Indeed, along with adhesion

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Table 1 Chemokines and their receptors

Subfamilies	Other names	Chemokine receptors
CC chemokines		
CCL1	TCA-3, I-309	CCR8
CCL2	MCP-1, MCAF, murine JE	CCR2, CCR10
CCL3	MIP-1α, LD78α	CCR1, CCR5
CCL4	MIP-1β, Act-2, HC21	CCR5, CCR8
CCL5	RANTES	CCR1, CCR3, CCR5
CCL6	C10, murine MPR-1	CCR1
CCL7	MCP-3	CCR1, CCR2, CCR3, CCR10
CCL8	MCP-2	CCR1, CCR2, CCR3, CCR5
CCL9/10	MIP-1γ, MPR-2, murine CCF-8	CCR1
CCL11	Eotaxin-1	CCR3, CCR2 antagonist
CCL12	Murine MCP-5	CCR5
CCL13	MCP-4, CKβ10	CCR1, CCR2, CCR3
CCL14	HCC-1	CCR1, CCR5
CCL15	HCC-2, MIP-1δ, MIP-5, LKN-1	CCR1, CCR3
CCL16	HCC-4	CCR1
CCL17	TARC	CCR4, CCR8
CCL18	MIP-4, DC-CK1, PARC, AMAC-1	Not known, CCR3 antagonist
CCL19	MIP-3β, ELC, Exodus-3	CCR7
CCL20	MIP-3α, LARC, Exodus-1	CCR6
CCL21	6Ckine, SLC, TCA-4, Exodus-2, CKβ9	CCR7
CCL22	MDC, STCP-1, ABCD-1	CCR4
CCL23	MIPIF-1, CKβ8	CCR1
CCL24	Eotaxin-2, MPIF-2, CKβ6	CCR3
CCL25	TECK	CCR9
CCL26	Eotaxin-3	CCR3, CCR10, CCR1,2,5 antagonist
CCL27	CTACK, ILC, murine Eskine	CCR10
CCL28	MEC	CCR10
CXC chemokines		
CXCL1	GROα, MGSAα, murine KC	CXCR2, CXCR1 (low affinity)
CXCL2	GROβ, MGSAβ, MIP-2α	CXCR2, CXCR1 (low affinity)
CXCL3	GROγ, MGSAγ, MIP-2β	CXCR2, CXCR1 (low affinity)
CXCL4	PF4	CXCR3
CXCL5	ENA-78	CXCR2, CXCR1 (low affinity)
CXCL6	GCP-2	CXCR1, CXCR2
CXCL7	NAP-2, CTA-III	CXCR2, CXCR1 (low affinity)
CXCL8	IL-8, NAP-1, MDNCF, murine MIP-2	CXCR1, CXCR2
CXCL9	MIG	CXCR3
CXCL10	IP-10, CRG-2	CXCR3
CXCL11	ITAC, β-R1, H174, IP-9	CXCR3
CXCL12	SDF-1, PBSF	CXCR4
CXCL13	BLC, BCA-1	CXCR5
CXCL14	BRAK, bolekine	Not known
CXCL15	Murine lungkine	Not known
CXCL16	—	CXCR6
C chemokines		
XCL1	Lymphotactin, SCM-1α, ATAC	XCR1
XCL2	SCM-1β	XCR1
CX_3C chemokines		
CX_3CL1	Fractalkine, neurotaxin, ABCD-3	CX_3CR1

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molecules, such as integrins and selectins, chemokines and their receptors act primarily as part of a complex molecular network that facilitates the selective movement of leukocytes into and out of injured tissue. The principal targets of chemokines are bone marrow-derived cells, as motility is an essential part of their function. Chemokine downstream signalling triggers integrin activation, which enables firm adhesion of leukocytes to endothelial cells. Focal actin polymerization of the leading edge of the cell leads to forward extension. At the rear end of the cell, focal activation of myosin II and contraction of myosin–actin complexes retracts the cell, allowing migration in the direction of the chemotactic gradient (Friedl and Weigelin, 2008; Thelen and Stein, 2008; Palmqvist *et al*., 2007).

Recent evidence allowed a better understanding of how xenobiotics can lead to immune diseases and the specific recruitment of monocytes, granulocytes and lymphocytes inside the injured tissue (**Figure 1**). After recognition by the receptors of the immune innate system, resident cells produce large quantities of colony-stimulating factors (CSFs) which induce the differentiation and the mobilization of myeloid cells that populate the bone marrow. These growth factors are a family of glycoproteins playing a major role in the proliferation, differentiation and survival of primitive haematopoietic stem and progenitor cells. These factors are well identified during inflammation and mainly include M (monocyte)-CSF, G (granulocyte)-CSF and GM (granulocyte/monocyte)-CSF (**Figure 1A**). They have been demonstrated to be effective in the emergence of differentiated monocytes, granulocytes and granulocytes/monocytes, respectively (Hamilton, 2008). Leukocyte extravasation usually occurs through a multistep process, involving first the selectins, then chemoattractant receptor signalling, followed by firm adhesion to vessel walls through the actions of integrins. Thereafter, leukocytes migrate along chemotactic gradients, by means of chemoattractant receptor signalling and the actions of adhesion molecules, particularly integrins (Mackay, 2008). Classical genetic approaches, such as knockout and transgenic experiments have elegantly identified unique immunological functions for these factors and demonstrated that chemokines dictated the specific influx of particular immune cells. For instance, it is well known that monocytes/macrophages mainly migrate through CCL2 gradient after highly expressing CCR2. In the same way, CXL1, 2, 3 and 8/CXCR1 and 2 are required for neutrophil accumulation, while the CCL11/CCR3 pair is essential for eosinophil recruitment. Recently, it has been shown that other circulating cells such as fibrocytes also require the CXCL12 chemokine and the CXCR4 receptor for their efficient migration into the inflamed tissue (**Figure 1A**) (Coelho *et al*., 2005; Schuh *et al*., 2003; Kunkel, 1999). CCR4 or the CCR4 ligands CCL17 or 22 and CXCR5 are determinant factors for the entry and the positioning of T and B lymphocytes in secondary lymphoid organs (**Figure 1B**) (Acosta-Rodriguez *et al*., 2007; Bromley *et al*., 2008). Migration of dendritic cells (DCs) from tissues to lymph nodes through a gradient of CCL19 and 21 is dependent on CCR7 (**Figure 1B**) (Randolph *et al*., 2005; Sigmundsdottir and Butcher, 2008). These general chemotactic mechanisms are probably also essential for lymphocyte recruitment during toxicant-induced inflammation. Indeed, it has been observed that DCs present in the tissue may be activated after toxic agent exposure and migrate to lymphoid tissue to present and activate lymphocyte responses (Ryan *et al*., 2007). However, it is still unknown whether this lymphocyte mobilization induced by nonprotein xenobiotics is similar to that triggered by specific antigens (**Figure 1B**). The main chemokines and receptors implicated in the recruitment of the principal leukocyte populations are summarized in **Table 2**.

1.3 Resident Cells as a Major Source of Chemokines

Recent evidence demonstrates that tissue-resident cells, including fibroblasts, epithelial cells and sentinel macrophages are capable of both chemokine release and receptor expression, strongly suggesting that these cells play an early and key role in controlling local inflammatory events (Filer *et al*., 2008; Palmqvist *et al*., 2007; Coelho *et al*., 2005). Indeed, all these cells can be significant sources of chemokine production during various inflammatory responses. For example, fibroblasts produce different patterns of chemokines in response to different inflammatory alarm stimuli. Human peritoneal, synovial or corneal fibroblasts are able to release CXCL8 and CCL2 as well as increased levels of the corresponding messenger RNAs (mRNAs), when stimulated or not with LPS or pro-inflammatory cytokines such as IL-1β and TNF-α (Filer *et al*., 2008; Witowski *et al*., 2001). As with fibroblasts, epithelial cells and macrophages from various tissues have been shown to respond to immune signals by secreting an array of chemokines and expressing various chemokine receptors known to participate in immune responses (Schuh *et al*., 2003; Panina-Bordignon and D'Ambrosio, 2003; Kunkel, 1999).

Based on the fact that a very large variety of exogenous (pathogen residues) and endogenous (inflammatory mediators, cellular components) molecules strongly induced chemokine expression, it has been postulated that inflammatory toxicants can also directly activate the production of chemokines by these resident cells. Interestingly, very different types of xenobiotics such as toxic inorganic particles (e.g. silica), antibiotics (e.g. bleomycin) and industrial solvents (e.g. carbon tetrachloride (CCl_4)) can all induce the release of numerous chemokines by macrophages, epithelial

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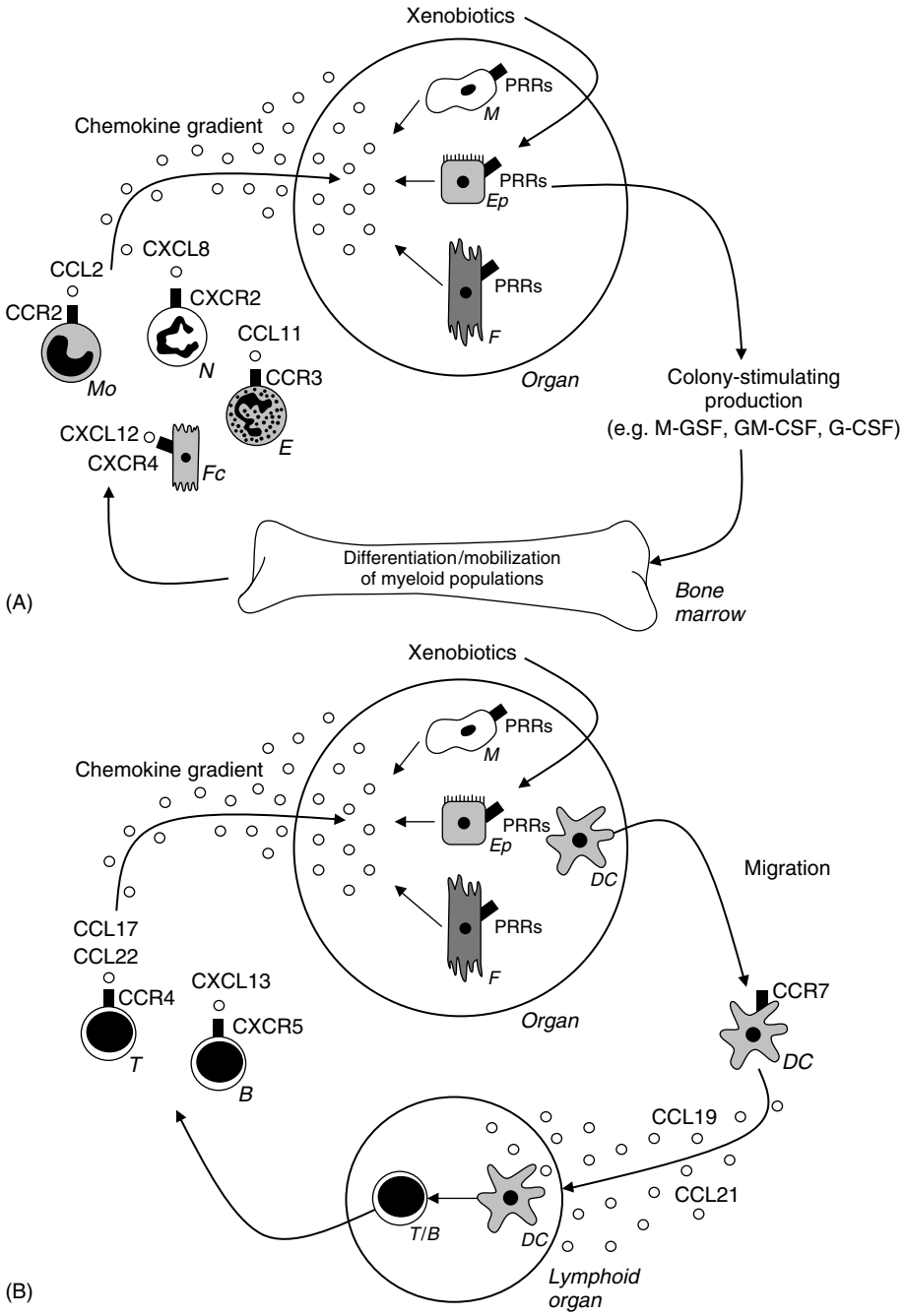

Figure 1 The role of chemokines in leukocyte infiltration in an inflamed organ after toxicant exposure. (A) After recognition of the xenobiotic by pattern recognition receptors (PRRs) of the innate immune system, tissue-resident cells such as macrophages (*M*), epithelial cells (*Ep*) and fibroblasts (*F*) release colony-stimulating factors such as granulocyte/monocyte colony-stimulating factor GM-CSF, granulocyte colony-stimulating factor G-CSF and monocyte colony-stimulating factor M-CSF. These factors communicate with the myeloid progenitors located in the bone marrow to differentiate and mobilize monocytes (*Mo*), neutrophils (*N*), eosinophils (*E*) and fibrocytes (*Fc*), and expand leukocytes that express CCR2, CXCR2, CCR3 and CXCR4 into the circulation. Chemokine receptor-expressing cells can thus traffic through the circulation and extravasate into the injured tissue in response to the specific ligands of these receptors (i.e. CCL2, CXCL8, CCL11 and CXCL12, respectively) also produced by the activated resident cells. (B) T cell (*T*) and B cell (*B*) expansion is orchestrated by xenobiotic-activated dendritic cells (*DC*) in the lymphoid organs. By expressing CCR7, these presenting cells migrate from the injured tissue to lymphoid organs through a gradient of CCL21 and CCL19 chemokines. After activation, T and B cell homing is then directed by a gradient of chemokines (i.e. CXCL17, CCL22 and CXCL13) and by the expression of CCR4 and CXCR5.

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Table 2 Main chemokine receptors and chemokines for the recruitment of leukocytes

Immune cells	Chemokine receptors	Chemokines
Monocytes/macrophages	CCR1, CCR2, CCR5, CCR8	CCL2–10
Dendritic cells	CCR7	CCL19 and 21
Neutrophils	CXCR1–2	CXCL1–3 and 8
Eosinophils	CCR3–5	CCL11 and 5
T lymphocytes	CCR4	CCL17 and 22
B lymphocytes	CXCR5	CXCL13
Fibrocytes	CXCR4	CXCL12

cells or fibroblasts. Indeed, it is well detailed that lung epithelial cells exposed to silica overproduce chemokines related to neutrophil tissue accumulation such as CXCL chemokines (Driscoll *et al*., 1993; 1996; 2001). This effect appears related to the capacity of the toxic particles to promote free-radical release and pro-inflammatory TNF-α production (Barrett *et al*., 1998; 1999; Desaki *et al*., 2000; Schins *et al*., 2000; Driscoll *et al*., 2001), and requires activation of p38 and extracellular signal-regulated kinase (ERK) signalling pathways as well as epidermal growth factor (EGF) receptor activation (Øvrevik *et al*., 2006; 2004). Silica-exposed pulmonary macrophages also release significant amounts of CXCL chemokines by similar activation pathways (Barrett *et al*., 1998; 1999; Kanj *et al*., 2006). These *in vitro* effects are not limited to noxious particles, since bleomycin or CCl_4 also induced the expression of CXCL8 or CXCL10 by endothelial cells and hepatocytes (Fichtner *et al*., 2004; Holden *et al*., 2000; Koniaris *et al*., 2001). The contribution of resident cells to the local cytokine/chemokine environment is thus an important consideration when assessing the deleterious activity of toxicants. However, it is important to keep in mind that all recruited leukocytes have the potential to actively participate in chemokine production as demonstrated for recruited and activated macrophages, neutrophils, eosinophils and lymphocytes trafficking through inflamed tissues (Schuh *et al*., 2003; Coelho *et al*., 2005).

1.4 Chemokines and Toxicant-Induced Diseases

Several lines of evidence support the view that the pathogenesis of some toxicant-induced inflammation and fibrosis involves uncontrolled immune processes. The fundamental concept usually put forward to explain the pathogenesis of these diseases is relatively simple and can be presented as follows: resident cells sense xenobiotics by innate immunity receptors and produce chemokines and CSFs which orchestrate specific influxes of inflammatory cells invading the toxicant-injured organ. The chronic recruitment of leukocytes exacerbates initial injuries and damages and destructs organ architecture as these cells are activated to release toxic mediators. By overproducing growth factors, chronically recruited leukocytes also pilot the exaggerated accumulation of mesenchymal cells and their connective tissue, leading to an unsolved fibrotic reaction (Huaux, 2007; Rimal *et al*., 2005; Fujimura, 2000). It is very intriguing that tissue inflammation and fibrosis appear after exposure to very diverse agents (chemical or not, inorganic or organic) and in different organs, suggesting that similar pathogenic pathways leading to these severe disorders exist and potentially involve a comparable network of mediators. This is particularly true for the chemokine network, since it is becoming evident that the pathophysiological responses to toxicants include a critical and general role of a set of chemokines implicated in differentiation, activation and mobilization of the inflammatory cells and the fibroblasts. This is particularly well illustrated by the macrophage-related chemokine CCL2 and its major receptor CCR2, which are both essentials for the development of lung or liver inflammatory and fibrotic reactions induced by silica, bleomycin or CCl_4.

In the following sections, we focus on the role of chemokines in regulating tissue inflammation and fibrosis after exposure to harmful agents by reviewing data from animal models of lung and liver toxicity.

2 CHEMOKINES REGULATE TOXICANT-INDUCED INFLAMMATION

Understanding how toxicants trigger immune cellular responses and chemokine production is crucial in order to elucidate what characteristics determine their harmful effects. New lines of evidence suggest that pattern recognition receptors (PRRs) recognizing viral and bacterial pathogens also sense and recognize xenobiotics. Indeed, recent studies have shown that scavenger receptors, essential for micro-organism recognition by the innate immunity, such as MARCO

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(macrophage receptor with collagenous structure), are responsible for the uptake of silica particles, free radical production and cytokine up-regulation, as well as cell death signalling in macrophages (Hamilton *et al*., 2006; Brown *et al*., 2006). The aryl hydrocarbon receptor (AHR), a ligand-dependent transcription factor best known for mediating the toxicity of dioxins, is essential for the establishment of T helper-17 (Th17) lymphocyte responses and the release of pro-inflammatory cytokines such as IL-22, strongly inducing the production of CXC chemokines and colony-stimulating factors necessary for granulocyte differentiation and recruitment (Veldhoen *et al*., 2008). In addition this receptor plays a central role in CCL1 and CXCL8 production in macrophage cultures after exposure to specific AHR ligands (Podechard *et al*., 2008; N'Diaye *et al*., 2006). After activation, the amplification cascade of chemokine expression is dependent on the inflammatory mediators released during the establishment of inflammation itself. Indeed, chemokine induction is also clearly mediated by the pro-inflammatory mediators produced at the site of inflammation such as the pro-inflammatory cytokines TNF-α and IL-1β. Importantly, endogenous molecules released during tissue and cell injury, such as fibrinogen, elastase and defensins, for example, are also capable of inducing chemokine production through PRRs such as Toll-Like-Receptors (Devaney *et al*., 2003; Smiley *et al*., 2001).

Because toxicants directly induce the release of chemokines by resident cells *in vitro,* toxicologists tested the relevance of these observations *in vivo.* First, all the toxic compounds reviewed here are able when administered in mice or rats to induce a marked expression of several chemokines. In the lung, bleomycin or silica markedly increase the expression by macrophages and epithelial cells of chemokines, orchestrating the recruitment of macrophages (CCL1 and CCL2), neutrophils (CXCL1 and CXCL2) or eosinophils (CCL11) (Smith *et al*., 1994; 1996; Smith, 1996; Driscoll *et al*., 1993; Keane *et al*., 1999; Huaux *et al*., 2005). Remarkably, these CXC and CC chemokines were also increased in the liver of animals treated with CCl_4 (Czaja *et al*., 1994; Kanno *et al*., 2005). The abrogation of these chemokines or their receptors by neutralizing antibodies or by using genetically deficient mice has generally led to a significant reduction of the leukocyte accumulation during the inflammatory stage. Driscoll and coworkers were the first to show that antibodies against CXCL2 reduced neutrophil infiltration in the lung of silica-exposed rats (Driscoll *et al*., 1996). On the same line, inhibition of CCR1, CCR2, CCL2 or CCL3 signalling in mice demonstrated that these elements are required for monocyte/macrophage recruitment in lung or liver after bleomycin or CCl_4 treatment, respectively (Smith *et al*., 1996; Smith, 1996; Baran *et al*., 2007; Zamara *et al*., 2007; Campbell *et al*., 2005). With a similar approach, the authors demonstrated the requirement of CCL11/CCR3 for effective eosinophil recruitment induced by the toxic antibiotic bleomycin (Huaux *et al*., 2005). However, additional studies concluded that the inhibition of one chemokine is not systematically associated with limitation of one particular type of leukocyte. Indeed, inhibition of CCL2 and CCL3 as well as CCR3 resulted in macrophage, eosinophil but also neutrophil depletion after injection of bleomycin in mice (Kimura *et al*., 2007; Ferreira *et al*., 2006; Ishida *et al*., 2007). Furthermore, reducing CXCL1, CXCL2 or CXCL3 activity with blocking antibodies or receptor antagonists was ineffective in limiting the lung accumulation of neutrophils induced by bleomycin (Russo *et al*., 2009; Keane *et al*., 1999). Additionally, overexpression of CXCL1 in the liver was associated with acute hepatotoxicity without a concomitant infiltration of neutrophils (Stefanovic *et al*., 2005). These findings illustrate the fact that the chemokine network is complex and very imbricate.

One of the interesting aspects regarding chemokines is the ability of pro-inflammatory cytokines to regulate the expression of chemokines. It has long been appreciated by *in vitro* approaches that early response cytokines such as TNF-α and IL-1β are potent inducing agents for the expression of a variety of chemokines. In an effort to better understand by which mechanisms chemokines are constantly overexpressed after toxicant exposure, researchers tested whether these *in vitro* findings were applicable *in vivo.* The pro-inflammatory cytokine TNF-α and the activation of nuclear factor kappa B (NFκB) appeared to be essential mediators of chemokine overexpression (i.e. CXCL2–3, CCL2–3) in silica-treated mice but also after bleomycin or CCl_4 treatments in animals (Hubbard *et al*., 2002; Pryhuber *et al*., 2003; Driscoll, 2000; Smith *et al*., 1996; Smith, 1996; Baran *et al*., 2007; Simeonova *et al*., 2001; Campbell *et al*., 2005; Czaja *et al*., 1994).

For decades, many human and animal studies have confirmed the involvement of various chemokine receptors or ligands in inflammatory disease models, from asthma to rheumatoid arthritis, sepsis and transplantation, which makes chemoattractant receptors promising targets of anti-inflammatory drugs (Mackay, 2008). Interestingly, some authors tried to also control and regulate xenobiotic-induced chemokine expression and inflammation through an anti-inflammatory strategy. First, it has been shown that the anti-inflammatory cytokine IL-10, well known to regulate exaggerated immune responses, is able to limit CXC chemokine expression after silica treatment, for instance (Driscoll *et al*., 1998). Also, other inhibitors have been proposed and studied with success in the liver after CCl_4 treatment, such as the CCL1 and CCL2 antagonist, SKL-2841, or cluster differentiation (CD)44 agonists (Kimura *et al*., 2007; 2008).

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3 CHEMOKINES ARE IMPLICATED IN XENOBIOTIC-INDUCED FIBROSIS

The inflammation induced by toxicants is often followed by a reparative phase in which polypeptide growth factors stimulate the recruitment and the proliferation of mesenchymal cells as well as regulate neovascularization and re-epithelialization of injured tissues. Tissue repair is accomplished by extracellular matrix deposition (mainly collagens I and III, fibronectin and proteoglycans) and tissue remodelling. During this phase, abnormal or uncontrolled reparative mechanisms may result in the development of fibrosis. Macrophages but also epithelial cells and recruited inflammatory cells are able, when appropriately activated, to release a number of potent polypeptide growth factors for mesenchymal cells, such as the fibrogenic cytokines TNF-α, IL-1 and transforming growth factor-β (TGF-β), and the growth factors such as platelet-derived growth factor (PDGF) and insulin-like growth factor (IGF) and fibronectin (Huaux, 2007; Rimal *et al*., 2005; Gulumian *et al*., 2006).

In vivo, it was first hypothesized that the participation of chemokines in fibrosis should be linked to their leukocyte chemotactic activity. Indeed, activated leukocytes are known to be potent sources of mediators modulating the biology of fibroblasts (Huaux, 2007). This postulate was supported by numerous studies documenting the reduced fibrotic process that went paired with reduced leukocyte infiltration. For instance, neutralizing antibodies directed against CCL2 or CCL3 not only reduced bleomycin-induced pulmonary mononuclear phagocyte accumulation but also fibrosis, clearly supporting the notion that leukocyte influx is linked to fibrosis (Smith *et al*., 1994; 1996; Smith, 1996). These conclusions were confirmed by neutralizing the main receptors of these two CC chemokines, that is, CCR1 and CCR2 (Tokuda *et al*., 2000; Baran *et al*., 2007). Again, similar mechanisms and mediators appear crucial in toxicant-induced liver fibrosis because CCL2 is intimately linked to the amplitude of fibrosis, and a treatment abrogating CCL2 blocked liver fibrosis as in the lung (Tsuruta *et al*., 2004; Kanno *et al*., 2005). Importantly, the authors of these studies argued that the limitation of fibrosis was associated with a reduced number of macrophages during the establishment of the disease. They also observed that activation and functions of these cells were also related to the chemokines/receptors. For instance, absence of CCR2 impaired the production of matrix metalloproteinase (MMP) by purified macrophages after bleomycin treatment (Okuma *et al*., 2004). Besides macrophage-related chemokines, those implicated in the influx of eosinophils and neutrophils seem to be important for fibrosis as well. For instance, CCR3 deficiency protected against bleomycin inducing lung fibrosis. Additionally, injection of the CCR3 ligand, CCL11, enhanced eosinophil and neutrophil recruitment and bleomycin-induced fibrosis (Huaux *et al*., 2005).

The beneficial antifibrotic activity provided by CXC or CC chemokine and receptor blockade was, however, not systematically accompanied with a similar reduction of leukocyte infiltration in several pioneer studies. This strongly suggested new profibrotic functions of chemokines with a minimum participation of inflammatory cells. For instance, CXCL2 depletion with antibody treatment reduced bleomycin-induced lung fibrosis without a change in the presence of pulmonary neutrophils (Keane *et al*., 1999). Similarly, lung fibrotic pathology was improved by treatment with the CXCR2 receptor antagonist DF2162 without interfering with neutrophil accumulation in the pulmonary parenchyma in bleomycin-treated mice (Russo *et al*., 2009). Again, observations made in the lung appear similar in the liver. Indeed, induced overexpression of CXCL1 in the liver of CCl_4-treated mice increased synthesis of Type I collagen without accumulation of neutrophils (Stefanovic *et al*., 2005). Finally, by studying the role of CCR4 in lung fibrosis after bleomycin treatment, Trujillo and coauthors demonstrated that this chemokine receptor plays a central role in fibrosis by regulating macrophage differentiation and activation instead of modulating lymphocyte recruitment (Trujillo *et al*., 2008).

Fibroblasts highly express chemokine receptors (Filer *et al*., 2008) and can therefore be directly activated by chemokines produced by the injured tissue after toxicant exposure, and may potentially contribute to uncontrolled fibroproliferative repair. Several studies, thus, explored the potential direct profibrotic functions of diverse chemokines expressed during the pathological inflammatory and fibrotic process induced by silica, bleomycin or CCl_4. Interestingly, addition of recombinant CXCL1 or CCL2 to lung, hepatic or skin fibroblasts in culture increased secretion and processing of Type I collagen (Gharaee-Kermani *et al*., 1996; Yamamoto and Nishioka, 2003; Stefanovic *et al*., 2005). In addition, Gharaee-Kermani and colleagues reported that CCR2 signalling regulates *in vitro* fibroblast responsiveness to TGF-β and fibrogenic cytokine expression (Gharaee-Kermani *et al*., 1996; 2003).

Not all chemokines possess profibrotic functions. Indeed, the CXCL10 and CXCL11 ligands of the CXCR3 receptor attenuate bleomycin-induced pulmonary fibrosis via inhibition of vascular remodelling (Burdick *et al*., 2005). In the same model of lung fibrosis, limitation of the tissue fibroproliferation by the activation of CXCR3 was mediated, in part, by the innate production of interferon (IFN)-γ, a well known antifibrotic cytokine (Jiang *et al*., 2004). Finally, other authors also observed that CXCL10 directly attenuated lung fibrosis by limiting migration and recruitment of fibroblasts in bleomycin-treated mice (Tager *et al*., 2004). The beneficial activity of CXCR3 ligands may represent a novel

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therapeutic strategy for pulmonary disease, which could have applicability to a wide range of fibrotic illnesses.

Altogether, these experimental approaches using knockout and transgenic experiments have clearly highlighted that chemokines possess very diverse and multiple functions during the establishment of inflammation and fibrosis and are certainly not only simple chemotactic factors.

4 MULTIPLE ROLES OF CHEMOKINES

It is now recognized that chemokines play important roles in several activities including maintenance of homeostasis, angiogenesis/angiostasis, cellular differentiation and activation, tumour growth and metastasis, lymphocyte homing and development of lymphoid tissue, and influencing the overall Type 1/Type 2 balance of immune response (Strieter *et al*., 2007; Coelho *et al*., 2005). In this context, a variety of investigations provided evidence that specific chemokines are important factors for angiogenesis and fibrocyte tissue accumulation after toxicant exposure.

Indeed, it has been reported that samples from lungs of bleomycin-treated mice have a marked angiogenic activity; that is, almost entirely attributable to overexpression of the angiogenic CXC chemokines CXCL2 and CXCL3 and the relative down-regulation of the angiostatic CXC chemokines CXCL10 and CXCL11. Moreover, when endogenous CXCL2 and CXCL3 were depleted or when exogenous CXCL10 or CXCL11 was administered to the animals during exposure to bleomycin, a marked attenuation of pulmonary fibrosis was observed that was entirely attributable to a reduction in angiogenesis in the lung (Keane *et al*., 1997; Strieter *et al*., 2007). Taken together, these findings support the notion that CXC chemokines are important angiogenic and angiostatic factors balancing the development of vascular remodelling, and are critical to promote fibrosis after exposure to bleomycin. Interestingly, neutralization of CXCL10 with antibodies stimulated hepatocyte proliferation and facilitated remodelling of hepatic cords in CCl_4-induced acute liver injury (Yoneyama *et al*., 2007). The exact function of CXCL10 in chronic responses to CCl_4 is not determined, but it can be supposed that this chemokine may facilitate uncontrolled liver remodelling and fibroproliferative repair as observed in the lung. It is still unknown whether chemokines regulating angiogenesis are also important in the development of fibrosis after silica administration.

Previously, circulating fibrocytes have been shown to express several chemokine receptors, and to respond chemotactically to the cognate ligands, thus suggesting their possible roles in recruitment of bone marrow progenitor cells (Lama and Phan, 2006; Gharaee-Kermani *et al*., 2007). Fibrocytes express CD34 and CD45, two leukocyte markers, and appear to have the capacity to differentiate to tissue fibroblasts and myofibroblasts. Induction of CCL21 and CXCL12 in bleomycin-injured lung tissue, and their respective cognate receptors, CCR7 and CXCR4 in fibroblasts from injured lungs, suggests recruitment of these extrapulmonary progenitor cells via these chemokines (Hashimoto *et al*., 2004; Phillips *et al*., 2004). This is supported by evidence that CXCR12 neutralizing antibodies (Phillips *et al*., 2004) or CXCR4 antagonists (Xu *et al*., 2007) reduce recruitment of fibrocytes and bleomycin-induced pulmonary fibrosis. The CCL3/CCR5 pair is apparently also important for fibrocyte infiltration during the experimental lung fibrosis (Ishida *et al*., 2007). In this study, recruited fibrocytes represented the main producers of the profibrotic cytokine TGF-β besides macrophages. Thus, bone marrow progenitor cells can be mobilized and recruited to tissues in response to chemokine signals released from the injured tissue (Gharaee-Kermani *et al*., 2007; Lama and Phan, 2006). It is uncertain whether fibrocytes may also play a pivotal role after silica or CCl_4 injection as observed with bleomycin. However, liver injury and fibrosis in mice is associated with the marked accumulation of collagen-producing fibrocytes, suggesting that these cells participate in the pathogenesis of the fibrotic disease in the liver, potentially through the regulation of chemokines (Kisseleva *et al*., 2006). The fact that the CXCR4/CXCL12 interaction plays a role in stem cell homing towards the CCl_4-damaged liver strongly supports the notion that fibrocytes are important during experimental liver fibrosis (Jung *et al*., 2006).

5 CONCLUSIONS

Chemokines represent a remarkable superfamily of immune mediators strongly implicated in the development of diseases. Although chemokines initially attracted attention as chemoattractants for immune cells, they are now known to play essential roles in cellular differentiation and activation. This view is also shared by toxicologists exploring the role of chemokines in the establishment of tissue inflammation and fibrosis induced by certain toxic agents. The exact cascade of events implicated in chemokine functions in these disorders is now better identified and can be summarized as follows: probably initiated by the activation of receptors of the innate immunity, toxicants induce tissue injury and expression of chemokines which initiate accumulation of numerous leukocytes into the tissue. The early pro-inflammatory cytokines produced conjointly amplify and maintain chemokine production. The persistence of this chemokine overexpression loop sustains chronic inflammation and plays pivotal roles in the development of abnormal fibroproliferative repair by

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modulating fibroblast functions, cellular differentiation, angiogenesis and fibrocyte accumulation.

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Oncology Biomarkers

Michael A. Tainsky

CONTENTS

1 INTRODUCTION

1.1 Overview

Tumour biomarkers are molecules often produced by the tumour itself or the host system in response to the tumour, and provide the biological material to determine the risk of getting cancer, to detect cancer, to classify cancer, or to provide insight into prognosis and therefore a therapeutic advantage. Tumour biomarkers include cancer-specific mutations or changes in gene expression or promoter methylation, which can result in alterations in protein expression. Because cancer cells shed DNA and RNA in the circulation, a phenomenon rarely seen in healthy individuals, tumour-specific genetic changes such as promoter methylation, gene mutations or circulating small RNAs are detectable in nucleic acids prepared from plasma or other body fluids. Biomarker proteins, either overly abundant or variant proteins, can be detectable in the circulation as the free, shed proteins or as novel autoantibodies to such proteins; the latter indicating that the host immune system can be exploited as biosensor of the disease. In addition, tumour-specific biochemical changes result in post-translational modification of proteins via glycosylation or phosphorylation, providing a variety of biomarker molecules. Cancer-related biochemical changes often effect measurable metabolic variations within a cell or organism, which may be powerful biomarkers.

Cancer biomarkers are discovered and utilized with a specific purpose in mind, such as the (i) early detection

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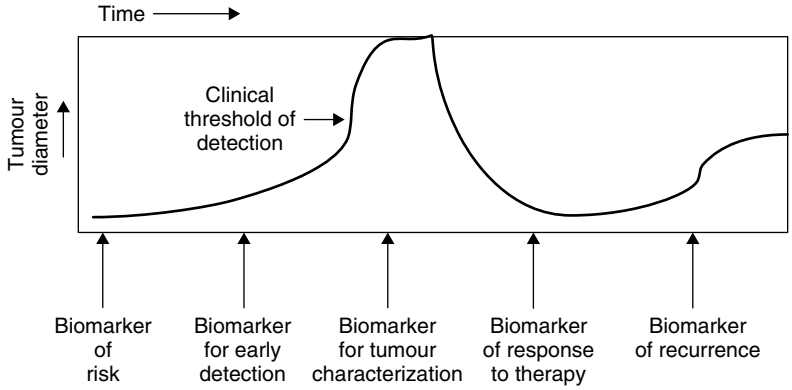

Figure 1 Different biomarkers for different stages of cancer progression. Biomarkers are employed for risk assessment, early detection, determining the mechanism of cancer development, response to therapy and cancer recurrence.

of cancer, (ii) diagnosis, (iii) prognosis, (iv) response to anticancer therapies, or (v) detection of cancer recurrence (**Figure 1**). Cancer cells provide the biomarker material that can lead to their own detection, which then provides the opportunity for their noninvasive detection in body fluids and tissues so as to reveal the presence of tumours or the level of tumour burden. Biomarkers for cancer diagnosis, prognosis and response to therapy became possible through elegant studies of specific carcinogenic mechanisms (McGuire, 1973; Heisterkamp *et al*., 1985; Slamon *et al*., 1987), leading to the development of clinical tests capable of predicting the optimal or targeted therapeutic approach for a given cancer. Alternatively, well-powered biomarker discovery projects have also led to clinical tests for therapeutic decisions for some cancers (Paik *et al*., 2004; 2006; Wolf *et al*., 2008; Lyman *et al*., 2007; van 't Veer *et al*., 2000; van de Vijver *et al*., 2002; Sotiriou *et al*., 2006).

There is an emerging expectation that panels of biomarker analytes rather than single markers will be required, to have sufficient sensitivity and specificity for the diagnosis or prognosis of cancer. This realization became apparent from the failure of single biomarkers to be adapted for clinical practice. The need for panels of biomarkers, specific for each cancer, is based on the experience from decades of molecular oncology research that has demonstrated molecular complexity of human carcinogenesis even within single cancer sites.

Biomarkers hold tremendous promise not only for diagnostic devices and to improve the effectiveness of clinical interventions, but also to stratify oncology patients to the most appropriate treatment. However, early detection must provide a patient with better survival, which ultimately can translate to more efficient delivery of healthcare. For example, early detection technologies applied to population-based screening for cancer of the cervix, colon and breast have resulted in reductions in disease-associated mortalities from cancers that are deadly if allowed to progress to an advanced stage. These screening approaches are technology driven but have proven that early detection will reduce the morbidity and mortality from cancer.

Biomarkers often can provide prognostic information of disease enabling intervention with the appropriate therapeutic agent and early decisions or corrections of cancer treatment. There has been a burst of novel technologies that are being used to discover and validate tumour biomarkers individually and where necessary in panels or combinations of biomarkers. Although biomarkers have the potential to provide personalized diagnostic, prognostic and therapeutic information for the individual patient, the devices to implement them clinically must ensure that healthcare providers have clear algorithms for step-by-step implementation for their patients.

2 BIOCHEMICAL ANALYTES AS ONCOLOGY BIOMARKERS

There are numerous analytical biochemistry approaches to identify and validate biomarkers. In the context of this chapter we will focus on three general areas of analytes, nucleic acids, and proteins and metabolites.

2.1 Nucleic Acid Biomarkers

2.1.1 RNA Expression Profiling

The identification of gene expression profiles for stage- or prognosis-specific classification of cancer patients is the future of biomarker-driven patient management. Gene expression profiles can be more effective than more standard methods of patient stratification such as histopathology biomarkers. To validate such biomarkers clinically using fresh or archived clinical samples, the RNA preparations must have minimal degradation or alternatively use gene measurement technologies that are not overly sensitive to some reduction in the RNA size.

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Gene expression profiling coupled with well-designed bioinformatics methods has greatly facilitated the identification of genes and pathways that regulate cellular changes critical to cancer development and molecular targets of personalized medicines. The microarray screening procedures are quite reproducible but require biological replicates or multiple samples from a particular class of sample to eventually be able to use the biomarkers for class prediction on an independent validation set. In addition, the microarray technologies tend to underestimate the true changes in gene expression, so that additional validation using reverse transcription polymerase chain reaction (RT-PCR) or northern blot analysis is necessary to have confidence in the results. In principle, the particular changes in gene expression between classes of samples may be less informative than the pathways they impact. There are numerous software approaches to pathways analysis for a given gene expression dataset. Those that weight the impact of the particular genes provide additional guidance on the identification of shorter lists of biomarkers (Okuda *et al*., 2008; Segota *et al*., 2008; Draghici *et al*., 2007; Khatri *et al*., 2007).

2.1.2 DNA Sequencing

DNA sequencing is commonly used in research to identify genetic changes in candidate genes. The use of DNA sequences as diagnostic markers is entering into clinical practice for the detection of DNA sequence variants, disease-specific single nucleotide polymorphisms or small insertions or deletions in genes. Somatic changes in DNA sequences are present in tumour tissue but not present in the normal tissue from the same person. The selection of somatic DNA sequence changes is thought to be a critical part of the carcinogenic process resulting in alterations in important growth regulatory genes. Oncogenic DNA sequence variants are useful diagnostic biomarkers and have provided specific molecular targets for cancer therapies, as the altered proteins resulting from these mutations are pharmacologic targets. Large-scale sequencing of numerous candidate genes is providing new biomarkers for studies of diagnosis and prognosis of lung, germ-cell, colorectal and breast cancers (Davies *et al*., 2005; Bignell *et al*., 2006; Ding *et al*., 2008; Leary *et al*., 2008; Marguerat *et al*., 2008). Somatic DNA sequence analysis has expanded recently from such candidate gene studies to genome-wide high-performance DNA sequencing, leading to whole genome analysis of DNA sequence variations in tumours (Ley *et al*., 2008).

2.1.3 Epigenomics

There are several normal cellular processes that are regulated in part by epigenetic modification through DNA methylation, including developmental imprinting, X chromosome inactivation and tissue-specific gene expression. Aberrant chromatin-remodelling mechanisms, such as DNA methylation and histone acetylation, are epigenetic modifications that have been observed in tumour tissues leading to the inhibition of tumour suppressor gene expression. Understanding these mechanisms may provide biomarkers of response for cancer chemotherapy. Drugs targeting such epigenetic changes have not yet been developed into gene-specific therapies. DNA methylation, histone deacetylase inhibitors and poly-adenosine diphosphate (ADP)-ribosylation inhibitors are in clinical trials and may provide novel tools to enhance classical chemotherapies or other targeted therapies, potentially activating tumour suppressors or other key checkpoint genes.

Aberrant promoter methylation has been found in growth suppressive genes in human tumorigenesis (Baylin *et al*., 2000; Baylin and Herman, 2000; Esteller *et al*., 2001; Feinberg *et al*., 2002). CpG (cytidine-phosphate-guanine) dinucleotides are typically methylated. In contrast, CpG islands, which are CpG-rich stretches of DNA ranging from 200 to 2000 bp in length in the regulatory regions of genes, are generally unmethylated. The transfer of a methyl group from *S*-adenosyl-L-methionine to the cytosines in CpG sites is catalysed by DNA-methyltransferases (DNMTs) (Lopatina *et al*., 2002). There are three well-characterized DNMTs: DNMT1, DNMT3A and DNMT3B. DNMT1, the most abundant DNMT, is mainly responsible for maintenance methylation, while DNMT3a and DNMT3b are responsible for *de novo* methylation (Lopatina *et al*., 2002).

In human cancers, the silencing of tumour suppressor genes through aberrant DNA methylation of a CpG island(s) in the promoters in these genes is a common epigenetic change (Baylin and Herman, 2000). There is an assortment of pathways from which genes have been shown to be hypermethylated in cancer cells, including DNA repair, cell cycle control, invasion and metastasis. The tumour suppressor genes *BRCA1* (hereditary breast cancer gene 1), $p16^{INK4a}$, $p15^{INK4b}$, $p14^{ARF}$, $p73$ and *APC* (adenopolyposis coli, colon-cancer-predisposing gene) are among those that are silenced by hypermethylation, although the frequency of aberrant methylation is somewhat tumour type specific (Esteller *et al*., 2001). Aberrant hypermethylation of DNA can be reversed with chemical agents that inhibit DNMTs, which in effect 'demethylate' DNA. A commonly used inhibitor of DNMT is 5-aza-deoxycytidine (5-aza-dC), a cytosine analogue. 5-aza-dC and related drugs work by substituting for cytosine during replication. DNMTs recognize and covalently bind 5-aza-dC in DNA. The covalently bound DNMT1 is unable to catalyse the transfer of methyl groups to the cytosine analogue because the substituted nitrogen base cannot be methylated. Consequently DNMT1 is depleted following several rounds of replication. This in turn results in DNA hypomethylation

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and the expression of genes that were silenced by methylation (Haaf, 1995; Kanai *et al*., 2001; Takebayashi *et al*., 2001). Higher throughput methods are being developed to identify larger panels of methylation biomarkers for disease detection and tumour progression (Adorján *et al*., 2002; Bibikova *et al*., 2006; Ordway *et al*., 2006). From such studies, panels of biomarkers for individual cancers are being developed for early detection and response to chemotherapy (Belinsky *et al*., 2005; Weaver *et al*., 2006; San-Miguel *et al*., 2005; Gonzalez-Paz *et al*., 2007; Hoque *et al*., 2006; Jiao *et al*., 2007; Hsu *et al*., 2007; Chim *et al*., 2007; Tsou *et al*., 2007; Lofton-Day *et al*., 2008; Ibáñez de Cáceres and Cairns, 2007; Grützmann *et al*., 2008; Rimel *et al*., 2009; Altimari *et al*., 2008; Frattini *et al*., 2008). There are going to be difficulties in developing standards for implementation in a clinical setting (Barker *et al*., 2006), particularly as a major direction of this field is the early detection of cancer using methylation of circulating tumour DNA in plasma (Esteller *et al*., 1999; Rouprêt *et al*., 2008; Jin *et al*., 2007; Hampton, 2007; Hoque *et al*., 2006).

2.1.4 MicroRNA

Another form of epigenetic modification of gene expression and post-translational alteration of protein expression is through microRNA (miRNA or miR)-related mechanisms. miRNAs are synthesized by RNA polymerase II as long transcripts that are processed to pre-miRNAs. Some miRNAs are transcribed by RNA polymerase III (Borchert *et al*., 2006). Two RNase III-related enzymes, Drosha and Dicer, process the maturation of the miRNAs (Lee *et al*., 2003). In the cytoplasm, Dicer processes the pre-miRNA into a 22-nucleotide double-stranded miRNA, and then one strand of the RNA is degraded. The other strand, as part of the RNA-induced silencing complex (RISC), targets the 3′ untranslated region of specific messenger RNAs (mRNAs), destabilizing the target mRNA(s) or repressing their translation (Lai, 2002). Screening for miRNA expression levels is routinely performed using array technologies to obtain a miRNome profile and validation/confirmation performed using northern blot, RNase protection assay or primer extension assay. Quantitative RT-PCR, *in situ* hybridization (Wienholds *et al*., 2005) and serial analysis of gene expression (SAGE) have also been applied to these small RNAs (Cummins *et al*., 2006).

Although the existence of miRNAs in humans has only been recognized for less than a decade, the flurry of activity has identified roles for them in normal development and numerous diseases including cancer, leading to a Nobel Prize in 2006. Currently more than 500 human miRNAs have been identified and they often are found in chromosomal clusters. There is substantial evidence for differential expression of miRNAs in a variety of cancers. miR-15a and miR-16-1 are found within a cluster in chromosome 13q14, which is frequently a deleted region in B cell chronic lymphocytic leukaemia (Calin *et al*., 2002; 2004). The miRNome may provide a better classifier of cancer type than the mRNA expression profile and therefore the miRNome appears to be a useful technology for the diagnosis and prognosis of cancer (Calin *et al*., 2004). miRNAs have been found to be repressed by epigenetic mechanisms in cancers (Lehmann *et al*., 2008; Lujambio *et al*., 2008) and, consistent with that observation, more miRNAs are down-regulated than up-regulated in cancer as compared to their normal tissues (Calin *et al*., 2004; Porkka *et al*., 2007). Because of their small size, miRNAs have tremendous potential as biomarkers because they are easily quantified in normal and cancer tissues as well as body fluids (Gilad *et al*., 2008; Chen *et al*., 2008; Mitchell *et al*., 2008; Lawrie *et al*., 2008). However, this field is in its infancy and no miRNA biomarkers are in clinical use.

2.2 Protein Biomarkers

Protein biomarkers hold a strong opportunity to be converted into clinical diagnostic tests. Protein biomarkers are often identified in basic science studies of cancer cells as overexpressed proteins. Given the proven ability of most manufacturers of clinical laboratory tests to adapt a protein-based immunoassay on to a standard clinical platform, the rapid translation of protein discoveries to a test should be quite efficient. The difficulty can be at the level of developing antibody pairs for sandwich immunoassays when multiple protein analytes must be assayed in a body fluid such as serum, plasma or urine. If these panels of tumour biomarker proteins form a unit that is used together to classify test subjects for early detection or recurrence and if each member of the panel is required for sufficient accuracy, failing to develop an antibody pair for any single member of the panel will nullify the utility of the clinical test (Zolg, 2006). Cancer-specific alterations in a protein may occur at the level of protein abundance or post-translational protein modification such as glycosylation or phosphorylation. If the protein being developed as a biomarker is present in a body fluid but only the post-translational modification is cancer specific, then antibodies to these specific changes represent a difficult challenge for the development of the antibody pairs. In addition, the issue of matrix complexity is formidable. The concentration range of plasma proteins comprises about nine orders of magnitude (Anderson and Anderson, 2002). Given that the abundant plasma proteins may be functionally related to the disease processes, depleting abundant plasma proteins may cause the unintentional but direct removal of some important protein analytes, or indirectly may remove key biomarker proteins that were associated with the

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abundant protein, thus unintentionally removing them from the serum or plasma.

There are a number of direct approaches to identify cancer-specific changes in proteins including abundance or post-translational modifications. Proteomic changes in cancer can be discovered by a combination of two-dimensional gel electrophoresis for separation of the proteins, with a variety of potential methods for their visualization, such as direct radioactive labelling, covalent attachment of fluorescent tags, and silver staining. Mass spectrometric analysis can then be used for the sequence identification of each protein spot (Chen *et al*., 2002; Roessler *et al*., 2006). This technology is reliable but lacking in the ability to characterize low-abundance elements of the cancer proteome. Generally about 2000–4000 proteins can be resolved and identified from a complex matrix such as serum or tissue. Because of redundancy of multiple isoforms of proteins, the actual identification of abundant proteins by two-dimensional gel electrophoresis with mass spectroscopic identification results in as much as 10-fold fewer actual protein determinations. The complexity can be diminished using enrichment procedures such as immunoaffinity chromatography for the removal of the most abundant proteins (Brand *et al*., 2006) or using less-complex matrices such as urine or lavage fluids. These latter body fluids offer a better source of tumour-specific proteins if the organ being tested for cancer is bathed in these fluids. However, given the low sensitivity of the detection methods, many have migrated to mass spectroscopy-based protein biomarker discovery for undirected high-throughput searches.

2.2.1 Proteomic Mass Spectroscopy

Proteomic mass-spectroscopy-based platforms have provided the ability to identify large numbers of novel proteins with the potential to be biomarkers. These studies are being performed in various biological matrices such as cultures of normal and tumour cells or human clinical samples (serum, plasma and urine). Proteomic biomarker discovery strategies involve the identification of markers using multidimensional protein purification technology followed by mass spectroscopy. Next the biomarkers must be prioritized based on criteria such as cancer relevance and the likelihood for clinical assay development. Lastly, validation of prioritized markers using multiple sample sources is critical, as some proteomic mass-spectroscopy studies have been biased by study design issues, such that day-to-day laboratory variation or the samples' origins can be more easily classified than the essential biological variation, such as cancer versus healthy. Proteomic mass-spectroscopy-based methods hold promise for the discovery of novel biomarkers that might provide new clinical tests, but to date their contribution to the diagnostic armamentarium has been disappointing (Zolg and Langen, 2004). The importance of experimental study design to overall process of biomarker discovery and validation has been underestimated and led to overestimation of accuracy for a set of small protein fragments in an ovarian cancer study using surface-enhanced laser desorption/ionization (SELDI) technology (Petricoin *et al*., 2002; Diamandis, 2004; Baggerly *et al*., 2005; Ransohoff, 2005). Effective studies using SELDI will require thorough attention to study design and independent validation in order to develop successful protein or peptide biomarkers for noninvasive testing.

Novel proteomic biomarker discovery technologies are being developed using quantitative isotopic labelling, improved mass-spectroscopy algorithms, and addressing the problems of sample preparation. Abundant proteins are being depleted and multidimensional prefractionation is being used to identify low-abundance protein biomarkers. Isotopic labelling with deuterated or ^{13}C isotopes of acrylamide to cysteine residues is being exploited for relative quantitation of proteins by matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) or liquid chromatography with tandem mass spectrometry (LC-MS/MS) high-resolution mass spectrometry (Martosella *et al*., 2005; Faca *et al*., 2006). These and similar mass-spectrometry-based technologies generate enormous datasets from complex patient discovery studies that challenge current data analysis procedures (Andreev *et al*., 2006; Rauch *et al*., 2006).

2.2.2 Antibody Microarrays

Multianalyte protein detection in complex matrices is also being approached using immunoassays on antibody microarrays that are highly parallel enzyme-linked immunosorbent assays (ELISAs). There are a few approaches for this type of biomarker discovery tool (Haab, 2005; Sanchez-Carbayo, 2006). One strategy is to utilize previously validated antibody pairs from sandwich ELISA tests and then multiplex the spotted capture antibodies on a surface and label the detection antibodies for the parallelized assay. As with other ELISA tests, assay interference must necessarily be addressed (Gonzalez *et al*., 2008). Another strategy is to choose candidate overexpressed proteins from other studies and develop pairs of antibodies suitable for antibody microarrays (Kopf and Zharhary, 2007; Kingsmore, 2006). The protein analytes can be measured in serum, plasma or urine for noninvasive biomarker assays or in liquefied extracts of tumour tissue for evaluation of prognostic biomarkers or potential therapeutic targets (Sanchez-Carbayo *et al*., 2006).

2.2.3 Cancer Autoantibodies

Antibodies in the serum of cancer patients are induced by the humoral immune response to overexpressed or

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mutant proteins. Cancer autoantibodies have been found to HER2 (human epidermal growth factor receptor 2, also called HER2/neu) protein (Disis *et al*., 1997) and p53 (Crawford *et al*., 1984), but because these antibodies have been detected in 5–10% of cancer patients (Disis *et al*., 2000; Mack *et al*., 2000), their sensitivity for diagnosing cancer is low. In fact, the sensitivity for p53 autoantibodies is also low even if the patient has p53 missense mutation. Autoantibodies to mucin 1 (MUC1) have been found in the sera of women with benign breast disease, as well as breast cancer at early and advanced stage (von Mensdorff-Pouilly *et al*., 1996). MUC1 autoantibodies also are found in patients with other cancers, also indicating a lack of their specificity (Gourevitch *et al*., 1995). A series of lung cancer two-dimensional gels and western blotting revealed autoantibodies to those proteins from patients' sera, (Chen *et al*., 2002).

Direct screening for autoantigens can be performed in order to discover the autoantibodies, using the serologic identification of antigens by recombinant expression (SEREX) technology. This method has resulted in the discovery of numerous antigens for breast cancer, including annexin XI-A, RPA32 (replication protein A: 32 kDa subunit), NY-ESO-1 and many other proteins (Scanlan *et al*., 2001; Sahin *et al*., 1995; Jäger *et al*., 2001; Tomkiel *et al*., 2002; Minenkova *et al*., 2003). SEREX technology is based on screening tumour-derived cDNA (complementary DNA) libraries made from a particular patient with serum obtained from the same patient. As a result, the identified tumour associated antigens (TAAs) in most cases do not show high reactivity with allogeneic patients' sera. SEREX cloning tends to identify antigens that were overexpressed in the tumour and are thus highly abundant in tumour-derived cDNA libraries. Thus less abundant mRNAs that may be coding for tumour-relevant antigens are generally missed. As a prokaryotic system is used in this immunoscreening, this approach misses autoantibodies against tumour-specific post-translational modifications such as glycosylation, which is frequently part of the epitope recognized in TAAs. The cDNA clones derived from these studies were not only found expressed in tumours, but also in normal testis tissues, so they have been called cancer testis (CT) antigens. Several modifications have been introduced to improve SEREX technology. The first variation involves established tumour cell lines instead of fresh tumour specimen as a source of cDNA, thus avoiding contaminating tumour specimens with normal cell RNAs, so that they won't be included in cDNA preparation. Also this avoids cDNA cloning of immunoglobulin sequences expressed by tumour-infiltrating B cells, giving rise to false-positives in the library screening (Chen *et al*., 1998). Second, the screening of sera from cancer patients on allogeneic cDNA libraries, including testicular cDNA libraries, may result in identification of TAAs and CT antigens that will show higher reactivity with serum IgGs (immunoglobulin Gs) obtained from many different cancers. A third modification involves a combination of representational difference analysis (RDA) and SEREX. RDA is a PCR-based subtractive hybridization method which can effectively isolate differentially expressed genes from a given cDNA population (tester) compared with another (driver) (Lisitsyn *et al*., 1993). This combined technology of RDA and SEREX has been applied for the identification of additional CT antigens in melanoma (Güre *et al*., 2000). However, these antigens have not yet provided sufficient sensitivity across a large enough cross-section of cancer patients to provide clinically useful early detection markers.

SERPA (serological proteome analysis), a combination of serology and proteomics technology, is considered to be a 'top-down' or intact protein analysis approach that involves separation of proteins on two-dimensional gel electrophoresis (2-DE), followed by western blotting, and finally identification by mass spectrometry. Klade *et al*. have combined 2-DE and serological analysis to develop the SERPA technology for the discovery of tumour biomarkers that have mounted humoral immune responses in cancer patients (Klade *et al*., 2001). Using this technology, Hanash *et al*. have reported humoral immune response to calreticulin in pancreatic cancer (Hong *et al*., 2004). In SERPA, three 2-DE gels are run with equal amounts of cell lysates that are made from fresh tumour specimens or cancer cell lines. Two gels are blotted onto nitrocellulose membranes and are processed with serum IgGs obtained from cancer patients and healthy individuals. The third gel (the preparative gel) is stained with Coomassie blue. After comparing the immunoreactive spots from the blot of the cancer patient with that of the control individual, spots that appear brighter or unique to the patient are excised from the Coomassie blue-stained gel. Next, peptide mass fingerprinting analysis is performed using MALDI-TOF MS. The advantage of this technology is that it is a less labour-intensive procedure than SEREX. Although sodium dodecyl sulfate (SDS)/urea is used for protein denaturation, post-translational modifications are still intact. Therefore SERPA provides more immunoreactively antigenic epitopes for serological testing than SEREX. Despite the success, this technology has its shortcomings. Two-dimensional gel electrophoresis is only effective in identifying highly abundant proteins in patients compared to controls. Also, 2-DE is unable to separate different proteins that can co-migrate on gels due to certain post-translational modifications. With the use of high-performance liquid chromatography (HPLC) to resolve tumour cell lysates into several thousand individual protein fractions in combination with multiplex Luminex xMAP technology for serological screening, SERPA can be modified to identify newer tumour diagnostic biomarkers in cancer patients.

Higher-throughput tumour antigen cloning can be accomplished using phage display technology coupled with serological identification of tumour antigens and

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profiling humoral immune responses in cancer patients on protein microarrays (Draghici *et al*., 2005; Chatterjee *et al*., 2006; Lin *et al*., 2007). A differential biopanning strategy was employed to immunoselect T7 phage tumour-derived cDNA libraries first with control serum IgGs (pooled from different age-matched normal healthy individuals) for the removal of common, nontumour antigens that bind to IgGs in normal sera. Next, the T7 phage remaining from the cDNA libraries are incubated with serum IgGs from cancer patients to enrich for phage clones bearing tumour antigens. These phage clones are then robotically printed on protein microarrays for immunoassays to identify circulating serum antibodies in those cancer patients mounting a humoral response against cancer antigens. These antigen arrays are then further processed with Cy3-labelled T7 monoclonal antibody that recognizes phage capsid protein, and Cy5-labelled secondary goat antihuman IgG that recognizes IgG from either patient or healthy control bound to the antigens on the arrays. After quantification of the fluorescent signals, statistical analysis is applied to the dataset of dye ratios and then further validated with an independent set of patients and controls (Chatterjee *et al*., 2006; Lin *et al*., 2007) using immunoassays on the antigen microarrays to validate the biomarkers as a diagnostic predictor of cancer. This approach has been adopted by others in the field of antigen biomarkers (Wang *et al*., 2005; Zhong *et al*., 2006). The strength of this approach is the development of large panels of biomarkers from the antigen space within the human serum proteome that are less sensitive to interpatient variations in the population. This methodology is capable of accurate detection of antibodies in the sera of test subjects. Like SEREX, a limitation is that the phage display technology cannot identify cancer-specific post-translational modifications such as glycosylation. Therefore, the serum antibody repertoire that this technology identifies is limited to amino acid epitopes of TAAs.

2.3 Metabolites and Metabolomics

Metabolomics is the global analysis of the small molecule metabolites produced by normal or abnormal cellular processes. In general, metabolomics is focussed on chemical profiles using a variety of analytical technologies. The separation technologies involve gas chromatography, HPLC and capillary electrophoresis, with detection and identification employing mass spectroscopy and nuclear magnetic resonance (NMR) spectroscopy. The human metabolome, however, cannot be defined by any single analytical method (Dunn and Ellis, 2005). There appear to be greater than 2000 metabolites that are accessible using current technologies, which results in a smaller range of potential molecular targets than genomics, transcriptomics or proteomics (Wishart *et al*., 2007). Metabolic patterns and biomarker classifiers for tumour staging and stratification have been developed for breast cancer (Bathen *et al*., 2007), prostate cancer (Cheng *et al*., 2005; Berger *et al*., 2007) and renal cell carcinoma (Sullentrop *et al*., 2002). Although in its infancy and not yet applied to clinical practice, the field of metabolomics should provide a readily accessible and easily measured set of analytes for the classification of cancers for early detection and prognostication. Given the well-known changes in the glycolytic pathways, apoptosis and phosphometabolic changes that occur upon carcinogenic progression, the changes in adenine nucleotide metabolic profiles provides a rich source for biomarker discovery (Mazurek and Eigenbrodt, 2003; Mazurek *et al*., 2002). The integration of metabolomics with other 'omics'-related technologies may provide more specific classifiers of alterations related to cancer stage, grade, response to therapy, and prognosis (Chung and Griffiths, 2007; Erb *et al*., 2008; Kim *et al*., 2008; Tomlins *et al*., 2006; Bathen *et al*., 2007).

3 BIOMARKERS FOR RISK PREDICTION

Cancer is a disease of somatic cell genetic damage resulting from the failure to recognize and repair mutations in DNA. Many germline mutations that predispose individuals and their relatives to cancer occur in genes that function in maintaining the integrity of the genome. The genes often code for proteins that are involved in DNA damage recognition or repair and also in genes involved in cell cycle checkpoint control. Those individuals born with one mutant allele of such genes are more prone to additional DNA damage and genomic instability and therefore accumulate these events leading to carcinogenic conversion of cells. Frequently the remaining wild-type allele of the cancer-predisposing gene is lost in the tumour cells. Evaluating the likelihood of developing cancer through these genomic biomarkers of risk is usually performed by DNA sequencing of candidate genes. Examples of such genes are shown in **Table 1**.

Genetic risk factors occur as mutations in DNA-repair and cell-cycle-control genes, leading to somatic genetic mutations and cancer. However, less than 10% of cancers can be traced to such Mendelian inheritance. Studies of genes responsible for this enhanced risk of developing certain cancers have led to a series of genetic biomarkers as germline mutations in cancer-causing genes (**Table 1**). The majority of inherited cancer syndromes result in particular types of cancers. These cancers are the indirect result of inherited germline mutations, due to the failure to repair somatic mutations in precursor cells leading to cancer development. The patterns of certain

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Table 1 Germline mutations in hereditary cancer syndromes: cancer-causing genes as biomarkers of risk

Syndrome	Germline mutation gene name (chromosomal locus)	Cancer phenotypes
Familial breast and ovarian cancer syndromes	*BRCA1* (17q21)	Breast cancer; ovarian cancer; colon cancer; prostate cancer
	BRCA2 (13q12.3)	Breast cancer; ovarian cancer; melanoma skin cancer; pancreatic cancer
Familial colon cancer syndromes:		
Familial adenomatous polyposis	*APC* (5q21–q22)	Intestinal polyps, osteomas, fibromas and sebaceous cysts; adenocarcinoma of the colon and rectum
Hereditary nonpolyposis colon cancer	*MLH1* (3p21.3) *MLH3* (14q24.3) *MSH2* (2p22–p21) *MSH6* (2p16) *PMS2* (7p22)	Intestinal polyps; adenocarcinoma of the colon and rectum; endometrial, ovarian, brain, skin and stomach cancers
Peutz-Jeghers syndrome	*STK11 (*19p13.3)	Intestinal hamartomatous polyps; mucocutaneous melanocytic macules; intestinal polyps; intestinal cancer
Neurofibromatosis Type I	*NF1* (17q11.2)	Malignant peripheral nerve sheath tumour; pheochromocytoma; meningioma; glioma; acoustic neuroma; optic neuroma; mental retardation; hypertension; hypoglycaemia
Li-Fraumeni syndrome	*p53* (17p13.1) *CHK2* (22q12.1)	Adrenal cortical carcinoma, brain tumours; breast adenocarcinoma; osteogenic sarcoma; leukaemia; lymphoma; rhabdomyosarcoma; sarcoma
Multiple endocrine neoplasia I	*Menin* (11q13)	Tumours of pancreatic islet cell, parathyroid, anterior pituitary and posterior pituitary; gastrinomas; insulinomas
Multiple endocrine neoplasia II	*RET* (10q11.2) *CDKN1B* (12p13.1)	Thyroid carcinomas, pheochromocytomas
von Lippel-Hindau disease	*VHL* (3p26–p25)	Retinal and central nervous system haemangioblastomas; pheochromocytomas; cysts of the pancreas and kidneys; renal cysts progressing to carcinomas
Familial retinoblastoma	*Rb1* (13q14.1–q14.2)	Retinoblastoma; osteogenic sarcoma; breast adenocarcinoma; bladder cancer

cancers within families with inherited predisposition to cancer will trigger genetics professionals to determine the appropriate genes for clinical testing. The clarity of these observations and determining the gene that should be tested is often complicated by the incomplete penetrance of these genetic factors, meaning that not all individuals who carry a germline mutation in a cancer-predisposing gene will develop cancer.

Genetic testing is generally performed on peripheral blood lymphocyte DNA because large amounts of DNA can be obtained relatively noninvasively. The mutations when identified are in one of the two alleles of that gene, and often the wild-type allele undergoes loss of heterozygosity in the tumour tissue, indicating a total loss of function in the tumours. Those individuals who test positive for a germline mutation in a cancer-predisposing gene are in need of enhanced medical surveillance and screening using biomarkers for the early detection of cancer.

The identification of genetic alterations that increase the risk for developing cancer is useful for predisposition testing with mutational screening and counselling as part of the established subspecialty of cancer genetics. Despite the promise of this predisposition genetic testing for inherited cancer risk, this information could lead to unforeseen negative consequences unless genetic analysis data are used properly. Not all healthcare professionals are knowledgeable in the interpretation of genetic testing

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results for cancer susceptibility. The complexities include interpreting family history data and choosing the correct gene to be sequenced.

In addition to well-executed molecular technologies for DNA testing, an accurate and detailed family history is the essential risk assessment tool in the evaluation of a hereditary cancer syndrome. Family history data include details of all cancers on both maternal and paternal sides within the family pedigree. Cancer-free relatives are included, because the frequency of both normal and affected relatives within a pedigree is a key factor in the evaluation of inheritance patterns. Family history data should be confirmed with medical and/or death records.

4 BIOMARKERS FOR EARLY DETECTION SCREENING

In order to detect cancer at its earliest stages when it is most curable, there is a need for biomarkers to detect individuals harbouring occult cancers. The expectation is that early detection tests will be pursued with particular targeted subsets of patients in mind who need more intensive diagnostic imaging after a positive test. One approach for accomplishing this objective is to detect molecular fingerprints of an organ in the process of developing a cancer and then to use the biomarkers to define a suitable treatment prior to a significant tumour burden. Effective biomarker identification depends on multiple levels of study design, each of which must be optimized to ensure the utility of a biomarker or panel of biomarkers specific to this clinical application, early detection. The success of biomarkers for the early detection of cancer is measured by the fact that they should not only detect disease early but also reduce the mortality from that cancer.

The choice of cellular tissue or body fluid for the early detection of cancer is clearly dictated by the breadth of the assay sought. A biomarker that can detect one cancer and rule out others will necessarily require a systemic body fluid such as plasma, serum or urine. Alternatively, if a biomarker is to have high sensitivity for a single cancer such breast cancer, then breast nipple aspirate fluid or ductal lavage fluid may provide a very specific matrix in which one would be less likely to find false positive results from other tissues. Likewise a test for a bladder cancer biomarker may reach a higher specificity if measured in urine rather than blood. Whether testing these matrices for early detection, diagnosis or prognosis of cancer, a variety of types of analytes and technologies are available, such as proteins, nucleic acids or metabolites.

4.1 Technological Issues

Noninvasive diagnostic approaches to detect early stage cancers may provide the clinician with evidence of cancer, but the criteria for a useful test depend on sensitivity and/or specificity of the test and how the results will affect the pathway of clinical intervention. The consequence of a positive result from an *in vitro* diagnostic cancer test may involve relatively invasive procedures to establish a true cancer diagnosis. Therefore the *in vitro* diagnostic test must be both specific, that is, rarely produce false positive results due to unrelated conditions, and relatively sensitive, that is, rarely produce false negative results, and only then such screening tests will offer life-saving potential for early detection and possibly a mechanism for personalized therapeutics using disease-related targets.

Various scenarios of diagnostic tests for the early detection of cancer have emerged using genomic, proteomic and metabolomic technologies. Because screening tests might involve panels of analytes, these technologies have opened a 'Pandora's Box' of questions on the steps in development of multianalyte clinical tests. Developing the reagents into a format that is amenable to a clinical laboratory is a rather daunting barrier to such a test. Even the development of a generic approach to measure dozens of analytes will require solutions to some unique optimization challenges, not the least of which is the informatics leading to a clinical decision. To ensure tests work properly during research and development, during production and in customer laboratories, kits require controls and calibrators for each analyte. A major barrier in multianalyte diagnostics will be the large number of controls and standards required for such a test. For nucleic acids-based tests these controls are more easily prepared; controls are particularly difficult for protein-based diagnostics. Controls for this approach may consist of cancer cell extracts, cancer patients' sera or other body fluids or alternatively panels of human recombinant proteins for each biomarker. Because patient material containing these biomarkers could be in short supply, the alternative approach of using recombinant proteins for each biomarker is preferable. However, preparing each of the recombinant proteins as controls, although feasible, is clearly a substantial technical challenge in that recombinant proteins will be missing critical cancer-specific post-translational modifications such as glycosylation or phosphorylation. A set of individual standards for multianalyte assays may not be possible, whether they are serum autoantibodies, circulating proteins or plasma genomic DNA or RNA targets. The danger is that the absence of true molecular standard calibrators may result in a test difficult to implement in clinical laboratories.

The true utility of a diagnostic test requires that the appearance of a positive test result must precede the development of late-stage or incurable cancer. However,

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there is no established method to define early detection. What constitutes early detection may vary among the different types of cancer. Diagnostics for any particular cancer may require either high specificity or high sensitivity, depending on the clinical pathway beyond the test. The prevalence of early stage disease and the costs of false positive and false negative results must be weighed against the benefits of early diagnosis. In balancing specificity vs. sensitivity, the intended goal of the diagnostic application must be part of the study design from discovery to implementation. An ideal screening test would have very high sensitivity, identifying nearly all individuals with disease. To accomplish this, the test may falsely identify many individuals who do not have cancer, resulting in lower specificity for the panel of biomarkers, leading to unnecessary, invasive, medical testing. Thus it is important that *in vitro* screening tests have both high sensitivity and high specificity. If test sensitivity is valued over test specificity, significant misclassification in the case of low-prevalence cancers may result in unnecessary and expensive medical follow-up. Conversely, valuing specificity over sensitivity may fail to detect cases of cancer. In light of all of these factors, a 'one-size-fits-all' approach to diagnostic standards for a multianalyte early detection screening test for cancer may be impossible. The balancing of specificity and sensitivity will depend on the nature of the clinical follow-up for each cancer. A high specificity imaging test following a high sensitivity biomarker screening test may be the best compromise solution.

4.2 Classical Screening Tests for Cancer

Carcinoembryonic antigen (CEA), first described in 1965 (Gold and Freedman, 1965), was among the first identified tumour biomarkers. CEA is a biomarker that is elevated in a variety of cancers including colorectal, breast, lung or pancreatic cancer. CEA in breast cancer detection has low diagnostic sensitivity and specificity (Hou *et al*., 1999; Clinton *et al*., 2003), with frequent false positives in normal individuals. High levels of CEA occur in many other cancers as well (Munjal *et al*., 1984). CEA is a better biomarker of ductal carcinomas than lobular carcinomas of the breast (Kuhajda *et al*., 1983). Because CEA can be found in the serum of patients with ductal carcinoma in situ, CEA may provide a biomarker indicative of an early stage of the cancer (De Potter *et al*., 1988) Mucins such as MUC1, or cancer antigen (CA) 15.3 and CA 27.29, MUC16 (CA 125) (Gion *et al*., 1999; Klee and Schreiber, 2004) and CA 549 (Dnistrian *et al*., 1991) have low sensitivity for presymptomatic diagnosis of breast cancer (Zamagni *et al*., 1992). With the advent of mass-spectroscopic protein identification in complex matrices such as serum and plasma, new biomarker proteins have emerged, including heat shock protein 27 (HSP27) and the transcriptional regulator 14-3-3 sigma, and the derivatives of the complement component C3a (Rui *et al*., 2003; Li *et al*., 2002; 2005). None of these biomarkers have become useful tools for breast cancer early detection.

A variety of ovarian tumour markers have been studied, and the most extensively investigated of these is CA125. This antigen was first recognized in 1981, using a murine monoclonal antibody developed in response to immunological challenge with an ovarian cancer cell line (Bast *et al*., 1981). CA125 levels were found to be increased in 50% of Stage I and 90% of Stage II ovarian cancers (Zurawski *et al*., 1988). Although sensitivity for Stage I disease using a simple cut-off of 30 U ml^{-1} was limited, it was apparent that CA125 was capable of detecting ovarian cancer preclinically. The use of a combination of markers to increase sensitivity and specificity has been extensively investigated and the marker that appears to exhibit the most complementarity to CA125 is OVX1, a monoclonal antibody developed using sequential immunization with three different ovarian cancer cell lines (Xu *et al*., 1991). OVX1 has been found to be elevated in 70% of patients with clinically evident ovarian cancer. In addition, 59% of patients with normal CA125 levels had increased OVX1, suggesting complementarity between the two markers. Although these results indicate improvement in sensitivity, preliminary data from different laboratories suggest that OVX1 may be unstable unless serum is rapidly separated, which could complicate its use in population screening if samples are sent by post. Another serum marker which exhibits complementarity to CA125 is macrophage colony stimulating factor (M-CSF). Among 25 patients with clinically evident tumours and a negative CA125, 56% had an elevated M-CSF serum level (Xu *et al*., 1991). A variety of other tumour markers have also been studied in ovarian cancer. Many of these have been shown to be of insufficient sensitivity or specificity regarding epithelial tumours. Among them, CEA has been reported to be elevated in 30–65% of epithelial tumours, mainly in patients with advanced stage disease (Schwartz *et al*., 1987). Cancer antigen 19-9 (CA19-9) is another carbohydrate antigen that can be found elevated in only 17–25% of patients with epithelial malignancies (Schwartz *et al*., 1987). Lipid-associated sialic acid (LSA) can be detected in serum of about 60% of patients with advanced stage disease (Patsner *et al*., 1988). The interleukins, IL-6 and IL-10, have been shown to be present in high levels in the ascites and serum of women with advanced stage epithelial cancer (Berek *et al*., 1991; Gotlieb *et al*., 1992). Measurement of serum levels of tumour-associated antigen CA125 (Bast *et al*., 1983), in conjunction with ultrasound screening as a second-line test, confers high specificity (Einhorn *et al*., 1992) but detects only approximately one half of early stage cases (Jacobs and Bast, 1989). Use of

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multiple serum markers may provide a more sensitive test. Complementarity has been demonstrated between CA125 and two novel markers. M-CSF (Kacinski and Bloodgood, 1989) and OVX1 (Xu *et al*., 1991) identify a percentage of patients with persistent ovarian cancer who had normal CA125 levels prior to second-look surgical staging procedures. Other new markers, such as tetranectin (TN) or cancer-associated serum antigen (CASA), have not provided additional discriminative value (Hogdall *et al*., 2000). Serum levels of epidermal growth factor (EGF) and its receptor are significantly different between ovarian cancer patients and healthy women and this may provide a potential diagnostic and/or prognostic marker useful for the management of recurrence and late-stage cancer (Baron *et al*., 1999). Homeobox protein B7 (HOXB7) was recently found to be a tumour antigen whose up-regulated expression could play a role in promoting growth of ovarian carcinomas (Naora *et al*., 2001). Urban *et al*. have characterized the behaviour of five serum tumour markers in a large cohort of healthy women (Urban *et al*., 2000). Serial measurements of CA125, HER2/neu, urinary gonadotrophin peptide, lipid-associated sialic acid and Dianon marker 70/K during six years of follow-up of 1257 healthy women at high risk of ovarian cancer showed that the individual-specific tumour markers behaved independently with substantial heterogeneity among high-risk but cancer-free women. None of these research findings has yet translated into a reliable early detection clinical test.

5 MOLECULAR DIAGNOSTICS FOR CANCER

Large-scale gene expression profiling holds tremendous promise for developing molecular portraits of subtypes of human tumours with different clinical outcomes that could not be subclassified upon initial clinical presentation. One of the possible problems in performing such studies is the number of samples as compared to the number of genes tested, the so-called 'curse of dimensionality'. Without an independent validation set, studies with many more genes than samples may result in over-fitting of the data, thus leading to a gene panel that is only accurate on the discovery sample cohort or at the one site of sample collection. Another issue is that there may be small numbers of genes whose expression discriminates cancer subtypes but they may not be drivers of the carcinogenic process and therefore may provide little survival information. Another not surprising issue is that independent studies can identify different panels of genes with similar discriminatory specificity and power. This is likely to be due to the technical differences such as the type of microarray used or the algorithm that identified the genetic classifier, and because there is redundant information among the individual attributes of gene expression profiles. These points are illustrated below.

One of the earliest applications of gene expression profiling to finding biomarkers for clinical oncology was a project on diffuse large-B-cell lymphoma. The International Prognostic Index (IPI), was established as a predictor of outcome in diffuse large-B-cell lymphoma based on clinical characteristics of age, tumour stage, serum lactate dehydrogenase levels, performance status and number of extranodal disease sites (Vose, 1998). However, the outcome in patients with diffuse large-B-cell lymphoma who have identical IPI values varies considerably. Gene expression profiling was pursued to develop risk-adjusted classifiers for possible therapeutic outcomes for diffuse large-B-cell lymphoma suitable for clinical practice. One group identified a panel of genes characteristic of normal germinal-centre B cells, whose expression when elevated indicated a significantly longer survival among patients with diffuse large-B-cell lymphoma than patients whose tumours had low levels of expression of these same genes (Alizadeh *et al*., 2000). Another group identified a panel of 13 genes that was independent of the IPI. Only 3 of these 13 genes were present in the data analysed by Alizadeh *et al*. (2000), and of those 3, 2 were associated with survival (Shipp *et al*., 2002). Another group used supervised analysis of gene-array data from 160 patients with diffuse large-B-cell lymphoma and developed a 17-gene predictive model which when applied to a set of similar lymphomas from 80 other patients could predict survival (Rosenwald *et al*., 2002). However, there was no overlap with either of the other two classifiers or survival. Another report identified 36 genes that predicted survival in diffuse large-B-cell lymphoma previously discovered from either individual studies or from large published microarray datasets. Using RT-PCR they ranked the genes for their predictive power for longer overall survival. Using a weak z value of ± 1.5 (p-value = 0.13), they found that 6 genes (*LMO2, BCL6, FN1, CCND2, SCYA3* and *BCL2)* in a univariate analysis provided a predictor of survival. This 6-gene model was as accurate as the 17-gene model of Rosenwald *et al*. and was independent of the IPI values in predicting outcome (Lossos *et al*., 2004). However, as yet there is no test in clinical practice derived from these studies.

6 BIOMARKERS FOR PROGRESSION AND SELECTION OF CANCER TREATMENT

In the area of cancer prognosis, the challenge is to weigh the impact of a number of variables, including systemic versus tissue biomarkers that can be used to predict

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outcome in patients with early-stage cancer. The expectation is that there is a subset of individuals who may not benefit from chemotherapy treatments and might be treated surgically with watchful waiting as a follow-up to surgery. Classical prognostic testing based on tumour tissue analyses was inexpensive and easy to perform. Some biological assays such as proliferative index, gene amplification and overexpression of oncogenes or their proteins are being employed in certain cancers as predictors of outcome. These tests have entered clinical practice, but far more complex test data are now appearing, often employing panels of genes whose expression levels are incorporated with the use of algorithms into the test reports. Not only can such tests predict outcome (Paik *et al*., 2004; 2006; Wolf *et al*., 2008; van 't Veer *et al*., 2000; van de Vijver *et al*., 2002; Sotiriou *et al*., 2006), but also can identify the tissue of origin in metastatic cancers of unknown primary (Ma *et al*., 2006; Dumur *et al*., 2008). A clinical practitioner will have to identify the appropriate test, or tests, that can predict when a therapeutic approach can improve the patient's outcome, or determine whether more classical diagnostic information alone is more clinically useful. A major goal of prognostic testing in cancer is to predict which patients will develop a recurrence of cancer and need treatment, and which will not require treatment. The prognostic value of a test is usually expressed in terms of relative risk (RR), which is the ratio of the risk of cancer recurrence in patients who have a positive test to the risk in those who test negative.

6.1 Biomarkers of Prognosis

With the advent of whole genome expression profiling, the opportunity to identify features of the transcriptome that indicated a good or poor survival became feasible. Likewise, proteomic profiling of primary tumour tissues could also provide a rich source of protein biomarkers that may distinguish differences in survival, but the impact of proteomic biomarkers is yet to be realized. Two gene-expression-based tests have been developed to determine the risk of recurrence in breast cancer patients with Stage I or II node-negative breast cancer. The first test, Oncotype DX™, is an RT-PCR-based assay performed on RNA extracted from paraffin-embedded tumour tissue. This test determines the expression levels of 21 genes, 16 of which are cancer-related genes and 5 are control reference genes. The Oncotype DX™ test specifically was developed to predict the risk of breast cancer recurrence in women with oestrogen receptor (ER)-positive, node-negative breast cancer, using the readily-available paraffin-embedded tumour tissue. The results are used to calculate a recurrence score that predicts the likelihood of cancer recurrence in patients treated with tamoxifen. Women with a low recurrence score need only treatment with tamoxifen, and those women with an intermediate- and high-risk score require additional treatment such as adjuvant chemotherapy.

The second multianalyte test for breast cancer recurrence is called MammaPrint®. Biomarkers of breast cancer subtypes were identified using patterns of gene expression measured by microarrays (Sørlie *et al*., 2001; Sorlie *et al*., 2003). Using hierarchical clustering of gene expression, large gene sets were able to identify five subtypes of breast cancer including basal-like, HER2-overexpressing subtype, two types of luminal cells, and a normal breast tissue-like subgroup. The basal-cell-type was often seen in carriers of BRCA1 mutations (Sorlie *et al*., 2003). These molecular classifiers were able to show that patients with the basal and HER2 subtypes had a good response to therapy. The genes associated with a complete response to chemotherapy in the basal subtype were different from those that defined the HER2-overexpressing subtype (Rouzier *et al*., 2005). This work has led to the 70-gene panel in the MammaPrint® technology. MammaPrint® involves an oligonucleotide microarray assay to analyse the expression of a panel of 70 genes, and is performed on fresh-frozen tumour tissue. MammaPrint® was developed to address early-stage breast cancers so as to evaluate them as having a high or low risk of future metastases. Those women categorized as having a high risk of metastasis are treated with more aggressive chemotherapy, while those categorized as low risk are spared unnecessary chemotherapy. The key to the successful development of these two tests was the establishment of a clear target population who would be candidates for each test, and focussing on establishing the parameters of the algorithms for each test on that population. Other prognostic tests for breast cancer are being developed using immunohistochemistry (IHC) or gene expression, but only MammaPrint® is currently approved by the US Food and Drug Administration (FDA).

6.2 Metastatic Cancer from Unknown Primary Tumours

About 10–15% of cancer patients present with metastases without an apparent site of origin at presentation with the cancer. In one-third of these patients no primary tumour can be identified histologically, and they are designated as having a cancer of unknown primary origin. This situation leaves tremendous ambiguity and often a failure to apply appropriate systemic combination chemotherapy. Clinical evaluation of metastatic lesions of unknown origin is unsuccessful in up to 60% of patients. In response to this clinical need, tissue-of-origin classification of uncertain

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primary cancers was developed using microarray-based gene expression profiling of a 1668-gene probe set to evaluate the similarity of tumour specimens to 15 known tissue profiles (Dumur *et al*., 2008). A molecular similarity profile of each test-tumour specimen's expression pattern is compared to 15 patterns from unique tissue types including bladder, breast, colorectal, gastric, germ cell, hepatocellular, kidney, non-small-cell lung, non-Hodgkin's lymphoma, melanoma, ovarian, pancreatic, prostate, soft tissue sarcoma, and thyroid. For each test specimen, an algorithm reduces dimensionality of the expression data into 15 separate 'Similarity Scores', one for each tissue type. This test, referred to as Pathwork^{DX}, is approved by the FDA for the application of tissue-of-origin testing of metastases from unknown primary cancers.

6.3 Circulating Tumour Cells

The presence of circulating tumour cells (CTCs) in the blood of cancer patients was recognized in the 1950s, but only recently has technology become available to exploit them as biomarkers for the detection or prognosis of cancer (Roberts *et al*., 1957). The detection of CTCs has been commercially developed in a device and can predict which women with metastatic breast cancer will have a better progression-free period and overall survival (Cristofanilli *et al*., 2005). This technology has also been applied to the prognosis of prostate, colorectal and gastrointestinal cancers (de Bono *et al*., 2008; Cohen *et al*., 2008; Hiraiwa *et al*., 2008). Molecular events driving cancer development, and likewise therapeutic targets such as EGF-receptor mutations, are detectable in CTCs (Maheswaran *et al*., 2008a), and CTCs provide a novel and very specific diagnostic tool for the future.

6.4 Biomarkers for Treatment Selection

Targeted anticancer therapies have resulted from years of research focussed on understanding the changes in molecular mechanisms and biomarker differences between cancer cells and normal cells. Classical chemotherapies have killed rapidly dividing cells, a feature of cancer cells. Because certain normal cells proliferate rapidly, chemotherapies result in significant side effects. By targeting therapy to a molecular mechanism known to differ between normal and cancer cells, targeted therapies have been developed that attack the cancer cells without affecting the normal cells, thus reducing side effects. Targeted therapies by definition provide molecular biomarkers of efficacy, that is, the mechanisms being targeted. Targeted therapies focus on the functions of the cancer cell predefined as dysregulated, thereby providing biomarkers for the choice of the appropriate targeted therapy. These molecules get into the cell and disrupt the particular molecular function of the cells that caused them to be neoplastic.

Once individuals are detected with early disease and potentially gene profiling can indicate the dysregulation of particular pathways, there is a need to identify subsets of patients for specific targeted cancer therapy based on their molecular profiling. Because, even within a single cancer histologic type, there are a variety of responses to therapy; identifying one or more critical biomolecules that drive the cancer phenotype in a patient will provide a personalized targeted therapeutic approach. These molecular targets for cancer therapy are the products of years of research. Given the rate of identification of candidate genes, the appearance of cancer-specific mutations and potential therapeutic targets will rapidly expand due to emergence of next-generation DNA sequencing capabilities as applied to tumour tissues (Scott *et al*., 2005; Cox *et al*., 2005; Davies *et al*., 2005; Stephens *et al*., 2005; Edkins *et al*., 2006; Ikediobi *et al*., 2006; Greenman *et al*., 2007; Campbell *et al*., 2008). The selection of target-positive patient populations provides efficiencies in more rapid assessment of drug performance. Also smaller patient populations can provide rapid objective evaluation of treatment outcomes.

6.4.1 EGF-Receptor Family

Biomarkers for targeted or personalized cancer treatment using small molecule therapies and antibody-based therapies by necessity requires years of research and development. For example, overexpression of the HER2 growth factor receptor protein, often resulting from gene amplification, occurs in approximately 25% of breast cancer patients. HER2-overexpressing breast cancers are more aggressive, often associated with shortened disease-free and poor overall survival (Slamon *et al*., 1987; 1989; Gusterson *et al*., 1992; Andrulis *et al*., 1998). The therapeutic monoclonal antibody trastuzumab (Herceptin®) was directed against an epitope on the extracellular domain of the HER2 growth factor receptor protein. This agent has clinical efficacy against HER2-overexpressing breast cancers used as a single agent or when used in combination with chemotherapy (Cobleigh *et al*., 1999; Slamon *et al*., 2001; Vogel *et al*., 2002; Seidman *et al*., 2002). An early obstacle to adoption of HER2 as a diagnostic target and ultimately as a molecular target of therapy was the development of reliable tests for its gene amplification and protein overexpression (Slamon and Clark, 1988; Press *et al*., 2002). Clear HER2 biomarker guidelines are now available for using targeted therapies

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in breast cancer (Wolff *et al*., 2007). Trastuzumab, the commercial name for this monoclonal antibody targeting HER2, is now the first-line treatment of patients with HER2-positive metastatic breast cancer. HER2 can also be targeted using small-molecule inhibitors of its tyrosine kinase activity. A critical issue is now the optimal selection of patients for each type of therapy: monoclonal antibody versus small-molecule inhibitor. The monoclonal antibodies do not cross the blood–brain barrier efficiently, and although the small-molecule inhibitors are able to cross the blood–brain barrier, they have a shorter half-life in the blood (Imai and Takaoka, 2006). Lapatinib (commercial name Tyverb®) is a dual specificity tyrosine kinase inhibitor of both EGF receptor and HER2, that inhibits the autokinase activity of these proteins by binding to the adenosine triphosphate (ATP)-binding sites (Nelson and Dolder, 2006). Lapatinib in combination with capecitabine in women with HER2-positive metastatic breast cancer has significant efficacy in metastatic breast cancers that progressed after failure on trastuzumab-based therapy plus an anthracycline and a taxane (Geyer *et al*., 2006). Unfortunately there is no clear set of serum biomarkers of response to trastuzumab plus chemotherapy to indicate when to change to the small-molecule inhibitor plus capecitabine.

There are a series of approved therapies targeting the epidermal growth factor receptor (EGFR) protein. Rather than simple overexpression of the wild-type growth factor receptor, the biomarker of efficacy for these agents is EGFR kinase domain mutation at amino acid 858, changing a leucine to an arginine (L858R), or less frequently one of multiple possible in-frame deletions in exon 19 (Bean *et al*., 2008; Godin-Heymann *et al*., 2008; Molina-Vila *et al*., 2008; Maheswaran *et al*., 2008b; Yamamoto *et al*., 2009). Tumours with these mutational biomarkers of efficacy are sensitive initially, but acquire resistance to gefitinib and erlotinib second-site mutations in EGFR. Somatic mutations in the tyrosine kinase domain of the *EGFR* gene are associated with sensitivity of lung adenocarcinomas to the EGFR tyrosine kinase inhibitors, gefitinib and erlotinib. Acquired drug resistance is frequently (50%) associated with acquisition of a new biomarker of resistance with a secondary somatic missense mutation substituting a methionine for threonine at amino acid position 790 (T790M).

6.4.2 bcr-abl Oncogene

The *BCR-ABL* oncogene is the result of a translocation of DNA sequences from human chromosomes 9 and 22, often called the Philadelphia Chromosome (Nowell and Hungerford, 1964; Nowell, 2007), which forms a new fusion transcript producing a fusion protein from *BCR* (break point cluster) and *ABL* (Abelson leukaemia viral oncogene homolog protein kinase) genetic sequences (Heisterkamp *et al*., 1985). This translocation and the BCR-ABL tyrosine kinase are present in 95% of patients with chronic myelogenous leukaemia (CML), and have been implicated as the cause of this disease. A chemical compound (Druker *et al*., 1996), designed to inhibit the ABL protein tyrosine kinase, was evaluated for its effects on cells containing the BCR-ABL fusion protein. Cellular proliferation and tumour formation by BCR-ABL-expressing cells were specifically inhibited by this compound. This compound (STI 571 or CGP 57148B (O'Dwyer and Druker, 2001), commercially known as Gleevec or Imatinib mesylate) was developed into a highly selective treatment of BCR-ABL-positive CMLs (Beran *et al*., 1998; Dan *et al*., 1998; Druker *et al*., 2001; Gora-Tybor *et al*., 1998; le Coutre *et al*., 1999; Sausville, 1999). Mutation in the kinase domain of the *BCR-ABL* gene alters drug binding, renders CML patients Gleevec-resistant and as such is a biomarker of resistance (O'Hare *et al*., 2007a). In other patients, increased BCR-ABL expression is an excellent biomarker of Gleevec resistance (Mahon *et al*., 2000). Gleevec is also relatively nontoxic and effective in gastrointestinal stromal tumours with activation of the c-Kit receptor (cell surface receptor that binds to stem cell factor), indicating that this receptor is a biomarker of efficacy for this drug (DeMatteo, 2002; Heinrich *et al*., 2000; Joensuu *et al*., 2002; Patel, 2002; Tuveson *et al*., 2001).

Additional oncogene biomarkers resulting from chromosomal fusions of tyrosine kinases, such as ABL and PDGFRA/B (platelet-derived growth factor receptor, alpha polypeptide/beta polypeptide), are associated with leukaemias resembling CML, and have potential for targeted therapeutics and personalized medicine. Gleevec has remarkably improved the outcome of BCR-ABL-containing CML, but because there are some CML patients with a poor response to Gleevec, biomarkers of response beyond detection of the *BCR-ABL* fusion transcript are necessary. Thus cytogenetic and molecular testing for BCR-ABL and other biomarkers during treatment provides important prognostic information (O'Hare *et al*., 2007b; Ross and Hughes, 2008; Wu *et al*., 2008).

6.4.3 Oestrogen and Progesterone Receptors

The expression of oestrogen and progesterone receptors has long been known as an excellent prognostic biomarker for breast cancer (McGuire, 1973). Numerous biochemical studies preceded its implementation as a biomarker and therapeutic target (Furr and Jordan, 1984; Jordan, 1984; Tate and Jordan, 1984; Tormey and Jordan, 1984). As a biomarker, oestrogen receptor positivity in breast cancers indicated that the cancer may respond to chemotherapy and chemoprevention with selective oestrogen receptor antagonists tamoxifen and raloxifene

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(Cummings *et al*., 1999; Fisher *et al*., 1989; 1998; Vogel *et al*., 2006), or respond in a postsurgical therapeutic setting. In women with advanced metastatic cancer, inhibitors of the enzyme aromatase, which is required for oestrogen biosynthesis, can increase the time to progression as compared to tamoxifen (Carpenter, 2008; Seo *et al*., 2008; Paridaens *et al*., 2008). In addition, most clinical trials revealed a survival advantage for treatment with aromatase inhibitors in early stage oestrogen receptor-positive breast cancer (Koeberle and Thuerlimann, 2007).

In summary, the use of biomarkers brings the era of personalized medicine for choice of cancer therapy to a new reality. It acknowledges the limitations of one-size-fits-all chemotherapy, and provides a rational basis for individualized cancer care.

7 SURROGATE BIOMARKERS OF TREATMENT OUTCOME AND RELAPSE

One area of biomarker use is the surveillance of cancer after treatment. The translocations associated with leukaemias and lymphomas are particularly useful molecular markers to determine the presence of minimal residual disease, and quantitative PCR assays of DNA can be used to determine whether the tumour burden is increasing. However, for most solid tumours there are few blood-based biomarkers of relapse. Some of the clinically utilized biomarkers are listed below, including prostate specific antigen (PSA), CA125, CEA, nuclear matrix protein 22 (NMP22) and CA19-9. For additional biomarkers of relapse see Nass and Moses (2007).

7.1 PSA

PSA is naturally secreted from normal prostate cells, but higher levels of PSA in serum have a correlation with the existence of prostate cancer. PSA is probably the only serum biomarker used in a primary-care setting. PSA was originally approved for the recurrence of prostate cancer but was used off-label so frequently that sufficient data arose to indicate its use presymptomatically. Other conditions can cause increased levels of PSA. Benign prostatitis, infections of the prostate gland, exercise that results in irritation of the surrounding tissues, and digital prostate examination by a doctor can result in a rise in PSA. The rate of PSA elevation and the fraction of free PSA generally determine the clinical pathway. PSA elevation above normal will often result in additional diagnostic testing to identify prostate cancer at an early stage. The clinical follow-up is usually digital rectal examination of the prostate, prostate biopsies and imaging. Even with such comprehensive clinical follow-up the majority of treated cases would probably result is no clinical cancer (Thompson and Ankerst, 2007). The high rates of over-diagnosis are evident from the fact that the lifetime chance of prostate cancer diagnosis is 18% but the likelihood of death from prostate cancer is only 3%. This low specificity is causing a considerable amount of unnecessary treatment, and better biomarkers for the presymptomatic detection of prostate cancer need to be found. Chromosomal translocations also involving ETS (avian erythroblastosis oncogene homolog)-family transcription factors have been observed in prostate cancer and should provide improvements in the diagnostic specificity for that disease using PSA (Tomlins *et al*., 2005; Rouzier *et al*., 2008).

7.2 Cancer Antigen 125 (CA125)

CA125, also known as MUC16, is a protein that binds a monoclonal antibody used for its testing. CA125 is a useful indicator of ovarian cancer recurrence, but as a biomarker for presymptomatic detection of ovarian cancer it has low sensitivity and low specificity. CA125 levels were found to be elevated in 50% of Stage I and 90% of Stage II ovarian cancers (Zurawski *et al*., 1988). However, multiple benign diseases, both gynaecological and nongynaecological conditions, can elevate serum levels of CA125 (Jacobs and Bast, 1989; Tuxen *et al*., 1995), and therefore the false-positive rate of CA125 is high. CA125 levels are elevated in people who have pancreatitis, kidney or liver disease, indicating its limited utility as a cancer diagnostic tool. CA125 is a good biomarker of recurrence of a previously treated ovarian cancer.

7.3 Carcinoembryonic Antigen (CEA)

CEA is synthesized during the development of the foetal gut, and is turned on in adult intestinal carcinomas and other cancers. CEA is a biomarker that is elevated in numerous cancers such as colorectal, breast, lung or pancreatic cancer. Although sometimes used as a screening test, there are several sources of false-positive tests, such as smoking. CEA testing after surgery for colon cancer is an effective way of determining the reduction of tumour burden and the recurrence of cancer after postoperative chemotherapy. However, there is no survival advantage of metastatic disease colon cancer between patients who went to a second-look surgery based on elevated CEA levels versus other criteria (Goldstein and Mitchell, 2005).

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7.4 NMP22

NMP22 is a specific nuclear matrix protein involved in DNA synthesis, RNA transcription, the regulation of gene expression, and mitosis. Bladder cancer results in elevation of intracellular NMP22 up to 25-fold, and the protein is shed into the urine. A urine test for NMP22 was approved in a number of formats, first for bladder cancer recurrence and later for presymptomatic testing for bladder cancer. The commercial test was reported to be 68% sensitive and 65% specific (Friedrich *et al*., 2002). Combining NMP22 with a telomerase enzyme assay, called TRAP (telomere repeat amplification protocol), resulted in 100% sensitivity and 96% specificity (Saad *et al*., 2002). However, no combination of biomarkers demonstrated sufficient sensitivity to detection of low stage and grade bladder tumours or carcinoma *in situ* to replace standard urethrocystoscopy (Van der Poel and Debruyne, 2001).

7.5 CA19-9

CA19-9 is a tumour biomarker that, like CEA or CA125, is not sufficiently specific for the presymptomatic detection of gastrointestinal cancers. Non-neoplastic conditions such as gallstones, cirrhosis, pancreatitis and cholecystitis induce elevated levels of CA 19-9. Although CA19-9 was discovered in colorectal cancer patients, CA19-9 has also been detected in the serum of patients with pancreatic, stomach and bile duct cancer (Steinberg *et al*., 1986). Researchers have discovered that, in those who have pancreatic cancer, high levels of CA19-9 are associated with advanced pancreatic cancer. CA19-9 is a useful biomarker to evaluate whether a patient is responding to cancer treatment for bile duct and pancreatic cancers. CA19-9 is also an indicator of recurrence of pancreatic cancer (Safi *et al*., 1998).

7.6 Chromosomal Translocations

Chromosomal translocations, particularly in leukaemias and lymphomas, can be used to determine the presence of residual disease. This approach was first applied to the early relapse or the detection of minimal residual disease in patients having chromosomal translocation for follicular lymphomas, t(14;18)(q32;q21) (Lee *et al*., 1987), allowing the detection of cells with the t(14;18) translocated DNA sequences at 1 cell in 100 000. PCR was also applied to acute T cell leukaemia/lymphoma using the t(10;14)(q24;q11) chromosomal translocation as the biomarker target (Kagan *et al*., 1990). The Philadelphia chromosome is a specific biomarker of CML and has been used to target therapy using the therapeutic tyrosine kinase inhibitor Gleevec. The presence of the translocation protein BCR-ABL-kinase is detected by PCR to monitor response to therapy and the presence of residual disease (Kim *et al*., 2004; Paschka *et al*.,

Table 2 Chromosomal translocations in leukaemias and lymphomas

Genes in translocation	Chromosomal target	Disease	References
IgH–BCL2	t(14;18)(q32;q21)	FL	Yunis *et al*. (1987); Lee *et al*. (1987)
MLL–AF4	t(4;11)(q21;q23)	ALL	Kersey *et al*. (1998); Uckun *et al*. (1998)
E2A–PBX1	t(1;19)(q23;p13)	PreB-ALL	Mellentin *et al*. (1990); Nourse *et al*. (1990); Kamps *et al*. (1990)
BCR–ABL	t(9;22)(q34;q11)	CML (AML)	de Klein *et al*. (1982); Lugo *et al*. (1990)
PML–RARA	t(15;17)(q22;q21)	APL	de The *et al*. (1990); Longo *et al*. (1990)
CBFB–MYH11	(inv)16p13q22	AML	Hébert *et al*. (1994); Poirel *et al*. (1995)
SIL–TAL1	Micro deletion 1p32	Paediatric T-ALL	Brown *et al*. (1990); Bernard *et al*. (1991)
TEL–AML1	t(12;21)(p13;q22)	Paediatric PreB-ALL	Romana *et al*. (1995); Raynaud *et al*. (1996)
AML1–ETO	t(8;21)(q22;q22)	AML	Tobal and Yin (1996); Muto *et al*. (1996)

FL—follicular lymphoma; ALL—acute lymphoblastic leukaemia; PreB-ALL—pre-B cell acute lymphoblastic leukaemia; CML—chronic myelogenous leukaemia; APL—acute promyelocytic leukaemia; T-ALL—T cell acute lymphoblastic leukaemia; AML—acute myeloid leukaemia.

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2003; Merx *et al*., 2002; Press *et al*., 2006). Likewise for follicular lymphoma, a common type of non-Hodgkin Lymphoma, PCR using the characteristic 14:18 translocation is used for diagnosis and the presence of residual disease (Bakhshi *et al*., 1985; Lee *et al*., 1987; Crescenzi *et al*., 1988). Interestingly, patients with evidence of minimal residual disease can remain in remission, which indicates that additional biomarkers are necessary to improve the predictive values of these assays. For follicular lymphoma, further molecular markers such as *ras* oncogene mutations and *p53* mutations may provide additional prognostic value (Hirt *et al*., 2008; Cikota *et al*., 2007).

A summary of leukaemia/lymphoma translocations commonly employed clinically as biomarkers of disease, response to therapy and relapse are shown in **Table 2** (Gabert *et al*., 2003).

Sarcomas are highly variable in cell-type of origin, growth properties and response to chemotherapy. Sarcomas often arise with specific chromosomal translocations that can be used to differentiate various subtypes. For a review of chromosomal biomarkers in sarcomas see Ludwig (2008), Nambiar *et al*. (2008), Riggi and Stamenkovic (2007) and Cooper (1996).

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Ethical Issues in Toxic Chemical Hazard Evaluation, Risk Assessment and Precautionary Communications

Claude Viau

CONTENTS

1 INTRODUCTION

Some key questions that require attention for the ethics of risk assessments are: Who needs to be protected? Against what? How? In this chapter, attention is focussed on man-made chemicals that may bring both benefits to the end users and to the companies that fabricate them, and harm to the same or other end users, the environment and possibly the same companies that manufacture the chemicals. In examining any risk situation, it is important to identify who stands to win and who stands to lose. Who will have a conflict of interest? Who will have a conflict of loyalty? Who will have a conflict of responsibility? Ethics in this context can be viewed as a question of striving for consistency between our knowing and our doing. One way of escaping from achieving consistency might be that we see the task as too demanding so that we take shortcuts to 'get a number out'. Another escape is we may consider that the context in which we perform the risk-assessment task will not allow us to do all we may deem necessary, so we 'adapt' our knowledge to the expected outcome. Discussion of the ethical issues will be elaborated from this background.

Many professional organizations have adopted codes of ethics to guide the proper behaviour of their members. The latter are considered accountable to their fellow members of the same organization, but mostly to the publics, who are potentially concerned and affected by their decisions or recommendations. As an illustration, the International Commission on Occupational Health (ICOH) clearly spells our some of the duties and moral obligations of its members, for example:

The risk assessment of occupational hazards must lead to the establishment of an occupational safety

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and health policy and of a programme of prevention adapted to the needs of undertakings and workplaces.
Source: (International Commission on Occupational Health, 2002).

A detailed analysis of this document would likely reveal that it embeds aspects of three theories of ethics: utilitarianism and consequentialism, deontological ethics and contractualism (Oughton, 2003). Consequentialism examines the moral value of an action based exclusively on its net consequences. By contrast, deontological ethics considers that consequences are not as central as respect for human autonomy and dignity in assessing the moral value of an action. Finally, contractualism insists on what is agreed upon by contracting parties under idealized conditions, insisting on the fair distribution of harms and benefits. The important aspect of any such analysis for the purpose of this chapter is to realize that in spite of all debates about the differences in these doctrines and their relative merits, there is large consensus on some key questions that the risk analyst ought to consider. Examples are: who is affected, how are risks and benefits distributed, and what possible courses of action are available to face the risk situation under study (Oughton, 2003)? In this chapter, I will emphasize areas of ethical questioning around risk assessment rather that attempting to circumscribe the epistemological contours of ethics as related to risk assessment. In agreement with Brownson *et al*. (2006), I will take the moral position that health scientists, to which category risk assessors belong, are obligated to contribute to the improvement of health in their area of expertise.

One of the immediate challenges in writing on the ethics of risk assessment is to define risk. Hatfield and Hipel (2002) outlined the controversy surrounding the very definition of this word in scientific journals devoted to this topic. For the purposes of this chapter, I will adopt the definition laid out by the International Organization for Standardization (ISO Technical Management Board Working Group on Risk Management Terminology, 2002): risk = the combination of the probability of an event and its consequences. I will consider that the 'event' has exposure to pollutants or drugs as at least one of its components and that 'consequences' are negative impacts on human health. This is not to say that ecological considerations are not in order, but simply that some focus is needed to discuss the topic of ethics in a short chapter. Finally, I will not explicitly consider Sandman's definition of risk (risk = hazard + outrage) (Sandman, 1993) although I fully acknowledge the importance of public representations of risks (Caux *et al*., 2007), and hope the reader will appreciate that due consideration is given in this chapter to a large range of factors that influence the outcome of a risk assessment.

2 SOME FUNDAMENTALS OF ETHICS

Most texts written on ethics mention the work of Beauchamp and Childress (2001). Although I have chosen to approach this chapter from an emergent rather than a theoretical perspective, this pillar of biomedical ethics deserves some consideration. The authoritative book by Beauchamp and Childress emphasizes four basic principles: autonomy, beneficence, nonmalfeasance and justice. Confronted with ethical dilemmas, physicians can analyse medical decisions through these lenses, which has the merit of allowing them to go beyond purely intuitive concepts of 'good' and 'bad' decisions. Transposing these concepts to public-health risk-assessment requires adaptation.

The autonomy of any individual can be respected insofar as that person has sufficient health literacy to appropriately weigh the advantages and disadvantages of the options proposed and assuming that the information is made available in a form that permits understanding by a lay person. A public-health context implies that the autonomy of a whole community must be considered (Chepesiuk, 2007), which certainly complicates the assessment of the health literacy and the development of a consultation process that ensures the best representation of the community's interests. Similarly, both beneficence and nonmalfeasance should be examined from a societal rather than an individual perspective (Vineis and Soskolne, 1993). Finally, the principle of justice should be understood as distributive or environmental justice, where the distribution of benefits and risks must be scrutinized from within-community and between-community perspectives (Roberts, 2000; Bauer, 2008).

3 ETHICAL BEHAVIOURS

In the early 1990s, Greenberg and Martell (1992) conducted a study among the members of the Society for Risk Analysis. About 25% of the membership of the society was surveyed and the response rate was near 30%. The questions were centred on observations made by members of the society of behaviours deemed unethical by self or other risk analysts. Among respondents, 51% reported having observed the 'deliberate overstatement of positive and understatement of negative results', 34% the 'deliberate failure to acknowledge data limits' and 33% the 'design of research to favour a specific result'. The authors discuss the bad policy decisions that can result from the use of unethical science. How well this study represents the frequency of professional misconduct among such professionals can be debated, but it highlights the relationship between available information, its interpretation, and the resulting public-health decisions that result from this process. The critical position of the risk assessor at the interface of the scientific

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information and the risk-management decision-process is discussed below. For further examination of ethical issues for toxicology professionals, see **Ethical, Legal, Social and Professional Issues in Toxicology**.

4 RISK ASSESSMENT AND THE RISK-MANAGEMENT PROCESS

Every risk analyst knows the so-called 'red book' published by the US National Academies Press in 1983 entitled *Risk Assessment in the Federal Government: Managing the Process*. This publication was a cornerstone of modern risk assessment and management (Committee on the Institutional Means for Assessment of Risks to Public Health NRC, 1983). It was followed more than a decade later by the publication of the US Government framework on risk management (The Presidential/Congressional Commission on Risk Assessment and Risk Management, 1997). The latter document schematically represents risk assessment as part of the encompassing iterative process of risk management, as illustrated in **Figure 1** where 'Risk' stands for risk assessment. Whereas the first document places risk assessment at the interface between information gathering and risk-management decisions, the second clearly shows how risk assessment is intertwined with how the risk question is defined, the selection of risk-management options and consideration for affected and involved parties, designated as stakeholders in the document and in **Figure 1**.

Although numerous other frameworks and schematic representations have been developed by various government and standards-making organizations (ISO Technical Management Board Working Group on Risk Management Terminology, 2002; Joint Standards Australia/Standards New Zealand Task Group on Environmental Risk Management, 2006), there would probably be a large consensus that this representation is typical of current approaches to risk managements in modern societies.

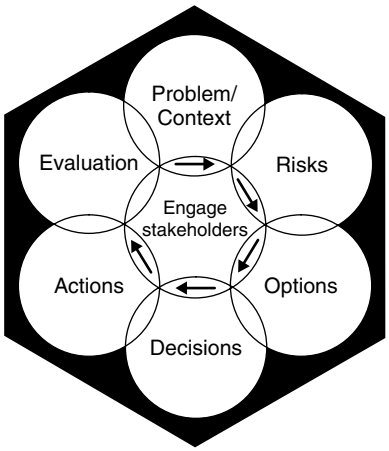

Figure 1 The Presidential/Congressional Commission on Risk Assessment and Risk Management schematic representation of their framework on risk management. 'Risk' means risk assessment in this diagram. (Reproduced from The Presidential/Congressional Commission on Risk Assessment and Risk Management, 2007.)

Many of the ethical questions related to hazard evaluation and risk assessment originate from the articulation between these various elements of the risk-management process. Some may argue that the best way to ensure an objective assessment of the risks is to isolate the scientific assessment from possible influences by other parts of the process. However, even scientific objectivity must be contextualized as Wing (2003) indicated:

> *Strong objectivity demands that scientists critically evaluate how the knowledge they create is shaped at every point by historical social forces. Strong objectivity is therefore not a static feature of scientific knowledge that, once attained, becomes a property of that knowledge.*

Furthermore, if the risk question is ill defined, no risk assessor can provide an appropriate answer to the actual situation that demands attention. A possible consequence here is to use science credentials and the general trust the public grants scientists (Eiser *et al*., 2008) to support a poorly defined risk question, whether intentionally or not.

5 THE RISK-ASSESSMENT INTERFACE

Figure 2 is a simplified illustration of the central role of risk assessment in the risk-management process. I will use this representation to discuss some of the potential ethical pitfalls that may affect risk assessment.

5.1 Sources of Information

In toxicological risk assessment, sources of information are numerous and originate from varied disciplines. The

Figure 2 The central role of risk assessment in the risk management process.

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obvious ones are animal toxicity data and epidemiological studies. The recent years have seen the emergence of genomics, proteomics and metabolomics as additional sources of information to the risk analyst. A recent report by the US National Research Council (Committee on Toxicity Testing and Assessment of Environmental Agents—National Research Council, 2007) proposes to redefine the way in which toxicity testing is conducted.

In this framework, the goals of toxicity testing are to identify critical pathways that, when perturbed, can lead to adverse health outcomes and to evaluate the host susceptibility to understand the effects of perturbations on the human population.Viewing toxic responses in that manner shifts the focus away from the apical end points emphasized in the traditional toxicity-testing paradigm, towards biologic perturbations that can be identified more efficiently without the need for whole-animal testing and towards characterizing host vulnerability to provide the context for assessing the implications of test results.

The potential consequences on the way risk assessments might be conducted in the future are not trivial. A single risk assessor rarely possesses expertise in all relevant areas of risk assessment, and the emphasis on mechanisms of action as core elements of toxicity testing will require an unprecedented breadth of scientific knowledge. Weighing the various elements of the scientific evidence currently is and will remain challenging. And as science is the main backing of numerous risk decisions, there is a moral obligation not to overlook any of the relevant scientific information that contributes to the risk assessment. Two typical scenarios can be envisaged.

In the first case, a risk assessor or risk-assessment team does an extensive review of the original literature on a specific risk question. For years now, there has been increasing incentive towards using a weight-of-evidence approach in medicine and related fields. Weed (2005) characterized the use of this concept in the context of risk assessment from a survey of the scientific literature between 1994 and 2004. He concluded that the use of the expression 'weight of evidence' is unclear and diversely employed by authors. He found that the expression was used in a purely metaphoric sense in roughly 50% of the cases. Without a clear definition and usage of this expression, it can falsely further undeserved scientific credibility to a risk estimate that may actually rest on foggy scientific evidence. In the words of Weed:

> *This metaphorical category of 'weight of evidence' also highlights an important problem in the current practice of risk assessment: lack of transparency; that is, a tendency to underreport, even omit, the details of the interpretative methodology used.*

In the second case, risk assessments may be conducted or may already have been performed by an independent committee composed of experts from various disciplines who deliver a consensus assessment to a decision-making organization, and may make this information available for future assessments of comparable situations. This process has the potential to produce a balanced judgement about risks. However, Irvine *et al*. (2004) warn that problems that are examined in a multidisciplinary setting are unlikely to admit a single solution. The authors therefore call for what they term dialogic ethics organized around an extended notion of collegiality. Generally, consensus opinions are considered preferable to a single-expert assessment because they increase the confidence of the recipient of the recommendation that multiple dimensions were considered and that biases are better circumscribed and controlled. However, consensus reports may also lead to a false sense of 'unanimity' among experts for the very reason that the recipient may not distinguish consensus from unanimity. This is what Hansson (2004) termed the 'consensus fallacy'. Recognizing that oftentimes expert committees strive for compromises whenever possible, a consensus opinion may well mask minority opinions that might inform decision-makers about important, perhaps otherwise inopportunely hidden, aspects of the risk assessment.

Figure 3 shows the classical 'continuum', or stepwise relationship, between exposure to a pollutant and the development of a disease (Committee on Biological Markers of the National Research Council, 1987). There exist two broad categories of factors that can modulate the progression of the biological events along the illustrated continuum. The lower portion of the figure shows that individual biological susceptibility factors may play such a role. It is worth insisting here on a truism: without exposure, the genetic-susceptibility factors cannot render an individual more or less susceptible to a pollutant-related disease. In other words, search for susceptibility factors must not be performed at the expense of a search for means to reduce exposures to pollutants (Vineis, 1997; Viau, 2005; Vineis *et al*., 2005).

In addition to objective scientific evidence, other aspects of the risks need to be cautiously considered. Those concerned by the risk assessment need to be involved in the risk-assessment process (Chapman, 2007; Hermansson and Hansson, 2007). **Figure 3** shows how the social environment may have a profound influence on the relationship between exposure to environmental hazards and disease. For example, it has been shown that a lower socioeconomic status augments the deleterious effects of exposure to fine particulates (Wheeler and Ben-Shlomo, 2005; Forastiere *et al*., 2007). This effect may be attributed namely to geographical distribution of both traffic emissions and of disease burden. More generally, lower socioeconomic status may be associated with insufficient health literacy to efficiently use available information on the relationship between health and pollution (Chepesiuk, 2007), with higher exposures, with poorer defence mechanism (e.g. unbalanced nutrition),

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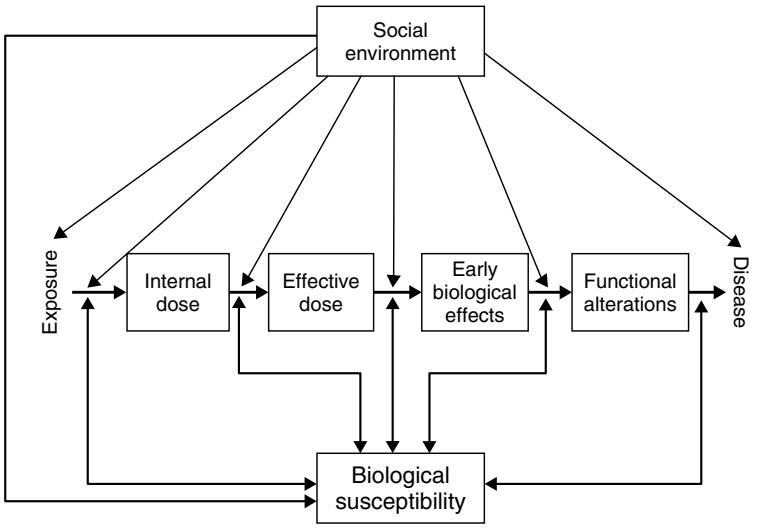

Figure 3 Modulating effect of the social environment on the classical relationship between exposure to an environmental hazard and disease.

with pre-existing health impairments and with limited access to health care. One might argue that these factors are intrinsically taken into account in the interindividual susceptibility factor used to elaborate reference doses or concentrations from no observed adverse-effect levels (NOAELs), obtained in animals or in humans. However, if a study of the social environment is not included in a risk assessment, are we assessing risks comprehensively enough to properly guide adequate public-health decisions and ensure environmental justice? Surely, the risk assessor must be involved in the examination of the influence of the social environment on environmental health issues to comprehend how such factors may modulate the continuum between exposure to pollutants and disease.

As Roberts (2000) stated:

> *If the central premise of the environmental justice movement is that minority and economically disadvantaged populations bear disproportionate health risks from pollution, then risk assessment should play a major role in identifying and rectifying inequitable situations.*

It is also appropriate to emphasize that in addition to its modulating effect, the social environment can itself be a cause of diseases, for example, through psychological stress that may affect the cardiac or central nervous system functions. It ensues that chemical factors may add up to or act synergistically with these social determinants in altering the health and wellbeing of members of a community. Contemplating toxicants as factors modulating the health effects of the social environment offers the risk analyst a different perspective altogether.

Another aspect that requires ethical thinking is scientific uncertainty. This topic will be further discussed in two other sections of this chapter dealing with risk management and the precautionary principle. From the standpoint of available information upon which to base a risk assessment, uncertainty needs to be recognized and fully discussed (Brody *et al*., 2007) although uncertainty may be seen by the public as an indication of either honesty or incompetence (Johnson, 2003). Judgements on risks are only exceptionally dichotomous ('safe' vs. 'dangerous'). Furthermore, as expert as they might be, judgements cannot be reduced to the abstract simplicity of an absolute risk estimate, as Horton (1998) asserted. Additionally, risk assessment along the safe–dangerous continuum should most of the time be considered as a range rather than a point estimate, reflecting the uncertainty of the estimate (Committee on Risk Assessment of Hazardous Air Pollutants, 1994). The complexity and uncertainty of assessing the risks associated with exposure to mixtures of chemicals should suffice to exemplify the uncertainty that surround many risk estimates (Cassee *et al*., 1998). Determining and revealing the boundaries of scientific (un)certainty should be an intrinsic part of risk assessment.

5.2 Context and Risk-Management Decisions

As already pointed out, defining the risk question shapes the way in which the risk assessment *per se* is performed (Hatfield and Hipel, 2002). It follows that for the risk assessment to be a true professional act, rather than a purely technical operation, risk assessors must ascertain that they fully understand the contours of the risk questions and that they can input into the process to help define these questions, based on their *a priori*

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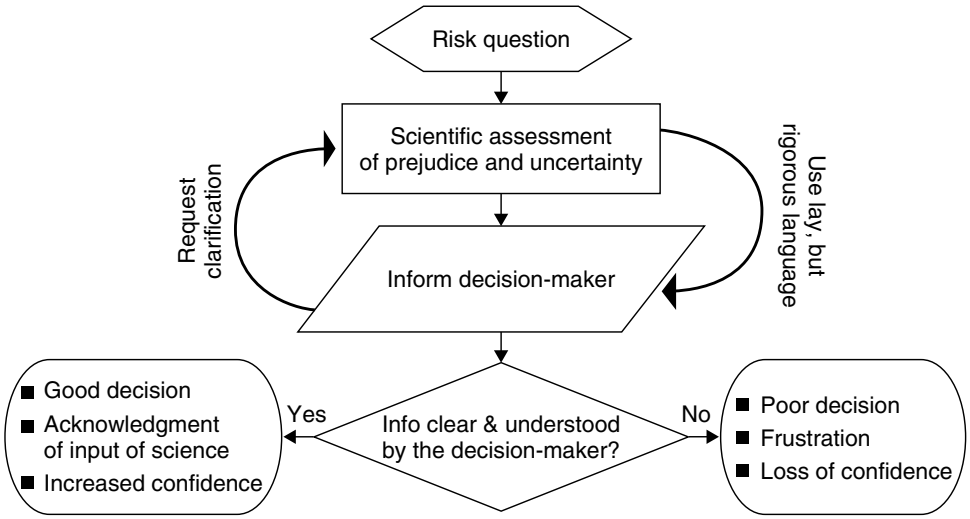

Figure 4 Communication of scientific risk assessment to decision-makers: an interactive process.

scientific knowledge. This actually contributes making the overall risk management process an iterative and interactive one. Science should not be used to bring credibility to pure ideology. Undocumented biases in the formulation of the risk question inevitably lead to biases in the risk assessment. Additionally, faced with complex questions, scientists tend to isolate elements of this complex system so as to better study, understand and explain the available information. By doing so, they may actually translate the complex system under study into scientifically manageable elements that, however, only remotely connect with reality. Put in plain language, scientists may end up looking only for the risks they are familiar with. It would appear adequate to acknowledge concerns about risk that are not addressed and to say why; additionally or alternately, risk assessors should indicate which concerns were considered, which were not, and how they both influenced their judgement.

It is interesting to observe how well-intentioned risk assessors can make radically different assessments based on the exact same scientific sources of information. Risk assessment is a value-laden process in which objectives, mandates, values and perspectives of the risk analysts influence the results of a risk assessment. Hatfield and Hipel (2002) have nicely illustrated this with the alachlor registration controversy in Canada in the 1980s. In this work, they showed how extrascientific factors led three groups to three different and contentious results, largely owing to the problem formulation and differences in the conceptualization of the system within which they were performing the risk assessment. The authors argue that discussion on the premise of the risk question and of its context can probably contribute to resolving some of these discrepancies. At least, an open discussion of the system parameters would have the merit of exposing the reasons for these discrepancies and, hence, of reducing suspicion of hidden agendas and ill-intention that may otherwise be expressed by the spectators of the scientific debate.

Once a scientifically sound and morally defensible risk assessment has been performed, there remains a key task to be accomplished: that of communicating the results to those concerned with the issue, including the decision-makers. All too often, this is seen as a one-way process: the risk assessor produces a report that is transmitted to those concerned and considers that step as the termination of his/her duties. **Figure 4** shows how inappropriately communicated or poorly understood scientific information can lead to poor decisions and nourish distrust and anger between parties. A typical comment from the decision-maker would be, 'I wish the scientists would provide me with useful and clear evidence upon which I can base a decision rather than an incomprehensible jargon cocktail'. Reciprocally, scientists might think, 'However clever and well-documented my recommendation might be, decision-makers always go with their own preconceived decisions that make no room for scientific evidence'. Two key points should be made here. Typically, scientists are not trained as science popularizers. They like to use scientific jargon that resonates in their knowledge sphere, but not in the nonspecialists' minds. Learning to communicate effectively is a challenge that involves making compromises between providing sufficient details to nuance one's expert opinion and 'getting to the point'. However, this is not an insurmountable endeavour and should be encouraged by the desire to make science serve its useful purpose. Conversely, the recipient of an expert opinion, especially if this person is a decision-maker, has the obligation to ensure that his/her opinion is based on the actual scientific assessment, not his/her biased interpretation of it.

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6 THE PRECAUTIONARY PRINCIPLE

6.1 Pandora's Box

The precautionary principle became popular as principle 15 of the Rio Declaration (United Nations Environment Programme, 1992):

> *In order to protect the environment, the precautionary approach shall be widely applied by States according to their capabilities. Where there are threats of serious or irreversible damage, lack of full scientific certainty shall not be used as a reason for postponing cost-effective measures to prevent environmental degradation.*

Numerous articles and editorials have been written in support of or against the precautionary principle. Some argue that this is nothing more than a policy tool to cover ignorance and favour protectionism (Tuomisto, 2004). Some consider it is an invalid principle for rational decision-making and untenable as a moral principle (Harris and Holm, 2002). Others believe it is an excellent example of a public-health decision-making approach that blends evidentiary and ethical considerations (Weed, 2004). Wandall (2004) compared the aims of pure science and risk assessment. She argues that it is crucial for the creation of new knowledge in pure science to try and avoid accepting hypotheses that may later turn out to be false. From that perspective, it is better to reject uncertain statements, even if they are proved true later. In risk assessment, she adds, it is vital to avoid concluding that a substance is harmless, when in fact it is not. This is a foundation of the precautionary principle. Weiss (2001) also insists on putting human-health protection at the forefront:

> *In essence, customary practice assumes that toxic agents and hazardous practices are entitled to more rights than the public and are innocent until proven guilty.*

Finally, Latour (2000) considers that the principal merit of the precautionary principle is to bring the scientific debate within the social arena rather than confining it to the experts' realm. The debate certainly has not come to a resolution yet and it would be both futile and presumptuous to hope to bring it to closure in this chapter. Suffice it to say that the precautionary principle does exist and is used by various organizations to support the decision-making process about risks. It has sufficient teeth that it has held in check the largest economic power on this planet around a number of issues (Whiteside, 2006). Furthermore, many people would probably agree that some form of guidance is required when important risk-management decisions must be taken in the face of large uncertainties, whether we call it the precautionary principle or not. It therefore probably deserves some attention.

6.2 The Muddled Boundaries of the Precautionary Principle

Just as the expression 'weight of evidence' was said to be largely diverted from its methodological sense in numerous scientific articles (Weed, 2005), many organizations and decision-makers pretend to base decisions on the precautionary principle in a purely metaphoric, and inappropriate, sense. Oftentimes, they actually mean 'based on a rigorous, and prudent, assessment of the risks, we have determined that . . . ' or, conversely, 'we have no scientific reason to take action here, but in consideration of public outrage, we have decided to . . . ' These misnomers largely contribute to the undermining of the precautionary principle as evolutionary thinking about risk management.

Principle 15 of the Rio Declaration and its numerous adaptations focus on two dimensions: the severity and irreversibility of a threat, and the uncertainty in the scientific assessment of the situation. Applied to toxic substances, severity and irreversibility refer to the harm that exposure to the substances might cause to people. Uncertainty concerns either the causal relationship between exposure and health outcomes, or the estimates of potential doses to which people might be exposed.

Figure 5 identifies various types of risk decisions based on appraisal of the severity and the uncertainty of a situation. The responsibility of assessing these parameters rests, at least in part, with the risk assessors. This is clear, at least as far as the scientific uncertainty is concerned, whereas severity is largely a value-based judgement that should involve those concerned by the risk situation. Admittedly, there can be no universal scale for severity. Amputation of a limb in a single industrial

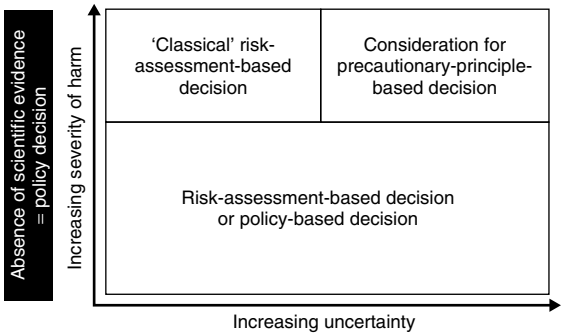

Figure 5 Labelling of various types of risk decisions based on the severity of a prejudice and the uncertainty of the assessment.

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worker would probably rank very high on the severity scale for this enterprise, whereas it would likely to be ranked much lower by an organization concerned with starvation or the propagation of deadly infections in a refugee camp of thousands of people. Although there can visibly be no universal rules for the application of the precautionary principle, an organization can still strive towards its consistent application. Building a consensus severity scale for potential outcomes within its mandate, establishing an uncertainty scale based on the weight of evidence, and mapping the various issues on a severity vs. uncertainty graph, such as the one shown in **Figure 5**, might constitute a first step towards consistency.

Two additional comments appear worthy at this point. First, there is nothing fundamentally wrong with taking a policy decision (lower portion of the graph) that is not based on scientific evidence, insofar as this is clearly spelled out and not erroneously labelled 'precautionary'. An example would be responsibly responding to public outrage that is not justified based on expert assessment of the scientific evidence. Second, there might be situations where all scientific evidence converges to indicate a given situation is not problematic (labelled 'Absence of scientific evidence' in **Figure 5**). Pretending a decision is based on the precautionary principle because 'absence of proof is not proof of absence' is nonsense. Sound application of the precautionary principle requires that there be 'some' credible indication of a severe threat based, for example, on structural similarity with other known toxic chemicals or partial epidemiological evidence. Otherwise, any decision based on the 'absence of proof is not proof of absence' principle is a policy, not a precautionary, decision.

7 RISK ASSESSMENT AND DIALOGUE ON ETHICAL ISSUES

I have attempted to identify areas in a toxicological risk-assessment process, including consideration of the precautionary principle, where a dialogue on ethical issues might be appropriate. One could term these potential 'ethical pitfalls'. This is illustrated in the flow diagram of **Figure 6**. I make no claim that this ought to be the only way risk assessments should be performed to be labelled 'ethical' or that the elements identified are comprehensive. The flow diagram illustrates a broad risk-management process and includes consideration of the precautionary principle, but focusses on areas that should directly involve the risk assessors. These are labelled E1–E6 and will be briefly examined below based on what has beendiscussed in this chapter. The abbreviation 'cMAD' stands for contextualized maximum allowable dose and can be interpreted loosely either in a standard setting sense of establishing a new reference dose or in determining whether an actual exposure situation is deemed acceptable, compared to a preset reference dose.

7.1 Scientific Certainty (E1)

Uncertainties arise when information is very scarce or when there are discrepancies among the findings of various pieces of independent and methodologically sound scientific investigations. However, scientific 'certainty' is a rather loose terminology in that it usually results from a professional judgement carrying some degree of subjectivity as to determining how certain is 'certain'. Hence, the judgement about certainty is not necessarily shared by other experts. In the context of toxicological risk assessment, it is possible that for a given substance, different reports will establish different levels of health effects, or establish different doses at which health effects may start to appear. There could also be dichotomous findings, where one group proposes an adverse health effect, while the other may propose that no adverse effects exist for the same exposure. Assessing (un)certainty based on a weight-of-evidence approach, supported by strong methodological bases and comparing one assessment to previous assessments to ensure consistency, should contribute to making this a reasonable process.

7.2 Severity of Harm (E2)

There might be a general consensus that life-threatening illnesses (e.g. cancer) or seriously debilitating and irreversible health effects (e.g. reproductive effects) are 'serious' health effects. However, the same effects that are mild or less than severe for a healthy individual may be perceived or experienced as very serious and even life-threatening by a sensitive individual (e.g. with a pre-existing health condition). Furthermore, from a public-health perspective, the number of potentially affected people also needs to be taken into account, as well as the distribution of the potential impact among various populations (Harrison, 2008). Once again, appreciation of the severity of harm will heavily depend on the organization's mission, but consistency can probably be achieved by mapping the potential consequences of the various risk situations encountered. Public involvement is essential here to avoid the biased judgement of one or more experts based on their own values.

7.3 Risk Acceptability (E3)

Substances with presumed no-threshold effects are treated differently from the ones where a threshold is deemed

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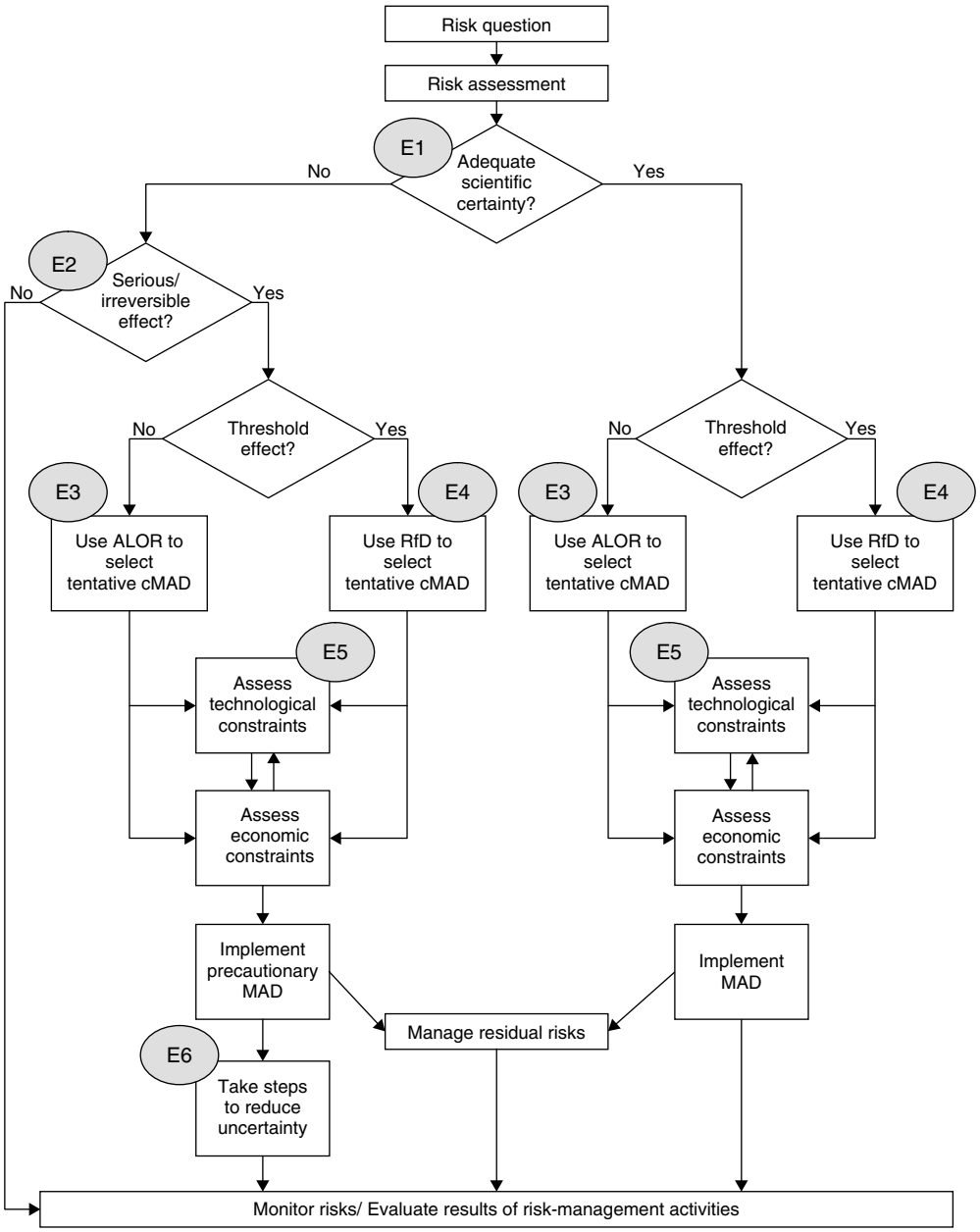

Figure 6 A risk-assessment flow diagram identifying potential ethical traps in the process. ALOR = acceptable level of risk; cMAD = contextualized maximum allowable dose; RfD = reference dose; E1–E6 = potential ethical pitfalls—see text for description.

to exist. For the nonthreshold chemicals, it is customary to estimate a 'residual' risk associated with exposure to one or more doses of chemicals. Since only a dose of zero corresponds to a null risk, a level of 'acceptable' risk needs to be defined for operational purposes. From a scientific perspective, risk acceptability should be a function of the severity of the potential hazard and of the probability that the risk materializes. Both these dimensions are profoundly conditioned by the perception of the risks by the public (Slovic *et al*., 2004). Some of the questions that need to be brought forward are: 'Acceptable to whom and with what consequences?' 'Who decides what is acceptable and on what basis?' One example will illustrate the complexity of these questions. It is 'generally accepted' that workers can be exposed in an occupational context to doses of chemicals that correspond to a potential risk of developing up to one cancer per thousand individuals. However, in the general population, the acceptable risk level is usually in the range of one per hundred thousand or one in a million. Justifications for the higher 'acceptable' risk levels in workers include the fact that they are healthier and stronger and they get benefits as salary from their job. Is this fair to people who are expected by society to

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contribute to its wellbeing (Ball, 2002)? Risks are more likely to be generally accepted for various reasons than they are truly acceptable.

7.4 Reference Doses (E4)

For threshold pollutants, reference doses (or concentrations) are typically established from the NOAELs obtained in an animal or epidemiological study. To account for interspecies and interindividual differences in susceptibility to develop toxicity from exposure to the studied pollutant, the NOAEL is divided by a series of so-called safety factors. Although in most cases this empirical and partially mechanistic approach has proved useful in protecting populations from the toxicity of pollutants, some expressed concerns about the general applicability of the factors (Hattis *et al*., 1999), in particular as they relate to the exposure of children (Landrigan *et al*., 2004). This brings back the initial questions asked at the beginning of this chapter: 'Who needs to be protected and against what?' The risk assessors would be well-advised to use NOAELs and safety factors as guidance, not absolute truth (Wennig, 2000).

7.5 Technological Constraints (E5)

Assessing the technological constraints or technological feasibility of lowering exposure to pollutants is not a risk-assessor's task *per se*. However, it is important to note that the engineers or other technical professionals examining the technical aspects of lowering exposures also have their own set of values that will influence their assessment and advice about what is feasible. It might therefore be appropriate, if not essential, that the risk-assessment issues be known to them before they conclude on the best 'possible technical solution'. The importance of this interaction between the risk assessors and the engineers may be even greater for the determination of 'ALARA' option, where the acronym stands for 'as low as reasonably achievable'. Alternative labels for such options have been 'as low as technically feasible' or 'as low as reasonably practicable'. All these expressions bear a heavy weight of value judgements. Comparable reasoning would also apply to economic assessment.

7.6 Uncertainty Reduction (E6)

A decision based on the precautionary principle must usually be considered temporary, awaiting further scientific evidence. It also obligates the decision-making body to take active steps in the reduction of uncertainty (World Trade Organization, 2008). Risk assessors of course have a role in identifying the knowledge gap to be filled and in determining when scientific information will be deemed sufficient to allow a more definitive risk-management decision. The reassessment of uncertainty must be performed in consistency with the initial assessment and with those made in other, comparable, cases.

8 CONCLUSION

Ethics is not static, but rather complex and adapting to the moving social context and values. It is therefore a paradoxical undertaking to try and 'conclude' this chapter on ethical issues in risk assessment and precautionary communications. It rather appears more appropriate to transform this conclusion into a series of invitations to risk assessors. An invitation to make their scientific knowledge contribute to the wellbeing of others. An invitation to openly acknowledge the limits of and the uncertainties in the scientific knowledge, as well as the contribution of social factors to chemical risks. An invitation to admit that most expert judgements are value-laden. An invitation to accept that those the risk-management process strives to protect need to have their say about the process. An invitation to strive for efficient translation of their knowledge to others, in order to contribute to wisdom in decision-making about risks. An invitation to make their knowing consistent with their doing.

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Food Allergy and Intolerance

Martinus Løvik

CONTENTS

1 INTRODUCTION TO FOOD ALLERGY: TERMS AND CONCEPTS

Allergy is a word meaning 'altered reactivity' and was first used by the Austrian physician Clements von Pirquet in 1906. Allergy as the word is used now represents an inappropriate, symptom-causing immune reaction against a substance foreign to the body. Allergy is a two-step process. First, upon exposure to the foreign substance, the allergic immune response develops (the 'learning phase', called *sensitization*), to establish a state of increased reactivity to the particular substance in the sensitized individual. Thereafter, upon prolonged or renewed exposure to the substance, the *allergic reaction* may develop ('triggering' or 'provocation' phase). The allergic reaction is a particular type of *inflammatory reaction* triggered by allergen-specific immune mechanisms. *Specificity* is very high—a change of one amino acid in the binding site (epitope) on the target molecule can

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be enough to completely abolish reactivity of an antibody. Another typical feature of immunity, including allergy, is *memory*—the heightened state of reactivity is very long-lasting, often life-long. Another important feature is that sensitization (the allergic state of immunity) is not always accompanied by clinical reactivity, and the word allergy should be reserved for the state with *clinical reactivity*. Sensitized individuals without clinical reactivity will be 'false positives' in common allergy tests.

Even among immunologists, the word allergy is used with somewhat different meanings. Traditionally, the word was used for hypersensitivity reactions (abnormally strong reactions) mediated by immunoglobulin E (IgE) antibodies (Type I reactions, according to the traditional Coombs and Gell classification) and by specific cell-mediated immune reactions (Type IV reactions). The IgE-mediated reactions are associated with allergic diseases like allergic rhinoconjunctivitis (e.g. hay fever), allergic asthma, allergic urticaria and food allergy, whereas the cell-mediated allergic reactions mainly are seen in the form of allergic contact dermatitis to low-molecular-weight substances, both man-made (chemicals) and natural (e.g. poison ivy). However, a few years ago, a new nomenclature was proposed, according to which all specific immune reactions causing hypersensitivity to substances foreign to the body should be called allergic, regardless of immune mechanism (Johansson *et al*., 2004). This way, allergy would mirror *autoimmunity*, the main difference being that allergy is directed against foreign (environmental) substances, whereas autoimmunity is directed against our body's own molecules. However, some experts feel that naming most immunopathology caused by exaggerated immune reactions against foreign substances allergy, brings confusion rather than more clarity to the field. Therefore, in the scientific literature, the term allergy is used by some authors in the old specific meaning, and by others in the newly proposed wider meaning.

Food allergy is allergy to foods and their components. Usually we talk about food allergy only after oral intake. However, one should be aware that allergy is systemic and is, in principle, expressed anywhere in the body where immune system cells or immunoglobulins are present. Food allergens can, if airborne, trigger airway diseases like asthma and rhinoconjunctivitis. This is sometimes a problem in the workplace, for example crab processing plants, where, in this setting, crab, which is a fairly common food allergen, will trigger airway disease. Similarly, fish allergics may get asthmatic attacks in fishmarkets because of fish proteins in the air. Further, skin contact with foods may trigger allergic skin reactions (e.g. contact urticaria) and on rare occasions even anaphylactic reactions (Lucas and Atkinson, 2008). Birch-pollen-allergic individuals may get signs of intestinal inflammation during the pollen season (Magnusson *et al*., 2003), and there is a close link between food allergy and atopic dermatitis (Hauk, 2008). Food allergy, skin allergy and airway allergy are therefore just different expressions of systemic allergy, with the organ of expression ('shock organ') largely determined by the route of exposure.

Only some 10–25% of people reporting they are food allergic have a diagnosable, IgE-mediated allergy (Altman and Chiaramonte, 1996). The remainder may have an intolerance that often is difficult to diagnose. Food intolerance is, with a few exceptions, a diagnosis made by exclusion of other possible causes for the symptoms. Often, the symptoms seem to be caused by psychological mechanisms (Lind *et al*., 2005). However, in some cases, symptoms seem to be caused by IgE-producing cells located in the intestinal mucosa, with negative allergy tests in skin and blood (Lin *et al*., 2002). Also, in particular in children, there seems to be food allergy caused by non-IgE, probably cellular, mechanisms (Jyonouchi, 2008). These reactions come much later after exposure than IgE-mediated reactions (days instead of minutes). Their mechanisms remain unclear, and some doubt the existence of these late food allergic reactions as an entity because they are so ill-defined, whereas many paediatricians consider them an important clinical problem.

The clinically severe, acute forms of food allergy are exclusively caused by IgE-mediated mechanisms. Therefore, both diagnostic work and governmental regulatory efforts with regard to food allergy are focussed on IgE-mediated allergy. This chapter on food allergy will largely deal with IgE-mediated allergy, and, in addition, gluten intolerance (which is difficult to classify, but most often is considered an autoimmune condition elicited by an external factor (gluten)), and two non-immune-mediated hypersensitivities (intolerances), namely lactose and fructose intolerance.

The target molecules of allergic reactions are called *allergens*. An allergen is by strict definition an antigen that is able to elicit an allergic reaction (Johansson *et al*., 2004). Allergens are nearly always protein molecules, but IgE responses against carbohydrate groups occur. Carbohydrate epitopes as targets for IgE-mediated reactions are, however, considered to infrequently be of clinical importance, but may cause 'false' test positivity. Low-molecular-weight molecules (chemicals and metals) may sometimes also be involved in IgE mediated allergy, and then require binding to a protein ('haptenization') for the low-molecular weight substance ('hapten') to become able to induce allergy (become 'complete allergens'). For food allergens, the nomenclature may seem a bit confusing, because both the food (e.g. eggs) and the molecules that are the targets of the allergic immune reaction (e.g. the egg protein lysozyme) are called allergens. However, in the regulatory context, it always is the food that is the regulated allergen (e.g. 'fish and products thereof').

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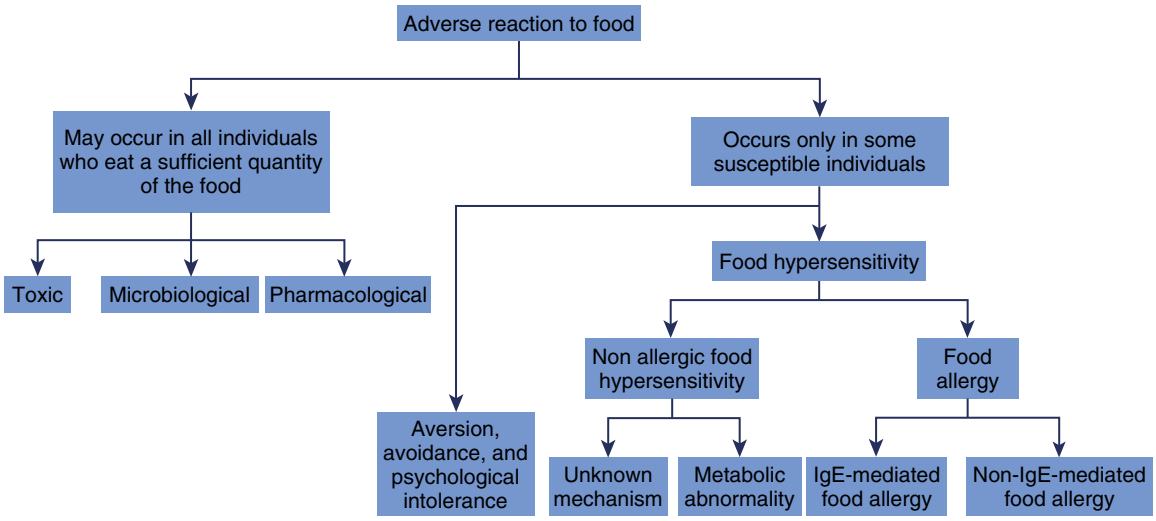

Figure 1 Classification of adverse reactions to food. See text below. (Reproduced, with permission, from ILSI Europe Concise Monograph series booklet Food Allergy (ISBN 1-57881-160-0).)

2 MECHANISMS AND SYMPTOMS OF FOOD ALLERGY

Adverse reactions to food can be grouped into those that, because of toxic, microbiological or pharmacological mechanisms, occur in all individuals who eat a sufficient quantity of the food, and those that occur only in some particularly sensitive individuals (**Figure 1**). The latter, apart from individuals in whom psychological mechanisms cause aversion and intolerance, suffer from food hypersensitivity. Food hypersensitivity consists of nonimmunological (nonallergic) hypersensitivity (intolerances because of metabolic conditions, for example enzyme deficiencies, or often conditions with unknown mechanisms) and food allergy. Food allergy consists of IgE-mediated food allergy, and non-IgE-mediated food allergy (Jyonouchi, 2008; Lin *et al.*, 2002). The most important and probably most common form of food allergy is IgE-mediated, and is linked to other IgE-mediated allergic diseases of the airways and the skin. Respiratory allergy, skin allergy and food allergy are closely related with regard to their development (environmental factors as well as genetic background), they share cellular and molecular mechanisms and they tend to occur together. IgE-mediated allergy has two phases, the sensitization phase and the provocation phase.

2.1 Sensitization

The sensitization phase represents an immune response, which results in the production of specific antibodies of the IgE class to the foreign protein (the allergen). The allergen is taken up by dendritic cells and presented in the context of Class II major histocompatibility complex molecules (MHC II) to T helper (Th) lymphocytes (**Figure 2A**). Two major classes of Th lymphocytes are recognized, Th1 and Th2 lymphocytes, which are important in determining the type of immune response that develops after antigen (allergen) stimulation. Additional classes are regulatory T lymphocytes (T_{reg}) (Akbari *et al.*, 2003; Bettelli *et al.*, 2008; Piccirillo, 2008; Stock *et al.*, 2006) and inflammation-promoting Th17 cells (Bettelli *et al.*, 2008; Chen and O'Shea, 2008; Steinman, 2007). The selection of a Th2-dominated response in favour of a Th1-dominated immune response is to a large extent decided by the antigen-presenting cells and the stimuli they get from the introduced foreign material and their microenvironment. Dendritic cells direct the Th cells by expressing costimulatory membrane molecules and secreting signal molecules (cytokines and chemokines) (Belkaid and Oldenhove, 2008) (**Figure 2A**).

Th2 lymphocytes direct B lymphocytes to produce antibodies of the IgE class, and thus promote an allergic immune response. The IgE antibodies will be distributed throughout the body in the blood and extravascular tissue fluid and will bind with their tail (Fc) part to high-affinity IgE receptors (FcεRI) on mast cells and basophil granulocytes, with their antigen-binding sites free. In particular, these cells will be located at the mucosal surfaces of the body (airways and gastrointestinal tract). The individual is now sensitized (**Figure 2A**, **2B**). If again exposed to the same allergen (provocation phase), the allergen will bind to IgE molecules sitting on the surface membrane of mast cells and thereby crossbind these molecules, leading to activation of the mast cell with release of their cytoplasmic granules with an array of potent mediators (histamine, leukotrienes, prostaglandins and others) causing the allergic inflammation (**Figure 2B**). Symptoms may be local or systemic, and can arise from different organs (**Figure 3**). Further, 6–12 hours after the early

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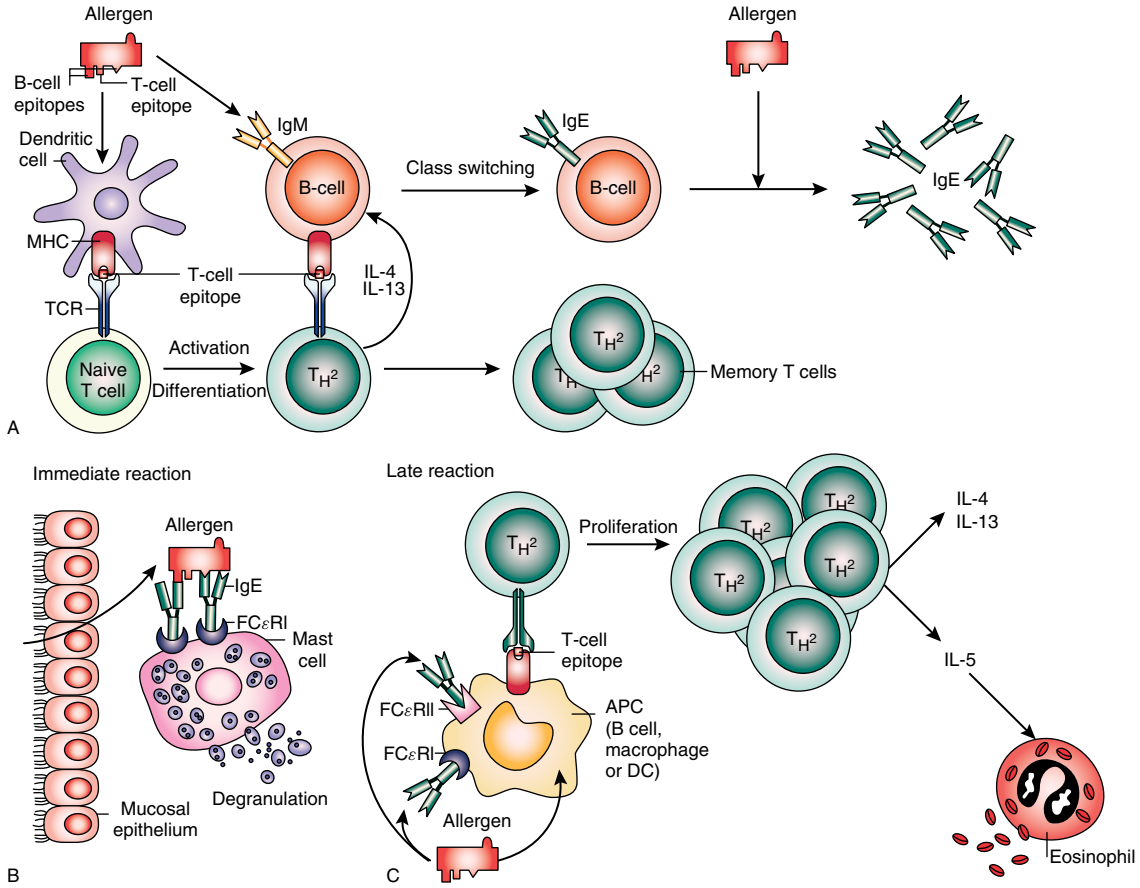

Figure 2 How allergens induce, maintain and trigger allergy. Upper panel (A) illustrates how an allergen at a mucosal surface is taken up by 'professional' antigen-presenting dendritic cells. After processing (digestion) of the protein allergen by the dendritic cell, a small allergen fragment, a T-cell epitope, is presented by dendritic cell MHC (major histocompatibility antigen) molecules on the surface of the dendritic cells, and is bound to the TCR (T cell receptor) of so-called naïve T cells (T cells not previously stimulated by an allergen). The T cells are activated and differentiate into so-called effector Th2 (in the figure T_H2)(T helper type 2) cells. These cells can differentiate into long-lived memory T cells that have specificity for the same T-cell epitope and rapidly will be activated into effector cells if they encounter the allergen again. Th2 effector cells interact with another class of lymphocytes, B cells. Naïve B cells carry IgM (immunoglobulin M) molecules on their plasma membrane, and the IgM molecules may mediate allergen uptake by the B cell if the allergen molecule displays a small part, a B-cell epitope, that matches the somewhat broad specificity of the IgM molecule. Similar to dendritic cells, B cells will then present T-cell epitopes of the allergen on their cell membrane MHC molecules. Upon recognition by Th2 cells with specificity for this epitope, the T cells will produce IL-4 (interleukin 4) and IL-13 (interleukin 13) signal molecules that stimulate the B cell to class switching. After immunoglobulin class switching, B cells will produce IgE (immunoglobulin E) molecules instead of IgM molecules, but with the same allergen specificity as before. Upon continued or later contact with the same allergen, the B cell will differentiate further and secrete large amounts of IgE molecules, all with the same allergen specificity. Lower left panel (B) shows how allergen, after penetration of the mucosal epithelial membrane, can cross-bind IgE molecules bound to the mast-cell membrane by FcεRI (IgE tail part (Fc) receptor one, the high-affinity Fcε receptor) molecules. The cross-binding of IgE molecules will activate the mast cell and cause the immediate release of cytoplasmic granules containing histamine and other mediator substances (degranulation), and an allergic inflammatory reaction will develop within minutes. This immediate reaction represents the first of two waves of allergic reaction upon exposure of an allergic individual to allergen. Lower right panel (C) illustrates the second wave of an IgE-mediated allergic reaction, the late reaction that develops 6–12 hours after allergen exposure. Allergen is taken up by an APC (antigen-presenting cell) either directly or by means of IgE molecules bound to cell membrane IgE high-affinity receptors (FcεRI) and IgE low-affinity receptors (FcεRII). The APC may be a B cell, a macrophage or a DC (dendritic cell). Allergen T-cell epitopes are then presented on MHC molecules to Th2 cells, which are stimulated to proliferate and produce interleukin 4, interleukin 13, and interleukin 5. Interleukin 5 attracts and activates eosinophil granulocytes, which are important contributors to the late reaction. (Reproduced, with permission, from Valenta, 2002.)

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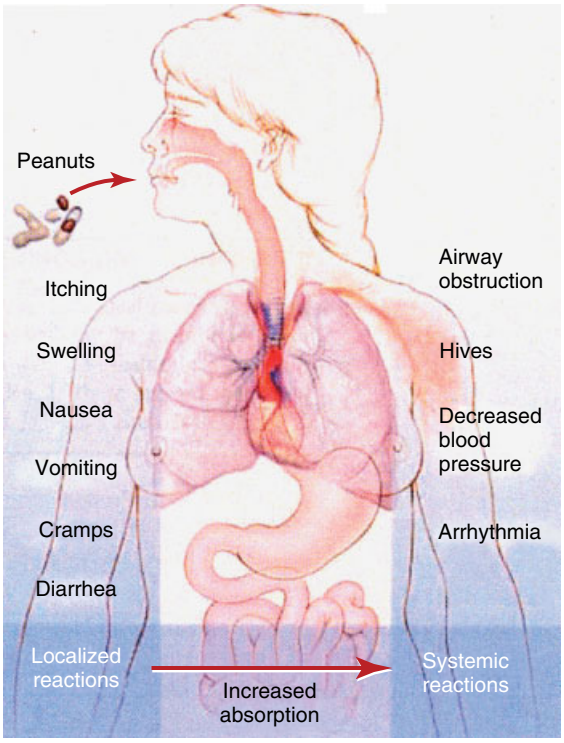

Figure 3 The development of clinical symptoms and signs after ingestion of allergen (here exemplified by peanut) by an individual with the particular allergy. Any combination of symptoms may occur, but systemic symptoms signal a more severe reaction that may develop into anaphylactic shock and cardiac arrest. (Reproduced, with permission, from Long, 2002.)

mast-cell response a second wave of inflammation will develop, with Th cells and eosinophil granulocytes as central actors (**Figure 2C**).

However, a Th2-dominated immune response is not sufficient to develop allergy. Various types of T_{reg} cells and other immunoregulatory cells are important players in the immune response. These cells may promote the production of the Th2-dependent antibody class immunoglobulin G4 (IgG4) instead of IgE, resulting in a 'nonallergic Th2-response', or they may, by other mechanisms, prevent the triggering of the allergic inflammation (Akbari *et al.*, 2003).

The assumed natural route of exposure for food allergic sensitization is the oral route. However, sensitization for IgE-mediated allergy may occur via the skin for certain chemicals and also for protein allergens, and the skin route appears in experimental systems to be strongly Th2- and thereby allergy-driving (Birmingham *et al.*, 2007; Strid *et al.*, 2004). The skin route may be of some relevance for food allergy, and some cases of peanut sensitization have been suggested to have been caused by the use of peanut oil for skin care (Lack, 2008), and cosmetics containing herbal products have also been suspected of inducing allergy. Crossreactivity between food-plant allergens and pollens does, in pollen-sensitized individuals, commonly result in the so-called oral allergy syndrome, or pollen food-allergy syndrome. This condition is caused by primary sensitization and clinical allergy to pollen, for example, birch pollen in Northern Europe. Ingesting foods which contain evolutionally conserved plant pan-allergens (see Section 5) will then cause an allergic reaction. Most often, this reaction is mild and limited to the oral cavity (oral allergy syndrome), but sometimes it may be stronger and even severe and systemic. Commonly, these crossreacting pollen allergens are heat sensitive and are destroyed by cooking, for example, birch-pollen-allergic individuals may react to raw carrots or apples, but not to boiled carrots or heat-treated apples. However, some of the allergens are heat-resistant, like the lipid transfer protein (LTP) (see Section 5). This allergen is involved in peach allergy, common in Southern Europe, and its development is possibly enhanced by inhalation of LTP-containing fuzz from the outside of the peach skin (Asero *et al.*, 2008a). As a result, hazelnut allergy in Northern Europe commonly is caused by crossreaction with heat-sensitive birch-pollen allergen, and the clinical reactions tend to be mild, while hazelnut allergy in Southern Europe is caused by crossreactivity between heat-resistant LTP proteins in peach and nuts, which tend to cause more severe clinical reactions.

What causes the development of food allergy is poorly understood. Clearly, there is a breakdown of normal immune regulation. Genetic predisposition is one important factor. Timing of antigen exposure in relation to development and maturation of the immune system is thought to be important. This also includes 'priming' via mother before birth, but it is uncertain whether and when prenatal exposure will result in tolerance and no allergy, and whether and when allergy development may be promoted instead. With regard to introduction of foods to the baby after birth, it has, for many years, been the dogma that early introduction of foods would promote allergy development, and the advice has been to introduce foods late, particularly so for commonly allergenic foods. A shift of the paradigm now occurs to be going on, because of recent evidence that early introduction of foods may be better in relation to allergy and may promote development of tolerance (Lack, 2008). However, to what extent this will affect advice given regarding the introduction of foods to babies, is not clear at present. The dose of food and the role of other food components and possible immune-stimulating factors ('adjuvants') in food is uncertain. Certain environmental factors, for example, cholera toxin and pertussis toxin, may promote a Th2 immune response at mucosal surfaces (Berin and Shreffler, 2008), while other factors, for example certain bacterial DNA motifs (CpG motifs) may prevent the development of a Th2 response and even turn an established Th2 response into a Th1-like response.

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In summary, the sensitization phase of allergy represents the immunization phase, making the individual ready to respond with an acute allergic reaction upon a new encounter with the relevant allergen. There is little specific knowledge about the conditions for food-allergy sensitization, and sometimes babies and small children experience a food-allergic reaction the first time (known) they ingest a particular food.

2.2 The Provocation Phase

Once a specific IgE has been produced and bound to the high-affinity receptors on mast cells, renewed encounter with the allergen may cause crossbinding of the specific IgE and mast-cell activation with granule exocytosis and mediator release (e.g. histamine, leukotrienes, cytokine and chemokines) (**Figure 2B**). The resulting allergic inflammatory reaction may be local or systemic, and may range from trival to life-threatening. There is no symptom specific for food allergy or for any particular food allergen. The IgE-mediated reactions usually cause symptoms that may start almost instantly, perhaps more often after a few minutes and usually within the first hour. Symptoms may be intestinal (nausea, vomiting, cramps, diarrhoea), cutaneous (urticaria, diffuse redness), from the upper airways and oral mucosa (itching, swelling, obstruction), from the lower airways (asthma symptoms) and the heart and vascular system (arrhythmia, decreased blood pressure) (**Figure 3**). A strong feeling of impending catastrophe is said to sometimes precede serious reactions. The degree of systemic involvement may vary, and any combination of symptoms is seen. Pre-existing asthma is a risk factor for severe food-allergic reactions, and some experts hold the notion that deaths are more often caused by respiratory failure than by cardiovascular collapse. Treatment is first of all intramuscular epinephrin (adrenalin), supplemented by antihistamines and corticosteroids and general emergency treatment. Severity of the reaction is dependent on personal factors including degree of sensitization, possible aggravating factors, food-matrix factors, and dose and potency of the allergen. It should be noted that the early, often almost immediate, reaction caused by preformed mediators released from mast-cell granules may be followed 6–12 hours later by a second wave of reaction, caused, at least in part, by the release of newly synthesized mediators (**Figure 2C**). The two waves of reaction may be separated by an almost symptom-free interval, and observation of sufficient length of patients with strong food-allergic reactions is therefore necessary.

2.3 Aggravating Factors

Food allergic reactions appear to be triggered more easily and become more severe if the person has ingested alcohol, or uses certain medications (e.g. nonsteroidal anti-inflammatory drugs (NSAIDs) and β-blockers). There are reports of patients with suspected food allergy, but a negative food challenge test to the food, who will, however, experience a reaction, if the food intake is combined with a bottle of beer and also exercise. Exercise is well documented to precipitate and aggravate food-allergic reactions, which in this case can come several hours after the food intake. Several food allergens have been reported to cause food-dependent exercise-induced anaphylaxis (FDEIA). Probably the most common allergen involved is wheat, with the ω-(5)-gliadins (part of the gluten molecule) representing the major group of proteins involved (Tatham and Shewry, 2008).

3 THE DIAGNOSIS OF FOOD ALLERGY

The diagnosis of food allergy rests on three pillars. These are: (i) the case history, (ii) tests for specific IgE in serum or skin reactivity to the food allergen in question (prick test) and (iii) food challenge to determine clinical reactivity to the food, preferentially in the form of a double-blind placebo-controlled food challenge (DBPCFC). Serum-specific IgG antibodies to the food have no diagnostic value in relation to allergy, and the intracutaneous test, which is used also by some medical institutions, tends to give a high number of false positives.

3.1 The Case History

Repeated symptoms upon intake of a food is an indication that food allergy to that food may be present, in particular if no other foods are eaten simultaneously (e.g. peanuts out of a bag), or if a few other foods eaten simultaneously vary from episode to episode, or are known to rarely cause food-allergy-like symptoms. However, often the culprit turns out to be different from the food suspected, for example, a patient thinks that they react to spice in hamburgers, but in reality react to the sesame seeds on the hamburger buns. Often the patient thinks she reacts to the most conspicuous or unusual component of the food, while the true cause is a common component. Also, it is important that often the causative component may not be visible ('hidden allergen') and may not be known to be present, for example, anchovies used to give more taste to liver paste may cause an allergic reaction in a fish-allergic individual, or casein used to give form and texture to salmon fillets may trigger an allergic reaction in a milk-allergic individual. Thus, pitfalls with regard to the case history are many. Symptoms may differ: perhaps most frequent are symptoms in the oral

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cavity (feeling of numbness, itching, pain, swelling of lips, tongue, and pharynx) and skin symptoms (redness, urticaria, angiooedema), but also gastrointestinal symptoms (vomiting, loose stools, belly pain) or respiratory symptoms (asthma-like) are not uncommon. Severe reactions may start with a perception of imminent catastrophe, and quickly lead to unconsciousness. Typical for an IgE-mediated allergic reaction is the quick onset of symptoms after food contact with the lips and food intake; often it is a matter of seconds, and a majority of reactions occur within 30 minutes. Thus, 'time to reaction' is an important element of the history. However, sometimes reactions may come somewhat later. In particular, in food-allergic reactions triggered by physical activity (exercise-induced food allergic reactions (FDEIA)), reactions may come a few hours after food intake (four hours is a limit reported by some, but reactions up to 24 hours after food intake have been claimed). If there appears to be inconsistencies and a varying relationship between intake of a food and suspected allergic reactions, one should consider whether varying the presence of cofactors may play a role (alcohol intake, physical exercise, mental stress, certain medications like NSAIDs, β-blockers, etc.). The presence of other IgE-mediated allergies (food, skin, respiratory) in the patient, siblings or parents is another important element in the history. Whereas, the case history sometimes may be very convincing and in practice allows an almost certain diagnosis of food allergy, in most cases additional evidence is needed.

3.2 Allergy Tests

Allergy tests fall into two main groups: serum-specific IgE and skin tests. *Serum-specific IgE determination* consists, as the name indicates, of the measurement of specific IgE antibodies to the (purified) allergen(s) in question. Several commercial systems are available, and these are generally well standardized and validated, and a very wide range of test allergens are available. Advantages are the high level of standardization, comparability with other labs and over time, and that the procedure is noninvasive (apart from the drawing of blood), in contrast to the skin test that entails exposure to the allergen, with a theoretical risk of boosting the allergy and a real risk of precipitating a clinical reaction (fortunately, it happens rarely). A disadvantage with specific IgE measurement is the relatively high cost, and that it may take time to get the answer if the blood specimen has to be sent away for analysis. *Skin tests* will, in practise, mean the so-called *skin-prick test* (*SPT*), in which a drop of fluid containing the allergen is placed on the skin (usually on the inner side of the forearm), whereupon the skin is pricked through this drop with a needle or special lancet so that a small amount of allergen penetrates into the skin. The reaction is read as area of swelling after 15 minutes. There are detailed international guidelines for the conduction of a SPT. Advantages are that the test is cheap and requires no special instruments. A variant (unstandardized) can be performed with the real food in question, by first pricking the lancet into the food (e.g. a fruit, piece of fish, etc.) and then pricking into the skin ('prick-to-prick test'). Disadvantages of the SPT include the exposure of the patient to the allergen, as discussed above, including the risk of precipitating an anaphylactic reaction, a certain subjective element in the evaluation of the test outcome, and influence by variable skin properties and conditions.

For both the determination of specific IgE, as well as the SPT, a critical point is the quality of the test allergens. This is often problematic and constitutes one major problem with allergy testing. Because some allergens are rather labile and easily destroyed by, for example, enzymatic activity or oxidation, and because they vary in their water and lipid solubility, components may be lost during preparation. Thus, there is much room for improvement of test allergen quality, and some discrepancies between different tests and between tests and clinical manifestation can be explained by test allergen differences. The ongoing introduction of recombinant allergen preparations is expected to bring great improvements, with regard to both specificity and reproducibility of allergy testing; however, natural allergens will also be needed, but may perhaps be used more for screening purposes.

It must be emphasized that both specific IgE and the SPT measure sensitization, not clinical allergy, and a 'positive' allergy test does not mean that clinical allergy is present. Sensitization is necessary, but not sufficient, for clinical allergy. Notably, depending on the test allergens and the tested population, as few as one fifth or one tenth of test positives may have clinical allergy, and, in general, the allergy tests discussed will grossly overestimate the number of food allergic individuals. Their negative predictive value is excellent, but their positive predictive value is poor. Whereas few individuals who test negative to an allergen will have an allergy to that food, a majority of those testing positive will have no allergic symptoms. However, a few individuals will test negative, but will, in spite of this, have the allergy. An explanation for these cases may be IgE-producing cells lodged in the mucosa, but little IgE reaching the blood (Lin *et al*., 2002).

3.3 Food Challenge

Food challenge is an obvious and important way to test for clinical food allergy. Optimally, this should be performed by a double-blind placebo-controlled protocol (DBPCFC). It is essential that the test food is properly disguised, for example in pancakes for children,

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that the allergens are not destroyed and that the placebo substance is not an allergen itself or otherwise has the capacity to trigger symptoms. The test food/allergen is given stepwise, beginning with a very low dose and giving a new higher dose after 15 or 30 minutes if no symptoms occurred after the previous dose. Ideally, one should start so low that there is no reaction to the first dose, as this may give an indication of the patient's individual threshold dose and contribute to the determination of a population threshold dose (see Section 8). The tolerated dose is usually given as the sum of the individual doses given without symptoms occurring. Symptoms are reported as subjective symptoms, and more reliably as objective symptoms. The dose interval from subjective symptoms occurring till objective symptoms appear is variable—sometimes 20- or 10-fold, sometimes less. There are several international and national guidelines for the DBPCFC. Variants of the challenge test are single-blinded challenges and open challenges; because of simplicity the latter are often used for screening, followed by DBPCFC for allergens found positive. The simplest challenge can be performed in the home. It consists of elimination and reintroduction of foods and food components, but the possibility of bias is large when one or both of the patient and the test administrator know if a suspected allergen is given. DBPCFC is often called the gold standard of food allergy testing. Still, both false positive and false negative results may occur, and, in addition, there are sometimes reactions to placebo. Furthermore, DBPCFC is not an allergy test in a strict sense, but a test for reactivity to the food. It should therefore always be performed in combination with allergy tests (specific IgE or SPT). The advantage with the DBPCFC is first of all its reliability with regard to demonstrating clinical reactivity to the food, with a relative absence of bias caused by expectations of the doctor and psychological factors of the patient. Disadvantages include its demand for high expertise and skill, it is time-consuming and expensive, and patients often fear reactions and discomfort they sometimes claim to experience for a long time after the test procedure. There is a risk of anaphylactic reactions; therefore, DBPCFC often is not used with patients having experienced severe reactions. The DBPCFC is not ethically uncontroversial, and practices differ in different countries for that reason, and because of local traditions and the resources needed.

3.4 Determination of Food-Allergen Exposure

An important part of the diagnostic workout of a suspected food allergic reaction is the determination of food-allergen exposure. The acute exposure assessment has two main components, the *history* of the incident (including reading of labels and getting information from the manufacturer) and *biochemical investigations* of food-allergen content.

For the history, it is important, not only to listen to the patient and others who were present, but also to actively try to find out if other items were consumed than those reported. The patient's story will often be biased towards one particular incriminating agent. Other meal components may be overlooked, like spices, sauces, toppings, snacks eaten before the meal, drinks and so on. The possibility of allergen contamination from foods present on the table, in the kitchen or in the storeroom, but not eaten must be kept in mind (Faeste *et al*., 2003). When relevant, the food package should be examined (labelling, batch number, etc.).

For the biochemical analysis, a sample of the food eaten (including stomach contents) is far better than a sample of the same food delivered from the kitchen or purchased in the store, because random contamination and small-scale manufacturing errors, for example, in the kitchen or a little bakery will be picked up only from a sample of the food eaten (or at least the same preparation batch). Purchased samples (preferably the same production batch as eaten by the patient) will have little value in picking up random contamination, but will be of value in relation to systematic contamination problems, and production and labelling errors. Food-allergen identification and quantification are highly specialized tasks, to be performed by experienced, competent laboratories. We refer to the special literature, and will here only briefly mention some main principles. First, one should be aware that protein denaturation (e.g. by heat, hydrolysis) may cause problems, in particular if immunological methods are to be used for allergen identification. An antibody reacting with the native protein may not bind the denatured protein, and there may be peptides present capable of triggering an allergic reaction even if no proteins or allergenic peptide fragments are detected in antibody-based assays. Further, extraction of the allergen may be a problem, and the food/allergen matrix may interfere with the analysis. Thus, needless to say, the preparation of food samples for analysis is a critical and demanding step. For the allergen assay, three major types of assay will be DNA-based assays, immunological assays (often enzyme-linked immunosorbent assay (ELISA) format) and spectrometric methods. DNA-based assays are suitable for screening and will demonstrate the presence of material from the particular source in question, for example, peanut, wheat or fish, but not the allergen itself. Immunological assays will demonstrate the presence of the allergenic protein, and can be more-or-less quantitative. One frequent problem is immunological crossreactivity (i.e. specificity problems). Increasingly, spectrometric methods are being used for protein/peptide analysis (Goggin *et al*., 2008). For research, and also legal purposes in relation to food fraud or diagnostic investigation of serious allergic reactions, other 'proteomics' methods may additionally be used.

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4 MAGNITUDE OF THE PROBLEM: EPIDEMIOLOGY OF FOOD ALLERGY

There are no symptoms that are specific for food allergy, and the diagnosis is often difficult and the procedures resource-demanding, as discussed above. Further, some 5–20 times more people think they have symptoms caused by food allergy than the number that can be verified by currently established medical diagnostic procedures (Altman and Chiaramonte, 1996; Young *et al*., 1994). Finally, the area of food allergy is a playground for many nonscientific self-appointed 'experts', laboratories and organizations, which contributes to the uncertainties about diagnosis and prevalence.

The common general statement about prevalence of food allergy is that 1–4% of adults and 4–8% of children suffer from food allergy. The prevalence of food allergy is age-dependent, gender-dependent and different in different geographical regions. There are few good epidemiological studies on this topic in the literature, first of all because of the complexity of establishing the medical diagnosis of food allergy. Data on the prevalence of seropositivity (specific food IgE to food allergens) and SPT positivity cannot be used as reliable indicators of prevalence, because test positivity (sensitization) is far more common than clinical allergy, and because the relationship between sensitization and clinical allergy will differ between different allergens and in different populations.

The literature on gender differences is scarce, but data suggest that food allergy is more common in females. This is supported by findings in the Norwegian Food Allergy Register, a reporting system for severe allergic reactions. Here, about 60% of the total of reported cases were females and 40% males (Løvik *et al*., 2004); interestingly, among prepubertal children, this appeared to be different with a predominance of boys, which resembles the situation with regard to asthma.

The prevalence of food allergy is highest in small children, and reported to be up to 8%. The most important allergens differ in children and adults. In particular, cows' milk and hens' egg allergies are important in small children and, to a large degree, responsible for the high prevalence of clinical allergy in this age group, but some 80% of the children will outgrow these allergies by school age. Peanut allergy is important in all age groups and is less frequently outgrown, whereas, for example, shrimp allergy is more often found in adults. In principle, all food allergies can be outgrown at all ages, a fact which is important to remember to avoid unnecessary life-long avoidance of allergens to which the person has developed tolerance. Once tolerance has developed in formerly clinically allergic individuals, it seems important that the allergen is eaten to help maintain and reinforce tolerance (tolerance is an active immunological nonresponse). When and how to test for the onset of tolerance to a food must be decided by a doctor, because of the risk of serious reactions.

There are some indications that the prevalence of food allergy in adults has been underestimated, and the upper limit of the probable prevalence rate is now sometimes said to be 4%. This is heavily based upon a study by Sicherer *et al*. (2004), where fish and shellfish allergy alone was reported in 2.3% of the population. Sicherer *et al*. (2003) and Sicherer and Sampson, (2000) reported prevalence rates of peanut and tree-nut allergy of 1.2 and 1.4%, respectively, in a US population. Emmett *et al*. (1999) reported a prevalence of 0.6% of peanut allergy in children 0–14 years of age, and somewhat less in adults. These studies were household surveys. Kanny *et al*. (2001), in a population study in France, found a prevalence of food allergy of 3.5%. Osterballe *et al*. (2005), in an unselected Danish population of children and adults found a prevalence of 3.2% for DBPCFC-verified food allergy. In the Norwegian Food Allergy Register (Løvik *et al*., 2007), the highest incidence of severe reactions reported is among young adults age 20–35 years.

There is a perception among allergologists that the prevalence of food allergy has been increasing, similar to respiratory allergies, but good studies documenting this have not been published. Studies of hospitalization, often cited in this context, may more reflect awareness among doctors and in the population, as well the function and organization of the health care system, than true changes in disease prevalence and severe reaction incidence.

Generally, the prevalence of food allergy appears to follow respiratory allergy. The prevalence of allergies to specific foods can vary geographically, depending on local food habits as well as pollen exposure. For example, buckwheat is important in Asia, rice in Japan, celery in Central Europe, sesame seeds in Israel and North Africa, and walnut and pecan in the USA (Metcalfe *et al*., 1996).

5 FOOD ALLERGENS

There are no known structural features that are characteristic of an allergen (Aalberse, 2000); allergens are defined by their function. An allergen is a molecule that can induce an allergic immune reaction against itself, that is for practical purposes in food allergy, an IgE response. The term 'food allergen' refers both to the complex whole food and to the chemically defined compounds that are responsible for allergenicity. In the regulatory context, one usually deals with foods or classes of foods as allergens (e.g. 'fish and products thereof'). However, the biochemical molecular entity causing most food allergies is protein. Some protein breakdown products, that is, peptide fragments, may conserve part of the allergenicity of the native protein and thus can also be considered as allergens. Food allergens are generally proteins of molecular weight of more than 9 kDa, but sometimes smaller

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peptides may cause a reaction. Allergenicity of a complex food is rarely due to a single protein component, but rather due to numerous different proteins which constitute the 'allergen repertoire' of the food. Due to the diversity and variability of the human IgE response, all of the allergenic proteins are not always recognized by all patients allergic to this food. Those allergens that are recognized by more than 50% of a population of patients allergic to the food are called *major allergens*. This concept relates only to the frequency of recognition by IgE antibodies, and it is not related to the severity of the clinical manifestations of an allergic reaction. Major allergens may constitute a small proportion of the total protein content of the food concerned; however, food allergens are often among the most abundant proteins in the food. Clinical reactions may be similar whether they are triggered by major or minor allergens.

5.1 The Allergenic Epitopes

The allergenicity of each single protein is due to a number of molecular immunoreactive structures, so-called IgE-binding epitopes. These epitopes comprise only about half a dozen to a dozen amino acids. Epitopes are widespread within the protein molecule. Similar to allergens, not all epitopes are recognized by all the patients allergic to the protein, and some epitopes are immunodominant while others are only recognized by a few patients. Thus, there is room for a tremendous variation between allergic individuals with regard to the precise targets of their allergic responses.

Epitopes are often described as belonging to one of two different classes, continuous or linear epitopes and conformational epitopes. The former consist of a continuous sequence of amino acids along the protein, and these epitopes tend to preserve their allergenicity even during (moderate) denaturation. Conformational epitopes, in contrast, are dependent on the 3D structure created by folding of the protein for their allergenicity, and will lose their allergenicity when protein folding is disorganized during denaturation. This obviously happens with so-called discontinuous epitopes, composed of amino acids in different parts of the protein molecule brought together by protein folding. It has been reported that, in children with milk allergy, reactivity against linear epitopes signifies reactivity that will persist even after a lower pH develops in gastric fluid as the child gets older. These linear IgE-binding epitopes, which may be located in hydrophobic parts of allergenic proteins can thus be used as markers of a persistent food allergy (Beyer, 2003; Chatchatee *et al*., 2001; Järvinen *et al*., 2002; Vila *et al*., 2001). The distinction between linear and conformational epitopes is, however, not absolute, and many 'linear' epitopes are recognized with much higher affinity in the folded protein compared to the isolated peptide.

Most allergenic epitopes consist of amino acids added during transcription/translation of DNA to RNA to protein (translational peptidic epitopes). However, epitopes may also be formed post-translationally, for example, by hydroxylation (hydroxyproline or hydroxylysine), deamidation (formation of glutamate from glutamine), phosphorylation or nitration (tyrosine) and also by addition of carbohydrate moieties.

Stability to processing and resistance to proteolysis by digestive enzymes have been considered a general characteristic of food allergens (Astwood *et al*., 1996). However, recent studies indicate that the relationship between resistance to digestion and allergenicity is less clear-cut than originally thought (Bannon *et al*., 2003; Díaz-Perales *et al*., 2003; Polovic *et al*., 2007).

Allergenicity may be destroyed by processing (e.g. heating) and digestion of the food. However, allergenicity may also be added, because some epitopes may be unmasked and become available after denaturation or hydrolysis of the protein. Further, new immunoreactive structures (neo-allergens) may be created during processing, including heat treatments, for example, by so-called Maillard reactions (Berrens, 1996; Besler, 2001; Besler *et al*., 2001; Davis *et al*., 2001; Hansen *et al*., 2003; Maleki *et al*., 2000; Mondoulet *et al*., 2005; Nakamura *et al*., 2005). For this reason, some but not other allergic individuals tolerate, for example, the cooked food, but not the raw food, but there are also examples that individuals react only to cooked food. Therefore, processing should not be relied on to reduce or remove the allergenicity of a food.

5.2 Crossreactivity

An antibody will match its antigenic epitope very specifically. However, there is some variation between antibodies with regard to how well they match their epitope, and how strongly they will bind. Different antigen molecules may share a very similar epitope, which may lead to the capacity of the same IgE antibody to bind to different allergens (albeit, as a rule, with different binding strength, depending on how good the match is). This phenomenon, that one antibody may bind to different allergens (strictly, to similar epitopes on different allergens), is called crossreactivity. This refers to identical antibody molecules, like monoclonal antibodies. In an immune serum (not purified or incompletely purified with regard to antibody specificities), there will be a potentially wide variety of antibodies, with specificities against a large number of epitopes, and with a potentially large number of antibody clones directed against each epitope. This serum will be able to react with different allergens. Therefore, one may say that crossreactivity, when talking about antigens, is due to highly similar epitopes being present on different (allergen) molecules, whereas

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crossreactivity of sera and antibody preparations means that antibodies with more or less different epitope specificities are present.

Importantly, serological crossreactivity between allergens (binding of IgE antibodies *in vitro*) does far from always mean that there is also clinical crossreactivity (also called crossallergenicity). For example, peanut-allergic individuals often display serological reactivity to bean proteins, but clinical crossreactivity (crossallergenicity) between peanuts and beans is seldom seen. However, even if crossreactivity between peanut and another legume, soya, rarely is accompanied by clinical symptoms, severe anaphylactic reactions have been reported in a few instances in peanut-allergic patients consuming soya uncontaminated by peanut, and the same applies to crossreactivity between peanut and the legume, lupin.

Many positive allergy tests, both with regard to specific IgE and SPT, are caused by crossreactivity. As a rule, reactivity against the primary sensitizing allergen ('the original allergy') is the strongest, whereas test responses due to crossreactivity tend to be weaker, but the pattern is not always clear. Crossreactivity may lead to clinical reactions, but clinical reactions due to crossreactivity (crossallergy) tend to be weaker than reactions triggered by the primary sensitizing allergen (but unfortunately, not always). Sometimes it is difficult to decide whether there is a case of true crossreactivity or a case of dual sensitization to two apparently crossreacting allergens. Crossallergies to foods may be due to primary sensitization via the skin or airways. A striking example of this is tree and grass pollen allergies leading to clinical allergies to many plant-derived foods (fruits, nuts, berries and vegetables). For example, in the Nordic countries, birch-pollen allergy is associated with allergy to a large number of plant foods, like nuts, apples, carrots and so on. These crossallergies are often (but not always) mild, and manifest themselves as the so-called oral allergy syndrome, with feeling of numbness, itching and swelling in the oral region (see Section 2 on Mechanisms and Symptoms of Food Allergy). Also, this leads to nut allergy in the Nordic countries generally causing milder clinical symptoms than nut allergy in Southern Europe, because in Southern Europe hazelnut allergy will more often be a primary allergy (and be directed against other nut allergens). Another example of crossreactivity due to a pan-allergen (see below) is the crossreactivity between rubber latex and tropical fruits (Wagner and Breiteneder, 2002).

Remarkably, because of crossreactivity (crossallergenicity), a person may experience an allergic reaction to an allergen never encountered before.

Isoallergens are, in common language, variants of the same allergen. Isoallergens are defined by the International Union of Immunological Societies (IUIS)/World Health Organization (WHO) Allergen Nomenclature Subcommittee as molecules from the same species having similar size and identical biological function, and having $\geq$67% amino-acid sequence identity (IUIS/WHO, 1994). Because of their similarity, isoallergens will have a high frequency of crossreactivity.

5.3 Allergen Families and Pan-Allergens

Crossreactivity is caused by similar epitopes being present on molecules in different foods (Aalberse *et al*., 2001). The more resemblance there is between the molecules, developmentally and functionally, the greater is the chance that there will be crossreactivity and crossallergenicity. Allergens are found in only a small subset of protein families (Aalberse, 2000; Chapman *et al*., 2007; Ivanciuc *et al*., 2008; Mills *et al*., 2004; Radauer *et al*., 2008). Plant allergens often are, for example, storage proteins, stress or defence proteins, or LTPs, and the chance that a protein is an allergen is increased if it belongs to such a class of proteins. Certain evolutionally conserved proteins that are allergens in many species are called pan-allergens. Such pan-allergens in plants are, for example, profilins (Asero *et al*., 2008b) and LTPs (Salcedo *et al*., 2004), in fish the muscle protein parvalbumin (Van Do *et al*., 2005), and in crustaceans, molluscs, mites and cockroaches, the muscle protein tropomyosin (Reese *et al*., 1999).

In sum, there are three levels of structures (whole food, protein and epitopes) involved in the interaction with IgE antibodies and responsible for the allergenicity of a given food. Different epitopes, and thereby different proteins and different foods, will respond differently to processing and digestion. Therefore, depending on the immunodominance of the different epitopes in a particular individual, the effect of processing on food allergenicity may also differ between food-allergic individuals. Similarly, crossreactivity of allergens will differ between individuals. Irrespective of this complexity, and the fact that about 200 food allergens have been described, eight allergenic foods are responsible for some 90% of clinical food allergy: peanut, soya, nuts, wheat, milk, egg, fish and shellfish ('the big eight'). Various national and international regulatory bodies may have somewhat expanded lists of allergens that are so important for public health that they should be subject to special treatment with regard to labelling (European Commission, 2007).

6 IS THE NEW FOOD AN ALLERGEN?—TESTING OF POTENTIAL ALLERGENICITY

No single factor has been recognized as a primary identifier of allergenicity. Therefore, in general, a number

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of factors are incorporated into the overall assessment of likelihood of allergenicity by a weight-of-evidence approach (Codex Alimentarius Commission, 2003; FAO/WHO, 2001). Factors considered include the source of the gene or protein, molecular structural similarity of the protein with known allergens, digestibility and specific IgE binding.

Identity or crossreactivity of a food protein with a known allergen may be suspected, for example, if the food is derived from an allergenic food by processing (high temperature, high pressure, enzyme treatment, etc.) or if it has been made by gene transfer from a known allergenic source. Identity or remaining allergenicity, as well as crossreactivity can be assessed by serum screening. In serum screening, binding of IgE to the protein in question in sera from individuals sensitized, either to the corresponding original source protein or relevant protein(s) from organisms more or less broadly related to it, is investigated. Immunochemical methods like dot-blot, ELISA, RAST (radioallergosorbent test) and Western blotting are used. Functional *in vitro* assays are based on activation by the putatively allergenic protein of basophils presensitized with IgEs of relevant specificity. Histamine release or cell-membrane activation markers and flow cytometry can be used (Ebo *et al*., 2008). However, the availability of a sufficient number of well-characterized sera is an important limiting factor. A final test of safety would be double-blind placebo-controlled (DBPCFC) challenge of individuals with the relevant food allergy. However, there are ethical constraints and it is difficult to find a sufficient number of well-characterized volunteers with the relevant allergy.

The assessment of *de novo* sensitization capacity is a difficult task. *In vitro* resistance of the protein to *pepsin degradation* is one component of the assessment (Astwood *et al*., 1996; Thomas *et al*., 2004) according to the Codex Alimentarius guidelines (Codex Alimentarius Commission, 2003; Ladics, 2004). However, opinions are divided with regard to the value of this assay, and it certainly is not a stand-alone assay. The rationale for the assay is that proteins that are rapidly broken down after ingestion are expected to be less likely to be able to sensitize. Many known food allergens show pepsin resistance, but there also are exceptions (Bannon *et al*., 2003; Díaz-Perales *et al*., 2003; Fu *et al*., 2002; Polovic *et al*., 2007). *Animal models* are discussed below; no validated animal models exist, but animal models may give supporting information. *Serum screening* to exclude crossreactivity of a novel protein with known allergens, if suspected, can be performed, but requires that a sufficient number of well-characterized sera are available. The rationale is that a protein that binds IgE, as a rule, will also be a sensitizer.

Bioinformatics or *in silico* methods are much focussed upon as a tool for allergenicity assessment, but there is general agreement that these methods alone cannot answer the question of allergenicity, although there has been rapid development in the field. The reason is that we do not know any structural features of a protein that makes it an allergen. Whether a 'new' protein will turn out to be an allergen cannot be predicted by bioinformatics. Therefore, bioinformatics allow only two questions to be answered: is the protein an existing allergen? Is the protein likely to crossreact with a known allergen? Most of the major food and respiratory allergens have been identified and cloned, and amino-acid sequences for a majority of these allergens have been incorporated into various databases (Brusic *et al*., 2003; Chapman *et al*., 2007; Gendel and Jenkins, 2006; Lucas and Atkinson, 2008; Mari *et al*., 2006). Information about the spatial structure of allergens is, however, only beginning to be mapped and incorporated into databases (Oezguen *et al*., 2008). A problem is that the criteria for including a new allergen into a database are not always clear and well-defined (Lucas and Atkinson, 2008). Examples of databases are the peer-reviewed Food Allergy Research and Resource Program (FARRP) database (*www.allergenonline.com*) and Allergome (*www.allergome.org*).

FAO/WHO (2001) proposed a risk assessment based upon a multiprocedural assessment scheme (**Figure 4**). According to the recommended protocol, a warning flag is raised if either of two criteria is met: a match of six consecutive amino acids to identify contiguous, linear epitopes, or an identity of more than 35% over an 80-amino acid window of the query protein to pick up conformational epitopes (FAO/WHO, 2001), in both cases based on comparison with a documented protein allergen ('sequence homology' in **Figure 4**). The use of 35% identity is 'conservative', because, for crossreactivity to occur, a high degree of homology most often is needed, likely in excess of 50–60% over significant spans of the new protein and the allergen (Aalberse, 2000). However, six amino-acid matches have been found to occur too often between unrelated proteins and therefore this is an unreliable criterion for prediction of allergenic potential (Gendel, 2002; Ladics *et al*., 2006; Silvanovich *et al*., 2006), and neither the Codex Alimentarius Commission nor the EFSA (European Food Safety Agency) adopted the six-amino acid criterion (Codex Alimentarius Commission, 2004; EFSA, 2004a). Eight contiguous amino-acid matches are often used instead, but the value of this approach is also questioned for similar reasons. One approach has been to identify motifs, that is, substructures in a protein connected to function and shared by allergens, and search these for similarity. However, motifs may be specific for protein families, for example tropomyosin, rather than for features connected with allergenicity (Kong *et al*., 2007; Li *et al*., 2004). In conclusion, several approaches alternative to the FAO/WHO protocol have been discussed, including similarity of entire allergens, computationally extracted

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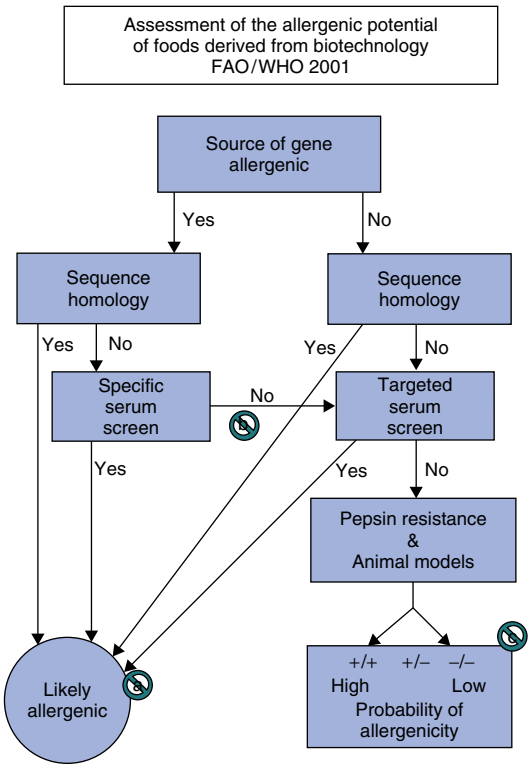

Figure 4 The FAO/WHO 2001 decision tree for possible allergenicity of a novel protein. Stop-sign explanations: (a) Any positive results obtained from sequence homology comparisons to the sequences of known allergens in existing allergen databases or from serum screening protocols, indicate that the expressed protein is likely allergenic. (b) The degree of confidence in negative results obtained in the specific serum screen is enhanced by the examination of larger numbers of individual sera. Conducting the specific serum screen with small numbers of individual sera when larger numbers of such sera are readily available should be discouraged. (c) When positive results are obtained in both the pepsin resistance and animal model protocols, the expressed protein has a high probability of becoming an allergen. If negative results are obtained in both protocols, the expressed protein is unlikely to become an allergen. When different results are obtained in the pepsin resistance and animal model protocols, the probability of allergenicity is intermediate, although rational explanations may be possible in some situations. (Reproduced, with permission, from FAO/WHO, 2001.)

motifs from allergens, similarity to experimentally verified IgE binding epitopes, and amino acid composition.

Finally, it must be remembered that in order to trigger mediator release from mast cells, two IgE-binding epitopes on the same protein molecule are necessary, so that neither a single match of six or eight amino acids between the new protein and the known allergen nor the presence of a conformational epitope may be predictive of capacity to trigger a clinical reaction. For the same reason, IgE binding will not always be associated with mast-cell mediator release or clinical symptoms.

7 ANIMAL MODELS

There is no validated animal model of food allergy for use in a regulatory context, but a number of experimental models are in use or under development for scientific, clinical and regulatory purposes. Basically, two types of model can be discerned, namely models of allergic sensitization and models of allergic reactions and anaphylaxis. Models of allergic sensitization will have as an outcome the specific IgE (or corresponding antibody isotype) response, determined on the protein level (e.g. ELISA) or as functional antibodies (e.g. passive cutaneous anaphylaxis (PCA) testing), or both. Models of allergic reactions will have as an outcome relevant immediate clinical symptoms upon allergen challenge and also atopic-dermatitis-like skin symptoms, symptoms of anaphylaxis, biochemical markers of reaction (e.g. serum histamine, mast-cell proteases, tryptase) or physiological measures of anaphylaxis (lung function and gas exchange, mucosal and vascular permeability, body temperature).

Animal models will, ideally, be helpful for several purposes. These include a number of issues that cannot be readily answered on the basis of human data, like the study of basic immunological mechanisms of food allergy, factors favouring or preventing the induction of food-allergic sensitization, factors favouring the elicitation or prevention of food-allergic reactions and anaphylaxis, the mechanisms of anaphylaxis, role of prenatal exposures and epigenetic factors, adjuvant factors and matrix factors (e.g. fat) in relation to allergy, testing of drugs and natural products for prevention of food-allergic sensitization or the triggering of food allergic reactions, vaccines for prevention or treatment of food allergy, testing of the allergenicity of novel foods, determination of allergenic potency of food components, including novel proteins, the reaction-triggering capacity of food components, immunological and clinical crossreactivity between food allergens, relationships between the serological response and clinical reactivity, and studies of different relevant routes of administration (peroral, inhalation, skin).

What constitutes an ideal animal model certainly will depend upon the purpose of the study and the questions asked, and it is likely that there will not be one single human-like model for universal use. Instead, different models will be the best ones and be used for different purposes. The following ideal criteria of an animal food-allergy model is not likely to be met by one model: IgE as the mast-cell triggering ('reaginic')

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antibody isotype, the presence of identified cell types and antibody isotypes homologous to those in man, sensitization induced by eating/peroral administration of the whole food with a minimum of manipulation (adjuvants, immune modulators), alternatively respiratory or skin routes of sensitization, same effect of adjuvants and cofactors as in man, a complete immune response similar to man (T cells and antibody isotypes other than IgE), IgE response to all known food allergens and not to nonallergens, epitope specificity and crossreactivities like in man, potency and potency 'hierarchy' similar to that in man, dose–response and thresholds for sensitization and triggering similar to man, same allergic-reaction mediators as in man (histamine, etc.), and signs, symptoms and other outcome markers similar to man. Clearly, no model will satisfy the ideal criteria listed, but the criteria are important when evaluating the suitability of a model for a given purpose (e.g. if the purpose is to determine allergenic potency for regulatory use, more weight should probably be given to factors related to sensitization (specific IgE) than to triggering of a reaction, although the latter are not irrelevant).

Food allergy occurs naturally in cats and dogs. In dogs, the prevalence has been reported to be about 1% (Day, 2005). There also are some claims that food allergy occurs in other domestic animals like the pig, but this has not been convincingly shown. In the dog, allergens reported to be involved include beef, chicken, milk, egg, wheat and soya bean. Cutaneous symptoms (atopic-dermatitis-like) are common. No spontaneous breed differences have been observed, but genetic factors appear important and atopic dog colonies have been developed with selective breeding.

Experimental animal models with induced food allergy include models in animals naturally developing food allergy (dog), and models in animals that are not known to naturally become food allergic (Helm, 2002; Helm *et al*., 2003; Knippels *et al*., 2004; McClain and Bannon, 2006). The latter include the guinea pig, swine, rats and mice. A problem is that mast-cell activating ('reaginic') antibodies, with the exception of swine, in contrast to man, are not limited to IgE, but include also IgG1a (guinea pig), IgG2b (rat) or IgG1 (mouse).

The guinea pig represents the oldest model for allergenicity testing, but has mostly been used with chemicals, cosmetics and other products, rather than foods or food proteins. There are claims that the oral route of immunization can be used without adjuvants. A problem is the dominating role of IgG1a rather than IgE as the mast-cell activating antibody.

The neonatal swine model (Helm *et al*., 2002) employs newborn piglets from White/Landrace sows. The piglets are sensitized ip (intraperitoneally) with cholera toxin as adjuvant, and challenged by intragastric gavage at one week intervals. Allergens employed have been peanut, chicken ovomucoid, milk, wheat flour and nuts (Helm *et al*., 2002; Rupa *et al*., 2008). This is a challenge model, and clinical outcomes are emesis, lethargy, diarrhoea and respiratory distress, and, in addition, histology of the intestinal mucosa. Symptoms in the piglets are said to be fairly similar to symptoms in man, the same being said about gastrointestinal anatomy and physiology, as well as immune function in the pig, and nutritional requirements of piglets, according to proponents of the swine model, resemble those of human babies. Thus, the swine model may represent a 'human-like' food-allergy challenge model.

The atopic inbred-dog model was introduced by Ermel, Buchanan and Frich (Buchanan and Frick, 2002; Ermel *et al*., 1997; Helm *et al*., 2003). Dogs bred with 'atopy' as a selection marker are injected repeatedly sc (subcutaneously) in the axilla with allergen and alum as adjuvant, up to day 85, and thereafter boostered with a larger dose of allergen with alum bimonthly. Skin tests are performed with Evans blue as a marker of vascular leakage upon allergen injection. Feed challenge is given at age 2.5 years. Clinical outcomes include vomiting, and number and quality of stools. Advantages of the dog model are said to be that the dog represents a species that naturally develops food allergy, and symptoms are 'human-like'. Allergens tested include wheat, barley, cows' milk, beef extract, peanut and tree nuts. Crossreactivity between walnut and Brazil nut was observed, and the allergen 'hierarchy' was reported to be similar to that in man. Thus, the dog also appears to represent a 'human-like' food-allergy challenge model.

Among rats, Brown Norway (BN) rats have been found more suitable for peroral sensitization with food allergens than strains like Wistar, Hooded Listar and PVG rats. Females are said to be more suitable than males for the food allergy model. The BN rat is a high IgE-responder strain, and the animals are immunized at age four to six weeks by daily dosing by gavage without adjuvant (Knippels and Penninks, 2002). Blood samples were drawn after 0, 35, 42 and 49 days, and serum anlysed by ELISA for specific IgG2b and IgE, as well as for functionality, by PCA. Oral challenge is given, and breathing frequency and blood pressure used as outcomes. Among the allergens used are ovalbumin (OVA), cows' milk, egg white, peanut, peanut allergen Ara h 1, shrimp allergen Pen a 1, potato allergen Sol t 1 and beef tropomyosin. Thus, the BN rat model is used both as a sensitization model and a challenge model. Other rat models have been used, for example based on ip immunization with carrageenan as adjuvant.

In the mouse, a variety of models have been used. The mouse has many well-known advantages as an experimental animal, both with regard to practical issues and science. 'Reaginic' antibodies capable of activating mast cells in the mouse are IgE and IgG1. Mostly, inbred BALB/c mice are used for sensitization models and inbred C3H/HeJ mice for challenge (anaphylaxis) models. The BALB/c mouse is a

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Table 1 Symptom score for peanut-allergic mice (Li *et al*., 1999)

Score	Symptoms
0	No symptoms
1	Scratching and rubbing around the nose and head
2	Puffiness around the eyes and mouth, diarrhoea, pili erecti, reduced activity and/or decreased activity with increased respiratory rate
3	Wheezing, laboured respiration and cyanosis around the mouth and tail
4	No activity after prodding, or tremor and convulsions
5	Death

good antibody responder, including IgE antibodies. The C3H/HeJ mouse because of a TLR4 receptor deficiency is a lipopolysaccharide (LPS) low-responder. Interesting percutaneous immunization models without adjuvant have been described (Birmingham *et al*., 2007; Strid *et al*., 2004), and investigators employing an ip immunization model in BALB/c mice have reported an allergen potency hierarchy similar to that in man (Dearman *et al*., 2003). In the ip immunization model, the epitope pattern, with β-lactoglobulin, as well as peanut, as allergens has been found to be similar to that in man (Adel-Patient *et al*., 2000; Li *et al*., 1999; 2001). Intranasal and subcutaneous immunization models have also been used. However, most used in the mouse is peroral immunization by gavage, employing cholera toxin as adjuvant, or, recently, acid-neutralizing medications or drugs. Serological outcomes are IgE and IgG1 antibodies (and also other isotypes) determined as protein by ELISA or as functional antibodies by PCA. Clinical outcomes in challenge experiments are clinical symptoms (**Table 1**), diarrhoea, body temperature, mucosal and vascular hyperpermeability, and histological evidence of affected intestinal mucosa and lung, as well as cellular, immunological and biochemical markers, including histamine and mast-cell proteases.

An advantage with the mouse is the detailed knowledge of its immune system and the availability of a wide range of immunological and biochemical reagents, as well as genetically variant mice. The anaphylaxis model in C3H/HeJ mice employs repeated immunizations by gavage up till about 40 days, with cholera toxin as adjuvant (Li *et al*., 1999; 2001). To trigger anaphylaxis, a larger dose of allergen without adjuvant is given. The model has been used with a number of allergens, including peanut, lupin and milk. Lately, a number of publications have documented the capacity of gastric acid neutralization (peroral suspensions or iv (intravenously) administered proton-pump inhibitors) to promote food allergy development to allergens (Untersmayr and Jensen-Jarolim, 2008).

8 REGULATORY ASPECTS OF FOOD ALLERGY—PRINCIPLES AND CONSIDERATIONS

8.1 Introduction

This chapter will discuss some regulatory principles and considerations regarding food allergy. We refer to national agencies and international organizations for the specific documents and regulations (e.g. Codex Alimentarius Commission, 2008; European Commission, 2007; FAO/WHO, 2001).

The development of a food allergic reaction is a two-step process. The first step is the sensitization process, whereby the immune system develops a specific IgE response against the food allergen, thus 'loading the gun'. The second step is a later exposure of the sensitized individual to the food allergen, eliciting the allergic reaction ('pulling the trigger'). The distinction between these two processes is important also in the regulatory context. Most regulatory measures are aimed at preventing the triggering phase, that is, exposure of the sensitized individual to the food allergen. Product labelling is a tool of major importance for this purpose, to make avoidance possible. Only in relation to novel foods, that is, foods that are new to the population (including food from genetically modified organisms), is the prevention of sensitization a regulatory concern.

8.2 The Sensitization Phase to Food Allergens in a Regulatory Perspective

For sensitization to food allergens, exposure to the food allergen (or cross-reacting allergens) is necessary. However, often, we do not know when this sensitizing exposure takes place, but it obviously can happen very early in life and possibly *in utero* or via mother's milk, because there are numerous cases of babies and small children reacting (sometimes fatally) to a food at the first known intake. Such cases are also fairly well documented for allergens for which unknown exposure of the baby through everyday use in the household could not so easily take place, for example, paranuts. It is a fact that very often, we do not know exactly when and how sensitization to food allergens is induced. Further, we do not know the conditions for sensitization in terms of doses and exposure distribution over time. This lack of knowledge constitutes one major obstacle to prevention of sensitization. Another major obstacle is the fact that the allergens most commonly causing severe food allergic reactions in the industrialized world (the big eight), also are among the most important (volume-wise and economically) and are nutritionally valuable foods,

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like milk, eggs, wheat, fish, peanuts and so on. To ban or strongly limit the use of these traditionally used staple foods with the aim of preventing sensitization and allergy, which either is mostly transient (milk and eggs in children) or affects less than 0.5–1% of the population for each food, would have dramatic social and economic consequences in relation to agriculture, fisheries, trade and food industries. Such a ban of the common allergenic foods also would cause problems with regard to providing a nutritionally adequate diet for the population and, in particular, children. Food tradition is also culturally important. Advice given in particular regarding nutrition in early childhood to tentatively reduce the risk of allergic sensitization is scientifically not very firmly founded. Consequently, prevention of sensitization by regulating the use of traditional allergenic foods is not an issue in regulatory work. This means that with regard to traditional foods, society accepts as a fact of life that a small proportion of the population gets sensitized and allergic, because we have no acceptable means of prevention.

Only with regard to new ('novel') foods, is the prevention of sensitization a regulatory issue. Examples of such foods (for Europe) are foods that have traditionally been used in other parts of the world (e.g. Pacific Islands), but not in Europe, for example nangai nuts (Sten *et al*., 2002), and also foods made by new industrial processes and foods made by genetic modification of organisms. Novel foods do not have a central role in society, and for healthy nutrition, that traditional allergenic foods have. Novel foods have, according to current regulations, to be evaluated for allergenicity to avoid the inadvertent introduction of a new allergen.

Experience is that 'new' foods introduced rarely introduce 'new' allergies, but may still be important food allergens because of crossreactivity with common traditionally used food allergens. Examples of this are lupin, which causes serious reactions in peanut-allergic individuals because of crossreactivity (Lindvik *et al*., 2008), and kiwi fruit (Lucas *et al*., 2003), which causes reactions in part because of crossreactivity with pollen allergens.

8.3 Incidence of Allergic Reactions

The main event of regulatory concern in food allergy is the triggering of allergic reactions. This is an acute event, precipitated by the intake of a sufficient dose of food on one single occasion. A food-allergic reaction, therefore, is comparable to an acute poisoning event; the amount of allergen consumed over a longer period is of limited interest, and then mostly in relation to sensitization and waning of the allergy. The fact that the dose of allergen of concern, in relation to triggering of a reaction, is not the accumulated dose over time, but instead the dose of allergen in one serving of the food, has implications for risk assessment and regulatory work.

The adverse event in relation to food allergy is the allergic reaction, a short-lasting event that cannot be well diagnosed afterwards. The key feature for diagnosis is the sum of clinical symptoms presented during the reaction. There are some biochemical markers of anaphylaxis, like serum *histamine* and *tryptase*, but, in particular, histamine is a short-lived marker present in parallel with the symptoms rather than after the reaction, and is, in practice, difficult to use. Tryptase is somewhat more persistent and can be measured in specimens taken, for example, from autopsied individuals. A key element is the assessment of allergen exposure before the reaction started, the history of allergy, the presence of relevant sensitization and a positive DBPCFC test (see Section 3). However, because the food allergic reaction is a short-lived phenomenon, leaving no lasting traces and, in addition, spans the full gradient of severity from hardly noticeable discomfort to dramatic death, our knowledge of the incidence of allergic reactions in the population to a particular allergen is extremely incomplete. Thus, discussing regulatory measures for foods on the market, we deal with an outcome, the incidence of which is very incompletely known. National or regional reporting systems for (severe) food-allergic reactions would give information of great value for assessing the public-health importance of food allergens (Løvik *et al*., 2004), and a few such reporting systems and registries have been established the last years. Ongoing work in international organizations may lead to more harmonization of food-allergic-reaction reporting systems, and stimulate to the establishment of reporting systems for severe food-allergic reactions in more countries (Björkstén *et al*., 2008).

8.4 Allergen Thresholds

The main regulatory measure to prevent food-allergic reactions is labelling. The basis of the labelling system is the listing of ingredients in the food. Additionally, in the European Union (EU), Annex IIIa (EFSA, 2004b; European Commission, 2007) identifies certain allergens that are of such importance that their presence should be indicated by labelling without exemptions granted for other foods. Labelling is mandatory, both in the presence of the allergenic food component itself and in the presence of 'products derived thereof', and there is no administrative threshold for the quantity of allergen necessary to trigger labelling. However, in the EU, it is possible to apply for exemption from labelling if allergens are no longer present in a product because of removal during the production process or, in principle, extreme dilution. Such exemptions have to be considered on an individual basis for a particular type of product, and

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a number of exemptions have been granted based on documentation that allergens are no longer present in the product in quantities sufficient to cause a problem. However, this has been decided by expert judgement. There have been no defined allergen thresholds and, as a matter of fact, the EFSA has concluded that, at present, there is not a sufficient scientific basis for establishing allergen thresholds (EFSA, 2004b).

Thresholds for labelling are most wanted by the food industry. However, in the population, individual allergen thresholds vary greatly between allergic persons, from less than 1 mg to many grams of the food (Taylor *et al*., 2002). Also, threshold variation from time to time for a given individual must be expected because of natural increase or waning of allergy strength, and because of varying presence of aggravating factors like exercise, mental stress, alcohol, medications and infections. Some individuals are extremely sensitive, and it will probably not be possible to find a common population threshold that will protect even the most sensitive. Instead, agreement on an administrative threshold that protects a great majority of food-allergic individuals may be the solution. Ongoing activities seek to determine thresholds for the foods for which information is best, like peanut.

8.5 Classification of Foods for Regulatory Measures

Novel foods, including genetically modified foods, have to be evaluated with regard to allergenicity. However, with regard to traditional foods, the classification of foods in relation to allergy may seem somewhat arbitrary. First, there are no general rules and recommendations at the national or international level with regard to criteria for which allergens should be labelled. There are a couple of hundred food allergens, many of them obviously of little importance because only one or a very few cases have been reported worldwide, and any regulatory measure would seem out of proportion to the problem. In the other end, some allergens, like peanut, fish and so on, obviously are of great importance and warrant the cost to society, manufacturers and consumers of labelling. The problem with regard to administrative measures is the 'intermediate' food allergens in the gradient from obviously important to obviously trivial. However, some attempts are currently being made to elaborate criteria and a framework for assessing the public-health importance of food allergens, based on the principles of evidence-based medicine (Björkstén *et al*., 2008). If successful and generally accepted, such criteria would make the evaluation of food allergens more similar in different countries and trade regions, would increase predictability in relation to administrative measures and increase transparency of the decision-making process. Key factors for the importance of food allergens are documented incidence of reactions, prevalence of clinical allergy and sensitization, potency of the allergen and exposure factors (extent and mode of use). The prevalence of sensitization to the allergen is a surrogate marker of limited value, because for some allergens (e.g. some legumes) sensitization is rather common, but reactions rarely occur, and, in general, sensitization grossly overestimates the number of people who would react to an allergen (Kagan *et al*., 2003).

Regulatory documents do not appear consistent in their grouping of food allergens. For example, in the EU's Annex IIIa (EFSA, 2004b; European Union, 2007), an 'allergen' that has to be labelled without exemptions is 'molluscs and products thereof'. Molluscs include more than 100 000 species. On the other hand, 'peanuts and products thereof', 'soya and products thereof' and 'lupin and products thereof' are listed separately, instead of combining them in a class called 'legumes and products thereof'. Various other legumes, like peas and beans are foods allergens of minor importance, and many molluscs certainly are also of little or no importance as allergens. Therefore, the grouping of food allergens to be labelled may seem inconsistent with regard to inclusion and limitation criteria. This may be due to the different importance of some foods, or it may reflect the history of allergen labelling.

9 GLUTEN INTOLERANCE

Gluten intolerance causing coeliac disease (gluten-sensitive enteropathy, sprue) is an autoimmune condition triggered by gluten protein, which is found in wheat, rye and barley. Gluten intolerance does not involve IgE and is not an allergy in the classical meaning of the word. Gluten intolerance has a range of different clinical expressions. A life-long gluten-free diet excluding wheat, rye and barley forms the basis for treatment of coeliac disease.

Including classical as well as oligosymptomatic and silent forms in children and adults, the prevalence of coeliac disease in Europe, as well as North and South America, is about 1:100–1:200 (Fasano and Catassi, 2008). Limited data from North Africa and Southwest Asia suggest a similar high prevalence for these areas (Accomando and Cataldo, 2004). The highest reported prevalence worldwide of 5.4% is found in the Sawahari population of North Africa (Catassi *et al*., 2001; Fasano and Catassi, 2008). In central Africa and the Far East, coeliac disease appears to be extremely rare (for references, see Dubois and van Heel, 2008). Disease prevalence mirrors the prevalence of coeliac-disease-associated genes (see below) and broadly also grain consumption (Fasano and Catassi, 2008). An environmental factor suggested to play a role is gastrointestinal viral infections in infancy (for references, see Dubois and van

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Heel, 2008). Countries with low infant gluten consumption (Denmark, Estonia, Finland) have a lower infant, as well as adult, incidence of coeliac disease than countries with a high infant gluten consumption, like Sweden (Mitt *et al*., 1998; Weile *et al*., 1995).

Gluten is the rubbery dough-forming protein which remains after the washing of wheat flour to remove starch. Gluten is a well-defined storage protein with a high content of glutamine and proline, and consists of glutenin and gliadin fractions. Glutenin is a high-molecular-weight fraction insoluble in alcohol, and the toxicity of this fraction in gluten intolerance has not been well characterized. The alcohol-soluble gliadin (mw 28 000–39 000) contains mainly monomeric low-molecular-weight proteins. Both fractions consist of numerous closely related protein components with repetitive peptide units. These repetitive peptide units are found in the α-, γ- and ω-subtypes of gliadin, which have all been shown to elicit the disease equally. A 33-mer peptide has been identified as a primary initiator of the inflammatory response in coeliac disease (Shan *et al*., 2002), and the amino acid sequences of several peptides that form potent T-cell epitopes in gluten intolerance have been characterized.

Wheat, rye and barley all contain gluten and glutenin proteins, collectively called prolamins. Maize, rice, buckwheat, millet and sorghum do not have any of these, whereas oats contain low amounts of prolamins. Consequently, while wheat, rye and barley have been documented to trigger coeliac disease (Wieser, 1996), maize, rice and buckwheat have been found not to be harmful. There is some disagreement about the coeliac toxicity of oats (Janatuinen *et al*., 2002; Lundin *et al*., 2003). Spelt (a primitive form of wheat) has often been claimed by coeliac disease patients to be better tolerated than 'modern' wheat, but spelt is just 'wheat spelt another way' without substantial differences that could explain a significant difference from modern wheat cultivars in relation to gluten intolerance. However, different wheat cultivars differ in their content of proteins, and the ratio of total gluten to gliadin can vary from 1.3 to 1.6. Complete acid hydrolysis abolishes the 'toxicity' of gluten, but partial hydrolysis and enzymatic peptic-tryptic degradation of gluten does not affect gluten intolerance-triggering properties because important peptide units are left unaffected. Heat treatment (baking) does not change gluten 'toxicity', but food technology processes affect extractability and detectability of gluten and thereby affect quantitative gluten measurements (Stern *et al*., 2001).

The normal daily intake of gluten is 15–20 g in the adult European population. The relationship between the amount of gluten ingested and the severity of clinical changes and histological abnormalities is still undefined (EFSA, 2004b). It appears that there is a threshold below which most, if not all, coeliac disease patients would tolerate trace amounts of gluten. However, individual variation and clinical heterogeneity make it difficult to find an acceptable threshold value (Stern *et al*., 2001). Challenge and dietary intake studies have shown that a daily intake of 100 mg of gliadin was sufficient to elicit typical coeliac changes in coeliac-disease patients, whereas a daily gliadin intake of 4–14 mg did not cause any small intestine mucosal damage (for references, see EFSA, 2004b). From a clinical point of view, a threshold value in terms of a daily allowable intake would be preferable to a product-centred mg kg^{-1} limit (EFSA, 2004b).

Cereals containing gluten and products thereof are among the foods on the EFSA 'allergen list', Annex IIIa, foodstuffs that always should be labelled without the exemptions granted most foods (European Commission, 2007). The new Codex Alimentarius Guidelines (Codex Alimentarius Commission, 2008) have a gluten level limit of 20 mg kg^{-1} for 'gluten-free foods', that is foods consisting of naturally gluten-free ingredients that do not contain any prolamin from wheat or *Triticum* grain species, such as spelt, kamut or durum wheat, rye, barley, oats and their crossbred varieties, as well as for foods in which gluten has been removed. Other foods consisting of ingredients with low gluten levels should be labelled 'low-gluten' if the gluten level is less than 100 mg kg^{-1} (effective from 2012) (Codex Alimentarius Commission, 2008).

The sensitivity, specificity and reproducibility of most methods for gluten analysis previously were unsatisfactory (Stern *et al*., 2001). Still, a European gliadin reference is now available, and immunoassays have improved (EFSA, 2004b). However, analytical problems due to heated or hydrolysed material and matrix effects have not been fully overcome.

The diagnosis of coeliac disease relies on small intestinal biopsy showing a severe mucosal lesion with inflammatory cells and flattening of the mucosa, with subsequent restoration of normal morphology and disappearance of symptoms on a gluten-free diet, which forms the cornerstone of coeliac-disease treatment (Fasano and Catassi, 2008; Green and Cellier, 2007; Polanco, 2008). Serological methods, like determination of autoantibodies against endomysium and gliadin antibodies are useful supplements to the diagnostic procedure, and particularly are useful for screening. Coeliac disease is strongly associated with the major histocompatibiliy antigens HLA-DQ2 and DQ8 (Fasano and Catassi, 2008; Marsh, 1992; Schuppan, 2000; Sollid, 2002). The HLA-DQA1*0501, DQB1*0201 gene is found in over 90% of patients. Gluten peptides are presented to immunocompetent cells by DQ2- and DQ8-positive antigen-presenting cells in the small intestine lamina propria. Tissue transglutaminase is released and will potentiate antigen presentation by deamidating or crosslinking gluten peptides (Schuppan, 2000), and T cell activation, cytokine production,

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inflammation and mucosal destruction occur. Secondary to the cellular immune reaction, production of antibodies against the important endomysial autoantigen tissue transglutaminase and against gliadin takes place. One result of the mucosal damage is malabsorption, which may lead to various deficiency-related symptoms.

The classical picture of coeliac disease with severe diarrhoea, vomiting and failure to thrive becoming manifest at 6–24 months of age is rarely seen today, as a shift from infant and childhood age to adolescent and adult age at diagnosis has taken place (Vivas *et al*., 2008). The classical form of coeliac disease with severe symptoms forms only the 'tip of the iceberg', with relatively silent and latent forms under the 'iceberg' waterline. Less specific deficiency symptoms, abdominal pain, liver disease, osteoporosis, arthritis and dermatologic and neuropsychiatric disease are common, and extraintestinal manifestations should always be considered (Fasano and Catassi, 2008; Vivas *et al*., 2008). There is in coeliac disease an increased risk of intestinal malignancy, and there is evidence that adherence to a strict gluten-free diet reduces this risk and also the risk of bone and autoimmune disease (Haines *et al*., 2008). In particular, coeliac-disease patients who do not respond to a gluten-free diet (so-called refractory coeliac disease, RCD), or rather a subgroup of these (Type 2 RCD) are at high risk of complications such as ulcerative jejunitis and enteropathy-type T-cell lymphoma (Ho-Yen *et al*., 2008). There is an association between coeliac disease and other cell-mediated autoimmune diseases, namely Type 1 diabetes, Graves' disease, rheumatoid arthritis and psoriasis, and, in particular, the association with Type 1 diabetes is striking (Dubois and van Heel, 2008; Farrell and Kelly, 2002; Neuhausen *et al*., 2008; Zhernakova *et al*., 2007). Type 1 diabetes and coeliac disease both appear to have shown rising incidence in recent years (for discussion and references, see Dubois and van Heel, 2008). The association is evident on the gene level (Dubois and van Heel, 2008), and one may speculate that there are also common causative environmental factors, possibly shared with allergic diseases that similarly have shown a marked increase the recent decades.

9.1 Dermatitis Herpetisformis

Dermatitis herpetiformis (DH) is a condition with cutaneous papulovesicular, pruritic lesions on extensor surfaces of the elbows, knees, buttocks, back and scalp. The lesions are precipitated by ingestion of gluten (Leonard *et al*., 1983). The skin eruptions are related to immunoglobulin A (IgA) deposits in the dermal papillae, a phenomenon that is pathognomonic for DH (Zone *et al*., 1996). The granular deposits of IgA are present in perilesional as well as unaffected skin. The concentration of IgA at the affected sites decreases with adherence to a gluten-free diet, and increases again if a gluten-containing diet is introduced (Leonard *et al*., 1983). The antigenic specificity of the IgA is not known, but it colocalizes with epidermal transglutaminase 3 (TG3) (not tissue transglutaminase 2, the target of immune response in coeliac disease) (Hull *et al*., 2008). DH is known nearly always to be associated with coeliac disease, but the intestinal inflammation process in DH is generally less severe than that seen in coeliac disease (Katz and Strober, 1978). Treatment consists of gluten-free diet and the drug dapsone.

10 LACTOSE INTOLERANCE

Lactose intolerance is caused by a reduced capacity to digest lactose, due to primary or secondary lactase (β-galactosidase) deficiency. It is not an allergic or immune-mediated disease.

Lactose is a disaccharide exclusively present in milk and milk products. Lactose cannot be absorbed as such, and is normally hydrolysed in the small intestine by lactase to give glucose and galactose. Galactose is converted into glucose in the liver. If lactose is not hydrolysed, it will exert an osmotic effect and cause accumulation of water and sodium in the intestinal lumen. This will increase the volume of the intestinal content and speed up its transit. Lactose will then reach the large intestine, where it is fermented by the colon microflora. Short-chain fatty acids and gases, including hydrogen and methane will be produced (Pouteau *et al*., 1998), and the fermentation products may cause clinical symptoms. These sometimes start as early as 15 minutes, and generally within 1–3 hours, after lactose ingestion, and consist of stomach and intestinal distention with variable pain, and then flatulence and borborygmia. In more severe cases diarrhoea may follow.

Lactase activity in the enterocytes is regulated primarily by genetic factors in healthy individuals, and is not induced by chronic lactose consumption (Lisker *et al*., 1975). Reduced lactase activity in the intestine can be due to an inherited deficiency in lactase that affects infants (MIM:223000; congenital lactase deficiency, also known as hereditary alactasia or disaccharide intolerance II); however, this form is rare. The most common form of lactase deficiency affects adults, because lactase activity normally is reduced 10- to 20-fold from infantile to adult levels at the age of 3–5 and up to 8–12 years in 75% of the world's population (Beja-Pereira *et al*., 2003). About 25% of the population of the world maintain infantile lactase levels in adulthood (lactase persistence; MIM:2231000) (Scrimshaw and Murray, 1988). Lactase deficiency is inherited as a recessive autosomal trait (Enattah *et al*., 2002), with no gender difference.

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In Europe, 'lactase persisters' are in the majority, and constitute 90–95% of the population in the Nordic countries. A gradient with decreasing prevalence of 'lactase persisters' can be observed as one goes further south in Europe and into Africa and Asia, with the exception of population groups traditionally relying on cattle, and specifically milk products, for food (Bersaglieri *et al*., 2004; Check, 2006; Gudmand-Hoyer *et al*., 1969; Gudmand-Hoyer and Jarnum, 1969; Sahi *et al*., 1983; Sahi, 1994; Swallow, 2003).

Lactose maldigestion thus is a normal physiological response to lactase intake in about 75% of the world's adult population. Symptoms will develop when the capacity of lactose activity is exceeded by the quantity of lactose present in the gut. Doses less than 10 g of lactose, corresponding to about 200 ml of milk (one to two glasses), are tolerated by most lactase-deficient adults. Clinical symptoms will occur in more individuals the higher dose of lactose ingested, and the number of intolerant individuals will decrease when the lactose dose decreases (Marteau *et al*., 2002; Vesa *et al*., 1996a; 2000). The lactose concentration reaching the colon depends largely on the lactose dose ingested, but also on how fast the lactose is consumed, the degree of dilution and the composition of the meal (matrix factors) (Martini and Savaiano, 1988). The relationship between the dose of lactose, the concentration of lactose that reaches the colon and maldigestion symptoms is certainly complex, but experiments have shown that a linear relationship exists in the range of typical serving sizes (6–20 g) (Hertzler *et al*., 1996; Savaiano, 2002). There appears to be some capacity of the colon microflora to adapt and regulate fermentation (Hertzler and Savaiano, 1996), and symptoms of intolerance are minimized in an acidified colon. Some lactase-deficient individuals appear not to have symptoms because their bacterial flora metabolize lactose (Campbell *et al*., 2005). Exogenous lactase added to the food or lactase produced, for instance, in fermented foods like yoghurts may enhance digestion of lactose (Marteau *et al*., 1990; Vesa *et al*., 1996b).

The gold standard in diagnosis of lactase deficiency is the measurement of lactase activity in biopsies of intestinal mucosa, but this is impractical for routine diagnosis. Commonly used is measurement of indirect phenotype markers, namely serum glucose (or galactose) levels after peroral lactose challenge, and H_2 or CH_4 in expiratory air (Arola, 1994). An SNP (single nucleotide polymorphism), C/T-13910 polymorphism, has increasingly been used in the diagnosis of lactase deficiency (Buning *et al*., 2005; Enattah *et al*., 2002). The C/C-13910 genotype corresponds to low or absent adult intestinal lactase activity, while the C/T-13910 and the T/T-13910 genotypes are associated with the presence of full lactase activity. However, some discrepancies between the different methods, including genotyping, and clinical symptoms have been observed in various studies.

In addition to the primary, genetically determined lactase deficiencies, secondary lactase deficiencies occur due to intestinal diseases like, for example, food allergy, coeliac disease and gastroenteritis. Depending on the triggering condition, secondary lactase deficiency will be transient. Secondary lactase deficiency must be considered in diagnostic work.

Iin the EU, products derived from milk have to be labelled according to the rules set down in Annex IIIa (European Commission, 2007). A variety of dietary 'lactose-free' or 'lactose-reduced' food items, which, with standard ingredients or manufacturing procedures would contain (higher levels of) lactose, are commercially available. Also, some milk products contain only low levels of lactose, for example some cheeses, and fermented milk products are in general considered to be better tolerated than nonfermented products.

11 FRUCTOSE INTOLERANCE

11.1 Introduction

Fructose is a monosaccharide common in human food, and a significant source of energy. High amounts are found in many fruits, sweets and confectionery, and some vegetables. Further, fructose is a component of sucrose. Sorbitol can be metabolized into fructose. Mean daily intake of free fructose has been reported to be about 15 g day^{-1}. If fructose in sucrose is added, total fructose consumption in heavy consumers may reach 150 g day^{-1}.

Fructose is not an allergen. However, different forms of fructose intolerance exist. Inborn errors of fructose metabolism involving different enzymes are often grouped into: (i) nonsevere conditions and (ii) severe conditions (EFSA, 2005).

11.2 Nonsevere Fructose Intolerance Conditions

Fructose malabsorption is common, but does not lead to severe symptoms and has been questioned as a disease entity (Rumessen, 1992). It is diagnosed by a breath hydrogen test after an oral fructose load. Fructose malabsorption is often encountered in patients with unspecific diarrhoea, abdominal pain and bloating. The gastrointestinal discomfort usually responds well to reduction of fructose in the diet, and there is no organ pathology (Gomara *et al*., 2008). In addition, there is a rare autosomal condition called isolated fructose malabsorption

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which has been linked to a deficiency of the GLUT5 transporter (Rumessen, 1992; Wasserman *et al*., 1996). Benign fructosuria is a deficiency of fructokinase, with no symptoms and no need for therapy (van den Berghe, 2000). In this condition, ingested fructose cannot be metabolized, and increased levels can be found in blood and urine.

11.3 Severe Fructose Intolerance Conditions

Hereditary fructose intolerance (HFI) is a potentially life-threatening condition, due to the absence of aldolase B (Ali *et al*., 1998; Odièvre *et al*., 1978). This leads to liver and kidney accumulation of fructose-1-phosphate and severe toxic symptoms due to inhibited glucose production and depletion of ATP. Prevalence worldwide has been reported to be 1:23 000. Heterogeneity of the condition is indicated by differences in clinical sensitivity to fructose, as well as different mutations of the affected aldolase B gene (Davit-Spraul *et al*., 2008), and some affected subjects remain undiagnosed for a long time. Babies typically do well during exclusive breast-feeding, but after introduction of sucrose, fruits and vegetables develop nausea, vomiting, lethargy and convulsions, together with hypoglycaemia. Severe liver disease and renal failure will follow. Diagnosis is made by assay of liver aldolase activity and DNA analysis. Therapy of HFI involves avoidance of all known dietary sources of fructose, sucrose and sorbitol. There is no scientifically established and generally accepted admissible level for fructose intake, but one recommendation is that a fructose-free diet should contain less than 1.5 g of fructose per day (Bell *et al*., 1987). Affected infants appear more sensitive to fructose than adults. Long-term prognosis on diet is good.

The very rare fructose-1,6-diphosphatase deficiency (FBPase deficiency) is also potentially life-threatening. The metabolic defect is, strictly, not in the fructose pathway, but in gluconeogenesis. About half the patients present in the first week of life with life-threatening acidosis, hypoglycaemia, hyperventilation, convulsions and coma. Acute treatment consists of glucose infusion and treatment of acidoses. Lipid storage causes hepatomegaly. Symptoms are precipitated by febrile conditions and fasting. Whereas treatment includes limitation of dietary fructose, complete avoidance is not necessary. With proper treatment, the long-term prognosis is good.

Fructose, sucrose and sorbitol can be analysed in food by enzymatic methods, as well as high-performance liquid chromatography (HPLC). The limit of detection is dependent on the complexity of the samples and matrix and extraction methods.

In the EU, there are no specific labelling requirements for fructose apart from those for food ingredients in general.

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Developmental Immunotoxicology

Rodney R. Dietert

CONTENTS

1 INTRODUCTION

The immune system serves as the bedrock in any consideration of risk of disease and wellbeing of the host. This is based predominantly on its two primary functions: (i) to identify, remove and/or destroy foreign invaders and (ii) to survey tissues and to maintain tissue integrity and homeostasis throughout the body. To perform these functions there are two relatively novel features found in the immune system that are lacking in many toxicological targets: (i) the immune system is fully dispersed throughout the body, with representation in most tissues, including the brain, and (ii) the system is designed to be largely mobile with cells responding to a variety of chemical signals through chemotaxis and relocation within the body.

The immune system is designed such that, when effective, responses against invading pathogens and emerging tumour cells are tailored to the task at hand, including the nature of the disease challenge, the temporal nature of the challenge and the specific location of the challenge. This range of potential responses coupled with a tailoring of an individual response is necessary, since during the course of a lifetime an individual will be exposed to pathogens ranging from small intracellular deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) viruses to large extracellular bacteria and parasites. It is increasingly recognized that inappropriate immune responses in scope, duration, location or specificity have the potential to be as problematic in terms of disease risk as are insufficient immune responses. For this reason, significant environmentally induced modulations of immune response potential represent a likely health risk.

The implications of the disperse nature of the immune system are that all routes of exposures and all local insults have the potential for producing immunotoxicity. Additionally, because the immune system is designed to mobilize cells to sites of tissue/cellular perturbation, local insults have the potential to involve immune cells far removed from the exposure route/location. Local insults (via dermal, oral, inhalation routes of exposure) can sometimes produce adverse immune changes elsewhere in the body (e.g. oral exposures increasing lung inflammatory responses). All of these characteristics of the immune system and its response to the environment have a potential impact on the nature and effectiveness of safety-testing strategies.

2 DEVELOPMENTAL IMMUNOTOXICOLOGY (DIT)

As a subdivision of immunotoxicology, developmental immunotoxicology (DIT) was recently defined as the effects on the immune system resulting from the exposure of the nonadult to physical factors (e.g. ionizing and ultraviolet radiation), chemicals (including drugs), biological materials, medical devices and, in certain instances, physiological factors, collectively referred to as

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agents (Luster *et al*., 2008). Clearly, this includes many different categories of environmental factors, including environmental contaminants, as well as drugs. While the focus of this chapter is on toxicants, Dietert and Piepenbrink (2008) suggested the value of considering an integrated womb-to-tomb approach to developmental immune protection given both the diversity of environmental factors (toxicants, diet, microbial exposure) that impact immune development and the life-long health ramifications that are integrally tied to effective immune function.

The study of DIT dates back to at least the 1970s (Luster *et al*., 1978), although a substantial amount of the research has been performed during the last decade. More recently, the focus has been on comparative sensitivity to xenobiotics of the developing immune system vs. that of the fully matured adult (Luebke *et al*., 2006a; 2006b). This has shifted discussion towards strategies to improve protection of the nonadult. DIT has been the topic of several workshops, symposia, reviews and books, where the focus has ranged from health effects to immune-assessment strategies and finally use of developmental immunotoxicity data in risk assessment (Dietert *et al*., 2000; 2002; Holladay and Smialowicz, 2000; Luster *et al*., 2003; 2005; Holsapple *et al*., 2004; 2005; Ladics *et al*., 2005; Dietert, 2005; 2008a; 2008b; 2008c; Holladay, 2005; Dietert and Piepenbrink, 2006; Dietert and Holsapple, 2007; Smialowicz *et al*., 2007; Burns-Naas *et al*., 2008).

The idea that a developing physiological system has critical developmental windows of vulnerability that increase the likelihood of environmentally induced dysfunction is not a concept restricted solely to the immune system. In fact, early-life events seem to exert an unexpectedly significant influence on the later-life functional capacity of several systems, including the immune, neurological, cardiovascular and endocrine systems.

3 EARLY-LIFE ORIGINS OF LATER-LIFE DISEASE: THE BROADER CONTEXT FOR DIT

The idea that foetal environmental programming plays a significant role in later-life disease risk, also known as the Barker hypothesis, has existed for more than a decade and was originally presented to connect intrauterine growth with later-life risk of cardiovascular disease (Barker, 1997). In recent years, this concept has been extended to include the neonatal period of maturation and to involve a broader array of environmental factors (toxicants, diet, infectious agents, physical stressors) impacting a wider range of diseases (Grandjean, 2008). Part of this foetal origin of disease concept is the notion that problematic early-life environmental exposures can cause a rewiring of specific physiological systems, thereby changing the course of ageing and lifelong disease risk. Environmentally induced prenatal–perinatal physiological rewiring has been considered for the immune (McDade, 2005; Phillips, 2006), endocrine (Kajantie, 2008), reproductive (Nijland *et al*., 2008), vascular (Gardiner, 2007) and skeletal (Lanham *et al*., 2008) systems.

Among the diseases with either pediatric or adult onset suggested to have early-life origins and environmental risk are: adult inflammatory bowel disease (Aspberg *et al*., 2006; Danese *et al*., 2007), allergic diseases (Prescott, 2003), Alzheimer's disease (Whalley *et al*., 2006), autoimmune diseases (Brown *et al*., 2006; Peng and Hagopian, 2006; Svensson *et al*., 2004), cardiovascular disease (Barker and Bagby, 2005; Louey and Thornburg, 2005), cerebral palsy (Jacobsson and Hagberg, 2004), lung disease (Landau, 2006), Parkinson's disease (Barlow *et al*., 2004), schizophrenia (Meyer *et al*., 2006; 2008; Ozawa *et al*., 2006), obesity (Krechowec *et al*., 2006), renal disease (Ingelfinger and Schnaper, 2005), metabolic syndrome (Gluckman and Hanson, 2004) and Type 2 diabetes (Jones and Ozanne, 2007).

This chapter considers the potential role of DIT (the prenatal, neonatal, juvenile and adolescent periods of development) in immune dysfunction and disease risks.

4 IMMUNE DEVELOPMENT AND CRITICAL WINDOWS OF IMMUNE VULNERABILITY FOR DIT

Immune maturational events occurring during the prenatal and early neonatal periods of life represent unique processes that lack exact equivalents in the adult. It is during these maturational events that the specialized immune-cell populations arise (e.g. T regulatory cells, Th17 cells, microglia, alveolar macrophages) seed the periphery and then further mature in response to additional gestationally timed physiological factors (e.g. surfactants). For this reason, each immune maturational event is a potential target of environmental disruption, with immune dysfunction as the likely adverse outcome. This dysfunction may take several forms, including not only immunosuppression resulting in an increased risk of infectious disease and cancer, but also misregulation producing an increased risk of allergic diseases and asthma, autoimmune diseases and inflammation-associated neurological disorders.

The novelty of the critical windows of vulnerability for the developing immune system has ramifications that impact hazard identification and risk assessment. First, because many of the key immune-cell maturation events are not replicated in the adult, it is not possible to effectively model whether a potential toxicant might be likely

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to disrupt prenatal immune events using adult animals or cells. Even *in vitro* effects of known toxicants are different among adult human vs. adult cord-blood-derived immune cells (Mainali and Tew, 2004). Therefore, strategies relying upon the use of adults to predict risk to the developing immune system following xenobiotic exposure or as a trigger for subsequent immunotoxicity testing in the nonadult are flawed and lack both sensitivity and specificity of modelling (Dietert and Piepenbrink, 2006).

The prenatal and neonatal process of immune maturation results in the spectrum of immune effector and regulatory cells that, together, mediate immune responses and regulate the nature, location, type and duration of responses. While genetic background is a factor in later-life immune potential, the environmental condition surrounding immune development plays a critical role in establishing postnatal immune capacity, and, in turn, influences later-life health and wellbeing. For this reason, identification of the risk of xenobiotic exposure to the developing immune system provides an important tool for enhanced disease prevention.

Several reviews have considered the sequence and timing of the novel events that occur during the maturation of the developing immune system, as well as the potential problems that can arise when these events are disrupted by xenobiotic exposure (Holladay and Smialowicz, 2000; Dietert *et al*., 2000; Landreth, 2002; Holsapple *et al*., 2003; Landreth and Dodson, 2005; Leibnitz, 2005; Dietert and Piepenbrink, 2006). More recently, the idea of immune-critical windows relative to DIT has been expanded to include not only those events that have systemic ramification for the offspring (e.g. T-cell clonal selection in the thymus), but also those having more specialized local/regional impact (e.g. microglia seeding, expansion and activation in the brain) (Dietert and Dietert, 2008; Luster *et al*., 2008).

Figure 1 shows a series of systemic and tissue-specific (or local) immune-critical developmental windows, which are maturational benchmarks that impact subsequent distribution and functional capacities of later-life immune-cell populations. Not surprisingly, xenobiotic-mediated disruptions during these intervals are likely to produce the most significant adverse immune

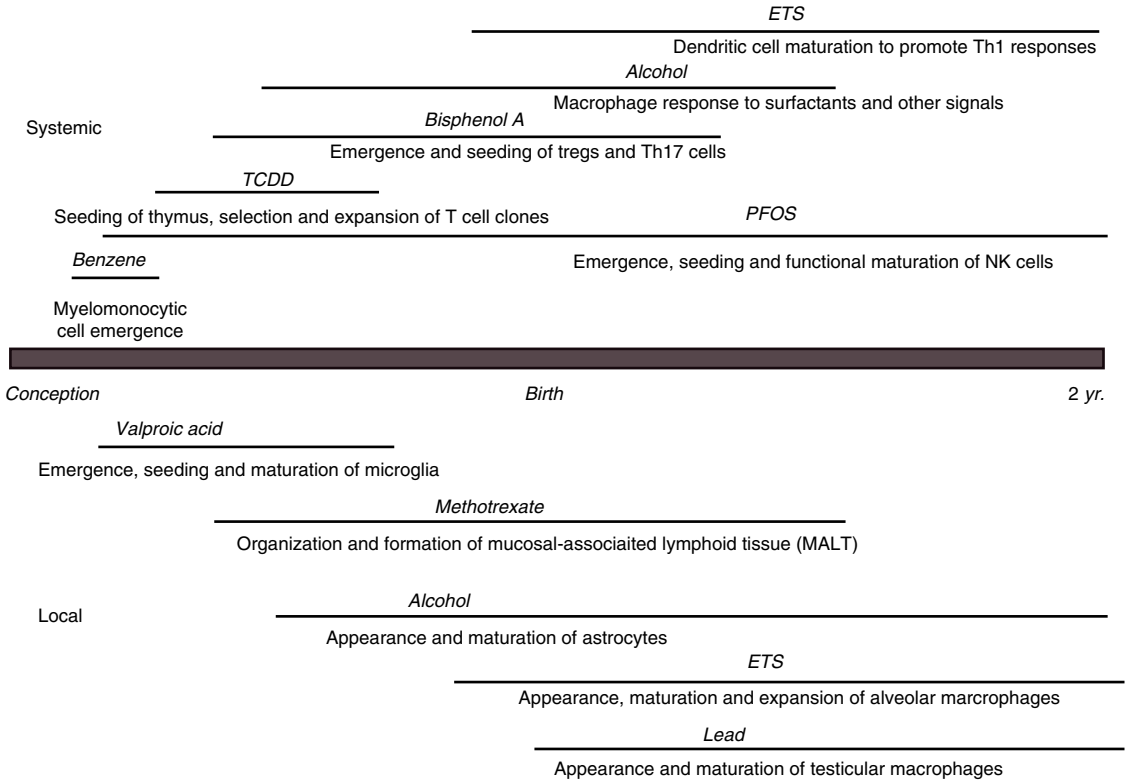

Figure 1 A timeline map of human critical development windows of immune vulnerability is shown from conception until two years after birth. It is divided into two sections. The top section shows six immune maturational processes that are systemic and represent key points of vulnerability for environmental disruption. The bottom section shows five immune maturational processes that pertain to a specific tissue (e.g. brain, lung, testes) or group of tissues (mucosal tissue). A chemical or drug reported to disrupt the specific maturation event is listed above each line segment for the event (e.g. alcohol for the maturation of astrocytes). (Adapted from information in Dietert and Piepenbrink, 2006; Dietert and Dietert, 2008.) Treg = T regulatory cells; Th17 = T helper 17 cell population (which produce Interleukin 17); ETS = environmental tobacco smoke; PFOS = perfluorooctane sulfonate; TCDD = 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

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Table 1 Examples of xenobiotics with reported early-life immunotoxicity

Chemical or drug	Sample reference
13-*cis*-Retinoic acid	Makori *et al*. (2002)
2,3,7,8-Tetrachlorodibenzo -*p*-dioxin	Hogaboam *et al*. (2008)
7,12-Dimethylbenzanthracene	Holladay and Smith (1995)
Acyclovir	Stahlmann *et al*. (1992)
Alflatoxin B1	Dietert *et al*. (1985)
Arsenic	Soto-Pena *et al*. (2006)
Atrazine	Rowe *et al*. (2008)
Benzene	Corti and Snyder (1996)
Benzo(a)pyrene	Holladay and Smith (1994)
Cadmium	Pillet *et al*. (2005)
Cannabinoids	del Arco *et al*. (2000)
Capsaicin	Santoni *et al*. (1995)
Chlordane	Barnett *et al*. (1987)
Chlorpyrifos	Navarro *et al*. (2001)
Cocaine	Rofael *et al*. (2003)
Ciclophosphamide	Kato *et al*. (2007)
Ciclosporin	Hussain *et al*. (2005)
Cypermethrin	Santoni *et al*. (1998)
Dexamethasone	Dietert *et al*. (2003)
Diazepam	Schlumpf *et al*. (1994)
Diesel exhaust	Fedulov *et al*. (2008)
Diethylstilbestrol	Luster *et al*. (1979)
Dimethoate	Institóris *et al*. (1995)
Dimethylbenz[a]anthracene	Holladay and Smith (1995)
di-*n*-Octyltin dichloride	Seinen *et al*. (1979)
Ethanol	Ping *et al*. (2007)
Ethylene glycol monomethyl ether	Holladay *et al*. (1994)
Genistein	Guo *et al*. (2006)
Heptachlor	Smialowicz (2002)
Hexachlorobenzene	Barnett *et al*. (1987)
Indomethacin	Kushima *et al*. (2007)
Lead	Miller *et al*. (1998)
Mercury	Silva *et al*. (2005)
m-2,3-Dimercaptosuccinic acid	Chen *et al*. (1999)
Methoxychlor	Chapin *et al*. (1997)
Nonylphenol	Karrow *et al*. (2004)
Ochratoxin	Thuvander *et al*. (1996)
Ozone	Selgrade *et al*. (2008)
Paracetamol	Shaheen *et al*. (2005)
Perfluorooctane sulfonate	Keil *et al*. (2008)
Polychorinated biphenyls	Heilmann *et al*. (2006)
T-2 toxin	Holladay *et al*. (1993)
Tobacco smoke	Ng *et al*. (2006)
7-Tributyltin oxide	Smialowicz *et al*. (1989)
Trichloroethylene	Peden-Adams *et al*. (2006)
Urethane	Luebke *et al*. (1986)

outcomes. An example of a chemical or drug that has been reported to disrupt the given immune maturational event is shown above each window. For many of these windows several xenobiotics are known to be capable of causing disruption. The idea of systemic critical windows for developmental immunotoxicity dates back for nearly a decade (Dietert *et al*., 2000; Holladay and Smialowicz, 2000; Bunn *et al*., 2001a; Landreth, 2002; West, 2002). However, a more recent expansion of this concept is that resident immune-inflammatory cells in different tissues (e.g. brain, lung, testes) may each have their own critical developmental windows where environmentally mediated disruption can contribute to later-life disease (Dietert and Dietert, 2008). This local or tissue-specific aspect of DIT becomes particularly important in cases where organ cell loss or tissue damage plays a role in specific childhood or adult diseases.

5 CHEMICALS AND DRUGS WITH REPORTED IMMUNOTOXIC ACTIVITY FOR THE DEVELOPING IMMUNE SYSTEM

Not surprisingly, a majority of immunotoxicology research and testing described in the literature is a result of adult animal exposures or the use of adult-derived cells. However, with the concern over early-life origins of later-life disease, DIT research has been increasing. A substantial spectrum of xenobiotics has been reported to produce adverse immune effects following exposure during prenatal, neonatal and/or juvenile periods of development (Dietert and Dietert, 2007). Some of these are connected with specific health concerns (Holladay, 1999; 2005; Gauthier *et al*., 2005a; Heilmann *et al*., 2006; Selgrade *et al*., 2006; Yeatts *et al*., 2006; Goodwin, 2007; Hamada *et al*., 2007; Ohshima *et al*., 2007; Luster *et al*., 2008; Selgrade, 2007; Hertz-Picciotto *et al*., 2008; Wang *et al*., 2007; Wang and Pinkerton, 2008).

Table 1 shows examples of chemicals and drugs with reported capacity to target the developing immune system. Among the most extensively studied xenobiotics affecting the developing immune system are: atrazine (Rooney *et al*., 2003; Rowe *et al*., 2006; 2008), dexamethasone (Eishi *et al*., 1983; Dietert *et al*., 2003; Mainali and Tew, 2004; Mainali *et al*., 2005), diethylstilbestrol (Fenaux *et al*., 2004; Besteman *et al*., 2005; Brown *et al*., 2006), oestrogenic and endocrine-disrupting compounds (Karrow *et al*., 2004; Yoshino *et al*., 2004; Guo *et al*., 2005a; 2006; Yang *et al*., 2008), ethanol (Gauthier *et al*., 2005b; Lazic *et al*., 2007), halogenated aromatic hydrocarbons (Arena *et al*., 2003; Camacho *et al*., 2004; Walker *et al*., 2004), heavy metals (Luster *et al*., 1978; Miller *et al*., 1998; Snyder *et al*., 2000; Sun *et al*., 2003;

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Dietert *et al*., 2004; Pace *et al*., 2005; Pilones *et al*., 2007), immunosuppressive drugs (Padgett and Seelig, 2002; Hussain *et al*., 2005; Prakash *et al*., 2007), mycotoxins (Dietert *et al*., 1983; Qureshi *et al*., 1995; 1998; Silvotti *et al*., 1997), organochlorine pesticides (Barnett *et al*., 1990; Theus *et al*., 1992; Chapin *et al*., 1997; Smialowicz *et al*., 2001; White *et al*., 2005), polycyclic aromatic hydrocarbons (Holladay and Smith, 1994; 1995; Rodriguez *et al*., 1999), tobacco smoke (Gilmour *et al*., 2006; Ng *et al*., 2006; Penn *et al*., 2007; Johansson *et al*., 2008; Wang *et al*., 2008) and tributyltin compounds (Smialowicz *et al*., 1989; Tryphonas *et al*., 2004). The diversity of xenobiotic factors affecting the developing immune system provides added incentive to ensure that safety-testing strategies would identify potential developmental immunotoxic outcomes. This is discussed in Section 9.

6 PREGNANCY AND ITS IMPLICATIONS FOR BOTH MATERNAL AND OFFSPRING IMMUNE STATUS

A successful pregnancy requires that the mother carry a semiallogeneic foetus to term without paternal antigen-derived immune rejection. T-cell function and cytokine balance play a critical role in protection of the foetus and maintenance of the pregnancy (Lim *et al*., 2000; Jin *et al*., 2006). Because of the need to suppress allogeneic-driven cell-mediated immune responses, a variety of systemic and uterine-local strategies are used to reduce the likelihood of rejection. These include both Th1 suppression and creation of a Th2-biased environment as well as local uterine efforts to reduce maternal immune stimulation by foreign antigens (Zhu *et al*., 2005; Saito *et al*., 2007; Zenclussen *et al*., 2007).

The creation of a Th2-preferred environment tracks with the likelihood of a successful full-term pregnancy. For example, evidence suggests that women who fail to adequately shift Th cytokines during pregnancy are more likely to have preterm deliveries (Malamitsi-Puchner *et al*., 2006). Additionally, a subset of women who repeatedly miscarry fail to suppress Th1 functional capacity and Th1 cytokine production during pregnancy (Jenkins *et al*., 2000; Wilson *et al*., 2004; Piccinni, 2006). The necessary shift in maternal environment and immune spectrum has implications for the mother and her immune function during the pregnancy (Poole and Claman, 2004).

The shift in immune balance is known to alter the severity of symptoms associated with maternal autoimmune diseases during the pregnancy. Those diseases dependent upon Th1 responses are usually reduced in symptom severity, while those connected with Th2 responses are increased. Pregnancy-associated changes have been reported for lupus (Dhar and Sokol, 2006) as well as rheumatoid arthritis (Doria *et al*., 2006; Ostensen and Villiger, 2007).

The Th2-biased environment of pregnancy also means that the offspring's developing immune system proceeds with a Th2 preference while *in utero*. Th1 responses are suppressed in both the mother and the developing child (McCracken *et al*., 2007). This means that with normal pregnancies, and in the absence of developmental immunotoxicity, children at birth have a Th2-bias capacity (Chen *et al*., 2006) that needs to be shifted towards a more useful balance of functions (Niers *et al*., 2007). These newborns possess immature response capacities that must undergo further maturation to achieve the needed spectrum of host defence. The maturational immaturity includes aspects of both the innate (Satwani *et al*., 2005; Levy, 2007; Sadeghi *et al*., 2007) and acquired (Upham *et al*., 2002; Krumbigel *et al*., 2005; Velilla *et al*., 2006) immune functions and is reflected in cytokine imbalances (Protonotariou *et al*., 2004; Rizos *et al*., 2007). Where developmental immunotoxicity intersects this process is the potential for xenobiotic exposure to interfere with the required perinatal–neonatal shifts in innate and acquired immune functional capacities. The default, should additional neonatal maturation not progress adequately after birth, is a greater likelihood of childhood allergic disease, risk of certain infections and misregulated inflammation (Dietert and Dietert, 2007).

7 GENDER AS A RISK FACTOR WITH DEVELOPMENTAL IMMUNOTOXICITY

Among the findings concerning adverse outcomes following the exposure of the developing immune system to xenobiotics is the importance of gender. Female and male offspring exhibit significantly different immunotoxicity profiles following exposure to a wide array of chemicals and drugs. Most of the studies conducted to date involve rodent models (mice and rats), but the significance of gender in environmentally mediated immune dysfunction also parallels gender differences seen in certain childhood diseases (Lleo *et al*., 2008; Ostman *et al*., 2008). In some cases, low-level exposure may produce immunotoxicity in only one gender for the immune parameters examined (Chapin *et al*., 1997). For other xenobiotics and exposures, both the nature of the adverse outcomes (Bunn *et al*., 2001b) and the postnatal timing for appearance (Rowe *et al*., 2008) can differ between males and females.

Among the xenobiotics reported to produce gender-specific DIT outcomes are atrazine (Rowe *et al*.,

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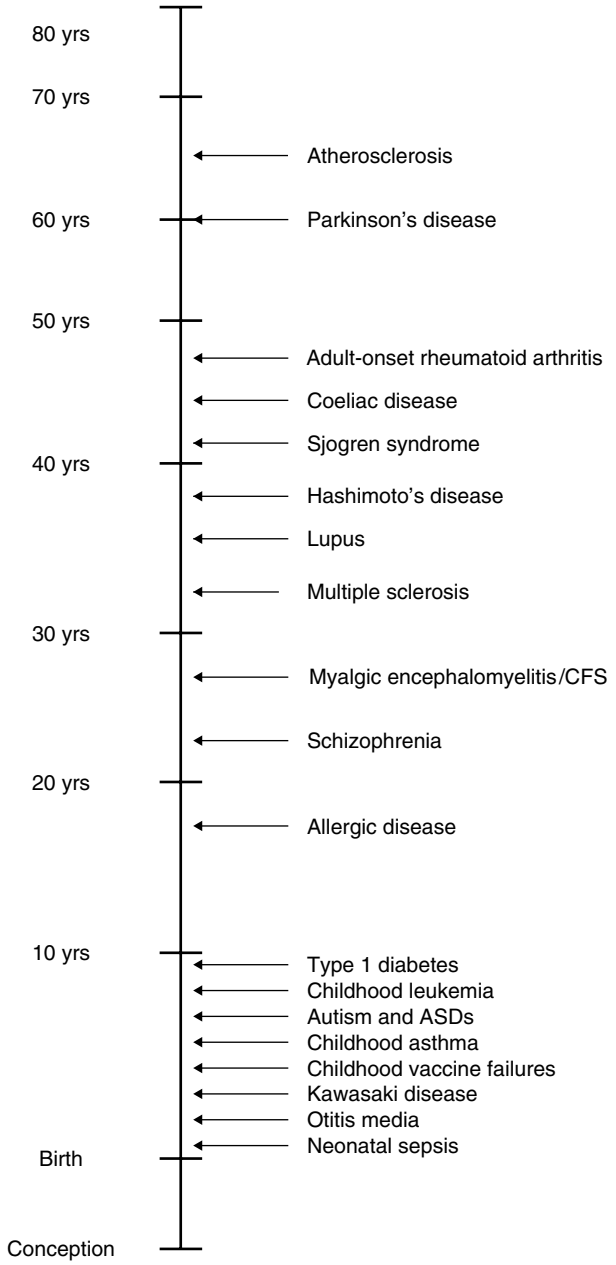

Figure 2 The figure illustrates a timeline of childhood- and adult-onset diseases or conditions that are either directly linked to early-life immune insult or have the potential to be caused by disruption of the developing immune system. As such, these diseases feature both environmental risk factors and immune dysfunction. The timeline presents the approximate average age of onset for each disease or condition.

2006), bisphenol A (Yang *et al*., 2008), cadmium (Pillet *et al*., 2005), chlordane (Blyler *et al*., 1994), cigarette smoke (Ng *et al*., 2006), diethylstilbestrol (Fenaux *et al*., 2004), ethanol (Redei *et al*., 1993), genistein (Guo *et al*., 2005a), lead (Bunn *et al*., 2001b), mercury (Silva *et al*., 2005), methoxychlor (White *et al*., 2005), nonylphenol (Guo *et al*., 2005b), NSAIDs (nonsteroidal anti-inflamatory drugs) (Kushima *et al*., 2007) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Gehrs and Smialowicz, 1999).

8 DIT AND ADVERSE HEALTH OUTCOMES

Research into immunotoxicity, immunity and health risks conducted in the 1980s and 1990s tended to focus on xenobiotic-induced immunosuppression, which was usually accompanied by reduced host resistance to infectious diseases (Selgrade *et al*., 1988; Johnson *et al*., 1989; Smialowicz *et al*., 1991; Luebke *et al*., 1994) This included early DIT studies (Stahlmann *et al*., 1992; Schlumpf *et al*., 1994). But with additional research into DIT and increasing concern over immune-inflammation-related pediatric health risks, immunosuppression is now recognized as only one of several adverse outcomes. In fact, even immunotoxicants known for more than a decade to produce immunosuppression (Faith and Luster, 1979; Gehrs *et al*., 1997) following prenatal and/or neonatal exposures were recently found to concomitantly increase the risk of autoimmunity (Fisher *et al*., 2005; Mustafa *et al*., 2008) and to cause increased risk of inflammation-mediated damage (Vorderstrasse *et al*., 2006; Hogaboam *et al*., 2008). For this reason, it is sometimes more useful to categorize outcomes associated with DIT as pertaining to immune dysfunction or misregulation vs. more restrictive labels. Xenobiotic disruption of immune maturation can produce a myriad of postnatal immune-dysfunction possibilities. These can include increased risk of allergic disease, including asthma, as well as autoimmunity in addition to immunosuppression and misregulated inflammation.

A timeline of childhood and adult onset diseases that are among those relevant to DIT are shown in **Figure 2**. These diseases are established as being directly connected to DIT, have data highly supportive of a connection or have features that make them likely candidates for DIT-influenced risk: immune dysfunction or environmental risk factors for the disease and early-life conditions that contribute to the later-life disease. Among the diseases of greatest interest are conditions associated with infectious agents (neonatal sepsis, otitis media), cancer (childhood leukaemia), allergic diseases (eczema, rhinitis, food allergies, coeliac) including asthma, autoimmune conditions (Type 1 diabetes, multiple sclerosis, systemic lupus erythematosus) and conditions associated with misregulated inflammatory damage-cell loss (schizophrenia, Parkinson's disease, autism).

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9 DEVELOPMENTAL IMMUNOTOXICITY TESTING

Immunotoxicity testing using adult animals has a long history spanning several decades (Dean *et al*., 1982; Van Loveren *et al*., 1988; Luster *et al*., 1992a; 1992b; 1994; Hinton, 2000). Effective approaches to adult immunotoxicity testing are based on a tier system for prioritization of testing. Usually the initial tier combines broad-scale functional and nonfunctional end points with subsequent tiers including parameters that provide more detailed information on specific functions (Luster *et al*., 1992a).

In contrast, consideration of developmental immunotoxicity testing has been a recent development that appeared this decade in association with recognition of two fundamental distinctions in toxicology between the developing and the mature immune system: (i) the developing immune system is more sensitive to toxic insult compared to that of the adult (Luebke *et al*., 2006a; 2006b) and (ii) a major disconnect exists in using adult immune exposure as a surrogate to predict risk of prenatal, neonatal or juvenile immunotoxicity (Dietert and Piepenbrink, 2006; Dietert, 2008a; 2008b; 2008c). For this reason, several recent research studies, workshops and reviews have considered effective strategies for developmental immunotoxicity testing (Miller *et al*., 1998; Ladics *et al*., 2000; 2005; Dietert *et al*., 2002; Holsapple, 2002; 2004; 2005; Smialowicz, 2002; Luster *et al*., 2003; Van Loveren *et al*., 2003; Barrow and Ravel, 2005; Dietert and Holsapple, 2007; Dietert, 2008a; 2008c).

The results of this recent literature reflect several points of general agreement, as well as several important points of departure regarding effective approaches to developmental immunotoxicity testing. First, most panels and authors agree that multiple parameters should be evaluated in any immune assessment following exposure of the nonadult and that they are likely to include a mix of functional and nonfunctional end points. This is similar to current immunotoxicity testing approaches in the adult. Additionally, most agree that effective use of animals is a high priority for any testing strategy. A third point of agreement is that for a general DIT screening, chemical or drug exposure across all developmental windows of the nonadult would be used. Finally, most authors agree that the data to date indicate that the developing immune system is more sensitive to xenobiotics than that of the adult.

Figure 3 illustrates a generalized testing schematic for developmental immunotoxicity assessment in the rat. Exposure to the test compound would occur across all developmental windows (prenatal, neonatal and juvenile). Nonfunctional assessment (e.g. histopathology and cell counting) could be conducted at various intervals between birth and adulthood with functional assays using a challenged immune system conducted in the juvenile and young adult.

The general points of agreement on developmental immunotoxicity safety testing reflect the considerable strides that have been made in research on xenobiotics and the developing immune system. However, there are also significant areas where suggested testing approaches diverge. These are: (i) whether use of a challenge

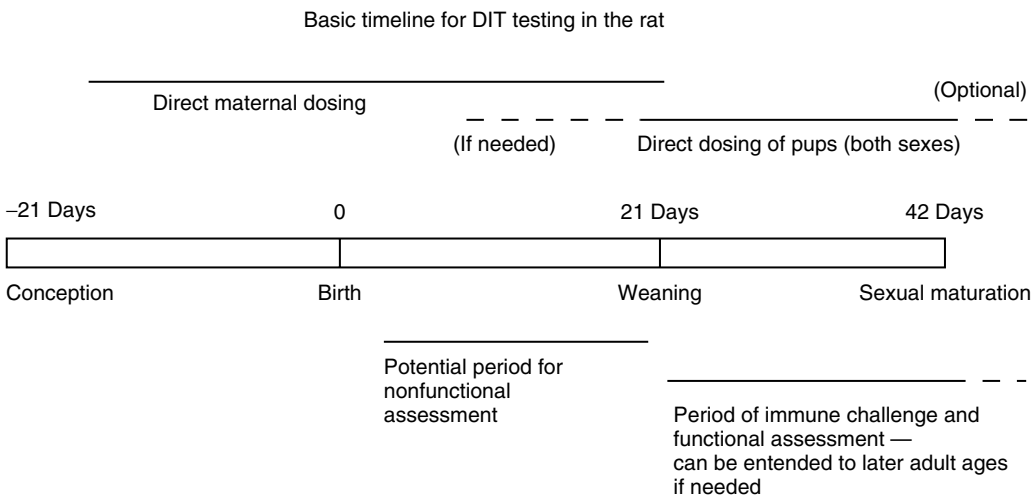

Figure 3 This illustrates a sample timeline for performing developmental immunotoxicity testing using the rat. Exposure to the xenobiotic occurs during all developmental windows of the nonadult and would include maternal dosing during gestation and lactation, with direct pup dosing beginning either postweaning or during lactation as is most relevant for human exposure. Assessment can be performed at various postnatal times. Nonfunctional assessment can begin prior to weaning and extend until the termination of the protocol. Immune challenge and functional assessment would occur after weaning. The protocol can be completed at 42 days after birth or extended longer as needed for the specific xenobiotics exposure, as well as the method of functional assessment. (Adapted from Dietert and Holsapple, 2007.)

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immune system (animals immunized or challenged with an infectious agent) is required for effective developmental immunotoxicity assessment, (ii) in the tiers, priorities to be given to measuring immune functional assays (e.g. T-dependent antibody response, cytotoxic T-lymphocyte response, delayed-type hypersensitivity response, natural killer cell activity) vs. nonfunctional parameters (e.g. histopathology, cell population counting) and (iii) whether an indication of adult immunotoxicity for a xenobiotic would be necessary before any developmental immunotoxicity testing would be triggered during safety evaluations.

The differences in DIT-related safety-testing philosophies and approaches are evidence in a comparison of Burns-Naas *et al*. (2008) and Dietert (2008a). In the former, histopathology is presented as the core evaluation tool for developmental immunotoxicity testing, with multifunctional testing either relegated to later tiers in the testing process and/or requiring positive indications of immunotoxicity before use. In contrast, Dietert (2008a) proposed that detection of immune dysfunction using multiple assays should be the highest priority in DIT safety assessment, with histopathology and other nonfunctional end points to be conducted in parallel as associated parameters. The latter paper also suggested the benefits of a specific linkage between developmental immunotoxicity safety-testing end points and the diseases of greatest public concern. Namely, that the specific DIT-associated parameters employed should be capable of detecting the immune dysfunctions characteristic of the most prevalent childhood diseases with environmental causes. Such an approach would establish a more direct linkage between immunotoxicity safety testing and the target diseases for anticipated risk reduction.

10 SUMMARY AND CONCLUSIONS

Immune status is well established as a critical factor in host protection against disease. Given the immune-health connection, it is not surprising that DIT is increasingly recognized as an area of research and testing that directly impacts the health and wellbeing of children and adults. Recognition of the developing immune system as an environmental target for postnatal disease fits within the larger multisystem paradigm of early-life origins for later-life disease.

A broad spectrum of chemicals, drugs and other environmental factors are known to disrupt immune development with adverse outcomes dependent upon the level and timing of the exposure. Additionally, the developing immune system is more sensitive to environmental insult than the fully matured immune system of the adult. Therefore, immunotoxic risk identified for an adult is unlikely to provide a good indication of the health risk to a foetus or child. For these reasons, developmental immunotoxicity research and testing have become integrally linked with strategies to reduce the incidence of those chronic childhood and adult diseases featuring immune dysfunction (e.g. allergies, asthma, Type 1 diabetes, multiple sclerosis, lupus, rheumatoid arthritis). Future DIT-related safety testing offers a significant opportunity to minimize problematic environmental exposures that could increase the risk for these diseases. This should result in more cost-effective approaches to address chronic immune-dependent diseases.

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Drugs of Addiction in the Domestic, Occupational and Sports Environment

Felix K. Adatsi

CONTENTS

1 INTRODUCTION

Throughout recorded history, humans have sought to use drugs for a variety of purposes. The use of wine or other alcoholic beverages for festive celebrations dates back to the Greeks and early Egyptians, and marijuana was used for medicinal purposes in China about 2737 BC. The men of old had a ritualistic use for drugs. Some in the human population still use drugs to express joy or sadness, for pleasure or to null pain, and others use them to gain a competitive advantage in activities which require stamina or endurance. Regardless of the nature of the motivating factor for the initial contact with a drug, if subsequent exposure to the drug becomes frequent and desirable, a gradual problem of addiction develops and becomes evident in the behaviour of the user.

Drug addiction has been described as a compulsion for drug use, even when the continued use goes to the detriment of the proper execution of basic human activities which are considered to be essential for normal social adjustment. The consequences of drug addiction are multifaceted and cut a swath of adverse effects measurable in the despondency, misery and eventual death of the individual. Activities, such as family relationships, work responsibilities and commitment to defined goals, are compromised, as ultimately is the health of the drug user (Hardman *et al*., 2001; Helms *et al*., 2006).

Drug use in modern societies is not a novel activity, but appears to be a natural outcome of our very existence. It should come as no surprise that where two or more people are gathered, drug use could emerge as an expected indulgent activity. It could begin as subtly as sharing a cigarette or a bottle of alcoholic

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beverage, and progress to the blatant craving for a drug or sharing a needle filled with heroin. However, unlike primitive societies, the extent of drug use in modern societies is measurable and the consequences are enormous. The 2007 National Survey on Drug Use and Health (NSDUH), an annual survey sponsored by the Substance Abuse and Mental Health Services Administration, issued the following statistics on the use of licit and illicit drugs in the United States (Substance Abuse and Mental Health Services Administration, (SAMHSA) Office of Applied Studies (OAS), 2008). In 2007, an estimated 19.9 million Americans aged 12 or older, representing 8.0% of the population, were current illicit drug users. Illicit drugs include marijuana/hashish, cocaine (including crack), heroin, hallucinogens, inhalants or prescription-type psychotherapeutics used nonmedically. Indeed, the World Health Organization has observed that illicit use of drugs has taken on global dimensions and the extent of the worldwide use of psychoactive substances is estimated at 2 billion alcohol users, and 185 million drug users (World Health Organization, 2009).

The consequences of drug abuse on the individual user are many. Individual drug users may become despondent and their value systems are easily compromised. Users schedule their activities around the drug use, and abandon stimulating family activities. In time, moral norms are compromised and the user becomes vulnerable to a variety of diseases, such as sexually transmitted diseases, hepatitis and HIV/AIDS because of the risk of sharing contaminated hypodermic needles. The effect of drug use on the family is often a disruption of the family structure and creation of varying levels of dysfunction in the family unit. If the user is an adult head of household, leadership roles become murky or nonexistent, and children suffer from lack of discipline and direction.

Currently, in the United States, nearly 7% of full-time adult employees and 9% of part-time employees use illegal drugs (Substance Abuse and Mental Health Services Administration, (SAMHSA) Office of Applied Studies (OAS), 2008). The profile of abusers includes law-enforcement personnel, medical personnel, sports figures, comedians, musicians and scientists. Employers with successful drug-free workplace programmes report improvements in productivity and morale. They also report a decrease in absenteeism, accidents at the workplace, attrition, loss of property and turnover. Sober workers are less likely to change jobs frequently or be late or absent from work. They are more productive, and less likely to file workers' compensation claims.

Several factors must be considered in classifying a drug as a drug of addiction in a particular society. Prominent among them are factors such as the potency of the drug, its prevalence and ease of acquisition in that environment, socioeconomic and political factors, and the overall prevailing culture of drug users in that society. Drug users play a significant role in this classification and it is perhaps their demand for a specific drug that ultimately calls attention to its abuse potential, probable classification as a drug of addiction and its subsequent control.

2 FACTORS INFLUENCING ADDICTION TO DRUGS

The psychosocial factors governing the initial exposure to or indulgence in drugs are complex. They may begin simply as consumers presenting themselves to a physician to seek relief from a lingering ailment, or desiring to be energized following a 'burn-out' feeling or just wishing to experiment with the drug. In the sports arena, subjects might be seeking to enhance their performance or gain a competitive advantage over other competitors in their field. In seeking to obtain relief by using the drug, the unsuspecting user soon becomes attached or 'hooked' to the drug. The effect of some of these drugs is short lived and the user soon develops tolerance to the drug. Overall, environmental factors, such as the availability of a drug, environmental stress, peer pressure, cultural influences and one's inherent susceptibility to drugs, will ultimately influence the selection or the user's propensity to the use of a drug (Kendler *et al*., 2008).

3 DRUGS OF ADDICTION

The main classes of drugs of addiction include stimulants, central nervous system (CNS) depressants and sedatives, opiates and opioid analgesics, hallucinogens and inhalants. Although some of the specific drugs might be grouped in a particular class based on their mode of action, they may cause users to exhibit symptoms which cross over into other classes. Assessment of the extent of addiction to specific drugs in each classification is based on reports from drug-testing laboratories, programmes dealing with self-reporting use of drugs and the frequency of detection from forensic drug-testing laboratories (Cone *et al*., 2008; Office of National Drug Control Policy (ONDCP), 2008; Quest Diagnostics, 2007). Sadly, the recognition and eventual classification of a drug as being a 'drug of addiction' by the scientific community will invariably lag behind the intended use of the drug. It is probably the pattern of inappropriate use imposed on the drug by users which will initiate that classification.

3.1 Stimulants

The drugs in this class include amphetamine, methamphetamine, methylphenidate (Ritalin), Methylenedioxy-

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methamphetamine ('MDMA', 'Ecstasy', 'XTC'), 3,4-methylenedioxymethamphetamine (DMA, 'Ecstasy', 'XTC'), cocaine (as a powder or as 'crack cocaine') and ephedrine. Drugs in this class are abused for their ability to cause excitement, impart a feeling of increased self-confidence, wellbeing and euphoria. Additionally, they offer a feeling of alertness and counter feelings of fatigue. The drugs in this class are popular among truck drivers and workers who must remain alert for jobs or activities requiring endurance and stamina (Logan, 2002). Amphetamines, methamphetamines, cocaine and MDMA are the drugs most commonly encountered within this context. Users who tap into these drugs, soon find that they reach a point of diminishing returns. The same drugs which energize them can also cause them to be fatigued and dysphoric. The central stimulatory effects or 'rush' which results from the use of stimulants is often followed by a 'crash' phase, during which there is depression of the senses (Dackis and Gold, 1985; Gawin and Kleber, 1986).

In general, stimulants such as cocaine and methamphetamine increase neuronal activity in the CNS, resulting in an increased level of the neurotransmitter dopamine (Isenschmid, 2002; Venton *et al*., 2006). This increased level of dopamine is thought to be responsible for the euphoric and excitatory properties of the drugs. With repeated use, stimulants cause some down-regulation of dopamine receptors and users feel an effect that is opposite to that experienced during the excitation phase. Users need to take more and more of the drug in order to experience the same effects they desire. Long-term abuse of these stimulants can result in addiction and users may display features such as violent behaviour, insomnia, auditory hallucinations, paranoia, and suicidal and homicidal intentions. Both cocaine and methamphetamine abuse have been responsible for many other adverse effects resulting in death (National Institute on Drug Abuse (NIDA), 2003; 2005; Kaye *et al*., 2008).

3.2 Central Nervous System (CNS) Depressants and Sedatives

Alcohol continues to be the main representative drug in this class of drugs, in terms of its availability and preponderant use. In many parts of the world, the use of alcohol as a beverage is considered a normal right of passage into adulthood. In Western societies it is so abundant that its use as a social beverage is freely condoned. Nevertheless, it remains a drug and its CNS depressant effects are sometimes taken as a paradigm by which other therapeutic drugs are compared. It is by far the most studied of all drugs in relation to its human effects and consequently the biological and medical effects literature on the subject is the largest single literature in medical science. The other drugs in this class include the barbiturates (amobarbital (Amytal), butalbital (Sandoptal), phenobarbital and secobarbital (Seconal)), benzodiazepines (alprazolam (Xanax), chlordiazepoxide (Librium), clonazepam (Klonopin), clorazepate (Tranxene), diazepam (Valium), estazolam (ProSom), lorazepam (Ativan), oxazepam (Serax) and triazolam (Halcion)) (Physicians' Desk Reference 2008). Other drugs in this class which are recognized for their sedative properties and popular with their demand are carisoprodol (Soma), chlorphenamine (chlorpheniramine, Chlortrimethon), diphenhydramine (Benadryl), gamma hydroxybutyrate (GHB), meprobamate (Miltown), tetrahydrocannabinol (marijuana) and zolpidem (Ambien). In spite of their classification as antihistamines, both chlorphenamine and diphenhydramine are also recognized for their sedative properties in moderate to high doses.

The general effects of these drugs are typified by those encountered in the use of alcohol (Jones, 2003; Garriot, 2003), which is clearly a prototype for the class. The effects and potential hazards posed by these drugs follow a dose–response relationship, with adverse effects increasing with increasing drug blood levels until physiological functions are overwhelmed, resulting in the death of the user. Most CNS depressants have similar pharmacological actions and act by interfering with neuronal activities in the brain. They enhance the actions of the neurotransmitter γ-aminobutyric acid (GABA). By increasing GABA activity, CNS depressants cause a decrease in nervous activities.

Adverse effects associated with this class of drugs span a wide range of psychomotor and cognitive, as well as behaviour changes (Woods and Winger, 1997). In general, learning, thinking and decision-making are impaired, as are activities requiring fine or gross motor skills, depending upon the amount of drug consumed. As CNS depressants, the adverse effects of these drugs also include an increase in reaction time, reduced vigilance, impairment of performance on divided-attention tasks, drowsiness, confusion and unsteady gait. For these reasons, use of CNS depressants drugs impairs driving skills (Ramaekers *et al*., 2004; Bedard *et al*., 2007; O'Hanlon *et al*., 1982; van Laar *et al*., 1992). These adverse effects increase in severity as more drug is consumed. Prolonged use of CNS drugs can cause addiction and despite their beneficial effects as antianxiety drugs, the barbiturates and benzodiazepines are capable of causing drug dependency and fatalities.

3.3 Opiate and Opioid Analgesics

Morphine and codeine, two naturally occurring opiates are found in this class. Other members of this class include buprenorphine (Subutex), dihydrocodeine

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(Synalgos), fentanyl (Duragesic, Sublimaze), heroin, hydrocodone (Vicodin, Lortab), hydromorphone (Dilaudid), meperidine (Demerol), methadone (Dolophine), oxycodone (Oxycontin), pentazocine (Talwin) and propoxyphene (Darvon).

The popularity of opioids among users and the addiction and entrapment of the user in a vicious cycle of dependence on opioids is due to their potent analgesic properties. They are used for the management of moderate to severe pain and chronic pain in cancer or terminally ill patients (Joranson *et al*., 2000; Manchikanti, 2006; Manchikanti and Singh, 2008; Merlo and Gold, 2008; Ballantyne, 2007). Opioids exert their action by binding to specific opioid receptors which are widely distributed throughout the brain, spinal cord and gastrointestinal tract. Three main types of receptors have been identified and are mu (μ), delta (δ) and kappa (κ) receptors (Hardman *et al*., 2001). Specific opioids bind to these receptors, based on their affinities, in their mode of action.

The effects of opioids in humans include euphoria, decreased anxiety, drowsiness, constipation and (pin-point pupil size), which may impair activities such as night driving, and cause life-threatening respiratory depression. A classic feature associated with the use of opioids is the development of tolerance and physical dependence (Hardman *et al*., 2001). For this reason, withdrawal symptoms can be severe when the cessation of drug use is abrupt. Symptoms of withdrawal include restlessness, vomiting, cold flashes, and muscle and bone pain. The extensive reliance on opioids predisposes the user to drug-related fatalities, and instances of accidental opioid fatalities can be found in the scientific literature (Denton *et al*., 2008; Graham *et al*., 2008; Hall *et al*., 2008).

3.4 Hallucinogens

Lysergic acid diethylamide (LSD) is perhaps the classic representation of drugs classifiable as hallucinogens. It enjoys this classification along with drugs, such as phencyclidine (PCP), ketamine, mescaline, and psilocybin/psilocin ('mushrooms'). Tetrahydrocannabinol (marijuana) has been classified as a CNS depressant, however some of its adverse effects warrant its inclusion in this class as well.

Hallucination has been defined as sensory perception without external stimulus of the relevant sensory organ (American Psychiatric Association, 1987). Many pharmacological substances are capable of producing hallucinogenic effects. However, use or mention of drugs such as LSD, peyote, psilocybin and psilocin ('mushrooms'), PCP and ketamine have become synonymous with experiences of a hallucinogenic nature. Most of these drugs are alkaloids and can be obtained from plants. It is no surprise that in certain cultures, chief priests used these plant materials as prized possessions for their hallucinogenic effects during social or religious rituals, and the ensuing induced states of detachment and 'mystical visions' they conferred on them was used to hold a magical sway over others in their tribe. These drugs produce profound drug-induced psychosis, distortions in the user's perception of reality and influence their perception of sound, colour, time and movement.

LSD is the most potent hallucinogenic drug known and produces significant mood- and perception-altering effects at very low doses (Hardman *et al*., 2001). Because of its effectiveness, it is dispensed illegally as 'microdots' or thin stamp-sized squares impregnated with the drug. Law-enforcement personnel seeking to apprehend users of this drug must be aware of these modes of trafficking, and among other clues on a suspect, look for sheets of 'blotter acid' papers or window panes which are thin squares of gelatin soaked with LSD. LSD produces tolerance and psychic dependence (Department of Health and Human Services (DHHS), National Institute on Drug Abuse (NIDA), 2001) and users must take progressively higher doses to achieve a desired effect.

Users of hallucinogenic compounds refer to their experiences with the drugs as 'trips'. A 'bad trip' is a collection of unpleasant and acute adverse effects of the drug which include nightmares, horrifying thoughts, fears of insanity and demonic transformations of surrounding objects (Department of Health and Human Services (DHHS), National Institute on Drug Abuse (NIDA), 2001; Hofmann, 1980). Users of the drug also experience highly intense effects from the senses. Sensory perceptions of smells, colour and sound are magnified. LSD acts on serotonergic 5-HT2 receptors and tolerance develops quickly to the drug (Drummer, 2002). Increasingly larger doses are needed to produce desired effects and this may ultimately lead to addiction to the drug.

PCP was developed for use as a surgical anaesthetic agent in the 1950s. Its use, however, was limited to veterinary medicine because of the extreme adverse effects, such as delirium and agitation experienced by patients when used in human medicine. Repeated use of PCP can result in addiction. Ketamine was developed to replace PCP as an anaesthetic agent and is similar in structure and mechanism of action to PCP. However, it is less potent and has a shorter duration of action. Both compounds are classified as dissociative anaesthetics. Users experience a variety of effects, including 'out-of-body' sensations, detachment from their environment and sometimes complete sensory detachment likened to a near-death experience. Use of ketamine induces amnesia and for this reason has been implicated in drug-facilitated sexual assaults on unsuspecting victims exposed to the drug (Department of Health and Human Services (DHHS), National Institute on Drug Abuse (NIDA), 2001).

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3.5 Inhalants

Included in this class of drugs are chemical substances which are gases or possess sufficient vapour pressures to exist in the gaseous state at room temperature. By their very nature, these chemicals are volatile substances and the major route of administration or exposure is by inhalation. They include gases (aerosols, butane, halothane, nitrous oxide, propane), nitrites (amyl nitrite, butyl nitrite, cyclohexyl nitrite) and volatile organic solvents (benzene, chloroform, correction fluids, electronic contact cleaners, ether, gasoline, lighter fluid, methylene chloride, paint thinners, perchloroethylene, toluene, trichloroethylene) (Klaassen, 2007; Balster, 1998). They are inexpensive and their ubiquitous presence as common household chemicals, promote their use by children and young adults. National surveys indicate that more than 22.9 million Americans have abused inhalants at least once in their lives (Substance Abuse and Mental Health Services Administration (SAMHSA) Office of Applied Studies (OAS), 2008).

Users who gravitate towards this class of drugs cut across a broad range of age groups, sexual orientation, citizenry and professional echelons. Those in the medical professions, academia and the food industry are likely to abuse nitrous oxide, partly because of its workplace availability. Young adolescent vagabonds seeking to experiment with drugs are likely to be hooked 'huffing' organic solvents or chemicals commonly found in commercial products such as correction fluids, lighter fluids, spray paints, nail-polish remover, dust off and paint thinners; commercial products which can be cheaply obtained (Kurtzman *et al*. 2001). Nitrites are used as sexual enhancers and are commonly abused by adults (Wu *et al*., 2005). Use of nitrites is common among male homosexuals who reportedly use them for heightened sexual pleasure. In general, these drugs produce a quick and rapid onset of effects and an equally rapid recovery and a sustained 'high' can be obtained by repeated inhalation or sniffing.

Inhalants generally exhibit behavioural effects similar to those of alcohol, barbiturates, benzodiazepines and other CNS depressant drugs (Balster, 1998; Klaassen, 2007). These effects include lightheadedness, loss of inhibition, euphoria, incoordination, drowsiness, dizziness, slurred speech, loss of memory, confusion, delusions and hallucinations. Repeated use of inhalants can lead to addiction, and high concentrations of chemicals in the lungs may result in seizures, coma and death. The volatility of the drugs in this class indicates that a major route of exposure is via the respiratory system. Once in the lungs, these volatile drugs are distributed over a large surface area and displace the air in the lungs. By depriving the body of oxygen, inhalant use may lead to a hypoxic condition. Hypoxia can damage brain cells and impair memory, thinking, learning and decision-making.

Long-term abuse of inhalants, especially those originating from the chlorinated hydrocarbon solvents such as methylene chloride and trichloroethylene, causes widespread and permanent damage to several organ systems including various parts of the nervous system and the brain. It results in the breakdown of the myelin sheaths surrounding nerve fibres. Prolonged destruction can result in paraesthesias, peripheral neuropathies, muscle spasms and tremors. The most serious outcome and hazard of inhalant abuse is a syndrome referred to as 'sudden sniffing death'. Manifestation of this syndrome is common with the abuse of compounds such as butane, isobutane, propane, chemicals in aerosols and 1,1-difluoroethane (Pfeiffer *et al*., 2006; Rohrig, 1997; Sugie *et al*., 2004; Xiong *et al*., 2004), and can result from a single heavy exposure to the inhalant by an otherwise healthy individual.

4 SOURCE AND AVAILABILITY OF DRUGS

4.1 Prescription Drugs

Since the mid 1900s, prescription drugs have increased in their availability to the general public. This increase is due to legitimate commercial production of these drugs and their distribution to hospitals, pharmacies, practitioners, and educational and research institutions (National Drug Intelligence Center, 2009). With this effluence came abuse. Some in the general population sought relief from the use of these drugs while others were tempted to experiment with them. The faint of heart found solace in the temporary relief offered by drugs perceived as 'painkillers' and a pattern of pilgrimage and return from the sanctuary offered by these drugs was established. In the United States, 'doctor shopping' constitutes a major source of availability and abuse of most prescription drugs. In this practice, the drug abuser visits many physicians and presents an ailment which may not exist or exaggerates an existing ailment, in order to obtain multiple prescriptions. Often the drug abuser proceeds to fill the prescriptions at different pharmacies to avoid detection.

Other avenues for access to prescription drugs involve the use of forged prescriptions, posing as a physician and calling in a fictitious prescription, break-ins into clinics and pharmacies, acquisition via internet pharmacy rings and flat-out theft from a sick neighbour, friend or relative. The diversion of prescription drugs can also come from unlikely sources. Unscrupulous medical personnel can author illegal prescriptions or collaborate with corrupt pharmacies to dispense drugs for profit. Finally, employees of pharmaceutical companies and commercial distributors steal prescription drugs from

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their respective inventories and resell the drugs for profit (National Drug Intelligence Center, 2009).

4.2 Cocaine, Heroine, Marijuana, Methamphetamine

These drugs are available through a variety of sources. Domestic production occurs in clandestine laboratories ('meth labs'), and smuggling across the borders accounts for the large amounts usually found in the southern United States. The predominant form of available methamphetamine in the United States is the powder form. Ice methamphetamine is another form of the drug found in the United States and is smuggled into the country primarily from its production sites in Guam and Mexico. Also available is the tablet form of the drug, which is produced in Asia, primarily in Burma (National Drug Intelligence Center, 2009). Powder methamphetamine is usually injected or snorted. Tablet methamphetamine can be ingested orally, smoked, snorted or mixed with water and injected.

4.3 Other Drugs

Drugs in this category arise mainly from syntheses in clandestine laboratories or as a result of novel discoveries and ingenuous ways to use chemicals already in existence. For example, the drug addict may come to perceive nail-polish solvent, γ-butyrolactone (GBL), or 1,4-butanediol (BD) as substances which produce desirable physiological and pharmacological changes, and switch the uses which have been conventionally attributed to these chemicals by society with the newly discovered excitable physiological changes they produce. Consequently, the chemicals jump the line and cross over from their conventional commercial applications to being chemicals which must be regulated because of their abuse potential. Other sources contributing to drugs in this category include the existence of the drug, such as alcohol, as a common beverage, by general acceptance by society. The same type of subjective paradigm exempts the consumption of coffee and tea by society, from being classified as an example of drug abuse. Both coffee and tea contain the stimulant caffeine which is physically addictive.

5 USUAL ROUTES OF EXPOSURE

The usual routes of exposure to many of the drugs listed include oral, intravenous, subcutaneous, intramuscular, inhalation or intranasal application and rectal administration (Hardman *et al*., 2001). In recent times, some in the human population have sought ways to maximize the effect of drugs and deferred to nontraditional methods (Wilson *et al*., 2005) of administering drugs to themselves. For example, rectal administration of drugs is gaining popularity among some of the youth who use this method to maximize the drug's effects and to avoid detection, particularly of drugs such as alcohol which is easily detected on the breath. Cocaine, oxycontin and many other stimulants have all been snorted or used via rectal administration.

6 TYPE OF SPECIMENS AND DETECTION

Blood is the biological specimen of choice to the forensic toxicologist because the presence of a drug in blood indicates that absorption of the drug has taken place. Furthermore, good correlations exist between the blood levels of most drugs and their pharmacological and/or behavioural effects on humans (Levine, 2003; Klaassen, 2007). It provides concrete chemical evidence to the forensic toxicologist in establishing a cause-and-effect relationship between the exposure of a drug and its effect, especially in drunk-driving cases. However, its acquisition for forensic purposes is invasive and hardly desirable for workplace testing.

Urine is the preferred and predominant sample collected at the workplace for testing. In the United States, the mandatory guidelines for workplace drug testing require the use of urine as the specimen of choice (National Institute on Drug Abuse (NIDA), 1988; Mandatory Guidelines for Federal Workplace Drug Testing Programs, 2004). It provides a noninvasive method of determining the presence or absence of a drug in the body of an individual. It is generally available in adequate amounts and contains relatively high concentrations of drugs and their metabolites. It is an easier specimen to analyse using automated immunoassay screening methods in the laboratory without the complication of interfering biochemical and protein substances present in blood. In certain types of acute exposure to a drug, a hidden limitation of urine may be shown in the time lag between exposure (e.g. oral ingestion), absorption, distribution, metabolism and excretion of the drug in the urine. If the lag time is substantial, the urine test result may be negative, although the drug may be building up in concentration in the blood and exerting its effect on the subject by virtue of the circulation of the drug-laden blood to the brain. A major limitation of urine is that it is a waste product of metabolism (Helms *et al*., 2006; Price and Wilson, 1992), and chemical substances found therein must be treated as such. Analysing this biological specimen might not fully answer the specific question on whether or not the individual producing the specimen

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was being directly influenced by the drug. However, results obtained from the analysis of a urine specimen will still clarify the forensic issues regarding exposure or use of the drug. For example, the impairing effect of certain drugs, such as alcohol, cocaine or marijuana, might wear off with the active part of the drug being undetectable in blood. Yet some of the active part of the drugs, as well as their metabolite(s), may be present in the urine long after the psychological effects wear off (Verstraete, 2004). The detection of the metabolites in the urine can be taken as evidence of exposure to the parent drug at some time in the past. In recent times, the detection of ethylglucoronide (Bergstrom *et al*., 2003; Rosano and Lin, 2008) and ethylsulfate (Helander and Beck, 2005) in the urine of parolees is gaining popularity as a means of monitoring these individuals for previous alcohol abuse even when they appear sober.

Advances in the analytical technology needed for the chemical testing of small specimen volumes containing low concentrations of drugs and their metabolites have led to the acceptance and increasing popularity of oral fluid (saliva) as a specimen for drug testing (Aps and Martens, 2005; Cone, 1993; Cone *et al*. 2007). In spite of its inherent limitations, saliva offers certain advantages over some traditional biological specimens. Collection is relatively simple, noninvasive and can occur under direct observation of the donor, thereby limiting the potential for adulteration. It can also be collected at any location and time without embarrassing the donor. Its value in alcohol testing for driving-related offences is usually controversial, primarily because of difficulties in connecting saliva alcohol levels to specific degrees of driving impairment. The same is true for drugs other than alcohol. The main challenge appears to arise from lack of harvesting a homogenous mixture of saliva because of differences in the amounts of glandular secretions from the parotid, sublingual and submaxillary glands and problems with oral contamination (Kidwell *et al*., 1998; Crouch, 2005). Additionally, another shortcoming is that certain drugs cannot be detected in saliva a short period after their use, even when the drugs might still be detectable in other biological matrices (O'Neal *et al*., 2000).

Another biological specimen that offers tremendous advantage when it comes to sample adulteration and timeline for the detection of a drug is hair (Cone, 1990; Inoue and Seta, 1992). Its collection, like urine and saliva, is noninvasive, simple and can occur at any time or location without trauma or embarrassment to the donor. Currently, it is the only method for drug testing that can provide up to a 90 day history of drug use in living individuals (Quest Diagnostics, 2007; Villain *et al*., 2004). This of course is unwelcome news to the drug user seeking to remain under the radar of a random workplace drug test programme or preparing for a job interview which may require passing a drug test.

Finally, the use of breath samples for forensic purposes has been well recognized (Garriot, 2003; Dubowski, 1994). Advances in breath testing have led to the manufacture and use of sophisticated breath-test instruments. Since the invention of the simple breathalyzer instrument in 1954, several generations of breath-testing instruments have been manufactured. Included in this group is the BAC Datamaster which is included on the list of instruments deemed satisfactory for the breath testing of alcohol (Conforming Products List of Evidential Breath Alcohol Measurement Devices, 2007). The singular use of breath as a biological sample for the determination of the bodily alcohol content in an individual is codified in legislation around the world. Consequently, its use as a probe for workplace alcohol testing was a foregone conclusion. Today, an investigation of alcohol abuse at the workplace begins, and in many cases, ends with the result of a breath test.

7 DRUGS COMMONLY ENCOUNTERED IN THE OCCUPATIONAL ENVIRONMENT

To some, a mental picture of the workplace is an environment consisting of a secured office setting, air conditioned and shining glass windows. An environment in which workers sporting a white shirt, tie, immaculately tailored suits and clutching laptops walk briskly from office space to office space and say very little as they go about their business. To others, a workplace is an outdoor environment; noisy, with loud music blaring, small talk and a lot of profanity through a haze of cigarette smoke. The choice of and affinity to drugs at these two locations will be different and the types of drugs consumed will be dictated by the prevailing culture of the environment. No single occupational environment, or one from which one derives a means of livelihood, is immune from the scourge unleashed by the indiscriminate use of drugs. From construction workers to truck drivers, pilots and physicians, educators and corporate executives, one encounters abuse of drugs either as prescription medications or in the form of illicit drugs (Wunsch *et al*., 2007; Merlo and Gold, 2008).

The drugs encountered in this environment cover a wide variety of available drugs and use is determined mainly by how much the user is willing to spend in acquiring the drug. Youthful workers might prefer marijuana, cocaine, methamphetamine and some of the newer drugs of addiction, such as prescription opiates. Older workers would use these same drugs, but may add to their cocktail, drugs commonly encountered decades ago, by virtue of their experience. Recently, drug screenings conducted in 2007 show more than a 50% decline in the percentage of positive tests for methamphetamine use at the workplace (Office of National Drug Control Policy

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(ONDCP), 2008; Quest Diagnostics, 2007). This decline may be due to several reasons, ranging from the lack of availability of the drug to the user, enforcement initiatives at the workplace, to a preference for a readily available alternative. In the United States, for example drugs such as ephedrine and pseudoephedrine, which traditionally could be obtained as an over-the-counter medication, are now difficult to obtain. With such cut in a major substrate in the synthesis of methamphetamine, the turnover rate from 'meth labs' is also reduced.

Among prescription drugs, opioids rank as one of the most abused drugs in the workplace. A major reason for this ranking is the human intolerance for pain. Increased reliance on computers at work, especially in developed nations, causes workers to be more prone to work-related musculoskeletal disorders as a result of repetitive typing or keyboard entries (Wilkens, 2003). Workers are more apt to complain about ailments such as carpal tunnel syndrome. Similarly, sedentary workers are more apt to complain about chronic back and neck pains. Some of these workers turn to opioids and other painkillers for relief. As the pain persists, so does the reliance on drugs for relief. Thus, an increase in the use of opioids due to liberal prescription has occurred, (Kuehn, 2007; Ballantyne and Mao, 2003) with the relevance and efficacy of some of these prescriptions being questionable.

8 DRUGS COMMONLY ENCOUNTERED IN SPORTS

The characteristic profile of the drug user in this group is an individual with a singular objective, to 'win-at-all-cost', and they must have something, such as a medal or a form of recognition, to show for the effort put forth. The usefulness of drugs to the athlete in this group is the real or perceived competitive advantage drugs offer the user over other participants. Adolescents and youths with eyes on playing professional sports and intent upon gaining a competitive advantage over their peers, begin to indulge in drugs in this group (National Survey on Drug Use and Health (NSDUH), 2002). Drugs encountered in this class must improve athletic performance and are therefore classified as performance-enhancing drugs (PEDs). PEDs include anabolic steroids, erythropoietin (EPO) and human growth hormones. Other drugs include amphetamine, taken for its psychic effect for alertness, increased confidence and decreased sense of fatigue. Methamphetamine is similarly taken for alertness, mood elevation, self-confidence and endurance. The appeal of amphetamine to the user is primarily to enhance performance rather than as a training drug. Nevertheless some gymnasts use the drug to suppress appetite and maintain their physical form. Marijuana use is also frequent in this arena, as is GHB, inhalants and narcotics. GHB on the other hand is abused primarily as a training drug and as an adjunct to body-building supplements (Baselt, 2004; Kleimenova *et al*., 1979). The main controlling body in human sport is the International Olympic Committee (IOC). Data generated by IOC-accredited laboratories, indicate that the most commonly abused drugs include those listed above, with steroid use leading in popularity among abusers (Mottram, 1999; Catlin *et al*., 2008).

9 DRUGS COMMONLY ENCOUNTERED IN BARS, CLUBS AND GYMS AND OTHER ENVIRONMENTS

The drugs encountered in this group are collectively termed 'club drugs'. They are generally drugs encountered at 'rave' parties. In their mode of action, these drugs cause stimulation of the senses and also have hallucinogenic effects on the user. Drugs encountered at these locations include 'Ecstasy', amphetamine, methamphetamine, methylphenidate (Ritalin), PCP, GHB, flunitrazepam (Rohypnol, 'Roofies'), ketamine ('Special K', 'Vitamin K') and LSD. The clientele in most bars, clubs and gyms is perhaps the most varied and fluid when compared with those in other environments. Typically, college students, young adults and middle-aged adults unwilling to give up the pleasures of their youth frequent these locations. It is perhaps an understatement to state that adventurous and risky behaviours can be found in most bars and clubs. For this reason, drug users who frequent bars are exposed to a large variety of stimulant drugs and drug-related health risks. Users are expecting to find newer and fast-acting drugs which will cause great stimulation, excitement and altered states, and alcohol is not a significant drug in most of these 'rave' clubs.

10 DRUGS COMMONLY ENCOUNTERED IN THE DOMESTIC ENVIRONMENT

In the domestic environment, alcohol is clearly the drug of choice. The factors leading to its choice include its relatively low cost and availability. It is easy to purchase low-quality alcoholic beverages containing high alcohol content in low-income areas, and around each corner in these areas is a liquor store or a pub. In many societies the consumption of alcohol as a beverage is not frowned upon, but rather extolled. Happiness, satisfaction and expression of joy for other forms of human achievement are celebrated with copious consumption of alcohol. Alcohol is even considered a panacea for human anguish.

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Many societies freely condone its consumption and those which write laws to check its use, write what might be considered from the broad perspective of the potential adverse effects of alcohol, protracted ones. In the United States, for example, one can purchase as much alcohol as one can afford, sample the many varieties which abound, and drink as much as can be physiologically tolerated. The restriction, for those with a clean record, is that they must be at least 21 years of age and cannot operate a motor vehicle after such consumption. Therein lies the potential for addiction to this drug. The frequent, plentiful and unrestricted consumption fosters an environment that is conducive to addiction to this drug. A consequence of this type of '*ad libitum'* indulgence in the drug is a myriad of adverse effects impinging on the functionality of family structure in the domestic environment.

This factor has also been largely responsible for the choice of 'crack cocaine' as the major abused drug over cocaine hydrochloride (cocaine powder) by poor inner-city populations. By converting cocaine into 'crack cocaine', these populations were able to smoke the drug and obtain the same 'high' at a price they could afford. The development of 'crack cocaine' lowered the economic barrier for the acquisition of cocaine by low-income populations and bridged the gap that existed between pleasures which hitherto were almost exclusively limited to a relatively small group of affluent population snorting cocaine (Helms *et al*., 2006). The next most frequently abused drug in this environment, and indeed in most of the other environments, is marijuana. Under peer pressure and a need to 'fit in', youths begin to experiment with drugs, which include marijuana, for the first time. In 2007, marijuana was the most commonly used illicit drug among persons aged 12 or older with an estimated 14.4 million users. Additionally, an estimated 2.7 million persons aged 12 or older used an illicit drug for the first time (Substance Abuse and Mental Health Services Administration, (SAMHSA) Office of Applied Studies (OAS), 2008). Correspondingly, most new smokers in 2007 were under age 18. Aside from these drugs, many other drugs, such as pain relievers, psychotherapeutics, inhalants and hallucinogens will also be encountered in the domestic environment. Overall, the prevalence of the various drugs in the various environments is listed in **Table 1** below.

11 SOCIOECONOMIC POLITICAL EFFECTS

Drug abuse has an insidious effect on society that could eventually lead to human resource depletion. Drug users who expose themselves to drugs via the intravenous route place themselves at risk of acquiring diseases such HIV/AIDS and certain types of hepatitis by contact with contaminated hypodermic needles. Pregnant mothers in this group place their unborn children at risk of acquiring the AIDS virus and other diseases. Drugs such as alcohol, 'crack cocaine' and heroin cross the placental barrier and adversely impact the developing foetus *in utero*. At term, these babies have low birth weights and are susceptible to congenital malformations, such as foetal alcohol syndrome, phocomelia and microcephaly. Many are addicted to the drugs used by their mothers and go through harrowing drug withdrawals at birth. They cry easily, are irritable and difficult to comfort, and many become mentally challenged individuals in life (Eriksson *et al*., 1979; Kelly *et al*., 2000).

Drug use has also been implicated in drug-facilitated sexual assaults (date rape) (Kintz *et al*., 2003; McCauley *et al*., 2009). Victims in this drug-related crime are typically adolescent girls. The drugs that are usually encountered are GHB and its congeners, fast-acting benzodiazepines such as flunitrazepam (Rohypnol), ketamine, and alcohol. Unsuspecting victims usually begin by consuming an alcoholic beverage with perpetrators who at some point during the encounter slip the drug into the victim's drink. Under the influence of these drugs, victims have reduced inhibitions and relaxation of voluntary muscles. In their physically incapacitated states they are unable to fight off perpetrators, and succumb to the sexual assault. They have lasting anterograde amnesia of events which occur while under the influence of the drugs (Schwartz *et al*., 2000) and are unable to clearly describe events leading to the assault, making it difficult to pursue and prosecute the perpetrator.

Use of drugs of addiction and trafficking threatens national security and the safety of citizens. Traffickers are able to infiltrate the higher echelon of society and corrupt those in positions of authority. Drug barons are formed within the society and conduct their drug activities with violence and without regard to innocent lives. Many societies in the world today succumb to the vengeance of drug use and some are on the verge of anarchy when it comes to instituting sustainable drug-enforcement initiatives.

Problems in the workplace related to the use of alcohol and other drugs of abuse cost American employers billion of dollars each year. The cost arises from the undue burden placed on the workplace by drug use. Workers who abuse drugs are three and half times more likely to be involved in a workplace accident, resulting in workers' compensation and disability claims. Additionally, healthcare costs are twice as high for employees with alcohol-abuse problems than for other employees. Other factors which might drive up the cost include an increase in absenteeism, which is estimated to cost 500 million lost workdays annually, and lack of productivity (U.S. Department of Health and Human Services (U.S. DHHS), 2008). The overall cost of drug abuse to society is equally very high. According to a study in 1992, the estimated economic cost of alcohol and drug abuse was $245.7 billion. Of this amount,

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Table 1 Occurrence and projected incidence of selected drugs at selected sites[a]

	Workplace	Sports	Clubs, bars, gyms	Domestic
CNS depressants				
Alcohol	Medium-High	Medium	High	High
Barbiturates: Butalbital Phenobarbital	High	Low	Medium	High
Benzodiazepines: Alprazolam Clonazepam Diazepam Lorazepam	High	Low	High	High
Carisoprodol	High	Low	Medium	High
Meprobamate	High	Low	Medium	High
Methocarbamol	Medium	Medium	Medium	High
Zolpidem	High	Low	Low	High
Doxepin	Medium	Low	Low	High
Diphenhydramine	High	Medium	Medium	High
Tramadol	High	High	Medium	High
Stimulants				
Amphetamines	Medium	High	High	High
Cocaine	High	High	High	High
Methamphetamine	Medium	High	High	High
MDMA	High	High	High	High
Methylphenidate	High	High	High	High
Opiates				
Fentanyl	High	Medium	Low	High
Heroin	Medium	Medium	High	High
Hydrocodone	High	High	High	High
Hydromorhone	Medium	High	High	High
Methadone	High	High	High	High
Meperidine	High	High	Medium	Medium
Oxycodone	High	High	High	High
Propoxyphene	High	High	High	High
Hallucinogens				
LSD	Low	Low	High	High
PCP	Low	High	High	High
Ketamine	Low	Low	High	Medium
Other drugs				
GHB	Low	High	High	Low
Inhalants	Medium	Medium	High	Medium
Marijuana	High	High	High	High
Tobacco	High	High	High	High

[a]High = Drugs that occur with great frequency and positivity; Medium = Drugs that occur with medium/average positivity; Low = Drugs that occur with minimal positivity. Ratings based on data culled from Quest Diagnostics (2007), Drug Abuse Warning Network (DAWN), (2005), Substance Abuse and Mental Health Services Administration, (SAMHSA) Office of Applied Studies (OAS), (2008), Cone *et al*. (2008), Office of National Drug Control Policy (ONDCP) (2008) reports and projections from recognized effects of drugs.

$97.7 billion was due to illicit drugs and those taken for nonmedical purposes (National Institute on Drug Abuse (NIDA), 2008a). The estimated cost represented a 50% increase over the cost estimate in 1985. It is conceivable that the projected cost at the present time would be a much higher figure. A more in-depth look at the breakdown of the cost to society indicates that more than half of the estimated costs were due to drug-related crimes, and about 20% to lost productivity of victims and incarcerated perpetrators of drug-related crimes. The other combined total costs arose from property damage, drug traffic control, legal and correction services, illness and premature deaths of drug users (National Institute on Drug Abuse (NIDA), 2008b).

Theultimate price to be paid by the drug user, whether intentional or accidental, is death. When it comes to the effects of drug abuse on mortality and morbidity, no single group of people in the human population is spared. In this regard, across the entire strata of society, drug abuse causes a 'levelling effect' of misery and

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eventual demise of all those who abuse drugs. From the incarcerated to the decorated, pop stars to movie stars, the ordinary sports enthusiast to sports legends, the ultimate price for drug abuse is the same. Several well-known celebrities succumbed to the adverse effects of drug use. In general, individuals abusing drugs have a higher risk of premature or drug-related deaths when compared with contemporaries who do not use drugs. Jonsson *et al*. (2007) found that drug abuse was directly or indirectly responsible for the deaths of 47% of deceased drug addicts in Sweden in 2002–2003. In the Swedish study, the main drugs detected were ethanol and morphine, and benzodiazepines to a lesser extent. Death due to the use of other illicit drugs is well documented in the literature. Methamphetamine use has been implicated in accidental deaths in Australia (Kaye *et al*., 2008) and use of opiates has been implicated in an overwhelming number of fatalities in the USA in recent years (Hall *et al*., 2008; Paulozzi, 2007). Many other drugs have also been implicated in accidental deaths or death by suicide. Among them are stimulants, such as cocaine and amphetamines (Graham and Hanzlick, 2008; Pilgrim *et al*., 2009), inhalants (Sugie *et al*., 2004) and a variety of CNS depressant drugs, such as alcohol (Theobald *et al*., 2001).

12 MEDICOLEGAL IMPLICATIONS

In societies where law and order are seen as instruments which aid in the settlement of disputes, innocence or guilt associated with an alleged drug use may be determined in a courtroom. As applied to the adverse effects and consequences of drug abuse in these societies, several professionals are usually involved in settling the matter. It is here that the role of the forensic toxicologist becomes important. In addition to acquiring a well-grounded educational training in the field, the forensic toxicologist must have an inquisitive mind, be familiar with a wide variety of drugs, and be conversant with current knowledge regarding the 'drug culture' of the jurisdiction of interest, including the flow, distribution and patterns of use of licit and illicit drugs (Adatsi, 2005). Whatever the requirements might be, once the elements of acceptability are met, ultimately the courts will be looking to the expert to provide direct testimony relating to the specific analyses performed and interpret data that has been generated elsewhere or to provide a spectrum of testimony impinging on what, how much and when exposure to a drug may have occurred. Many judicial systems, including the American judicial system, can be a hostile arena for scientists who agree to serve as expert witnesses. For this reason, a great deal of preparation is needed prior to appearing in court (Furst, 1997; Nowicki and Bright, 2004). Of necessity is adequate knowledge of the discipline in which testimony is sought, and prior communication with the attorney requesting the services of the expert witness. Above all, the expert witness must bear in mind the following famous quote:

> *If the law has made you a witness, remain a man of science. You have no victim to avenge, no guilty or innocent person to convict or save, you must bear your testimony within the limits of science.* ~ *Dr. P.C.H. Brouardel, 19th Century French medicolegalist*

A prominent manifestation of the medicolegal aspect of drug abuse is in the area of drug-related driving offences. In many societies where the automobile is part and parcel of human activity, its operation by the citizenry is considered a privilege. Therefore, safe, proper and normal driving in these societies has a legal component and is regulated. The operation of a motor vehicle is a complex activity requiring the proper functioning of the senses of the CNS. It requires that the driver integrate several functional parameters that are integrated at the level of the CNS. It is a divided-attention task that requires the simultaneous execution of activities requiring thinking clearly, making critical decisions, and monitoring and prioritizing incoming sensory cues. It also requires paying attention to the driving scene, and reacting to perceived events quickly and appropriately. These activities are integrated at the level of the CNS. Many drugs, including alcohol, which affect the CNS or are themselves CNS depressants, impair the proper functioning of the CNS, thereby impairing the driver's ability to operate a motor vehicle in a safe manner.

Drugged driving via drugs other than alcohol is also a public concern that endangers the driver, passengers and other drivers. In 2007, an estimated 12 998 people were killed in alcohol-impaired driving crashes in the United States (National Highway Traffic Safety Administration (NHTSA), 2008). Coming in as a close second to alcohol-related fatalities is the involvement of marijuana. Overall, marijuana has been found to be a prevalent illicit drug in impaired drivers, fatally injured drivers and motor-vehicle crash victims (National Institute on Drug Abuse (NIDA), 2008a; Ramaekers *et al*., 2004; Bedard *et al*., 2007). Many other drugs, from stimulants to CNS depressants have also been implicated in poor driving (Holmgren *et al*., 2007; Jones *et al*., 2008; Logan, 1996; Logan and Couper, 2001; National Institute on Drug Abuse (NIDA), 2008b). Many prescription drugs, such as the benzodiazepines and opiates, come with warnings against the operation of machinery, including motor vehicles, until such time that the user is able to tolerate the drug and can unambiguously determine that they can safely operate a motor vehicle safely (Physicians' Desk Reference (PDR), 2008).

There is a multiplicity of punitive consequences which follow the single misstep of driving under the influence of drugs, as measured by the established rules of proper

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conduct within the society of the drug user. Take the case of our hypothetical drug user who spent an evening celebrating a promotion at the job, by drinking with friends at a local beer bar somewhere in the United States of America. Reportedly, he spent most of the evening drinking evenly over a period of three hours. When the bar closed in the early hours of the next morning he got into his car and started to drive home. Quite imaginably, his driving was affected by the 12 beers he reportedly consumed while in the bar. A few drivers with whom he shared the road that night called the police to report his erratic driving. However, before the police got to him, he crashed his vehicle into another, killing its occupant. The criminal aspect of this accident earned him a 15 year sentence for 'operating under the influence of liquor, causing death', this being the applied penalty, pursuant to the statutory violation of the privilege offered him to drive under the laws of the country of his residence.

While in prison, an assessment was made of the grief, and damage and disruption of the life he caused others by virtue of the crash. Here comes the next punitive multiple in the series of consequences which follow this act of bad judgement. The financial liability phase is enormous and spreads to many areas. His own assets, the responsibility of the bar, rehabilitation back into society and the guilt that he is behind the agony and pain being suffered by others for the loss of a loved one in the accident he caused come into focus. Survivors of the deceased mounted a suit against the bar for serving a drunken individual with alcohol, even when it was clear that he may have been visibly intoxicated with alcohol. Serving him with additional alcohol enhanced his drunken state and increased the probability that he might be involved in a traffic accident if he operated a motor vehicle. In the ensuing civil litigation, the action of the bar, in furnishing more alcohol to a visibly intoxicated individual, was judged to contribute to the occurrence of the traffic accident. Subsequently, judgement was against the bar to bear some financial responsibility for the fiscal needs of the surviving next-of- kin of the deceased.

13 CONCLUSION

There is no aspect of our human existence that is not affected by the adverse effects resulting from the use of drugs of addiction in the domestic, occupational and sports environment. Drug abuse is a menace that inflicts damage to society, reduces human resources and threatens national security. It costs billions of dollars in preventive initiatives and treatment programmes. The choice of one drug over another as an addictive drug is governed by its availability, ease of procurement, and cultural and environmental factors, as well as the user's inherent susceptibility to that drug. The list of drugs in each category is by no means an exhaustive one and not intended to represent a comprehensive compilation of all drugs which could belong to that class. Instead it highlights the current knowledge about those drugs commonly encountered and those showing a high abuse pattern.

The types of drugs likely to be found or abused in any environment will be greatly influenced by the culture and complex social orientation of that society. Conceivably, the popularity of today's abused drugs will wane in the years to come. However, it is doubtful that conventional drugs of abuse will totally disappear or lose their appeal to the user. Unfortunately, some of the same drugs which were popular several decades ago will continue to remain on the user's menu and can easily revert to being drugs of choice if vigilance on their control is relaxed. In time, other drugs synthesized for legitimate use or those synthesized in clandestine laboratories will become available and some will be diverted into the wrong hands and gain popularity as drugs of abuse.

Given its long history as being an integral part of human existence, drug addiction will continue to plague society in the years to come, and efforts to identify and curb the use of 'drugs of addiction' will persist. There will always be those drugs which must be consumed to prolong life and assure survival, and those consumed for pleasure or whatever else. As familiarity with each drug becomes well established, a new paradigm will be established to classify drugs into subgroups based on their preponderance, appeal and location of use. Sadly, in this regard, it is the drug user who will perhaps always be a step ahead of the scientific community, as well as society at large, in the recognition or classification of a drug as being a new drug of addiction.

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Glutathione and Glutathione-S-Transferase in Detoxification Mechanisms

Xianchun Li

CONTENTS

1 INTRODUCTION

All aerobic organisms, including bacteria, plants, insects and mammals, are subject to oxidative stress resulting from overproduction of reactive oxygen species (ROS), such as free radicals (e.g. superoxide, nitric oxide (NO) and hydroxyl radicals (OHs) and other reactive species (RS) (e.g. hydrogen peroxide, peroxynitrite and hypochlorous acid) (Łukasik and Goławska, 2007). ROS are produced endogenously as a result of aerobic metabolisms, such as oxygen-consuming respiration in the mitochondria and oxygen-producing photosynthesis in the chloroplast. The ROS produced may react with biomolecules, such as DNA, RNA, proteins and lipids, resulting in cell damage and death (Apel and Hirt, 2004; Reed, 1995). Moreover, aerobic organisms also face toxic challenges from various exogenous toxic and/or pro-oxidant xenobiotics present in their diet or environment. Xenobiotics may exert toxic effects by directly disrupting the normal functions of the organisms and/or by generating ROS upon entry to the body or oxidation (Ahmad, 1992; Kalyanaraman *et al*., 1987; Reed, 1995).

To cope with the inevitable oxidative stress from aerobic metabolisms, toxin-rich diets or the environment, aerobic organisms have evolved an integrated detoxification system capable of scavenging ROS and degrading toxic xenobiotics, as well as complex regulatory machinery capable of inducing certain components of the detoxification system when encountering a particular ROS or xenobiotic (Apel and Hirt, 2004; Li *et al*., 2007). This detoxification system heavily depends upon reduced glutathione (γ-glutamyl-cysteinyl-glycine; GSH), an endogenous antioxidant molecule, and glutathione-S-transferase (GST), an antioxidant/detoxification enzyme. GSH and GST are ubiquitous in all living organisms and often exert synergistic actions in detoxifying both ROS and xenobiotics. This chapter presents the current understanding of GSH and GST enzymes, with emphasis on their roles in detoxification, their reaction mechanisms and regulatory control.

2 GLUTATHIONE

2.1 *De Novo* Synthesis of Glutathione

GSH is a water-soluble linear tripeptide of L-glutamine, L-cysteine and glycine (**Figure 1**). It is ubiquitously distributed in the different cellular compartments, including almost 90% in the cytosol (1–10 mM),

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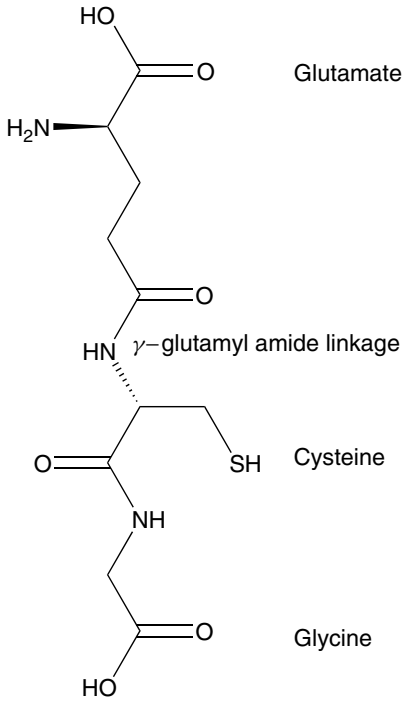

Figure 1 Structure of reduced glutathione. GSH is a linear tripeptide of L-glutamine, L-cysteine and glycine. The electron-donating sulfhydryl (-SH) group on the cysteinyl portion allows GSH to participate in two types of metabolic reactions: oxidation-reduction (redox) and conjugation reactions. The peptidic γ-linkage between the γ-carboxyl group of glutamate and the amino group of cysteine protects GSH from hydrolysis by intracellular peptidases.

approximately 10% in the mitochondria (5–10 mM) and a small percentage in the endoplasmic reticulum and the nucleus (Dalle-Donne *et al*., 2008; Lu, 2000). But all cellular GSH is synthesized within the cytoplasm in a two-step enzymatic process (**Figure 2**; Bukowska, 2004; Franco *et al*., 2007; Wu *et al*., 2004). In the first step, a peptidic γ-linkage is formed between the γ-carboxyl group of glutamate and the amino group of cysteine catalysed by γ-glutamylcysteine synthetase (GCS), also known as glutamylcysteine ligase (GCL). This peptidic γ-linkage protects GSH from hydrolysis by intracellular peptidases. In the second step, GSH synthetase (GS) catalyses the reaction between amine residue of glycine and the cysteine carboxyl from γ-glutamylcysteine dipeptide to form GSH (**Figure 2**; Bukowska, 2004; Franco *et al*., 2007; Wu *et al*., 2004). Both enzymatic steps consume one molecule of adenosine triphosphate (ATP) per catalytic cycle. As GSH levels rise, they self-limit further GSH synthesis because GSH can competitively inhibit the GCS activity. Otherwise, cysteine availability is usually the rate-limiting factor (Dickinson and Forman, 2002; Franco *et al*., 2007; Wu *et al*., 2004).

2.2 Roles of GSH in Detoxification Against Reactive Species and Toxic Xenobiotics

GSH is the most abundant intracellular nonprotein thiol (RSH) molecule. Its high electron-donating sulfhydryl (-SH) group on the cysteinyl portion combined with high intracellular concentration (millimolar levels) generate great reducing power. This characteristic allows GSH to participate in a wide variety of metabolic protection and/or detoxification processes, including oxidation-reduction (redox) reactions and nucleophilic displacement or addition-type conjugation reactions (Franco *et al*., 2007; Meister, 1995a; 1995b; Wu *et al*., 2004). When participating in redox reactions, GSH acts as an antioxidant, and scavenges a wide range of RS, including ROS and reactive nitrogen species (RNS), which are generated endogenously or from exogenous toxic and/or pro-oxidant compounds. As an antioxidant, GSH can directly react with NO to form the relatively stable S-nitrosoglutathione (GSNO) ($GSH + NO + O_2 \rightarrow GSNO + O_2^-$), which can be inactivated by its conjugation to proteins through S-nitrosylation reactions ($GSNO + \text{Protein-SH} \rightarrow \text{Protein -SNO} + GSH$). GSH can also effectively scavenge a number of ROS, including the superoxide anion ($\bullet O_2^-$), $\bullet OH^-$, singlet oxygen ($^1O_2$) and protein and DNA radicals directly, by donating electrons and becoming oxidized to the thyil radical (GS$\bullet$) (**Figure 2**; Franco *et al*., 2007). Moreover, GSH can function as an essential cofactor for GSH peroxidases (GPXs), peroxiredoxins (PXRs), phospholipid hydroperoxide GSH peroxidases (PHGPXs) and GST, to catalytically detoxify cells from hydroperoxides (e.g. H_2O_2), peroxynitrite ($OONO^-$) and lipid peroxides (LOOH) (**Figure 2**; Mates, 2000; Valko *et al*., 2007).

In these direct and indirect (enzyme-catalysing) redox reactions, RS are reduced or inactivated mainly through the generation of disulfide bonds between two GS molecules to form glutathione disulfide (GSSG) (**Figure 2**). This disulfide linkage is reversible and one GSSG molecule can be rapidly reduced to two GSH molecules by the NADPH-dependent glutathione reductase (GR) (**Figure 2**). Changes in the intracellular thiol–disulfide (GSH/GSSG) balance within the cell can be used as an indicator of the redox status of the cells. Under normal physiological conditions, the resulting GSSG can be rapidly reduced to GSH by GR (**Figure 2**), keeping GSSG at ~1% of total GSH concentration (Forman *et al*., 2002; Wu *et al*., 2004). Under severe oxidative stress, GR alone may not be able to keep the cellular GSSG level at ~1% of total GSH concentration and the increased GSSG accumulation can be actively extruded through specific transporters and degraded extracellularly (discussed below).

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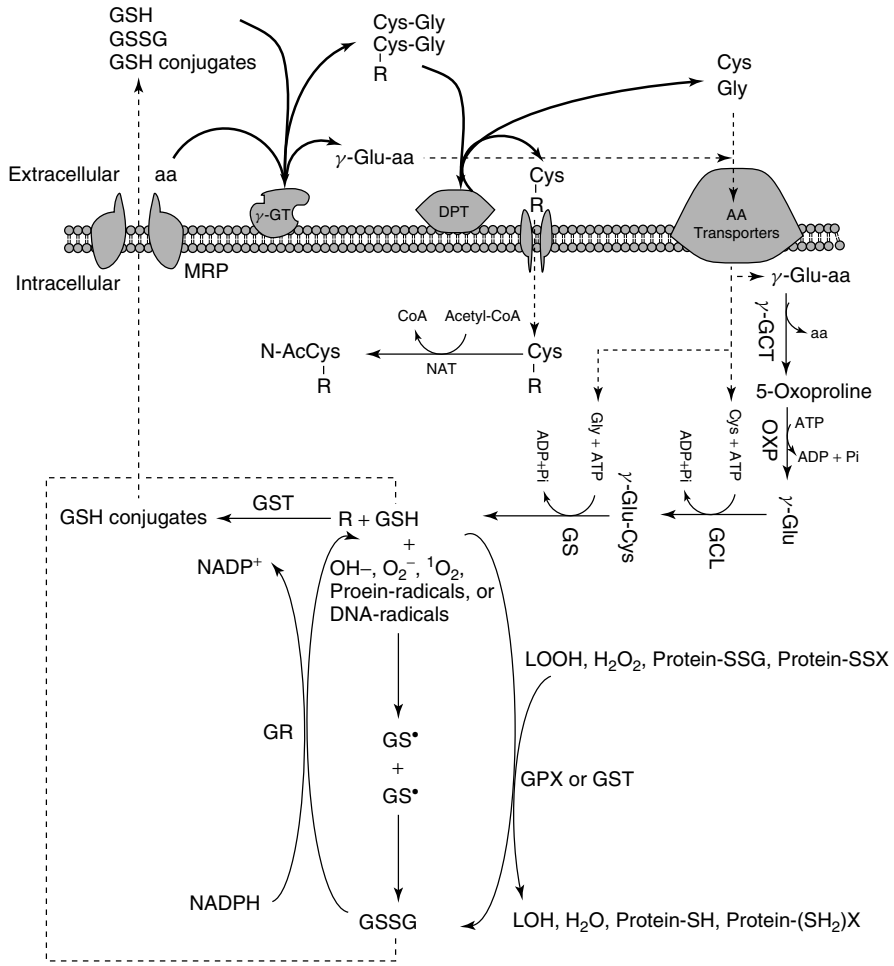

Figure 2 GSH biosynthesis, its utilization and recycling from GSSG and GSH conjugates. GSH is synthesized within the cytoplasm in a two-step enzymatic process catalysed by glutamylcysteine ligase (GCL) and GSH synthetase (GS). GSH can directly or catalytically (by GSH peroxidase (GPX) or glutathione-S-transferase (GST)) reduce various reactive species and oxidants through formation of glutathione disulfide (GSSG). GSH can also conjugate with endogenous and exogenous electrophiles (R), which is largely catalysed by GST. While GSSG can be reduced enzymatically by the NADPH-dependent glutathione reductase (GR) to regenerate GSH within the cells, GSH conjugates must be exported into the extracellular space by ATP-dependent multidrug-resistance protein (MRP) transporters to avoid deleterious effects. MRP may export some GSSG to the extracellular space as well, when the cellular GSSG concentration is too high. The extracellular GSH conjugates, GSH and GSSG are metabolized by γ-glutamyl transpeptidase (γ-GT) and dipeptidase (DPT) to γ-glutamyl-amino acid (γ-Glu-aa), cysteine (Cys), glycine (Gly) and cysteine conjugates ($\overset{\text{Cys}}{\underset{\text{R}}{|}}$, breakdown products of GSH conjugates). The first three products are transported back into the cells by specific amino-acid transporters to serve as substrates for GSH biosynthesis. The cysteine conjugates are taken up into the cells and acetylated by intracellular *N*-acetyltransferases (NAT) to mercapturic acid derivatives ($\overset{\text{N-AcCys}}{\underset{\text{R}}{|}}$) and excreted. Metabolic pathways are depicted by solid lines, whereas pathways showing transport are dashed.

When participated in nucleophilic displacement or addition-type conjugation reactions, GSH plays an essential role in the detoxification and elimination of a great variety of exogenous toxic xenobiotics (e.g. arene oxides, unsaturated carbonyls, organic halides), drugs and endogenous electrophiles (e.g. oestrogen, melanins, prostaglandins and leukotrienes). In these conjugation reactions, the sulfhydryl group of GSH acts as a nucleophile and either displaces another atom (e.g. Cl) or group (e.g. nitro) or attacks an electrophilic site in xenobiotics, endogenous compounds or their reactive metabolism intermediates, to form GSH conjugates (**Figure 2**; Blair, 2006; Franco *et al*., 2007; Meister and Anderson, 1983). GSH conjugation reactions may be spontaneous, but primarily are catalysed by GST, a family of Phase II detoxification enzymes (Meister and Anderson, 1983; Wu *et al*., 2004). To some substrates, GSH conjugation can retain activity

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of the parent compounds or even activate the parent compounds (Blair, 2006; Monks *et al*., 1990). For example, directly toxic GSH conjugates may be formed from vicinal dihaloalkanes via formation of electrophilic sulphur mustards. Precursor organic thiocyanates and nitrosoguanidines (*N*-methyl-*N*′-nitro-*N*-nitroguanidine) may release toxic agents on GSH conjugation (Monks *et al*., 1990). But, in general, GSH conjugation is considered as an important detoxification mechanism because most of the GSH conjugates produced are more water soluble and less toxic than the parent electrophiles.

2.3 Catabolism of GSSG and GSH Conjugates and Replenishment of GSH

GSH-dependent protection and detoxification of cells against various RS and toxic foreign compounds typically leads to rapid depletion of GSH and formation of GSSG and GSH conjugates (**Figure 2**). While GSSG can be reduced enzymatically by the NADPH-dependent GR to regenerate GSH within the cells, GSH conjugates must be exported into the extracellular space by ATP-dependent transporters to avoid deleterious effects. This is because γ-glutamyl transpeptidase (γ-GT), the only enzyme that can break down the γ-glutamyl amide bond in GSH, GSH conjugates and GSSG, is expressed mainly on the apical surface of cells and located on the external side of the plasma membrane (**Figure 2**). Under severe cellular oxidative stress, too much GSSG may be produced, so that some of them must be transported to the extracellular space as well, for further catabolism (Blair, 2006; Franco *et al*., 2007; Meister and Anderson, 1983). The efflux of GSH conjugates and GSSG (also GSH) across the plasma membrane largely depends on the multidrug-resistance protein (MRP, encoded by ABCC genes), a subfamily of the ATP binding cassette transporters (ABC transporters) (**Figure 2**; Hammond *et al*., 2001; Ballatori *et al*., 2005; Franco *et al*., 2007). The MRP transporters act as cotransporters of organic anions (OA^-) and GSH, GSH conjugates or GSSG. Such MRP-mediated efflux of GSH, GSH conjugates and GSSG is ATP-dependent and requires the presence of GSH (Ballatori *et al*., 2005; Cole and Deeley, 2006; Franco *et al*., 2007). In addition, GSH conjugates may also be transported to the extracellular space by the Ral-regulated effector protein (RLip76, RALBP1), a 76 kDa Ral-binding, Rho/Rac-GAP and Ral effector protein (Awasthi *et al*., 2002; 2003; Sharma *et al*., 2001; 2004). The efflux of GSH conjugates and GSSG confers drug resistance to tumour cells and can protect normal cells from toxic insults (Franco *et al*., 2007).

Two extracellular enzymes, the γ-GT and the cysteinyl glycinase, a dipeptidase (DPT), catalyse the catabolism of GSH, GSSG and GSH conjugates in the extracellular milieu (**Figure 2** and **Figure 3**; Dickinson and Forman, 2002; Franco *et al*., 2007; van Klaveren *et al*., 1997). First, the γ-GT cleaves the γ-glutamyl amide bond to cysteine and transfers the glutamyl residue from GSH, GSH conjugates or GSSG to other amino-acid acceptors (usually cystine), thus producing the common product γ-glutamyl-amino acid (γ-Glu-aa) and substrate-specific products cysteinylglycine (cys-gly, GSH), cys-gly conjugates (GSH conjugates), cys-gly and GSH (γ-Glu-$^{\text{Cys-Gly}}_{\text{Cys-Gly}}$, GSSG) or cystinylbisglycine ($^{\text{Cys-Gly}}_{\text{Cys-Gly}}$, GSSG) (**Figures 2** and **3**; Franco *et al*., 2007; Jones *et al*., 1979). The substrate-specific products are further cleaved by the extracellular DPT, to the common product glycine and the substrate-specific products cysteine (GSH), cysteine conjugates (Cys-R; GSH conjugates) or cystine ($^{\text{Cys}}_{\text{Cys}}$, as an amino-acid acceptor; GSSG). The glycine and cysteine formed are then transported back into the cells by specific amino-acid transporters to serve as substrates for *de novo* intracellular GSH biosynthesis (**Figure 2**). The γ-Glu-aa is also transported back into the cells where it is enzymatically converted to glutamate for GSH biosynthesis through the activity of the γ-glutamyl cyclotransferase (γ-GCT) and the 5-oxoprolinase (OXP) (**Figure 2**). On the other hand, the cysteine conjugate, a breakdown product of GSH conjugates, is taken up into the same cells or into other cell types, where it is acetylated by intracellular *N*-acetyltransferases (NATs) to a mercapturic acid derivative and excreted.

2.4 Homeostatic Regulation of GSH Content

GSH content is under tight homeostatic control, both intracellularly and extracellularly, to maintain a reduced cellular environment. This is achieved by a dynamic balance between GSH synthesis, the utilization of GSH for detoxification against RS, drugs and toxic xenobiotics, and its recycling from GSSG and GSH conjugates. Under normal physiological conditions whereby utilization of GSH is relatively low (only against endogenous RS and electrophiles), the GSH homeostasis system would attempt to keep the cellular GSH content and GSH/GSSG ratio under their upper limit. This is accomplished by the basal levels of the *de novo* synthesis and recycling of GSH, and GSH inhibition of the activity of the rate-limiting enzyme γ-GCL (Dickinson and Forman, 2002; Estrela *et al*., 2006; Franco *et al*., 2007; Wu *et al*., 2004). Upon oxidative stress or electrophilic stimuli, cellular GSH is rapidly used and depleted for protection of cells against these toxic insults, leading to quick and short-term falls in intracellular GSH associated with increased GSSG/GSH conjugate levels (Rahman *et al*., 2005). Accordingly, the GSH homeostasis system would quickly replenish the GSH

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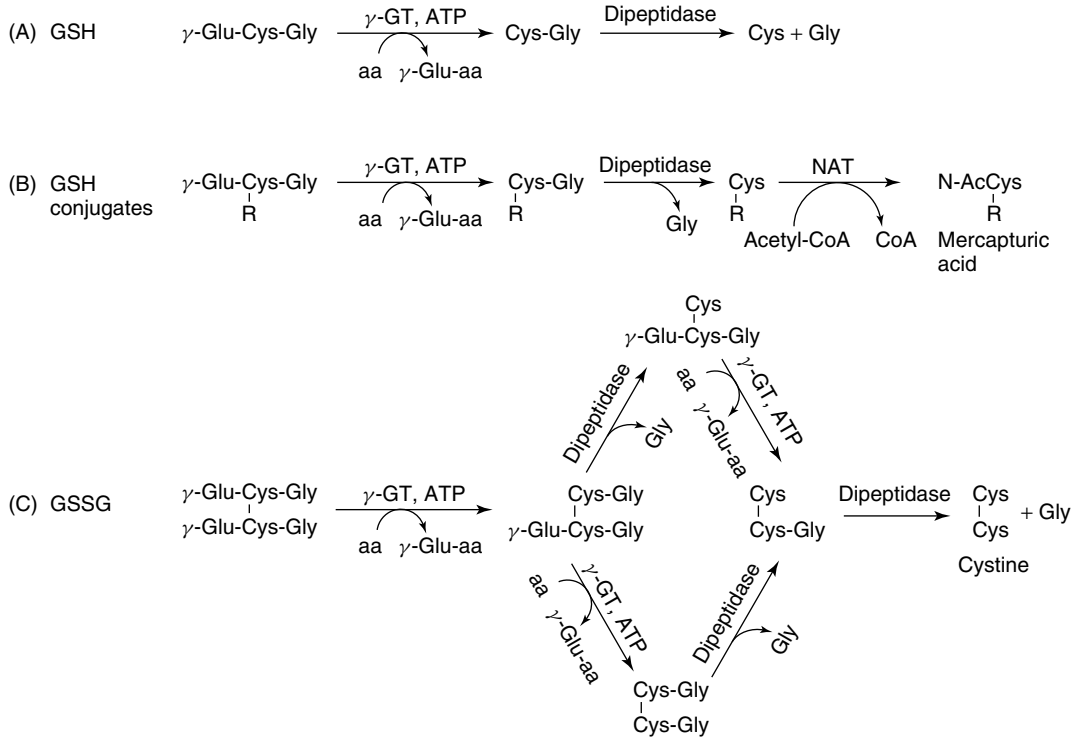

Figure 3 Catabolism reactions and products of (A) GSH, (B) GSH conjugates and (C) GSSG. Cys, cysteine; Gly, glycine; γ-Glu-aa, γ-glutamyl-amino acid; NAT, *N*-acetyltransferases; γ-GT, γ-glutamyl transpeptidase; R, electrophiles.

content and restore the GSH/GSSG ratio back to their normal levels. Depending on cell types and the oxidative and electrophilic stimuli, this can be achieved by increases in *de novo* GSH biosynthesis, GR-mediated GSSG reduction, efflux of GSSG/GSH conjugates, extracellular catabolism of GSSG and GSH conjugates (see **Figure 2**) and/or their combinations. In fact, a large number of compounds, including those forming GSH conjugates and those generating RS, have been shown to cause initial short-term falls in intracellular GSH levels, followed by increases in GSH levels, through induced transcription of the genes involved in *de novo* GSH biosynthesis (γ-GCL, GS) (Dickinson and Forman, 2002; Rahman *et al*., 2005; Shih *et al*., 2003; Trachootham *et al*., 2008; Wild *et al*., 1999; Wu *et al*., 2004; Zhu *et al*., 2008a; Zipper and Mulcahy, 2000), GSSG reduction (GR) (Harvey *et al*., 2008; Ling *et al*., 2008; Shih *et al*., 2003; Zhu *et al*., 2008a; 2008b; 2008c), efflux of GSSG/GSH conjugates (MRP) (Shih *et al*., 2003; Trachootham *et al*., 2008) and/or extracellular catabolism of GSSG and GSH conjugates (γ-GT) (Maher *et al*., 2007; Zhang *et al*., 2005; 2006a).

Recent studies from vertebrate systems indicate that it is the nuclear factor-erythroid-2-related factor 2 (Nrf2)-Kelch-like ECH-associating protein 1 (Keap1) signalling cascade that transduces oxidative and electrophilic stress signals into up-regulation of the above GSH homeostasis gene (γ-GCL, GS, GR, MRP, γ-GT) (**Figure 4**; Harvey *et al*., 2008; Ling *et al*., 2008; Maher *et al*., 2007; Rahman *et al*., 2005; Shih *et al*., 2003; Surh *et al*., 2008; Trachootham *et al*., 2008; Zhang *et al*., 2005; 2006a; Zhu *et al*., 2008b; 2008c). As a member of the NF-E2 family of the basic leucine zipper transcription factors, Nrf2 increases transcription of its target genes by heterodimerizing with small Maf proteins and then binding to a cis-acting element known as the antioxidant responsive element (ARE) or electrophile responsive element (EpRE) within the 5′-regulatory regions of these genes (Motohashi *et al*., 2004). All the GSH homeostasis genes are its target genes, because they have at least one copy of ARE in their 5′- regulatory regions (Erickson *et al*., 2002; Harvey *et al*., 2008; Ling *et al*., 2008; Maher *et al*., 2007; Moran *et al*., 2002; Rahman *et al*., 2005; Zhang *et al*., 2006a). Under normal physiological conditions, Nrf2 is captured in the cytoplasm by the actin-binding protein repressor Keap1 (**Figure 4**; Kobayashi and Yamamoto, 2005; Surh *et al*., 2008; Trachootham *et al*., 2008). Keap1 acts as a substrate adaptor of Cul3-based E3 ubiquitin ligase and targets Nrf2 for rapid degradation by the ubiquitin proteasome (Trachootham *et al*., 2008). Consequently, all GSH homeostasis genes are expressed at their basal levels due to lack of Nrf2-Maf heterodimers for binding to ARE enhancers in the 5′-regulatory regions of these genes (**Figure 4**). Upon oxidative or electrophilic stimuli, the tight Keap1–Nrf2 complex is dissociated due to oxidation or covalent modification of the critical

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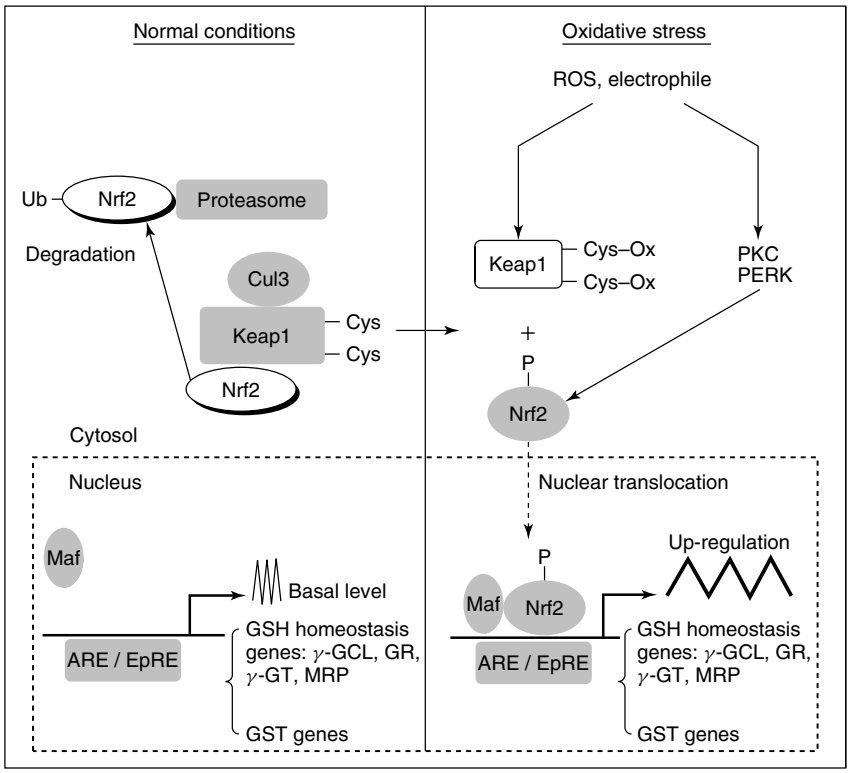

Figure 4 Redox and electrophilic regulation of GSH homeostasis and GST expression. Under normal conditions, Nrf2 is captured in the cytoplasm by the Keap1-Cul3 repressor complex for degradation by proteasome, and GSH homeostasis and GST genes are expressed at basal levels. Upon oxidative stress or electrophilic stimuli, Nrf2 is released from the Keap1-Cul3 repressor complex due to the oxidation/modification of the critical cysteine thiols of Keap1 or phosphorylation of Nrf2 by kinases such as PKC or PERK. The free Nrf2 is translocated to the nucleus, where it heterodimerizes with Maf proteins and then binds to the antioxidant responsive element (ARE) or electrophile responsive element (EpRE) in the 5′ promoter region of GSH homeostasis and GST genes. As a result, transcriptions of GSH homeostasis and GST genes are coordinately up-regulated (induced), leading to a concomitant increase in cellular GSH levels and GST enzymes. Grey, Active forms of the molecules. γ-GCL, glutamylcysteine ligase; GR, glutathione reductase; γ-GT, γ-glutamyl transpeptidase; MRP, multidrug-resistance protein transporter; GST, glutathione-S-transferase. (Modified from Trachootham *et al*., 2008.)

cysteine (Cys-151, Cys-273 and Cys-288) thiols of Keap1 by ROS and electrophiles, or phosphorylation of serine or threonine residues of Nrf2 by the upstream kinases, such as protein kinase C (PKC) and PERK (proline-rich extensin-like receptor protein kinase) (Surh *et al*., 2008; Trachootham *et al*., 2008). Either way, Nrf2 is released from the Keap1, stabilized and translocated into the nucleus, where it forms heterodimers with Maf and up-regulates transcriptions of the GSH homeostasis genes (**Figure 4**). As a result, cellular GSH content and the GSH/GSSG ratio are quickly returned to their normal levels.

3 GLUTATHIONE TRANSFERASE

3.1 Classification and Nomenclature

Glutathione transferases (EC 2.5.1.18), also known as glutathione-S-transferases, are a superfamily of multifunctional enzymes that evolved from a thioredoxin-like ancestor via multiple rounds of gene duplication and subsequent diversification (Hayes and McLellan, 1999; Ranson and Hemingway, 2005). Based on their cellular location, GSTs are grouped into three families: mitochondrial GST, microsomal GSTs, now designated membrane-associated proteins involved in eicosanoid and glutathione metabolism (MAPEG) and cytosolic GSTs. Microsomal GSTs bear no obvious resemblance to mitochondrial and cytosolic GSTs in terms of amino-acid sequence, molecular weight, or tertiary and quaternary structures (Ji *et al*., 2002; Torres-Rivera and Landa, 2008). For example, while mitochondrial and cytosolic GSTs exist as heterodimers or homodimers of subunits of 199–244 (23–27 kDa) and 226 amino acids respectively, microsomal GSTs may exist as monomers, homodimers, heterodimers, homotrimers or multimers of subunits of 14–17 kDa (137–150 amino acids) (Blanchette *et al*., 2007; Hayes *et al*., 2005; Ji *et al*., 2002; Kim *et al*., 2008; Morgenstern *et al*., 1985).

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Further classification of cytosolic and mitochondrial GSTs has been an evolving process, initially based on their elution orders from various affinity columns, substrate specificities, immunological properties or isoelectric points, which resulted in different classification/nomenclature systems in different phyla (Blanchette *et al*., 2007; Öztetik, 2008; Ranson and Hemingway, 2005). These biochemical-feature-based classification/nomenclature systems were gradually abandoned, largely because of substrate overlap and failure to reflect subunit composition (Ranson and Hemingway, 2005). Subsequently, a unified classification/nomenclature system that is largely based on the amino-acid identity of each subunit and subunit composition, and to a lesser extent, structural properties (catalytic site and dimer interface), has been proposed to classify and name both mammalian (Mannervik *et al*., 1992) and nonmammalian (Chelvanayagam *et al*., 2001; Edwards *et al*., 2000) mitochondrial and cytosolic GSTs. This system classifies each subunit to a different class, designated by a Greek letter, with members in each class sharing 40% or higher amino-acid identity (Chelvanayagam *et al*., 2001; Edwards *et al*., 2000; Mannervik *et al*., 1992). By this united criteria, the mitochondrial and cytosolic GST subunits currently characterized from all organisms are grouped into ∼15 classes, designated alpha (A), beta (B), delta (D), epsilon (E), phi (F), lambda (L), kappa (K), mu (M), omega (O), pi (P), rho (R), sigma (S), theta (T), tau (U) and zeta (Z) (Blanchette *et al*., 2007; Torres-Rivera and Landa, 2008). Mitochondrial GSTs are quite distinct from cytosolic GSTs and are all grouped into the Kappa class. Among the 14 cytosolic GSTs classes, the mu, alpha, pi, theta, sigma, zeta and omega classes are ubiquitous and can occur in any organism. The remaining seven cytosolic classes are organism-specific GSTs, found only in certain kingdoms or phyla, such as lambda, phi and tau in plants; delta and epsilon in insects; rho in fishes and beta in prokaryotes (Blanchette *et al*., 2007; Torres-Rivera and Landa, 2008; Ranson and Hemingway, 2005).

Under the unified classification/nomenclature system, the name of each GST subunit (or gene) is composed of the initials of the species scientific (nonmammalian species) or common name (mammalian species), followed by the acronym GST, a capital letter for the initial of the class name and a numeral for the individual gene, which is assigned according to the order of gene discovery in each class in that relevant species (Chelvanayagam *et al*., 2001; Li *et al*., 2007; Mannervik *et al*., 1992; Ranson and Hemingway, 2005; Öztetik, 2008). When naming a GST enzyme, two numbers with a dash in between are added after the class name to indicate the subunit composition of the enzyme. Thus AgGSTD2 is the second member of the *Anopheles gambiae* Delta class of GST subunits to be identified. AgGSTD2-2 represents a homodimer enzyme of two AgGSTD2 subunits, whereas AgGSTD1-2 would be a heterodimer GST enzyme made up of a subunit of AgGSTD1 and a subunit of AgGSTD2. And hGSTA2-2 represents a homodimeric GST enzyme of two human GSTD2 subunits.

3.2 Roles of GST in Detoxification against Reactive Species and Toxic Xenobiotics

The functions of GSTs are extremely versatile; this is manifested by the types of reactions they catalyse, the diversity of their substrates, and their noncatalytic activities. As a superfamily of Phase II detoxification enzymes, GSTs function primarily as GSH transferases to catalyse the conjugation of electrophilic endobiotics and xenobiotics (or their metabolites) to GSH (see **Figure 2**; Blanchette *et al*., 2007; Hayes *et al*., 2005; Li *et al*., 2007; Ranson and Hemingway, 2005; Sharma *et al*., 2004). Some GSTs also function as selenium (Se)-independent GSH peroxidase (**Figure 2**), GSH-dependent isomerase and GSH-dependent DDT dehydrochlorinase (DDTase). In addition, GSTs also have noncatalytic activities, functioning as binding and carrier proteins i.e. ligadins of various toxic compounds, modulators of the mitogen-activated protein (MAP) kinase signalling pathway via protein−protein interactions (Blanchette *et al*., 2007; Hayes *et al*., 2005; Sharma *et al*., 2004). As discussed below, most of these activities are essential for cell survival and detoxification against oxidants, RS and toxic electrophiles.

GST plays a pivotal role in cellular protection and detoxification by functioning as GSH transferases. As described in **Figure 2**, GSH confers protection by participating in two types of metabolic reactions: redox reactions for inactivating various oxidants/RS and scavenging the free radicals, and conjugation reactions for detoxifying toxic electrophilic compounds. Both types of reactions, in most cases, require catalysis of antioxidant/detoxificative enzymes. GSTs are the only enzymes that catalyse GSH conjugation reactions with endogenous and exogenous electrophiles. And GSH conjugation is a key stage in the conversion of lipophilic compounds to water-soluble metabolites that are more readily eliminated from the body (Ranson and Hemingway, 2005). Catalysis of GSH conjugations with various electrophiles is the basic function of GSTs. Almost all GSTs can function as GSH transferases although different GSTs may differ in the substrate specificities. Endogenous harmful electrophiles detoxified by GST-mediated GSH conjugations include epoxides, leukotrienes, the arachidonic acid oxidation products oxyeicosanoids (15-deoxy-Δ12,14-prostaglandin J2), the quinone-containing metabolites (aminochrome, dopachrome, noradrenochrome and adrenochrome) from catecholamine oxidation, the lipid peroxidation products,

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α, β-unsaturated aldehydes (2-alkenals, acrolein, crotonaldehyde, 4-hydroxy-2-alkenals, 4-hydroxy-2(E)-nonenal, 4-oxo-2(E)-nonenal), the cholesterol peroxidation products, cholesterol-5,6-oxide, epoxyeicosatrienoic acid and 9,10-epoxystearic acid, the oestradiol catechol metabolites, 2-hydroxy-oestradiol and 4-hydroxy-oestradiol and oxidized DNA bases (Hayes *et al*., 2005; Blair, 2006). The GSH conjugates formed are further metabolized and finally excreted as mercapturic acid derivatives in mammals (**Figure 2**).

A wide range of exogenous xenobiotics and/or their Phase I metabolites are detoxified by GST-mediated GSH conjugations. These include phytochemicals, therapeutic drugs, industrial intermediates, insecticides, herbicides, environmental pollutants and carcinogens (Hayes *et al*., 2005; Ranson and Hemingway, 2005; Li *et al*., 2007). Dietary phytochemicals that are metabolized by GST-mediated GSH conjugation reactions in animals include flavonoids, organothiocyanates (allyl thiocyanate, benzyl thiocyanate and 2-phenylethyl isothiocyanate) and α,β-unsaturated carbonyls (trans-2-octenal, *trans*-2-nonenal, 2, 4-hexadienal, *trans,trans*-2, 4-heptadienal, *trans,trans*-2, 4-nonadienal and *trans,trans*-2, 4-decadienal) (Li *et al*., 2007). Adriamycin, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), busulfan, carmustine, chlorambucil, cisplatin, crotonyloxymethyl-2-cyclohexenone (COMC-6), cyclophosphamide, ethacrynic acid, melphalan, mitozantrone and thiotepa are among the cancer chemotherapeutic drugs detoxified by GSTs (Hayes *et al*., 2005). GST-mediated GSH conjugations are implicated in the detoxification and resistance of some of the organophosphate insecticides and several herbicides including atrazine, fluorodifen, pentachloronitrobenzene (PCNB), propachlor, chlorimuron ethyl (Li *et al*., 2007; Öztetik, 2008). A large number of epoxides, such as the antibiotic fosfomycin and those formed from environmental carcinogens aflatoxin B1, 1-nitropyrene, 4-nitroquinoline, polycyclic aromatic hydrocarbons (PAHs) and styrene, by the actions of cytochrome P450 monooxygenases in the liver, lung, gastrointestinal tract and other organs, are detoxified by GSTs (Hayes *et al*., 2005). GSTs have been also documented to detoxify the ultimate carcinogenic bay- and fjord-region diol epoxides produced from chrysene, methylchrysene, benzo[c]chrysene, benzo[g]chrysene, benzo[c]phenanthrene, benzo[a]pyrene, dibenz[a,h]anthracene and dibenzo[a,l]pyrene, as well as the carcinogenic heterocyclic amines found in cooked protein-rich food (Hayes *et al*., 2005).

In addition to catalysing the conjugation of electrophilic substrates to GSH, many GST enzymes, such as the mitochondrial kappa (K) class GSTs and the cytosolic alpha (A) class GSTs also have a high Se-independent GSH peroxidase activity that allows them to catalyse some of the redox reactions that GSH participates in (Hurst *et al*., 1998; Prabhu *et al*., 2004; Sharma *et al*., 2004; Torres-Rivera and Landa, 2008; Yang *et al*., 2002a). By functioning as Se-independent GPXs, these GSTs provide protection against lipid peroxidation by terminating lipid peroxidation cascade through the reduction of fatty acid hydroperoxides (FA-OOH), and phospholipid hydroperoxides (PL-OOH) (Hayes *et al*., 2005; Sharma *et al*., 2004; Yang *et al*., 2001; 2002b; Zhao *et al*., 1999). Since these GST enzymes (e.g. hGSTA1-1 and hGSTA2-2) can use membrane PL-OOH, such as 1-palmitoyl-2-(13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoyl)-L-3-phosphatidylcholine and phosphatidylcholine hydroperoxide, as substrates *in situ*, they can protect cell membranes at the site of damage (Hayes *et al*., 2005; Hurst *et al*., 1998; Prabhu *et al*., 2004; Sharma *et al*., 2004; Yang *et al*., 2001; 2002a). Furthermore, as oxidative stress and subsequent lipid peroxidation are the common secondary effects of many drugs and xenobiotics, GSTs with Se-independent peroxidases play a crucial role in protection against xenobiotics by reducing lipid hydroperoxides. For example, GSTs with GSH peroxidase activity are implicated in pyrethroid resistance in insects (Vontas *et al*., 2001; 2002) and herbicide resistance in plants (Edwards *et al*., 2000).

Some GSTs can effectively detoxify the organochlorine insecticide DDT by functioning as GSH-dependent DDTase. A number of insect GSTs have been shown to catalyse the dehydrochlorination of the DDT to the noninsecticidal metabolite DDE by using GSH as a cofactor rather than as a substrate (Chen *et al*., 2003; Li *et al*., 2007; Lumjuan *et al*., 2005; Ortelli *et al*., 2003; Ranson *et al*., 2001; Syvanen *et al*., 1996). Besides catalysing conjugation, reduction and dehydrochlorination reactions, GSTs also contribute to intracellular and circulatory transport, sequestration, and disposition of endogenous lipophilic compounds and xenobiotics by acting as binding and transport proteins of hydrophobic nonsubstrates (Hayes *et al*., 2005; Li *et al*., 2007). This nonenzymatic binding activity can reduce the amount of toxic compounds that reaches their target sites (Kostaropoulos *et al*., 2001) and speed up their elimination.

3.3 Structure and Catalytic Mechanism of Cytosolic GSTs

To understand how GSTs catalyse the conjugation reaction of GSH with various electrophiles, three-dimensional (3D) structures have been determined for nearly all cytosolic classes of GSTs (Armstrong, 1997; Öztetik, 2008; Ranson and Hemingway, 2005; Sheehan *et al*., 2001). Although different classes of GSTs share low levels of sequence identity, their overall 3D structures are very similar, with each subunit of a dimeric enzyme consisting of two distinct

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domains (Domains I and II) that are separated by a short variable linker region of 5–10 residues (Öztetik, 2008). Domain I resides in the N-terminal one-third of the protein, consists of a β-α-β-α-β-β-α structural motif and provides the binding site for GSH (thus called the G-site) (Armstrong, 1997; Sheehan *et al*., 2001). Domain II, on the other hand, is located in the C-terminal two-thirds of the protein, consists of a variable number of α helices and contains the binding site for hydrophobic substrates (called the H site). Overall, the amino-acid sequences of GSTs are relatively conserved in the N-terminal Domain I, but are quite variable in the C-terminal Domain II (Blanchette *et al*., 2007; Torres-Rivera and Landa, 2008). This is probably because the N-terminal G site always binds and interacts with the same substrate GSH, regardless of GST classes. In contrast, the C-terminal H site often binds to, not one, but a number of different hydrophobic substrates, depending on GST classes and subunit composition. It is the H site that determines the substrate specificity of each GST enzyme.

While cytosolic GSTs are crystallized as a homodimer or heterodimer, at least some of them, if not all, exist as an equilibrium mixture of monomers and dimers in solution (Hearne and Colman, 2006; Huang *et al*., 2008; Vargo *et al*., 2004). The fact that the monomeric form of several human GST enzymes possesses at least partial activity as compared with the dimer (Hearne and Colman, 2006; Huang *et al*., 2008; Vargo *et al*., 2004) demonstrates that each subunit of a dimeric enzyme represents an independent catalytic unit. In other words, all the catalytic reactions between the common substrate GSH and various hydrophobic substrates take place at the subunit level. Nonetheless, dimerization is thought to stabilize the tertiary structure and the catalytic site of each subunit (Erhardt and Dirr, 1995; Huang *et al*., 2008) and to increase the efficiency of the enzyme (Hegazy *et al*., 2004).

The catalytic reactions that occur in each subunit include the G-site–GSH binding, the activation of GSH by the catalytic essential amino acid in the G site, the H-site–hydrophobic-compound binding, and the nucleophilic attack of the activated GS anion on the hydrophobic compound. The key step is the activation of GSH, that is, the ionization of the SH group of GSH by the catalytic essential residue in the G site (Atkins *et al*., 1993; Dirr *et al*., 1994; Liu *et al*., 1992). Depending on the GST class, the catalytic essential residue can be tyrosine (M, P, A and S classes), serine (F, U, D, E, T and Z classes) or cysteine (DHARs, L, B and O class) (Agianian *et al*., 2003; Frova, 2006; Ranson and Hemingway, 2005; Sheehan *et al*., 2001). The catalytic serine and tyrosine function as hydrogen-bond donors via their hydroxyl group to catalyse the ionization of the SH group of GSH, lowering its pK_a to a value of approximately 6–7 from its normal value of around 9.0 (Armstrong, 1997; Sheehan *et al*., 2001). This gets GSH ready for the final conjugation between GSH and the hydrophobic substrate (Torres-Rivera and Landa, 2008). In contrast, the catalytic cysteine residue forms mixed disulfides with GSH, rather than promoting the formation and stabilization of the reactive thiolate anion of GSH. Those GST enzymes bearing a catalytic cysteine residue at the G site often have great peroxidase activity, but poor GSH conjugation activity (Öztetik, 2008).

3.4 Regulation of GST Expression

GST enzymes are expressed at a basal level in normal conditions. Upon exposure to low levels of oxidative stress or a diverse range of endogenous and exogenous harmful electrophiles, expression of GST enzymes is strongly induced (**Figure 4**; Blanchette *et al*., 2007; Cheng *et al*., 2001; Dinkova-Kostova *et al*., 2001; Singhal *et al*., 1999; Surh *et al*., 2008). The electrophilic compounds that induce expression of GST genes are often the hydrophobic substrates of GST enzymes, which include lipid peroxidation products (4-hydroxynonenal (4-HNE)), chemoprotective agents (oltipraz, sulforaphane, 3H-1,2-dithiole-3-thione), phytochemicals (cruciferous sulforaphane, furanocoumarins, indoles, flavonoids, α,β-unsaturated carbonyls and glucosinolates) and various toxicants or pro-oxidants (t-butylhydroquinone, butylhydroxyanisole, thiazoles) (Fukuda *et al*., 1997; Kim and Lee, 2007; Li *et al*., 2007; Surh *et al*., 2008; Wadleigh and Yu, 1987; 1988; Zhang *et al*., 2006b; Zhu *et al*., 2008a). These inducers belong to nine distinct chemical classes, including oxidizable diphenols and quinones, Michael reaction acceptors (olefins or acetylenes conjugated to electron-withdrawing groups), isothiocyanates, hydroperoxides, trivalent arsenic derivatives, divalent heavy metal cations (Hg^{2+}, Cd^{2+}), vicinal dithiols, 1,2-dithiole-3-thiones, and carotenoids and other conjugated polyenes (Dinkova-Kostova *et al*., 2001). Nonetheless, they all can covalently modify sulfhydryl groups by alkylation, oxidation or reduction, which is responsible for the initial 'sensing' of these inducers by the actin-binding protein repressor Keap1, which contains several critical cysteines (Cys-151, Cys-273 and Cys-288) for modification (see **Figure 4** and Section 2.4 for details; Dinkova-Kostova *et al*., 2001; Hayes *et al*., 2005).

Interestingly, induction of GST genes is often paralleled with induction of GSH homeostasis genes (γ-GCL, GS, GR, MRP, γ-GT) (**Figure 4**; Li *et al*., 2005; Shih *et al*., 2003; Surh *et al*., 2008; Zhu *et al*., 2008a; 2008b; 2008c). Such a coordinated induction of GSH homeostasis genes and GST genes (also other Phase 2 detoxification genes) leads to a concomitant increase in cellular GSH levels and GST enzymes, which enables the cell to survive exposure to harmful xenobiotics and oxidative stress (Cao *et al*., 2003; Cheng *et al*.,

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2001; Dinkova-Kostova *et al*., 2001; Li *et al*., 2005; Singhal *et al*., 1999). Thus, it is not a surprise that GST genes, like GSH homeostasis genes, also contain ARE/EpRE (consensus sequence TGACNNNGC) in their 5′-promoter regions and are regulated by the very same Nrf2-Keap1 signalling pathway (see **Figure 4** and Section 2.4 for details; Hayes *et al*., 2005; Kim and Lee, 2007; Surh *et al*., 2008).

4 CONCLUDING REMARKS

GSH and GSTs are two primary lines of defence that protect human and other organisms from both acute and chronic toxicities of electrophiles and reactive oxygen/nitrogen species. In humans, a wide range of pathological conditions such as AIDS, ischaemia, neurodegenerative, metabolic and inherited diseases, as well as ageing, are associated with reduced/increased GSH levels or deterioration of GSH homeostasis (Estrela *et al*., 2006; Franco *et al*., 2007). Genetic polymorphisms of human GST enzymes (GSTA1, GSTM1) may be associated with increased cancer risk (Reszka *et al*., 2006). As specific GST isozymes (e.g. glutathione S-transferase P (GSTP)) are overexpressed in a wide variety of tumours resistant to drugs, GSTs have emerged as a promising therapeutic target for the development of novel cancer drugs (Burg *et al*., 2006; Tew, 2007; Townsend and Tew, 2003; Townsend *et al*., 2005; Zhao and Wang, 2006). The medical implications of GSH and GSTs are beyond the scope of this chapter and interested readers are encouraged to read the aforementioned reviews.

Much of our knowledge on the structure, catalytic properties and biological functions of GST enzymes are obtained from studies of mammalian cytosolic GSTs. Further work is required to elucidate the catalytic mechanisms and biological functions of the mitochondrial and microsomal GSTs. Historically, much work on GSH and GSTs has been focussed on the roles of GSH and GSTs in cytoprotection against oxidative stress and toxic compounds. More recently, emphasis has been placed on the contribution of GSH and GST enzymes to signalling pathways and the transcriptional regulation of GSH homeostasis genes and GST genes. Although the ARE/Nrf2-Keap1 pathway is elucidated as a key player in controlling transcription of GST and GSH homeostasis genes in mammals, it is yet unclear whether the nonmammalian counterparts are similarly regulated.

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Xenobiotic-Induced Inflammation: Pathogenesis and Mediators

Peter J. M. Clements

CONTENTS

1 INTRODUCTION

Inflammation is an extremely complex and fascinating adaptive process by which fluid, electrolytes, plasma proteins and leukocytes accumulate in the extravascular spaces, in response to a variety of noxious stimuli. These inducers of inflammation include trauma, infectious agents, neoplastic cells and xenobiotics. Such stimuli act via complex pathways of intracellular signalling and intercellular mediators to orchestrate the inflammatory process. The cardinal clinical signs of inflammation, redness (rubor), heat (calor), swelling (tumor) and pain (dolor) were initially described 2000 years ago by Celcus, with loss of function (functio laesa) added by Virchow in the nineteenth century.

Inflammation can be divided into acute and chronic phases, characterized by differing morphological and biochemical patterns. It is initially a vascular phenomenon requiring blood supply to deliver leukocytes and plasma proteins from the blood to the site of injury/invasion to destroy micro-organisms, degrade particulates, dilute/flush away toxins and destroy infected, damaged or neoplastic cells, then remove the debris and prepare the site for tissue healing/regeneration.

Due to the influx of inflammatory exudates and the effects of various mediators and effector cells, a properly regulated inflammatory response is beneficial in fulfilling the functions described above. However, dysregulated or prolonged inflammation can lead to ongoing tissue destruction and attempted repair, both of which may result in functional compromise to the affected area or to the whole body, depending on the organ affected.

An understanding of inflammation at the molecular level has led to the development of many therapeutic molecules in order to combat or reduce it. However, inflammation may also be induced by xenobiotics. This can be due to physicochemical properties of the xenobiotic, for example pH or high concentrations at the site of administration, or to local activity in cells at or close to the site of administration. This gives rise to inflammation localized to the point of entry into the body, which may be the alimentary tract (e.g. oral administration), nasal cavity/respiratory tract (intranasal/inhaled delivery), vascular system (intravenous injection/infusion), skin/subcutis (subcutaneous injection), skeletal muscle (intramuscular injection) or

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body cavity (peritoneal infusion/injection or intrathoracic administration due to a dosing accident). Inflammation can also be induced/enhanced by variations in experimental dosing techniques. There are risks inherent to a particular form of delivery, for example intravenous injection, or to specific vehicles (e.g. oily depot injections), in addition to direct effects of the xenobiotic being administered.

Xenobiotics may also act directly on cells/tissues, possibly via the production of toxic metabolites, causing cellular injury or death (e.g. via oxidative damage). They may also cause injury indirectly through an exaggerated pharmacology. The site(s) of induced inflammation will then depend on the pharmacodynamics, metabolism or accumulation of the particular xenobiotic (or xenobiotic metabolite) and the ability of the cells to adapt to its presence. Alternatively, xenobiotics may also cause inflammation by interacting with cells of the innate and/or adaptive immune systems, causing the release of proinflammatory cytokines or hypersensitivity reactions, or even by increasing susceptibility to infection by local commensal or pathogenic bacteria, for example in the alimentary or respiratory tract.

In tandem with a knowledge of cell biology, pharmacodynamics and toxicokinetics, examining the complex molecular mechanisms involved in inflammation can give insight into particular ways a xenobiotic may act (or be predicted to act) to induce inflammation. Mechanisms which apply to xenobiotics administered to animals on toxicology studies may or may not also be relevant in the clinical setting.

2 INFLAMMATION

A variety of exogenous and endogenous stimuli, including the effects of xenobiotics, can induce inflammation directly or indirectly. The activity of these *inducers* is recognized or *sensed* by the body, which then acts via a complex, interrelated series of inflammatory *mediators* to bring about changes in tissues and cells, which act as the *effectors* of the inflammatory process. Different patterns of inducers, mediators and effectors will influence the nature and morphologic appearance of the inflammatory response. A summary of the main processes involved in inflammation is shown in **Figure 1**.

Since the main function of inflammation in a tissue involves the delivery of fluid, soluble mediators and effector cells from the systemic circulation, it requires the vascular system for this delivery. Mediators and inflammatory cells may also be delivered to the lymph node draining the site of inflammation, via the lymphatic vessels. Initially, blood flow to the arterioles and capillaries at the site of injury is increased by the vasodilatory/modulatory actions of chemical mediators including vasoactive amines, prostaglandin D2, leukotriene B4 and nitric oxide, a process known as *active hyperaemia*. This produces an increase in hydrostatic pressure and a slowing in blood flow, or even stasis. Increases in the permeability of capillaries and postcapillary venules follow, due to changes in the intercellular junctional complexes of activated endothelial cells (ECs). This results in the selective leakage of plasma fluid and proteins such as albumin and fibrinogen into the extracellular space, a process known as *transudation*. In addition to the above mediators, this phase is influenced by bradykinin, other leukotrienes, platelet-activating factor (PAF), substance P and components of the complement cascade (C3a, C5a). Fibrinogen is an important component of the clotting cascade and polymerizes to form fibrin strands in the perivascular extracellular space. This forms a scaffold which promotes the chemotaxis and migration of inflammatory cells, confines the inciting stimulus/inflammatory response and provides an initial framework for the subsequent healing process. Once activated, ECs along with adjacent mast cells and fibroblasts alter the expression of their surface adhesion molecules (integrins, selectins) and produce cytokines/chemokines (interleukins (ILs)/chemotactic proteins), which act in concert to facilitate the recruitment of leukocytes (neutrophils, lymphocytes, monocytes/macrophages) into the perivascular extracellular space, via an ordered, multistep process called *transmigration*. Leukocytes in the slow-moving plasma of hyperaemic dilated capillaries/venules are able to marginate towards the vessel wall and roll along it, slowing to become firmly adherent, before passing between activated ECs into the perivascular *exudate*. The presence of significant amounts of protein, with or without inflammatory cells, signifies an *exudate*. If endothelial cells themselves are damaged (e.g. by toxins, trauma), the 'pores' created in the vessel walls become large enough to allow the passive extravasation of blood cells, and haemorrhage occurs (which is stemmed by the formation of a fibrin/platelet clot by the clotting cascade). As leukocytes exit the circulation and enter the surrounding tissues, they migrate along a concentration gradient of chemoattractant signals released by microbes, foreign materials, necrotic or neoplastic cells, which serves to appropriately target their activities. Neutrophils are often the predominant type of white blood cell in exudates in the early stages of inflammation. Exudates containing large numbers of viable and nonviable neutrophils are termed *purulent*, or the material itself as *pus*. If a marked neutrophilic response occurs with significant tissue destruction, then *abscess* formation may occur, which may become walled off from the body by the progressive production of fibrous tissue, if the inducing stimulus is not removed and resolution cannot therefore occur. Neutrophils contain prominent cytoplasmic granules composed

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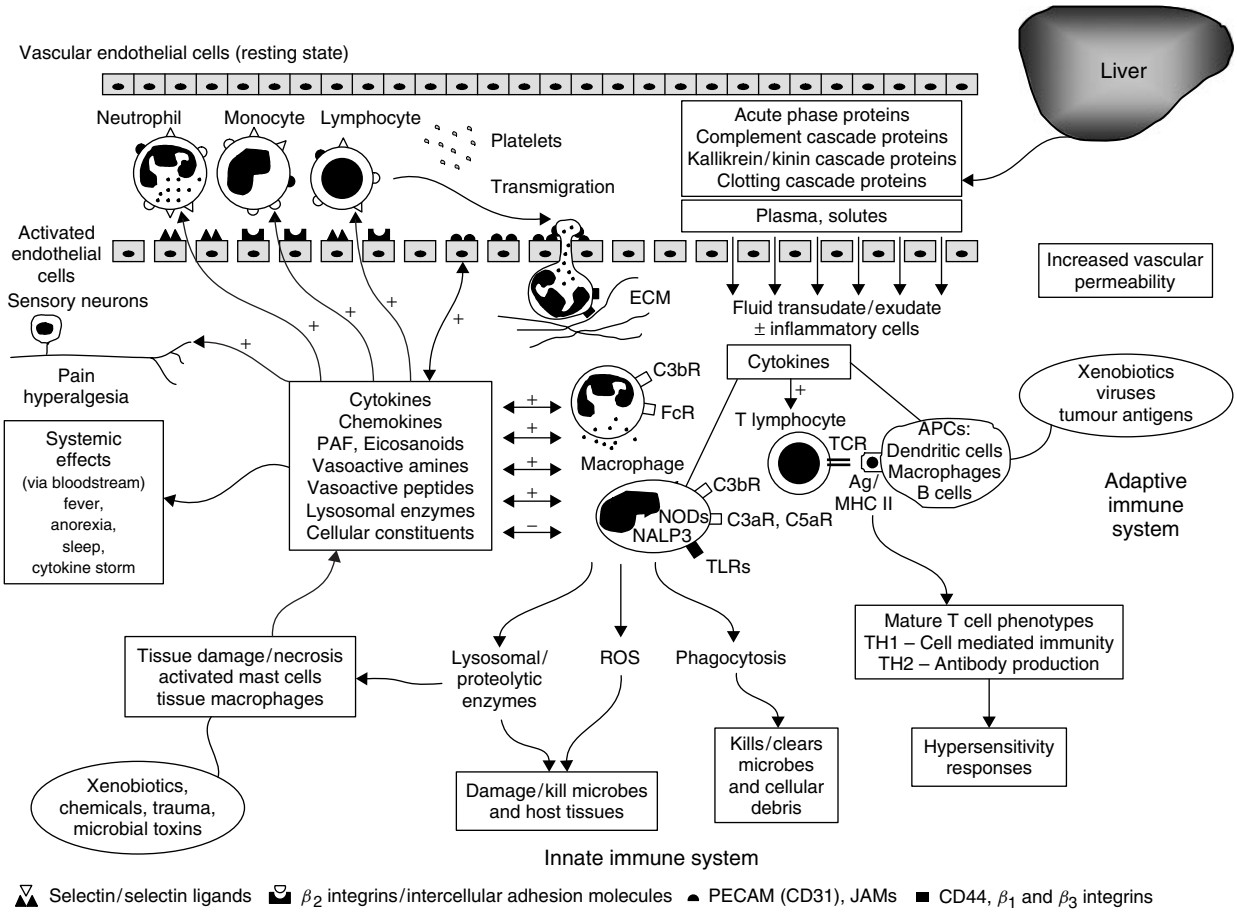

Figure 1 The major processes and classes of mediators involved in inflammation. For detailed explanation, refer to the main text. Expression of cell surface/cytoplasmic proteins as depicted is illustrative and is not as restricted to different cell types as shown. Stimulation of fibroblasts and production of new blood vessels by growth factors/angiogenic factors, to produce vascularized fibrous tissue important in both healing and chronic inflammation, are not shown. Ag—antigen; APCs—antigen presenting cells; C3aR, C3bR, C5aR—receptors for different complement components; ECM—extracellular matrix components; FcR—receptor for Fc portion of immunoglobulins (e.g. IgG); JAMs—junction-associated molecules; MHC II—major histocompatibility complex class II; NALP3—example of an inflammasome; NOD—nucleotide oligomerization domain-like proteins (cytoplasmic pattern-recognition receptors); PAF—platelet activating factor; PECAM—platelet endothelial cell adhesion molecule; ROS—reactive oxygen species; TCR—T cell receptor; TLRs—toll-like receptors.

of various enzymes (lysozyme, cathepsins, matrix metalloproteinases, myeloperoxidases), antimicrobial peptides, designed to kill microbes and degrade foreign material/damaged cells via phagocytosis or secretion of enzymes into the exudates. Monocytes/macrophages and lymphocytes also function to provide a link between the process of inflammation and the innate/adaptive immune response, to remove the inciting cause(s) of inflammation by clearing the debris formed by tissue/microbial destruction. The systemic release of cytokines such as tumour necrosis factor (TNF), IL-1, IL-6 and high-mobility group box 1 (HMGB1) protein also leads to clinical signs of fever and nausea. In order to avoid a potentially detrimental, uncontrolled inflammatory process, cytokines (transforming growth factor beta (TGFβ), IL-10, growth factors), lipoxins and intercellular signalling systems also serve to modify and dampen the inflammatory response and to initiate tissue repair. Once the stimulus has been removed, this allows the proliferation of fibroblasts (fibroplasia, fibrosis), blood vessels (angiogenesis) and epithelial tissues as required in the process of healing.

With minimal tissue damage, resolution may be possible with no permanent morphologic or functional changes. If significant tissue destruction has occurred, then healing through repair with phagocytosis and fibrosis/granulation tissue formation may occur. This will result in a fibrous tissue scar. However, if inciting stimuli/inducers are not successfully removed, for example if a microbe or foreign material persists by evading destruction (e.g. mycobacteria, fungi, parasites, asbestos fibrils, oily vehicles), if exposure to a

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xenobiotic is prolonged or repeated, or if an autoimmune reaction occurs, the inflammatory process becomes chronic. This shows distinct morphological appearances and patterns of cytokine/chemokine expression whereby acute inflammation, tissue destruction and repair occur simultaneously. The adaptive immune response will also become involved. Chronic inflammation shows a predominance of lymphocytes, plasma cells and macrophages with granulomatous inflammation rich in macrophages and/or multinucleated giant cells. Associated stimulation of fibroblasts, mesenchymal and epithelial cell types represents ongoing, but incomplete tissue repair. Tissue damage/dysfunction may be exacerbated by the continued destruction of tissue by effector cells and/or the indirect effect of functional compromise due to reduced functional mass or fibrosis (e.g. myocardial fibrosis, hepatic cirrhosis).

2.1 Fluidic/Exudative Phase

Normal fluid dynamics is an active process, and the balance between the amount of water and small molecules within blood (and lymphatic) vessels is influenced by

1. hydrostatic pressure within the vessel. This is the pressure of fluid in the circulation and may be influenced by fluid volume, driven primarily by sodium ions and vascular tone.
2. the colloid osmotic pressure exerted by plasma proteins held within the vessel by capillary walls. This may be influenced by capillary wall permeability, which normally limits egress of proteins, and the concentration of plasma proteins in the circulation (of which the liver is a major source of synthesis).
3. any impedance or obstruction to onward vascular flow, which will tend to increase the pressure upstream.

At the arterial end of a capillary bed, there is normally a net outflow of fluid (water plus electrolytes and small plasma proteins) which remains neutral across the capillary bed with a net inflow via intercellular pores at the postcapillary venules and lymphatics. During the fluidic/exudative phase of inflammation, there is a net fluid outflow at all levels of an inflamed, hyperaemic vascular bed, into the perivascular interstitium. This causes heat, redness and swelling of the affected area and is designed to dilute the stimulus and deliver the appropriate plasma proteins and cells to the site in order to neutralize it. This is achieved by an increase in blood flow to the site through vasodilation of arterioles and capillaries. Activated ECs effect an increase in permeability of the capillaries and postcapillary venules. Fluid, electrolytes, plasma proteins (such as albumin, fibrinogen) and soluble inflammatory mediators pass through as transudation occurs and blood flow slows. Leukocytes then emigrate into the interstitium, which now becomes filled with exudate, acting to contain the stimulus. This is facilitated by chemical mediators, reduced blood flow and altered expression of cell adhesion molecules. The swelling caused by extravasation of fluid/plasma proteins into the extracellular space, plus the influence of algesic chemical mediators (bradykinin, prostaglandin E2) also stretches/sensitizes sensory neural receptors causing pain.

2.1.1 The Importance of Endothelial Cells

Vascular ECs are important in regulating the passage of fluid, electrolytes and cells across the blood-vessel wall. This will vary according to the site of the vascular bed. ECs are polarized, having distinct luminal and basal surfaces, and mediate transmural transport via transcellular pathways (utilizing cytoplasmic vesicles, caveolae or paracellular pathways via cell junctions). Endothelial cells can mediate an extremely rapid increase in vascular permeability in the following ways:

1. Opening of endothelial gaps via junctional complexes between adjacent ECs. This can be mediated by means of EC contraction via actin/myosin filaments, mainly in the cytoplasm of postcapillary venules, where there are high densities of receptors for vasoactive mediators including histamine, serotonin, bradykinin, angiotensin II and leukotrienes. This is the most prevalent mechanism to increase vascular permeability. In addition, reorganization of cytoskeletal microtubules/microfilaments in ECs of postcapillary venules and capillaries also opens junctional complexes, and is induced by cytokines such as IL-1 and TNF, or by hypoxia. These effects are dynamic, lasting between 15 and 30 minutes after a single stimulus.
2. Injury to ECs causing degeneration/necrosis and detachment. This could be caused directly by chemicals, burns, chemotherapeutics or microbial cytotoxins, at any level of the vascular bed (i.e. arterioles, capillaries or venules). Alternatively, ECs may be killed by the action of activated leukocytes, which occurs mostly in venules, or pulmonary capillaries. It is a late response, mediated by the release of lysosomal proteolytic enzymes and reactive oxygen species (ROS) from adherent, activated leukocytes, particularly neutrophils, as they transmigrate out of venules as part of the inflammatory process. Death of ECs, the presence of inflammatory mediators, release of tissue factor and exposure of basement membrane (BM) collagen activate platelets and the clotting and complement cascades.

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3. Increased EC transcytosis. This has been associated with vascular endothelial growth factor (VEGF) and may be relevant to histamine and other chemical mediators.
4. Leakage from new blood vessels. Stimulation by angiogenic factors including VEGF results in new blood-vessel formation, which is an important process in normal tissue growth, healing and neoplasia. Immature ECs/blood vessels have a higher permeability than mature ones, which may predispose to perivascular fluid accumulation.

2.2 Cellular Phase

In this phase of acute inflammation, neutrophils, monocytes and lymphocytes are delivered from the circulation to the site of accumulated protein-rich fluid exudate in order to facilitate removal of the inducing stimuli, that is, the killing of bacteria, neoplastic/damaged cells and/or the removal of necrotic cells. This is achieved by

- release of proteolytic enzymes (including matrix metalloproteinases, elastases and cathepsins) from cytoplasmic granules or lysosomes,
- release of chemical mediators,
- generation and release of ROS, and
- phagocytosis.

2.2.1 Leukocyte Migration

Leukocyte migration (also known as extravasation) occurs primarily through capillaries and postcapillary venules, along a chemotactic gradient. It comprises an orderly series of stages which has been termed the leukocyte adhesion cascade. These stages include capture, rolling, activation by chemokines, firm adhesion and migration through the endothelium. As leukocytes migrate from the vasculature, all the different stages of this process may be observed concurrently, from marginated cells 'pavementing' (lining up adjacent to one another) at the endothelial surface, to cells passing through the vessel wall, to those which have entered the perivascular extracellular matrix. Inhibition of any of these steps reduces the number of leukocytes migrating into the perivascular extracellular matrix.

Vasodilation and increased vascular permeability result in a local slowing or even stasis of blood flow. This allows leukocytes to marginate, that is, move to the periphery of the vascular lumen, and come into close apposition with the vascular ECs. This facilitates capture and subsequent rolling of leukocytes along the EC luminal surface. Intercellular adhesion molecules (ICAMs) on the cell surface of leukocytes and luminal surface of ECs play a key role in the migration process, their expression and binding affinity being influenced by chemokines and cytokines. Binding of adhesion molecules to their respective receptors mediates not only leukocyte exit from blood vessels, but also passage into and within the extracellular matrix (ECM).

Cellular adhesion molecules can be divided into different families: the integrins, immunoglobulin superfamily (including selectins and ICAMs) and others, such as the hyaluronic acid receptor CD44 (cluster of differentiation 44). They have a variety of receptor specificities which often overlap, coordinating their interactions to achieve an initially temporary, increasingly stable association, anchoring leukocytes firstly to the ECs, then the scaffolding of the ECM.

The selectins, members of the immunoglobulin superfamily of cell-surface molecules, are important in the initial stages of leukocyte migration. They are expressed on leukocytes (L-selectin) and ECs/platelets (E-selectin/P-selectin). The other members of the immunoglobulin superfamily, the cellular adhesion molecules along with members of the integrin family, play a significant role later in the process of firm adhesion and migration through the endothelium. The capture of leukocytes is mediated by interactions between complex carbohydrate moieties covalently bound to leukocyte surface membrane glycoproteins (e.g. Sialyl Lewis X, E-selectin ligand, P-selectin glycoprotein ligand) and their cognate receptors on ECs (P-selectin and E-selectin proteins). Different selectins are also expressed on leukocytes (L-selectins), which bind to receptors on ECs such as glycoprotein cell adhesion molecule-1 (GlyCAM-1) and others. In the early phase of leukocyte capture these interactions are temporary, helping to maintain a close proximity between leukocytes and ECs. As these interactions become more numerous they result in sustained contact. Leukocytes then roll along the EC surface, propelled by the pressure of the blood flow. Inflammatory mediators also play an important role in facilitating leukocyte migration. Pro-inflammatory cytokines including TNF, IL-1 and IL-6 and the phospholipid-derived PAF are secreted by resident tissue macrophages, mast cells and ECs themselves, in response to inducers of inflammation. These mediators, along with others such as chemokines (concentrated at the EC surface and ECM, bound to proteoglycans), complement proteins C3a and C5a and platelet-derived growth factor (PDGF) enhance the ability of leukocytes such as neutrophils to bind to ECs in two ways:

- Activating leukocytes by promoting the proteolytic cleavage of L-selectin and β_2 integrins. This removes L-selectin, allowing rapid surface expression of the β_2 integrins (Mac-1(macrophage antigen-1)) and other heterodimeric combinations of CD11a, b, c with CD18) in a low-affinity state. Proteolytic cleavage of these integrins causes a

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conformational change which creates a high-affinity binding state.

- Activating ECs by up-regulating the expression of the receptor for β_2 integrins, intracellular adhesion molecule-1 (ICAM-1) and other ICAM molecules on the luminal surface of activated ECs. ECs are also stimulated by additional inflammatory mediators, including histamine, thrombin and PAF, to redistribute P-selectin from cytoplasmic granules (Weibel-Palade bodies) to their luminal cell surface. These enhanced interactions, plus the presence of chemokines on the EC surface slow the rolling of the neutrophils and facilitate a firm adhesion of leukocytes to the ECs in preparation for the final stages of transmigration.

The presence of a chemokine gradient in the inflammatory exudate is an important factor in leukocyte transmigration or diapedesis. Neutrophils and other leukocytes exit postcapillary venules by moving through interendothelial spaces at the EC intercellular junctions. Platelet endothelial cell adhesion molecule-1 (PECAM-1, CD31), which is present on EC and leukocyte surfaces, is one of a number of ICAMs which are involved in this process. PECAM-1 molecules interact in a homophilic fashion, that is, they bind to other PECAM-1 molecules expressed on adjacent cells. Junctional adhesion molecules (JAMs) are also involved in this process, as are interactions between β_2 integrins/ICAM-1 and E-selectin/leukocyte-expressed ligands. Leukocyte pseudopodia extend between ECs and contact the subjacent BM. Penetration of the endothelial BM is probably achieved by leukocyte collagenase secretion with subsequent exposure to ECM proteins (fibrin, fibronectin, vitronectin, proteoglycans, collagen). Binding to these ECM proteins is mediated partly by the hyaluronic acid receptor (CD44) expressed on lymphocytes, by β_3 integrins expressed on monocytes/macrophages and also by β_1 integrins. In this way, leukocytes (neutrophils, lymphocytes, eosinophils, monocytes and macrophages) are delivered to the site(s) of inflammation from the vasculature in the inflammatory exudates. They are now able to migrate along the chemokine gradient(s) and attempt to neutralize/remove the origin of inflammatory inducers.

2.3 Inducers of Acute Inflammation

Acute inflammation can be stimulated by both exogenous and endogenous factors. As with sensors and mediators of inflammation, there is functional redundancy. Different inducers will stimulate similar functional pathways, and some inducers may also act in a fashion similar to mediators.

2.3.1 Exogenous Inducers

These may be divided into microbial and nonmicrobial groups. Microbes include viruses, bacteria, protozoa (and also metazoan parasites), which stimulate inflammation via common pathogen-associated molecular patterns (PAMPs) and specific virulence factors. PAMPs include lipoprotein, peptidoglycan and lipoteichoic acid from Gram-positive bacteria; lipopolysaccharide (LPS) from Gram-negative bacteria and spirochaetes (e.g. *Leptospira*); single- and double-stranded RNA, unmethylated CpG (cytosine-phosphate-guanine) DNA (from bacterial DNA) and bacterial flagellin from uropathogenic bacteria. Therefore, PAMPs constitute a limited, defined set of conserved molecular patterns carried by micro-organisms of different classes for which cognate receptors (pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs), C-type lectin receptors and numerous cytosolic Nod-like receptors (NLRs)) have evolved in their hosts. PRRs detect the presence of microbes or other endogenous ligands (see later) and, when activated, lead to the release of inflammatory cytokines (TNF, IL-1) and interferon (IFN). This release occurs via intracellular pathways mediated by a variety of different kinases (including p38, the mitogen-activated protein (MAP) kinase pathway) and transcription factors (including nuclear factor NFκB). An alternative method of signalling employed by certain NLRs is to oligomerize, forming an inflammasome molecule which activates the enzyme caspase-1, also resulting in release of inflammatory cytokines. Damage to the basement membrane separating epithelial cells from mesenchymal cells allows access by pathogens, or even commensal bacteria, to tissue macrophages where they can be recognized via TLRs, leading to the release of inflammatory mediators.

In contrast to PAMPs, virulence factors are pathogen-specific and are not directly sensed, rather they trigger inflammation by their adverse effects on host cells/tissues. For example, Gram-positive exotoxins which form cell membrane pores stimulate inflammation via the resulting efflux of intracellular K^+ ions. This efflux is sensed by one of the NLRs, called the NALP3 (NACHT, leucine-rich-repeat and pyrin-domain-containing protein 3) inflammasome, which leads to pro-inflammatory cytokine release. This mechanism is discussed in more detail later.

Nonmicrobial exogenous inducers include allergens, physical agents (foreign-body material (e.g. hair shafts, plant material), suture materials, implants); mechanical stimuli, including trauma, thermal or freezing injury and electromagnetic radiation of various wavelengths; chemical agents, including acids, alkalis and toxins/poisons; and nutritive factors such as ischaemia or vitamin deficiencies. Both microbial and nonmicrobial inducers may

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damage epithelia, for example via irritation. Expulsion and clearance of pathogens/irritants via epithelial barriers, such as the respiratory epithelium via the mucociliary escalator, or the gastric mucosal barrier, are important methods of protection, and breakdown often induces inflammation as the presence of inducers in an inappropriate tissue compartment is sensed. Xenobiotics are classified as exogenous factors although their effects may be varied, including direct chemical, foreign body or toxic effects at the site of application, or systemic absorption and toxicity in target tissues. The presence of foreign materials such as silica or asbestos stimulates accumulation of macrophages to attempt digestion. These cells may fuse to form multinucleated giant cells within granulomas in unsuccessful attempts to digest the foreign material. Resistant microbes such as mycobacteria or fungi also induce a similar macrophage response. Other particulates including diesel exhaust and carbon black are phagocytosed by macrophages which become enlarged and accumulate in the lung, associated with a chronic inflammatory reaction. Reactive metabolites of xenobiotics may be generated *de novo* by particular target cell populations, such as hepatocytes. Xenobiotic activation by Phase I metabolic enzymes may lead, for example, to oxidative cellular damage. Similarly to the effects of microbial toxins (virulence factors), this could lead to the release of products of cell injury and death, which are themselves endogenous inducers. However, the source of reactive metabolite(s) is still external. In addition, a xenobiotic (or its metabolite) may be immunogenic *per se,* or act as a hapten to stimulate an immune response which is directed at cells containing/expressing the xenobiotic at the cell surface. There is evidence to indicate that certain types of skin rashes may be caused in this way (e.g. penicillins, lamotrigine, carbamazepine).

2.3.2 Endogenous Inducers

These are derived internally, and include the factors released from injured or necrotic cells. These are less well defined than exogenous factors, although the way in which they induce inflammation appears to be related to the breakdown in compartmentalization of cells and their components/enzymes/signalling molecules. Such sequestration is normally maintained via the action of various different natural barriers such as epithelia, cell membrane, basement membrane and vascular endothelium. Cellular components such as adenosine triphosphate (ATP), K^+ ions, uric acid, HMGB1 protein and S100 Ca^{2+}-binding proteins (A8, A9, A12), heat shock proteins (HSP60 and HSP70) and endoplasmic reticulum proteins are released by membrane disruption occurring during necrotic cell death. ATP activates nociceptors, sending a neurosensory signal, and it also binds purinoceptors (e.g. $P2X_7$) on the macrophage cell surface. This causes K^+ ion efflux, one of the signals, in addition to the presence of uric acid (urate) crystals, known to activate the NALP3 inflammasome, leading to pro-inflammatory cytokine release. HMGB1 binds to the RAGE (receptor for advanced glycation end-products), which is expressed on vascular endothelia and elsewhere. This RAGE-binding cooperates with multiple TLRs to induce inflammation, a signalling mechanism which is also used by S100 proteins that operate via TLR4. Macrophages stimulated by bacterial LPS, which acts via TLR4, also release HMGB1, demonstrating multiplicity/cooperativity between inducers of the inflammatory process. There is also evidence to suggest that in addition to passive release from necrotic cells, intracellular proteins may be secreted (via a mechanism independent of the endoplasmic reticulum–Golgi pathway) by intact, but stressed cells. This mechanism appears to be regulated by inflammasomes and is mediated by activated caspase-1. Damage to vascular endothelium may significantly influence the extent of both the fluidic/exudative and cellular phases of inflammation. One contributing inducer of inflammation is the exposure of BM collagen and extracellular matrix components. This activates Hageman factor (factor XII), a sensor of vascular damage, which is a key player in the proteolytic cascades of the kallikrein–kinin, coagulation, fibrinolytic and complement systems, generating a variety of inflammatory mediators. Contact with collagen also activates platelets, which produce various inflammatory mediators, including eicosanoids such as thromboxanes, and serotonin.

Endogenous inducers important in chronic inflammation include crystals of monosodium urate and calcium pyrophosphate dehydrate (causing gout and pseudogout in humans), oxidized lipoproteins (high- and low-density) and advanced glycosylation end-products (AGEs), which can accumulate with age and under hyperglycaemic and pro-oxidative conditions. Macrophages detect and phagocytose crystals beyond a certain size, which triggers NALP3 inflammasome activation and IL-1 secretion. Breakdown products of the extracellular matrix also stimulate inflammation. Hyaluronate is a glycosaminoglycan normally present in the ECM as a high-molecular-weight polymer, which is biologically inert. Upon tissue injury, hyaluronate is broken into low-molecular-weight fragments which activate TLR4 via a pathway thought also to involve ROS. Additional ECM-derived inducers include heparan sulfate, fibronectin and fibrinogen. Endogenous inducers may also be secreted, such as surfactant protein A, present in the lung, which stimulates inflammation by binding to surfactant protein receptor 210.

Other endogenous inducers act to cause autoinflammatory responses, that is, those directed at, or which cross-react with, the body's own 'self' antigens, reactions to which are normally suppressed by the immune system. These antigens may derive from degenerate, dysplastic or neoplastic cells, or from xenobiotics, to induce hypersensitivity reactions.

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2.4 Sensors and Mediators of Inflammation

Sensors of inflammation are those molecules which recognize the presence of inducers and act via intracellular signalling pathways, to stimulate the production of certain inflammatory mediators.

2.4.1 Sensors of Exogenous and Endogenous Inducers

Pattern recognition receptors are proteins which may be secreted, cell-surface, or intracellular, and have evolved to detect a conserved set of microbe-associated inducers of inflammation known as common pathogen-associated molecular patterns (PAMPs). PRRs are also engaged by specific virulence factors such as Gram-positive bacterial exotoxins, and particulates such as silica and asbestos. Importantly, endogenous inducers such as heparin sulfate, urate crystals, S100 protein and efflux of cellular K^+ ions also have cognate PRRs. PRRs are encoded as part of the germline DNA and are not subject to the somatic gene rearrangements which occur for highly variable recognition molecules in the adaptive immune system, such as T cell receptors (TCRs) and immunoglobulins.

PRRs comprise a variety of sensing molecules. Secreted PRRs include acute-phase proteins such as C-reactive protein (CRP) and serum amyloid protein (SAP), which are synthesized by the liver and present in the plasma. They bind to microbial membranes and, along with mannan-binding lectin (which binds to microbial mannose sugars), they activate complement by the Alternative/Lectin pathways. They can also label microbial membranes for recognition/killing by immune cells, a process called opsonization.

PRRs are also expressed on cell membranes, to detect extracellular inducers. TLRs are a large family of transmembrane proteins which bind a wide variety of exogenous and endogenous ligands. Macrophage mannose receptor binds mannose residues stimulating phagocytosis, whilst CD14 acts as a cofactor for TLRs in detecting LPS and peptidoglycan. PAMPs such as lipoprotein, peptidoglycan and lipoteichoic acid (from Gram-positive bacteria), LPS (from Gram-negative bacteria, *Leptospira*), single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA), unmethylated CpG DNA, and bacterial flagellin from uropathogenic bacteria bind to the extracellular domains of TLRs in the plasma membrane.

Other PRRs are found in the cytoplasm, not on the cell surface. These include C-type lectin receptors, Nucleotide-binding oligomerization domain-like receptors (NLRs) and dsRNA-activated protein kinase. They bind numerous intracellular ligands, including peptidoglycans and dsRNA (characteristic of certain viruses).

When activated by ligand-binding, PRRs lead to leukocyte activation, the release of inflammatory cytokines (TNF, IL-1, IL-18, IL-33) and IFN by a variety of intracellular signalling pathways. For example, activation of TLR4 can be achieved by the binding of (i) exogenous bacterial inducers such as LPS (with the protein cofactors CD14 and MD2 (lymphocyte antigen 96)), or (ii) endogenous inducers such as hyaluronate breakdown fragments, which signal tissue damage. Ligand-binding stimulates intracellular signalling through the cytoplasmic domain of TLRs via a variety of different adapter proteins, including MyD88 (myeloid differentiation primary response gene 88), Toll/IL-1 R adapter protein (TIRAP) and TNF-receptor associated factor 6 (TRAF-6). These adapter proteins link to different kinase pathways, including the mitogen-activated protein kinase pathway (MAP kinase) and TNF-receptor associated kinase pathway (TAK), to activate transcription factors p38, c-Jun/AP-1 and NFκB in the nucleus. The transcription factors mediate pleiomorphic cellular effects, including up-regulation of inflammatory cytokine gene expression (TNF, IL-1, IL-6), defensin and antimicrobial peptide release, stimulation of leukocyte phagocytosis, and dendritic cell activation (which enhances antigen presentation and crosslinks with the adaptive immune system). IFN-β expression is up-regulated by a mechanism independent of the MyD88 adaptor, via a different kinase pathway and interferon regulatory factor-3, although there is cross-talk between these different pathways.

An alternative method of signalling is employed by certain cytoplasmic PRRs responding to cytosolic inducers. NLRs are often involved in recognition of cytosolic PAMPs. Upon ligand-binding, NLRs oligomerize, forming a multisubunit inflammasome molecule which activates the enzyme caspase-1. (This formation of a multisubunit, caspase-activating signalling protein is analogous to the oligomerization of the protein APAF-1 (apoptotic peptidase activating factor-1) to form the apoptosome, one of the processes mediating apoptosis, via a different caspase (−9), which is stimulated by loss of mitochondrial cytochrome C.) Activation of caspase-1 is known to be essential for processing, maturation and release of cytokines IL-1β, IL-18 and IL-33. There is also evidence to suggest that in addition to passive release from necrotic cells, inflammasome-activated caspase-1 mediates secretion of intracellular proteins by intact cells, via a mechanism independent of the endoplasmic reticulum–Golgi pathway. This may be an important mechanism whereby damaged cells initiate inflammation.

One particular type of NLR-inflammasome senses cellular components such as cellular K^+ ions and crystalline monosodium urate and calcium pyrophosphate dehydrate. The presence of these inducers is sensed by the NALP3 inflammasome, one of the NLRs formed by the oligomerization of the protein NALP3. Present

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in macrophages, activation of this inflammasome stimulates caspase-1 activation and pro-inflammatory cytokine release (e.g. IL-1). The response to crystals, which is essentially a foreign-body reaction, is also caused by exposure of macrophages to particulate xenobiotics such as asbestos and silica: the NALP3 inflammasome is activated, stimulating cytokine release.

Oxidized lipoproteins (high- and low-density) and AGEs, can accumulate with age and under hyperglycaemic and pro-oxidative conditions. Like HMGB1, AGEs, bind RAGE, which can induce inflammation either directly by intracellular signalling from the cytoplasmic domain of RAGE itself, or via the activation of TLRs and kinase pathways as described above.

Breakdown products of the extracellular matrix can also stimulate inflammation via PRRs. Hyaluronate is a glycosaminoglycan normally present in the ECM as a high-molecular-weight polymer, which is biologically inert. Upon tissue injury, hyaluronate is broken into low-molecular-weight fragments which activate TLR4 via a pathway thought also to involve ROS.

Hageman factor and platelets are important sensors in inflammation. Vascular damage exposes BM collagen (and other extracellular matrix filaments). This activates Hageman factor (factor XII), which is a key player in the proteolytic cascades of the kallikrein–kinin, coagulation, fibrinolytic and complement systems, generating a variety of inflammatory mediators. Contact with collagen also activates platelets, which produce various inflammatory mediators, including eicosanoids such as thromboxanes, and serotonin.

Control of inflammation, which is important in preventing the pathogenic effects of dysregulated, exaggerated responses, also occurs at the level of sensing via PRRs such as the TLRs. Heparan sulfate proteoglycans are expressed ubiquitously on cell surfaces and in the ECM. They bind to and regulate a variety of inflammatory mediators (TNF, IL-2, IL-6, chemokines), but they also attenuate inflammation by binding to inducers such as LPS. They are cleaved from cell surfaces by metalloproteinases associated with ongoing inflammation, and act to bind to LPS and mediators thus modulating the inflammatory process.

2.4.2 Chemical Mediators of Inflammation

Chemical mediators are generated when the presence of inflammatory inducers is sensed. These mediators function as intra/intercellular signalling molecules. They interact in a complex and coordinated fashion to orchestrate the inflammatory response, by influencing their own levels and the functions of various effector cells/tissues. Many of these mediators have overlapping effects on the vasculature and the recruitment of leukocytes, and significant mediators are summarized in **Table 1**. They are derived

1. from the plasma, where they are usually present as inactive precursors synthesized and secreted by the liver, requiring activation to exert biological effects (e.g. clotting, kinin, complement proteins), or
2. from cells present at the site of inflammation, notably mast cells, basophils, platelets, neutrophils and macrophages. They may either be synthesized *de novo* (e.g. prostaglandins, cytokines) upon cellular activation, or preformed and released from cytoplasmic granules or vacuoles (e.g. histamine, serotonin, lysosomal enzymes).

These mediators bind receptors on target effector cells. The effect on these activated cells is often to stimulate the release of further inflammatory mediators, thus forming a potentiating functional cascade.

Mediators already circulating in the plasma generally form part of a proteolytic cascade and require cleavage for activation. Therefore, once the clotting, kinin and complement pathways are initiated, they progress very rapidly, in seconds to minutes. The release of preformed mediators also confers an extremely short response time for the acute inflammatory response: they can be active within seconds of an inducing stimulus.

Mediators which are synthesized *de novo* upon cell activation are released over a longer time-span (hours), as they occur as a result of altered gene transcription and protein expression (e.g. cytokine synthesis, up-regulation of ICAM-1 on vascular ECs) or activation of cellular enzyme pathways (lipid mediators such as prostaglandins, leukotrienes, PAF).

The speed with which the acute inflammatory reaction occurs is rapid. This reaction is also rapidly regulated due to the short half-life of the mediators and the presence/balance of both pro-inflammatory and suppressive molecules. Inherent rapid decay, enzymatic inactivation by kinases or proteases, removal by scavenging systems such as antioxidants, and competition by endogenous inhibitors (e.g. complement inhibitors) all contribute to the short functional half-life of inflammatory mediators. This is important, as acute inflammation can be a destructive process and, if allowed to progress unchecked, can result in significant tissue damage.

Mediators of inflammation, although they express a significant degree of functional overlap, may be classified into different groups on the basis of their biochemical properties.

2.4.3 Vasoactive Amines

Histamine and serotonin (5-hydroxytryptamine) are important, preformed amine molecules which are released from mast cells and platelets upon degranulation, which is an all-or-nothing response. They have complex effects upon the vasculature, depending on the site of the vascular bed, causing increased vascular permeability, vasodilation or vasoconstriction. The

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Table 1 Summary of inflammatory mediators and their major effects

Mediator	Vasomodulator (dilation or constriction)	Increase vascular permeability	Leukocyte recruitment/activation/chemotaxis	Other
Plasma protein cascades				
Kinin	√	√	—	Stimulates pain, eicosanoid synthesis
Kallikrein	—	—	√	Hageman factor (XII) senses exposed collagen/extracellular matrix proteins
Clotting/fibrinolysis	—	(√)	(√)	
Complement				
C3a, C5a	—	√	√	Can induce mast cell degranulation
C3b	—	—	—	Opsonin
Acute phase proteins	—	—	—	Microbicidal, opsonins, inhibit proteases
Vasoactive amines	√	√√	(√)	
Substance P	√	√√	√	
Cytokines				
IL-1	√	√	√	Fever
TNF (α)	√	√	√	Acute phase protein synthesis
IFN (γ)	—	—	—	Macrophage activation, Th1 response
Chemokines	—	—	√	
Eicosanoids				
Prostaglandins	√	√	—	Pain, reduce inflammation (PGE_2)
Thromboxanes	√	—	—	Thrombosis
Leukotrienes				
LTB_4	√	(√)	√√	
LTC_4, D_4, E_4	√	√√	√	Stimulate T lymphocytes
Lipoxins	—	—	—	Decrease leukotriene synthesis
Resolvins	—	—	—	Antagonize leukotriene receptors
Platelet activating factor	√	√	√√	
HMGB1	—	—	—	Macrophage activation, anorexia, malaise
Defensins/collectins	—	—	—	Microbicidal, stimulate mast cell degranulation
Reactive oxygen intermediates/nitric oxide	√	√	—	Inhibit mast cell-induced inflammation, regulate chemotaxis

Relative activity shown as: (√), √ or √√. Modified from Ackerman, 2007.

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induced vascular effects last for minutes to hours. Histamine effects are mediated via 7-transmembrane (7-TM) domain histamine receptors on leukocytes, ECs and smooth muscle, of which there are various subtypes (e.g. H_1, H_2, H_3). Vasodilation (active hyperaemia) is mediated via binding of H_1 and H_2 receptors on ECs, whereas increased vascular permeability, due to EC contraction (cytoskeletal reorganization) and gap junction formation, is primarily an H_1-mediated effect. Leukocyte activation is also mediated via H_1 and H_2 receptors. Histamine also mediates neural/vagal reflexes, bronchial constriction, release of prostaglandin $F_{2\alpha}$, pain/itching, tachycardia and eosinophil chemotaxis. Serotonin has similar effects to histamine and is also a neurotransmitter. Stimulators of mast cell and platelet degranulation (and hence vasoactive amine release) include contact with BM collagen; thrombin (a product of the clotting cascade, binding to protease-activated receptors (PARs)); adenosine diphosphate (ADP) (released from injured vascular ECs); complement fragments C3a, C4a, C5a; substance P and IgE. If release occurs as a result of binding of antigen to surface IgE on sensitized mast cells (as in Type I hypersensitivity reactions), the profound degree of vascular permeability induced by these amines can cause anaphylactic shock and circulatory collapse.

2.4.4 Vasoactive Peptides

This class of mediators may be preformed and released from secretory vesicles, as for the tachykinins ('fast'-kinins) substance P and neurokinins A and B, or generated from inactive plasma/extracellular fluid precursors as for bradykinin ('slow'-kinin) and fibrinopeptides A and B, fibrin degradation products.

Tachykinins are synthesized by sensory (afferent) neurones and are involved in asthma and allergic reactions. Release of substance P from neurones can be caused by stimulation of vanilloid receptors, which directly induces inflammation and pain. Tachykinins cause similar effects, including vasoconstriction, vasodilation, increased vascular permeability, leukocyte activation and chemotaxis. Substance P also acts directly on mast cells, basophils and eosinophils causing degranulation and release of vasoactive amines (histamine, serotonin).

The other vasoactive peptides, notably bradykinin, which is the major functional product of the kinin cascade, are generated by enzymatic proteolysis. Proteolysis is initiated by activated Hageman factor, kallikrein, thrombin or plasmin, which links the kinin pathways with clotting, fibrinolytic and complement cascades. Vasoactive peptides cause vasodilation and increased vascular permeability, either directly, or by stimulating mast cell degranulation and the release of vasoactive amines (histamine, serotonin). They are also proalgesic (pain-stimulating, a cardinal clinical sign of inflammation), cause smooth muscle contraction/bronchoconstriction and increased arachidonic acid metabolism (resulting in the production of various lipid mediators).

Hageman factor (also known as factor XII in the clotting cascade) is a key inflammatory mediator which coordinates the vasoactive peptide and complement responses by linking the plasma mediator systems of the kinin, fibrinolytic, clotting and complement cascades. As mentioned earlier, Hageman factor is also a sensor of vascular damage (it is activated by the presence of BM collagen and extracellular matrix components) and an inducer of inflammation by stimulating the clotting and kinin cascades.

Regulation of vasoactive peptides is achieved by further proteolysis of bradykinin by the enzyme kininase, whilst earlier in the pathway, plasma kallikrein is inactivated by another protease, serum α_2-macroglobulin, which is one member of a family of serpin proteases. The serpin proteases include α_1-antitrypsin, α_1-antichymotrypsin and antithrombin III, which regulate the clotting and complement systems. Feedback inhibition of tachykinin production is achieved by histamine binding to (inhibitory) H_3 receptors present on nerve fibres synthesizing substance P. In addition, binding of substance P to its receptor (neurokinin 1 receptor (NK1 R)) leads to a reduction in NK1 R expression on target cells (mast cells, ECs, epithelial cells, macrophages), thus decreasing their sensitivity.

2.4.5 Complement

The complement cascade is a pro-inflammatory complex of proteolytic pathways activated via several routes, which generates a variety of intermediates and products that mediate or effect key purposes in inflammation, such as microbicidal effects, chemotaxis, opsonization (labelling of cells for recognition/phagocytosis by innate and adaptive immune systems). The complement cascade can be activated by the presence of (i) antigen-antibody complexes (Classical pathway, demonstrating cross-talk between the innate and adaptive immune systems in acute inflammation), (ii) lectins bound to mannose groups on microbial surfaces (lectin pathway), or (iii) microbial surfaces or products such as lipopolysaccharides (Alternative pathway). It generates the so-called membrane attack complex (C5-9, a multisubunit effector of inflammation), which is inserted into plasma membranes (e.g. bacterial or host cells) forming a pore and causing cell lysis due to unregulated influx of water, electrolytes and small molecules. Several pathways of activation generate complement fragments C3a, C4a and C5a (known as anaphylatoxins). These promote recruitment of granulocytes (neutrophils, eosinophils, basophils), monocytes and induce mast cell degranulation, thus causing vascular effects via the release of vasoactive amines/substance P. Fragment C3b, which is deposited on the surface

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of microbes/cells which activate complement, labels these cells for phagocytosis via recognition of C3b by C3b-receptor expressed on phagocytic cells (e.g. macrophages). Cross-talk between major plasma protein cascades (clotting, kinin, complement, fibrinolysis) also stimulates inflammation by the recognition of thrombin by PARs expressed on leukocytes causing cell activation.

2.4.6 Lipid Mediators

Collectively, these are the eicosanoids and PAF. They are derived from phospholipids in the inner layer of the plasma membrane. Following cell injury or activation by other inflammatory mediators, cytosolic phospholipase A_2 is activated by intracellular Ca^{2+} ions and generates arachidonic acid (eicosatetraenoic acid) and lysophosphatidic acid, which are the precursors of the eicosanoid and PAF classes of lipid. These are synthesized in ECs, leukocytes and platelets in response to physical stimuli and chemical mediators, including complement fragment C5a. Arachidonic acid can also be released extracellularly by the action of soluble phospholipase A_2 (which is inhibited by corticosteroids, accounting for a major component of the anti-inflammatory effect of these therapeutic compounds). Lipid mediators function as autocoids, that is, local hormones, mediating inflammation by acting both as intercellular and intracellular messengers that act on nearly every effector of the inflammatory response (i.e. smooth muscle cells, ECs, platelets, mast cells, eosinophils, epithelial cells, neutrophils and macrophages).

Arachidonic acid is a 20-carbon essential fatty acid. It is metabolized to generate the eicosanoids either by cyclo-oxygenases (COX1 and COX2), forming prostaglandins and thromboxanes, or by lipo-oxygenases, forming leukotrienes and lipoxins.

Cyclo-oxygenase isoenzymes are either constitutively expressed in nearly all tissues (COX1) or may be induced locally by inflammation (COX2), for example in leukocytes and ECs. These enzymes have been major therapeutic targets for anti-inflammatory drugs such as aspirin, ibuprofen, or celecoxib, which is selective for COX2. COX enzymes generate prostaglandin H_2 (PGH_2), which is further metabolized by different enzymes to generate a variety of different mediators. Prostaglandin E_2 (PGE_2) causes vasodilation and, with PGD_2 (a major product of activated mast cells), affects vascular permeability. PGE_2 is a potent hyperalgesic and inducer of fever, along with cytokines. Platelets are major producers of thromboxane A_2 (TXA_2), which is prothrombogenic, and antagonizes the antithrombogenic properties of PGI_2.

Prostaglandin receptors are expressed on a variety of effector cells including eosinophils, basophils, monocytes, dendritic cells and T lymphocytes (coupling inflammation with innate immunity). There are different receptors for different eicosanoids, which orchestrate phenotypic changes such as leukocyte migration, degranulation and release of mediators. They also constitute therapeutic targets to reduce inflammation. For example, PGD_2 receptors are divided into two types, DP1 and DP2 (also known as CRTH2). They are G-protein-coupled receptors which signal through elevation of second messengers cyclic adenosine monophosphate (cAMP) and intracellular Ca^{2+}. In contrast to DP1 (and similar to chemokine receptors—see later), DP2 also signals through inositol triphosphate (IP_3).

Leukotrienes are products of the 5-lipoxygenase (5-LOX) pathway which requires an activating cofactor (5-lipoxygenase activating protein (FLAP)) to colocalize with its substrate, arachidonic acid, forming 5-hydroperoxyeicosatetraenoic acid (5-HPETE). The enzyme activity generates a by-product hydroxyeicosatetraenoic acid (HETE) and an intermediate, leukotriene A_4 (LTA_4), which itself is a substrate for either leukotriene A_4 hydrolase, or leukotriene C_4 synthetase, generating LTB_4 (mainly in neutrophils and macrophages) or LTC_4 (mainly in eosinophils and mast cells and also macrophages). LTD_4 and LTE_4 are formed more slowly upon secretion of LTC_4. Leukotrienes are pro-inflammatory in several ways. Along with the 5-LOX by-product, 5-HETE, they are chemotactic to leukocytes. LTB_4, is most potently chemotactic to the cells responsible for its production, neutrophils and macrophages, and stimulates leukocyte ICAM expression, chemokine production and neutrophil degranulation. This serves to concentrate these cell types at the site of inflammation where they release their destructive enzymes/lysosomal contents. Leukotrienes increase vascular permeability, notably through the action of LTC_4, LTD_4 and LTE_4. These leukotrienes also cause vasoconstriction and bronchospasm and stimulate T-lymphocyte proliferation. Their receptors are also expressed on a variety of cell types including leukocytes (including lymphocytes, and also dendritic cells, again linking with innate immunity), ECs and vascular smooth muscle. Leukotriene receptors, such as the receptor for LTB_4 (called BLT1) are 7-TM domain, G-protein-coupled receptors, similar to chemokine receptors.

PAF is generated via the acetylation of lysophosphatidic acid and transfer of phosphocholine in a variety of cells including ECs, platelets, leukocytes and mast cells. It has potent biological activity and mediates vasodilation, vasoconstriction, increased vascular permeability, platelet activation/aggregation and leukocyte recruitment/adhesion/chemotaxis. PAF effects are mediated by binding a G-protein-coupled receptor expressed in target cells including ECs, leukocytes and smooth muscle. PAF stimulates release of vasoactive amines from platelet granules and via mast cell degranulation. In addition, PAF increases eicosanoid synthesis and neutrophil oxidative burst following phagocytosis.

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Lipoxins are products of the 12-LOX pathway active in platelets, using as its substrate LTA_4 transferred by direct contact from leukocytes, particularly neutrophils. This generates lipoxins A_4 and B_4 (LXA_4, LXB_4). Lipoxins are generally anti-inflammatory, inhibiting neutrophil adhesion and chemotaxis and decreasing production of pro-inflammatory cytokines by a wide variety of cell types. They may also endogenously regulate the pro-inflammatory leukotrienes, since levels of lipoxins and leukotrienes show an inverse relationship. Such regulatory activity is shown by prostaglandins PGE_2 and PGI_2, which can stimulate lipid-mediator class switching in leukocytes from LTB_4 synthesis to LXA_4. Metabolism of eicosanoids derived from omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) gives rise to an additional lipid series called resolvins and protectins. These act via chemokine-like receptors and antagonize leukotriene receptors to attenuate signalling (e.g. via NFκB) and reduce the production of TNF and other pro-inflammatory signals. With the lipoxins, resolvins and protectins stimulate nonphlogistic macrophage recruitment from plasma (i.e. without the elaboration of pro-inflammatory mediators) and phagocytosis of micro-organisms/apoptotic cells, whilst increasing the exit of phagocytes via the lymphatics. This is important in the clean-up of microbes/damaged tissue in preparation for resolution/healing. They also inhibit neutrophil and eosinophil infiltration/chemotaxis, showing dual pro-resolution in addition to anti-inflammatory activity.

Regulation of lipid inflammatory mediators is also achieved through the short half-life of these lipid metabolites (either via decay or enzymatic destruction). An autoregulatory function may also exist for PAF. The target cells for PAF activity not only synthesize PAF, but can also degrade it via PAF acetyl hydrolase, thus limiting its pro-inflammatory activity.

2.4.7 Cytokines

This comprises a large group of soluble, short-acting protein mediators produced mainly by activated lymphocytes and macrophages, but also by a wide variety of cells including other leukocytes, ECs, epithelial cells and fibroblasts, which modulate the functional phenotype of target cells (effectors of inflammation). Most members of this group belong to the interleukin class of molecules and have a wide spectrum of effects on different cell types, that is, they are pleiotropic. For example, IL-12 mediates growth and differentiation of not only T lymphocytes, but also B lymphocytes and natural killer (NK) cells. Much is known about the major function of cytokines in adaptive immunity and in addition they function as inflammatory mediators, also playing key roles in innate immunity. This is another example of the functional interplay between inflammation and both arms of the immune system at the biochemical level.

Depending on the distribution of their cognate receptors, cytokines can act in one or all of three ways. Firstly in an autocrine fashion, binding to receptors expressed on the surface of the cells secreting the cytokine, as occurs for activated T cells, potentiating their own growth. Secondly, in a paracrine fashion by binding to receptors expressed on cells in the immediate vicinity, and thirdly in an endocrine fashion, by acting on targets distant from the site of production. The systemic acute-phase responses of fever, decreased appetite (anorexia), sleep and increased production of acute-phase proteins induced by IL-1, IL-6 and TNF are examples of endocrine effects important in inflammation.

Cytokines are important in mediating acute and chronic inflammation. TNF, IL-1 and IL-6 are the principal functional cytokines which promote leukocyte recruitment and inflammatory responses, but others also have key roles to play in innate and adaptive immunity against intracellular pathogens (IL-12, IFN-γ). They can activate macrophages (IFN-γ), eosinophils (IL-5), neutrophils and ECs (TNF). IFN-γ is produced mainly by activated T lymphocytes and NK cells and is an important cytokine involved in macrophage activation. TNF and IL-1 are in turn produced primarily by activated macrophages, illustrating the importance of this cell type in mediating acute inflammation. Macrophages are not the sole source of these cytokines: for example, lL-1 is secreted by activated lymphocytes and other cell types. Production can be stimulated by a variety of inducers as mentioned previously (including stimulation of TLRs and subsequent MAP kinase activation, or via inflammasome pathways). The principal functional effects of TNF and IL-1 are on ECs, leukocytes, fibroblasts and the induction of systemic acute-phase reactions. In ECs, TNF and IL-1 induce cellular activation, which involves an increase in transcription and expression of genes encoding cell adhesion molecules, cytokines, chemokines, growth factors, cytoskeletal remodelling, enzymes involved in the production of lipid mediators (eicosanoids) and nitric oxide. This increases the 'stickiness' and thrombogenicity of ECs and increases vascular permeability, facilitating leukocyte adhesion and transmigration. TNF also primes neutrophils, increasing their responsiveness to other inflammatory mediators; it enhances IL-1 and IL-6 produced by leukocytes (autocrine/paracrine effects) and stimulates fibroblasts to proliferate and remodel collagen (promoting both collagen degradation and synthesis).

Cytokines are also important mediators in the adaptive immune response. Lymphocyte activation, proliferation and maturation to different functional phenotypes is mediated largely by interleukins (IL-2, 4, 5, 7, 9, 10, 12–29). IL-2 is a key growth factor for T cells; differentiation of T cells to Th1 (T helper 1) or Th2 phenotypes is mediated by IL-12 and IL-4, respectively; IL-15 stimulates NK cell growth and activity. Interleukins (IL-4, IL-10) are also important in modulating interactions of

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T cells with dendritic cells (antigen-presenting cells) which are important in antigen recognition at epithelial surfaces. Other cytokines (erythropoietin, various colony-stimulating factors, IL-3) function as stimulators of haematopoiesis.

Activation of NK cells, which are important effector lymphocytes involved in antibody-dependent killing of target cells, is also mediated by cytokines. NK cells can be stimulated by the haematopoietic cytokine Flt3 (fms-like tyrosine kinase-3) and also IL-4, IL-12, IL-15 and IL-21, which induce cell differentiation, expression of the IgG receptor required for target recognition (CD16) and IFN-γ release (which activates macrophages). IL-21 also controls the extent of this activation by initiating a delayed programmed cell death in the activated NK cells.

Cytokines form two main biochemical structural classes (I and II) and bind to their cognate receptors, which have similarly evolved into two classes and are expressed on the target cell surface. Many of the Class I cytokines (including IL-2 to 7, IFN and erythropoietin) bind their cognate receptors and signal through a pathway involving janus kinases (JAKs) and signalling molecules (STAT, signal transducer and activator of transcription). The activated STATs dimerize, translocate to the nucleus and stimulate pro-inflammatory gene transcription, mediating cell activation, proliferation and cytokine production. Consider the T lymphocyte as a target cell, for example: stimulation via IL-2, leading to proliferation and differentiation is a key event in switching on the adaptive immune response and is blocked when the IL-2 receptor is inhibited (e.g. by a monoclonal antibody (mAb)). Different isoforms of JAKs and STATs convey subtly different signals and result in different phenotypes: for example, JAK1 is important in lymphocyte development and STAT6 mediates Th2 responses. Signalling via the JAK/STAT pathway can also be inhibited intracellularly by various mechanisms: at the level of the cytokine receptor, by proteins called suppressors of cytokine signalling (SOCS); at the level of kinase-mediated STAT activation by phosphatases which cleave the activating phosphate groups; and at the level of gene transcription by an inhibitory protein, PIAS (protein inhibitor of activated STAT), which binds to activated STATs in the nucleus.

TNF-receptors, in contrast, are homotrimers which act via a different pathway. Similar to TLRs, TNF-receptor binding increases nuclear levels of the transcription factor NFκB, but it acts through a different pathway, via phosphorylation of IκB (inhibitory protein κB). IκB normally inhibits NFκB activity by binding to it and trapping it in the cytosol. Phosphorylation of IκB releases it from binding, allowing NFκB translocation to the nucleus, where it can function as a transcription factor. In activated cells, transcription is increased for a wide variety of inflammatory genes, including pro-inflammatory cytokines like IL-1, as well as genes mediating cell adhesion, proliferation and apoptosis.

HMGB1 functions in a cytokine-like manner. This nuclear protein is one of a range of factors released from necrotic cells. It binds to RAGE, which is expressed on vascular endothelia and macrophages. The RAGE-HMGB-1 conjugate cooperates with multiple TLRs to stimulate the production of other cytokines. HMBG1 is also secreted by activated macrophages and is an important mediator of elements of the acute-phase response such as fever, anorexia and malaise.

2.4.8 Chemokines

Chemokines are often considered as a subgroup of cytokines which function primarily as chemotaxins, promoting lymphocyte, neutrophil and macrophage migration. Chemokines are produced from almost all cell types and fall into structurally distinct subfamilies, most of which are represented by the CC and CXC families, depending on the position of cysteine residues. Activated macrophages, and tissue cells, are major sources of CXC chemokines, whilst CC chemokines are produced largely by T lymphocytes. Other important chemokines include the C-chemokine (lymphotoxin) and the CX3C chemokine, fractalkine. Cytokines functioning as chemotaxins in inflammation are often termed 'chemotactic protein' or 'inflammatory protein', with the cell target or source specified in the name (e.g. granulocyte chemotactic protein-2, macrophage inflammatory protein-1). Another chemokine, CXCL12 has been implicated in recruitment of macrophages to hypoxic tissues. Other 'homing' chemokines are also responsible for mediating lymphocyte partitioning to specific areas of lymphoid tissue, conferring the characteristic anatomical structure of lymph nodes, or splenic white pulp. These are usually expressed constitutively in lymphoid tissues. The main functional difference between these chemokines is in the population of cells attracted by their presence, which is largely determined by receptor distribution. Receptor distribution is itself a function of the cell type and differentiation state, which are relevant to partitioning of different lymphocyte types and mature vs. immature cells in lymphoid tissue. Activation state of the target cell (e.g. in inflammation) also influences the level of receptor expression and will determine how sensitive a cell is to chemotactic signals. Chemokine receptors are 7-TM domain proteins (the same class of receptor as the vasoactive amines histamine, dopamine and serotonin), which are specific to different chemokine families but will often bind several different chemokines within each family. For example, the CCR1 receptor binds CCL chemokines 3, 7, 9, 15, 16 and 23. Such receptor promiscuity is another example of functional overlap for inflammatory mediators. Receptor–ligand binding signals through a receptor-coupled G protein, which in

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turn activates phosphodiesterases (PDEs), generating second messengers such as cAMP and IP_3. These second messengers mediate intracellular calcium levels via protein phosphorylation and can also mediate gene expression by activating transcription factors. Regulation of signalling is achieved by the internalization and degradation of receptor–ligand complexes.

2.4.9 Acute-Phase Proteins

These are plasma proteins which are mostly synthesized by the liver. Concentrations of these proteins may increase dramatically as part of the inflammatory response. These proteins function as part of the innate immune system. Some have microbicidal and opsonizing properties which act to remove inflammatory inducers such as microbes and cell debris. They also function as mediators of inflammation, and some can be measured in blood as circulating, clinical pathologic indicators of inflammation. Acute-phase proteins include CRP, fibrinogen, haptoglobin, α_1-acid glycoprotein, α_1-antitrypsin, complement proteins (C3 and C4) and serum amyloid A protein (SAA). Their production is stimulated by pro-inflammatory cytokines: CRP and fibrinogen are increased by IL-6, whilst IL-1 and TNF increase levels of SAA. Since the principal source of these proteins is the liver, it is important to note that increases in acute-phase proteins are caused by sufficiently high levels of these cytokines in the systemic circulation, not as a local effect. Complement proteins are involved in inflammation by the generation of factors C3a and C4a, which mediate recruitment of granulocytes (neutrophils, eosinophils, basophils) and monocytes, and induce mast cell degranulation. In contrast, protease inhibitors such as α_1-antitrypsin act to limit inflammation by inhibiting the activity of proteolytic enzymes released primarily by neutrophils, thereby reducing the extent of tissue damage caused by these enzymes.

2.4.9.1 Defensins and Collectins

Defensins are cationic proteins with antimicrobial properties, which also function as inflammatory mediators. Alpha and beta defensins, along with anionic peptides including histatins and dermacidins, are produced by epithelial cells and neutrophils. They are active against microbes such as bacteria, fungi and some enveloped viruses, probably by forming membrane pores. Defensins also stimulate mast cell degranulation, function as chemotaxins, stimulate cell activation (including T lymphocytes, macrophages and dendritic cells), cell proliferation, cytokine synthesis/release (e.g. IL-8) and wound repair.

Collectins are fibrillar proteins with a lectin-binding domain which can bind and aggregate bacteria/viruses but which can also mediate inflammatory responses. Collectins, like surfactant proteins A and D, found in lung surfactant, can stimulate macrophages and regulate production of TNF and ROS.

2.4.9.2 Reactive Oxygen Intermediates and Nitric Oxide

The production of various free radicals is a significant effector of inflammation in activated neutrophils and macrophages, related to the killing of microbes or infected/neoplastic cells. Reactive oxygen intermediates may also be generated by the metabolism of xenobiotics. These highly reactive molecules, which include superoxide anion, hydroxyl radicals and reactive nitric oxide intermediates, notably peroxynitrite, will react biochemically with a variety of different cellular molecules (proteins, lipids, nucleic acids), generating various cell molecule intermediates which are themselves highly reactive, and propagate further damaging reactions. Cell membrane phospholipids will be attacked, forming lipid peroxides; DNA adducts and strand breaks may be formed; protein crosslinking and oxidation of signalling molecules may occur. These changes compromise membrane and nucleic acid integrity, gene regulation/cell cycle, protein structure/function and intracellular signalling, possibly resulting in the release of additional inflammatory mediators/cell necrosis. If affected cells are ECs, then vascular permeability may be increased. Intracellular antioxidant systems exist to combat the generation of ROS. These include enzymes such as catalase, superoxide dismutase, glutathione peroxidase, proteins such as transferrin and melatonin, and essential dietary factors such as vitamins A, C, E, lycopenes and flavonoids. These defend against ROS by reducing the free radicals, but may be depleted by excessive/prolonged generation of ROS, as may occur with certain xenobiotics, and significant cell damage ensues. The presence of superoxide intermediates may be indicated by measurement of reduced glutathione levels, or by immunohistochemistry to detect the presence of inducible nitric oxide synthase (iNOS), glutathione *S*-transferase Pi, or 8-hydroxydeoxyguanosine (8-OHdG).

Nitric oxide itself is a powerful, though short-lived inflammatory mediator, which is produced by the enzyme nitric oxide synthase (NOS). There are different forms of NOS: neural, inducible and endothelial. The action of endothelial NOS in generating NO derivatives causes vascular smooth muscle relaxation. NO also exhibits anti-inflammatory activity by inhibiting mast cell-induced inflammation, platelet aggregation/adhesion, by oxidizing lipids and by regulating leukocyte chemotaxis.

2.4.9.3 The Acute-Phase Response and Cytokine Release Syndrome

The acute-phase response is an important systemic manifestation of malaise associated with inflammation, and is due to the (endocrine) activity of IL-1, TNF and IL-6, acting mainly on sensory nerve fibres, ECs

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(particularly in the brain) and macrophages/microglia (influencing prostaglandin levels, notably PGE_2). This response influences the hypothalamus/various brainstem nuclei, inducing fever, decreased appetite and increased sleep/unconsciousness. Neutrophilia (release of neutrophils into the circulation) and stimulation of the corticosteroid endocrine axis are also part of the acute-phase response. Haemodynamic shock, an effect associated particularly with TNF, is circulatory hypotension (reduced blood pressure) as a result of systemic effects on vascular resistance (also increased vascular permeability), and is associated with increased heart rate and acidosis (decreased blood pH). TNF also promotes mobilization of lipids and proteins (catabolism); along with suppressed appetite this can lead to sustained weight loss and anorexia (cachexia). Other mediators, such as HMGB1 proteins, feature in the acute-phase response.

The signs/symptoms typical of the acute-phase response, mediated largely by cytokines, are also known as a 'cytokine storm'. At the molecular level, this is characterized by an excessive release of pro-inflammatory cytokines. Historically, it has been described in graft-versus-host disease, as part of the pathogenesis of pancreatitis in man, following significant tissue trauma and associated with certain viral pathogens and particularly with sepsis. Depending on the particular inducer(s) and pattern of cytokines expressed, clinical manifestations can vary, but the broad pathogenesis is one of massive cytokine release leading to widespread inflammatory effects such increased vascular permeability and hypovolaemic shock. Certain Gram-positive bacterial toxins with very high biological potency have been termed superantigens. Such superantigens are able to activate T lymphocytes by binding cell surface major histocompatibility complex (MHC) class II/TCR molecules, but in a way that is not restricted to antigen-specificity. Thus, large numbers of immune-competent cells can be activated in an antigen-independent fashion, leading to systemic release of pro-inflammatory cytokines including IL-1, TNF and IFN. Septicaemia and endotoxic shock are not dissimilar in their pathogenesis. Specific bacterial-toxin cytotoxic effects and/or the specific LPS-mediated activation of TLRs represent differences in the specific pathogeneses, but the end-result is a similar systemic inflammatory reaction, with significant release of cytokines and other inflammatory mediators including eicosanoids, PAF and kinins. The various effects of these mediators, discussed earlier, lead to vasodilation/vasoconstriction, leakage of plasma proteins into the extracellular space, activation of the complement, kinin and clotting cascades and sequestration of activated leukocytes and platelets. Activation of the plasma protein cascades may lead to disseminated intravascular coagulation and depletion of factors which may then predispose to haemorrhage. Hypoperfusion may result in local tissue hypoxia which is also pro-inflammatory, resulting in production of the chemokine CXCL12 (or stromal-derived factor-1), which can recruit macrophages to the injured tissue. If severe/widespread, these changes may result in circulatory shock and multiple organ failure, which can be fatal even if aggressively treated. The superantigen/superagonist mechanism is also related to xenobiotic-induced inflammation, as some protein therapeutics (notably the anti-CD28 monoclonal antibody TGN1412) can cause this phenomenon, which has led to significant morbidity in the context of clinical trials.

2.5 Effectors of Inflammation

The action of inducers and mediators of inflammation is to alter the phenotype of effector cells. The changes in these cells, which may be in the synthesis/expression of certain molecules or mediators, change in cell shape, migration, phagocytosis or release of cytoplasmic granules, are responsible for the functional alterations which characterize an inflammatory response.

2.5.1 Endothelial Cells

The importance of vascular ECs in mediating vascular permeability and leukocyte transmigration has already been mentioned. Via changes in cell morphology and expression of cell adhesion molecules (including the selectins, ICAM-1, PECAM-1 and JAMs), synthesis of cytokines, and so on, they function as important effectors, themselves responding to inducers and mediators in order to regulate local blood flow, ensure the accumulation of a cell-rich transudate at the site(s) of inflammation and form new blood vessels if required as part of the healing process. This is mediated by various growth factors, including VEGF.

2.5.2 Mast Cells

Mast cells are generally located adjacent to small blood/lymphatic vessels, particularly close to epithelial surfaces, and are important in the initiation and early stages of the inflammatory response. They express cell surface receptors for the Fc-region of IgE and become coated with IgE molecules, the antigen-specific regions of which face the extracellular environment. Crosslinking of these IgE molecules by allergens or parasites (e.g. Type I hypersensitivity response) stimulates degranulation and the release of the potent mediators contained therein. Mast cell degranulation can also be induced by substance P released from sensory nerve endings and from macrophages, defensins and complement factors C3a, C4a, C5a. Granules contain preformed substances including TNF, vasoactive amines (histamine, serotonin), proteoglycans (e.g. heparan), neutral proteases, tryptase, chymase and stem cell factor. Proteolytic enzymes can

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degrade protein toxins/mediators and also extracellular matrix, which contributes to remodelling but can also cause damage, particularly if degranulation is excessive. Tryptase is also mitogenic to epithelial cells, which contributes to healing. Mast cells can also synthesize various inflammatory mediators such as leukotriene C_4, PAF, prostaglandin D_2, cytokines and chemokines (e.g. eosinophil chemotactic factor, which can play a major role in recruitment of eosinophils associated with mast cell activation).

2.5.3 Neutrophils

These cells are derived from the bone marrow and are circulated in the blood. They are a key effector cell in acute inflammation, functioning principally to kill/phagocytose bacteria or foreign materials (recognized by membrane-expressed C3b or Fc receptors) and to release preformed lysosomal enzymes (proteases, phosphatases, phospholipase A2, myeloperoxidases), antimicrobial molecules (lysozyme, lactoferrin, defensins) and inflammatory mediators (leukocyte adhesion molecules, defensins, plasminogen activators) from their cytoplasmic granules to degrade protein/extracellular matrix and enhance the inflammatory reaction. Cytoplasmic granules can be classified as 'specific' and 'azurophil', based on their appearance, and their contents differ slightly. Both types of granules are discharged into phagosomes to degrade microbes/foreign material, or they can be released into the surrounding ECM. The enzymes either degrade cellular constituents or generate superoxide bursts (e.g. myeloperoxidase) designed to kill phagocytosed microbes. However, these enzymes when released into the ECM will also cause local tissue/cellular damage or liquefaction, which will itself stimulate the inflammatory process and may lead to the formation of pus/an abscess.

Increased production and release from the bone marrow (and in rats, notably the spleen) is stimulated by cytokines (IL-1, TNF, IL-3 and granulocyte/macrophage colony stimulating factor (GM-CSF)) and increased levels of circulating neutrophils (plus increases in acute-phase proteins such as fibrinogen and CRP) can be clinical pathological indicators of an ongoing inflammatory response. Neutrophils are short-lived, only surviving a few days once they leave the systemic circulation. They express receptors for many different classes of inflammatory mediator. In addition to C3b and Fc receptors, they also express receptors for cytokines, substance P, PAF and chemokines. Ligand binding of these receptors and cell surface adhesion molecules (e.g. selectins and β_2-integrins such as Mac-1 (CD11b/CD18 heterodimers)) activates neutrophils by mechanisms discussed earlier, resulting in up-regulation of those cell surface molecules involved in transmigration from the blood stream, activation of eicosanoid pathways and cytoskeletal changes facilitating formation of pseudopodia for cell movement (chemotaxis), phagocytosis and degranulation.

2.5.4 Eosinophils

With neutrophils, eosinophils are collectively termed granulocytic cells. They are also recruited from the circulation to sites of inflammation, and are particularly prominent in inflammatory reactions to allergens or parasites and in locations such as the alimentary tract. Eosinophil chemotactic factor (from mast cells), histamine, C5a, cytokines (IL-4, IL-5, IL-13) and other chemokines (CCL5, CCL11 or eotaxin) are all involved in the recruitment and activation of eosinophils. Their heterogeneous cytoplasmic granules are notably eosinophilic (in contrast to neutrophils in most species); primary granules contain major basic protein (MBP), which is toxic to host cells/parasites and stimulates vasoactive amine release (e.g. histamine). Secondary granules (specific granules) contain a cationic protein with similar properties to MBP and also microbicidal peroxidase, neurotoxin and various proteases (e.g. collagenases, metalloproteases) which degrade the ECM. Excessive activation of eosinophils is associated with significant cell/connective tissue damage. A variety of cytokines are also produced by activated eosinophils, including ILs 1–6, 8, 10, 12, 16, TGF, GM-CSF, chemokines and eicosanoids, notably LTC_4. Catalase/phosphatase enzymes are also released from eosinophil granules, which influence the inflammatory response by inactivating leukotrienes.

2.5.5 Monocytes/Macrophages

Monocytes are produced in the bone marrow and circulate in the blood. Independent of inflammation, these cells migrate to tissues, where they differentiate to become tissue macrophages or histiocytes; these cells adopt a surveillance function and if activated by a local insult can become important sources of mediators early in the inflammatory response. Dendritic cells are also part of the monocyte/macrophage cell lineage and are recruited preferentially to lymphoid tissues and external interfaces like skin and mucous membranes of the gut and respiratory tracts. Monocytes can also become activated and recruited from the circulation as part of the inflammatory response, differentiating to become macrophages upon transmigration into tissue. Activation signals for macrophages include those mediated via TLRs (binding of microbial inducers, or from contents of damaged/necrotic cells possibly by RAGE complexing); via cytokines (particularly IFN-γ from activated T lymphocytes and IL-12 associated with cell-mediated immunity/Th1 responses, and IL-4 and IL-13 associated with Th2 responses). These macrophages are important sources of inflammatory mediators such as cytokines

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(TNF, IL-1) and play an important part in phagocytosis of microbes and foreign materials which may be recognized by C3b receptors or Fc receptors in the macrophage surface. This recognition also serves to activate macrophages and is important in the innate immune response, for example, in the lung, where macrophages phagocytose particulates which are carried beyond the mucociliary escalator of the respiratory epithelium (e.g. environmental 'nuisance dusts', or lactose used in inhalation studies).

Different forms of activation result in different phenotypic changes, ranging from phagocytosis, to a respiratory burst and the release of ROS/nitric oxide to kill cells/microbes, to up-regulation of MHC expression for antigen presentation, and to release of IL-1 and TNF. Inert particulates, foreign materials or resistant microbes (e.g. fungal hyphae, mycobacteria) can continue to stimulate a macrophage response, via cytokines, microbial proteoglycans, integrins and other cell surface receptors mediating cell fusion. These signals, which may in part be sensed intracellularly via the NALP3 inflammasome, result in a macrophage-rich granulomatous inflammatory infiltrate or granuloma, which may contain macrophages fused into multinucleated giant cells in an attempt to remove the inducing stimulus.

Macrophages are also important in causing tissue damage and in stimulating fibrosis. ROS, nitric oxide proteases, eicosanoids and chemokines from activated macrophages will either damage tissue directly, or attract neutrophils. This is important in removing microbes and damaged or necrotic tissue to make way for healing. Activated macrophages also release growth factors including PDGF, fibroblast growth factor (FGF) and TGFβ. These stimulate fibroblasts to produce collagen and also angiogenesis, both of which are required for the healing process/production of granulation tissue.

In addition to their role in innate immunity, macrophages are also key effector cells for the adaptive immune system, and influence chronic inflammation by antigen presentation (a function both of dendritic cells and activated macrophages), T lymphocyte help resulting in the proliferation of reactive T cells, which may stimulate cell mediated immunity (Th1 phenotype) or the differentiation/proliferation of B lymphocytes and antibody production (Th2 phenotype).

2.5.6 Lymphocytes and Plasma Cells

These cells are important in adaptive immunity and are a feature of chronic inflammation, generally being recruited to tissues at 24–48 hours, later than neutrophils. Naive lymphocytes locate from the circulation to specific compartments of the lymphoid tissues, exiting the circulation via the high endothelial venules. This process is mediated by chemokines (CCL19 and 21, CXCL12 and 13) and ICAMs acting via their cognate receptors expressed on the lymphocytes. Mucosal lymphoid tissue targets cells using a specific adhesion molecule, mucosal adressin cell adhesion molecule (MadCAM). Mature, activated lymphocytes are recruited to sites of inflammation in a similar fashion to other leukocytes.

Once located at the site of inflammation, T lymphocytes expressing the classical α/β TCRs and CD4 on their cell surface (helper T lymphocytes) orchestrate chronic inflammation according to different phenotypes, Th1, Th2 or Th0. Th1 responses (cell-mediated responses) are facilitated by a pattern of cytokines, including IL-12 released by dendritic cells, which results in the recruitment of macrophages via release of IFN-γ and TGFβ from the activated T cells. Th2 responses (humoral responses) are characterized in particular by IL-4, IL-5 and IL-10 from Th2-type CD4 lymphocytes, which stimulate B lymphocytes to produce antibodies and will also activate eosinophils and mast cells. These responses may be seen where the basis of stimulation is allergic. Th1 and Th2 phenotypes are not mutually exclusive although each tends to inhibit the other. This lack of phenotype exclusivity is due in part to the presence of Th0 lymphocytes, which are able to secrete patterns of cytokines common to both phenotypes. The predominant form of T lymphocyte response can also be influenced by dendritic (antigen-presenting) cells and another class of T lymphocyte, the regulatory T cell which can inhibit the dendritic cells themselves. Lymphocytes expressing CD8 cell surface molecules (and classical TCR heterodimers) are known as suppressor/cytotoxic T cells. These cells can kill target cells expressing foreign antigens (e.g. virus- or xenobiotic-derived) via specific recognition of these antigens in conjunction with MHC Class I.

B lymphocytes are responsible for antibody production (and differentiation into a mature plasma cell antibody-secreting phenotype), but these cells can also stimulate other lymphocytes by taking up and presenting antigen.

NK cells are a subset of lymphocytes which do not express TCRs or surface immunoglobulins but do bear Fc receptors (CD16 molecules) and a variety of other costimulatory and inhibitory molecules. These cells contain cytoplasmic granules and are important in the recognition and removal of cells/microbes coated with IgG and cells expressing stress-induced or viral proteins. They are regulated by a variety of cytokines and also secrete cytokines themselves, including TNF and IFN-γ.

This participation of the adaptive immune system influences the nature of the induced chronic inflammatory response and is important in some types of xenobiotic-induced inflammation, where the xenobiotic acts to induce a specific immune response which may either cross-react with endogenous antigens (molecular mimicry) or else target cells expressing the (complexed) xenobiotic at the cell surface.

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2.5.7 Fibroblasts

These cells secrete collagen, other ECM components, cytokines, chemokines and proteases (matrix metalloproteases) and are important physiologically in providing stromal support/scaffolding/compartmentalization in normal tissues. In inflammation, fibroblasts play a significant role in the regulation, production, vascularization and remodelling of an intercellular matrix. This is important both in containing a chronic inflammatory response (e.g. abscess formation) and in the production of bridging fibrovascular (granulation) tissue which may fill a tissue defect caused by damage/inflammation as part of the healing process. Growth factors such as PDGF, FGF, TGFβ and VEGF, produced by macrophages and other cell types in inflammation, activate fibroblasts.

References to recent reviews and articles covering the morphological, functional and molecular aspects of inflammation, at a level of detail which is beyond the scope of this chapter, are included in the 'Further Reading' section.

3 XENOBIOTIC-INDUCED INFLAMMATION

Xenobiotics can cause inflammation by a variety of mechanisms with differing pathogeneses and morphologic patterns of change. Xenobiotic-induced inflammation may be associated with

- the point of entry or administration of the xenobiotic
- systemic toxicity in different target tissues
- the indirect effects of pharmacology
- intracellular or extracellular accumulation
- the induction of cytokine release
- stimulation of an adaptive immune response.

In many cases xenobiotics will cause cytotoxicity/necrosis in target cells which may result in the release of cellular contents in abnormal tissue compartments. As previously discussed, the presence of these cell contents acts as an inflammatory inducer which is sensed and leads to the release of inflammatory mediators. Cellular damage may occur as a result of physicochemical properties of a xenobiotic which may be influenced by the site of administration/experimental procedure. It may also occur as a result of direct cellular toxicity, which may arise through a number of different mechanisms. These mechanisms may serve to exacerbate inflammation by generating pro-inflammatory mediators such as nitric oxide derivatives or ROS; however a similar endpoint of cellular degeneration and/or necrosis is achieved. In addition, cells may be injured in an indirect fashion as a result of exaggerated pharmacology. Xenobiotics may also cause inflammation by acting on cells/signalling pathways which are themselves involved in the processes of inflammation and immunity. They may do this by excessively stimulating pro-inflammatory pathways leading to cell activation and release of inflammatory mediators, or alternatively by inducing an adaptive immune response which may result in chronic inflammation.

3.1 Inflammation Associated with the Point of Entry

Administration of xenobiotics can be an important factor in initiation of inflammation. Xenobiotics may exacerbate inflammation as a result of the route of administration, for example intravenous administration, which may itself be influenced by variations in operator experience, technical equipment or duration of administration. Intravenous administration will cause a degree of trauma to the vessel (and overlying subcutis/skin), which will be associated with a degree of perivascular haemorrhage, fibrin and inflammatory cells, as a result of the activation of pro-inflammatory processes and release of mediators. Duration of intravenous access is also a factor. Indwelling venous cannulae can cause prolonged irritation and activation of the clotting cascade, leading to inflammation and thrombus formation. Over time, chronic thrombi may show fibroplasia/fibrosis ('organization') and mineralization in control animals (**Figure 2**) in addition to those receiving xenobiotic. Therefore, it is always important to review the variation of background inflammation/haemorrhage to reveal exacerbation of the inflammatory response, which may be caused by irritant xenobiotics. Irritant effects include necrosis of the vessel wall/surrounding tissues, with enhanced oedema, inflammatory cell infiltration and thrombus formation. Barbiturate anaesthetic agents are examples of irritants which can cause significant vascular and extravascular tissue damage and even sloughing of necrotic tissue due largely to the high (alkaline) pH of formulations. Intraperitoneal injection can also cause inflammation of the peritoneal cavity and serosal surface of abdominal viscera by chemical irritation in rats and dogs (Brady *et al*., 2004; Kanter *et al*., 1994). Accidental deposition of oral formulations into the pleural cavity as a result of dosing accidents and oesophageal perforation will cause significant, often fatal intrathoracic inflammation. Introduction of fluid into the pleural space will interfere with lung ventilation and perfusion, but inflammation is related to the irritant effect of oral formulations when exposed to the sensitive pleural membranes and also the efflux of food material through the perforated oesophagus.

Oral administration results in the deposition of xenobiotic into the stomach, directly if administered by gavage tube. If this compromises the normal defence of the gastric mucosal barrier (which constitutes the superficial mucous layer and the gastric epithelium), or the relatively

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Figure 2 Inflammation in the heart, vasculature and lung. (A) Normal rat heart showing close apposition of normal myofibres (original magnification ×200). (B–E) Progression of acute inflammation in rats following a single subcutaneous dose of isoprenaline. (B) Early changes in acute inflammation. Note the central necrotic myofibre (arrow), interstitial oedema (seen as increased clear space between cardiomyocytes) and predominantly neutrophilic inflammatory cell infiltrate (original magnification ×200). (C) Note the predominantly mononuclear inflammatory cell infiltrate into the interstitium and phagocytosis of necrotic cardiomyocytes (original magnification ×200). (D) Later stage (48 h postdosing) showing the presence of mononuclear inflammatory cells and remaining cardiomyocyte cytoplasmic debris, but note the plump spindle-shaped nuclei in the interstitium, indicating activation of interstitial cells including fibroblasts. (E) At this more advanced stage, healing by fibroplasia/fibrosis is becoming more established. Necrotic cardiomyocyte debris has been phagocytosed, removed and the interstitium is expanded by plump, proliferating interstitial cells, with some remaining mononuclear inflammatory cells (lymphocytes/monocytes/macrophages). Collagen is being laid down by the fibroblasts (original magnification ×400). (F) Inflammation in a canine arteriole. This is an example of idiopathic canine polyarteritis ('Beagle Pain Syndrome'), a spontaneous condition. Note the margination (arrow) and transmigration of inflammatory cells through the arteriolar wall and into the perivascular interstitium (original magnification ×200). (G) Inflammation related to experimental procedure. Vena cava from a control rat with an indwelling cannula. The site of the cannula is shown by the ovoid clear area in the lower right quadrant of the image. It is surrounded by a large thrombus showing features of chronic inflammation, with fibroplasia, lymphohistiocytic inflammatory cell infiltrate and multifocal necrosis/mineralization (arrows). The thrombus is adherent to the vessel wall on the right hand side and there is a narrow crescentic space on the left, containing red blood cells, which represents the remaining lumen of the vena cava (original magnification ×20). (H) Chronic inflammation in the rat lung associated with phospholipid accumulation in alveolar macrophages. Alveoli are filled with vacuolated macrophages, cellular debris, eosinophilic proteinaceous material and cholesterol crystals (needle like clefts in the section). There is a mixed inflammatory cell infiltrate and alveoli have become lined by Type II pneumocytes (cuboidal epithelium). There is also perialveolar fibrosis (arrows).

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hardy squamous epithelium of the rodent forestomach, then it will expose the subepithelial compartment to inflammatory inducers including the normally acid conditions of the stomach contents, which will produce further damage, exacerbating the inflammation. This may occur as a topical/irritant effect of direct administration. Physicochemical factors such as pH and buffering capacity of administered formulations will be important considerations but administration of ethanol, hyperosmolar glucose, salt solutions and also stress, protein restriction or starvation have been reported to induce inflammation/ulceration in the rat forestomach and/or glandular stomach (Goldberg, 1973; Puurunen *et al*., 1980). Inflammation and the milieu of inflammatory cytokines, particularly IL-1, IL-2 and TNF, can enhance both antigen presentation by dendritic cells/macrophages, and the induction of an adaptive immune response. This is illustrated in the experimental induction of alimentary tract hypersensitivity to tribenzenesulfonic acid (a sulfonamide) by administration of alcohol, which breaks down the mucosal barrier and induces inflammation (Bertram, 2002). The hypersensitivity response causes granulomatous inflammation in the colonic mucosa. The mechanisms of hypersensitivity will be discussed later.

Oral gavage administration may also give rise to necrosis and inflammation in the upper respiratory tract. In rats, lesions in the nasal cavity of multifocal olfactory and respiratory epithelial necrosis and inflammation have been associated with oral but not parenteral administration of xenobiotics (Pino *et al*., 2006). Changes are also usually present in the nasopharynx and represent reflux or convection of oral formulation in animals which are obligate nasal breathers. This is also considered to be a result of topical irritancy of oral formulations to the more sensitive nasal epithelia.

Delivery by the inhalation route may also cause toxicity related to topical irritancy. Due to anatomical factors, the nasal airways and larynx are common sites of irritancy. Many inhaled xenobiotics cause degeneration/necrosis and inflammation in the nasal cavity and airways including formaldehyde, cigarette smoke, ammonia, ozone, chlorine (Harkema *et al*., 2006). The regional distribution of lesions, including inflammation, reflect local dose, water solubility, epithelial susceptibility and local metabolism. Squamous epithelium is more resistant than respiratory or olfactory epithelia. Erosion or ulceration of squamous epithelium causes inflammation due to compromise of this epithelial barrier and presumably exposure of inflammatory inducers either to other keratinocytes or to the underlying tissues. Oxidants like chlorine and ozone are less irritant but can cause inflammation associated with the less resistant transitional epithelium: exudation of neutrophils into the epithelium and airway is a feature and may be linked to the rapid increase in mucus secretion. With continued exposure to ozone, a chronic inflammatory reaction develops. Formaldehyde affects the respiratory epithelium, and the continued cycle of inflammation and attempted repair, with epithelial cell replication (presumably as part of the growth factor-driven inflammation/repair process) is thought to play a part in the induced nasal tumours (Harkema *et al*., 2006). In the larynx, inhaled irritants can also cause respiratory epithelial compromise, such as loss of cilia and degeneration associated with inflammation. Susceptible sites are usually at transitions from squamous to other, more susceptible types of epithelium, including respiratory epithelium of the ventrolateral epiglottis, and the ventral pouch (Lewis, 1991). A protective hyperplastic/metaplastic response is associated with irritancy.

Route of administration may also influence concentrations of xenobiotic in local tissues and therefore, target-tissue toxicity. A good example of this is the 'topical' effect of anti-inflammatory agents following oral administration, which can lead to gastric erosion/ulceration and inflammation. Oral anti-inflammatory drugs (including alcohol, aspirin, corticosteroids), histamine H_2 receptor antagonists and proton pump inhibitors have been shown to alter the make-up of normal gastric mucous layer, reducing the content of protective sulfomucins and prostaglandin PGE_2 (Greaves, 2007). This compromise of the normal protective effects of the gastric mucous layer may predispose to mucosal damage. The nonsteroidal anti-inflammatory drugs (NSAIDs) indomethacin (indometacin) and ibuprofen produce gastric mucosal erosion and ulcers with associated inflammation (Shriver *et al*., 1975). The initial stages are neutrophil-independent, then tissue damage is exacerbated by neutrophil influx. In the early stages, NSAIDs are readily absorbed into the mucosa, a process which is dependent on the physicochemical properties of the NSAID (versus its antiprostaglandin effect). Important factors in the pathogenesis of mucosal damage include lipid solubility, acidity, which mediate interaction with cell membrance phospholipids and their ability to uncouple mitochondrial oxidative phosphorylation once 'trapped' in the cell (Bjarnason *et al*., 2003), in addition, the pharmacological activity of COX inhibition blocks the production of protective prostaglandins (mucus content and mucosal blood flow mediated by vasodilators such as PGE_2). The inhibition of COX enzyme is also associated with an increase in the 5-LOX pathway, generating the pro-inflammatory leukotrienes (e.g. LTB_4). The penetration of cellular constituents, LTB_4, gastric acid and bacteria attract neutrophils (e.g. via TLRs/PRRs) which themselves cause tissue damage and exacerbate the inflammatory reaction by the release of lysosomal enzymes including myeloperoxidase, the generation of superoxide radicals, eicosanoids such as LTB_4, proteases and other inflammatory mediators (Bertram, 2002). This reaction can progress to form penetrating ulcers and peritonitis due to the presence of powerful inflammatory inducers (irritant

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materials, inflammatory cells, bacteria) and mediators in the peritoneal cavity.

Certain inhaled xenobiotics cause degenerative/inflammatory lesions in olfactory epithelium of the nasal cavity due to high local concentrations, coupled with metabolism to generate products toxic to the overlying epithelium. Xenobiotics including 3-methylfuran when given by inhalation, or systemic acetaminophen, can cause necrosis of the olfactory epithelium as a result of metabolism by the cytochrome P450 enzymes expressed in this epithelium (Harkema *et al*., 2006).

Inflammation caused by adenoviral vectors also varies depending on the route of administration. Adenovirus causes pneumonitis by inhalation, hepatitis by intravenous injection and myositis by intramuscular injection (Schnell *et al*., 2001). Viral capsid has been shown to be important in stimulating this initial inflammatory response via the release of pro-inflammatory cytokines (Liu and Muruve, 2003) and is discussed in more detail later.

3.2 Inflammation Caused by Systemic Toxicity

Xenobiotics can cause direct toxicity to target cells by a number of different mechanisms, leading to cellular dysfunction and death. If this results in the release of inflammatory inducers then it will result in inflammation. The site and severity of inflammation will depend on the target cell population, which is itself dependent upon a combination of absorption, level of expression of the pharmacologic target, metabolism and cellular adaptation mechanisms. It may be restricted to a single tissue or organ or, if the vasculature is affected, inflammation may occur in a variety of different locations.

3.2.1 Organ- and Tissue-Specific Inflammation

Paraquat causes acute damage to Type I and II alveolar epithelial cells via redox cycling, depletion of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and the generation of ROS, notably hydroxyl radicals. Following systemic administration, paraquat is concentrated in the lungs by a receptor-mediated uptake mechanism (Haschek *et al*., 2002), and the high oxygen levels present here contribute to the severity of damage (Lock and Wilks, 2001). Although damage does not directly affect the pulmonary capillaries, they are intimately associated with alveolar epithelial cells which are an important component of the blood–air barrier. Alveolar epithelial necrosis leads to alveolar oedema, haemorrhage and extensive alveolar, interstitial and perivascular inflammatory cell infiltration which can be fatal (Clark *et al*., 1966). Surviving animals show chronic inflammation with extensive fibroplasia and attempted alveolar epithelial regeneration (Kimborough and Gaines, 1970). Interstitial inflammation in the lung also occurs as a result of ozone-induced damage, treatment with chemotherapeutic drugs such as bleomycin and busulfan, and hypersensitivity reactions, with fibroplasia a feature of chronic lesions (Haschek *et al*., 2002). Bleomycin also induces pulmonary interstitial pneumonia and fibrosis, and TNF (α) has been implicated in the pathogenesis of these changes to the end-stage of fibrosis (Piguet and Vesin, 1994).

The distribution of the pharmacological target of a xenobiotic may influence the distribution of induced inflammation. In rats, inflammation of the caecum associated with indomethacin inhibition may indicate a protective effect of normal COX2 expression. COX2 is present at high levels in macrophages and other interstitial cells at the ileocaecal junction. Inhibition by xenobiotics or in transgenic mice with the *COX2* gene deleted may have a preferentially deleterious effect on these cells, accounting for the distribution of the observed ulcerative/inflammatory lesions (Haworth *et al*., 2005). Necrosis of lymphoid tissue may also occur as a direct effect of xenobiotic administration, leading to inflammation and haemorrhage. Inhibitors of p38 kinases (involved in the MAP kinase signalling pathway) may cause acute intestinal toxicity in dogs. The primary change of lymphoid necrosis and inflammation is considered to lead to mucosal haemorrhage visible macroscopically at necropsy. Affected animals show signs of neutrophilia and fever, which are associated with systemic effects of various inflammatory mediators. High levels of expression of p38 kinase have been demonstrated in canine B lymphocytes (but not in rodents), and inhibition is thought to cause a species-specific necrosis of B lymphocytes and inflammation in the dog (Davis, 2008).

The nature of the target tissue may also influence the pathogenesis of the inflammatory response. The acinar cells of the exocrine pancreas contain proenzymes which are normally activated when they reach the lumen of the gastrointestinal tract. Xenobiotic-induced inflammation can be caused by a variety of drugs including corticosteroids, antibiotics, anti-inflammatory agents and anti-HIV drugs (Trivedi and Pitchumoni, 2005). Although the precise mechanisms are unclear, when these cells are damaged by xenobiotics, there is release and activation of proteases/lipases contained within. These enzymes then exacerbate tissue damage and thus increase the generation of inflammatory inducers.

Xenobiotics which generate reactive metabolites can also lead to cell injury/necrosis and ensuing inflammation. The site of inflammation may then depend on the presence of metabolic enzymes required for activation and/or the endogenous protective mechanisms which may

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be more or less effective. Carbon tetrachloride is metabolized preferentially by centrilobular hepatocytes, generating trichloromethyl radicals, which bind covalently to lipids and proteins causing oxidative stress, lipid peroxidation, structural damage to membranes and enzyme inhibition, notably that of microsomal ATPase. Cells undergo ballooning degeneration, and necrosis, releasing cellular enzymes (e.g. alanine aminotransferase, which is measured in blood samples) and many other cellular constituents including ATP, K^+ ions, and so on, which act as inducers of inflammation.

Kupffer cells (specialized hepatic macrophages) are important in detecting these inducers and mediating influx of neutrophils (Edwards *et al*., 1993), in addition, the oxidative stress induced by carbon tetrachloride increases NFκB levels in the nucleus, which also promotes expression of pro-inflammatory cytokines (Liu *et al*., 1995). Cytokines, including tumour necrosis factor alpha (TNF-α), IL-1β, IL-6 and iNOS, plus growth factors such as PDGF isoforms, are up-regulated and play an important part in acute and chronic inflammation caused by carbon tetrachloride (Lee *et al*., 2008; Borkham-Kamphorst *et al*., 2008). Apelin is a protein which is important in the pathogenesis of carbon tetrachloride-induced inflammation and fibrosis. It enhances vascular dilation after endothelial nitric oxide synthesis via the extracellular receptor kinase (ERK) pathway, and may also initiate angiogenesis. In rats, apelin expression is regulated by TNF and it is increased in hepatic stellate cells in inflamed/fibrotic liver (Principe *et al*., 2008).

Systemic absorption of compounds and their distribution around the body can lead to inflammation related to bioactivation in the skin. Phototoxicity results from the absorption of visible light by xenobiotics (e.g. 8-methoxypsoralen, sulfonamides, fluoroquinolones) or endogenous porphyrins accumulating due to hepatotoxicity. Absorption of light triggers complex reactions resulting in the formation of free radicals which cause dermoepidermal erythema, oedema and necrosis (Klein-Szanto and Conti, 2002). Inflammation is induced, which is exacerbated if ulceration of the epidermis occurs, leading to exposure of subepithelial cells to (self-) trauma, environmental irritants, microbes, cellular debris and the effects of proteolytic enzymes from infiltrating neutrophils.

Xenobiotic-mediated inhibition of excretory mechanisms can also cause cellular toxicity and inflammation. Interference with the normal bile salt transport and excretion mechanisms can lead to hepatocellular damage, necrosis and subsequent inflammation. Drugs including rifampicin, ciclosporin (cyclosporine) and troglitazone directly inhibit ATP-dependent taurocholate transport by competitively binding to BSEP (bile salt export protein), the major hepatocellular bile salt efflux system (Pauli-Magnus *et al*., 2005). In mice, administration of lithocholic acid also causes cholestasis, hepatocellular necrosis and inflammation (Staudinger *et al*., 2001). Necrosis of hepatocytes will release cellular constituents which can act as inflammatory mediators. Damaged/stressed cells may secrete cellular proteins by a non-Golgi-mediated pathway (Medzhitov, 2008) but it is not known if this is part of the pathogenesis of cholestasis. Bile salts are themselves irritant and may induce inflammation if they escape their normal tissue compartments as they accumulate.

Route of excretion can also influence the pathogenesis of inflammation in target tissues. Cyclophosphamide is an alkylating agent which causes haemorrhagic inflammation of the bladder. Hepatic metabolism of cyclophosphamide generates acrolein, which is excreted in the urine and enters into the uroepithelial cells. Acrolein then activates intracellular ROS, nitric oxide production and cytokine (TNF, IL-1) expression (directly or through NFκB and c-Jun/AP-1), depleting cellular antioxidant defence mechanisms such as glutathione, and driving peroxynitrite production. Finally, the increased peroxynitrite level damages lipids (lipid peroxidation), proteins (protein oxidation) and causes DNA strand breaks, leading to activation of poly(adenosinediphosphate-ribose) polymerase (PARP), a DNA repair enzyme. PARP overactivation results in the depletion of oxidized nicotinamide adenine dinucleotide phosphate (NADP) and ATP, and consequently in necrotic cell death. Such cell damage/death will (i) cause haemorrhage if ECs are affected, and (ii) release intracellular contents into the mucosa and the urine. These pro-inflammatory cytoplasmic constituents and cellular proteases, can then cause further tissue damage and exacerbate the inflammatory reaction (Korkmaz *et al*., 2007). Renal tubular excretion of xenobiotic and crystal formation in the kidney tubules is an example of extracellular accumulation and is discussed later.

3.2.2 Vascular Lesions at Multiple Sites

Drug-induced vascular injury can act via a variety of mechanisms, which may be complex/multifaceted, but which directly affect the vessel and which lead to perivascular inflammation. Necrotizing vasculitis may be induced by a number of xenobiotics (e.g. phenylpropanolamine, methamphetamine), usually in small-to-medium calibre arterioles (Van Vleet *et al*., 2002). Whilst the mechanism is largely unclear, necrosis which would immediately increase vascular permeability and liberate cellular inducers of inflammation is associated with an initial neutrophil-rich infiltrate and thrombus formation. PDE IV inhibitors have been developed as anti-inflammatory/antiasthma drugs (Souness *et al*., 2000), but cause vascular injury and inflammation in preclinical studies. The proposed mechanisms include increases in intracellular cAMP and nitrate stress, physical stress due to local vasodilation and also activation of pro-inflammatory pathways. In

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rats, mesenteric vessels showed plasma insudation (in the vessel wall) and exudation, arterial haemorrhage and fibrinoid necrosis/mural fibrin, inflammatory cell infiltration in vessel walls and mesenteric oedema. Morphological evidence of mast cell activation, degranulation and EC activation were also observed (Zhang *et al*., 2008b). Similar morphological changes are also seen in monkeys (Losco *et al*., 2004). In rats, PDE IV inhibitors (SCH 351591 and SCH 534385) caused activation of mast cells, ECs, macrophages and the formation of the potent oxidant peroxynitrite (which is a reaction product of nitric oxide and superoxide anions) in ECs and macrophages (Zhang *et al*., 2008b). Peroxynitrite formation may be a common pathway in drug-induced vascular injury and is thought to contribute to oxidative damage of macromolecules (e.g. DNA) and the induction of apoptosis in ECs and vascular smooth muscle cells (Mihm *et al*., 2000). It is also important in inflammation (Pacher *et al*., 2007), mediating L-selectin shedding, up-regulation of Mac-1 (CD11b/CD18) on neutrophils and increased expression of E-selectin, P-selectin, ICAM-1 and VCAM-1 (vascular cell adhesion molecule) on ECs (Zhao *et al*., 2004; Sohn *et al*., 2003), thus enhancing the association of ECs and neutrophils (Sohn *et al*., 2003). Induction of peroxynitrite in ECs may therefore be an early inducer of inflammation in PDE IV inhibitor-induced vascular injury. Mast cell activation/degranulation is also pro-inflammatory via the release of various pro-inflammatory mediators, including vasoactive amines, proteases, VEGF (which increases vascular permeability) and bFGF (basic fibroblast growth factor, important in fibroplasia). All of these will act to increase vascular permeability, exudation, recruitment of leukocytes and activation of fibroblasts present in inflammation. In the rat, the vascular changes were reflected in circulating biomarker changes indicative of inflammation, including CRP, haptoglobin, thrombomodulin, α_1 acid glycoprotein, IL-6 and VEGF (Weaver *et al*., 2008).

Similar morphological changes have been reported for PDE III inhibitors in dogs (Clemo *et al*., 2003). These vasoactive compounds have also been shown to increase the local production of iNOS, nitrotyrosine and peroxynitrite by mast cells and ECs, which are molecular/cellular events associated with the vascular necrosis/apoptosis and inflammation observed in mesenteric blood vessels of rats (Zhang, 2008b).

3.3 Inflammation as an Indirect Consequence of Pharmacology

A wide variety of agents cause direct toxic damage resulting in myocardial necrosis, including ionophores, gossypol, allylamine and fungal toxins, all of which induce inflammation (Van Vleet *et al*., 2002). The myocardial injury caused by β adrenergic stimulation is an example of cell degeneration/necrosis and inflammation as a primary result of exaggerated pharmacology. Although multiple mechanisms have been proposed for the action of isoprenaline (isoproterenol), it is generally accepted that the pharmacologically mediated tachycardia results in increased oxygen demand (Dhalla *et al*., 1992), and this is well documented in the rat as a model of an infarct-like myocardial lesion. The temporal progression of inflammatory changes is shown in **Figure 2**. Myocardial necrosis occurs primarily in the left ventricle and is associated with elevations in circulating myocardial proteins such as cardiac troponins (York *et al*., 2007) and an inflammatory response which is initially characterized by oedema and neutrophil infiltration, followed by monocytes/macrophages which remove cardiomyocyte debris, and healing by formation of fibrous tissue by adjacent interstitial fibroblasts/perimysial cells. The release of cellular components from degenerate/necrotic cells would be expected to induce an inflammatory response and myocardial gene expression analysis has shown that IL-6 is up-regulated early in this process, along with MAP kinase and NFκB signalling, which are implicated in inflammatory signalling, necrosis and apoptosis (Mikaelian *et al*., 2008). Increases in iNOS are also correlated with lesion severity (Zhang *et al*., 2008a). Together these data highlight the prominence of pro-inflammatory processes in the pathogenesis of cardiomyocyte injury in this model.

Vasodilating antihypertensive agents, such as minoxidil and hydralazine, cause myocardial necrosis associated with a fall in blood pressure. Hypotension, hypoperfusion and tachycardia cause myocardial necrosis and subsequent inflammation (Van Vleet *et al*., 2002).

NSAIDs can cause interstitial nephritis and papillary necrosis in the kidney in rats and dogs. Whilst these compounds are anti-inflammatory, the mechanism of inhibition of prostaglandin synthesis via effects on cyclo-oxygenase enzyme activity is thought to cause a variety of changes. Interstitial nephritis is observed as a chronic change: the mechanism is not known, though it may be due to an inflammatory reaction driven by other eicosanoids not produced via COX pathways (i.e. leukotrienes, lipoxins). A cell-mediated specific immune response may also occur since the predominant infiltrating lymphocytes are CD8+ T cells with low numbers of B cells. In the medulla, the mechanism underlying papillary necrosis is thought to be an alteration of blood flow related to the vascular effects of changes in normal prostaglandin synthesis. Interstitial cells are the earliest cell type affected, followed by congestion, local tissue hypoperfusion, hypoxia, endothelial degeneration and necrosis (Khan and Alden, 2002). These lesions are often characterized by a rim of infiltrating inflammatory cells at the border between the hypoxic/ischaemic tissue and adjacent tissue with a functional blood supply, or as vascular supply is restored

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following degeneration/necrosis (Gopinath *et al*., 1987). This indicates that an intact vascular supply is required to transport plasma/inflammatory cells to the site of inflammation, which may be stimulated by a combination of factors including damage to ECs, the presence of cytoplasmic components from necrotic cells and the release of mediators associated with tissue hypoxia such as hypoxia-induced factor.

3.4 Inflammation as a Result of Intracellular or Extracellular Accumulation

Accumulation of xenobiotics in cells may result in cytotoxicity but can also stimulate inflammation without causing cell death.

3.4.1 Particulate Accumulation

Inert xenobiotics, such as silica, asbestos, carbon black and para-aramid fibrils, cause chronic and/or granulomatous inflammation in the lung when inhaled (Medzhitov, 2008; Levy, 1995; Nikula *et al*., 1995; Warheit, 1995). In rats, the macrophage/chronic inflammatory response to inhaled particulates is particularly marked (Levy, 1995). These materials accumulate and are associated with an increase in the numbers of pulmonary macrophages. The lack of 'self'-receptors (e.g. MHC molecules, CD47) and physical nature and size inhibit phagocytosis (Medzhitov, 2008). Xenobiotic particulates such as asbestos and silica have been shown to regulate inflammation via activation of the NALP3 inflammasome in macrophages. This is associated with granulomatous inflammation/granuloma formation (Cassel *et al*., 2008; Dostert *et al*., 2008). In addition it is also known that inhaled diesel particles and carbon black accumulate in macrophages in the rat lung. This accumulation appears to inhibit macrophage scavenging and motility and is probably associated with a similar mechanism to silica, since they both cause a similar pulmonary macrophage accumulation and chronic inflammation when administered by inhalation to rats (Nikula *et al*., 1995; Levy, 1995). Accumulation of particulates in macrophages has also been associated with additional molecular evidence of inflammatory processes. Following exposure to indium phosphide powder, oxidative stress, increased macrophage levels of iNOS, COX2 expression and oxidative DNA damage (8-OHdG formation) have all been demonstrated in rats showing alveolar granulomatous inflammation and proteinosis (Gottschling *et al*., 2001). Continuing exposure to inhaled particulate with ongoing macrophage accumulation and associated chronic granulomatous inflammation is thought to lead to pulmonary squamous metaplasia, atypical hyperplasia and neoplasia (Gottschling *et al*., 2001).

3.4.2 Phospholipid Accumulation

A different form of intracellular accumulation is caused by phospholipid accumulation, or phospholipidosis (PLD), which is commonly associated with cationic amphiphilic compounds across diverse pharmacologic targets (Kacew *et al*., 1997; Nonoyama and Fukuda, 2008). PLD is generally indicative of an adaptive response of the target cell to an inhibition in the normal pathway of lysosomal function and subsequent lysosomal accumulation of xenobiotic in multilamellar bodies. Uncommonly, PLD can lead to cytotoxicity and associated inflammation, although there is little evidence to suggest that this is a direct result of PLD, rather accumulation of the drug/metabolites (Reasor and Kacew, 1996). Examples include amiodarone and chloroquine. Despite the absence of lung inflammation in preclinical species, in the clinic, amiodarone has been associated with PLD in alveolar macrophages, ECs and other cell types. This was associated with chronic inflammation/fibrosis, causing obliteration of airways. Various mechanisms are postulated, from cytotoxicity, hypersensitivity to generation of free radicals and/or modulation of the inflammatory response, with the major metabolite desethylamiodarone being implicated (Reasor and Kacew, 1996; Malhotra *et al*., 2003).

Spinosad® (spinosyn A and spinosyn D), an insecticide, causes PLD (vacuolation on standard light microscopy) in a variety of cell types, some associated with cellular degeneration/inflammation on long-term administration (Yano *et al*., 2002). These inflammatory changes were seen in the lung and thyroid gland, the two most sensitive tissues for PLD. In the lung, alveolar histiocytosis (macrophages were vacuolated) was associated with chronic inflammation (visible macroscopically). In the thyroid, chronic inflammation was associated with vacuolated epithelial cells, necrotic cellular debris and the production of fibrous tissue (Yano *et al*., 2002). Here, the inflammation is associated with tissue necrosis. In long-term rat studies where pulmonary inflammation is associated with PLD, the presence of nuclear pyknosis and cholesterol clefts, indicative of foamy alveolar macrophage necrosis, may be observed (**Figure 2**), providing a link between inflammation and cytotoxicity. Similarly, Elmiron® (pentosan polysulfate sodium), a synthetic glycosaminoglycan, also caused multifocal alveolar chronic active inflammation associated with vacuolated macrophages and cholesterol clefts (NTP (National Toxicology Program), 2004).

Accumulation of phospholipid in bile duct epithelium in rats treated with an antirheumatism agent causes apical vacuolation at low doses, but at increased severity is associated with necrosis of bile duct epithelial cells. Epithelial cell death will cause the release of cellular components which are capable of acting as inducers of inflammation. In addition, these components need to be exposed to sensing mechanisms, which indicates

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that there is also compromise of the bile ductular BM, allowing egress of necrotic cellular debris, and probably small amounts of bile, into the ECM. Bile itself is an irritant which will also act as an inducer of inflammation if not properly compartmentalized. The precise mechanism is not clear, but bile duct epithelial necrosis is associated with an inflammatory cell infiltration (Nonoyama and Fukuda, 2008). Aminoglycoside antibiotics cause acute renal toxicity due to tubular necrosis. Mitochondrial enzyme inhibition and peroxidation of phospholipids have been associated with this effect (Mingeot-Leclercq and Tulkens, 1999). If the tubular BM remains intact, little or no inflammatory response is observed; however at its most severe, tubular necrosis can lead to an inflammatory cell infiltration.

3.4.3 Crystal Formation

Extracellular accumulation of uric acid also induces inflammation. Cytotoxic drugs, diuretics, salicylates, ethambutol and nicotinic acid are associated with arthritis. These xenobiotics cause hyperuricaemia and the deposition of uric acid crystals in joints. Uric acid/urates are known to induce inflammation. The mechanism of inducing inflammation appears to be similar to that triggered by environmental xenobiotic particulates. Macrophages detect and phagocytose crystals beyond a certain size, which triggers NALP3 inflammasome activation and IL-1 secretion (Martinon *et al*., 2006; Medzhitov, 2008). Crystal formation in renal tubules, with associated tubular dilation, and inflammation of the tubules, renal interstitium and renal pelvis, are caused by oxalate and sulfonamide administration (Gopinath *et al*., 1987). Although the mechanism is unclear, tubular dilation may compromise the normal BM integrity, and the inflammatory reaction can be marked with tissue damage and mediator release presumably caused by inflammatory cells, particularly neutrophils.

3.5 Systemic Inflammation due to the Induction of Cytokine Release

Induction of excessive systemic release of pro-inflammatory cytokines (the so-called 'cytokine storm') is an important mechanism by which xenobiotics can cause hypovolaemia and shock.

Immunostimulatory mAbs designed to stimulate the immune response are being developed as cancer therapies. However, these can also cause autoimmunity and systemic inflammation. As these mAbs are designed to be as humanized as possible in their protein sequences, they are usually immunogenic in preclinical species, in addition to their pharmacologic activity which should be maintained in relevant species used for toxicity studies. MAbs can increase co-stimulation of T lymphocytes, such as anti-CD28, which results in the activation, cytokine up-regulation and proliferation of these cells (Melero *et al*., 2007). Classically, T lymphocyte activation requires specific interactions between the TCR and antigen presented in association with MHC Class I or II, plus a host of additional co-stimulatory (or inhibitory) molecules (including CD40, CD28) which modulate the signal and response. Some anti-CD28 mAbs can stimulate T lymphocyte activation in the absence of the primary TCR/MHC-mediated signal. This so-called 'superagonist' effect was observed when such a mAb (TGN1412) was administered to normal volunteers on a clinical trial (Suntharalingam *et al*., 2006), but only low levels of cytokine release were observed in primates on toxicity studies (Dayan and Wraith, 2008). Administration of TGN1412 caused cytokine release syndrome ('cytokine storm'), with increased circulating levels of TNF-α, IL-1β, IL-12 and IL-6. It is likely that neutrophils, monocytes and plasma cells were also affected, contributing to the pathogenesis of T lymphocyte activation, culminating in multiorgan failure due to massive angioedema and disseminated intravascular coagulation (Suntharalingam *et al*., 2006; Melero *et al*., 2007; Mehrishi *et al*., 2007) as a result of increased vascular permeability and activation of the clotting/fibrinolytic plasma protein cascades.

Other mAbs, such as the anti-CD20 rituximab, have also been associated with cytokine release, possibly mediated by crosslinking of antibody to target-cell receptors followed by the engagement and activation of complement by binding to the biologically active Fc region of the antibody (Winkler *et al*., 1999).

Administration of adenoviral vectors (generally based on human adenovirus Type 5) in gene therapy can also result in the activation of the innate immune system and an acute cytokine response in a variety of species from mice, through nonhuman primates, to man. Elevation of TNF, IL-6 and IL-12 is observed in mice as an early event following intravenous injection of adenovirus, concomitant with macrophage, dendritic cell and EC activation, plus increased chemokine production (Zhang *et al*., 2001; Liu *et al*., 2003). This initial reaction is mediated, at least in part, by the presence of adenoviral capsid (Liu *et al*., 2003). A similar response occurs in nonhuman primates, following intraportal administration, causing thrombocytopaenia, disseminated intravascular coagulation, petechial haemorrhages, elevated TNF, IL-12, IL-6, and macrophage/dendritic cell activation, with sinusoidal neutrophilic aggregates observed in the liver (Schnell *et al*., 2001). This neutrophilic infiltration pays a significant role in the hepatotoxicity associated with adenovirus vectors (Muruve, 2004). Pathways involving TLRs, although important in microbial detection/inflammation, have not been specifically implicated, although common signalling pathways appear to be involved. The adenovirus capsid binds to its receptor on the cell surface, along with the β_2-integrin CD11b, which activates

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pro-inflammatory signalling, including the p38-MAP kinase and extracellular signal-regulated kinase (ERK) pathways, leading to NFκB-mediated up-regulation of pro-inflammatory cytokines, chemokines and cellular adhesion molecules. The stimulation conferred by this initial innate inflammatory response can be sufficient to induce an adaptive immune response in the absence of viral replication (Liu and Muruve, 2003; Muruve, 2004). In man, intravenous administration of adenoviral vector has also been associated with a systemic inflammatory response, elevation of serum IL-6 and IL-10, with disseminated intravascular coagulation and fatal multiple organ failure (Raper *et al*., 2003).

The initiation of adaptive immune responses is controlled by innate immune (pro-inflammatory) signals. Adjuvants are included in vaccines specifically to stimulate the adaptive immune system to respond to the relevant antigen and generate a protective, specific immune response. Immunostimulatory adjuvants, like Freund's, containing microbial components (such as LPS, unmethylated CpG DNA), act via the stimulation of TLRs to initiate a pro-inflammatory cytokine response which in turn enhances interactions between antigen-presenting cells and T lymphocytes (Medzhitov, 2008). Granulomatous hepatitis has also been reported in man following treatment with bacillus Calmette-Guérin (BCG) as an immune modulator (Lamm *et al*., 1986). Aluminium adjuvants, commonly referred to as 'alum', are particulates which stimulate immune responses via the induction of pro-inflammatory cytokines. These particulates aggregate, are readily phagocytosed by macrophages and have been shown to stimulate IL-1b and IL-18 production *in vitro*. Similar to silica and asbestos, alum adjuvants activate the NALP3 inflammasome, resulting in the release of these cytokines (Eisenbarth *et al*., 2008). Bacterial elements of DNA plasmids contain unmethylated CpG DNA and can therefore also exert a pro-inflammatory effect. Probably acting via TLRs, these can stimulate release of TNF, IL-2, IL-6 and IFN-γ (macrophage activating factor) from effector leukocytes, which may contribute to the pathogenesis of glomerulonephritis in mice, caused by the production of IgG antibodies to bacterial DNA (Dempster and Haworth, 2008).

3.6 Inflammation due to Adaptive Immune Responses

The morphological hallmark of autoimmune disease and allergy is inflammation. However, there are no specific morphological features which facilitate distinction between chronic inflammation as a consequence of specific antigen-mediated responses and other types of chronic inflammation. Vasculitis or dermoepidermal inflammation may be suggestive of a hypersensitivity response, and immunohistochemistry is important in defining the lymphocyte subsets present.

3.6.1 Hypersensitivity Responses

Many different xenobiotics can cause hypersensitivity responses, and notable examples include aspirin, cephalosporins, penicillins, sulfamethoxazole, halothane, lamotrigine and carbamazepine (Park *et al*., 1998). Hypersensitivity responses are classically categorized into four different types. These types are not mutually exclusive and a particular xenobiotic can cause multiple types of reaction. For example, penicillin causes reactions in all four categories (Naisbitt *et al*., 2000). The underlying prerequisite is that there is an adaptive immune response towards the xenobiotic in question and that antidrug antibodies (ADAs) and/or T lymphocytes specific to cells expressing drug-associated antigens are produced. Specific adaptive immune responses will give rise to immunologically based inflammatory reactions upon (re-) exposure to the drug (or its haptenated metabolites).

- Type I hypersensitivity: if ADAs in the form of IgE are present on the surface of mast cells, then rapid mast cell activation and degranulation is stimulated by antigen binding. This results in the release of the plethora of vasoactive/pro-inflammatory mediators including vasoactive amine, eicosanoids, PAF, cytokines, chemokines. These immediate responses cause allergic (anaphylactic) reactions and can result in hypotensive shock if the vasodilation/plasma transudation is sufficiently severe/widespread.
- Type II hypersensitivity: if ADAs target antigen expressed on the cell surface, then cytotoxicity of the target cells may occur via the recruitment of NK cells and phagocytic cells (e.g. macrophages) or complement fixation and assembly of a membrane attack complex (C5-9). These interactions are mediated via Fc receptors.
- Type III hypersensitivity is associated with the formation of soluble antigen/antibody complexes which are deposited throughout tissues, notably the walls of small blood vessels. Again, Fc receptors are targeted by complement and phagocytes which can exacerbate inflammation (vasculitis/arteritis) by the production of ROS.
- Type IV (cell-mediated) hypersensitivity reactions take longer to evolve, requiring antigen presentation in association with MHC. This stimulates a T lymphocyte (Th1) response recruiting macrophages (and the release of pro-inflammatory Th1 cytokines) and mediating specific cytotoxicity via TCR-mediated recognition of cells expressing antigen complexed with MHC Class I (CD8+ cytotoxic T cells). These reactions often affect the skin (Naisbitt *et al*., 2000).

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3.6.2 Generation of Xenobiotic-Specific Antigens

Metabolism of the xenobiotic is usually a key step in antigen formation for small molecules, as the generation of reactive intermediates is required for protein binding. Macromolecules may be antigenic in themselves, but small molecules (usually <1 kDa) are not immunogenic *per se* and require to be linked to larger (cell-derived) ones in order for immune recognition to occur (Park *et al*., 1987). For example, benzylpenicillin is metabolized to penicillenic acid, which is then conjugated to the anime group of proteins, which then becomes antigenic either directly, or when a xenobiotic-conjugated peptide is presented in conjunction with MHC to T lymphocytes (Naisbitt *et al*., 2000). Different patterns of inflammation can reflect target cells where this metabolism/conjugation occur and may be initiated/enhanced by the presence of so-called danger signals. These are co-stimulatory signals, such as pro-inflammatory cytokines, which increase both antigen presentation and T lymphocyte activation (Matzinger, 1994). Halothane can cause liver necrosis and inflammation associated with the presence of ADAs and autoantibodies in patients (Uzmalinoglu *et al*., 1970; Kenna *et al*., 1984; Homberg *et al*., 1985). Studies in the guinea pig provided evidence of protein adducts presented to T lymphocytes by Kupffer cells (Furst and Gandolfi, 1997).

3.6.3 Hypersensitivity Reactions in Specific Tissues

Immune-mediated inflammation in the skin is also associated with some of the xenobiotics listed above. The skin reactions are usually accompanied by systemic signs of inflammation/immune activation such as fever and lymphadenopathy. There is a dermal and epidermal infiltration of T lymphocytes: both helper (CD4+) and cytotoxic (CD8+) phenotypes have been demonstrated for sulfonamides, phenytoin and carbamazepine. Cytotoxic T lymphocytes lyse target cells via pore-forming perforins (Mauri-Hellweg *et al*., 1995; Naisbitt *et al*., 2000). Benzo[a]pyrene (BAP) is a polycyclic aromatic hydrocarbon (PAH) which is metabolized to a reactive diol epoxide. This metabolite binds cellular macromolecules including protein and DNA (Klein-Szanto and Conti, 2002). Binding to the latter is associated with genotoxicity characteristic of this class of xenobiotics, but contact hypersensitivity also associated with PAHs may reflect binding to the former. PAHs can up-regulate various cytokines/chemokines including IL-1, IL-2 and TNF. BAP has been demonstrated to increase expression of the chemokine CCL1 (N'Diaye *et al*., 2006). These may provide important co-stimulatory signals to promote allergic contact hypersensitivity seen in man.

Hypersensitivity vasculitis occurs as a result of immune activation and inflammation. Lesions are characterized by a predominantly lymphohistiocytic infiltrate in the absence of medial/fibrinoid necrosis and thrombus formation (in contrast to acute xenobiotic -induced vascular necrosis/injury discussed earlier). Immune-mediated vascular injury, similar to other hypersensitivity responses, shows no dose-relationship and has been associated with antimicrobials (penicillin, ampicillin, sulfonamides, tetracyclines) and other xenobiotics, including propylthiouracil, phenylbutazone (Van Vleet *et al*., 2002; Naisbitt *et al*., 2000). Hypersensitivity reactions may also be associated (e.g. for penicillin) with immune-mediated arthritis (Woodard *et al*., 2002).

Some of the mAbs designed to interfere with co-inhibitory signals similar to the CD28 signal, such as cytotoxic T lymphocyte-associated antigen-4 (anti-CTLA-4), have also caused transient, reversible autoimmune effects. Clinically these manifest most frequently as skin itchy (pruritic) eczema/rashes and inflammatory bowel syndromes with moderate to severe diarrhoea, although inflammation of the pituitary gland and uveal tract of the eye have also been described (Blansfield *et al*., 2005). No autoantigens have yet been identified, and it may be that the inflammatory bowel syndromes could be due to inappropriate immune responses to commensal flora (Melero *et al*., 2007).

Adenoviral vectors, in addition to the initial inflammatory/innate response stimulated by capsid-mediated pro-inflammatory cytokine release, induce a delayed, specific immune-mediated inflammation two to seven days later in mice (Liu *et al*., 2003). This phase requires viral gene transcription in infected cells, and is associated with adenovirus-specific cytotoxic T lymphocyte infiltrates in the liver and a second peak of cytokine mRNA (messenger RNA) expression (e.g. TNF). Chronic inflammation is thus induced as a consequence of a specific, cell-mediated immune response to the viral vector.

3.7 Conclusions

Xenobiotics can induce inflammation by a variety of different mechanisms, many of which result in cell injury, degeneration and/or necrosis, either directly or indirectly, by parent or metabolite. This cytotoxicity may be related to pharmacology or to the generation of toxic intermediates in the affected cells. Xenobiotics may also activate inflammatory/immune cells to release inflammatory mediators in nonspecific ways or by stimulating a specific immune response. The pattern of morphologic and functional changes induced by these mechanisms differs according to the target cell population affected and persistence of inducing stimuli. Inflammation itself can also cause tissue damage, resulting in an exacerbation of the initial insult, and can enhance adaptive immune responses. Much is known about the molecular events involved in inflammation itself and has been

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shown to apply to inflammation induced by xenobiotics. It is important to note that superantigen-mediated cytokine release, or immune-mediated mechanisms of inflammation may be poorly predicted by preclinical toxicity studies. A detailed knowledge of the pathogenesis and molecular mechanisms involved in inflammation can facilitate the design of *in vitro* or *in vivo* studies to predict or investigate these responses associated with xenobiotic administration.

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Medicolegal and Ethical Issues in the Practice of Toxicology: A British Perspective

Denis A. D'Auria

CONTENTS

1 INTRODUCTION

A legal system provides a country with a means of resolving disputes, where the parties may be individuals, companies, public bodies or even the Government itself. In achieving this end, the Law also relies on science to provide advice to the courts on issues in contention at any time. The courts are unlikely to decide disputes involving scientific and medical issues unless they receive appropriate advice from a suitably authoritative source.

Toxicological advice might be critical to the outcome of a case concerning a work-related accident involving an acute release of a chemical or indeed chronic release. In product liability claims, a toxicologist will advise parties about issues that might relate to causation. Toxicological advice may assist in resolving labour disputes concerned with working conditions related to toxic substances in use in the workplace.

It is said that law brings order to everyday life and affects the very fabric of our society. It is inevitable, that toxicologists and the practice of toxicology will be heavily influenced by law and this chapter will explore some of those areas of interaction, using the law in England and Wales as a basis for the discussion. The law in Scotland is different. However, since most UK law especially in the fields of public health, occupational health and safety and toxic substances control is European in origin, it will include a brief review of relevant European Union-wide law and where appropriate, mention will be made of key principles drawn from relevant other jurisdictions. This review is not exhaustive, but it begins with a brief overview of the legal system in England and Wales.

2 SOURCES OF LAW IN ENGLAND AND WALES

To provide something of a background, this section will briefly review the legal system and its operation.

2.1 The Common Law and Precedent

The United Kingdom is a parliamentary democracy. The principal legislative power lies with parliament, which is responsible for the provision of law through statute. However, not all statutes are applicable throughout the United Kingdom and may be limited to one or more jurisdictions, for example, England, Wales, Scotland and Northern Ireland.

The law in England and Wales has never been codified. To deal with this situation, the traditional approach has been that the courts seek to identify similar cases that have occurred in the past and argue whether or not these can be distinguished from the current case before the court. The doctrine of precedent, as it is known, enables the judiciary to make legal rules without reference to parliament. This form of law is known as common law and is based on historical practices of the judges, journeying between towns and cities to deal with cases in each place.

The outcome was based on what decisions were made in similar cases elsewhere in the jurisdiction, as well as who rendered the judgement in what court. Thus, precedent value was on similar circumstances being judged in the same level or higher court. This apparent complexity confers the advantage of significant flexibility, which is of immense value in areas of contract and civil liability.

Criminal law is about keeping order in society, as opposed to resolving disputes between parties, although others argue that the disputants are the criminals on the one hand and society on the other. The resolution of such disputes becomes so important for the good of society that the prosecutions are pursued by the state in the name of the people. As one might expect, criminal law is generally enacted in statute.

Within the legal system therefore, judges serve two quite different roles. They declare and develop the common law by analysing the legal arguments put before them. To do this, they are entitled to resolve both questions of law and questions of fact. They also interpret the meaning of acts of parliament and regulations, though to a much lesser extent than one might have originally believed.

2.2 Binding Precedent

Much legal analysis is based on distinguishing the present case from those similar cases that have been heard previously. As indicated above, this process is far from

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simple and indeed it can only work if one is bound by former decisions. This is the nature of so-called binding precedent. For example, the opinions of those judges sitting at the top of the hierarchy of the courts count for more than those in the lower courts. The highest court in the United Kingdom is the Judicial Committee of the House of Lords. The decision of this court binds all lower courts. Decisions of the next echelon, the Court of Appeal binds all lower courts, but not the House of Lords, which is of course, superior to it. Ultimately, every judge in a parliamentary democracy must yield to elected representatives, who can overturn any judgement by simple statute.

2.3 Primary and Delegated Legislation

Current legislative practice is to establish broad principles in a parent act giving power to a minister to make regulations. Once drafted and finalized, these are laid before parliament and are only debated if objections are raised.

Both primary and delegated legislation carries binding legal obligations and sanctions. If this continues for decades, the law can become overly complex and impenetrably obscure. For these reasons, regulations, which are made under powers in a parent act, are reviewed and updated periodically. An example of this is the Control of Substances Hazardous to Health Regulations first brought into force in 1984 and updated at various times, most recently in 2002.

2.4 The Approved Code of Practice

A newer device relevant to making regulations an effective tool for enforcement is the Approved Code of Practice. These are drafted by the appropriate department of government and are attached to acts of parliament or enacted regulations. They direct courts and tribunals to refer to them in attempting to interpret relevant acts and regulations. They are particularly used in the occupational health and safety field. When consulting these publications, which are widely available, care must be taken to ensure that the document being studied is indeed an Approved Code of Practice, which is normally clearly stated on the front cover or in the introduction. They are not themselves law, being described as quasi-legislation, but they are effective in providing advice in readily understandable terms. Failure to follow the advice contained therein is regarded as prima facie evidence of a breach of law. However, it may be open to a defendant to prove that the countermeasures adopted, had the same effect as those recommended in the Approved Code of Practice.

2.5 Civil and Criminal Law

As mentioned above, criminal law provides for sanctions against those who offend against society.

Criminal prosecutions are brought by public officials such as the Crown Prosecutions Service or Health and Safety Executive inspectors. In Scotland, it is the procurator fiscal.

Unless qualified for legal aid, the defendant bears the costs of the defence. Penalties awarded to those found guilty range from fines at various levels, through community service, to imprisonment. Both companies and individuals can be subject to criminal sanction. Money paid in fines, is, for the sake of clarity, retained in the Court Service and is not paid out in compensation.

The principal function of civil law is to compensate the victim of a noncriminal wrongdoing. The claimant (formerly known as the plaintiff; or the pursuer in Scotland) is the individual who is harmed by the unlawful act. They sue for compensation for what they have suffered in the civil courts or occasionally in the tribunals.

The claimant may also seek a court order similar to an injunction (or an interdict in Scotland). This will be addressed to the defendant and direct them to return the situation to legality. Breach of any such order will be a contempt of court and will be punished according.

2.6 Sources of Law in the European Union

The United Kingdom is now a member of the European Union and as such has ceded much of its legislative power in certain fields to it. The power to make new law is given to the Council of Ministers and the European Commission. One minister from each member state attends Council meetings. Depending on the items set out in the agenda, this may be the Foreign Minister, Agriculture Minister and so on. The administrative arm of the Union is the Commission, located in Brussels. The judicial power is conferred on the Court of Justice of the European Community, based in Luxembourg. Procedures in this Court are very different from those in common-law jurisdictions. There is reliance on written representations and limited oral examination. The Court bases its procedures on French administrative law (*le droit administratif*). A key official of the Court is the Advocate General who provides advisory opinions to the judges.

Each member state sends one judge to the Court. Its principal task is to interpret the treaties and any secondary

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legislation made with its authority. European Court of Justice rulings must be recognized and enforced by the courts of member states, but there is no appeal from its decisions.

2.7 Legislation in the European Union

Secondary legislation takes the form of regulations, directives and decisions. Regulations are mandatory. They have the force of law throughout the community, without the need to be ratified by the legislatures of each member state. Directives are binding as to the results to be achieved, but leave the choice of method of achieving the objectives to the member states. They, therefore, require domestic implementing legislation. The Consumer Protection Act 1987 was the main statute that implemented the principles set out in the Product Liability Directive.

These directives are frequently delayed in their implementation by member states. It is therefore not unusual for the Commission to bring the erring member state before the European Court of Justice for failure to implement a directive.

In Marshall vs. Southampton Health Authority (1986), the Court of Justice held that an individual employed by a government could sue that government under the provisions of an unimplemented directive as though it had been implemented, a privilege not available to nongovernment employees. In other words, the directive is directly enforceable in emanations of the state without 'domesticization' by implementing legislation.

The Court of Justice further developed the law in Francovitch vs. Italy (1992) when it was held that an individual may, in some circumstances, sue a government for damages for failure to enact a directive within the specified period.

Decisions are rulings, given by the Commission, in individual cases and may be addressed to a state, an organization or an individual. However, they are binding only upon the state, organization or individual to whom they are addressed.

Recommendations are persuasive, but not legally binding. The process of harmonization amongst the European Union states encompassed occupational health schemes. A case before the European Court of Justice (Grimaldi vs. Fonds des Maladies Professionelles, 1990) highlighted the plight of expatriate workers who developed conditions compensatable under the law of their homeland, but not in the country in which they are working. The Commission had made recommendations about compensation and called upon member states to introduce legislation to provide compensation to those affected by conditions with a proven causal link to work. However, the European Court of Justice held that the recommendation cannot confer rights on individuals, but must be taken into account by national courts when interpreting domestic legislation. A further recommendation included a list for governments to consider and called for immediate legislation to deal with scientifically recognized, occupationally related conditions. Almost 50% of that list was chemicals, and specifically metals, including cadmium, chromium, mercury, manganese and nickel.

The European law, therefore, affects issues of public health and safety, as well as toxic-substance control. Nowhere is this more obvious than in the new regulations that came into force, the so-called, REACH Regulations, which will be discussed next.

3 THE REACH REGULATIONS: A EUROPEAN UNION APPROACH

The so-called REACH Regulations are set to have a major impact on the European Communities chemical manufacturers, suppliers and users, not only in the European Union, but far beyond its borders. The acronym stands for 'Registration, Evaluation and Authorisation Regulations'. It is probably the European Union's most comprehensive and complex piece of legislation yet produced.

3.1 Background

Chemical-control laws have existed in Europe for many decades. One of the early directives applied to classification, packaging and labelling, dating from Council Directive 67/548/EEC (1967). In addition, there are over 40 additional Community-level laws which also regulate chemical safety. A principal advantage of the REACH Regulations is that it simplifies the complexity and confusion surrounding the prior legal framework for chemical-substances control and incorporates it into a single piece of legislation, dealing, at the same time, with the difficulties with implementation and enforcement. Cynics amongst us would wish to add a qualifier, 'allegedly'. The regulations run to over 800 pages and there is currently a United Kingdom government-sponsored consultation over enforcement, which is likely to involve a minimum of three separate agencies, an enforcement environment where multiple agency jurisdiction is not popular. Obligations involving information and registration were not introduced until 1981, but these requirements were only applied to substances brought to the market since then. The difference is not small, since an estimate of over 100 000 substances in significant production was left largely unreviewed. In the absence of testing and a review of usage, it is impossible to determine whether or not these chemicals are indeed being handled responsibly. Manufacturers may have sketchy knowledge of environmental and human

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safety. Material safety data sheets may overstate the case. Frequently, downstream users receive inadequate information about the substances and their associated risks. Indeed, they may not be equipped to recognize the significance of those risks. This lack of information often results in environmental and health-protection problems. It is a widely held belief that chemicals cause a considerable measure of ill health. A lack of information makes it difficult to deal with the focus of public-health concerns. Many chemical entities and others of a more complex nature have been the focus of controversy, including bisphenol A, phthalates, brominated flame retardants and perfluorooctanoic acid.

The previous regulatory system was not conducive to innovation because of the different rules that apply to existing and new substances. REACH Regulations eliminates this discrepancy and establishes a single, and indeed, unique, legal framework for all chemicals in use in the European Community territory.

3.2 The REACH Regulations: An Overview

These regulations are to be distinguished from the normal sets of regulations in the British legislative environment. These are of EU origin. REACH Regulations has been described as one of the European Union's largest legislative projects. Without a doubt extensive, it is also complex and difficult to understand. However, that difficulty is primarily a function of its length.

The new regulations provide a unified framework for the control of toxic substances on the European market. The main purpose of the REACH Regulations is to improve environmental and health protection standards. Whilst these are most laudable objectives, one of industry's principal concerns is the high cost of compliance.

The basic principles of the REACH Regulations are set out below.

3.2.1 Registration

The centrepiece of the regulations is the requirement for registration. REACH Regulations will provide systematic information about relevant chemicals. The regulations define a substance broadly; it includes chemical elements and raw compounds in either natural or manufactured forms, including process-related impurities.

A manufacturer or importer of a substance is required to submit a registration dossier to the new European Chemical Agency based in Helsinki in Finland, if at least 1 tonne (the equivalent of 1000 kg) is manufactured or imported.

There are, however, certain exemptions to the registration procedure. For example, pharmaceutical preparations for human or veterinary use, or substances added to foodstuffs or feeds, as these are already regulated at the Community level to protect environmental and human health in equivalent ways.

The annexes to the REACH Regulations contain two lists of chemicals that are exempted from registration requirements. Annexe IV contains substances about which available information is sufficient to indicate that they pose only a minimal risk. This list includes such substances as carbon, nitrogen, water and sunflower oil. The list in Annexe V contains substances for which registration is considered irrelevant or unnecessary and includes minerals, ores and noble gases.

Manufacturers and importers must include in their information dossiers data about the chemical identity of the substance in question, its physicochemical, toxic and ecotoxicological properties, its classification and labelling, and the ways in which it is used. For chemicals with an annual production of 10 tonnes, a chemical safety report must be provided.

Not surprisingly, the data to be provided in the dossier is determined by the annual production or importation levels. The largest group is produced or imported in quantities of 1–10 tonnes per annum. There are four categories set out in the REACH Regulations:

- 1–10 tonnes per annum;
- 10–100 tonnes per annum;
- 100–1000 tonnes per annum;
- Excess of 1000 tonnes per annum.

The greater the quantities in question, the more stringent are the information requirements for the dossier. Only data on the substance's physicochemical properties, and on a few acute impacts on the environment and human health must be submitted for production or import of 1–10 tonnes per annum. Extensive testing is required if levels in excess of 1000 tonnes are produced or imported.

The aim of registration is to offer manufacturers the opportunity of proving that their product is safe in use. Testing requirements follow logically and are not inflexible protocols tiered rather than tailored to risk. Certain tests can be waived where appropriate to the circumstances. If, for example, there is no relevant exposure for expected employees or consumers, chronic toxicity testing may be waived.

Registration imposes obligations on manufacturers and importers, since they are the parties responsible for the safety of their chemicals. Importers and manufacturers therefore must seek to prove that their products are indeed what they claim to be, that is, safe to use. A second objective is that the results of testing may ultimately lead to the adoption of available control measures for safe use of the substances along the supply chain.

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There is a suggestion prima facie, that this is a reversal of the burden of proof. As legalistic as this might sound, this is not so. It is illustrative of a basic principle. Under the old system, authorities of the member states determined the risks associated with any substance, with a view to justifying any protective measures which were to be prescribed. This process had to be of limited effectiveness, given the vast numbers of chemicals involved in modern business, limited competent staff available and resources of the authorities concerned.

Implementation of REACH Regulations will lead eventually to the development of a broad information base, which will be of great value to all stakeholders. New findings will be disseminated to stakeholders at relevant times. Manufacturers may be encouraged to improve their products, and users, their handling procedures.

There may also be a legal impact outside the general area of toxic substances control. For example, water- and soil-quality regulations are based on toxicological hazard criteria. Data in REACH Regulations will facilitate actions relative to such related areas. The position is, to some extent, analogous to the relationship between the regulations for consumer protection and occupational health and safety.

REACH Regulations introduces obligations for the transfer of information about toxic substances, properties and risk management measures throughout the supply chain. Under these requirements suppliers of a substance or a preparation must provide downstream customers with a relevant data sheet if the substance or preparation is classified as:

- Dangerous
- Persistent, bioaccumulative and toxic (PBT)
- Very persistent and very bioaccumulative (vPvB).

As under previous requirements, safety data sheets need to include information about potential hazards, first-aid measures to be taken in response to exposure cases, measures to be taken in case of fires, requirements pertaining to handling, storage, disposal and transport, physicochemical and toxicological properties. REACH Regulations therefore differs remarkably from the requirements under Section 5 of the UK Health and Safety at Work Act, and so on, since many of these dossiers will be subject to professional scrutiny and published information will hopefully be more likely to be based on firm evidence. In addition, the information requirements have a slightly different emphasis. Hopefully, the effect will be to have less 'over-anxious' reporting of hazards and precautions.

Under REACH Regulations, safety data sheets must incorporate exposure scenarios, based on the uses identified by the party who was subject to their registration obligations. These exposure scenarios are to include information about what types of exposure are to be expected by downstream users and about relevant risk-management procedures to be adopted in each case.

A party subject to registration obligations needs access to information about experience gained with use of the respective substance or substances. For this reason, REACH Regulations introduces requirements whereby downstream users in the supply chain must provide information to the manufacturers and others located upstream. In cases where the downstream user does not wish to inform a manufacturer, then they are not obliged to do so. One reason may be, for example, loss of competitive advantage. Whilst this might appear a gap in the flow of information, the use information must be provided to the European Chemicals Agency.

3.2.2 Evaluation

The European Chemicals Agency is the new Community institution responsible for the technical, scientific and administrative aspects of REACH Regulations. The Agency will be responsible for evaluation, together with the relevant national authorities. These procedures for evaluation are particularly worthy of note.

Two procedures can be distinguished, known as dossier evaluation and substance evaluation. The dossier procedure involves the Agency checking whether or not a registration dossier conforms to all applicable requirements, for example, whether or not it contains all necessary tests, or whether the data supplied are of adequate quality. Substances that provide reasons for concern are assessed thoroughly by the competent authority in the member state from which the registration originated. Such evaluations can result in the issue of substance prohibitions or restrictions required by the European Chemicals Agency.

3.2.3 Authorization

REACH Regulations provides for authorization procedures for substances of special concern. This group includes those classified as PBT and vPvB, as well as those with carcinogenic, mutagenic and reprotoxic properties. How precisely these will be applied in practice remains to be seen and in particular, whether or not one returns to a mechanism similar to the Delaney clause. Additional substances can be subjected to this authorization procedure if initial assessments indicate that serious impacts on human health or the environment are likely. The regulations indicate that this is particularly likely in the event of a substance being found to have endocrine-disruptive properties.

Workloads arising from this procedure, no one is in any doubt, will be significant. A practical management device, which will be applied, will limit the workload by use management. Thus, only uses of those substances that are listed in the annex to the regulations will be subject to authorization procedures. At a time to be decided,

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nonauthorized uses of chemicals on that list will be prohibited. The structure of the authorization procedure has given cause for considerable discussion. As a result authorizations will not be limited in time, but will be regularly reviewed to determine whether conditions for authorization are still being fulfilled. Thus, the door is ajar for the application of future research.

Within the REACH Regulations framework, the issue of substitution has, of course, become significant. Every application for authorization must include both an analysis of possible substitute substances or procedures, as well as an analysis of its technical and economic feasibility. Decisions on authorizations, similarly, will take into account the possibilities for substitution. This, of course, will be of relevance only in situations where risks are not being adequately controlled.

The concept of adequate control applies only to substances for which there is a 'derived no-effect level' (DNEL) or a 'predicted no-effect concentration (PNEC) value' cannot be determined from Annex I of REACH Regulations. Substitution obligations will not be imposed, therefore, in all cases. The authorization procedure itself will also provide incentives for substitution. After all, authorization procedures create costs and uncertainties for business. The market's response to a chemical which fails authorization, or perhaps incurs heavy restriction, might well illicit considerable negative publicity for a supplier. In contrast, alternatives which do not require authorization may develop considerable market benefits. There seems, therefore, a considerable business advantage to investment in REACH Regulations obligations.

Prohibitions or restrictions on substances and their usage are permitted under REACH Regulations. These measures certainly existed under earlier chemical control regimes. The procedures under REACH Regulations, however, are considerably simplified. Restrictions are intended to act as a safety net and their implementation is far more rapid than has been the case in the past.

3.3 The Impact of REACH Regulations

It is likely that REACH Regulations will bring advantages for innovation. Substance information collected within the registration procedure will be deposited in the European Chemical Agency's database, but unlike the Community's former ECDIN (Environmental Chemicals Data and Information Network) project, this will be available on the internet and to the public, with the exception of confidential information placed with the Agency during the process. Published information will include commercial designation, physicochemical properties, results of toxicological and ecotoxicological studies, and relevant DNEL and PNEC levels. Composition of commercial preparations, and information about relationships between manufacturers or importers and downstream users will not be included on grounds of confidentiality and commercial sensitivity.

A legislative initiative, such as this, will inevitably raise many concerns. The impact of REACH Regulations that is of most concern is the financial burden on industry. Although there may be business incentives for investing in obligations under REACH Regulations, some studies have also considered the benefits from environmental and human-health protection. Some Community-sponsored studies indicate potential savings within the healthcare sector of 50 billion euros in the next 30 years (Council Directive 67/548/EEC, 1967; European Commission, 2003). Such savings result from decreased rates of skin disorders, allergies and cancer. Environmental benefits were predicted as significant, but were not quantified in these studies.

In the past, special regulation was applied whenever it was deemed necessary. Historically, priorities were determined rather more on the detection of chemicals in new media or biological samples. The right priorities, it must be admitted, were not always made. The availability of key information at an early stage in the life cycle of the chemical is not believed to provide more explicit and valid bases for future regulatory action.

Decision-making in this area has always depended on advances in chemical analysis. Organochlorine compounds could not easily be detected until electron capture became available. The availability of analytical methodology and relevant information will both be regarded as significant contributors to accurate and reliable regulatory decision-making in the future. This must be potentially huge, given advances in microelectronics and improvements in the sensitivity of analytical methods.

Perhaps, the most meaningful impact of REACH Regulations will be its ability to introduce what is essentially a preventive regime to toxic-substance control. No longer will it be necessary for a substance to cause a problem before it is subject to regulation. REACH Regulations therefore, employs the combined benefits of anticipatory regulation and effective monitoring.

The 15 year ongoing testing process will have a significant impact in the science sector, specifically, toxicology and ecotoxicology, as well as industrial hygiene. Impact and exposure data worth in excess of an estimated 10 billion euros will become available. The insights gained from these effect-monitoring exercises will lead potentially to increased understanding of underlying pathogenic mechanisms and structure–activity relationships.

During implementation of REACH Regulations, there will be an increased demand for animal testing, but also it is hoped, an enhanced understanding of alternatives to animal experimentation. It is likely too, that an impetus will be given to computer simulation, QSAR (quantitative structure–activity relationship)-driven assessments

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and *in vitro* methods as the only techniques available to complete the necessary workload. The forthcoming databases may contribute to the validation of alternative methodologies. All such methods, however, will have clear limitations and should only be used within an appropriately defined context to achieve results consistent with the underlying philosophy of REACH Regulations. It is hoped that appropriate research will be stimulated to underpin the REACH Regulations concept and its future applications.

Industry will respond by promoting the development of safe chemicals and their application. REACH Regulations will provide the data for the emergence of a chemical market with minimal adverse impact on human health and environment. The availability of REACH Regulations data may provide a significant competitive advantage. Surprisingly, REACH Regulations is becoming viewed almost as a 'brand' for a safe chemical, as contributing to a competitive advantage for European manufacturers.

REACH Regulations simplifies the framework for moving research chemicals to actual applications and eventually to market. As it requires a systematic approach, this perhaps forms its greatest benefit. In keeping with its regulatory intent, existing substances will be subject to a broad range of prohibitions, restrictions and conditional authorizations, resulting in pressure to move towards substitution whenever practical.

The coupling of globalization and rapid information exchange reshapes the regulatory environments in many of the EU states and is likely to be continued as the implementation of REACH Regulations gathers pace. Moreover, the publicly available information, the wealth of new assessments and the exposure evaluations required will have an impact across the EU. It is, perhaps, for this reason that regulations, rather than a directive, have been chosen as the most appropriate legislative instrument.

REACH Regulations is likely to have a far-reaching effect on legal systems through its impact on liability. Assessment of liability may vary throughout different legal systems. However, issues of predictability of chemical damage remain key to these considerations. Such information will be readily available. When these requirements are violated, a defence against employee or consumer legal action becomes less plausible. This may affect producers and users both within and external to the EU and will be especially true for REACH Regulations substances under prohibition.

The European Chemical Agency will check dossiers for completeness and quality. When grounds for concern exist, the Agency can require intensive testing and can impose additional requirements. Through this process, contaminants may emerge which may help clarify decision-making going forward. The intervention of professionally qualified staff becomes important to the philosophy of REACH Regulations.

An authorization procedure is expected to apply to a substance producing a high level of risk. The list contained in Annex XIV will contain about 1000 substances and will provide candidates for environmental analysis. The outcome of this process will further refine the list.

3.4 Enforcement

It is said that there is no law without enforcement, and truly REACH Regulations cannot fulfil its declared intent without enforcement. REACH Regulations can only be enforced by competent national authorities. Each authority has a number of responsibilities under the regulations:

- Provision of advice to stakeholders through a 'help desk' facility
- Enforcement of compliance within their jurisdiction, at least reacting to 'whistle-blowing'
- Conduct of in-depth evaluation of selected prioritized substances and preparation of draft decisions
- Propose harmonized classification and labelling
- Identification of substances of very high concern for authorization
- Proposal of restrictions
- Nomination of candidates for membership of the European Chemical Agency's, committees on risk assessment and socioeconomic analysis
- Appointment of members to the forum for information exchange and meetings to monitor enforcement matters
- Liaison with enforcing organization in relation to downstream responsibilities.

Currently, the Department of the Environment, Food and Rural Affairs (DEFRA) is the lead government department on REACH Regulations for all policy matters, including enforcement, acting with the Department of Business Enterprise and Regulatory Reform (BERR), the Environment Agency, the Health and Safety Executive and the devolved administrations of Wales, Scotland and Northern Ireland. A second consultation paper, setting out the proposed process for enforcement and enforcing authorities, is currently under way. Briefly, it is proposed that elements concerning registration will be enforced by the Health and Safety Executive as competent authority. Supply-chain-related matters are likely to be enforced by the Health and Safety Executive and the Health and Safety Executive of Northern Ireland up until the point of retail sale. Thereafter, enforcement will be carried out by trading standards and consumer protection agencies. Use-related duties will be enforced by a range of enforcing authorities, using the existing regime, and enforcing authorities for health, safety and environment

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legislation. The consultation will close in August 2008 and a report is expected by December 2008. It is likely that the report will set out powers, as well as specify the REACH Regulations offences and penalties for noncompliance.

The REACH Regulations will have significant impact on science, industry and commerce from the start of the phasing-in period. However, the adverse impacts perceived by industry are being pragmatically addressed through the phased implementation. Moreover, it has the promise of reduced environmental damage and improved human- health protection. Those promises will not be achieved without an effective and efficacious enforcement system, in the absence of which this immensely complex piece of legislation will fall into just another piece of Brussels bureaucracy.

4 THE TOXICOLOGIST AND EXPERT EVIDENCE

The evidence of experts may be crucial to the resolution of a dispute that is the subject of the case being heard by the court. Lay witnesses may only testify as to the facts that they have observed. As experts have special knowledge, they are permitted to give opinion evidence. A lay witness cannot say that a man was drunk, only that he was observed walking unsteadily and smelling of alcohol. A toxicologist is equally not permitted to say that a man was drunk, but he can say that he was able to estimate a blood alcohol of 140 mg per 100 ml in his laboratory and this was consistent with unsteady gait. Thus, expert witnesses are allowed, and even expected, to express an informed opinion on the matters referred to them. The toxicologist could, therefore, be justified in informing the court that the behaviour was consistent with the measured blood alcohol concentration.

4.1 Historical Background

The legitimacy of experts giving opinion evidence was settled once and for all in Folkes vs. Chadd (1782) where the defendants argued that the plaintiff's expert, one Thomas Smeaton, an engineer, gave an opinion, whereas the jury had to base their verdict on fact. The trial judge, Lord Mansfield, thought differently:

> *Mr Smeaton's … opinion is deduced from the facts, which are not disputed—the situation of banks, the course of tides and of winds and the shifting of sands … [he] understands the construction of harbours, the causes of their destruction and how remedied. In matters of science, no other witnesses can be called. … Of this, such men as Mr Smeaton alone can judge. Therefore, we are of the opinion that his judgement, formed on the facts, was very proper evidence.*

Lord Mansfield's decision achieved two things. First, he acknowledged the usefulness of expert witnesses and their evidence. Second, he accorded them a special place in legal proceedings, whilst at the same time, removing from them all vestiges of the role they had in deciding technical matters. Their place was in the witness box and not alongside the judge.

The increasing technical nature of civil disputes has led to an increase in the use of expert evidence. Increasingly, experts are called upon to act as advisors to barristers in drafting pleadings, in assessing the merits or otherwise of the case, and in directing cross-examination. In this section, the role of the expert in UK law will be reviewed. Toxicologists may be involved in many areas of law, but notably personal injury arising out of chemicals, occupational health and safety, product liability, employment law and a lot more besides. They may well be involved in criminal matters, including drink driving, traffic law, drug enforcement, criminal-liability issues, rape and murder, as well as criminal sanctions arising out of breaches of health and safety law.

External to the judicial forum, toxicologists may be involved in risk-assessment exercises and their subsequent management, as well as alternative dispute resolution (ADR) through expert determination and even mediation.

Before turning to the principles of the expert evidence, a brief review of some legal aspects might be of value.

4.2 The Law of Tort

The area of personal injury is a part of the law of tort, which means a civil wrong. This covers, generally, two specific areas, that of negligence and breach of statutory duty. The majority of tort actions are in negligence. However, the plaintiff, or more correctly, in today's terminology, the claimant, may only recover damages when there is negligence on the part of the defendant. Thus, the classic tort formula is as follows:

- The defendant owes a duty to the claimant
- There has been a breach of that duty
- Damage must have been incurred as a result of that breach.

The first element is usually easy to determine, for example, in the context of, say, employer/employee relationships. The second element is usually the subject of expert evidence.

The third element has a number of aspects to consider. First, the resultant damage must be directly caused by the putative chemical. Second, that damage must be

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direct and not too remote. Third, the damage must be foreseeable.

There is extensive case law setting the parameters of the tort formula, as well as its subelements concerning damage. These however, are beyond the scope of this chapter.

Once the elements of the formula are satisfied, the judge will award the damages. Similarly, the calculus used is complex, being covered by a complex mixture of common sense, obscure rules and case law. Thankfully, it too goes beyond the scope of this chapter.

4.3 The Ikarian Reefer Principles

The principles of expert evidence have been set out in a classic case by Mr. Justice Cresswell (National Justice Compania Naviera SA vs. Prudential Assurance Co Ltd, 1993). These principles have become generally accepted as the classic statement of good practice. They were eventually incorporated into the Civil Procedure Rules, 1998, which swept away the old-style approach to expert evidence and is now the underpinning set of rules in UK practice. We will examine these principles in turn.

4.3.1 The First Principle

The first principle is set out in Mr. Justice Cresswell's judgement:

> *Expert evidence presented to the court should be, and should be seen to be, the independent product of the expert, uninfluenced as to the form or content by the exigencies of litigation.*

Few would object to this principle at first glance. However, form cannot be uninfluenced by litigation. The content demanded by judges today will contain certain elements, such as the report author's curriculum vitae to lay out the groundwork for the oral evidence of the person who will be subject to examination-in-chief and cross-examination in the witness-box. Similarly, the precise nature of the proceedings will direct the report-writer to address certain matters upon which the expert evidence is required. It is usual practice for lawyers to set the questions for the experts, the result being a sharply focussed report, with greater relevance to the court's needs. The involvement of lawyers in the drafting of the report was the subject of comment by Lord Denning, the Master of the Rolls (Whitehouse vs. Jordan, 1980). He was commenting on a joint report of two claimants' experts:

> *In the first place, their joint report suffers, to my mind, from the way it was prepared. It was the result of long conferences between the two professors and counsel in London and it was actually 'settled' by counsel. In short, it wears the colours of a special pleading, rather than an important report. Whenever counsel 'settle' a document, we know how it goes: 'We had better put this in'; 'We had better leave this out'; and so forth.*

The case went on appeal to the House of Lords, where Lord Wilberforce (Whitehouse vs. Jordan, 1981) commented on this judgement in the following words:

> *While some degree of consultation between experts and legal advisers is entirely proper, it is necessary that expert evidence presented to the court, should be and be seen to be, the independent product of the expert, uninfluenced as to form or content by exigencies of litigation.*

Thus the principle of independence is confirmed by the UK's highest court. Whilst it is quite proper for a lawyer to ask for a passage to be omitted, whether or not the expert should accept this instruction depends on the implications. Asking for a passage to be omitted is perfectly justified if the issues covered are not included in the pleadings. It would not be so justified, if removal of the passage distorted the report-writer's true meaning. The difficulty arises when lawyers ask 'catch-all questions' and the expert must report on an issue which may bring the case to an end. In this situation, the expert must remain true to their belief, on the evidence available, but be content to have avoided significant public expenditure.

It is also permissible, for lawyers, for a draft report to be clarified or for material to be developed further or the content to be arranged differently. This is sometimes needed to assist in presenting the case to the judge, whilst the expert is undergoing oral examination in court. The expert is best advised to confine themselves, as far as possible, to the questions posed by the lawyers. Throughout the scrutiny of their report by counsel, the expert has a duty to maintain a proper balance of truth.

4.3.2 The Second Principle

Cresswell sets out the second principle in the following terms:

> *An expert witness should provide independent assistance to the Court by way of objective unbiased opinion in relation to matters within his expertise (See Polivitte Ltd—v- Commercial Union Assurance). An expert witness in the High Court should never assume the role of an advocate.*

The fact that this warranted a comment at all suggests that biased opinions and reports that bordered on advocacy were common occurrences. Certainly there were 'hired guns', but these are now far fewer than before.

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There are a number of expert bodies beginning to develop professional standards among experts so that the court may have confidence in their integrity.

Giving unbiased opinions is not equivalent to giving 'independent assistance' for the court. Experts are appointed by parties and paid by one of them or jointly. They are engaged by the parties to provide oral evidence, advice on case management and trial litigation. A more balanced case was put forward by Garland J in: Polivitte Ltd vs. Commercial Union Assurance Ltd (1987)

> *I have almost considered the role of an expert to be two-fold: first, to advance the case of the party calling him, so far as it can properly be advanced on the basis of information available to the expert in professional exercise of his skill and experience; and secondly, to assist the Court, which does not possess the relevant skill and experience in determining where the truth lies.*

4.3.3 The Third Principle

The third principle is set out in the following words:

> *An expert witness should state the facts or assumptions upon which his opinion is based. He should not omit to consider the material facts which detract from his concluded opinion.*

This principle is fine, provided that it is qualified by a statement limiting it to the questions posed to the expert. An expert is not required to speak out or report issues that are not pleaded in the case. This can place experts in very difficult situations. It is a common mistake that is easily avoided.

4.3.4 The Fourth Principle

That experts should stay within their expertise is a statement of the obvious, but it is very difficult in modern science and toxicology is no different. The fourth principle is:

> *A witness should make it clear when a particular question falls outside his experience.*

This principle has become something of a *cause célèbre* in the UK. The problem arises when, say, toxicologists have to consider toxicological evidence. Mere skill in assessing epidemiological data may be sufficient. But toxicologists may find themselves in difficulty when discussing epidemiological literature. The issue appeared in a Scottish case before a senior judge, the Lord Ordinary (Main vs. McAndrew Wormald Ltd, 1988). The case concerned a man, now deceased, who had been exposed to asbestos during his lifetime. During the appeal, it was held that it was permissible for experts to refer to literature and in particular, to rely on epidemiological literature, though they were neither epidemiologist nor statisticians.

In his judgement, Lord Nimmo Smith in McTear vs. Imperial Tobacco Ltd (2005), delivered a full résumé of the expert evidence in a matter where the pursuer (the claimant in England and Wales) sought damages against a tobacco company for the death of her husband from lung cancer. He failed to accept the decision in the Main case as authority for introducing evidence not within the expertise of those experts so instructed. He said that there was still a requirement that published material fell within the expertise of the witness and that would be decided on the facts of the case. He highlighted the need for epidemiology to be fully and properly tested.

4.3.5 The Fifth Principle

The fifth principle was enunciated in Cresswell 's judgement in the following words:

> *If an expert's opinion is not properly researched before he considers that insufficient data is available, then this must be stated with an indication that the opinion is no more than a provisional one. In cases where an expert witness who has prepared a report could not assert that the report contained the truth, the whole truth and nothing but the truth, without some qualification, that qualification should be stated in the report.*

This latter notion was drawn from the remarks of Staughton LJ in Derby vs. Weldon (1994), which are as follows:

> *I do not think that an expert witness, or any other witness, obliges himself to volunteer his views on every issue in the whole case when he takes an oath to tell the whole truth. What he does oblige himself to do is to tell the whole truth about those matters which he is asked about.*

This principle improves, therefore, the transparency, as well as the management of the litigation. It reminds the witnesses of the need to tell the truth and in this simple admonition attempts to remove the last vestiges of a 'combat' metaphor. This is not a fight, just an attempt to dispense justice.

4.3.6 The Sixth Principle

The sixth principle is set out as follows:

> *If after the exchange of reports, an expert witness changes his view on a material matter having read the*

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other side's expert's report or for any other reason, such a change of view should be communicated (through legal representatives) to the other side without delay and when appropriate, to the court.

Curiously, it is apparent that when this judgement was written, disclosure of the change of opinion to the other side was not mandatory. Counsel had up to the last minute to decide whether to summon a witness or to rely on a report. However, as stated, the principle was an aspiration of Cresswell J, the judge, and has now been incorporated into current practice.

4.3.7 The Seventh Principle

The seventh and last principle is set out in the following terms:

Where expert evidence refers to photographs, plans, calculations, analyses, measurements, survey reports or other similar documents, these must be provided to the opposite party at the same time as the exchange of reports.

This, of course, is another statement of good practice aimed at promoting transparency.

5 THE CIVIL PROCEDURE RULES

The Ikarian Reefer principles set out above are all regarded as good practice and have been met with approval by the judiciary in their incorporation into the Civil Procedure Rules, 1998. Prior to this date, there was considerable judicial unease about the behaviour of several experts in civil cases. The need for experts was certainly recognized, but they were seen as a source of excessive expense, delay and redundancy.

This brought the old subject to the attention of Lord Woolf, who chaired a committee providing a report to Government, seeking to improve access to justice and reducing the costs of litigation (Lord Woolf, 1996). The Civil Procedure Rules, 1998 apply throughout the civil, as distinct from the criminal, jurisdiction in the UK. For example, they apply in County Court, the High Court and the Court of Appeal, as well as in lesser tribunals. Their aim is to increase cooperation between the parties in the conduct of proceedings and thus, lead to the swift dispensing of justice.

Exposure to lawyers, in both reality and the media causes one to hear such phrases as: 'the judge's track record'; 'beating my learned colleague'; 'my strategy' or 'the tactics for the case'. The lay person would be forgiven if this has led them to the impression that pursuing a legal action is nothing more than a game of skill. Lord Donaldson, Master of the Rolls (MR), has some advice for anyone harbouring such thoughts (Davies vs. Eli Lilley & Co, 1987):

Litigation in this country is conducted 'cards upon the table'. Some people from other lands regarded this as incomprehensible. 'Why,' they ask, 'should I be expected to provide my opponent with the means of defeating me?' The answer, of course, is that litigation is not a war, or even a game. It is designed to do real justice between the appearing parties and if the court does not have all the relevant information, it cannot achieve this objective.

Most people seek a 'fix' to their grievance. Very few deliberately set out to provoke litigation. Consequently, it may be a considerable time before the notion of legal action becomes relevant. At this stage, an expert report may help by assessing the strength of the case or providing advice on settlement terms to the claimant.

5.1 Experts Under the CPR

The Civil Procedure Rules (CPR) strictly limit the number of experts called by each side, often to one medical and one other. Consequently, some lawyers appoint so-called 'shadow' experts to assist in assessing the case and even settling the pleadings.

Communications between expert and legal advisor are, of course, privileged and should not be disclosed without consent.

The functions of the expert are set out below:

- To act as advisor to one party and provide a preliminary report
- To act as an expert witness and provide a written report to the court
- To help draft pleadings
- To advise on documents for, or provided during, disclosure
- To comment on draft questions for the opposition's experts
- To narrow outstanding issues in discussions with experts
- To advise on negotiating a settlement.

Once litigation has commenced, the CPR apply, especially if a report is made to the court. If the expert was previously involved in the case as an advisor, fresh instructions must be issued.

Under Rule 35.4 of the CPR, the court's permission is required to appoint experts. This usually follows an early case conference or a pretrial review. These are normally conducted by a district judge and occur at intervals during the pretrial process. Under Rule 35.5 (i), the report must be written.

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Today, the expectation of the court is that a single expert will be appointed to advise the court. The authority to do this is set out under Rule 35.7. There is opposition to this relatively new departure in expert-witness management from the legal profession. If parties cannot agree to the so-called 'single joint expert' under Rule 35.8, the court is empowered to select an expert from the list provided by the parties or to direct appointment by some other means.

Objections are usually dealt with by the district judge. Occasionally the Court may make other directions to the expert, which will be set out in a written order. These might include, for example, permitting the parties to ask written questions, or more often to direct that experts meet. The single joint expert will have their neutrality and independence recognized and is therefore unlikely to be attacked in court. Communications between a single joint expert and the parties are not privileged for obvious reasons.

If parties agree and there are sufficient reasons to support the view a district judge may permit the appointment of separate experts for each side. The days of the 'professional' expert witness are long gone. An expert with standing in their primary profession as a toxicologist, or whatever, are more likely to be appointed than one who was not.

In the case of party-appointed experts, each party bears their own costs with respect to their experts. In the case of a single joint expert, the parties are jointly and severally liable for the costs.

Changes in the justice system in the UK now permit lawyers to accept instructions on the basis of what is known as conditional fee arrangements. Broadly speaking, lawyers accept cases and legal expenses insurance covers the costs of the litigation. Naturally, for the lawyers, the strength of the case becomes crucial. Experts should not accept instructions under these arrangements and their fees should be guaranteed in full. To do otherwise would bring their independence into doubt.

It is clear that the court is determined to control expert evidence and therefore, places considerable emphasis on the written report. The powers to restrict expert evidence are set out in Rule 35.4. The court's permission is required under 35.4.1. Applications for employment of expert evidence must identify the specific field of expertise and where practical, the expert, as set out in Rule 35.4.2 (a-b). Lastly, the report must, of course, be written.

5.2 Accepting Instructions

The importance of the written report is underlined by the often-quoted advice that experts should never accept instructions for which they cannot deliver the report in due time. To do so risks judicial displeasure, which is inevitably not conducive to contentment. In deciding whether or not to accept instructions, the reader might wish to consider:

- Are the issues in dispute their professional knowledge, experience and competence?
- Is the reader confronted by any relevant personal or professional matters?
- Is the reader's evidence likely to help the court?
- Can the reader meet the timescales involved within their personal and professional schedules? Booked holidays are not an excuse acceptable to a judge.

If the answers to these questions are in the affirmative, then the report can be accepted.

5.3 Writing the Report

The report should be written in simple language and with wide margins. This permits marginal notes by both the judge and counsel. A suggested format is set out below, though not all sections are relevant or necessary on all occasions. A case reference number should be included on the cover sheet. Qualifications and experience of the writer should be included, as well as any relevant literature, any detailed experiments carried out and a list of those who assisted in their conduct. Paragraphs should be numbered to facilitate reference. This writer's personal preference is for numbering sequentially. The report should contain a table of contents and be page-numbered. Finally, it should contain the expert's declaration, which is set out below. As can be seen, this is a statement of compliance and truth.

The report format is designed to be judge-friendly. Being what is today called 'CPR-compliant', the use of this format is not a surreptitious marketing tool, but a characteristic that is sought by judges. If the report is not CPR-compliant, this may well influence the weight given by the judge to the report in assessing the evidence.

Once the report is completed and submitted, questions may be asked from either side to clarify or seek to correct factual issues. If appointed as a single joint expert, the replies need to be copied to the other party.

As far as the format of the report is concerned, the following sections are needed:

- Cover sheet
- Table of contents
- Headers—name of expert, date, field
- Chronology
- Introduction
- Background to dispute
- Issues for consideration—expert's instructions
- Method of enquiry
- Documentation

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- Discussion/commentary
- Conclusion
- Opinion
- Declaration.

There are various versions of the expert's declaration and the one below has been used by the writer for some years:

I, … (Name), declare that:

I understand that my duty included in my providing written reports and giving evidence is to help the court and that this duty overrides any obligation to the part who has engaged me.

I confirm that I have complied with my duty.

I believe that the facts I have stated in this report are true and that the opinions I have expressed are correct.

I have endeavoured to include in my report those matters of which I have knowledge, or of which I have been made aware, that might adversely affect the validity of my opinion.

I have indicated the sources of all information I have used.

I have not, without forming an independent view, included or excluded anything which has been suggested to me by others and, in particular, my instructing lawyers.

I will notify those instructing me immediately and confirm in writing if, for any reason, my existing report requires any correction or qualification. I understand that:

- My report, subject to any corrections before swearing as to its correctness, will form the evidence to be given under oath;
- I may be cross-examined on my report by a cross-examiner, assisted by an expert;
- I am likely to be the subject of public adverse criticism by the judge, if the court concludes that I have not taken reasonable care in trying to meet the standards set out above.

I have not entered into any arrangement where the amount or payment of my fees is in any way dependent on the outcome of the case.

Signature:
Name in print:
Consultant Toxicologist
Qualifications and Affiliations
Date and Place of Signature

5.4 Case Management

Thereafter, involvement of experts will depend on the outcome of the pretrial review, conducted in the High Court by a master or a district judge. This review seeks to limit, or conversely, extend the expert evidence. By this approach, the court seeks to avoid unnecessary evidence or witnesses. From this point on, the report cannot be edited. The practice of side letters has also been swept aside.

At this point, the court directs deposition and exchange of documents. Disclosure of documents used to be the basis of an application. Nowadays, it is the outcome of a pretrial review, which, in effect, is a court order. It applies to all documents under the control of the parties upon which they intend to rely. The documents in one party's possession may affect their own case adversely or another party's. Conversely, it may support it. In essence, the disclosure is no longer automatic and its implementation is strictly controlled in both its nature and extent, by the court.

5.5 Conference in Counsel's Chambers

At some time prior to trial, there will be a meeting of the principal individuals involved in each counsel's chambers. This is termed a 'conference' and may involve a wide-ranging discussion of evidence and specifically reports, approaches to the case, liability, risks and so on. The conference will be attended by each party concerned, any insurance representative, instructing solicitors and, usually, as many experts as are involved. This helps everyone concerned to get a better view of the case's strengths and weaknesses. The presence of the experts ensures that all parties are fully informed about the risks involved. More particularly, the discussion may throw up issues for discussion, at the next, and often final, stage of the process—the meeting of experts.

5.6 The Meeting of Experts

The notion of the meeting of experts prior to a case is not new, they were often held under the 'without prejudice' rule. They were usually controlled by lawyers, either through the agenda or even chairing the meeting. They were exceptional. Under the CPR, they are very much more routine and help move the case towards agreement. The agenda is usually set by the experts and not the lawyers. Lawyers are not usually present at the meeting, but they may ask for specific issues to be discussed. Unless the case is particularly complicated, a meeting need not necessarily take place. Under these circumstances, the experts can rely on an extended telephone conversation.

The aim of this exercise is to agree a memorandum of what issues are agreed between the experts and what are

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not. Thus, in most cases, experts have concluded their work at this point and advise the court. This avoids the unnecessary expense of serving a summons and bringing them to court, not to mention the significant additional costs involved.

5.7 Summary

Whilst it is very much a personal matter whether one wishes to enjoy this work or not, there are certain important considerations. First, involvement in expert-witness work helps develop a rigorous approach to problem-solving skills. Second, it improves research and critical appraisal skills. Third, one has made a contribution, which can be seen to influence policy. Fourth, one's knowledge can be subject to the rigorous scrutiny of a barrister, trial lawyer or even a judge. Notwithstanding the financial rewards, involvement in expert evidence work is very much worthwhile.

6 WHISTLE-BLOWING AND THE PUBLIC INTEREST

Consider the toxicologist, employed by a chemical supplier. His employers have decided to begin importing a pesticide that is to be used for aerial spraying of crops. During his preparation of the REACH Regulations dossier, his team becomes aware of two issues. First, there have been reports of outbreaks of unexplained neurological disorders in three countries, where the pesticide has been used. Second, there is some laboratory evidence of ecotoxicity.

He considers the evidence significant and notifies his senior management of his very serious concerns. However, the response to his briefing paper comes back indicating that the company executive management has pinned their forecasts for company profits on the import of this substance and need therefore, to bring it as quickly as possible to the European market place. His senior manager tells him in no uncertain terms that his loyalty to their mutual employer is expected and the likelihood of his concerns actually coming to fruition is generally regarded as minimal. The dossier is required and he is told not to include the adverse human-health effects or ecotoxicity.

Our company toxicologist is left in something of a moral dilemma. His own moral compass will probably encourage him to lean more towards disclosure on the grounds of public safety. However, he will also ask about his relationship with the company and the small matter of the confidentiality clause that the company inserted into his contract. Then there are other matters.

He needs to support this wife and three children. With his youngest child joining his older siblings at university in the next academic year, he cannot afford to lose his job. When the dossier is sent to the Health and Safety Executive and the European Chemical Agency, there is no mention of neurological disorder or ecotoxicological issues.

6.1 An Ethicist's View

How should he have behaved? He has tried talking to his senior management, but to no avail. What should he do now? Should he distance himself from the situation and accept it as a *fait accompli,* a situation beyond his ability to intervene? Should he disclose the true situation to the Health and Safety Executive, the competent authority and/or the Agency in Helsinki? He wants to do the right thing, but what is the correct way to handle the situation and how does he get there? A philosopher and applied ethicist, Steare, has suggested a simple framework to help decide what is right (Steare, 2007). Steare sets out the following five questions:

- What are the rules?
- How do we act with integrity?
- Who is this good for?
- Who could be harmed?
- What is the truth?

A useful starting point for our toxicologist might be to apply these questions to the situation.

The rules and laws about public disclosure are drafted to cover a large range of situations. They are therefore, of necessity, indistinct. Breaching the company confidentiality agreement may not be illegal, but this does not mean that it is right. If the law is silent on the subject, an ethical statement might help.

The preamble to the Code of Ethics adopted by the Society of Toxicology on 31 January 1985 and revised on 1 June 2005, reads:

> *The Society of Toxicology is dedicated to developing and communicating knowledge to improve the health and safety of living beings and to promote the environment upon which we depend. … To obtain this objective, each member must maintain high ethical standards, recognise a duty to share this knowledge with the public and be a thoughtful advocate for human, animal and environmental heath. To this purpose, this Code requires a personal commitment.*
>
> (Society of Toxicology, 1985).

The Code of Practice attached to the Victorian Occupational Health and Safety Act, 2004, requires the professional occupational health and safety member to point out to senior management the problem, potential

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consequence and the member's ethical and legal obligations. The professional member is then exhorted to inform the third party, having sought the advice of senior colleagues and informed their senior management of his action. Professional members are then protected by the Whistleblowers Protection Act (2001). There appears to be added weight to the protections of our whistle-blowing toxicologist in the State of Victoria, where they have the very clear protection of a legal framework.

The Eurotox Code of Practice requires it members to communicate:

> *in a timely and responsible manner with regard to the significance and credibility of available data, abstain from professional judgements influenced by conflict of interest, economic as well as "academic" in connection with special interest groups…*
>
> (Eurotox, 2009).

This has been a brief review of several ethical statements. It seems that the principal theme remains constant, however, that a professional toxicologist should not participate in any attempt to distort the meaning of any scientific communication either by action or inaction. The professional view seems, therefore, to argue in favour of public disclosure.

One might expect professional toxicologists to act with integrity. What precisely does that mean? Every individual has a series of values or principles, which drives their view of life. This is often called, by the psychoanalyst, their 'world view'.

These principles guide us to what is right. When our behaviour is consistent with our 'world view', we act with integrity. If those principles are just 'talk', we behave with hypocrisy. Law and rules of ethical behaviour are imposed from external sources, whether state or professional organizations, but our integrity comes from within.

We now come on to more familiar ground, as we have to deal with the calculus of benefit or harm. For each course of action, whom would our toxicologist harm and whom would he benefit? The difficulties of carrying out this calculus are the limits that need to be set. It is only really helpful if they are balanced across the widest possible range of stakeholders and thus, our toxicologist needs to identify that range of stakeholders before setting out the harms and benefits. This, of course, is the fundamental thinking behind the so-called 'stakeholder analysis'.

Lastly, there is the matter of truth. One is reminded of Mahatma Gandhi's famous quotation:

> *There is no God higher than truth.*

Disclosure does not depend on whether these reports are convincing or not. The actual decision-making lies outside the company and should not be usurped by it. If the reports were flawed, our toxicologist should point this out and a critique of the evidence be included. Most readers would agree that the right thing is to disclose.

However, increasingly, society is recognizing the value of this activity called whistle-blowing as a form of internal policing and, as we have seen, is giving the disclosure, under certain circumstances, statutory protection. In the UK, this is provided generally by the Public Disclosure Act, 1998. This section will provide an overview of the Act and whistle-blowing in general.

6.2 Whistle-Blowing: The UK Position

The media will have provided a general appreciation of the nature of whistle-blowing through their coverage of high-profile cases. At its most simplistic, whistle-blowing occurs when an employee or other worker provides certain types of information, usually to the employer or a regulator, which has come to their attention through work. The whistle-blower is not usually directly personally affected by the danger or illegality. Whistle-blowing occurs, therefore, when a worker raises concern about danger or illegality that affects others, for example, members of the public.

The key piece of legislation is the Public Interest Disclosure Act, 1998. In general, workers should be able to make disclosures about wrongdoing to their employer, so that problems can be identified and resolved quickly within the organization. The Act mainly takes the form of amendments to the Employment Rights Act, 1996 and contains provisions about the kinds of disclosures which are protected and the persons offered protection by the Act.

6.2.1 Protected Persons

The provisions introduced by the Act seek to protect most workers from being subject to detriment by their employer. Detriment may include denial of promotion, facilities or training opportunities, which the employer might otherwise have offered. If dismissed, employees may make an application before an employment tribunal for unfair dismissal, if they have made a protected disclosure.

Contracted workers are not employees and may not therefore apply for unfair dismissal before an employment tribunal. They may, however, complain of being subject to detriment.

With limited exceptions, the provisions protect the following categories:

- Those working under contracts of employment
- Those who work personally for someone else, a so-called 'worker's contract', but are not self-employed in the proper meaning of the word

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- Home workers;
- Certain agency workers
- National Health Service practitioners
- Certain categories of trustees.

The list of protected persons may be revised at any time, usually through an Order in Council. This is a flexible way of updating a statute without the necessity of drafting a bill or finding time within the parliamentary session. When seeking information, therefore, it is important to obtain the current position as the scope of these measures can equally be changed by case law.

6.2.2 Qualifying Disclosures

Qualifying disclosures are disclosures of information, which the worker reasonably believes tend to show that a specified event took place at some time in the past, was happening in the present or is likely to happen in the future. These events include:

- A criminal offence
- A breach of a legal obligation
- A miscarriage of justice
- A danger to the health and safety of any individual
- Damage to the environment
- Deliberate covering up of information tending to show any of the above five matters.

Most of these will be relevant to the toxicologist, with a possible exception of the miscarriage of justice.

The employee making the disclosure must have a reasonable belief that the information tends to show one of the events identified above for it to constitute a 'relevant failure'. That belief, however, need not be correct. The worker must, however, demonstrate that it was reasonable to hold that belief at the time of disclosure, given all the circumstances.

Even if the relevant failure took place oversees, or where the relevant law was not that of the United Kingdom, the Act still applies. If the worker committed an offence in making a disclosure, as might be the case if he were statutorily forbidden to do so, for example, under the Official Secrets Act, 1989, the disclosure does not qualify for protection.

Any information acquired, relevant to potential litigation, is privileged and should not be disclosed. If that information was disclosed by a legal advisor, in the course of managing a case, it would be protected. However, if it was made by a secretary, it would not.

6.2.3 Protected Disclosures

A qualifying disclosure would be a protected disclosure when it is made:

- To the worker's employer; either directly to the employer or by procedures authorized by the employer for that purpose
- To another person whom the worker believes to be solely or mainly responsible for the relevant and
- The worker acts in good faith.

Disclosure to the employer may well lead to rapid resolution of the problem by the individual best placed to do so. However, an authorized procedure may be adequate. Many companies now have whistle-blowing policies, designed to facilitate confidential disclosures by staff, often through the human-resource department or mediated via a nominated director. A slight variation of this occurs in some companies in Europe who appoint, say, an external academic, as an ombudsman. This particular mechanism works extraordinarily well. Simple procedures that are easy to use, readily accessible and promoted by the company are more likely to result in internal disclosure to the employer in preference to externally. In drawing up these procedures, negotiators must take account of the best way to secure trust and confidence in their staff, to ensure that their procedures will be used. For these reasons, joint negotiation will ensure participation by the workforce and their representatives.

6.2.4 Making a Qualifying Disclosure to a Prescribed Person

Under the Public Interest Disclosure Act, workers concerned about wrongdoing or relevant failures can make disclosures to a person or a body which has been prescribed for the purpose of receiving disclosure about the matters concerned. A qualifying disclosure will become protected, provided that:

- The disclosure is made in good faith
- The individual making the disclosure reasonably believes that the disclosure and any allegation arising from it are substantially true
- They reasonably believe that the matter falls within the description of matters for which the person or body has been prescribed.

In the case of our toxicologist, the Health and Safety Executive might be responsible for the people receiving the pesticide, storing, transporting and applying it. In respect of the community impacted by the spraying, the Environment Agency would be appropriate. Space does not permit detailing the persons/bodies and the relevant areas of concern. An interested reader is referred to the original Act. If a disclosure is made to a lawyer in the course of obtaining legal advice, provided it is qualified, it will be protected without further condition. If the employee is on the staff of a government organization, including a nondepartmental public body, any qualifying disclosure made under the conditions set out above, to a

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government minister, will be treated as protected, whether made directly or through government officials.

6.2.5 Serious Failures

Conditions are varied when making a qualifying disclosure about an exceptionally serious failure. It would be a protected disclosure if the person:

- Makes the disclosure in good faith
- Reasonably believes that the information disclosed, any allegation contained therein is substantially true
- Does not act for personal gain.

It must be reasonable for the disclosure to be made in all circumstances, having regard, in particular, to the person to whom the disclosure is made. The relevant failure must be exceptionally serious as a matter of fact and not simply that the person making the disclosure reasonably believed that this was so.

6.2.6 Conditions for the Protection of Disclosures

Qualifying disclosures become protected when the appropriate conditions are met. First, the person making the disclosure must:

- Make the disclosure in good faith
- Reasonably believe that the information and any allegations contained therein are substantially true
- Not act for personal gain.

In addition, one or more of the following conditions must be met:

- The individual reasonably believed that he would be subject to a detriment by his employer, if the disclosure were to be made to his employer or to a prescribed person
- In the absence of an appropriated prescribed person, the person making the disclosure reasonably believed that disclosure to the employer would result in the destruction or concealment of information about wrongdoing
- The person making the disclosure had previously disclosed substantially the same information to his employer or to a prescribed person
- It must be reasonable for the disclosure to be made in all the circumstances.

In reaching decisions about statutory protection, the employment tribunal needs to consider a large number of factors to determine whether or not the disclosure was reasonable. The first might be the identity of the person to whom the disclosure was made. Thus, it may be reasonable, under the circumstances, to disclose to a professional body with responsibility for professional standards in a particular field, for example, toxicology and medicine. Second, an obvious consideration is the seriousness of the relevant failure and whether or not this is continuing, or is likely to recur. It may be significant if the disclosure breaches the employer's duty of confidentiality to others, for example, confidentiality about a client and proposed purchases which might directly impinge on market advantage, a recognized concern under REACH. Third, an employment tribunal might consider what action might have been taken had disclosure been made previously to the employer or a prescribed person. Last, whether or not the worker complied with any internal procedures, approved by the employer, may be considered in the context of the tribunal.

6.2.7 Health and Safety Matters

The Employment Rights Act, 1996 provides, in Sections 44 and 100, protection for employees who, in certain circumstances, raise concerns about, or take action in connection with health and safety matters. Under these provisions it would be unfair to dismiss employees who act to protect themselves or others from serious and imminent danger. The new provisions under the 1998 Act, therefore, simply complement those contained in the earlier 1996 Employment Rights Act.

6.2.8 Contractual Duties of Confidentiality

Our toxicologist was concerned about the confidentiality clause in his employment contract. Any provision in an agreement between an employer and an employee that prevents the latter from making disclosures protected by the 1998 Act is void. This applies to any agreement between the two parties and includes, therefore, a contract of employment or separate agreement and this, in turn, includes agreements settling claims under the Public Interest Disclosure Act, 1998.

6.2.9 Dispute Resolution

The Employment Dispute Resolution Act came into force on 1 October 2004. It requires employers to follow a minimum three-step process to promote internal resolution of the dispute, whether or not it concerns dismissal, disciplinary or grievance issues. Where both parties have failed to adhere to these procedures, employment tribunals will impose financial penalties. However, it differentiates between a grievance and protected disclosures. A formal grievance is not a necessary prelude to making a protected disclosure. The statutory minimum grievance procedures only apply to a protected disclosure if the employee actually intends that the disclosure constitutes the substance of a grievance to raise with his employer.

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6.2.10 Employment Tribunals and Remedies

The provisions of the Public Interest Disclosure Act protect both employees and workers. Both can complain that they have been subjected to detriment by their employer for making a protected disclosure. An employee who is dismissed can make a claim for unfair dismissal. In the case of a worker who is not an employee, whose contract was terminated in view of his protected disclosure, a complaint can be made of having been subjected to detriment. The complaint must be made within three months, but the time is extended in connection with the statutory dispute-resolution process. It can also be heard if the tribunal believes it was not reasonably practicable to have the complaint made within the three month timeframe and it has nonetheless been made within a time period which they consider to be reasonable.

For unfair dismissal claims, interim relief is available, where the complaint is made within seven days from the effective date of termination. Where such a complaint is upheld, it is open to the tribunal to order reinstatement or redeployment, as well as payment of compensation. If a worker's claim is regarded as well founded, the tribunal may make a declaration to that effect and order compensation.

7 CLAIMS MANAGEMENT

The advent of increased access to justice and other aspects of deregulation have led to the evolution of a claims-management industry. One particular measure of interest is the ability to refer cases to other solicitors for a fee. This is not reflected, for example, in the medical profession. Nightly, on television, there are advertisements encouraging those who have had an accident or developed an illness attributable to work to pursue the person or company responsible on a 'no win, no fee' basis. Viewers are given the impression that claimants have nothing to lose and the potential of a huge financial windfall to come. They have become, in the UK, the pariahs of the law. Claims farmers have become the butt of much criticism, particularly when the Lord Chancellor blames the rise of compensation on their activity.

Many of the cases they offer to bring on the part of clients have little merit and are not really likely to get to trial. Some claims farmers exploit their client's misfortunes to encourage them into pursuing the flimsiest of law suits. Advertisements are to be found in shopping centres, on television, on the back of passing busses and on the pavements of our cities.

Many claims farmers are not lawyers, but entrepreneurs who refer to a captive panel of solicitors, who cut their cost for a guaranteed flow of cases. The essence, of course, is to reduce costs and this is often achieved through the use of nonlawyers and administrative staff to carry out the routine, day-to-day and low-level work, referring to professional lawyers when necessary. In the UK, they are often seen representing clients in tribunals, which is perfectly legal.

The worst kind of claim farmer is the one who encourages their client to take out expensive cost-insurance to cover the eventuality of having to pay their opponent's costs. In the event of a case collapsing, the claimant is left with costs, which may outweigh any compensation that may have been obtained.

In the USA, the descriptor 'ambulance chaser' is given to those lawyers or their representatives who haunt hospital corridors for people who are victims of accidents or injury, so that they can sue the person liable on their behalf. In many ways, one can describe this activity as fundamentally more honest than appears the case in the UK. Once instructed, ambulance chaser or not, the legal advisor in the USA works on their client's behalf, in stark contrast to many claims farmers operating today in the UK.

In any period of legal reform, changes must be made sustainable and maintain confidence in the justice system. The Lord Chancellor spoke at a conference of personal injury lawyers in 2007 (Eurotox, 2009). He referred to inadequate and generally suboptimal information given by claims-management companies to claimants, indicating a frequent failure to include low-cost options. He pointed out that the most serious complaints came from the unregulated companies—those who used hard selling techniques, cold calling and advertised in family-practitioner surgeries and healthcare facilities. In short, the industry was placed well and truly in his regulatory sights.

Before examining the claims-management industry and the Compensation Act, a brief review of how we arrived at the present position is relevant.

7.1 Access to Justice

Section 58 of the Courts and Legal Services Act 1990 allowed the use of conditional fee agreements in such types of cases as specified by the Lord Chancellor. These, however, were subject to requirements made by Section 58 (10), which exclude all criminal and family law proceedings from these arrangements.

Conditional fee agreements allow clients to agree with their lawyers, that the lawyer will not receive all or part of their usual fee or expenses if the case is lost. If it is won, the client pays an up-lift to the lawyers in addition to the usual fee.

In 1995, conditional fee agreements were permitted for a limited range of cases, including personal injury and

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cases before the European Court of Human Rights. The maximum up-lift in the event of success was set at 100%. In addition, the Law Society recommended that lawyers should voluntarily limit the up-lift to 25% of the damages obtained if that was lower than the 100% up-lift of the fee.

At the same time, insurance policies were developed, which allowed the client to take one insurance to cover the costs of the other party and the client's own costs, other than their lawyers' fees. If the case should be lost, the up-lift and the premium were taken from any damages that might be recovered by the client. In July 1998, the Government extended the availability of conditional fee agreements to all civil cases, excluding family law.

Since the introduction of fees, the common law has been developed and two important cases are worthy of note. In Thai Trading Company (a Firm) vs. Taylor (1998), Lord Falconer (2007), the Court of Appeal held that there were no longer any public policy grounds to prevent lawyers agreeing to work for less than their normal fees in the event that they were unsuccessful, provided that they did not try to receive more than their normal fees, if they were successful. The latter was only permissible in those proceedings in which conditional free agreements were allowed. In another case (Thai Trading Company (a Firm) vs. Taylor, 1998) the Vice Chancellor held that it was also lawful for a conditional fee agreement to apply in a case which was to be resolved by arbitration under the Arbitration Act, 1950, even though these were not formally court proceedings, provided all the requirements specified by regulation as to form and content of conditional fee agreements were complied with. In addition, it is now possible for someone contemplating litigation to take out an insurance policy in the event that the case is lost, to cover the costs of the other party, as well as their own legal costs, including their lawyers' fees, if these were not subject to a conditional fee agreement. Some of these policies had indeed been developed to support the use of conditional fee agreements, but others had been used to meet lawyers' fees charged in the traditional way. The Act makes premiums paid for protective insurance recoverable in costs. The principles behind the Government's desire are very simple. They are designed to increase access to justice for, in the words of the eminent judge, Lord Denning, 'the man on the Clapham omnibus'(Lord Chancellor's Department, 1998).

7.2 The Compensation Act 2007

From the above, it is clear that many claims management businesses were found to engage in adverse practices to the detriment of the consumers, the claimants and the public interest. These included aggressive marketing techniques applied at sensitive times, encouragement of a frivolous claim, which contributes to the perception of a compensation culture, giving consumers misleading information about options available to them, providing poor quality advice and dropping claims when they did not appear financially lucrative. Following a report by the Better Regulation Task Force (2004), the Government stepped in with the Compensation Act, 2007. The Act received the Royal Assent on 25 July 2006 and came into force on 23 April 2007.

Within the Act, the definition of claims-management regulation is so wide that it can apply to almost any claim for compensation. It is now suitably restricted by a Statutory Scope Order, which narrows the definition to specified activities and sectors. Any person carrying out the activities listed below connected to making a claim for compensation for: personal injury; industrial injuries disablement benefit; criminal injuries; employment; housing disrepair; or financial products or services must be authorized to do so. The activities regulated include:

- Advertising for seeking out or direct marketing to persons who may have a claim
- Advising a claimant or potential claimant in relation to his claim or cause of action
- Referring details for a fee, of a claim or claimant, or a cause of action or potential claimant to another person, including a person having a right to conduct litigation
- Investigating or commissioning the investigation of circumstances, merits or origins of a claim with a view to using the results in pursuing the claim
- Representing a claimant, whether in writing or personally, to a tribunal body or person.

Toxicologists are most likely to be caught in the penultimate category. A statutory order exempts certain persons from the need to be regulated if they are already regulated for the management of claims services, as for example, are lawyers and insurers. Charities, not-for-profit agencies and independent trades unions are also exempted.

7.3 Issues for the Toxicologist

It is likely that toxicological evidence is potentially featured in personal injury, criminal cases, employment and industrial injury disablement cases. Depending on the circumstances, expert evidence is more likely to be required for the first three categories. Indeed, there is an argument to suggest that cases are more likely to need toxicological expertise because the area is so little understood. Paradoxically, a rigorous attention to expert evidence is sometimes lacking in these cases, which leads

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to difficulties later in the case. Therefore, one possibility is that expert evidence may render the case nonviable and this would not be what a claims-management company might wish for their own purposes.

Environmental medicine, (formerly known as clinical ecology and also sometimes called nutritional medicine) is a convincing mix of orthodox clinical medicine and toxicology. For those practitioners who sincerely hold beliefs in relation to the ideas of clinical ecology, there is an overriding obstacle, which is simply that there is relatively little conventional evidence underpinning their theory and more particularly, their practice. There will be an immediate clash between conventional, clinical and toxicological evidence, on the one hand, and those who practise environmental medicine on the other.

Dealing with unregulated claims management companies can place the toxicologist at risk from two areas. First, one's professional reputation can be damaged through simple association with a company who is criticized in the press for being the subject of enforcement procedures, under the Compensation Act. Second, such companies may have a risk of going out of business with relatively little notice. They may be placed in that position, not only as a result of business pressure, cash flow difficulties and the like, but also as a result of enforcement procedures. The result, of course, is that the toxicologist may not get paid.

When clients instruct a lawyer and their claim is managed under a conditional free agreement, this should have no effect on the fee paid to a toxicologist. The fee paid to a toxicologist for expert advice should under no circumstances be determined by the potential success or otherwise of the case. In UK law, the toxicologist is appointed to advise the Court, not the parties. If the fee is to be influenced by the conduct of the case, then it would be hard to defend the expert's impartiality.

Many lawyers maintain in-house panels of experts, including toxicologists, with whom they build up a relationship, each party developing an understanding of the other's 'needs' and 'wants'. This is to be applauded and will be of considerable benefit to both claimant and defendant. However, it would be wrong for the toxicologist either to reduce their fee or extend credit terms beyond their usual terms of business in return for an entry on their database or a promise of increased referrals.

8 SOCIAL RESPONSIBILITY AND PUBLIC STATEMENTS

Many codes of ethical standards reflect the normality of our society in seeking to give back something positive to the community in which we live. This thought is encapsulated in the tendency for large corporations to have spin-off charitable foundations or active social participation. Social responsibility is often seen as a fundamental necessity in today's marketing strategy. This being so, it is not surprising to have these notions reflected in codes of ethics for toxicologists (Society of Toxicology, 1985).

It is true to say that the public has a colossal appetite for information about toxicology, whether it concerns adverse effects on human health or the environment. Given also the range of toxicological issues from acrylamide in food to cranberry juice, the stage is set for a rigorous exchange of information with the public. However, this brings with it duties on the part of toxicologists which are not necessarily explicitly set out ethical standards.

At the very essence of the relationship between toxicologists and society is their professional and scientific responsibility to the community in which they work and live. They apply and make public their knowledge of toxicology in order to contribute to human welfare. They are concerned about and work to mitigate the cause of adverse health effects in humans and the environment.

It is an ethical imperative that toxicologists should at all times work to advance the cause of human welfare and the science of toxicology. Toxicologists should also strive to avoid misuse of their work, to comply with the law and to encourage the development of law and social policy.

Tacit acceptance of misunderstanding by journalists and the media should not be tolerated and therefore, toxicologists should make themselves available to journalists to help them to avoid this. There are several organizations that promote this sort of dialogue and university press offices can play a major role in this. Credibility might be enhanced on the part of the toxicologists by the occasional acceptance of *pro bono* work. In dealing with statements made by others, toxicologists must retain professional responsibility for any statements made on their behalf. They must prevent others making deceptive statements and correct any that are made, whenever practicable.

Toxicologists should avoid false or deceptive statements, avoiding assertions which lack any credible evidence base. Where it is limited or flawed, these should be explained. Public statements may be false, deceptive, misleading or fraudulent, because of what they state, convey or suggest, or because of what they omit, concerning research practice or other work activities or other persons or affiliations.

False or deceptive statements which specifically should be avoided include:

- Training, experience or competency
- Personal academic degrees
- Credentials
- Institution or association affiliations
- Services offered

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- Scientific bases for, or results of, or degree of success of their services
- Their fees
- Publication of research findings
- Providing participants with information.

One should only claim as credentials, earned degrees and those used as the basis for licensure or specialist accreditation. All statements made to the press and media must be based on the appropriate toxicological literature and practice, whether they are on or off the record. Unless experienced in dealing with journalists, care should be taken in making 'off the record' comments, despite conventions that protect this form of communication. It is always worthwhile assuming that 'off the record' comments may well find their way into print, justified by reported speech. Statements should always be consistent with the toxicologist's integrity and the speciality's ethical principles.

Following assistance to the press or other media with a particular story, journalists should never be left with the impression that they now have a special relationship with the toxicologist.

9 ALTERNATIVE DISPUTE RESOLUTION

ADR may be defined as a range of procedures which serve as alternatives to the adjudicatory procedures of litigation and arbitration, for the resolution of disputes generally, but not necessarily involving the intervention and assistance of a neutral third party who helps to facilitate such a resolutions. Arbitration, originally an alternative procedure, is now generally viewed as being closer to litigation in its approach, and part of mainstream practice, leaving the term ADR to refer mainly, but not necessarily exclusively, to consensual rather than adjudicatory processes.

9.1 Types of ADR

The development of ADR in the UK has its principal origins in the dissatisfaction of many people with the way in which disputes are traditionally resolved. This is reflected in the criticisms of the courts and perhaps, more commonly, the legal profession, as well as sometimes a sense of alienation from the entire justice system. There are three primary categories of dispute resolution, which include both the traditional and alternative approaches. Negotiation is probably the most common, with disputants resolving their difficulties by communication. There are various styles and theories of negotiation, for example, positional bargaining or principled negotiation, but it is usually a skill learned by experience. Adjudication is a dispute process in which a neutral has, and exercises, authority to hear the respective positions submitted by the disputants and to make a decision on their dispute which will be binding on them. This may be by litigation or:

- Arbitration: a privately chosen neutral is paid by disputants and follows rules employed by statute or arbitral organization
- Administrative or statutory tribunal: in matters such as industrial injury benefit, criminal injury compensation and others
- Expert determination: in which the parties appoint an expert to consider their issues and to make a binding decision or an appraisal without an enquiry following an adjudicatory process
- Private judging: a process not yet widely used in the UK, where cases are referred to a referee, chosen by the parties to decide some or all of the issues or to establish specific facts.

Mediation is the third category. It is a process by which disputants engage the assistance of a neutral third party to act as a mediator, a facilitating intermediary, who has no authority to make binding decisions, but uses various procedures, techniques and skills to help parties resolve their dispute by negotiated agreement without adjudication. The mediator is a facilitator who may, in some models of practice, make an appraisal of some merits of each party's case, but does not make a binding decision. The theory remains that the parties resolve their own dispute. Mediation is by far the commonest form of ADR practised in the UK today.

9.2 Hybrid Processes

Each of these primary processes can be used in its own right without adaptation. But, the experienced ADR practitioner will devise a permutation of various procedures which makes a better fit with all the nuances with all the parties' needs in a case without being constrained by prescribed rules.

Certain common combinations have evolved in this way and form a distinct category which is known as hybrid processes. The so-called 'mini-trial' is a form of evaluative mediation, which is followed by negotiation, and the so-called 'med-arb', which commences with mediation, but continues with arbitration in the event of the dispute not being resolved, are two examples.

Two particularly interesting forms include the neutral fact-finding expert and the early neutral evaluation. The former involves an investigation by a neutral expert into certain specific issues of fact, technical matters and law and thereafter, if required, a mediatory role and eventual participation in an adjudicatory process. The

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latter requires a neutral evaluator to meet the parties at an early stage of the case in order to make a confidential assessment of the dispute, partly to help them narrow and define the issues and partly to promote efforts to arrive at a settlement.

9.3 Issues for Toxicologists

A toxicologist may be involved in a number of these processes from time to time, especially if they undertake a great deal of medicolegal work. Processes of particular interest to the toxicologist will be expert determination, neutral fact-finding and early neutral evaluation. They may also be involved in providing expert evidence for the purposes of arbitration or, indeed, as an adjunct to mediation.

Mediation, and indeed arbitration, requires special training. Mediation training is relatively short and can well be regarded as an important life skill. Whilst toxicologists trained in mediation are rare, the combination can be particularly useful in mediation involving complex toxicological issues. However, the success of mediations does not usually depend specifically on the employment of a subject specialist.

Instructions to the toxicologist may not make it clear at the outset whether or not the toxicologist is to be involved in an ADR process or simply in the provision of an expert opinion. If, given the circumstances, an ADR process is a possibility, then the toxicologist would be well advised to seek clarification from those appointing him.

9.4 Expert Determination

In this ADR process, an expert is appointed by parties to undertake a specific resolutive function, for example determining safety measures are adequate for workers exposed to a toxic substance. The expert's functions and authority arise from contract and are different from those of an arbitrator. In these circumstances, the toxicology expert may be liable in negligence if the case is not in the context of a legal action. If it were, they would have immunity for their opinions. The opinion of an arbitrator may be appealed to the courts.

An expert determination can be set aside on certain grounds and these have been the subject of judicial comment, as in Campbell vs. Edwards, where Lord Denning MR, stated:

> *It is simply the law of contract. If two persons agree that the price of property should be fixed by a valuer on whom they agree, and he gives that valuation honestly and in good faith, they are bound by it. If there were fraud or collusion, of course, it would be very different. Fraud or collusion unravels everything.*
> (Campbell vs. Edwards, 1976)

Thus, in principle, the parties are bound by their own agreement that the task to be undertaken should be entrusted to the person whom they have nominated. The parties:

> *... made the [expert] their judge on that point. They thought proper to confide in his judgement and skill and must abide by it unless they could have made it plainly appear that he had been guilty of some gross fraud or partiality.*
> (Belchier vs. Reynolds, 1978)

In other words, the agreement to refer the dispute to an expert is as binding on both parties as any other term of their contract.

There is some precedent for predicting the court's responses to challenges for expert determination. First, the court would need to determine what parties have agreed to remit to the expert, that is, to what his or her terms of determination will be considered. If the expert has departed from his contractual instruction in any material respect, it will be open to either party to contend that the determination is not binding on the grounds that the expert had not done what he was appointed to do. If, on the other hand, the expert has carried out his contractual functions and instructions, then the parties would not have a basis for challenging his determination (Nikko Hotels (UK) Ltd vs. MEPC plc, 1991; Jones vs. Sherwood Computer Services Ltd, 1992). It is clear from these authorities that it is in everyone's interest to specify as clearly as possible in the terms of reference what they require the expert to undertake and if possible, the means by which they are to arrive at their determination. This will hopefully result in a consonance between the wishes of the parties and the means by which the expert achieves them. The process clearly has value in circumstances which lend themselves to independent determination, for example, safe use of chemicals, toxicological safety and so on.

9.5 Early Neutral Fact-Finding Expert

This simple process is not in wide use in the UK, but bears some similarity with 'shadow experts' under the CPR. A neutral expert investigates legal or technical issues and submits a nonbinding report. This may assist the parties to reassess their position and may assist the court if it is agreed between the disputants to be so used. Alternatively, another expert may be appointed for litigation purposes.

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The initial purpose of the neutral expert is to investigate the technical matters and report to the parties jointly. If the parties require, the neutral's role can be changed to that of a facilitator of subsequent negotiations. The process is suitable for cases with technical issues which would benefit from neutral expert appraisal and reporting. These may include occupational health and safety, product liability and related matters. It is not appropriate where there are no specialist issues requiring expert involvement. Neither is it appropriate where witness credibility is at issue and would render technical appraisal irrelevant.

9.6 Early Neutral Evaluation

This is a process which began in the USA in 1985. An experimental programme involved cases being selected and referred to a third party lawyer for evaluation some six months prior to the commencement of litigation. This process is rarely encountered in any formal sense in the UK, but it is used from time to time. The idea is somewhat analogous to the 'advisor' under the CPR in the UK.

The mandate of the early neutral evaluator is, not only to look at the strengths and weaknesses of the case, but also to consider how best to conduct the litigation rapidly and economically.

Initially the scheme was designed to force parties to confront the merits of the case more carefully and to develop an efficient appraisal to the issues and to discovery. Facilitation of a settlement was later added to the scheme. The evaluator was given the authority at the evaluation session to help the parties, in joint or separate meetings, to explore the possibilities of settlement. Another function is to consider alternatives to litigation to help in resolving the case effectively and expeditiously. The material used in the evaluation is 'privileged', as is the record of discussions in sole or joint sessions.

Positive benefits include making parties and their lawyers examine the case more effectively and systematically, giving them a better understanding of the issues, improving communications and creating opportunities for early settlement discussions.

In the UK, it is rarely used under the description of the early neutral evaluation. However, it is occasionally used to deal with some disputes characterized, again, with high scientific and technical content. Communication of the science and technical issues by a credible expert can often diffuse the impact of the dispute.

10 CONCLUSIONS

This chapter has been a review of the areas in which the professional skills of the lawyer and toxicologist overlap. The result has been something of an eclectic mix. Some areas have been excluded, such as occupational health and safety legislation and in particular, the Control of Substances Hazardous to Health Regulations. The reason for their exclusion is that they are the province of laboratory managers rather than toxicologists in the formal sense. Similarly, although toxicologists can be very much involved in criminal law, fewer of them become engaged in this area unless they are forensic specialists and the nature of their tasks is fairly self-evident. The areas that have been covered reflect a fascinating area which can offer considerable challenge to the toxicologist interested in making toxicology truly a science that serves our community. It has been a cook's tour, but hopefully, with sufficient depth for the interested reader.

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Space Toxicology: Toxicological Risk Management of Human Health during Space Exploration

Noreen N. Khan-Mayberry and John T. James

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1 INTRODUCTION

We leave as we came, and God willing as we shall return, with peace and hope for all mankind.
Eugene Cernan (Commander of last Apollo mission), December 17, 1972

Human exploration of space came at just the right time for many of us to be real-time witnesses to the achievement of placing human footprints in the ancient dusts of the lunar surface. For millions of earthlings, this achievement inspired us and radically changed how we viewed the moon. This sojourner of the night was no longer the mysterious, unreachable orb that it had been since man first looked skyward. It had become part of who we were and where we could go; it had become a target for human exploration. After the remarkable series of Apollo missions in the late 1960s and early 1970s, the space-faring nations failed to seize the momentum and pulled back to achieve a series of low-earth-orbit missions. These missions were most remarkable for the bridges that were built between nations that were not politically similar. The first of these was the Apollo–Soyuz demonstration project in 1975, and much later this was followed by the Shuttle–Mir (see **Figure 1**) missions of the late 1990s, and now the International Space Station (ISS) has become an outpost for many countries to explore the cosmos. Most recently, mankind's heart has returned to the romance and challenge of walking again on the moon and reaching out for the first time to another planet.

The unfolding of human space exploration involves many serious risks to those bold enough to step forward and become astronauts or cosmonauts. More often than we would like, those risks have turned into tragedy before the eyes of millions of people watching launches and landings on television. Among the risks that must be managed in space are those of a harmful exposure to toxic compounds such as carbon monoxide (CO), fixatives or reactive dusts. Toxic compounds have presented major problems during a few missions. These include the accumulation of carbon dioxide (CO_2) during the ill-fated Apollo 13 mission, the exposure of the Apollo crew to nitrogen tetroxide during the descent of the capsule, the fires aboard Mir in the late 1990s and the noxious products from regeneration of the metal-oxide canisters aboard the ISS.

Space toxicology has much in common with its parent discipline; however there are unusual facets of the subdiscipline of space toxicology that distinguish it from other subdisciplines:

1. Exposures to compounds in space are continuous and can go on for months from the accumulation of slowly released toxicants or from accidental releases of compounds that are difficult to remove from the air. We must also pay attention to the possibility that toxic compounds can be created in the air revitalization systems (ARSs) from relatively less harmful compounds.
2. Astronauts undergo an array of physiological changes that make them potentially more susceptible to the effects of toxic compounds. These may be caused by reduced gravity, stress or isolation. The space-adaptation effects include loss of red-cell mass, loss of bone, cardiovascular sensitivity and effects on the immune system. In addition, radiation exposures can be much greater during spaceflight when the mission goes beyond low-earth orbit.
3. Exposures to compounds in space are often continuous for the duration of the mission; however, routine activities or accidents can elevate exposures for a period of hours or a few days. Exposures are best viewed as a steady-state component perturbed by episodes of elevated pollutants. The crewmembers have extremely limited escape options when compared to earth-based populations, although there is always a contingency for abandonment of the vehicle if the risk to the crew were clearly life-threatening. Submariners are the only earth-based population with similar exposures to space crews, and their escape options are also somewhat limited.
4. Space habitats and vehicles are equipped with systems to scrub the air, and also recover and purify the water. These air and water systems are intimately linked because water-soluble air pollutants are inevitably recovered in the water and these pollutants must be removed before the crew consumes the water. Certain air pollutants can poison filter systems in either the air scrubbers or the water purifiers, so the space toxicologist must be alert to this possibility.
5. The ethnic and racial population of humans undertaking space missions has broadened to include almost all earth-based groups. With the noteworthy exception of the historic flight of Senator John Glenn at the age of 77 years, all persons launched into space have been middle-aged, and known to be in good health. The ethnic diversity means that the space toxicologist must be aware of the possibility that certain crewmembers may be unusually susceptible to pollutants. On the other hand, the fact that all crewmembers are healthy adults and not pregnant gives the space toxicologist a bit more flexibility in interpreting exposures that on earth could be harmful to the elderly, the unborn or the infirm.

In this chapter, we provide a resource of information for toxicologists interested in the field of space

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toxicology, as well as those with a special interest in the future of space exploration.

2 HISTORY OF SPACE TOXICOLOGY

> *We choose to go to the moon. We choose to go to the moon in this decade and do the other things, not because they are easy, but because they are hard, because that goal will serve to organize and measure the best of our energies and skills, because that challenge is one that we are willing to accept, one we are unwilling to postpone, and one which we intend to win, and the others, too.*
>
> President John F. Kennedy (35th President, United States of America)

The National Aeronautics and Space Administration (NASA) recognized from the earliest days of human spaceflight that maintenance of air quality required stringent control of materials (Osbon, 1963) and that standards for two weeks of continuous exposure were needed for lunar missions (Edgerley, 1964). Within a few years, the Space Science Board had set provisional limits on a handful of compounds for emergencies (one hour exposure), 90 days of exposure and 1000 days of exposure (NAS (National Academy of Sciences), 1968). The documentation of each value was limited. A much longer list of compounds was considered by the Panel on Air Quality in Manned Spacecraft for exposures of one hour, 90 days and six months (Committee on Toxicology, 1972); 10 minute, 'special area', exposures were set for six compounds. These limits were suitable for missions to the lunar surface and for prolonged stays in Skylab, which orbited the earth in the 1970s; however, as the concept of a reusable, earth-orbiting space-shuttle vehicle matured, limits were established for seven and 30 days of exposure based on recommendations from the National Academy of Sciences (Dietlein, 1976).

In the 1990s the approach to setting spacecraft limits was expanded to include one hour and 24 hour exposure guidelines for contingency (emergency or accidental) exposure scenarios. NASA asked the National Research Council (NRC) to form a committee specifically for spacecraft guidelines. The committees formed were first the Subcommittee on Spacecraft Maximum Allowable Concentrations (SMACs) for air standards, and later the subcommittee on Spacecraft Water Exposure Guidelines (SWEGs) for water consumption. These committees consist of appropriate peers in air and water quality from various discipline specialties within the field of toxicology and human-risk assessment. While NASAs space toxicologists set the air-exposure values, the NRC subcommittee on SMACs stringently reviews and works in collaboration with NASA toxicologists, who present and defend all values to the committee, before approval and publication. During this process, long-term chronic exposure limits expanded from seven and 30 days to include 180 days and, in the new millennium, 1000 days air exposure values were added, to respond to longer-duration missions. NASA toxicologists later endeavoured to develop water-consumption guidelines. These water guidelines are also peer reviewed and formalized using the Subcommittee on SWEGs. The water consumption standards are one day, 10 days, 100 days and 1000 days.

Figure 1 Space Shuttle on launch pad preparing for launch to the International Space Station. (Reproduced from NASA.)

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3 THE DISCIPLINE OF SPACE TOXICOLOGY

Perchance, coming generations will not abide the dissolution of the globe, but, availing themselves of future inventions in aerial locomotion, and the navigation of space, the entire race may migrate from the earth, to settle some vacant and more western planet.... It took but little art, a simple application of natural laws, a canoe, a paddle, and a sail of matting, to people the isles of the Pacific, and a little more will people the shining isles of space. Do we not see in the firmament the lights carried along the shore by night, as Columbus did? Let us not despair or mutiny.

Henry David Thoreau

What is the difference in the effect, once it is encountered by a space-faring explorer as opposed to someone on Earth? Once man decided to venture past our protective earth's atmosphere and explore space, astronauts would interact with chemicals (liquid, vapour or particulate). Thus, NASA would find it a great medical and life-support technology challenge, as it still does, to sustain life and human functions in an optimal manner for the tasks required for space exploration (Gardner, 1999). While NASA had early space guidelines for air quality, in the 1990s NASA began to consider how the mode of action of a chemical would differ when introduced into a physiologically altered human. Exploring beyond the outer atmosphere and gravitational field of our planet to the moon and throughout our solar system presents us with a number of complex and unique environments. Many of these distinct worlds all present a rich collection of chemical, geological and possibly biological systems that we journey to discover and explore, not only for the sake of curiosity, but for the desire to gain further scientific understanding of a vast and unexploited milieu. To accomplish the intricate and exciting goals of man working and living in space, space toxicology has materialized with the primary objective of protecting man from potentially harmful environmental contaminants that are inevitable in the living and working environment of astronauts. The following are key considerations of space toxicology, some of which may also apply to the parent discipline of toxicology:

1. Chemicals, chemical by-products and their mixtures must come in contact with tissue(s) and or organ(s) for which they have specific ability to cause damage (Ballantyne *et al*., 2000).
2. Chemicals behave differently in zero-gravity and reduced-gravity environments, which often dramatically increases the ability of a chemical to come into physical or structural contact with tissue(s) and/or organ(s).
3. The observed toxic effect or end point should be quantitatively related to the degree of exposure (concentration and duration) to the chemical(s) or chemical mixture(s). (Ballantyne *et al*., 2000).
4. The target of space toxicology studies and assessments is to determine the potential for harmful effects in the human that is living in an altered physiological state in a closed-loop environment for time-based exposures.
5. Acceptable risk criteria set by NASA for human spaceflight are also considered for off-nominal or accidental acute exposures.

Space toxicology is a unique and targeted discipline for spaceflight, space habitation and occupation of celestial bodies, including planets, moons and asteroids. Astronaut explorers face distinctive health challenges and have access to limited resources for rescue and medical care during space operations. The inherent changes in health caused by spaceflight, such as stress factors, anaemia and bone loss may also modify overall susceptibility to toxic effect. The ultimate goal of space toxicology is to protect the health of the astronaut by assessing potential chemical exposures during spaceflight and setting safe limits that will protect the astronaut, in a physiologically altered state, against chemical exposures.

When you look at the stars and the galaxy, you feel that you are not just from any particular piece of land, but from the solar system.

Kalpana Chawla (late US Astronaut STS-107)

The space environment is quite distinctive from our Earth's environment. Space is a near-perfect vacuum allowing stars and planetary bodies to move in accordance to gravitational forces on various scales. The atmospheric protection found on Earth is not present in the space environment. These and other distinct environmental conditions of space influence the potential toxic effect of chemical exposures and complicate the steps that must be taken to effectively maintain astronaut health. Altered gravity (reduced or zero gravity) and the closed-loop living environment further complicate the task of safeguarding crew health, since the habitat (vehicular or settlement) must be carefully regulated and monitored so that harmful amounts of toxicants do not build up within the enclosed volume. Keeping the quality of air and water consumed safe and healthy is an increasingly complex task as longer-term space expeditions are undertaken.

Astronaut explorers are a diverse group selected from the world population. NASA stringently screens the health of potential crewmembers prior to induction into the astronaut corps. This elite group of healthy individuals is selected to withstand the physiological, mental and environmental changes that are inherent to space exploration. Prior to selection for specific spaceflights,

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individuals are screened for susceptibility biomarkers with respect to mission specific and/or innate adverse reactions resulting from spaceflight adaptation. One example is an increase in probability of renal-stone formation during space habitation; therefore individuals with a history of renal stone formation may be excluded (James, 2008). The health screening process minimizes the interindividual variability among this population.

The fundamental nature of space toxicology is that it is a discipline concerned with studying and predicting the potential of chemicals and chemical mixtures, in air and water, to produce harmful effects in humans, in an altered gravity environment. Space toxicology is a study of the interaction between chemicals in microgravity and humans in order to determine the potential for chemical(s) to produce injury which results in adverse health effects and to investigate the type, frequency, mechanism of action and production factors influencing their development and pervasiveness and the permanence or reversibility of these adverse effects. The definition of this discipline may be stated succinctly; however, implementation of this science can be complex.

The space toxicologist will assess the potential for adverse health effects by taking into consideration the amount of individual chemicals, as well as the content of each chemical in mixtures, human physiological changes, route of exposure, chemical by-products (if these by-products are formed prior to exposure, such as pyrolysis products) and potential contact time in order to assign toxicity hazard rating. All chemical-containing materials must be assessed before they are allowed to be utilized during spaceflight operations. The broad scope of chemical-containing materials, including liquids, gases and particulates, and types of chemical exposure scenarios, must all be deliberated in order to assign toxicity assessment values, as well as in setting spaceflight standards. Spaceflight standards are set for a number of chemicals for human exposure in air and water. These standards are time-weighted averages and consist of short-term and long-term permissible exposure guidelines. These guidelines should not be exceeded during nominal space operations, since they are intended to protect astronauts from acute and chronic health effects.

4 TOXICOLOGY STANDARDS FOR HUMAN SPACE EXPLORATION

Since the beginning of manned spaceflight, NASA has considered the overall health of astronaut crew members as vital for individual performance and well-being, as well as overall mission success. This was the impetus for the birth of space toxicology. Humans interacting with chemical constituents in enclosed or closed-loop environments are intrinsic to spaceflight; therefore these chemical exposures must be controlled and maintained at a level that is not detrimental to crew health. Despite engineering efforts, hundreds of chemicals will potentially be encountered in the closed-loop environment during spaceflight. Some chemicals that must be considered are those produced by the crew members, as well as those originating from vehicular components, payload experiments and use of utility compounds.

Most environmental pollutants in spacecraft do not have appropriate regulatory limits. NASA cannot apply earth-based standards to spaceflight since they do not consider the various physicochemical changes of spaceflight. Therefore when setting spacecraft standards, the space toxicologist must prioritize amongst the chemicals that crewmembers are likely to be exposed to and develop a standard that can be applied to acute and chronic exposures.

4.1 Spacecraft Maximum Allowable Concentrations

The first time the spacecraft maximum allowable concentrations (SMACs) were compiled into a formal NASA publication, the document only listed the seven-day SMAC values and did not provide any rationale for these concentrations (Coleman and James, 1990). As the Space Station Programme was being defined and redefined, it became apparent that exposures of 180 days could be anticipated, so NASA once again turned to the NRC for assistance in setting values for contingencies (one hour and 24 hours) and long-duration exposures up to 180 days (James, 1995). The need for contingency SMACs was validated because real-time, on-board air analysers were being developed to quantify pollutants accidentally released into the air (Limero *et al*., 2000) or from accidental combustion of polymers (Beck *et al*., 1991). A combination of short-term exposure limits and on-board analysers, along with flight rules, facilitated the management of toxicological events when they occurred. An array of SMACs was set for approximately 50 compounds and thoroughly documented in four volumes from the NRC (National Research Council (NRC), 1994; National Research Council (NRC), 1996b; National Research Council (NRC), 1996a; National Research Council (NRC), 2000a). A few years ago it was recognized that revised standards were needed for many compounds and 1000-day standards were essential in anticipation of long duration missions to Mars, hence a fifth volume containing revised values and 1000-day SMACs was published (National Research Council (NRC), 2008).

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4.2 NASA Acceptable Risk for SMAC Values

For contingency chemical release events one and 24 hour SMACs are utilized; NASA has defined acceptable risk as a concentration of a substance in air that may be tolerated during the performance of specific tasks during emergency conditions lasting for less than one hour or less than 24 hours (National Research Council (NRC), 1992; Khan-Mayberry, 2008b). The one and 24 hour SMACs could include reversible effects that do not impair judgment and do not interfere with proper responses to the emergency, such as shutting a valve, closing a hatch, removing a source of heat or ignition, or using a fire extinguisher (National Research Council (NRC), 1992; Khan-Mayberry, 2008b). For example, exposure at the one and 24 hour levels may produce effects such as increased respiratory rate from increased CO_2, headache or mild central nervous system (CNS) effects from CO, and respiratory tract and eye irritation from ammonia or sulphur dioxide (National Research Council (NRC), 1992; Khan-Mayberry, 2008b).

In contrast to one and 24 hour SMACs, which are intended to guide exposures during emergencies, SMACs lasting up to 180 days, and now 1000 days, are intended to provide guidance for operations during those time periods (180 days in an environment like the Space Station and 1000 days for extended stays on the lunar, Martian or other planetary surfaces) including transit time. Accounting for accumulation, detoxification, excretion and repair of toxic injuries is important in determining long-term SMACs (National Research Council (NRC), 1992; Khan-Mayberry, 2008b). Whether a material has a cumulative effect must be taken into account for long-duration SMACs. Neuropathologic regeneration or repair of toxic injuries occurs more readily in intermittent than in continuous exposures, making repair important in setting long-term SMACs (National Research Council (NRC), 1992; Khan-Mayberry, 2008b).

4.3 Spacecraft Water-Exposure Guidelines (SWEGs)

Water is a precious resource during human spaceflight and as NASA attempts to recover as much water as possible from humidity condensate and urine, the need for spaceflight-specific standards became apparent. NASA had been using the Public Health Service Environmental Protection Agency's maximum contaminant levels (MCLs) as guidelines for definition of acceptable water quality; however, experts agreed that unique limits were needed for people engaged in spaceflight because of the different exposure conditions in space (National Research Council (NRC), 2000a). Some of the problematic compounds in spacecraft water were of no concern for earth-based populations, so limits had never been set for these compounds. In cooperation with the NRC, NASA has developed spacecraft water-exposure guidelines (SWEGs) for approximately 30 compounds for ingestion periods of one day and 10 days, contingency values, along with 100 and 1000 days for long-term ingestion (National Research Council (NRC), 2000b). These should not be applied to earth-based activities and are not appropriate, because the astronaut population is to a good extent different than earth-based populations and the risks acceptable to NASA tend to be quite different than for other populations. At present, the only water component with a SWEG that can be quantified in orbit is total organic carbon, but NASA plans to be able to monitor many more compounds during exploration missions involving prolonged stays on distant celestial bodies. Presently, the SWEGs serve as design goals for engineers as they develop water purification systems to ensure a supply of potable water throughout missions.

4.4 NASA Acceptable Risk for SWEG Values

For contingency events one and 10 day SWEGs are utilized; NASA has defined acceptable risk as a consumption of a chemical dissolved in water that may be tolerated during the performance of specific tasks during rare emergency conditions lasting for time periods up to 24 hours or 10 days contingent upon the specific event (National Research Council (NRC), 2000a). The one day SWEG is expected to prevent irreversible hazardous health effects and degradation in crew performance. This contingency value allows for temporary discomfort providing there is no adverse effect on judgement, performance or the ability to respond to an emergency. The longer-term SWEGs are meant to prevent immediate or delayed adverse health effects and degradation in crew performance that would result from continuous exposure for up to 1000 days. These long-term SWEGs are written to provide guidance for oral (consumption) exposure during nominal operations (National Research Council (NRC), 2000a).

5 IDENTIFIABLE SOURCES OF TOXICANTS

There are a substantial number of particulate and volatile space contaminants that have been identified and quantified by NASA's toxicology laboratory. Chemicals that are of greatest concern when assessing for potential toxicity are those that target organs or organ systems

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that are already compromised by spaceflight-induced physiological changes. The primary sources of contaminants come from human metabolism and materials off-gassing. Off-gassed components are from nonmetal surfaces which contribute steady low-level compounds to the breathable airspace. This chronic exposure is of particular interest to the space toxicologist, since the closed-loop environment does not provide ventilation of the air to the external environment. Acute exposures from evolving off-nominal catastrophic events such as fires, accidental release of chemicals from instrumentation or payload experiments may occur. Utility compounds such as lubricants, adhesives and personal hygiene products also contribute to the array of air pollutants. Therefore chemicals brought into the space environment are assessed for potential toxicity prior to approval for spaceflight use.

5.1 Metabolic Sources of Contamination

The enclosed volumes of inhabited space vehicles and habitats may contain 'pockets' of obstructed flows and poorly ventilated areas. This amplifies the concern for concentrated areas of isolated chemical release. The crewmember could potentially enter a 'pocket' inadvertently and receive an acute adverse exposure if the environment is not properly controlled. Metabolism is usually the greatest source of low-level contamination aboard spacecraft (Coleman and James, 1994). Potential opportunities for build-up of toxic levels of metabolic contaminants occur during nominal space operations, such as maintenance activities in restricted or confined areas, gathering of several crewmembers in small areas to perform experiments and other day-to-day activities.

CO_2 is the most commonly thought of contaminant introduced into the cabin atmosphere as a result of metabolic production. According to calculations by NASA (National Aeronautics and Space Administration) (1987) each crewmember produces 0.76–1.15 kg (1.67–2.58 lb) of CO_2 per day, contingent upon physical activity (operational tasks and exercise) (Coleman and James, 1994). Production of CO_2 in humans is through oxidative metabolism of carbohydrates, fatty acids and amino acids (Wong, 1996). This CO_2 is quickly released into the space environment via respiratory elimination.

Metabolically manufactured acetaldehyde is also introduced into the space habitat or vehicle. It was estimated that acetaldehyde is produced at a rate of about 83 μg day^{-1} per human (Olcott, 1972; Wong, 1994b). Acetaldehyde is oxidized primarily in the liver to acetic acid by aldehyde dehydrogenase, aldehyde oxidase and xanthine oxidase (Wong, 1994b).

Ammonia is introduced into the closed-loop volume via metabolism. While NH_3 is mainly eliminated in urea, a minor elimination pathway is via exhalation (Wong, 1994a). Ammonia production occurs by catabolism of amino acids, amines, nucleic acids, glutamine and glutamate in peripheral tissues and from dietary amino acids (Cornell, 2008). High-protein meals and stringent exercise can increase ammonia production and metabolism (Cornell, 2008) and should be considered if NH_3 levels ever become elevated without a known source of contamination.

There is no way to prevent these types of contaminants from entering spacecraft or space habitats, so the ARS Air Revitalization System is utilized to sustain good air quality, as well as utilizing preventative measures, such as limiting how long crew members are in small, tight or confined spaces.

5.2 Off-Gassing

An off-gassed product is a compound evolved as a gas from a material or assembled article. Polymeric materials are known to release gases via breakdown of the polymer and volatilization of the products, or through the liberation of gases trapped during the process of manufacturing or cleaning of materials (Coleman and James, 1994). In order to minimize the risk of toxic contamination to the crew by flight articles, NASA requires that off-gas acceptability testing be performed as a mandatory step before flight acceptance is conferred (NASA (National Aeronautics and Space Administration), 1991b).

Alcohols, aldehydes, aliphatic hydrocarbons, CO, chlorinated hydrocarbons and siloxanes may be a result of materials off-gassing (Coleman and James, 1994). At the end of an off-gas test (72 hours, 120°F) the atmosphere of the sealed test chamber is determined by analytical instrumentation that allows for separation, identification and quantification of all off-gassed products according to NASA standards. An individual flight article, the assembled flight articles or multiple numbers of a flight article are determined for acceptance by their toxic hazard index or toxicity (T) value. The T value is defined as, 'the dimensionless ratio of the projected concentration of each off-gassed product to its seven day SMAC value and summing the ratios for all off-gassed products without separation into toxicological categories' (International Organization for Standardization, 2000).

The T-value calculation:

$$T_{\text{total}} = C_1/\text{SMAC}_1 + C_2/\text{SMAC}_2 + \ldots + C_n\text{SMAC}_n \quad (1)$$

C_1 = concentration of contaminant 1 and SMAC_1 = 7d SMAC of contaminant 1.

The T_{total} for any test item should not exceed 0.5 if it is to be deemed acceptable for flight (Coleman and James, 1994; International Organization for Standardization, 2000). If the test article does not meet this criterion,

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it may undergo treatment to reduce off-gassing levels, such as 'baking' the article at an elevated temperature before undergoing additional off-gas testing. NASA can also assign a rating that allows a certain mass of the material to be used. NASA may opt to replace the failed material with another type of material that has passed off-gas requirements.

In years past, NASA has attempted to summarize the off-gassing from all materials inside a module, but this has proven to be inaccurate. Beginning with the ISS, NASA has tested each US module on the ground for aggregate off-gassing. This is because first entry into sealed modules that are attached to the ISS can pose a distinct toxicity hazard. While modules are assembled with materials determined to be safe by off-gas testing, once assembled, new volatile chemicals could be introduced via adhesives, sealants or other chemicals used in assembly process, therefore it is necessary to off-gas-test assembled modules with at least 75% by mass of its launch contents. These modules are also sealed for many days (typically 100 days) prior to launch. The off-gas test should be at least one fifth of the time between the last clean air purge and the first entry of the crew. Results of a whole-module off-gas test can be used to predict the level of pollution the crew will encounter when first entering the module.

5.3 Payload Chemicals

Chemicals are also introduced into the habitable space environment through payload experiments. Potentially toxic or known toxic chemicals are routinely employed in biological and chemical studies. There are a vast number of chemicals used in payloads such as buffers, fixatives, acids, preservatives and proteins. The space toxicologist must assess every chemical contained in a payload and assign a toxicity hazard level (THL) value based upon its inherent toxicity, the amount that could possibly be released into the cabin volume and the ability of the crew to remediate the contaminant from the area.

5.4 Thermodegradation

Even a small fire or pyrolysis event can pose a serious and frightening hazard inside a sealed spacecraft. NASA utilizes fire-resistant materials on its spacecraft, especially in the insulation of wiring; the most commonly used insulations are Teflon and Kapton. While unlikely to burn, these polymeric materials pose unique concerns due to the highly toxic materials that are released during pyrolysis. These highly toxic chemicals include hydrogen cyanide (HCN), hydrogen fluoride (HF), hydrogen chloride (HCl), CO and nitrogen dioxide (NO_2). While NASA has restrictive flammability standards in place to safeguard against the introduction of thermodegradation contaminants, most nonflammable materials that undergo smouldering from high heat will release highly toxic pyrolysis products (NASA (National Aeronautics and Space Administration), 1991b; Coleman and James, 1994). It is noted that in the absence of gravity, convection does not occur, so combustion products are not transported from the fire and combustion events may be oxygen-poor leading to high production of CO.

Despite best efforts during spaceflight, there are a few examples of pyrolysis products being released. Arcing and pyrolysis of wire insulation made of Kapton and Teflon sleeves; electrical-wire fusion of Kapton–poly tetrafluoroethylene electrical-wire insulation; thermodegradation of electronic components of data display units, and thermodegradation of Delrin housing and Stepan foam insulation of a refrigerator unit. The refrigerator incident in particular caused the crew members to temporarily seal off this area and enter a different module for fresh air (Coleman and James, 1994). Fortunately none of the documented thermodegradation events released life-threatening levels of toxic products; however, they highlight the concern of potential hazards associated with these types of incidents (Coleman and James, 1994).

5.5 Combined Effect of Radiation

Radiation itself can be a toxic hazard to space explorers. High levels of ionizing radiation can have deleterious and even fatal effects if not properly managed (Eckart, 1996; Gardner, 1999). Radiation has the ability to potentiate the toxicity of volatile and other airborne chemicals (Gardner, 1999). Radiomimetic chemicals and drugs must be carefully evaluated during spaceflight (Coleman and James, 1994). Much more research on radiation effects on chemicals in microgravity is needed in order to fully elucidate radiation's role in the potential toxic effect of chemicals in physiologically compromised astronauts.

6 UNUSUAL PHARMACOKINETICS OF COMPOUNDS IN ASTRONAUTS

Absence of gravitational forces (or altered gravity) must be considered when assessing pharmacokinetics, chemical reactivity, behaviour and settling times (particulate). Studies have shown that chemical kinetics are altered during microgravity (Berinstain *et al*., 1999). Due to the known physiological changes in the body resulting in changes in fluid shift, organ shape and enzymatic activity, it can be expected that pharmacodynamics (measurement of quantifiable drug responses as a function of drug concentration) and pharmacokinetics (time course of a

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chemical/drug and its metabolites in the body) of chemical compounds would be altered Derendorf (1994). It is stated by Levy (1991) that studies of pharmacodynamics of drugs on animal and human test subjects subjected to physiological perturbations will lead to an increase in the understanding of chemical reactions and the biological system. Derendorf (1994) further discussed the mechanisms that will affect drug efficacy including clearance (excretion), change in enzyme activity and renal changes, binding (absorption) to tissues and proteins, blood flow (distribution) and bioavailability (potential efficacy). These variables are arduous to predict (and a source of much debate amongst regulatory toxicologists) under normal terrestrial conditions, making the need for further research in this area very useful for long-term space exploration. In 2004, Graebe *et al*. summarized the current state of knowledge in regard to pharmacokinetics and pharmacodynamics. This article addressed the lack of data in these areas and summarized current procedures in place to predict the effect of drugs in microgravity. Prior to flight, astronauts test all medications that are frequently used during spaceflight before mission assignment to identify potential side effects, performance-related problems and individual therapeutic effects; this evaluation tool is not precise for determination of how these chemicals react in space, but they have proven to be useful in the absence of true data (Graebe *et al*., 2004).

7 ASTRONAUTS AS A SUSCEPTIBLE POPULATION

We hope someday, having solved the problems we face, to join a community of galactic civilizations.
President Jimmy Carter, 1977 (39th President of the United States of America)

Astronauts engaged in spaceflight must physiologically adapt to the absence of gravity and the stresses associated with intense work and isolation. Post-flight physiological evaluations of astronauts and flight experiences have revealed a number of ways that astronauts are changed from their earth-based health status. The mechanism of these changes is often not understood, but the target organ or system involved in change is clear. A potentially toxic compound that adversely affects the same system or organ may be more potent in space because of the spaceflight-induced changes. That is, the crew is more sensitive to the toxic compound than they would be on earth.

7.1 Haematotoxicants

Based on immediate, post-flight measurements after missions of varying duration, the loss of approximately 10% of red-cell mass occurs within a week of in-orbit operations and persists at this level of loss throughout the time a crewmember is in orbit (Huntoon *et al*., 1989). In setting SMACs, experts agreed that exposure of crewmembers to a compound that reduces red-cell mass or other red-cell parameters should not have an exposure limit as high as would be estimated for earth-based exposures. The mechanism of the spaceflight reduction in red-cell mass is not clearly elucidated and often the mechanism of action of a given haematotoxicant is unknown. Expert judgment suggested that a reduction of the standard for an earth-based exposure of threefold would be sufficient to protect astronauts in orbit, even with their spaceflight-induced loss of red-cell mass. This factor has been applied in setting SMACs for 2-ethoxyethanol, benzene, nitromethane, indole and isoprene.

7.2 Cardiotoxicants

Episodic reports of cardiac arrhythmias during spaceflight, and especially during extravehicular activities (EVAs), suggest that exposures to cardiotoxicants on orbit should be more constrained than for earth-based populations (Buckey and Buckey, 2006). Arrhythmias, such as preventricular contractions, have been reported during stressful times, such as during EVA, and also at times when the crew was known to be potassium-depleted (Leguay and Seigneuric, 1981). Such arrhythmias can have serious and sudden medical consequences; therefore, the experts engaged to set exposure limits determined that a factor of five was appropriate for reduction of any earth-based exposure standard for cardiotoxicants to one suitable for spaceflight. Compounds for which this factor was used include the following: bromotrifluoromethane (shuttle fire extinguishant), CO and several Freons.

7.3 Immunotoxicants

A number of changes in the immune system during and immediately after spaceflight have been demonstrated; however, these cannot be uniformly characterized as adverse effects. Methodological differences appear to play a major role in the outcome variations of research investigations and clinically significant functional changes have not been demonstrated (Borchers *et al*., 2002). Many factors may contribute to immune changes, including isolation, confinement, loss of gravity, radiation, sleep irregularities and poor nutrition (Sonnenfeld and Shearer, 2002). Postflight immune changes include altered cytokine production, natural-killer (NK) cell function, monocyte function and granulocyte function,

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whereas, in-flight studies have demonstrated reactivation of latent herpes viruses and altered cell-mediated immunity (Crucian *et al*., 2008). Experiments on animals flown in space and on humans in bed-rest studies have also contributed to our understanding of the potential effects on the spaceflight-stressed immune system. Collectively, the term immunologists use to describe these changes is 'immune system dysregulation'. Such effects clearly suggest that any exposure limit for an immunotoxicant ought to be reduced to compensate for the possibility that an astronaut's immune system is compromised. The safety factor for this concern has been set at three by NASA and the NRC Committee on Toxicology.

7.4 Ethnic Diversity in Sensitivity to Toxicants

Science, for hundreds of years, has spanned the differences between cultures and between countries.
Laurel Clark (US Astronaut)

Human spaceflight involves persons from a wide variety of racial and ethnic groups. In setting exposure limits for certain toxicants we must account for differences that could make certain groups more susceptible to those toxicants. NASA does not use any general safety factor for intraspecies variability, as some agencies do, because the astronauts comprise a healthy, middle-aged population; however, factors are used when data show that a specific subpopulation is sensitive to the toxic effects of a compound. When the SMACs were set for ethanol, the issue of the 'flushing response' that occurs in certain Asian subpopulations after oral consumption of ethanol was considered as a separate end point (James, 1996). This enabled the SMAC to be protective of this subpopulation of astronauts. For other compounds a factor of three has been used when a sensitive subpopulation is known or postulated to exist. For example, the formaldehyde SWEG was reduced by a factor of three due to the potential for increased sensitivity of a subpopulation related to genetic polymorphisms in formaldehyde dehydrogenase (McCoy, 2007).

8 AIR REVITALIZATION AND WATER RECOVERY

The air- and water-quality systems are critical for ensuring the safety of air and water. As stated by Waligora *et al*. (1994), astronauts are not expected to dedicate significantly more time towards life support activities than they would on Earth; therefore the broad goal in the development of life support systems is to provide an unassuming, reliable unit that requires minimal crew involvement.

8.1 Air Revitalization System

Air quality is of particular importance for protecting the health of astronaut explorers in a closed loop. Coleman and James (1994) describe air 'scrubbing' as having particular importance after a release of toxic chemicals or after thermodegradation. The ARS is the primary tool for keeping air pollutants at safe levels and plays a pivotal role in health, safety and mission success. NASA has a long-standing process for the development for the ARS that has been utilized on board manned spacecraft and space habitats such as Skylab and the ISS (NASA (National Aeronautics and Space Administration), 2008b). Several options exist for providing a practical design for the ARS, each option affects crew comfort and safety, reliability, weight and cost (Waligora *et al*., 1994). Multiple iterations of the ARS have been engineered throughout the history of manned spaceflight, from Project Mercury through to the ISS. All of the ARS systems show a spectrum of process technologies and hardware for purification and management of spacecraft cabin atmospheres (NASA (National Aeronautics and Space Administration), 2008b). NASA describes the primary processes for air revitalization or scrubbing as separation or reaction processes. Separation processes that have been employed by NASA include physical adsorption, absorption and filtration. Chemical adsorption, oxidation, reduction and electrochemistry are some of the reactive processes employed. The major function of the ARS is removing trace contaminants such as volatile organic compounds (VOCs), particulate matter (PM) and CO_2 from the cabin atmosphere. The ARS serves as resource recovery and recycling, and atmospheric gas production, distribution and storage, such as CO_2 reduction to methane and carbon products, and atmospheric gases from *in situ* resources (NASA (National Aeronautics and Space Administration), 2008b).

The course taken for the removal of CO_2 during the Mercury, Gemini, Apollo and Shuttle Programmes employs consumable LiOH canisters. The current US Shuttle vehicles, use two LiOH canisters for CO_2 absorption. Each canister contains 2.3 kg of LiOH and at least 85 g of charcoal (Coleman and James, 1994; NASA (National Aeronautics and Space Administration), 1989). The LiOH canisters, in addition to CO_2, are also able to adsorb HF, HCl, HCN, NO_2 and other gases produced by fire (Coleman and James, 1994). The charcoal in these canisters traps VOCs released by crew metabolism and off-gassing. These canisters must be replaced at 6–12 hour intervals depending upon the size of the crew

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(Coleman and James, 1994). NASA has operated molecular sieves regenerated by vacuum swing and/or thermal swing for CO_2 removal during the Skylab and ISS Programmes (NASA (National Aeronautics and Space Administration), 2008b). NASA has gained some insight through the use of immobilized amines that are regenerated by a vacuum swing process. However, even though the process has been demonstrated on multiple Shuttle missions, this procedure is still being studied and has not been employed for routine use on manned spacecraft (NASA (National Aeronautics and Space Administration), 2008b).

Granular activated carbon (GAC) and a variety of oxidation catalysts is used by NASA for the removal of trace chemical contaminants. The GAC is normally derived from coconut shells. Specialized forms of GAC are specially treated to remove contaminants such as NH_3 and CH_2O (NASA (National Aeronautics and Space Administration), 2008b). GAC, along with thermal catalytic oxidation, is used to eliminate CH_4 and CO for long-duration missions. Shorter missions employ an ambient-temperature catalytic oxidizer (NASA (National Aeronautics and Space Administration), 2008b).

Mechanical filtration is utilized to remove PM commonly found during spaceflight. The ISS utilizes high-efficiency particulate air (HEPA) filters that remove respirable range particles while the shuttle uses a filter rated for 40 microns nominal and 70 microns absolute size particles (NASA (National Aeronautics and Space Administration), 2008b).

8.2 Water Recovery System

Clean water is vital for the protection of crewmembers from waterborne contaminants found in potable and hygiene water. For long-duration missions, it would be impossible to carry all of the water needed by crewmembers living in space, therefore the water revitalization and recovery systems are in place to supply this precious commodity. During early spaceflight missions, water supplies were transported on board for short-duration flights during the Mercury and Gemini eras. Apollo flights introduced water created as a by-product of electrical power fuel cells. This water was used for consumption and food reconstitution; however, personal hygiene was kept to a minimum (Waligora *et al*., 1994; Sauer and Calley, 1973. Skylab did not employ fuel cells, since the mission length did not make their use feasible. The Shuttle uses fuel cells to supply water for consumption, food reconstitution and personal hygiene (Waligora *et al*., 1994). Recycled water is needed for housekeeping, personal hygiene and consumption; each of these tasks increase in criticality as flight duration increases (Waligora *et al*., 1994). The water required is estimated at 2.2 l per person per day for drinking, hygiene and food preparation, 1 l per person per day for water regeneration and up to 0.3 l per person per day for flushed water; these basic needs require the capability to recover and recycle safe water during sustained presence in space. Water reclamation systems are designed to supply specified amounts of water at a specific quality (National Research Council (NRC), 2000a).

NASA has found that the hundreds of chemicals identified in the closed-loop atmosphere may end up in the recycled water. The in-orbit water recovery hardware on the ISS has humidity condensate collection systems, as well as equipment for the recovery of humidity condensate (NASA (National Aeronautics and Space Administration), 2008a). All of the CO_2-removal equipment in use on the ISS has been engineered towards working in harmony with the optimal closed-loop ARS; this system operates by reacting CO_2 with H_2 produced by an onboard water-electrolysis unit. The interaction between these processes recovers a significant amount of water (NASA (National Aeronautics and Space Administration), 2008a). Future plans for urine treatment include regenerative environmental-control life-support system (ELCSS) hardware. This equipment will be deployed on the ISS and will have the capacity to increase the recovery of wastewater by processing pretreated urine in a vapour compression distillation (VCD) subsystem (NASA (National Aeronautics and Space Administration), 2008a). This water processing assembly (WPA) will treat urine distillate and humidity condensate via combined adsorption and ion-exchange processes, and thermal catalysis. Once in use on the ISS, NASA estimates that approximately 93% of wastewater will be recovered to potable standards, leaving the remaining wastewater as a concentrated brine solution (NASA (National Aeronautics and Space Administration), 2008a).

9 SPACE TOXICOLOGY ASSESSMENTS

Space toxicology combines our understanding of the adverse health effects of environmental pollutants with our conception of how these pollutants behave differently in space and how humans may be more sensitive to exposures during times of space exploration. Our goal when assessing toxicity is to manage the health risks that would potentially be derived from toxic exposures during spaceflight, habitation of outposts and exploration of celestial bodies. Within this century we hope this will include a sustained presence on our moon, followed by placing explorers in outposts on Mars, the tiny moons of Mars and perhaps nearby asteroids. Toxicological risks must be assessed and managed within the context of extreme isolation, continuous exposures, reuse of air and water, limited rescue options and the need to use highly

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Figure 2 Astronaut conducting a spacewalk: the life-support system contains several batteries. (Reproduced from NASA.)

toxic compounds for propulsion. As we begin to explore other celestial bodies *in situ*, other toxicological hazards, such as inhalation of reactive mineral dusts, must also be assessed for risk.

Space toxicology assessments are risk-assessment tools used by NASA engineers, flight surgeons and flight directors to ascertain the potential toxicity of a chemical, chemical mixture or chemical by-product being flown in space. Data on chemical and biological materials to be flown in the pressurized volumes of habitable spacecraft and habitats are needed by space toxicologists to assess the toxicity and assign hazard levels (NASA (National Aeronautics and Space Administration), 2006). These levels may be used as a means to decide whether or not a chemical-containing payload will be safe enough to fly during manned spaceflight missions. It is up to the toxicologist to determine the chemical THL in the specified space vehicle or habitat module in which the proposed chemical(s) will be housed during spaceflight or space habitation. If this includes more than one risk scenario, each hazardous scenario must be assessed and rated separately. THLs are assessed only for chemicals, and the space toxicologist must understand the mission environment and be aware of all chemical hazards contained in the habitable volume of the spacecraft or space habitat in order to address the concurrent exposures to a variety of airborne chemicals.

Any apparatus that is scheduled to enter the pressurized or habitable volume and contains chemicals or off-gassed chemical contaminants must be assessed for health hazards. Payload experiments often contain toxic chemicals involved in materials processing, tissue fixation, fluid behaviour and other types of experiments (Coleman and James, 1994). The vast and varied numbers of chemicals that will be introduced into the habitable volume by a multitude of instrumentation and operational equipment, all required for spaceflight operations, must be evaluated for their potential to introduce chemicals in the closed-loop environment. Use of materials that have been deemed safe by off-gas testing is not always possible when designing new instrumentation or devices necessary for specific flight tasks. At times the toxicologist will also come across unusual toxicity requests for compounds not intentionally flown in space (e.g. CdO dust from corroded connectors).

Batteries contain toxic or corrosive chemicals that serve as electrolytes or active ingredients. Several batteries are used to power devices such as laptops and space-suit life-support systems (see **Figure 2**) aboard space vehicles and habitats. There are several hundred batteries currently in use on the ISS. Each type of battery in use must be assessed for toxicity. For the Space Shuttle, all toxicological assessments for batteries are housed in one NASA document (NASA (National Aeronautics and Space Administration), 1991a).

The THL rating scale is numerical and has unique criteria for each number. The scale ranks from 0 (nonhazardous) to 4 (catastrophic). A THL of 0, or nonhazardous, is defined in NASA guidelines as a chemical that may be a gas, solid or liquid, which may or may not be containable, that will cause zero to no greater than slight irritation that will last less than 30 minutes, which requires no therapy and causes no systemic effects.

The critical or level 1 THL is defined as a gas, solid or liquid, which may or may not be containable, that results in slight to moderate irritancy that lasts greater than 30 minutes and will require therapy. The systemic effects will be minimal with no potential for lasting internal tissue damage.

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All THLs rated 2–4 are considered catastrophic hazards and distinguished as follows. A catastrophic level 2 THL is either a solid or nonvolatile liquid that can be contained, cleaned and removed by crewmembers, which has no systemic effects and causes moderate to severe irritation that has the potential for long-term performance decrement and will require therapy. It also may cause permanent eye damage.

The catastrophic level 3 THL is a solid or nonvolatile liquid that can be contained, cleaned and removed by crewmembers, that would cause appreciable effects on perception, coordination, memory or the potential for long-term (delayed) critical injury (cancer) or may result in internal tissue damage. Irritancy alone does not constitute a level 3 hazard.

The highest THL, catastrophic level 4 is a gas, volatile liquid or fumes if released or escapes, that are not containable, that would result in moderate to severe irritancy. THL 4 exposures have the potential for long-term decrement of crew performance, would cause appreciable effects on perception, coordination, memory or the potential for long-term (delayed) critical injury (cancer) or may result in internal tissue damage. If the chemical is an eye-only hazard, there may be a risk of permanent eye damage. THL 4 exposures will require therapy for the affected crewmembers.

NASA is committed to not flying or limiting any toxicity level 4 hazards from being used in the crew's habitable volume during manned spaceflight. However, certain THL 4 chemicals may be deemed critical for spaceflight and therefore every effort is made to isolate THL 4 chemicals from the crewmembers during spaceflight.

The THLs have become essential for all manned spaceflight operations. These toxicity ratings are utilized by a number of groups throughout NASA in support of planning, design and decision-making for each mission. Hardware may be designed to ensure THL levels are not exceeded, payload developers must ensure that their payload does not introduce a catastrophic hazard into the habitable volume, safety engineers make containment and labelling decisions based in part upon THLs for the protection of ground personnel and crewmembers. NASA flight surgeons make preflight and real-time decisions for operations decisions and treatment, if necessary, of crewmembers in the event of off-nominal exposures and releases.

10 RISK-BASED ENVIRONMENTAL MONITORING

The space toxicologist will assess risk by taking into consideration the amount of individual chemicals or, if applicable, the content of each chemical in mixtures, human physiological changes, route of exposure, chemical by-products (if deemed appropriate) and potential contact time, in order to assign toxicity hazard rating. All chemical-containing materials must be assessed before they are allowed to be utilized during spaceflight operations. The broad scope of chemical-containing materials, including liquids, gases and particulates, and chemical exposure scenarios, must all be deliberated in order to assign toxicity assessment values, as well as in setting spaceflight guidelines or standards. Spaceflight guidelines/standards are set for a number of chemicals for limiting human exposure in air and water. These values are time-weighted averages and consist of short-term and long-term permissible exposure limits. These quantities should not be exceeded during nominal space operations since they are intended to protect astronauts from acute and chronic health effects.

10.1 Predictable Risks

Since we know the compounds present aboard any spacecraft, we can make plausible predictions about which of them might become a toxic hazard. At the present time NASA uses an array of instruments to manage predictable risks of air pollution in the ISS or Shuttle. These instruments provide real-time and archival (returned to earth for analysis postmission) monitoring of chemical contaminants. Propellants are monitored using a gold-salt method in the air lock (a small compartment through which astronauts must go to gain access to the vehicle exterior) in case some of this material contaminates the EVA suits and is brought back into the air lock (Bacal *et al.*, 2008). If propellants are detected, then flight rules dictate procedures for removing residual propellants, such as baking out in sunlight or brushing off the offending crystals. Ammonia can be monitored inside the ISS using detector tubes in the remote possibility that this heat-exchange material breaches several barriers and migrates from the external thermal loops into the cabin. Again, flight rules dictate procedures based on ammonia-monitoring results. CO_2 is monitored using a hand-held infrared spectrometer, referred to as the carbon dioxide monitoring kit (CDMK), to determine if pockets of this anthropogenic gas have formed or if whole-module analysers are accurate (Limero *et al.*, 2000).

10.2 Unpredictable Risks

We can expect combustion events to occur episodically in spaceflight; in that sense these events are predictable; however, the toxic threat caused by a fire is unknown without specific air monitoring. NASA employs a hand-held device known as the compound specific analyser—combustion products (CSA-CP) using electrochemical sensors for CO, HCN and HCl to assess the

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Figure 3 Crewmember operating and air monitor on the Shuttle. (Reproduced from NASA.)

toxic risk from a fire if it occurs, and to assess the air revitalization as air quality is recovered after an event. Flight rules, based on the results of monitoring, govern the crew actions in dealing with a fire. Because the ISS has become a sprawling complex, four such analysers are deployed in selected modules throughout the ISS to deal with any combustion event.

Volatile organic compounds can accumulate slowly in spacecraft atmospheres or be suddenly released as a result of accidents. Such unpredictable sources require a broad-spectrum capability to monitor VOCs at trace levels (see **Figure 3**). For several years the ISS has depended on a volatile organics analyser (VOA) based on gas chromatography/ion mobility spectrometry to monitor the atmosphere *in situ* (Limero *et al*., 2003). This large instrument proved valuable in characterizing the impact of nominal regeneration of Metox canisters and in characterizing the pollutants released when seals in the Elektron (an oxygen generator) were overheated during a repair (James, 2003). Since the VOA is well past its design lifetime and shows signs of imminent failure, replacement instruments are being developed. One promising instrument based on commercial technology is a gas chromatograph/differential mobility spectrometer that is hand-held (Limero *et al*., 2008).

11 DUST AS A TOXICITY HAZARD

11.1 Nuisance Dust

During ordinary spaceflight operations dust is seldom a problem aboard the ISS or the Shuttle. HEPA filters remove virtually all particles that could be a problem aboard the ISS. This does not mean that the ventilation ducts do not occasionally become obstructed with 'dust bunnies'. When this happens, the ability of the air revitalization to remove pollutants can be significantly reduced. Improved materials management has reduced the early problems of floating lint and debris aboard the Shuttle. When new modules are brought to the ISS, the crew is warned to wear eye protection when first entering it because the dust and debris that had settled during ground-based processing becomes airborne in the absence of gravity.

11.2 Reactive Dust

In addition to nuisance dust, spacecraft can sometimes have reactive dust in the air. The prime source for this is the LiOH dust that can come from canisters designed to remove CO_2 from the air by reacting with it. Aboard the Shuttle a single canister lasts for approximately one day depending on the size of the crew. The ARS contains two canisters in parallel that can be changed out on an alternating schedule. Occasionally, if the canisters are old, LiOH dust will have contaminated the outside of the canisters and the person changing the filter may receive a slight, irritating exposure to this reactive dust. Some crews report that when a new filter has been placed in line they sense a very slight degree of mucosal irritation for a few minutes, presumably from LiOH dust swept into the airstream as it passes through the interior of the fresh reaction bed.

11.3 Lunar Dust

During the Apollo flights to the moon, and especially when the lunar lander returned to the command module while it was orbiting the moon, the absence of gravity enabled the accumulated dust to float freely in the

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Figure 4 Apollo astronaut exploring the lunar surface during the Apollo missions. The open design contributed to lunar dust contamination of the suits of the astronauts. The main sources of lunar dust contamination came from exploration on foot and taking lunar samples. (Reproduced from NASA.)

air. Typically, the dust proved to be no more than a nuisance to the crew; however, in at least one instance an allergic-like reaction occurred to the dust. By the time the crew received these exposures to the dust it had been aged in the habitable environment for many hours or several days. Lunar geologists have described the surface of pristine lunar dust as highly reactive due to solar wind bombardment and micrometeoroid impacts (see **Figure 4**). Lunar dust also has a very large surface area for a given dust grain size. These properties suggest that fresh lunar dust could be more toxic than the aged dust encountered by the Apollo crews. NASA has investigated the toxicological properties of lunar dust stimulants (Lam *et al*., 2002) and is in the process of testing authentic lunar dust. The problem is confounded by the fact that there are different types of lunar dust (mare and highland) and each of these types can be in various degrees of 'maturity'. In anticipation of prolonged stays on the lunar surface, NASA is developing a database from which evidence-based exposure standards can be established.

11.4 Martian Dust

Martian dust is likely to present a very difficult problem for the first humans to reach that exotic environment. Planet-wide dust storms have been observed in the past few years and small 'dust devils' have been photographed as they dance across the Martian surface raising dust spouts and etching the terrain with grooves. The chemical reactivity of Martian dust was demonstrated by experiments aboard the Viking spacecraft (Oyama, 1977). In fact, it was this reactivity that led some scientists to propose that this was evidence of life (Oyama and Berdahl, 1977). At the time of writing NASA has landed a spacecraft in a crater near the north pole of Mars and is testing surface samples of dust *in situ* to assess its reactivity. The results could elucidate the type and magnitude of the reactivity of Martian dust. Of course we face the potential problem of the heterogeneity of the dust in various locations; however, planet-wide dust storms may have resulted in a relatively uniform surface covering by the dust.

12 THE VISION FOR SPACE EXPLORATION

This cause of exploration and discovery is not an option we choose; it is a desire written in the human heart.

President George W. Bush (43rd President, United States of America)

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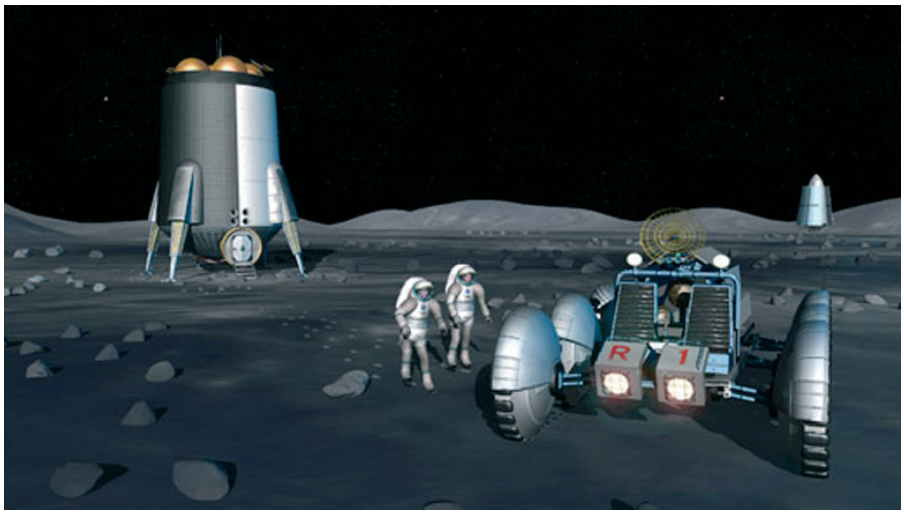

Figure 5 Computer depiction of future lunar missions. (Reproduced from NASA.)

We end with a brief look ahead to the future of space exploration. Space toxicology will support the programmes of the future and embark upon supporting crew health as we venture to distinct and uncharted locations. The future of space operations involves intense isolation, greater challenges with air and water reclamation, reuse and conditioning, chronic chemical exposures, exposure to reactive mineral dusts and limited return and rescue options.

In 2004, NASA published its *Vision for Space Exploration*; this document outlined the United States' plans for retiring the shuttle, completing the ISS and returning to the moon and exploring beyond. The goal to advance scientific, economic and security interests is described as fundamental for the United States (NASA (National Aeronautics and Space Administration), 2004). In order to accomplish this incalculable ambition, NASA has embarked upon multiple projects. These include implementing a human and robotic programme for exploration of the solar system and beyond, extending the human presence across the solar system by returning to Moon in preparation for human exploration and habitation of other celestial destinations (see **Figure 5**), such as Mars, its moons and perhaps nearby asteroids, developing technologies and infrastructures to support future exploration and promoting international and commercial participation (NASA (National Aeronautics and Space Administration), 2004). As NASA prepares to return to the lunar surface in 2018, space toxicologists labour to support these efforts through an array of projects. These include (but are not limited to):

- Supporting the successful retirement of the Shuttle programme with continued toxicology analysis and real-time support during missions.
- Working with international partners to ensure the maintenance of a safe living and working environment on the ISS
- Conducting lunar dust toxicity research and developing guidelines and risk criteria for human exposure (Khan-Mayberry, 2008a).
- The adaptation and development of next-generation environmental monitoring equipment, to improve our real-time monitoring capabilities of air and water quality
- Providing subject-matter expertise and toxicological analysis in support of the design and development of spacecraft, spacesuit and habitat designs supporting all new manned spaceflight programmes.

Space exploration entails cooperation with the best and brightest across technical disciplines from public, educational and private industries; this keeps our field of space toxicology interesting and filled with diverse opportunities for those with an interest in collaborative efforts.

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Long-term Adverse Health Consequences from Exposure to Products Generated from Major Disaster Sites

Matthew P. Mauer

CONTENTS

1 INTRODUCTION

Major disasters have been a constant reality since ancient times. From the destruction of Pompeii in AD 79, to the Chicago fire in 1871, to the World Trade Center (WTC) disaster in 2001, major disasters have repeatedly caused disruption, illness and death. A variety of natural and human-made events may be characterized as major disasters. Some examples include the cataclysmic eruption of Krakatoa in 1883, the San Francisco earthquake in 1906, the explosion of the atomic bomb at Hiroshima in 1945, the deadly smog events in Donora in 1948 and in London in 1952, the toxic gas release at Bhopal in 1984, the nuclear disaster at Chernobyl in 1986, the Oklahoma City bombing in 1995, the Indian Ocean tsunami in 2004 and Hurricane Katrina in 2005.

By definition, disasters are emergencies of a severity and magnitude resulting in deaths, injuries, illness and/or property damage that cannot be effectively managed by the application of routine procedures or resources (Landesman, 2001). Disaster has also been defined as a situation of massive collective stress (Kinston and Rosser, 1974). Leonard and Teitelman (1991) have defined a disaster as 'any community or regional event that disrupts community function and activities and causes concern for the lives, health and property of the citizens of a community'.

Epidemiological approaches to disasters usually focus on the immediate post-impact period with emphasis on surveillance of injuries, outbreaks of infectious and communicable diseases, illness due to environmental hazards, and mortality (Logue *et al*., 1981). However, it is important to recognize that disasters may result in long-term health consequences, including effects on mortality and morbidity one or more years after the event (Logue *et al*., 1981). The physical health of survivors of major disasters may be adversely affected for years, particularly in technological disasters, such as those involving chemicals and radiation (Noji, 2005). Thus, epidemiological studies of disasters must account for several issues, including characterization of the population at risk and/or exposed to the disaster, estimation of the exposure to the disaster, identification of short-term adverse health outcomes, and identification of long-term

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adverse health outcomes (Dominici *et al*., 2005). It has been noted that evidence for long-term consequences of disasters, both physical and emotional, through the use of cohort studies with sufficient follow-up time is somewhat lacking (Ibrahim, 2005). Galea (2007), in discussing the findings of one study of long-term health effects after a disaster, stated that 'thinking only about the acute population-health consequences of disasters is essentially considering only the tip of the iceberg'. Furthermore, he explained that persistent illness after a disaster may result in an increase in use of physician services and changes in the patterns of need for medical care, which can extend well beyond the immediate aftermath of a disaster. He went on to state that 'recognition that physical symptoms may persist for several years after exposure to a disaster, then, has implications for clinicians—perhaps primarily for primary care providers'. Long-term health effects can thus result in substantial personal, economic and societal burdens. When one considers that long-term health effects may include lingering, chronic effects as well as latent diseases such as cancer, the importance of studying such effects after a major disaster becomes clear.

This chapter will review reports of long-term health effects from a selection of major disasters, from several different disaster categories. Specifically, this review will cover the physical effects of exposures, as opposed to psychological effects. While not all major disasters for which pertinent literature exists can be covered, those discussed have been selected to provide an overview of a wide range of exposures. For some more recent disasters, such as the WTC disaster, there is a large and growing body of evidence regarding long-term health effects. For other disasters, the evidence is more limited. Overall, this review is intended to offer insights into the spectrum of long-term health effects that may be considered when planning epidemiological studies in the aftermath of future disasters. In addition, by illuminating some of the limitations of our knowledge in this area, this review may help encourage future disaster planners and researchers to emphasize the study of long-term health consequences from major disasters.

2 TYPES OF DISASTERS

According to the web site of the United States Department of Homeland Security's Federal Emergency Management Agency (FEMA), there are 17 categories of disaster (**Table 1**). Disaster types can also be grouped into 'natural disasters' and 'technological disasters' (Landesman, 2001). Natural disasters are unpreventable and, for the most part, uncontrollable. They include earthquakes, floods, heat waves, hurricanes, landslides, thunderstorms, tornadoes, tsunamis, volcanoes, wildfires and winter storms. Technological or human-made disasters are also unpredictable, may be unpreventable, and may have limited physical damage while having long-term effects (Landesman, 2001). Technological disasters include dam failure, fire, hazardous materials incidents (chemical, biological or radioactive), nuclear power plant emergencies and terrorism. They may also include explosions, building or bridge collapses, transportation crashes, and breaks in water, gas or sewer lines (Landesman, 2001).

Table 1 Types of disasters

Chemical emergencies
Dam failure
Earthquake
Fire
Flood
Hazardous material
Heat
Hurricane
Landslide
Nuclear power plant emergency
Terrorism
Thunderstorm
Tornado
Tsunami
Volcano
Wildfire
Winter storm

Reproduced from FEMA, 2008.

3 TERRORISM-RELATED DISASTERS

3.1 The World Trade Center Disaster—11 September 2001

The WTC disaster represents one of the worst technological disasters in history. The 11 September 2001 terrorist attacks in New York City resulted in the destruction of the WTC towers and the destruction of or damage to many other surrounding buildings. The magnitude of the destruction and resulting wreckage was unprecedented (**Figure 1**). Tragically, thousands lost their lives, including passengers on the involved commercial aircraft, community residents, office workers, and heroic rescue and response personnel.

On the morning of 11 September 2001, each of the WTC towers (North Tower and South Tower) sustained a direct hit from a jet airliner laden with jet fuel. The combustion of over 90 000 l of jet fuel in the two airliners initiated fires that reached a maximum temperature of 750–800 °C, resulting in the collapse of the towers (Eagar and Musso 2001). The collapses destroyed an estimated 1.2 million tonnes of construction material, an estimated 50 000 desktop computers,

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Figure 1 Lower Manhattan, New York City, just after the collapse of the first World Trade Center tower, 11 September 2001. (Reproduced from USEPA.)

Figure 2 The cloud of dust and debris enveloping lower Manhattan immediately after the collapse of the World Trade Center towers, 11 September 2001. (Reproduced from USEPA.)

300 mainframe computers, and large amounts of plastics, PVC-coated copper cable, office furnishings, fluorescent lights and other materials (Nordgren *et al*., 2002). Two utility substations containing approximately 500 000 l of polychlorinated biphenyl (PCB)-contaminated transformer oil were also destroyed. In addition, approximately 380 000 l of heating and diesel fuel, and fuel from several thousand automobiles stored in subterranean structures, were burned in the postcollapse fires (Nordgren *et al*., 2002).

The collapse of the towers produced an immense dust cloud, which enveloped much of lower Manhattan, penetrating many buildings in the area (Chen and Thurston, 2002) (**Figure 2**). Fires at the site continued to burn for approximately three months, until late December 2001. Emissions from the site from 11 September 2001 through early 2002 affected lower Manhattan as well as immediately adjacent areas in Brooklyn and New Jersey (Lioy *et al*., 2002; Wolff *et al*., 2005). The most intense exposures occurred during the collapse and in the following two days. Thereafter, the fires weakened and much of the resuspendable dust was washed from outdoor surfaces by rain (Lioy *et al*., 2006). Reports indicated that, from late September onward, the fine-particle mass levels in the area were generally within the limits of the United States Environmental Protection Agency (US EPA) National Ambient Air Quality Standard in effect at that time, averaged over a 24-hour period (Thurston and Chen, 2002). However, intermittent smoke plumes from ongoing fires, and resuspended dust from debris-removal activities, continued to impact the area for months (Lioy *et al*., 2006; Cahill *et al*., 2004) (**Figure 3**). For weeks, an acrid cloud hung over lower Manhattan until the fires were extinguished (Landrigan *et al*., 2004). An additional issue of concern was the dust that had infiltrated offices, businesses and residential buildings (Lioy *et al*., 2006).

Figure 3 Plumes of smoke rise from the debris at Ground Zero—fires burned at the site for over three months after 11 September 2001. (Reproduced from NOAA.)

3.1.1 Exposures

The dust and combustion products generated from the destruction of the World Trade Center consisted of a complex mixture of a variety of compounds. Lioy *et al*. (2002) studied three bulk samples of the total settled dust and smoke, collected from weather-protected locations east of the WTC site in September 2001. The samples consisted primarily of construction materials, soot, paint and glass fibres. Hydrocarbon levels indicated partially burned or unburned jet fuel, plastic, cellulose and other ignited materials. Analyses identified metals, radionuclides, ionic species, asbestos, polycyclic aromatic hydrocarbons (PAHs), PCBs, polychlorinated dibenzodioxins, polychlorinated dibenzofurans, pesticides, phthalate esters, brominated diphenyl ethers and other hydrocarbons. The vast majority of particles in the samples were greater than 10 μm in diameter (Lioy *et al*.,

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2002). McGee *et al*. (2003) studied the toxicity of fine particulate matter ($PM_{2.5}$) from samples collected on 12 and 13 September 2001. Their analyses indicated that the main components were construction materials such as cement, concrete aggregate, ceiling tiles and wallboard. Their results showed high levels of calcium and sulphur, and much lower levels of transition metals and other elements (McGee *et al*., 2003). Larger size dust particles were found to be caustic and alkaline, which could be irritating to mucous membranes (Chen and Thurston, 2002).

Cahill *et al*. (2004) analysed aerosols generated at a collection site roughly 1.8 km from the WTC site and 50 m above ground level. Samples were collected between 2 and 30 October 2001. The results demonstrated that WTC-derived plumes occasionally swept over lower Manhattan, resulting in intense aerosol impacts lasting several hours at any given site (Cahill *et al*., 2004). Size fractions above 1 μm consisted of finely powdered concrete, gypsum and glass, with sootlike coatings and anthropogenic metals, but little asbestos. The very fine size range was dominated by sulfuric acid and organic matter, including PAHs and their derivatives, and glasslike silicon-containing aerosols (Cahill *et al*., 2004). The authors concluded that 'a person could, in a few hours, be subject to materials in amounts and composition that they would not have had to endure in years of typical ambient conditions'.

Pleil *et al*. (2004) predicted PAH air concentrations on 14 September 2001 that were among the highest reported from outdoor sources. They concluded that the initial high concentrations resulted from the fires that diminished over the next 100 days. During most of that period, the PAH levels were primarily from diesel sources, with levels slowly declining to background levels (Pleil *et al*., 2004). Wolff *et al*. (2005) reported that the highest levels of polycyclic aromatic hydrocarbon-deoxyribonucleic acid (PAH-DNA) adducts were found in women whose blood was collected closest to 11 September 2001.

Olson *et al*. (2007) investigated concentrations of volatile organic compounds (VOCs) from four locations near the WTC site between September 2001 and January 2002. They found that concentrations of total VOCs and most individual VOCs decreased from the time period when fires were burning to the time period after the fires were finally extinguished, in late December 2001.

Edelman *et al*. (2003) reported on biomonitoring results from New York City firefighters who responded to the WTC disaster. Blood and urine specimens from 321 firefighters were analysed for 110 potentially fire-related chemicals. Specimens were collected three weeks after the disaster. Most chemical concentrations were found to be generally low and not outside of reference or background ranges. However, Special Operations Command firefighters had more than twice the level of urinary 1-hydroxypyrene, a PAH metabolite, than other exposed firefighters or control firefighters. Heptachlorodibenzodioxins and heptachlorodibenzofurans were found in significantly higher levels in exposed firefighters when compared with control firefighters. Firefighters on site during the collapse of the towers had significantly higher urinary antimony levels than firefighters who arrived on site postcollapse, or controls. The urinary antimony adjusted geometric mean for Special Operations Command firefighters was twice as high as for other exposed firefighters or controls. In addition, lead levels were significantly higher in exposed firefighters than in controls, though the increase was considered to be far below clinically significant levels (Edelman *et al*., 2003).

Tao *et al*. (2008) reported on biomonitoring conducted for New York State personnel who responded to the WTC disaster. Specifically, they reported on results of testing for eight perfluorochemicals including perfluorooctanesulfonate (PFOS), perfluorooctanoic acid (PFOA), perfluorohexanesulfonate (PFHxS) and perfluorononanoic acid (PFNA). These compounds are used in soil- and stain-resistant coatings on upholstery, carpets, leather, floor waxes, polishes and in firefighting foams, and were potentially released during the collapse of the towers. Plasma samples from 457 responders were analysed based on reporting of lower-respiratory symptoms (symptomatic vs. asymptomatic), and based on exposure groupings determined using an exposure assessment method (Herdt-Losavio *et al*., 2008). The exposure groupings were: more dust exposed (MDE), less dust exposed (LDE), more smoke exposed (MSE) and less smoke exposed (LSE). PFOS, PFOA, PFHxS and PFNA were consistently detected in almost all samples. PFOA and PFHxS concentrations in WTC responders were approximately twice as high as concentrations reported for a USA general population. Concentrations of PFHxS were significantly higher in the MDE group than in the LDE group. Concentrations of PFNA were significantly higher in the MSE group than in the LSE group. These results suggest that perfluorochemicals were released as a result of the collapse of the WTC towers, and that individuals exposed to the resulting smoke and dust are at risk for a higher body burden of these compounds, though the potential health implications of these findings are unknown (Tao *et al*., 2008).

3.1.2 Health Effects

A number of health effects have been reported in different cohorts with WTC exposures, including responders, community residents and office workers. Though not a focus of this review, it should be noted that a major health impact in this population was psychological effects, including symptoms of post-traumatic stress disorder (PTSD) and depression (Galea *et al*., 2002; Trout *et al*., 2002; Tapp *et al*., 2005; Brackbill *et al*., 2006; Perrin *et al*., 2007; Mauer *et al*., 2007).

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3.1.2.1 Effects in Response Personnel

Prezant *et al*. (2002) provided the first reports of clinical findings in New York City firefighters who were exposed at the WTC site. They defined the term 'World Trade Center cough' as a persistent cough that developed after exposure to the WTC site and that was accompanied by respiratory symptoms severe enough to require medical leave for at least four weeks. They defined exposure based on time of arrival at the disaster site and found that 8% of firefighters with a high level of exposure, 3% of firefighters with a moderate level of exposure, and 1% of firefighters with a low level of exposure exhibited WTC cough. Symptoms of dyspnoea were present in 95%, 87% had gastro-oesophageal reflux disease, and 54% had nasal congestion. Of the firefighters who underwent post-bronchodilator spirometry, 63% demonstrated a response to bronchodilator and 24% of those who had challenge testing demonstrated bronchial hyper-reactivity. Chest radiography was unchanged from baseline in 96% of the firefighters with WTC cough. Among 78 of those subjects with normal findings on chest radiography who underwent high-resolution computed tomography (CT) during inspiration and expiration, 51% demonstrated air trapping and 30% demonstrated bronchial wall thickening. There was little evidence of parenchymal changes. The authors concluded that 'intense, short-term exposure to materials generated during the collapse of the World Trade Center was associated with bronchial responsiveness and the development of cough. Clinical and physiological severity was related to the intensity of exposure'. Banauch *et al*. (2003) followed with a report on spirometry and methacholine challenge testing in a representative sample of New York City firefighters with no history of respiratory disease or current smoking, stratified by exposure intensity (high, moderate and control). Exposure was defined based on time of arrival at the disaster site. They found that hyper-reactivity of the airways at one, three and six months postcollapse was associated with exposure intensity. Six months postcollapse, highly exposed firefighters were 6.8 times more likely than moderately exposed workers and controls to be hyper-reactive. In addition, hyper-reactivity persisted in 55% of those who were hyper-reactive at one and three months. The authors concluded that 'development and persistence of hyperreactivity and reactive airways dysfunction were strongly and independently associated with exposure intensity.' Banauch *et al*. (2006) conducted a longitudinal study of pulmonary function in 12 079 New York City Fire Department rescue workers who were employees on or before 11 September 2001. Spirometries from 1 January 1997 through 11 September 2002 were analysed. Adjusted average forced expiratory volume in one second (FEV1) and forced vital capacity (FVC) were analysed to compare results during the first year after, and the five years before, 11 September 2001. They found that WTC responders experienced a substantial reduction in adjusted average FEV1 during the year following 11 September 2001 (372 ml). This FEV1 decrement equalled 12 years of ageing-related decline in FEV1. In addition, exposure intensity as characterized by initial arrival time at the disaster site correlated linearly with FEV1 reduction in an exposure–response gradient. They reported that similar findings were observed for adjusted average FVC. The authors concluded that 'World Trade Center exposure produced a substantial reduction in pulmonary function in New York City Fire Department rescue workers during the year following 9/11/2001.' Izbicki *et al*. (2007) reported on their investigation of sarcoidosis or 'sarcoid-like' granulomatous pulmonary disease. During the five years after 11 September 2001, they evaluated enrolees in the Fire Department of New York (FDNY) World Trade Center Monitoring and Treatment Program who had chest radiographs suggestive of sarcoidosis. They conducted chest CT imaging, pulmonary function tests (PFTs), provocative challenge tests, and biopsy. They compared annual incidence rates to the 15 years prior to the WTC disaster. They found pathologic evidence consistent with new-onset sarcoidosis in 26 patients. During the first year after the disaster 13 were identified (incidence rate of 86/100 000) and 13 were identified during the next four years (average annual incidence rate of 22/100 000), as compared to 15/100 000 during the 15 years before the disaster. They concluded that 'post-World Trade Center the incidence of sarcoidosis or "sarcoid-like" granulomatous pulmonary disease was increased among FDNY rescue workers.'

Salzman *et al*. (2004) reported on a cohort of police first-responders who were evaluated through January 2002. Respiratory symptoms occurred in almost 78%, but resolved or improved by the time of the evaluation in three-fourths of subjects. The most commonly reported symptom was cough (62.5%). Spirometry results were abnormal in approximately 29% and the abnormalities were mild. Buyantseva *et al*. (2007) reported on lower-respiratory symptoms in New York City police officers at 1 month and 19 months after the WTC disaster. They found that prevalence of cough was the same at both the initial and follow-up assessments (43.5%). However, the prevalence of phlegm, shortness of breath and wheeze all increased significantly from the initial to the follow-up assessments.

Herbert *et al*. (2006) reported results from an ongoing medical monitoring programme for WTC responders in 2006. Among all responders in this cohort ($N = 9442$), almost half reported any new or worsened lower-respiratory symptom while working at the WTC site. Almost two-thirds reported any new or worsened upper-respiratory symptom. The most prevalent lower-respiratory symptom was dry cough (28.7%). The most common upper-respiratory symptom type was nasal-related symptoms (48.4%). Among those responders reporting any respiratory symptom while performing WTC work (68.8%), symptoms persisted to the time of examination in 59%. Abnormal spirometry was observed

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in 29%, with low FVC in 21% and obstruction in 6%. Among nonsmokers, 28% had abnormal spirometry in comparison with 13% in the general United States population. PFT abnormalities and respiratory symptoms were associated with early arrival at the disaster site. The authors concluded that 'World Trade Center responders had exposure-related increases in respiratory symptoms and PFT abnormalities that were persistent up to 2.5 years after the attacks.'

Mauer *et al*. (2007) reported results from an evaluation of health effects in a cohort of New York State personnel who responded to the WTC disaster. They found that nearly half reported any new or worsening lower-respiratory symptom. A similar, though slightly lower percentage of study participants reported any new or worsening upper-respiratory symptom. The most common lower-respiratory symptom was dry cough (30.5%) and the most common upper-respiratory symptom was throat irritation (27.9%). Almost 14% reported acid stomach or heartburn symptoms. Some respiratory symptoms were associated with having been caught in the cloud of dust on 11 September 2001. PFT results revealed abnormalities in only a small percentage of this cohort, and clinical laboratory test results (blood chemistry and complete blood count) did not reveal any significant abnormalities in the overall cohort. This cohort deployed somewhat later than first-responders. Thus, they probably experienced moderate overall levels of exposure in comparison to groups such as the firefighters and New York Police Department (NYPD) first-responders. The authors concluded that 'this cohort generally reported fewer symptoms than most other more highly exposed responder cohorts. Results suggest that being present when the buildings collapsed was associated with reported symptoms.'

Skloot *et al*. (2004) reported that, in a cohort of ironworkers who responded to the WTC disaster, lack of a respirator with a canister was a risk factor for large airways dysfunction, as determined by forced oscillation (FO) testing. The FO data also showed a substantially higher prevalence of abnormal airflow resistance after exposure than was seen with spirometry. Oppenheimer *et al*. (2007) reported that symptomatic individuals with presumed WTC exposure and normal spirometry results demonstrated elevated airway resistance and frequency dependence of resistance determined by Impulse Oscillometry (IOS), elevated reactance area on IOS and frequency dependence of compliance (FDC), and reversibility of these functional abnormalities after bronchodilator. They concluded that these abnormalities likely reflect dysfunction in airways more distal to those evaluated by spirometry.

Herbstman *et al*. (2005) reported on a study of respiratory health effects in 183 WTC clean-up and recovery workers, conducted in December 2001. They stated that approximately one-third reported cough and one-quarter reported phlegm. Prevalence rates of symptoms were related to the number of days spent working at the WTC. The prevalence rates of upper-airway symptoms exceeded those of lower-respiratory symptoms. Tapp *et al*. (2005) described symptoms in a cohort of New York City transit employees, 7.5 months after the WTC disaster. They found that workers in the dust cloud at the time of the collapse had significantly higher risk of persistent lower-respiratory and mucous membrane symptoms. Wheeler *et al*. (2007) described newly diagnosed asthma among WTC site workers and volunteers in the World Trade Center Health Registry. They found that newly diagnosed asthma was reported in approximately 4% of this population. The risk of newly diagnosed asthma was 12-fold higher than the expected three-year risk in the general population. Earlier arrival time, longer duration of work, exposure to the dust cloud, and working on the pile were all significant risk factors. Among workers who arrived on 11 September 2001, longer delays in use of respiratory protection were associated with increased asthma risk. Tao *et al*. (2006) investigated respiratory health among clean-up workers 20 months after initial exposure, in comparison with a group of workers who were never at the WTC site. They found that the clean-up workers were more than three times as likely to report any lower-respiratory symptoms. In a 2008 case report, de la Hoz *et al*. (2008a) reported the clinical characteristics of 10 symptomatic WTC workers who were diagnosed with vocal cord dysfunction. Most of the patients had hoarseness, dyspnoea not associated with bronchial hyper-reactivity, or dyspnoea associated with asthma. The authors concluded that 'vocal cord dysfunction appears to be part of the spectrum of airway disorders caused by occupational exposures at the World Trade Center site.'

3.1.2.2 *Effects in the Community*

Trout *et al*. (2002) evaluated concerns related to health effects and occupational exposures among a population of office workers in a building close to the disaster site, three months after the disaster. Constitutional and mental health symptoms were evaluated in comparison with a group of office workers in Dallas, Texas. Symptoms, including cough, shortness of breath, wheeze, chest tightness, and others, were reported more frequently in the New York City workers. Fagan *et al*. (2002) reported results from a telephone survey of Manhattan residents five to nine weeks after the disaster. Among residents with asthma (13%), 27% reported experiencing more severe asthma symptoms after 11 September 2001. Increased severity was more common in residents who reported psychological distress and/or difficulty breathing due to smoke and debris from the disaster. Szema *et al*. (2004) reported on a retrospective review of charts from 205 paediatric patients with established asthma from a clinic in Chinatown, for the year before and the year after 11 September 2001. They found that,

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after 11 September 2001, these children had significantly more asthma-related clinic visits and received significantly more prescriptions for asthma medications. Those who lived within 5 miles (8 km) of the disaster site had more clinic visits after the disaster. In addition, mean %predicted expiratory flow rates declined for the patients who lived within 5 miles (8 km) of the disaster site, during the three months after the disaster.

Reibman *et al*. (2005) investigated lower-respiratory symptoms and screening spirometry results in residents in an 'exposed area' and in a 'control area', approximately one year after the disaster. They found that new-onset lower-respiratory symptoms were more frequent among residents from the 'exposed area', as were persistent new-onset lower-respiratory symptoms. No differences in screening spirometry results were detected. In the same study population, Lin *et al*. (2005) reported on upper-respiratory symptoms. They found that residents in the affected area reported higher rates of new-onset upper-respiratory symptoms after 11 September 2001. Most of these symptoms were persistent one year later. They also found that residents in the affected area who were previously healthy had significantly more respiratory-related unplanned medical visits and more new medication use after the disaster. Wagner *et al*. (2005) reported on their investigation of the asthma status of Medicaid managed care enrolees who may have been exposed as a result of the disaster. They conducted a mailed survey among enrolees living in New York City. Of survey respondents, 45% reported worsened asthma after the disaster. Those who reported worsened asthma were more likely to have utilized health services for their asthma. Residence in lower Manhattan and Western Brooklyn was associated with worsened asthma. Brackbill *et al*. (2006) reported on health effects among survivors of collapsed and damaged buildings (excluding those involved in rescue and recovery), from data in the World Trade Center Health Registry. Almost two-thirds were caught in the dust and debris cloud. More than half of those studied (56.6%) reported experiencing new or worsening respiratory symptoms after the disaster. In addition, almost one-quarter reported heartburn/reflux. Survivors caught in the dust and debris cloud were more likely to report any respiratory symptom, severe headaches, skin rash/irritation, hearing problems/loss, heartburn and diagnosed stroke. The authors concluded that 'two to three years after 11 September, survivors of buildings that collapsed or that were damaged as a result of the World Trade Center attack reported substantial physical and mental health problems.'

Lederman *et al*. (2004) reported on their assessment of the impact of gestational stage at the time of the disaster, and distance from the WTC in the four weeks after 11 September, on birth outcomes among 300 nonsmoking women who were pregnant at the time of the disaster. They found that term infants born to women who were pregnant at the time of the disaster and who were living within 2 miles (3.2 km) of the disaster site during the month after the event demonstrated significant decrements in term birth weight and birth length, after controlling for sociodemographic and biomedical risk factors. Furthermore, women in the first trimester of their pregnancy at the time of the disaster delivered infants with significantly shorter gestation and a smaller head circumference, regardless of the distance of their residence or worksite from the disaster site. The authors concluded that 'the observed adverse effects suggest an impact of pollutants and/or stress related to the World Trade Center disaster and have implications for the health and development of exposed children.' Perera *et al*. (2005) described their efforts to learn whether PAHs, as measured by benzo[*a*]pyrene-DNA adducts (PAH-DNA adducts), from the WTC disaster affected birth outcomes in pregnant women, and their newborns. They found no independent foetal growth effects due to PAH-DNA adducts, but adducts in combination with *in utero* exposure to environmental tobacco smoke were associated with decreased foetal growth. An assessment of possible effects on child development was reported by Perera *et al*. (2007). They found a significant interaction between PAH-DNA adducts and *in utero* exposure to environmental tobacco smoke on mental development index score at three years of age. They concluded that 'results suggest that exposure to elevated levels of PAHs in conjunction with prenatal environmental tobacco smoke exposure may have contributed to a modest reduction in cognitive development among cohort children.'

3.1.3 Summary

The evidence in the literature suggests that WTC-related exposures have resulted in a variety of health outcomes, including lower- and upper-respiratory effects, gastro-oesophageal reflux disease, psychological effects, and possibly adverse birth outcomes and sarcoidosis in some cohorts. Some of these health symptoms have been persistent over time in some populations. The precise reasons for this are still unclear. With the exception of sarcoidosis, interstitial lung diseases have not been reported in any WTC cohorts, but single case reports of eosinophilic pneumonia (Rom *et al*., 2002), bronchiolitis obliterans (Mann 2005) and granulomatous pneumonitis (Safirstein *et al*., 2003) have been published (Prezant, 2008). In a study of the range of clinical characteristics in a group of workers representative of the widest possible range of occupations at the WTC site, de la Hoz *et al*. (2008b) reported on five predominant diagnostic categories in their analysis: upper-airway disease (78.5%), gastro-oesophageal reflux disease (57.6%), lower-airway disease (48.9%), psychological illness (41.9%), and chronic musculoskeletal illness (17.8%). A combination of the first three categories was the most frequent pattern of presentation (29.8%). The main respiratory health

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consequence from the WTC disaster has been the 'World Trade Center Cough Syndrome', which involves chronic rhinosinusitis, asthma and/or bronchitis, often complicated by gastro-oesophageal reflux dysfunction (Prezant, 2008).

Lower-respiratory effects have been consistently reported in both response workers and in community residents. Studies have indicated that exposed cohorts have experienced reduced lung function with declines in FEV1 and FVC, significant responses to bronchodilators, and significant responses on provocative challenge tests (Prezant *et al*., 2002; Banauch *et al*., 2003; 2006; Salzman *et al*., 2004; Herbert *et al*., 2006). In addition, Oppenheimer *et al*. (2007) concluded that IOS and FDC abnormalities likely reflect dysfunction in airways more distal to those evaluated by spirometry.

The precise nature of the pulmonary effects experienced by those with WTC exposures is complex, and has not yet been fully elucidated. Typically, a pattern representing obstructive impairment might be expected from an irritant-induced airway injury. However, an unexpectedly high proportion of WTC responders have demonstrated declines in FEV1 and FVC with preservation of the FEV1/FVC ratio (Mendelson *et al*., 2007). Herbert *et al*. (2006) reported that the most common spirometric abnormality seen among their responder cohort was a low FVC. These findings could be interpreted to represent restrictive impairments, such as might be found with parenchymal lung disease (Mendelson *et al*., 2007; Herbert *et al*., 2006). However, these findings may result from air trapping, causing a 'pseudorestrictive' pattern (CDC 2004; Herbert *et al*., 2006). Herbert *et al*. (2006) reported that 19% of WTC workers and volunteers demonstrated a partial reversal of their low FVC following bronchodilator, which can be seen with low FVC due to air trapping. Prezant *et al*. (2002) reported that among responders who underwent high resolution computed tomography, half demonstrated air trapping. Mendelson *et al*. (2007) reported results from high-resolution computed tomography scans in 29 WTC workers with lower-respiratory symptoms. Air trapping was demonstrated in 25 of the 29 patients, and there was a significant correlation between air trapping and duration of dust exposure. They concluded that 'air trapping from small airways disease may account for some of the reported clinical and pulmonary function abnormalities in World Trade Center dust-exposed workers.'

Further evidence of the potential lower-respiratory effects of exposure to WTC dust was provided in a study of the effects of WTC-derived $PM_{2.5}$ on the respiratory tract in mice (Gavett *et al*., 2003). The authors found that 'exposure to high levels of WTC $PM_{2.5}$ can promote mechanisms of airflow obstruction in mice.' They concluded that 'high-level exposure to WTC $PM_{2.5}$ could cause pulmonary inflammation and airway hyper-responsiveness in people.'

3.1.4 Ongoing Concerns

Looking towards the future, the primary concerns for populations with a history of WTC exposure relate to possible long-term and persistent health effects, as well as onset of health effects with longer latency periods, such as cancer. Some commonly reported health effects, such as upper- and lower-respiratory symptoms, have demonstrated persistence over time. Aerodigestive inflammatory syndrome, which consists of 'World Trade Center' cough, irritant asthma or reactive airways dysfunction syndrome (RADS), rhinosinusitis or reactive upper-airways dysfunction syndrome (RUDS), and gastro-oesophageal reflux disorder, is a major consequence of exposure (Lioy *et al*., 2006). These effects must be monitored in the future. Furthermore, ongoing studies to help clarify the precise physiological mechanisms responsible for the respiratory effects of WTC exposure will be necessary.

A variety of compounds that could potentially cause cancer were present in the dust and smoke resulting from the disaster. For most cancers, the typical latency period is approximately 12–25 years, though it can range from as short as three years (radiation- or toxin-induced leukaemias) to as long as 40 years (asbestos-induced mesothelioma) (Rugo, 2004). Thus, studies to investigate cancer incidence among WTC responders and residents will become increasingly important as more time passes. Further studies of possible adverse developmental effects in children born to mothers who were pregnant at the time of their WTC exposure may also provide important information.

4 CHEMICAL DISASTERS

4.1 Bhopal Gas Leak Disaster—2–3 December 1984

What has been referred to as the 'World's Worst Industrial Disaster' (Dhara and Dhara, 2002) occurred on the night of 2–3 December 1984 in Bhopal, the capital city of one of the largest states in India. The Department of Relief and Rehabilitation, Government of Madhya Pradesh, has stated that more than 200 000 persons were exposed to a toxic cloud of methyl isocyanate gas, and that more than 6000 deaths resulted. Some independent agencies estimated the death toll to be as high as 15 000 to 20 000 deaths. It is estimated that approximately 50 000 persons are suffering from long-term health effects (Dhara *et al*., 2002).

4.1.1 Exposures

Methyl isocyanate is an intermediate product in manufacturing carbaryl, which is a carbamate pesticide. This

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pesticide was being manufactured at the Union Carbide plant in Bhopal when the accident occurred. Dhara and Dhara (2002) reported that the toxic cloud was released from the plant due to the introduction of water into a methyl isocyanate storage tank resulting in an uncontrollable reaction. Safety systems, such as a flare tower and a caustic soda scrubber, failed to contain the reaction. Approximately 27 tonnes of methyl isocyanate escaped from two tanks within 1–2 hours. The gas was not able to disperse quickly due to adverse atmospheric conditions (inversion and low wind speed). It was reported that the gas cloud enveloped the area and moved slowly. No air monitoring occurred at the time of the incident or later, but the estimated mean methyl isocyanate concentration in the cloud was 27 ppm. That concentration is 1400 times higher than the United States Occupational Safety and Health Administration's workplace standard (Dhara and Dhara, 2002).

Dhara and Dhara (2002) reported that the acute irritant effects of the exposure resulted in panic, anxiety and disorientation, which caused people to run out of their homes and into the gas cloud, resulting in even greater exposures. The primary exposures involved the eyes and the respiratory tract, though some skin exposure and presumably ingestion exposure, due to the chemical dissolving in saliva, also occurred. Most of the immediate mortality that followed the gas leak was attributed to direct, irritative toxicity to the lower respiratory tract, and resulting hypoxia (Cullinan *et al*., 1996).

4.1.2 Health Effects

4.1.2.1 General Effects

Cullinan *et al*. (1996) reported on health effects approximately 10 years postdisaster among a representative sample of gas-exposed persons in comparison with a reference group of unexposed persons from the same city. A random sample of those surveyed also underwent neurological testing. Exposed persons reported more general health problems, more episodes of fever, more adverse pregnancy outcomes and more respiratory symptoms than unexposed persons, with a strong gradient by exposure category. Persons in high-exposure categories reported neurological symptoms more frequently, and this was generally confirmed with neurological testing. Ocular symptoms also showed a similar pattern.

Ocular studies, conducted nine months to two years after exposure, revealed chronic irritant symptoms including persistent eye watering, burning, itching and redness. The primary chronic lesions included conjunctivitis, deficient tear secretion, and persistent corneal opacities (Andersson *et al*., 1986; Raizada and Dwivedi, 1987; Khurrum and Ahmad, 1987). A three-year postexposure follow up survey was conducted among previously surveyed exposed and unexposed Bhopal residents (Andersson *et al*., 1990). The results indicated an increased risk of eye infections, hyper-responsive phenomena, excess cataracts, and resolution of corneal erosions in exposed persons. This constellation of symptoms was characterized as 'Bhopal Eye Syndrome'. The authors stated that 'in its response to methyl isocyanate, the eye should be considered a sentinel organ for more general phenomena in the body.' In summary, Dhara and Dhara (2002) stated that 'the single acute exposure seems to have resulted in a chronic inflammatory process' in the eye.

4.1.2.2 Respiratory Effects

Kamat *et al*. (1992) reported on exposed subjects who were followed with clinical, lung function, radiographic and immunologic tests up to 24 months postdisaster. Dominant symptoms included exertional dyspnoea, cough, chest pain, sputum, and muscle weakness. Radiographically, interstitial deposits were found to be persistent changes. PFTs showed primarily restrictive changes with small-airway obstruction. The small-airway findings persisted. They also found signs of alveolitis in bronchoalveolar lavage (BAL) fluid on fibre optic bronchoscopy. In 11 cases, positive methyl isocyanate-specific antibodies to IgM (immunoglobulin M), IgG and IgE were found. The authors felt that earlier evidence of interstitial restrictive lung pathology with small-airway disease was confirmed. They went on to explain that 'the airway component seems to have progressed, leading to a fluctuating course with persisting disability.'

Vijayan *et al*. (1995) reported on initial BAL results in a group of exposed persons, approximately five years postdisaster, and repeat lavage results (approximately 2.7 years postdisaster) in a group of exposed persons who had the test done previously. All subjects had persistent respiratory symptoms. They found that among those undergoing repeat lavage, lower-respiratory tract inflammation had progressed from macrophage alveolitis to macrophage-neutrophilic alveolitis. Among those undergoing lavage for the first time, five years postdisaster, macrophage-neutrophilic alveolitis was the common finding. The authors suggested that, because alveolar macrophages and neutrophils are capable of promoting lung injury and fibrosis, inhaled toxic gas might produce permanent damage to pulmonary parenchyma. They concluded that 'the persistence of clinical, radiological, and pulmonary function abnormalities in this study as well as in previous studies may be due to the consequences of macrophage-neutrophilic alveolitis.'

A study of the relationship between lung inflammation, changes in lung function, and severity of exposure among Bhopal victims included BAL and PFTs, conducted one to seven years after the disaster (Vijayan and Sankaran, 1996). The PFTs included FEV1, FVC and FEV1/FVC ratio. The authors found that total lung inflammatory cells and absolute numbers of

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macrophages and lymphocytes increased significantly as severity of exposure increased. FEV1/FVC ratio was significantly lower as severity of exposure increased. BAL neutrophils showed significant negative correlations with FEV1 %predicted, while neutrophil percentage was negatively correlated with FEV1/FVC ratio. Absolute lymphocytes were negatively correlated with FVC %predicted. Radiographic abnormalities were associated with decline in FEV1 %predicted. The authors concluded that 'pulmonary function abnormalities occur in gas-exposed subjects as a consequence of an abnormal accumulation of lung inflammatory cells.' Furthermore, they suggested that, because pulmonary function decline was associated with radiographic abnormalities, injury following toxic-gas exposure can lead to irreversible lung damage.

Cullinan *et al*. (1997) reported on respiratory morbidity 10 years postdisaster. Respiratory symptoms (cough or phlegm for greater than three months, dyspnoea, wheeze in past year) were significantly more common among exposed persons versus unexposed persons. The frequency of symptoms decreased as exposure decreased, with exposure characterized by distance lived from the plant. Pulmonary function was also assessed, including FEV1, FVC, FEV1/FVC ratio and forced expiratory flow (FEF) between 25 and 75% of vital capacity (FEF25–75). All pulmonary function indices were lower among those in the higher-exposure categories. The authors concluded that 'the symptoms reported, and the differences in lung function detected, are compatible with chronic airflow limitation and particularly with disease of the small airways.'

Dhara *et al*. (2002) reported on long-term health effects in survivors of the Bhopal disaster, nine years later. They found that for subjects reporting any versus no exposure, prevalence ratios were elevated for most respiratory and nonrespiratory symptoms. Respiratory symptoms included shortness of breath, cough for at least three months, asthma and phlegm. Mucous membrane symptoms included nose pain, eye redness and eye pain. Systemic symptoms included fever in the last 12 months, fatigue, chest pain, taste problems and loss of appetite.

4.1.3 Summary

Chronic inflammatory ocular symptoms were reported to be persistent for several years after the Bhopal disaster. One author dubbed the symptoms 'Bhopal Eye Syndrome'. However, the primary persistent health effects noted in survivors of the Bhopal gas leak involved the lower respiratory tract. Persistent lower-respiratory effects included declines in PFTs with indications of small-airways disease. Studies reported findings of alveolitis, abnormal accumulation of inflammatory cells in the lungs, and interstitial lung disease, up to 10 years postdisaster.

4.2 Seveso Accident—10 July 1976

On 10 July 1976, substantial amounts of 2,3,7,8-tetrachlorodibenzo-*para*-dioxin (TCDD) were accidentally released from a chemical plant near the town of Seveso, Italy, impacting an area with a large residential population. The accident occurred due to an uncontrolled exothermic reaction during the production of trichlorophenol. A fluid mixture entraining approximately 2900 kg of organic matter was released through an exhaust pipe. This mixture included a substantial amount of TCDD. The exhaust was then driven by prevailing winds as far as 6 km south of the chemical plant (Pesatori *et al*., 2003).

4.2.1 Exposures

Pesatori *et al*. (2003) indicated that the most heavily contaminated area covered approximately 87 ha, and average levels of TCDD in soil samples ranged between 15.5 and 580.4 $\mu g\,m^{-2}$. This has been designated zone A and had approximately 750 inhabitants. A further contamination zone with levels not exceeding 50 $\mu g\,m^{-2}$ covered 270 ha. This has been designated zone B and had approximately 5000 inhabitants. A third area with levels generally below 5 $\mu g\,m^{-2}$ covered 1430 ha. This has been designated zone R, with approximately 30 000 inhabitants. Bertazzi *et al*. (1997) reported that a total of 180 families comprising more than 700 individuals were evacuated from zone A and that residents in zones B and R had to comply with regulations aimed at avoiding continuing exposure through consumption of local crops, dairy food and meat products.

4.2.2 Health Effects

Bertazzi *et al*. (1993) described an evaluation of cancer incidence over a 10-year period after the accident. This and subsequent follow-up studies used the population of a surrounding noncontaminated area as a reference group. In zone B, hepatobiliary cancer was significantly elevated, especially in those living in the area for over five years. Males had a significant increase in lymphoreticulosarcoma, and women had a significant increase in multiple myeloma. Results from a 15-year follow-up mortality study (Bertazzi *et al*., 1997) indicated that, in zone B, males demonstrated significantly increased mortality from rectal cancer, pleural cancer and leukaemia. Women demonstrated significantly increased mortality from multiple myeloma. They found no increase for all-cancer mortality.

A 20-year follow-up mortality study also reported no increase in all-cancer mortality (Bertazzi *et al*., 2001). In zone B, males demonstrated significantly increased mortality from rectal cancer. Lymphohaematopoietic neoplasms were significantly increased in both genders. This was also demonstrated for Hodgkin's disease.

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Females demonstrated a significant increase in diabetes. In zone A, chronic rheumatic heart disease and chronic obstructive pulmonary disease were significantly increased. The authors concluded that 'results support evaluation of dioxin as carcinogenic to humans and corroborate the hypotheses of its association with other health outcomes, including cardiovascular- and endocrine-related effects.'

Consonni *et al*. (2008) reported results from a 25-year follow-up mortality study. They found a significant excess of lymphohaematopoietic neoplasms in zone B, and a significant excess of non-Hodgkin's lymphoma and multiple myeloma in zone A. They also reported a significant increase in chronic rheumatic heart disease and chronic obstructive pulmonary disease in zone A, as well as a significant increase in diabetes among females in zone B. The authors concluded that 'a toxic and carcinogenic risk to humans after high TCDD exposure is supported by the results of this study.'

Warner *et al*. (2007) report on results from the Seveso Women's Health Study, which assessed reproductive health among 363 women who had resided in zone A or zone B and who had adequate stored sera collected soon after the accident. They examined the relationship between 1976 serum TCDD levels and ultrasound-detected ovarian follicles among 96 women in the menstrual follicular phase, and serum hormone levels among 129 women in the menstrual luteal phase at follow-up. The authors concluded that the results provided no clear evidence of an association of high exposure to TCDD with quality of ovarian function, approximately 20 years postaccident.

4.2.3 Summary

The long-term mortality studies that have reported results up to 25 years postaccident suggest that high exposure to TCDD is associated with mortality due to some types of cancer, including lymphohaematopoietic neoplasms overall and rectal cancer in males. Results also suggest that mortality due to some chronic cardiac, pulmonary and endocrine conditions might be associated with high TCDD exposure in this cohort.

5 NUCLEAR DISASTERS

5.1 Chernobyl Accident—26 April 1986

The Chernobyl nuclear power plant, located in Ukraine approximately 10 km south of the border with Belarus, was the scene of the worst disaster in the history of the nuclear industry. On 26 April 1986 two explosions destroyed the core of unit 4 of the plant and the roof of the building (Hatch *et al*., 2005). A complete meltdown of the reactor took place, with an estimated 150–200 million curies of radioactive materials subsequently released into the atmosphere over the next 10 days (Tronko *et al*., 1999). The International Atomic Energy Association classified the event as a 'major accident', and the release of radionuclides greatly exceeded those in all previous atomic power station accidents (Tronko *et al*., 1999). Fallout levels were extremely nonuniform (Likhtarov *et al*., 2006). During the first 10 days of releases, wind direction changed frequently, so all areas surrounding the reactor received some fallout. Rainfall occurred in an irregular pattern, also contributing to varying degrees of deposition (Hatch *et al*., 2005). Over 450 different radionuclides were released over Ukraine, Belarus and Russia, which were the most contaminated areas (Tronko *et al*., 1999; Hatch *et al*., 2005; Howe, 2007). The radioactive cloud spread to the Baltic States, as well as Scandinavia and other parts of Western Europe. Contamination of the ground was found to some extent in every country in the Northern Hemisphere, even on the North American continent (Tronko *et al*., 1999; Hatch *et al*., 2005; Howe, 2007).

5.1.1 Exposures

There were three primary cohorts exposed to radioactive contamination as a result of the Chernobyl disaster. Emergency response workers or clean-up workers, also referred to as liquidators, included approximately 600 000 individuals. Roughly 240 000 liquidators worked at the reactor site and within 30 km of the site in 1986 and 1987, when doses were highest (Cardis *et al*., 2006; Howe, 2007). Residents who were evacuated or relocated during 1986 included approximately 116 000 individuals, in Ukraine, Belarus and Russia. An additional 220 000 residents were relocated after 1986 (Cardis *et al*., 2006; Howe, 2007). Residents in contaminated areas that were not evacuated include more than 5 million individuals (Cardis *et al*., 2006; Howe, 2007). Doses ranged from 10 mSv among the least exposed, nonevacuated residents to 100 mSv for liquidators in 1986 and 1987 (Cardis *et al*., 2006).

Releases from the site included gases, aerosols and finely fragmented nuclear fuel particles (Hatch *et al*., 2005). Exposures included short-lived radioactive iodines, most importantly iodine-131 (^{131}I), external exposure from radionuclides deposited on the ground, and ingestion of radioactive caesiums, particularly caesium-134 (^{134}Cs) and caesium-137 (^{137}Cs) (Cardis *et al*., 2006; Nuclear Energy Agency, 2002; United Nations Scientific Committee on the Effects of Atomic Radiation, 2000). The most important exposures for liquidators were from external radiation, primarily from gamma-emitting radionuclides (Hatch *et al*., 2005). The most important pathways of exposure for residents

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in contaminated areas were external exposure from radionuclides deposited on the ground and internal exposure from ingestion of milk and other foods contaminated with ^{131}I, ^{134}Cs and ^{137}Cs. In the first few months, the thyroid was the most exposed organ, due to ^{131}I, with the primary exposure pathway being from pasture to cow to milk (Hatch *et al*., 2005). Adequate prophylactic measures against radioiodine exposure with ingestion of stable iodine salts were not instituted after the disaster (Tronko *et al*., 1999).

Following either internal or external radiation exposure, children are the most vulnerable exposed group, because thyroid sensitivity to radiation is high and they have a longer lifespan to develop effects (Tronko *et al*., 1999; Hatch *et al*., 2005).

5.1.2 Health Effects

5.1.2.1 Cancers

Antonelli *et al*. (1996) reported on a study of thyroid cancer among 64 children aged 4–16 years in an area of Belarus. Previously, among children under age 15 in this area, there was one case of thyroid cancer observed from 1981 to 1985, 21 cases from 1986 to 1990 and 143 cases from 1991 to 1994. Among the 64 subjects, more than 90% were less than 6 years of age. The authors reported that there appeared to be no correlation between aggressiveness of the tumour and the age of the subjects. Astakhova *et al*. (1998) conducted a case–control study to test a possible association between the Chernobyl disaster and an increase in thyroid cancer among children in national therapy centres in the Minsk area of Belarus. Cases were matched with two sets of controls and the results indicated a strong relationship between thyroid cancer and estimated radiation dose. Tronko *et al*. (1999) reported data from a registry of thyroid carcinoma cases at the Institute of Endocrinology and Metabolism in Kiev. During the five years prior to the disaster, 59 cases of thyroid carcinoma were identified among children and adolescents 18 years of age or younger. The total number of cases in Ukrainian children and adolescents between 1986 and 1997 was 577. The group most affected included children who were less than six years of age in 1986. Morphologically, the tumours were generally aggressive with a high frequency of lymph node metastases, venous invasion and extrathyroidal spread. Davis *et al*. (2004) reported on a case–control study of radiation exposure and thyroid cancer risk among children and adolescents aged 0–19 years at the time of the disaster, residing in the more highly contaminated areas of the Bryansk area of Russia. They found a statistically significant trend of increasing risk with increasing dose, suggesting that exposure to radiation from Chernobyl is associated with risk of thyroid cancer and that the relationship is dose dependent. Likhtarov *et al*. (2006) conducted an ecological study to investigate the relationship between ^{131}I thyroid dose and thyroid cancer. The study included 301 907 individuals who were between the ages of 1 and 18 at the time of the disaster and were living in an area in Northern Ukraine. Cases included 232 thyroid cancers diagnosed between 1990 and 2001. The estimated relative risk per Gray was significantly elevated. The authors concluded 'in broad terms, these estimates are compatible with results of other studies from the contaminated areas, as well as studies of external radiation exposure.' Tronko *et al*. (2006) studied a cohort of over 13 000 individuals younger than 18 years of age who lived in the most heavily contaminated areas in Ukraine at the time of the disaster. They identified 45 pathologically confirmed cases of thyroid cancer during the 1998–2000 screening, as compared to 11.2 cases that would have been expected in the absence of the disaster. The authors concluded that exposure to radioactive iodine was strongly associated with increased risk of thyroid cancer among those exposed as children and adolescents.

Inskip *et al*. (1997) conducted thyroid examinations for 2000 liquidators from Estonia, nine years after the disaster. The workers had been sent to the site between 1986 and 1991 and served for an average of three months. The authors found that the workers did not experience a significantly increased risk of nodular thyroid disease associated with exposure to external radiation. They concluded 'possible reasons for the apparent absence of effect include low radiation doses, the protracted nature of the exposure, errors in dose measurement, low sensitivity of the adult thyroid gland, or the insufficient passage of time for a radiation effect to be expressed.' Rahu *et al*. (2006) studied two cohorts of liquidators, from Estonia and Latvia, between 1986 and 1998. Each cohort was followed using nationwide population and mortality registries. A statistically significant excess of thyroid cancer cases was found, though there was no evidence of a dose response. The authors discussed the possibility that the excess cases observed might be due to screening.

Risk for leukaemia was investigated in several studies. Konogorov *et al*. (2000) estimated the radiation-induced risk of leukaemia in 162 684 liquidators using data from the Russian National Medical and Dosimetric Registry. They found no significant association between risk of leukaemia and various measures of exposure. The International Consortium for Research on the Health Effects of Radiation (2006) reported on a population-based case–control study that investigated whether acute leukaemia was increased among children from Belarus, Russia and Ukraine who were *in utero* or less than six years of age at the time of the Chernobyl disaster. They found a statistically significant increase in leukaemia risk with increasing estimated radiation dose to the bone marrow. This finding was most evident in children from Ukraine, but not found in children from Belarus or Russia. The authors felt this may, at least in part, represent an overestimate of risk in

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Ukraine. They concluded that the study provided no convincing evidence of an increased risk of childhood leukaemia related to exposure to Chernobyl radiation. Ivanov (2007) presented preliminary results from a study using the Russian National Medical and Dosimetric Registry. He reported that, during the first 10 years after the disaster, the relative risk of leukaemia was statistically significant among liquidators with higher exposure doses in comparison with liquidators with lower exposure doses. Hatch *et al*. (2005) stated that 'to date, the available data support a measurable increase of leukaemia among cleanup workers.' Howe (2007) evaluated empirical studies for evidence relating to Chernobyl radiation exposure and leukaemia risk. He concluded that 'possibly apart from Russian cleanup workers, no meaningful evidence of any statistical association between exposure and leukaemia risk as yet exists.'

With regard to solid cancers, other than thyroid, Hatch *et al*. (2005) reported that little could be said, most likely due to the longer minimum latency period and lower risks per dose. Cardis *et al*. (2006) stated that 'apart from the large increase in thyroid cancer incidence in young people, there are at present no clearly demonstrated radiation-related increases in cancer risk.' They noted that studies of such cancers are few, and have methodological limitations. Furthermore, due to inadequate latency, they felt it was too early to evaluate the full radiological impact of the disaster. Ivanov (2007) reported preliminary results indicating that in 1991–2001, among Russian liquidators, the excess relative risk for incident solid cancers was statistically insignificant.

5.1.2.2 Genetic Effects

A number of studies have looked at genetic effects from exposure to Chernobyl-related radiation. While the clinical implications of these effects may be uncertain, these findings still merit discussion. Weinberg *et al*. (1997) reported on an analysis of DNA isolated from the blood of experimental and control subjects, involving offspring born before and after the disaster, and their parents. They used genome fingerprinting simple sequence repeat-anchored polymerase chain reaction (PCR) amplification and random amplified polymorphic DNA PCR. They detected new bands that appeared in children born after the disaster, while no such bands appeared in children born in the same family before the disaster or in children from control families who were not exposed to the radiation.

Dubrova *et al*. (1997) conducted an analysis of germ-line mutation rate at human minisatellites among children born in heavily contaminated areas of Belarus. Data confirmed a two-fold higher mutation rate in exposed families in comparison with nonexposed families from the United Kingdom. Within the Belarus cohort, mutation rate was significantly greater in families with higher parental radiation dose. Weinberg *et al*. (2001) screened for the appearance of new fragments using multisite DNA fingerprinting among children born to liquidator families conceived after parental exposure. They found a seven-fold increase in the number of new bands in comparison with controls. There was a strong tendency for the number of new bands to decrease with elapsed time between exposure and offspring conception. The authors concluded 'these results indicate that low doses of radiation can induce multiple changes in human germ-line DNA.' Livshits *et al*. (2001) measured the frequency of inherited mutant alleles at seven hypermutable minisatellite loci in children born to liquidators and controls. There was no significant difference in the frequency of inherited mutant alleles between the two groups. The exposed group was then divided into those who were conceived within two months of their father having worked as a liquidator and those who were conceived at least four months after their father stopped working as a liquidator. The frequencies of mutant alleles were higher for the majority of loci for the former group than for the latter group.

Dubrova *et al*. (2002) reported that a statistically significant increase in mutation rate was found in the germ line of exposed fathers, whereas the maternal germ-line mutation rate in exposed families was not elevated. They concluded that elevated minisatellite mutation rate can be attributed to post-Chernobyl radioactive exposure. Kiuru *et al*. (2003) compared minisatellite mutation rates of children born to Estonian liquidators after the disaster with those of their siblings born prior to the disaster. They found that the minisatellite mutation rate was not significantly elevated among children born after the accident. Slebos *et al*. (2004) also studied whether radiation exposure can cause paternal genetic mutations that are transmitted to offspring, among families of liquidators. They found that children conceived before and children conceived after their father's exposure showed no statistically significant differences in mutation frequencies. Their results did suggest a modest increase in germ-line mutations in tetranucleotide repeats.

5.1.3 Summary

There is evidence that exposure to radiation from the Chernobyl disaster resulted in elevated risk for thyroid cancer among children and adolescents. There is some suggestion of elevated risk for leukaemia among clean-up workers or liquidators, though the evidence for this is more equivocal. There is inadequate evidence for an association with other cancers, though until recently the latency period would be considered inadequate. There is also some suggestion in the literature that exposure to Chernobyl radiation has resulted in genetic mutations in some offspring conceived after or born after parental

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Figure 4 Mount St. Helens erupts, 18 May 1980. (Reproduced from USGS.)

exposure, though the potential clinical significance of these changes is uncertain.

6 VOLCANIC DISASTERS

6.1 Mount St. Helens Eruption—18 May 1980

Mount St. Helens, dormant since 1857, catastrophically erupted on 18 May 1980, releasing approximately 4 km^3 of rock and ash into the atmosphere, and covering much of Washington State with a layer of fine volcanic ash up to several centimetres deep (Vallyathan *et al*., 1983; Baxter, 1986). Volcanic ash erupted to an altitude of 60 000 ft (over 18 km) and was deposited eastward, impacting not only Washington State but also Idaho and Montana (**Figure 4**). In Spokane County, Washington, volcanic ash persisted in depths up to 2 inches (5 cm) for several weeks after the initial event (Kraemer and McCarthy, 1985). Subsequent eruptions and resuspension of ash by wind and human activities resulted in exposure of over 1 million people in the northwestern USA (Vallyathan *et al*., 1983).

6.1.1 Exposures

Mineralogical analysis demonstrated that the majority of the ash was composed of silicate minerals of the plagioclase class (Fruchter *et al*., 1980; Green *et al*., 1982). Most of the ash particles were within the respirable range, or less than 10 μm in diameter (Green *et al*., 1982). Dollberg *et al*. (1986) reported that crystalline silica was identified as being present in the Mount St. Helens volcanic ash at levels of 3–7% by weight.

6.1.2 Health Effects

There were some variable reports of cellular effects of the volcanic ash from Mount St. Helens. Volcanic ash was reported to inhibit superoxide anion release from zymosan-stimulated rat alveolar macrophages (Castranova *et al*., 1982). Because superoxide is an antibacterial substance, this result might suggest that exposure to volcanic ash could impair antibacterial host defence mechanisms by adversely affecting the ability of alveolar macrophages to protect the lung from infection (Castranova *et al*., 1982; Green *et al*., 1982). Martin *et al*. (1986), in a review of the existing literature, suggested that evidence indicated that antibacterial defences against pyogenic organisms and viruses were not impaired by ash exposure. Green *et al*. (1982) reported that *in vitro* biologic tests using various concentrations of ash indicated the ash was mildly fibrogenic, which was confirmed *in vivo*. Vallyathan *et al*. (1983) reported on the pulmonary response among rats to intratracheal administration of 1 or 10 mg of ash. At 28 days, the intra-alveolar aggregates of mononuclear cells had condensed to form granulomas. The granulomas enlarged in size from 28 to 180 days, with progressive increase in the amount of collagenous tissue. The authors concluded 'volcanic ash may pose a risk for pneumoconiosis in heavily exposed human populations.' Adler *et al*. (1984) reported that their studies of the *in vitro* interactions of ash with epithelial cells of rodent respiratory airways suggested that volcanic ash from Mount St. Helens interacted minimally with respiratory mucosal cells. In their review, Martin *et al*. (1986) stated 'the data indicate that volcanic ash, even in high concentrations, causes little toxicity to lung cells *in vitro* and *in vivo*, as compared to the effects of free crystalline silica, which is known to be highly fibrogenic.' They did allow that volcanic ash is not entirely inert, probably due to the low concentrations of free crystalline silica present in the ash. They concluded 'humans without lung disease who are exposed to volcanic ash are probably at very little risk for pulmonary toxicity unless the exposure is extremely heavy and prolonged.'

In a study of childhood asthma hospitalization rates in Spokane County, Washington, Kraemer and McCarthy (1985) reviewed medical records from seven hospitals for children up to 19 years of age. They reported a nearly two-fold increase in paediatric asthma admissions over seasonally expected rates from April through June 1980. They also reported an increase in asthma admissions during the weeks following the eruption, when levels of suspended volcanic ash particulates were at their highest. Buist *et al*. (1986a) reported results from a four-year follow-up of a cohort of loggers exposed over an extended period to varying levels of volcanic ash from Mount St. Helens. They found that a significant, exposure-related decline in FEV1 occurred during the first year after the eruption. However, by the fourth year

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the decline was no longer significant. A similar pattern was noted for self-reported symptoms including cough, phlegm and wheeze. The authors stated that 'findings are consistent with the hypothesis that the inhaled ash caused mucus hypersecretion and/or airway inflammation that reversed when the exposure levels decreased.' In a review conducted by Buist *et al*. (1986b), they reported that long-term or chronic effects of exposure to the volcanic ash included irritation and inflammation of the upper and lower respiratory tracts while low-level chronic exposures continued, or after intense short-term exposure.

Olenchock *et al*. (1983) studied the effects of volcanic ash exposure on the level of humoral immune factors IgG, IgA, IgM, C3, C4 and antinuclear antibody (ANA) in volcanic-ash-exposed loggers in comparison with nonexposed loggers. C3 and C4 were significantly lower among exposed loggers shortly after the eruption and also one year later. Exposed loggers also demonstrated a significant decrease in serum IgG and IgA levels one year after the event. The authors concluded that 'exposure to volcanic ash may affect humoral immunologic parameters.'

6.2 Soufriere Hills, Montserrat Eruption—19 July 1995

In July 1995, the Soufriere Hills volcano on the Caribbean island of Montserrat began erupting, and it has continued to be active since that time. The eruptions have deposited large quantities of ash, particularly on the southern part of the island that is downwind of the volcano. The capital, Plymouth, was evacuated in April 1996, though the port was kept open and people were allowed into the area during safe periods to clear ash from their properties (Searl *et al*., 2002). All activities in Plymouth ceased after a major eruption on 25 June 1997 when 19 people were killed. Continuing high levels of volcanic activity during the summer and fall of 1997 resulted in the evacuation of areas in the central part of the island. Since 1997, approximately 4500 of the original 12 000 residents have remained on the island, in the northern areas (Searl *et al*., 2002). The eruptions have involved the growth of a lava dome in the old crater that periodically produces large ash emissions, often in the form of pyroclastic flows, when it collapses or explodes (Forbes *et al*., 2003).

6.2.1 Exposures

The remaining population of the island has been exposed to ash from the periodic ash falls as well as from resuspension of ash particles by wind and human activity. Searl *et al*. (2002) reported that individual exposures were related to occupation. The highest exposures occurred among gardeners, cleaners, roadworkers and police at roadside checkpoints. Individual exposures for some of these workers in 1997 included concentrations of cristobalite (silicon dioxide) that exceeded the American Conference of Governmental Industrial Hygienists (ACGIH) recommended occupational exposure limits. Analysis of the ash in 1996 demonstrated high levels of crystalline silica (15–20% by weight), as well as substantial quantities of particles smaller than 10 μm (13–20% by weight) (Searl *et al*., 2002). The silica was present as cristobalite (Baxter *et al*., 1999). Freshly erupted ash also contained water-soluble sulfates, chlorides and fluorides adsorbed on particle surfaces. The surface acidity of these particles could result in irritant effects on the airways (Searl *et al*., 2002).

6.2.2 Health Effects

Forbes *et al*. (2003) studied the effect of ash exposure on the respiratory health of children on Montserrat. They found that children aged 12 and under who had ever lived in areas with moderate or heavy ash exposure had a significantly higher prevalence of reported wheeze ever, and wheeze in the past 12 months, in comparison with children who had always lived in low exposure areas. They also found that the risk for exercise-induced bronchoconstriction, defined as a 10% decline in peak expiratory flow rate, was almost four-times higher in children who currently lived in areas with moderate or heavy ash exposure in comparison with children who currently lived in low-exposure areas. In a report for the Institute of Occupational Medicine, Cowie *et al*. (2002) gave results from an October 2000 survey of workers from occupations with potentially high exposures to volcanic ash. They also conducted spirometry and chest radiography. They found that the risks of reporting symptoms of asthma and breathlessness were significantly associated with exposures from heavy ash clearing activities. In addition, among men, FVC and FEV1 results were significantly reduced in subjects who had ever worked as gardeners or roadworkers. There were few signs of abnormalities on the chest radiographs. The results suggested exposure to volcanic ash had some mild effect on respiratory health among the Montserratian workers, though there was no evidence of an accelerated or severe silicotic reaction to ash exposure. There was no evidence of any effect of residential exposure or exposure from domestic cleaning tasks on study participants' health. Cowie *et al*. (2003), in another report for the Institute of Occupational Medicine, calculated risks of silicosis for six different population groups and four locations on the island. They reported that risk estimates for most groups are 1 in a 100 or less after up to 20 years of volcanic activity. The exceptions to this included gardeners (2–3 per 100) and children

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(5 per 100). The authors did note that the risk calculation methods used could lead to over-estimation of risk.

6.3 Summary

Studies related to the two examples of volcanic disasters presented here suggest there may be two types of exposure most likely to result in ongoing, potentially long-term respiratory health effects. First, individuals with ongoing exposure to ash falls and/or resuspended ash may experience lower-respiratory symptoms such as shortness of breath, cough and wheeze, as well as declines in PFT results. This may be most prominent for those with higher levels of exposure, such as certain occupational groups, and in more vulnerable populations, such as children. These effects may eventually resolve if exposure ceases. The other exposure group of concern involves those individuals with the heaviest, most acute and intense exposures. It is plausible, though not yet confirmed, that these individuals may experience lingering respiratory symptoms and declines in pulmonary function. Some individuals with these high exposures may also be at some, probably low, risk for the development of silicosis.

Hansell (2003) has noted that despite the fact that over 455 million people worldwide live within exposure range of a volcano active within recorded history, there has been relatively little epidemiological research published on the health effects of volcanic emissions. The International Volcanic Health Hazard Network was created in 2003 to help determine the health effects of volcanoes, as there has been limited long-term monitoring of exposed populations (Jones, 2006). One reason more studies are needed is because there may be important differences between exposures and thus effects from different volcanoes as well as between different events involving the same volcano, in terms of eruption pattern, gaseous emissions, base composition of ash, compounds adsorbed onto ash particles, the percentage of respirable particles, and toxicological activity (Hansell, 2003). Using the two examples presented in this chapter, most respirable ash in Montserrat has been generated from pyroclastic flows, with cristobalite concentrations measured at up to 20% by weight. However, Montserrat ash derived from phreatic explosions has had lower concentrations of cristobalite (8.6% by weight) (Housley *et al*., 2002). In comparison, cristobalite concentrations were noted to be approximately 4.2% by weight for ash from the Mount St. Helens eruption (Baxter *et al*., 1999). As noted by Hansell (2003), other volcanoes, such as Sakurajima, Japan, may have ashfalls accompanied by sulphur dioxide emissions (Uda *et al*., 1999) or, in the case of Kilauea, Hawaii, may involve predominantly sulphur dioxide emissions (Mannino *et al*., 1996).

7 CONCLUSIONS

This review presents epidemiological evidence for long-term adverse health consequences from a selection of major disasters, which represent some of the more thoroughly studied events in history. While it provides an overview of outcomes expected from several types of disasters, it also illustrates the gaps in knowledge that exist for some of these events. For instance, studies of health effects related to the World Trade Center disaster have been numerous and are expected to continue for many years. This represents one of the most thoroughly studied disasters in history, though even for these studies there have been significant methodological obstacles (Savitz *et al*., 2008). Conversely, long-term health effects from volcanic disasters have not been as thoroughly studied, despite their regular occurrence throughout history.

Among disaster types not covered in this review, floods and hurricanes offer another brief example of the relative lack of data that exists regarding long-term adverse health consequences of disasters. While floods account for approximately 40% of natural disasters, and may become even more frequent and severe due to global warming and increasing urbanization and deforestation (Landesman, 2001), knowledge of long-term health effects of flooding is less complete than is knowledge of immediate post-event effects (Reacher *et al*., 2004). Some studies have attempted to evaluate this issue (Bennet, 1970; Abrahams *et al*., 1976; Tapsell and Tunstall, 2008), but overall the literature is somewhat lacking. Hurricane disasters have not generated many long-term health studies either. Hurricane Katrina resulted in a truly monumental disaster including failure of the levees in New Orleans and subsequent flooding of 80% of the city (Rabito *et al*., 2008). There have been a number of reports of short-term, post-event health effects (Centers for Disease Control (CDC), 2006; Tak *et al*., 2007; Rath *et al*., 2007; Cummings *et al*., 2008; Rabito *et al*., 2008). However, to date there are few published reports of long-term physical health effects among populations affected by this disaster.

For the majority of disaster types, there is relatively little information available regarding long-term health outcomes among disaster victims (Cook *et al*., 2008). In many cases this may be due to inherent methodological challenges in tracking displaced populations, accurately identifying responder and community cohorts, identifying exposures of concern, and in establishing causal links between such exposures and specific health outcomes, both in the short term and long term. Savitz *et al*. (2008) note that the unanticipated nature of a disaster makes it more difficult to carefully define the population of interest, measure exposure and prepare for longitudinal health studies. In some cases, there may be insufficient resources available to develop and sustain longitudinal studies. Many organizations responsible for disasters do

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not have an adequate process or authority structure for coordinating long-term population health surveillance that may last for months or years after a disaster (Fenig and Cone, 2005).

Though the World Trade Center disaster was a unique and unprecedented event, the epidemiological response that has been ongoing, in the form of health monitoring and longitudinal studies, can serve as an example of how, given adequate resources, postdisaster research may be effectively pursued despite ever-present methodological challenges. Ideally, national and local health services should be equipped to provide health surveillance systems and resources in the weeks, months and years after a disaster (Cook *et al*., 2008). Disaster response planning should include the study of long-term health impacts as an integral part of the overall response plan for disasters, and efforts should be made to ensure the availability of resources for such response activities. However, even without adequate resources, any attempt to identify affected populations and track postdisaster health impacts over time may provide valuable knowledge, given the relative lack of published data on this issue for many disaster types.

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Toxicology and Disasters.

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Biological Correlates of Low-Level Electromagnetic-Field Exposure

Jitendra Behari

CONTENTS

1 INTRODUCTION

During the last four decades or so there has been growing concern about the effects of electromagnetic radiation on biological systems. This is because of electronic devices, for communication, the air surveillance system, industry, and diagnostic and therapeutic purposes in medicine, emitting microwaves/radiofrequency (RF) waves. In view of this, the importance of the electromagnetic radiation pervading the environment is now increasingly realized. This has added to the list another pollutant in the environment (electropollution) after air, water, soil and noise. The effects are broadly classified into two: thermal and nonthermal, though the line of demarcation between the two is not very clear. While the former leads to an increase in body temperature, the latter does not. The nonthermal effects are attributed to the induced electromagnetic fields (**Figure 1**).

While the issue is still being debated, devices emitting these radiations have found their use in everyday life. These principally include mobile phones and microwave ovens, to name a few. Microwaves are emitted by mobile phones of the GSM type (global system for mobile

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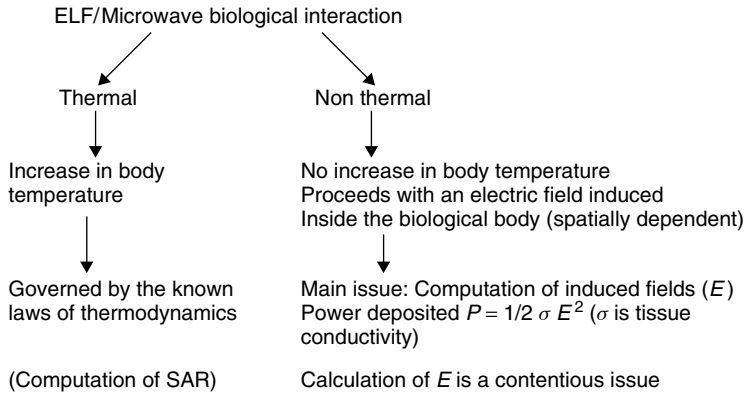

Figure 1 The boundary line between thermal and nonthermal effects is thin and not yet well defined. ELF: extremely low frequency; SAR: specific absorption rate; E_i: induced field; P: power deposited; σ: tissue conductivity.

telecommunication). It is an extremely low frequency (ELF) modulated pulsed microwave carrier. This is not the case for analogue radio and television. It can be argued that digital mobile phones transmit information in bursts of microwaves. This raises a question regarding its negative effect on human health. It has been observed that the two low-frequency sine waves (8.3 and 217 Hz, respectively) act on the composite pulsed GSM signal.

There are reports indicting adverse health effects of cell phones which emit electromagnetic radiation, with a maximum value of 50% of their energy being deposited when held close to the head. Also there is a likelihood that fields emanating from base stations (though very weak), would create a health hazard, though the issue is still being debated. It would therefore be useful to explore and understand the mechanism of electromagnetic-field effects over a wide frequency band at the existing level of RF/microwave exposure from various devices and in the environment.

While the thermal effects of microwaves are not commonly detected, nonthermal effects, if undetected, may have negative effects on human health. It is understood that RF electromagnetic fields (EMFs) from base stations to which humans are exposed (far-field exposures) are well below the allowed values. In a recent survey, it was reported that around 4% of people claim that they are sensitive to RF EMFs to some degree (Eltiti *et al*., 2007). The biological effects also depend upon the intermittency of the exposure, may be accumulative, and may include membrane interactions that affect the ion fluxes, the modulation of neuronal impulse activity and possibly induce arrhythmia in isolated heart. Behavioural effects and those on reproductive patterns are also possible at low levels of microwave exposure. There are important biomedical considerations associated with long-term exposure to any environmental factor capable of tissue interactions. These include effects specifically attributable to wave type, duration, intermittency, frequency of recurrent exposure, interactions involving simultaneous exposure to multiple factors, age at onset of exposure and may also reflect ethnicity. While examining the biological effects, it was realized that it is not the external field, but the fields within the tissue and the body that are important.

Taking cognisance of the above, several governments have imposed limitations on the fields radiated by power systems. Biologists and the physicians have conducted many clinical tests over many years and have proposed a set of maximum values for the radiated fields, depending on the frequency.

2 THE PHYSICS OF THE PROBLEM

Biological bodies are inhomogeneous, having tissue-specific dielectric properties and a complex shape, making the computation of the induced field rather difficult. The fields induced inside the body depend upon, but are not limited to, the following parameters:

1. The location of the field with respect to the surroundings, for example, if there are metallic objects around, the person is grounded or otherwise
2. Polarization of the incident wave with respect to the orientation of the human body
3. Size of the human body (L) with respect to the wavelength (λ) of the incident radiation (L/λ)
4. The portion of the body
5. The electrical properties of the tissue in question.

In free-space propagation of an electromagnetic field, the power density is given by:

$$\text{Power density} = E^2/1200\,\Pi \text{ mW cm}^{-2} \qquad (1)$$

where E is the electric field strength.

Figure 2 illustrates how the average body specific absorption rate (SAR) is size and frequency dependent (free-field exposure level of 10 mW cm^{-2}, Gandhi

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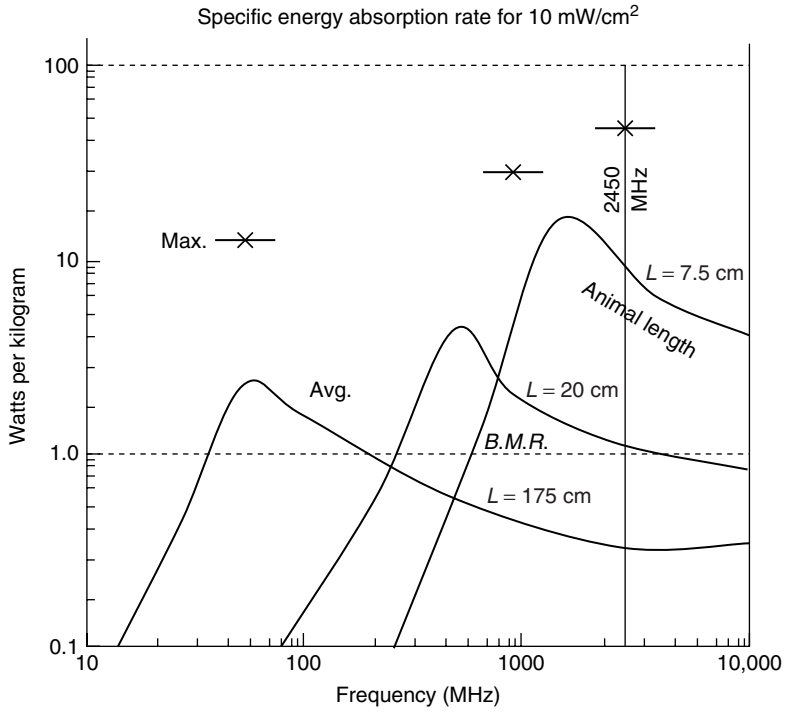

Figure 2 Average SAR in prolate spheroidal models of biological subjects exposed to electromagnetic radiation as a function of frequency. (Reproduced from Gandhi *et al.*, 1979. © IEEE.)

et al., 1979). The absorption characteristics for a prolate spheroridal model representing a man, 175 cm long, oriented parallel to an incident electric field (maximum absorption) is shown. It indicates that the average SAR, for a man model, exposed to radiation at frequencies from 10 to 10 000 MHz, first increases as the square of the frequency, reaching a peak of $2\,W\,kg^{-1}$ at about 70 or 80 MHz, then gradually approaches an asymptotic value of approximately $0.16\,W\,kg^{-1}$. In the upright position, the grounded body has longitudinal resonance around 35 MHz (Gandhi and Rozzell 1975). In the transverse and anteroposterior axes, maximum absorption occurs at frequencies from 135 to 163 MHz. This configuration may be obtained in a far-field exposure. However, near-field patterns of energy absorption, as in the case of a user of a mobile phone near to the head, will be dominated by regional peaks determined by the proximity of the transmitting antenna to specific body parts and their relative orientation.

When a smaller body (simulating a rat), 20 cm long is exposed, the maximum absorption shifts towards a higher frequency, 650 MHz, and when a still smaller 7.5 cm long body (simulating a mouse) is exposed, the maximum absorption is within the microwave frequency range (2450 MHz). From simple biophysical considerations, each body therefore has a characteristic resonant frequency, depending upon the length of the long axis. Correspondingly, for the same level of incident exposure, the average value of the SAR is dependent upon the length of the body, the degree of decoupling decreasing the average value of SAR by more than an order of magnitude. It is suggestive that absorbed RF energy can be converted into other forms of energy and can cause interference with the functioning of biological systems. A significant portion of this energy is converted into heat (absorption). However, the effects are frequency dependent. At frequencies, below 100 kHz, the induced fields can even stimulate nervous tissue.

3 THE HEAD AS A RECIPIENT OF ELECTROMAGNETIC ENERGY

The waves falling on the biological objects may not necessarily penetrate through the entire body, but are limited to a distance determined by the skin depth. The depth to which microwaves can penetrate inside the tissue depends on the electric and magnetic properties of the tissue and of the microwave frequency. In general, at a given frequency, the lower the water content of the tissue, the greater the wave penetration and vice versa. As of now, reliable data are available on the dielectric constants of biological tissues and the assessment of biointeraction mechanisms can be quantified to an appreciable extent.

Out of all the organs, the head is the targeted recipient of electromagnetic energy from many wireless systems (e.g. mobile phones), whose impulses are also transmitted to other organs and parts. Thus, its physical and biological properties have been well investigated. For the

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head, the resonant frequency is a function of head size, decreasing from around 700 MHz in infants to 400 MHz in adults (Gandhi and Hagmann, 1977). Early studies using thermalizing far-field exposure levels identified sites for preferential deposition of electromagnetic energy (hot spots) within the head. These hot spots occur inside lossy spheres, as models for brain tissue, with radii $0.1 < r > 8$ cm for frequencies between 300 MHz and 12 GHz (Kritikos and Schwan, 1972; 1976). However, at lower frequencies and much higher frequencies, heating occurs primarily at the surface of the sphere facing the source. Heat conduction and convection from a central brain hot spot have been modelled, with inclusion of a factor for blood flow, which accounts for heat removal (Kritikos and Schwan, 1979). For a spherical head 10 cm in diameter, exposed to a 1.0 GHz, 10 mW cm^{-2} field with a central hot spot 2 cm in diameter, the expected temperature rise would be 0.5 °C, with normal blood flow and heat conduction (Adey, 1997).

For a given incident field flux, the heating potential in the hot spot decreases rapidly with increasing head radius (Kritikos and Schwan, 1972; 1975; 1976). Thus, in man, the expected temperature rise would be lower at this 1.0 GHz field frequency than seen in the 10 cm model. There are strong differences in convective capacities between cerebral grey and white matters, attributable to substantially higher densities of blood-vessel capillary beds in central cortical grey matter (Ranck, 1964). For the human head, simulated as a sphere 15.0 cm in diameter with three layers representing brain, fat and skin, a 2.45 GHz (or higher frequency) field is rapidly absorbed at the surface of the head.

4 DOSIMETRY

At lower frequencies (<100 kHz), many biological effects are quantified in terms of current density in tissue and this parameter is most often used as a dosimetric quantity. At higher frequencies, many (but not all) interactions are due to the rate of energy deposition per unit mass. This is why the SAR is used as the dosimetric measure at these frequencies. It is expressed as W kg^{-1}. The SAR is thus the absorbed power by the absorbing mass. The most obvious approach towards dosimetric analysis is to experimentally determine the SAR distribution in phantoms simulating animal and human bodies, as well as in real cadavers. One way of determining the local or whole-body SAR is by temperature measurements. The SAR is proportional to the temperature increase only when the effects of heat diffusion can be neglected.

With these limitations in mind, the SAR concept has proven to be a simple and useful tool in quantifying the interactions of RF/microwave radiation with living systems, enabling comparison of experimentally observed biological effects for various species under various exposure conditions. This provides (as of now) the only means, however imperfect, of extrapolating the animal data into potential hazards to humans exposed to RF radiation, and facilitating, planning and effectively executing therapeutic hyperthermic treatment (Stuchly and Stuchly, 1996).

The SAR is defined as the time derivative of the incremental energy dW absorbed by or dissipated in an incremental mass dm contained in a volume element dV of a given density ρ (WHO, 1993; NCRP, 1981). An equivalent method is to take a temperature measurement. The SAR is proportional to the temperature increase ΔT, when the effects of thermal conduction, convection and radiation are negligible, in the time interval Δt.

$$\mathrm{SAR} = -\left(\frac{\mathrm{d}}{\mathrm{d}t}\right)\left(\frac{\mathrm{d}W}{\mathrm{d}m}\right) = \left(\frac{\mathrm{d}}{\mathrm{d}t}\right)\left(\frac{\mathrm{d}W}{\rho(\mathrm{d}V)}\right) = C\frac{\Delta T}{\Delta t} \tag{2}$$

The same can also be evaluated, using the Poynting vector theorem for sinusoidal varying electromagnetic fields:

$$\mathrm{SAR} = \left(\frac{\omega\varepsilon_0}{2\rho}\right) - |E_\mathrm{i}|^2 = \left(\frac{\varepsilon}{2\rho}\right) - |E_\mathrm{i}|^2 \tag{3}$$

where $|E_\mathrm{i}|^2$ is the peak value of the internal electric field (in V m^{-1}). SAR is also dependent upon if the wave type, that is, square, sine or triangular. The power of the square is larger than the other two. The average SAR is defined as the ratio of the total power absorbed in the exposed body to the mass in which it is absorbed, which is not necessarily that of the total body. The local SAR refers to the value within a defined unit volume or unit mass, which can be arbitrarily chosen.

To better understand experiments in microwave exposure, as well as their relation to safety standards; it is useful to refer to the diagram in **Figure 3**. In this diagram, with log–log coordinates of power (or power density or SAR) on the ordinate and time on the abscissa, it is possible to draw the threshold for various effects and hazards. For example, to heat a finite sample to a given temperature, the threshold is a constant SAR for long periods of time, while, for short periods of time, during which no heat is lost from the sample, the threshold curve is a line of constant specific absorption (SA = SAR − time) which is at 45° from the horizontal. The intersection of the two lines, constant SAR and constant SA, determines the applicable thermal time constant and associated 'average time' in setting exposure standards (Osepchuk and Petersen, 1996).

As evident by Equations (2) and (3), both approaches (temperature/field) have some common requirements. Any measurable field disturbance caused by the intrusion of the probe must be avoided. A further requirement is that any signal, picked by any part of the probe, other

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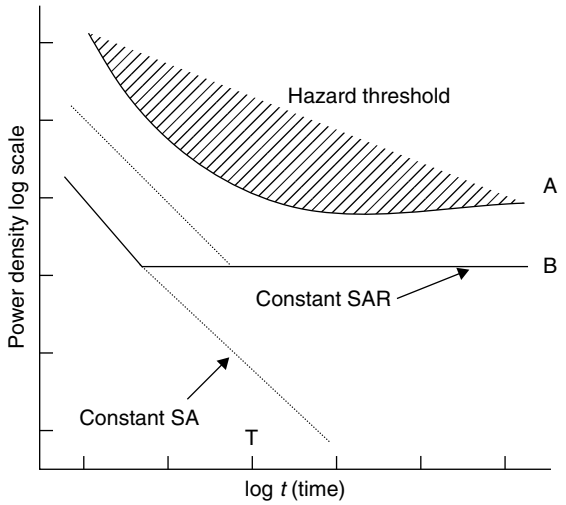

Figure 3 Thresholds for various effects and hazards expressed as a function of time. (Reproduced from Osepchuk and Petersen, 2001. © IEEE.)

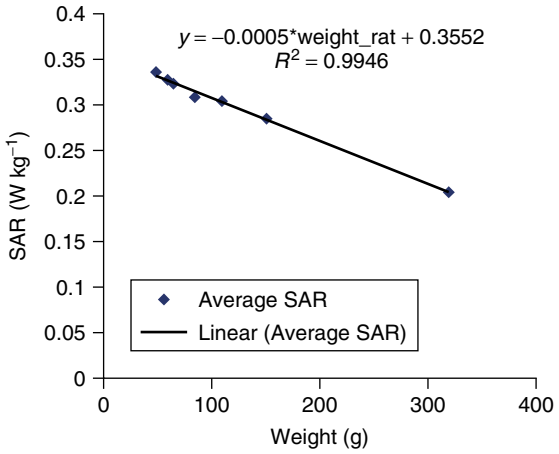

Figure 4 The linear-regression model of the SAR as a function of weight of the rat.

than the signal itself should be sufficiently suppressed. Further, the spatial resolution should be better than the smallest spatial dimension of any local field maxima or minima, in order to enable accurate assessment of the SAR distribution. Determination of the SAR distribution requires measurements in the tissue volume of interest. In larger volumes, with greatly nonuniform distributions, SAR varies from place to place, demanding measurements in several places, and the location of the sensor must be precisely known.

In animal experimentation (particularly in the developing stage) it is clear that as the animal age increases (and hence the weight) the SAR values change and dosimetric estimation becomes uncertain. On the basis of theoretical formulation from Durney *et al*. (1979), weight/age increases for the rat as shown in **Figure 4**. It is found that SAR in the foetus is strongly dependent on the geometrical relationship between the foetus and the EM source, while the average SAR for the foetus is always lower than the RF safety guidelines under the exposure conditions (Togashi and Ryder, 2007). The single-value concept of SAR thus has many uncertainties.

Also, the concept of SAR is not sufficient when one is looking for biological effects not dependent upon absorption. This raises questions about using these parameters for evaluating effects that may be of another nature. The possibility of nonthermal effects is thus a controversial one and the problem of dosimetry becomes much more complex. SAR may be a valid quantitative measure of interactions other than absorptive ones, when the mechanism is dependent on the intensity of the electric field, except, however, when the direction of the field is of importance with respect to the biological structure. Similarly, the SAR concept may not be sufficient for direct interactions with the E field. Further, there is always a level of uncertainty about perceived field strength and actual RF exposure.

The extrapolation of results from animal experiments (mostly) to humans is used in a reduced form, termed frequency scaling. The approach essentially enables us to use results from one model experiment and to predict results in another biological object, differing only by a scale factor. The frequency-scaling principle can be applied to determine the equivalence between the exposure of a man of height l_m and an animal of a length l_a, with both man and animal having the same orientation with respect to the exposure field, using the relationship:

$$f_m l_m = f_a l_a \tag{4}$$

where f_m and f_a are the frequencies at which man and animal are exposed, respectively (Durney and Christensen, 2000). It should be mentioned that the SAR distribution in the two cases will be similar in the two bodies (but may not be the same). This may be valid for low losses, which is not always be the case for biologic structures.

5 BIOLOGICAL EFFECTS

While considering the biological effects of electromagnetic-field exposure, the evidence for nonthermal effects has been gathered under two headings: (i) *in vitro*: altered cell responses and (ii) *in vivo*: results of chronic exposure in animals. The data available on biological effects of electromagnetic-field exposure are immense and at times contradictory. These effects may not always produce adverse health impacts. There is an element of uncertainty across various mobile frequency bands and also on whether the target under investigation is brain or muscle tissue. Living systems are

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electrochemical systems that use EMFs in everything from protein folding, through mobile communications, to nervous system function. If we expose a living system to a weak external signal it will interact and may interfere with normal physiological functioning (Parker and Winters, 1992). It is this aspect of EMF–biological interaction that has aroused interest in recent years. Some of the physiological systems that have been targeted for investigation are discussed below.

5.1 The Blood–Brain Barrier (BBB)

The blood–brain barrier (BBB) protects the mammalian brain from potentially harmful compounds in the blood. It is a selectively permeable, hydrophobic barrier that is readily crossed by small, lipid-soluble molecules. It serves, not only to restrict entry of toxic polar molecules into the brain, but also as a regulatory system that stabilizes and optimizes the fluid environment of the brain's intracellular compartment. A dysfunctioning BBB allows influx of normally excluded hydrophilic molecules into the brain tissue. This might lead to cerebral oedema, increased intracranial pressure and, in the worst case, irreversible brain damage. Opening of the BBB may subject the central nervous system (CNS) to assault from extraneous micro-organisms. It is thus a natural defence system that maintains the physicochemical environment of the brain within certain narrow limits that are essential for life.

The BBB has thus been a subject of investigation because of its vicinity to the radiation source and its central function in the human brain. Because of its penetration, exposure of an electromagnetic field could cause significant alteration in BBB behaviour. The emission intensity of sodium fluorescein in brain tissue exposed to an EMF for 30 minutes was larger than that without such exposure (Williams *et al*., 1984). In a study on the transport of D-mannitol across the BBB, it was observed that a higher power (larger SAR) generally yielded larger permeability, and a pulse wave was more effective in permeability enhancement than a continuous wave (CW) (Oscar and Hawkins, 1977). Further, for higher electromagnetic frequencies, the conductivity was larger, with a correspondingly higher SAR (Equation 3), hence producing a greater permeability. Larger modulation also induces greater permeability. In addition, the conductivity at a depth of 50% was larger than that for 0% for an AM (amplitude modulation) wave with a modulation of 16 Hz (Albert *et al*., 1987). A twofold increase in the BBB permeability of sucrose was obtained after exposure to an EMF of 1.8 GHz over four days (Schirmacher *et al*., 2000). From all these observations, it can be concluded that permeability enhancement was not because of temperature increase, as it was within the variation of 1 °C in daily rhythm.

This selective permeability has the disadvantage that agents and drugs that are effective in treating diseases in other parts of the body may not be able to gain entry into the brain to combat infection. The ability to selectively open the BBB suggests the possibility of using microwave regional hyperthermia to facilitate chemotherapy for brain tumours and facilitate the delivery of anticancer drugs such as methotrexate. This drug is often used for high-dose chemotherapy.

A series of investigations on BBB permeability have revealed changes at a very low level of microwave exposure. As a specific example, effects of microwave fields on the BBB have been investigated by exposing male and female Fisher 344 rats in a transverse electomagnetic (TEM) line chamber to 915 MHz CW microwaves, as well as pulse-modulated waves with repetition rates of 8, 16, 50 and 200 per second. The SAR varied between 0.016 and 5 W kg^{-1}. The rats were not anaesthetized during the two hours of exposure. The results show that both continuous and pulsed microwaves have the potential to open up the BBB for albumin passage, with no significant difference between the two (Salford *et al*., 1994).

5.2 The Nervous System

Due to the proximity of a mobile phone to the head, most research efforts have been concentrated on estimating the potentially toxic effect of RF on the CNS. This is largely because the nervous system is the body's main control and integrating network. Its activities are divided into two parts: sensing and then responding. In the first category it serves two functions: sensing intrinsic and extrinsic (environmental) stimuli, and interpreting and integrating these signals. In the second phase, it responds to interpretation by initiating actions in the form of muscular contractions or glandular secretions. The nervous system of vertebrates is composed of two primary parts: the central nervous system composed of brain and spinal cord and the peripheral nervous system, composed of all nerves outside the CNS. When the nerves or brain are perturbed, for example by RF radiation, morphological, electrophysiological and chemical changes will take place. These include transient minor effects on a electroencephalogram (EEG), on sleep structure and on cognitive processes in human subjects (Mann *et al*., 1997. A significant change in these can lead to a change in overall behaviour. It has been further reported that different areas of the brain have different sensitivities to RF radiation (Lai *et al*., 1989; Ray and Behari, 1988). Exposure of the brain to a mobile phone may lead to an increase in temperature to the extent of 0.1 °C (Van Leeuwen *et al*., 1999; Wainwright, 2000), after taking into consideration the high blood-perfusion rate.

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This, in contrast to skin blood perfusion, was significantly elevated after exposure to mobile phone frequencies (Monfrecola *et al*., 2003). Under some conditions, such as when the handset operates at full transmitter power because of a long distance to the next base station, local SAR values in the brain are reported to be in the range of $1\,W\,kg^{-1}$ or more (Huber *et al*., 2003).

6 THE ROLE OF CALCIUM

6.1 Effects on Metabolism

Lerchl *et al*. (2008) have shown that chronic RF exposure (383, 900 and 1800 MHz) leads to frequency-dependent increase of body weight in Djungarian hamsters at moderate SAR values. These authors have concluded that this is indicative that metabolic processes may be affected by such exposures. It is possible that molecular processes involved with the brain energy metabolism of the rat may be perturbed by radiation in the microwave frequency region. It has been reported that there are divalent iron atoms and two divalent copper atoms in the molecules of the respiratory chain (Lehninger, 1965). Each of these is essential to the respiratory function at the molecular level. Charged particles like these, if not otherwise constrained, undergo translational motion in an electric field. Such a group of particles would possibly respond to an oscillating electric field over a wide range of frequencies, starting from natural RF oscillations up to the highest values to which natural process react (Sanders *et al*., 1984). Nicotinamide adenine dinucleotide (NADH), adenosine triphosphate (ATP) and creatine phosphate (CP) are key compounds in brain energy metabolism. ATP is a key compound in energy metabolism because it is the carrier of energy for the processes in living cells. NADH is oxidized to produce ATP in the mitochondria, while brain ATP concentration is maintained at the expense of CP. When demand for ATP is higher than the mitochondrial production capacity, CP is rapidly converted to ATP to sustain ATP levels and a significant decrease in CP levels is observed prior to any decrease in ATP. The changes in these compounds are frequency dependent.

Another effect on metabolism of electromagnetic radiation is the leakage of free calcium ions, either through the cell's external membranes or those surrounding internal 'calcium stores'. This can have dramatic effects on many aspects of metabolism and explains results like the stimulation of growth and increased risk of cancer, symptoms suffered by electrosensitive humans. Calcium concentration inside the cell controls the rate of many metabolic processes, for example, the activity of many enzyme systems and the expression of genes. Ca^{+2} ions use special channels to cross the membrane along the concentration gradient. The concentration of calcium ions in the cytosol is normally kept about a thousand times lower than that outside by metabolically driven ion pumps in the membranes. Electromagnetic-field stimulation increases membrane leakiness, causing unregulated amounts of extra calcium to flood in. The actual effects depend on the programmed state of the cell at that instant of time; for example, the growth rate, the repair rate after injury or the rate of healing can be enhanced. On the other hand, if there are mutant precancerous cells present, it may also lead to tumour promotion.

6.2 Calcium Release from Cell Membranes

Ca^{+2} ions are able to bind without causing deformations to either membrane proteins or soluble proteins of the cytoplasm or organelles. They have several ion-binding sites and, by changing their configuration as a function of the occupied site, they are capable of exciting a specific target enzyme. The Ca^{2+} ion has been identified in relaying electrochemical messages to the cell surface and thus is the most critical target for microwave exposure. It is known that Ca^{2+} signalling has extreme sensitivity to coherent excitation by very low field energies within specific amplitude and frequency windows (Gartzke and Lange, 2002). This causes a large electrostatic force to be generated at the cell surface. The lowest level of exposure could be $0.00015\,W\,kg^{-1}$ ($0.08\,\mu W\,cm^{-2}$) with a 16 Hz signal modulated at 240 MHz (Schwartz *et al*., 1990). The mechanism of action of gonadotrophin releasing hormone (GnRH) on the anterior pituitary gonadotrophin reveals that Ca^{2+} functions as a second messenger in the GnRH-activated signal-transduction cascade (Jennes *et al*., 1995; Haisenleder *et al*., 2001; Liu *et al*., 2003).

Ca^{+2} ions use special channels to cross the membrane along the concentration gradient. These voltage-gated channels are normally closed in excitable cells, but they open in response to an action potential. There has always remained a question of whether the cell membrane (calcium ion channel), is directly involved in electromagnetic-field interactions (Liburdy, 1992). This is important because the receptor sites and ion channels are located in the cell membrane and are first involved in the signal-transduction processes. Another question is if alterations in the calcium fluxes are propagated down the signal-transduction cascade to alter events such as gene activation. Further, changes in the calcium ion second messenger are linked to induction of c-myc messenger RNA, a mid-stage signal-transduction marker, which, in turn, travels down the signal-transduction cascade to alter gene activation events and cell proliferation.

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6.3 Brain Function and EMF Effects

The role of calcium in the control of brain function has been under investigation and was found to be influenced by a range of modulating frequency. A statistically significant increase in net $^{45}Ca^{2+}$ transport was observed for modulating frequencies of 6–16 Hz, followed by a fall over the frequency range 20–35 Hz (Bawin *et al*., 1978). The nonlinear effect of modulated waves on chicken cerebral tissue has also been demonstrated with $^{45}Ca^{+2}$. The animals were exposed to 0.8 mW cm^{-2} at 147 MHz, amplitude modulated by a sinusoidal signal (0.5–35 Hz). The existence of frequency windows was confirmed (Blackman *et al*., 1979) for an incident power flux of 1 mW cm^{-2}; there was a positive response when the modulation lay between 6 and 12 Hz, but little response at 0.5 and 20 Hz. A power window was also shown to exist at constant frequency; when the chicken cerebral tissue was submitted to a 450 MHz carrier wave modulated at 16 Hz there was a significant increase in power levels of 0.1 and 1 mW cm^{-2}, while no effect was observed for power levels of 0.05 and 5 mW cm^{-2}. Similar results in terms of frequency and power windows were reported by Kunjilwar and Behari (1993) who exposed rats to 112 MHz, amplitude modulated at 16 Hz (SAR 0.1 W kg^{-1}), and reported changes in acetylcholineste rase activity and Na^+/K^+ phosphatase activity (Behari *et al*., 1998). They observed no significant changes when the CW was changed to 73.5 and 36.75 MHz with a modulating frequency of 76 Hz. The limits of the frequency and power windows were 6–20 Hz and 0.1–1 mW cm^{-2}, respectively (Gandhi, 1982). The carrier frequency was shown to be less than 1 GHz, but itself had little effect, while the modulating frequencies were more controlling. For avian brain tissue under an AM wave, conductivity at a modulation of 16 Hz was 1.2 times that for 3 Hz (Albert *et al*., 1987). For human brain, conductivity also increases with an FM modulation increase from 9 to 16 Hz (Blackman *et al*., 1979).

Bawin *et al*. (1975) have found that exposing brain tissue to weak VHF (very high frequency) radio signals modulated at 16 Hz released calcium ions bound to the surfaces of cells. Blackman *et al*. (1982) concluded that weak fields were often more effective than strong ones. Without these ions, cell membranes are weakened and are more likely to tear under the stresses and strains imposed by the moving cell contents. Although the resulting holes are normally self-healing, they will increase leakage while they are open and this can explain the bulk of the known biological effects of weak electromagnetic fields. Leaks in the membranes surrounding lysosomes can release digestive enzymes, including DNAse (an enzyme that destroys DNA). Panagopoulos *et al*. (2007) showed that exposing adult *Drosophila melanogaster* to a mobile phone signal for just six minutes a day for six days broke the DNA in the cells that give rise to eggs into fragments, and half of the eggs died. Diem *et al*. (2005) also found significant fragmentation after exposing cultured rat and human cells to a simulated mobile phone signal for 16 hours. They showed that exposing human cells for 24 hours to simulated phone signals gave DNA fragmentation similar to that due to the gamma rays emitted from a radioactive isotope.

7 ELECTROMAGNETIC-FIELD EXPOSURE AND ENZYME ACTIVATION

7.1 Ornithidine Decarboxylase (ODC)

Ornithidine decarboxylase (ODC) is an enzyme essential for cell growth and DNA synthesis. It is a rate-limiting enzyme in the synthesis of polyamines. Polyamines (putrescine, spermadine, spermine, cadaverine) are long-chain molecules that are positively charged (polycationic). They have the highest charge/mass ratio of any biomolecule. High ODC activity occurs in the unregulated growth of tumour cells, as in the malignancy prostate cancer (**Figure 5**).

RF exposure has been shown to induce ODC activity, levels of which are often elevated during cell growth and tumour promotion. The exposure of mouse fibroblasts to amplitude modulated microwaves at an SAR of 3 W kg^{-1} increase ODC activity (Penafield *et al*., 1993), but to a much lower level than treatment with a chemical promoter. Litovitz *et al*. (1993) have examined the modulation frequency dependence of ODC activity in cultured cells (fibroblasts), determining the minimal duration that a single ELF modulation frequency must be sustained (coherence time) in order to elicit an ODC response. Using a 915 MHz field, switching modulation frequencies from 55 to 65 Hz at a coherence time of one second or less abolished enhancement of ODC responses, while coherence times of 10 seconds or longer produced full enhancement. These microwave coherence effects and pulsed effects are similar to those observed in ELF fields.

In an investigation in murine L929 cells, ODC was activated by an AM wave at 845 MHz, though it was not affected by frequency modulation (FM) at the same frequency (Penafield *et al*., 1993). ODC synthesizes polyamines, which promots the permeability of superoxide dismutase (SOD) across animal cell membranes (Poduslo and Curran, 1996).

7.2 Protein Kinase C Activity

Protein kinase C (PKC) plays a key role in a variety of pathologic states, including oncogenesis (Harvey

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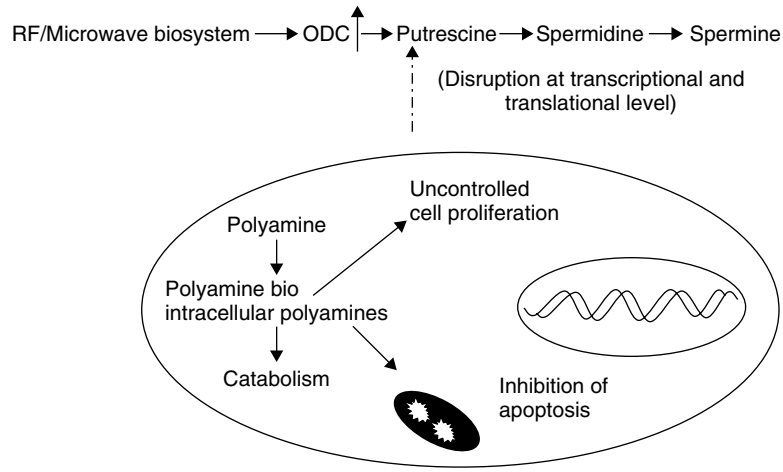

Figure 5 Mode of RF/microwave interaction with biological system in tumour promotion.

and French, 2000; Nishizuka, 1986), and in mediating cellular responses to extracellular stimuli involved in proliferation, differentiation, apoptosis and exocytotic release in a number of non-neuronal cells (Ohkusu *et al*., 1986). Several lines of evidence suggest that PKC modulates ion conductance by phosphorylating membrane proteins such as channels, pumps and ion-exchange proteins, besides its role in extrusion of Ca^{2+} immediately after its mobilization into cytosol. The enzyme has also been implicated in phosphorylation of several neuronal proteins, which are thought to regulate neurotransmitter release and long-term potentiation in memory formation (Suzuki, 1994).

The activation of this enzyme is thought to be biochemically dependent on Ca^{2+}. Tumour-promoting phorbol esters have a structure very similar to diacylglycerol and activate protein kinase C directly, both *in vitro* and *in vivo* (Castagna *et al*., 1982). It has been variously reported that protein kinase C is the receptor for tumour promoters (Parker *et al*., 1984). Tumour promoters, such as phorbol esters, increase the affinity of the enzyme for Ca^{2+}, resulting in its full activation at physiological Ca^{2+} concentration. TPA has a specific membrane receptor in the cell membrane (Hunter *et al*., 1984; Niedel *et al*., 1983). In order to stimulate cell proliferation in cells, growth factor and PKC are needed to induce the signal pathways. Byus *et al*. (1984) reported a decrease in the activity of this enzyme following exposure of human lymphocytes for a period of 15–30 minutes to 450 MHz, amplitude modulated at 16 Hz.

8 GENOTOXIC EFFECTS

The photon energy of RF radiation (10^{-3}–10^{-6} eV) is much less than the energy required to break chemical bonds (hydrogen bonds ~0.5 eV) and therefore it is believed that this radiation does not damage DNA. However, it is possible that certain cellular constituents are altered by exposure to EMF, such as free radicals. Free radicals are able to interact with DNA or other cellular components and are involved in cell regulatory processes.

8.1 Electromagnetic Fields and DNA

- Since nerve cells do not divide and are not likely to become cancerous, more likely consequences of DNA damage in nerve cells are changes in function and cell death, which could either lead to or accelerate the development of neurodegenerative diseases.
- EMF-induced DNA damage could be blocked by treating rats with antioxidants, including melatonin, immediately before and after exposure. Melatonin is a hormone secreted from the brain's pineal gland. As a potent antioxidant, it effectively eliminates free radicals inside cells.
- The effect of RF radiation on DNA could conceivably be more significant on neurons than on other cell types, because these nerve cells have a low capability for DNA repair.

DNA damage may lead to increased mutation frequency and could be linked to gene mutations. A gene is a section of DNA containing the information needed to make a particular protein or enzyme. There is also a section that can turn the gene on or off in response to outside signals. The growth of an organism from a fertilized egg involves a hugely complex pattern of switching genes on and off that regulates growth, cell division and differentiation into specific tissues. DNA damage can sometimes give unregulated growth to form

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tumours. However, the effect may not be immediate and may take years before it is fully replicated.

In most studies, genotoxic effects have been investigated after short-term exposure (Moulder *et al*., 1999; Vijayalaxmi and Obe, 2004). Diem *et al*. (2005) reported DNA strand breaks (by comet assay) in human diploid fibroblasts and cultured rat granulose cells after RF field exposure (1800 MHz, SAR 1.2 or 2 W kg^{-1}; different modulations; duration 4, 16 and 24 h; intermittent 5 minutes on/10 minutes off or CW), but it is not clear if continuous exposure of nonmodulated or modulated 1800 MHz was used. A statistically significant increase in micronucleus formation and in chromosomal aberrations were observed in fibroblasts as well. Speit *et al*. (2007) used CW with intermittent exposure (1800 MHz, SAR 2 W kg^{-1}), applied using the same cell system and reported negative results. Nikolova *et al*. (2005) reported, after six hours, but not after 48 hours RF field exposure, a low and transient increase in DNA strand breaks in embryonic stem-cell-derived neural progenitor cells.

When mice were exposed to 2.45 GHz fields at an SAR of 1.18 W kg^{-1} for two hours per day for 120, 150 and 200 days, structural genomic rearrangement were observed in brain and testes cells (Sarkar *et al*., 1994). Lai and Singh (1995), (1996) reported that rats exposed to pulsed (two-second pulses, 500 pulses per second) or CW 2.45 GHz fields with SARs of 0.6 or 1.2 W kg^{-1} for two hours increased the number of single- and double-strand breaks in brain cell DNA when assayed four hours after RF exposure. Lai and Singh (1997) also reported that treatment of rats immediately before or after exposure with either melatonin (1 mg kg^{-1}) or PBN (*N*-*t*-butylphenylnitrone, 100 mg kg^{-1}) blocks the formation of DNA breaks by RF fields. These experiments challenge the belief that RF fields are unable to break molecular bonds. Epigenetic events may be involved. Paulraj and Behari (2006) studied the same phenomena at other frequencies and low dose level, and confirmed DNA strand break; they also concluded that the hippocampus is the targeted site of such interactions.

While damage to DNA strands has been confirmed by several workers, it is argued that its repair is an ongoing process and the damaged chromosomes can be reconstituted. However, this proposition is not without risk. There is no guarantee that these will replicate in the manner they were originally present. Pieces may be left out (deletions), joined in backwards (inversions), swapped between different parts of the chromosome (translocations) or even attached to the wrong chromosome. The effect may also be frequency dependent. In most cases, the new arrangement can work for a while, if most of the genes are still present, and any metabolic deficiencies can often be made good by the surrounding cells. However, things may be different when it comes to meiosis. During meiosis, the chromosomes line up in pairs (one from each original parent) along their entire length so that corresponding parts are adjacent and can be exchanged. Malformed pairs are torn apart in the later stages of meiosis so that eggs or sperms that have an incomplete or unbalanced set of genes may not function properly, and so reduce fertility and other physiological functioning. There is a possibility that this may lead to permanent genetic damage, which although it may not be visible in the first generation, may be evident thereafter.

8.2 Fertility Patterns

For some time there has been a debate about whether exposure to RF electromagnetic fields is associated with an elevated risk in reproduction. Radio waves from mobile phones may have an effect on the process of spermatogenesis. According to one study, lifestyle can decrease semen quality (Kilgalton and Simmons, 2005) and prolonged use of mobile phones can have negative effects on sperm motility chacteristics (Fejes *et al*., 2005). It has been shown that sperm DNA damage is not repaired, because of the chromatin structure (Singh and Stephens, 1998).

Concerns are growing about the possible hazardous effects of RF electromagnetic waves (EMW) emitted by household electronic gadgets on human health. Nakamura *et al*. (2003) found that exposure of pregnant rats to 2.45 GHz CW microwaves at 2 mW cm^{-2} power density for 90 minutes decreased uteroplacental blood flow and increased progesterone and $PGF_2\alpha$ (placental growth factor). Dasdag *et al*. (2003) reported a decrease in seminiferous tubule diameter in male rat testes after exposure. They used a commercially available 890–915 MHz global signal module with 0.141 W kg^{-1} whole-body SAR. More recently, Aitken *et al*. (2005) found significant damage to mitochondrial and nuclear genomes in epididymal spermatozoa of mice, when exposed to RF 900 MHz EMW, 12 hours a day for seven days. Several authors (Fejes *et al*., 2005; Ji-Geng *et al*., 2007; Kesari and Behari, 2008) have also observed that carrying the mobile phones near reproductive organs for a longer time may have negative effects on sperm motility and male fertility.

Forgacs *et al*. (2006) have reported that repeated whole-body 1800 MHz GSM-like microwave exposure (0.018–0.23 W kg^{-1}) below the maximum permissible exposure (MPE) level recommended by the International Commission for Nonionizing Radiation Protection (ICNIRP) is able to increase the serum testosterone level, RBC (red blood cells) and VPRC (volume of packed red blood cells) in male mice, although still within physiological limits. These authors further reported that the unaltered *in vitro* steroidogenic capacity of Leydig cells obtained from microwave-exposed mice suggest that Leydig cells are not the primary targets of the applied microwave exposure or the direct action is transient only and confined to the period of exposure. This suggests that

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the main target of action is probably located at the higher regulation level of the hypothalamic-pituitary-gonadal axis. It is known that prolonged use of GSM mobile phones may lead to reduced melatonin production (Burch *et al*., 2002; Jarupat *et al*., 2003). Melatonin exerts an antigonadotrophic effect mainly at the level of the hypothalamus and pituitary (Jackson *et al*., 1984; Bittman *et al*., 1985; Vanecek, 1998) and directly decreases the testosterone secretion in Leydig cells too (Valladares *et al*., 1990; Kus *et al*., 2002).

Sun *et al*. (2005) investigated the effects of electromagnetic radiation emitted by computers on human sperm quality and did not find any adverse effects. Reactive oxygen species (ROS), such as superoxide anions ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2) may influence the structural integrity and function of sperm, such as motility, capacitation and sperm–oocyte fusion (Griveau *et al*., 1995). Spermatozoa are particularly vulnerable to oxidative stress because their plasma membrane is rich in polyunsaturated fatty acids (PUFAs) and membrane bound NADPH oxidase. ROS have been shown to correlate with reduced male fertility (Iwasaki and Gagnon, 1992), cause perioxidative damage to the sperm plasma membrane (Hughes *et al*., 1996), and induce both DNA strand breakages and oxidative base damage in human sperm (Kodama *et al*., 1997). A decrease in total antioxidant capacity of seminal plasma has been correlated with a reduction in sperm quality, such as concentration, motility and morphology (Smits *et al*., 1998).

The issue that biological effects of RF radiation are genotoxic is still not fully resolved, though some of the available results do not discount such a possibility. Some *in vitro* data show effects that are negative, while others are positive, close to the guidelines levels for safe exposure.

9 NONGENOTOXIC EFFECTS

Several studies reported the effect of RF fields on cell-cycle kinetics, but in the majority of the investigations no effects were detected (Vijayalaxmi *et al*., 2001; Higashikubo *et al*., 2001; Zeni *et al*., 2003; Miyakoshi *et al*., 2005; Lantow *et al*., 2006c). Alteration of cell proliferation was described in only a few reports (Pacini *et al*., 2002; Capri *et al*., 2004b).

Apoptosis is an important mechanism of protection against cancer. Several studies have reported RF field effects on human peripheral blood mononuclear cells (Capri *et al*., 2004a), lymphoblastoid cells (Marinelli *et al*., 2004), epidermis cancer cells (Caraglia *et al*., 2005), human Mono Mac 6 cells (Lantow *et al*., 2006c) and Molts 4 cells (Hook *et al*., 2004). No difference in apoptosis induction was detected between sham-exposed and RF-field-exposed cells (Hook *et al*., 2004). On the other hand, Marinelli *et al*. (2004) have reported a better survival rate of T lymphoblastoid leukaemia cells exposed to 900 MHz nonmodulated RF fields and Caraglia *et al*. (2005) found apoptosis induction in human epidermoid cancer cells after exposure to 1.95 GHz fields. The REFLEX study (Nikolova *et al*., 2005) reported no effects of RF fields on cell cycle, cell proliferation, cell differentiation, apoptosis induction, DNA synthesis and immune cell functionality. These authors described some effects on the transcript level of genes, after RF exposure, related to apoptosis and cell-cycle control; however, these responses were not associated with detectable changes in cell physiology. Analysis of whole-genome compementary DNA arrays show alterations in gene expression after various RF exposure conditions using different cell types, but no consistent RF signature, such as stress response, could be identified (Remondini *et al*., 2006).

Heat-shock proteins act primarily as molecular chaperones to eliminate unfolded proteins, which can also appear from cellular stress. This stress response can be induced by many different external factors, including temperature, chemicals, oxidative stress, heavy metals, ionizing and nonionizing radiation and ultrafine carbon black particles. Hsp70 has been shown to interfere with postmitochondrial events to prevent free-radical-mediated apoptosis (Gotoh *et al*., 2001). An increased expression level of Hsp70 can thus offer protection against stress. Heat-shock proteins are also involved in oncogenic processes (Jolly and Morimoto, 2000; Inoue *et al*., 1999; French *et al*., 2001). Some investigators have described increased heat-shock-protein levels after RF exposure (Leszczynski *et al*., 2002; Kwee *et al*., 2001; de Pomerai *et al*., 2000). However, these results are controversial, because there are negative findings also (Cotgreave, 2005). It may be that data on Hsp70 awaits further confirmation, at different frequencies and power levels.

Nikolova *et al*. (2005) described modulation in gene regulation after RF-field exposure at an SAR of $1.5\,W\,kg^{-1}$ in p53-deficient embryonic stem cells. Proteomic analyses of human endothelial cell lines showed RF fields induce changes in the expression and phosphorylation state of numerous proteins, including Hsp27.

Another important parameter is to measure neurotransmitters in various parts of the brain (Zhou *et al*., 1986). Neurons transmit information across the synapses using a range of neurotransmitters. Calcium plays an essential role in this, because a small amount of calcium must enter the neuron every time before it can release its neurotransmitters. If the electromagnetic field causes extra calcium to leak inside the cell (neuron), it increases the background level and may trigger an early release of neurotransmitter. It has been shown that the excitation of acupuncture points by microwaves (0.2–3 GHz) may produce an efficient analgesic effect, as shown by

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the corresponding increase in the pain threshold (Teng *et al*., 1989). Furthermore, respective variations in pain threshold and neurotransmitter release in the center of pain reception in the brain are proportional (Van der Vorst *et al*., 1992).

Particular interest has also been expressed in the biological effects of pulsed modulated RF and microwaves on the ear. The human ear can perceive pulsed modulated radiation between 200 MHz and 6.5 GHz as a buzzing or clicking noise, depending on the modulation characteristics. The effect is attributed to the thermoelastic expansion of the brain tissue, following the small ($\ll 1\,^{\circ}C$), but rapid, increase in temperature on the absorption of the incident energy, generating a sound wave in the head that is detected by the hair cells (cochlea) (Sienkiewicz *et al*., 2005). However, its clinical potential is yet to be fully utilized.

10 MECHANISM OF ENERGY TRANSFER AND AMPLIFICATION

10.1 Macromolecular Resonance

Electromagnetic-field effects in biology and their variability are suggestive of the possibility of resonance effects. So far resonance conditions are characterized in terms of narrow 'windows', of frequency and amplitude ranges only (Blackman *et al*., 1995).

There are two broad situations: when collisional perturbations are very short and when they are long in comparison with the field period. In the former case, every collision is quite effective in interrupting the molecule's radiation absorption–emission process. In a fluid with a high rate of collisions, a collisional broadened relaxation-type spectrum results. In the later case, whene collisions are of long duration compared to the EMF period, the spectrum becomes resonant. The collision time in a macromolecular fluid is a function of temperature, and therefore the EMF frequency determines the kind of spectrum (rotational or resonant). Water is a broad-band attenuator of microwave (MW) fields, which also shields other possible bimolecular absorption processes. Experiments suggest resonant absorption in growing cells at 41 GHz fields and in DNA at 11 GHz (Grundler *et al*., 1977; Azanza and Del, 1994). However, the significance of these resonance frequencies in biological systems is yet to be established.

The nonthermal, pulsed microwave effects are connected with the transfer of information from the irradiating field to the live organism, through the latter's ability to identify certain frequencies of radiation, then its ability to absorb energy from the field (Hyland, 2001). The intensity of radiation needed for this recognition is many orders of magnitude below even that currently associated with nonthermal effects.

10.2 Stochastic Resonance

Fields millions of times weaker than the membrane potential gradient of $10^7\,V\,m^{-1}$ modulate cell processes to surface-stimulating molecules. This may underline the ability of biological systems to amplify small signals in noisy environments. Viewed in this manner; it is possible to imagine an information-processing system in which the presence of an ongoing noise-like activity enhances information-handling capacity. Low-level effects are attributed to the direct interaction between neuron membranes and the local electric fields. Power levels of 0.1 and $1\,mW\,cm^{-2}$ correspond to free-space values of 61 and $194\,V\,m^{-1}$, respectively. This field is negligible compared to the static transmembrane potential of $\sim$90 mV ($\sim 10^7\,V\,m^{-1}$) across a membrane of thickness 4 nm. However, this is larger than the field due to slow brain waves ($1\,V\,m^{-1}$) or from terrestrial fields (10^{-3}–$10^{-6}\,V\,m^{-1}$) (**Figure 6**).

10.3 Cell-Surface Glycoproteins as Possible Sites for EM-Field Interactions

Cells in tissue are separated by narrow fluid channels, typically not more than 150 A° wide, that act as windows on the electrochemical senses surrounding each cell. These channels are preferred pathways for intrinsic and environmental electromagnetic fields in tissue, since they offer a much lower electrical impedance than cell membranes. Functional measures of brain electrical impedance are thus an index of conductance in this extracellular space and have been correlated with brain tissue physiological states in health and disease (Adey *et al*., 1963; 1965; Porter and Bocke, 1965). These intercellular channels are also the sites of electrochemical sensors that protrude as protein strands from within the cell membrane. They form a strongly negatively charged glycocalyx on the cell surface (**Figure 7**). They act as specific receptors for hormones, antibodies, neurotransmitter molecules, certain chemical cancer promoters and, maybe, EMF signals. Their amplified signals to the cell interior elicit enzymatic responses regulating metabolism, messenger functions and cell growth. McConnell (1975) noted that intrusion of the phospholipid tails are constrained and they behave more rigidly.

It is now established that cell Ca^{2+} signalling has extreme sensitivity for coherent excitation by field energies within specific amplitude and frequency windows (Schwartz *et al*., 1990). Membranes are identified as sharply partitioning each cell from its neighbour, by reason of their high content of fat molecules (phospholipids). The structure is identified as double layer of phospholipid molecules, the plasma

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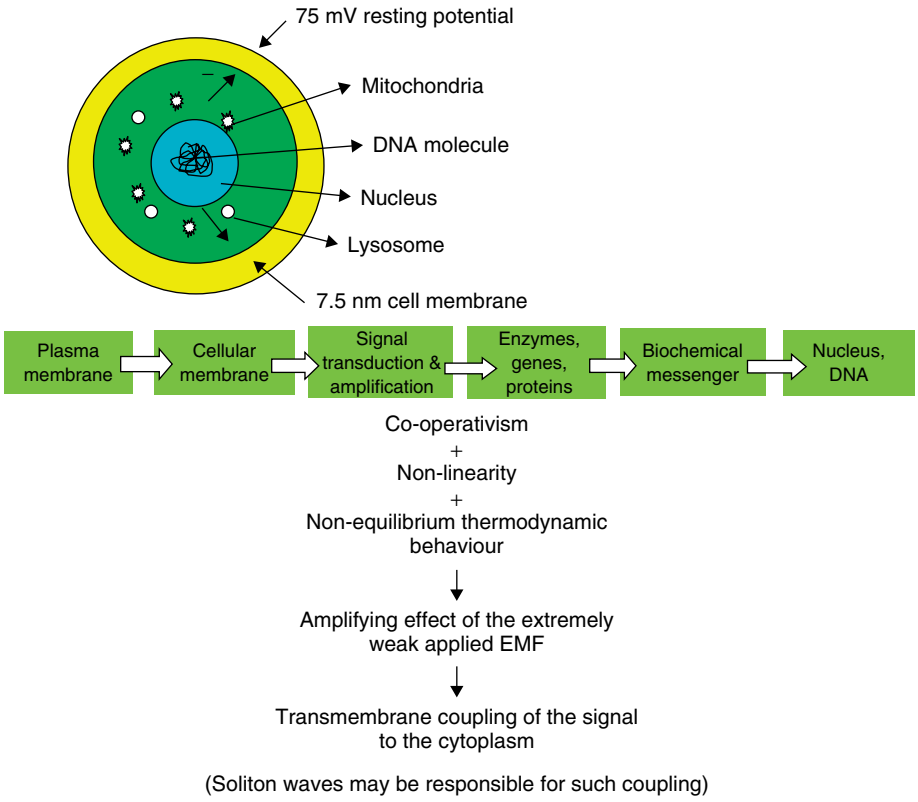

Figure 6 An idealized excitable cell at rest in the human body. 75 mV across a thickness of 7.5 nm corresponds to an electric field of 10 million V m^{-1}.

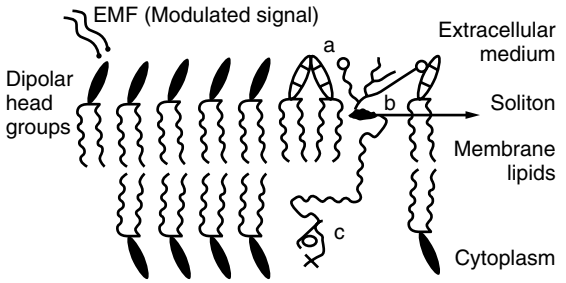

Figure 7 Model of the interaction between an EM field and the cellular system. a: glycocalyx; b: soliton; c: intracellular spacing. (Reproduced with permission from Lawrence and Adey, 1982. © Maney Publishing.)

membrane. The cell membrane is thus a physical and an electrical barrier. Since generally accepted physiological findings have revealed sensitivities to induced tissue electric gradients from ELF environmental fields and from ELF-modulated fields, it is anticipated that power amplification mechanisms are operative in transductive coupling of these weak stimuli from fluid surrounding cells to the cell interior.

In search for a general mechanism of interaction of EM field with cells, Na^+-K^+ ATPase, the 'ion-pump' enzyme in cell membranes has been studied (Behari *et al.*, 1998; Blank and Soo, 1992; 1991). Changes in enzyme activity in either electric or magnetic fields show that the most important factor affecting signal transduction is the level of enzyme activation, the maximum biological (ATP-splitting) activity of the enzyme under a given set of conditions (i.e. ion concentration, temperature). Enzyme function involves communication between ATP-splitting catalytic sites and ion-binding sites in different parts of the molecule in a coordinated sequence; ion-binding sites in enzymes are essential and are probably brought about by charge movements during function. Such movements have been observed in the microsecond range. Ion fluxes via effects on ion pumps such as Na^+K^+ ATPase in human red blood cells exposed to RF and microwave radiation (Allis and Sinha-Robinson, 1987; Liu *et al.*, 1990; Christiansen, 1989), pointed out dependence of the optical properties on membrane excitation. States may determine the stability of dark soliton propagation as a means of transmembrane signalling. This is summarized in **Figure 6**.

11 BIOMARKERS OF TUMOUR PROMOTION

From physical considerations it is suggestive that RF exposure is not mutagenic and is therefore unlikely to initiate cancers. Animal studies have demonstrated

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that microwaves and RF fields can be called cocarcinogenic since they increase chemically induced skin cancer (Szmigielski *et al*., 1982a). Cellular studies have verified that these carcinogenic effects are mediated by DNA, since relatively weak fields increase DNA synthesis (Liboff *et al*., 1984) and can modulate DNA repair (Meltz *et al*., 1987). Some studies have also demonstrated that electric fields could break or nick DNA strands (Tamiya *et al*., 1988), and the change in secondary structure (conformation) of DNA, a phenomena associated with cancer (Kitchin *et al*., 1989). It should be mentioned that these field emissions were largely at low EM field strengths, the safety limits for the public at large.

Intracellular processes occurring under the influence of power-line magnetic fields, related to free radicals and signal transmission, may determine the biological effects. An uncontrolled oxygen radical release (oxidative stress), may cause protein oxidation, enzyme inactivation and lipid peroxidation within the cellular membranes, causing structural and functional abnormalities. SOD plays a key role in the system protecting the body from destructive free-radical activity. A decrease in SOD and glutathione peroxidase activities, with an increase in catalase (CAT) activity, has been reported in powerline frequency-generated magnetic fields (Kula *et al*., 2002) and in RF-field exposure alike (Kesari and Behari, 2008). It is possible that magnetic fields interact with the free radicals formed, rather than inducing them.

Szmigielski *et al*. (1982b) and Szudzinski *et al*. (1982) reported that chronic exposure of mice to RF (2.45 GHz, 2–3 or 6–8 $W\,kg^{-1}$, two hours a day, six days a week for up to 12 months; 40 or 100 animals per group) accelerated the development of sarcoma colonies in the lung after subcutaneous injection of sarcoma cells; mammary tumours in mice having a normally high incidence of these tumours by painting the skin with a known chemical carcinogen. Szmigielski *et al*. (1988) reported that exposure to RF fields (2.45 GHz CW,4–5 $W\,kg^{-1}$, two hours a day, 5–6 days a week for a few months; 40 animals per group) increased the number of chemically induced hepatomas and sarcomas, and increased the number of skin tumours in mice given a subcarcinogenic dose of benzo[a]pyrene. The authors suggested that the acceleration of tumour development may have resulted from a direct effect on immunocompetent cells. However, no such effect was reported when exposed to a low-level microwave field after doses of 7,12-dimethylbenz[o]anthracene, a known carcinogen (Paulraj, 2000).

These results and other investigations are not without contradictions. Salford *et al*. (1993) reported no effect on the progression of tumour cells injected into rat brain after exposure to continuous or pulsed 915 MHz RF fields (CW exposure, 1 W per pulse,1.67 $W\,kg^{-1}$, 0.41 $W\,kg^{-1}$ for 217 Hz modulation, pulse width 0.52 ms, 2 W per pulse, 62 animals per group). Santini *et al*. (1988) found that the progression of subcutaneously implanted melanoma cells in mice was unaffected by daily exposure to pulsed or CW exposure to RF fields (2.45 GHz CW and pulsed, 10 $W\,m^{-2}$, 1.2 $W\,kg^{-1}$, 2.5 hours a day, six days a week for the lifetime of the animal). Wu *et al*. (1994) reported no effect of RF exposure (2.45 GHz exposed three hours a day, six days a week for five months; average SAR 10–12 $W\,kg^{-1}$) on chemically induced colon cancer in mice. Rotkovska *et al*. (1993) found that the exposure of mice to low-level RF fields from police radar (34 GHz) did not affect biological parameters that could initiate any pathologic process. Repacholi *et al*. (1997) reported a 2.4-fold increase in the incidence of lymphomas in transgenic mice exposed to up to 900 MHz fields pulsed at 217 Hz with pulse widths of 0.6 seconds. The time to lymphoma development was also shorter in the RF-exposed animals.

The studies undertaken so far do not show any significant variation in the melatonin levels due to continuous RF-EMF exposure (Vollrath *et al*., 1997; De Seze *et al*., 1999; Sukhotina *et al*., 2006).

It can be concluded that there are various indicators of tumour promotion due to electromagnetic-field exposure, including

1. Increase in ODC and PKC activity
2. Increase in Ca^{2+} efflux
3. Altered antioxidant enzyme activity
4. DNA strand breakage (single and double)
5. Na^{+}/K^{+} phosphatase activity
6. Decreased melatonin levels. ↓ Cancer promotion (Stress development/RF/MW syndrome)

It is quite intriguing that the mode of EMF interaction with biological system is multifold. There is ample evidence that low-level electromagnetic fields cause DNA strand breakage in brain cells and sperm. The application of pulsed electromagnetic fields to induced fracture and osteoporosis in rats causes accelerated healing (Behari, 1991) and deceleration in ovariectomized and neuroectomized rat (bones) (Lochan *et al*., 2005; Jayanand *et al*., 2003).

Some recent investigations (Prakash, 2008) have revealed that the synergistic effects of nanoparticles and pulsed electromagnetic radiation can prevent microgravity-induced osteoporosis. This is evidenced by an increase in bone mineral content, bone mineral density and morphological changes. Results indicate that the extremely low frequency field components of clinical pulsed elecromagnetic field (PEMF) signals are far more capable of stimulating bone remodelling activity than higher-frequency components. This is because the pulsed signals carry a multiplicity of messages to the biological systems compared to continuous ones. This in contrast to other low-level field effects, which are often implicated in tumour promotion. In contrast to high power, the use of weak electromagnetic fields to study

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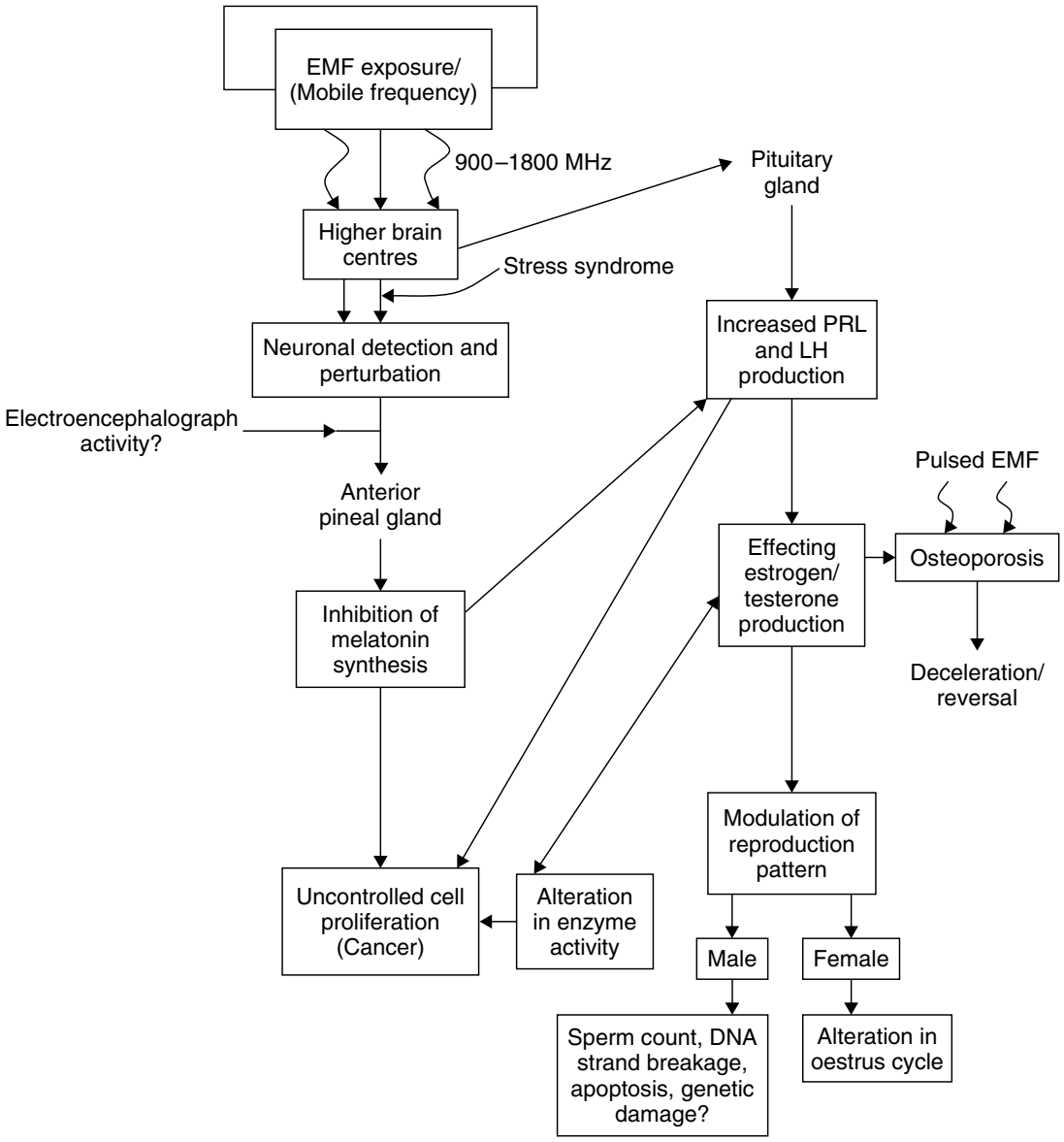

Figure 8 Proposed model of EMF exposure: influence on brain cells, promotion of the growth of initiated tumour cells, osteoporosis and reproduction patterns.

the sequences and energetic events that couple humoral stimuli from surface receptor sites to the cell interior has identified cell membranes as a primary site of interaction with these low-frequency fields in pericellular fluid. A summary of the modes of EMF biointeraction is shown in **Figure 8**.

Long-term biological consequences of repeated microwave irradiation depend on the parameters of irradiation. Experiments have shown that changes in cholinergic receptors, after repeated microwave exposure, also depend on the endogenous signals in the brain. The microwave effects could be blocked by pretreatment, before each session of daily exposure, with the narcotic antagonist naltrexone. It is apparent from these results that endogenous opiods may play a mediating role in some of the neurological effects of microwaves (Lai, 1992). The microwave effects can be summarized as follows: (i) microwaves enhance morphine-induced catalepsy in the rat; (ii) narcotic antagonist blocks a transient increase in body temperature after microwave exposure; (iii) microwave-induced changes in high-affinity muscarinic cholinergic receptors in the brain after repeated sessions of microwave exposure can be blocked by pretreatment with a narcotic antagonist and (iv) the three major subtypes of opiod receptors, μ, δ and κ are involved in mediating the effect of microwaves in the brain.

The hypothesis that low-intensity pulsed microwave exposure can be a source of stress has been investigated by giving low intensity, single and repeated exposure to benzodiazepine receptors in three areas of brain: the cerebral cortex, the hippocampus and

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the cerebellum (Lai, 1992). The cerebral cortex and hippocampus are known to play significant roles in stress responses and, along with the cerebellum, contain high concentrations of benzodiazepine receptors. The authors investigated the effects of single (45 minutes) and repeated (10 × 45 minute sessions daily) microwave exposure (2.45 GHz, 1 mW cm^{-2}, average whole-body SAR 0.6 W kg^{-1}, pulse 500 ppm with pulse-width 2 ms) on the concentration and affinity of benzodiazepine in the cerebral cortex, hippocampus and cerebellum of the rat. The mechanism by which acute stress affects benzodiazepine receptors in the brain is not yet fully understood.

The similarity of the effects of microwaves and those of established sources of stress led to the speculation that microwave irradiation is a 'stressor' (Salford *et al*., 1994). The effects of absorption of electromagnetic energy on brain tissue and cell membranes depend on the type of electromagnetic field (modulation). These effects appear to be important at frequency and amplitude modulations below 300 Hz, although conclusive evidence has yet to emerge. The effect of these frequencies is important to examine, as these fall close to EEG frequencies, though their direct coupling is unlikely (Takashima *et al*., 1978). Whole-body exposure of rats at 30 mW cm^{-2} (SAR 25 W kg^{-1}) causes an increase in EEG activity (the sum of EEG wave frequency bands) immediately after exposure and a slight increase in delta waves (0.5–4.0 Hz) of the EEG recording (Thuróczy *et al*., 1994).

12 HEALTH IMPLICATIONS AND POSSIBLE REMEDIATION

The increasing use of electromagnetic fields for a variety of purposes (domestic, occupational and industrial) raises the problem of their health implications. Various functional properties of DNA (including DNA synthesis (Liboff *et al*., 1984) and repair (Meltz *et al*., 1987) are altered by EM fields. Direct effects on DNA may be mediated by its conformational state. Conformational states, in turn, are controlled by hydrogen bonds, which hold together the folded secondary structure of biomolecules (Takano *et al*., 1999). Mobile phones speed up DNA rewinding, suggesting an increase in hydrogen-bond formation. If mobile phone radiation is found to have effect on hydrogen bonds, it will have serious implications in biology. This hypothesis, however, contradicts other data suggesting that the primary site of EMF biointeraction is the plasma membrane and/or the ions that bind to various protein receptors within the plasma, conditions requiring a complex interaction with the EM field from the mobile phone. In systems where two EM fields interact, the strength and orientation between the two fields is critical in order to obtain resonance conditions.

Some technologies have emerged, utilizing a concept that one type of EM field can reverse or neutralize the biological effects produced by another type. Industry-frequency magnetic fields are important because of the biophysical influence on the cell. (Kula and Drozdz, 1996; Kula *et al*., 2000). One of the major molecular effects of magnetic fields is their influence on the nuclear spins of paramagnetic molecules (Grissom, 1995). This mechanism plays an important role when in the course of a chemical reaction the chemical bond is disrupted and two molecules with unpaired electrons are formed (a pair of radicals). Depending on their electron spin orientations, radical recombination or diffusion and formation of free radicals (e.g. oxygen radicals) may take place. A DC field neutralizes the effect of a pulsed magnetic field on nitrite outgrowth in neuronal cellular studies (Blackman *et al*., 1996). Another related study has been the use of incoherent noise fields to neutralize magnetic-field effects on ODC induction (Farrell *et al*., 1998). In the case of mobile-phone frequency exposures, it has been demonstrated that they increase the number of fruit-fly offspring, as well as some biochemical markers. It also increases the levels of transcription factors which are known to activate DNA synthesis. This effect can be reversed when a commercially available aluminium shield is added to the mobile phone. Syldona (2007) have demonstrated that mobile-phone-induced changes in DNA rewinding are completely reversed when an Aulterra Neutralizer is added to the mobile phone. This effect is seen 77% of the time, yielding a statistically significant result. The evidence supports a set of sequential interactions between ELF and ELF-modulated RF fields and some regulatory mechanisms.

Some experiments suggest that the Aulterra Neutralizer contains a highly paramagnetic mineral. A magnetoresistance response (Meissner effect) is induced when the neutralizer is in the presence of a magnetic field generated from mobile phones. Thus, paramagnetic shielding (in addition to ferromagnetic shielding) is probably the reason for the ability of the Neutralizer. RF exposure causing increased DNA damage in cells is cumulative. Various evidence suggests that responses of the central nervous system to this radiation could lead to a stress response (Lai, 1992; Lai *et al*., 1987). Stress effects cumulate over time and involve first adoption and then an eventual breakdown of homeostatic processes when they persist. The results of major reports (WHO (World Health Organization), 2005; Seitz *et al*., 2005; Rubin *et al*., 2005) seem to support these findings.

In leukocytes, physiological activation is associated with the onset of phagocytosis and leads to increased formation of ROS. These cells exert a wide variety of functions including the regulation of the immune response (pro- and anti-inflammatory processes), scavenging of senescent cells, phagocytosis of infected or

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malignant cells, wound healing, repair and detoxification. The generation of free radicals enhances important physiological processes, for example, signal-transduction processes of various membrane receptors and further immunological functions. An imbalance between excessive formation of ROS and the limited antioxidant defence system may take place. However, several authors (Lantow *et al*., 2006a; 2006b; Simko *et al*., 2006) reported no increase in free-radical level.

13 GUIDELINES FOR SAFETY LIMITS

The question of laying down the criteria for safe exposure is a problematic one, because the dose needs to be assessed, not just as external field intensity, but also as cumulative exposure, as well as SAR, for specific anatomical sites. This demands the need to have an accurate knowledge of RF exposure. This radiation is termed a silent killer; its impact is not immediately visible. Any epidemiological studies over a long period (10 years or more) are difficult to carry out and control. Moreover, the basic restrictions are expressed in quantities that are internal to the body and are not measured, such as SAR. On the other hand, the reference levels are expressed (measured) in the free-space situation, such as for the electric field. Such data are not always available.

Nonetheless, attempts to draw up safety limits and their criteria have long been in operation. RF exposure limits are defined by the ICNIRP (1998) and are set to $0.08\,W\,kg^{-1}$ for the general public and $0.4\,W\,kg^{-1}$ for occupational exposure. The guidelines for whole-body exposure on humans is based on responses to animals at levels sufficient to produce behavioural changes, but not thermal damage to the tissue. It is possible that, because of the large interspecies differences among the small animals (rodents) and primates, the former may not be representative of human responses under identical exposure conditions over the whole body.

However, the latest basic restriction for localized exposure is $2\,W\,kg^{-1}$ for most parts of the body (Lin, 2006). For the extremities (arms and legs distal from the elbow and knees, respectively, including the fingers, toes, hands and feet) and for pinnae, the basic restriction expressed in terms of SAR is $4\,W\,kg^{-1}$. The value of SAR is obtained by averaging over some specified time periods (i.e. 6–30 minutes) and by averaging over any 10 g of tissue.

For frequencies between 3 and 100 GHz, the basic restrictions are the same as the derived limits of the MPE. The value of the MPE is obtained by averaging over some specified time periods that vary from 2.5 to 30 minutes for different frequencies.

The new Institute of Electrical and Electronics Engineers (IEEE) standard includes several major differences from the 1991 edition. First and the foremost, the IEEE standard instituted an exclusion for the pinnae or the external ears by relaxation of the abovementioned basic SAR restriction from 2 to $4\,W\,kg^{-1}$. Further, the SAR value has been raised from $1.6\,W\,kg^{-1}$ averaged over any 1 g of tissue, to $2\,W\,kg^{-1}$ over any 10 g of tissue. This effectively means relaxing the criteria, because deposition of energy over 10 g would be inherently lower, compared to the SAR over 1 g. This revised choice (10 g in the shape of a cube) would permit depositions of RF or microwave energy in different parts of the eye that exceed the basic SAR restriction by a large margin, while keeping the SAR for the entire eye below the prescribed limit ($2\,W\,kg^{-1}$).

In the 1991 edition, the MPE between 30 and 300 MHz, was $10\,W\,m^{-2}$. The new MPE in terms of power density is $2\,W\,m^{-2}$ between 30 and 400 MHz, the region most susceptible for human exposure. It ramps up from 2 to $10\,W\,m^{-2}$ between 400 and 2000 MHz. For frequencies greater than 2000 MHz, the MPE is $10\,W\,m^{-2}$. Also, the designated frequency bands and the MPE are different. In the 1991 edition, they were $10\,W\,m^{-2}$ between 30 and 300 MHz. The ramp up from 10 to $100\,W\,m^{-2}$ took place between 300 MHz and 3000 MHz. For frequencies greater than 3000 MHz, the MPE was $100\,W\,m^{-2}$ (**Figure 9**). In general, the new IEEE standards are more restrictive between 30 MHz and 100 GHz. Furthermore, new IEEE guidelines state that the maximum spatial power density should not exceed 20 times the square of the allowed spatially averaged peak values for frequencies below 400 MHz, and should not exceed $40\,W\,m^{-2}$.

The major differences also include the tissue mass and the time period over which the SAR values are to be averaged, and the applicable frequency bands for the MPE. Also, the most significant difference is the exclusion of pinnae from the head by IEEE, which made it possible to allow a higher local SAR value for the basic restriction at $4\,W\,kg^{-1}$. In the ICNIRP guidelines, pinnae are not excluded and are treated as an integral part of the human head.

Moreover, localized SAR values in the ICNRP guidelines are averaged over any 10 g mass of contiguous tissue. ICNIRP guidelines do not specify a cubic volume of tissue as the averaging mass. In addition, all SAR values are averaged over a six minute period in the ICNIRP guidelines, in contrast to the 2.5–30 minutes stipulated in the new IEEE standards.

The IEEE standards are not identical to the ICNIRP guidelines. This may also be true for frequencies used in mobile communications and wireless devices and systems. Global harmonization of RF exposure standards for the general public would be a future goal. Some of the points of contradiction, as mentioned above, will continue to be a point of investigation, and should also include more accurate assessment of SAR values, more reliable

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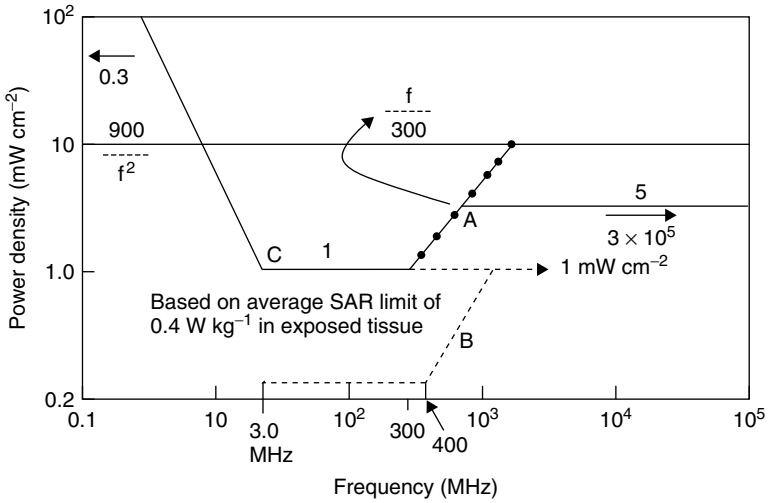

Figure 9 (A) New IEEE (2006) guidelines; (B) ANSI C 95.4 Subcommittee Human Exposure Guide (based on whole body exposure); (C) Old IEEE Guidelines.

(reproducible) biological results and an assessment of their impact on health.

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Susceptibility of Neonatal Rats to Xenobiotics

Mutsuko Hirata-Koizumi, Ryuichi Hasegawa, Akihiko Hirose and Makoto Ema

CONTENTS

1 INTRODUCTION

Children, particularly neonates, are in the process of rapid development and growth (Scheuplein *et al*., 2002). Such developing organisms experience many complex, integrated events involving the regulation of cell growth, differentiation and morphogenesis. Many environmental factors, including nutritional adequacy (e.g. protein, vitamin and folic-acid availability), maternal smoking and alcohol consumption, prescription drugs and chemical contamination with substances such as lead and organic mercury, can interfere with these events, through mutagenesis or through altered cell division, hormone activity, enzyme function or energy sources, which may exert significant irreversible adverse effects by disrupting normal development. On the other hand, the immaturity of immunological, metabolic and elimination functions, especially during the early postnatal period (Alcorn and McNamara, 2002; Holsapple *et al*., 2003), can lead higher susceptibility to chemical toxicity, compared with adults. When the effects of chemicals on human health are evaluated, the possible greater risk to such sensitive groups should be fully taken into account.

Current risk assessments of xenobiotics, especially industrial chemicals, are usually performed based on animal studies. In order to cover different developmental stages, multiple types of toxicity studies are conducted. In repeated-dose toxicity studies for the evaluation of general toxicity, dosing of chemicals to rodents begins as soon as possible after weaning and any case, before the animals are nine weeks old (OECD, 1998; 2008). Prenatal developmental toxicity studies involve the administration of chemicals to pregnant animals, and developmental toxicity, including teratogenicity, is evaluated (OECD, 2001a). In reproductive toxicity studies, chemicals are usually given to rats from the premating period thorough the mating, gestation and lactation periods (OECD, 1983), with, in multiple generation studies, direct administration to pups after weaning (OECD, 2001b). Combination of the results from these studies would appear to enable the evaluation of health effects of chemical exposure in any period during the lifetime. However, evaluation of the effects of chemical exposure during the early postnatal period may be inadequate because preweaning animals are only exposed to chemicals indirectly through maternal milk during nursing, or directly through small amounts of food or water containing chemicals in the late lactation period in cases of test substance administration via diet or drinking water, in reproductive toxicity studies (OECD, 1983; 2001b). Quantitative evaluation is impeded, because it is unclear how much test material is ingested by preweaning animals, in such studies. For chemicals that are not excreted in maternal milk, toxic effects from direct chemical exposure during the early postnatal period would not be detected. In the recently adopted Organization for Economic Cooperation and Development (OECD) test guidelines for developmental neurotoxicity, it is noted that direct dosing of pups

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should be considered in those cases where there is a lack of evidence of continued exposure to offspring through the maternal milk (OECD, 2007). In order to evaluate the toxic effects and dose–response during the preweaning period, it is necessary to administer chemicals directly to neonatal animals. Although various factors such as ethical limitations, limited human and economic resources, or handling difficulties, do not make it easy to conduct such studies and there has been a lack of sufficient information regarding the susceptibility of neonatal animals to the toxicity of chemicals; some information has been accumulated in recent years.

In this chapter, special attention is directed to the susceptibility to chemical toxicity during the early postnatal period from the standpoint of risk assessment for susceptible groups such as neonates and infants. We introduce comparative analysis of the toxic susceptibility to 20 chemicals between neonatal rats and young rats as examples, and discuss the possible causes of differences and the adequacy of uncertainty factors for the risk assessment of neonates or infants. In the comparative analysis, preweaning rats were directly administered with chemicals, and the toxic susceptibility evaluated. For detailed information on the methods and results on 18 chemicals, please refer to our previous publication (Hasegawa *et al*., 2007). We re-evaluate the neonatal susceptibility to 20 chemicals, by adding the recent new data on two chemicals (Hirata-Koizumi *et al*., 2007; 2008a; 2008b; 2008c) to it, in this chapter.

2 Comparative Analyses of the Toxic Susceptibility of Neonatal and Young Rats to 20 Chemicals

In this section, we summarize comparative analysis results of the toxic susceptibility in neonatal and young rats for 20 chemicals. Here, 'neonatal rat study' means an 18-day repeated-dose toxicity study targeting the preweaning period, in which male and female rats were administered with chemicals by oral gavage from postnatal days 4 to 21. The results of neonatal rat studies were compared with the findings in young rats, mainly the results of 28-day repeated oral-gavage studies using five- or six-week-old young rats ('young rat study'). For comparison of the toxic susceptibility between rats of differently ages, presumed no observed adverse effect levels (pNOAELs) were estimated for 20 chemicals. The pNOAEL is defined as the most likely no adverse-effect dose. In most cases, this value is equal to the no observed adverse-effect level (NOAEL), the highest dose at which no adverse effects are observed, but it is not always adequate for an accurate comparison of the toxic susceptibility, because the NOAEL is dependent on the dose spacing. Therefore, in some cases, a more realistic no adverse-effect dose was estimated as a single value or range.

Table 1 shows the pNOAELs of 20 chemicals in neonatal rats and young rats, and their ratios (pNOAEL for young rats/pNOAEL for neonatal rats). Of the 20 chemicals, pNOAEL ratios were estimated for 18 chemicals. The mean ratio was 2.35, and all values demonstrated differences of fivefold or less. The ratios showed that neonatal rats were clearly more susceptible (twice or greater) to 11 chemicals (61.1%), and less susceptible (half or less) to six chemicals (33.3%). Exceptionally, tetrabromobisphenol A (TBBPA) showed toxicity only in the kidneys of neonatal rats, and the nephrotoxicity is considered to be a highly specific toxicity in neonatal rats rather than a difference in the sensitivity to the same toxic end point between ages.

2.1 Chemicals with Higher Susceptibility in Neonates

2.1.1 Nitrophenols (4-Nitrophenol and 2,4-Dinitrophenol)

The major toxic effect of 4-nitrophenol and 2,4-dinitrophenol was clinical signs such as convulsions, a decrease in locomotor activity and prone/lateral positioning, followed by death, in both neonatal rats and young rats (Koizumi *et al*., 2001). In the neonatal rat study, 4-nitrophenol induced deaths at 230 mg kg^{-1} and above, and most of the rats exhibited convulsions before death. This chemical also induced one death in 36 animals in the 160 mg kg^{-1} group, and no adverse effects were observed at 110 mg kg^{-1} or less. In the young rat study, 4-nitrophenol caused the death of most males and females at 1000 mg kg^{-1}, and was not toxic at 400 mg kg^{-1} except for male-rat-specific renal toxicity, which is considered not to be relevant for other species. The pNOAELs for 4-nitrophenol were concluded to be 110 mg kg^{-1} day^{-1} in neonatal rats and 400 mg kg^{-1} day^{-1} in young rats.

As with 2,4-dinitrophenol, clear clinical signs of toxicity and death were found at 30 mg kg^{-1} in the neonatal rat study and at 80 mg kg^{-1} in the young rat study (Koizumi *et al*., 2001). Administration of 20 mg kg^{-1} 2,4-dinitrophenol also lowered the body weight of neonatal rats. This effect was not observed in the young rat study, so it appears that this may be because rapid weight gain occurs during the neonatal period (Koizumi *et al*., 2001; 2002; 2003; Hirata-Koizumi *et al*., 2005a; 2008b; Takahashi *et al*., 2006). Such higher susceptibility regarding neonatal body-weight gain was also found for 2,4,6-trinitrophenol (Takahashi *et al*., 2004), 1,1,2,2-tetrabromoethane (Hirata-Koizumi

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Table 1 Comparison of pNOAELs for 20 industrial chemicals in neonatal and young rats

Chemical name	Neonatal rats (mg kg^{-1} day^{-1})	Young rats (mg kg^{-1} day^{-1})	Ratio[a]
4-Nitrophenol	110	400	3.6
2,4-Dinitrophenol	10	20	2.0
3-Aminophenol	80	240	3.0
2-Chlorophenol	40[b]	200	5.0
4-Chlorophenol	100	100	1.0
3-Methylphenol	60–80[b]	300	4.0–5.0
3-Ethylphenol	100	300	3.0
4-Ethylphenol	30	100	3.3
2-*t*-Butylphenol	20	100	5.0
2,4-Di-*t*-butylphenol	5	20	4.0
p-(α,α-Dimethylbenzyl)phenol	30	100	3.3
1,3,5-Trihydroxybenzene	100	300	3.0
2,4,6-Trinitrophenol	40–50[b]	20	0.4–0.5
(Hydroxyphenyl)methylphenol	100	40	0.4
Trityl chloride	60	12	0.2
1,3-Dibromopropane	50	10	0.2
1,1,2,2-Tetrabromoethane	50	6	0.1
HDBB	0.5[c]	0.1[d]	0.2
DBHCB	—[e]	—[f]	—
TBBPA	40	—[g]	—

This table reproduces the data in **Table 2** of Hasegawa *et al*., 2007, by adding new data on HDBB and DBHCB.
HDBB, 2-(2′-hydroxy-3′,5′-di-*t*-butylphenyl)benzotriazole; DBHCB, 2-(3′,5′-di-*t*-butyl-2′-hydroxyphenyl)-5-chlorobenzotriazole); TBBPA, Tetrabromobisphenol A.
–Appropriate values were not able to be given
[a]Ratio of the pNOAEL for young rats to that for neonatal rats, showing comparative toxic susceptibility of neonatal rats to young rats.
[b]A realistic no adverse-effect dose was speculated because the NOAEL selected from established dose levels was considered to be inadequate for precise comparison.
[c]In the neonatal rat study, histopathological examination was conducted only for the liver and heart, and the haematology and developmental parameters, such as reflex ontogeny and external and sexual development were not examined.
[d]The value was estimated based on the results of a 52-week repeated-dose toxicity study using young rats because adverse effects were observed at the lowest dose in a 28-day study (young rat study). Since the estimated pNOAEL was markedly different between sexes, the lower value was given.
[e]The pNOAEL could not be estimated because adverse effects were observed even at the lowest dose of 250 mg kg^{-1} in the neonate.
[f]The pNOAEL could not be estimated because the young rat study was conducted only at a single dose.
[g]No accurate values for the pNOAEL could be generated because any adverse effects were not observed, even at the highest dose.

et al., 2005a) and 2-(3′,5′-di-*t*-butyl-2′-hydroxyphenyl)-5-chlorobenzotriazole (DBHCB) (Hirata-Koizumi *et al*., 2008c), as discussed below. Since the pNOAELs of 2,4-dinitrophenol are considered to be 10 mg kg^{-1} day^{-1} in neonatal rats and 20 mg kg^{-1} day^{-1} in young rats, the toxicity of nitrophenols in neonatal rats seems to be 2.0–3.6 times greater than in young rats.

2.1.2 3-Aminophenol

In the neonatal rat study, tremors, depression of body-weight gain and hypertrophy of thyroid follicular epithelial cells were observed at the highest dose of 240 mg kg^{-1}, and no definitive toxicity was found at 80 mg kg^{-1} (Koizumi *et al*., 2002). In the young rat study, in addition to the similar changes observed in neonates, anaemia and hyaline droplets in the proximal epithelium of kidneys were found at 720 mg kg^{-1}. Although slight pigmentation in the renal proximal tubular epithelium was observed at 240 mg kg^{-1}, this is not considered to be an adverse effect because of the lack of changes in haematological parameters at this dose. It is concluded that the pNOAEL was 80 mg kg^{-1} day^{-1} in neonatal rats and 240 mg kg^{-1} day^{-1} in young rats, and, therefore, the susceptibility of neonatal rats to 3-aminophenol was three times higher than that of young rats.

3-Aminophenol induced anaemic changes only in young rats. Similar anaemic effects only in young rats were found for 2,4,6-trinitrophenol and 1,3-dibromopropane (Hirata-Koizumi *et al*., 2005a;

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Takahashi *et al*., 2004), as described below. Although methaemoglobin levels were not determined in the young rat studies, it was reported that methaemoglobin reductase levels in young rats were distinctly lower than in neonates (Gruener, 1976; Lo and Agar, 1986), which may be the reason for the higher susceptibility to anaemic effects in young rats.

2.1.3 Chlorophenols (2-Chlorophenol and 4-Chlorophenol)

Chlorophenols mainly induced behavioural toxicity both in neonatal and young rats (Hasegawa *et al*., 2005). In the neonatal rat study of 2-chlorophenol, tremors, hypoactivity and abnormal gait were observed at 300 mg kg^{-1}. Basophilia of the renal tubules was also observed at that dose. Tremors were also found at 50 mg kg^{-1}, but only once during the dosing period and in one of 12 females. Therefore, the pNOAEL for neonatal rats was estimated to be 40 mg kg^{-1} day^{-1}, a little below the 50 mg kg^{-1}. In the young rat study of 2-chlorophenol, in addition to the behavioural changes observed in neonatal rats, slight centrilobular hypertrophy of hepatocytes were found at the highest dose of 1000 mg kg^{-1}. The pNOAEL was concluded to be 200 mg kg^{-1} day^{-1}, and therefore, neonatal rats are considered to be five times more susceptible to 2-chlorophenol toxicity than young rats.

In the case of 4-chlorophenol, clinical signs, including tremors, bradypnoea/tachypnoea and hypoactivity, were found at 300 mg kg^{-1} and above in neonatal rats and at 500 mg kg^{-1} mg/kg in young rats (Hasegawa *et al*., 2005). There were no other adverse effects in either study. The pNOAELs for 4-chlorophenol were concluded to be 100 mg kg^{-1} day^{-1} in both neonatal and young rats; the toxic susceptibility to this chemical was similar regardless of age.

2.1.4 3-Methylphenol

In the neonatal rat study, various clinical signs, such as deep respiration, hypersensitivity on handling, tremors under contact stimulus and depression of body-weight gain were observed at 300 mg kg^{-1} (Koizumi *et al*., 2003). At 100 mg kg^{-1}, the hypersensitivity and tremors were also noted in a small number of males only on single days during the dosing period. The pNOAEL is considered to be slightly lower than 100 mg kg^{-1}, at which the incidence of clinical signs was very low. In the young rat study, clear clinical signs and depression of body-weight gain, as observed in neonatal rats, appeared at 1000 mg kg^{-1}. Since the pNOAEL for young rats was considered to be 300 mg kg^{-1} day^{-1}, differences in the toxic susceptibility to 3-methylphenol could be concluded to be four to five times.

2.1.5 Ethylphenols (3-Ethylphenol and 4-Ethylphenol)

In the neonatal rat study of 3-ethylphenol, the only adverse effect was a slight lowering of body weight at the highest dose of 300 mg kg^{-1} (Takahashi *et al*., 2006). On the other hand, in the young rat study, 3-ethylphenol caused clinical signs of toxicity such as staggering gait and prone/lateral positioning, increase in liver weight, with alteration of the related blood biochemical parameters, and hyperplasia of the squamous cells in the forestomach at 1000 mg kg^{-1}. Although the toxicity profiles of both ages were not similar, it is considered to be due to inadequate high-dose setting in the neonatal rat study. The pNOAELs for 3-ethylphenol were concluded to be 100 mg kg^{-1} day^{-1} in neonatal rats and 300 mg kg^{-1} day^{-1} in young rats.

4-Ethylphenol caused more severe changes than 3-ethylphenol. In neonatal rats, deaths, clinical signs of toxicity, such as hypoactivity and deep respiration, and inhibition of body-weight gain were observed at 300 mg kg^{-1} (Takahashi *et al*., 2006). Since the delay in righting reflex was found even at 100 mg kg^{-1}, the pNOAEL for neonatal rats was concluded to be 30 mg kg^{-1} day^{-1}. In the young rat study, 4-ethylphenol caused clinical signs of toxicity, lowering of body weight and slight hepatic effects at 1000 mg kg^{-1}, and pathological changes in the forestomach at 300 mg kg^{-1} and above; therefore, the pNOAEL was concluded to be 100 mg kg^{-1} day^{-1}. These results show that neonatal rats have three times higher susceptibility to the toxicity of ethylphenols than young rats.

Following administration of 3- and 4-ethylphenols, squamous cell hyperplasia in the forestomach was observed in young rats, but not in neonatal rats. In the neonatal rat study of 4-ethylphenol, this change was not found even at high toxic doses. *p*-(α,α-Dimethylbenzyl)phenol and (hydroxyphenyl)methylphenol also affected the forestomach, only in young rats (Hasegawa *et al*., 2005), as discussed below. Generally, phenols have similar toxicological effects, due to their actions as extremely corrosive protoplasmic poisons (Manahan, 2003; Bloom and Brandt, 2001). In neonatal rats, the epithelium of the gastrointestinal tract would be more quickly renewed than in young rats because of more active body metabolism in the developing process. In addition, the low capacity of neonates to secrete gastric acid might explain the lower sensitivity to the effects on the forestomach.

2.1.6 t-Butylphenols (2-t-Butylphenol and 2,4-Di-t-butylphenol)

In the neonatal rat study of 2-*t*-butylphenol, clinical signs of toxicity, such as ataxic gait and decrease in

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locomotor activity, and slight hypertrophy of hepatocytes with increased liver weight, were observed at 60 mg kg^{-1} and above (Hirata-Koizumi *et al*., 2005b). The pNOAEL was concluded to be 20 mg kg^{-1} day^{-1}. In the young rat study, similar clinical signs of toxicity and increase in the liver weight were found at 500 mg kg^{-1}, and the pNOAEL was concluded to be 100 mg kg^{-1} day^{-1}.

As for 2,4-di-*t*-butylphenol, various histopathological findings were detected in the liver and kidneys, both in neonatal and young rats (Hirata-Koizumi *et al*., 2005b). Since slight hepatic changes were observed at 40 mg kg^{-1} in neonatal rats and at 75 mg kg^{-1} in young rats, the respective pNOAELs were concluded to be 5 and 20 mg kg^{-1} day^{-1}. Based on these findings, the susceptibility of neonatal rats to 2-*t*-butylphenol and 2,4-di-*t*-butylphenol was thought to be four to five times higher than that of young rats.

2.1.7 *p*-(α,α-Dimethylbenzyl)phenol[a]

In the neonatal rat study, dilation of renal tubules and papillary ducts were observed at 100 mg kg^{-1} and above (Hasegawa *et al*., 2005). In the 300 mg kg^{-1} group, such changes were accompanied with increased level of urinary volume or blood urea nitrogen, and no complete recoveries were found in the renal changes after a nine-week recovery-maintenance period. Toxic effects on reproductive organs were also noted in neonatal rats; testicular weight at 300 mg kg^{-1}, and ovarian weight at 100 mg kg^{-1} and above were lowered, and increased numbers of atretic follicles were found in the ovaries of 300 mg kg^{-1} group. After the recovery-maintenance period, in addition to similar follicular changes, decreased numbers of corpora lutea and hypertrophy of uterine endometrial epithelium were detected. In the young rat study, renal effects, as observed in neonatal rats were found at 300 mg kg^{-1} and above. In addition to deaths, lowering of body weight, squamous hyperplasia in the forestomach and bile-duct proliferation in the liver were found in the 1000 mg kg^{-1} group. No effects were detected on the reproductive organs in young rats. The pNOAELs were concluded to be 30 mg kg^{-1} day^{-1} for neonatal rats and 100 mg kg^{-1} day^{-1} for young rats; therefore, the susceptibility of neonatal rats to *p*-(α,α-dimethylbenzyl)phenol was considered to be 3.3 times higher than that of young rats.

2.1.8 1,3,5-Trihydroxybenzene[b]

In neonatal rats, hypertrophy of thyroid follicular cells was observed with increased thyroid weights at 500 mg kg^{-1} (Hasegawa *et al*., 2005). Although the liver weights were also increased at this dose, it was not accompanied by any histopathological alterations. The pNOAEL is concluded to be 100 mg kg^{-1} day^{-1} in neonatal rats. In the young rat study, similar effects on the thyroids and liver were found at 1000 mg kg^{-1}, and the pNOAEL is concluded to be 300 mg kg^{-1} day^{-1}. Based on these findings, the susceptibility of neonatal rats to 1,3,5-trihydroxybenzene appears to be three times higher than that of young rats.

2.2 Chemicals with Lower Susceptibility in Neonates

2.2.1 2,4,6-Trinitrophenol

In the neonatal rat study, the only observed adverse effect was a slight lowering of body weight at the highest dose of 65.1 mg kg^{-1} (Takahashi *et al*., 2004). In the preliminary dose-range-finding study conducted over the same dosing period, deaths and clinical signs of toxicity, as well as lowered body weight, were found at 81.4 mg kg^{-1}. The pNOAEL for neonatal rats was considered to be 40–50 mg kg^{-1} day^{-1}, slightly lower than 65.1 mg kg^{-1}. In young rats, haemolytic anaemia (i.e. decreased red blood cells and haemoglobin, extramedullary haematopoiesis and haemosiderin deposition in the spleen) and testicular toxicity (i.e. atrophy of seminiferous tubules in the testis and decrease in numbers of sperm in the epididymis) were observed without any deaths or changes in body weight at the highest dose of 100 mg kg^{-1}. In the prior 14-day dose-finding study, 2,4,6-trinitrophenol caused deaths at 500 mg kg^{-1}. The pNOAEL for young rats was concluded to be 20 mg kg^{-1} day^{-1}; therefore, it is considered that young rats have 2.0–2.5 times higher susceptibility to the toxicity of 2,4,6-trinitrophenol than neonatal rats. This is quite the opposite result from that found in the comparative studies for other phenols (see above).

2,4,6-Trinitrophenol demonstrated a qualitatively different toxicity in neonatal and young rats. Lowering of body weight only seen in neonatal rats would come from the rapid weight gain during the early postnatal period in the control group, and anaemic effects only in young rats might be due to lower methaemoglobin reductase levels, as mentioned above (see Sections 2.1.1 and 2.1.2). In the rat testes, the seminiferous cords contain only undifferentiated Sertoli cells and gonocytes at birth (Sjöberg *et al*., 1986). The Sertoli cells proliferate rapidly during the early postnatal period, and then slow down and cease multiplying by approximately postnatal day 20 (Orth, 1982; Orth, 1984; Toppari *et al*., 1996). Most gonocytes have differentiated into spermatogonia at about 10 days of age, and the first primary spermatocytes appear at days 12 or 13 after birth (Sjöberg *et al*., 1986). At 24 days of age, the first meiotic divisions are observed, but not until 44 days of age are the mature spermatids found in the testes. The testicular toxicity of 2,4,6-trinitrophenol in young

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rats might be due to the effect on the maturation of spermatids.

2.2.2 (Hydroxyphenyl)methylphenol[c]

In the neonatal rat study, the highest dose of 100 mg kg^{-1} did not cause any changes, but in the preliminary dose-range-finding study performed using the same method of administration, half the animals of the 200 mg kg^{-1} group died and the final body weight was lowered in this group (Hasegawa *et al*., 2005). Histopathological examination was not conducted in this preliminary study. In the young rat study, one female became moribund and the final body weights of males were decreased at 1000 mg kg^{-1}. All animals of this dose group showed squamous hyperplasia in the limiting ridge of the forestomach, and two-thirds of the animals featured centrilobular hypertrophy of hepatocytes. Hepatic pathological changes were also found with low incidences at 200 mg kg^{-1}. The pNOAELs were concluded to be 100 mg kg^{-1} day^{-1} in neonatal rats and 40 mg kg^{-1} day^{-1} in young rats, showing a 2.5-times higher susceptibility in young rats.

2.2.3 Trityl Chloride[d]

Similar hepatic effects were observed in neonatal and young rats (Hasegawa *et al*., 2005). In the neonatal rat study, centrilobular hypertrophy of hepatocytes was observed with increased liver weight in the 300 mg kg^{-1} group. In the preliminary dose-range-finding study, in addition to more evident increase in the liver weight, one female death and a lowering of body weight were found in the 600 mg kg^{-1} group. In the young rat study, centrilobular hypertrophy of the hepatocytes accompanied with increased liver weight was observed at 60 mg kg^{-1} and above. In the 300 mg kg^{-1} group, soft faeces and mucosal thickening of the cecum were found in most animals with more extensive hepatic changes. Based on these findings, the pNOAELs were concluded to be 60 mg kg^{-1} day^{-1} in neonatal rats and 12 mg kg^{-1} day^{-1} in young rats, showing that young rats have five times higher susceptibility to the toxicity of trityl chloride than neonatal rats.

2.2.4 Bromoalkanes (1,3-Dibromopropane and 1,1,2,2-Tetrabromoethane)

1,3-Dibromopropane caused lowering of body weight and centrilobular hypertrophy of hepatocytes at 150 mg kg^{-1} in neonatal rats (Hirata-Koizumi *et al*., 2005a). The pNOAEL was concluded to be 50 mg kg^{-1} day^{-1}. In young rats, in addition to findings similar to those observed in neonates, slight anaemic changes, including decreased haemoglobin and increased reticulocyte ratio were found at 250 mg kg^{-1}. Since centrilobular hypertrophy of the hepatocytes was also observed at mg kg^{-1}, the pNOAEL for young rats was considered to be 10 mg kg^{-1} day^{-1}.

In the neonatal rat study of 1,1,2,2-tetrabromoethane, increased liver weight with slight elevation of blood total protein at the highest dose of 50 mg kg^{-1} was not considered adverse because of the lack of histopathological changes (Hirata-Koizumi *et al*., 2005a). In the preliminary dose-range-finding study, lowering of body weight and a marked increase in the liver weight was noted at 200 mg kg^{-1}, but histopathological examination was not conducted in this preliminary study. In the young rat study, while body weight was not affected, even at the highest dose of 200 mg kg^{-1}, centrilobular hypertrophy of the hepatocytes was observed in the liver at 20 mg kg^{-1} and above. Hypertrophy of follicular cells was also found in the thyroids at 60 mg kg^{-1} and above. The pNOAELs were considered to be 50 and 6 mg kg^{-1} day^{-1} in neonatal and young rats, respectively. Based on these results, it can be concluded that young rats have 5–10 times higher susceptibility to the toxicity of 1,3-dibromopropane and 1,1,2,2-tetrabromoethane than neonatal rats.

2.2.5 Benzotriazole Ultraviolet Absorbers (2-(2′-Hydroxy-3′,5′-di-t-butylphenyl) benzotriazole[e] and 2-(3′,5′-Di-t-butyl-2′-hydroxyphenyl)-5-chlorobenzotriazole)[f]

In the young rat study of 2-(2′-hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole (HDBB), toxic effects were observed mainly in the liver, such as hypertrophy and vacuolar degeneration of the hepatocytes, focal necrosis and bile duct proliferation (Hirata-Koizumi *et al*., 2007). HDBB also affected the heart, kidneys, thyroids and blood. These adverse effects were found even at the lowest dose of 0.5 mg kg^{-1} in males, but in females, they were detected only at 12.5 mg kg^{-1} and above. In the 52-week repeated-dose toxicity study using young rats, the pNOAEL of HDBB was concluded to be 0.1 mg kg^{-1} day^{-1} in males and 2.5 mg kg^{-1} day^{-1} in females, based on histopathological changes in the liver (Hirata-Koizumi *et al*., 2008a). There was a marked gender-related difference in the HDBB toxicity in young rats. In the neonatal rat study, hepatocellular findings were found at 2.5 mg kg^{-1} and above in both sexes (Hirata-Koizumi *et al*., 2008b). Interestingly, the gender-related difference was not detected in the toxic susceptibility of neonates to HDBB. The pNOAEL of HDBB for neonatal rats is concluded to be 0.5 mg kg^{-1} day^{-1} in both sexes. These findings suggest that the toxic susceptibility of males to HDBB is five times higher in young rats than neonatal rats, but conversely, five times higher susceptibility of neonates was found for females.

Similar phenomena were found for the structural analogue, 2-(3′,5′-di-*t*-butyl-2′-hydroxyphenyl)-5-chlorobenzotriazole (DBHCB) (Hirata-Koizumi *et al*., 2008c).

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In young rats to whom 250 mg kg^{-1} DBHCB was administered, the relative liver weight increased to more than twice that of the control in males, while the change in relative liver weight was less than 10% in females. On histopathology, hypertrophy of the hepatocytes was observed in males, but not in females. In neonatal rats, inhibition of body-weight gain and histopathological changes in the liver were observed, as well as increased liver weight and blood biochemical changes at 250 mg kg^{-1} and above in both sexes. These findings clearly show the lack of gender-related differences in the DBHCB toxicity in neonatal rats. Unfortunately, it is not possible to compare the susceptibility of neonatal and young rats to the DBHCB toxicity because the dose setting in both studies were inadequate to estimate the pNOAELs.

2.3 Neonatal-Specific Toxicological Profiles

2.3.1 Tetrabromobisphenol A[g]

In the neonatal rat study, tetrabromobisphenol A (TBBPA) induced nephrotoxicity characterized by the formation of polycystic lesions at 200 mg kg^{-1} and above, while some deaths occurred at 600 mg kg^{-1} (Fukuda *et al*., 2004). After the nine-week recovery-maintenance period, such nephrotoxic lesions were still present. The pNOAEL was concluded to be 40 mg kg^{-1} day^{-1}. On the other hand, in the young rat study, the kidneys showed no histopathological changes even at the highest dose of 1000 mg kg^{-1}, and no chemically related changes were found in other examined parameters, either. In order to investigate the specificity of the nephrotoxicity induced by TBBPA in neonatal rats, an additional 18-day repeated-dose toxicity study using young rats was conducted at the higher doses. The results revealed no histopathological changes in the kidneys up to 6000 mg kg^{-1} in young rats. These findings clearly indicate that the nephrotoxicity of TBBPA is specific for neonatal rats, although the toxic dose level was relatively high.

Such specific renal toxicity in neonatal rats has also been reported for other chemicals, such as chlorambucil (Kavlock *et al*., 1987), 2,3,7,8-tetrachloro-1,4-dibenzodioxine (Couture-Haws *et al*., 1991) and difluoromethylornithine (Gray and Kavlock, 1991). In the kidneys of rats, nephrogenesis occurs at a rapid pace between birth and 8 days and is complete by 11 days of age, and tubular differentiation continues until the time of weaning (Zoetis and Hurtt, 2003). Developing renal tubules during the early postnatal period might show higher sensitivity to the induction of renal polycystic lesions associated with hyperplasia of the tubular epithelium in response to cellular damage.

2.3.2 p-(α,α-Dimethylbenzyl)phenol[a]

Although the major target of *p*-(α,α-dimethylbenzyl)phenol was the kidney in both neonatal and young rats, this chemical also affected the reproductive organs, but only in neonates (Hasegawa *et al*., 2005) (see Section 2.1.7). In females, ovarian follicular changes did not show recovery, and additional changes were found in the ovaries and uterus after the recovery-maintenance period. Follicle stimulating hormone (FSH) is a major survival factor for early antral follicles, the stage during which a majority of follicles undergo atresia under physiological conditions (Chun *et al*., 1996; McGee and Hsueh, 2000). Deprivation of gonadotropins by hypophysectomy leads to atresia and apoptosis of developing follicles, whereas FSH treatment of cultured early antral follicles prevents the spontaneous onset of follicular apoptosis (Nahum *et al*., 1996; Chun *et al*., 1996). In the neonatal rat study, *p*-(α,α-dimethylbenzyl)phenol decreased the number of the FSH-positive pituitary cells. In rats, ovarian follicles become subjected to strong gonadotropin control during the second week of postnatal life (Beckman and Feuston, 2003); therefore, decrease in FSH stimulation during this critical period might be associated with the specific ovarian toxicity of *p*-(α,α-dimethylbenzyl)phenol in neonatal rats.

3 POSSIBLE CAUSES OF THE DIFFERENCES IN TOXIC SUSCEPTIBILITY OF NEONATAL AND YOUNG RATS

Higher susceptibility of neonatal rats was found for 11 phenolic compounds, most of which induced clinical signs, suggestive of the effects on the central nervous systems, as the major toxicity. From available published data, several factors could be hypothesized as potential contributors to the increased susceptibility in neonates. For example, it has been reported that 2,4-dinitrophenol is eliminated much more rapidly from the serum in mature rabbits than in neonates (Gehring and Buerge, 1969), and this might be due to immature hepatic metabolic or renal excretory functions during the neonatal period (Gow *et al*., 2001; Rich and Boobis, 1997; Zoetis and Hurtt, 2003). For 4-nitrophenol, more concrete data are obtained. This chemical is mainly excreted as the glucuronide and sulfate in urine in young/adult rats (Machida *et al*., 1982). Since uridine diphosphate (UDP)-glucuronyltransferase and sulfotransferase activities for 4-nitrophenol are lower in microsomes or cytosols prepared from the livers of neonatal rats (Matsui and Watanabe, 1982), a higher level of 4-nitrophenol would be expected in the blood of neonatal rats than young rats treated with this chemical. A similar

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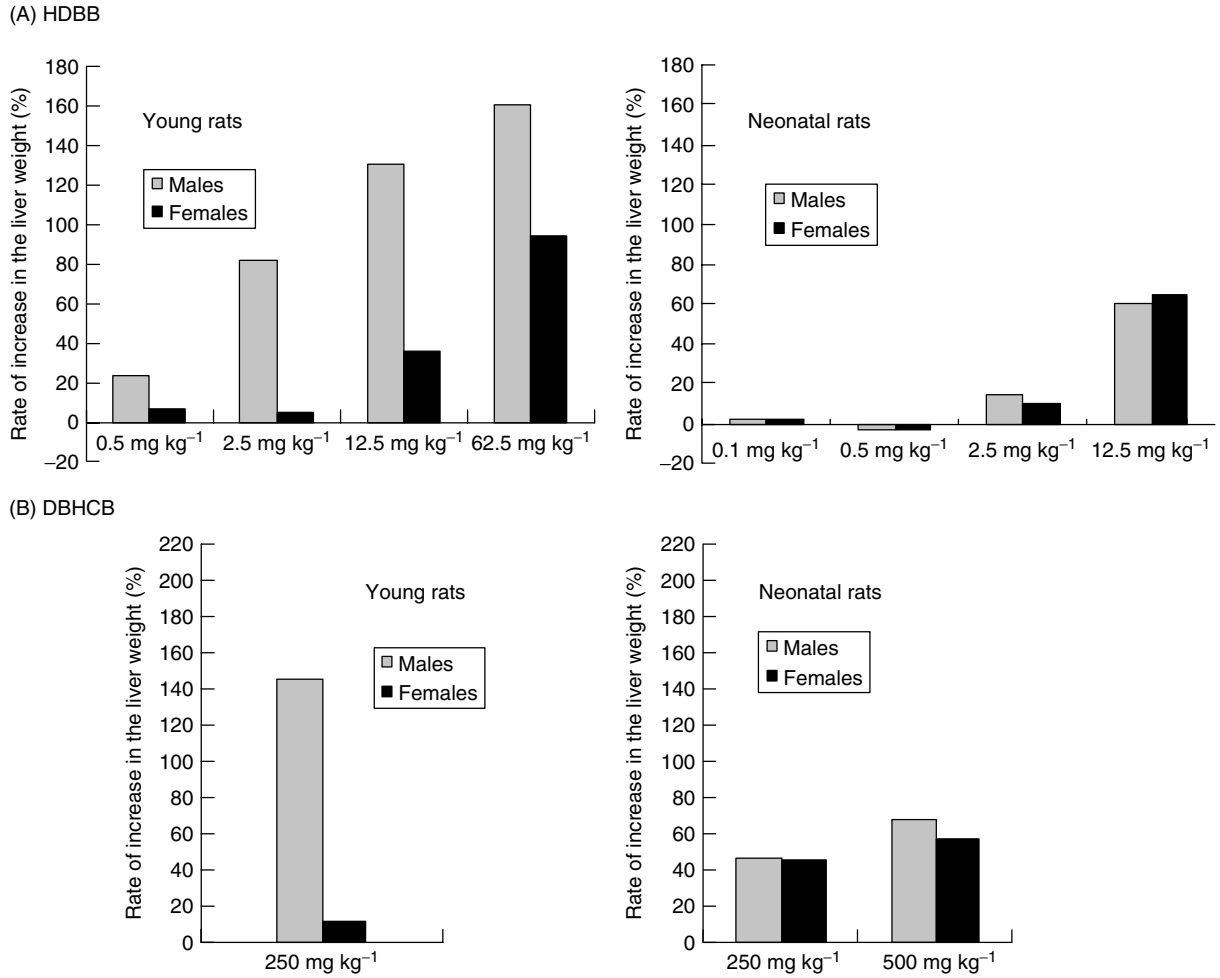

Figure 1 Rate of increase in the liver weight in young and neonatal rats administered with HDBB or DBHCB. (A) HDBB was given by gavage to young rats for 28 days (Hirata-Koizumi *et al.*, 2007), and to neonatal rats from postnatal days 4 to 21 (Hirata-Koizumi *et al.*, 2008b). (B) DBHCB was given by gavage to young rats for 28 days, and to neonatal rats from postnatal days 4 to 21 (Hirata-Koizumi *et al.*, 2008c).

phenomenon could occur for 3-methylphenol, because this chemical is known to be predominantly conjugated with glucuronide or sulfate and excreted in the urine (Bray *et al.*, 1950). In this manner, the higher toxic susceptibility of neonatal rats might be associated with the higher level of administered chemicals in the blood. In rats, it is also known that the blood–brain barrier does not completely form during the early postnatal period (Vidair, 2004), which might result in further elevation of phenolic compounds in one of the possible target organs, the brain.

While the above-mentioned 11 phenols are considered to have direct actions on their target organs, six chemicals showing lower susceptibility of neonatal rats may need metabolic activation to exert toxic effects. For example, following administration of 1,3-dibromopropane to adult rats, mercapturic acid conjugates were detected as the major urinary metabolites (James *et al.*, 1981; Jones and Wells, 1981; Onkenhout *et al.*, 1986). The chemical structures of the metabolites suggest that oxidative biotransformation by cytochrome P450 would occur before or after glutathione conjugation, as is common for dihaloalkanes (Zoetemelk *et al.*, 1986; Tornero Velez *et al.*, 2004; IARC (International Agency for Research on Cancer), 1999a; 1999b; Guengerich *et al.*, 1991; ATSDR (Agency for Toxic Substances and Disease Registry), 1992). In the metabolic process, it is considered that some active metabolites, including episulfonium ions and bromoaldehydes, are generated, and they might contribute to the emergence of 1,3-dibromopropane toxicity. Similarly, it has been suggested that 1,1,2,2-tetrabromoethane requires an oxidative metabolism by cytochrome P450 to produce active intermediates (Kennedy *et al.*, 1993; Ahr *et al.*, 1980; Hales *et al.*, 1987; Wolf *et al.*, 1977). In neonatal rats, expression levels and activities of hepatic cytochrome P450 and glutathione S-transferase is low, especially in the early days after

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birth (Borlakoglu *et al*., 1993; Cresteil *et al*., 1986; McCusker *et al*., 1989); therefore, production of active metabolites would be significantly lower in neonatal rats.

Benzotriazole ultraviolet absorbers showed interesting results in that the marked gender-related differences in the toxicity in young rats was not observed in neonatal rats. A comparison of the degree of change in the liver weight clearly shows this phenomenon (**Figure 1**). Sexual variations in the toxic susceptibility of young rats have been documented for many other chemicals (Muraoka and Itoh, 1980; Knuckles *et al*., 2004; Agarwal *et al*., 1982; Carlson and DuBois, 1970; Coleman *et al*., 1990; Stern *et al*., 2007; Wang *et al*., 2001), and the various causes are indicated mainly for toxicokinetic determinants. In particular, many mechanistic studies have been reported on the metabolic enzymes cytochrome P450s (Waxman and Chang, 2005). In rats, a subset of hepatic cytochrome P450s is expressed in a sex-dependent fashion, and interestingly, the difference between sexes is not apparent until puberty. If such metabolic enzymes have an intimate involvement in the toxic activation or detoxication of benzotriazole ultraviolet absorbers, a lack of sex differences in the toxicity in neonatal rats could be explained. On the other hand, recently, it has been shown that certain key transporter-protein mRNAs are expressed differentially between sexes in sexually mature rats and that certain of these differences develop during the period of sexual maturation (Buist *et al*., 2002; 2003; Buist and Klaassen, 2004; Kudo *et al*., 2002). Another possible explanation for benzotriazole ultraviolet absorbers exerting equivalent effects on the liver of male and female neonatal rats might be the contribution of these transporters to the uptake or excretion.

4 DOSE–RESPONSE CURVES IN NEONATAL RATS AS COMPARED WITH YOUNG RATS

Comparison of the pNOAELs revealed that neonatal rats were clearly more susceptible to 11 chemicals and less susceptible to six chemicals, as mentioned above (see **Table 1**). In order to compare the clear toxic doses in neonatal rats with those in young rats, we also estimated the presumed unequivocally toxic level (pUETL), which is defined as the clear toxic dose giving similar severity for both neonatal and young rats, for 20 chemicals, and compared the values between ages. **Table 2** shows the pUETLs of neonatal and young rats, and their ratios (pUETL for young rats/pUETL for neonatal rats). The comparison of pUETLs showed different results from that of the pNOAELs. Especially for 2,4,6-trinitrophenol, (hydroxyphenyl)methylphenol, 1,3-dibromopropane and 1,1,2,2-tetrabromoethane, while the pNOAELs were higher in neonatal rats than in young rats, the pUETLs were paradoxically lower in neonates. These findings suggest a difference in the slope of the dose–response curve between neonatal and young rats.

In **Table 3**, ratios of the pUETL to the pNOAEL in neonatal rats and young rats are shown for each chemical. The values for 17 chemicals were obtained, and among them, 2,4,6-trinitrophenol, (hydroxyphenyl)-methylphenol, trityl chloride, 1,3-dibromopropane and 1,1,2,2-tetrabromoethane demonstrated much larger ratios in young rats than in neonatal rats. The dose–response curve of these chemicals must be much steeper in neonatal rats than in young rats, as shown in **Figure 2A**. A major reason for variation in shapes of the dose–response curve is unclear. One possible explanation might be a low capacity for protection against deleterious oxidative stress in the neonatal rats, when the toxic chemical burden crosses a threshold in the liver. Actually, it has been reported that hepatic activities of superoxide dismutase, catalase and glutathione peroxidase are extremely low in neonatal rats, compared with the adults (Yoshioka *et al*., 1982). Other 12 chemicals showed almost the same pUETL/pNOAEL ratios in both ages. The dose–response curve would show a parallel shift from right (young rats) to left (neonatal rats) or in the opposite direction, as shown in **Figure 2B**.

5 INCORPORATION OF NEONATAL SUSCEPTIBILITY INTO RISK ASSESSMENT

The aim of dose–response assessment in risk assessment is to derive reference values, such as acceptable daily intakes (ADIs) or tolerable daily intakes (TDIs). The points of departure, the NOAELs or benchmark dose lower bounds (BMDLs) are usually based on the results of toxicity studies using experimental animals. In such toxicity studies, uniform experimental animals are typically used as the study population. Risk assessment needs to target sensitive subpopulations, such as neonates and infants.

In current risk assessments, two uncertainty factors are generally applied to the NOAELs or BMDLs, one for extrapolation from animal to human and the other for potential variation in sensitivity among members of the human population, each with default values of 10-fold. There are some data which allow consideration of whether the 10-fold factor for intra-human variability is adequate for the risk assessment of neonates or infants (Dourson *et al*., 2002; Hasegawa *et al*., 2007), as follows. In human data analyses, most studies targeted comparison of toxicokinetic/pharmacokinetic parameters (Ginsberg *et al*., 2002; Naumann, 2001; Renwick, 1998;

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Table 2 Comparison of pUETLs a for 20 industrial chemicals in newborn and young rats

Chemical name	Neonatal rats (mg kg^{-1} day^{-1})	Young rats (mg kg^{-1} day^{-1})	Ratio[a]
4-Nitrophenol	230	600–800	2.6–3.5
2,4-Dinitrophenol	30	80	2.7
3-Aminophenol	240	720	3.0
2-Chlorophenol	200–250	1000	4.0–5.0
4-Chlorophenol	300	500	1.7
3-Methylphenol	300	1000	3.3
3-Ethylphenol	—[b]	—[b]	—
4-Ethylphenol	200–250	1000	4.0–5.0
2-*t*-Butylphenol	100–150	500	3.3–5.0
2,4-Di-*t*-butylphenol	100	500	5.0
p-(α,α-Dimethylbenzyl)phenol	300	700–800	2.3–2.7
1,3,5-Trihydroxybenzene	500	1000	2.0
2,4,6-Trinitrophenol	65	100	1.5
(Hydroxyphenyl)methylphenol	140–160	1000	6.3–7.1
Trityl chloride	400–500	300	0.6–0.8
1,3-Dibromopropane	150	250	1.7
1,1,2,2-Tetrabromoethane	200	300–400[c]	1.5–2.0
HDBB	12.5[d]	2.5[e]	0.2
DBHCB	—[f]	—[f]	—
TBBPA	—[b]	—[b]	—

This table reproduces the data in **Table 2** of Hasegawa *et al*., 2007, by adding new data on HDBB and DBHCB.
–Appropriate values were not able to be given.
[a]Ratio of pUETL for young rats to that for neonatal rats.
[b]The pUETL values were not able to be given because clear toxic effects were not observed even at the highest dose in neonatal or young rats.
[c]These range values were estimated on the basis of all relevant toxicity data, including single-dose toxicity data in young rats (the lowest mortality dose was 722 mg kg^{-1} for males and 852 mg kg^{-1} for females) (MHLW (Ministry of Health, Labour and Welfare of Japan), 2003).
[d]In the neonatal rat study, histopathological examination was conducted only for the liver and heart, and haematology and developmental parameters, such as reflex ontogeny, and external and sexual development were not examined.
[e]Since the pUETLs were markedly different between sexes, the lower value was given.
[f]The pUETL could not be estimated because the young rat study was conducted only at a single dose.

Renwick *et al*., 2000; Skowronski and Abdel-Rahman, 2001), and only Glaubiger *et al*. (1981) analysed the comparative susceptibility to the overall toxicity between children and adults, showing that the variance is quite small, on the basis of comparing maximum tolerated doses for 17 anticancer drugs. For animal data analyses, Charnley and Putzrath (2001) demonstrated that younger animals are less susceptible to 47% and more susceptible to 40% of the tested chemicals in carcinogenesis studies. Calabrese (2001) also reported that younger animals have greater sensitivity than older animals in 54% (more than 10-fold in 14%) by LD_{50} (median lethal dose) ratio analyses for 313 chemicals. All these reports suggested that the sensitivity of children/younger animals against adults to most chemicals may be within 10-fold, meaning that the 10-fold uncertainty factor as a human variability might be enough to cover the sensitivity of children/younger animals, but these analytical results did not target the early postnatal periods. Sheehan and Gaylor (1990) and Goldenthal (1971) targeted this period, and compared the LD_{50} values between neonatal and adult animals for a large number of chemicals. However, these analyses are also inappropriate for evaluating an adequacy of the uncertainty factors because the factors are usually applied to the NOAELs or BMDLs estimated from repeated-dose results.

The above mentioned comparative analysis on 20 chemicals is the first trial to evaluate the susceptibility of neonatal rats to chemicals based on the results of repeated-dose toxicity studies, and the analytical results show the importance of studying the early life-stage sensitivity to toxic insult. When toxicity studies using neonatal animals determine the critical effects of chemicals, then ADIs or TDIs should be estimated based on their findings. When such neonatal studies do not determine any critical effects, then the ADIs or TDIs are appropriately estimated based on the critical effects found in other studies, and then the neonate is protected. It is when toxicity studies for the neonatal period have not been conducted that the uncertainty factors must be invoked to protect the neonate. The analytical results on 20 chemicals suggest that an uncertainty factor of 10-fold for human

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Table 3 Comparison of pUETL/pNOAEL ratios for 20 industrial chemicals in neonatal and young rats

Chemical name	Neonatal rats	Young rats
4-Nitrophenol	2.1	1.5–2.0
2,4-Dinitrophenol	3.0	4.0
3-Aminophenol	3.0	3.0
2-Chlorophenol	5.0–6.3	5.0
4-Chlorophenol	3.0	5.0
3-Methylphenol	3.8–5.0	3.3
3-Ethylphenol	—	—
4-Ethylphenol	6.7–8.3	10.0
2-*t*-Butylphenol	5.0–7.5	5.0
2,4-Di-*t*-butylphenol	20.0	25.0
p-(α,α-Dimethylbenzyl)phenol	10.0	7.0–8.0
1,3,5-Trihydroxybenzene	5.0	3.3
2,4,6-Trinitrophenol	1.3–1.6	5.0
(Hydroxyphenyl)methylphenol	1.4–1.6	25.0
Trityl chloride	6.7–8.3	25.0
1,3-Dibromopropane	3.0	25.0
1,1,2,2-Tetrabromoethane	4.0	50.0–66.7
HDBB	25.0	25.0
DBHCB	—	—
TBBPA	—	—

–Appropriate values were not able to be given because pNOAELs or pUETLs were not estimated in neonatal and/or young rats.

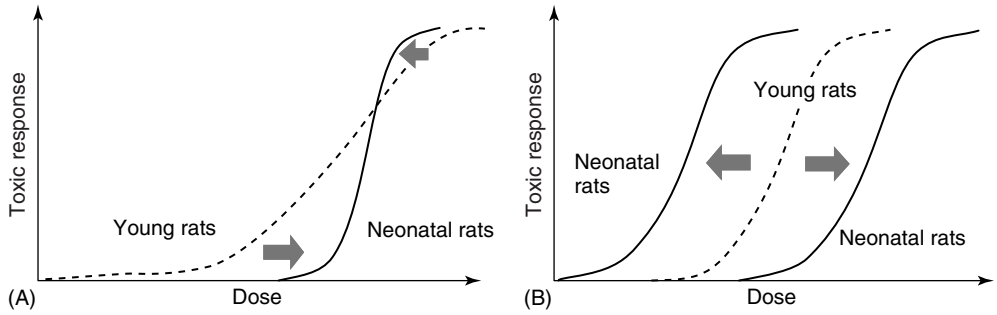

Figure 2 Illustration of two patterns of shift of dose–response curves from young to neonatal rats. (A) Change in the slope: a steeper shaped curve in neonate rats than young rats. (B) Parallel shift from right (young rats) to left (neonatal rats) or in the opposite direction.

variability can be considered appropriate for risk assessment, unless knowledge of particular toxicity in neonates or infants is present, or if not present is discountable due to other credible information on the chemical. Finally, it should be noted that these repeated-dose studies targeting neonatal animals are available only for a limited group of chemicals, and therefore conducting further studies and the gathering information for a greater variety of chemicals are strongly desired.

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NOTES

In this chapter, we use IUPAC names for most chemicals, but more general names are used for the others. These compounds are indicated by superscript lowercase letters, and the IUPAC names are given below.

[a] 4-(2-Phenylpropan-2-yl)phenol
[b] Benzene-1,3,5-triol
[c] Methylenediphenol
[d] [Chloro-di(phenyl)methyl]benzene
[e] 2-Benzotriazol-2-yl-4,6-di-*tert*-butylphenol
[f] 2-(5-Chlorobenzotriazol-2-yl)-4,6-di-*tert*-butylphenol
[g] 2,2′,6,6′-Tetrabromo-4,4′-isopropylidenediphenol

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Empirical Methods and Default Approaches in Consideration of Exposure Duration in Dose–Response Relationships

George M. Woodall, Jeffrey S. Gift and Gary L. Foureman

CONTENTS

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1 INTRODUCTION

Consideration of the components of toxicity in risk assessment settings has perhaps been limited by one of toxicology's basic guiding principles—that the dose makes the poison. Toxicity is necessarily a function of exposure which has components not only of total dose, but also of time or duration (e.g. dose rate). The consideration of duration as an element of toxicity in risk-assessment settings has a long, but nonetheless scanty history, especially in comparison to dose–response relationships. It is likely that at least part of this imbalance is due to the risk-assessment scenarios often being for a lifetime at a presumably average exposure concentration, where the evaluation is often of different doses all applied in a continuous manner such that duration is not a variable. The importance and occurrence of shorter-term exposure scenarios, where time and dose are essentially equal covariables, are of increasing interest, not only for occupational settings **Occupational Toxicology**, but for scenarios affecting the general population (e.g. evacuation and re-entry in emergency-response scenarios) **Acute Toxicity**; **Chemical Incidents-Emergency Planning, Preparedness, Response and Recovery. Community Considerations**.

Probably the most commonly cited and utilized relationship between exposure concentration and exposure duration (time) leading to a response is the mathematical relationship $C \times t = k$, which has come to be known as Haber's Law (Haber, 1924). Haber established that a simple relationship of concentration multiplied by the duration of exposure (usually in minutes) equalled a constant (k) when applied for short durations to warfare gases used in World War I. Later studies established that the relationship also worked with a number of solvent chemicals (Flury and Wirth, 1934). A more utilitarian form of this relationship was developed by ten Berge *et al*. (1986) where the equation is refined and expressed as $C^n \times t^b$ allowing for empirical derivation of the relationship between C and t (**Figure 1**) to explain deviations from Haber's rule seen with some common chemicals (e.g. ammonia). Haber's rule can be seen as a special case of the ten Berge model, where the exponents of concentration (n) and time (b) are both unity. **Figure 1** also shows the outcome (note a log–log scale) of setting the value of $n = 1$ and to a relatively large value of 3. Time/duration relationships different from that of Haber's rule (i.e. where $n = 1$) would result from this range of 'n's. Extrapolations to a given duration under these different relationships could produce remarkably disparate values.

It is well established that many levels of complexity exist in relating dose to toxicity **Environmental and Endogenous Factors Determining the Expression of Toxicity**; **Dose-Response Analysis in Experimental Toxicology and Risk Assessment**; however, it is perhaps

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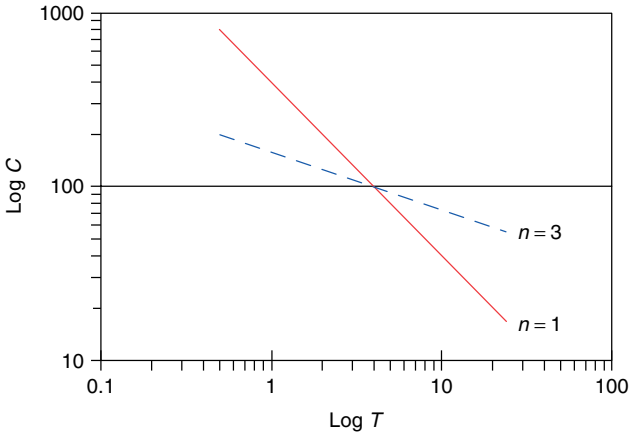

Figure 1 The concentration (C) times duration (t) relationship $C^n \times t = k$ on a log–log plot. The exponent n is actually a composite variable from the probit equation relating concentration and time, $Y = b_0 + b_1 \ln C + b_2 \ln t$, with $n = b_1/b_2$. (Reproduced from ten Berge *et al.*, 1986.)
Plots for different values of n are illustrated.

not as well appreciated that many of the same complexities are in play in relating exposure duration to toxicity. The complexities for dose and toxicity are founded in the areas of kinetics, dynamics, repair, adaptation and so on. Only recently has it become understood that these same elements affect the relationship between exposure duration and toxicity (Rozman and Doull, 2000; Rozman, 2000). Examples of these complicating factors include comparisons between one-time-only exposures to recurrent exposures at different intervals (daily, weekly, etc.), in relating the length of time from exposure to an adverse response and in extrapolations along the temporal continuum (short to long durations, or vice versa). There are, additionally, numerous examples in the literature where responses are not accurately defined by the $C \times t = k$ prediction, likely to be due to various complexities, obvious or latent (ten Berge *et al.*, 1986; Sommerville *et al.*, 2006; Dalbey *et al.*, 1998; Rajini *et al.*, 1993; Boyes *et al.*, 2003).

The association of time/duration of exposure to kinetics, dynamics, repair and so on has been demonstrated convincingly for several chemical compounds. For example, Stahl *et al.* (1992) noted that 1,2,3,4,6,7,8-heptachlorodibenzodioxin (HpCDD), toxicity (death up to 70 days after a single acute exposure) observed a strict $C \times t = k$ relationship, whereas the closely related 2,3,7,8-tetrachlorodibenzodioxin (TCDD), did not. Rozman (1999) demonstrated that these differences are due in large measure to the observation that the biological $t_{1/2}$ of a single dose to HpCDD (at 200 days) essentially established and maintained a near steady-state condition for an adequate duration (70 days) within which toxicity occurred; whereas with TCDD ($t_{1/2}$ of 20 days), a single exposure did not. As such, the difference in toxicokinetics between these two related chemicals resulted in a confounding of an underlying $C \times t = k$ relationship. This confounding was affirmed by experimentally establishing a $C \times t = k$ relationship for TCDD with augmented dosing (once every four days) that maintained a 'quasi' steady state (i.e. an effective dose over an adequate duration). These investigations have presented solid evidence affirming that $C \times t = k$ may be operable when all variables, complexities and confounders are known and can be controlled (e.g. where the timescale in steady state is long compared to the timescale of toxicity) and therefore, useful as a predictive model under these circumstances. The fact remains, however, that exposures dealing with or related to a $C \times t$ relationship will likely not occur under such controlled conditions and that knowledge of contributing complexities, such as kinetics, will be lacking. Under these usually occurring conditions of limited understanding of variables, more pragmatic approaches for estimating the $C \times t$ relationship are required.

In cases where more detailed data have been available, physiologically based toxicokinetic (PBTK) models have been applied in elucidating $C \times t$ relationships. The nature of these models allows for extensive and expandable incorporation of numerous parameters allowing for consideration of biological and kinetic sources of complexity, all in an integrated manner. The few duration studies available with these highly parameterized models demonstrate that they can closely approximate empirical information, including instances that do not follow the Haber relationship (Simmons *et al.*, 2005; Bruckner *et al.*, 2004; Lu *et al.*, 2008). These models thus affirm the complexity of $C \times t$ relationships and imply that consideration of variables, such as those related to physiology and biochemistry, allows for at least partial resolution of this complexity. Development of such models, however, is labour intensive, requires knowledge about the toxicologically active form of the chemical (parent or metabolite), the identity of the known or assumed target tissue and, of course, rare duration–response data against which the model may be evaluated. Thus this approach, although having considerable scientific merit, is likely not to be a practical alternative in exploring the $C \times t$ relationship for a majority of chemicals. Again, simpler methods and approaches are needed.

Acute lethality is the toxicity end point for which $C \times t = k$ data is most often available for modelling (and on which much of the historical basis for $C \times t$ relationships are established). In most risk-assessment applications, however, the concern will be for toxicity at a much lower severity than mortality. Application of the $C \times t = k$ relationship for lethality to other nonlethal end points engenders considerable uncertainty, unless the mechanism causing the toxicity is the same or similar enough for both end points. With certain end points, such as sensory-based irritation, scientific justification for application of lethality-based results may be lacking altogether (Shusterman *et al.*, 2006). End-point-specific

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$C \times t$ data and information would thus be desirable, as would a better understanding of the $C \times t$ relationship for longer durations and/or repeated exposures.

Methods and approaches to evaluate the $C \times t$ relationships from empirically observed nonlethal responses are especially relevant for acute exposure-duration scenarios. This work presents case studies, along with, for each, various methods and approaches for evaluating the $C \times t$ relationship, with varying amounts of observed data, with lethal and nonlethal end points, as well as with relevant ancillary information affecting this relationship. The methods include application of the US Environmental Protection Agency's (EPA) version of severity-based categorical regression, CatReg (US EPA, 2006; *http://epa.gov/ncea/catreg*), the EPA's version of the ten Berge software (US EPA, 2008; *http://www.epa.gov/ncea/bmds/dwnldu.html#berge*) and benchmark dose (BMD) software (US EPA, 2009; *http://ww.epa.gov/ncea/bmds/*), and methods (US EPA 2000; *www.epa.gov/nceawww1/pdfs/bmds/BMD-External_10_13_2000.pdf*) as well as a proposed default approach for evaluation of the $C \times t$ relationship in minimal data situations. The case studies also highlight the practical utility, strengths and limitations of these methods and approaches. They are provided as a resource for the reader to evaluate $C \times t = k$ with varying levels of data and ancillary information. Future directions for research on $C \times t$ relationships and duration extrapolation are also discussed.

2 CASE STUDIES AND APPROACHES TO C x t EVALUATION

2.1 Phosgene Case Study

2.1.1 General

An acid chloride, phosgene is a highly reactive poisonous gas with known and investigated progression of effects leading to lethality via pulmonary collapse. Much has been written on local pulmonary interactions of phosgene, particularly with the surfactant system, that mixture of lipids and proteins situated at the air–liquid interface of the alveolus necessary for maintaining alveolar stability and preventing pulmonary oedema (Jugg *et al*., 1999; Pauluhn *et al*., 2007). The induction of surfactant abnormalities following phosgene exposures is presumed to be a key pathophysiological event leading to pulmonary oedema and sequelae (Pauluhn *et al*., 2007). Protein levels in bronchoalveolar lavage (BAL) fluid have been shown to be among the most sensitive end points characterizing these early, acute effects of phosgene exposure (Hatch *et al*., 1986; Sciuto, 1998; Pauluhn, 2006a; 2006b; 2006c).

In their study of the $C \times t$ relationship of pulmonary responses to high lethal exposures to phosgene Rinehart and Hatch (1964) concluded that 'there was no indication that concentration contributed more than exposure time to the magnitude of change in pulmonary performance over the range of $C(x)T$ values studied'. The work of Pauluhn (2006a; 2006b) indicates that this should be approximately true for both lethal and nonlethal exposures, with the latter referring specifically to end points indicative of a transient perturbation of the blood–air barrier (Pauluhn, 2006b).

2.1.2 Issue: C × t and End Point

Phosgene has a relatively robust database of $C \times t$ information, especially on target tissue end points of the respiratory tract. The issue examined in this case study is comparison of two approaches on data utilization in estimating the $C \times t$ relationship. One approach involves utilization of all available data, inclusive of a range of target-tissue end points measured in several different species and multiple studies, some of which employ and report on only a single duration; this approach was implemented using the US EPA version of categorical regression software, CatReg (US EPA, 2006). In contrast, the second approach is the use of a single data set in a single species (rat) examining the timecourse for expression of a single highly sensitive respiratory tract end point, BAL-fluid protein levels in a refined dose–response analysis using BMD analysis. These analytical methods and tools are described only generally here, with references given to more in-depth methodological sources.

2.1.3 Methods

2.1.3.1 CatReg: Severity-Based Categorical Regression

The US EPA developed CatReg to perform categorical regression analysis with the categorical component representing response severity (Guth *et al*., 1997; Strickland and Guth, 2002; US EPA, 2006). Response severity data are obtained from judgment-based evaluations and assignments of the responses observed in various toxicity studies. It is these individual categories of responses that may be evaluated by CatReg with respect to concentration and duration and other variables of the data.

For example, the severity response category scheme used by CatReg for the phosgene database was as follows: no adverse effect assigned severity 0; a mild adverse effect assigned severity 1; a severe adverse effect assigned severity 2; lethality assigned severity 3. The assigned severity category and other information associated with the specific response, such as the gender, species and, of course, the concentration and duration of exposure, all may be recorded for each individual response. An attribute of this severity assignment is that it can be applied to responses irrespective of study, species,

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or gender. Outputs from CatReg analysis of such data may then be used to define $C \times t$ relationships for a specific category of severity derived simultaneously from a large number of different studies that may comprise many different species and end points. Outputs may also be examined from the perspective of any subset of the entered data, such as examining the $C \times t$ relationship for a severity 1 (mild adverse) effect in a specific species or the $C \times t$ relationship for a category severity 2 effect either for an individual species or for all species with both genders.

Regression lines generated in CatReg concentration–duration plots commonly have a negative slope with respect to increasing durations, reflecting the general tenet that exposures of greater durations and lower concentrations result in a similar severity of response. The regression lines generated by CatReg are actually probability functions relating the likelihood of observing an effect for a specific severity category over a range of exposure durations. For example, the regression line presented in this example analysis of phosgene represents the 10% (extra) risk of observing a particular severity category or higher over a range of concentrations (C) and durations (T) and for that reason are designated as 'ERC-T10%' lines for any given severity category. These lines are estimations of the probability (or likelihood) than an effect of a certain severity will occur at various concentrations and durations of inhaled phosgene at this level of risk.

Other outputs from the CatReg regression important to the $C \times t$ relationship include model estimates for the coefficients of both concentration (β_1) and duration (β_2) for a given severity category that are analogous to coefficients in the ten Berge equation (see legend to **Figure 1**). When the concentration and time terms in the model are log transformed, β_1/β_2 represents n in $C^n \times t$.

Further in-depth specifics in the statistical and procedural aspects underlying CatReg are available at the web site (*http://cfpub.epa.gov/ncea/cfm/nceatools_human.cfm*) and via a number of supporting references (Guth *et al*., 1997; Dourson *et al*., 1997; Strickland and Guth, 2002; Brown and Strickland, 2003; Brown and Foureman, 2005; US EPA, 2006).

2.1.3.2 Benchmark Dose

The BMD approach is an alternative to the NOAEL–LOAEL (no/lowest observed adverse-effect level) approach for estimating a threshold-type dose in data sets. Rather than the risk assessor designating a single dose in a data set as an NOAEL, based on some measure of response, BMD fits a mathematical model to the entire dose–response data set for any given end point. The model allows the risk assessor to designate a certain level of response (e.g. 10% extra risk or one standard deviation from the mean, SD) and then to estimate the dose that corresponds to that benchmark response (BMR), in the context of the entire range of dose–response available. This feature allows all dose–response groups to be compared on a common basis (i.e. that exposure required to produce the same BMR) rather than being restricted to designation of an actual dose, as in the NOAEL–LOAEL, for which response information may be judged only from contiguous doses. The terms BMD_{10} and BMD_{SD}, usually refer to the central estimate of the dose at the BMR, with the corresponding statistical 95% lower bound on dose designated as $BMDL_{10}$ and $BMDL_{SD}$, respectively. The analysis done here for each concentration–duration point is with the central estimate, actually the maximum likelihood estimate (MLE), the BMD. When the BMD approach is applied to air concentrations, as in the next example, the parallel term benchmark concentration (BMC) is used. Also, extra risk, the type usually used by the US EPA, is defined as the estimated increased risk over background ($P_d - P_0$) divided by the maximum risk with background excluded ($1 - P_0$).

2.1.4 Phosgene Concentration–duration Information and Analysis

The following tables are included to demonstrate the type of data utilized in these two analytical methods.

Although not exhaustive in its data listing, **Table 1** serves to demonstrate the variety and scope of data available on the respiratory tract on the $C \times t$ relationship for phosgene. As can be seen, these data comprise information from end points ranging in adversity from alterations in cofactor levels (decreased nonprotein sulfhydryls) to lethality from five different species. The table also indicates the severity categorization assigned for the CatReg analysis, ranging from phosgene exposure without effect, severity 0, up to lethality, severity 3.

Table 2 shows the end-point-specific analysis on BAL-fluid protein data obtained from phosgene-exposed rats two days post-exposure by Diller *et al*. (1985). US EPA benchmark dose software (BMDS) models and a BMR level of one SD increase of BAL protein levels were used to estimate the exposure concentrations giving a 1 BMC_{SD} over the range of response between the control value and the values noted at the exposure durations listed in **Table 2**. Pauluhn (2006b) reported that 'the $C \times t$-dependent changes of phosgene-induced pulmonary injury appear to be described best by elevation in BAL fluid protein concentration one day following exposure', as opposed to BAL fluid protein or other end point measurements taken as late as three months post-exposure. Using duration-specific dose–response data sets describing the relationship between BAL-fluid protein concentration and exposure concentration, benchmark measures were estimated for the various exposure durations. The slope of the resultant BMC-duration plot was then used to derive the value of n in C^n. Further

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Table 1 Example phosgene inhalation studies by species, end points, effect levels and range of severity categorization

References	Species	Duration (h)	NOAEL (mg/m^3)	LOAEL (mg/m^3)	End point(s)	Severity category (0–3)
Hatch *et al*. (1986)	Rat, Mouse, Guinea pig, Rabbit	4	— 0.4 0.8 0.8	0.4 0.8 2.0 2.0	Bronchoalveolar lavage fluid (BAL) protein	0–1
Diller *et al*. (1985)	Rat	4 8.3 5.5 1 0.33 0.17	— — — — — —	0.4 0.4 0.6 4 10 20	Histology, BAL protein BAL protein BAL protein Histology, BAL protein BAL protein BAL protein	0–2
Slade *et al*. (1989)	Guinea pig	4	—	1	BAL protein	0–1
Currie *et al*. (1987)	Rat	4	—	2	Lung weight, lavage protein, cells	0–2
Franch and Hatch (1986)	Rat	4	1	—	Lung weight, G6PD-nase, nonprotein sulfhydryls	0–2
Madden *et al*. (1991)	Rat	4	4	—	Lavage cells, viability, prostaglandins, leukotrienes	0–2
Burleson and Keyes (1989)	Rat	4	0.4	2	Pulmonary NK cell activity	0–2
Jaskot *et al*. (1991)	Rat	4	—	2	Lung weight, nonprotein sulfhydryls, GSH enzymes	1–2
Ghio and Hatch (1992)	Rat	1	—	2	BAL protein, cells; permeability	1
Ghio *et al*. (1991)	Rat	1	—	2	BAL protein, cells, leukotrienes, death	1–3
Ehrlich *et al*. (1989)	Rat	4	—	4	Lung weight, total cells, macrophages, lymphocytes and neutrophils in lung cell populations, cytotoxic T-lymphocyte activity	1–2
Currie *et al*. (1985)	Rat	4	—	4	Lung weight, mitochondrial activity, ATP concentration, Na-K-ATPase activity	1–2
Long and Hatch (1961)	Rat	0.5	—	4	CO uptake, oxygen consumption, respiratory rate	0–2
Ghio and Hatch (1996)	Rat	0.5	—	8	BAL protein, cells; permeability, death	0–3
Price *et al*. (1979)	Rat	0.5	5.7	64.2	Death	3
Box and Collumbine (1947)	Rat Mouse	0.17 0.17	— —	151 151	Death Death	3 3
Durlacher and Bunting (1947)	Dog	0.5	—	290	Death	3

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Table 2 BMC_{SD} estimates (from a linear polynomial model) for the response of increased lung-lavage fluid protein ($\mu g\,ml^{-1}$) in male rats two days post-exposure to various phosgene exposure durations. The interpretation of these data is (upper column) that a BAL-fluid level of 107 (69+38) $ug\,ml^{-1}$ is estimated to result from exposure to an air concentration of 6.1 mg phosgene m^{-3} for 20 min (0.33 h)

Exposure ($mg\,m^{-3}$)	BAL fluid, $\mu g\,ml^{-1}$, mean ± SD		Duration (h)	BMC_{SD} ($mg\,m^{-3}$)
	Control	Exposed		
10.1	69 ± 38	355 ± 255	0.33	6.1
4.0	117 ± 97	288 ± 29	0.83	1.6
0.4	69 ± 38	90 ± 39	4.17	0.71
0.4	117 ± 97	255 ± 78	8.3	0.24
0.4	69 ± 38	301 ± 133	8.3	0.16

These data are from **Table 2** in Diller *et al*., 1985.

specifics on the analysis of the Diller data via BMC are given in **Table 2**.

As can be seen from these tables, both the extent of data and the evaluation of the response of the Diller *et al*. (1985) data differed between the CatReg and BMD analyses. In the CatReg analysis, all animals from all duration-exposure groups were included in the analysis (see the listing for Diller in **Table 1**), nearly all of which were noted and recorded as being at a response level at or below severity 1 (mild adverse) defined as a 2× increase over concurrent control BAL-fluid protein levels. In the BMC analysis, however, the shortest dose/duration group of 10 minutes (0.17 hours) was eliminated from the analysis (see explanation below). Also, as noted above, the threshold level was set for a specific significant response at 1 SD from the concurrent control value. The reasoning for these differences is based upon additional information gained from the test species and about the end point.

In his analysis of breathing patterns of rats exposed (nose-only) to relatively high phosgene levels ($40\,mg\,m^{-3}$ and higher), Pauluhn (2006a) noted a marked and reproducible transient depression in ventilation parameters 10–15 minutes from the start of exposure. Accordingly, it was considered justifiable to exclude results for these short exposure durations from his analysis and the same data (10 minutes or 0.17 hours) were excluded from this end-point-specific BMC analysis of the Diller data. Regarding the BAL end point, Hatch *et al*. (2001) noted BAL-fluid protein levels in rodents were relatively insensitive indicators of mortality, whereas other reports suggest BAL-fluid protein to be a much more sensitive indicator of mortality in humans (Pittet *et al*., 1997). It was thus considered appropriate to further refine the response level at which this end point may be considered to have an effect, hence the assignment and determination of 1 BMC_{SD} in the BMC analysis for the BAL-fluid protein end point vs. the mildly adverse criterion described above for the CatReg analysis.

Figure 2 shows the composite CatReg solution for nearly all phosgene concentration–duration data for respiratory tract end points and is inclusive of those data shown in **Table 1**. As would be anticipated, the placements of the ordinate values at any given point on the duration axis for severity 3 (lethality) data points are higher in concentration than the values for severity 2, which is likewise higher than for severity 1, with severity 0 data points having the lowest ordinate values. The ERC-10% curve for a severity 1 response is shown with confidence limits and is situated lower on the axis (and further from the actual severity 1 data points) than would a higher-probability curve, such as an ERC-30% or ERC-70%, which would be progressively nearer to the severity 1 data. Also as anticipated, the placement of data points for any given severity with increasing duration define a negative slope. This observation was confirmed by the CatReg regression analysis and solution for the severity 1 respiratory effects (the ERC-T10% curve along with its confidence bounds) for combined species (rats, guinea pigs and mice) in **Figure 2**. These observations indicate that components of both concentration and duration have a role in characterizing the $C \times t$ relationship to phosgene responses.

2.1.5 Comparison of Results

The overall and comprehensive analysis made possible by the nature of the CatReg method is highlighted in **Figure 2**. Data points from over 20 studies, representing five different species for many different respiratory end points each of which has been judged with respect to severity, are all directly represented on **Figure 2**. The normalization of these data through the severity categorization scheme discussed above demonstrates the meta-analytic nature and value of CatReg for this type of analysis. This example case study does not show the versatility of CatReg in utilizing capacities to further sort, filter and analyze all data through a spectrum of permutations. Rather, the data were utilized here collectively to estimate the probability of a severity 1 effect, along with its statistical boundaries through the $C \times t$ relationship represented by these data. Direct outputs from CatReg (i.e. β_1/β_2) were used to determine the value of n (in C^n) at 1.4. A meta-analysis such as this can provide an investigative starting point, but may need to be refined

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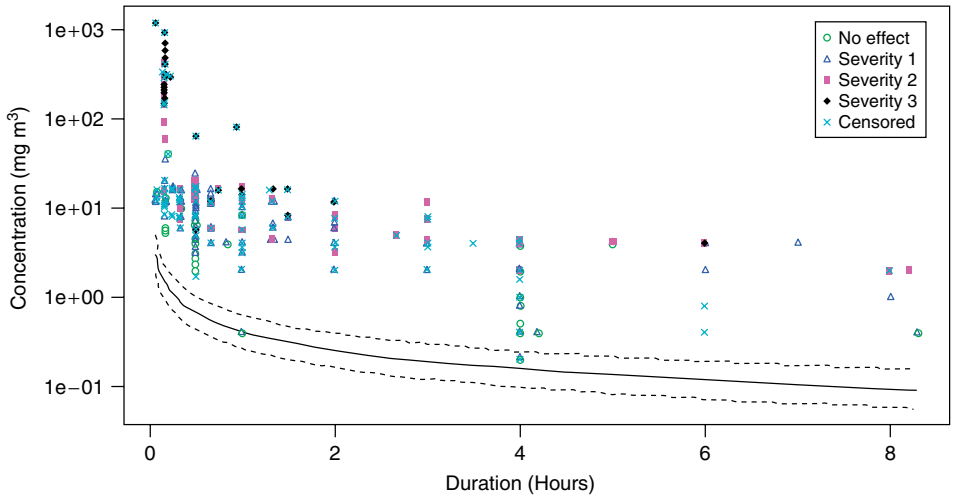

Figure 2 A sample concentration–duration plot from the CatReg analysis of respiratory tract effects from phosgene exposure. The effects have been graded for severity (grades 0–3 with 0 being 'no effect' and 3, lethality). The cumulative odds model, log-probit link function, provided the best fit to rat, mouse and guinea-pig respiratory effects (for details of on these models see the latest CatReg Software Documentation, US EPA, 2006). Data points may represent more than one subject, depending on the number of subjects in an experimental group responding with a particular severity. Groups with individuals in more than one severity category are represented by more than one symbol per the key shown above. When a response does not clearly fit into a single severity category, either because insufficient information is available or because the adversity of the effect is uncertain, the effect is 'censored' over two or more categories. The mathematical approach used for censoring is detailed in the CatReg Software Documentation (US EPA, 2006). Solid line = ERC-T10% for mild adverse effects (severity 1); Dotted lines = 95% one-sided lower and upper bound confidence limits.

based on biological information or sensitivity analyses to remove data (e.g. time points, test species or end points) that confound rather than inform or may be less relevant to the potential for human lung irritation from acute phosgene exposures, as is seen in the accompanying BMD analysis of the Diller *et al.* (1985) data.

Figure 3 shows the analysis for n on the calculated BMC_{SD} using the selected end point of BAL-fluid protein data from the rat study by Diller *et al.* (1985). As explained above, this analysis is based on specific information, further refined using ancillary information and shows that: (i) BAL is the most sensitive end point in inhalation exposures to phosgene, (ii) BAL end point information one to two days post-exposure is most representative of damage and (iii) ventilatory parameters in the early stages of exposure (10–15 minutes for rats) are unstable. The value for n in this refined analysis, at 1.0 differed from that determined with the more broad inclusive CatReg analysis.

The values of n derived from these two approaches both indicate that duration is a major determinant in the $C \times t$ relationship of nonlethal effects from acute inhalation exposure to phosgene. Both of these values are relatively far from those values of n (i.e. >1) that indicate a predominance of concentration over duration (e.g. note the decreased slope of the $C \times t$ line at $n = 3$ in **Figure 1** above). It is also to be noted that phosgene was one of the agents used to establish Haber's rule at $n = 1$ (see Pauluhn *et al.*, 2007). The most likely reason the CatReg analysis with phosgene presented in this review is not totally concordant with Haber's rule is that, as discussed above, some data included in this meta-type analysis may have an inordinate or unexpected influence on the results. Different species may have competing kinetic and dynamic influences, different end points may vary in their sensitivity and different exposure regimens used in studies can cause variability. In this case, the reflex apnea seen in rodents exposed to high concentrations of irritating chemicals for up to 15 minutes led to the decision to drop the 10 minute duration from the BMC analysis; this refinement was not done with the CatReg analysis. Consideration of these factors in assessing risks from exposure to environmental chemicals is the reason this report was written.

2.2 Ethylene Oxide Case Study

2.2.1 General

At room temperature and pressure, ethylene oxide (EO) is a colourless, highly reactive gas. EO serves as a feedstock in the manufacture of ethylene glycol and surfactants, and is used as a sterilant for heat-sensitive materials and products, especially in the healthcare industry (e.g. in hospitals). Trace amounts of EO are produced from the combustion of carbon-based compounds, but the main sources of potential exposures for humans are from industrial applications or its use as a sterilizing agent.

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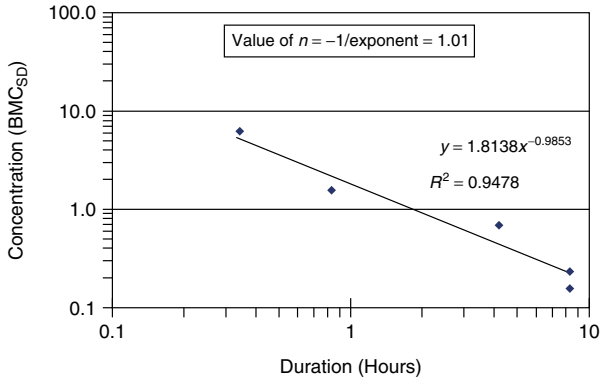

Figure 3 A log–log plot of benchmark dose estimates for increases in lung-lavage protein levels after various durations of exposure to phosgene. (Reproduced from Diller *et al.*, 1985.) The dose–response data at each exposure duration were analyzed using a linear model to obtain the benchmark for a response level of 1 standard deviation from concurrent controls (BMC_{SD}). These BMC_{SD} estimates are plotted against duration and fit to a model in the form of $C = m \times t^b$. This model was transformed to the $C^n \times t = k$ equation where values of m are related to k (where $m = k^{1/n}$) and values of b are related to n (where $b = -1/n$). The value for the exponent n in this analysis is $-0.9853 = -1/n$, such that $n = 1.01$. The actual transformation is: $C^n \times t = k$; $C^n = (kt^{-1})$; $C = (kt^{-1})^{1/n}$; $C = k^{1/n} \times t^{-1/n}$.

Health effects associated with exposure to EO include potential cancers of the haematopoietic system; developmental and foetal toxicity; dermal, ocular and respiratory tract irritancy and effects on neurological function (Agency for Toxic Substances and Disease Registry (ATSDR), 1990).

2.2.2 Issues

The data sets available for concentration–duration analysis with EO include several based on the lethality end point, and one study that also substantiated nonlethal developmental effects in mice from single exposures at varying concentrations and exposure durations, showing a $C \times t$ relationship. These data sets allow for demonstrating: (i) the application of existing software implementing the $C^n \times t$ approach and (ii) the consequences of different values of n in an applied scenario.

2.2.3 Methods

All calculations of the value of n in this section (referring to the exponent n in the $C^n \times t = k$ equation) were determined using the statistical software DoseResp developed by ten Berge (2006), a version of which is available at the following web sites, *http://www.epa.gov/ncea/bmds/dwnldu.html#berge* or *http://home.planet.nl/~wtberge/doseresp.html*.

2.2.4 Data and Analysis

Three sets of inhalation lethality data were available for EO and used in this analysis. A composite data set on rats was constructed from the studies by Jacobson *et al.* (1956), Nachreiner (1991) and Nachreiner (1992). This composite data set comprised rats of two strains (Wistar and Sprague–Dawley) exposed over a relatively wide range of EO concentrations (882–6161 ppm) at two durations (60 or 240 minutes). A second composite data set was created from the two Nachreiner studies alone, in which only Wistar rats were used. The third data set was from the developmental study in mice by Weller *et al.* (1999). In this study, pregnant mice were allocated to exposure groups (50–70 in each concentration/duration group). There were five time points (90, 105, 120, 180 and 360 minutes) for observations of lethality available for analysis in the Weller study, with exposure concentrations ranging from 350 to 1800 ppm. Further specifics on these data sets are available in **Table 3** below. The point to be noted here is that this approach to elucidating $C \times t$ requires relatively little data, as compared to other approaches such as CatReg.

2.2.5 Results and Implications

Qualitatively, the values of the exponent n derived from these three data sets are consistent in that they are between 1 and 2 (**Table 3**). As mentioned before, the value of n gives an indication of the relative role and prominence of duration or concentration in the effect observed. As shown in **Figure 1**, values of $n > 1$ (e.g.

Table 3 Summary of $C \times t$ data for ethylene oxide

Group size	End point	$C^n \times t = k$ data			n
		No. of ($C \times t$) combinations	Duration range (min)	Concentration range (ppm)	
5–10	Lethality	23	60 or 240	882–6161	1.2 ± 0.06
5	Lethality	17	60 or 240	1021–6161	1.4 ± 0.13
14–70	Lethality	8	90–360	350–1800	1.7 ± 0.2

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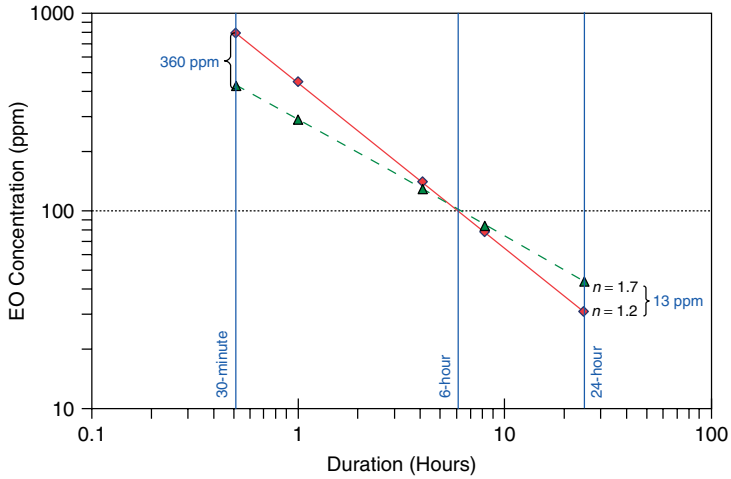

Figure 4 Comparison of results in extrapolating using different values of n in the equation $C^n \times t$. The figure compares two different values of n derived from lethality results in animals exposed to ethylene oxide. The extrapolation depicted is from a known effect level (NOAEL of 100 ppm), duration (six hours), and extends to a shorter (30 minutes) duration and to a longer (24 hours) duration using the values of n indicated. The ppm values noted at the ends of the two curves represent the divergences in concentrations at the durations indicated.

3) define increasingly shallow slopes on the $C \times t$ relationship indicating that the effect noted has a prominent concentration component with duration being a less determinative factor. Values of $n < 1$ yield increasingly steeper slopes on the duration–concentration curve thereby indicating duration to be more determinative in elicitation of the response. Thus the EO results for n consistently indicate duration to be a major determinant in the concentration–duration relationship for EO, at least at high concentrations and with mortality as an end point.

Quantitatively, however, parameters expressed as exponents by their very mathematical nature may be expected to be critical and highly sensitive elements in characterizing relationships. As such, it is instructive to examine the results for n obtained for the EO data in **Table 3** from the aspect of its intended utility (i.e. quantitatively extrapolating to different intersections of the concentration–duration curve). **Figure 4** is constructed to demonstrate both the sensitivity and the manner in which extrapolations would be affected by seemingly small differences in n. This example assumes observation of an NOAEL occurring at 100 ppm, six hour exposure to EO, with the requirement to extrapolate to an NOAEL concentration at a shorter duration of 30 minutes. Application of the most disparate values of n from **Table 3**, 1.2 and 1.7, shows the quantitative differences as a consequence of this difference in n. The higher concentration value dictated by the lower value of n (1.2) results in an extrapolated value of 793 ppm. This is over 360 ppm higher than the value of 431 ppm obtained with the higher value of n (1.7). If the direction of this extrapolation is changed from six hours to 24 hours (i.e. from a shorter to a longer duration), the resultant concentrations due to these different values of n at 24 hours are 31 ppm for $n = 1.2$ and 44 ppm for $n = 1.7$. (The maths for the former condition would be $C^n \times t = k$, $(100^{1.2}\text{ ppm}) \times 6$ hours $= 1507$ ppm/hour $= 3014 \times 0.5$ hours $= (793^{1.2}\text{ ppm}) \times 0.5$ hours; $(100^{1.7}\text{ ppm}) \times 6$ hours $= 15071$ ppm/hour $= 30142 \times 0.5$ hours $= (431^{1.7}\text{ ppm}) \times 0.5$ hours.)

For scenarios such as health-based risk assessment, projecting exposures, through a highly uncertain process such as extrapolation, bears considerable liability, especially if it leads to higher and higher concentrations. In this example of extrapolating from a longer to a shorter duration, the marginally lesser value of n of 1.2 resulted in a much higher extrapolated concentration, nearly two-fold higher (763/431 ppm), than did the greater value of n at 1.7. To complicate this situation further, it can be seen that the converse of this situation occurs when the extrapolation proceeds from shorter to longer duration. Here the slightly greater value of n at 1.7 resulted in a 1.4 fold higher (44/32 ppm) value than did the lesser value of n of 1.2.

This example serves to point out further considerations in performing duration extrapolation, including the consequences of the existing differences in the value of n derived from the $C^n \times t$ approach, and further, the temporal direction in the application of different values of n. In applying these results to this example of EO, for example, justification could be made for applying an n of 1.7 when extrapolating from longer to shorter duration and for applying an n of 1.2 when extrapolating from a shorter to a longer duration. This leads us to a discussion of an approach to avoid underestimation of risk when faced with conflicting data or uncertainty.

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2.3 The '*n*' Boundary Approach

2.3.1 General Issue: Variability and Minimum Data Default

A general issue common to most all concentration–duration data sets and their analysis, including these case studies, is their attendant uncertainty and variability reflected in the exponent n. The type of data necessary for elucidating the actual relationship and the nature of the variability would require multiple pairing of various concentration–duration experiments, data that is currently a rarity. For both phosgene and the EO case studies discussed here it is demonstrated that uncertainty/variability in n can lead to quite widely disparate results in exposure levels derived for a common duration and that the greater the extent of extrapolation, the intended application of these analyses, the greater the resultant differences. With EO, for example, extrapolation with the relatively small variability of the estimated n over a moderate range of duration (0.5–6 hours) resulted in concentration values that were nearly doubled. Yet another complicating aspect of the uncertainty/variability is that the outcome of extrapolation is influenced by the temporal direction of the extrapolation (i.e. from longer to shorter or from shorter to longer durations). The EO case study demonstrated clearly that extrapolations from longer to shorter durations resulted in increasingly higher differences in concentrations related to the value of n, with the converse relationship between values of n occurring in the extrapolation from shorter to longer durations. Extrapolating health-based values to high concentrations that have not been examined directly or well characterized may have increasing liability.

2.3.2 Toxicity Information

The data sets examined by ten Berge (1986) did not examine variability or uncertainty around estimation of an individual n. These authors, however, did report the range over which n varied in those data sets examined as being 1–3. This range of values has, in general, been affirmed by others (e.g. see Sommerville *et al.*, 2006).

2.3.3 Analysis and Results

A plot of these values defining this range of 1–3 is given in **Figure 1** above. Examination of these simulations (relative to a fixed time point) reveal how the $n = 3$ curve is shifted towards paralleling the x (duration) axis, that is, produces relatively small changes in C through a range of values for t. From this figure it can also be seen how the $n = 1$ curve is shifted towards paralleling the y (concentration) axis instead and yields relatively large changes in C through a smaller range of values for t. Thus these curves each address a different liability of duration extrapolation. The $n = 3$ curve serves to restrict extrapolation to higher concentrations when proceeding from longer to shorter durations. The $n = 1$ curve provides for a diminution of the $C \times t$ product when proceeding from shorter to longer durations.

A modification of **Figure 1** is presented below as **Figure 5**, highlighting the aspects and points discussed above. Curves corresponding to $n = 1$ and 3 are shown based on a hypothetical NOAEL of 100 ppm at a duration of four hours, which forms the intersection between

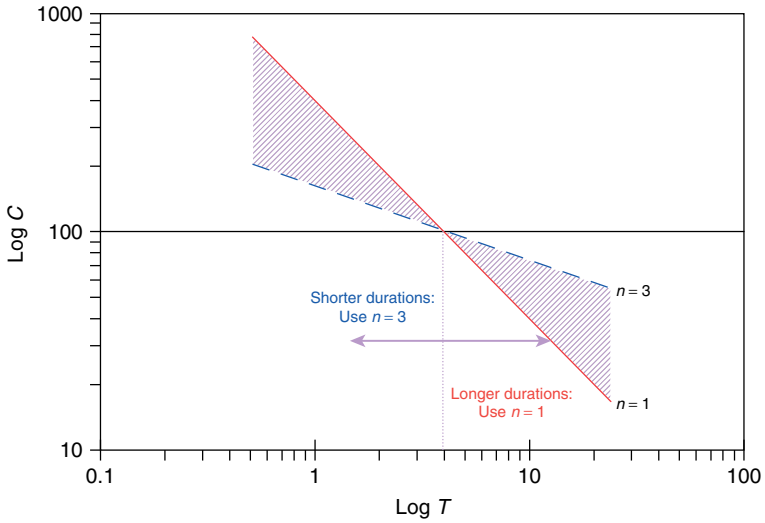

Figure 5 Duration extrapolation with a composite n in $C^n \times t = k$. The solid line corresponds to the $C \times t = k$ relationship at $n = 1$ (steep) and the dashed line at $n = 3$ (more shallow), which intersect at the duration indicated on the x-axis. The recommendation is to use the lower boundary of the shaded portion between these straight lines, using the portion of the line for $n = 3$ for shorter durations and the portion of the line for $n = 1$ for longer durations when extrapolating from observed data.

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these two curves. The areas of intersection between these curves have been crosshatched and may be described as follows. The area to the left of the common duration intersection is bounded on the lower portion by the $n = 3$ curve and on the upper portion by the $n = 1$ curve and corresponds to the progressively higher concentrations that would result from utilizing values of $n < 3$ in extrapolating from longer to shorter durations. The area to the right of the common duration intersection is bounded on the lower portion by the $n = 1$ curve and on the upper portion by the $n = 3$ curve. This area corresponds to the progressively lower concentrations that would result from utilizing values of $n > 1$ in extrapolating from shorter to longer durations. As discussed above, it is these hatched areas of extrapolation that are typically most problematic in defining a concentration–duration relationship, especially for purposes such as risk assessment and establishing health-effect reference values.

When represented in this manner it can be readily seen that, unless there are data to help elucidate the correct value of n, these problematic areas could be excluded from the extrapolation process by adopting a composite curve for extrapolation that would consist of a value of $n = 3$ for shorter durations and a value of $n = 1$ for longer durations. In extrapolating from longer to shorter durations, application of the lower portion of the $n = 3$ curve can be seen to result in less rapid rates of concentration increase than the corresponding portion of the $n = 1$ curve. In extrapolating from shorter to longer durations, application of the value of $n = 1$ curve can be seen to result in more rapid rates of concentration decrease than the corresponding portion of the $n = 3$ curve.

2.3.4 Implications and Use of Default

This composite curve would have considerable utility. As the values used are at the practical limits of n (1 and 3), the composite curve could be used to project and define the upper range of concentration and duration values that may be considered for purposes of investigating or establishing health-effect end points. This curve could also be reasonably adapted to serve as a manner by which a concentration–duration relationship could be established for a minimal-data situation. This approach has been adopted by the National Academies of Science's National Research Council (NRC, 2001) as a default procedure for establishing and bounding concentration–duration curves under conditions of minimal information (see *http://www.epa.gov/oppt/aegl/pubs/sop.pdf*).

3 DISCUSSION/SUMMARY

Considerable scientific attention has been given to both concentration and duration as aspects of dose in acute settings using the concept of toxic load (TL). The application of the TL concept in characterizing the amount of toxic material received and in the production of specified responses in a population has recently been reviewed (Sommerville *et al.*, 2006). Proponents of TL acknowledge the wide range of variables, including variance of the tested species/individuals due to underlying physiological and biochemical differences, batch effects, experimental error and character of exposure that may complicate the accurate estimation of a TL relationship. Efforts to more accurately estimate TL has lead to a series of models put forth or developed to consider one or more of these variables. TL models are all based more on empirical observations than on basic biological theories, including models described by Griffiths and Megson (1984) and Ride (1995). Also, sophisticated PBTK models highly parameterized to accommodate many of these variables can accurately predict empirical data, thereby implying the confounding role of these variables in the concentration–duration relationship (Bruckner *et al.*, 2004; Simmons *et al.*, 2005).

Rozman (2000) and others (Rozman *et al.*, 1996; Rozman and Doull, 2000; 2001; Doull and Rozman, 2000) have presented evidence that, in the absence of, or by mitigating, the confounding variables, such as those acknowledged by the proponents of TL, Haber's product $C \times t$ (i.e. $C^1 \times t = k$) may be the most precise relationship between the concentration and time components. With that acknowledgement, however, is counterposed that exposure to environmental chemicals routinely occurs under less than ideal conditions for matching a simple $C \times t$ relationship and those factors that force deviation from the ideal and are influencing the $C \times t$ relationship in some manner are not likely to be accounted for in real-world situations.

Whether acute exposures to toxic airborne agents occur to humans under occupational or nonoccupational scenarios, or to laboratory animals under controlled conditions, all are likely to be inclusive of elements of variability that will induce variance from the precise relationship between concentration and duration. It is on this basis that these case studies of empirical data are presented, along with the various methods and techniques for analysis and evaluation of their $C \times t$ relationship.

It may be instructive at this point to consider again the procedures employed and discussed above in addressing the situation when minimal information is available on which to derive $C \times t$ relationships, referred to herein as the n-boundary approach. Viewed generally, application of this approach reasonably advocates the use of values of n that are dependent on the temporal direction of the extrapolation; designating higher values of n such as 3 when extrapolating to shorter duration (to higher concentrations) and lower values of n such as 1 when

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Table 4 A summary of empirical methods and approaches for use in characterizing the $C \times t$ relationship of toxicity responses

Method/ approach	Source	Description	Data requirement	Attributes	Limitations
PBTK models	Open literature	Highly sophisticated models capable of estimating internal dose at target tissues associated with response. Used for various purposes, including $C \times t$ relationships	Highly parameterized for a single data set	Highly sophisticated, accommodates many variables to characterize definitive $C \times t$	Information intensive. Requires many parameters to develop as well as duration-dose data for validation of model.
CatReg	*http://epa.gov/ncea/catreg*	US EPA version of categorical regression based on severity. Results from different studies, species and gender may be normalized based on severity category. Concentration– duration analysis conducted for any severity category	May utilize many data sets in an integrative analysis	Meta-analytic feature of categorization allows for extensive compilation and integration of data sets.	Requires extensive data analysis and documentation.
ten Berge (software)	*http://www.epa.gov/ncea/bmds/dwnldu.html#berge*—Excel least-square equations used for calculating trendlines from response data	Software version of ten Berge model	A single multipoint concentration–duration data set	Ease in application.	Analysis limited to single data sets.
BMD analysis	*http://epa.gov/ncea/bmds*	US EPA version of benchmark dose software	Preferred minimum of three data sets from which individual benchmark concentrations may be derived for three different durations.	For refined analysis of dose–response data and common response level when available for individual durations	Analysis limited to single data sets
n-Boundary	This work and *http://www.epa.gov/oppt/aegl/pubs/sop.pdf*	A version of $C^n \times t$ where the value of n is set based the temporal direction of extrapolation. Design and intent is to extent limit extrapolations to higher concentrations for shorter durations and to lower concentrations for longer durations	Minimum data. May be applied with a single initiating $C \times t$ data point for which response is defined	Ease in application. Results in low or conservative estimates of concentration, that is, those that likely err on the side of safety	High attendant uncertainity.

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extrapolating from shorter to longer durations (to lower concentrations), in the absence of any chemical-specific information to help inform these extrapolations. This approach and reasoning, in which extremes of extrapolations resultant from temporal directions are minimized through assignment of values of n, could be considered as a means to accommodate data-based uncertainty and variability in the value of n such as encountered in these examples. The values of 1 and 3 used for n above were based on the historical range reported, mainly from the historical study of ten Berge *et al*. (1986) and others. Another option for a range of values that could be considered to fit this purpose would be to examine a range of empirically derived n values for use in extrapolation such that the higher or highest values of n be used in extrapolating from longer to shorter durations and the lower or lowest value of n be used in extrapolating from shorter to longer values. With the EO case study, for example, the n value of 1.7 would be used for extrapolating from shorter to longer durations and the n value of 1.2 would be utilized for extrapolating from shorter to longer durations.

As has been emphasized, however, any and all results from such procedures for evaluation of the $C \times t$ relationship need to be evaluated from the aspect of the entire database and information available for both reasonableness and appropriateness. As demonstrated above with phosgene, this evaluation applies to specifics of end points reported such that actual data sets may be justifiably modified for evaluating and refining the $C \times t$ relationship. This evaluation may apply to the overall character of certain end points. For example, results analyzing the response of sensory-based irritation (in humans), gives clear indications that exposure concentration has a proportionally greater effect for this end point than does exposure duration (see review by Shusterman *et al*., 2006). Other observations with this response indicates that this characterization may be even further complicated with time-effects disappearing or even reversing.

The methods and approaches presented in this work for evaluating $C \times t$ relationships are listed in **Table 4**. This listing of methods and approaches is provided as a set of tools, each tool useful for approaching specific situations requiring resolution and evaluation of $C \times t$ relationships. These tools are designed especially for use in acute inhalation scenarios and involving both lethal and nonlethal end points. It is also acknowledged that these approaches and methods are based largely on mathematical and statistical principals and only peripherally on any underlying biological models. It is hoped and anticipated, however, that application of these methods to various data sets may help in the syntheses of new hypotheses, whose testing would support existing biological theories on $C \times t$ relationships or provide a basis to formulate new ones.

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Toxicological Considerations in Relation to the Regulatory Safety Evaluation of Medical Devices

Raju Kammula

CONTENTS

1 INTRODUCTION

Medical devices and their component materials are potential sources of toxins that may produce undesirable local and/or systemic toxic responses when used clinically. The evaluation of toxic responses of medical devices using various toxicological test methods is also called biocompatibility evaluation of medical devices. Biocompatibility evaluation of medical devices is carried out to determine that a device performs as intended and presents no significant harm to the patient or user. This assessment is generally considered to be the determination of the biological interaction of the medical-device materials with host tissues. This interaction should not result in a toxic, injurious or immunological response in the person receiving the device. Therefore a safe medical device

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should be biologically compatible (biocompatibility) with the patient by not producing toxic responses.

Historically, evaluating the toxicity (biocompatibility) of medical devices has been a complex task. This complexity arises from the fact that devices are made of diverse range of materials and have various intended uses, with body contact ranging from transient skin contact to permanent implantation. The biocompatibility of medical devices is demonstrated by testing of device materials and their leachable chemicals and degradation products, using toxicological test methods.

The safety and effectiveness of medical devices marketed in the United States (USA) is regulated by the Center for Devices and Radiological Health (CDRH) of the US Food and Drug Administration (FDA). The medical devices marketed in the European Union (EU) are required to comply with EU Medical Devices Directive 93/42/EEC (EU, 1993), which specifies requirements for safety assessment. There are several national and international standards that address the toxicological evaluation of medical devices. In recent years the FDA—in particular, the CDRH uses and accepts toxicological data generated using the national and international biocompatibility standards to evaluate the safety of medical devices. The EU and Japan (Ministry of Health Labor and Welfare: MHLW, 2003 Notification No. 0213001) also uses and accepts toxicological data, generated using international standards.

This chapter is an introduction to a relatively new and rather complicated field in toxicology—the toxicological testing of medical devices. It discusses the toxicological considerations to establish the safety of medical devices to meet the requirements of regulatory agencies. The guidelines for testing of medical devices are discussed and a general description of the various test procedures are given. Developments in the field of biocompatibility regarding international harmonization and the potential for new methodologies are also addressed.

2 REGULATION OF MEDICAL DEVICES

In US the FDA is the regulatory agency primarily responsible for the regulation of medical devices. The Federal Food, Drug, and Cosmetic Act (1938) provided the first authority to the FDA to regulate devices because of the concerns about fraudulent or quack devices. The statue did not provide provisions for premarket clearance of medical devices, because of the belief that such products played little role in health care. As device technology developed, and devices such as Intrauterine devices, heart valves, vascular grafts, renal dialysis, and so on, became more important in health care, it was realized that devices should be subject to more comprehensive regulation, including premarket review. The 1976 amendments to the US Food, Drug and Cosmetic Act, provided FDA with authority to require that medical devices intended for human use demonstrate reasonable assurance of safety and effectiveness—including material toxicity (biocompatibility). The Safe Medical Devices Act (1990) and the Medical Device Amendments (1992) further amended the Act. Additional modifications resulted from the Food and Drug Administration Modernization Act (FDAMA) of (1997), which, among other important reforms, allowed FDA to recognize consensus standards including biocompatibility standards established by national and international standards-development organizations.

Medical devices to be marketed in the EU are required to comply with EU Medical Devices Directive 93/42/EEC (EU, 1993), which specifies requirements for safety assessment issues. The purpose of the directive is to promote a single European market for trade in medical devices, while ensuring that users and patients are not exposed to unnecessary risks.

3 CLASSIFICATION OF MEDICAL DEVICES IN THE UNITED STATES

The Medical Device Amendments of (1976) put into place a classification scheme for medical devices. The various types of devices are placed into one of three classes, depending on the degree of risk they present and the level of regulatory control needed to provide reasonable assurance of their safety and effectiveness.

Medical devices designated as Class I require the lowest level of regulatory control, and this designation is intended for those devices for which there is sufficient information to conclude that safety and effectiveness can be ensured by general controls alone. General controls provide enforcement authority for misbranding, adulteration, registration and listing, banned-device authority, consumer notification and recall, product reporting and good manufacturing practice (GMP).

Medical devices designated as Class II are devices for which general controls alone are not sufficient to ensure safety and effectiveness, but for which there is sufficient information to establish special controls to provide this assurance. Therefore, in addition to the provisions for general controls, these devices are subject to one or more special controls that may include performance standards, guidelines, patient registries or postmarket surveillance.

Class III devices entail the highest level of regulatory control. For such devices, there is insufficient information to demonstrate that either general or special controls can provide a reasonable assurance of safety and effectiveness. Because of a lack of valid scientific data, these devices present the most risk and are therefore subject to

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general controls, as well as premarket approvals (PMAs) prior to marketing.

The requirements for medical devices to enter into commercial distribution in the USA vary according to the regulatory class of the device. Premarket notification (510(k)) and PMAs are the primary pathways to market for new devices, yet their regulatory requirements differ. All Class III devices are subject to PMAs, and must demonstrate a reasonable assurance of safety and effectiveness, as shown by valid scientific evidence. A significant-risk device that is not yet cleared for marketing through a 510(k) or approved through the PMA process can be tested in humans only when an investigational device exemption (IDE) application has been approved by the agency.

4 TYPES OF REGULATORY SUBMISSIONS TO THE FDA

4.1 Premarket Notification (510(k))

Unless exempt, Class I and Class II devices are subject to Section 510(k) of the FDAMA, which requires anyone wishing to introduce a device into commerce to notify the FDA at least 90 days in advance. A 510(k) notification is used to determine whether a new device is, or is not, substantially equivalent to a pre-amendments device or to a reclassified postamendments device. Substantial equivalence is based on an assessment of the device's intended use, technical characteristics and factors of safety and effectiveness. A new device that has been found to be substantially equivalent may be marketed immediately, whereas a new device that is not substantially equivalent may be marketed only through an approved PMA application. Devices reviewed under a 510(k) notification are not 'approved' by the FDA; rather, these devices are cleared for marketing as being substantially equivalent to legally marketed devices (Premarket Notification 510(k), 2008).

4.2 Premarket Approval (PMA)

All devices placed into Class III are subject to PMA requirements. A class III device is 'for use in supporting or sustaining human life or for a use, which is of substantial importance in preventing impairment of human health, or presents a potential unreasonable risk of illness or injury'. PMA is a process of scientific review to ensure the safety and effectiveness of Class III devices. A PMA submission must contain valid scientific evidence from which it can be concluded that there is reasonable assurance of the safety and effectiveness of the device under its conditions of use.

A PMA submission must have sections, among others, devoted specifically to laboratory, animal and clinical data. The laboratory and animal data sections must contain detailed descriptions of all laboratory and animal testing for the safety and effectiveness of the device performed by or for the applicant. This includes descriptions of materials of components, methods and results of analysis of contaminants and leachables and all *in vitro* and animal toxicology tests. Information on nonclinical laboratory studies must include a statement that each study was conducted in compliance with the Good Laboratory Practice (GLP) regulation (Good Laboratory Practice Regulations, 2008) or a statement of reason for noncompliance (Premarket Approval Application (PMA), 21 CFR 814, 2008).

4.3 Investigational Device Exemptions (IDEs)

A significant-risk device that is not yet cleared for marketing through a 510(k) or approved through the PMA process can be tested in humans only when an IDE application has been approved by the agency. A significant-risk device is one that presents a potential for serious risk to the health or welfare of a patient and is: (i) an implant, (ii) used in supporting or sustaining human life or (iii) substantially important in diagnosing, curing, mitigating or treating a disease, or in preventing impairment of human health. An IDE application must contain sufficient evidence of the device's safety, including results from toxicity tests and a reasonable expectation of effectiveness to warrant its testing in humans. Although non-significant-risk devices do not require an approved IDE application, they are still subject to the IDE regulation (Investigational Device Exemption (IDE), 21 CFR 812, 2008).

4.4 Product Development Protocols (PDPs)

Section 515(f) of the Federal Food, Drug and Cosmetic Act provides this alternative to the PMA process for Class III devices. An approved product development protocols (PDPs), established early in the device's development, defines the types of data and specific safety and performance levels that must be attained for market clearance. Marketing may commence following FDA verification that the device meets all safety and performance levels. This process allows FDA to effectively regulate Class III medical devices from initial development to marketing (Product Development Protocol (PDP), 21 CFR 814.19, 2008).

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4.5 Humanitarian Device Exemption

The Safe Medical Devices Act of 1990 provides an incentive for the development of humanitarian-use devices (HUDs). An HUD is a device that is intended to benefit patients by diagnosing or treating a disease or condition affecting fewer than 4000 individuals in the United States per year—circumstances under which a device manufacturer's research and development costs for a product could exceed its market returns.

The humanitarian device exemption (HDE) application is similar in form and content to that for PMAs, but is exempt from the effectiveness requirements. Specifically, an HDE application is not required to include the results of scientifically valid clinical investigations demonstrating that the device is effective for its intended use. However, the application must contain sufficient information, including preclinical toxicology data, for the FDA to determine that the device does not pose unreasonable risks and that the probable benefits to health outweigh the risks (Humanitarian Use Devices, 21 CFR 814, sub part H, 2008).

5 BIOCOMPATIBILITY EVALUATION OF MEDICAL DEVICES

Biocompatibility evaluation of medical devices is performed to determine the potential toxicity of device materials resulting from contact of the device with the body. The device materials should not—either directly or through the release of their material constituents—produce adverse local or systemic effects, be carcinogenic, or produce adverse reproductive and developmental effects. Therefore, evaluation of any new device intended for human use requires data from systematic testing to ensure that the benefits provided by the final product will exceed any potential risks posed by device materials.

One of the first steps in the manufacture of a medical device involves the selection of suitable biocompatible materials. This is an essential step because the types of tests required for evaluation of a device depend on the physical and chemical nature of its materials in addition to the nature of the device's exposure to the body. A specific material may appear suitable on the basis of its physical properties, cost and availability, but might contain toxic chemical components. Therefore, it is prudent to perform full chemical characterization of the candidate materials at an early stage to eliminate those that have potential to release toxic leachable chemicals, and select those that are sufficiently biocompatible or nontoxic for their intended use.

Detailed chemical characterization of the device/materials, which includes identification and quantification of potential leachable chemicals and their potential toxicity will aid in the toxicity risk assessment of the device. It will also facilitate in many cases the elimination of some of the toxicological tests to evaluate the biocompatibility of the device. The International Standards Organization (ISO) standard 10993, Part 18 gives detailed guidance on performing chemical characterization of medical-device materials (ISO, 2005).

Many believe that the FDA and other regulatory agencies, in addition to regulating devices themselves, approve the materials used in medical products and maintain a list of approved or acceptable biomaterials. In a very few instances, biomaterials are used as final products (e.g. injectable collagen, certain dental composites or bone cements); in such cases the FDA regulates these materials as devices. But, in general, the FDA neither approves materials nor maintains a list of approved materials (Kammula and Morris, 2001).

The suggested toxicologic/biological tests to evaluate the biocompatibility of medical devices include cytotoxicity, acute systemic toxicity, subchronic and chronic toxicity; irritation to skin, eyes and mucosal surfaces; sensitization; haemocompatibility; short-term implantation effects; genotoxicity; and carcinogenicity and effects on reproduction, including developmental effects. However, in certain cases, depending on the varying characteristics and intended uses of devices, as well as the nature of their body contact, these general tests may not be sufficient to demonstrate the safety of some specialized devices. Additional tests for specific target-organ toxicity—such as neurotoxicity and immunotoxicity—may be necessary for some devices. For example, a neurological device in direct contact with brain parenchyma and cerebrospinal fluid (CSF) may require an animal implant test to evaluate its effects on the brain parenchyma, propensity to induce seizure, or effects on the functional mechanism of the choroid plexus and arachnoid villi to secrete and absorb CSF. Thus, the specific clinical application of the new device determines which tests are most appropriate (FDA, 1995).

6 GUIDANCES AND STANDARDS TO EVALUATE BIOCOMPATIBILITY OF MEDICAL DEVICES

In 1986, the FDA, Health and Welfare Canada, and Health and Social Services UK issued first guidance to evaluate the biocompatibility of medical devices, the Tripartite Biocompatibility Guidance for Medical Devices. This Guidance has been used by FDA reviewers, as well as by manufacturers of medical devices, in selecting appropriate tests to evaluate the adverse biological responses to medical devices. Since that time, the ISO, in an effort to harmonize biocompatibility testing, developed a standard for biological evaluation of medical

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Table 1 The ISO standard 10993, consists of the following parts[a], under the general title Biological Evaluation of Medical Devices

—Part 1: Evaluation and testing
—Part 2: Animal welfare requirements
—Part 3: Tests for genotoxicity, carcinogenicity and reproductive toxicity
—Part 4: Selection of tests for interactions with blood
—Part 5: Tests for *in vitro* cytotoxicity
—Part 6: Tests for local effects after implantation
—Part 7: Ethylene oxide sterilization residuals
—Part 8: Selection and qualification of reference materials for biological tests
—Part 9: Framework for identification and quantification of potential degradation products
—Part 10: Tests for irritation and sensitization
—Part 11: Tests for systemic toxicity
—Part 12: Sample preparation and reference materials
—Part 13: Identification and quantification of degradation products from polymeric medical devices
—Part 14: Identification and quantification of degradation products from ceramics
—Part 15: Identification and quantification of degradation products from metals and alloys
—Part 16: Toxicokinetic study design for degradation products and leachables
—Part 17: Establishment of allowable limits for leachable substances
— Part 18: Chemical characterization of materials

[a]Future parts will deal with other relevant aspects of biological testing.

devices (ISO 10993). The scope of this 18-part standard is to evaluate the effects of medical device materials on the body (**Table 1**). The first part of this standard 'Biological Evaluation of Medical Devices: Part 1: Evaluation and Testing', provides guidance for selecting the tests to evaluate the biological response to medical devices. Most of the other parts of the ISO standard deal with appropriate methods to conduct the biological tests suggested in Part 1 of the standard.

The ISO Standard, Part 1, uses a similar approach to Tripartite Guidance in style, format and content. It uses a tabular format (matrix) for laying out the test requirements based on the various factors discussed above. It sets forth several fundamental principles similar to those in Tripartite Guidance (**Table 2**) for the evaluation of medical devices and provides a framework for their application. This part of the standard divides medical devices into three main categories: surface devices, externally communicating devices and implant devices. Each category is further divided into subcategories according to the type of contact the patient is exposed (**Table 3**). The selection of appropriate tests for a device in a given category depends also on the duration of contact. Three different time periods are given: limited contact (<24 hours), prolonged contact (24 hours to 30 days) and permanent contact (>30 days). The standard lists a series of biological tests that must be considered for each category in a test matrix to demonstrate a particular device is safe for its intended use (**Table 3** and **Table 4**). These tests include *in vitro* cytotoxicity, dermal irritation and intracutaneous reactivity, skin sensitization, acute systemic toxicity, subchronic toxicity, chronic toxicity, carcinogenicity, mutagenicity, implantation and haemocompatibility.

Parts 2 to 18 of ISO 10993 contain guidelines and test methods to conduct various recommended tests. In some cases, parts 3 to 18 are specific and prescriptive, whereas others provide general directions and crossreference more standards and published test methods.

Most of the participating countries in the ISO harmonized the requirements or modified the standards to mesh with the regulatory infrastructure and their national standards. The EU retained many of the standards as harmonized, since these standards meet the EU Medical Devices Directive 93/42/EEC (EU, 1993), which specify requirements for safety assessment. Japan adopted ISO 10993-1 as published, but made some changes in the conduct of the individual biological tests (MHLW, 2003).

7 THE FDA BIOCOMPATIBILITY REQUIREMENTS AND USE OF STANDARDS

Section 204 of the FDAMA of 1997, allows the FDA to recognize consensus standards established by national and international standards-development organizations. There are several national and international

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Table 2 Principles for the evaluation of medical devices

1. The selection and evaluation of any material or device intended for humans requires a structured biological evaluation programme. The biological evaluation shall be planned, carried out and documented by knowledgeable and experienced individuals capable of making informed decisions.
2. The evaluation programme shall include documented informed decisions that assess the advantages/disadvantages and relevance of: (i) the physical and chemical characteristics of various candidate materials; (ii) any existing toxicology and other biological safety data on product and component materials, breakdown products and metabolites; (iii) any history of clinical use or human exposure data and (iv) test procedures.
3. In the selection of materials to be used in device manufacture, the first consideration should be fitness for the purpose with regard to characteristics and properties of the material, which include chemical, toxicological, physical, electrical, morphological and mechanical properties.
4. The following should be considered for their relevance to the overall biological evaluation of the device: (i) the material(s) of manufacture; (ii) intended additives, process contaminants and residues; (iii) leachable substances; (iv) degradation products; (v) other components and their interactions in the final product; (vi) the performance and characteristics of the final product and (vii) physical characteristics of the final product, including but not limiting to, poracity, particle size, shape and surface morphology.
5. The choice of tests and the data required in a biological evaluation, and their interpretation, should take into account the chemical composition of the materials, including the conditions of exposure as well as the nature, degree, frequency and duration of exposure of the medical device or its constituents to the body, enabling the characterization of devices to facilitate the selection of appropriate tests. The rigour necessary in the biological evaluation is principally determined by the nature, degree duration and frequency of the exposure and the toxicological hazards identified for the material.
6. All potential biological hazards should be considered for every material and final product, but this does not imply that testing for all potential hazards will be necessary or practical. The range of possible biological hazards is wide and can include short-term effects such as acute toxicity, irritation to the skin, eye and mucosal surfaces, haemolysis and thrombogenicity, as well as long-term or specific toxic effects such as subchronic and chronic toxic effects, sensitization, genotoxicity, carcinogenicity and effects on reproduction including teratogenicity.
7. Selection of any *in vivo* and *in vivo* tests should be based on end-use applications. Appropriate good laboratory practices should be applied in the conduction of tests and competent informed persons should evaluate the test data. Whenever possible, *in vitro* screening should be carried out before *in vivo* tests. Test data, complete to the extent that an independent analysis could be made, shall be retained.
8. The materials or final product should be considered for biological re-evaluation if any of the following occurs: (i) any change in the source or in the specification of the materials used in the manufacture of the product; (ii) any change in the formulation, processing, primary packaging, or sterilization of the product; (iii) any change in the final product during storage; (iv) any changes in the intended use of the product and (v) any evidence that the product may produce adverse effects when used in humans.
9. The biological evaluation should take into account the nature and mobility of the chemical constituents in the materials used to manufacture the device and other information, other non-clinical test, clinical tests and post-market experience for an overall assessment.

consensus standards that address the biological evaluation of medical devices to evaluate their toxicity. Such standards are typically developed by consensus, with the participation of industry, healthcare professionals and academic and government scientists.

The CDRH recognizes those standards that help provide a reasonable assurance of the safety and effectiveness of medical devices and do not conflict with any legislation or regulation under which the Center operates. Therefore, information submitted regarding conformance with recognized standards would have a direct bearing on the evaluation of biological safety made during the review of IDEs, HDEs, PMAs and PDPs. In the case of 510(k)s, information on conformance with CDRH-recognized consensus standards may help establish substantial equivalence of a new device with a legally marketed device.

If a manufacturer elects to conform to a recognized consensus standard to satisfy premarket review requirements, the manufacturer must submit either a declaration of conformity to the standard or state how conformance to that standard will be achieved. When acceptance criteria are clearly specified by a standard, the FDA's need to review actual data is eliminated. However, the manufacturer must maintain all records relating to the conformity to the standard for a period of two years after approval, or for the expected lifetime of the device, whichever is longer.

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Table 3 Initial biocompatibility evaluation tests for consideration per ISO part 1 guidance and FDA G95-1

Medical device categorization by			Biological effect							
Nature of body contact		Contact duration A-limited (<24 h) B-prolonged (>24 h −30 d) C-permanent (>30 d)	Cytotoxicity	Sensitization	Irritation or Intracutaneous reactivity	Systemic toxicity (acute)	Subchronic toxicity (subacute toxicity)	Genotoxicity	Implantation	Haemocompatibility
Category	Contact									
Surface device	Skin	A	X	X	X	—	—	—	—	—
		B	X	X	X	—	—	—	—	—
		C	X	X	X	—	—	—	—	—
	Mucosal membrane	A	X	X	X	—	—	—	—	—
		B	X	X	X	O	O	—	O	—
		C	X	X	X	O	X	X	O	—
	Breached or compromised surface	A	X	X	X	O	—	—	—	—
		B	X	X	X	O	O	—	O	—
		C	X	X	X	O	X	X	O	—
External communicating device	Blood path, indirect	A	X	X	X	X	X	—	—	X
		B	X	X	X	X	—	—	—	X
		C	X	X	O	X	X	X	O	X
	Tissue/bone/dentin communicating[a]	A	X	X	X	O	—	—	—	—
		B	X	X	X	X	X	X	X	—
		C	X	X	X	X	X	X	X	—
	Circulating blood	A	X	X	X	X	—	O	—	X
		B	X	X	X	X	X	X	X	X
		C	X	X	X	X	X	X	X	X
Implant device	Tissue/bone	A	X	X	X	O	—	—	—	—
		B	X	X	X	X	X	X	X	—
		C	X	X	X	X	X	X	X	—
	Blood	A	X	X	X	X	X	—	X	X
		B	X	X	X	X	X	X	X	X
		C	X	X	X	X	X	X	X	X

This table is a framework for the development of an assessment programme and is not a checklist.
X indicates data end points that can be necessary for a biological safety evaluation, based on a risk analysis, where existing data are adequate, additional testing is not required (ISO 10993-1).
O additional tests, may be applicable in U. S. (FDA Bluebook Memorandum #G95-1)
[a]Tissue includes tissue fluids and subcutaneous spaces.
Reproduced from the FDA, 1995.

Conformance with recognized consensus standards in itself, however, may not always represent a sufficient basis for regulatory decisions. A specific device may raise additional safety or effectiveness issues not addressed by a recognized consensus standard, or FDA regulations may require additional information beyond what the consensus standard provides. Under such circumstances, conformity with a recognized standard will not satisfy all requirements for investigating or marketing the product in the United States.

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Table 4 Supplementary biocompatibility evaluation tests for consideration per ISO part 1 guidance and FDA G95-1 guidance

Medical device categorization by			Biological effect					
Nature of body contact Category	Contact	Contact duration A-limited (<24 h) B-prolonged (>24 h –30 d) C-permanent (>30 d)	Chronic toxicity	carcinogenicity	Reproductive and developmental	Biodegradation	Toxicokinetics	Immunotoxicology
Surface device	Skin	A	—	—	—	—	—	—
		B	—	—	—	—	—	—
		C	—	—	—	—	—	—
	Mucosal membrane	A	—	—	—	—	—	—
		B	—	—	—	—	—	—
		C	O	—	—	—	—	—
	Breached or compromised surface	A	—	—	—	—	—	—
		B	—	—	—	—	—	—
		C	O	—	—	—	—	—
External communicating device	Blood path, indirect	A	—	—	—	—	—	—
		B	—	—	—	—	—	—
		C	X	X	—	—	—	—
	Tissue/bone/dentin communicating[a]	A	—	—	—	—	—	—
		B	—	—	—	—	—	—
		C	X	X	—	—	—	—
	Circulating blood	A	—	—	—	—	—	—
		B	—	—	—	—	—	—
		C	X	X	—	—	—	—
Implant device	Tissue/bone	A	—	—	—	—	—	—
		B	—	—	—	—	—	—
		C	X	X	—	—	—	—
	Blood	A	—	—	—	—	—	—
		B	—	—	—	—	—	—
		C	X	X	—	—	—	

This table is a framework for the development of an assessment programme and is not a checklist.
X indicates data points that can be necessary for biological safety evaluation, based on a risk analysis, where existing data are adequate, additional testing is not required (ISO, 2003).
O additional tests, which may be applicable in the USA FDA, 1995.
[a]Tissue includes tissue fluids and subcutaneous spaces.
Reproduced from FDA, 1995.

For example, as noted above, even though the FDA have recognized Part 1 of ISO 10993-1, dealing with biological test selection, the standard does not address several tests required to evaluate devices contacting nervous-system tissue and devices that may affect the immune system. Therefore, many of these devices, if tested according to ISO 10993-1 alone, could raise additional safety issues not addressed by the standard.

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Figure 1 Decision flowchart for reviewers performing biocompatibility evaluations for the 510(k) process. (Reproduced from FDA, 1995.)

The modifications documented in the FDA's Blue Book Memorandum (FDA, 1995), address these additional safety concerns.

The Blue Book Memorandum contains an FDA-modified matrix (**Tables 3** and **4**) designating additional tests that may be needed for various device categories—and recommending specialized testing for neurotoxicity and immunotoxicity of certain devices. It also includes a decision flowchart for the selection of toxicity tests for 510(k)s (**Figure 1**).

The flowchart was designed to help FDA reviewers to determine whether the biocompatibility information provided in a 510(k) submission for a device under review is sufficient. The flowchart helps reviewers in the determination of substantial equivalence by directing them to specific review criteria about device materials, their chemical composition, manufacturing processes and sterilization methods. The chart also advises reviewers to consult device-specific guidances and, if necessary, to seek assistance from senior toxicologists in the

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Center, as well as to consider information from other sources, such as master files and published literature. In addition, reviewers are advised to consider any scientific or risk-analysis justifications for not conducting some of the suggested tests. Finally, if the data and analyses provided do not resolve all the questions concerning the biocompatibility of a subject device, the chart advises reviewers that additional toxicology testing is required and that such testing should be conducted according to the FDA-modified test matrix (FDA, 1995).

The FDA has also recognized other parts of ISO 10993, several of which contain protocols for carrying out the tests suggested in Part 1. In addition, the FDA has recognized several standards developed by the United States Pharmacopeia (USP) and the American Society for Testing and Materials (2008) that present specific protocols for conducting various biological tests. A list of these recognized standards and the dates they were recognized, together with information detailing the extent of their recognition by the FDA, can be obtained from the CDRH web site (*http://www.fda.gov/cdrh/stdsprog.html*). Also available on the site is supplemental information on the data requirements or modifications for particular standards that may be necessary to support the regulatory requirements of premarket submissions. Often, recognized standards entail test protocols, which the Center may recognize; however, if the standard does not include any acceptance or rejection criteria for the assessment of test results, it is necessary for the manufacturer to submit the actual data for assessment by the Center's review staff (FDA, 1995).

8 TOXICOLOGY TESTING OF MEDICAL DEVICES

This section will address the toxicology testing methods commonly used to evaluate biocompatibility of medical devices and their materials. For detailed methods and protocols for the tests discussed, the reader should consult the numerous published references.

Medical devices and their component materials are potential sources of toxins that may produce undesirable local and systemic effects when used clinically. Therefore, evaluation of any new device intended for human use requires systematic testing to ensure that the benefits provided by the final product will exceed any potential risks produced by device materials.

Medical devices are primarily made from high-molecular-weight polymers, metals and ceramics held together by screws, clips, adhesives and heat seals. They, in general, tend to be solids and do not achieve their intended activity by chemical action within or on the body. Most components of medical devices contain mixtures of polymers, and additives, such as catalysts, antioxidants, colourants, plasticizers, stabilizing agents and lubricants. In addition they also contain residual products of sterilization.

The toxicological testing of device materials has unique constraints, which limit the applicability of classical protocols and procedures. Classical protocols are designed to obtain quantitative dose–response data on pure chemicals and drugs. In the case of synthetic device materials, complete chemical composition is often not available. In addition, synergistic reactions among the components can cause a toxic reaction, even though the individual additives are considered nontoxic. In general, the toxicity testing of device/materials is directed towards the assessment of the potential toxicity of the device/material under specific conditions, particularly under end-use conditions. The relative significance of acute, subacute, subchronic and chronic toxicological responses to the health of patients has been established for drugs and chemical agents, and it is in that context that empirical toxicological data generated on the devices and their materials should be assessed.

Logically, the toxicity testing should take place at two points in the development of medical devices. First, as soon as the material is identified as a candidate material and second, as soon as end-use configuration of the material is determined and the final device is available for testing. As stated earlier, end-use testing of a device will carry certain constraints unique to the final configuration and application of the device. Sterilization may degrade the device material or react with nontoxic additives in the device to increase the toxic potential of the device; therefore all toxicity testing should be done on the final sterilized device.

The process of obtaining the toxicity profile of a medical device may require various modifications to standard or classic toxicity testing procedures, as well as utilizing new and innovative approaches. In practice the toxicity profiles of medical devices is obtained by conducting a battery of toxicity tests which include acute, subchronic and chronic toxicity; irritation to skin, eyes and mucosal surfaces; sensitization; haemolysis; thrombogenicity; genotoxicity; carcinogenicity; and effects on reproduction, including developmental effects. However, depending on varying characteristics and intended uses of devices, as well as the nature of contact, these general tests may not be sufficient to demonstrate the safety of some specialized devices. Additional tests for specific target-organ toxicity, such as neurotoxicity and immunotoxicity may be necessary for some devices. Also, for some implanted devices, a material-mediated pyrogenicity test in rabbits is suggested. The specific clinical application of the new device determines which tests are more appropriate (FDA, 1995).

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9 TEST ARTICLE AND SAMPLE PREPARATION

Before selecting the test methods it is important to decide what the test article really is, since medical devices are very complex, with many components, primarily made from high-molecular-weight polymers, metals and/or ceramics held together by screws, clips, adhesives and heat seals. The ideal test sample is the intact medical device that has been processed and sterilized in the same manner as the medical device that will be used in humans. It is however, not always practical to use the intact medical device because of the design constraints inherent in the biological test methods. In cases where a medical device comprises several components made from different materials, the ideal and practical procedure from a toxicological point of view would be to test extracts of the components separately. In some cases, however, an evaluation under normal-use conditions is mimicked by using the device or a piece of the device directly. When extracts are tested, the extraction media should constitute both polar and nonpolar solvents to ensure the extraction of components of widely different solubility properties. The most commonly used extraction media are physiological saline, vegetable oil, dimethylsulfoxide and ethanol. Other extraction media such as polyethylene glycol (PEG) or aqueous dilutions of ethanol may be selected in certain cases. For *in vitro* cytotoxicity testing, complete cell-culture medium is most often employed.

The extraction temperatures vary depending on the physicochemical properties of the device materials. Some leachable compounds may be chemically altered at high temperatures and it is now generally recommended that extraction be conducted at 37 °C, simulating body temperature, for 72 hours. However, depending on the nature of the device materials, higher temperatures (50 °C for 72 hours, 70 °C for 24 hours and 121 °C for one hour) have been used to exaggerate extraction process. For *in vitro* cytotoxicity tests, extraction at 37 °C for 24 hours is usually recommended, since certain constituents of the media are relatively labile.

The amount of leachable substances released in to the extraction media is also related to the surface area and thickness of the product to be extracted. The ratio of surface area to the volume of the extracting fluid varies depending on the size and shape of the product. When surface area cannot be determined due to the configuration of the specimen, it is recommended to use of 0.1 g of elastomer or 0.2 g of plastic or other polymer for every 1 ml of extracting fluid. Generally moulded elastomeric closures are tested intact. For a better understanding of extraction principles and methods, refer to the US Pharmacopeia (2008a) and ISO 10993 Part 12 Sample preparation and reference materials (ISO, 2006e).

10 TOXICITY TESTS FOR BIOLOGICAL EVALUATION

10.1 Cytotoxicity

The aim of *in vitro* cytotoxicity tests is to detect the potential ability of a device or its materials to induce sublethal or lethal effects, such as lysis of cells (cell death), the inhibition of cell growth, colony formation and other effects, as observed at the cellular level. Three types of cell-culture assays that are commonly used to screen medical devices and materials; the elution test, the direct-contact test and the agar diffusion test. These methods are known to be sensitive, economical and quick to conduct. Several types of cell cultures are available for testing; two cell strains, L-929 and Wl-38, are most commonly used. The ISO 10993 standard, Biological Evaluation of Medical Devices—Part 5: Tests for *in vitro* cytotoxicity (ISO, 1999a), gives a detailed description of all three methods. In the elution test, an extract (elute) of the material is prepared and added in varying concentrations to the cell cultures. Growth inhibition is a widely used parameter, but others may also be used. In the direct-contact test, pieces of test material are placed directly on top of the cell layer, which is covered only by a layer of liquid cell-culture medium. Toxic substances leaching from the test material may depress the growth rate of the cells or damage them in various ways. In the agar diffusion test, a piece of test material is placed on an agar layer covering a confluent monolayer of cells. Toxic substances leaching from the material diffuse through the thin agar layer and kill or disrupt adjacent cells in the monolayer. The selection of an appropriate test method is based on the physical and chemical properties of the test material and the potential clinical use of the device.

There is usually a good qualitative correlation between results from cell-culture tests and studies performed *in vivo* with respect to cytotoxicity vs. primary tissue effects. It is important to recognize, however, that although cell-culture toxicity is, in general, a good and sensitive indicator of primary tissue compatibility, exceptions may arise in cases where leaching substances cause tissue damage *in vivo* through more complex mechanisms. At present, the *in vitro* cytotoxicity assays are used as screening tests and considered primarily as supplements to the various *in vivo* tests.

10.2 Irritation

The ISO 10993 standard, Biological Evaluation of Medical Devices-Part 10: Tests for irritation and delayed-type hypersensitivity (ISO, 2002) describes irritation tests for both single and repeat-use exposure to a device. Based on the route of exposure irritation,

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tests are subclassified into, skin, intracutaneous, eye and mucus membrane irritation tests. The preferred animal species for irritation tests is the albino rabbit, whose highly sensitive, light skin makes it possible to detect even very slight skin irritation caused by a substance. Skin-irritation tests of medical devices are performed with two extracts obtained with polar and nonpolar solvents or with the device material itself. In the single-exposure test, rabbits are treated for several hours, whereas for the cumulative or repeat-use test, the same procedure is repeated for several days. All extracts and extractants are applied to intact skin sites. Skin reaction is seen as redness or swelling and is graded according to a specified classification system.

In the primary skin irritation study, patches of device materials are applied to fur-free skin of six rabbits. However, for most topically used devices, three rabbits consistently showing little or no response is adequate proof that dermal irritation is not a potential hazard. If the results are equivocal, three additional rabbits can be added, for analysis of a large sample (Derelanko *et al*., 1993). For medical-device materials, the occluded patches should stay for 24 hours. The treated skin should be observed for erythema and oedema at 1, 24, 48 and 72 hours after the removal of the patches. A primary skin-irritation index is calculated by adding all scores and dividing by the number of dosed sites.

The intracutaneous injection test, described in the US Pharmacopeia (USP, 2008b) is widely used. This test, conducted in rabbits, simulates the irritation which can occur in humans upon exposure of breached skin to a foreign irritating device or when a device is introduced subcutaneously. The test measures signs of inflammation as erythema, oedema, ulceration and necrosis. Device extracts are injected into the skin and the degree of irritation is measured at 24, 48 and 72 hours. Skin reactions are scored and evaluated, based on the severity of erythema and oedema.

Mucus membrane irritation assays are used to evaluate dental and personal hygiene devices (Chvapil *et al*., 1979; Eckstein *et al*., 1969). Eye irritation assays are used in evaluating contact lenses, and other devices that contact the ocular tissue (FDA, 1988). ISO 10993-10 (ISO, 2002) provides detailed guidance for mucusmembrane irritation assays.

10.3 Sensitization

Sensitization assays are described in ISO 10993 standard, Biological Evaluation of Medical Devices—Part 10: Tests for irritation and delayed-type hypersensitivity (ISO, 2002). The sensitization reaction is also known as allergic contact dermatitis, which is an immunologically mediated cutaneous reaction. This is in contrast to irritant contact dermatitis (skin irritation)—a skin reaction caused by the primary and direct effect of a substance on the skin. In animals, the sensitization reactions manifest themselves as redness (erythema) and swelling (oedema).

The preferred animal species for sensitization testing is the albino guinea pig. All available guinea-pig methods have certain features in common: an induction (sensitization) phase, when the potential allergen is presented to the organism, followed by a rest period, and a subsequent challenge phase to determine whether or not sensitization has occurred. One of the most recognized and validated assays is the guinea-pig maximization test (GPMT). The GPMT (Magnusson and Kligman, 1969) is widely used for assessing the sensitizing potential of medical devices/materials. In the maximization test, both polar (saline) and nonpolar (vegetable oil) extractions are prepared from devices or component parts of the device according to methods described in ISO 10993-12 (ISO, 2006e) and USP 31 (US Pharmacopeia, 2008b). During the induction phase of the test, the animals are treated with intradermal injections and topical application of the extract alone, the adjuvant alone, and the extract and adjuvant mixture. A minimum of 10 animals are used with each extract and a minimum of five animals are maintained as untreated controls. A week later, the same site is pretreated with a surfactant, sodium lauryl sulfate to eliminate any skin barrier to a topical patch of the material extract to be applied at the same site on the following day. Two weeks after the second induction, the test animals, as well as animals in the control group for each extractant, are challenged with a topical application of the appropriate extract to the flank and covered with an occlusive patch for 24 hours. Skin reactions at the test site are rated for erythema and oedema at 24, 48 and 72 hours after application of the extract. A positive reaction at the test site not observed at the control site could be indicative of sensitization reaction.

The second commonly used method is the repeated-patch test published by Buehler (1965). In this test the material to be evaluated is cut into 2.5×2.5 cm sections for each patching. Liquids, gels and pastes are applied as a 0.5 ml or 0.5 g dose. A group of 10 animals is patched either three times in one week or once a week for three weeks. Each treatment consists of six hours occluded patching. After a week recovery period, all test and control animals are challenged by topical patching. Any response, such as redness and swelling seen in the test animals, but not seen in control animals is indicative of sensitization reaction.

Predictive tests in guinea pigs are important tools in identifying the possible hazards to a population repeatedly exposed to a substance. Nevertheless, results from sensitization tests in guinea pigs have to be evaluated carefully. A positive test result in this assay may rate a substance as a stronger sensitizer than it appears to be during actual use. On the other hand, a negative result in such a sensitive assay ensures a considerable safety margin regarding the potential risk to humans.

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Recently, an alternative test method, the mouse local lymph node assay (LLNA), was developed to replace the use of guinea pig sensitization test to identify potential sensitizing materials (Kimber *et al*., 2002). The basic principle underlying the LLNA is that sensitizers induce proliferation of lymphocytes in the lymph node draining the site of chemical application, and this proliferation is proportional to the dose applied, providing a means of obtaining an objective, quantitative measurement of sensitization.

This method has been validated for chemicals, both in the USA and Europe by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and European Committee on the Validation of Alternative Methods (ECVAM) respectively. However, it has not been validated for chemical mixtures and metals; therefore, at present its utility in the evaluation of medical devices/materials is limited.

10.4 Acute Systemic Toxicity

Acute systemic toxicity is an adverse effect occurring within a short time (less than 24 hours) after administration of a single dose of a substance. Systemic toxicity assays for medical devices are listed in ISO 10993 standard, Biological Evaluation of Medical Devices—Part 11: Tests for Systemic Toxicity (ISO, 2006d). The standard covers tests for single (acute) and repeated-dose toxicity and contains a list of recommendations for other standard tests for oral, dermal, intravenous, intraperitoneal and inhalational routes of administration. Single-dose (acute) systemic toxicity tests are designed to determine if the leachable materials induce mortality, body-weight changes or clinical signs of toxicity. The acute-toxicity test in mice, which is described in the US Pharmacopeia 31 (2008c), is commonly used to evaluate medical devices. In this test, the test material is extracted in 0.9% saline, 5% ethanol in saline, vegetable oil and PEG 400. The first two extracts are injected intravenously and vegetable oil and PEG 400 extracts are injected intraperitoneally into mice. Animals are observed for a minimum of three days. Adverse reactions generally appear within the first 24 hours after dosing. Clinical signs such as lethargy, hyperactivity, convulsions, body-weight loss and death are some of the parameters monitored and compared to a vehicle control group of mice.

Determining acute systemic toxicity is usually an initial step in the assessment and evaluation of the toxic characteristics of a substance. By providing information on health hazards likely to arise from short-term exposure, the acute systemic toxicity test can serve as a first step in the establishment of a dosage regimen in subchronic and other studies, and can also supply initial data on the mode of toxic action of a substance.

10.5 Genotoxicity

Genotoxicity assays are described in ISO 10993 standard, Biological Evaluation of Medical Devices—Part 3: Tests for Genotoxicity, carcinogenicity and reproductive toxicity (ISO, 2006a). Genetic toxicology tests are used to investigate device materials for possible mutagenic effects—that is, damage to the body's genes or chromosomes. Mutagenesis can occur as a result of interaction between mutagenic agents and the genetic material of the organism. Mutations can be classified into two general types: gene mutations and chromosomal mutations. Gene mutations are changes in nucleotide sequences at one or several coding segments within a gene; chromosomal mutations are morphological alterations or aberrations in the gross structure of the chromosomes. The tests that are used to evaluate the genetic toxicity are diverse and include *in vitro* tests using micro-organisms and cells from multicellular animals, as well as *in vivo* tests using insects, plants and mammals. Both *in vitro* and *in vivo* tests can be further grouped, on the basis of the end point detected, into three major groups: (i) forward and reverse mutations (e.g. point mutation, deletion mutation, etc.), (ii) clastogenicity assays detecting structural and numerical changes in chromosomes (e.g. chromosome aberrations, micronuclei, etc.) and (iii) the assays that identify DNA damage (e.g. DNA strand breaks, unscheduled DNA synthesis (UDS), etc.). For medical devices, the ISO standard recommends *in vitro* testing in one nonmammalian system (bacterial) and two mammalian cell systems, for effects on DNA, gene mutations and chromosomal aberations. Assays may be performed with extracts of the device materials. Physiological saline and dimethyl sulfoxide are recommended as extracting media. The extracts are prepared at 37 °C for a minimum of 24 hours using the highest possible surface area (ISO, 2006a). The ISO 10993-3 standard (ISO, 2006a) recommends the test methods published by the Organization for Economic Cooperation and Development (OECD) for chemicals.

The standard recommends the Ames assay, the most widely used genotoxicity test, which uses *Salmonella typhymurium* strains for screening potential carcinogens (OECD, 1981). This is one of the established tests in which large number of chemicals representing a variety of chemical classes have been tested. It is a very sensitive assay to determine the potential of a test material to induce the genetic mutation of histidine reversion using five strains of *Salmonella typhymurium* (TA 98, TA 100, TA 1535, TA 1537 and TA 102) that are sensitive to DNA-damaging agents in the presence or absence of the S-9 mammalian microsomal enzyme.

The standard also recommends several *in vitro* mammalian cell systems to detect gene and chromosome mutations. It specifically recommends the mouse lymphoma assay (OECD, 1997c), incorporating colony

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number and size determination in order to cover both end points (clastogenicity and gene mutations). It also recommends an *in vitro* mammalian cytogenetic test for clastogenicity. If any of the *in vitro* tests are positive, the standard recommends an *in vivo* test on the basis of most appropriate end point identified in the *in vitro* tests. The *in vivo* tests have advantage of taking into account absorption, distribution and excretion, processes that are not functional *in vitro* systems. The most commonly used *in vivo* tests are the micronucleous test in rodents (OECD, 1997a), the metaphase analysis in rodent bone marrow (OECD, 1997b) and the UDS test with mammalian liver cells *in vivo* (OECD, 1997d).

10.6 Implantation

Implantation tests are designed to assess acute and long-term *in vivo* tissue reaction to device materials. It is considered one of the most pertinent and direct tests to evaluate device materials and their leachable components. When a device material is implanted into a muscle or subcutaneous tissue, leachable components from the implant diffuse into surrounding tissue and the severity of the tissue response depends on the toxic nature of the leachables. Tissue samples taken from the implanted site are evaluated by macroscopic and microscopic examination for the nature and degree of inflammatory responses around the implant site. These responses are compared with the tissue response caused by an established standard reference material. Most of the implantation methods used are based on the original US Pharmacopeia rabbit muscle method (US Pharmacopeia, 2008f) which lasted only three to five days, the implant tests used to study the effects of various device materials may be conducted for any length of time up to the lifespan of the animal. The biological parameters assessed include: the presence of inflammatory cells, such as polymorphonuclear leukocytes, lymphocytes, plasma cells, macrophages and giant cells. In addition, tissue changes such as necrosis, neovascularization, fibrosis and fatty infiltration are evaluated.

In cases where an implanted material degrades *in vivo*, a delayed response may occur; for this reason subchronic and chronic implantation tests are carried to study the effects of potential degradation products of the material.

ISO 10993 standard, Biological Evaluation of Medical Devices—Part 6: Tests for local effects after implantation (ISO, 2006c), contains a description of general guidelines and more detailed descriptions of methods for implantation in subcutaneous tissue, muscle and bone. The standard gives guidance on the implantation of solid degradable and nondegradable materials, as well as nonsolid materials such as liquids, pastes and particulates.

10.7 Haemocompatibility

The purpose of haemocompatibility testing is to look for possible undesirable changes in the blood caused directly by a medical device or chemicals leaching from a device. Undesirable effects of device materials on the blood may include haemolysis, thrombus formation, alterations in coagulation parameters and immunological changes. ISO 10993 standard, Biological Evaluation of Medical Devices—Part 4: Selection of tests for interaction with blood (ISO, 2006b) gives guidance for evaluating interaction of medical devices with blood. The standard lists recommended test methods to evaluate blood–device interactions for devices externally communicating with the blood path, for devices communicating with circulating blood and for implanted devices in the cardiovascular system. Haemocompatibility test methods are categorized by the primary process or system being measured, as thrombosis, coagulation, plates and platelet functions, haematology and immunology.

10.8 Subchronic Toxicity

Subchronic toxicity is the potentially adverse effect that can occur as a result of the repeated daily dosing of a substance to experimental animals over a period of time, up to 10% of their lifespan. In the assessment and evaluation of the toxic characteristics of a chemical, the determination of subchronic toxicity is carried out after initial information on toxicity has been obtained by acute testing, and provides data on possible health hazards likely to arise from repeated exposures over a limited time. Such testing can furnish information on target organs and the possibilities of toxin accumulation, and provide an estimate of a no-effect exposure level that can be used to select dose levels for chronic studies and establish safety criteria for human exposure. The route of exposure and the length of exposure depends on the animal model to be used. No standard protocols exist for subchronic evaluation of medical devices. The ISO standard 10993 Part 11: Tests for systemic toxicity (ISO, 2006d), references several classical toxicology methods that have been established for the safety evaluation of drugs and chemicals. Modifications to the established methods are necessary to make them applicable to devices.

In subchronic toxicity studies using established methods for drugs and chemicals, one or two animal species are dosed daily, usually for a period of three to six months; the rat is the standard animal species

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of choice. The animals are given the test substance in increasing doses. The dose level of the low-dose group should be at the level of human exposure. When extracts of medical devices are employed, one dose level (the highest practically applicable volume) is often sufficient, since strong toxicity is generally not expected. Therefore, in general, subchronic studies are conducted with one test and one control dose level.

One key element to keep in mind in the design of subchronic test protocols for medical devices is that the treatment or implant should mimic the end use. In repeated exposure (extracts) or constant exposure (implants) studies, evaluation of both local and systemic adverse effects should be part of the study design. The implant studies that have both test and control materials implanted in the same animal to study the local effects of the device are not acceptable as true systemic subchronic studies.

10.9 Chronic Toxicity

ISO 10993, Part 11: Tests for systemic toxicity (ISO, 2006d), gives guidance to conduct chronic systemic toxicity of medical devices, but it provides no test protocols or references to conduct chronic toxicity studies. Chronic toxicity is defined as effects of either single or multiple exposures to medical devices, materials and/or their extracts during a major period of the lifespan of the test animal (greater than 10% of animal lifespan). For implanted devices the standard suggests that the chronic toxicity test protocol may be expanded to include an implantation test protocol to evaluate both chronic systemic and local effects. Chronic implanted studies are required for all devices in contact with the body for 30 days or longer, regardless of exposure site (**Table 4**), and every attempt should be made to ensure that test conditions approximate the end-use conditions.

10.10 Carcinogenicity

The objective of carcinogenicity studies is to observe test animals over a major portion of their lifespan to detect any development of neoplastic lesions (tumour induction) during or after exposure to various doses of a test substance.

ISO 10993 Standard, Biological Evaluation of Medical Devices, Part 3: Tests for genotoxicity, carcinogenicity and reproductive toxicity (ISO, 2006a), gives guidance on carcinogenicity assays for medical devices. There are very few medical devices for which this test can be justified.

The ISO standard suggests three situations where a carcinogenicity assay should be considered:

1. Reabsorbable materials and devices, unless there are significant and adequate data on human use and exposure
2. Materials and devices where positive results have been obtained in genetic toxicity testing on mammalian cells
3. Materials and devices introduced into the body and/or its cavities with a permanent or cumulative contact for 30 days or longer, except when significant and adequate human-use history is available.

Carcinogenicity testing in drugs and chemicals is normally conducted with oral or inhalation dosing. For implants and medical devices, some modifications to the standard procedures may be necessary. These modifications are primarily in the test sample preparation and dosing.

Carcinogenicity studies are usually performed in rodents, starting at six to eight weeks of age and continued for the lifetime of the species, which is approximately two years. OECD Guidance 451 (OECD, 1981) provides general guidance for testing of chemicals, which can be applied for extracts of medical devices. In these studies, mice and rats are dosed with device extracts every day for 18–24 months. At the completion of the dosing period, all surviving animals are sacrificed and their organs and tissues examined microscopically for the presence of tumours. An increased incidence of one or more category of tumours in the dosed group would indicate that the product tested has the potential to induce tumours and could be considered a possible carcinogen in humans.

The American Society for Testing and Materials (ASTM) Standard F1439-03 gives specific protocols for the performance of lifetime bioassays for the tumorigenic potential of implant materials (ASTM, 2008). These lifetime implant studies are generally conducted in rodents by subcutaneous implantation of device materials. However, these studies have raised some concern, because the data is not predictive of human experience with the same materials. In rodents, solid implanted materials (synthetic polymers, glass and metal films), independent of their chemical composition, induced tumours (primarily sarcomas). This phenomenon, which often manifests itself in rodents, is known as solid-state or foreign-body carcinogenesis, and is due to the size, shape and surface characteristics of the implant and duration of implantation. This phenomenon does not occur in guinea pigs and chickens. Because of the issues associated with carcinogenicity studies in rodents, the FDA advises manufacturers to initiate discussions with the appropriate review division in the Office of Device Evaluation, CDRH, prior to the initiation of expensive, long-term testing of any new device materials, to ensure the proper testing with the appropriate animal species (FDA, 1995).

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11 REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

ISO 10993 Standard Biological Evaluation of Medical Devices, Part 3: Tests for genotoxicity, carcinogenicity and reproductive toxicity, (ISO, 2006a) gives guidance for conducting reproductive and developmental toxicity tests. The standard recommends these tests for: (i) long-term contact devices contacting reproductive tissue or the ebmryo/foetus (intrautrine and contraceptive devices); (ii) resorbable materials and devices that are used in women of child bearing age.

None of the traditional methods for the study of reproductive effects have been widely used with devices. The available protocols for chemicals and drugs to study reproductive and teratological studies can be used for device extracts, but should be modified for implants based on the nature of device materials and intended use. The standard recommends OECD guidelines 414 (OECD, 2001) and 415 (OECD, 1983) to conduct teratogenicity and one-generation reproductive toxicity studies. Since these guidelines are not intended for medical devices, the standard recommends appropriate modification to the protocols based on the nature of the device materials and intended use.

11.1 Biodegradation

Biodegradation is defined as an alteration of the medical device material involving the release of degradation products, as well as loss of integrity or performance of the device in a physiological environment. Biodegradation assays are described in ISO 10993 standard, Biological Evaluation of Medical Devices—Part 9: Degradation of materials related to biological testing (ISO, 1999b). The test methods for degradation studies are given in parts 13, 14 and 15 of ISO 10993 Standards, for polymers (ISO, 1998), ceramics (ISO, 2001) and metals (ISO, 2000) respectively. Part 9 of the standard (ISO, 1999b) describes methods for studying *in vitro* degradation and techniques for identification and quantification of *in vivo* degradation products from implanted medical devices.

11.2 Toxicokinetics

Toxicokinetic studies evaluate the kinetics of absorption, distribution, biotransformation and excretion of toxic chemicals in the whole organism. In the medical device evaluation it is applied to leachable and extractable chemicals, as well as biodegradation products from device materials. The leachable and extractable components include additive chemicals, monomers and low-molecular-weight components of polymeric materials. In addition they also include residual chemicals from sterilization processes (e.g. ethylene oxide). The ISO 10993 Standard Part 16: Toxicokinetic study design for degradation products and leachables (ISO, 1997) gives guidance on evaluating the biological disposition of extractable chemicals *in vitro* and biodegradation *in vivo*.

11.3 Pyrogenicity

Pyrogenic reactions are generally associated with bacterial endotoxins that may remain on the devices after sterilization. However, certain materials are able to cause febrile reactions in humans and animals, similar to endotoxins from Gram-negative bacteria. *In vitro* and *in vivo* tests have been developed to determine the presence of endotoxins. The bacterial endotoxins test, also known as the Limulus amoebocyte lysate (LAL) test, measures the concentration of endotoxins using either a turbidimetric or calorimetric end point (US Pharmacopeia, 2008d). The LAL test would not detect certain device materials that are pyrogenic. In such cases, the rabbit pyrogenicity test is used. The rabbit pyrogen test involves measuring the rise in temperature of rabbits over a three hour interval following the intravenous injection of the extract from of the device material (US Pharmacopeia, 2008e). The sample passes the test if no rabbit shows an individual rise in temperature of 0.5 °C or more above its respective control temperature. ISO 10993 Part 1 (ISO, 2003), does not require this test, however, the FDA requires rabbit pyrogenicity tests for all implanted devices to evaluate the material-mediated pyrogenicity (FDA, 1987).

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Phototoxicology

J. Frank Nash

CONTENTS

1 INTRODUCTION

Exposure to sunlight is essential to human life. The beneficial/healing properties of sunlight are documented throughout the history of mankind (Zanolli, 2003). Examples of physiological and psychological benefits of sunlight, alone and in combination with exogenous test materials, are diverse, ranging from the production of pre-vitamin D and psychological benefits in patients with seasonal affective disorder to treatment of psoriasis, that is, PUVA therapy. However, as is the case with any test material/pharmacological agent, too much sunlight has deleterious effects on human skin (Ichihashi *et al*., 2003). Moreover, such harmful effects may be exacerbated in the presence of exogenous test materials. It is this latter context which gives rise to the field of phototoxicology.

Phototoxicology, in the most basic terms, is the study of the biological consequences resulting from the interaction of light and a chromophore. In most cases, the chromophore is an exogenous test material but it can also be an endogenous compound. There exist a variety of *in vitro* and *in vivo* test methods that may be used to access acute and chronic phototoxicological hazard potential of a material/chemical.

It should not be surprising that in the twenty-first century, the assessment of light with exogenous chromophores is an important part of a human safety evaluation, particularly for those products applied topically to sun-exposed parts of the body. What may be surprising is that the field of phototoxicology has been developed, in part, out of 'crisis', or in a reactionary manner to clinical findings in humans, rather than a pragmatic, systematic approach. For example, in the early 1960s, tetrachlorosalicylanide (TCSA) was incorporated into hand soap, which lead to many cases of photoallergenicity (Harber *et al*., 1966; Sams, 1968). Likewise, there is a long list of drugs and other ingredients which are known to produce photoirritation in humans; many of these discovered after human exposure. Finally, in the 1990s, severe photo-related toxicities were observed in patients treated with fluoroquinolones (Norrby and Lietman, 1993; Urbach, 1997; Ferguson and Dawe, 1997). Borne out of these clinical experiences and other similar events were many of the photo-methods and activities in regulatory communities aimed at

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proactively determining phototoxicological potential of compounds.

The objectives of this paper are to: (i) review methods available to assess phototoxicological hazard potential of test materials, and (ii) recommend an approach for conducting a phototoxicological risk assessment.

2 PHOTOTOXICITY: MECHANISTIC OVERVIEW

Any consideration of phototoxicology starts with photochemistry and the Gotthaus-Draper principle: *only absorbed light is active photochemically or photobiologically.* For a molecule to have a direct photobiological effect, it must absorb photons and dispense with this gain of energy in some manner. **Figure 1** provides an oversimplified mechanistic pathway of this process. In this illustration, a chromophore absorbs photon energy and becomes excited. In one case, this excited-state molecule rapidly (i.e. in picoseconds) returns to ground state, harmlessly emitting energy as, for example, heat or phosphorescence. However, in some cases, the singlet excited state forms a longer-lived triplet state which loses energy at a slower rate and may form photoproducts or free radicals. This latter sequence is thought to play an important role in direct photobiological/toxicological effects.

Beyond the direct photochemical events, any impact of a xenobiotic on ultraviolet radiation (UVR)-induced responses in the skin would be attributed to some secondary mechanism or response modifier; for example, changing structure/function of the skin thereby increasing the 'dose' of light or suppressing the immune response. Thus, understanding the potential mechanism(s) of the interaction between UVR and a chemical is critical when considering the phototoxicological impact of a response and, arguably, most important in human risk assessment.

3 DEFINITION OF LIGHT

The common point of initiation for any biological response to light is absorption of photon energy by a chromophore. The probability of light absorption is dependent on the molecular structure of the chromophore and the wavelengths of light. For the purposes of phototoxicology, the wavelengths of light of greatest interest are nonionizing UVR (290–400 nm), visible radiation (400–760 nm) and, to a lesser extent, near-infrared (760–1300 nm). The solar UVR spectrum is divided somewhat arbitrarily into UVC (100–290 nm), UVB (290–320 nm) and UVA (320–400 nm). Because wavelengths below 290 nm are absorbed by the ozone layer and do not reach the surface of the earth, UVC radiation from sunlight is of little concern when considering phototoxicological potential of test materials.

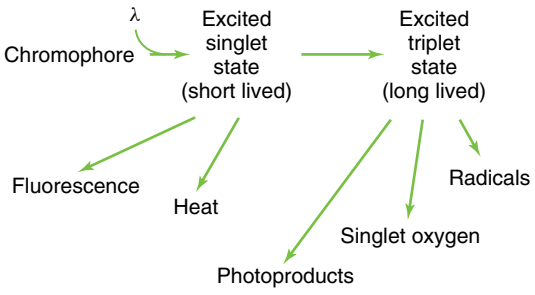

Figure 1 Mechanistic model of phototoxicity.

Because light is a dependent variable in all phototoxicological studies, it is critical to understand a few basic terms. First, *spectrum* is used to describe a range of continuous wavelengths of light coming from a source, for example xenon arc solar simulator or sun. Because energy of UVR is inversely proportional to its wavelength and different wavelengths have vastly different biological effects (e.g. 1000× difference in potency between 300 and 400 nm UVR on erythema in human skin), it is essential that the spectrum of light is known. Joule (J) is the unit of energy used to express the amount of light energy. *Irradiance* is the rate of delivery of energy per unit area expressed as $mW\,cm^{-2}$ or $W\,m^{-2}$, and *fluence* is the amount of energy delivered to an area of tissue, in $mJ\,cm^{-2}$ or $J\,m^{-2}$. Fluence, commonly referred to as the 'dose', and irradiance are related: fluence ($J\,m^{-2}$) = irradiance ($W\,m^{-2}$) × time (s). These terms and very simple relationships are most helpful when interpreting phototoxicological studies or in preparing a photo-related risk assessment.

4 DETERMINATION OF UV/VISIBLE LIGHT ABSORPTION

Determination of the UV/visible absorption spectrum is a starting point when considering the need for phototoxicological testing. The purpose for such a determination is to have some knowledge of the wavelengths at which a chemical may absorb energy and its molar extinction coefficient (MEC). Such information provides insights regarding the wavelengths at which a test material is affected by light, that is, photodegradation or photoactivation. At a minimum, these data can be used to better tailor the design or interpretation of phototoxicological studies, in the event that such investigations are necessary.

The absorption spectrum of any molecule in solution can be empirically determined using a spectrophotometer. This UV/visible absorption spectrum of a solution is a function of the concentration of all absorbing species,

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the path length of the spectrophotometric cells and the MEC of each species. This absorption spectrum is unique for any given molecule and is the probability that a photon will be absorbed plotted against the wavelength. The absorption spectrum for a molecule shows regions of absorption referred to as 'absorption bands'. Importantly, the vehicle used will have some influence on this absorption spectrum. This point has ramifications with regard to phototoxicology.

Whereas there is no one single approach for measuring the absorption of UV/visible light of a molecule or product, the Organisation for Economic Co-operation and Development (OECD) Guideline for testing Chemicals entitled 'UV-VIS Absorption Spectra (Spectrophotometric Method)' (*http://lysander.sourceoecd.org/vl=788910/cl=13/nw=1/rpsv/ij/oecdjournals/1607310x/v1n1/s1/p1*) may be used as a starting point or reference. It is worth noting that there are other methods for determining the absorption spectrum of a compound or test product.

The most comprehensive and important work related to the interpretation of absorption data was performed by Henry (2006) and reported at the American Society for Photobiology meeting (Henry, 2006). In this study, the MEC of 37 known photoirritants representing multiple chemical classes was determined. All known human photoirritants had at least one absorption band with an MEC $>10001\ mol^{-1}\,cm^{-1}$. Stated differently, compounds with an MEC $<10001\ mol^{-1}\,cm^{-1}$ over the range of 290–760 nm are unlikely to have phototoxicological concerns. As Henry (2006) noted, however, many compounds have MEC $>10001\ mol^{-1}\,cm^{-1}$ and, even so, this says nothing about the biological impact of the test material. Nonetheless, determination of absorption profiles is relatively cheap and reproducible, and, as such, serves as a convenient starting point in considering phototoxicological potential of a test material.

5 OVERVIEW OF PHOTOIRRITATION

Photoirritation is the name used to describe the event produced by the interaction between UV or visible radiation and a chemical resulting in an acute, adverse skin reaction (Allen, 1993; Gould *et al*., 1995). This acute adverse reaction is also referred to as a 'phototoxic' response or 'cutaneous phototoxicity' or 'part of a photosensitization reaction', which has lead to some confusion. For the purpose of this review, the term photoirritation will be used.

In the context of phototoxicology, photoirritation is a high-frequency reaction with a rapid onset (e.g. minutes to hours following exposure to light). Symptoms include erythema (redness), burning, prickling, pain and pigment changes (i.e. hyper or hypopigmentation). Whereas photoirritation is typically not a serious (i.e. life-threatening) adverse event, it can be quite serious and painful. In most cases, the symptoms are reversible, resolving within days. However, pigmentary changes may take several months to resolve.

A photoirritation reaction can be evoked in all subjects, provided the concentration of chemical and 'dose' of light are appropriate. The action spectrum for most photoirritant reactions is 320–400 nm; that is, UVA light. Classes of compounds known to be phototoxic in humans are psoralens, sulfonamides, phenothiazines, tetracyclines, fluoroquinolones, coal tar derivatives, anthracene, acridine, porphyrins and dyes (Moore, 2002; Stein and Scheinfeld, 2007). Generally, such chemicals are often polycyclic with many unsaturated bonds, absorb long-wave UVA (320–400 nm) or visible light and become photoactivated, accounting for their phototoxic properties. Photoirritation is prevented by protection from or avoiding light exposure.

5.1 Approaches Used to Assess Photoirritation

5.1.1 *In Vitro*

Many photoirritation *in vitro* methods have been proposed and evaluated for both screening and mechanistic purposes (Lovell and Sanders, 1990; Spielmann *et al*., 1995; Pape, 1997; Lovell and Jones, 2000). During the 1990s, the European Centre for the Validation of Alternative Methods (ECVAM) together with the European Cosmetics trade association (Colipa) undertook the task of validating an *in vitro* photoirritation assay (Spielmann and Liebsch, 2002). The 3T3 *n*eutral *r*ed *u*ptake *p*hoto*t*oxicity (3T3 NRU-PT) assay was carefully selected based on its sensitivity and selectivity using known human photoirritants. The 3T3 NRU-PT assay was evaluated extensively and shown to be highly predictive of human clinical phototoxicity (Spielmann *et al*., 1998).

Table 1 presents an overview of the 3T3-NRU-PT assay. Presently, this study consists of incubating 3T3 mouse fibroblast cells with various concentrations of the compound of interest. Following this incubation, the cells are exposed to $5\ J\,cm^{-2}$ UVA. The %inhibition of growth is compared against cells exposed to chemical but not to UVA light. A photoirritation inhibitory factor (PIF) and mean photo effect (MPE) are calculated using IC_{50} (half maximal inhibitory concentration) and weighted values from concentration–response curves, respectively. Based on the validation study results, a test substance with a PIF < 2 or an MPE < 0.1 predicts: 'no phototoxicity'. A PIF > 2 and < 5 or an MPE > 0.1 and < 0.15 predicts: 'probable phototoxicity', and a PIF > 5 or an MPE

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Table 1 OECD Test No. 432: *in vitro* 3T3 neutral red uptake phototoxicity test

3T3 NRU-PT	Comment
General design	Mouse fibroblast, 3T3 cells (monolayer) Apply various concentrations of solubilized test material Expose to 5 J cm^{-2} UVA (320–400 nm) Add neutral red dye Measure neutral red uptake Use known positive and negative control materials
Measures	%Inhibition of growth as compared to vehicle
Data interpretation	Calculate photoirritation inhibition factor (PIF) value using IC_{50} values. PIF $\geq$ 5.0 predicts 'phototoxicity'; PIF $<$ 2 predicts 'no phototoxicity'; PIF $>$ 2 and $<$ 5 predicts 'probable phototoxicity' Calculate mean photo effect (MPE) using weighted average of concentration response curves. MPE $>$ 0.15 predicts 'phototoxicity'; MPE $<$ 0.1 predicts 'no phototoxicity'; MPE $>$ 0.1 and $<$ 0.15 predicts 'probable phototoxicity'
Key references	(Spielmann *et al*., 1994; 1995; 1998)

$>$ 0.15 predicts: 'phototoxicity' (Peters and Holzhutter, 2002).

The 3T3-NRU-PT assay has been accepted by the European Union (Spielmann *et al*., 1995; 1998) and endorsed by ECVAM's Scientific Advisory Committee. Moreover, at the request of the European Commission DG (Directorate General) Enterprise, their experts, the Scientific Committee on Cosmetic non-Food Products (SCCNFP), proposed the use of the assay as the standard method for testing the UV light-absorbing cosmetic ingredients or mixtures of ingredients for phototoxic (photoirritation) potential. This led to the formal acceptance of the assay in the European Directive (Commission Directive 2000733/EC of April 25 2000), and in 2004 publication of an OECD Test Guideline No. 432.

As with any method, the 3T3-NRU-PT assay is not perfect. The test material used in the 3T3-NRU-PT assay must be soluble. This is an issue for many topically applied finished products. To overcome this challenge, organotypic skin cultures have been evaluated with some success (Edwards *et al*., 1994; Liebsch *et al*., 2005). The assay does not provide potency or 'rank order' of photoirritation potential. As well, the 3T3-NRU-PT assay is a hazard-based study and it is difficult to extrapolate results, for example positive 'photoirritation', to human risk. This has been particularly problematic for pharmaceutical test materials, many of which may have photoirritation potential as determined in 3T3-NRU-PT assays, but pose little or no human risk as a result of exposure. Notwithstanding these concerns, the 3T3-NRU-PT assay represents a useful and relevant means of evaluating photoirritation potential and, after the absorption profile is determined, the next step in a phototoxicological assessment.

5.1.2 *In Vivo Animal Models*

Photoirritation testing has been done in several animal species including mice, rabbits and guinea pigs (Harber, 1981; Nilsson *et al*., 1993; Lambert *et al*., 1996). From the preceding discussion, *in vitro* photoirritation studies have several shortcomings. To this end, the *in vivo* guinea pig photoirritation test (Nilsson *et al*., 1993) is preferred and may be useful if (i) the test material cannot be tested *in vitro*; (ii) a No Observed Effect Level (NOEL) is needed for human risk assessment; or (iii) the test is required by a regulatory agency.

An overview of the guinea pig photoirritation assay is summarized in **Table 2**. Briefly, guinea pig skin is exposed to the test chemical. After 2 h of exposure to the test or control material, half of the test sites are exposed to 10 J cm^{-2} UVA while the remaining sites are shielded. Visual grading is performed at 1, 24 and 48 h after UVA exposure. An incidence and severity index are calculated, comparing treated sites to control (untreated) or vehicle-treated site.

The guinea pig photoirritation test can be used to evaluate systemically administered test materials. For such studies, measures of plasma and/or skin concentrations of test material may be helpful in determining the time between drug and light exposure to maximize the interaction (Horio *et al*., 1994).

In addition to the guinea pig, mouse models have been developed for *in vivo* photoirritation testing (Gerberick and Ryan, 1989a; Shimoda *et al*., 1993; Marutani *et al*., 1993). The mouse models have been used to assess the photoirritation potential of topical or systemically-administered test materials. In the mouse ear-swelling model (Gerberick and Ryan, 1989a), the end point is easily quantitated, that is, measurement of swelling, and is more objective than models based on subjective evaluation of skin changes. Both mouse and

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Table 2 *In vivo* guinea pig photoirritation test method

In vivo phototoxicity test	Comment
General design	Albino guinea pigs (n = 18), hair removed 2 h exposure to test and control materials using Hilltop chamber patches Expose half of the test sites to 10 J cm^{-2} UVA, keep other half covered Grade all sites at 1, 4, 24 and 48 h after exposure Use 8-MOP as control
Measures	Visual skin grading by trained personnel evaluating incidence and severity indexes
Data interpretation	Determination of photoirritation is based upon the differences in skin response at the UVA-exposed and UVA-shielded test sites after exposure to the respective application of the test material in comparison to controls
Reference	(Nilsson *et al.*, 1993)

guinea pig models have been shown to identify known human photoirritants.

5.1.3 In Vivo Human Testing

The method used to evaluate photoirritation in humans is described in **Table 3**, following the procedure of (Kaidbey and Kligman, 1978). This clinical phototoxicity test method can be used to assess compounds/products administered topically or systemically. In the latter case, the study is modified by administration of the chemical at the standard therapeutic dosage either once or repeatedly over several days. This method differentiates between reactions that are induced by (i) UV, (ii) test material or (iii) test material + UV (true photoirritation response).

Specific wavelengths of UVR may be used depending on the absorption profile of the test material, or a standardized artificial light source, filtered to emit UVA, may be used. In each subject, a minimum erythema dose (MED) is determined for the light source, generally on the back of the subjects, before dosing and at various points during dosing. A photoirritation response is considered positive if a significant decrease in the ratio of MED before and after dosing is observed (Ferguson and Johnson, 1993; Ferguson and Dawe, 1997).

5.2 Summary of Photoirritation Testing

The *in vitro* 3T3 NRU-PT assay should be the first test used in assessing photoirritation potential of a test material. This assay will provide a yes/no answer regarding the photoirritation *potential* of the test material. Animal models of photoirritation are available to define NOEL and for testing materials incompatible with *in vitro* conditions. Finally, human photoirritation testing may be used to confirm the absence of photoirritation response under specific exposure conditions. Considering the frequency and human relevance of photoirritation, testing for this adverse event should be conducted in standard phototoxicological risk assessments.

6 OVERVIEW OF PHOTOALLERGY

Photoallergy is a cell-mediated immunologic reaction to a chemical that has been made antigenic by the interaction with light (Allen, 1993; Gould *et al.*, 1995). This reaction is analogous to allergic contact dermatitis, but differs in that the test material requires activation by light to elicit the response. Clinically, photoallergenic skin responses resemble photoirritation reactions with a similar action spectrum. There are several differences between photoallergy and photoirritation, including (i) onset, with photoallergic responses taking at least 24 h to develop, (ii) distribution of lesion, with some 'bleedover' to unexposed skin in photoallergic responses, and (iii) histologically, the photoallergic response is characterized by epidermal oedema and vesicle formation and a dense perivascular infiltrate. Photoallergenic responses are less frequent than photoirritation reactions.

Many of the known human photoallergens are also photoirritants (Stein and Scheinfeld, 2007). Antimicrobial agents, fragrances, plant derivatives, sunscreens and some drugs have been reported to produce photoallergy in humans (Bryden *et al.*, 2006). Specifically, halogenated phenolic compounds, coumarins, musk ambrette, promethazine, chlorpromazine, ketoprofen and *p*-aminobenzoic acid have been reported to be photoallergenic in humans. As is the case for photoirritation, these chemicals absorb light and become photoactivated. The photoactivated molecule may be identified as foreign by the immune system or it may serve as a hapten, interacting with a larger biomolecule such as protein which may then initiate an immunologic response. As is the case for photoirritation, photoallergic responses can be prevented by protection from or avoiding light exposure.

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Table 3 Human photoirritation and photoallergy test methods

Human photoirritation and photoallergy tests	Comment
General design	**Photoirritation** Ten subjects that meet inclusion/exclusion criteria. Test material patched for 24 h prior to UV exposure UV exposure: UVB = 1/2 MED; UVA = 10 J cm^{-2} Three test sites: 1. test material + UV exposure 2. nonirradiated control site (test material only) 3. irradiated control site (no test material) Test sites read at 10 min, 24 h and 48 h after UV exposure **Photoallergy** Twenty-five subjects that meet inclusion/exclusion criteria. Test material patched for 24 h prior to UV exposure Induction phase –24 h patch followed by UV exposure, twice per week for 3 wk –UV exposure: UVB = 3×MED; UVA = 4 J cm^{-2} –nonirradiated control site (test material only) –irradiated control site (no test material) –final induction followed by 2-wk rest period Challenge phase –single 24 h patch application followed by UV exposure –UV exposure: UVB = 1/2 MED; UVA = 4 J cm^{-2} –naive site –three test sites (see photoirritation) Sites read at 24, 48 and 72 h following UV exposure
Measures	Visual reading by trained personnel; 0–4 scale for objective irritation with additional notations for sensory response
Data interpretation	For the interpretation of results, it is important to compare the response of the test material + UV exposed site to both control sites—test material only and UV only. If the response pattern is not clearly indicative of sensitization vs. clinical irritation, it may be necessary to follow up with a rechallenge and/or prospective/provocative use test.
References	(Kaidbey and Kligman, 1978; 1980; Kligman and Kaidbey, 1982)

6.1 Approaches Used to Assess Photoallergenicity

6.1.1 In Vitro

There are no *in vitro* assays for assessment of photoallergy. The closest *in vitro* approach is the determination of binding to serum albumin following UV exposure (Lovell and Jones, 2000). This photobinding technique has been evaluated but, at this time, has not been advanced as a method for validation (Moser *et al*., 2001). Beyond photobinding, there are knowledge-based screens such as DEREK (Deductive Estimation of Risk from Existing Knowledge) with structural alerts for photoallergens and photoirritants (Barratt *et al*., 2000).

Very recently, an *in vitro* photoallergy test using THP-1 cells exposed to UVA has been reported (Hino *et al*., 2008). THP-1 cells, derived from human monocytic leukaemia cells, express co-stimulatory molecules CD86 and CD54 as well as major histocompatibility complex (MHC) class II molecule. When THP-1 cells are cultured with known allergens, these surface markers are expressed and can be measured. This approach has been modified by Hino *et al*. by exposure of THP-1 cells to test material followed by UVA, and reported to identify select photoallergens. This promising approach may be useful as a means of identifying potential photoallergens and/or distinguishing photoirritants from photoallergens.

6.1.2 In Vivo Animal Models

At present, photoallergy can be assessed preclinically using *in vivo* methods. The preferred animal species is the guinea pig (Ichikawa *et al*., 1981), although the mouse ear model is used also (Gerberick and Ryan, 1990). As presented in **Table 4**, the guinea pig photoallergy

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Table 4 *In vivo* guinea pig photoallergy test methods

In vivo photoallergy test	Comment
General design	Albino guinea pigs (n = 25–45), hair removed Inject Freund's complete adjuvant to define test sites Remove stratum cornea by tape stripping test sites 2 h exposure to test and control materials using Hilltop chamber patches Expose half of the test sites to 10 J cm^{-2} UVA, cover other half repeat on days 2, 4, 7, 9 and 11 (excluding injection of Freund's adjuvant) On day 21 conduct primary challenge Grade at 24 and 48 h after UVA exposure Use 8-MOP and musk ambrette as controls
Measures	Visual grading by trained personnel Incidence and severity indexes determined
Data interpretation	Determination of photoallergy is based upon the differences in skin reactions at the UVA-exposed and UVA-shielded test sites after exposure to the respective challenge dose of test material in comparison to controls
Key references	(Ichikawa *et al.*, 1981; Gerberick and Ryan, 1989b)

assay consists of two phases: the induction and challenge phases (Harber *et al.*, 1982; Gerberick and Ryan, 1989b). During the induction phase, test sites are injected with Freund's adjuvant to designate the test areas. These test sites are repeatedly tape stripped to remove the stratum corneum, followed by application of the test or control material for 2 h. After treatment with the test materials, half of the test sites are exposed to 10 J cm^{-2} UVA while the remaining sites are shielded. This procedure is repeated, except for Freund's adjuvant injection, on days 2, 4, 7, 9 and 11. On day 21, nonirritating doses of test or control materials are applied to the sites for 2 h, followed by exposure to 10 J cm^{-2} UVA. Visual grading is undertaken at 1, 24 and 48 h following UV exposure, and incidence and severity indexes calculated in comparison to controls.

More recently, Ullrich, Vohr and colleagues have introduced a modification of the local lymph node assay (LLNA) termed photo-LLNA or UV-integrated model for the differentiation of skin reactions (UV-IMDS) (Vohr *et al.*, 1994; 2000; Homey *et al.*, 1995; Neumann *et al.*, 2005). In brief, groups of mice are treated with test material or vehicle on the dorsum of both ears on three consecutive days. Immediately after application of test material mice are exposed to 10 J cm^{-2} UVA light. For the comparison of induction and challenge responses, mice are treated on the shaved back with test material or vehicle alone on three consecutive days (induction-phase treatment). Twelve days after the last induction-phase exposure, mice are challenged with test material or vehicle on the dorsum of both ears for a further three days (challenge-phase treatment). Lymph nodes are excised 24 h after the last challenge-phase treatment. Lymph node weight, cell counts and stimulation indices, which were defined as the ratio of mean lymph node cell counts from groups treated with test compound to corresponding results of control groups, are determined. This approach holds promise and may replace current models for photoallergenicity testing. It will be necessary for a more comprehensive evaluation of photo-LLNA, together with refinement of the testing procedure, to advance this test method.

6.1.3 *In Vivo Human Models*

Table 3 provides an overview of the method used for clinical photoallergy testing of topical compounds based on the work of Kaidbey and Kligman, (1980). The design of this photoallergy test is based on human repeat-insult patch testing, designed for confirmatory evaluation of contact sensitizers. This method can be adapted for testing chemicals administered by other routes by replacing the patch induction phase with dosing of the subjects with the therapeutic dose and route over a similar period of time. The chemical can be administered by patch testing for the challenge phase.

6.2 Summary of Photoallergenicity Testing

Photoallergy is a rare event even in a phototoxicological context. It can be distinguished from photoirritation based on incidence, time of onset after exposure, clinical appearance and distribution of reaction relative to site of product application. There are no validated *in vitro* approaches for evaluating photoallergenic potential of test materials. The *in vivo* guinea pig photoallergenicity study is a mainstay for such testing. However, the UV-IMDS (i.e. photo-LLNA), may become a replacement for the guinea pig at some point in time. Like photoirritation, there is a human clinical photoallergenicity test that

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is used to confirm the absence of photoallergenicity. Determination of photoallergenicity is, in general, not a routine evaluation in phototoxicological risk assessment unless there is a positive photoirritant evaluation.

7 OVERVIEW OF PHOTOGENOTOXICITY AND PHOTOCARCINOGENICITY TESTING

To date, the area with the most uncertainty in phototoxicology risk assessment is photogenotoxicity and photo co-carcinogenicity testing. This is largely attributable to the lack of documented human photo co-carcinogens, aside from the combination of 8-methoxypsoralen (8-MOP) and UVA (PUVA) therapy used to treat psoriasis (Stern, 1984; Stern and Laird, 1994). Even if data from rodent photo co-carcinogenicity studies are considered, as is often the case in toxicology when human data are not available, the database remains small and controversial with few positive and even fewer negative substances having been tested. The lack of an appropriate dataset of rodent photo co-carcinogens and non-photo co-carcinogens makes validation of a short-term *in vitro* test, such as photogenotoxicity, at best challenging if not unachievable (Jacobs *et al*., 1999). As such, the use of existing models of photogenotoxicity and photo co-carcinogenicity, are research tools with uncertain relevance in a human phototoxicological risk assessment.

7.1 Photogenotoxicity/Photo Co-carcinogenicity: Parallels to Genotoxicity and Carcinogenicity Testing

When considering the carcinogenic potential of a chemical, it is often helpful to initially evaluate its genotoxic properties. If a compound is genotoxic, it is potentially carcinogenic (Ennever and Rosenkranz, 1987; Sato and Tomita, 2001). The same theoretical relationship exists between photogenotoxicity and photo co-carcinogenicity. For instance, the only known human photo co-carcinogen, that is, 8-MOP, is photochemically activated, leading to DNA lesions (Hara *et al*., 2007), which may be responsible, in part, for its long-term tumorigenic properties (Forbes *et al*., 1976). However, the strength of the relationship between photogenotoxicity and photo co-carcinogenicity cannot be accurately determined due to the absence of known human photocarcinogens or co-carcinogens. Nevertheless, the cause and effect relationship is logically sound and theoretically appealing.

An important difference between standard genotoxicity/carcinogenicity testing and photogenotoxicity/photo co-carcinogenicity testing is that in the latter cases we examine the effects of two agents and their potential interactions, namely UVR, a known carcinogen, and the test material. Such interaction may be direct, for example photochemical activation of the test substance to a genotoxic substance, or indirect, for example pseudo-photoclastogenic (Lynch *et al*., 2008). The potential for a test substance to have indirect effects that enhance the activity of UVR is quite possible in both photogenotoxic and photo co-carcinogenicity studies. The possibility that indirect mechanisms account for positive results complicates interpretation of these studies, even if some indirect effects may be relevant to human risk assessment, for example immunosuppression.

7.2 Photogenotoxicity Testing

Photogenotoxicity is defined as the genotoxic effect induced by and dependent on the concomitant exposure to a chemical and solar-simulated UV (Brendler-Schwaab *et al*., 2004). Two photogenotoxicity approaches have been used. One is an *in vitro* photomutagenicity assay and the other is an *in vitro* photocytogenetics assay. The photomutagenicity assay measures small DNA changes that result in gene mutation. The photocytogenetics assay measures larger DNA changes that result in chromosome damage.

The current tests used to assess photogenotoxicity of test materials are based on existing genotoxicity methods with the inclusion of UVR exposure. The studies are: (i) photo-Ames, a test for point mutations (Chetelat *et al*., 1993); (ii) photo-chromosome aberration using mammalian cells such as Chinese Hamster Ovarian (CHO) and V79 cells (Itoh *et al*., 2002); and (iii) photo-comet assay using human keratinocytes and V79 cells (Chetelat *et al*., 1996). The conduct and interpretation of these studies is detailed in reviews by Brendler-Schwaab, Müller, Kasper (Müller *et al*., 1998; Kersten *et al*., 1999; Gocke *et al*., 2000; Brendler-Schwaab *et al*., 2004) and Meunier *et al*. (2002).

It is important to point out that methods for measuring photogenotoxicity are not yet as standardized around the world as routine tests; there are, however, ongoing international efforts to harmonize photogenotoxicity methods. Most important, the predictive validity of photogenotoxicity testing has not been established and, as such, the value in human phototoxicological risk assessment is uncertain.

7.3 Photo Co-carcinogenicity Testing

Photo co-carcinogenicity testing is defined here as the study of how chemicals modulate the development of

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skin tumours induced by solar-simulated UVR. Up to this point, the assessment of acute or subacute interactions of a drug and UVR has been considered. In contrast, modulation of photo co-carcinogenicity represents a chronic toxicity that is not amenable to human testing.

The induction of UVR-induced tumorigenesis has been studied for several decades using hairless mice, a species that develops a nonmelanoma skin cancer equivalent to human squamous cell carcinoma (Forbes, 1981). What has been demonstrated is that tumour induction is dependent on the dose, the time or duration of exposure and the wavelength of UVR (Forbes *et al*., 1978; 1981). For murine nonmelanoma skin cancers, the wavelengths of importance are in the short-wave UVB (290–320 nm) and are up to 1000 times more potent than long-wave UVA in inducing skin tumours in mice.

In the process of understanding the relationship between UVR and skin cancer development, there have been a number of experiments that have shown that drugs can modify this interaction *vis-à-vis* multiple mechanisms (Mukhtar *et al*., 1999). These mechanisms include direct photoactivation of a chemical resulting in DNA adduct formation, and the universe of indirect mechanisms including promotion, immunosuppression, skin irritation, dietary changes, and so on, all of which can modify UVR-induced skin cancer formation (Forbes and Urbach, 1974).

Forbes and colleagues have introduced test methods for evaluating the photo co-carcinogenicity potential of test materials (Forbes *et al*., 1993; 2003; Sambuco *et al*., 2003). The animal model used in the photo co-carcinogenicity study is the SKH1 albino hairless mouse. The hairless mouse has the advantage that it does not require clipping, hair being a very good UV protectant. Furthermore, hairless skin allows easy detection of skin lesions as they arise (Forbes and Sambuco, 1998). The key design characteristics include use of a UV solar simulator that emits the full spectrum of UVR present in sunlight. In one version, the test substance is applied before UVR on Monday, Wednesday and Friday and after UVR on Tuesday and Thursday for 40 weeks with no treatment on Saturday and Sunday, followed by a 12-week observation period without dosing. The reason for this pattern of exposure is that, in theory, it allows detection of photochemically activated substances, as well as those that may modulate photocarcinogenesis but are photo-labile. Generally, separate groups receiving either high or low UVR serve as benchmarks for groups that are dosed with low UVR and the test substance. The repetitive low doses of UVR will induce tumours in all mice, and the higher UVR dosing regimen will induce tumours with a shorter latency. The end point measures that are evaluated generally are lesion (i.e. squamous cell papillomas and/or carcinomas), tumour prevalence (incidence), tumour yield (multiplicity) and time to tumour (latency). This is not a model for basal cell carcinoma, the most common human skin cancer, or melanoma, the most deadly and aggressive skin cancer in humans.

The key finding in the SKH1 albino hairless mouse photosafety study is whether the test substance alters the time of appearance of tumours or increases the number of tumours per mouse (Molefe *et al*., 2005). Agents that cause tumours to appear more rapidly or in increased numbers are defined as 'photo co-carcinogens'. Agents that delay the onset of tumours or decrease the number of tumours are photo-protective (e.g. sunscreens). Strictly speaking the photo co-carcinogenesis protocol discussed here is a co-carcinogenesis design, because the test substance is administered along with a known carcinogen (i.e. UVR). The assay is also referred to as simply photocarcinogenesis study.

As an experimental paradigm, there is some validity in assessing chemical modification of photocarcinogenicity. However, when considering risk assessment and using this as a model or requesting this for evaluation of chemicals, consideration of the limitations is necessary. As with all *in vivo* rodent carcinogenicity assays, there are likely to be both qualitative and quantitative differences between responses observed and possible effects in humans. The SKH1 hairless mouse is fairly sensitive to UVR-induced tumour formation, and its epidermis is at most one-third as thick as human epidermis, allowing for increased penetration of both UVR and topically applied chemicals. Importantly, there are multiple mechanisms by which chemicals might act as co-carcinogens with UVR. These include photochemical activation, inhibition of DNA repair, immunosuppression, altered optical properties of the skin, and tumour promotion. The photo co-carcinogenicity study may provide little mechanistic understanding and be heavily dependent on the exposure conditions used in the study.

A common problem associated with all *in vivo* tests used in cancer hazard identification is the uncertainty of extrapolating results from animal models to humans. This is a particular problem when nongenotoxic effects such as tumour promotion are involved in the tumorigenic mechanism. In general, skin tumour promotion in laboratory animals shows substantial strain and species specificity, which makes interspecies extrapolation of results uncertain, and no human skin tumour promoters have been unequivocally identified. Therefore, effects observed in the hairless mouse photo co-carcinogenicity study may not be predictive of human responses.

Another problem is that there is only one chemical that has been clearly identified as a human photo co-carcinogen, namely 8-MOP. The immunosuppressive agents, such as ciclosporin and azathioprine, are human skin carcinogens, and probably exacerbate the risk of UVR-induced skin cancer, but other factors such as papillomavirus infection may contribute to the mechanism of increased cancer risk. The lack of chemicals that are known human photo co-carcinogens means that the

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photo co-carcinogenicity assay can not be readily validated for human risk assessment and its predictivity is uncertain.

7.4 Summary of Photogenotoxicity and Photo Co-carcinogenicity Testing

In vitro photomutagenicity or photoclastogenicity assays may be used in an attempt to understand the photogenotoxicity potential of a test material. Despite all the limitations currently associated with these testing schemes, *in vitro* photogenotoxicity is addressed using relatively inexpensive tests that hold the most promise as a means to quickly and inexpensively assessing potential mutagenic/clastogenic effects of chemicals. If *in vivo* photo co-carcinogenicity testing is required, there is currently a single model using SKH1 albino hairless mice that has a history at least within the US Food and Drug Administration (FDA). Again, it cannot be overemphasized that the current rodent photo co-carcinogenicity study results may have little or no bearing on human heath risk. As such, we cannot fully assess what the relevance of the rodent studies is to human health risk.

8 REGULATORY GUIDANCE FOR PHOTOTOXICOLOGICAL ASSESSMENT

Prior to 2000, there were discussions occurring in the USA and Europe regarding approaches to be used for evaluation of phototoxicological potential of drugs and cosmetic products. This culminated in guidelines issued by the FDA in the USA (*www.fda.gov/cder/guidance/3640fnl.htm*) and European Agency for the Evaluation of Medicinal Products (EMEA) (*www.emea.europa.eu/htms/human/humanguidelines/nonclinical.htm*). These guidance documents provide a decision tree for photosafety testing, focussed on pharmaceuticals. In general, the FDA and EMEA approaches share a similar yes/no decision tree but differ rather significantly in the area of photogenotoxicity and photo co-carcinogenicity testing. The FDA has not found photogenotoxicity results to be particularly robust and, as such, has leaned towards *in vivo* photo co-carcinogenicity testing (Ellis, 1998; Jacobs *et al*., 1999). On the other hand, the EMEA has requested *in vitro* photogenotoxicity data in lieu of *in vivo* photo co-carcinogenicity studies. Nonetheless, and consistent with Section 7 of this chapter, the EMEA has recently reconsidered some photogenotoxicity studies, with their Committee for Proprietary Medicinal Products/Safety Working Party (CPMP/SWP) stating:

> *Oversensitivity and the occurrence of "pseudo-effects" with* in vitro *models recommended by the current guideline, in particular the mammalian cell test for photo-genotoxicity have become a major problem. Therefore the use of these test models for regulatory purposes can no longer be justified and need to be replaced by more appropriate approaches.*[a]

In summary, there are regulatory guidelines for photosafety testing which provide a structured approach in the evaluation of test materials. It would be prudent to seek some additional perspective from regulators before embarking on expensive and time-consuming testing as there are still some uncertainties in the value of some data.

9 SUMMARY

At the second ECVAM workshop on phototoxicity testing (Spielmann *et al*., 2000), a structured flowchart was developed which provides guidance in phototoxicological evaluation of test materials. Now, it is suggested that the basic approach towards human phototoxicological risk assessment should include, first the determination of the absorption spectrum and second, photoirritation testing, using *in vitro* 3T3 NRU-PT (OECD 432) methods and, if needed, *in vivo* preclinical or, for confirmatory testing, human. These data will answer the two basic questions: (i) does the test material absorb UV? and (ii) is the material photoactivated, and what is the biological consequence (i.e. photocytotoxicity)? If a material is photoactivated, there may be the need to understand the photoallergenic potential and long-term consequences of use/exposure. In the case of photoallergenicity, there are animal models that exist and, in the future, perhaps an *in vitro* test may emerge. To understand potential photogenotoxicity and photo co-carcinogenicity, there are experimental approaches including adaptations of standard *in vitro* genotoxicity tests and a photo co-carcinogenicity study using SKH1 hairless mice. For photogenotoxicity and photo co-carcinogenicity testing, there remains a level of uncertainty regarding the outcome of such studies. This is particularly true as it relates to risk assessment. Finally, there are regulatory guidance documents for photosafety testing which may require some testing to forego or address labelling questions related to photosafety.

Phototoxicology is an important consideration for all test materials. However, given the dependence of such events on exposure to UVR, common sense and practical approaches should be considered in the context of testing needs. Clearly a material that is not exposed to UVR should not be a phototoxicological risk. By the same token, systemically administered materials are not exempt

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from testing as they may represent a phototoxicological risk. In the end, the end points and test methods identified herein should provide the tools needed to conduct a robust phototoxicological risk assessment.

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NOTES

[a.] Concept paper on the need for revision of the Note for Guidance on Photosafety Testing (CPMP/SWP/398/01). The proposed guideline will replace the Note for Guidance on Photosafety Testing CPMP/SWP/398/01.

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Biomarkers of Exposure to Carcinogens

Paul T.J. Scheepers

CONTENTS

1 INTRODUCTION

In human exposure assessment a biomarker is a parent substance, metabolite or addition product (adduct) that can be measured in blood, urine or exhaled air. Sometimes hair, skin and nails are also used as tissues for isolation of biomarkers. For isolation of DNA adducts there is some experience with the use of exfoliated epithelial cells isolated from the buccal mucous or from urine. A biomarker provides information on the internal exposure to a substance, in contrast to external exposure monitoring that can be provided by personal air sampling or skin-contamination testing.

The use of biomarkers in a human biological monitoring campaign can be a good way to evaluate exposure to carcinogenic substances on the workplace or in the general environment.

If the parent compound is used as a biomarker of exposure, information on uptake of a substance from various different sources and via different routes can be obtained. Use of a metabolite or adduct to DNA or proteins will also provide information on bioavailability and metabolic activation.

Even if the biomarker is not identical to the substance a person is exposed to, it may still contain chemical-structure information, such as an addition product that can be linked to this parent substance. This makes a biomarker very useful for assessment of exposure.

The half-life of biomarkers determines the moment of sample collection relative to time of exposure. Elimination from the lungs is usually fast (minutes to hours), elimination from plasma and urine is fast (hours to days), elimination from blood cells is much longer (weeks to months) and the half-life of adducts to macromolecules depends on many factors, but may vary from hours to days for most DNA adducts to weeks or months for some specific DNA adducts and their repair products (Henderson *et al*., 1989). Adducts to proteins may have a longer lifespan (weeks to months), depending on the turnover of the protein or of the cell, such as in the case of haemoglobin (Hb) in erythrocytes (Scheepers, 2008).

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It must be noted that the excretion pattern is not just dependent on the terminal half-life of a substance, but is also dependent on the pattern of exposure and the *porte d'entrée*. Biomarker levels will show a different pattern in workers as opposed to subjects exposed in the general environment. Inhalation exposure will show a pattern of excretion that is different from that after dermal absorption.

There are many specific chemicals that are very persistent due to specific properties like high lipophilicity or noncovalent binding to proteins (e.g. halogenated substances like polychlorinated biphenyls (PCBs), dioxins and also some metals like cadmium and lead).

Metabolites and adducts are usually mechanism-based and provide useful information on the formation of metastable intermediates that are involved in genotoxic activity. Most biomarkers reflect systemic bioavailability rather than a target dose, which sometimes limits the interpretation in terms of assessment of cancer risk.

Some biomarkers have been used to study exposure to carcinogenic substances in the general population or to reconstruct exposures after chemical incidents involving carcinogenic substances.

Based on evaluations by the International Agency for Research on Cancer (IARC) to 2003 it is estimated that workers are exposed to carcinogenic substances in 18 occupations and industries. These exposures involve 28 substances classified as definite human carcinogens, 27 probable carcinogens and 113 agents classified as possible occupational carcinogens (Siemiatycki *et al*., 2004).

In this chapter parent compounds will be discussed in Section 2, metabolites in Section 3, adducts to proteins in Section 4 and DNA adducts and their products of enzymatic repair in Section 5. In Section 6 the use of biomarkers in human biological monitoring studies will be discussed.

2 PARENT COMPOUNDS

For those substances that can exert their toxicity without any metabolic activation, a parent compound is suitable as a biomarker. For the large group of volatile organic compounds (VOCs), the primary toxic effect may be neurotoxicity, whereas a metabolite may represent a genotoxic substance. For this reason parent substances are often determined as relevant biomarkers of exposure in body fluids and exhaled air. Biological monitoring of metals will also be discussed.

2.1 Body Fluids

VOCs can be determined from blood by headspace analysis. In this analysis of the sample the analyte is exchanged between the liquid and gas phase until equilibrium. In the gas phase the concentration is determined and the concentration in the liquid is then calculated using a blood–air partitioning coefficient. The advantage of such an approach is a clean matrix for gas chromatographic analysis. Parent organic compounds and inorganic compounds can also be analysed from urine if they are readily soluble in water. An important group of carcinogens that is normally determined as a parent compound from body fluids is metals (Nordberg *et al*., 2007).

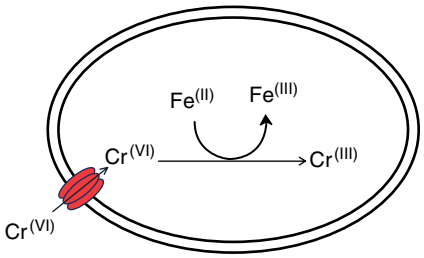

Figure 1 Cr(VI) enters the erythrocyte through an ion transporter for phosphate and sulfate. In the red blood cell Cr(VI) is reduced to Cr(III) by Fe(II) in oxyhaemoglobin that is converted to methaemoglobin. Cr(III) cannot pass through the membrane, causing accumulation of chromium in the cell. (Reproduced from Lewalter *et al*., 1985. © Springer.)

2.1.1 Blood

Most organic substances can be analysed from full blood or plasma. For volatile organic substances a headspace analysis may be used. In plasma, metals can be free or bound to metallothioneins, for example. Metals may also be determined from different types of blood cells, such as erythrocytes and granulocytes. An interesting application is the determination of chromium from erythrocytes, for example, in workers who are exposed chromate-containing primers or by inhalation of fumes from stainless-steel welding. Chromates containing hexavalent chromium may enter blood cells using ion channels (**Figure 1**). In this way chromium becomes trapped in the erythrocyte and the systemic exposure to hexavalent chromium can be evaluated by isolation of the erythrocytes and determination of the total chromium content. Also, the plasma levels of chromium in welders are related to the type of metal that is welded and the composition of the electrodes used (Scheepers *et al*., 2008).

2.1.2 Urine

Urine can also be analysed for the parent substance, such as in the case of organic solvents (Fustinoni *et al*., 1999), but if the carcinogenicity involves enzymatic activation, a downstream metabolite may be selected as a biomarker

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(see Section 3). Urine is also a good matrix for detection of metals. Some knowledge about speciation and kinetics is needed in order to interpret urinary metal excretion.

In the analysis of arsenic, speciation between inorganic and organic arsenic is useful because of differences in the origin and toxicity. Inorganic As can be derived from drinking water and is a known human carcinogen, whereas organic arsenic compounds, with a much lower toxicity, originate primarily from the diet, in particular seafood (Buchet *et al*., 1996).

In biological monitoring of chromium, kinetics are important. Stainless-steel welders are exposed to welding fume particles that contain chromates. Chromium leaches very slowly into the circulation due to the sparse water solubility of some of the chromates that are deposited deeply in the lungs (Schaller *et al*., 2007). This explains why the preshift urine sample of a welder may still contain considerable levels of chromium compared to a nonexposed worker (Scheepers *et al*., 2008). In this case exposure during the work shift can be evaluated by comparing preshift and postshift values of urinary chromium excretion.

2.2 Exhaled Air

End-exhaled air is defined as the last 300–400 ml at the end of a prolonged exhalation and is equivalent to the alveolar fraction. It can be easily and noninvasively collected in both adults and children. In this air volume the chemical substance equilibrates almost instantly with arterial blood, ruled by the blood–air partition coefficient. With this parameter, the concentration in the arterial bloodflow that takes the substance to the brain can be calculated.

End-exhaled air can be collected in a ~100 ml container. This container must be equipped with a one-way valve to prevent ambient air being drawn in if the subject starts to inhale at the end of the deep exhalation. There are commercial systems such as the BIO-VOC that can be used for the routine collection of end-exhaled air samples in adults and children (**Figure 2**). In the analysis, compared with body fluids, exhaled air is a very clean matrix that enables ultrasensitive detection of VOC down to pmol l^{-1} for chlorinated hydrocarbons.

3 METABOLITES

Biotransformation leads to detoxification for most chemical substances. However, metabolism may also lead to formation of reactive intermediates and more- or less-stable metabolites that can exert a specific toxic effect that may be different from the toxicity of the parent compound.

Figure 2 Exposure to benzene can be investigated using breath analysis. End-exhaled air was collected by children attending primary school in Turkey. The volatile organic solvents are transferred to a solid phase by the investigator directly after sample collection. In this way the organic compounds are well preserved for later analysis. (Reproduced with permission from J. Konings.)

As a parent compound, styrene can be determined in end-exhaled air or urine (Teixeira *et al*., 2008). Styrene is metabolized to styrene-7,8-oxide, which is a genotoxic metabolite. Styrene-7,8-oxide and phenylethyleneglycol can be determined in blood (Christakopoulos *et al*., 1993). In urine, mandelic acid, phenylglyoxylic acid, phenylglycine and 4-vinylphenol are possible urinary biomarkers (**Figure 3**). It is also possible to determine the secondary metabolites 1- or 2-phenyl-2-hydroxyethylmercapturic acid derived from a glutathione conjugate (Fustinoni *et al*., 2008). Some metabolites or conjugates are less useful because they are not specific for styrene. For example, benzoic acid (not shown in **Figure 3**) is not specific for styrene exposure since this substance also used as a food additive (E210). Hippuric acid, besides being a reaction product of benzoic acid, is also a conjugate formed following exposure to toluene.

4 PROTEIN ADDUCTS

Peptides and proteins contain numerous nucleophilic groups that can be targets for electrophilic attack by reactive intermediates to form adducts (**Figure 4**). These are mostly electrophilic, but not only alkylating substances. Törnqvist *et al*. (2002) point out that not all of these adducts are exclusively of post-translational origin, like N-terminal valines, that are much used as biomarkers (**Table 1**).

Intracellular proteins such as Hb follow zero-order kinetics, leading to a steady-state level that is determined by the turnover of the cells. In the case of Hb, the lifespan

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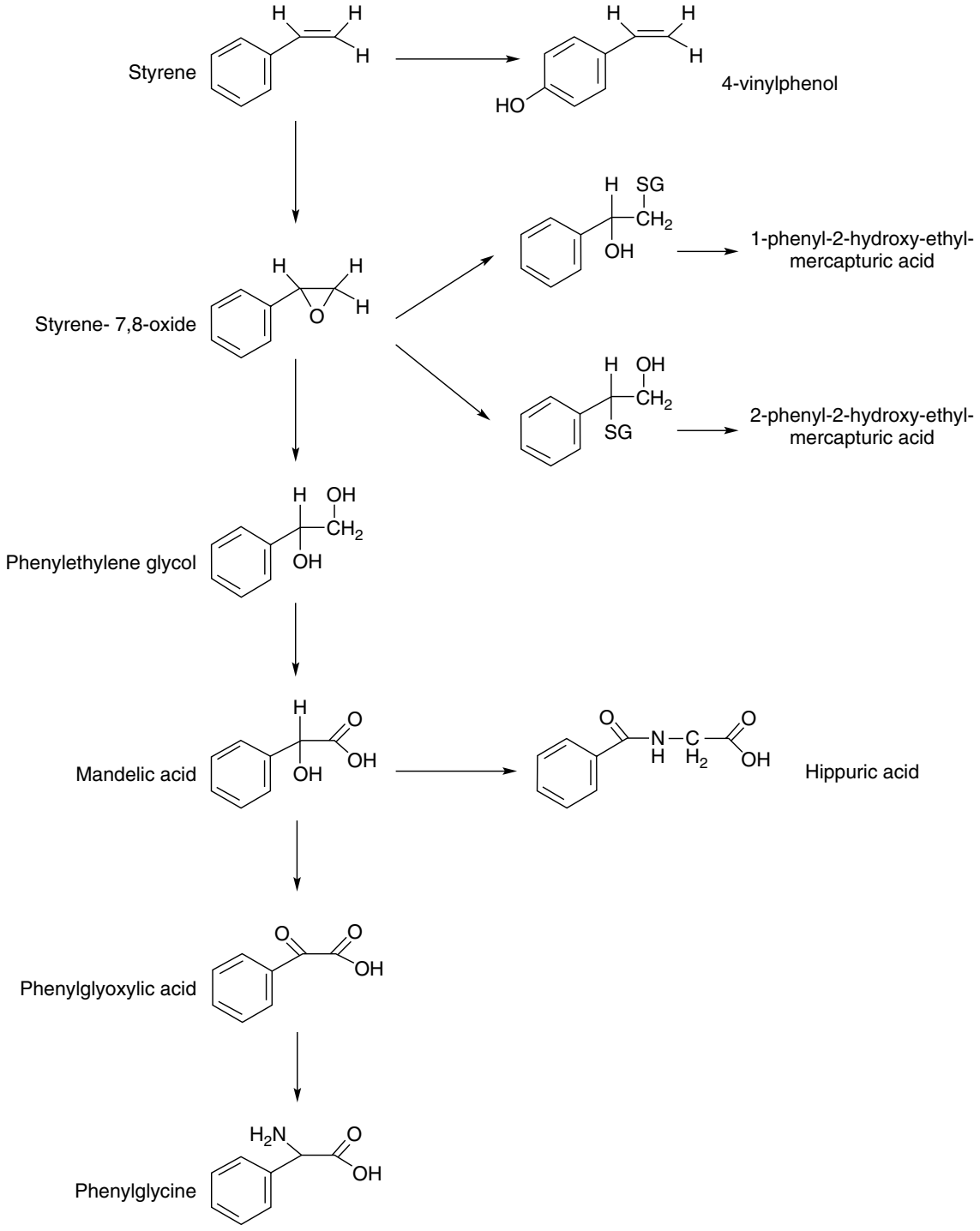

Figure 3 Part of the biotransformation pathway of styrene. This scheme shows the many options for selection of biomarkers. (Modified from Fustinoni *et al*., 2008.)

of the erythrocytes is 126 days in humans (Bishop and Surgenor, 1964), causing a linear decrease of the adduct after cessation of exposure with a half-life of 63 days in humans. This kinetic pattern was observed in studies following the adduct levels after withdrawal from smoking, although the levels of 4-aminobiphenyl (ABP) Hb adducts declined faster than expected (Maclure *et al*., 1990). The formation and decay of adducts to proteins free in solution, such as serum albumin (SA), follow first-order kinetics. For adducts to SA in humans this results in a half-life of 20 days (Bishop and Surgenor, 1964). Adducts to SA and Hb follow these kinetics as long as they are chemically stable. So far, a mechanism of enzymatic repair of adducts to SA and Hb has not

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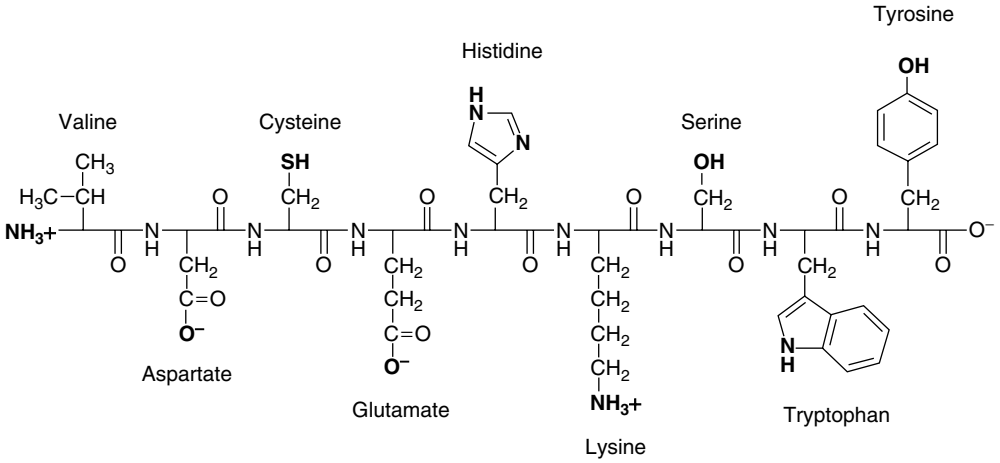

Figure 4 Hypothetical peptide showing amino acids in their predominating form at pH ~7. Atoms shown in bold represent free bases with nucleophilic reactivity. In this peptide valine is at the N-terminal position. (Modified from Törnqvist *et al*., 2002.)

been reported. Some studies have shown the formation of adducts to proteins with a longer lifespan than SA and Hb, such as histones. So far these adducts have not been detected in humans *in vivo* (Scheepers *et al*., 2008).

5 DNA ADDUCTS AND DNA-REPAIR PRODUCTS

5.1 Addition Products

Similar to proteins, the DNA bases also contain nucleophilic targets for covalent binding of electrophilic intermediates (**Figure 5**). For a long time, sensitive detection of these products relied on isolation of DNA from peripheral lymphocytes. Some attempts to use other tissues for isolation of DNA adducts have been successful, such as exfoliated bladder epithelial cells that are excreted with urine (Talaska *et al*., 1996) and exfoliated epithelial cells that can be retrieved from swipes of buccal mucous (Romano *et al*., 1999). With ^{32}P-postlabelling it is possible to detect one adduct in 10^8 nucleotides. Identification of the adduct relies on coelution of the isolated adduct from human blood samples with laboratory-synthesized adduct standards, preferably using a liquid chromatography system for separation. Nowadays the sensitivity of mass spectrometry (MS)-based techniques is in the same range as for the ^{32}P-postlabelling method (Singh and Famer, 2006).

In contrast to protein adducts, most DNA structure modifications are not stable, due to the activity of enzymatic repair (base and nucleotide excision repair, O^6-alkylguanine-DNA alkyltransferase, glycosylases, etc.). This means that some adducts may disappear after several hours to a few days. However, some bulky adducts, such as those formed by polycyclic aromatic hydrocarbons (PAHs) may be more stable. These adducts have been successfully used to characterize both occupational (Van Schooten *et al*., 1995; Binkova *et al*., 1998; Hemminki *et al*., 1997) and environmental exposures (Hemminki *et al*., 1990; Perera *et al*., 2005) to PAHs. These DNA-adduct levels also correlate well with tobacco smoking (Phillips *et al*., 1988; Talaska *et al*., 1991).

Phosphotriester adducts also appear more persistent because there are no known enzymatic repair systems in eukaryotic cells, and the development of methods for this type of adduct could yield interesting novel biomarkers for long-term exposure (Scheepers, 2008).

5.2 Products of DNA Repair

After repair, N-7-guanine and N-3-adenine adducts are excreted in urine, which offers an opportunity to use these enzymatic repair products as biomarkers of short-term exposure. Adducts of ethylene oxide to N-7-guanine have been chemically characterized by LC-ESI-MS/MS (liquid chromatography-electrospray ionization tandem mass spectrometry) (Marsden *et al*., 2007). Such repair products have also been used to characterize exposure to aflatoxin B1 (Ross *et al*., 1996; Qian *et al*., 1994). If determined in urine these products do not provide information on the tissue where the adduct was formed.

5.3 Other Approaches

Reactive oxygen species can be formed during inflammatory processes resulting from exposure to

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Table 1 Substances for which hemoglobin adducts were observed in humans

Substance	Adduct	Involved amino acid	Background (pmol g^{-1} globin)[a]		Reference
			Nonsmokers	Smokers	
Acetaldehyde	Acetaldehyde	Lys	—	—	Lin *et al*. (1993)
Acrylamide	*N*-2-carbamoylethyl	N-terminal val	30	100	Bergmark (1997)
Acrylonitrile	*N*-2-cyanoethyl	N-terminal val	<2	80	Bergmark (1997); Pérez *et al*. (1999)
3-Aminobiphenyl	3-Nitrosobiphenyl	Cys	ND	14.1	Sarkar *et al*. (2006)
4-Aminobiphenyl	4-Nitrosobiphenyl	Cys	44.3	220	Sarkar *et al*. (2006)
Benzene	*S*-(Phenyl)	Cys	—	—	Bechtold and Strunk (1996)
1,3-Butadiene	*N*-(2,3,4-Trihydroxy butyl)	N-terminal val	—	—	Osterman-Golkar *et al*. (1996); Sorsa *et al*. (1996)
Chrysene	r-1,t-2,3-t,c-4-Tetra hydroxy-1,2,3,4-tetrahydrochrysine	—	—	—	Day *et al*. (1990)
N,*N*-Dimethyl formamide	*N*-Methylcarbamoyl	N-terminal val and lys	—	—	Mraz *et al*. (2006)
NNK[b]	4-Hydroxy-1-(3-pyridil) -1-butanone	Asp, glu or terminal carboxylate	—	—	Carmella *et al*. (1993)
Ethylene oxide	*N*-2-Hydroxyethyl	N-terminal val	20	100	Törnqvist *et al*. (1986)
Glycidol	2,3-Dihydroxypropyl	N-terminal val	2–7	~10	Hindso-Landin *et al*. (1996); Hindso-Landin *et al*. (1997)
Propylene oxide	2-Hydroxypropyl	N-terminal val	2	4	Törnqvist and Ehrenberg (1990)
Styrene	*S*-2-Hydroxy-2-phenylethyl and 2-hydroxy-1-phenylethyl	Val	2.19	3.55	Teixeira *et al*. (2008)
	2-Hydroxy-2-phenylethyl and 2-hydroxy-1-phenylethyl	Cys	—	—	Fustinoni *et al*. (1998)

[a]Unknown; ND, not detected.
[b]Nitroamine-4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.
Based on Törnqvist *et al*. (2002).

fine particles. Some reactive intermediates of chemical exposures may also be involved in redox cycling or auto-oxidation, generating reactive oxygen species. Especially when transient metals are also present, reactive hydroxyl radicals can be formed. Such events can cause oxidative damage to DNA that is normally effectively repaired by nucleoside and base-excision repair enzymes, but a residue of repair products may be excreted. These repair products can be used as biomarkers for oxidative damage. Of several repair products 8-hydroxydeoxyguanine (8-OHdG) has been most used as a biomarker. This approach is valuable to assess general exposure to oxidants, but cannot be used to study the contribution of specific agents (Battershill *et al*., 2008).

Chromosome aberrations, sister chromatid exchanges (SCEs) and micronuclei can be related to cancer, but they are not specific to a particular chemical exposure. Also, the determination of urinary mutagenic activity in the *Salmonella typhimurium* assay (Bos and Jongeneelen, 1988) is nonspecific to the causal agents and therefore not suitable in most studies of exposure assessment.

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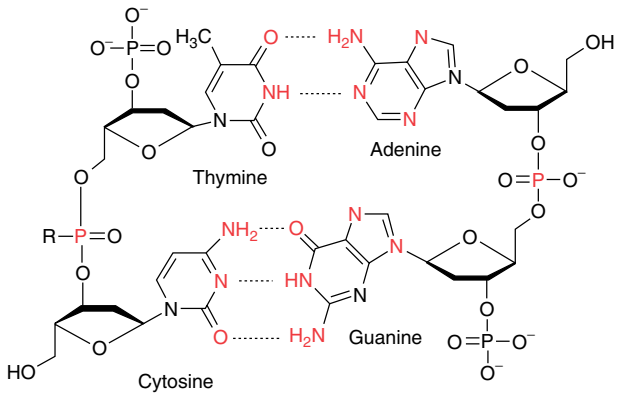

Figure 5 General structure of DNA with the four DNA bases and R representing the xenobiotic adduct-forming substance, in the case of a phosphotriester adduct.

6 GUIDELINES FOR HUMAN BIOLOGICAL MONITORING OF EXPOSURE TO CARCINOGENIC SUBSTANCES

6.1 Study Design

6.1.1 Research Question

Human biological monitoring can be used for scientific research, routine monitoring or to find answers to *ad hoc* questions.

Scientific research can be done to validate a biomarker in a volunteer study, that is, a study of kinetics of excretion of a biomarker, or a field validation to assess the suitability of a biomarker for the purpose of exposure characterization. Once validated, biomarkers can be used as quantitative exposure parameters in epidemiological studies.

Well-validated biomarkers can be used in long-term monitoring programmes to reveal trends in exposure that can help to support policy-makers. Examples of such programmes are NHANES (2008) and GerES (2008) (see list of references for web addresses of these projects).

Biological monitoring can be used to reconstruct exposure after chemical incidents in industry or any incidents that are marked by unintended release of chemicals into the environment. Biomarkers of exposure can also be used to evaluate the effectiveness of remedial actions in a workplace, such as a change in work procedures or introduction of personal protective equipment.

6.1.2 Study Population

The study population may be selected at random from the general population. In occupational settings the subjects who are at risk are often clearly identifiable by their job title. For some study aims it may be useful to compare two groups that are different with respect to one or more determinants of exposure, or that may be different in health status. Before selecting study subjects, a list of inclusion and exclusion criteria should be drafted.

6.2 Design

6.2.1 Follow-up Study

Sequential observations can be a good way to find out about work-related sources of exposure. In the setting of occupational exposure a pre-exposure or background value may be obtained on the morning before the start of the first shift of a workweek (preshift spot sample) and subsequent samples may be collected on the same day or on subsequent days during a working week, including a postshift sample at the end of the workweek. Using this design, a great number of individuals can be followed parallel in time. This strategy can be extended by including reference subjects. The reference subjects should have characteristics very similar to the subjects from the index group (age, social economic status, gender, smoking status), but with a suspected lower exposure. In practice, persons with mild, but specific, health complaints may be compared with persons that did not report any of these symptoms, to study the possible role of chemical exposure.

An intervention study is often used to evaluate a change in time. This can be a change in the determinants of emissions of toxic substances or a change in the determinants of exposure. In an intervention study, the initial situation (before the change) is compared to a new situation, using well-standardized methods. To rule out a temporal covariate, it is recommended to use a crossover design. Two possible designs for intervention studies are illustrated in **Figure 6**.

6.2.2 Confounders and Effect-modifiers

Besides the exposure to a carcinogenic substance, several host factors may have an influence on the biomarker level in an individual (**Table 2**). Some dietary factors may have a direct influence as sources of (co)exposure. For other factors it is possible to adapt the study design. For example, the results in smokers and nonsmokers can be analysed in separate groups. However, if factors are less frequent or the influence on biomarkers is not well understood, it is recommended to reduce their influence by standardization.

6.2.3 Questionnaires

The characteristics of study subjects can be registered in a questionnaire. Information about gender, age and biometry of the subjects, as well as some questions

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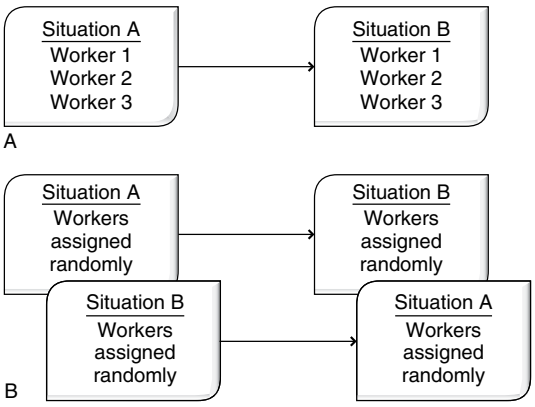

Figure 6 Study design to evaluate a transition from situation A to situation B. All workers can be followed at the same time (a). In order to rule out the influence of a temporal covariant (e.g. season or workday in the week) or the effect of sequence (e.g. a learning effect), the population can be randomly divided in subgroups (b). Each worker is assigned to one of the groups at random. Half of the population is followed in the transition from situation A to B, whereas the other half is followed in a transition from B to A.

about physical exercise, diet and alcoholic beverages, and use of medication is obtained using standardized questions. Sometimes outliers in biomarker levels can be explained using the information from the questionnaire.

Table 3 contains examples of some coexposures that could interfere with the primary exposure of interest. In the case of collection of repeated spot urine samples, breath samples or blood samples, some questionnaires have to be completed (repeatedly) at each moment of sample collection.

6.3 Ethics Issues

In this section some general guidelines are given regarding ethics issues that need consideration during the processes of preparation and performance of human biological monitoring studies. Information will be provided for the study protocol that is needed in order to obtain approval from the ethics committee that is responsible for the evaluation of scientific studies involving human subjects.

6.3.1 Informed Consent

An information and consent procedure is normally used in biomedical research to inform human subjects about the purpose of the study, their role in the study and their rights (e.g. the possibility to discontinue their participation and their own and external access to personal data). This procedure should be in line with the Declaration of Helsinki (World Medical Association, 2000).

Table 2 Factors that may influence the observed level of a biomarker

Factor	Influence	Remedy
Circadian rhythm	Temporal variation in composition of body fluids	Standardize times of sample collection
Body fat	Influence on toxicokinetics	Register body mass index or a more specific measure of body fat
Meal	Influence on blood lipid content	Collect samples before rather than after a meal
Smoking	Direct influence on levels of several biomarkers (sometimes indirect by induction of metabolic activity)	Study influence of smoking in a separate group and standardize for this effect. Register the amount smoked per day
Alcohol	Inhibition or induction of metabolic activity	Register the number of alcoholic beverages per day
Medication	Inhibition or induction of metabolic activity	Register use of prescribed and nonprescribed medication
Diet	Direct influence on levels of several biomarkers	Register use of certain food components, standardize by asking volunteers to refrain from consumption of certain specific food components
Food supplements	Direct influence on levels of several biomarkers	Register the use of certain specific food supplements like creatine monohydrate
Physical exercise	Influence on toxico-kinetics	Register level of physical exercise and evaluate impact by physiologically based pharmacokinetic (PBPK) model

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Table 3 Possible sources of coexposure in the general population

Chemical marker	Source
Antineoplastic agents	Treatment with these agents
(Total) arsenic	Consumption of seafood
Chlorinated hydrocarbons	Evaporation from recently dry cleaned clothes
Nickel/chromium	Orthopaedic or dental implants
PAH	Treatment of psoriasis or eczema with coal-tar ointment or use of coal-tar containing shampoo; consumption of broiled, fried, smoked and barbecued meat and fish
Phthalates	Leaching from plastics
Platinum	Exhaust fumes from gasoline-powered vehicles with platinum-containing three-way catalysts

6.3.2 Sample Coding

Coding is a measure to ensure confidential handling of information, protecting the individual and enabling blinded analysis. It should be specified by whom, where and how the samples are coded, how the code forms are kept in an archive and what links between code and study outcome exist (Knudsen, 2004; 2005). Persons who have access to the code list must be identified. It should also be clear for how long the code lists will be kept and who is responsible for their destruction.

6.3.3 Protection of Privacy of Study Subjects

During the study, the privacy of the study subjects should be respected. Normally, the information identifying a person will be kept in the medical file by the physician directly responsible for the medical health of the study subjects (occupational physician or general physician). Also databases containing the results from questionnaires should be made anonymous. If explicitly requested individual study subjects will be granted access to their individual study results.

6.3.4 Obtaining Ethics Approval

To obtain ethics approval a request should be submitted to a national, regional or local review committee recognized by the (health) authorities. The purpose of the study and the study protocol should be outlined. The study protocol includes the method of recruitment of study subjects, inclusion/exclusion criteria of study subjects, description of activities that may lead to exposure to specific carcinogenic substances studied, study design, specification of the analytes, future use of samples and protection of privacy of the study subjects. Usually all information and documents that will be provided to the study subjects should also be reviewed by the ethics committee.

6.4 Communication

6.4.1 Introduction to the Study

Depending on the purpose of the study and the size of the study population, a study can be introduced personally by a physician or during a meeting by oral presentation. It is useful to supply written information to the study subjects in any case. The most important points that should be addressed are presented in **Table 4**.

6.4.2 Obtaining Informed Consent and Enrollment

The purpose of informed consent is to make sure that persons participate in a study purely on a voluntary basis. Participants should not feel obliged to take part based on (material or immaterial) obligation to the investigator. Also, they should be informed of all details concerning the study and their participation, and be convinced that no information is withheld from them. They should have the opportunity to ask questions and let additional information influence their decision to take part. The informed consent form states that even after taking the decision to participate and signing the form, the participants may decide to discontinue participation without the obligation to explain to the investigator about it.

6.5 Collection, Transport and Storage

In this section the collection, transport and storage of blood, urine and exhaled air samples will be discussed in general terms. More specific information can be found in biological applications datasheets that have been made available on the internet for specific carcinogenic substances (BIOMONECS, 2006).

6.5.1 Blood

Blood can be collected from the forearm by vena puncture. If only 5–10 µl of blood is required, a skin prick with a sterile lancet to collect blood from a vein in the finger of the study subject may suffice. For metals, special tubes with low metal content are needed. If plasma and blood cells are to be used, an anticoagulant should be

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Table 4 Issues to be included in information provided to the study population

Subject	Contents
Purpose	The purpose of the study should be clearly stated.
Parameters	The parameters that will be investigated should be specified and the relationship of these parameters with the goal of the study should be clarified.
Role of participants	The role of the participants should be made clear, specifying in detail when and how samples will be collected, how and when questionnaires will be completed and what other requests are made to the participants. It should be made clear that participation is on a voluntary basis.
Privacy	Clarify how the privacy of the participants will be protected during and after the study. Will the data collection be anonymous and who will keep the biological samples and the data, and for what period of time? Also restrictions and procedures for future use of biological samples or data (by third parties) should be described.
Recruitment	The procedure for recruitment of participants should be explained (e.g. the use of inclusion/exclusion criteria) and the informed consent procedure should be explained.
Results	The moment and the way of presenting the study outcome to the participants should be made clear. It is important to inform the participants of any plans to disseminate the information to health authorities or to the scientific community or by uploading the data into a specific computer database.
Compensation	It should be made clear to what extent participants will be compensated for efforts and expenses.
Financial support	It should be stated how the study is financially supported and which parties are involved.
Contact	Names and contact information of the study coordinator(s).

added. If using an anticoagulant the tubes should be thoroughly mixed directly after blood collection.

If isolated red or white blood cells are needed, it is recommended that the cells be precipitated by centrifuge and the cells rinsed with a sterile solution of 0.9% sodium chloride within eight hours of collection. Alternatively, lysis may be required to terminate any biochemical activity in the blood cells prior to storage. Isolated cell fractions and plasma may be stored at $-20\,^{\circ}C$ to $+4\,^{\circ}C$, depending on the stability of specific analytes. For blood cells to be used in biological assays, the addition of freezing medium with stabilizer and freezing at $-80\,^{\circ}C$ is common.

6.5.2 Urine

With a spot urine sample an aliquot of urine is collected from one void, for example, a preshift and postshift sample in workers. A sample collected just after awakening is a well-defined concentrated preshift sample.

For postshift urine collection, the study subjects should take off any contaminated (work) clothes and wash their hands before collecting a urine sample to avoid contamination of the urine sample or the urine container.

For 24 hour urine, all urine should be collected. As an alternative, the study subject can be asked to collect a part of each void (e.g. midstream), but in that case the total volume of each void should also be registered. Collection of 24 hour samples is not recommended, unless the study subjects are well instructed and collection of such samples is needed because of the study purpose. This may be required after an accidental high exposure or in a study where volunteers are exposed under controlled conditions, for example, to study excretion kinetics.

Prelabelled polypropylene and polyethylene bottles can be used with a wide opening that can be sealed with a screw cap. When analysing metals it is generally recommended to rinse the urine container with a 3% nitric acid solution prior to use. Urine bottles should be filled to a maximum of three-quarters of the container volume when storing at temperatures below $0\,^{\circ}C$.

In spot urine samples, adjustment for the sample density is recommended. The most common correction is by determination of the urinary creatinine content. A normal creatinine level is around $1\,g\,l^{-1}$. Values as low as $0.05\,g\,l^{-1}$ or higher than $2.5\,g\,l^{-1}$ should be treated as an outlier. In that case the creatinine level is not reliable and may be indicative of an error during sample collection or abnormal kidney function.

On-site treatment of the samples is only needed to protect the analyte or the integrity of the sample. Micro-organisms may appear in urine samples due to contamination from the ambient environment or due to an infection of the bladder or urethra. Samples may be stored at $-18\,^{\circ}C$. If the samples are stored at $4\,^{\circ}C$ for not much longer than 24 hours no preservation is needed. Organic preservatives, such as natrium azide or citric acid may interfere with the analytical procedure and therefore it is useful to verify any interference with the analysis in advance. Inorganic preservatives that may be used are acid solutions (analytical grade solutions of hydrochloric acid, nitric acid or sulfuric acid) until a pH of approx. 2 is reached. Use of a 6 M solution of hydrochloric acid is

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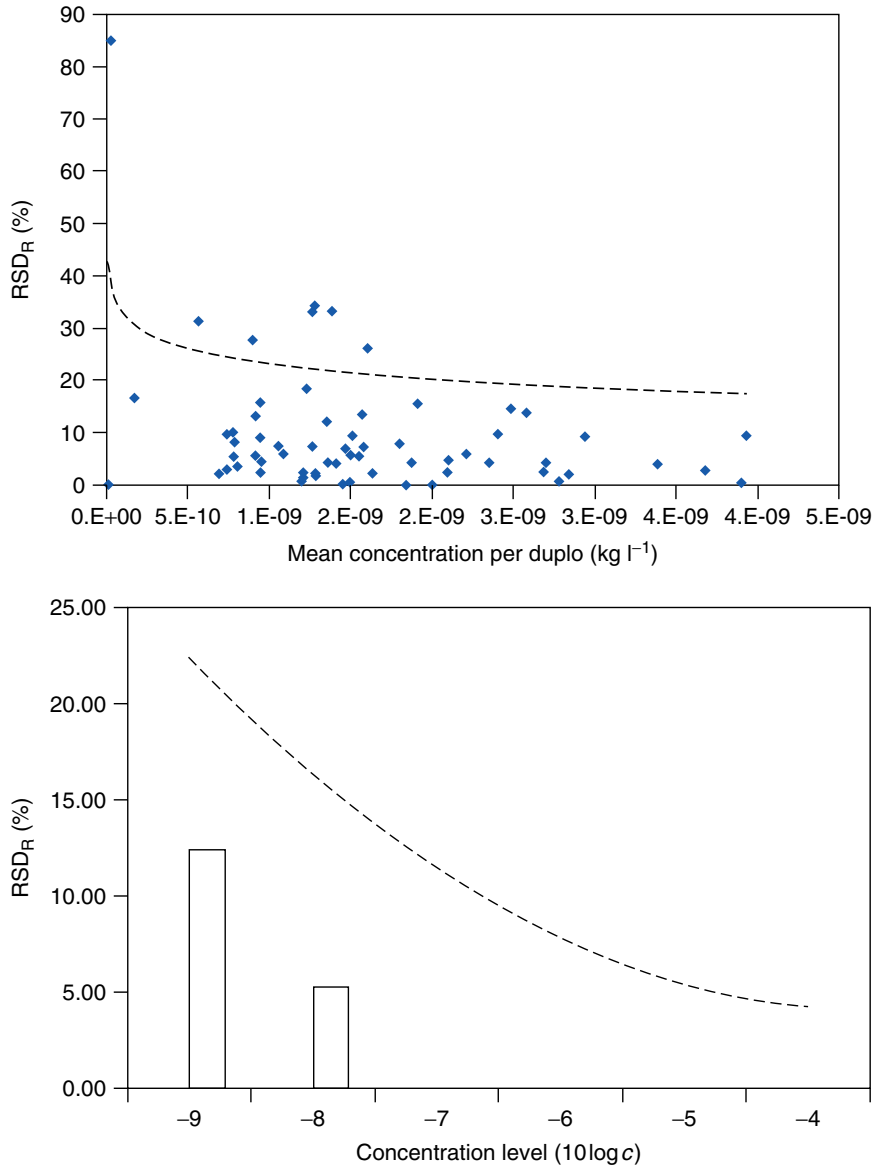

Figure 7 RSD_R in relation to concentration of nickel (μg l^{-1}) in background samples. The dotted line represents the RSD_R required according to IUPAC (1990) in both panels. The raw data are presented in the upper panel. For seven samples the RSD_R exceeds the required value. The lower panel shows that the aggregated data fulfill the IUPAC requirement.

effective as a preservative for storage at 4 °C. A warning text should be printed on the label if a preservative is added to the urine bottle in advance.

After storage at 4 °C or lower, a precipitate may appear, consisting of salts and sometimes protein sediment that could capture some of the analyte inside. It is recommended to gradually warm up the sample to 37 °C in a water bath, and homogenize the sample thoroughly before analysing.

6.5.3 Exhaled Air

End-exhaled air is recommended as a well-defined fraction that can easily be collected in the field. For collecting an end-exhaled sample, a breath sampler can be used (**Figure 2**). The study subject should be instructed to take the sample after a standardized period of time following the end of exposure. The sample should be collected in a clean environment. Breath sampler and solid sorbent tubes may be reused after cleaning. When cleaning, instructions from the supplier of these materials should be followed and materials should be tested before use, to avoid carryover of organic analytes from one sample to another. Storage of breath samples in the gas phase is not recommended because analytes may be lost due to leakage of the breath-sample container, adsorption of the analyte onto the sample container and/or by chemical reactions of the analytes with other components in the breath sample. It is recommended to transfer the organic substances from the breath sampler to a solid sorbent

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Table 5 Background values of nonsmoking adults

Substance	Biomarker	N	0.95 percentile	
			$\mu g\,l^{-1}$[a]	$\mu g\,g^{-1}$ creatinine[a]
Aniline	Aniline in urine	60[ee]	22.1	23.8
Arsenic	Arsenic in urine	64	20.4	23.0
Benzene	Benzene in end-exhaled air	65	$8\,ng\,l^{-1}$	—
	S-Phenyl-mercapturic acid in urine	64	7.3	6.5
	t,*t*-Muconic acid in urine	64	0.32	0.33
Butylbenzylphthalate (BBP)	MBP[b] in urine	64	116	146
	MBzP[c] in urine	64	29.7	28.0
Cadmium	Cadmium in urine	64	0.36	0.39
Chloroform	Chloroform in end-exhaled air	60[ee]	$0.1\,ng\,l^{-1}$	—
Chromium	Chromium in urine	64	0.39	0.42
Diethylhexylphthalate (DEHP)	5-oxo-MEHP[d] in urine	64	82.6	72.3
	MEHP[d] in urine	64	25.1	26.3
	5-Hydroxy-MEHP[d] in urine	64	99.6	109
	5-Carboxy-MEHP[d] in urine	64	142	152
Nickel	Nickel in urine	64	3.3	3.3
Platinum	Platinum in urine	60[ee]	$30\,ng\,l^{-1}$	$35\,ng\,g^{-1}$
Polycyclic aromatic hydrocarbons	1-Hydroxypyrene in urine	64	0.20	0.17
Tetrachloroethylene	Tetrachloro-ethylene in end-exhaled air	54[ee]	$0.2\,ng\,l^{-1}$	—
o-Toluidine	*o*-Toluidine in urine	60[ee]	0.50	0.62
Trichloroethylene	Trichloro-ethylene in end-exhaled air	54[ee]	$0.1\,ng\,l^{-1}$	—

[a]Unless otherwise indicated.
[b]MBP = mono-*n*-butyl.
[c]MBzP = mono-benzyl phthalate.
[d]MEHP = mono-(2-ethylhexyl) phthalate.
[e]No subjects from United Kingdom.

within a few hours of sample collection. Once the organic analytes are transferred to a solid sorbent, the sample can be stored at room temperature in a clean environment for a prolonged period of time. It is recommended to seal the sorbent tubes in a gas-tight plastic bag to avoid contamination during transportation and storage.

6.6 Chemical Analysis

6.6.1 Performance Characteristics

The most important characteristics are precision and sensitivity. Precision is expressed in reproducibility and repeatability. Reproducibility is usually expressed as a relative standard deviation (RSD). The criterion for acceptable reproducibility can be taken from a consensus publication from IUPAC (Pocklington, 1990) as the between-laboratory reproducibility relative standard deviation (RSD_R) that can be calculated from $2^{(1-0.5\log c)}$ where c is the concentration expressed as a decimal fraction (**Figure 7**). The within-laboratory method performance, expressed as the repeatability relative standard deviation (RSD_r) is about one-half to two-thirds of the RSD_R (Pocklington, 1990).

The sensitivity of a method is determined by the limit of detection (LOD) and quantification limit of quantification (LOQ). The LOD is defined as the lowest amount of analyte in a sample which can be detected, but not quantified. The lowest amount of analyte in a sample which can be quantitatively determined with specified precision and accuracy under certain experimental conditions is called the limit of quantification.

6.6.2 External Quality Assurance

The quality of the analysis should be evaluated using reference standards. For biomarkers only few certified

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reference materials are available from the Community Bureau of Reference (BCR) in Brussels. Round robins are useful for intercomparison of lab performance using matrix-mixed standards for different biological materials.

6.7 Interpretation of Results

6.7.1 Background Values

Some studies provide background values for biomarkers in the general population, such as for metals by Brune *et al*. (2003). In 2006, background values were established as part of a EU project (**Table 5**). For this campaign, samples were collected in 2003 and 2004 in Belgium, Denmark, Germany, Sweden, the Netherlands and the United Kingdom in male and female (1:1), mostly white-collar workers, 18–65 of age. All subjects were nonsmokers or smokers who stopped smoking more than six months prior to sample collection.

6.7.2 Biological Limit Values

The EU has a system of biological limit values (BLVs), although not many BLVs have been published so far. The ACGIH has a system of biological exposure indices (BEIs) that is updated each year (ACGIH, 2008). In addition BAT values established by Deutsche Forschungsgemeinschaft (2008) can also be used.

6.8 Dissemination of Results

It is preferred to collaborate with a physician, who is authorized to keep the study outcome and the key to the person code list (two-way verification of person identity). In this way it is possible for the physician to disclose the data to an individual subject if needed. This may be done if the outcome is unexpected and may be indicative of a risk to the health of the subject.

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Cardiac Toxicity of Anthracyclines

Richard D. Olson and Barry J. Cusack

CONTENTS

1 INTRODUCTION

The first doxorubicin with broad-spectrum activity used in the treatment of cancer was doxorubicin (DOX) (adriamycin). It was isolated by Federico Arcamone and coworkers in 1968 from microbes (*Streptomyces peucetius var.caesius*) in the soil near the Adriatic Sea, hence the name adriamycin. DOX has been used in treatment of breast cancers, acute lymphoblastic leukaemias, sarcomas and small-cell carcinoma of the lung. Its efficacy has made DOX one of the most important chemotherapeutic agents to increase childhood cancer survival.

Anthracyclines, including DOX, daunorubicin (DNR) and epirubicin, have troublesome side effects that decrease their clinical utility and prevent their use in some clinical settings. Anthracyclines produce mucousitis, nausea, myelosuppression and alopecia. These side effects are acute and reversible, although neutropenia is frequently the dose-limiting side effect in clinical practice. However, this class of antineoplastic agents is most known for an irreversible, chronic cardiotoxicity that limits the total, cumulative dose. It may be fatal.

Anthracyclines can elicit an early (acute) and delayed (chronic) cardiotoxicity (Bristow *et al*., 1978; Buzdar *et al*., 1985). The acute cardiotoxicity can manifest with electrocardiogram (ECG) changes including ST-T changes, widening of the QRS complex and decreases in voltage. Dysrhythmias, pericarditis, myocarditis and reversible decreases in cardiac contractility have been reported clinically. These acute changes are usually not considered a major clinical problem (Bristow *et al*., 1978), although some researchers suspect that both the acute and chronic injury may summate yielding the overall clinical picture of anthracycline cardiotoxicity. The chronic cardiotoxicity can be fatal (Buzdar *et al*., 1985) and has necessitated lowering the recommended total cumulative dose, especially in combination with other antineoplastic drugs, such as herceptin and taxanes in treatment of breast cancers (Dresdale *et al*., 1983; von Hoff *et al*., 1979; Watts, 1991). Cardiotoxicity of anthracyclines is identified

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by echocardiography or radionuclide angiography, but the most sensitive technique is myocardial biopsy.

2 MODELLING ANTHRACYCLINE CARDIOTOXICITY: TARGETS OF INJURY

2.1 Acute *In Vitro* Cardiotoxicity

Acute and chronic animal models of anthracycline cardiotoxicity have yielded important insights into how anthracyclines injure the heart. Perhaps the earliest model of anthracycline cardiotoxicity was the isolated contracting cardiac muscle fibre or tissue. These included isolated contracting atria and ventricular muscle preparations studied *in vitro*. As a result of these studies, we now know the subcellular targets and anthracycline structures responsible for anthracycline cardiotoxicity in this model.

The hallmark of DOX cardiotoxicity in the isolated rabbit atria is a selective decrease in contractility of the first contraction following a rest interval, compared to contractility of repetitive contractions at a steady-state contraction rate of 1 Hz (Shadle *et al*., 2000; Burke *et al*., 2002). The first contraction following the rest interval is normally enhanced because of an increase in sarcoplasmic reticulum (SR) activator calcium (Ca^{2+}) during the rest interval. Thus, interventions or drugs that selectively impair SR Ca^{2+} function, such as caffeine, selectively impair enhanced contractility of the rested contraction compared to contractility of the repetitive contractions at steady state, which is less dependent on SR Ca^{2+} stores (Bers, 1985; Bose *et al*., 1988; Bouchard *et al*., 1989; Bouchard and Bose, 1989). DOX and DNR, like caffeine and ryanodine, selectively impair postrested contractions, suggesting that anthracyclines cause cardiotoxicity in this model by impairment of SR function (Shadle *et al*., 2000; Burke *et al*., 2002).

Anthracyclines, like caffeine, also impair the force–frequency relationship of cardiac muscle. For example, in rabbit atria or papillary muscle preparations, the increase in contractility that occurs at higher rates of stimulation is selectively decreased compared to contractility at slower rates of contraction (steady state). The enhanced contractility that occurs at higher rates of contraction (treppe) results, in part, from increased SR Ca^{2+} stores (Shadle *et al*., 2000; Burke *et al*., 2002).

Further evidence for the idea that anthracyclines target SR comes from comparative studies with neonatal rabbit atrial preparations (Burke *et al*., 2002). Neonatal rabbit atria have immature and relatively nonfunctional SR, and activator Ca^{2+} comes primarily from transsarcolemmal Ca^{2+} sites (Burke *et al*., 2002; Klitzner and Freidman, 1989). Thus, agents that selectively impair SR function should have attenuated effects on contractility. Indeed, caffeine and anthracyclines elicit dramatically reduced impairment of contractile function in the neonatal-rabbit isolated atrial preparation (Burke *et al*., 2002).

In isolated SR preparations from dog heart, caffeine and anthracyclines have very similar effects on Ca^{2+} release (Shadle *et al*., 2000; Olson *et al*., 2000). Both caffeine and anthracyclines cause Ca^{2+} release from Ca^{2+}-loaded SR preparations, an effect blocked by ruthenium red, indicating that anthracycline- and caffeine-induced Ca^{2+} release is mediated through the Ca^{2+} release channel. These effects by anthracyclines on SR are not free-radical mediated, but require the quinone moiety since they occur rapidly and free radicals are not detected in the isolated SR preparation (Shadle *et al*., 2000; Olson *et al*., 2000). Replacement of the quinone with an imino (DNR vs. 5-imino DNR) abolishes the anthracycline impairment of cardiac function and SR Ca^{2+} release (Shadle *et al*., 2000; Olson *et al*., 2000).

In summary, in the isolated cardiac and SR preparations *in vitro*, anthracyclines cause toxicity by targeting the SR and impairing cardiac Ca^{2+} metabolism through a mechanism requiring the quinone moiety, but probably independent of redox cycling and free-radical generation.

2.2 Chronic Cardiotoxicity

Is the SR a target in the more clinically relevant chronic model of anthracycline cardiotoxicity? At the onset of chronic cardiotoxicity, cardiac SR begins to swell and vacuoles appear, suggesting dysfunction in activator Ca^{2+} sequestration, storage or release, and chronic DOX cardiotoxicity leads to impairment of cardiac Ca^{2+} transients (Olson and Mushlin, 1990; Singal *et al*., 1987; **Figure 1**). The decrease in Ca^{2+} transients appears to result, at least in part, from loss of SR activity. (Dodd *et al*. 1993) found that the density of the cardiac SR Ca^{2+} release channel was reduced by 50% in a chronic rabbit model of DOX cardiotoxicity, suggesting that the SR might be an important target in DOX-induced disruption of cardiac Ca^{2+} homeostasis. Arai *et al*. (1998) demonstrated that chronic DOX administration in rabbits down-regulated the gene expression of cardiac SR Ca^{2+} release channel and SR Ca^{2+} pump, phospholamban and messenger RNA. Both SR Ca^{2+} release channel density and messenger RNA (mRNA) concentration significantly regress with left-ventricular fractional shortening (FS) assessed by M-mode echocardiography to predict the degree of cardiac dysfunction (**Figure 2**). These data suggest that SR is an important subcellular target in chronic DOX cardiotoxicity.

How might chronic anthracycline administration lead to a decrease in protein concentrations of SR Ca^{2+} release

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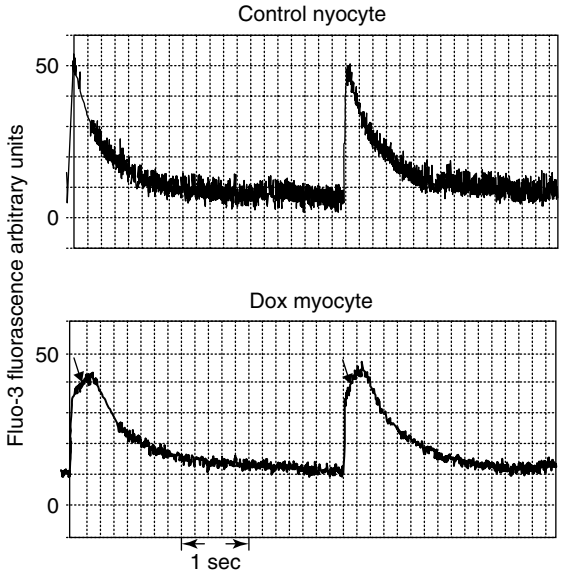

Figure 1 Examples of fluo-3 calcium transients elicited by electrical stimulation (0.25 Hz, 37 °C) in isolated adult ventricular myocytes obtained from a pair-fed control (top tracing) and from a chronic doxorubicin-treated rabbit (bottom tracing). The control myocytes show a typical very rapid upstroke of $[Ca^{2+}]_i$ to a peak with a subsequent decline. In this example, there is some high-frequency tape recorder noise superimposed on the calcium transient. The bottom tracing shows an abnormal calcium transient contour, which we found typical of doxorubicin-treated male rabbit myocytes. The initial upstroke is somewhat slower than in the control myocytes, and is followed by a very distinctive and unusual secondary slowing in the upstroke of the calcium transient (see arrow) which results in a significant delay in the time to peak of the calcium transient. (In collaboration with Dr. William H. Barry, University of Utah, SLC, Utah.)

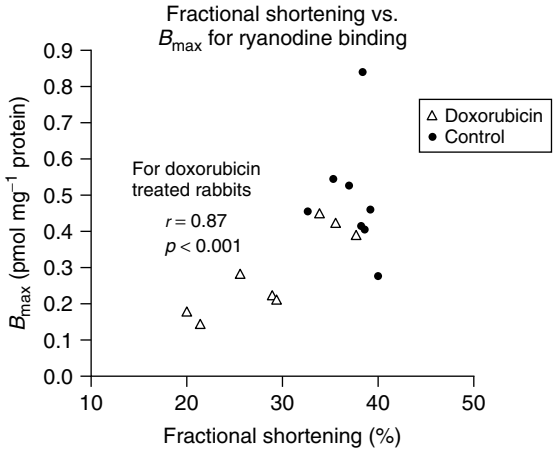

Figure 2 Correlation between the binding of [^{3}H]-ryanodine to the SR calcium release channel and left-ventricular fractional shortening in rabbits chronically treated with doxorubicin and their pair-fed, saline-treated controls.

channels and associated proteins? Although the answer to this question is currently unknown, one possibility relates to decreases in thyroid hormone in the chronic model of DOX cardiotoxicity.

Triiodothyronine (T3), the active cellular form of thyroid hormone (thyroxine, T4), is a powerful effector of Ca^{2+}-handling proteins and contractile function of the heart. Circulating T4, released from the thyroid gland in response to thyroid stimulating hormone (TSH), is converted into T3 primarily by iodothyronine 5′monodeiodinase in the circulation and liver. In the myocyte, T3 binds to nuclear T3 receptors and the complex then binds to thyroid hormone response elements on genes to regulate transcription. The SR Ca^{2+} pump (SERCA2) gene contains thyroid-hormone-receptor-responsive elements and both the SR Ca^{2+} pump and Ca^{2+} release channel are under the regulatory influence of T3 (Jiang *et al*., 2000; Muller *et al*., 1997; Rohrer and Dillman, 1988; Pennock *et al*., 2000). Indeed, thyroid hormone modulates the abundance of SR Ca^{2+}-release-channel receptors and the degree of [^{3}H]-ryanodine binding to the Ca^{2+}-release-channel receptors (B_{max}) in a rabbit model (Jiang *et al*., 2000).

Cardiotoxic doses of DOX in the chronic rabbit model caused nearly an 80% reduction in free triiodothyronine (fT3) up to three months after cessation of DOX administration (**Figure 3**). This was not due to caloric restriction from anorexia, since age-matched pair-fed control rabbits had normal fT3 levels (**Figure 3**). The data from these studies suggest that the role of T3 in mediating changes in SR proteins in the chronic model of DOX cardiotoxicity needs further evaluation.

In summary, it appears that the SR is a target of injury in both the acute and chronic animal model of anthracycline cardiotoxicity. Other sites, including mitochondria, have also been suggested to be injured, perhaps leading to apoptosis and myocyte dropout that occurs in anthracycline cardiotoxicity.

3 RISK FACTORS FOR ANTHRACYCLINE CARDIOTOXICITY

3.1 Anthracycline/Herceptin Drug Interaction

The HER2 (ErbB2) proto-oncogene encodes a 185 kDa transmembrane tyrosine kinase that is a member of the epidermal-growth-factor family and has important effects on cell growth, differentiation and cell survival (Slamon *et al*., 2001; Seidman *et al*., 2001; 2002; Ewer *et al*., 1999; Feldman *et al*., 2000). HER2 appears to have

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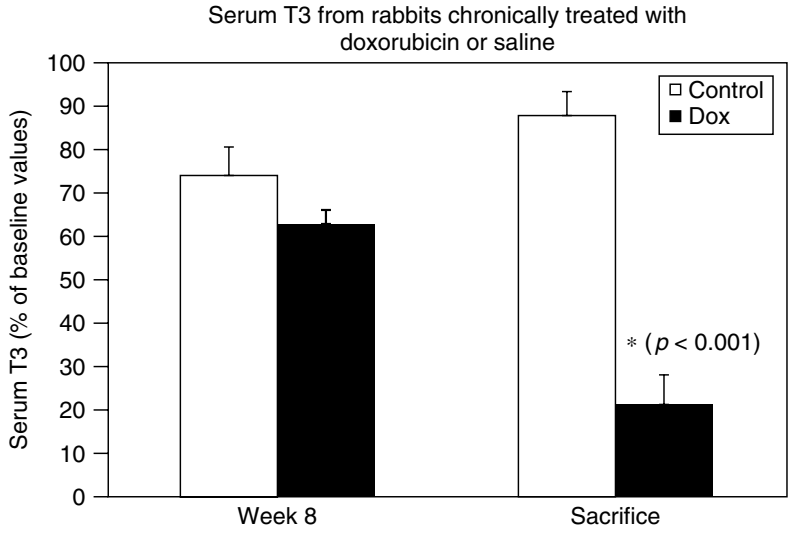

Figure 3 Serum concentrations of free triiodothyronine (T3). The T3 values were obtained eight weeks after beginning DOX or saline injections and at sacrifice, and expressed as a percentage of pretreatment baseline values. Values are mean ± SEM. *$p < 0.001$ vs. control.

an important role in the adult heart. Ozcelik *et al*. (2002) conditionally mutated HER2 in cardiomyocytes of mice. Most of the mutated mice reached adulthood, but developed a severe dilated cardiomyopathy. Crone *et al*. (2002) also developed a conditional knockout HER2 mouse model in which the protein expression was inhibited by 70% compared to control mice. At three months of age, there was no difference in body weight or heart rate between the wild-type and left ventricular end diastolic diameter. However, echocardiographic analysis of hearts from the mutated mice showed decreased FS and enlarged end diastolic dimensions (left ventricular end diastolic diametersLVEDDs) compared to the wild-type ($FS = 38 \pm 3\%$ vs. $19 \pm 2\%$; $LVEDD = 3.7 \pm 0.1$ mm vs. 4.6 ± 0.2 mm). Hearts from the conditional knockout mice also had increased apoptosis and overexpression of the anti-apoptotic gene *Bcl-xL* partially protected the increase in LVEDD and decrease in FS, suggesting that apoptosis may be important in development of heart failure. These studies have led to the speculation that HER2 may mediate cell survival by blocking apoptosis and cell-death injury. Impairment of these pathways may create a susceptibility for increased anthracycline-induced cardiotoxicity (Chien, 2000; 1999).

Approximately 45 000 women will die of breast cancer in the US this year (Slamon *et al*., 2001). Of women with breast cancer, 25–30% overexpress HER2 (Slamon *et al*., 2001; Seidman *et al*., 2001; 2002; Ewer *et al*., 1999; Feldman *et al*., 2000). Higher levels of HER2 is associated with a worse prognosis, which prompted researchers to target the HER2 protein in women with breast cancer (Slamon *et al*., 2001; Seidman *et al*., 2001; 2002; Ewer *et al*., 1999; Feldman *et al*., 2000). Thus, a humanized monoclonal antibody to the protein was developed (herceptin, traz) and when used in combination with other forms of chemotherapy, increased median time to disease progression from 4.6 months (chemotherapy alone) to 7.4 months and a lower death rate at one year (22% vs. 33%; $p = 0.008$) (Slamon *et al*., 2001). Cardiac toxicity was the most severe side effect, which occurred in 27% of women treated with traz, an anthracycline and cyclophosphamide and 13% treated with traz and paclitaxel (cardiac toxicity occurred in 8 and 1% of patients that received only the anthracycline and paclitaxel treatment arm, respectively (Slamon *et al*., 2001). Retrospective review of other studies (Seidman *et al*., 2002) confirmed that the risk of cardiac dysfunction was highest when anthracyclines were used in combination with traz and has led to the reduced clinical use of anthracyclines with traz in treatment of metastatic breast cancer overexpressing HER2. This is unfortunate because anthracyclines and traz yield the best therapeutic benefit (Slamon *et al*., 2001).

3.2 Gender-Related Risk for Cardiotoxicity

Recent data reveals that anthracycline cardiotoxicity is more pronounced in female than male cancer patients. Females have a 300% greater risk of DOX cardiotoxicity than males, which increases to 500% if females are postpubescent, suggesting a contribution by sex hormones (Lipshultz *et al*., 1995; Sibler *et al*., 1993). These recent clinical findings suggest that DOX cardiotoxicity is a significant women's health issue and has

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mandated reducing the cumulative dose of DOX to 360 mg m^{-2}, when used with taxanes or eliminating altogether the combined use of DOX with herceptin (Seidman *et al*., 2002; Gianni *et al*., 1995; Dombernowsky *et al*., 1996). Unfortunately, these recommendations are at the expense of reduced effectiveness in treating breast cancer.

The mechanism of this gender-related difference in risk for developing DOX cardiotoxicity remains enigmatic. One explanation for the gender-related differences in cardiotoxicity may relate to differences in cardiac and plasma pharmacokinetics of anthracyclines. Females had half the average plasma clearance of DOX than males, which might lead to higher cardiac levels of DOX and intracardiac conversion to the cardiotoxic metabolite, doxorubicinol (DOXOL) (Paz-Ares *et al*., 1995; Wasan and Kwong; 1997).

What might contribute to gender differences? Gender differences in risk for DOX cardiotoxicity suggest that sex hormones might contribute. Oestrogens alter gene expression of many enzymes. For example, oestrogen causes an increased expression of cardiac nitric oxide synthase (NOS; endothelial, eNOS and neuronal, nNOS) (Weiner *et al*., 1994; Andersen and Stender, 2000; Hayashi *et al*., 1997; Chen *et al*., 2003; Nuedling *et al*., 1999; Fraser *et al*., 2000; Wang and Abdel-Rahman, 2002; Xu *et al*., 1999). In addition, oestrogens cause the inactive form of eNOS to dissociate from a scaffolding caveolae protein in sarcolemma (a scaffolding caveolae protein in sarcolemma), yielding the active form. Recent studies demonstrate that DOX is a substrate for all three isoforms of NOS (nNOS, eNOS and inducible, iNOS) (Garner *et al*., 1999). DOX binds to the reductive domain of NOSs and undergoes a semiquinone redox cycling to generate superoxide anions and other free radicals. This interaction of DOX with eNOS has been demonstrated to cause cytotoxicity, including impairment of iron metabolism, apoptosis and lipid peroxidation, and has been suggested to mediate DOX cardiotoxicity (Kalivendi *et al*., 2001; Vasquez-Vivar *et al*., 1999; Duquaine *et al*., 2003). Since cardiac levels of eNOS and nNOS are expressed at higher levels in females than males, DOX might cause a selective gender-related increase in cardiac oxidative damage and free-radical injury contributing to the gender differences in DOX cardiotoxicity.

3.3 Age as a Risk Factor

Old age is a major risk factor for cardiotoxicity (Marchandise *et al*., 1989; Palmeri *et al*., 1986; Rabinovitch *et al*., 1989; von Hoff *et al*., 1979). Palmeri and coworkers studied patients with normal resting left ventricular ejection fractions (LVEFs) and noted that age is a significant predictor of final LVEF after completion of DOX therapy (Palmeri *et al*., 1986). Rabinovitch and colleagues also found that age was a significant predictor of LVEF during DOX therapy (Rabinovitch *et al*., 1989). The high risk of cardiotoxicity is not due to an altered incidence of coronary heart disease in older cancer patients (von Hoff *et al*., 1979). Early peak concentrations of DOX are increased with age (Robert and Hoerni, 1983), likely due to a highly significant decline in distribution clearance [CL(d)] with increasing age ($p < 0.0005$) (Li and Gwilt, 2003). This may contribute to the enhanced risk of cardiotoxicity with age, given that previous studies have indicated that peak plasma anthracycline concentrations are more important than total area under the curve (AUC) as predictors of cardiotoxicity (Bielack *et al*., 1989). There is no information on the effect of age on DNR kinetics in man.

What biochemical pathways may predispose to age-dependent anthracycline cardiotoxicity? The activity of rat heart antioxidant enzymes, superoxide dismutase and cytosolic Cu–Zn superoxide dismutase (SOD) declines with age (Ji *et al*., 1991). Myocardial a product of lipid peroxidation, increases with age in homogenates and mitochondria (Ji *et al*., 1991; Sawada and Carlson, 1987). Mitochondria and homogenate from senescent heart generates more O^{2-} and H_2O_2 (Ji *et al*., 1991; Sawada and Carlson, 1987). Lipid peroxidation is increased in Fischer 344 rat heart with age (Phaneuf and Leeuwenburgh, 2002). In addition, activity of aconitase, which is also a major target of superoxide and peroxynitrite disruption of the [4Fe–4S] cluster, declines with age in the heart muscle. Thus, the aged heart may be 'primed' for damage from free-radical insult from agents such as anthracyclines.

4 ANTHRACYCLINE PHARMACOKINETICS

The original anthracycline agents, DOX and DNR, have a similar structure (**Figure 4**), with a planar tetracyclic anthraquinone ring linked by a glycosidic bond to the amino sugar, daunorubicinol. DOX and DNR are extensively metabolized, undergoing reduction of the carbonyl group to respective alcohol metabolites DOXOL and daunorubicinol (DNROL). Carbonyl reduction is catalysed by ubiquitous cytosolic carbonyl reductases (Lovless *et al*., 1978). The other major metabolic pathway involves reductive cleavage of the daunosamine sugar moiety to yield anthracycline aglycones, that are without antitumour activity (Lovless *et al*., 1978). Concentrations of DOX and DNR in tissues are much higher than in plasma. Following cellular uptake, at least in whole-heart perfusion models, the greatest concentration of anthracycline is in the nucleus and mitochondria. There is considerable conversion of parent compounds to DOXOL

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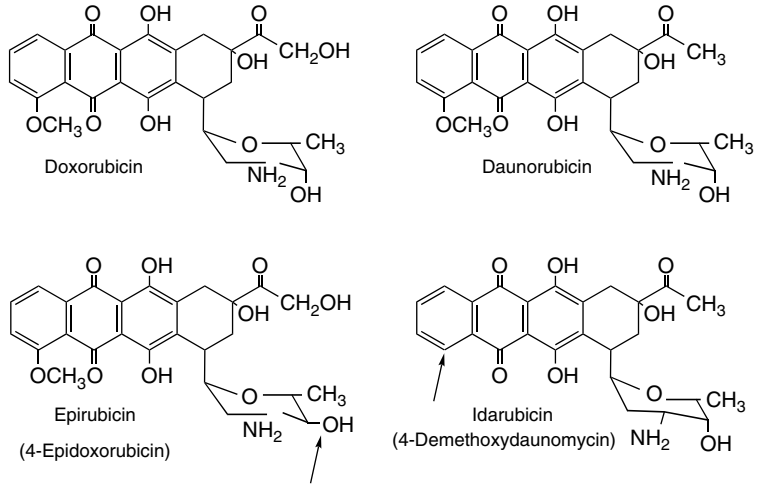

Figure 4 Structure of four anthracyclines in current use. For epirubicin and idarubicin, arrows point to the sites where these drugs differ, respectively from doxorubicin and daunomycin (daunorubicin).

and DNROL, quantitatively the most important metabolites in man (Takanashi and Bachur, 1976), and in rabbits (Cusack *et al*., 1988).

5 MECHANISMS OF ANTHRACYCLINE CARDIOTOXICITY

5.1 C-13 Alcohol Metabolite

The major metabolite of anthracyclines in most species is the C-13 alcohol metabolites DNROL and DOXOL, generated by ubiquitous cytosolic carbonyl reductases. DOXOL is more potent in inhibiting cardiac SR Ca^{2+} ATPase, the Ca^{2+} release channel and in inhibiting contractile function *in vitro* (Olson *et al*., 1988). The alcohol metabolite can be formed from parent drug in human-heart cytosol and can have important deleterious effects on iron metabolism, (Minotti *et al*., 1998). Chronic DOX cardiotoxicity was more severe in a transgenic mouse model that overexpressed human carbonyl reductase in the heart, with increased formation of cardiac DOXOL (Forrest *et al*., 2000). Mice with reduced expression of the carbonyl reductase enzyme that converts the parent anthracycline to the C-13 alcohol metabolite had resistance to acute DOX cardiotoxicity (Olson *et al*., 2003). The cardiac concentration of DNROL, but not DNR was inversely related to contractility (d*S*/*dt*) in left ventricular trabeculae carneae in chronically treated Fischer 344 rats (Cusack *et al*., 2002). Thus, formation and accumulation of the alcohol metabolite may play an important role in free-radical generation, disruption of iron metabolism and of SR Ca^{2+} transport, and may be an important mediator of anthracycline cardiotoxicity.

5.2 Free-Radical Stress

A leading hypothesis is that anthracyclines, such as DOX and DNR and their alcohol metabolites, contain the functional quinone moiety that undergoes enzymatic one-electron reduction to semiquinone radicals, a step that occurs in myocardial microsomes, the sarcotubular system (Doroshow, 1983a) and mitochondria (Doroshow and Davies, 1986). Semiquinone radicals of DOX and DNR, in the presence of molecular oxygen, are rapidly reoxidized to quinones, generating superoxide (O_2^-). The alcohol metabolite DOXOL (100 nM) also can produce O_2^- (Gervasi *et al*., 1986). A redox cycle is set up, with consumption of molecular oxygen, continuous production of O_2^-, which is dismutated to hydrogen peroxide (H_2O_2). This can diffuse through the cytoplasm and, if not metabolized by catalase, is converted, in the presence of iron, to the highly reactive, cytotoxic hydroxyl radical (•OH) by Haber–Weiss and Fenton reactions (Doroshow, 1983b; Doroshow and Davies, 1986). Both •OH and other radicals, such as O_2^- can cause peroxidation of lipids, generation of cytotoxic aldehydes (Luo *et al*., 1997), oxidation and inactivation of enzymes and other proteins, DNA damage (Monti *et al*., 1995) and ensuing cellular damage.

5.3 Iron

This subject is very ably reviewed by others (Minotti *et al*., 2004; Xu *et al*., 2005). Several lines of evidence suggest a significant role for iron in the pathogenesis of anthracycline cardiotoxicity. Overloading of dietary iron in Sprague–Dawley rats caused a significant increase in myocardial iron in those treated with

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single dose DOX ($6\,mg\,kg^{-1}$) and sacrificed two to three weeks later, compared to those receiving regular chow (70 ± 3 vs. $52 \pm 4\,\mu g\,g^{-1}$ tissue (Panjrath *et al*., 2007). There was clear evidence of increased cardiotoxicity in the iron-overloaded compared to the normal-diet group treated with DOX. In the rats treated with DOX, measures of apoptosis, including ^{99m}Tc annexin-V uptake and cleaved caspase-3 staining were increased in iron-overloaded animals and electron microscope (EM) histopathology was increased. Knockout *Hfe*–/– mice used as models of haemochromatosis, when treated with DOX, 5 mg kg^{-1} every two weeks for six doses, demonstrated increased iron uptake in the heart, with increased staining in cardiomyocytes (Miranda *et al*., 2003). DOX treatment caused increased mitochondrial damage in *Hfe*–/– animal than in controls. Furthermore, iron chelation is a proven method of significantly reducing the risk of anthracycline cardiotoxicity in animals using dexrazoxane (ICRF-187) (Cusack *et al*., 2006) and salicylaldehyde isonicotinoyl hydrazone (SIH) (Sterba *et al*., 2007). Dexrazoxane has been proven as cardioprotective in randomized clinical trials in humans (Jones, 2008), and is used clinically in limited situations (Cvetkovic and Scott, 2005). Due to concern that it might affect chemotherapy of other tumours, its use indication remains confined to treatment of metastatic breast cancer. These studies conducted *in vivo* suggest that iron toxicity appears to enhance anthracycline cardiotoxicity, whereas iron depletion appears protective.

Iron is a highly important element for cell metabolism, and iron levels are tightly regulated. Soluble ferric iron (III) is transported in the blood, bound to transferrin receptor. Uptake into cells is mediated by donation of iron to the soluble transferrin receptor (TfR1), is endocytosed into endosomes and then released into the cytoplasm. Ferritin is a major storage protein for nonmetabolic iron storage. Normal cellular iron flux is tightly regulated by the binding of iron regulatory proteins (IRP1 and IRP2) to the untranslated cis-sequences of iron responsive elements (IREs) of ferritin and transferrin receptor1 (TrF1) mRNA (Hentze *et al*., 2004; Papanikolaou and Pantopoulos, 2005). Low cellular iron increases binding of IRPs to these IREs stabilizes TfR1 mRNA and inhibits ferritin translation, thereby increasing TrF1 and reducing the ferritin levels. This effect augments the cellular labile-iron pool. When cellular iron becomes replenished, the bifunctional IRP1 is inactivated by reassembling the 4Fe–4S cluster in the protein so that IRP1 loses the ability to bind to IRE and acquires aconitase function. In iron-replete cells, IRP2 activity is lost in a different fashion by proteasomal degradation. The resultant loss of IRP/IRE binding decreases TrF1 and augments ferritin. This effect diminishes the cellular labile iron pool. Intracellular storage sites for iron include ferritin and lysosomes (Xu *et al*., 2005).

DOX can directly bind to iron, forming drug–iron complexes that can cycle between iron (II) and iron (III) states (Xu *et al*., 2005) and this leads, in the presence of reducing agents, to formation of the anthracycline semiquinone and of superoxide O_2^-. In addition, the anthracycline quinone can accept electrons from reductases, with formation of H_2O_2. This can lead, in the presence of reactive iron, to formation of the highly reactive hydroxyl radical; a process that is preventable by iron chelators or antioxidants (Rajagopalan *et al*., 1988). Accumulation of reactive metabolites leads to cellular damage and apoptosis. The cardiac myocyte is considered vulnerable to oxidative pathology due to the relative paucity of antioxidant enzyme activity.

Seminal studies by Minotti *et al*. (1998) found that metabolism of DOX or DNR to the C-13 alcohols DOXOL or DNROL occurred in human heart biopsies. However, formation of the C-13 alcohol delocalized Fe^{2+} from the aconitase 4Fe–4S cluster in human heart cytosol preparations, to release a twofold excess of low-molecular-weight Fe^{2+}, which was then available for further oxidation reactions (Minotti *et al*., 1998). Furthermore, DOX irreversibly inactivated IRP1 by an effect of DOX–Fe(II) complexes to form a 'null protein' that could neither bind to IRE RNA nor reconstitute aconitase, with ensuing dysregulation of iron homeostasis. In reactive oxygen species systems, DOX inactivated both IRPs through a sequential action of DOXOL and reactive oxygen species (ROS) on IRP1 or an independent action of ROS on IRP2 (Minotti *et al*., 2001). Thus iron metabolism in the cell can be extensively dysregulated by anthracyclines. Other investigators demonstrated that, in bovine endothelial cells, DOX in submicromolar concentrations, not only increased iron uptake, but also increased IRP1 activity and increased apoptosis (Kotamraju *et al*., 2002). Antioxidants and iron chelators opposed these effects of DOX. They also observed that the toxic effect of anthracycline on endothelial cells was abrogated by the TfR antibody, emphasizing the additional importance of TfR function and iron transport in the cytotoxicity (Kotamraju *et al*., 2002).

Subsequent investigations have demonstrated a more complicated picture, however. Kwok and Richardson (2002) demonstrated that a concentration-dependent reduction in active IRP1–IRE binding following six hours of incubation; the effect was not seen after 24 hours incubation, however. In contrast to Minotti *et al*. (2001), they did not observe any effect of DOXOL on IRP1–IRE binding. The observed loss of IRP1 activity was explained by the formation of DOX –iron or DOX–copper complexes that caused a decrease in active IRP–RNA binding reversibly by the reducing agent βmercaptoethanol. In other studies using H9c2 embryonic rat heart cell cultures, DOX and DNR produced a bell-shaped, dose-related effect on IRP1–IRE binding, so that binding was increased at lower concentrations of anthracycline and decreased

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towards control levels at higher concentrations of anthracycline (Minotti *et al*., 2001). Others have noted activation of IRP–IRE binding with DOX treatment in endothelial cells (Kotamraju *et al*., 2002), thought due to effect of ROS. Thus the effect of anthracycline on IRP function remains controversial, with most reports suggesting a decrease in IRP–IRE binding.

The question remains whether IRP–IRE binding changes are applicable in the clinically relevant models of chronic anthracycline cardiotoxicity *in vivo*? We have demonstrated that IRP1–IRE binding was not altered in a model of chronic DNR cardiotoxicity in the Fischer 344 rat (Cusack *et al*., 2006). In this study, young and old Fischer 344 rats were treated with DNR 2.5 and 1.67 mg kg^{-1} iv weekly for six weeks to reach a total cumulative dose of 15 and 10 mg kg^{-1}, respectively. Rats were sacrificed two weeks after the last dose. Impairment of contractility was observed in left atrial tissue in DNR compared to control animals. However, no effect on active or total IRP1–IRE binding was detected. This observation has been corroborated by Corna *et al*. (2006), who demonstrated that acute cardiotoxicity in young male mice four days after injection with 15 mg kg^{-1} DOX occurs in the mouse, independent of IRP1 effects. They found that IRP2 activity was down-regulated, with unchanged IRP1 activity. Furthermore, ferritin L and H expression was increased and TfR expression decreased. Rat hearts demonstrated evidence of toxicity with elevated malondialdehyde (MDA) and thioreduxin levels as measures of oxidative damage and of brain natriuretic peptide (BNP) levels as indicator of functional stress. Similar findings were noted in IRP1 –/– mice, suggesting that the cardiac toxicity, IRP activity, and ferritin and TfR changes did not require IRP1. The results also suggest that IRP2 is more important as a mediator of iron cellular flux than IRP1. Thus, the effect of DOX on IRP–IRE appears controversial and varies according to conditions of treatment (concentration and duration of anthracycline exposure) and cells used in experiments.

Lyosomal activity is also involved in anthracycline-mediated iron toxicity. Using neonatal rat cardiomyocytes and cancer cell lines, incubated with DOX, Kwok and Richardson (2003) noted a marked rise in uptake of ^{59}Fe into ferritin in all cell lines. The same effect was achieved by other redox-cycling agents such as, and appeared due to impaired ability to release ^{59}Fe from ferritin. Since ferritin is catabolized by lysosomes, this raises the possibility that anthracycline's interaction with ferritin may be mediated by effects on lysosomes. Depression of lysosomal activity using enzyme inhibitors, enzyme inhibitor or lysosomotropic agent, such as lysosomotropic agent and methylamine, caused a several-fold increase in ^{59}Fe-ferritin accumulation in human SLK-Mel-28 melanoma cells (Kwok and Richardson, 2004). Proteasomal inhibition has similar effects. DOX also caused a significant increase in ferritin accumulation of ^{59}Fe. Thus, cardiotoxicity may be related to effects of anthracycline on lysosomal ferritin release of iron. Anthracyclines also act as atypical cytosolic iron chelators, paradoxically causing an increase in ferritin without any increase in TfR1 or Ndrg1 protein levels (proteins regulated by iron levels), leading to increased ferritin-iron content (Xu *et al*., 2008). These studies demonstrate that the effect of anthracyclines are complex and pleiotropic, but overall suggest that anthracyclines directly bind to iron, and exert abnormal effects on IRP metabolism and ferritin-iron uptake and binding.

5.4 The Ubiquitin Proteasome System (UPS)

About 20% of protein turnover occurs in lysosomes. Another 80% occurs via the ubiquitin/proteasome system (UPS), which has high specificity and is involved in degrading abnormal, misfolded, mutated or oxidized protein, and also in controlling cellular homeostasis by proteolytic effects on key regulatory proteins (Powell, 2006). Degradation is an ATP-requiring process that occurs via two successive steps (Glickman and Ciechanover, 2002). The first is conjugation of several ubiquitin molecules to the substrate protein by a process controlled by three ubiquitin ligase enzymes that act in concert, with an initial ubiquitin activating step, followed by ubiquitin conjugation to the enzyme E2, and then by a final step whereby the protein is bound specifically by an ubiquitin ligase enzyme E3, the latter being the rate-limiting step in ubiquitination. The resultant polyubiquitinated protein is then degraded by a 26S proteasome complex into small peptides, releasing reusable ubiquitin. The proteasomal core is a cylindrical shaped structure containing four stacked rings, each containing seven subunits. It is called the 20S proteasome. A regulatory complex called the 19S proteasome activator attaches to the 20S proteasome. The combination is termed the 26S proteasome when either one or two 19S regulatory complexes are attached to the 20S proteasome. The 20S proteasome is comprised of two inner rings, each containing seven homologous β subunits. The outer two rings contain homologous α subunits aligned over the corresponding β subunits. The proteolytic activity resides within the inner β subunits. Three main activities have been described, chymotryptic, tryptic and postglutamyl peptide hydrolytic-like or 'caspase-like' activities.

5.4.1 UPS and the Heart

There is mounting evidence that dysfunction of the UPS is involved in different forms of cardiac pathology, including cardiac ischaemia and cardiomyopathies.

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In ischaemia, proteasomal dysfunction causes accumulation of oxidized and ubiquitinated protein and prodeath proteins, with ensuing cell damage (Powell, 2006). Failure to remove endoplasmic reticulum (ER) abnormal proteins can produce stress and contributes to proteasomal dysfunction and cardiac damage in ER stress-related cardiomyopathies (Powell, 2006). Inhibition of proteasomal function by a drug approved for treatment of myeloma, a drug approved for treatment of myeloma, may also cause cardiac damage as reported in the US package insert by Millennium Pharmaceuticals for a drug approved for treatment of myeloma. Myocardial pathological changes were observed in monkeys treated with bortezomib. Overt, severe heart failure with slow recovery over months has been described following treatment with bortezomib (Voortman and Giaccone, 2006).

5.4.2 Effects of Anthracyclines on the UPS

DOX can have a direct effect on the proteasome, at least in the neoplastic cell. DOX (4 μM for 24 hours) caused apoptosis in erythroleukaemic K562 cells, associated with altered subunit composition of 26S proteasomes, considered due to post-translational modification and altered phosphorylation state of specific subunits, combined with increased peptidase and ribonuclease activities (Tsimokha *et al*., 2007). In an L1210 mouse leukaemia cell line, DOX binds to a proteasome that appears to function as a carrier to translocate DOX from the cytoplasm to the nucleus (Kiyomiya *et al*., 2002) and determines the action on poly(ADP-ribose) polymerase. In proliferating human leukaemia cells, DOX caused an increase in nuclear proteasomal activity that was significantly reduced by inhibition of a poly(ADP-ribose) polymerase (PARP) enzyme (Ciftci *et al*., 2001). In neonatal rat cardiomyocytes, DOX depletes an important cofactor for transcription factors, an important cofactor for transcription factors including an important cofactor for transcription factors regulators of expression of cardiac genes that govern growth and homeostasis, regulators of expression of cardiac genes that govern growth and homeostasis (Poizat *et al*., 2000). DOX was found to do this by increased proteasomal proteolysis of p300. Neither a proteasome substrate (a proteasome substrate) nor ubiquitinated protein levels were altered acutely by DOX (1 μM), suggesting that the effect of DOX at this concentration on p300 was selective. Kumarapeli *et al*. (2005) studied the effect of high-dose acute DOX effect in transgenic mice that expressed a surrogate protein (a full length, protease-specific substrate for the UPS) that is a full length, protease-specific substrate for the UPS, requiring activity of ubiquitinylation, and activity of both the 20S and 19S moieties of the 26S proteasome for proteolysis. Following injection of high-dose DOX (25 mg kg^{-1}), the cytosolic levels of the GFPdgn were decreased due to increased proteasomal degradation (inhibited by proteasomal inhibitors proteasomal inhibitors). Interestingly, proteasomal peptidase chymotrypsin and caspase-like activities were reduced, suggesting that the effect of DOX on proteolysis of GFPdgn was on the part of the UPS outside the β-ring of the 20S proteasome, where peptidase activity is located. It is not known whether the effect of DOX in increasing proteasomal degradation of the surrogate protein GFPdgn was specific, since effects on other substrates were not measured. It should be remembered that these findings were observed in acutely treated mice at six and 24 hours after a high dose of DOX. These results cannot necessarily be extrapolated to conditions of chronic cardiotoxicity when the heart is exposed to repeated lower concentrations of anthracycline under 2 mg kg^{-1} over several weeks duration.

These investigators subsequently showed that, in cultured UPS reporter and also in neonatal cardiomyocytes, DOX both increased the proteolysis of the UPS reporter (UPS reporter) and opposed the proteasome inhibitor-induced accumulation of endogenous substrates of the UPS (e.g. c-Jun and β-catenin) (Liu *et al*., 2008). DOX appeared to cause *in vitro* degradation of GFPu and c-Jun by the reconstituted UPS by increasing proteasomal function. DOX also increased the peptidase activities of purified 20S proteasomes at drug concentrations of 1–5 μM. The effects of DOX on proteasomal function may be mediated by ubiquitination. For example, they demonstrated that DOX increased the levels of an E3 ligase involved in the proteasomal degradation of HSP 70 (an E3 ligase involved in the proteasomal degradation of HSP 70) while decreasing HSP 70. Analogous observations have been reported; in cultured neonatal rat cardiomyocytes, DOX (0.3 μM) enhanced proteasomal-dependent downregulation of an E3 ligase involved in the proteasomal degradation of HSP 70 an important survival protein in cardiomyocytes, an important survival protein in cardiomyocytes (Ito *et al*., 2007). Anthracyclines also may influence activity of ubiquitinating ligases. DOX, both after single doses *in vivo* (15 mg kg^{-1}) and in neonatal rat cardiomyocytes *in vitro* (0.1 μM for 24 hours), causes up-regulation of a muscle-specific ubiquitin ligase that plays a role in cardiac muscle atrophy, a muscle-specific ubiquitin ligase that plays a role in cardiac muscle atrophy (Yamamoto *et al*., 2008). In aggregate, these studies suggest a role for anthracyclines in increasing proteasomal activity with down-regulation of important factors for cellular maintenance and function, such as β-catenin, c-Jun, HSP 70 and NFAT5, or by up-regulation of ubiquitin ligases such as atrogin-1 that promote muscle atrophy. Proteasomal inhibition may play a different role in reducing DOX cardiotoxicity. DOX caused topoisomerase IIβ-mediated DNA double-strand breaks in H9C2 embryonic cardiomyocytes that was opposed by proteasomal inhibitors bortezomib and MG132 (Lyu *et al*., 2007).

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However, not all studies show enhancement of proteasomal activity by anthracyclines. Fekete *et al.* (2005) found that both DOX and DNR inhibited proteasomal 26S activity in lysates from ECV 304 cells with an IC_{50} (half maximal inhibitory concentration) value of 14 μM for DNR. Kiyomiya *et al.* (2002) demonstrated that DOX (5 μM) caused apoptosis of L1210 cells and also inhibition of purified proteasomal peptidase activity. It also is possible that chronic treatment with anthracycline may have a different effect on UPS machinery due to effects of chronic oxidative stress (Friguet *et al.*, 2000; Keller *et al.*, 2000) and effects of lipid peroxidation products (Bulteau *et al.*, 2001; Okada *et al.*, 1999) which can reduce UPS activity. Reduced proteasomal activity due to oxidization can be reversed by dithiothreitol (DTT), a known antioxidant (Ferrington *et al.*, 2005). It is not known currently whether chronic anthracycline cardiotoxicity is associated with altered proteasomal function or ubiquitination.

6 PREVENTION OF CARDIOTOXICITY

The recognition that anthracyclines can cause a cumulative dose-related cardiotoxicity has lead to numerous approaches to reduce such risk. Success in preventing cardiotoxicity depends on the ability to identify significant risk factors for cardiotoxicity and also having measures that can reliably detect cardiotoxicity. Particular attention is required in treatment of these groups of patients. The most powerful predictor of cardiotoxicity is lifetime exposure to anthracyclines. Since the risk of cardiotoxicity increases rapidly with doses of DOX above 450 mg m^{-2}; such doses are not recommended. However, some patients develop cardiotoxicity, even with doses below 300 mg m^{-2}. In order to detect such patients, close monitoring has been recommended.

6.1 Monitoring for Cardiotoxicity

Measures to detect subclinical cardiotoxicity include endomyocardial right ventricular biopsy for histological examination, functional measurements using echocardiography or cardiac radionuclide angiography, and assay of biochemical markers (e.g. atrial natiuretic peptide, brain natiuretic peptide, troponin I, troponin T) of cardiac stress or damage (Jannazzo *et al.*, 2008). Guidelines have been promulgated for treatment in children, using both echocardiography and radionuclide angiography measures of cardiac contractility, and endomyocardial biopsy to determine specificity, if needed (Cheitlin *et al.*, 2003; Klocke *et al.*, 2003; Steinherz *et al.*, 1992). Some, but not all, studies of systolic and diastolic function have found that monitoring during the course of therapy may predict eventual cardiac function (Jannazzo *et al.*, 2008). However, there is still no satisfactory evidence-based consensus on the correct sequence or combination of measures to use that has gained wide acceptance in clinical practice.

6.2 Methods for Reduction of Cardiotoxicity

6.2.1 Modification of Anthracycline Delivery and Formulation

Methods to reduce cardiotoxicity include obvious adjustments, such as dose limitation of anthracyclines to avoid the risk associated with cumulative doses over 450 mg m^{-2} (Swain *et al.*, 2003). This may be adequate in many treatment protocols, but foils some therapeutic strategies, for example retreatment with anthracyclines in recurrent disease. Administration of anthracycline by prolonged infusion prevents achievement of high plasma, and by inference, high cardiac concentrations of parent drug or metabolite. Administration over six hours (Shapira *et al.*, 1990), or over 48–96 hours (Legha *et al.*, 1982) can lower cardiotoxicity, although progressive decline in contractility can still be observed (Speyer *et al.*, 1985), and the dependability of antitumour action of the prolonged schedules has not been established (Bielack *et al.*, 1989). Due to this concern and the cumbersome and more expensive nature of prolonged infusion, this approach has not gained wide acceptance.

Modification of the drug delivery can also be accomplished using pegylated liposomal formulations of DOX or DNR. This formulation, using stealth nanocarriers that permit decreased distribution of DOX in normal tissues and increased uptake in tumour tissue, resulted in decreased cardiotoxicity in at least two randomized clinical trials (van Dalen *et al.*, 2006). There is no evidence that tumouricidal effect was compromised. Palmar-plantar erythrodysesthesia syndrome can complicate use of liposomal anthracyclines (Hui and Cortes 2000). Pegylated DNR also has proven useful, especially for treatment of Kaposi sarcoma, with improved risk–benefit ratio with respect to cardiotoxicity (Petre and Dittmer, 2007).

6.2.2 Anthracycline Analogues

Anthracycline analogues, such as epirubicin have been developed to reduce the risk of cardiotoxicity. However, a comprehensive systematic review did not demonstrate a significant reduction in the risk of cardiotoxicity from epirubicin compared to DOX (van Dalen *et al.*, 2006). Furthermore, recent data from the National Cancer Institute indicated that the risk of cardiotoxicity occurred at lower than previously considered doses (Ryberg *et al.*, 2008). Idarubicin, which is more lipophilic than other

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anthracyclines and can also be given orally, may be associated with reduced cardiotoxicity, but there are no good randomized comparative trials to document this (Cersosimo, 1992).

6.2.3 Antioxidants

The premise that anthracyclines cause cardiotoxicity by free-radical damage has led to many clinical studies using antioxidants in an attempt to offset this risk. Clinical studies using acetylcysteine, phenetylamines, coenzyme Q10, l-carnitine, a potent antioxidant in addition to its β-blockade properties (which is a potent antioxidant in addition to its β-blockade properties) and the combination of vitamin E, vitamin C and a potent antioxidant in addition to its β-blockade properties have been recently included in a Cochrane review (van Dalen *et al*., 2008). There was insufficient evidence to conclude that any of these agents provided significant protection from anthracycline cardiotoxicity.

6.2.4 Dexrazoxane

On the other hand, a putative iron chelator, a putative iron chelator does reduce the risk of anthracycline cardiotoxicity (van Dalen *et al*., 2008), substantiating the rationale of the role of iron toxicity in the pathogenesis of anthracycline cardiotoxicity. In that meta-analysis, the reduction in the risk of clinical heart failure (relative risk (RR) 0.18; 95% confidence interval (CI) 0.10–0.32; $p < 0001$) or both clinical and preclinical heart failure (RR 0.29; CI 0.2–0.41; $p < 0.0001$) were highly significant. Studies have included both patients with breast cancer and sarcoma, both children and adult patients, and both DOX and epirubicin, suggesting a robust and versatile effect of prevention by dexrazoxane (Lopez *et al*., 1998; Marty *et al*., 2006; Swain *et al*., 1997b; Venturini *et al*., 1996; Vici *et al*., 1998; Wexler *et al*., 1996). There are caveats with the use of this agent, however: myelotoxicity, with lower nadir white cell counts, was increased in Phase 1 studies (van Dalen *et al*., 2008); in one trial there was a decreased response rate to the antitumour effect of DOX in combination with dexrazoxane in comparison with DOX alone (Swain *et al*., 1997b). However, the systematic review did not demonstrate any difference in response rate, progress-free survival or overall survival. Swain *et al*. (1997a) also observed that, in patients with breast cancer who have received $300\,mg\,m^{-2}$ DOX, dexrazoxane was highly protective against cardiotoxicity in those who continue to receive DOX treatment. Based on these observations and concern about possible effects of dexrazoxane on tumour response, myelosuppression and occurrence of secondary malignant disease (Tebbi *et al*., 2007), the current Food and Drug Administration (FDA) indications for dexrazoxane include use in patients with advanced breast cancer who have received $300\,mg\,m^{-2}$ DOX. Further studies of the use of dexrazoxane are needed to better document its role in children, in leukaemias and its protection against long-term cardiotoxicity.

6.2.5 Renin-Angiotensin II Axis Blockade

Several studies endorse the utility of reduction of angiotensin II activity as a measure to prevent anthracycline cardiotoxicity. Studies in mice indicate that acute and chronic DOX treatment in an angiotensin II type 1a receptor blocker knockout mice or mice treated with an angiotensin II type 1a receptor blocker, an angiotensin II type 1a receptor blocker, causes less cardiotoxicity than in DOX-treated wild-type mice (Toko *et al*., 2002). In that study, measures of echocardiographic functional parameters, histological myofibrillar loss and apoptosis were attenuated in the treatment compared to control groups of mice, both after a single dose of DOX, $20\,mg\,kg^{-1}$, and after weekly DOX, $1\,mg\,kg^{-1}$, for 12 weeks. Similarly, the angiotensin II receptor blocker, angiotensin II receptor blocker, protected against acute DOX cardiotoxicity in humans treated with DOX-containing regimens for lymphoma, as measured by echocardiography (left ventricular dimensions) and electrocardiographic (QTc measurements) outcomes (Nakamae *et al*., 2005). Angiotensin-converting enzyme (ACE) inhibition also confers cardioprotection against anthracycline cardiotoxicity. Chronic treatment with DOX in rats produced a significant lengthening of the QαT interval, and depression of the cardiac positive inotropic effect induced by iv ACE inhibitor, both of which were significantly ameliorated by the ACE inhibitor, ACE inhibitor (Sacco *et al*., 2001). In separate experiments, zofenopril did not impede the antineoplastic activity of DOX. In long-term studies in rabbits treated with one dose of DOX, lisinopril treatment for 10 weeks prevented both late-onset increased ventricular atrial natriuretic peptide (ANP) expression, myocyte loss and measures of apoptosis (Boucek *et al*., 2003). Thus, angiotensin II blockers or ACE inhibitors may prove beneficial in reducing the risk of cardiotoxicity and deserve further clinical evaluation.

Anthracycline cardiotoxicity is considered to be mediated, at least in part, by apoptosis, activating apoptotic pathways and also causing apoptotic morphological changes of cardiomyocytes, at least *in vitro* (Takemura and Fujiwara, 2007). Trophic factors with antiapoptotic effects may therefore be beneficial in ameliorating anthracycline cardiotoxicity. stimulates production of erythrocytes, which stimulates production of erythrocytes, also has cytoprotective effects, considered to derive from its ability to oppose apoptosis in cardiomyocytes (Parsa *et al*., 2003). Studies in neonatal ventricular myocytes, using clinically relevant concentrations of erythropoietin and DOX, demonstrated that erythropoietin effected antiapoptotic morphological changes, with

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reduction in caspase-3 activity and in the number of apoptotic cells, assayed using annexin-V staining and DNA laddering (Kim *et al*., 2008). In addition, impairment of cardiac function following chronic dosing ($5\,mg\,kg^{-1}$ ip weekly × 3), was offset by erythropoietin pretreatment. Further studies indicated that this was due to activation of the P13K-dependent cytokine trophic factor. Another cytokine trophic factor, cytokine trophic factor, stimulates production of platelets, and has antiapoptotic properties, mediated by the Akt prosurvival pathway. Thrombopoietin reduced apoptotic changes in H9C2 myotube cells treated with DOX and also offset cardiotoxicity, as measured by histological, apoptotic and functional parameters, following single-dose injection of DOX, $20\,mg\,kg^{-1}$ ip in mice (Li *et al*., 2006). Whether either erythropoietin or thrombopoietin will emerge as clinical treatments is conjectural at this point, but nevertheless they are of interest in that they suggest that antiapoptotic measures may be successful in ameliorating anthracycline cardiotoxicity.

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Photoallergy

Rūta Dubakienė

CONTENTS

1 INTRODUCTION

Photosensitivity is the broad term used to denote abnormal adverse reactions to ultraviolet radiation (UVR) or visible light (Epstein, 1983; 1999). The forms of photosensitivity are phototoxicity and photoallergy. Phototoxic disorders have a high incidence, whereas photoallergic reactions are much less frequent in the human population. Several hundred endogenous and exogenous substances can be transformed into antigens by sunlight and may invoke phototoxic or photoallergic reactions (Hasan and Jansen, 1996; Dubakiene and Kupriene, 2006). Phototoxic and photoallergic reactions can be diagnosed separately on the basis of pathogenesis, clinical characteristics and histology (Lankerani and Baron, 2004; Ichihashi, 2007). Drug photosensitivity is a major problem, since the abnormal reactions seriously limit or exclude the use of drugs (Takiwaki *et al*., 2006). Standardized detection of the photosensitizing properties of particular substances should be considered prior the drugs being introduced in therapy or products made available on the market.

2 HISTORICAL REVIEW

Photosensitive reactions have been recognized for thousands of years. In ancient Egypt, India and Greece psoralen-containing plant extracts were combined with exposure to sunlight to treat skin diseases. As early as 2000 BC in Egypt, the juice of *Ammi majus*, which grows throughout the Nile River valley as a weed, was rubbed on patches of vitiligo, after which patients were encouraged to lie in the sun. Reports of dermatitis following contact with parsnips and angelica came from both England and the United States (Stowers, 1897; White, 1897). Freund (1916) observed characteristic hyperpigmented lesions that he attributed to *eau de cologne* containing bergamot oil. Unfortunately, none of the authors recognized the necessity of UVR for the reaction. In 1932, Oppenheim reported that the combination of meadow grass exposure and UVR could result in a striate arrangement of itching blisters 24–48 hours later. It was found that plant furocoumarins caused photosensitization (Kuske, 1938). Jensen and Hansen (1993) reported that UVR between 320 and 380 nm caused

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the maximum reaction. Klaber (1942) introduced the term 'phytophotodermatitis' to emphasize the necessity of plants and light to cause the reaction. European scientists pioneered photosensitivity disorder research during the 1960s. In 1967, Danish researchers attributed strange skin lesions on women to perfumed soap. In the same year, British researchers discovered that sandalwood oil in sunscreens and cosmetics caused photoallergies. Shortly thereafter, French scientists demonstrated that bergamot oil in sunscreens caused photosensitivity disorders. German researchers isolated photoreactive agents in colognes, perfumes and oral contraceptives. American scientists, in 1972, linked sunlight-activated aniline compounds (found in drugs, varnishes, perfumes, shoe polish and vulcanized rubber) to hives and skin conditions such as dermatitis and dandruff.

Most recently the Food and Drug Administration has listed medications that increase sensitivity to light and can cause a wide variety of health problems known as photosensitivity disorders. An increased list of photoreactive agents found in several hundred of substances, chemicals and drugs is published annually (Bryden *et al*., 2006; Pigatto *et al*., 2008). In order to avoid photosensitive reactions, attempts are made to determine the photosensitizing properties of such substances before drugs are introduced in therapy or products made available on the market (Wahie *et al*., 2007). Moreover, the standard set of photoallergens has to be updated periodically (Bakkum and Heule, 2002).

3 INCIDENCE AND PREVALENCE

The prevalence of photoallergy in the general population is uncertain. Almost 400 medications have been reported to cause photosensitivity and a half of them photoallergy. Only a small number, however, induce reactions frequently or have been well studied. In evaluations performed at photodermatology centres in New York, Melbourne, Singapore and Detroit, documented photosensitivity induced by a systemic drug occurred in 5 to 15% of the referred patients (Fotiades *et al*., 1995; Chong and Khoo, 2004). In studies performed in the United States, the United Kingdom, Europe, Australia and India, the percentage of photopatch-tested patients who had clinically relevant reactions leading to a diagnosis of photoallergic contact dermatitis ranged from 1.4 to 12%, with the value in most series being around 10% (Berne and Ros, 1998; Moore, 2002; Scheuer and Warshaw, 2006; Neumann *et al*., 2000; Neumann and Lehmann, 2003; Bryden *et al*., 2006; Sharma *et al*., 2007; Katsarou *et al*., 2008). The racial incidence of drug-induced photosensitivity reactions is unknown. Photosensitivity reactions can occur even in races with heavily pigmented skin (Kontos *et al*., 2002). Men are more likely to have photoallergic reactions than women. Photosensitivity is associated with death only in rare individuals who are exposed to large amounts of sunlight after taking large doses of psoralens (McKenna, 2004). Although mortality is rare, drug-induced photosensitivity can cause significant morbidity in some individuals, who must severely limit their exposure to natural or artificial light. Carcinogenic potential due to prolonged exposure to these photosensitizing drugs has been suggested (Stern *et al*., 1998; Lim and Stern, 2005), however, its clinical relevance remains to be determined. There is a need for randomized, controlled trials of strategies for prevention, control and treatment of photosensitivity disorders. Additional data are needed for a better assessment of the risk of photosensitivity associated with many drugs (Ferguson, 2002). Standardization of the test procedure is needed to compare the test results of different institutions.

4 PHOTOSENSITIZERS

Photosensitizers or photoreactive agents are chemicals that induce a photoreaction. The chemicals may be therapeutic, cosmetic, industrial or agricultural (Fitzpatrick *et al*., 2001). Following interaction of UVR with a chemical present in sufficient amounts in the skin, one of several reactions, phototoxic or photoallergic, may occur in susceptible patients (Vassileva *et al*., 1998; Stein *et al*., 2007). Medications that trigger reactions can be topical, oral and, on rare occasions, inhaled (Reid, 1996; Pérez-Pérez *et al*., 2007; Sánchez-Pedreño *et al*., 2001).

Mostly, photoallergic reactions result from the topical administration of the agent under the action of sunlight, with a spectrum within the UVA and visible light range or UVB range (Allen, 1993; Stege and Krutmann, 1996; Lugovic *et al*., 2007; Belhadjali *et al*., 2002; Agin *et al*., 2008). Everyday items, such as perfumes, soaps, deodorants, lotions, hair sprays and styling creams, artificial sweeteners, petroleum products, tattoos and certain foods may contain photoreactive agents and induce photoallergy (Schauder *et al*., 1996; Monfrecola *et al*., 2000; Uetsu *et al*., 2000; Emmert *et al*., 2007). Sunscreens help to reduce the effects of UVR, although some sunscreens contain ingredients that cause photosensitivity themselves (Collins and Ferguson, 1994; Cook and Freeman, 2001). Currently, the benzophenones are frequently used in high-protection-factor sun-barrier preparations, and appear to have a particular ability to induce such responses (Darvay *et al*., 2001). Usage of products containing photoreactive agents can aggravate existing skin diseases (eczema, herpes, etc.) and also precipitate or worsen autoimmune diseases (lupus erythematous, rheumatoid arthritis). Further photopatch test series should be regularly reviewed and updated, as the relevance of individual photoallergens changes over time.

Medications containing photoreactive agents include antibiotics, nonsteroidal anti-inflammatory drugs,

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Table 1 Common photosensitizers

Class	Medication	Photoallergic reaction	Phototoxic reaction
Antibiotics	Tetracyclines (doxycycline, tetracycline)	No	Yes
	Fluoroquinolones (ciprofloxacin, ofloxacin, levofloxacin)	No	Yes
	Sulfonamides	No	Yes
Nonsteroidal anti-inflammatory drugs (NSAIDs)	Ibuprofen	No	Yes
	Ketoprofen	Yes	Yes
	Naproxen	No	Yes
	Celecoxib	Yes	No
Diuretics	Furosemide	No	Yes
	Bumetanide	No	No
	Hydrochlorothiazide	Yes	Yes
Retinoid	Isotretinoin	No	Yes
	Acitretin	No	Yes
Hypoglycemics	Sulphonylureas (glipizide, glyburide)	Yes	No
Photodynamic therapy (PDT) prophotosensitizers	5-Aminolevulinic acid	No	Yes
	Methyl-5-aminolevulinic acid	No	Yes
	Verteporfin	No	Yes
	Photofrin	No	Yes
Neuroleptic drugs	Phenothiazines (chlorpromazine, fluphenazine, perazine, perphenazine, thioridazine)	Yes	Yes
	Thioxanthenes (chlorprothixene, thiothixene)	No	Yes
Antifungals	Terbinafine	No	No
	Itraconazole	Yes	Yes
	Voriconazole	No	Yes
Other drugs	*p*-Aminobenzoic acid (PABA)	Yes	Yes
	5-Fluorouracil	Yes	Yes
	Amiodarone	No	Yes
	Diltiazem	No	Yes
	Quinidine	Yes	Yes
	Hydroxychloroquine	No	No
	Coal tar	No	Yes
	Enalapril	No	No
	Dapsone	Yes	No
	Oral contraceptives	Yes	No
Sunscreens	PABA	Yes	No
	Cinnamates	Yes	No
	Benzophenones	Yes	No
	Salicylates	Yes	No
Fragrances	Musk ambrette	Yes	No
	6-Methylcoumarin	Yes	No

cardiovascular drugs, diuretics, antidiabetic drugs, antidepressants, antipsychotics, antihistamines, skin agents and others (Horio *et al*., 1978; Ophaswongse and Maibach, 1993; Schürer *et al*., 1991; Torinuki, 1995; Alvarez-Fernández *et al*., 2000; Trujillo *et al*., 2001; Johnston, 2002; Adams and Gadenne, 1999; Torinuki, 1995; Alvarez *et al*., 2003; Gubinelli *et al*., 2003; Marzano *et al*., 2003; Llambrich and Lecha, 2004; Landers *et al*., 2003; Morison, 2003; Vachiramon and Wattanakrai, 2005; Takiwaki *et al*., 2006; Langan and Collins, 2006). Common photosensitizers presented in **Table 1**. An adverse effect of several anti-infective agents and their derivatives is photosensitivity reactions. Most of them are cyclic and tricyclic hydrocarbons,

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frequently containing an alternate double-bond isoprene or naphthyridine nucleus (Vassileva *et al*., 1998). Fluoroquinolones, antibacterial agents, are well known to cause photosensitivity as an adverse effect, and their crossreactivity has been clinically documented (Tokura *et al*., 1998; Yazici *et al*., 2004; Oliveira *et al*., 2000; Willa-Craps *et al*., 1995; Kumar and Freeman, 1996). Empirical studies suggest pefloxacin and fleroxacin to be the most potent photosensitizers, while enoxacin, norfloxacin and ofloxacin are less potent (Vassileva *et al*., 1998). Tetracyclines are an example of the phototoxic hazards of antibiotics. Among them, chlorine derivatives most frequently cause phototoxicity (Vassileva *et al*., 1998). Sulfa-derived drugs (sulfonamide antibacterials, hypoglycaemics, diuretics) have been well-known causes of photosensitivity reactions since Epstein (1939) first reported photoallergic contact dermatitis following intradermal injection of sulfanilamide (Vassileva *et al*., 1998). Celecoxib, a new-generation nonsteroidal anti-inflammatory drug, a selective cyclooxygenase-2 (COX-2) inhibitor containing a sulfonamide substitute, is now widely used clinically, due to its advantage of a lower rate of gastrointestinal irritation. However, Yazici *et al*. (2004) reported a photoallergic drug eruption associated with the introduction of celecoxib. Skin patch testing showed positive reaction to 1% celecoxib without crossreactivity to 1% sulfamethoxazole-trimethoprim (Yang *et al*., 2004).

Photoallergic contact dermatitis from topical ketoprofen (KTP), a nonsteroidal anti-inflammatory agent, is a well-known side effect. Ketoprofen clinically evokes the allergic type of photo contact dermatitis when applied to the skin and irradiated with UVA (Pigatto *et al*., 1996; Le Coz *et al*., 1998; Matthieu *et al*., 2004; Foti *et al*., 2008; López-Abad *et al*., 2004; Atarashi *et al*., 2007; Asensio *et al*., 2008; Devleeschouwer *et al*., 2008). The arylpropionic acid derivatives (APADs), ketoprofen and tiaprofenic acid (TPA) can provoke photoallergic dermatitis (Fernández-Jorge *et al*., 2008). Their chemical structures share the same elements, the benzoyl radical and the thiophene ring. The finding that the vast majority of the ketoprofen photoadduct can be accounted for by means of antibenzoyl antibodies strongly supports the view that the drug binds preferentially via the thiophene ring, leaving the benzene ring more accessible. By contrast, selective recognition of SUP–protein photoadducts by anti-thenoyl antibodies evidences a preferential coupling via the benzene ring, leaving the thiophene moiety more distant from the protein matrix. As a result of research by (Lahoz *et al*., 2001), we have been able to identify a common substructure that is present in TPA–albumin and KTP–albumin photoadducts. The greatest structural similarities were found between TPA and SUP as they share the same benzoylthiophene chromophore. It appears that the benzoyl radical is the key structure for photosensitivity and the photo crossreactivity of ketoprofen, suprofen (SUP) and TPA (Sugiura *et al*., 2002). It was reported that the crossreactivity applied to fenofibrate and oxybenzone, but not to APADs without a benzophenone moiety, which might therefore be used in such patients (Le Coz *et al*., 1998). Crossreactivity of the substituted benzophenones, oxybenzone and sulisobenzone, occurred only with the former in less than 30% of the patients, and a high frequency (69%) of contact allergy to the fragrance mix was found (Matthieu *et al*., 2004). Dermatologists should be aware of the severity of photoallergic reactions to ketoprofen and the risk of cross-sensitization (Sugiura *et al*., 2002; Ota *et al*., 2007; Girardin *et al*., 2006).

4.1 Photosafety of New Active Substances

The goal of photosafety is to detect the adverse effects of pharmaceutical products in the present of light. This type of testing is relevant for substances that enter the skin via dermal presentation or systemic circulation. Despite increasing numbers of new drugs reaching the marketplace, one might expect, with modern preregistration drug screening, that the chance of a drug with a significant phototoxic potential reaching the marketplace would be much less than it was, see in **Figure 1**. Four different end points should be specifically addressed in photosafety testing: phototoxicity, photoallergy, photogenotoxicity and photocarcinogenicity. From a regulatory perspective it is much easier to request a phototoxicity study, both in the laboratory and in the clinical setting, if the drug under development is a known member of a photoactive group. It is much less easy with a completely novel molecule. In that situation, if early-phase *in vitro* and human-usage studies fail to demonstrate such an effect, it may be left to postmarketing surveillance to detect any problem. The less commonly the drug produces photosensitivity the more it needs to be watched out for, and the mistake of incorrectly placing a drug photosensitivity presentation into another diagnosis, such as an idiopathic photodermatoses should be avoided (CPMP 2002). Photosafety testing is warranted for those chemicals that absorb light of wavelength 290–700 nm and are either topically/locally applied or reach the skin or eyes following systemic exposure.

5 PATHOLOGICAL MECHANISM

The pathogenesis of contact photoallergy and contact allergy is similar except that UVR radiation is necessary for the induction and elicitation phases of a photoallergic response (Gerberick and Ryan, 1990a). A number of individual agents and drug families have the ability to change a subject's sensitivity to artificial or solar irradiation (Fotiades *et al*., 1995; Neumann *et al*., 2000;

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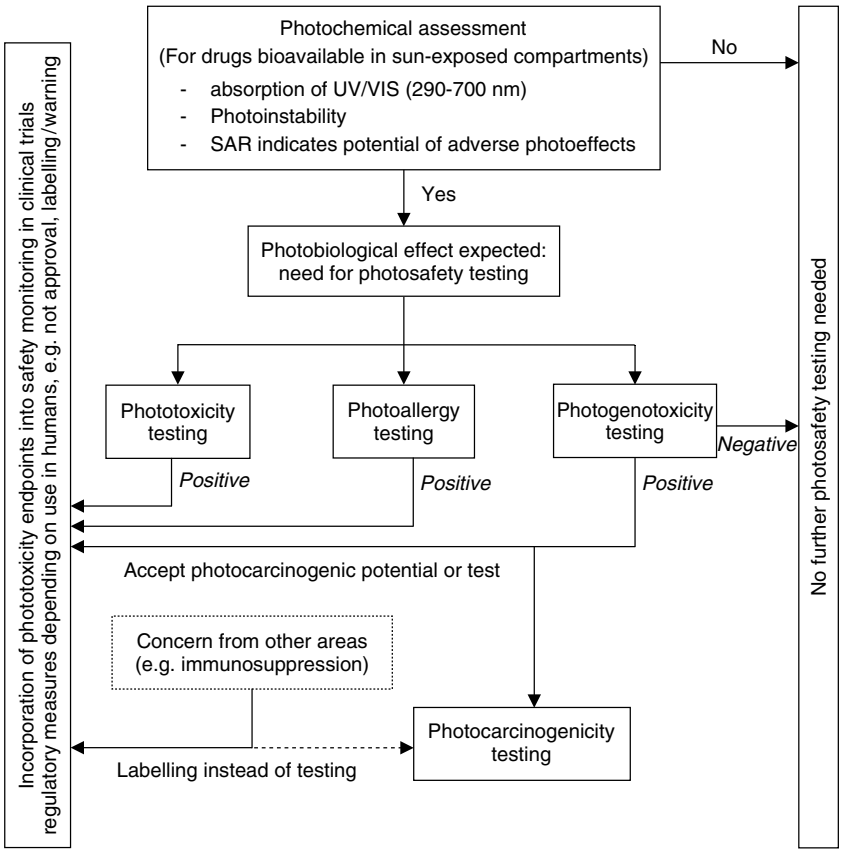

Figure 1 Assessment of photosafety of new active substances.

Moore, 2002; Ferguson, 2002; Rodríguez *et al*., 2006; Scheuer and Warshaw, 2006). Absorption of light energy alters photosensitizing chemicals producing a hapten, which attaches to a protein carrier and causes hypersensitivity (Ferguson, 2002). Drug-induced photoallergy is an immune adverse reaction to the combined effect of drugs and light. From a mechanistic point of view, it first involves covalent binding of the drug to protein, resulting in the formation of a photoantigen. Hence, determination of the structures of drug–protein photoadducts is of great relevance to understand the molecular basis of photoallergy and crossimmunoreactivity among drugs (Lahoz *et al*., 2001).

Wavelengths within the UVA (320–400 nm) range and, for certain compounds, within the visible range, are more likely to cause photosensitivity reactions; UVA (315–430 nm) and sometimes the visible region, particulatly, will produce a photochemical effect with damage to cellular components within the skin, resulting in the clinical presentation, although occasionally UVB (290–320 nm) can also be responsible for such effects. UVB wavelengths are most efficient at causing sunburn and nonmelanoma skin cancer. Photosensitivity reactions may be more specifically categorized as phototoxic or photoallergic. The most common of these are is phototoxic, and other significant, but less common mechanisms induce photoallergic reaction (Darvay *et al*., 2001). In patients who present with photosensitivity, it is often difficult to differentiate phototoxic from photoallergic reactions; distinguishing characteristics are summarized in **Table 2**.

Photoallergic reactions are cell-mediated immune responses in which the antigen is a light-activated drug. Photoactivation results in the development of a metabolite that can bind to protein carriers in the skin to form a complete antigen. The reaction then proceeds exactly as for other cell-mediated immune responses. Specifically, Langerhans cells and other antigen-presenting cells take up the antigen and then migrate to regional lymph nodes. In these locations, the Langerhans cells present the photoallergen to T-lymphocytes that express antigen-specific receptors. The T-cells become activated and proliferate, and they return to the site of photoallergen deposition. In the skin, the T -cells orchestrate an inflammatory response that usually has an eczematous morphology if the photoallergen is applied topically, or the characteristics of a drug eruption if the photoallergen is administered systemically.

Phototoxicity is a form of photosensitivity that is not dependent on an immunologic response. Phototoxic reactions are dose dependent and will occur in almost any one who takes or applies an adequate amount of the offending agent and UVR, but the dose necessary

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Table 2 Distinguishing characteristics of phototoxic and photoallergic reactions

Feature	Phototoxicity	Photoallergy
Incidence	High	Low
Pathophysiology	Direct tissue injury; not immunologically mediated	Type IV delayed hypersensitivity response; immunologically mediated
Amount of agent required for photosensitivity	Large	Small
More than one exposure to agent required	No	Yes
Distribution	Sun-exposed skin only	Sun-exposed skin, may spread to unexposed areas
Onset of reaction after exposure to agent and light	Minutes to hours	24–72 h
Clinical presentation	Exaggerated sunburn: erythema, oedema, vesicles, bullae; burning, stinging; frequently resolve with hyperpigmentation	Dermatitis: usually eczematous lesions and usually pruritic
Histologic features	Necrotic keratinocytes, epidermal degeneration; sparse dermal infiltrate of lymphocytes, macrophages, neutrophils	Spongiotic dermatitis, dermal lymphohistiocytic infiltrate

to induce such a reaction varies among individuals. Phototoxic reactions can appear on first exposure to the agent and demonstrate no cross-sensitivity to chemically related agents.

5.1 Risk Factors

The photobiological risk of photoallergic reaction depends on environmental and individual factors. Environmental risk factors are summarized in **Table 3**. Photoallergic reactions are quite unpredictable. They can occur in persons of any age, but are more common in adults than children, possibly because adults are usually exposed to more medications and topical agents (Dubakiene and Kupriene, 2006). The degree of photosensitivity varies among individuals; with the same exposure, not everyone will have a photoreaction. A person who has a photoreaction after a single exposure to an agent may not react to the same agent after repeated exposures; on the other hand, a person who is allergic to one chemical may develop photosensitivity to related chemical (cross-sensitivity). The crossreactivity patterns between the four clinically most important allergens: methyl methacrylate (MMA), 2-hydroxyethyl methacrylate (2-HEMA), 2-hydroxypropyl methacrylate (2-HPMA) and ethyleneglycol dimethacrylate (EGDMA) were studied in guinea pigs. The results of this study offer new insights into the development of MMA hypersensitivities and common cross-sensitization patterns in clinical practice (Rustemeyer *et al*., 1998). It is assumed that skin-protein conjugation at the 5- or 6- positions of the coumarin ring is important to elicit crossreactivity with esculetin or daphnetin groups (Masamoto, 2001).

5.2 Experimental Models

A wide range of experimental models for photoallergy testing existed. The potentials of *p*-aminobenzoic acid, 4-isopropyldibenzoylmethane and homosalate (three sunscreens), and two known human photoallergens (musk ambrette and tetrachlorosalicylanilide) to cause photoallergy, phototoxicity and/or contact sensitization were determined using a guinea pig photoallergy model (Gerberick and Ryan, 1989). In addition, the photoallergic potentials of various compounds were assessed using a mouse ear-swelling test (MEST) model, which offers the advantage of being quantifiable and more objective than models based on subjective evaluation of erythematous skin reactions (Gerberick and Ryan, 1990b). At present, photoallergy testing is mainly conducted using guinea pig models. Alternative test methods which may take into consideration better objectivity in photoallergic hazard identification and animal welfare are currently under development. *In vitro* tests monitoring photochemical reactions, that is, photo-adduct formation and photo-oxidation of biomolecules, can be useful screening tools. *In vivo* models under development (modified local lymph node assays (LLNAs) and MEST-assay) may become valuable in the future. However, the validation of these tests is still limited. Photoallergy testing should be performed to the state of the art.

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Table 3 Risk factors for photoallergy

Gardening	Brushing against *Dictamnus* spp. or *Ruta* Cultivating celery, parsnip or parsley Clearing weeds with a 'weed wacker' or 'string trimmer' Pruning or harvesting figs Growing *Angelica* for herbal medicine, cake decorating (when candied), tonic and flavouring in wines
Canning or processing fruits and vegetables	Canning celery or stocking celery in grocery stores Making lemonade or limeade, especially if selling it outside Squeezing lime juice for margaritas and other drinks
Hiking/jogging/walking	Through fields and riverbanks *Heracleum* spp. Rolling in the meadow Hiking in southern California and Baja California, Mexico (*Cneoridium dumosum*) Coast spice bush
Medications and cosmetics	Application of 'tan promoters' or perfumes containing bergamot oil (berlock dermatitis) Excessive UV exposure after taking or applying psoralens for PUVA Application of rue (*Ruta* spp.) as an insect repellent
Play	Making peashooters with *Heracleum mantegazzianum* (giant Russian hogweed) or Other *Heracleum* spp. Playing amongst rue bushes and Umbelliferae Fighting with parsnips/celery
Ingestion	Ingestion of excessive psoralens (especially celery) before using a tanning parlour (UVA) Ingestion of *Chlorella* (Japan)
Clothing	Wearing leis of *Pelea anisata* (Hawaii) Figure leaf

Adapted from *http://www.telemedicine.org/botanica/bot5.htm*.

Early attempts to study drugs with human volunteers had an attractive simplicity of approach. Recently, a more sophisticated, less traumatic approach for volunteers involves a carefully selected healthy population who are randomized to receive active drug, placebo or positive control following careful monochromatic and polychromatic solar-simulator baseline testing. A double-blind, randomized, parallel-group comparative study investigated the phototoxic potential of gemifloxacin mesylate, a potent, novel fluoroquinolone (FQ) antimicrobial was conducted (Vousden *et al*., 1999). In a single-centre, double-blind, randomized study, 30 adults (20 men, 10 women) received oral levofloxacin (500 mg qd × 5 d) or placebo. No subject experienced an immediate wheal-and-flare reaction. Levofloxacin has a low photosensitizing potential when administered to healthy subjects (Boccumini *et al*., 2000). A study comparing the phototoxic potential of sitafloxacin, a novel FQ with three others: sparfloxacin, enoxacin, levofloxacin and placebo in Caucasian volunteers has been conducted (Dawe *et al*., 2003). With good-quality instrument control and careful minimal erythema dose (MED) determination, pre- and on-drug photoallergy values can be compared.

5.3 DNA-Based Testing

Phototoxic responses after administration of photosensitive pharmaceutics have been recognized as undesirable side effects, and predicting potential hazardous side effects is gaining importance as new drugs are introduced to the market (Onoue and Tsuda, 2006). An assay using established cell lines, human A431 epidermal cells and mouse 3T3 fibroblasts, has been developed to predict the phototoxic potential of compounds. The test determines the viability of the two cell lines in response to UV light in both the presence and absence of the test compound (Duffy *et al*., 1987–1988). Onoue *et al*. (2008) developed effective analytical tools to predict the photogenotoxic potential of pharmaceutical substances with the use of pBR322 DNA, a plasmid DNA. Data, taken together with the results of the ROS (reactive oxygen species) assay, suggest that singlet oxygen might act as a major toxic species in quinone-induced photogenotoxicity. The structural analysis of plasmid DNA by capillary gel electrophoresis (CGE) after exposure to UVA/B in the presence of photosensitizers could be automated, allowing easy, fast and highly reliable prediction of

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the photogenotoxic potential of a large number of drug candidates (Onoue *et al*., 2008).

6 CLINICAL MANIFESTATION

Photoallergic reactions primarily occur on the areas of the skin that are exposed to UVR, but may spread beyond these to other areas. It is likely that the majority of mild episodes of drug-induced photosensitivity pass either unnoticed by the patient or are assumed to be mild sunburn. Those patients who reach hospital dermatologist outpatients have usually either been severely affected or undiagnosed (Yashar and Lim, 2003). According to the model of administration of the photosensitizer, photoallergic reactions can be contact photoallergic dermatitis or photoallergy induced by systemic agents. **Table 4** summarizes differences between phototoxic and photoallergic contact dermatitis.

The onset of a photoallergic reaction is usually delayed for 24 hours or even several days, and recovery is often slower than from a phototoxic reaction, with the reaction sometimes persisting for some time after the offending product has been discontinued. This reaction presents as an eczematous eruption with erythema, papules and vesicles, pruritis, weeping, oozing and crusting, and, later, scaling and lichenification. Hyperpigmentation does not occur in photoallergic reactions. Photoallergic reactions may also be macular, bullous or acute urticarial lesions and may develop within minutes after exposure to UVR.

7 DIAGNOSIS AND TESTING

7.1 Physical Examination

Photoallergic reactions typically develop in the sun-exposed areas such as portions of the head and face, arms, legs, and torso. Specifically, it would spare areas underneath the chin, underneath the lips, nasolabial fold, and postauricular area (Yashar and Lim, 2003). Shaded areas such as those under the hair fringe, upper eyelid, below the nose, upper lip, submental area, post-auricular area, web spaces of finger and flexures tend to be spared (Hearn, 2005). However, in severe cases lesions may extend to covered areas. Pruritic eczematous eruption is suggestive of photoallergic reactions. Erythema and vesiculation are present in the acute phase. Lichenification and scaling are the hallmarks of chronic photosensitivity. An uncommon variant of polymorphous light eruption also can present with small blisters. Morphological clues in differential diagnosis of phottoallergy is presented in **Table 5**.

In addition, exposure to known photosensitizers is of the main importance during the history taking. Intervals between sun exposure, the development of lesions, and the duration of the lesions offer some clues on the diagnosis. The age of onset and connective tissue disease also help in narrowing the differential diagnosis.

7.2 Phototesting

Phototesting with UVA, UVB and, sometimes, visible light is helpful in diagnosing photosensitivity disorders. This test is performed by treating small areas of skin on the back or inner aspect of the forearms with gradually increasing doses of light. The minimum dose of light required to produce uniform erythema over the entire irradiated site after 24 hours is called the minimum erythema dose (MED). Patients with phototoxic reactions have a reduced MED to UVA or, in some instances, UVB.

7.3 Photopatch Tests

In 1984, the German, Austrian and Swiss Photopatch Test Group was founded to standardize the photopatch-test procedure and to investigate photoallergic reactions, as

Table 4 Differences between photoallergic and phototoxic contact dermatitis

Feature	Photoallergic	Phototoxic
Incidence	Low	High
Occurrence on first exposure	No	Yes
Onset after ultraviolet exposure	Usually 24–48 h (6–72)	Minutes to days
Dose dependence		
Chemical	Not crucial	Important
Radiation	Not crucial	Important
Clinical morphologic appearance	Eczematous	Erythematous and bullous hyperpigmentation
Histology	Spongiotic dermatitis	Necrotic keratinocytes
Diagnosis	Photo-patch testing	Clinical history and psychical

Adapted from Deleo, A. V. (2004). Photocontact dermatitis. *Dermatology Therapy*, **17**, 279–288.

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Table 5 Morphological features in differential diagnosis of phottoallergy

Morphological features	Differential Diagnoses
Erythema	Sunburn
	Drug phototoxicity
	Lupus erythematosus
	Dermatomyositis
Urticaria	Solar urticaria
	Erythropoietic protoporphyria
	Porphyria cutanea tarda
	Drug-induced urticaria
Oedema	Solar urticaria
	Polymorphic light eruption
	Lupus erythematosus
Papules	Polymorphic light eruption
	Systemic lupus erythematosus
	Actinic prurigo
Blisters	Polymorphic light eruption
	Hydroa vacciniforme
	Porphyria cutanea tarda
	Drug phototoxicity
	Phototoxic contact dermatitis
Eczema	Chronic actinic dermatitis
	Photoaggravated eczema
	Lupus erythematosus
	Dermatomyositis drug photoallergy
	Photoallergic contact dermatitis
Scars	Discoid lupus erythematosus
	Actinic prurigo
	Hydroa vacciniforme
	Porphyria cutanea tarda
	Erythropoietic protoporphyria
No rash	Polymorphic light eruption
	Sine eruptione
	Erythropoietic protoporphyria
	Drug induced phototoxicity

[a]Adapted from *http://www.nsc.gov.sg/showpage.asp?id=357*; Yashar and Lim, 2003; Hearn, 2005.

well as the epidemiology of photoallergy, in central Europe (Neumann *et al*., 2000). Therefore, in the first test period from 1985 to 1990, 32 test substances were applied on the backs of patients suspected to be photosensitive. After evaluation of these data, some substances were dismissed, and others were additionally integrated into the test tray. Thus a modified test tray comprising 26 test substances was used for the second test period (1991–1997). The British Photodermatology Group (1997) published a workshop report in 1997 with guidelines on photopatch-testing technique. This recommended UVA as the radiation source and suggested compounds for a series of photocontact allergens. No firm recommendations were made for timing of irradiation or patch-test readings. Photopatch testing is an important tool in the diagnosis of photoallergic contact dermatitis:

- Suspected photoallergens are applied to the back in two sets
- One set is removed after 24 hours and irradiated with $5-10\,J\,cm^{-2}$ UVA
- Both sets of patch tests are evaluated for a positive reaction after 48 hours
- Erythema, edema and/or vesiculation at an irradiated site indicate a positive reaction
- A positive reaction at both sites is interpreted as allergic contact dermatitis
- A positive reaction at the unirradiated site with a stronger one at the irradiated site should be interpreted as both allergic dermatitis and an photoallergic contact dermatitis reaction to the same compound.

There still exist a variety of protocols presently in use, with little information as to which method is most reliable or discriminatory.

7.4 Laboratory Tests

Laboratory tests are required to exclude porphyria cutanea tarda, by assessing urine porphyrin levels, which are elevated in porphyria cutanea tarda and within the normal range in pseudoporphyria and drug-induced photosensitivity, and to determine antinuclear antibody (ANA) and anti-Ro (SSA) antibody levels.

Histologically photoallergic reaction is similar to contact dermatitis. Epidermal spongiosis with a dermal lymphocytic infiltrate is a prominent feature. The presence of necrotic keratinocytes is suggestive of photoallergy rather than allergic contact dermatitis.

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Significance of Biochemical Markers in Applied Toxicology

Fernando Gil and Antonio F. Hernández

CONTENTS

1 INTRODUCTION

Although the presence of a xenobiotic in the environment always represents a risk for living organisms, it is necessary to consider key factors that can influence the host reaction to chemical compounds, including their physicochemical properties, routes of exposure (inhalation, oral and/or dermal absorption) and a number of host characteristics including age, race, gender, health status, genetic susceptibility and previous exposure to the same or other chemicals.

One of the methods to assess and quantify the exposure to xenobiotics and its potential impact on organisms is biological monitoring using biomarkers. A biomarker can be broadly defined as any biological index capable of being measured, reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical or biological. The measured response is associated with or indicative of a defined and observable biological end point that can range from the morphological level down to the molecular level, and which indicates events in the process leading to clinical disease (Ward and Henderson, 1996). Biomarkers, therefore, provide information on the nature and the effect of an exposure, as well as on the susceptibility of individuals or populations to the toxic effects of such an exposure (Rockett and Kim, 2005). Ideally, biomarkers will be expressed across a large section of the population, and can be monitored quickly, easily, conveniently and with minimal cost (Rockett *et al*., 2004).

A US National Institutes of Health (HNIH) Study Group defined a biomarker as a characteristic that is objectively measured and evaluated as an indicator of normal physiologic processes, pathologic processes, or pharmacologic responses to a therapeutic intervention (Biomarkers Definitions Working Group, 2001). Biomarkers are essential tools in monitoring studies, which include environmental monitoring, biological monitoring, biological effect monitoring and health surveillance, as well as drug development processes. Biomarkers are also becoming increasingly important in toxicology and human health. Their use in the evaluation of disease risk has increased markedly in the last decades. The discovery, validation and analysis of biomarkers requires highly sensitive and selective analytical technologies. Recent advances in technology have added several new tools to the biomarker screening toolkit and improved the throughput of existing quantitative assays (Orhan, 2007). Genomics, proteomics and metabonomics have provided a wealth of data in the search for predictive, specific biological indicators.

However, biological monitoring cannot be applied for assessing exposure to xenobiotics that exhibit their toxic effects immediately at the sites of first contact (caustic agents, lung irritants) and for those chemicals poorly absorbed, because of the lack of detection in biological matrices. Furthermore, the complexity and accessibility of certain organ systems (for example nervous system) do not allow for a rapid and easy development of sensitive, specific and reliable biomarkers.

When assessing the usefulness of a particular exposure biomarker, one must consider analytical and toxicokinetic aspects in order to establish its validity. For optimal analytical quality, standardization is needed: sampling, handling and measurement procedure, and quality assurance methods. Life events, such as physiological (pregnancy, senescence) or pathological processes (e.g. liver or kidney disease) may also affect the toxicokinetic

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of a particular xenobiotic (Grandjean *et al*., 1994; Gil, 2005).

When a quantitative relationship is established between internal dose and adverse effects, and the internal-dose effects and internal-dose response relationships are known, then biological monitoring allows for a direct health risk assessment and thus for an effective prevention of the adverse effects (Gil, 2005). Although the effects of environmental chemicals have traditionally been detected by means of biomarkers of exposure or effects, nowadays proteomics offers a new approach using a set of high-throughput methodologies with a wide dynamic range that makes possible the discovery of novel biomarkers (Sheehan, 2007).

The ideal biomarkers (Grandjean *et al*., 1994; Gil, 2000) should be detected early and should warn of adverse effects before they become irreversible. In all cases, they should comply with the following characteristics: sample collection should be easy; analysis should be robust (rapid, simple, reliable and accurate); they must be specific for a particular type of exposure, tissue and/or disease. Furthermore they should be sensitive (detectable at low levels), capable of reflecting subclinical and reversible changes, have a preventive character and be ethically acceptable.

Only a few biomarkers fit these characteristics well. Biomarkers may range from those that are highly specific, such as aminolevulinic acid dehydratase (ALAD), an enzyme of the haem pathway which is inhibited only by lead, or the inhibition of acetylcholinesterase (AChE), which is specific for organophosphorus (OP) and carbamate insecticides, to those that are nonspecific, such as effects on the immune system or on DNA that can be caused by a wide variety of xenobiotics (polycyclic aromatic compounds, chemotherapeutic drugs, organochlorine pesticides, etc.) (Walker *et al*., 1996).

2 CLASSIFICATION OF BIOMARKERS

Biomarkers have generally been classified into four major categories: internal dose, biologically effective dose, preclinical biologic effects and susceptibility, with some overlap between them (Committee on Biological Markers of the National Research Council, 1987). Of the four categories, internal dosimeters have been most widely used, particularly in the workplace, to determine exposure to carcinogens and other toxicants. Analyses of the biological material provide information on the various steps inside the exposed individual that bridge the gap between an external exposure to a toxic agent and a possible resulting occupational or environmental disease (**Figure 1**) (Perera and Weinstein, 2000).

2.1 Biomarkers of Exposure

Biomarkers of exposure can be used to confirm and assess the exposure of individuals or populations to a particular substance, providing a link between external exposures and internal dose. Biomarkers of exposure represent the measure of internal dose of the toxic compound or metabolite in a wide variety of biological samples (blood, urine, exhaled air, sputum, saliva, hair, fingernails and faeces) (Van Cauteren *et al*., 1996). Their quantitative measurement facilitates the determination of dose–response relationships.

The internal dose reflects the amount of the toxicant or its ultimate metabolite at the critical site in the target organ and may also mean the amount of a chemical stored in one or in several body compartments or in the whole body. This usually applies to cumulative toxic agents (e.g. solvents, organochlorine compounds, metals) (Lauwerys, 1993; Greim, 2001).

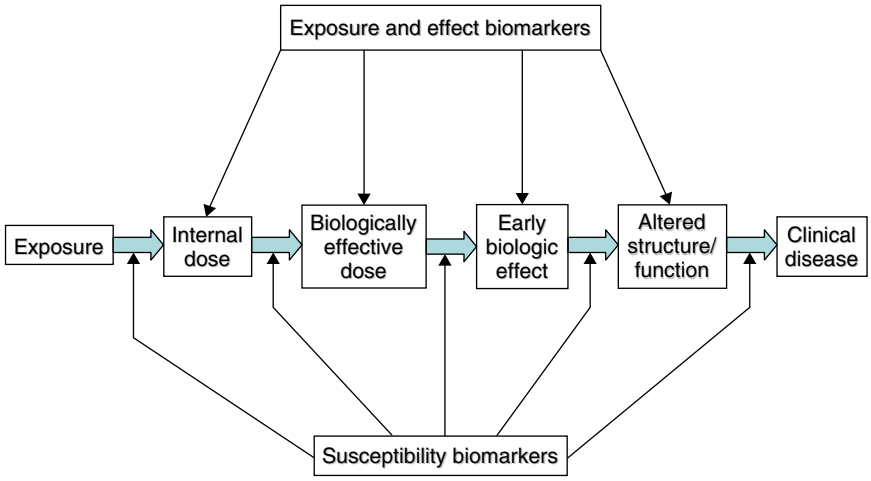

Figure 1 Fate of xenobiotics within the body. (Reproduced from Perera and Weinstein, 2000. © Oxford University Press.)

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Biomarkers of internal dose take into account individual toxicokinetic differences of the compound in question and indicate the actual levels of the compound within the body and in specific tissues or compartments. However, since their half-lives range from a few hours to decades their interpretation and applicability to different study designs varies accordingly. Examples include: urinary levels of 1-hydroxypyrene from exposure to polycyclic aromatic hydrocarbons (PAHs), DDT (dichloro-diphenyl-trichloroethane), polychlorinated biphenyls (PCBs), dioxins and furans in serum or adipose tissue biopsies; styrene levels in exhaled air or blood; and phenylglioxilic acid, its metabolite, in urine due to workplace and environmental contamination (Perera and Weinstein, 2000).

Internal dose exposure can also be measured by determining adducts with cellular macromolecules such as proteins, amino acids, DNA or its bases. The concentration of the ultimate reactants that interact with macromolecules is determined by the rate of metabolic activation and inactivation. Individuals with different rates of enzyme activities involved in biotransformation will show different adduct levels. Thus, these markers reflect both exposure and metabolic capacity of the individuals (Greim, 2001). Haemoglobin (Hb) and serum albumin are the preferred monitor molecules because they are accessible in large amounts. They are chemically stable and are not prone to repair mechanisms as are DNA adducts. Because of the long lifespan of Hb (120 days) and the long half-life of serum albumin (20 days), these adducts accumulate in the human body, making them very sensitive parameters for human biomonitoring (Angerer *et al*., 2007).

Although markers of internal dose are valuable supplements to conventional methods of assessing exposure, markers of internal dose do not indicate the extent to which a given compound has interacted with critical cellular targets. Therefore, assays have been developed to measure the 'biologically effective dose' of a compound; that is, the amount that has reacted with critical cellular macromolecules, usually DNA, or a surrogate, such as specific proteins in the blood (Perera and Weinstein, 2000).

2.2 Response or Effect Biomarkers

Response or effect biomarkers are indicative of biochemical changes within an organism as a result of xenobiotic exposure and interaction with biological targets. They reflect a measurable biochemical, physiological, behavioural or other alteration within an organism that, depending upon the magnitude, can be recognized as associated with an established or possible health impairment or disease. Wide interindividual variations in the response to equivalent doses of chemicals are usually observed.

These biomarkers are the most studied and include changes in enzyme activities (usually inhibition), DNA adducts in target tissues, modifications in some parameters of blood composition, protein increases and appearance of autoantibodies against a xenobiotic or a particular cellular fraction.

Biomarkers of effect can be used to document either preclinical alterations or adverse health effects elicited by external exposure and absorption of a chemical, so that they reflect early biological effects. Thus the linkage of biomarkers between exposure and effect contributes to the definition of dose–response relationships. Significant examples of biomarkers of effect classified according to the organ or system targeted by the toxicant are considered below. This review is focussed on biomarkers available for human health assessment. Although there exist other biomarkers which can be applied in environmental toxicology (i.e. metallothioneins, heat stress proteins, etc.) or forensic toxicology (biochemical markers of alcohol or drug consumption), such biomarkers go beyond the scope of this review.

2.2.1 Nephrotoxicity Biomarkers

Acute kidney injury is a common condition with significant associated morbidity and mortality. Unfortunately, standard metrics used to diagnose and monitor kidney injury, such as blood urea nitrogen and serum creatinine, are insensitive and nonspecific, resulting in delayed diagnosis and intervention. The prevention of renal diseases requires the use of sensitive tests capable of detecting effects at a stage when they are still reversible. Chronic exposure to certain nephrotoxic compounds (solvents, heavy metals, halogenated hydrocarbons, organic and therapeutic agents) may cause progressive degenerative changes in the kidney. However, the clinical signs of renal damage are not apparent until the injury is extensive and irreversible, probably due to a large reserve capacity of this target organ (Bernard and Hermans, 1997; Mueller *et al*., 1997; Finn *et al*., 1997). Because of the complexity of the nephron and its regional sensitivity to xenobiotics, it is important to co-localize sites of marker release with pathological lesions (Buchet *et al*., 1990; Mutti *et al*., 1992; Roels *et al*., 1993; Bernard *et al*., 1994a; 1995a; 1995b; Hotz *et al*., 1995; Voss *et al*., 2005; Marchewka *et al*., 2006; Nortier *et al*., 1997; Guder and Hofmann, 2008). Probably, the main limitation is that these biomarkers do not permit the detection of effects on all areas or segments of the kidney or nephron (for example effects on the papilla or the distal tubule).

- Glomerulus: the determination in urine of a high-molecular-weight protein (such as albumin or transferrin) has been used for the early detection of glomerular barrier defect. Fibronectin and laminin

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fragments are also biomarkers of glomerular damage. Higher levels of free sialic acid points to disturbances in glomerular filtration.

- Proximal tubules: the increase in low-molecular-weight proteins in urine (such as retinol-binding protein, β_2-microglobulin, α_1-microglobulin or α-amylase), assumed to be freely filtered, reflect tubular dysfunction. Brush border markers include alanine aminopeptidase and γ-glutamyltransferase, as well as the efficiency of the nephron resorption process through concentration of β_2-microglobulin in urine and its filtracy fraction of elimination.
- More recent biomarkers include carbonic anhydrase, alkaline phosphatase, alanine aminopeptidase, adenosine deaminase-binding protein and glutathione *S*-transferase-α (α-GST). The last of these is a hallmark of early changes in the proximal tubular integrity for biomonitoring adverse effects induced by the chronic exposure to certain heavy metals such as lead and cadmium (Garçon *et al*., 2004).
- Thick ascending limb of the loop of Henle: measurement of Tamm-Horsfall glycoprotein.
- Distal tubules: determination of GST-π.

Nephrotoxic damage can be also detected by measuring the urinary activity of kidney-derived components including enzymes, antigens, and so on. Lysosome-derived enzymes, such as *N*-acetyl-β-D-glucosaminidase (NAG), isoenzyme (NAG-B), β-galactosidase and β-glucuronidase are also used for monitoring proximal tubule insults. The B isoenzyme of lysosomal NAG has been proposed as an index of nephrotoxicity because of its high activity in the kidney and its stability in urine.

Although the kidney is one of the main targets of xenobiotic-induced toxicity, early detection of renal damage is difficult. Recently, several novel biomarkers of nephrotoxicity have been identified by transcription profiling, including kidney injury molecule-1 (Kim-1, a transmembrane glycoprotein which is up-regulated following nephrotoxic injury), lipocalin-2, tissue inhibitor of metalloproteinases-1 (Timp-1), clusterin, osteopontin (OPN) and vimentin. They appear to be sensitive and tissue-specific biomarkers that will improve detection of early acute kidney injury following exposure to nephrotoxicants, when compared to traditionally used biomarkers. However, it is not known if these cellular marker molecules may also be useful to predict chronic nephrotoxicity or to detect nephrotoxic effects, although induction of Kim-1 messenger RNA expression was the earliest and most prominent response observed *in vitro*, supporting the use of this marker as a sensitive indicator of chronic kidney injury (Rached *et al*., 2008).

2.2.2 Biomarkers of Blood System Disease

Alterations of the haem biosynthetic pathway represent the most studied biomarkers of effect. ALAD is an enzyme involved in the haem synthesis in bone marrow erythroblast, and the assay is highly specific for lead exposure and effect. The inhibition of ALAD has been shown to be a reliable marker of effect for lead in studies on humans and certain animals (eagles, starlings, ducks and geese) (Scheuhammer, 1987; Peakall, 1992a; Melancon *et al*., 1992; Walker *et al*., 1996). In addition, this biomarker has also proven to be very sensitive in epidemiological studies on pesticide applicators (Panemangalore *et al*., 1999; Hernández *et al*., 2005).

The most commonly used biomarkers in the blood are those dealing with Hb or its biosynthetic pathway. Some chemicals are capable of reacting with Hb and give rise to intermediate compounds that can be analysed for biomonitoring purposes. One example is carbon monoxide (CO), which binds Hb with high affinity, generating carboxyhaemoglobin (CO-Hb), which shows a dose–response relationship with clinical disturbances, particularly in acute poisoning. In contrast, its usefulness in chronic exposure has not been sufficiently evaluated. Another example is nitrites, which may oxidize Hb, rendering methaemoglobin (Met-Hb), which could also be measured by spectrophotometric methods. On the other hand, certain organochlorine compounds may cause the formation of excess amounts of hepatic highly carboxylated porphyrins. Only a few agents have been involved in chemical-induced porphyria, such as hexachlorobenzene, lead and PCBs (Gil and Pla, 2001). It has been mentioned above that reactive intermediates generated by oxidative metabolism in Phase I reactions can bind covalently to Hb. However, they must be considered as biomarkers of internal exposure rather than biomarkers of effect. Some examples of chemicals that may produce Hb adducts are ethylene oxide, acrylamide, 3-amino-1,4-dimethyl-5OH-pyrido-indole, 4-aminobiphenyl, 2,6-dimethylaniline, and so on (Gil and Pla, 2001).

2.2.3 Hepatotoxicity Biomarkers

Liver toxicity is caused by a number of chemicals that are metabolized by the cytochrome P450 (CYP450)-dependent mixed-function oxidase system to reactive intermediates. Liver injury biomarkers include various peripheral proteins released in response to a cellular damage or, locally, proteins that are significantly altered within the liver. These include both circulating cytosolic, mitochondrial or canalicular membrane markers, and the up-regulation or depletion of radical scavengers, modulators and stabilizers of intracellular damage (Amacher, 2002).

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Some of the many enzymes found in hepatocytes can be measured in the serum and are used as tests of liver function. They can be divided into two categories: enzymes that primarily reflect cholestasis, such as the alkaline phosphatase, 5′-nucleotidase and γ-glutamyl transpeptidase, and those that primarily reflect hepatocellular necrosis, such as the aminotransferases (Reichling and Kaplan, 1988). Ornithine transcarbamylase (OTC) is a hepatic enzyme involved in ammonia elimination via the urea cycle, and can be measured in serum as a diagnostic marker of hepatic disorders due to its localization in periportal mitochondria (Ishikawa *et al*., 2003). The ratio of serum OTC to alanine aminotransferase (ALT) is a potent indicator for the diagnosis and prognosis of hepatocellular carcinoma (Murayama *et al*., 2007).

An examination of the current state of hepatotoxic biomarkers indicates that serum F protein, arginase I and GST-α levels, all measured by enzyme-linked immunosorbent assay (ELISA), may show utility and provide additional information relative to serum aminotransferase values (Ozer *et al*., 2008).

2.2.4 Respiratory Toxicity Biomarkers

Pneumotoxic agents, for example 4-ipomeanol, 3-methylfuran, trichloroethylene and carbon tetrachloride, produce disease of the respiratory system. Clara cells are particularly sensitive to toxic lung injury and they contain indeed most of the lung CYP450 activity, which confers on them a high xenobiotic metabolizing activity (Bernard *et al*., 1992; Hermans and Bernard, 1996). Clara cell protein (CC16 or CC10) is a lung biomarker, measurable in serum, bronchoalveolar lavage (BAL) fluid and sputum. This 15.8 kDa protein is secreted along the tracheobronchial tree, particularly in the terminal bronchioles where Clara cells are localized. This protein increasingly appears to protect the respiratory tract against oxidative stress and inflammation. This protective role is confirmed by studies on transgenic mice, showing that CC16 deficiency is associated with an increased susceptibility of the lung to viral infections and oxidative stress. The determination of CC16 in serum is a new noninvasive test to detect Clara cell damage or increased epithelial permeability in various acute and chronic lung disorders. This test may provide a useful tool to noninvasively evaluate the damage and increased permeability to proteins of the air–blood barrier associated with some pathology (Broeckaert and Bernard, 2000). Serum concentrations of CC16 are also decreased in subjects with chronic lung damage caused by tobacco smoke and other air pollutants, as a consequence of the destruction of Clara cells. Serum CC16 is also decreased in several occupational groups chronically exposed to silica, dust and welding fumes, and lung diseases (cancer, asthma and patients with chronic obstructive pulmonary disease) (Bernard *et al*., 1994b; 1994c).

In addition to CC16, other ‘ideal’ biological markers of effect have been identified; namely, tumour necrosis factor-α and interleukin-8 (both of them released by monocytes), reactive oxygen species (released by neutrophils), 8-isoprostanes (serum), total equivalent antioxidant capacity, glutathione, glutathione peroxidase activity, GST activity and platelet-derived growth factor (serum) (Gulumian *et al*., 2006).

Increased levels of some enzymes in BAL fluid may indicate an increased permeability of the alveolar/capillary barrier. Lactate dehydrogenase (LDH) is a cytoplasmic enzyme that is found extracellularly only in the presence of lysed or damaged cells. β-Glucuronidase or similar lysosomal hydrolytic enzymes are excellent markers for the toxicity of inhaled particles. These particles are phagocytosed by macrophages, and the enzymes are released from activated or lysed macrophages.

2.2.5 Neurotoxic Biomarkers

The complexity of the nervous system, together with problems associated with the determination of precise targets for neurotoxic action, is responsible for the limited advances seen in biomonitoring of neurotoxicity. Additionally, the inaccessibility of brain tissue in humans limits neurochemical measurements in neurotoxicity studies (Costa and Manzo, 1995).

Developing accessible biomarkers of neurotoxic effects which are readily applicable to human populations poses a challenge for neurotoxicology. Proteomics is one of the fastest growing areas in science and is particularly applicable to the development of biomarkers of neurotoxicity. The most significant and useful example of a specific biomarker of neurotoxicity is the inhibition of AChE in blood samples and brain caused by OP or carbamate insecticides. This biomarker has been used not only in human toxicology but also in ecotoxicology as proof of cause of death (Fairbrother and Bennett, 1988; Peakall, 1992b). AChE activity in brain may remain depressed for several weeks after the toxic exposure, and the depression is reasonably well correlated with the effects. However, blood AChE has the advantage of easy sampling since there is no need of animal sacrifice (Hill, 1988), and clearly brain enzyme cannot be sampled in life in humans. Butyrylcholinesterase (BuChE) or plasma nonspecific pseudocholinesterase only reflects exposure to OPs or carbamate insecticides, but not neurotoxicity.

Several OP compounds (tri-*o*-cresyl phosphate, methamidophos, leptophos) induce a delayed polyneuropathy (organophosphate induced delayed polyneuropathy—OPIDP) that is accompanied by, and probably causally related to, the inhibition of neuropathy target esterase (NTE) in the nervous system (Lotti, 1995). This enzyme is also present in lymphocytes; therefore measurement of NTE in lymphocytes has been proposed as an example of neurotoxic biomarker for biomonitoring purposes,

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although it has not been applied successfully to epidemiological studies.

The glial reaction to nervous system damage, often termed gliosis, is a hallmark of all types of nervous system injury. As such, the development and implementation of gliosis biomarkers represents a broadly applicable approach for neurotoxicity safety assessment. Recent studies have demonstrated that neuroantibodies can be used as biomarkers of neuropathy development. Serum autoantibodies against neuronal cytoskeletal proteins (e.g. neurofilament triplet, glial proteins such as myelin basic protein and glial fibrillary acidic protein) have been reported as peripheral markers of neurodegeneration that may be applicable to humans and experimental studies, including OPIDP (O'Callaghan and Sriram, 2006; El-Fawal and McCain, 2008; El-Fawal and O'Callaghan, 2008).

Another example of a biomarker of neurotoxicity is monoamine oxidase (MAO), an active enzyme that plays a key role in inactivating catecholamines. MAO-B is a microsomal isoenzyme of MAO that acts on a broad spectrum of phenylethylamines. The amino acid sequence of the enzymes from human cerebral cortex has been shown to be identical to platelet MAO-B, so the activity of the platelet MAO-B should reliably reflect that of MAO-B in the nervous system, and has the advantage of easy accessibility. MAO-B activity in platelets has been used as a biomarker of effect of styrene and perchloroethylene occupational exposures, which is known to cause dopamine depletion (Checkoway *et al*., 1994; Mutti and Franchini, 1987).

2.2.6 Biomarkers of Cardiotoxicity

There is an overwhelming weight of evidence that considers cardiac troponin (cTn) as the preferred, *de facto*, translational, safety biomarker for myocardial injury in cardiotoxicity. In addition to being the gold standard for cardiac injury in humans, it has been widely used for clinical assessment and monitoring of cardiac toxicity in subjects being treated for cancer. No other biomarker of myocardial injury comes close to cTn in effectiveness, including creatine kinase isoenzyme MB (CK-MB), lactate dehydrogenase isoenzyme 1 (LDH-1) and LDH-2, myoglobin and FABP3 (heart fatty acid binding protein-3). In addition to the use of cTn for monitoring active myocardial degeneration, there is growing evidence that measurements of brain natriuretic peptide (BNP) may be effective for monitoring drug-induced left ventricular dysfunction (O'Brien, 2008).

The role of air pollution in exacerbating heart disease has only recently become a focus of attention. Particulate matter has been linked to increased levels of systemic inflammation biomarkers such as C-reactive proteins (CRPs). Daily variation of ambient pollution is correlated with rises and falls in CRP levels. Increased CRP levels have been associated with increased morbidity and mortality in individuals with coronary artery disease (Sandhu *et al*., 2005).

2.2.7 Biomarkers of Reproductive Toxicity

To monitor reproductive health and identify adverse effects at the earliest possible levels, it is necessary to develop a network of biomarkers covering all stages and aspects of reproductive development and function. Various biomarkers of reproductive development and health have been identified, including those associated with pubertal development, adult reproductive health and pregnancy outcome (Rockett and Kim, 2005). Biomarkers for the male reproductive system may include physiological indicators of impaired testicular function, sperm number or cytogenetics characteristics.

Measures of hormonal status (follicle stimulating hormone (FSH), luteinizing hormone (LH) and testosterone) can be readily obtained from blood and, in the case of testosterone, from urine and saliva. However, these levels are greatly influenced by circadian rhythms and demonstrate large interindividual and intra-individual variability. Biomarkers for the male reproductive system are rather easily accessible and some are reasonably well validated; such markers are less well developed for the female reproductive system (Osorio and Windman, 2005; Windman and Osorio, 2005).

Inhibin B has been proposed as a potential biomarker of testicular toxicity to compliment traditional end points. This glycoprotein is produced predominantly by Sertoli cells and regulates pituitary FSH release by a negative-feedback loop. Systemic inhibin B concentrations seem to reflect the extreme ends of spermatogenic status, with high levels of inhibin B observed in normal, fertile individuals and lower levels of inhibin B in individuals with severe damage to the testis as a result of germ cell depletion. In epidemiological studies, inhibin B combined with FSH measurements has a higher positive predictive value for detecting male infertility than either alone (Stewart and Turner, 2005).

2.2.8 Immunotoxicity Biomarkers

The immune system protects the organism against infectious micro-organisms and the growth of some tumours. An important variety of factors may modify the immune function, such as drugs (nonsteroidal anti-inflammatory or vitamin complexes), biological parameters (sex, age, pregnancy), and other factors (diet, alcohol consumption, circadian rhythms, stress, nutritional state and sleep disturbances).

Different xenobiotics may stimulate or suppress the immune system. Dioxin, PCBs, immunotherapeutic drugs and other toxic agents including some OP compounds (parathion, chlorpyrifos, malathion and diazinon) can

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affect the immune system and can lead to decreased resistance to infections or tumours. These chemicals can also alter the course of autoimmunity or induce hypersensitivity reactions.

Several biomarkers have been proposed to assess immunotoxicity. They include full blood count, antibody-mediated immunity (immunoglobulin concentrations in serum), phenotypic analysis of lymphocytes by flow cytometry (surface markers), cellular immunity (including delayed-type hypersensitivity on skin and natural immunity to blood group antigens), markers of inflammatory response (CRP) and antibodies (autoantibodies to cellular organules). Examination of nonspecific immunity including interleukins analysis or measurement of complement components can also be used. For example, changes in the relative abundance of different lymphocyte subpopulations (suppressor and helper T cells) have been used as biomarkers for the immune suppression (Van Loveren *et al*., 1995; Kimber, 1995; Melancon, 1995).

2.2.9 Biomarkers of DNA Damage

The interaction of a xenobiotic with genes can lead to DNA mutations and consequent alterations in the descent. The damage of DNA has been studied in both humans and animals. Some xenobiotics (PAHs, aromatic amines, heterocyclic amines, mycotoxins, alkylating chemotherapeutic agents and nitrosamines) have reacting groups that can interact with nucleophilic targets, increasing the risk of cancer. DNA adducts are being used to assess the genotoxic potential of chemicals. Certain DNA adducts produced by interactions are promutagenic, and replication of the damaged DNA could lead to DNA changes that may result in altered gene expression or mutated gene products. Thus, biomarkers such as metabolic polymorphisms, urinary metabolites, unscheduled DNA synthesis, DNA single-strand breaks, chromosomal aberrations and protein and DNA adducts are used in the evaluation of cancer risk.

Molecular biology techniques permit the detection of covalent interactions of xenobiotics with proteins and other macromolecules. Human DNA adduct formation (covalent modification of DNA with chemical carcinogens) has been shown to correlate with the incidence of a carcinogenic process and is a promising biomarker for elucidating the molecular epidemiology of human cancer (Meyer and Bechtold, 1996; Shugart, 1996; Poirier and Weston, 1996).

Cytogenetic biomarkers in peripheral blood lymphocytes, such as chromosomal aberrations, sister chromatid exchanges and micronuclei, have long been applied in surveillance of human genotoxic exposure and early effects of genotoxic carcinogens. The relevance of chromosomal aberrations as a biomarker has been further emphasized by epidemiological studies suggesting that a high frequency of chromosomal aberrations is predictive of an increased risk of cancer (Norppa, 2004).

At this time, the study of DNA adducts is not feasible for routine analysis. The biological monitoring of human and animal DNA adducts includes ^{32}P-postlabelling and, recently, immunoassays using adduct-specific antibodies. The major drawback is the lack of specificity of the antibodies used in the assay which cross-react with a number of xenobiotic-related adducts (Dunn *et al*., 1987; Chang *et al*., 1994; Poirier, 1997). Other analytical techniques such as gas chromatography-mass spectrometry (GC-MS) and synchronous fluorescence spectroscopy are being used to measure DNA adducts. Peripheral blood lymphocytes are a readily accessible source of human cells that are known to contain DNA adducts.

2.2.10 Biomarkers of Gene Expression

During the first stages of cancer there is a significant increase in gene-expression biomarkers related to the specific type of cancer. The development of xenobiotic-related tumours is associated with the aberrant expression of genes that encode proteins involved in cellular growth.

Biomarkers of gene expression include growth factors (platelet-derived growth factor, transforming growth factor α, transforming growth factor β, etc.) and oncoproteins (transmembrane and epidermal growth factor receptors, oncogene proteins such as p21 and p54 and tumour suppressor gene proteins such as nuclear phosphoprotein p53) (Poirier, 1997; Brandt-Rauf, 1997). p53 has many anticancer mechanisms: it can activate DNA repair proteins when DNA has sustained damage; it can also hold the cell cycle at the G_1/S regulation point for DNA damage recognition and reparation; and if the DNA damage proves to be irreparable it can initiate apoptosis, the programmed cell death.

These biomarkers may be studied in biological fluids such as serum, plasma, urine and bronchoalveolar liquid by ELISA, radioimmunoassay (RIA) or immunoblotting.

2.2.11 Biomarkers of Oxidative Damage

Oxidation and the production of free radicals are an integral part of human metabolism, producing oxidative stress which is related to many diseases, including cancer and heart disease. The use of biomarkers for oxidative stress may provide evidence of a causal relationship between oxidative damage to macromolecules (DNA, lipids and proteins) and cancer. A wide variety of functional assays, both *in vivo* and *ex vivo*, include various measures of DNA oxidation (oxidized DNA bases such as 8-hydroxy-2′-deoxyguanosine (8-OHdG), autoantibodies to oxidized DNA, modified comet assay), lipid oxidation (thiobarbituric acid-reactive substances, exhaled pentane/ethane, low-density lipoprotein resistance to oxidation, isoprostanes) and protein oxidation (protein carbonyls) (Hwang and Kim, 2007).

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Some pollutants, such as heavy metals, pesticides, PAHs, halogenated aromatic hydrocarbons and solvents, are capable of developing oxidative damage by generating free radicals. These molecules damage membrane lipids, DNA and proteins, and effect changes to antioxidant enzymes. Although there are considerable gaps in our knowledge of cellular damage, response mechanisms, repair processes and disease aetiology in biological systems, free radical reactions and the production of toxic reactive oxygen species are known to be responsible for a variety of oxidative damages leading to adverse health effects and diseases. In response to oxidative stress, changes in the antioxidant systems, as adaptive responses, and modified macromolecules can serve as biomarkers.

The antioxidant enzyme systems (glutathione reductase, catalase, superoxide dismutase and peroxidase activities) and nonenzymatic systems (reduced glutathione, ascorbate, α-tocopherol) play a defensive role against oxidative stress by-products. Malondialdehyde and 8-OHdG have been proposed as oxidative stress markers. Macromolecules that may be affected by free-radical damage include lipids, proteins and nucleic acids (Hoffman *et al*., 1989; Di Giulio *et al*., 1989; Hogson and Levi, 1994). The depression of the key antioxidant enzymes and the elevated lipid peroxidation are important mechanisms underlying the oxidative damage.

2.3 Biomarkers of Susceptibility

Polymorphisms of relevant xenobiotic-metabolizing enzymes may be used as markers of toxicological susceptibility. A growing number of genes encoding enzymes involved in biotransformation of toxicants and in cellular defence against toxicant-induced damage to the cells have been identified, leading to increased knowledge of allelic variants of genes and genetic defects that may result in a differential susceptibility of individuals towards environmental toxicants (Their *et al*., 2003).

Techniques based on polymerase chain reactions (PCRs), using DNA isolated from lymphocytes and other cells, allow the detection of genotypes of known polymorphisms involving a variety of xenobiotic-metabolizing enzymes.

Markers of polymorphisms of activating systems and detoxicating systems can be distinguished according to two different steps of metabolism. Polymorphisms of activating systems can be determined by measuring the activity of CYP450 isoenzymes implicated in the first step of the biotransformation process. CYP450s are a huge gene superfamily of haem proteins involved in xenobiotic as well as endobiotic metabolism. They play a critical role in adaptation to environmental changes for survival of the living organism. In addition, the huge environmental loads of human-made chemicals are biotransformed into bioactive or detoxified forms by CYP450s. Thus, CYPs have been used for biomonitoring of environmental pollutants, screening of their metabolisms and exploring remedies. In particular, the induction or inhibition of CYP450s has been applied to exposure monitoring of environmental toxicants, which are biotransformed by CYP450s. Associated with CYP450s there are a wide range of enzyme activities referred to as mono-oxygenase activities (Paine, 1995; Van Cauteren *et al*., 1996; Yi *et al*., 2007).

CYP450 Phase I enzymes can produce highly reactive DNA-damaging intermediates during the normal process of converting chemical carcinogens to excretable forms. Polymorphic variations in the sequences of CYP1A1, CYP1A2, CYP1B1 and other P450s exist in the human population, and specific forms have been associated with increased cancer risk in various populations. Individual variation in 'Phase II' detoxifying enzymes, such as glutathione *S*-transferase M1 (GSTM1), can also contribute to individual susceptibility. About 40% of the population have a deletion at this locus which has been linked to increased risk of bladder and lung cancers. Another common genetic factor related to increased cancer risk is the inheritance of a homozygous recessive mutation in the *N*-acetyltransferase gene resulting in the inability to efficiently detoxify aromatic amines via *N*-acetylation. The mutation is carried by approximately 50% of the population. It has been demonstrated that slow acetylators are at increased risk of bladder cancer, especially those occupationally exposed to aromatic amine bladder carcinogens. In contrast to its protective effect in bladder cancer, the fast acetylator phenotype is associated with an increased risk of colon cancer, demonstrating the complexity of gene–environment interactions with respect to cancer susceptibility. Moreover, combinations of metabolic polymorphisms are increasingly being linked to increased cancer risk. Acquired or inherited variations in the efficiency of fidelity of DNA repair can also influence individual susceptibility to cancer (Perera and Weinstein, 2000).

The active metabolites of certain organophosphorothioate insecticides can be hydrolysed and inactivated by paraoxonase1 (PON1). Studies suggest that high levels of serum paraoxonase are protective against poisoning by OP pesticides whose active metabolites are substrates of this enzyme (Costa *et al*., 2003). Paraoxonase activity can be used as a biomarker of susceptibility in humans because it exhibits an important polymorphism. Kinetic analysis of the two human PON1-192 isoforms revealed that the catalytic efficiency (V_{max}/K_m) of hydrolysis of oxons is what determines the '*in vivo*' protection of PON1 against exposure to a specific OP. Therefore, the Q/R polymorphism at position 192 confers different enzyme levels and catalytic

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efficiency in a substrate-dependent manner, suggesting that certain individuals may be more vulnerable to the toxic effect of OP exposure (Furlong, 2007).

3 CONCLUSION

In conclusion, the selection of appropriate biomarkers is of critical importance because of the opportunity for greater precision in the assessment of risk to individuals or population subgroups, with the consequent implications for mitigation and health protection. The advent of new '-omic' technologies (genomics, proteomics and metabonomics) promises to provide immense opportunity for the discovery and development of new markers of cellular and tissue integrity. Such biomarkers should be easily measurable at low concentrations in accessible tissues such as blood and urine. In addition, they should be specific and sensitive indicators of target-organ toxicity and markers of subclinical alterations, and thus may be useful in diagnostic and preventive strategies. In the near future it is necessary to develop or validate the analytical methods for evaluation of the selected biomarkers.

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From Diet to Detox: Controversial Therapies in Autistic Spectrum Disorders

Michael J. Fitzpatrick

CONTENTS

1 INTRODUCTION

An academic review article entitled 'Stem cell therapy for autism' provoked a flurry of international publicity in summer 2007 (Ichim *et al*., 2007). The authors, based in a biotech company in Arizona and in a clinic in Costa Rica, proposed treating autistic children with a combination of stem cells derived from bone marrow and umbilical cord blood cells. Before long, reports appeared in the US press that children with autism were being brought by their parents to Mexico and China to get stem cell treatments (which are illegal in the USA—and Britain) at great expense.

The promise that stem cells, cells which retain the primitive capacity to divide and differentiate into specialized cell types, will deliver treatments for hitherto intractable diseases has beguiled medical science for decades. Though there have been some achievements, such as the use of bone marrow stem cells in the form of 'transplants' to treat leukaemia, progress has been slow and the clinical yield disappointing. The theoretically more promising—and more controversial—area of embryonic stem cell research has so far produced no clinical benefits. While serious research continues, entrepreneurs have promoted stem cell treatments for a wide range of conditions, including cerebral palsy, multiple sclerosis, Parkinson's disease and many more, far in advance of any rational therapeutics. The quest by biotech companies for a wider market for stem cells has led them to take a growing interest in treating autism.

There is no scientific rationale for stem cell treatment in autism. The authors of this review speculate that autism is caused by 'neural hypoperfusion' and associated with 'immune dysregulation' and chronic inflammation of the gastrointestinal and central nervous systems. They claim that stem cells and cord blood can stimulate new blood vessel formation and correct the immune and inflammatory problems. But there is no good evidence of a deficient blood supply to the brain in autism, nor any consistent pattern of immune dysfunction. Nor is there evidence that stem cell treatment is effective in autism—or that it is safe.

Stem cell therapy follows a series of treatments that have been hailed as wonder cures in autism. These include holding therapy in the 1970s, facilitated communication and auditory integration therapy in the 1980s and secretin, the pancreatic hormone that was hailed as a wonder cure in 1998. In turn these interventions attracted large numbers of desperate parents—and invariably led them, and their children, to disappointment (Fitzpatrick, 2004).

Over the past decade a network of practitioners has emerged offering a range of what are characterized as 'unorthodox biomedical' interventions for children with autism. These treatments are, like stem cell therapy and treatment with hyperbaric oxygen (another popular method), almost exclusively derived from therapies already in widespread use in the alternative health sector or in other fields of medical practice. Self-styled 'unorthodox biomedical' practitioners often have close links with private laboratories offering esoteric tests and with commercial suppliers of their recommended medications and other products. Widely publicized claims by campaigning parents that their children have 'recovered' from autism as a result of biomedical treatments have

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ensured a growing demand (McCarthy, 2008). Here we look more closely at some widely used biomedical interventions, examining the theories justifying their use and the evidence of their therapeutic value in autism.

2 BIOMEDICAL INTERVENTIONS

- **Diets**: Gluten free/casein free (GFCF), Feingold, Ketogenic, Specific carbohydrate.
- **Supplements**: Vitamins, minerals, antioxidants, amino acids, enzymes, essential fatty acids.
- **Detox and immune system treatments**: Chelation, intravenous immunoglobulins (IVIGs), transfer factor, colostrum.
- **Medication**: Antibiotics, antifungals, antivirals, anti-inflammatories.

3 SPECIAL DIETS

The most widely used unorthodox biomedical intervention in autism is the 'gluten-free, casein-free' (GFCF) diet, based on the exclusion of wheat and dairy products. The dietary approach to autism has emerged since the 1980s, out of the wider trend for explaining a range of contemporary disorders, among both adults and children, as the result of the changing nutritional practices of modern society. As the notions of 'health foods' and 'healthy eating' have moved from the counter-cultural fringe to the mainstream, diverse diets have been promoted, not merely in the cause of weight loss, but for their claimed therapeutic benefits. The GFCF diet is the focus of numerous parent groups, web sites and recipe books and a flourishing commercial sector provides a wide range of suitable food products and cooking ingredients (Lewis, 1998; Seroussi, 2002).

Wheat and milk occupy a significant place in the environmentalist fable of the Neolithic fall from primeval grace, when human beings first cultivated grasses and domesticated cattle, initiating a transformation in diet that paved the way towards the degenerative diseases of modernity. Though there is considerable anthropological evidence for the notion that robust hunter-gatherers were displaced by sickly farmers and, ultimately, by even more diseased city-dwellers, the misanthropic outlook of contemporary environmentalists leads them to overlook the dramatic advances in human health associated with the industrialization of food production over the past century. When today's infants are weaned from breast milk on to cereals and dairy products, this process is regarded as a reprise of humanity's tragic Neolithic transition. The result, from the perspective of the gurus of ecological and environmental medicine, is another series of epidemics—of cancer and heart disease, food allergies and intolerances. Modern diets are also blamed for causing irritable bowel syndrome and diverse combinations of otherwise inexplicable symptoms. Though coeliac disease ('gluten enteropathy' resulting from an autoimmune reaction to dietary gluten) is rare, wheat intolerance has become widely recognized.

In a book entitled *They Are What You Feed Them,* Alex Richardson, a nutritionist and campaigner around issues of diet and children's health, argues that an 'epidemic' of developmental disorders, including attention deficit hyperactivity disorder, dyslexia and dyspraxia, autistic spectrum disorders and behaviour, conduct and oppositional defiant disorder, now affects 'one in four' of all children (Richardson, 2006). She attributes this epidemic to the fact that 'children's diets have changed out of all recognition during the past few decades'. Her book carries denunciations of 'the appalling nutritional quality of much food', claims about 'shocking' dietary imbalances and their 'devastating effects' on children's behaviour, mood and learning, and polemics against 'junk food'. Yet Dr. Richardson offers little information about how children's diet has changed and less evidence of how these changes might have produced such a profusion of disturbed behaviours. Her conclusion is that the supposed epidemic supposedly resulting from dietary changes can only be tackled by drastic changes in children's eating habits, avoiding additives, sugar and 'bad carbs' (refined carbohydrates) and polyunsaturated 'trans' fats, and consuming 'good carbs' (complex carbohydrates) and omega 3 'essential' fatty acids.

Richardson's explanation for what she characterizes as an 'autism epidemic' is that it results from 'the combination of two things: on the one hand, increasing exposure to potential toxins, from synthetic chemicals, heavy metals, and other environmental contaminants, and, on the other, decreasing intake of many essential nutrients needed to "defuse" and get rid of those toxins.' (Richardson, 2006).

A fashionable aversion to wheat and dairy products has converged with a number of questionable theories to produce the notion that the GFCF diet is an appropriate intervention for children with autism. In the 1960s, the American psychiatrist Curtis Dohan suggested that the low prevalence of schizophrenia among the islanders of the South Pacific might be attributable to a diet low in wheat, rye, barley and oats. He went on to recommend treating schizophrenics with a diet restricting cereals and dairy products. In the 1970s, Benjamin Feingold, a paediatric allergist in California, linked hyperactivity in children to salicylates and additives (including sweeteners and preservatives) and recommended excluding such products from children's diets. Feingold's theory and variations on his exclusion diet have had a wide popular appeal among middle-class parents of difficult children. The Californian psychologist and autism campaigner Bernard Rimland, the pioneer of unorthodox

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biomedical treatments in autism, was an early supporter of Feingold's approach.

In the late 1970s, American neuroscientist Jaak Panksepp noticed parallels between the behaviour of experimental animals addicted to opiates and autistic children (Panksepp, 1979). Both showed indifference to pain, diminished social interest and extreme persistence in stereotypical behaviours. On the basis of this analogy, he formulated his 'opioid excess' theory of autism, hypothesizing that its distinctive features might be attributable to an excess of endorphin (or 'opioid') neurotransmitters. In the early 1980s, the Norwegian biochemist Kalle Reichelt and his colleagues claimed that they had identified abnormal peptides with an opioid effect in the urine of patients with schizophrenia and autism (Reichelt *et al*., 1991). They further claimed that these peptides were the result of the incomplete breakdown of certain proteins in cereal and dairy products: gliadomorphin (from gliaden or gluten in cereal grains) and casomorphin (from casein in milk) (Reichelt *et al*., 1993). They proposed that, in children with autism, bowel inflammation resulting from gluten sensitivity allowed opioid peptides to enter the circulation and produce autistic symptoms, by acting on opiate receptors in the brain. They further postulated that, during pregnancy, circulating opioid peptides derived from the maternal diet could pass across the placenta and, at a critical stage, damage the developing brain of the foetus, producing autism as a result. The opioid excess theory was invoked by the former gastroenterology researcher Andrew Wakefield (in the highly controversial 1998 *Lancet* paper that launched the scare linking autism to the measles, mumps and rubella vaccine) to provide the link required by his hypothesis between the 'leaky gut' he attributed to 'autistic enterocolitis' and the supposedly neuropsychiatric features of 'regressive autism' (Wakefield *et al*., 1998).

In subsequent studies, Reichelt and his team claimed that a GFCF diet produced a significant improvement in the behaviour of autistic children (as well as relieving bowel symptoms) (Knivsberg *et al*., 1995). This approach has been popularized in Britain by the pharmacy lecturer Paul Shattock and his colleagues in Sunderland, who provide urine testing for opioid peptides with primitive chromatographic techniques, as the basis for dietary intervention (Shattock, 1995; Whiteley and Shattock, 1997). The GFCF diet has been promoted by the autism biomedical movement in the USA and a number of commercial laboratories offer urine testing (Shaw, 2002).

When researchers have used more rigorous methods, opioid peptides have proved elusive (Hunter *et al*., 2003). Wright and colleagues tested the urine of 155 age- and sex-matched controls and failed to find any association between urinary peptides and autism (Wright *et al*., 2005). The opioid excess theory received a decisive blow with the publication of a study led by the London paediatrician and autism specialist Hilary Cass and colleagues (Cass *et al*., 2008). Using a combination of liquid chromatography and mass spectrometric techniques they failed to detect opioid peptides in the urine of 65 boys with autism and 158 controls. The authors suggested that the findings of earlier researchers in this field were unreliable because they used techniques that were neither sensitive nor specific enough to support the conclusions that were drawn from their use. Their conclusion was that 'given the lack of evidence for any opioid peptiduria in children with autism, it can neither serve as a biomedical marker for autism, nor be employed to predict or monitor response to a casein and gluten exclusion diet'.

Though numerous anecdotal accounts claim dramatic benefits from the GFCF diet, these have not been confirmed by scientific study. It is widely recognized that such studies are difficult to conduct and those that have been undertaken have generally been small in scale and methodologically unsatisfactory. A systematic review carried out for the New York State Department of Health found that only 1 out of 16 studies provided useful evidence (New York State Department of Health, 1999). Their conclusion was that 'special diets, including elimination diets, are not recommended as a treatment for autism in young children'. The report noted that there were 'no known advantages to special elimination diets for children with autism' and expressed concern that 'they may cause the child to get inadequate nutrition and can be expensive' (New York State Department of Health, 1999).

Similar concerns were expressed in a survey carried out by the Cochrane group in the UK (Milward *et al*., 2004). These authors found only one study, carried out by Ann-Mari Knivsberg in Norway, that was of a satisfactory standard (Knivsberg *et al*., 2002). Knivsberg and colleagues measured the impact of the GFCF diet on 20 children with autism in four areas—cognitive skill, linguistic ability, motor ability and autistic traits—and found significant benefit only in the last. Their conclusion was that 'these regimes are not without cost in terms of inconvenience and extra financial cost, as well as limits on food choices of the affected family member, and one cannot recommend the use of such diets on the basis of one small trial alone'. A pilot study, carried out in Florida in 2006, tested the GFCF diet using a randomized, double-blind procedure, measuring urinary peptides and autistic symptoms in 15 children over a three month period (Elder *et al*., 2006). The results showed no significant benefit from the diet, though the researchers noted that 'families reported improvements that were not empirically supported by our work'. For the authors, the fact that some parents opted to continue the GFCF diet even after learning the results of the study suggested that it had a powerful placebo effect.

Though biomedical campaigners are as ready to proclaim the safety of GFCF diets as they are to claim their benefits, medical authorities have expressed some

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concerns. Neurologists Susan Hyman and Susan Levy warn that children on such diets may become deficient in calcium, vitamin D, iron and protein: 'the child on a restricted diet may be at risk for rickets, and supplemental calcium and vitamin D are indicated' (Hyman and Levy, 2000). On the basis of wrist X-rays of 75 boys with autism, Hediger and colleagues warned of the danger of osteoporosis from a GFCF diet (Hediger *et al*., 2008).

Special diets may also impose a substantial burden on the whole family. Their advocates insist that they must be followed with military discipline:

> *One thing must be made clear right from the beginning of the diet: your child has to stick to the diet rigidly. Evidence has suggested that there tends not to be any middle ground where the strictness of the diet is concerned, it is all or nothing.*
>
> (Whiteley and Shattock, 1997)

Furthermore, 'compliance needs to be as near to 100%' as possible, for 'at least six months'. The penalty for failure is regression: 'research has shown that children who break the diet do generally tend to regress behaviourally'.

4 SUPPLEMENTS

The identification of vitamin deficiency diseases in the early twentieth century, and the recognition of the association of oxidative stress with cell death and tissue necrosis in chronic degenerative diseases in recent decades, have stimulated a multibillion dollar trade in vitamins and antioxidants, and related minerals and enzymes. The beguiling notion that if a deficiency of something causes illness, then the administration of a 'megadose' of the same substance is likely to improve health has seized the popular imagination.

The use of vitamins in autism can be traced back to the era of 'metabolic psychiatry' in the 1960s, when neurotransmitter theories were advanced to explain schizophrenia and depression. In this period some researchers suggested that neurotransmitters might be a factor in the causation of autism. Indeed, as the report of the British Medical Research Council's 2001 inquiry into the aetiology and epidemiology of autism observes, 'over the years virtually every neurotransmitter system has been implicated in the pathogenesis of autistic spectrum disorders' (Medical Research Council, 2001). A number of investigators have identified biochemical deficits in children with autism, in relation to mechanisms of oxidative stress and methylation pathways. The most popular supplements among families with autistic children, according to a survey carried out by Bernard Rimland's Autism Research Institute in California, are vitamin B_6 (pyridoxine) and magnesium, dimethylglycine and vitamin C (ascorbic acid), all of which are believed to play a role in the synthesis of neurotransmitters as well as having antioxidant properties (Adams *et al*., 2008). A recent vogue is for injections of vitamin B_{12}: injections always have a more potent placebo effect than oral medications, perhaps explaining their popularity among parents (though their children may not be so enthusiastic).

Many more amino acids and compounds have been recommended as supplements for children with autism. These include tryptophan, tyrosine, cyproheptadine, D-cycloserine and carnosine (Levy and Hyman, 2005). Products promoted as antioxidants include glutathione and vitamin E (tocopherols and tocotrienols). Vitamin A is said to activate the immune system, producing significant, often immediate, improvements in visual perception, attention and language in children with autism. Supplements of magnesium, zinc, calcium, selenium and molybdenum are all recommended. In addition to protecting against coronary heart disease, omega-3 fatty acids derived from fish oils have been claimed to improve academic performance in children, to reduce delinquency in adolescents and to enhance cognitive function in the elderly. They are said to reduce symptoms in people with schizophrenia, depression and, inevitably, autism. Global demand has risen to a level that threatens stocks of fish.

While great benefits are claimed for all these supplements in anecdotal reports and in small, poorly conducted studies, proper scientific confirmation is lacking. Though the profile of adverse effects is generally low, blanket claims that these supplements are 'natural' and therefore safe must be treated with scepticism. Excessive doses of most vitamins can produce significant side effects; most notably, vitamin B_6 is well known to cause peripheral neuropathy.

5 DETOX AND CHELATION

The familiarity of the term 'detox', now universally recognized as an abbreviation of 'detoxification', reflects the popular acceptance of a concept that only a decade ago was confined to the world of clinical pharmacology (Marks, 2006). In common with other terms that have made the transition from an esoteric scientific discipline into the public realm, the concept of detoxification has also undergone a transformation in meaning. In the laboratory or clinical setting, detoxification meant the removal of a toxic or poisonous substance from the body. Toxicity may be the result of an overdose, accidental or intentional, of some drug—common culprits in the past were digoxin, salicylates, barbiturates and paracetamol. Occupational exposure to industrial or agricultural chemicals or heavy metals (lead, mercury) occasionally causes acute or chronic poisoning. Wars and political conflicts, and

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domestic disputes, sometimes involve the deployment of toxic substances, such as mustard gas and arsenic. The shift of detoxification towards wider public exposure began with its extension to the treatment of problems of alcohol and drug dependency, with regimes of gradual withdrawal and replacement therapies.

'Detox' treatment programmes are now promoted in newspapers and magazines and are available in a variety of forms in pharmacies and alternative health clinics. These treatments are based on the assumption that common nonspecific symptoms of malaise and fatigue are the result of the 'toxic' effects of modern lifestyles, especially junk food and (generally unspecified) environmental pollutants. The fact that the metaphor of 'toxicity' can be extended to include the stress-inducing effects of 'toxic' relationships and 'toxic' workplaces confirms its wide explanatory scope. All these features of toxicity are deemed to require a quasi-medical 'detox' treatment, which may involve various vitamins and supplements together with the consumption of large volumes of water, to provoke a therapeutic diuresis. Practitioners who have reinterpreted autism as a disorder resulting from the unique susceptibility of some children to environmental toxicity now recommend treating these children with 'detox' regimes.

We can distinguish three aspects of 'detox' interventions in autism. The first follows from the conviction that children with autism suffer from an impaired capacity to process environmental toxins—often attributed to a genetic susceptibility. Biomedical practitioners believe that the impaired detoxification system in autism requires treatment. Fortuitously, the same range of supplements, including vitamins, antioxidants and coenzymes, that we have discussed already, is also believed to correct oxidative stress and support the key detoxification pathways.

The second—and more controversial—mode of 'detox' is the use of 'chelation' treatments to remove toxic heavy metals by binding them to a water-soluble agent that enables them to be harmlessly excreted. Chelation was first used to treat victims of poison gas in the First World War and subsequently to deal with acute poisoning by a range of toxins, such as lead, arsenic and mercury. In recent years, chelation has come to be used by alternative health practitioners to treat a wide range of chronic conditions, including medical and psychiatric disorders which have been attributed to environmental toxins, particularly heavy metals, such as mercury in dental amalgam. Chelation has been most popular in coronary heart disease, in which it has been promoted as an alternative to surgical treatments (such as by-pass grafting and angioplasty).

In February 2001, Rimland's Autism Research Institute convened a conference of 25 physicians in Dallas Texas. This conference produced a 'mercury detoxification consensus position paper' which was updated in February 2005 (Autism Research Institute, 2005). Endorsing the—entirely unsubstantiated but increasingly popular—proposition that autism is 'a form of mercury poisoning' resulting from exposure to the mercury-containing preservative thimerosal in some vaccines, this conference recommended the use of a number of chelating agents. Even if autism were the result of neurological damage from chronic mercury exposure—an unsubstantiated proposition—chelating agents which do not cross the blood–brain barrier could not remove it, or indeed any other heavy-metal toxin, from the brain. As a treatment for autism, chelation lacks either a coherent theoretical rationale or empirical evidence of efficacy.

The death in 2005 of a five-year-old boy with autism, who had been brought by his parents from England to the USA for a range of alternative treatments, while undergoing chelation therapy at a private clinic near Pittsburgh, provoked widespread condemnation of this technique (Fitzpatrick, 2009). However, it did not appear to deter the demand for chelation therapies among parents of autistic children—or the willingness of practitioners to provide such therapies.

6 IMMUNE THERAPIES

The third aspect of detox is the treatment of the immune system in autism. The 'immune system' is another example of a concept that has acquired a popular status as metaphor far beyond its traditional meaning in medical microbiology (Fitzpatrick, 2004). Though the notion that the immune system is in some way disordered in autism is widely held in the biomedical movement, the concept tends to be used in a vague and often contradictory way. For example, some suggest that the immune system is 'suppressed' or 'underactive', resulting in an increased susceptibility to infection or immunizations or other environmental factors. Others suggest that autism is characterized by an excessively vigorous, 'up-regulated' or 'overactive' immune response, manifested in processes of autoimmunity, in which the body's defence mechanisms play a destructive role. Neither conception has any coherent scientific basis.

Over the past decade there have been a small number of reports claiming to have identified specific autoantibodies in autistic children, and also claiming dramatic benefits from immunological treatments. Three researchers in the USA—Vijendra Singh, Sudhir Gupta and H. Hugh Fudenberg—have become widely known among parent organizations.

In different studies, Professor Singh's team in Utah have observed raised levels of antibodies to measles virus, to MMR (measles, mumps and rubella vaccine) and to 'basic myelin protein'—in the lining of nerve fibres—in autistic subjects (Singh *et al*., 1998; 2002). However, British authorities considered Singh's findings 'not credible' (Dyer, 2002). The signs of an inflammatory

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process that generally accompany autoimmune conditions have never been demonstrated in autistic children and 'demyelination'—the characteristic damage to the myelin sheath surrounding nerve fibres seen in multiple sclerosis—is not a feature of autism. However, on the basis of his contested findings, Professor Singh recommends treating autistic children with a range of immunological treatments, including steroids, IVIG, plasmapheresis, and with sphingomyelin (a product of cattle brains containing myelin) (Singh, 1999; 2000).

Sudhir Gupta, at the University of California at Irvine, has also found a range of immunological deficits in children with autism, for which he recommends treatment with IVIG. In a widely quoted study, he gave 10 autistic children IVIG at four-weekly intervals for six months and observed a 'marked improvement in a number of autistic characteristics, including eye contact, calmer behaviour, speech, echolalia, and so forth' (Gupta *et al*., 1996). The small size of this study, the lack of a control group and the highly subjective character of the assessment of behavioural changes all make it impossible to draw any firm conclusions from this research.

Long retired from his career in clinical immunology and once barred from medical practice, Dr. H. Hugh Fudenberg recommends the use of 'transfer factors', which are said to transfer immunity to autism from a donor to the autistic recipient (Fudenberg and Wilson, 1978). Transfer factors are 'protein immunomodulators' derived either from cows' colostrum (the fluid expressed before lactation is established after calving) or from human white blood cells (dialysable lymphocyte extract, DLyE), preferably taken from a closely related, nonautistic donor. In a widely quoted paper, he presented the results of treating 22 autistic children with DLyE; 21 showed a significant improvement and '10 became normal in that they were mainstreamed in school and clinical characteristics were fully normalized' (Fudenberg, 1996). This treatment is not only very expensive, but carries considerable risks of transmitting blood-borne infections and other adverse reactions.

There have been some extravagant claims made for the explanatory power of autoimmune theories. For example, Professor Singh declares that he 'firmly believes' that 'up to 80% (and possibly all) cases of autism are caused by an abnormal immune reaction, commonly known as autoimmunity' (Singh, 1999). But scientific theory must be grounded in evidence, not belief, and scientists are obliged to suspend belief until they can substantiate their hypotheses. Professor Singh believes that autoimmune mechanisms explain not only autism, but 'obsessional compulsive disorder, multiple sclerosis, Alzheimer's disease, schizophrenia, major depression, etc.' (Singh, 2000). Professor Gupta also takes a broad view of the scope of autoimmune theories: he is also involved in research along similar lines into chronic fatigue syndrome and ageing.

Professor Singh recommends immunological treatments for all the conditions listed above; Professor Gupta believes that IVIG is the treatment of choice for a range of autoimmune disorders; Dr. Fudenberg hails his 'transfer factor' as an effective therapy for attention deficit disorder, HIV/AIDS, chronic fatigue immunodeficiency syndrome, Alzheimer's disease, Gulf War syndrome 'and others'.

A third common feature is that these authorities state particularly impressive results in autism. Professor Singh claims that there is 'enormous potential for restoring brain function in autistic children and adults through immunology' (Singh, 1999); in Professor Gupta's series of cases, every one was said to have improved on treatment; Dr. Fudenberg's claim of a cure rate approaching 50% is the most spectacular of all (Fudenberg, 1996).

The New York State review was dismissive of these immunologists' claims (New York State Department of Health, 1999). In a systematic review, the authors found only two articles (those of Gupta and Singh quoted above) that presented information on behavioural and functional outcomes. It found that 'both of these studies had serious methodological flaws and cannot provide acceptable scientific evidence about the efficacy of immune therapies for treating children with autism' (New York State Department of Health, 1999). It recommended that IVIG and other immunological therapies should not be used for children with autism, emphasizing not only lack of proven efficacy, but that these treatments also posed 'significant health risks' from allergic reactions and transmission of HIV, and hepatitis B and C. The New York report also noted that there was 'no adequate scientific evidence that children with autism have any type of immunologic problems or that they have any immunologic test results that are significantly different than results for the general population' (New York State Department of Health, 1999).

7 MEDICATION

One of the most popular diagnoses in the world of alternative health is that of systemic candidiasis. Sometimes characterized as 'candidal overgrowth' of the gastrointestinal tract, this diagnosis is invoked to explain a wide range of digestive and psychological symptoms. The notion that candida—a normal commensal in the human gut—can have extensive pathological effects is another by-product of the 1970s school of orthomolecular psychiatry. Pioneered by the Alabaman psychiatrist Orion Truss, the theory was popularized by another psychiatrist—Bill Crook from Tennessee (Crook, 1988). Dr. Crook suggested that treating candidiasis might improve symptoms in autism, and this approach has been pursued with enthusiasm by the unorthodox biomedical movement. Children with autism have been treated with

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yeast-free diets, probiotics and antifungal medications (nystatin, fluconazole).

The theory of candidal overgrowth in autism is one aspect of a wider belief that 'gut dysbiosis', a disturbance of the normal balance of intestinal organisms, plays an important role in autistic symptoms and behaviour. The (ill-defined) concept of gut dysbiosis reflects the idea of a Manichean struggle between good and evil, which permeates the outlook of contemporary alternative health practitioners, who believe that we have friends and enemies among carbs, cholesterol and fats as well as intestinal bacteria. Another flourishing alternative health trade—this one in digestive enzymes and probiotics—has found a new wave of customers among parents of children with autism.

One of the ironies of the unorthodox biomedical world is that antibiotics find a place in its lists of both causes of autism and treatments for it. On the basis of one study in which 11 autistic children were treated with vancomycin, an antibiotic which is not absorbed in transit through the gastrointestinal system and thus acts only within the digestive tract itself, and where 80% were said to have benefited, this drug has been widely promoted (Sandler *et al*., 2000). Another irony here is that colitis is a well-recognized adverse effect of vancomycin. Though viral causes of autism (notably measles) have not been substantiated, antiviral medications such as valaciclovir (which is effective against members of the herpes family of viruses, which have never been implicated in autism) is widely recommended.

The question of whether children with autism experience excessive digestive symptoms—or even a distinctive form of gastrointestinal disturbance—remains controversial. Different studies conducted in different ways and setting the threshold of symptomatic significance at different levels have yielded widely discrepant results (Black *et al*., 2002; Valicenti-McDermott *et al*., 2006). However it has long been recognized that children with autism and learning difficulties are often inclined towards idiosyncratic dietary habits and have a tendency to become constipated (sometimes resulting in faecal impaction and overflow diarrhoea). The particular difficulties involved in the toilet training of children with autism mean that their parents have a more intimate familiarity with the movements of their children's bowels for much longer than parents of typically developing children. This may be a factor in the high levels of concern expressed by some parents about gastrointestinal issues in autism and may have contributed to the popularity of Dr. Wakefield's concept of 'autistic enterocolitis' in the biomedical movement.

Dr. Wakefield and his colleagues have a lower threshold for diagnosing gastrointestinal disorder in children with autism than mainstream gastroenterologists (MacDonald and Domizio, 2007; Baird *et al*., 2008). Though they suggest that the pathological appearance of 'autistic enterocolitis' is more subtle than that of familiar inflammatory bowel diseases (such as Crohn's or ulcerative colitis), they recommend treating 'autistic enterocolitis' in a similar way. Dr. Bryan Jepson, a colleague of Dr. Wakefield working at his private clinic in Texas, outlines a treatment protocol that includes drugs such as sulfasalazine and mesalazine, corticosteroids and immune modulators (including methotrexate and azathioprine, and the anti-TNF (tumour necrosis factor) drug infliximab)—all powerful drugs with a formidable list of side effects (Jepson and Johnson, 2007).

8 CONCLUSION

The quest to 'defeat autism' that is promoted by campaigners who attribute the increasing prevalence of autism to environmental factors (notably vaccines) and pursue 'unorthodox biomedical' interventions has a range of damaging effects. It has led thousands of parents in the USA and the UK into futile litigation based on unsubstantiated vaccine–autism links. Many parents are spending large sums of money, not to mention family energies, on unproven tests and treatments, often provided by controversial practitioners. Children with autism are being subjected to treatments that are unlikely to be beneficial, but may be dangerous.

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Toxicogenomics: An Overview with Special Reference to Genetic and Genomic Approaches to the Identification of Toxic Effects

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1 INTRODUCTION AND HISTORICAL PERSPECTIVES

The word "toxicology" is derived from the Latin words "toxikon" meaning poison and "logos" meaning "discourse." In other words, toxicology can be literally defined as "the science of poisons." However, a more accurate and technical definition of the term can be stated as "toxicology is the branch of science which deals with the nature of poisons, their effect on living systems, detection and remedies thereof."

The history of toxicological studies can be traced back to the beginning of mankind. Indeed, when men were foragers, even before learning how to cultivate crops, they had learnt (by bitter experience, of course) that not all flesh or fruits or vegetables are edible. Consumption of some of them does produce maladies, ranging from mild irritation to death, although it was not a formal study of a subject in the way we understand it today. Further, when the aborigines of South America started using curare in blow pipes thousands of years ago, it was certainly an application of toxicological knowledge. Poisons have played stellar roles in a large number of royal feuds since the very early ages, especially in Greece as well as India. There is ample evidence in early medical testaments from Egypt and India that shows that several poisons, their symptoms and applications were well known more than 5000 years back. In fact, as early as sixteenth century, Paracelsus propounded the very basic axiom of toxicology, which states that "All substances are poisons; there is none which is not a poison. The right dose differentiates a poison from a remedy."

Like all other forms of sciences, the study of toxicology was at a complete standstill during the dark ages. However, with the advent of Renaissance, the study of toxicology flourished once again as more and more scholars recognized the fact that there is a direct relationship between exposure and disease outcomes. This was the age when scientists (notably Bernardino Ramazzini) began to understand that occupational exposure to mercury and lead can lead to the development of specific ailments, which actually heralded the study of occupational toxicology. However, it was not until the middle of the nineteenth century that the study of toxicology took

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a definite shape. Because of industrial revolution, thousands of new chemicals were being synthesized and mining and metallurgical procedures were also rampant, thereby leading to exposure to several detrimental hazardous chemicals and resulting in ailments and mortality. This necessitated the development in the field of toxicology to account for such deaths.

The twentieth century witnessed the blossoming of toxicology. This process was accelerated to a great extent by the dramatic improvement in the instrumentation, analytical techniques, bioassays and biomarker biology. Today, toxicology is a multi-dimensional discipline encompassing a broad area and involving several techniques and specializations. By the second half of the twentieth century, toxicology was a well-defined science, based on its firm principles, and had a unique identity of its own. The workers in the field had started exploring fresh areas such as the effect of genetic composition of an individual on the susceptibility to a particular toxin and so on. They were also undertaking ambitious initiatives such as deciphering the effects of toxic mixtures and synergistic effects of more than one toxic compound, which was not possible earlier. Advances in technology meant that new drugs could be designed to combat the toxic challenges and their efficacy could be tested in a better way utilizing newer improved model systems. Study of toxicology proved to be an extremely interesting field which was becoming mature. However, the best was yet to come!

2 FROM TOXICOLOGY TO TOXICOGENOMICS

In 1990, Human Genome Project was initiated, which aimed at identifying all the genes in human DNA and sequence the entire human genome; it was completed in 2003. This project also witnessed rapid technological advances in different sectors of biological sciences, notable of which were unprecedented progress in the development of automated instruments, data storage, computational efficacy and information transfer. This major breakthrough had its impact on toxicology as well. In the post-genomic era, this discipline has developed in leaps and bounds with the advent of techniques such as whole genome sequencing and the easy availability of large computational platforms. There has been a paradigm shift from classical genetic approaches towards a more holistic genome-based approach that is revolutionizing the way toxicology had been studied so far. With the help of these techniques, it is now possible to elucidate how an organism reacts to a particular toxin at DNA, RNA and protein levels. It is also possible to study the effects of poisons on different tissues and organs and at different stages of life. Instead of looking individually at single genes or mutations thereof, it is now possible to screen the entire genome at a single go. Thus, these techniques have immense potential: they are opening up new avenues for the study of toxic materials, which were hitherto considered impossible and/or improbable. In this chapter, we are going to take a cursory glance at these genomic techniques as they apply to the study of toxicology, and understand how the interaction of classical genetic and state-of-the-art genomic approaches has enriched this field and added new dimensions to the study of toxins and their effects.

3 TOXICOGENOMICS

3.1 Definition

The term “toxicogenomics” is a combination of two individual terms “toxicology” and “genomics.” Thus, it is apparent that the word “toxicogenomics” refers to the study of toxicology utilizing genomic principles and techniques. In other words, “toxicogenomics” is a sub-discipline of toxicology that encompasses techniques which aim at elucidating how genomes respond to toxicants. The NIEHS National Centre for Toxicogenomics (NCT) formally defines toxicogenomics as, “The collection, interpretation, and storage of information about gene and protein activity in order to identify toxic substances in the environment, and to help treat people at the greatest risk of diseases caused by environmental pollutants or toxicants.” Thus, toxicogenomics addresses the effects of toxins at the transcription level, that is, at the level of RNA and proteins (i.e., at the level of gene function), rather than at the level of DNA itself. In this chapter, we will be looking closely at how this technique is utilized to elucidate the mechanism of toxic action at the RNA level and how the predictions therefrom can be validated using classical genetic procedures.

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3.2 Genomic components

3.2.1 Microarray

The genomic components involved in toxicogenomics include all of transcriptomics, proteomics and metabonomics. However, here, we will concern ourselves solely with the transcriptomic technique. The other "omics" technologies will be dealt with in other chapters (see Application of Proteomics to Study Mechanisms of Toxicity and Dose Response Relationships of Chemical Exposure, Metabolomics in Systems Toxicology: Towards Personalized Medicine and Application of Metabonomic Approach in Target Organ Toxicity).

Transcriptomics looks at the expression of several genes simultaneously at the transcription level, that is, at the RNA level. This technique is based on the following three principles:

1. Almost all the cells of an individual carry the same genetic information, but in any particular cell type, only a certain fraction of all those genes are active, depending on the internal and external (i.e. environmental) conditions and cues. Thus, under any given set of circumstances, only a specified set of genes will be actively transcribing, whereas others would be shut down.
2. Unstable RNA can be isolated from any tissue at any given time and converted to corresponding cDNA (which is much more stable and easier to work with) using an enzyme called reverse transcriptase.
3. DNA is known to bind to other DNA molecules with complementary sequences. This property is exploited to hybridize cDNA for specific genes to probes having complementary sequence, with high degree of fidelity and accuracy.

In principle, transcriptomic technique begins with isolating mRNAs from the tissue of interest and then converting them to corresponding cDNAs by means of reverse transcription. The cDNA thus generated is amplified using polymerase chain reaction and then labelled with fluorescent dyes and subsequently hybridized to its respective probe attached to specific positions on a solid support (usually glass slides). Following hybridization and wash to remove non-specifically bound or free molecules, fluorescent signals are visualized and quantitatively measured with the help of software designed specifically for the purpose. A set of several control and experimental samples is then statistically analysed to determine the pattern of alteration of gene expression upon toxic insult.

In practice, the process of DNA microarray can be divided into the following steps:

I. Preparation of DNA chip
II. Isolation of mRNA
III. Conversion on mRNA to cDNA
IV. Labelling
V. Hybridization
VI. Washing
VII. Image acquisition
VIII. Image analysis and data generation
IX. Analysis of data

We will briefly look into each of these processes in the following sections.

Preparation of DNA Array

Unlike other forms of hybridization techniques such as Western blot or Northern blot, which are performed on membranes (nitrocellulose or nylon), DNA microarray uses solid support to eliminate material loss caused by diffusion and absorption and to provide a firm attachment of the probes. Typically, glass slides are used for this purpose. Each slide consists of few thousands of DNA probes arrayed on a support and designed to bind to specific DNA molecules with high degree of fidelity. Specific DNA probes are attached (spotted) each on a specific location of the glass slide generating a spot grid using high-throughput robotics, imparting a high level of accuracy and resulting in good reproducibility of the result obtained. Several spotting techniques are available nowadays, with upgradations for each of them being devised almost every day. The simplest of these techniques involves "printing" the probes (generated by classical methods of nucleotide synthesis) onto the glass slides coated with a positively charged material (such as epoxy-silane, amino-silane), with the aid of a mechanized printhead. Other techniques which involve synthesis of probes on the chip itself are also becoming popular these days. Affymetrix Inc. (Santa Clara, CA) utilizes the process of photolithography to synthesize high-density short oligonucleotide (each being 70–80 bases long) arrays directly on the slide surface; this technology is known as "Gene Chip." Whatever be the method of spotting, the final

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product is a solid support (like a glass slide) on which probes for several genes have been spotted at specific locations on a pre-designed grid. Such readymade DNA arrays are widely available commercially and they allow the users to choose from a wide range of products which may be organism specific, tissue specific, developmental stage specific or disease specific.

Isolation of mRNA

Proper isolation of mRNA from the samples is of paramount importance for generating good-quality data in transcriptomic studies. Several commercial kits are available these days for the isolation of total RNA/mRNA from a wide variety of tissues. The choice of kits depends on the source tissue to be used and the amount of tissue available. However, almost all the kits used routinely are based on the "single-step" method of isolation of total RNA by acid guanidinium thiocyanate–phenol–chloroform extraction, described by Chomczynski and Sacchi (1987). This method is based on the fact that when extracted with an acidic solution containing guanidinium thiocyanate, sodium acetate, phenol and chloroform, followed by centrifugation, total RNA remains in the upper aqueous layer whereas DNA and protein remain in the lower layers and thus get separated. The aqueous layer is then retrieved and total RNA is precipitated with isopropanol with good quality and high yields (Chomczynski and Sacchi, 2006).

mRNA is isolated from total RNA taking advantage of the fact that almost all (histone RNA being the notable exception) have a poly(A) tail at the 3′ end. Spin column chromatographic technologies have evolved targeting this poly(A) tail to bind to cellulose columns loaded with oligo(dT) probes. On binding and subsequent wash, the unbound fraction consisting of RNAs which are non-polyadenylated (and hence mostly non-coding RNAs), as well as other impurities, is removed. The bound mRNA is then eluted by washing the column with suitable buffers. These days, newer and improved techniques are being devised, which utilize superparamagnetic microbeads to bind to poly(A) tails of the mRNA and thus can directly isolate highly pure mRNA from the cell lysate itself. These techniques are much more superior in terms of sensitivity and yield.

Conversion of mRNA to cDNA

One of the most important and critical part of microarray technique is the conversion of mRNA to the corresponding cDNA. This process basically consists of two different components. The first is the synthesis of single-stranded cDNA from mRNA. In the second step, the single-stranded DNA formed is converted to double-stranded cDNA. The first step in this process is known as "first strand cDNA synthesis." Conversion of mRNA into the corresponding single-stranded cDNA is achieved with the help of an enzyme called reverse transcriptase, which has the ability to catalyse the conversion of RNA into DNA in the presence of nucleotides and Mg^{2+}. In practice, reverse transcriptase isolated from Moloney murine leukemia virus (MMLV) or avian myeloblastosis virus (AMV) is used for this purpose. The problem with this process is that a pre-requisite for reverse transcription is a short stretch of double-stranded sequence, whereas mRNA is single-stranded. However, this apparently insurmountable obstacle is overcome by using a oligo(dT) primer, which binds efficiently to the 3′ poly(A) tail of the mRNA and thus provides a convenient start site for reverse transcriptase. This process thus results in the generation of double-stranded mRNA–cDNA hybrid molecules. Hence, before the single-stranded cDNA can be used to generate double-stranded cDNA molecules, the mRNA bound covalently to the single-stranded cDNA needs to be removed. This can be achieved by alkaline treatment or boiling. However, these days a much more elegant approach is adopted (Gubler and Hoffman, 1983) known as "replacement synthesis." In this method, RNase H is used to nick the RNA strands at non-specific sites in mRNA ss cDNA hybrids, generating short RNA fragments attached covalently to the ss cDNA. In the next step, DNA polymerase I utilizes these short RNA fragments to act as primer and catalyse the synthesis of the second cDNA strand. DNA polymerase I subsequently removes the short RNA stretches by means of its 5′–3′ exonuclease activity. Finally, DNA ligase (typically T4 ligase is used) joins the newly synthesized cDNA strands in an energy-dependent manner. The double-stranded cDNA is now ready to be processed further. The advantage of using reverse transcription is that the remaining process is carried out using cDNA which is much more stable and easier to handle than RNA is.

Labelling

To estimate the level of transcripts, one should be able to visualize them. This is accomplished by labelling the transcripts with specific dyes/

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fluorophores. The amount of fluorophore binding to transcripts bears a particular stoichiometric ratio and hence they will emit a signal (usually fluorescence) of specific strength when scanned. The amount of signal generated is directly proportional to the amount of transcript present.

Several approaches have been successfully employed to label cDNA and depend on the design of the experiment and the objectives of the study. In one approach, experimental and control cDNA are separately labelled with two different fluorophores and hybridized to the same slide. In the other approach (usually Affymetrix and other DNA chip-based methods), cDNA from one sample is hybridized to probes on one slide and hence these are essentially one flurophore system.

Labelling of cDNA can either be direct or indirect. In the direct method (often called as "nick translation"), the cDNA generated is first treated with DNase to introduce single-stranded nicks at random sites. DNA polymerase I subsequently removes the nucleotides by 5′–3′ exonuclease activity. DNA polymerase I then substitutes the removed nucleotide with dNTP supplied externally (of which any one nucleotide is labelled with one of several possible labels such as biotin, dioxigenin, fluorescein, etc.). The final nick can be sealed by DNA ligase to give the labelled product. However, it has been shown that this method can sometimes be inefficient as the enzyme efficacy is reduced while using bulky tagged nucleotides as substrates.

The indirect method of labelling involves two distinct steps. In the first step, during cDNA synthesis from mRNA, dTTP is substituted by its analogue aminoallyl-dUTP. This analogue is recognized by the reverse transcription system as dTTP and is normally incorporated into the cDNA. The special property of this analogue is that it contains primary aliphatic amino groups, which have the ability to bind with fluorescent dye molecules such as Cy3 and Cy5 (Cy stands for Cyanine), which contain activated *N*-hydroxysuccinimide. In the next step, the analogue containing cDNA is incubated with these dyes to generate labelled cDNA ready to be hybridized to their respective probes.

Hybridization

Once the cDNA has been labelled, the next step is to hybridize the labelled samples to the microarray slides. Prior to hybridization, the microarray slides have to be heated to temperatures of 95 °C in deionized water and then dehydrated in ethanol. This is done to denature the probes on the microarray slide, so that they can bind to the cDNA samples. The slides are then rehydrated in pre-hybridization buffers (which usually contain 5× saline sodium citrate (SSC) and 0.1% sodium dodecyl sulfate (SDS)). Labelled cDNA is denatured by heating for a brief period at 95 °C and applied to the microarray slide. The entire set-up is then allowed to stand for 18–23 h at 42–50 °C to allow proper hybridization of cDNA to corresponding probes on the slide. Proper care has to be taken to ensure that the slide does not get dry during this process.

Washing

After hybridization, washing is a very critical step, which ensures removal of loosely bound or non-specifically bound dyes and probes. Improper washing can result in the generation of false-positive results caused by non-specific binding or false-negative results caused by high background signals. Slides have to be washed in a series of buffers containing 0.1% SDS and diminishing concentrations of SSC (2×SSC followed by 0.1×SSC) with slow shaking. The washing is usually carried out at room temperature, but sometimes it can also be carried out at 60 °C, depending upon the nature of the experiment. The slides are finally rinsed briefly in 0.1×SSC and dried by centrifugation. The slides are now ready to be scanned and are stored in dark until further analysis. These days fully automated hybridization systems are available, allowing for superior hybridization and better wash, leading to generation of better quality data and higher reproducibility because of reduction in background noise.

Image Acquisition

The raw data obtained from a microarray experiment are in the form of images. For fluorescently labelled systems using two-dye system, such as Cy3 and Cy5, specialized laser scanners are available, which scan the slides at two different wavelengths corresponding to the emission wavelengths of the two dyes. The lasers illuminate the slides at wavelengths corresponding to the excitation wavelength of each of the two dyes, one by one. This results in excitation of the dye molecules, which then emit fluorescence at specific wavelength (550 nm for Cy3 and 660 nm for Cy5). These fluorescent signals are then perceived by the detector to generate a digital map of the fluorescent intensities for each pixel. In this manner, two 16-bit TIFF images are generated

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for the entire slide, one corresponding to the signal intensity of each dye. It is of paramount importance to optimize the settings for image acquisition to generate a high-quality image while preventing photobleaching owing to application of high laser intensity.

Image Analysis And Data Generation

Once the two different images are formed, the image analysis software (several are commercially available, ScanAlyze, GenePix, ImaGene being a few) converts the 16-bit images into 8-bit images and then creates a 24-bit overlay RGB image. This helps in the further quantitative analysis of the pixel intensities to generate data about relative abundance of each transcript. For example, let us consider that in one microarray experiment, the control cDNA has been labelled with Cy3 (green), whereas the experimental cDNA has been labelled with Cy5 (red). After hybridization, wash and scanning, two images were generated, one for Cy3 and the other for Cy5. After overlaying of the two images, four kinds of outcomes may occur:

1. some spots will be totally green – indicates that only control cells express that transcript;
2. some spots will be totally red – indicates that only experimental cells express that transcript;
3. some spots will be perfectly yellow – indicates that both the control and experimental cells express that transcript in exactly equal amount;
4. some spots will be yellowish with tinge of red or green – indicates that these transcripts are unequally expressed in control and experimental cells. Greenish tinge indicates that they are expressed more in control cells, whereas reddish tinge indicates that they are expressed more in experimental cells.

The function of the software then is to quantify the ratio of the each pair of transcripts in terms of numerical values. However, in doing so, it has to take into account several complicated confounding factors, including background signal, local background signal intensity (which might be totally different from the overall background signal), shape and dimension of the spot, and so on. Nowadays, improved versions of softwares are commercially available, which allow the user to do all kinds of manipulations to generate good quality numerical data from raw images. However, there is no perfect software yet which can do all the necessary calculations and corrections. Different softwares use different algorithms and each has its own set of pros and cons; thus, there are always some loopholes and probability of errors creeping in while generating numerical data as well as in subsequent analysis. It is therefore very important to plan beforehand the experiments and identify the probable loopholes before reaching the stage of data analysis, so that the errors can be minimized at each step.

Analysis of Data

Data analysis is a crucial step in any experiment and microarray is no exception to this general rule. In microarray experiments, in particular (as in any other "omics" technique), the challenge of data analysis is further complicated by the huge amount of data generated per experiment. To extract meaningful conclusions from this deluge of data, it is extremely essential to implement the right statistical tools and algorithms. The choice of analytical methods depends on the study objectives and the experimental design (see Chapter Integrative Analysis of Microarray Data – A Path for Systems Toxicology).

Whatever be the algorithm and tool used, one problem every microarray experiment has to deal with is the inter-experiment variability. To nullify such variability and avoid false-positive or false-negative results, one has to normalize the ratios obtained (for each set of transcript from each set of experiment) against some common standard reference. This allows the comparison of results obtained from different experiments and gives an accurate measure of the reproducibility of the experimental set-up. Several approaches for normalization have been implemented till date, but the most favoured one perhaps is to normalize the data against a group of putative housekeeping genes whose transcript levels have been proved to remain fairly constant within an acceptable range under normal circumstances. Thus, using these transcripts as positive controls, one can eliminate, to a large extent, the possibility of generating false results and hence false conclusions therefrom. Another level of normalization is absolutely imperative for experiments employing multiple arrays. In this case, it is not sufficient to normalize the results for inter-experiment variability, but also the variability between different microarray platforms, as the difference in processing of the samples has to be normalized before the

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results can be meaningfully correlated and interpreted. In these cases, clustering analysis along with principal component analysis or multi-dimensional scaling has proved to be beneficial (Hess *et al*., 2001).

In conclusion, it can be said that a properly planned, executed and analysed microarray study will generate results which are robust and meaningful and can lead to identification of genes, and molecular signatures thereof, in response to toxic effects and thus herald the emergence of a new era in the study of toxicology.

3.2.2 miRNA Expression Profiling

Micro RNA (miRNA) are short stretches of single-stranded RNA molecules (21–23 nucleotides long), described first in 1993, that play negative regulatory role in controlling gene expression. Till date about 500 miRNAs have been identified and they have been shown to be key players in different biological processes as also in disease development through regulation of the transcriptional pattern of other genes. So, they might easily regulate the toxic effects of different agents as well. Hence, the transcriptomic studies of levels of miRNAs have become a lucrative area in modern-day toxicogenomic research.

The principle of miRNA expression study is just the same as normal transcriptomic study. miRNA can be isolated using a variety of commercially available kits. The process begins with the isolation of total RNA from the tissue of interest followed by enrichment of the small RNA (20–30 nucleotide size) fragment using a glass filter fibre approach. In the technique, total RNA is allowed to pass through two successive glass filters under differing ethanol concentrations. This leads to the immobilization of large RNAs on the first filter under conditions of low ethanol concentration, whereas the small RNAs successfully pass through it. However, with addition of more ethanol, the latter get immobilized as they are passed through a second glass filter. The small RNAs are then eluted with appropriate buffers to obtain a small RNA-enriched sample (Microarray Core Facility, UT, Southwestern Medical Center at Dallas). Other gel electrophoresis-based methods of miRNA isolation are also becoming popular these days.

The miRNAs thus isolated are then labelled according to the same indirect method as we have discussed in case of cDNA in the previous section. The first step involves the addition of 3′ amine-modified tails to miRNA, to which a variety of dyes can be attached in the subsequent steps. Cy3, Cy5, Alexa Fluor and digoxigenin are some of the dyes commonly used in this process.

Once the sample is labelled, it can then be hybridized to the customized array consisting of probes for each miRNA. Hybridization usually takes about 12–14 h to complete at 42 °C, followed by washing with high-stringency and low-stringency wash buffers. The slide is then dried and stored in dark until analysis. The analysis, as well as image acquisition, data generation and data analysis, is also very similar to what we have discussed in the previous section.

3.2.3 Genetic Polymorphism Analysis

Another technique that has gained tremendous momentum in the post-genomic era is the study of effect of genetic polymorphism(s) on susceptibility to toxins. Genetic polymorphism may be defined as the existence of several forms of DNA sequences at a specific locus in the genome of a particular species at a frequency of 1% or more. Thus, the same gene may have slightly different compositions in different individuals. Although the high level of abundance of polymorphic forms of a gene indicates that a minor change in sequence would not result in a drastic change in the function or phenotypic effects of the gene (or else, it would not have been stabilized in a population at such a high frequency, but would have been eliminated or reduced to a rare mutation caused by negative selection pressure), it might result in subtle changes in gene function that might affect the response of an individual towards a toxic insult. Genetic polymorphisms may be synonymous (does not cause a change in the amino acid sequence of the protein product) or non-synonymous (cause a change in the amino acid sequence of the protein product). Most of the genetic polymorphisms are changes in a single nucleotide (hence termed as single nucleotide polymorphism or SNP), but there are examples of polymorphisms that involve more than one nucleotide (as entire gene deletion in case of *GSTT1* and *GSTM1*). It has been estimated that human genome consists of several thousands of such genetic polymorphisms. With detailed knowledge about the occurrence of such genetic polymorphisms (well documented in literature and stored in

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databases) in our genome, we are now in a position to ask ourselves what is the effect of presence of such variations. In fact, polymorphisms in different genes have been associated with susceptibility to develop different diseases (mostly through association studies which measure the relative frequency with which a particular polymorphism occurs together with the trait of interest in a population). Toxicology has also benefited from the same approach and several association studies have been carried out to find out whether specific polymorphism(s) in the genes involved in metabolism of toxicants are associated with susceptibility to the toxicant concerned.

Association studies usually involve a case–control design where the occurrence of a particular polymorphism in case and control groups is determined. The case and control are defined on the basis of a phenotypic outcome (such as a disease occurrence), where the case has an outcome, but not the control. The two groups are selected in such a way so as to eliminate all other sources of probable confounding variables, and evenly matched except in their outcome variable. Genomic DNA is isolated (usually from blood) and the genotype for each subject is determined by polymerase chain reaction (PCR)-sequencing or PCR-RFLP (Restriction fragment length polymorphism) method. The occurrence of the polymorphism of interest is then evaluated in both the study groups and is expressed as odds ratio (what are the odds for a subject with the polymorphism of interest to develop the disease outcome compared with a subject having the other allele).

One of the most important issues any toxicologist seeks to address is what makes an individual susceptible to a particular poison. Individual susceptibility depends on several factors, but is, to a considerable extent, genetic in nature and is present inherently in individuals, coded in the form of triplets of bases in the genome. In a great majority of cases, this susceptibility is polygenic and usually each gene (or variation thereof) is a low-penetrance one. It is thus the interplay of several low-penetrance genetic variations that determine the ultimate susceptibility of each individual towards a particular toxicant. Classical genetic techniques are inadequate to determine the susceptibility of an individual to a toxicant in such cases, where several low-penetrance variants cumulatively bring about the final degree of susceptibility of a subject. Yet, delineating the high-susceptibility groups or populations is one of the most important issues in toxicology. This is where the genetic polymorphism studies have a tremendous impact. By definition, genetic polymorphism studies look into the minute variations in the genome which are expected to cause subtle effects rather than a drastic one on the phenotype. Thus, association studies can quantitate the effect of such minute genetic variations in the low-penetrance candidate genes and the degree of susceptibility they possess to the individual who possesses them. These days, genome-wide association studies (GWAS) have become possible, and are gradually becoming an important strategy (Shi, Wang and Huang, 2009) in complex toxicological studies. GWAS permit a comprehensive scan of the genome in an unbiased fashion and thus have the potential to identify totally novel susceptibility factors (Shi, Wang and Huang, 2009). Thus, genetic polymorphism analysis has become a very important tool in the field of toxicology, primarily as it permits easy yet accurate screening of susceptible individuals in a population who are highly prone to the adverse effects of a toxicant. This has hitherto not been possible with the classical genetic techniques such as genome-wide linkage studies the toxicologists had at their disposal.

The genetic polymorphism study has become widespread because of its elegant and simple study design, economy, ease of analysis and ability to adjust for probable confounding factors by statistical methods. With the advent of cost-effective and high-throughput modes of DNA sequencing, study of genetic polymorphism to account for susceptibility (to toxins in our case) has gained immense popularity. This approach thus promises to assist researchers to identify highly susceptible groups among those exposed to different toxins, be environmental or occupational, with high degree of accuracy.

3.3 Advantages and Disadvantages

The major advantage of all these genomic processes is that they are extremely high-throughput systems. These are very fast processes which can generate data on several thousand points (genes, miRNA or polymorphisms) at a single shot. In fact, toxicogenomic experiments can lead to the unravelling of putative pathways, molecular targets and thereby mechanisms of polygenic and complex disease aetiology.

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From the foregoing discussion, it might appear that toxicogenomics is all about advantages. However, all these approaches also have several limitations. Primarily, even with the most advanced technology we have at our disposal these days, it is not possible to spot all the genes of an organism on a single slide. Thus, on each microarray platform, only a specific subset of the entire genome is represented. Hence, this method is not completely exhaustive yet with respect to the entire genome. Genes which transcribe rarely are more probable to be absent in the arrays than those which are transcribed abundantly, and this might cause loss of important information in some cases. Also, with the huge amount of data generated by transcriptomic experiments, it is essential to weed out the fallacious results with utmost caution to reach truly meaningful conclusions. This necessitates the possession of sophisticated data analysis systems and computational platforms, which are not always easily available. Besides, this procedure can often be exorbitantly costly for many researchers.

However, perhaps the most important point is that the results obtained using toxicogenomic techniques (be it microarray or genetic polymorphism study) are largely predictive rather than definitive. This is because of the fact that there exists a high degree of variability within and between experiments. The variability in microarray can result from several sources and at several stages. Differences in sample quality, manual handling, efficiency of RNA extraction, dye incorporation and hybridization efficacy, and washing all bring about variation in each successive step of the experiment. The problem is compounded further by variations resulting from other sources such as temporal differences in printhead structure as it keeps on printing different spots on a slide, local variations in hybridization in the same slide, as also variation between different slides. Most of these variations can be addressed statistically using various mathematical algorithms, yet some amount of error does creep in. It is practically impossible to eliminate all sources of variation, no matter whatever be the mathematical normalization performed. This obviously results in a degree of approximation while reaching conclusions from transcriptomic experiments, and thus every transcriptomic experiment needs to be further cross-validated before spelling out a definitive hypothesis.

The same is also true for genetic polymorphism study. Although the results can be adjusted for confounding factors, there is no denying of the fact that any association is a statistical inference based on observation and might just be due to chance. So, these associations also need to be validated at the functional level before they can be accepted to be universally true.

4 GENETIC COMPONENTS

As we have already discussed, a transcriptomic technique (including that used to study toxicological effects) has a high predictive value. Thus, it helps the workers to narrow down from an imponderable number of genes that might be implicated to a relatively few with high probability of involvement in the process. However, even within those genes predicted to play a part, it is absolutely necessary to vindicate the biological roles they play in bringing about susceptibility or resistance to the toxicant. It is in the validation of their roles that genetic techniques play a big role. By definition, genetic techniques are adept at looking into the action of one gene at a time. Hence, having found out a few candidate genes by transcriptomic methods, the worker can substantiate whether they play any role at all and, if so, what that role is. We will hence be looking at a few such genetic approaches in the upcoming sections.

4.1 Molecular Biology Techniques

4.1.1 Real-Time Polymerase Chain Reaction

One of the best methods used routinely nowadays to quantitate the mRNA expression level is known as real-time PCR (RT-PCR). In this technique, fluorescent reporter molecules are employed to monitor the amount of product formed during each cycle of an amplification reaction. The procedure begins with isolation of total RNA (or mRNA) from the tissue of interest and utilization of reverse transcriptase PCR to generate cDNA of the desired DNA fragment using specific primers. The specific cDNA thus generated is then used in the next step of amplification reaction. The basic rationale for this method involves detection of fluorescence whose level is (directly or indirectly) proportional to the increment in the level of DNA as it is being synthesized during the process of amplification. The

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technique consists of determining the number of amplification cycles required for the fluorescent signal to attain a minimum threshold level at which the signal can be detected (C_T). It is evident that if the initial number of copies of specific DNA fragment is more, the number of amplification cycles required to reach C_T will be less compared with the same DNA fragment with less number of initial copy numbers, or in other words, the starting amount of nucleic acid is inversely proportional to the C_T. The quantity of DNA in the sample can then be obtained by interpolation of its C_T value versus a linear standard curve of C_T values obtained from a serially diluted standard solution (Walker, 2002). The greatest advantage offered by this method is that the measurement of amplification products is carried out in "real time" enabling the quantification during the exponential phase of the reaction itself (Ginzinger, 2002) and not at the end of the PCR when the reaction has long been in the plateau phase. This is because, during the exponential phase, the efficiency of amplification is maximum (copy number being almost doubled in every successive cycle at this phase), while being least affected by reaction-limiting conditions (Walker, 2002); hence, the method is highly sensitive and accurate.

Several methods are available to achieve fluorescent signals in proportion to the DNA product. We will be discussing three techniques that are widely used at present. In the indirect "Taqman" system, in addition to normal forward and reverse primers required for amplification, an additional fluorescence resonance energy transfer (FRET) probe is employed, which is an oligonucleotide (complementary to the DNA of interest) consisting of a reporter molecule at the 5′ end and a quencher molecule at the 3′ end. During the amplification process, because of the proximity of the quencher and the reporter, no fluorescence is detected. However, during each extension cycle, as the Taq DNA polymerase progresses, the 5′–3′ exonuclease activity cleaves the reporter dye from the probe, and once separated from the quencher, the reporter dye emits its characteristic fluorescence. The direct method uses a fluorescent dye known as SYBR Green. The peculiarity of this dye is that it binds to double-stranded DNA but not to single-stranded DNA. On binding, this dye fluoresces brightly. This binding and resulting fluorescence, being proportional to the amount of double-stranded DNA, allow for sensitive and accurate measurement of the amount of double-stranded DNA formed at the end of each amplification cycle. The other direct method widely in use is the "molecular beacon," which consists of a hairpin probe which maintains dye and quencher molecules in proximity and hence prevents the reporter molecule from fluorescing in the unbound state. Binding of the probe at a target sequence-specific region to its complementary strand on the amplified target DNA separates the two fluorochromes, thereby alleviating the FRET interference and allowing the reporter to fluoresce (Walker, 2002).

4.1.2 Northern Blotting

Northern blotting is a molecular biology technique widely applied to evaluate the expression level of specific mRNA. This method also starts with the extraction of RNA from cells/tissue of interest. The isolated RNA is then separated according to size by running on an agarose gel under denaturing conditions. For sample denaturation, agents such as formamide (which lowers the annealing temperature of the probe–RNA interaction, preventing RNA degradation at high temperatures), formaldehyde, methylmercuric hydroxide, glyoxal and dimethyl sulfoxide are used, but not alkali, as they tend to degrade RNA. The RNA thus separated are then transferred to a positively charged nylon membrane, immobilized and then hybridized to probes, specific for the RNA of interest. The probe can be radiolabelled cDNA or RNA or non-isotopically labelled DNA. Following hybridization and subsequent wash, the signals generated by specific binding of probe to RNA of interest can be detected by suitable detectors and then quantified using commercially available softwares for densitometric analyses. The main advantages of this process are that it is extremely sensitive and can detect minute quantities of RNA and the alternatively spliced transcripts of the same gene.

4.2 Cytogenetic Techniques

A large number of toxicants exert their effects on living systems by adversely affecting the integrity of the genome. They induce genetic damage to different extent, which subsequently leads to malfunctioning of the genetic information encrypted in the form of nucleotide sequences and thus results in the development of phenotypically observable maladies in the long run. So, while studying toxic

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effects, one of the first parameters one needs to ascertain is whether these agents are genotoxic. Cytogenetic methods provide easy, accurate and reliable methods for qualitative and quantitative analysis of genetic damage induced by toxicants. Also, these studies provide strong counter validation of the results obtained from genomics studies. In this chapter, we will be discussing about a few such routinely used cytogenetic techniques.

4.2.1 Single-Cell Gel Electrophoresis (SCGE) Assay

Commonly known as comet assay, this is an easy, reliable and fast technique that is employed to quantify the genetic damage at the single-cell level. The assay works on the principle that strand breakage of the super-coiled duplex DNA leads to the reduction in the size of the large molecule and these strands can be stretched out by electrophoresis. Also, under highly alkaline conditions, there is denaturation, unwinding of the duplex DNA and expression of alkali-labile sites as single-strand breaks. When such fragmented DNA is placed in an electric field, the negatively charged DNA fragments migrate towards the anode. This migration is a function of the size of the fragments (inversely proportional), and also the number of broken ends of DNA (directly proportional) (Indian Institute of Toxicological Research, 2006). Thus, greater the amount of damage induced, larger will be the number of broken ends, and smaller will be the size of the fragments generated. Hence, the DNA fragments (belonging to a particular cell) will move farther away from the undamaged part, thereby generating a comet-like structure, where the "head" is formed by the undamaged DNA whereas the "tail" is formed by the broken fragments. Lengthier the tail, greater is the number of broken fragments, smaller is the size of fragments generated and more is the damage induced. The comets can be visualized by staining them with DNA-binding fluorescent dyes such as ethidium bromide or SYBR Green and the damage can be quantified using image analysis softwares.

4.2.2 Chromosomal Aberration Assay

Chromosomal aberration (CA) assay is employed to determine qualitativelyand also quantitatively the genetic damage caused by toxicant(s) at the chromosome level. Structural chromosome aberrations may be induced via DNA breaks by various types of toxic agents. CA assay allows the visualization of such structural anomalies in peripheral blood lymphocytes. This assay can also be performed using cell cultures, but cultured cell lines tend to lose and gain chromosomes spontaneously, showing a high and unpredictable rate of chromosome aberrations, which makes them an unsuitable choice for this assay. This assay consists of setting up of primary cultures of peripheral lymphocytes (from human or mice) in appropriate culture medium and stimulating the cells to undergo division by adding a mitogen such as phytohaemagglutinin-M. Two hours prior to harvesting, a spindle inhibitor (such as colchicine or colcemid) is added to arrest the dividing cells at metaphase (as this is the phase in cell cycle when chromosomes are most condensed and hence best visible). The cells are then harvested by centrifugation, treated with hypotonic solution to allow the cells to swell and become turgid, fixed and washed. Permanent slides are then prepared with cell suspensions, by allowing them to drop on chilled glass slides. They are then allowed to dry under humid conditions, stained with Giemsa and examined by light microscopy. Suitable negative controls are a pre-requisite for proper evaluation of baseline genetic damage in the study population to account for false-positive results. Positive controls in the form of S9 mix or mitomycin C can also be used. In this process, the cells are usually harvested at 48 h because that produces a mixture of cells in the first and second division cycles, which is important to detect both chromatid and chromosomal aberrations. However, for a few toxins which are known to delay cell cycle (such as arsenic), harvesting is usually carried out after 72 h of culture. Observations consist of scoring 50–100 well-spread metaphase plates per culture in duplicate. The frequency of aberrant cells in the experimental cultures compared with control cultures gives an accurate estimation of the genotoxic potential of the toxicant. The major advantages of this process are that it is very easy to perform, the sample can be obtained with minimum invasiveness and the analysis is fast, accurate and also amenable to automation to a considerable extent.

4.2.3 Micronucleus Assay

Micronucleus assay (MN) is a convenient tool to determine visually the DNA-damaging potential of a toxin at the cellular level. Several kinds of toxic exposure lead to the fragmentation of DNA as we have seen earlier. These DNA fragments (mostly

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acentric) are not pulled towards the pole of the daughter nuclei during anaphase. They hence persist in the cytoplasm as minute nuclear fragments and are known as micronuclei. The frequency of micronucleus induction provides a reliable estimate of the toxicity of the agent concerned. The assay protocol is very similar to that of CA when peripheral blood lymphocytes are used for the purpose (harvested at 72 h), the only difference being the addition of cytochalasin B at 44 h of culture instead of a spindle inhibitor. The function of cytochalasin B is to prevent cytokinesis by generating binucleate daughter cells. However, this assay has the added advantage of being performed on a large number of other cell types including buccal mucosa, nasal mucosa, urothelial cells and so on, which can be obtained by non-invasive methods.

5 INTERACTION BETWEEN GENOMIC AND GENETIC APPROACHES

In this section, we will be looking at a few examples of how genetic and genomics approaches together have led to the unravelling of the mechanisms as well as effects of a few toxicants. These examples will clearly illustrate the importance of employing both approaches as and when necessary to have a close look at the mode of toxic actions.

Benzene toxicity has been studied widely. It has been shown using cytogenetic techniques that benzene exposure leads to significant levels of genetic damage including an increased induction of aneuploidy (Zhang *et al*., 1999). Genetic polymorphism studies have demonstrated that SNPs in double-strand break repair pathway genes, such as *WRN*, *TP53* and, *BRCA2*, are involved in susceptibility to benzene-induced haematotoxicity (Shen *et al*., 2006). Microarray experiments were employed to detect the changes in gene expression patterns in peripheral blood mononuclear cells (PBMC) on benzene exposure, using a comprehensive human array, theU133A/B Affymetrix GeneChip set. Pairwise *t*-statistic along with bootstrapping control was used to statistically analyse the results obtained and led to the identification of 29 known genes with differential patterns of expression on benzene exposure in PBMC. Further validation by RT-PCR confirmed the differential expression of *CXCL16*, *ZNF331*, *JUN* and *PF4* on benzene exposure (Forrest *et al*., 2005).

Arsenic is another such chemical whose toxic effects have now been reported from about 35 countries in the world and is the cause of global concern. In West Bengal, India alone, 25 million individuals are estimated to be exposed chronically to arsenic through contaminated drinking water. Scientists across the world have been trying to unravel the mechanism of arsenic toxicity and susceptibility thereof. They have adopted both classical cytogenetic as well as modern molecular techniques to elucidate the mechanisms involved in chronic arsenic toxicity and susceptibility. Our group has shown that induction of genetic damage is one of the potent mechanisms by which arsenic exerts its toxic effects on human beings. We have used all the three parameters of CA, MN and comet assay to elucidate the elevated level of genetic damage induced by arsenic (Basu *et al*, 2002, 2005; Mahata *et al*., 2003; Banerjee *et al*., 2008). It has been shown using genetic polymorphism studies that SNPs of DNA repair pathway genes such as *ERCC2* and *p53* are involved in imparting high risk of development of arsenic-induced pre-cancerous skin lesions (De Chaudhuri *et al*., 2006; Banerjee *et al*., 2007). Implication of other genetic polymorphisms such as *APE1*, *XRCC3* and *NBS1* with arsenic-induced skin lesions has also been shown in different populations (Andrew *et al*., 2003; Thirumaran *et al*., 2006; Applebaum *et al*., 2007; Breton *et al*., 2007). Further cytogenetic studies have elucidated that individuals having the risk genotypes of *ERCC2* and *p53* have a significantly elevated level of genetic damage as measured by CA compared with those having the non-risk genotypes (Banerjee *et al*., 2007; De Chaudhuri *et al*., 2008). Thus, these studies validate the observations obtained from genome-based genotyping studies (Lu *et al*., 2001). Recently, employing cytogenetic techniques such as comet assay and challenge assay, it has been shown that deficiency in DNA repair capacity is one of the paramount factors in arsenic susceptibility (Banerjee *et al*., 2008). Thus, a combination of molecular biology and classical cytogenetic approaches has helped to elucidate the mode of arsenic toxicity at several levels in the process delineating the high-susceptibility groups.

6 CONCLUSION

Thus, from the forgoing discussion, it is evident that both genetic as well as genomic approaches have their own advantages and disadvantages. Generally

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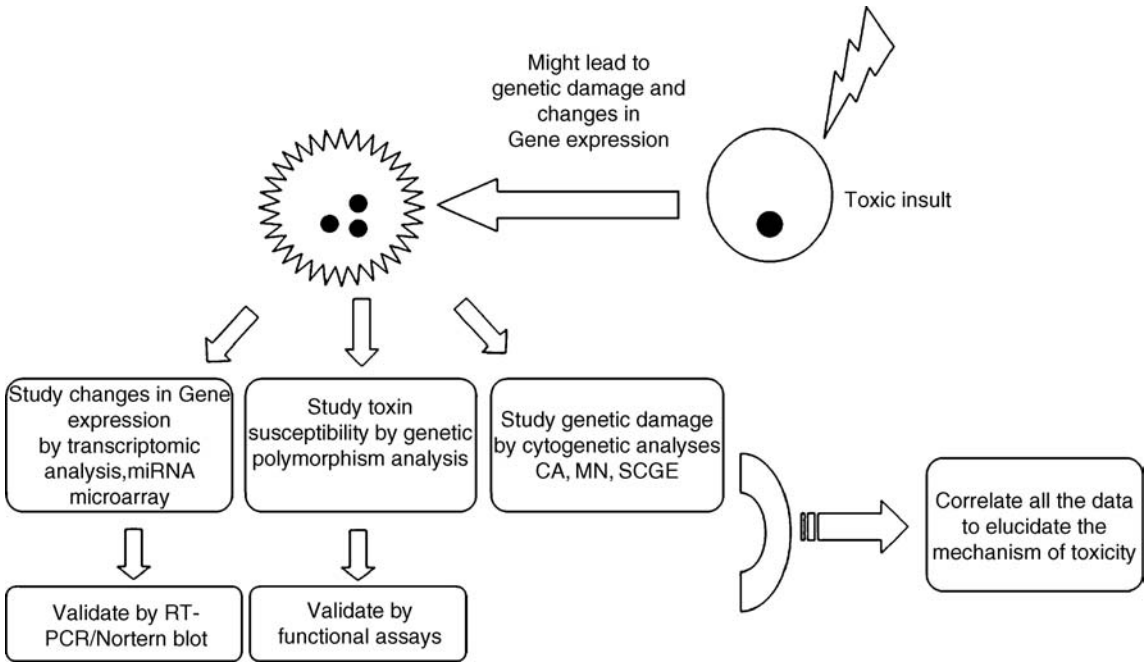

Figure 1. Interaction of genomic and genetic approaches helps to elucidate the mechanism of toxic action and also identify the highly susceptible individuals at the extreme end of the continuum who are at highest risk as a result of toxic exposure.

speaking, genomic techniques lead to the generation of large amount of data in a short span of time and enable us to look at how the entire genome works at a single go. Thus, armed with the knowledge of human genome, it is now easy to look at how the genome is being modulated at the functional level by the effect of a toxicant. However, with this huge amount of data come the problems of low reproducibility, data storage and retrieval, proper analysis and drawing of meaningful conclusions from them. It is in here that the molecular biology and classical cytogenetic techniques prove to be invaluable. Although they look at one gene at a time, the results obtained are usually more robust and simple to analyse. Thus, the ideal way to study the toxic effects seems to be a combination of the two approaches. Genomic analyses predict a specific set of genes with altered expression that might be involved in the mechanism of toxic action. The main function of genomics would be to shortlist from a large number of possible players to a relatively fewer number of genes whose probability of being involved in the toxic mechanism is the highest. Besides, genomic approaches can also lead to the identification of pathways involved and also the probable therapeutic targets for a toxicant. The function of the molecular and classical cytogenetic systems would then be to validate these predictions by examining the effects of toxicant on each of these probable genes as predicted by genomic techniques. Hence, together, genetic and genomic approaches provide the researchers with a powerful tool to evaluate the potential mode of action as well as possible remedies for any toxicant (Figure 1).

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Toxicogenomics and the Evolution of Systems Toxicology

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Toxicogenomics – Applications in Systems Toxicology

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1 INTRODUCTION

A significant increase in mining, refining and manufacturing, as well as the use of products containing chemicals, has taken place during the last one and a half century. Although chemicals are of immense benefit to mankind, some of them, including many commonly used chemicals, pose a serious threat to human health. Significant quantities of various chemicals are present as contaminants in the air we breathe, the water we drink and the food we eat. In addition, significant quantities of various toxic chemicals are found in the environment as well as in various occupational settings as contaminants. Therefore, every human being is at risk of exposure to toxic chemicals capable of resulting in potential adverse health effects. Virtually every organ/tissue in the body has been identified as a target for toxicity resulting from such exposure to toxic chemicals. In fact, chemical exposure has been identified as a major aetiological factor for several diseases in humans, including cancer in various organs and tissues (Loeb and Harris, 2008; Sorahan, 2009). As human exposure to potentially toxic chemicals is almost unavoidable, it has become increasingly necessary to have the capability to effectively detect human exposure to toxic chemicals and, if possible, to predict or even determine the potential for adverse health effects resulting from such exposures. Equally important is the requirement to determine the potential mechanisms underlying the toxicity of chemicals because this may provide an opportunity to develop strategies to intervene or even prevent toxicity resulting from these exposures. This understanding, if possible, should not be confined to a single tissue or organ in the body, but rather be applicable to toxic effects taking place in all organs and organ systems in the body.

Great progress has been made in the last several decades in determining the potential of chemicals to cause toxicity as well as elucidating the potential mechanisms underlying their toxicity. A variety of approaches may be employed to determine whether a chemical is toxic or not. The ultimate goal of a successful toxicity test has to be its ability to predict most accurately the potential of a chemical to cause toxicity in a human being following exposure to the chemical in question. The desired features of an ideal toxicity test are: (i) sensitivity – the test should be highly sensitive so as to detect subtle or pre-clinical toxicity much before the onset of any clinical symptom of toxicity; (ii) non-invasiveness – the test should be applicable in situations of human monitoring for chemical exposure and the resulting toxicity and this is possible at best only if the test can be conducted non-invasively using easily accessible bio-fluids such as peripheral blood, urine, etc.,

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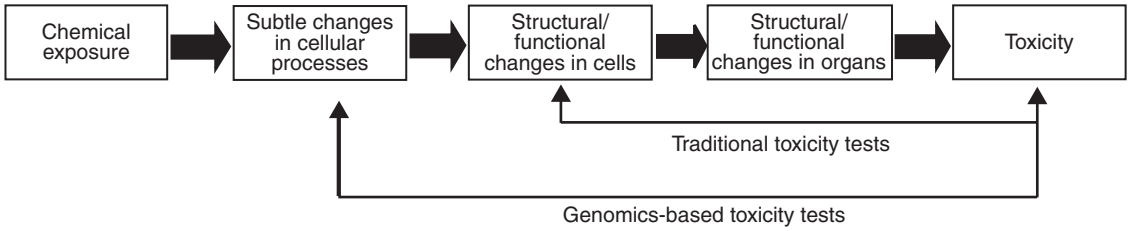

Figure 1. Continuum of events in chemical toxicity. Compared with the traditional toxicity tests, genomics-based toxicity determination can detect toxicity before the onset of clinical symptoms of toxicity.

that may serve as surrogate(s) for target organ(s); (iii) simple – the test should be simple so that it can be performed easily without the need for highly sophisticated equipments or significant technical expertise; (iv) specificity; (v) cost-effectiveness; and (vi) reproducibility. Being able to accurately predict the potential of chemicals to cause toxicity immediately following exposure has the unique advantage of implementing strategies to prevent the onset of toxicity so as to protect the exposed individuals from developing adverse health effect(s).

Various *in vitro* (cell/tissue culture) and *in vivo* (experimental animals) toxicity tests have been developed and are currently being employed to determine the potential of chemicals to cause toxicity (Boulmedarat *et al.*, 2005; Kikkawa *et al.*, 2006). These tests rely heavily on various endpoints pertaining to structural and/or functional damage to cells/organs induced by the toxic chemical. Although the various tests that are currently being employed are capable of detecting toxicity induced by chemicals, they lack one or more of the features described above that would make them ideal to predict and/or determine toxicity. For example, live animals can be exposed to toxic chemicals and histological/biochemical changes taking place in the organ(s) or blood can be determined as indicators of target organ toxicity (Amin *et al.*, 2004; Ohbayashi *et al.*, 2007;Hard, Flake and Sills, 2009). It is to be emphasized that the biochemical and histological changes in the blood and in the target organs, respectively, of the exposed animals are not predictors of toxicity, but rather indicators of injury/damage that has already taken place in response to exposure to the chemicals. Such changes are noticeable only when significant damage, including structural and/or functional damage, to the target organ(s) or tissue(s) has already taken place. Therefore, many of the traditional toxicity testing procedures lack the high sensitivity required to detect early, subtle toxicity (toxicity not associated with clinical symptoms) following exposure to very low concentrations of the toxic chemical (Figure 1). Lack of adequate sensitivity make many of the currently available toxicity tests less desirable in situations such as monitoring human exposure to low concentrations of toxic chemicals routinely present in our environment or the workplace.

2 SYSTEMS TOXICOLOGY

Upon entering the biological system, toxic chemicals may interact with various cellular components and result in perturbation of the cellular processes vital for normal functioning of the system. The biological system, in turn, may respond to the chemical-induced perturbation of cellular processes by activating the cellular response system. It has been proposed that the balance between the chemical-induced perturbation of the biological system and the system's response to the perturbation will determine whether exposure to the chemical may eventually result in toxicity or not. It has been hypothesized that alterations in the global gene expression profile may precede the toxicity induced by chemicals in a biological system. Therefore, determination of the differential gene expression profile of a biological system that has been exposed to a toxic chemical may provide important information regarding not only the potential of the chemical to cause toxicity but also the mechanisms underlying the toxicity. The global gene expression profiling may provide a complete understanding of all the biological processes that are affected in a cell, a tissue, an organ, an organ system and all organ systems of an organism in response to exposure to a toxic chemical. Such an understanding is essential

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to develop an ideal toxicity test capable of predicting/determining the potential of a chemical to cause toxicity as well as to elucidate the mechanisms underlying the toxicity. Recent advances in genomics and bioinformatics and their application in toxicology have facilitated the development of a new toxicology research area commonly referred to as toxicogenomics. Toxicogenomics is promising with respect to obtaining a complete understanding of the response of an organism to exposure to a toxic chemical. The three main areas of toxicogenomics are transcriptomics, proteomics and metabonomics. Toxicogenomics represents the complete understanding of the response at the level of transcripts (transcriptomics), proteins (proteomics) and metabolites (metabonomics). Integration of the cellular profiles of transcripts, proteins and metabolites with various traditional toxicological endpoints may provide a comprehensive view of the response of a biological system to exposure to toxic chemicals and this has emerged as a new toxicology branch commonly referred to as systems toxicology.

Systems toxicology may be defined as the understanding of all toxicological interactions taking place in a biological system under stress (chemical exposure). Thus, all these interactions between the exposure of the system to the toxic chemical and the ultimate elimination of the toxic chemical and/or its metabolites from the system may be considered as various aspects of systems toxicology. These include:

1. absorption and distribution of the chemical within the biological system;
2. metabolism of the chemical taking place in the biological system resulting in the generation of toxic and/or non-toxic metabolites;
3. interaction of the toxic chemical and/or its toxic metabolites with the cellular target(s) of toxicity within the biological system;
4. alterations in the expression profile of genes (transcripts) that may be related to the toxicity of the chemical;
5. alterations in the expression profile of proteins that may be related to the toxicity of the chemical;
6. histological changes in target organs that may be considered as structural manifestations of toxicity;
7. alterations in the biochemical constituents such as the release of enzymes from target organs into the bloodstream, which may be considered as functional and/or structural manifestations of toxicity;
8. response of the biological system such as the activation of cellular defence system to defend against the toxicity of the chemical; and
9. elimination of the chemical and/or its metabolites from the biological system.

The knowledge obtained from systems toxicology has implications in various aspects of chemical toxicity such as:

1. assessing the risk of toxicity and adverse health effects following exposure to potentially toxic chemicals present in the environment and workplace;
2. determining the safety and toxicity of drug molecules;
3. developing biomarkers to monitor human exposure to potentially toxic chemicals;
4. determining the mechanism(s) underlying the toxicity of chemicals; and
5. developing strategies to prevent the toxicity of chemicals.

As the objective of systems toxicology is to obtain a complete understanding of the response of a biological system to exposure to a toxic chemical, toxicogenomics (transcriptomics, proteomics and metabonomics) seems to have the potential to provide the data required for such an understanding. Of the three areas of toxicogenomics, transcriptomics has enjoyed the most significant growth to date, and therefore the impact of transcriptomics will be further discussed in this chapter.

3 TRANSCRIPTOMICS – TECHNICAL ASPECTS

Transcriptomics or transcript profiling deals with determination of the abundance of all transcripts (mRNA) that are expressed in a cell at a given time. Transcriptomics may be considered as a snapshot of all the expressed transcripts in a biological sample at a given time. It is assumed that exposure of cells to toxic chemicals results in a biological response, and a full understanding of the cellular response to the chemical exposure is important

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in predicting, determining and understanding the toxicity of the chemical. This can be achieved by determining the abundance of all expressed genes (transcripts) in the chemical-exposed sample and comparing it with that of a control, unexposed sample. Several techniques are currently available to determine the mRNA expression profile of cells, including northern hybridization, PCR amplification, subtractive hybridization, serial analysis of gene expression, differential display and microarray analysis. Microarray analysis has the unique advantage of simultaneously determining the expression profile of thousands of genes present in biological samples and has been the method of choice to determine the global gene expression profile in biological samples. Microarray analysis of global gene expression profile is a complex process consisting of several sequential steps. The first step in the microarray analysis of gene expression profilings is the isolation of high-quality total RNA (it is not necessary to purify the mRNA) from the control and chemical-exposed biological samples. Subsequently, the mRNA is reverse transcribed to synthesize cDNAs. The cRNAs synthesized from the cDNAs are labelled with fluorescent dye, for example cy3, and the resulting targets are hybridized to the probes which are arrayed on the microarray. The sequence-specific hybridization of the labelled targets with the probes present on the microarray is subsequently detected by scanning the arrays with a fluorescence scanner and the intensity of the hybridization signal obtained is considered as an indication of the abundance of the targets (transcripts) expressed in the sample. The resulting data can be analysed using appropriate computational and statistical programs to determine the transcript profile of the sample. Comparison of the transcript profile of the chemically treated sample with the appropriate control sample will provide information about transcripts that are differentially expressed as a result of the chemical exposure. Appropriate bioinformatic analysis of the differentially expressed genes, integration of the differential gene expression profile with traditional toxicity endpoints and careful interpretation of the resulting data in light of the existing toxicology literature should provide a complete understanding of the events taking place in the biological system in response to exposure to the toxic chemical and in turn may contribute to systems toxicology (Figure 2).

Transcriptomics research has enjoyed considerable growth over the past several years. The discovery of microarray technology and its impact on transcriptomics studies have revolutionized toxicology research. The amount of RNA required for microarray studies has decreased significantly over the years and this has facilitated the use of this powerful technique even in difficult-to-obtain precious samples such as human tissues. Microarrays and the reagents and equipments required for analysis of global gene expression profiles are currently available from several manufacturers, and this has resulted in a significant reduction in the cost associated with the analysis. Currently, the microarray-based transcriptomics profiling technique has become an affordable technique that can yield reliable, reproducible and biologically relevant toxicity data. The impact of transcriptomics in toxicology research is illustrated by the number of research papers published in recent years that describe gene expression data (Figure 3). To date, microarray-based transcriptomics studies have been conducted in a wide variety of species that include nematodes (Menzel *et al.*, 2009), fish (Baker *et al.*, 2009), amphibians (Langerveld *et al.*, 2009), rodents (Courter *et al.*, 2008; Xu *et al.*, 2008), birds (Gust *et al.*, 2009), dogs (Higgins *et al.*, 2003), non-human primates (Marvanova *et al.*, 2003) and humans (McHale *et al.*, 2007). Transcriptomics-based toxicogenomics studies have been employed to study the toxicity of chemicals targeting various organs including brain (Glover *et al.*, 2009), eye (Yang *et al.*, 2007), nose (Roberts, Thomas and Dorman, 2008), lungs (Rouse *et al.*, 2008), heart (Carney *et al.*, 2006), kidney (Amin *et al.*, 2004), reproductive organs (Fukushima *et al.*, 2005; Valdez *et al.*, 2009), immune system (Baker *et al.*, 2009) and skin (Gerecke *et al.*, 2009). However, most of the transcriptomics studies reported so far have involved chemicals that are primarily hepatotoxic in nature or target liver as the primary target organ (Reilly *et al.*, 2001; Uehara *et al.*, 2008;Blomme, Yang and Waring, 2009; Sano *et al.*, 2009). This is mainly because liver is the primary organ responsible for metabolizing toxic chemicals (Lee, 2003), and furthermore, liver toxicity has been cited as a major reason for withdrawal of new drugs from the market (Barros and Martin, 2008). Studies investigating hepatotoxicity of chemicals will be discussed in detail to illustrate the potential application of transcriptomics in studying the systems toxicity of chemicals.

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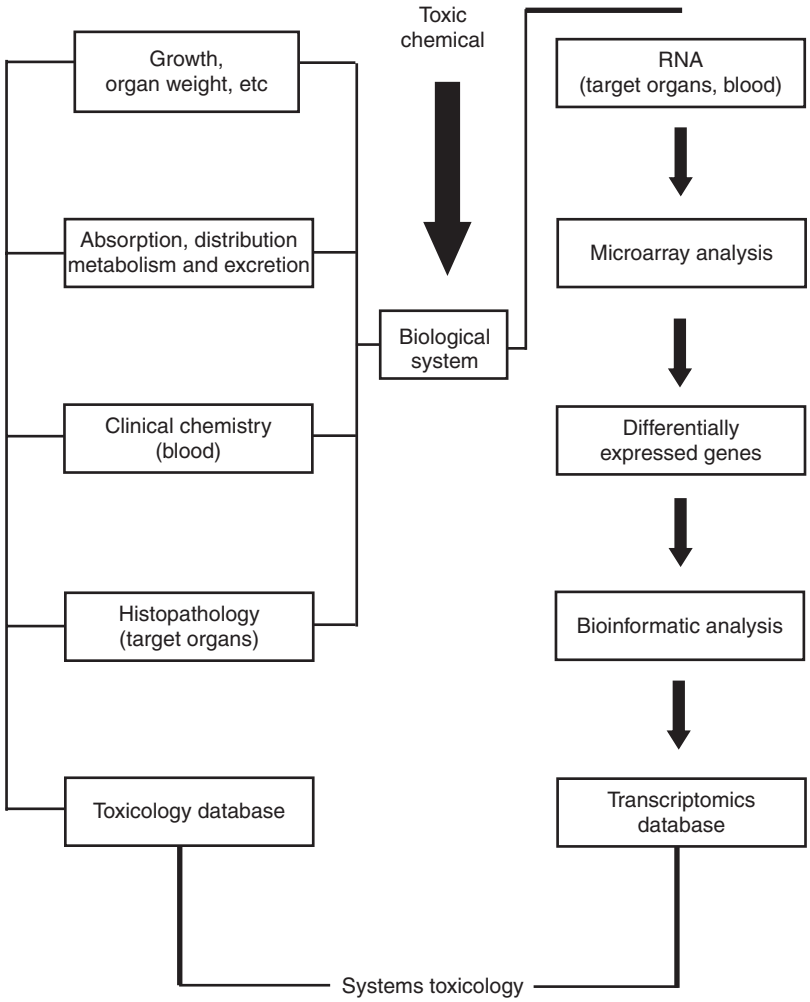

Figure 2. Integration of transcriptomics with traditional toxicity endpoints.

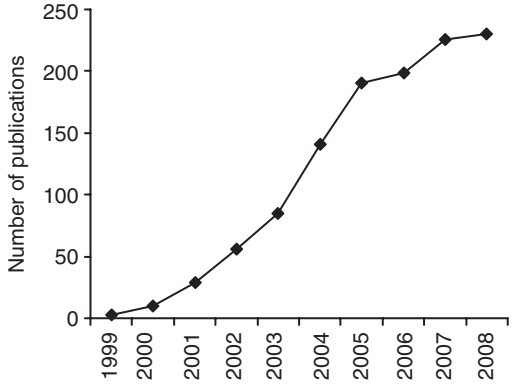

Figure 3. Impact of transcriptomics data in toxicology. The Pubmed database was queried using the keywords "microarray" and "toxicology," and the number of publications from 1999 to 2008 is presented.

4 HEPATOTOXICITY DETERMINATION BY TRANSCRIPTOMICS

The application of microarray-based transcriptomics studies in systems toxicity is best illustrated in the case of chemicals that target liver as their primary target organ. Hepatotoxic chemicals have been employed as model chemicals by several investigators to demonstrate the potential application of microarray-based global gene expression profiling to study the toxicity of chemicals (Bulera *et al.*, 2001; Hamadeh *et al.*, 2002a). So far, microarray-based transcriptomics studies have been conducted to classify hepatotoxic chemicals, determine mechanisms of hepatotoxicity and predict hepatotoxicity.

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In a study conducted earlier by Waring *et al.* (2001), rats were treated with 15 different hepatotoxic chemicals including allyl alcohol, amiodarone, Aroclor 1254, arsenic, carbamazepine, carbon tetrachloride, dimethylnitrosamine, dimethylformamide, diquat, etoposide, indomethacin, methapyrilene, methotrexate, monocrotaline and 3-methylcholanthrene. The various chemicals selected in this study are all known to result in hepatotoxicity such as necrosis, DNA damage, cirrhosis, hypertrophy and hepatic carcinoma. Hepatotoxicity was determined by histopathology of liver and clinical chemistry evaluation of blood. In addition, global gene expression profiles of liver were determined by microarray analysis. Overall, a good correlation was noticed between histopathology, clinical chemistry and gene expression profiles induced by the hepatotoxic agents employed. The authors also attempted to cluster the various hepatotoxic compounds employed in the study based on their hepatotoxicity (histopathology and clinical chemistry results) and liver gene expression profiles. The results demonstrated that various hepatotoxic compounds employed in the study clustered together in fairly good agreement based on the observed changes in clinical chemistry, histopathology and gene expression profile. In some cases, liver gene expression profiles outperformed the traditional hepatotoxicity endpoints (histopathology of liver and blood clinical chemistry) in clustering the hepatotoxic chemicals. The authors, based on the results of their study, concluded that microarray-based global gene expression profiling could be employed as a highly sensitive and reliable technique to identify hepatotoxic chemicals in rat.

Whether gene expression profiling can be employed to distinguish (classify) hepatotoxic chemicals that differ in their mechanism of toxicity has been investigated (Hamadeh *et al.*, 2002b). Hepatotoxicity was induced in rats by administering either peroxisome proliferators (clofibrate, Wyeth 14 643 and gemfibrozil) or an enzyme inducer (phenobarbital). Hepatotoxicity and liver gene expression profiles of the rats were determined by histopathology and microarray analysis, respectively. The microarray data were subjected to several computational analyses to determine whether hepatotoxic chemicals exhibit gene expression patterns distinguishable based on their mechanism of toxicity. The results of this study demonstrated that the peroxisome proliferators exhibited a gene expression pattern that was clearly distinguishable from that of the enzyme inducer, suggesting the potential application of microarray-based gene expression profiling to classify hepatotoxic chemicals based on their mechanism(s) of toxicity.

A major advantage of gene expression profiling in toxicity studies is its superior sensitivity to detect target organ toxicity compared with the traditional toxicity endpoints such as histology and clinical chemistry. Several studies have indicated that gene expression changes indicative of hepatotoxicity are detectable well before the onset of clinical or histopathological changes associated with hepatotoxicity. In a study conducted by Heinloth *et al.* (2004), rats were administered either sub-toxic (50 and 150 mg/kg b.w.) or toxic (1500 mg/kg b.w.) doses of acetaminophen. At time intervals of 6, 24 and 48 h following administration of the chemical, groups of rats were killed and the induction of hepatotoxicity was determined on the basis of histopathology of liver and clinical chemistry of blood. At each time interval, RNA was isolated from the liver and global gene expression profile was determined by microarray analysis. At all time intervals, the rats administered the sub-toxic doses of acetaminophen exhibited normal histology and clinical chemistry, suggesting either the absence of hepatotoxicity in the rats or the inability of these traditional hepatotoxicity endpoints, because of their poor sensitivity, to detect subtle toxicity induced by the chemical. Similarly, rats administered the overtly toxic dose of acetaminophen (1500 mg/kg b.w.) did not exhibit alterations in either histology or clinical chemistry at the earliest time interval of 6 h following administration of the chemical. However, significant hepatotoxicity, as indicated by alterations in liver histology and blood clinical chemistry, was noticed in rats administered 1500 mg acetaminophen/kg b.w. at later time intervals of 24 and 48 h. Microarray analysis of global gene expression profiling demonstrated significant differential expression of several genes including those involved in energy metabolism and stress response in the liver of the rats administered 1500 mg acetaminophen/kg b.w. for 24 and 48 h. Interestingly, many of the genes which were found differentially expressed at time intervals of 24 and 48 h in the rats administered the overtly toxic dose of 1500 mg/kg b.w., and are therefore considered as indicators of acetaminophen-induced hepatotoxicity, were also

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found differentially expressed in the rats administered the sub-toxic doses of the chemical. It is worth emphasizing that the rats administered the sub-toxic doses of acetaminophen did not exhibit any histological or clinical changes indicative of toxicity. Taken together, these results demonstrated the superior sensitivity of gene expression changes to detect hepatotoxicity compared with the traditional histological and clinical markers of toxicity. Similar observations supporting the superior sensitivity of gene expression profiling compared with the traditional hepatotoxicity endpoints in detecting hepatotoxicity have been reported by other investigators (Hamadeh *et al.*, 2002b).

The results of a recent study carried out by Huang *et al.* (2008) further support the findings that gene expression profiling can be employed to predict hepatotoxicity as well as understand the mechanisms of chemical toxicity in the liver. Rats were administered individually one of the seven hepatotoxic chemicals that are known to induce necrosis. Microarray analysis of the global gene expression profile in the rats identified several differentially expressed genes in the chemical-treated rats compared with the controls. Using a Random Forrest classifier with feature selection, a group of 21 genes were identified as predictors of necrosis induced by the hepatotoxic chemicals. The selected predictor genes were able to predict necrosis induced by the model hepatotoxic chemicals such as acetaminophen, carbon tetrachloride and allyl alcohol with an accuracy of 90%, 80% and 60%, respectively. Pathway and network analysis of the necrosis predictor genes identified inflammation and apoptosis as major biological processes responsible for necrosis induced by the selected hepatotoxic chemicals.

5 TRANSCRIPTOMICS STUDIES USING SURROGATE TISSUES

Toxicogenomics is certainly a viable and attractive approach to study the systems toxicity of chemicals in humans. However, a major limitation in studying the toxicity of chemicals in human beings is the difficulty associated with obtaining samples of target organs to study. Because of ethical and/or other practical issues such as the health and safety of the individuals involved, it is not possible to obtain samples of target organs or tissues from human beings to determine chemical toxicity. A practical solution to this problem is the potential use of surrogate tissues to study the toxicity of chemicals in target organs that are not easily and safely obtainable. Some surrogate tissues most commonly employed to study target organ toxicity are blood, urine, saliva, sweat, tear, cerebrospinal fluid, milk, broncho-alveolar lavage, sputum, stool, breath condensate, nail, hair, buccal cells and placenta (Burczynski and Rockett, 2006). From the surrogate tissues listed above, it is clear that many of them are not actually tissues in a biological sense. Any biospecimen that can be used to determine or study the biological processes taking place in target organs that are not easily obtainable can be considered a surrogate tissue. Of all the surrogate tissues employed to study target organ toxicity, blood is not only the most frequently used but also the most suitable one (Burczynski, and Rockett 2006). Blood may be considered as the internal environment for the various organs and tissues in the body that are often the targets for chemical toxicity. Very often, alterations in the biological processes taking place in the target organs and tissues in response to their exposure to toxic chemicals are reflected by alterations that are similar to a certain extent in the blood. Alterations in the activity of specific enzymes or the absolute amount of certain biochemical molecules or even the amount of the parent compound and/or its metabolite(s) present in the circulating blood or blood cells may provide valuable information regarding the target organ toxicity potential of chemicals; therefore, one or more of these indicators may be used as surrogate markers of toxicity. Activities of transaminases (aspartate and alanine aminotransferase) in the blood are routinely employed as reliable surrogate markers of hepatotoxicity (Heinloth *et al.*, 2004; Beyer *et al.*, 2007; Bushel *et al.*, 2007). Other surrogate markers of toxicity are blood urea nitrogen (BUN) for nephrotoxicity (Fang *et al.*, 2008; Roomi *et al.*, 2008), serum acetyl cholinesterase activity for neurotoxicity induced by organophosphorus insecticides (Brahmi *et al.*, 2006), blood level of hormones for toxic chemicals targeting endocrine system (De Angelis *et al.*, 2009) and so on.

Although these surrogate toxicity markers have been of immense help to determine the target organ toxicity of chemicals, all or most of them suffer from significant shortcomings such as not being specific for the target organ(s) involved or not being sensitive enough to detect toxicity in a pre-clinical

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condition. For example, significant structural damage, such as necrosis, to the hepatocytes is necessary for transaminases to be released from the liver into the peripheral blood and then measured as markers of hepatotoxicity. Furthermore, an elevation in the serum level of transaminases need not always be an indicator of chemical toxicity taking place in the liver. For example, significant elevations in serum transaminases have been reported under conditions other than exposure to hepatotoxic chemicals (Harrison, Bahar and Payne, 2002; Ramakers *et al.*, 2009).

As presented earlier in this chapter, gene expression changes taking place in target organs are known for their superior sensitivity as markers of toxicity compared with the traditional biochemical and histological markers of toxicity. Therefore, it seems reasonable to investigate whether gene expression profiling to determine target organ toxicity can be performed using easily available surrogate tissues such as blood. This concept has recently gained popularity, and there have been few studies reporting the potential application of blood gene expression profiling as sensitive surrogate indicators of target organ toxicity. As in the case of target organs, most of the toxicogenomics studies investigating the potential application of blood transcriptomics as surrogate markers of target organ toxicity have been conducted using model hepatotoxic chemicals. In a classical study, Bushel *et al.* (2007) of the National Institute for Environmental Health Sciences (NIEHS) demonstrated the usefulness of blood transcriptomics as sensitive indicators of hepatotoxicity induced by the model hepatotoxic drug acetaminophen in rats. Rats were given either a subtoxic (150 mg/kg b.w.) or two overtly toxic (1500 and 2000 mg/kg b.w.) doses of acetaminophen. Blood and liver obtained from the control and acetaminophen-administered rats were analysed to determine hepatotoxicity induced by the chemical. The parameters employed included liver histology, various haematological parameters (total and differential white blood cell counts, red blood cell count, platelet count, hematocrit and haemoglobin content), blood level of transaminases and global gene expression profiling in blood. Based on the results obtained, these authors demonstrated that blood gene expression profiles may be employed as surrogate markers of hepatotoxicity induced by acetaminophen. Furthermore, the results demonstrated that blood gene expression profiles exhibited superior sensitivity as hepatotoxicity markers compared with the traditional toxicity endpoints such as histological, haematological and biochemical changes.

The potential application of blood transcriptomics as appropriate surrogate marker of target organ toxicity was further demonstrated by the results of a comprehensive study published recently (Lobenhofer *et al.*, 2008). These investigators employed a series of eight well-characterized hepatotoxic chemicals that are known to exhibit similarities and differences in the type and location of necrosis induced in the target organ (liver) to demonstrate the potential use of blood transcriptomics as surrogate marker of hepatotoxicity. The study was designed to provide a means to generate mechanistic and predictive measures of toxicity by integrating multiple data streams obtained from liver (target organ) and blood (surrogate tissue). The hepatotoxic chemicals selected were administered in rats at three doses (low, medium and high) for three time intervals (6, 24 and 48 h) to result in sub-toxic to severe hepatotoxicity (necrosis). Toxicity was determined based on established histological, biochemical and haematological parameters in liver (the target organ) and blood (surrogate tissue). In addition, gene expression profiling of liver and blood was performed using two separate microarray platforms. A support vector machine (SVM) approach was employed to identify hepatotoxicity-specific classifiers for the target organ and the surrogate tissue. The classifiers thus obtained were used to group the samples based on principal component analysis and hierarchical clustering to determine whether the animals exposed to each of the hepatotoxicants could be separated into different compound groups. Results of the study demonstrated that the classifiers derived from both liver and blood transcriptomic data were able to accurately classify samples on the basis of the hepatotoxic chemical administered. It is noteworthy to mention that the classifiers generated from the blood data outperformed those generated from the liver data in classifying the hepatotoxicants.

Results of recent studies carried out in our laboratory (Umbright *et al.*, 2009) further support the concept that blood gene expression profiling may be employed as a suitable, alternate approach to detect and distinguish target organ toxicity induced by toxic chemicals. Rats were administered a single, acute dose of a well-characterized hepatotoxic (acetaminophen) or a

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neurotoxic (methyl parathion) chemical. Administration of acetaminophen resulted in overt hepatotoxicity as evidenced from significant elevation in blood transaminases' activities. Similarly, administration of methyl parathion resulted in neurotoxicity as evidenced from significant inhibition of acetylcholinesterase activity. Microarray analysis of the global gene expression profile of leucocytes obtained from the blood samples of rats identified distinct gene expression profiles capable of detecting and distinguishing hepatotoxicity and neurotoxicity induced by the model chemicals administered. The gene expression markers of target organ toxicity were detectable in the blood earlier than the appearance of the commonly used clinical markers of toxicity, further supporting the superior sensitivity of gene expression markers compared with traditional toxicity endpoints to detect target organ toxicity (Figure 4). The ability of the marker genes to detect and distinguish hepatotoxicity and neurotoxicity was further confirmed using the blood samples of rats administered additional model hepatotoxic (thioacetamide, carbon tetrachloride and dimethylnitrosamine) and neurotoxic (ethyl parathion and malathion) chemicals.

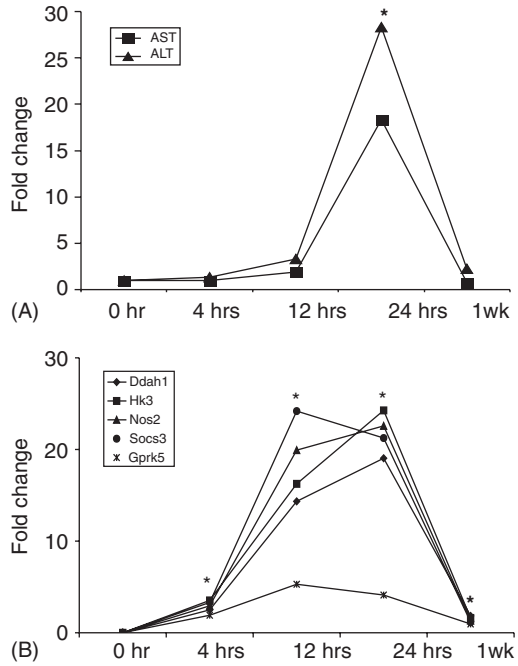

Figure 4. Gene expression profiling is more sensitive than the traditional toxicity endpoints. A single acute toxic dose of acetaminophen was administered in rats. At various time intervals ranging from 4 h to 1 week following administration of the chemical, blood was analysed for toxicity based on the activities of AST and ALT (a) and expression of marker genes for hepatotoxicity (b). Significant alterations in the expression of the selected hepatotoxicity marker genes were observed in the blood before any significant change in the activities of transaminases, suggesting the superior sensitivity of gene expression changes as indicators of target organ toxicity (Reproduced with permission from Umbright *et al*. (2009)).

6 TRANSCRIPTOMICS STUDIES – CHALLENGES

The past 10–15 years have witnessed significant progress in the application of transcriptomics in studying the toxicity of chemicals. Nevertheless, microarray-based transcriptomics research still faces many challenges, and further attention to these issues or challenges is required to enhance the potential of transcriptomics in toxicogenomics and systems toxicology. Following is a description of some of the major challenges facing transcriptomics and the progress that has been achieved.

6.1 Confirmation of Microarray Data

In spite of the high-throughput potential, transcriptomics data are usually error-prone. It is therefore possible to notice several false positives and false negatives among the hundreds if not thousands of differentially expressed genes that are identified in a chemically treated sample based only on microarray determination of gene expression profiles. It is often necessary to confirm the microarray data to eliminate at least false positives as much as possible. Appropriate statistical methods to minimize the false discovery rate (FDR) are usually applied while analysing the microarray data (Benjamini and Hochberg, 1995). Another approach is to confirm the microarray data by more quantitatively reliable supplementary techniques such as real-time PCR or northern hybridization. However, considering the hundreds if not thousands of differentially expressed genes that may be identified from a typical microarray experiment, it is a challenging task to confirm the microarray data by these cumbersome supplementary techniques. It is worthy to mention that microarray technology has improved considerably since its introduction in the mid-1990s and it has

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become a more reproducible and reliable technique capable of detecting differentially expressed genes with significant accuracy. A reasonably good correlation has been noticed in the gene expression profile data obtained by microarray and real-time PCR analysis (Joseph, He and Umbright, 2008; Umbright *et al.*, 2009).

6.2 Comparability of Transcriptomics Data

A major roadblock in microarray-based transcriptomics research is the comparability of expression profiling data obtained from multiple sources (Irizarry *et al.*, 2005; Larkin *et al.*, 2005). As described earlier in this chapter, microarray analysis of gene expression profiling is quite complex and technologically challenging. Each step in the analysis can potentially contribute to variability in the data. Depending on the microarray platforms employed (one channel vs. two channels, oligonucleotide vs. cDNA, in-house fabricated vs. commercially available), the same biological sample may yield different gene expression profiles. It is worth mentioning that significant refinement in microarray technology has taken place since its introduction in the mid-1990s and this has facilitated reasonably good concordance in microarray data obtained using different microarray platforms (Liew *et al.*,2006). Each step in the microarray analysis procedure, such as RNA isolation, reverse transcription and synthesis of labelled cRNA targets, hybridization and washing of chips, and acquisition and analysis of data, can contribute to variations in microarray data. Variability in the microarray data generated by different investigators can have implications in the ultimate utility of the microarray data generated. Therefore, it is necessary to take precautions or even to develop standards for conducting microarray experiments so as to minimize the variability associated with the technical aspects of microarray experiments. Similar to the guidelines recommended by MIAME (minimum information about microarray experiment) for documentation of microarray data (Brazma *et al.*, 2001), standards for conducting microarray experiments may be developed to minimize the variability associated with the microarray data generated in different laboratories.

Several studies have been conducted in the past to address the variability or comparability issue of microarray data. The Toxicogenomics Research Consortium (Bammler *et al.*, 2005) has identified some of the potential sources of variability in microarray data. In a study conducted by investigators of the Toxicogenomics Research Consortium, microarray data were generated by seven participating laboratories using two standard RNA samples and 12 microarray platforms. It was noticed that reproducibility for most microarray platforms was generally good when the data were generated by the same participating laboratory. Most of the variations were noticed when the data were generated by different laboratories. Reproducibility among the participating laboratories increased markedly when standardized procedures were implemented for RNA isolation, target labelling, microarray hybridization, data acquisition and processing. It was also noticed that the variability was least when the analysis was performed based on biological themes.

The contribution of biological variability in addition to technical issues associated with the quality of microarray data has been demonstrated by the results of a study conducted by the investigators from seven geographically dispersed research centres in the USA (Beyer *et al.*, 2007). Several precautions were taken in an attempt to avoid, or at least minimize, the variations in the data generated by the participating laboratories. The study involved microarray analysis of the gene expression profile of the liver of mice administered a toxic dose of acetaminophen. Even though administration of acetaminophen in mice was conducted independently in the participating laboratories, they all received mice, feed, bedding and acetaminophen from centralized sources. Furthermore, all participating laboratories were required to adhere to a standard protocol for treatment, treatment termination, specimen collection and processing. The specimens collected in the individual laboratories were sent to a centralized facility for measurement of endpoints [liver histology, serum alanine aminotransaminase (ALT) activity and liver gene expression profile]. Administration of acetaminophen, in general, resulted in hepatotoxicity as evidenced from histological changes (necrosis) in the liver and elevation of ALT activity in the blood of the mice. However, animal-to-animal variability was noticed with respect to the liver toxicity induced by acetaminophen, and this was more

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prominent among the laboratories than within an individual laboratory. A corresponding variation in the number of significantly differentially expressed genes was noticed among the laboratories. The variability observed in gene expression profiles was significantly reduced when the differential gene expression data were anchored to the liver injury phenotype (the importance of anchoring differential gene expression profiles to toxicity phenotype is further discussed elsewhere in the chapter).

The Carcinogenicity Working Group of the Predictive Safety Testing Consortium, a collaboration between several pharmaceutical industries and the US Food and Drug Administration (FDA), has tested (Fielden *et al.*, 2008) two published hepatic gene expression signatures (Nie *et al.*, 2006;Fielden, Brennan and Gollub, 2007) developed to predict carcinogenic potential of chemicals in rats. The microarray data obtained from two short-term rat studies using over 150 compounds (carcinogenic and non-carcinogenic) were subjected to a meta-analysis for predicting carcinogenicity. It was found that despite significant differences in the study designs and microarray platforms employed in obtaining the data, the signatures proved to be relatively robust and more accurate than expected by chance (Fielden *et al.*,2008). However, a considerable loss (15–30%) in accuracy of the predictive signature was noticed when the signature generated by the individual laboratories was further validated. A loss of 15–30% accuracy is substantial and could result in false classification of a potentially carcinogenic chemical as non-carcinogenic or vice versa. The loss in accuracy of predictability was attributed mainly to the differences in experimental conditions, treatment protocols and microarray platforms employed.

The studies presented above unequivocally support the observation that variability is an integral part of microarray data. Furthermore, they have identified some of the potential sources such as technical issues and biological variation that are responsible for the variability observed. Standardization of procedures employed to obtain the microarray data and incorporation of biological themes (phenotypic anchoring) when analyzing the microarray data can significantly reduce variability in the microarray data generated by different investigators. Such improvements can result in more reliable and toxicologically relevant microarray data that can be used to determine the toxicity of chemicals including those to which significant human exposure may take place.

6.3 Biological Relevance of Transcriptomics Data

Another major challenge facing transcriptomics-based systems toxicology is determining the toxicological relevance of the transcriptomics data. Typically, microarray analysis of gene expression profiling may result in the identification of hundreds or even thousands of genes that are significantly differentially expressed in a biological system in response to its exposure to a toxic chemical. Some of these changes may be detected by chance and as such may have no toxicological relevance, whereas others may be related to the chemical-induced perturbation of the biological system and therefore are expected to be toxicologically relevant. What would be the criteria to determine whether a certain gene expression profile is toxicologically relevant or not? Toxicology research conducted over the past several decades has identified several cellular events that are considered reliable endpoints of toxicity. For example, exposure of a rat to hepatotoxic chemicals results in liver damage that can be detected by histological analysis of the tissue. The damaged liver cells in turn release enzymes, including transaminases, (aspartate and alanine transaminases) into the bloodstream, resulting in elevated serum levels of these enzymes. In general, a good correlation has been noticed between the serum level of transaminases and the liver damage or toxicity induced by hepatotoxic chemicals. In a similar way, it is possible to establish a relationship between differentially expressed genes and the established histological or biochemical markers of toxicity. This is commonly referred to as phenotypic anchoring (Paules, 2003; Powell *et al.*, 2006). Phenotypic anchoring is defined as establishing a correlation or relationship between the toxicogenomics (transcriptomics, proteomics and metabonomics) data and the toxicity phenotype as determined by traditional toxicity endpoints. As many experimental details as possible are required for the successful anchoring of a gene expression profile to a toxicity phenotype. For example, information regarding the dose of the chemical administered may suggest whether a

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particular gene expression profile is an indicator of subtle toxicity or overt toxicity. Similarly, the time elapsed between exposure to the chemical and the determination of the expression profile may suggest whether a certain profile is a marker for early or late toxicity.

The importance of phenotypic anchoring in understanding the toxicological relevance of gene expression data is best illustrated in the case of hepatotoxicity. In a study by Heinloth *et al.* (2004), the prototypical hepatotoxic drug, acetaminophen, was administered in rats at doses of 0, 50, 150 and 1500 mg/kg b.w.. The control and the drug-administered rats were sacrificed at time intervals of 6, 24 and 48 h following administration of the chemical. Administration of acetaminophen at the selected doses and durations resulted in different grades of toxicity ranging from no toxicity (lower doses and early time points) to severe toxicity (higher doses and late time intervals) as revealed by the results of liver histopathology and blood clinical chemistry. Microarray analysis of the global gene expression profile in the liver resulted in the identification of a large number of differentially expressed genes in the acetaminophen-administered rats compared with the controls. Hierarchical clustering of the differentially expressed genes at any dose or time showed that there were clusters of genes similarly regulated at all three doses of the chemical, including the lower doses that did not result in any detectable change in liver histology or blood clinical chemistry. Bioinformatics analysis of the differentially expressed genes revealed that genes involved in energy-producing biochemical pathways were up-regulated whereas those involved in energy-consuming pathways were down-regulated in the acetaminophen-administered rats including those administered the lowest, non-toxic dose of the chemical. Biochemical analysis revealed significantly reduced ATP levels in the livers of the acetaminophen-administered rats compared with the controls, further supporting the microarray data. Similarly, Powell *et al.* (2006) have successfully anchored gene expression signatures in acetaminophen-administered rats to conventional biomarkers of oxidative stress. Thus, phenotypic anchoring has demonstrated the capability of a gene expression profile to detect and predict toxicity as well as to determine the mechanisms underlying the toxicity of chemicals. In addition, as discussed above, phenotypic anchoring of differentially expressed genes also demonstrated the superior sensitivity of gene expression profiles as indicators of toxicity compared with traditional toxicity endpoints.

6.4 Establishing Standards in Acquiring and Processing Transcriptomics Data

As described above, a major challenge facing transcriptomics-based toxicogenomics research is the difficulty in comparing data generated by different investigators. Although the contribution of factors such as biological variability of experimental samples in this regard is unavoidable, there are a number of factors, especially those related to acquiring and processing the transcriptomics data, that can be controlled so as to minimize the differences in the transcriptomics data generated by various investigators. For example, standardization of techniques such as isolation of RNA from biological samples, synthesis of labelled targets, microarray hybridization and washing, and microarray scanning to obtain gene expression signals may minimize the technical variability associated with transcriptomics data, facilitating better comparability of microarray data among investigators. Similarly, standardization of data analysis procedures may enhance the comparability of transcriptomics data obtained from different laboratories. Different investigators, for example, have adopted different criteria to select significantly differentially expressed genes. Although some investigators have applied correction for FDR (Bushel *et al.*, 2007; Huang *et al.*, 2008), others have not (Reilly *et al.*, 2001; Minami *et al.*, 2005). Differentially expressed genes have been selected solely on the basis of fold-change in expression (Reilly *et al.*, 2001; McHale *et al.*, 2007) or *p*-value (Huang *et al.*, 2008). There are also cases where a combination of fold change in expression and statistical significance of the data has been employed to select significantly differentially expressed genes (McHale *et al.*, 2007; Elferink *et al.*, 2008; Umbright *et al.*,2009). Thus, depending on the selection criteria employed (fold change in expression, statistical significance or a combination of both), considerable differences in the genes selected as significantly differentially expressed must be expected. This, in turn, may result in corresponding differences in the results obtained from further downstream analysis

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of gene expression data such as determination of the involvement of specific pathways, networks and biological functions in the toxicity of the chemical being investigated. Thus, adopting standards for acquiring and analysing microarray data may reduce the variability in the data generated by different investigators, facilitating better comparison of the transcriptomics data. It is encouraging to notice that the standardization efforts by various groups such as the Microarray Quality Control Consortium and the Toxicogenomics Resource Consortium have resulted in considerable improvements in the reproducibility of transcriptomics data (Bammler *et al.*, 2005; Irizarry *et al.*, 2005; Larkin *et al.*, 2005; Shi *et al.*, 2006; Beyer *et al.*, 2007).

6.5 Translation of Transcriptomics Data from Experimental Models to Humans

Most of the toxicogenomics studies reported so far have employed cell cultures or experimental animals as models to derive microarray data relevant to the toxicity of chemicals. The ultimate goal of any toxicity study employing experimental models is to derive data that are relevant and applicable to situations of human exposure to chemicals resulting in potential toxicity. The role of confounding factors potentially capable of influencing the gene expression profile is more significant in the case of humans compared with the experimental models. Experimental models such as animals are relatively homogeneous in nature and factors such as species, strain, diet, exposure to other chemicals, etc. can be controlled in them to minimize the confounding effects of these factors on gene expression profile. In contrast, individuals who may be exposed to toxic chemicals present in the environment or the workplace are very heterogeneous in nature and may be subjected to a large number of confounding factors such as race, age, sex, life style factors and exposure to other chemicals that may potentially influence the gene expression profile. It needs to be thoroughly investigated whether toxicogenomics data derived from well-controlled experiments involving cell culture and animal models can be translated to highly variable situations of human exposure to toxic chemicals. In a recent study published by Bushel *et al.* (2007), a rat experiment was conducted to identify a discriminatory blood gene expression profile for hepatotoxicity induced by acetaminophen. The investigators were able to identify acetaminophen-overdosed patients from the controls by employing human orthologues for the rat discriminatory genes they had identified. Although this study has demonstrated the potential translation of transcriptomics data derived from experimental animals to humans under conditions of exposure to relatively large doses of acetaminophen capable of causing overt liver toxicity, it is not known whether the same can be applied to situations of human exposure to low concentrations of toxic chemicals present in the environment as well as the workplace.

7 DISCLAIMER

The findings and conclusions in this report are those of the author(s) and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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System Toxicology Approaches for Evaluating Chemical Carcinogenicity

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1 DEVELOPMENT OF SYSTEMS BIOLOGY AND TOXICOGENOMIC APPROACHES TO STUDY EFFECTS OF CHEMICALS

The systems biology approach derives knowledge on biological functions from evaluating interactions among various parts of biological systems especially molecular and metabolic pathways. The emphasis on pathways and their interactions for understanding biological processes provides the differentiation from traditional approaches that concentrated on mostly understanding of function of an individual gene and/or protein. The development of systems biology was enabled by a wide application of microarray technologies capable of evaluating gene expression patterns (mRNA levels) encompassing the whole genome. Although gene expression changes of one or several genes have been analyzed before, the new aspect introduced with genomics and all other '-omics' methods was that all definable entities of an '-ome', corresponding to a certain class of molecules in biology were now studied globally. In a broader sense, protein expression and metabolite changes may also be considered as systems biology enablers.

Toxicogenomics encompasses the analysis of gene expression changes under the influence of chemical stressors/toxic compounds in a biological model. Fundamental assumptions of toxicogenomics are that all toxicological relevant effects are accompanied by gene expression changes (Farr and Dunn, 1999), and that similar toxicological mechanisms cause comparable expression changes, with potential exceptions being acute necrotic effects. From a technical point of view, toxicogenomics was possible because of the development of whole genome microarrays, which allow interrogation of the expression level of essentially all known genes and/or sequenced transcripts of a species of interest. Microarrays are available in different designs, yet a few major ones have eventually penetrated the market (Ahmed, 2006). Those microarrays based on oligonucleotides, either printed on a solid support, attached to beads, or synthesized *in situ* onto a wafer chip, seem to be preferred over printed cDNA arrays, probably caused by ease of production or reproducibility, although a comparison between these major microarray types have revealed high repeatability between and within platforms, and high correlation between microarray results and other quantitative gene expression measurements. Any discordance observed could be related back to differences in probe sequences. This examination had been conducted by the MicroArray Quality Control (MAQC) consortium, with participation by microarray platform providers, the US FDA National Center of Toxicological Research, and others (Guo *et al*., 2006; Shi *et al*., 2006). Also, a comparison of microarray platform results to those obtained by real-time polymerase chain reaction

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(RT-PCR or Q-PCR) as well-known quantitative gene expression technology revealed high correlation for relative gene expression changes between the two methods thus validating the use of microarrays for gene expression analysis (Canales *et al*., 2006). In addition to MAQC, further public consortia have addressed issues, such as reproducibility of microarray results, standard practice for assays and analysis, relevance of microarray results to conventional end-points, and robustness of statistical models on diverse data sets and are formulating recommendations on these issues which are still developing especially those concerning data analysis (Mattes, 2008; Shi *et al*., 2008).

With respect to the amount of starting material, improved protocols and amplification methods have been developed which allow even small numbers of cells or pieces of tissue to be analyzed for their expression profiles. Defined tissue sections of interest isolated by laser-capture microdissection (LCM) were thereby successfully subjected to gene expression profile analysis (Caretti *et al*., 2008; Paul *et al*., 2008; Stemmer *et al*., 2009; Stemmer *et al*., 2006). Thus, highly standardized microarrays with reproducible performance and reasonable sensitivity are now available for toxicogenomic investigations of various biological models when standardized protocols are closely followed and the results are appropriately analyzed (Shi *et al*., 2008).

A toxicogenomic study in general has three major components (the biological model, the technological platform, and data analysis and interpretation) leading from application of a substance to a mechanistic explanation or hypothesis, to biomarker candidates, or to prediction of a potential toxicity. The choice of the biological model is crucial. It requires selection of an appropriate *in vitro* or *in vivo* model in relation to the issue to be investigated, rigorous standardization especially for *in vitro* models, and an optimized study design with respect to doses and time-points. In addition, it should allow correlating the expression profiles with conventional toxicological end-points or other emerging biomarkers which thus have to be measured in parallel. As described above, the technology is available, characterized by comparability of technical platforms when using data normalized to controls, by data quality and reproducibility, by highly standardized protocols, and by the possibility to validate the microarray results with independent methods (e.g. Q-PCR). After collection of all data, the challenge then lies in applying adequate analysis methods to identify truly deregulated genes versus noise or to reach a biological interpretation. Most challenging is still the use of such data for prediction of a certain mode of toxicity which is highly dependent on a well-populated reference database. These analysis issues will be dealt with the following paragraphs.

Toxicogenomics may be generally used in two areas of toxicology, that is, as mechanistic or predictive tool. Mechanistic toxicogenomics encompasses the assignment of functional categories to significantly deregulated genes in a biological model, and their relation to possible mechanisms of toxic action. This then leads to generation of mechanistic hypotheses, and may allow uncovering mechanistic similarities between different toxins. Predictive toxicogenomics, as just mentioned, relies on a database of expression profiles from samples represented by, for example, organs or cells following treatment with compounds of pre-defined toxic classes. Then marker genes are selected and classifiers are calculated by statistical or other algorithms to allow classification of unknown samples with respect to potential induction of these toxicity classes. These potential applications of toxicogenomics derive from considerations of the groups of genes likely deregulated following compound treatment (Figure 1): (i) if the compound is from research in a pharmaceutical company or otherwise has a specific receptor, genes related to the target of this compound will likely be changed, if the target is expressed in the tissue under investigation. These genes can help to increase the knowledge about the compound's pharmacodynamic action; (ii) if toxicity developed after compound treatment, genes related to the primary mechanism of toxicity will be deregulated. These can be employed for analysis of the affected pathways, to elucidate mechanisms of toxicity and thus to evaluate the human relevance of the toxicities observed in animal studies; (iii) genes related to the toxicity phenotype developing in response to primary injury will be found affected. These may be used as marker or signature genes for toxicity classification; and (iv) simply compound-specific genes with no specific relation to pharmacodynamics or toxicity have also to be expected. The genes likely best suited to serve as biomarkers for toxicity are those with a mechanistic basis of their deregulation.

Workflows for mechanistic and predictive toxicogenomics will be described in general. A mechanistic study starts with treatment of a biological

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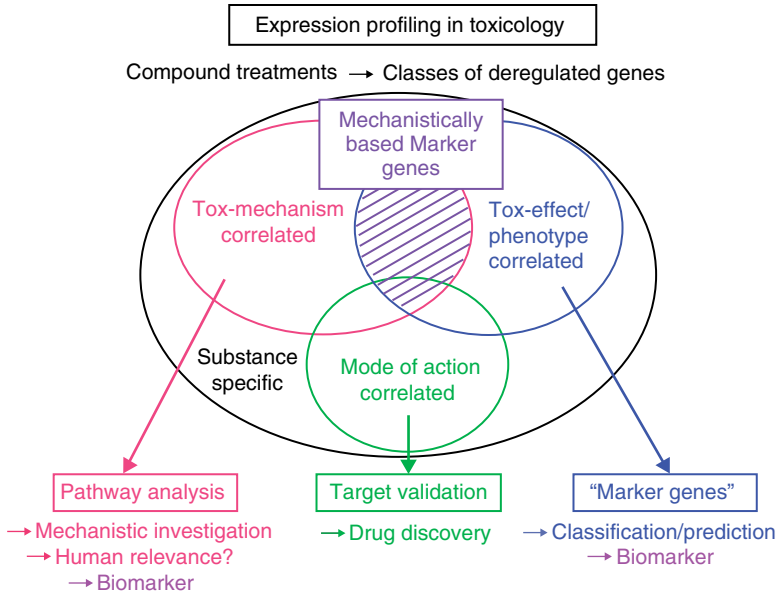

Figure 1. Expected classes of deregulated genes in toxicogenomics experiments.

model, for example, rats *in vivo*, with one or several compounds to be investigated and potentially model compounds, depending on the scientific question. Usually several time-points and/or doses are applied, with at least three, preferably five or more biological replicates per treatment group. In case of several time-points, time-matched controls treated with vehicles should be included. As mentioned above, classical end-points like clinical chemistry parameters and histopathological observations, and potentially other biomarker measurements should be planned for. Tissues to be processed for expression profiling should be frozen and stored appropriately. After obtaining the expression profiles on a certain platform, they should be subjected to rigorous quality control before being considered further.

The analysis (Figure 2) may begin with a principal components analysis (PCA) to obtain an overview of the data, for example, whether changes in gene expression can be seen upon treatment, whether these become altered during the duration of treatment, and whether there is variability between the replicates compared with differences between treatment groups. PCA is a classical means of dimensionality reduction and visualization of multivariate data. It involves a mathematical procedure that transforms a number of possibly correlated variables (here genes) into a smaller number of uncorrelated variables called principal components. The first principal component accounts for as much of the variability in the data as possible, and each succeeding component accounts for as much of the remaining variability as possible. Restriction to the first two or three principal components is often sufficient for capturing dominant structures of the data and uncovering important relationships between individual experiments or groups of experiments. Each sphere in a PCA represents the expression profile of one sample represented by values for the three first principal components, each corresponding to combinations of genes with similar expression patterns.

An important step is the selection of genes significantly deregulated between treated and control groups. The exact methods applied are dependent on the study design, the magnitude of the gene expression changes, and the variability between replicates estimated from, for example, a PCA. Suggested methods are parametric tests like *t*-tests between treated and control groups, or one-way analysis of variance (ANOVA) for the factors, such as dose and time. Non-parametric rank test may also be employed. These tests should be combined with an *n*-fold deregulation cutoff between the mean or median gene expression intensities of the treated and

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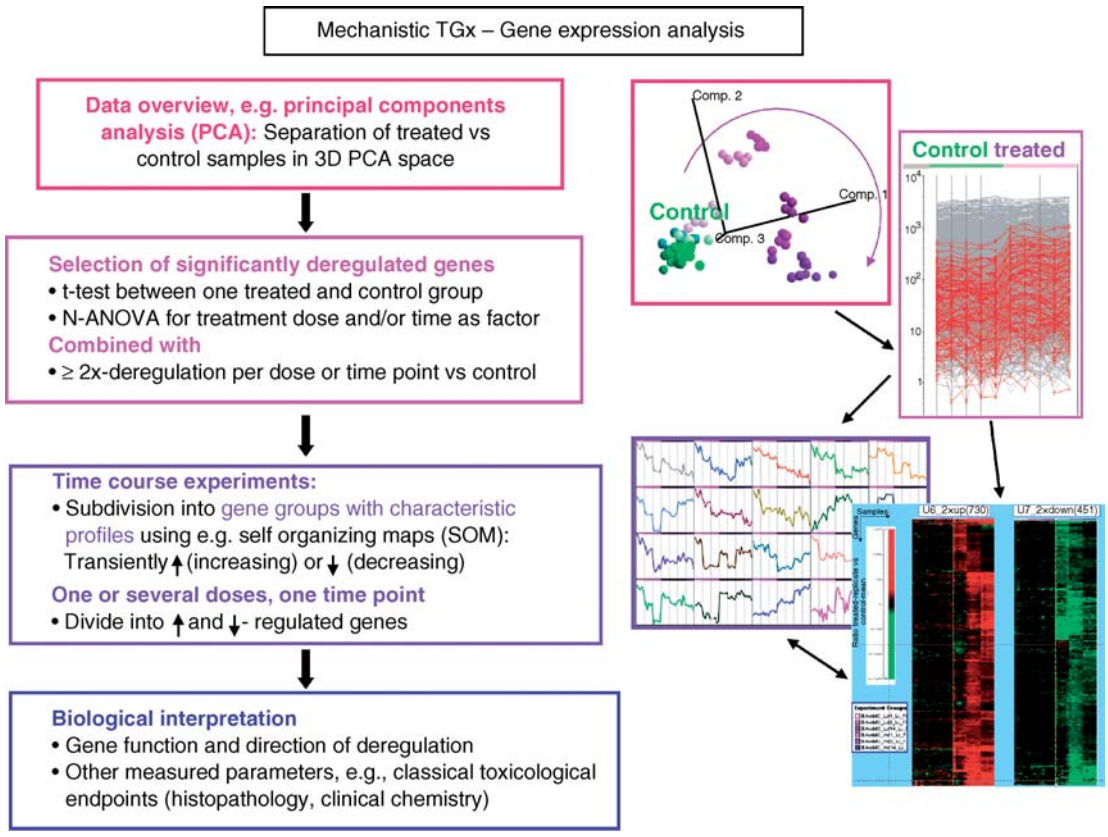

Figure 2. Gene expression analysis in mechanistic toxicogenomics.

control replicate groups (Shi *et al*., 2008). The cut-offs chosen for *P*- and *Q*-values should not be ≥ 0.01. Similarly, *n*-fold thresholds should be at least 1.5×, preferably 2×. Furthermore, depending on the microarray platform, low-level expressed genes may be excluded. One has to keep in mind that all these methods are approximations to the biology affected by a certain treatment and that by strict statistical criteria they may not be applicable because of relatively low number of replicates in comparison to the high number of variables (genes). Despite these limitations, experience has demonstrated that meaningful interpretations can be derived from carefully selected genes (Ellinger-Ziegelbauer *et al*., 2005; Blomme, Yang and Waring, 2009; Marin-Kuan *et al*., 2006).

To reach such interpretations from deregulated genes, it is advisable to subdivide them into groups with characteristic profiles in case of time–course experiments by applying clustering methods, such as self-organizing maps (SOM). If only one time-point is available, the genes should at least be subdivided into up- and down-regulated genes. Then, the main task is the interpretation of these genes in the context of their biological function within the organ or cell type under investigation, their direction of deregulation, information about their expression regulation, and observations from all other measured parameters. Pathway softwares and knowledge bases can provide help, but gene by gene interpretations in the context of the study specifics are likely required, using knowledge stored in public databases, such as Entrez Gene and Pubmed available at the Entrez website (http://www.ncbi.nlm.nih.gov/sites/gquery), or comparison with deregulation patterns available in experiments uploaded to public microarray databases like ArrayExpress, GEO or CEBS. Gene promoter analyses, if feasible, may also be applied. The result of a comprehensive interpretation of the deregulated genes can be a mechanistic hypothesis for an observed pathology induced by one or

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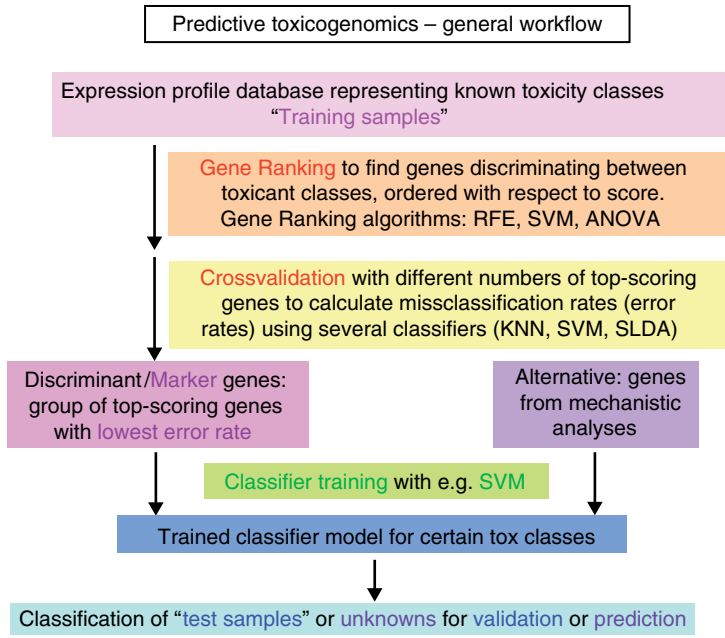

Figure 3. General workflow for predictive toxicogenomics.

a class of compounds, an estimation of the relevance of this pathology for humans, biomarker candidates characteristic of this pathology, or an idea for establishment of a screening assay to avoid this phenotype for further compound development.

Toxicogenomics may also be used in a "**predictive**" setting that aims to predict or classify a compound with respect to its potential toxicity or mode of action based on the expression profiles of a set of marker genes, also called gene signature. Reasons to engage in predictive toxicogenomics could be to have a tool for improved candidate selection with enhanced throughput, or to reduce the number of animal studies provided that the prediction allows replacing longer-term assays at least for compound ranking, or helps with Go/No Go decisions in compound development before longer-term animal studies are conducted. Before engaging in predictive toxicogenomics one should consider whether the classifier should be diagnostic or strictly predictive. The former would then allow classification of samples which already show the phenotype, which may be accomplished with organ gene expression profiles, but also with protein or metabolite signatures in non-invasive samples, whereas the latter would classify samples at lower dose or earlier than appearance of the toxicity to be predicted.

A general workflow to start a predictive toxicogenomic exercise is depicted on Figure 3. As introduced above, the core part is a well-populated database of expression profiles obtained from, for example, organs or cells after treatment of the respective biological model with compounds inducing the toxicity phenotype to be classified, and with a set of negative control compounds, representing the positive and negative compound class, respectively. More than one positive class may be defined (Kondo *et al*., 2009; Ellinger-Ziegelbauer *et al*., 2008; Natsoulis *et al*., 2005; Raghavan *et al*., 2005; Ruepp *et al*., 2005; Thomas *et al*., 2009). A prior mechanistic analysis of some of the model compounds can be helpful to characterize biological pathways participating in the toxicity to be classified, and to select optimal doses and timepoints. The profiles are then divided into a training set to be used for classifier calculation, and a test set to allow evaluation of the classifier for prediction accuracy parameters. One should bear in mind that a training class should represent at least 5–7 compounds, depending on the number of molecular mechanisms which can contribute to a certain toxicity phenotype. After definition of the positive and negative training classes, marker or signature genes may be selected by gene ranking with the goal to

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find genes discriminating between toxicant classes, ordered with respect to a score indicating the contribution of that gene to discrimination of positive and negative training profiles. A variety of methods can be employed for gene ranking, for example, simple statistic tests, such as *t*-tests or ANOVA, or algorithm like recursive feature elimination (RFE) or support vector machine (SVM). In general, too few genes, despite potential great discriminating power, will not be enough to separate different compound classes well because they may not represent all characteristic expression profiles, whereas too many genes will add noise to a set of clear profiles. Therefore, the optimal set of genes is derived from the score-ordered, that is, ranked gene list by internal cross-validation with different numbers of top-scoring genes to calculate miss-classification rates (error rates) using several classifiers, for example, SVM, K-nearest neighbor (KNN), or sparse linear discriminant analysis (SLDA), among others. During cross-validation, the data are repeatedly split into two parts: one part is used to train the classification algorithm, and the other part to test it. Thus, the internally created test profiles are back-classified, which through iteration enables the calculation of a miss-classification rate. The marker gene set to go forward is then that group of top-scoring genes with the lowest error rate.

For the following classifier calculation, either a gene set derived by gene ranking or a group of genes obtained from a mechanistic analysis may be used. Any appropriate algorithm, for example, those used for cross-validation, may be applied to derive a classifier model from the marker expression profiles of the positive and negative training classes. For evaluation, the initially retained test set is subjected to classification with the model obtained with the training set. As the toxicity class for the test samples is known, true and false-positive and false-negative prediction rates, overall prediction accuracy and other parameters can be calculated. If these quality measures are deemed appropriate, the classifier can then be used to classify the expression profiles of unknown samples. It is important to understand that such a classification only indicates a likelihood or potential for a certain toxicity and should therefore be used for compound ranking, or compound flagging in case of a clearly positive signal to guide further development watching out for the predicted toxicity phenotype.

2 ASSESSMENT OF CANCER RISK ASSOCIATED WITH EXPOSURE TO CHEMICALS

Chemical carcinogenesis is a multi-step process featuring alteration of genome integrity detected as pivotal gene mutations and chromosome damage, malignant transformation of cells, and ultimately development of cancer after exposure to chemical agents. Because of the association between DNA damage and cancer development, the pre-clinical safety evaluation paradigm for drugs and chemicals consists of assessing their genotoxicity, for example, their potential to cause DNA damage, and carcinogenicity, the latter comprising the ability to produce tumors in animals upon long-term exposure (Figure 4).

The standard genotoxicity testing battery (Muller *et al*., 1999) consists of (i) a bacterial gene mutation assay, (ii) an *in vitro* mammalian mutation and/or chromosome damage assay, and (iii) an *in vivo* chromosome damage assay. The test battery allows for relatively simple, accurate and economical hazard identification associated with exposure to chemicals, namely potential to cause DNA damage resulting in mutations at the gene and chromosome levels. The potential of chemicals to induce tumors (carcinogenicity) in animals and to extrapolate cancer risk to humans is performed using the chronic 2-year bioassay in rats and mice developed at the NCI in the early 1960s (Weisburger, 1983). The protocol typically requires a life-time, 2-year exposure of mice and rats to three concentrations of tested agent, plus controls (Chhabra *et al*., 1990). Recently, the cancer susceptible transgenic mice models, such as p-53+/−, Hras2 and Tg.AC have been evaluated and considered as an alternative approach for the mouse bioassay (Bucher and Portier, 2004; MacDonald, 2004).

Because of the mechanistic associations of DNA damage and cancer, and the practical limitations of carcinogenicity assays, genotoxicity results have been used as a surrogate for carcinogenicity data, and in case of pharmaceuticals, are required for initiation of clinical trials. Carcinogenicity assessment for compounds negative in genotoxicity testing is typically required for marketing approval of pharmaceuticals (Jacobs and Jacobson-Kram, 2004). Although the link between DNA damage and cancer is well documented, the potential to predict the

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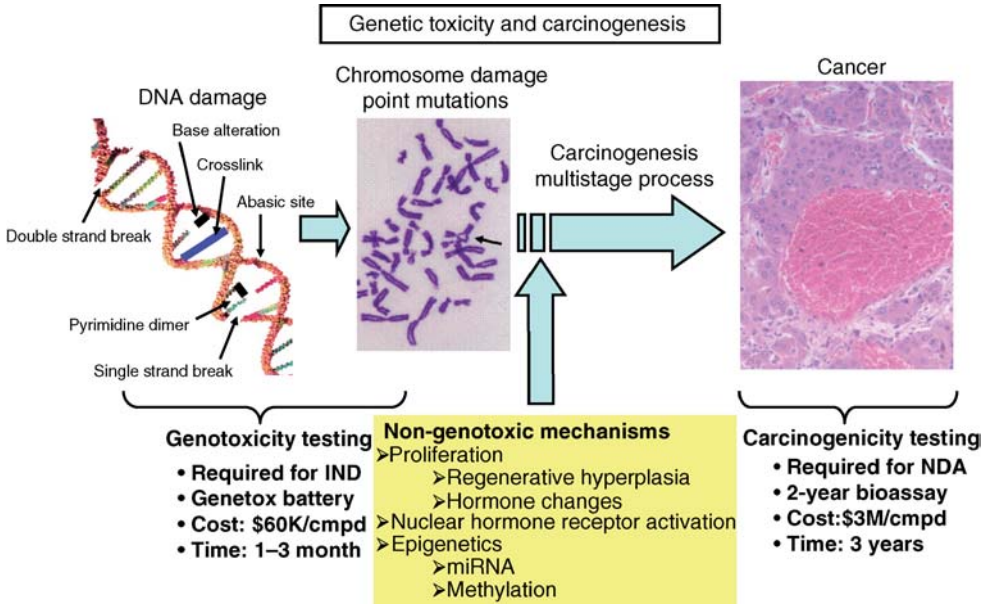

Figure 4. Genotoxicity and carcinogenicity testing. The standard genotoxicity testing allows for relatively simple and accurate detection of DNA damage caused by treatment with tested agents. However, carcinogenicity testing evaluates the potential to induce tumors in animals.

outcome of a carcinogenicity study using genotoxicity test data is rather imprecise. The most comprehensive analysis of genotoxicity and carcinogenesis data showed that the genotoxicity testing battery is highly sensitive for detection of carcinogens, detecting 93% of carcinogens. However, the testing paradigm features low specificity (Kirkland *et al*., 2006; Kirkland *et al*., 2005). This is supported by the fact that 50% non-carcinogens among marketed pharmaceuticals had some positive genotoxicity findings (Snyder and Green, 2001). The discrepancy is caused by limitations of genotoxicity end-points and assays, such as insufficiency of certain *in vitro* assays to model the *in vivo* target organ situation and the complexity of carcinogenic mechanisms. In case of carcinogenicity studies, the analysis of several databases showed that 50% of compounds tested positive in at least one species or sex (MacDonald, 2004; Hoffmann and Hartung, 2006). This high incidence and other research questioned the relevance for humans of tumors induced in rodents (Knight, Bailey and Balcombe, 2006; Ward, 2008). The International Life Sciences Institute (ILSI) Risk Science Institute (RSI) developed a framework to address this issue (Holsapple *et al*., 2006) which suggests that knowledge of key events and identification of a mode of action (MOA) in rodent bioassays provide a more rational basis for human cancer hazard and risk assessment. Therefore, the development of alternative short-term experimental approaches with better cancer-predictive potential at least partially including the investigation of MOA(s) is very important.

With respect to risk and relevance of positive findings of carcinogenicity studies, two basic mechanistically distinct subgroups called genotoxic and non-genotoxic carcinogens are discriminated (Bolt and Degen, 2004). In theory, a single mutation can initiate tumor development. Therefore, the risk associated with exposure to DNA-damaging compounds, genotoxic carcinogens, such as agents forming DNA adducts, is typically estimated using linear extrapolation methods. This implies that even low-exposure levels are associated with a certain cancer risk. In contrast, non-genotoxic carcinogens induce tumors via a variety of non-DNA-damaging mechanisms, such as affecting mitogenesis and regenerative processes, inhibition of apoptosis, modifying endocrine functions or immunosuppression (Williams, 2001). In this case, the cancer risk could be considered as non-linear, exhibiting a threshold dose. Thus, exposure levels that do not

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trigger the above processes might be considered as cancer risk-free.

The classification of genotoxic and non-genotoxic carcinogenic mechanisms is a crucial component of cancer risk assessment for chemicals and drugs. However, investigating carcinogenic mechanisms represents an experimental and scientific challenge. The genotoxicity testing battery provides critical information on the ability of compounds to cause DNA damage in mainly *in vitro* cell systems yet the relevance of some findings to cancer risk has been questioned (Kirkland *et al*., 2006). There is substantial evidence that some genotoxic chemicals may exhibit a thresholded dose–response curve in the *in vitro* systems (Henderson, Albertini and Aardema, 2000). Mechanisms producing thresholded responses in genotoxicity assays can be characterized as DNA non-reactive, including inhibition of enzymes involved in protein and DNA synthesis or DNA repair, inhibition of Na/K transport, inhibition of topoisomerases, and inhibition of processes leading to an imbalance of DNA precursors, energy depletion, production of reactive oxygen species, lipid peroxidation, and nuclease release from lysosomes (Scott *et al*., 1991). To differentiate a true threshold dose–response curve from reaching an assay detection limit, the understanding of underlying mechanisms is critical (Kirkland and Muller, 2000) Currently, the mechanistic investigation requires lengthy experimental follow-up strategies with uncertain outcome, which for instance lead to significant delays in the introduction of new medicines to patients. Frequently, the assessment of genotoxicity is complemented with a variety of other genotoxicity assays and end-points. For instance, DNA adduct breakage detection assays, such as the Comet assay and micronucleus tests in various cell and tissue types *in vitro* and *in vivo* provide a "weight of evidence approach" (Anonymous, 2004; Dearfield and Moore, 2005; Thybaud *et al*., 2007). The fact that only one-third of marketed drugs found to be positive or equivocal for carcinogenicity were also positive for genotoxicity indicates the existence of a considerable number of non-genotoxic carcinogens. Thus, understanding underlying mechanisms, that is, differentiation of DNA-reactive versus DNA-non-reactive mechanisms, is essential for critical risk assessment of positive findings of both genotoxicity and carcinogenicity studies (MacDonald, 2004; Jacobs and Jacobson-Kram, 2004). Furthermore, the overall toxicological profile of a given compound including additional *in vitro* and/or *in vivo* studies in combination with an evaluation of the weight of evidence might be necessary for both positive and negative compounds in the genotoxicity testing battery (Thybaud *et al*., 2007; Kasper *et al*., 2007). This concept is also recognized by the current FDA guidance for industry (Anonymous, 2004). However, until now, no adequate surrogate test model for evaluating non-genotoxic carcinogenicity is available.

Despite an array of established *in vitro* and *in vivo* genotoxicity testing methods, gaining insights into genotoxic mechanisms with respect to the oncogenic potential of the test compound *in vivo* is often difficult. As broad mechanisms-based approaches are not available the development of experimental approaches enabling mechanism-based risk assessment is extremely important. This is not only true for pharmaceuticals, but may comprise all kinds of chemicals as also fully recognized by the EU REACH initiative (Combes, Barratt and Balls, 2006). In addition, such approaches, even if some of them would still employ animals, would help to contribute to the 3R concept because they will enable faster and more educated evaluation of the genotoxic and carcinogenic potential of chemicals using fewer animals. Also, *in vitro* tests for genotoxicity producing less false-positive findings will reduce the necessity of follow-up in *in vivo* models. The 3R concept, which is concerned with the reduction, refinement and replacement of animal studies, has become the scientific basis of institutions serving the development of alternatives to animal experiments (Spielmann, 2003).

3 TOXICOGENOMIC ANALYSIS OF THE STRESS RESPONSE TO GENOTOXINS AND CARCINOGENS *IN VITRO*

The response to stress turns on a complex network of molecular interactions including alteration of mRNA levels of various genes leading to activation or repression of pathways involved in repair, survival and/or cell death. Treatment of bacterial, yeast and mammalian cells using ionizing radiation and prototypical DNA-damaging agents led to identification of DNA-damage inducible genes. Those genes included ones involved in DNA repair,

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cell growth arrest, cellular signaling and apoptosis. Gene expression-based approaches employing single promoter–reporter biosensors sensitive to DNA damage in prokaryotic and mammalian cells have been utilized since the pre-genomic era for detection of genotoxicity (Aubrecht and Caba, 2005). Furthermore, arrays of such biosensors have been developed and proven useful for investigation of molecular mechanisms of toxicity (Aubrecht *et al*., 1999; Todd *et al*., 1995). The stress response to genotoxicants in mammalian cells is quite complex and involves hundreds to possibly thousands of gene expression modulations (Amundson *et al*., 2005; Burns and El-Deiry, 2003). The wide availability of cDNA and/or oligonucleotide microarrays has enabled studying underlying molecular networks of genotoxic mechanisms on a genomic level (reviewed in Aubrecht and Caba, 2005). As a technology, microarray applications in toxicogenomics have matured, for example, the MAQC project shows inter- and intra-platform reproducibility of gene expression measurements (Shi *et al*., 2006).

Historically, ionizing radiation that features simple and accurate dosimetry without complex uptake and metabolism as is the case for toxicants, has been used as a model treatment for studying the genotoxic stress response at gene expression level (Amundson *et al*., 2001; Amundson, Do and Fornace, 1999; Fornace *et al*., 1999). The cellular response to radiation includes cell-cycle arrest and apoptosis both triggered via the p53 pathway, and activation of MAPK cascades, NF-κB and the AP-1 transcription complex (Amundson, Bittner and Fornace, 2003; Liu *et al*., 1996; Snyder and Morgan, 2004). Individual data sets (gene expression profiles) obviously share common features. Exposure of cells to ionizing radiation typically results in the alteration of mRNA levels of genes involved in cell cycle (CDKN1A, GADD45, Cyclin E) apoptosis (BAX, BCL-XL), DNA repair (XPC, DDB2, GADD45) and many others believed to be involved in intracellular signaling pathways and various physiological processes (FOS/JUN, MDM2, FRA-1 IL-8, HSP70) (Amundson, Bittner and Fornace, 2003; Snyder and Morgan, 2004). Although a majority of the experiments has been performed with relatively high doses of radiation, significant gene expression changes were also reported at very low levels of exposure *in vitro* (Amundson, Do and Fornace, 1999). Expression patterns consisting of stress-response genes similar to *ex vivo* experiments were reported in humans after total body irradiation procedures (Amundson *et al*., 2004).

Other prototypical agents have been tested as well. For instance, gene expression changes elicited by the well-characterized DNA-damaging compound, cisplatin, were clearly distinguishable from the gene expression profile of non-steroidal anti-inflammatory agents (Burczynski *et al*., 2000). Genomic analysis of human fibroblasts treated with ionizing radiation, UV radiation, and the oxidative stress inducer, *tert*-butyl hydroperoxide, at equitoxic doses was used to illustrate the ability of toxicogenomics to differentiate individual agents (Heinloth *et al*., 2003). Agent-specific gene expression profiles have both common and distinct features, with ionizing radiation appearing to be more closely related to oxidative stress than to UV radiation (Todd *et al*., 1995; Heinloth *et al*., 2003).

Differentiating between DNA-reactive and DNA-non-reactive mechanisms of genotoxicity provides a crucial component of managing risk of compounds with positive findings in genotoxicity assays (Kirkland and Muller, 2000). The application of emerging toxicogenomics approaches in genetic toxicology has been extensively reviewed (Aubrecht and Caba, 2005). There is increasing evidence suggesting that the toxicogenomic analysis of genotoxic stress response could differentiate DNA-reactive and DNA-non-reactive mechanisms of genotoxicity including cytotoxicity in TK6 (Amundson *et al*., 2005; Islaih *et al*., 2004) and L5178Y cells (Dickinson *et al*., 2004; Hu *et al*., 2004). One of the most comprehensive studies to date evaluated stress-specific gene expression profiles of 13 DNA-damaging and cytotoxic (non-DNA-damaging) agents in p53-proficient TK6 cells and in the p53-deficient isogenic cell line (Amundson *et al*., 2005). This study provided stress-specific gene expression profiles that included a total of 1451 responsive genes. DNA-damaging treatments featured a strong involvement of p53-dependent pathway whereas no apparent p53-dependent response was elicited by the cytotoxic stresses (Amundson *et al*., 2005). As in case of ionizing radiation, reported data display considerable amount of variability mainly caused by differences in cell systems and treatment protocols. Interestingly, the gene expression profiles showed only a modest alteration of a relatively small number of DNA repair genes by DNA-reactive treatments where the majority of inducible genes mediate regulatory processes involved in cell-cycle

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control, apoptosis, senescence, and inflammation. This is in line with the previous reports (Amundson and Fornace, 2003; Kastan and Bartek, 2004). A further extensive study similarly used TK6 cells to investigate the expression alterations after treatment with 14 anticancer drugs representing DNA-reactive and DNA-non-reactive *in vitro* genotoxins (Le Fevre *et al*., 2007). A molecular signature composed of 28 genes involved mainly in signal transduction and cell-cycle pathways allowed the classification of the drugs according to their mechanisms of action. Mitotic spindle inhibitors most clearly separated from the other compounds during expression profile clustering.

After treatment with DNA-reactive genotoxins, induction of typical p53-regulated genes, such as CDKN1A (p21) and GADD45A show an approximate linear dose response with no apparent threshold. In studies with cisplatin in a p53-deficient cell line, a linear relationship between the induction of gene expression of relevant genes (GADD45A, FOS) and DNA damage (adduct formation and DNA-protein cross-linking) was shown (Dickinson *et al*., 2004). When DNA adducts were measured and compared with gene expression changes following benzo[a]pyrene diol epoxide (BDPE) exposure of p53-proficient TK6 cells, DNA adducts are observed at lowest concentrations suggesting that assessing adduct formation is more sensitive at detecting genetic damage than measuring gene expression changes (Akerman *et al*., 2004). Although most stress responses are proportional to dose, unique responses at low doses have been demonstrated (Coleman *et al*., 2005).

Responses to DNA damage take place at the transcriptional, translational or molecular interaction level. As key components of the DNA repair machinery might be already present at the site of the DNA damage to counteract the effects of low-grade injury, it has been suggested that substantial DNA damage is required to invoke a global DNA damage response, which includes cell-cycle arrest, gene expression, etc. (Rouse and Jackson, 2002). This has to be taken into account with regard to experimental design, including dose setting and time-points. Biologically relevant stress genes that provide insights into the genotoxic mechanisms are immediate-early responding genes with robust changes in gene expression within several hours after treatment and prior to the onset of appreciable cytotoxicity (Amundson *et al*., 2005). Although it has been shown that transcriptional responses might be detected at sub-toxic levels, in order to avoid sensitivity issues of the analytical technology, selecting reasonable exposures that induce robust responses should be considered.

In addition to the mechanistic investigations of DNA-reactive vs. DNA-non-reactive genotoxins based on expression profiling *in vitro*, gene expression responses in cell lines to genotoxic versus non-genotoxic carcinogens have been reported. Preliminary results by Van Delft *et al*. on time-dependent gene expression deregulations upon exposure of HepG2 cells to benzo(a)pyrene, a genotoxic carcinogen, demonstrated up-regulation of phase I-metabolizing enzymes (CYP 1A1, CYP 1B1) within hours after exposure whereas responses by cell-cycling controlling genes (p53-responsive GADD45, CDKN1A) causing growth arrest, became apparent at 12–18 h after B(a)P challenging. Molecular pathways, capable of discriminating between genotoxic and non-genotoxic carcinogens in this *in vitro* model refer to apoptosis (van Delft *et al*., 2005) whereas the carcinogenic potential of polycyclic aromatic hydrocarbons (PAHs) appears related to their capability of modulating cholesterol biosynthesis and fatty acid synthesis, additionally to apoptosis (Staal *et al*., 2006) indicating considerable specificity of genomic responses because discrimination of carcinogens belonging to the same chemical class appeared feasible.

A further study used the rat hepatoma cell line MH1C1 to investigate carcinogens *in vitro*. When treated with the chemicals for 3 days at a non-toxic dose, analysis of gene expression changes allowed a set of genes to be identified differentiating carcinogens from non-carcinogens, encompassing 26 genes (Tsujimura *et al*., 2006). Gene clusters contained metabolism-related genes encoding transferases, oxidoreductases, hydrolases, and lyases as examples. Also, many genes that encode DNA-binding proteins related to translation and transcription were selected, and regulation of cell proliferation and signaling pathways appeared affected.

4 TOXICOGENOMICS ANALYSIS OF CARCINOGENIC MECHANISMS *IN VIVO*

The pathogenesis of neoplasia is a multistage process which consists of at least three operationally

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defined stages beginning with initiation followed by a promotion and then a progression phase (Oberemm *et al*., 2009). Its full complexity can only be studied in an *in vivo* model representing all aspects of exposure and target organ biology. The fact that the gene expression profile analysis provides only a snapshot of molecular processes at the time the sample was collected has implications on experimental design in regard of time-points of analysis and dose. Recently, investigation of the gene expression profile over a 50-week time course after treatment of rats for 7 weeks with *N*-nitroso-morpholine, a genotoxic hepatocarcinogen, showed, that during the first 8 weeks the gene expression profile in the liver was characteristic of genotoxic carcinogens (Oberemm *et al*., 2009). Later on, very few genes were found to be deregulated in the non-dissected liver, which likely represent genes very strongly deregulated in the progressing cell clones. This suggests that an obvious time-point to measure early effects of different carcinogen classes in a whole tissue, by, for example, gene expression profiling, would be the time directly after start of exposure to a carcinogen, because at the earlier times the molecular responses to a particular compound may be similar in all cells, whereas later on the cells may respond differently because of having acquired different mutations, or because of the absence of any adverse mutations. With defined carcinogens applied at doses which later on should induce tumors, such an investigation may allow to define a set of molecular pathways, that when affected early on in a certain constellation may later lead to tumor development, permitting the prediction of a long-term end-point.

With regard to experimental design, evaluating dose–response relationships using one or few compounds may be seen as "vertical" investigations. However, examinations dealing with many compounds of a certain toxicant class dosed mainly at one level could be seen as "horizontal" ones. The former represent a fundamental concept in toxicology required in the later phase of risk assessment, whereas the latter, searching for mechanistic similarities between compounds inducing the same toxicity may be very helpful for hazard identification and characterization covering different mechanisms. The vast majority of the published studies so far could be described as horizontal investigations aiming for mechanistic interpretations.

The suitability of toxicogenomic analysis for investigating molecular mechanisms of carcinogenicity after application of carcinogens *in vivo* has been explored only recently. As the liver is considered a major target organ for rodent carcinogenicity, the published studies have focused mainly on investigating early responses to carcinogen and/or non-carcinogen exposure in the rat liver. Nakayama *et al*. (2006) treated rats for 28 days with four genotoxic rodent liver carcinogens and with their non-carcinogenic isomers, and found distinctive gene expression deregulations in the liver with the former, but not the latter. Kramer *et al*. (2004) treated male rats for 5 days at three dose levels with two genotoxic and five non-genotoxic rodent hepatocarcinogens, and two non-carcinogens and analyzed the liver expression profiles on cDNA arrays. They found that down-regulation of transforming growth factor-β-stimulated clone 22 (TSC-22) and up-regulation of NAD(P)H cytochrome P450 oxidoreductase (CYP-R) CYP-R correlated well with the estimated carcinogenic potential, which was based on the dose used in context with the previously reported carcinogenic potency. Yet only TSC-22 deregulation could be confirmed with Q-PCR. To unveil molecular mechanisms of non-genotoxic carcinogenesis, Nie *et al*. (2006) selected genes differentially expressed between livers of rats after 1-day treatment with 24 non-genotoxic carcinogens and 28 non-carcinogens. Half of the deregulated genes could be organized into five networks, all of which were linked to the proto-oncogene c-myc, whose activation by mutation or over-expression is known to initiate or promote tumor progression. The expression profiles of this study were also employed to select a gene signature for prediction of other profiles. In an even more extensive study, Fielden *et al*. derived a multigene biomarker for non-genotoxic hepatocarcinogens after treatment of rats with 100 non-genotoxic hepatocarcinogens and non-hepatocarcinogens. An understanding of potential modes of action for non-genotoxic hepatocarcinogenicity was possible by closer examination of this multigene marker profile in the context of hepatic tumorigens of known mechanisms (Fielden, Brennan and Gollub, 2007).

With respect to extrahepatic tissues, a combined transcriptomics and metabonomic analysis after treatment of mice for 13 weeks with a genotoxic and a non-genotoxic liver and lung carcinogen, two non-carcinogens and two vehicle

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controls identified biomarkers which could discriminate between carcinogenic and non-carcinogenic treatments (Thomas *et al*., 2007) An extension of this investigation with further lung carcinogens and non-carcinogenic controls identified mainly genes involved in oxidative stress and/or detoxification responses as discriminating for lung carcinogens after 13 weeks of treatment (Thomas *et al*., 2009). Short-term effects of two renal carcinogens on the gene expression profiles in the rat kidney were examined in wild-type rats and Eker rats heterozygous for a dominant germline mutation in the tuberous sclerosis 2 (Tsc2) tumor suppressor gene after treatment with the genotoxic carcinogen aristolochic acid (AA) or the non-genotoxic carcinogen ochratoxin A (OTA) for up to 14 days (Stemmer *et al*., 2007). The response to AA was similar in both rat strains, representing as a DNA damage response and up-regulation of certain phase I and II genes. In contrast, OTA deregulated more genes in Eker than in wild-type rats, indicating a clearer demarcation of affected pathways, including oxidative stress responses and both pro- and anti-proliferative responses. Gene deregulations pointing to cell-structure remodeling and fibrotic changes were strongly affected in Eker, but essentially not affected in wild-type rats. With respect to different carcinogen classes, this latter study in rat kidney with just two carcinogens supported findings in the rat liver.

Ellinger-Ziegelbauer *et al*. (2005) reported about an effort to derive gene expression profiles that could discriminate between genotoxic and non-genotoxic carcinogens based on several members of each class. A functional analysis of the genes deregulated after short-term treatment of rats with genotoxic and non-genotoxic hepatocarcinogen revealed distinct gene expression profiles for the two classes of carcinogens. After short-term treatment of rats for up to 14 days with doses reported to induce liver tumors and which did induce other histopathological changes as expected for these compounds, the liver expression profiles were measured on RGU_34A microarrays (array express accession no. E-TOXM-16). Significantly deregulated genes were first extracted on a per compound basis and then functionally categorized with respect to carcinogen class (Ellinger-Ziegelbauer *et al*., 2005). Interpretation of these data suggested that DNA damage response and survival/proliferation signaling seems to be specific for genotoxic carcinogens, whereas responses to oxidative DNA or protein damage, or to general oxidative stress, and regeneration and cell-cycle progression are characteristic features of non-genotoxic carcinogens. The expression profiles of two of the characteristic gene groups clearly show (Figure 5) that a DNA damage response represented by the up-regulation of p53 target genes (p21, CCNG1, and possibly MGMT) is a response most strongly induced by the genotoxic carcinogens which is in line with their potential to cause direct DNA damage. The weaker up-regulation of these genes by the non-genotoxic carcinogens may be due to oxidative stress induction, because other oxidative stress responsive genes were also up-regulated by these compounds (Ellinger-Ziegelbauer *et al*., 2005), and because p53 can be activated by both, DNA damage and oxidative stress. In addition, this could also indicate secondary DNA damage upon ROS formation after treatment with non-genotoxic carcinogens, potentially indicated by the increased expression of apurinic/apyrimidinic endonuclease 1 APEX1 (Figure 5). The increased expression of genes involved in cell-cycle progression appears to be characteristic for the non-genotoxic carcinogens.

Thus, an in-depth mechanistic analysis revealed that characteristic expression signals do exist in rat liver which in short-term studies, may differentiate genotoxic from non-genotoxic carcinogens and which are based on well-defined functional gene categories that can be related to the generally accepted model of tumorigenesis, including initiation and promotion. For the former, a strong DNA damage response at the gene expression level suggested direct DNA modification in case of the genotoxic carcinogens, whereas gene expression responses to oxidative stress of different origin indicated possible secondary oxidative DNA damage in case of the non-genotoxic carcinogens. Early promotion may be assisted by enhanced expression of genes mediating survival or proliferation signaling by the genotoxic carcinogens, and by transient or delayed induction of genes involved in cell-cycle progression by the non-genotoxic carcinogens. This analysis also revealed that neither a single gene nor a single category will be sufficient for discrimination of genotoxic versus non-genotoxic carcinogens. Therefore, a systems biological approach may be necessary to analyze these complex data sets in a concise and integrated manner with emphasis on the pathways involved.

In a following study, these authors expanded the number of compounds and used supervised

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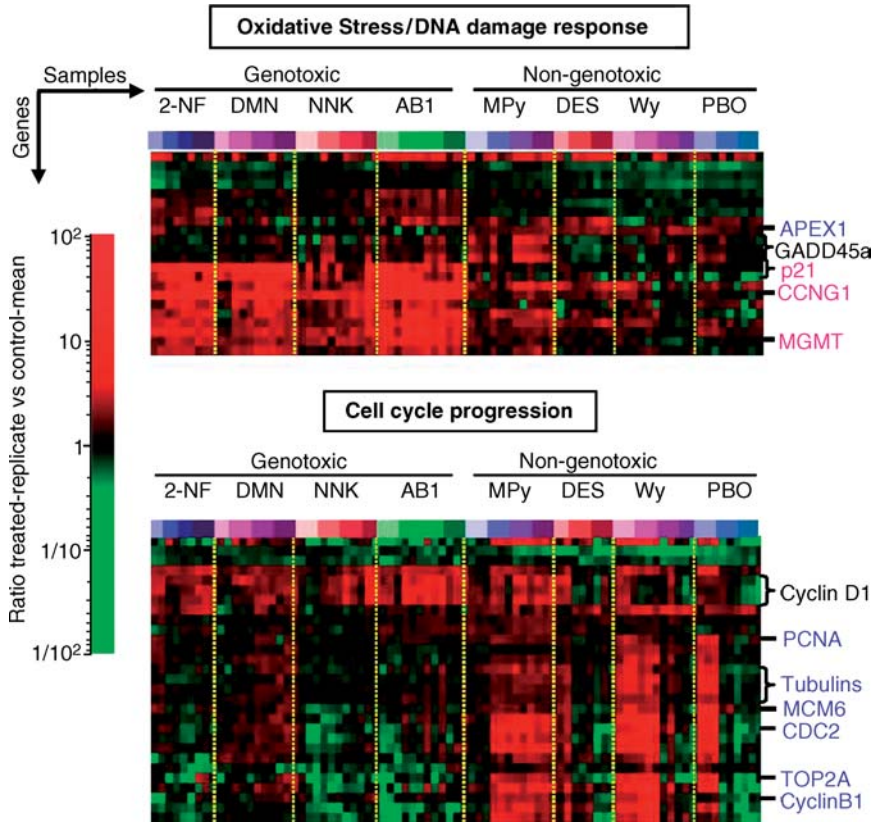

Figure 5. Deregulation of characteristic functional gene groups by genotoxic and non-genotoxic hepatocarcinogens. Two subgroups of the genes, representing an oxidative stress/DNA damage response and cell-cycle progression, originally selected to be significantly deregulated after treatment of rats with at least one of the carcinogens analyzed (indicated above each heatmap) were subjected to one-dimensional cluster analysis using Euclidian as distance metric. The heat maps show ratios of gene expression calculated by dividing the expression values in the single-treated replicate samples through the mean expression value of all corresponding time-matched control replicates. Red illustrates up-regulation and green represents down-regulation versus the control, as indicated by the color scale on the left. The color codes directly above the heat maps designate the different time-points per compound, representing d1, d3, and d7 for DES and PBO, and d1, d3, d7, and d14 for all other compounds. Shown on the right are examples of genes deregulated by genotoxic carcinogens (pink), by non-genotoxic carcinogens (blue), or by both types of carcinogens (black). APEX1, apurinic/apyrimidinic endonuclease 1; GADD45a, growth arrest and DNA-damage-inducible protein alpha; CCNG1, yclin G1; MGMT, O6-methylguanine-DNA methyltranferase; PCNA, proliferating cell nuclear antigen; MCM6, mini chromosome maintenance deficient 6; CDC2, cell division cycle 2 protein kinase; 2-NF, 2-nitrofluorene; DMN, dimethylnitrosamine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; AB1, aflatoxin B1; MPy, methapyrilene HCl; DES, diethylstilbestrol; Wy, Wy-14643; PBO, piperonylbutoxide.

learning algorithms to extract biomarkers discriminating genotoxic from non-genotoxic carcinogens and from non-carcinogens. These biomarkers were then used to classify further compounds with reasonable accuracy, thus strengthening the concept that toxicogenomics may also be used to predict a potential for hepatocarcinogenicity from short-term studies (Ellinger-Ziegelbauer *et al*., 2008).

Most of the studies mentioned above were "horizontal" investigations using several compounds at one dose level. Only few reports have been published with respect to evaluating dose–response relationships of gene expression changes induced by carcinogens. For instance, the gene expression profiles were correlated with the carcinogenic potency of a compound derived from the incidences of animals with liver tumors at a specific dose level, as known from the original 2-year bioassay (Kramer. *et al*., 2004). This aided in identification of correlating gene expression profiles. In another more detailed study, a method was established which allowed to calculate mean benchmark doses (BMD)

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for individual cellular processes as revealed by the functions of the genes deregulated at certain dose levels (Thomas *et al*., 2007). The analysis was based on gene expression changes in the rat nasal epithelium flowing acute exposure to four concentrations of formaldehyde for 6 h, in a range previously shown to induce tumors. The described method could potentially aid in applying information from gene expression profile changes to risk assessment by revealing the molecular changes associated with chemical exposure and providing a possibility to identify reference doses at which particular cellular processes are altered.

5 CONCLUSIONS

The recent progress in technologies and molecular sciences has enabled interrogating complex networks of cellular pathways on genomic level in response to treatment with chemicals. The promise of toxicogenomics is to provide insights into the molecular action of different classes of toxicants via analyzing gene expression profiles. In this review, we focus on evaluating the potential of emerging toxicogenomic science to evaluate mechanisms of genotoxicity and carcinogenicity *in vitro* and *in vivo* test systems. Furthermore, we provide future directions and perspective on applicability of such approaches in hazard identification and risk assessment paradigms.

Although the published datasets *in vitro* and *in vivo* exhibit significant variability, common features emerge in respect of molecular pathways suggesting the need for a system toxicology approach for data analysis. The DNA damage-responsive p53 pathway is extensively activated both by DNA-reactive genotoxicants *in vitro* and genotoxic carcinogens *in vivo*, confirming and extending the molecular knowledge of what has been known before (Figure 6). The ability to detect triggering of relevant biological pathways in response to DNA damage in the *in vitro* assay systems and in animals *in vivo* provides opportunity for development of translational biomarkers for genotoxic carcinogenicity including examining relevant target tissues. With respect to other compound classes, called *in vitro*-defined DNA-non-reactive genotoxicants and *in vivo* non-genotoxic carcinogens, the published studies have shown the involvement of generic pathways in their mechanisms of action, such as general stress response, signaling and cell-cycle genes *in vitro*, or various oxidative stress response and cell-cycle genes *in vivo*. Yet given the current state of knowledge, there appears no direct *in vitro–in vivo* relationship as it seems to exist for the DNA-reactive genotoxicants and genotoxic carcinogens. Nevertheless, the data represent a first proof of concept that a distinction of DNA-reactive versus DNA-non-reactive genotoxins *in vitro* and genotoxic versus non-genotoxic carcinogens *in vivo* may be feasible via toxicogenomics.

Although promising, more work needs to be carried out in order to fully realize the potential and value of toxicogenomic analysis in genotoxicity and carcinogenicity testing. In order to apply toxicogenomics widely for differentiation of genotoxic and carcinogenic mechanisms, it will require evaluating large sets of agents representing a broad range of toxic effects. These investigations will help to better define carcinogen classes, and potentially subdivide especially the non-genotoxic carcinogen class into several subclasses based on mechanistic insight with clearer relation to the DNA-non-reactive genotoxicants. The fact that the gene expression profile analysis provides only a snapshot of molecular processes at the time the sample was collected appropriate study designs have to be developed for studies comparing large sets of compounds across mechanistic classes. The further development of bioinformatic and statistical methods will be necessary to refine pathways distinguishing DNA-reactive from DNA-non-reactive genotoxins *in vitro* and genotoxic from non-genotoxic carcinogens *in vivo*. As the *in vivo* studies were largely limited to liver, investigating other target organs for carcinogenicity including potential target cell populations from which the tumor(s) develop is important. Furthermore, the potential of other 'omics' technologies to explore transcriptional regulations (epigenetics, micro-RNA) as well as more downstream events (proteomics, metabolomics) for evaluating mechanisms of genotoxicity and carcinogenicity should be fully captured. Finally, in order to explore the potential of toxicogenomic analysis to provide translatable biomarkers of exposure and suitable for risk assessment, there is an obvious data gap in regard of dose–response and time–course relationships, which needs to be addressed.

Despite the limitations and data gaps, the published data clearly indicate the potential practical application of toxicogenomics as a tool for

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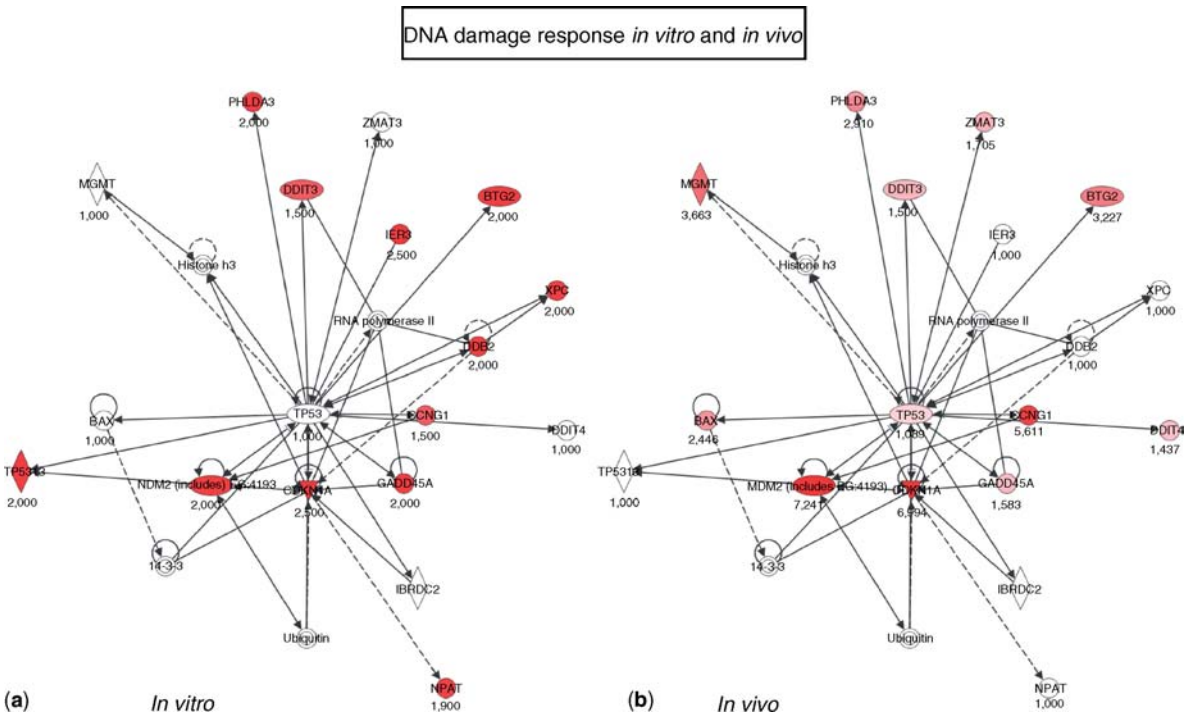

Figure 6. Common network of genes responsive to treatment with DNA-reactive agents *in vitro* (a) and genotoxic carcinogens *in vivo* (b). A network (Ingenuity pathway software) was generated from common DNA damage-responsive genes in the *in vitro* studies with DNA-damaging treatments (a) and in the rat livers after treatment with genotoxic hepatocarcinogens *in vivo*. The grey to black color is a qualitative indication of the extent of up-regulation observed on average in the *in vitro* (left) and *in vivo* (right) system. The *in vitro* data are an estimated qualitative mean of the expression data reported for human p53 wild-type cell lines (TK6, NH32) in response to genotoxic agents (Amundson *et al*., 2005; Islaih *et al*., 2004; Akerman *et al*., 2004) summarized in Aubrecht and Caba (2005). When induction was reported in all papers with deregulation factors >2, the induction factor for this qualitative figure was set at 2.5, when clear induction was reported in all papers, it was set to 2, and when induction was reported in some papers it was set to 1.5. The *in vivo* data are average gene expression deregulations for five genotoxic hepatocarcinogens as reported in the supplemental table of Ellinger-Ziegelbauer *et al*. (2008).

providing mechanistic context to results obtained with both genotoxicity and carcinogenicity assays. For instance, application of toxicogenomics to exclude DNA-reactive mechanisms for compounds positive in the *in vitro* chromosome damage assays would greatly facilitate risk assessment of these findings. This approach could be applied in the pre-clinical R&D phase of developing novel chemical entities for discriminating hazardous carcinogens. Moreover, gene expression profiling *in vivo* after short-term treatment has potential to further increase the confidence in DNA-non-reactive mechanisms for compounds with positive genotoxicity and/or carcinogenicity findings because in this model, actual responses to a compound in the target organ could be measured. As in most cases, compounds applied are pro-carcinogens which must be metabolized into an ultimate carcinogen *in vivo*, and whose metabolism cannot easily be reproduced *in vitro*, investigations of compound-dependent gene expression deregulations in several potential, metabolically highly active target organs may reveal whether the compound is metabolized to a genotoxic carcinogen in a particular organ, or even through the integrative action by several organs.

Toxicogenomics has a potential to significantly improve cancer risk assessment for drugs and environmental chemicals. The development of toxicogenomic approaches is challenging involving substantial resources and time. It requires designing of appropriate testing protocols and validation. Therefore, collaborative efforts that include scientists from academia, industry and regulatory agencies, such as the ILSI HESI Genomics

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Committee, the Critical Path Initiative in the US or the Innovative Medicines Initiative in Europe are essential for developing experimental protocols and testing paradigms.

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Effect of p53 Genotype on Gene Expression and DNA Adducts in ENU-Exposed Mice

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1 INTRODUCTION

The potent mutagen, *N*-ethyl-N-nitrosourea (ENU), is a direct-acting alkylating agent with a well-characterized DNA adduct profile. ENU binds to nucleophilic oxygen atoms found in nucleotides, including O^6-guanine, O^2- and O^4-thymine, through an S_N1 mechanism. O^6-Ethyldeoxyguanosine (O^6-Et-dG) is formed at the highest frequency of the oxygen adducts and the mutagenicity and toxicity of this adduct are well characterized. O^6-Et-dG adducts, as well as O^4-ethyldeoxythymidine and O^2-ethyldeoxythymidine, result in base mispairing during DNA synthesis and the induction of transition mutations as determined by sequence analysis of ENU-induced mutants (Beranek, 1990).

O^6-Et-dG adducts are primarily repaired by the protein, O^6-methylguanine methyltransferase (Mgmt), that is encoded by the *Mgmt* gene in mice (Gerson, 2004). The ethyl group is transferred from the guanine of DNA to a cysteine residue on Mgmt, which then undergoes suicide inactivation. The results of both *in vivo* and *in vitro* experiments have led to the suggestion that the expression of *Mgmt* is under the regulatory control of p53 (Grombacher, Eichhorn and Kaina, 1998; Rafferty *et al*., 1996), which is encoded by the *Trp53* gene in mice. When mice lacking a functional *Trp53* gene were exposed to ionizing radiation (IR), which also induces *Mgmt* expression, hepatic *Mgmt* gene expression was not induced (Rafferty *et al*., 1996). In subsequent experiments with mouse fibroblasts and rat hepatoma cell lines, those lines lacking wild-type *p53* were unable to induce *Mgmt* expression (Grombacher, Eichhorn and Kaina, 1998). Furthermore, when *p53*-deficient mice, in which *Mgmt* was over-expressed, were exposed to *N*-methyl-*N*-nitrosourea (MNU), they were less susceptible to the tumourigenic properties of MNU than were p53-deficient mice (Reese, Allay and Gerson, 2001).

Although the role of Mgmt in the repair of O^6-alkylguanine is well documented, the expression kinetics of *Trp53*, *Mgmt* and other genes in the alkylation damage and repair pathways have

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not been fully addressed. Thus, to provide additional insight into the relationship between DNA alkylation and genes regulated by p53, we conducted an *in vivo* kinetics study utilizing the p53 mutant mouse model (Donehower *et al*., 1992). $P53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$ TSG*p*53® mutant mice were exposed to ENU and sacrificed at 4 and 24 h after exposure. The livers were harvested for gene expression and DNA adduct analysis. The liver was selected because of the well-characterized mutagenic and carcinogenic effects of O^6-alkylguanine in that tissue (Major and Collier, 1998). O^6-Et-dG levels, which served as a biological dosimeter, were determined by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (HPLC-ES-MS/MS). Expression levels of 84 p53-related genes, representative of the pathways under p53 control, were determined by the use of a quantitative PCR (qPCR) RT^2 p53-pathway targeted array® (SABiosciences, Frederick, MD, USA). As *Mgmt* was not present on the array, the levels of this gene were determined by hydrolysis probe Taqman® qPCR (Applied Biosystems Foster City, CA).

The levels of $p21^{(waf1)}$ expression were measured by Taqman® probes to confirm RT^2 array accuracy. In addition to determining changes in expression of individual genes, an analysis that focused on significant changes in the expression of defined gene clusters, grouped according to their cellular function in relation to *p*53, was performed.

2 MATERIALS AND METHODS

2.1 Animals and Animal Care

Male mice having $p53^{+/+}$*(p53N5-W)*, $p53^{+/-}$ (TSG*p53*®N5-T) and $p53^{-/-}$ (TSG*p53*®N5-N) genotypes were obtained from Taconic Farms (Germantown, NY, USA) at 4–6 weeks of age. The animals underwent quarantine and an acclimation period at the NCTR Animal Care Facility before the initiation of the experiment, at which time the animals ranged in age from 8 to 9 weeks. All mice were housed two per cage in standard polycarbonate mouse cages with bonnets and hardwood chip bedding. Millipore-filtered tap water and chow (NIH-31, Purina Rodent Chow, pellets) were provided ad libitum. The animal room was maintained at a temperature of $23 \pm 3\,^{\circ}C$, a relative humidity of $50 \pm 20\%$ and a 12-h light/dark cycle. The animal room received 10–15 air changes per hour. All procedures were approved by the NCTR Institutional Animal Care and Use Committee.

2.2 Chemical Exposure

N-Ethyl-N-nitrosourea (Sigma Aldrich Inc., St. Louis, Mo, purity > 99.1%) was diluted in phosphate-buffered saline (PBS; pH 6.0) such that each animal received a single dose of 150 mg kg^{-1} in no more than 0.1 ml of diluent interperitoneally (i.p.). Control animals were injected i.p. with 0.1 ml of PBS. Six mice from each p53 genotype were injected with 150 mg kg^{-1} of ENU, and six control mice from each genotype were injected with PBS. At 4 and 24 h after injection, three mice from each genotype from the ENU-exposed group and three mice from each genotype from the control group were sacrificed by CO_2 euthanasia. One animal from the wild-type group treated with ENU died after the chemical administration at 4 h and was subsequently removed from the analysis. The median lobe of the liver was placed in RNA*later* (Ambion, Austin, TX, USA) for RNA extraction; the remaining lobes of the liver were flash frozen in RNALater and stored at $-80\,^{\circ}C$ for subsequent analysis.

2.3 RNA Isolation and Reverse Transcription

Qiagen AllPrep DNA/RNA mini kits (Qiagen, Valencia, CA, USA) were used to extract total RNA from liver tissues stored in RNA*later* (Ambion). Approximately 30 mg tissue stored in RNA*later*, Ashville, NC solution was minced and homogenized by using a FastPrep FP120 homogenizer (Thermo, Savant, Ashville, NC) for 40 s in 600 μl lysis buffer (RLT buffer and 1% β-mercaptoethanol) in a lysing matrix D tube (MP Biomedical, Santa Ana, CA). The liver samples were kept on ice before and after the homogenization step. The homogenates were transferred to clean 1.5 ml tubes and centrifuged at 10 000 *g* for 3 min, and the supernatants were removed and placed in a second clean tube. An equal volume of 50% ethanol was added to the supernatants and mixed by pipetting. Total RNA and DNA were isolated according to the AllPrep kit (Qiagen) instructions and included treatment of the RNA with DNase I (Qiagen). From each sample,

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1.0 μg of RNA was reverse transcribed with random hexamer primers using the high-capacity cDNA Reverse Transcription Kit™ (Applied Biosystems) and the cDNAs produced were used without further purification as a template for qPCR.

2.4 Gene Expression Analysis

Gene expression profiles were constructed using the RT^2 profiler PCR array® mouse p53 signalling pathway (SABiosciences, Frederick, MD, USA). The reactions were performed using the Applied Biosystems 7500 Fast-Real Time PCR System. The results were analysed with the 7500 Fast System SDS Software. The $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001) was used to calculate the normalized C_t values:

$$2^{-(\text{GOIexp}-\text{RGexp})-(\text{GOIcntr}-\text{RGcntr})}$$

where GOI represents the C_t value for the gene of interest, RG represents the reference gene C_t value, exp represents the experimental ENU-treated animal and cntr represents the PBS-treated control animals.

2.5 Hydrolysis Probe qPCR

The levels of *Mgmt* and *p21^{Waf1}* mRNA transcripts in the livers of both the control and ENU-exposed mice were determined by real-time quantitative PCR. The qPCR utilized Taqman® primer and probes (Applied Biosystems) designed to detect *Mgmt* and *p21^{Waf1}* mRNA along with *Actb* and *Gapdh* internal reference gene mRNA. Reactions were performed in duplicate using a 7500 Fast-Real Time PCR System (Applied Biosystems). The cDNA samples were from the same cDNA reactions described above and used in the RT^2 SuperArray reactions.

2.6 DNA Adduct Analysis

Hepatic nuclei were prepared from the median lobe of the mouse livers. DNA was isolated from the nuclei, and the hepatic levels of O^6-et-dG were assessed by HPLC-ES-MS/MS (Churchwell, Beland FA and Doerge, 2006).

2.7 Statistical Methods

The gene set enrichment analysis (GSEA) statistical procedure was used to determine functionally related sets of genes that were expressed at different levels (enrichment and/or depletion) when compared among experimental groups (Mootha *et al*., 2003). The common approach to the GSEA is to generate a list of differentially expressed genes and then compare the number of genes in the list with the total number of genes in the set to determine the significance for an over-representation in the gene set (Draghici *et al*., 2003). A two-sided multivariate analysis of variance (MANOVA) method (Tsai and Chen, 2009) was used to identify which pathways were differentially expressed among the treatment groups, genotypes and two time points because the MANOVA method was found to perform better than the existing gene set analysis methods. In addition to gene set analysis, we also used two-way ANOVA with an interaction term for each gene to detect the main effect and interaction between time and genotypes. Changes in hepatic O^6-et-dG levels were assessed by ANOVA, using time and genotype as fixed factors.

3 RESULTS

3.1 RNA Quality Control

The purity and yield of the extracted total RNA and DNA for the adduct analysis were determined by measuring the absorbance at 260 and 280 nm using a NanoDrop (ThermoFisher, Waltham, MA), and the RNA integrity number (RIN) was determined with a Bioanalyzer 2100 (Agilent, Santa Clara, CA). RNA samples used in these experiments exhibited the ratio of 260/280 > 1.8 and RIN values ranging from 7.0 to 9.5, which indicated minimal degradation of the RNA.

3.2 O^6-Et-dG Levels

O^6-Et-dG was detected in DNA from the livers of each of the treated mice (Figure 1). At 4 h after exposure, the level of O^6-et-dG was not significantly different among the three genotypes of ENU-treated mice. At 24 h, O^6-et-dG levels were significantly reduced from those detected at 4 h in the livers of ENU-treated mice with the *p53$^{+/+}$* ($P < 0.05$)

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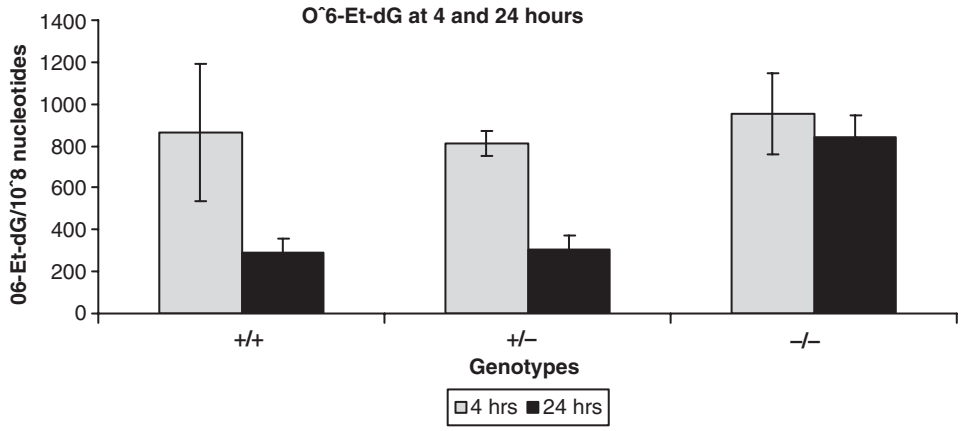

Figure 1. O^6-ethyldexoyguanosine levels in $p53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$ mice at 4 and 24 h post-ENU exposure. The DNA adduct, O^6-ethyldeoxyguanine (O^6-Et-dG), was detected in the livers from each of the treated mice. At 4 h after exposure, the level of O^6-et-dG was not significantly different among the three genotypes of mice. At 24 h, O^6-et-dG was detected in the liver DNA from each of the three genotypes. In contrast to the results at 4 h, a significant effect of genotype was detected. O^6-Et-dG levels in the $p53^{+/+}$ and $p53^{+/-}$ mice were significantly reduced from those found at 4 h in the livers of mice with the corresponding genotype. No significant difference in the level of O^6-et-dG between 4 and 24 h for mice with the $p53^{-/-}$ genotype was detected. Vertical bars represent mean of three animals and the standard error of the measurements.

and $p53^{+/-}$ ($P = 0.002$) genotypes. No significant difference was detected in the level of O^6-et-dG between 4 and 24 h for ENU-treated mice with the $p53^{-/-}$ genotype. The level of O^6-et-dG in control mice was equal to or less than 0.6 ± 0.1 adducts/10^8 nucleotides.

3.3 Mgmt Expression

Expression levels of *Mgmt* were analysed in livers of mice from each of the *p53* genotypes at 4 and 24 h post-ENU exposure (Figure 2). At 4 h, there were no significant changes in *Mgmt* levels among the different *p53* genotypes: $p53^{+/+}$ and $p53^{-/-}$ ($P = 0.09$), $p53^{+/+}$ and $p53^{+/-}$ ($P = 0.75$) and $p53^{+/-}$ and $p53^{-/-}$ ($P = 0.17$). However, at 24 h, *Mgmt* expression was up-regulated in the livers of the $p53^{+/+}$ (2.27-fold increase) and $p53^{+/-}$ (1.35-fold increase) mice. In mice with the $p53^{-/-}$ genotype, a 0.68-fold decrease was observed 24 h after ENU exposure. The *Mgmt* expression levels were significantly different among genotypes [$p53^{+/+}$ and $p53^{-/-}$ ($P = 0.001$), $p53^{+/+}$ and $p53^{+/-}$ ($P = 0.016$)] but not significantly different between $p53^{+/-}$ and $p53^{-/-}$ ($P = 0.06$; Table 1).

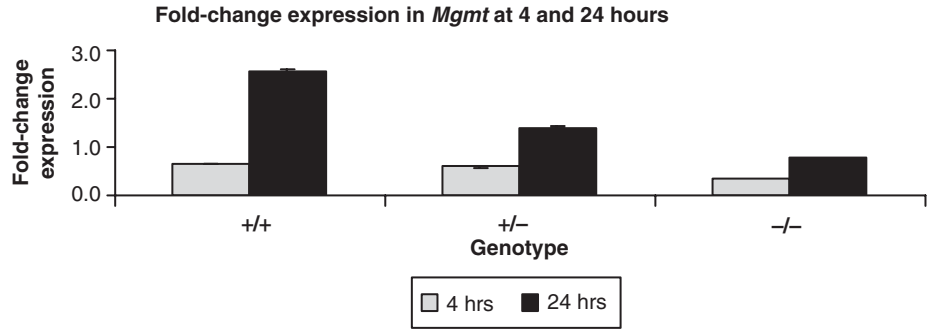

Figure 2. Changes in the expression levels of the *Mgmt* gene at 4 and 24 h post-ENU exposure in $p53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$ mice. At 4 h, no difference among genotypes in the expression of *Mgmt* gene was detected. In contrast, at 24 h, a 2.5-fold increase in *Mgmt* expression was found in mice with the $p53^{+/+}$ genotype. Fold changes of 1.25 and 0.8 were found in the $p53^{+/-}$ and $p53^{-/-}$ mice, respectively. Vertical bars represent mean of three animals and the standard error of the measurements.

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Table 1. Effects of genotype and time after exposure on *Mgmt* expression in the livers of ENU-exposed mice.

	4 hr						24 hr					
	WT		HE		KO		WT		HE		KO	
Summary statistic	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
	0.67	0.05	0.58	0.06	0.34	0.09	2.27	0.09	1.35	0.08	0.68	0.25
Genotype comparison	WT vs. KO		WT vs. HE		KO vs. HE		WT vs. KO		WT vs. HE		KO vs. HE	
	0.0919		0.7458		0.1683		0.0008[a]		0.0162[a]		0.0594	
Time comparison 4 4 hr vs. 24 24 hr	WT		KO		HE							
	0.0009[a]		0.3053		0.0017[a]							

[a] Significant.

3.4 p21^{Waf1} (Cdkn1a) Expression

The *p21*^{Waf1} (CDK-interacting protein 1; wild-type *p53*-activated fragment 1) gene encodes the *p21*^{Waf1} tumour suppressor protein, which promotes cell cycle arrest in the G_2/M phase. *P21*^{Waf1} expression was measured using a Taqman® assay to validate the expression changes observed using the Superarray pathway-specific plates (Figure 3). At 4 h, *p21*^{Waf1} mRNA levels from the ENU-exposed *p53*$^{+/+}$ and *p53*$^{+/-}$ mice were more than 100-fold greater than the levels detected in the unexposed mice. Exposure to ENU resulted in an increase of approximately 20-fold in *p21*^{Waf1} expression levels in *p53*$^{-/-}$ mice compared with the controls. Expression levels of *p21*^{Waf1} were similar for *p53*$^{+/+}$ and *p53*$^{+/-}$ genotypes ($P = 0.9$) but were significantly different when comparing *p53*$^{-/-}$ with *p53*$^{+/+}$ ($P = 0.02$) and *p53*$^{+/-}$ ($P = 0.01$). At 24 h, *p21*^{waf1} expression was reduced compared with the levels observed at 4 h in tissue for each genotype. Expression of *p21*^{Waf1} increased approximately 44-fold in the ENU-exposed *p53*$^{+/-}$ mouse and 57-fold in the ENU-exposed *p53*$^{+/+}$ mouse compared with their controls. In contrast, only a minimal increase was detected in the *p53*$^{-/-}$ mouse after ENU exposure compared with the controls. Expression levels of *p21*^{Waf1} were significantly different between *p53*$^{+/+}$ and *p53*$^{-/-}$ ($P = 0.002$), and *p53*$^{+/-}$ and *p53*$^{-/-}$ ($P = 0.008$) genotypes, but not between *p53*$^{+/+}$ and *p53*$^{+/-}$ ($P = 0.34$) genotypes at 24 h. These results are consistent with those obtained with the targeted arrays (see below; Table 2).

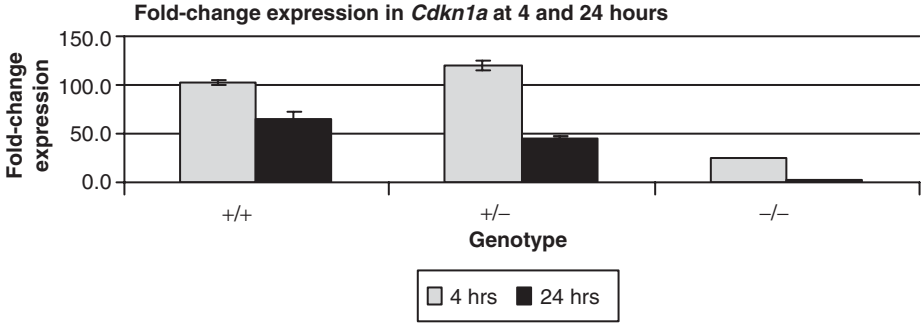

Figure 3. Changes in the expression levels of the *Cdkn1a* (*p21*^{Waf1}) at 4 and 24 h post-ENU exposure in *p53*$^{+/+}$, *p53*$^{+/-}$ and *p53*$^{-/-}$ mice. At 4 h, the expression of *Cdkn1a* (*p21/Waf1*) increased approximately 100-fold in the ENU-exposed *p53*$^{+/+}$ and *p53*$^{+/-}$ mice. In ENU-exposed *p53*$^{-/-}$ mice, the increase was approximately 25-fold. At 24 h, ENU exposure resulted in a 45- to 60-fold increase in expression in the mice with the *p53*$^{+/+}$ and *p53*$^{+/-}$ genotypes, respectively. In contrast, only a small increase of approximately 2.5-fold was detected in mice with the *p53*$^{-/-}$ genotype at 24 h. Vertical bars represent mean of three animals and the standard error of the measurements.

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Table 2. Effects of genotype and time after exposure on $p21^{Waf1}$ expression in ENU-exposed mouse liver.

	4 h						24 h					
	WT		HE		KO		WT		HE		KO	
Summary statistic	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
	105.89	21.21	115.42	15.58	24.59	5.33	57.38	10.17	43.95	4.00	2.09	0.82
Genotype comparison	WT vs. KO		WT vs. HE		KO vs. HE		WT vs. KO		WT vs. HE		KO vs. HE	
	0.024[a]		0.890		0.010[a]		0.002[a]		0.349		0.008[a]	
Time comparison 4 h vs. 24 h	WT		KO		HE							
	0.099		0.014[a]		0.011[a]							

[a] Significant.

3.5 P53 Pathway-Specific qPCR Arrays

The effect of *p53* genotype on gene expression in the liver of mice at 4 and 24 h after exposure to ENU was determined using an 84-gene targeted array of genes known to be regulated by p53. The 84 genes were grouped into the following five major p53-regulated gene groups based on their listed gene ontology function: (i) apoptosis including induction of apoptosis, anti-apoptosis, regulation of apoptosis and genes related to apoptosis; (ii) cell cycle including cell cycle arrest, negative regulation of the cell cycle, regulation of the cell cycle and genes related to the cell cycle; (iii) cell growth, proliferation and differentiation; (iv) DNA repair; and (v) transcription factors.

3.5.1 Gene Expression Changes in the Wild-Type ($p53^{+/+}$) Mice 4 h after exposure to ENU

At 4 h post ENU exposure, 33 apoptosis genes were analysed for changes in expression as a result of ENU exposure. There were three genes involved in the induction of apoptosis (Table 3), where more than a fourfold increase in expression was observed: *Casp2* was up-regulated 71.74-fold, *Prkca*, *protein kinase C*, was up-regulated 13.59-fold, and the proapoptotic gene, *Pmaip* (Michalak *et al.*, 2008), was up-regulated 71.05-fold. The expression levels of three anti-apoptotic genes were investigated and an increase in expression was found only for *Bcl-2* (26.57-fold); *Birc5* and *Dapk1* had expression levels below the limit of detection. There were additionally seven genes related to or regulating apoptosis where a greater than fourfold increase in expression was observed as shown in Table 3. No genes involved in apoptosis were down-regulated in the liver of $p53^{+/+}$ mice.

Exposure to ENU increased the expression levels of five of the seven genes involved in cell cycle arrest. $P21^{Waf1}$ (*Cdkn1a*), *Cdkn2a*, *Chek1*, *Msh2* and *Sesn2* exhibited greater than fourfold increases in expression as did *Brca1*, which negatively regulate the cell cycle (Table 4). Ten of the 21 genes involved with or related to cell cycle regulation had greater than fourfold increases in expression, including *Brca2*, *Ccne2*, *E2f1*, *Jun*, *Sfn*, *Ccng1*, *Cdc25a*, *Cdc2a*, *Chek2* and *Mdm2*.

Of the genes analysed involved in regulating cell growth, proliferation and differentiation (Table 5), more than a fourfold change in up-regulation was displayed by seven genes: *Brca1*, *Cdkn1a*, *Pmaip1*, *Zmat3*, *Btg2*, *Egr1* and *Mdm2*. Three of the 14 genes regulated by p53 and involved in DNA repair were up-regulated greater than fourfold (Table 6), including *Msh2*, involved in base excision repair in response to O^6-Me-dG (Yoshioka, Yoshioka and Hsieh, 2006).

3.5.2 Gene Expression Changes in the Heterozygous ($p53^{+/-}$) Mice after 4 h ENU exposure

Only one apoptosis gene, *Tnfrsf10b*, a member of the tumour necrosis factor receptor superfamily (Table 3), was highly up-regulated (21.07-fold), whereas other gene, *Prkca*, involved in the induction of apoptosis was substantially down-regulated (0.26-fold). The apoptotic pathway of $p53^{+/-}$ mice was shown to be significantly different ($P = 0.011$) when compared with the $p53^{+/+}$ after ENU exposure. The following sub-groups, induction of apoptosis, genes related to apoptosis, negative regulation of the

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Table 3. Effects of genotype and time after exposure on the expression of the apoptosis functional gene groups in the livers of ENU-exposed mice.

		4 h						24 h					
		WT		HE		KO		WT		HE		KO	
Function	Gene	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Induction of apoptosis	*Atm*	2.53	[a]	0.45	0.09	0.29	0.07	0.74	0.14	1.01	0.09	0.72	0.20
	Bax	2.56	1.41	1.62	0.12	0.72	0.11	2.23	0.33	2.28	0.15	0.86	0.09
	Casp2	71.74	70.34	1.73	0.37	1.63	0.19	1.32	0.30	0.99	0.20	0.65	0.16
	Dapk1	BLD	BLD	0.57	0.09	BLD	BLD	0.79	0.17	1.04	0.08	1.37	0.49
	Foxo3a	1.17	0.04	1.83	0.45	1.43	0.63	1.12	0.22	0.80	0.04	1.68	0.74
	Myc	2.95	1.44	0.76	0.21	5.35	2.78	2.27	1.41	1.52	0.57	2.90	0.74
	Pmaip1	71.05	68.63	2.22	0.94	NA	NA	1.06	0.23	1.39	0.37	0.61	0.18
	Prkca	13.59	13.39	0.26	0.08	0.19	0.04	0.50	0.05	0.53	0.03	0.63	0.17
	Pten	0.86	0.53	1.09	0.25	1.19	0.32	0.98	0.21	0.96	0.05	1.08	0.34
	Tnf	BLD	BLD	BLD	BLD	2.09	0.81	BLD	BLD	BLD	BLD	BLD	BLD
	Trp63	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD
Anti-apoptosis	*Bcl2*	26.54	26.09	0.66	0.08	1.86	0.80	0.65	0.10	0.97	0.20	0.89	0.25
	Birc5	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD
	Dapk1	BLD	BLD	0.57	0.09	BLD	BLD	0.79	0.17	1.04	0.08	1.37	0.49
Genes regulating and related to apoptosis	*Apaf1*	11.98	8.36	1.83	0.46	1.22	0.23	1.75	0.29	1.87	0.25	0.71	0.28
	Bid	1.26	0.18	0.89	0.18	0.83	0.20	1.03	0.20	1.25	0.11	1.19	0.26
	Brca1	107.45	*	0.97	0.30	0.71	0.21	0.85	0.22	0.65	0.30	3.54	0.30
	Btg2	9.34	1.79	3.28	0.50	4.32	1.27	3.30	0.23	2.56	0.21	2.26	0.79
	Casp9	2.90	1.13	1.40	0.26	1.00	0.08	1.14	0.18	1.30	0.16	0.85	0.24
	Cradd	2.22	1.21	0.48	0.05	0.31	0.03	0.74	0.10	1.03	0.14	0.55	0.08
	Fadd	6.79	5.57	1.00	0.18	1.89	0.49	1.14	0.34	0.84	0.07	1.82	0.64
	Mcl1	1.09	0.47	1.11	0.17	1.52	0.41	0.87	0.12	1.11	0.10	0.79	0.14
	Bag1	3.04	1.57	0.78	0.12	1.14	0.18	0.93	0.18	1.06	0.10	0.98	0.22
	Bnip3	0.85	0.69	0.53	0.07	0.42	0.07	0.73	0.06	1.34	0.10	0.55	0.07
	E2f1	40.21	39.35	1.00	0.22	0.81	0.25	1.37	0.42	0.95	0.16	1.32	0.21
	Fasl	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD
	Nfkb1	1.44	0.54	0.74	0.11	1.10	0.37	0.87	0.14	0.88	0.05	0.98	0.27
	Sirt1	3.14	0.61	1.45	0.31	2.25	0.61	0.88	0.17	1.06	0.08	0.98	0.33
	Tnfrsf10b	140.44	107.51	21.07	2.12	0.55	0.13	21.35	5.37	6.78	1.93	BLD	BLD
	Traf1	51.94	50.43	0.41	0.13	1.27	0.34	0.84	0.08	0.80	0.06	0.92	0.20
	Trp53	1.87	0.71	1.17	0.13	1.89	0.24	0.92	0.13	0.95	0.06	1.11	0.38
	Trp53bp2	2.05	0.21	2.30	0.74	2.00	0.61	1.08	0.10	1.00	0.09	1.62	0.60
	Trp73	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD

BLD, below level of detection.

[a]RNA could be obtained from only one animal for this data point.

cell cycle and genes related to the cell cycle were significantly different than wild type ($P < 0.05$; Table 7).

In the 34-gene cell cycle gene group (Table 4), the expression of three genes increased more than fourfold, including *Cdkn1a* (90.68-fold increase) and *Sesn2* (7.02-fold increase), both involved with cell cycle arrest. *Ccng1* was up-regulated 4.72-fold; that, along with *Mdm2*, regulates the negative feedback attenuating p53 activity through its degradation (Jensen *et al*., 2003; Kimura and Nojima, 2002). No genes were down-regulated more than fourfold. The group of genes analysed associated with the cell cycle in $p53^{+/-}$ mouse liver also showed statistically significant difference ($P = 0.006$) when compared with the $p53^{+/+}$ mouse liver (Table 7).

There were no significant changes in expression of the 18 cell growth, proliferation and differentiation group genes analysed except *Cdkn1a* (116.22-fold; Table 5). The functional group analyses revealed no differences in gene expression between wild-type and heterozygous genotypes ($P = 0.06$; Table 7).

The same was observed in the DNA repair gene group (Table 6): there were no genes with a fourfold or greater change in expression after ENU exposure.

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Table 4. Effects of genotype and time after exposure on the expression of the cell cycle functional gene groups in the livers of ENU-exposed mice.

		4 h						24 h					
		WT		HE		KO		WT		HE		KO	
Function	Gene	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Cell cycle arrest	*Cdkn1a*	116.23	8.54	149.8	31.59	90.68	53.58	60.70	8.36	39.33	17.39	2.31	0.09
	Cdkn2a	86.40	84.00	1.00	0.21	BLD	BLD	0.69	0.40	0.83	0.33	0.75	0.27
	Chek1	89.51	86.03	1.28	0.14	NA	NA	0.46	0.18	2.10	0.46	0.68	0.24
	Gadd45a	2.23	1.02	0.60	0.06	0.72	0.10	1.39	0.13	1.34	0.34	1.55	0.08
	Msh2	16.05	14.93	0.66	0.08	0.57	0.06	0.84	0.04	1.00	0.13	1.13	0.07
	Rprm	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD
	Sesn2	5.96	0.31	7.02	1.97	2.30	0.46	2.54	0.28	1.96	0.81	1.45	0.08
Negative regulation of the cell cycle	*Atm*	2.53	[a]	0.45	0.09	0.29	0.07	0.74	0.14	0.72	0.20	1.01	0.09
	Brca1	107.45	[a]	0.97	0.30	0.71	0.21	0.85	0.22	3.54	0.30	0.65	0.30
	Pten	0.86	0.53	1.09	0.25	1.19	0.32	0.98	0.21	1.08	0.34	0.96	0.05
	Rb1	1.63	0.71	0.73	0.17	0.52	0.09	1.06	0.08	0.67	0.18	0.96	0.05
	Trp53	1.87	0.71	1.17	0.13	1.89	0.24	0.92	0.13	1.11	0.38	0.95	0.06
	Trp63	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD
	Trp73	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD
	Wt1	BLD	BLD	BLD	BLD	0.69	0.19	1.35	0.98	BLD	BLD	0.95	0.16
Regulation of the cell cycle	*Brca2*	9.64	8.67	0.78	0.25	0.46	0.06	1.34	0.15	1.49	0.65	1.22	0.12
	Ccne2	5.92	4.37	1.12	0.23	0.66	0.07	1.36	0.17	1.06	0.20	1.98	0.20
	Cdk4	1.00	0.25	0.76	0.11	0.76	0.11	0.85	0.15	1.03	0.16	1.17	0.14
	E2f1	40.21	39.35	1.00	0.22	0.81	0.25	1.37	0.42	1.32	0.21	0.95	0.16
	E2f3	3.97	3.22	1.18	0.19	1.04	0.09	0.87	0.11	1.06	0.18	1.09	0.04
	Esr1	2.81	2.02	0.49	0.04	0.52	0.05	1.36	0.35	0.78	0.20	1.43	0.36
	Jun	5.70	0.93	2.03	0.61	6.30	2.35	0.95	0.10	1.85	0.94	0.77	0.14
	Kras	1.32	0.38	1.09	0.13	0.85	0.17	0.74	0.05	0.57	0.10	1.06	0.12
	Sfn	38.30	37.23	1.15	0.24	1.98	0.60	1.11	0.18	1.17	0.37	0.85	0.09
Other genes related to the cell cycle	*Ccnb2*	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD
	Ccng1	7.59	5.63	4.72	0.78	0.58	0.09	4.11	0.35	0.54	0.07	4.21	0.94
	Ccng2	2.40	0.42	1.31	0.30	1.55	0.39	0.96	0.15	0.91	0.24	0.92	0.09
	Ccnh	1.49	0.34	0.64	0.09	0.59	0.08	0.85	0.16	0.77	0.12	1.21	0.19
	Cdc25a	12.20	10.77	1.68	0.13	0.96	0.19	0.84	0.16	0.25	0.05	0.83	0.09
	Cdc25c	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD
	Cdc2a	7.12	4.55	1.40	0.13	1.27	0.30	0.46	0.09	0.68	0.14	0.46	0.08
	Chek2	14.08	[a]	0.50	0.09	0.43	0.08	1.10	0.12	1.17	0.27	1.26	0.20
	Mdm2	7.08	1.70	3.12	0.73	1.10	0.12	1.83	0.36	0.71	0.17	2.10	0.46
	Numb	1.04	0.14	1.06	0.23	1.27	0.53	1.11	0.22	1.21	0.40	0.96	0.09
	Pttg1	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD
	Cul9	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD

BLD, below level of detection.
[a]RNA could be obtained from only one animal for this data point.

The expression levels of the functional group were not significantly different ($P = 0.16$) when either mutant genotype was compared with the wild type. No significant effect on the genes encoding for transcription factors was detected ($P = 0.19$; Table 7).

3.6 Gene expression changes in the null ($p53^{-/-}$) mice after 4 h

When the apoptosis-associated genes were analysed, *c-Myc* was up-regulated by 5.35-fold and *Prkca* was down-regulated by 0.19-fold. The expression of the apoptosis-associated gene group in $p53^{-/-}$ mice was significantly different ($P < 0.001$) when compared with the wild-type mice after ENU exposure (Table 7).

When the genes related to the cell cycle functional group were analysed, a 149.81-fold increase in expression of the cell cycle arrest-mediating *Cdkn1a* gene was accompanied by a 6.30-fold increase in expression of the oncogene c-*Jun* (Table 4). The expression of the cell cycle-associated gene group from $p53^{-/-}$ mice was significantly different ($P = 0.002$) from the wild-type mice (Table 7).

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Table 5. Effects of genotype and time after exposure on the expression of the cell growth, proliferation and differentiation functional gene groups in the livers of ENU-exposed mice.

		4 h						24 h					
		WT		HE		KO		WT		HE		KO	
Function	Gene	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Cytokines	*Fasl*	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD
	Il6	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD
	Tnf	BLD	BLD	BLD	BLD	2.09	0.81	BLD	BLD	BLD	BLD	BLD	BLD
Cell growth, proliferation and differentiation	*Esr1*	2.81	2.02	0.49	0.04	0.52	0.05	1.36	0.35	1.43	0.36	0.78	0.20
	Brca1	107.45	[a]	0.97	0.30	0.71	0.21	0.85	0.22	0.65	0.30	3.54	0.30
	Cdk4	1.00	0.25	0.76	0.11	0.76	0.11	0.85	0.15	1.17	0.14	1.03	0.16
	Cdkn1a	116.22	8.54	149.80	31.5	90.68	53.5	60.69	8.36	39.33	17.3	2.30	0.09
	Myc	2.95	1.44	0.76	0.21	5.35	2.78	2.27	1.41	1.52	0.57	2.90	0.74
	Pcna	2.89	1.12	0.99	0.20	1.35	0.21	0.86	0.14	0.78	0.07	1.08	0.43
	Pmaip1	71.05	68.63	2.22	0.94	BLD	BLD	0.87	0.14	0.88	0.05	0.98	0.27
	Tnf	BLD	BLD	BLD	BLD	2.09	0.81	BLD	BLD	BLD	BLD	BLD	BLD
	Trp53	1.87	0.71	1.17	0.13	1.89	0.24	0.92	0.13	0.95	0.06	1.11	0.38
	Zmat3	15.86	11.00	2.80	0.54	1.68	0.45	1.08	0.10	1.00	0.09	1.62	0.60
	Btg2	9.34	1.79	3.28	0.50	4.32	1.27	3.30	0.23	2.56	0.21	2.26	0.79
	Hif1a	1.40	0.28	0.99	0.17	0.97	0.13	0.77	0.14	1.14	0.13	0.59	0.09
	Myod1	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD
	Nf1	1.67	0.98	0.64	0.12	0.72	0.40	0.86	0.14	0.78	0.07	1.08	0.43
	Wt1	BLD	BLD	BLD	BLD	0.69	0.19	0.87	0.14	0.88	0.05	0.98	0.27
	Egr1	13.26	8.71	1.19	0.24	1.81	0.91	1.50	0.81	0.53	0.11	0.73	0.25
	Jun	1.86	0.48	1.85	0.58	2.78	1.34	1.15	0.17	0.86	0.03	1.53	0.58
	Kras	1.32	0.38	1.09	0.13	0.85	0.17	1.10	0.22	0.96	0.10	1.02	0.19
	Mdm2	5.96	3.98	1.55	0.25	1.56	0.28	1.83	0.36	2.10	0.46	0.71	0.17
	Numb	1.04	0.14	1.06	0.23	1.27	0.53	0.99	0.08	1.56	0.23	0.95	0.20
	Pttg1	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD

BLD, below level of detection.
[a]RNA could be obtained from only one animal for his data point.

In the cell growth, proliferation- and differentiation-associated gene group (Table 5) Cdkn1a (90.68-fold), c-*Myc* (5.35-fold), *Btg2* (4.32-fold) and c-*Jun* (2.78- fold) were all up-regulated. The expression of this gene group in the null mouse was significantly different ($P = 0.007$) from the wild type (Table 7).

No significant difference ($P = 0.2$) in the expression of the DNA repair gene group was found when the $p53^{-/-}$ mice were compared with the wild type (Table 7). The p53 pathway-related transcription factor gene c-*Jun* was up-regulated, as previously mentioned. Among the sub-groups induction of apoptosis, genes related to apoptosis, negative regulation of the cell cycle and genes related to the cell cycle were significantly different when the mutant genotype was compared with the wild type ($P < 0.05$). The sub-groups regulation of apoptosis (0.025), regulation of cell cycle (0.016), regulation of cell proliferation (p=0.048) and other genes related to cell growth, proliferation and differentiation ($P = 0.002$) were significantly different when compared among genotypes.

3.6.1 *Gene Expression Changes in the $p53^{+/+}$ Mice after 24 h ENU Exposure*

At 24 h, the only apoptosis-related gene (Table 3) that was up-regulated more than fourfold was *Tnfrsf10b* (21.35-fold increase); no genes were down-regulated more than fourfold compared with the controls. In the cell cycle-associated gene group, two genes were up-regulated more than fourfold (Table 4). These were *Cdkn1a*, which increased 60-fold, and *Ccng1*, which increased fourfold. None of the genes in the DNA repair and transcription factor gene groups were significantly changed except *Wt1* (21.35; Table 6).

3.6.2 *Gene Expression Changes in the $p53^{+/-}$ Mice after 24 h*

Of the apoptosis-associated genes, only *Tnfrsf10b* expression increased substantially (6.78-fold) after

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Table 6. Effects of genotype and time after exposure on the expression of the DNA repair and transcription factor functional gene groups in the livers of ENU-exposed mice.

		4 h						24 h					
		WT		HE		KO		WT		HE		KO	
Function	Gene	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
DNA repair	*Apex1*	1.20	0.19	0.82	0.10	1.21	0.23	0.86	0.24	0.99	0.12	1.05	0.17
	Atm	2.53	*	0.45	0.09	0.29	0.07	0.74	0.14	1.01	0.09	0.72	0.20
	Atr	2.08	1.26	0.51	0.12	0.49	0.07	0.94	0.22	1.80	0.27	0.64	0.16
	Brca1	107.45	*	0.97	0.30	0.71	0.21	0.85	0.22	0.65	0.30	3.54	0.30
	Brca2	9.64	8.67	0.78	0.25	0.46	0.06	1.34	0.15	1.22	0.12	1.49	0.65
	Dnmt1	BLD	BLD	BLD	BLD	BLD	BLD	0.93	0.21	0.7	0.03	1.04	0.11
	Ercc1	3.19	1.85	1.09	0.18	1.00	0.23	1.10	0.22	0.96	0.10	1.02	0.19
	Lig4	2.81	2.02	0.49	0.04	0.52	0.05	1.36	0.35	1.43	0.36	0.78	0.20
	Msh2	16.05	14.93	0.66	0.08	0.57	0.06	1.14	0.34	0.84	0.07	1.82	0.64
	Pcna	2.95	1.44	0.76	0.21	5.35	2.78	0.81	0.18	0.95	0.07	0.88	0.21
	Pttg1	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD
	Rev3l	2.80	1.81	1.09	0.26	0.89	0.23	0.84	0.16	0.91	0.04	0.90	0.34
	Xrcc4	1.55	0.63	0.48	0.03	0.40	0.04	2.54	0.28	1.45	0.08	1.96	0.81
	Xrcc5	1.10	0.11	0.64	0.12	0.72	0.22	1.11	0.18	0.85	0.09	1.17	0.37
Transcription factor	*Cdkn2a*	86.40	84.00	1.00	0.21	BLD	BLD	0.69	0.40	0.75	0.27	0.83	0.33
	E2f1	40.21	39.35	1.00	0.22	0.81	0.25	1.37	0.42	0.95	0.16	1.32	0.21
	E2f3	3.97	3.22	1.18	0.19	1.04	0.09	0.87	0.11	1.09	0.04	1.06	0.18
	Ep300	1.86	0.48	1.85	0.58	2.78	1.34	1.15	0.17	0.86	0.03	1.53	0.58
	Esr1	3.19	1.85	1.09	0.18	1.00	0.23	1.10	0.22	0.96	0.10	1.02	0.19
	Foxo3a	1.17	0.04	1.83	0.45	1.43	0.63	1.36	0.35	1.43	0.36	0.78	0.20
	Hif1a	2.23	1.02	0.60	0.06	0.72	0.10	0.77	0.14	1.14	0.13	0.59	0.09
	Jun	5.70	0.93	2.03	0.61	6.30	2.35	0.95	0.10	0.77	0.14	1.85	0.94
	Myc	1.32	0.38	1.09	0.13	0.85	0.17	0.74	0.05	1.06	0.12	0.57	0.10
	Nfkb1	1.44	0.54	0.74	0.11	1.10	0.37	0.86	0.14	0.78	0.07	1.08	0.43
	Rb1	1.04	0.14	1.06	0.23	1.27	0.53	0.87	0.14	0.88	0.05	0.98	0.27
	Rela	12.01	11.23	0.87	0.24	1.43	0.49	1.11	0.22	0.96	0.09	1.21	0.40
	Stat1	0.80	0.41	0.70	0.09	1.21	0.53	0.93	0.11	0.92	0.03	0.68	0.12
	Trp53	1.87	0.71	1.17	0.13	1.89	0.24	0.92	0.13	0.95	0.06	1.11	0.38
	Trp63	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD
	Trp73	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD
	Wt1	BLD	BLD	BLD	BLD	0.69	0.19	21.35	5.37	6.78	1.93	BLD	BLD

BLD, below level of detection.

[a]RNA could be obtained from only one animal for this data point.

ENU exposure (Table 3). Two-way ANOVA of the apoptosis pathway did not reveal any significant differences in the expression of these genes in the $p53^{+/-}$ mouse ($P = 0.13$) compared with the $p53^{+/+}$ mouse 24 h post-ENU exposure (Table 7).

In the cell cycle group, *Cdkn1a* was up-regulated by approximately 39-fold and this change was accompanied by a fourfold change in *Ccng1* (Table 4). The expression of the group of genes regulating cell cycle was not significantly different ($P = 0.154$) from the level detected in mice with the $p53^{+/+}$ genotype (Table 7).

None of the cell growth genes analysed were differentially regulated greater than fourfold in $p53^{+/-}$ mice at 24 h post-ENU exposure except *Cdkn1a* (39.33-fold; Table 5). Expression profiles of the cell growth genes in the $p53^{+/-}$ mouse were not significantly different ($P = 0.4$) from the same gene group analysed in $p53^{+/+}$ mice (Table 7).

Analysis of the DNA repair gene group expression profiles did not reveal (Table 7) any statistically significant differences ($P = 0.13$) between the $p53^{+/-}$ and $p53^{+/+}$ mice. No sub-groups showed significant differences compared with the wild type ($P > 0.05$; Table 7).

3.6.3 Gene Expression Changes in the $p53^{-/-}$ Mice after 24 h ENU Exposure

No significant changes in the expression were detected in the apoptosis gene group analysed in $p53^{-/-}$ mice at 24 h post-ENU exposure (Table 3). The expression of the apoptosis pathway in the $p53^{-/-}$ mouse was significantly different ($P = 0.03$)

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Table 7. The *P*-values for each gene function group from gene set enrichment analysis, MANOVA and pair-wised genotype comparison, at two time points after ENU exposure.

	4 h				24 h			
Gene groups	MANOVA	WT vs. KO	WT vs. HE	KO vs. HE	MANOVA	WT vs. KO	WT vs. HE	KO vs. HE
Apoptosis	<0.001	<0.001	0.011	0.005	0.016	0.025	0.191	0.006
Induction of apoptosis	0.011	0.006	0.028	0.004	0.018	0.028	0.391	0.032
Anti-apoptosis	BLD	BLD	BLD	BLD	0.442	0.272	0.694	0.347
Regulation of apoptosis	0.013	0.025	0.047	0.090	0.031	0.031	0.491	0.010
Other genes related to apoptosis	0.001	0.002	0.009	0.003	0.092	0.615	0.070	0.003
Cell cycle	0.001	0.002	0.006	0.033	0.001	0.033	0.154	0.010
Cell cycle arrest	0.007	0.064	0.015	0.019	0.020	0.016	0.187	0.045
Negative regulation of the cell cycle	0.058	0.037	0.464	0.037	0.024	0.030	0.654	0.008
Regulation of the cell cycle	0.027	0.016	0.071	0.122	0.034	0.289	0.132	0.026
Other genes related to the cell cycle	0.003	0.002	0.003	<0.001	0.024	0.024	0.309	0.016
Cell growth, proliferation and differentiation	0.005	0.007	0.056	0.033	0.027	0.030	0.364	0.010
Regulation of cell proliferation	0.052	0.048	0.094	0.001	0.008	0.021	0.238	0.004
Cell differentiation	0.095	0.067	0.014	0.794	0.097	0.130	0.189	0.149
Other genes related to cell growth, proliferation and differentiation	0.005	0.002	0.009	0.050	0.066	0.136	0.430	0.016
DNA repair	0.201	0.206	0.158	0.277	0.001	0.036	0.130	0.007
Transcription factors	0.100	0.171	0.190	0.058	0.170	0.283	0.479	0.067

BLD, below level of detection.

compared with the wild type after ENU exposure (Table 7).

No cell cycle genes were expressed at a level of greater than fourfold at 24 h post-ENU exposure (Table 4). The cell cycle gene expression profiles in the $p53^{-/-}$ mice were significantly different ($P = 0.03$) when compared with the cell cycle gene expression profiles in $p53^{+/+}$ (Table 7).

The cell growth, proliferation and differentiation genes did not show significant changes in their expression profiles (Table 5), but the gene group exhibited significant difference ($P = 0.03$) from the wild type (Table 7).

The DNA repair pathway analysed in the liver of $p53^{-/-}$ mice was significantly different ($P = 0.036$) compared with the wild type. Among the sub-groups, induction of apoptosis, regulation of apoptosis, cell cycle arrest, negative regulation of the cell cycle, other genes related to the cell cycle and regulation of cell proliferation, and differentiation showed significant differences compared with the wild type ($P < 0.05$; Table 7).

4 DISCUSSION

The tumour suppressor protein p53 is well known to function in the DNA damage response (Meek, 2009). Acting as a transcription factor, p53 regulates the activity of several DNA damage response pathways after exposure to a toxic agent. In this study, we sought to provide further insight into these responses by exposing TSG^{TM}*p53* mice with $p53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$ genotypes to the classic mutagen ENU and characterizing the *Trp*53 response with the use of targeted real-time qPCR expression arrays. In this study, we have found that, as expected, exposure to ENU resulted in the strong differential expression of genes that are important in establishing and regulating the cell cycle checkpoints in the $p53^{+/+}$ mice. Substantial increases in *p21* mRNA, which functions in the establishment of the G1/S and G2/M checkpoints, were detected at 4 h after exposure in the mice of each genotype. This was somewhat surprising because $p21^{Waf1}$ gene expression is activated by p53 and it was expected that proportionally lower levels of $p21^{Waf1}$ mRNA would be observed in the $p53^{+/-}$ and the $p53^{-/-}$ mice as a result of gene dosage. mRNA levels decreased in tissues from each of the genotypes by 24 h and the level was very low in the $p53^{-/-}$ mice. This suggests that in the early response to DNA damage, other transcription factors may be activating $p21^{Waf1}$, and that the activity declines over time. This concept is supported by studies (Moustakas and Kardassis, 1998) that demonstrate multiple alternate binding sites for the *p21* promoter (Abbas and Dutta, 2009; Macleod *et al*., 1995).

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Other genes that contribute to the integrity of the damage-sensing response and the integrity of the checkpoints, *Cdkn2* (the p53-Arf pathway for damage sensing), *Chek 1* and *Chek 2* (important in the G2/M pathway), *Brca2*, *E2f1* and *Cdc25a*, were highly up-regulated in the $p53^{+/+}$ mice at 4 h after exposure, but not in the $p53^{+/-}$ or $p53^{-/-}$ mice. At 24 h, the expression level of each of these genes was not significant in the $p53^{+/+}$ mice liver, which suggests that the DNA damage processing was nearing completion and the cells could return to the cycle.

In addition to the cessation of the cell cycle, the effectors of apoptosis were up-regulated in the $p53^{+/+}$ mice as part of the early response to ENU exposure. *Pmaip1* (*Noxa*), *E2F1*, *Apaf-1*, *Brca1*, *Traf1* and *Tnfrs10b* (*DR5*, *Killer*) were each highly induced in the $p53^{+/+}$ mice. The increased expression of *E2F1* may relate to its role both in the control of cell proliferation through its interaction with *Rb* and in the signalling of cells into the mitochondrial-associated apoptosis pathways. *Pmaip1* (*Noxa*) codes for a BH_3-domain-only protein (Oda *et al*., 2000) which localizes to the mitochondria activating the release of cytochrome *c* from the mitochondrial membrane (Seo *et al*., 2003) and mitochondrial fragmentation (Woo *et al*., 2009). E2F1 up-regulates the transcription of the BH3-only proteins of the Bcl-2 family, including *Noxa*. Furthermore, E2F1 regulates the transcription of *Apaf-1* and an E2F1-binding site has been identified in the *Apaf-1* promoter (Furukawa *et al*., 2002). The up-regulation of this pathway may provide a link between the control of cell replication and apoptosis (Rogoff and Kowalik, 2004).

Also up-regulated in response to ENU were *Tnfrsf10b* (*DR5/Killer*) and *Traf-1*. DR4 and DR5 are membrane-bound receptors that bind ligands such as Trail and initiate apoptosis through the activation of caspase-8 (Srivastava, 2001; Wang, 2008). Traf-1 is an adapter protein in the TNF protein family and interacts with certain apoptotic effector proteins (Zapata and Reed, 2002).

Our results are consistent with the onset of apoptosis through a DNA damage-induced mitochondria-dependent pathway in the livers of the ENU-exposed $p53^{+/+}$ mice. However, a different pattern was observed in the $p53^{+/-}$ and $p53^{-/-}$ mice. With the exception of *DR5*, which was expressed in the $p53^{+/-}$ mice and functions in a p53-independent manner (Wang *et al*., 2008), ENU exposure did not increase the expression of these genes in the $p53^{+/-}$ or $p53^{-/-}$ mice. These results are consistent with other studies that indicate that apoptosis is compromised in cells that lack functional p53. At 24 h, there were a minimal number of genes that were differentially expressed in each genotype, which suggests that the apoptosis pathway was down-regulated in the $p53^{+/+}$ mice.

It should be noted that two genes with anti-apoptotic activity, *Bcl-2* and *Prkca*, were up-regulated at 4 h in the ENU-exposed $p53^{+/+}$ mice. This was surprising because Bcl-2/Bax ratios and the dimerization of Bcl-2 with itself or the heterodimerization of Bcl-2 with Bax lead to suppression of apoptosis. Furthermore, Prkca is also an inhibitor of apoptosis in that it phosphorylates the Bcl-2 homodimer, an event which actively suppresses apoptosis (Ruvolo *et al*., 1998; Villar *et al*., 2009). What this may suggest is that a delay exists in the down-regulation of the signalling pathways that inhibit apoptosis, providing time for the DNA damage processing pathways to function.

In this study, we measured the levels of O^6-et-dG as a marker for alkylation damage. However, it should be noted that alkyl DNA adducts can be repaired through several Mgmt-independent repair pathways. O^6-Et-dG is repaired through both the Mgmt pathway and the mismatch repair (MMR) pathway. At 4 h, the MMR gene, *Msh2*, was highly up-regulated in the $p53^{+/+}$ mice, but slightly repressed in the $p53^{+/-}$ and the $p53^{-/-}$ mice. The difference in expression between the genotypes may reflect the fact that *Msh2* is up-regulated by *E2F1* (La Thangue, 2005) which was also highly expressed in the $p53^{+/+}$, but not in the $p53^{+/-}$ or $p53^{-/-}$ mice. Increased expression of *Msh2* in the $p53^{+/+}$ mice was accompanied by a slight increase in the levels of the DNA repair genes (*Ercc1*, *Xrcc4*, *Xrcc5* and *Lig4*). At 24 h, *Msh2* mRNA levels were not differentially expressed in any of the genotypes.

In contrast to *Msh2*, no differential expression of *Mgmt* was detected at 4 h in the $p53^{+/+}$, $p53^{+/-}$ or $p53^{-/-}$ mice. This finding was accompanied by no significant difference in the level of O^6-et-dG at 4 h in any of the genotypes. However, at 24 h, *Mgmt* expression increased and the level of O^6-et-dG decreased in the $p53^{+/+}$ and $p53^{+/-}$, but no differences were observed in the $p53^{+/-}$ or $p53^{-/-}$ mice. These results are consistent with a model in which MMR is activated as part of the early response to ENU alkylation, but that the initiation of the repair of the major mutagenic adducts, O^6-Et-dG,

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is delayed until after 4 h in the $p53^{+/+}$ mouse. A different pattern is observed for the $p53^{+/-}$ mouse. That is, MMR is not activated, as evidenced by the lack of increased expression of *Msh2* and the repair of O^6-et-dG is shifted solely to *Mgmt*. In the $p53^{-/-}$ mouse, neither pathway appears to be intact, which may be a major factor in the development of hepatocellular carcinoma after ENU exposure.

In conclusion, exposure to ENU results in the activation of the DNA damage response pathway. As part of the initial response, the DNA damage detection and response mechanisms are activated to insure that damaged cells are either removed from the tissue or not replicated. The cell cycle checkpoints, the apoptosis pathways and MMR are up-regulated and an integrated decision is made to repair or destroy cells with DNA damage. In liver cells with only one functional copy of *p53*, the apoptosis response and MMR are either absent or delayed and the integrity of DNA is dependent upon the activity of *Mgmt*. Loss of both alleles results in a compromised DNA damage response, and the level of DNA damage that remains at the later time point may reflect the initial steps in the formation of hepatocellular carcinoma.

It should be noted that these conclusions are based upon the responses based on the intact tissue. We recognize that individual cell types within the tissue may have an expression profile that differs from the average response. Experiments are planned in which the expression profiles for individual cell types can be developed

5 DISCLAIMER

The views presented in this study are those of the authors and do not necessarily reflect those of the Food and Drug Administration.

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Application of Ecotoxicogenomics for Understanding Mode of Action of Chemicals and Species Extrapolation

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1 ECOTOXICOGENOMICS

Toxicogenomics is a combination of toxicology and genomics (Lovett, 2000) and has been established as an important field in toxicology. It intends to understand and evaluate toxic effects of chemical substances on the genome of study organisms (genomics). Genomics is an omics technology and generally also includes transcriptomics, which analyzes the effects of chemicals on the transcriptome. Transcriptomics has been developed with DNA microarray technology (Schena *et al*., 1995), and typically toxicogenomics means the use of DNA microarray for toxicological purpose. Toxicogenomics was originally developed for the evaluation of chemical risks posed to humans and has been mainly developed by using cultured human cells, but also model animals such as rats and mice. This new field has been expected as a sensitive and predictive tool for evaluating the toxicity of chemicals.

Generally, hazardous effects of chemicals on organisms are results of abnormalities in certain tissues caused by differential gene expression triggered by chemical exposure. Thus, genomic approaches are expected to reflect changes that phenotypically occur at later stages and are therefore being used as a sensitive and predictive tool for the hazardous evaluation of chemicals. In addition, toxicogenomics has the potential to identify sensitive, so far unknown biomarkers that might have the potential to predict toxicity because a number of gene expression profiles can be examined without any prepossession.

One of the important points of toxicogenomics is that this technology can be applied to other organisms as well as model species, because the same strategy can be applied irrespective of species once their RNA can be obtained. There are often differences and species-specific arrays are being used. Toxicogenomics, which was originally based on human and model animals, is strongly supported by genomic information resources, such as gene annotation, gene function, biological response, and metabolism, combined with accumulated biological knowledge and observation. Therefore, observed changes in gene expression may also clarify the molecular mechanisms that lead to the hazardous effects of toxicants. However, genomic challenges in non-model organisms are still limited and are desired to expand. To evaluate the risk

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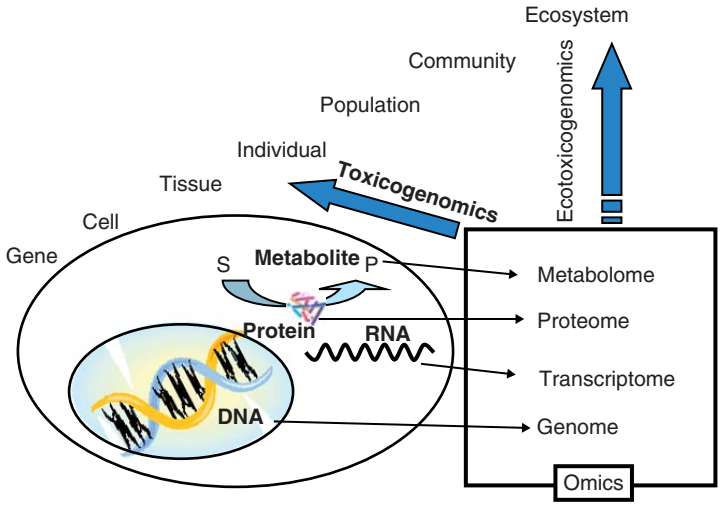

Figure 1. Scheme of -omics and ecotoxicogenomics.

of environmental chemicals to individual species and ecosystems and to understand their modes of action, the application of toxicogenomics has been expanded to organisms used in environmental studies and is named ecotoxicogenomices (Snape *et al*., 2004; Iguchi *et al*., 2006, 2007, 2008). Recently, many genomic challenges have been started in non-model organisms (Crump *et al*., 2002; Gracey and Cossins, 2003; Larkin *et al*., 2003; Williams *et al*., 2003; Volz *et al*., 2006). Limited genomic information is available for non-model species. The application of genomics to sentinel, non-model organisms that form an ecosystem is expected to determine the risk of chemicals that are suspected to be hazardous, on a scale ranging from individuals to ecosystems (Figure 1) and to contribute to the protection of ecosystems. So far, however, the advancement of ecotoxicogenomics is often limited by the lack of genomic information available for such species.

2 OVERVIEW OF CHEMICAL POLICY IN JAPAN

As for protecting human health from direct impacts of hazardous chemicals, there are several laws such as Labor Safety & Health Law for worker's safety, and Food Sanitation Law for consumer's health, and so on. Indirect impacts of chemicals on human health via the environment are covered by environmental legislations such as the Air Pollution Control Law, Waste Disposal Law, Water Pollution Control Law, and Chemical Substances Control Law (CSCL). CSCL has been amended in 2003 to also cover the risk management of chemicals to wildlife in the environment.

If a company that has a plan of initiating manufacturing and/or importing new chemicals of more than 1 t yr^{-1}, the company is required to notify the government in advance with data of the chemicals. In several specific cases such as intermediate chemicals for products, export-only chemicals, or chemicals whose amount of manufacture/import is less than 1 t yr^{-1}, notification of those new chemical substances is exempted. In such cases, the government verifies the cases and monitors by on-site inspection or reporting from enterprises. The government evaluates the chemicals by existing data, including the ones provided by the company. The evaluation is to check three properties (persistency, bioaccumulation, and toxicity either to humans or to living organisms) of the notified chemicals. Then, based on the evaluation result, the hazardous chemicals of concern are subject to regulation by classification into several categories according to their chemical properties.

Existing chemicals are mainly assessed by the government, and based on the test result, they may be subject to regulation as three types of monitoring

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chemicals: persistency, bioaccumulation, and toxicity either for human life or for wildlife.

To protect wildlife, acute toxicity data have to be collected using three species including algae (*Pseudokirchneriella subcapitata*), crustacea (*Daphnia magna*) and fish (medaka, *Oryzias latipes*) for the registration of chemicals. These three species are consisted as a small food web because algae support the *Daphnia* and *Daphnia* support fish as foods. Acute toxicity data are not sufficient to understand the functional mechanism of chemicals, and therefore the establishment of ecotoxicogenomics methods for these three species is beneficial for future risk assessment and risk management of chemicals. As the beginning of our ecotoxicogenomics work, the medaka genome project had been completed and commercial microarrays for medaka were already available, and we therefore focused on *Daphnia* and algae.

3 EXPECTED OUTPUT OF ECOTOXICOGENOMICS

Ecotoxicogenomics has great advantages compared with conventional ecotoxiclogical approaches. To evaluate hazardous effects of chemicals on non-model organisms, conventional approaches could only give one parameter such as EC_{50} or LC_{50}. Although these parameters are valuable for the estimation of toxic concentrations, they are insufficient to characterize the toxicity of a compound because characteristic feature of chemicals cannot be obtained only from these concentrations. Even if two chemicals have the same EC_{50}, it does not mean the same properties in terms of toxicity at all.

Genomic approaches however give many parameters with gene annotations and these values are very useful to characterize chemicals. If two chemicals have similar effects on the expression of genes, one can expect that these two chemicals have similar effects in terms of toxicity. This advantage of genomics is also useful in ecotoxicogenomics, especially for small aquatic organisms, because pathological approaches that generally give important information in toxicity cannot be applied.

4 APPLICATION OF ECOTOXICOGENOMICS TO *DAPHNIA*

In order to apply toxicogenomic approaches to ecotoxicology, species selection is important. As a first step, species that satisfy the following criteria can be considered as good models for ecotoxicogenomics: (i) they are an important species in ecological networks, (ii) they can be easily sampled from the field, (iii) they can be cultivated in laboratories to allow controlled studies of chemical exposure, and (iv) their genetic background can be evaluated. The last two criteria are critical points for the selection of species because it is difficult to elucidate the cause of gene expression changes by comparing samples obtained from different field sites. Consistently, organisms that have huge genome sizes are also not appropriate for genomic approaches.

Table 1. Selection criteria of species for ecotoxicogenomics.

1. Easy to breed
2. High breeder (mature about 5 days after birth and lay eggs every 3 days)
3. Parthenogenesis (easy to obtain genetically clone individuals)
4. Translucent (easy to observe internal organs without dissection)
5. *In vitro* development is possible (developmental process can be observed under the microscope)
6. Database of chemical effects is available
7. ESTs and genome database are available
8. Need for the environmental risk assessment in the respective countries

Among many species in the environment, *Daphnia magna* is an organism that satisfies the criteria described above. In addition, *D. magna* has further advantages as indicated in Table 1. Therefore, *D. magna* is a suitable organism for the application of ecotoxicogenomics.

According to genetic information available, we have also obtained EST sequences of *D. magna* (Watanabe *et al*., 2005) and the genomic sequencing of the related *Daphnia*, *D. pulex*, is currently in progress (DGC: http://daphnia.cgb.indiana.edu/).

5 EXAMPLE OF *DAPHNIA* FOR ECOTOXICOGENIMICS

In order to study genome-wide gene expression profiles, we previously established a DNA microarray containing 10 K genes (Watanabe *et al*., 2007) (Figure 2). Several sets of oligonucleotide sequences were designed per one gene and one or two oligonucleotide sequences were selected. This microarray was used to analyze gene expression

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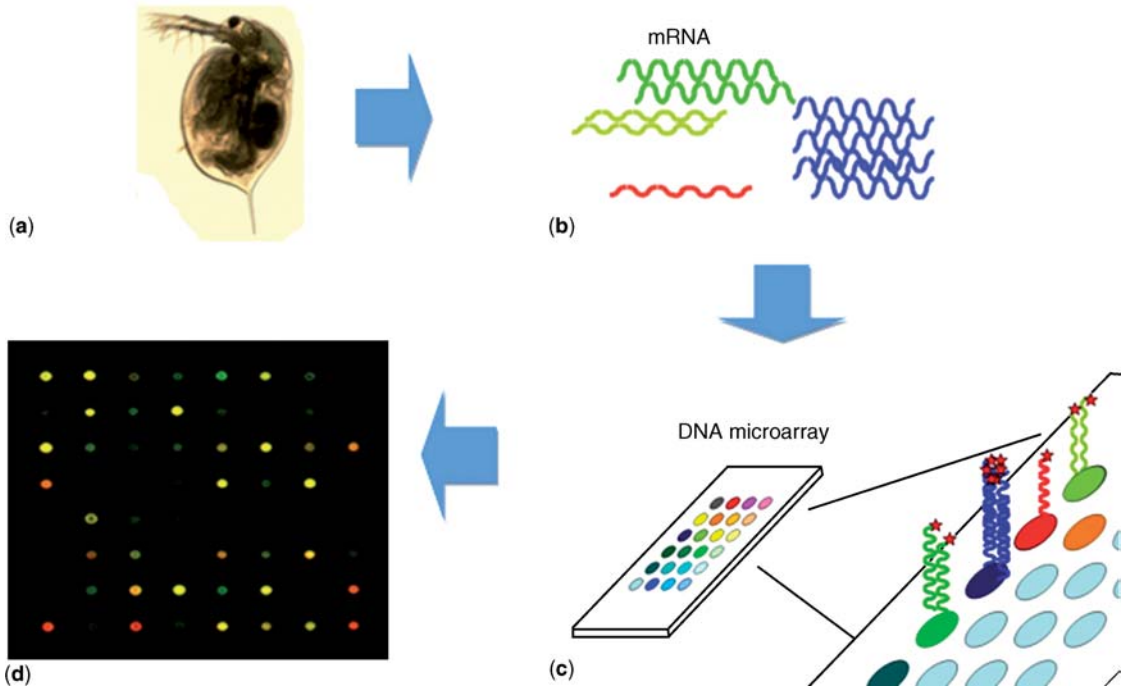

Figure 2. Experimental flow of ecotoxicogenomics. (a) Exposure of a chemical to *Daphnia magna* for 24 h; (b) extraction of RNA; (c) hybridization with array; (d) and scanning the hybridized spots.

changes in *Daphnia* following exposure to several chemicals (Watanabe *et al*., 2007, 2008; Connon *et al*., 2008). Figure 3 shows part of the result. Hierarchical clustering analysis showed that chemicals could be categorized into approximately groups, which indicates that some chemicals have similar effects on the organism. Before the application of the DNA microarray, it was impossible to categorize the chemicals in terms of their biological effects on aquatic organisms. Thus, the application of DNA microarray for the evaluation of biological effect caused by chemical exposure enabled us to understand chemical toxicity in biological terms.

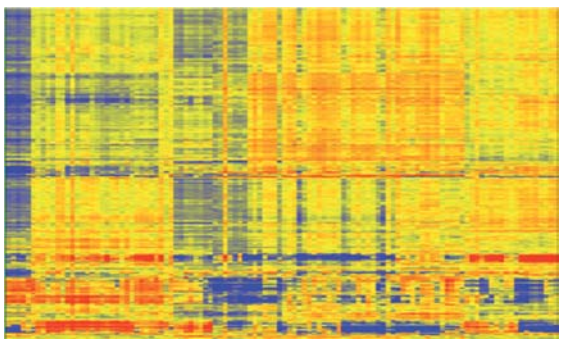

Figure 3. Hierarchical clustering of the gene expression pattern after chemical exposure. Chemicals were exposed to daphnia for 24 h and DNA microarray analysis was performed (Watanabe *et al*., 2007). Red and blue indicate activated and repressed gene, respectively. Each column corresponds to one DNA microarray analysis.

6 APPLICATION OF ECOTOXICOGENOMICS TO OTHER ORGANISMS

Ecotoxicogenomics approaches can be applied to a variety of other organisms in the environment as long as genetic information is available. For example, algae are suitable organisms for the application of ecotoxicogenomics. Similar to *Daphnids*, they can be easily maintained and exposed to chemicals and used for the Chemical Safety Law in Japan. Especially, a phytoplankton, *Pseudokirchneriella subcapitata*, has long been used in ecotoxicology and assigned as an organism for chemical toxicity

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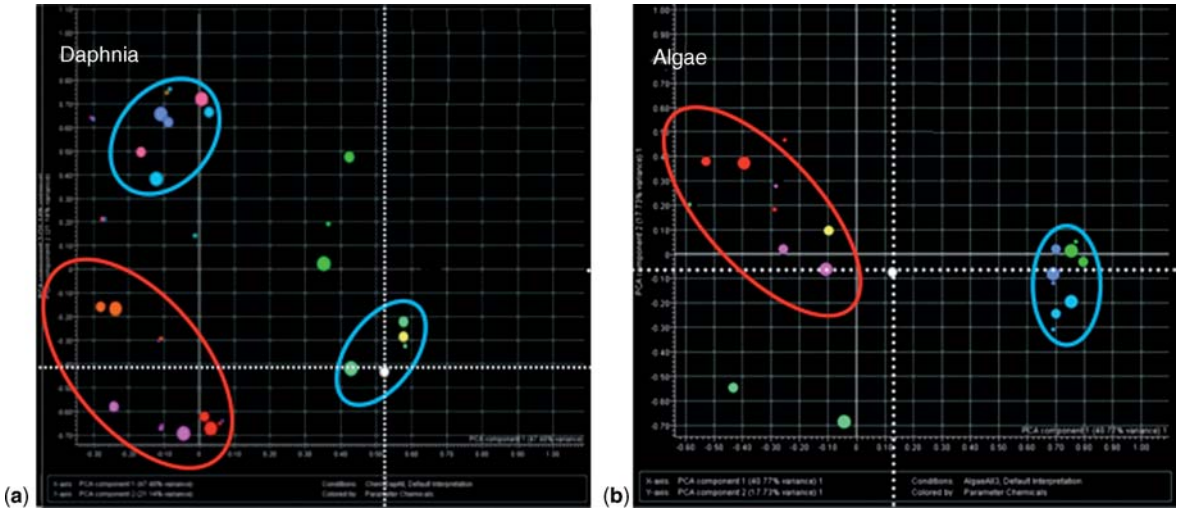

Figure 4. Principal component analyses (PCA) of gene expression changes after exposure of chemicals. After chemical exposure to *Daphnia* (a) or algae (b) for 24 h, gene expression changes were analyzed using DNA microarray. PCA was performed on differentially expressed genes. Two dimensions of the components are indicated. Four chemicals (different color dots) with three concentrations (same color dots) were used. Larger dots denote higher concentration.

testing in the Organization for Ecological Cooperation and Development (OECD). EST sequences were obtained from the cDNA library of *P. subcapitata* and an oligo microarray was designed based on the available sequence information (Watanabe *et al*., unpublished data). One of the advantages of genomic approach is that the same procedure can be applied after purifying RNA from organisms. Thus, hierarchical clustering and other analyses can be performed based on the gene expression profile of the algae and chemical effects can be grouped using the same chemicals for these two species. These groups can be presented in another way of analysis such as principal component analysis (PCA) as shown in Figure 4. These analyses showed that some chemicals (circled in red) have similar effects on both *Daphnia* and algae whereas other chemicals (circled in blue) have different effect on *Daphnia* and algae. By taking into account their gene annotations, it is expected to evaluate chemical effects on other species. Thus, once genomic information can be obtained, genomic approaches are useful to determine similarities and differences between chemical effects. These analyses indicate that genomic approaches can be effectively applied to organisms in the environment. Currently, we are integrating the microarray data of chemicals effects on three species: algae, *Daphnia* and medaka.

7 FUTURE PERSPECTIVES

If chemicals can be classified by their biological effects, this will be helpful for the prediction of hazardous effects of new chemicals. Accumulation of DNA microarray data will make it possible to determine whether chemicals of similar structure are causing the same biological effect. Quantitative linkage between chemical structure and biological effect will be useful for the prediction of chemical effects. Quantitative structure–activity relationship (QSAR) has been known to be a practical tool for the prediction of chemical toxicity. However, conventional QSAR did not consider biological effects of the chemicals and a wide variety of chemicals should be considered when extracting characteristic structures. Combination of biological effects and chemical structure may facilitate the classification and prediction of chemicals in terms of biological effects.

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A Community-Based Participatory Research/Translational Biomedical Research Strategy for Personalizing Nutrition, Medicine, and Healthcare

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1 INTRODUCTION

Physiological and physical variations in humans are caused by single nucleotide polymorphisms [estimated at ~0.1% (Frazer *et al.*, 2007)], copy number variation [~0.7% (Henrichsen, Chaignat and Reymond, 2009)], and epigenomic influences (Bock *et al.*, 2008). The combination of these variations alters the metabolism of foods and the effect of food components on the expression of genetic makeup (rev. in Kaput, 2008; Kaput JaR, 2006; Kaput and Rodriguez, 2004; Vakili and Caudill, 2007). Although gene–nutrient or gene–environment interactions are complex and challenging to study, advances in experimental technologies for analyzing genomes, proteins, metabolites, and transcripts are allowing quantitative analyses of the relationships among genetic makeup, environment, and health outcomes (Corella *et al.*, 2009).

Current experimental designs, however, rely on assessing differences between cases and controls, or between groups who are exposed to a toxin versus those who are not exposed, or who consume some specified nutrient versus those who do not. Experimental designs that rely on the average response of individuals in one group or population versus another group or population yield the attributable fraction (AF) – "the proportional reduction in average disease risk over a specified time interval that would be achieved by eliminating the exposure of interest from the population" whereas other factors remain unchanged (Rockhill, Newman and Weinberg, 1998). The population attributable fraction specific for a population is frequently used by the commercial enterprises and the public as an individual risk factor (Vineis and Kriebel, 2006). As individuals may differ genetically, physiologically, and nutritionally from the population averages, the AF can only be considered an estimate of the risk for an individual. Although genetic association studies that include nutrient intake data explain more of the data (Corella *et al.*, 2009), the amount of phenotypic variation explained by the gene–nutrient interactions is small and may not apply to individuals

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of different genetic or cultural backgrounds. Novel algorithms are being developed to use combinations of individual genetic profiles, population incidence rates of disease, genotype frequency, effect sizes for genetic variants, and models of joint effects among genetic variants on lifetime risk estimates to develop "personalized" risk factors (Yang *et al.*, 2009). However, the risk assessment result varied between 6 and 21% depending upon the assumptions in the calculations (Yang *et al.*, 2009).

Intervention studies also yield information for medical treatments or recommendations for nutritional intakes. Although nutrigenomic and nutritional intervention studies provide preliminary information about optimum diets (Kornman *et al.*, 2007), the small number of individuals in many of the studies and their undetermined genetic ancestry precludes using the information to predict responses in other individuals. The underlying reason for not being able to use information determined solely from genetic association studies is that many if not most genes interact with other genes (or their protein or mRNA products interact). Epistatic (gene–gene) interactions have been shown to alter the influence of individual SNPs on measured phenotypes (e.g., Tuo *et al.*, 2006; Mannila *et al.*, 2006; Hamon *et al.*, 2006; Adjers *et al.*, 2005; Helgadottir *et al.*, 2006; Smith *et al.*, 2008). These specific examples illustrate the need for developing new approaches to study the interaction of genetic makeup and environmental factors that include whole-genome analyses. We have previously described challenges to analyzing gene–nutrient interactions that include the genetic diversity of human populations, complexity of diets and cultures, the intricacies of physiological processes that depend on gene–environment interactions (Kaput *et al.*, 2007a; Kaput *et al.*, 2007b), and the need for new experimental designs that are not based on population studies (Kaput, 2008). These challenges are reviewed in the context of an emerging strategy for human population studies: the use of community-based participatory research methods that have great potential to provide the path for developing recommendations for improving personal and public health (McCabe-Sellers *et al.*, 2008). These new experimental designs are expected to generate more reliable data, but may also address health disparities that have continued to persist in developed and developing countries in spite of several decades of very competent basic science research (Hebert *et al.*, 2009).

2 GENETIC DIVERSITY OF HUMAN POPULATIONS

The promise of personalized healthcare was a natural consequence of the human genome (Venter *et al.*, 2001; Lander *et al.*, 2001) and the subsequent analyses of human genetic variation (Frazer *et al.*, 2007; Consortium, 2003; Consortium, 2004); http://hapmap.ncbi.nlm.nih.gov/). More recent studies have further characterized human populations in geographical or political areas of the world (Table 1) resulting in detailed maps of the genomic architecture of human populations. The Human Genome Diversity Project (http://www.stanford.edu/group/morrinst/hgdp.html) and Human Variome Project (HVP; http://www.variome.org/) are expanding analyses of sequence variations by including individuals in many other human populations. The newly initiated 1000 Genomes project (http://www.1000genomes.org), which is being organized by an international consortium, will employ genome-wide re-sequencing and targeted coding region sequencing in a total of approximately 1500 individuals from three human populations: Europeans, Africans, and Asians (Lang, 2008; Siva, 2008). Each of these populations will be represented by a number of subpopulations consisting of approximately 100 individuals including Yoruba in Ibadan, Nigeria; Japanese in Tokyo; Chinese in Beijing; Utah residents with ancestry from northern and western Europe; Toscani in Italy; and other populations to be determined. The goal of this international effort is to characterize alleles with frequencies of approximately 1% genome-wide and less than 1% in coding regions. Except for the HVP, the phenotype of individuals sequenced in these projects will not be analyzed. Hence, these data will be used for improving selection of reagents and designs for genome-wide association studies and will not be directly focused on identification of disease-causing genes or genes that interact with environmental factors. Resequencing of more genomes is now possible because new sequencing technologies are reducing the cost and increasing the throughput (Metzker, *in press*). Creating a more in-depth coverage of human genetic diversity is necessary for identifying causative SNPs or other genetic variation and to account for epistasis (see above and (Sankararaman *et al.*, 2008; Musani *et al.*, 2007)).

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Table 1. Genetic architecture of selected world populations.

Populations	Individuals	#/type markers	Ref	NutriAlert article
Africa	2432	1327 Microsatellite/INDEL	(Tishkoff *et al.*, 2009)	http://www.nugo.org/nutrialerts/40018
121 African				
4 African-American				
60 non-African				
Asia	1928	54 794 Autosomal SNPs	(Abdulla *et al.*, 2009)	
		50 K XBA Affymetrix Array		
73 Asian				
2 non-Asian				
Europeans				
36 Countries	1387	197 146 SNPs	(Novembre *et al.*, 2008)	http://www.nugo.org/nutrialerts/40263
		Affymetrix 500K		
23 recruitment sites	2457	309 790 autosomal SNPs	(Lu *et al.*, 2009)	http://www.nugo.org/nutrialerts/38843
		Affymetrix 500K		
Iberian Peninsula				
23 Sites	1140 males	Y chromosome markers	(Adams *et al.*, 208)	http://www.nugo.org/nutrialerts/39298
		ABI3100		
Iberean Penisula				
Balearic Islands				
North Africa				
Sepharadic Jewish				
India				
25 sites	132 Indian samples	560 123 Autosomal SNPs	(Reich *et al.*, 2009)	http://www.nugo.org/nutrialerts/40584
		Affymetrix 6.0		
Japan				
8 regions in Japan	7003	140 387 autosomal SNPs	(Yamaguchi-Kabata *et al.*, 2008)	
		Perlagen		
African	60			
European	60			
East-Asian	90			
Mexico				
7 Mexican States	300	99 953 SNPs	(Silva-Zolezzi *et al.*, 2009)	http://www.nugo.org/nutrialerts/40018
		100 K Affymetrix		
Native Americans				
24 Native North, Central, South	422	678 autosomal microsatellite	(Wang *et al.*, 2007)	
2 Siberian	14			
Pacific Islanders				
41 Pacific	952	687 autosomal microsatellites and 203 indels	(Friedlaender *et al.*, 2008)	
Singaporean				
Chinese	99	1 584 040 SNPs	(Teo *et al.*, 2009)	
Malay	98	1 580 905 SNPs		
Indian	95	1 583 454 SNPs		
		Illumina 1 M and Affy SNP 6.0		

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3 COMPLEXITY OF BIOLOGICAL PROCESSES IN DISEASE AND HEALTH

Health and disease processes result from a complex interaction among genes and environment, the result of which is that physiological processes produce variable responses and measurements (Kaput *et al.*, 2007a; Kaput *et al.*, 2007b) and others (Brown *et al.*, 2006; Wolford and Vozarova de Courten, 2004; Gardiner, 2004; van Ommen *et al.*, 2009). Such complexity results from the many genes and pathways that make small contributions to the overall phenotype, the epistatic (gene–gene) interactions that may alter the expression of an analyzed SNP, and epigenetic effects on gene expression caused by variable histone modifications and DNA methylation status. Dietary chemicals directly or indirectly affect or regulate many of these steps (Kaput JaR, 2006; Kaput and Rodriguez, 2004). For example, the substrates for epigenetic mechanisms are derived from the one-carbon metabolism pathway. Interactions exist between genes of the folate/methionine cycle and co-factors that are derived from the diet (Klerk *et al.*, 2002; Gellekink *et al.*, 2005; Rossell *et al.*, 2006; Picard *et al.*, 2004; Blander and Guarente, 2004; Hsiao, Deroo and Archer, 2002; Eberharter and Becker, 2002; Consortium, 2004; Porto *et al.*, 1998; Waterland and Jirtle, 2003). Changes in epigenetic regulation may occur throughout life, but fetuses and developing children may be particularly susceptible to unbalanced nutrition (Dolinoy *et al.*, 2006; Szyf, 2007; Szyf, 2009).

Genetic analyses, whether DNA resequencing or genotyping, coupled with "deep" phenotyping (Tracy, 2008) using proteomic (Kussmann, 2007), metabolomic (Gibney *et al.*, 2005), and transcriptomic (Garosi *et al.*, 2005) technologies, will generate detailed genetic and physiological data for each individual. The use of these technologies is likely to overcome the diversity of challenges of analyzing human genetic heterogeneity (Kaput *et al.*, 2007a; Kaput *et al.*, 2007b) in population-based study designs.

4 NUTRIENT ASSESSMENTS AND STUDY DESIGNS

As the "omic" technologies mature and individual genome data become increasingly available, a fundamental challenge for developing an understanding of complex biological processes is the difficulty in measuring food and nutrient intakes (Rutishauser, 2005; Tucker, 2007). Novel methods, such as photographs of before and after food servings (Kikunaga *et al.*, 2007) and omic analyses linking food exposure to defined biomarkers, may overcome these limitations (see below). A secondary, but no less important challenge is that many population-based studies measure biological complexity at a single point in time using a limited set of biomarkers (with the notable exception of longitudinal studies such as the Framingham Heart Study – http://www.nhlbi.nih.gov/about/framingham/).
These one-time point measures may or may not provide an accurate assessment of a given condition or biomarker, essentially because long-term changes in the nutrient, physical, immune, or psychological environment could alter biomarker levels observed at any single time-point.

5 DEVELOPING THE PATH TO PERSONALIZED HEALTH INTERVENTIONS

Two related concepts have emerged from analyses of the challenges for understanding health and disease and the research of the last quarter century: (i) analyses of genetic diversity between and within populations (see Table 1) demonstrates that it is not possible to have true genetic matches between cases and controls (Lu *et al.*, 2009); and (ii) the development of omics technologies may fundamentally change the design of human studies, particularly human intervention studies. Instead of pre-selecting or assigning individuals to a case or control group, these new technologies can be used to more completely genotype and phenotype an individual, and then sort individuals of like genotype–phenotype–environment exposure (including diet) into groups using dimensionality reduction or classification algorithms (Kaput, 2008; Kaput and Dawson, 2007). This strategy can be used in intervention studies when each individual is analyzed before and after an intervention or exposure to a xenobiotic (e.g., diet, drug), when individuals with the most different phenotypes are compared genetically such as post-menopausal and pre-menopausal breast cancer (Hall *et al.*, 1990), or when individuals can be selected based on variations within functional genes (and not just variants used for genetic mapping) and phenotypes can be compared subsequently. As the full spectra of human genetic and

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phenotypic variation has not been well characterized, the first groups tested would often be those most different in short-term interventions, in established phenotypes, or with the greatest differences in genetic makeups – that is, to determine the extent of the range of variation within the human population. The key aspect of this concept is that membership in the group at each extreme is based on some quantitative measure of phenotype or genotype. Once maximum differences among phenotypes or among genotypes are determined, groups between the extremes can be determined. Although many if not all biological traits are continuous with no discrete breaks in the phenotypic or genetic continuum, such "binning" is a standard for medical practice that uses clinical measurements to group individuals into treatment options and for statistics that rely on tertiles, quartiles, quintiles, etc., to determine structure within experimental data. This approach differs from standard population study designs in that the binning is performed prior to physiological analyses if the genetic variation is predetermined or prior to genetic analyses if different phenotypes are identified. Many human studies discern the groups after experimental data are acquired. A variation of this comparative strategy demonstrated its utility: Holmes *et al.* showed that individuals from different ethnic populations could be clustered based on urinary metabolite levels (Bernini *et al.*, 2009) and blood pressure measurements (Holmes *et al.*, 2008). Although genetic analyses (e.g., Jorde and Wooding, 2004; Tishkoff and Verrelli, 2003; Tishkoff and Kidd, 2004) have shown that variation is greater within ancestral populations (e.g., within Europe) compared with between populations (European versus Asian), the predominant "nutrient"-related alleles (i.e., genes involved in nutrient metabolism) in a population coupled with local cultural food availability and habits may explain the ability to cluster individuals into groups specific to an environment. Hence, creating bins of similar metabolic responses appears feasible.

6 TRANSLATIONAL AND COMMUNITY-BASED PARTICIPATORY RESEARCH

Developing this strategy requires novel approaches to extensively analyze individuals genetically and phenotypically over time to determine how the individual responds to their environment (or intervention). Translational research (Zerhouni, 2007; Zerhouni, 2006) of drug responses or medical treatments is one approach for pharmacogenomic research. That is, monitoring differences in individual responses to drugs for therapeutic use and adverse reaction due to genetic variation and these combined effects could explain decreased or increased burden of disease (e.g., Hosford *et al.*, 2004; Roses, 2000; Srivastava, 2003; Pirmohamed *et al.*, 2004; Holmes *et al.*, 2009).

An extension of the translational research model that may also be used to implement this comparative approach has historical roots in the 1940s but has been emerging from medical practice and from sociological/nutritional research efforts. Participatory research describes a continuum of similar but slightly different approaches (Cornwall and Jewkes, 1995). Some have termed these approaches as community-based participatory research (e.g., Horowitz *et al.*, 2008; Boyer *et al.*, 2005;O'Fallon, Tyson and Dearry, 2000; Israel, 2005), participatory action research, academic–community-based participatory research, or primary care research (e.g., Mold and Peterson, 2005; Beasley *et al.*, 2007; Hueston *et al.*, 2006). The slightly different wording indicates the degree of involvement of community residents in the phases of the research process. Community-based research occurs when the researcher brings the research proposal to the community and asks them to participate with no or little input or decision-making authority (Cornwall and Jewkes, 1995). A consultative approach adopts some of the input from the community members. Collaborative research occurs when the community and researchers work together to design and implement the study, but the overall process is managed by researchers. Many CBPR studies in the 2000s are modeled in this approach. The collegiate model occurs when all parties work together drawing upon different skills while mutual learning takes place but is led by the community members, not the researchers (Cornwall and Jewkes, 1995). Although CBPR has been gaining much interest in the social and nutritional sciences fields (Chen, Jones and Gelberg, 2006; Plumb *et al.*, 2008; see Table 2), relatively few studies have used this method for biomedical research (Boyer *et al.*, 2007; Wells *et al.*, 2006).

The collaborative CBPR method is a cyclic process whereby the participants provide information and biological samples on an ongoing basis, and the biomedical researcher provides existing knowledge

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Table 2. Examples of participatory research programs in the United States.

Location	Program	Populations	Focus	Partners	Ref or Website
Chicago, IL	REACH CSEDCAAC	African American Latino	African American and Latino organizations; disparities in health and access to health care; reducing diabetes	Six provider organizations Three provider/professional organizations Three government agencies Seven community-based organizations One religious group Two business groups One consumer group	(Giachello *et al.*, 2003)
Alaska	CANHR	Native Americans SW Alaska	Obesity as a risk factor for chronic diseases: T2DM and cardiovascular disease	The University of Alaska Fairbanks National Institutes of Health National Center for Research Resources National Indian Health Service Yukon-Kuskokwim Health Corporation Yup'ik community	(Boyer *et al.*, 2005; Mohatt *et al.*, 2007)
Little Rock, AR	Arkansas Special Populations Access Network	Delta, Central AR, and NW AR	Community-based network for cancer control activities	National Cancer Institute University of Arkansas Medical School	http://crchd.cancer.gov/spn/aspan-spcn-publication.html
Marvell, AR	Delta Nutrition Intervention Research Initiative	Lower Mississippi Delta	Community-based intervention Biomedical research, micronutrients, and obesity	Alcorn State University Arkansas Children's Hospital Research Institute Pennington Biomedical Research Center, Southern University and A&M College The University of Arkansas at Pine Bluff, The University of Southern Mississippi Agricultural Research Service (ARS) of USDA, Little Rock, AR. Mississippi Cooperative Extension Service; College of Public Health of the University of Arkansas for Medical Sciences Members from the Delta communities of Marvell, AR; Hollandale, MS; and Franklin Parish, LA. Division of Personalized Nutrition and Medicine, FDA/NCTR	http://www.ars.usda.gov/main/site_main.htm?modecode=62-51-05-00 (McCabe-Sellers *et al.*, 2008)
Detroit, MI	REACH (Racial and Ethnic Approaches to Community Health) Detroit Partnership	African American Latino	Type 2 diabetes and its complications	Six community-based organizations Detroit Health Department Henry Ford Health System University of Michigan School of Public Health	(Kieffer *et al.*, 2004; Feathers *et al.*, 2007)

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Table 2. (*Continued*)

Location	Program	Populations	Focus	Partners	Ref or Website
Detroit, MI	ESVHWP - HEED	Eastside area	Prevent/delay T2DM	Detroit Department of Health and Wellness Promotion Eastside Parish Nurse Network Friends of Parkside Henry Ford Health System Islandview Development Corporation Village Health Workers Warren/Conner Development Coalition University of Michigan School of Public Health	(Schulz *et al.*, 2005)
Los Angeles, CA	CHIC	Low-income LA	Public participation, assessment of the community context, practical trial methods, and health information technology	University of California Los Angeles (UCLA), UCLA Robert Wood Johnson Clinical Scholars, UCLA Family Medicine Research Center, RAND Health, Los Angeles County Department of Health Services, Los Angeles County Department of Mental Health, Los Angeles Unified School District, The Department of Veterans Affairs Greater Los Angeles Healthcare System, Community Clinic Association of Los Angeles County, Healthy African American Families, QueensCare Health and Faith partnership, CDC and NIH	(O'Fallon, Tyson and Dearry, 2000; Wells *et al.*, 2006) www.communitytrials.org/chic-index.htm
Minnesota	IDEAL	Statewide	Diabetes prevention	Minnesota Department of Health Health Partners (managed care)	(Desai *et al.*, 2003)
Baltimore, MD		African-American elementary school-age children who were graduates of a school-based asthma education program	A multifaceted environmental intervention strategy for asthmatic children	East Baltimore Community, Baltimore City Health Department, Baltimore City Department of Education, Clinical Research Unit at Johns Hopkins University, Johns Hopkins Health System Bloomberg School of Public Health Environmental Protection Agency National Institute of Environmental Health Sciences	(Swartz *et al.*, 2004)

(*Continued*)

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Table 2. (*Continued*)

Location	Program	Populations	Focus	Partners	Ref or Website
Pittsburgh, PA	Family SCORE (Family strategies concentrating on risk evaluation)	African-Americans, European-Americans, and others (other ethnicities not specifically identified)	Risk-factor assessment in children to help identify families who are at increased risk for CVD	University of Pittsburgh Urban League of Pittsburgh Pittsburgh Theological Seminary Children's Hospital of Pittsburgh Pennsylvania Department of Health Excellence in Partnerships for Community Outreach and Research on Disparities in Health at the Center for Minority Health, National Institutes of Health/National Center on Minority Health and Health Disparities	www.pediatrics.org/cgi/content/full/118/6/e789
Raleigh, NC	Project DIRECT (Diabetes Interventions Reaching and Educating Communities Together)	African-Americans, European-Americans, and others (other ethnicities not specifically identified)	Reducing the burden of diabetes in the vulnerable high-risk African-American population in southeast Raleigh	Centers for Disease Control and Prevention North Carolina State Department of Health and Human Services Wake County Human Services, Southeast Raleigh Community North Carolina State and Central Universities, University of NC at Chapel Hill Wake Forest University School of Medicine Morehouse School of Medicine Preventive Medicine and Community Health Mel & Enid Zuckerman Arizona College of Public Health	(Din-Dzietham *et al*., 2004)
Lancaster, PA	Amish Family Diabetes Study; Amish Family Osteoporosis Study: Amish Family Calcification Study: Amish Family Longevity Study: and Heredity and Phenotype Intervention)	European-Americans (Amish Population)	Genes that cause diabetes, obesity, osteoporosis, hypertension, and cardiovascular disease	University of Maryland, National Institutes of Health, the Amish community	www.medicalalumni.org/bulletin/fall_2004/lead1.html

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Table 2. (*Continued*)

Location	Program	Populations	Focus	Partners	Ref or Website
Somerville, MA	SUS (Shape Up Somerville)	African-Americans, European-Americans, Hispanic-Americans, Asian-Americans, and other	Determine whether BMI *z*-scores could be decreased among children in grades 1–3	Centers for Disease Control and Prevention, Blue Cross and Blue Shield of Massachusetts, Inc., Blue Cross and Blue Shield of Massachusetts HMO Blue, Inc., United Way of Massachusetts Bay, The United States Potato Board, Stonyfield Farm, Dole Food Company, Tufts University, City of Somerville, Control communities	(Economos *et al.*, 2007; Economos *et al.*, 2009)

as well as results from the ongoing study. However, CBPR does not mandate that all community members become "subjects or participants" in the research, but rather that they are involved in the design, implementation, analysis, and interpretation of the research. The collaborative CBPR model is best suited for biomedical research because of the expertise needed for the analytical procedures for analyzing parameters most relevant to the community, such as their unique dietary and environmental factors (McCabe-Sellers *et al.*, 2008). The community and biomedical partners continually inform each other as the research is conducted and applied. Collaborations are formed between and among the participants and the biomedical partners to design, implement, evaluate, and publish the research. This means that community members are not merely the objects of research but highly engaged in the research process (Ndirangu *et al.*, 2008). Unlike community-based research, CBPR requires a collaborative assessment with key informants and representation from across the community (Ndirangu *et al.*, 2007). CBPR is a slower process than the more traditional intervention research methods but has gained in momentum because developing trusting relationships and enhancing empowerment or ownership promises to be a more sustainable and therefore more effective approach to promoting health through behavioral changes (Israel *et al.*, 1998; Israel *et al.*, 2003; Kone *et al.*, 2000). The goal of CBPR is to build knowledge and understanding of health research for the community and individual and to relay the knowledge attained into action-oriented interventions through behavioral and policy change and to improve quality of life and public health for community members (O'Fallon, Tyson and Dearry, 2000). CBPR is a general approach to the practice of public health and research concerning the social and environmental determinants of health (Ali, Olden and Xu, 2008). We previously described all aspects of developing a CBPR partnership using the 12-year history of the USDA–Delta NIRI research program (McCabe-Sellers *et al.*, 2008).

7 APPLICATIONS TO PERSONALIZING RESEARCH FOR PERSONAL HEALTHCARE

The concept of using CBPR methods for biomedical research is that the participants participate in the research activities from assisting in the planning to helping in the recruitment of other community members. The research can become "personalized" because one individual is assessed and informed. The applications are therefore more immediate than population-based methods and targeted to the community and individual. The development of relationships among researcher and community is challenging but of critical importance for those who have typically been excluded from research studies or those who suffer from culturally based health disparities (Chen, Jones and Gelberg, 2006; Plumb *et al.*, 2008; Boyer *et al.*, 2007; Wells *et al.*, 2006). Most importantly, individuals in socio-economically disadvantaged populations will not benefit from the advances in health research unless their genotypes and cultural environments are included in biomedical research studies. As

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genetic and omic data developed from population studies cannot yet be reliably associated with health outcomes in individuals, the initial information flows between researcher and community collaborator focused on nutritional assessments and dietary advice. As more gene–nutrient or omic–nutrient associations are proven, the information flow will include more biomedical data and results.

8 CONCLUSIONS

The challenges to developing personalized nutrition and medicine applications are the genetic diversity of human populations, complexity of diets and cultures, nutrient variation within the food supply, and the intricacies of physiology dependent on gene–nutrient interactions that differ among individuals. Developing novel strategies to address, account, and analyze these variables is a major challenge for the biomedical research community (Kaput, 2008). Importantly, the traditional nutritional or genetic population-based designs identify risk factors that may not necessarily apply to the individual. An approach to reach the goal of personalizing healthcare is to identify groups within populations with similar metabolic profiles based on similar genetic profiles. Identifying these groups *after* characterizing them with omic technologies (i.e., deep phenotyping) may yield an understanding of the full range of genetic and phenotypic variation in the human population. Community-based participatory research and primary care research provides a path to that goal. Although any one community or primary care facility will not encompass the full range of genetic makeups or phenotypes, replicating this approach in populations throughout the world and using harmonized study designs will allow for combined and comparative data analyses.

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DISCLAIMER

This work has been approved for publication by these agencies but it does not necessarily reflect official agency policy.

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Role of the Endocannabinoid System in Systems Toxicity

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1 INTRODUCTION

Cannabinoids are present in the plant of *Cannabis sativa* and include the most popular compounds used as drugs of abuse. More than 60 cannabinoids have been detected in *Cannabis sativa* plant, of which, besides Δ^9-tetrahydrocannabinol (Δ^9-THC) as the main psychoactive compound, cannabigerol, cannabichromene, cannabidiol, and cannabinol are also present in sufficient amounts (Smita and Sushil Kumar, 2007). Additionally, during the last decade research has highlighted the mechanisms of action of some of the cannabinoids, mainly due to the discovery of molecular targets for such molecules in vertebrates. Such targets include the G-protein-coupled cannabinoid (CB) receptors and their endogenous ligands, the endocannabinoids. The mechanism of action of cannabinoids is best investigated for Δ^9-THC and other CB agonists. The mode action of other cannabinoids of therapeutic interest, for example, cannabidiol (CBD), as well as the carboxy metabolite of Δ^9-THC (11-nor-9-carboxy-Δ^9-THC) is less well established. The majority of Δ^9-THC effects are mediated through agonistic actions at CB receptors (Grotenhermen, 2004). Some non-CB-mediated effects of Δ^9-THC and synthetic derivatives have also been described, for example, some effects on the immune system and some neuroprotective effects.

The discovery of the CB receptor and the availability of highly selective and potent cannabinomimetics led to the rapid identification of a family of lipid transmitters that serve as natural ligands for the CB1 receptor. The two major endogenous cannabinoids are neutral arachidonic acid (AA) derivatives *N*-arachidonoylethanolamine (anandamide or ANA) and 2-arachidonoylglycerol (2-AG). In each case, the carboxylic acid moiety of AA is replaced with a neutral functionality (Kingsley and Marnett, 2007). The pharmacological properties of the endocannabinoids were found to be very similar to those of the synthetic cannabimimetics. The subsequent description of a complex biochemical pathway for the synthesis and release of endocannabinoids completed the scaffold of a new signaling system termed the "endocannabinoid system." It recently became clear that, at least in mammals, the functions of this endocannabinoid signaling system are not limited to the brain, but extended to the whole organism. Endocannabinoids are generally considered to be released from cells "on demand", as no evidence exists for their storage in secretory vesicles, and also several of their biosynthetic enzymes are found in the plasma membrane. Moreover, endocannabinoids act locally on their receptors, possibly due to their high lipophilicity, and are immediately inactivated under physiological conditions (Di Marzo, 2008).

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Several pathways might exist for the formation and catabolism of ANA and 2-AG. ANA originates from a phospholipid precursor, *N*-arachidonoyl-phosphatidyl-ethanolamine (NArPE). NArPE is then transformed into ANA by four possible alternative pathways, the most direct of which is catalyzed by an *N*-acyl-phosphatidylethanolamine-selective phosphodiesterase (NAPE-PLD). 2-AG, when serving as an endocannabinoid, is produced almost exclusively by the hydrolysis of diacylglycerols (DAGs) via *sn*-1-selective DAG lipases (DAGLs) α and β. After their cellular re-uptake, ANA is metabolized by fatty acid amide hydrolase (FAAH) and 2-AG via monoacylglycerol lipase (MAGL). 2-AG is also metabolized to some extent by other recently identified lipases, the $\alpha\beta$-hydrolases 6 (ABH6) and 12 (ABH12), as well as FAAH. Both ANA and 2-AG, possibly under conditions where MAGL or FAAH activity is suppressed, might become substrates for cyclo-oxygenase (COX-2) and give rise to the corresponding hydroperoxy derivatives. The ANA and 2-AG hydroperoxy derivatives can then be converted to prostaglandin ethanolamides (prostamides) and prostaglandin glycerol esters, respectively, by various prostaglandin synthases. These metabolites, although inactive at CB receptors, appear to act at new binding sites for which pharmacological evidence exists. However, the physiological relevance of these pathways is still not fully understood. ANA also interacts with several non-CB receptors, the best established of which is the transient receptor potential vanilloid subtype 1 (TRPV1) channel, where the endocannabinoid binds at an intracellular site. Recently, ANA and 2-AG were reported to activate GPR55, an orphan G-protein-coupled receptor. There is also evidence for interaction of endocannabinoids with peroxisome-proliferator-activating receptor (PPAR)-α and -γ, although at high concentrations. However, CB1 and CB2 receptors are certainly the most-studied molecular targets for ANA and 2-AG, which activate them with different affinity. ANA behaves as a partial agonist at both CB1 and CB2 receptors, but has higher affinity for the CB1 receptor. The intrinsic activity of ANA at CB1 receptors is four- to 30-fold higher than at CB2 receptors. However, 2-AG is a complete agonist at both CB1 and CB2 receptors but it exhibits less affinity than ANA for both CB1 and CB2 receptors (Howlett *et al*., 2002). Importantly, in the brain CB1 receptors are often expressed in presynaptic terminals so that endocannabinoids synthesized from postsynaptic neurons can travel backwards (retrograde signaling) and inhibit neurotransmitter release. Apart from CB antagonists, so far specific blockers have only been developed for FAAH, MAGL, and DAGLs. Figure 1

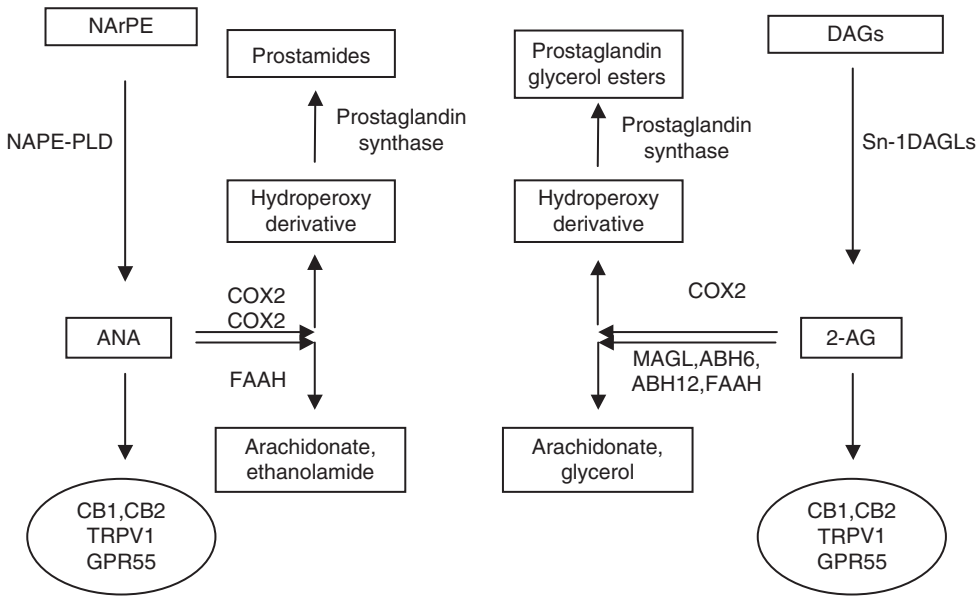

Figure 1. Main biochemical pathways for synthesis, degradation, and cellular actions of ANA and 2-AG.

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illustrates the main biochemical pathways of the two major endocannabinoids, ANA and 2-AG.

In a relatively short time since their discovery, endocannabinoids have been implicated in the regulation of a range of physiological processes in mammals. There are now several examples in almost each of the major therapeutic areas of interest in which alterations in the endocannabinoid system are associated with various disease states. In particular, changes in tissue concentrations of ANA and 2-AG have been noted in many symptoms or disorders, including pain, inflammation, cancer, obesity, immunological, metabolic, cardiovascular, gastrointestinal, and hepatic disorders (Di Marzo, 2008). It is clear that the multitude of physiological (and pathological) processes involving CB receptors give rise to a number of therapeutic targets. Cannabinoids have been used for medicinal purposes for a very long time and the debate regarding whether or not "medicinal marijuana" should be an acceptable form of treatment continues. A key issue will always be the presence of unwanted psychotropic effects of centrally acting cannabinoids, which can be minimized by the use of carefully controlled formulations, although never removed. The reported acute toxicity of cannabis and other cannabinoids remains very low as no one has ever died due to direct and immediate consequence of their recreational or medical use. However, the activation of the cannabinoid system through Δ^9-THC, other phytocannabinoids, and synthetic cannabinoids exerts numerous actions associated with inflammation, cellular atypia, and molecular dysregulation of the tracheobronchial epithelium (Sarafian *et al*., 2005). A toxic side effect of cannabis use that merits attention is the referred short-term effect on the cardiovascular system: increase of heart rate and decrease of blood pressure. Chronic toxicity studies in animals reported alterations in both male and female sexual hormone levels, although there were no adverse affects on human fertility, or chromosomal and genetic damage. Previous studies revealed that Δ^9-THC induced mitochondrial injury in both primary lung epithelial cells and in the A549 cell line (Sarafian *et al*., 2003), by disrupting mitochondrial function and cell energetics (Sarafian *et al*., 2005). The potential contribution of THC to airway injury, by exposing primary cultures of human small airway epithelial (SAE) cells to THC (0.1–10.0 $\mu g\,ml^{-1}$) for either 1 or 7 days, was further examined. THC induced a time- and dose-dependent decrease of cell viability, as also ATP levels, and mitochondrial membrane potential. The same authors further observed alterations at the levels of caspase-8, catalase, Bax, early growth response-1, cytochrome P4501A1 (CYP1A1), metallothionein 1A, PLAB, and mRNA. After 7 days of exposure, the mRNA levels of heat shock proteins (HSPs) and the pro-apoptotic protein Bax were decreased, whereas those of GADD45A, IL-1A, CYP1A1, and PTGS-2 were significantly increased. Such findings suggest a contribution of THC to various cellular functions such as DNA damage, inflammation, and apoptosis. The identification of the different components of the endocannabinoid system supported targets for CB signaling modulation. Thus, it may be possible to target receptors that are not present in the brain (by the topical application of CB1 receptor agonists or the use of specific CB2 receptor agonists). An alternative approach would be to bolster up existing CB signals rather than overlaying new ones using, for example, inhibitors of endocannabinoid uptake and metabolism (Fowler *et al*., 2005). Specific agents that can modulate endocannabinoid system have been developed (Table 1).

2 AIM OF THE REVIEW

This study gives a general perspective of the endogenous cannabinoid system and focuses on its role in the development of system toxicity. Extended discoveries during the last decade regarding the mechanism of action of endocannabinoids and their significant role in pathophysiological conditions have led to the development of drugs capable of modulating their dynamics. The major findings of the reported data implicating the endogenous or exogenous ligands (agonists and antagonists) of CB receptors are presented in Table 2.

2.1 Immune System Modulation

Endocannabinoids are believed to control immune functions and play a role in immune homeostasis. Immune cells express both CB1 and CB2 receptors, secrete endocannabinoids, and have functional cannabinoid transport and breakdown mechanisms. The expression levels of CB2 receptors in immune cells are 10–100 times greater than those of CB1 ones. It has been shown that endocannabinoids

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Table 1. Agents able to modulate the endocannabinoid system according to their mechanism of action.

Agent	Mechanism of action	Reference
Δ^9-THC	CB1/2 agonist[a]	(Zani *et al*., 2007)
CP-55 940	CB1/2 agonist	(Gkoumassi *et al*., 2009)
R-(+)-WIN-55, 212-2	CB1/2 agonist	(Marchalant *et al*., 2009)
ANA	CB1/2 agonist[a]	(Koura *et al*., 2004)
R-(+)-MANA	CB1/2 agonist	(Gustafsson *et al*., 2009)
ACEA	CB1/2 agonist	(Schuelert and McDougall, 2008)
HU-210	CB1/2 agonist	(Michalski *et al*., 2007)
AM-251	CB1 antagonist	(Fonseca, Correia-da-Silva and Teixeira, 2009)
LY 320135	CB1 antagonist	(Muthian *et al*., 2004)
AM 630	CB2 antagonist	(Di Filippo *et al*., 2004)
SR141716A	CB1 selective antagonist	(Izzo *et al*., 2001)
AM 1241	CB2 selective agonist	(Shoemaker *et al*., 2007)
JWH133	CB2 selective agonist	(Feizi *et al*., 2008)
HU-308	CB2 selective agonist	(Qian *et al*., 2009)
SR144528	CB2 selective antagonist	(Eisenstein *et al*., 2007)
AM 404	ANA inhibitor	(Borsani *et al*., 2007)
OL-135	FAAH inhibitor	(Kinsey *et al*., 2009)
URB 597	FAAH inhibitor	(Jayamanne *et al*., 2006)
URB 532	FAAH inhibitor	(Patel *et al*., 2005)
OMDM106	FAAH inhibitor	(Ortar *et al*., 2007)
AM1172	ANA selective inhibitor	(Hillard *et al*., 2007)
VDM11	ANA selective inhibitor	(Murill-Rodríguez *et al*., 2008)
AA-5-HT	FAAH selective inhibitor	(Capasso *et al*., 2005)
PAL	FAAH selective inhibitor	(Capasso *et al*., 2005)
JZL184	MAGL selective inhibitor	(Long *et al*., 2009)
UCM 707	ANA transporter blocker	(Hasanein and Soltani, 2009)

ANA, anandamide; R-(+)-MANA, R-(+)-methanandamide; ACEA, arachidonoyl-2A-chloroethylamide; AA-5-HT, *N*-arachidonoyl-serotonin; PAL, palmitoylisopropylamide.

[a] Partial.

modulate T- and B-cell proliferation and apoptosis, macrophage-mediated killing of sensitized cells, activation, inflammatory cell cytokine production, chemotaxis, and migration. There is also evidence that endocannabinoids biosynthesis, uptake, and degradation occur in macrophages and leukocytes. Such findings support the role of endocannabinoids as local modulators of immune and inflammatory reactions (Pestonjamasp and Burstein, 1998; Bisogno *et al*., 1997). Maccarrone *et al.* (2000) investigated the ability of the human mast cell-1 (HMC-1) line to take up and hydrolyze ANA and how the degradation of this lipid might be controlled. Intact HMC-1 cells were able to accumulate [^{3}H]ANA in a time- and concentration-dependent manner whereas [^{3}H]ANA uptake was almost completely inhibited by the specific ANA transport inhibitor AM404. The uptake was enhanced dose-dependently when incubations were carried out in the presence of nitric oxide (NO) donors, sodium nitroprusside (SNP) (100 mM), or *S*-nitroso-*N*-acetylpenicillamine (SNAP) (0.5, 2.5, 5, and 10 mM). The authors concluded that HMC-1 cells have a specific ANA transporter and an FAAH, characterized for the first time. This suggests an inverse relationship between NO and ANA, which is noteworthy if one recalls that NO is a pro-inflammatory agent. A major finding of this investigation is that HMC-1 cells have negligible basal levels of FAAH activity, which becomes remarkable in the presence of specific 5-lipoxygenase inhibitors. This is noteworthy because hydroperoxides generated from ANA by lipoxygenase have been shown to be potent inhibitors of FAAH. Because HMC-1 cells express an active 5-lipoxygenase, as shown by Macchia *et al*. (1995), it can be suggested that ANA used as a substrate to assay FAAH is converted instead by 5-lipoxygenase into hydroperoxides, which inhibit FAAH activity. As it has been shown (Maccarrone *et al.*, 2001), lipopolysaccharide (LPS) down-regulated FAAH expression and increased ANA levels in human peripheral blood mononuclear cells (PBMCs). Treatment of PBMCs with LPS (0, 10, 25, 50, 100, and 150 $\mu g\,ml^{-1}$)

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Table 2. Effects of CB receptors endogenous and exogenous ligands in various toxicity models.

	Compound	Main findings	Reference
Immune system modulation			
Pathophysiology			
NMDA neuronal damage[a]	ANA	Protected from inflammatory damage	Eljaschewitsch *et al.* (2006)
Molt-4 human tumor cells[b]	ANA	Apoptosis induction	McKallip *et al.* (2002)
ConA-induced hepatitis in mice[a]	ANA	Decreased liver injury	Hegde *et al.* (2008)
Mechanism of action			
OKT3-stimulated human PBMCs, T- and B-lymphocytes[a]	ANA	Inhibited T- and B-cell proliferation	Schwarz *et al.* (1994)
B9 and FDC-P1 murine cells[b]	ANA	Stimulated DNA synthesis related to proliferation	Derocq *et al.* (1998)
Rat basophilic leukemia cells[b]	ANA, PEA	Down-modulated cell activation	Facci *et al.* (1995)
Isolated PBMCs[b]	ANA, MethANA	Failed to inhibit superoxide production	Kraft *et al.* (2004)
Mice[a]	HU-210, HU-211	Suppression of immune response	Titishov *et al.* (1989)
Induction of inflamation			
Pathophysiology			
Rat model of cerulean-induced acute pancreatitis[a]	ANA	Increased or decreased induction of pancreatitis depending on the time of administration	Dembiński *et al.* (2008)
Rat model of toxin A-induced intestinal inflammation[a]	ANA, 2-AG	Increased inflammatory	McVey *et al.* (2003)
Rat model of cerulean-induced acute pancreatitis[a]	ANA, AM251	ANA increased while AM 251 inhibited the disease severity	Dembiński *et al.* (2006)
Closed head injury[a]	2-AG	Protection and reduced BBB disruption	Panikashvili *et al.* (2006)
Mouse model of intestinal inflammation[a]	Cannabinol, CP 55,940	Augmentation of anti-transit effects	Izzo *et al.* (2001)
Induction of fibrosis			
Pathophysiology			
Isolated HSCs from healthy human and rats livers[b]	ANA	Induced necrosis mediated with ROS	Siegmund *et al.* (2005)
HSCs pre-treated with URB597[b]	ANA	Induced necrosis mediated with ROS	Siegmund *et al.* (2006)
BDL-induced cirrhosis in rats[a]	ANA	Induction of intrahepatic vascular resistance	Yang *et al.* (2006)
Cirrhotic patients[a]	ANA	No correlation between plasma levels and systemic hemodynamics or portal hypertension	Fernández-Rodriguez *et al.* (2004)
Primary HSCs and hepatocytes[b]	2-AG	Induced necrosis mediated with ROS	Siegmund *et al.* (2007)
Genetically obese rats[a]	Rimonabant	Eliminated hepatic steatosis	Gary-Bobo *et al.* (2007)
Non-alcoholic steatohepatitis[a]	Rimonabant	Decreased liver injury	Banasch *et al.* (2007)
Mouse model of CCl_4, TAA, BDL-induced liver fibrosis[a]	SR141716A	Decreased fibrosis	Teixeira-Clerc *et al.* (2006)
Rat model of mesenteric resistance arteries in rats with CCl_4-induced cirrhosis[a]	ANA	Induced relaxation	Domenicali *et al.* (2005)
Mechanism of action			
Mouse model of ethanol-induced steatosis[a]	2-AG	Stimulated the expression of lipogenic genes	Jeong *et al.* (2008)
Human hepatic myofibroblasts[b]	2-AG, MethANA	Promoted cytotoxic effects	Julien *et al.* (2005)

(*Continued*)

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Table 2. (*Continued*)

	Compound	Main findings	Reference
IR injury			
Pathophysiology			
Mouse model[a]	ANA, 2-AG	Increased levels were correlated with hepatic IR injury	Bátkai *et al.* (2007)
Rat model of low-flow IR[a]	ACEA, JWH-015	Reduced infarct size	Calignano *et al*. (1998)
Isolated rat hearts in IR injury[b]	ANA, 2-AG, PEA, SR144528	Perfusion with PEA or 2-AG but not ANA decreased myocardial damage	Lepicier *et al*. (2003)

[a] *in vivo*.

[b] *in vitro*.

reduced, in a dose-dependent manner, FAAH activity. FAAH inhibition reached statistical significance ($P < 0.05$) at 25 mg ml^{-1} LPS and a maximum at 100 mg ml^{-1}. As an irreversible FAAH inhibitor also increased the ANA levels (Maccarrone *et al*., 2001), the observed stimulatory action of LPS on ANA levels was considered due to FAAH inhibition. Recently, it has been suggested that ANA is released by CNS tissue as a mechanism that controls and limits immune response in healthy and injured brain (Eljaschewitsch *et al*., 2006). There is a 3.7-fold (5 pmol mg^{-1} w.w.) and 1.9-fold (5 pmol mg^{-1} w.w.) increase of ANA concentrations in inflammatory lesions of patients with active and silent multiple sclerosis (MS), respectively, compared with those noted in healthy controls (2.7 pmol mg^{-1} w.w.). The levels of other endocannabinoids, such as 2-AG and palmitoylethanolamide (PEA), were only slightly influenced in MS patients. The authors also addressed the question regarding the ANA accumulation as a direct consequence of neuronal damage or a result of immune cell invasion. In living brain tissue, a 2.8-fold increase in ANA concentration was detected after induction of specific excitotoxic neuronal damage by *N*-methyl-D-aspartate (NMDA) that was potentiated up to a 13.2-fold increase after invasion of murine microglial cell line (BV-2) microglial cells. BV-2 microglial cells did not induce ANA production in a resting state. Finally, the influence on inflammatory damage to neurons was examined. Incubation with 2 mM ANA completely abolished inflammatory damage but not the primary excitotoxic damage, whereas incubation with ANA alone did not increase neuronal damage. Schwarz *et al*. (1994) investigated the effects of ANA on immune function and compared its potency to that of synthetic cannabinoids. Human PBMCs, T- and B-lymphocytes were prepared and proliferation assays were performed with PBMCs stimulated with murine anti-CD3 antibody, phytohaemaglutinin (PHA) (OKT3) or PHA. The results showed that ANA inhibits accessory cell–dependent T-cell proliferation and accessory cell–independent proliferation of B cells. In all isolates, 10^{-5} M ANA had no effect on cell proliferation, but at 10^{-4} M, it invariably blocked proliferation completely. The inhibitory effects of 3×10^{-5} M ANA ranged from 30 to 80% in cells from different donors. The two synthetic cannabinoids, Δ^8-THC and CP55940, showed similar effects and completely inhibited T-cell proliferation at concentrations of 3×10^{-5} and 10^{-5} M, respectively. Δ^9-THC was of equal potency as Δ^8-THC in inhibiting proliferation of OKT3-stimulated PBMC. Role of endocannabinoids when present at low doses has been also implicated in the migration of B cells along with induction of some cytokines (Titishov *et al*., 1989). In this study, the effects of synthetic agonists HU-210 and HU-211 on the inductive and productive phases of the primary humoral immune response due to sheep red blood cell immunization were investigated in mice. Animals treated with HU-210 (0.01, 0.05, 0.1, and 0.5 mg kg^{-1}) exhibited a dose-dependent suppression of both phases of the primary humoral immune response in the hemolytic plaque assay, the hemagglutination titer, and the ratio of the spleen weight to final body weight. Mice treated with HU-211 (0.01, 0.05, 0.01, 0.5, and 1.0 mg kg^{-1}) did not exhibit dose-dependent immune suppression. ANA seems to potentiate the growth of murine IL-6-dependent lymphoid B9 and the IL-3-dependent myeloblastic FDC-P1 cell lines (Derocq *et al*., 1998). The effect of ANA was examined at optimal concentrations of 0.5–1 μM on B9 and FDC-P1 cells, cultured with increasing amounts of IL-6 and IL-3, respectively. In the absence of any growth factor, ANA

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by itself did not significantly influence the DNA synthesis in both cell lines. However, ANA greatly enhanced (by two- to threefold) the rate of DNA synthesis, when the respective growth factors were present in the culture medium of both cell lines. The stimulating effect of ANA was concentration-dependent with a maximal activation obtained with 0.5–1 μM for B9 and 0.25–0.5 μM for FDC-P1 cells costimulated with either IL-6 50 U ml^{-1} or IL-3 50 ng ml^{-1}, respectively. The addition of 1 μM ANA increased the cell number by 2.2-fold, indicating that the enhancement of DNA synthesis was related to a true proliferative response. In order to examine whether ANA can attenuate ConA-induced hepatitis, exogenous ANA (10 mg kg^{-1}) was administered into mice (Hegde *et al*., 2008). ConA and ANA treatment resulted in decreased AST and ALT levels (12 h ConA challenge) and less liver damage (48 h ConA challenge) compared with ConA and vehicle-injected mice, suggesting that ANA can effectively block ConA-induced hepatitis. There was also a significant decrease in inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-9, IL-12, and IL-17, and in chemokines such as KC, monocyte chemoattractant protein-1, and eotaxin after 12 h ANA treatment. It is noteworthy that ANA treatment in ConA-injected mice failed to decrease IL-2 levels compared with ConA and vehicle-injected ones. Furthermore, PEA, unlike ANA, was highly effective in reducing antigen-evoked [^{3}H]serotonin release from rat basophilic leukemia cells of the secreting subline 2H3 (RBL-2H3) cells (Facci *et al*., 1995). As with the active cannabinoids, in three experiments ANA (12.5 μM) reduced the down-modulatory activity of PEA by 50–100%. These findings indicate that, although both PEA and ANA bound to CB2 receptors *in vitro*, only PEA could down-modulate cell activation.

Dentritic cells (DCs) play a major role in antigen presentation and the development of antigen-specific T-cell responses. 2-AG has been suggested to act as a chemotactic molecule capable of recruiting DCs during innate immune response. Immature bone marrow-derived DCs and/or DCs matured by exposure to peptidoglycan (PGN) were assayed for their capacity to migrate *in vitro* toward various concentrations of 2-AG (1, 5, and 10 μM) (Maestroni, 2004). The authors showed that this effect was real chemotaxis, as 2-AG was ineffective in absence of a concentration gradient, that is, when it was present in both upper and lower wells of the chemotactic chamber. The CB2 receptor antagonist SR 144528 inhibited completely the chemotactic effect of 2-AG, indicating that the observed effect was CB2 mediated. A recent study has shown that ANA failed to inhibit superoxide production in neutrophils, although other synthetic cannabinoid treatment exhibited the inhibition property (Kraft *et al*., 2004). Isolated human PBMCs were incubated with ANA and MethANA at concentrations between 10^{-3} and 10^3 nM. Neither the spontaneous nor the *N*-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced oxygen radical production of human PBMCs was significantly affected under these experimental conditions. In this respect, ANA did not significantly differ from its non-hydrolyzable derivative MethANA. When higher concentrations of ANA, up to 100 μM, were used, the respiratory burst reaction remained unaltered. Finally, it has been demonstrated that ANA can induce apoptosis in immune cells with malignant phenotype. Molt-4 human tumor cells, when cultured for 4 h in the presence of various concentrations of ANA, were found to undergo significant levels of apoptosis as quantified using the TUNEL assay, at concentrations of 20 μM or greater (McKallip *et al*., 2002).

2.2 Induction of Inflammation

Fatty acid profiles and endocannabinoid concentrations were determined in different tissues to examine the possible impact of (n-3) LCPUFA on the dysregulated endocannabinoid system in Zucker rats. The visceral adipose tissue (VAT) ANA concentration was lower in the fish oil (FO) (25 pmol g^{-1}) and krill oil (KO) (15 pmol g^{-1}) groups than in control (38 pmol g^{-1}), whereas 2-AG was similar in FO (330 pmol g^{-1}) and significantly lower than control (330 pmol g^{-1}) only in the KO-supplemented rats (100 pmol g^{-1}). Endocannabinoid concentrations in subcutaneous adipose tissues (SAT) did not differ among the three groups. Liver and heart endocannabinoids were similarly affected in the KO-supplemented rats. ANA concentrations were ∼25% of, and 2-AG concentrations ∼200% of, those of control in both tissues. In FO-fed rats, liver but not heart ANA concentrations were less than in control. Heart, liver, VAT, and SAT FAAH activities did not differ among the experimental groups. Conversely, MAGL activity was significantly lower in the VAT of the FO and KO groups, and in the

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heart tissue of the KO group, compared with control. However, liver MAGL activity tended to be lower, although non-significantly in both groups compared with control (Batetta *et al*., 2009). 2-AG appears to protect acute head trauma by exerting anti-inflammatory effects and by counteracting blood–brain barrier (BBB) damage. In this study, 2-AG ($5\,mg\,kg^{-1}$) was administered immediately after closed head injury (CHI) and determined the BBB permeability 4 h after the trauma. In the left, injured hemisphere 2-AG robustly reduced BBB disruption, as evidenced by 40% decrease in dye levels extracted from the brain ($P < 0.01$), whereas in the right hemisphere, not affected by CHI, no changes in dye levels were noted. As the proteolytic enzymes TACE and MMP are implicated in the disruption of the BBB, the authors furthermore examined whether these enzymes are up-regulated after CHI, and whether the protective effect 2-AG on the BBB is mediated by inhibition of these enzymes. Such results show that TACE expression increased between 2 and 4 h after CHI ($P < 0.01$); however, 2-AG had no effect on TACE and MMP-9 levels (Panikashvili *et al*., 2006). Some recent studies indicate that ANA increases the severity (Dembiński *et al*., 2006) whereas another study exhibits some therapeutic effects (Michalski *et al*., 2007) in acute pancreatitis. Dembiński *et al*. (2006) checked whether the administration of a natural endogenous CB1 receptor agonist (ANA; 0.8, 1.5, or $3.0\,\mu mol\,kg^{-1}$ per dose i.p.) or CB1 antagonist (AM 251; $4\,\mu mol\,kg^{-1}$ i.p.) affects the cerulein-induced acute pancreatitis in Wistar rats. According to this study, ANA pretreatment increased the severity of cerulein-induced pancreatitis evident by increased pancreatic tissue damage, serum concentration of IL-1β and serum activity of pancreatic enzymes (lipase, amylase, and poly-C ribonuclease). Opposite effect was observed after administration of the selective CB1 receptor antagonist, AM 251, which inhibited the development of cerulein-induced acute pancreatitis, as well as reversed the injurious effect of ANA in the same model. The observation that stimulation of CB1 receptors during induction of acute pancreatitis enhances the severity of this disease is in agreement with a previous study by Matsuda *et al*. (2005), who found that administration of CB1 receptor antagonist prolongs the survival of rats with severe necrotizing acute pancreatitis evoked by injection of sodium taurocholate into the bilio-pancreatic duct. Additionally, clinical case report indicates that cannabis may induce acute pancreatitis (Grant and Gandhi, 2004). However, Michalski *et al*. (2007) reported that cerulein-induced acute pancreatitis is associated with up-regulation of ligands and receptors of the endocannabinoid system in the pancreas and administration of a synthetic agonist of CB1 and CB2 receptors, HU210, exhibits some therapeutic effects in experimental as also in clinical acute pancreatitis. Dembiński *et al*. (2008) examined the reason for these opposite confusing results and showed that the influence of ANA on the severity of acute pancreatitis depends on the phase of the inflammation. Experiments were carried out in two series on male Wistar rats and acute cerulean-induced pancreatitis. Administration of ANA before induction of acute pancreatitis has increased the severity of acute pancreatitic injury because it enhanced the pancreatitis-evoked increase in serum activity of pancreatic digestive enzymes (lipase amylase and poly-C ribonuclease), IL-1β, and pancreatic MPO activity. However, administration of ANA after induction of acute pancreatitis results in opposite effects regarding the severity of both symptoms and pancreatic tissue damage (Michalski *et al*., 2008). These authors evaluated endocannabinoids and CB receptor levels as well as the potential function of cannabinoid activation and antagonism by synthetic cannabinoid derivatives and their respective antagonists in either chronic pancreatitis human tissues or derived pancreatic stellate cells (PSC). It was suggested that activation of the endocannabinoid system induces a quiescent phenotype of chronic pancreatitis-derived PSC by down-regulating the production of extracellular matrix (ECM) proteins and inflammatory cytokines. This effect was accompanied by marked changes in PSC appearance toward a less mesenchymal-like phenotype (Michalski *et al*., 2008).

In recent studies, it has been reported that endocannabinoids may be also involved in intestinal inflammation. In a mouse model of intestinal inflammation induced by oral croton oil administration, CB receptor agonists cannabinol and CP 55 940 reduced upper gastrointestinal motility. However, the anti-transit effects of both agonists were augmented during intestinal inflammation (Izzo *et al*., 2001). Both CP 55 940 ($0.03 \pm 10\,nmol\,mouse^{-1}$) and cannabinol (i.p.; $10 \pm 3000\,nmol\,mouse^{-1}$) administration produced a dose-related inhibition of transit and they had a lower ED_{50} value

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compared with the corresponding treatment in control mice. In the same study, ANA and 2-AG were detected in control tissues ($36.4 + 6.1$ and $44.1 + 4.0$ nmol g tissue^{-1}, respectively) and this production was not significantly modified in the small intestine of croton oil-treated mice ($29.2 + 1.6$ and $35.6 + 2.5$ nmol g tissue^{-1}, respectively). By contrast, a significant increase in ANA amidohydrolase activity was observed in croton oil-treated mice. Increased levels of ANA, but not 2AG, have been reported in inflamed colon tissue in ulcerative colitis patients, a finding replicated in a mouse model of colonic inflammation (D'Argenio *et al*., 2006) induced by 2,4-dinitrobenzene sulfonic acid (DNBS) and 2,4,6-trinitrobenzene sulfonic acid (TNBS) in Wistar rats and C57/BJ mice, respectively. Treatment of rats with TNBS caused an almost threefold enhancement of colon ANA, but not 2-AG, levels, limited to the submucosa, with no effect on mucosal endocannabinoid levels. Treatment of mice with DNBS caused a robust enhancement of colon ANA – but not 2-AG – levels, which were already significant after 3 days and reached maximum after a week (fourfold elevation). The same study also included data from human mild-to-moderate ulcerative colitis patients ($n = 8$). A more than twofold elevation of ANA, but not 2-AG, levels was found in mucosal biopsies from patients with untreated ulcerative colitis compared with controls. ANA levels significantly correlated with disease clinical activity, whereas no correlation was found between endocannabinoid levels and endoscopic and histologic figures (D'Argenio *et al*., 2006). The converse was described in a model of allergic inflammation, which was associated with increased levels of 2AG, but not ANA (Oka *et al*., 2005). These authors investigated in detail the possible pathophysiological roles of the CB2 receptor and 2-AG in acute inflammation in the mouse ear, induced by the topical application of 12-*O*-tetradecanoylphorbol 13-acetate (TPA). The amount of 2-AG was markedly augmented following the topical application of TPA (14.4 ± 2.0 pmol μmol^{-1} of lipid P), versus the amount in the vehicle (acetone)-treated controls (4.2 ± 2.0 pmol μmol^{-1} of lipid P) (Oka *et al*., 2005). These findings propose that endocannabinoids levels vary according to the nature of inflammation. A potential cause for these variations would probably originate by the effect of the pathological condition on the levels of enzymes involved in endocannabinoids metabolism and the different activity of such enzymes to the metabolism of endocannabinoids group of compounds.

In another model of acute intestinal inflammation induced by administration of toxin A from *Clostridium difficile* to isolated segments of the rat ileum, McVey *et al*. (2003) have shown that toxin A treatment resulted in increased ANA and 2-AG ileal concentrations and that administration of these substances essentially duplicated the inflammatory effects of toxin A, suggesting that one or both of these endocannabinoids may mediate the inflammatory effects of toxin A. Intraluminal injection of ANA induced dose-dependently intense inflammation in the rat ileum, whereas administration of 2-AG duplicated the effects of ANA on luminal fluid accumulation. However, two synthetic cannabinoid agonists, WIN 55,212-2 mesylate and HU 210, and palmitoylethanolamide did not affect the rat ileum at similar doses. The endocannabinoid-induced inflammation is capsazepine-sensitive, indicating that it is mediated by the capsaicin VR1 receptor. This is confirmed by the fact that ANA also stimulates the capsazepine-sensitive release of SP in the ileum. Both toxin A-induced inflammation and ANA and 2-AG release are potentiated by pretreatment with inhibitors of FAAH (McVey *et al*., 2003).

A growing body of evidence suggests that endocannabinoid signaling plays a critical role in hemorrhagic or endotoxic shock. Wang *et al*. (2001) succeeded in the measurement of around 20 pmol of ANA in the medium of LPS-stimulated mouse macrophage-like RAW264.7 cells, and of around 18 and 30 pmol ml^{-1} of ANA and 2-AG in the sera of some patients with endotoxic shock. In contrast, approximately 4.0 pmol ml^{-1} of ANA and 10 pmol ml^{-1} of 2-AG were detected in normal human serum. The increased amounts of ANA and 2-AG in the sera of the patients with endotoxic shock further supported the notion, established on data obtained from a rat model, that ANA and 2-AG are endogenous mediators of shock-induced hypotension (Wang *et al*., 2001). In addition, the recruitment of inflammatory cells into the intima is crucial for the development and progression of atherosclerosis. Steffens *et al*. (2005) demonstrated that oral treatment with a low dose of THC inhibits atherosclerosis progression in the apolipoprotein E knockout mouse model, through pleiotropic immunomodulatory effects on lymphoid and myeloid cells.

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Oral administration of THC ($1\,mg\,kg^{-1}\,day^{-1}$) resulted in significant inhibition of disease progression. Lymphoid cells isolated from THC-treated mice showed diminished proliferation capacity and decreased interferon-gamma secretion. Macrophage chemotaxis was also inhibited *in vitro* by THC. All these effects were completely blocked by a specific CB2 receptor antagonist.

2.3 Induction of Fibrosis

The endocannabinoid system appears to activate the fibrogenic cells and contribute to the ECM accumulation in the liver. However, the underlying mechanisms are still not well characterized.

The CB1 and CB2 receptors are markedly up-regulated in the cirrhotic human liver, predominantly in hepatic myofibroblasts within fibrotic septa. Experimental studies in which CB1 and CB2 receptors were blocked by either genetic and/or pharmacological manipulations have provided important information on the EC system-dependent regulation of both pro- and anti-fibrogenic responses in the liver (Teixeira-Clerc *et al*., 2006; Julien *et al*., 2005). Indeed, mouse models of liver fibrosis induced by carbon tetrachloride (CCl_4), thiacetamide (TAA), or bile duct ligation (BDL) in either CB1-deficient animals or those treated with the CB1 receptor antagonist rimonabant (SR141716A) presented a significant decrease of fibrosis induction compared with their wild-type littermates. In this study, Teixeira-Clerc *et al*. (2006) investigated the consequences of CB1 receptor inactivation on progression of fibrosis in the three established experimental models. After chronic CCl_4 intoxication, extensive fibrotic septa with some nodules in wild-type mice were noted. That was not confirmed in CCl_4-treated *Cnr1*$^{-/-}$ mice, which showed limited formation of fibrotic septa. Fibrosis area was decreased by 37% in CCl_4-treated *Cnr1*$^{-/-}$ compared with wild-type mice. Similarly, the fibrosis area was reduced by 30% or 35% in *Cnr1*$^{-/-}$ mice exposed to TAA or BDL, respectively. Moreover, treatment with SR141716A strongly reduced fibrosis area by 41% and 34% in the BDL and TAA models, respectively. Hepatic expression of transforming growth factor-β1 (TGF-β1) was also significantly decreased in *Cnr1*$^{-/-}$ or SR141716A-treated mice, compared with wild-type ones. Finally, the number of liver fibrogenic cells was reduced in *Cnr1*$^{-/-}$ and in SR141716A-treated mice, as shown by decreased expression of smooth muscle actin (β-SMA). These results show that genetic or pharmacological antagonism of CB1 receptors reduces the fibrogenic response associated with chronic liver injury, independently of the offending agent. By contrast, CB2-deficient mice present a significant increase in fibrosis following exposure to CCl_4 (Teixeira-Clerc *et al*., 2006). Julien *et al*. (2005) provided the first evidence for the expression of CB2 receptors in the cirrhotic but not in normal human liver and their up-regulation in hepatic fibrogenic cells. Moreover, the authors showed that activation of CB2 receptors triggers growth inhibition and apoptosis in hepatic myofibroblasts and activated HSCs. In contrast, strong CB2 immunostaining was noted in different cells' populations in fibrotic septa, irrespective of the etiology of cirrhosis. CB2 receptor expression was also found in non-parenchymal, ductal epithelial, as well as inflammatory cells in cirrhotic tissues. Accordingly, CB2 receptors were also expressed in cultured human hepatic myofibroblasts. CB2 receptors were also detected in fully activated rat HSC, whereas they were faintly expressed in non-activated ones. THC dose-dependently (0–1000 nM) inhibited DNA synthesis elicited by $20\,ng\,ml^{-1}$ BB homodimeric platelet-derived growth factor (PDGF-BB), with a half-maximal inhibition occurring in the presence of $300\,nmol\,l^{-1}$ of the compound. In these conditions, THC had no cytotoxic effect on human hepatic myofibroblasts. Increasing THC concentrations produced cytotoxic effects toward serum-deprived human liver myofibroblasts, as shown by cell rounding, shrinkage, and detachment. Consistently, THC induced a time-dependent activation of caspase-3, with a maximal increase in activity peaking after 4–6 h; 2-AG (0–20 μM) and methanandamide (0–4 μM) dose-dependently inhibited DNA synthesis and promoted cytotoxic effects. However, growth inhibitory and cytotoxic effects of the compounds were insensitive to pre-treatment with the CB2 receptor antagonist SR 144528. These results therefore suggest that ANA and 2-AG inhibit DNA synthesis and elicit apoptotic effects of human hepatic myofibroblasts by a CB2-independent pathway (Julien *et al*., 2005).

Recent experimental studies in isolated cell models also showed that ANA induces necrosis while 2-AG induces apoptosis in activated HSCs via

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increased ROS generation. However, these effects of EC are likely to be independent of the interaction with CB1 and CB2 receptors (Siegmund *et al*., 2005, 2007). Siegmund *et al*. (2005) isolated primary HSCs by a two-step collagenase perfusion from surgical specimens of healthy human livers ($n = 3$) or from livers of male Sprague–Dawley rats. ANA treatment caused a dose-dependent (25–100 μmol l^{-1}) increase in intracellular Ca^{2+}, which was detectable as early as 3 min after addition of ANA. Pre-treatment with either the extracellular Ca^{2+}-chelators EDTA and BAPTA-tetrapotassium salt or the intracellular chelator BAPTA-AM diminished ANA-induced cell death by 70%, 61%, and 62%, respectively ($P < 0.001$). ANA also caused a marked, dose-dependent (25–100 μmol l^{-1}), and extremely rapid increase in ROS formation. Pre-treatment with the antioxidant GSH completely abrogated the generation of ROS by ANA and reduced ANA-mediated cell death by 40%, indicating that ROS also contribute to ANA-induced cell death (Siegmund *et al*., 2005). The same group (Siegmund *et al*., 2007) investigated the effects of 2-AG on primary HSCs and hepatocytes. 2-AG (0.5, 1, 5, 10, 25, 50, and 100 μM) induced cell death in activated rat HSCs starting at concentrations of 5 μM and induced almost 80% cell death after 18 h of treatment with 25 μM 2-AG. In contrast, primary rat hepatocytes showed no sign of cell death even at the maximum dose used (100 μM 2-AG). After 2-AG stimulation, HSCs generated extremely high amounts of ROS in a dose-dependent manner (0.3, 1, 5, 10, 25, 50, and 100 μM) with substantial induction of ROS formation at submicromolar 2-AG concentrations. Taken together, these data indicate that, at least in experimentally induced liver fibrosis, CB1 and CB2 receptors have opposing effects on fibrogenesis.

The effect of ANA in activated HSCs was further confirmed when Siegmund *et al*. (2006) investigated the ability of FAAH to modulate ANA-induced cell death using isolated primary rat hepatocytes by pre-treating hepatocytes with URB597. To induce liver injury, $FAAH^{-/-}$ and $FAAH^{+/+}$ mice underwent double ligation of the common bile duct. ANA induced an extremely rapid onset of cell death in primary rat HSCs with more than 40% cell death occurring after 2 h at 25 and 50 μM. After 24 h, more than 80% of HSCs underwent cell death, being propidium iodide (PI) fluorescence positive. On the contrary, no alteration in cell death rate after ANA treatment, even at the highest doses used (100 μM), was noted in hepatocytes. URB597 sensitized hepatocytes to the effects of ANA with more than 40% cell death and strong PI uptake, suggesting that FAAH is indeed critically involved in the resistance to ANA. Similar data were obtained using another FAAH inhibitor, oleoyl ethyl amide. Pre-treatment with URB597 also increased ANA-induced ROS production in hepatocytes, whereas URB597 alone did not increase ROS. In contrast to ANA, methANA was capable of inducing cell death in rat hepatocytes ($P < 0.05$). To further investigate the role of FAAH in ANA-induced cell death, the authors constructed an FAAH-expressing adenovirus (AdFAAH), allowing to overexpress FAAH in HSCs. Infection with AdFAAH resulted in a strong expression of FAAH and high levels of FAAH activity in HSCs. AdFAAH induced almost complete resistance to ANA-mediated death up to doses of 25 and 50 μM with less than 20% cell death and no increase in PI uptake. At 100 μM ANA, AdFAAH conferred partial protection to HSCs. In contrast, HSCs infected with the GFP-expressing control virus (AdGFP) remained sensitive to ANA-induced cell death and showed a strong uptake of PI and leakage of GFP as signs of necrotic cell death (Siegmund *et al*., 2006). Additionally, several investigations have now demonstrated that the EC system is highly up-regulated in liver cirrhosis and is implicated in the pathogenesis of severe complications of the disease including portal hypertension, hyperdynamic circulatory syndrome, and cirrhotic cardiomyopathy. The existing data suggest that further experimental model studies are awaited to answer a series of open questions, such as the identification of the EC or other ligands that interact with the CB1 and CB2 receptors, the cellular targets, and the molecular mechanisms that modulate the fibrogenic process.

The EC system appears to be involved in all the events that contribute to portal hypertension and influences also the dynamic component. ANA causes a dose-dependent increase in intrahepatic vascular resistance in the isolated perfused rat liver (Yang *et al*., 2006, 2007). Yang *et al*. (2006) evaluated the possible roles of ANA in the intrahepatic microcirculation of cirrhotic livers and its interaction with the production of vasoconstrictive eicosanoids using Sprague–Dawley rats while cirrhosis was produced by BDL. In the vehicle-pre-incubated group, ANA

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produced concentration-dependent (1.44×10^{-10} to 1.44×10^{-3} M) increases in portal perfusion pressure (PPP) in all cases. Magnitudes of the increases in PPP were significantly greater in cirrhotic than in normal livers for each ANA concentration. Pre-incubation with the Cox inhibitor, indomethacin, significantly attenuated the increased response of cirrhotic livers to ANA (a rightward shift of the concentration–response curves of PPP, $P < 0.05$). However, pre-incubation with the platelet-derived growth-5-lipoxygenase (PDGF-5LOX) inhibitor, piriprost, did not significantly alter the PPP to ANA in cirrhotic livers. However, in normal livers, concentration–response curves of PPP to ANA were not affected by pre-incubation with either indomethacin or piriprost. The ANA-induced increase in PPP was found concomitantly with a significant increase in thromboxane B2 (TXB2) and Cys-LT production in the perfusate. In response to ANA administration, cirrhotic livers exhibited a significantly greater increase in intrahepatic vascular resistance and production of TXB2 and Cys-LTs than normal livers. In a similar study, the same group (Yang *et al*., 2007) evaluated the chronic effects of AM251 on the intrahepatic microcirculation in cirrhotic rats. At 3 weeks after surgery, both BDL and sham-operated rats were randomly assigned to receive either vehicle or AM251 (3 mg kg^{-1} of body weight day^{-1}) for 7 days. The authors state that in a preliminary study, 7 days of sequential doses of AM251 (1, 3, 5, and 7 mg kg^{-1} of body weight day^{-1}) were given intraperitoneally to all animals. It was found that 1 mg kg^{-1} of body weight day^{-1} did not significantly affect portal venous pressure (PVP), whereas 3 mg kg^{-1} of body weight day^{-1} resulted in a significant reduction in PVP. The administration of 5 or 7 mg kg^{-1} of body weight day^{-1} did not decrease PVP further. Thus, in chronic AM251-treated cirrhotic rats, a decrease in portal venous pressure was associated with the decrease in intrahepatic vascular resistance and superior mesenteric artery blood flow. Furthermore, acute infusion of AM251 resulted in a decrease of blood flow and an increase of resistance.

Batkai *et al*. (2001) showed that rats with biliary cirrhosis have low blood pressure, which is elevated by the CB1 receptor antagonist SR141716A. The low blood pressure of rats with CCl_4-induced cirrhosis was similarly reversed by SR141716A, which also reduced the elevated mesenteric blood flow and portal pressure. The results of this study implicate ANA and vascular CB1 receptors in the vasodilated state in advanced cirrhosis and indicate a novel approach for its management whereas elevated concentration of ANA has been observed in monocytes. Increased ANA levels were also found in the plasma of cirrhotic patients (Fernández-Rodriguez *et al*., 2004), but no correlations were found between plasma levels and systemic hemodynamics, portal hypertension, and hepatic and renal dysfunction. Plasma ANA levels were higher in patients than in healthy subjects; however, they were not different between patients with and without ascites. Furthermore, no correlation was observed between ANA levels and the systemic vascular resistance and cardiac indices. In addition, no correlation was found between these levels and the Child–Pugh' score, the glomerular filtration rate (GFR), the renal blood flow, the renal vascular resistance, the plasma renin activity, and aldosterone concentration or the hepatic venous pressure gradient.

The cellular mechanisms that mediate the vasodilating effect of the EC in cirrhosis have been further elucidated by Domenicali *et al*. (2005) in a model of mesenteric resistance arteries isolated from rats with CCl_4-induced cirrhosis complicated with ascites. ANA (10 μM) produced a concentration-dependent (10^{-8} to 5×10^{-4}) relaxation of phenylephrine pre-contracted mesenteric arteries. Incubation with the TRPV1 antagonist, capsazepine (5 μM), and the CB1 receptor antagonist, SR141716A (3 mM), did not significantly alter the maximal relaxation. When both antagonists were incubated together, there was stronger inhibition of the response to ANA at the concentrations used. Nevertheless, the limited solubility of ANA precluded the use of higher concentrations and the construction of a full concentration–response curve. In addition, preliminary data indicate that, in a rat model of pre-ascitic CCl_4-induced cirrhosis, the administration of the CB1 receptor antagonist rimonabant dose-dependently reduces the incidence and accumulation of ascites after 2 weeks of treatment at the doses of 3 or 10 mg kg^{-1} day^{-1} (Domenicali *et al*., 2008). Ascites formation (V: 90%; R3: 54%; R10: 10%) and volume [from 5.5 ± 0.8 ml (V) to 1.6 ± 0.3 ml (R3) and 0.5 ml (R10)] ($P < 0.05$) were reduced. During week 1, rimonabant significantly improved sodium balance, an effect that became even more evident during week 2. Both treated groups showed lower cardiac output and higher MAP and peripheral vascular resistance.

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The liver fibrosis score was reduced by about 30% in the R10 ($P < 0.05$ vs. V) but not in R3. MAP was inversely correlated with sodium balance ($R = -0.77$; $P < 0.001$), whereas no significant correlations were found between fibrosis score and either sodium balance or hemodynamics. Rimonabant also appears to improve renal sodium balance and delays decompensation in pre-ascitic cirrhosis (Domenicali *et al.*, 2009). A significant reduction in ascites formation (group 1: 54%; group 2: 10%; vehicle: 90%) and volume (group 1: 1.6 ± 0.3 ml; group 2: 0.5 ml; vehicle: 5.5 ± 0.8 ml) occurred in treated rats. Rimonabant significantly improved sodium balance during week 2 (group 1: 0.98 ± 0.08 mmol; group 2: 0.7 ± 0.08 mmol; vehicle: 3.05 ± 0.11 mmol). Both treated groups showed lower cardiac output and higher mean arterial pressure, peripheral vascular resistance, and renal blood flow ($P < 0.05$). Liver fibrosis was reduced in group 2 by 30% ($P < 0.05$ vs. vehicle). Mean arterial pressure inversely correlated with sodium balance ($R = -0.61$; $P = 0.003$), but not with fibrosis score. However, steatosis of genetically obese Zucker rats can be dramatically reduced by chronic CB1 blockade. It was known that fat retention might lead to inflammation, facilitating cell injury and deposition of fibrotic tissue. Once-daily treatment of the obese (fa/fa) rats with rimonabant (30 mg kg^{-1} orally for 8 weeks) completely eliminated this hepatic steatosis. The liver of the obese (fa/fa) rats treated with rimonabant was found to be histologically comparable to that of lean animals. In contrast, the severe hepatic steatosis persisted in the pair-fed obese (fa/fa) rats. Furthermore, rimonabant treatment of obese (fa/fa) rats strongly reduced the elevated plasma levels of liver injury enzyme markers ALT, GGT, and ALP. In contrast, submitting obese (fa/fa) rats to pair feeding did not affect the plasma levels of these enzymes, which remained high (Gary-Bobo *et al.*, 2007).

It was recently reported that SR141716 (10 mg kg^{-1}) treated C57BL/6N mice were resistant to the hepatic steatogenic induced effect of ethanol. Similar to the rimonabant-treated wild-type mice, $CB1^{-/-}$ ones were resistant to the steatogenic effects of ethanol and were also resistant to its hepatotoxic effects. Furthermore, it resulted in a significant increase in the hepatic expression of the gene encoding the CB1 receptor and in the hepatic levels of 2-AG, but not ANA. This latter change occurred selectively in HSCs. Once produced, the 2-AG stimulates the expression of the lipogenic genes SREBP-1c and FAS and, thus, the deposition of fat in neighboring hepatocytes by binding overly expressed CB1 receptors in a paracrine fashion. In stellate cells from ethanol-fed compared with pair-fed mice, the gene expression of DAGLb was significantly increased whereas the expression of DAGLa and monoglyceride lipase (MGL) remained unchanged. These findings suggest that the increased 2-AG content of stellate cells from ethanol-fed mice is related to increased biosynthesis of 2-AG (Jeong *et al.*, 2008). Another report describes the successful treatment of non-alcoholic steatohepatitis (NASH) with rimonabant in 21-year-old Caucasian woman with severe obesity (Banasch *et al.*, 2007). Three months of rimonabant treatment (20 mg day^{-1}) were associated with a marked reduction in plasma of ALT, AST, and liver GGT enzyme levels by 74%, 72%, and 72%, respectively. In addition, non-invasive *in vivo* monitoring of hepatic mitochondrial function by 13C-MeBT indicated a distinct improvement of hepatic metabolic capacity (92% change in week 12 compared with baseline).

2.4 Ischemia/Reperfusion Injury

Hepatic ischemia/reperfusion (IR) injury is a significant clinical problem involved in the liver failure associated with circulatory shock, hepatic surgery, and liver transplantation.

Bátkai *et al.* (2007) used pharmacological agonist JWH-133, and antagonist/inverse agonist of cannabinoid CB2 receptors SR144528, as well as CB2 receptor knockout ($CB2^{-/-}$) mice to study the role of the endocannabinoid system in an *in vivo* model of liver IR injury. Interestingly, the hepatic ANA and 2-AG contents correlate with the plasma concentrations of transaminases and pro-inflammatory cytokines. In addition, the authors studied the effects of JWH133 on TNF-α-induced ICAM-1 and VCAM-1 expression in human liver sinusoidal endothelial cells (HLSECs) and adhesion of human neutrophils to HLSECs *in vitro*. The authors demonstrated that pre-treatment of mice with a CB2 receptor agonist JWH133 decreases inflammatory cell infiltration, tissue and serum TNF-α, MIP-1α, and MIP-2 levels, tissue lipid peroxidation, and expression of adhesion molecule ICAM-1. CB2 activation also attenuates the TNF-α-induced ICAM-1 and VCAM-1 expression

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in HLSECs and adhesion of human neutrophils to HLSECs *in vitro*. These findings suggest that activation of the hepatic EC system following IR can limit the degree of tissue injury by stimulating the CB2 receptors. In contrast, $CB2^{-/-}$ mice developed increased IR-induced tissue damage and proinflammatory phenotype. Reperfusion, but not ischemia alone, triggers a marked elevation of the plasma ANA and 2-AG levels (Kurabayashi *et al*., 2005), as well as of the hepatic ANA, 2-AG, and *N*-oleoyl-ethanolamine (OEA) concentrations, which are produced by all the major cell types of the liver (hepatocytes, Kupffer cells, endothelial cells). Such findings also suggest that an IR-induced activation of hepatic endocannabinoids may limit the extent of tissue injury via stimulation of CB2 receptors. Furthermore, various studies have been performed, again mainly in isolated rat heart models, to clarify the potential cardioprotective role of endocannabinoid and ANA-related mediator signaling in I/R injury. Perfusion with PEA or 2-AG, but not ANA, decreased myocardial damage in isolated rat hearts, whereas CB2 antagonism with SR144528 totally abolished the beneficial effects of PEA and 2-AG (Lepicier *et al*., 2003). The CB1 antagonist rimonabant only partially blocked the effect of 2-AG. However, selective agonists for both CB1 and CB2 receptors, ACEA and JWH-015, also reduced the infarct size in this isolated rat heart model of low-flow IR. The effect of SR144528 on PEA-mediated actions is surprising because it is considered to act through CB1- and CB2-independent pathways. Previous findings may suggest that the CB2 antagonist SR144528 may also antagonize some of the effects mediated by PPAR-α agonists. Indeed, it has been described that the analgesic effects of PEA and other PPAR-α ligands are antagonized by SR144528 (Calignano *et al*., 1998; LoVerme *et al*., 2006). The above-described findings are somewhat conflicting with a subsequent study published by Underdown *et al*. (2005). In an isolated rat heart model of no-flow ischemia and reperfusion, ANA perfusion reduced the infarct size, which could be blocked by either CB1 or CB2 antagonism, using rimonabant or SR144528, respectively (Underdown *et al*., 2005). However, CB1 and CB2 agonists (ACPA and JWH-133) could not mimic the effect of ANA.

Endocannabinoids acting via CB1/CB2-dependent or independent mechanisms have also been implicated in the protection conferred by various forms of pre-conditioning (including ischemic) of the myocardium. In a whole animal model of myocardial IR injury induced by coronary occlusion/reocclusion in anesthetized mice, the published evidence points to the protective role of CB2 but not CB1 receptor activation (Lamontagne *et al*., 2006). In a recent study using non-selective CB agonist WIN55212–2 and CB2 antagonist AM630 in a mouse model of myocardial IR, the reduction of leukocyte-dependent myocardial damage could be attributed to CB2 receptor activation because the protection afforded by WIN55212–2 could be prevented by AM630, but not by CB1 antagonist AM251 (Di Filippo *et al*., 2004).

ANA is likely to play an important role in the control of vascular tone, as supported also by the observation that both rat endothelial cells and macrophages can release it (Randall *et al*., 1996; Deutsch *et al*., 1997). Randall *et al*. (1996) suggested that ANA is a candidate for endothelium-derived hyperpolarizing factor (EDHF). To address this question, the effects of SR141716A were investigated against endothelium-dependent relaxations both in rats and in isolated perfused rat superior mesenteric arterial bed. Relaxations of the mesentery responses to ANA (300 nmol; $n = 5$) were unaffected by removal of the endothelium but were antagonized by 1 mM SR141716A and reduced in the presence of high K (60 mM) buffer. Deutsch *et al*. (1997) showed that ANA, via CB1 receptors, stimulates renal endothelial cells to release NO, which is an important mediator of the hemodynamic and neurological actions of ANA in the renal vasculature. In this preparation, NO release was more sensitive to ANA (10 nM) than were the blood-perfused juxtamedullary afferent arterioles, where 1 μM was needed to elicit significant vasodilation. ANA (1 μM) also stimulated NO release from cultured renal microvascular endothelial cells. NO release from cultured endothelial cells was inhibited by *N*-nitro-l-arginine methyl ester (L-NAME). Sugiura *et al*. (1998) also examined whether or not 2-AG can be released from human umbilical vein endothelial cells and human aortic smooth muscle cells. About 35% of [^{3}H]AG was released into the medium from either control or thrombin-stimulated cells. It was found that 2-AG is the predominant species of AG in endothelial cells: about 80–90% of AG was 2-AG in both control and thrombin-stimulated cells. It was also found that the level of 2-AG was markedly elevated in thrombin-stimulated cells (195% of control).

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3 CONCLUSIONS

Different studies emphasize participation of the endogenous cannabinoid system in physiological and pathological conditions. Additionally, for the same condition different studies report up- or down-regulation of the CB receptor-mediated signaling exerting either protective or harmful effects. Alterations in ANA and 2-AG tissue levels have been recorded in inflammation, immunological, cardiovascular, and hepatic disorders. Of interest, changes in endocannabinoid tone in the same direction often accompany opposing symptoms, or levels of ANA and 2-AG change in different or even opposing ways during the same condition. It is becoming increasingly clear that, within a certain tissue, the endocannabinoid system is affected in more than just one way by a given stressful or pathological stimulus, depending on the nature and duration of this stimulus, subsequently leading to more than one functional outcome. For this reason, the data existing so far implicate these mediators in disease states and target organ toxicity but further studies are needed.

RELATED ARTICLES

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Integrative Analysis of Microarray Data: A Path for Systems Toxicology

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1 AFFYMETRIX MICROARRAYS

The Affymetrix GeneChip® 3′ IVT arrays consist of probes of 25 base pair length synthesized with a photolithographic method on quartz wafers (Dalma-Weiszhausz *et al*., 2006; Fodor *et al*., 1991). For a tissue sample, dye-labeled RNA is hybridized to the array. Samples are compared by using different arrays as experimental replicates. The array used for hybridization is scanned to generate an image with light spots for probes dominantly present among the RNA. The scanned image hybridization values are derived for each probe.

Probes are organized in pairs. The perfect match probe (PM) has full complementarity to the target sequence. The mismatch probe (MM) is similar to its corresponding PM probe but the 13th nucleotide is changed to its base pair complement. The MMs are introduced with the intention of measuring local background signal.

Transcript sequences are drawn from public databases such as UniGene, RefSeq, or Ensembl. Transcript abundance is measured by several probes with different sequences that are distributed across the gene sequence. This set of probes constitutes a probe set unique to a gene. Probe sets comprise 4–11 probes depending on the chip platform. For example, in the popular mouse 430 2.0 array, there are 11 probes in one probe set. An annotation of probe sets to genes is provided by Affymetrix (Affymetrix, 2009). But with new releases in the sequence databases probes may become obsolete, either because they do not hit the target sequences anymore or because specificity is reduced by single-nucleotide polymorphisms. Obsolete probes are supposed to introduce noise. In the R/BioC package "customCDF ", the probes are remapped to gene sequences and are reorganized in new probe sets (Dai *et al*., 2005).

2 STANDARDIZED PROCESSING OF AFFYMETRIX EXPRESSION ARRAYS

Integration and meta-analysis of different data sets requires standardized data analysis (Jensen and Steinmetz, 2005). One possible framework for automation of the processing is the R/BioConductor framework (Gentleman *et al*., 2004; R Development Core Team, 2005). Within this framework, biologically motivated corrections of the primary data are performed using statistical models. The processing comprises three steps: determination of test cases, pre-processing and data evaluation, see Figure 1.

Most experiments have a case control design comparing two groups of samples. Without loss of generality these groups are denoted as treatment and control and constitute a test case. For both groups,

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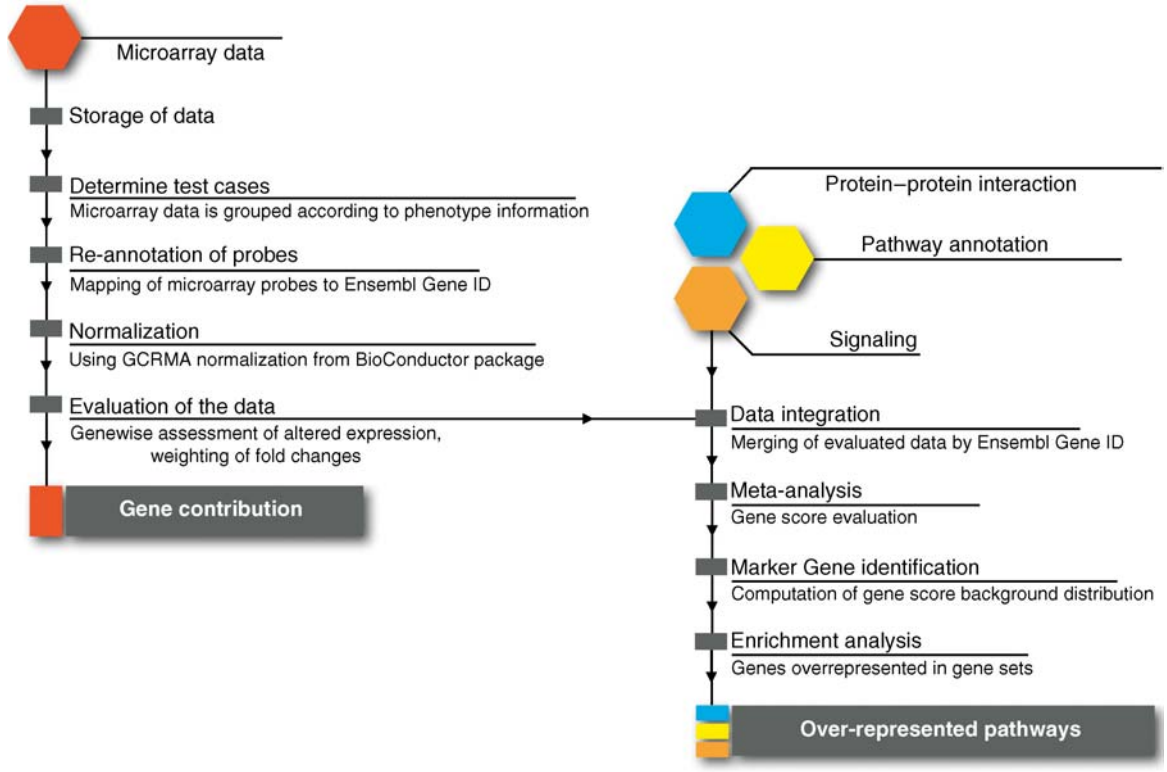

Figure 1. Data workflow for the meta-analysis.

replicates should be generated for solid statistical analysis. For a test case, the generated chips are normalized with each other.

Preprocessing accounts for three major influence factors in the hybridization data: background expression, probe binding affinity and measurement variation (Clevert and Rasche, 2009). After years of research, several methods are available for pre-processing. As an example, GC-corrected robust multiarray analysis (GC-RMA) is presented as a three-step procedure: background correction removing unspecific intensities from scanner images, reduction of non-biological differences between chips, and summarization of probe intensities to probe set expression values (Wu and Irizarry, 2004). Probe hybridizations consist of technical artifacts such as chemical background, optical background, or cross-hybridization. GC-RMA corrects for such effects taking into account the GC content of the probe sequences, that is, the number of G or C nucleotides in the sequence. A higher GC content is associated with a higher binding affinity of the probes because of three instead of two covalent bindings for single nucleotides. GC-RMA contains a position-specific model correcting the binding affinity between probes.

Between chips, unwanted effects are introduced by RNA extraction, pipetting, temperature fluctuations, hybridization efficiency and more. To reduce these effects, the quantile normalization is implemented in GC-RMA (Bolstad *et al.*, 2003).

Finally, probe intensities are summarized into probe set expressions. GC-RMA uses median polish (Irizarry *et al.*, 2003). Median polish proposes a linear model of a baseline hybridization with two factors: a probe effect and an array effect. The model is fitted robustly with a median decomposition.

An advantage of the probe set design is the possibility of calculating a presence tag, that is, the probability that the corresponding gene is duly expressed and active in the sample under study. Unexpressed genes confuse the results with low intensities, leading to high, unmotivated fold changes. The presence tag, or detection *p*-value, is

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based on a comparison of raw perfect match values to corresponding mismatch values. Using a robust Wilcoxon test the result are *p*-values for subsequent interpretation.

The computation of gene expressions and presence tags is followed by a gene-wise evaluation of treatment versus control expressions. Expressions are assessed by three criteria: presence, variation, and alteration (Rasche, Al-Hasani and Herwig, 2008). For the approach of a meta-analysis as presented in this chapter, the three criteria are condensed into a single score for every gene. The score s_i of the i-th gene is computed as follows:

$$s_i = \begin{cases} |\log 2\,(r_i)| \left(1 - \frac{e_i}{r_i}\right)(1 - p_i), & p_i \leq 0.1 \text{ and } e_i/r_i \leq 1 \\ 0, & \text{else} \end{cases}.$$

Here, r_i is the fold change, p_i is the average detection *p*-value, and e_i is the standard error of the ratio derived from the experimental replicates of the study. Thus, the fold change is weighted with its reproducibility across the experimental replicates and with the likelihood of the gene being expressed in the study's target samples.

3 STORAGE OF EXPRESSION ARRAY DATA SETS

The use of microarray techniques generates large amounts of raw and processed data. A data repository capable of storing all information is a crucial requirement for an integrative analysis approach. BASE (BioArray Software Environment) is a MIAME (Minimum Information About a Microarray Experiment) compliant platform that provides an integrative framework for data storage, management and analysis (Brazma *et al*., 2001; Vallon-Christersson *et al*., 2009). It offers a web browser user interface, a laboratory information management system (LIMS) for arrays and biomaterials, features batch import/export functionality of data, and integrates tools for data analysis and visualization. Furthermore, it is possible to write extensions and plug-ins to add further functionality to the whole system, such as data transformation, data viewing, and analysis modules. BASE is written in Java and data are stored in a relational database (e.g., MySQL) in flat files. All software necessary to run a local installation is freely available. This and similar databases can be efficiently used for storing and querying microarray data.

4 EXPERIMENT ANNOTATIONS AND TEST CASE SETUP

Heterogeneous annotations on biological, technical, and hierarchical levels are necessary to define the initial test cases for a meta-analysis and are fundamental for analysis. Each microarray hybridization experiment is accompanied by biological and technical annotations. Biological annotations comprise information about the sample, for example, species, cell line, tissue source and condition. Depending on the experimental focus, this list will need to include additional fields. For example, the investigation of the effect of chemical compounds on different cell lines implies that information about compound dosage and time points are given. Technical annotations comprise information surrounding the hybridization, for example, labeling, RNA/DNA extraction protocol, array platform, quality control data, and personnel involved. This information is necessary for quality control and correct grouping of raw data.

The following meta-analysis approach illustrates a typical microarray data analysis. It investigates the effect of chemical compounds on diverse biological materials. All test cases are defined by species, biological target (e.g., tissue, cell line), compound, dosage and time. As an additional point, we introduce the meta-analysis of different compounds and different experimental platforms in order to derive a generalized picture of compound action.

5 SCORING OF TOXICITY RELEVANCE BY META-ANALYSIS OF MICROARRAY DATA SETS

Necessary for any meta-analysis is the consolidation of the different identifier types, species, or arrays (Rasche, Al-Hasani and Herwig, 2008). The Ensembl database provides a stable reference for microarray studies (http://www.ensembl.org; version 55). As comparability of chip studies is hindered by the total number of probes and pre-processing issues between manufacturers, the analysis in this chapter constrains on studies using Affymetrix arrays. Expression results from the arrays are mapped to Ensembl by the customCDF annotations (Dai *et al*., 2005). The whole data analysis is performed in R/BioC (Gentleman *et al*., 2004; R Development Core Team, 2005).

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Table 1. Data sources.

Compound name	CAS	Species	Tissue (no. test cases)	Data source	Exp. Type
2-Amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP)	105650-23-5	Human	Lung (4)	E-MIMR-102	*In vitro*
Bis-(2-chloroethyl) sulfide (sulfur mustard)	505-60-2	Rat	Lung (6)	GSE1888	*In vivo*
4-Nitroquinoline 1-oxide (4-NQO)	56-57-5	Mouse	Lymphoma (9)	E-TOXM-1	*In vitro*
Ochratoxin A (OTA)	303-47-9	Rat	Kidney (5), Liver (5)	GSE2852	*In vivo*
Carbonyl chloride (phosgene)	75-44-5	Mouse	Lung (8)	GSE2565	*In vivo*
			Total no. 37		

The effect of compound exposure to biological systems is manifold. In this meta-analysis approach, we investigated the influence of five compounds on several cell lines and tissues, identifying a group of genes related to the overall toxicity of the chemicals under study. Furthermore, we explored functional information and identified pathways associated with this core set of genes.

All studies are listed in Table 1. 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) is a heterocyclic carcinogen in the human diet and has been studied in human lung adenocarcinoma A549 cells at several time points using the Affymetrix HG U95Av2 platform (Giard *et al*., 1973). Male Sprague–Dawley rats were exposed to the cytotoxic chemical sulfur mustard at several time points and lung tissue extracts hybridized on the Affymetrix RAE230A platform (Dillman *et al*., 2005). Transcription profiling of mouse lymphoma L5178Y cells treated with the tumorigenic compound 4-nitroquinoline *N*-oxide at several time points was performed on the Affymetrix MG_U74A platform (Newton, Aardema and Aubrecht, 2004). Ochratoxin A is a common food-contaminating, potentially carcinogenic and weakly mutagenic mycotoxin studied at several time points in male Fischer 344 (F-344) rats using the Affymetrix RG_U34A platform (Marin-Kuan *et al*., 2006). Phosgene is a highly toxic gas that was tested at several time points on male CD-1 mice using lung tissue extracts for transcription profiling on the Affymetrix MOE430A platform (Sciuto *et al*., 2005). Hybridization raw data and experimental information from these studies were downloaded from GEO (Barrett *et al*., 2009) or ArrayExpress (Parkinson *et al*., 2007). In total, 37 test cases were defined, representing the five compounds at investigated time points and dosages.

As described above, the gene expression alteration in every study is quantified with a score. For every gene, the scores from individual studies are summarized constituting a gene score. These gene scores now introduce a ranking among the total set of 14 035 analyzed genes, highlighting genes with special toxicological relevance. To assess the significance of the gene scores, a background distribution of scores is computed. In a bootstrap approach, random scores are drawn from each study. Genes with a score above the 99.9% threshold of the background distribution are called significant, and for this study, a cut-off score value of 6.041 was computed. This procedure results in a core set of 956 genes that show significant toxicity relevance in the data sets under study.

6 IDENTIFICATION AND EVALUATION OF TOXICOLOGIC MARKER GENES

The 10 genes with highest scores are listed in Table 2. For example, *EGR1* encodes a C2H2-type zinc-finger protein that functions as a transcriptional regulator, targeting genes involved in differentiation and mitogenesis. *FOS* is one of the four Fos gene family members that encode leucine zipper proteins. These proteins can form, among others, the transcription factor complex AP-1, which controls several processes including cell proliferation, differentiation, transformation, and apoptosis. *BTG2* encodes a member of the BTG/Tob protein family. This gene is an anti-proliferative tumor suppressor, the encoded protein being involved in the regulation of the G1/S transition of the cell cycle.

These examples illustrate the general cellular response to diverse carcinogenic and toxic compounds leading to the activation of MAPK signaling pathways and DNA damage response pathways affected by genotoxic mode of actions of the different compounds.

To further validate the functional context of the marker genes with respect to biological networks, enrichment analyses based on the hypergeometric distribution assess whether these genes are over-represented. By this procedure, the 956 genes

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Table 2. Top 10 highest scored genes.

General meta-analysis			Meta-analysis ochratoxin A		
Rank	HUGO	Score	Rank	HUGO	Score
1	EGR1	28.41	1	SLC6A18	11.04
2	GCLC	25.94	2	DBP	10.28
3	MT1H	21.28	3	OAT	8.98
4	MT1A	19.05	4	PREP	8.15
5	FOS	18.9	5	HAAO	8.01
6	DNAJB1	18.83	6	HBB; HBD	7.95
7	MT1CP	17.44	7	IGFBP1	7.46
7	MT1DP	17.44	8	CYP2D6	7.37
9	DUSP1	15.27	8	CYP2D7P1	7.37
10	BTG2	15.24	10	SLC2A5	7.08

identify 339 significantly over-represented pathways with a *p*-value below 0.05 out of a total of 1830 pathways. The 10 most significant pathways are listed in Table 3.

Twenty-two significant genes are over-represented inside the *HIF-1-α* transcription factor network consisting of 63 members. This network functions as a master transcriptional regulator of the adaptive response to hypoxia and is involved in embryonic vascularization, tumor angiogenesis, and pathophysiology of ischemic disease. Further cancer-related pathways include Aurora A signaling (rank 22, *p*-value 2.43E−05), p53 signaling pathway (rank 35, *p*-value 1.51E−04), and pathways involved in cell maintenance such as metabolic pathways, cell cycle, and biological oxidations (rank 16, *p*-value 1.49E−05).

Table 3. Data sources.

CPDB pathway ID	*p*-value	Description
CPDB_PID_543	6.08E−011	*HIF-1-α* transcription factor network
CPDB_KEGG_65	7.01E−011	Metabolic pathways
CPDB_Reactome_221	4.50E−009	Glutathione conjugation
CPDB_KEGG_223	7.02E−008	Glutathione metabolism
CPDB_Reactome_188	3.00E−007	Integration of energy metabolism
CPDB_KEGG_329	5.06E−007	Metabolism of xenobiotics by cytochrome P450
CPDB_KEGG_709	7.03E−007	Fatty acid metabolism
CPDB_BioCarta_894	8.44E−007	oxidative stress-induced gene expression via *NRF2*
CPDB_Reactome_232	1.72E−006	Phase II conjugation
CPDB_KEGG_1822	3.49E−006	Cell cycle

In summary, we see that the five compounds under study increase the activity of genes and pathways involved in cell metabolism and maintenance. This example demonstrates that a meta-analysis of different compounds can create a general view on cellular response to genotoxic compounds and the effects on carcinogenesis.

7 CASE STUDY: OCHRATOXIN A

Another application of the meta-analysis can be achieved by looking at different experimental results for the same compound. To show the specific effect of ochratoxin A on the gene expression level, we conducted a separate case study. In total, 10 test cases were defined in this approach, five based on kidney extracts and five based on liver extracts. The computed cut-off score value of 3.736 defines a core set of 85 genes, whereas 66 of these genes overlap with the gene set from the main analysis. This amounts to 19 unique genes that show significant ochratoxin A relevance.

Among these genes is the methyltransferase Guanidinoacetate N-methyltransferase (*GAMT*). A defect in this gene has been related to neurologic syndromes and muscular hypotonia. Furthermore, another specific marker is *CYP4F2* that encodes a member of the cytochrome P450 superfamily of enzymes. These proteins catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids, and other lipids.

Enrichment analysis identifies 67 significant pathways with a *p*-value below 0.05. Besides basic cellular processes such as biological oxidation and metabolic pathways also enriched in the multi-compound study, some pathways stand out in the context of ochratoxin A treatment. One example

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is the post-translational protein modification pathway, one of the later steps in protein biosynthesis, consisting of eight genes whereof two genes are significant. This enrichment analysis-based conclusion is confirmed by a study indicating that ochratoxin A causes immunosuppression and immunotoxicity by, among other factors, inhibition of protein synthesis (Al-Anati and Petzinger, 2006).

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Application of Systems Biology in Neurotoxicological Studies During Development

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1 INTRODUCTION

Systems biology has been defined as the iterative and integrative study of biological systems as they respond to perturbations (Auffray *et al*., 2003). In this review chapter, the value of systems biology to enhance the understanding of complex biological processes such as neurodegeneration in the developing brain is explored. High-throughput/data content molecular biology approaches including genomics, proteomics, and metabolomics provide the fundamental data necessary for the building blocks of biological systems. As these databases grow and become linked together as integrative modules, they provide the intermediate components necessary for the systems biology approach. It is the appropriate placement of these biological modules into a proposed mechanistic flow scheme, thus allowing for the development of integrated computational models, that is the overall goal. However, the development of these mathematical models often lags behind the initial definition of the system and remains to be accomplished for the current example.

For toxicology, it is essential that quantitative correlations of exposure (i.e., dose, time intervals, and outcome) be integrated into the computational model (Henry, 2003). In addition to knowledge about the proximate toxicant and its mechanism of action, the primary toxicological effect or phenotypic anchor must also be utilized (Waters, Olden and Tennant, 2003). At the systems biology level, quantitative simulations can be conducted and predictions of the model can be tested. The outcome of these iterations is systematically incorporated back into the model to improve its design and refine its predictive capacities. The interconnectivity of a system at this level determines its state and extends its predictive power (Jazwinski, 2002).

The goal of systems biology is to predict the functional outcomes of component-to-component relationships using computational models that allow for the directional and quantitative description of the complete organism in response to environmental perturbations (Waters *et al*., 2003). Systems biology approaches can also be used as effective tools for dissecting the mechanisms underlying toxicological phenomena associated with exposure to toxicants. It is the development of predictive models that integrate responses across different organizational levels that is the focus of this review chapter.

The developing nervous system may be more or less susceptible to neurotoxic insults depending on the stage of development. Because of the complexity and temporal features of the manifestations of developmental neurotoxicity, this area of toxicology can benefit from a systems biology approach. DNA microarray technology

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simultaneously detects the perturbation in tens of thousands of genes in one single experiment and is rapidly becoming incorporated into toxicological research. Combined with bioinformatics tools, it has been widely used to investigate the mechanisms of toxicant action, to search for novel biomarkers of toxicity and to build molecular signatures or models for predictive toxicology (Boverhof and Zacharewski, 2006; Waring *et al*., 2001; Waters and Fostel, 2004). This chapter focuses on representative general anesthetics, including ketamine, as an example of the application of the systems biology approach to outline how specific receptor subunit and intracellular signaling events are involved in potential anesthetic-induced neurotoxicity. The biochemical and molecular mechanisms as well as gene expression profiles that underlie potential anesthetic-induced neurotoxicity, particularly during a sensitive developmental stage, are explored.

Four steps of a systems biology approach reported by Leroy Hood's group (Auffray *et al*., 2003) will be discussed in this context. First, available information on the biological system of interest will be described and a preliminary model of how the system functions will be formulated. Second, where possible, the genes and proteins expressed in the described pathways will be defined. Third, kinetic experiments providing information across important periods of development will be considered. Fourth, various global datasets will be integrated to determine whether they support the model. Discrepancies will be identified and hypotheses-driven studies will be conducted in order to address them. Thus, data generated via iteration of the third and fourth steps will be used to reformulate the model in light of new data.

2 ROLE OF GLUTAMATERGIC TRANSMISSION AND NMDA RECEPTOR DYSFUNCTION IN ANESTHETIC-INDUCED NEURODEGENERATION

The amino acid L-glutamate is generally recognized as the major excitatory neurotransmitter of the mammalian central nervous system (CNS), and glutamate receptors play a major role in fast excitatory synaptic transmission. Glutamate promotes neuronal migration, differentiation, and plasticity during development and throughout life (Komuro and Rakic, 1993). Malfunctions of the glutamate system can affect neuroplasticity and cause neuronal toxicity. In the case of anesthetic-induced neurodegeneration, many glutamate-regulated processes seem to be perturbed. Abnormal neuronal development, abnormal synaptic plasticity, and neurodegeneration have been proposed as mechanisms that underlie anesthetic-induced neuronal cell loss. It is becoming clear that some of the most important functions of the nervous system, such as synaptic plasticity and synaptic formation, critically depend on the behavior of NMDA receptors, and that neurological damage caused by a variety of pathological states can result from exaggerated or inappropriate activation of NMDA receptors (Choi, 1988; Olney *et al*., 2000).

The general anesthetic ketamine blocks subtypes of glutamate receptors: the NMDA receptors. As a dissociative anesthetic, ketamine is commonly used to produce analgesia in children in emergency departments (Kohrs and Durieux, 1998). Clinically, ketamine's role in pediatric anesthesia is well established. However, recent studies have found that ketamine may cause dose-dependent, widespread apoptotic neurodegeneration in immature rat and monkey brains (Ikonomidou *et al*., 1999; Jevtovic-Todorovic *et al*., 2003; Slikker *et al*., 2007). The window of vulnerability appears restricted to the phase of rapid synaptogenesis, also known as the brain growth spurt.

Glutamatergic transmission is mediated by receptor families that are classed as ionotropic (iGluRs) or metabotropic (mGluRs). iGluRs are ligand-gated ion channels that can be sub-classified into the following groups based upon their ligand-binding properties: NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptors (AMPA), kainate receptors (KA), and, more recently, delta receptors. NMDA receptors appear to be heteromeric complexes (Monyer *et al*., 1992). NMDA-R1 (Grin1) subunits can form homo-oligomeric receptors that are functional, and the presence of this subunit is required to produce detectable NMDA-activated channel currents *in vitro* (Buller *et al*., 1994). NMDA-R2 (Grin2A-D) subunits produce functional receptors only when co-expressed with NMDA-R1 (Monyer *et al*., 1992), and co-expression of NMDA-R1 with NMDA-R2 subunits increases their responsiveness to NMDA and yields different functional properties (Buller *et al*., 1994).

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Proposed mechanisms for the developmental neurotoxicity caused by ketamine include a compensatory up-regulation of NMDA receptors and subsequent over-stimulation of the glutamatergic system by endogenous glutamate via this receptor up-regulation (Slikker *et al*., 2007; Wang *et al*., 2005, 2006). This results in dysregulation of calcium signaling, oxidative stress (Wang *et al*., 2000), and activation of the NF-kB signaling pathway (Wang *et al*., 2006). However, the exact molecular mechanisms underlying ketamine-induced apoptotic neuronal cell death remain elusive.

To better understand the molecular pathogenesis of ketamine-induced developmental neurotoxicity, systems biology approaches were carried out to examine the changes in gene expression profiles in developing brains, which have been shown to display a high susceptibility to ketamine-induced apoptotic neuronal cell death.

3 SYSTEMS BIOLOGY AND ANESTHETIC-INDUCED NEUROTOXICITY

3.1 Systems Biology Approach and Ketamine-Induced Neurodegeneration

Condensed or fragmented DNA was assayed using TUNEL staining as previously described (Johnson *et al*., 1998). The repeated ketamine exposure produced elevated neuronal cell death as indicated by an increased number of TUNEL-positive cells in the developing (PND 7) rat brain (Figure 1). In controls (Figure 1a), only a few TUNEL-positive cells were observed. However, numerous darkly stained TUNEL-positive cells exhibiting typical nuclear condensation and fragmentation were observed (Figure 1b) in ketamine-treated rat pups.

In order to understand the underlying mechanism of ketamine-induced neurodegeneration, brain tissues from the frontal cortical levels, where the most severe neuronal damage was expressed, were selected for RNA isolation and microarray analysis. Consistent with the TUNEL assay and previous *in vivo* data, a total of 32 genes were found to be involved in apoptosis, and among them, 15 genes were up-regulated and 17 genes were down-regulated (Table 1) in animals exposed to six injections of 20 mg kg^{-1} ketamine, compared with the controls (Shi *et al*., 2010). The apoptosis-related genes are a group of genes that have two distinct modes of operation: pro-apoptosis or anti-apoptosis. In response to various inducers such as stressful stimuli or sustained elevation of intracellular calcium levels, the ultimate fate of the brain cell is determined by the roles of these apoptosis-related genes in regulating the life/death cell balance. The mechanism(s) underlying the ketamine-induced neuronal cell death have not been fully elucidated. However, present microarray

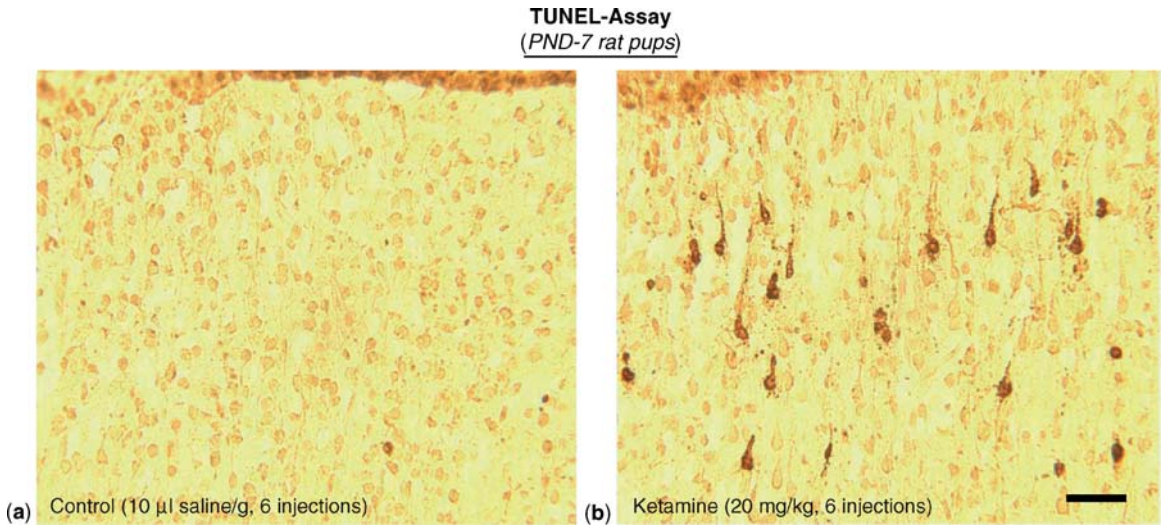

Figure 1. Ketamine-induced neurodegeneration in PND 7 rats assessed by TUNEL labeling. Representative photographs indicate that TUNEL-positive cells are more numerous in layers II and III of the frontal cortex in the ketamine-treated rat brain (b). Only a few TUNEL-positive cells were observed in the control (saline-treated) rat brain (a). Scale bar = 60 µm. Reproduced from Shi *et al.* (2010) © Elsevier.

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data indicate that the majority (approximately two-thirds) of up-regulated genes were pro-apoptotic in nature, including Agt, Clu, Gjb6, Hrk, Igfbp3, Inpp5d, Jun, Mal, Rassf5, and Txnip. Meanwhile, from these up-regulated genes, expression of *Alb*, *Apoe*, and *Cryab* may provide inhibition of apoptosis (Mao *et al*., 2004). It has been reported that silencing the expression of *Bag3* (Bcl-2-associated athanogene 3) leads to reduced protein levels of Bcl-XL, Mcl-1, and Bcl-2 in colon cancer cells and increased apoptosis (Jacobs and Marnett, 2009). As a critical gene, *Cebpb* [CCAAT/enhancer-binding protein (C/EBP)] acts as a major regulator of metabolic homeostasis and is involved in many cellular processes, such as differentiation, growth, immune responses, neoplastic growth, development of the reproductive system, and pro- and anti-growth pathways (Gade *et al*., 2008).

However, in genes that have been down-regulated (17 genes), about one-half are anti-apoptotic genes. The over-expression of *Acvr1c* has been shown to suppress the apoptotic effects, and *Amigo2* acts as an anti-apoptotic factor (Hashimoto *et al*., 1998; Ono *et al*., 2003). *Bnip3* encodes cellular proteins that interact with Bcl-2. In cortical cells, cyanide induces a rapid up-regulation of *Bnip3* expression, followed by a caspase-dependent cell death (Prabhakaran *et al*., 2007). Down-regulation of *Cd24*, *Cdc2a*, and disruption of the *Rasa1* gene in early embryonic mice induce apoptosis of neuronal cells (Lapinski *et al*., 2007; Smith *et al*., 2006). In neocortical and hippocampal tissues, apoptotic effects can be demonstrated following *Ahr* activation (Kajta *et al*., 2009) and this gene was found to be upregulated in our studies. These observations may imply that the frontal cortex is the brain region most vulnerable to ketamine-induced neurotoxicity during development, and the neuronal survival in the early phases of the apoptotic cascades mostly depends on the balance between the pro- and anti-apoptotic factors of the apoptosis-related genes.

It should be mentioned that the present microarray analysis did not detect regulatory effects of some popular apoptotic genes, such as from the caspase-3 and Bcl-2 gene families (Slikker *et al*., 2007; Wang, Fridley and Johnson, 2005, 2006), in ketamine-induced neurotoxicity. This all may be related to the timing of sampling for the microarray analyses; analyses at different times after ketamine exposure may result in different observations.

Table 1. Apoptosis-related genes identified by GOFFA.

	Gene symbols	Gene names
1	*Acvr1c*	Activin A receptor, type IC
2	*Ahr*	Aryl hydrocarbon receptor
3	*Alms1*	Alstrom syndrome 1
4	*Amigo2*	Adhesion molecule with Ig like domain 2
5	*Atp7a*	ATPase, Cu^{2+} transporting, alpha polypeptide
6	*Bnip3*	BCL-2/adenovirus E1B 19 kDa-interacting protein 3
7	*Bub1b*	Budding uninhibited by benzimidazoles 1 homolog, beta
8	*Cd24*	CD24 antigen
9	*Cdc2a*	Cell division cycle 2 homolog A (S. pombe)
10	*Inhba*	Inhibin beta-A
11	*Myc*	Myelocytomatosis oncogene
12	*Ntf3*	Neurotrophin 3
13	*Pak7_predicted*	p21 (CDKN1A)-activated kinase 7 (predicted)
14	*Pdia2_predicted*	Protein disulfide isomerase-associated 2 (predicted)
15	*Rasa1*	RAS p21 protein activator 1
16	*Tnfrsf11b*	Tumor necrosis factor receptor superfamily, member 11b
17	*Unc5c*	unc-5 homolog C (C. elegans)
18	*Agt*	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)
19	*Alb*	Albumin
20	*Apoe*	Apolipoprotein E
21	*Bag3*	Bcl-2-associated athanogene 3
22	*Cebpb*	CCAAT/enhancer-binding protein (C/EBP), beta
23	*Clu*	Clusterin
24	*Cryab*	Crystallin, alpha B
25	*Gjb6*	Gap junction membrane channel protein beta 6
26	*Hrk*	Harakiri, BCL-2-interacting protein (contains only BH3 domain)
27	*Igfbp3*	Insulin-like growth factor-binding protein 3
28	*Inpp5d*	Inositol polyphosphate-5-phosphatase D
29	*Jun*	Jun oncogene
30	*Mal*	Myelin and lymphocyte protein, T-cell differentiation protein
31	*Rassf5*	Ras association (RalGDS/AF-6) domain family 5
32	*Txnip*	Thioredoxin-nteracting protein

Genes 1–17 were down-regulated, and genes 18–32 were up-regulated.

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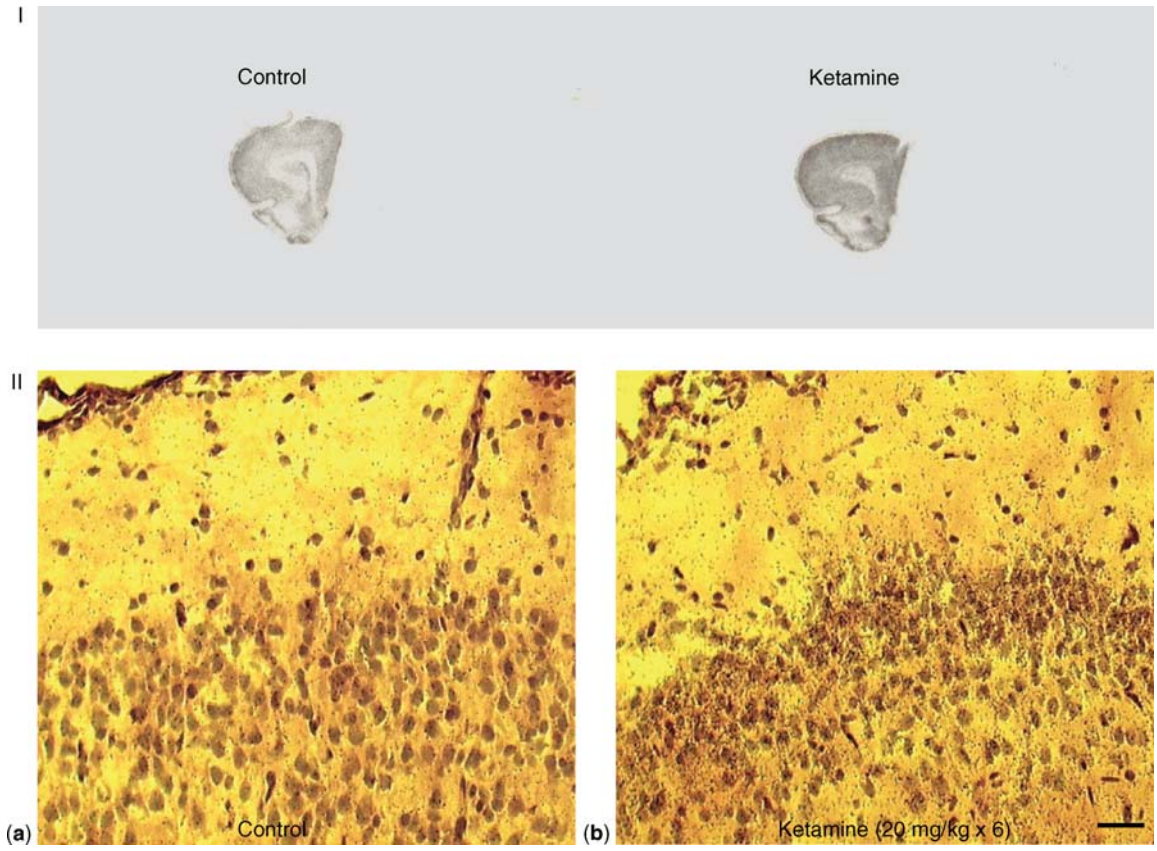

Figure 2. NMDA receptor NR1 subunit mRNA abundance in PND 7 rats. *In situ* hybridization was performed on rat brain sections (coronal) using a ^{35}S-labeled oligonucleotide probe specific for the NMDA receptor NR1 subunit. Panel I shows a general view of NR1 *in situ* hybridization signals in frontal cortical areas from both control and ketamine-treated rats. Panel II illustrates that the autoradiographic density (labeling) for NR1 subunit mRNA was higher in ketamine-treated (20 mg kg^{-1} × 6 injections) rat brain frontal cortex (b) compared with control (a). Scale bar = 90 μm. Reproduced from Shi *et al.* (2010) © Elsevier.

3.2 Systems Biology Approach and Ketamine-Induced Alteration in NMDA Receptor Expression

Because ketamine is an NMDA receptor antagonist, it was postulated that the localization of the most severe neurodegeneration in the frontal cortex might correspond to alterations in NMDA receptor expression levels. To dissect the underlying mechanisms, an oligonucleotide probe complementary to the mRNA encoding the NMDA receptor NR1 subunit was used for *in situ* hybridization. In both controls and ketamine-treated (20 mg kg^{-1} × 6 injections) rats, NMDA receptor NR1 subunit mRNA was prominent. The autoradiographic density (labeling) for NR1 subunit mRNA was up-regulated in the frontal cortex of rats treated with ketamine compared with control (Figure 2). Meanwhile, the gene expression of the NMDA receptor subunit gene, *Grin1* (*NR1*), was significantly up-regulated in ketamine-treated rat pups as detected in microarray experiments and subsequently confirmed with TaqMan analyses (Table 2) (Shi *et al.*, 2010). The

Table 2. Selective validation of the microarray results by Q-PCR.

Gene symbols	Fold-change (Q-PCR)	Fold-change (microarray)
Grin1 (NR1)	1.8*	1.5*
Grin2a (NR2A)	1.5*	1.2
Grin2b (NR2B)	1.0	0.9
Grin2c (NR2C)	1.7*	1.5*
Grin2d (NR2D)	1.2	1.1

* $P < 0.05$, as compared with the control.

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NMDA receptor NR1 subunit is widely distributed throughout the brain and is the fundamental subunit necessary for NMDA channel function. In these studies, by using *in situ* hybridization techniques to detect the relative densities of NMDA receptor NR1 subunits, a potential parallel relationship between enhanced apoptosis and NMDA receptor expression levels was examined. Our *in situ* data provided direct evidence that repeated ketamine exposure results in a substantial increase in autoradiographic density (labeling) of NR1 subunit mRNA in the frontal cortex and hippocampus. These data indicate that ketamine-induced pathological change is closely associated with a remarkable up-regulation of NMDA NR1 subunit mRNA.

It is possible that increased expression of *Grin1* (*NR1*) was accompanied by an altered expression of other glutamate receptor subunits. Our microarray analyses (Table 2) revealed an increase in *Grin2a* (*NR2A*; 1.5-fold) and *Grin2c* (*NR2C*; 1.7-fold), but no significant effects were observed in *Grin2b* (*NR2B*) or *Grin2d* (*NR2D*). It should be noted that NMDA-R2 subunits produce functional receptors only when co-expressed with NMDA-R1 (Monyer *et al*., 1992) and heteromeric complexes increase the responsiveness to NMDA and yield different functional properties (Buller *et al*., 1994). Our findings are consistent with those of previous *in situ* hybridization and immunoblotting data that demonstrated a compensatory up-regulation of NMDA-R1 and NMDA-R2 receptors following prolonged exposure to NMDA receptor antagonists (Slikker *et al*., 2007; Wang *et al*., 2005; Zou *et al*., 2009).

NMDA receptor density has been shown to increase in cultured cortical neurons after exposure to the NMDA receptor antagonists D-AP5, CGS-19755, and MK-801, but not after exposure to the AMPA/kainate receptor antagonist CNQX (Williams, Dichter and Molinoff, 1992). In the present study, no significant changes were detected in the gene expression patterns of AMPA or kainate receptors after repeated ketamine exposure. These findings support our previous pharmacological data showing that application of the non-NMDA receptor antagonist, CNQX, or nifedipine (an antagonist of the L-type voltage-sensitive calcium channel) did not produce a significant protective effect against ketamine-induced neuronal apoptosis (Wang *et al*., 2005). We hypothesize that continuous blockade of NMDA receptors by ketamine causes a compensatory up-regulation of NMDA receptors and this up-regulation makes neurons bearing these receptors more vulnerable, after ketamine withdrawal, to the excitotoxic effects of endogenous glutamate, because this up-regulation of NMDA receptors allows for the accumulation of toxic levels of intracellular calcium even under normal physiological conditions. This hypothesis is also supported by data from previous *in vitro* studies where it was shown that co-administration of antisense oligonucleotides that specifically target NMDA receptor NR1 and NR2A subunit mRNAs were able to block the neuronal damage induced by ketamine or PCP (Wang, Fridley and Johnson, 2005, Wang *et al.*, 2005, 2006).

4 PHARMACOKINETIC OUTCOMES AND PHYSIOLOGICAL PARAMETERS IN ANESTHETIC-INDUCED NEUROTOXICITY IN THE DEVELOPING BRAIN

Plasma concentration of ketamine is a critical parameter associated with neuronal cell death in animals. In perinatal monkeys, steady-state plasma concentrations of ketamine were achieved following 6–12 h of anesthesia. The ketamine levels in monkeys were 10–25 $\mu g\ ml^{-1}$, which is 5–10 times higher than those observed in humans (2–3 $\mu g\ ml^{-1}$) (Slikker *et al*., 2007). However, the dose of ketamine used was necessary to maintain anesthesia in this experimental animal model. Monkeys at various stages of development require different ketamine plasma concentrations to maintain anesthesia. Postnatal day (PND) 35 animals required higher plasma concentration of ketamine to maintain the same plane of anesthesia as the PND 5 animals (Figure 3). An important observation from the perinatal monkeys was that the plasma concentrations of ketamine were the highest in the PND 35 monkeys even though no evidence of increased neuronal cell death was observed as compared with control animals of the same age. In the PND 5 animals, where neuronal cell loss was evident, the plasma levels averaged approximately 10 $\mu g\ ml^{-1}$, which is only 3–5 times higher than the plasma levels observed in humans (Slikker *et al*., 2007).

Physiological parameters including percent oxygen saturation, exhaled carbon dioxide, body temperature, heart rate, blood pressure, glucose, and hematocrit were monitored in the experimental monkeys and all parameters were maintained within normal ranges. Monitoring and control of these parameters is essential for any animal model and is

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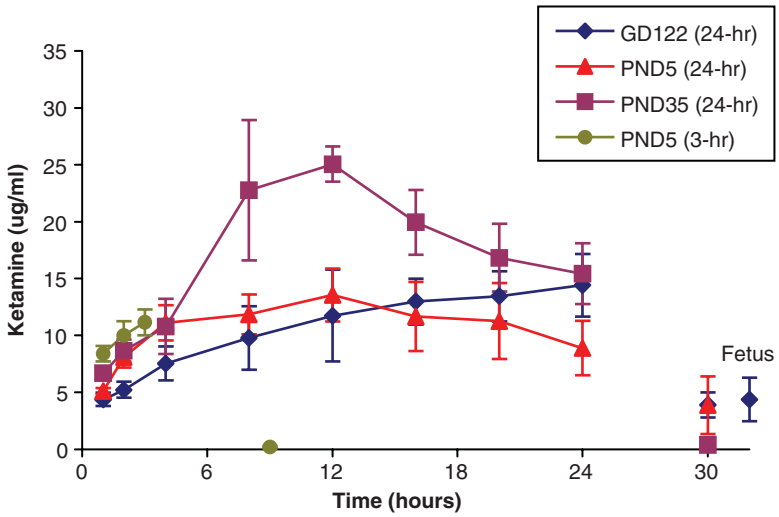

Figure 3. Plasma concentrations of ketamine in pregnant (GD122) or infant (PND5 or PND35) monkeys. Data points represent mean plasma concentrations ($\mu g\,ml^{-1}$) ±SEM for ketamine (a) from monkeys infused with 20–50 $mg\,kg^{-1}\,h^{-1}$ ketamine for 3 or 24 h, followed by a 6-h withdrawal period. Fetal plasma concentrations (GD122) at the time of C-section of pregnant animals are indicated (Fetus). $N = 3$ animals per time-point. Reproduced from Slikker *et al.* (2007) © Oxford University Press.

made possible by using a nonhuman primate animal model. These parameters are carefully controlled during pediatric anesthesia, but it is very difficult to monitor them in rodent models. Because prolonged hypoperfusion can lead to cerebral hypoxia and ischemic-related cell death, it is necessary that no evidence of abnormal blood pressure or oxygen saturation was observed. In this nonhuman primate model study, the percent oxygen saturation averaged 94% or above for all study groups.

5 CONCLUSION

This chapter provides an overview of our efforts to apply a systems biology approach to understand a particular toxicological problem: potential anesthetic (e.g., ketamine)-induced neurodegeneration in the developing nervous system. Systems biology, as adopted for toxicology, is referred to as systems toxicology and involves the study of system perturbations caused by chemicals or stressors. By monitoring alterations in gene, protein, cell signaling, and pharmacokinetic outcomes, it is hoped to define the affected system(s) in an integrative manner.

In our studies with ketamine, alterations of 32 genes were associated with apoptotic pathways (Shi *et al.*, 2010). Perturbations of genes associated with NMDA-type glutamate and other receptor signaling pathways were identified by Ingenuity Pathway Analysis software. Q-PCR confirmed that NMDA receptor genes including *Grin1* (*NR1*), *Grin2a* (*NR2A*), and *Grin2c* (*NR2C*) were significantly up-regulated. The ketamine-induced up-regulation of NMDA receptor *Grin1* (*NR1*) mRNA signaling was further confirmed by *in situ* hybridization. In addition, elevated plasma concentrations of ketamine (pharmacokinetic experiments) appear to be a critical parameter associated with neuronal cell death in animals.

Although not yet fully delineated, the working model for anesthetic (e.g., ketamine)-induced neurodegeneration during development involves the modulation of normally occurring brain sculpting mechanisms that control CNS development. Exposure of the developing mammal to anesthetics (such as ketamine) perturbs the endogenous NMDA receptor system and results in enhanced neuronal cell death. These observations support our working hypothesis that prolonged ketamine exposure produces up-regulation of NMDA receptors and subsequent over-stimulation of the glutamatergic system by endogenous glutamate, triggering enhanced apoptosis of developing neurons (Figure 4).

Although many more studies are needed in order to build a quantitative model, some general pathways have been identified using the carefully crafted four steps of a systems biology approach. Further elucidation of the precise pathway and developmental stages of susceptibility to anesthetic agents awaits additional studies.

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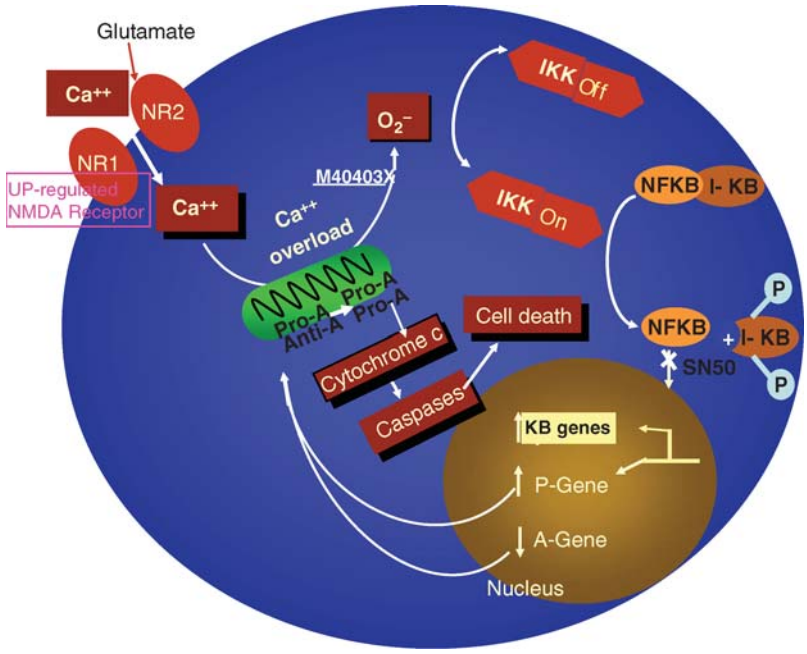

Figure 4. This cartoon illustrates our working model of potential anesthetic (e.g., ketamine)-induced neuronal cell death. Excessive activation of up-regulated NMDA receptors results in a calcium overload that exceeds the buffering capacity of the mitochondria and interferes with electron transport in a manner that results in an elevated production of reactive oxygen species, and the dissociation of some transcription proteins, such as NF-kB, and their transport into the nucleus. In the nucleus, these transcription factors bind to several DNA sequences of several known genes. The consequence of this binding is not completely understood, but the loss of the balance of pro- and anti-apoptotic genes is apparent: the diminished formation of anti-apoptotic heterodimers in favor of pro-apoptotic homodimers. These homodimers are thought to create mitochondrial membrane pores through which cytochrome *c* can leak into the cytoplasm where it can activate caspases that play a critical role in the ultimate demise of the neuron. Modified from McInnis *et al*. (2002) © American Society for Pharmacology and Experimental Therapeutics.

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Mitochondria-Specific Mouse Gene Array and its Application in Toxicogenomics

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1 MITOCHONDRIA AND THEIR FUNCTIONS

Mitochondria are the powerhouses of cells that are responsible for the generation of more than 90% of the cellular energy by the process of oxidative phosphorylation. In addition, mitochondria are intricately involved in other key metabolic pathways, such as fatty acid oxidation, the Krebs cycle, the urea cycle, heme biosynthesis, steroid biosynthesis, as well as calcium signalling (Schatz, 1995; Pozzan and Rizzuto, 2000). It is also becoming increasingly evident that mitochondria are the 'cellular decision points' for apoptosis (programmed cell death), a process that serves as a major defence mechanism to remove unwanted and potentially dangerous cells (Petite and Kroemer, 1998). Collectively, these functions highlight a critical role of mitochondria in the life and death of the cell.

A distinctive feature of mitochondria is that these are the only subcellular organelles besides the nucleus that contain their own genome. Mitochondrial DNA (mtDNA) is a double-stranded, circular molecule of approximately 16 kb in size that encodes 13 polypeptides and the transcriptional machinery of two ribosomal RNAs (rRNAs, 12S and 16S) and 22 transfer RNAs (tRNAs) required for intra-mitochondrial synthesis of these polypeptides (Clayton, 1992). The large degree of complexity of mitochondria is due to the fact that among over 1000 proteins located within the mitochondria, only 13 proteins are encoded by the mtDNA (Taanman, 1999). These constitute 7 out of approximately 45 subunits in complex I, 1 out of 11 subunits in complex III, 3 out of 13 subunits in complex IV and 2 out of 16 subunits in complex V, all of which are critical components of oxidative phosphorylation. However, the remaining 76 proteins of oxidative phosphorylation are encoded by the nuclear DNA. In addition, proteins involved in different metabolic pathways within mitochondria, proteins responsible for the transport of ions and solutes across mitochondrial membranes, and proteins essential for mtDNA transcription, translation, replication and repair are all encoded by the nuclear DNA. These proteins are synthesized in the cytosol and are subsequently translocated into the mitochondria (Neupert *et al*., 1990). This strongly indicates that mitochondrial function is not solely controlled by mtDNA, but significantly influenced by nuclear genes. The precise coordination between the mitochondria and the nucleus is therefore vital to meet the energy needs of the cell as well as for proper functioning of metabolic pathways within mitochondria. In view of this, changes in expression levels of mitochondrial genes and/or nuclear genes associated with mitochondrial function may result in altered mitochondrial activity, which, in turn, could be disastrous to cells and, therefore, to tissue and organ function.

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2 MITOCHONDRIAL DYSFUNCTION AND IMPLICATIONS IN DISEASES AND DRUG TOXICITIES

Tissues such as brain, liver, heart, respiratory systems, skeletal muscle, optic nerve, kidney and endocrine glands greatly depend on mitochondria for their high energy demands. When performing a primary role of energy production, these organelles also serve as a major source of reactive oxygen species (ROS) that can potentially induce damage to DNA, RNA, proteins and membranes, leading to cellular demise. Although mitochondria possess antioxidant defence systems to protect themselves against these toxic radicals, imbalance between production and detoxification of ROS can result in oxidative stress within mitochondria, leading to various pathologies in tissues.

2.1 Degenerative Diseases and Disorders

It is becoming more evident that subtle functional changes in mitochondria can manifest into insidious pathologies in neurons because these cells significantly depend on mitochondrial energy. Oxidative stress due to altered mitochondrial activity has been suggested as a major factor associated with the development of a number of neurological disorders including Alzheimer's, Parkinson's and Huntington's diseases, as well as amyotrophic lateral sclerosis (Jenner, 1996; Beal, 1998; Schapira, 1999; Manfredi and Beal, 2000). Like the brain, the heart immensely relies on mitochondrial energy for its contractile function. Impaired mitochondrial bioenergetics and changes in mitochondrial morphology in cardiomyocytes have been related to cardiovascular diseases such as dilated and hypertrophic cardiomyopathy, ischaemic cardiomyopathy, myocardial infarction and the pathogenesis of atherosclerosis (Lesnefsky *et al*., 2001; Ballinger *et al*., 2002; Marin-Garcia and Goldenthal, 2002; Monteiro *et al*., 2003). Defects in mitochondria have also been implicated in different types of cancers (Carew and Huang, 2002). Altered expressions, as well as activity, of mitochondrial respiratory chain complexes have long been suspected in the development and progression of cancer. Warburg's (1956) hypothesis suggests a shift from the oxidative phosphorylation to glycolysis to generate energy as a result of functionally impaired mitochondria in cancer cells. Mutations in mtDNA have been indicated as one of the features of cells in the breast, ovarian, brain, liver and colon cancers (Penta *et al*., 2001; Delsite *et al*., 2002; Tamori *et al*., 2004; Lu, Sharma and Bai, 2009; Akouchekian *et al*., 2009), which could render mtDNA-encoded components of oxidative phosphorylation complexes malfunctioning. Bearing in mind that mitochondria are significantly involved in apoptosis, their contribution in cancer development is not surprising.

There is considerable evidence that indicates mitochondrial involvement in obesity (Hojlund *et al*., 2008). In addition, mitochondrial dysfunction has been recognized in the pathogenesis of type-2 diabetes, a metabolic disorder characterized by insulin resistance in the initial stages that eventually progresses to impaired insulin secretion by the pancreas. Substantial evidence indicates altered mitochondrial dynamics responsible for insulin resistance (Zorzano, Liesa and Palacin, 2009) whereas a genetic defect in cytosolic–mitochondrial interaction has been reported as an underlying cause of impaired insulin secretion by the pancreas (Gerbitz, Gempel and Brdiczka, 1996; Petersen *et al*., 2003). A number of studies have also described association between mutations in mtDNA and development of type-2 diabetes (Kadowaki *et al*., 1994; Kameoka *et al*., 1998). Progressive accumulation of somatic mutations in mtDNA during lifetime leading to altered mitochondrial activity has been suggested to play a critical role in the ageing process (Linnane *et al*., 1989). In addition, functional decline in mitochondrial electron transport activity has been shown in animal models at various ages in different tissues (Sugiyama *et al*., 1993; Desai *et al*., 1996; Rafique, Schapira and Coper, 2004; Ferguson *et al*., 2005; Choksi and Papaconstantinou, 2008).

2.2 Mitochondrial Disorders

Constitutively, impaired mitochondrial activity can affect virtually every tissue and organ in the living organism. It can affect tissues individually or various combinations of tissues and organ systems. Tissues with the highest energy needs, such as the brain, heart and skeletal muscles are particularly vulnerable to the defects in mito-

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chondrial bioenergetics that may manifest into neuropathies, cardiomyopathies and myopathies, often in combination and frequently in association with dysfunction in other organs (Scheffler, 2000). Mitochondrial disorders are characterized by a wide range of mutations in mtDNA that result in a variety of symptoms affecting multiple organs (Wallace, 1992). These disorders include mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy with ragged red fibres (MERRF), chronic progressive external opthalmoplegia (CPEO), Leber's hereditary optic neuropathy (LHON), Kearns–Sayre syndrome (KSS) and neuropathy, ataxia and retinitis pigmentosa (NARP).

2.3 Drug- and Chemical-Induced Toxicities

A growing body of evidence suggests mitochondria as potential target organelles of different drug- and chemical-induced toxicities (Arnaudo *et al*., 1991; Adachi *et al*., 1993; Lewis and Dalakas, 1995; Foli *et al*., 2001; Kohler and Lewis, 2007; Santos *et al*., 2007; Desai *et al*., 2008). Chemotherapeutic drugs, such as bleomycin, *cis*-platin and doxorubicin, have been shown to induce mitochondrial dysfunction through different mechanisms (Adachi *et al*., 1993; Shen *et al*., 1995; Desai *et al*., 2000, 2001; Santos *et al*., 2007). Also, mitochondrial toxicity is a serious side-effect of anti-retroviral drugs, in particular the nucleoside reverse transcriptase inhibitors (NRTIs), such as zidovudine, stavudine and didanosine, used to treat human immunodeficiency virus-1 (HIV-1) infections (Foli *et al*., 2001). These drugs inhibit mitochondrial DNA polymerase gamma, an essential enzyme for mtDNA replication, resulting in depletion of mtDNA content, and/or cause mtDNA strand breaks by incorporating into mtDNA during DNA synthesis (Bienstock and Copeland, 2004; Cote, 2005; Kohler and Lewis, 2007). However, other mitochondrial targets can be significantly affected by these drugs (Desai *et al*., 2008, 2009). Moreover, mitochondrial dysfunction has been indicated as an early event occurring during exposures to environmental toxins, such as aflatoxin B1 and ochratoxin that are known to cause hepatic and renal cancer, respectively (Aleo, Wyatt and Schnellmann, 1991; Sajan, Satav and Bhattacharya, 1996). Similarly, mitochondria have been linked to neurotoxicity caused by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 3-nitropropionic acid, chemicals used to mimic symptoms of Parkinson's and Huntington's diseases, respectively, in animal models (Beal, 2003; Ayala *et al*., 2007). Adverse effects of MPTP and its toxic metabolite 1-methyl-4-phenylpyridine on brain mitochondrial electron transport chain complexes have been demonstrated *in vitro* as well as *in vivo* (Desai *et al*., 1996; Gu *et al*., 2003).

3 POSSIBLE MECHANISMS OF MITOCHONDRIAL DYSFUNCTION

Mechanisms responsible for altered mitochondrial function are diverse. Mutations in mitochondrial genes and/or nuclear genes encoding mitochondrial proteins, altered transcription of these genes, faulty translation into proteins as well as interference with the coordination between mitochondrial and nuclear genomes can adversely influence mitochondrial function. Given that nuclear-encoded mitochondrial proteins are transported to mitochondria, proper transport of these proteins, assembly of proteins, in particular complexes of oxidative phosphorylation, and incorporation in the inner mitochondrial membrane are critical for normal mitochondrial activity. Maintenance of mitochondrial membrane potential is also equally important in maintaining mitochondrial function. One or a combination of all of these factors can substantially affect mitochondrial function. These can alter bioenergetics and other key metabolic pathways, impair membrane transport of ions and solutes, and/or disturb mitochondrial calcium homeostasis that may account for drug toxicities or various pathologies in tissues. Interference in mitochondrial biogenesis, a dynamic process critical for cell survival during changing metabolic environments, may also have implications in mitochondria-related drug-induced toxicities and diseases.

4 NEED FOR DEVELOPMENT OF A HIGH-THROUGHPUT MOLECULAR SCREENING TOOL IN MITOCHONDRIAL RESEARCH

As explained earlier, mounting evidence suggests mitochondrial dysfunction as an underlying cause of

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a number of diseases and various drug-related toxicities. Mitochondria are complex cellular organelles that possess intricate networking of various molecular pathways. Understanding of the mitochondrial function in drug toxicities and diseases therefore requires a comprehensive approach to determination of the many aspects of mitochondrial function. The knowledge of the mechanistic basis of mitochondrial dysfunction is essential in the development of effective treatment strategies and the designing of novel interventions for minimizing the severity or delaying the progression of neurodegenerative diseases, such as Alzheimer's, Parkinson's and Huntington's diseases. Also, gaining insights into the molecular events associated with altered mitochondrial activity is important in obesity, particularly in children, as childhood obesity leading to early-onset diabetes has reached epidemic proportions in the United States. Evidence of irreversible cardiomyopathy induced by potent chemotherapeutic drugs, such as anthracyclines, and myopathy and hepatic steatosis as a result of exposure to anti-HIV drugs further emphasizes the need for the development of a high-throughput technology to delineate the mechanisms of impaired mitochondrial activity during toxic insults so that new treatment regimens can be designed to lessen drug toxicities. The use of advanced molecular technologies to get an improved perspective of mitochondrial activity will also prove valuable in understanding complex diseases such as mitochondrial disorders for which currently there is no cure.

Despite several approaches that are pursued thus far, the role of mitochondria in diseases and drug toxicities has still not been adequately elucidated. This could in part be due to utilization of seemingly insensitive molecular techniques that generated limited information. One of the most significant achievements in genomics in recent years is the innovation of DNA microarray technology that allows simultaneous measurement of expression levels of hundreds to thousands of genes in one experiment. Because of its high-throughput potential, it is rapidly emerging as an essential genomic tool in the study of xenobiotic-induced toxicities and in disease pathogenesis. Using this technology, a mitochondria-specific mouse oligonucleotide microarray (MitoChip) has been developed which is discussed in more detail below.

5 DEVELOPMENT OF MITOCHONDRIA-SPECIFIC MOUSE OLIGONUCLEOTIDE MICROARRAY (MitoChip)

The MitoChip was the first mitochondria-specific mouse oligonucleotide microarray and was developed at the Center for Functional Genomics at the National Center for Toxicological Research (NCTR) for profiling of the expression of 542 genes involved in mitochondrial structure and function (Desai *et al*., 2007). It consists of both mitochondrial and nuclear genes involved in oxidative phosphorylation, mtDNA transcription, replication and repair, mitochondrial biogenesis, mitochondrial fusion, the Krebs cycle, fatty acid β-oxidation, heme biosynthesis, steroid biosynthesis, lipid metabolism, apoptosis and mitochondrial membrane transport. It also includes nine housekeeping genes as positive controls and nine Arabidopsis plant genes as negative controls. Oligonucleotides corresponding to each gene are 50 bases long and were designed and synthesized by the MWG Biotech, Inc., Ebersberg (Germany).

5.1 Printing and Post-Processing of Arrays

The mouse oligonucleotides are printed on poly-1-lysine-coated glass slides (Erie Scientific, Portsmouth, NH, USA) using an OmniGrid® 100 microarrayer (GeneMachines, San Carlos, CA, USA) in a printing room maintained at 23 °C and 50% humidity. Printed arrays are baked for 2 h at 80 °C followed by cross-linking with 300 mJ of UV. Arrays are further treated with bovine serum albumin, fraction V solution containing sodium dodecyl sulphate (SDS), according to the procedure by Erie Scientific (www.eriesci.com) with minor modifications. The processed arrays are stored dry in drykeeper desiccator (without desiccant) (Sanplatec Corp, Osaka, Japan) maintained at 10% humidity until utilized in experiments.

5.2 Isolation and Determination of Quality of RNA

The quality of RNA is extremely important in microarray experiments as it influences the labelling efficiency of fluorescent dye molecules during

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generation of high-quality targets. Total RNA is extracted from tissues using Qiagen RNeasy kits (Qiagen, Valencia, CA, USA). The purity and yield of the extracted RNA are determined spectrophotometrically by measuring optical density at wavelengths of 260 and 280 nm. The quality of extracted RNA is evaluated using the RNA 6000 LabChip and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Only RNA with RNA integrity number higher than 8, which indicates minimal RNA degradation and a ratio of 28S to 18S ribosomal RNA close to 2, is considered suitable for microarray experimentation.

5.3 Preparation of Fluorescently Labelled cDNA (Indirect Labelling Method) and Hybridization on MitoChip

The standard operating procedure used for preparation of cDNA targets is based on the protocol described previously (Hegde *et al*., 2000) with a few modifications. The indirect labelling method involves the incorporation of a nucleoside triphosphate analogue containing a reactive aminoallyl group followed by coupling with a fluorescent dye molecule to the aminoallyl group. Initially, DNase-treated RNA is reverse transcribed using random hexamer primers (Invitrogen, Carlsbad, CA, USA) and SuperScript II reverse transcriptase (Invitrogen) in the presence of reverse transcription labelling mixture containing deoxyribonucleotide triphosphates (dNTPs; Invitrogen) and aminoallyl-deoxyuridine triphosphate (aa-dUTP; Ambion, Austin, TX, USA), generating aminoallyl-cDNA. Upon purification, the aa-cDNA pellet is coupled with fluorescent cyanine dyes (Cy3 and Cy5; GE Healthcare, Piscataway, NJ, USA) to generate labelled targets. Samples and Universal mouse reference RNA (Stratagene, La Jolla, CA, USA) are labelled with Cy5 and Cy3, respectively. Before hybridization, labelled cDNA is scanned spectrophotometrically (200–700 nm) to ensure optimal dye incorporation per sample for adequate signal intensity. A mixture of Cy5-labelled treated sample and Cy3-labeled Universal mouse reference RNA in a salt-based hybridization buffer (Ocimum Biosolutions Inc., Gaithersburg, MD, USA) is pre-warmed at 42 °C before hybridization. Hybridization is carried out under a lifterslip (Erie Scientific) placed on the MitoChip in a closed hybridization cassette (TeleChem International Inc., Sunnyvale, CA, USA) in a water bath at 42 °C for 18 h. Following hybridization, the array is washed in three different buffers containing different concentrations of sodium chloride, sodium citrate and SDS, pre-warmed at 30 °C.

5.4 Scanning of Hybridized Array

The hybridized array is scanned with a GenePix 4000B scanner (Axon Instruments, Union City, CA, USA) at 10 μm resolution using appropriate gains on the photomultiplier tube (PMT) to obtain the highest intensity with <1% saturated pixels. The resulting image is analysed by measuring the fluorescence of hybridized targets on spotted oligonucleotides on the slide using the GenePix Pro 6.1 imaging analysis software (Axon Instruments). The data are further entered into the NCTR-designed database, ArrayTrack™, for storage and further analysis (Tong *et al*., 2004).

5.5 Statistical Analysis

The expression level of each gene is estimated as a $\log_2$ ratio, which is a ratio of the median fluorescence of Cy5 (the sample) relative to the median fluorescence of Cy3 (the reference standard). Data generated using commercial whole-genome arrays are normalized using Loess or median intensity normalization. However, normalization and analysis of the mouse MitoChip data are challenging because of the relatively low density of probes. Therefore, average expression of the housekeeping genes and Arabidopsis genes on the array is used as the normalizing covariate to analyse the expression data of mitochondria-related genes (Parrish and Delongchamp, 2006).

Gene expression data are further categorized into different gene ontologies (GO) based on molecular function, cellular compartment and biological process using the Mouse Genome Informatics website (www.informatics.jax.org). This allows meaningful biological interpretations of the massive amount of data generated in a microarray experiment. Effects of the treatment on each GO term (molecular function or cellular process) is determined by taking into account the change in expression level of each gene evaluated for GO (Delongchamp *et al*., 2006). Differential expression levels of genes are also

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verified by quantitative real-time PCR to increase confidence in the MitoChip microarray data (Livak and Schmittgen, 2001).

6 VALIDATION AND UTILITY OF THE MitoChip

In an effort to validate the MitoChip, our laboratory has been involved in conducting several experiments aimed at elucidating the mechanism(s) of impaired mitochondrial function during exposures to drugs/chemicals. These agents include: (i) anti-HIV drugs, nucleoside reverse transcriptase inhibitors (zidovudine and lamivudine) and non-nucleoside reverse transcriptase inhibitors (nevirapine); (ii) chemotherapeutic drugs (flutamide and cis-platin); and (iii) a dietary supplement for weight loss (usnic acid) in different mouse models. This review will discuss results from the experiments conducted in C3B6F$_1$*trp*53 mice treated with zidovudine (AZT) and lamivudine (3TC), as well as B6C3F$_1$ mice treated with usnic acid.

6.1 Nucleoside Reverse Transcriptase Inhibitors and Mitochondrial Dysfunction

Much progress has been made to find a cure for the acquired immunodeficiency syndrome (AIDS) since the first cases of AIDS were reported in the United States in 1981. Several effective anti-retroviral drugs have been developed to treat HIV infections. These include NRTIs, nucleotide reverse transcriptase inhibitors (NtRTIs), non-NRTIs (NNRTIs), protease inhibitors and fusion inhibitors. However, a major breakthrough in the treatment of AIDS has been the development of the highly active anti-retroviral therapy (HAART) combination regimen. Since its implementation in 1997, not only the incidence of AIDS has decreased but the survival in AIDS patients has substantially increased. Among different classes of anti-retroviral drugs that comprise the HAART regimen, NRTIs remain a major component of the combination therapy.

3′-Azido-3′-deoxythymidine (AZT, zidovudine) was the first NRTI approved by the US Food and Drug Administration (FDA) to treat HIV-1 infections. Although current guidelines by the Centers for Disease Control and Prevention recommend HAART combination regimen as standard care for treating HIV-1 infections, AZT monotherapy is prescribed to HIV-1-infected pregnant women during labour until delivery and to newborns for the first 6 weeks of life to reduce mother-to-child transmission of the HIV-1 virus (Connor *et al*., 1994; Abrams, 2004; Mofenson, 2004; Center for Disease Control (CDC) and Prevention, 2009). Besides, AZT in combination with lamivudine (2′,3′-dideoxy-3′-thiacytidine; 3TC) is the most commonly used NRTIs during pregnancy (Barbaro *et al*., 2005; Watts, 2006). It is also one of the effective dual NRTI combinations recommended to HIV-1-infected adults (CDC, 2008). Nonetheless, the success of the NRTIs also brought challenges of drug-induced adverse events that are believed to be due to mitochondrial dysfunction. These side-effects include metabolic (dyslipidemia and lactic acidosis), musculoskeletal (myopathy and osteopenia), hepatobiliary (hepatotoxicity and pancreatitis) and haematological complications (bone marrow suppression) (Moyle, 2000) that may significantly interfere with the quality of life and, in some cases, may even be life-threatening. To date, tremendous efforts have been invested in understanding the molecular basis of impaired mitochondrial function during exposure to NRTIs, in particular AZT alone or in combination with other NRTIs during transplacental or perinatal exposures in humans and in different animal models. These clinical and animal studies have examined effects of NRTIs on mitochondria at the molecular as well as ultrastructural levels. A few of these *in vitro* and *in vivo* studies conducted in different model systems are summarized in Table 1.

A widely accepted mechanism underlying NRTI-induced mitochondrial dysfunction is inhibition of DNA polymerase gamma, an enzyme necessary for replication of mtDNA (Lewis and Dalakas, 1995). Supporting this hypothesis are studies that demonstrated a decline in mtDNA content in skeletal muscle of HIV-1-infected adults on long-term AZT therapy (Dalakas *et al*., 1990; Arnaudo *et al*., 1991; Casademont *et al*., 1996), in skeletal and heart muscle of foetal *Erythrocebus patas* monkeys exposed transplacentally to AZT alone or in combination with 3TC (Gerschenson *et al*., 2000, 2004; Divi *et al*., 2007a), and in skeletal muscle of rats administered AZT postnatally (Lewis *et al*., 1992). Effect of long-term exposure to AZT on mtDNA was also evident *in vitro* in human HeLa cells that exhibited severe depletion in mtDNA contents

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Table 1. *In vitro* and *in vivo* studies conducted to examine mitochondrial dysfunction during exposures to NRTIs.

Investigation	Drugs/mode of exposure/animal model	Reference(s)
Effect on mitochondrial morphology		
Change in number, size and structure of mitochondria in skeletal, cardiac muscle or liver	AZT in humans	Dalakas *et al*. (1990); Pezeshkpour, Illa and Dalakas (1991); Chen *et al*. (1992); Tomelleri *et al*. (1992); Peters *et al*. (1993); Simpson *et al*. (1993) Cupler *et al*. (1995)
	AZT in rats	Lewis *et al*. (1992)
	AZT or AZT/3TC *in utero* in human, monkeys, mice	Gerschenson *et al*. (2000, 2004); Bishop *et al*. (2004); Divi *et al*. (2004, 2007)
	AZT/3TC perinatal[a] in mice	Bishop *et al*. (2004)
	AZT in human myotubes	Semino-Mora, Leon-Monzon and Dalakas (1994)
Effect on mtDNA		
Depletion in mtDNA in skeletal or cardiac muscle, cord blood lymphocytes, or umbilical cord tissue	AZT or AZT/3TC *in utero* in monkeys, mice	Gerschenson and Poirier (2000); Gerschenson *et al*., (2000, 2004); Divi *et al*. (2007)
	AZT or 3TC or AZT/3TC perinatal[a] in mice	Chan *et al*. (2007)
Depletion in mtDNA in skeletal muscle	AZT in human	Dalakas *et al*. (1990); Arnaudo *et al*. (1991); Casademont *et al*. (1996)
	AZT in rats	Lewis *et al*. (1992)
AZT incorporation into mtDNA in skeletal muscle, liver, kidney or placenta	AZT *in utero* in mice	Olivero *et al*. (1997)
	AZT/3TC *in utero* in monkeys	Gerschenson *et al*. (2004)
Mutations in mtDNA tRNA genes in heart muscle	AZT *in utero* in mice	Walker *et al*. (2004)
Oxidative damage to mtDNA in skeletal muscle or liver	AZT in mice	de la Asunción *et al*. (1998, 1999)
Effect on mitochondrial bioenergetics		
Altered OXPHOS[b] enzyme function in skeletal or heart muscle	AZT or AZT/3TC *in utero* in monkeys	Gerschenson and Poirier (2000); Gerschenson *et al*. (2000, 2004)
	AZT in human	Mhiri *et al*. (1991)
	AZT or AZT/3TC perinatal[a] in human	Blanche *et al*. (1999)
	AZT in rats	Lamperth *et al*. (1991)
	AZT in human HeLa cells	Divi *et al*. (2007)
Respiration, calcium loading capacity, or membrane potential in isolated heart or liver mitochondria	AZT or ddI[c] in rats	Lund and Wallace (2004)
	AZT in rats	Elimadi *et al*. (1997)

[a] Perinatal indicates exposure to drugs both *in utero* and neonatal.

[b] oxidative phosphorylation.

[c] didanosine.

(Divi *et al*., 2007b). Unlike nuclear DNA, mtDNA lacks introns and protective histones. Also, DNA polymerase gamma has a relatively high error rate during replication and there is a limited DNA repair in the mitochondria. These factors render the mtDNA vulnerable to oxidative damage by ROS, resulting in mutations. Increased levels of 8-oxo-7, 8-dihydro-2′-deoxyguanosine, a marker of oxidative damage to DNA, have been reported in mtDNA in skeletal muscle of mice treated with AZT (de la Asunción *et al*., 1998). Mutations in mtDNA-encoded tRNAs were also found in the hearts of mice exposed perinatally to AZT, 3TC or AZT/3TC (Chan *et al*., 2007). Interestingly, AZT-induced mutations in mitochondrial tRNA genes persisted in heart muscle even late in life in mice exposed to the drug *in utero* (Walker *et al*., 2004).

A number of studies also have demonstrated significant influence of AZT on mitochondrial bioenergetics (Lamperth *et al*., 1991; Mhiri *et al*., 1991; Elimadi *et al*., 1997; Blanche *et al*., 1999; Gerschenson *et al*., 2000; Lund and Wallace, 2004).

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As far as respiratory chain complexes in skeletal muscle mitochondria are concerned, long-term exposure to AZT resulted in inhibition of succinate-cytochrome *c* reductase activity in AIDS patients (Mhiri *et al*., 1991; Tomelleri *et al*., 1992) whereas activity of rotenone-sensitive NADH cytochrome *c* reductase was significantly lowered in rats (Lamperth *et al*., 1991). Altered respiration was also evident in heart and liver mitochondria in rats treated with AZT (Elimadi *et al*., 1997; Lund and Wallace, 2004). Although transplacental exposure to AZT/3TC decreased relative activities of respiratory chain complexes I and IV in skeletal muscle and complex I in heart muscle in *Erythrocebus patas* monkeys (Gerschenson *et al*., 2004), transplacental exposure to AZT alone in *Erythrocebus patas* monkeys caused depletion of complex I activity but increases in activities of complexes II and IV in skeletal muscle (Gerschenson and Poirier, 2000; Gerschenson *et al*., 2000), suggesting different mechanisms of AZT-induced mitochondrial toxicity. Abnormal activities of complexes I and IV were also observed in different tissues in children exposed to AZT *in utero* and after birth (Blanche *et al*., 1999).

Collectively, these findings provide convincing evidence that AZT alone or a dual combination of AZT and 3TC substantially alter mitochondrial function and that the mechanism underlying mitochondrial toxicity appears to be complex. Also, it is apparent that several mitochondrial targets may be involved in NRTI-induced toxicity. These findings are significant; however, these data do not explain the effects of NRTIs on other critical mitochondrial pathways, such as fatty acid β-oxidation and the Krebs cycle involved in energy metabolism, to understand the basis of altered mitochondrial function.

6.1.1 Evaluation of Mechanisms of Mitochondrial Dysfunction During Exposures to NRTIs Using MitoChip

Is the mechanism of NRTI-induced mitochondrial dysfunction a result of inhibition of DNA polymerase gamma or multi-factorial?

The first validation study performed for MitoChip was in collaboration with the National Toxicology Programme (NTP) at NCTR. This study involved treatment of female C3B6F$_1$*trp*53(+/−) haplodeficient and C3B6F$_1$*trp*53(+/+) wild-type mice with 240 mg/kg body weight (bw)/day AZT or a combination of AZT and 3TC at 160 and 100 mg/kg bw/day, respectively. These mice were transplacentally exposed from gestation day (GD)-12 to GD-18 followed by continued dosing in infant mice from postnatal day (PND)-1 to PND-28. Transcriptional profiling using MitoChip revealed no significant difference in expression levels of hepatic mitochondria-related genes between *p53* (+/−) haplodeficient and *p53* (+/+) wild-type mice (Desai *et al*., 2008). A striking finding was the greater effect of the combination regimen on expression levels of hepatic mitochondria-related genes than AZT alone in both genotypes. To elucidate the mechanism of mitochondrial dysfunction, knowledge of the interaction between mitochondrial and nuclear genomes is crucial because coordination between the two genomes determines the activity of mitochondria. One of the advantages of using the MitoChip is that it provides better insight into the cross-talk between mitochondrial and nuclear genomes under perturbed conditions such as NRTIs exposures.

In female C3B6F$_1$*trp*53 mice, AZT/3TC caused a significant decline in expression levels of mtDNA-encoded genes of complex I (NADH ubiquinone dehydrogenase), subunit 2 and 5 (ND-2 and ND-5) and complex IV (cytochrome *c* oxidase), subunits 1 and 2 (Co-I and Co-II) in conjunction with a severe down-regulation of 16 out of 19 tRNAs (84%) evaluated on the MitoChip (Table 2). In contrast, only 58% of the tRNAs were down-regulated by AZT alone. Interestingly, these changes in hepatic mitochondrial genes in AZT/3TC-treated mice were associated with significant up-regulation of a majority of differentially expressed nuclear genes of complexes I and IV, suggesting a compensatory mechanism to maintain the function of these complexes. Similarly, nuclear genes of complexes III and V were also up-regulated in these mice. The effect of NRTIs on mtDNA has been demonstrated by Chan *et al*. (2007) who showed greater damage to mtDNA in heart tissue of CD-1 mice perinatally exposed to AZT/3TC compared with the damage noted in mice treated with AZT or 3TC alone. Corroborating our genomic findings is a study that reported substantially altered activities of complexes I and IV in *Erythrocebus patas* monkeys exposed to AZT/3TC *in utero* (Gerschenson *et al*., 2004). However, important as these results are, they lack information on consequences of AZT/3TC exposure in terms of other mitochon-

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Table 2. Genes significantly affected by AZT and AZT/3TC treatments in the liver.

GenBank accession ID	Functional category	AZT 240 mg/kg bw/day		AZT + 3TC 160 + 100 mg/kg bw/day	
		FC	*P*	FC	*P*
Oxidative phosphorylation (combined effect of complexes I–V)			NS		**0.015**
NADH ubiquinone dehydrogenase (complex I) (29)			NS		**0.049**
NM_010886	Complex 1, alpha subcomplex, 4 (Ndufa4)	1.164	0.119	1.255	0.008*
NM_028177	Complex 1, alpha/beta subcomplex, 1 (Ndufab1)	1.098	0.232	1.176	0.017*
NM_027175	Complex 1, alpha subcomplex, assembly factor 1 (Ndufaf1)	1.112	0.174	1.167	0.020*
NM_025597	Complex 1, beta subcomplex 3 (Ndufb3)	0.871	0.088	0.866	0.031*
NM_025316	Complex 1, beta subcomplex 5 (Ndufb5)	0.851	0.044*	0.739	0.0001*
NM_023172	Complex 1, beta subcomplex 9 (Ndufb9)	1.061	0.525	1.197	0.026*
NM_025523	Complex 1, subcomplex unknown, 1 (Ndufc1)	1.103	0.211	1.258	0.002*
NM_145518	Complex 1, Fe-S protein 1 (Ndufs1)	1.157	0.067	1.175	0.016*
AU018363	Complex I, subunit 2, mitochondrial (mt-Nd2)	1.161	0.129	0.819	0.017*
AU018713	Complex I, subunit 5, mitochondrial (mt-Nd5)	0.836	0.164	0.681	0.002*
Succinate ubiquinone dehydrogenase (complex II) (3)			NS		**0.043**
NM_023374	Succinate dehydrogenase Ip subunit (Sdhb)	1.226	0.038*	0.780	0.004*
Ubiquinol cytochrome c reductase (complex III) (7)			**0.030**		**0.002**
NM_026219	Ubiquinol-cytochrome *c* reductase binding protein (Uqcrb)	1.082	0.490	1.261	0.021*
NM_025407	Ubiquinol-cytochrome *c* reductase core protein 1 (Uqcrc1)	1.256	0.011*	1.248	0.003*
AK075856	Ubiquinol-cytochrome *c* reductase core protein 2 (Uqcrc2)	0.548	0.000*	0.604	0.000*
Cytochrome c oxidase activity (Complex IV) (13)			NS		**0.001**
NM_007747	Cytochrome *c* oxidase, subunit Va (Cox5a)	0.908	0.282	0.789	0.004*
NM_009942	Cytochrome *c* oxidase, subunit Vb (Cox5b)	1.140	0.260	1.267	0.019*
NM_007748	Cytochrome *c* oxidase, subunit VI a, polypeptide 1 (Cox6a1)	1.186	0.049*	1.189	0.016*
NM_053071	Cytochrome *c* oxidase, subunit VIc (Cox6c)	1.159	0.073	1.223	0.005*
NM_007749	Cytochrome *c* oxidase, subunit VIIc (Cox7c)	1.115	0.239	1.268	0.005*
AU018394	Cytochrome *c* oxidase I, mitochondrial (mt-Co1)	0.976	0.844	0.489	0.0001*
AU019143	Cytochrome *c* oxidase II, mitochondrial (mt-Co2)	0.927	0.328	0.775	0.001*
AB042432	Cytochrome *c* oxidase III, mitochondrial (mt-Co3)	1.108	0.370	1.253	0.023*
ATP synthase (complex V) (14)			NS		**0.022**
NM_020615	ATP synthase, H + transporting, F1 complex, gamma polypeptide 1 (Atp5c1)	1.157	0.120	1.220	0.013*
NM_175015	ATP synthase, H + transporting, F0 complex, subunit c (subunit 9), isoform 3 (Atp5g3)	0.934	0.389	0.844	0.016*
NM_027862	ATP synthase, H + transporting, F0 complex, subunit d (Atp5h)	1.048	0.614	1.209	0.020*
NM_016755	ATP synthase, H + transporting, F0 complex, subunit f (Atp5j)	1.098	0.238	1.265	0.002*
NM_020582	ATP synthase, H + transporting, F0 complex, subunit f, isoform 2 (Atp5j2)	1.027	0.792	1.228	0.022*
NM_138597	ATP synthase, H + transporting, F1 complex, O subunit (Atp5o)	0.963	0.669	0.861	0.045*

(*Continued*)

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Table 2. (*Continued*)

GenBank accession ID	Functional category	AZT 240 mg/kg bw/day FC	AZT 240 mg/kg bw/day *P*	AZT + 3TC 160 + 100 mg/kg bw/day FC	AZT + 3TC 160 + 100 mg/kg bw/day *P*
Fatty acid/Lipid metabolism (30)			**0.023**		**0.007**
NM_025826	Acyl-CoA dehydrogenase, short/branched chain (Acadsb)	1.117	0.257	1.210	0.023*
AF179975	Fatty acid CoA ligase, long chain 6 (Acsl6)	0.705	0.000*	0.768	0.001*
NM_013495	Carnitine palmitoyltransferase 1a, liver (Cpt1a)	1.347	0.007*	1.396	0.001*
NM_007760	Carnitine acetyltransferase (Crat)	1.230	0.027*	1.260	0.004*
NM_025797	Cytochrome *b*-5 (Cyb5)	1.195	0.220	1.463	0.004*
NM_019779	Cytochrome P450, family 11, subfamily a, polypeptide 1 (Cyp11a1)	0.846	0.136	0.785	0.013*
NM_010023	Dodecenoyl-CoA delta isomerase (3,2 trans-enoyl-CoA isomerase) (Dci)	0.940	0.448	0.868	0.042*
NM_053119	Enoyl CoA hydratase, short chain, 1 (Echs1)	1.049	0.599	1.206	0.019*
NM_007997	Ferredoxin reductase (Fdxr)	1.236	0.035*	1.223	0.014*
NM_178878	Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), alpha subunit (Hadha)	1.310	0.006*	1.299	0.001*
NM_145558	Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), beta subunit (Hadhb)	1.262	0.020*	1.057	0.437
NM_033134	Inositol polyphosphate-5-phosphatase E (Inpp5e)	1.168	0.065	1.186	0.015*
Krebs cycle (15)			NS		**0.003**
NM_080633	Aconitase 2 (Aco2)	1.298	0.017*	1.171	0.058
NM_026444	Citrate synthase (Cs)	1.255	0.023*	1.150	0.065
BC006702	Dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex) (Dlst)	1.106	0.171	1.166	0.015*
NM_010209	Fumarate hydratase 1 (Fh1)	0.999	0.989	1.199	0.025*
NM_173011	Isocitrate dehydrogenase 2 (NADP+) (Idh2)	1.151	0.091	1.242	0.004*
NM_029573	Isocitrate dehydrogenase 3 (NAD+) alpha (Idh3a)	1.215	0.043*	1.226	0.011*
NM_008323	Isocitrate dehydrogenase 3 (NAD+), gamma (Idh3g)	0.816	0.038*	0.778	0.003*
NM_023374	Succinate dehydrogenase Ip subunit (Sdhb)	1.226	0.038*	0.780	0.004*
Mitochondrial fusion (4)			NS		**0.022**
NM_024200	Mitofusin 1 (Mfn1)	1.105	0.229	1.152	0.042*
NM_133201	Mitofusin 2 (Mfn2)	1.129	0.151	1.178	0.022*
NM_133752	Optic atrophy 1 homologue (Opa1)	1.082	0.286	1.171	0.015*
Mitochondrial DNA replication (5)			NS		**0.030**
NM_153796	Twinkle (Peo)	0.991	0.914	0.870	0.042*
NM_021028	Thymidine kinase 2, mitochondrial (Tk2)	1.261	0.010*	1.166	0.026*
Membrane transport proteins (57)			**0.005**		**0.0006**
NM_022026	Aquaporin 9 (Aq9)	1.274	0.014*	1.242	0.007*
NM_030054	Mitochondrial solute carrier protein (Mscp)	1.283	0.053	1.280	0.020*
NM_016714	Nucleoporin 50 (Nup50)	1.666	0.004*	1.242	0.086
NM_027324	Sideroflexin 1 (Sfxn1)	1.159	0.136	1.178	0.045*
NM_053196	Sideroflexin 2 (Sfxn2)	1.140	0.171	1.222	0.015*
NM_053198	Sideroflexin 4 (Sfxn4)	1.157	0.113	1.164	0.044*
NM_153150	Solute carrier family 25, member 1 (Slc25a1)	1.169	0.044*	1.099	0.114
NM_024211	Solute carrier family 25, member 11 (oxoglutarate carrier), (Slc25a11)	1.337	0.018*	1.027	0.755

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Table 2. (*Continued*)

GenBank accession ID	Functional category	AZT 240 mg/kg bw/day		AZT + 3TC 160 + 100 mg/kg bw/day	
		FC	*P*	FC	*P*
NM_172436	Solute carrier family 25, member 12 (Aralar carrier) (Slc25a12)	0.875	0.143	0.777	0.003*
NM_011017	Solute carrier family 25, member 15 (ornithine transporter) (Slc25a15)	1.106	0.644	1.453	0.045*
NM_026071	Solute carrier family 25, member 19 (deoxynucleotide carrier) (Slc25a19)	0.774	0.002*	0.806	0.001*
AK077159	Solute carrier family 25, member 2 (ornithine transporter) (Slc25a2)	0.756	0.002*	0.789	0.001*
NM_020520	Carnitine/acylcarnitine translocase (Slc25a20)	1.252	0.074	1.236	0.038*
NM_181328	Solute carrier family 25), member 29 (palmitoylcarnitine transporter (Slc25a29)	1.046	0.723	1.297	0.019*
NM_133668	Solute carrier family 25, member 3 (phosphate carrier) (Slc25a3)	0.919	0.254	0.803	0.002*
NM_026542	Solute carrier family 25, member 39 (Slc25a39)	1.172	0.039*	1.035	0.539
AK078077	Solute carrier family 25, member 4 (adenine nucleotide translocator) (Slc25a4)	1.895	0.033*	1.618	0.041*
NM_007451	Solute carrier family 25, member 5 (Slc25a5)	1.189	0.020*	1.105	0.077
NM_016917	Solute carrier family 40, member 1 (iron-regulated transporter) (Slc40a1)	1.313	0.050*	1.415	0.004*
NM_011591	Translocator of inner mitochondrial membrane 17b (Timm17b)	1.330	0.008*	1.228	0.013*
NM_016897	Translocase of inner mitochondrial membrane 23 homologue (Timm23)	1.173	0.052	1.281	0.001*
NM_011694	Voltage-dependent anion channel 1 (Vdac1)	1.241	0.027*	0.606	0.0001*

NS, not significant. Number in parenthesis indicates total number of genes evaluated for the functional category. Fold change (FC) is relative fold change in the expression level of each gene calculated as a ratio of average expression level of treated samples to control samples. Fold changes with *P*-values ≤ 0.05 corresponding to each gene were considered significant and are marked with an asterisk. *P*-values in bold represent treatment effect on overall function, which is the combined effect of drug-induced change in expression level of each gene associated with functional category. Gene names are associated with gene symbols in parenthesis.

drial targets. MitoChip, on the other hand, provided information on AZT- and AZT/3TC-related changes in expression levels of genes associated with oxidative phosphorylation as well as shed some light on their influence on other mitochondrial pathways and processes (Desai *et al*., 2008). As an example, exposure to AZT/3TC was associated with changes in expression levels of genes involved in mitochondrial fusion. The mitochondrial fusion is a dynamic process that regulates mitochondrial size and morphology (Legros *et al*., 2002). Furthermore, it has been suggested that it may serve as a protective mechanism in response to altered bioenergetics (Chen and Chan, 2005). In view of this, up-regulation of genes, mitofusins (Mfn) 1 and Mfn 2 and optic atrophy 1 homologue (Table 2), involved in mitochondrial fusion in AZT/3TC-exposed mice may indicate an adaptation to altered mitochondrial respiratory function to sustain cell survival in AZT/3TC-treated mice.

Although one of the proposed mechanisms of NRTI-related mitochondrial toxicity is the result of direct inhibition of DNA polymerase gamma (Lewis and Dalakas, 1995; reviewed in Lewis, Day and Copeland, 2003), neither AZT nor AZT/3TC altered expression levels of polymerase gamma or its accessory subunit polymerase gamma 2 in the liver of C3B6F$_1$*trp*53 mice (Desai *et al*., 2008). However, the expression level of twinkle, a mitochondrial helicase, was significantly decreased only by AZT/3TC, suggesting that other genes essential in mtDNA replication may play an equally important role in influencing mtDNA levels during NRTI treatments. Consistent with this concept was lower mtDNA copy number in human osteosarcoma cells due to reduced expression of twinkle caused by RNA interference (Tyynismaa *et al*., 2004).

One of the adverse effects of NRTIs is lipodystrophy, a condition characterized by abnormalities in fatty (adipose) tissue associated with total or

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partial loss of body fat, which could in part be related to defects in mitochondrial fatty acid metabolism. Thus far, studies investigating NRTI-mediated changes in fatty acid metabolism are inadequate to delineate the basis of lipodystrophy. Results obtained using the MitoChip have however aided in the understanding of the consequences of AZT and AZT/3TC exposures on the fatty acid β-oxidation pathway in C3B6F$_1$*trp*53 mice. Expression levels of genes associated with the initial step of dehydrogenation (acetyl-Coenzyme A dehydrogenase, short/branched chain) and the second step of hydration (enoyl-Coenzyme A hydratase, short chain, 1) of short-chain fatty acids were increased only by AZT/3TC, whereas hydroxyacyl-coenzyme A dehydrogenase-α that performs a dual role of dehydrogenation and hydration of long-chain fatty acids was induced by both AZT and AZT/3TC. Moreover, the expression level of hydroxyacyl-coenzyme A dehydrogenase-β, which is responsible for 3-ketoacyl-coenzyme A thiolase activity on long-chain fatty acids, was increased only by AZT treatment but not by AZT/3TC. Based on these findings, it appears that genes involved in the oxidation of short/branched-chain and long-chain fatty acids were influenced by AZT/3TC, whereas AZT treatment alone affected genes involved in the oxidation of only long-chain fatty acids. Both AZT- and AZT/3TC-treated mice also exhibited increased transcriptional level of carnitine palmitoyltransferase 1a, required for the transport of long-chain fatty acids. In view of these observations, up-regulation of genes associated with the oxidation of long-chain fatty acids may be in response to the enhanced transport of long-chain fatty acids. Collectively, these changes may imply altered fatty acid metabolism during exposure to NRTIs. If these changes signify increased fatty acid oxidation, then increased transcriptional levels of several genes associated with the Krebs cycle could be correlated to changes in fatty acid oxidation in both AZT- and AZT/3TC-treated mice (Table 2).

Each mitochondrion has its own DNA. However, mitochondrial proteins encoded by nuclear DNA are synthesized in the cytoplasm followed by their transport to different mitochondrial compartments by translocases of the outer and inner mitochondrial membranes. In addition, several other membrane translocators are essential for the transport of ions and solutes for mitochondrial activity. The use of the MitoChip also provided important information concerning AZT- and AZT/3TC-related changes in the transcriptional levels of various membrane transporters in the liver of C3B6F$_1$*trp*53 mice. The AZT- and AZT/3TC-mediated down-regulation of the deoxynucleotide carrier Slc25a19 may be of particular importance because of its role in the transport of deoxynucleotides, required for the synthesis of mtDNA into the mitochondrial matrix (Dolce *et al*., 2001). This change may partly influence the mtDNA levels by limiting the dNTP pools within the matrix. Moreover, AZT/3TC-mediated differential expression of sideroflexins (Sfxn1, 2 and 4) involved in the transport of components required for iron utilization into or out of mitochondria may have implications in conditions such as anaemia, one of the early adverse events observed in HIV-1-infected patients on NRTIs (Shah, 2006; Sivadasan *et al*., 2009).

It is important to note that NRTI-mediated changes in the expression levels of mitochondria-related genes were subtle. However, taken together, these relatively small changes in the expression levels of genes associated with a molecular function or a pathway may be biologically significant as these genes act synergistically. The results clearly indicate a greater influence of AZT/3TC (160/100 mg/kg) on mitochondria than of AZT (240 mg/kg) alone in the liver of C3B6F$_1$*trp*53 mice. This AZT/3TC-induced altered transcriptional profiles of mitochondria-related genes could be the result of synergistic action of 3TC with AZT based on the findings from the previous study in our laboratory that showed no effect of perinatal exposure to 160 mg/kg AZT on the transcriptional levels of hepatic mitochondria-related genes in both haplodeficient and wild-type C3B6F$_1$*trp*53 mice (unpublished data). Lamivudine (3TC) monotherapy is not recommended to HIV-1-infected patients; therefore, animals treated with 3TC alone were not included in this study. Altogether, these results underline the fact that NRTI-mediated changes in expression levels of genes related to mitochondrial function is a consequence of drug action on multiple mitochondrial targets and not exclusively due to inhibition of DNA polymerase gamma as previously postulated. Other factors such as genes involved in mtDNA replication (twinkle) and/or transport of substrates for mtDNA synthesis (Slc25a19) can equally influence mtDNA synthesis during NRTI exposure. The extent of the data generated by MitoChip clearly demonstrates its utility as a valuable genomic tool in understanding different aspects of mitochondrial function at the genome level during NRTI exposures, providing data for the generation of new hypotheses about

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mitochondrial function. Such information is vital in designing safer treatment strategies or developing novel interventions to minimize the severity of drug-related side-effects in HIV-1-infected patients.

Is DNA polymerase gamma-mediated mechanism of mitochondrial dysfunction during NRTI exposure tissue-specific?

There is considerable evidence that indicates depletion of mtDNA levels in the heart and skeletal muscle of *Erythrocebus patas* monkeys exposed to 10 and 40 mg/kg of AZT *in utero* (Gerschenson and Poirier, 2000) and in skeletal muscle of AZT-treated rats (Lewis *et al*., 1992). This could partly be related to inhibition of DNA polymerase gamma. Supporting this notion was a study in our laboratory that showed a modest, but statistically significant dose-related decline in the mRNA expression level of DNA polymerase gamma in the skeletal muscle of male and female $B6C3F_1$ infant mice treated short term (PND-1 to PND-8) with 10, 50, 100 and 200 mg/kg AZT (Desai *et al*., 2009; Table 3). It is noteworthy that despite the small sample size ($N = 3$ per group), AZT-related decrease in the expression level of DNA polymerase gamma was significant in $B6C3F_1$ infant mice. Based on body surface area (mg/m^2), these doses correspond to human infant doses of 1.1, 5.5, 11.0 and 22.0 mg/kg, respectively. Interestingly, the effect of AZT on DNA polymerase gamma was evident in skeletal muscle of infant mice even at doses lower than a therapeutic dose (8 mg/kg/day). The change in DNA polymerase gamma was also associated with a significant dose-related decrease in the transcriptional levels of membrane transporters for deoxynucleotides (Slc25a19) and phosphates (Slc25a3), which, in turn, may affect mtDNA content in skeletal muscle by altering the transport of substrates essential for mtDNA synthesis. In addition, the expression of a gene encoding mitochondrial ribosomal protein L17, involved in mitochondrial genome maintenance, was significantly altered in skeletal muscle of these mice. Although mtDNA content was not measured in the skeletal muscle in infant mice, lower expression levels of genes involved in mtDNA replication and maintenance as well as transporters of substrates required for mtDNA synthesis together could change mtDNA levels in skeletal muscle.

Although significant decreases in the transcriptional level of DNA polymerase gamma were observed in skeletal muscle of infant $B6C3F_1$ mice at AZT doses lower than a therapeutic dose, the expression level of DNA polymerase gamma remained unchanged in the liver of $C3B6F_1$*trp*53 mice during perinatal exposure to 240 mg/kg AZT, 2.4-fold the recommended dose in human infants. Changes in the hepatic transcript level of DNA polymerase gamma were also not seen in another experiment in which adult male and female $B6C3F_1$ mice were postnatally exposed to AZT at doses as high as 600 mg/kg/day (4.8-fold human equivalent dose) for 12 weeks. In this experiment, AZT treatment neither changed the expression levels of genes involved in mtDNA replication nor altered the mtDNA copy number in the liver (unpublished data; partly presented in Table 3). It is important to mention that these results were derived from both adult and infant mice of different strains ($C3B6F_1$*trp*53 and $B6C3F_1$), receiving different AZT doses, and under different exposure conditions (perinatal and postnatal). However, based on observations that short-term exposure to AZT doses lower than a therapeutic dose resulted in a decrease in the expression level of DNA polymerase gamma in skeletal muscle of infant mice, whereas long-term AZT treatment at doses higher than two- and four-fold human equivalent doses in $C3B6F_1$*trp*53 and $B6C3F_1$, respectively, showed no effect on the transcription level of DNA polymerase gamma in the liver, one can hypothesize that DNA polymerase gamma-related mitochondrial toxicity during AZT exposure might be tissue-specific.

6.2 Weight-Loss Dietary Supplement, Usnic Acid, and its Effect on Mitochondria

Herbal dietary supplements are often regarded as safe remedies by consumers. Nonetheless, these products can result in severe adverse effects and can interfere with the efficacy of prescription drugs. Usnic acid is a lichen metabolite that exhibits antimicrobial (Lauterwein *et al*., 1995), antiviral (Scirpa *et al*., 1999) and anti-inflammatory (Vijayakumar *et al*., 2000) properties. Because of such broad-spectrum beneficial biological characteristics, this natural product is widely used in the treatment of a number of ailments (Rafanelli *et al*., 1995; Ingolfsdottir, 2002). The therapeutic antimicrobial and anti-inflammatory activities of usnic acid are due to its ability to uncouple mitochondria (Abo-Khatwa, Al-Robai and Al-Jawhari, 1996; Ingolfsdottir, 2002) and the same property is the

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Table 3. Effect of AZT on genes involved in mtDNA synthesis and repair in liver and skeletal muscle.

		AZT doses (mg/kg body weight/day)												
		Liver				Skeletal muscle								
		C3B6F$_1$*trp*53 mice[a]		B6C3F$_1$ mice[b]		B6C3F$_1$ mice[c]								
GenBank accession ID	Gene description (function)	240 mg		600 mg		10 mg		50 mg		100 mg		200 mg		Dose-related response
		FC	*P*	FC	*P*	FC	*P*	FC	*P*	FC	*P*	FC	*P*	
NM_025301	Mitochondrial ribosomal protein L17 (mtDNA maintenance)	1.089	0.373	1.159	0.213	0.677	0.000*	0.774	0.006*	0.725	0.001*	0.674	0.000*	0.004
NM_017462	Polymerase (DNA directed), gamma (mtDNA replication)	0.946	0.217	1.006	0.898	0.857	0.000*	0.900	0.012*	0.886	0.005*	0.864	0.001*	0.035
NM_026071	Solute carrier family 25 (deoxynucleotide carrier), member 19 (transport of deoxynucleotides)	0.774	0.002*	1.070	0.202	0.959	0.230	0.957	0.208	0.934	0.059	0.910	0.008*	0.011
NM_133668	Solute carrier family 25 (phosphate carrier), member 3 (Transport of phosphates)	0.919	0.254	1.094	0.254	0.829	0.005*	0.874	0.036*	0.840	0.009*	0.812	0.002*	0.019

[a] Fold change (FC) represents relative fold change in the expression level of each gene calculated as a ratio of average expression level of treated samples to control samples. Fold changes with *P*-values ≤ 0.05 corresponding to each gene were considered significant and are marked with an asterisk.

[b] Mice treated with 240 mg/kg AZT from gestational day12 to 18 followed by continued treatment from postnatal day (PND) 1 to 28 and humanely sacrificed at 1 h following the last dose ($N = 3$ per group).

[c] Mice treated with 600 mg/kg AZT for 12 weeks beginning at 8 weeks of age and humanely sacrificed at 24 h following the last dose ($N = 4$ per group).

Mice treated with 10, 50, 100 and 200 mg/kg AZT from PND 1 to 8 and humanely sacrificed at 1 h following the last dose ($N = 3$ per group).

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rationale for which it is marketed as a dietary supplement to lose weight. However, to achieve significant weight reduction, relatively high doses of usnic acid are required that can potentially result in serious side-effects. Its use became a safety concern when several cases of liver injury were reported in consumers who ingested dietary supplements containing usnic acid or sodium usneate for weight loss (Favreau *et al*., 2002; Frankos, 2004).

Animal studies investigating usnic acid-mediated effects on mitochondria not only are limited, but have primarily concentrated on measuring mitochondrial respiration or ATP production (Abo-Khatwa, Al-Robai and Al-Jawhari, 1996; Han *et al*., 2004; Pramyothin *et al*., 2004). In primary mouse hepatocytes, 2 μM (+)-usnic acid caused partial inhibition of state 3 respiration with a modest uncoupling of mitochondria, whereas a total inhibition of respiratory states 3 and 4 occurred at 5 μM (Han *et al*., 2004). Altered oxygen consumption, ADP/O ratio and ATP generation have been also reported in isolated rat liver mitochondria as well as in cultured mouse hepatocytes treated with hepatotoxic doses of (+)-usnic acid (Abo-Khatwa, Al-Robai and Al-Jawhari, 1996; Han *et al*., 2004; Pramyothin *et al*., 2004). Usnic acid-mediated morphological damage to hepatic mitochondria was also evident in rats treated intra-peritoneally with 200 mg/kg for 5 days (Pramyothin *et al*., 2004). Although several factors may be involved in usnic acid-induced liver injury, oxidative stress has been believed to be primarily responsible for liver toxicity based on changes observed in levels of hydrogen peroxide, lipid peroxidation and reduced glutathione in rat hepatocytes exposed to usnic acid (Han *et al*., 2004; Pramyothin *et al*., 2004).

Although these investigations have contributed to the understanding of the side-effects associated with dietary use of usnic acid, the precise molecular mechanism underlying usnic acid-induced liver toxicity still remains to be elucidated, probably due to lack of adequate knowledge concerning changes in overall mitochondrial activity. Critical mitochondrial pathways, such as fatty acid β-oxidation and the Krebs cycle that usnic acid might influence, have not been examined. Therefore, the MitoChip was used to investigate the interactions between mitochondrial pathways to understand the role of mitochondria in usnic acid-induced hepatotoxicity in a mouse model.

6.2.1 Determination of Molecular Changes Induced in the Mitochondria by Usnic Acid

In a collaborative study with the NTP at NCTR, transcriptional profiling of mitochondria-related genes was investigated in female $B6C3F_1$ mice administered (+)-usnic acid in rodent feed ad libitum for 14 days beginning at 8 weeks of age (Joseph *et al*., 2009). Groups of mice received 60, 180 and 600 ppm usnic acid/kg bw/day, doses that correspond to 15, 50 and 200 mg usnic acid/kg bw/day, respectively, based on the mean body weight and daily feeding measurements. The results obtained from the transcriptional analysis of 542 mitochondria-related genes showed a remarkable effect of usnic acid on genes associated with electron transport chain complexes, mitochondrial tRNAs, fatty acid/lipid metabolism, the Krebs cycle and apoptosis at the highest dose of 600 ppm (200 mg/kg) compared with controls.

A striking finding was a significant increase in expression levels of genes associated with all four complexes (I–IV) of the electron transport chain whereas complex V that is responsible for the production of ATP remained unaffected by usnic acid, suggesting usnic acid-mediated uncoupling. Tight coupling of electron transport with oxidative phosphorylation requires an adequate proton gradient across the inner mitochondrial membrane. Also, proper interaction between mitochondrial and nuclear genomes is essential, in particular for complexes I, III, IV and V, as these multi-subunit proteins are encoded by both mitochondrial and nuclear genomes. In usnic acid-treated mice, 30 out of 82 genes (37%) involved in oxidative phosphorylation were up-regulated in the liver. These included eight mitochondrial genes (five of complex I, one of complex III and two of complex IV) and 17 nuclear genes of complexes I–IV in association with increased expression of 10 mitochondrial tRNAs, suggesting enhanced mitochondrial electron transport activity. Considering this observation, one may argue that increased electron transport would result in a higher proton gradient across the inner mitochondrial membrane thereby, driving complex V to generate more ATP. In contrast, expression levels of genes of complex V remained unaltered by usnic acid. This may be due to the translocation of lipophilic usnic acid through mitochondrial membranes into the matrix, where it is

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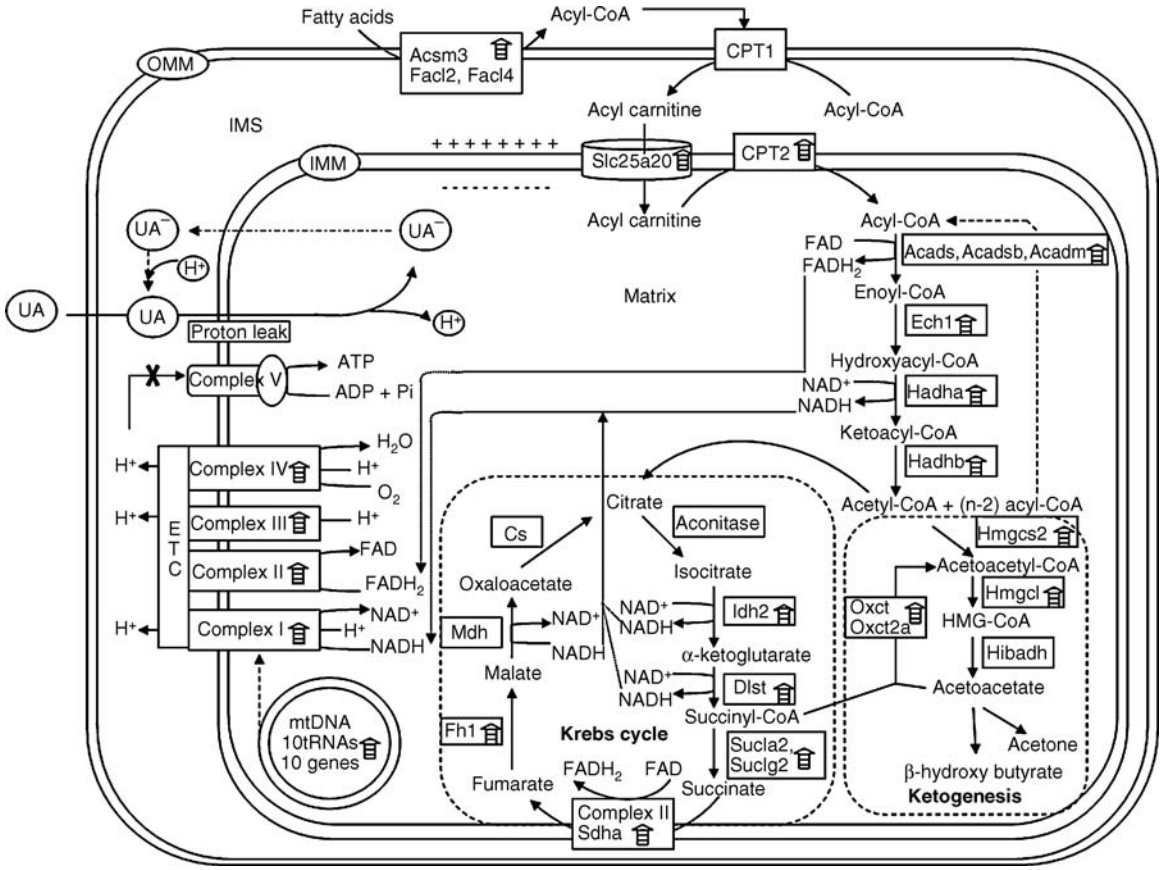

Figure 1. Schematic representation of UA-mediated effects on mitochondrial functions in the mouse liver. UA is a lipophilic compound that can easily pass through mitochondrial membranes into the matrix, releasing a proton into the matrix. The resulting usniate anion can diffuse back into the IMS where it can bind to a proton to re-form UA, which diffuses back into the matrix. The resulting cycle causes a proton leak that eventually can dissipate the proton gradient across the inner membrane, causing uncoupling. The insufficient proton motive force may not be able to activate complex V that in turn may alter ATP levels. However, complexes I–IV of the ETC can be induced by UA to create sufficient proton gradient across the IMM to activate complex V. This can also be associated with up-regulation of mitochondrial genes. This further may lead to activation of fatty acids to form acyl-CoAs followed by their increased transport into the matrix. Within the matrix, higher levels of acyl- CoA may induce the fatty acid β-oxidation pathway resulting in a final product, acetyl-CoA, which then can enter the Krebs cycle for continued oxidation to CO_2. However, if the amount of acetyl-CoA exceeds the capacity of the Krebs cycle, the level of acetyl-CoA can partly be diverted to ketogenesis followed by ketone body degradation to maintain steady-state level of ketone bodies. +++++ and – – – – – – indicate acidic and alkaline side of the IMM, respectively. Hatched arrows in the boxes indicate results of the present study. ADP, adenosine diphosphate; ATP, adenosine triphosphate; CoA, coenzyme A; cDNA, complementary DNA; CPT1, carnitine palmitoyltransferase 1; CoA, coenzyme A; Cs, citrate synthase; ETC, electron transport chain; FAD, flavin adenine dinucleotide (oxidized form); $FADH_2$, flavin adenine dinucleotide (reduced form); H^+, proton; Hibadh, 3-hydroxyisobutyrate dehydrogenase; IMM, inner mitochondrial membrane; IMS, inter-membrane space; Mdh, malate dehydrogenase; mtDNA, mitochondrial DNA; NAD+, nicotinamide adenine nucleotide (oxidized form); NADH, nicotinamide adenine nucleotide (reduced form); O_2, oxygen; OMM, outer mitochondrial membrane; Pi, inorganic phosphorus; rRNA, ribosomal RNA; tRNA, transfer RNA; UA, usnic acid; UA^-, anionic form of usnic acid.

ionized to usniate anion by releasing a proton. The resulting usniate anion then diffuses back into the inter-membrane space, where it binds to a proton (accumulated during electron transport through complexes I–IV) and translocates back into the matrix as usnic acid. The resulting proton shuttling cycle can potentially dissipate the proton gradient across the inner mitochondrial membrane, causing uncoupling (Figure 1). This mechanistic pathway explains the induction of genes associated with

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complexes I–IV, with no significant effect on complex V. This may be a compensatory mechanism to re-form the proton gradient necessary to maintain ATP synthesis by complex V. Although ATP levels were not measured in the liver of B6C3F$_1$ mice, it is important to note that a decline in ATP synthesis has been reported in isolated rat liver mitochondria exposed to (+)-usnic acid at concentrations higher than 4.5 μM (Pramyothin *et al*., 2004).

Up-regulation of genes that encode complexes I–IV in B6C3F$_1$ mice was also associated with increased expression of genes involved in fatty acid β-oxidation. These included genes associated with activation of fatty acid to acyl-coenzyme A (acyl-coenzyme A synthase, medium-chain family member 3, fatty acyl-coenzyme A ligase, long chain 2 and 4), translocation of acyl-coenzyme A into the matrix (carnitine palmitoyltransferase 2, carnitine acetyltransferase), oxidation of acyl-coenzyme A to enoyl-coenzyme A (acyl-coenzyme A dehydrogenase, short chain, short/branched chain and medium chain), hydrolysis of enoyl-coenzyme A to l-3-hydroxyacyl-coenzyme A and further oxidation to 3-ketoacyl-coenzyme A (hydroxyacyl-coenzyme A dehydrogenase-α and β and l-3-hydroxyacyl-coenzyme A dehydrogenase, short chain) (Figure 1). Collectively, these changes imply increased fatty acid oxidation in usnic acid-treated mice, explaining the rationale for the use of usnic acid as a weight-loss dietary supplement. Surprisingly, however, the decrease in body weights of these mice was not significant compared with controls. These mice also exhibited increased expression of genes associated with the Krebs cycle [isocitrate dehydrogenase 2 ($NADP^+$), isocitrate dehydrogenase 3, beta subunit and 3 (NAD^+) gamma subunit, succinate dehydrogenase Ip subunit, succinate-coenzyme A ligases]. It can be suggested that higher levels of acetyl-coenzyme A as a result of enhanced fatty acid oxidation may account for increased transcription of genes in the Krebs cycle. Altogether, these changes indicate an adaptive response to energy deficits due to usnic acid-mediated uncoupling.

Several pro-apoptotic genes [B-cell leukaemia/lymphoma 2 (Bcl2)-associated X protein, Bcl2-like 13, Bcl2/adenovirus E1B 19KDa-interacting protein 1, NIP3 (Bnip3), Bnip3-like, direct inhibitor of apoptosis (IAP)-binding protein with low isoelectric point (pI), mitochondrial carrier homologue 1, programmed cell death 8, sphingosine-1-phosphate phosphatase 1, voltage-dependent anion channel 1] were also induced by usnic acid treatment in B6C3F$_1$ mice. Usnic acid-mediated loss of mitochondrial coupling might contribute to increased sensitivity of the mitochondrial permeability transition (Scorrano, Petronilli and Bernardi, 1997), promoting apoptosis. Consistent with these findings was the observation of greater apoptotic changes observed by immuno-histochemical analysis in the liver of usnic acid-treated mice compared with controls (Ali *et al*., 2008).

Mitochondrial damage is often a trigger for apoptosis. However, hepatic mitochondria from B6C3F$_1$ mice treated with 600 ppm usnic acid (200 mg/kg) appeared to have retained sufficient mitochondrial function to maintain a healthy adaptive response to the uncoupling actions of usnic acid. Histological evaluation also showed no indication of liver damage in these mice. Alternatively, intra-peritoneal injection of 200 mg/kg usnic acid for 5 days caused structural damage to liver mitochondria as well as elevated levels of serum transaminases, indicating hepatic injury in rats (Pramyothin *et al*., 2004). Different routes of administration, oral versus intraperitoneal, may have resulted in different responses to 200 mg/kg usnic acid in liver mitochondria. It can be postulated that higher concentrations and/or long-term exposures to usnic acid might impair hepatic mitochondrial function in B6C3F$_1$ mice that can manifest in liver injury.

The application of the MitoChip has generated substantial information that allowed a clear understanding of the interactions between different mitochondrial pathways in the liver of B6C3F$_1$ mice during usnic acid exposure. It has also provided an important mechanistic insight into the molecular events that may have implications for usnic acid-induced liver toxicity. Results from this investigation were the first to provide extensive knowledge of hepatic mitochondrial function at the transcriptional level in B6C3F$_1$ mice administered usnic acid. Such information has the potential to be useful in determining the human risk of exposure to low levels of usnic acid that are present in dietary supplements.

7 CONCLUSIONS

The mouse MitoChip is the first mitochondria-specific microarray for gene expression profiling and was developed at the Center for Functional Genomics at NCTR, FDA. It has been shown to be

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useful in understanding the potential mitochondrial toxicity during drug exposures in mice. One of the advantages of the MitoChip is that it provides information regarding the interaction between two genomes at the transcriptional level, the nuclear genome and the mitochondrial genome. To determine the utility of this new genomic tool in toxicology, it was used to examine the molecular basis of mitochondrial dysfunction in the liver and skeletal muscle in different mouse models exposed to NRTIs such as AZT and 3TC. The data obtained in female C3B6F$_1$*trp*53 mice demonstrated a pronounced hepatic mitochondrial dysfunction as a result of perinatal exposure to AZT/3TC. A much reduced response was observed in mice treated with AZT alone. Besides generating comprehensive information on the transcriptional levels of genes involved in oxidative phosphorylation, MitoChip technology also provides insights into other critical mitochondrial pathways and processes, such as mitochondrial fusion. Changes observed in the transcription of genes involved in mitochondrial fusion led to the understanding that this may serve as a compensatory mechanism in response to energy deficit as a result of altered oxidative phosphorylation in AZT/3TC-treated mice. A widely accepted theory of NRTI-related mitochondrial toxicity is the inhibition of DNA polymerase gamma, resulting in depletion of mtDNA content. However, our data indicated that NRTI-related inhibition of DNA polymerase gamma may be tissue-specific. This was based on findings that the expression of DNA polymerase gamma was decreased in skeletal muscle but remained unchanged in the liver of mice treated with AZT. These studies also revealed that NRTI-mediated mitochondrial toxicity is multi-factorial, and that inhibition of DNA polymerase gamma may not be exclusively responsible for mitochondrial dysfunction. The use of the MitoChip has also been helpful in providing vital information on oxidative phosphorylation and its interaction with other mitochondrial pathways during exposure to usnic acid. Such information has implications in defining the role of mitochondria in usnic acid-induced hepatotoxicity that was observed in several individuals who consumed it for weight reduction. Collectively, these studies demonstrated the utility of the MitoChip as an important genomic tool to determine the molecular basis of various drug-induced toxicities associated with impaired mitochondrial function. Mechanisms associated with mitochondrial toxicity may differ with different drugs. Therefore, transcriptional analysis of mitochondria-related genes will be a valuable approach to enable characterization of genomic profiles pertinent to different toxicants or agents targeting mitochondria. The comprehensive toxicogenomics approach described here using the MitoChip is a promising new method for gaining an improved perspective of mitochondrial involvement during toxic exposures. Knowledge of the basis of mitochondrial dysfunction could be the key in the design and development of effective treatment strategies for minimizing drug-related toxicities and diseases associated with altered mitochondrial function. Finally, application of the MitoChip approach in genomic research holds great promise in revealing molecular links between mitochondria and a number of diseases and drug-induced toxicities associated with altered mitochondrial function.

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DISCLAIMER

The findings and conclusions in this report are those of the author and do not necessarily represent the views of the FDA.

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Integration of Systems Toxicology into Drug Discovery

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1 INTRODUCTION

Systems toxicology encompasses a variety of molecular, cellular and physiologically based experimental approaches to quantify and integrate large-scale highly parallel biological information on whole-body responses to xenobiotics for the purposes of toxicity prediction and human and environmental risk assessment of chemicals and therapeutic products. The application of systems toxicology implies the integration of knowledge from multiple systems, which may include the integration of biochemical, cellular and tissue responses measured *in vitro* or *in vivo*. The integration of multiple systems necessarily requires a consideration of how molecular changes impact higher-order processes and ultimately how these changes are manifested at an organismal level. This approach is expected to improve our ability to deduce more complex biological phenomena, such as physiology or pathology, from an integration of their component parts. This approach may also permit formal prediction of biological phenomena from the evaluation of more easily measured molecular responses. Taken together, systems toxicology has the potential to improve our ability to understand mechanisms of drug toxicity and predict the potential of drugs to cause toxicity, thereby improving efficiencies in drug development and our ability to assess human risk.

The subject of systems toxicology has primarily focused on the application of toxicogenomics, or global mRNA expression profiling, for understanding mechanisms of toxicity and identifying biomarkers predictive or diagnostic of adverse effects (Nuwaysir *et al*., 1999). Therefore, this chapter will focus on the use of toxicogenomics, the most established technology for measuring system-wide molecular changes, for enabling toxicological assessment at an early stage in drug discovery research. Other "omic" technologies concerned with protein or metabolite profiling (proteomics and metabolomics, respectively) have also been discussed in this context (Wetmore and Merrick, 2004; also see Use of Proteomic and Metabolomic Techniques in Ecotoxicological Research); however, their use has been limited owing to the need for more technology platform development and fewer published examples of demonstrated success. As such, they will not be reviewed here; however, many of the concepts of toxicogenomics could be applied to other "omic" technologies, generating multi-dimensional biological data. Time will tell whether such technologies provide value above and beyond toxicogenomics for mechanistic understanding or biomarker identification.

2 DISCOVERY TOXICOLOGY

Numerous reports have documented the increasing cost of drug development and the observed decline

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in productivity as represented by the number of new chemical entities (NCEs) that have been marketed in recent years (Frantz and Smith, 2003). The Tufts Center for Drug Development has estimated the cost of developing an NCE at $802M to $1.1 billion (DiMasi, Hansen and Grabowski, 2003). A large proportion of these costs are related to the cost incurred by developing compounds that ultimately fail as a result of the trial and error approach of drug discovery research. As a result of the poor success rate for developing successful drugs, one only needs to slightly improve productivity to have a pronounced effect on cost savings. For example, it has been estimated that a 12% increase in success rate could save between $221 and $242 million per approved drug (DiMasi, Hansen and Grabowski, 2003). The poor success rate and the failure of industry to realize gains in productivity brought on by advances in technology and our increased understanding of disease has been the subject of great debate and has motivated many to take steps to address gaps in the critical path for bringing effective and safe therapies to market (Woodcock and Woosley, 2008). This requires industry to alter their strategy and take steps to advance and adopt new technologies that will enhance productivity, whereas regulatory agencies must also adapt to begin to accept alternative solutions to the current, yet antiquated and costly, approach to drug discovery and development.

In response to these challenges, pharmaceutical companies have attempted to improve a number of critical aspects of drug discovery and development that contribute to the high rate of late-stage attrition. By far, one of the greatest sources of late-stage attrition is toxicity, which is encountered in both human and animal toxicity testing (Kola and Landis, 2004). Our ability to assess safety in humans is necessarily limited to clinical trials using a relatively small number of subjects, and in post-marketing surveillance where our statistical power to detect adverse effects may be improved, although not always adequately controlled for. Quite often adverse effects in humans are identified at later stages in human clinical testing when millions of dollars have already been invested. Nonetheless, we are limited to evaluating potential toxicity using animal models prior to investment in human clinical trials. These animal evaluations are still performed at relatively late stages after many years of drug discovery research and compound optimization have been performed.

While the timing of safety evaluation in humans or animals is clearly not optimal given the price of failure due to toxicity, also consider that our ability to predict human toxicity using animal models is quite low. For example, retrospective industry experience estimates that rodent toxicology studies only predict 43% of human toxicities, non-rodent studies predict 63% and the combination of both species predicts approximately 71% of human toxicities (Olson *et al.*, 2000). These results are somewhat misleading, however, as they fail to capture the toxicity correlates of drug candidates that are never tested in humans due to unacceptable animal toxicity. This has the potential to result in safe and effective drugs being halted in their development due to animal toxicities that may never materialize in humans. Unless mechanistic data demonstrate clearly that the compound in question is an animal-specific toxicant, compounds that are overtly toxic in pre-clinical studies are discontinued from further development. Thus, despite the reported poor predictivity, the true predictivity of animal studies is actually unknown in most cases. To further exacerbate the challenge, the increasingly competitive landscape in the pharmaceutical industry and the increased scrutiny over the safety of therapeutic products also raise the bar for developing successful new therapies. Despite the acknowledgement that animal models are not highly predictive of human toxicity, they remain the best tools to judge the likely safety profile of drug candidates before human safety can be empirically defined.

An emerging approach to deal with the late-stage attrition afflicting the pharmaceutical industry is to consider compound safety at an earlier stage in the drug discovery pipeline before formal development begins (Kramer, Sagartz and Morris, 2007). By considering safety at this early stage, compound selection criteria can be enforced and medicinal chemistry approaches altered to improve the quality of the compounds being advanced into later and costly stages of drug development. Advanced knowledge of unintended pharmacology and toxicity acquired through *in silico*, *in vitro* or *in vivo* approaches creates an opportunity to shift attrition-based decisions upstream in the process to points where corrective action can still be taken at a relatively low cost with relatively minimal investment in time. Therefore, the cost savings from discovery toxicology come from shifting, rather than reducing, attrition.

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A typical workflow for a drug discovery project team is depicted in Figure 1. Discovery research begins with the identification and validation of a target of interest, followed by high-throughput screening of potentially hundreds of thousands of compounds. Hits stemming from high-throughput screening are further evaluated for potency and drug-like properties including *in vitro* pharmacokinetic properties (e.g., metabolic stability, permeability, solubility, P450 inhibition) and physico-chemical considerations (Lipinski *et al*., 2001). Drug-like molecules exhibiting reasonable potency are further characterized for on-target efficacy, selectivity and *in vivo* pharmacokinetics. The lead scaffolds exhibiting the best combination of factors are selected for further optimization to generate a lead candidate suitable for *in vivo* toxicity evaluations. Compounds exhibiting a favorable combination of potency, efficacy, animal and predicted human pharmacokinetics, with reduced secondary pharmacology and suitable *in vivo* toleration, are chosen as clinical candidates to enter development. Development candidates are then thoroughly evaluated in a battery of toxicity tests to ensure their safe use in first-in-human (FIH) clinical trials. These studies typically include genotoxicity, safety pharmacology (cardiovascular, respiratory and central nervous system tests) and general *in vivo* toxicological evaluations in one rodent (i.e., rat) and one non-rodent species (i.e. dog or non-human primate).

The application of discovery toxicology in drug discovery research is illustrated in Figure 2. At the screening stage, very little compound is available to support direct experimentation in most toxicological assays. As a result, *in silico* approaches to identify potential toxicophores and unfavorable physico-chemical properties (Valerio, 2009) can be used as a means of identifying chemical scaffolds that may be problematic. Additionally, *in cerebro* evaluation of the pathways and biological and physiological effects of the target can be carried out to identify potential target-mediated adverse effects that may manifest *in vivo*. This proactive assessment of the target can aid in the design of experiments to evaluate possible target liabilities and enable early risk assessment around a target.

At the hit-to-lead stage, a more thorough characterization of the compounds is undertaken to identify potential off-target toxicities. More material is usually available to facilitate a moderate-throughput assay to evaluate interaction with unintended targets such as the hERG (potassium) channel and other ion channels that may present a cardiovascular risk (Hancox *et al*., 2008); with DNA that may present a risk for genotoxicity (Custer and Sweder, 2008) and with other closely related targets that may present a risk for unwanted pharmacology (Krejsa *et al*., 2003). Additionally, cytotoxicity and mechanism-based toxicity assays can be used to identify additional hazards that are known to commonly plague small molecule drugs (Abraham *et al*., 2008; O'Brien *et al*., 2006). Characterizing hits for potential hazards can be used to rank and prioritize the most promising lead scaffolds for further optimization before initiating more resource-intensive *in vivo* studies.

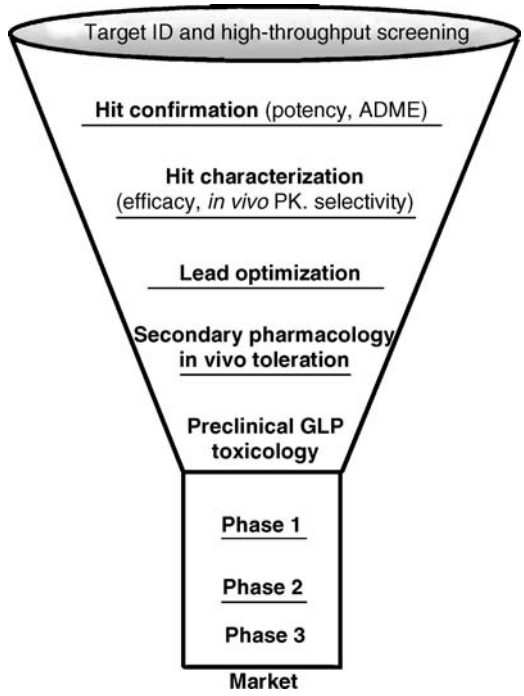

Figure 1. General drug discovery and development flow scheme for small molecule therapeutics.

At the lead optimization stage, medicinal chemistry attempts to modify the lead scaffold to improve a variety of features including potency and pharmacokinetics, and reduce any safety hazards identified through the hit characterization phase (i.e. hERG inhibition). As lead candidates mature, compounds can be tested in *in vivo* toleration assays, usually a rat and a non-rodent such as dog or non-human

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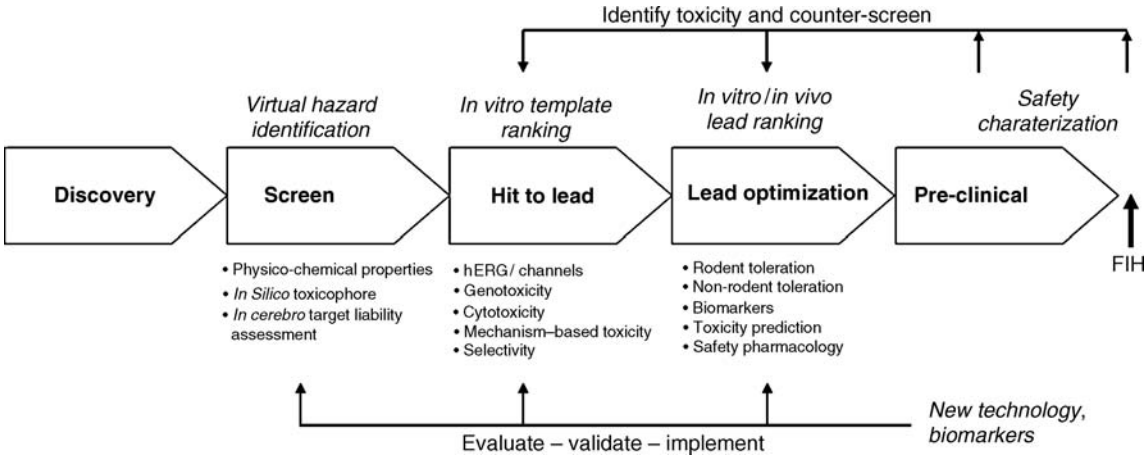

Figure 2. General discovery toxicology flow scheme.

primate, and non-good laboratory practice (GLP) safety pharmacology assays to select lead candidates for further pre-clinical testing in GLP toxicology studies. At this stage, biomarkers and systems toxicology approaches such as toxicogenomics can be used to further characterize and predict potential hazards that may emerge under longer-term dosing conditions, as detailed below.

Although a generic workflow as illustrated in Figure 2 is useful as a starting point to guide programme strategy, it must be recognized that each programme is unique and should be evaluated in light of its own clinical goals, patient population, the competition and any relevant regulatory considerations that will impact safety assessment. For example, anti-depressants targeting noradrenaline transporters may dictate an early evaluation of cardiovascular parameters in telemetrized rats before more resource-intensive rat toxicity studies are initiated. If rodents are not considered a sensitive species and toxicity in non-rodents are of heightened concern, then short-term intravenous infusions coupled with accessible biomarkers may facilitate a screening strategy that minimizes compound and animal usage at an early stage. Quite often lead compounds and their toxicity *in vivo* will dictate a screening strategy for prioritization and selection of backup molecules. In such cases, it is valuable to utilize investigative techniques, including toxicogenomics as described below, to rapidly identify mechanisms of toxicity to guide the application of *in vitro* screening assays (Gross and Kramer, 2003).

As compounds and lead series progress into *in vivo* models, including GLP toxicology studies, unanticipated toxicities may emerge that require a mechanistic understanding to enable human risk assessment. Such findings may halt further development and efforts will be concentrated on identifying follow-on compounds that are devoid of the toxicity. These efforts will benefit from a suitable *in vitro* or *in vivo* assay to provide an efficient means of screening additional compounds to avoid repeating the same mistake twice. It is here that toxicogenomics can positively influence drug discovery by providing a means to diagnose or predict toxicity, or aid in the understanding of mechanisms of toxicity. This information can be used in lead optimization to help understand the relevance of the findings for humans, and facilitate the screening and differentiation of molecules to aid in compound selection.

3 PREDICTIVE TOXICOGENOMICS AND APPLICATIONS TO DISCOVERY TOXICOLOGY

Initially, the first microarray experiments were limited in the number of genes evaluated and the analytical software consisted primarily of Microsoft Excel. These early investigations usually consisted of counting the number of genes that were up- or down-regulated in response to drug treatment, with little to no biological relevance ascribed to the changes other than determining the magnitude and direction of the observed fold changes (Farr

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and Dunn, 1999). With the increasing quality of the gene annotation, and the increased throughput and size of the microarrays being used, the focus shifted to using the technology to understand biology and mechanisms of drug action. This was still a manual process that required extensive curation to annotate the genes and studying the literature to derive mechanistic insight; however, the approach yielded useful information and reinforced the concept that gene expression changes needed to be anchored to pathological effects for accurate interpretation (Hamadeh *et al*., 2002b). True hypothesis testing with microarrays was still in its infancy.

The next advancement in toxicogenomics came with the observation that small gene sets could discriminate among mechanistically distinct drug classes (Bushel *et al*., 2002; Hamadeh *et al*., 2002a). These and other similar studies identified small gene sets that could mechanistically classify similar toxicants from one another and classify uncharacterized samples into pre-defined classes of similar mechanism (Thomas *et al*., 2001). These early proof-of-principle studies were critical to establish the foundation for subsequent experimental approaches and illustrate the significance of reference gene expression data sets for interpretation. These types of experiments provided the momentum for public and private efforts to construct large reference databases of toxicogenomic data to enable compound classification and the development of predictive gene sets or signatures (Ganter *et al*., 2005; Waters *et al*., 2003; Williams-DeVane, Wolf and Richard, 2009). Many pharmaceutical companies embarked on extensive internal efforts to establish or license for use gene expression databases to develop and apply signatures for lead optimization (Steiner *et al*., 2004; McMillian *et al*., 2005; Ganter *et al*., 2005).

There are many publications describing the use of toxicogenomics for the prediction of drug-induced toxicity in the rodent, which is the primary *in vivo* screening tool of choice in the pharmaceutical industry. A few chosen examples are highlighted in Table 1. Most of the published efforts have focused on the prediction of liver and kidney toxicity, with few efforts focused on the prediction of cardiotoxicity and other tissues. This likely reflects the greater incidence of drug-induced toxicities in these organs, in addition to the well-described mechanisms of toxicity in these organs. These approaches typically utilize tissues from rats following short-term (i.e., 24 h to 14 days of repeated daily doses) treatment to identify gene expression changes that are statistically associated with the pathology (Steiner *et al*., 2004; Thukral *et al*., 2005; Brown *et al*., 2002), or in some cases precede the histological appearance of tissue injury (Fielden *et al*., 2005). These putative biomarkers are then evaluated independently, although not always, against other toxicants with the goal of evaluating how accurately the biomarker can diagnose or predict the pathological event. Although histological diagnosis of tissue injury will remain the gold standard for evaluating drug-induced toxicity, the ability to predict the onset of tissue injury before it is observed microscopically can aid in the early identification of toxic effects that may not manifest until longer-term studies are conducted. As a result, toxicogenomics could be applied in the early stages of lead candidate selection testing as a means of predicting longer-term safety. Together with other endpoints, including traditional histology and clinical pathology, toxicogenomics data could

Table 1. Examples of predictive toxicogenomic approaches *in vivo*.

Predicted endpoint	Model	Study design	Reference
Hepatotoxicity			
Various liver pathologies	Rat	6-h to 14-day oral repeat dose	Steiner *et al*. (2004)
Oxidative stress/peroxisome proliferators/macrophage activators	Rat	24-h single oral dose	McMillian *et al*. (2005)
Acute hepatotoxicity	Rat	24-h single oral dose	Zidek *et al*. (2007)
Nephrotoxicity			
Proximal tubular toxicity	Rat	7-day oral repeat dose	Thukral *et al*. (2005)
Renal tubular toxicity	Rat	28-day oral repeat dose	Fielden *et al*. (2005)
Renal tubular toxicity	Rat	28-day oral repeat dose	Kondo *et al*. (2009)
Cardiotoxicity			
Cardiomyopathy	Rat	7-day intraperitoneal dose	Brown *et al*. (2002)

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aid in the selection of clinical candidates prior to development.

In addition to the prediction of future tissue injury, toxicogenomics data can be used to help diagnose tissue injury. Quite often, gene expression changes are readily observed when tissue damage has occurred. This often manifests as an increase in the expression of genes controlling the inflammatory response to injury, or adaptive changes to respond to unfolded proteins, oxidative stress or other cellular responses to maintain homeostasis. These expression changes are not definitive proof of tissue damage, but when used in combination with histological data can contribute to a weight of evidence in the assessment of potential toxicity that may be deemed equivocal when evaluated by traditional means. The contribution of toxicogenomics data to confirm pathological diagnoses is also particularly valuable in early short-term *in vivo* screening studies. Many histological changes occur with limited frequency and can easily be missed when only a few animals per treatment group are to be evaluated.

Although numerous success stories have been published, it is likely that many failures have also been identified in the course of toxicogenomics research that is less frequently communicated in the scientific literature. Foster *et al*. (2007) offer a valuable retrospective evaluation of how toxicogenomics has, and has not, impacted pre-clinical testing and lead optimization. Their findings indicate that significant transcriptional changes were more often observed prior to the histological structural change in the tissue, which further suggests that gene expression signatures can be predictive (i.e., pre-monitory). Their survey data also indicate that mechanistic data were obtained for 30% of the targets evaluated. Despite routine use of toxicogenomics in lead optimization, it is clear that the technology is not capable of diagnosing or predicting all types of tissue toxicity or for all compound classes and mechanisms, and that a certain degree of false negatives is expected. When combined with traditional data, however, it is likely that the overall number of false negatives progressing through development will be low. Perhaps of greater concern, though, is the perception of false positives. Although gene expression changes themselves are not considered adverse, a gene expression-based prediction of toxicity in the absence of histological changes could unnecessarily impugn an otherwise safe compound if misinterpreted. This concern has perhaps curtailed the more widespread use and acceptance of toxicogenomics across regulated industries. Nonetheless, their use is continuing and is further being explored for different applications, particularly within the chemical industry (Ankley *et al*., 2006).

Carcinogenicity in rodents has been extensively studied with toxicogenomics. The goal of toxicogenomics is to identify biomarkers that are predictive of carcinogenic outcome in the 2-year rodent bioassay (Table 2). Much of the focus has been on predicting carcinogenicity induced by compounds that act via non-genotoxic mechanisms as a result of the lack of *in vitro* and *in vivo* assays that can adequately predict these mechanisms of carcinogenicity and recapitulate the multi-stage process of carcinogenicity. The diversity of mechanisms described to contribute to tumour formation also makes prediction difficult, as they are hard to model with short-term assays. The need to predict carcinogenicity in rodents and humans has motivated

Table 2. Examples of toxicogenomic approaches *in vivo* for predicting carcinogenicity.

Predicted endpoint	Model	Study design	Reference
Liver tumour promoter	Rat	Modified Ito medium term rat liver bioassay	Shibutani *et al*. (2002)
Genotoxic and non-genotoxic carcinogens	Mouse	14-day, 1-, 3- and 6-month dietary exposure	Iida *et al*. (2007)
Genotoxic carcinogens	Rat	14-day oral repeat dose	Ellinger-Ziegelbauer *et al*. (2004)
Genotoxic and non-genotoxic hepatocarcinogens	Rat	5-day oral repeat dose	Kramer *et al*. (2004)
Genotoxic and non-genotoxic carcinogens	Rat	14-day oral repeat dose	Ellinger-Ziegelbauer *et al*. (2005)
Non-genotoxic carcinogens	Rat	24-h single oral dose	Nie *et al*. (2006)
Liver and lung carcinogens	Mouse	90-day oral repeat dose	Thomas *et al*. (2007)
Non-genotoxic hepatocarcinogens	Rat	5-day oral repeat dose	Fielden, Brennan and Gollub (2007)
Non-genotoxic hepatocarcinogens	Rat	28-day oral repeat dose	Uehara *et al*. (2008)
Lung carcinogens	Mouse	90-day repeat dose	Thomas *et al*. (2009)

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researchers over the past 2 to 3 decades to develop *in vitro* and alternative short- or medium-term *in vivo* assays that attempt to provide a less resource-intensive means of identifying chemicals with the potential to induce tumours through non-genotoxic mechanisms (Ito, Tamano and Shirai, 2003; Mauthe *et al*., 2001; Cohen, 2004). The underlying premise of toxicogenomics for carcinogenicity prediction is that gene expression changes in the target tissue precede and contribute to tumour development, and that these changes can be monitored after a short-term *in vivo* treatment to predict longer-term carcinogenic outcomes.

With the availability of numerous well-studied genotoxic and non-genotoxic carcinogens, numerous approaches were taken to analyse gene expression changes in the mouse and rat following carcinogen treatment (Table 2). Because of the high prevalence of liver tumours in rodents and the wealth of existing literature on the subject, hepatocarcinogenicity became the focus of many research efforts. Early studies using microarrays and other genome-wide expression profiling technologies focused on the identification of single genes as predictive biomarkers. Instead, unique patterns of multiple genes were found to be necessary to differentiate genotoxic from non-genotoxic compounds (Ellinger-Ziegelbauer *et al*., 2004, 2005), and it was quickly appreciated that no single gene by itself could adequately predict a heterogeneous class of compounds (Iida *et al*., 2003, 2005).

The application of supervised classification algorithms and the availability of larger genomic data sets using paradigm carcinogenic compounds lead to the identification of signatures that were found to have broader applicability for the classification of non-genotoxic carcinogens from short-term rodent studies (Table 2). One of the first large efforts to identify gene signatures to predict carcinogenicity was by Nie *et al*. (2006). Using hepatic cDNA microarray data from the livers of rats treated with a single dose of test compound for 24 h, Nie *et al*. identified a set of candidate biomarkers that were estimated to have an accuracy of approximately 89%. Testing on independent samples confirmed that the signature could predict other non-genotoxic carcinogens. The utility of this signature for predicting non-genotoxic compounds that induce tumours outside the liver has not been widely evaluated; however, initial results indicate that it may have potential. Ellinger-Ziegelbauer (2008) applied a similar approach by applying a number of gene selection and classification algorithms to identify a three-class predictor of genotoxic hepatocarcinogenicity, non-genotoxic hepatocarcinogenicity and non-hepatocarcinogenicity using rat liver gene expression data. The gene selection approaches provided gene sets enriched in functional categories related to cancer, in addition to being able to predict carcinogenic class with a reported accuracy of about 75–88%. Although these proof-of-concept results are encouraging, as with other efforts there are key variables that can have dramatic impacts on the overall utility and interpretation of the data that may prohibit broader use. As changes in gene expression are dynamic, typically dose-dependent and subject to secondary influences due to organ toxicity, a range of doses and time-points would be ideal to capture the optimal window of expression changes that reflect carcinogenic outcome. This can be difficult when testing compounds prospectively in lead optimization; therefore, a signature that will accurately predict carcinogenicity with one or many doses and be robust to target organ effects would be ideal.

Work by Fielden, Brennan and Gollub (2007) also demonstrated the value of a large database of gene expression profiles for generating and validating gene expression signatures of non-genotoxic hepatocarcinogenicity. They used hepatic gene expression data from 5-day repeat dose rat studies to predict non-genotoxic hepatocarcinogenicity. To adequately test the accuracy of the model, the signature was tested against hepatic gene expression data from rats treated for up to 5 days with 47 independent compounds distinct from those used in the training set. This resulted in a sensitivity of 81% and a specificity of 84%. By comparison, the signature was superior in accuracy relative to more traditional endpoints that have previously been proposed and evaluated as predictors of carcinogenic potential, including increased liver weight, hepatocellular hypertrophy, hepatic necrosis, serum alanine aminotransamine activity and induction of cytochrome P450 genes (Allen *et al*., 2004; Elcombe *et al*., 2002). Additionally, the gene expression signature was found to be useful for understanding the mode of action when the gene expression profile of the test compound was compared against non-genotoxic carcinogens of known mode of action, such as P450 inducers, peroxisome proliferators, steroidal compounds with oestrogenic and androgenic activity,

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and hepatotoxic carcinogens. These results indicate that hepatic gene expression data from short-term rat studies can be used to predict non-genotoxic hepatocarcinogens, but also to understand the compounds' mode of action to aid human risk assessment. This is highlighted by the flow chart in Figure 3, which illustrates how the early prediction of carcinogenic effects can be incorporated into drug discovery toxicology. It is also worth pointing out that some efforts using genomics and metabonomics to predict lung tumour incidence have also seen success (Thomas *et al*., 2009, 2007); however, it appears long-term treatment (i.e. 90 days) is necessary for prediction, which may reduce its utility in early *in vivo* screening studies.

Although gene sets have been identified to predict pathological changes in the profiled tissue (Table 1), it is clear that microarray data will not become a replacement to clinical pathology or histopathology measurements. Safety assessment will continue to rely on traditional measures of toxicity and base decisions on the weight of evidence to judge risk. Although investigative and mechanistic toxicology has become integrated into pre-clinical development, there is still a need for more proactive approaches to complement the classical reactionary approaches. Although toxicogenomics is being incorporated in early *in vivo* screening studies to aid in compound selection, it is expected that the validation of *in vitro* models in toxicogenomics will further push this technology upstream in lead optimization (see below).

4 MECHANISTIC TOXICOGENOMICS AND APPLICATIONS TO DISCOVERY TOXICOLOGY

Although the interpretation of toxicity endpoints in pre-clinical studies is well understood, the predictivity of the findings in animal models for humans is often less clear. This discrepancy is not only due to underlying differences in biology and pathophysiology across species, but also because of intrinsic differences in pharmacokinetics and metabolism properties. Many examples have been published demonstrating rodent-specific toxicities due to differences in drug metabolism (Mutlib *et al*., 2000), thyroid hormone regulation (Wu and Farrelly, 2006) or bladder tumour formation (Cohen and Lawson,

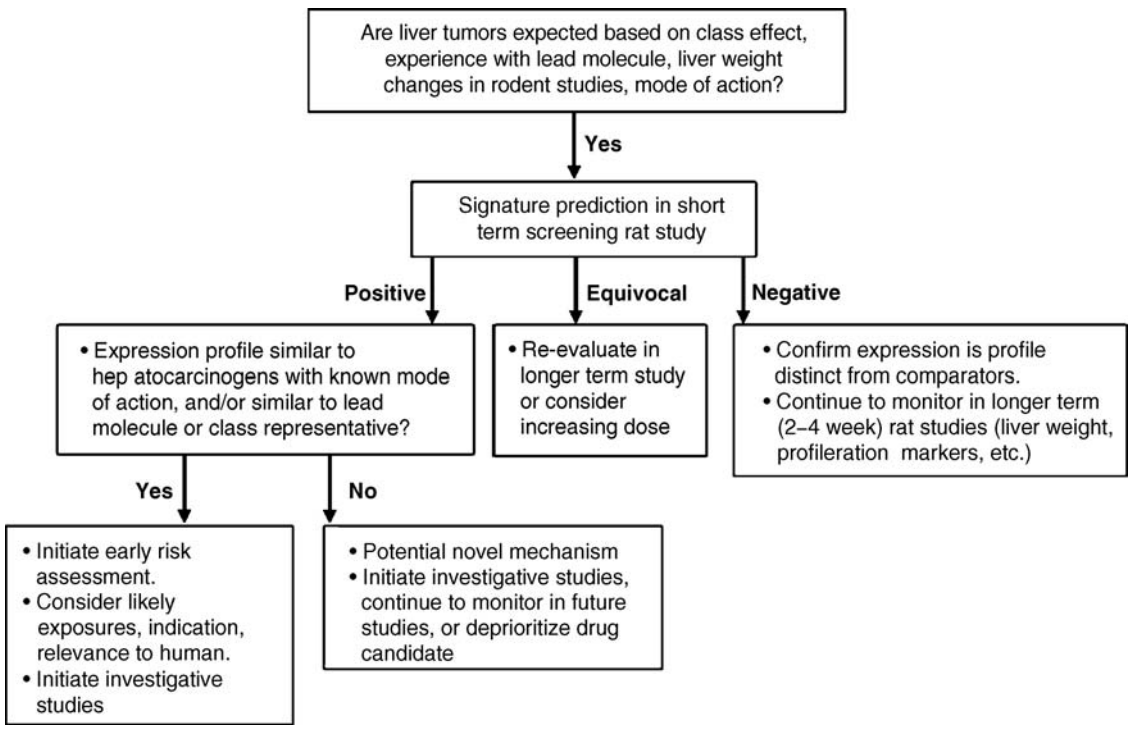

Figure 3. Flow scheme illustrating the application of toxicogenomic signatures for carcinogenicity prediction in drug discovery toxicology.

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1995), for example. Compound toxicity in animals is observed frequently in routine screening studies; however, when the mechanism of toxicity and human relevance are unknown, development of that compound is halted in the interest of time and money and to focus resources on identifying suitable backups. Therefore, an opportunity is lost to learn about the mechanism of toxicity of a drug and potentially its class. By understanding the mechanism of toxicity in rodent models, a risk assessment can be made to understand the relevance of the finding for humans. Additionally, by obtaining a more thorough understanding of the mechanism of toxicity, one can apply the new knowledge to guide the selection of *in vitro* screening strategies or *in vivo* biomarkers to evaluate backup molecules, and ultimately to modify the chemistry of the candidate drug to avoid toxicity in future molecules. Improvements to human risk assessment and the selection of the right drug candidates for development is not made by applying regulatory toxicity studies earlier in discovery, but by obtaining a more thorough understanding of the mechanisms of toxicity prior to human studies, and understanding the potential hazards to design a pre-clinical testing strategy tailored for each programme. This upfront investment in understanding mechanisms of toxicity should also lead to a more informed application and interpretation of *in vivo* and *in vitro* models to predict human toxicity. With that in mind, it is useful to consider how toxicogenomics can contribute to the understanding of mechanisms of toxicity.

Microarray data, like other toxicological and pathological data, require the perspective of experience to draw meaningful conclusions. In traditional toxicology studies, clinical pathology and histopathology changes are interpreted relative to not only a vehicle control group but also an extensive knowledge base of historical data reflected in pathologists' experience or the companies' data warehouses. By comparison, sufficient experience and historical microarray data are often not available and so meaningful interpretation of gene expression data can be difficult. Individual genes that are regulated up or down may lead to more questions than answers when viewed in isolation. However, after a gene expression change has been observed hundreds of times across many diverse physiological, pharmacological, toxicological and pathological conditions, the biological significance of the gene expression change can begin to be understood for reliable interpretation. This approach would include the inclusion of negative and positive controls in the comparison to the test compound. Ideally, the genes that are perturbed by the test compound can be linked to known mechanisms of toxicity. This can often be evaluated by overlaying the expression changes induced by a toxic treatment, and appropriate positive and negative control treatments, on to canonical pathways that are known to be affected or initiated by certain mechanisms of toxicity. This can be performed by using a number of commercial and publically available software packages (Table 3). Example include Nrf2-regulated genes that are known to be up-regulated in response to oxidative stress (Li and Kong, 2009), P450 genes that are known to be up-regulated in response to bile acid perturbation or by direct nuclear receptor activation (Guo *et al*., 2003; Slatter *et al*., 2006), or inflammatory genes up-regulated in response to immune-mediated damage (McMillian *et al*., 2004). By extending this approach, a battery of mechanistic changes can be quickly assessed to rule in, or rule out, certain mechanisms of toxicity that may contribute to an observed toxicity. As with other hypotheses, a weight of evidence approach is typically recommended before relying on such gene expression changes to guide project strategy. It is useful to evaluate the sensitivity and specificity of any changes by comparison to reference compounds with or without the toxicity under study, and by use of compounds that are known to act through the hypothesized mechanism of toxicity. Once validated by this rigorous approach, a suitable diagnostic biomarker set reflective of the mechanism of toxicity can be used to screen backup candidates. These experimental design considerations highlight

Table 3. Examples of commercial and publically available software packages for evaluating gene expression data and pathways.

Software	URL
Public	
ArrayXPath	http://www.snubi.org/software/ArrayXPath/
BioCarta	www.biocarta.com/genes/index.asp
David	david.abcc.ncifcrf.gov/
KEGG	www.g-language.org/data/marray/
GenMAPP 2	www.genmapp.org/
Commercial	
GeneGo, MetaCore	www.genego.com
Ingenuity, pathway Analysis	www.ingenuity.com
Ariadne, Pathway Studio	www.ariadnegenomics.com

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the value of a large historical database of gene expression experiments to strengthen statistical and biological inferences made on isolated expression changes from new test samples. This approach is vital to hypothesis testing when trying to understand mechanisms of toxicity.

Ideally, a thorough understanding of the mechanism of toxicity is desired; however, the resources and time required for such investigations are often not realistic or even essential to guide assay development for backup selection. For example, hepatocellular toxicity may be initiated by disruption of mitochondrial function as a result of inhibition of components of the electron transport chain. However, in practice, a relatively simple and rapid *in vitro* evaluation of mitochondrial respiration can often be used as a surrogate in the absence of knowledge of what mitochondrial enzyme is affected. Again, suitable use of negative controls and toxic compounds known to mediate toxicity through a similar mechanism of action is crucial to extrapolate *in vitro* effects to likely *in vivo* outcomes. This pragmatic approach will ensure implementation of a relevant and timely screening approach.

5 APPLICATION OF *IN VITRO* TOXICOGENOMICS IN DISCOVERY TOXICOLOGY

Applying toxicogenomics to cell-based systems represents another means of facilitating differentiation of compounds at a much earlier stage of drug discovery than *in vivo* testing would permit due to limited compound supply. Furthermore, it may offer the ability to predict *in vivo* toxicity (Ryan, Stevens and Thomas, 2008) as a means of ensuring that resources are allocated to chemical scaffolds that are least likely to be plagued by toxicity. The use of toxicogenomics at this stage has not been as widely used owing to the technical challenges of reproducibility and the questionable concordance to *in vivo* systems (Boess *et al*., 2003; Hultin-Rosenberg *et al*., 2006). However, research in this area continues and promising results in niche areas have demonstrated cases where toxicogenomics in cell-based systems would provide practical advantages when compared with *in vivo* models. An example of *in vitro* toxicogenomics is the prediction of phospholipidosis using human HepG2 cells (Sawada, Takami and Asahi, 2005; Sawada, Taniguchi and Takami, 2006). This liability frequently affects the liver, lung or macrophages and is often induced by compounds that are cationic and amphiphilic in nature, such as amiodarone and tricyclic anti-depressants (Massey *et al*., 1995; Xia *et al*., 2000). Such compounds cause the appearance of concentric lamellar bodies, which is the distinguishing feature of this histological abnormality. This effect is thought to be due to lysosomal phospholipase inhibition (Reasor and Kacew, 2001). Sawada, Takami and Asahi (2005) attempted to identify biomarkers of phospholipidosis by treating human HepG2 cells with known inducers of phospholipidosis and negative controls, and evaluating gene expression on a microarray to identify genes that were consistently regulated in response to phospholipidosis inducers. These evaluations lead to the selection of 12 genes that were subsequently measured on a real-time PCR platform and assessed for reproducibility, sensitivity and specificity against a large independent test set (Sawada, Taniguchi and Takami, 2006). The results were encouraging and demonstrate how an *in vivo* histological endpoint could be predicted by a simple cell-based system. Other approaches evaluating lipid-related pathologies, such as drug-induced steatosis, have also shown promise (Boess *et al*., 2007). Some efforts have evaluated cell-based assays and toxicogenomics as a means to predict hepatocarcinogenicity; however, the results suggest more work is needed to overcome limitations with system variability (Mathijs *et al*., 2009). Nonetheless, efforts have demonstrated that genotoxic carcinogens can be differentiated from non-genotoxic carcinogens (van Delft *et al*., 2004, 2005).

Although *in vitro* sciences continue to mature and contribute to our mechanistic understanding of drug-induced toxicity, the ability of toxicogenomics in cell systems to predict *in vivo* responses has not been adequately demonstrated to replace or supplement the use of short-term *in vivo* models in the early evaluation of drug candidates. Advances in cell models, such as the use of three-dimensional culture systems or co-culture systems, may improve the translatability of *in vitro* gene expression measurements (Dash *et al*., 2009).

6 CONCLUSIONS

Systems toxicology has emerged from the successful application of toxicogenomics and other technologies that enable genome-wide evaluation of biological phenomena. Application of toxicogenomics to *in vitro* and *in vivo* models has helped

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facilitate the identification of biomarkers of toxicity, as well as enable a greater understanding of drug action, including mechanisms of toxicity. Drug discovery research is a complex and highly expensive process that has suffered from late-stage failures owing to unanticipated animal and human toxicity. Discovery toxicology attempts to shift attrition earlier in the drug discovery and development process by thoughtful application of *in silico*, *in vitro* and *in vivo* assays to identify potential hazards and facilitate prioritization of compounds for advancement. The application of toxicogenomics to the early characterization of drug candidates increases our ability to predict potential toxicities before they are detected using traditional means. Additionally, toxicogenomics can be used to understand mechanisms of drug toxicity and guide assay development for screening new molecules. Improving the efficiency and predictivity of these early steps in drug discovery research should ultimately decrease our late-stage failure rate, and thus our use of more expensive and longer-term animal studies. Improvements in this process will require collaborative efforts to validate genomic approaches in pre-clinical safety testing and further our understanding of mechanisms of drug toxicity. Additional efforts to capture historical data and learn from our past successes and failures can also guide our interpretation of pre-clinical data to support human risk assessment. The overall desired outcome is to speed the rate and reduce the cost at which safer compounds are advanced into human testing, while also reducing our reliance on longer-term animal studies for optimizing compounds.

RELATED ARTICLES

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Systems Toxicology: A Valuable Tool for Early Prediction and Mechanistic Assessment of Liver Toxicity of Food-Related Products

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1 INTRODUCTION

The high metabolic activity of the tissue and high levels of exposure to xenobiotics make the liver a major target organ for toxicants. This combination makes liver toxicity the leading cause of drug withdrawal from clinical development and an important parameter in safety evaluation of drugs. Traditional pre-clinical hepatotoxicity testing *in vivo* involves changes in liver-specific enzyme levels in blood and histopathology primarily in livers of small rodent models. However, animal studies are expensive, time-consuming and involve differences in biodiversity and metabolism. Therefore, alternative models and more sensitive methods are valuable tools for toxicological risk assessment. The focus of this review is to assemble published information on systems technology, a rapidly developing new discipline, for the purpose of early prediction and mechanistic assessment of liver toxicity of food-related products.

2 SYSTEMS TOXICOLOGY

Systems toxicology is a rapidly developing new branch of toxicology. It integrates classical toxicology with the new 'omics' technology. It provides an experimental approach for global assessment of the genomic, proteomic and metabolomic profiles of a biological system (Craig *et al*., 2006). Recently, the omics technology has been recognized as a valuable tool for predictive and mechanistic toxicology (Fielden and Zacharewski, 2001) of new drugs during their pre-clinical safety evaluation (Nicholson *et al*., 2002).

3 OMICS TECHNOLOGIES

Systems toxicology makes use of data derived from three omics technologies: genomics, proteomics and metabolomics. Genomics technology measures global gene expression profiles at the RNA and DNA

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levels. It is relatively older among the three omics technologies and, therefore, is more advanced and better studied compared with the proteomics and/or metabolomics. When genes interact with the environment, they can produce toxicity and disease. In recent years, DNA microarray technology has been used extensively for measuring global gene expression profiles and identifying molecular mechanisms of action in response to toxicant exposure both *in vivo* and *in vitro*. Microarray technology allows analysis of thousands of gene expressions simultaneously and provides information on molecular mechanisms of toxicity. It is a very useful tool to measure large-scale gene expression profiles (Stears, Martinsky and Schena, 2003). For example, it has been used to identify biomarkers of hepatotoxicity and the mechanisms associated with it (Dai *et al*., 2006; Kussmann, Raymond and Affolter, 2006; Mutlib *et al*., 2006; Nie *et al*., 2006; Tugendreich *et al*., 2006; Fielden, Brennan and Gollub, 2007; Eun *et al*., 2008). Quantitative real-time polymerase chain reaction (qRT-PCR) technology is also a useful tool for gene expression analysis. It uses selected genes that are representative of various biological pathways. This technology has been proven to provide reproducible data generated in multiple laboratories across three continents (Ellinger-Ziegelbauer *et al*., 2009). Both genomic technologies, microarray and qRT-PCR, have been used to identify biomarkers of toxicity.

Proteomics technology measures global protein profiles in tissues and body fluids in early response to toxicant exposures. This technology is relatively new, is rapidly developing and has not been used to its full potential in toxicology. It can identify protein biomarkers and provides mechanisms of toxicity of chemicals that induce changes in the cellular protein levels (Amacher *et al*., 2005). Dail *et al*. (2008) have used this technology to identify a proteomic screening method for hepatotoxicity. In this study, they used proteomic assays to investigate the early stages of phenobarbital hepatotoxicity in rats exposed to a dose below the detection limits of liver injury measured by the classical liver toxicity tests. This study clearly demonstrated that proteomic technology could detect the early stages of liver toxicity which could not be detected by the classical tests. The study identified cytochrome P450 2B2 as a biomarker for liver toxicity. Heijne *et al*. (2003) have shown that it offers information complementary to genomics. More recently, the protein microarrays are becoming popular to identify biomarkers of toxicity. Spisak *et al*. (2007) have reviewed the application of protein microarray technology for identification of biomarkers of liver toxicity. This technology provides a very sensitive method to detect protein biomarkers of hepatotoxicity. Protein microchips used in conjunction with mass spectrometers can detect less than 1 μg protein. This technology is used for serum tumour marker profiling and mRNA expression verification (Spisak *et al*., 2007).

Metabolomics (or metabonomics) technology measures global profiles of the metabolites in biological systems. Like proteomics, the metabolomics technology is relatively new and rapidly developing. This technology makes use of liquid chromatography, mass spectrometry and proton nuclear magnetic resonance (NMR) spectroscopy to determine global metabolic profiles of tissues and/or body fluids (Schnackenberg and Beger, 2006a, 2006b; Schnackenberg, 2007). Metabolomics can provide comprehensive analysis of endogenous and exogenous cellular metabolite profiles (Robertson, 2005).

4 INTEGRATION OF OMICS TECHNOLOGIES

Recently, attempts have been made to integrate various omics technologies to produce very powerful and useful tools for predictive and mechanistic toxicology. Craig *et al*. (2006) used this approach to study mechanism of cancer development in rat liver induced by methapyrilene. Rantalainen *et al*. (2006) integrated the proteomic and metabolomic data from a mouse model representing human prostrate cancer. Schnackenberg *et al*. (2006) used integrated genomic, metabonomic and proteomic data to evaluate the acute effects of valproic acid in the mouse liver. Integration of bioinformatics and omics technology data has been used for better understanding of mechanisms and pathways of diseases and drug safety (Schnackenberg and Beger, 2006a, 2006b; Schnackenberg, 2007). Using such tools, Skordi *et al*. (2007) elucidated the mechanism of ethionine toxicity in rat liver. Dumas *et al*. (2007) have applied the integration of genomic and metabolic data to map mammalian metabolic phenotypes in diabetic rats. Recently, Li and Chan (2009) have developed the "three-stage-integrative-pathway-search (TIPS) framework" for integration of gene expression and toxicity measurements to identify liver toxicity gene targets and

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pathways using HepG2 cells as an *in vitro* model. More and more such reports are expected to be available in this rapidly developing area of research.

5 SYSTEMS TOXICOLOGY *IN VIVO*

Systems toxicology has been applied to animal models to evaluate the liver response to exposure of hepatotoxicants. Genomic technology was used by Minami *et al*. (2005) to evaluate hepatic gene expression profiles by both microarray and real-time RT-PCR analysis. In this study, they exposed rats to the known hepatotoxins, acetaminophen, bromobenzene, carbon tetrachloride, dimethyl amine and thioacetamide, by a single intraperitoneal injection. All the test chemicals exhibited direct correlation between the biochemical and toxicogenomic parameters in the rat liver. In another study, Chung *et al*. (2006) used microarray technology to study D-galactosamine-induced liver injury in mice. They identified genes associated with injury and regeneration stages of the mouse liver. Nie *et al*. (2006) and Fielden, Brennan and Gollub (2007) used the gene expression profiles to study mechanisms of tumour induction in liver by non-genotoxic carcinogens. Zidek *et al*. (2007) developed a predictive screening system for acute hepatotoxicity by analysing differential gene expression profiles of well-known hepatotoxic and non-hepatotoxic compounds with a bead-based illumina oligonucleotide microarray containing 550 liver specific genes.

Proteomic technology was used by Dail *et al*. (2008) to determine the early responses of the rat liver exposed to phenobarbital, an inducer of liver metabolizing enzymes and a non-genotoxic liver carcinogen in rats. This study identified protein biomarkers associated with the known effects of this compound. Rats were exposed to phenobarbital at a dose of 80 mg kg^{-1} day^{-1} or a vehicle control for 5 days. The level of exposure of phenobarbital in this study was below the detection limit of liver damage at a very early stage determined by the classical tests for liver damage. Of the 3342 proteins identified by global mass spectrometric proteomic analysis, expression of 121 proteins was significantly increased and that of 127 proteins significantly decreased in the test samples compared with the controls. The greatest 167-fold increase was for cytochrome P450 (CYP) 2B2. Increased metabolism, CYP 2B2 induction and cell division were associated with phenobarbital toxicity. Their proteomic CYP results agreed well with the results determined by the traditional liver toxicity methods. This study helps to demonstrate the use of global proteomic technology as a screening tool for toxicity studies.

Metabolomic technology was applied to liver toxicity studies by Ekman *et al*. (2006). In this study, rats were exposed to triazole fungicides, myclobutanil or triadimefon, daily by gavage at a dose of 175 mg $kg^{-1}day^{-1}$ for 14 days to determine endogenous metabolite profiles of the liver and blood serum using proton NMR spectroscopy. The results of this study clearly show that metabolomic technology is a good tool to evaluate liver toxicity of fungicide class of chemicals. In another study, Van Ginneken *et al*. (2007) used metabolomics to study hepatic steatosis in mouse induced by starvation. They observed major changes in the lipid profiles of the rat liver after 24 h fasting and identified a unique triacylglycerol as the biomarker for hepatic steatosis. This technology was used to determine metabolite profiles of ethionine in rat liver (Skordi *et al*., 2007). Integrated genomic and metabolic data have been used to study metabolic phenotypes in diabetic rats (Dumas *et al*., 2007). These studies demonstrate the excellent potential of metabolomic technology for evaluation of hepatotoxicity of xenobiotics *in vivo*.

5.1 Inter-Laboratory Comparison of Systems Toxicology *In Vivo*

Genomics technology was used by Beyer *et al*. (2007) to compare a multi-laboratory study of acetaminophen hepatotoxicity in mouse along with clinical chemistry and histological analysis. Data were generated individually by seven laboratories using a standard protocol. Greater than 20% necrosis was observed in livers of treated animals and correlated well ($r_{\text{Pearson}} = 0.846$) with serum alanine aminotransferase (ALT) activity. Gene expression data were coupled with classical toxicological data. Comparison of data generated from different laboratories showed that the gene expression profiling accompanied by histopathology and clinical chemistry data provided accurate information on hepatotoxicity.

Proteomics and metabolomics are relatively new disciplines compared with the genomics. Therefore, inter-laboratory comparison of data generated from

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multiple laboratories using these technologies has not been possible. However, such studies are anticipated to be available in the near future.

6 SYSTEMS TOXICOLOGY *IN VITRO*

Systems toxicology has been applied to *in vitro* systems to evaluate the hepatotoxic potential of xenobiotics. Primary hepatocytes and hepatocyte cell lines are the frequently used *in vitro* models for hepatotoxicity studies (Beekman *et al*., 2006; Sahu, Ruggles and O'Donnell, 2006). *In vitro* models are particularly useful for mechanistic toxicity investigations. Fielden, Brennan and Gollub (2007) have used *in vitro* systems and genomic microarray technology to identify biomarkers of predictive and mechanistic toxicology of genotoxic and non-genotoxic hepatocarcinogens.

Attempts have been made to apply systems toxicology to various *in vitro* systems such as primary hepatocytes, hepatocyte cell lines and lymphoblastoid cell lines to evaluate hepatotoxic potential of xenobiotics. The results of these studies clearly demonstrate that this technology is a very useful tool for evaluating early response of the liver to various toxic insults. These studies show that *in vitro* systems toxicology can be used to evaluate a range of adverse health effects in the liver from drug toxicity, carcinogenesis to phospholipidosis (PLD), a lysosomal storage disorder characterized by excessive intracellular accumulation of phospholipids. For example, Sawada, Takami and Asahi (2005) and Sawada, Taniguchi and Takami (2006) used human hepatoma cell line HepG2, ArrayPlate and real-time PCR assays to screen PLD, induced by more than 50 cationic amphiphilic drugs including antidepressants and cholesterol-lowering drugs. This study showed a direct correlation between both the assays. Atienzar *et al*. (2007) used HepG2 cells and gene expression analysis to screen PLD-inducing potential of a large number of drugs such as acetaminophen, amiodarone, erythromycin, phenobarbital, tamoxiphen and valproic acid. Nioi *et al*. (2007) used HepG2 cells and gene expression profiles to characterize PLD induced by amiodarone, amitriptyline, fluoxetine, tamoxifen and loratadine. By adding a fluorescently labelled phospholipid (LipidTox) to the HepG2 growth media, they correctly identified 100% of the PLD-positive and PLD-negative compounds. They found this assay less time-consuming, more sensitive and of higher throughput than the gene expression analysis. Boess *et al*. (2003) used primary rat hepatocytes, gene expression profiling and traditional biochemical assays to evaluate hepatotoxicity of two serotonin receptor (5-HT6) antagonists. They analysed the CYP 2B2, CYP 2B Exon 9 and CYP 3A1 genes. This study concludes that the *in vitro* gene expression analysis combined with traditional biochemical information is a very powerful tool for evaluating potential heaptotoxicity of compounds and the mechanisms of their actions. Using the RT-PCR technology, Ellinger-Ziegelbauer *et al*. (2009) generated reproducible gene expression profiles of human lymphoblastoid cell line TK6 induced by model genotoxic agents, cisplatin, etoposide, taxol and NaCl.

Bjork and Wallace (2009) investigated structure–activity relationships of the environmentally persistent peralkyl acids, perfluorooctanoic acid and perfluorooctane sulfonic acid, known for activation of peroxisome proliferator-activated receptor alpha (PPARα). They determined the PPARα-dependent liver toxicity by quantitative reverse transcription PCR (RT-PCR) *in vitro* using rat and human primary hepatocytes and human hepatoblastoma cell line, HepG2. Their study demonstrates that the PPARα -dependent gene expression profiles by peralkyl acids in rat primary hepatocytes are not observed in either primary or transformed human liver cells in culture, indicating that the PPARα -dependent liver toxicity in rodents may not extrapolate to humans.

There are limitations in the ability of *in vitro* systems to model *in vivo* conditions. Intrinsic limitations of the *in vitro* model can affect reproducibility of the omics results. Gene expressions can be affected by the *in vivo* cellular environment and cell–cell interactions. Therefore, choice of an *in vitro* model comparable to the *in vivo* situation is a limiting factor for systems toxicology *in vitro*. Also the extrapolation of the data derived from *in vitro* systems to the animals and humans is a critical factor. Dere *et al*. (2006) compared the gene expression changes induced by 3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in Hepa1c1c7 mouse hepatoma cells *in vitro* and C57BL/6 mouse liver tissue *in vivo* using the microarray and real-time RT-PCR techniques. A direct comparison of their untreated *in vitro* and *in vivo* systems revealed a good correlation ($R = 0.75$) between the basal levels of the two systems, illustrating that many genes were basally

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expressed to similar levels. However, the TCDD treatment resulted in a number of divergent gene responses across both the systems. TCDD-induced genes both *in vitro* and *in vivo* include xenobiotic and oxidoreductase enzymes such as abhydrolase domain containing 6 (Abhd6), cytochrome P450 family 1 subunt a polypeptise 1 (Cyp1a1), dehydrogenase/reductase (SDR family) member 3 (Dhrs3), NAD(P)H dehydrogenase quinone 1 (Nqo1), prostaglandin-endoperoxide synthase 1 (Ptgs1), UDP-glucose dehydrogenase (Ugdh) and xanthin dehydrogenase (Xdh). This study demonstrated inherent differences in TCDD-induced gene expression profiles between the two systems, highlighting the limitations of the *in vitro* test system.

6.1 Inter-Laboratory Comparison of Systems Toxicology *In Vitro*

Using genomic technology, Ellinger-Ziegelbauer *et al*. (2009) have successfully demonstrated that systems toxicology provides reproducible data generated in nine different laboratories. The study used a common protocol for individual experiments, but each laboratory used its own standard operating procedures for cell culture and RNA analysis. They evaluated gene expression profiles of human lymphoblastoid cell line TK6 treated with four model genotoxic agents, cisplatin (DNA-reactive agent), etoposide (topoisomerase inhibitor), taxol (mitotic spindle poison) and NaCl (cytotoxic clastogen) by RT-PCR technology. They observed very reproducible and characteristic gene expression profiles associated with these toxic agents. No inter-laboratory comparison of systems toxicology using liver cells *in vitro* has been reported.

In an inter-laboratory study, Beekman *et al*. (2006) evaluated gene expression profiles of the well-known non-genotoxic hepatocarcinogen, methapyrilene, in primary rat hepatocytes by microarray technology. The genomic data generated by four different laboratories were compared. Genes indicative of good biomarkers of hepatotoxicity were identified following complex data analysis which included the experimental and statistical variability.

Inter-laboratory comparison of data generated from multiple laboratories using proteomics and metabolomic technologies is not available at the present time. However, they are relatively new and rapidly developing technologies compared with the genomics, and therefore reports on comparative inter-laboratory studies may not be far way.

7 APPLICATIONS OF SYSTEMS TOXICOLOGY

Systems toxicology has the promise of providing a very useful tool for exploratory toxicology with potential for multiple applications. Genomics has already found a wide application in toxicology in the last few years. DNA microarrays used for global gene expression analysis have been a powerful tool in toxicology (Eun *et al*., 2008). More recently, protein microarrays are becoming popular for protein expression profiling of toxicity (Spisak *et al*., 2007). A few important applications of systems toxicology for evaluation of hepatotoxicity are discussed below.

7.1 Mechanistic Hepatotoxicity

Systems toxicology is a rapidly developing new tool for "mechanistic toxicology" (Fielden and Zacharewski, 2001). Gene expression profiles represent cellular response to exposure of toxicants. Therefore, systems toxicology by profiling the gene expression determines early information on the mechanisms of toxicity at the molecular level. It identifies potential biomarkers of toxicity (Gatzidou, Zira and Theocharis, 2007). Because systems toxicology is a new rapidly developing field, it is difficult to discuss the mechanisms of hepatotoxicity determined by this technology when only a few limited studies are reported. However, few available studies indicate that oxidative stress plays an important role (Xu *et al*., 2004; Hussain *et al*., 2005; Oberdorster, Oberdorster and Oberdorster, 2005; Sayes *et al*., 2005; Nel, Xia and Li, 2006).

Oxidative stress, induced by reactive oxygen species, is a common mechanism of toxicity. Hepatocytes respond and adapt to oxidative stress. McMillian *et al*. (2005) investigated drug-induced oxidative stress in rat liver by gene expression profiling. Different classes of hepatotoxins such as reactive metabolites, peroxisome proliferators and

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macrophage activators all produce oxidative stress and hepatotoxicity, but each class has distinctive gene expression profiles. These distinctive gene sets provide useful biomarkers of hepatotoxicity. This study demonstrated that the transcription factor, Nrf2, regulates the genes involved in oxidative stress induced by hepatotoxicants. It is also known to be involved in the induction of phase II drug metabolism enzymes (Kong *et al*., 2001). Nanomaterials have been shown to induce oxidative stress in the liver leading to hepatotoxicity (Xu *et al*., 2004; Hussain *et al*., 2005; Oberdorster, Oberdorster and Oberdorster, 2005; Sayes *et al*., 2005; Nel, Xia and Li, 2006).

Proteomics technology can provide information on mechanisms of toxicity of chemicals that induce changes in the cellular protein levels by measuring global protein profiles in biological systems (Amacher *et al*., 2005; Spisak *et al*., 2007; Dail *et al*., 2008). Using this new technology, Dail *et al*. (2008) have identified serum proteins associated with oxidative stress in the rat liver induced by phenobarbital at a dose level below the detection limits of classical serum hepatic enzymes. Schnackenberg *et al*. (2006) applied an integrated omics approach to understand the acute hepatotoxicity of valproic acid in the mouse liver. They evaluated the integrated genomic, metabonomic and proteomic microarray data generated from the analysis of urine, serum and liver tissue from mouse exposed subcutaneously to $600\,mg\,kg^{-1}$ of valproic acid. The results of this study showed changes, induced by valproic acid, in the liver glycogenolysis pathway. Glycogenolysis is important for maintaining glucose levels, and therefore proteomics is a useful tool for studying mechanisms of this biological system.

Kim *et al*. (2009) used metabolomic technology to examine mechanisms of liver injury in mouse induced by ethanol. This study concluded that oxidative stress is responsible for the ethanol-induced liver toxicity. Duan *et al*. (2009) used proteomic and metabolic analysis of the rat liver following severe burn injury. They observed changes in hepatic protein expression profiles and metabolic patterns indicative of oxidative stress. The results of this study suggest that burn injury produces oxidative stress in the liver. More and more mechanistic studies using the relatively new proteomic and metabolomic technologies are expected to be available not in the distant future.

7.2 Predictive Hepatotoxicity

Systems toxicology is a new rapidly developing tool for "predictive toxicology" (Fielden and Zacharewski, 2001). The major application of this new discipline is the identification of genes predictive of toxicity. Gene expression profiles provide early molecular information on toxicity that cannot be detected by the conventional endpoints of toxicity. It is a good tool for predicting toxicity and, therefore, has been extensively applied in drug discovery (Waring *et al*., 2002).

Hamadeh *et al*. (2002a, 2002b) used DNA microarray to examine liver gene expression profiles of rats exposed to the hepatocarcinogen methapyrilene. They observed the gene expression analysis to be more sensitive than classical histopathology to predict early hepatotoxicity. This observation led Kier *et al*. (2004) to develop microarrays with "tox genes" to detect hepatotoxicity. Ruepp *et al*. (2005) developed a model to predict early stages of chemical hepatotoxicity based on liver gene expression profiles, histopathology and clinical chemistry.

Dai *et al*. (2006) treated rats with over 50 known heptotoxic and non-hepatotoxic agents for 3 days. The majority of the rats treated with hepatotoxins did not show liver toxicity after the end of the treatment period as evaluated by the traditional hepatotoxic endpoints. However, gene expression profiling clearly demonstrated liver toxicity of hepatotoxic agents compared with the non-hepatotoxic agents. This study clearly established a strong correlation between gene expression changes and liver toxicity at very early stages when the classical toxicity endpoints are not sensitive enough to detect it.

In another study, Mutlib *et al*. (2006) treated mice with the hepatotoxin, *N*-methylformamide, and its corresponding deuterium-labelled analogues resistant to metabolic processing. They measured gene expression changes in the livers of treated animals. They showed a direct correlation between the gene expression changes and the production of the reactive metabolite methyl isocyanate.

Studies on genotoxic (Ellinger-Ziegelbauer *et al*., 2004) and non-genotoxic (Fielden, Brennan and Gollub, 2007) hepatocarcinogens have demonstrated characteristic gene expression profiles indicative of early events leading to liver carcinogenesis in rats. Eun *et al*. (2008) have successfully identified large-scale characteristic molecular signatures of hepatotoxicity and the

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mechanisms associated with it by gene expression analysis. These studies indicate that genomic toxicology is a useful tool for detecting and predicting hepatotoxic as well as hepatocarcinogenic potential of materials in spite of the differences in their mechanisms of action.

Proteomics technology has been used successfully for identification of protein biomarkers to predict hepatotoxicity and liver cancer (Craig *et al*., 2006). Spisak *et al*. (2007) have used protein microarrays for biomarker discovery.

Studies of Dail *et al*. (2008) on hepatocarcinogen phenobarbital have demonstrated that the results obtained from the global mass spectrometric proteomic analysis compared very well with the results obtained by the traditional liver toxicity methods. The level of exposure to phenobarbital in this study was below the detection limit of liver damage at a very early stage determined by the classical tests. They have concluded that the proteomic technology can be used as a screening tool for hepatotoxicity studies.

Metabolomic technology is a useful tool for detecting and predicting liver toxicity of potential hepatotoxicants (Ekman *et al*., 2006). This technology has been used by Van Ginneken *et al*. (2007) to predict hepatic steatosis in mouse.

The above studies demonstrate that the systems toxicology is a powerful tool to predict hepatotoxicity. It can be particularly useful for high-throughput screening of potential hepatotoxicants (Fielden and Kolaja, 2006; Craig *et al*., 2006). It is a valuable tool for screening of food-related products to predict their potential liver toxicity.

7.3 Biomarkers of Hepatotoxicity

Systems toxicology is an excellent tool for discovery of biomarkers (Kussmann, Raymond and Affolter, 2006; Gatzidou, Zira and Theocharis, 2007). There is a strong need for drug industries to use this sensitive new rapidly developing technology for identifying biomarkers of early detection of drug toxicity during pre-clinical and clinical evaluation (Robertson, 2005; Schnackenberg *et al*., 2006; Espandiari *et al*., 2007).

Fielden, Brennan and Gollub (2007) used liver gene expression profiling by microarray technology to develop biomarkers for non-genotoxic hepatocarcinogens. They collected hepatic gene expression data from rats treated for 5 days with 47 test chemicals. Each chemical was evaluated in the short-term *in vivo* study using only three rats. They observed that this short-term *in vivo* rodent model was more sensitive, more accurate and provided quicker results compared with the traditional models for risk assessment of non-genotoxic hepatocarcinogens (Fielden, Brennan and Gollub, 2007). However, one possible limitation of this study may be the very small number of only three animals which may affect the statistical significance of the results considering the individual variability among the animals. Real-time quantitative RT-PCR (qRT-PCR) has also been used for identification of biomarkers of toxicity (Ellinger-Ziegelbauer *et al*., (2009). Both genomic technologies, microarray and qRT-PCR, have been used for identification of biomarkers of liver toxicity (Dai *et al*., 2006; Mutlib *et al*., 2006; Nie *et al*., 2006; Tugendreich *et al*., 2006; Eun *et al*., 2008). These studies show that genomic technology is a valuable sensitive tool for screening hepatotoxic as well as hepatocarinogenic potential of suspected test agents, although their biomarkers and mechanisms of action may be different. Genomic biomarkers provide early prediction and mechanistic assessment of hepatotoxicity and hepatocarcinogenicity.

Proteomics technology has been used successfully for identification of protein biomarkers for hepatotoxicity (Craig *et al*., 2006). Spisak *et al*. (2007) have used protein microarrays for identification of biomarkers.

Dail *et al*. (2008) have used global mass spectrometric proteomic analysis to determine biomarkers for hepatotoxicity. This technology offers information on biomarkers complementary to genomics (Heijne *et al*., 2003).

This technology was used to identify protein biomarkers for early detection of liver fibrosis and cirrhosis in rats (Low *et al*., 2004; Xu *et al*., 2004). Using proteomics and bioinformatics data, Xu *et al*. (2004) identified a serum protein that is downregulated in cirrhotic rat liver. This technology has also been used to identify serum protein biomarkers of hepatotoxicity induced by viruses. Using ProteinChip arrays, Poon *et al*. (2005) observed fibrotic protein changes in the serum of patients suffering from hepatitis B, but these proteins were not identified. He *et al*. (2003) used proteomics to identify seven serum proteins as biomarkers of liver injury induced by hepatitis B virus.

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Metabolomic technology is a useful tool for determining biomarkers of hepatotoxicity (Ekman *et al*., 2006). This technology has been used to identify a unique triacylglycerol as the biomarker for hepatic steatosis in mouse (Van Ginneken *et al*., 2007). More such studies are expected to be available not in the distant future.

Schnackenberg *et al*. (2006) used integrated genomic, metabonomic and proteomic microarray data to identify biomarkers for liver toxicity induced by valproic acid in mouse. Their studies identified two proteins, glycogen phosphorylase and amylo-1,6-glucosidase, as biomarkers of liver toxicity. These two proteins were increased in animals exposed to valproic acid compared with the vehicle controls. Both of these proteins are involved in converting glycogen to glucose. This study suggests a perturbation in the glycogenolysis pathway induced by valproic acid.

The studies reported so far clearly demonstrate that the systems toxicology is a powerful tool for identifying biomarkers for predictive or diagnostic hepatotoxicity. The validated biomarkers can be applied to predictive and mechanistic hepatotoxicity (Olden, 2006).

McBurney *et al*. (2009) used genomic, proteomic and metabolomic technology to identify biomarker of liver toxicity in rats exposed to drugs entacapone and tolcapone. In this study, the molecular analysis of liver tissue and plasma samples by the genomic, proteomic and metabolic techniques demonstrates that omics technology is a valuable tool for evaluating liver toxicity. It is anticipated that more and more such studies will be undertaken in near future. Already biomarker sets developed from large reference databases such as DrugMatrix are available commercially.

7.4 Species and Gender Differences in Hepatotoxicity

Systems toxicology is a useful tool for evaluating species differences in hepatotoxicity. Dail *et al*. (2008) applied the proteomic analysis to identify the protein biomarker, cytochrome P450 2B2, in the rat liver exposed to phenobarbital at dose level below the detection limits of classical serum hepatic enzymes. They have used these proteomic data obtained from the rats for intrapolation to the humans to explain why phenobarbital is a non-genotoxic hepatocarcinogen in rats but not in humans. This study has demonstrated that systems toxicology is a very useful tool for investigating cross-species intrapolation of data.

Systems toxicology can be successfully applied to evaluate the gender effects in hepatotoxicity at very early stage when classical liver toxicity tests are not sensitive enough to detect toxicity. Gender difference plays an important role in the toxic responses of the liver (Treinen-Moslen, 2001). Females are pre-disposed to hepatotoxicity, and concomitant agents that induce cytochrome P450 enzymes also increase individual susceptibility (Stedman, 2002). Drug-induced liver failure is more common in women than in men (Russo *et al*., 2004; Carey *et al*., 2008). Cocaine causes more liver damage in male mice than in female mice (Visalli, Turkall and Abdel-Rahman, 2004). Gender differences in P450, especially CYP2A, expression is higher in female pigs compared with the male pigs (Gillberg, Skaanild and Friis, 2006). These studies, which show gender effects in hepatotoxicity measured by classical methods, are ideal for revisiting by the systems technology to identify biomarkers of hepatic gender effects at the very early stages of liver toxicity that cannot be detected by classical tests.

Gender difference in hepatotoxicity has been demonstrated using *in vitro* systems. Delongchamp *et al*. (2005) used gene expression profiles of donated male and female human liver tissues and primary human hepatocytes to evaluate gender differences in hepatotoxicity. They tested a total of 31 100 genes and found that the gene expression of only 224 genes differed between sexes. The observed gender differences in gene expressions were small. False discovery rates exceeded 80% for every set of genes selected, making it impossible to identify specific genes with gender differences. They observed high inter-individual variability in phenotypes and genotypes of human liver as well as large variations in individual consumption of various diets. This study suggests that gene expression profiling alone may not be able to identify gender differences in hepatotoxicity. More recently, Silkworth *et al*. (2008) have used DNA micrarry technology to study gender effects of the liver carcinogen arochloro 1254 in rats. They observed significantly altered gene expression profiles by this hepatocarcinogen.

Proteomic and metabolomic toxicology are relatively new, and therefore gender differences in

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hepatotoxicity studies have not been reported using these technologies. More research is needed for evaluation of gender effects in liver toxicity. Systems toxicology will be useful to study gender-specific liver toxicity at the molecular level. It can identify genomic, proteomic and metabolomic biomarkers related to sex-specific liver toxicity.

7.5 Food Safety

Systems toxicology is useful for improving food safety assessment (Spielbauer and Stahl, 2005). In a review article, Stierum *et al*. (2005) have reported that systems technology was used to evaluate hepatotoxicity of food additives such as butylated hydroxytoluene, curcumin, propyl gallate and thiobendazole. Arbillaga *et al*. (2009) has tested the toxicity of ochratoxin A (OTA), a mycotoxin found as a contaminant in cereals, in human renal proximal tubular epithelial cell line HK-2 using gene expression profiles. The comparison of their gene expression analysis with the cellular oxidative stress level and oxidative DNA damage indicates a DNA non-reactive mechanism of OTA toxicity.

Systems toxicology offers valuable molecular tools for the detection and identification of foodborne pathogens. Liver is an important site of host–pathogen interactions. Identification of genes that affect susceptibility to foodborne pathogens provides insights into the pathogenesis of food-related diseases. Genomic technology has been used for detection of foodborne pathogen *Escherichia coli* in beef (O'Hanlon *et al*., 2005). This technology has been applied to investigate host–microbe interactions (Cummings and Relman, 2000) and acute hepatitis C virus infection in liver (Bigger, Brasky and Lanford, 2001). Plumet and Gerlier (2005) have developed a sensitive and specific assay based on real-time RT-PCR to quantify individual measles virus RNA. This can be useful for a reliable detection of measles virus in clinical samples. Cai *et al*. (2005) have assessed the efficacy of nitazoxanide and paromomycin against two strains of the *Cryptosporidium parvum* in HCT-8 cells *in vitro* using quantitative real-time RT-PCR assay. Both compounds displayed dose-dependent inhibitions.

Very few reports are available on proteomics or metabolomics of hepatitis caused by foodborne viruses. However, one has to recognize that both these technologies are relatively new. He *et al*. (2003) used proteomics to compare hepatitis B virus-negative and -positive serum samples. This study identified changes in seven serum proteins that can be used as biomarkers of hepatitis B virus-induced liver injury. More and more such proteomic and metabolomic studies are expected to be available in the near future.

7.6 Food Safety Evaluation and Quantitative Health Risk Analysis

Toxicological profiles and mechanisms of toxicity of a potential toxicant are important information for its health risk assessment. Systems toxicology provides toxicological profiles and molecular mechanisms of toxicity of potential toxic agents. The greater understanding of the molecular basis of toxic exposure provides a more scientific basis for health risk analysis. Therefore, systems toxicology will play a very useful role in the risk evaluation of toxicants (Oberemm, Onyon and Gundert-Remy, (2005); Boverhof and Zacharewski, 2006). Oberemm, Onyon and Gundert-Remy (2005) have discussed the potential use of genomics in safety evaluation and risk assessment. Normally, an expensive 2-year rodent bioassay is used for risk assessment of non-genotoxic hepatocarcinogens, but Fielden, Brennan and Gollub (2007) have developed a short-term *in vivo* rodent model which is more sensitive, more accurate and provide quicker results compared with the traditional models for risk assessment of non-genotoxic hepatocarcinogens. Ku *et al*. (2007) and Thybaud, Le Fevre and Boitier (2007) have successfully applied genomic technology for genotoxicity hazard and risk assessment.

Concentration-dependent omics data from *in vitro* models may not be directly useful for extrapolating health risk in humans, but information on relative toxicities is useful for risk estimation. Identification of type and extent of adverse health effects associated with a toxic exposure is important for hazard and risk assessment. Systems toxicology provides large amounts of such information in a relatively short period of time. Therefore, proper validation of the omics techniques is necessary for proper use of omics-generated information for risk evaluation of food-related products.

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8 CONCLUSIONS

This review of literature clearly demonstrates that systems toxicology is a valuable tool for early prediction and mechanistic assessment of liver toxicity. It provides accurate molecular information for toxicity hazard and risk assessment in a relatively short period of time (Oberemm, Onyon and Gundert-Remy, 2005; Boverhof and Zacharewski, 2006; Fielden, Brennan and Gollub, 2007; Ku *et al*., 2007; Thybaud, Le Fevre and Boitier, 2007). This technology will find very useful applications in food safety (Stierum *et al*., 2005; Arbillaga *et al*., 2009). It is an excellent tool for quick screening of hepatotoxic potential of food-related products that occur naturally or that are added deliberately to the foods and dietary supplements. The published reports show the value of omics technologies in combination with classical histopathology and biochemical assays providing a powerful tool for accurate assessment of "predictive" and "mechanistic" hepatotoxicity. The systems toxicology *in vitro* offers a powerful cost-effective tool for faster high-throughput toxicity testing. It can be effectively used for high-throughput screening of food-related products for potential hepatotoxicity.

Global proteomic (Dail *et al*., 2008) and metabolomic (Zivkovic and German, 2009) analysis identifies more carcinogenic biomarkers for prediction and mechanistic assessment of potential hepatotoxicity of food-related products. Metabolomic technology is a useful tool for the assessment of nutritional status of patients with diet-related diseases (Zivkovic and German, 2009).

In conclusion, the systems toxicology has been recognized recently as an excellent tool for rapid high-throughput screening for predictive and mechanistic toxicology. Therefore, it can be judiciously used for high-throughput screening of early detection, prediction and mechanistic assessment of liver toxicity caused by food-related products, food additives, foodborne microbial pathogens, herbal products and/or dietary supplements as well as for their risk assessment.

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RELATED ARTICLES

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Mass Spectrometry-Based Proteomics in Systems Toxicology

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1 INTRODUCTION

The advance of novel technologies and instrumentation has significantly changed the methods and focus of scientific research in the past few decades. The human genome project, largely driven by high-throughput DNA sequencing has brought new avenues of "omics" into biomedical research and enabled both hypothesis- and discovery-driven research. The former tends to focus on smaller numbers of genes, transcripts, or proteins to answer a pre-defined question that is based on previous experimental results. In contrast, the discovery-driven approach mainly focuses on broad questions, global characteristics of molecular networks (e.g., transcripts, proteins, and metabolites), and systematic analysis of biological functions at higher levels (e.g., a cell, tissue, or organism). Such studies are not directed by predisposed knowledge or assumptions, and the general goal is to use relatively high throughput approaches to acquire considerable data to elucidate the system under study. This type of discovery-driven research is exemplified by systems biology (Ge, Walhout and Vidal, 2003; Kitano, 2002) and pioneered by genomics, in which a few tens of thousands of genes are analyzed in a high-throughput fashion on a DNA microarray (Schena *et al*., 1995). However, at the current time, information in the genome cannot predict the full cellular phenotypes and functions of proteins. As such, the concept of proteome was coined and defined as the total protein components expressed in a cell, tissue, or organism at a specific time and physiological state (Wasinger *et al*., 1995; Humphery-Smith, Cordwell and Blackstock, 1997).

Although great efforts in the area of proteomics have been made, many challenges remain. It is well-known that a cellular proteome is complex, but the degree of complexity is uncertain. It has been estimated that protein-coding genes total approximately 20 500 in human (Clamp *et al*., 2007), a number similar to that in mouse or *Caenorhabditis elegans* (Mouse Genome Sequencing Consortium, 2002; The *C. elegans* Sequencing Consortium, 1998). However, human beings are distinct from mice and roundworms. Assuming as many as 14 000 genes are expressed within the human cell at any single time and three different mRNA transcripts are expressed per gene, then the cell has 42 000 transcripts for protein synthesis. This estimate is very conservative considering the presence of splice variants, single nucleotide polymorphisms, and so on. The protein precursor translated from a single transcript can be processed in many different ways to form a mature protein, including truncations, modifications, protein splicing, and so on. There are more than 300 different types of known modifications with some more commonly occurring such as phosphorylation, glycosylation, methylation, acetylation, and ubiquitination. For some modifications,

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the exact type of modifier may be different. For example, there are a variety of different glycoforms for glycosylation. Assuming a single protein has four potential phosphorylation sites, in itself, it may give rise to sixteen different isoforms ranging from all the sites phosphorylated to none of them being phosphorylated. If each of the 42 000 possible protein products contained only four possible sites of phosphorylation, these would give rise to 672 000 different protein species. Obviously, this number is extremely conservative and only one specific type of modification is considered in this calculation. Considering the possibility of multiple types of modifications in a single protein, the estimate suggests that the human proteome would be most likely to contain well over 1 000 000 distinct protein species. The above inference could provide a hint to understand the complexity of the human proteome within a single tiny cell. This large number of protein species is well beyond the current capacity of state-of-the-art proteomic technologies resulting in a consistent undersampling of the proteome regardless of the analytical method chosen. Other challenges in proteome analysis include transit protein modification and complex formation, dynamic protein synthesis and turnover, huge dynamic range of protein abundance levels, and so on.

The enormous complexity of proteomes has spurred development and advancement in this field and many new areas are emerging along with the development of systems biology or systems toxicology (Slikker *et al*., 2007). One such emerging field is the combined application of proteomics and toxicology leading to the development of a new discipline, termed toxicoproteomics. Although it is in an early stage, toxicoproteomics is an attractive area and possesses many opportunities for exciting studies (Merrick, 2008). The field is concentrated on the proteomic study of toxicity caused by toxic substances, including drugs, toxins, environmental stressors, chemicals, and any other materials that may cause significant pathological responses (e.g., engineered nanomaterials (Poland *et al*., 2008)). The discipline needs to combine knowledge and methods of toxicology, pathology, and other expertise to discover toxicity biomarkers and define, at the proteome level, the toxicity pathways, modes-of-action, and key molecular events.

One of the major efforts in toxicoproteomics research is to elucidate pathological responses to a specific toxicant at the protein molecule level. As such, toxicoproteomics requires systematic analysis of protein functions associated with toxicant exposures, including, but not limited to, qualitative and quantitative measurements of protein expression, protein modifications (including natural and exogenic modifications), protein-protein/toxicant interactions, protein structures, protein activity, and subcellular localization. The research also needs to develop novel technologies and approaches for better measurements of toxicity at the molecular level, improve our understanding of toxicity mechanisms, and gain knowledge in organisms' responses to toxic substances for the promotion of public health. The major aims of toxicoproteomics research include: (i) development of proteomic technologies for efficient analysis of proteins; (ii) identification and validation of protein biomarkers for early detection of toxicity and disease; (iii) elucidation of molecular mechanisms of toxicity; (iv) generation of toxicoproteomics knowledge for better risk assessment.

Toxicoproteomics, as well as other proteomics branches, have been driven by the advances in analytical technologies and instrumentation. Far before the invention of the term "proteome" or "proteomics" in 1994 (Wasinger *et al*., 1995; Humphery-Smith, Cordwell and Blackstock, 1997), protein analysis at the "proteome" level actually had been conducted for decades using high-resolution two-dimensional electrophoresis (2-DE) (O'Farrell, 1975). The development of proteomics can be attributed to the continuous improvement in intact protein separation using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) or 2-DE and the realization of mass spectrometry (MS) analysis of proteins/peptides using the soft ionization techniques matrix-assisted laser desorption/ionization (MALDI) (Tanaka *et al*., 1988; Karas and Hillenkamp, 1988) and electrospray ionization (ESI) (Fenn *et al*., 1989) in late 1980s. In the past two decades, the field grew rapidly as a result of significant progression in high-resolution analytical separation (e.g., capillary high-performance liquid chromatography with nanoflow separation, nano-HPLC), nano-ESI, application of stable isotope dilution MS (SID-MS), and increase of MS sampling speed and performance. Consequently, two basic approaches, gel-based and solution-based proteomic analyses, have been developed for global quantitative measurement of protein abundance changes. It should be noted that protein microarrays,

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as a novel technology, have been developed and applied to systems biology for many years (Zhu *et al*., 2001). Owing to very few applications of this technique to toxicological studies, this chapter mainly focuses on MS-based proteomic technologies in systems toxicology (Aebersold and Mann, 2003; Wetmore and Merrick, 2004; Monsinjon and Knigge, 2007).

2 TWO-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS

The extremely high complexity of proteome samples requires proteins to be separated prior to any further characterization so that low abundance molecules can be detected. When the word "proteomics" is mentioned, the first thought that enters most people's minds is an image of dense protein spots separated on a 2D-PAGE gel. So far in proteomics, 2D-PAGE has been the most commonly used protein separation technique with high resolution for intact proteins. The technique can be tracked back to 1975, when O'Farrell, Klose, and Scheele achieved the milestone of successful implementation of high resolution 2D-PAGE based on predecessors' 2D protein separation concept, invention of polyacrylamide gel, isoelectric focusing (IEF), and sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) (O'Farrell, 1975; Klose, 1975; Scheele, 1975). Since then, 2D-PAGE has been further improved and optimized in sample preparation, use of immobilized pH gradient (IPG) gel (Bjellqvist *et al*., 1982) as the first dimensional IEF separation, protein spot visualization, and development of 2D image analysis tools. The reproducibility of protein separation and quantitation of 2D-PAGE has been significantly improved and the modern 2D-PAGE can resolve thousands of proteins in a single gel. All these advances establish the fundamentals of 2D-PAGE in conjunction with MS protein identification as the cornerstones of proteomics.

The principle of 2D-PAGE is two-dimensional orthogonal protein separation based on the charge state and size of each protein. The first dimensional separation is IEF, which separates proteins according to their isoelectric points (p*I*) and currently is achieved using IPG gels across a certain range of pH (e.g., pH 3–10) (Görg *et al*., 2000). After IEF is completed, the IPG gel is conditioned in an SDS buffer and then transferred onto an SDS slab gel for SDS-PAGE. Electrophoresis in the presence of SDS, which denatures the proteins and eliminates charge-to-mass differences, is for the separation of proteins based on their molecular weight. The separation power of 2D-PAGE is able to resolve the proteins differing by a single charge, thereby allowing *in vivo* modifications such as phosphorylation and certain mutations to be detected. Fortunately, the technique allows relative quantitation of the proteins from different proteomes.

A 2D-PAGE-based quantitative proteomic analysis usually includes several steps: protein extraction, running the 2D gels, gel visualization, 2D image acquisition and analysis, excision of 2D spots of interest, in-gel protein spot digestion, and MS analysis for protein identification (Figure 1). For quantitative comparison of two distinct cell populations (e.g., control vs. treated cells), the proteome samples are prepared by extracting proteins from different populations of cells separately using the same lysis buffer. The samples with equivalent protein amounts are then subjected to 2D-PAGE for fractionation. The 2D gels are visualized by stains such as the commonly used Coomassie Blue or silver. The resulting images are then digitalized and analyzed using 2D image analysis software such as Delta2D, ImageMaster, Melanie, PDQuest, and so on. Since a 2D image contains hundreds to thousands of protein spots, the spots must be accurately detected and aligned among the analyzed gel images so that the spot intensities of the same protein across different gels can be compared for relative quantitation (Yu *et al*., 2001). Usually, protein spots showing higher intensities on the gel from either the control or treated cells are excised and then digested in-gel by an enzyme such as trypsin. The resulting peptides are extracted from the gel and analyzed by MS or tandem MS (MS/MS) (more detailed discussion later) to acquire the raw data for identification of the protein(s) within the gel spot. Protein identification is accomplished using software (e.g., Sequest and Mascot) designed to compare the mass spectrometry data against a database comprised of a large number of protein sequence entries (Eng, McCormack and Yates, 1994; Yu *et al*., 2000).

One of the merits of 2D-PAGE is that multiple samples can be separated in parallel using the same device, which saves time overall in the whole procedure of proteomic analysis of multiple samples. However, it is still tedious and labor intensive

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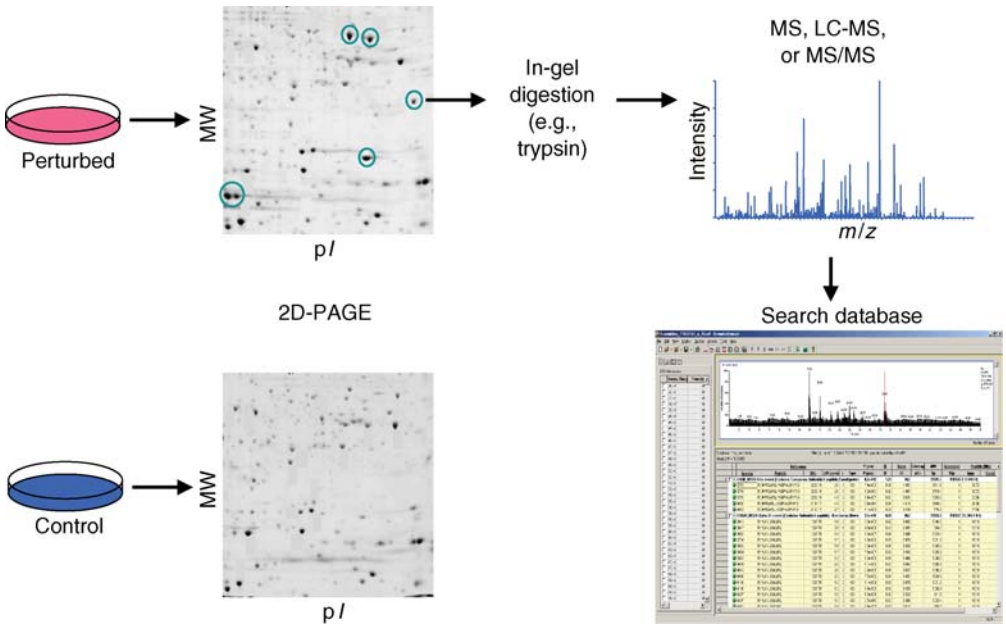

Figure 1. Schematic workflow of quantitative proteomics using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry. Comparative proteome samples are resolved on separate 2D gels. After staining and image analysis, protein spots showing higher intensities on the gel from either the control or treated cells are excised and then digested in-gel by an enzyme such as trypsin. The resulting peptides are extracted from the gel and analyzed by MALDI-MS, LC-MS or tandem MS (MS/MS). The MS or MS/MS data are then searched against an appropriate database to identify the protein(s) within the gel spot. MW, molecular weight; p*I*, isoelectric point; MALDI, matrix-assisted laser desorption/ionization.

when tens of samples need to be compared. The other advantage of 2D-PAGE-based proteomics is that the downstream MS identification is performed only for the differentially displayed protein spots. This makes MS analysis simple and straightforward. Nonetheless, the technique has been criticized as well for its drawbacks. The most commonly used gel visualization methods such as Coomassie Blue and silver staining have limited dynamic ranges and the sensitivity is relative low (e.g., low nanograms for silver staining) compared to the state-of-the-art LC-MS approaches (e.g., femtomoles routinely, attomoles to zeptomoles in some cases) (Belov *et al*., 2000; Shen and Smith, 2005; Hanke *et al*., 2008). This makes many low abundance proteins undetectable in the gel but identifiable using MS. Procedures involving the use of aldehydes for silver staining may not be compatible to the MS analysis of gel spots (Yan *et al*., 2000). The use of SYPRO stains such as SYPRO Red, SYPRO Orange, and SYPRO Tangerine dyes for gel visualization is a simple one-step procedure (Steinberg *et al*., 1996). SYPRO Ruby staining has a linear dynamic range of over three orders of magnitude with sensitivity comparable to the most sensitive silver staining techniques (Berggren *et al*., 2000). SYPRO staining is compatible to MS analysis; however, UV transilluminator or similar imaging systems are required to visualize the gel spots. To enhance the detection of low abundance proteins, "ultrazoom" gels with narrow pH gradients in the first dimension can be used for loading large amounts of protein (Hoving, Voshol and van Oostrum, 2000; Wildgruber *et al*., 2000). Another issue with 2D-PAGE is poor gel-to-gel reproducibility. The procedure must be controlled strictly to obtain well-reproducible gel images. To overcome this problem, 2D difference gel electrophoresis (2D-DIGE) has been developed to visualize and compare multiple proteomes in a single 2D gel (Unlu, Morgan and Minden, 1997). In 2D-DIGE, multiple fluorophores (e.g., Cy2, Cy3, and Cy5) are used to label different proteomes to be compared, and the labeled samples are combined and run on a single 2D gel. The 2D image of each sample is acquired using excitation and emission wavelengths unique to the dye used for that sample, and the relative abundance of each protein in each sample can then be measured. A merged 2D image can be generated to display differentially expressed

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proteins in different colors by superimposing the separately acquired images of compared samples. Other shortages of 2D-PAGE include insufficient separation of membrane proteins because of solubility issues, exclusion of proteins with extremely high/low molecular weight or isoelectric points, and potential co-migration of multiple proteins into the same spot.

3 MASS SPECTROMETRY

Mass spectrometry is another cornerstone that has driven the proteomics revolution. The technology is at a prominent position in the field of proteomics owing to its rapid identification of proteins and the capability for relative and absolute protein quantification. After approximately a century's development, the sensitivity afforded by current MS enables peptides/proteins to be successfully identified at the range of low femtomole (fmol, 10^{-15} mol) to attomole (amol, 10^{-18} mol) (Hanke *et al*., 2008), and at the zeptomole levels (zmol, 10^{-21} mol) in particular cases (Belov *et al*., 2000; Shen and Smith, 2005). Mass measurement accuracy for high-resolution MS can be routinely achieved to 1–5 ppm and even high ppb (parts-per-billion) (Olsen *et al*., 2005; Williams and Muddiman, 2007). Such high mass measurement accuracy and sensitivity greatly increase the database-search-based peptide identification confidence levels for both high and low abundance proteins.

3.1 Ionization Methods

Peptides or proteins must be ionized before they are introduced into the mass spectrometer for mass measurement. A mass spectrometer can be thought of as two distinct parts: the ionization source and the mass analyzer. The ionization source is the region of the instrument in which the sample is ionized and desorbed into the gas phase. A mixture of positively and negatively charged molecular ions is generated after ionization. For peptide and protein analysis, MALDI (Tanaka *et al*., 1988; Karas and Hillenkamp, 1988; Karas *et al*., 1987) and ESI (Fenn *et al*., 1989; Dole *et al*., 1968) are the two most common ionization methods. The breakthrough of identifying proper organic matrices for effective laser desorption/ionization of large molecules (10–20 kDa) opened a new era of using MALDI for the analysis of biological macromolecules such as proteins together with the ESI application to proteins during the same time.

In MALDI, the sample is mixed with a saturated solution of matrix, for example, α-cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB), and 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), which are commonly used for peptide/protein analysis. A small volume of the mixture (e.g., 1–2 μL) is deposited onto a MALDI plate, and the sample is integrated into a crystal lattice after it is dried owing to the high matrix-to-sample concentration ratio. In the MALDI source region, the sample is irradiated by using a laser pulse (e.g., nitrogen laser at 337 nm) and the matrix absorbs most of the photon energy provided by the laser. The matrix-absorbed energy is then transferred to the analytes (e.g., peptides), which are subsequently ejected from the plate surface into gas phase. MALDI typically generates singly charged ions whatever the analyte is a peptide or a large protein, thus the generated ions have high mass-to-charge (*m/z*) ratios. Thus this type of ionization method is coupled prevalently with a time-of-flight (TOF) analyzer that can measure ions at a large *m/z* range.

The implementation of ESI for biological macromolecules has made a dramatic impact on the advance of proteomics. ESI allows liquid separation techniques to be coupled online with MS for real-time MS data acquisition. In ESI, the sample dissolved in solution flows through an emission tip toward the ionization source region by direct infusion, HPLC, or capillary electrophoresis, and so on. A high voltage is applied to the emission tip (spray tip), so the sample solution is charged and produces submicrometer-sized droplets upon exiting the spray tip. The liquid droplets contain ions produced from the sample of interest as well as the solvent. At this point, the analytes may be ionized; however, the charged droplets need to undergo further desolvation by either passing through a heated capillary or a curtain of nitrogen gas. The ions of analytes at the gas phase are then transmitted to the mass analyzer for mass measurement. In contrast to MALDI, ESI has a strong capability of generating multiply charged ions from biological molecules such as proteins, peptides, DNA, and RNA. This attribute enables high molecular weight peptides/proteins to be measured in the mass analyzers with limited *m/z* ranges since the instrument measures a molecule's *m/z* ratio.

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3.2 Mass Analyzers

The mass analyzer is the region where the *m/z* ratios of gas phase ions created in the source region are measured. Several different mass analyzers have been applied to proteomic analysis. Many types of mass spectrometers have been developed resulting from different combinations of mass analyzers, ionization sources, collision cells, ion optics, and so on. Each of them has unique features in terms of speed, sensitivity, resolution, mass accuracy, capabilities of tandem MS, etc. The selection of instrument to be utilized is based on the research focus. The details of each type of mass spectrometer were described previously (Yu, Conrads and Veenstra, 2005).

A quadrupole mass analyzer (Yost and Boyd, 1990) has been historically coupled with ESI. While single quadrupole mass spectrometers have limited utility in proteomics, the triple quadrupole instrument has been widely applied to protein analysis since it is composed of three quadrupole regions and has true MS/MS capability. The instrument is quite versatile in the detection of product ion, precursor ion, and neutral loss. These characteristics together with high measurement speed and sensitivity make it the choice for selected ion monitoring (SRM) in targeted proteomic analysis (more discussion below).

The TOF mass analyzer (Cotter, 1997), as implied in its name, measures the *m/z* values of ions based on the time they take to fly in the field-free drift tube. Attractive features of TOF analyzers include high resolution, mass accuracy, and so on. The TOF instrument in combination with MALDI (MALDI-TOF) has long been used for protein identification by peptide fingerprinting (Shevchenko *et al*., 2000). The recently developed MALDI-TOF/TOF (Medzihradszky *et al*., 2000; Suckau *et al*., 2003) has two TOF tubes separated by a collision cell and the first TOF tube is used for precursor ion selection when MS/MS is performed. The hybrid quadrupole-time-of-flight (Q-TOF) mass spectrometer (Chernushevich, Loboda and Thomson, 2001) can be considered either as the addition of a quadrupole and a collision cell to the TOF analyzer, or as the replacement of the third quadrupole (Q3) in a triple quadrupole MS by a TOF analyzer. This instrument has the advantages of quadrupole's ion selectivity and sensitivity and TOF's high resolution and mass accuracy in both MS and MS/MS modes; thus it has had a tremendous impact in proteomics.

Quadrupole ion trap mass analyzers (Stafford *et al*., 1984) have multiple capabilities of ion collection, isolation, storage, and ion manipulations such as collision-induced dissociation (CID) and ion-ion reactions. These characteristics enable an ion trap analyzer to perform multiple stage tandem MS (i.e., MS^n) for molecular structure elucidation. Compared to the conventional three-dimensional (3D) ion traps that operate with a 3D quadrupole field, the new stand-alone 2D ion trap (i.e., linear ion trap) mass analyzers have higher injection efficiencies and higher ion storage capacities (Schwartz, Senko and Syka, 2002; Hager, 2002; Douglas, Frank and Mao, 2005). Although ion traps normally have poor mass accuracy and low resolution, the highly efficient tandem MS has made the instrument a true "work-horse" in global proteomic studies.

A new type of ion trapping mass analyzer, Orbitrap, was developed recently (Makarov, 2000; Hardman and Makarov, 2003). An Orbitrap mass analyzer consists of an outer barrel-like electrode and a coaxial central spindle-like electrode to which electrostatic fields are applied. The Orbitrap analyzer radially traps ions about the central electrode. Unlike the ion trap analyzer, it measures the frequencies of harmonic oscillations of trapped ions along the axis of the electric field. The mass spectrum is obtained by a fast Fourier transform of the measured ion frequencies. The newly improved design of Orbitrap can provide higher field strength and higher frequencies of ion oscillations and thus higher resolving power (e.g., FWHM 350 000 at *m/z* 524) (Makarov, Denisov and Lange, 2009). Orbitrap mass analyzers are popular in proteomics owing to the high resolution and mass accuracy (typically <5 ppm) (Hu *et al*., 2005).

A Fourier transform ion cyclotron resonance (FTICR) mass analyzer (Comisarow and Marshall, 1974) measures the cyclotron frequencies of all of the ions in the ICR cell, which is placed in a magnetic field, and uses a Fourier transform to convert these frequencies into *m/z* values. It has provided the highest resolution (Solouki *et al*., 1997), mass accuracy (Bruce *et al*., 1999), and sensitivity (Hakansson *et al*., 2001) for peptide and intact protein measurements so far achieved; however, these performance parameters depend on the strength of the magnet field present. Although FTICR-MS is not used as widely as other types of MS in proteomics owing to the expense and technical challenges, its unique capabilities will make this type of instrument play important roles in global proteomic analysis, especially in the analysis of intact proteins.

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MS performance is expected to continue to improve as development continues.

4 PROTEIN IDENTIFICATION BY MASS SPECTROMETRY

Several approaches have been developed for the identification of proteins using MS. Ideally protein identification should be performed at the intact protein level so that protein isoforms, for example, as a result of different modifications, can be distinguished. This strategy for the analysis of intact proteins is called the top-down approach. However, there are many challenges to identify intact proteins at the current stage, including difficulties in LC separation of high molecular weight proteins and low efficiency in protein fragmentation during MS/MS analysis. In addition, high mass accuracy should be obtained for high confident protein identification, which is usually achievable using high-resolution mass spectrometers such as FTICR-MS. Currently the approach is mainly for the analysis of single proteins or simple protein mixtures (Garcia *et al*., 2007; Ouvry-Patat *et al*., 2008), and only a limited number of applications have been reported using online HPLC separation coupled with MS for the analysis of a few tens or hundreds of intact proteins (Chi *et al*., 2007; Bunger *et al*., 2008).

Alternatively, identification of a protein can be relied on the identification of peptides that are unique to the protein. The procedure is called a bottom-up approach, in which peptides are identified using MS from the enzymatic digest of a protein. For well-separated proteins such as a single isolated protein or the protein spots from 2D-PAGE, digestion of the protein or 2D spots using an enzyme (e.g., trypsin) results in a mixture of peptides that can be analyzed by MS (mostly by MALDI-TOF owing to its high speed and mass accuracy) to acquire a spectrum of masses (m/z) of the peptides, referred to as a "peptide map" or a "peptide fingerprint" (Berndt, Hobohm and Langen, 1999). This ensemble of experimental peptide masses is then compared to theoretical peptide maps derived from a protein database to identify the correct protein (Fenyo, 2000). When a complex protein mixture or a proteome sample is analyzed, peptide fingerprinting may not provide a clear identification and a more detailed analysis needs to be performed. In these instances, the sequence of a peptide is identified by MS/MS in which individual peptide ions are isolated and fragmented by CID within the mass spectrometer (Hunt *et al*., 1986). During CID, the fragmentation occurs primarily on the peptide bonds and this process can be controlled so one does not obliterate the analyzed peptide into its constituent amino acids. Therefore, the resulting spectrum contains various lengths of peptide fragments (i.e., *b*- and *y*-series of fragment ions) so that partial or even complete primary sequence information is retained (Figure 2). The ensemble of these fragment ions (or product ions) together with the mass of the intact peptide can be compared with the theoretical MS/MS mass spectra calculated from the protein sequences in the database using software programs to identify the peptide sequence (Eng, McCormack and Yates, 1994). This MS/MS approach is the most popular for the identification of proteins from complex peptide mixtures. To avoid the use of MS/MS and increase the throughput of protein identification, Smith *et al*. (2000) developed the approach named Accurate Mass Tags (Conrads *et al*., 2000), lately called Accurate Mass and Time Tags (AMT) (Strittmatter *et al*., 2003) for peptide identification solely based on the peptide mass or in conjunction with LC retention time. An AMT database must be developed and validated by MS/MS data prior to its direct use for protein identification. Accurate mass measurement of peptides ($\sim$1 ppm or less) is critical for correct identification of peptides if the AMT database is established solely based on peptide masses and thus the successful application of this approach is limited to a few laboratories. When LC retention time constraint is applied, the mass measurement accuracy could be modest.

For tandem MS of peptides and proteins, CID is efficient for the majority of peptides generated by enzymatic digestion; however, the technique is not efficient for intact proteins and some modified peptides such as phosphopeptides. Recently, electron capture dissociation (ECD) (Axelsson *et al*., 1999) and electron transfer dissociation (ETD) (Syka *et al*., 2004) have been developed to achieve efficient peptide backbone fragmentation for large peptides and even proteins. Large peptides/proteins are usually multiply charged during ESI, which is favorable to ECD or ETD and thus the techniques have great potential for the analysis of intact proteins (Bunger *et al*., 2008; Ge *et al*., 2009). In contrast to CID, both ECD and ETD generate *c*- and *z*-types of peptide fragment ions, and the fragmentation process does not result in evident loss of phosphate groups from phosphopeptides (Stensballe *et al*., 2000). An

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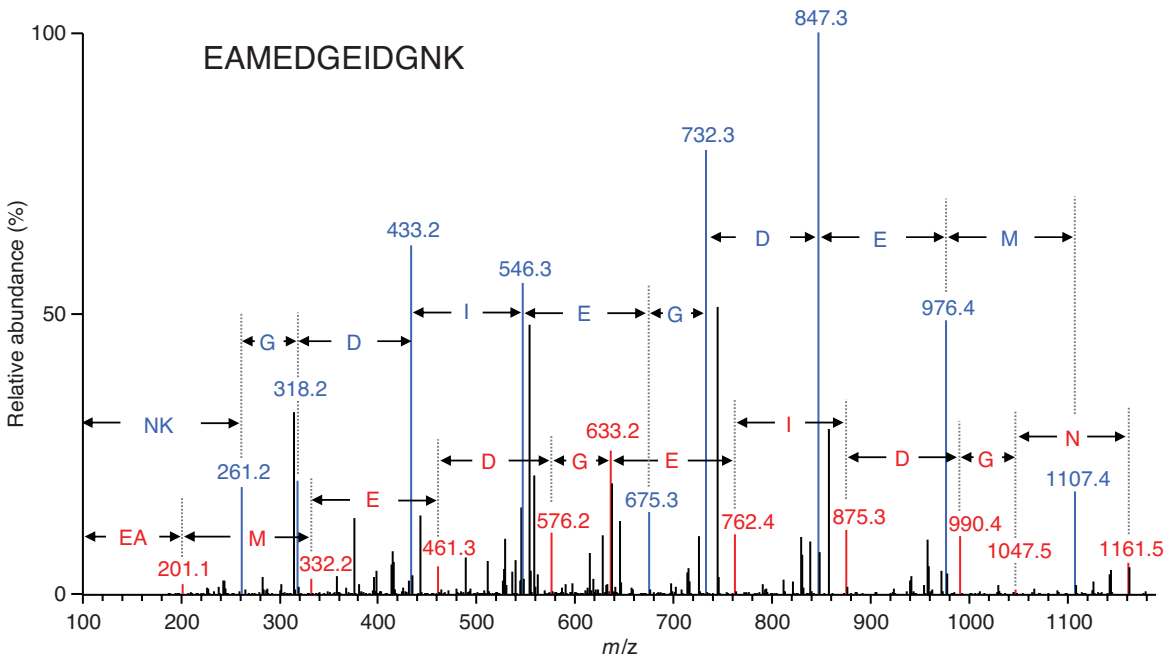

Figure 2. Collision-induced dissociation (CID) of the peptide EAMEDGEIDGNK from human Nucleolin. Shown in the MS/MS spectrum are the *b*- (red) and *y*-series (blue) of fragment ions, from which partial primary sequence can be determined by comparing the mass differences between either *y*-ions or *b*-ions in the spectrum with the calculated molecular masses of the amino acid monomers within the peptide.

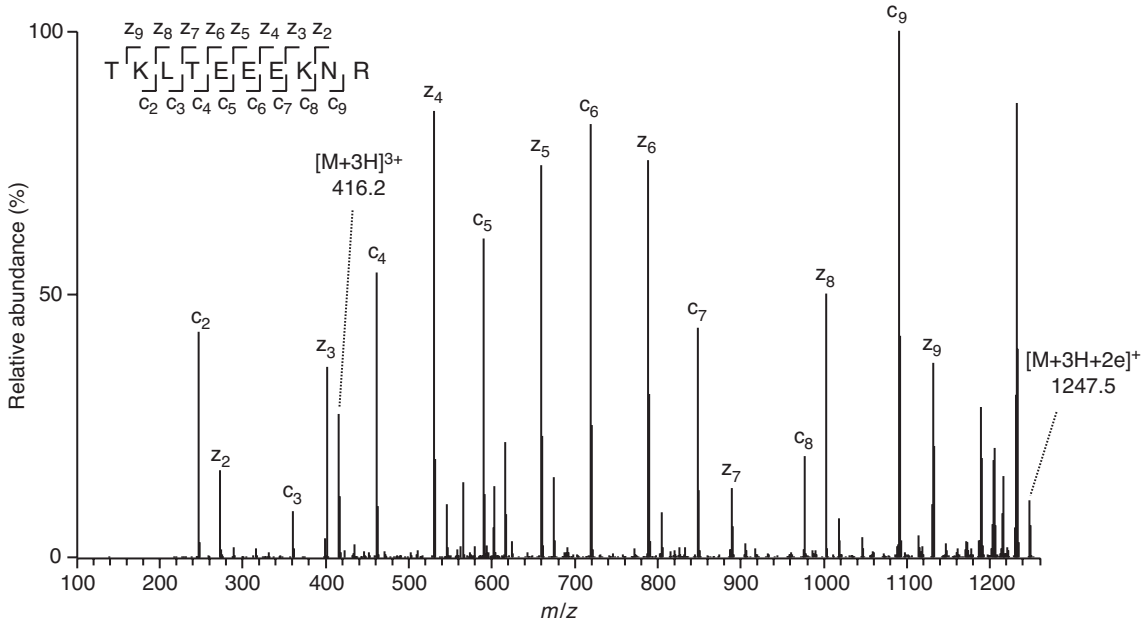

Figure 3. Electron transfer dissociation (ETD) tandem mass spectrometry analysis of the peptide TKLTEEEKNR from bovine alpha-S2-casein. Shown are *c*- and *z*-types of fragment ions generated during ETD. $[M + 3H]^{3+}$, peptide molecular ions; $[M + 3H + 2e]^{+}$, peptide molecular ions acquired two electrons but not subjected to fragmentation.

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example of ETD MS/MS spectrum of a peptide is shown in Figure 3. Currently, ECD has been implemented on FTICR-MS (Axelsson *et al*., 1999) while ETD has been on ion trap MS (Syka *et al*., 2004) and the hybrid ion trap-Orbitrap MS (McAlister *et al*., 2007). The utility of ETD in phosphoproteome studies has been demonstrated from Hunt's lab that reported 1252 phosphosites on 629 proteins identified in a single experiment from enriched phosphopeptides within 30 μg of total yeast protein (Chi *et al*., 2007). As mentioned earlier, good performance of ETD or ECD fragmentation is limited to the peptides with high charge states (e.g., ≥+3) (Swaney, McAlister and Coon, 2008). The protein digestion methods that generate relatively large peptides are preferred, for example, the use of Lys-C, Lys-N, and Glu-C proteinases or microwave D-cleavage digestion (Taouatas *et al*., 2008; Hauser *et al*., 2008). To increase the charges of tryptic peptides during ESI, m-nitrobenzyl alcohol (m-NBA) has been added to HPLC mobile phase (Kjeldsen *et al*., 2007). Alternatively, for enhanced identification of tryptic peptides, a data-dependent decision tree algorithm (Swaney, McAlister and Coon, 2008) can be employed in real-time acquisition of CID and ETD spectra in a single analysis, in which the type of data is dependent on the precursor charge state and *m/z*. The approach greatly increases peptide identifications for proteome samples. Other fragmentation techniques such as sustained off-resonance irradiation (SORI) fragmentation and infrared multiphoton dissociation (IRMPD) may be used to provide complement or better fragmentation for certain applications (Bogdanov and Smith, 2005; Siuti and Kelleher, 2007).

5 SOLUTION-BASED QUANTITATIVE PROTEOMICS TECHNOLOGIES

Solution-based proteomic analysis has increased popularity in recent years to circumvent the use and overcome some shortages of gel-based proteomics. In solution-based approaches, proteome samples (e.g., cell lysate) may or may not be fractionated using gel-free separation techniques such as HPLC. The total lysate or fractionated proteins are enzymatically digested, and the resulting peptide mixtures are subjected to further fractionation using liquid separation techniques and/or analyzed by online LC-MS-MS/MS (Figure 4). The above approach is named multidimensional protein identification technology (MudPIT) (Washburn, Wolters and Yates, 2001) and is generally for qualitative analysis or characterization of complex proteomes. However, it is amenable to quantitative comparison of protein abundance changes across different samples. For quantitative proteome analysis, all the compared proteome samples are processed and analyzed by MS in parallel, and the acquired LC-MS-MS/MS data are used to identify the peptides and proteins within each sample. Then the peptide ion chromatographic peaks are aligned and intensities are normalized. The abundance of each identified peptide/protein is compared across analyzed samples so that differentially expressed proteins can be identified. Since there is no stable isotope labeling involved in the above procedure, this quantitative proteome analysis method is called a label-free approach (Wiener *et al*., 2004). However, sample preparation and instrument analysis should be rigorously controlled for this approach to minimize any variations produced during sample preparation and LC-MS/MS analysis of individual samples. Label-free quantitative proteomic analysis has been increasingly applied to the identification of potential biomarkers from many diseased tissues or biofluids (Ong and Mann, 2005; Wang *et al*., 2008).

It is also convenient to incorporate stable isotope labeling into the solution-based proteomic procedure to achieve relative protein quantitation for the proteome samples to be compared (Figure 4). The labeling can be conducted prior to or after protein extraction (i.e., pre-extraction or post-extraction). As implied in the name, the pre-extraction method mainly refers to metabolic labeling, which is usually performed during cell culturing using either $^{14}N/^{15}N$ enriched media (Conrads *et al*., 2001) or stable isotope-coded amino acids (Chen, Smith and Bradbury, 2000; Veenstra *et al*., 2000; Ong *et al*., 2002). For some isotope-coded elements (e.g., deuterium), the labeled peptides are not co-eluted with the natural peptide counterparts from the reversed-phase LC column, which potentially introduces quantitation errors. Therefore, nitrogen and carbon elements are recommended since light and heavy versions of the labeled peptides are basically co-eluted. A representative example of metabolic labeling for quantitative proteomics is the method named stable isotope labeling by amino acids in cell culture (SILAC) (Ong *et al*., 2002). In this technique, cells are cultured either in the

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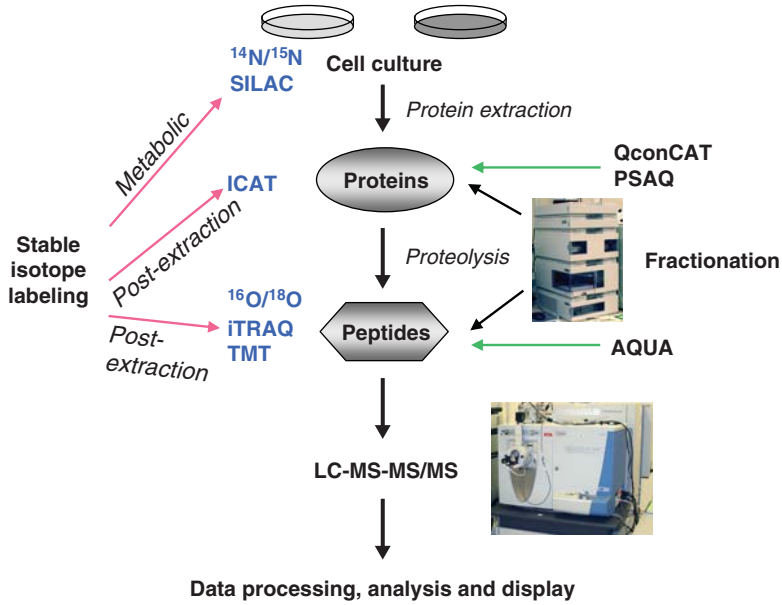

Figure 4. Solution-based proteomic approaches for relative and absolute protein quantitation. In the label-free quantitative proteomic approach, each proteome sample is prepared and enzymatically digested separately, and the resulting peptides are analyzed using LC-MS-MS/MS. Pre-fractionation using liquid separation techniques such as HPLC can be incorporated at the protein and peptide levels to increase the proteome coverage. Alternatively, stable isotope labeling can be performed at different stages of sample preparation (left) to achieve relative protein quantitation. For absolute quantification, isotope-coded peptide or protein standards (AQUA, QconCAT, or PSAQ) can be spiked into the peptide or protein samples with known quantities (right). The abundance of the peptide being analyzed is then compared with that of the isotope-coded standard. AQUA, absolute quantification; ICAT, isotope-coded affinity tag; iTRAQ, isobaric tag for relative and absolute quantitation; PSAQ, protein standard absolute quantification; QconCAT, absolute quantification using concatenated signature peptides; TMT, tandem mass tag.

normal media or in the media containing heavy isotope-coded amino acids such as ^{13}C-labeled lysine and arginine, which are then incorporated into proteins during protein synthesis and cell proliferation. A schematic approach of quantitative proteome analysis using SILAC and multi-dimensional separation is shown in Figure 5. The advantage of metabolic labeling is that the compared samples can be pooled early to avoid variations in downstream preparation of multiple samples. Recently, SILAC was successfully performed to label whole animals (e.g., mice) with $^{13}C_6$-lysine, and complete protein labeling was demonstrated for all the organs tested (Kruger *et al.*, 2008). The SILAC-mice provide a powerful tool for biological and medical research, allowing protein levels to be measured from whole organs all the way down to sub-cellular organelles and individual proteins. When metabolic labeling is not applicable, stable isotopic labeling can be performed after protein is extracted. Examples of most notable post-extraction isotope labeling include isotope-coded affinity tags (ICAT) (Gygi *et al.*, 1999; Yu *et al.*, 2004) and some isotope-coded amine-reactive reagents such as the isobaric multiplexing tagging reagents for relative and absolute protein quantitation (iTRAQ) (Ross *et al.*, 2004) and Tandem Mass Tags (TMT) (Thompson *et al.*, 2003). While ICAT is mainly for pair-wise comparison of proteomes, iTRAQ and TMT can be used to label multiple samples. ICAT is specifically designed to label Cys residues and the labeled Cys-containing peptides (after digestion) can be isolated via biotin-avidin affinity (Gygi *et al.*, 1999). Since only Cys-containing peptides are analyzed by LC-MS/MS, the complexity of the sample from ICAT labeling is significantly decreased and thus the potential to identify low abundance proteins is increased. In the above post-extraction labeling approaches, the labeling is completed via chemical reactions. However, stable isotopes can also be incorporated during enzymatic digestion of proteome samples. For example, trypsin-catalyzed $^{16}O/^{18}O$ exchange is such a type of labeling in which two oxygen atoms of the C-terminal carboxylate

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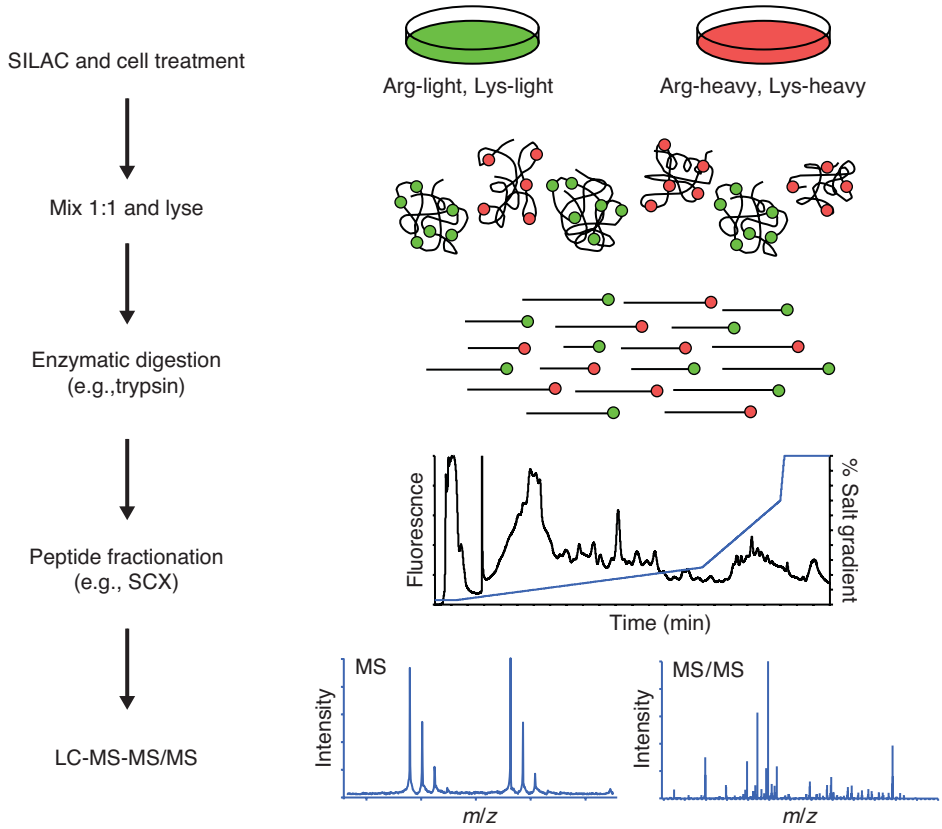

Figure 5. Quantitative proteome analysis using stable isotope labeling by amino acids in cell culture (SILAC), multi-dimensional LC separation, and mass spectrometry (MS). Two populations of cells are cultured in normal and stable isotope-coded arginine and lysine amino acids (Arg-light and Lys-light versus Arg-heavy and Lys-heavy). One population of cells is then stimulated and the other serves as a control. Cells are then mixed 1:1, lysed, and digested with trypsin. The resulting peptides are then fractionated by HPLC such as strong cation exchange (SCX). Each fraction is analyzed by reversed-phase liquid chromatography coupled on-line with tandem mass spectrometry (LC-MS-MS/MS). The MS scans are used to reconstruct the chromatographic peaks of the pair of labeled peptides, and relative quantitation is performed by comparing the peak areas of the peptides.

group of each peptide are replaced by ^{18}O atoms offered by ^{18}O-water via trypsin-catalyzed oxygen exchange mechanism (Schnolzer, Jedrzejewski and Lehmann, 1996; Yao *et al*., 2001). The enzyme-catalyzed $^{16}O/^{18}O$ labeling can be combined with ICAT to improve protein coverage of quantitation (Blonder *et al*., 2006).

Strategies have also been developed to absolutely quantify protein abundances and associated changes in proteome samples. Gygi's lab developed the absolute quantification (AQUA) method (Gerber *et al*., 2003) in which the stable-isotope labeled (heavy) peptides were synthesized and spiked in known quantities into the proteome sample to be analyzed. By comparing the peptides derived from the proteome sample with the "heavy" peptides (i.e., internal standards), the quantities of measured peptides can be calculated. To mix the isotope-coded molecules with proteome samples as early as possible and for more accurate quantitation, stable isotope labeled artificial proteins of concatenated signature peptides were synthesized (i.e., QconCAT) recently, which allowed the standards to be spiked into samples before trypsin digestion (Beynon *et al*., 2005). To further improve the quantitation, isotope-labeled full-length protein standards called Protein Standard Absolute Quantification (PSAQ) were *in vitro* synthesized and introduced into proteome samples (Brun *et al*., 2007). The advantage of this method is that the isotope-coded protein standards can be spiked at the earliest time and processed in the same way

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(e.g., pre-fractionation) as the analyzed sample to a maximum degree so that absolute quantitation can be performed robustly. For each quantitative approach, it should be noted that high-resolution mass spectrometers (e.g., FTICR-MS and Orbitrap-MS) are expected to achieve better quantitation and higher confidence of protein identification. The power of these types of instrument could be demonstrated particularly when intact protein biomarkers are analyzed in conjunction with isotope-labeled standards and efficient fragmentation techniques.

6 TOXICITY BIOMARKER DISCOVERY

Tissue and organ toxicity as a result of exposure to environmental toxicants or drug treatment remains a major public health issue. For example, many incidences of organ toxicity in liver, kidney, heart, brain, and so on, have been reported to be associated with chemotherapeutic agents. Cardiotoxicity (manifestations of heart failure, ischemia, hypotension, hypertension, edema, bradycardia, etc.) (Yeh, 2006) is one adverse event that can be caused by a wide range of non-targeted anticancer therapeutic agents (Floyd *et al*., 2005). It has been reported that doxorubicin, the most commonly used anthracycline anticancer drug, exerts a dose-limiting cardiotoxicity that effects up to 49% of patients treated for lymphoma (Hershman *et al*., 2008). Although better therapeutic efficacy and less organ toxicity have been expected for the new generation of targeted anticancer therapies (Shawver, Slamon and Ullrich, 2002), recent clinical practice and investigations suggest that these types of "magic bullets", including trastuzumab, imatinib, and sunitinib, often induce adverse effects on the heart in a small population of patients (Sengupta *et al*., 2008; Kerkela *et al*., 2006; Chu *et al*., 2007).

To prevent the development of adverse events and complications of disease management, it is critical to detect toxicity at the earliest stage. Early toxicity detection relies on the biomarkers that are indicative of the development of toxicity, and these detection tools should be very sensitive and specific. For cardiotoxicity, the most practical monitoring tool currently in clinical use is serial assessment of left ventricular function with echocardiography or radionuclide ventriculography (Sengupta *et al*., 2008). However, echocardiography has been found to be associated with limitations such as poor reproducibility and high variability (Telli *et al*., 2007). Protein biomarkers could be invaluable tools in early detection of disease, toxicity and organ injury. Quantitative proteomic technologies can be used to discover potential toxicity biomarkers and these technologies have been employed for many years to identify disease biomarkers (Anderson, 2005). Proteomic biomarker discovery has focused on biofluids such as human serum and plasma that can be collected by relatively non-invasive means. However, there is a wide range of protein abundance (10–11 orders of magnitude) within sera or plasma (Rifai, Gillette and Carr, 2006) yet one can expect that many important biomarkers, particularly at early stages of disease or toxicity, would enter the blood stream at very low levels. Therefore, it has been challenging to identify biomarkers from blood. Nonetheless, investigators have focused on blood as the biomarker source since it represents a collection from all organs and cell types, and can be collected in large volumes and in a relatively non-invasive manner (compared to sampling tissue, for example). Urine represents another type of body fluid for biomarker research. It is easily collectable and has shown utility particularly for biomarkers of nephrotoxicity and injury. Since proteins altered in neurotoxicity and neurological diseases may not enter the blood stream or urine, the focus has been on the cerebrospinal fluid (CSF). More recently, efforts have been made to identify biomarkers using solid tissues and interstitial fluids as starting materials since these types of samples may theoretically contain biomarkers with higher abundance. Once biomarker candidates are identified, a more focused approach for body fluids can be made. Another drawback in the search for biomarkers of toxicity is the relatively low throughput of the current proteomic platforms. This needs to be increased since time- and dose-dependent toxicology studies usually generate hundreds or even thousands of samples.

7 BIOMARKER VALIDATION AND QUALIFICATION

Biomarkers must be qualified by regulatory agencies (e.g., US Food and Drug Administration) prior to routine clinical application specified in labels for drugs approved by the FDA. Biomarker candidates have to be biologically qualified and the analytical test has to be validated. Clinical qualification can be quite challenging since it usually requires

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prospective studies and large numbers of clinical samples (Rifai, Gillette and Carr, 2006) that are independent from those used at the biomarker discovery stage. Baseline variations of measured biomarkers as a result of different ages, lifestyles, nutrition, sex, and so on, should be determined. Other challenges include proper patient recruitment for prospective studies, simultaneous measurement of multiple biomarkers, high stringency in biomarker measurement, high specificity and sensitivity, and so on.

For routine use of such biomarkers, assays must be available and validated. The assay should provide robust, specific, accurate, and precise analytical measurements along with adequate dynamic range. This kind of analytical validation should be closely associated with the specific biomarkers measured and include many aspects of the validation process, including sample handling, instrument analysis and data processing. For a specific protein biomarker, antibody-based assays such as enzyme-linked immunosorbent assay (ELISA) might be readily developed. However, in most cases it is likely that multiple biomarkers need to be assessed and thus there is an urgent need for multiplex assays that are capable of measuring tens or even hundreds of biomarkers in a single assay. It would be a great challenge to multiplex antibody-based assays since not all antibodies are available for the discovered biomarker candidates and development of a number of antibodies is a time-consuming and expensive process. Emerging proteomic technologies, for example, MS-based multiple reaction monitoring (MRM) (Anderson and Hunter, 2006), are being developed toward realization of these platforms as valid tools for protein biomarker validation and disease diagnosis as well as toxicity detection.

Selected reaction monitoring (SRM) or MRM (when multiple product ions are monitored) has great potential as an MS-based tool for protein biomarker validation and qualification owing to its high selectivity, sensitivity, dynamic range, and throughput. These features allow high-throughput measurement of biomarkers and absolute quantification can be performed potentially when standards are incorporated. In the biomarker discovery stage, the current proteomic workflow and throughput permit only 10–20 samples to be analyzed per experiment as discussed by Carr *et al.*, but thousands of proteins can be quantitated (Rifai, Gillette and Carr, 2006). In the validation and qualification stages, however, hundreds or even thousands of samples but a small number of proteins (e.g., 1–10) should be analyzed (Rifai, Gillette and Carr, 2006). Therefore, the MRM approach potentially is the method of choice for protein biomarker validation and qualification. MRM is usually performed in a triple quadrupole MS, in which the first quadrupole (Q1) is used as a filter to select a peptide (i.e., biomarker) with a predefined specific *m/z* value. The selected peptide ions are then transmitted to the second quadrupole (Q2) for CID to generate fragment ions. Ions of one or more specific fragments are filtered in the third quadrupole (Q3) and then detected (Figure 6). The process to detect a single peptide with one fragment ion transition only takes several milliseconds. Therefore, hundreds and even thousands of biomarkers can be quantified in a single analysis using the state-of-the-art MS. It should be noted that development of a specific MRM assay is a time-consuming process. MRM was able to directly quantify plasma proteins at high hundreds of ng ml^{-1} range with in-run coefficients of variation (CVs) of 2–22% and a dynamic range of 4.5 orders of magnitude (Anderson and Hunter, 2006). When stable isotope dilution (SID) or immunoaffinity enrichment such as Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA) is applied, the limits of detection (LODs) and limits of quantification (LOQs) have been achieved to the low ng ml^{-1} range in plasma (Keshishian *et al.*, 2007; Anderson *et al.*, 2004). With continual improvements in MS detection, the LODs are expected to be at pg ml^{-1} levels. A recent study showed that the interlaboratory CVs of MRM for plasma proteins ranged from 4–14% for the samples prepared on one site and the CVs increased to 10–50% for the samples prepared in each individual lab (Addona *et al.*, 2009). That the large number of biomarkers can be quantified by MRM along with its high sensitivity potentially makes this technique the major tool for candidate biomarker verification although the performance of the current technology is not as stringent as that currently required and approved in assays by US Food and Drug Administration (Addona *et al.*, 2009).

8 PROTEOMIC ANALYSIS OF TOXICITY MECHANISMS

Although the initiating event leading to organ damage may differ between drugs, environmental

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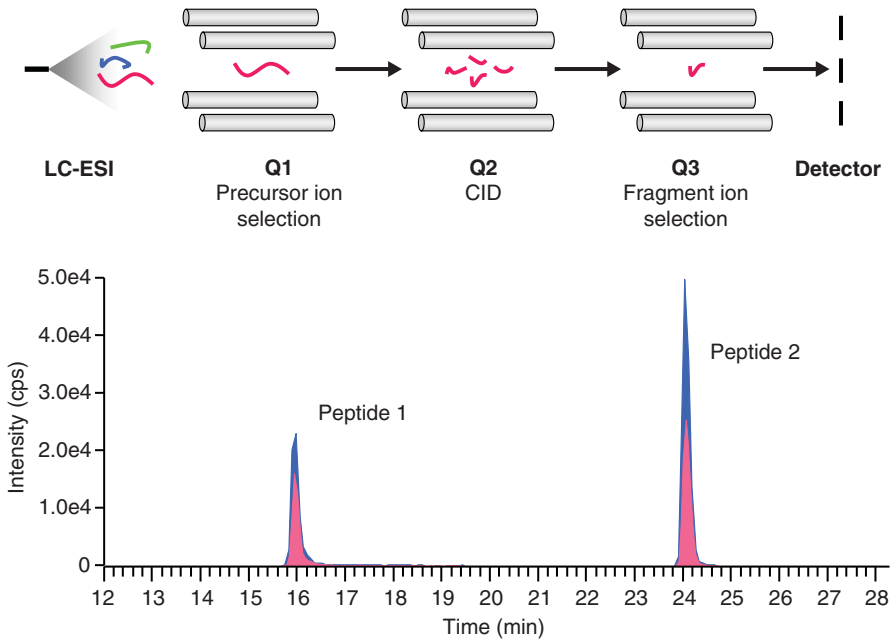

Figure 6. Schematic diagram of multiple reaction monitoring (MRM) for targeted analysis of peptides on a triple quadrupole mass spectrometer. In selected reaction monitoring (SRM) or MRM, the first quadrupole (Q1) is used as a filter to select a peptide with a predefined specific *m/z* value. The selected peptide ions are then transmitted to the second quadrupole (Q2) where they are fragmented by CID. Ions of one or more specific fragments are filtered in the third quadrupole (Q3) and then detected (upper panel). The lower panel is stable isotope dilution-multiple reaction monitoring (SID-MRM) quantitative assays of two targeted peptides. Shown are the extracted ion chromatograms (XIC) peaks of MRM Q1/Q3 ion pairs for natural (blue) and heavy (red) isotope-coded peptides.

toxicants and diseases, cells and organs have limited ways to respond to insults and thus there may be similarities downstream of the initiating event. Elucidation of toxicity mechanisms can provide essential information for better disease prevention, diagnosis and treatment. The unique capabilities of proteomic technologies are able to provide valuable information to unravel the cellular events related to the toxicity mediated by: (i) on-target effects of drugs interacting with tissues other than the selected organ; (ii) off-target effects. As an example of the former, some chemotherapeutic agents target tyrosine kinases that are ubiquitous and present on tissues such as the heart leading to unwanted cardiotoxicity (Force, Krause and Van Etten, 2007). In contrast, acetaminophen (discussed below) induces toxicity in the liver through off-target effects. Phenotypically similar organ toxicity could be caused by different compounds with diverse initiating mechanisms. Therefore, systematic analysis of toxicity at the molecular level using quantitative proteomic approaches could reveal toxicity mechanisms and biomarkers unique for a particular compound as well as those shared by multiple compounds. Both can be valuable depending on the question being asked.

Proteomic approaches have been applied to a variety of systems for the understanding of toxicity mechanisms. Main areas of mechanistic studies using proteomics include quantitative protein profiling of drug- or environmental toxicant-induced organ toxicity, toxicity pathway mapping, assessment of chemical/metabolite-induced protein modifications, and measurement of oxidative responses (Fountoulakis *et al.*, 2000; Ruepp *et al.*, 2002; Dowling and Sheehan, 2006; Andringa *et al.*, 2008; Moon *et al.*, 2008). For example, proteomic studies have provided some insights on the hepatotoxicity caused by overdose of acetaminophen, *N*-acetyl-*p*-aminophenol (APAP). A 2D-PAGE-based proteomic analysis of liver from an APAP-induced mouse hepatotoxicity study revealed altered expression of about 35 proteins and many of these are known targets for covalent modification by *N*-acetyl-*p*-benzoquinoneimine (NAPQI), a metabolite of APAP (Fountoulakis *et al.*, 2000).

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Proteomic analyses of mitochondrial proteins of the liver exposed to APAP indicated that the abundance of chaperone proteins (Hsp10 and Hsp60), ATP synthase subunits, and beta-oxidation pathway proteins was decreased (Ruepp *et al*., 2002), and the protein thiol content of catalase (a key enzyme in hydrogen peroxide metabolism) was modified (Andringa *et al*., 2008). These studies suggest that quantitative proteome analyses of protein expression changes, protein modifications, and specific subcellular contents would provide novel insights into the mechanisms of drug-induced toxicity.

Since many environmental toxicants and therapeutic drugs induce oxidative responses of biological systems, proteomic investigation of protein oxidation is another approach for a mechanistic study of toxicity. Acute exposure of MDMA (3,4-methylenedioxymethamphetamine, ecstasy), as a result of abuse, causes damage to many organs including the liver and brain. A proteomic study was designed to specifically enrich and identify oxidatively modified mitochondrial proteins for the mechanistic understanding of rat liver damage caused by MDMA (Moon *et al*., 2008). Exposure of MDMA resulted in a great inhibition of the activities of mitochondrial aldehyde dehydrogenase, 3-ketoacyl-CoA thiolases, and ATP synthase. In addition, a significant increase of protein oxidation was observed for the proteins involved in energy supply, fat metabolism, antioxidant defense, and chaperone activities (Moon *et al*., 2008). The above data suggest that oxidative inactivation of a class of mitochondrial enzymes likely contributes to MDMA-induced mitochondrial dysfunction and subsequent liver damage. In contrast, oxidation of a single protein at specific sites may contribute to oxidative stress-mediated cell death. A proteomic study of oxidative stress caused by the oxidant taurine chloramine (TnCl) resulted in the identification of more than 20 oxidized proteins (Klamt *et al*., 2009). Further study revealed that oxidation of cofilin (an actin-regulatory protein) causes it to lose its affinity for actin and to translocate to the mitochondria, where it induces swelling and cytochrome c release. Oxidation of cofilin by TnCl results in the formation of intramolecular disulphide bonds and Met sulphoxide, but only Cys oxidation causes cofilin to induce mitochondrial damage. Oxidant-induced apoptosis is independent of Bax activation and requires both oxidation of cofilin Cys residues and dephosphorylation at Ser3 within the protein (Klamt *et al*., 2009). The above studies demonstrate that proteomics is a powerful tool to elucidate the mechanisms of chemical compound- or drug-induced cell and tissue damage.

9 CONCLUSIONS

System biological approaches to understanding toxicity require proteomic methods and many quantitative proteomic technologies are currently available to meet these needs to some extent. These technologies can be classified into two basic approaches: gel-based and solution-based approaches. Mass spectrometry is the core technology for both approaches, providing the capabilities of protein identification and quantitation. Global proteome quantitation using either stable isotope labeling or label-free approaches enables not only the discovery of toxicity biomarkers but also the understanding of toxicity mechanisms. Targeted proteomic approaches such as MRM could play important roles in biomarker validation and quantitative analysis of targeted proteins and pathways. Stable isotope labeled peptide/protein standards are novel tools for both relative and absolute protein quantification. Analytical and clinical validation of biomarkers is essential to qualify biomarkers for clinical application. With further development of novel proteomic tools and an increase in sample throughput, the full spectrum of mass spectrometry-based proteomic research will greatly advance systems toxicology and an understanding of the mechanisms of cellular injury.

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Applications of Proteomic Technologies to Toxicology

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1 INTRODUCTION

Proteins are the major targets in cells or tissues for toxicant exposure, and play central roles in a system view of toxicological processes because they are ultimately the functional molecules for many cellular processes. To understand the toxic mechanisms and/or modes of action, we need to understand the toxic process at proteome level (proteomics), and to integrate the disparate proteomic and toxicology data. The linking of conventional toxicological research with functional proteomics has resulted in the emergence of toxicoproteomics. This systematic proteomic approach to investigating toxic processes and mechanisms could give information on integrated responses of tissues to toxic stress over time, on variability between exposed groups, and may provide data necessary for building predictive computational models. Proteomics is a high throughput approach that permits the simultaneous analysis of thousands of proteins. It can often detect molecular changes before the appearance of visible morphological changes and thus reduce toxicity testing time. For clinical toxicology, it is feasible that biomarkers may be detected for use as early indicators of later disease. Therefore, proteomics is an essential component of systems toxicology. The challenge will be to differentiate between specific and unspecific changes in the proteome, and to decide on the relevancy of such changes for further toxicological research. The major objectives of toxicoproteomics include elucidation of the molecular mechanisms of toxicity and xenobiotic interactions with biological systems, and identification of protein targets for application as signatures or biomarkers of a specific toxicity for chemical toxicity testing, diagnosis and monitoring of disease onset and progression, drug development, and human health risk assessment.

Proteomics was originally defined as the high throughput separation, display and identification of proteins (Anderson and Anderson, 1998; Balckstock and Weir, 1999). Traditional proteomics mainly focuses on protein expression levels in cells and/or tissues, which consistently change in response to the exposure of toxicants. Protein expression profiling of cells (Jin *et al*., 2004), tissues (Witzmann *et al*., 1999), and body fluids (Son *et al*., 2003) exposed or treated with toxicants for identification of toxic mechanisms, toxicity pathways, drug targets, toxicity and disease biomarkers, is probably the most popular application of proteomics to toxicological research. It provides a powerful means for defining relative protein levels, a molecular signature for any physiological, pathological, or toxicological process, as well as

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for disease development. Chowdhury and Aposhian (2008) identified 75 and 52 differentially expressed proteins in the livers and urinary bladders, respectively, of hamsters exposed to sodium arsenite in drinking water. In a study of protein expression profiles of human neuroblastoma (NB) cell line SH-SY54 using two dimensional gel electrophoresis (2-DE) and mass spectrometry (MS), GRP75 protein, a potential brain cancer biomarker, was found to be dramatically unregulated in the NB cells (Hsu, 2008). In a proteomic analysis of human prostate cancer cells exposed to benzo[a]pyrene (BaP) using 2-DE and MS, 26 differentially expressed proteins were identified (Chaudhary, Pechan and Willett, 2007). Among those proteins, some proteins such as acid leucine-rich nuclear phosphoprotein and cyclophilin A were previously reported to be changed at protein expression levels in other cancers. Proteomics-based immunochemical analysis of snake venom toxicity and immunoresponse provides valuable information for the selection and design of *in vitro* assessment of preclinical efficacy of antivenom, development of antivenom drugs, and treatment of snakebite injury (Gutiérrez, 2009).

As proteomics studies progress, the goals of proteomics are not only to identify proteins and analyze protein expression levels in mixtures, but also to derive more information on functional status of proteins from the samples that are analyzed. Modern proteomics can address the functional status of proteins, for instance through protein posttranslational modifications (PTMs), protein-protein interactions, protein intracellular location, and the rates of protein synthesis and degradation. Proteomics becomes a comprehensive study of proteins and their biological functions within a given biosystem. Generally, proteins are converted to their mature form through a complicated process of PTMs (Khidekel *et al*., 2004). Many of the PTMs are regulatory and reversible, and control biological functions in cells (Khidekel *et al*., 2004; Ptacek, 2005). For instance, phosphorylation can confer enzymatic binding or signaling transduction activities (Ptacek, 2005). Phosphorylation on serine, threonine and tyrosine residues is extremely important modulator of protein functions (Nagao and Yanagida, 2002). Altered phosphorylation of the proteins such as myosin light chain 1 has been reported in the failing heart (Abdelaziz *et al.*, 2005). The detailed phosphorylation status of six isomers of stathmin was characterized in hepatocellular carcinoma (HCC) (Muller *et al*., 1999). In addition to protein expression and PTMs, studies of protein interactions comprise another major application of proteomics to toxicology, which is becoming increasingly important. The tandem affinity purification (TAP) method (Bouveret *et al*., 2000) combined with LC/MS has helped improve the examination of protein complexes, which provides critical insight into protein function within biological processes (Rout *et al*., 2000).

In summary, applications of proteomics to toxicology mainly focus on relating changes at protein expression levels, PTMs, and protein interactions in cells or tissues after exposure to toxicants, with adverse health outcomes. The ability of proteomics to efficiently identify protein expression, PTMs, and protein interactions yields in-depth information on protein structure and function, which, in turn, provide valuable information for the understanding of toxic processes, pathways and mechanisms. Implementation of proteomics into toxicology has recently been catalyzed by the report of the US. National Research Council (NRC) on Toxicity Testing in the twenty-first century (National Research Council, 2007), which outlined the promise that new technologies, including proteomics, could dramatically increase the efficiency and accuracy in evaluating both chemical toxicity and adverse human health outcomes. Substantial efforts have been made towards the development of alternative methods for safety studies that do not require using laboratory animals. In the EU, timelines for phasing out many standard tests using laboratory animals were established in amendments to the cosmetics directive. Also, the new European Chemicals Legislation (REACH) favors alternative methods to conventional *in vivo* testing, if validated and appropriate. In the fields of safety and risk assessment it is expected that the development and validation of *in silico* methods, testing batteries, and tiered testing systems will be needed to overcome limitations in available resources and limited knowledge of the biological processes involved in complex biological endpoints (Lilienblum *et al*., 2008). To accomplish these goals, innovative toxicoproteomic strategies and high throughput experimental systems for low-cost and efficient identification of toxicity pathways and biomarkers, and for chemical toxicity testing need to be established. The huge amount of proteomic data and specific toxicological endpoints need to be integrated for an effective and comprehensive understanding of modes of action and

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mechanisms of chemical induced toxic responses and disease processes.

2 PROTEOMICS TECHNOLOGIES

The specific technologies that have been used for toxicoproteomic investigations are numerous and include 2-DE and MS (2DE-MS), multi-dimensional protein identification technology (MudPIT), and MS stable isotopic labeling such as Isotope-Coded Affinity Tags (ICAT) methodology, retentive chromatography-mass spectrometry (RC-MS) such as Surface-enhanced laser desorption/ionization (SELDI), and protein arrays (Figure 1). These technologies used for toxicology investigations could be generally combined into three main technology platforms: 2-DE gel-based, mass spectrometry (MS)-based, and non-gel/non-MS-based proteomic methods. In the following discussion of key proteomic technologies methodologies, we do not attempt to be exhaustive. Rather, we focus on their most important features and the recent applications of these proteomic platforms to toxicology research.

2.1 2-DE Gel-Based Proteomics

2.1.1 Protein Expression Profiling Using 2-DE

In 2-DE gel electrophoresis, proteins are separated first on the basis of isoelectric point, and then in a second dimension on the basis of molecular mass (Bischoff and Luider, 2004; Hanash, 2003; Gygi *et al*., 2000). 2-DE is the most powerful technique for protein separation. Thousands of proteins can be efficiently resolved on a single 2-DE gel. For each protein, the isoelectric point, molecular weight (MW), and the related quantities can be easily determined. The information content of the 2-DE gel data is high. 2-DE is also the most powerful technique for protein visualization and quantitation. Separated proteins and their modified forms can be visualized after gel staining by silver (Gharahdaghi *et al*., 1999), fluorescent dyes such as Sypro Ruby (Lopez *et al*., 2000; Berggren *et al*. 2000) and Coomassie Blue (Westermeier, 2006). Differences between samples can be compared and relative quantities can be determined by directly measuring the ratios of protein spot intensities in independent gels (Lilley, Razzag and Dupree, 2002). The software for protein quantification of 2-DE gel images is sophisticated and reliable after years of extensive development. Other advantages of 2-DE gel electrophoresis include easy gel storage and protein identification. In a recent study from our laboratory, proteins spots in 2-DE gels stained by Sypro Ruby and stored for two years could still be clearly visualized and accurately identified by MS. 2-DE has been the workhorse in the application of proteomics to toxicology especially in differential display analysis to find proteins that are differentially expressed between control and chemical-exposed samples. Figure 2 shows a typical 2-DE gel-based proteomic workflow for toxicological research. In this

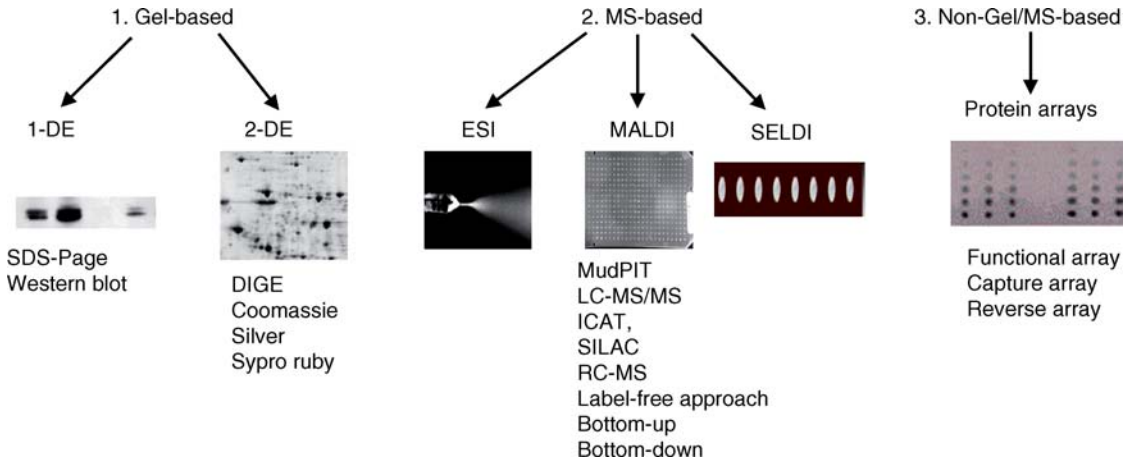

Figure 1. Introduction on major proteomic technology platforms for toxicology research.

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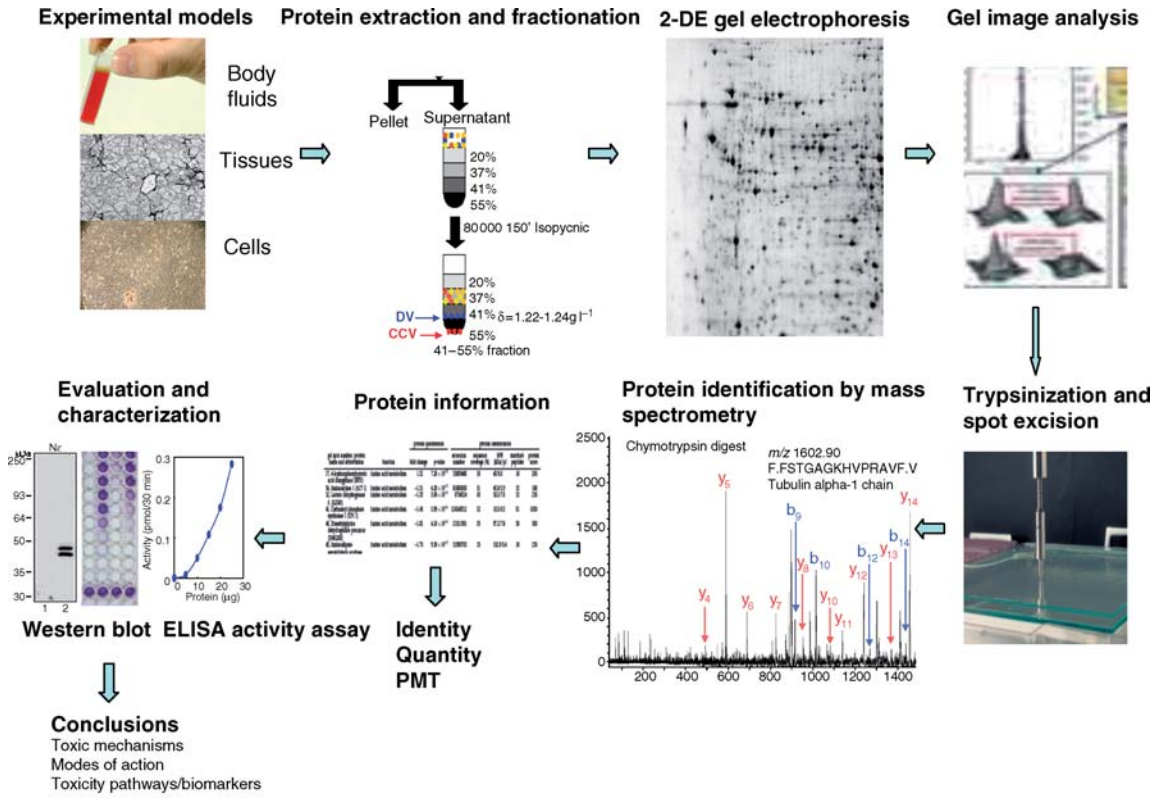

Figure 2. 2-DE proteomic workflow in toxicology research.

workflow, 2-DE provides a powerful means for protein separation and MS for obtaining peptide mass fingerprints or sequences for protein resolved on 2-DE gels. The development of mass spectrometry for the sensitive identification of gel-separated proteins in late 1980s, coupled with large-scale expressed sequence tag databases and computational methods for the correlation of the results of mass spectrometric analysis with the content of sequence databases, has greatly facilitated global protein analysis. With this platform, differentially expressed protein can be efficiently detected by 2-DE and identified by MS. If the proteins are shown to change consistently in samples treated with or exposed to toxicants, they may be associated with a toxic process. A comprehensive profiling of changes in protein expression of soluble proteins in livers from mice treated with the mouse liver tumorigen propiconazole to uncover the pathways and networks altered by this fungicide has been performed recently using 2-DE and MS (Ortiz *et al*., 2010). This study has revealed a series of alterations in livers induced by propiconazole: nuclear receptor activation, metabolism of xenobiotics, metabolism of biochemical intermediates, biosynthesis of biochemical intermediates, and oxidative stress in mouse liver, and provides novel insights into toxic mechanisms and/or modes of action of propiconazole. The information generated is valuable and necessary as well for human health risk assessment of this environmental chemical. Using 2-DE and MS, 682 distinct proteins in 90 lung adenocarcinoma specimens were identified. A 20-protein profile was shown to be able to predict survival among stage 1 lung cancer patients (Azad *et al*., 2006). 2-DE and MS also aid in analysis of small pathological samples obtained by minimally invasive biopsy (MacKeigan *et al*., 2003). Although only a small portion of the proteome in cells or tissues has been analyzed in these studies, pronounced changes in its composition that are associated with toxic mechanisms and toxicity pathways have been found.

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2.1.2 Protein PTM Profiling Using 2-DE

2-DE gel analysis provides a unique opportunity for identifying PTMs and isomeric forms of proteins. PTMs can be readily localized in 2-DE gels because they appear as distinct horizontal or vertical clusters of protein spots. Below, we will briefly discuss 2-DE gel-based proteomic approaches for analysis of protein phosphorylation, oxidation, and glycosylation, which may be the three most important functional attributes for the studies of chemical-induced toxicity. Significant progress has been made in the analysis of protein phosphorylation during the past several years (Wolf-Yadlin *et al*., 2007). Advanced techniques have been developed to isolate, purify, and enrich phosphorylated proteins or peptides (Schulenberg, Beechem and Patton, 2003; Schulenberg *et al*., 2004). Selective phosphoprotein staining with fluorescence dyes following 2-DE gel electrophoresis is commonly used to identify the potentially phosphorylated protein targets (Schulenberg, Beechem and Patton, 2003; Schulenburg *et al*., 2004; Kinoshita *et al*., 2006). With this new method, the phosphoproteins could be detected with good sensitivity and visualized with a variety of fluorescent gel image scanners. A combination of Western blotting with 2-DE is another common approach for the detection of phosphorylated proteins. Approximately 100 phosphorylated proteins were detected and 21 phosphorylated proteins were identified by MS from murine fibroblast cells stimulated with tumor necrosis factor-α (Kaufmann, Bailey and Fussenegger, 2001). Several studies on the role of protein oxidation in chemical induced toxicity and disease have been published (Delfino, Sioutas and Malik, 2005). Utilizing a 2-DE gel based proteomic approach combined with Western blotting and cell toxicity assays, an integrated approach for the systematic measurement and identification of protein oxidation in the livers from propiconazole-treated mice was developed (Bruno *et al*., 2009). This study identifies 17 carbonylated proteins and establishes a novel mode of propiconazole-induced toxicity in mouse liver that primarily involves oxidative damage to cellular proteins. Compared to traditional approaches, 2-DE gel-based proteomic approaches give much greater scope for detection and identification of oxidized proteins, and offer greater sensitivity for detecting oxidative changes. Xiao *et al.* (2003) demonstrated that diesel exhaust particles (DEP) induced a hierarchical oxidative stress response in which some proteins may serve as markers for oxidative stress during particulate matter (PM) exposure. In addition to protein phosphorylation and oxidation, the specific functional implications of protein glycosylation have been well studied. Protein glycosylation is one of the most important fields of study on PTMs due to the involvement of such modification in a variety of important biological activities such as carcinogenesis (Alam, Singha and Singh, 2005). More than half of human proteins are glycosylated by N- and O-linked glycans. Glycosylation patterns of proteins vary under different conditions. Heterogeneous glycoforms of the same protein may have different properties or biological activities (Dwek, 1996). The analysis of protein glycosylation is thus of interest in many areas such as diseases (Ohtsubo and Marth, 2006) and environmental toxicology. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is the most toxic environmental pollutant and a potent liver carcinogen. In order to understand the toxic mechanisms of TCDD, multiple proteomic approaches were used to identify differential profiles of protein glycosylation and phosphorylation in human Chang liver cells treated with TCDD (Kim, 2008). In this study, the several glycosylations on cathepsin B, HSP60 and two other proteins were found to be increased and identified. The toxic effects of TCDD may require the down regulation of cathepsin B activity, and enhanced glycosylation of cathepsin B may antagonize existing cathepsin B activity. These results suggested the roles of protein glycosylation in regulation of chemical toxicity in a biological system. Using 2-DE and MS, a total of 80 glycoproteins from human hepatocellular carcinoma cell lines were identified (Zhou *et al.*, 2007). The focus of glycoproteomics has recently changed from global glycosylation profiling to characterization of site-specific glycosylation (Kaji *et al*., 2006). In addition to the detection of PTMs individually, a technique capable of detecting phosphorylation, glycosylation, and total protein expression in a single 2-DE gel using three different fluorescent dyes has been reported (Ge *et al*., 2004). This novel platform was applied to the proteomic analysis of pregnancy-induced refractoriness to breast cancer using a rat breast cancer model system. Differential analysis of enriched rat mammary epithelial cell proteins from susceptible and refractory animals resulted in the identification

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of multiple proteins with altered phosphorylation, glycosylation, or expression patterns.

2.1.3 Limitations of 2-DE Based Proteomics Approaches

Although 2-DE gel electrophoresis is a powerful tool for discovery-phase toxicology research, not all expressed proteins can be displayed in a single gel. Low-abundance proteins, very large and small proteins, basic and acidic proteins and hydrophobic proteins present their own special challenge for 2-DE gel electrophoresis (Krapfenbauer *et al*., 2001). Regulatory and signaling proteins involved in important toxicity pathways are usually expressed at low levels or are hydrophobic. Efficient display of these proteins in 2-DE gel, to some extent, is the key to the success of a proteomic project. To overcome the limitations of 2-DE gel electrophoresis, a large effort has been made to improve the capabilities of 2-DE gel systems (Elrick *et al*., 2006), protein sample fractionation techniques, and new 2-DE gel based proteomics strategies, such as narrow 2D gel (ZOOM gel) (Tang and Speicher, 2005) and the Difference In Gel Electrophoresis (DIGE) system (Wu, 2006). To enrich low-abundant or specific groups of proteins, protein fractionation prior to 2-DE gel electrophoresis has been widely used (Issaq *et al*., 2002; Meistermann *et al*., 2006; Murayama *et al*., 2001; Guimaraes de Araujo and Huber, 2007; Lenstra and Bloemendal, 1983; Righetti *et al*., 2005). In addition, a proteomic technology platform combining different protein fractionation techniques and gel running systems in order to maximize the capabilities of 2-DE gel systems for protein separation and display has been reported (Ge *et al*., 2004). A combination of different protein fractionation techniques with different protein gel electrophoresis systems has demonstrated a significant capability of 2-DE gels in protein separation including those proteins, which are usually excluded by conventional 2-DE gel systems.

2.2 MS-Based Proteomic Technologies

The ability of mass spectrometry to identify even small amounts of protein from complex mixtures is a primary driving force in current proteomics. This gel-free mass spectrometric analysis of complex protein mixtures with much higher throughput and sensitivity enables a true proteome dynamic and comprehensive capture of cellular responses at any moments. Therefore, it has become an indispensable proteomic method for toxicology research. Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are the two techniques that have been widely used for mass spectrometric analysis of proteins or peptides (Bantscheff *et al*., 2007; Yates, Ruse and Nakorchevsky, 2009). MALDI techniques immobilize protein samples in an energy-absorbing matrix in a chip or plate. It is usually coupled to time-of-flight (TOF) analyzers that measure mass of intact peptides, and is widely used to identify proteins by peptide mass mapping. It is well-suited for identification of small proteins, and has many advantages such as simplicity, mass accuracy, high resolution, and sensitivity. Recently, MALDI ion sources have been coupled to quadrupole ion-trap mass spectrometers and two types of TOF instruments for fragmentation of MALDI-generated precursor ions for peptide sequence. ESI is mainly coupled to ion traps and triple quadrupole instruments and used to generate fragment ion spectra, collision-induced (CID) spectra. It provides information about peptide sequence in addition to peptide mass. It is ideal for the analysis of both large and small proteins since it is efficient for obtaining fine structural information from the molecules.

To improve efficiency, accuracy, and quantification capabilities of MS in identification of proteins from complex mixtures, various combinations of protein and peptide separation schemes have been coupled with MS instrument before peptide mixtures are applied for mass spectrometric analysis. The most popular peptide separation approaches are 2D or 3D chromatographic separation of peptide mixtures obtained from tryptic digestions of protein samples. Multidimensional Protein Identification Technology (MudPIT) system is a 2D (strong cation exchange and reversed phase) liquid chromatography (LC) separation method that can be coupled with online MS to produce thousands of tandem mass spectra (Yates, Ruse and Nakorchevsky, 2009). It has been developed as a method to analyze the highly complex samples necessary for large-scale proteome analysis by electrospray ionization, tandem mass spectrometry (MS/MS), and database searching. Bottom-Up approaches are often applied on protein mixtures in comparative proteomics using the MudPIT methodology (Yates, Ruse and

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Nakorchevsky, 2009). The term Bottom-Up reflects the reconstruction of the primary structure of the proteins from the little pieces of sequences of the peptides that could either be identified in databases or derived from the analysis of their mass spectra. It usually begins with direct digestion of protein mixtures to complex peptide mixtures, and then peptide analysis by LC-MS. MudPIT/LC-MS was applied to the analysis of potential susceptibility factors in drug-induced liver disease (Welch *et al*., 2005) and effects of chronic morphine exposure on the synaptic plasma-membrane subproteome of rats (Prokai, Zharikova and Stevens, 2005). It can also increase the detection of PTMs especially protein phosphorylation (Zhai *et al*., 2008). Within the bottom-up approach, protein phosphorylation on a highly enriched protein is characterized with the goal of location of a specific phosphate group or detection of a phosphorylation site within complex mixtures.

To add a quantitative dimension to peptide LC-MS/MS experiments, stable-isotope tags are introduced to proteins via metabolic labeling using heavy salts or amino acids or via chemical reactions using isotope-coded affinity tags or similar reagents (Han *et al*., 2001; Tam *et al*., 2004; Whetstone *et al*., 2004). So far, isotope-tags that are specific for sulhydryl groups, amino groups, N-linked carbohydrates, phosphate ester groups, and for active sites of serine and cysteine hydrolases have been developed (Zhang, Yan and Aebersold, 2004). For example, the ICAT reagent usually has a thiol-specific reactive group adjacent to an alkyl linker, which contains either nine [^{12}C] or nine [^{13}C] atoms - thus resulting in a mass difference of 9 daltons between the control versus the corresponding experimental version of the same tryptic peptide (Han *et al*., 2001; Tam *et al*., 2004; Whetstone *et al*., 2004). One advantage of the ICAT approach is that the *in vitro* incorporation of a stable isotope into one of the two samples enables the analysis of control and experimental samples simultaneously. Using ICAT and 2-DE LC-MS/MS, 644 proteins were qualitatively identified and 261 proteins were quantified from miscrodissected hepatocellular carcinoma (HCC) tissues (Li, 2004). Fifty-one out of 560 identified proteins showed a two-fold or greater change in expression in response to salinity stress in yeast (Li, Steen and Gygi, 2003). The major limitation of the isotope tagging approach is that it requires substantial time to analyze a large number of peptides, and therefore it is not suitable for high-throughput proteomic analysis.

Retentate chromatography-MS (RC-MS) is an analytical chemistry technique that combines the physical separation capabilities of LC with the mass analysis capabilities of MS. One of the most popular RC-MS is SELDI. SELDI has been widely applied to diagnosis and classification of disease, most notably for cancer characterization using patient sera (Yang *et al.*, 2005). Only a few microliters of serum are needed for each analysis. Yang *et al.* (2005) explored an application of serum SELDI proteomics patterns to distinguish lung cancer patients from healthy subjects. The correlation between pathological changes within an organ with the proteomic patterns in serum was also reported (Petricon, 2002; Lin *et al.*, 2006). SELDI-TOF MS has been used to identify clusters of proteins differentially present in normal tissue and early stage ovarian cancer, which are able to discriminate between healthy individuals and early stage ovarian cancer patients with specificity of 95% and sensitivity of 100%. Three potential biomarkers to distinguish glioblastomas from oligodendrogliomas were identified by SELDI. Using protein chip SELDI-TOF MS system, a 11.7 kDa ovarian cancer biomarker was detected (Moshkovskii *et al.*, 2005), and a prostate cancer-specific biomarker of amyloid A was found (Le *et al*., 2005). Altered expression of serum proteins in diabetic rats treated with ginsenoside-Re were detected using SELDI-TOF MS (Cho, 2006). This was the first study demonstrating that C-reactive protein could be altered by ginsenoside-Re treatment and that ginsenoside-Re may improve diabetes and its complications through suppression of inflammation.

So far, MS analyses of protein sequences have been successfully used in toxicological research for protein identification, identifying PTMs, and studying protein interactions. As a quantitative method, MS has been used to analyze protein expression and PTM profiles to define protein status and to infer their cellular functions. Pandey *et al.* (2000) isolated newly-phosphotyrosine-modified proteins using an antibody against phosphotyrosine and cells stimulated with epidermal growth factor. Peng, 2003 have used affinity purification to capture the ubiquitinated proteins in yeast cells. More than 1000 ubiquitinated proteins and 100 ubiquitation sites were identified by MS. MS-based proteomics have made and will continue to make a unique contribution to identification and mapping of PTMs in order to address important biological questions.

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MS-based proteomics has had a significant impact on the analysis of protein interactions too. To study protein interactions, one protein is often used as an affinity reagent to isolate its binding partners. In yeast, 232 stable complexes were isolated and protein constituents were identified by MALDI MS (Gavin, 2002). This study provides valuable information on phosphatases, kinases, and other protein interaction networks and signaling pathways. MS has also been extensively used to analyze PTMs especially to determine the type and site of a modification on single and purified proteins. Olsen *et al.* (2006) have detected 6600 phosphorylaton sites and 2244 proteins in epidermal growth factor stimulated Hela cancer cells for the study of phosphorylation dynamics and mapping of entire signaling cascades. Large-scale MS analysis of other large complexes and organelle such as nucleolus has been reported (Lam, Trinkle-Mulcahy and Lamond, 2005). More than 400 nucleolar proteins have been identified by MS. Compared with the *in vitro* techniques such as two-hybrid system and protein array, MS-based methods are able to detect protein interactions *in vivo*. All these studies on identification and mapping of signaling cascades provide a solid molecular basis for further analysis of toxic processes and mechanisms of these organelles or biosystems.

2.3 Non-gel/non-MS-Based Proteomic Technologies

Protein array analysis is a promising way to assess protein interactions systematically, and represents a promising new and complementary proteomic tool to 2-DE and mass spectrometry-based approaches (MacBeath and Schreiber, 2000). In this approach, individually purified proteins are separately attached to a surface and then analyzed for their activities. It provides a multiplexed approach for identifying protein-protein interactions, protein substrates, transcription factor protein-activation, as well as identifying the targets of biologically active small molecules (Arenkov and Kukhtin, 2000; Haab, 2005). The most popular protein-based chips are the antibody arrays that directly separate proteins from each other by antibody binding to specific protein targets. Using the protein microarray, it has been possible to demonstrate significant differences in the serum protein profiles of lung cancer patients when compared with healthy individuals and patients with chronic obstructive pulmonary disease (Gao, 2005). Sera from disease-free individuals and pancreatic cancer patients were successfully classified, and patients and normal control subjects were successfully discriminated using a novel protein array (Honda, 2005). In a study to evaluate the potential of proteomics as a tool for ecological biomarker discovery using protein arrays and SELDI TOF, mussels were exposed to environmentally relevant concentrations of oil, alkylphoenols, and polycyclic aromatic hydrocarbons (PAHs). The results indicated that protein expression profiles could be used to distinguish control from exposed organisms, types of exposure, and degree of exposure (Bjornstad *et al.*, 2006). Various protein-array formats allow rapid and high throughput investigation of protein activity and interaction on a proteomic scale. Liu, Patricelli and Cravatt (1999) created probes that specifically target serine hydrolases. These probes contain a fluorophosphonate-reactive group, which binds to most members of the serine hydrolase superfamily. In addition to protein arrays, peptides can also be analyzed using peptide microarrays (Volkmer, 2009).

3 APPLICATIONS OF PROTEOMIC TECHNOLOGIES TO TOXICOLOGY

Proteomics has recently been used in the studies of mechanistic toxicology, clinical toxicology, pharmacoproteomics, safety assessments of chemicals, and environmental toxicology. A general summary of the major applications of proteomics to toxicology is shown in Figure 3.

3.1 Mechanistic Toxicology

Alterations in the proteome may provide a highly sensitive means of detecting toxicity responses because toxicants or chemicals frequently alter the profile of proteins in the sample by inducing changes in the expression levels of specific proteins or by altering protein structure via modulation of posttranslational modifications. Protein profiling itself provides little information about the underlying mechanisms of toxic response. However, once the differentially expressed or modified proteins or pathways are identified, biological function can be

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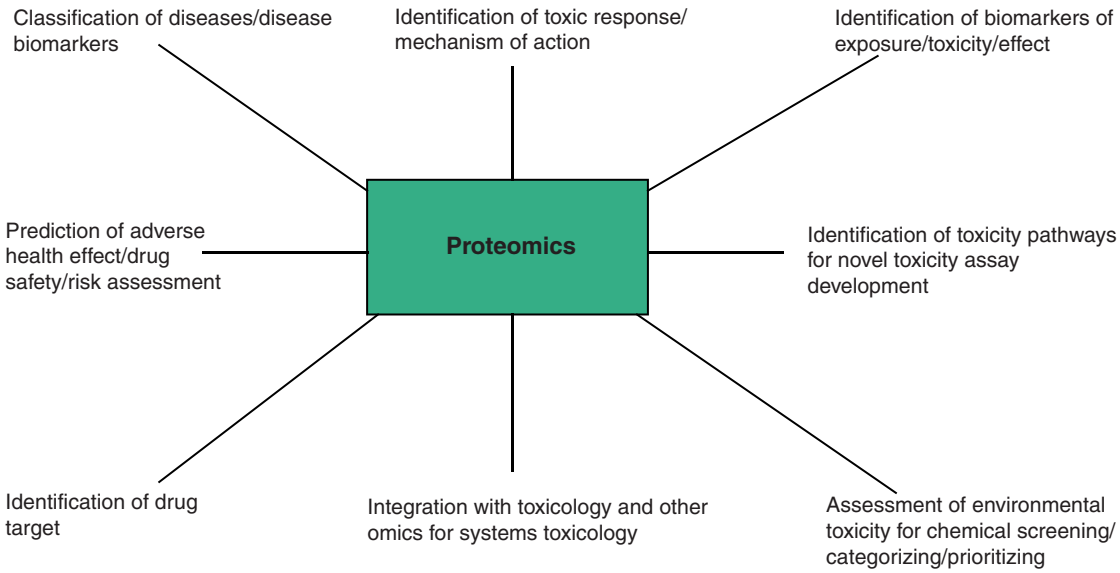

Figure 3. A summary of the applications of proteomics to toxicology.

inferred by mapping components to known biological pathways and verified in follow-up functionality studies. Therefore, the altered protein profile provides a sensitive indicator of toxicant exposure, disease state, and altered cellular metabolism for characterization of adaptive changes of cells and organisms to toxic stressors. Because of this, molecular characterization of the proteome using proteomics represents a powerful tool for developing a complete understanding of toxic events and processes starting with the interaction of a toxicant with a cell, through physiological and tissue/organ changes, resulting in disease development.

In the clinical application of proteomics to toxicology, it was found that shorter survival times of patients with B-cell chronic lymphocytic leukemia may be associated with the altered expression of some redox enzymes, heat shock protein 27, and protein disulphide isomerases, which may confer resistance to cytotoxic drugs to the cancer cells (Alaiya, Al-Mohanna and Linder, 2005). A study on proteome response to ochratoxin A (OTA), a naturally occurring mycotoxin, suggested that coordinated regulation of molecular networks is involved in the OT-induced cytotoxicity, and that proteome response can be an indicator of neurodegeneration (Yoon *et al.*, 2009). A total of 488 proteins associated with Alzheimer's disease were identified. These proteins included well-known components of plaques such as amyloid beta, alpha1-antichymotrypsin, and apolipoprotein E. Protein expression profiling of arsenic-treated human cells and animal tissues has revealed a mechanism of cellular stress response in which several heat shock protein family members including Hsp60, 70, and 90 are up-regulated (Lau, He and Chiu, 2004; Welsh and Gaestel, 1998). All of these studies suggest that proteomics methods can provide valuable information on toxic mechanisms and pathways that are essential parts of mechanistic toxicology.

Proteomics is a powerful tool for whole proteome profiling to identify substance specific alterations in protein expression patterns after *in vivo* treatment. In combination with other toxicology tools and OMICS including transcriptomics and metabolomics, it is a promising approach for generating toxicity signatures, which are needed to foster predictive toxicology. The potential of such novel toxicology is to predict adverse health effects after short-term exposure of laboratory animals. It will lead to a reduction in the duration of screening studies and reduction in the number of required animals per dose group (Hengstler *et al.*, 2006). Carcinogenic potential is conventionally measured using a two-year study, incurring notable expense in both animal and human resources (Perera *et al.*, 1991). It is therefore of great interest to identify biomarkers of carcinogenicity that can be detected

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in short term. Proteomic profiling of liver samples after one day of exposure to the carcinogen N-nitrosomorpholine (NNM) revealed significant up-regulation of proteins involved in response to cellular stress induced by NNM (Fella *et al*., 2005). Several interesting cancer-related proteins or biomarkers were identified by proteomic analysis of animal tissues with 18 weeks of exposure to the carcinogen. An investigation on the identification of new proteomics biomarker signatures predicting hepatic carcinoma/adenoma, as well as hepatotoxicity has also been reported (Fella *et al*., 2005).

3.2 Clinical Toxicology and Disease Related Research

Most diseases are manifested through changes in protein levels or functions, and existing drug therapies typically aim to interfere with the activity of a specific protein. Proteomics has advanced disease research. The emphasis of the application of proteomics to clinical toxicology is in understanding disease pathways and mechanisms, identifying protein targets or biomarkers for detection, progression, and therapy of diseases. They have also facilitated the differentiation of normal from diseased states correctly by characterizing protein expression profile, PTMs and signaling pathways altered in diseased cells or tissues. As toxicoproteomics progresses, more emphasis will be placed on its capability to identify diagnostic biomarkers of the presence and staging of diseases, as illustrated in ovarian cancer (Petricoin and Liotta, 2002), lung cancer (Tang *et al*., 2006) and rheumatoid arthritis (de Seny, 2005).

The detection of protein profiles associated with disease states dates back to the very early application of 2-DE gel electrophoresis to analysis of clinical materials (Tyers and Mann, 2003). The robust and high throughput MS has recently revolutionized protein based diagnosis (Rodland, 2004). Protein arrays have been extensively used for clinical research (Witte and Nock, 2004). These proteomics technologies have allowed scientists to simplify the study of diseases by using different tissues and cell populations, blood, and other biofluids. It has recently been reported that only the phosphorylated form of stathmin is highly correlated with leukemia, illustrating the power of proteomics in understanding disease mechanisms (Sowell *et al*., 2009). An extensive 2-DE-based proteomic analysis of 21 HCC patients identified 60 proteins that demonstrated significant changes in the expression level between nontumorous and tumorous tissue (Kim, 2003). Recently, proteomics methods for analysis of small amounts of diseased tissues have been developed. Laser capture microdissection (LCM) is an ideal technology for extracting disease cells from specimens in which the exact morphologies of both captured cells and surrounding tissues are preserved (Emmert-Buck *et al*., 1998). LCM preserves the molecular composition and architecture of the cells so that direct comparison of the protein profiles can be made in a micro compartment of the same sample. To date, LCM in combination with 2-DE is one of the most widely used tools for identification of disease biomarkers (Roy and Shukla, 2008). A combination of LCM and SELDI to analyze prostate cancers, a protein present in 94% of prostate cancers was identified (Roy and Shukla, 2008). Proteomics was used to define tumor subsets among resected lung specimens to distinguish between primary adenocarcinomas and primary squamous cell carcinomas with 98% accuracy (Conrad *et al*., 2007). To increase pancreatic cancer survival rates, clinicians are actively seeking an assay capable of classifying samples as normal or carcinogenic when cancer is at its early stage. Using LC-MS/MS and ELISA microarrays for the identification of breast cancer biomarkers, 64 altered proteins were identified from nipple aspirate fluid (Woodbury, Varnum and Zangar, 2002), and 15 of the identified proteins had been reported to be potential markers for breast cancer. Another interesting area in clinical toxicology is quantitative proteomic analysis of host-pathogen interactions. It has been reported that two state-of-art quantitative proteomics approaches, metabolic labeling SILAC and chemical isobaric tagging iTRAQ were used to study changes on macrophage protein expression in response to exposure to M. tuberculosis lipids. 166 macrophage differentially expressed proteins involved in immune response, oxidation, and reduction as well as in other cellular processes were identified (Shui *et al*., 2009).

3.3 Pharmacoproteomics

It is well-known that most drug targets are proteins. The application of proteomics within

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the pharmaceutical industry has been defined as pharmacoproteomics. The major proteomics applications in the drug industry are drug target identification and validation, identification of biomarkers of drug efficacy and safety from readily accessible biological fluids, and investigation of toxic mechanisms/modes of action of drugs. The use of pharmacoproteomics promises to reduce the investment of time and money required for drug development.

3.3.1 Drug Target Identification

The application of proteomics to the pharmaceutical industry has been a powerful tool for candidate selection and detection of new targets for desired effects and should have the same power for preventive strategies in individualized medicine. Target identification involves identifying altered protein expression, posttranslational modification, and activity. There are about 400 known protein drug targets and the number could rise to more than 10 000. Proteomics research holds the key to identifying those thousands of new drug targets since it is a powerful tool for identification of hundreds to thousands of protein expression changes, PTMs, and activities in model systems during disease progression. Using proteomics approaches, four biochemical pathways relevant to Type 2 Diabetes Mellitus (T2DM) were identified, including up regulation of protein biosynthesis, down regulation of insulin secretion, energy use, and metabolism (Lu *et al*., 2009). Proteomics analysis of whole cell homogenates, cytosolic, and myofilament subfractions of ischemic/reperfused rabbit hearts elucidated abundance changes in multiple proteins involved in stress response and energy metabolism in mitochondria (Jäger, Jungblut and Müller-Werdan, 2002). In 2-DE and mass spectrometric analysis of platelet-dense granules from abdominal aortas of aneurysm patients, 40 proteins involved in several important cardiovascular disease pathways and some atherosclerosis-associated proteins were identified (Hernández-Ruiz, 2007). Using the 2-DE proteomics platform to analyze atherosclerotic carotid arteries, the effect of atorvastation on secretome from plaques was observed (de la Cuesta *et al*., 2009). 2-DE and MS was used to analyze a rat model of endothelin-induced cardiac hypertrophy (Macri and Rapundalo, 2001), tyrosine phosphorylation status in a study of lymphocytes with Scott syndrome (Imam-Sghiouar, 2002), and protein glycosylation in pancreatic adenocarcinoma tumor cells (Tarbé *et al*., 2005). Once a protein target has been identified and validated, the expression and purification of the target for crystallization frequently follows. The knowledge of the crystal structure of the protein can provide very important drug binding information for the production of drug targets. Such a structural proteomic approach has been used for the development of drugs for HIV, influenza, and some other infectious diseases (Schmid, 2002). The most rapid impact that toxicoproteomics is likely to make in the near future is through the discovery and validation of biomarkers of disease and disease progression.

3.3.2 Pharmacological Safety

In an early study, a proteomic profile database describing xenobiotic effects on protein expression in rodent liver was built using proteomic techniques (Anderson *et al*., 1996). Using this proteomic database, it was possible to detect, clarify, and characterize a broad range of liver toxicity mechanisms. The use of proteomics gave insights into the molecular mechanisms of the pharmacological action of a hypoglycemic agent (Arce *et al*., 1998). The results strongly suggested the association of the chemical toxic effects with microsomal proliferation and induction of the P450 enzyme system. The proteomic study from Edvardsson *et al*. (1999) confirmed the agonistic mechanisms of action of the peroxisome proliferator activated receptor (PPAR). Proteomic analysis of HK-2 cells exposed to oxalate and calcium oxalate crystals provides novel insights into the molecular mechanisms of renal injury and stone formation (Fu, Xu and Yu, 2005). Proteomics, especially when combined with conventional methods such as histopathology and clinical chemistry, offers the prospect of new insights into toxic mechanisms. Cyclosporin A, an immunosuppressant drug, is toxic to kidney. In a study for clarification of toxic mechanisms of cyclosporin A, a cytosolic calcium-binding protein, calbindin was found to be altered and associated with cyclosporin A-induced renal impairment (Aicher *et al*., 1998). When the monoclonal anti-calbindin antibody was used to perform enzyme-linked immunosorbent assays and immunohistochemistry with renal tissues from rat, dog, monkey, and human, a close correlation between calbindin decrease and nephrotoxicity was

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confirmed. In a proteomic analysis of drug candidate toxicity and safety, the safety profiles of all of the 20 compounds were successfully predicted (Gao, 2004). Craig (2006) has used a combination of genomic, proteomic, and metabolomics approaches to study the effects of methapyrilene in male rats. The hepatotoxic mechanisms of this drug at multiple levels in a biosystem were uncovered. In another study, cytokeratins were identified as nonspecific indicators of renal damage in the urine of gentamicin-treated rats (Kennedy, 2002). In a proteomic analysis of fatty liver disease, changes in protein expression associated with steatosis were detected prior to the onset of overt toxicity. The protein changes including upregulation of pyruvate dehydrogenase and phenylalanine hydroxylase were detected only 6 h after the exposure of rats with the steatosis-induced compound (Meneses-Lorente *et al.*, 2006, 2004). In summary, proteomics has been successfully used for the assessment of efficacy and safety of drug compounds.

3.4 Safety Assessment of Chemicals

Decades ago, safety measurements for chemicals were generally to limit exposure and to define the circumstances of use in production and transport. This strategy is valid for working places and is still implemented into modern safety science (Foth and Hayes, 2008a). However, the evaluation and control of dangerous substances is very complex because they are widely distributed in consumer products and impact the environment. Safe handling of industrial chemicals needs information on sensitization potential, organ toxicity after repeated exposure, germ cell mutagenicity, carcinogenesis, reproductive toxicity, behavioural toxicity, and fate in the environment (Foth and Hayes, 2008b).

The challenges to fully characterizing the toxicity of hundreds and thousands of chemicals using conventional toxicity methods have long been recognized. Complete toxicity testing of these chemicals by standard protocols requires the use of multiple mandatory tests that far exceed available resources time, experienced personnel, laboratory capacity and money. The situation can be improved by applying screening technologies to predict the rank order toxicity of a group of chemicals to establish priorities for further testing.

Use of high-throughput methods that allow efficient and economical screening of large numbers of chemicals is in an urgent need. The belief that a specific group or class of chemicals will induce specific protein changes provides a basis for the application of proteomics to predictive toxicology. Such changes or signatures could be used to screen novel compounds. Proteomics permits proteome wide assessment of protein changes associated with chemical exposure and toxicity. Compounds that belong to the same class reveal discernible protein signatures with higher degree if similarity to each other than to the protein signatures corresponding to exposure to compounds from a different class. Several early studies on the changes in the protein expression patterns for chemical toxicity screening have been reported (Anderson *et al.*, 1995; Witzmann, Fultz and Lipscomb, 1995). The effects of perfluoro-n-octanoic acid and perfluoro-n-decanoic acid on the expression of a set of heat shock and glucose-regulated proteins were analyzed. This study demonstrated that 2-DE electrophoresis stress protein patterns could be used for future toxicological screening. It has been reported that the stress response to perfluorocarboxylic acids is tissue-, toxicant-, and stress protein class-specific and dose-related, and the 2-DE stress protein pattern is suitable to future toxicologic screening applications based on the fingerprints of the abundant stress-response proteins (Witzmann, Fultz and Lipscomb, 2005). A proteomic study has recently evaluated the biological responses to multi-walled carbon nanotubes using human monoblastic leukemia cells. This proteomic approach provided a promising new method for safety evaluation of nanomaterial toxicity (Haniu *et al.*, 2010). The identification of responding proteins or protein changes can provide valuable information on toxicity pathways or toxicity signatures, which are important for chemical screening and development of novel chemical toxicity assays. Proteomics has the potential to detect the "fingerprints" of a specific toxic effects and identification of protein expression signatures that predict carcinogenic potentials of chemicals.

3.5 Environmental Toxicology

The major application of proteomics to environmental toxicology is the clarification of toxic modes of action and identification of toxicity pathways

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describing key toxic events and processes starting with the interaction of a pollutant with a cell, through physiological and tissue/organ changes, resulting in disease development. In an early study, the effects of jet fuel vapor on protein expression in the rat lungs, kidney, and liver was investigated, and possible risk of the fuel vapor exposure to human health was suggested (Drake *et al*., 2003). In order to investigate the multiple effects of PAH on protein expression, proteomics analysis of MCF-7 cells treated with benzo[a]pyrene and dibenzo[a]pyrene and standard extracts from coal tar and diesel exhaust were performed. The similarities and differences in the acute response to individual PAH were compared. Interestingly, each PAH generated unique patterns of alterations in protein expression (Hooven and Baird, 2008). Recently, an overview and illustrative study to assess the effects of endocrine disrupting chemicals (EDC) in fishes has been reported (Ankley *et al*., 2009). In the study, a systematic approach including genomics and proteomics was used to delineate toxicity pathways for 12 model EDC with different known or unknown toxic modes of action. This study established the basis of toxicity prediction approaches for species, end point, and chemical extrapolation. In addition to these toxicoproteomics studies using animal and human tissues and cells, body fluids including serum and urine have also been used for the study of toxicity of environmental chemicals. An innovative proteomic technique for qualitative and quantitative assessment of urinary protein modulation associated with acute uranium exposure in rats has been reported (Malard *et al*., 2009). Fourteen differentially expressed proteins involved in nephrotoxicity were identified, suggesting the value of urinary proteomics for profiling of uranium toxicity. A combination of genomic, proteomics, and bioinformatics approaches have also been used to study toxic mechanisms of environmental chemicals.

Recently, environmental toxicity biomarker discovery has been a popular area of applications of proteomics to environmental toxicology research. Biomarkers of exposure and toxicity are cellular indicators of the physiological state and changes during a toxic or diseased process at a specified time. By comparing the protein profile of chemically treated cells or tissues to a database containing profiles induced by known toxicants, protein biomarkers of exposure and toxicity may be identified. Using SELDI-TOF-MS, 20 altered plasma proteins (Harezlak *et al*., 2008), and a human beta-defensin-1 (HBD-1) peptide (Hegedus, 2008) were found to be associated with arsenic exposure. These biomarkers have great potential value and predictive accuracy that could be used for exposure and toxicity assessment in humans. A biomarker could also provide information about potential physiological effects and help to determine the nature of the toxicity and toxic injury. In 2-DE gel analyses of metal toxicities, both cytokeratin 8 and cytokeratin 14 were significantly altered in cells exposed to arsenite and cadmium, respectively, suggesting these two proteins might play important roles in metal-mediated apoptotic processes (Gonsebatt *et al*., 2006). The results obtained from several proteomic analyses of 2,3,7,8-tetrachlorodibenzo-p-dioxin toxicity suggest that TCDD affects the liver and immune systems (Bruno, 2002; Lee *et al*., 2005; Pastorelli, 2006). Using SELDI and LC-MS, three inflammation biomarkers, calgranulin A, calgranulin B and anaphylatoxin C3a were identified in bronchoalveolar lavage fluid of rats exposed to diesel exhaust particles (DEP), suggesting that one of the major mechanisms through which DEP cause toxicity is inflammation (Lewis *et al*., 2007). Detection and profiling of small molecular changes on the protein level at early time points could lead to early biomarkers, which could enhance the predictive value of alternative short-term chemical toxicity including carcinogenicity assays. A number of inflammatory response proteins that may be associated with the early phases of inflammation in response to DEP exposure were identified. The results show that histopathology studies at different time points correlate the histopathology changes with protein expression changes.

One of the major challenges in environmental toxicology is to decide whether identified hazardous chemicals will affect human health and the environment. To date, many of the chemical toxicity testing approaches are primarily based on endpoints from animal toxicity studies. Thus, an appropriate toxicity data extrapolation needs to be performed to correct the differences in toxic responses as well as ages, health status and living environment within the exposed group between animals and humans. Another big challenge is to extrapolate dose and species responses of environmental chemical exposure. Toxicity repair, adaptation, and mitigation of a biological system may lead to a robust tolerance

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against very low exposure levels of environmental stressors. It is well known that different species react differently from one another, and within a species, sensitive individuals may react differently from the majority of subjects. To extrapolate the data regarding differences in species and doses, a clear understanding of similarities or differences in proteome profiles and toxicity pathways is critical, but this information is sparse.

4 CONCLUSIONS AND FUTURE DIRECTIONS

Toxicity pathways, toxic processes, and mechanisms are enormously complex. Simultaneous measurement of proteomics parameters in a given system under defined toxicity conditions, and integration of the proteomic data with toxicity end points is the trend of current toxicology, a new perspective for molecular toxicology. The combined analysis of multiple data sets may be able to define the links between proteomic data and particular toxic processes or diseases under investigation. Integration of proteomics data with classical toxicology end points and clinical observation allows for more sensitive and earlier detection of adverse health effects, precise identification of toxicity signatures, and development of models for toxicity.

Today, modern toxicology is committed to the 3Rs concept, which was defined first by Russel and Burch in 1959. It states the need to refine, to reduce, and to replace animal testing whenever possible. For this reason, innovative proteomic toxicology focuses on the development and improvement of short- and medium-term toxicity studies. For this, a variety of human cell culture systems instead of animal models will be generated and extensively used for toxicoproteomic research (Oberdorster *et al.*, 2005). The cell culture based test system could help overcome cost and throughput limitations associated with animal testing, thereby, enabling the efficient investigation of toxicity profiles of toxicants. A human cell culture *in vitro* toxicity testing system, combined with a minimum number of *in vivo* animal systems may be the optimal approach for future applications of proteomics to the evaluation of toxicity. Recent analytical developments have made the global and simultaneous assessment of multiple biomolecules such as DNA, RNA, proteins, and metabolites possible. These OMICS approaches will be integrated and the results of this integration are expected to be able to reveal patterns of regulation within and among networks, and provide a systems level description of an organism's responses to any chemical or disease. *In vitro* toxicity testing systems and systems toxicology approaches represent two important trends of current and future toxicological research.

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Application of Quantitative Proteomic Approaches to Toxicology

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1 INTRODUCTION

The genome provides a static blueprint of the molecular make up of biological systems in organisms. Unlike the genome, however, the protein complement of a cell or organism is very dynamic. Proteins interact with each other and also with other molecules including DNA, RNA and lipids to maintain homeostasis. In addition, proteins undergo post-translational modifications (PTMs), exist as different isoforms, shuttle from one cellular compartment to other and are sometimes expressed in a tissue-specific manner (Prasad *et al*., 2009). Biological systems respond to external stimuli through cell-surface receptors in the extracellular space that ultimately relay information to the transcriptional machinery in the nucleus resulting in modulation of expression of specific sets of genes (Kandasamy *et al*., 2010).

Quantitative proteomic technologies coupled with high-resolution mass spectrometry can be used effectively to unravel key biological roles of proteins by differential profiling and measuring their dynamic changes in expression levels and PTMs (Chaerkady *et al*., 2008; Chaerkady *et al*., 2009; Chaerkady and Pandey, 2008). Especially, phosphorylation and glycosylation status can be monitored using specific affinity enrichment methods (Chaerkady *et al*., 2008; Harsha *et al*., 2008). As proteins are the functional units which are directly involved in biological processes, differential proteomic analyses provide better insights into dynamics of molecular changes specific to disease and normal status. In this study, we review important applications of quantitative proteomic tools in environmental toxicology.

2 QUANTITATIVE PROTEOMIC TECHNOLOGIES TO STUDY ENVIRONMENTAL TOXICITY

Quantitative mass spectrometry of complex proteomic samples using *in vitro* or *in vivo* stable isotope-based labeling has become the method of choice for identification of differentially regulated proteins. Table 1 lists various approaches in common use today for quantitative proteomics along with their salient features. The high reliability of these methods is a consequence of high-resolution mass spectrometers, availability of multiplexed labeling methods and robust multidimensional protein and peptide separation tools (Chaerkady *et al*., 2009; Molina *et al*., 2009). Stable isotope-labeling methods are advantageous because the chemical nature of the peptides and

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proteins from different samples of interest remains the same thereby allowing for pre-fractionation of combined samples prior to mass spectrometric analysis. There are several stable isotope-labeling methods available for quantitative proteomic analysis. These include *in vivo* labeling methods, such as stable isotope labeling with amino acids in cell culture (SILAC) (Ong *et al*., 2002; Harsha, Molina and Pandey, 2008) and *in vitro*-labeling methods, such as iTRAQ (Keshamouni *et al*., 2006), ^{18}O labeling (Chaerkady *et al*., 2008), and ICAT (Shiio and Aebersold, 2006).

Table 1. Approaches for quantitative proteomics.

Method	Features
Stable isotope labeling with amino acids in cell culture (SILAC)	Metabolic labeling using heavy amino acids for *in vivo* differential labeling of proteins
Isobaric tags for relative and absolute quantitation (iTRAQ)	Isobaric tags (up to eight different tags) for labeling-free N-terminal amino groups and ε-amino group of lysine residues allows robust labeling of all peptides
Tandem mass tags (TMT)	Isobaric tags (up to six different tags) for labeling free N-terminal amino group and ε-amino group of lysine residues allows robust labeling of all peptides
Acrylamide labeling	Differential labeling of cysteine residues using ^{13}C-labeled acrylamide
Isotope-coded affinity tags (ICAT)	Differential labeling of cysteine residues
^{18}O labeling	Trypsin incorporates two ^{18}O atom at C-terminal carboxyl groups of peptides during digestion of proteins
Label-free quantitation	Normalization of chromatograms and retention times followed by mass spectral counting and/or peak area measurements
Absolute quantitation (AQUA) using multiple reaction monitoring	Targeted quantitation of peptides by analyzing pre-selected peptide ions and calculating abundance based on a corresponding heavy peptide

Stable isotope labeling with amino acids in cell culture (www.silac.org) is a simple metabolic-labeling approach where "light" or "heavy" forms of amino acids (e.g., arginine or lysine) are incorporated into proteins. The heavy form of amino acids contain substituted stable nuclei (e.g., ^{13}C, ^{15}N) and thus cells grown in heavy media behave exactly like the control-cell population grown in the presence of natural amino acids. After several cell divisions, light amino acids are replaced by heavy amino acids. Figure 1 illustrates a SILAC-based strategy for quantitation and identification of proteins from three different samples. The SILAC strategy has become a routine technique in many areas of cell biology (Harsha, Molina and Pandey, 2008). It has been applied to globally characterize the effects of antitumor agents (Harsha *et al*., 2008; Zhang and Neubert, 2009), phosphoproteomes (Hilger *et al*., 2009), and cellular differentiation (Molina *et al*., 2009; Prokhorova *et al*., 2009). It has also been used to study secreted proteins in cell-based systems (Molina *et al*., 2009; Grønborg *et al*., 2006). Several signaling pathways have been dissected using specific enrichment of the phosphoproteome such as in insulin and epidermal growth factor receptor signaling (Kruger *et al*., 2008; Guha *et al*., 2008). Thus, the SILAC approach can be adopted for toxicological studies in global profiling, signaling, and PTM analysis, which can now be used to compare up to five different conditions simultaneously (Molina *et al*., 2009).

Molecular effects of exposure to toxicants can be tested using standard and appropriate cell lines using quantitative proteomics. The secretome of cells can be a good source of potential biomarkers (Chang *et al*., 2009; Srisomsap *et al*., 2010; Zhong *et al*., 2008). Analysis of the secretome provides a good complementary approach to search for potential biomarkers in serum because these low abundant proteins are often masked by high amounts of other proteins in serum. It is advantageous to use a cell line because the secreted proteins can be easily isolated from culture media. In addition, using the SILAC labeling allows differential labeling of secreted proteins from control and treated cells. Secreted biomarkers of pancreatic and esophageal cancers have been investigated using cell lines (Harsha *et al*., 2008; Grønborg *et al*., 2006). This strategy can also be easily adapted to investigate biomarkers in environmental toxicology.

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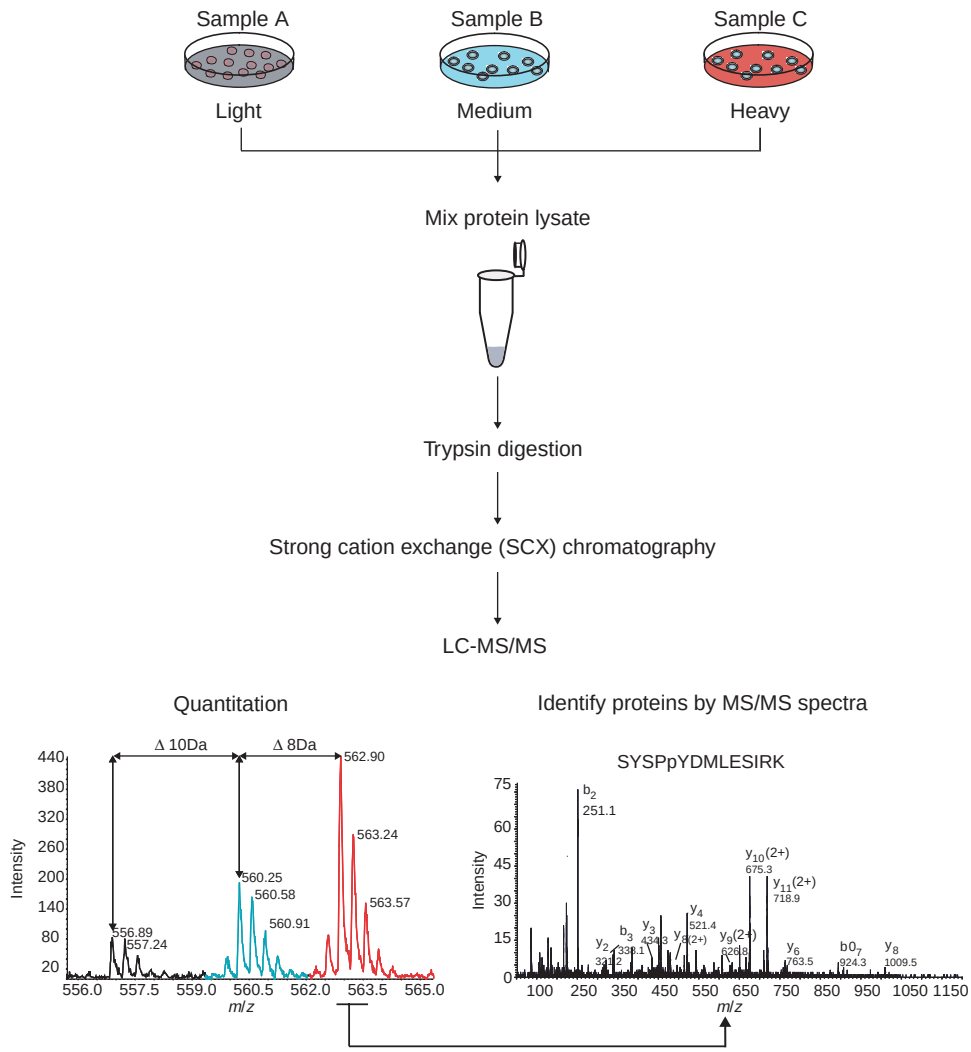

Figure 1. Schematic of SILAC strategy for comparing expression of proteins in three different states. Cells are grown separately in media containing light, medium, or heavy amino acids. Upon completion of labeling, cells are exposed to various conditions and equal amounts of protein lysates are mixed. The samples are then processed at protein and peptide levels before mass spectrometric analysis. Relative intensities (extracted ion chromatograms) of peptide ions provide expression levels of the peptides under different conditions.

There are a number of quantitative proteomic methods available for *in vitro* protein profiling, among them, iTRAQ method has been used most extensively. The iTRAQ reagents are made up of a set of primary amine-specific isobaric reagents that allow the identification and quantitation of proteins. The current iTRAQ method allows simultaneous labeling of up to eight different samples. In an eight-plex method, the isobaric tags consist of reporter ions of 113, 114, 115, 116, 117, 118, 119, and 121 Da. In the iTRAQ method, all peptides with one or more primary amine groups are uniformly labeled increasing the reliability of quantitation. Unlike other labeling methods, in the iTRAQ strategy, the MS signal of a peptide from different samples is summed and hence even peptides of low abundance can be quantitated. This is important because the iTRAQ reporter ions used for quantitation are released only during fragmentation. Figure 2 illustrates a typical scenario of iTRAQ-based quantitation and identification of proteins. Table 2 catalogs several published proteomic

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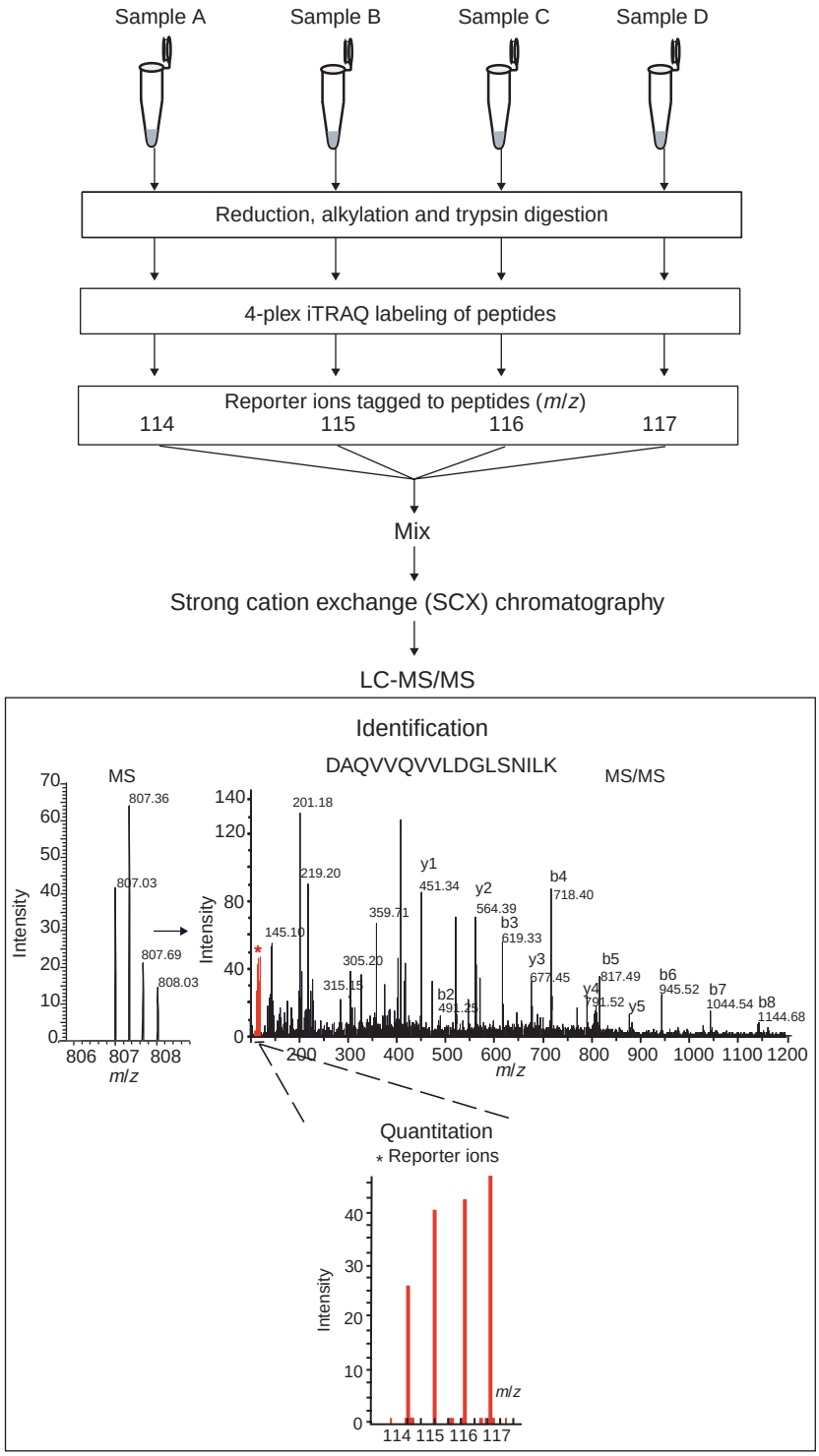

Figure 2. Schematic of iTRAQ strategy for comparing expression levels of proteins in four different states. Peptides are labeled with reagents containing tags that yield specific reporter ions upon CIS fragmentation. Relative intensities of these reporter ions are observed in the low mass region at 114.1, 115.1, 116.1, and 117.1 *m*/*z* values. The relative abundance of any peptide in the four different samples is calculated from the relative intensities of these reporter ions.

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Table 2. Published studies on application of quantitative proteomics in toxicology.

Proteomic approach	Toxicant	Sample analyzed	Potential biomarkers	Reference
SELDI-TOF MS	Benzene	Serum	↓ PF4 and ↓ CTAPIII	(Vermeulen *et al*., 2005)
Two-dimensional gel electrophoresis	Cyclosporine A	Kidney	↓ *CALB1*	(Aicher *et al*., 1998)
Two-dimensional gel electrophoresis and MALDI-TOF MS	Aerosolized JP-8 jet fuel	Kidney	↑ *EZR* and 7 other proteins ↓ *HMGB1* and 16 other proteins	(Witzmann *et al*., 2000)
Two-dimensional gel electrophoresis and MALDI-TOF/TOF MS	Microcystin	Liver	↑ suclg2, anxa4 and five other proteins ↓ amy2a, cpb1 and 8 other proteins	(Wang *et al*., 2010)
Two-dimensional gel electrophoresis and ESI-Q-TOF	North sea oil spiked with alkyl phenols and polycyclic aromatic hydrocarbons	Plasma	↑ fga, apob ↓ trf and hpx are among 137 differentially expressed proteins	(Bohne-Kjersem *et al*., 2009)
Two-dimensional gel electrophoresis and MALDI-TOF MS	Tetrabromobisphenol-A	Liver	↑ hsp70, gp96 ↓ pgam1a, bhmt	(De Wit *et al*., 2008)
SELDI/MALDI-TOF/TOF MS and LC-MS/MS	A group of 16 chemicals	Liver, kidney, urine, blood	↑ Crp, B2m, Hp and 4 other proteins in plasma. ↑ Apoa2, Gsta3, Pebp2, B2m in liver.	(Collins *et al*., 2010)
Two-dimensional gel electrophoresis and MALDI-TOF MS	Carbon nanotubes	U937 cell line	↑ *HNRNPA2B1*, *ANXA2*, *VASP* and 16 others ↓ *SNRPA1*, *PSMB1* and 24 others	(Haniu *et al*., 2010)
Two-dimensional gel electrophoresis and MALDI-TOF/TOF MS	Perfluorododecanoic acid	Testis	↑ *IDO* ↓ *UQCRH*, *COX6A1*, *SOD1*, *YWHA*, *AKAP4*	(Shi *et al*., 2010)
iTRAQ labeling and LC-MS/MS	Docetaxel	PC3-Rx and PC3 prostate carcinoma cell lines	↑ *GDF15*, *A1BG*, *VTN*, *RNPEP*, *FETUA* ↓ *CYR6*, *MASPIN*, *AGR2*	(Zhao *et al*., 2009)

studies, where different quantitative proteomic methods have been applied to toxicology.

Because of widespread contamination in food, water, and household items, arsenic toxicity is quite common in some areas of the world. Arsenic accumulation in the body can affect many organs leading to cancer (skin, lung, liver, and bladder), cardiovascular disease, diabetes, and reproductive defects (Banerjee *et al*., 2008; Banerjee *et al*., 2009; De Chaudhuri *et al*., 2008; Ghosh *et al*., 2008; Huang *et al*., 1999). Although the effects of its toxicity have been studied well, no detailed investigation has been carried out to understand the biological process and early markers of exposure at the protein level. We have used a quantitative proteomic approach for identifying specific differentially expressed proteins in HepG2 liver cells in response to arsenic exposure. Protein extraction was performed from HepG2 liver cells grown for 24 and 48 h with or without exposure to arsenic by adding sodium arsenite to the media. After digestion with trypsin, peptides were labeled using four-plex iTRAQ-labeling strategy as illustrated in Figure 2. Labeled peptides were pooled and fractionated using strong cation-exchange chromatography followed by high-resolution tandem mass spectrometry. Mass spectrometry-derived data were searched against a human protein database. Several proteins that were differentially regulated in response to arsenic exposure were identified. These included HSP70-1B, which was elevated ~four fold

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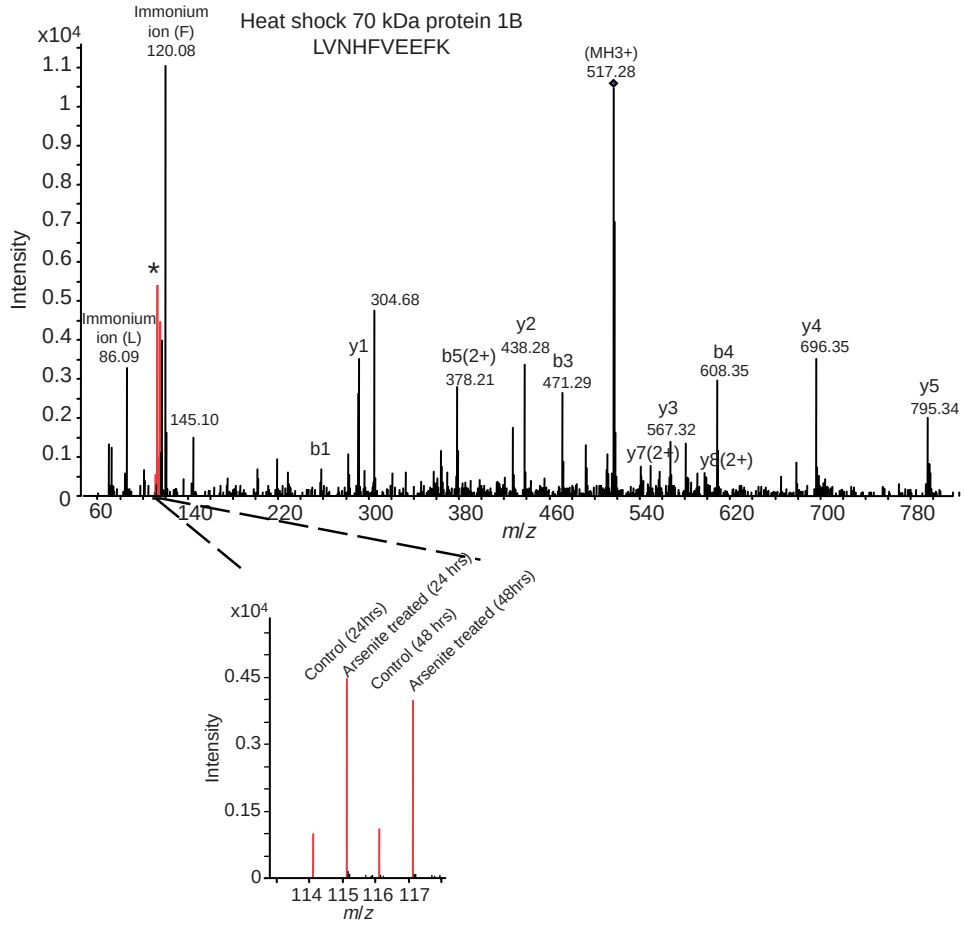

Figure 3. iTRAQ-based quantitation of up-regulation of HSP701B upon arsenic exposure. The figure shows the MS/MS spectrum of a representative iTRAQ-labeled peptide (LVNHFVEEFK) derived from *HSP701B*. A zoomed in view of the relative intensities of the reporter ions from four different conditions (denoted by an asterisk in the MS/MS spectrum) is shown. The peptides from 24 and 48 h untreated controls were labeled using iTRAQ reagents with 114.1 and 116.1 mass tags, respectively, whereas peptides from arsenic-treated samples for 24 and 48 h were labeled with iTRAQ reagents with 115.1 and 117.1 mass tags, respectively.

in arsenic-treated cells. Figure 3 shows the MS/MS spectrum and reporter ion intensities of a representative peptide from the HSP70-1B protein. Similar investigations could be carried out to identify protein signatures from other biological samples, such as serum or urine obtained from arsenic-exposed patients. The candidate biomarkers identified from these studies can be validated using tissue microarrays and/or ELISA in a separate set of patients. Results from such studies will help to elucidate the key molecules involved in response to arsenic toxicity.

3 QUANTITATIVE PROTEOMICS TO IDENTIFY ALTERED LEVELS OF PROTEIN PHOSPHORYLATION

Phosphorylation of proteins can be differentially induced in response to exposure to toxic compounds even though overall expression levels of these proteins remain unchanged. A knowledge of the exact phosphorylation sites that are inducibly regulated can lead to the development of valuable biomarkers or therapeutic targets (Bose *et al.*, 2006). In addition, a set of hyper- and hypophosphorylated

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molecules can indicate activation or repression of specific signaling pathways associated with exposure to toxicants. Identification of such signaling pathways associated with exposure to a particular toxicant can aid in discerning the molecular mechanisms involved in the deleterious effects associated with toxicants (Harsha *et al*., 2008). Enrichment of phosphorylated proteins/peptides is an essential step in the identification of differentially induced phosphorylation events. TiO_2 and anti-phosphopeptide antibody-based methods are commonly used for the enrichment of phosphopeptides. Quantitative proteomic strategies such as iTRAQ or SILAC can be combined with phosphopeptide enrichment methods in order to profile the phosphoproteomes. TiO_2 affinity chromatography approach has been used in many phosphoproteomic studies (Boja *et al*., 2009; Mayya *et al*., 2009; Olsen *et al*., 2010). Phosphopeptides/proteins can be enriched from affected tissues (e.g., skin lesions, tumors) obtained from patients or cells exposed to toxicants. Phosphopeptides isolated from controls can be labeled differently than those from test samples. Once labeled, these phosphopeptides can be pooled and be analyzed by LC-MS/MS and quantitated using dedicated software. Identification of differentially phosphorylated proteins and aberrantly regulated signaling pathways will help in understanding the molecular dynamics associated with the exposure to toxicants.

4 GLYCOPROTEOMIC PROFILING OF SERUM HELPS IN IDENTIFICATION OF GLYCOSYLATED BIOMARKERS

Identifying biomarkers that are detectable in the serum is beneficial as blood is an easily accessible compartment for diagnostics (Chaerkady and Pandey, 2008). Membrane proteins and glycoproteins represent most likely candidates that are shed into the circulation; analysis of these sub-proteomes provides an attractive option for biomarker discovery (Kristiansen *et al*., 2008). However, abundant proteins in serum that include albumin and immunoglobulins, often interfere with the characterization of low-abundant proteins by mass spectrometry. Depletion of abundant proteins can be achieved by using conventional spin column strategies or more advanced multiple antigen removal system columns. The glycoproteome component can be enriched in order to detect the less abundant glycosylated proteins using lectin affinity chromatography (Chaerkady *et al*., 2008; Kristiansen *et al*., 2008). A combination of several different lectins (e.g., concanavalin A, wheat germ agglutinin, and jacalin) is generally preferred for enrichment purposes. Owing to different binding specificities, the use of multiple lectins in the enrichment step can capture a more diverse set of glycoproteins. For identification of *N*-linked glycopeptides, the extracted tryptic peptides can be treated with PNGaseF, which removes *N*-linked glycans. A deamidation reaction occurring during cleavage at the glycan-linked asparagines, converts asparagine to aspartic acid, which increases the mass by 1 Da that is very easily identified by mass spectrometry (Chaerkady *et al*., 2008; Kristiansen *et al*., 2008). Over-expressed proteins and/or glycoproteins in the serum of patients which are specifically associated with exposure to environmental toxicants, such as arsenic or lead, can lead to discovery of biomarkers.

5 DEVELOPMENT OF MASS SPECTROMETRY AS A DIAGNOSTIC AND MONITORING TOOL

Advances in proteomic technologies have ushered in the possibility of using mass spectrometers as diagnostic tools in addition to their established role as a discovery platform. Improved precision of nanoflow liquid chromatography combined with the increased sensitivity of mass spectrometers allow a more comprehensive analysis of biological samples for discovery of biomarkers and inclusion of PTMs in biomarker screening. As explained above, iTRAQ- and SILAC-based differential proteomics methods have been widely employed to discover candidate biomarkers in several human diseases (Chaerkady *et al*., 2008; Harsha *et al*., 2008; Harsha, Molina and Pandey, 2008; Grønborg *et al*., 2006). Immunohistochemistry and ELISA are commonly used methods for validation of candidates identified from discovery screens. However, a major limitation of these techniques as validation platforms is the non-availability of antibodies for newly discovered biomarkers. Multiple reaction monitoring (MRM)

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using triple quadrupole mass spectrometers can be a reliable alternative to immunochemical methods for validation purposes (Anderson and Hunter, 2006; Wolf-Yadlin *et al*., 2007). Using triple quadrupole mass spectrometers, specific peptide ions can be selected based on their mass to charge (m/z) ratios, which provides high sensitivity and increased accuracy that is often comparable to antibody-based detection methods. Several peptides can be analyzed simultaneously, which implies that a number of biomarkers can be assessed in a single experiment. The use of proteotypic peptides (experimentally derived peptides which serve as unique identifiers of a given protein or isoform in tandem mass spectrometry experiments) can further increase the sensitivity and specificity of MRM analyses (Gupta *et al*., 2009; Kuster *et al*., 2005; Kuzyk *et al*., 2009; Picotti *et al*., 2010).

6 CONCLUSIONS

Quantitative proteomics using high-resolution mass spectrometry coupled with state-of-the-art stable isotopic-labeling technologies has already proven to be a robust platform for proteomic profiling. Dose–response studies in toxicology can be performed at the proteome level to study responses to diverse toxicants that could provide molecular signatures for diagnosing and monitoring environmental safety hazards or drug toxicities. Apart from being a discovery tool, we anticipate that mass spectrometry will evolve into a reliable and sensitive platform for validation and diagnosis in clinical settings.

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Use of Proteomic and Metabolomic Techniques in Ecotoxicological Research

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1 INTRODUCTION

Over the last decade, the environmental sciences have witnessed an incredible movement towards the utilization of high through-put molecular tools that are capable of detecting simultaneous changes of hundreds and even thousands of molecules and molecular components after exposure of organisms to different environmental stressors. These techniques have received lots of attention because they not only offer the potential to unravel novel mechanisms of physiological and toxic action, but they also are amenable for the discovery of biomarkers of exposure and effects (for some recent reviews see Viant, Rosenblum and Tjeerdema, 2003; Rochfort, 2005; Dowling and Sheehan, 2006;Lin, Viant and Tjeerdema, 2006; Calzolai *et al*., 2007; López, 2007; Nesatyy and Suter, 2007; Nunn and Timperman, 2007; Samuelsson and Larsson, 2008; Tjeerdema, 2008; Viant 2007, 2008). In this chapter we will review the state of knowledge of two of these holistic tools in ecotoxicological research: proteomics and metabolomics. We will follow this review with a presentation of three of our own case studies utilizing proteomic and metabolomic tools for the assessment of effects and mechanisms of toxicity in different animal models exposed to a variety of environmental pollutants. We will end with some final conclusions and research needs.

1.1 Proteomics

Proteomics is defined as the simultaneous analysis and quantification of cellular or extracellular protein abundance. Compared to genomics, proteomics can provide functional mechanistic information since mRNA is a "disposable" message and only a limited amount of it gets translated into proteins. In addition, proteomic techniques can capture changes in the activity of proteins measured as post-translational modifications (see also Mechanistic Investigation of EMD335823s Hepatotoxicity using Multiple Omics Profiling Technologies , Systems Biology: Integrating '-Omics'-Oriented Approaches to Determine Foodborne Microbial Toxins, Application of Quantitative Proteomic Approaches to Toxicology and Application of Proteomics to Study Mechanisms of Toxicity and Dose Response Relationships of Chemical Exposure). One major drawback of using proteomics in ecotoxicological research is that the genome of most organisms used for these studies is largely unknown. This results in the identification of a limited number of proteins. However, *de novo* sequencing is becoming an approachable alternative when working with

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organisms that have poorly annotated gene and protein databases. This is achieved by a variety of mass spectrometry (MS)-based techniques such as high-resolution liquid chromatography -MS (LC-MS/MS) as described in more detail below.

The first proteomic studies conducted in ecotoxicology employed the study of protein expression signatures (PES) (e.g., Bradley *et al*., 2002; Shrader *et al*., 2003). Since these studies do not report peptide sequences, they provide little information regarding mechanisms of toxicity and will not be discussed in this chapter. In addition, studies that have used ProteinChip® array technology and have not reported protein identifications are not included in this review (e.g., Knigge, Monsinjon and Andersen, 2004; Bjørnstad *et al*., 2006; Larsen *et al*., 2006). A summary of proteomic studies conducted in tissues or whole organisms collected after exposure to different contaminants under laboratory controlled conditions is summarized in Table 1. Field studies that have utilized proteomics to evaluate responses of free-ranging organisms are summarized under Section 1.3. It can be seen that most studies separate proteins based on their molecular weight and charge using two-dimensional electrophoresis (2-DE). Later staining of gels results in the visualization of up to several hundred gene products. Proteins can also be separated by LC and only a limited number of ecotoxicology studies have used this approach (Table 1). Compared to 2-DE, multi-dimensional LC is more automated, faster, and can produce very accurate data on protein quantification when coupled with isotopic labeling techniques. In addition, very large, very hydrophobic and/or very basic proteins are difficult to separate using gel techniques (López, 2007). Regardless of the technique used for protein separation, identification is conducted using MS.

From Table 1 some findings of interest include: strong gender-specific responses and lack of correspondence between gene and protein responses. Female zebrafish (*Danio rerio*) exposed to flame retardants responded with changes at the protein level that were different to those observed in males (Kling *et al*., 2008). Similarly, Wei *et al*. (2008) reported marked gender differences in protein profiles in rare minnows (*Gobiocypris rarus*) exposed to perfluorooctanoic acid. In terms of the relationship between transcription of mRNA and protein abundance, studies have reported a general lack of correspondence between the two. For instance, Wei *et al*. (2008) reported discrepant results between mRNA and protein levels in rare minnows exposed to perfluorooctanoic acid. Likewise, we found a similar result in largemouth bass (*Micropterus salmoides*) exposed to a variety of different contaminants as discussed under Section 2.1.

1.2 Metabolomics

Animals are known to produce hundreds of metabolites (low molecular-weight compounds, <1 KDa) that are involved in general metabolic reactions or required for the maintenance, growth, and normal function of an organism. Host and environmental factors influence the type and abundance of these metabolites. For instance, several studies have shown a change in metabolite profiles after exposure to specific environmental stressors, such as disease (Moalemiyan *et al*., 2006), temperature (Rosenblum *et al*., 2005), nutritional quality (Nikiforova *et al*., 2005), and as reviewed in this chapter, toxicants. Thanks to recent technological advances, it is now possible to profile the metabolome of an organism, a method also known as "metabolomics". Metabolomics can be defined as the quantitative systematic analysis study of the metabolome, the total quantitative collection of metabolites present in a cell or organism (Fiehn, 2001) (see also Chapters Systems Biology: Integrating '-Omics'-Oriented Approaches to Determine Foodborne Microbial Toxins and Application of Metabonomic Approach in Target Organ Toxicity). The size of the metabolome is unknown, but it has estimated to range from a minimum of 600 (yeast) to hundreds of thousands (plants) of metabolites (Samuelsson and Larsson, 2008).

Metabolomic techniques can simultaneously measure the concentration of hundreds of metabolites from a single individual, which are later interpreted using pattern recognition algorithms that determine differences on metabolite profiles across individuals. A major advantage of this high-throughput approach is that by examining all metabolites present in a biological system, there is no bias associated with the choice of metabolites to be studied. In addition, metabolomics offers several advantages over other "omic" techniques. First, since metabolites are the end-products of gene expression and protein activity and are crucial to cellular regulatory processes, metabolomics provide a closer link to functional physiological processes

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Table 1. Summary of laboratory controlled studies that have utilized proteomic techniques in ecotoxicological research.

Species	Exposure experiment	Age and gender of organisms	Tissue	Proteomic method	Proteins of interest[a]		Reference
					Up-regulated	Down-regulated	
Invertebrates							
Clam, *Chamalea gallina*	Aroclor 1254 (10–1000 $\mu g\ l^{-1}$); $CuCl_2$ (0.1–5 $mg\ l^{-1}$); Tributyltin (TBT, 0.3–3 $\mu g\ l^{-1}$); Arsenic (As, 0.1–10 $mg\ l^{-1}$) × 7 d	Age and gender not reported	Whole animals	2-DE-MALDI-TOF-MS	Tropomyosin (Aroclor 1254, Cu) Light chain myosin (Aroclor 1254, Cu) Actin (TBT, As)	Actin (Cu)	Rodriguez-Ortega *et al*. (2003)
Blue Mussel, *Mytilus edulis*	Crude oil 0.5 $mg\ kg^{-1}$; mixture of 0.5 +0.1 $mg\ kg^{-1}$ alkylphenols + 0.1 $mg\ kg^{-1}$ PAHs × 21 d	Adults, gender not reported	Gill	2-DE-MALDI-TOF-MS/LC-MS/MS	Heavy metal binding protein	Actin Tropomyosin Fructose-bisphosphate aldolase	Manduzio *et al*. (2005)
Chinese mitten crab, *Eriocheir sinensis*	Cadmium, 50–500 $\mu g\ l^{-1}$ × 3–30 d	Intermolt males	Gill	2-DE-MS/MS	Glutathione-*S*-transferase	Glutathione-*S*-transferase Alpha tubulin Crustacean Ca-binding protein 23	Silvestre *et al*. (2006)
Vertebrates							
Fishes							
Rainbow Trout, *Oncorhynchus mykiss*	Zinc, 3.5 μM × 6 d	Fingerlings, mixed genders	Gill	SELDI	Apolipoprotein A-I Proto oncogene protein c-fos Na^+/H^+ exchanger	Ovarian cystatin Glucose-6-phosphate dehydrogenase Growth regulator	Hogstrand *et al* (2002)
Japanese Flounder, *Paralichthys olivaceus*	Cadmium, 10 $mg\ kg^{-1}$ × 24 h	Juvenile (3 m), gender not reported	Brain	2-DE-MALDI-TOF-MS	Endoglucanase Creatine kinase	Transferrin	Zhu *et al*. (2006)
Zebrafish, *Danio rerio*	Tetrabromo-bisphenol-A, 0.82 $mg\ l^{-1}$ × 14 d	Adults 1:1 sex ratio	Liver	DiGE	Heat shock 70kDa	Betaine homocysteine methlytranferase	De Wit *et al*. (2008)

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					Phosphoglycerate mutase 1			
	Brominated flame retardants, 10-100 nmol g^{-1} feed × 21 d	Adults (5 m), mixed genders	Liver	2-DE-MALDI-TOF-MS	Peroxiredoxin 6 (♂) Alpha-enolase 1 (♀) Betaine-homocysteine methyltransferase (♀)	Transketolase (♀) TPA-transferrin (♀) Ironregulatory protein 1 (♀)	Kling *et al.* (2008)
	Perfluorooctа-ne sulfonate, 0.5 mg l^{-1} × 4–192 h post-fertlization	Embryos	Whole embryos	2-DE-MALDI-TOF-MS	Peroxiredoxin 2 Eukaryotic translation elongation factor 1 gamma Tyrosine recombinase	None	Shi *et al.* (2009)	
	Hexabromocy-clododecane (HBCD, 0.05-187 μM); Tetrabromo-bisphenol-A (TBBPA, 0.05-220 μM); Mix (2-100 μM of each) × 72 h	Liver cells	Liver cells	2-DE-MALDI-TOF-MS/FT-ICR	Transaldolase (Mix) Transketolase (TBBPA) Aldehyde dehydrogenase 2b (HBCD)	Hsp 70 9B (Mix) Eucaryotic elongation factor 1 (HBCD) Laminin receptor 1 (Mix)	Kling and Förlin (2009)	
Largemouth bass, *Micropterus salmoides*	Toxaphene (TOX, 100 μg g^{-1}); $CdCl_2$ (0.00067 μg g^{-1}); Phenanthrene (PHE, 50 μg g^{-1}); PCB 126 (2.5 μg g^{-1}); Atrazine (ATRZ, 3 μg g^{-1}) × 96 h	Adult males	Liver	2-DE-MALDI-TOF-MS	ATP synthase (Cd) Parvalbumin β (PCB) Brain type fatty acid binding protein (PHE)	Receptor protein kinase C (Cd) Na/K ATPase subunit isoform 1b (ATRZ) Betaine-homocysteine methyltransferase (PHE)	Sanchez *et al.* (2009a)	
Japanese medaka, *Oryzias latipes*	Microcystins, 1 μg μl^{-1} g body weight × 5–72 h	Adults, gender not reported	Liver	2-DE-ESI-QTOF	Methyltransferase Transmembrane 4 Apolipoprotein A1 Ferritin	None	Mezhoud *et al.* (2008a)	

(*continued*)

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Table 1. (*Continued*)

Species	Exposure experiment	Age and gender of organisms	Tissue	Proteomic method	Proteins of interest[a]		Reference
					Up-regulated	Down-regulated	
	Microcystins, 1 $\mu g\,ml^{-1} \times$ 30–60 min	Adults, gender not reported	Liver	2-DE-MALDI-TOF/ESI-QTOF	Acidic ribosomal phosphoprotein PO Phenylalanine hydroxylase Enolase	None	Mezhoud *et al.* (2008b)
	Microcystin-LR, 1 $\mu g\ \mu l^{-1} \times 2\,h$	Adults, mixed genders	Liver	2-DE-ESI-QqTOF-MS/MS	Apolipoprotein A1 Transferrin Complement C3-1	Aldehyde dehydrogenase 2 ATP synthase Thiosulfate sulfurtransferase	Malecot *et al.* (2009)
Rare minnow, *Gobiocypris rarus*	Perfluoroocta-noic acid, 3–30 $mg\,l^{-1} \times 28\,d$	Adults (~9 m), mixed genders	Liver	2-DE-MALDI-TOF/TOF-MS	Fatty acid binding protein (♂) Peroxiredoxin (♂) Methionine sulfoxide reductase B (♀)	Glutathione peroxidase 1 (♂) ATP synthase (♂) Regucalcin (♀) Phenylalanine hydroxylase (♀)	Wei *et al.* (2008)
Fathead minnow, *Pimephales promelas*	17β-trenbolone (TB, 0.05–5 $\mu g\,l^{-1}$), flutamide (FL, 50–500 $\mu g\,l^{-1}$),Mix (0.5 TB + 500 FL) × 48 h	Adult females	Liver	2-DE-LC-QqTOF-MS/MS	Vitellogenin (FL) GAPDH (TB) Heat shock 70 (Mix, FL)	Fatty acid binding protein 10 (all) Aldolase B (FL) Superoxide dismutase (TB, FL)	Martyniuk *et al.* (2009)
Amphibians							
African clawed frog, *Xenopus laevis*	Aroclor 1254, 0.1-1.0 $mg\,kg^{-1} \times 72\,h$	Tadpoles (stage 35/36)	Whole tadpoles	2-DE-LC-MS/MS	Enolase Peroxiredoxin Aldehyde dehydrogenase	Myosin heavy chain alpha Actin alpha 1 skeletal muscle	Gillardin *et al.* (2009)

[a] Only the most significant changes in protein expression are presented. Readers should refer to the original articles for a full description of protein changes.

2-DE = Two-dimensional electrophoresis; DiGE = Differential in-Gel electrophoresis; ESI = Electrospray ionization; FT-ICR = Fourier transform ion cyclotron resonance; LC = Liquid cromatography; MALDI = Matrix-assisted laser desorption; QqTOF = Quadrupole time-of-flight; SELDI = Surface enhanced laser desoprtion/ionization; TOF/MS = Time-of-flight mass spectrometry.

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compared with transcriptomics and proteomics. This is because changes at the gene and protein level do not always end in phenotypic effects. Another great advantage of metabolomics is that it can be applied to widely diverse species with relatively little time required for re-optimizing protocols for a new species (i.e., it is species-independent). This is because metabolites in primary metabolism are highly conserved, compared to gene sequences which are much more diverse.

A summary of ecotoxicological metabolomic studies published to date are presented in Table 2. This table only summarizes laboratory-controlled experiments. Field studies that have utilized metabolomics to evaluate responses of free-ranging organisms are summarized under Section 1.3. As can be seen from Table 2, the majority of the metabolomic studies have utilized ^{1}H nuclear magnetic resonance (^{1}H NMR) analyses. ^{1}H NMR spectroscopy is considered a broad-based method for detecting metabolites because it detects proton-containing molecules and the majority of known metabolites contain protons (Ong *et al*., 2009). Other advantages of this technique are that results are highly reproducible, it requires minimal sample amount and preparation, and it can be run using samples collected non-destructively (e.g., urine). Despite constant improvement, major limitations of NMR-based metabolomic techniques include its relatively low sensitivity that results in an examination of a limited number of metabolites (Keun, Beckoner and Griffin, 2002; Wang, Bollard and Keun, 2003). Since biological samples are quite complex, the likelihood of characterizing a comprehensive metabolome in a biological system may prove difficult. An alternative approach is the use of LC or gas chromatography (GC) for separation of metabolites and MS for their quantification and identification. Indeed, two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GCxGC/TOF-MS) has been successfully used to characterize metabolite profiles in complex biological mixtures (Welthagen *et al*., 2005; Mohler *et al*., 2006; Ralston-Hooper *et al*., 2008). Advantages of GCxGC/TOF-MS over ^{1}H NMR include the ability of the former to deconvolute chromatographic peaks for identification as well as accurate quantification (Marriott and Shellie, 2002). Disadvantages of GC/MS are that it only detects volatile metabolites and samples need to be prepared for derivatization. Despite its sensitivity, LC/GC-MS based metabolomics has not been widely utilized to study the impacts of environmental contaminants or stressors on organisms. Since the number of metabolites produced by an organism is quite extensive, one instrument or technique will not be able to detect and identify all metabolites. Thus an approach that capitalizes on the advantages of the different metabolomic tools available will produce a more comprehensive view of the metabolome of cells and organisms.

Based on the studies presented in Table 2, a few interesting metabolomic findings are worth mentioning here. (i) Several studies have demonstrated that metabolomics can be more sensitive than traditional endpoints. For example, Viant *et al*. (2005) showed that when Japanese medaka (*Oryzias latipes*) embryos were exposed to trichloroethylene, NMR metabolomics was close to 20 times more sensitive compared to traditional endpoints (developmental abnormalities, hatching success and mortality). (ii) Very small samples can be collected non-destructively (urine, 10 – 30 μl) for successful metabolomic analysis (Ekman *et al*., 2007). (iii) Metabolomics data have resulted in good correlation with traditional toxicity endpoints. Metabolite changes in Japanese medaka embryos exposed to the herbicide dinoseb correlated to changes in growth, survival, and development (Viant *et al*., 2006). Using earthworms exposed to different types of pollutants, Guo *et al*. (2009) showed that metabolic responses were related to changes in reproduction measured as cocoon production rates. (iv) Visualization of non-overlapping clusters of metabolite profiles along a Principal Component Analysis (PCA) grid has been used to differentiate among different modes of toxic action. For instance, Japanese medaka embryos exposed to dinoseb responded with a dose-response increase in metabolite changes (Viant *et al*., 2005; Viant, Pincetich and Eerderna, 2006a). Furthermore, embryos exposed to the higher dose also showed a shift in metabolite profiles, opposite to control and low dose groups. The authors speculate that this separation is related to differences in modes of toxic action at different doses, and that a higher dose not only perturbs the same or similar biochemical pathways to a greater degree, but rather induces an entirely different metabolic condition (Viant, Pincetich and Eerderna, 2006; Viant *et al*., 2006).

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Table 2. Summary of laboratory controlled studies that have utilized metabolomic techniques in ecotoxicological research.

Species	Exposure experiment	Age and gender of organisms	Tissue	Metabolomic method (extraction)	Metabolites interest[a]		Reference
					Up-regulated	Down-regulated	
Invertebrates							
Abalone, *Haliotis rufescens*	Pentachlorophenol, 1.2 mg l^{-1} × 4–18 h	Three years old, gender not reported	Foot muscle	^{31}P NMR	None	Phosphoarginine Adenosine triphosphate	Tjeerdema et al. (1991, 1993)
Earthworm, *Eisenia veneta*	3-trifluoromethylaniline, 0.0001–1 mg cm^{-2} × 72 h	Not reported, hermaphrodites	Whole worms	^{1}H NMR	Alanine Glycine Glucose	2-hexyl-5-ethyl-3-furansulfonate	Warne *et al.* (2000)
	2-fluoro-4-methylaniline (2F4MA), 3,5-difluoroaniline(35DFA) 1–100 μg cm^{-2}; 4-fluoroaniline (4FA, 25–250 μg $cm^{-2)}$ × 72 h	Adults, hermaphrodites	Whole worms	^{1}H NMR	Inosine monophosphate (2F4MA, 35DFA)	None	Bundy *et al.* (2002)
Earthworm, *Lumbricus rubellus*	Copper, 40–60 mg kg^{-1} × 110 d	Juveniles and adults, hermaphrodites	Whole worms	^{1}H NMR	Histidine	None	Gibb *et al.* (1997)
	Copper, 10–480 mg kg^{-1} × 70 d	Adults, hermaphrodites	Whole worms	^{1}H NMR	Betaine Glycine Phospho-ethanolamine	Glucose Malate Succinate	Bundy *et al.* (2008)
	Pyrene, 10–640 mg kg^{-1} × 42 d	Adults, hermaphrodites	Whole worms	^{1}H NMR/GC-MS (2M:1C)	Alanine Leucine Tyrosine	Lactate Tetra-, hexa-, octodecanoic acids	Jones *et al.* (2008)
	$CdCl_2$,(8–750 mg kg^{-1}; Atrazine (ATRZ, 5–100 mg kg^{-1}; Fluoranthene (FA, 14–1400 mg kg^{-1}) × 28 d	Adults, hermaphrodites	Whole worms	^{1}H NMR	β -hydroxybutyrate (ATRZ) Nicotinic acid (Cd)	Succinate (Cd)	Guo *et al.* (2009)
Amphipods *Diporeia* spp.	Atrazine, 30 μg l^{-1} × 28 d	Mixed ages and genders	Whole organisms	GCxGC/TOF-MS (2M:1C)	Heptacosane	L-Aspartic acid	Ralston-Hooper *et al.* (2008)

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					9,12-Octadecadienoic acid			
					Urea			
Blue mussel, *Mytilus edulis*	Lindane (LD, 5 $\mu g\,l^{-1}$);				Alanine (LD)	Succinate (LD)		
	Atrazine (ATRZ, 5 $\mu g\,l^{-1}$) × 30 d	Adults, non-reproductive mixed genders	Foot muscle	^{1}H NM	Leucine (ATRZ) Aspartate (LD)	Taurine (LD)		Tuffnail *et al.* (2009)
Vertebrates								
Fishes								
Rainbow trout, *Oncorhynchus mykiss*	17α -ethinylestradiol, 2–20 $ng\,l^{-1}$ × 14 d	Juveniles, mixed genders	Plasma	^{1}H NMR (1M:3C)	Polyunsaturated fatty acid Phosphatidylethanolamine Phosphatidylcholine	Alanine Cholesterol		Samuelsson *et al.* (2006)
Chinook salmon, *Oncorhyncus tshawytscha*	Dinoseb (DB, 50–750 $\mu g\,l^{-1}$); diazinon (DZ, 10-100 $mg\,l^{-1}$); esfenvalerate (ESF, 1–100 $\mu g\,l^{-1}$) × 96 h	Eyed embryos	Whole embryos	^{1}H NMR (6% Perchloric acid)	Lactate (DZ) Glucose (DZ) Creatine (DB)	Adenosine triphosphate (DB) Phosphocreatine (DB)		Viant *et al* (2006a)
Japanese medaka, *Oryzias latipes*	Trichloroethylene, 0.9–8.8 $mg\,l^{-1}$ × 1–8 d	Embryos (10–12 h post-fertilization to 8 d post-fertilization)	Whole embryos	^{1}H NMR (6% Perchloric acid)	Histidine Isoleucine Glucose	Tyrosine Glutamate Glycogen		Viant *et al.* (2005)
	Dinoseb, 50–500 $\mu g\,l^{-1}$ × 110 h	Embryos (stage 19)	Whole embryos	^{1}H NMR (6% Perchloric acid)	Lactate	Adenosine triphosphate Phosphocreatine Tyrosine		Viant *et al.* (2006b)

(*continued*)

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Table 2. (*Continued*)

Species	Exposure experiment	Age and gender of organisms	Tissue	Metabolomic method (extraction)	Metabolites interest[a] Up-regulated	Down-regulated	Reference
Fathead minnow, *Pimephales promelas*	Vinclozolin, 60-450 $\mu g\,l^{-1} \times 21$ d	Adult males	Urine	^{1}H NMR	Lactate Acetate Creatinine	Alanine Monounsaturated fatty acids Hippurate	Ekman *et al.* (2007)
	17α -ethynylestradiol, 10-100 $ng\,l^{-1} \times$ 1-8 d and 8 d post-exposure	Adults, mixed genders	Liver, plasma	^{1}H NMR	Phosphatydilcoline (♂ mostly, but also ♀, liver) Cholesterol (♀, liver) Glutamate (♂, liver)	Di-Triglycerides (♀ mostly, but also ♂) Glycogen (♂, liver) Cholesterol (♂ mostly, but also ♀, plasma, liver)	Ekman *et al.* (2008, 2009)
Stickleback, *Gasterosteus aculeatus*	Dibenzanthracene, 0.01-50 $\mu g\,l^{-1} \times 4$ d	Males, age not reported	Liver	^{1}H NMR (2M:2C:1.8W)	Malonate Glutamate Taurine	None	Williams *et al.* (2009)
Mammals							
Bank vole, *Clethrionomys glareolus*	Arsenic, 28 mg kg^{-1} feed, × 14 d	Adult males	Kidney	HRMAS ^{1}H NMR	None	Lipid triglycerides	Griffin *et al.* (2001)

[a] Only the most significant changes in metabolite expression are presented. Readers should refer to the original articles for a full description of metabolite changes.

^{1}H NMR = ^{1}H Nuclear magnetic resonance spectrometry; ^{31}P NMR = ^{31}P Nuclear magnetic resonance spectrometry; HRMS = High resolution magic angle spinning; GC-MS = Gas chromatography – mass spectrometry.Extractives: M = Methanol; C = Chloroform; W = Water.

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1.3 Proteomic and Metabolomic Field Studies

Proteomics and metabolomics have also been applied for the analysis of animals naturally exposed to environmental pollutants. These field studies are summarized in Table 3. Although the number of studies is quite limited, a main outcome of these studies has been the ability of protein and metabolite profiles to predict site of origin. For instance, NMR metabolomic profiles in earthworms sampled from sites contaminated with different types and levels of metals were good predictors of site (Bundy *et al.*, 2007). More importantly, these authors determined that most of the metabolite differences across sites were due to differences in the concentration of zinc. The same can be said about protein profiles being predictive of the field site of origin (Knigge, Monsinjon and Andersen, 2004). In this latter study, the authors combined sets of protein markers in a tree-building algorithm and were able to correctly classify samples from these sites with an accuracy of 90%. These are examples of how these techniques can prove to have significant impact as diagnostic tools under field conditions.

2 CASE STUDIES

In this section, we will present some examples of proteomic and metabolomic application in ecotoxicological research. We will describe the methods in some detail, but in some cases we have directed the reader to published articles that describe the methods in more detail. The studies presented include: (i) Analysis of proteomic responses in fish exposed to different types of contaminants using GeneGo™; (ii) Comparison of proteomic and metabolomic responses in aquatic invertebrates exposed to herbicides; and (iii) Use of metabolomics to characterize egg quality in fish-eating birds exposed to persistent organic pollutants.

2.1 Analysis of Proteomic Responses in Largemouth Bass Exposed to Pollutants with Differing Modes of Action using GeneGo™

In many cases, genomic and proteomic experiments result in a large amount of data indicating the over-expression or suppression of genes and proteins. Interpretation of this high-throughput data can be very difficult and time consuming if analyzed manually. Many efforts have been put forward to develop data mining software to provide solutions to this problem. GeneGo Inc., a privately owned company founded in 2000, developed MetaBase, the most comprehensive manually curated database available in systems biology. MetaBase compiles all human known information related to protein interactions, networks, signaling pathways, toxicogenomics, and metabolomics. MetaCore is the platform that utilizes MetaBase database to built networks, canonical pathways, and predict toxicity and diseases. MetaCore utilizes sophisticated statistical algorithms to predict which pathways, cellular processes or networks are most likely being affected by the experimental conditions. In addition, thresholds and *p* values can be adjusted to the experiments' need. While GeneGo is based on human proteins/genes for the analyses, experimental data from other organisms can also be used via orthologs. Organisms supported via orthologs include mouse, rat, bovine, chimpanzee, dog, zebrafish, chicken, fruit fly, mosquito, *Arabidopsis*, rice, blast of rice, fission yeast, and baker's yeast. It is GeneGo's goal to expand this list within a few years. Using this type of software for the analysis of genes and proteins derived from fish (in our case largemouth bass) has of course some limitations. For instance, vitellogenin is an egg-yolk precursor protein present in all egg-laying vertebrates including fish, but does not have a human homologue. Nevertheless, we believe that use of this software is still very helpful for elucidating mechanisms of toxicity in non-model species.

2.1.1 Materials and Methods

Exposure Experiments

Seven groups of 12 adult (1 year old) largemouth bass were exposed to one of five chemicals (cadmium chloride [$CdCl_2$], atrazine, phenanthrene, toxaphene, and polychlorinated biphenyl, PCB 126) or one of two control vehicles (water or fish oil) via intraperitoneal injection (1 ml injectate volume) using a 3 ml syringe with a 1.5 inch, 26 gauge needle on the ventral side, anterior of the anal fin. Cadmium chloride and atrazine were dissolved in water, while phenanthrene, toxaphene, and PCB 126

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Table 3. Summary of field studies that have utilized proteomics or metabolomic techniques for ecotoxicological research.

Species	Contamination of field sites/location	Age and gender of organisms	Tissue	Method	Major findings	Reference
Proteomics						
Rainbow trout, *Oncorhynchus mykiss*	Sewage discharge/Grabo, Sweeden/	Juveniles, mixed genders	Liver	2-DE-FT-ICR	Total of four proteins changed in expression in relation to animals collected from the reference site.	Albertsson *et al.* (2007)
Blue mussels, *Mytilus edulis*	Organic chlorines, polyaromatic hydrocarbons, heavy metals, dioxins, furans, polychlorinated biphenyls/Gothenburg, Sweeden	Age and genders not reported (~5 cm in size)	Digestive gland	2-DE/LC ESI-MS/MS	Contaminated sites could be distinguished from the reference based on the expression of 13 proteins involved in amino acid metabolism, detoxification, β-oxidation, and protein folding and degradation.	Amelina *et al.* (2007)
Smallmouth bass, *Micropterus dolomieu*	Polychlorinated biphenyls, arsenic, carbon disulfide, mercury, nitrogen, phosphorous, sewage discharge/Shenadoah River, Virginia, USA	Adults (~2.5 y), mixed genders and intersex males	Anterior kidney	2-DE-MALDI-TOF-MS	Protein expression differed among the three sites studied with proteins involved in immune and stress responses being the most important.	Ripley *et al.* (2008)
Goldfish, *Carassius auratus*	Sewage discharge and high temperatures/Gaobeidian Lake, Beijing, China	Adult males	Liver	2-DE-MALDI-TOF-MS	Several proteins related to energy metabolism and oxidative stress were up-regulated in fish from the polluted site.	Wang *et al.* (2008)
Metabolomics						
Earthworms, *Lumbricus rubellus, L. terrestris, Eisenia andrei*	Cadmium, zinc, lead, smelting works UK	Juveniles and adults, hermaphrodites	Whole worms	^{1}H NMR	Earthworms from the most contaminated site had an increased concentration in maltose.	Bundy *et al.* (2004)
Earthworms, *L. rubellus*	Metal-contaminated soils UK	Adults, hermaphrodites	Whole worms	^{1}H NMR	Individual sites could be resolved on the basis of NMR spectral profiles. Zinc was identified as the major metal causing metabolic changes in earthworms.	Bundy *et al.* (2007)

2-DE = Two-dimensional electrophoresis; ESI = Electrospray ionization; FT-ICR = Fourier transform ion cyclotron resonance; LC = Liquid cromatography; MALDI = Matrix-assisted laser desorption; TOF = Time-of-Flight; MS = Mass spectrometry; ^{1}H NMR = ^{1}H Nuclear magnetic resonance spectrometry.

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were dissolved in fish oil (Zeigler Brothers, Harleysville, PA, USA). Target body burdens for cadmium chloride, atrazine, phenanthrene, toxaphene, and PCB 126 were 0.00067, 3.0, 50, 100, and 2.5 $\mu g\ g^{-1}$, respectively. These target body burdens were intended to be sufficiently high enough to elicit sublethal physiological responses but not overt toxicity based on a literature review (Eisler and Belisle, 1996; Jarvinen and Ankley, 1999; Shailaja and D'Silva, 2003). Immediately after injection, fish were transferred to aerated, 75.7 l flow-though glass tanks (4 fish per tank) held at 25 °C with a 16:8 hr (light:dark) photoperiod for 96 h. Fish were not fed during this time. After the exposure period, fish were euthanized via emersion in a lethal concentration (500 mg l^{-1}) of MS-222 (tricaine methanesulfonate, Finquel®, Redmond, WA, USA). Livers were removed and immediately frozen in liquid nitrogen. To validate the concentration of the specified toxicants in exposed fish, two whole-body fish samples per treatment and control group were saved and submitted for chemical analyses as described in Sanchez *et al.* (2009).

Proteomic Analyses

Liver tissue from three individuals per treatment and control were prepared for protein separation. Briefly, 0.05 g of liver tissue from each individual was immersed in lysis buffer (16 M urea, 4 M thiourea, 2% CHAPS, 0.8% ampholyte, 65 mM DTT) and mechanically homogenized with a 7 mm × 95 mm saw tooth stainless steel generator probe (Omni International, Marietta, GA, USA). Samples were then placed in a 37 °C water bath for 15 min, sonicated with 10, 1 sec bursts and returned to the water bath. This process was repeated 4 times. Samples were centrifuged at 1600 × g for 10 min to remove any debris. The supernatants were then collected and moved to a clean 1.5 ml Eppendorf tube (Eppendorf AG, Hamburg, Germany). Proteins were precipitated in a 1:1 (v/v) solution of 25% trichloroacetic acid at 4 °C for 4 hr. Precipitated samples were centrifuged and the supernatant removed leaving the protein pellets intact. Protein pellets were washed twice using 200 µl of 100% cold acetone and then centrifuged for 5 min at 16300 × g. The remaining acetone was evaporated using a Sevant SPD 131DDA SpeedVac Concentrator (Thermo Electron Corporation Milford, MA, USA). Dried pellets were then diluted in 400 µl of rehydration buffer (8M urea, 2M thiourea, 4% CHAPS, 65mM DTT, 0.8% ampholytes). A Bradford protein assay (1976) to determine protein concentration and a test of conductivity with a TwinCond meter (Spectrum Technologies, Inc., Plainfield, IL) were conducted.

Nonlinear IPG strips (18 cm, pI 3-10, BioRad Laboratories, Inc., Hercules, CA, USA) were rehydrated with 300 g total protein in 350 µl rehydration buffer (8 M urea, 2 M thiourea, 2% CHAPS, 0.8% ampholyte, 65 mM DTT) overnight and proteins separated in 1st dimension for 24.5 hr using an Investigator Running System (Genomic Solutions, Inc., Ann Arbor, MI) at 20 °C (100,000 Vh with a maximum current of 80 uA per strip). After focusing, strips were equilibrated for 30 min in reducing buffer (0.375 M Tris-HCl solution pH8.8, 6 M Urea, 20% glycerol, 2% SDS containing 2% DTT) followed by re-equilibration for an additional 30 min in a alkylation buffer (0.375 M Tris-HCl solution pH 8.8, 6M Urea, 20% glycerol, 2% SDS, and 2.5% iodoacetamide). Strips were then loaded onto 12% SDS-PAGE and proteins separated in SDS running buffer (25 mM tris, 190 mM glycine, 0.1% SDS) for 16 h or until the tracking dye neared the bottom of the gel (225 mV at constant current). Gels were removed, fixed in acidic solution (50% methanol, 7% acetic acid), stained with Coomassie Brilliant Blue G-250 (Amresco Inc., Solon, OH) for 48 hr, and destained with washing solution (20% methanol, 5% acetic acid) until the gel background was clear (approximately 48 hr). Gels were then scanned into a BioRad GS-800 Densiometer (BioRad) and images analyzed with PDQuest software (BioRad). Matchsets were created for each treatment and their appropriate control ($CdCl_2$ and atrazine treatments vs. H_2O control; phenanthrene, PCB 126 and toxaphene vs. oil control). Within each matchset, gel images were aligned, the optical densities of spots averaged across treatments and compared to controls. Gel images were normalized to the total density of the gel image to remove variance based on saturated spots or differential staining. Only gels displaying acceptable protein separation (i.e., minimum 150 spots matched to master gel) were included in the subsequent statistical analysis (three gels were included in the $CdCl_2$, atrazine, phenanthrene, and H_2O control groups and two gels were included in the toxaphene, PCB 126 and oil control groups). Spots identified in treatment groups as differing (t-test, $\alpha = 0.05$) from

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controls were manually excised, washed with 50% acetonitrile 100 mM ammonium bicarbonate (ABC) buffer, dried, and swelled in 50 mM ABC buffer. Proteins within each gel spot were trypsin-digested (15 ng l^{-1} trypsin) overnight at 37 °C. Peptides were extracted from the gel spots with 5% trifluoroacetic acid/60% acetonitrile and dried in a SpeedVac. Peptides were then resolubilized in 0.01% trifluoroacetic acid, cleaned with C18 ZipTips (Millipore, Bellerica, MA, USA), plated onto a MALDI plate with an α -Cyano-4-hydroxycinnamic acid matrix, and analyzed in a 4800 Plus MALDI TOF/TOF™ Analyzer mass spectrometer (Applied Biosystems, Framingham, MA, USA). MS/MS spectra were searched against ray-finned fishes NCBI databases using GPS Explorer software (Applied Biosystems) utilizing MASCOT (Matrix Science Ltd., Boston, MA, USA) to identify excised proteins. Total ion score confidence intervals (C.I. %) above 95% were considered as significant database matches.

Data Analysis and Upload using GeneGo™

Differentially expressed proteins obtained after each exposure were analyzed using GeneGo. Using the GeneGo Data Manager, experimental data (which included fold change in relation to controls) were uploaded in the format of EntrezGene (Locus Link). Human was selected as the organism to allow GeneGo to find homologue genes. From this point on, all analyses were done with respect to the human homologues of the corresponding genes. Datasets from each exposure experiment were compared using the "compare experiment workflow" tool. Threshold was left as the default value of 0.005. The threshold value corresponds to the minimal positive expression or the maximum negative expression for the protein list. Since the proteins included in the data file had already been selected as significant based on their *p*-values, no *p*-values were included in the data file for GeneGo analysis.

For enrichment analyses, we sorted the gene ontology database (i.e., networks, maps, and cellular processes) by statistical significance, with *p*-values corresponding to the degree of intersections between the experimental genes and the genes from the networks in the database. Taking into account sampling without replacement, the probability of intersections between the experimental data and a network/pathway being random follows a hypergeometric distribution:

$$P(r, n, R, N) = \frac{C_R^r \cdot C_{N-R}^{n-r}}{C_N^n} = \frac{C_n^r \cdot C_{N-n}^{R-r}}{C_N^R} = \frac{R! \cdot (N-R)!}{N!} \cdot \frac{n! \cdot (N-n)!}{r! \cdot (R-r)!} \cdot \frac{1}{(n-r)!(N-R-n+r)!} \quad (1)$$

In equation (1), N corresponds to all nodes in the MetaCore database of interactions whereas R corresponds to the subset of nodes coming from the data set. The n represents the amount of nodes in a particular network module (i.e., nodes related to a cellular process), while r is the number of nodes from the data set that are present among the n nodes.

Finally, we also utilized the "toxicity analysis workflow" tool as another approach for examining mechanisms of toxicity for the chemicals under study. The toxicity analysis uses a licensed database on MetaCore that contains organ-specific toxicogenomics and drug response information. The ontologies were sorted by statistical significance.

2.1.2 Results and Discussion

A detailed description of proteome changes in largemouth bass exposed to different contaminants is presented in Sanchez *et al*. (2009). Overall, changes were observed in the expression of proteins associated with cellular ion homeostasis (toxaphene), oxidative stress (phenanthrene, PCB 126), and energy production including glycolysis ($CdCl_2$, atrazine) and ATP synthesis (atrazine). When these data were analyzed using GeneGo, 11, 16, 5, 15, and 4 significantly altered proteins were considered for pathway analysis for the atrazine, $CdCl_2$, PCB 126, phenanthrene, and toxaphene treatments, respectively. A summary of unique (present only in each exposure) and overlapping proteins is presented in Figure 1. As can be seen from this figure, expression of several proteins was uniquely associated with a particular contaminant. Phenanthrene and $CdCl_2$ stood out as the chemicals that induced the largest number of unique proteins (total of 8 proteins each), followed by atrazine (6), toxaphene (2) and PCB 126 (1) (see Figure 1 and Appendix 1 for a name list of all these proteins). Some proteins were also shared between treatments. For instance, expression of FABP7, HSPA1A, and HSPAIB was

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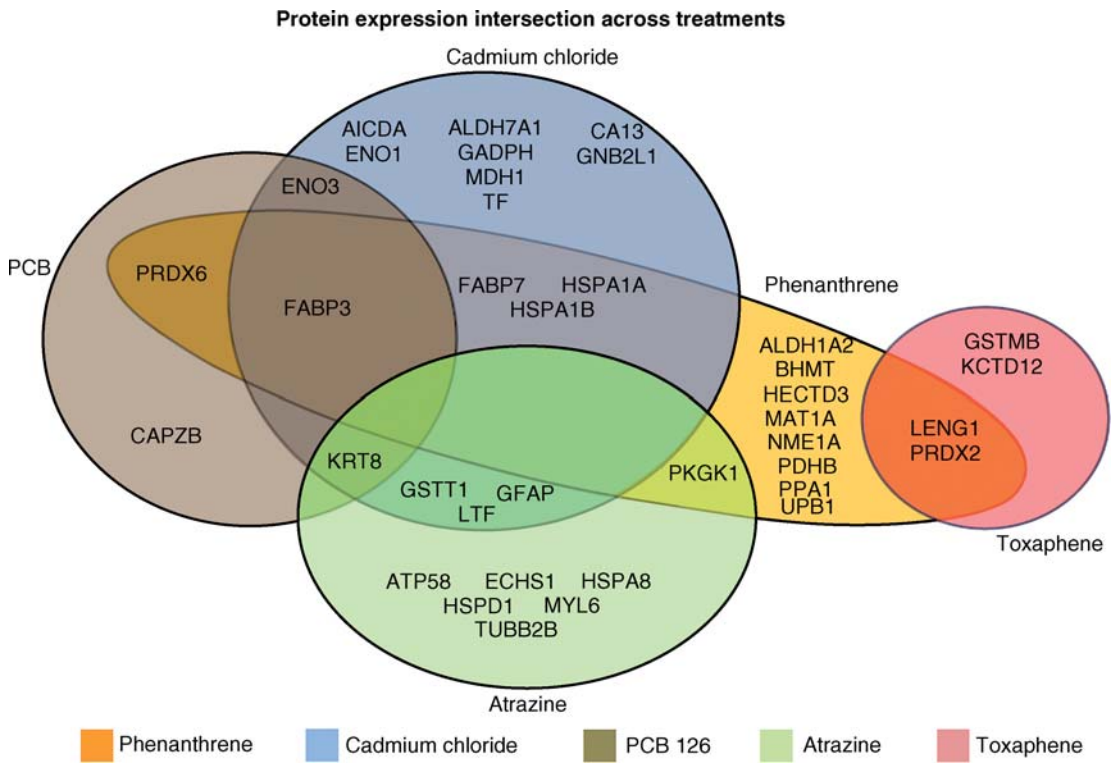

Figure 1. Comparison of protein expression data from livers of largemouth bass exposed to phenanthrene (50 μg^{-1}), cadmium chloride (0.00067 μg^{-1}), PCB 126 (2.5 μg^{-1}), atrazine (3 μg^{-1}), and toxaphene (100 μg^{-1}) after a 96 h exposure. Protein expression data were analyzed using GeneGo. Appendix 1 lists the names of the genes shown on this figure.

affected by both $CdCl_2$ and phenanthrene. Similarly, expression of KRT8 was affected by atrazine, PCB 126, and $CdCl_2$. Several other instances of overlapping expression across treatments can be seen from Figure 1.

Pathway analysis of protein data sets indicated that phenanthrene, $CdCl_2$, and PCB 126 significantly affected processes associated with liver toxicity (Figure 2). Proteins designated as similar among these three treatments were found to be involved in liver toxicity end point processes such as ischemia-induced cellular changes, hypoxia, apoptosis via mitochondrial membrane dysfunction, inflammation, and steatosis (Figure 2). Unique proteins from individual treatments were also found to affect liver toxicity endpoint processes such as hypoxia ($CdCl_2$), peroxisomal proliferation (phenanthrene), cell cycle progression of mitosis (PCB 126), and oxidative stress (phenanthrene).

We also had the opportunity to input gene expression data onto GeneGo from these same liver samples that were produced using largemouth bass microarrays developed by EcoArray® (see Sanchez *et al.* (2009) for a detailed description of the genomic analyses conducted). We wanted to see what, if any, overlap existed between changes in the gene and protein expression in these tissue samples. Within each treatment, protein data sets exhibited lower *p*-values suggesting higher effects at the protein level compared to the gene level after 96 hr of exposure to the abovementioned chemicals. In addition, we observed little overlap of significantly affected pathways within chemical treatments determined from gene and protein data sets. Indeed, phenanthrene was the only treatment that showed an overlap of significant pathways when gene and protein data sets were compared. Common pathways included thymidine triphosphate (TTP) metabolism, deoxyguanosine triphosphate (dGTP) metabolism, and deoxycytidine triphosphate (dCTP)/deoxyuridine triphosphate (dUTP) metabolism (Figure 3).

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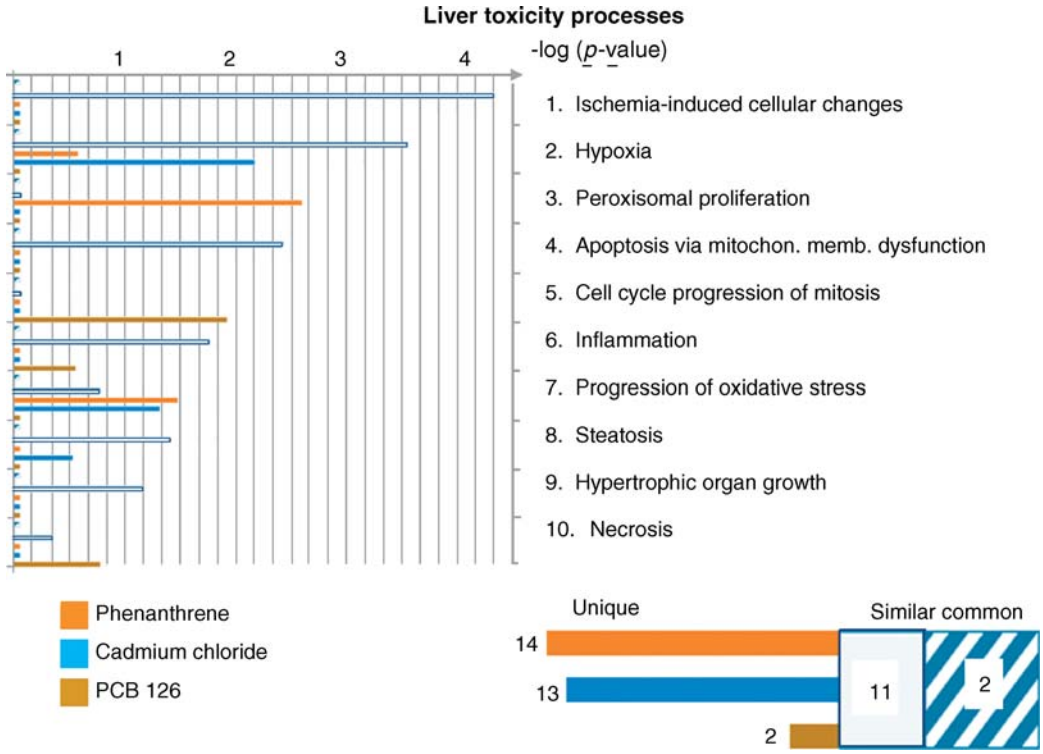

Figure 2. Liver toxicity processes induced by phenanthrene (50 μg^{-1}), cadmium chloride (0.00067 μg^{-1}), and PCB (2.5 μg^{-1}) in largemouth bass after a 96 h exposure. Protein expression data was analyzed using GeneGo.

While the lack of intersection between gene and protein responses may appear anomalous, it has been widely documented that mRNA levels do not typically reflect cognate protein levels (Anderson and Seilhamer, 1997; Gygi *et al*., 1999). Based on the typical absence of an mRNA/cognate protein correlation, Steiner and Anderson (2000) suggest that the two approaches (i.e., gene and protein profiling) be viewed as complementary and used in parallel to determine meaningful physiological effects. For example, the authors highlight the usefulness of genomic profiling when attempting to determine the expression changes of low abundance transcripts, which are typically difficult to monitor at the protein level. Further, they highlight the advantages of proteomic approaches that enable a researcher to observe and evaluate post-translational modifications that are not detectable at the mRNA level of expression. Taken together, a more complete snapshot of physiological processes and/or responses to chemical stressors is possible.

As such, it is interesting that we observed an overlap of pathways derived from gene and protein data sets for the phenanthrene treatment. In particular, we determined that pyrimidine deoxyribonucleotide (dCTP, dUTP, TTP) metabolism was affected. Polycyclic aromatic hydrocarbons (PAHs) have been shown to alter pyrimidine incorporation (Hellman, d'Argy and Ullberg, 1984) and benzo(a)pyrene, in particular, has been shown to affect pyrimidine metabolism in mice (Wu *et al*., 2009). PAHs have also been implicated in causing G to T nucleotide transversions via adduction in mammalian (Mass *et al*., 1996) and fish tissues (Amanuma *et al*., 2002). Imbalances in dNTP concentrations in cells are potentially mutagenic or lethal (Mathews, 2006). This analysis provides novel evidence that pyrimidine metabolism in fish hepatic tissues is affected by phenanthrene exposure and that this effect is determinable by pathway analyses of genomic and proteomic data.

Groupings of chemical treatments based on similarly affected proteins indicated that phenanthrene was the most toxicologically nonspecific chemical among those evaluated while toxaphene was shown to induce the most disparate toxicological

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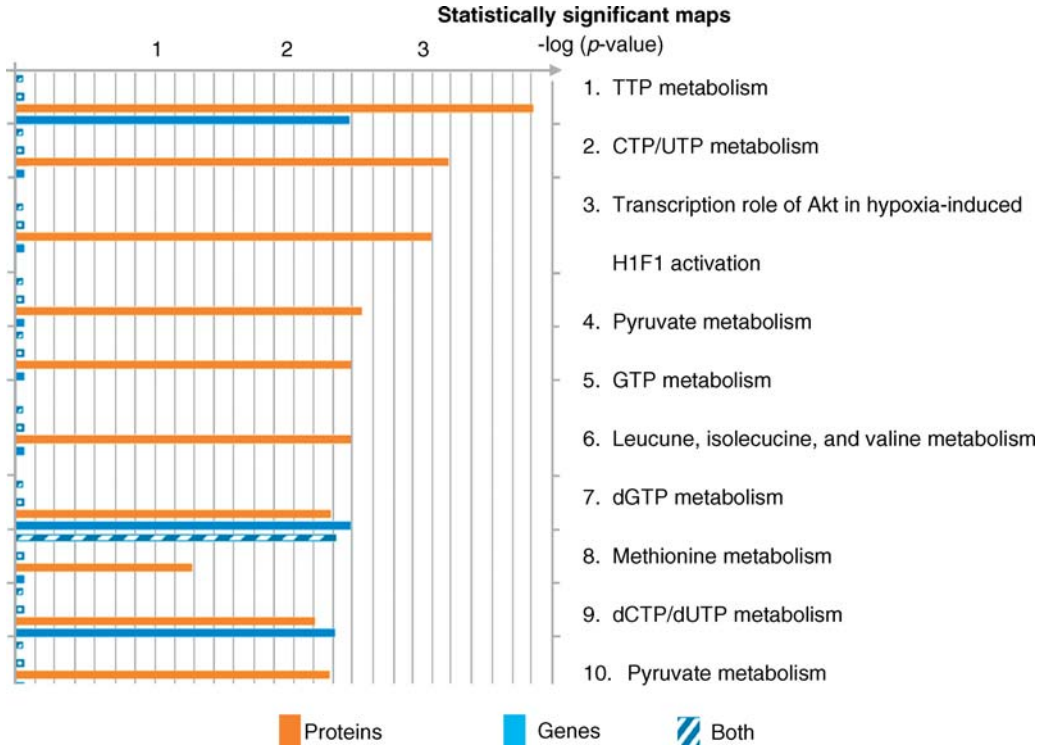

Figure 3. Metabolic pathways affected by largemouth bass exposed to phenanthrene (50 μg^{-1}) for 96 h. Livers were collected and both genomic and proteomic analyses conducted (see text for more details). Phenanthrene was the only chemical out of the five tested that showed an overlap of significant pathways between gene and protein data. Common pathways included thymidine triphosphate (TTP) metabolism, deoxyguanosine triphosphate (dGTP) metabolism, and deoxycytidine triphosphate (dCTP)/deoxyuridine triphosphate (dUTP) metabolism.

response based on this analysis. Toxaphene is an alicyclic organochlorine insecticide whose mode of toxic action involves binding at the picrotoxinin site in the γ-aminobutyric acid (GABA) complex, which prevents nerve Cl^- influx (Lawrence and Casida, 1983; Bloomquist and Soderlund, 1985; Coats, 1990). In a study of channel catfish (*Ictalurus punctatus*), Desaiah and Koch (1977) documented a reduction in the function of Mg^{2+}-ATPase and Pałecz, Komuński and Gabryelak (2005) noted a reduction in Na^+K^+-ATPase function in freshwater mussels (*Unio tumidus*). The specificity with which toxaphene tends to target ion channels and ion fluxes may be reflected in its uniqueness among tested chemicals analyzed herein.

In summary, we have shown that bioinformatics software developed primarily for mammalian animal models can be utilized for the analysis of gene and protein data collected from non-mammalian organisms. Although we were unable to find human homologues for all the genes and proteins that were significantly affected in largemouth bass exposed to different chemicals (overall match of 80%, range of 50–100%), the results obtained make toxicological as well as biological sense (e.g., evidence of liver toxicity and of pyrimidine toxicity after phenanthrene exposure and general lack of overlap between gene and protein responses, respectively). Thus, this approach should be helpful for elucidation of mechanisms of toxicity utilizing data derived from non-mammalian vertebrates.

2.2 Proteomic and Metabolomic Responses in *Hyallela azteca* Exposed to Atrazine and its Metabolite DEA

Atrazine remains one of the most controversial herbicides on the market today. It has elicited endocrine disrupting effects in vertebrate (Diana *et al*., 2000; Wiegand *et al*., 2001; Hayes *et al*.,

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2002; Stoker *et al*., 2002) as well as invertebrates species (Schober and Lamber, 1977; Dodson *et al*., 1999). However, the mechanism of action (MOA) of atrazine remains unclear especially in invertebrate species. Incorporating a global approach, which utilizes genomics, proteomics, and metabolomics would result in valuable information on the MOA of atrazine. In this case study, we examined the proteomic and metabolomic profiles of chronically exposed *Hyalella azteca* to atrazine and one of its metabolites, deethyl-atrazine (DEA) in order to better understand how these contaminants impact freshwater invertebrates.

2.2.1 Materials and Methods

Exposure Experiments

H. azteca organisms used during the 42 d exposures were purchased from Aquatic BioSystems Inc. (Fort Collins, CO). *H. azteca* juveniles ($\leq$7 d old) were collected from cultures raised in the Purdue Ecological Risk Assessment (PERA) laboratory and allowed to acclimate for 7 d before test initiation. Ten organisms were placed in 70 ml of 60:40 tap/deionized water at 25 °C and allowed to acclimate for 24–48 h prior to the initiation of toxicity tests. After acclimation, water was spiked with known volumes of aqueous stock solutions to achieve target concentrations of 30 or 0 $\mu g\,l^{-1}$ atrazine and DEA. Organisms were fed 200 µl of *Pseudokirchneriella subcapitata*, yeast, cerophyll, and tetramin (YCT) daily. Water changes were performed three times per week by removing 10% of the water volume and replacing it with fresh solution. At 42 d, organisms were classified as either males by the presence of a large gnathopod or females by the presence of eggs in the marsupial plates as previously described (Ralston-Hooper *et al.*, 2009). Organisms were then flash frozen and stored at −80 °C until proteomic and metabolomic analysis could be performed.

Metabolomic Analyses

For metabolomic analysis, three *H. azteca* organisms per sample were used and wet weights ranged from 0.0036 – 0.0129 (average 0.0064) g per sample. Samples were first homogenized for 20 s in 300 µl of methanol and 150 µl of water using a 7 mm × 95 mm saw tooth stainless steel generator probe (Omni International, Marietta, GA, USA) and then sonicated using 10, 1 s bursts. In order to extract non-polar metabolites, 450 µl of chloroform was added to the sample, thoroughly vortexed, and then centrifuged at 3,000 g for 20 min. The polar and non-polar phases were then collected and separated by placing each fraction in new vials. Each extract was dried using a Sevant SPD 131DDA SpeedVac Concentrator (Thermo Electron Corporation Milford, MA, USA). Once dried, the methanol:water extracted samples (polar metabolites) were prepared for GCxGC/TOF-MS analysis (Ralston-Hooper *et al.*, 2008). The non-polar fraction or chloroform extracted samples were re-suspended in mobile phase (50% water, 25% Methanol, 25% Acetonitrile) and injected onto the LC/TOF-MS for analysis. The HPLC was an Agilent 1100 (Agilent Technologies, Santa Clara, CA, USA) equipped with a micro well plate autosampler, capillary, and nano-pumping device. Reverse phase liquid chromatography was used to analyze the samples. A zorbax-C8 column (Agilent Technologies, Santa Clara, CA) with 2.1 × 150 mm, 5 µm dimensions was used for the separation. Solvent A consisted of water +0.1% piperidine. Solvent B contained acetonitrile:methanol (50:50 v/v) +0.1% piperidine. The flow rate was 300 $\mu l\,min^{-1}$ and 10 µl of sample was loaded on the column. The gradient was as follows: time 0 50% B held for 5 min, ramped to 95%B over 20 min, held at 95% B for 20 min, ramped down to 50% B over 5 min, and held for 10 min. The MS analysis was done using a time-of-flight instrument. The samples were evaluated with negative polarity electrospray ionization. The capillary voltage was set to 3500 V, desolvation gas 9.0 $l\,h^{-1}$, desolvation temperature 350 °C, and nebulizer 40 psi. The mass range scanned was 100–1800 with a scan rate of 1. The fragmentor was 175 V, skimmer 65 V, and octapole RF of 250 V. The samples were evaluated with Agilent Masshunter version 01.03 and downstream alignment done with Agilent GeneSpring MS software version 01.02. Metabolites were identified by blasting m/z values against the human metabolome database (HMDB) using negative ion mode (Wishart *et al.*, 2007). See Figure 4 with some examples of mass spectra utilized for metabolite identification. Chromatographic peaks were then aligned, matched, normalized, and compared.

Analysis of variance (ANOVA) and Tukey means separation post hoc testing were used to detect significant metabolite differences among the three treatment groups and were performed using SAS

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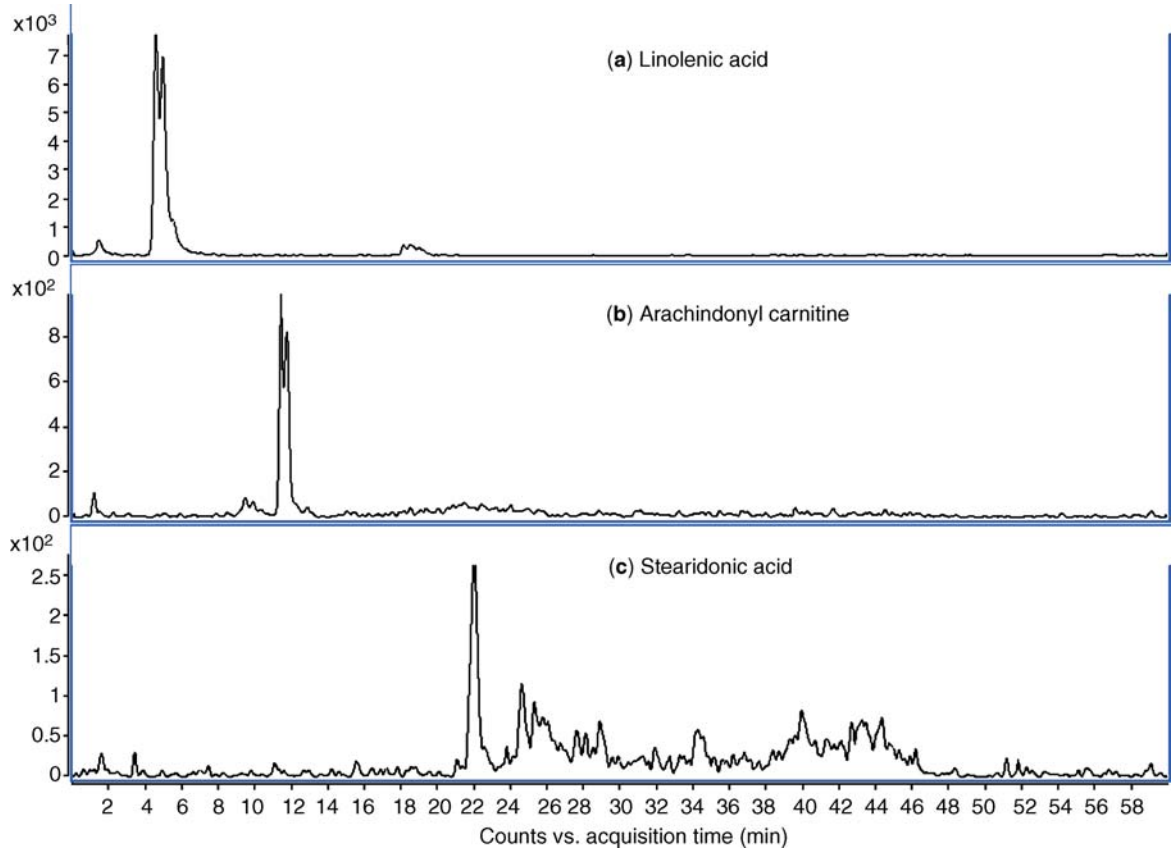

Figure 4. Time-of-Flight-Mass-Spectral chromatograms of identified metabolites associated with β-oxidation of fatty acids in atrazine and DEA exposed *Hyalella azteca*.

statistical software version 9.1 (Statistical Analysis Software, Cary, NC, USA). Unsupervised divisive cluster analyses (DIANA) were performed using R statistical software version 2.8.1 (R Foundation for Statistical Computing, Vienna, Austria).

Proteomic Analyses

For proteomic analysis, *H. azteca* whole body tissues from three individuals exposed to 30 $\mu g\,l^{-1}$ atrazine and DEA were prepared for protein separation (average 0.009 g wet weight). Proteomic analysis was performed similar to that of fish tissue, which was described previously under "Proteomic Analyses" in Section 2.1.1.

2.2.2 Results and Discussion

Metabolites and proteins were identified that suggests atrazine and DEA may disrupt energy metabolism in atrazine and DEA exposed organisms. Metabolomic analysis yielded an up-regulation of carnitines and fatty acids when compared to control organisms whereas proteomic analysis resulted in a down-regulation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), enolase, and ATP synthase as well as other proteins associated with energy production (Table 4). GAPDH, enolase, and ATP sythase are commonly reported glycolic enzymes identified during 2-DE research (Petrak *et al*., 2008). GAPDH is an enzyme used during glycolysis that catalyzes the synthesis of 1,3-bisphosoglycerate, a high-energy intermediate used for the synthesis of ATP. Enolase is responsible for the catalysis of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate (PEP) during the final step of glycolysis. When analyzed independently of metabolomic data, this down-regulation would suggest disruption in glycolysis. Ultimately,

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Table 4. Identified proteins and metabolites when two dimensional electrophoresis and liquid chromatography (LC) and two dimensional gas chromatography (GCxGC) time-of-flight mass spectrometry (TOF-MS) were performed on *Hyalella azteca* exposed to 30 $\mu g\,l^{-1}$ atrazine (ATZ) and desethylatrazine (DEA) for 42 days.

Accession or HMDB #[a]	Protein or metabolite identification	Biological function	Regulation
Proteins			
gi|13702286	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Glycolysis	↓ ATZ, DEA female DEA male
gi|158451635	Enolase	Glycolysis	↓ ATZ, DEA female
gi|25144756	ATP sythase	ATP synthesis	↓ ATZ, DEA female
gi|156972297	Creatine kinase	ATP synthesis	↓ ATZ, DEA female
gi|46849485	Phosphoglycerase kinase DEA female	Glycolysis	↓ ATZ female, male
Metabolites			
HMDB06455	Arachidonyl carnitine	Acyl carnitine	↑ ATZ, DEA female, male
HMDB03073	Linoleic acid	Fatty acid	↑ ATZ female, male
SHMDB06769	Stearidonic acid	Fatty acid	↑ ATZ, DEA female
HMDB00674	Dipalmitoylphosphatidic acid	Fatty acid	↑ ATZ, DEA female, male

[a] Human metabolome database (HMDB) accession.
[#] Or National Center for Biotechnology Information (NCBI) database accession number.

disruptions in glycolysis would result in a decrease in ATP thus reducing the overall energy status of exposed organisms. However, these organisms were physically larger than control organisms (data not shown, but see Ralston-Hooper *et al*., 2009) and did not experience high incidences of mortality when chronically exposed (Ralston-Hooper *et al*., 2009). Additionally, research has reported increases of these enzymes when exposed to various environmental contaminants (Dihazi *et al*., 2005; Tanguy *et al*., 2005; Gunnarsson, Nordberg and Selstam, 2007) lending to the difficulty of putting these proteins into biological context.

However, metabolomic analysis of polar and non-polar metabolites indicated an up-regulation of carnitines, fatty acids, glycerols, and urea when compared to control organisms, indicating β-oxidation of fatty acids. The mitochondrion catalyze *β*-oxidation of fatty acids and is the major process by which fatty acids are oxidized to generate energy (ATP) (Reddy and Hashimoto, 2001). The physiological role of carnitines is to facilitate the transfer of fatty acids across the mitochondrial membrane that results in generation of ATP (Arrigoni-Martelli and Caso, 2001). In general, an increase in fatty acids or carnitines results in increased energy of the exposed organism.

When proteomic and metabolomic data are used in conjunction, a clearer picture emerges. It appears that atrazine and DEA do indeed reduce glycolysis in exposed invertebrates. However, since invertebrates are comprised mostly of lipids, *H. azteca* organisms can rely on β-oxidation of fatty acids in order to meet their energy demands.

Overall, this case study demonstrates the advantages of utilizing both proteomic and metabolomic data to understand how environmental contaminants impact exposed organisms.

2.3 Use of Metabolomics to Characterize Egg Quality in Fish-Eating Birds Exposed to Persistent Organic Pollutants

Maternal exposure to environmental contaminants can impact vitellogenesis and thus the nutritional quality of developing avian eggs. For instance, alterations of fatty acid profiles in yolk (Stanton *et al*., 2003) and protein contents in albumin (Fernie *et al*., 2000) have been reported in avian eggs exposed to several chlorinated organics, including PCBs. Since effects of contaminant exposure are most pronounced in early-life stages, we hypothesize that exposure of great blue heron (*Ardea herodias*) embryos to these contaminants can result in failure to hatch or fledge. The goal of the current study was to evaluate sublethal effects of environmental stressors by studying the metabolomic profile of great blue heron eggs naturally exposed to PCBs, polycyclic aromatic hydrocarbons (PAHs), and organochlorine pesticides (OCPs) using a metabolomics approach. A review of the literature indicates this is the first metabolomics study conducted in wild birds.

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2.3.1 Materials and Methods

Samples

Great blue heron eggs were collected from six colonies located across the southern, central, and northern regions of Indiana during the springs of 2005 and 2006 (Baker and Sepúlveda, 2009). Eggs collected from southern Indiana (Brown County, N39°16′3″, W86°15′46″) were expected to be less contaminated than those from the central region (Tippecanoe and Carroll Counties, N41°16′50″–N41°37′7″, W86°57′3″–W87°09′5″), and eggs from the central region were expected to be less contaminated than those from the northern region (Lake and Porter Counties, N40°16′5″–N40°24′4″, W86°44′8″–W87°00′3″) (refer to Baker and Sepúlveda, 2009 for more detailed information about the colonies sampled). Half of each of the yolk samples collected from the eggs was sent to the University of Florida Analytical Toxicology Core Laboratory for quantification of PCBs, PAHs, and OCPs. Remaining yolk samples were retained for GCxGC/TOF-MS metabolomic analyses.

Metabolomic Analyses

Wet weights for great blue heron egg yolk samples ranged from 0.44–0.97 g. GCxGC/TOF-MS metabolomic procedures were described in detail in "Proteomic Analyses" (Section 2.1.1.).

2.3.2 Results and Discussion

Heron eggs contained an overall mean of 3,101 (range 164–27,703) $\mu g\,kg^{-1}$ PCBs, 7.20 (range 2.76–13.48) $\mu g\,kg^{-1}$ PAHs, and 2,869 (range 262–11,896) $\mu g\,kg^{-1}$ OCPs. Principle component analyses (PCA) identified no significant metabolites ($p > 0.05$) based on level of exposure to PCBs, OCPs, or the total contaminant load. However, PCA results did indicate differences in metabolite profiles for eggs exposed to varying PAH concentrations; based on the range of values observed, eggs were divided into three exposure categories: low ($<7\,\mu g\,kg^{-1}$), medium (7–10 $\mu g\,kg^{-1}$), or high ($>10\,\mu g\,kg^{-1}$) PAH concentrations (Figure 5). Fourteen significant metabolites were identified based on PAH concentration; of these, eight were identified to metabolite type with sufficient confidence (i.e., similarity value >700) but three were by-products of the derivatization process and are not listed (Table 5). Overall, metabolites were up-regulated in eggs with low PAH concentrations and down-regulated in eggs with high PAH concentrations.

Additional analyses were performed to determine the potential relationship between the region where eggs were collected and metabolomic profiles. When analyzed based on the region (southern, central, or northern) of the State of Indiana from which the eggs were collected, six significant metabolites were identified, three of which were identified to metabolite type with sufficient confidence (Table 5); eggs from the southern region expressed significant up-regulation of metabolites, whereas metabolites were not detected in the northern region and were not significantly different in the central region. Of the significant metabolites observed, three metabolites were identified as significant based on both PAH concentration and region: oleic acid, octadecanoic acid, and androstan-17-one.

The relationship between PAH concentration and metabolomic profiles suggests that eggs exposed to low versus medium/high PAH concentrations have metabolomic differences (Figure 5). PAH exposure has impacted overall health and reproduction of many vertebrate species such as loggerhead sea turtles (*Caretta caretta*) (Alam and Brim, 2000) and brown bullheads (*Ameiurus nebulosus*) (Steyermark *et al*., 1999). More importantly, exposure to PAH mixtures has resulted in decreased hatchability, increased mortality, and severe deformities in mallard duck (*Ana platyrhynchos*) embryos (Hoffman and Gay, 1981) as well as significant effects on survival and deformity rates of snapping turtle (*Chelydra serpentina*) embryos (Van Meter, Spotila and Avery, 2006). Differences in metabolite profiles may help explain the reported adverse effects of exposed embryos. For example, in egg-laying vertebrates, such as great blue herons, embryonic growth and survival are profoundly dependent on egg nutritional composition, or more specifically, its fatty acids concentration. Two fatty acids, oleic acid and octadecanoic acid, were identified in eggs exposed to all levels of PAHs; however, eggs exposed to low PAH concentrations had significantly higher levels of these fatty acids. This suggests that higher concentrations of PAHs reduced fatty acid content of the exposed embryos, which could lead to increased mortality or developmental effects.

Interestingly, ergost-5-en-3-ol, cholesta-3,5-diene, and androstan-17-one, all sterol structures, were greater in eggs associated with low PAH

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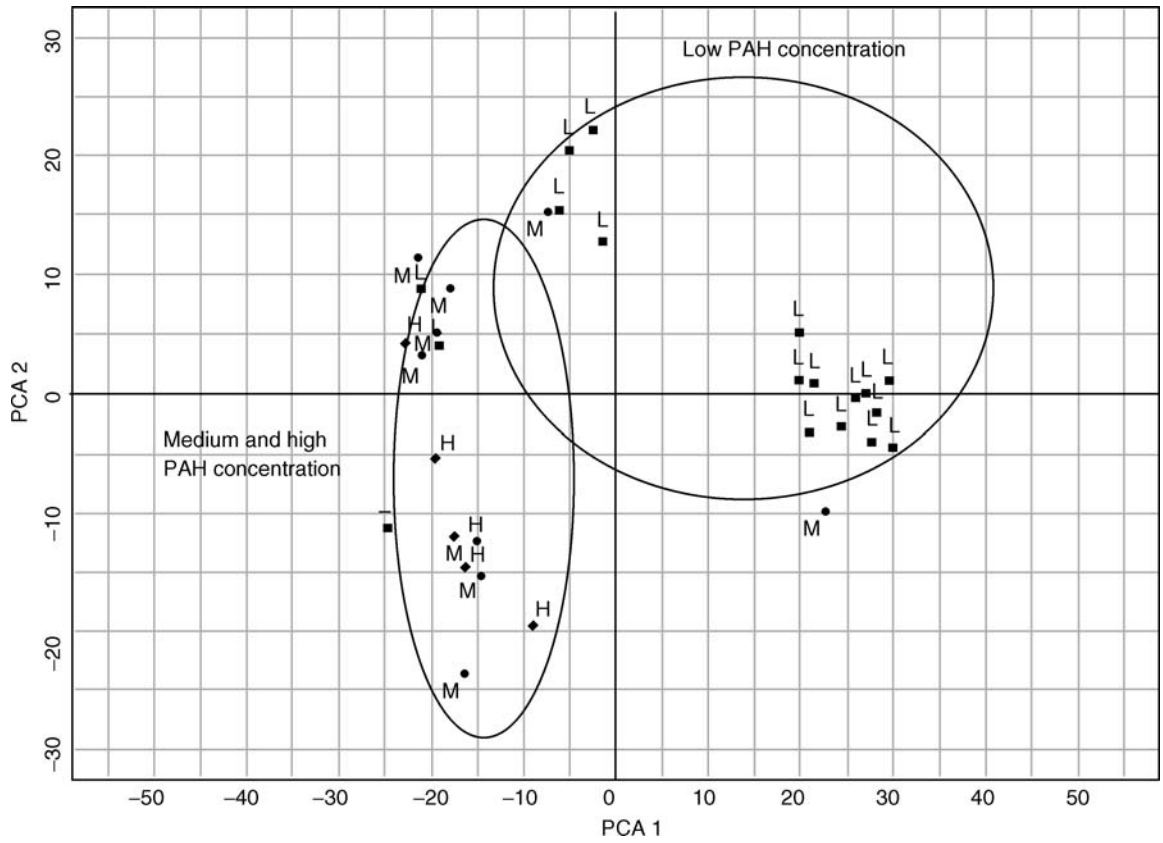

Figure 5. Results of principal component analysis indicating metabolic differences between great blue heron eggs with low (L ■ < 7 μg kg^{-1}), medium (M ● <7 μg kg^{-1}) and high (H ♦ > 10 μg kg^{-1}) polycyclic aromatic hydrocarbons (PAH) concentrations.

Table 5. Significant metabolites identified by relative polycyclic aromatic hydrocarbon (PAH) concentration (low, medium, high) and region from which eggs were collected. Significant differences in metabolite expression for each analysis are indicated by capital superscripts. Up-regulation or down-regulation of metabolites is indicated by ↑and ↓, respectively. NS = Metabolite not identified as significant.

Metabolite ID	Metabolite function	Similarity Value	Analysis	
			PAH concentration	Region
Oleic acid	Fatty acid	818	Low $\uparrow^{A}$ Medium $\uparrow^{B}$ High $\downarrow^{BC}$	South $\uparrow^{A}$ Central $\uparrow^{B}$ North $\downarrow^{C}$
Androstan-17-one, 3-ethyl-3-hydroxy	Steroid hormone	785	Low $\uparrow^{A}$ Medium $\uparrow^{B}$ High $\downarrow^{BC}$	South $\uparrow^{A}$ Central $\uparrow^{AB}$ North $\downarrow^{B}$
Octadecanoic acid	Fatty acid	736	Low $\uparrow^{A}$ Medium $\uparrow^{B}$ High $\downarrow^{BC}$	South $\uparrow^{A}$ Central $\uparrow^{AB}$ North $\downarrow^{B}$
Ergost-5-en-3-ol	Sterol	740	Low $\uparrow^{A}$ Medium $\uparrow^{AB}$ High $\downarrow^{B}$	NS
Cholesta-3,5-diene	Cholesterol	856	Low $\uparrow^{A}$ Medium $\uparrow^{AB}$ High $\downarrow^{B}$	NS

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concentrations. The biological context of these metabolites is not clear. For example, high concentrations of sterol lipids may be detrimental to egg development, since sterols such as ergost-5-en-3-ol are not as readily absorbed as cholesterol, which may interfere with nutritional status and membrane formation in the developing egg (Child and Kuksis, 1983; Salen *et al*., 2000). Also, higher levels of ergost-5-en-3-ol were identified in unfertilized eggs of brown trout (*Salmo trutta lacustris* L) (Lehtinen *et al*., 1999). However, these sterol structures may also play an important role in lipoprotein transport or steroid synthesis so higher concentrations may not necessarily have adverse effects on egg development. Most interestingly, the steroid, androstan-17-one, was identified in eggs exposed to low PAH concentrations. Maternally derived hormones such as androgens have been implicated in egg laying behavior, hatching, and clutch size of American coot (*Fulica americana*) eggs (Reed and Vleck, 2001). In general, chicks that hatched from eggs with higher androgen levels had behavioral as well as growth advantages over chicks hatched from eggs with lower androgen levels.

In general, it appears that eggs exposed to various PAH levels have different metabolomic profiles. Eggs exposed to lower PAH levels may indeed have an advantage over those exposed to higher PAH concentrations. Furthermore, these compounds where not only associated with PAH levels but were also found in higher concentrations in eggs collected from nests in northern Indiana verses those from central and southern Indiana colonies. However, it is also possible that we not only observed metabolite differences due to PAH concentration, but year of sample collection may also be playing a role. These eggs were sampled over multiple years, which could result in metabolomic profiles differences due to changes in maternal resources or environmental factors, which vary from year to year (e.g., temperature and rainfall). Overall, this research demonstrates the feasibility of utilizing GCxGC/TOF-MS to successfully identify differentially expressed metabolites in egg yolks of birds. To our knowledge, this is the first metabolomic study conducted in wild birds.

3 SUMMARY AND CONCLUSIONS

Proteomics and metabolomics are powerful tools in environmental toxicology. They offer great potential as biomarker discovery tools as well as for increasing our understanding of the cellular mechanisms involved in stress response. Thus, the major potential of these tools is based on their ability to predict adverse impacts of stressors to invertebrates, fish, and wildlife. Because organisms are rarely exposed to a single chemical, these high-throughput mechanistically-based tools can also improve our knowledge of the effects of mixtures. However, several major challenges remain before these tools can be successfully applied for environmental risk assessment. Some of these challenges include:

1. Linking expression profiles to phenotypic and population-level effects. Integration of "omic" data with phenotypic changes and ecologically relevant endpoints is a must if these tools are to be implemented by regulatory agencies as predictors of risk to free-ranging animals. The use of "omic" approaches in existing risk assessment approaches would allow for a high-throughput screening of simultaneous changes in thousands of molecules and thus provide a more rapid evaluation of the toxicity of chemicals.
2. More field studies that utilize "omic" approaches are needed. As discussed at the beginning of this chapter, few field studies have employed proteomic and metabolomic tools to evaluate exposure and effects of biota to pollutants. Results are promising in that multivariate analysis of protein and metabolite profiles are predictive of site and thus specific contaminant exposure. Field studies that can tie protein and metabolite changes to sublethal effects represents a major data gap.
3. All current "omic" techniques provide only a static snapshot of the molecular profile at a particular point in time. More studies are needed that add a temporal component and evaluate changes in proteins and metabolites over several time points as well as during embryo and larval development.
4. A major challenge will involve separating "biological noise" with effects elicited by specific stressors. It is known that factors such as gender, age, and physiological condition can affect the profile of proteins and metabolites. These types of effects must be taken into consideration during experimental design and data interpretation.
5. Serious weaknesses remain in the areas of data analysis and integration of "omic" databases.

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Although there have been considerable advances in this field over a very short period of time, most of these have been over equipment development, with less attention being paid to data analysis. Likewise, very few ecotoxicological studies have integrated "omic" data collected from the same organisms. This is of great importance because toxicological responses are under regulation of a complex array of genes, proteins, and metabolites. Integration of "omic" databases would help in the identification of genes that are linked to particular toxic outcomes.

6. A big challenge is to annotate the proteomes and metabolomes of species routinely used in ecotoxicological research. In proteomics research this gap could be overcome in the short-term with the use of *de novo* sequencing approaches.
7. Another important task is the standardization of general protocols, including experiments, data handling, and reporting. The Metabolomic Standards Initiative (MSI) was recently launched with the goals of standardizing chemical analysis, data processing, ontology, and minimum reporting guidelines for anyone conducting metabolomics work.
8. Finally, it should be pointed out that only a limited number of taxonomic groups have been evaluated utilizing proteomic and metabolomic tools. For example, only a handful of such studies have been conducted with amphibians and mammals. No studies have been published with birds (with the exception of the study presented in this chapter) or reptiles.

Regardless of all the pressing challenges, there is no doubt that these holistic measures of organism response to environmental stressors will continue to make a great impact in the ecotoxicological field.

APPENDIX 1

Summary of genes shown under Figure 1.

Gene symbol	Protein name (*Homo sapiens*)
FABP3	Fatty acid binding protein 3, muscle and heart
FABP7	Fatty acid binding protein 7, brain
HSPA1A	Heat shock 70 kDa protein 1A
HSPA1B	Heat shock 70 kDa protein 1B
LENG1	Leukocyte receptor cluster (LRC) member 1
PGK1	Phosphoglycerate kinase 1
PRDX2	Peroxiredoxin 2
PRDX6	Peroxiredoxin 6
ALDH1A2	Aldehyde dehydrogenase 1A2
BHMT	Betaine-homocysteine methyltransferase
HECTD3	Probable E3 ubiquitin-protein ligase HECTD3
MAT1A	S-adenosylmethionine synthetase isoform type-1
NME1	Nucleoside diphosphate kinase A
PDHB	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial
PPA1	Inorganic pyrophosphatase
UPB1	Beta-ureidopropionase
ENO3	Beta-enolase
GFAP	Glial fibrillary acidic protein
GSTT1	Glutathione S-transferase theta-1
KRT8	Keratin 8
LTF	Lactotransferrin
AICDA	Activation-induced cytidine deaminase
ALDH7A1	Alpha-aminoadipic semialdehyde dehydrogenase
CA13	Carbonic anhydrase 13
ENO1	Alpha-enolase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GNB2L1	Guanine nucleotide-binding protein subunit beta-2-like 1
MDH1	Malate dehydrogenase, cytoplasmic
TF	Transferrin
GSTM3	Glutathione S-transferase mu 3 (brain)
KCTD12	Potassium channel tetramerisation domain containing 12
ATP5B	ATP synthase subunit beta, mitochondrial
ECHS1	Enoyl-CoA hydratase, mitochondrial
HSPA8	Heat shock cognate 71 kDa protein
HSPD1	Heat shock protein 60 kDa, mitochondrial
MYL6	Myosin light polypeptide 6
TUBB2B	Tubulin beta-2B chain
CAPZB	Capping protein (actin filament) muscle Z-line, beta

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Application of Proteomics to Study Mechanisms of Toxicity and Dose–Response Relationships of Chemical Exposure

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1 INTRODUCTION

Toxicoproteomics, the application of proteomic technologies in toxicology, enables studies of the adverse effects of xenobiotic substances in relation to changes in protein profiles. Through the application of technologies that provide global protein expression information, it should be possible to determine the biological response to a toxic insult. As a result, critical proteins and hence pathways in biological systems that are important in toxicological responses may be identified. Identifying proteins whose expression is associated with specific toxic end points is an initial step in predicting, characterizing and understanding toxicity. Subsequent analysis of protein function and the pathways of protein expression changes represent additional methods to generate hypotheses relevant to developing a better understanding of the mechanisms of toxicity. The application of proteomics promises biomarkers for early detection and new toxicity endpoints to facilitate the identification and characterization of toxicity and will provide a better understanding of the mechanisms of toxicity of compounds. It is anticipated that toxicoproteomics will facilitate a means that will provide more sensitive and earlier detection of adverse effects at low doses, which may lead to more accurate determinations of the no observed effect concentration (NOEC) of a given compound in toxicity studies and hence improved risk assessment.

The application of proteomic technologies in toxicology and their usefulness for biomarker discovery have been reviewed previously (Kennedy, 2002; Wetmore and Merrick, 2004). Toxicoproteomics applied to the study of mechanisms of toxicity has been increasingly adopted in recent years and, in some cases, has been integrated with other 'omics' technologies; these have been the subject of some reviews, although much of the focus has been on the application of genomics (Ellinger-Ziegelbauer *et al.*, 2008; Blomme, Yang and Waring, 2009). In this report, proteomic technologies and their latest applications in studies of the mechanisms of toxicity and dose–response relationships are reviewed and discussed.

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2 PROTEOMIC TECHNIQUES

A number of different techniques have been developed to investigate protein profiles in complex samples. Here, some of the main techniques that have been applied in toxicoproteomic studies are described and the relative merits and limitations of the various approaches considered.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), often in conjunction with matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-ToF MS), is a very commonly applied approach. Relative quantification of stained protein spots seen in 2D gels by image analysis may be used to locate proteins of interest which are then identified following tryptic digestion and analysis by MS, often MALDI-ToF MS. This widely used approach has the advantage of being available to most laboratories. Many protein spots can be resolved in a 2D gels and the visual appearance of a stained gel provides a ready readout of the data, although, in practice, meaningful interpretation requires the use of dedicated software. Often post-translational modifications that cause a shift in p*I* are apparent and may be confirmed by subsequent MS analysis. However, this approach has a number of drawbacks, including a rather limited dynamic and molecular mass range, difficulty in detecting low-abundance proteins, poor resolution of membrane-bound, highly acidic or basic and low-molecular-weight proteins, and a need for careful sample preparation to remove salts and other contaminants. The technique has a low throughput and is laborious. Some of the disadvantages have been addressed through the application of 2D-differential in-gel electrophoresis (2D-DIGE), which uses multiple fluorescent dyes to label protein samples prior to running the gel. This allows multiple samples to be co-separated and visualized in a single 2D gel, and makes quantification easier. However, the limited number of DIGE dyes available allows only a few samples to be analysed at any one time.

Instead of attempting to separate and quantify proteins before digesting them with trypsin to identify proteins by MS, an alternative approach is to first digest the protein mixture and then attempt to separate and quantify the peptides present. The principle behind this approach is that it is easier and more reproducible to separate and quantify peptides than proteins. Large-scale analysis of highly complex protein mixtures from whole cells, organs, organelles or biofluids has been achieved in this way (Fang *et al*., 2006; Ono *et al*., 2006; Cutillas and Vanhaesebroeck, 2007). Separation of peptides prior to MS analysis, typically by tandem mass spectrometry (MS/MS), has been achieved by reverse-phase LC or a combination of cation-exchange chromatography and reverse-phase LC (Le *et al*., 2006), an approach that has been termed multi-dimensional protein identification technology (MudPIT) (Paoletti, Zybailov and Washburn, 2004). Although this approach has been shown to provide increased protein coverage compared with 2D-gel-based approach (Domon and Aebersold, 2006), it is technically more demanding, requires robust and reproducible ion-exchange as well as reverse-phase LC, and produces large, extremely complex datasets that need specialized software to interpret.

Elements of both of these approaches are combined in the so-called GeLC-MS approach. In this case, samples are resolved by simple one-dimensional sodium dodecylsulphate–PAGE to separate proteins on the basis of their relative mass, the entire gel is then cut into a series of slices and the mixtures of proteins contained within each are digested with trypsin before being analysed by LC-MS (Rezaul *et al*., 2005; Nicholas *et al*., 2006). The procedure has a number of advantages including the use of a high-resolution method to fractionate proteins and making LC separation of the resultant tryptic peptides less demanding.

Although knowledge of the protein constituents of organs, tissues and cells under any given time or condition is useful, it often does not allow researchers to address issues of biological importance. In order to answer questions of biological relevance, it is often necessary to establish the dynamics involved, for example, to determine how the expression levels of proteins vary in response to an externally applied stimulus, such as exposure to a toxicant. A variety of comparative and quantitative proteomic techniques have been developed to address this important issue. It should be appreciated that quantification of any analyses by MS is best achieved using heavy isotope-labelled internal standards. This is a well-established approach (Gevaert *et al*., 2008; Kito and Ito, 2008) and can be applied to determine the absolute quantification of specific peptides (Goshe and Smith, 2003; Tao and Aebersold, 2003) or a variety of peptides in a multiplexed format (Beynon *et al*., 2005; Yan and Chen, 2005;Morano, Zhang and Fricker, 2008). However,

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such approaches require knowledge of the proteins of interest and usually this is not the starting point of a proteomic experiment. More usually, it is the relative level of expression of proteins or peptides that is determined. A number of labelling approaches have been developed that can accomplish this (Chen *et al*., 2007). Ideally, labelling should be applied early in the protocol so that variation due to sample handling before the labels are attached and the samples combined for subsequent fractionation and analysis is minimized. The labels attached are chemically similar but can be distinguished by either their physical characteristics or mass, and so by comparing their levels on specific proteins or peptides a measure can be produced indicative of the relative amounts of the labelled proteins or peptides. The most commonly applied labels include the use of the DIGE fluorescent tags, stable isotope labelling by isotope-coded affinity tags (ICAT) (Smolka *et al*., 2001; Turecek, 2002) and stable isotope labelling with amino acids in cell culture (SILAC) (Chen *et al*., 2005; Asara *et al*., 2006). Another technique termed isobaric tags for relative and absolute quantification (iTRAQ) is also available, although this can only be applied to labelling of peptides after trypsin digestion (Zieske, 2006). The usefulness of the labelling approach is limited by the number of different tags that are available and this compromises experimental design often to a simple one-to-one comparison, for example, of samples derived from a pre- and post-treatment. Commercially sourced labels are costly, which may be a factor in the design of more complex experiments. Consequently, analysis of comparative levels is often limited to consideration of fold differences above an arbitrary threshold as often insufficient samples are analysed to allow the use of statistical analysis to compare between treatments.

As an alternative, a variety of label-free quantitative approaches have been developed (Colinge *et al*., 2005; Heller *et al*., 2007; Stalder, Haeberli and Heller, 2008; Seibert *et al*., 2009). In comparison with labelling approaches, label-free analyses are often based on more unique identified peptides (Patel *et al*., 2009). Label-free approaches are not limited by the number of different labels available and are not costly. This means that more samples can be analyzed. Consequently, experiments can be designed to include a suitable number of samples per group to facilitate appropriate statistical analysis.

Surface-enhanced laser desorption/ionization (SELDI)-ToF-MS provides an alternative approach to label-free proteomic analysis. This technique combines the use of an interactive chip with MALDI-ToF MS. Complex mixtures of proteins are applied to one of several interactive chips under conditions where a fraction of proteins bind to the surface. The interaction on the chip surface may be ionic, hydrophobic, hydrophilic, metal ion chelating or affinity-based and the conditions of binding may be manipulated by varying the buffering conditions employed. The bound proteins are then subsequently analysed by ToF-MS under conditions that ionize but do not fragment the proteins. The mass of each protein ion is determined from its ToF and relative quantification is based on the ion current intensity. Application of SELDI-ToF-MS requires minimal sample preparation and only small amounts for analysis; it is rapid and hence is amenable to the analysis of tens or hundreds of samples in a single run (Petricoin, Ornstein and Liotta, 2004). This allows the design of suitably scaled experiments to be performed and comparisons based on appropriate statistical analyses. This technique has been widely applied to identify potential biomarkers for prognosis, diagnosis and treatment of human diseases in patients, particularly in plasma or serum samples (Wulfkuhle *et al*., 2001; Carter *et al*., 2002; Laronga *et al*., 2003). SELDI-ToF-MS is sensitive and useful for the detection of protein ions with masses of less than 20 kDa, but above this mass, the resolution of the proteins becomes increasingly poor. The MS data output is not suitably informative to identify protein ions of interest. To achieve this, it is necessary to use other methods, typically purification followed by trypsin digestion and MALDI-ToF MS or LC-MS/MS, to identify the proteins (Abdul-Salam *et al*., 2006; Zhu, Edwards and Boobis, 2009).

3 MECHANISMS OF TOXICITY

Although toxic effects may result from exposure to numerous man-made and naturally occurring chemicals, the mechanism(s) of toxicity involved remain largely unknown. Proteomic studies may aid in the elucidation of the underlying mechanisms of toxicity. In the case of pharmaceuticals, sensitive markers may be used in screens for toxicity early in the development process and could ultimately provide sensitive surrogate markers for clinical studies. In

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the case of industrial chemicals and environmental contaminants, an increased understanding of the mechanisms involved is expected to help in the identification of responsive biomarker proteins that may be used to aid risk assessment.

3.1 Drug-Induced Toxicity

The inability to predict with any accuracy the pharmacological and toxicological effects of drug candidates in pre-clinical studies has contributed to the decline in the number of new drugs which make it to market and the rise in cost associated with drug development. Identifying molecular interactions associated with therapeutic and toxic outcomes early in the development of drugs is of great importance. Traditional mechanism-elucidation strategies are narrow, often focusing solely on the identification of a single molecular target. An approach that is able to establish the spectrum molecular effects following exposure to a toxic compound and the biochemical consequences that ensue would be of great benefit.

Understanding the mechanisms of toxicity for drugs is critical in forming a rational plan for developing efficient screens for toxicity. Often safety assessment in the pharmaceutical industry is performed in the later stages of the drug discovery process, in part because the tools for early safety screening are lacking. A better understanding of the mechanisms involved may change this practice.

Proteins are the principal targets of drug discovery. The common aims of proteomics in the drug development process include the identification of molecular targets, investigations into mechanisms of drug action or toxicity and the identification of biomarkers of efficacy and toxicity from readily accessible biological fluids. Biomarkers of toxicity may be used to screen compounds in pre-clinical studies for target organ toxicities as well as, later on, in the development process during clinical trials. The application of proteomics might be expected to lead to a greater understanding of processes involved and explain why particular organs are often affected.

Hepatotoxicity is commonly found not only during the discovery and development of new therapeutic molecular entities but also once drugs have been in widespread use (Holt and Ju, 2010). Indeed, hepatotoxicity is the focus of much published work that aims to explore mechanisms of drug-induced toxicity (Russmann, Kullak-Ublick and Grattagliano, 2009). For instance, Dail *et al.* (2008) have illustrated the value of toxicoproteomics in comparison with traditional methods by studying the hepatic response to phenobarbital (PB) toxicity. PB is a well-characterized xenobiotic known to cause hepatotoxicity in the rat. A proteomic approach using LC-MS was applied to identify proteins altered after exposure to PB for 5 days at a dose that did not cause liver damage (as evident by a lack of release of hepatic enzymes into the serum). Protein changes included those affecting P450-mediated metabolism, cell cycle, apoptosis and cellular proliferation. Many alterations in the hepatic protein profile corresponded well to known effects of PB elucidated in previous studies (Dail *et al.*, 2007), and at the same time revealed a large amount of additional information which may help explain the mechanism of PB liver toxicity. The authors suggest that proteomic analysis performed in this way may be useful for the identification of chemicals with carcinogenic potential at an early stage and propose that it may, in combination with systems biology modelling, form the basis of a new screening methodology that can be incorporated into toxicological studies.

The mechanism of hepatotoxicity following acetaminophen (APAP) overdose in mice has been examined by 2D-DIGE. APAP is known to be converted into the reactive metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI), and it is this chemical that is responsible for hepatotoxicity (Ruepp *et al.*, 2002). Using 2D-DIGE treatment with APAP was shown to cause changes in mitochondrial proteins, suggesting a decrease in energy production pathways, an effect that was consistent with a change in the morphology of the mitochondria, as well as the release of chaperonin proteins. Others (Yamamoto *et al.*, 2007) have observed using 2DE-MS and 1H-NMR that the effects of APAP on both human hepatocyte transplanted chimeric mice and normal mice involve pathways of lipid and fatty acid metabolism, glycolysis and energy metabolism, oxidative stress and the tricarboxylic acid cycle.

3.2 Chemical Contaminants in the Environment

Many chemicals released into the environment have the potential to disrupt the normal function of exposed organisms, either through an effect on the

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endocrine system or by targeting specific organs. Proteomics have been applied to study the toxicity of such chemicals, particularly those that may cause endocrine disruption, hepatotoxicity and lung toxicity.

Exposure to oestrogens or chemicals with oestrogen-like properties may induce changes in both reproductive and non-reproductive organs, including some persistent molecular alterations (Morgan, Schulkin and Pfaff, 2004; Verdier-Sévrain *et al*., 2006; Warri *et al*., 2008; Roy *et al*., 2009). Identification of oestrogen-responsive genes and proteins allows an understanding of the molecular mechanisms of oestrogenic activity. Label-free quantitative GeLC-MS has been used to examine the effect of 17β-oestradiol on the human breast cancer cell line, MCF-7, and has demonstrated up-regulation of 55 proteins that together describe an up-regulated pathway consistent with the events of cell proliferation (Zhu, Boobis and Edwards, 2008a). There are many chemicals in the environment that have oestrogenic activity and they are derived from a number of different potential sources including phyto-oestrogens (e.g., genistein), industrial contaminants (e.g., bisphenol A) and organochlorine pesticides (e.g., endosulfan). SELDI-ToF-MS has been used to show that genistein, bisphenol A and endosulfan cause protein profile changes similar to that of 17β-oestradiol in MCF-7 cells. The most responsive proteins were identified as histones H2A, H2B, H3 and H4 (Zhu, Edwards and Boobis, 2009).

Possible mechanisms of hepatotoxicity due to exposure to the environmental pollutants 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Sarioglu *et al*., 2006, 2008) and benzo[a]pyrene (BaP) (Shen, Liu and Yu, 2008), as well as microcystins in cynobacteria-contaminated drinking water (Fu, Yu and Xu, 2009), suggest the involvement of a wide range of pathways including regulation of transcription, cell cycle, apoptosis, transport, signal transduction, metabolism, protein degradation and cell differentiation. In particular, the roles played by p53 and PP2A A subunit have implicated the ubiquitin-proteasome pathway in response to microcystins. Interestingly, up-regulation of voltage-dependent anion channel-selective protein 2, which was dependent on the presence of a functional aryl hydrocarbon receptor and an inhibitor of the activation of the proapoptotic protein BAK and the mitochondrial apoptotic pathway, implies an unrecognized mechanism by which TCDD may affect cellular homeostasis and survival (Sarioglu *et al*., 2008). These results aid in understanding hazardous effects of these compounds as well as providing the possibility of the establishment of potential biomarkers.

The potential risk of environmental nanoparticles on human health, particularly in the lung, has been the subject of increased concern in recent years (Asgharian and Price, 2007). Nano-particles present possible dangers, both medically and environmentally. The concerns are that due to their small size they are able to pass through cell membranes in organisms, they have a high surface-to-volume ratio, which can make them very reactive or catalytic, and their interactions with biological systems are largely unknown (Sayes, Reed and Warheit, 2007; Rupp, 2009). Ultrafine carbon black has been suggested to cause oxidative stress and lung injury, but mechanisms have not been clearly demonstrated. A GeLC-MS approach has been used to examine the proteins present in bronchoalveolar lavage fluid following ultrafine carbon black-induced lung injury. This was found to cause an increase in leukaemia inhibitory factor receptor and epidermal growth factor receptor, suggesting an effect on the rate of epithelial shedding from the lung (Chang *et al*., 2007).

In another study the response of rat lung epithelial cells to exposure to acrolein was examined. Acrolein is a highly toxic unsaturated aldehyde that can be formed from the breakdown of certain pollutants in outdoor air or from burning tobacco or gasoline. This chemical was found to produce changes in a diverse range of proteins including those included in all proliferation and apoptosis (Sarkar and Hayes, 2009).

3.3 Food Additives/Contaminants

Two-dimensional PAGE and MALDI-ToF-MS or LC-MS/MS have been used to investigate the effect of feeding rats a betaine-supplemented ethanol diet and have shown the prominent up-regulation of betaine homocysteine methyltransferase-1, methionine adenosyl transferase-1 and glycine *N*-methyltransferase in the liver (Kharbanda *et al*., 2009). Up-regulation of these methionine metabolic pathway enzymes is a protective mechanism by which betaine restores a normal metabolic ratio of liver *S*-adenosylmethionine to *S*-adenosylhomocysteine. Ethanol also caused down-regulation of carbonic anhydrase-III protein

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levels which were not restored by betaine supplementation. One function of carbonic anhydrase-III is to moderate the effects of oxidative stress; therefore, reduction in its levels may contribute to ethanol-induced redox stress.

Surface-enhanced laser desorption/ionization-ToF-MS has been used to examine protein profiles of plasma and urine in rats treated with the neurotoxicants such as acrylamide and methylmercury. These chemicals may be present in food, acrylamide being formed from certain cooking processes and methylmercury as a contaminant from industrial sources. Protein ions that vary in intensity with dose and duration of treatment were found at levels of exposure that did not cause neuropathology in rats, suggesting their possible use as protein biomarkers in serum and urine for the assessment of sub-clinical neurotoxicity (Fang, Boobis and Edwards, 2007).

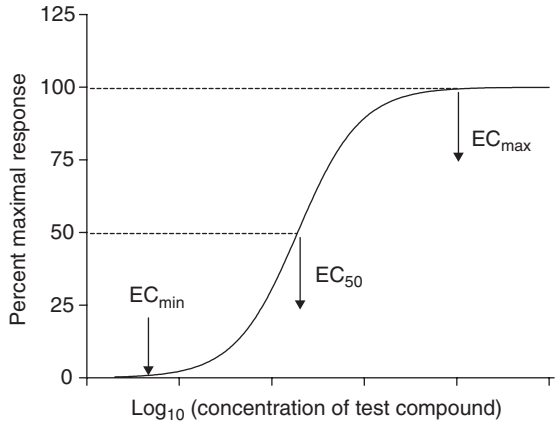

Figure 1. An idealized dose–response curve. EC$_{min}$ is the highest concentration that produces no detectable effect. EC$_{max}$ is the lowest concentration that produces a maximal effect. EC$_{50\%}$ is the concentration that produces a 50% maximal effect.

4 DOSE–RESPONSE RELATIONSHIPS

Proteins that are found to be responsive to toxicants may serve as biomarkers. A useful biomarker would need to possess a number of properties including being mechanistically relevant, sensitive and dose-responsive so that the information can be used to make meaningful risk assessments. Three important parameters can be generated from a dose–response curve. These are the concentrations of the toxicant that produce a maximal response or EC$_{max}$, a half maximal response or EC$_{50}$ and no detectable effect or EC$_{min}$ (Figure 1). The EC$_{max}$ value is important as it indicates the threshold concentration above which no further response to dose can be achieved. The EC$_{50}$ value provides an accurate measure of the relative responsiveness of a given toxicant. The EC$_{min}$ value is the highest concentration that produces no detectable effect and is otherwise known as the NOEC. This concentration is important as it provides the basis for risk assessment in combination with an appropriate estimate of a safety margin (Eason and O'Halloran, 2002; Janssen and Heijerick, 2003; Rietjens and Alink, 2006; Van Wijngaarden *et al*., 2007). The introduction of concepts such as thresholds of toxicological concern (TTC) and dose-dependent transitions in mechanisms of toxicity has emphasized the complexities associated with dose–response characterization of chemical exposure, especially low-level exposure to mixtures such as environmental co-exposures (Holsapple and Wallace, 2008). The concept of TTC acknowledges that there are doses of toxicants that are safe (doses that do not elicit a toxic response) and that all chemicals can elicit a toxic response (if the dose is high enough). An improved understanding of dose-dependent transition in mechanisms of toxicity and the need for better study design with suitable choice of doses, particular in the region of the dose transition, will impact on the risk-assessment process (Slikker *et al*., 2004).

Often current testing strategies do not precisely capture transitions in the dose–response relationship, leaving unanswered the critical question of where within the dose–response relationship the transition occurs with respect to other critical reference points, for example actual human exposure or the experimentally derived NOEC. An area of major difficulty is estimating where on the dose–response curve human exposure is likely to be. The central role played by proteomics, more generally as speculated, is emphasized as is the value of dose–response curves in exploring low-level exposure.

Studying dose–response relationships of chemical exposures using proteomics approaches is an extremely challenging task because of complexities arising from the large number of samples to be analysed and the techniques to be used for identification of protein biomarkers to indicate these relationships. For these reasons, there are only a few publications on this aspect. Our laboratory has employed SELDI-ToF-MS to determine dose–response relationships

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of chemical exposure on human cells and animals. It has been reported (Zhu, Edwards and Boobis, 2008b) that dose–response relationships of mixtures of oestrogenic compounds such as 17β-oestradiol, genistein, bisphenol A and endosulfan on human breast cancer MCF-7 cells exhibit simple concentration additivity and by measuring the levels of histone proteins is indicative biomarkers of the effects, (Zhu, Edwards and Boobis, 2009). This is perhaps one of the most successful applications of SELDI-ToF-MS in the determination of dose–response relationships of chemical mixtures, partially because the protein biomarkers (histones H4, H2B, H2A and H3) that indicate these relationships have molecular weights around 15 kDa, which are readily detected by SELDI-ToF-MS. As sample preparation for SELDI-ToF-MS is relatively quick and simple, it is possible to analyse many samples in a single batch and so produce sufficient data to construct reasonable dose–response curves, in this case allowing comparison between the effects of several compounds with oestrogenic activity (Figure 2). In another study conducted in our laboratory, SELDI-ToF MS was used to profile rat serum and urinary proteins in response to treatment with neurotoxicants. Proteins that responded to treatment with both acrylamide and methylmercury in a dose-dependent manner were found (Fang, Boobis and Edwards, 2007) and could potentially be used to produce dose–response curves in appropriately designed experiments. However, as the proteins in this study were not identified, mechanistic information is lacking at present. This would need to be addressed to underpin the relevance of proteins to the toxic effect of compounds.

Other methods to identify dose-responsive proteins can also be applied. Kleno *et al*. (2004) have described the application of 2D-DIGE combined with the use of principal component analysis and partial least squares regression to find patterns of hepatotoxic change following treatment of rats with hydrazine at different doses and times. Altered expression profiles in proteins involved in lipid metabolism, Ca21 homeostasis, thyroid hormone pathways and stress response were found. The authors claimed that several of the identified proteins have not previously been implicated in hydrazine toxicity and may thus be regarded as new potential biomarkers of induced liver toxicity (Kleno *et al*., 2004). The robustness of the patterns of changes will need further study to assess their specificity. Also 2D-DIGE is too cumbersome to be applied usefully to routine dose–response assessments. Ideally, amongst the responsive proteins one or two may be found that represent accurately the response of the liver to the toxin.

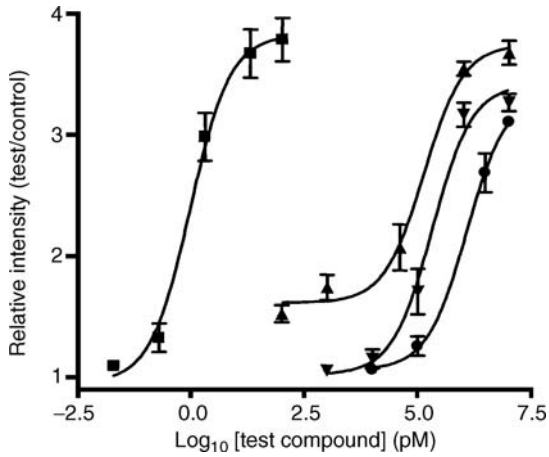

Figure 2. Dose–response curves of oestrogenic effect using histone H4 as a responsive protein to oestrogenic chemicals: 17β-oestradiol (■); genistein (▲); bisphenol A (▼); endosulfan (●).

5 CHALLENEGES AND PERSPECTIVES

The application of proteomics to understand the mechanisms of toxicity by drugs, environmental chemicals and food additives is still at an early stage and a number of challenges exist.

An ideal proteomic approach would enable comprehensive characterization of the proteome of a target organ or tissue in a high-throughput manner. Currently, the proteomic techniques involved are complex, costly and time-consuming. At present, there is no standardized or agreed methodology or platform in place. This is understandable as different methods may be applicable in different circumstances and not one approach is likely to answer all questions asked. However, different methods do vary in their sensitivity and reproducibility and this needs to be appreciated when interpreting such data. The sensitivity of MS instruments may be improved but they are probably close to their maximum detection limits. Methods that increase reproducibility mostly based on the well-established use of heavy isotope labels are being improved (Pan *et al*., 2008). If current technical limitations can be addressed,

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it will allow workers to perform more suitably designed experiments. Too often, the quality of the proteomic data is limited by the ability to analyse only a handful of samples. Thus, sample numbers are often inadequate making dose–response experiments impractical. At present, there are very little confirmatory data and this needs to be addressed. Techniques such as proteomics that produce a host of changes need to be considered carefully as a proportion of the data will be falsely positive. Standardization of the processes of data handling and interpretation, using statistically-based methods, would be helpful. The development of the tools needed for productive proteomics data analysis is still in its infancy.

It is difficult to attach toxicological significance to observed changes in protein levels. Often it is not possible to determine whether a pattern of protein-expression changes is representative or associated with an adverse event. One way to approach this is to combine information with various studies in a toxicoproteomics database. Integrating protein expression data with traditional toxicity endpoints will provide an increasingly valuable source of information. It should also be possible to combine this information with data from other disciplines such as genomics and metabonomics ('omics' technolgies) as well as epigenetics to develop a systems toxicology approach. The resultant information may be used to elucidate mechanisms of toxicity and biomarkers related to the mechanisms, as well as the identification of potential molecular targets for therapeutic intervention by drugs.

6 CONCLUSIONS

The field of toxicoproteomics is still evolving. The application of proteomics to toxicology is not without its limitations and may not be suitable in all circumstances, but it is anticipated that toxicoproteomics will contribute meaningfully to furthering our understanding of the process of toxicity.

RELATED ARTICLES

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Metabolomics in Systems Toxicology: Towards Personalized Medicine

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1 INTRODUCTION

Toxicology assessments are routinely used in pre-clinical animal studies to evaluate the effects of a potential new drug candidate. Despite the rigorous testing of new drug candidates in the screening and pre-clinical developmental phases, there are still some drugs that have toxicity issues that arise in the clinical testing phase and post-market. The failure of a drug candidate during the clinical testing phase and post-market results in patients that have been treated with a drug that is not completely safe and is an enormous loss to the pharmaceutical company in terms of both time spent in development and overall costs involved with research development (R&D). It is estimated that the cost of developing a new drug candidate is over 1 billion dollars and rising (Harrigan and Yates, 2006) whereas the time for development, testing and ultimate approval by the US FDA averages 8–10 years. However, only one in five new drug candidates actually make it through the approval process, with half failing in phase III clinical testing. This means patients were given drugs that were either not effective or possibly toxic. Therefore, novel and early translational biomarkers of toxicity are needed that will reduce the time and cost associated with drug discovery and approval.

It is clear that the current set of clinical chemistry biomarkers used to diagnose organ injuries have shortcomings. Table 1 shows a list of the currently used clinical chemistry markers and the tissue primarily associated with that marker. These markers are either late biomarkers, meaning that they increase only after significant organ damage has occurred, as is the case for blood urea nitrogen (BUN) and creatinine for renal injury, non-specific in the case of lactate, or not very specific as in the case of alanine aminotransferase (ALT), which can increase due to causes other than liver injury.

Table 1. Clinical chemistry measurements and the tissue primarily associated with that marker.

Plasma measurement	Tissue marker
Albumin	Liver and kidney
Alkaline phosphatase (AP)	Liver
Alanine aminotransferase (ALT)	Liver
Aspartate aminotransferase (AST)	Liver
Bilirubin	Liver
Bile Acids	Liver
Blood urea nitrogen (BUN)	Kidney
Cholesterol	Liver
Creatinine	Kidney
Glutamate dehydrogenase (GDH)	Liver
Glucose	Liver and Kidney
Glutathione-*S*-transferase (GST)	Liver
Lactate	Non-specific
Lipocalins	Multiple
Neutrophils	Multiple
Platelets	Multiple
Sorbitol dehydrogenase (SDH)	Liver

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Therefore, the pharmaceutical industry and regulatory agencies have invested significant energy and monetary efforts towards the identification of novel biomarkers of toxicity that can be applied in both pre-clinical and clinical studies. The omics technologies, which comprise transcriptomics, proteomics and metabolomics, are promising platforms for the discovery of new translational biomarkers of toxicity and health status. Additionally, the data acquired using multiple 'omics' platforms may be integrated to obtain a more comprehensive understanding of the toxicity in terms of the molecular pathways affected. Metabolomics and systems biology will not only benefit the field of toxicology but also play a large role in furthering the concept of personalized medicine. Because the metabolome encodes an individual's metabolic phenotype in addition to their genotype, the pre-dose spectrum may be useful in determining how a particular individual will respond to a drug regimen. This chapter will start with a general discussion regarding the role of metabolomics in systems toxicology, followed by some discussion related to metabolomics standards and quality control. Furthermore, examples of pre-clinical and clinical applications of metabolomics in toxicology will be discussed in some detail with an emphasis on recent works in the field. This will be followed by reviews on dynamics and metabolic fluxes, which are specialized areas of metabolomics. This chapter will also cover the role of metabolomics in personalized medicine, which plays an important role in directing medical science towards a greater mechanistic understanding of health, disease and treatment. The significance of metabolomics in the investigation of the complex relationships between drug–drug and drug–nutrition interactions elucidating health maintenance and disease development will be discussed before ending the chapter with a discussion on challenge tests in metabolomics.

2 ROLE OF METABOLOMICS IN SYSTEMS TOXICOLOGY

2.1 Omics Technologies and Systems Toxicology

Systems toxicology has been defined as "the study of the perturbation of biological systems by chemicals and stressors, monitoring changes in molecular expression and conventional toxicological parameters, and iteratively integrating response data to describe the functioning of an organism" (Waters and Fostel, 2004). A systems toxicology approach involves the integration of traditional toxicology endpoints (i.e., clinical chemistry and histopathology data) with data acquired using omic technologies. The omics platforms include genetics, transcriptomics, proteomics and metabolomics, and provide snapshots of the system at various time-points (Fiehn *et al.*, 2000). Figure 1 shows how genetics, transcriptomics, proteomics and metabolomics, are connected and are used to define systems biology or systems toxicology. Genetics relates the inherited genetic code of a patient or other animal species in pre-clinical and clinical testing with health status and responses to medical treatments. Transcriptomics studies changes at the level of mRNA expression; proteomics investigates alterations in protein translations and metabolomics evaluates modifications in the small molecule metabolites following a toxic insult or after a change in health status with the onset of disease. Often the effects of external factors such as environment, age, nutrition, physical exercise, gut microflora and others are neglected when evaluating omics endpoints but these external factors need to be considered as they can affect all the omics analyses. Many metabolites are under homeostatic control in the cell through feedback networks that are shown as dashed lines in Figure 1. Metabolomic profiles are primarily connected with downstream changes in transcription and

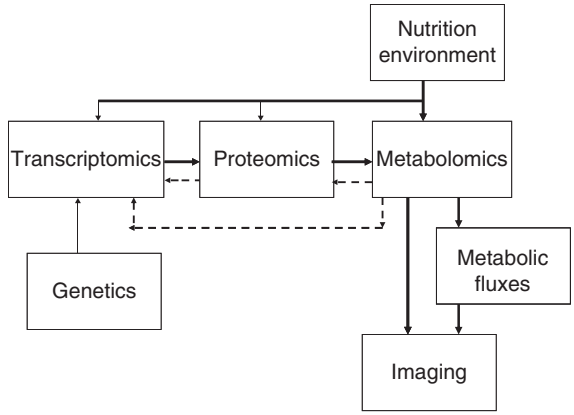

Figure 1. A schematic connection of genetics, transcriptomics, proteomics and metabolomics in systems toxicology.

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proteomic changes and also with nutrition and other environmental factors. Because metabolic changes are related to many different inputs, understanding the changes in the metabolome can be challenging. One way is to correlate these changes to transcriptomic or proteomic changes but this is limited by the fact that genomics and proteomic alterations occur on different time scales and usually only one time point is evaluated per animal, which makes understanding dynamical responses in most systems biology experiments hard to achieve. Another way to evaluate the changes in the metabolome is through evaluating flux analysis, which employs isotope-labelled tracers such as U-^{13}C6 glucose to analyse the incorporation of ^{13}C from glucose into metabolites in downstream metabolic pathways. Additionally, some metabolic changes and metabolic fluxes can be evaluated by MRI imaging (Kanamatsu *et al.*, 2007). Metabolomics, metabolic flux analyses and imaging can play a major role in both toxicology studies and the advancement of personalized medicine.

A systems-level understanding of a drug's mechanism of action or toxicity can be obtained through evaluation of the complex interactions between genes, proteins and metabolites together with traditional toxicology and histopathology endpoints. The ability to better understand mechanism(s) and mode(s) of action is beneficial on several fronts. First, a better mechanistic understanding of drug-induced toxicity will allow for the rapid screening of compounds under development earlier in the pipeline. Second, the non-invasive translational biomarkers can be used in pre-clinical and clinical studies. Third, the ability to identify new biomarkers using a systems toxicology approach may make it feasible to identify a susceptible subset of the population that may benefit by avoiding specific drugs.

To implement a systems toxicology approach, a strong bioinformatics component is required. The bioinformatics element is necessary to elucidate biomarkers, pathways and mechanism(s) from the diverse, highly dimensional omics datasets. Furthermore, it is required that traditional clinical endpoints be tied in to the data analysis to generate models and derive mechanism(s) and mode(s) of action. The genetics, transcriptomics, proteomics and metabolomics datasets are usually analysed to determine mechanism and biomarkers of toxicity and disease status. However, there is rarely a direct correlation between the gene transcript for instance and a specific protein or metabolite. The bioinformatics component can assess all the datasets and changes at all levels to fully elucidate the molecular pathway that is operating under a given set of conditions whether it be due to a toxic response or altered health status.

The metabolite changes noted in biofluids and tissue samples are the result of many influences including altered gene expression and environmental changes. However, changes at the transcript level do not cause instantaneous alterations in protein levels or metabolite concentrations; rather there is a time lapse before or after gene activity is altered. Furthermore, there is not necessarily a one-to-one correlation with a particular transcript and associated metabolite(s). Therefore, in order to establish modes of toxicity or mechanisms of disease, it is important to characterize these components at multiple time-points, doses and associated toxicological effects. By evaluating changes in the transcripts, proteins and metabolites at different times, a more complete analysis of pathway perturbations can be accomplished to qualify a particular biomarker or pathway. Once a marker has been proposed, it needs to be evaluated at different times to determine how the biomarker level progresses with recovery or as the disease progresses. Because unfocused metabolomics data are generally obtained more rapidly than the other omics technologies, this platform can be used in studying development and design for systems analyses by determining time-points of interest for the other omics platforms.

Technologies and methods such as metabolic profiling have the potential to identify toxicity early in the drug discovery process, thus protecting patients and saving medical institutions and pharmaceutical companies' resources such as time and finances. As noted previously, the estimated cost and time for developing a new chemical entity (NCE) in 2006 was high with a large attrition rate especially at the phase III clinical testing stage (Harrigan and Yates, 2006). Therefore, a major effort of omics research in toxicology has been in pharmaceutical drug discovery with the goal of identifying new biomarkers of drug toxicity that can be used in pre-clinical and clinical studies (Robertson, 2005). Identification of biomarkers that can accurately detect toxicity in clinical studies remains a high priority because the traditional

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markers do not have the accuracy and sensitivity required. More accurate toxicity biomarkers that can be used in pre-clinical drug development and later in clinical trials have the potential to impact pharmaceutical drug discovery by lowering both the cost and time associated with the development and marketing of a NCE, thus improving patients' access to high-quality therapeutics. For example, metabolomics methods were used in a pre-clinical study at Merck on a compound known to cause hepatotoxicity in several species (Mortishire-Smith *et al.*, 2004). Statistical analyses showed that the treated group was distinguished from the control group by the relative depletion of tricarboxylic acid cycle intermediates and the appearance of medium-chain carboxylic acid. *In vitro* experiments with this compound showed it caused defective metabolism of fatty acids. Metabolomics therefore provided mechanistic insight into the hepatotoxicity of a drug. This type of knowledge may be applied in future drug discovery to identify compounds that will also cause hepatotoxicity in the same species.

Metabolomics along with systems biology data also has the potential to further our understanding of toxicity and increase our ability to predict idiosyncratic drug-induced toxicity, which accounts for 6–10% of all adverse drug reactions (Ju and Uetrecht, 2002). An idiosyncratic reaction is defined as "any adverse effect that cannot be explained on the basis of the known mechanisms of action of the drug and occurs mostly unpredictably in susceptible individuals only, irrespective of dosage" (Zaccara, Franciotta and Perucca, 2007). Idiosyncratic adverse drug reactions (IADRs) are not generally diagnosed due to their rare occurrence but are of great concern for the pharmaceutical industry, regulatory agencies and consumers (Zaccara, Franciotta and Perucca, 2007). Furthermore, cause–effect relationships are difficult to establish because IADRs may be produced by many factors that are unique to an individual (Navarro and Senior, 2006). Some of the specific risk factors proposed include underlying disease, differences in nutrition, polypharmacy, individual drug metabolism and bioactivation, inhibition of cell function, age, physical activity, genetics and inherited metabolic defects, and the innate immune system (Ulrich, 2007). These risk factors will also have an impact on an individual's overall metabolome, which may be useful for the prediction of idiosyncratic reactions on a case-by-case basis. Generating more complete omic analyses in many individuals will be necessary to test classification algorithms of IADRs in clinical studies. Analyses of biofluids by metabolomic and proteomic methods would add critical data for the development of personalized medicine. The USFDA has started a biomarker qualification process, which aims to evaluate data for new drug-independent biomarkers and determine whether these biomarkers can be qualified (Goodsaid and Frueh, 2007).

2.2 Metabolomics Platforms

The most commonly used platforms for metabolomics studies have been proton nuclear magnetic resonance spectroscopy (1^{H} NMR) (Clarke and Haselden, 2008; Wei *et al.*, 2008, 2009; Park *et al.*, 2009; Powers, 2009; Um *et al.*, 2009) and mass spectrometry (MS) (Clarke and Haselden, 2008; Dorman *et al.*, 2008; van Vliet *et al.*, 2008). The increase in sensitivity of NMR with the availability of higher field strength instruments along with the introduction of the cryoprobe has made it possible to measure hundreds of metabolites within several minutes. NMR offers other advantages including the fact that all the protons within a molecule are detected the same and the data are highly quantitative by nature. NMR data are also highly reproducible in both intra- and inter-laboratory experiments with less than 2% variation between laboratories using spectrometers with different field strengths. However, there is a great deal of overlap in the metabolite chemical shift resonances, so that it requires 2D NMR experiments or additional techniques such as LC or MS to uniquely identify a metabolite of interest. Furthermore, metabolites present at submicromolar concentrations are not detected by NMR.

Magic angle spinning (MAS) NMR technology makes it possible to analyze intact tissue samples in a non-destructive manner. This technique can be applied to analyse tissue biopsy samples in the clinical setting and allows for a direct link between the biofluid metabolic profile and histopathology. MAS NMR spectroscopy has been demonstrated in metabolic profiling studies of drug toxicity, disease and health. The biochemical composition of intact liver tissue from mice administered acetaminophen (APAP) was

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investigated using MAS NMR (Coen *et al*., 2003). The results indicated that there was a possible mitochondrial inability to utilize pyruvate along with an impairment of fatty acid beta-oxidation in the liver. The effects of tamoxifen in endometrial cells were evaluated by Griffin *et al*. (2003) by high-resolution MAS NMR. Several metabolites were affected and together indicated altered RNA transcription and cell membrane turnover. Hydrazine is a compound known to have both hepatotoxic and neurotoxic effects in animals. Therefore, the effects of hydrazine on liver, brain and kidney tissue from Sprague–Dawley rats were evaluated by MAS NMR (Garrod *et al*., 2005) and showed many changes in the endogenous metabolites, including an increase in alanine in all tissues, that may be indicative of a general inhibition of alanine transaminase activity.

Historically, MS and gas chromatographic (GC) methods combined with pattern recognition have been applied for metabolic profiling studies but these approaches targeted specific chemical classes (Lehnert and Hunkler, 1986; Bamforth *et al*., 1999; Chace, Kalas and Naylor, 2002). More recent studies use MS not only to profile specific classes of chemicals but to profile the entire biofluid or tissue sample. Continuing advances in sensitivity and reproducibility of MS technology have led to its resurgence as a popular tool for metabolic profiling. MS detection is generally coupled with an initial separation step usually accomplished by high-performance liquid chromatography (HPLC) or GC methods. Ultra-performance liquid chromatography (UPLC) is another method that has been coupled to MS in metabolomics experiments because of its higher sensitivity and speed over HPLC. MS methods offer greater sensitivity than NMR and the ability to measure metabolites at concentrations down to the picogram level. Because biofluids and tissues contain a wide range of molecules including acidic, basic, amphoteric and neutral components, the ionization method and acquisition parameters will directly influence the types of metabolites that can be measured, making it possible that several experiments will need to be run. For example, if an electrospray source is used, it may be necessary to collect data in both positive and negative ionization modes. Ion suppression or enhancement can also affect quantitation and reproducibility of the MS data. Finally, LC/MS data require reproducibility in both chromatographic retention time and *m/z* mass measurement. Column, flow rate, voltage and gradient conditions can all affect the LC/MS data, making it extremely difficult to make direct comparisons between laboratories.

Although most studies have used either NMR or MS for metabolic profiling, the application of both technologies allows for a more complete characterization of the changes in metabolome that occur in biofluids or tissues. The use of both techniques in tandem on a sample set provides a powerful means of elucidating changes in the metabolome as a result of drug efficacy, toxicity or disease status. These changes can be assessed in terms of the molecular pathways being perturbed and allow for the elucidation of the mechanism(s) at work under a particular set of conditions.

3 METABOLOMICS STANDARDS AND QUALITY CONTROL

There are two types of metabolomics experiments: one is an unfocused approach (a global profiling) that usually involves chemometric analysis of NMR or MS data to determine new biomarkers or patterns of toxicity or health status. The other is a focused metabolic profiling approach when preselected chemical classes (e.g., vitamins, amino acids, bile acids, steroids or lipids) are evaluated and related to health status. In either case, standards and quality control measures are required during NMR, LC/MS or GC/MS data acquisition.

A major challenge in the interpretation of NMR and MS spectral data is in the identification of peaks of interest. Although early studies simply focused upon spectral features that differed between classes, it has become a necessity to identify the chemical that gives rise to a particular spectral feature especially when trying to put forth that feature as a biomarker (Trygg, Holmes and Lundstedt, 2007; Wishart, 2008a). Identification is complicated by the fact that there are few public databases available (Wishart, 2008b). It has been reported that a high-field proton NMR spectrum may contain from 2 to 3000 peaks of which 10% are known and an MS spectrum may contain up to 15 000 peaks of which 5% are known (Clarke and Haselden, 2008). However, these are only estimates and the number of peaks detected and identified depends highly upon the specific analytical technique and sample preparation. The generation of appropriate databases is

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a major challenge for metabolomics studies especially with regards to MS in which the spectral data depends highly upon the analytical conditions and chromatography.

In order to generate appropriate databases, there needs to be an agreed-upon set of standards for different types of experiments, samples, etc. The goal of standardization is to optimize the amount and quality of the biological information generated in metabolomics experiments by defining the use of chemometrics and quantitative approaches, defining the best-practice procedures and defining the minimum reporting requirements, such as has already been performed for microarray analysis (Brazma *et al*., 2001). Although the metabolomics standards initiative was formed in 2005 and, in 2007, this group published reporting standards for the metabolomics society and publication in the journal *Metabolomics*, there still remains no set of standards for metabolomics analyses that have been accepted by other journals and the medical community at large (Sansone *et al*., 2007).

Scalbert *et al*. (2009) recently published a review article that addressed some of the issues with MS-based metabolomics approaches. Many of the issues raised apply not only to MS approaches but to NMR approaches as well. In some cases, the issues raised are more problematic in terms of metabolomics studies with human subjects because animal studies can be more stringently controlled. The problems described include: (i) protocols that are not optimized for metabolomics studies, such as uncontrolled diet and time of sample collection; (ii) variations between individuals that are larger than treatment effects; (iii) variation in methods of sample collection and storage; (iv) profiling methods that do not sufficiently cover the metabolome; (v) spectral quality; (vi) MS data that cannot be easily compared between studies or laboratories; (vii) inconsistent results due to data extraction method; (viii) overfitting of data and incomplete reporting; (ix) failure to adequately identify metabolites; and (x) difficult interpretation of changes in metabolomics profiles. The authors suggest solutions and recommendations to address the aforementioned issues, with the biggest commonality among the solutions being related to the need to develop standardized procedures for sample collection, analysis and interpretation that are widely disseminated among the metabolomics community.

4 PRE-CLINICAL AND CLINICAL EXAMPLES OF METABOLOMICS IN TOXICOLOGY STUDIES

4.1 Pre-clinical Studies

There have been numerous examples of the use of metabolomics for toxicology studies. Much of the early work in metabolomics was focused on its use in the pharmaceutical industry as a tool for safety assessment in drug discovery (Griffin and Bollard, 2004; Robertson, 2005). One of the initial and most well-known efforts aimed at applying metabolomics technologies to toxicology was the Consortium for Metabonomic Toxicology (COMET), which was initiated by Jeremy Nicholson at Imperial College (London, UK) along with six pharmaceutical companies. The consortium investigated approximately 150 known toxicants to generate an expert system that could be applied in future drug development studies to determine whether a compound under development had the potential to be toxic especially with regards to liver and kidney function. The final reports of the consortia indicated that models predictive of liver and kidney toxicity were successfully developed and are now being applied by the pharmaceutical companies involved in the consortium in pre-clinical drug development studies (Lindon *et al.*, 2003, 2005; Ebbels *et al.*, 2007; Nicholson, Keun and Ebbels, 2007).

In a review by Robertson (2005), the advantages of metabolomics in toxicology are discussed with regards to its potential impact in the pharmaceutical industry especially in pre-clinical toxicology. Several applications of metabolomics in pharmaceutical development are discussed including screening, biomarker identification and elucidation of mechanism(s) of toxicity. In terms of screening, it has been envisioned that metabolomics can play a role not only as a generic screen of potential toxicity but also as an early screen for biomarkers of toxicity from a compound known to have a particular effect. Robertson *et al.* (2001) have shown metabolomics to be a useful screening tool for drug-induced vasculitis, which usually requires tissue biopsies to detect.

Biomarker discovery has also been a major effort in metabolomics research. The current clinical chemistry measurements generally lack sensitivity and specificity and often only show a significant change when tissue damage has occurred. In contrast, metabolomics along with the other omics

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technologies offers the potential to discover earlier biomarkers of toxicity that have greater sensitivity and specificity. To date, the major focus of biomarker discovery in the pre-clinical setting has been towards the identification of markers of liver and renal toxicity. The early identification of liver toxicity is of interest because it represents the major reason that a drug fails in the later stages of development or is removed from the shelf post-market. There are many recent examples where metabolomics has been used to investigate potential new biomarkers of specific organ toxicity as described below.

Lee *et al.* (2007) used a GC/MS-based approach to investigate the toxicological effects of nonylphenol (NP), an endocrine disrupter. Rats were injected with vehicle, 50 or 250 mg NP/kg for 4 days and urine samples were collected. Two potential urinary biomarkers of NP toxicity were identified related to steroid metabolism. In addition to biomarkers of toxicity that are altered post exposure, it has also been shown that metabolomics studies may be useful in discovering biomarkers of susceptibility. In one study, the metabolic pattern in the urine NMR spectrum prior to dosing rats with APAP correlated with the degree of liver damage found after drug administration (Clayton *et al.*, 2006). Similar results were found in a study of the hepatotoxicant, galactosamine, wherein models built using the pre-dose spectra were able to predict a toxic outcome, which has 0.99 correlation with the post-dose days 2 and 3 clinical parameters ALT and AST (Schnackenberg *et al.*, 2007).

Metabolomics in systems biology also has the ability to elucidate mechanisms of toxicity. A well-designed systems toxicology approach will be able to identify mechanisms and pathways of interest. One of the issues in pre-clinical and clinical research is that often a mechanism is not identified. Metabolomics offers the ability to not only help to identify mechanisms but also discover corresponding biomarkers that can then be used to classify compounds based upon similar mechanisms. Nicholls *et al.* (2007) provided a mechanistic hypothesis to the neurotoxic effects of hydrazine. Their metabolomics analyses revealed an increased level of 2-aminoadipate, which is an intermediate in the metabolism of lysine and tryptophan (Candito *et al.*, 1995) and related to the kynurenic acid levels in the brain. Kynurenic acid is an important endogenous metabolite that antagonizes the actions of glutamate and aspartate and prevents the neurotoxic action of quinolinic acid (Moroni *et al.*, 1988). An NMR-based metabolomics study identified markers that were associated mechanistically with phosphodiesterase type 4 inhibitor-induced vascular lesions in rats (Slim *et al.*, 2002).

One pathway of interest related to drug toxicity is the *trans*-sulfuration pathway, which describes the production of glutathione from S-adenosylmethionine (SAMe). There have been numerous reports that have indicated a role of glutathione in detoxification following administration of a toxic drug dose (Comporti, 1987;Jaeschke, Knight and Bajt, 2003; Martin, Racz and Forkert, 2003; Kaminskas, Pyke and Burcham, 2004; Hudson *et al.*, 2005; McMillan and McMillam, 2006;Zegura, Lah and Filipic, 2006; Lotkova *et al.*, 2007;Saravanan, Sindhu and Mohanakumar, 2007). Our previous studies have shown a decrease in SAMe, which is the primary source of the sulfur group that is donated to produce glutathione, following acute or chronic dosing with APAP (Schnackenberg *et al.*, 2009). These results indicate that the trans-sulfuration pathway may be of great importance in terms of toxicity following administration of a drug that can be biotransformed to a reactive metabolite through cytochrome P450 oxidation. Following formation of the reactive metabolite, a cellular oxidative stress response is initiated to detoxify the reactive species and remove it from the system. Figure 2 shows the pathway from SAMe to glutathione (GSH) and eventually *N*-acetyl-cysteine (NAC) conjugates in the *trans*-sulfuration pathway and how this pathway is directly connected to one-carbon metabolism and folic acid including the roles of vitamin B_{12} and vitamin B_6. Vitamin B_{12} donates its methyl group to homocysteine, which is then converted back to methionine, an important source of methyl groups for several reactions involving SAMe. SAMe is the primary source of sulfur for glutathione (GSH) production which is often used for detoxification of reactive metabolites. Drug–NAC conjugates are the breakdown products of the detoxified drug–GSH conjugates through the reduction of the reactive drug metabolites with GSH. We and others have observed correlations between drug–NAC conjugate levels with clinical chemistry and histopathology results (Wagner *et al.*, 2006, 2007). Reactive metabolites have been reported to be necessary in most drug-induced toxicities but are not sufficient by

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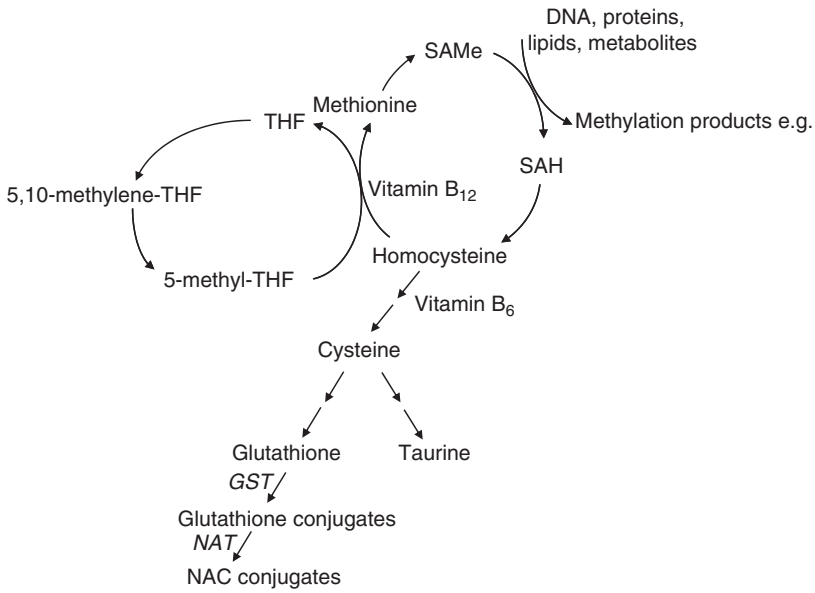

Figure 2. The *trans*-sulfuration metabolic pathways (related to drug toxicity) from SAMe to glutathione and eventually to drug conjugation with GSH.

themselves to cause the toxicity (Walgren, Mitchell and Thompson, 2005). Detection of endogenous or xenobiotic mercapturic acids (MAs) would be useful to monitor the patient more closely in the clinic and possibly supplement the patient with sulfur-containing metabolites such as NAC.

Based on the previous results, perturbations in the *trans*-sulfuration pathway were further assessed through the evaluation of creatine [byproduct of the conversion of SAMe to *S*-adenosylhomocysteine (SAH)], taurine (byproduct of the synthesis of glutathione in the *trans*-sulfuration pathway) and SAMe (Schnackenberg *et al.*, 2009). The results showed that urinary levels of creatine were increased on day 1 or 2 in eight high-dose liver toxicity studies and the taurine concentration in urine was increased in three of five liver toxicity studies whereas SAMe was found to be reduced in four of five liver toxicity studies. Transcriptomic results showed that some genes involved in the *trans*-sulfuration pathway were disturbed, which validated the metabolomics data that *trans*-sulfuration pathway from SAMe to glutathione was perturbed due to the administration of hepatotoxicants. In another liver toxicity study, we utilized the high-throughput LC/MS-based metabolomic assay to investigate APAP and its metabolites excretion profile in urine from rats treated with a single dose of 0.2% carboxymethylcellulose (CMC, vehicle control), 400 or 1600 mg APAP/kg body weight (Sun *et al.*, 2008;Sun, Schnackenberg and Beger, 2009). The results showed that urinary APAP–NAC had high and statistically significant correlations with clinical chemistry data, creatine and histopathology data at 48 h post-dosing. SAMe concentration alterations were also detected as inversely proportional with urinary APAP–NAC concentration changes in this study. A high concentration of urinary SAMe indicated low level of urinary APAP–NAC. All these liver toxicity studies suggest that metabolomics techniques are very useful to access the potential toxicity of a drug through drug metabolite pharmacokinetic profile studies from reactive metabolites and endogenous metabolites involved in *trans*-sulfuration pathway. Additionally, metabolites involved in one-carbon metabolism and vitamins B_6 and B_{12} may be important indicators of overall toxicity status following a toxic insult as noted in Figure 2.

Metabolomics approaches were used to discover biomarkers of nephrotoxicity in Sprague–Dawley rats treated with the nephrotoxins gentamicin, cisplatin or tobramycin (Boudonck *et al.*, 2009). Non-targeted metabolomics analysis was performed on urine and kidney samples collected from rats after 1, 5 and 28 dosing days. Increases in urinary levels

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of polyamines and amino acids were observed in drug-treated rats after one dose. The changes were seen prior to any observable histological kidney damage and conventional clinical chemistry indications of nephrotoxicity. After prolonged chronic dosing, there was a progressive loss of branched-chain amino acids in urine and a concomitant decrease in amino acids and nucleosides in kidney tissue. The increase of amino acids in urine is consistent with previous studies (Macpherson, Moscarello and Goldberg, 1991; Ghiculescu and Kubler, 2006; Portilla *et al*., 2006; Niemann and Serkova, 2007; Xu *et al*., 2008). Aminoaciduria may have to do with diminished renal reabsorption by the tubules or increased permeability of the glomerular membranes (Macpherson, Moscarello and Goldberg, 1991; van de Poll *et al*., 2004).

To date, there have been few reports of integrated systems biology studies. This may have been due in part to the complexity of integrating and understanding data generated on the three different platforms. In two studies of bromobenzene-induced hepatotoxicity, changes in gene expression, protein expression and select metabolites were analysed (Heijne *et al.*, 2003, 2004). A systems biology study of valproic acid (VPA)-induced hepatotoxicity in CD-1 mice indicated a perturbation in the glycogenolysis pathway (Schnackenberg *et al.*, 2006). Craig *et al.* 2006 utilized a systems toxicology approach to investigate the hepatotoxicity of methapyrilene, a histamine antagonist that causes periportal liver necrosis in rats. The integrated genomics, proteomics and metabolomics results indicated altered energy use as well as oxidative stress following dosing. Recently, Bundy *et al.* (2008) reported a systems toxicology study using cDNA transcript microarrays and NMR-based metabolic profiling to investigate the response to copper in earthworms. Both the microarray and metabolomics results indicated a disruption in energy metabolism especially with regards to carbohydrate use and oxidative phosphorylation. The Liver Toxicity Biomarker Study (LTBS) was developed to investigate drug-induced liver injury using omics technologies (McBurney *et al.*, 2009). The differences in molecular events caused by two compounds with similar structure and mechanism of action but with different hepatotoxic potential were evaluated (McBurney *et al.*, 2009). The first phase of the study using entacapone and tolcapone as the model compounds yielded potential markers and, more importantly, illustrated the value of a systems approach.

4.2 Clinical Studies

There have been numerous examples of clinical metabolomics in toxicology assessments. Following organ transplant, some patients have toxic reactions to cyclosporine, which is an immunosuppressive drug frequently prescribed to prevent rejection. Several studies have investigated the metabolic changes in urine to detect biomarkers of cyclosporine cytotoxicity. Serkova and Christians (2005) investigated the metabolic effects of cyclosporine using an NMR-based approach. Spectra from urine were acquired following dosing with cyclosporine alone and in combination with other immunosuppressants, sirolimus or everolimus, to determine the effects of co-administration on the nephrotoxic effects of cyclosporine. Treatment with cyclosporine resulted in increased levels of glucose, hydroxybutyrate, creatine, creatinine, trimethylamine-*N*-oxide (TMAO) and cholesterol and a decreased concentration of glutathione. Co-administration of cyclosporine with sirolimus resulted in an enhanced toxicity, whereas co-administration with everolimus showed some protection against the cyclosporine toxicity based upon the acquired metabolic profile. Metabolomic approaches have been used to access other aspects of health. For example, Constantinou *et al.* (2004) developed rapid methods for detection of inborn errors of metabolism using metabolomics methods. It was also shown that metabolomics could be used to detect and assess the severity of coronary heart disease in a clinical setting (Brindle *et al.*, 2002). Brindle *et al.* (2003) also showed a link between serum metabolic profile and hypertension.

5 DYNAMICS AND METABOLIC FLUXES

A major initiative of systems biology is to obtain a better understanding of the integrated omics data (Kell, 2006). Much of the omics emphasis is on the individual collection of transcript, protein or metabolite changes with very little value placed on the system as a whole and the dynamical interaction of transcripts, proteins and metabolomics. As metabolomics data can be obtained easily over

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multiple time-points, it offers an opportunity to gain dynamical systems biology information. Figure 3 shows the nine possible states for transcript levels and metabolites at any point in time. Most of the time, many transcripts and endogenous metabolites in tissues and blood are under homeostatic control, which is the centre of the diagram. However, at any one time, genes can be expressed at higher or lower levels and metabolites can be either up- or down-regulated. If a gene and the genes' corresponding metabolite are significantly increased, this would be an in-phase increase in the pathway at that time-point. Conversely, if a gene and a metabolite directly associated with that gene are both reduced, this would be an in-phase decreased pathway at that time-point. As the metabolites are downstream of the gene changes, the temporal response of the gene to associated metabolite changes would reveal whether the system is overdamped (tightly controlled), underdamped (loosely controlled) or critically damped. Diabetes is an example of an underdamped system between glucose and insulin. These are not the only scenarios, there will be time-points where the genes are up but the metabolites associated with that gene are down, which could represent a slow response to return the metabolites to their homeostatic levels. Alternatively, the metabolites in a gene pathway may be increased while the gene is decreased and this would be an alternative out-of-phase response. Gene transcripts and metabolites may be out of phase because of different time lag responses and the timing and effectiveness of feedback loops. Usually, metabolites are directly affected by more than one gene, so there is no one-to-one correspondence and the system biology response is connected to timing and nutritional value of dietary intake.

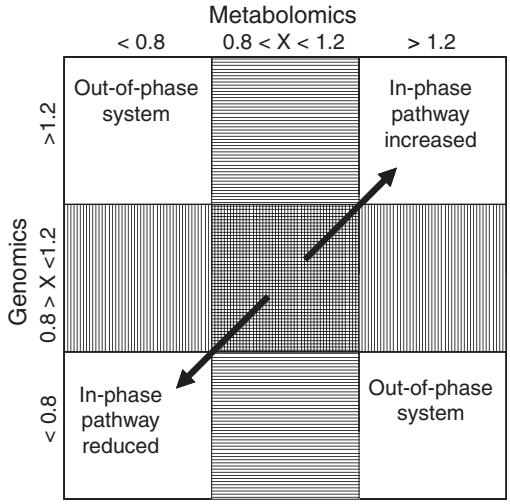

Figure 3. A dynamic relationship between transcript levels and metabolites at any time-point.

In the four cases described above, omics data represent snapshots in time of genes, proteins and metabolites that are changing in response to an agent and they will usually return to homeostatic levels provided no permanent organ damage or health status changes have occurred. However, the one time-point may not be enough to determine whether the system is underdamped, overdamped or critically damped. The dynamical route back to homeostasis will be different for each gene, protein and metabolite, which is why one time-point systems biology analyses are so hard to reproduce and mechanistically interpret. One of the biggest challenges for systems biology will be connecting transcriptomics, proteomics and metabolomics data to form consistent integrative mechanistic understanding of toxicity and health status. If one finds in-phase correlations between transcripts, proteins or metabolites in tissue or blood, this is usually a significant, interpretable finding. One way to improve the chance of finding usable metabolomics biomarkers is to use biofluids such as urine which is collected over a time period (usually 24 h). The metabolomics analysis of urine allows biomarkers to be built up over time, and therefore there is less dependence on a single time-point from one animal but the down side is there is less likely to be a significant in-phase correlation of the metabolite biomarkers with gene transcripts or proteins detected in tissues.

A potential way to better understand the dynamics of system biology at this level is through the analysis of metabolic fluxes (Marin *et al*., 2004). Metabolic flux analysis involves a labelled metabolite (usually ^{13}C-labelled glucose) and measurements of ^{13}C-labelled products over time. In this way, the kinetic analysis (turnover) of the metabolites can be determined. The addition of transcriptomics or proteomics flux data will increase our dynamic understanding of specific pathways (Zhao *et al*., 2009).

Metabolic flux analysis of ^{13}C-labelled glucose has been used to determine the turnover of select metabolites to develop biomarkers of toxicity, efficacy and disease states that can be used in

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pre-clinical and clinical studies (Lee *et al*., 1998; Boren *et al*., 2003; Marin *et al*., 2004; Boros *et al*., 2005; Beger *et al*., 2009; Kominsky *et al*., 2009). We have used ^{13}C glucose fluxes to evaluate the effect of VPA (Beger *et al*., 2009). Briefly, we observed that ^{13}C-labelled glucose deposited as liver glycogen or as ribose of RNA was decreased by VPA treatment and incorporation of ^{13}C via acetyl CoA into plasma cholesterol was significantly lower at 60 min. The flux markers, as well as decreased glucose re-absorption and an increased disposal via urine, all serve as early flux biomarkers of VPA-induced adverse metabolic effects in mice. These observations are consistent with our previous metabolomics analysis of VPA (Schnackenberg *et al*., 2006).

6 METABOLOMICS IN PERSONALIZED MEDICINE

Personalized medicine is based on the concept that a person's genetic information can be used to predict whether that individual will respond favourably or adversely to a drug or medical therapy (Nebert and Vesell, 2005; van der Greef, 2005;van der Greef, Hankemeier and McBurney, 2006; Schnackenberg and Beger, 2006). Based on this genetic information, health care professionals would be able to determine the optimum drug and drug regimen for each patient. The ability to tailor therapies to an individual would result in more effective medical treatments when avoiding or reducing adverse drug reactions. Considerable effort has been made to use genetic information, especially with regard to single nucleotide polymorphisms (SNPs), for individualizing medical therapies (Nebert, Jorge-Nebert and Vesell, 2003; Nebert and Vesell, 2005). However, most SNPs tests do not predict an individual's response because a single gene acts in concert with other genetic and environmental factors; that is, SNPs are context specific (Helgadottir *et al*., 2006). A person's phenotype results from the action of many genes and their interactions with environmental factors that include, for example, nutrient intake over the lifetime of the individual. Other external factors such as diet, antioxidant status, energy status, gut microbiota, health status, alcohol consumption, BMI and age will influence the expression of one's genetic potential. Therefore, the ability to accurately assess an individual's genotype and phenotype will be a critical component in determining the correct drug treatment or the potential response following a therapeutic intervention. The phenotypic make-up of an individual may provide a more relevant and current picture of how an individual may respond under a given set of circumstances. For instance, many drugs and alcohol are known to interact with other drugs adversely (Gorard, 2006;Phillips, Barker and Eguchi, 2008). Metabolomics has the potential to provide information about the patient's genotype and phenotype because it represents the interaction of gene and environment and this interaction can be monitored over time.

The omics platforms have generated data that can be related to mechanisms of disease or drug toxicity and drug efficacy (Fiehn, 2001; German *et al*., 2004). These research platforms will lead to new opportunities for personalized health because they add additional details that can improve our current knowledge about the health and disease continuum. A major advantage of metabolomics is that it can be collected from a patient's biofluid samples, which can be gathered over a period of time or throughout the lifetime of the patient. The drawback of metabolomics is that the markers from biofluids are less likely to be directly related to a specific tissue (Jordan and Cheng, 2007;Spratlin, Serkova and Eckhardt, 2009) and one may need additional information such as imaging to determine the source of the biofluid biomarkers. Of the omics platforms, metabolomics has the potential to greatly impact personalized health due to its ability to provide complementary information to one's genetic profile. This information contains information on phenotype characteristics such as age, nutritional status, polypharmacy, alcohol usage and other phenotype characteristics that can be combined with genetics and related to drug efficacy, drug toxicity and drug clearance. Therefore, biomarkers will need to be utilized based on a patient's age, gender, genetic and other characteristics. The metabolic phenotype information has been referred to as "metabotype" of individuals or populations (Holmes, Wilson and Nicholson, 2008). The pool of metabolites detected in biofluids and tissues at a given time will be affected not only by genetic and environmental factors but also by factors such as homeostasis, which tends to limit metabolic changes to short periods. Specific ways on how to deal with homeostasis will be discussed later. Regardless, the

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combination of genetic and phenotypic factors is likely to be the most descriptive aspect in determining whether an individual may experience an adverse drug reaction or respond favourably to a drug. Furthermore, the ability to link changes in the metabolite profile with genetic factors using a bioinformatics approach should elucidate mechanism(s) of drug efficacy and toxicity. Combining these platforms will lead to a more precise characterization of an individual's genotype and phenotype characteristics, which should enhance the probability of success for personalized medicine (Hall *et al*., 2002; Griffin, 2003; Ippolito *et al*., 2005).

Metabolomics has the capability to capture the metabolic profile at a given point in an individual's life, which is dynamic throughout the lifespan. We have shown in two pre-clinical age-related toxicity studies that there were similar metabolic urinary changes in control rats as a function of age (Espandiari *et al*., 2007, 2008). We observed changes in ketone bodies, metabolites involved in the Krebs cycle, amino acids and hippurate as a function of age in control rats in these studies. In the clinic, it will be necessary to associate these point-in-lifetime measurements with phenotype conditions that will affect how a patient responds to a medical treatment to develop the concepts and biomarkers needed for personalized medicine. For instance, how does antioxidant status affect drug toxicities that are caused by reactive metabolites and oxidative stress? Are the effects of antioxidants age or disease dependent? Analysing an individual's metabolic profile will be an essential component for detecting drug elimination and drug metabolism pathways, and assessing the effectiveness or toxicity of a particular medical treatment. It has been hypothesized that genomics, transcriptomics, proteomics, metabolomics and omic responses to challenges may be applied for sorting a patient population into those who would respond favourably, those who would not respond or those who would have an adverse response to a given medical treatment. For those patients classified as non-responders or at risk of adverse reactions, lower doses or alternate treatments could be used.

The incorporation of metabolic profiling data into the concepts and methods of personalized medicine will present substantial challenges given the vast genetic and dietary variation among the human population. The analyses of complex gene–environment interactions will require genetic and metabolic characterizations for each individual in greater depth than has been performed in any study to date. Such in-depth analyses will need to be carried out in several large studies to determine the validity of genetic and metabolic biomarkers due to the tremendous amount of genotype–phenotype interactions possible. Among the confounding factors for linking metabolic biomarkers to effects in specific organs is the fact that the concentrations of endogenous metabolites in biological fluids are linked to dietary intake. Because the majority of endogenous metabolites are used in most cells, very few metabolites can serve as a biomarker specific to a select organ. One approach that may address this complexity is to use patterns of metabolites as markers. Another approach is to use stable non-radioactive labelled tracers such as U-13C6 glucose to analyse downstream endogenous metabolic products related to specific pathways (Boros, Brackett and Harrigan, 2003; Harrigan *et al*., 2006; Maguire *et al*., 2006). In addition to providing pathway information, metabolic fluxes may also be associated with evaluating drug efficacy or toxicity for a given patient non-invasively using techniques such as magnetic resonance imaging (MRI) or magnetic resonance spectroscopy (MRS) (Wang and Yan, 2008).

Recent literature has shown that genetics and environmental factors, including the gut microbiota, interact to produce outcomes following a drug intervention or dietary alteration (Nicholson, Holmes and Wilson, 2005). The review described how the composition of the gut microbiota can be affected not only by diet but also by other environmental factors such as stress, which can lead to altered metabolism. Therefore, it is necessary to understand the function of the gut microbes to understand how they might also affect human health. NMR-based metabolomics has shown that even on a controlled diet, there is variation in the urine and plasma samples collected from healthy male subjects (Lenz *et al*., 2003). Within the subject population, minor variability was observed between inter-individual plasma samples but significant variability was observed between urine samples from different individuals over a 2-week study. The inter-individual variability in urine samples may have been due to dietary and gut microflora differences as noted in the review (Nicholson, Holmes and Wilson, 2005). Although the intra-subject variation in urine samples was much less, clear diurnal effects were noted. The alterations in urinary metabolites

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may reflect each individual's effort to maintain homeostasis in the plasma. This study reiterates the necessity of understanding the effects of diet and gut microbiota compositions because these may directly affect urinary metabolite biomarkers of drug toxicity. In another study, dietary and cultural effects on the metabolic profile have been examined and shown to influence the metabolic profile in urine (Lenz *et al*., 2004). In particular, cultural differences were noted between Swedish and British volunteers primarily based upon the dietary preferences, with the Swedish subjects excreting large amounts of trimethylamine-*N*-oxide due to their preference for a fish diet. This study indicates the strong influence of diet and stresses on the importance of dietary and/or lifestyle restrictions during clinical trials where possible. Dumas *et al*. (2006) also examined the metabolic profiles of individuals from several different regions in a large-scale epidemiological study and showed cross-population differences related to genetics, diet and gut microbiota composition using NMR. Holmes *et al*. (2007) recently reported an epidemiological study that addressed the large-scale population use of prescription and non-prescription drugs. NMR-based analysis was used to screen populations for self-administered drugs. The metabolic profiles were significantly influenced by dietary habits of the countries and this information may play a role in personalized medicine. Assfalg *et al*. (2008) showed that the day-to-day variability in the urinary metabolic profile could be removed by analysing multiple samples from the same individual over several months. Various algorithms identified invariant components, which were not only distinct but also reproducible for each healthy individual over the time of the study. Therefore, it appears that a process for eliminating daily metabolic "noise" from single individual and individual metabolic phenotypes now exists. These studies indicate that for an individual, the metabolic profile is relatively stable unless perturbed by drugs or changes in health status.

There are two potential general applications of metabolic profiling data in relation to personalized health. Metabolites and proteins from biofluids can be analysed before a patient receives a medical treatment and used with a metabolomic and protein phenotype database to discover biomarkers associated with favourable response to the drug. Some of these metabolic markers can be used as susceptibility markers to help identify patients who may develop IADRs, which has been called pharmacometabotyping or pharmacometabonomics (Clayton *et al*., 2006). Alternatively, biofluid samples can be obtained during drug treatment for analyzing pharmacodynamics and drug clearance as well as drug efficacy and toxicity markers. Clayton *et al*. (2009) recently published a proof of principle pharmacometabonomics study conducted in human subjects. Samples were collected from 99 healthy male volunteers that ranged in age from 18 to 64 years. Pre-dose urine samples were collected and then samples from 0 to 3 h and 3 to 6 h post-dosing with a therapeutic dose of APAP. Analysis of the pre-dose and post-dose spectra indicated that subjects with high pre-dose levels of *p*-cresol had a lower capacity to sulfonate APAP. It is known that saturation of phase I sulfonation and glucuronidation pathways during APAP dosing can lead to the increased formation of *N*-acetyl-*p*-benzoquinone imine (NAPQI), the toxic metabolite of APAP. A patient with lower capacity for APAP sulfonation could be more susceptible to forming NAPQI and having an adverse event. In addition to demonstrating the pharmacometabonomic approach in human subjects, this study also demonstrates the role of the gut microbiome and how it might be manipulated to improve drug efficacy and reduce adverse events.

Phase I and phase II clinical trials may be the optimal settings for evaluating metabolites in biofluids because control of diet and other patients' activities can be implemented. The ability to control diet or other confounding factors such as time of sample collection would aid the interpretation of the metabolic analyses. Dimensionality reduction algorithms (Motsinger and Ritchie, 2006) could be employed to analyse these metabolomic datasets with data from genetic analyses of candidate genes (Bauchet *et al*., 2007;Zembrzuski, Callegari-Jacques and Hutz, 2006). Such analyses may identify the critical biomarkers or pathways that need to be evaluated in phase III and IV trials besides identifying individuals that should or should not participate in phase III and IV trials. Generating "training sets" of single or multiple metabolites would allow for validation of biomarkers in phase III trials and potentially provide valuable information for the drug label.

The concept of using metabolomic data to determine susceptibility prior to dosing has been described as "pharmaco-metabotyping" (Clayton *et al*., 2006). This landmark paper demonstrated the

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importance of phenotype analyses in predicting a toxic outcome following administration of APAP. The pre-dose urine spectra correlated with the degree of liver damage found after administration of APAP. The authors reported higher levels of pre-dose taurine correlated with lower degrees of liver injury, which can be explained in terms of the known protective effects of taurine or the availability of inorganic sulfate that is used to form APAP sulfate. We have produced similar pharmacometabonomic models in a study of the hepatotoxicant galactosamine. Models built using the pre-dose spectra of four rats were able to predict a toxic outcome and clinical chemistry parameters (Schnackenberg *et al*., 2007). Analysis of the models and spectra revealed that most of the metabolites in the Krebs cycle were down-regulated in the urine from rats that had increased susceptibility to galactosamine-induced liver toxicity. A GC/MS-based pharmacometabonomic approach demonstrated that metabolite pools resulting from physiological and environmental changes play a major role in how different interventions are closely associated with variation in pre-dose (baseline) urinary metabolic profiles of rats (Li *et al*., 2007). These studies have shown that many factors can impact a patient's pharmacometabonome and metabolomics should play a role in personalized medicine (Haselden and Nicholls, 2006).

7 DRUG–DRUG AND DRUG–NUTRIENT INTERACTIONS

Metabolomics can also be applied to study polypharmacy, which is described as the concurrent use of multiple medications in a single patient. Polypharmacy is of special interest for the FDA due to the fact that drug–drug interactions can increase the negative consequences of a drug, i.e. its potential toxicity (Phillips, Barker and Eguchi, 2008). Detecting and monitoring drug intake and changes in metabolism in polypharmacy studies can be particularly problematic due to the easy access to many over-the-counter drugs in addition to those that have been prescribed. Metabolomics can provide a suitable analytical platform to monitor the exogenous metabolic profile in an individual. Drug–drug adverse interactions are becoming more prevalent, especially in the elderly where the number of polypharmacy patients (those taking more than five drugs concurrently) is increasing (Gorard, 2006). This is a problem because the use of multiple medications increases the risk for drug-related adverse events and drug interactions. For example, many antidepressants are metabolized by CYP2D6 or inhibit CYP2D6 and therefore can lead to drug interactions that can increase the possibility of adverse events when multiple antidepressants are taken simultaneously (Pollock *et al*., 1996). Many HIV antiretrovirals are metabolized by CYP2D and have shown adverse drug interactions (Dickinson, Khoo and Back, 2009). Additionally, the use of multiple medications creates a more complicated drug regimen for the medical staff and patient, potentially making compliance more difficult. Similarly, it has been demonstrated that patients who perceive they are taking too many medications are less likely to comply with their drug regimen. Furthermore, taking multiple medications may confound the effects of one or more of the drugs and the likelihood of drug interactions increases with the number of drugs taken simultaneously. In a patient taking multiple medications, a prescriber may not be able to distinguish which medications are helping, and which are causing adverse events in the patient. In addition, each drug could lower the availability of certain metabolites such as SAMe, glutathione or vitamins that are needed for protection against potentially harmful drugs (Sun *et al*., 2008; Wopereis *et al*., 2009). Most drug–drug interactions are primarily caused by one drug either inhibiting or inducing the enzymes that metabolize the other drug. When this occurs, idiosyncratic reactions could occur that may be responsible for idiosyncratic drug toxicities. Metabolomics can be used in monitoring polypharmacy and for personalized medicine because it has the ability to monitor multiple drug levels based on xenobiotic and endogenous metabolite levels at the same time. Therefore, metabolomics can provide information on pharmacokinetics and the underlying metabotype of the patient that is being medically treated.

Polypharmacy does not only refer to a prescribed drug regimen but can be expanded to include the concurrent use of any of the following: prescribed drugs, over-the-counter drugs, alcohol or street drugs. The death rate in the United States due to fatal medication errors (FME) combined with alcohol and/or street drugs rose 3196% from 1983 to 2004 whereas the death rate due to non-domestic FME not involving alcohol or street drugs increased only 5%.

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During this time, the number of per capita prescriptions increased 73.6%, so drug adverse interactions with alcohol and illegal drug stressors are most likely to be responsible for this alarming increase in FMEs (Phillips, Barker and Eguchi, 2008). Again, metabolomics could play a role in understanding these adverse drug interactions by monitoring multiple drug levels and many important endogenous metabolites.

Drug–nutrient interaction is another area where metabolomics can play a role. Nutritional needs vary with specific medication, gender, gut microflora and age (Blumberg, 1997; Drewnowski and Warren-Mears, 2001; McKevith, 2005; Kennedy, 2006; Baker, 2007; Rivlin, 2007), making it necessary to monitor nutrient intakes and physical activity over long periods of time, which is a distinct challenge for analysing the nutrient–drug interactions. Many biologically relevant nutrients in a diet are present at low concentrations and can be affected by chronic exposures to drugs and malnutrition. Because the effects of an unbalanced diet are cumulative, these nutritional imbalances can become biologically relevant and are known to play a role in the development of certain diseases such as cancer and diabetes (Block *et al*., 2000; Krebs-Smith and Kris-Etherton, 2007; Moore *et al*., 2008). To date, metabolomics has the capability to detect hundreds of metabolites simultaneously, and it is expected that similar nutrition interactions will be found not only for disease states such as cancer and diabetes but also for identifying nutrient interactions in drug toxicity.

Technological advances in analytical instrumentation make it feasible to analyse hundreds of metabolites accurately, providing the ability to assess the effect of certain nutritional factors on personalized health. The relatively new field of nutrigenomics (Whitfield, German and Noble, 2004; Gibney *et al*., 2005; Kaput *et al*., 2005; Zeisel *et al*., 2005;Kussmann, Raymond and Affolter, 2006; Trujillo, Davis and Milner, 2006) analyses gene–nutrient interactions using transcriptomics, proteomics and metabolomics technologies to better understand the effects of nutrition on health status and drug toxicity. The liver is the primary organ responsible for processing both dietary and xenobiotic components and for phase I and phase II drug metabolism. Many liver cytochrome P450 enzymes can be either activated or suppressed by bioactive nutritional components or other drugs, and these interactions can affect drug metabolism and drug clearance from the liver (Walter-Sack and Klotz, 1996;Harris, Jang and Tsunoda, 2003; Murray, 2006; Murray and Petrovic, 2006; Nekvindova and Anzenbacher, 2007). Therefore, nutritional and metabolic status may be an important factor in determining whether a toxic reaction occurs in the liver and, if so, the capability of the patient to overcome the toxic episode (Kaput *et al*., 2007).

Many nutrigenomic studies have demonstrated that diets interact with gene variants to produce different phenotypic outcomes (Veech, Eggleston and Krebs, 1969; Djuric *et al*., 2001; Duffy *et al*., 2001). For example, some ingredients in foods inhibit or activate gene expression (Harris, Jang and Tsunoda, 2003; Milner, 2004; Ross, 2007). Grapefruit juice (Bailey *et al*., 2000;Bailey, Dresser and Bend, 2003; Arayne, Sultana and Bibi, 2005; Saito *et al*., 2005; Sica, 2006) has been shown to affect many drugs through inhibition and/or inactivation of CYP3A4 in the intestine and at high enough doses in the liver. Recent reports suggest that the active ingredients in grapefruit juice inhibit intestinal CYP3A4 activity (Bailey *et al*., 2000;Bailey, Dresser and Bend, 2003). Other citrus juices have also been shown to affect drug metabolism of certain compounds (Saito *et al*., 2005). Metabolomics has the potential to identify the ingredient(s) in grapefruit juice and nutrients that alter CYP3A4 activities. Garlic is shown to have the ability to induce CYP3A4, which can interact with drugs such as saquinivir (an HIV protease inhibitor) and decrease exposure (Harris, Jang and Tsunoda, 2003). Other effects of some chemicals in food include delaying gastric emptying time, solubilizing the drug by food and digestive fluids, complex interactions between the drug and food components, altered hepatic blood flow and modulation of drug-metabolizing enzymes (Harris, Jang and Tsunoda, 2003).

Other nutrients can also alter drug pharmacodynamics; a prime example in this case is the effect of foods rich in vitamin K and warfarin activity. The liver uses vitamin K to produce prothrombin and this can change the effectiveness of warfarin. Foods that are high in vitamin K can decrease the effects of warfarin, whereas consuming less vitamin K can increase the effect of warfarin (Suttie, 1990; Booth and Centurelli, 1999; Merli and Fink, 2008). Warfarin activities can also be affected by alcohol (Sellers and Holloway, 1978; Weathermon and Crabb, 1999) and many herbal supplements

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(Izzo and Ernst, 2001; Hu *et al*., 2005; Shalansky *et al*., 2007). In one study, a longitudinal analysis of the risk of warfarin-related bleeding events with the co-administration of complementary and alternative medicine was evaluated. The risk of bleeding was associated with use of cayenne, ginger, willow bark, St John's wort and coenzyme Q(10) (Shalansky *et al*., 2007). Moon, Wang and Morris (2006) recently reviewed the effects of flavonoids on cytochrome P450 and other phase II enzymes involved in the metabolism of xenobiotics. The flavonoids are prevalent in vegetables, nuts, fruits and beverages including coffee, tea and red wine (Hollman and Katan, 1997). Flavonoids play a potentially protective role against cancer (Shih, Pickwell and Quattrochi, 2000) and can inhibit the activities of many enzymes including cytochrome P450 enzymes involved in phase I metabolism (Moon, Wang and Morris, 2006). In addition to the effect on phase I drug metabolism, flavonoids have also been shown to influence enzymes involved in phase II drug metabolism (Harris, Jang and Tsunoda, 2003; Moon, Wang and Morris, 2006). Dietary effects of flavonoids have been noted for enzymes including uridine diphosphate glucuronoyltransferases (UGTs), glutathione-*S*-transferases (GSTs) and quinone reductase (Moon, Wang and Morris, 2006).

The influence of nutrients on drug metabolism is exemplified by the various antioxidants in the diet, which apparently ameliorate toxic reactions to some drug compounds (Waters *et al*., 2001; Han *et al*., 2006). APAP is responsible for 50% of all drug-induced acute liver toxicities in adults (Lee, 2004; Amar and Schiff, 2007). Glutathione and taurine are sulfur-containing antioxidants that can reduce or prevent APAP-induced drug toxicity by binding to NAPQI, the toxic metabolite of APAP (Bessems and Vermeulen, 2001). Our previous studies have shown that the precursor for glutathione, SAMe, is also significantly lowered in urine samples after dosing with APAP (Sun *et al*., 2008; Schnackenberg *et al*., 2009). Therefore, dietary components that would increase the levels of antioxidants and SAMe could potentially help protect a subject from APAP toxicity. Pre-treatment of mice with the peroxisome proliferator, clofibrate, have been shown to protect against APAP-induced hepatotoxicity. Clofibrate-mediated hepatoprotection is associated with increased VANIN-1 and increased cystamine synthesis; cystamine is a potent antioxidant capable of ameliorating APAP toxicity in rodents and humans (Moffit *et al*., 2007).

Antioxidants may play a role in drug toxicities and health status. Ascorbic acid (vitamin C) is an important antioxidant that is involved in immune system functions, synthesis of carnitine and catecholamines, and absorption of iron in the body (Sies, Stahl and Sundquist, 1992; Arrigoni and De Tullio, 2002). Because vitamin C is only obtained through the diet, its concentration will vary greatly from person to person and from day to day within an individual. Vitamin D and calcium supplementation in diets have been shown to significantly reduce cancer incidence, and associated serum level of 25-hydroxyvitamin D was noted to be a predictor of cancer risk. (Lappe *et al*., 2007). Vitamin E has been shown to protect against cisplatin toxicity (Salganik, 2001; Leonetti *et al*., 2003; Ali and Al Moundhri, 2006; Ajith *et al*., 2007). Hence, metabolomic methodologies are contributing essential data in studies designed with nutrigenomic concepts and may improve the ability to link dietary exposures with disease development and individual responses to drug therapy.

8 METABOLOMICS OF CHALLENGE TESTS

One problem facing metabolomics is that much of the metabolome is under homeostatic control in the tissue and blood, so subtle temporal changes may be masked by evaluating the samples at the wrong time-point. One proposed way around the homeostatic control issue is to challenge a patient's system and record how they respond at several time-points using clinical chemistry parameters and new technologies such as metabolomics and proteomics (van Ommen *et al*., 2009). An oral glucose tolerance test (oGTT) represents a challenge to a patient and is how type-2 diabetes is commonly diagnosed. Generally in an oGTT, only blood glucose and insulin are monitored over time but new technologies can measure many other relevant changes occurring concurrently in blood. Two groups have recently measured metabolomics responses to oGTTs and shown that many other metabolites are altered during an oGTT challenge. Zhao *et al*. (2009) reported decreases in saturated and monosaturated free fatty acids and acylcarnitines, and increases in bile acids and lysophosphosphatidylcholines. Wopereis *et al*. (2009) reported that metabolites in the glutathione

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synthesis pathway showed time-dependent suppression in response to the oGTT challenges, which may be linked to inflammatory modulation that may be further linked to insulin signalling. Another group has reported the lipid response of three people with the same body mass index who were provided a controlled meal that had high lipid content for three consecutive days. They reported that the intra-individual variation within each subject across the 3 days was lower than the inter-individual differences (Zivkovic *et al*., 2009). This lipid challenge study along with the two oGTT metabolic profiling studies showed that detecting metabolic phenotype is possible and could play a major role in personalized medicine.

The ultimate goal of systems toxicology is to integrate data obtained from genomics, proteomics, metabolomics and imaging platforms to better understand the causes and progression of drug toxicity or disease. As an integral component of systems toxicology, metabolomics will have a major impact on personalized healthcare based upon the fact that metabolomics can identify biomarkers with real biological endpoints and provide a global systems interpretation of biological effects, including drug–drug and drug–nutrient interactions, for example.

9 CONCLUSIONS

Metabolomics is relatively new platform compared with transcriptomics and proteomics. Metabolomics requires multiple analytical platforms to detect as many of the metabolites in a tissue or biofluid sample as possible. Due to the wide range of concentrations and tremendous chemical and structural diversity of metabolites, one analytical platform is insufficient. Currently, it has been estimated that only 5–10% of the peaks detected by metabolomics analytical technologies are determined and therefore a lot of work still needs to be done to determine the remaining unknown peaks. Nonetheless, the use of metabolomics in toxicity and personalized medicine studies has the potential to aid systems toxicology studies in providing phenotype and metabolic pathway information. Metabolomics has the ability to deliver non-invasive biomarkers that can be detected early before major organ damage occurs. Pharmacometabolomics is extremely promising in providing markers for a patient's susceptibility to drug toxicity, and will play a pivotal role in personalized medicine. To validate metabolomics biomarkers, it will be important to apply the developed metabolomics standards initiative (MSI). However, significant work is still needed for broader acceptance of the quality control standards for metabolomics (Sansone *et al*., 2007). New sensitive and specific surrogate markers or accepted biomarkers of drug toxicity will facilitate the development of safer drugs at reduced developmental costs by removing drugs earlier in the drug development process. Most endogeneous metabolomic biomarkers of drug toxicity are relatively easily translated so that they can be used in both preclinical and clinical trials. Metabolomics should be used in personalized medicine studies because it can provide complementary information to a patient's genetic code. Genotype, nutritional status and the environment will be critical factors in determining a patient's potential outcome, which is information encoded in his/her pre-dose or pre-treatment metabolic profile. Finding the link between genetics, transcriptomics, proteomics, nutrition and gut microflora in relation to patient health or disease status is an essential component of the FDA's critical path to personalized medicine.

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The views presented in this article do not necessarily reflect those of the US Food and Drug Administration.

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Species-Metabolite Relation Database KNApSAcK and Its Multifaceted Retrieval System, KNApSAcK Family

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1 INTRODUCTION

Comprehensive metabolomics is clearly distinct from conventional metabolism studies in the sense that it addresses whole cellular activities rather than just focusing on enzymes, reactions, or metabolites in a specific pathway. Metabolomics research currently confronts a problem associated with high-throughput data acquisition technologies, including mass spectrometry, which have facilitated simultaneous detection and quantification of a large variety of metabolite-derivative peaks without appropriate assignment of metabolites (Hall, 2006). To assign the metabolites in the peaks of spectra, we need to survey natural products reported in the literatures, which is a very daunting data collection process. Therefore, in order to feasibly translate peak information to metabolite, we have developed a metabolite database concerning species–metabolite relations called KNApSAcK (Shinbo *et al*., 2004). There are at least three publicly available databases concerning natural products: PubChem (Wheeler *et al*., 2008), KEGG (Kanehisa *et al*., 2008) and KNApSAcK (Shinbo *et al*., 2006). The PubChem database comprises records for over 19.6 million compounds with over 11 million unique structures including small molecules, with particular emphasis on diagnostic and therapeutic agents. The huge amount of information, however, makes it inconvenient to assign metabolites to spectral peaks because no distinction is made on the origin of compounds, for example whether they are synthetic or natural compounds. In KEGG, the metabolic pathways are constructed by interspecies gene relations such as orthologues and paralogues, so metabolite–species relations can be obtained via information on enzymes; however, the KEGG database mainly focuses on metabolites related to well-established metabolic pathways and includes only about 13,000 metabolites. On the other hand, the relationships between metabolites and their biological origins have been addressed systematically in the KNApSAcK database. Here, KNApSAcK database makes it possible to assign metabolites to spectral peaks tractably. In the present study, we review the current

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status of KNApSAcK database and its application to metabolomics and introduce multifaceted retrieval system KNApSAcK Family.

2 KNApSAcK DB FAMILY (http://kanaya.naist.jp/KNApSAcK/)

Accumulation of species–metabolite relationships from scientific literature has been started since 2004 by six individuals (K.K, Y.N, H.A, Y.S., M.A and S.K). So we call this project KNApSAcK by taking and rearranging the initial letters of the family names of the members (Shinbo *et al*., 2004). The additional letters pc means the database can be accessed and used by a personal computer in the situation that databases including all phytochemical metabolites still need to be constructed through collaboration among scientists who have undertaken the task of constructing databases for plant secondary metabolites.

The KNApSAcK package when installed on the user's computer provides tool for analysing his/her own datasets of mass spectra that are prepared according to a particular format, as well as for retrieving information on metabolites by entering the name of a metabolite, the name of an organism, molecular weight or molecular formula. A list of metabolites that are associated with a taxonomic class can be obtained by searching with the taxonomic name, from which information on individual metabolites can be retrieved. Detailed manual can be obtained from the following URL: http://kanaya.aist-nara.ac.jp/KNApSAcK/Manual/KNApSAcKManual.html, and Web Version of Keyword search is provided at http://kanaya.naist.jp/knapsack_jsp/top, which makes it possible to link any website to KNApSAcK core database as indicated in the following examples. (i) Information on the metabolite assigned to C00000001 (a C_ID) can be retrieved by http://kanaya.naist.jp/knapsack_jsp/info.jsp?sname=C_ID&word=C00000001 and the reported metabolites in *Bacillus* (an organism) can be retrieved as http://kanaya.naist.jp/knapsack_jsp/info.jsp?sname=organism&word=bacillus.

Currently we have accumulated 76,357 species–metabolite relations involving 37,693 metabolites (last update: August, 27, 2009) and noted a power law relationship between the number of species and the associated number of metabolites (Figure 1). In a previous study (Shinbo *et al*., 2006), we observed the power law distribution for the number of species and the number of metabolites in each species. It is noteworthy that the metabolic pathways of individual organisms also follow the power law, i.e., the probability $P(k)$ that a metabolite interacts with k other metabolites in the metabolic pathway decays as a power law, following $P(k) \sim k^{-r}$, where r is constant (Ravasz and Somera, 2002).

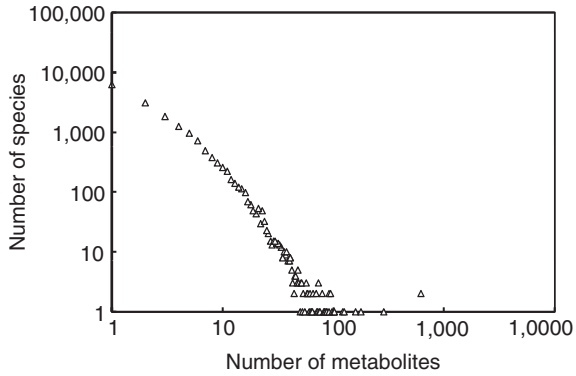

Figure 1. Power law distribution in the metabolite and species.

3 KNApSAcK FAMILY (http://kanaya.naist.jp/KNApSAcK_Family/)

Figure 2 shows the main window of KNApSAcK family, which consists of seven parts. The "Pocket" includes search system for species and metabolites related to human life such as edible plants in Japan ("Lunch Box"), herb teas ("Tea Pot", in progress), traditional Japanese medicine ("KAMPO"), poisonous plants ("Poison", in progress), and bio-fuel resources ("Fuel", in progress). "KNApSAcK from around the world" (at the top center, with the world map) includes medicinal and edible plants utilized in each country. Figure 3 shows the main window of "KNApSAcK from around the world" which consists of 119 nations and 7356 pairwise relationships between medicinal/edible plants and nations accumulated in 4538 species from scientific literature records (last update: Sept. 16, 2009). The list of plants in a certain country is obtained by clicking on the national flag of the country. By typing the scientific name of a species (e.g. *Brassica* or

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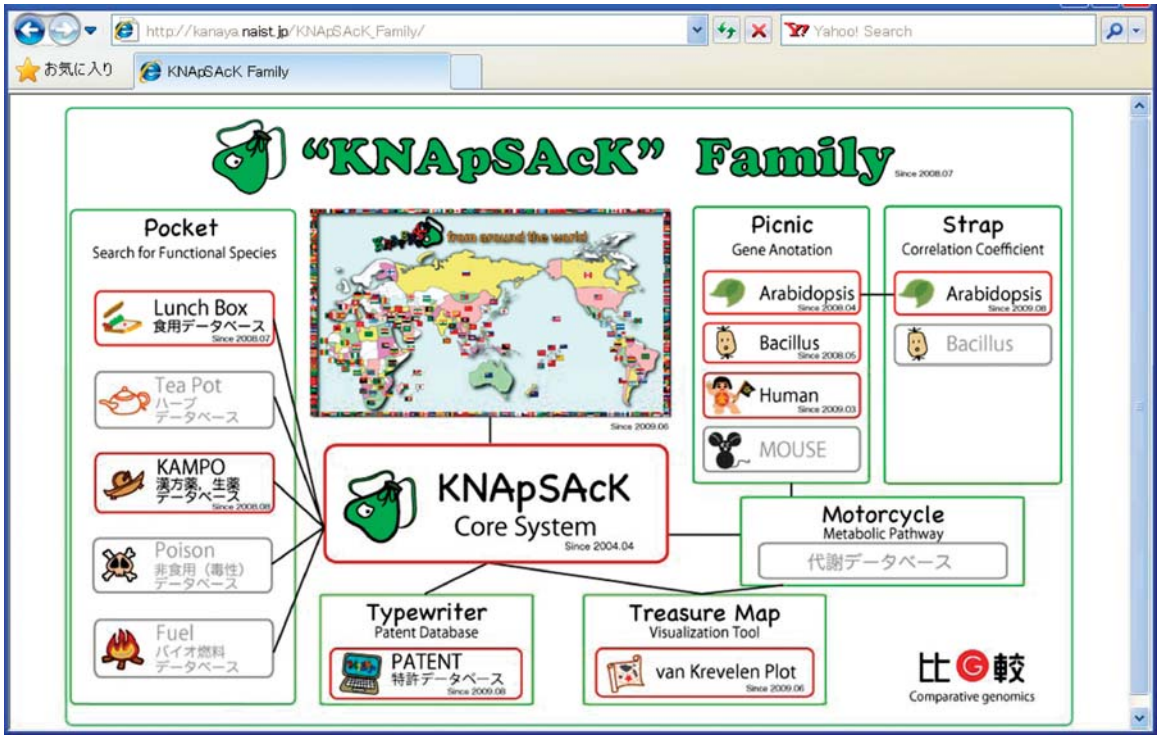

Figure 2. Main window of "KNApSAcK" family (http://kanaya.naist.jp/KNApSAcK_Family/).

Brassica hirta) in the text box and then clicking the List button, the national flags of the countries which utilize the plant start blinking. Then, information for metabolites contained in the species can be obtained if "KNApSAcK" icon is displayed for the species.

As shown in Figure 4, a power law relationship is also observed between the number of medicinal species and the number of metabolites. Figure 4 shows the number of medicinal plants utilized in individual countries. The 10 most utilized medicinal plants in the world are *Psidium guajava* (32 nations), *Ricinus communis* (17), *Zea mays* (15), *Ruta chalepensis* (15), *Ruta graveolens* (14), *Glycyrrhiza glabra* (14), *Zingiber officinale* (13), *Punica granatum* (13), *Foeniculum vulgare* (13), and *Apis indica* (13).

"Typewriter" includes patent information in Japan related to plants, and "Treasure Map" is a visualization system for metabolites by van Krevelen plot, which is also connected to the KNApSAcK core system. Gene annotation system called "Picnic" and highly correlated genes for a targeted gene can be searched using "Strap".

4 UTILIZATION OF KNApSAcK DATABASE IN SCIENTIFIC RESEARCH

The usefulness of KNApSAcK DB has been reported in several papers (Kikuchi and Kakeya, 2006; Want *et al.*, 2007;Saito, Hirai and Yonekura-Sakakibara, 2007; Moco *et al.*, 2007; Hummel *et al.*, 2007; Fardet *et al.*, 2008; Dunn, 2008; Oikawa *et al.*, 2008; Hagel and Facchini, 2008; Tohge and Fernie, 2009) We have accumulated 76,357 records (species–metabolite pairs) encompassing 37,693 metabolites on August 2009. The total number of secondary metabolites for which molecular structures have been elucidated is speculated to be 50,000 (De Luca and St Pierre, 2000), of which around 75% have been compiled in the KNApSAcK database. Furthermore, it is reported that the total number of secondary metabolites produced by entire plants together is more than 200,000 (Dixon and Strack, 2003). So KNApSAcK project is important for systematization of diversity of chemical structures and for mining useful natural compounds having industrial and medicinal significance.

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Figure 3. Main window of "KNApSAcK from around the world" (http://kanaya.naist.jp/KNApSAcK_Family/).

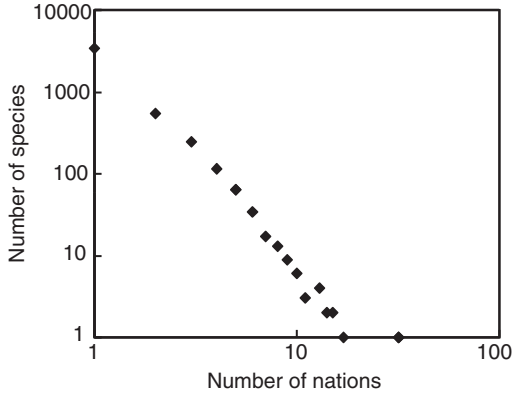

Figure 4. Power law distribution in the nation and utilized medicinal species.

Information on metabolites in the database can be searched by the name of the metabolite or organism, molecular weight, and molecular formula. Estimation of metabolites from an accurate mass peak is the first step of non-targeted analysis in metabolomics. In KNApSAcK DB, we can retrieve metabolites by a mass spectra data set consisting of *m/z* and corresponding intensities in the individual experimental conditions taking ionization mode into consideration. Using this function, a list of candidate metabolites is feasibly constructed for any species including model plants, because metabolites reported to closely related species as well as a target species are simultaneously retrieved by molecular weight in ionization mode (*m/z*). A number of researchers utilized this function to examine metabolites in various organisms such as tomato fruit (Iijima *et al*., 2008; Mintz-Oron *et al*.,

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2008), *Arabidopsis thaliana* (Oikawa *et al*., 2006; Nakamura *et al*., 2007; Yonekura-Sakakibara *et al*., 2007;Ohta, Sihbata and Kanaya, 2007; Böttcher *et al*., 2008; Malitsky *et al*., 2008; Kai *et al*., 2009), strawberry (Hanhineva, 2008), cabbage (Hounsomea *et al*., 2009), rice (Kind, Scholz and Fien, 2009), turmeric (Xie, Ma and Gang, 2009), *Arabidopsis lyrata* ssp. *petraea* (Davey, Woodward and Quick, 2008), oriental folk medicine *Angelica acutiloba* (Tianniam, Bamba and Fukusaki, 2009) and bacterium *Escherichia coli* (Takahashi *et al*., 2008). Metabolomics protocols incorporating KNApSAcK DB were proposed by Giavalisco *et al*. (2008), Overy *et al*. (2008), Oishi *et al*. (2009), Matsuda *et al*. (2009), Draper *et al*. (2009), and Sawada *et al*. (2009). Some biodatabases such as MetHouse (Gaida and Neumann, 2007, http://msbi.ipb-halle.de/), KEGG (http://fire3.scl.genome.ad.jp/dbget-bin/www_bfind?knapsack), Omicspace (http://omicspace.riken.jp/) and PRIMe (http://prime.psc.riken.jp/, Akiyama *et al*., 2008) are linked to KNApSAcK DB via web version (http://kanaya.naist.jp/knapsack_jsp/top.html/). Search extension transformation of Wiki into a relational system was proposed using species–metabolite relation in KNApSAcK database (Arita and Suwa, 2009). Evolutional interpretation of the diversity of flavonoid species was also carried out based on the DB (Takamoto and Arita, 2009).

5 CONCLUSION AND REMARKS

Considering that the KNApSAcK system covers a large number of species, not just only few model organisms, KNApSAcK DB has strong potential to contribute to metabolomics research society by way of applying it not only to simple metabolite search but also to further metabolomics analysis. We aim to accumulate 50,000 metabolites in total by 2010.

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Biomarkers for the Assessment of Acetaminophen Induced Liver Injury

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1 INTRODUCTION

Drug-induced liver injury (DILI) is the leading cause of acute liver failure in the United States and Europe (Fontana, 2008; Williams *et al*., 2010), and acetaminophen (APAP) is the leading cause of drug-related liver transplants within the United States accounting for 49–50% of all reported cases (Russo *et al*., 2004). DILI has also led to the failure of development of new drugs, preventing their future use in clinical trials, and removal of approved therapeutics from the market (Martin-Murphy, Holt and Ju, 2010). The liver becomes a primary target for adverse drug reactions because of its central role in biotransformation and excretion of foreign compounds, and its location within the circulatory system providing preferential exposure to a wide variety of substances administered orally.

APAP is a commonly available single ingredient or combination over-the-counter and prescription analgesic and antipyretic drug worldwide (Hinson, Roberts and James, 2010). APAP has been believed to be safe and effective when consumed at doses according to label; however, recent investigations have raised concerns regarding safety even when taken according to label (Watkins *et al*., 2006). It is also known that severe liver injury and liver failure can occur when the recommended dose of APAP has been exceeded by taking one or more over the counter or prescription medications that contain APAP. APAP is metabolized in liver by cytochrome P-450 enzymes; mainly by CYP2E1 isozyme (Hinson, Roberts and James, 2010). The parent drug or metabolites are also conjugated with glutathione, sulfate or glucuronide to produce water-soluble compounds. Consequently, the compounds can then be excreted from the body in bile or urine. Liver damage due to APAP has been attributed to *N*-acetyl-*p*-benzonaphthoquinone (NAPQI), a reactive metabolite generated during the metabolism of APAP by CYP enzymes (Han *et al*., 2010). NAPQI is a highly reactive metabolite, which reacts with protein and non-protein thiols. At low doses, NAPQI is mainly detoxified by glutathione (GSH) in hepatocytes. However, the GSH pool in hepatocytes is severely depleted at high, hepatotoxic doses of APAP. Because of this, NAPQI will be able to bind with thiols in amino acid residues of proteins affecting cellular protein targets such as mitochondrial proteins resulting in mitochondrial dysfunction. A loss of cellular ATP occurs, which leads to necrosis. GSH depletion itself can also induce apoptosis in hepatocytes.

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There is an urgent need for clinical tests that will help distinguish DILI from other common causes of liver injury (Watkins, 2009), and to enable clinicians to identify specific drugs that are responsible for DILI in the instances of poly-pharmacy. In addition, new tests are required that can differentiate patients, who may develop DILI from those who can be treated safely. At present, serum enzyme levels (e.g., alanine amino transferase, ALT; aspartate amino transferase, AST) are used in the clinic as gold standards for evaluating hepatic injury. However, these markers may not be specific to liver damage and more importantly these markers do not indicate specific types of liver pathology. Furthermore, invasive biopsy and histopathological evaluations can be painful, time consuming, and result in some level of risk to the patient.

Emerging insights into the mechanisms underlying DILI suggests that desired biomarkers possibly exist, which can be discovered through the application of new technologies for analysis of noninvasive biological fluids, such as blood and urine. For example, circulating miRNAs (Wang *et al*., 2009) in mice have been identified as potential biomarkers for liver injury, and plasma from rats exposed to APAP and D-Galactosamine have liver-specific mRNA not found in vehicle control groups (Wetmore *et al*., 2010).

Clayton *et al.* (2009) used a pharmacometabonomic approach to show that the pattern of endogenous metabolites in urine collected prior to and following dosing with APAP could be used to determine biomarkers for drug fate. ^{1}H nuclear magnetic resonance (NMR) based metabonomics analysis was applied to urine samples obtained from 99 human healthy nonsmoking male volunteers between 18 and 64 years old. After collecting predose urine samples, subjects were given a single dose of 1000 mg of APAP. Post dose urine samples were collected over two consecutive periods (0–3 h and 3–6 h). Analysis of ^{1}H NMR data have shown that the individuals having high levels of p-cresol sulfate in their predose urine had low post dose urinary ratios of APAP-sulfate to APAP-glucuronide. The study has concluded that, in individuals with high bacterially generated p-cresol generation, competitive O-sulfonation of p-cresol reduced the effective systemic capacity to sulfonate APAP. This study further showed the possibility of predicting those who are susceptible to AILI.

Using metabolomics analysis of human urine samples, Winnike *et al.* (2010) demonstrated urinary profiles obtained shortly after the start of APAP treatment, could distinguish responders from nonresponders; response being determined by measurement of ALT at later time-points. While statistical analyses revealed that predictive metabolites included those derived from the toxic metabolite NAPQI the authors concluded that the inclusion of endogenous metabolites was necessary for significant prediction.

It has recently been shown that hepatotoxicants can produce compound specific changes in the peripheral blood (PB) transcriptome in rats (Lobenhofer *et al*., 2008; Miyamoto *et al*., 2008; Wang *et al*., 2009). Transcriptomic and metabolomics analysis of PB samples obtained from six healthy human adults treated with 4 g bolus dose of APAP and from three receiving placebo have been used to characterize changes in the transcriptome and serum metabolome (Fannin *et al*., 2010). In addition, blood was also collected from individuals on each of the clinical days (predose and at the following post dose time points: 30 min, 90 min, 2, 3, 4, 5, 6, 8, 12 h) for the measurement of clinical chemistries and complete blood count. Traditional liver chemistries did not indicate liver injury. However, 48 h after exposure, treated subjects showed marked down regulation of genes involved in oxidative phosphorylation/mitochondrial function that was not observed in the placebos. Unbiased analysis of the serum metabolome revealed an increase in serum lactate from 24 to 72 h postdosing in the treated subjects alone. The single 4 g APAP dose produced a transcriptome signature in PB cells characterized by down-regulation of oxidative phosphorylation genes accompanied by increased serum lactate. Similar gene expression changes were observed in rats and several patients after consuming hepatotoxic doses of APAP.

The ability to predict the risk of idiosyncratic DILI by using *in vitro* covalent binding (CB) levels has been investigated by using 12 drugs positive to DILI (including APAP) and 12 negative drugs (Usui *et al*., 2009). In this investigation, each drug was incubated with liver microsomes in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH), UDP-GA (UDP-Glucuronic Acid), or GSH. The CB level alone was not sufficient for assessment of DILI risks, but when the CB level was multiplied by the maximum daily dose

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(which may reflect maximum hepatic exposure) the positive and negative outcomes groups did discriminate. Taken together, these findings suggested that the combination of CB level and daily dose may estimate the risks of idiosyncratic DILI.

A liquid chromatography/mass spectrometry (LC/MS) based metabolomic assay and NMR spectroscopy, using Sprague-Dawley rats dosed with APAP (vehicle, 400 mg kg^{-1}, and 1600 mg kg^{-1}) was used to investigate a drug's excretion kinetic profile in urine (Sun, Schnackenberg and Beger, 2009) to obtain drug toxicity information. Ultra performance liquid chromatography/ mass spectrometry (UPLC/MS) and NMR were used to evaluate the excretion kinetics of major drug metabolites. They found that N-acetyl-L-cysteine acetaminophen (APAP-NAC) had statistically significant correlations with clinical chemistry data, endogenous metabolite concentrations, and histopathology data.

Cheng *et al*. (2009) exposed humanized mice for pregnane X receptor (PXR) to rifampacin and APAP and measured CYP3A4 and conducted metabolomics on plasma and serum, using the human PXR and CYP3A4 double transgenic (TgCYP3A4/hPXR) mouse line. They found that toxicity was coincident with depletion of hepatic glutathione (GSH) and increased production of hydrogen peroxide, suggesting increased oxidative stress upon hPXR activation. Moreover, mRNA analysis demonstrated that CYP3A4 and other PXR target genes were significantly induced by rifampicin treatment. Urinary metabolomic analysis indicated that two APAP derived metaoblites (cysteine-APAP and S-(5-acetylamino-2-hydroxyphenyl)mercaptopyruvic acid) as the major contributors to the toxic phenotype. Quantification of plasma APAP metabolites indicated that the APAP dimer formed coincident with increased oxidative stress. In addition, serum metabolomics revealed reduction of lysophosphatidylcholine in the APAP-treated groups. These findings demonstrated that human pregnane X receptor (hPXR) was involved in regulation of APAP-induced toxicity through CYP3A4-mediated hepatic metabolism of APAP in the presence of PXR ligands.

Profiling of major known APAP metabolites in urine and serum (Chen *et al*., 2008), using wild type and CYP2E1-null mice, LC-MS based method, and various assays, revealed that the ratio of metabolites derived following CYP2E1 conversion of APAP to metabolites derived from direct conjugation with APAP decreased with increasing APAP doses administered 24 h after administration of APAP at 200 mg kg^{-1} and 400 mg kg^{-1} dose levels, serum ALT and AST activities in wild-type mice were dramatically increased in both treatment groups studied but their activities in CYP2E1-null mice were unaffected by 200 mg kg^{-1} and slightly increased by 400 mg kg^{-1} treatment. It further indicated that the CYP2E1-null mice were significantly resistant to APAP-induced hepatotoxicity. Measurement of hepatic glutathione and hydrogen peroxide levels in the study revealed the importance of oxidative stress in determining the consequence of APAP overdose. Overall, the oxidative stress elicited by CYP2E1-mediated APAP metabolism might significantly contribute to APAP-induced toxicity. Furthermore, it has also been revealed that mechanisms involving P450 isozymes other than CYP2E1 were also contributing to APAP-toxicity by identifying novel APAP metabolites.

Using the LC/MS based metabolomic analysis of the serum from wild type and CYP2E1 null mice the same group (Chen *et al*., 2009) further showed the accumulation of long-chain acylcarnitines in serum from wild-type mice dosed with APAP at levels to induce hepatotoxicity; while these long-chain aceylcarnitines did not elevate in the CYP2E1 null mice. This observation, in conjunction with the increase of triglycerides and free fatty acids in the serum of APAP-treated wild-type mice, suggested that APAP (and not a metabolite derived from APAP) disrupted fatty acid beta-oxidation (Chen *et al*., 2009). A time-course study further indicated that both wild-type and CYP2E1-null mice had elevated serum acylcarnitine levels within the early hours of APAP treatment. While serum palmitocarnitine levels remained high in wild-type mice after 2 h of APAP treatment, its level in CYP2E1-null mice has slowly returned to basal levels at the end of 24 h. This result indicated that the homeostasis of acylcarnitines in both mouse lines was significantly affected by 400 mg kg^{-1} APAP treatment, and the increase of serum palmitoylcarnitine was an early event in the APAP-induced toxicity.

Distinct from serum aminotransferase activity and hepatic glutathione levels, the pattern of serum acylcarnitine accumulation suggested that acylcarnitines may function as complementary biomarkers for monitoring the APAP-induced hepatotoxicity.

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Overall, serum metabolomics of APAP-induced hepatotoxicity revealed that the CYP2E1-mediated metabolic activation and oxidative stress following APAP treatment could cause irreversible inhibition of fatty acid oxidation, potentially through suppression of PPARα -regulated pathways.

Urinary metabolic perturbations associated with acute and chronic acetaminophen-induced hepatotoxicity was investigated (Sun *et al*., 2008) using NMR spectroscopy and ^{1}H NMR to determine biomarkers of APAP induced hepatotoxicity. Acute and chronic doses of APAP were administered to male Sprague-Dawley rats. NMR and UPLC/MS were able to detect both drug metabolites and endogenous metabolites simultaneously. The principal component analysis (PCA) of NMR or UPLC/MS spectra showed that metabolic changes observed in both acute and chronic dosing of acetaminophen were similar. Histopathology and clinical chemistry studies were performed and correlated well with the PCA analysis and magnitude of metabolite changes. Depletion of antioxidants (e.g., ferulic acid), trigonelline, S-adenosyl-L-methionine, and energy-related metabolites indicated that oxidative stress was caused by acute and chronic acetaminophen administration. Similar patterns of metabolic changes in response to acute or chronic dosing suggest similar detoxification and recovery mechanisms following APAP administration.

Toxicological responses to APAP overdose were examined using 2-dimensional gel electrophoresis (2DE)-based proteomics and (1) H-nuclear magnetic resonance (NMR)-based metabonomics to evaluate human hepatocytes transplanted in chimeric mice (Yamamoto *et al*., 2007). Proteins expressed in chimeric mouse liver that correlated with the presence of histopathological findings were identified that involve lipid/fatty acid metabolism, glycolysis and energy metabolism/production pathways. In addition, oxidative stress-related proteins showed altered expression. The metabonomic analysis of urine and plasma revealed alterations of endogenous metabolites which were the intermediates involved in the tricarboxylic acid (TCA) cycle, as previously observed in normal mice.

A metabolome differential display method based on capillary electrophoresis time-of-flight mass spectrometry (Soga, 2007) to profile liver metabolites following acetaminophen-induced hepatotoxicity has globally detected 1,859 peaks in mouse liver extracts and highlighted multiple changes in metabolite levels, including an activation of the ophthalmate biosynthesis pathway. The work confirmed that ophthalmate was synthesized from 2-aminobutyrate through consecutive reactions with gamma-glutamylcysteine and glutathione synthetase. Changes in ophthalmate level in mouse serum and liver extracts have been closely correlated and ophthalmate levels increased significantly in conjunction with glutathione consumption. Overall, their results provided a broad picture of hepatic metabolite changes following acetaminophen treatment. It was also found that serum ophthalmate is a sensitive indicator of hepatic GSH depletion, and may be a new biomarker for oxidative stress.

Human subjects taking 50 mg and 500 mg of APAP had increased levels of mercapturic acids (MA) as determined via constant neutral loss (CNL) scanning using linear ion-trap MS (Wagner *et al*., 2006) and suggested APAP-MA as an interesting class of marker compounds formed during the APAP detoxification process, even at low-doses. The authors in the study further suggested that the method could easily be transferred to other metabolites such as glucuronides and sulfates in the area of toxicotikentcs.

Gene chip array (Affymetrix) data from liver tissue and high resolution ^{1}H NMR spectra from intact liver tissue, tissue extracts and plasma (Coen *et al*., 2004) identified the major metabolic change as comprised of a decrease in hepatic glucose and glycogen in intact tissue, coupled with an increase in lipid content, with increases in the levels of glucose, pyruvate, acetate and lactate in plasma, and increases in alanine and lactate in the aqueous tissue extracts. Collectively these data provided evidence for an increased rate of hepatic glycolysis. The metabolic observations were consistent with the altered levels of gene expression relating to lipid and energy metabolism in liver, which both preceded and were concurrent with the metabolic perturbations.

An integrated metabonomics study using high-resolution ^{1}H NMR spectroscopy investigated the biochemical composition of intact liver tissue (using magic angle spinning), liver tissue extracts, and blood plasma samples obtained from control and acetaminophen-treated mice (Coen *et al*., 2003). Metabolic effects in intact liver tissue and lipid

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soluble liver tissue extracts from animals treated with the high dose level of acetaminophen included an increase in lipid triglycerides and monounsaturated fatty acids together with a decrease in polyunsaturated fatty acids, indicating mitochondrial malfunction with concomitant compensatory increase of peroxisomal activity. In addition, a depletion of phospholipids was observed in treated liver tissue, which suggested an inhibition of enzymes involved in phospholipid synthesis. There was also depletion in the levels of liver glucose and glycogen. In addition, the aqueous soluble liver tissue extracts from high dose animals also revealed an increase in lactate, alanine, and other amino acids, together with a decrease in glucose. Plasma spectra showed increases in glucose, acetate, pyruvate, and lactate. These all observations provide evidence for an increased rate of glycolysis. These findings could indicate a mitochondrial inability to use pyruvate in the citric acid cycle and also reveal the impairment of fatty acid beta-oxidation in liver mitochondria of such treated mice.

Alterations in metabolic profiles of serum from rabbits administered APAP have been investigated in a ^{1}H NMR based metabonomics approach (Zira *et al*., 2009) in order to study the effect of antidote, *N*-acetylcysteine (NAC), and other proposed antidotes (silybinin (SIL), cimetidine (CIM) and SIL/CIM). A single dose of APAP (2 g kg^{-1} b.w., i.g.) or doses of APAP combined with the antidotes SIL, CIM, and NAC was administered to rabbits. Serum ALT and AST levels and histopathological findings were used to estimate AILI and to monitor the effect of antidotes in the treatment of AILI. Metabolomics data of serum samples obtained from the controls and treated animals revealed the separation of APAP-treated, APAP-antidote treated, and control animals from each other and the variation of metabolic profiles in each group.

Our laboratory has also used metabolomics of urine to identify non-invasive biomarkers of APAP induced liver injury, and examined the relationship of alterations in changes in urinary metabolite levels with changes observed for the right, left, and median lobe of the liver tissue. APAP was used in this metabolomics study because it is the most common cause for DILI that results in acute liver failure, and because considerable literature is available to compare results from our investigation with other metabolomic investigations as well as with other biomarker investigations.

2 MATERIALS/METHODS

2.1 Study Design and Sample Collection

Male Sprague Dawley rats were obtained from Charles River, Raleigh, NC, and arrived at 8 weeks of age. They were provided food (Purina rodent chow 5002) and water ad lib and housed in microisolator cages for 2 weeks. At least six rats per dose and time point were administered by gavage 0, 10, or 1500 mg kg^{-1} APAP (dissolved in CMC/Tween) per kilogram body weight (mg kg^{-1}) per day for up to 9 days. Urine was collected over dry ice (0–6 h and 6–24 h) from rats in all glass metabolism cages prior to the first dose (pre-dose), and again following day 1 (day 1 urine) and following Day 8 (day 9) of dosing, and stored at -70° C. The focus of the metabolomics analysis was on urine collected 6–24 h following exposure to APAP doses.

2.2 Serum Enzymes and Histopathology

Groups of rats were sacrificed following the 24 h urine collection on day 1 (termed the day 1 group) or day 9 (termed the day 9 group) for the collection of blood and liver. Blood was processed to serum for the measurement of ALT and AST using standard commercial kits and procedures. Sections of the right, median, and left lobes of the liver were collected and stained with H&E stain. Histopathologic assessment was made by evaluating approximately 20 histological parameters of the liver by Experimental Histopathology Laboratories (EPL), Inc. (Research Triangle Park, NC). The extensive list of histological diagnoses in this investigation was utilized to correlate to the metabolomics profile with DILI. The individual histological observations were given a "score" of 1–4 to indicate the magnitude of the effect as determined by the semiquantitative histological observation. A score of 1 indicates a minimal effect while a score of 4 indicates the greatest change noted for each histological observation.

2.3 Metabolomics; Sample Preparation, data acquisition, and processing

Urine samples were prepared for NMR analysis by mixing an aliquot (~630 μL) of urine with 70 μL

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of a solvent buffer solution containing two internal standards (4,4-dimethyl-4-silapentane-1-sulfonic acid, DSS, and imidazole) for line shape analysis and spectral assignment and quantitation using the library in NMR Suite 6.0 Professional software. The left lobe, right lobe, and median lobe were individually prepared for NMR analysis by homogenization of each liver lobe (3:1 by weight; water:liver) and extraction with acetonitrile. Following extraction, the sample was dried under N_2(g) and reconstituted in 630 μL D_2O with 70 μL of the solvent buffer solution (DSS and imidazole). NMR spectra were acquired on a Varian Inova 600 MHz instrument located in the NMR facility at Duke University, Durham, NC. ^{1}H NMR spectra were acquired using the first increment of a NOESY sequence, with a 100 ms mixing time, 1 s relaxation delay, a spectral width of 12 ppm, and 32 transients. The water resonance was suppressed using resonance irradiation during the relaxation delay. All spectra were acquired at 25 °C, and the quality of each NMR spectrum was assessed for the level of noise and alignment of identified markers. Spectra were assessed for missing data and underwent quality checks.

NMR data was processed using a traditional binning approach (Lindon, Nicholson and Holmes, 2007) by automated integration (increments of 0.04 ppm) over the spectral window (producing 237 bins after excluding urea and the region of water suppression), and normalized to the total spectral intensity. NMR data were also processed using Chenomx NMR Suite 6.0 Professional software, which deconvolutes the entire spectrum based on chemical shift and coupling patterns, and then matches signals to a reference library of approximately 300 low molecular weight metabolites (Sumner *et al*., 2009; Sumner *et al*., 2010a, 2010b, 2010c). The internal standards (DSS and imidazole) were used as the references for performing the library matching. This software contains an internal library adjustment for increments in chemical shift based on pH variations (Weljie *et al*., 2006). A concentration determination for each metabolite was made by relative integration of the analyte to the internal standard, where the library of concentrations was developed to account for differences in integral values as related to the relaxation time of the signal (Weljie *et al*., 2006). This method has some advantageous over binning in two major ways. First, small increments in pH can result in portions of metabolite signals aligning with different bins when using the binning approach, while deconvolution circumvents this problem. A second advantage is that this analysis depends on the concentration of each metabolite, while the binning approach results in situations where each metabolite has multiple signals that fall within separate bins. For subsequent data reduction of the data derived from library matching, the concentration of each metabolite was normalized to total creatinine.

2.4 Data Reduction and Visualization

Data captured by NMR (metabolite id and concentration; or bin region and integral value) were transferred to software for data reduction and visualization using SIMCA P+ 12.0 (Umetrics AB, Umeå, Sweden). Several approaches were taken to analyze the NMR data to provide the best set of analytes that could distinguish the study groups (day 1 vehicle, day 1 low dose, day 1 high dose, day 9 vehicle, day 9 low dose, day 9 high dose) and that could provide the best correlation between the metabolomic profiles and the histopathological findings. For analysis with binned data, the integrals were normalized to the total integral for each spectrum. For analysis using data from library matching, the concentration of each metabolite was normalized to creatinine. Principal component analysis (PCA), partial least squares projection to latent structures discriminant analysis (PLS-DA) and orthogonal partial least squares projection to latent structures discriminant analysis (OPLS-DA) were conducted using SIMCA-P 12.0 for the binned and metabolite data. Loadings and variable importance plots were examined to determine the bins or metabolites that best correlated with dose groups, and to find the bins and metabolites that best correlated with histopathological findings. Subsequently, PCA using only the bins or analytes selected from the loadings and variable importance plots was conducted to demonstrate that the analysis with the subset of bins or metabolites could provide clear separation of the dose groups, and could provide correlation with the histopathological findings. Metabolites identified as important for the separation of dose groups or for correlation with histopathological findings were mapped to biochemical pathways.

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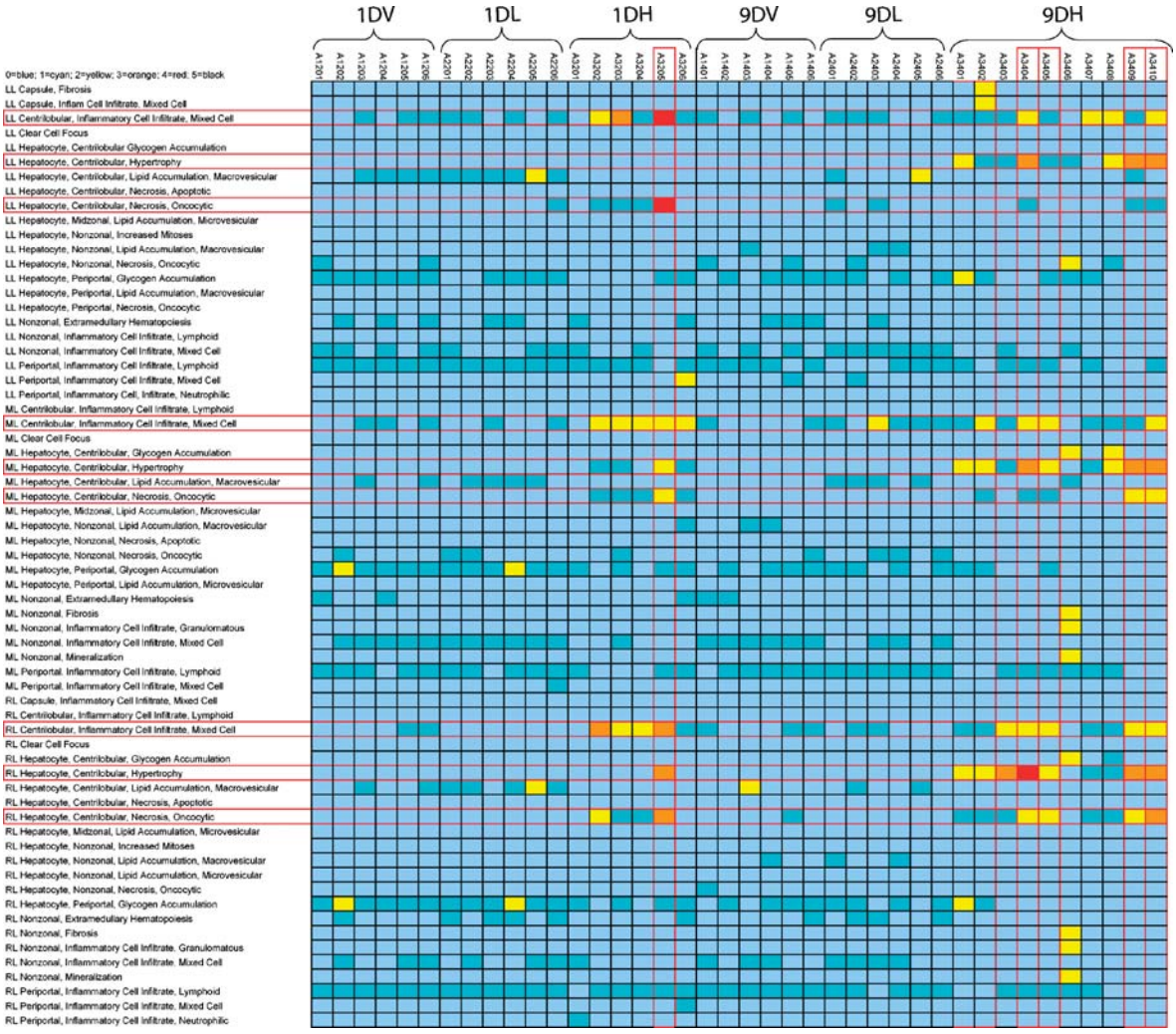

Figure 1. Histopathology findings for the left, median, and right liver lobes of rats administered 0, 10, or 1500 mg kg^{-1} APAP for up to 8 days. Centrilobular inflammatory cell infiltrate (mixed cell), centrilobular hypertrophy, and centrilobular necrosis (oncolytic) comprise the major incidence of pathologies. Abbreviations: 1DV, 1 Day vehicle group; 1DL, 1 Day low dose group; 1DH, 1 Day high dose group; 9DV, 9 Day vehicle group; 9DL, 9 Day low dose group; 1DH, 9 Day high dose group. Color codes for severity of incidences: blue, no effect; teal, minimal; yellow, mild; orange, moderate; red, severe.

2.5 Library matching

Full library matching using the NMR Suite 6.0 Professional Software (Chenomx, Edmonton, Alberta, Canada) provided a list of metabolites (and their respective concentrations) that demonstrated a match within each of the urine spectra. The concentration for each metabolite was normalized to the creatinine concentration for each urine sample and PCA analysis was conducted using the concentration based data. For liver extracts, the concentrations of the metabolites were normalized to DSS concentration.

3 RESULTS AND DISCUSSION

3.1 Histopathology of Liver

An extensive panel of liver pathologies was investigated by using approximately 20 histological parameters (Figure 1) for each of the three lobes evaluated. Centrilobular necrosis, centrilobular

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Table 1. Liver pathology incidences (necrosis, inflammation, and hypertrophy). LL, left lobe; ML, middle lobe; RL, right lobe).

Histopathology	1500 mg kg^{-1} after 1 day exposure	1500 mg kg^{-1} after 9 days exposure
LL Centrilobular, Inflammatory Cell Infiltrate	5/6	9/10
LL Hepatocyte Centrilobular Hypertrophy	0/6	9/10
LL Hepatocyte Centrilobular Necrosis, Oncolytic	4/6	3/10
ML Centrilobular, Inflammatory Cell Infiltrate	5/6	9/10
ML Hepatocyte Centrilobular Hypertrophy	4/6	9/10
ML Hepatocyte Centrilobular Necrosis, Oncolytic	5/6	5/10
RL Centrilobular, Inflammatory Cell Infiltrate	5/6	9/10
RL Hepatocyte Centrilobular Hypertrophy	1/6	9/10
RL Hepatocyte Centrilobular Necrosis, Oncolytic	4/6	9/10

inflammation, and centrilobular hypertrophy were the major pathological entities identified in treated animals. After one day of exposure to APAP, centrilobular necrosis and inflammation treated with a high dose (1500 mg kg^{-1}) were the two lesions most frequently noted and had the highest severity grade in rats treated with the high dose (1500 mg kg^{-1}) (Table 1). However, it should be noted that not all animals had these lesions with one animal not having these lesions in any of the three liver lobes evaluated (Figure 1). Hypertrophy was also frequently noted in the livers of animals receiving 1500 mg kg^{-1} although this lesion was in general less severe and less common than necrosis and inflammation (Table 1). Hypertrophy was most prominent in the median lobe. Necrosis, inflammation and hypertrophy were noted in similar incidence and severity in the 10 mg kg^{-1} group as compared to the control indicating that there was no histological effect noted in 1 day in this dose group. The findings after 8 days of exposure to APAP were similar to those after one day of exposure; the high dose group (1500 mg kg^{-1}) had increased incidences of severities of centrilobular necrosis, inflammation, and hypertrophy when compared to the control group. Similar to the results after 1 day, there was no histological difference between low dose and control. In the high dose group, the incidence and severity of hypertrophy was greater after 9 days of APAP exposure as compared to 1 day of exposure (Table 1, Figure 1). The liver pathology due to APAP is complex with several histological types of injury being present in the high dose groups with variability among the lobes sampled for the 1-day and the 9-day study. Thus, any non-invasive marker profile cannot be specific to one specific pathologic entity, but rather an indicator of acetaminophen induced liver injury (AILI).

3.2 Serum clinical chemistry data

The serum ALT and AST enzyme levels in the plasma were elevated in some but not all rats dosed with 1500 mg kg^{-1} APAP for 1 day or 8 days compared with vehicle controls (Figure 2a and b). Some animals receiving 1500 mg kg^{-1} of APAP had serum enzyme concentrations similar to control animals while other animals had very high serum enzyme levels for both ALT and AST. There was not a consistent correlation between serum enzyme elevation and histopathology findings.

3.3 Metabolomics of urine

Metabolomics analysis was conducted on urine (6–24 h) collected from rats administered by gavage 0, 10, or 1500 mg kg^{-1} APAP for 1 or 8 days. PCA was conducted using binned data normalized to the total intensity, and excluding the region of water suppression (Figure 3). PCA analysis of the bin data shows good separation of the groups by dose and duration. At both day 1 and 9, the high dose is clearly distinguished from the low dose and control, which are similar.

Targeted profiling using Chenomx software (Weljie *et al*., 2006) was performed for urine samples with subsequent multivariate analysis including PCA, PLS-DA, and OPLS-DA. PCA of all urine samples (Figure 4a) as well as PLS-DA analysis of 1 day (Figure 4b) and 9 day samples (Figure 4c) showed a clear separation of vehicle and low dose groups from high dose groups after both 1 day and 9 days dosing. The low dose groups (1 day and 9 day) were closely grouped with those of

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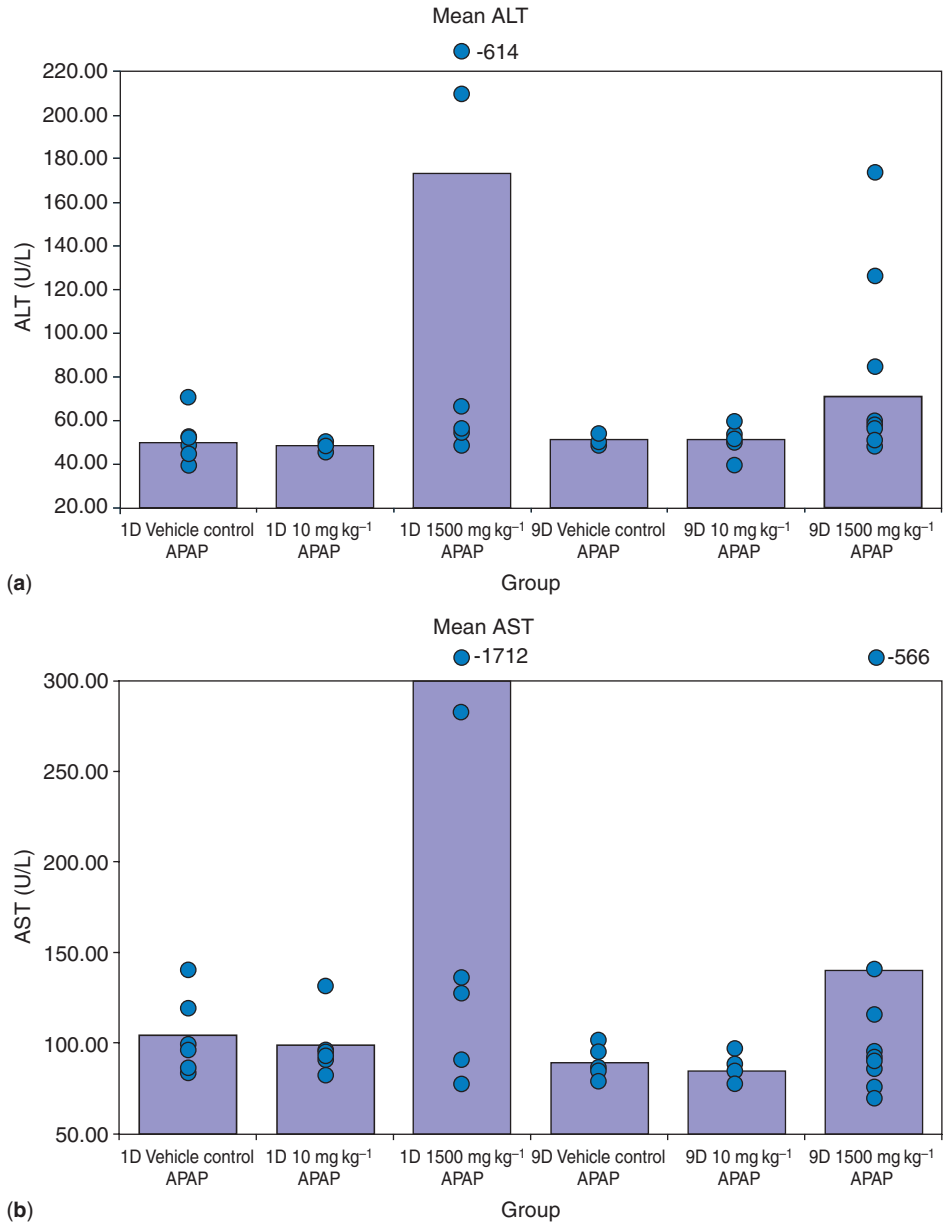

Figure 2. Analysis of serum ALT (a) and AST (b) indicates an average increase in serum enzyme levels in the rats administered a 1500 mg kg^{-1} APAP dose compared with vehicle control, although the level of serum enzyme elevation was not consistent with the severity of liver injury.

vehicle indicating that this relatively low dose of APAP (10 mg kg^{-1}), even after nine days of exposure, did not introduce significant perturbation on the metabolite profiles. A multivariate data analysis using concentration data normalized to time-matched vehicle control was performed to improve separation between groups and the resultant PLS-DA score plot is shown in Figure 5. A clear separation between the low and high dose groups for 1 day and 9 day urine samples is demonstrated.

Loadings plots and variable importance plots were evaluated to determine the subset of

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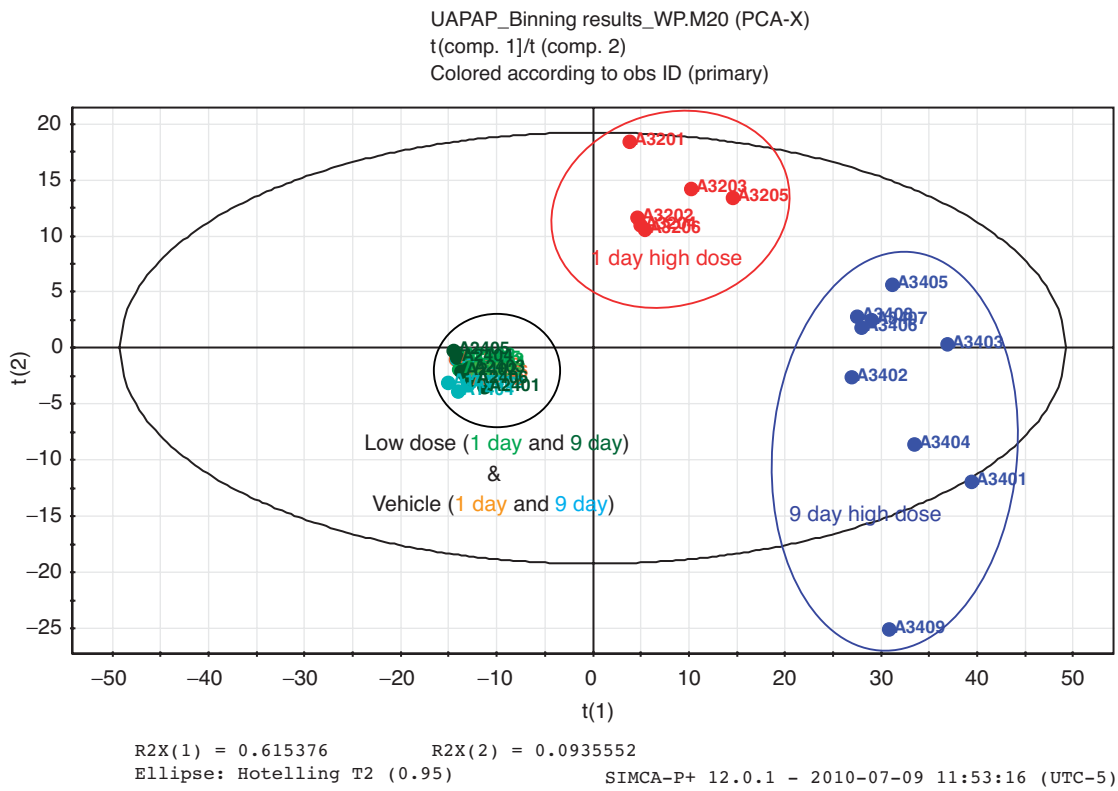

Figure 3. PCA analysis of binned NMR data obtained for urine (6–24 h) samples collected following 0 mg kg dose (orange, 1 day; teal, 9 days), 10 mg kg^{-1} dose (green, 1 day; and dark green, 9 days), and 1500 mg kg^{-1} dose (1 day, red); 9 days, (blue) with APAP.

metabolites that are responsible for the separation of different dose groups from the time-matched vehicle controls. Subsets of metabolites responsible for separating different dosing groups (for example, 1 day high dose group from 1 day vehicle group, 9 day high dose group from 9 day vehicle group, 9 day high dose group from 1 day high dose group) were identified. The urinary metabolites that best separated these groups were determined through evaluation of the variable importance plots, contribution plots, loading plots and are shown in the Table 2. PCA analysis was then conducted using the subsets of metabolites that were most responsible for the separation of these groups (Figure 6).

3.4 Metabolomics analysis of liver extracts

The left lobe, right lobe, and median lobes of liver were individually homogenized (3:1 by weight; water:liver), extracted with acetonitrile, dried under $N_{2(g)}$, and reconstituted with D_2O and a solvent buffer containing DSS and imidazole. Metabolomics analysis of the liver was conducted as described for urine. Multivariate data analysis was conducted independently for the a) right lobes; b) left lobes; or c) median lobes.

Multivariate data analysis **(**OPLS-DA) of all liver samples from both 1 day after exposure and 9 days of exposure demonstrated the separation of the study groups. Subtraction of time matched control values from the APAP exposed groups is shown in Figure 7a, also demonstrating the separation of 1-day samples from 9-day samples. Analysis of the 1-day samples using PLS-DA showed that the separation of groups by dose was greater than the separation of groups by lobe of liver (Figure 7b). This same trend was apparent for the 9 day group (Figure 7c). Multivariate analysis (PCA and PLS-DA) was performed for each individual lobe separately following subtraction of the time-matched vehicle control. For the left lobe (Figure 8a), the profiles for

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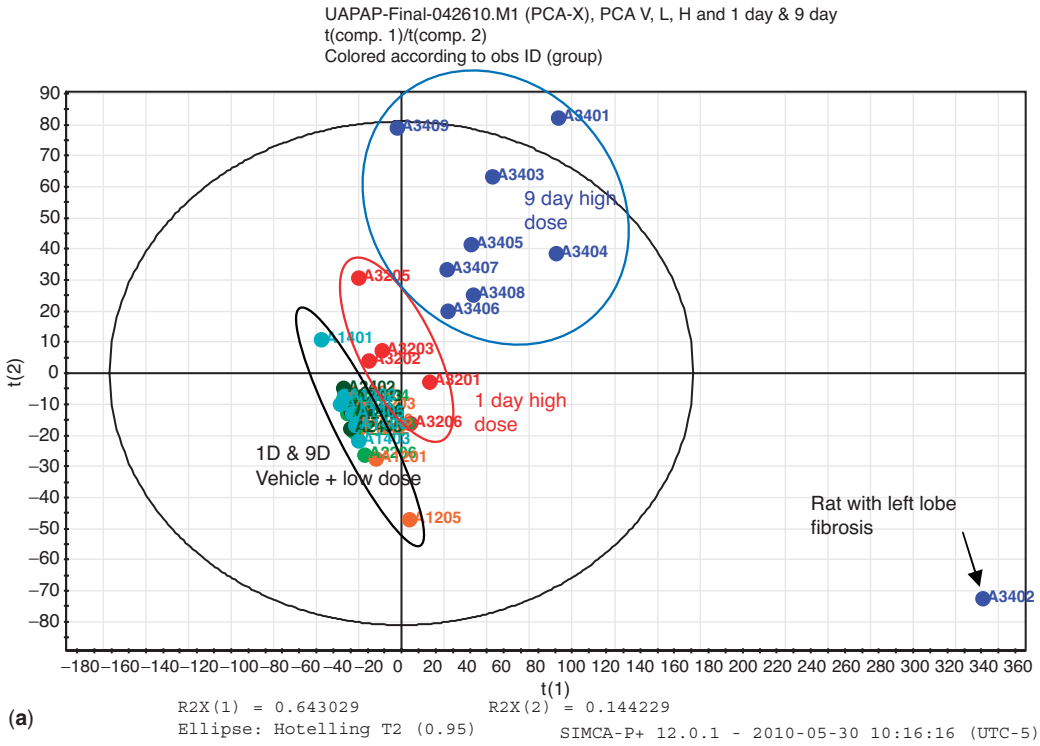
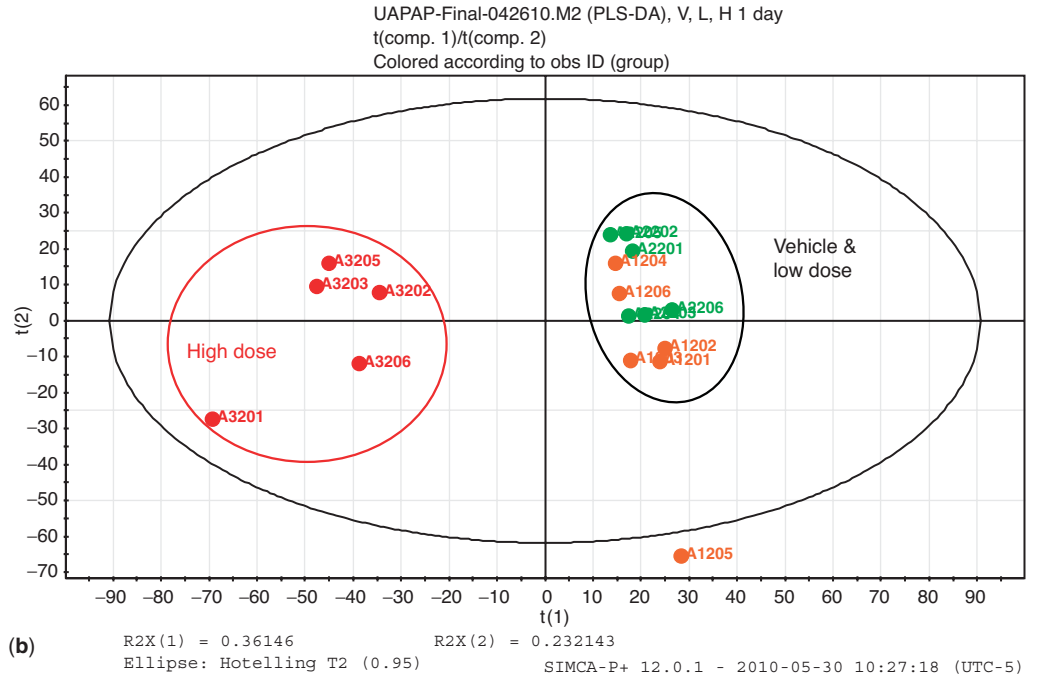


(a)



(b)

Figure 4. PCA using concentration data of urinary metabolites that were obtained from Chenomx profiling shows (a) separation of the high-dose groups (1 day, red; 9 days, blue) from vehicle control (1 day. orange; 9 days, teal) and low-dose (1 day, green; 9 days, dark green) groups, (b) separation of 1-day high-dose group (red) from time-matched vehicle control (orange) and low-dose (green), and (c) a separation of 9-day high-dose group (blue) from time matched vehicle control (teal) and low-dose (dark green).

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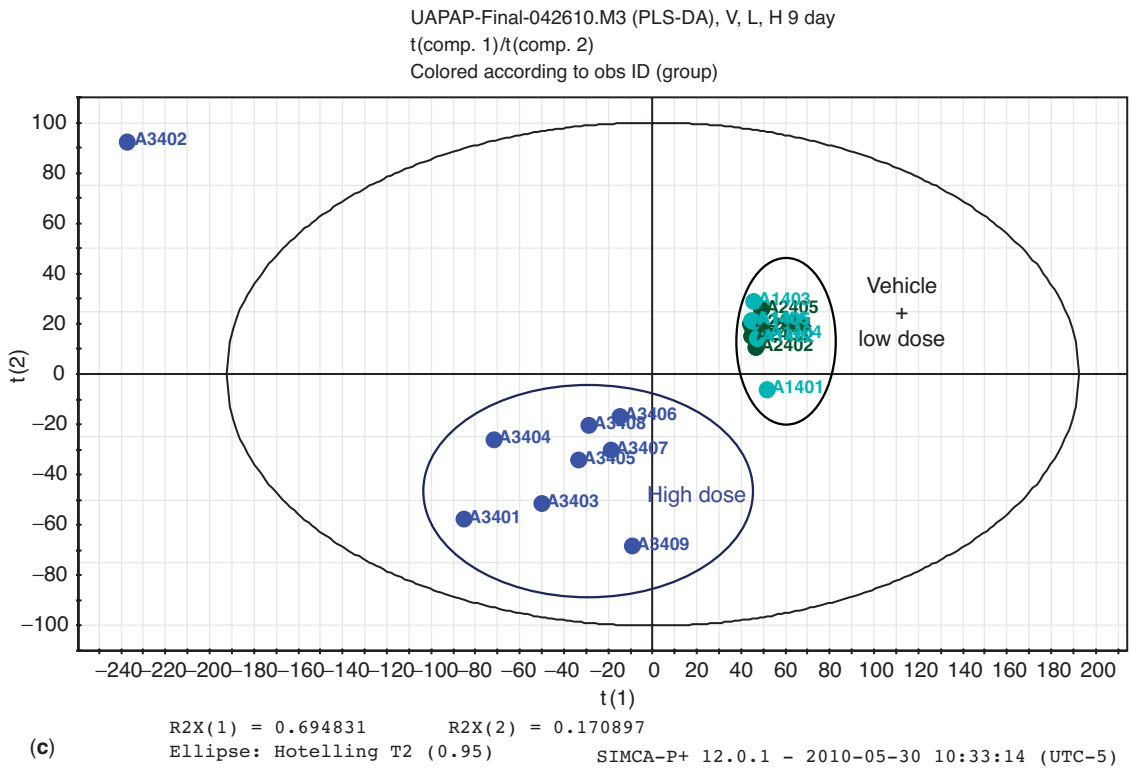

(c)

Figure 4. (*Continued*)

the 1-day (green) and 9-day (dark green) low dose groups were distinct from the 1-day (red) and 9-day (blue) high dose groups. The high dose groups in the right lobe (1 day and 9 days, Figure 8c) were more separated from the other study groups, when compared to the median (Figure 8b) and left (Figure 8a) lobes. PCA and PLS-DA analysis of data obtained for all lobes at each day (1 day and 9 day) were performed separately. It was noted that the median lobe was separated from left and right lobes at day 1 (Figure 9) while the right lobe was more separate from left and median lobes at the day 9 time point (Figure 10). The separation of median lobe from the left and right lobe for the 1-day study is consistent with more prominent centrilobular hypertrophy in the median lobe (1-day study). Lobe variation in transcriptional profiles has been demonstrated after APAP administration (Irwin *et al.*, 2005).

Subsets of metabolites in liver extracts were determined that separate the high dose groups from the control groups in each liver lobe (left, median, and right) by evaluating the variable importance, contribution, and loading plots of multivariate data analyses and are shown in the Tables 3, 4, 5. It was possible to identify a common subset of metabolites that separate the dose groups in all three lobes, as well as additional metabolites that separate groups in each individual lobe. Multivariate analysis (PCA and PLS-DA) were performed using these subsets and two representative score plots are shown for the analyses using the common subset and a subset that is found to be specific to the middle lobe in Figures 11 and 12, respectively.

3.5 Defining pathways relevant to the urinary and liver markers

Metabolites that define AILI were mapped to metabolic pathways. Our metabolomics study on both urine and liver extracts suggests that interruptions in amino acid metabolism (glycine, glutamine, glutamate, tyrosine), glutathione metabolism, citric acid cycle, Krebs cycle, purine and pyrimidine metabolism, benzoate metabolism, norepinephrine,

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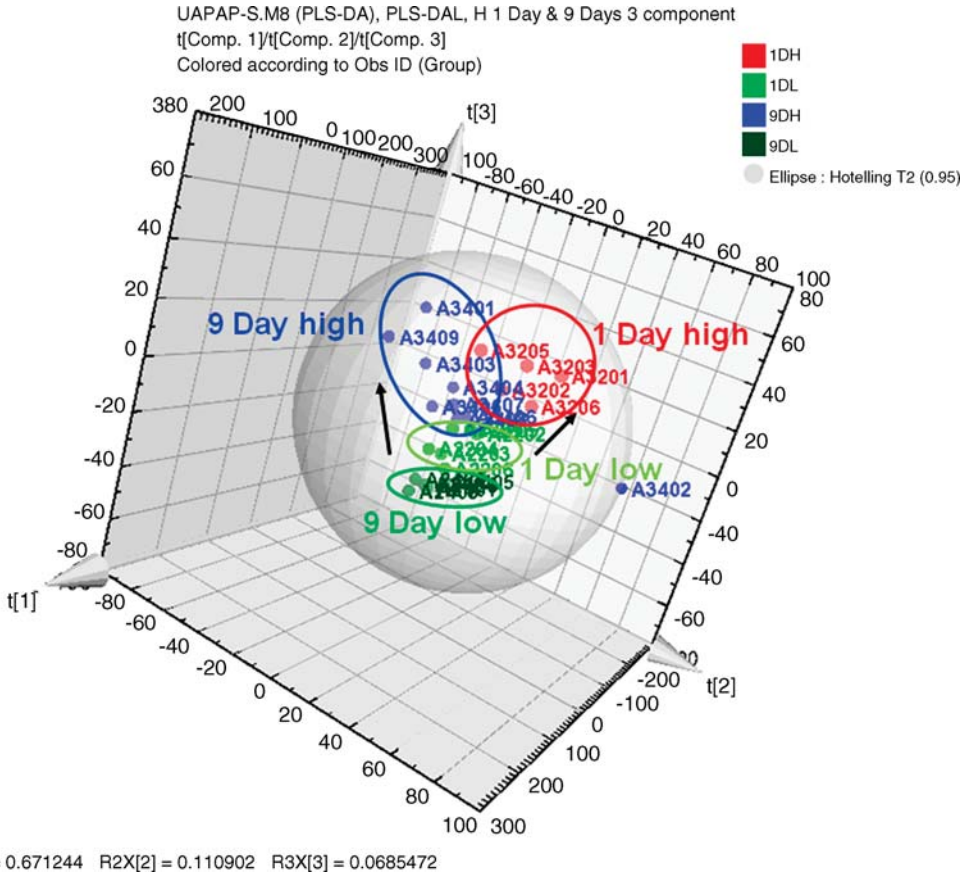

Figure 5. PLS-DA using concentration data of urinary metabolites subtracted from the time matched vehicle controls shows a separation of high-dose groups (1 and 9 day) from the low-dose groups.

mitochondrial fatty acid β-oxidation, metabolism of nicotinamide, urea cycle, and AMP metabolism.

Consistent with our investigations were earlier metabonomics (and metabolomics) studies, which showed perturbations in cellular metabolism and functions in liver corrolarry with the presence of AILI (Coen *et al*., 2003; Coen *et al*., 2004; Sun *et al*., 2008; Zira *et al*., 2009; Bollard *et al*., 2010). Metabonomics and transcriptomics studies using an animal model of AILI showed dose- and time-dependant biochemical changes in energy and lipid metabolism (Coen *et al*., 2003; Coen *et al*., 2004) and an increase in lipid triglycerides, monounsaturated fatty acids, with a decrease in polyunsaturated fatty acids- also indicating mitochondrial malfunction and related compensatory increase of peroxisomal activity. A depletion of phospholipids in liver tissue in the animals treated with APAP and perturbation of amino acid, glucose and glycogen metabolism were also observed.

Bollard *et al*. (2010) showed differences in levels of urinary metabolites measured for rats following partial heptectomy compared with matched controls. This included elevated levels of taurine, hypotaurine, creatine, guanidoacetate, betaine, N,N-dimethylglycine, and bile acids and decreased levels of triglycerides. In addition, in the liver, triglycerides, cholesterol, alanine, and betaine were elevated after partial hepatectomy while choline and its derivatives were decreased (Bollard *et al*., 2010). Analysis of urine from rats following acute or chronic dosing with APAP (analyzed by both NMR and UPLC/MS) showed multiple time dependent metabolic changes in acetaminophen drug metabolites, antioxidants, and energy-related metabolites,

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Table 2. Subsets of urinary metabolites that separate the high-dose Groups (1 and 9 day) from time-matched vehicle controls.

1 Day high dose		9DH-1DH-aging		9DH-9DV	
Increased	Decreased	Increased	Decreased	Increased	Decreased
Phthalate	Cis-Aconitate	Oxalacetate	Citrate	Oxalacetate	cis-Aconitate
Phenylalanine	Glycerate	Gentisate	Methylamine	Thymine	5,6-Dihydrouracil
Protocatechuate	Cysteine	Protocatechuate	Sarcosine	Protocatechuate	2-Oxoglutarate
3-Chlorotyrosine	5-Aminolevulinate	Thymine	Dimethylamine	Glutamine	Isocitrate
Thymine	3-Hydromuconate	Phenol	2-Oxoglutarate	Gentisate	Cysteine
Methylamine	Fructose	Pyroglutamate	Phenylacetylglycine	4-Hydroxyphenylacetate	Uracil
γ-Methylhistidine	O-Phosphoethanolamine	Ethylene glycol	Cytosine	Phenol	Dimethylamine
Tyrosine	Uracil	Acetate	4-Pyridoxate	Acetate	3-Hydromuconate
3,4-Dihydroxymandelate	Serine	Tyrosine	Succinylacetone	Tyrosine	N-Carbamoylaspartate
4-Hydroxyphenylacetate	S-Sulfocysteine	3,4-Dihydroxymandelate	Uracil	3-Chlorotyrosine	4-Pyridoxate
4-Hydroxybenzoate	N-Carbamoyl-β-alanine	Methylmalonate	Hippurate	Pyroglutamate	Betaine
Phenylacetylglycine	trans-Aconitate	Pyruvate	Quinolinate	4-Hydroxybenzoate	Hippurate
Salicylurate	Threonine	3-Hydroxybutyrate	3-Phenylpropionate	Ethylene glycol	5-Aminolevulinate
Glutamine	Trigonelline	3-Hydroxyisovalerate		Vanillate	Caprylate
Vanillate	N-Acetylaspartate	Cystine		Phenylalanine	Sarcosine
3,5-Dibromotyrosine	Methylmalonate	Galactonate		3,4-Dihydroxymandelate	Trimethylamine N-oxide
Glycylproline	Lysine	π-Methylhistidine		Salicylurate	Caprate
π-Methylhistidine	Lactate	Glutamate		τ-Methylhistidine	Trigonelline
Tropate		Acetone		π-Methylhistidine	Fumarate
Phenol		O-Phosphoserine		Phthalate	Citrate
Indol-3-acetate					
Creatine					

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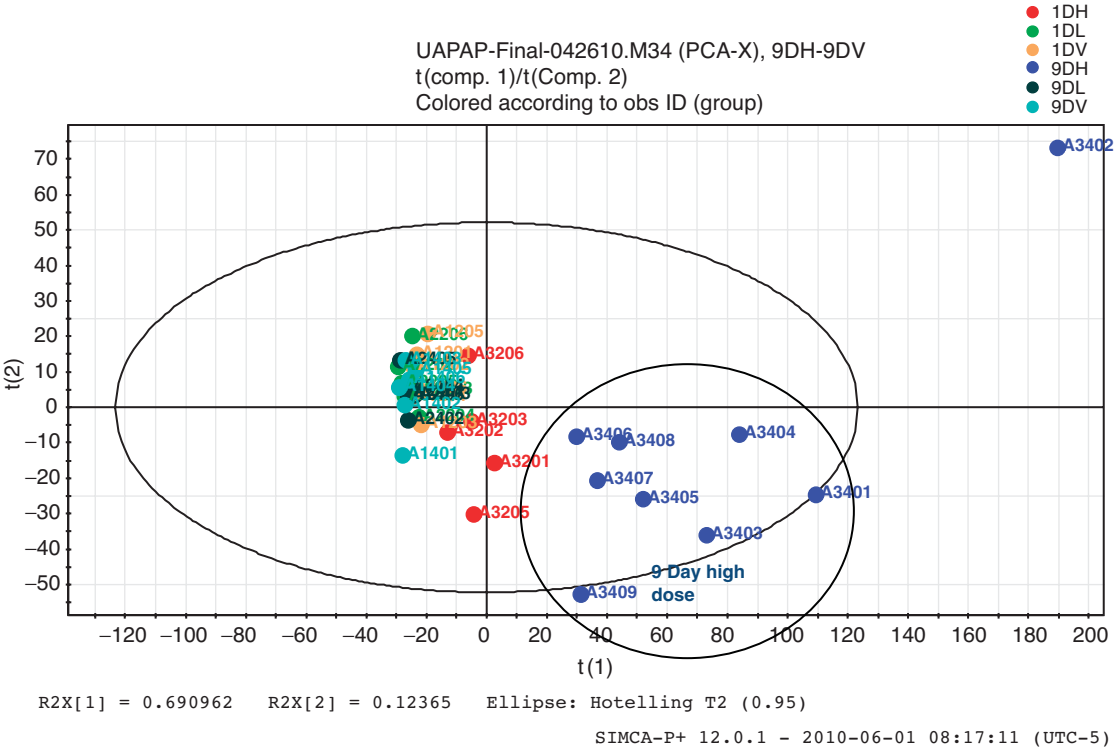

Figure 6. PCA of urine data (concentrations) obtained by using the subset of metabolites that are responsible for separation of the 9-day high-dose group from the time-matched vehicle control (1-day vehicle, orange; 1-day low, green; 1-day high, red; 9-day vehicle, teal; 9-day low, dark green; 9-day high, blue).

in the urine of rats when compared with control samples (Sun *et al*., 2008).

Reactive oxygen species and reactive nitrogen species in normal hepatocytes are critical for normal physiological processes, including oxidative respiration, growth, regeneration, apoptosis, and microsomal defense (Diesen and Kuo, 2010a, 2010b; Han *et al*., 2010). When the levels of these species exceeds the normal antioxidant capacity of cells, oxidative stress occurs, leading to various damaging effects to all liver cells. AILI is shown to deplete glutathione pool in the liver cells, reduces ATP production by perturbing pathways involved (Soga *et al*., 2006; Hinson, Roberts and James, 2010). Recent gene expression profiling studies (Boorman *et al*., 2005a, 2005b; Irwin *et al*., 2005; Powell *et al*., 2006; Toyoshiba *et al*., 2006; Bushel *et al*., 2007) on APAP-induced toxicity have also shown that there is differential expression of genes in the liver after APAP exposure when compared to controls. The differentially expressed genes in the other studies included those responsible for oxidative stress, and energy metabolism (Heinloth *et al*., 2004; Powell *et al*., 2006). Studies using blood and liver gene expression profiles found that the genes that discriminate the subtoxic and nontoxic dose levels from toxic levels were genes involved in the activation of immune or inflammatory responses against an external stimulus. Examples of overrepresented categories were defense response, immune response, response to stress,regulation of phagocytosis, regulation of endocytosis, response to bacteria, and inflammatory response (Bushel *et al*., 2007). To summarize, there is impairment in energy metabolism, protein synthesis, and mitochondrial function in AILI. It further suggests that the activation of cellular signaling and immunity could be the major cause of liver

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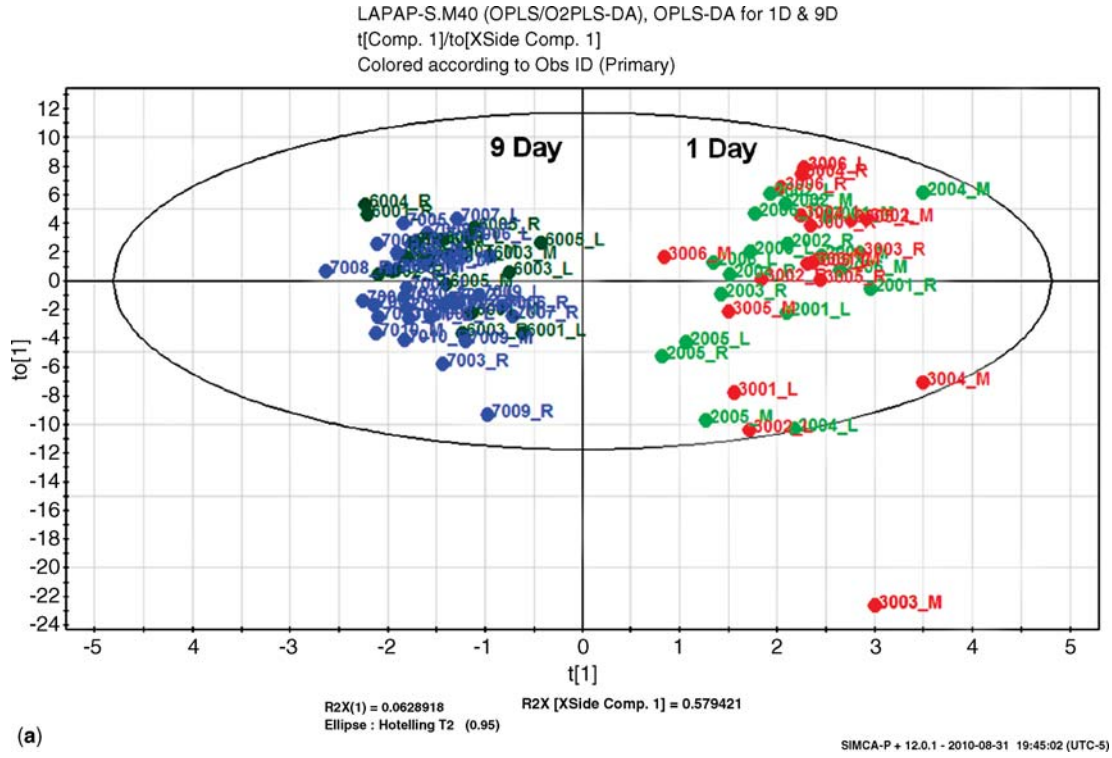

Figure 7. (a) OPLS-DA analysis of liver metabolite data (concentrations) subtracted from the time-matched vehicle controls show a separation of 1-day samples from 9-day samples; (b) PLS-DA of urine samples obtained after 1-day exposure shows a separation caused by dose (1-day vehicle, orange; 1-day low, green; 1-day high, red); and (c) PLS-DA of urine samples obtained after 9 days exposure shows a separation caused by dose (9-day vehicle, teal; 9-day low, dark green; 9-day high, blue).

damage due to APAP intoxication. Perturbation in amino acid metabolism and the changes in amino acid levels are accounted for the disturbed protein synthesis and protein synthesis because of the necrosis and other liver damaging effects (Zira *et al.*, 2009).

4 CONCLUDING REMARKS

Our investigation has demonstrated a promising non-invasive urinary marker profile that could be further evaluated for the early detection of APAP induced liver injury. Early diagnosis of reversible liver injury induced by APAP would greatly enhance treatment options and benefits. Our studies reveal that the liver from rats with AILI has a significant perturbation of amino acids metabolism (glycine, leucine, arginine, alloisoleucine, asparagine, alanine, and aspartate) purine and pyrimidine metabolism, glycolysis, Krebs cycle, cysteine metabolism, and glutathione), and that there is a common subset of metabolites that define liver injury irrespective of the lobe. Furthermore, there are subsets of metabolites that are specific to defining the biochemical profile of each lobe. Urinary metabolites consistent with the presence of AILI suggested interruptions in some of the same pathways (amino acid metabolism, energy metabolism, purine, and pyrimidine metabolism) and indicate the possibility of developing a non-invasive surrogate marker for AILI that may correlate with the lobe response. Using a metabolomics approach for the validation of the marker profile will enable the determination of any additional metabolites that should be included in a marker profile to reveal AILI related histopathologies in human investigations.

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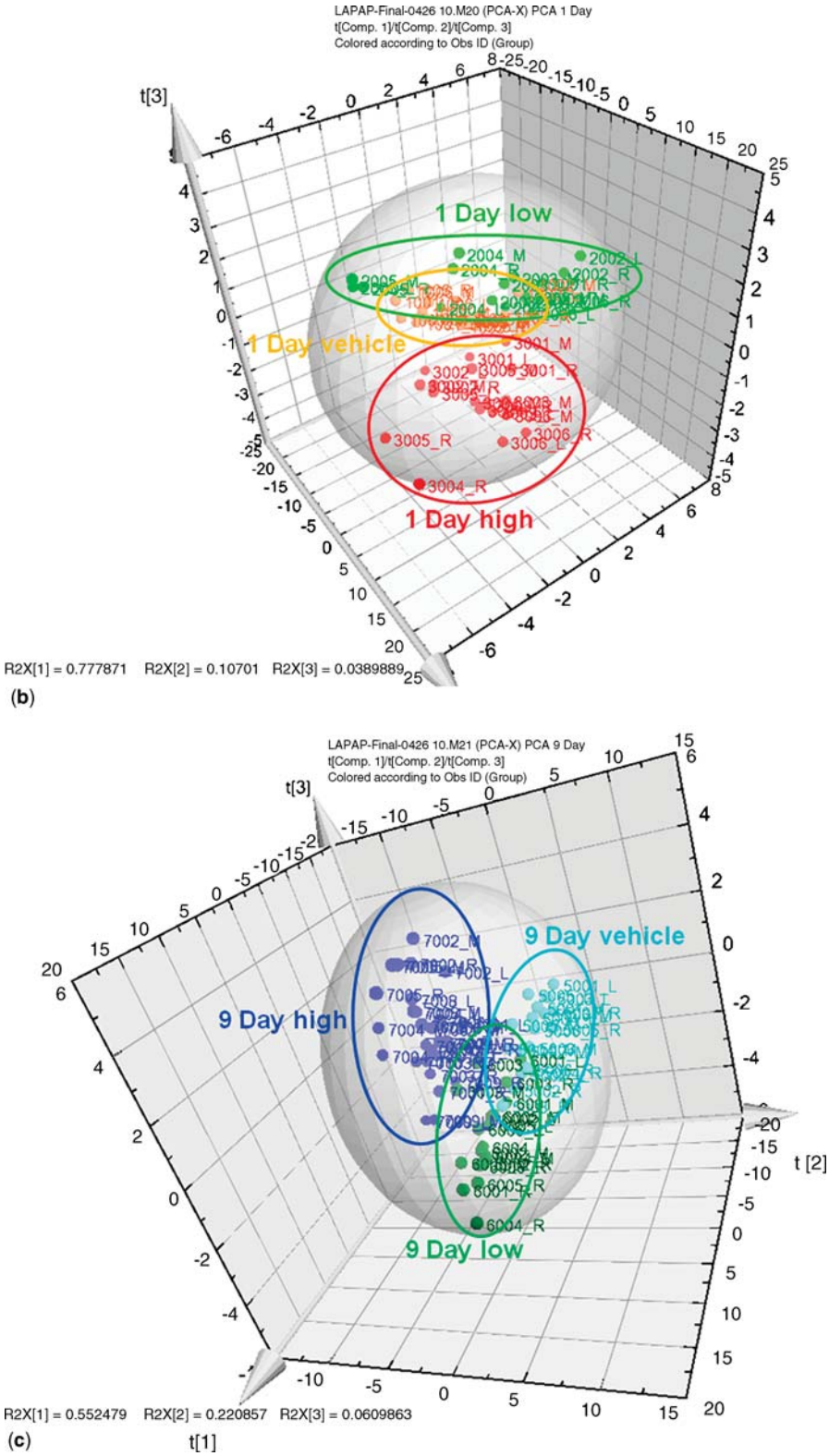

Figure 7. (*Continued*)

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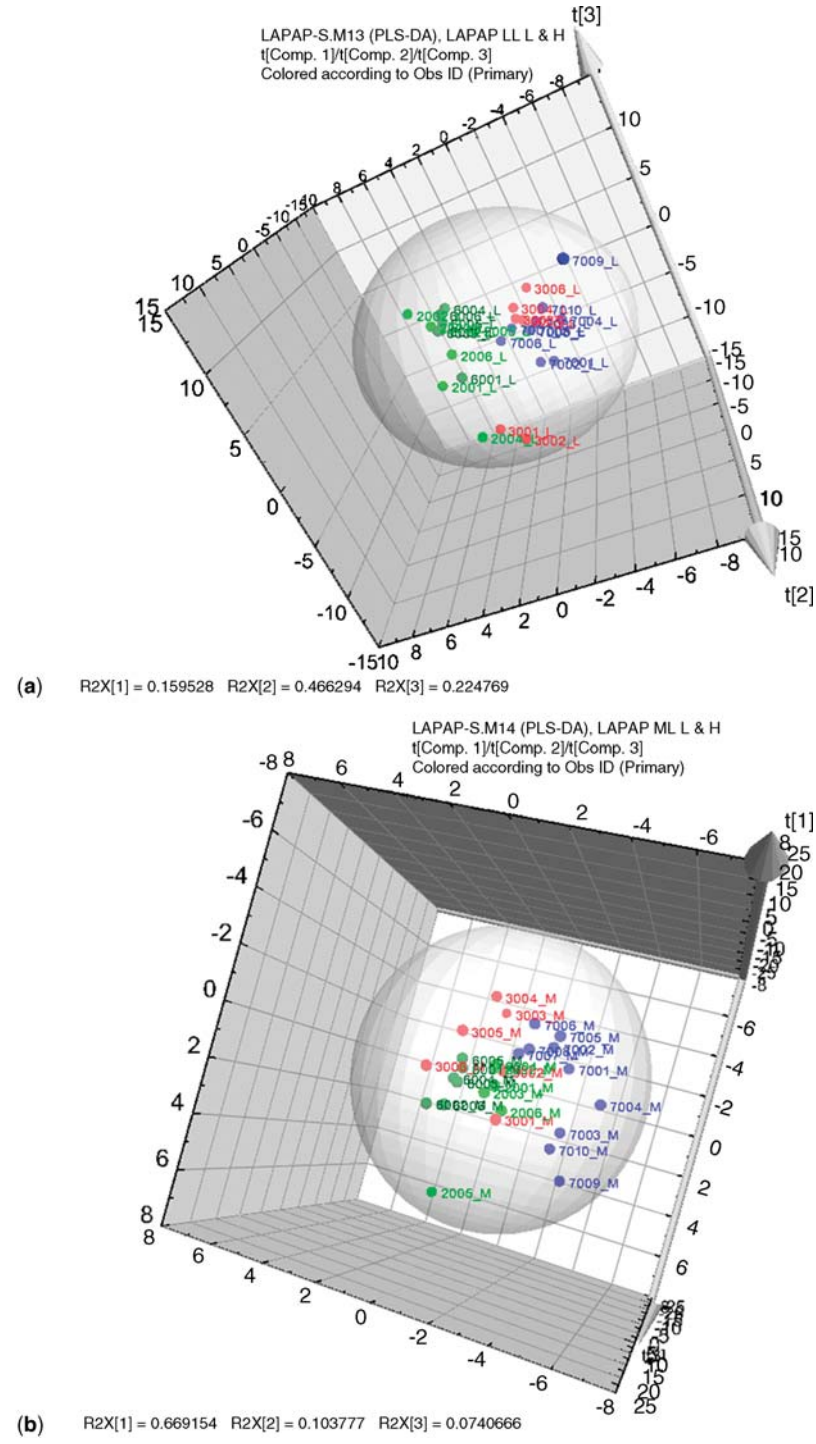

Figure 8. (a) PCA analysis of concentration data of metabolites found in left liver lobe after subtracting from the time-matched vehicle controls shows separation of the dose groups. (b) PCA analysis of concentration data of metabolites found in median liver lobe after subtracting from the time-matched vehicle controls shows separation of the dose groups. (c) PCA analysis of concentration data of metabolites found in right liver lobe after subtracting from the time-matched vehicle controls shows separation of the dose groups (1-day low – green, 9-day low – dark green, 1-day high – red, 9-day high – blue).

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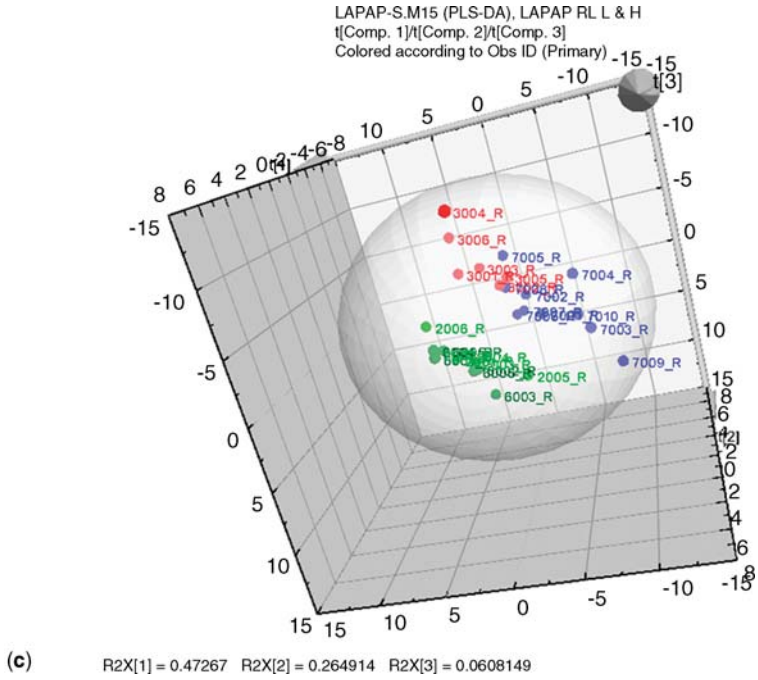

Figure 8. (*Continued*)

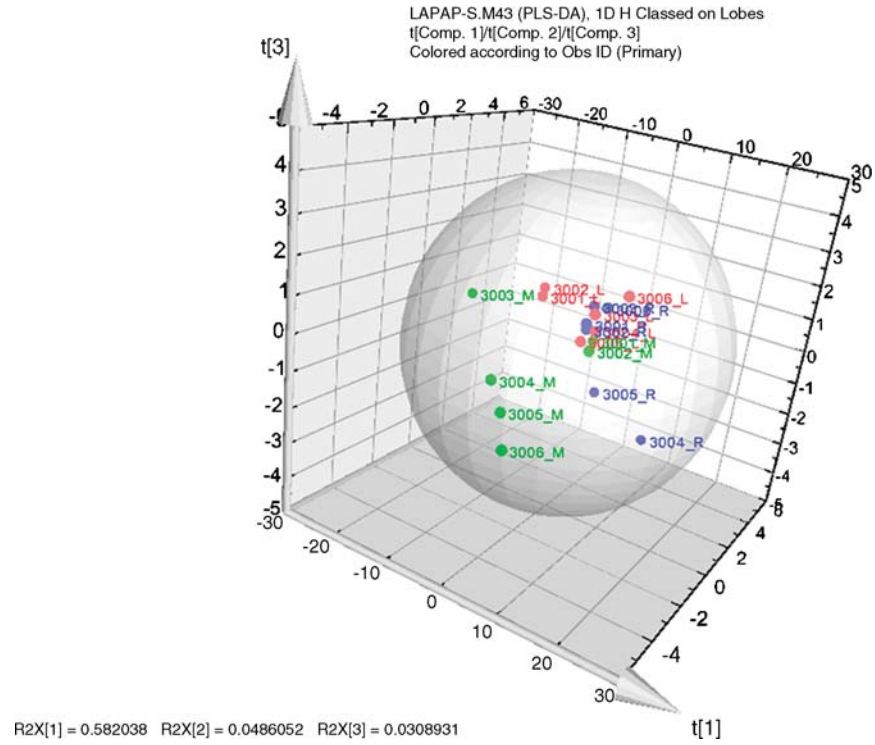

Figure 9. PLS-DA of liver samples from left, median, and right lobes obtained from the rats treated with high-dose APAP after 1-day exposure to APAP (left lobe, red; median lobe, green; right lobe, blue).

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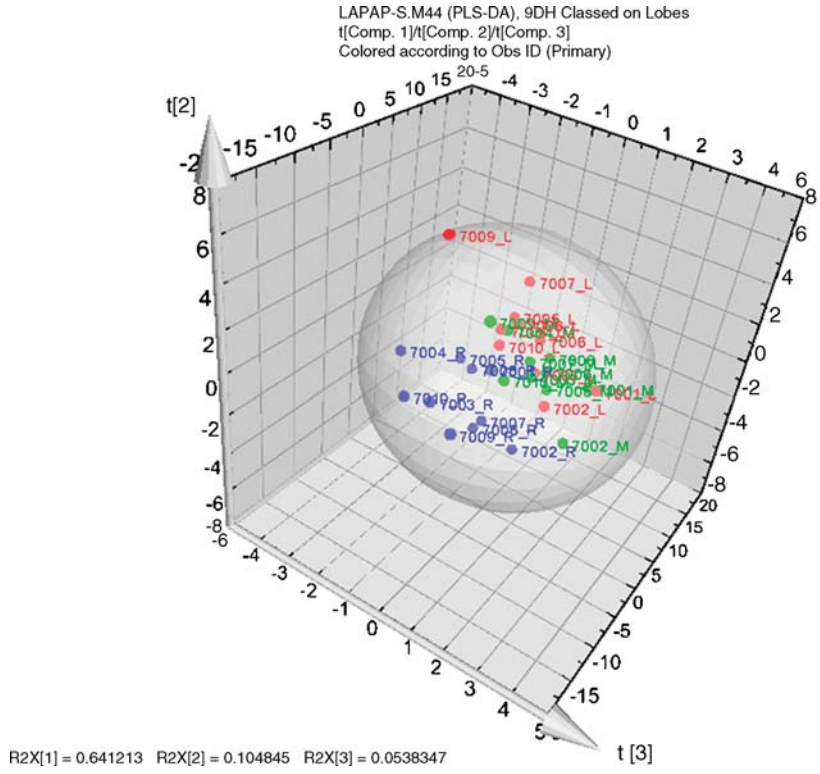

Figure 10. PLS-DA of liver samples from left, median, and right lobes obtained from the rats treated with high-dose APAP after 9 days exposure to APAP (left lobe, red; median lobe, green; right lobe, blue).

Table 3. Subsets of liver metabolites that separate the high dose group (1 day) from time-matched vehicle control. Metabolites marked in bold are increased in all lobes, those in *italics* are decreased in all lobes, and normal font indicates a lobe dependence.

Left Lobe		Median Lobe		Right Lobe	
Increased	Decreased	Increased	Decreased	Increased	Decreased
Homoserine	*Glutathione*	**Oxalacetate**	*β-Alanine*	**Cystine**	*β-Alanine*
Glutamate	*β-Alanine*	**Cystine**	*Glutathione*	**Oxalacetate**	*Glutathione*
O-Phosphoserine	*Glutarate*	**Glycerol**	*Butanone*	**O-Phosphoserine**	*Glutarate*
Carnitine	*Butanone*	**Glutamate**	*Glutarate*	Glucose	*Butanone*
Cystine	*Betaine*	**Glucitol**	*Betaine*	**Glycerol**	*Betaine*
O-Phosphocholine	N,N-Dimethylglycine	**Guanidoacetate**		**Ribose**	
3-Phenylpropionate	Trimethylamine N-oxide	**Serine**		**Homoserine**	
Oxalacetate		**Mannitol**		**Guanidoacetate**	
Glucitol		Allantoin		Allantoin	
Ribose		**Ribose**		**O-Phosphoethanolamine**	
Glycerol		**O-Phosphoserine**		**Mannitol**	
N-Carbamoyl-β-alanine		Taurine		**Glutamate**	
O-Phosphoethanolamine		Glucose		Taurine	
Mannitol		**Homoserine**		Carnitine	
Serine		Galactitol		**Glucitol**	
Alanine		**O-Phosphoethanolamine**		**Serine**	
Glucarate		**Glycylproline**		Methylmalonate	
N-Acetylglycine		S-Sulfocysteine		**Glycylproline**	
Glycylproline		Methylmalonate		Glucarate	
Guanidoacetate		Lactose		Galactitol	

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Table 4. Subsets of liver metabolites that separate the 9-day high-dose group from 1-day high-dose group after removing the effect of aging of the animals. Metabolites marked in bold are increased in all lobes, those in *italics* are decreased in all lobes, and normal font indicates lobe dependence.

Left Lobe		Median Lobe		Right Lobe	
Increased	Decreased	Increased	Decreased	Increased	Decreased
Pyroglutamate	*Guanidoacetate*	Creatinine	Glucose	2-Aminobutyrate	Glucose
2-Aminobutyrate	Choline	2-Oxoglutarate	*Guanidoacetate*	**Alloisoleucine**	Phenylacetate
Leucine	*Cystine*	Ornithine	Galactitol	**Arginine**	Homoserine
Arginine	Fumarate	Isoeugenol	Glucitol	Glutarate	Methanol
Alloisoleucine	Glycerol	2-Hydroxyisocaproate	*Taurine*	**Hypoxanthine**	N-Acetylglycine
Hypoxanthine	Glucitol	**Thymine**	Glycerol	**Leucine**	*Guanidoacetate*
Glutaric acid monomethyl ester	Ribose	**Tiglylglycine**	*Cystine*	**Pyroglutamate**	*Taurine*
Phenol	3-Hydromuconate	Glutathione	Xanthine	**Thymine**	2-Aminoadipate
2-Hydroxyphenylacetate	Gentisate	**Alloisoleucine**	Ethylene glycol	**Tiglylglycine**	*Cystine*
Ornithine	Cis-Aconitate	**Arginine**	Mannitol		N-Acetylglutamate
Glutamine	*Taurine*	**Leucine**	Serine		Xylose
Lysine	Urocanate	**Hypoxanthine**	Allantoin		Allantoin
2-Oxoglutarate	Acetoacetate	**Pyroglutamate**	3-Methylxanthine		2-Octenoate
Isoeugenol	O-Phosphoethanolamine		Alanine		Galactitol
Fucose	Ferulate		Caprylate		Aspartate
Tiglylglycine	Succinylacetone		Xylose		Xanthine
3-Hydroxy-3-methylglutarate	Quinolinate		Isocitrate		2-Hydroxy-3-methylvalerate
2-Hydroxyisocaproate	Galactose		Ribose		Malate
Thymine	Glycerate		S-Sulfocysteine		3-Methylxanthine
Asparagine	Formate		cis-Aconitate		

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Table 5. Subsets of liver metabolites that separate the high-dose group (9 days) from time-matched vehicle control. Metabolites marked in bold are increased in all lobes, those in *italics* are decreased in all lobes, and normal font indicates lobe dependence.

Left Lobe		Median Lobe		Right Lobe	
Increased	Decreased	Increased	Decreased	Increased	Decreased
O-Phosphoserine	*Glutathione*	**O-Phosphoserine**	*Glutathione*	**O-Phosphoserine**	Glucose
Glycylproline	*Betaine*	**Glycylproline**	Glucose	**Pyroglutamate**	*Glutathione*
Homoserine	*Trimethylamine N-oxide*	**Homoserine**	*N,N-Dimethylglycine*	**3,5-Dibromotyrosine**	Glucitol
3,5-Dibromotyrosine	*N,N-Dimethylglycine*	**3,5-Dibromotyrosine**	*Betaine*	**Arginine**	*2-Phosphoglycerate*
Oxalacetate	*Butanone*	**Pyroglutamate**	*Trimethylamine N-oxide*	**Glutamate**	*Betaine*
Pyroglutamate	Levulinate	**Glutamate**	*2-Phosphoglycerate*	**Glycylproline**	Taurine
Arginine	*Uridine*	**Glucarate**	Lactate	Glutamine	*Trimethylamine N-oxide*
Cysteine	*Maleate*	**Arginine**	*Butanone*	**Cysteine**	S-Sulfocysteine
Glucarate	*2-Phosphoglycerate*	Glycine	Levulinate	**2-Aminobutyrate**	*N,N-Dimethylglycine*
Glycine		Oxalacetate	Isocitrate	**Glucarate**	Serine
Allantoin		**Cysteine**	Methylmalonate	**Homoserine**	Galactitol
Glutamate		Glutamine	*Uridine*	**Leucine**	Glycerol
2-Hydroxyisocaproate		**2-Aminobutyrate**	*Maleate*	**Lysine**	*Butanone*
Leucine		Carnitine	Methanol	**N-Carbamoylaspartate**	Galactose
2-Aminobutyrate		**Lysine**	Methylamine	Alloisoleucine	Mannitol
Threonine		**Leucine**	cis-Aconitate	Ornithine	*Maleate*
Alloisoleucine		2-Hydroxyglutarate	*β*-Alanine	Hypoxanthine	Guanidoacetate
N-Carbamoylaspartate		**N-Carbamoylaspartate**	2-Hydroxyisobutyrate	2-Hydroxyvalerate	1,6-Anhydro-*β*-D-glucose
Lysine		2-Hydroxyisocaproate		2-Hydroxyglutarate	cis-Aconitate
2-Hydroxyvalerate		Hypoxanthine		Threonine	*Uridine*

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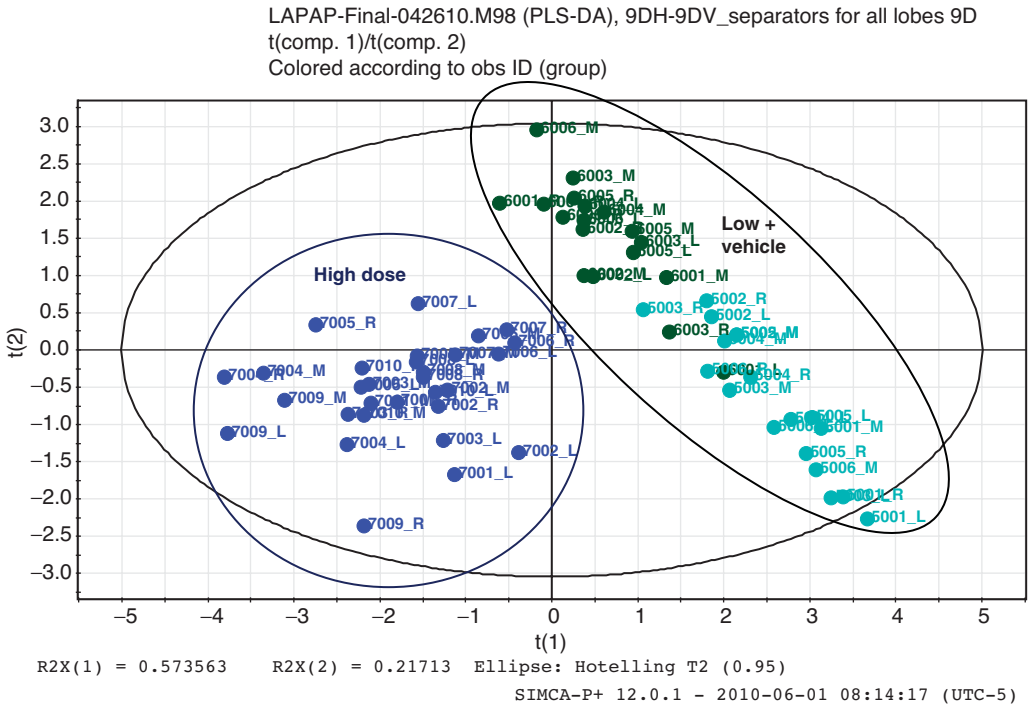

Figure 11. PCA of liver data (concentrations) obtained by using the subset of metabolites that are responsible for separation of the 9-day high-dose group from the time-matched vehicle control irrespective of the lobe (9-day vehicle, teal; 9-day low, dark green; 9-day high, blue).

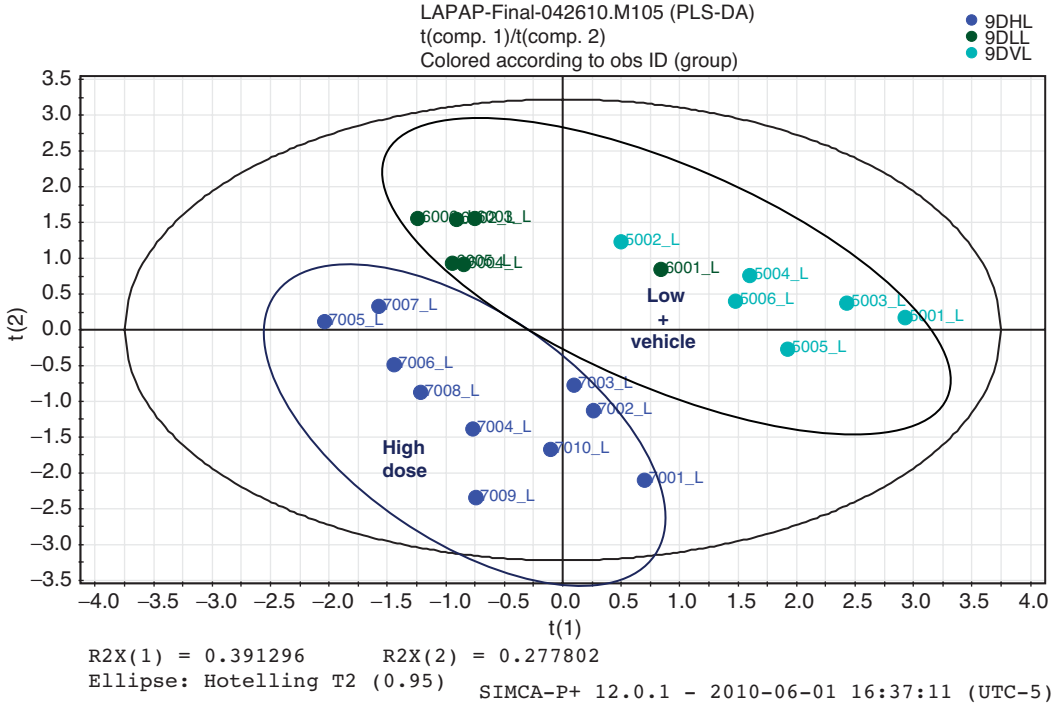

Figure 12. PCA of liver data (concentrations) obtained by using the subset of metabolites that are responsible for separation of the 9-day high-dose group from the time-matched vehicle control for the left lobe (9-day vehicle, teal; 9-day low, dark green; 9-day high, blue).

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Application of Metabonomic Approach in Target Organ Toxicity

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1 INTRODUCTION

Toxicology studies the adverse effects of diverse agents, such as drugs, industrial-, agricultural- and biological-origin chemicals, on living systems. Upon entering the organism, these agents could induce functional and structural alterations in cells, tissues and organs. A toxic substance could interact at similar subcellular sites in different organs, although the level of toxicity in each one would depend on differences in the absorption, distribution, metabolism and response of a specific cell type to the toxic effect (Zimmerman, 1999).

Toxicological studies are usually based on histopathological evaluation and blood biochemistry measurements. Recently, conventional toxicological approaches combined with new technologies, such as the "omics" ones (genomics, proteomics and metabonomics), are used for both the characterization and the understanding of the mechanisms underlying toxicity (Gatzidou, Zira and Theocharis, 2007). Genomics represents the study of the genome of an organism including the alterations occurring during a toxic insult. The proteome, the entire complement of proteins, including the modifications made to a particular set of proteins, produced by an organism or system is termed as proteomics. Based on the definition given by Nicholson, Lindon and Holmes 1999, "Metabonomics is the dynamic multiparametric response of living systems to pathophysiological stimuli or genetic modifications." The term metabonomics has been used to describe the study of the global metabolite profiles in cells, tissues or biofluids of an organism under physiological or pathological conditions. Metabolomics is usually coupled with statistics and especially pattern recognition techniques such as principal component analysis (PCA), providing information concerning low-concentration metabolites that could not be measured using conventional biochemical methods.

Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS), coupled with either liquid or gas chromatography (LC-MS and GC-MS, respectively), are the most commonly used methods that lead the metabolic profiling studies. Both NMR and MS have the ability to measure simultaneously variations in the levels of metabolites. Although MS is more sensitive than NMR, it is not very often used due to the discrimination required before the sample reaches the MS. NMR spectroscopy is a method of choice for the metabonomic studies because it is quick, requires little or no sample preparation and is not catastrophic for the sample. High-resolution NMR of biofluids, tissues or cells can give valuable information concerning alterations in the levels of metabolites under pathological conditions, including toxicity.

The purpose of the present study is to record the existing data in the recent literature concerning

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metabonomics and its application in toxicology, focused on different metabolic alterations occurring post-toxin-induced injury in target organs (liver, kidneys, heart and brain). Target organ toxicity studied using the metabonomic approach provides a holistic picture of the alterations leading to the discovery of metabolites that could be correlated with the toxins and their mechanism of action.

2 METABONOMICS AND TARGET ORGAN TOXICITY

The existing metabonomic studies on target organ toxicity categorized for different organ and mode of toxic effect (acute and chronic) are summarized in the following section. Descriptive tables regarding the animal, dose, route of administration, type of experimentation (time- and/or dose-dependent) used, the examined biofluids and methodology applied are also provided (Tables 1–3).

2.1 Liver Toxicity

The liver is more susceptible to toxins because it is the major site for xenobiotic metabolism. To that end, many experimental animal models are used to determine whether the exposure to different substances poses any risk either to animals or to humans. The metabonomic approach has been widely applied in the models of hepatotoxicity induced by chemicals or drugs (Table 1).

Acetaminophen (paracetamol, APAP) is a well-known mild analgesic drug, safe in therapeutic doses, that induces centrilobular hepatic necrosis at high doses. It is regularly used as an experimental hepatotoxin because it provides a model of acute liver failure (Zimmerman, 1999).

A dose- and time-dependent metabonomic study was performed to characterize the biochemical alterations in mice after APAP administration (Coen *et al*., 2003). ^{1}H-NMR spectra of intact liver tissue, tissue extracts (aqueous and lipid) and blood plasma samples were obtained from APAP-treated [50,150 and 500 mg kg^{-1} of body weight (BW), intraperitoneally (i.p.)] and control animals, killed at 15, 30, 60, 120 and 240 min after the drug administration. PCA was applied to cluster the cases based on their common metabolic profile recorded by NMR. The decreased hepatic glucose and glycogen levels and the increased lactate levels in the aqueous soluble liver extract reflected a rise in the rates of glycogenolysis and glycolysis during APAP-induced injury. Furthermore, the enhanced lactate levels, glucose and glycogen noted in plasma samples indicated mitochondrial malfunction leading to incapacity to use pyruvate in the citric acid cycle. The elevated triglyceride levels appearing in spectra of intact liver tissue, lipid-soluble liver extract and blood plasma can be attributed to an energy shift resulting from APAP toxicity. Histopathological examination was in concert with the results obtained from the lipid-soluble liver extracts presenting impairment of mitochondria that could lead to decreased energy levels and lipid accumulation in the liver.

Based on this experimental model, the APAP-induced liver injury was further investigated using the combination of metabonomics and transcriptomics, in an effort to better understand the time-dependent biochemical consequences (Coen *et al*., 2004). Alterations in the levels of metabolites, as glucose, choline/phosphocholine, lipoproteins, lactate and acetate, were parallel with those observed in gene expression [Insulin-like growth factor binding protein 1 (IGFBP-1), phospholipase, lipoprotein lipase], suggesting modifications in lipid metabolism, mitochondrial dysfunction and energy metabolism as a result to the toxic insult (Coen *et al*., 2004).

More recently, metabolic profile alterations in serum samples post-acute APAP-induced liver injury in rabbits were monitored using ^{1}H-NMR spectroscopy, by our group (Zira *et al*., 2009a). The effect of the established antidote *N*-acetylcysteine (NAC) and the proposed antidotes silybinin (SIL), cimetidine (CIM) and SIL/CIM was also examined using the same methodology. Animals were administered with a single dose of either APAP (2 g kg^{-1} BW, intragastrically) or APAP combined with the antidotes SIL [100 mg kg^{-1} BW intramuscularly (i.m.), 30 min after APAP administration], CIM (50 mg kg^{-1} BW, i.m.) and NAC (150 mg kg^{-1} BW, i.m.) and were killed at 24 h post-treatment. Elevated lipid levels (LDL/VLDL, *N*-acetylglycoproteins, CH=CH) in the APAP-treated group compared with controls confirmed the previously mentioned observation of the energy metabolism shift due to the toxic agent. Co-administration of APAP with CIM, SIL/CIM and NAC decreased the lipid levels compared with

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Table 1. Summary of the experimental models examined in liver toxicity.

	Liver toxicity							
	Experimental animals	Dose used	Root of administration	Time-dependent experiment	Dose-dependent experiment	Biofluids examined	Methods	References
APAP acute	Mice	50, 150, 500 mg kg^{-1} BW	i.p.	Yes	Yes	Tissue, plasma	NMR	Coen *et al.* (2003)
	Mice	50, 150, 500 mg kg^{-1} BW	i.p.	Yes	Yes	Tissue, plasma	NMR, transcriptomics	Coen *et al.* (2004)
	Rabbits	2 g kg^{-1} BW	i.g.	No	No	Serum	NMR	Zira *et al.* (2009)
	Rats	400, 1600 mg kg^{-1} BW	Oral	Yes	Yes	Urine	NMR, UPLC/MS	Sun *et al.* (2008)
	Rats	6, 25, 110, 450, 1800 mg kg^{-1} BW	Oral	Yes	Yes	Urine	NMR	Schoonen *et al.* (2007)
APAP chronic	Rats	200, 400,800 mg kg^{-1} BW	Oral	Yes	Yes	Urine	NMR, UPLC/MS	Sun *et al.* (2008)
	Rats	2 g kg^{-1} BW	Oral	No	No	Urine	NMR	Kim *et al.* (2008)
Hydrazine acute	Rats	75, 90, 120 mg kg^{-1} BW	Oral	No	Yes	Urine, plasma	NMR	Nicholls *et al.* (2001)
	Rats, mice	30 and 90 mg kg^{-1} BW (rats), 100 and 250 mg kg^{-1} BW (mice)	Oral	Yes	Yes	Urine	NMR	Bollard *et al.* (2005)
	Rats	20, 30 mg kg^{-1} BW	Oral	No	Yes	Serum	NMR, genomics, proteomics	Klenø *et al.* (2004)
CCl_4 acute	Rats	1 ml kg^{-1} BW	i.p.	Yes	No	Tissue, plasma	NMR	Zira *et al.* (2007)
	Rats	1 ml kg^{-1} BW	Oral	Yes	No	Urine	LC-MS	Lin *et al.* (2009)
	Rats	1 ml kg^{-1} BW	Oral	Yes	No	Urine	NMR	Kim *et al.* (2008)
CCl_4 chronic	Rats	0.05–0.5 ml	Oral	No	Yes	Tissue	NMR	Harvey *et al.* (1999)
D-Galactosamine	Rats	0.8 g kg^{-1} BW	i.p.	Yes	No	Urine	NMR	Kim *et al.* (2008)
ANIT acute	Rats	150 mg kg^{-1} BW	Oral	Yes	No	Tissue	NMR	Waters *et al.* (2002)
	Rats	0, 50 75, 100, 150 mg kg^{-1} BW	Oral	Yes	Yes	Urine	NMR	Azmi *et al.* (2005)
TAA acute	Rats	150 mg kg^{-1} BW	Oral	Yes	No	Tissue, plasma, Urine	NMR	Waters *et al.* (2005)
	Rats	400 mg kg^{-1} BW	i.p.	No	No	Tissue, serum	NMR	Zira *et al.* (2009)
TAA chronic	Rats	300 g l^{-1}	Oral	Yes	No	Tissue, serum	NMR	Constantinou, Theocharis and Mikros (2006)
Bromobenzen acute	Rats	1.5 g kg^{-1} BW	Oral	Yes	No	Tissue, plasma, Urine	NMR	Waters *et al.* (2007)
	Rats	2, 8, 32, 125, 500 mg kg^{-1} BW	Oral	No	Yes	Urine	NMR	Schoonen *et al.* (2009)
Realgar chronic	Rats	1.0 g kg^{-1} BW	i.g.	Yes	No	Urine, serum, tissue	NMR	Wei *et al.* (2005)
Methapyrilene chronic	Rats	50 mg kg^{-1}, 150 mg kg^{-1} BW	Oral	No	Yes	Tissue,	NMR, genomics, proteomics	Craig *et al.* (2007)
Ethionine	Rats	80 mg kg^{-1}, 800 mg kg^{-1} BW	Oral	Yes	Yes	Urine, liver	NMR	Skordi *et al.* (2006)
Allyl formate	Rats	75 mg kg^{-1} BW	i.p.	Yes	No	plasma, Urine, tissue	NMR	Yap *et al.* (2006)
PFDoA chronic	Rats	0.02, 0.05, 0.2, 0.5 mg kg^{-1} BW	Oral	—	Yes	Tissue, serum	NMR	Ding *et al.* (2009)

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the APAP group. The decreased glucose and the increased lactate levels revealed the augmented rate of glycogenolysis and glycolysis. Alterations in the levels of other metabolites such as alanine, valine, leucine and isoleucine were related to the degree of liver damage because the protein synthesis was disturbed and necrosis caused protein degradation. The beneficial effect of the antidotes SIL, CIM and NAC were in agreement with histopathological and serum biochemical findings.

An NMR and UPLC/MS metabonomic analysis of urine obtained from either acutely or chronically, orally, APAP-treated rats was also performed (Sun *et al*., 2008). Acute APAP intoxication was achieved by a single dose of either 400 or 1600 mg kg^{-1} BW. Chronic APAP intoxication was achieved by treatment with 200, 400 and 800 mg kg^{-1} BW APAP a day for seven consecutive days. Urine samples were collected at different time-points. In contrast to the acute APAP intoxication, clinical chemistry and histopathology in the chronic toxicity study did not reveal any evidence of toxicity. Regenerative changes occurred 48 h post-acute administration as demonstrated by both histopathology and metabonomics. Krebs' cycle intermediates (citrate, pyruvate, succinate and 2-OG), as well as glucose, trimethylamine N-oxide (TMAO), taurine and betaine, were found reduced after both acute and chronic APAP treatment. Additionally, an increase in acetate levels suggested a switch in the energy metabolism, due to the APAP intoxication.

Hydrazine is a metabolite of many pharmaceutical substances such as isoniazid (anti-tuberculosis) and hydralazine (anti-hypertensive) and its hepatoxic, neurotoxic, carcinogenic and mutagenic effects have been widely studied (Zimmerman, 1999).

The biochemical effects induced by hydrazine orally administered in rats at doses of 75, 90 and 120 mg kg^{-1} BW have been investigated (Nicholls *et al*., 2001). ^{1}H-NMR spectra were obtained in blood plasma samples and urine pre- and post-hydrazine treatment. The increased urinary levels of both creatine and taurine have been correlated with the reduced liver function. ^{1}H-NMR blood plasma spectra revealed an increase in tyrosine levels that have been previously reported as a consequence of hydrazine administration (Runge-Morris, Iacob and Novak, 1998).

The "omics" technologies were combined to investigate the mechanisms of hydrazine-induced hepatotoxicity in rats (Klenø *et al*., 2004). A single oral dose of hydrazine (30 and 20 mg kg^{-1} BW) was administered in rats that were sacrificed at 48 and 168 h post-dosing. Hydrazine administration caused changes in genes, proteins and metabolites that can be related to glucose and lipid metabolism and to oxidative stress.

Furthermore, interspecies variations, between rats and mice administered with hydrazine, have been studied (Bollard *et al*., 2005). Comparison of the ^{1}H-NMR spectra presented common metabolic changes in both rats and mice: a drop in succinate and citrate levels and an increase in creatine and 2-aminoadipate were noted in urine samples, the latter being related to neurological effects.

Acute carbon tetrachloride (CCl_4) administration in rats causes centrilobular hepatic necrosis (Jin *et al*., 2006) and steatosis followed by a regenerative process (Theocharis *et al*., 2001; Taniguchi *et al*., 2004), whereas chronic administration leads to cirrhosis and induces hepatic carcinoma in experimental animals (Zimmerman, 1999).

The time-dependent acute CCl_4 intoxication in rats was examined by ^{1}H-NMR-based metabonomics (Zira *et al*., 2007). Animals were administered with 1 ml CCl_4 kg^{-1} BW (i.p.) in a mixture with corn oil 1 : 10 (v/v) and were sacrificed at 0 (controls), 12, 24, 36, 48, 60 and 72 h post-dose. Alterations in the levels of lactate, glucose, LDL/VLDL, glutamine, glutamate and UFA/PUFA were associated with shift in the energy metabolism, increased rates of glycolysis/glycogenolysis, disturbed protein synthesis and lipid accumulation caused by CCl_4 treatment. The changes in the examined metabolites reached their maximum between 24 and 36 h (toxic phase) but gradually regained the control levels at 72 h (regenerative state). The pathways disturbed are in agreement with the findings of another studythat comprised a GC/MS-based metabonomic analysis on plasma in mice (Huang *et al*., 2008).

An integrated LC-MS metabonomics approach was applied on urine to monitor the metabolic alterations caused by a single dose of CCl_4 (1 ml kg^{-1} BW) in rat urine (Lin *et al*., 2009). Urine samples were collected at day 0 (controls) and at days 1–6. Oral administration of CCl_4 increased serum biomarkers AST and ALT and caused restorable liver damage evident by biochemical assays and histopathological examination. The decrease noted in the levels of citric acid was due to TCA

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dysfunction under oxidative stress caused by CCl_4. ATP insufficiency resulted in increased free creatine levels, which is degraded to creatinine, giving elevated urinary levels.

The alterations caused in the hepatic metabolism during CCl_4-induced cirrhosis were also investigated (Harvey *et al*., 1999). Rats were administered with weekly oral doses of CCl_4 (ranging from 0.05 to 0.5 ml) for 10 weeks. The animals were sacrificed 3 weeks after the last CCl_4 administration. ^{31}P- and ^{1}H-NMR spectra were obtained in perchloric acid-extracted liver tissues. Based on the ^{1}H- and ^{31}P-NMR experiments, alterations in all detectable metabolites in CCl_4-treated compared to untreated rats were noted. Cirrhotic rats presented a significant increase in the Pi level and Pi : ATP ratio and a significant decrease in ATP levels and ATP : ADP ratio, pointing to a poorer energy state. A significant decrease in NAD was apparent in cirrhotic rat livers, illustrating alterations in either the biosynthetic and/or the degradative pathway. Elevated levels of phosphocholine and phosphoethanolamine were presented in rats and were attributed to the stimulation of membrane biosynthesis in response to CCl_4 membrane damage.

1-Napthylisothiocyanate (ANIT) is a known toxin that induces reproducible and dose-dependent intrahepatic cholestasis by injuring biliary epithelial cells (Wu *et al*., 2005; Tanaka *et al*., 2009). ANIT, when administered acutely, leads to necrosis of portal bile ducts and inflammation of portal areas, whereas prolonged administration leads to bile ductal proliferation and cirrhosis induction (Zimmerman, 1999; Kodali *et al*., 2006).

Changes in the liver metabolites levels of ANIT-treated rats, sacrificed at different time-points post-treatment, were examined using ^{1}H-NMR spectroscopy of aqueous and lipid-soluble extracts and intact liver tissue (Waters *et al*., 2002). Rats were administered with a single ANIT dose of 150 mg kg^{-1} BW, p.o., and were sacrificed at 3, 7, 24, 31 and 168 h later on. Glucose and glycogen levels were decreased being characteristic of ANIT-induced toxicity. The declined levels of fumarate suggested alterations in the citric cycle. ^{1}H-NMR spectra and PCA of the aqueous liver extract presented an increase in the resonances assigned to inosine, adenosine, uridine, adenine, uracil and the nucleotide 5′ monophosphates GMP and TMP. Such observations may be attributed to ANIT or its metabolites that inhibit the enzymes involved in nucleotide metabolism. The elevated choline and phosphocholine levels observed in the ^{1}H-NMR aqueous soluble liver extract could be explained by the membrane breakdown and the accelerated lipid metabolism.

^{1}H-NMR spectroscopy was also employed to investigate the effects of ANIT and distinguish them from the effects of 1-napthylisocyanate (NI) and 1-napthylamine (NA), two main products of ANIT metabolism (Azmi *et al*., 2005). ^{1}H-NMR spectroscopy of rat urine from ANIT-treated animals revealed changes in the metabolites in a dose-dependent manner for all doses used. A single oral dose of ANIT (0, 50, 75, 100 and 150 mg kg^{-1} BW), NI and NA (75 mg kg^{-1} BW) was administered to rats. Decreased citrate, succinate, 2-oxoglutarate (2-OG), dimethylglycine and hippurate urine levels were noted. In addition, elevated urine levels of glucose, lactate, acetate, creatine, taurine and bile acids were recorded at the different time-points examined. TMAO and choline levels declined at first and increased later compared with controls. Elevated glucose and TMAO levels and decreased LDL/VLDL, choline and lactate levels were observed in plasma samples. ^{1}H-NMR of the intact tissue revealed dose-dependent decrease of glucose and glycogen levels together with increased lipid resonances, TMAO, choline and glycerophosphocholine levels. Following NI and NA administration, ^{1}H-NMR spectra of urine, blood plasma and intact tissues revealed that similar metabolite alterations occurred after treatment with either compound; however, the authors concluded that ANIT is the most toxic followed by NI and NA.

Thioacetamide (TAA) causes centrilobular hepatic necrosis and nephrotoxic damage when administered acutely (Waters *et al*., 2005) and bile duct proliferation leading to liver cirrhosis after chronic administration (Constantinou, Theocharis and Mikros, 2006).

Waters *et al*. have studied liver and kidney toxicity induced by acute TAA administration (150 mg kg^{-1} BW in water, sacrificed at 3, 7, 24, 31 and 79 h post-dosing) using the metabonomic approach (Waters *et al*., 2005). ^{1}H-NMR spectra of intact liver tissue, blood plasma and urine were recorded and their results were correlated with clinical chemistry and histopathological examination. ^{1}H-NMR spectroscopy of blood plasma from animals treated with a single TAA dose revealed an increase in choline and creatine levels at all time-points

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examined and a rise in the amino acids valine, glutamine, leucine, isoleucine, glutamate, lysine and alanine that could be associated with the elevated levels of 3-D-hydroxybutyrate and acetoacetate. Citrate and LDL levels were decreased while acetate was augmented. ^{1}H-NMR spectra of intact tissue revealed a decrease in the glucose, glycogen, TMAO and betaine levels. The signals assigned to lipids, choline and phosphocholine were elevated accompanied by a rise of isoleucine, leucine and creatine levels. The depletion in hepatic glucose and glycogen levels was correlated with the elevated plasma lactate, suggesting increased rates of glycogenolysis and glycolysis. The elevated lipid levels monitored in the ^{1}H-NMR of the intact liver demonstrate the drug-induced lipid accumulation, a common response to toxicity.

Rat serum and liver tissue extracts from TAA-induced fulminant hepatic encephalopathy (FHE) rats (Theocharis *et al*., 2003) were studied using NMR-based metabonomics, by our group (Zira *et al*., 2009b). Rats were administered with acute dose of TAA (400 mg TAA kg^{-1} BW, i.p., at three consecutive time intervals of 24 h) and were sacrificed 1 h after the last TAA administration. NMR analysis revealed an overall decrease in the hepatic levels of lactate, acetate, valine, leucine, isoleucine, choline, glutamine, glutamate, glutathione, $N^+(CH_3)_3$ of the choline headgroup, glucose and glycogen in treated animals as a result of TAA-induced FHE. Serum lipoproteins in TAA-treated rats were also decreased, suggesting induction of lipid peroxidation.

Moreover, the NMR-based metabonomic approach of chronic TAA-induced liver injury, representative of early and late fibrosis and cirrhosis, has been studied (Constantinou, Theocharis and Mikros, 2006). Animals were chronically treated with TAA (300 mg l^{-1} in drinking water) and were sacrificed at the end of the first, second and third months from the onset of treatment. The changes in the metabolic profile were recorded by ^{1}H-NMR spectroscopy in liver tissue extracts (aqueous, lipid) and serum. A decrease in the resonances assigned to glucose and glycogen combined with the increased lactate levels in serum during the second and third months suggest a rise in the rates of glycogenolysis and glycolysis. The augmented hepatic lactate may be attributed to an energy shift towards ketone body formation. ^{1}H-NMR spectra of the aqueous liver extract revealed a decrease in the glucose levels that could be related to the observed deficiency of Krebs' cycle intermediates (e.g., fumarate). In addition, the observed increase in glycerophosphorylcholine and choline could be characteristic of lipid catabolism and the depletion of hepatic TMAO may suggest a TAA-induced block in the N-oxidation pathway of choline and glycerophosphorylcholine.

Bromobenzene administration causes hepatic centrilobular necrosis along with kidney and lung necrosis. The bromobenzene-induced hepatotoxicity was studied using the metabonomic approach (Waters *et al*., 2006). The alterations caused after oral bromobenzene administration (1.5 g kg^{-1} BW, in corn oil) were monitored by ^{1}H-NMR spectroscopy of intact rat liver tissue, blood plasma and urine. ^{1}H-NMR urine analysis presented a rise in creatine along with a decrease in taurine levels, indicating hepatic necrosis with glutathione reduction. A number of metabolites involved in energy generation (glucose, glycogen, Krebs' cycle intermediates) were altered pointing to increased energy metabolism. The changes observed in the levels of amino acids, in either liver tissue or blood plasma, were related to the amino acid and nucleotide metabolism. Lipid degradation was verified by increased choline/phosphocholine levels together with decreased liver lipid and plasma LDL. 5-Oxoproline, a new metabolite associated with the γ-glutamyl cycle, was detected in rat liver, plasma and urine and following bromobenzene administration.

Realgar is an ore crystal containing more than 90% tetra-arsenic-tetrasulfide (As_4S_4) and has been used as a traditional Chinese medicine. However, it is known that arsenic is a poison and carcinogen possibly through chromosomal abnormalities, oxidative stress, altered growth factors, cell proliferation and altered DNA repair (Wei *et al*., 2009).

The time-dependent toxicological effects of realgar in male Wistar rats after intragastric administration (1 g kg^{-1} BW) were investigated using the metabonomic approach. Animals were treated with realgar for a time period of 21 days, and ^{1}H-NMR spectra of urine, serum and liver tissue aqueous extracts were recorded (Wei *et al*., 2009). Metabonomics revealed alterations in the levels of metabolites related to energy metabolism. Decreased levels of glucose and glycogen represent elevated rates of glycolysis and glycogenolysis. Decreased levels of pyruvate and lactate followed

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by elevated urinary excretion of Krebs' cycle intermediates (citrate, succinate and α-ketoglutarate) suggested an expansion in Krebs' cycle pool. Alteration in the levels of ATP/ADP in aqueous soluble liver extract also suggested disturbance in energy metabolism. The increased levels of choline-containing metabolites after realgar treatment were possibly caused by realgar-induced membrane toxicity. Furthermore, the decreased levels of alanine and glucose in both liver and serum imply a delay in the glucose-alanine cycle and the drop in the levels of glutathione is the way the organism protects cells from the free radicals created by the toxic agent.

Methapyrilene is a histamine antagonist causing periportal liver necrosis in rats, and its effect was investigated using a combination of genomics, proteomics and metabonomics (Craig *et al*., 2005). Male rats were orally administered with methapyrilene for 3 days with a subtoxic dose of 50 mg kg^{-1} day^{-1} or a toxic dose of 150 mg kg^{-1} day^{-1} (causing liver necrosis). Administration of methapyrilene caused a number of dose-related changes in gene, protein and metabolite profiles. Alterations in the expression of genes associated with stress (e.g., heat shock protein expression at gene and protein levels) and apoptosis, and in the metabolic pathways of glucose, fatty acids, choline metabolism and urea, were noted. Hepatic glucose and glycogen levels were decreased, as recorded by ^{1}H MAS NMR of intact liver, in agreement with the decrease noted in the gene and protein levels of intermediates related to carbohydrate metabolism. These observations suggest increased glycolysis and decreased gluconeogenesis that could be further supported by the decline of fructose 1,6-bisphosphatase and pyruvate carboxylase. The elevated triglycerides levels observed in ^{1}H MAS NMR along with the altered proteins involved in the phenylalanine catabolism and the synthesis of ketone bodies suggested impairment in mitochondrial fatty acid β-oxidation.

Ethionine, an analogue of the amino acid methionine, induces fat accumulation, steatosis and pancreatitis post-acute administration while post-chronic administration it induces bile duct proliferation, cirrhosis and hepatic carcinoma (Lombardi, 1976; Zimmerman, 1999).

The ethionine-induced liver injury in rats was recently investigated using NMR-based metabonomics (Skordi *et al.*, 2007). Animals were orally treated with a single low and high dose of ethionine (80 and 800 mg kg^{-1} BW, respectively). Rat urine was collected 7 and 17 h after dosing and over a 7-day period at 7- and 17-h intervals; the animals were sacrificed on days 2 and 8, respectively. MAS NMR of the intact liver presented accumulation of triglycerides, increased diacyl glycerol and increased hepatic bile acid concentration, which indicate liver damage caused by ethionine administration. Ethionine also interferes with the methylation reactions, γ-glutamyl cycle and energy metabolism (decreased levels of TCA intermediates, citrates, 2-OG, fumarate and succinate).

Allyl formate is a toxic agent that causes periportal hepatotoxicity (Obata, Egashira and Yamanaka, 1989; Yap *et al*., 2006). ^{1}H-NMR-based metabonomics were used to study the alterations induced in rats following allyl formate treatment. Spectra from intact liver tissue, blood plasma and urine were recorded for controls and rats administered with a single dose of allyl formate (75 mg kg^{-1} BW, i.p.) and sacrificed at days 2 and 8 post-treatment (Yap *et al*., 2006). Allyl formate administration induced hepatic lipidosis with significant increases in liver lipids and lower hepatic glucose, glycogen, taurine and TMAO levels. The decreased hepatic levels of glucose and glycogen also suggested increased energy demand. Furthermore, the reduced hepatic and urinary TMAO levels could be related to reduced food intake, disruption of liver function or intestinal bacteria. Moreover, the increased plasma tyrosine suggested impaired protein synthesis in the liver.

Peroxisome proliferators (PPs) are industrial/commercial chemicals presenting tumor promoter activity in rodents in a mechanism not completely clarified (Kawashima *et al*., 1995; Reo and Adinehzadeh, 2000). Among the PPs, perfluorododecanoic acid (PFDoA), a member of the perfluorocarboxylic acids (PFCAs) extensively used in food packaging, cosmetics, surfactants, surface protectors and fire-retardant foams, lacks promoter activity (Reo and Adinehzadeh, 2000; Prevedouros *et al*., 2006).

NMR-based metabonomics was applied to study the effect of PFDoA chronic exposure (110 days) on rat liver. PFDoA was orally administered via gavage at doses of 0.02, 0.05, 0.2 and 0.5 mg kg^{-1} BW/day (Ding *et al*., 2009). Increased lipid concentrations in liver tissue and decreased lipids and lipoproteins in serum suggested alterations in lipid metabolism due to PFDoA-induced hepatotoxicity. The increased levels of acetate and ketone bodies (3-HB and

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acetoacetate) were a sign of lipid peroxidation. Elevated levels of sugars, lactate, pyruvate, acetate and creatine suggested alterations in carbohydrate and energy metabolism after PFDoA administration.

A combined study of the effect of APAP and bromobenzene was conducted to compare NMR urine analysis with histopathological and biochemical parameters (Schoonen *et al*., 2007). A single dose of each of the toxic agents at five different concentrations (2, 8, 32, 125 and 500 mg kg^{-1} BW for bromobenzene and 6, 25, 110, 450 and 1800 mg kg^{-1} BW for APAP) was administered to rats and urine samples were collected at days 1 and 2 post-treatment. Changes in the plasma AST and ALT levels at both time-points post-treatment at the highest dosages are in agreement with the centrilobular necrosis induced by each of these compounds alone. NMR data presented an increase in lactate, acetate, pyruvate, glucose and 3-hydroxybutyrate, attributed to increased rates of glycolysis and disrupted oxidative phosphorylation from fatty acids, caused by APAP. Bromobenzene administration produced decrease citrate and α-ketoglutarate levels, which could also be attributed to a decreased oxidative phosphorylation.

In addition, the metabonomic approach was used in a combined study to discover potential biomarkers of hepatotoxicity induced by three agents, CCl_4, APAP and D-galactosamine (Kim *et al*., 2008). A single dose of either CCl_4 (1 ml kg^{-1} BW, orally) or D-galactosamine (0.8 g kg^{-1} BW, i.p.) was administered and urine was collected at days 1 and 2. APAP (2 g kg^{-1} BW, orally) was administered for 2 days and urine was collected 24 h after last treatment. NMR spectroscopic data combined with PCA from rats treated with CCl_4 present alterations in the levels of citrate, allantoin, taurine, 2-OG, acetate, lactate, betaine, succinate, benzoate and hippurate. PCA and NMR spectroscopic data from rats treated with APAP present alterations in the levels of citrate, allantoin, taurine, 2-OG, acetate, lactate, betaine, succinate, benzoate and hippurate, among other metabolites. Finally, in rats treated with D-galactosamine, the levels of 2-OG, acetate, taurine, citrate, lactate, allantoin, succinate, benzoate, betaine and hippurate were altered. Certain metabolites were altered in all three hepatotoxins. Decreased levels of citrate, 2-OG and succinate suggest hepatic necrosis. The elevated levels of taurine could be associated with the reduced hepatic function.

2.2 Kidney Toxicity

The kidney, after the liver, is one of the main target organs of xenobiotic-induced toxicity. Unfortunately, early detection of toxicant-induced kidney toxicity is still inadequate, yet the application of the "omics" technology could provide new biomarkers for kidney toxicity. The available data regarding metabonomics and kidney toxicity are depicted in Table 2.

Aristolochic acid (AA) is a chemical compound present in plants of the *Aristolochia*, *Bragantia* and *Asarum* species and is considered to be responsible for acute renal failure (ARF) associated with interstitial fibrosis, hyperproteinemia, severe anaemia, uraemia and carcinoma (Liu *et al*., 2003; Chen *et al*., 2007). Chen *et al*. used the metabonomic approach to identify the characteristic metabolic profile associated with AA-induced nephrotoxicity

Table 2. Summary of the experimental models examined in kidney toxicity.

	Kidney toxicity							
	Experimental animals	Dose used	Root of administration	Time-dependent experiment	Dose-dependent experiment	Biofluids examined	Methods	References
Aristocholic acid acute	Rats	50 and 96 mg kg^{-1} BW	i.p.	Yes	No	Urine	LC-MS	Chen *et al.* (2006)
Doxorubicin acute	Rats	5 and 15 mg kg^{-1} BW	i.v.	Yes	Yes	Urine	NMR	Park *et al.* (2009)
Gentamycin chronic	Rats	120 mg kg^{-1} BW	Subcutaneous	Yes	No	Urine	NMR	Lenz *et al.* (2005)
	Rats	60 and 120 mg kg^{-1} BW	Subcutaneous	Yes	Yes	Urine	NMR, GC-MS	Sieber *et al.* (2009)
Cyclosporine chronic	Rats	10 and 25 mg kg^{-1} BW (alone or combined with Rapa)	Oral	Yes	Yes	Urine	NMR	Klawitter *et al.* (2008)

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(Chen *et al.*, 2006). Rats were divided into three groups receiving either 0.9% saline solution (controls), or a single dose of AA (50 mg kg^{-1} BW on day 0) or CAM (*Caulis Aristolochiae manshuriensis*, 30 g kg^{-1} day^{-1}, equivalent with 96 mg kg^{-1} day^{-1} AA). Urine samples were collected pre-dose (−12–0 h) and 1–12, 12–24, 48–60, 84–96 and 132–144 h post-dose. Histopathological examination and plasma biochemistry confirmed severe kidney damage after the AA administration at high dosage. Both AA and CAM presented similar biochemical perturbations as noted by the LC-MS metabonomics analysis. Increased adenosine levels led to the accelerated formation of homocysteine, suggesting AA-induced renal dysfunction. The elevated levels of serine and 5-CH_3-tetrahydrofolate indicate elevated rates of the folate cycle. The up-regulated D-serine might be an indication of the perturbation of the D-serine metabolism after the AA administration. The depleted levels of arachidonic acid could be possibly attributed to the blocked phospholipase A(2)-catalysed release of arachidonic acid caused by AA.

Doxorubicin (adriamycin) possesses therapeutic activity against human neoplasms; however, its clinical use remains limited due to cardiac and renal toxicity. ^{1}H-NMR-based metabonomics have been used to evaluate urinary metabolic alterations in rats treated with doxorubicin (Park *et al.*, 2009). A single intravenous (i.v.) injection of 5 and 15 mg doxorubicin hydrochloride per kg BW was administered to rats and urine samples were collected pre- and post-dose. PCA and particularly the mean scores plots showed that low- and high-dose -groups presented a dose-dependent shift. The low-dose group presented its maximum shift from controls at 48 h and returned to control levels. However, the high-dose group has not reached control levels at 168 h, suggesting irreversible toxicity. Animals treated with the high dose of doxorubicin presented either moderate recovery or no recovery towards control levels. Elevated urinary levels of taurine and creatine suggested hepatic toxicity. Increased urinary levels of creatine were apparent in the high-dose group at 168 h post-treatment, indicating inhibition of creatine kinase by doxorubicin. Elevated levels of *N*-methylnicotinamide in moderate recovery group animals of the high dose provided evidence for recovery from the toxicity. Increased phenylacetylglycine levels were the result of the doxorubicin-induced phospholipidosis in the liver. Urinary levels of TMAO in the low-dose group reached their maximum at 48 h post-dose and then recovered to control levels, suggesting that osmotic stress in the renal medulla resulted in metabolite alterations. The concomitant elevation in lactate, alanine and glucose levels (known as markers of renal cortical injury) indicated progression of renal toxicity induced by doxorubicin. In the high dose, both moderate- and no-recovery animals presented elevated lactate, alanine and glucose levels that were not altered compared with controls at 168 h. The excessive proteinuria might be provoked by doxorubicin, resulting in renal damage. Furthermore, the intermediates of the TCA cycle (citrate, 2-2-OG and succinate) were decreased, suggesting increased energy metabolism.

The application of HPLC-MS and ^{1}H-NMR spectroscopy to the study of the effects of the aminoglycoside antibiotic gentamycin was also reported (Lenz *et al.*, 2005). Male Wistar rats were treated with gentamycin at a dose of 120 mg kg^{-1} BW per day (subcutaneously), in the form of two separate doses of 60 mg kg^{-1} twice daily 8 h apart, for seven consecutive days. Urine samples were collected at day 1 and daily thereafter for 9 days. In addition to the metabonomic analysis, gentamycin-induced nephrotoxicity was also estimated using histopathological kidney examination and standard plasma and urine biochemical analysis. A decrease in TMAO and betaine and a gradual increase in the levels of citrate, glucose and lactate were noted, typical of gentamycin-induced nephrotoxicity.

The effect of gentamycin was studied using a combined ^{1}H-NMR and GC-MS metabonomics approach in order to find novel urinary markers for early detection of chemically induced nephrotoxicity (Sieber *et al.*, 2009). Male Wistar rats were treated with gentamycin at doses of 60 and 120 mg kg^{-1} BW per day (subcutaneously), for seven consecutive days. Urine samples were collected at 24-h intervals, whereas blood was withdrawn 24 and 72 h after the first dosing. Apart from the metabonomic approach, clinical chemistry (in urine and plasma), enzyme-linked immunosorbent assay (ELISA) in urine, immunohistochemistry and quantitative RT-PCR were also performed. Based on the metabonomic analysis, a clear dose- and time-dependent separation of gentamycin-treated rats compared to controls was observed. Decreased levels of citrate, hippurate, trigonelline and 3-indoxysulfate and increased

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levels of glucose, lactate and dimethylglycine were observed. Creatine was not detected in controls; on the contrary, it was excreted in urine of gentamycin-treated rats. Enhanced urinary levels of creatine have been associated with hepatotoxicity; however, in the present study, this might be the result of drug-induced oxidative modification of mitochondrial or cytosolic creatine kinases, enzymes susceptible to oxidative stress. Increased lactate levels may be attributed to the mitochondrial toxicity induced by gentamycin.

The immunosuppressant calcineurin inhibitor cyclosporine (CsA) is used to protect against allograft rejection (Ojo *et al.*, 2003). However, extended use of calcineurin inhibitors is associated with a high risk of complications that can affect both graft and patient survival. CsA toxicity, acute or chronic, involves oxidative stress, apoptosis, metabolic changes and an increase in vascular resistance, resulting in decreased renal blood flow (Humes *et al.*, 1985; Sullivan, Hak and Finn, 1985; Campistol and Grinyó, 2001; Chapman, O'Connell, Nankivell, 2005). CsA combined with other drugs [proliferation signal inhibitors such as sirolimus (known as rapamycin, Rapa)] presents additive or synergistic immunosuppressive activity, allowing for lowering CsA doses. The time-dependent effects of CsA and Rapa, alone or in combination, on the rat kidney were studied (Klawitter *et al.*, 2008). Rats were divided into nine treatment groups that received orally CsA alone or with Rapa: I, controls (skim milk for 6/28 days); II, CsA 10 mg kg^{-1} day^{-1} for 6 days; III, CsA 25 mg kg^{-1} day^{-1} for 6 days; IV, Rapa 1 mg kg^{-1} day^{-1} for 6 days; V, CsA 10 mg kg^{-1} day^{-1} + Rapa 1 mg kg^{-1} day^{-1} for 6 days; VI, CsA 25 mg kg^{-1} day^{-1} + Rapa 1 mg kg^{-1} day^{-1} for 6 days; VII, CsA 10 mg kg^{-1} day^{-1} for 28 days; VIII, Rapa 1 mg kg^{-1} day^{-1} for 28 days; IX, CsA 10 mg kg^{-1} day^{-1} + Rapa 1 mg kg^{-1} day^{-1} for 28 days. On days 5 and 27, rats were placed in metabolic cases for the 24-h urine collection. On the final day, 2 h after the time of last administration, the animals were sacrificed. All rats receiving CsA for 28 days with or without Rapa presented impairment of renal function and histomorphologic alterations typical of CsA-induced toxicity. Compared with controls animals treated with CsA after 6 days presented decreased levels of citrate, succinate and 2-OG and increased levels of acetate, lactate, trimethylamine and hippurate. CsA alone or combined with Rapa, after 28 days, increased urinary lactate and glucose and decreased Krebs' cycle intermediates, suggesting that the proximal tubulus cells cannot use lactate and glucose as energy substrates; therefore, they substract urinary Krebs' cycle intermediates. Although Rapa alone does not induce nephrotoxicity, this study proves that even though serum creatinine levels are not altered changes in kidney histology are apparent after 28 days, typical of tubular system atrophy.

2.3 Heart Toxicity

The cardiovascular system is a potent target of toxicity because drugs and a wide range of compounds in industry and domestic life present a potential hazard. The recent metabonomics studies concerning heart toxicity are summarized in Table 3.

Table 3. Summary of the experimental models examined in heart and brain toxicity.

	Heart toxicity							
	Experimental animals	Dose used	Root of administration	Time-dependent experiment	Dose-dependent experiment	Biofluids examined	Methods	References
Doxorubicin acute	Rats	20 mg kg^{-1} BW alone or combined with Oleu)	i.p.	No	No	Tissue	NMR	Andreadou *et al.*, (2009)
Ischemia/ Reperfusion	Rabbits	—	—	No	—	Plasma	NMR	Constantinou *et al.*, (2007)
	Brain toxicity							
	Experimental animals	Dose used	Root of administration	Time-dependent experiment	Dose-dependent experiment	Biofluids examined	Methods	References
3-Nitropropionic acid chronic	Rats	5 and 10 mg kg^{-1} BW	i.p.	No	Yes	Tissue, plasma	NMR	Tsang, Haselden and Holmes (2009)

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Doxorubicin (adriamycin, DXR) presents dose-dependent cardiotoxicity (Cole *et al*., 2006). The alterations caused by DXR administration on male Wistar rats were examined in order to identify and quantify metabolites that could be considered as potential biomarkers (Andreadou *et al*., 2009). Furthermore, the effect of the antioxidant oleuropein (Oleu) on the DXR-induced cardiotoxicity was studied. Animals were administered (i.p.) with a single dose of saline (control group), DXR (20 mg kg^{-1} BW), and a combination of Oleu and DXR at different dose regimens (rats treated with 100 or 200 mg Oleu/kg/BW, 2 days before, on the same day and 2 days after the administration of DXR and rats treated with 100 and 200 mg Oleu/kg/BW immediately after DXR administration and for two consecutive days). DXR-treated group presented elevated levels of acetate that could be attributed to the decarboxylation of pyruvate by reactive oxygen species produced by DXR. Furthermore, acetate accumulation may be responsible for the inhibition of fatty acid β-oxidation induced by DXR. Increase in succinate levels in the DXR group compared with the control group was noted, probably due to a rapid inactivation of mitochondrial succinate dehydrogenase by DXR. A decrease in valine, leucine and isoleucine levels may be attributed to the DXR-induced reduction of ATP production through inhibition of β-oxidation of fatty acids and Krebs' cycle impairment, using amino acids as an energy reservoir. On the other hand, Oleu at both doses tested reduced both succinate and acetate accumulation, indicating that energy consumption is restored in DXR-induced cardiotoxicity. PCA of the examined groups did not separate the two groups treated with 100 mg Oleu from the DXR group. In contrast, the two groups administered with 200 mg Oleu were well separated from the controls and the DXR group but without any distinction between the two groups.

The plasma perturbations caused in the metabolite levels during heart ischaemia–reperfusion (I/R) in rabbits were also examined (Constantinou *et al*., 2007). Furthermore, the effect of melatonin (a natural hormone of the indole family) and of a novel synthetic indole derivative was examined. Animals were grouped into six main groups: baseline, controls, melatonin-(I/R), ischaemic pre-conditioning, melatonin-pre-conditioning and indole derivative. Alterations in the plasma levels of LDL/VLDL, unsaturated fatty acids, glucose, alanine, glutamine, glutamate, phenylalanine, tyrosine and histidine were observed compared with controls. The lactate/glucose ratio was increased in the reperfusion group compared with baseline, whereas the lactate + alanine/acetate ratio was decreased, suggesting an increase in the anaerobic metabolism during reperfusion and an increased consumption of lipids, respectively. Both ratios remained unchanged during reperfusion when hearts were subjected to pre-conditioning, indicating that glycolysis followed the aerobic pathway and lipid consumption was not increased. PCA grouped all reperfusion samples away from baseline; still, reperfusion samples that were subjected to pre-conditioning were placed among the baseline ones. Melatonin-treated rats formed their own cluster away from beseline; however, no clustering could be achieved between melatonin–ischaemia and melatonin–reperfusion animals. Furthermore, rats treated with the synthetic indole were discriminated from melatonin-treated ones and the baseline; nevertheless, no discrimination between I/R was apparent.

2.4 Brain Toxicity

The risk of neurotoxic damage from exposure to environmental pollutants and industrial chemicals placed the nervous system in the category of target organs for toxic injury, although limited studies exist in this field (Table 3).

3-Nitropropionic acid (3-NP) is a neurotoxin, naturally produced by certain legumes and fungi (Gustine, 1979). It is extensively used in laboratories because it gives rise to behavioural and histological features similar to Huntington's disease (HD) (Tsang, Haselden and Holmes, 2009).

The dose-dependent alterations caused by 3-NP in selected brain regions were examined (Tsang, Haselden and Holmes, 2009). Sprague–Dawley rats were administered with 3-NP i.p. at doses of 0 (controls), 5.0 (low dose) and 10 mg kg^{-1} day^{-1} (high dose, which was reduced to 7.5 mg kg^{-1} from day 3 onwards) for 14 consecutive days. In general, controls presented an increase in body weight, whereas 3-NP-treated rats presented a decrease in body weight on the final day of the dosing. In all brain regions, a dose-dependent increase in the levels of succinate and decreases in *N*-acetylaspartate and taurine levels were noted, after treatment with 3-NP. Region-specific alterations included a decrease in

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γ-aminobutyric acid (in the brainstem, ventral striatum and frontal cortex) and alterations in lipids occurring in the globus pallidus and dorsal striatum. Increased lactate levels (in the cortex and dorsal striatum) in the rats treated with 3-NP suggest a deficiency of oxidative metabolism. ^{1}H-NMR data of the aqueous extracts of the frontal cortex revealed decreased levels of *N*-acetylaspartylglutamate, whereas the lipid extract revealed a dose-dependent decrease of phosphatidylcholine and an increase of glycerol. The alterations in the levels of choline and phosphocholine were associated with changes in the membrane composition and with decreased anabolic and/or increased catabolic activity in the ventral striatum.

3 CONCLUSIONS

The "omics" technologies, especially metabonomics, have been widely applied to the investigation of target organ toxicity. Especially when coupled with NMR spectroscopy metabonomics has been characterized as a powerful tool that studies simultaneously the fluctuations of low-molecular-weight metabolites that could be further characterized as biomarkers. Nevertheless, the target organ toxicity studies performed, using the metabonomic approach, remain limited to experimental animal models (Tables 1–3), and to our knowledge, no clinical toxicological data have been recorded up to now.

In the metabonomic studies stated, a complete separation of experimental from control samples was achieved. However, no clear biomarker(s) of toxicity was established in any of them. Additionally, the existing data are not sufficient to predict either the toxic and recovery phases or a state of organ failure where the recovery is not possible. The reason for unclear conclusions from the existing data that simply report differences between toxic and healthy states is the lack of databases that could collect, combine and extrapolate all useful information from each study.

As previously demonstrated the existing data on metabonomic approach and target organ toxicity are mainly related with different models of acute and/or chronic liver injury. Further use of metabonomics is imperative in other models of tissue injury such as lung, heart, brain and gastrointestinal tract. For better exploitation of the results from metabonomic studies, the creation of databases is imperative. Such databases would comprise all metabolites altered during the toxic insult so that the toxic agents could be clustered in groups based on common alterations.

The metabonomic approach in combination with data obtained from genomics and proteomics studies could provide valuable information regarding the underlying mechanisms of target organ toxicity.

RELATED ARTICLES

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Toxicoinformatics for Systems Toxicology

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1 AN INTRODUCTION TO TOXICOINFORMATICS

Systems biology by its nature is a data intensive enterprise. The idea behind systems biology and systems toxicology is to integrate data from varying and disparate data sources to create a clearer understanding of how chemicals affect organ systems, entire organisms, and ecosystems.

Generally, when we think of toxicoinformatics and its impact on systems toxicology, we think about the creation of biological networks. To others, the term systems toxicology really focuses on mathematical modeling of biological phenomena. And to others, the focus is on the integration of biological networks with mathematical models. This chapter discusses systems biology with respect to network biology, and next steps after creating a gene expression network.

No matter how you define systems toxicology, one requirement will always remain: how do we manage this data? When working in a team science environment, the next question is generally "how do we exchange data?" This chapter attempts to address many of these issues, and gives some guidance on how teams can manage and organize their data.

2 DATA MANAGEMENT

Data management concerns the acquisition, handling, storage, and ways of making data available. This includes ensuring data security, the creation of databases and data warehouses, and making these databases and warehouses available to yourself or others for later use in data analyses. This chapter highlights important topics in each of those areas; however, readers should consult specialty texts on these topics for more depth.

2.1 Data Security

Usually, when we think of data security, we envision antivirus software, firewalls, and data encryption to prevent unintended release of our sensitive information. However, data security includes ensuring data that are copied from the source to the destination is a bona fide copy of the original (e.g., copying data from a laboratory computer to your office computer). This section discusses the more practical elements of data encryption, especially their use in ensuring copies of data are bona fide copies of the original.

2.2 Digitally Signing Data

Generally, data encryption is a means to ensure that only the intended recipient can read a piece of data, such as a data file. Encryption works by applying some algorithm to a set of data to transform it. When the intended recipient (could be yourself) wants to read the file, they need to use the decryption algorithm. Both the encryption algorithm and the decryption algorithm require the use of a key. The

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algorithms use the key to either encrypt or decrypt the file. The encryption and decryption algorithm typically use different keys.

One of the most common encryption schemes in use today is the asymmetric cryptosystem. There are numerous examples of asymmetric cryptosystems that we use every day, and probably do not realize it. For instance, when you purchase a product online from your favorite retailer, most of us look for the "closed padlock" icon in our web browser.

First, the entity operating the server contacts a certificate authority (CA) to obtain a certificate. This digital certificate contains a public and a private key, and is signed by the CA. When a client (e.g., web browser) sends an SSL connection request to the server, the server responds by sending its certificate with only the public key and the CA's signature. Most web browsers will check the CA's signature to ensure authenticity. This is performed by decrypting the signature using the CA's public key, and comparing that decrypted signature to a plaintext (non-encrypted) signature contained in the certificate. The key here is that when the CA signed the certificate, they encrypted the plaintext key using their private key, and this encrypted key is the signature. Because of the relationship between a public and private key pair, the only way for the decrypted signature to match the plaintext key is if the proper private key was used to encrypt the signature (remember, the signature was encrypted using the private key by the CA, and the client is decrypting the signature using the public key). If the signatures check out, then the client encrypts a random key using the server's public key. The only way to decrypt this random key is to use the private key on the server. From here, all transmissions are encrypted using the random key – this is a form of symmetric encryption.

As we have seen in this example, asymmetric encryption methods allow data to be signed and encrypted. This is useful for data transmissions between parties through an insecure channel, such as the internet, but this is overkill if we want to ensure data going from one hard drive to another is the same.

2.3 File Signatures/Fingerprints

One very common problem in systems biology today is ensuring that data coming from one source are actually a bona fide copy of the original. As the size of files from instruments and machines continues to increase, so the number of data will transfer problems. Consider for instance the ubiquitous practice of compressing large datasets. Depending upon the algorithm, problems in data transmission can either be a nuisance (e.g., loss of some bytes) or catastrophic (e.g., unable to read the file). In other cases, if we were to have an issue in transmitting a text file, we may lose the file structure (this can be fixable), or may lose data. In all of these cases, the concern is that we lose data.

One way to ensure that data are a bona fide copy of the original is to compare the checksums, also known as file signatures or fingerprints, of the original file and copy. If the checksums are identical, then you know the files are the same. However, if the checksums are different, then the file contents are different. The most commonly used checksum algorithms are MD5, SHA-1, and the SHA-2 family (includes SHA-256 and SHA-512). The MD5 and SHA256 checksums can be calculated in Linux using the md5sum and sha256sum utilities. In Windows, you can calculate MD5 and SHA-1 using the File Checksum Integrity Verifier (FCIV) available from Microsoft (http://support.microsoft.com/kb/841290).

The process of checking the checksums is rather simple: you run the appropriate program on the file of interest on the original computer. You keep track of the checksum value it returns. For instance, for a database I created recently, the MD5 and SHA-256 checksums are:

- MD-5: 026616c0b802605d3c0df47fe742e65c
- SHA-256: cd6b3eee03e3da12dcd6b67013bfe4d c6d7b75999c40b0833e93774b90b0db65

Then you calculate the checksum of the copied file, and compare the checksum of the original file and the checksum of the copied file. If they are different, then you know the copied file is corrupted. For instance, when I distribute the database file to colleagues, I ask that they verify that the checksum value for their copy is the same as the ones listed above.

2.4 Creating Databases and Data Warehouses

Databases are combinations of hardware and software that facilitate data storage and retrieval.

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Building databases is more of an art than a science; however, as the amount of data that we need for understanding our systems continues to increase, investigators will soon understand why spreadsheets are not appropriate for making sense of their data.

The most basic rule of designing a database is to reduce, as much as possible, any data redundancy. The corollary to this is to not to go so far as to make your database difficult to use. For instance, if we were to create a database for an organization, such as the US National Toxicology Program, we may need to create a table storing vendor information. This table would contain the vendor's name, street address, city, state, and zip code. Remembering the first rule stated above, we might believe that city, state, and zip code may become redundant within the table (i.e., multiple vendors may be in the same zip code, and thus, in the same city and state). Thus, we might want to create a separate table that incorporates the US Postal Service's zip code database, which associates zip codes with the city and state. This would be an ideal situation if we were also going to design a graphical user interface front-end, such as a website to enter this information. However, if our use case is instead that our users are going to be uploading legacy data from spreadsheets or text files, it might be better to just allow the city, state, and zip code to remain in the vendor table.

The second most important rule in designing databases is to make sure each table has a unique key or identifier. We call this unique key the primary key and it serves as a means to hook tables together. For instance, if we want to create a database to manage animal end-point assays, such as histopathology data, we would need to capture information about the animals (e.g., sex, age, species, strain, and laboratory identifier), their cage (e.g., bedding, cage mates, access to and type of food and water), and other environmental factors (e.g., light–dark cycle, humidity, temperature), in addition to tables to manage the end-point data itself. One solution is to create an animal table, a cage table, an environmental factors table, and a table for the end-point data. For this solution to work, a unique identifier must be assigned to each animal, each cage, each environmental factor, and each end-point data point. Next, when we construct the animal table, we include a column for the cage identifier. The cage table will include a column for the environmental factor identifier. The end-point data table will include a column for the animal identifier.

By including these identifiers in the tables, we are able to perform queries against the database where the tables are joined together. The simplest way to think of table joining is to imagine replacing the foreign unique identifier, known as a foreign key, in a table (e.g., the cage table contains an animal identifier foreign key) with its associated values in its primary table. For instance, imagine replacing all instances of the animal identifier = 1 in the cage table with the data in the animal table associated with animal identifier = 1 (e.g., the strain, species, age, sex, and laboratory identifier).

Data warehouses are slightly different from databases with respect to the scope of data contained. Databases are intended to store the raw data from projects, experiments, and studies. As a result, databases can become massive. If, users are only interested in aggregated data (e.g., group mean values and standard deviations), then it may be more useful to create a data warehouse.

A data warehouse only stores aggregated information. Data warehouses have a similar design feel as databases in that they should not contain redundant information. However, the underlying table designs for a data warehouse may differ substantially from the database. Again, the primary motivator for design should always be the intended usage of the database or data warehouse.

Querying databases and data warehouses is carried out using the Structured Query Language (SQL). SQL is a relatively straightforward language for investigators to learn. The exact ways in which to use SQL and how to write SQL queries is beyond the scope of this book chapter, so readers should consider reading an SQL reference. One can be found in the "References" section of this chapter (Viescas and Hernandez, 2008).

3 NETWORK MODELING AND GENE REGULATORY ELEMENT IDENTIFICATION

Systems toxicology datasets are much larger than those that many toxicologists find familiar. Consider that the average gene expression microarray will provide data for 10 000–40 000 probes. Multiply that by a number of time-points in a time–course experiment, or several doses in a dose–response dataset, along with biological replication, and the data quickly number in the hundreds of thousands to millions of data points.

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Most investigators when approached with massive datasets, such as these, will naturally turn to pattern recognition techniques to identify some type of underlying structure. This typically results in agglomerative hierarchical clustering heat maps, k-means clustering, and self-organizing maps to visualize patterns or identify unique patterns. Although these methods are useful for visualizing correlative structures within the data, new methods have been emerging that focus more explicitly on constructing gene regulatory networks.

3.1 Gene Network Modeling

The goal of gene network modeling is typically to discover some hidden regulatory structure underlying gene expression across dose, time, or developmental stage. For instance, an investigator may be interested in the gene regulatory network underlying exposure to a particular drug. In this case, the investigator may perform a dose–escalation, a time–course study, or a combination of both. By identifying genes that are "connected" to each other in the network, the investigator may hypothesize that these connected genes, or some subset of these connected genes, are regulated by the same transcription factor(s). Following up on these hypotheses may lead to new data that can enable the creation of systems toxicology mathematical models that drive further understanding of the system as a whole.

The first step in constructing a gene network is to identify an appropriate gene expression microarray normalization method. In the past, this step was often overlooked; however, it has recently been recognized that normalization methods that are appropriate for identifying differential gene expression may not be appropriate for constructing gene networks (Lim *et al*., 2007). Additional work needs to be performed in this area on real biological and simulated biological networks before any definitive advice on specific normalization methods can be made.

Data quality issues may also hamper network construction; thus, investigators are encouraged to ensure they are using data from the highest quality microarrays available (Burgoon *et al*., 2005). For instance, especially when combining data from multiple studies, differences in the overall signal intensity of arrays may create false-correlative structures, while simultaneously destroying true correlations. This is another area that requires further study.

After data normalization and quality items have been addressed, investigators must choose what metric they will use for generating the network. Rather than focus on complicated and computationally intensive methods, such as Bayesian networks and dynamic Bayesian networks, this chapter focuses on simpler correlation and mutual information-based methods that are relatively common today, and are significantly less computationally intense.

The correlation methods are the Pearson's and Spearman's correlations. The Pearson's correlation is the correlation most of us are used to:

$$\text{Correlation} = \frac{\text{cov}(X, Y)}{\text{SD}(X)\text{SD}(Y)} \tag{1}$$

where SD is the standard deviation. The Spearman's correlation, also known as the Spearman's rank correlation, is defined as:

$$p = 1 - \frac{6\sum d_i^2}{n(n^2 - 1)} \tag{2}$$

where d_i is the difference between x_i and y_i.

In R, the Pearson's and Spearman's correlations are calculated using cor(gene_expression_matrix, method = "pearson") and cor(gene_expression_matrix, method = "spearman"), respectively. The rows in the input matrix are the individual microarrays, whereas the columns are the genes or probesets (i.e., the input matrix is the transpose of the gene expression matrix). If using any of the standard microarray analysis packages, this gene expression matrix is usually part of an expression set, and can be assigned to a variable using something like:

$$\text{gene\_expression\_matrix} \leftarrow \text{exprs(expression\_set)} \tag{3}$$

After applying the methods, a new square matrix is created, where the rows and columns are the genes or probesets from the original data matrix. Thus, the rows and columns in this square matrix represent the correlation between the genes or probesets. The resulting square matrix is referred to as a correlation

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matrix. Most investigators calculate the absolute value of the correlation values in the square matrix.

Similarly, mutual information methods take the same type of matrix as input as the correlation methods. Mutual information is calculated as:

$$I(X;Y) = \sum_{y \in Y} \sum_{x \in X} p(x, y) \log_2 \left(\frac{p(x, y)}{p(x)p(y)} \right) \quad (4)$$

where the mutual information $I(X;Y)$ is measured in bits, $p(x)$ and $p(y)$ are the marginal probabilities, and $p(x, y)$ is the joint probability. The output of the mutual information methods is typically a square matrix, similar to what is constructed by the correlation methods.

To create a graph, typically this square matrix is converted to an adjacency matrix, where all correlation and mutual information values above a given threshold are converted to 1, and all other values are converted to 0. Each gene or probeset is both a row and a column. Thus, the diagonal of the matrix is set to 1 (because a gene or probeset is always correlated with itself and the mutual information is always 1). The graph will consist of nodes (genes or probesets) and edges between these nodes. An edge is drawn between nodes if the corresponding value in the adjacency matrix is 1. For instance, if two genes, cyp1a1 and cyp1b1, have a value of 1 in the adjacency matrix, then the graph will have an edge connecting these two nodes.

Generally, graphs for the correlation methods are drawn based on the adjacency matrix. Similar mutual information graphs can also be drawn; however, several groups have begun exploring methods of "pruning" these mutual information graphs to get rid of spurious, or false, edges. Discussion of these multitudes of methods is beyond the scope of this chapter. Instead, investigators are encouraged to monitor the results from the ongoing DREAM competition (http://wiki.c2b2.columbia.edu/dream/index.php/The_DREAM_Project).

DREAM is a competition where groups to test their method against other methods for constructing biological networks. Thus far, the competition has not identified any particular methods that consistently outperform the others. It remains unclear what the poorly performing methods are (the competition organizers only disclose those methods that perform the best within the competition). Perhaps the most significant open question is whether the more complicated mutual information pruning methods (e.g., ARACNE) outperform the simpler correlation and mutual information based methods (i.e., using the adjacency matrix resulting from the correlation or mutual information matrices).

Once you have a gene expression network, the next question is generally "what do I do with this network?" One possible next step is the use of network structures to identify possible gene regulatory binding sites. The way this works is given below:

- Perform community analysis of the network.
- Obtain the region approximately 5KB-10KB upstream of each gene's transcription start site.
- Perform a supervised position weight matrix search using position weight matrices from a database such as TRANSFAC (Frericks *et al*., 2008; Liu, Taylor and Edenberg, 2006; Wingender, 2004) or JASPAR (Frericks *et al*., 2008; Hannenhalli and Wang, 2005).
- Identify those gene regulatory elements that are over-represented in the community versus random sequences or all of the genes in the network (Lo *et al*., 2010).

Community analysis is a network theoretic method for identifying genes in the network that are more connected with each other, than they are with the rest of the network. Several methods exist for identifying communities in networks, and this remains an open-research question in network theory. If performing network analysis in R, I suggest investigators try using the igraph library, which contains several community analysis methods. The spin-glass method has worked well in my previous applications.

Once the investigator has identified the community, they need to obtain the region upstream of each gene's transcription start site (TSS). For species where the genome is well known and annotated, these data can be obtained easily from the UCSC Genome Browser or possibly a model organism genome database. Once these sequences have been obtained, the investigator should obtain a list of position weight matrices, from databases such as TRANSFAC or JASPAR, and perform an analysis of these upstream gene regions.

Then the investigator needs to perform an enrichment analysis. This can be as simple as counting the frequency of the each binding site within the upstream regions for all of the genes

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in the community. Then, generally, we would scramble these upstream regions and perform the position weight matrix searches again. This process is repeated thousands of times and a distribution of the random sequence frequencies is calculated. Then, the investigator can calculate the likelihood of finding a hit from each position weight matrix due strictly to chance.

From here, the investigator can now generate new hypotheses regarding the regulation of the genes within each community. Using this new information, the investigator can begin to plan chromatin immunopreciptation experiments, to further identify how the genes are regulated. Once a more complete picture of the transcriptional regulation of the genes is created, along with further analysis for putative co-regulators, the investigator can begin to think about creating mathematical models of the transcriptional response.

4 CONCLUDING REMARKS

By combining all of these data into a database and including data from other sources, including pathology, and data across multiple organs, investigators can begin to create a more complete picture of the entire system. Over time, as more data become available and more investigators make these data publicly available, we as a community will be able to achieve the true aims and goals of systems biology. However, until we begin making our data more available, and public databases become available and easily searched, using cutting-edge web technologies, such as the Semantic Web, we will not be able to fully realize the true potential of systems biology.

RELATED ARTICLES

Toxicogenomics and the Evolution of Systems Toxicology

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Chemoinformatics and its Applications

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1 INTRODUCTION

Advancements in the fields of medicinal and pharmaceutical chemistry have depended on the convention trial and error process. As this process is slow and tedious, even after several years of research, it was feasible only to identify few chemicals with drug-like property. Also, it was not experimentally feasible to understand why certain molecules have the drug-like property and few others related to these molecules do not have that property. Only recently, more systematic investigation of drug-like compounds in biological system, called as "chemical biology," has evolved.

2 CHEMOINFORMATICS

The field of "chemoinformatics" involves the use of information technology to manage chemical information and solve chemical problems, with specific emphasis on manipulation of structural information and utilization of chemical databases. This field deciphers the chemical information needed for drug discovery. For the first time, Frank Brown defined the term "chemoinformatics" as the utilization of chemical information resources to transform data into information and knowledge for the intended purpose in making better decisions faster in the area of drug lead identification, organization and also general purpose such as chemical library construction and enrichment. The basic goal of this field is to transform data into knowledge through information processing. The new discipline of chemoinformatics covers the application of computer-assisted methods to chemical problems such as information storage and retrieval, the prediction of physical, chemical or biological properties of compounds, spectra simulation, structure elucidation, reaction modelling, synthesis planning and drug design.

High-throughput screens (HTS) of small molecules for drug discovery and chemical genomics have evolved for analysing protein functions and cellular networks. At the same time, structures of millions of drug-like molecules and related information are freely available in the online compound database projects, such as PubChem, ChemBank, ChemMine, ChemDB. To search and analyse the vast amounts of available compounds and screening information, and to assemble diverse screening libraries, efficient compound analysis is very difficult. Chemoinformatics-based tools are very useful to solve the chemical problems efficiently.

Chemical informatics typically handles the massive amounts of data and manages the data using information systems that assist chemists in making sense of the data, often accurately predicting the properties of chemical substances (Figure 1).

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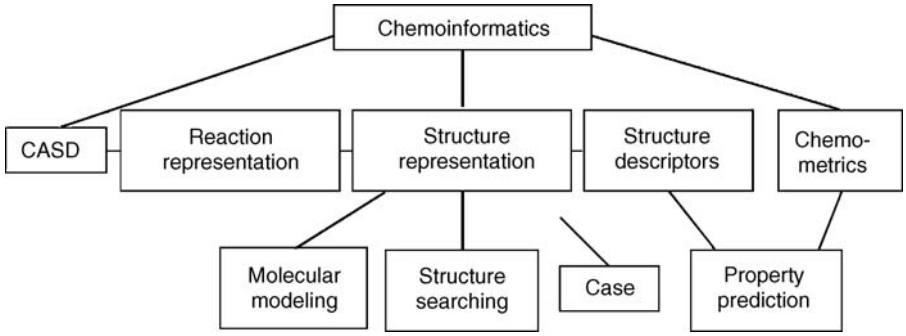

Figure 1. High-throughput screens of small molecules for drug design.

3 CHALLENGES IN CHEMOINFORMATICS

Much of the current attention in chemoinformatics is centred on the drug industry. When a potential drug is developed, libraries of compounds are screened for activity with one or more relevant assays using high-throughput screening techniques. Candidate compounds are then evaluated for binding, potency, selectivity and functional activity. Pharmaceutical companies now use sophisticated modelling techniques to predict the toxicity of compounds long before they are tested on human beings in order to save cost and time involved and avoid sacrifice of animals, and can easily reject the chemical compounds.

Two most important challenges of chemoinformatics are:

- To extract knowledge from large-scale raw HTS databases in shorter time periods.
- To be able to provide efficient *in silico* tools to predict adsorption distribution, metabolism, excretion and toxicity (ADMET) properties.

Chemical informatics may concentrate on molecular modelling, chemical structure coding and searching, chemical data visualization or a number of other areas of specialization. Chemoinformatics system should give information about structure [e.g., Simplified Molecular Input Line Entry System (SMILES)], molecular connectivity (e.g., SDfile), 3D chemical structure, pharmacophore, structure similarity, molecular similarity and diversity, chemical property, HTS and activity fingerprint, lead identification and optimization, combinatorial library design and synthesis and metabolic profile.

4 CHEMOINFORMATICS TASKS

The following tasks in chemoinformatics research are common:

- analysis of HTS data;
- similarity search of chemicals;
- design of combinatorial libraries;
- design of focused libraries;
- comparison of the similarity/diversity of libraries;
- virtual screening;
- docking;
- *de novo* design;
- pharmacophore perception;
- prediction of affinities, physicochemical properties and pharmacokinetic properties;
- establishment of QSAR models which can be interpreted and guide the further development of a new drug.

Tools which are commonly used in chemoinformatics include: statistical tools (e.g., recursive pairing), data analysis tools, visualization tools, Chemically-Aware Web Language (CML).

The applications of chemoinformatics in drug discovery are compound selection, virtual library generation, virtual screening, structure–activity relationship (SAR) on HTS data, sequential screening and *in silico* ADMET.

5 CHEMICAL DATA COLLECTION TECHNOLOGY

There are two important driving forces for chemical data collection: high-throughput screen and combinatorial library. Combinatorial chemistry and

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high-throughput screening are data-dependent and data-rich technologies. When making combinatorial libraries of chemical compounds, you need information on the molecular components, their biological effects and information on how to prepare the compound. There is also data collection technology for managing and storing the libraries. In high-throughput screening, the test results need to be captured, stored and then analysed. This is followed by applying chemoinformatics tools to collect, manage, analyse and disseminate the chemical information needed for drug discovery.

6 ALGORITHMS IN CHEMOINFORMATICS SYSTEM

Chemoinformatics systems employ a wide variety of algorithms for indexing and retrieving chemical compounds in databases, generating all isomers with a constitutional formula, or for computer-assisted organic synthesis. All these tasks involve three classes of algorithm for chemical graphs:

- the canonical coding problem, for generating a unique representation of a chemical compound;
- the automorphism partitioning problem (also known as constitutional symmetry perception, graph symmetry or topological symmetry), for detection of equivalent atoms and bonds in a molecule; and
- the graph isomorphism problem, for determining whether two connection tables represent the same chemical compound.

7 STEPS INVOLVED IN CHEMOINFORMATICS SYSTEMS:

Several steps are involved in most of the chemoinformatics systems. Outline about the few important steps are given below:

- In chemical documentation process, unique chemical structure representations (one-, two- and three-dimensional) are developed for chemical data management and searching.
- The computer generation of chemical compounds consistent with given structural constraints is used both in synthesis design and in structure elucidation.
- Artificial intelligence systems for synthesis design use canonical codes and constitutional symmetry information to generate and evaluate reaction paths.
- The synthesis by computer-aided molecular design of new compounds that conform to various physical property requirements can reduce the time and effort required using traditional empirical approaches. This process generates chemical structures compatible with experimental and structural restrictions.
- Canonical coding and symmetry perception are used to uniquely generate all possible isomers that adhere to the design constraints.
- Quantitative SARs (QSAR) are used to model the biological effect of a set of compounds. It makes extensive use of structure generation, substructure search and symmetry perception algorithms.
- Modern drug-design procedures make extensive use of large combinatorial libraries and *in silico* screening of chemicals, both using algorithms based on canonical coding, graph isomorphism and constitutional symmetry perception.

8 CHEMOINFORMATICS SOFTWARES/TOOLS

8.1 Chemoinformatics Tool Kit

It consists of similarity search, clustering, cluster and active compound inspection. Similarity searching measures: Tanimoto, Cosine, Euclidean, Hamman, Tversky or composite similarity or dissimilarity measures.

Clustering measures output Ordered Pair, Sparse Matrix or Square Matrix.

9 REPRESENTATION OF CHEMICAL STRUCTURE

The representation of chemical structures must follow chemical structure theory. IUPAC has long been involved in the development of systematic and standard procedures for naming chemical substances on the basis of their structure. The aim of the IUPAC Chemical Identifier Project (IChIP) is to establish a unique label. The IUPAC Chemical Identifier (IChI) would be a non-proprietary identifier for chemical substances that could be used in printed

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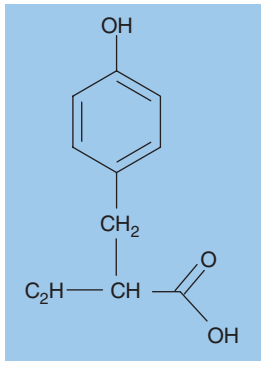

Figure 2. Connections between atoms.

and electronic data source. New systems have been introduced to represent the chemical compounds, such as:

- structure diagrams;
- nomenclature;
- line notations; and
- connection table.

9.1 Structure Diagram

- Atoms represented as $C_8H_9NO_3$:
  - o connections between atoms (Figure 2)
  - o bond types (Figure 3)
  - o charges (Figure 4)

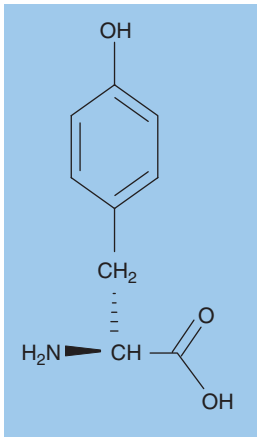

Figure 3. Bond types.

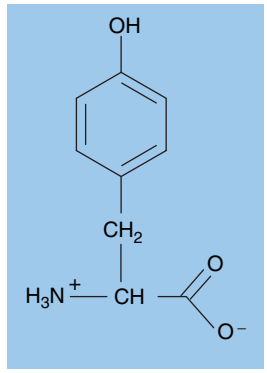

Figure 4. Charges.

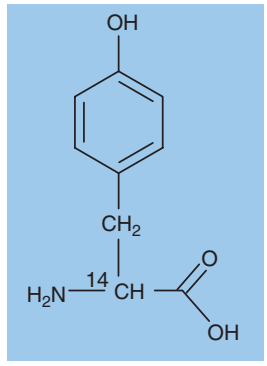

Figure 5. Isotopes.

  - o isotopes (Figure 5)
  - o 3D coordinates for atoms (Figure 6)

- The following is the list of commonly used computer programs that allow input/editing of structure diagrams:

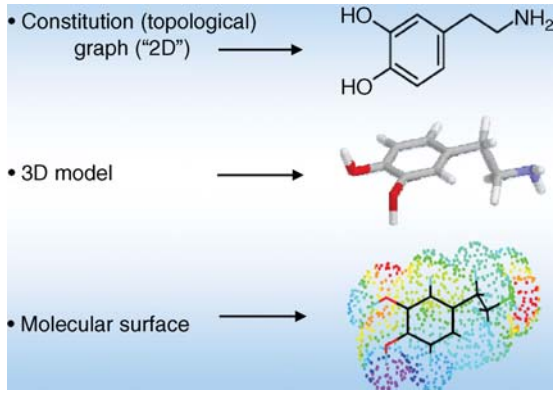

Figure 6. Three-dimensional orientation.

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- ISIS/Draw (MDL) http://www.mdl.com/downloads/downloadable/index.jsp
- ChemDraw (CambridgeSoft), Chemsketch http://www.cambridgesoft.com/products/
- GRINS/JavaGRINS (Daylight) http://www.daylight.com/products/javatools.html
- MarvinSketch http://www.chemaxon.com/marvin/

9.2 Chemical Nomenclature

The name is used to identify any chemical substance that is potentially important. In this, chemical structure is represented as text string. Another form of chemical nomenclature is the trivial name. Trivial names are usually short and easy to pronounce but do not usually give much information about the structure. *Systematic* names are usually long and difficult to pronounce but usually describe structure in considerable detail.

9.3 Line Notations

In this form of chemical representation, structures are shown as compact linear string of alphanumeric symbols. This has the advantage of easy handling by computer; also, compact storage can be easily transmitted over a network. Simultaneously, this representation allows rapid manual coding/decoding by trained users and is much faster for input than using a structure drawing program.

9.3.1 Simplified Molecular Input Line Entry System

Dave Weininger developed SMILES representation of chemical molecules. SMILES contain the same information as might be found in an extended connection table. The primary reason SMILES is more useful than a connection table is that it is a linguistic construct, rather than a computer data structure. They are true language, albeit with a simple vocabulary (atom and bond symbols) and only a few grammar rules. SMILES representations of structure can in turn be used as "words" in the vocabulary of other languages designed for storage of chemical information (information about chemicals) and chemical intelligence (information about chemistry).

With standard SMILES, the name of a molecule is synonymous with its structure; with unique SMILES, the name is universal, that is, anyone in the world who uses unique SMILES to name a molecule will choose the exact same name.

One other important property of SMILES is that it is quite compact compared with most other methods of representing structure. Typical SMILES will take 50–70% less space than an equivalent connection table, even binary connection tables. A database of 23 137 structures, with an average of 20 atoms per structure, uses only 1.6 bytes per atom when represented with SMILES. In addition, ordinary compression of SMILES is extremely effective. The same database cited above was reduced to 27% of its original size by Ziv-Lempel compression (i.e., 0.42 bytes per atom).

SMILES representation has found wide use among researchers and chemical information programmers. Some of the areas where it is employed include as keys for database access, as a mechanism for researchers to exchange chemical information, as an entry system for chemical data and as a part of languages for artificial intelligence or expert systems in chemistry.

SMILES denote a molecular structure as a graph with optional chiral indications. This is essentially for representing the two-dimensional picture drawn by chemists to describe a molecule.

9.3.2 SMILES Specification Rules

SMILES notation consists of a series of characters with no spaces. In its notation, hydrogen atoms may be omitted (hydrogen-suppressed graphs) or included (hydrogen-complete graphs) and the aromatic structures may be specified directly or in Kekulé form.

There are five generic SMILES encoding rules, corresponding to specification of atoms, bonds, branches and disconnections.

Atoms

Lowercase letters specify atoms in aromatic rings; for example, aliphatic carbon is represented by the capital letter C and aromatic carbon by lowercase c. As attached hydrogens are implied in the absence of brackets, the atomic symbols represented in Table 1 are valid SMILES notations.

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Table 1. Showing few valid SMILES notations.

C	Methane	(CH_4)
N	Ammonia	(NH_3)
S	Hydrogen sulphide	(H_2S)
O	Water	(H_2O)
Cl	Hydrochloric acid	(HCl)

Table 2. Showing valid SMILES notations for denoting bonds.

C=C	Ethane	(CH_2=CH_2)
O=C=O	Carbon dioxide	(CO_2)

Bonds

Single, double, triple and aromatic bonds are represented by the symbols –, =, # and:, respectively. Adjacent atoms are assumed to be connected to each other by a single or aromatic bond (single and aromatic bonds may always be omitted) (Table 2).

Branches

Branches are specified by enclosing them in parentheses, and can be nested or stacked. In all cases, the implicit connection to parenthesized expression (a "branch") is to the left (Table 3).

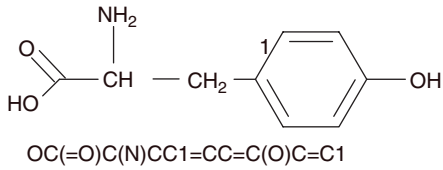
Disconnections

Disconnected compounds are written as individual structures separated by a "." (period). Adjacent atoms separated by dot (.) imply that the atoms are not bonded to each other. This is true whether or not the atoms are in the same connected component.

For example: C1.C1 specifies the same molecule as CC (ethane)

Simplified SMILES encoding rules are as follows (Figure 7):

- Atoms are shown by atomic symbols: B, C, N, O, F, P, S, Cl, Br and I.



OC(=O)C(N)CC1=CC=C(O)C=C1

Figure 7. Sample SMILES notation developed by Dave Weininger (daylight).

- Hydrogen atoms are assumed to fill spare valencies.
- Adjacent atoms are connected by single bonds.
- Double bonds are shown as "="; triple bonds are "#."
- Branching is indicated by parentheses.
- Ring closures are shown by pairs of matching digits.

9.3.3 Wisswesser Line Notation

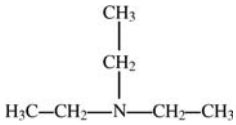
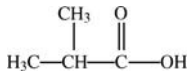
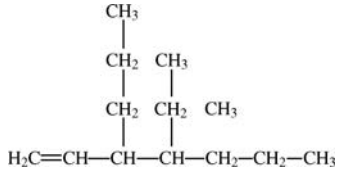
This representation format was invented by William J. Wisswesser in the early 1950s. This notation was the first comprehensive line notation capable of representing arbitrarily complex molecules correctly and compactly. It is a fragment-oriented description of a molecule, which is similar to how a chemist thinks of the molecule. Wisswesser line notation (WLN) is a very brief way to describe a compound, to search for compounds based on chemist-defined criteria (instead of indexer-defined criteria) and, when combined with cleverness, has provided new ways to deal with chemical data.

For example, sorting a list of compounds gives a way to do similarity searches because the parent fragment (which is the most important part for most chemists) is given first.

$$1H = CH4 \ \textit{Methane}$$

$$2H = CH_3 - CH_3 \ \text{Ethane}$$

Table 3. Showing SMILES notations for branching patterns.

		
CCN(CC)CC	CC(C)C(=O)O	C=CC(CCC)C(C(C)C)CCC
Triethylamine	Isobutyric acid	3-propyl-4-isopropyl-1-heptene

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$$3H = CH_3 - CH_2 - CH_3 \text{ Propane}$$

$$QVR\ BG\ CG\ DG\ EG\ FG = C_7HCl_5O_2 \text{ Pentachlorbenzoate}$$

Wisswesser line notation was the first line notation to feature a canonical form; that is, the rules for WLN meant there was only one "correct" WLN for any particular molecule. Scientists and researchers well versed in WLN were able to write molecular structure in a line format and communicate molecular structure to one another and to computer programs. Unfortunately, WLN's complexity prevented widespread adoption.

As the rules for correct specification of WLN are huge, encoding those rules into a computer proved difficult, and the rules for the canonicalization were computationally intractable. WLN provided a way to describe a molecule, but it did not produce a canonical name. That is, it did not include a set of rules, which could be applied to a molecule to get the same name every time.

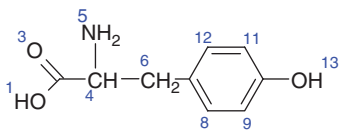
9.3.4 *Representation of Organic Structure Description Arranged Linearly (ROSDAL) (Beilstein)*

Structure in Figure 7 is linearly represented as follows:

$$1O - 2 = 3O, 2 - 4 - 5N, 4 - 6 - 7 = -12 - 7, 10 - 13O$$

9.3.5 *Sybyl Line Notation (Tripos)*

SYBYL line notation (SLN) is a powerful way to represent molecular structures, reactions, libraries of structures, molecular fragments, formulations, molecular queries and reaction queries. Nearly any chemical structure imaginable, including macromolecules, pharmaceuticals, catalysts and even combinatorial libraries, can be represented as an SLN string (refer Figure 8).



OHC(=O)CH(NH2)CH2C[1]=CHCH=C(OH)CH=CH@1

Figure 8. Sample of SLN format for a molecule.

$$OHC(=O)CH(NH2)CH2C[1]=CHCH=C(OH)-CH=CH@1$$

9.3.6 *InChI*

InChI is the latest and most modern of the line notations. It resolves many of the chemical ambiguities not addressed by SMILES, particularly with respect to stereocentres, tautomers and other "valence model problems."

9.4 Connection Table

Most of the early (and some modern) representations of molecules were in a connection table, literally, a table enumerating the atoms, and a table enumerating the bonds and which atoms each bond connected.

9.4.1 *Redundant Connection Table (Figure 9)*

1.	O	1	2 1		
2.	C	0	1 1	3 2	4 1
3.	O	0	2 2		
4.	C	1	2 1	5 1	6 1
5.	N	2	4 1		
6.	C	2	4 1	7 1	
7.	C	0	6 1	8 2	12 1
8.	C	1	7 2	9 1	
9.	C	1	8 1	10 2	
10.	0	9 2	11 1	13 1	
11.	C	1	10 1	12 2	
12.	C	1	11 2	7 1	
13.	O	1	10 1		

9.4.2 *MDL Connection Table*

This is a proprietary file format developed by MDL available for download at the following website: http://www.mdl.com/downloads/public/ctfile/ctfile.jsp. It is a standard for exchange of datasets, and several different flavours and versions are known among researchers that include the following:

- Molfile (single molecule)
- SDfile (set of molecules and data)
- RGfile (Markush structure)
- Rxnfile (single reaction)
- RDfile (set of reactions with data)

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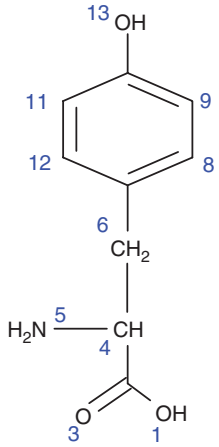

Figure 9. Structure corresponding to redundant connection table.

Chemoinformatics represents molecules as objects in a chemical space defined by molecular descriptors. Among thousands of descriptors, fragment descriptors occupy a special place.

- Fragment descriptors represent selected subgraphs of a 2D molecular graph.
- Structure–property approaches use their occurrences in molecules or binary values (0, 1) to indicate their presence or absence in the given graph.

The unique properties of fragment descriptors are related to the fact that:

- any molecular graph invariant (i.e., any molecular descriptor or property) can be uniquely represented as a linear combination of fragments descriptors;
- any symmetric similarity measure can be uniquely expressed in terms of fragment descriptors;
- any regression or classification structure–property model can be represented as a linear equation involving fragment descriptors.

10 LIST OF SOME OF THE SIGNIFICANT CHEMOINFORMATICS DATABASES

- The Accelrys' Biotransformation, Metabolism databases is marketed by Accelrys (San Diego, USA).
- ACD/Labs Physico-Chemical Property databases are marketed by Advanced Chemical Development, Inc.(Toronto, Canada).
- The AQUASOL dATAbASE of Aqueous Solubility, sixth edition, is marketed by the University of Arizona (Tucson Arizona, USA).
- The Available Chemical Directory (ACD) is marketed by MDL Information Systems Ltd (San Lenandro, USA).
- The Cambridge Structural Database (CSD) is maintained and distributed by the Cambridge Crystallographic Data Centre (Cambridge, UK).
- CASE and MultiCASE, MultiCASE, Inc. (Beachwood, OH, USA).
- The Derwent World Drug Index (WDI) and Derwent Drug File are published by Thomson Derwent (London, UK).
- MDL Drug Data Report (MDDR) is marketed by MDL Information Systems Ltd (San Lenandro, USA).
- The Physical Properties Database (PHYSPROP) is marketed by Syracuse Research Corporation (SRC) (North Syracuse, USA).
- The Protein Data Bank (PDB) is operated by Rutgers, The State University of New Jersey; the San Diego Supercomputer Center at the University of California, San Diego; and the Center for Advanced Research in Biotechnology of the National Institute of Standards and Technology, and funded by NSF, NIH and the Department of Energy.
- Toxicological Data Network (TOXNET) is a collection of databases, published by various services, and made accessible free of charge over the Internet by National Library of Medicine (Bethesda, USA).
- The WOrld of Molecular BioActivities database (WOMBAT) is published by Sunset Molecular Discovery LLC (Santa Fe, USA).
- PubChem.
- eMolecules.
- Zinc.
- Chemspider.

10.1 QSAR

Quantitative structure–activity relationship (sometimes QSPR: quantitative structure–property relationship) is the process by which chemical structure is quantitatively correlated with a well-defined

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process, such as biological activity or chemical reactivity. It is the calculation of QSAR and QSPR values and is employed to predict the activity of compounds from their structures. In this context, there is also a strong relationship to chemometrics. Chemical expert systems are also relevant because they represent parts of chemical knowledge as an *in silico* representation.

For example, biological activity can be expressed quantitatively as in the concentration of a substance required to give a certain biological response. Additionally, when structures or physicochemical properties are expressed by numbers, one can form a mathematical relationship, or QSAR, between the two. The mathematical expression can then be used to predict the biological response of other chemical structures.

Quantitative structure–activity relationships represent an attempt to correlate structural or property descriptors of compounds with activities. These physicochemical descriptors (otherwise called as molecular descriptor) include parameters to account for hydrophobicity, topology, electronic properties and steric effects, and are determined empirically or, more recently, by computational methods. Activities used in QSAR include chemical measurements and biological assays. QSAR currently are being applied in many disciplines mostly pertaining to drug design and environmental risk assessment (Figure 10).

10.2 Hammett Equation

The Hammett equation in organic chemistry describes a linear free energy relationship relating reaction rates and equilibrium constants for many reactions involving benzoic acid derivatives with *meta*- and *para*-substituents to each other with just two parameters: substituent constant and reaction constant (Figure 11). This equation was developed and published by Louis Plack Hammett in 1937 as a follow-up to qualitative observations in a 1935 publication.

The basic idea is that for any two reactions with two aromatic reactants only differing in the type of substituent, the change in free energy of activation is proportional to the change in Gibbs free energy. This notion does not follow from elemental thermochemistry or chemical kinetics and was introduced by Hammett intuitively.

The basic equation relates the equilibrium constant, K, for a given equilibrium reaction with substituent R and the reference K_0 constant when R is a hydrogen atom to the substituent constant σ, which depends only on the specific substituent R and the reaction constant ρ, which depends only on the type of reaction but not on the substituent used.

$$\log \frac{K}{K_0} = \sigma\rho$$

10.3 Taft Equation

The Taft equation is a linear free energy relationship used in physical organic chemistry in the study of reaction mechanisms and in the development of QSAR for organic compounds. Robert W. Taft developed it in 1952. It is a modification to the Hammett equation. Although the Hammett equation accounts for how field, inductive and resonance effects influence reaction rates, the Taft equation also describes the steric effects of a substituent.

The Taft equation is written as:

$$\log\left(\frac{k_s}{k_{\mathrm{CH3}}}\right) = \rho^*\sigma^* + \delta E_s$$

where $\log(k_s/k_{CH3})$ is the ratio of the rate of the substituted reaction compared to the reference reaction, σ^* is the polar substituent constant that describes the field and inductive effects of the substituent, E_s is the steric substituent constant, ρ^* is the sensitivity factor for the reaction to polar effects and δ is the sensitivity factor for the reaction to steric effects.

An important aspect of chemoinformatics is the development of QSARs or SARs, which are used to predict chemical behaviour from molecular structure. Behaviours that are predicted in this way include physical properties (e.g., boiling point, vapour pressure, aqueous solubility, hydrophobicity, dipole moment), reactivity (e.g., hydrolysis, oxidation rates) and bioactivity (e.g., inhibition of an enzyme, antibiotic activity, toxicity). These methods are typically based in chemical thermodynamics coupled with statistical methods.

There are four chemical problems that should be solved by chemoinformatics:

- store a molecule;
- find exact molecule;

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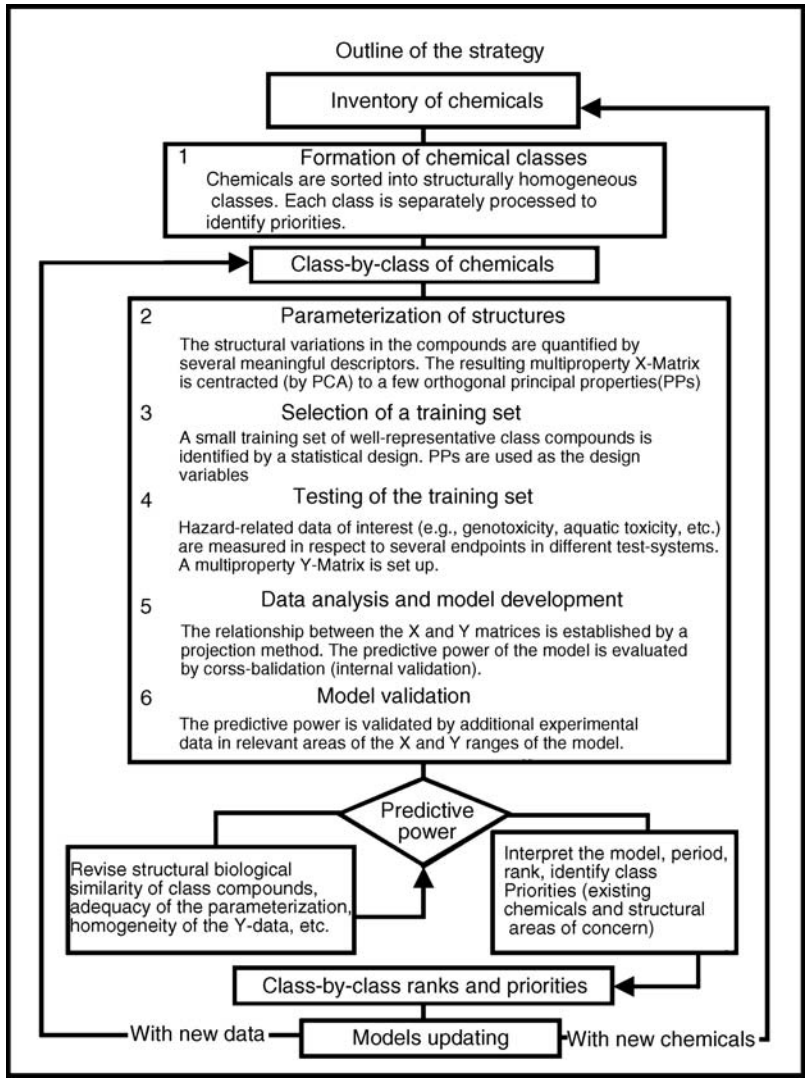

Figure 10. Strategy adopted for QSAR analysis.

- substructure search;
- similarity search.

Among these similarity search and substructure search are very difficult tasks in chemoinformatics.

10.4 Similarity Search

Many chemical information systems can find molecules similar to a given molecule, ranked by similarity. There are several ways to measure molecular similarity, such as SMILES, WLN, connection table, etc.

10.5 Substructure Search

Structural similarity searching and clustering of small molecules play an important role in drug discovery and chemical genomics.

A good similarity search would find the target structure accurately and find the substructure of it. There are many ways to measure similarity.

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Substituent	para effect	meta effect
Amine	–0.66	–0.161
Methoxy	–0.268	+0.115
Ethoxy	–0.25	+0.15
Dimethylamino	–0.205	–0.211
Methyl	–0.170	–0.069
None	0	0
Fluor	+0.062	+0.337
Chlorine	+0.227	+0.373
Bromine	+0.232	+0.393
Iodine	+0.276	+0.353
Nitro	+0.778	+0.710
Cyano	+1.000	+0.678

Source : Hammet 1937

Figure 11. Hammet's constants.

10.6 2D Topology

The best-known and most widely used similarity metrics compare the two-dimensional topology; that is, they only use the molecule's atoms and bonds without considering its shape. Tanimoto similarity is perhaps the best known, as it is easy to implement and fast to compute.

10.7 3D Configuration

One of the most important uses of similarity is in the discovery of new drugs, and a molecule's shape is critical to its medicinal value. 3D similarity searches compare the configuration (also called the "conformation") of a molecule to that of other molecules. The "electronic surface" of the molecule is the important bit – the part that can interact with other molecules. 3D searches compare the surfaces of two molecules, and how polarized or polarizable each bit of the surface is.

3D similarity searches are uncommon, for two reasons: they are difficult and they are slow. The difficulty comes from the complexity of molecular interactions – a molecule is not a fixed shape, but rather a dynamic object that changes according to its environment. And the slowness comes from the difficulty: to get better results, scientists employ more and more complex programs.

10.8 Physical Properties

The above 2D and 3D similarities are based on the molecule's structure. Another technique that compares the properties – either computed or measured or both – and declares that molecules with many properties in common are likely to have similar structure. It is the idea of QSAR taken to the database.

QSAR-based methods ultimately go with the application of statistical methods:

- GFA
- clustering
- PCA
- PLS
- simulated annealing

10.9 Statistical Methods

10.9.1 Clustering

"Clustering" is the process of differentiating a set of things into groups where each group has common features. Molecules can be clustered using a variety of techniques, such as common 2D and/or 3D features. Note that clustering is not a similarity metric but it may use various similarity metrics when computing clusters. It is included here because it can be used as a "cheap substitute." That is, when someone wants to find compounds similar to a known compound, you can show them the group (the cluster) to which the compound belongs. It allows you to pre-compute the clusters, spending lots of computational time up front, and give answers very quickly (Figure 12).

10.9.2 Non-Hierarchical Clustering

In this method, compounds are placed in clusters without a hierarchical relationship between the clusters. Several non-hierarchical methods are available:

- single pass
- relocation
- nearest neighbour method.

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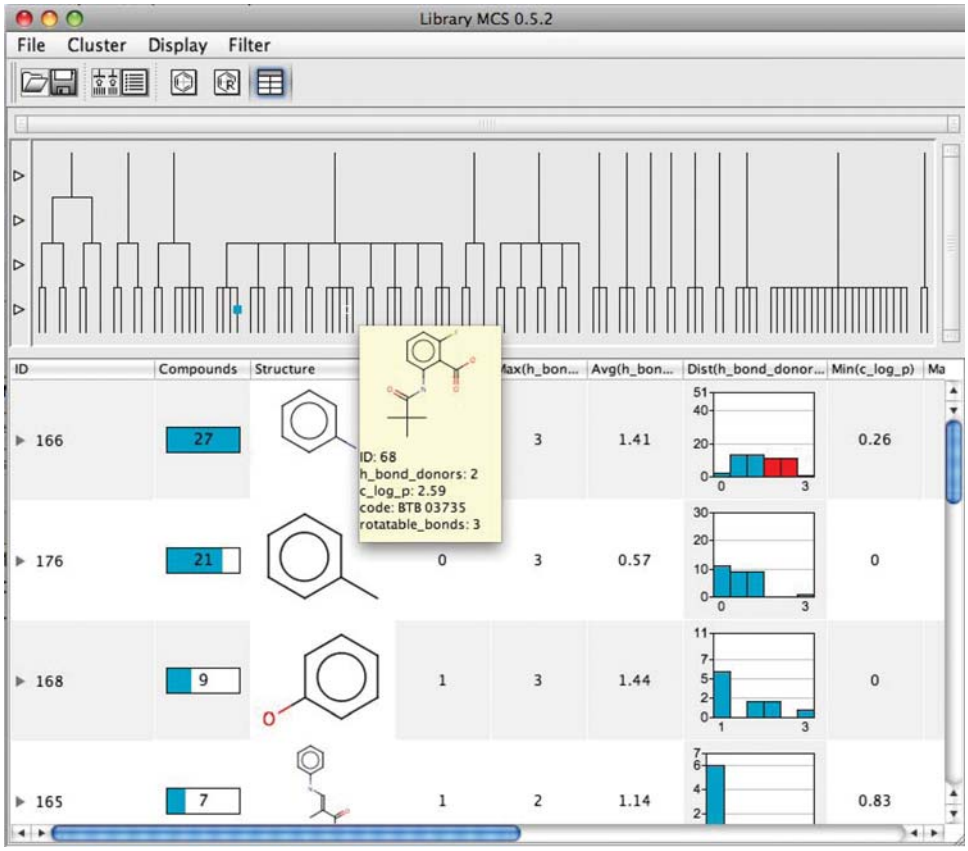

Figure 12. Clustered group of chemicals (Library MCS tool view).

11 CONCLUSIONS

Chemoinformatics has developed over the past 40 years to a mature discipline that has applications in all areas of chemistry. Chemists have to become more efficient in planning their experiments and extract more knowledge from their data. Chemoinformatics can help in this endeavour, and so many researchers and scientists in pharmaceutical chemistry and medicinal chemistry are focusing on implementing chemical informatics in their research works. There are still many problems that await a solution, and therefore we still will see many new developments in chemoinformatics.

RELATED ARTICLES

Toxicogenomics and the Evolution of Systems Toxicology

Structure-Activity Relationships for Carcinogenic Potential

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Biomarker Discovery: Introduction to Statistical Learning and Integrative Bioinformatics Approaches

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1 INTRODUCTION

Biomarker detection seems a straight forward objective. Making use of high-throughput technology as part of a study where participants have been in contact with different doses of the xenobiotic in question may reveal indicators of the belonging physiological reactions and, for example, allow for characterizing susceptibility groups. However, a biomarker is not only a measurable, differentially regulated molecule. It has to demonstrate generalizability in the sense that a *new* sample can be measured and correctly classified or characterized on the basis of the biomarker's values. There are strong requirements towards reproducibility and validity which have to be tested for each candidate biomarker prior to its possible first clinical use. This is especially important in a setting of different individuals with distinct genetic background and living conditions. Moreover, screening tens of thousands of molecules for their biomarker qualities – using only in the order of hundred samples, often less – involves the intrinsic problem of overfitting, yielding candidate biomarkers with poor generalizability.

This chapter wants to bridge between the toxicologist's physiological approach to analyze studies to detect molecular biomarkers and the statistical bioinformatics view on biomarker discovery. Towards this objective we first define the term biomarker and outline its role in toxicology research in Section 2. Throughout the following sections we make use of a famous gene expression dataset by Golub *et al.* (1999) as well as the statistical programming language `R` (Ihaka and Gentleman, 1996) and its packages collection, the Bioconductor suite (Gentleman *et al.*, 2004) to reproducibly demonstrate different approaches, possible stumbling blocks and their solutions (Ruschhaupt *et al.*, 2004). In Section 3 we exemplify the search for univariate molecular biomarkers, including discussion of sample selection, sample size, comparison of three shrinkage approaches as well as how to deal with multiplicity issues. In section 4 we introduce the leave-one-out cross validation approach to estimate a candidate biomarker's prediction error rate. Section 5 introduces the multivariate biomarker panel (biosignature) and exemplifies three statistical learning methods using the Golub gene expression dataset (Golub *et al.*, 1999). In Section 5.5 we compare uni- and multivariate feature selection results for the example of a random forest analysis. If a priori information about the molecular features is

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available (functional gene-sets, covariance structure, regulatory topological information) it can be used to enhance both detection and efficiency of biomarker signatures – we approach related objectives in Section 6. In Section 7 we introduce the concept of integrative bioinformatic analysis and its application to biomarker discovery. Section 8 reasons about the circumstances for using biomarkers to infer the underlying causes for the biomedical phenomenon under study, and in section 9 we summarize the approaches to biomarker discovery presented in our chapter.

2 BIOMARKERS IN TOXICOLOGY?

2.1 What is a Biomarker?

A biological marker (biomarker) is defined as "a characteristic that is objectively measured and evaluated as an *indicator* of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention" (Biomarkers Definitions Working Group, 2001). Hence, in the current context of toxicology we would understand a biomarker also as indicating responses to contact with chemicals in humans and animals. We propose to use "indicator" in this chapter for features which alone or in combination consistently allow classification of an individual to a unique group with defined characteristics regarding the traits of interest in toxicology, such as, for example, susceptibility to adverse effects. These features can, but do not have to, be functionally related to the unique group characteristics (Jacobsen *et al.*, 2008).

Classical examples of biomarkers include the tuberculin skin test (TST) for presence of tuberculosis infection (reviewed in Jacobsen *et al.*, 2008), pro-inflammatory indicators such as TNFα, IL2 and IL6 (Dinarello, 2000), or specific antigen titers – measured with antibodies – to indicate presence of specific pathogens.

There are a number of different molecular species available as candidate biomarkers which belong to the OMICs levels of gene expression: features of transcriptome (including miRNAs), proteome (including cytokines, antigens, antibodies, and hormones), or metabolome. Effects of xenobiotics are conceivable in each of these levels, also in parallel.

2.2 Importance of Biomarkers in Toxicology

As in toxicology biomarkers should be eligible for use in screenings, time series and dilution series exposure studies for safety evaluation and risk assessment, they need to be characteristics which are easily and reproducibly measurable. Such biomarkers could then be used to characterize different types of physiological answers (Cariello *et al.*, 2005; Morgan *et al.*, 2005), groups of susceptibility or even indicate the beginning of adverse events before their clinical onset, which may be too severe to allow exhaustive clinical studies (for example see Watterson *et al.*, 2009).

The hope in toxicology is that an optimum integration of clinical and chemical measurements, and biomarker responses would lead to an improved understanding of adverse effects and their significance in both human and ecological risk assessment (Eason and O'Halloran, 2002). It is, however, not necessarily the causal background of detected biomarkers itself that might contribute to such improvements, but the improved characterization of the involved samples or classification abilities, as shown later in this chapter.

3 FINDING BIOMARKERS

3.1 Biomedical and Clinical Studies to Detect Biomarkers

Toxicology, the study of the adverse effects of chemicals on living organisms, represents an interdisciplinary science that requires both expertise on the chemical nature of the studied compounds and precise knowledge of the normal cellular mechanisms that can be compromised by toxic substances. In the clinical context, the contribution of toxicology is primarily the identification of adverse effects, ideally at an early stage of pharmaceutical drug development. Such side-effect biomarkers anticipated in a clinical study are based on various toxicological information gleaned from non-clinical studies. Such prior studies involve experiments *in vitro*, for the cell culture level as well as in the animal model. On these levels prior to a clinical study, different molecules are conceivable as biomarkers when compared to the clinical study situation. This is due to the different sources of variation and biases (confounders) in these situations (as an example consider tissue heterogeneity

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as analyzed by Jacobsen *et al.*, 2006). Nevertheless, a biomarker in the animal model may correctly point to problems of tolerance or toxicity, even if not accessible in the situation of a clinical study involving patients. Most valuable conclusions are to be expected from study designs which include well-established principles of clinical studies in epidemiology and medical biometry (Repsilber *et al.*, 2005; Feng *et al.*, 2004; Wacholder *et al.*, 1992). In fact, drug development may be discontinued if no biomarkers can be detected using conventional clinical laboratory methodology; therefore, new approaches for finding biomarkers are needed. The use of molecular toxicological methods using OMICs technology is expected to be an effective future approach (Naraoka *et al.*, 2008; Bolt and Stewart, 2009).

OMICs technologies (transcriptomics, proteomics, metabolomics) when applied in clinical studies have the advantage that they benefit from the usually higher sample sizes, still for biomarker detection this approach has a core problem: Usually the number of samples n, that is, molecular profiles available for biomarker screening (usually one hundred or less), is much smaller than the number of features p to choose from (usually hundreds to tens of thousands). This so-called "small n large p"-problem leads to some important implications for biomarker candidate detection – which are going to be illustrated in the following together with existing approaches to their solution. For illustration, and to enable practical "hands-on" experience by reproducing the analyses we introduce, we make use of an example gene expression dataset.

3.2 A Prominent Illustrative Example: The Golub 1999 Gene Expression Dataset

Golub *et al.* (1999) issued and analyzed a gene expression study involving 47 patients with acute *lymphoblastic* leukemia (ALL) and 25 patients with acute *myeloid* leukemia (AML). Gene expression of these samples were measured using Affymetrix Hgu6800 microarrays. Data on the expression of 7129 genes (Affymetrix probes) are publicly available (Golub data website, 2009). Here, and in the following, `R` code chunks are integrated in the text such as to exemplify our explanations with a "hands-on-example." The reader may install the `R` software from http://www.r-project.org/cran and enjoy the reproducibility of these analyses (Ruschhaupt *et al.*, 2004): `R` script inputs are with lightblue, results with orange backgrounds:

```
library(golubEsets) # load public Golub dataset
data(Golub_Merge)
dat ← exprs(Golub_Merge) # extract gene expression matrix
index.ALL ← (1:ncol(dat))[Golub_Merge@phenoData$ALL.AML == "ALL"]
index.AML ← (1:ncol(dat))[Golub_Merge@phenoData$ALL.AML == "AML"]
all.names ← c()
all.names[index.ALL] ← paste("L", 1:length(index.ALL),sep='')
all.names[index.AML] ← paste("M", 1:length(index.AML),sep='')
colnames(dat) ← all.names
rownames(dat) ← featureNames(Golub_Merge)
DAT ← as.data.frame(dat)
class(DAT)
dim(DAT)
genes ← rownames(DAT) # retrieve Affymetrix identifiers
```

```
heatmap(as.matrix(DAT),labRow="")
```

As a common first data-mining step, we perform a two-way clustering (independently for samples and features), using hierarchical clustering with average linkage algorithm and Euclidean distance measure (Eisen *et al.*, 1998; Datta and Datta, 2006). The results is shown in Figure 1, a so-called *heatmap*.

It is already illustrating that the two classes, ALL and AML are not detected as separate clusters. Hence, we have to begin searching for valid candidate biomarkers.

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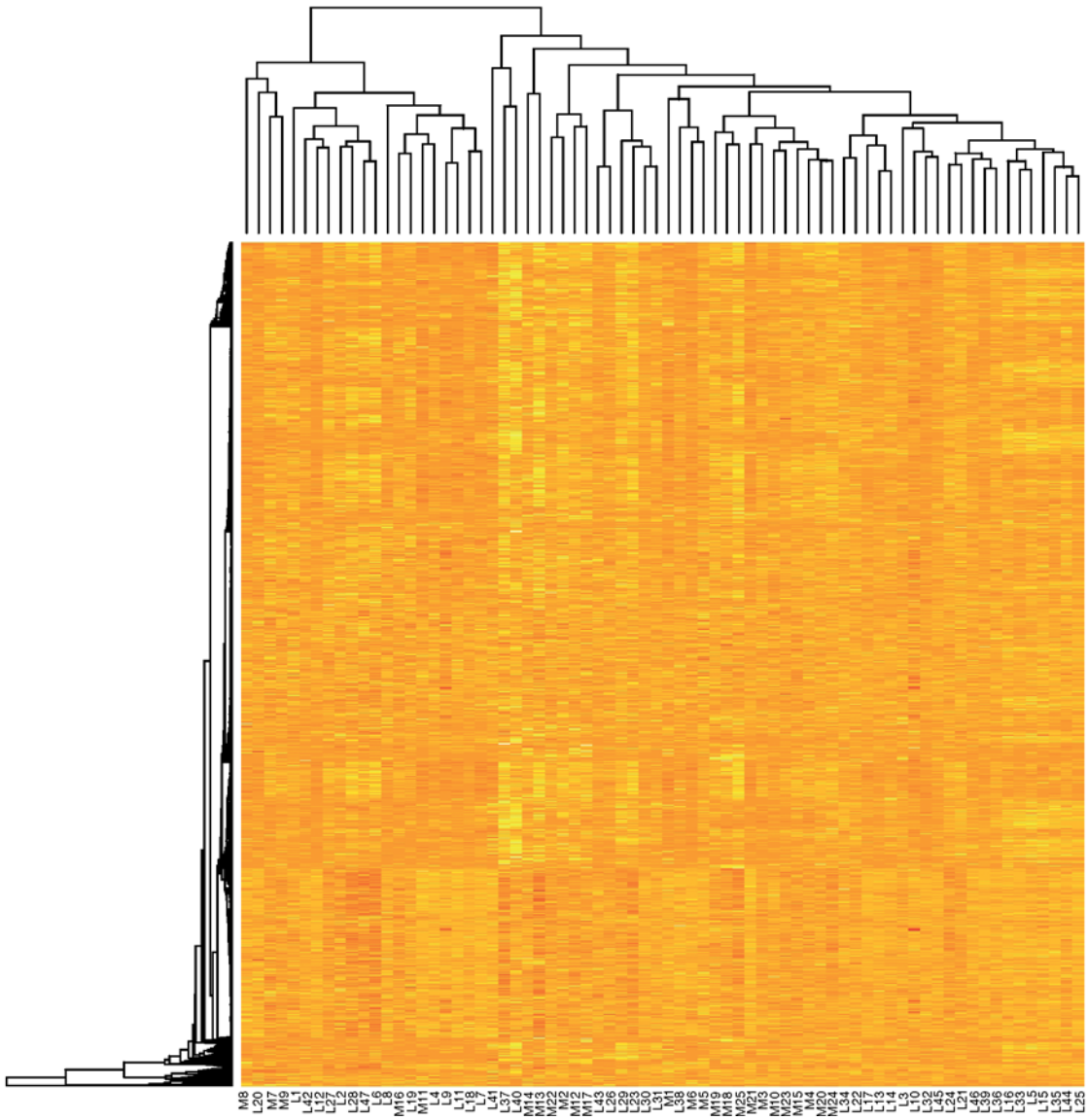

Figure 1. Heatmap of the Golub dataset. Genes as well as samples are clustered using hierarchical clustering, Euklidean distance measure and average linkage algorithm.

3.3 Normalization of OMICs Datasets – Necessity and Danger

OMICs datasets have to be prepared prior to uni- or multivariate analysis for biomarker detection. This preparation is necessary to ensure that biases from technical sources are removed (normalization), as well as for choosing a scale for which the screened features will be comparable (variance stabilization).

For different OMICs technologies there exist numerous approaches towards these objectives (see for example Smyth and Speed, 2003; Lisec *et al.*, 2006).

Without normalization, the screening study is at risk to deliver wrong candidates – which will fail in a validation study – as known technical error sources are not accounted for. However, such biases may be rather specific for the dataset under analysis. The

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corrections applied to correct for biases in the current study may not be the same for a new sample – possibly measured in another lab. After all attempts to make OMICs profiles comparable over labs, technological platforms and experimental designs – this issue has not yet been fully resolved, and it is to be seen as a major stumbling block for valid biomarker detection (Members of the Toxicogenomics Consortium, 2005; Moreau *et al.*, 2003). *Validity* with regard to a biomarker in this context would mean the ability to reproducibly measure it in different labs and come to coherent classification predictions.

3.4 A First Approach to Detect Univariate Candidate Biomarkers: The Classical *t*-Test

In the case of the illustrative Golub dataset, we base our presentation on a normalized dataset. As a first step, we look for single gene expression signals which would allow to classify a new sample as belonging either to the class AML or ALL.

For a biomarker two properties are key:

1. showing a *large* difference Δ between the classes
2. showing *small* variation σ within the classes

For a good biomarker the quantity $T = \Delta/\sigma$ (test-statistics) should be *large* in the sense that differences between groups are sufficiently high compared to individual variation. To learn about what *sufficiently high* means, we take a look on the illustrative example dataset by Golub *et al.* (1999). We begin with gene-wise t-tests and have a look at those candidates ranked top regarding their large test-statistics (and corresponding small p-values):

```
p.values ← apply(DAT,1,function(x){
   t.test(x[index.ALL],x[index.AML])$p.value
 })
ttest.ranking ← names(sort(p.values,decreasing=FALSE))
ttest.ranking[1:4]
```

```
[1] "X59417_at"    "M92287_at"    "M31523_at"    "M31211_s_at"
```

Feature "X59417_at" is ranked top regarding its p-value. (The following features are at positions two, three and four: "M92287_at," "M31523_at" and "M31211_s_at.") It is, however, conceivable, that when tens of thousands of features are screened – especially in studies with small sample sizes – there are possibly many with very small values for σ just as a random result. These features would get high values for T – and add to the number of false positives.

3.5 Improvements for Detection of Univariate Biomarkers: Shrinkage Approaches

There has been several similar proposals how to deal with the problem of random small variances: A very first, but continuously used, approach is to use a constraint for values of $\Delta > \theta$, where θ usually is some log fold-change – like for example $\theta = 1$, in the case of log-2 values corresponding to a minimum fold-change of two-fold. One could argue that changes in gene expression have to reach a certain limit to be *relevant* (not only *significant*). In any case, there is no alternative to validating candidate molecules using independent samples and an independent technique, such as for example quantitative Real-Time PCR (VanGuilder *et al.*, 2008) in the case of RNA or Westernblot (Renart *et al.*, 1979) for protein candidates. However, certainly, threshold values ("cutoffs") for relevant fold-changes, are gene-specific values. This leads us back to alternative estimates for the variances.

Towards preventing too small values for the estimated variances, Tusher, Tibshirani and Chu (2001) proposed a shrinkage approach, where they add a so-called fudge factor a_0 to the denominator of the ranking statistics: $T_{\text{Tusher}} = \Delta/(a_0 + \sigma)$. Here, a_0 is chosen according to some optimality criterion, but in principle, if σ becomes arbitrarily small, a_0 prevents T_{Tusher} from getting too large. For similar penelized versions of the t-test statistics see also Lönnsted and Speed (2003) or Efron *et al.* (2001). Here, we exemplify SAM (significance analysis of microarrays) by Tusher, Tibshirani and Chu (2001) for our illustrative example:

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```
library(siggenes)
cl ← rep(0,ncol(DAT)) # contains the class labels: 0: ALL, 1:AML
cl[index.ALL] ← 1
sam.out←sam(DAT, cl ,B=100,rand=123)
sum.sam.out ← summary(sam.out , 2.4)
sam.ranking ← rownames(sum.sam.out@mat.sig)
sam.ranking[1:10]
```

```
 [1] "X59417_at"        "M92287_at"        "M31523_at"        "U05259_rna1_at"
 [5] "X95735_at"        "M84371_rna1_s_at" "M11722_at"        "M31211_s_at"
 [9] "X17042_at"        "J05243_at"
```

The first three top ranked features in this case are the same as for the t-test, however, "M31211_s_at," which was ranked position 4 for the standard t-test, has now been shifted to position eight.

Smyth (2004) further developed the approach by Lönnsted and Speed (2003) and generalized the penelized t-test approach towards linear modelling and arbitrary contrasts. Their ideas are implemented in the R-package limma (Smyth, 2005). Let us have a look at their analysis in the case of the Golub dataset:

```
library(limma)
design ← cbind(Grp2vs1=cl)
design ← model.matrix(~factor(design))
fit ← lmFit(DAT, design=design)
eb ← eBayes(fit ,proportion=0.5)
raw.p.smyth ← eb$p.value[,2]
smyth.ranking ← genes[order(raw.p.smyth ,decreasing=FALSE)]
smyth.ranking[1:10]
```

```
 [1] "X95735_at"   "X17042_at"   "M23197_at"   "M84526_at"   "L09209_s_at"
 [6] "U46499_at"   "M27891_at"   "M16038_at"   "M22960_at"   "M63138_at"
```

Jaffrezic *et al.* (2007) take a mixed model approach to estimating the variances of the features in the ranking. They describe a simulation study where – for different benchmark datasets – they are able to outperform the suggestions by Tusher, Smyth and co-workers. Here comes the implementation for our example:

```
source("SMVar-adjFunctions.R") # see Appendix for this R-function!
genes ← as.data.frame(genes)
res ← SMVar.q(X=DAT,y=cl ,geneNumbers=genes ,threshold=1.0)
```

```
[1] "7129 differentially expressed genes"
```

```
raw.p.jaffrezic ← res[["Adj.p-value"]][order(res[[1]])]
jaff.ranking ← res$genes
jaff.ranking[1:10]
```

```
 [1] X59417_at        M92287_at        M31523_at        U05259_rna1_at
 [5] M31211_s_at      M84371_rna1_s_at M11722_at        J05243_at
 [9] X17042_at        M89957_at
7129 Levels: A28102_at AB000114_at AB000115_at AB000220_at ... Z97074_at
```

The top ranked features for all four approaches to find differentially expressed genes are different, figure 2 gives the details.

As for the Golub dataset we are dealing with comparatively large sample sizes, variance estimates used by the *t*-test can be considered stable. For the three shrinkage methods we observe different levels of concordance with the t-test ranking: Results for the approach by Jaffrezic *et al.* (2007) are closest, for the approach by Smyth, Yang and Speed (2003) we find the largest deviations.

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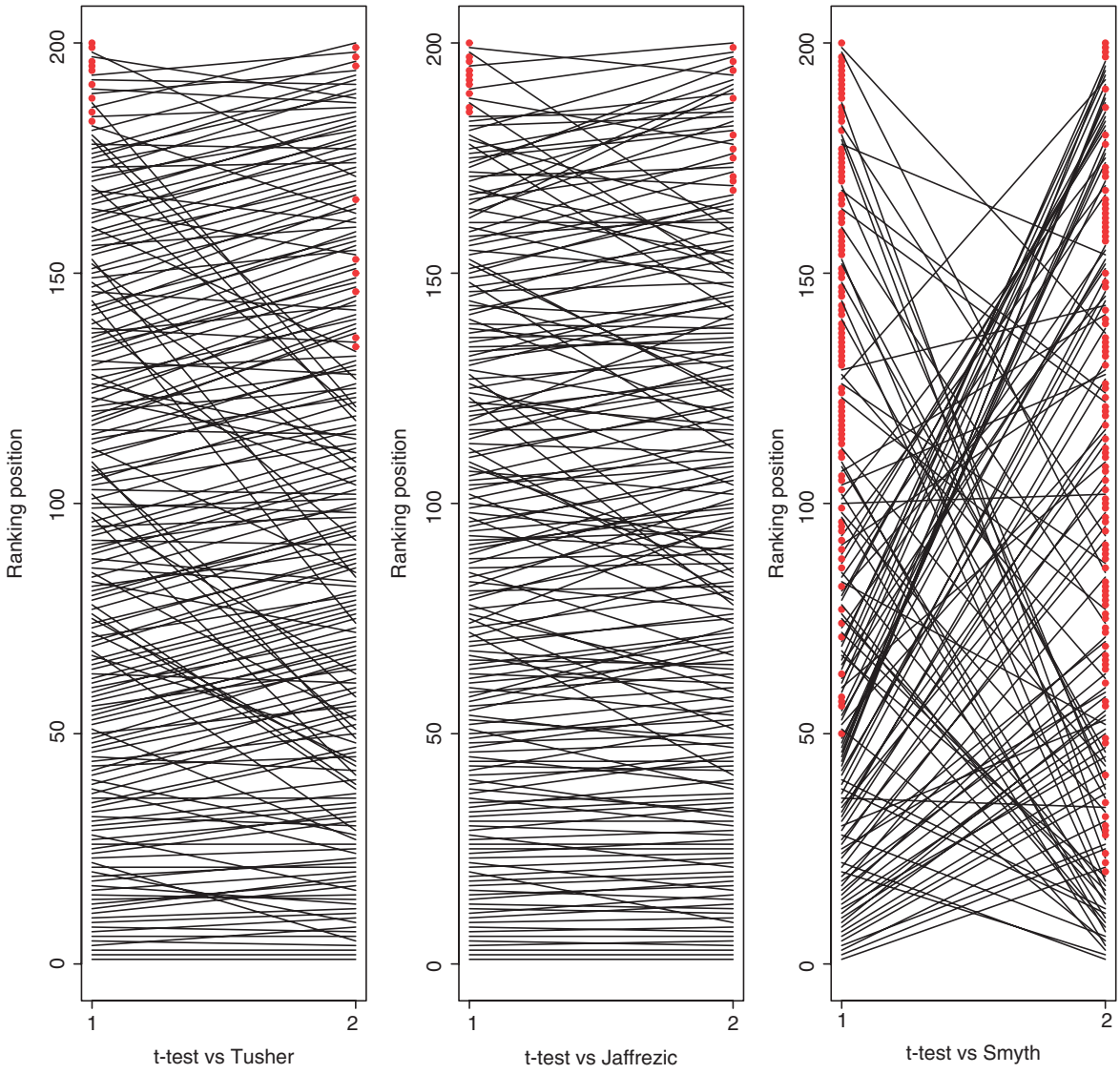

Figure 2. Comparison of ranking lists obtained by classical *t*-test and the approaches by Tusher, Tibshirani and Chu (2001), Jaffrezic *et al*. (2007), and Smyth, Yang and Speed (2003).

3.6 Role of Multiplicity During Screening for Biomarkers

If multiple statistical tests are used to rank features with respect to their p-values, one has to be aware of the fact that with fixing the error of type I – to falsely reject the null hypothesis of no differential expression – the number of false positives in a screening experiment involving tens of thousands of features can be a relevant number (the "multiple testing problem"): For example for a significance level of $\alpha = 0.05$ and $p_{\text{features}} = 10000$ we expect 500 false positive candidates for differential gene expression if no feature would be differentially regulated. The so-called false discovery rate, FDR, controls the expected proportion of incorrectly rejected null hypotheses. There are numerous approaches to estimate false discovery

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rates for gene expression experiments, for example, as proposed by Benjamini and Hochberg (1995), Benjamini and Yekutieli (2001), or by Storey and Tibshirani (2003), to name some examples, see Pounds (2006) for a review. Considering these multiplicity corrections is certainly of value for the detection of biomarkers, as these results may hint to the actual rate of truly differential expression in an experiment. For example, if there are no features left as candidates with an FDR smaller than 0.2, using such a result may lead to inreproducible results for any biomarker candidates selected from the corresponding ranking list. Whereas for the case with hundreds of candidates on FDR values all below 0.01, we can expect a lot of ongoing true differential expression.

Let us examine to which of these cases our illustrative dataset by Golub *et al.* (1999) belongs (based on the p-values resulting from the approach by Jaffrezic *et al.* (2007) and the FDR correction approach by Storey and Tibshirani, 2003):

```
library(qvalue)
q ← qvalue(raw.p.jaffrezic)$qvalue
sum(q<0.01)
```

```
[1] 731
```

Hence, in the Golub dataset we expect a lot of differentially expressed genes. This is also what we await, since with ALL and AML tumors we are comparing two different cell types.

3.7 Sample Size Considerations for Biomarker Screenings

A crucial question concerning experimental design is the assessment of study group sizes for biomarker discovery (Feng *et al.*, 2004). Different variables influence the appropriate size of samples to provide the basis for statistically valid analyses. These include technical and biological variances. Both parameters can be predicted by pilot surveys including repeated measurements of the same samples for assessing technical variance, and different samples within the study groups for assessing biological variance. Based on these pilot experiments the number of comparisons required to reach a certain power at a given significance level can be predicted. Important in this context is the problem of multiple testing which markedly increases the proportion of false positives when analyzing thousands of genes (proteins, metabolites, or lipids) in parallel, as discussed above. Ultimately, independent verification (e.g. by quantitative PCR of *new independent* samples) is the only way to exclude false-positive candidates and, consequently, no threshold for differential gene expression (e.g., 2-fold) assures exclusive selection of true positives. In conclusion, the experimental design of global OMICs analyses requires decisions prior to the set-up of experiments which strongly influence possibilities of analysis and interpretation of such results (Jacobsen *et al.*, 2008; Repsilber *et al.*, 2005).

How good is the top ranked single candidate for differentially gene expression – as biomarker for classification? We address this question in the following section.

4 QUALITY OF BIOMARKER CANDIDATES

Biomarker quality for classification is the best characterized by classification error proportion tested on a validation dataset (Martens and Næs, 1989). The principle of validation is to confront the biomarker candidate with samples which were not used (during training) to find this candidate. Such samples could be newly measured (external validation) or left out from the training process of a dataset already available (internal validation). A systematic way to efficiently use all available data is a special form of the internal validation – the cross-validation. One possible form of cross-validation is the "leave-one-out" cross validation: Each sample of the dataset is left out once and used as test case (when the remaining data are used for training). For a discussion of different forms of cross-validation we refer to Vapnik (1995).

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We will now demonstrate a leave-one-out (LOO) cross-validation to estimate the classification error rate using the best t-test candidate of the Golub dataset:

```
pred ← c() # empty vector to store predictions of left out cases
for(cv in 1:length(cl)){
   print(paste(round(cv/length(cl)*100,digits=2),"%"))
   train.X ← DAT[,-cv]
   train.Y ← cl[-cv]
   p ← apply(train.X,1,function(x){
     t.test(x[train.Y==0],x[train.Y==1])$p.value
   })
   ind ← order(p,decreasing=FALSE)[1] # select best candidate from training
   mean.0 ← mean(as.numeric(train.X[ind,train.Y==0]))
   mean.1 ← mean(as.numeric(train.X[ind,train.Y==1]))
   cut ← 0.5*(mean.0 + mean.1) # assuming identical variances in both groups
   if(mean.1 > mean.0){
     pred[cv] ← sum(DAT[ind,cv] > cut)
   } else { pred[cv] ← sum(DAT[ind,cv] < cut)}
 }
sum(cl!=pred)/length(cl) # proportions of prediction errors
```

```
[1] 0.2222222
```

Hence, we have to expect a classification error of 22%. Is this already the optimum, or could we expect a better classification from combinations of single biomarkers to so-called biomarker panels or biosignatures (cf. Jacobsen *et al.*, 2008)?

5 DETECTING BIOMARKER SIGNATURES: STATISTICAL LEARNING

5.1 Biomarker Signatures – Better Biomarkers?

Significant differential expression of a *single* feature often is insufficient for a good biomarker (see for example the various examples in Eriksson *et al.*, 1999). Biomarkers should allow consistent classification of an individual to a predefined group with distinct features. In principle a *single* gene would suffice as a biomarker but, obviously, a biomarker *signature* (i.e., a combination of several single biomarkers) has two advantages: First, it is more robust with respect to outlier results for a single marker part of the signature. Second, it enables capturing multi-dimensional predictive patterns with dependencies between multiple features. The latter makes biosignatures appropriate for use in highly complex systems as in the case of "OMICs"-studies, where we have to suspect such multivariate dependencies ("patterns").

5.2 The Basic "Learning" Cycle

Classification tools have been developed for analyzing multiple features to reveal the optimal biosignature for discrimination, as, for example, various types of discriminance analysis, partial least squares approaches, boosting logistic regression, support vector machines, or random forests (Kapetanovic *et al.*, 2004; Vapnik, 1995). In principle, classification tools follow the same basic procedure: In a first step the chosen algorithm specific model is parametrized using a training data set where the correct classification for each sample together with the candidate gene values is provided. During this training step the program "learns" how to assess the different candidates and identify the minimal and the most efficient gene cluster as biosignature. In a second step of evaluation, an independent test data set is used to validate the capacity of the chosen candidate molecular profile data to correctly classify *new independent* individual samples.

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5.3 Overfitting

Various publications on this topic stress the immense importance of sound cross-validation approaches to escape the threats of *overfitting* in multivariate classification (Feng *et al.*, 2004; Simon *et al.*, 2003). Serious overfitting problems arise if the number of features p, e.g. transcripts measured during a microarray gene expression experiment, far exceeds the number of samples n (isolates from different patients). In cases like this, all samples can always be correctly classified, but perhaps without having found any general feature pattern. Therefore, the generalizability of any feature panel, like a biomarker signature, has to be optimized in a cross-validation approach – and validated with external data. An alternative to external validation for cases which already use cross-validation during learning (for optimization of internal parameters) is *double* cross-validation: In each *external* cross-validation run the dataset is split into training and testset. The training set is then split again into *internal* training and internal test sets. To each external cross-validation run belong several internal cross-validations for optimization of the learned biosignature.

5.4 Statistical Learning for the Golub Data Example

Here, we continue our illustrative sample study of the famous gene expression study by Golub *et al.* (1999). We compare different methods of Statistical Learning using appropriate cross-validations and compare with the results from using *single* biomarkers for classification. Methods for supervised learning eligible for classification in OMICs datasets are countless. We chose frequently applied methods, freely available on the Biocondcutor platform (Gentleman *et al.*, 2004, 2005): Random Forests (Breiman, 2001), Powered Partial Least Squares Discriminant Analysis (PPLS-DA, Indahl *et al.*, 2009; Nguyen and Rocke, 2002; Eriksson *et al.*, 1999; Boulesteix and Strimmer, 2007), and K-nearest neighbor classification (Ripley, 1996; Venables and Ripley, 1999). We begin our illustration with a cross-validation of Random Forest classifications for the Golub data:

```
library(randomForest)
pred.RF ← c()
print(length(cl))
for(cv in 1:length(cl)){
   if(cv%%10==0) print(cv)
   train.X ← t(DAT[,-cv])
   train.Y ← as.factor(cl[-cv])
   trainedRF ← randomForest(x=train.X,y=train.Y)
   pred.RF[cv] ← predict(trainedRF,newdata=t(DAT[,cv]))
 }
```

Cross-validation error rate for the Random Forest example (given in %) is

```
sum(pred.RF - cl - 1)/length(cl)*100
```

```
[1] 1.388889
```

Next example, an implementation of the PPLS-DA for our dataset, first the internal cross-validation for optimizing the number of latent variables:

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```
library(cppls)
library(MASS)
library(multicore)
inner.cv ← function(k){
   ## create inner training set and inner test set
   inner.index ← sample(1:nrow(train.X),
                        size=round(nrow(train.X)*0.9),
                        replace=FALSE)
   inner.train.X ← train.X[inner.index,]
   inner.train.y ← train.y[inner.index]
   inner.train.data ← list(x=inner.train.X,
                           y=dummy(inner.train.y))
   inner.test.X ← train.X[-inner.index,]
   inner.test.y ← train.y[-inner.index]
   plsda ← cppls(y~x, ncomp=ncomp,
                 data=inner.train.data,
                 method="cppls", lower=0, upper=1)
   lda.x ← inner.train.X %*% plsda$loading.weights[,1:ncomp]
   lda.data ← list(x= lda.x, y= inner.train.y)
   lda.res ← lda(y~x,data=lda.data)
   ## predict inner test set:
   test.data ← list(x= inner.test.X
                    %*% plsda$loading.weights[,1:ncomp])
   inner.pred ← predict(lda.res,newdata=test.data)$class
   err ← 1 - sum(inner.pred==inner.test.y)/length(inner.test.y)
   return(err)
 }
```

And, second, the external cross-validation implementation for this case:

```
n.CV.inner ← 10
ncomp.max ← 10
pred.pplsda ← ncomp.opt ← outer.errs ← c()
loading.weights ← list()
err.prop ← matrix(NA,nrow=ncomp.max,ncol=n.CV.inner)
for(cv in 1:length(cl)){## outer cross-validation loop
   print(paste("cv=",cv,"of",length(cl),"␣->␣",date()))
   train.X ← t(DAT[,-cv])
   train.y ← as.factor(cl[-cv])
   ## optimizing ncomp:
   for(ncomp in 1:ncomp.max){
     print(paste("ncomp=",ncomp))
     res.cv.inner ← mclapply(1:n.CV.inner,# here: inner CV!
                             FUN=inner.cv,mc.cores=2)
     err.prop[ncomp,] ←  as.numeric(res.cv.inner)
   }## ncomp-loop
   means ← apply(err.prop,1,mean)
   ncomp.opt[cv] ← (1:ncomp.max)[means==min(means)][1]
```

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```
    ## fit optimal pplsda-model (ncomp=ncomp.opt):
    outer.train.data ← list(x=train.X,y=dummy(train.y))
    pplsda.opt ← cppls(y~x, ncomp=ncomp.opt[cv],
                       data=outer.train.data,
                       method="cppls", lower=0, upper=1)
    loading.weights[[cv]] ← pplsda.opt$loading.weights
    lda.data.opt ← list(x=train.X %*%
              pplsda.opt$loading.weights[,1:ncomp.opt[cv]],
             y= train.y)
    lda.res.opt ← lda(y~x,data=lda.data.opt) # discriminant analysis
    ## test optimal model on outer test data:
    test.X ← t(DAT[,cv,drop=FALSE])
    test.y ← cl[cv]
    outer.test.data ← list(x=test.X %*%
                pplsda.opt$loading.weights[,1:ncomp.opt[cv]])
    outer.pred ← predict(lda.res.opt,
                         newdata=outer.test.data)$class
    outer.errs[cv] ← 1 - sum(outer.pred==test.y)/length(test.y)
  }## end external cv
```

```
sum( outer.errs)/length(cl)*100
```

```
[1] 2.777778
```

And the K-nearest neighbour classification:

```
library(class)
pred.knn ← c()
for(cv in 1:length(cl)){
    train.X ← t(DAT[,-cv])
    train.Y ← as.factor(cl[-cv])
    test ← t(DAT[,cv])
    pred.knn[cv] ← knn(train.X, test, train.Y, k = 10, prob=TRUE)
  }
sum((pred.knn-1)-cl)/length(cl)*100
```

```
[1] 9.722222
```

Even a simple learning method like *k*-nearest neighbors has a better classification error rate in the leave-one-out cross-validation than the single feature approach. Note that, the parameter k from the nearest neighbors fitting (which we set to $k = 10$ in the last code chunk) could have been optimized taking an internal cross-validation approach (alike that we demonstrated for the PPLS-DA).

5.5 Feature Selection: The Biosignature from Statistical Learning

When a statistical learning machine is trained, we are of course interested which of the features were mainly used for prediction. A biomarker signature should not contain thousands of features, but a few – of the order of 25 – to be able to build a cheap

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testing device for field trials. As an example for such a testing device consider microfluidic cards (Ricco *et al.*, 2002). Dutkowski and Gambin (2007) propose to *combine* feature selection lists from different algorithms to arrive at what they call a "consensus biomarker selection." For our Golub dataset example, and the objective to predict the status "AML" or "ALL" from a gene expression signature, we exemplify the feature selection capabilities using the Random Forest approach again. The algorithm for feature selection in this context often is the *Gini-index*, which describes the increase in classification error (from cross-validation) as variable importance.

Here comes our "hands-on" example regarding the Golub dataset:

```
library(randomForest)
pred.RF.imp ← c()
for(cv in 1:length(cl)){
   train.X ← t(DAT[,-cv])
   train.Y ← as.factor(cl[-cv])
   trainedRF ← randomForest(x=train.X,y=train.Y,importance=TRUE)
   ## now select 20 most important features:
   imp.index ← order(trainedRF$importance[,4],decreasing=TRUE)
   trainedRF.imp ← randomForest(x=train.X[,imp.index[1:20]],
                                y=train.Y,importance=TRUE)
   pred.RF.imp[cv] ← predict(trainedRF.imp,# RF using 20 best features
                             newdata=t(DAT[imp.index[1:20],cv]))
 }
```

Figure 3 shows all feature importances for the Golub dataset classification using random forests. From these values it is now possible to pick the 20 most important.

Here we compute the cross-validation error rate for random Forest prediction using only the 20 most "important" (Gini index) variables:

```
sum(abs(cl - (pred.RF.imp -1)))/length(cl)*100
```

```
[1] 2.777778
```

This is a little worse than the random forest error rate using all features, but still comparable to PPLS-DA and much better than for the best univariate candidate.

Figure 4 compares random forest feature importance with the classical t-test ranking – there are new features which become important for the multivariate pattern.

Also for PLS-based algorithms, there are powerful possibilities to use feature selection (Le Cao *et al.*, 2008; Chong and Jun, 2005).

6 USING A PRIORI INFORMATION

A main problem in detecting single biomarker molecules taking a screening approach using one (or more) OMICs technologies is the high number of variables (features) from which to choose.

As pointed out by Feng, Prentice and Srivastava (2004), a special form of including a priori knowledge is the so-called "hypothesis driven approach" (as opposed to the purely "data driven approach"): If it is possible, based on information from pilot studies or other prior experiences, to focus on a special tissue, or cell type, background variance (other tissues or cell types) is significantly reduced and, hence, the power to detect promising biomarker candidates enhanced.

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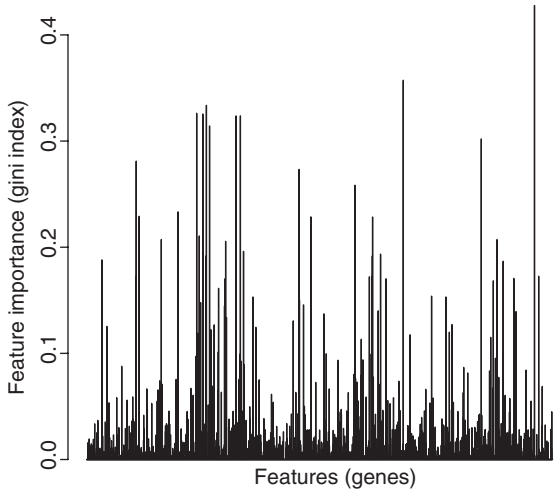

Figure 3. Feature importance plot (Gini index) for Random Forest learning applied to the Golub data. Some features show especially large importance, most are unimportant.

We want to stress the importance of using such possibilities, especially regarding limited sample sizes for the first phases studies for drug discovery.

Another possibility to exploit prior knowledge would be the notion that genes (and also proteins, miRNAs, or metabolites) do not act on their own but together with other features. Prior knowledge of such *functional relationships* between features should be a possibility to reduce the problem given above.

There are several possibilities of including prior information about functional grouping structures of features of which we want to highlight three in the subsequent subsections: Gene set enrichment analyses and related approaches, first steps to consider network structures in classification analyses, and an example to use knowledge about covariance structures in multivariate classification. A functional grouping, or "gene-set," sometimes "pathway," can have several forms: It could be for example a Gene Ontology (GO) term (Ashburner *et al.*, 2000; Falcon and Gentleman, 2007; Alexa *et al.*, 2006), a "molecular signature" (Broad Institute (MIT & Harvard), 2009), or some other functional group of features as available from one of the many "pathway databases" (Kanehisa and Goto, 2000; Bader and Donaldson, 2009). If a specific functional set representation for analyzing an OMICs experiment has been targeted, the next step is a database-assisted mapping of array

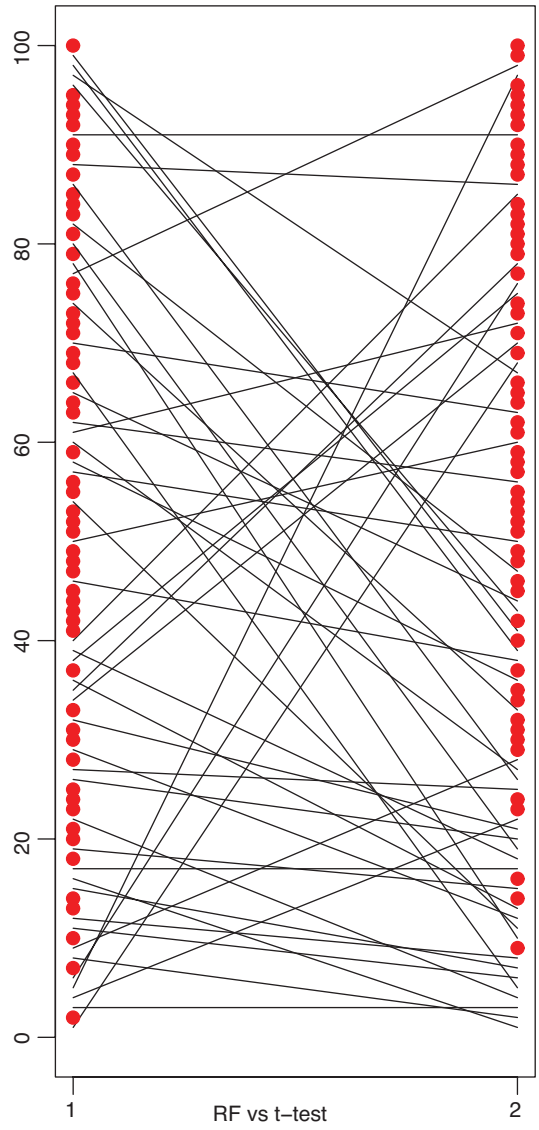

Figure 4. Comparison of an univariate feature ranking (t-test) and an example for a statistical learning method's feature ranking (Random Forest). The best t-test ranked features are also important for the Random forest prediction, but there is not much coherence in general.

features to gene identifiers used in the targeted representation of the functional sets under investigation (Durinck *et al.*, 2005).

Here, we map Affymetrix features of the Golub dataset on Human Gene Symbols to be able to use "molecular signatures" of Broad Institute (MIT &

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Harvard) (2009) in three different gene set enrichment analyses, as explained below:

```
library(biomaRt)
human ← useMart("ensembl", # connect to Ensembl database
                dataset = "hsapiens_gene_ensembl")
affy.genes ← as.character(genes[[1]])
res.query ← getBM(attributes = c("affy_hugenefl","hgnc_symbol"),
                filters ="affy_hugenefl",
                values=affy.genes,
                mart = human)
HGS ← unique(res.query$hgnc_symbol)[-1] ## no 1 is empty
DAT.HGS ← matrix(NA,nrow=length(HGS),ncol=ncol(DAT))
for(hg in 1:length(HGS)){
   h ← HGS[hg]
   affyids ← res.query$affy_hugenefl[res.query$hgnc_symbol==h]
   index ← (1:length(affy.genes))[affy.genes %in% affyids]
   DAT.HGS[hg,] ← apply(DAT[index,],2,mean)
 }
```

The result is a new dataset, "DAT.HGS," for which features are human gene symbols.

6.1 Gene Set Enrichment and Related Approaches

Ackermann and Strimmer (2009) give a comprehensive overview, a *taxonomy*, of existing methods to analyze gene set enrichment. Such methods can be used to test either of three possible null hypotheses:

1. Is a list of differentially expressed single features over-represented in a given set of genes? (e.g., Backes *et al.*, 2007; Falcon and Gentleman, 2007)
2. Is a set of genes differentially regulated? (e.g., Hummel *et al.*, 2008; Goeman *et al.*, 2005, 2004)
3. Is a special set of features more differentially regulated than all other sets of genes in the collection? (e.g., Subramanian *et al.* 2005, Efron and Tibshirani 2007)

Here comes the analysis according to Efron and Tibshirani (2007):

```
library(GSA)
geneset.obj ← GSA.read.gmt("c2.all.v2.5.symbols.gmt") # read in gene sets
GSA.res ← GSA(x=DAT.HGS,y=c1+1,genenames=HGS,
                genesets=geneset.obj$genesets, method='maxmean',
                resp.type="Two␣class␣unpaired", nperms=1000)
```

```
GSA.listsets(GSA.res, geneset.names=geneset.obj$geneset.names,
              FDRcut=0.2,maxchar=200)$negative[,2:4]
```

	Gene_set_name	Score	p-value
[1,]	"YAGI_AML_PROGNOSIS"	"-1.4315"	"0"
[2,]	"GOLUB_ALL_VS_AML_DN"	"-4.5843"	"0"
[3,]	"PARK_RARALPHA_MOD"	"-1.0089"	"0"
[4,]	"VERHAAK_AML_NPM1_MUT_VS_WT_UP"	"-1.2842"	"0"

```
GSA.listsets(GSA.res, geneset.names=geneset.obj$geneset.names,
              FDRcut=0.2,maxchar=200)$positive[,2:4]
```

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```
      Gene_set_name                                Score     p-value
[1,] "GOLUB_ALL_VS_AML_UP"                        "3.4794" "0"
[2,] "DNA_DAMAGE_SIGNALING"                       "0.5801" "0"
[3,] "HADDAD_HSC_CD10_UP"                         "1.2333" "0"
[4,] "ZHAN_MMPC_PC"                               "1.2626" "0"
[5,] "HADDAD_HPCLYMPHO_ENRICHED"                  "1.1587" "0"
[6,] "PARK_MSCS_BOTH"                             "1.0204" "0"
[7,] "ZHAN_TONSIL_PCBC"                           "0.8334" "0.001"
[8,] "ZHAN_MULTIPLE_MYELOMA_SUBCLASSES_DIFF"      "1.4857" "0.001"
[9,] "ZHAN_MMPC_EARLYVS"                          "0.7251" "0.001"
```

Observe the genesets "GOLUB_ALL_VS_AML_DN" and "GOLUB_ALL_VS_AML_UP" as controls. Next, we demonstrate the "globaltest" alternative:

```
library(globaltest)
rownames(DAT.HGS) ← HGS
globaltest.res ← globaltest(X=DAT.HGS,
                            Y=cl,genesets=geneset.obj$genesets)
```

```
library(globaltest)
library(multtest)
head(globaltest.res)
```

```
Global Test result:
Data: 72 samples with 5949 genes; 6 gene sets
Model: logistic
Method: Asymptotic distribution

     Genes Tested Statistic Q Expected Q  sd of Q    P-value
[1,]    12      5     4.3363    0.42562  0.36733 1.6982e-05
[2,]    33     23   143.2700   10.30000 13.52400 1.1256e-04
[3,]    47     24    29.0080    3.91170  3.64070 8.8112e-04
[4,]   103     58   118.7300   11.60700  9.80150 2.4077e-05
[5,]    11      6    79.1210   15.54000 20.10900 1.9252e-02
[6,]   121     49    26.1500    4.23560  3.41520 1.1416e-03
```

```
p.raw ← p.value(globaltest.res)
p.raw[is.na(p.raw)] ← 1
gt.q ← mt.rawp2adjp(p.raw,proc="BY") # multiple testing correction
sum(gt.q$adjp[,2] < 0.05)/length(p.raw)
```

```
[1] 0.5951374
```

```
geneset.obj$geneset.names[head(gt.q$index,n=15)]
```

```
 [1] "PARK_RARALPHA_MOD"                "TZD_ADIP_DN"
 [3] "ROSS_PML_RAR"                     "ST_INTEGRIN_SIGNALING_PATHWAY"
 [5] "BYSTRYKH_HSC_CIS_GLOCUS"          "LIAN_MYELOID_DIFF_GRANULE"
```

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```
 [7] "TGFBETA_EARLY_UP"                    "GOLUB_ALL_VS_AML_DN"
 [9] "TGFBETA_ALL_UP"                      "PASSERINI_ADHESION"
[11] "HOFFMANN_BIVSBII_BI"                 "ADIP_VS_FIBRO_UP"
[13] "YAGI_AML_PROG_ASSOC"                 "HADDAD_HSC_CD7_UP"
[15] "HADDAD_CD45CD7_PLUS_VS_MINUS_UP"
```

Last, the enrichment analysis using the hypergeometric distribution. This method relies on a specific list of differentially expressed genes (candidates) as analysis result prior to gene-set enrichment – we take the top 100 candidates from the *t*-test comparison of AML and ALL profiles:

```
p.val.ttest <- apply(DAT.HGS,1,function(x){
   return(t.test(x[cl==0],x[cl==1])$p.value)
 })
top100 <- HGS[order(p.val.ttest,decreasing=FALSE)[1:100]]
ref.genes <- HGS
## Enrichment computation:
thresh.l <- 15
gs.pval <- gs.name <- gs.SetSize <- gs.k <- c()
gs.l <- gs.expect <- c()
count <- 1
for (i in 1:length(geneset.obj$genesets)){
   k <- sum(top100%in%geneset.obj$genesets[[i]])
   l <- sum(ref.genes%in%geneset.obj$genesets[[i]])
   m <- length(ref.genes)
   n <- length(top100)
   k.expect <- n*l/m
   if(k.expect < k & thresh.l < l){
     gs.pval[count] <- phyper(k, l, m-l, n, lower.tail=FALSE)
     gs.name[count] <- geneset.obj$geneset.names[i]
     gs.SetSize[count] <- length(geneset.obj$genesets[[i]])
     gs.k[count] <- k
     gs.l[count] <- l
     gs.expect[count] <- k.expect
     count <- count + 1
   }
 }
## Adjust for multiple testing:
p.corr.gs <- mt.rawp2adjp(gs.pval,proc="BY")
which(p.corr.gs$adjp[,2] < 0.05)
```

```
 [1]  1  2  3  4  5  6  7  8  9 10 11 12 13 14 15 16 17 18 19 20 21
```

```
gs.name[p.corr.gs$index[1:21]]
```

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```
 [1] "GOLUB_ALL_VS_AML_UP"
 [2] "HADDAD_HSC_CD10_UP"
 [3] "HADDAD_HPCLYMPHO_ENRICHED"
 [4] "LEE_TCELLS2_UP"
 [5] "KLEIN_PEL_DN"
 [6] "HSA04662_B_CELL_RECEPTOR_SIGNALING_PATHWAY"
 [7] "ZHAN_MMPC_PC"
 [8] "MMS_MOUSE_LYMPH_HIGH_4HRS_UP"
 [9] "CMV_IE86_UP"
[10] "HADDAD_HSC_CD7_UP"
[11] "HADDAD_CD45CD7_PLUS_VS_MINUS_UP"
[12] "CROMER_HYPOPHARYNGEAL_MET_VS_NON_UP"
[13] "BCRPATHWAY"
[14] "ZHAN_MMPC_EARLYVS"
[15] "SASAKI_TCELL_LYMPHOMA_VS_CD4_UP"
[16] "SASAKI_ATL_UP"
[17] "PROTEASOME"
[18] "HDACI_COLON_BUT12HRS_DN"
[19] "RUTELLA_HEMATOGFSNDCS_DIFF"
[20] "E2F3_ONCOGENIC_SIGNATURE"
[21] "REN_E2F1_TARGETS"
```

Within the molecular signature pathways "c2.all.v2.5.symbols.gmt" Broad Institute (MIT & Harvard), 2009, "GOLUB_ALL_VS_AML_UP" and "GOLUB_ALL_VS_AML_DN" appear as controls throughout. As in this dataset two different cell types – which are the bases of the AML and ALL tumors respectively – are compared, we expect a rather large number of differentially expressed pathways.

6.2 A Systems Biological Approach to Classification: Considering Network Structures

Using a priori knowledge about functional groups of genes (pathways, molecular signatures, etc.) for most approaches does not depend on any topological regulatory structures between the features of a functional group. However, some approaches promise change: Draghici *et al.* (2007) exemplify how the analysis of pathways could profit from including more topological details of the underlying regulatory network. Schadt (2009) points out that in the context of common human diseases, the disease states can be considered emergent properties of molecular networks, as opposed to the core biological processes associated with a disease being driven by responses to changes in a small number of genes. Promising implementations of systems biological approaches for classification algorithms are underway, but currently in experimental status. As example, we want to refer to the work of Sæbø and Martens (Sæbø *et al.*, 2008, 2006), who propose to implement a third matrix for the PLS-algorithm, which could supply information about prior knowledge on covariance structures of features. Such covariance structures could in principle also be deduced – and be dependent – on topological information about regulatory networks.

7 THE INTEGRATIVE BIOINFORMATICS APPROACH

In the previous sections we were concentrating on describing the extension of the classical univariate approach, based on *t*-test and its relatives from classical statistical testing, to multivariate methods. These combine several features to biomarker panels, also called biosignatures, for classification. A further extension is now straightforward, the combination of features from different levels of gene expression – variation on the genomic level (measured as variation in genetic markers

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(polymorphisms)), on the transcriptome, proteome, and metabolome may be combined: There is a major motivation to use several OMICs levels at the same time, which relates to a data quality as well as the multiplicity problem inherent to most high-throughput studies: Each experimental technique will not only measure the biological variation it was designed for, but also add technical variance and possibly a methodological bias to the results. Together with the high-throughput character of genome-wide experiments, this probably results in high rates of false positive discoveries. There are also technologically biased results, which among other effects are partly responsible for false positives (Searls, 2003; Steinfath *et al.*, 2007). Here, if data from two or more different platforms, different levels of gene expression or biological organization, point to the same biomarker candidates, their relevance can be established with greater confidence. Such integration can also be seen as a means of verification of isolated results, or as if gathering additional degrees of freedom to be able to test a given hypothesis together with the high level of signal errors typical for high-throughput experimental data (Aitchison and Galitski, 2003).

Frequently, measured data are not independent across different experiments, but data obtained from distinct platforms are more likely to be independent observations. Based on these motivations, Aitchison and Galitski (2003) describes integrated data analysis as a way of going "from inventories to insights" and propose the following general approach:

The first level of integrated data analysis is also referred to as "data integration" (storage, organization in databases, mapping of feature annotations, joint normalization) to distinguish it from the following components. Data integration itself cannot explain the dynamical behavior of the biological system and is not a replacement for a dynamical qualitative or quantitative model. However, data integration is needed to increase the information available for the individual unit, the feature of interest, by adding more measured values, thus making systems features well distinguished from an otherwise overwhelmingly noisy background. A second component of integrated data analysis takes a pattern finding and correlation analysis point of view. Here, the goal of analysis is to find common patterns in data of different origin (see for example Patil and Nielsen 2005). Finally, as a third component of integrated data analysis, the formulation of quantitative as well as conceptual system models.

Practically, during integrative analysis, not only additional features can be included for a classification task, but also the information about which of these features from the different OMICs levels belong functionally together. Such information could be extracted systematically from appropriate biological databases (Kanehisa and Goto, 2000; Bader and Donaldson, 2009; Broad Institute (MIT & Harvard), 2009). Durinck *et al.* (2005) demonstrate how to use biomaRt (Durinck *et al.*, 2009) and Bioconductor (Gentleman *et al.*, 2004) as to build and use the powerful link between biological databases and OMICs data analysis.

8 CAUSALITY AND BIOMARKERS

It can be helpful if toxicologists also identify the mechanisms responsible for adverse effects. This knowledge can be particularly relevant when therapeutic and adverse effects are due to different mechanisms and can therefore contribute to a rational design of safer drugs. Examples are the recent reviews on mechanisms of telomere maintenance (Liew and Norbury, 2009) and genomic instability (Florl and Schulz, 2008). Schadt (2009) hopes that integrating large-scale, high-dimensional molecular and physiological data holds promise not only for defining the molecular networks that directly respond to genetic and environmental perturbations that associate with disease but also for causally associating such networks with the physiological states associated with disease. Also Klipp *et al.* (2005) and Searls (2005) point to the necessity to integrate the analysis of different levels of biological organization to identify functional biological themes revealing how biological molecules interact with each other. As shortly discussed above, involving causative reasoning into biomarker discovery is related to a "hypothesis driven approach," which can enhance power of biomarker detection through focussing and minimization of disturbing variation (and biases) (Feng *et al.*, 2004).

Besides the hopes that integrative biomarker signatures may help elucidating causal reasons for certain clinical phenotypes, we want to point to the problem of inseparability of first and second order effects: Candidate biomarkers may reflect

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only second order effects of some prior impact, as these second order effect often are measurable in more features and also of larger magnitude than the first order effects which are the first steps of the whole system's reaction. Often such – causative – first order effects are near to under detection level, think for example of the well-known systems of phage λ and its repressor which is present only in a few copies per cell.

9 SUMMARY

Our chapter on Biomarker discovery gives a statistical bioinformatics view of the field, combined with a "hands on"-example to reproduce on the reader's own desktop. It should be straight ahead to implement and apply the collection of methods mentioned here to the specific dataset in a systems toxicological approach, to

- find single biomarkers for classification from OMICs screening studies
- combine single markers to biosignatures with empowered prediction using several methods of statistical learning
- estimate classification errors using appropriate validation techniques
- approach an integrated analysis by either collecting data on different OMICs levels – or by taking into account existing systems biological a priori knowledge on gene-sets or regulatory topologies

We show that integrated data analysis as the combination of disparate experimental data (experiments, platforms, but in particular from heterogeneous biological OMICs-levels) can be a useful approach for the validation of analysis results on the one hand, and to establish biological models for the functional interplay of the different levels of biological organization aiming at a functional and holistic understanding.

Our hope is that this methodological collection, which is mostly based on the authors' experiences with transcriptome and metabolome studies in a molecular medical background will prove its applicability for the systems toxicologist hunting for valid biosignatures.

Supplements

R-scripts implementing the approach proposed by Jaffrezic *et al.* (2007), to store as file "SMVar-adjFunctions.R":

```
#Implemented by Guillemette Marot (guillemette.marot@jouy.inra.fr)

SMVar.q ← function(X,y,geneNumbers,minrep=2,threshold=0.05)
 {
   ## read expression data (4 develop stages, 5 repl each (Cols))
   stage1 ← as.data.frame(X[,y==0])
   stage2 ← as.data.frame(X[,y==1])
   listcond ← list(stage1,stage2)

   if (minrep<2){print("warning: minrep must be ≥ 2")}
   nbcond=length(listcond)
   nbgenes=dim(geneNumbers)[1]
   for (i in 1:nbcond){
     condi=listcond[[i]]
     assign(paste("nbrep",i,sep=""),dim(condi)[2])
     assign(paste("ddl",i,sep=""),
            get(paste("nbrep",i,sep=""))-1)
     assign(paste("invddl",i,sep=""),
            1/get(paste("ddl",i,sep="")))
     indcond=apply(condi,1,
```

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```
    FUN=function(x){sum(is.finite(x))≥ minrep})
  assign(paste("indcond",i,sep=""),indcond)
}

globind=indcond1
for (i in 2:nbcond)
  {globind=globind & get(paste("indcond",i,sep=""))}

globind=as.vector(which(globind==FALSE))

if (length(globind)>0)
  {
    for (i in 1:nbcond)
      {
        listcond[[i]]=listcond[[i]][-globind,]
      }
    geneNumbers=geneNumbers[-globind,]
    nbgenes=nbgenes-length(globind)
    print(paste(c("warning:",(length(globind)),
                  "genes␣with␣too␣many␣missings␣deleted"),
                collapse="␣"))
  }

calcmRSS=function(datacond)
  {
    mean←apply(datacond, 1,
               FUN = function(x) mean(x[is.finite(x)]))
    RSS←apply(datacond - mean, 1,
              FUN = function(x) sum(x[is.finite(x)]^2))
    datacond←cbind(mean,RSS,datacond)
    datacond
  }
listcondRSS=lapply(listcond,calcmRSS)
```

```
for (i in 1:nbcond){
  lnevari=log(listcondRSS[[i]][,2]*get(paste("invddl",i,sep="")))
  lnevari[which(is.infinite(lnevari))] ← NA
  mui=1/length(lnevari)*sum(lnevari,na.rm=TRUE)
  taui2=var(lnevari,na.rm=TRUE)-2/get(paste("ddl",i,sep=""))
  lambdai=taui2/(taui2+2/get(paste("ddl",i,sep="")))
  assign(paste(c("sigma2",i),collapse=""),
         exp(mui+lambdai*(lnevari-mui)))
  assign(paste(c("varsigma2",i),collapse=""),
         (get(paste(c("sigma2",i),
                    collapse="")))^2*1/(1/taui2+
                                        get(paste("ddl",i,sep=""))/2))
}
for(j in 1:(nbcond-1)){
    for(k in (j+1):nbcond){
      assign(paste(c("deltag",j,k),collapse=""),
```

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```
                (listcondRSS[[k]][,1]-listcondRSS[[j]][,1]))
          teststat=get(paste(c("deltag",j,k),collapse=""))/
  sqrt(get(paste(c("sigma2",j),collapse=""))/get(paste("nbrep",j,sep=""))+
       get(paste(c("sigma2",k),collapse=""))/get(paste("nbrep",k,sep="")))
  ddlt=(2*(get(paste(c("sigma2",j),collapse=""))+get(paste(c("sigma2",k),
           collapse="")))^2)/(get(paste(c("varsigma2",j),
           collapse=""))+get(paste(c("varsigma2",k),collapse="")))
          Studentpval=2*(1-pt(abs(teststat),ddlt))
          cond1=rep(j,nbgenes)
          cond2=rep(k,nbgenes)
          stat=data.frame(geneNumbers,teststat,Studentpval,
            ddlt,get(paste(c("deltag",j,k),collapse="")),cond1,cond2)
          colnames(stat)=c(names(geneNumbers),
                     "TestStat","StudentPValue","DegOfFreedom",
                     "LogRatio","Cond1","Cond2")
          assign(paste(c("stat",j,k),collapse=""),stat)
        }
      }

    globstat=data.frame()
    for(j in 1:(nbcond-1)){
      for(k in (j+1):nbcond){
        globstat=rbind(globstat,get(paste(c("stat",j,k),collapse="")))
      }
    }
    n=length(names(geneNumbers))


    library(qvalue)
    globstat$StudentPValue[is.na(globstat$StudentPValue)] <- 1
      ##adjpvalStud <- qvalue(globstat$StudentPValue)$qvalue
    adjpvalStud <- p.adjust(globstat$StudentPValue,method="none")
    globstatStud=data.frame(globstat,adjpvalStud)
    names(globstatStud)=c(names(globstat),"Adj.p-value")
    globstatStud=globstatStud[order(globstatStud[,(n+7)]),]
    genesdiffStud=globstatStud[which(globstatStud[,(n+7)]<= threshold),
      c(1:length(names(geneNumbers)),(n+4):(n+7))]
    print(paste(length(unique(genesdiffStud[,1])),
                "differentially␣expressed␣genes",sep="␣"))

    invisible(globstatStud)
  }
SMVar.paired.q <- function(logratio,geneNumbers,
                           minrep,method="BH",threshold=0.05)
  {
```

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```
  if (minrep<2){print("warning: minrep must be ≥ 2")}
  nbgenes=dim(geneNumbers)[1]
  nbrep=dim(logratio)[2]
  ddl=nbrep-1
  invddl=1/ddl
  globind=apply(logratio,1,
    FUN=function(x){sum(is.finite(x))≥ minrep})

  globind=as.vector(which(globind==FALSE))

  geneNum=data.frame(gene=geneNumbers)

  if (length(globind)>0)
    {
      logratio=logratio[-globind,]
      geneNum=data.frame(gene=geneNumbers[-globind,])
      nbgenes=nbgenes-length(globind)
    }

  mean? apply(logratio, 1,
              FUN = function(x) mean(x[is.finite(x)]))
  RSS? apply(logratio - mean, 1,
             FUN = function(x) sum(x[is.finite(x)]^2))
  lnevari=log(RSS*invddl)
  mui=1/length(lnevari)*sum(lnevari)
  taui2=var(lnevari)-2/ddl

  lambdai=taui2/(taui2+2/ddl)
  sigma2=exp(mui+lambdai*(lnevari-mui))
  varsigma2=sigma2^2*1/(1/taui2+ddl/2)

  deltag=mean
  teststat=deltag/sqrt(sigma2/nbrep)
  ddlt=(2*sigma2^2)/varsigma2
  Studentpval=2*(1-pt(abs(teststat),ddlt))
  stat=data.frame(geneNum,teststat,Studentpval,ddlt,deltag)
  colnames(stat)=c(names(geneNum),"TestStat",
             "StudentPValue","DegOfFreedom","LogRatio")

  n=length(names(geneNum))
  adjpvalStud=p.adjust(stat$StudentPValue,'none')
  statStud=data.frame(stat,adjpvalStud)
  names(statStud)=c(names(stat),"Adj.p-value")
  statStud=statStud[order(statStud[,(n+5)]),]
  genesdiffStud=statStud[which(statStud[,(n+5)]≤ threshold),
    c(1:length(names(geneNum)),(n+4),(n+5))]
  invisible(statStud)
}
```

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Mechanistic Investigation of EMD335823s Hepatotoxicity Using Multiple Omics Profiling Technologies

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1 INTRODUCTION

Development of a new drug is time-consuming, expensive and complex, and the possibility of failure to reach the market is high. Therefore, any advance in technology that can shorten development times and/or more accurately reflect the liabilities of a drug once it enters the general population would be of tremendous benefit.

So far, classical toxicology, with up to 2-year animal carcinogenicity studies, followed by traditional investigations (clinical chemistry, urinalysis, pathology, etc.) of biological endpoints is the gold standard in the drug development process. Beside the fact that these long-term studies need a lot of animals, we also have the problem that such observations do not provide information about a drug's mode of action, which is however important to know in order to better evaluate any adverse effects associated with drug exposure (Guerreiro *et al*., 2003). As drugs are expected to induce/alter a multitude of molecular/cellular pathways, it makes more sense to elucidate these on a molecular basis. Omics technologies (genomics, proteomics, and metabonomics) permit simultaneous monitoring of hundreds or thousands of macro and small molecules together with the promise to allow functional monitoring of multiple key cellular pathways simultaneously (MacGregor, 2003). In addition, changes in gene, protein, and metabolite expression are often more sensitive and characteristic of the toxic response or process than currently employed endpoints of pathology. Consequently, these expression changes do have the potential to indicate toxicity already at lower doses and/or earlier timepoints (Guerreiro *et al*., 2003). Toxicogenomics and, to a smaller degree, toxicoproteomics are already known as powerful tools for compound classification, mechanistic studies, and detection of toxicity biomarkers (Ellinger-Ziegelbauer *et al*., 2008; Geurts *et al*., 2005). However, recent publications (Craig *et al*., 2006; Kleno *et al*., 2004; Ruepp *et al*., 2002; Coen *et al*., 2004) have demonstrated that systems toxicology/systems biology can be of even more benefit in elucidating a drug's mode of action. Classical toxicology together with genomics, proteomics, and metabonomics has been shown to work synergistically in order to allow the generation of hypotheses about a drug's mechanism of toxicity. This understanding could therefore provide information on whether observed changes are species specific or predictive for human risk.

The FP6-PredTox project, a pilot project for the innovative medicines initiative (IMI), was started to help improve the drug development process through a series of public–private collaborations. The major aim was to find new technologies/biomarkers that

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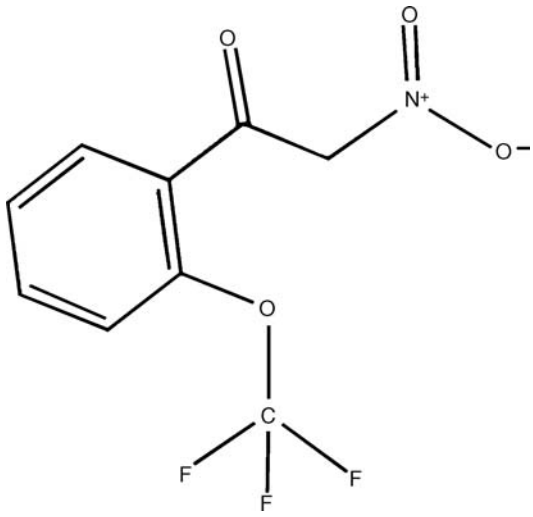

Figure 1. Structure of EMD 335823, an aldose reductase inhibitor.

could better predict drug safety earlier during development. The 14 participating companies offered test substances that could not enter the market, in part because of their hepato- or nephrotoxicity.

These substances have been analyzed in a systems toxicology approach with a combination of classical toxicology together with genomics [both whole genome (Affymetrix) and focused (Illumina DASL^{TM} technology for gene expression profiling from FFPE tissues)], proteomics (2D-DIGE), as well as metabonomics (both NMR- and LC/MS-based). The substance we focus on here, EMD 335823 (Figure 1), was developed by Merck KGaA as an aldose reductase (AR) inhibitor.

Aldose reductase is a monomeric, nicotinamide adenine dinucleotide phosphate-oxidase (NADPH)-dependent enzyme that is a member of the aldo-keto reductase family and is highly distributed in many tissues, such as nerve, lens, inner medulla of the kidney or renal cortex, etc. It catalyzes the reduction of aldo sugars and other saturated and unsaturated aldehydes, and constitutes the first step of the polyol pathway where glucose is reduced to the sugar alcohol sorbitol (Figure 2). As sorbitol does not easily cross cell membranes and its subsequent conversion to fructose via sorbitol dehydrogenase is slow, it accumulates inside cells resulting in changes in osmotic pressure, alterations in the redox state of pyridine nucleotides, and depleted intracellular levels of myoinositol. For example, too much sorbitol trapped in eye and nerve cells can damage these cells, leading to retinopathy and neuropathy, two common symptoms that can be found in diabetic patients (Boulton *et al*., 1985; Ranganathan *et al*., 1993). AR inhibitors are a class of drugs that have been shown to be effective in preventing the development of various diabetic complications because they prevent or slow the action of AR.

2 METHODS

2.1 Animal Study, Dosing, and Sample Collection

The animal study was performed on 10-week-old male Wistar rats (Han:Rcc_WIST) from RCC Ltd. (Füllingsdorf, Switzerland) with an average body weight of 170–200 g. Five rats were used for each treatment group and time-point, which were individually housed in Makrolon type-3 cages (‘Lignocel‘Schill AG, Muttenz, Switzerland), except on days −4/−3, 1/2, 3/4, and 12/13 when they were housed individually in metabolism cages (Tecniplast, Buguggiate Italy). Animals were kept under standard laboratory conditions with a light/dark cycle of 12 : 12 h. The test item EMD335823 was applied by oral gavage on a daily basis for up to 14 days. The compound was given in two different doses: a low (no observed adverse effect level, NOAEL) dose (15 mg kg^{-1}) and a high (maximal tolerated dose, MTD) dose (350 mg kg^{-1}). These doses were chosen based on the data from a previous 2-week pilot study. Animals were sacrificed on days 1, 3, and 14 after a 24-h fasting period.

2.2 Toxicogenomics

2.2.1 Affymetrix Whole Genome Array

Liver, kidney, and blood were profiled by toxicogenomics. RNA from target organ tissues was extracted using QIAGEN RNeasy® Mini Kit followed by DNA digestion using the RNase-free DNase Set (Qiagen GmbH, Hilden, Germany). For RNA extraction from blood samples, the samples

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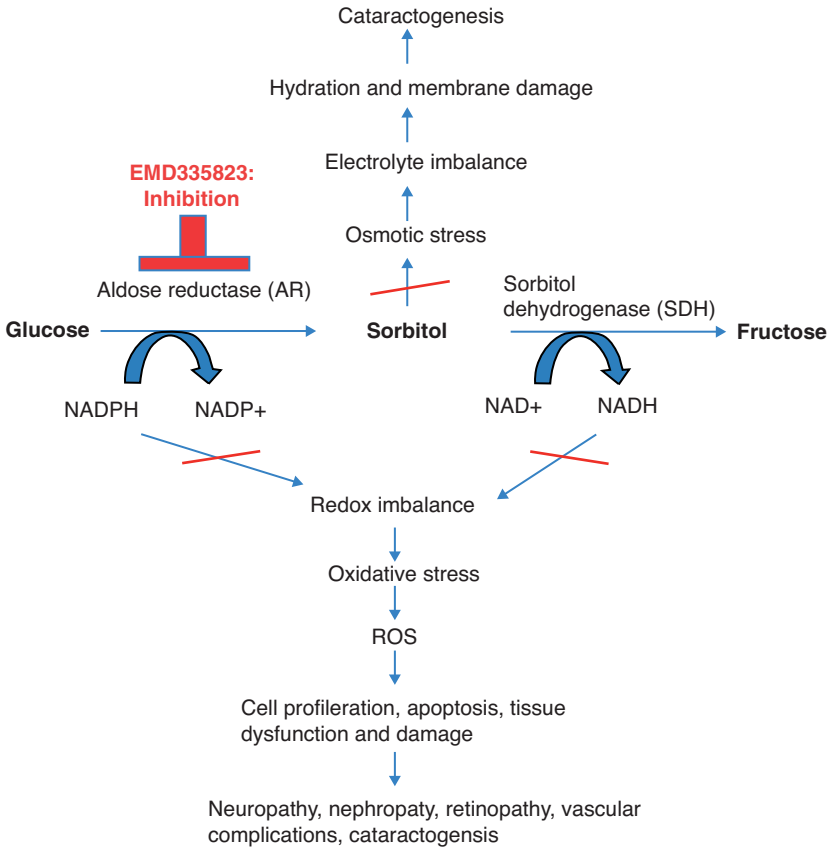

Figure 2. In the polyol pathway, glucose is reduced to sorbitol via aldose reductase, and afterwards, aldehyde dehydrogenase converts sorbitol into fructose. EMD 335823 inhibits aldose reductase and prevents from too high levels of sorbitol and resulting effects in diabetic patients (adopted from Srivastava, Ramana and Bhatnagar, 2005).

were collected from each animal and placed in special tubes containing PAXgene stabilizing solution (PAXgene® blood RNA tubes; PreAnalytiX Hombrechtikon, Switzerland). After collection, the tubes were gently inverted 10 times and left for 3 h at room temperature (RT) for cell lysis. Furthermore, RNA isolation was performed following the handbook from the PAXgene® Blood RNA System (PAXgene® Blood RNA Kit handbook).

The resulting RNA from both tissue and blood samples was processed using Affymetrix kits following the standardized protocol as provided by the supplier. For blood gene expression analysis, however, additional globin reduction reagents (provided by GeneLogic, Gaithersburg, MD, USA) were used. *In vitro* transcripts (cRNAs) were hybridized onto Affymetrix RAE 230 plus microarrays. Microarray quality was evaluated using the Genedata Expressionist® Refiner software (Genedata, Basel, Switzerland), and RMA-condensed data (provided by Genedata) were used for further evaluation using different software. A first overview on the data was given by principal component analyses (PCA) to test the data sets for any general differences. Statistical tests such as *t*-test and way ANOVA (factors: time, dose, time/dose interaction) have been applied in order to derive *P*-values (<0.05), and the ratio of medians (>1.5 and <-1.5) between different timepoints and/or doses using Genedata Expressionist® Analyst software (Genedata). Biological interpretation of pre-selected gene lists was subjected to pathway and network analyses employing ingenuity

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pathways analysis (IPA) from Ingenuity® Systems as well as GeneGO's MetaCore™ Pathway software.

2.2.2 DASL™ Assay

When using formalin-fixed paraffin-embedded (FFPE) tissue specimen for toxicogenomic assessments, special platforms are needed because RNA derived from formalin-fixed tissue is highly degraded (Bibikova *et al*., 2008). The DASL™ assay (Illumina Inc., San Diego, CA, USA) is one technology enabling the analysis of poor-quality RNA and was used in this study.

The FFPE tissue samples, used for transcription profiling, were derived from livers of treated and control animals. Livers were transferred to 10% buffered formalin for fixation and afterwards embedded in paraffin. Paraffin blocks were stored at RT until needed. Five sections of one block with a thickness of 5 μm were taken for gene expression analyses. FFPE tissue was deparaffinized by transferring the sections into xylol, followed by an ethanol wash. Afterwards, the RNA was extracted using the High Pure RNA Paraffin Kit (Roche, Mannheim, Germany) following the protocol suggested by the manufacturer. After isolation, the RNA was quantified using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA quality was checked via gel electrophoresis using the total RNA 6000 Nano Chip assay on an Agilent 2100 Bioanalyzer (Agilent Technologies GmbH, Waldbromm, Germany). Furthermore, as a quality check, the RPL13a gene was analyzed with TaqMan qPCR as recommended by Illumina.

Total RNA was processed/labeled according to the manufacturer's protocol. One thousand five hundred thirty-six oligonucleotide probe groups are multiplexed together in one reaction. In this study, three probe groups per gene were used, corresponding to 512 toxicologically relevant genes (Zidek *et al*., 2007).

DASL™ data were extracted with the BeadStudio software (Illumina Inc.) and summarized to one expression value per gene. These data were exported and further analyzed in Genedata Expressionist® Analyst software (Genedata). PCA was carried out for a general overview, and putative differentially detected genes were determined using *t*-test ($P \leq 0.05$), ANOVA, and fold regulation (>1.5 and <-1.5). GeneGo's MetaCore™ software was used to support mechanistic interpretation.

2.3 Toxicoproteomics

2.3.1 2D-DIGE

Liver tissue of 14-day high-dose and control animals was processed for 2D-DIGE analysis at NovoNordisk. Protein extracts from individual control and treated animals were labeled with Cy3 and Cy5, respectively. Additionally, a mix of protein extracts from control and treated animals was labeled with Cy2 (internal standard for the normalization of spot abundances). Mixtures of Cy2, Cy3, and Cy5 samples were run using two-dimensional gel electrophoresis. The first dimension was performed with IPG strips pH 5.5–6.7 using a Multiphor II apparatus. For the second dimension, IPG strips were transferred onto uniform polyacrylamide gels. Gels were run in an Ettan Dalt II electrophoresis unit. Scanning was performed using a Typhoon Trio+ (GE Healthcare, Munich, Germany), and global fluorescence intensities of the scanned images were normalized by adjusting the exposure times to the average pixel values acquired. DeCyder software version 6.5 (GE Healthcare) was used for image analysis. Significantly modulated spots were determined by performing a Student's *t*-test with the following thresholds: $P \leq 0.01$ and fold change ≥ 1.3. Protein identification of deregulated spots was carried out by MALDI-MS and MALDI-MS/MS. Protein identification and statistics of the modulated and identified proteins have been used for interpretation using IPA from Ingenuity® Systems.

2.4 Metabonomics

2.4.1 NMR Analysis

Urine and serum samples were analyzed at LipoFIT Analytic GmbH (Regensburg, Germany) or at the University of Wuerzburg, following standardized protocols (Völkel *et al*., 2005; Mally *et al*., 2007). Spectra were phase-corrected, calibrated, baseline-corrected (no negative values), normalized to the total integral, and binned in 0.04 (urine only), 0.01, and 0.001 ppm bins. Lists of statistical values per matrix and analytical technique, including two-way ANOVA *P*-values and

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Benjamini–Hochberg *Q*-values for the effects "dose," "time," and "dose–time interaction," and *t*-test *P*- and *Q*-values between treated and control replicates for each dose–time-point combination were prepared. For the PCA analysis, the data were Pareto-scaled.

For qualitative multivariate analysis NMR raw data (acquired at 600 MHz with the noesygpr1d pulse sequence from the Bruker library) were binned into 0.04-ppm-wide buckets, with the region from δ 4.40 ppm to δ–6.20 ppm excluded to avoid distortion by water suppression. The spectra were normalized to the total integral. Multivariate data analysis was performed using SIMCA P+ 11.5 software. All models were constructed with mean-centered and Pareto-scaled data.

2.4.2 LC-MS Analysis

Urine and serum samples were analyzed at Metabometrix Ltd. (London, UK) using LC-MS based on standardized protocols. Lists of statistical values per matrix and analytical technique, including *P*- and Benjamini–Hochberg *Q*-values from two-way ANOVA for the effects "dose," "time," and "dose–time interaction," as well as *t*-test *P*- and *Q*-values between treated and control replicates for each dose–time-point combination were generated.

3 RESULTS AND DISCUSSION

3.1 Clinical Investigations and Pathological Outcome

Investigation of clinical pathology parameters showed that high dose-treated animals (only 1-day-treated animals) had increased blood urea nitrogen (BUN), creatinine, sodium, and phosphorus levels. In addition, decreased total cholesterol, triglycerides, fatty acids, relative β-globulin, and absolute β-globulin levels were recorded. In liver, there were no microscopic findings of toxicological relevance on day 1, but in animals treated for 3 and 14 days, hepatocellular hypertrophy was recognized, which was in correlation with the enlargement of the livers seen, especially in day 14 animals. In addition, animals treated with high dose for 14 days showed clear test item-induced findings. Animals of this group were affected to a varying degree, with animals 42 and 45 showing the severest lesions: marked bile duct inflammation with bile duct and liver cell necrosis. In animal 44, the necrosis was only limited to the bile ducts in combination with marked bile duct inflammation. Bile duct inflammation, liver cell necrosis, and bile duct necrosis were considered to be adverse responses.

3.2 Toxicogenomics

3.2.1 Affymetrix Whole-Genome Arrays and DASL™ Assay

Global gene expression analysis was carried out for liver, kidney, and blood samples. A first PCA with all animals showed that there is a moderate treatment-related effect shown by a clear separation of high-dose animals from the vehicle control and low-dose animals (Figure 3a). A further PCA analysis for the detection of time-related effects especially indicates two animals of the high-dose 14 day treatment group (A42 and A45) behaving differently from the others (Figure 3b) corroborating the histopathological outcome that these animals had the most severe effects. Both effects could also be detected by analyzing degraded RNA with the DASL™ assay (Figure 3c). The PCA from the Affymetrix data additionally displayed one of the 3 day high-dose-treated animals (A29) clustering closer to the high-dose animals, which was the only rat of this treatment group showing signs of hepatotoxicity (hepatocellular hypertrophy). The data showed very large differences between individual animals in their response to EMD 335823, especially at high doses. This high variation was also reflected in the clinical chemistry and histopathology data. Based on these highly variable data, global gene interpretation proved to be difficult.

In addition, analysis of gene expression data from vehicle control animals alone suggests that day 1 animals may have been treated differently than the 3 and 14 day animals. ANOVA analyses of Affymetrix, as well as DASL™ data, from vehicle-treated samples clearly showed a distinct number of deregulated genes (e.g., 446 genes for Affymetrix) at different time-points with the over-representation of gene groups such as lipid biosynthesis and response to starvation (Figure 4). This result, based on both array technologies, leads to the assumption that day 1 animals were not starved overnight before sacrifice. This also correlates well with the pathological

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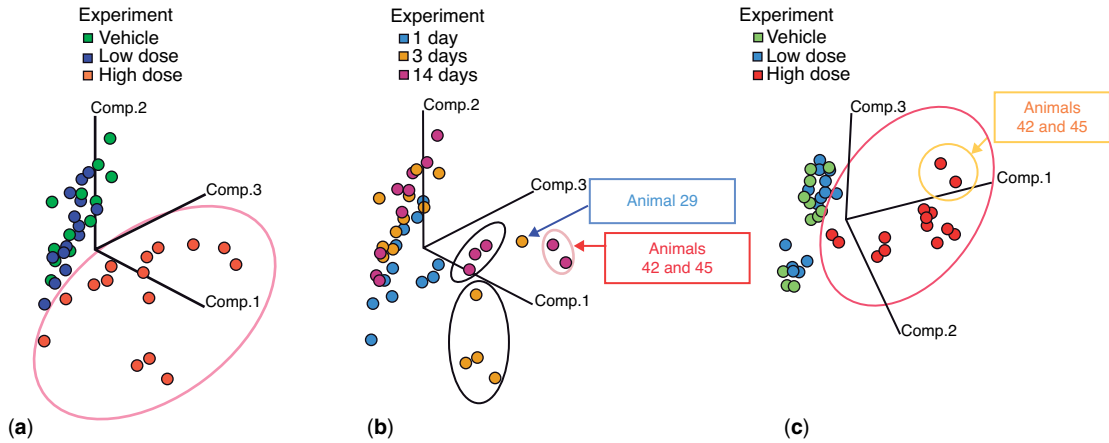

Figure 3. PCA of all liver gene expression data [Affymetrix (a, b) and DASL™ assay (c)]. (a) Clear separation of high-dose animals from control and low-dose animals can be achieved in a dose-dependent manner; (b) Day 3 high dose (yellow circled) and day 14 high dose (purple circled) animals showed effects after treatment with EMD335823, with two animals from day 14 high-dose group (42 and 45; showing most severe effects in histopathology) behaving differently from the others; (c) DASL™ assay data also showed clear separation of high-dose animals (red) from control and low-dose animals in a dose-dependent manner. Additionally, animals 42 and 45 separated from the rest of the high-dose group.

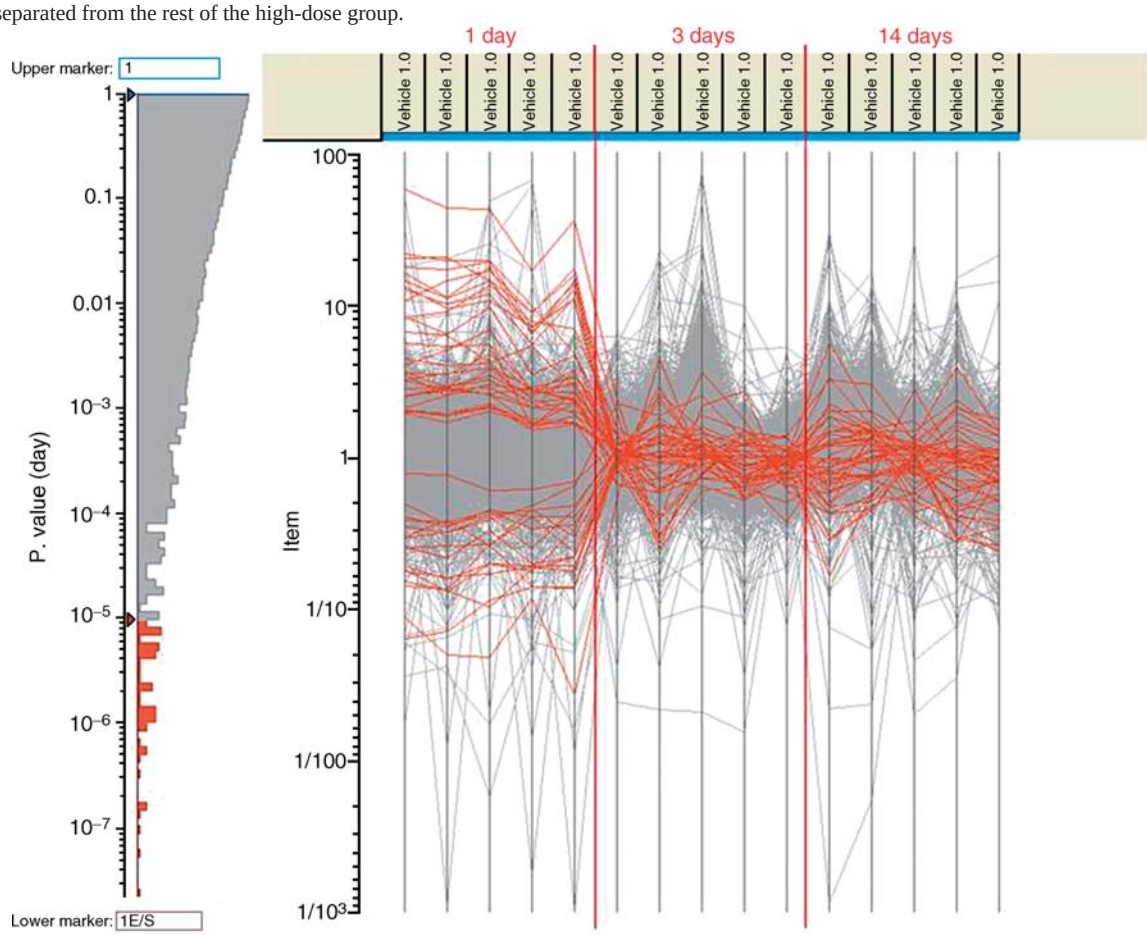

Figure 4. ANOVA on untreated "vehicle" samples: effect of experiment duration, gene expression from Affymetrix whole genome analysis.

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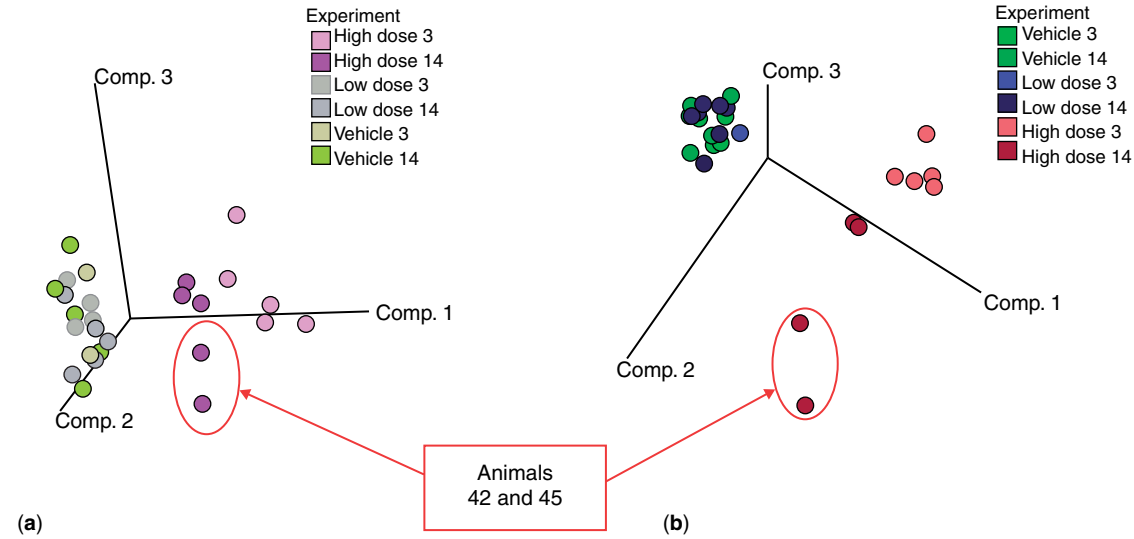

Figure 5. PCA for Affymetrix data (a) and data from the DASL™ assay (b) for days 3 and 14 animals only. The resulting data sets display a much clearer separation between high-dose and low-dose/control animals. Again, animals 42 and 45 are separated from the rest of the high-dose animal group.

finding of high glycogen content in the hepatocytes from this group, and observed glycogen depletion on days 3 and 14 animals indicating overnight starvation (as originally planned).

This fact has obvious implications for further data analysis. As such, data were re-analyzed excluding the day 1 animal samples. The PCA shown in Figure 5 shows that the dose-related effects are more obvious than before and interpretation of the data was made easier.

For analysis of the DASL™ data, only vehicle- versus high-dose-treated animals were compared, focusing on the 14-day treatment. By analyzing the deregulated genes (fold change ≤ 1.5 and ≥ -1.5 *t*-test: $P \leq 0.05$) glycolysis and gluconeogenesis were significantly deregulated, indicating a direct damage of hepatocytes, and confirming the histopathological necrosis of bile duct and hepatocytes. The data suggest that pathways of cell proliferation and immune response, including inflammation, were affected, which could be linked to the inflammation observed of the bile duct. In addition, bile acid synthesis was activated. The specific bile acid transporter abcc3 was strongly up-regulated in day 14 high-dose animals, which was confirmed by analysis of the Affymetrix data. Furthermore, many deregulated pathways corresponding to lipid and fatty acid metabolism were observed, correlating well with Affymetrix gene expression data.

After the application of a two-way ANOVA for "dose," "time," and "dose–time interaction," lists of significantly deregulated genes have been extracted from the whole-genome Affymetrix data set. These probe sets were separated into up- and down-regulated genes and graphically displayed with a one-dimensional clustering heat map (Figure 6). Characteristic profiles are obvious after exposure to high doses, with increasing number of gene expression changes after 3 days. Over-represented categories in this data set included protein folding, protein transport, and cell redox homeostasis, which indicates that oxidative stress and xenobiotic metabolism, as well as AR inhibition, are going on. In addition, several toxicologically relevant pathways showed a tendency to increase over time, such as acute phase response, tissue regeneration, fibrinolysis, bile acid synthesis, and bile acid transport.

Probe sets of all three time-points deregulated by at least 1.5-fold with *P*-values of less than 0.05 were uploaded into GeneGo's MetaCore™ pathway software. The most significantly affected canonical pathways are shown in Figure 7. As fatty acid metabolism and lipid metabolism are highly over-represented within these data sets, we suggest that

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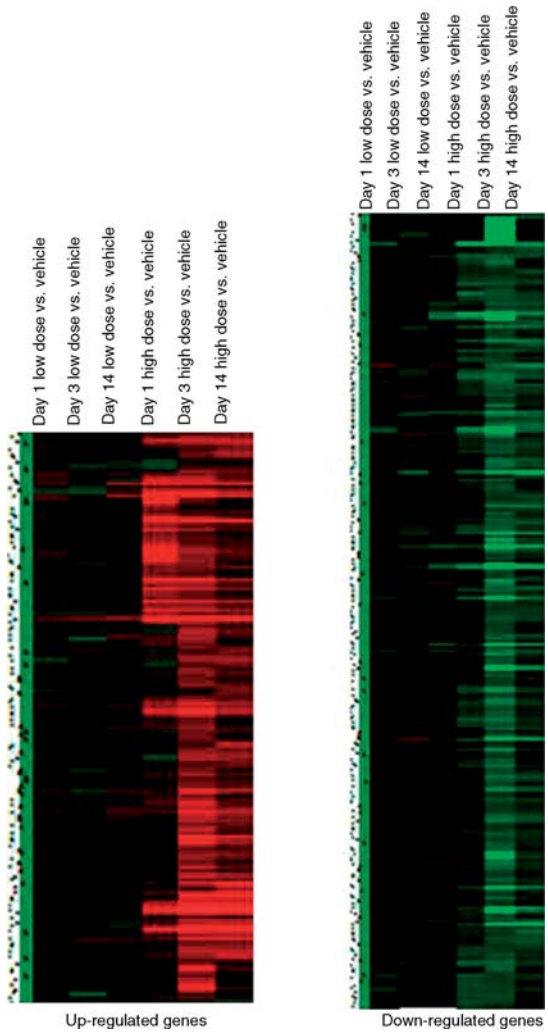

Figure 6. One-dimensional clustering heat map of up- and down-regulated genes from whole-genome Affymetrix data set. All three time-points and doses are given versus their matched vehicle. Red indicates up-regulated genes, green indicates down-regulated genes, and non-regulated genes are marked with black.

this is a major effect of our compound at both dose levels. The increase in fatty acid β-oxidation is also known to be significantly affected by PPARα agonists and to positively influence dyslipidemia (Cariello *et al.*, 2005).

This analysis revealed effects on multiple genes involved in bile acid biosynthesis (Figure 8), especially in high-dose animals, and furthermore some effects also in tryptophan metabolism. This effect can be based on the fact that a large number of genes are common between the fatty acid metabolism and tryptophan metabolism pathways, such as many aldehyde dehydrogenases and cytochrome P450s. However, tryptophan is also a key amino acid in the biosynthesis of nicotinamide adenine dinucleotide (NAD^+) (Murray *et al.*, 2000). A subtype of PPAR ligands increases NAD^+*in vivo* in the rat liver by alteration of the tryptophan–NAD^+ pathway (Shibata *et al.*, 1996; Shin *et al.*, 1998, 1999). Interestingly, Delaney *et al.* (2005) have proposed using urinary metabolites from the tryptophan–NAD^+ pathway as a marker of peroxisome proliferation.

Categorization of all the resulting canonical pathways that were common to both time-points (days 3 and 14) and doses (low and high) outlined the following networks (networks are prioritized based on the number of fragments of canonical pathways on the network): cellular lipid processing, very long chain fatty acid metabolism, lipid metabolic processing, lipid transport, cholesterol metabolic processing, acetyl-CoA biosynthetic process from pyruvate, cell surface receptor linked signal transduction, and stress response. All these gene expression changes seem to be related to the compound's mechanism of action because the inhibition of the target molecule AR leads to an increased use of the glycolysis pathway in connection with the citrate cycle and lipid metabolic processes. On one hand, the stress response can be interpreted as a general stress response reflecting a general hepatic damage as it was also shown by the pathological investigations. But it could also be due to the livers response to the pharmacological action of the molecule. AR is not only the first and rate-limiting enzyme of the polyol pathway, but it has also a detoxifying function in the body by reducing glutathione (GSH)-conjugated aldehydes formed during lipid peroxidation, such as 4-hydroxyononenal-3-yl-glutathione (HNE)-GSH. In case of AR inhibition, we also assume that reduction of aldehydes is diminished as well and concentrations of 4-hydroxy-2(*E*)-nonenal (HNE)-conjugated and unconjugated molecules can increase in the organ over time. This suggests that AR inhibition *in vivo* leads to an increase in downstream products of lipid peroxidation. In addition, according to the literature, significant increases in HNE concentrations in the tissue have been detected

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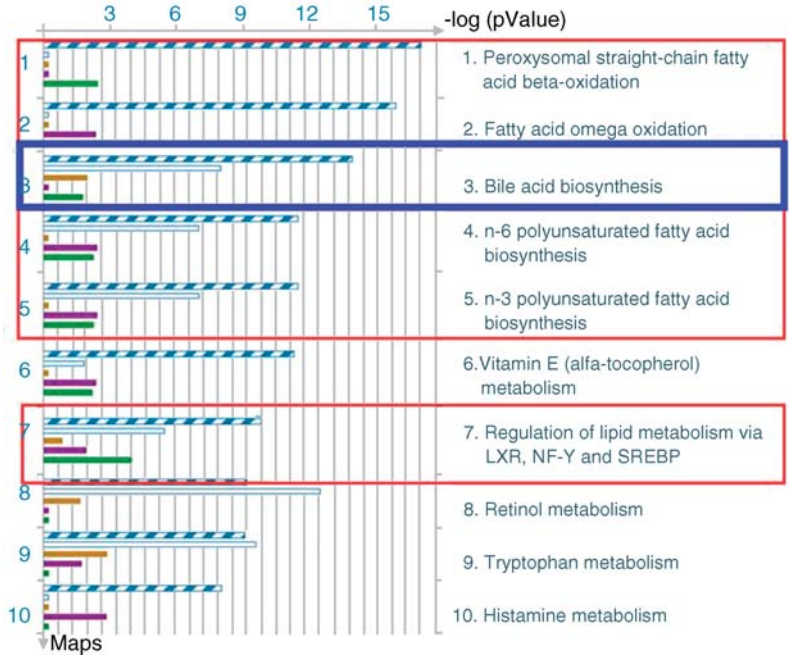

Figure 7. Distribution by canonical pathway maps given by GeneGo's MetaCore™ software. Colored bars indicate single time-points and doses (green: high dose day 1; brown: high dose day 3; violet: high dose day 14) whereas striped bars represent common genes across all samples.

in combination with higher frequencies of apoptotic cells (Rittner *et al.*, 1999).

Overall, the gene expression data (and histopathology, clinical chemistry) of all animals showed a large variation in animal response, especially at a high dose. Therefore, animals 42 and 45 (the most severely affected animals of the day 14 high-dose group) were analyzed separately. In comparison with the other three animals of the high-dose group, approximately 680 probe sets were found to be differentially expressed specifically by animals 42 and 45. Examination of these deregulated genes displayed an obvious decline of the effect on lipid/fatty acid metabolism on days 3 and 14 low- and high-dose animals. Instead, a much clearer picture of hepatotoxicity was seen as it was indicated by the following toxicologically related processes: (i) acute phase response/inflammation↑, (ii) protein damage (necrosis)↑, (iii) transport (necrosis)↑↓, (iv) oxidative stress↑, (v) regeneration↑↓, (vi) proliferation↑↓, (vii) regulation of transcription↑↓, and (viii) apoptosis↑↓. All these processes can be directly related to the observed histopathology.

3.3 Toxicoproteomics

3.3.1 2D-DIGE

Overall, 244 spots were found to be modulated with 130 spots up-regulated and 114 down-regulated. One hundred fifty-four spots were used for further protein identification by MALDI-MS and MALDI-MS/MS, resulting in 72 proteins with unique accession numbers (37 up-regulated and 35 down-regulated). Modulated proteins were analyzed using IPA from Ingenuity® Systems. Even though only samples from day 14 were processed, it was clearly observed that not only cell damage and response to drug treatment were prominent but also regeneration processes had been initiated.

From the IPA "Tox Functions," three main important processes were affected: liver cholestasis, liver steatosis, and liver proliferation (Figure 9). The first two effects fit to the known toxicity and pharmacological action of EMD 335823, namely bile duct toxicity and strong effects on lipid metabolism. As only day 14 samples were processed with 2D-DIGE, it seems that the liver proliferation shown above is likely to be a treatment-related effect rather than an

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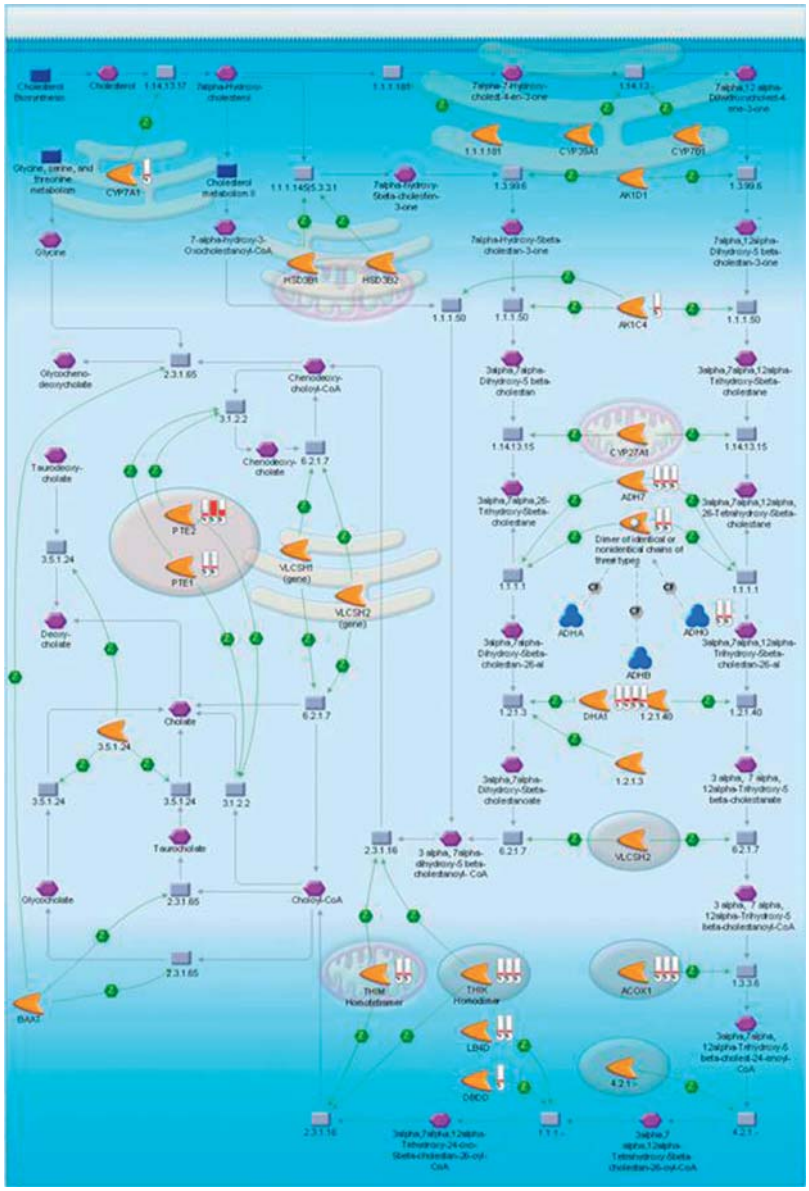

Figure 8. Overview map on the bile acid biosynthesis pathway as given from GeneGo's MetaCore™. Several genes are up-regulated (indicated by red bars beside the genes) within the high-dose-treated animals.

acute response. This was also confirmed by other proteins that were involved in inflammatory and regeneration processes (shown in Figure 10a).

The up-regulation of fatty acid metabolism was identified as the most significantly affected pathway, indicating that this is a major effect of our compound. This assumption is supported by the top 10 up-regulated proteins given in Figure 10b. Additionally, proteins were identified to be connected, either directly or indirectly, to PPAR alpha/RXR alpha activation as well as PXR/RXR activation. These proteins (e.g., Acot1↑, Acot2↑, MAPK1↑, ASL↓) could indicate that EMD335823 acts via PPAR regulation.

A comparison of protein IDs from 2D-DIGE with results from whole-genome gene expression

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Figure 9. Most significantly deregulated "Tox Functions" based on 2D-DIGE protein changes. Liver cholestasis and liver steatosis are effects that fit to the known toxicity and pharmacological action of EMD 335823.

analyses using IPA resulted in 10 overlapping genes and proteins with the same direction of deregulation. Most were related to detoxification or xenobiotic metabolism, as well as PPARα /RXR activation (Table 1). This shows that there is a good concordance between genomics and proteomics even when only a limited number of identified proteins are available.

3.4 Metabonomics

3.4.1 NMR and LC/MS Analysis of Urine and Serum Samples

A general overview on the NMR urine data as well as serum data revealed, in the same way as the genomics data, a clear separation of high dose from

Most significantly affected canonical pathways

- Fatty acid metabolism
- LPS/IL-1 mediated inhibition of RXR function
- Oxidative stress response mediated by Nrf2
- Aryl hydrocarbon receptor signaling
- Bile acid biosynthesis

(a)

The top ten upregulated proteins:

- Fatty acid metabolism:
  – Delta(3,5)-Delta(2,4)-dienoylCoA isomerase
  – Carnitine O-palmitoyltransferase 2
- Detoxification/Xenobiotic metabolism:
  – Aldo-keto reductase family 7
  – Epoxide hydrolase
- Stress response:
  – T-complex protein 1 subunit gamma
- Others:
  – Selenocysteine lyase
  – Carboxyl esterase isoenzyme
- PPARα regulation/fasting over 24h:
  – Acyl-coenzyme A thioesterase 1
  – Acyl-coenzyme A thioesterase 2

(b)

Figure 10. Results from IPA analysis of 2D-DIGE data of day 14 animals. Most significantly affected pathways (a) indicate fatty acid metabolism as the most prominent effect of our compound. This is also given by the top 10 up-regulated proteins (b).

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Table 1. Common genes (Affymetrix) and proteins (2D-DIGE) found deregulated by EMD335823 in liver.

Name	Gen Pept/Uni Prot/Swiss Prot accession (A1)	Affymetrix (A2)	Fold Change (A1)	Fold Change (A2)
CRYL1	Q811X6	1376051_at	1.61	2.97
PGD	Q7TP11	1371646_at	1.64	1.81
AKR7A3	Q68FZ3	1368121_at	4.1	4.88
HAL	Q5EBB8	1387308_at	−1.83	−1.91
ASL	Q4QRB8	1368916_at	−1.34	−1.96
KYNU	P70712	1398282_at	−1.43	−2.03
GSTA4	P14942	1372297_at	1.94	1.79
ME1	P13697	1370870_at	2.87	3.01
OAT	P04182	1367729_at	−1.86	−2.85
ACOT2	O55171	1388210_at	4.66	3.08

control but no clear separation between the different time-points. The best separation was again seen for high-dose animals in comparison with control animals for days 3 and 14, and confirmed by analysis of LC/MS data (Figure 11a) whereas on day 14 both animals 42 and 45 again showed the most pronounced separation (Figure 11b). In addition, serum metabonomics also showed a clear separation of day 1 animals from day 3 and day 14 animals, which could also give a hint on the effect of non-starved animals at day 1 (Figure 11b).

Outliers were removed from the model in a subsequent PCA analysis, which results in a clearer picture of high-dose animals being separated from controls at all time-points (data not shown).

To identify altered bins, an orthogonal partial least squares discriminant analysis (OPLS-DA) model was constructed using control and pre-dose versus low- and high-dose animals (outliers animal 43 pre-dose and animal 45 day 12 were excluded). Similar to PCA analysis, treated animals were separated from control and pre-dose samples in a dose-dependent manner (data not shown). S-plots, which combine the contribution or magnitude (covariance P) with the effect and reliability (correlation P_{corr}) of the variables, were used to identify regulated bins. A cut-off value of 0.05 for the covariance and 0.5 for the correlation was chosen; therefore, modulated bins are located at the extreme ends of the S-shaped curve (Figure 12a).

These qualitative analyses carried out for all three time-points revealed a decrease in citrate and *2*-oxoglutarate (Figure 12b) and potential changes in the excretion of trimethylamine-*N*-oxide (TMAO), betaine, and creatinine. Up-regulated bins include aromatic metabolites (around 7.0–8.0 ppm) that

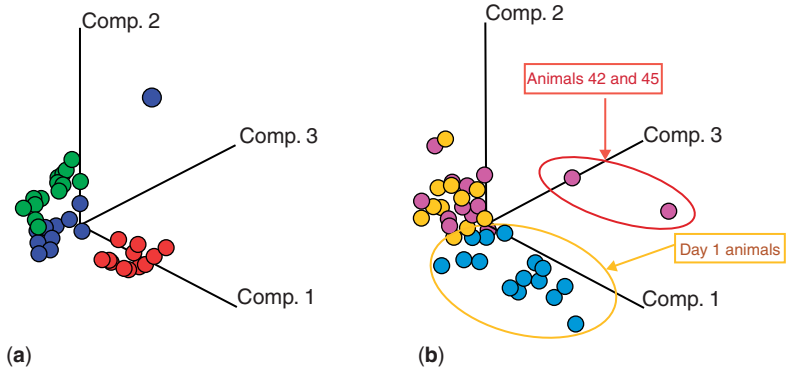

Figure 11. PCA plot NOESY Presat pulse sequence for urine LC/MS data (binned 0.04 ppm) and serum NMR data (binned 0.01 ppm). (a) PCA plot for urine LC/MS data colored by dose (green: vehicle; blue: low dose; red: high dose). A clear separation can be detected between high-dose animals mainly at days 3 and 14 and control animals. (b) PCA plot for serum NMR data colored by time-points (blue: day 1/2; yellow: day 3/4; pink: day 14/15). Animals 42 and 45 show the most pronounced separation confirming pathological findings.

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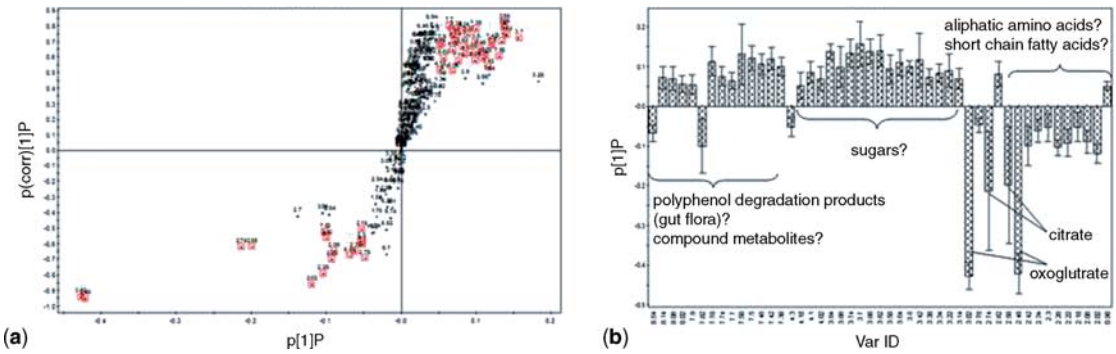

Figure 12. (a) S-plot or S-shaped curve with all time-points showing regulated bins highlighted by red squares; (b) up- and down-regulated bins with jack-knifed confidence intervals (all time-points combined).

may represent drug metabolites, aromatic amino acids, or polyphenol degradation products originating from altered gut microflora and CH_2 and CH_3 groups of aliphatic amino acids and short-chain fatty acids (around 1.3–2.3 ppm).

The Chenomx Suite NMR software (Chenomx Inc., Edmonton, Canada) includes a database with more than 250 endogenous compounds, enabling quantitative analysis of the 16 major urinary metabolites, based on integration of NMR signals. Spectra of high-dose animals of days 1 and 3 could not be integrated because the signals were too small, probably due to very dilute urine. Consistent with the qualitative data analysis, a decrease in urinary concentrations of 2-oxoglutarate and citrate was observed in treated animals. Although these changes were not always statistically significant because of large variability between animals, the decrease of these molecules could imply a general increase in energy metabolism (Thomas, Holmes and Robertson, 2005). Phenylacetylglycine was significantly increased on day 12. This may be derived from altered gut microflora, although it has previously (controversially) been discussed as a marker of phospholipidosis (Reasor and Kacew, 2001; Sawada *et al*., 2005). Methylamine, formate, 1-methylnicotinamide, and TMAO were significantly increased in high-dose animals on day 12. Formate is part of the C_1-metabolism and carbon from ^{14}C-marked formate occurs in transmethylation products. 1-Methyl-nicotinamide is a metabolite of nicotinamide. As already mentioned with the toxicogenomics results, 1-methyl-nicotinamide is an end product of the tryptophan–NAD^+ pathway in rats. Urinary excretion has previously described as enhanced in rats after treatment with peroxisome proliferators (Delaney *et al*., 2005; Cariello *et al*., 2005).

3.5 Mechanistic Investigations

Based on the analysis of gene, protein, and metabolite profiles, several different potential mechanisms of action of EMD 335823 could be postulated. (i) A significant up-regulation of genes (e.g., ACOX 1, CTE1, CYP4a) and proteins specifically regulated by PPARα. This leads to the assumption that this compound is probably a PPARα activator. PPARs are nuclear receptors that control a variety of genes involved in several pathways of lipid metabolism, cellular differentiation, and glucose homeostasis. PPARα mainly acts on lipid and lipoprotein catabolism genes, predominantly in the liver (e.g., fatty acid β-oxidation). This would fit with some primary effects that were observed in treated animals within this study. However, this has to be further evaluated and confirmed via other techniques (e.g., a transactivation assay studying the interaction of the compound with the PPARα receptor *in vitro*). (ii) Oxidative stress, mediated by the transcription factor Nrf2, is postulated. This basic leucine-zipper transcription factor, nuclear factor erythroid 2 p45-related factor 2 (Nrf2), deregulated by EMD 335823, is a cellular oxidative stress sensor and anti-apoptosis factor, which regulates many antioxidative, cytoprotective genes and has been shown to play a pivotal role in protecting cells from

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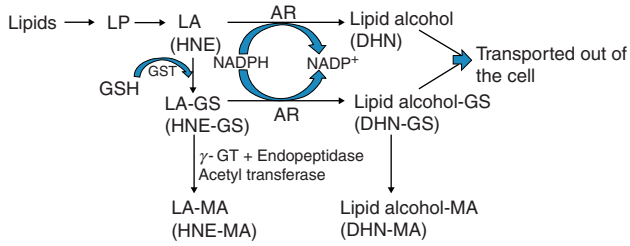

Figure 13. A schematic representation of the role of aldose reductase in the detoxification of free radicals and lipid aldehydes. LP, lipid peroxide; LA, lipid aldehyde; AR, aldose reductase.

oxidative stress. A number of genes normally associated with stress response were up-regulated and included genes such as GSH-dependent antioxidant enzymes for the reduction of reactive metabolites and detoxification, such as glutathione peroxidase 2 (GPX2), glutathione *S*-transferases (e.g., GST A2) or UDP-glucuronosyltransferases (e.g., UGT1A6), heme oxygenase-1 (Hmox1), metallotheonein (Mte1a), and NAD(P)H dehydrogenase, quinone 1 (Nqo1) (Harvey *et al*., 2009). (iii) We could confirm the inhibition of the pharmacological target. AR is involved in biotransformation of reactive aldehydes and corresponding GSH conjugates, which are formed during lipid peroxidation (Figure 13). Therefore, we can hypothesize that reduced detoxification of toxic lipid peroxidation products caused by inhibition of AR by EMD 335823 can lead to liver injury. GSH-derived metabolites of HNE and 1,4-dihydroxy-2-nonene (DHN), namely 4-hydroxyononenal-3-yl-mercapturic acid (HNE-MA) and 1,4-dihydroxynonenal mercapturic acid (DHN-MA) (also Figure 13), were found in liver, blood, and urine samples by LC-MS.

Even though the results showed a dose-dependent increase of both metabolites, the ratio of DHN-MA/HNE-MA was decreased. The fact that the ratio decreases with higher concentration of EMD335823 leads to the assumption that we had an increase in the inhibition of our target molecule AR. As it is shown in Figure 13, DHN-MA can only be produced via AR, and therefore with decreasing AR function we also have a decreased ratio of DHN-MA/HNE-MA. HNE-MA is still being produced in high amounts via acetyl-transferase reflected by an up-regulation of this gene. Additionally, HNE-protein adducts were detected by immunoblot/immunohistochemistry (IHC), agreeing with this proposed hypothesis. Furthermore, AR is an enzyme catalyzing the first step of the polyol pathway. The inhibition of AR will result in the increase of glucose metabolism via glycolysis and consequently result in alterations in fatty acid metabolism. Whether the changes in lipid metabolism seen in this study are purely related to the pharmacological action or are in fact a result of hepatocyte damage is difficult to distinguish. However, AR is a high-K_m enzyme and therefore its activity is very low at normal glucose concentrations. Accordingly, the flux through the polyol pathway as an alternative to glycolysis only increases significantly when very high glucose levels saturate hexokinase, the enzyme responsible for the transformation of glucose into glucose-6-phosphate, the first metabolite of glycolysis (Cárdenas, Cormish-Bowden and Ureta, 1998; Beyer and Hutson, 1986). In addition, these types of effects are commonly seen with hepatotoxic compounds. Disturbances in lipid metabolism could result in lower energy resources in the liver, which may also contribute to the pathogenesis of liver damage. Based on this, it more likely seems to be a sign for hepatotoxic damage instead of being a result based on the pharmacological action of EMD335823.

4 CONCLUSION

In a previous 2-week rat study with EMD335823 (other doses), clear toxicity could be detected in the liver already after 2 days even with the low dose. These effects included large increases in alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), and alkaline phosphatase (PAL) as well as effects on the bile ducts (hyperplasia, necrosis). In this study, similar effects were observed in the liver. Additionally, animals of the 14-day high-dose group showed hepatocellular hypertrophy and

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multifocal necrosis. Hepatocyte and biliary necrosis in the liver were considered to be test-item related and of adverse character.

Different technologies for genomics, proteomics, and metabonomics were applied in order to find out which single technology or even a combination of technologies would generate the best information to carry out in-depth mechanistic investigations.

Gene expression analysis was the most powerful technology and showed good concordance to the histopathological outcome. Even analysis of FFPE tissues revealed reliable gene expression data, which confirmed data from fresh frozen samples and histopathology. The other technologies were able, at least to some extent, to corroborate these gene expression changes. In all cases, the majority of deregulations observed were linked to an alteration in the liver's lipid/fatty acid/cholesterol metabolism. There was an adaptive detoxification response, with many phase I and II enzymes being up-regulated. This may help explain the hepatocellular hypertrophy observed in all high-dose animals (day 14). The large number of genes involved in translation/transcription (major categories when day 1 animals were removed from the analysis and seen specifically in animals 42 and 45) could also be linked to this hepatocellular hypertrophy.

There were several clear effects on the bile duct system, followed by deregulation of genes representing tissue reorganization and bile duct proliferation, probably as a result of necrotic processes. When looking specifically at the two animals (42 and 45) that showed the greatest bile duct hyperplasia/hepatocyte necrosis/inflammation histopathologically, a specific gene expression pattern was observed. Indeed, these two animals also showed an effect in the blood gene expression profile that may be linked to the more severe effects on the liver.

No gene expression changes were clearly identified that occurred at a lower non-toxic dose. However, there were some indications that a compound-specific signature was visible at an earlier time-point, when no histopathological changes were observed (high dose). It is feasible that this was only an adaptive response to stress (and/or related to the hypertrophy seen at earlier times). The large number of genes deregulated and the fact that the pharmacological actions of the compound may have "masked" the more subtle changes related to the underlying hepatotoxicity highlight the difficulty of analyzing such large datasets.

2D-DIGE identified several proteins that were significant and relevant to the compound's proposed mechanisms of toxicity. This is based not only on the number of deregulated proteins but also on their biological effect. Strong effects on carbohydrate metabolism (e.g., glucose metabolism) can be interpreted as a result of the known pharmacological effect of the substance. Several proteins were also involved in fatty acid β-oxidation, which is a known effect of PPARα agonism. The deregulation of proteins that are known to be under regulation of PPARα (e.g., Acot2, AHCY, ASL) further supports the suggestion that EMD 335823 is a PPARα activator. A comparison of deregulated proteins and genes resulted in 10 overlapping items with the same deregulation, with an excellent concordance between them. Even though this is only a small number (based on the limited number of identified proteins) the deregulation of biologically relevant mechanisms, such as detoxification, xenobiotic metabolism or PPARα /RXR activation, further supports the suggested mechanism of toxicity. However, it is clear that 2D-DIGE alone would not have been able to provide enough knowledge about the compound's mechanism of action.

Metabonomics also showed its potential usefulness with the identification of several effects that were not prominent using genomics or proteomics. Multivariate data analysis was able to discriminate treated from pre-dose and control animals in a dose-dependent manner. Metabolic changes included altered excretion of Krebs cycle intermediates, TMAO, and metabolites presumably derived from gut microflora. These changes are frequently observed in response to toxic insults and may represent signs of general toxicity. However, it should be emphasized that this analysis is limited by the software package applied and lacks information on potential changes of minor metabolites. In concordance with genomics and proteomics, it was clear from both urine and serum metabonomic analysis (NMR and LC/MS) that animals 42 and 45 were more severely affected than the others.

Due to the limited numbers of proteins and metabolites identified, these technologies probably would not have identified all the relevant mechanisms if used in isolation. The performance of global gene expression profiling greatly enhanced the understanding and the ability to interpret the other data types. Therefore, we can conclude that the use of toxicogenomics is essential for

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future mechanistic studies. However, the additional "omics" profiling technology data show the added benefit of such a multiple approach, enabling a more detailed analysis when all are used in conjunction.

"Omics" profiling technologies, in addition to traditional toxicology, greatly improved the overall toxicity study for this hepatotoxic compound. It must be noted that a lot of time is required to reach a more molecular understanding of the mechanism, even with the availability of advanced/novel bioinformatics tools. Especially from the gene expression data, there are distinct indications that molecular changes are occurring at earlier time-points, before any visible pathological changes are seen. This shows that there is the potential for shortening existing animal study designs, with the added benefit of reducing animal welfare issues. In addition, using FFPE samples would also allow the possibility to carry out retrospective analyses without the need for new animal studies. Even if these "omics" analyses take some time in themselves, they might have a great benefit for supporting the drug development process in the future.

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Application of DNA Microarray in Studies of Herbal Dietary Supplements*

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1 INTRODUCTION

Since the US Congress passed the Dietary Supplement Health and Education Act (DSHEA) in 1994, the use of herbal dietary supplements has become increasingly popular in the United States. It has been reported that over 70 million Americans used alternative medical therapies in 2002, and that herbal therapies were among the most common, with sales of approximately $5 billion each year in the United States (Tindle *et al*., 2005). Many herbs have been used in constrained regions or countries of the world for many years, and recently have gained global attention. The American Herbal Products Association (AHPS) estimates that there are about 3000 species of plants in as many as 50 000 different products sold as herbal supplements in the United States (Zurer and Hanson, 2004). Despite many human toxicity reports associated with the use of herbal dietary supplements, safety issues concerning potential side effects of herbal products remain to be addressed adequately. The mechanisms of toxicity remain unclear for many herbal products and the data on the identification of toxic ingredients are also largely lacking. Thus, assessment of the safety of herbal dietary supplements is critically important for human health protection (Coppens *et al*., 2006; FDA, 2004; Fu *et al*., 2009; Kaplowitz, 1997; Rietjens *et al*., 2008).

Assessment of safety or efficacy of an herbal product should take into account the quality of the material, because it may not contain the correct plant species as claimed or it may be contaminated with other herbs, microorganisms, pesticides, metals, or even adulterated with unlabeled ingredients, such as synthetic drugs. Thus, quality control is a fundamental procedure in the standardization of medicinal herbs, including herbal dietary supplements. High-quality and contaminant-free raw herbal plants are a pre-requisite for quality assurance and safety of the herbal products, for example, herbal dietary supplements. Authentication of plant species used for preparation of herbal dietary supplements is the first step in achieving safety assurance.

Scientific efforts have been made to identify the risk factors and underlying mechanisms of herbal products-induced toxicity (Clayton *et al*., 2007; Fu *et al*., 2008; Guo *et al*., 2008; Han *et al*., 2004). Given the fact that the majority of herbal products are mixtures of many chemical constituents whereas pharmaceutical drugs normally contain a single pure entity with a well-understood chemical structure, there are challenges and complexities regarding determination of toxic mechanisms and evaluation of risks for such mixtures.

Toxicogenomics, which combines toxicology with genomics or other high-throughput molecular profiling technologies (NAP, 2005), has been

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applied broadly in toxicant or toxin characterization, toxicity prediction, biomarker discovery and identification, toxicant classification, environmental chemical/compound prioritization, and underlying mechanism elucidation (Boverhof and Zacharewski, 2006; Dix *et al*., 2007; Waring *et al*., 2001a, 2001b; Waters and Fostel, 2004). Although this approach has been applied to study drugs or chemicals since the application of DNA chips from the late 1990s (Afshari, Nuwaysir and Barrett, 1999), the use of a toxicogenomic approach for mechanistic study of toxicity associated with herbal dietary supplements has only recently been initiated.

In our laboratories, we have applied DNA microarray technology to study the alterations in gene expression and associated molecular pathways and networks, responsible for herbal dietary supplement-induced liver toxicity (Guo *et al*., 2006, 2009; Mei *et al*., 2007, 2008). We also proposed that through subsequent studies, the hypothesized mechanism of toxicity based upon the DNA microarray results can be further confirmed and validated (Guo *et al*., 2010b). In this chapter, we highlight, and provide examples of, applications of DNA microarrays in product authentication and mechanistic studies related to the use of herbal dietary supplements.

2 APPLICATION OF DNA MICROARRAY FOR SAFETY ASSURANCE OF HERBAL DIETARY SUPPLEMENTS

Quality control is a fundamental procedure in the standardization of herbal plants and herbal products, including herbal dietary supplements for human consumption (Larimore and O'Mathuna, 2003). Herbal products exhibit great variability due to different environmental and cultivation conditions. Herb samples prepared from different parts of a plant (e.g., from leaves, stems, or roots) can result in great differences in their composition. Harvest of the herbal samples from different geographic regions and seasons may also result in products with drastically different constituents, including those with beneficial or toxic effects. Authentication and a well-designed sampling plan are of utmost importance for safety assurance of herbal dietary supplements because this can prevent misuse and careless or intentional adulteration of raw plant materials. A number of methodologies for authentication of herbal plants have been established. These include plant taxonomic identification, morphological and microscopic examination, and fingerprint chromatographic marker characterization. DNA-related techniques, such as DNA molecular marker characterization, have been increasingly used over the past two decades (Joshi *et al*., 2004), and most recently, DNA microarray-based methods have been recognized because of the capability of detecting multiple molecular markers simultaneously and rapidly. The combination of DNA-based methods with conventional taxonomic methodologies is revolutionizing the field of plant authentication.

Morphological and histological approaches are frequently used for authentication, although they are not sufficiently precise if the herbs are intentionally substituted or adulterated by plants with similar morphological shape and tissue constructs. Chemical fingerprint uses the spectroscopic pattern and/or chromatographic profile of constituents as markers for quality assurance of a herbal plant. UV–visible absorption and infrared spectroscopy are commonly used to determine spectroscopic patterns; and HPLC is used to determine chromatographic profiles, although gas chromatography/mass spectrometry, liquid chromatography/mass spectrometry, and liquid chromatography/mass spectrometry/mass spectrometry are also utilized. Recently, chemical fingerprinting, representing a group of constituents with both known and unknown identities in the parent herb, has become increasingly popular and is a widely accepted method for quality control of medicinal herbs (Chen *et al*., 2007). However, the multi-chemical constituents in a herbal plant could interfere with authentication, and deliberate adulteration also hinders authentication.

DNA sequencing based on particular markers has also been applied to plant authentication (Carles *et al*., 2001). This approach requires distinct DNA sequences that are unique to each species of herbal plants. Sequences such as the 5S rDNA spacer and the internal transcribed spacer (ITS) located between 16S and 26S ribosomal DNA (rDNA) vary from species to species and can be used for the purpose of authentication at the species level; sequences such as the nuclear 18S DNA are conserved among species and therefore suitable for discrimination at the plant family level (Chavan, Joshi and Patwardhan, 2006; Wenzig and Rudolf Bauer, 2009; Zhang *et al*., 2007). Although the DNA-sequencing-based approach has been used widely, the low-throughput

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property of the technology, especially when dealing with a large number of samples and multiple markers, has been recognized as a limitation. The rapid development of plant DNA sequence databases makes it possible to develop probes for construction of DNA microarrays for identifying and authenticating herbal plants. Expanded use of DNA microarray technology will dramatically increase the throughput and enhance the accuracy of plant identification for quality control of herbal products.

In order to generate DNA microarrays for such purposes, distinct DNA sequences need to be employed to synthesize a corresponding probe on a gene chip. When the sample is analyzed, the resulting probes have to be able to detect the targeted complementary DNA sequences. Examples of studies using DNA microarray technology in plant authentication are summarized below.

Ding *et al*. (2002) designed a microarray of the ITS1-5.8S-ITS2 regions from 28 *Dendrobium* species, two other orchids, and two non-orchids. Determination was made based on the distinctive hybridization profiles. The data showed that 24 *Dendrobium* species may be differentiated from one another. The four species, *D. officinale* and *D. hercoglossum*, *D. nobile* and *D. moniliforme*, were differentiated by 5S rDNA arrays. Thus, all 28 species could be distinguished by a simple microarray based on either the ITS or the 5S DNA sequence. This study demonstrated that ITS microarrays could be used to establish the identities of the various *Dendrobium* species and to authenticate the medicinal *Dendrobium* from adulterant orchids (Ding *et al*., 2002; Zhang *et al*., 2007).

A silicon-based DNA microarray was developed for the identification of toxic traditional Chinese medicinal plants (Carles *et al*., 2005). Species-specific oligonucleotide probes were derived from the 5S ribosomal RNA gene and also from the leucine tRNA gene of a number of toxic herbs, including *Aconitum carmichaeli*, *A. kusnezoffi*, *Alocasia macrorrhiza*, *Croton tiglium*, *Datura inoxia*, *D. metel*, *D. tatula*, *Dysosma pleiantha*, *Dy. versipellis*, *Euphorbia kansui*, *Hyoscyamus niger*, *Pinellia cordata*, *P. pedatisecta*, *P. ternata*, *Rhododendron molle*, *Strychnos nux-vomica*, *Typhonium divaricatum*, and *T. giganteum*. These probes were immobilized via dithiol linkage on a silicon chip and each chip contained probes corresponding to 20 plant species. The genomic target sequences were amplified and fluorescently labeled by asymmetric polymerase chain reaction (PCR) before hybridization. By this approach, multiple toxic plant species were identified by parallel genotyping. Based on the results, the authors concluded that the DNA chip-based authentication of herbal plants may be useful and feasible for screening toxic plant species (Carles *et al*., 2005).

A DNA microarray was developed specifically for the identification of various *Panax* plants and drugs (PNX array) (Zhu, Fushimi and Komatsu, 2008), as a method for the authentication of *Panax* plants and drugs as well as their derived health foods. A PNX array was developed on the basis of specific polymorphic nucleotides of the nuclear 18S rRNA gene of 13 *Panax* taxa determined by DNA sequencing. Thirty-five specific oligonucleotides were designed, synthesized, and printed as probes on a glass slide, which contained 33 probes corresponding to the species-specific nucleotide substitutions and two probes as positive and negative controls. The species-specific probes were 23–26 nucleotides in length and the species-specific substitution nucleotide was located at the central part. Partial 18S rRNA gene sequences amplified from *Panax* plants and drugs, as well as their derived health food products, were fluorescently labeled as targets to hybridize to the PNX array. After hybridization, distinct fluorescent patterns were generated and the specific patterns were used to identify *Panax* species. Based on the results, this newly developed PNX array offered a rapid and reliable method for the authentication of *Panax* plants and drugs as well as their derived health foods.

3 APPLICATION OF DNA MICROARRAY IN MECHANISTIC STUDIES OF TOXICITY INDUCED BY HERBAL DIETARY SUPPLEMENTS

3.1 Complexity of Toxicity Induced by Herbal Dietary Supplements

There are a wide variety of complex toxicities associated with the use of herbal dietary supplements. First, adverse reactions may be caused by plant material contamination, adulteration, or misuse. One example of misusing plant material in dietary supplements occurred in the early 1990s and involved a rapidly progressive interstitial nephropathy in Belgian patients who used Chinese herbs as

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part of a weight-loss program (Cosyns *et al*., 1994; Vanherweghem *et al*., 1993). It was later discovered that *Stephania tetrandra* (*fangji* in Chinese), one of the herbs in the weight-loss formula, had been inadvertently replaced by *Aristolochia fangchi*, perhaps as a result of confusion between "*fangji*" and "*fangchi*" (Cosyns, 2003; Vanherweghem *et al*., 1993). The latter (*fangchi*) contains aristolochic acids that were found to induce DNA adducts, mutations, and tumors in humans and animals (Arlt, Stiborova and Schmeiser, 2002; Chen *et al*., 2006; Cosyns, 2003; Mei *et al*., 2006).

The second type of herbal dietary supplements-related toxicity can be considered as intrinsic to the product, that is, toxic effects are from the plant itself. The plant may contain toxic constituents, or the metabolites of some constituents are toxic. For example, *Senecio* species are used in herbal medicines and as food worldwide. Riddelliine is an abundant component in *Senecio*. Toxicity and carcinogenicity studies have shown that riddelliine is metabolized to two major metabolites, 6,7-dihydro-1-hydroxymethyl-5*H*-pyrrolizine (DHP) and riddelliine *N*-oxide (Yang *et al*., 2001). Further studies showed that DHP-derived DNA adducts were formed both *in vivo* and *in vitro* after exposure to riddelliine (Chou *et al*., 2003a, 2003b); G:C → T:A transversions were the major type of mutation induced in the liver of riddelliine-treated rats (Mei *et al*., 2004a, 2004b); and riddelliine caused liver tumors in male mice and both sexes of rats, mononuclear cell leukemia in rats, and lung neoplasms in female mice (Chan *et al*., 2003).

The third type of herbal dietary supplements-related toxicity relates to its idiosyncratic nature. The toxicity occurs with low frequency and it is normally associated with patients who show hypersensitivity reactions or some specific populations with genetic make-ups that make them more prone to the toxic effects (Kaplowitz, 2005). Lastly, herb–drug interactions are generally accepted to be a major cause for some herbal dietary supplements-associated toxicity. It has been shown that herbal dietary supplements can induce or inhibit the activities of drug-metabolizing enzymes, thus affecting both the pharmacokinetics and pharmacodynamics of co-administered drugs (Driver, Baxter and Williamson, 2009; Kaplowitz, 1997). This may result in decreased pharmacological effects or increased toxic effects of the co-administered drugs (Zhou *et al*., 2003). A well-studied example of an herbal dietary supplement causing herb–drug interaction is St. John's wort, which is widely used to treat depression, anxiety, and insomnia. Numerous clinical reports have shown that St. John's wort interacted with conventional drugs such as carbamazepine, benzodiazepines, cyclosporine, chlorzoxazone, and many others (Driver, Baxter and Williamson, 2009; Wenzig and Rudolf Bauer, 2009). Through extensive studies, it was found that St. John's wort induced the activity of cytochrome P450 3A4 (CYP3A4), the drug-metabolizing enzyme involved in metabolizing the majority of drugs. In addition, CYP2C19, CYP2C8, and CYP2E1 were also induced by St. John's wort. Comprehensive induction and inhibition studies of drug-metabolizing enzymes can provide the molecular basis for elucidating the mechanisms of interaction between herbal dietary supplements and drugs, and also the needed information to make informed safety decisions on the use of dietary herbal supplements. Applying microarray technology may be an effective initial approach for obtaining the whole spectrum of drug-metabolizing gene alterations caused by dietary herbal supplements (see detailed information in Guo *et al*., 2010b).

3.2 Pathway Analyses after Treatments with Herbal Dietary Supplements

The process of determining how genes and proteins interact and influence biological pathways, networks, and cellular physiology is facilitated by the field of genomics, which allows the simultaneous molecular analysis of thousands of genes. In many toxicogenomic studies, gene expression profiling using DNA microarrays has been identified as a key component to addressing biological and toxicological questions. Once altered genes are identified, the perturbed pathways and networks can be generated using pathway analysis software and database information. For instance, Ingenuity Pathway Analysis (IPA) software (www.ingenuity.com) may be used to determine the most relevant biological pathways of the affected genes. This analysis identifies the pathways from the IPA library of canonical pathways that are most significantly altered in the differentially expressed gene data set. The significance of the association between the data set and the canonical pathway is measured in two ways: (i) a ratio of the number of genes from the data set

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that map to the pathway divided by the total number of genes that map to the pathway and (ii) Fischer's exact test, in which a *P*-value is calculated to determine the probability that the association between the genes in the data set and the canonical pathway is explained by chance alone.

Kava-containing products remain popular and their consumption is dramatically increasing, despite the fact that kava has been banned in several Western countries following reports of hepatotoxicity (CDC, 2002). *Ginkgo biloba* is one of top five sold products in health food stores in the United States with a total sale exceeding $100 million (Chan, Xia and Fu, 2007), even though some ingredients identified in *Ginkgo biloba* are known mutagens (NTP, 1998). Using microarray technology and the IPA database, we investigated the gene relationships, functional clustering, and mechanisms involved in kava and *Ginkgo biloba* exposure in animal models (Guo *et al*., 2009, 2010a, 2010b), as summarized below.

F344 male rats or B6C3F1 male mice were gavaged with kava extract at the doses of 0.125–2 g kg^{-1} body weight/day for 14 weeks. B6C3F1 male mice were gavaged with *Ginkgo biloba* extract at the doses of 0.2–2 g kg^{-1} day^{-1} for 104 weeks. Pathway analyses were performed based on the differentially expressed genes observed in the highest dose group of three studies using DNA microarray (Guo *et al*., 2009, 2010a, 2010b). Table 1 lists the canonical pathways altered by these three treatments, with a cut-off of $P < 0.01$. After 14 weeks of a diet containing 2 g kg^{-1} kava, 42 and 31 molecular pathways were changed in the livers of rats and mice, respectively (Table 1). The finding of similar pathways alterations in mice and rats after the same chemical treatment may suggest similar effects and toxicities. For example, kava altered 17 canonical pathways in rat and mouse, including xenobiotic metabolism signaling, metabolism of xenobiotics by CYP, glutathione metabolism, nuclear factor erythroid 2 (NRF2)-mediated oxidative stress response, and lipopolysaccharide (LPS)/Interleukin (IL)-1-mediated inhibition of retinoid X receptor (RXR) function (Table 1).

A living organism has a series of pathways and enzymatic activities, including xenobiotic metabolism signaling, metabolism of xenobiotics by cytochrome P450, and glutathione metabolism, which are designed to detoxify and eliminate a xenobiotic (a foreign chemical compound such as a drug or toxin). These processes can be divided into three main phases: modification, conjugation, and excretion. The phase I enzymes, such as the CYPs, act to modify the xenobiotic compounds by adding polar groups through oxidation or reduction. Sometimes, the process of metabolism gives rise to metabolites that are more toxic than the original compound. A well-known example is the procarcinogen, benzo[*a*]pyrene. Its carcinogenetic effect depends on enzymatic metabolism of benzo[*a*]pyrene to the ultimate mutagen, benzo[*a*]pyrene diol epoxide. Benzo[*a*]pyrene is first oxidized by CYP 1A1 to form (+)benzo[*a*]pyrene-7,8-epoxide, and it is further metabolized by epoxide hydrolase to form (−)benzo[*a*]pyrene-7,8-dihydrodiol. The ultimate carcinogen, (+)benzo[*a*]pyrene-7,8-dihydrodiol-9,10 epoxide, is formed after another reaction with CYP1A1 (Pfeifer *et al*., 2002; Shou, Gonzalez and Gelboin, 1996). In phase II, the electrophilic and nucleophilic intermediates are conjugated by transferases, such as the glutathione *S*-transferase family, with charged species such as sulfate, glutathione, or glucuronic acid. These reactions result in bulkier and more polar metabolites that may then be actively removed from the cell by phase III transporters, such as members of the multidrug resistance protein transporter family.

In kava-treated rats, 72 drug-metabolizing genes were significantly changed by 2 g kg^{-1} kava treatment. Among them, 19 genes were associated with phase I metabolizing enzymes, 21 genes with phase II enzymes, and 32 genes with phase III transporters (Guo *et al*., 2009). In addition, 16 genes were commonly regulated by the three highest doses of kava treatments (0.5, 1.0, and 2.0 g kg^{-1}), including dose-dependent increases for Cyp1a1, 1a2, 2c6, 3a1, and 3a3. In this study using kava-treated mice, we found 95 drug-metabolizing-enzyme-associated genes significantly altered, including 28 phase I metabolizing enzymes genes, 29 phase II genes, and 38 transporters (Guo *et al*., 2010c). Based on the results of microarray and Taqman assays, genes whose expression was significantly altered included Gstal, Gsta2, Cyp1a1, Cyp1a2, Cyp2a5, Cyp2b20, Cyp2c55, and Cyp3a11. Cyp1a1 is primarily expressed in extra-hepatic tissues, with low amounts being expressed in the liver. The Cyp1a1 isozyme can metabolize a number of xenobiotics, including those with flat and planar structures, which include the highly toxic and tumorigenic polycyclic aromatic hydrocarbons (PAH) (Conney,

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Table 1. Canonical pathways altered significantly ($P < 0.01$) by kava and *Ginkgo biloba* in mice or rats.

	P-value		
Canonical pathway	kava-rat[a]	kava-mouse[b]	Ginkgo-mouse[c]
Acute phase response signaling	1.41E-08	1.62E-05	
Alanine and aspartate metabolism	5.01E-05		
Aminosugar metabolism	7.08E-04		1.38E-03
Androgen and estrogen metabolism	7.59E-07		
Antigen presentation pathway	1.62E-07		
Arachidonic acid metabolism	4.57E-07	1.95E-04	2.57E-07
Aryl hydrocarbon receptor signaling	1.00E-10	5.13E-07	9.77E-06
B-cell receptor signaling	5.25E-03		
Bile acid biosynthesis		6.76E-03	
Biosynthesis of steroids	5.13E-04		
Butanoate metabolism		1.02E-03	
C21-Steroid hormone metabolism	7.08E-03		
Circadian rhythm signaling	2.51E-05		
Citrate cycle		1.70E-04	
Complement system	1.15E-09		
D-Glutamine and D-glutamate metabolism	5.75E-03		
Fatty acid elongation in mitochondria		1.82E-03	
Fatty acid metabolism	7.94E-03	1.41E-06	4.57E-09
FGF signaling	2.19E-03		
Fructose and mannose metabolism	6.31E-06		
Galactose metabolism	3.16E-05		
Glutamate metabolism	1.38E-03		
Glutathione metabolism	2.82E-10	3.63E-09	4.57E-04
Glycerophospholipid metabolism	3.63E-03		
Glycine, serine, and threonine metabolism		3.24E-03	
Glycolysis/gluconeogenesis	1.00E-04	6.17E-03	
Hepatic cholestasis	2.14E-04		
Hepatic fibrosis/hepatic stellate cell activation	5.75E-04		
Histidine metabolism		8.71E-03	
Hypoxia signaling in the cardiovascular system	1.02E-03		
Inositol metabolism	4.27E-03	9.77E-04	
Interferon signaling	6.46E-04		
Linoleic acid metabolism	1.58E-05	1.17E-03	3.09E-06
LPS/IL-1-mediated inhibition of RXR function	1.58E-13	3.39E-09	2.04E-07
LXR/RXR activation	9.77E-05		
Lysine degradation		8.13E-04	
Metabolism of xenobiotics by cytochrome P450	3.16E-14	6.31E-15	1.26E-11
Methane metabolism		6.03E-03	
Mitochondrial dysfunction	1.00E-05		
Nicotinate and nicotinamide metabolism	8.71E-04		
Nitrogen metabolism	3.02E-08		
NRF2-mediated oxidative stress response	2.51E-17	2.75E-07	5.37E-03
One-carbon pool by folate		5.01E-03	
Pentose and glucuronate interconversions	2.19E-06		
Pentose phosphate pathway	6.31E-03	1.78E-04	
Phospholipid degradation	3.47E-04		
Propanoate metabolism		5.50E-03	
Protein ubiquitination pathway	3.98E-13	3.98E-04	
PXR/RXR activation		7.76E-05	1.74E-03
Pyruvate metabolism	4.17E-03	6.17E-04	
Retinol metabolism	1.51E-05	9.33E-05	
Starch and sucrose metabolism	2.19E-08		
Tryptophan metabolism	3.09E-03	7.41E-07	1.74E-07
Valine, leucine, and isoleucine degradation		1.51E-04	
Xenobiotic metabolism signaling	1.26E-21	1.58E-13	1.91E-04
β-alanine metabolism		4.90E-04	

[a] F344 rats were treated with kava 2 g kg^{-1} day^{-1} by gavage for 14 weeks.
[b] B6C3F1 mice were treated with kava 2 g kg^{-1} day^{-1} by gavage for 14 weeks, and the data were from Guo *et al*. (2010c).
[c] B6C3F1 mice were treated with *Ginkgo biloba* 2 g kg^{-1} day^{-1} by gavage for 104 weeks, and the data were from Guo *et al*. (2010a).

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2003; Shou *et al*., 1996; Martignoni, Groothuis and de Kanter, 2006). Therefore, induction of the Cyp1a1 gene by kava treatment in mice and rats would enhance metabolic activation of the hazardous environmental PAHs and jeopardize human health. Our results demonstrated that many drug-metabolizing genes were altered in response to kava treatment, which is consistent with the report that kava-induced herb–drug interaction through modulation of metabolizing enzymes is a highly possible cause of hepatotoxicity (Fu *et al*., 2008).

Chemical compounds may induce oxidative stress due to an imbalance between the production of reactive oxygen and the detoxification of reactive intermediates. The NRF2-mediated oxidative stress-response pathway plays a critical role in protecting cells from endogenous and exogenous stresses, and is involved in antioxidative response, detoxification of xenobiotics, and proteome maintenance. Under normal physiological conditions, the transcription factor NRF2 is localized in the cytoplasm and interacts with Keap1. Upon oxidative stress, NRF2 is released from Keap1, translocated to the nucleus, and subsequently activates its various downstream target genes (Kensler, Wakabayashi and Biswal, 2007). The target genes show a wide spectrum of functions including inactivating oxidants, increasing the levels of glutathione, and enhancing toxin export via transporters to improve cell survival. As expected, NRF2 knock-out mice are more susceptible to acetaminophen-induced hepatocellular injury (Chan, Han and Kan, 2001) and benzo[a]pyrene-induced tumor formation (Ramos-Gomez *et al*., 2001). This susceptibility is partly due to a reduced level in the expression of detoxification enzymes (Aleksunes and Manautou, 2007; Kensler, Wakabayashi and Biswal, 2007). Activation of detoxification enzymes plays a pivotal role in protecting cells from oxidative insult when cells encounter toxin challenge.

Chronic *Ginkgo biloba* treatment in mice altered 12 canonical pathways (Table 1), including xenobiotic metabolism signaling, metabolism of xenobiotics by cytochrome P450, glutathione metabolism, NRF2-mediated oxidative stress response, and LPS/IL-1 mediated inhibition of RXR function. For example, the NRF2-mediated oxidative stress response pathway was identified with 23 genes involved. Most of these genes were up-regulated whereas two genes were down-regulated (Guo *et al*., 2010). In response to *Ginkgo biloba* treatment, NRF2-regulated genes, including various Gst genes that are involved in GSH-conjugate formation, were altered. The Gst genes (Gsta2, Gsta4, Gstms, and Gstp1) and Ugt1a2 were up-regulated following *Ginkgo biloba* treatment. In turn, the resulting enhancement of GST enzymes is used for neutralizing the electrophiles and the process generally is considered an important detoxification mechanism. The NADPH-dependent enzyme NQO1 was also up-regulated, which has a cell protective role by detoxification of xenobiotic carbonyls and quinones. *Ginkgo biloba* treatment also elevated Gsr (glutathione reductase) gene expression, and GSR regulates cellular GSH homeostasis by catalyzing the reduction of GSSG to GSH using NADPH as a reducing cofactor (Harvey *et al*., 2009). *Ginkgo biloba* treatment reduced the activity of Sod3, which is one of the superoxide dismutases (SODs) that are the most important line of antioxidant enzyme defense systems against reactive oxygen species (ROS), and particularly superoxide anion radicals (Zelko, Mariani and Folz, 2002).

3.3 Common Mechanisms for Classes of Components in Herbal Dietary Supplements

Using the pathway analysis knowledge databases, the differential expressed genes that are identified in one or more studies may be used to elucidate molecular mechanisms, providing an improved understanding of toxicity and identifying common mechanisms that could define classes of chemicals. For example, we compared the expression of genes and biological functions in rat liver after treatment with carcinogens (comfrey and riddelliine). Comfrey (*Symphytum officinale*) is a perennial plant and has been consumed by humans as a vegetable, a tea, and a herbal medicine for more than 2000 years. It has also been demonstrated that comfrey is hepatotoxic and carcinogenic in experimental animals and hepatotoxic in humans, due to a mixture of pyrrolizidine alkaloids (PAs). PAs exist in many plants and many of them cause liver toxicity and/or cancer in humans and experimental animals. Riddelliine is one of the tumorigenic PAs and has been studied as a prototype PA. Riddelliine is a major component of *Senecio*, which has been used in herbal medicines or food. The toxicity

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and carcinogenicity of riddelliine have been studied by the NTP (Chan *et al*., 1994, 2003; NTP, 2003), and the mechanism of riddelliine-induced tumorigenicity in experimental animals has been studied at the National Center for Toxicological Research (NCTR/FDA) (Chou *et al*., 2003a, 2003b, 2003c; Mei *et al*., 2004a, 2004b; Yang *et al*., 2001a, 2001b). To examine the possible PA-induced effects on gene expression caused by comfrey, the gene expression profiles in the livers of comfrey-treated rats were compared with the gene expression profiles from rats treated with the purified PA riddelliine. The correlation of the gene expression and biological functions related to carcinogenesis between the two treatments was explored.

Groups of six Big Blue Fisher 344 rats were fed a diet containing 8% comfrey root for 12 weeks or gavaged with riddelliine at 1 mg kg^{-1} body weight five times a week for 12 weeks. Gene expression was investigated using Applied Biosystems Rat Whole Genome Survey Microarrays (Applied Biosystems, Foster City, CA, USA), and the biological functions were analyzed with Ingenuity Pathway Analysis software (IPA, Redwood City, CA, USA). Although there were large differences between the significant genes and biological processes that were altered by comfrey and riddelliine, there were a number of common genes and functional processes that were related to carcinogenesis. Particularly, there were strong correlations between the two treatments for fold-change alterations in expression of drug-metabolizing and cancer-related genes (Guo *et al*., 2007).

Biological processes were examined for the genes altered due to comfrey and riddelliine exposures individually, and the software placed the biological processes into functional processes or categories such as cancer and cell death. After a cut-off with a *P*-value less than 0.01, we observed that there were 83 and 118 functional processes for riddelliine and comfrey treatments, respectively. Among them, 46 of the functional processes were altered by both comfrey and riddelliine, which was about half of the functional processes altered by riddelliine and one-third of the functional processes altered by comfrey. The top categories for these functions included cancer, cell death, cell morphology, cell-to-cell signaling and interaction, and tissue development. These results suggest that common mechanisms may be responsible for the toxicity of both comfrey and riddelliine.

The alteration of cancer- and cell-death-associated genes by both comfrey and riddelliine treatments is likely due to the PAs. Microarray analysis showed that comfrey altered many more genes involved in cancer-related pathways than riddelliine. The expression of 387 cancer-related genes was significantly altered after comfrey exposure, and the expression of 84 cancer-related genes was significantly changed by riddelliine treatment. Among them, there were 42 genes whose expressions were significantly altered by both riddelliine and comfrey treatments (Guo *et al*., 2007), which may indicate the PAs' carcinogenic insults. Figure 1 shows a good correlation between the $\log_2$ fold changes in gene expression caused by the two treatments; only two of the genes were regulated in the opposite directions. The correlation coefficient (R^2) was 0.65, indicating good agreement between the two treatments. These common cancer-related genes include genes involved in apoptosis and cell death, invasion, cell growth, cell morphology, and cell cycle.

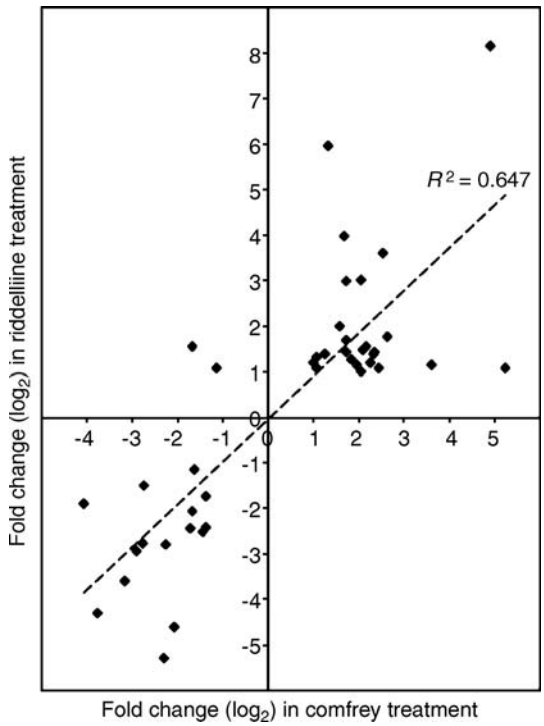

Figure 1. Comparison between $\log_2$ fold changes of cancer-related genes altered by comfrey and riddelliine treatments. The rats were treated with 8% comfrey roots in diet and 1 mg kg^{-1} day^{-1} riddelliine by gavage for 3 months, and the cancer-related genes were commonly regulated by both treatments.

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These results also suggest that the carcinogenicity of comfrey is due to PAs.

Most of genes involved in apoptosis (Cdkn1c, Egr1, Fas, Gdf15, Hgf, Hrasls3, Hspa1a, Lama5, Smox, Tnfsf10) were up-regulated and may be explained by the removal of cells damaged by PAs. For example, early growth response 1 (Egr1), whose expression increased 12- and twofold over the control with comfrey and riddelliine treatments, respectively, has the ability to function in numerous capacities, including differentiation, growth, growth inhibition, and apoptosis depending on the cell type and the stimulus. We also observed that comfrey and riddelliine significantly up-regulated Hspa1a, the gene encoding heat shock protein (HSP) 70 family members. High levels of inducible HSP70s prevent stress-induced apoptosis and block caspase activity, mitochondrial damage, and nuclear fragmentation (Mosser *et al*., 1997). These genes may all be involved in repair and protection of the cell against the damage caused by the PAs.

As PAs require metabolic activation to exert their biological and toxicological effects, study and comparison of these genes altered by comfrey and riddelliine can provide valuable information about common mechanisms on PAs' biotransformation and metabolism. The results showed that there were 45 and 87 drug-metabolizing genes up- or down-regulated by the treatment with riddelliine and comfrey, when a cut-off of twofold change and $P < 0.01$ was used as the criteria to select genes (Guo *et al*., 2007). There were more drug-metabolizing genes altered by comfrey treatment than those by riddelliine treatment, probably because comfrey is a mixture of many substances in addition to PAs, and these other substances may be involved in the induction of other drug-metabolizing genes. About 50% of drug-metabolizing genes altered by the treatment with riddelliine were also found altered by the treatment with comfrey. It should be noted that the expressions of 22 of these genes were altered by both comfrey and riddelliine, with the changes in the same direction and a correlation coefficient of 0.72 (Guo *et al*., 2007). Although the process of drug metabolism is complicated, these results suggest that there is a common mechanism of drug metabolism involved in these two chemical treatments.

Comparative studies also showed that comfrey and riddelliine induced similar profiles of DHP-derived DNA adducts (Chou and Fu, 2006) and similar mutation spectrum profiles (Mei *et al*., 2006) in the liver. Gene expression analysis indicated strong correlations between the gene expression fold-change alterations in drug-metabolizing genes and cancer-related genes caused by both agents (Guo *et al*., 2007). Cumulatively, these results suggest that comfrey induces liver tumors by a genotoxic mechanism, that comfrey and riddelliine may share common mechanisms of drug metabolism and carcinogenesis, and that PAs contained in comfrey are the main active components responsible for genotoxicity and tumor induction in rat liver.

4 CONCLUSIONS

With the increased usage of herbal dietary supplements comes an increased demand for product standardization and authentication, as well as a better understanding of the molecular mechanisms underlying their functional and toxicological activities. It is critically important to establish reliable and fast methodologies for safety assurance of herbal dietary supplements. This review provides an overview of methods and proposals for studying mechanisms of toxicities induced by herbal dietary supplements.

To date, most of proposed mechanisms for herbal dietary supplements-induced toxicity focused on herb–drug interaction mediated by drug-metabolizing enzymes. Because of the high degree of correlation between gene expression and enzyme activity for phase I enzymes (Iyer and Sinz, 1999; Roymans *et al*., 2004), the simple and rapid measurement of gene expression instead of the complex and time-consuming measurement of enzyme activity is an attractive alternative for exploring the changes of phase I enzymes induced by herbal dietary supplements. DNA microarray technology is a powerful tool to monitor simultaneously relative expression levels of thousands of genes, including the induction or inhibition of drug-metabolizing enzyme genes after treatment with an herbal dietary supplement or its raw herbal plant. This rapid approach can provide valuable information about the changes in expression of the biologically potent phase I enzymes that can be useful in predicting potential herb–drug interactions. Moreover, microarray technology is also a powerful methodology for exploring toxicology mechanisms induced by herbal dietary supplements because of the comprehensive definition of gene

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expression changes and the ability to generate testable hypotheses based on these data.

DNA microarray technology is an effective strategy for global gene expression profiling. However, understanding the functional relevance of gene expression profiles is a challenge and follow-up experiments are normally required. Although DNA microarrays offer a promising avenue for the study of herbal dietary supplement toxicity, it is anticipated that subsequent functional studies will be necessary to validate possible mechanisms. After obtaining initial information at the gene level, conventional methodologies, such as determining protein expression by immunoblotting and determining enzyme activity by quantitative metabolism, can be followed, and well-designed experiments that pinpoint particular pathways or mechanisms can be performed to investigate the molecular basis of mode of actions.

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Unravelling Sex Differences in Drug-Induced Liver Injury*

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1 INTRODUCTION

Hepatotoxicity results in a significant number of drug candidates failing during the clinical stage of development and, even more harmful to the patients and drug manufacturer, to be withdrawn from the market post approval. With the increasing use of combinations of prescription and over-the-counter drugs, herbal remedies, dietary supplements and alcohol, the potential for adverse events is increasing dramatically. Consequently, drug-induced liver injury (DILI) has been identified by the FDA Critical Path Initiatives as a key area of focus in which better evaluation tools and safety biomarkers are needed (http://www.fda.gov/ScienceResearch/SpecialTopics / CriticalPathInitiative / CriticalPathOpportunitiesReports/ucm077248.htm#topic1). To help meet this goal, one effort underway by members of the Systems Biology Division at the National Center for Toxicological Research (FDA) is the development of a liver toxicity knowledge base (LTKB) to provide a content-specific resource for the regulatory and research communities (http://www.fda.gov/AboutFDA/CentersOffices/nctr/WhatWeDo/ResearchDivisions/ucm079059.htm).

In 1993, FDA published a guideline for the study and evaluation of sex differences in drug development (http://www.hhs.gov/ohrp/humansubjects/guidance/58fr39406.htm). [although "gender differences" are used more widely than "sex differences," the latter is more accurate as it cannot be influenced by cultural factors (Howard *et al.*, 2001). Thus, the term "sex difference" is used throughout this review.) Sex difference is observed in the prevalence of various liver diseases. For instance, autoimmune hepatitis (Beland, Lapierre and Alvarez, 2009) and alcoholic liver disease (ALD) (Van Thiel *et al.*, 1989) are much more common in females than in males. Women tend to develop more severe ALD with less consumption than men, a scenario that can be recapitulated by animal experimentation. However, cirrhosis is more prevalent among men and post-menopausal women in general. In patients with chronic hepatitis C, faster progression to fibrosis

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is seen in men compared with women, regardless of age (Poynard *et al.*, 2001). Furthermore, non-alcoholic fatty liver disease (NAFLD), the most common aetiology of chronic liver diseases in the United States, is less prevalent among women during their reproductive years, although following menopause, the prevalence of NAFLD in women increases and exceeds that of men (Suzuki and Abdelmalek, 2009).

Given the sex differences observed in patients with various liver diseases, it seems pertinent to examine responses in animal studies to begin to understand sex-based susceptibilities to drugs and toxicants. Toxicology studies with oxamniquine in several laboratory animal species revealed a sex-biased hepatotoxicity with females being more sensitive than males (Gregory *et al.*, 1983). A National Toxicology Program (NTP) study conducted at the National Center for Toxicological Research demonstrated that fumonisin B_1 induced liver tumours in female but not in male mice (Howard *et al.*, 2001). Dehydroepiandrosterone (DHEA) is a naturally occurring steroid secreted from the human adrenal cortex and has been used as a drug and, by some, as a dietary supplement aimed at preventing age-related changes. Studies in Sprague–Dawley rats have shown that females are more susceptible than males to the development of hepatocellular tumours after exposure to DHEA. However, the reverse was seen when the same drug was tested in F-344 rats (Mayer and Forstner, 2004). Studies with acetaminophen (APAP) in rats have demonstrated both age- and sex-related differences, with males and old age being susceptibility factors (Tarloff *et al.*, 1996). In clinical and epidemiological studies, however, APAP-related DILI was seen more often in women than in men (Russo *et al.*, 2004; Ostapowicz *et al.*, 2002).

These studies demonstrate the complexity in trying to unravel apparent differences in susceptibility because differences in age, sex, reproductive status, genetics and drug under investigation, as well as cross-species extrapolation, may confound interpretation. Furthermore, different mechanisms (e.g. drug metabolism, generation of toxic metabolites, immune/inflammatory reactions and repair process) may be involved in different stages of DILI. Therefore, sex or other factors may differentially impact the disease course of DILI. Most importantly, care must be taken when evaluating results from human studies regarding sex-based susceptibilities to drugs and other agents. Rarely are the authors able to obtain information related to the number of females and males taking a particular drug or exposed to an environmental agent. Thus, although it may be true that the 'average' patient experiencing DILI is female, it is not clear whether that is because, for example, of a higher percentage of women versus men being exposed to the agent.

Recently, a great deal of effort has been placed in the field of -omics technologies, particularly genomics, with the hope of discovering novel insights into the mechanisms of various diseases and toxicity. The genomic approach has become an active field in drug development and research to determine inter-individual variability in drug response including study of sex-biased phenomena in disease and medicine. For example, a number of ALD studies have employed gene expression microarray technologies (Sharma *et al.*, 2008; Tadic *et al.*, 2002). These high-throughput genomic studies not only confirm some findings of the previous studies based on conventional approaches but further our knowledge for sex-driven liver injury and toxicity at molecular and functional levels.

The processes involved in DILI are complex. Sex-based differences (e.g. hormones, body weight, fat, organ blood flow, diet, environment, etc.) apparently are important sources of individual variability. However, the systematic evaluation of sex-biased phenomena, particularly related to increased female sensitivity, has received limited attention. In this review, we analysed the published studies to understand the basis for the often stated claim that women are more susceptible to DILI than men and to identify any factors important to sex-biased DILI. We then discuss the findings for sex differences related to disease and drug response at the molecular level with emphasis on the use of genomic approaches such as gene expression microarrays.

2 SEX DIFFERENCE IN DRUG-INDUCED LIVER INJURY

A number of clinical studies have demonstrated increased female sensitivity to DILI for some drugs in several studied populations, but contrary findings have also been observed. One major difficulty with assessing sex-specific sensitivity in the human population is the often unknown or, at best, imprecise exposure information, which is required for

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assessing true sex-specific differences of DILI. Additional complication in analysis is that clinical diagnosis of DILI is challenging, sometimes inconclusive and difficult to confirm (Wilke *et al.*, 2007). This may improve with the establishment of an international consortium to standardize the diagnosis in a clinical setting (Fontana *et al.*, 2009). In this analysis, we reviewed studies which applied standardized causality assessment tools, although their case identification strategies and applied causality assessment tools are different. We first analysed five large population studies (Table 1) to gain a general picture on sex-biased DILI. Next, we focused our analysis on specific factors (e.g. drugs, acute liver failure and age) that could contribute sex-biased phenomena significantly.

Table 1 summarizes five clinical and epidemiological studies conducted in four different countries. There was no clear evidence for sex-biased DILI for the cases submitted to the Spanish Hepatotoxicity Registry between 1994 and 2004 (Table 1, Study 1) (Andrade *et al.*, 2005) and cases from an out-patient hepatology clinic in a Swedish University hospital (Table 1, Study 4) (De Valle *et al.*, 2006). In contrast, female dominance in DILI population was observed for the patients from the Mayo Clinic Hospital in the United States (Table 1, Study 5) (Carey *et al.*, 2008) and in some age groups (Table 1, Studies 2 and 3).

Drug could be a potential factor for sex-biased DILI. It has been reported that female sex is more prevalent among patients with APAP-induced acute liver failure (Russo *et al.*, 2004; Ostapowicz *et al.*, 2002). As shown in Table 2, female dominance in DILI has been observed for other drugs. For example, clinical trials of tolcapone, a catechol-*O*-methyl transferase inhibitor for the adjunctive treatment of Parkinson's disease, demonstrated the elevation in alanine aminotransferase (ALT) or aspartate aminotransferase (AST) in up to 3.7% of patients and female patients more frequently suffered this adverse event than male patients by a ratio of 5 : 2 (Olanow and The Tasmar Advisory Panel, 2000; Olanow and Watkins, 2007). Flucloxacillin is widely used in many European countries and Australia for treatment of staphylococcal infection. Its use has been associated with a characteristic cholestatic hepatitis. A couple of previous studies reported female dominance among patients with flucloxacillin-induced DILI, although none of them show statistical significance (Daly *et al.*, 2009). Halothane caused severe cases of DILI in thrice as many females as males (Bjornsson *et al.*, 2005). However, it is also evident that not all the drugs exhibit a female-dominant DILI. For example, disulfiram was found to cause severe DILI in three men with no women reporting this toxicity (Bjornsson *et al.*, 2005). As mentioned previously, a major complicating factor in assessing sex-specific DILI in many reports is that they are not generated from controlled clinical studies with known gender participation rates.

There is some evidence of sex bias in terms of reported liver failure cases (Table 3). More cases of APAP-induced acute liver failure in women than men have been noted in some studies (Larson *et al.*, 2005; Ostapowicz *et al.*, 2002). Of patients who survive DILI, women seem to be more prone to develop autoimmune liver disease (Bjornsson and Davidsdottir, 2009). The FDA Adverse Event Reporting System (AERS, 2009) database was queried for cases of liver failure associated with drug use for the time period April 2008 through March 2009. During this time period, the number of females versus males experiencing liver failure associated with drug use was 948 versus 916, respectively. Of these, 103 female and 37 male cases were associated with APAP exposure. Therefore, it would appear that female sex is predominant among patients with APAP-induced acute liver failure, which parallels previous studies (Ostapowicz *et al.*, 2002). Limitations with the AERS data include the fact that adverse events are typically under-reported; some cases had to be omitted because the sex was not identified, many cases involve exposure to multiple drugs and the incidence of adverse events in the US population cannot be calculated. However, they do provide some insight into the sex distribution of hepatic failures for those cases that are reported.

It is generally acknowledged that DILI occurs more often in older aged than younger patients. However, the sex dependency related to age is less clear. For examples, in Study 2 (Table 1) (Lucena *et al.*, 2009), there were more females than males in the 40–59 age group but not after the age of 60. This finding seems contrary to that reported in Study 3 (Sgro *et al.*, 2002) that shows a female dominance in the age group >50 years old. As shown in Table 3, both studies conducted in the United States with APAP as a main cause (Russo *et al.*, 2004; Ostapowicz *et al.*, 2002) of DILI show more women at a younger age (around 30 years old). The study conducted in Spain with amoxicillin clavulanate

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Table 1. Summary of findings for sex-difference in drug-induced liver injury in human population.

Population	Inclusion criteria and causality assessment	Patterns of DILI	Drugs	Main findings
Study 1 • A Spanish population 1 [Andrade *et al.*, 2005) • No. cases = 461 (M/F = 244/217) • Age range = 13–88 years • Submitted to the Spanish Hepatotoxicity Registry between 1994 and 2004	• Inclusion: International Consensus meeting • Causality: Council for International Organizations of Medical Sciences (CIOMS) scale	Injury is characterized using the R = ALT/ALP ratio • Hepatocellular ($R > 5$) • Cholestatic ($R < 2$) • Mixed injury ($2 < R < 5$)	• 505 drugs involved • Anti-infective group of drugs involved most DILIs • Amoxicillin clavulanate accounted for 12.8% cases	• No clear evidence for sex-biased DILI across all the age groups • Female has a higher percentage in hepatocellular injury and fulminant hepatic failure
Study 2 • A Spanish population 2 (Lucena *et al.*, 2009) • No. cases = 603 (M/F = 310/293) • Age range = 13–88 years • Submitted to the Spanish Hepatotoxicity Registry between 1994 and 2007	• Inclusion: International Consensus meeting • Causality: Council for International Organizations of Medical Science (CIOMS)/Roussel Uclaf Causality Assessment Method scale	Injury is characterized using the R = ALT/ALP ratio • Hepatocellular ($R > 5$ or ALT $> 3 \times$ ULN) • Cholestatic ($R < 2$ or ALP $> 2 \times$ ULN) • Mixed injury ($2 < R < 5$)	• Amoxicillin clavulanate is the single drug with the highest number of cases • Overdose-induced DILI were excluded	• Patients aged > 60 years accounted for 46% of the cases with M/F = 158/118 • Patients aged 40–59 years had a female predominance
Study 3 • A French population (Sgro *et al.*, 2002): • No. cases = 34 (M/F = 12/22) • Age range = 25–80 years • The population selected from a region where the patients hardly go elsewhere for medical care • Study year: 1997–2000	• Inclusion: International Consensus meeting • Causality: French regulatory authorities	Injury is characterized using the R = ALT/ALP ratio • Hepatocellular ($R > 5$) • Cholestatic ($R < 2$) • Mixed injury ($2 < R < 5$)	Anti-infectious, psychotropic, hypolipidemic agents and non-steroidal anti-inflammatory drugs involved most cases	• No clear evidence for sex-biased DILI for the younger group (<50 years) • Female is dominant in the group of >50 years for DILI (M/F = 6/17)
Study 4 • A Swedish population (De Valle *et al.*, 2006) • No. cases = 77 (M/F = 34/43) • Age range = 16–86 years • A diagnoses database in an out-patient hepatology clinic in a Sweden University hospital was analysed between 1995 and 2005	• Inclusion: International Consensus meeting • Causality: International Consensus meeting	Types of DILI include hepatocellular, cholestatic and mixed	Antibiotics were the most common agents followed by non-steroidal anti-inflammatory drugs	• No clear evidence for sex-biased DILI • Sex difference does exist for some drugs • Female has a higher percentage in hepatocellular injury

(*Continued*)

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Table 1. (*Continued*)

Population	Inclusion criteria and causality assessment	Patterns of DILI	Drugs	Main findings
Study 5 • An US population (Carey *et al.*, 2008) • No. cases = 40 • The DILI patients were identified from the Mayo Clinic Hospital	• Inclusion: International Consensus meeting • Causality: International Consensus meeting	Injury is characterized using the R = ALT/ALP ratio • Hepatocellular ($R > 5$ or ALT > 2 × ULN) • Cholestatic ($R < 2$ or ALP > 2 × ULN) • Mixed injury ($2 < R < 5$)	27 of 40 cases were related to acetaminophen	• Female is predominant in DILI • The hepatocellular injury occurs in all acetaminophen-related DILI

Table 2. Sex difference in drug induced liver injury for various drugs.

Population	DILI definition	Female/male ratio
Tolcapone (Olanow and The Tasmar Advisory Panel, 2000) • No. cases = 1535 treated with tolcapone, 3.7% affected • Age range = 15–78 years • Phase IIIa clinical trial studies during a period of approximately 6 months by Roche Laboratories	>3 × ULN in ALT or AST	5/2
Flucloxacillin (Daly *et al.*, 2009): • No. cases = 51 • Mean onset age = 63.1 years • Cases were collected between October 2004 and December 2007 sponsored by International Serious Adverse Events Consortium	The cases were defined by (i) clinically apparent jaundice or bilirubin 440 mmol/l (after exclusion of cases due to hemolysis), (ii) ALT > 5× ULN; or (iii) ALP > 2× ULN plus any elevated bilirubin above ULN	36/15
Halothane (Bjornsson *et al.*, 2005) • No. cases = 16 • Mean onset age = 59 years • Cases were collected between 1966 and 2002 from the Swedish Adverse Drug Reactions Advisory Committee (SADRAC)	Cases of DILI that resulted in death or liver transplantation were selected based on meeting the International Consensus Criteria (RUCAM)	12/4
Disulfiram (Bjornsson *et al.*, 2005) • No. cases = 3 • Mean onset age = 37 years • Cases were collected between 1966 and 2002 from the Swedish Adverse Drug Reactions Advisory Committee (SADRAC)	Cases of DILI that resulted in death or liver transplantation were selected based on meeting the International Consensus Criteria (RUCAM)	0/3

as the main causal agent (Andrade *et al.*, 2005) (Study 1 of Table 1) also demonstrated female dominance for the study population of much older ages (mean age: 53 years). However, for a Sweden population (Table 3) (Bjornsson *et al.*, 2005), most of the patients with acute liver failure were > 60 years old, and there was no clear evidence of female preference. It is noted that the drugs under investigation in these studies were dissimilar, which may account for the observed differences in age susceptibility. As shown in animal studies, age effects vary with the drug under investigation. It seems that although the DILI incidence occurs more in the older age groups, sex differences are not clear when all drugs are considered.

From the human studies summarized above, it is clear that sex-biased DILI is dependent on many factors including the drug under evaluation, age and pattern of hepatic injury. Unfortunately, most of these factors are difficult to control in a setting

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Table 3. Sex-biased acute liver failure.

Population	Main drugs	Prevalence in female (%)
A Spanish population 1 (Andrade *et al.*, 2005) • No. cases = 18 • Age range = 14–83 years • The patients selected from the Spanish Registry between 1994 and 2004	Amoxicillin clavulanate	89
A US population (Russo *et al.*, 2004) • No. cases = 270 • Age range = 3–63 years • Cases were identified from the United Network for Organ Sharing liver transplant database in 1990–2002	Acetaminophen alone, or in combination with another drug, accounted for 49% of cases	76
A US population (Ostapowicz *et al.*, 2002) • No. cases = 308 • Age range = 15–78 years • Cases were identified from the consortium effort (The US Acute Liver Failure Study Group) between 1998 and 2001	Acetaminophen	73
A Swedish population (Bjornsson *et al.*, 2005) • No. cases = 103 • Age range = 12–91 years • Cases reported to the Swedish Adverse Drug Reaction Advisory Committee from 1996 to 2002	Halothane, paracetamol (APAP), flucloxacillin, sulfamethoxazole, trimethoprim, diclofenac	43

of clinical or epidemiological research without having a better understanding of potential mechanisms. Moreover, the differences in ethnicity of populations with unknown underlying diseases further complicate the observations and comparative analyses. For example, in a study using isolated human liver microsomes and cultured hepatocytes, Hispanics had about twice the average activity of CYP2A6, CYP2B6 and CYP2C8, and half the activity of CYP1A2 when compared with Caucasians and African Americans (Parkinson *et al.*, 2004). Thus, sex-specific sensitivity to hepatotoxicity needs to be better defined in patients by further evaluating in clinical as well as animal studies with an interdisciplinary collaboration. In summary, evidence suggesting DILI-related sex differences is less conclusive in literature.

3 SEX DIFFERENCES IN LIVER GENE EXPRESSION PROFILES UNDER PHYSIOLOGICAL CONDITIONS

As depicted in Figure 1, the development of DILI is a complicated process. It is believed that females may have a higher sensitivity than males for clinically relevant hepatic adverse reactions. This is biologically plausible because women differ from men both genotypically and phenotypically. Several mechanisms have been suggested but the exact systems involved have not emerged. For example, there are apparent differences between the sexes in pharmacokinetics and pharmacodynamics (Neutel and Walop, 2005). In addition, females typically have a higher percentage of body fat than males, leading to preferential accumulation of hydrophobic drugs. Further increasing the potential for female predominance in DILI is the fact that doses are typically not adjusted for body weight, so females often receive higher exposures than men. One or more of these factors could lead to a greater sensitivity to DILI in females compared with males. In addition, it is generally acknowledged that variation in drug metabolism plays a primary role in sex differences in pharmacokinetics (Waxman and Holloway, 2009).

Some studies are demonstrating a genetic link to DILI. For example, studies with flucloxacillin suggest that the HLA-B*5701 genotype is a major determinant of DILI (Daly *et al.*, 2009). A study examined the role of glutathione-*S*-transferase (GST), a phase II detoxifying enzyme, on DILI and found that GSTM1 and GSTT1 gene deletions

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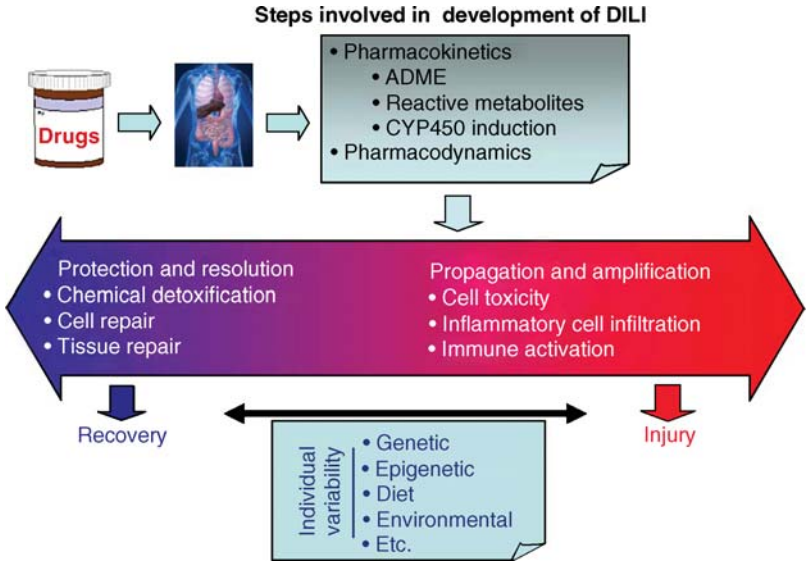

Figure 1. Steps involved in the development or avoidance of DILI.

were associated with susceptibility for several drugs (Lucena *et al.*, 2008). Similar to the genetic links to DILI above, sex differences in liver gene expression are considered a major contributing factor for the differential sex sensitivity to liver diseases and toxicity. For decades, efforts in unravelling sex-associated hepatic genes were largely based on the gene-by-gene approach. The higher-throughput DNA microarray techniques were introduced to address this issue (Tadic *et al.*, 2002). Liver genes showing sex differences, under both physiological and pathological conditions, have thereafter been greatly expanded and novel mechanisms for liver functions and dysfunctions have been revealed with unprecedented rapidity.

In 1932, it was reported that female rats were much more sensitive to the anaesthetic drug amytal sodium than males (Nicholas and Barron, 1932). Later studies revealed that the increased sensitivity was due largely to sex-biased expression of cytochrome P450s (Cyp), phase I drug-metabolizing enzymes. A large number of genes in the liver, besides Cyps, exhibit sex-specific expression. However, a comprehensive picture is still lacking. DNA microarray studies provide a powerful means to survey sex-associated gene expression (Rinn *et al.*, 2004; Mendrick, 2008; Mattes *et al.*, 2006; Boedigheimer *et al.*, 2008; Yang *et al.*, 2006). These studies greatly expanded the list of genes differentially regulated in each sex and a few of these found in the liver are listed in Table 4. A total of 4809 female-predominant liver genes and 4441 male-predominant liver genes have been identified (Yang *et al.*, 2006). It is apparent that such extensive differences could not have been revealed without the introduction of DNA microarray techniques and other emerging molecular biomarker technologies.

Because of differences in species/strains of animals, different microarray platforms and methods of data analysis, there exist great variations in the exact numbers and identities of sex-biased liver genes. However, consensus does exist. For example, differential expression of Cyp genes was unanimously observed (Rinn *et al.*, 2004). Genes related to steroid metabolism were also identified in most studies. These genes have in fact long been recognized by conventional techniques. Far beyond its ability to 'cover' the well-known differentially expressed genes, microarray techniques have made it possible to identify a wealth of new genes that display sharp differences between males and females, such as those involved in protein-tyrosine kinase activity, complement and coagulation cascades, cytokine signalling and solute carrier proteins. Over 70% expressed liver genes are sexually dimorphic in 24-week-old mice (Yang *et al.*, 2006). These extensive sex differences in liver gene expression profiles were

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Table 4. Numbers of hepatic genes differentially expressed between males and females under physiological conditions.

GEO/PMID no.	Organism	Fold change cutoff	Female-predominant	Male-predominant	Functions
GSE1146	Rat	>1.5	39	49	Drug/steroid metabolism
GSE1148	Mouse	>3.0	2	4	Drug metabolism
GSE5072	Mouse	>1.5	753	850	Steroid/oxidative/xenobiotic metabolism, etc.
GSE7169(70)	Mouse	>1.5	1437	1045	Drug/steroid metabolism
GSE2814	Mouse	>1.0	4809	4441	Steroid/lipid/steroid metabolism; protease inhibitor activity, etc.
		>1.2	737	994	
		>2.0	61	81	
		>3.0	30	35	
GSE2497	Mouse	>1.5	21	22	Drug/steroid metabolism
PMID = 14684613	Rat	>1.5	4	24	Glucose (/lipid) oxidation/synthesis, amino acid turnover, drug metabolism
PMID = 16026598	Human	>1.5	165	321	Drug/steroid metabolism

obviously not expected and they might provide better explanations for the sexual dimorphism in both liver physiology and pathology. These results may also warrant further studies aiming at exploring the unknown sex-biased liver phenotypes.

The sex difference in hepatic gene expression is largely due to the difference in hormone exposure. It is generally believed that growth hormone (GH) secreted from the pituitary gland plays a critical role in controlling sex-associated liver gene expression. The pattern of GH secretion differs between sexes. Males have a pulsatile pattern with a large release every couple of hours. Between releases, circulating GH is at a negligible level. In stark contrast, females have nearly continuous release of GH; however, the peak concentration of GH is much lower than the peak level in males (Waxman and Holloway, 2009; Mode and Gustafsson, 2006). The combination of the sex-specific patterns of GH release combined with the differences in GH peak concentration causes differential activation of the target transcription factors, which directly control the expression of a variety of liver genes, thus leading to sex-biased hepatic gene expression patterns. This general picture was originally derived from the expression profiles of several of the best studied Cyps and now DNA microarray data have not only confirmed but also further extended or refined this view.

As early as 2002, researchers reported that GH altered the expression level of genes in the rat liver in a sex-specific manner (Gardmo, Swerdlow and Mode, 2002). In a study, Ahluwalia, Clodfelter and Waxman (2004) compared more fully the effects of GH treatment on sex-biased gene expression. It was shown that continuous GH treatment of hypophysectomized male rats induced an increase of 73% of the female-predominant hepatic genes and a decrease of 90% of the male-predominant liver genes; however, among genes up-regulated or down-regulated by GH, only 10% were not sex-dependent. It was clearly demonstrated that GH treatment of male rats almost completely abrogated the sex differences in hepatic gene expression. This study firmly established that GH is a critical determinant for sexual dimorphic liver gene expression.

The GH signalling pathway is very complex and, although there remain unidentified components, the key events and factors have been characterized in the downstream gene regulation pathway. In brief, GH binds the GH receptor on the cell surface, inducing a conformational change that activates Janus kinase 2 (JAK2), a GH receptor-associated tyrosine kinase. JAK2 phosphorylates the GH receptor on several tyrosine residues. These serve as binding sites for various downstream signalling molecules that have SH2 (phosphotyrosine-binding) domains. One key factor is the binding of signal transducer and activator of transcription (STAT). Once bound, JAK2 phosphorylates the bound STAT, leading to activation and subsequent translocation to the nucleus where it binds DNA response elements and activates gene transcription (Waxman and Holloway, 2009; Mode and Gustafsson, 2006). Two different types of STAT, i.e. STAT5a and STAT5b, have received extensive attention. It has been suggested

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that the pulsatile GH in males activates STAT5b with each pulse, whereas the constant GH level in females leads to partial desensitization or lack of full elicitation of STAT5b. This differential activation of STAT5 might constitute the molecular basis for the sexual dimorphic gene expression pattern in the liver. Microarray studies using STAT5 knockout animals have been used to test these hypotheses (Clodfelter *et al.*, 2006, 2007). In STAT5a knockout mice, only 15% female-predominant genes, such as Cyp2a12, Cyp2d22, Cyp3a16 and Cyp8b1, were decreased in female mice but not in males, indicating that STAT5a mainly plays an important role in controlling a portion of sex-biased hepatic genes in females. On the other hand, in STAT5b knockout mice, 90% of the male-biased genes were decreased and 61% of the female-biased genes were increased in males, essentially abolishing the male pattern of liver gene expression in males; however, 90% of sex-biased genes were hardly influenced in females, showing that STAT5b mainly plays a role in maintaining hepatic gene expression in males. A more detailed review of this literature can be found in an article by Waxman and Holloway (2009).

As with the DNA microarray technology itself, some controversies exist on the reliability of the results in profiling sex-associated genes in the liver. Delongchamp *et al.* (2005) argued that the extent of sex differences in human hepatic gene expression was fairly small, with the majority of fold-change falling below 1.4, and that this kind of minor difference is unlikely to be detected if gene selection were based on their *P*-values. This view appears incompatible with a majority of published investigations, but does highlight the importance of interpreting DNA microarray data in a cautious manner. Better designed and/or analysed microarray data might provide more convincing conclusions. As most raw DNA microarray data are now deposited in freely accessible public databases such as Gene Expression Omnibus (GEO), meta-analysis of the related data might be a convenient way to cross-validate the results and reach more balanced conclusions.

4 SEX DIFFERENCES IN LIVER GENE EXPRESSION PROFILES UNDER PATHOLOGICAL CONDITIONS

The incidence, treatment response and clinical outcome of many liver diseases display sharp sex differences. For example, men are more prone to developing hepatocellular carcinoma (HCC) and a more virulent form of acute hepatitis C than women (Altekruse, McGlynn and Reichman, 2009; Ozaras and Tahan, 2009). Many of these sex differences are also reproducible, and sometimes more striking, in animal models. For example, in mice treated with diethylnitrosamine, only 10–30% of females, but nearly 100% of males, developed HCC (Naugler *et al.*, 2007). In contrast, women are more susceptible to autoimmune hepatitis (Beland *et al.*, 2009). This sex bias in disease prevalence and severity would support the possibility of a sex-based predisposition to the development of DILI. However, the molecular pathogenesis of many diseases is ill defined.

Hepatocellular carcinoma is the most prevalent type of primary tumour in the liver, yet, to the best of our knowledge, there is only one report using sex-matched human samples in profiling liver gene expression in HCC (Takemoto *et al.*, 2005). The authors found that 27 genes were differentially expressed between males and females in human liver tumour tissues, among which 12 were male-predominant and 15 were female-predominant. These genes are either involved in antioxidant functions (such as Sod1) or related to cell cycle control and cell differentiation (such as Fgfr1∼3). Very interestingly, genes that display physiological sex differences were not detected in this study, indicating that the tumour *per se* can change the expression pattern more dramatically than sex itself.

To understand the sex-specific pathogenesis of HCC in humans and aid in the design of sex-biased therapy in treating this common type of liver cancer, an animal model that mimics human HCC was also studied using DNA microarrays. Gene expression profiling was studied for male-predominant HCC mice that were developed by knockout of the thioredoxin-interacting protein gene. This model is expected to be a good mimic of human HCC (Sheth *et al.*, 2006). In another animal model of HCC, DNA microarrays were used to characterize the hepatic gene expression profiles of liver-gender disrupted male mice (Rogers *et al.*, 2007). Male A/JCr mice were infected by *Helicobacter hepaticus* to facilitate the development of masculine-biased HCC, and castration was carried out to disrupt the gender-pattern of hepatic gene expression. It was found that 13% of all liver genes were castration-sensitive in normal males and 17% were castration-sensitive in HCC males, with a 29% overlap in both groups

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of genes. The overlapped genes might be of critical importance in the male-predominant manner of HCC pathogenesis. Further bioinformatics analysis revealed that pro-inflammatory cytokine pathways played a central role in the altered expression of sex-biased genes. IFN-γ was then selected for in-depth biological experimentation, and it was confirmed that this cytokine alone could recapitulate the *H. hepaticus*-associated gene expression alterations. It was concluded that the liver of male mice is more vulnerable than that of the female to cytokine-mediated disruption of sexually dimorphic hepatic gene expression profiles, and are therefore more prone to develop HCC after *H. hepaticus* infection (Rogers *et al.*, 2007).

Both halogenated aromatic hydrocarbons (HAHs), such as 2,3,4,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and polychlorinated biphenyls (PCBs), such as Aroclor 1254, are well-known liver carcinogens. Female rats seem to be more susceptible to these two agents with respect to liver tumorigenesis but the mechanisms are poorly understood. In a recent study by Silkworth *et al.* (2008), DNA microarray experiments were carried out to address this issue. It was demonstrated that TCDD and Aroclor 1254 significantly disrupted the expression pattern of a panel of sex-associated hepatic genes, particularly those involved in steroid metabolism. The disruptive effects were more dramatic in female rats than in males. This differential vulnerability to sex-gene disruption might underlie the sexually dimorphic response toward chemical carcinogens.

Inorganic arsenic is also a well-recognized carcinogen. It has been shown that adult male mice tend to develop HCC after transplacental exposure, but females do not. The mechanisms of such differential susceptibility have recently been explored by DNA microarray methods (Liu *et al.*, 2006). It was demonstrated that nearly 50 biological networks were disrupted in the males with HCC. One remarkable change was the feminization pattern of liver metabolic enzyme genes in the male, as evidenced by the marked increase of many female-dominant P450s including Cyp2a4, Cyp2b9 and Cyp2d9, and sharp down-regulation of several male-dominant genes such as Cyp7b1, Cyp2f2 and Cyp41. It was proposed that such disruptive effects on sex-associated genes could contribute a significant part in the pathogenesis of male-exclusive transplacental arsenic HCC.

5 FUTURE ASSESSMENT OF SEX DIFFERENCES IN DILI AND THE ROLE OF GENOMICS

Genomics has already provided a wealth of knowledge about liver genes that are regulated in a sex-specific manner. The ability to profile tens of thousands of genes at a single time point enables powerful comparisons of the systems that are differentially regulated in each sex under homeostatic conditions. Genomics extends these assessments to genes and systems that are differentially regulated in each sex after drug exposure. These changes can then be correlated with sex-specific sensitivity to a drug to determine the critical factors responsible for the increased sensitivity.

Pre-clinical toxicity studies are conducted in typical laboratory species (mice, rats, rabbits, canines and non-human primates) to assess the safety of new drug candidates. They generally involve multiple doses of a drug so that an accurate dose response can be constructed. Classical endpoints of toxicity are used such as clinical observations, body weight, feed consumption, clinical pathology (haematology, clinical chemistry, coagulation and urinalysis), gross pathology and histopathology. In addition, toxicokinetic assessments are increasingly being included in these studies. As these studies often include both sexes, toxicogenomics can be seamlessly integrated to study sex differences in liver gene expression after drug exposure.

A major advantage of integrating genomics in preclinical studies is that it helps in correlating sex-specific differences in liver gene expression with differential sex sensitivity based on the classical endpoints of toxicity collected in the study. In addition, if toxicokinetic samples are collected, powerful correlations can be made between gene expression, sensitivity and drug kinetics. The biggest hurdles in integrating this technology are cost and additional sample processing. However, costs are continuously decreasing as the technology matures and laboratories successfully integrate the additional sample processing steps into traditional preclinical study designs.

Genomics holds the promise of providing tremendous insights into potential sex-specific sensitivities in the human population. The main challenges are robust trial design and identification of appropriate biomarkers in easily collected samples. Clinical trials conducted under good clinical practices (GCPs)

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are likely to provide the best opportunity for the successful integration of genomics for the assessment of sex-specific sensitivity to DILI. These trials are typically well designed, conducted and documented, allowing the best chance for drawing robust correlations between the types of collected data. As with preclinical studies, including genomic analysis in clinical trials would allow changes in gene expression to be correlated with adverse effects, greatly facilitating the assessment of sex-specific differences in DILI.

The major hurdle with integrating genomics in clinical trials for assessing sex-specific DILI will be identifying appropriate biomarkers that can be measured in easily collected samples. The gold standard would be assessing gene expression in the liver; however, obtaining biopsy samples for every DILI case would be impossible. Blood is an easily and routinely collected clinical sample that holds promise. Circulating white blood cells have been shown to identify patients exposed to high levels of APAP (Bushel *et al.*, 2007). Recent studies have detected both liver-specific mRNA and liver-enriched microRNA in peripheral blood after hepatotoxicant exposure (Miyamoto *et al.*, 2008; Wang *et al.*, 2009). Assessment of free mRNA or microRNA in the blood would be analogous to measuring serum ALT, a liver-specific enzyme released into the circulation after liver injury.

Integrating genomics in both preclinical and clinical studies will greatly facilitate the assessment of sex-specific sensitivity to DILI and the genes and systems involved with the differential sensitivity. In addition, identifying translational biomarkers (biomarkers in preclinical studies that can be used in clinical studies and vice versa) will increase the ability to prevent more serious injury to individuals in clinical trials that may be especially sensitive to a drug. This will in turn reduce the number of DILI cases and protect human health.

6 CONCLUDING REMARKS

With the introduction of DNA microarray technology, we have witnessed remarkable progression in profiling sexually dimorphic hepatic genes associated with liver physiology and disease. In males and females, the excretion of GH is pulsatile and constant, respectively, which in turn triggers the full or partial activation of transcription factor STAT5, thus leading to the differential expression of a panel of liver genes. STAT5a is responsible for sex-related hepatic genes in females, and STAT5b is responsible for sex-associated hepatic genes in males. Sex differences in liver gene expression are extensive, and disruption of the sex-dependent gene expression pattern is related to various liver disorders such as liver cancer and ALD. However, the existence of true female-predominant human DILI in terms of incidence is unclear as discussed above. To support or refute this belief, more preclinical and clinical studies need to be performed to understand the extent of this perceived gender bias where drugs are concerned. Future microarray studies involving both sexes and reproductive lifecycle would be useful to obtain new insights into the mechanisms of any sex-biased hepatotoxicity and response to injury. We have developed LTKB at NCTR/FDA and our focus is to assess sex differences in DILI, a part of the overarching efforts emphasized by the FDA's Office of Women's Health.

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Commonality and Stochasticity in Systems Toxicology

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1 INTRODUCTION

Gene chip analysis, a microarray technology, is a type of "molecular microscopy" based on global gene expressions. Regarding its function, gene chip analysis, however, does not simply visualize "something" by this microscopy. The principles of "cells" and "pathology" were visualized and discovered with the development of photomicroscopy. It is not essential that photomicroscopy enabled the visualization of "objects," that is cellular structures, and provided pathological findings *per se*, but that photomicroscopy enabled the realization of concepts and/or principles explaining those "objects". Conceptually, gene chip analysis is not simply visualizing global gene expressions, but it is supposed to establish principles that have never been recognized in the past in the field of toxicology. What would they be?

When one treats 100 mice with a carcinogenic chemical compound, 46% of the mice develop pulmonary neoplasms, 36% acute myeloid leukaemias (AMLs) and the rest soft tissue tumours and angiogenic tumours. Although this treatment protocol is highly repeatable, generally, one cannot identify which mice would develop pulmonary tumours or angiovascular neoplasms. Why can such a particular outcome not be predicted? It may be speculated that those 100 mice may still be "different" despite their inbred genetic background. However, as we describe later, these uncertainties in each individual are based on a probabilistic hit; that is, a biological test at the individual level produces a probabilistic random outcome in mice whose genetic backgrounds are prohibitively identical. Each signalling pathway may be probabilistically different among individual mice, and probabilistic incidences of the routes of signalling pathways may be analysed computationally. A new concept of biological variability, i.e. stochasticity, is presented below.

When two holes as targets are hypothesized, a bullet can hit only one, not both at once. However, two or more bullets may hit at once in biology. The uncertainty law was originally established to account values in physics on the basis of the probabilistic quantum law. However, probabilistic stochasticities have also been focused on in the field of biology over the past decade, because biological scientists have realized the stochasticities in various biological reactions as quantum biology. Let us consider what happens next when multi-target holes exist and these holes are linked to individually different molecular signalling pathways; the induction of probabilistic adverse signalling by a hit would be multiplied. This is a stochastic probabilistic phenomenon in biology. Namely, this is the molecular probabilistic part of epigenetics. To understand xenobiotic responses in systems biology, a new method of analysing toxicological data called

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"systems toxicology' based on genomic expression has been established.

Stochastic and different gene expression profiles were analysed for individual Purkinje cells in the cerebellum examined by single-cell microarray (Yagi K.). Thus, the overall cerebellar function in each mouse shows different probabilistic gene expression profiles, indicating individual differences among mice. If the development of individual mice were probabilistic, then cloned animals represented by the well-known sheep Dolly would not have an endemic infectious risk caused by their epigenetic inhomogeneity in immune responses. One problem associated with these conditions is the possible disappearance of stochasticity in the calculation of average values, because commonality tends to erode such a random stochasticity.

Previous toxicological data have been analysed frequently with their average endpoints focused on the commonality. However, probabilistic stochasticity may be analysed as specific stochastic clusters that elucidate other aspects of biological diversity in future "systems toxicology".

2 STEADY-STATE GENE EXPRESSION DURING AGEING

The Gompertzian formula, established by Benjamin Gompertz 1825 almost 185 years ago denotes human death rate during a unit time interval that increases linearly and exponentially with lifetime. The implications of Gompertzian linearity are considered to be based on the fact that lifespan may involve a function based on the integral of various life-threatening factors, such as neoplastic diseases, cardiovascular diseases and senile nephrosclerosis during ageing (the risk-integration theory) (Linzbach, 1972; Hruza, 1972). Namely, this linearity is based on a system in which each term of lifespan-linked diseases is independent of each other and is multiplicatively, not additively, linked to terms (equal diseases) (Equation 1).

$$N'(t) = -rN(t)\log[N(t)/K] \qquad (1)$$

where $N(t)$ is the number of individuals at time t, r is the intrinsic growth rate and K is the number of individuals in equilibrium. Namely, the risk across a lifespan is the total risk integration obtained by multiplication of the risk of a disease by that of another disease (Hirabayashi and Inoue, 2007).

According to the concept above, the age-related increase in the incidence of malignant neoplasms is believed to be based on the age-related exponential increase in genomic instability. However, a more important concept of Gompertzian linearity is that deaths from not only malignant neoplasms, but also cerebrovascular and other non-neoplastic diseases, are supposed to be concomitantly incorporated into an increase of curves as individual risk parameters, which is possibly based on an increase of genomic instability. The increases in risks associated with genomic instability and other factors with age would be studied in systems biology, whereas those increases in risks under toxicological impact are supposed to be studied in systems toxicology.

When one examines 100 senescent mice, senescent phenotypes may be observed in nearly all these mice. However, there may also be probabilistic phenotypes observed at different incidences individually. When one compares the phenotypes of these senescent mice with those of less senescent young adult mice by gene chip microarray, two different categories of gene expression profiles are recognized; that is, one corresponds to common ageing profiles (CAPs) and the other corresponds to stochastic ageing profiles (SAPs). The former were selected by two-way analysis of variance (ANOVA), and the latter were selected by principal component analysis (PCA) among others.

2.1 Common Ageing Profiles

Gene expression profiles for bone marrow cells from 2-month-old (five young mice) and 21-month-old (five old mice) male mice of the C3H/He strain are shown on the left and right panels of Figure 1, respectively, for comparison. In these linear configurations, expression intensities of the same genes in each mouse were connected to each other in the order of expression intensity from the top to the bottom as compared with those of the third mouse in the young mouse group (left panel). The characteristic differences between the young mouse group and the old mouse group are as follows. First, the gene expression order tends to be down-regulated in the 21-month-old mice (right panel). Second, the ranges of gene expression intensities in the 21-month-old senescent mice are broader than those in the 2-month-old young mice. When one compares the five 2-month-old mice with the five 21-month-old

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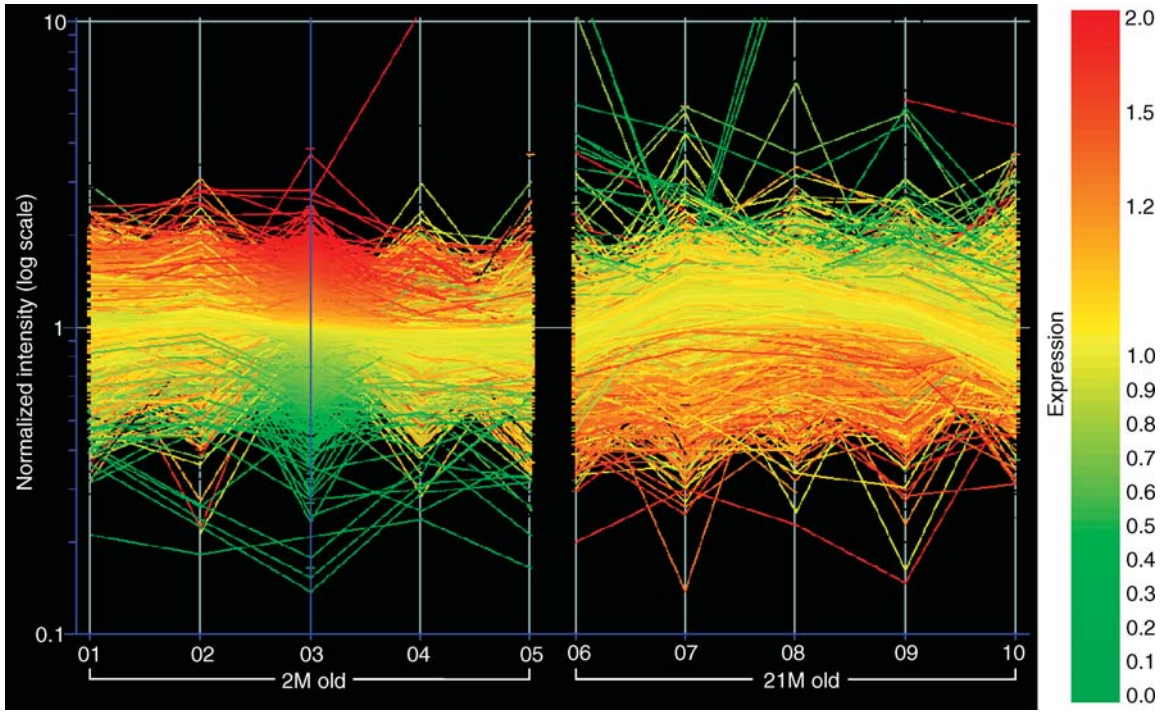

Figure 1. Linear configurations of gene expression profiles for bone marrow cells from 2- and 21-month-old mice: microarray profiles differentiate bone marrow cells from 2-month-old mice from those from 21-month-old mice. The five individual data points in the left panel are for bone marrow cells from 2-month-old C3H/He mice. The other five individual data points in the right panel are for bone marrow cells from 21-month-old C3H/He mice. Along the gene expression level scale from the highest (red) to the lowest (green) for No. 03 mouse, the same genes are connected and indicated by the same intensity of gene expression. Accordingly, genes over-expressed in 2-month-old mice are relatively under-expressed in the 21-month-old mice. Unless otherwise indicated, all the obtained microarray data presented in this chapter were normalized and analysed using GeneSpring GX 7.3.1 (Agilent Technologies Inc., Santa Clara, CA, USA).

mice concomitantly using a dendrogram without supervised information, both groups are not clearly separated from each other, probably because neither age-related common expression nor stochastic expression are only for minor genes as compared with 40 000 other probe sets.

Next, when one compares gene expressions in 2-month-old bone marrow cells with those in 21-month-old bone marrow cells by ANOVA, the finding depends on the range of confidential gains applied; 122 probe sets were chosen to be included in the list of CAPs (Figure 2). Among the 122 probe sets, as compared with gene expressions in 2-month-old bone marrow cells, those in 21-month-old bone marrow cells showed decreased expression levels of *cyclin D1* (Ghiaur *et al*., 2006; Matsumura *et al*., 1999), *cyclin G2* (cell cycle repressor; Arachchige Don *et al*., 2006; Horne *et al*., 1996), *FoxP* (monocyte differentiation and macrophage function; Shi *et al*., 2008), *Pax5* (B-cell differentiation; Nutt *et al*., 1997;Singh, Medina and Pongubala, 2005) and β-*catenin* (stem cell maintenance; Kim *et al*., 2009), as well as unstabilized self-determinant immunoglobulin-related genes. Along with these alterations in common gene expression levels, decreased expression levels of genes involved in the cell cycle in haematopoietic progenitor cells, increased expression levels of genes related to oxidative stress and an increased instability of genes for dormancy in haematopoietic progenitor niches are observed. However, *RUNKs*-related regulatory changes in haematopoietic progenitor cells were not observed (Figure 3).

Furthermore, genes interacting with the above-mentioned genes, as confirmed by the analysis of surrounding genes, were found to be involved in cell cycle regulation [e.g., *Trp53* (Hirabayashi *et al*., 2002b; Livingstone *et al*., 1992), *bcl2* (Zinkel, Gross

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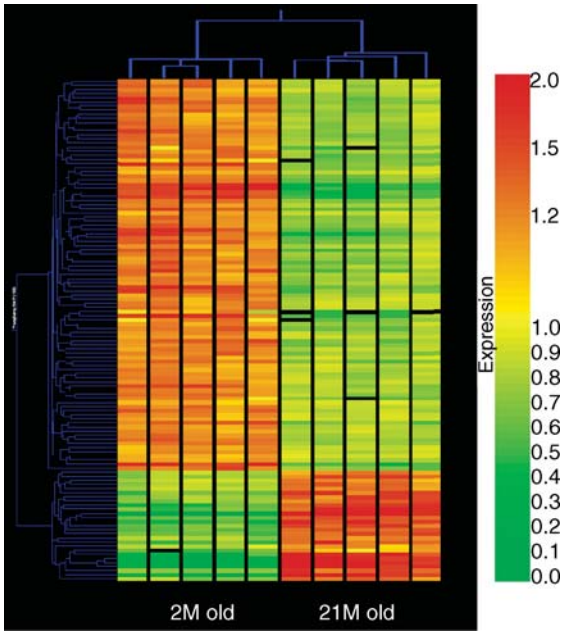

Figure 2. Probe sets selected to discriminate between age groups: One hundred and twenty-two probe sets (genes) were selected from gene expression profiles by ANOVA for bone marrow cells of C3H/He mice to discriminate between 2- and 21-month-old mice using dendrogram.

and Yang, 2006) and *myc* (Hirabayashi *et al*., 1992; Kelly *et al*., 1983)]. Oxidative stress responses [e.g. *FoxO* (Furukawa-Hibi *et al*., 2002), *Hif1* (Gorlach and Bonello, 2008) and *cyclin G2*] were also found to interact with *TNF-α* , *interferon-γ* and *PPAR-γ*. These findings imply that genes related to the maintenance of haematopoietic progenitor niches, including antioxidative notch signalling, and genes for maintaining immunogenicity are generally over-expressed in senescent mice (Appel *et al*., 2005; Hu *et al*., 2008; Zhang *et al*., 1995). In conclusion, in CAPs, senescence-related homeostasis is generally maintained and alterations of genes are limited within the range of homeostatic maintenance.

2.2 Stochastic Ageing Profiles

The characteristics of gene expressions, as shown in SAPs, in 21- and 2-month-old bone marrow from five mice each were found to be more exaggerated than those of gene expressions of those probe sets in CAPs. The CAPs show that the expressions of *cyclin D1*, *cyclin G2* and *FoxP* were down-regulated in 21-month-old bone marrow, and the SAPs also show that these genes were generally down-regulated. When the CAPs were overlapped with the SAPs, alteration was observed, for example increase in $p27^{kip1}$ expression level, decrease in *FoxO* expression level and increase in *FoxD3* expression level in the senescent group. Along with these alterations, *Hif1* expression was up-regulated in some bone marrow cells but also down-regulated in other bone marrow cells along with the down-modulation of MnSOD expression. There was no change in *Fas* expression, but the expression level of *Fas ligand* (FasL) decreased, and that of *Bcl2* increased in some bone marrow cells in the senescent group.

When one examines the relationships between CAP- and SAP-related signalling in more detail using the signalling network database, stochastic gene expressions scattered in the networks occasionally merged downstream towards the CAP genes stochastically. *Cyclin D1*, for example, that is one of the CAP members decreased during the senescence, receives several different negative signalling from the surrounding stochastic genes during senescence. Namely, *cyclin D1* is commonly down-regulated as follows: by negative signalling owing to the down-modulation of *NF-κ B* and *AP-1* and non-essential steady-state signalling from *KLF4* and *eIF4F*, in one case; by the down-regulation of *AP-1* and *NF-κ B* directly, and competitive relatively milder up-regulation by *eIF4F* and the consequent up-regulation by *KLF4* owing to double cancellation of *KLF4* upstream of *KLF5* in another case; by the same bilateral regulation as observed in the first case except for controversial down-regulation by *eIF4F* in the fourth case; and by prominent up-regulation by *eIF4F* and *Ap-1*, but with a strong negative up-regulation of *KLF4* owing to negative *KLF5* cancellation signalling, and down-regulation by *NF-κ B* in the last case [*NF-κ B* (Hinz *et al*., 1999), *AP-1* (Bakiri *et al*., 2000), *KLF4* (Shie *et al*., 2000), *eIF4F* (Barnhart *et al*., 2008), *KLF5* (Dang *et al*., 2002)].

Accordingly, xenobiotic responses may start from various xenobiotic stochastic gene expressions. Signals related to such responses may spread to surrounding genes and, finally, may occasionally merge with CAP member genes. Thus, the CAPs may receive different signalling with up-regulation and/or down-regulation stochastically differing from one case to another. Therefore, common

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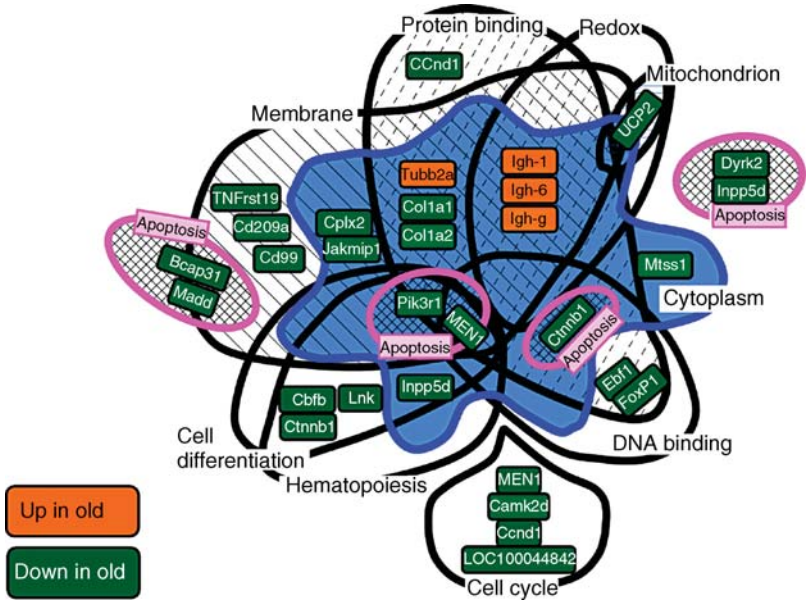

Figure 3. Up-regulation or down-regulation of genes from senescent mice identified by gene ontology: one hundred and twenty-two probe sets (genes) selected by ANOVA from genes expressed in bone marrow cells from five 2-month-old mice and five 21-month-old mice were analysed using gene ontological database to discriminate between both age groups [Bcap31: B-cell receptor-associated protein 31; Cbfb: core binding factor β; Camk2d: calcium/calmodulin-dependent protein kinase II, delta; Ccnd1: cyclin D1; Cd209a: CD209a antigen; Cd99: CD99 antigen; Col1a1: collagen, type I, α 1; Col1a2: collagen, type I, α 2; Cplx2: complexin 2, Ctnnb1: catenin (cadherin-associated protein), β1; Dyrk2: dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase 2; Ebf1: early B-cell factor 1; Foxp1: forkhead box P1; Igh-1: immunoglobulin heavy chain I; Igh-6: immunoglobulin heavy chain complex; Igh-g: immunoglobulin heavy chain (gamma polypeptide); Inpp5d: inositol polyphosphate-5-phosphatase D; Jakmip1: janus kinase and microtubule interacting protein 1; LOC100044842: similar to cyclin fold protein 1; Madd: MAP-kinase-activating death domain; Men1: multiple endocrine neoplasia 1; Mtss1: metastasis suppressor 1; Pik3r1: phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 α); Sh2b3/Lnk: SH2B adaptor protein 3/similar to lymphocyte-specific adaptor protein Lnk; Tnfrsf19: tumour necrosis factor receptor superfamily, member 19; Tubb2a: tubulin, β2a; Ucp2: uncoupling protein 2 (mitochondrial, proton carrier)].

gene expressions seem to be conciliatorily regulated by positive and negative signals from various stochastic genes differing from one case to another.

3 XENOBIOTIC RESPONSES

In this section, two aspects of xenobiotic responses examined by gene chip microarray analysis will be focused on: responses to benzene exposure and radiation. Senescent changes described in the first section can be categorically considered to be a type of xenobiotic response to "time", in which essential biological responses during ageing are based on a long-term relationship between living organisms and environmental xenobiotic materials (including oxidative stresses and photostimulation, among others). In this section, we focus on more active xenobiotic responses examined by gene chip microarray analysis so that a general principle on xenobiotic responses in systems biology may be established.

Whole-body exposure of mice to benzene (repeated, intermittent) and ionizing gamma-irradiation (single dose) was carried out. The reason that benzene exposure was chosen is its potential use as a model of xenobiotic relationship, which is mediated by aryl hydrocarbon receptors (AhR) expressed only on primitive haematopoietic progenitor cells in bone marrow tissue. Another reason for choosing radiation exposure was to elucidate the particular mechanism underlying the late effect after a single dose of exposure.

Tissues for gene chip microarray analysis were sampled and processed 28 days after the last exposure, in order to observe the earliest long-term responses to the late effect; not the acute reversible responses.

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3.1 Benzene

3.1.1 Background

The haematopoietic toxicity of benzene is mediated by AhR (Yoon *et al*., 2002), and is thus specifically induced in haematopoietic progenitor cells in bone marrow because AhRs are only specifically expressed in progenitor cells at the steady state (Garrett and Gasiewicz, 2006; Harper, Riddick and Okey, 2006; Hirabayashi *et al*., 2002a; Hirabayashi and Inoue, 2010). Intermittent exposure by inhalation or gavage 6 h/day or once a day, 5 days/week, for 26 weeks is designed so as not to induce aplastic anaemia, using an appropriate concentration of benzene for exposure, that is 300 ppm (Cronkite *et al*., 1982, 1984; Kawasaki *et al*., 2009; Li *et al*., 2006; Snyder *et al*., 1980) in the present study, which produces 30–50% haematopoietic neoplasms (HPNs) depending on the mouse strain used. Gene chip microarray analysis was performed 28 days after 2 weeks of intermittent gavage exposure to benzene at 150 mg/kg b.w. to elucidate benzene-specific gene expression profiles, from which we can further define gene expression in terms of early plausible responses such as possible leukaemogenesis. Gene chip microarray analysis immediately after benzene exposure has been separately reported elsewhere (Yoon *et al*., 2003). Analysis on 28 days after 2 weeks of the last exposure to benzene was supposed to exclude acute signals such as those of cellular death and apoptosis, or those of advanced late effects. Because benzene exposure may lead to the development of human myeloid leukaemias (Aksoy, Erdem and DinCol, 1974; Delore and Borgomano, 1928; IARC, 1982; Yin *et al*., 1987), the incidence of myeloid leukaemias in the C3H/He mouse strain, which is prone to myeloid leukaemia (Seki *et al*., 1991; Yoshida *et al*., 2007; Yoshida *et al*., 1996), was compared with that in the C57BL/6 mouse strain, which is prone to malignant lymphoma (Hirabayashi *et al*., 2007a, 2007b; Kawasaki *et al*., 2009; Li *et al*., 2006), and gene expression profiles of both strains were examined. Interestingly, benzene exposure induced specific gene expressions in both strains on one hand, whereas individual mice expressed different stochastic and probabilistic gene expression profiles related to various gene ontologies including cellular functions plausible to leukaemogenesis on the other hand.

In conclusion, gene expressions related to maintaining functional haematopoiesis, and gene expressions balancing between the acceleration and deceleration of cell kinetics were regulated as the common gene expression profiles, whereas further up-regulation or down-regulation of individual genes was probabilistically observed as the stochastic gene expression profiles.

3.1.2 Long-Term Benzene Inhalation and Leukaemogenesis

In the protocol of intermittent inhalation of 300-ppm benzene mentioned above, the incidence of leukaemias induced was maximum because mice tended to develop aplastic anaemia and die at an exposure of over 300 ppm (Maltoni *et al*., 1989). Cumulative incidences of HPNs during the lifetime of mice after the intermittent benzene exposure protocol for 26 weeks are shown in Figure 4 (a and b: survival curves; c and d: incidences of whole HPNs; e and f: types of HPNs in C57BL/6 and C3H/He mice). Interestingly, when one looks at Figure 4f, only two cases (9%) of AMLs are observed in the higher-concentration exposure group, 300 ppm, on the left most column. Because much higher incidences of AMLs were observed in *Trp*53-deficient C3H/He mice in a previous report (Kawasaki *et al*., 2009), induction of myeloid leukaemia by benzene exposure was considered to be plausible not only in human but also in murine species. Further details on the incidences and histopathological findings of the study are described elsewhere (Kawasaki *et al*., 2009).

3.1.3 Benzene-Induced Strain Differences in Global Gene Expressions

Common Gene Profiles

The benzene dose for exposure by gavage equivalent to the above-mentioned inhalation exposure dose is 150 mg/kg b.w., which induces equivalent incidences of HPNs during the lifetime (French *et al*., 2001; NTP, 1986). Gene chip microarray analysis was performed 2 weeks after intermittent gavage exposure to the same amount of benzene. The 45 101 probe sets obtained were analysed using Affymetrix GeneChip® Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA, USA) and two-way ANOVA with false detection ratio (FDR) for the two strains with and without benzene exposure. Statistically, the number of significant benzene-exposure-specific probe sets (genes) whose *P*-values were lower than 0.05 was

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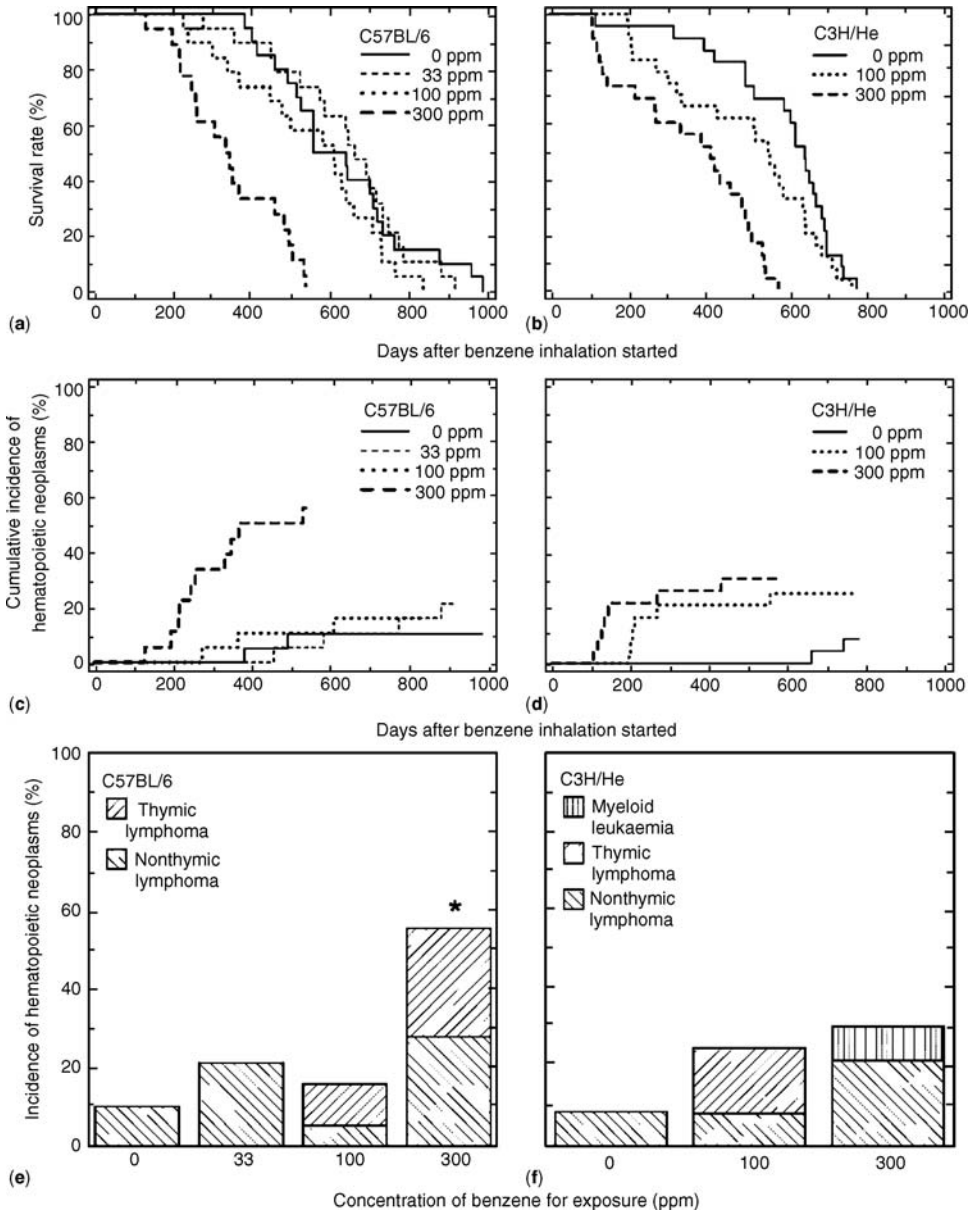

Figure 4. Survival rates, cumulative incidences of haematopoietic malignancies and their histological types in two strains after benzene inhalation. (a, b) Survival rates (%) are plotted on the vertical axis and survival time (days) after benzene inhalation on the horizontal axis for C57BL/6 mice (a) and for C3H/He mice (b). Bold dotted line for 300 ppm exposure group, regular dotted line for 100 ppm exposure group, fine dotted line for 33 ppm exposure group and solid line for sham exposure control. Statistical significance determined by log rank test: (a) 0 vs. 300 ppm, $P = 4.7 \times 10^{-6}$; 33 vs. 300 ppm, $P = 8.3 \times 10^{-6}$; 100 vs. 300 ppm, $P = 4.9 \times 10^{-4}$; (b) 0 vs. 300 ppm, $P = 5.6 \times 10^{-6}$; 100 vs. 300 ppm, $P = 4.9 \times 10^{-3}$. (c, d) The illustration shows the cumulative lifetime incidences of all haematopoietic malignancies (%) in C57BL/6 mice (c) and C3H/He mice (d). Bold dotted line for 300 ppm exposure group, regular dotted line for 100 ppm exposure group, fine dotted line for 33 ppm exposure group and solid line for sham exposure control. Statistical significance determined by log rank test: (c) 0 vs. 300 ppm, $P = 2.7 \times 10^{-4}$; 33 vs. 300 ppm, $P = 4.5 \times 10^{-4}$; 100 vs. 300 ppm, $P = 1.8 \times 10^{-3}$; (d) 0 vs. 300 ppm, $P = 7.0 \times 10^{-3}$. (e, f) Incidences of HPN histological types are shown in bar graphs for the C57BL/6 strain (e) and C3H/He strain (f). The incidence of HPNs is shown on the ordinate axis versus benzene exposure dose on the abscissa axis of each graph for the C57BL/6 strain (0, 33, 100 and 300 ppm) and C3H/He strain (0, 100 and 300 ppm). Histological types, such as AML, thymic lymphoma and non-thymic lymphoma, are designated by inset legends in each figure. The incidence in only the 300-ppm exposure group shows statistically significant difference (*$P < 0.05$) by Fisher's exact test.

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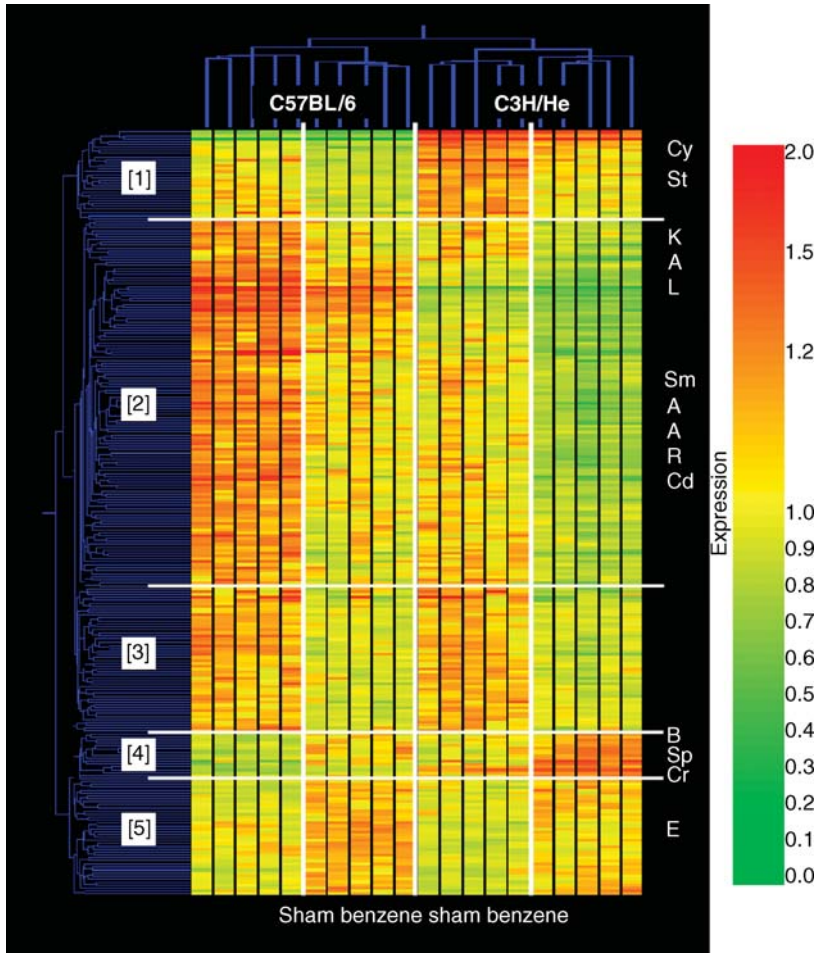

Figure 5. Probe sets selected to determine strain differences in response to benzene exposure: Two hundred and fifty-eight probe sets (genes) were selected from gene expression profiles by two-way ANOVA for bone marrow cells, from C57BL/6 and C3H/He mice (five mice each) with and without benzene exposure, and applied to a dendrogram to discriminate genes relevant to strain differences by benzene-specific gene expression intensities. Gene abbreviations on the right are listed in Table 1.

258. A dendrogram consisting of these 258 probe sets for the two strains is shown in Figure 5. In this figure, five categories were identified without supervision of information, in terms of four different components (two strains with and without benzene exposure). Benzene exposure induced the down-modulation of gene expressions in categories [1], [2] and [3], and benzene exposure induced the up-modulation of gene expressions in categories [4] and [5]. Among them, the C3H/He strain was dominant during the steady state in category [1], as compared with category [2], in which gene expression levels during the steady state were higher in C57BL/6 than in C3H/He. In category [3], gene expression levels in the steady state were higher than those in benzene exposure without significant differences between strains. In categories [4] and [5], both strains showed minimum gene expression levels in the steady state, but C3H/He showed higher expression level in category [4] whereas C57BL/6 showed a higher expression level in category [5] after benzene exposure. Representative probe sets of each category are shown in Table 1.

Category [1] consists of *Cryl1* and *Sltm*. The former is reported to be associated with genomic instability (Chen *et al.*, 2003; Etienne *et al.*, 2007), whereas the latter is associated with suppression of apoptosis (Chan *et al.*, 2007). Thus,

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Table 1. List of genes shown in Figure 5.

Affymetrix systemic name	Gene initials used	Common name	Genbank ID	Descriptions
1447112_s_at	Cy	*Cryl1*	C85932	Crystallin, lambda 1
1424452_at	St	*Sltm*	BC019992	SAFB-like, transcription modulator
1435329_at	K	*Kdm2a/Fbxl11*	BE690994	Lysine (K)-specific demethylase 2A/F-box and leucine-rich repeat protein 11
1420947_at	A	*Atrx/rad 54*	BB825830	α-Thalassaemia/mental retardation syndrome X-linked homologue (human)
1423521_at	L	*Lmnb1*	AA270173	Lamin B1
1429658_a_at	Sm	*Smc2*	BI684556	Structural maintenance of chromosomes 2
1450051_at	A	*Atrx/rad 54*	BB825830	α-Thalassaemia/mental retardation syndrome X-linked homologue (human)
1420946_at	A	*Atrx/rad 54*	BB825830	α-Thalassaemia/mental retardation syndrome X-linked homologue (human)
1449292_at	R	*Rb1cc1*	BE570980	RB1-inducible coiled-coil 1
1434045_at	Cd	*Cdkn1b* (*p27 kip1)*	BB354528	Cyclin-dependent kinase inhibitor 1B (P27)
1420975_at	B	*Baz1b/WSTF*	BB253608	Bromodomain adjacent to zinc finger domain, 1B/Williams syndrome transcription factor (WSTF)
1424875_at	Sp	*Spg20*	BB040507	Spastic paraplegia 20, spartin (Troyer syndrome) homologue (human)
1420849_at	Cr	*Crnkl1*	AV143435	Crn, crooked necklike 1 (*Drosophila*)
1435054_at	E	*Eme1*	BG064903	Essential meiotic endonuclease 1 homologue 1 (*S. pombe*)

their expressions may be specifically induced by benzene exposure, which supports a plausibility of the predominant induction of HPNs in the C3H/He strain. In category [4], the genes that are up-regulated after benzene exposure are *WSTF*/*Baz1b* [Williams-Beuren syndrome transcription factor, also known as *BAZ1B*; a component of the *WICH* complex (*WSTF-ISWI* ATP-dependent chromatin-remodelling complex)], *Crnkl1* (Crn, crooked neck-like 1) and *Spg20* [spastic paraplegia 20, spartin (Troyer syndrome) homologue], whose functions are related to an enhanced double-strand break repair (Xiao *et al*., 2009), suppression of cell cycle (Chung *et al*., 2002) and up-regulation of potential suppressive factors (Bakowska *et al*., 2005), respectively.

The up-regulation of the repair of DNA damage associated with cell cycle suppression induced by benzene exposure was determined solely on the basis of the up-regulation of *Eme1*/*MUS81* (essential meiotic endonuclease 1 homologue 1, a component of *Mus81-Eme1* structure-specific endonuclease) (Hanada *et al*., 2006) in category [5] and the down-modulation of *Lmnb1* (lamin B1) (Fiume *et al*., 2009) in category [2]. However, many genes are identified to react with xenobiotics, which may lead to neoplastic changes, such as the down-modulation of $p27^{kip1}$ to release from cell cycle suppression (Nakayama *et al*., 1996; Payne and Kemp, 2003; Polyak *et al*., 1994), the down-modulation of *Kdm2a* to increase genomic instability (Frescas *et al*., 2008), the down-modulation of *Atrx*/*Rad54* to suppress DNA repair (Roy *et al*., 2008), the down-modulation of *Smc2* (structural maintenance of chromosome 2) to suppress double-strand break repair (Schar, Fasi and Jessberger, 2004) and the suppression of *Rb1cc* (*RB1*-inducible coiled-coil 1) to increase genomic instability caused by suppression of *Rb1*-inducible genomic stabilization (Ikebuchi *et al*., 2009). These are common trends of gene expression profiles for each strain or, in the case of both strains, plausible for induction of benzene-specific HPNs.

Stochastic Gene Profiles

In contrast to these common trends of gene expressions in these two strains, or each strain, each mouse often shows stochastically unique gene expressions, as shown in Figure 6. The five mice in each group show different expression levels: one signalling pathway comes down from *TCF* (T-cell-specific transcription factor) to *cyclin D1*, another pathway from *Bcl-6* to *cyclin D1* and another from *E2F* to *cyclin D1* in different expression intensities from mouse no. 1. Thus, three signalling pathways coming down to *cyclin D1* are different from one mouse to another among the five mice in this group (Bakiri *et al*., 2000; Hrzenjak *et al*., 2006; Liu and Habener,

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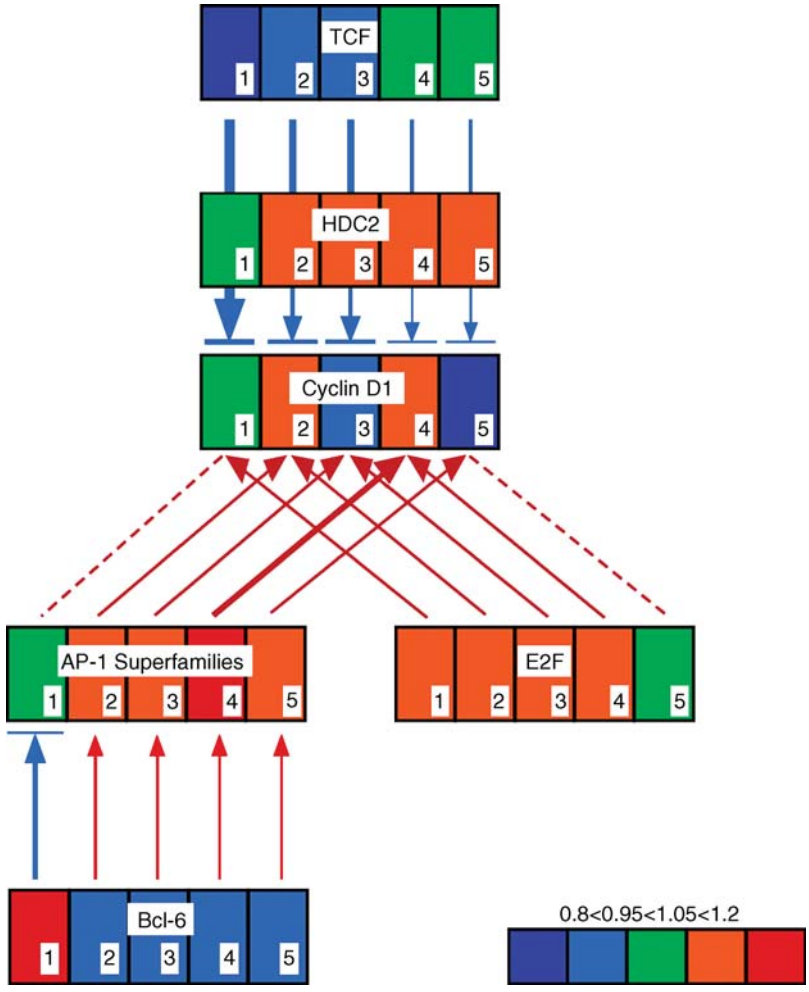

Figure 6. Sample stochastic gene expressions in three pathways upstream of *cyclin D1* and differences in gene expression levels among mice: To maintain the stochasticity of expression characteristics, gene expression profiles of the sham-control group were compared separately with individual expression profiles of the benzene-treated group by PCA followed by the selection of genes with high contribution scores from certain PCA components. A union gene list generated from the above-mentioned 878 probe sets (genes) for C57BL/6 mice was subjected to genetic interaction analysis (KeyMolnet Lite Version 4.8; Institute of Medicinal Molecular Design, Inc., Tokyo, Japan) to determine the interaction among the genes in the list and a sample stochastic gene expression three pathways upstream of *cyclin D1* is selected. Gene expression level in each C57BL/6 mouse was designated by scale for an expression level shown by fold changes in graded expression level. The gene abbreviations in the figure are as follows: *TCF*, T-cell-specific transcription factor, KMMC (KeyMolnet ID number) 03078; *HDAC2*, histone deacetylase 2, KMMC 05302; *cyclinD1*, cyclin D1, KMMC 00061; E2F, transcription factor E2F, KMMC 00143; *AP-1*, activator protein 1, KMMC 00101; *Bcl-6*, B-cell lymphoma 6, KMMC 03878.

2008; Shen *et al.*, 2008; Vasanwala *et al.*, 2002; Ye *et al.*, 2009). The stochasticity of mechanisms underlying these different stochastic signalling is not known yet, but each upstream signalling pathway towards *cyclin D1* from *AP-1* superfamilies and *E2F* is different from one mouse to another, as shown by the different expression levels of *cyclin D1* in these mice; thus, the epigenetic impact on cell cycling should be different from one mouse to another. These stochastic differences in gene expression levels in signalling pathways after benzene exposure are supposed to be linked to different pathological endpoints of mice. Thus, if a large number of experimental samples were used, each signalling pathway would be composed of a large number of gene clusters. Those possible probabilistic

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signalling pathways would be established as a probabilistic signalling network in systems biology. Those gene clusters and pathways therefore predict probabilistic toxicological endpoints simultaneously. If a large number of experimental data would be obtained to elucidate those clusters in the near future, essential data obtained in the early phase may computationally predict future endpoints.

3.2 Ionizing Radiation

3.2.1 Background

Two radiation doses, 0.6 and 3 Gy, were used to compare gene expression profiles of irradiated mice with those of a non-irradiated control. ^{137}Cs-gamma-ray or X-ray at 3 Gy, in general, produces the maximum incidence of myeloid leukaemias in C3H/He mice because the risk of radiation-induced leukaemogenesis may be identified by the integral between the area under the stem/progenitor cell survival curve and the area above the curve for the minimum number of mutated stem/progenitor cells for the development of at least one case of leukaemia as a function of radiation dose (Hirabayashi and Inoue, 2007).

The maximum ratio of one curve to the other curve, in general, is calculated as 3 Gy, which produces about 30% leukaemias. With decreasing dose of radiation exposure on the other hand, the incidence of haematopoietic malignancy decreases, and the dose at which 5% haematopoietic malignancies is induced is estimated to be about 0.6 Gy. Five mice each for radiation exposure at 3, 0.6 and 0 Gy were examined by gene chip microarray analysis 28 days after radiation, by which time acute responses would have ceased and only long-term responses remained.

The common gene profiles and the stochastic gene profiles were focused, similarly to those in the previous section, on the ageing effect of steady-state gene expression. First, the common gene expressions were obtained by one-way ANOVA without FDR (the common gene profiles). Second, those common genes with specific functions in haematopoietic stem cells listed by Ivanova *et al*. 2002 were identified. Third, the union genes from individual significant expression profiles of each radiation-dose group and with specific functions listed by Ivanova and coworkers were selected by comparing their expressions with the general expressions of the non-irradiated group (the stochastic gene profiles).

For the common gene profile, 520 genes (probe sets) were selected by the Welch *t*-test with the confidence level set at a *P*-value smaller than 0.005, and their relationships were analysed using a dendrogram and shown in Figure 7. This dendrogram shows branches for dose-specific clusters and dose-dependent clusters. In this study, dose-response-dependent clusters were used to unsupervisedly select 226 probe sets from the 520 probe sets. The functions of genes were overviewed focusing on their effects on the irradiated recipients, and representative functional genes were identified, such as their effects on cell cycle regulation, DNA repair, apoptosis, induction of genomic and/or chromosomal instability, abnormal cellular proliferation and differentiation, dysfunction of suppressor genes and consequent enhancement of induction of neoplastic plausibility and, lastly, dysregulation and dysfunction of haematopoiesis.

The characteristics of the common and stochastic gene expression profiles that overlapped with those in Ivanova's list were focused on.

3.2.2 Common Gene Expressions

DNA Damage and Abnormal Repair

Genes that induce DNA damage and abnormal repair inhibit nucleotide excision repair owing to the down-modulation of the *Rad23* homologue *mHR23a* (Ng *et al*., 2003) and increase sensitivity to 5-fluorouracil and possibly also to ionizing radiation owing to the down-modulation of *UPB1* [ureidopropionase, beta (Thomas *et al*., 2008)], which is also related to hypotonia and dystonic movement (Moolenaar *et al*., 2001; Ohse *et al*., 2002). Concomitantly, as a type of cell cycle modulation, an attenuated *Trp*53 function owing to the down-modulation of *Setd7* was observed, inducing the plausible up-regulation of the pathway leading to tumourigenesis (Ivanov *et al*., 2007), namely enhancement of DNA damage as well as *Chk1*-dependent S-phase arrest owing to the down-regulation of *Setd8* (Jorgensen *et al*., 2007) and a delayed transition from the G2- to S-phase owing to *cyclin A/cdk 2* dysfunction induced by *SCAPER* (S-phase cyclin A-associated protein in the ER) down-modulation (Tsang *et al*., 2007). The down-modulation of *CT alpha*/*Pcyt1a* (phosphate cytidylyltransferase 1, choline, alpha

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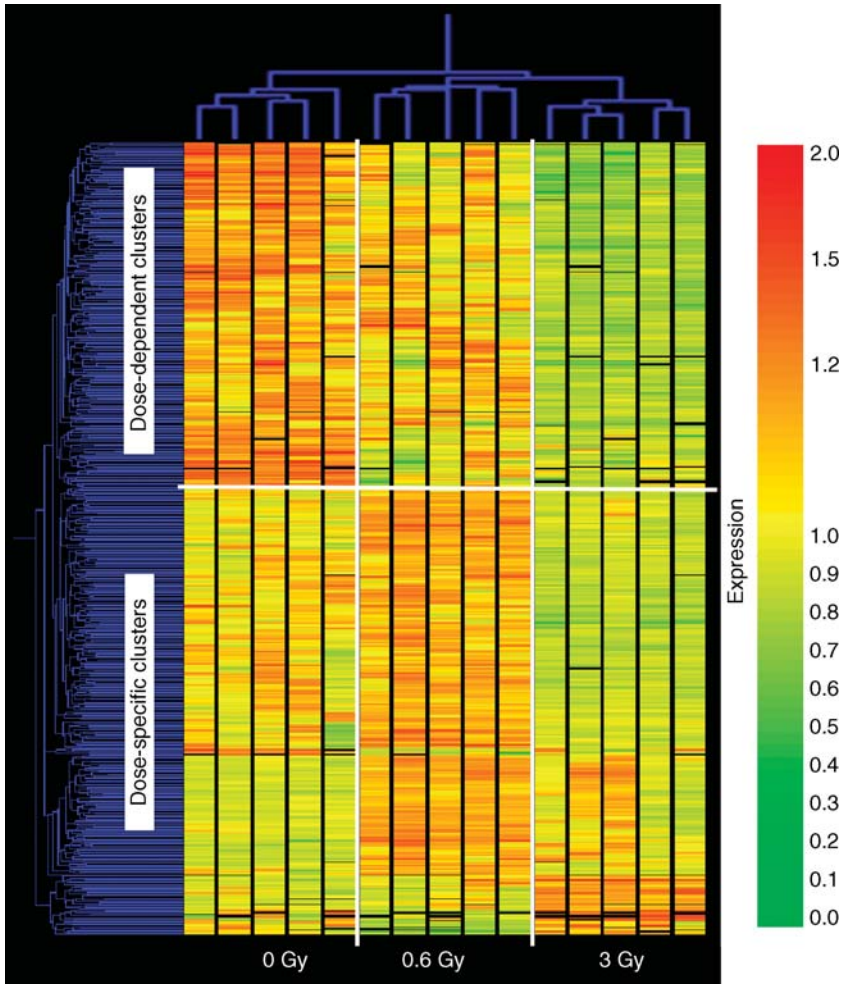

Figure 7. Welch *t*-test selected 520 probe sets from gene expression profiles for bone marrow cells from C57BL/6 mice with/without graded dose of gamma-ray irradiation, and dose-dependent cluster genes and dose-specific cluster genes discriminated by dendrogram: 520 probe sets (genes) were selected by Welch *t*-test ($P < 0.005$) from gene expression profiles among groups of bone marrow cells from C57BL/6 mice (five mice each) irradiated with 0.6 or 3.0 Gy ^{137}Cs-gamma-ray or mice without irradiation 28 days before gene chip microarray analysis, and applied to a dendrogram to discriminate dose-dependent cluster genes and dose-specific cluster genes.

isoform) induces cell cycle arrest (Banchio, Schang and Vance, 2004). The down-modulation of *G2E3* (G2/M-phase-specific E3 ubiquitin ligase) induces cell cycle dysregulation owing to consequent DNA damage (Brooks, Banerjee and Crawford, 2007), and the down-modulation of *Steap3* (STEAP family member 3)/*Tsap6* (tumour suppressor-activated pathway 6) induces downstream *Trp*53 to suppress the up-regulation of the cell cycle (Passer *et al*., 2003). Furthermore, the down-modulation of *SmarcA2* (SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 2) induces cell cycle dysregulation (Zraly, Marenda and Dingwall, 2004), and the inhibition of S-phase transcription of *CT alpha*/*Pcyt1a* in turn inhibits phosphorylation during the cell cycle, resulting in the attenuated phosphorylation of *Cdk2* (Banchio, Schang and Vance, 2004).

Apoptosis-Related Gene Expressions

Concerning the alterations of apoptosis-related gene expressions, three types of apoptotic change, namely enhancement of apoptosis, suppression of apoptosis and apoptotic regulatory failures, are observed. *Atrx* (α thalassaemia/mental retardation syndrome X-linked homologue)/*Rad54* functions as

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a radiation-induced helicase, which is a biomarker of breast cancer (Roy *et al*., 2008). With decreasing expression level of *Atrx/Rad54*, *Trp*53-dependent apoptosis is up-regulated (Seah *et al*., 2008). *Plagl2* (pleiomorphic adenoma gene-like 2) functions as a suppressor gene, collaborating with *Hif1* downstream of the *Hif1* pathway (Mizutani *et a*l., 2002), and with the down-modulation of *Plagl2*, apoptosis is down-modulated. Furthermore, apoptosis is suppressed by the down-modulation of *Steap3*/*Tsap6*, which consequently induces cell cycle arrest (Passer *et al*., 2003). Furthermore, the down-modulation of *Xiap* (X-linked inhibitor of apoptosis), which interacts with *Chek1* (Galvan, Kurakin and Bredesen, 2004), activates apoptosis and increases the level of reactive oxygen species (ROS) (Resch *et al*., 2008), which further activates apoptosis. *Rbm5* enhances the splicing regulation of antiapoptotic *caspase 2* (Fushimi *et al*., 2008), leading to *Rbm5* modulation that in turn suppresses apoptosis. There are apoptotic regulatory failures, such as that induced by a decreased expression level of *Rock1* (Rho-associated, coiled-coil-containing protein kinase 1), which is induced by the down-regulation of *caspase 3* (Ongusaha *et al*., 2008) and induces the down-regulation of *Fas*-mediated apoptosis via phosphorylation of *erzin* and *moesin* by *Rock1* itself (Hebert *et al*., 2008). Similarly, concerning apoptosis regulatory failure, when *Tr4* (testicular orphan nuclear receptor 4)*/NR2C2* (nuclear receptor subfamily 2, group C, member 2) is down-regulated, radiation-induced apoptosis seems to occur (Kim *et al*., 2007). The down-regulation of *Irf8* (interferon regulatory factor 8)/*ICSBP* (interferon consensus sequence-binding protein) regulates the induction of apoptosis and the expression of *Fas* (Yang *et al*., 2007). Furthermore, *Map3K5* is an apoptosis-regulating signal kinase of *ASK-1* (Ichijo *et al*., 1997), which usually binds to *thioredoxin* (*Trx*) (Saitoh *et al*., 1998). When *ASK-1* is activated along with the transformation of the *Trx* oxidative form by ROS, it then induces apoptosis (Fujino *et al*., 2007).

Instability of Genomic DNA and Chromosomes

Radiation induces regeneration failure and instability of genomic DNA and chromosomes. There are several notable findings on induction of genomic instability by the alteration of the *SMC* family (Losada and Hirano, 2005). First, genomic instability related to *ATM* failure is induced by the down-modulation of *SMC1a* (Kitagawa *et al*., 2004). Second, chromosomal instability is also induced by the down-modulation of both *SMC2* and *SMC4*, which form the core of codesin functioning in chromosome assembly and segregation (Losada and Hirano, 2005).

Interestingly, the down-regulation of *Bach1* (BTB and CNC homology 1, basic leucine zipper transcription factor 1) induces accelerated ageing owing to the cancelation of the suppression of *Trp*53-dependent premature senescence possibly owing to oxidative stress. Along with this cancelation, genomic instability is also induced by *Trp*53 dysfunction (Dohi *et al*., 2008). In addition, the down-modulation of *Supt16h* (suppressor of Ty16 homologue)/*Cdc68* and *FUS* (fused sarcoma)/*TLS* (translocated in liposarcoma) induces genomic instability owing to oxidative changes in *ATM* after ionizing-radiation exposure (Gardiner *et al*., 2008; O'Donnell *et al*., 2004). Furthermore, not only genomic instability but also chromosomal instability is induced by the down-modulation of *SMC2* and *SMC4* (Losada and Hirano, 2005). *Akap9* (A kinase anchor protein 9), which is normally involved in chromosomal rearrangement in thyroid cancer cells after radiation (Ciampi *et al*., 2005), induces the radiation-dose-dependent down-modulation of gene expression also in the bone marrow and, thus, chromosomal rearrangement as well. *Baz2b* (bromodomain adjacent to zinc finger domain, 2B) generally functions in chromatin remodelling in collaboration with *Acf1* (Jones *et al*., 2000); however, the down-modulation of *Baz2b* after ionizing radiation induces chromatin instability. The down-modulation of *CTCF* (CCCTC-binding factor), in collaboration with *rad21*, results in the suppressive regulation of orchestration of the down-regulation of the chromatin loop structure in the B-cell system by an epigenetic mechanism (Degner *et al*., 2009).

Cellular Proliferation Arrest

Concerning cellular proliferation arrest, progressive epithelial maturation is down-regulated in the small intestine by the down-regulation of *Cbfa2T2* (core-binding factor, runt domain, alpha subunit 2, translocated to 2)*/MTGR1* (myeloid translocation gene-related 1) (Amann *et al*., 2005). *Zbtb7a* (zinc finger and BTB domain containing 7a), a suppressor gene belonging to the *Rb* family, also called *Pokemon*, attenuates the suppression of *ckap4/p63* (Jeon

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et al., 2008), which negates the induction of cellular proliferation. On the other hand, regarding cellular differentiation arrest, the down-modulation of *CUL5* (cullin 5) suppresses cellular differentiation via the down-modulation of *E3* ubiquitination (Baxter *et al*., 2009). Furthermore, the down-regulation of *MEF2C* induces the dysregulation of differentiation of pluripotent progenitor cells as well as B cells (Gekas *et al*., 2009). However, the down-regulation of a regulator of nuclear receptor-mediated repression, *Ncor1* (nuclear receptor corepressor 1), also induces cellular differentiation arrest (Hermanson, Jepsen and Rosenfeld, 2002). The down-regulation of LIM and senescent cell antigen-like domain 1 (*lims1*) in megakaryoblast induces cellular differentiation abnormality (Chen, Hu and Shivdasani, 2007). Concerning cellular differentiation abnormality during the developmental stage, the down-regulation of *Arid5b* (AT-rich interactive domain 5B)*/Mrf2* (modulator recognition factor 2)/*desrt* induces growth retardation and failure of reproductive organ development (Lahoud *et al*., 2001). Thus, it is plausible that the down-regulation of *Arid5b* induces impairment of regeneration of the bone marrow after radiation exposure. Regarding the radiation effect on the tumour suppressor gene *NFATc3* (nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 3), the gene generally suppresses T-cell leukaemia cells as a tumour suppressor (Glud *et al*., 2005); however, the down-regulation of *NFATc3* suppresses T-helper cell type 2 (Th2)-mediated IL-13 production (Klein *et al*., 2006), thereby suppressing cellular proliferation. *MALAT1* (metastasis-associated lung adenocarcinoma transcript 1) is a *Trp*53-type suppressor gene and non-coding RNA, which is reported to be often up-regulated in malignant neoplasms (Guffanti *et al*., 2009). This up-regulation is supposed to mutate *MALAT1*. However, the down-modulation of non-mutated *MALAT1* may suppress cellular proliferation and differentiation in the bone marrow. *Bin1* (bridging integrator 1) is also a tumour suppressor, which induces proapoptotic cellular death (Cassimere, Pyndiah and Sakamuro, 2009). The down-modulation of *Bin1* cancels proapoptotic tumour suppression, which is considered to be a response to DNA damage for regeneration. *Irf8*/*ICSBP*, reported as a *Fas*-mediated apoptosis inducer (Yang *et al*., 2007) whose expression level is also reported to decrease during ageing (Stirewalt *et al*., 2009), suppresses apoptosis by radiation-induced down-modulation of itself, which may lead to leukaemogenic plausibility (Holtschke *et al*., 1996), as described above.

Tumourigenic/Leukaemogenic Plausibilities

Similarly to the down-modulation of *Irf8*, several genes show high tumourigenic/leukaemogenic plausibilities. The down-modulation of *Rasa1*, a tumour growth suppressor (Yang *et al*., 2009), results in the down-regulation of the suppressor function of *Rasa1*. *Topors* (topoisomerase I-binding protein)/*p53BP3* (p53-binding protein) is a *Trp*53-binding protein (Zhou, Wen and Ao, 1999) that stabilizes *Trp*53 by SUMOylation (Weger, Hammer and Heilbronn, 2005) and catalyses *Trp*53 by its down-regulation, resulting in an increase in tumourigenic plausibility. The down-regulation of *Gimapb* (a *GTPase*, *IMAP* family member 6) is observed in non-small cell carcinoma of the lung and is supposed to induce the dysregulation of neoplasms (Shiao *et al*., 2008). Thus, this down-regulation in the bone marrow after radiation exposure may promote leukaemogenesis. The down-regulation and dysregulation of *Jarid1-A* leads to leukaemogenic plausibility (Wang *et al*., 2009).

Haematopoiesis After Radiation Exposure

Lastly, concerning specific effect of radiation exposure on haematopoiesis, the most prominent effect is the suppression of haematopoiesis owing to the down-regulation of *Runx* via a G-protein gene, *Gna13* (guanine-nucleotide-binding protein, alpha 13), induced by the down-regulation of *Runx2* (Teplyuk *et al*., 2008). Another haematopoietic impairment is induced by the down-modulation of *Musashi homologue 2* (*MSI2*) through the attenuation of maintenance of stem cell identity (Siddall *et al*., 2006). The attenuation of maintenance of stem cell identity and also maintenance of T-cell identity in a haematopoietic microenvironment is induced by a decrease in the expression level of *Ccr9* (chemokine C-C motif receptor 9) (Chamberlain *et al*., 2008; Lai and Kondo, 2007; Wright *et al*., 2002). Similarly, the down-modulation of *c-cbl* (casitas B-lineage lymphoma) induces the instability of haematopoietic stem cell proliferation (Rathinam *et al*., 2008; Slape *et al*., 2008). *OLFM4* (olfactomedin 4) is a myeloid developmental marker (Chin *et al*., 2008). The down-modulation

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of *OLFM4* suppresses its own haematopoietic function. Furthermore, the suppression of *chd4* (chromodomain helicase DNA-binding protein 4) induces chromatin remodelling (Srinivasan *et al*., 2006). The suppression of *Irf8*/*ICSBP*, which is induced during ageing (Stirewalt *et al*., 2009), induces chronic myeloproliferation in collaboration with *Meis1*, which promotes chronic myeloid leukaemia (CML) of bone marrow cells (Hara *et al*., 2008). The suppression of *Clec2i* (C-type lectin domain family 2, member i)/*Clr-g* in turn suppresses T-cell proliferation and IL-2 production (Tian *et al*., 2005). Lastly, when *Trpm7* is down-modulated, thymocyte development at the double-negative stage (i.e. negative for both CD4 and CD8) is disrupted during thymopoiesis, which may have significance in leukaemogenic plausibility (Jin *et al*., 2008).

3.2.3 Common Gene Profiles Overlapped With Those in the List of Ivanova et al.

Ivanova *et al*. (2002) established the stem-cell-specific gene expression profiles from the cDNA library obtained from immature haematopoietic stem-cell compartment sorted by flow cytometry. It is of interest to compare the common gene expression profile of the bone marrow after ionizing radiation with those stem-cell-specific gene expression profiles listed by Ivanova and coworkers. Eighteen probe sets (17 genes) showed overlapping. Among them, eight probe sets (seven genes) showed irradiation-dose-dependent down-regulation (dose-dependent cluster), eight probe sets were commonly up-regulated only in the 0.6-Gy-irradiated group and one probe set was commonly up-regulated only in the 3-Gy-irradiated group (dose-specific clusters). The dose-dependent genes were *Sltm* (RIKEN cDNA 5730555F13 gene), *Mef2c* (myocyte enhancer factor 2C), *Centd3* (RIKEN cDNA E030006K04 gene), *Serpinf1* [serine (or cysteine) proteinase inhibitor, clade F, member 1], *Slc12a2* (solute carrier family 12, member 2), *Rora* (retinoic acid receptor (RAR)-related orphan receptor α) and *Runx2* (runt-related transcription factor 2). The dose-specific genes in the 0.6-Gy-irradiated group were *Cox8a* (cytochrome *c* oxidase, subunit VIIIa), *Uba1* (ubiquitin-activating enzyme E1, Chr X), *Fkbp1a* (FK506-binding protein 1a), *Crebl1* (cyclic adenosine monophosphate (cAMP)-responsive element-binding protein-like 1), *Ivns1abp* (influenza virus NS1A-binding protein), *Ppap2c* (phosphatidic acid phosphatase type 2c), *Krt10* (keratin, type I cytoskeletal 10), *Terf2* (telomeric repeat binding factor 2) and *Lass4* (longevity assurance homologue 4). Only one gene, *Snrpc* (U1 small nuclear ribonucleoprotein 1C), was up-regulated in the 3-Gy-group.

3.2.4 Stochastic Gene Profiles Overlapped With Those in the List of Ivanova et al.

Each gene expression profile for the 0.6- and 3-Gy-irradiated individual mice is compared with gene expression profiles for five mice of the non-irradiated group by PCA, and union genes were collected to establish the stochastic gene profiles. Genes showing overlapping of their stochastic expression gene profiles with those in Ivanova's list were analysed. From the comparison between the 0- and 3-Gy-irradiated groups, 65 genes were selected, which included *Acadl* (acyl-coenzyme A dehydrogenase, long-chain), *Apc* (adenomatosis polyposis coli), *Ccnd1* (cyclin D1), *Ctnnd1* [catenin (cadherin-associated protein), delta 1], *Dhcr7* (7-dehydrocholesterol reductase), *Dll1* (delta-like 1), *Hspa9* (heat-shock protein 9), *Igbp1* [immunoglobulin (CD79A)-binding protein 1], *Myb* (myeloblastosis oncogene), *Mycbp2* (MYC-binding protein 2), *Tnf* (tumour necrosis factor) and *Wnt6* (wingless-related MMTV integration site 6) among others. The expressions of these genes were individually stochastic in not only 3-Gy-irradiated mice but also non-irradiated mice. Interestingly, two-thirds of these 65 genes were individually up-regulated in the non-irradiated group. From the comparison between the 0- and 0.6-Gy-irradiated groups, 36 genes were selected, which included *Baalc* (brain and acute leukaemia, cytoplasmic), *Bmp2* (bone morphogenetic protein 2), *Cdc14b* (CDC14 cell division cycle 14 homologue B), *Gja1* (gap junction protein, alpha 1/connexin 43), *Gstm1* (glutathione *S*-transferase, μ1), *Lamc1* (laminin, γ1), *Sox3* (SRY-box-containing gene 3) and *Upp1* (uridine phosphorylase 1) among others. The expressions of these genes were interestingly also individually stochastic in not only 0.6-Gy-irradiated mice but also non-irradiated mice. Furthermore, most of these 36 genes were individually up-regulated from the non-irradiated group to the 0.6-Gy-irradiated group. Whereas most of the genes, including *Runx2* and *Mef2c*, responsible for maintaining haematopoiesis were

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down-regulated after radiation exposure specifically in the 3-Gy-irradiated mice in the case of the common gene expressions, more than one-half of genes whose functions are considered to be acceleration of the cell cycle or elimination of oxidative stresses, such as *Cyclin D1* and *Hspa9*, are, in contrast, up-regulated in the case of stochastic gene expressions.

4 HAEMATOPOIETIC NEOPLASMS

In toxicological pathology, the possible carcinogenicity/leukaemogenicity of a chemical compound is assayed by comparing neoplastic and pre-neoplastic histopathological findings between experimental group(s) treated with the test chemical and the control group treated solely with a vehicle. This is based on the conceptual principles on the development of neoplasms and pre-neoplastic histopathological findings established over the past hundred years of history of pathology since the time of Ludwig Aschoff.

When one analyses such neoplasms using a gene chip microarray technique, it should not be used to simply support findings obtained by conventional histopathological technologies and methods, not only because of unavailable databases to link such tumour-specific gene expression profiles to findings of such conventional pathological technologies and methods, but also because of further possible potentials of gene chip microarray methods. Namely, a gene chip microarray, for instance, provides not only gene expression profiles for pathological endpoints that are comparable to neoplastic endpoints related to pathological diagnosis, but also gene expression profiles for toxicological endpoints, which are linked to multiple clusters that predict epigenetic pathways before irreversible morphological fixation to pathological conditions (Table 2). In the latter case, the type of genes that would be profiled in each cluster is not fully established owing to the lack of concept and insufficient databases for toxicological predictable profiles. However, a probabilistic predictability by the latter approach may open the door to systems toxicology, which is an attractive and powerful new toxicological methodology established after the completion of the whole-genome reading project established in 2000 [*C. elegans* (*C elegans* Sequencing Consortium, 1998), *Drosophila* (Adams *et al*., 2000), mice (Waterston *et al*., 2002) and humans (Lander *et al*., 2001)].

Table 2. Gene expression profiles: pathological endpoints and toxicological endpoints.

Pathological endpoints
Definitive profile (commonality)
Deterministic
Diagnostic
Toxicological endpoints
Epigenetic profile (stochasticity)
Probabilistic
Predictable

Gene expression profiles for neoplastic tissues and surrounding tissues both consist of profiles equivalent to those for the senescent animals, owing to the fact that such neoplastic tissues developed from equally senescent cells in the tissues. Sometimes, cancer tissues for microarray analysis are obtained by microdissection. However, gene expression profiles obtained from cancer tissues by microdissection still consist of common gene expression profiles linked to senescence as the major phenotype. Most of the gene expression profiles obtained from cancer tissues correspond to those of senescent animals. Namely, when one recalls the historical experiment conducted by Beatrice Mintz (Dewey *et al*., 1977), one can realize the fact that the major gene expression profiles from cancer tissue analysed using microarray techniques are largely those without carcinogenic phenotypes. On the other hand, one may be only able to obtain a profile for a definitive pathological endpoint for diagnosis, but fail to obtain epigenetic toxicological profiles for predicting possible tumourigenic plausibility. Microdissection excludes pre-neoplastic epigenetic changes; thus, it is unable to provide predictive cancer-specific plausible gene expression profiles from the cancer tissue, and we need another molecular strategy to replace microdissection techniques to select plausible or definitive cancer-specific gene expression profiles from most non-neoplastic gene expression profiles.

What would be an essential molecular biological strategy to replace microdissection techniques to select expression profiles related to tumourigenicity? The strategic changes in the toxicological paradigm occurring from the histological definition to the molecular biological/toxicological definition of toxicology are one of the essential topics of this section, that is to make gene expression profiles of the surrounding tissues clusterized into stochastic union genes computationally. In this section,

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gene expression profiles for two types of myeloid leukaemia, one developed in the bone marrow after whole-body radiation exposure (1.5–3.0 Gy) and the other developed spontaneously in animals without radiation exposure, are chosen as the analytical models for microarray analysis. Methodologically, common gene profiles are those of genes expressed commonly among the experimental groups, excluding genes expressed in the non-treated, 21-month-old control group. Stochastic gene expression profiles, on the other hand, are those of genes expressed in individuals of the experimental group, differentiated from genes expressed in the non-treated 21-month-old control group. In the former, a number of senescent genes including housekeeping genes are appropriately removed. In the common gene profiles, genes are expressed in all members of the group but are not always expressed at a high level, whereas in the latter, gene expression profiles cannot be differentiated using a dendrogram between the experimental group and the control group. However, the profiles can be differentiated by PCA without specific supervised analysis of a signalling cascade, because radiation-induced myeloid leukaemias and spontaneous myeloid leukaemias, used as models in this section, show epigenetic and stochastic characteristics in terms of the mechanism of leukaemogenic progression. Namely, the characteristics of expression profiles are not obtained for genes commonly expressed but for genes differently and stochastically expressed.

As mentioned above, PCA and/or equivalent analytical tools define each individual gene expressed by a Euclidean vector, which has been realized owing to the remarkable progress of microarray techniques with improved repeatability. It is speculated that future progress in the field of one-cell microarray analysis may clearly elucidate epigenetic individual differences during the developmental stage and open the road to stochastic toxicology.

4.1 Radiation-Induced Myeloid Leukaemias Versus 21-Month-Old Bone Marrow Cells

In this section, radiation-induced myeloid leukaemias are focused on as an analytical model for use in gene chip microarray analysis. Radiation-induced myeloid leukaemias may develop in over 300 radiation-fragile sites with probabilistic random hitting, and they may develop as an effect of epigenetic and stochastic radiation causing deterministic DNA damage. Thus, the model is appropriate for analysing general systems toxicology from two viewpoints, that is commonality and stochasticity. The development of myeloid leukaemias in C3H/He mice clearly shows treatment-dependent specificity with a lower background incidence in the non-treated control, that is 1% spontaneous myeloid leukaemia; thus, C3H/He mice were utilized in the present study.

Myeloid leukaemias were selected from those developed from mice exposed to 1.5–3.0 Gy, where the ratio of the integral becomes comparatively higher between the radiation survival curve for haematopoietic stem/progenitor cells along the radiation exposure and the curve for the minimum number of mutated stem/progenitor cells for the development of at least one case of leukaemia as a function of radiation dose. The detailed experimental procedure is described elsewhere (Hirabayashi *et al*., 2009).

Figure 8 shows linear configurations of all the expressed genes that had been connected for over five non-treated 21-month-old bone marrow cells without developing any particular haematopoietic diseases (Nos 01 to 05 in the left panel) and six cases with radiation-induced myeloid leukaemias that developed after radiation exposures (Nos 06 to 11 in the right panel). When one focuses on one case, No. 03 for example, and categorizes genes in the order of expression level from the highest (red line) to the lowest (green line), the order of genes from the reddish line to the greenish line in each case, as observed in No. 03, is essentially comparable to that of the five individual cases in the left panel (Nos 01 to 05). Namely, the order of gene expression levels in the left panel is essentially comparable among the cases in the group (i.e. up-regulated genes in the case of No. 03 designated by greenish lines are nearly identical to those in the cases from Nos. 01 to 05). However, the order of gene expression profiles for radiation-induced leukaemia cases shown from Nos 06 to 11 is the opposite across these cases compared with those for No. 03. Namely, genes designated by reddish lines, which are highly expressed in No. 03, tend to appear in the low-expression-level zone with increased diversity and degree of individual differences in the cases of radiation-induced leukaemias from Nos 06 to 11.

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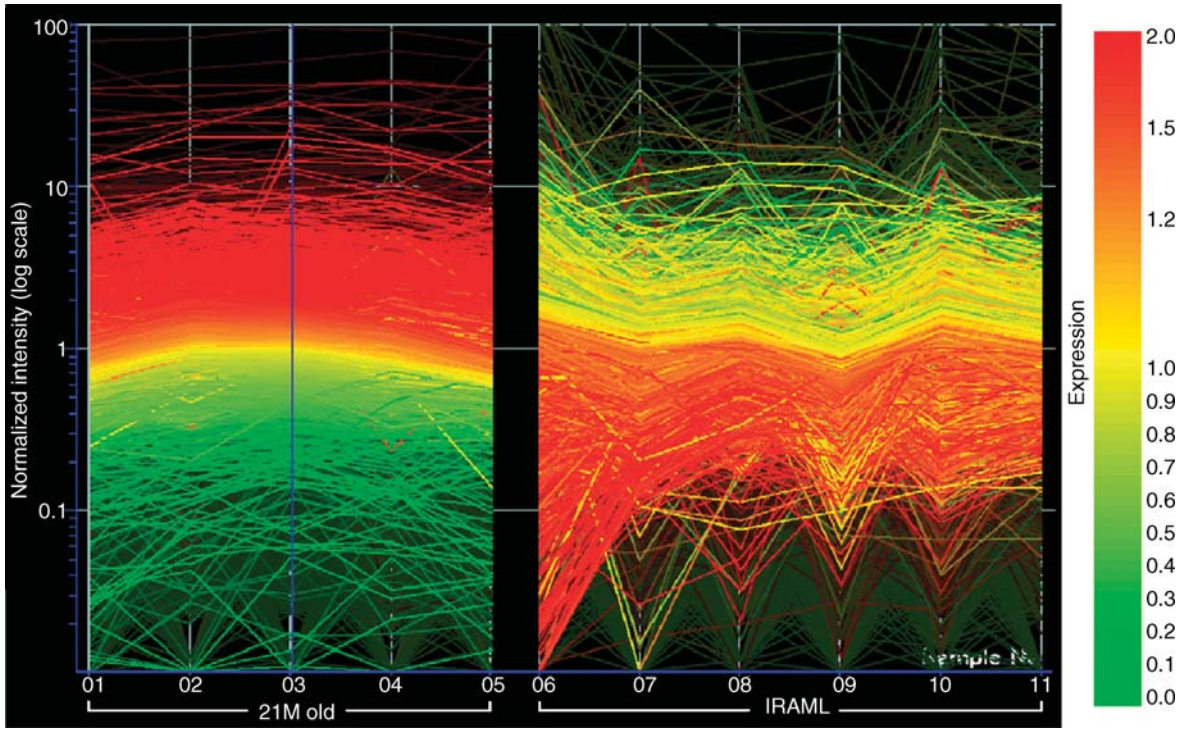

Figure 8. Linear configurations of gene expression profiles for 21-month-old bone marrow cells and radiation-induced myeloid leukaemias: microarray profiles differentiate 21-month-old bone marrow cells from radiation-induced myeloid leukaemias. The five individual data points in the left panel are for bone marrow cells from 21-month-old C3H/He mice. The other six individual data points in the right panel are for 3 Gy-radiation-induced myeloid leukaemias in C3H/He mice. Along the scale for gene expression level from the highest (red) to the lowest (green) for No. 03, the same genes of the other individuals are connected and shown by the same expression intensity colour. Accordingly, genes over-expressed in the 21-month-old mice are largely repressed in radiation-induced myeloid leukaemia cases.

Furthermore, the reddish line configurations, that is genes that are highly expressed in the 21-month-old bone marrow, are largely located below 1.0 for radiation-induced myeloid leukaemia cases. In contrast, the greenish line configurations, that is genes that are highly expressed associated with radiation-induced leukaemias, show a low expression level in 21-month-old bone marrow cells.

When one compares these gene expressions in 21-month-old bone marrow cells with those in radiation-induced myeloid leukaemias by PCA (Figure 9a), an individual gene expression in 21-month-old bone marrow cells and all gene expression profiles in radiation-induced myeloid leukaemia cases are differentiated clearly not only by component #1 but also by the eigenvector value of several components. Using components #1, #2 and #3, we can separate both groups in the three-dimensional expression diagram shown in Figure 9b. Gene expression profiles of 21-month-old bone marrow cells showed lower individual commonality than those of 2-month-old bone marrow cells (Figure 1). However, when 21-month-old bone marrow cells were compared with those of radiation-induced myeloid leukaemias, both gene expression level and commonality increased in the latter (Figure 8). These trends observed in the radiation-induced myeloid leukaemias are further extended to spontaneous myeloid leukaemia cases, as described in the next section, largely owing to the increase in the stochasticity of gene expressions.

Each gene expression profile of each individual radiation-induced myeloid leukaemia case was compared with a set of gene expression profiles of the group of 21-month-old mice by individual PCA. Union gene repertories from all PCA data with a contribution score of over 1.0 provided

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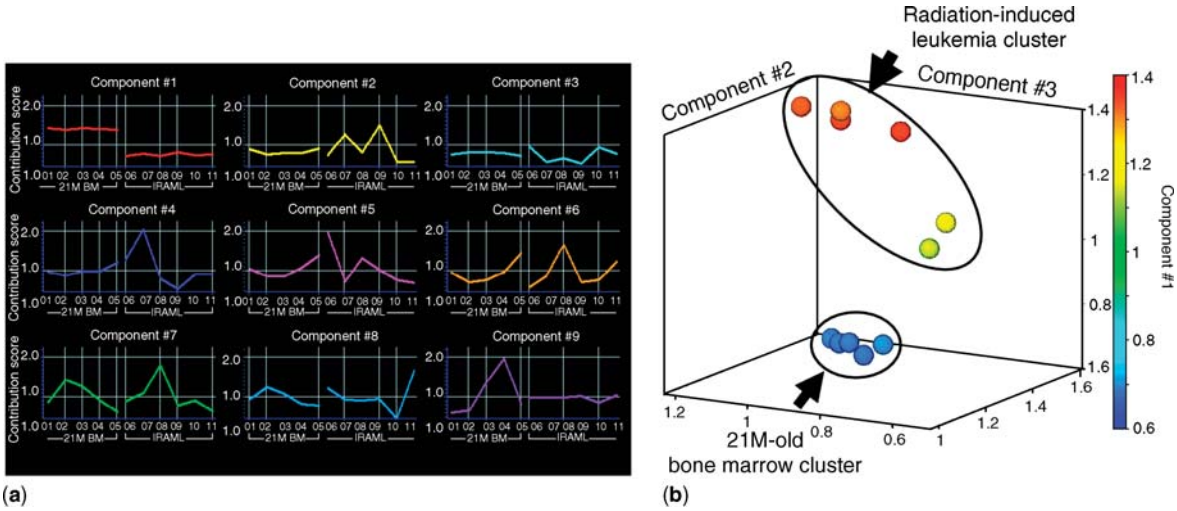

Figure 9. PCA differentiates radiation-induced leukaemias from 21-month-old bone marrow cells: three-dimensional expression clustering from results of PCA of six cases of radiation-induced myelogenous leukaemias and five cases each of 21-month-old bone marrow cells are shown in the three-dimensional contribution scores for components #1, #2 and #3, which discriminate the radiation-induced myelogenous leukaemia cluster from the 21-month-old bone marrow cell cluster. The line graph in the left panel shows actual contribution scores converted from each eigenvector value (a), which were used for the clustering of three-dimensional expressions shown in the right panel (b). Note that the contribution scores of the radiation-induced myelogenous leukaemia clusters in (a), except for components #1 and #9, comparatively diverge from those of 21-month-old bone marrow cell clusters in (a).

242 probe sets. Among them, genes with leukaemogenic/tumourigenic plausibility are tabulated in Table 3.

4.2 Spontaneous Myeloid Leukaemias Versus 21-Month-Old Bone Marrow Cells

Gene expression profiles for spontaneous myeloid leukaemias are shown by linear configurations for six mice in the right panel of Figure 10. As compared with those for 21-month-old bone marrow cells in the left panel of Figure 10, which is the same as the left panel of Figure 8, the individual expression profiles for these spontaneous myeloid leukaemia cases vary markedly from each other.

It is of interest that the expression profiles for radiation-induced myeloid leukaemias in the right panel of Figure 8 show a slightly higher commonality than the expression profiles for the spontaneous myeloid leukaemias shown in the right panel of Figure 10. Genes that are up-regulated, as shown by reddish lines in the left panels of Figures 8 and 10, are generally down-regulated in the radiation-induced myeloid leukaemia cases, as shown in the right panel of Figure 8. On the other hand, the reddish lines show different levels among cases of spontaneous myeloid leukaemias, whose detailed underlying mechanisms have been analysed and reported elsewhere (Hirabayashi *et al*., 2009).

As observed in the right panel of Figure 10, the expression profiles for spontaneous myeloid leukaemias show marked stochasticity. However, when one compares these expression profiles with those for 21-month-old bone marrow cells and extracts their characteristic common genes by PCA, these gene groups that are up- or down-regulated, and essentially are different from the common genes observed in the radiation-induced myeloid leukaemia cases, were selected, except for four genes, *MLLT7/Foxo4* (folkhead box O4)*/AFX*, *Cald1*, *Txnl2* (*Thioredoxin-like 2*)*/Glxn3* (*glutaredoxin 3*) and *ApoE* (183 probe sets were selected on the basis of the contribution score of component #1 of over 0.9 or under −0.9). First, several genes considered to function as suppressor genes are elucidated to play a role of tumourigenic/leukaemogenic promoters. Namely, myeloid/lymphoid or mixed lineage leukaemia 3 (*Mll3*) was up-regulated, thus functioning as a leukaemogenic promoter (Ruault *et al*., 2002; Tan and Chow, 2001).

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Table 3. Genes with leukaemogenic/tumourigenic plausibility selected from union gene repertories, in all PCAs analyses with contribution scores over 1.0; 242 probe sets, which are generated from gene expression profiles of individual cases of radiation-induced myeloid leukaemias compared with a set of gene expression profiles of 21-month-old mice obtained by individual PCA.

Affymetrix systemic name	Common name	Genbank ID	Descriptions
1415874_at	*Spry1*	NM_011896	Sprouty homologue 1 (*Drosophila*)
1417602_at	*Per2*	AF035830	Period homologue 2 (*Drosophila*)
1417851_at	*Cxcl13*	AF030636	Chemokine (C–X–C motif) ligand 13
1418062_at	*Eef1a2*	NM_007906	Eukaryotic translation elongation factor 1 alpha 2
1418547_at	*Tfpi2*	NM_009364	Tissue factor pathway inhibitor 2
1418597_at	*Top3a*	NM_009410	Topoisomerase (DNA) III alpha
1419417_at	*Vegfc*	NM_009506	Vascular endothelial growth factor C
1420034_at	*Ppp2r2d*	AU019644	Protein phosphatase 2, regulatory subunit B, delta isoform
1421462_a_at	*Lepre1*	NM_019783	Leprecan 1
1423259_at	*Idb4*	BB121406	Inhibitor of DNA binding protein 4
1424041_s_at	*C1s*	BC022123	Complement component 1, s subcomponent
1424228_at	*Polr3h*	AK019868	Polymerase (RNA) III (DNA directed) polypeptide H
1424295_at	*Dppa3*	AY082485	Developmental pluripotency-associated protein 3
1425278_at	*Ube4a*	BC021406	Ubiquitination factor E4A, UFD2 homologue (*S. cerevisiae*)
1425366_a_at	*Hus1*	AF076845	Hus1 homologue (*S. pombe*)
1425608_at	*Dusp3*	BC016269	Dual-specificity phosphatase 3 (*vaccinia virus phosphatase VH1-related*)
1425750_a_at	*Jak3*	L40172	Janus kinase 3
1427833_at	*Spi16*	U96702	Serine protease inhibitor 16
1427843_at	*Kua*	AB012278	Transmembrane protein 189
1434369_a_at	*Cryab*	AV016515	Crystallin, alpha B
1435132_at	*Disp1*	AI505698	Dispatched homologue 1 (*Drosophila*)
1436682_at	*Tmsb10*	AW259435	Thymosin, beta 10
1437106_at	*Jarid1a*	BM246184	Jumonji, AT-rich interactive domain 1A (Rbp2-like)
1437307_at	*Senp8*	BG069815	SUMO/sentrin-specific peptidase 8
1437473_at	*Maf*	AV284857	Avian musculoaponeurotic fibrosarcoma (*v-maf*) AS42 oncogene homologue
1437863_at	*Bche*	BB667762	Butyrylcholinesterase
1443229_at	*Atad2*	AV319821	ATPase family, AAA-domain-containing 2
1443952_at	*Nr1d1*	BI525006	Nuclear receptor subfamily 1, group D, member 1
1447897_x_at	*Anapc11*	AV019615	Anaphase-promoting complex subunit 11 homologue (*yeast*)
1448169_at	*Krt18*	NM_010664	Keratin 18
1449481_at	*Slc25a13*	BC016571	Solute carrier family 25 (mitochondrial carrier, adenine nucleotide translocator), member 13
1449700_at	*Igbp1*	C81413	Immunoglobulin (CD79A)-binding protein 1
1450173_at	*Ripk2*	NM_138952	Receptor (TNFRSF)-interacting serine-threonine kinase 2
1450199_a_at	*Stab1*	NM_138672	Stabilin 1
1450208_a_at	*Elmo1*	NM_080288	Engulfment and cell motility 1, ced-12 homologue (*C. elegans*)
1450297_at	*Il6*	NM_031168	Interleukin 6
1452905_at	*Meg3*	AV015833	Maternally expressed protein 3
1453227_at	*Rhobtb3*	BG801497	Rho-related BTB-domain containing protein 3
1455158_at	*Itga3*	BI664675	Integrin alpha 3
1455297_at	*Spin2*	BG070258	Spindlin family, member 2
1455985_x_at	*Shmt2*	AV213251	Serine hydroxymethyltransferase 2 (mitochondrial)
1456975_at	*Taok1*	BM238077	TAO kinase 1
1457040_at	*Lgi2*	BE947711	Leucine-rich repeat LGI family, member 2
1457311_at	*Camk2a*	AW490258	Calcium/calmodulin-dependent protein kinase II alpha
1458047_at	*Tnfsf13b*	BB667811	Tumour necrosis factor (ligand) superfamily, member 13b
1458381_at	*Clic5*	BB028501	Chloride intracellular channel 5
1458641_at	*Braf*	BM217816	Braf transforming gene
1459868_x_at	*Il11ra1*	AV313111	Interleukin 11 receptor, alpha chain 1
1460170_at	*Ext2*	NM_010163	Exostoses (multiple) 2
1460666_a_at	*Ebf3*	NM_010096	Early B-cell factor 3

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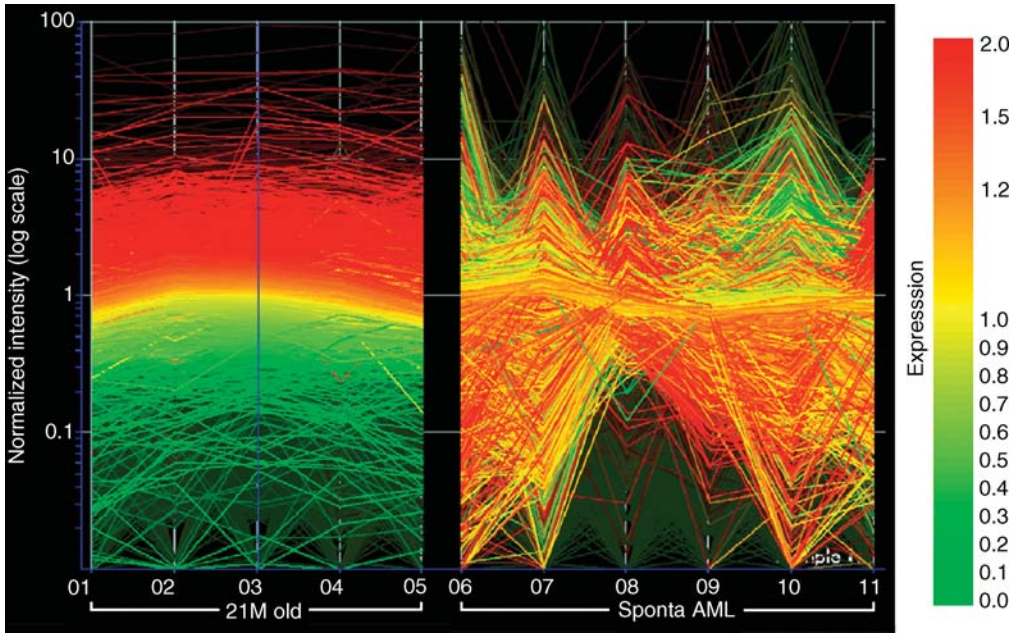

Figure 10. Linear configurations of gene expression profiles for 21-month-old bone marrow cells and spontaneous myeloid leukaemias: microarray profiles differentiate 21-month-old bone marrow cells from spontaneous myeloid leukaemias. The five individual data points in the left panel are for bone marrow cells from 21-month-old C3H/He mice. The other six individual points in the right panel are for spontaneous myeloid leukaemias in C3H/He mice. Along the scale for gene expression level from the highest (red) to the lowest (green) for No. 03, the same genes are connected and shown by the same intensity colour of gene expression. Accordingly, genes over-expressed in the 21-month-old mice are stochastically absent from one case to the other in spontaneous myeloid leukaemias.

MLLT7/Foxo4/AFX, which activate apoptosis (Tang *et al*., 2002) and are also known to be partner genes of fusion with *MLL* in acute leukaemias (Borkhardt *et al*., 1997), were down-regulated; thus, this would be functioning also as a promoter of development of leukaemias/lymphomas. Generalized down-regulation of *Pten* was observed, whose relevance to the development of HPNs has been reviewed elsewhere recently (Chalhoub and Baker, 2009). Namely, owing to the cancelation of suppression of the pathway from *PiP2* to *PiP3* by *Pten*, the accumulation of *PiP3-activated Akt* initially leads to the inhibition of $p27^{kip1}$ and consequent cell cycle progression and then inhibits apoptotic factors, such as *Bad* and *FoxO*, leading to the attenuation of stem cell quiescence/maintenance. Thus, the down-regulated *Pten* functions as a leukaemogenic promoter. Second, some genes are related to cell cycle regulation and cellular proliferation and considered to function in tumour proliferation and invasion. Namely, *caldesmon 1* (*Cald1*), known to be activated specifically during mitosis and also known as a novel *K-cyclin* (*D-like cyclin*)-*cyclin-dependent-kinase* substrate that is thought to regulate the actin cytoskeleton and consequently control cell shape, adhesion, cytokinesis and motility (Cuomo *et al*., 2005), is up-regulated. Thus, the acceleration of cell cycle by *Cald1* may contribute to oncogenicity. *Nr2C2* (nuclear receptor subfamily 2, group C, member 2), known as an orphan nuclear receptor, which is the same as *Tr4* and activates NF κ B in lymphoid follicles and the thymus (Kim *et al*., 2007), is up-regulated; thus, this may function in cellular proliferation. *Mif* (macrophage migration inhibitory factor), known to play a role in leukaemic cellular invasion and proliferation (Talos *et al*., 2005), is up-regulated. *FUS* (fusion, derived from t (12;16) malignant liposarcoma)/*Tls*, known to be a target of the signalling pathway of *ATM* (ataxia-telangiectasia mutated) (Gardiner *et al*., 2008) and also fuses with *ERG* in t(16;21) AML (Ichikawa, 1994), is up-regulated; thus, the up-regulation of *FUS* may induce a dysregulation of *Cdk1* with a *FUS*-mediated oncoprotein (Pan *et al*., 2008). Third, a few genes contribute to the induction of genomic instability and splicing abnormality, which

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may contribute to neoplastic development. Namely, *tetra-, tri-peptidyl repeat* domain 3 (*TTC3*), which is the critical region of Down's syndrome (Tsukahara *et al*., 1998) and is involved in age-related increase in replication/transcription error (Zheng *et al*., 2008), is up-regulated, thus possibly inducing genomic instability.

The last set of genes characteristically up-regulated includes genes protecting neoplastic and leukaemic cells such as *Txnl2*/*Glxn3*, which are known to function similarly to thioredoxin in the cytoplasm (Cha and Kim, 2009), are up-regulated and thus considered to contribute to the survival of leukaemic cells. Ribosomal protein *L23* (*RPL23*) is an MDR promoter (Shi *et al*., 2004) and may thus contribute to the resistance of leukaemic cells to multidrugs. *Apolipoprotein E* (*ApoE*), which functions in the survival of chronic lymphoid leukaemia (CLL) cells (Weinberg *et al*., 2008), is up-regulated and is thus considered to be a leukaemia endpoint marker.

5 CONCLUSIONS

In response to the report of the National Research Council (NRC), "Toxicity Testing in the 21st Century: A Vision and A Strategy (Tox 21) (NRC, 2007)" investigations on new methodologies to predict xenobiotic effects of chemical compounds worldwide in the 21st century have been carried out. In Tox21 on the new dimension of toxicity testing (Schmidt, 2009), it is reported that "On the ground floor of the National Institutes of Health (NIH) Chemical Genomics Center (NCGC) in Rockville, Maryland, a ten million dollar automated laboratory spends all day and all night screening chemicals at speeds no team of human researchers could ever match. In a week, depending upon the nature of the assay, it can yield up to 2.2 million molecular data points derived from thousands of chemicals tested at 15 concentrations each." They are prioritizing chemicals by incorporating more biological aspects into the prioritization. As Robert J. Kavlock of the National Center for Computational Toxicology declared, the project may accomplish a better job selecting and prioritizing the right chemicals for only a limited number of animal testings than by other criteria such as production volume, the likelihood of human exposure or structural similarity to other chemicals with known liabilities.

Current goals of the programme are not to simply compile data on physical and chemical characteristics but rather to evaluate perturbations in toxicity signalling pathways as databases, as mentioned in the NRC report (NRC, 2007).

"Systems toxicology" is "systems biology" applied to general toxicology, which is to elucidate a universal concept of biological interactions between living organisms and xenobiotics by global assays of transcriptomics, proteomics and other various applied omics studies, on various biological phases in *in vitro* cellular responses, in developmental, pubertal and senescent stages, and at the ontological or phylogenical level.

In this chapter, the aim was to focus on systems toxicology to incorporate a new biological concept that distinguishes commonality and stochasticity from the xenobiotic responses when one incorporates data from gene chip microarray computational toxicology into systems toxicology. The multiplicity of biological reactions was better understood when common gene expression profiles and stochastic gene expression profiles are differentiated. We propose the following. First, age-related changes that are xenobiotic responses to "time" as a xenobiotic in which essential biological responses during ageing are based on a long-term relationship between living organisms and environmental xenobiotic materials with oxidative stresses and photostimulation among other. Second, we focus on xenobiotic responses, in addition to senescence, using two sample models, benzene and ionizing radiation, in which common gene expression profiles and stochastic gene expression profiles are clearly defined.

Lastly, murine myeloid leukaemias induced by radiation exposure and spontaneous leukaemias were compared with respect to their differences in gene expression profiles specifically based on individual differences in commonality and stochasticity. No sample model of strong genotoxic chemicals was introduced, which tends to provide deterministic common gene expression profiles in the present study. Stochastic biological responses are phenomenologically ordinary individual differences. However, these are based on post-transcriptomic biological diversity linked to ultimate stochastic toxicology, which is only elucidated by systems toxicology. The authors hope that their proposed concept in this chapter will help readers to recognize biological and toxicological stochastic diversities in the new era of post-genomic science.

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Immunotoxicogenomics: A Systems Approach

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1 INTRODUCTION

Immunotoxicity can be defined as the adverse effects of toxicants (chemicals and drugs) on the immune system. It can be divided into two types: direct immunotoxicity, which deals with direct toxic effects on cells of the immune system and results in reduced resistance to infections and increased incidence of neoplasms; and indirect immunotoxicity, in which case the xenobiotic itself is recognized as foreign, resulting in allergy and sometimes autoimmunity. Xenobiotics that are able to induce allergy are named sensitizers or allergens and can be divided into contact sensitizers and respiratory sensitizers. Exposure to contact sensitizers may result in allergic contact dermatitis, whereas exposure to respiratory sensitizers may result in occupational asthma. While validated animal models exist for the prospective identification of contact sensitizers, with the local lymph node assay currently being used most often, the prospective identification of respiratory sensitizers lacks a validated model and human data are used instead (in retrospect). The advent of non-animal testing for sensitizing capacity stimulated by legislation such as the European Cosmetics Directive and the REACH program provides major opportunities for developing assays to detect contact sensitizers and, along similar lines, respiratory sensitizers. Similar to the situation for contact sensitizers, such assays may be *in silico* quantitative structure-activity relationships (QSAR), *in chemico* (peptide reactivity), *in vitro* (cell-based assays including those relying on genomics and signal transduction), or a combination of these assays (Vandebriel and Van Loveren, 2010).

Toxicogenomics employs "omics" technologies such as genomic-scale mRNA expression (transcriptomics), cell and tissue-wide protein expression and modification (proteomics), and metabolite profiling (metabolomics), in combination with bioinformatics methods and conventional toxicology. In relation to chemical hazard and risk assessment, toxicogenomics provides tools to improve the understanding of mechanisms of toxicity, identify biomarkers of toxicity and exposure, reduce uncertainty in grouping of chemicals for assessments, (Q)SARs, inter-species extrapolation, and effects on susceptible populations. Moreover, toxicogenomics can provide alternative methods for chemical screening, hazard identification, and characterization. Because of its sensitivity, altered gene expression profiles may already be seen at doses that are relevant to human exposure, not necessarily producing adverse effects. Distinguishing adaptive from adverse effects is an issue that remains to be resolved.

Systems toxicology is defined here as the study of the interactions of all elements in a given biological

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system, under stress or toxicant perturbation, to achieve a mechanistic understanding of the toxicological response. It involves integration of chemical- or stressor-affected biological systems (cells, tissues, organs, or entire organisms) at the molecular level, monitoring changes in molecular expression and conventional toxicological parameters, and iteratively integrating response data to describe the functioning organism (Waters and Fostel, 2004). In the field of systems toxicology, the analysis of complex biological responses to chemical exposure is facilitated by toxicogenomics. Because data integration results in additional knowledge of the system as a whole, systems toxicology offers the promise of developing novel reliable toxicity biomarkers and more accurate risk assessment. Software tools aiming to improve risk assessment based on gene expression data have been developed, such as Gene Ontology (GO)-Quant (Yu *et al*., 2006) and BMDExpress (Yang, Allen and Thomas, 2007). Both models describe dose–response relationships from microarray experiments within GO categories.

In this chapter, we first discuss the data that propose the oxidative stress pathway as the major one induced by contact sensitizer exposure. Second, we discuss the (less abundant) data that have led to the proposition of the PTEN pathway as the major one induced by respiratory sensitizer exposure. Third, we investigate the chemical characteristics that determine whether a sensitizer is a contact or respiratory sensitizer that have only recently begun to emerge. Fourth and finally, we hypothesize how the two different pathways may result in the *in vivo* observation of preferential Th1 and Th2 responses, respectively.

Identification of the oxidative stress response pathway as the major one induced by contact sensitizers has resulted in non-animal assays to identify them, and it is envisaged that such assays will be developed for respiratory sensitizers in the near future, thereby improving risk assessment within a systems toxicology framework.

2 OXIDATIVE STRESS RESPONSE TO CONTACT SENSITIZERS

The oxidative stress response pathway, illustrated in Figure 1, is first discussed briefly. Kelch-like ECH-associated protein 1 (Keap1) is a sensor protein that contains highly reactive Cys residues. In the absence of electrophiles (haptens), the basic leucine zipper transcription factor nuclear factor–erythroid 2-related factor 2 (Nrf2) is associated with Keap1. Keap1 anchors the Nrf2 in the cytoplasm, targeting it for ubiquitination and proteasomal degradation. Upon electrophile binding Keap1 dissociates from Nrf2, resulting in translocation of Nrf2 to the nucleus, where Nrf2 forms a heterodimer with small MAFs. This heterodimer binds to antioxidant response elements (ARE), inducing transcription of genes that contain ARE in their promoter.

Natsch (2010) recently reviewed the evidence for a major role of the oxidative stress response pathway after contact sensitizer exposure. In this section, we discuss the relevant transcriptomics data. An inventory of all genes that are dependent on the oxidative stress response is taken as a starting point. Gene profiling after *KEAP1* gene knock-down in the keratinocyte cell line HaCaT revealed upregulation of 22 genes (MacLeod *et al*., 2009).

Dendritic cells (DC) are pivotal in the immune response, uniquely able to induce the differentiation of naive T cells. DC maturation has been widely used as a model to detect contact sensitizers. Cell surface markers such as CD86 are used to measure maturation and hence sensitizing capacity. DC can be cultured from monocytes isolated from peripheral blood mononuclear cells (monocyte-derived DC or moDC) and cord blood ($CD34^+$ DC), but myeloid cell lines such as THP-1 and MUTZ-3 are also used.

Expression profiling of moDC after exposure to the contact sensitizer dinitrobenzene sulfonic acid showed upregulation of ARE-responsive genes (Ryan *et al*., 2004). From the above-mentioned list of 22 genes, aldo-keto reductase 1C2 (*AKR1C2*), malic enzyme 1 (*ME1*), phosphogluconate dehydrogenase (*PGD*), and thioredoxin reductase (*TXNRD1*) were significantly upregulated after exposure.

Three MAPK signaling pathways exist in mammals, involving extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38MAPK (p38). Haptens activate ERK1/2 and p38 (Becker *et al*., 2003), and activation of ERK1/2 and p38 by the contact allergen trinitrochlorobenzene could be inhibited by the thiol antioxidant *N*-acetylcysteine (NAC); this inhibitor also prevented binding of the hapten to protein (Bruchhausen *et al*., 2003).

Exposure of DC to sensitizers but not to non-sensitizers altered the redox state of the cells, as

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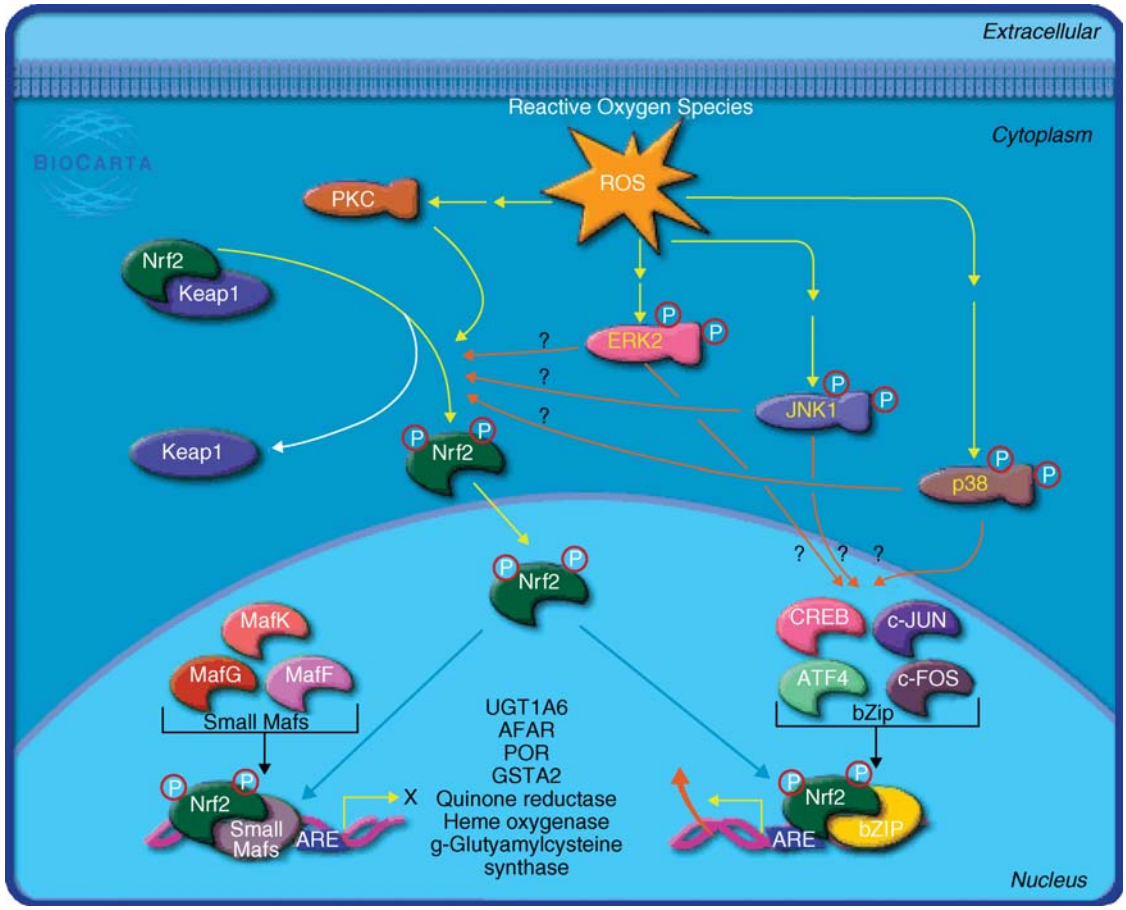

Figure 1. The oxidative stress response pathway. Upon hapten binding to Cys residues of Keap1, the Keap1/Nrf2 heterodimer dissociates and Nrf2 translocates to the nucleus. In the nucleus, Nrf2 forms a heterodimer with small MAFs, after which this heterodimer binds to ARE sites, thereby inducing transcription of ARE-dependent genes such as *HMOX1* and *NQO1*. Reproduced with permission from Biocarta (http://www.biocarta.com/pathfiles/h_arenrf2Pathway.asp) (accessed 24 November, 2009).

measured by a reduced ratio of intracellular oxidized (GSSG) versus reduced (GSH) glutathione, and this altered ratio was accompanied by p38 activation (Mizuashi *et al*., 2005). Suppressed GSSG/GSH ratio, p38 activation, and increased CD86 expression were all reduced by NAC treatment. Thus, altered oxidant/redox state is an important effect of sensitizing agents.

Exposure of $CD34^+$ DC and the THP-1 cell line to sensitizers but not irritants resulted in the increased expression of heme oxygenase-1 (*HMOX1*) and NAD(P)H:quinone oxidoreductase-1 (*NQO1*). Pre-incubation with NAC inhibited CD86 expression as well as expression of *HMOX1* and *NQO1*, showing the role of oxidative stress in this response (Ade *et al*., 2009).

From the above-mentioned list of 22 genes, aldo-keto reductase 1C1 (*AKR1C1*), ferritin heavy 1 (*FTH1*), and *NQO1* were upregulated in moDC and the MUTZ-3 cell line, after exposure to the contact sensitizer cinnamic aldehyde (Python, Goebel and Aeby, 2009).

In conclusion, each of the DC genomics studies showed affected genes that overlap with the MacLeod set of 22 genes. In order to improve comparison of the studies, complete gene profiles should be compared instead of only comparing lists of affected genes. In any case, there is significant overlap in the affected genes, suggesting robustness of the antioxidant response pathway.

A novel assay whose development was led by the identification of the oxidative stress response

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pathway uses an ARE reporter construct (Natsch and Emter, 2008). The reporter construct contains eight ARE sequences upstream of a luciferase gene. After testing 102 chemicals, the accuracy was found to be 83% with only 4 out of 30 non-sensitizers inducing (low) luciferase activity.

In conclusion, the past few years have seen the emergence of the oxidative stress response pathway as the major pathway induced by contact sensitizers. Comparing entire sets of array data should greatly improve the comparisons between the various studies discussed in this section. An important approach that is still missing is to knock down genes involved in the oxidative stress response pathway and evaluate the effects on downstream parameters (signal transduction, DC maturation, and cytokine production) after sensitizer exposure (functional genomics). In any case, the identification of the oxidative stress response pathway provides new avenues to develop *in vitro* assays to detect contact sensitizers, including high-throughput reporter assays.

3 PTEN PATHWAY RESPONSE TO RESPIRATORY SENSITIZERS

The first successful attempt to identify respiratory sensitizers *in vitro* used the bronchial epithelial cell line BEAS-2B (Verstraelen *et al*., 2009). The phosphatase and tensin homolog deleted on chromosome 10 (PTEN) pathway, illustrated in Figure 2, was the most specific one induced. PTEN, a multifunctional phosphatase, is best known as a major tumor suppressor that dephosphorylates phosphatidylinositol-3,4,5-triphosphate (PIP3). This inositol phospholipid, which is produced by phosphatidylinositol-3-kinase (PI3K), activates the signaling kinase AKT. AKT plays an important role in anti-apoptosis, proliferation, and oncogenesis. Interestingly, PTEN activity decreased in a murine model of ovalbumin-induced airway hyperresponsiveness and bronchial inflammation, and these clinical effects, as well as VEGF levels, were reduced by administration of an adenovirus containing PTEN cDNA (Kwak *et al*., 2003; Lee *et al*., 2006). Peroxisome proliferator activated receptor γ (PPARγ) agonists as well as an adenovirus containing PPARγ cDNA had a similar effect and this effect was mediated through PTEN (Lee *et al*., 2005). Moreover, in a murine model of toluene diisocyanate (TDI)-induced asthma, adenovirus containing PTEN cDNA reduced the pathophysiological features of airway disease (Kim *et al*., 2007a). In summary, PTEN plays a pivotal role in airway inflammation and hyperresponsiveness, and TDI-induced asthma. Importantly, the clinical picture of TDI-induced asthma is exemplary for the clinical effects of respiratory sensitizers, implicating that the pathway discovered by Verstraelen *et al.* (2009) in an *in vitro* model is of clear relevance to the situation in exposed humans, giving the model additional value.

It is as yet not clear whether and to what extent the oxidative stress response is involved in the cellular response to respiratory sensitizers. The ARE reporter assay was found to be negative for respiratory sensitizers, suggesting that these compounds do not induce an oxidative stress response. A model for a role of PTEN in determining cellular susceptibility to the oxidative stress response has recently been proposed (Sakamoto *et al*., 2009). Although this model includes Nrf2-dependent induction of ARE-responsive genes such as *HMOX1* and *NQO1*, the underlying pathway is likely to be different from the oxidative stress pathway because it is unresponsive to NAC pre-treatment.

4 CONTACT AND RESPIRATORY SENSITIZERS HAVE DIFFERENT CHEMICAL CHARACTERISTICS

A recently developed *in silico* model identified two categories of respiratory sensitizers, based on a "reactivity threshold": one category with extreme electrophilicity without the capacity of cross-linking, and a second with a lower but still significant electrophilicity, combined with cross-linking capacity (Enoch, Roberts and Cronin, 2009). This report presents the first mechanism-based *in silico* prediction of respiratory sensitizers. Importantly, it is the first step in unraveling the rather enigmatic difference in chemical characteristics between contact and respiratory sensitizers. The results from Enoch, Roberts and Cronin (2009) suggest that electrophilicity is the (most important) factor in the distinction between the two types of sensitizers.

Another, less well established difference is not in the haptens themselves but in the amino acids they bind to. Theoretical considerations on respiratory sensitization provide some clues to the nature

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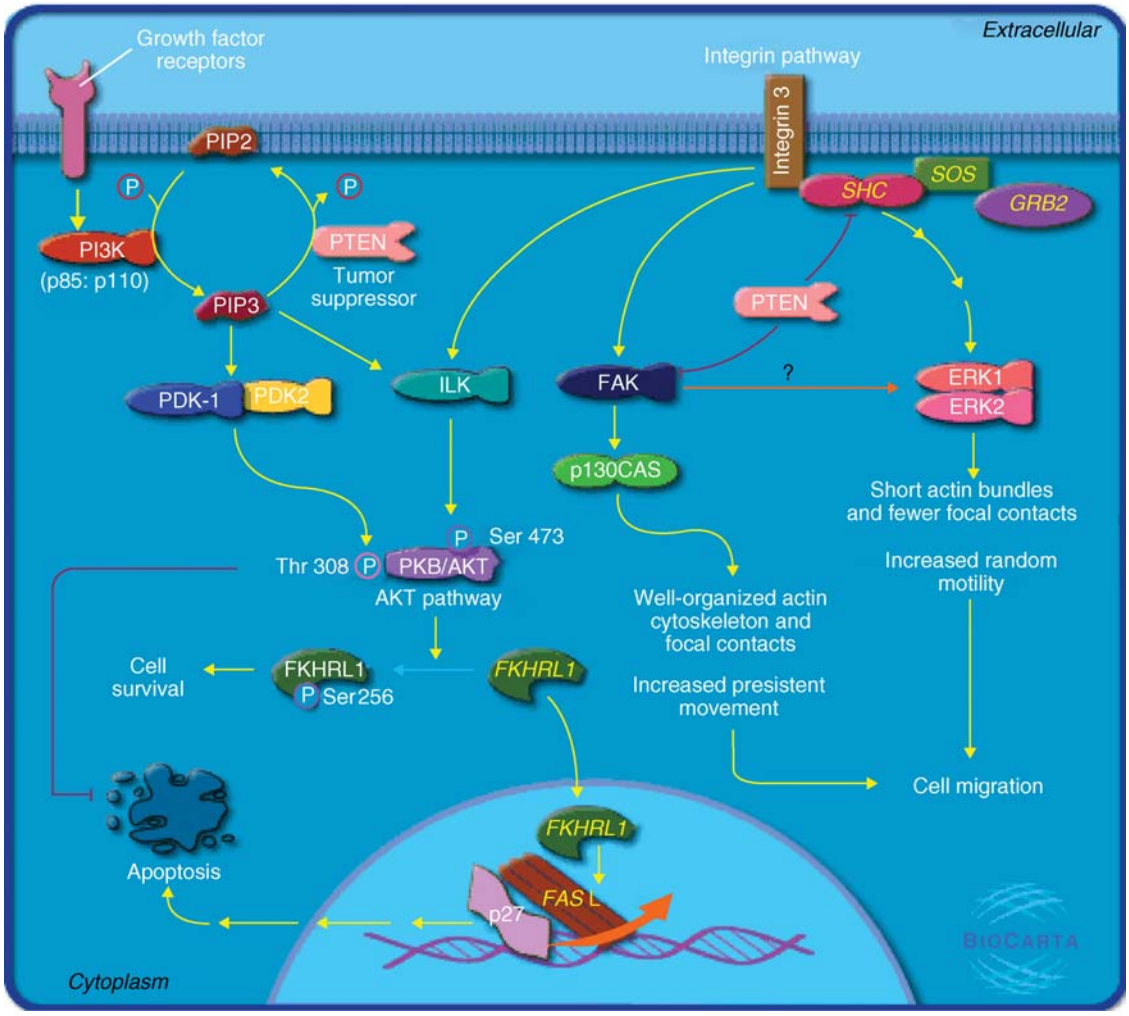

Figure 2. The PTEN pathway. PI3K activity results in PIP3 production. PIP3 activates the signaling kinase AKT. Activation of the AKT pathway inhibits apoptosis and increases cell survival. PTEN dephosphorylates PIP3, thereby inhibiting the AKT pathway. Reproduced with permission from Biocarta (http://www.biocarta.com/pathfiles/h_ptenPathway.asp) (accessed 24 November, 2009).

of respiratory sensitizers; the airways provide an oxidizing environment, so respiratory sensitizers do not bind to cysteine but to lysine instead and (even then) only 0.1% of lysine is reactive at physiological pH. This suggests that respiratory sensitizers are hyper-electrophilic. To produce enough epitopes bi- or multifunctionality is helpful (D.W. Roberts, personal communication). Clearly, the Enoch, Roberts and Cronin (2009) results are in line with these considerations.

Although it is now well established that contact sensitizers bind to Cys residues on Keap1, it is unknown which protein(s) is the target of respiratory sensitizers. As they may bind to lysine and not cysteine residues, it is unlikely that Keap1 is involved, at least not in a similar way as for contact sensitizers. If and how such mechanisms of respiratory sensitizer binding fit in the PTEN pathway is unknown.

5 DIFFERENT IMMUNE RESPONSES SKEWED IN VIVO BY CONTACT AND RESPIRATORY SENSITIZERS

We are beginning to understand the pathway(s) resulting from the response to contact sensitizers, and may thus ask the question as to how this may

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eventually result in a Th1 response, with the knowledge that respiratory sensitizers induce a Th2, or mixed Th1/Th2, response (Dearman, Basketter and Kimber, 1995, 1996; Vandebriel *et al*., 2000, 2003; Van Och *et al*., 2002). A possible explanation may be that activation of Nrf2 by contact sensitizers results in increased glutathione levels (MacLeod *et al*., 2009). These increased glutathione levels would in turn skew Th1 responses (Peterson *et al*., 1998; Kim *et al*., 2007b). Treatment of lymph node cells from animals sensitized with the contact allergen dinitrofluorobenzene (DNFB), with antibodies to heat shock protein (HSP) 27 and HSP 70, reduced IFN-γ and IL-17 production, while increasing IL-4 and IL-10 production (Yusuf *et al*., 2009). HSPs are produced in response to, among others, oxidative stress. It may thus be suggested that oxidative stress by inducing HSP production results in Th1 skewing.

A separate line of evidence for Th1 skewing activity of the oxidative stress response is the observation that ovalbumin-sensitized and -challenged Nrf2$^{-/-}$ mice showed increased production of the Th2 cytokines IL-4 and IL-13 compared with wild-type controls (Rangasamy *et al*., 2005). Moreover, expression of the Th1 cytokine IFN-γ, but not the Th2 cytokine IL-4, decreased with aging in the ears of DNFB-sensitized and ear-challenged Nrf2$^{-/-}$ mice (Kim *et al*., 2008).

The fact that respiratory sensitizers do not, or to a lesser extent, induce the Th1 response would suggest that respiratory sensitizers do not, or to a lesser extent, induce the oxidative stress response, in line with the observation that they are negative in the ARE reporter assay (Natsch and Emter, 2008).

6 CONCLUSIONS AND FUTURE DIRECTIONS

Toxicogenomics is of great benefit to understand mechanisms of toxicity, identify biomarkers of toxicity and exposure, reduce uncertainty in grouping, and improve risk assessment. Using the oxidative stress pathway as an example, we hope to have shown that a systems toxicology approach is more advanced than one which employs toxicogenomics alone because research questions can be approached in different ways, in this case not only by genomics (*in vitro* exposure of DC) but also, *in vivo* (contact hypersensitivity), by signal transduction (role of Nrf2 and NAC) and reporter assays. With regard to respiratory sensitizers, our knowledge is still limited to an affected pathway, so different approaches should be taken here as well, but it is encouraging that the pathway involved has been shown to play a major role *in vivo* in murine models for protein- and low-molecular-weight chemical-induced asthma. In the near future, a systems toxicology approach for respiratory sensitization should result in reporter assays.

For contact sensitization, risk assessment seems to be feasible, for instance by comparing results from non-animal assays (*in vitro*, *in chemico*, and *in silico*) with EC3 values from the local lymph node assay (Vandebriel and Van Loveren, 2010). It is still remote for respiratory sensitization, partly because a validated *in vivo* model and quantitative data are lacking. To be able to identify respiratory sensitizers we propose to develop non-animal assays on the basis of human data (physiology, pathology, toxicology, genomics, etc.). This proposition also holds for risk assessment of respiratory sensitization. We anticipate that development of non-animal assays as well as risk assessment will depend on a systems toxicology framework.

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Systems Biology: Integrating '-Omics'-Oriented Approaches to Determine Foodborne Microbial Toxins

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1 INTRODUCTION

Maintaining food safety is becoming ever more difficult in this time of global markets, free trade and the constant development of new products that use novel methodologies. Most foodborne illnesses are caused by pathogenic micro-organisms in the food, such as bacteria, viruses, parasites, toxigenic moulds or microalgae, which produce a variety of toxins. According to the U.S. Federal Food, Drug, and Cosmetic Act (FFDCA) Sec 402(a)(1-6), food is considered adulterated if it bears or contains any poisonous or deleterious substances.

It is extremely difficult to detect specific microbes and the toxins microbes produce in contaminated food. The food industries and regulatory agencies have historically relied on conventional, well-established microbiological techniques involving pre-enrichment of pathogenic species on selective media to detect individual bacterial colonies. Biochemical or immunological methods are then used to identify the pathogens (de Boer and Beumer, 1999). Although these methods are commonly used and accepted, they are equally labour-intensive and time-consuming, and can not always distinguish whether micro-organisms are viable or non-viable. However, new systems biology techniques have enabled researchers to detect minute signals generated by microbes amid the noise of most food matrices.

Being able to detect and type pathogenic and spoilage micro-organisms at an early stage will allow us to protect our food more effectively. Traditionally, plasmid typing, pulse-field gel electrophoresis, ribotyping and random-amplified polymorphic DNA analysis were established as molecular typing methods during the genomic era (Kathariou, 2002). In recent decades, the post-genomic revolution has enabled us to track microbial toxins by exploring regulatory control of genes, proteins, and primary and secondary metabolites of microbial pathogenic circuits. The new systems biology techniques make it possible to explore the global expression of genes (genomics), proteins (proteomics) and low-molecular-weight metabolites (metabolomics) in pathogenic microbes, and study genomic-scale or cell-wide processes by combining high-throughput experimental tools. This article presents an overview of these techniques and how they can help industry and regulatory agencies to detect microbial toxins in food and feed.

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Table 1. An overview of microbial toxin properties and characteristics.

Property	Exotoxin	Endotoxin
Chemical nature	Protein	Lipid A of lipopolysaccharide
Relationship to cell	Release from the cell	Integrated part of the cell wall
Gram stain	Produced by both Gram-positive and Gram-negative micro-organisms	Produced by only Gram-negative micro-organisms
Mode of action/target sites	Many types of exotoxin based on structure and function: cytotoxin, enterotoxin or neurotoxin	One type of endotoxins
Specificity	High degree: specific receptors on host target cells	Low degree: diverse range of host cells and systems affected
Symptoms	Specific/conditional effects in host	Diverse range of effects in host: fever, vomiting or diarrhoea
Toxoidable	Can be denatured to remove toxicity	Can not be denatured; chemical composition prohibits molecular modification

2 MICROBIAL TOXINS AND DETECTION

Bacteria are harmless, even helpful and actually have been proven to be beneficial to life on earth, yet there are still a few pathogenic bacteria that have the potential to cause infectious disease by producing toxins (Fischbach and Walsh, 2009; Liu and Nizet, 2009). Many foodborne diseases have been described by *Shigella*, *Escherichia coli* and *Campylobacter* as potential causative agents in raw and undercooked meat and poultry or raw milk and untreated water-borne bacteria (Wright, Danyluk and Otwell, 2009; Todd *et al*., 2008). The growth of common micro-organisms is unrestrictive and their pathogenecity depends on virulence factors, which determines the fate of bacterial toxins.

A bacterial toxin can be defined as a colloidal proteinaceous poisonous substance produced by the metabolic activities of a living organism, unstable, toxic to the tissues and capable of inducing antibody formation. There are over 220 known bacterial toxins, divided into exotoxins and endotoxins. Endotoxins (cell-associated substances) originate inside the cell, whereas exotoxins are produced outside the cell and absorbed by the host (Calandra, (2001); Driscoll, Brody and Kollef, 2007; Liu and Nizet, 2009). Endotoxins are generally enzymes produced by Gram-positive and Gram-negative bacteria. There are many more known exotoxins than endotoxins; exotoxins are known to range in toxicity, but form a class of poisons that is among the most potent, per unit weight, of all toxic substances. The properties and characteristics of endotoxins and exotoxins are compared in Table 1.

Because microbial toxins have different structures and functions, it is impossible to use a single standard technique to detect and analyse specific toxin. Conventional methods currently used for the routine detection of pathogens include enrichment techniques followed by plating methods or immunochemical and nucleic acid-based methods (Notermans and Wernars, 1991; de Boer and Beumer, 1999; Nagy *et al*., 2006). However, these techniques are time-consuming and often limited because they are designed to identify one specific toxin. Any successful toxin detection or screening system must be able to handle the challenges presented by emerging/mutated pathogens and little-known biohazards.

3 FUNCTIONAL 'OMICS' AND DETECTION OF MICROBIAL TOXINS

The high-throughput experimental approaches used in systems biology can analyse all the components of a biological system simultaneously (Ishii *et al*., 2007) and predict the systematic behaviour of cells holistically (Figure 1). This integrative approach allows researchers to study the complex metabolic and regulatory networks of pathogenesis in the microbial system, which may lead to the design of new toxin detection devices. Now that 527 bacterial and 47 archael genomes have been fully sequenced and abundant information is available about the genomics of micro-organisms such as *Escherichia coli* (http://genomeonline.org) (Liolios *et al*., 2009), the next challenges are determining the function

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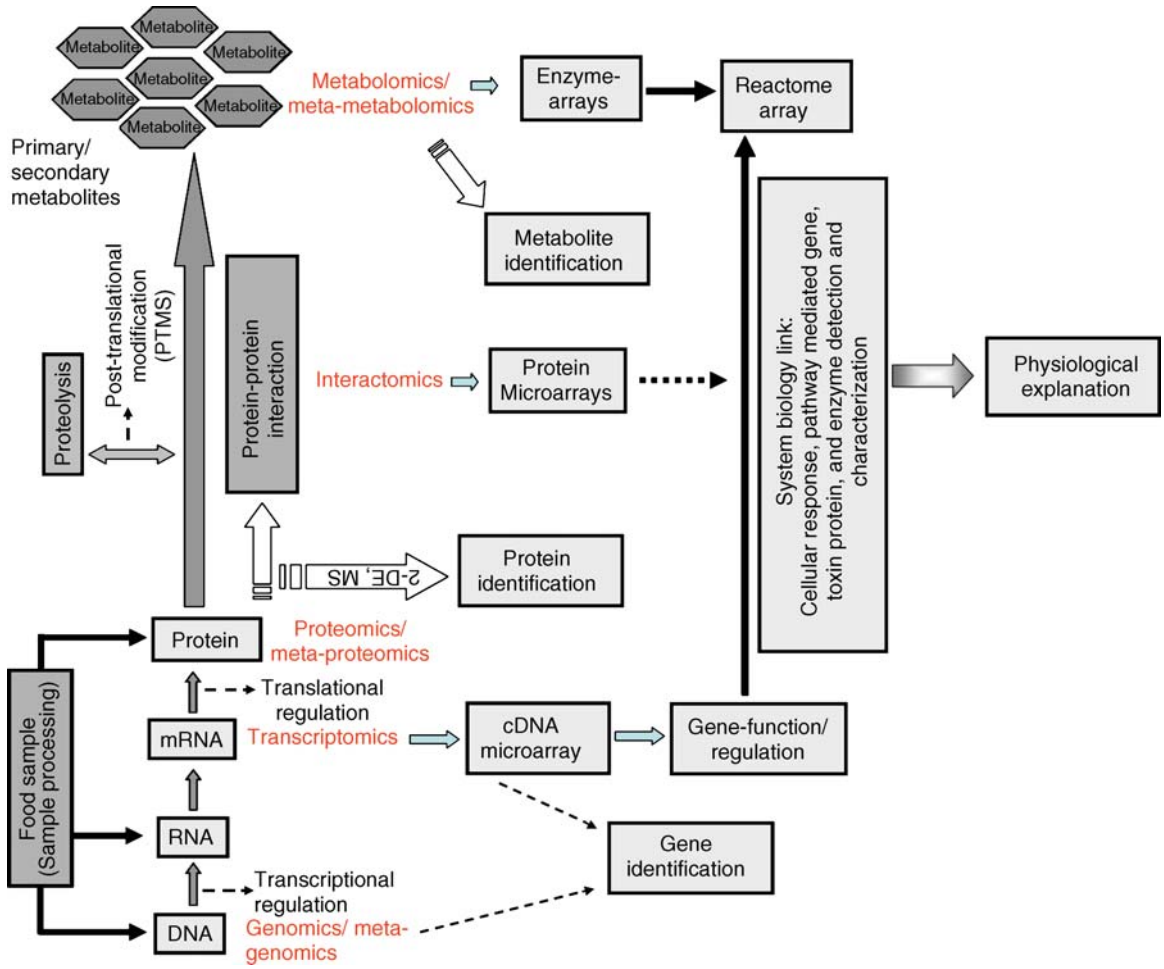

Figure 1. Integration of 'omics'-based approaches to detect microbial toxins in the post-genomics era. The enriched microbial DNA from a variety of food and related substances will end up on DNA microarrays (transcriptomics). Moving forward, extraction of whole-cell proteins and metabolites will activate systems biology route of proteomics and metabolomics, respectively, using 2DE, protein microarray and MS-based platforms. The metabolite-based enzyme arrays will further provide reactome arrays that may link to identify cellular responses, pathway-mediated genes and specific toxin proteins towards physiological explanation of specific microbe. 2DE, 2D electrophoresis; MS, mass spectrometry; PTM, post-translational modification.

of each gene and understanding the metabolic and regulatory networks within each biological system. The field of functional '-omics' consists of high-throughput global experimental approaches that make use of the information and reagents provided by structural genomics to assess gene function (Hieter and Boguski, 1997). This field has seen considerable growth in recent years, encompassing areas such as transcriptomics (global gene expression, i.e. mRNAs), proteomics (global protein expression) and metabolomics (global expression of primary and secondary metabolites) (Figure 1).

3.1 Genomics

The genome is the genetic information that flows from the DNA of an organism to the mRNA; genomics refers to analysing the expression of a large number of genes through relative or semi-quantitative amounts of RNA in biological samples. Genomics techniques and high-throughput methods can be used to facilitate the identification of potential virulence factors and their relative genes.

Traditionally, transposon mutagenesis has been used to inactivate genes in a selected bacterial

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strain and the resulting insertion mutants have been screened for attenuation in an appropriate animal infection model in order to identify novel virulence factors. A variation on this method, signature-tagged mutagenesis (STM), incorporates unique DNA tags; this method allows mass screening of mutants in animal infection models (Hensel *et al*., 1995), but is limited to a single bacterial strain and a particular infection model. Therefore, it identifies only highly specific virulence factors, with limited extrapolation to generic virulence determinants of other pathogens. Another technique, *in vivo* expression technology (IVET), involves finding genes that are up-regulated *in vivo* (Mahan *et al*., 1993), and identifies genes other than those required for virulence. Both STM and IVET have the potential to identify genes in a pathogen that also exist in non-pathogenic strains. Whole-genome computational comparisons of related pathogenic and non-pathogenic strains can suggest genes that potentially play a role in virulence (Heermann and Fuchs, 2008; Lanie *et al*., 2007); for example, Garbom *et al*. 2004 examined hypothetical genes conserved in six human microbial pathogens in order to identify novel genes associated with virulence in *Yersinia pseudotuberculosis*.

Now that complete genome sequences for human pathogens are available, they can be used to facilitate genome organization studies, genome comparisons and functional genomics, as well as to develop microarrays (Ishii *et al*., 2007). Today's nucleotide sequencing technologies can produce complete sequences for a large number of genomes, which facilitates high-throughput gene identification (Hall, 2007; MacLean, Jones and Studholme, 2009). Determining the functions of these genes is one of the most important current challenges for molecular biology. In laboratory studies, researchers have recently estimated the minimal gene set required for bacterial cell viability (Baba *et al*., 2006; Liberati *et al*., 2006; Gallagher *et al*., 2007; French, Bell and Ward, 2008). However, such studies can identify only those bacterial genes required for viability under specific laboratory growth conditions. Both STM (Hensel *et al*., 1995) and transposon-site hybridization (TraSH) (Sassetti, Boyd and Rubin, 2001) use hybridization to identify genes that have been disrupted by transposon insertions. They have also been used to identify "niche-specific" virulence genes in pathogenic bacteria through negative selection (Andrews-Polymenis, Santiviago and McClelland, 2009). Transposon-mediated differential hybridization (TMDH) is a newer technique that has been used in one study to analyse approximately one million mutants and identify essential genes in *Staphylococcus aureus* (Chaudhuri *et al*., 2009).

The Scalar Analysis of Library Enrichments (SCALEs) is another genomics approach that uses DNA and gene-chip data to provide access to new genome-scale patterns (Struble and Gill, 2009; Warner, Patnaik and Gill, 2009). Global transcription machinery engineering (gTME) and gene-disruption methods such as transposon insertion and site-specific homologous recombination are also being used to build microbial libraries of the phenotypes by analysing their desirable traits (Warner, Patnaik and Gill, 2009).

The availability of many completed and/or in-progress genome sequences of microbes that cause foodborne diseases (listed at http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi) has paved the way for functional genomics approaches including analysis of the bacterial transcriptome, proteome and metabolome (Kuipers, 1999; De Vos, 2001). Table 2 summarizes a comprehensive list of genomics databases for microbial study with respective descriptions. Based on microbial categorical identification, the genomes of Gram-negative bacteria including *Salmonella*, *Escherichia coli* and *Campylobacter* have been described, along with the use of functional genomics tools to study stress response and host–pathogen interaction (Wells and Bennik, 2003). A comprehensive literature review of genomics on microbial food safety discussing the foodborne pathogens *Listeria monocytogenes* and *Bacillus cereus* has been given previously (Abee, van Schaik and Siezen, 2004). The completed genome sequences have been utilized to develop high-throughput analytical methods such as DNA microarrays for gene expression analysis (transcriptomics).

3.2 Transcriptomics

The term "transcriptome" refers to the subset of genes that are transcribed in an organism. It dynamically links the genome, the proteome and the cellular phenotype together. Complete genome sequences can be used in comparative genomics to conduct subtractive hybridization, which can

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Table 2. General genomics databases for microbial study.

Database	Description	Website
GTD	Genomic target database	http://iioab-dgd.webs.com/
AssociationDB	Explore genome-wide association studies	http://genetik.charite.de/
BNDb	An integrated proteomics and transcriptomics database	http://www.bndb.org/
Comparative genometrics (CG)	Conduct comparative biometric analysis of chromosomes of different organisms	http://www2.unil.ch/comparativegenometrics/
DEG	Find information about genes essential to life in prokaryotes and eukaryotes	http://tubic.tju.edu.cn/deg/
DNAlive	A tool for the analysis and graphical display of structural and physical characteristics of genomic DNA	http://mmb.pcb.ub.es/DNAlive/
EMGlib	Search for annotated genomic information of completed prokaryote genomes	http://pbil.univ-lyon1.fr/emglib/emglib.html
Entrez genome	Search for genomic sequences from completely sequenced organisms and those for which sequencing is in progress	http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome
FusinDB	Analyse prokaryotic gene fusion events	http://igs-server.cnrs-mrs.fr/FusionDB/
Genome Information Broker (GIB)	Conduct comparative genomics studies using extensive collection of microbial genomes and Arabidopsis genome	http://gib.genes.nig.ac.jp/
Genomes OnLine Database (GOLD)	Access information regarding complete and ongoing genome and metagenome projects around the world	http://genomesonline.org/index2.htm
HGT-DB	Search prokaryotic genomes for putative horizontally transferred genes	http://genomes.urv.es/HGT-DB/
ICDS-database	Search for interrupted coding sequence detected in 116 complete prokaryotic genomes	http://www-bio3d-igbmc.u-strasbg.fr/ICDS/
Integr8	Explore the biology of organisms with completely deciphered genomes and proteomes	http://www.ebi.ac.uk/integr8/EBI-Integr8-HomePage.do
Integrated microbial genomes (IMG) system	Search and analyse microbial genomes from a comprehensive database by the DOE-Joint Genome Institute (JGI)	http://img.jgi.doe.gov/cgi-bin/pub/main.cgi
MBGD	Conduct comparative analysis of completely sequenced microbial genomes	http://mbgd.genome.ad.jp/
NCBI genomic biology	Provides several genomic biology tools and resources, including organism-specific pages that include links to many web sites and databases relevant to that species	http://www.ncbi.nlm.nih.gov/Genomes/
PHIDIAS	Information related to pathogen–host interactions	http://www.phidias.us/
TIGR microbial database	Retrieve genetic information of published microbial genomes and chromosomes and those in progress	http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi
The comprehensive microbial resource (CMR)	Search for genomic information on all of the publicly available, complete prokaryotic genomes	http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi
IBM's bioinformatics and pattern discovery group	Access to a large number of software tools for various bioinformatics tasks	http://cbcsrv.watson.ibm.com/Tspd.html

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characterize novel and unique gene sequences using DNA microarrays. The DNA microarray is a high-throughput tool with tremendous potential for detecting pathogenic microbes (Rasooly and Herold, 2008; Puttamreddy *et al*., 2008), but has certain limitations (Peplies, Glockner and Amann, 2003; Zhou, 2003). Researchers use DNA microarrays to study gene expression by identifying genes that are differentially expressed under different conditions or certain genetic perturbations (such as comparing virulent and non-virulent strains). The results are used to determine the expression patterns of genomic signature sequences, which usually represent genomic characteristics such as genes coding for rRNA, toxins, virulence factors, antibiotic resistance determinants or shared conserved indels (insertions/deletions) (Gupta and Griffiths, 2002; Gupta, 2004). One can identify micro-organisms or their important characteristics by finding these differentially expressed signature sequences.

The DNA microarray is based on a straightforward concept extrapolated from the seminal work of Edwin Southern. Southern blots (DNA–DNA hybridization) and Northern blots (RNA–DNA hybridization) use complementary gene sequences to determine the presence or absence of certain DNA or RNA with radiological, fluorogenic or chemiluminescent detectors. Traditional Northern and Southern blots identified DNA or RNA targets of interest using small numbers of specific probes, but the current convention is to refer to the known sequence as the "probe" and the unknown mixture of sequences as the "target". DNA microarrays are coated glass microscope slides with thousands of DNA samples spotted on them in a precise pattern. There are two principal types of microarrays based on the nature of the target: DNA (including cDNA, PCR products, oligonucleotides or plasmids) or RNA (Celis *et al*., 2000; Ye *et al*., 2001) based on two different spotting methods: mechanical microspotting and photolithography (Kumar *et al*., 2000). In addition, there are three major functional classes of microarrays: (a) functional gene arrays (FGAs), in which probes target genes that encode enzymes involved in key processes; (b) phylogenetic oligonucleotide arrays (POAs), which contain oligonucleotide probes that target taxonomic genes (e.g., the 16S rRNA gene); and (c) community genome arrays (CGAs), which provide the complete genomic DNA of multiple cultured strains or species (Zhou, 2003; Wu *et al*., 2006).

With the completed genome sequences of foodborne pathogens available (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi), it is now possible to analyse the expression of all genes in each genome under various pathogenic conditions using whole-genome DNA microarrays (Call, Brockman and Chandler, 2001; Shen and Higgins, 2006; Liu *et al*., 2007; Siddique, Sharma and Al-Khaldi, 2009; Weckx *et al*., 2009). Such genome-wide expression analysis provides important data for identifying regulatory circuits in these organisms. In the past, DNA microarrays have been used to evaluate microbial communities in raw milk (Giannino *et al*., 2009): oligonucleotide arrays based on a PCR method were developed for taxonomic markers and species-specific virulence genes for simultaneous identification of lactic acid bacteria and foodborne pathogenic bacteria from raw milk. Foodborne pathogens from the above studies showed a highly specific hybridization patterns with the genomic DNA from *Campylobacter jejuni*, *E. coli*, *Salmonella thyphimurium*, *Listeria monocytogenes* and *Yersinia enterocolitica* (Giannino *et al*., 2009).

Bacillus anthracis spores, which have highlighted the importance of the biodefence system in the United States, are extremely stable and able to survive under very harsh conditions. Liu and Ream 2009 determined 27 germination- and sporulation-related genes using real-time transcriptase PCR assays under different milk intervention strategies. This study revealed no overlapping in gene expression under heat treatment (ABC transporter, GTP pyrophosphokinase, germination protein gerN, stage II sporulation protein P and spore germination protein GerHC), milk pasteurization, and milk pasteurization and microfiltration strategies (stage II sporulation proteins E and A, and stage IV sporulation protein A).

Staphylococcus aureus is a major pathogen that causes staphylococcal infection and food poisoning; it produces five major classical types of staphylococcal enterotoxins (SEA, SEB, SEC, SED and SEE) as well as new SEs or SE-like superantigenes (Sags). Multiple strains of *S. aureus* harbour more than one SE gene, and identification of SEs involved in food poisoning can delay identification of the specific pathogen in a pathogenic outbreak. Lin, Chiang and Tsen 2009 developed a chromogenic microarray method that allows simultaneous detection of classical SE genes and a new SE gene (seg), which is phylogenetically highly related to SEB and

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SEC toxins. In addition, studies to detect enterotoxinogenic *S. aureus* in milk or beef homogenate containing 10–10^4 target cells per millilitre or gram of the sample revealed all six enterotoxin genes.

High-throughput genome sequencing of *Salmonella*, a prevalent foodborne pathogen, has expanded our knowledge of the evolution of epidemic isolates, allowing the creation of complete microarrays. This has improved the throughput of IVET, used to uncover promoters active during infection. In another method, STM, pools of mutants are subjected to selection. Andrews-Polymenis, Santiviago and McClelland 2009 reviewed changes in a population that was monitored on a microarray. A pathogen detection microarray was developed using various 7-mer oligonucleotides specifically targeting *Escherichia coli* O157:H7, *Salmonella enterica*, *Listeria monocytogenes* and *Campylobacter jejuni* pathogens (Suo *et al*., 2009). To achieve high-detection sensitivity and specificity, these DNA microarrays were designed by targeting specific DNA amplification instead of whole-genome random amplification. With a detection sensitivity of 1×10^{-4} ng (∼20 copies) genomic DNA, these microarrays were reported to effectively screen single or multiple pathogens in food samples, providing important genotypic information of pathogenic virulence (Suo *et al*., 2009). In another study, a rapid DNA-microarray-based detection method claimed to identify 10 different pathogenic bacteria (*Enterobacter sakazakii*, *Salmonella enterica*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Serratia marcescens*, *Acinetobacter baumannii*, *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Escherichia coli* O157) associated with powdered infant formula contamination, based on the 16S-23S rRNA gene internal transcribed spacer (ITS) sequences and wzy (O antigen polymerase) gene (Wang *et al*., 2009).

Besides the detection of foodborne pathogens, the success rate of DNA microarrays was tested by another event of epidemic outbreak surveillance (EOS). A custom DNA microarray was designed with the capability of detecting 20 natural (including avian flu) and bio-threat agents with strain-level resolution. The trial exercise on 10 000 samples showed the feasibility of implementing microarrays as a screening tool for EOS regardless of food or biothreat (Uttamchandani *et al*., 2008). These microarray-based platforms for pathogen detection are now on the market from Akonni Biosystems (http://www.akonni.com) and Verdus Laboratories (http://www.vereduslabs.com).

The study of functional expression of the genomic information of microbial pathogenicity using transcriptomics is powerful; however, its scope is limited by not accounting for post-transcriptional processes (Dharmadi and Gonzalez, 2004), a problem which has yet to be resolved. Other tools in '-omics' approaches such as proteomics and metabolomics, with the association of bioinformatics, offer complementary transcription studies to obtain the complete physiological descriptions of individual microbial toxins.

3.3 Proteomics

Proteomics relies heavily on protein characterization at a given time and under certain conditions in prokaryotic or eukaryotic cells. It is also dependent on the sensitivity of instruments for accurate mass determination and the availability of databases for protein identification. Because toxin proteins can mediate microbial pathogenesis, proteomics is a key part of system-level studies and it is the primary tool used to identify cellular interactions beyond the mRNA level (Graham, Graham and McMullan, 2007). Proteomics complements DNA microarray technology for monitoring gene expression and analysing global gene expression patterns at the protein level. Based on the typical workflow shown in Figure 2, discussion will focus on the most common methodologies for identifying toxins generated by food pathogens.

3.3.1 Gel-Based Detection of Microbial Toxins

In proteomics, the invention of 2-DE by O'Farrell and Klose made it possible to explore thousands of proteins at once using an extensive separation technique based on the charge in the first dimension and SDS–PAGE size fractionation in the second dimension (O'Farrell, 1975; Klose, 1975). Nowadays, Immobilized pH gradient (IPG) strips and advanced bioinformatics have remarkably improved the reproducibility and comparability of 2-DE gels. In microbes, proteins can be identified from unique cell type and quantified using 2-DE followed by software-aided quantification tools and mass spectrometry techniques (Watt *et al*., 2003;

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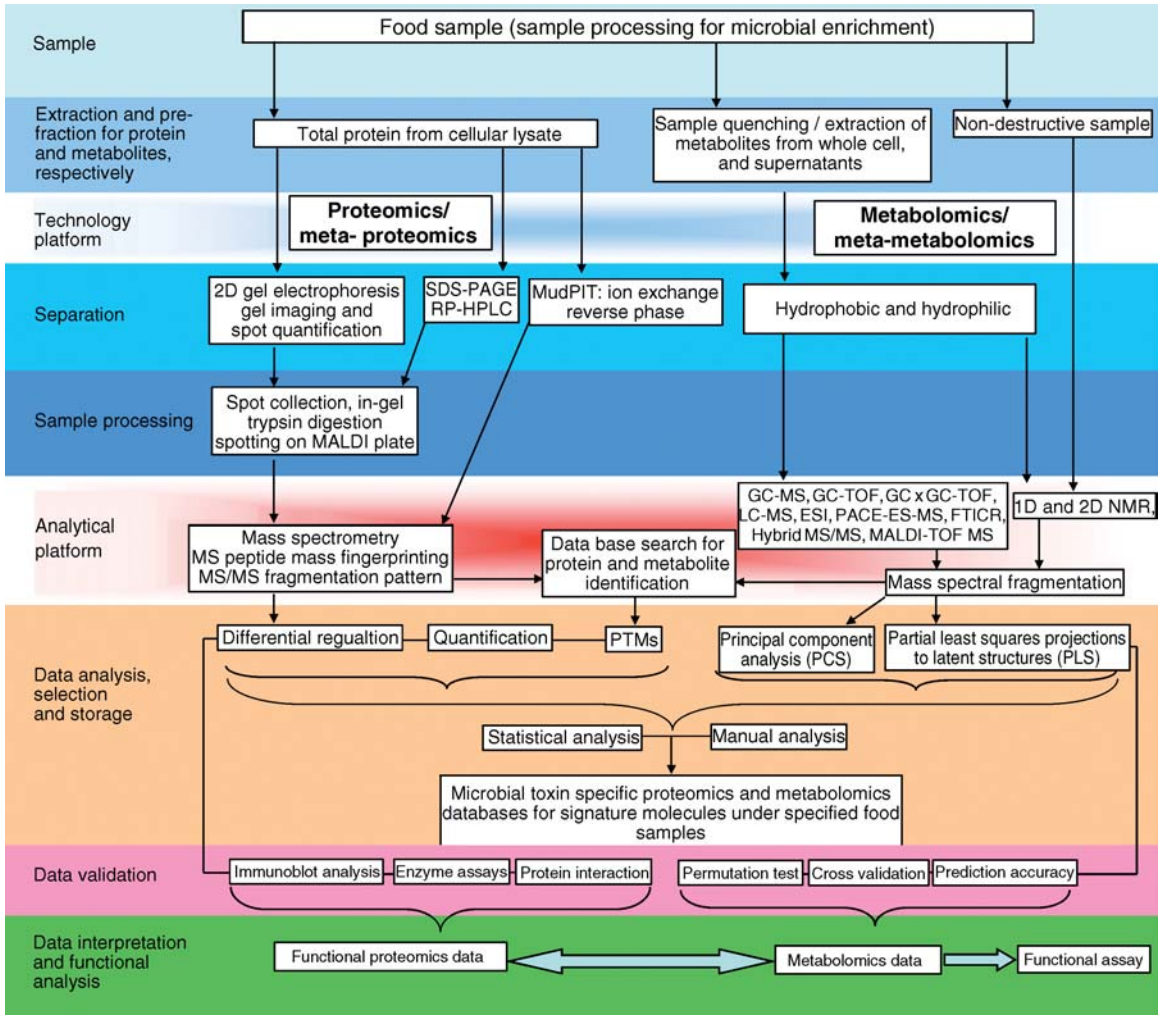

Figure 2. Typical workflow for functional 'omics' approaches, proteomics and metabolomics expression profiling experiments. A variety of methods have been established to identify and/or profile proteins and metabolites. SDS–PAGE, Sodium dodecyl sulphate–polyacrylamide gel electrophoresis; RP-HPLC, reverse phase-high pressure liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization-time or flight; MudPIT, multidimensional protein identification technology; MS, mass spectrometry; GC-MS, gas chromatography–mass spectrometry; GC-TOF, gas chromatography–time-of-flight; NMR, nuclear magnetic resonance; LC-MS, liquid chromatography–mass spectrometry; ESI, electron spray ionization; FT ICR, Fourier transform ion cyclone resonance; PTM, post-translational modification.

Singh, 2006). Similar to transcriptomics, comparative proteomics studies assist in the identification of possible enzymatic bottlenecks in microbial pathogenesis (Oosthuizen *et al*., 2002).

The physiological state of an organism leads to a differential expression in genes and proteins. This expression induces a chain of enzymatic reactions that regulates the synthesis and secretion of many toxin proteins that are tightly controlled by regulatory elements sensitive to the environmental signals. 2-DE can explore the regulatory elements responsible for microbial pathogenesis. A dairy-associated toxigenic bacterium, *Bacillus cereus* is a well-known enterotoxin-producing food-poisoning organism and is regarded as one of the most important organisms that can contaminate pasteurized milk and milk products (Christiansson, Bertilsson and Svensson, 1999). Biofilms of this bacterium may serve as a chronic source of microbial contamination, thereby compromising food

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quality. High-resolution 2-DE revealed 15 and seven uniquely expressed proteins between the 2- and 18-h-old biofilm proteomes of planktonic cells. Induced expression of catabolic ornithine carbamoyltransferase, lactate dehydrogenase and YhbH, a member of the sigma 54 modulation protein family, was obtained in response to environmental stresses, which indicates that this protein plays an important role in regulation of the biofilm phenotype (Oosthuizen *et al*., 2002).

An opportunistic pathogen, *Enterobacter sakazakii* has been implicated in foodborne diseases in neonates and infants. To understand the dry stress condition, *E. sakazakii* was investigated using 2-DE under two different types of osmotic stress conditions (Riedel and Lehner, 2007). A total of 80 differentially expressed protein spots were noted. Protein affiliation revealed that a number of differentially expressed proteins from desiccated and hyperosmotic-grown samples had the same functional category, but were regulated in opposite directions. The protein pattern under high salt concentration reflected that the protein modulated was involved with central metabolic pathways, whereas the adaptation of cells in a desiccated state represents an accumulation of proteins serving in a protective role for the micro-organism.

Campylobacter jejuni is a leading cause of food- and waterborne illness. Seal *et al*. 2007 studied a robust versus a poor chicken-gastrointestinal-colonizing isolate of *C. jejuni* on 2-DE, which revealed the specific expression of an outer membrane fibronectin binding protein, serine protease and a putative aminopeptidase in the soluble portion of the robust colonizer A74C. Several other proteins, including a cysteine synthase and aconitate hydratase, were detected specifically in the poor colonizer *C. jejuni* NCTC 11168-PMSRU isolate, demonstrating that proteomics is a useful tool for characterization of phenotypic variation among *Campylobacter* sp. The membrane-associated proteome of the *C. jejuni* gastrointestinal isolate (JHH1) was investigated by 2-DE and 77 proteins were identified, of which 44 were predicted to be membrane proteins. A total of 28 proteins were significantly abundant in or unique to JHH1, including eight proteins involved in chemotaxis signal transduction and flagellar motility, the amino-acid-binding surface antigens CjaA and CjaC, and four outer membrane proteins (OMPs) (Cordwell *et al*., 2008). Another proteomics analysis of pathogenesis on 2-DE identified consistent changes with modulation of essential metabolic genes, up-regulation of stress tolerance proteins and increased expression of major OMP (MOMP) and flagellar filament protein (FlaA). Among down-regulated proteins, the majority were metabolic proteins, including fructose bisphosphate aldolase (Gba), fumarate hydrotase (FumC), succinyl-CoA synthetase alpha chain (SucD) and aspartate-semialdehyde dehydrogenase (Asd) (Svensson *et al*., 2009).

Clostridium perfringens, a foodborne pathogen associated with the production of 13 different extracellular toxins, was investigated under cooked meat medium using a combination of 2-DE and MS. A total of 11 prominent proteins were over-expressed in the cells of *C. perfringens* ATCC13124 grown in cooked meat medium (Alam *et al*., 2009). In a 2-DE-based proteomic analysis, an enterohaemorrhagic *E. coli* (EHEC) O157 stress-sensitive variant of F2 (designated MP37) reflected decreased culturability, which revealed that MP37 in the viable but non-culturable (VBNC) had decreased levels of some oxidation-responsive factors (AhpCF, Ace F), but had increased levels of outer membrane protein W (OmpW) (Asakura *et al*., 2007). In pathogenesis, micro-organisms usually employ autoinducer molecules that can modulate virulence expressions, biofilm development and bioluminescence. The role of the universal autoinducer LuxS/Al-2 molecule was investigated in *E. coli* O157:H7 on 2-DE. The key finding of the study revealed that only three proteins appear to be under the direct influence of the LuxS/Al-2 system (Soni *et al*., 2007). Interestingly, these studies support identifying the protein expression in *E. coli* O157:H7 that may vary depending on the food matrix.

3.3.2 *Non-Gel-Based Detection of Microbial Toxins*

Despite its visual characterization and powerful separation of bio-molecules, 2-DE-PAGE has downsides, like other technologies. Its limitations include difficulty in resolving proteins with extremes in isoelectric point (p*I*, <4 and >8), molecular mass, stain-based resolution and inability to reveal hydrophobic proteins. Advancements implementing analytical tool MS have helped to modernize the proteomics area. MS deduces the composition of molecules by determining the particular peptide mass that results from certain combinations of

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amino acids. To study these combinations of amino acids, proteins are routinely digested with proteases to generate peptides small enough for MS analysis, referred to as PMF. In this process, the controlled fragmentation of a peptide yields a series of overlapping fragment ions that differ by the mass of a particular amino acid, which allows the full or partial amino acid sequence of the peptide to be deduced. This phenomenon is called MS/MS or tandem MS, as it typically uses one MS analyser to select ions for fragmentation and a second to measure the fragment ions. Over the past 5 years, the gel-free techniques (Figure 2) such as liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and protein isotope labelling have become popular in proteomics (Singh, 2006; Wolff *et al*., 2007; Gevaert *et al*., 2007).

Members of the genus *Cronobacter* are opportunistic pathogens for neonates and are often associated with contaminated milk powder formulas. Carranza *et al*. 2009 analysed surface-associated and whole-cell proteins by two complementary proteomics approaches, 1D-SDS–PAGE combined with LC-ESI-MS/MS and 2D-LC-MALDI-TOF/TOF MS, that led to the identification of 832 proteins corresponding to 19% of the theoretically expressed proteins involved in central metabolic pathways, translation, protein folding and stability. Other identified proteins were putative virulence factors, macrophage infectivity potentiator, a superoxide dismutase protecting the pathogen against reactive oxygen species and an enterobactin-receptor protein.

Campylobacter sp. was investigated under both gel- and non-gel-based proteomics approaches. The protein biomarkers were observed in the matrix-assisted laser desorption/ionization time-of-flight mass spectra (MALDI-TOF-MS) of cell lysate of five strains of *C. upsaliensis* and one strain of *C. helveticus* by "bottom-up" proteomics techniques. Fagerquist 2007 reported these protein biomarkers: periplasmic protein, 10 kD chaperonin, protein of unknown function (DUF465), D-methionine-binding lipoprotein MetQ, phnA protein, DNA-binding protein HU, thioredoxin, cytochrome *c* family protein, helix-turn-helix domain protein, asparigenase family protein and several ribosomal and conserved hypothetical proteins. Interestingly, the proteomic identification of the protein biomarkers of the non-genomically sequenced *C. upsaliensis* and *C. helveticus* identified homologous protein amino acid sequences to that of the sequenced strain. Based on MALDI TOF-TOF, Fagerquist *et al*. 2009 developed web-based software for the rapid identification of protein biomarkers of several micro-organisms.

Various fungi, oomycetes, viruses and bacteria may cause diseases in plants that can be devastating to the food supply. Padliya and Cooper 2006 reviewed MS-based proteomics for the detection of plant pathogens. *Fusarium* infections in maize are a global issue. A LC-MS/MS method was developed to detect beauvericin and the four enniatins A, A1, B and B1 in maize and maize silage (Sorensen *et al*., 2008). A form of direct sample analysis on a microchip using SELDI-TOF-MS is another promising analytical technique for specific samples (Issaq *et al*., 2002); it represents a novel approach to biomarker discovery combined with liquid chromatography. Although SELDI analysis has been useful for identifying potential biomarkers in clinical research, some have questioned its reproducibility and specificity (Jacobs and Menon, 2004; Diamandis, 2004). Another emerging technique, Fourier transform ICR (FT-ICR) MS, may allow multistage MS experiments (MS*n*) with an enhanced detection limit of 30 zmol for a 10-kDa protein (Laskin and Futrell, 2005).

Adding ESI and LC to MS has opened new analytical windows for the detection and identification of potential contaminants in the environment. A direct analysis of a peroxisomal protein pattern associated with marine contaminants in mussels *Mytilus galloprovincialis* was performed using ESI MS/MS to identify various epoxide hydrolases, peroxisomal antioxidant enzymes and SOX (Mi *et al*., 2005). Another ion source that has been developed allows the protein chips to be analysed using a hybrid triple Quadrupole/TOF MS providing additional sequencing capabilities (Seibert *et al*., 2004). With this new source, proteins can be tryptically digested directly on the arrays and the resulting fragments identified by tandem MS. An absolute quantitative mass spectrometry was developed to investigate the *Staphylococcal* enterotoxins involved in food poisoning outbreaks using QTOF-MS (Hennekinne *et al*., 2009).

3.3.3 Interactomics

In micro-organisms, many proteins take part in a multicomponent protein aggregation under

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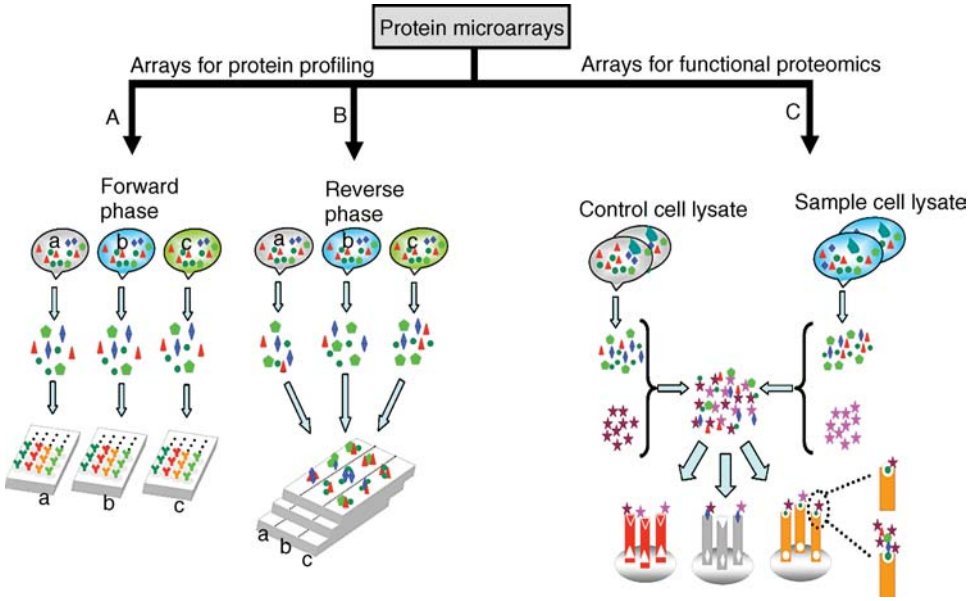

Figure 3. Different types of protein microarray platforms for microbial toxin detection. (a) In a forward-phase protein microarray platform, complex protein mixtures from different sources can be analysed on one microarray containing thousands of captured molecules. The analyte can be detected using direct fluorescent labelling or by a second specific antibody. (b) The reverse-phase protein microarray can be used to analyse whole repertoire of a protein sample that represents the physiological state of an organism. The protein sample can be immobilized onto a nitrocellulose membrane with glass backing. (c) Functional proteomics could further be achieved by designing a differential protein display platform. The control and different physiological state cellular lysate samples conjugated to distinct fluorescent molecules. Equal amounts of protein samples were mixed together and incubated simultaneously on an antibody microarray. The target molecules can be detected by their respective specific antibodies with modulated expression of proteins that may directly reflect in overlay of the colour intensities.

physiological response that affects the entire complex protein interaction network. Detection of interactive partner proteins is the prime task of second-generation proteomics, that is functional proteomics. The growing demand in genomics and proteomics for the analysis of gene and protein function is enormously enhancing the need for microarray-based assays, which are fast, accurate and sensitive. Other than the DNA microarray, as a next-generation tool, antibody-based protein microarrays are increasing in popularity (Angenendt, 2005; Kreutzberger, 2006; Kingsmore, 2006; Ehricht *et al.*, 2009) and it has been verified that this technology is capable of filling the gap between transcriptomics and proteomics (Liu and Zhu, 2005; Kerschgens, Egener-Kuhn and Mermod, 2009).

Protein arrays or biochips are in the developmental stage and can be modified for use in the field to analyse specific protein toxins in an array affixed to a platform (Figure 3). Technically, two types of protein arrays have been developed so far: focused protein profiling and arrays for functional studies. Based on the sample application, the first category can be subdivided into two groups: forward-phase protein microarrays (Figure 3A) and reverse-phase protein microarrays (Figure 3B). The forward-phase protein microarrays use sample incubation on the array so that different analytes can be detected simultaneously, as in antibody microarrays. The reverse-phase arrays, one of the latest phase microarray platforms, feature samples immobilized on a nitrocellulose membrane backing with glass (Figure 3B). Using these arrays, a large collection of probes can easily be screened within a single-step reaction (Labaer and Ramachandran, 2005). The functional protein microarray can further be designed by conjugating the cell lysate sample with the fluorescent molecules (Figure 3C). Differential expression in the target molecule can be detected by overlay of the colour intensities.

Protein Array-Based Detection of Microbial Toxins

Analytical assays are of the utmost importance to ensure food safety. In the past, many diagnostic

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tests were performed based on the cell culture, plate assays, molecular technique (e.g., PCR) or immunoassay methods (e.g., ELISA – enzyme-linked immunosorbent assay) where molecules of interest could be detected in complex food samples. As the expression of most genes, proteins and biomolecules in living cells (prokaryotes or eukaryotes) is environmentally responsive, it is desirable to monitor multiple biomarkers simultaneously to predict the microbial state of action in advance.

Data obtained from protein, protein-domain and peptide microarrays can be combined to understand microbial pathogenesis and detect specific toxins. Rao *et al*. 2004 compared immobilized antibody microarrays with the Luminex flow cytometry system for detection of bacterial and viral proteins and reported lower levels of detection in the latter system, supporting the protein microarray for miniaturization. A fluorescent-labelled antibody-based array biosensor was developed to detect toxins, bacteria and viruses at low levels within 20 min in food, environmental matrixes and clinical samples. The detection limit was 25 pg ml^{-1} in a buffer of ovalbumin egg-contaminated sample and 1.3 ng ml^{-1} (13 ng g^{-1}) was obtained in non-egg pasta extract (Shriver-Lake, Taitt and Ligler, 2004). A variety of food allergen proteins from milk, eggs, hazelnuts, peanuts, shellfish and sesame were detected using biosensor immunoassays in food samples (Yman *et al*., 2006). The allergen detection limit was down to 1–12.5 μg g^{-1} in food samples.

A microarray sandwich immunoassay biosensor was tested to detect *E. coli* O157:H7. The array platform was developed using biotinylated capture antibodies on streptavidin-coated microarray slides. The capture and reporter antibodies on this array platform detected 10^6 cells ml^{-1} under optimized experimentation (Gehring *et al*., 2006). Huelseweh, Ehricht and Marschall 2006 developed a protein chip for the ArrayTube™ platform with easy and cheap handling technology, using the classical procedure of a sandwich-enzyme-linked immunosorbent assay and signal amplification by streptavidin-poly-horseradish peroxidase. With efficient detection limits, this chip was demonstrated to detect a variety of viruses (Venezuelan equine encephalitis virus, St Louis encephalitis virus, West Nile virus, yellow fever virus, Orthopox virus species), bacteria (*Francisella tularensis*, *Yersinia pestis*, *Brucella melitensis*, *Burkholderia mallei* and *Escherichia coli* EHEC O157:H7) and toxins (*Staphylococcus* enterotoxin B, ricin). It was possible to detect five of the "dirty dozen" agents on one single protein chip in parallel. Protein microarray technology is a complementary method for the study of protein *in vitro* as well. Many studies performed using protein-microarray-based platform with wide-ranging potential for biodefence were reviewed by Uttamchandani *et al*. 2008. These studies are equally important to detect foodborne pathogens and ensure the safety of the food chain. Further research on 2-DE-based, MS-based and array-based identification of proteins/enzymes may help us to evaluate the interaction of food matrices and molecules in controlling pathogen survival and virulence in foods.

Although array-based proteomics is an ambitious approach, it raises serious challenges as well. In contrast to captured molecules from DNA chips, which can easily be predicted and synthesized from complementary base pairings, captured molecules from a protein array need to be generated and screened for sensitivity and specificity. Immobilization of proteins on the array without damaging their tertiary structure is also crucial in protein microarrays because the proteins often occur in complexes. Limitations also exist in sensitivities to microarray-based protein expression, as the high background from non-specific binding could interfere with signal intensities. In addition, when the direct fluorescent-labelling approach is used, strong signals within a microspot may derive from a high concentration of a single protein molecule (Figure 3C) or from co-immunoprecipitated complex.

Because the regulatory interactions within any organism exist as a complex network on multiple levels, any individual alteration that might be detected via 2-DE or protein arrays will not necessarily change the way the microbe functions. One must conduct unbiased (metabolite) analyses when creating a gene or protein profile in order to precisely define an organism's biochemical functions. These analyses connect the gene sequence to the metabolic function (metabolome) and work together with existing genomics techniques (transcriptomics). Integration of metabolomics, proteomics and transcriptomics is still a challenge for researchers in situations where low-molecular-weight primary and secondary metabolites play an important part in the toxicity of a microbe.

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3.4 Metabolomics

In the course of its life, a microbial cell will excrete hundreds of different primary and secondary metabolites that vary according to environmental factors. Metabolomics is an extension of targeted metabolite analysis to include the study of the entire metabolic content of a cell at a given time (called the metabolome). It involves three strategies: (a) metabolite fingerprinting, (b) metabolite profiling and (c) metabolome analysis.

Although DNA microarrays and proteomics can produce vast amounts of data for gene and protein expression, metabolomics has far simpler data sets, which makes data analysis and interpretation easier. A typical workflow of most common methodologies used to identify toxins has been shown in Figure 2.

Metabolites are low-molecular-weight organic compounds (<1000 Da) that are involved in general metabolic reactions or required for an organism's maintenance, growth and normal functioning. Like proteins, metabolites are expressed in a context-dependent way, and in different microbial physiological states, metabolite concentrations are directly related to protein expression (Raamsdonk *et al*., 2001); this can facilitate descriptions of those metabolic states in different growth phases. Quantifying all of the metabolites in a cellular system is the ultimate principle for any experiment in metabolomics. To achieve this, automation and miniaturization technologies have been developed that can isolate and characterize metabolites, including tools for sampling, extracting specific molecular classes, maintaining storage temperature, preparing samples and analysing those samples (Buchholz *et al*., 2002;van der Werf, Jellema and Hankemeier, 2005).

To quantify metabolites inside and outside the cell, metabolomics frequently utilizes high-throughput analyses such as nuclear magnetic resonance (NMR) and gas or liquid chromatography paired with mass spectrometry (GC-MS and LC-MS) (Buchholz *et al*., 2002; Issaq *et al*., 2009). After samples are detected using LC-MS, NMR analysis is often performed along with MS/MS experiments. Fractionation and NMR analysis are not often straightforward because the samples are usually complex and in limited quantity; profiling metabolic composition requires a combination of sensitive equipment, such as LC using MS/MS and high-resolution MS detection. One promising new system includes a hybrid MS detector such as a linear ion-trap combined with an FT; however, it is limited to identifying certain isomers (Mano and Goto, 2003; Tolstikov and Fiehn, 2002).

Metabolomics depends on the translation of information from large sets of metabolome data into genotypic and phenotypic characteristics of particular organisms. Unbiased statistical multivariate data analysis (MVDA), pattern recognition, chemometrics and biometrics tools are used to perform this. Because biological systems are multivariate and biomolecules are interdependent, scientific hypotheses can be produced by MVDA analysis through mathematically reducing parameters in data sets and visualizing their clustering behaviour (Dillon and Goldstein, 1984;Glassey, Montague and Mohan, 2000).

Various filamentous fungi and yeasts from the genera *Saccharomyces*, *Penicillium*, *Aspergillus* and *Fusarium* are well known to be involved in food spoilage by degradation or toxin contamination. There are several reviews that aim to compile extensive knowledge of metabolite production by fungi (Cole and Schweikert, 2003a, 2003b; Cole, Jarvis and Schweikert, 2003). Using metabolomics-based approaches, Smedsgaard and Nielsen 2005 described an intelligent screening strategy (ISS) for the efficient discovery of microbial products and exploitation of micro-organisms. These microbial products could be useful to determine specific bacteria from sites of toxin contamination. The micro-organisms *Campylobacter jejuni* 81-176 and *Campylobacter coli* VC167 were functionally characterized using focused and targeted metabolomics approaches, respectively (McNally *et al*., 2006; McNally *et al*., 2007). These studies were designed to demonstrate the usefulness of metabolomics for targeting compounds within the bacterial metabolome to assign functions to genes, identify metabolic intermediates and elucidate novel biosynthetic pathways. A capillary electrophoresis-TOF-MS method with integrated high-confidence analyte identification for metabolomics was developed to elucidate metabolic changes in *E. coli* (Timischl *et al*., 2008). A comprehensive global metabolic profiling of *E. coli* was examined in chemostate culture conditions in the laboratory (Winder *et al*., 2008).

To detect specific foodborne pathogens, complete profiles of their metabolomes need to be carried

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Table 3. Bioinformatics tools to analyse gene expression.

Name	Developer	Classification	URL
ArrayAssist	Stratagene, USA	K means; hierarchical clustering; self-organizing maps; principal component analysis	http://www.stratagene.com/softwaresolutions/
Avadis Prophetic	Strand Genomics, USA	K means; hierarchical; self-organizing maps; random walk; eigenvalue	http://avadis.strandgenomics.com/
BRB ArrayTools	National Cancer Institute, USA	Hierarchical clustering; multi-dimensional scaling	http://linus.nci.nih.gov/BRB-ArrayTools.html
Cleaver 1.0	Stanford University, USA	K means; principal component analysis	http://classify.stanford.edu
Cluster 2.11	Lawrence Berkeley National Lab, USA	Hierarchical clustering, self-organizing maps (SOMs); K means clustering; principal component analysis	http://rana.lbl.gov/EisenSoftware.htm
GeneCluster	Broad Institute, Massachusetts Institute of Technology, USA	Self-organizing maps	http://www.broad.mit.edu/cancer/software
GeneLinker Platinum	Improved Outcomes Software, Canada	K means; hierarchical clustering; self-organizing maps; principal component analysis	http://www.improvedoutcomes.com/
GenePattern	Broad Institute, Massachusetts Institute of Technology, USA	Hierarchical clustering; self-organizing maps	http://www.broad.mit.edu/cancer/software
Genesis	Graz University of Technology, Austria	K means; hierarchical clustering; self-organizing maps; principal component analysis	http://genome.tugraz.at/Software/Genesis/Genesis.html
GeneSpring	Agilent Technologies, USA	K means; hierarchical clustering; self-organizing maps; principal components analysis	http://www.agilent.com/chem/genespring/
GeneTraffic	Stragene, USA	K means; hierarchical clustering; self-organizing maps; principal component analysis	http://www.stratagene.com/softwaresolutions/
MultiExperiment Viewer	The Institute for Genomic Research, USA	K means; hierarchical clustering; self-organizing maps; principal component analysis	http://www.tm4.org/mev.html
Partek Predict	Partek, USA	Principal component analysis	http://www.partek.com/

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Table 4. Major proteomics database resources of microbiology research.

Website resources	Description
http://www.mpiib-berlin.mpg.de/2D-PAGE/	Proteomics Database system for Microbial Research
http://webclu.bio.wzw.tum.de/prompt	PROMPT: Protein mapping and comparison Tool
http://ca.expasy.org/sprot/hamap/	HAMP: High-quality automated and manual annotation of microbial proteomics
http://gelbank.anl.gov/	GELBANK
http://david.abcc.ncifcrf.gov/conversion.jsp	Database for annotation, visualization and Integrated discovery (DAVID)
http://genolist.pasteur.fr/	GenoList
http://fields.scripps.edu/sequest/	SEQUEST
http://www.doe-mbi.ucla.edu/Services/GDAP	GDAP
http://www-lecb.ncifcrf.gov/2dwgDB	2DWG Image Meta-database
http://bioinformatics.icmb.utexas.edu/OPD	Open Proteomics Database
http://www.systemsbiology.org	Systems Biology Institute
http://www.expasy.ch/ch2d/	Swiss 2DPAGE
http://mipsgsf.de	MIPS
http://www.bind.ca	BIND
http://www.sbeams.org	SBEAMS
http://www.expasy.ch/ch2d/2d-index.html	Index
http://dip.doe-mbi.ucla.edu	For interacting proteins (DIP)
http://www.ebi.ac.uk/intact	Protein–Protein interaction database (IntAct)
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein	NCBI protein database
http://www.wzw.tum.de/proteomik/lactis/	DynaProt 2D
http://www.cebitec.uni-bielefeld.de/groups/brf/software/brifep	BRIGEP-BRIDGE based browser
http://compbio.mcs.anl.gov/sentra/	SENTRA (Signal transduction proteins)
http://www.boutell.com/gd	GD library for 2D gels

Table 5. Major metabolomics database and pathway resources of microbiology research.

Website resources	Description
http://marvis.gobics.de/	MARVIS: Tool for clustering and visualization of metabolome markers
http://rosy.tu-bs.de	ROSY – the *ROseobacter* SYstems biology database
http://www.systomonas.de	System Biology of *Pseudomonas*
http://www.genome.ad.jp/kegg/	KEGG: GeneBank based pathways flow chart
http://ch.expasy.org/enzyme/	ENZYME: Enzymatic nomenclature
http://metlin.scripps.edu/	METLIN: Metabolites correlation solution
http://MetaCyc.org/	MetaCyc
http://BioCyc.org/	BioCyc: Cellular metabolic networks
http://www.ncgr.org/pathdb/	PathDB: Pathways search tools
http://www.biocarta.com/genes/allPathways.asp	BioCarta: Designing the Pathways maps
http://csbdb.mpimp-golm.mpg.de	Golm metabolom database: Gas chromatography-MS based metabolite profiling
http://wit.mcs.anl.gov/WIT2	WIT: Metabolic reconstruction
http://compbio.mcs.anl.gov/puma2/cgi-bin/met_recon.cgi	PUMA2: Evolutionary analysis of metabolism
http://www.brenda.uni-koeln.de/	BRENDA: Enzymatic information
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=pccompound	PubChem: Organic compound search with Enzyme and protein sequence link
http://ch.expasy.org/tools/pathways/	Pathways chart: Roche Biochemical Pathways
http://www.genome.jp/kegg/pathway.html#environmental	KEGG: Pathway database
http://www.genome.ad.jp/ligand/	LIGAND: A composite database comprising three sections: compound, reaction and enzyme

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out that characterize specific toxins. Specifically, enterotoxin and exotoxin activity can be profiled to characterize the neighbouring or corresponding enzymes based on qualitative and quantitative estimation. However, no report has been published so far that represents a global metabolome study to detect foodborne pathogens from given food matrices.

3.5 Bioinformatics

The tools of functional transcriptomics, proteomics and metabolomics are decisive for the description of microbial virulence, but they generate a great amount of data that may be difficult to analyse without a bioinformatics (computational tools) approach. Bioinformatics plays a key role in integrating and analysing the obtained data in DNA microarray transcriptomics. Reconstruction of metabolic potential using bioinformatics tools and databases, followed by targeted experimental verification and exploration of metabolic and regulatory network properties, are among the top challenges for those interested in improving the exploitation of food pathogens.

In microbial biotechnology, bioinformatics has been used for computation analysis of laboratory data, identification of protein coding segments and development of genomics, proteomics and metabolomics data. Bioinformaticians aimed to design computational methods to determine and predict biomolecules based on three major approaches: (a) to combine mathematical modelling and search techniques; (b) to explore, match and analyse high-level functions using mathematical modelling; and (c) to use computation search alignment techniques to compare new biomolecules within each functional '-omics' approach.

The powerful tools of systems biology (transcriptomics, proteomics and metabolomics) can be verified by *in silico* pathways based on the information biomolecules generated. The exponential growth of such verifications is summarized here in comprehensive lists of genomics (Table 2), transcriptomics (Table 3), proteomics (Table 4) and metabolomics (Table 5) databases for microbial study with respective descriptions. A comprehensive resource that can fulfil the requirements for gene-protein-based and metabolite-based detection of foodborne pathogens is still needed, however.

4 OMICS AND FUTURE IMPLICATIONS – REACTOME

After it became possible to integrate computational biology with DNA and protein microarrays, 2-DE and sophisticated analytical chemistry tools (Figure 2), biological information could be interpreted in advance. A comprehensive transcriptome, proteome and metabolome study would be especially useful in understanding microbial pathophysiology in different phases of growth and locating contamination sources in food. Thus, a new method is needed to connect functional products of genes (metabolites) with cognate enzymes/proteins and produce global descriptions of metabolic responses. An array-based "reactome" could contain a detailed, quantitative profile of the metabolic activity of a cell population—a "metabolic bar code" for a specific micro-organism—and serve as a connection between the genome and metabolome by centralizing the proteome. A metabolome–genome link has already been established in one study by determining the metabolic profiles of *Pseudomonas putida* strain (KT2440) and *Streptomyces coelicolor* strain (M145) on a reactome array (Beloqui *et al*., 2009). By connecting genes and metabolites and determining possible enzymes/proteins on the array, the reactome can be applied towards the detection of foodborne pathogens.

Towards fulfilling the goal of early and faster detection of foodborne pathogens, genomics data are necessary to complement the proteomics and metabolomics approaches. However, proteomics and metabolomics would retain their central positions in functional transcriptomics and genomics. A study published by Trauger *et al*. 2008 correlates the transcriptome, proteome and metabolome in the environmental adaptation of *Pyrococcus furiosus*, a hyperthermophile. Analysing low-molecular-weight metabolites related to proteins, not mRNAs, is essential in determining the nature and site of a toxin, which is presumably the final destination of gene expression. A faster and more reliable detection method for the individual toxin products, the enzymes and/or metabolites, can only be discovered via a joint transcriptomics, proteomics and metabolomics approach (Figure 1).

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The recently developed bioinformatics tools at http://www.systomonas.de and http://rosy.tu-bs.de may also help to correlate microbial metabolic physiology, genetic profiling and protein profiling on a single platform.

5 CONCLUSION

A variety of microbial toxins have now become diversified in food and related substances due to ongoing microbial tolerance of antibiotics and other environmental factors. Our understanding to trace diversified microbial toxins is limited because of complex microbial physiology. The challenges in understanding of these toxins can be met by integrating functional '-omics'-based technologies such as genetic engineering, transcriptomics, proteomics and metabolomics. In addition, "reactome" offers remarkable, unique platform ahead to understand and detect microbial toxins. The gene-oriented changes are now feasible to explore through proteomics that can determine the microbial virulent state in adulterated food. The expression pattern of intra- and extracellular metabolites from unique gene through protein expression route would promise to predict organisms' metabolic path towards toxin products. The continued technological advancements could ultimately lead to comprehensive approaches in which gene expression, protein and metabolites are analysed to elucidate the functioning of entire organism.

RELATED ARTICLES

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Epigenomic Analysis in Toxicology

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1 INTRODUCTION

The epigenome, the totality of epigenetic modifications in a cell, plays a fundamental role in development, differentiation, and reprogramming (Law and Jacobsen, 2010). Like the genome and unlike transcriptome and proteome in a cell, the epigenome is replicated upon somatic DNA replication (Ushijima *et al*., 2003; Laird *et al*., 2004; Riggs and Xiong, 2004; Margueron and Reinberg, 2010). At the same time, unlike the genome, the epigenome undergoes dynamic changes during development, differentiation, and reprogramming (Bird, 2007; Cedar and Bergman, 2009). In other words, the epigenome is established as a consequence of interactions between the genome and environmental input (Gan *et al*., 2007), and serves as a cellular memory once established.

From a toxicological viewpoint, agents that induce aberrations in the epigenome are of serious concern. Once an aberrant epigenome is established by some factors, the aberrant epigenome is inherited at somatic cell divisions even if the aberrant status is hazardous to the cell or host. It is well established now that aberration of the epigenome can be causally involved in cancer development and progression (Jones and Baylin, 2007), and it is expected that aberration of the epigenome could be involved in a broader range of acquired disorders (Jones *et al*., 2008; Robertson, 2005). This chapter will introduce what is the epigenome, how it is altered in cancers and other disorders, what induces epigenetic alterations, and essential techniques for epigenome analysis.

2 EPIGENETIC MODIFICATIONS AND EPIGENOME

Epigenetic modifications include DNA methylation and histone modifications. DNA methylation is well known for its high fidelity at somatic cell replication (Ushijima *et al*., 2003; Laird *et al*., 2004; Riggs and Xiong, 2004), and thus is considered as the central player in maintenance of long-term cellular memory in mammalian cells. Histone modifications are more diverse, and individual modifications seem to have their own roles and fidelity in somatic cell replication.

2.1 DNA Methylation

DNA methylation in epigenetics refers to physiological methylation at the 5 position of cytosines at some CpG sites (Figure 1a). This methylation is different from pathological DNA methylations at O^6 and $N7$ positions of guanines, which are abnormal adducts produced by alkylating agents and important in the field of toxicology. DNA methylation at CpG sites is characterized by its inheritance upon somatic cell division, and critical roles in regulation of gene transcription.

2.1.1 Maintenance of DNA Methylation Statuses

When a CpG site is methylated, cytosines on both strands are methylated (Figure 1b). At DNA

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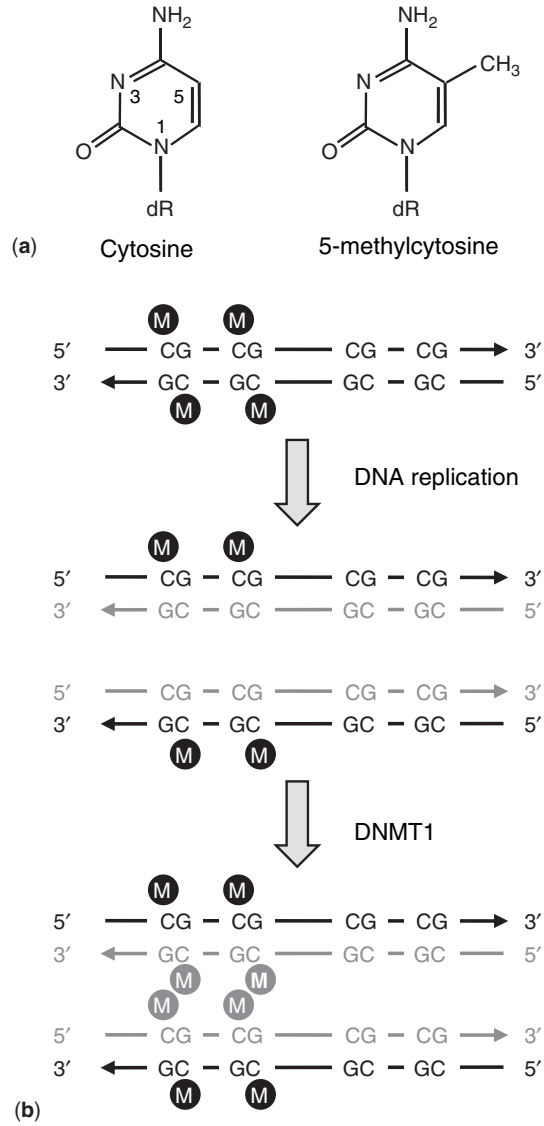

Figure 1. Characteristics of DNA methylation: (a) structure of 5-methylcytosine; (b) maintenance of DNA methylation at somatic cell replication. DNMT1 restores fully methylated statuses by methylating hemi-methylated CpG sites at DNA replication. Methylated or unmethylated statuses are inherited with high fidelity.

replication, cytosines in a newly synthesized DNA strand do not contain methyl groups, and hemi-methylated CpG sites are temporarily formed. However, a maintenance methylase, DNA methyltransferase 1 (DNMT1), associated with a replication fork (Hermann, Goyal and Jeltsch, 2004), restores those hemi-methylated CpG sites into fully methylated CpG sites. DNMT1 has much lower activity on unmethylated CpG sites, and unmethylated CpG sites are kept unmethylated. Therefore, DNA methylation patterns are replicated at somatic DNA replication with a high fidelity (~99.9%), especially in CpG islands (Ushijima *et al*., 2003; Laird *et al*., 2004; Riggs and Xiong, 2004).

DNA methyltransferases are essential machineries to establish and maintain DNA methylation. As mentioned above, DNMT1 has the major role in maintaining DNA methylation upon DNA replication, and homozygous knockout of *Dnmt1* is lethal in mid-gestation (Li *et al*., 1992). In contrast, two *de novo* methylases, DNMT3A and DNMT3B, are involved in establishment of genome-wide DNA methylation patterns (Okano, Xie and Li, 1998; Hermann, Goyal and Jeltsch, 2004). While Dnmt3a cannot methylate nucleosomal DNA, Dnmt3b can (Takeshima *et al*., 2006). Homozygous knockout of *Dnmt3a* causes lethality after birth (Okano *et al*., 1999), and Dnmt3a is essential in establishment of genomic imprinting (Kaneda *et al*., 2004). Homozygous knockout of *Dnmt3b* causes lethality before birth, and germline mutations of *DNMT3B* cause a recessive inherited disorder, ICF syndrome, in humans (Okano *et al*., 1999).

2.1.2 *Gene Silencing Caused By DNA Methylation of Promoter CpG Islands*

DNA methylation of a CpG island in a gene promoter region has been known to be consistently associated with transcriptional repression of its downstream gene (Baylin and Ohm, 2006; Ushijima, 2005). This was further supported by recent genome-wide analyses of DNA methylation and gene expression (Weber *et al*., 2007; Rauch *et al*., 2009; Yamashita *et al*., 2009). As a mechanism how DNA methylation of a CpG island in a promoter region causes silencing of its downstream gene, the role of nucleosome formation, as discussed below, is currently believed to be important (Li *et al*., 2007), in addition to induction of inactive histone modifications and inhibition of binding of methylation-sensitive transcription factors.

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2.1.3 *Gene Body Methylation and Increased Transcription*

Recent genome-wide analyses also showed that methylation of CpG islands in gene bodies is often associated with increased gene transcription (Hellman and Chess, 2007; Rauch *et al*., 2009; Yamashita *et al*., 2009). Since the association is much weaker than that between methylation of promoter CpG islands and gene repression, the association observed in gene bodies is considered to have no direct cause-consequence relationship.

2.2 Nucleosomes and Histone Modifications

DNA in the nucleus is not naked, and forms nucleosome structures along with core histones. The critical importance of histone modifications and nucleosomes in transcriptional regulation is now recognized.

2.2.1 *Nucleosome and Nucleosome-Free Region in Promoters*

A core nucleosome is made of 146 ± 2 base-pair stretches of DNA around the histone octamer for 1.65 turns in a left-handed superhelix (Figure 2) (Luger *et al*., 1997). A linker is made of a short stretch of DNA and linker histone H1, and connects two nucleosomes. Thus one nucleosome contains a core and a linker, and is approximately 200 base-pairs long. The histone octamer consists of two of each of four core histone proteins, H2A, H2B, H3, and H4. It is known that DNA in nucleosomes is resistant to micrococcal nuclease and *Sss*I methylase activity, which is experimentally important.

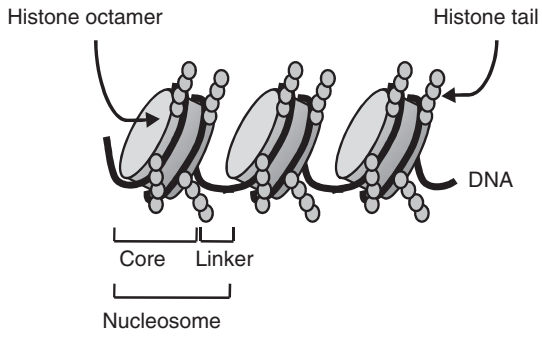

Figure 2. Structure of nucleosome. DNA wraps around the histone octamer, forming a core nucleosome. Core nucleosomes are connected by a linker, and the core and linker forms a nucleosome. Histone tails protrude from the histone octamer.

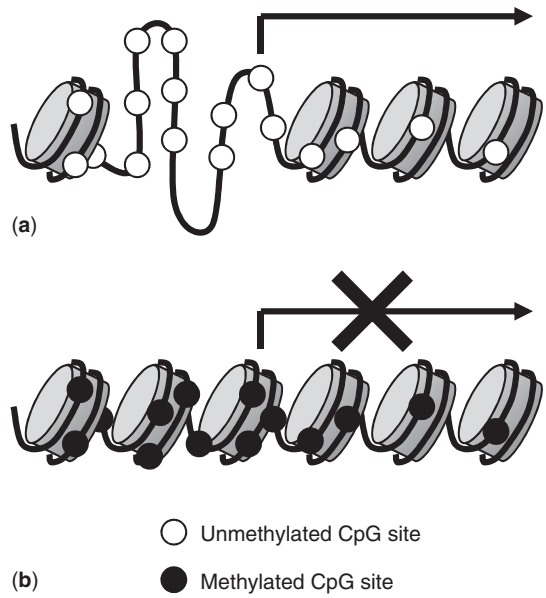

Figure 3. Nucleosome-free region (NFR) and its role in transcription: (a) an approximately 200 bp region upstream of a transcription start site (TSS) lack a nucleosome, and is designated as a NFR. RNA polymerase II and other transcription factors are considered to bind to the NFR; (b) if a NFR in a promoter CpG island is methylated, a nucleosome is formed, and transcription from the NFR is markedly impaired.

It is now known that an approximately 200 bp region just upstream of a transcription start site (TSS) lacks a nucleosome, forming a nucleosome-free region (NFR) (Figure 3a) (Lee *et al*., 2004; Li *et al*., 2007; Ozsolak *et al*., 2007). When a NFR of a CpG-rich promoter is unmethylated, no nucleosomes are formed there, and transcription can be initiated. In contrast, if a NFR is methylated, a nucleosome is formed in the region, and transcription is markedly impaired (gene silencing by promoter methylation) (Figure 3b) (Lin *et al*., 2007).

2.2.2 *Histone Modifications and Their Roles in Transcription Regulation*

All core histones are composed of a histone fold domain and a structurally undefined tail region (Zheng and Hayes, 2003). Tail regions of histones

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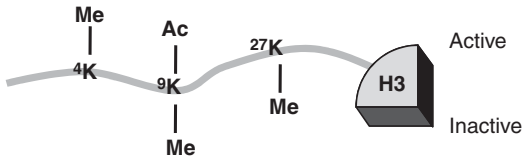

Figure 4. Representative histone modifications. Methylation of H3K4 and acetylation of H3K9 are associated with increased gene transcription, and methylation of H3K9 and H3K27 are associated with gene silencing.

H2A, H2B, H3, and H4 protrude from the histone octamer, and their chemical modifications play important roles in gene regulation (Ruthenburg *et al*., 2007). Histone acetylation can be observed on the tails of four kinds of histones, and is usually associated with active gene transcription. Histone acetyltransferases (HATs) acetylate histones, and histone deacetylases (HDACs) deacetylate histones (Minucci and Pelicci, 2006). Four classes of HDACs are known, and HDAC1, HDAC2, and HDAC4 are considered to be good targets of HDAC inhibitor drugs.

In addition to histone acetylation, histone methylation at specific lysine and arginine residues is now known to have specific meanings (Figure 4) (Ruthenburg *et al*., 2007). Especially, methylation of lysine 4, 9, and 27 of histone H3 (H3K4, H3K9, and H3K27, respectively) is associated with active or inactive gene transcription (Barski *et al*., 2007). At transcription start sites, trimethylation of H3K4 (H3K4me3) is strongly associated with active transcription, and H3K27me3 is associated with silencing of a group of genes (Barski *et al*., 2007; Kondo *et al*., 2008). In transcribed regions, H3K4me1, H3K4me2, and H3K4me3 are associated with active transcription, H3K27me2 and H3K27me3 are associated with inactive transcription, and H3K9me2 and H3K9me3 are weakly associated with inactive transcription. The methylation statuses of histones are finely regulated by histone methyltransferases (Kouzarides, 2007; Hublitz, Albert and Peters, 2009) and demethylases (Shi, 2007; Klose and Zhang, 2007).

2.3 Interplay Between DNA Methylation and Histone Modifications

DNA methylation and histone modifications are often dependent upon each other. For example, DNA methylation is recognized by multiple proteins, such as MeCP2 and MBDs, and these proteins recruit histone deacetylases (Richards and Elgin, 2002) and a histone methyltransferase, SUV39H1 (Fujita *et al*., 2003), which is known to be involved in formation of a heterochromatin structure (Stewart, Li and Wong, 2005). Deacetylated histones are known to be positively charged and to associate tightly with DNA, inhibiting accession of transcription complexes to DNA. On the other hand, H3K9me3 is recognized by heterochromatin protein 1 (HP1), and HP1 recruits DNMT3A and DNMT3B (Fuks, 2005). It helps that inactive histone modification is re-enforced by DNA methylation.

3 EPIGENOME ALTERATIONS IN CANCERS AND OTHER DISORDERS

Epigenomes of normal cells are precisely established and maintained according to developmental stages (Meissner *et al*., 2008; Rauch *et al*., 2009). The vast majority of CpG islands are kept unmethylated, and repetitive sequences, which consist of more than 40% of the genome (Lander *et al*., 2001), are heavily methylated. In cancer cells, an altered epigenome, characterized by "global hypomethylation and regional hypermethylation", is observed (Figure 5).

3.1 Global Hypomethylation

Global hypomethylation, defined as a decrease in 5-methylcytosine content in the genome, is proposed to be present in almost all types of cancer cells (Feinberg and Tycko, 2004). Global hypomethylation is closely associated with hypomethylation of repetitive sequences (Feinberg and Tycko, 2004; Kaneda *et al*., 2004), but can involve demethylation of normally methylated CpG islands. Demethylation of normally methylated promoter CpG islands leads to aberrant transcription of cancer-testis antigen genes, such as melanoma antigen genes (*MAGE*s) (de Smet *et al*., 1999), and potentially oncogenes. Also, hypomethylation of a differentially methylated region (DMR) of *IGF2*, known as loss of imprinting, can lead to increased expression and tumor development (Cui *et al*., 2002). A mouse strain with global hypomethylation demonstrated increased rates of chromosomal loss

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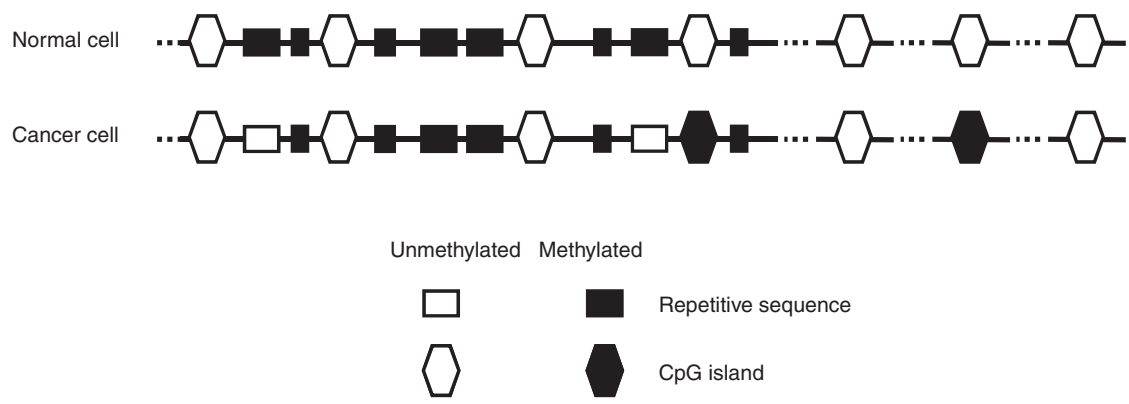

Figure 5. Epigenomic alterations in cancers. Normally methylated repetitive sequences are hypomethylated, and a fraction of normally unmethylated CpG islands are methylated.

(Chen *et al*., 1998) and increased incidences of lymphomas, colonic microadenomas and liver tumors (Chen *et al*., 1998; Eden *et al*., 2003; Yamada *et al*., 2005). At the same time, global hypomethylation led to suppression of macroscopic tumors of the intestine (Laird *et al*., 1995; Yamada *et al*., 2005).

3.2 Regional Hypermethylation – Aberrant Methylation of CpG Islands

"Regional hypermethylation" denotes methylation of CpG islands that are normally unmethylated. If such methylation is induced in the promoter CpG island of a tumor-suppressor gene, the gene is permanently silenced, and the silencing can be causally involved in cancer development and progression (Baylin and Ohm, 2006; Jones and Baylin, 2007). Now, many tumor-suppressor genes involved in various cellular processes, such as cell cycle regulation (*CDKN2A*), WNT signalling (*SFRP* family and *CDH1*), and DNA repair (*MLH1* and *MGMT*), are known to be inactivated by promoter methylation (Baylin and Ohm, 2006). In some cancer types, such as gastric cancers, tumor-suppressor genes are inactivated more frequently by promoter methylation than by mutations (Ushijima and Sasako, 2004). Importantly, CpG islands aberrantly methylated in cancers are not limited to those in promoter regions, and can be present in CpG islands in gene bodies. Methylation of such regions is often associated with increased gene expression (Ushijima, 2005; Rauch *et al*., 2009; Yamashita *et al*., 2009).

3.3 Driver Methylation and Passenger Methylation

Now it is known that several hundred to one thousand promoter CpG islands are methylated in cancer cells (Rauch *et al*., 2009; Yamashita *et al*., 2009). Most of the genes methylated in cancers have no or only low expression, have H3K27me3 modification, and lack stalled RNA polymerase II in normal counterpart cells (Takeshima and Ushijima, 2010; Takeshima *et al*., 2009). Therefore, it is considered that most of the promoter CpG islands aberrantly methylated in cancers are not causally involved in carcinogenesis, but methylated in association with it. As mutations are classified as driver and passenger mutations, methylation causally involved in carcinogenesis is designated as "driver methylation", and methylation that simply accompanies the process is designated as "passenger methylation".

3.4 Aberrant Histone Modifications

Histone modifications are also known to be altered in cancers. The global decrease in acetylation of lysine 16 and trimethylation of lysine 20 of histone H4 is known as a hallmark of cancer cells (Fraga *et al*., 2005a). A global decrease in H3K4me1, H3K9me2, and H3K9me3 and acetylation of histone H3 and H4 are reported in prostate cancer cells (Ellinger *et al*., 2010; Seligson *et al*., 2009). A decrease in H3K4me2, H3K9me2, and acetylation of H3K18 is present in pancreatic cancers,

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and is the most significant predictor of overall survival (Manuyakorn *et al*., 2010). In addition to these alterations, EZH2, a histone methyltransferase involved in H3K27me3, is known to be overexpressed in breast and prostate cancer cells (Kleer *et al*., 2003; Varambally *et al*., 2002). In accordance with EZH2 overexpression, H3K27me3 is increased in many genes in prostate cancer cells (Kondo *et al*., 2008). Since H3K27me3 is involved in gene silencing independently of DNA methylation (Kondo *et al*., 2008), it is expected that H3K27me3 can be causally involved in gene silencing of tumor-suppressor genes.

3.5 Epigenetic Field for Cancerization

Aberrant DNA methylation is present in non-cancerous tissues of cancer patients, forming an epigenetic field for cancerization (epigenetic field defect) (Figure 6) (Ushijima, 2007). High levels of methylation of specific CpG islands, if appropriately selected, are observed in non-cancerous tissues of cancer patients, but not in the corresponding tissues of age-matched individuals (Maekita *et al*., 2006). The methylation level is correlated with risk of cancer development (Nakajima *et al*., 2006), and the accumulation can be considered to be associated with cancer development. In an animal model, it was clearly demonstrated that aberrant DNA methylation was induced as a result of exposure to an environmental factor, and accumulation is associated with cancer development (Niwa *et al*., 2010). Epigenetic field defects are now attracting attention as a target for cancer risk diagnosis and cancer prevention.

3.6 Comparison Between Point Mutations and Aberrant DNA Methylation

Aberrant DNA methylation of promoter CpG islands, especially in NFRs, is now accepted as an equivalent of inactivating mutations, such as inactivating point mutations and chromosomal losses. However, when compared with point mutations, sharp contrasts have been clarified (Table 1) (Ushijima and Asada, 2010). The number of alterations in a cancer is estimated to be approximately 80 for mutations and several hundred to 1,000 for methylation (Gao *et al*., 2008; Hayashi *et al*., 2007; Keshet *et al*., 2006; Rauch *et al*., 2008; Wood *et al*., 2007; Yamashita *et al*., 2009). The fraction of cells with alterations in non-cancerous (thus polyclonal) tissues is very small for mutations (usually at 1×10^{-5}/cell) and can be large for methylation

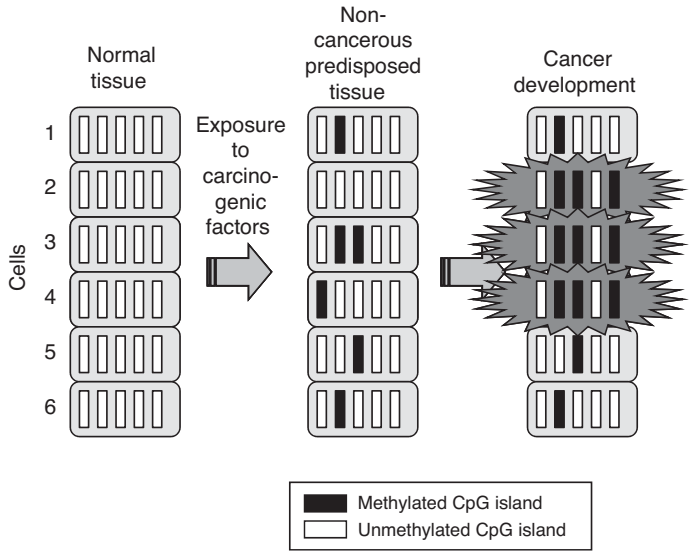

Figure 6. Epigenetic field for cancerization. By exposure to carcinogenic factors, methylation of various, but specific genes, involving both passenger and driver genes, is induced in normal appearing tissues. However, the accumulation level is correlated with cancer risk, and the status is designated as an epigenetic field for cancerization or epigenetic field defect.

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Table 1. Comparison between aberrant DNA methylation and point mutations.

	Point mutation	DNA methylation	References
Number of alterations per cancer cell	~80	Several hundred to 1,000	(Gao *et al*., 2008; Hayashi *et al*., 2007; Keshet *et al*., 2006; Rauch *et al*., 2008; Wood *et al*., 2007; Yamashita *et al*., 2009)
Frequency of alterations of a specific gene in non-cancerous tissues	10^{-5}/cell up to 10^{-3}/cell	0.1 to several % up to several 10%	(Maekita *et al*., 2006; Nagao *et al*., 2001)
Target gene	Random	Specific	(Costello *et al*., 2000; Keshet *et al*., 2006; Loeb, 2001; Wood *et al*., 2007)
Reversibility	Irreversible	Reversible	(Gan *et al*., 2007; Issa and Kantarjian, 2009; Jones and Taylor, 1980; Loeb, 2001; Meissner *et al*., 2008; Wood *et al*., 2007)

Modified from (Ushijima and Asada, 2010).

(up to several 10% of cells) (Maekita *et al*., 2006; Nagao *et al*., 2001). Regarding target genes, mutations are induced mostly in random genes, but methylation is induced in specific genes depending on tissues and inducers (Costello *et al*., 2000; Keshet *et al*., 2006; Loeb, 2001; Wood *et al*., 2007; Nakajima *et al*., 2009; Oka *et al*., 2009). Although mutations are essentially irreversible, methylation is potentially reversible, and is now used as a therapeutic target (Gan *et al*., 2007; Issa and Kantarjian, 2009; Jones and Taylor, 1980; Loeb, 2001; Meissner *et al*., 2008; Wood *et al*., 2007). As discussed in Section 4, inducers of aberrant DNA methylation are markedly different from those of mutations.

3.7 Possible Involvement of Epigenomic Alterations in Acquired Disorders Other Than Cancers

Epigenomic alterations are known to be responsible for some inborn disorders other than cancers, such as Rett syndrome (inborn mutations of *MeCP2*), ICF syndrome (inborn mutations of *DNMT3B*), and Beckwith-Wiedemann syndrome (imprinting disorder). From toxicological viewpoints, involvement of epigenomic alterations in acquired human disorders other than cancers is of great interest. As described above, aberrant methylation of specific genes can be present in up to several 10% of cells in non-cancerous tissues, different from mutations. Even if one of 10^5 cells in a tissue had lost expression of specific genes by mutations, it does not harm the function of the tissue. However, it is well expected that, if 10% of cells in a tissue had lost expression of specific genes by methylation, it could harm the function of the tissue.

Epigenomic differences become larger as monozygotic twins grow older, and this could explain different disease susceptibility between twins (Fraga *et al*., 2005b). Monozygotic twins with and without multiple sclerosis had exactly the same genome and transcriptome, but a slightly different epigenome (Baranzini *et al*., 2010). Glucocorticoid receptor is reported to be aberrantly methylated in the hippocampus of suicide victims (McGowan *et al*., 2009). Activating epigenetic changes are induced in the nuclear factor kappaB (NF-κB) subunit *p65* gene in aortic endothelial cells after transient high glucose, and the epigenetic changes and altered gene expression persists during subsequent normoglycemia (El-Osta *et al*., 2008). Involvement of epigenetic alterations in autoimmune disorders and atopic disorders is also proposed (Maciejewska Rodrigues *et al*., 2009; van Panhuys, Le Gros and McConnell, 2008). The mechanistic basis and evidence in human and animal studies strongly indicate that epigenomic alterations are involved in common acquired human disorders.

4 INDUCERS OF EPIGENETIC ALTERATIONS

Epigenetic alterations play a major role in cancer development, as described, and possibly in other disorders (Robertson, 2005; Ushijima and

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Asada, 2010). Nevertheless, only limited information is available on the factors that induce epigenetic alterations, including aging, inflammation, virus infection, one carbon metabolism, and chemicals (Ushijima and Okochi-Takada, 2005). These inducers are also in a sharp contrast with those of mutations, such as mutagenic chemicals, radiation, and ultraviolet light. Little information is available on how epigenetic alterations are induced.

4.1 Interpretation of Changes in Epigenetic Modifications

Epigenetic modifications can regulate gene transcription, but can be regulated by it at the same time. Therefore, we have to be cautious in interpreting the meaning of changes of epigenetic modifications. An agent may target epigenetic modifications first, and the epigenetic changes can then lead to permanent changes in gene expression (left panel in Figure 7). This change of epigenetic modifications corresponds to mutations, and can be designated as epigenetic alterations. Inducers of epigenetic modifications are of great concern from a toxicological viewpoint.

At the same time, an agent may induce gene expression changes first, and then the expression changes can lead to changes in epigenetic modifications, such as histone acetylation statuses (right panel in Figure 7). Such changes in epigenetic modifications might be inherited upon cell division, or might not be inherited. It is often observed that DNA methylation of a CpG island in an exon is induced when expression of the gene is reduced, or that DNA methylation of the CpG island is reduced when its expression is induced (see sections 2.1.2 and 2.1.3).

Even limited to regions within a promoter CpG island, methylation outside a NFR is often observed while the NFR is kept unmethylated (Graff *et al*., 1997; Issa *et al*., 2001; Abe *et al*., 2002). A gene is usually kept to be transcribed even if regions outside the NFRs are methylated (Ushijima, 2005). This shows that methylation outside NFRs is relatively easily induced, but does not cause gene silencing.

4.2 Aging

Issa *et al*. (1994) first reported that a *Not*I site in exon 1 of estrogen receptor (*ESR*) was methylated in normal colon mucosa in association with aging (Issa *et al*., 1994). The age-dependent methylation

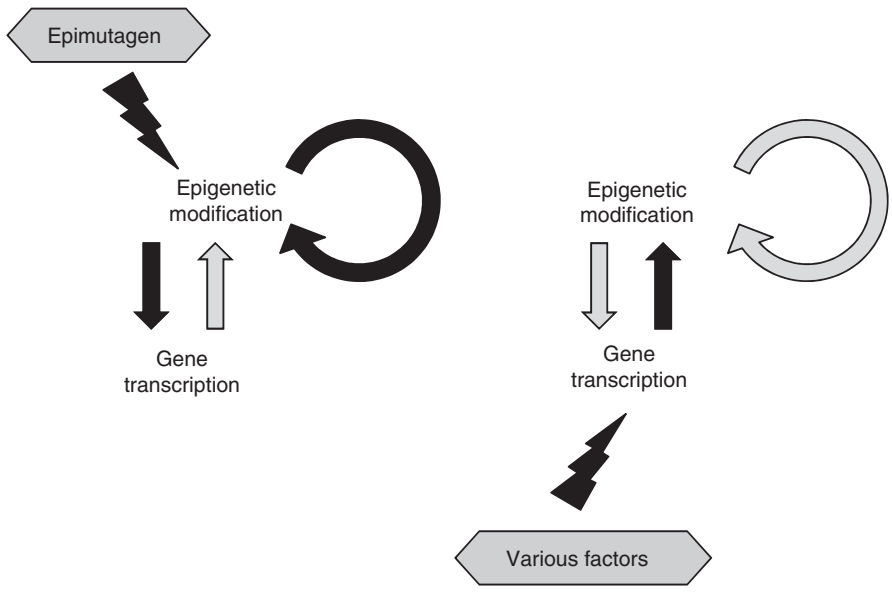

Figure 7. Direct and indirect effects on epigenetic modifications by exogenous factors. *bona fide* epimutagens (defined in Section 4.6) target epigenetic modifications first, and their alterations are inherited and lead to changes in gene transcription. On the other hand, many chemicals induce changes in gene transcription first, and the changes can be accompanied by changes in epigenetic modifications.

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was later confirmed by many investigators using human and animal samples (Abe *et al*., 2002; Waki *et al*., 2003). It was later shown that age-dependent methylation takes place in specific CpG islands (type A CpG islands) (Ahuja *et al*., 1998; Toyota *et al*., 1999). However, it is often observed that, even within the same CpG island, only peripheral regions are methylated but its central regions, which correspond to NFRs in promoter CpG islands, are kept unmethylated (Graff *et al*., 1997; Issa *et al*., 2001; Abe *et al*., 2002). This suggests that mechanisms for methylation induction are different between NFRs in central regions of CpG islands. As a mechanism of age-dependent methylation, an increase in the cumulative number of cell proliferations is considered to give a higher chance of induction of "aberrant" DNA methylation (Issa *et al*., 2001; Issa *et al*., 1994).

4.3 Chronic Inflammation

Among the poorly characterized inducers, the best-characterized inducer is possibly chronic inflammation. Aberrant DNA methylation is known to be present in colonic tissues with long-standing ulcerative colitis (Hsieh *et al*., 1998; Issa *et al*., 2001; Toyota *et al*., 2002), in the liver with chronic hepatitis (Kondo *et al*., 2000), and in gastric tissues exposed to *Helicobacter pylori* (*H. pylori*) infection (Maekita *et al*., 2006; Park *et al*., 2009). In addition to these associations, we recently demonstrated using an animal model that inflammation triggered by *H. pylori* infection, not *H. pylori* itself, is indeed the cause of methylation induction (Niwa *et al*., 2010). Exact mechanisms of how chronic inflammation induces aberrant DNA methylation are still unknown, but expression levels of *Tnfa*, *Il1b*, *Cxcl2*, and *Nos2* are well correlated with methylation induction.

4.4 Viral Infection and Exogenous DNA

It was noted decades ago that viral DNA is methylated upon infection into mammalian cells (Doerfler *et al*., 1995). It was shown that cells transgenic for an adenovirus type have methylation of not only the transfected viral DNA but also cellular DNA (Muller, Heller and Doerfler, 2001), and the presence of exogenous DNA was suggested to induce methylation of even endogenous genes. The Epstein-Bar (EB) virus infection is occasionally associated with human gastric cancers, and such cancers are known to have more methylated CGIs than gastric cancers without EB virus infection (Kang *et al*., 2002; Chang *et al*., 2006). As a potential mechanism, it was recently reported that DNMT1 is activated by EBV latent membraneprotein 2A (Hino *et al*., 2009). Liver tissues infected by Hepatitis virus C have methylation of multiple genes (Nishida *et al*., 2008). An adult T-cell leukemia virus was also shown to induce methylation of endogenous genes (Yasunaga *et al*., 2004). All these indicate that viral infection and exogenous DNA are inducers of aberrant DNA methylation of endogenous genes.

4.5 Disturbances in One Carbon Metabolism

Disturbances in one carbon (methyl group) metabolism, due to deficiency of folate, vitamin B_{12}, or choline, can influence DNA methylation status by limiting availability of the methyl donor, *S*-adenosylmethionine (Poirier, 2002). In animal experiments, methyl supplementation in maternal diet during pregnancy affected methylation levels of a transposable element of offsprings, and the resultant phenotype persisted for a life time (Waterland and Jirtle, 2003). In human, malnutrition during intrauterine and neonatal periods is known to be associated with the development of obesity, type 2 diabetes, and other related co-morbidities (Kalhan, 2009). This strongly indicates that disturbances of one carbon metabolism can induce changes in DNA methylation, which predispose individuals to disease conditions.

4.6 Chemicals

Some chemicals are considered to induce epigenetic alterations, and are designated as "epimutagens" (Holliday, 1991; MacPhee, 1998; Holliday and Ho, 2002). One of the most well characterized epimutagens is a DNA demethylating agent, 5-aza-2′-deoxycytidine (5-aza-dC), which is widely used in laboratories and has now been approved as a therapeutic drug for myelodysplastic syndrome (Jones, 1985; Issa *et al*., 2005; Issa and Kantarjian, 2009). 5-Aza-dC is incorporated into DNA strands and

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Table 2. List of chemicals reported to alter epigenetic statuses.

Action	Chemical	Characteristics	Reference
DNA hypermethylation	Butyrate	Short-chain fatty acid	(Boffa, Mariani and Parker, 1994)
	4-(*N*-Nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK)	Tobacco-specific carcinogen	(Pulling *et al*., 2004)
	Phenobarbital	Antiepileptic agent	(Bachman, Phillips and Goodman, 2006)
	Vinclozolin	Antiandrogenic compound	(Anway *et al*., 2005)
	Diethylstilbestrol	Synthetic estrogen	(Bromer *et al*., 2009)
DNA hypomethylation	5-Azacytidine, 5-Aza-2′-deoxycytidine	Cytidine analog	(Egger *et al*., 2004)
	5-Fluoro-2′-deoxycytidine	Cytidine analog	(Jones and Taylor, 1980)
	5,6-Dihydro-2′-azacytidine	Cytidine analog	(Curt *et al*., 1985)
	Zebularine	Cytidine analog	(Cheng *et al*., 2003; Holleran *et al*., 2005)
	Ethionine	Methionine analog	(Shivapurkar, Wilson and Poirier, 1984)
	Arsenic compound	Metal compound	(Zhao *et al*., 1997;Reichard, Schnekenburger and Puga, 2007)
	Valproic acid	Antiepileptic agent	(Detich, Bovenzi and Szyf, 2003)
	Procainamide	Antiarrhythmic agent	(Lee *et al*., 2005; Segura-Pacheco *et al*., 2003)
	Procaine	Anesthetic agent	(Villar-Garea *et al*., 2003)
	Hydralazine	Antihypertensive agent	(Segura-Pacheco *et al*., 2003)
	6-Mercaptopurine	Anticancer agent	(Hogarth *et al*., 2008)
	6-Thioguanine	Anticancer agent	(Hogarth *et al*., 2008)
	Psammaplins A	Antibiotic agent	(Pina *et al*., 2003)
	(-)-Epigallocatechin-3-*O*-gallate (EGCG)	Major polyphenol from green tea	(Fang *et al*., 2003)
	RG108	DNMT inhibitor	(Brueckner *et al*., 2005)
	SGI-1027	DNMT1 inhibitor	(Datta *et al*., 2009)
	Bisphenol-A	Synthetic estrogen	(Bromer *et al*., 2010)
Alterations of histone modifications	Butyrate	short-chain fatty acid	(Stadtman and Barker, 1949)
	Trichostatin A	Microbially derived compound	(Yoshida *et al*., 1990)
	Valproic acid	Antiepileptic agent	(Kramer *et al*., 2003)
	Suberoylanilide hydroxamic acid (SAHA)	Hydroxamic acid	(Kelly *et al*., 2003)
	Depsipeptide	Microbially derived compound	(Furumai *et al*., 2002)
	Nickel compound	Metal compound	(Chen *et al*., 2006)
	Chromium compound	Metal compound	(Zhou *et al*., 2009)
	Arsenic compound	Metal compound	(Zhou *et al*., 2009)
	Cobalt compound	Metal compound	(Li *et al*., 2009)
	Cocaine	Crystalline tropane alkaloid	(Maze *et al*. 2010

traps DNMT1, which is subsequently degraded by proteasome (Ghoshal *et al*., 2005). This leads to depletion of DNMT1 in a cell, and passive DNA demethylation is resultantly induced. There are many other chemicals reported to induce changes in epigenetic modifications (Table 2), but their direct action or indirect action through gene expression changes should be carefully evaluated.

5 EPIGENOMIC ANALYSIS IN TOXICOLOGY

Epigenomic alterations are deeply involved in carcinogenesis and possibly in other disorders. In addition, there are a large number of non-mutagenic carcinogens (Snyder and Green, 2001), some of which exert their carcinogenic action by inducing

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cell proliferation. It seems reasonable to consider a possibility that some of the non-mutagenic carcinogens exert their action by epigenetic mechanisms. In this context, epigenomic analysis seems essential in toxicology, which has just started. Unfortunately, few reliable and sensitive methods specifically designed for toxicological analysis have been reported yet, and ordinary procedures for epigenetic and epigenomic analysis are used also for toxicological analysis. Their brief principles and efforts in development of convenient assay systems are described.

5.1 Principles of DNA Methylation Analysis

Methods can be divided into those for analysis of specific genomic regions and those for genome-wide analyses. DNA methylation at specific genomic regions is analyzed mainly based upon two principles of methylation detection; methylation-sensitive restriction enzymes, and bisulfite modification of DNA (Figure 8). Some restriction enzymes, such as *Hpa*II and *Sma*I, have recognition sequences with CpG sites, and cannot cleave if the CpG site is methylated. Bisulfite

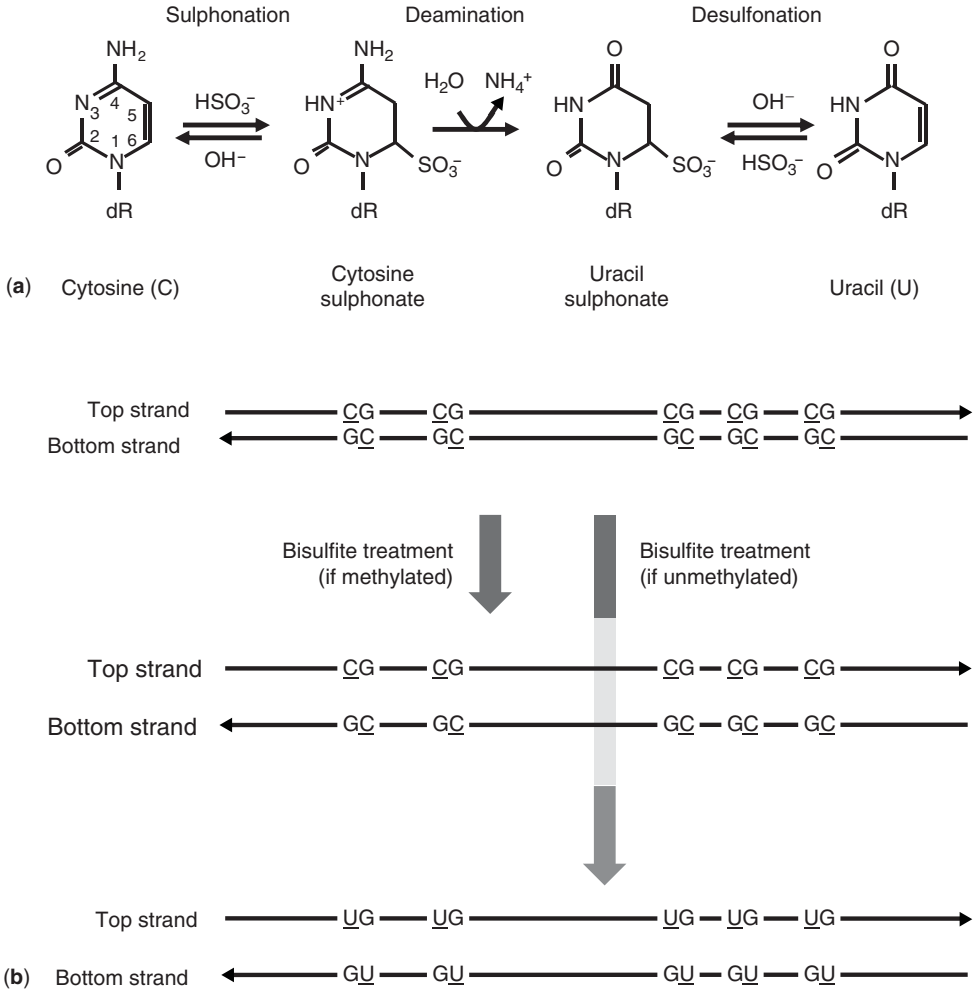

Figure 8. Principle of bisulfite modification: (a) chemical reactions for unmethylated cytosine; (b) sequence changes produced by bisulfite modification of methylated and unmethylated DNA. Different sequences are produced from methylated and unmethylated DNA, and the difference can be detected by various modalities.

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modification takes advantage of different efficiency in converting cytosine to uracil, which is very efficient for unmethylated cytosines but very slow for methylated cytosines. After bisulfite conversion, the top and bottom strands are no longer complementary. Methylated and unmethylated DNA will produce different sequences after the conversion, and the difference can be detected by various techniques, such as sequencing, allele-specific PCR, restriction digestion, and pyrosequencing. Depending upon the purpose of experiments, appropriate techniques should be selected, considering the required amount of DNA, flexibility in selection of CpG sites to analyze, how quantitative the method is, technical complexity, and the cost.

Genome-wide analyses are generally composed of a step of detection of DNA methylation and another step of genome-wide analysis (Ushijima, 2005; Laird, 2010). The methylation detection can be performed using affinity-based methods, such as use of anti 5-methylcytidine antibody and affinity column with methylated DNA binding domains, but also using methylation-sensitive restriction enzymes and bisulfite conversion. The detection step can be performed using microarray or next-generation sequencers.

5.2 Principles of Histone Modification Analysis

Methods for histone modification analysis can be divided into: (i) those for analysis of global contents of histone modifications within a cell; (ii) those for analysis of histone modifications for a defined genomic region; (iii) those for histone modifications of defined genomic regions in a genome-wide manner. Global contents of histone modifications within a cell are mainly analyzed by immunohistochemistry and Western blotting. In contrast, histone modifications in defined genomic regions are analyzed by chromatin immunoprecipitation (ChIP). All of these methods are based upon the recognition of histone modifications by antibodies, and their specificity is critical for successful analysis.

The ChIP method can detect physical interactions between histones containing a specific modification and genomic DNA within a cell (Figure 9). The ChIP method is composed of four steps including: (i) preparation of fragmented chromatin from cells; (ii) immunoprecipitation by using a specific antibody; (iii) purification of immunoprecipitated (IP) DNA; (iv) analysis of IP DNA (Lee *et al*., 2006). Fragmented chromatin is usually prepared by cross-linking DNA and histones by formaldehyde, followed by a fragmentation step by sonication or micrococcal nuclease. Immunoprecipitation is performed using a specific antibody, and then the immuno-complex of chromatin and antibody is collected and purified. IP DNA is analyzed by PCR of a specific genomic region, or by microarray or next-generation sequencers for a genome-wide analysis (Barski *et al*., 2007; Lee *et al*., 2006; Wang *et al*., 2008).

5.3 Screening Methods for Epimutagens

A major reason why only a limited number of chemicals are reported to have epigenetic actions (see Section 4.6) is the lack of easy-to-use assay systems for chemicals' capacity to induce epigenetic alterations. For mutagens, there are various *in vitro* assays, using bacterial cultures or mammalian cells, and also *in vivo* assays using genetically-engineered animals (MacGregor, Casciano and Muller, 2000) (Table 3). In contrast, very limited assay systems are available for epimutagens. To construct an assay system for epimutagens, considerations should be given to what target genomic region is used as a marker for epigenetic effects, such as DNA demethylation and methylation, and what reporter

Table 3. Characteristics of assay systems for mutations and epigenetic alterations.

	Mutation assays	Assays for epigenetic alterations
Bacterial system	*Reversion in S. typhimurium* (Ames test)	Essentially impossible
Mammalian cell	*HPRT* or *TK* mutations Chromosome aberration test Mouse lymphoma assay Measurement of UDS	Under development (see text)
In vivo Assay	Micronucleus test Mouse specific locus test Tg mice for a marker gene (Big Blue, gpt-Δ, Muta-mouse etc.)	Not available yet

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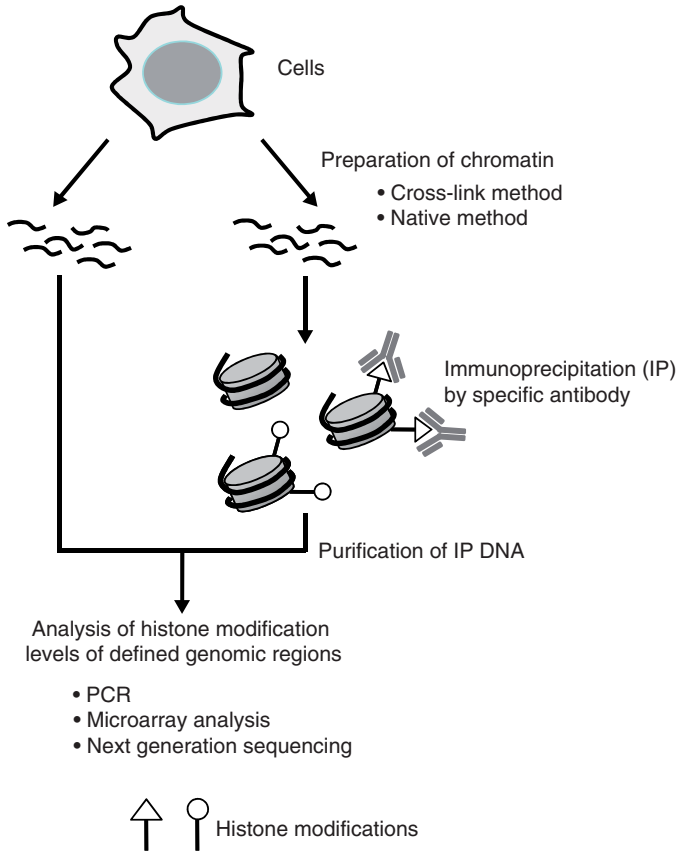

Figure 9. Principle of chromatin immunoprecipitation (ChIP). Fragmented chromatin is prepared, and then immunoprecipitated (IP) by using a specific antibody. DNA purified from the IP chromatin is used for analysis of histone modification levels for defined genomic regions by several technologies such as PCR, microarray, and next generation sequencing.

system is used. For screening purposes, a convenient and reliable assay system is essential.

So far, assay systems only for DNA demethylating agents have been reported. Three systems have been reported using a promoter of an exogenous gene and a reporter gene (Biard *et al*., 1992; Cervoni and Szyf, 2001; Fan *et al*., 2005). Among these, Fan *et al*., 2005 successfully identified 5-bromo-2′-deoxyuridine (BrdU) as an anti-silencing agent without changing DNA methylation status. These exogenous promoters have a concern that they have epigenetic modifications different from endogenous genes. From this aspect, two assay systems are reported using a promoter of an endogenous gene (Okochi-Takada *et al*., 2004; Oyer *et al*., 2009). In addition to these efforts to use specific exogenous and endogenous promoters, hypomethylation of repeat sequences is also proposed as a precursor of toxicity (Carnell and Goodman, 2003).

6 EPILOGUE

Epigenomic alterations are important for cancer and possibly for other disorders. Nevertheless, epigenomic toxicology has just started, and scientists are not armed well yet. Application of findings in epigenetics and epigenomics to toxicology is now an exciting task.

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Sequencing Technology Advances and Toxicology

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1 INTRODUCTION

The toxicogenomics tool kit has traditionally included quantitative-PCR, microarrays, and capillary sequencing. The first generation Sanger (Sanger, Nicklen and Coulson, 1977) sequencing method developed over 30 years ago has been the core chemistry for nucleic acids sequencing. In first generation capillary sequencing a single reaction generates a single nucleic acids sequence (Turner *et al*., 2009). In second generation methods, fluidics, PCR, and massively parallel sequencing is combined to generate reads from millions of miniature parallel reactions (Figure 1). Third generation methods comprise of truly single molecule sequencing and massively parallel sequence detection with no PCR amplification. The next generation sequencing methods are transforming the tool kit for toxicogenomics by allowing much improved whole-cell transcriptome quantization, micro-RNA analyses, and whole-genome sequencing (Turner *et al*. 2009).

2 PCR, QUANTITATIVE PCR AND DIGITAL PCR

The polymerase chain reaction (PCR) is a proven tool in toxicology because of its ability to amplify specific genes or gene segments directly. Although traditional PCR with gel-based quantization of nucleic acids is very sensitive, it is also time-consuming and only semi-quantitative. Real-time PCR employing techniques such as TaqMan is significantly more quantitative when the goal is to measure two or more nucleic acids. In real-time PCR, quantization is conducted in the PCR exponential phase, where there is a linear relationship between the log of target DNA quantity and the number of PCR cycles to reach a threshold of fluorescence (Heid *et al*., 1996; de Kok *et al*., 1998). The number of PCR cycles required to reach a fluorescence (ΔRn) threshold value is the cycle threshold (Ct). The Ct value for each sample is proportional to the log of the initial amount of target DNA (Higuchi *et al*., 1993). Since Ct values are proportional to the log of the copy number of target DNA, transformations are needed using a calibration curve. The calibration curve can be used to estimate the copy number of target DNA from the corresponding Ct values. The major limitation of real-time PCR based quantization is that Ct value based quantization is not a true quantity, but requires a calibration curve with known reference samples. In addition, the accuracy of quantization assumes that PCR efficiency is the same in all samples including the reference samples; this is often not the case due to PCR inhibitors in some biological samples and toxicology studies. Digital PCR and Digital Gene Expression Analyses are single molecule nucleic acids amplification technologies, which offer a solution to many of the problems faced by Real Time PCR methods.

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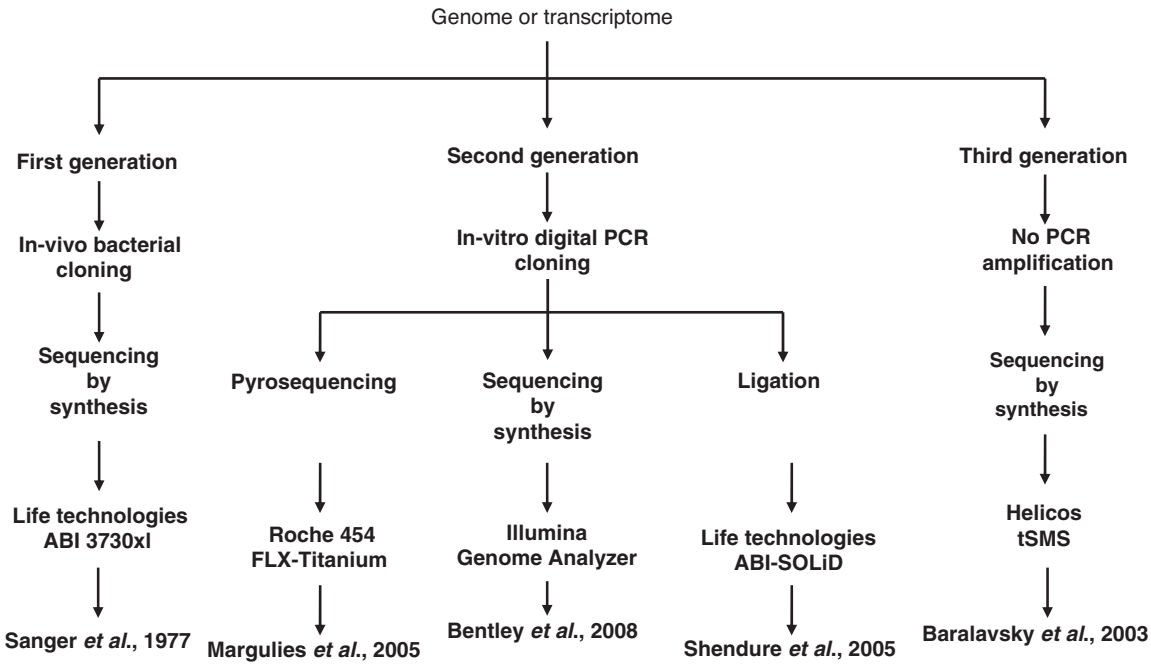

Figure 1. Overview of the next generation sequencing platforms.

3 DIGITAL PCR AND DIGITAL GENE EXPRESSION ANALYSES

Digital Polymerase Chain Reaction (digital PCR) is a refinement of conventional polymerase chain reaction methods that can be used to directly quantify and clonally amplify nucleic acids including DNA, cDNA, or RNA. Theoretically, PCR exponentially amplifies nucleic acids, and the number of amplification cycles and the amount of PCR end-product should allow the computation of starting quantity. However, many factors complicate this calculation, creating uncertainties and inaccuracies. These factors include: initial amplification cycles may not be exponential; PCR amplification eventually plateaus after an uncertain number of cycles; and PCR amplification efficiency in a sample of interest may be different from that of reference samples.

Digital PCR overcomes these difficulties by transforming unreliable exponential data from conventional PCR to digital signals that simply indicate whether or not amplification has occurred. Digital PCR is achieved by capturing each individual nucleic acid molecule present in a sample within many separate chambers (or regions or beads) prior to PCR amplification. A count of chambers containing detectable levels of PCR end-product is a direct measure of the absolute nucleic acids quantity. Digital Gene Expression Analyses refers to next generation sequencing of RNA. Digital Gene Expression Analysis methods originate from digital PCR where each captured individual RNA molecule is sequenced in parallel.

4 SEQUENCING TECHNOLOGY

4.1 Roche 454 Sequencing

In 454 sequencing the nucleic acids molecules are ligated to adaptors such that amplification with one set of primers becomes possible. The adaptors are also used to attach the template and daughter amplicons onto 20 μm beads. PCR amplification of each template is conducted in millions of micelles in an emulsion with goal of having one bead with one template and PCR reagents in each micelle. The target ratio of bead to template is 1:1. During PCR a cloned template is generated on the bead surface using an innovative solid surface PCR approach. After PCR the beads are recovered and deposited into plates called Pico Titer Plates (PTP). Each bead with a

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clonal population of DNA is deposited into one well in the PTP, which also contains packing and enzyme beads. Each DNA clone on each bead is sequenced in parallel using pyro-sequencing (Margulies *et al*., 2005). The 454 sequencer is capable of generating greater than one giga-base (1 000 000 000 bases) of sequence data per day.

4.2 Life Technologies Solid Sequencing

The template amplification step with SOLiD is similar to that of 454 using beads and emulsions to retain the clone of daughter DNAs on the bead surface. However the beads used in SOLiD are much smaller than 454 with a diameter of $\sim$1 μm and bound to slides at a very high density of 100 000 000 beads. The 100 000 000 beads with DNA clones are sequenced in parallel (although many beads could be filtered out during data analyses due to more than one clone captured per bead). Sequence detection in SOLiD is conducted 8 nucleotides at a time by ligation. To the bead bound clones a sequencing primer is hybridized and 16 8-base probes are introduced. Using an innovative approach of stepwise ligation, imaging and cleavage, sequencing is achieved by generating the color profile of every fifth di-nucleotide (Shendure *et al*., 2005). The Life Technologies SOLiD sequencer is capable of generating greater than two giga-bases (2 000 000 000 bases) of data per day.

4.3 Illumina Genomic Analyzer Sequencing

The Illumina Genome analyzer uses an innovative approach to generate template clones for sequencing. An adapter DNA is ligated onto the template ends. The modified template is then introduced into a flow cell where a random lawn of forward and reverse primers are attached to the interior bottom surface. The template DNA adapter hybridizes to one of the primers and a complementary copy attached to the base surface is generated. The end of the complementary copy folds over and hybridizes to another primer, which in turn is extended to create a new daughter DNA. These new daughter DNA clones act as templates in isothermal PCR to create a surface-attached, clustered clone of the original template.

The double stranded DNA clusters are converted to single strand clusters by selectively removing one strand and the flow cell is transferred to a genome analyzer. In the genome analyzer the clusters are sequenced with reversible florescent terminator deoxyribonucleotides (Bentley *et al*., 2008). During each sequencing cycle, one nucleotide is read with each clone cluster fluorescing in one color. The Illumina sequencer is capable of generating greater than 1.5 gigabases (1 500 000 000 bases) of data per day.

4.4 Helicos Sequencing

The Helicos method is a third generation method that is true single molecule sequencing without PCR amplification of the template. Sample preparation for sequencing is straight forward and fast requiring only the addition of the poly-A tail and fluorescent label. The poly-A tail hybridizes to poly-T strands in the flow cell chamber. The substrate for sequencing is each template and not a clone cluster as is the case with the methods from Roche, Life Technologies and Illumina. Therefore, the flow cell can be densely packed with billions of template molecules. Sequence detection is conducted by adding fluorescent nucleotides, one at a time, and querying one nucleotide per cycle. Sequencing of millions of template molecules occur simultaneously (Braslavsky *et al*., 2003). The Helicos sequencer is capable of generating greater than 2.5 gigabases (2 500 000 000 bases) of sequence data per day.

4.5 Other Next Generation Sequencing

In addition to the currently available next generation sequencing systems by Roche, Life Technologies, Illumina and Helicos there are a number of other very promising platforms. Companies with platforms that are in various stages of readiness include Affymetrix, Base4innovation, Complete Genomics, Dover Systems, Genome Corporation, Intelligent Bio-Systems, NABsys, Oxford Nanopore Technologies, Pacific Biosciences, Reveo, Sequenom, and VisiGen Biotechnologies. The number of researchers and companies involved in the development of next generation sequencing methods illustrates the enthusiasm and promise the new methods. The second generation and third generation sequencing methods will results in very

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high levels of data yield and much reduced cost making the $1000 whole genome sequencing goal a reality (Service, 2006).

5 APPLICATIONS OF SEQUENCING IN TOXICOLOGY

Whole genome sequencing has resulted in a biology revolution by facilitating the study of entire cellular systems with the whole cell and sometimes the entire organism in focus. Although many organism model systems have been sequenced and there appears to be consensus that we are in a post genome era, the use of sequencing has only just begun (Pushkarev, Neff and Quake, 2009).

6 SEQUENCE VARIANT SCREENING, PHARMACOGENOMICS AND TOXICOLOGY

Pharmacogenomics is the study of mutations and polymorphism and their effects on drug selection, optimal dose, adverse events, and efficacy. The application of pharmacogenomics principles offers the possibility of personalized medicine with drug therapy delivered based on known susceptibilities. With reduction in adverse events more drugs may be made available by reducing the use of trial error methods. It is also expected that the cost of clinical trials could be reduced.

In the current model of drug development over 75% of prospective drugs fail due to problems with safety and efficacy and also problems with manufacturing (Lesko and Woodcock, 2004). With wider use of pharmacogenomics methods, drugs such as Vioxx, which was taken off the market due to adverse events can be avoided. Individuals susceptible to adverse events could be excluded from taking a particular drug making many more drugs available for non-susceptible patients. The Food and Drug Administration (FDA) has been working on the "Critical Path" to help make product development more predictable and less costly (Lesko and Woodcock, 2004). The FDA has also developed pharmacogenomics data submission guidelines to assist and partner with researchers to better serve the needs of the emerging technologies.

Next generation sequencing has reduced the cost of sequencing the human genome from millions of dollars to approximately $100 000. It is expected that within the next five years the cost will be further reduced to $10 000. Advances in throughput and reduced cost will make whole genome sequencing a reality in clinical trials to identify subgroups of individuals more prone to adverse events and also to better optimize dose. One of the key advantages of next generation sequencing in clinical trials is the ability to potentially whole genome profile polymorphisms and mutations without prior assumptions of which genes modify drug efficacy.

7 MICROBIAL GENOMICS

Next generation sequencing has opened new avenues of exploration in microbial genomics (Hall, 2007). There are many important toxicology implications as microbial-genome diversity is better understood and characterized in the context of their human hosts.

A study of *Streptococcus* strains (Tettelin *et al*., 2005) has shown that after sequencing eight genomes approximately 33 new genes are discovered with each new strain sequenced. This has given rise to the pan genome concept which suggests that most microorganism are made of a core set of essential genes and a set of optional genes that could differ from individual to individual. This genomic diversity is made more complex with mutations and polymorphisms within each gene and their influence on drug susceptibility and host interactions. In light of the pan genome, the sequence of a single reference genome (or transcriptome) maybe a poor representation of the genetic diversity in pathogen populations. To better prepare for emergence of drug resistant or more virulent strains it is increasingly clear that we need deep genome and transcriptome data for a representative population in addition to a reference sequence. One of the most straight forward applications of next generation sequencing is microbial mutation screening. Mutation screening needs to be carried out at the population level to identify correlations between genotype and phonotype with statistical rigor. While whole genome and transcriptome sequencing is still expensive, prices are projected to fall dramatically. Due to the relatively small size of most microbial genomes, next generation sequencing will find fast applications in microbial toxicology.

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Metagenomics aimed at characterizing microbial diversity in particularly complex environments such as the gut or skin and how this ecology is influenced by drugs is also an important application of next generation sequencing. There are a number of reports using more traditional methods already examining the metagenomics of the gut (Gill *et al*., 2006; Manichanh *et al*., 2006; Zhang *et al*., 2006). With advances in sequencing, metagenomics studies are becoming easier to conduct and will provide detailed insights into interaction between very complex microbial environments and toxicological outcomes.

8 TRANSCRIPTOMICS, NEXT GENERATION SEQUENCING, AND MICROARRAYS

Microarray technology has made it possible to study thousands of RNA molecules expressed in one experiment. Many advances in toxicogenomics can be attributed to microarray technology. It can be postulated that next generation sequencing can replace most microarray studies (Kato, 2009). In second generation sequencing the level of a particular transcript is inferred by the number of times a specific sequence is identified. Use of second generation sequencing for gene expression studies is referred to as Digital Gene Expression Analyses (Okubo *et al*., 1992). In microarrays, the level of a transcript is determined by the level of hybridization of one target RNA compared to a differently colored reference RNA and it is difficult to compare data from different series experiments (Kato, 2009). It is expected that many of the drawbacks of microarray methods including inter-experiment variation, platform to platform variation and need for extensive reference samples can be overcome with sequencing. For sequencing, RNA is isolated from the cell or tissue of interest and reverse transcribed into cDNA. The cDNA is sequenced and sequence counts used to directly measure RNA levels. It has been reported that changes in expression observed by next generation sequencing is greater than that of microarrays and quantitative PCR (’t Hoen *et al*., 2008) and that the results were highly reproducible with little variation with different sequencer runs (Marioni *et al*., 2008; ’t Hoen *et al*., 2008). Microarray data consist of continuous signals, while sequence sequencing consensus data comprise absolute number of reads (Marguerat, Wilhelm and Bahler, 2008). The digital, countable nature of next generation sequencing data makes it highly suitable for gene expression analyses. The advantages of next generation sequencing over microarrays also include almost unlimited dynamic range and the ability to profile expression without prior assumptions of which genes are expressed. In a comparative study five microarray platforms and digital gene expression analyses it has been shown that next generation sequencing offers major advances in robustness, resolution and inter-lab portability over microarrays (’t Hoen *et al*., 2008).

9 CANCER AND PRENATAL DIAGNOSTICS

Next generation sequencing has found many useful clinical and research applications in detection of cancer and also prenatal genetic abnormalities. In cancer, tumor DNA is split into the circulatory system and mutated DNA can be readily detected by sequencing serum DNA. In prenatal diagnostics, fetal DNA can be detected in the mother’s serum and sequenced. Two very important studies showing the utility of next generation sequencing in prenatal diagnostics have already been published (Fan *et al*., 2008; Chiu *et al*., 2008). The overall sequencing method for cancer and prenatal diagnostics is similar. First DNA from serum is isolated and prepared for sequencing by ligating adapters. A significant amount of DNA in serum from a pregnant mother or a cancer patient originates from the fetus or tumor respectively. The prevalence of mutations, aneuploidy or allele imbalance can be used for cancer and prenatal illness diagnostics.

10 ETHICS

Genetic diagnostics done at very high throughput levels with the new sequencing methods can be hampered by abuse and discrimination both real and imagined (ten Bosch and Grody, 2008). Current fears about genetic testing have largely been about predictive testing such as that for familial cancer. With next generation sequencing hundreds of genes or even the whole genome can be analyzed simultaneously. A person seeking a test for one illness may discover susceptibility to potentially many illnesses.

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Table 1. Comparison of next generation sequencing systems.

	PCR	Sequencing	Read length	Read fidelity	Bases read per day	Temp-lates #	Sample Preparation Time
ABI 3730xl	Bacterial cloning	Sequencing by synthesis	1000	>99.9%	1 MB	96	
Roche 454	Bead Capture Emulsion PCR	Pyro-sequencing	~500	99.5%	~1 GB	>1 million	3 days
ABI SOLiD	Bead Capture Emulsion PCR	Ligation-Sequencing	~50	99.9%	~2 GB	>40 million	4 days
Illumina	Microfluidic Isothermal Solid Surface Bridge PCR	Reversible termination Sequencing by synthesis	~50	98.5%	~1.5	>85 million	2 days
Helicos	No PCR	Reversible termination Sequencing by synthesis	~50	99%	~2.5	>100 Million	1 day

A wise course of action with the new very promising methods is to exercise caution by providing extensive pre-test counseling, informed consent and maintenance of stringent privacy rules. It will also be wise to avoid overly restrictive measures to protect patients that may ultimately reduce patient access to the technology (ten Bosch and Grody, 2008). With careful consideration of the potential benefits a proper balance could be struck as next generation sequencing transitions to the clinical setting from the research arena.

11 TECHNICAL CHALLENGES

For all sequencing platforms, ease of sample preparation, read length, read quality, and data processing are very important measures. In all the next generation sequencers, except that from Roche, read lengths are short with about 50 bases read per template (Table 1). When read lengths are short, whole genome sequencing for organisms with large genomes can be difficult and data processing is quite challenging. Short reads are useful for comparative studies to identify polymorphisms after a reference genome has been assembled and for transcriptomics studies. For de novo sequencing, in both read length and quality, Sanger capillary sequencing outperforms all the next generation sequencing platforms (Table 32.1). However as protocols get optimized and data handling software improves the quality measures of next generation sequencing will surpass that of capillary sequencing even for de novo sequencing. In the near term, the major challenge for next generation sequencing is data management and analysis of sequence data (Turner *et al*., 2009).

RELATED ARTICLES

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Next-Generation Sequencing: A Revolutionary Tool for Toxicogenomics

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1 INTRODUCTION

DNA sequencing is a fundamental way for deciphering a broad range of biological phenomena at the molecular level. Although the development of DNA sequencing technologies has a rich and diverse history (Hutchison, 2007; Shendure and Ji, 2008), the dideoxy sequencing established by Sanger, Nicklen and Coulson 1977 has dominated the industry and research community for almost three decades and currently remains the gold standard for decoding DNA sequences.

Many biochemical and technical improvements have endowed modern Sanger sequencers with very low error rates (∼0.001%), relatively long read-length (up to ∼1000 bp), and high-throughput and robust performance. However, the cost of Sanger-based approaches for large sequencing projects remains expensive (on the order of $0.50 per kilobase). This limitation incentivized the National Human Genome Research Institute (NHGRI) of the National Institutes of Health (NIH) to initiate a funding program in 2004 to develop novel technologies that will enable extremely low-cost, high quality DNA sequencing. With a stated goal of reducing the cost of sequencing mammalian-sized genomes by four orders of magnitude to approximate $1000 per genome in 10 years (http://grants.nih.gov/grants/guide/rfa-files/RFA-HG-09-011.html), the initiative has propelled the development and commercialization of novel next-generation sequencing (NGS) technologies.

In 2005, 454 Life Sciences (now part of Roche Applied Science, http://454.com/about-454/index.asp) developed and commercialized its Genome Sequencer (GS) system for ultra-high throughput DNA sequencing (Margulies *et al.*, 2005). As the first commercialized NGS technology, the Roche/454 sequencing system provided a compelling case study for the establishment of a novel, revolutionary technology that enables scientists to carry out massively parallel DNA sequencing reactions at a relatively small cost, as well as at a much faster speed compared to using conventional Sanger's technologies. For example, the sequencing of the first human genome in the Human Genome Project (Collins *et al.*, 2004; Lander *et al.*, 2001) with automated Sanger technology took some 13 years at a cost of about $2.7 billion. In contrast, sequencing a human genome with the Roche/454 sequencer took but five months and approximately $1.5 million (Wheeler *et al.*, 2008). This great stride was enabled by ultra-high throughput, simplified *in vitro* sample preparation, and miniaturization of sequencing chemical reactions (Rothberg and Leamon, 2008).

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Illumina, Life Technologies, and Helicos BioSciences quickly followed Roche's lead and launched their own NGS platforms. Concurrently, new DNA sequencing technologies have been advancing at other companies and research institutions, such as Pacific Biosciences (http://www.pacificbiosciences.com), VisiGen Biotechnologies (http://visigenbio.com), Sequenom (http://www.sequenom.com), Complete Genomics (http://www.completegenomics.com), and the Center for Computational Genetics at Harvard Medical School (http://arep.med.harvard.edu/gmc).

Differing in sequencing chemistry as well as technical details, all NGS platforms share a similar technical strategy – miniaturization of individual sequencing chemical reactions to boost sequencing throughput (Metzker, 2010). The miniaturization of sequencing reactions, coupled with other technical breakthroughs, such as overcoming the bottlenecks of library preparation and template preparation (Rothberg and Leamon, 2008) enables millions of simultaneous individual sequencing reactions. Only a single fragment of DNA is sequenced in each miniaturized chemical reaction, but millions of them are spatially arranged so that individual reactions are isolated from one another, and distinctly detected by laser scanning or other approaches. The results are prodigious volumes of short read sequence data, unprecedented detail and resolution of sequence complexity, with consequential challenges in storing, managing, analyzing, and interpreting such wealth of data.

In this chapter, we first describe the fundamental principles of four commercially available NGS platforms, that is, Roche/454 GS FLX, Illumina Genome Analyzer *II*x, Life Technologies SOLiD, and Helicos HeliScope. We then discuss the general difficulties to be overcome in the analyses of NGS data. Next, we outline some main applications of NGS technologies and, finally, we compare the analysis of a toxigenomics study using NGS data with that using microarray data.

2 NEXT-GENERATION SEQUENCING TECHNOLOGIES

2.1 Roche/454 Pyrosequencing

The Roche/454 GS FLX system (Figure 1) relies on pyrophosphate detection (Nyren, Pettersson and Uhlen, 1993) and emulsion PCR (Tawfik and Griffiths, 1998). A library of DNA templates is prepared by a highly efficient *in vitro* DNA amplification method known as emulsion PCR (Figure 1b), where sheared DNA fragments are ligated to specific oligonucleotide adapters, resulting in each DNA fragment binding to a fragment-carrying bead. The beads are then captured in separate emulsion droplets that function as amplification reactors to produce some 10 million clonal copies of the DNA template that are needed for sufficient light signal intensities (Fuller *et al.*, 2009). On completion of the emulsion PCR amplification, the emulsion is disrupted, and the beads containing clonally amplified template DNAs are enriched, and then the beads are again separated by limiting dilution and deposited into individual picotiter-plate wells. The picotiter-plates serve as sequencing reactors to let individual enzymatic sequencing reactions occur without interference from adjacent wells. Visible light emitted from the subsequent pyrosequencing reactions (Ronaghi *et al.*, 1996) are detected by an imaging charge-coupled device (CCD) that is bonded to a fiber-optical bundle. During each cycle of a pyrosequencing reaction, a single species of unlabeled nucleotide is supplied to the reaction mixture to all beads on the chip, so that the complementary strand of DNA is sequentially synthesized. With the incorporation of each base in the growing chain, an inorganic pyrophosphate group is released that is converted to ATP by sulfurylase. During sequencing, the ATP molecule is next used by luciferase to convert luciferin to oxyluciferin, producing a light pulse (Figure 1c). Detecting the light emissions together with knowing the nucleotide identity in each step allows the incorporated base to be determined. Through a series of such pyrosequencing reaction cycles, the sequence of the DNA template carried by individual beads is determined.

Because there is no terminating moiety preventing multiple consecutive incorporations in a given pyrosequencing reaction cycle, the length of homopolymers in sequence reads must be inferred from light signal intensity, with a higher intensity corresponding to more repeats. The error rate of calling consecutive repeats increases when the length of the homopolymers is greater than 3–4 repeating bases. Thus, the main error type for the Roche/454 system is insertions and deletions (or indels), other than substitutions (Shendure and Ji, 2008).

Compared to other NGS platforms, the strength of the Roche/454 system is its longer sequence reads.

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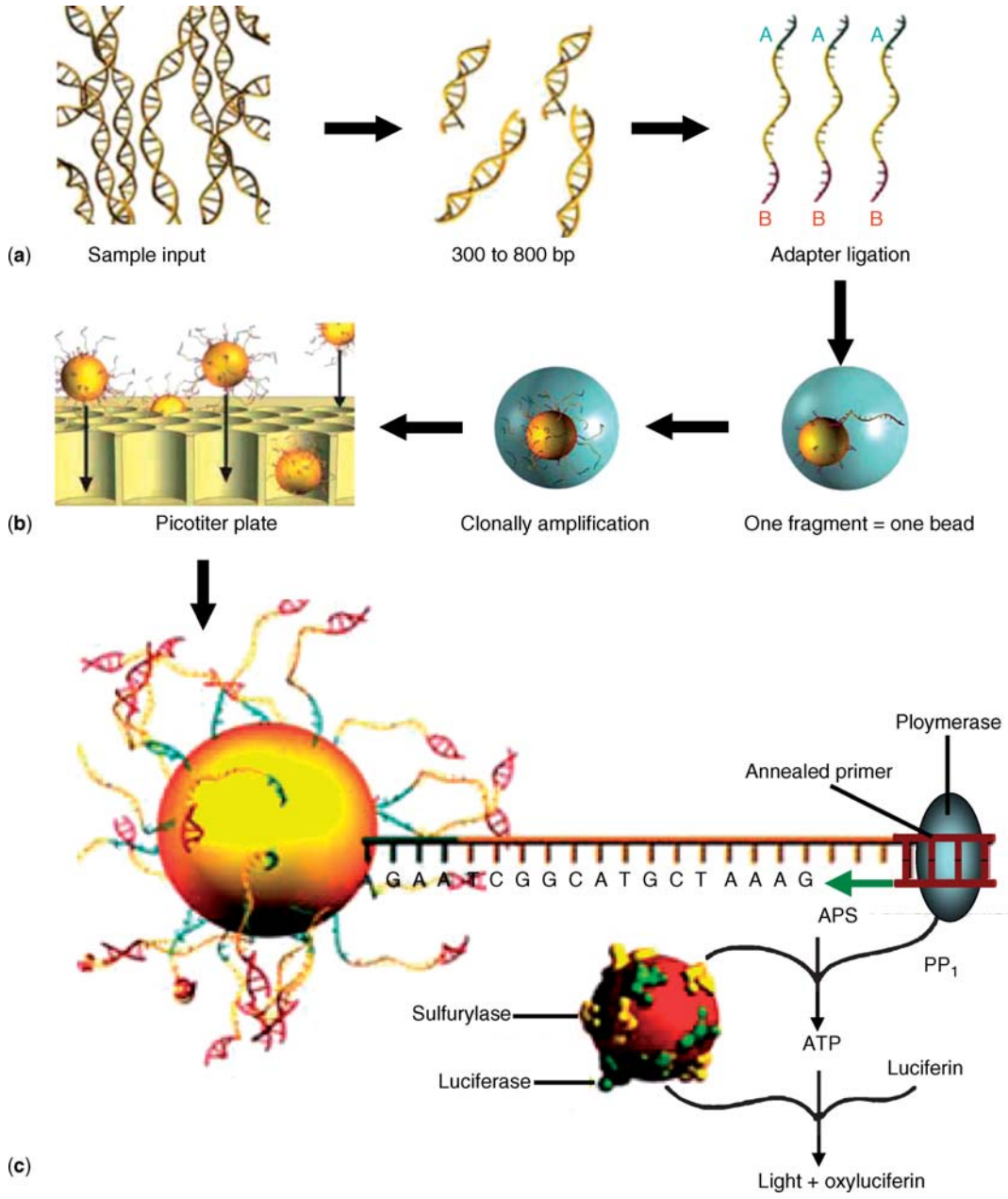

Figure 1. Overview of the Roche/454 GS FLX system workflow: (a) fragmentation of DNA and ligation to Roche specific adapters (denoted as blue A and orange B); (b) clonal amplification by emulsion PCR; and (c) real time sequencing-by-synthesis.

The Roche/454 GS FLX, with its newest chemistry, termed GS FLX Titanium series reagents, can generate more than one million individual sequence reads with read length over 400 base pairs over a 10 h time span (http://www.454.com/products-solutions/system-benefits.asp). Although its per-base cost is much higher than that of other NGS platforms (e.g., Life Technologies/SOLiD and Illumina/Genome Analyzer *II*x), the Roche/454 system is best suited for certain applications such as *de novo* sequencing of new genomes, for which long read length is critical for *de novo* genome assembly.

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2.2 Illumina Sequencing Technology

The Illumina GA system is the first short read sequencing platform and currently dominates the NGS market (Metzker, 2010). The first GA system was launched by Solexa in 2006, which was subsequently acquired by Illumina in early 2007 (http://www.illumina.com/technology/sequencing_technology.ilmn).

The Illumina GA system (Figure 2) uses an array technique to achieve cloning-free DNA amplification. Reversible terminator chemistry is the defining characteristic that provides massively parallel sequencing of millions of DNA fragments at low cost. DNA samples are randomly sheared into fragments that are then end-repaired to generate 5′-phosphorylated blunt ends. The Klenow fragment of DNA polymerase is then used to attach a single "A" base to the 3′ end of the DNA fragments, which prepares the DNA fragments for ligation to oligonucleotide adapters (Figure 2a). After ligation to adapters at both ends, the DNA fragments are denatured, and single-stranded DNA fragments are attached to reaction chambers that are optically transparent solid surfaces called a flow cells. To obtain sufficient light signal intensity for the reliable detection, attached DNA fragments are extended and amplified by bridge PCR amplification (Figure 2b). The bridge PCR amplification can create an ultra-high density sequencing flow cell containing hundreds of millions of clusters that, in turn, contains some 1000 copies of the same DNA template. These templates are finally sequenced through the sequencing-by-synthesis technique that applies reversible terminators with removable fluorescent dyes.

For sequencing and DNA synthesis, the reaction mixtures comprising primers, DNA polymerase, and four reversible terminator nucleotides, each labeled with a different fluorescent dye, are supplied to the flow cell. In each sequencing cycle, a specific terminator is incorporated according to sequence complementarity in each template DNA strand in a clonal cluster. After incorporation, the identity (base calling) and the position of the specifically incorporated terminator on the flow cell is determined according to the fluorescence dye emission, and the signal recorded using a CCD camera. In the following cycle, the reversible terminator is unblocked and the fluorescent dye label is removed

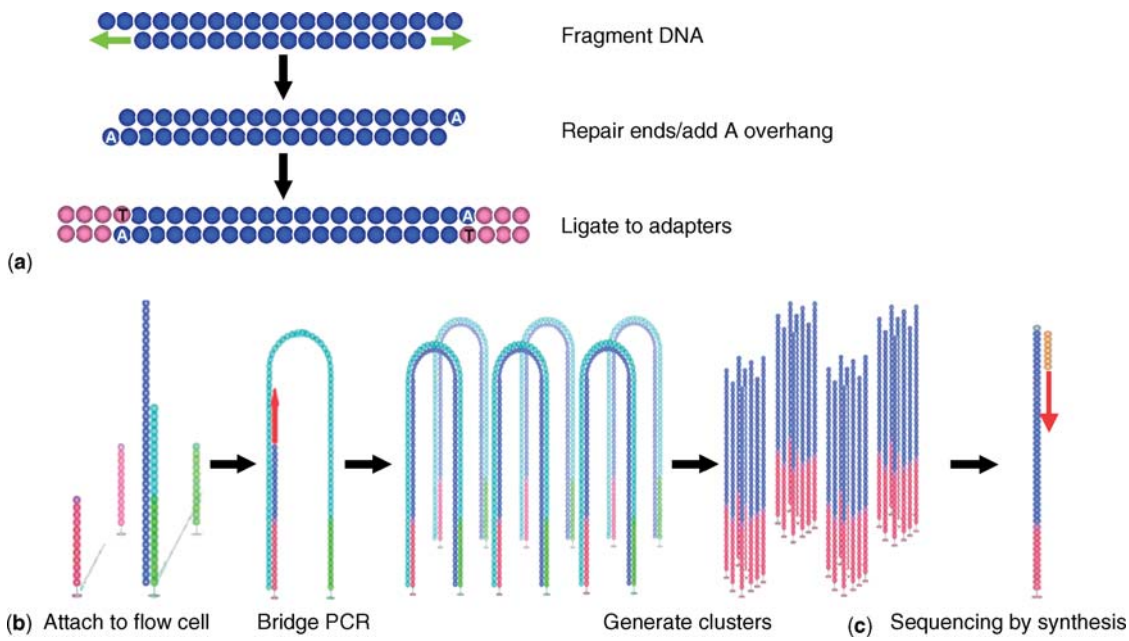

Figure 2. The Illumina Genome Analyzer sequencing strategy: (a) DNA templates are prepared by fragmentation of DNA, end repair, and ligation to adapters; and then are amplified by; (b) bridge PCR in which single-stranded fragments are randomly bound to the inside surface of the flow cell channels and are amplified to generate clusters with some 1000 copies of the same DNA template; and finally DNA templates are sequenced with; and (c) reversible terminator-based sequencing-by-synthesis technique.

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from the base, so that a new nucleotide can be incorporated and a new base can be detected using the same strategy. This repetitive sequencing-by-synthesis process takes about 2.5 days to generate 50 million reads per flow cell, with a read-length of some 36 bases. The overall sequencing output of the Illumina GA system is more than one billion base (Gb) pairs per analytical run (Bentley *et al.*, 2008).

The upgraded GA *II* that is capable of sequencing both single-read and paired-end (sequencing both ends of the template molecules) libraries, generating some 1.5 Gb of outputs per day, corresponding to 80–100 million reads per flow cell. Moreover, the Illumina's newest version of GA, HiSeq 2000 extends throughput up to 200 Gb per run and two billion 100+ bp paired-end reads.

In a giving cycle of sequencing, any modified nucleotide could be incorporated with a decreased or an increased efficiency, resulting in an under- or over- incorporation and a heterogeneous mixture of synthesis lengths, and concomitant degradation of signal purity and precision. In addition, chemical cleavage of terminating moieties and florescent dye labels are subject to incompletion. Therefore, the Illumina's sequencing strategy generates much shorter reads and its most common error type is substitutions (Shendure and Ji, 2008). The base-call error rate increases with read length due to "dephasing noise" (Dohm *et al.*, 2008). In addition, an underrepresentation of AT-rich and GC-rich regions (Dohm *et al.*, 2008; Hillier *et al.*, 2008; Metzker, 2010) has been observed.

2.3 Life Technologies/SOLiD

The SOLiD (Supported Oligonucleotide Ligation and Detection) system is a short-read sequencing platform relying on ligation chemistry. This platform was developed by Life Technologies based on the strategies described by Shendure *et al*. (2005) and Mckernan *et al*. (2008).

Library construction for the SOLiD system is similar to Roche/454 technology, in which DNA is stochastically sheared into fragments that are subsequently ligated to oligonucleotide adapters (Figure 3a), attached to beads, and clonally amplified by emulsion PCR. After denaturing templates, the template-carrying beads are enriched to separate desired beads from undesired. The templates on the selected beads then are 3′ modified for the purpose of covalent attachment to the slide. Then, 3′ modified beads are deposited onto a derivitized-glass flow cell surface to generate a dense, disordered array (Figure 3b). Sequencing reactions are started by hybridizing a primer oligonucleotide complementary to the adapter at the adapter-template junction (Figure 3c). Unlike the Roche/454 sequencing approach, the sequencing-by-synthesis in the SOLiD system is driven by a DNA ligase rather than a DNA polymerase. Briefly, in the ligation chemistry, a mixture of partially degenerated oligonucleotide octamers is competitively hybridized to the DNA fragments as probes, and a universal primer is oriented to provide a 5′ phosphate group for ligation. The specificity of the probe ligated to a primer is determined by the 4th and 5th bases of the probe that are complementary to the template, and the identities (base callings) of the 4th and 5th bases of probes are characterized by one of four florescent labels at the end of the octamer, so that the interrogation of the 4th base and 5th base is achieved. After ligation, the ligated octamer oligonucleotides are cleaved off after the fifth base and the fluorescent label is removed, so that the next hybridization and ligation cycle can proceed. In such a way, the bases 4 and 5 in the template are determined in the first cycle, and the bases 9 and 10 in the second cycle, and so on. The ligation-sequencing can also be carried out in the same way with another primer offset by one base in the adapter, so the bases 3 and 4, 8 and 9, . . ., in the template can be determined (Figure 3d). By any given five-cycle rounds, each base is interrogated twice with two different fluorescent labels, resulting in significantly reduced base-call error rate (http://www3.appliedbiosystems.com/AB_Home/applicationstechnologies/SOLiDSystemSequencing/OverviewofSOLiDSequencingChemistry).

The current version, SOLiD 3 Plus system, is capable of using both fragments and mate-paired libraries, and of generating in one run over 60 Gb sequence data and one billion reads with read-length up to 50 bases.

By using ligation-based sequencing-by-syntheses, the SOLiD system mitigates homopolymeric sequencing error. The dominant error type is substitutions. Furthermore, according to the manufacturer (http://www3.appliedbiosystems.com/AB_Home/applicationstechnologies/SOLiDSystemSequencing/SOLiD-Accuracy), an

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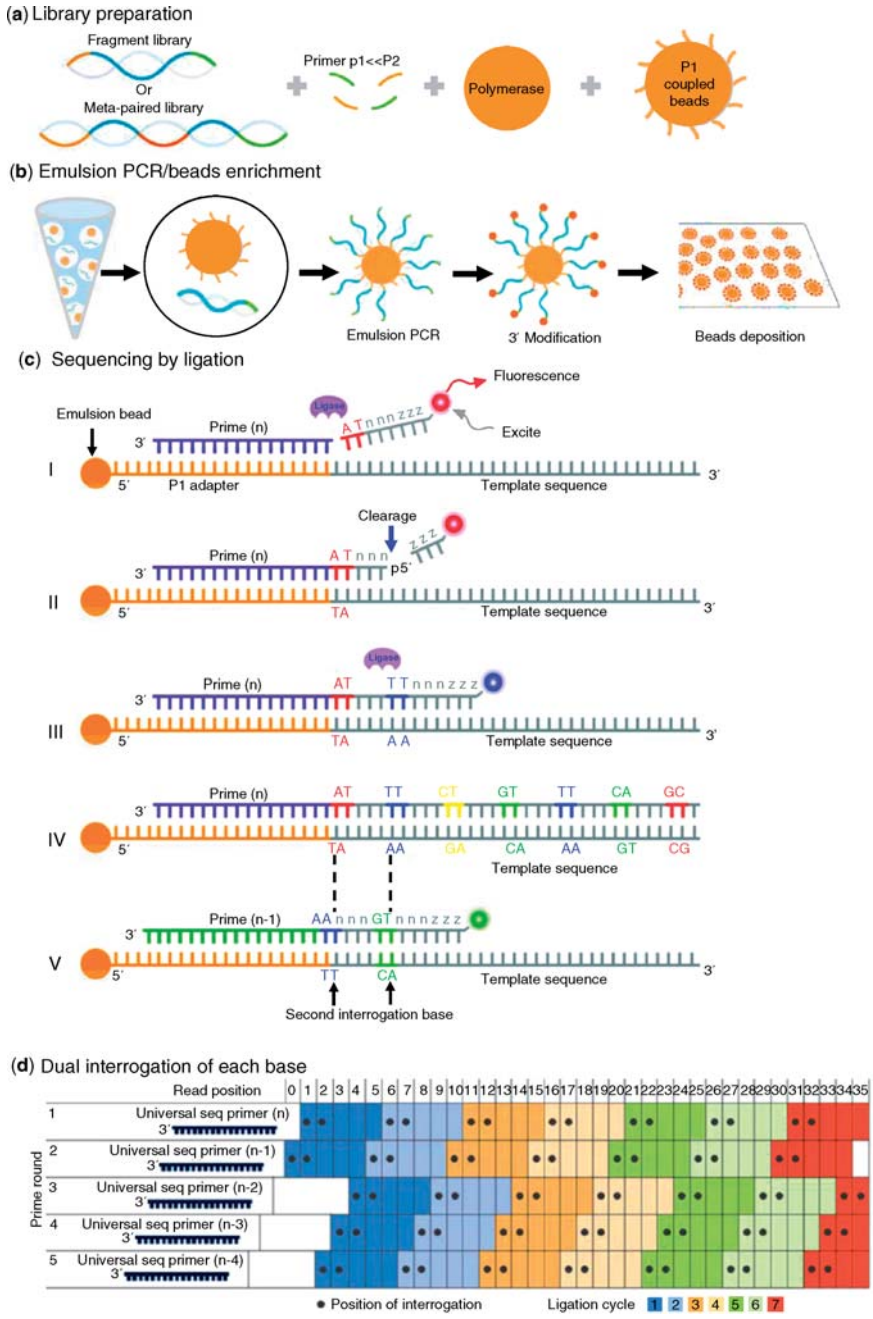

Figure 3. Outline of the Life Technologies SOLiD sequencing: (a) preparation of libraries by ligation of two different DNA adapters, P1 and P2, to the 5′- and 3′- ends of DNA fragments, respectively; (b) emulsion PCR is performed, and DNA templates on enriched beads undergo a 3′ modification, and are deposited onto a flow cell to generate a dense, disordered array; (c) sequencing by ligation: (I) primers hybridize to the P1 adapter on template-carrying beads; a set of four fluorescently labeled di-base probes compete for ligation to the primers. Specificity of di-base probes is achieved by interrogating every 1st and 2nd bases in each ligation reaction. Each ligation is followed by fluorescence detection, after which, (II) the fluorescent label is cleaved and (III–IV) multiple cycles of ligation, detection and cleavage are performed with the number of cycles determining the eventual read length. Following a series of ligation cycles, the extension product is removed and (V) the template is reset with a primer complementary to the n−1 position for the second round of ligation cycles. For each DNA template, (d) a total of five rounds of primer reset are needed, through which, virtually each base is interrogated in two independent ligation reactions by two different primers.

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over all accuracy of 99.94% can be achieved by using the two-base encoding system that can recognize and eliminate two-thirds of measurement errors.

2.4 Helicos HeliScope Genetic Analysis System

The HeliScope Genetic Analysis System, developed by Helicos BioSciences (http://www.helicosbio.com) in 2007, is the first commercialized single-molecule DNA sequencer. It is based on the True Single Molecule Sequencing (tSMS) technology stemmed from the work by Braslavsky *et al.* (2003) and relied on the cyclic interrogation of a dense array of sequencing features. By directly sequencing single molecules of DNA or RNA without requiring clonal amplification like other systems, the Helicos' tSMS technology significantly increases the speed and decreases of the cost of sequencing.

In the HeliScope system (Figure 4), a DNA library is constructed by random fragmentation of DNA samples, and 3′ end ploydenylation of DNA fragments with the adenosine terminal transferase (Figure 4a). Denatured poly-A fragments are captured on a flow cell surface by hybridization to surface-tethered poly-T oligomers to yield a disordered array of primed single molecule sequencing templates. In each cycle of sequencing (Figure 4b), DNA polymerase and one of four fluorescently labeled nucleotides are supplied to the flow cell. The template dependent incorporation of single dye-labeled nucleotide is imaged with a CCD camera to make a base calling. Followed by dye-label cleavage and washing, the next cycle of nucleotide extension and imaging is repeated. Each sequencing cycle consists of the successive addition of polymerase and a different type of dye-labeled nucleotide. The total number of sequencing cycles performed ranges from 25 to 55, resulting in read-lengths from 25 to 55 bases. The HeliScope instrument is currently capable of imaging billions of single molecules per run and producing over 1 Gb of usable sequence data per day.

Similar to the Roche/454 platform, the HeliScope system is asynchronous, meaning that some DNA strands will fall behind or ahead of others in a sequence-dependent manner, and some DNA templates just fail to incorporate by chance on a given cycle; therefore, base substitution error is likely to occur. However, the substitution error rate is quite low (0.01–1% with one pass and 0.001% with two passes). On the other hand, there are no terminating moieties present on the labeled nucleotides, so homopolymers could be problematic. Helicos has since developed a Virtual Terminator technology to correct the homopolymer errors, increasing sequencing accuracy (Bowers *et al.*, 2009). In general, as a result of incorporation of unlabeled bases, deletion is the dominant error type in the HeliScope system. The deletion error rate is 2–7% with one pass and 0.2–1% with two passes (Harris *et al.*, 2008).

There are important differences among the aforementioned NGS technologies in terms of costs, advantages, limitations, and practical aspects of use for specific applications. For example, the Illumina and the Life Technologies platforms are particularly well suited for variant discovery by resequencing the human genome (Metzker, 2010), where a reference genome is available. The Roche/454 sequencer may be preferable for *de novo* sequencing due to its longer read-length. The Helicos platform is well suited for RNA-Seq that is relying on tag counting (Wang, Gerstein and Snyder, 2009) or direct RNA sequencing (Ozsolak *et al.*, 2009). Table 1 provides a summary of the characteristics of the NGS platforms from the four manufacturers mentioned above.

3 ANALYSES OF NGS DATA

3.1 Overview of NGS Data Analyses

The new massively parallel sequencing technologies promise to refine and advance science across many fields. Moreover, the now tractable costs enable the powerful systems to reach increasing numbers of hands, thereby, broadly accelerating science. However, the realization of many promises is predicated on progress in overcoming obstacles in handling massive datasets and in developing tools to check and assure sequence quality, conduct sequence alignment and assembly, and biologically interpret and draw inferences from the data. NGS experiments generate immense volumes of short-read sequence data (Voelkerding, Dames and Durtschi, 2009) (Table 1). Data acquisition for such volumes is problematic alone, requiring

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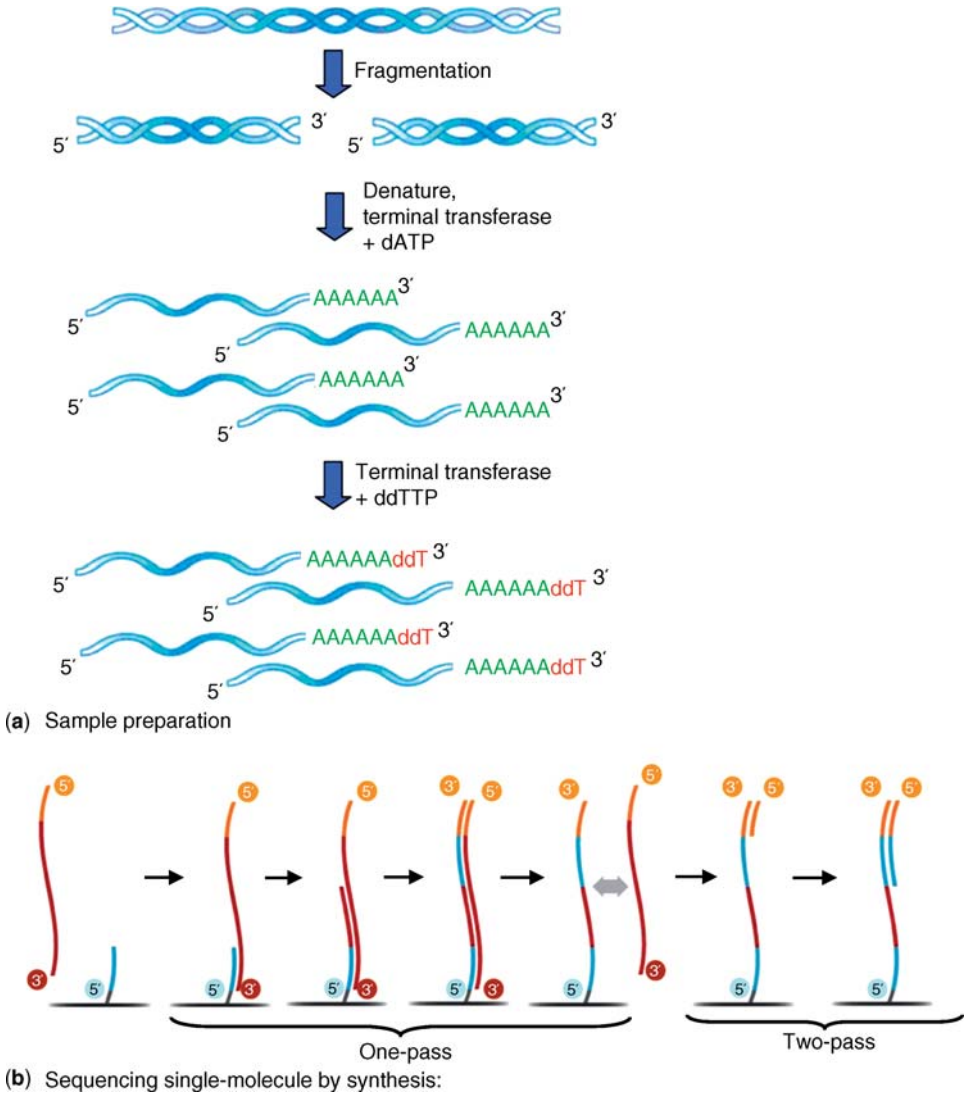

Figure 4. The Helicos true single molecule sequencing (tSMS) system: (a) DNA templates are prepared by fragmentation, 3′ poly (A) tail addition, labeling, and blocking by terminal transferase. In contrast to other NGS technologies, no ligation or PCR amplification is required; and (b) sequencing single-molecule by synthesis: the flow cell is incubated with a mixture of polymerase and one fluorescently labeled nucleotide (C, G, A or T); then the mixture is rinsed away and fluorescent labels are detected. The fluorescent group is finally removed from the incorporated nucleotide and the process continues through each of the other three nucleotides. In such multiple four-base nucleotide addition and detection cycles the sequence of each fragment is determined.

an infrastructure with high bandwidth pipelines between processes that will be computationally intensive.

Translating such volumes of short-read data to biological results can be described as requiring three analysis stages, as depicted in Figure 5. In the first stage, images from NGS sequencers are analyzed and converted into sequence reads using the manufacturer's base-calling system. The reads are filtered and aligned in the second stage. Depending on the intended biological application as well as considerations of the cost, labor intensity and time requirement, the alignment can be done by *de novo* assembly or by mapping to a reference sequence

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Table 1. The characteristics of next-generation sequencing platforms.

Product	Manufacturer	Read length (bases)	Number of reads (M/run)	Data volume (Gb/run)	Run time (days)	Price (US$)	Comments
GS FLX Titanium	Roche/454	~400	> 1	0.4 ~ 0.6	~0.5	500 000	Longer reads and fast run times; high reagent cost; high error rate in homopolymer repeats
Genome Analyzer IIx	Illumina 2 × 35 2 × 50 2 × 75 2 × 100	1 × 35 225 ~ 250 225 ~ 250 225 ~ 250 225 ~ 250	225 ~ 250 16.0 ~ 18.0 22.5 ~ 50.0 34.0 ~ 38.0 45.0 ~ 50.0	8.0 ~ 9.0 ~4 ~5 ~7.5 ~9.5	~2	540 000	Currently the most widely used system
HiSeq 2000	Illumina 2 × 50 2 × 100	1 × 35 2000 2000	1000 75 ~ 100 150 ~ 200	26 ~ 35 ~4 ~8	~1.5	NA	
SOLiD 3 plus	Applied Biosystems	50 or 2 × 50	~500 or 1000	25 ~ 30 or 50–60	3.5–4.5 for 35 bp, 6–7 for 50 bp; or 8–9 for 2 × 35 bp; 12–14 for 2 × 50 bp	595 000	Two-base encoding provides inherent error correction
HeliScope Genetic Analysis System	Helicos BioSciences	30 ~ 35	600 ~ 800	21 ~ 28	8	999 000	Non-bias single-molecule sequencing, high error rate

that can be a complete genome, subsets of a genome (e.g., expressed genes and individual chromosomes of interest), a transcriptome, or an exome. In the final third stage, mapped and unmapped reads can be used to answer specific biological questions, such as the profiling of expression of genes, exons or isoforms; the discovery of novel transcripts, genes, splice variants, or single nucleotide polymorphisms (SNPs); and, the detection of transcription factors, methylation status, and histone modifications.

3.2 NGS Quality Control

The rapid expansion of applications of NGS technologies in solving biological, biomedical, and clinical problems makes the topic of NGS quality control including data quality, reliability, reproducibility, and biological relevance more and more important because of the inherent relatively high error rate in raw sequence data. It is preferable to establish an early consensus of standardized benchmarks for sequencing quality metrics (Editorial, 2008) to avoid the future dilemmas when comparing data from different NGS platforms, such as occurred for microarray platforms the past few years (Shi *et al.*, 2006; Shi *et al.*, 2008). The third phase of the MicroArray Quality Control (MAQC) (Shi *et al.*, 2006) project, also called sequencing quality control (SEQC) is such an endeavor that is aimed at assessing the technical performance of NGS platforms. The SEQC project plans to generate benchmark datasets with reference samples and evaluate advantages and limitations of various NGS platforms and bioinformatics strategies in RNA and DNA sequencing.

3.3 Bioinformatics Tools for NGS Data Analyses

Currently, a number of bioinformatics tools are available for analyzing NGS data (Table 2) that can be grouped in four general categories: (i) base

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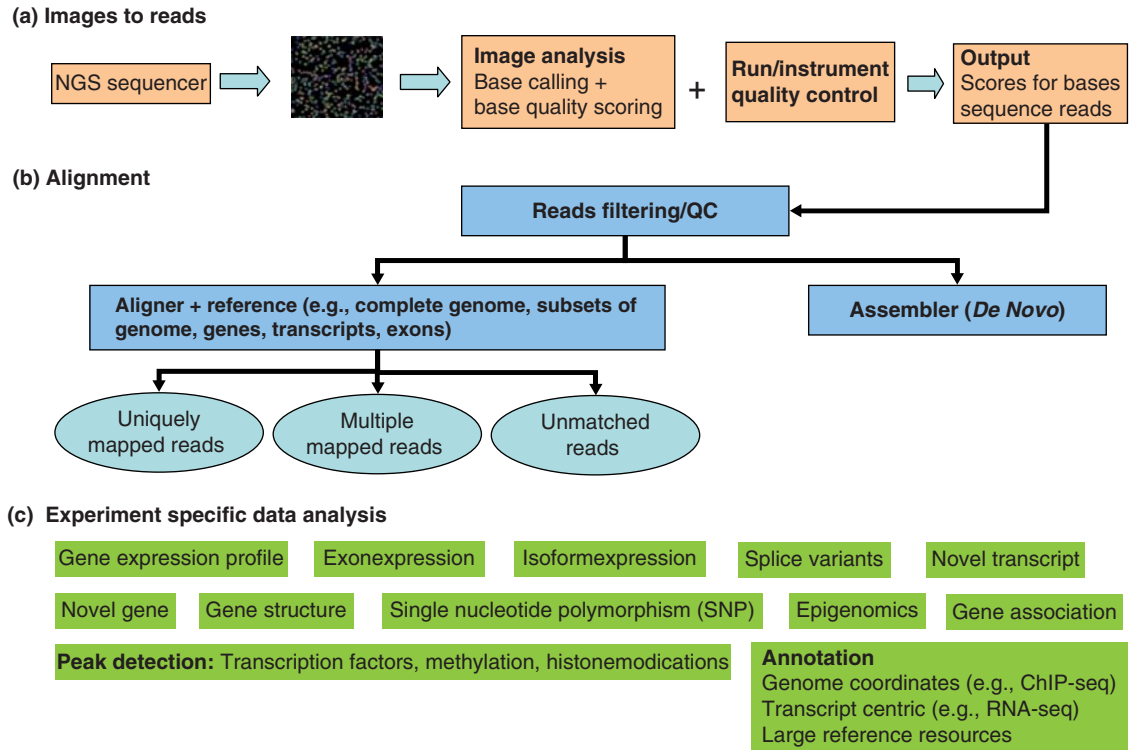

Figure 5. A typical workflow for the analyses of NGS data: (a) conversion of images to sequence reads; (b) alignment of sequence reads (map to a reference or *de novo* assembly); and (c) experiment-specific downstream analyses that depends on the applications.

calling and polymorphism detection, (ii) alignment of reads to a reference, (iii) *de novo* assembly, and (iv) genome browsing and annotation. However, these current tools have some limitations, and many challenges and questions remain. Efficient data analysis pipelines are still needed for many applications and the relative advantages and limitations of existing tools need to be objectively evaluated.

For base calling, most researchers simply use the calls generated with the data-pipeline software provided by manufacturers, but alternative approaches implementing more advanced statistical methodologies are also being developed. For example, Erlich *et al*. (2008) created an Alta-Cyclic approach that uses machine learning to reduce noise factors, substantially improving the number of accurate reads. Rougemont *et al*. (2008) proposed an algorithm using model-based clustering and probability theory to improve base-call quality by identifying and removing ambiguous bases from read ends. However, these improvements must be evaluated for cost effectiveness given the need for substantial investment to handle large volumes of raw image data (Voelkerding, Dames and Durtschi, 2009).

Proper alignment is mandatory to render NGS data biologically meaningful. Because of the short read-length, relatively high error rate in base calling, and a huge volume of data, alignment of data from NGS platforms is much more difficult than that from Sanger sequencing platforms (Trapnell and Salzberg, 2009). One limitation of aligning and assembling of reads is that a large portion of reads cannot be uniquely aligned to a reference when sequence reads are too short and the reference is too complex (Voelkerding, Dames and Durtschi, 2009). In addition, the chance of unique alignment or assembly is reduced not only by the presence of repeat sequences in complex genomes, but also by shared homologies within closely related gene families and pseudogenes (Voelkerding, Dames and Durtschi, 2009).

Conventional alignment solutions like BLAST (http://blast.ncbi.nlm.nih.gov) (Altschul *et al*.,

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Table 2. Bioinformatics tools for next-generation sequencing data analyses.

Program	Functions	Author	URL
454 Analysis Tools	Integrated solution	Roche	http://www.454.com
Bfast	Alignment	(Homer, Merriman and Nelson, 2009)	http://bfast.sourceforge.net
Bowtie	Alignment	(Langmead *et al.*, 2009)	http://bowtie-bio.sourceforge.net
BreakDancer	Variation detection	(Chen *et al.*, 2009)	http://www.nature.com/nmeth/journal/v6/n9/extref/nmeth.1363-S4.zip
CLC Genomics Workbench	Integrated solution	CLCBio	http://www.clcbio.com
Cross_match	Alignment	Phil Green	http://www.phrap.org
EagleView	Assembler viewer	(Huang and Marth, 2008)	http://bioinformatics.bc.edu/marthlab/EagleView
ELAND	Alignment	Anthony J. Cox, Illumina	http://www.illumina.com
Exonerate	Alignment	(Slater and Birney, 2005)	http://www.ebi.ac.uk/~guy/exonerate
Galaxy	Integrated	(Taylor *et al.*, 2007)	http://main.g2.bx.psu.edu
Genomatix	Integrated solution	Genomatrix	http://www.genomatix.de
GMAP	Alignment	(Wu and Watanabe, 2005)	http://www.gene.com/share/gmap
JMP Genomics	Viewer and statistical analysis	SAS Institute	http://www.jmp.com
LookSeq	Viewer	(Manske and Kwiatkowski, 2009)	http://www.sanger.ac.uk/Software/analysis/lookseq
MapView	Viewer	(Bao *et al.*, 2009)	http://evolution.sysu.edu.cn/mapview
MAQ	Alignment and assembly	(Li, Ruan and Durbin, 2008a)	http://maq.sourceforge.net
Matlab Bioinformatics	Alignment and statistical analysis	The Mathworks™	http://www.mathworks.com/
MUMmer	Alignment	(Ossowski *et al.*, 2008)	http://mummer.sourceforge.net
NextGENe	Integrated solution	Softgenetics	http://softgenetics.com
PeakSeq	Chip-Seq	(Rozowsky *et al.*, 2009)	http://www.gersteinlab.org/proj/PeakSeq
PIQA	Integrated pipeline	(Martinez-Alcantara *et al.*, 2009)	http://bioinfo.uh.edu/PIQA
Rtracklayer	Viewer	(Lawrence, Gentleman and Carey, 2009)	http://bioconductor.org/packages/2.5/bioc/html/rtracklayer.html
SAM	Sequence Assembly Manager	(Warren *et al.*, 2005)	http://www.bcgsc.ca/platform/bioinfo/software/sam
SeqMap	Alignment	(Jiang and Wong, 2008)	http://biogibbs.stanford.edu/~jiangh/SeqMap
SeqMan NGen	Integrated solution	DNAStar	http://www.dnastar.com
SHORE	Mapping and analysis pipeline	Korbinian Schneeberger *et al.*	http://www.1001genomes.org/downloads/genomemapper_singleref.html
ShortRead	Input, quality assessment	(Morgan *et al.*, 2009)	http://www.bioconductor.org/packages/2.3/bioc/html/ShortRead.html
SHRiMP	Alignment	(Rumble *et al.*, 2009)	http://compbio.cs.toronto.edu/shrimp
Slider	Alignment and SNP detection	(Malhis *et al.*, 2009)	http://www.bcgsc.ca/platform/bioinfo
SOAP	Alignment and analysis	(Li *et al.*, 2008b)	http://soap.genomics.org.cn
SSAHS	Alignment	(Ning, Cox and Mullikin, 2001)	http://www.sanger.ac.uk/Software/analysis/SSAHA
Vmatch	Alignment	Stefan Kurtz (Delcher *et al.*, 1999)	http://www.vmatch.de
ZOOM	Alignment	(Lin *et al.*, 2008)	http://www.bioinformaticssolutions.com

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1990) and BLAT (http://genome.ucsc.edu/cgi-bin/hgBlat) (Kent, 2002) are efficient to align long reads such as those generated by Sanger sequencing, but inadequate to handle NGS short reads. Recently, a variety of sequence alignment algorithms and software packages have been developed specifically for processing a large number of short reads. Table 2 provides an overview of such programs. The algorithms implemented in these software packages vary with the applications, but they include sequence alignment, *de novo* assembly, alignment viewing, and variant discovery. However, the state-of-the-art in short-read alignment and assembly remains the trade-off between speed and accuracy, with a tradeoff needing to be made between ideal alignment and computational efficiency.

4 APPLICATIONS OF NGS TECHNOLOGIES

Over the past five years, the NGS technologies have markedly accelerated multiple research areas, making feasible experiments that previously were not affordable or even technically feasible. Novel fields and applications in biology, life sciences and biomedicine are becoming reality. In this section, we describe some major applications of NGS.

4.1 *De novo* Sequencing or Resequencing of Genomes

The ultra-high throughput and low cost of NGS technologies have made sequencing numerous whole genomes tractable. NGS platforms have been used for *de novo* sequencing many bacterial genomes (Chaisson and Pevzner, 2008; Margulies *et al*., 2005), viral genomes (Harris *et al*., 2008), the giant panda genome (Li *et al.*, 2010c), and resequencing human genomes at dramatically increased speed and decreased cost (Li *et al.*, 2010b; Lin *et al.*, 2008; Pushkarev, Neff and Quake, 2009; Wheeler *et al*., 2008). These applications have demonstrated the power of NGS technologies for *de novo* sequencing or sequencing of personal genomes that will be critical toward moving to the realm of personalized genomics and medicine.

4.2 Target Genomic Resequencing

Resequencing of genomic sub-regions or gene sets is fundamental in basic and clinical research seeking causative and predisposition mutations within populations (Dahl *et al.*, 2007; Ding *et al.*, 2008; Okou *et al.*, 2007). The target resequencing strategy involves comparative analysis of candidate genes or genomic sub-regions from two groups of people with different phenotypes, and requires a high level of accuracy to identify low frequency causative SNPs and structural variants/mutations of diseases that are implicated by linkage studies and whole-genome wide association studies (Porreca *et al.*, 2007; Yeager *et al.*, 2008). Traditional capillary electrophoresis methods provide the highest accuracy and are the best suited for analyzing a limited set of amplicons in a large number of patient samples. However, this is burdensome in cost and labor for investigating a large number of genes or large sub-regions. In contrast, NGS technologies are highly advantageous in terms of both cost and labor, as evidenced by numerous recent studies (Albert *et al.*, 2007; Chou *et al.*, 2010; Hodges *et al.*, 2007; Li *et al.*, 2009b; Okou *et al.*, 2007).

4.3 Chromatin Immunoprecipitation Followed by Sequencing (ChIP-Seq)

ChIP-Seq is a strategy that combines ChIP (chromatin immunopreciptation used to determine the location of DNA binding sites for proteins) technique with the NGS technologies to directly sequence DNA fragments to interrogate DNA-protein interactions, and was an early application of NGS (Barski *et al.*, 2007; Johnson *et al.*, 2007; Mikkelsen *et al.*, 2007). By directly sequencing DNA fragments that interact with proteins, ChIP-Seq provides substantially improved data than microarray-based ChIP-chip method that is the most commonly used for genome-wide profiling of DNA-binding proteins, histone modifications or nucleosomes (Park, 2009). Compared to ChIP-chip, ChIP-Seq has higher resolution, fewer artifacts, greater coverage, and a larger dynamic range. ChIP-Seq can also be used to identify the cistrome of DNA-associated proteins and precisely map global binding sites for any protein of interest (Kaufmann

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et al., 2010; Ouyang, Zhou and Wong, 2009; Visel *et al.*, 2009).

4.4 Next-generation RNA Sequencing (RNA-Seq)

Applying NGS technologies to sequence RNA or complementary DNA (cDNA) reverse transcribed from the RNAs offers an alternative methodology for high-throughput transcriptome analysis (Marioni *et al.*, 2008; Wang, Gerstein and Snyder, 2009; Wilhelm *et al.*, 2010). In a typical RNA-Seq experiment, RNAs or cDNAs are first directly sequenced with NGS technologies; and then the sequence reads are mapped to a reference genome to construct a whole-genome transcriptome map (Wang, Gerstein and Snyder, 2009); finally, the transcripts (genes of interest) are characterized (e.g., alternative splicing) and quantified (Wang, Gerstein and Snyder, 2009).

Thanks to the deep coverage and base level resolution provided by next-generation sequencing instruments, RNA-Seq provides researchers with efficient ways to measure transcriptome data experimentally, allowing them to get information such as how different alleles of a gene are expressed, detect post-transcriptional mutations or identifying gene fusions.

By directly sequencing the entire transcriptome without prior knowledge of transcribed regions and at deep coverage and base level resolution, RNA-Seq is revolutionary in its abilities to provide precision in measuring transcriptome data (Li *et al.*, 2010a; Marioni *et al.*, 2008). The far higher resolution improves discovery of novel transcripts, differential allele expression, alternative splice variants, post-transcriptional mutations and isoforms compared with more conventional Sanger sequencing and microarray-based approaches (Chepelev *et al.*, 2009; Hittinger *et al.*, 2010; Jiang and Wong, 2009; Perkins *et al.*, 2009; Richard *et al.*, 2010; Sultan *et al.*, 2008; Tang *et al.*, 2009; Trapnell, Pachter and Salzberg, 2009; Wilhelm *et al.*, 2010). Recent studies (Guttman *et al.*, 2009; Li *et al.*, 2009a; Pan *et al.*, 2008; Porreca *et al.*, 2007; Wang *et al.*, 2008) that used RNA-Seq to characterize the RNA populations have provided more complicated pictures of RNA regulation and expression, through alternative splicing, alternative polyadenylation, and RNA editing. These findings have expanded our traditional view of the extent and complexity of gene expression (Licatalosi and Darnell, 2010), and advanced our understanding of mechanisms of RNA expression regulation in both eukaryotic (Jacquier, 2009) and prokaryotic (Sorek and Cossart, 2010) genomes.

4.5 Comparison Between RNA-Seq and Microarrays

To evaluate the technical performance of NGS technologies on quantifying the expression level of transcripts, we recently used data generated from a rat toxicogenomics study to compare the performance of NGS (Illumina Genome Analyzer *II*) with a microarray-based approach (Affymetrix Rat Genome 230 2.0 arrays) to detect differentially expressed genes (DEGs) (Su *et al.*, 2010). The RNA samples were the same as those used in the MAQC-*I* (Shi *et al.*, 2006) validation study, for which the microarray data already existed (Guo *et al.*, 2006). Eight RNA samples, four treatment and four control, were collected from the kidneys of rats treated/or not-treated (controls) with 10 mg kg^{-1} body weight carcinogen aristolochic acid (AA) (Guo *et al.*, 2006) and then sequenced with an Illumina Genome Analyzer *II* platform. The RNA sample from each rat was sequenced in one lane, generating over 16 million 36 bp reads per sample. Figure 6a shows a scatter plot of NGS log2 FCs (Fold Changes) versus microarray log2 FCs for 11,202 common genes; the Pearson's correlation coefficient (r) is 0.52. Figure 6b plotted the 4169 DEGs that were detected by either NGS or microarrays, where in both cases differential expression of genes was determined by the same two criteria, FC > 1.5 and p-value < 0.05. The 3322 red points and 372 magenta points represent genes detected only by the NGS and microarray, respectively, whereas the 522 blue points and 402 green points represent DEGs commonly selected and either up-regulated, or down-regulated, respectively. One circled point represents a singular common gene with differential expression in the opposite directions. Among all 4619 DEGs, the Pearson's coefficient for log2 FCs is 0.65 (Figure 6b). Although 71% of the DEGs (Figure 6d) selected from microarray data were also selected from NGS data, only 22% of the DEGs selected from the NGS data were selected from the microarray data, supporting conjecture that NGS is

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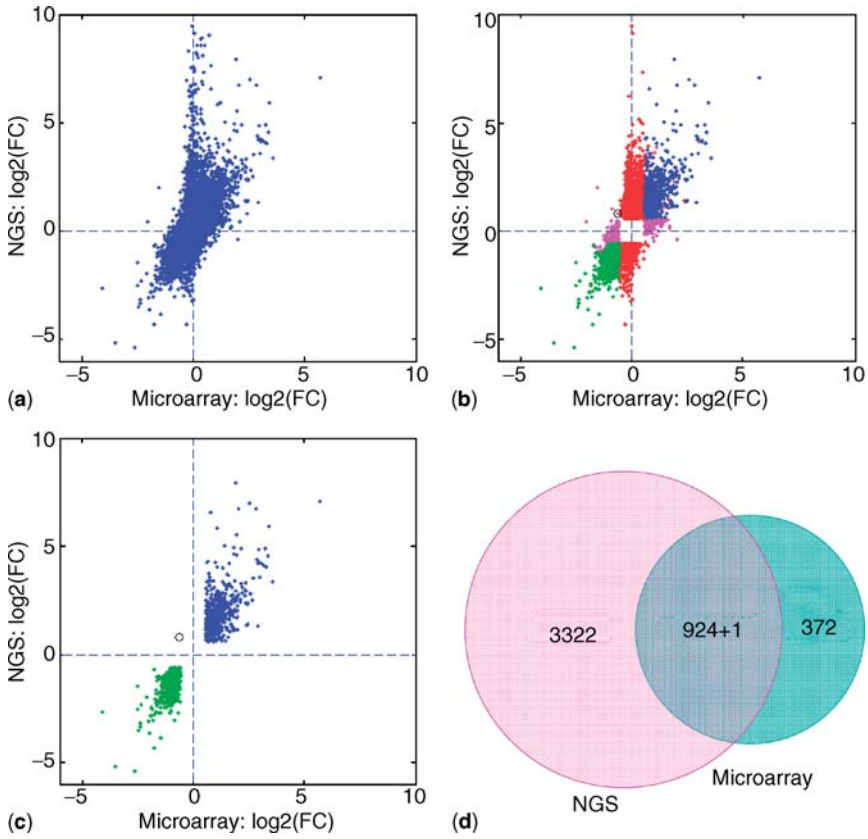

Figure 6. Comparison between NGS and microarray data, based on the log2 fold changes (FCs): (a) all 11 202 common transcripts; (b) 4619 differentially expressed genes (DEGs) identified from either NGS or microarray data; (c) 925 DEGs commonly selected from both NGS and microarray data. For each platform, FCs were calculated by comparing four aristolochic acid treated samples to four control samples and DEGs were selected with an FC cut-off of 1.5 and p-value < 0.05. The magenta and red spots in panel (b) are DEGs determined only by microarrays and NGS, respectively. The blue and green spots in (b) and (c) represent up- or down-regulated DEGs commonly determined by NGS and microarrays. The circle in (b) and (c) denotes a gene up-regulated in NGS but down-regulated in microarrays. The Pearson's correlation coefficients (r) between NGS log2 FCs and microarrays log2 FCs are 0.51, 0.65, and 0.91 in (a), (b), and (c), respectively. The Venn diagram in panel (d) shows the overlap between NGS and microarray DEG lists of panel (b). Of 925 common DEGs (b), 924 are altered in the same directions (c).

more sensitive than microarrays in detecting DEGs under the same selection criteria. The log2 FCs for 925 genes commonly selected by the NGS and microarrays are shown in a scatter plot in Figure 6c with $r = 0.91$. Only one out of 925 genes disagrees in regulated directions. Hence, the concordance between genes commonly detected as differentially expressed by both platforms is very high.

5 FUTURE PERSPECTIVE

NGS technologies are substantially impacting basic genomics research, and many more and far-reaching impacts are anticipated. Over the past few years, NGS technologies have demonstrated their immense potential for enabling scientific advancement in an ever-increasing diversity of biological and medical research areas (Sultan *et al.*, 2008). In the next several years, NGS technologies are anticipated to transition into broader areas including disease etiology, new drug development, clinical-diagnostics, personalized medicine and nutrition, as well as toxicogenomics. Requisite to continuing successful transition will be further sequencing cost reduction, improved read accuracy, more streamlined sample preparation, and perhaps more importantly, computer-based analytics for data

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acquisition, management, validation, analyses, and biological interpretation.

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Systems Toxicology Modeling for Prediction in Humans

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1 INTRODUCTION

Increasing social and economic pressures to develop more effective, safer, and cost-effective medicines make meeting increasingly stringent target product profiles (TPP) and timelines the norm rather than the exception. These very pressures further demand that the way (pre-)clinical experiments are conducted is changed as well. Developments in biological understanding and technology have enabled us to meet some of these expectations. In this chapter, a methodology is proposed for integrating the available knowledge across experiments to predict toxicity in man, thereby making drug development more efficient.

The anticipation is that by utilizing available *in vitro* and *in vivo* (animal) data it will be possible to predict how a molecule will behave in humans. This will allow avoidance of unnecessary human exposure to unsafe or inefficacious drugs as well as reducing lengthy experiments at irrelevant doses by implementing more meaningful designs to deliver more relevant results. Secondly, animal experiments will be designed in such a way that they deliver relevant information – replacing some experiments by simulation and refining the experiments that are conducted to deliver the results required to move to the next phase.

A key piece of information that will enable efficient evaluation of safety and efficacy in man concerns the dose range and regimen to be evaluated and which drug to combine with. The dose and regimen will depend on a molecule's pharmacokinetics, efficacy, and adverse effects. In many ways, these properties will have been evaluated in various experiments before a drug is dosed to humans. Interestingly enough, while the experiments today are largely conducted under a great deal of scrutiny (obeying numerous SOPs and guidelines) – combining all the information and subsequently deciding on a dose and regimes remains a fairly arbitrary process.

Today, we readily board a new plane based on thousands of hours in computer simulation models, we let our politicians decide environmental policies guided by mathematical models that are humblingly complex, and our savings are managed by computer models, yet drug development remains an empirical process, in particular the safety assessment of drugs.

Most experiments conducted today *in vitro*, in animals or man, will have been described by some mathematical equation. Assume for a moment that

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any mathematical model that describes some process to which a drug is subjected characterizes that response in parameters that are unique to the physiology of the species tested and also to the drug tested. We can then take this model, add the drug-specific parameters, and replace the species-specific parameters with those of a different species to predict the time course of response in the new species. There are several assumptions in these previous statements, such as the process being similar and the drug-specific parameter being truly drug specific. For example, the regulation of body temperature in rats and humans involves the serotonin-1A receptor; however, it is not so in mice. The validity of the general assumptions therefore needs to be checked case by case. A drug-specific parameter such as affinity is – depending on the receptor – dependent on the species used, something that also needs to be taken into account. However, the concept of scaling results remains valid if the results of any experiment are understood in the context of its environment, be it a Petri dish or the animal physiology. Most often the information collected can be used for reliable predictions.

Key is the understanding of the context in which the drug is evaluated; the mathematical model is merely a tool, a common language to ensure a consistency in our understanding and predictions, but also to visualize highly complex, interdependent non-linear phenomena. This chapter will therefore focus on the following elements to predict in humans: (i) a drug's pharmacokinetics, (ii) drug's toxicity in organs, and (iii) the resulting whole-body toxicity. The pharmacokinetics are essential to the pharmacological process – the "fight" of the body to rid itself off the drug versus the efforts of the drug to distribute itself determine how long the drug will be at the site of action. The second timeline will be driven by the physiology of the actual organ, be it a target of toxicity or pharmacological response. The final timeline will be driven by the overall physiology, or the interaction of the entire system.

Pharmacokinetic information, that is, to predict what might happen to the drug when it is administered to humans, plays a significant role in determining whether a drug will succeed in the clinic (Reigner *et al.*, 1997; Dickins, 2004; Leahy, 2004). This has led to a keen interest in the pharmaceutical industry to develop reliable, high-throughput methodologies to gather PK information. Over the past decade, these methods have been implemented early on in the drug discovery setting to remove compounds with unsuitable PK profiles and only advance those that are predicted to perform well in humans. An accurate prediction of human PK is the basis of a correct calculation of safety margin in human trials for modeling a target or for a given compound. In this chapter, different methods for predicting human PK based on physiochemical properties, *in vitro* and *in vivo* experiments, will be reviewed.

The second part on organ toxicity will focus on the drug-induced prolongation of the QT interval. QT prolongation has become a major regulatory and commercial concern in drug development as the costs associated with late-stage failures, market withdrawals, and patient liabilities have spiralled upwards. Consequently, as a reaction, much work has been invested into computational, simulation, and experimental methods for anticipating QT and proarrhythmic toxicity, thereby allowing drug development programs to mitigate or manage the liability (Sanguinetti and Mitcheson, 2005). To date, regulatory agencies and sponsors are hesitant to embrace such models until clear understanding and agreement on the key proarrhythmia mechanisms emerge (Lawrence *et al.*, 2008). In contrast to *in vitro* and *in vivo* models of cardiac electrophysiology, mathematical system models are presented, which explicitly describe the underlying biophysical processes that are responsible for the genesis of the cardiac action potential. As such, they can be used for two important purposes – to integrate lower-level data to project higher-level possible observations not yet measured and to help interpret and reconcile differences in outcomes or observations across multiple systems.

The final part illustrates the power of combining different models to come to a whole-animal toxicity model. In the example, a model for chemotherapy-induced neutropenia, which represents one of the major limitations during treatment, will be evaluated. The particular example nicely illustrates the high degree of non-linearity in concentration and toxicodynamic-time profiles, which means that mathematical approaches can far exceed our ability to anticipate the events that may occur in humans based on empirical correlations alone.

The three different sections outlined above aim to show that by utilizing available *in vitro* and *in vivo* (animal) data it is possible to get a better understanding and ultimately predict how the molecule

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will behave in humans at the early stages. These approaches will allow for the evaluation of safe and efficacious doses in humans in an efficient manner. At the same time, it should also help us design more adequate experiments, utilizing less number of animals.

2 PREDICTING HUMAN PHARMACOKINETICS

2.1 Introduction

The primary aim of pre-clinical studies with new drugs is to enter suitable candidates into human clinical trials as quickly and safely as possible. This knowledge, combined with technological advances in high-throughput chemistry enabling numerous compounds to be generated rapidly, has led to a keen focus within the pharmaceutical industry to develop reliable, high-throughput methodologies to gather PK information. In the last decade, these advanced methods have been implemented early on in the drug discovery setting to remove compounds with unsuitable PK profiles and only advance those that are predicted to perform well in humans. The result has been a dramatic shift away from unfavourable PK characteristics being a common reason for attrition of new chemical entities (Theil *et al*., 2003). An accurate prediction of human PK is based on a correct calculation of safety margin in human trials for modeling a target or for a given compound.

The PK profile of a drug is progressively built up using data from a variety of sources and includes considerations such as bioavailability, clearance, and temporal features, such as half-life (Dickins, 2004; Leahy, 2004; Willmann *et al*., 2003). This accumulating information plays a crucial role in the optimization, selection, and development of new drugs from the initial stages of development through to phase 3 clinical trials. Existing methods to generate human PK data fall into three broad categories: *in vivo*, *in vitro*, and *in silico*, and can also be classified into empirical or physiological. Classical empirical methods, such as *in vivo* pre-clinical studies in animals and subsequent allometric scaling to humans, have been utilized for many years to predict human PK parameters. More recently, approaches to human PK prediction of small molecules are increasingly reliant on data generated from *in vitro* studies. With the continuing impetus to improve PK prediction further, strong interest has been sparked into use of *in vitro–in silico* physiologically based PK (PBPK) models (Reigner *et al*., 1997; Poulin and Theil, 2000; Poulin, Schoenlein and Theil, 2001; Poulin and Theil, 2002a; Poulin and Theil, 2002b; Nestorov, 2003). These approaches combine physicochemical properties of compounds and *in vitro* measures, together with information on tissue composition and blood flow, to predict human PK profiles and are increasingly used in compound selection and to simulate clinical outcomes, such as the likely impact of drug interactions and disease, before a drug is administered to man.

2.2 Methods

2.2.1 Construction of PBPK Models

The following section explores some of the key issues associated with the development of PBPK models. More details on the construction of such models are available in a number of reviews (e.g., Clewell, 2008).

Physiologically based pharmacokinetic models divide the body into compartments, including the eliminating organs (e.g., kidney and liver) and non-eliminating tissue compartments (such as fat, muscle, brain, etc.), which are connected by the circulatory system (Figure 1).

The models use physiological and species-specific parameters (such as blood flow rates and tissue volumes) to describe the pharmacokinetic processes. A list of pharmacokinetic parameters with their corresponding definition is provided in Table 1. These physiological parameters are

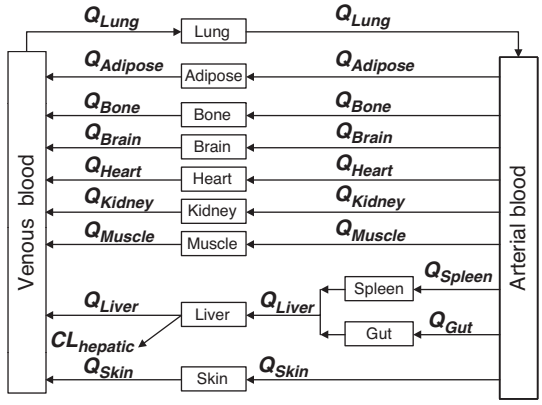

Figure 1. PBPK model.

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coupled with compound-specific parameters such as physicochemical and biochemical parameters (e.g., tissue/blood distribution coefficients and metabolic clearance) to predict the plasma and/or tissue concentration versus time profiles of a compound in an *in vivo* (animal and/or human) system.

Once a model has been developed, the concentrations in the various tissues can be determined by using the mass balance equation below:

Drug concentration in tissue = rate of drug distribution into tissue − rate of drug distribution out – rate of drug elimination within the tissue.

Depending on the drug and tissue, the distribution is perfusion-rate- or diffusion-rate-limited. Perfusion-rate-limited kinetics tend to occur with relatively low molecular weight, hydrophobic drugs that have no problem crossing the lipid barrier of the cell wall. In this case, the process limiting the penetration of the drug into the cells is the rate at which it is delivered to the tissue, so that blood flow is the limiting process. In contrast, diffusion-rate-limited kinetics occurs with more polar drugs that do not freely dissolve in the lipid of the cell membrane and, therefore, have difficulty in penetrating into the cell. In this case the diffusion of drug across the membrane, which is independent of blood flow, becomes the limiting process.

Table 1. Definition of main pharmacokinetic parameters.

PK parameter	Definition	Range of values (human)
AUC	Area under the plasma concentration–time curve following intra- or extravascular administration of the compound	
CL	Total clearance This parameter represents the elimination (metabolism and excretion) of the compound from the body	
CL_H	Hepatic clearance This parameter represents the elimination of the compound through metabolism in the liver and/or biliary excretion	0–20 ml $min^{-1}kg^{-1}$ low: <6 ml $min^{-1}kg^{-1}$ intermediate: between 6 and 14 ml $min^{-1}kg^{-1}$ high: >14 ml $min^{-1}kg^{-1}$
V_d and V_{dss}	Volume of distribution The volume of distribution (V_d) corresponds to the theoretical volume in which the drug would need to distribute to be at plasma concentration. $V_d = C_0$/Dose (iv) V_{dss} corresponds to volume of distribution at steady state	From 0.1 L kg^{-1} (distribution restricted to vascular space) to >50 L kg^{-1} (high volume due to extensive binding)
$T_{1/2}$	Half-life This parameter represents the time taken for the drug concentration in the plasma to decrease by 50% $T_{1/2}$ is a function of the clearance and volume of distribution, and reflects how often a drug needs to be administered. $T_{1/2} = 0.693V_d/CL$	Few minutes to several months, depending on the drug
F_{abs}	Fraction absorbed It is the fraction or percentage of the dose administered, which becomes available in the portal vein after intestinal absorption. $F_{abs} = F/(1 - CL_H/Q_H)$, where Q_H represents hepatic blood flow.	0–100%
F	Bioavailability It is the fraction or percentage of the dose administered, which becomes available in the systemic circulation. Oral bioavailability is obtained by comparison of dose-normalized exposure obtained after oral versus intravenous administration: $F = (AUC_{po}/AUC_{iv}) \times (Dose_{iv}/Dose_{po})$	0 to 100 %

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For perfusion limitation, the rate of change of drug concentration in a tissue where no elimination occurs can be described as:

$$V\frac{dC}{dt} = Q\left(C_{in} - \frac{C_{in}}{K_p}\right) \quad (1)$$

where V is the physical volume of the tissue, C is the drug concentration in tissue, Q is the blood flow to the tissue, C_{in} is the drug concentration entering the tissue, and K_p is the partition of the drug between blood and tissue.

When diffusion rate limitation occurs, diffusion to and from the extracellular space must be taken into account:

$$V_e\frac{dC_e}{dt} = Q\,(C_{in} - C_e) - P(C_e - C_i) \quad (2)$$

where P is the membrane permeability coefficient, C_e is the free extracellular drug concentration, C_i is the free intracellular drug concentration, and V_e is the anatomical extra cellular volume.

With organs such as liver, where elimination can occur, the rate of elimination must be included in the mass-balance equation. The rate of elimination is described by the equation:

$$\text{Rate of elimination} = CL_H C_t \quad (3)$$

where CL_H is the hepatic clearance of the drug, and C_t is the concentration in tissue (in this case the liver).

This term can then be inserted into the mass balance equation, such as that for perfusion limitation:

$$V\frac{dC}{dt} = Q\left(C_{in} - \frac{C_{in}}{K_p}\right) - CL_H C_t \quad (4)$$

A recent excellent review of whole-body physiological models is provided (Nestorov, 2003). The whole-body model applied in a drug discovery context is described more fully elsewhere (Poulin and Theil, 2002a).

Physiologically based components for prediction of distribution and metabolism may be combined in a whole-body model and linked to an oral absorption model. Thus, plasma and tissue concentration versus time profiles after intravenous and oral administration may be simulated based upon limited data and/or *in silico* estimates (Poulin and Theil, 2002b). In addition, the whole-body model provides a framework for incorporation of additional processes when these can be estimated.

2.2.2 Models to predict distribution

For each organ included in the PBPK model, estimates of tissue/plasma partitioning are required to quantify the distribution for the compound under study.

The tissue plasma partitioning can be estimated in silico using mechanistic tissue composition models (Poulin and Theil, 2000; Poulin, Schoenlein and Theil, 2001) or can be determined experimentally. Semi-empirical methods to estimate tissue plasma partitioning based on *in vivo* V_{ss} were also reported (Ballard *et al*., 2003). These methods are reviewed below:

Tissue composition-based models have been developed to estimate tissue distribution based on *in vitro* and in silico data (Poulin and Theil, 2000; Poulin, Schoenlein and Theil, 2001). These models have greatly extended the applicability of the PBPK approach to early compound research and development. Briefly, these equations assume that the compound distributes homogenously into the tissue and plasma by passive diffusion where two processes are accounted for: (a) non-specific binding to lipids estimated from compound lipophilicity data [$\log P$ (octanol : water distribution coefficient of the unionized drug) and $\log D$ (octanol : water distribution coefficient of the ionized + unionized drug measured at a given pH)] and (b) specific reversible binding to common proteins present in plasma and tissue estimated from the plasma f_u (f_{up}, fraction unbound in plasma). These models have been reviewed in detail (Poulin and Theil, 2000).

2.2.3 Models to Predict Clearance

Models to predict clearance have been reviewed recently and a summary is provided below.

Hepatic Metabolic Clearance

Both empirical and physiologically based approaches have been developed to predict the *in vivo* hepatic metabolic clearance in animals and humans (Lave *et al*., 2002). Because of the improved availability of animal and human liver samples, the number of predictions that include *in vitro* data and are based on *in vitro* to *in vivo* (IVIV) physiologically based direct scaling approaches

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has markedly increased during the last decade. The attractiveness of these approaches is that they do not require extra information from preclinical studies in animals.

The strategy that allows this extrapolation of *in vitro* clearance to the *in vivo* situation can be summarized as follows: the first step is to obtain CLH,int (hepatic intrinsic clearance) from *in vitro* data; reliable *ab initio* prediction of metabolic clearance for a wide variety of compound structures is not currently available. Thus, *in vitro* CLH,int values determined from various systems (e.g., hepatocytes, liver microsomes, recombinant CYPs), by substrate depletion or metabolite formation, are normalized for cell, microsomal protein, or enzyme concentration to obtain CLint in units of $\mu l\ min^{-1}\ 10^{-6}$ cells or $\mu l\ min^{-1}\ mg^{-1}$ protein and corrected for any non-specific binding. The second step consists of scaling the activity measured *in vitro* to the whole liver. This is achieved by using hepatocellularity numbers (hepatocytes), microsomal recovery (microsomes), or inter-system extrapolation factor (recombinantly expressed CYPs) values. Microsomal recovery and hepatocellularity scaling factors have been reported in the literature for various species including man (Wilson *et al*., 2003). Successful examples of IVIVE using recombinantly expressed CYPs have also been reported (Clausen and Bickel, 1993). However, the accuracy of such clearance estimates relies significantly on the availability of well-defined values for inter-system extrapolation factors (Bickel *et al*., 1987). The third stage involves the use of a liver model that, in addition to CLH,int, incorporates the effects of hepatic blood flow and blood binding, to convert the estimated CLH,int into a CLH. The main liver models used, in decreasing frequency, are: the venous equilibrium (well-stirred) model >the dispersion model >the undistributed sinusoidal (parallel-tube) model (see Figure 2, Lave *et al*., 1999).

Predictions of *in vivo* hepatic metabolic clearance based on *in vitro* data have been reported extensively over the past 30 years (e.g., Ritschel and Hammer, 1980; Yata *et al*., 1990; Yokogawa *et al*., 1990; Kaliszan, Nasal and Turowski, 1996; Reinoso, Telfer and Rowland, 1998; Ballard, Leahy and Rowland, 2000; Ballard *et al*., 2003; Lombardo *et al*., 2002; Wajima *et al*., 2003). The literature indicates variable success with a general trend for underprediction (Abraham, Chadha and Mitchell, 1994) for drugs metabolized by cytochrome P450 (CYP) and UDP-glucuronosyltransferase (UGT), particularly when human liver microsomes (HLM) are employed as the enzyme source (Sawada *et al*., 1984; Nestorov, Aarons and Rowland, 1998; Luco, 1999; Bjorkman, 2002). Accurate prediction requires careful scrutiny of the quality of both the *in vitro* and *in vivo* data, the use of accurate scaling factors, and correction for non-specific microsomal binding (Clausen and Bickel, 1993). Underprediction might arise from the release of membrane long-chain unsaturated fatty acids, which act as potent competitive inhibitors of several UGT and CYP enzymes (Houston, 1994; Houston and Carlile, 1997; Lave *et al*., 1997; Zuegge *et al*., 2001).

Several studies have assessed the predictive performance of physiologically based IVIV scaling based on microsomes or recombinant systems expressing human CYPs or hepatocytes. For example, using Simcyp computer modeling software, to predict median drug clearances for 15 drugs, the predicted values of median clearances fell within twofold of observed values for 78% of the drugs when microsomal binding was disregarded and for 100% when it was considered (Clausen and Bickel, 1993). Overall, the predicted fold variability fell within twofold of the observed variability for 80% (oral) and 67% (intravenous) of the drugs (Clausen and Bickel, 1993). In another study (Mahmood and Balian, 1996), using the standard biological scaling factors alone resulted in a systematic five- to sixfold underprediction of *in vivo* CLH,int. Further empirical adjustments of hepatocyte and microsome human *in vitro* intrinsic clearance values provided more accurate predictions of hepatic clearance with more robust models resulting from incorporation of *in vitro* binding for both hepatocytes and microsomes.

Interestingly, several reports on relatively small numbers of compounds have indicated that data generated from hepatocyte incubations containing serum may yield more accurate, direct estimates of CLH,int, *in vivo* and CLH (Hoener, 1994; Houston, 1994; Houston and Kenworthy, 2000), perhaps by removing the inhibitory effects of long-chain unsaturable fatty acids through serum binding. Combining the results based on cryopreserved human hepatocytes in these studies to obtain an extended data set of 26 compounds, encompassing a 50-fold range of clearance, a range of protein binding from minimal to 90%, and metabolized by a variety of phase I and phase II enzymes, the clear-

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ance of 77% of the compounds was predicted within a twofold error in the absence of serum. In the presence of serum, prediction accuracy rose to 85%.

Renal Clearance

Renal excretion involves glomerular filtration and tubular secretion by active transport, both of which occur in the proximal nephron, and subsequent predominantly distal reabsorption, much driven by the extensive reabsorption of water. Despite a detailed knowledge of these factors, and the development of models of renal clearance (Lave *et al*., 1999; Pahlman *et al*., 1999), prediction of human renal clearance has remained essentially empirical, principally because allometric scaling of animal renal clearance has been relatively so successful (Lin, 1995; Ishizuka *et al*., 1999). Still, as with transporters in other organs, species-specific differences in affinity and efficiency of renal uptake and efflux transporters exist (Grass and Sinko, 2002), suggesting that when taken into account some improvement over allometry should result. That said, for some drugs either filtration (especially for large polar compounds) or extensive reabsorption (especially for lipophilic compounds) predominates, processes virtually non-existent in the liver, thereby dampening the impact of any species differences in secretion on renal clearance. For such drugs, *in silico* prediction should suffice. With only unbound drug filtered at the glomerulus, owing to size exclusion of proteins, and with filtration driven by arterial pressure, glomerular filtration is readily predicted as the product of glomerular filtration rates and fraction of drug in plasma unbound. Attempts have been made, with some success, to predict tubular reabsorption based on the physicochemical properties of the molecule (log P, pK_a), taking into account urine flow and pH when, as commonly occurs, reabsorption is passively driven (Norris *et al*., 2000; Agoram, Woltosz and Bolger, 2001). Moreover, when passive tubular reabsorption predominates, renal clearance tends to be low and generally a small component of total clearance, so that even an appreciable error in prediction of the extent of reabsorption is of minor concern. When secretion is a substantial component of renal clearance, allometric scaling tends to underpredict human values (Parrott and Lave, 2002). A model of renal clearance for compounds that undergo extensive active reabsorption, as commonly occurs for many vitamins and essential nutrients, has been developed (Rostami-Hodjegan, 2004).

Biliary Clearance

Common approaches to optimize the key ADME properties of new chemical entities (NCEs) during drug discovery are mainly based on solubility, lipophilicity, permeability, and metabolic stability. The optimization of compound properties for improved metabolic stability often results in more hydrophilic and less membrane-permeable compounds, thereby contributing to a shift in the elimination routes from metabolism toward non-metabolic processes. Thus, besides hepatic metabolism, it is increasingly recognized that hepatic uptake and efflux transport processes have a significant impact on hepatic clearance of many NCEs. For such compounds, hepatic distribution may become sufficiently slow to become permeability-rate-limited and assumptions of perfusion-rate-limited distribution and well-mixed compartments are no longer valid. These permeability limitations, often associated with active transport processes, need to be adequately accounted for in order to better predict clearance from *in vitro* data. This is best performed with physiologically based models by the addition of permeability and by the incorporation of more complex liver models, which include active uptake into the liver, active efflux into the bile, biliary elimination, and enterohepatic recirculation, in addition to metabolism. However, this improvement to current modeling methodologies requires the availability of all the appropriate quantitative input data as well as validation of the corresponding *in vitro* models and the proper IVIV scaling approaches.

Various approaches have been proposed to scale hepatobiliary clearance based on *in vitro* data. A holistic approach, where all processes (uptake, metabolism, and secretion into bile) are integrated, uses hepatocytes in sandwich culture in a single *in vitro* tool to estimate the overall processes driving hepato-biliary clearance (Carlile, Zomorodi and Houston, 1997; Lave *et al*., 1997; Naritomi *et al*., 2001). Whereas the rank order of global biliary clearances in rat was consistent from *in vitro* to *in vivo*, most of the *in vivo* values were underpredicted by more than twofold. A very limited number of drugs have been evaluated for biliary clearance using sandwich-cultured human hepatocytes. Only one was predicted within twofold whereas the two others were underpredicted by 2.2- and 4.6-fold (Carlile, Zomorodi and Houston, 1997).

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Another approach, which is entirely mechanistic, involves the determination of intrinsic clearances for each process independently from each other and the integration of these processes in a mechanistic mathematical model to derive the hepatobiliary clearance. The current limitation of this approach is the ability to measure each process *in vitro*, especially efflux transport, as well as the availability of validated scaling factors for individual transporters.

2.2.4 Models to Predict Oral Absorption

Although, in some cases, a simple, first-order model can adequately describe oral absorption, more physiologically representative approaches have been developed and shown to be useful for predicting the fraction absorbed, for example, a compartmental approach such as the Advanced Compartmental Absorption and Transit (ACAT) model (Agoram, Woltosz and Bolger, 2001) and a model describing the GI tract as a tube (Willmann *et al*., 2003). Simulation of oral absorption using physiologically based models represents another challenge because of the many processes involved such as the release, dissolution, degradation, metabolism, uptake, and absorption of a compound as it transits through the different segments of the digestive tract (Semino, Lilly and Andersen, 1997). Oral absorption simulations require *in vitro* and *in silico* input data such as solubility, permeability, particle size, partition coefficient (log *P*), ionization constant (pK_a), and dose.

Physiologically based models to predict oral absorption in animals and humans have recently been reviewed (Agoram, Woltosz and Bolger, 2001; Grass and Sinko, 2002), and several models are now developed to a degree that they are commercially available. The commercial models have not been published in detail because of proprietary reasons, but in essence, they are transit models segmenting the gastrointestinal tract into different compartments with the kinetics of transit, dissolution, and uptake described by differential equations. For example, the model underlying GastroPlus™ is known as the ACAT(Figure 3) and is a semi-physiologically based transit model, consisting of nine compartments corresponding to different segments of the digestive tract. Oral absorption simulations take *in vitro* and *in silico* input data such as solubility, permeability, particle size, log *P*, pK_a (ionization constant), and dose.

2.2.5 PBPK Modeling Strategy for Prediction to Man

A PBPK modeling strategy has been proposed recently and is illustrated on Figure 4.

The proposed modeling strategy attempts to evaluate the data generated in drug discovery and early development in a more integrated way, by combining *in silico* and *in vitro* prediction methods for absorption, distribution, and hepatic clearance to estimate the oral PK profile in humans.

The strategy consists essentially of two major steps. The first step is to predict specific ADME parameters, and then combine their input in a PBPK model to estimate, in a second step, the overall PK after oral and intravenous administration. Before a prediction in humans is attempted, a comparison of the predicted PK parameters and the concentration/time profiles with *in vivo* data from animals (rat, dog or monkey) is performed as a model validation. The rat is usually used because it is by far the most commonly used animal species for the ADME characterization of compound candidates and because tissue composition models are available in rats and humans. A satisfactory prediction for the rat suggests that the prediction in humans might be successful, although this cannot be considered as a full validation.

Thus, the PK in the rat is used to confirm that the PBPK model selected can be used for prediction of human PK. In a first step, disposition PK in the rat after intravenous administration is predicted with a PBPK model by using estimates of tissue:plasma partition coefficients, and hence the derived V_{ss} as well as hepatic clearance as the assumed major

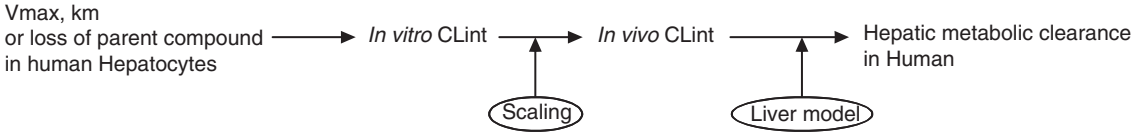

Figure 2. *In vitro* and *in vivo* scaling strategy for hepatic metabolic clearance. The arrows link the different steps of the scaling procedure.

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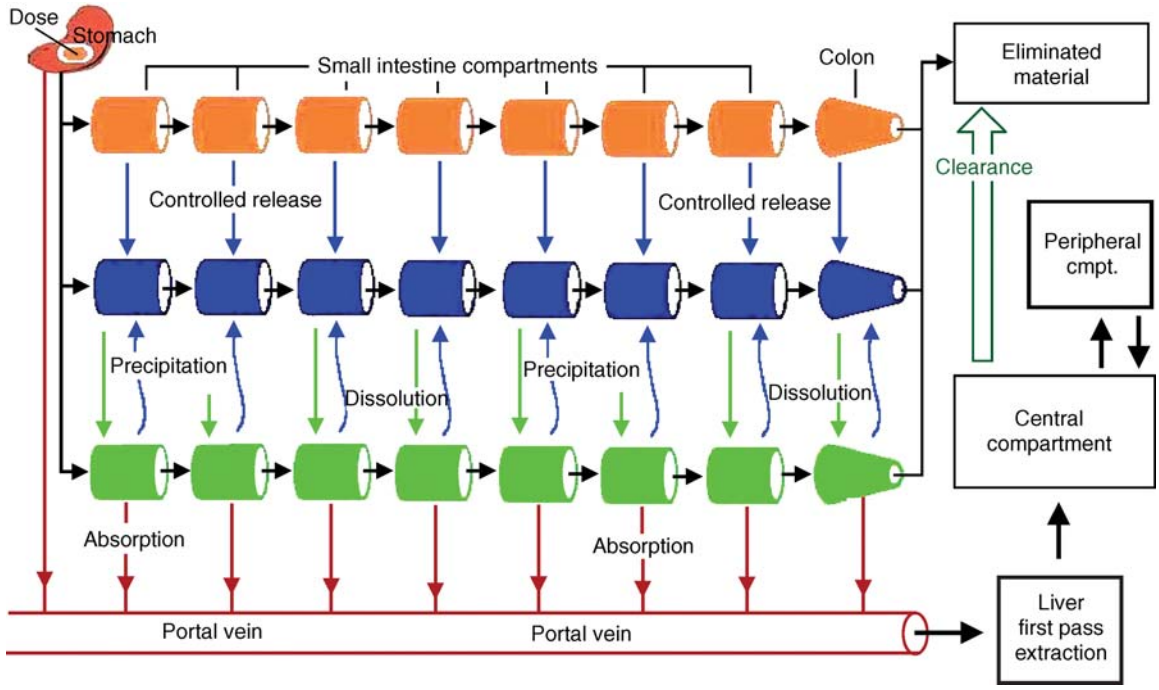

Figure 3. ACAT model. The arrows represent the movement of the compound through the various compartments of the model. Reproduced from Agoram, Woltosz and Bolger (2001) © Elsevier.

component of clearance. If the predicted plasma concentration/time profile in rat describes the experimental data adequately, the intravenous PK in humans is simulated in a second step. In case of major deviations between predicted and experimental concentration/time profile in the rat, model refinements are necessary prior to prediction of human PK.

During the drug discovery and development process, more and more data become available and the data obtained are characterized by higher quality. The constantly increasing data quantity and quality potentially improve the model predictions as shown in Figure 5.

2.2.6 Commercial Tools Available

A number of user-friendly software packages bringing PBPK modeling to the non-expert are available and have been reviewed recently (Clewell, 2008).

The possible benefits from having PBPK software packages available for scientists who are non-modelers have been a topic of interest for many years. Such a tool would need to meet many criteria to be broadly useful. This tool would need to be user-friendly, fast, flexible, and requiring little mathematical expertise from the user. The import of data into the tool and the export of results from the tool would need to be fast and automatable. The required input would be clearly defined and difficult to enter incorrectly, making consistent use by scientists of varying background possible. The tool would ideally be applicable through all stages of preclinical through clinical development. The pharmaceutical industry now has many tools that meet some or all of these criteria. Tools using physiology-based whole-body models for the simulation of the PK behavior, including absorption, distribution, and elimination, include GastroPlusTM (Simulations Plus Inc., www.simulations-plus.com), Simcyp (Simcyp, www.simcyp.com/), PK-Sim® (Bayer Technology Services, www.pksim.com), MEDICI-PK (AT Computing in Technology GnbH, www.cit-wulkow.de/pdf/Broschueres/MediciPK/Medici-web2.pdf), and Cloe PK® (Cyprotex, www.cyprotex.com).

GastroPlusTM, which is well known in the pharmaceutical industry for its capabilities in predicting oral absorption in preclinical species based on the

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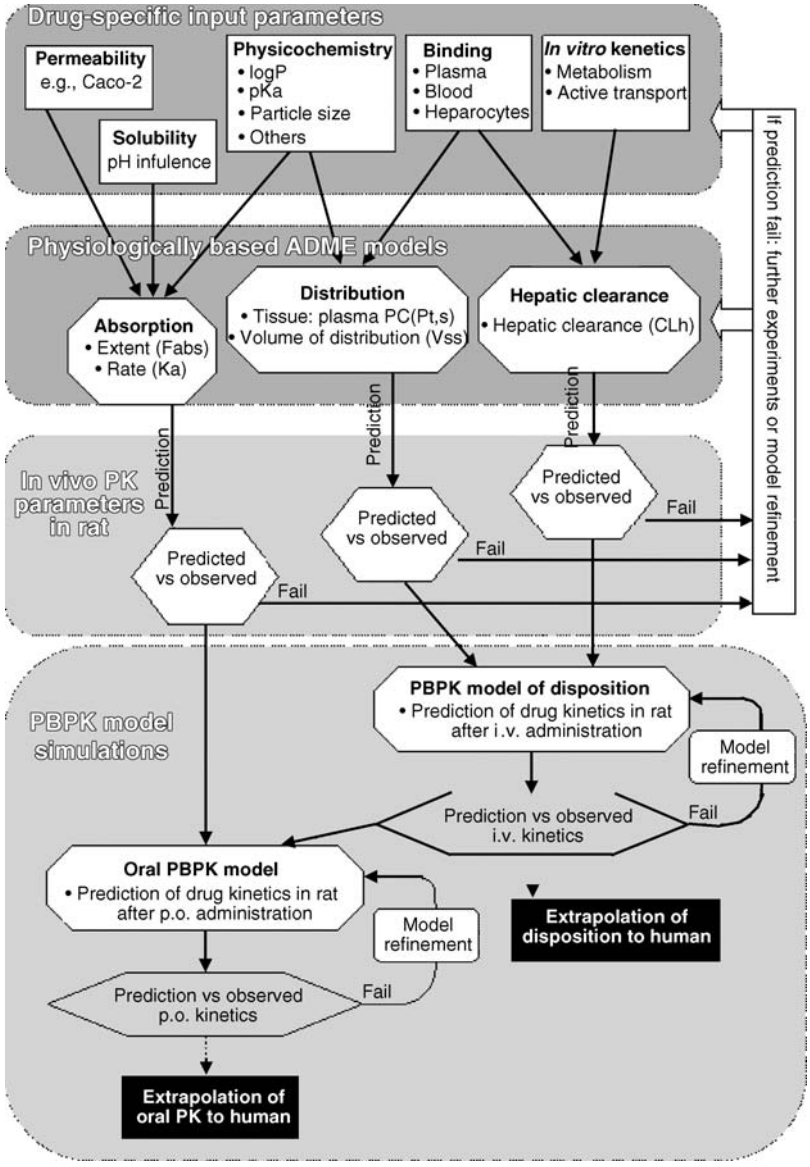

Figure 4. Strategy for PBPK predictions. Reproduced with permission from Theil *et al*. (2003) © Elsevier.

ACAT model, has recently expanded to include the capability of PBPK modeling. In the most recent version, GastroPlusTM includes a physiological intestinal model for humans in fed and fasted state as well as models for the rat, dog, cynomolgus monkey, and mouse. Several additional modules are available for parameter optimization, for combining the absorption model with physiological and compartmental PK models, and for PD models. The software also includes clinical trial simulation capabilities and a powerful method of performing sensitivity analysis. Although currently only PBPK modules for the rat and humans are available, the user can create PBPK models for other species by creating input files with appropriate physiological parameters. The performance of GastroPlusTM to predict absorption was evaluated (Parrott and Lave, 2002). In this study, GastroPlus was compared with iDEATM, another software to predict absorption that is no longer commercially available.

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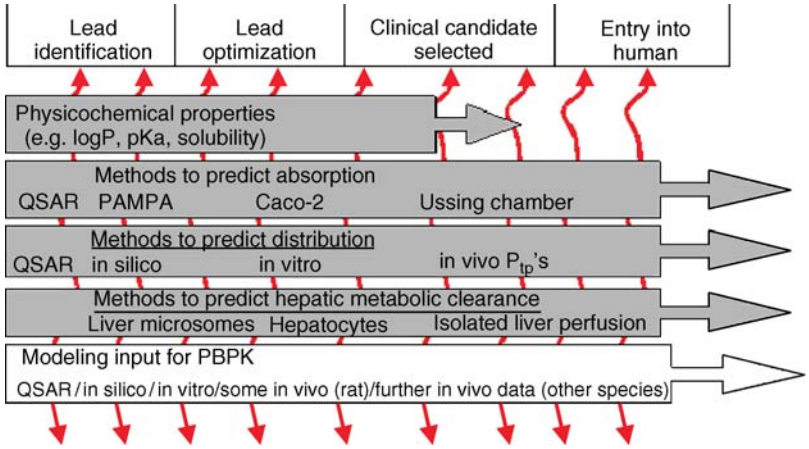

Figure 5. Modeling approach within drug discovery and early nonclinical development. Reproduced with permission from Theil *et al*. (2003) © Elsevier.

Another tool for predicting human PK, Simcyp (Simcyp Ltd, Sheffield, UK), was previously known as a drug–drug interaction clinical trial simulator but has expanded to include PBPK modeling capabilities. Simcyp utilizes fundamental scaling procedures (Houston, 1994), for the prediction of *in vivo* hepatic clearance (CLH) from *in vitro* metabolism data. These *in vitro* metabolism data can be obtained from individual cytochrome P450s (CYP) in human-expressed recombinant systems. These are used for predicting the behavior not only in average individuals, but also in whole populations or in special subpopulations. In order to predict drug–drug interactions involving CYP, Simcyp utilizes the relationship between the inhibitor concentration at the active site *in vivo* and the inhibition constant (K_i) determined *in vitro*. Competitive inhibition, induction, and/or mechanism-based inactivation mechanisms can be investigated within this software, according to the principles described elsewhere (Ito *et al*., 1998; Tucker, Houston and Huang, 2001). Simcyp not only predicts the mean value but also simulates the extremes in the population by applying a Monte Carlo approach. This software comes with a database of physiological properties for specific populations and of PK information for specific drugs on the market (e.g., the CYP3A4 substrate midazolam, the CYP3A4 inducer rifampicin, and the CYP3A4 inducer and inhibitor ritonavir).

Yet another tool, PK-Sim, developed as a user-friendly tool for PBPK modeling, has PBPK models in the mouse, rat, dog, monkey, and humans. In the model, tissue concentrations depend on the blood flow rate to the tissue, the rate of permeation into the tissue, active transport processes into and out of the tissue, and the rate of metabolism within a tissue. Although this detailed description allows significant flexibility and complexity, the user-friendly interface also allows for simple simulations whereby the more complex features are not utilized. In PK-Sim, the GI tract is described as a single tube with physiologically relevant dimensions and spatially varying properties such as pH and surface area. The software has Monte Carlo capabilities, and simulations examining the impact of inter-individual variability can be performed using the population PK module. Physiological parameters for people from 0 to 80 years old are provided, allowing simulations of PK in the young and the elderly. Also, the software has the capability of linking PK to PD behavior with a PK/PD module.

In a fresh approach to PBPK modeling, the new software MEDICI-PK was developed using a novel software concept aimed at providing increased flexibility while maintaining user-friendliness. Instead of adopting the traditional approach whereby user-friendliness is made possible by adopting a static model, in MEDICI-PK the system of differential equations is generated automatically when starting a simulation. This software allows for predictions of parent compound and multiple metabolites, and interactions (metabolic and otherwise) between compounds (Huisinga, Telgmann and Wulkow,

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2006). MEDICI-PK has PBPK modeling capabilities for multiple species (e.g., humans, mouse, and dog) that can be extended by the user, and also contains PD modeling capabilities.

Cloe-PK® is a commercial tool aimed at prediction of human PK at the earliest stages of drug discovery. The PBPK model includes 14 organs and tissues, clearance by metabolism and renal excretion, and diffusion-limited uptake in tissues. In each of the organ compartments, the capillary, intracellular, and extracellular spaces are explicitly included. Using *in vitro* and *in silico* data as input, this software can predict human IV and oral AUC from clinical trial data within two- and 3.75-fold, respectively (www.cyprotex.com/products/pk_howwell.htm). Studies on the accuracy of the model for predicting PK behavior in the rat and humans have been published (Brightman *et al*., 2006), and illustrate the utility of this software for predicting PK and ranking compounds based on minimal data and *in silico* parameters.

These software packages are exceptionally useful tools, and we expect them to be increasingly used in the pharmaceutical industry. However, their implementation may require an M&S expert to determine appropriate applications and methods of use and to perform validation exercises to convince the scientists involved in the preclinical development of drugs that the packages do indeed work. Additionally, in-house PBPK expertise and models will still be necessary because these standard tools do not allow the flexibility to simulate every situation that will arise. For example, currently some generic PBPK tools do not yet have the capability to simulate exposures to a parent compound and a metabolite, which could be of interest for simulating the PK of prodrugs or investigating possible PK interactions between the parent and metabolite. Also, generic PBPK software packages have sophisticated capabilities that may require an expert modeler to determine appropriate ways to use (and not misuse) the features (e.g., GastroPlus has the capability of simulating enterohepatic cycling, and PK-Sim can incorporate saturable and non-saturable metabolism in multiple tissue compartments). In addition, the availability of such tools simplifies the technical use of PBPK models; however, a good understanding of the models and underlying equations is still mandatory in order to guarantee good interpretation of output. The different technologies for PBPK modeling discussed have different strengths, making them useful for different purposes within the research and development process in the pharmaceutical industry (see Table 2).

2.3 Applications and Limitations of the Current Methodology

2.3.1 PBPK for Extrapolation of Pharmacokinetics to Man

A number of publications illustrate the potential of PBPK, both for predicting human pharmacokinetics and for examining species differences in pharmacokinetics.

Table 2. Commercial tools available.

Process simulated	Absorption	Metabolism an drug–drug interaction	Whole-body simulations
Software/Company website	GastroPlus (SimulationsPlus, http://www.simulationsplus.com/) IDEA[a] (Lion Bioscience, http://www.lionbioscience.com/)	SimCyp (SimCyp, http://www.simcyp.com/)	Cloe PK (Cyprotex, http://www.cyprotex.com/) pkEXPRESS[1]: (Lion Bioscience, http://www.lionbioscience.com/) PK-Sim (Bayer Technology Services, http://www.pk-sim.com) SimCyp (SimCyp, http://www.simcyp.com/)
Description	Semi-physiological absorption models Input data: structural descriptors, permeability, solubility, *in vitro* ADME data	Predictions in whole populations or in special subpopulations Input data: *in vitro* metabolism data on recombinant CYP	Physiological models including permeability limitations for PK-Sim. Input data: physicochemical and *in vitro* ADME properties. The coming versions of Gastroplus and Simcyp will include PBPK into their new release.

[a] No longer commercially available.

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Successful predictions were also reported in the case of epiroprim (Luttringer *et al*., 2003). This was achieved by combining PBPK and allometric scaling. Epiroprim, an antimicrobial agent from the diaminopyrimidine family, was considered a challenging compound for the current extrapolation methods because of large interspecies differences in its pharmacokinetic properties mainly related to mixed elimination pathways, namely hepatic metabolism and biliary excretion of unchanged compound. For the prediction of distribution in man, the tissue:plasma partition coefficients were predicted using the human tissue composition model based on *in silico* input parameters. The human clearance was predicted by using allometric scaling combining animal intrinsic *in vivo* blood clearance and *in vitro* hepatocyte data in animals and humans. Under these circumstances, PBPK led to reasonable predictions of the *in vivo* disposition profile of epiroprim in rat and humans.

Lumped physiologically based models (in which the tissues are subdivided into groups, according to their kinetic properties) have been used to predict human plasma concentration–time curves for the melatonin agonist S20098 (Bogaards *et al*., 2000). By combining *in vitro* metabolism data obtained from human cytochrome P450 enzymes with partition coefficients in fat and liver, the range of plasma concentrations observed in humans could be successfully predicted. In another study (Lave *et al*., 2002), a lumped physiologically based pharmacokinetic model originally developed by Arundel for the rat (Ballard *et al*., 2003) was extended and applied to humans for 10 extensively metabolized compounds. Overall, the results showed that this model might be used to predict concentration versus time profiles in humans. Irrespective of the compounds' pharmacokinetic and physicochemical characteristics, the average error for the predicted pharmacokinetic parameters [CL (total clearance), V_{dss} (volume of distribution at steady state), and $T_{1/2}$ (half-life)] of the 10 compounds was less than twofold (Lave *et al*., 2002).

Recently, empirical and PBPK approaches were compared for the prediction of human pharmacokinetics using 19 diverse compounds taken from recent clinical development projects at Roche (Parrott *et al*., 2005). The original set of compounds included in this analysis represented all the compounds developed at Roche that went into clinical development between 1998 and 2002. From this set of biological compounds, prodrugs and compounds that were not absorbed were excluded. The remaining compounds ($n = 19$) were used in the analysis. The compounds selected covered a wide range of physicochemical and PK properties. All compounds were lipophilic with $\log P$ values ranging between 1.2 and 6.6, with a mean of 4.0. Five compounds were acids with a $pK_a < 8$, seven were bases with a $pK_a > 6$ and seven were neutral or weakly ionized at physiological pH. Half-life in man ranged from short (0.50 h) to long (80 h). Elimination pathways included hepatic metabolism, renal excretion, biliary excretion, or a combination of these. Fraction unbound in plasma (fup) values ranged from extensive (0.00060) to low (0.89). Distribution consisted of both passive and active processes and varied from limited to widespread. In the rat, absorption ranged from 15 to 100%. Predicted values (pharmacokinetic parameters and plasma concentrations) were compared to observed values in order to assess the accuracy of the prediction methods.

Based on the strategy proposed, a prediction would have been made prospectively for approximately 70% of the compounds. The accuracy for these compounds in terms of the percentage of compounds with an average-fold error of less than twofold was 83, 58, 83, 67, 92, and 100% for clearance, volume, half-life, C_{max} [maximal (peak) plasma concentration], AUC (area under the plasma concentration–time curve), and T_{max}, respectively. For the other 30%, unacceptable prediction accuracy was obtained in animals; therefore, a prospective prediction would not have been made using PBPK. The accuracy for these compounds in terms of the percentage of compounds with an average-fold error of less than twofold was 40, 40, 60, 0, 40, and 80% for clearance, volume, half-life, C_{max}, AUC, and T_{max}, respectively. In general, for compounds that were cleared by hepatic metabolism or renal excretion, and whose absorption and distribution were governed by passive processes, the prediction accuracy was very good (average fold error less than twofold) using PBPK. Significant mispredictions were achieved when other elimination processes (e.g., biliary elimination) or active processes were involved. PBPK prediction accuracy was significantly improved when any non-linearities in clearance were incorporated; when both microsomal and plasma binding were included in the scaling of microsomal data; when intestinal metabolism was incorporated for CYP3A4 substrates; and when

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solubility was measured in physiological media for low-solubility compounds. The poorer predictions or the compounds for which a prediction was not attempted with PBPK were often as a result of processes that were not incorporated into the model due to the lack of *in vitro* input parameters to describe processes such as biliary excretion or enterohepatic recirculation.

The prospective use of PBPK approaches was also described recently (Parrott *et al*., Molecular Pharmaceutics). In recent years, the strategy for prospective predictions of human pharmacokinetics has been applied prospectively and clinical data have been obtained for 10 compounds. Figure 6 shows the correlations of predicted AUC and C_{max} for these compounds in single ascending dose studies. Eighty percent of AUC predictions and 60% of C_{max} predictions are within twofold of the observed mean values.

All in all, there is growing evidence that physiologically based models show improved prediction accuracy for extrapolation of human pharmacokinetics over more empirical methods. The PBPK approach is based upon solid physiological principles and so can often be extended in a rational way to include additional relevant information and data. PBPK model also offers a mechanistic framework that contributes to an improved understanding of the compound's properties, which ultimately should result in better prediction accuracy. The variability and uncertainty associated with the predictions also require some special attention. As is apparent from the literature and available clinical data for the compounds studied, there is a large degree of variability in the population in terms of both physiology (tissue volumes, blood flows, transit times, etc.) and biochemistry (plasma binding, CYP expression, activity, etc.). In order to produce predictions that are more realistic of the target population this variability together with any uncertainty (variations due to assumptions, hypotheses, handling of system, etc.) ideally must be accounted for. The incorporation of these factors can be translated into a measure of confidence in the prediction.

2.3.2 *Early Pharmacokinetic and Pharmacodynamic (PKPD) Predictions in Man Based on PBPK and Preclinical Data*

In a recent study, a mechanistic PBPK and pharmacokinetic/pharmacodynamic approach was reported for the prediction of the human response using the example of a immunomodulator (Meno-Tetang and Lowe, 2005). The example was based on the immunomodulator FTY720 (the first sphingosine-1-phosphate receptor agonist) acting by stimulating the sequestration of lymphocytes into lymph nodes in order to remove cells from blood circulation. A physiology-based pharmacokinetic model

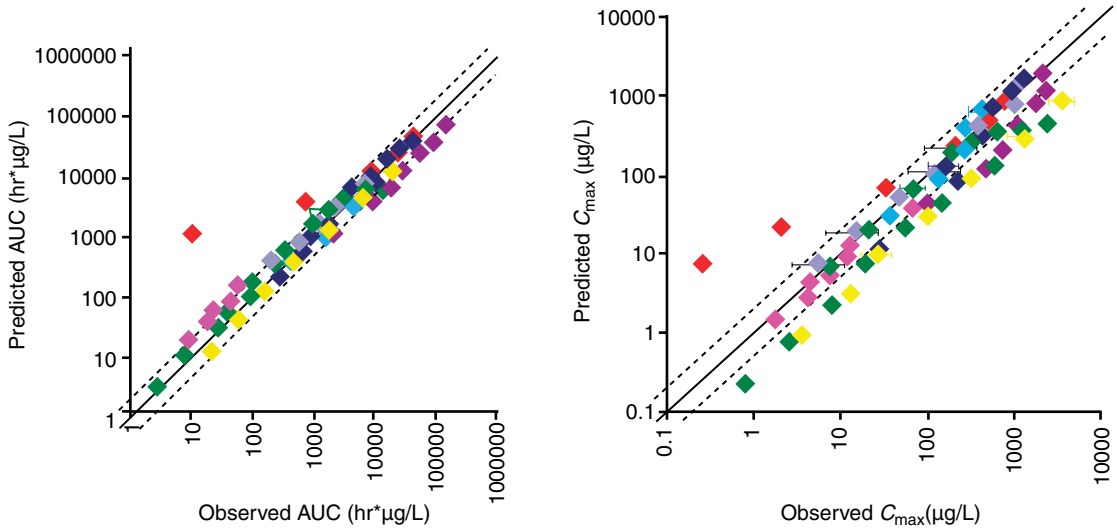

Figure 6. Predicted and observed AUC and C_{max} in humans for 10 diverse compounds. Each compound is represented by a single color and appears multiple times to represent different dose levels.

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incorporating permeability limitations in thymus, brain, and lymph nodes was connected to an indirect response model of the lymphocyte system to characterize the cell trafficking effects. Permeability was obtained after fitting *in vivo* tissue data in rats. The IC50 of FTY720 was estimated from monkeys; man was assumed to be similar to monkeys. To make predictions of the pharmacodynamic behavior for man, systemic exposure was obtained from a physiology-based model, and an estimate of lymphocyte turnover in man was obtained from preclinical species using allometric scaling. Predictions compared well with clinical results.

2.3.3 PBPK Applied for Clinical Candidate Selection

In contrast to empirical techniques, an approach based upon a mechanistic framework can lead to greater insights into the behavior of compounds and offers more potential in the early stages of drug development because it does not require animal *in vivo* data in several species as is needed for allometric scaling. Therefore, PBPK has a great potential to assist clinical candidate selection where numerous factors need to be considered and data related to the pharmacokinetics and pharmacodynamics of the compounds need to be combined and compared in a rational way.

An example showing the utility of such an approach was provided recently (Parrott *et al*., 2005). In this example, *in vitro* and *in vivo* data for five potential clinical candidates were combined in PK/PD models to estimate the effective human doses and associated exposures and aid in the selection of the most promising compound. To ensure that the decision was based upon significant differences between the compounds, estimates of variability and/or uncertainty were carried out in the modeling of the PK and PD. The five compounds were from the same structural class and showed similar physicochemical properties. Molecular weights were in the range 406–472 and all compounds were largely unionized at physiological pH. Log *P* values ranged from 2.1 to 2.9 and permeability was good for all compounds whereas solubility ranged from 2.2 to 0.009 mg ml^{-1}. *In vitro* receptor binding and plasma protein binding data in rats and humans and *in vivo* pharmacokinetic and pharmacodynamic data in rats were also available for all compounds.

In the first step, the scaling of intrinsic clearances determined in rat hepatocytes was compared to *in vivo* clearance. When taking account of non-linearity the estimated hepatic metabolic clearance values were in reasonable agreement with observed total clearances, which ranged from 7 to 35 ml min^{-1} kg^{-1}, and it was considered reasonable to estimate the expected clearances in humans by similar scaling of human hepatocyte data. The error around the mean predicted human clearance was based on the variability seen in different batches of human hepatocytes.

Tissue-composition-based prediction of volume on the basis of lipophilicity and plasma protein binding showed an average fold-error of 2.2 and the correlation of predicted versus observed volume for the five compounds was poor. For the prediction of volume of distribution, it was assumed that the volume in 1 kg^{-1} in humans was the same as the observed volume in the rat (ranging from 0.9 to 2.8 l kg^{-1} for the five compounds). Due to the uncertainty in the prediction of volume the error range associated with this parameter was set as a uniform distribution over a twofold range.

The effect versus plasma concentration relationship in the rat pharmacodynamic data was shown to be a direct one and was well described by a simple E_{max} model. Based on preclinical models for efficacy, a 90% effect was considered as the target for therapeutic effect. The effect versus plasma concentration relationship in the rat pharmacodynamic data was shown to be a direct one and was well described by a simple E_{max} model. A 90% effect was considered as the target for therapeutic effect. Finally, the human C90 (human concentration corresponding to 90% effect) was estimated by accounting for the different affinities of each compound for the rat and human receptors and also for the different free fraction unbound in rat and humans.

$$\text{Thus, C90\_man} = \text{C90\_rat}(\text{Ki\_man}/K\text{i\_rat}) \times (\text{fu\_rat}/fu\text{\_man}) \quad (5)$$

Finally, absorption in the rat was shown to be well predicted by GastroPlus™ because good agreement was seen between simulated and observed oral profiles, and so the predicted human pharmacokinetics and pharmacodynamics were combined in a GastroPlus™ model of humans to allow for estimation of the effective steady-state doses and

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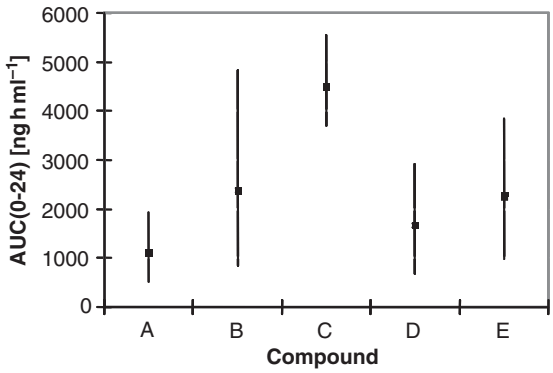

Figure 7. Simulated AUC at steady state for five potential clinical candidate compounds. Symbols give the mean prediction whereas lines indicate a 95% confidence range. Reproduced with permission from Parrott *et al*. (2005) © John Wiley & Sons, Inc.

exposures after repeated oral dosing. Incorporation of variability and uncertainty was achieved using some stochastic simulations. The predicted effective doses and associated exposures including error ranges on the predictions were then provided for the various potential clinical candidates (see Figure 7).

This example showed that the PBPK approach assists a sound decision on the selection of the optimal molecule to be progressed by integrating the available information and focusing the attention onto the expected properties in humans. Importantly, the method is also able to include estimates of variability and uncertainty in the predictions.

2.3.4 PBPK to Prioritize Compounds Prior to In Vivo Experiments

Drug discovery is increasingly "data rich" with high-throughput chemistry generating numerous compounds that are rapidly screened for pharmacological and pharmacokinetic properties. Determination of *in vivo* PK is considerably more costly than *in vitro* screening, and so there is interest in optimizing resources by using simulation to prioritize compounds. However, acceptance of this approach requires extensive validation. The practical value of generic physiologically based models of pharmacokinetics at the early stage of drug discovery was evaluated (Germani *et al*., 2005; Parrott *et al*., 2005). In the study reported below, the validation was carried out with rat data but the ultimate aim is to run these predictions in man.

In the study by Parrott and coworkers, a generic PBPK model was applied to predict plasma profiles after intravenous and oral dosing to the rat for a set of 68 compounds from six different chemical classes. The compounds were selected without particular bias and so are considered representative of current discovery compounds. The physicochemical properties of the compounds are rather different from those of marketed compounds; in particular, they have high lipophilicity (mean log *P* of 4) and low aqueous solubility as well as a tendency to neutrality at physiological pH. The more extreme property values can present measurement difficulties, and so for consistency, all predictions were made on the basis of calculated lipophilicity and protein binding whereas *in vitro* measurements of intrinsic clearance in hepatocytes, ionization, solubility, and permeability were used for all compounds.

In the first stage, distribution was predicted with tissue-composition-based equations and the estimated tissue partition coefficients were combined with clearance estimated by direct scaling of hepatocyte intrinsic clearance in a PBPK model as described earlier.

In the second stage, GastroPlus™ was used to simulate oral absorption and oral profiles were produced by feeding this predicted input into a compartmental disposition model fitted to the mean observed IV data.

For IV dosing simulations, 60% of the clearance and volume predictions were within twofold of the observed values and the ranking of compounds by predicted versus observed parameters showed correlation coefficients of 0.8 and 0.6 for clearance and volume, respectively. For oral dosing simulations, 40% of the predicted AUC values were within twofold of observed values and the mean fold-error was 4.1. For compounds with measured solubility less than 0.012 mg ml^{-1} only 10% of predictions were within twofold whereas for compounds with higher solubility 70% were within twofold (Parrott *et al*., 2005) (see Figure 8).

Overall, this study indicated that generic simulation of pharmacokinetics at the lead optimization stage could be useful to predict differences in pharmacokinetic parameters of threefold or more based upon minimal measured input data. Fine discrimination of pharmacokinetics (less than twofold) should not be expected due to the uncertainty in the input data at the early stages. It is also apparent that verification of simulations with *in vivo* data for a few compounds of each new compound class was required to allow an assessment of the error in

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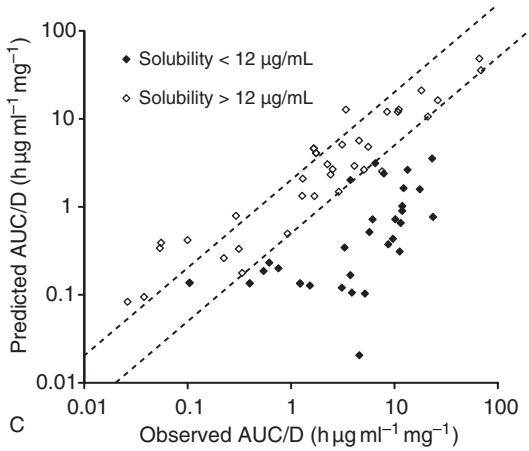

Figure 8. Predicted versus observed AUC/dose for 68 Roche compounds. Reproduced with permission from Parrott *et al.* (2005) © John Wiley & Sons, Inc.

prediction and to identify invalid model assumptions.

The value of PBPK for simulating the First-Time-In-Animal Study was also illustrated previously (Germani *et al.*, 2005). In this study, the model was used to predict the plasma pharmacokinetics obtained in 24 rat and two mouse pharmacokinetic studies. This study was restricted to the prediction of disposition profiles (i.e., profiles observed after intravenous administration of the compounds). The administered compounds were synthesized as part of a number of different discovery programs. The simulated profiles were generally in agreement with the observed ones. Plasma clearance and mean residence times were well predicted: average fold errors were 2.7 and 2.5, respectively, with approximately one half of the cases predicted within a twofold of observed. Some overprediction of plasma clearance was observed in the range of low clearance values, in case of compounds with $\log P = 5$ and protein binding of 99. On the other hand, slight underestimations were observed for molecules with $\log P$ near zero and protein binding of 99.9%. The error in the volume of distribution at steady state (V_{ss}) was higher, but still acceptable (average fold error was 3.7).

2.4 Discussion

For extrapolation of human pharmacokinetics, physiologically based models show improved prediction accuracy over more empirical methods. The PBPK approach is based upon solid physiological principles and can be extended to include additional relevant processes. Also, besides satisfactory prediction capabilities, an approach based upon a mechanistic framework can lead to greater insights into the behavior of compounds and offers more potential in the early stages of drug development because it does not require animal *in vivo* data in several species as is needed for allometric scaling.

When applied at the clinical candidate selection phase the PBPK approach assists a sound decision on the optimal molecule to be progressed by integrating the available information and focusing the attention onto the expected properties in humans. Early prediction is essential to assess the safety of a novel drug target or of novel compounds. Importantly, the method is also able to include estimates of variability and uncertainty in the predictions, which should be considered as an important piece of information for decision making.

The results on the use of generic simulation prior to *in vivo* studies indicate that some caution is required because certain chemical classes were poorly predicted, and it is recommended that generic PBPK models should only be applied for prioritization after verification of the simulations with *in vivo* pharmacokinetics for a few compounds of a given chemical class. Such verification will help to identify invalid model assumptions or missing processes where additional data are needed. In addition, for poorly soluble compounds, the use of aqueous solubility is shown to be inadequate for reliable prediction of oral absorption in physiologically based models.

Important limitations of the generic PBPK approach are realized for compounds that have significant active distribution/absorption processes, where biliary elimination is a major component of the elimination process or where the assumptions of flow-limited distribution and well-mixed compartments are not valid and permeability-limited distribution is apparent. These drawbacks could be addressed by the addition of permeability barriers for some tissues and by the incorporation of a more complex liver model that addresses active uptake into the liver, active efflux into the bile, biliary elimination, and enterohepatic recirculation. However, this will require the availability of appropriate input data for quantification of the various processes involved as well as validation of the corresponding

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in vitro to *in vivo* scaling approaches. Finally, the physiologically based methods illustrated in the chapter are used for prediction of small molecules, whereas the prediction of biologics and antibodies is still based on empirical allometric scaling.

Overall, these three examples illustrate how PBPK models can already add value at various stages of the preclinical compound research and development process, although one has to keep in mind a number of limitations. Their use is growing and the potential will be fully exploited as powerful and user-friendly software continues to make these models accessible to non-specialists.

3 MULTISCALE MECHANISTIC MODELS OF CARDIAC ELECTROPHYSIOLOGY FOR ASSESSING QT PROLONGATION AND PROARRHYTHMIC RISK IN DRUG DEVELOPMENT

3.1 Introduction

Over the past decade, drug-induced prolongation of the QT interval in the clinical electrocardiogram has become a major regulatory and commercial concern in drug development as the costs associated with late-stage failures, market withdrawals, and patient liabilities have spiralled upwards. The danger of proarrhythmia accompanying QT prolongation (Figure 1a), in particular the appearance of a ventricular arrhythmia known as torsades de pointes (TdP, Figure 1b), impacts all therapeutic drug classes (Haverkamp *et al*., 2000). In fact, QT concerns are the frequent cause of clinical holds, NDA withdrawals, delays in approval, and restricted labeling (Malik and Camm, 2001). Although QT prolongation was a well known and, for many years, a desired property of antiarrhythmic therapeutics, it was not well appreciated as a dangerous side effect until associated with terfenadine, a non-sedating antihistamine in widespread use. The molecular target mediating terfenadine's QT prolonging effect was identified as the delayed rectifier potassium current I_{Kr} encoded by hERG (human ether-a-go-go related gene) (Roy, Dumaine and Brown, 1996) whose inhibition delays cellular repolarization and induces ectopic early afterdepolarizations. Further cementing this connection between I_{Kr} or hERG blockers and QT prolongation was the finding that fexofenadine (the active metabolite of terfenadine) was devoid of hERG inhibitory activity and also lacked QT prolonging effect clinically (Scherer *et al*., 2002). The result was the initiation of an ongoing tidal wave of research, regulatory concern, and scrutiny over the drug development approach to QT prolongation and how to detect it as early and reliably as possible.

Although generally correlative, the relationship between hERG inhibition, QT prolongation, and incidence of TdP is far from perfect, with a considerable number of false positives (hERG inhibition without TdP) and false negatives (TdP with weak or no hERG inhibition) (Redfern *et al*., 2003). Although a variety of preclinical experimental models are available for assessing the potential of a drug candidate to prolong the QT interval, they are not true surrogates for the human heart. None of the preclinical models (*in vitro* or *in vivo*) have been proven to be fully predictive for QT prolongation and, more importantly, for assessing proarrhythmia and TdP risk (Hammond *et al*., 2001). As a reaction, much work has been invested into computational, simulation, and experimental methods for anticipating QT and proarrhythmic toxicity, thereby allowing drug development programs to mitigate or manage the liability (Sanguinetti and Mitcheson, 2005). Perhaps, not surprisingly, most of the experimental models perform best when comparing compounds within a chemical and/or target class (Cavero, Lux and Steinhoff, 2007), but suffer when comparing across classes. To advance the comparison, Polak, Wisniowska and Brandys (2009) undertook a large-scale curation and meta-analysis of hERG inhibition data. Although this surely helps develop computational chemistry methods and guide teams in reducing the liability, these compounds are not necessarily QT prolonging or torsadogenic. Thus, the continued focus on hERG may miss the overall goal of reducing proarrhythmic risk in development. Correlations of QT prolongation with behavior in the rabbit heart clearly provide value; however, they do not elucidate true torsadogenic risk or provide a mechanistic basis for any effect observed (Dumotier *et al*., 2008).

Current safety assessments of drugs only involve repolarization assays of normal tissue rather than proarrhythmic animal models that challenge current perceptions of appropriate surrogates for TdP in man and call into question existing strategies for preclinical risk assessment (Thomsen *et al*., 2006). Regulatory agencies and thus sponsors are hesitant

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to embrace such models until clear understanding and agreement on the key proarrhythmia mechanisms emerge (Lawrence *et al*., 2008). In contrast to *in vitro* and *in vivo* models of cardiac electrophysiology, mathematical system models explicitly describe the underlying biophysical processes that are responsible for the genesis of the cardiac action potential. As such, they can be used for two important purposes: to integrate lower-level data to project higher-level possible observations not yet measured, and to help interpret and reconcile differences in outcomes or observations across multiple systems.

In silico models are created from and validated by reference data from many of the same experimental paradigms they seek to replicate. These *in silico* models can be used to investigate repolarization and QT issues from the scale of a single-ion channel up to the whole heart (for a historical perspective, see Noble, 2001). Importantly, *in silico* model hypotheses can be tested in the setting of various simulated electrophysiological backgrounds and clinical scenarios (e.g., gene mutations in ion channels). Certainly, the mechanistic association between preclinical data and the clinical environment is not entirely clear. However, because the goal of preclinical cardiac safety screening is to identify a signal for QT prolongation and determine the risk associated with that signal, it is important to examine how the novel technique of *in silico* modeling can be used to efficiently accomplish this aim. *In silico* modeling relates quantitative ion channel data to possible clinical outcomes, fills experimental parameter space, integrates across species, highlights study design issues, and explores the impact of risk factors. Indeed, *in silico* modeling in combination with *in vitro* and *in vivo* experimentation appears to be a compelling approach to assessing QT prolongation and proarrhythmia risk at the preclinical stage.

3.2 Review of Cellular and Tissue Models of Cardiac Electrophysiology

Systems models of cardiac electrophysiology can be categorized into those at the cellular level and those at the tissue or organ level. Cellular models describe the electric activity of a single cell using the mathematics of ordinary differential equations that relate the transmembrane potential difference and the function of different ion channels, exchangers, and pumps both on the cell surface and within the cell. These models describe the movement of ions between the cytoplasm and extracellular fluid through ligand or voltage-gated channels (e.g., potassium flux through the I_{Kr} channel), and also between various intracellular compartments (e.g., calcium release from sarcoplasmic reticulum). Importantly, novel voltage-clamp and current-clamp experiments have been used to extract required parameters for describing the mathematics of a particular ion current. In this manner, an integrated *in silico* model of the cardiac cell is assembled from individual "parts" (e.g., ion currents) that are developed with and validated by quantitative experimental data.

Such models, first introduced in the seminal work of Hodgkin and Huxley (1952) to describe the action potential in the giant squid axon, were subsequently adapted to describe the action potential in a cardiac Purkinje fiber (Noble, 1962) and in cardiac ventricular myocardium (Beeler and Reuter, 1977). With further accumulation of experimental data, a growing body of ever-improving cellular models has been created for different types of tissues (ventricular, atrial, sinoatrial node or Purkinje fiber) as well as species (mouse, rabbit, dog, pig, man). In the modern era, the Luo–Rudy model for the mammalian ventricular myocyte (Luo and Rudy, 1991, 1994) and its progeny (Zeng *et al*., 1995; Shaw and Rudy, 1997; Viswanathan, Shaw and Rudy, 1999; Faber and Rudy, 2000) have played a central role in the field. We also recognize the human ventricular cell models developed by ten Tusscher *et al*. (2004) for faithfully implementing heterogeneity for epicardial, mid-myocardial, and endocardial cell types using detailed data from these regions (Figure 9d).

Tissue and whole-heart models describe mathematically the passive diffusion of electrotonic current in tissue composed of (at least partially) excitable cells and, together with the dynamic model of cellular action potential, they belong to the well-studied class of reaction–diffusion systems. These models are broadly categorized as monodomain or bidomain depending on the representation of the intracellular and extracellular spaces. Monodomain models homogenize the two spaces into a single one and only describe the transmembrane potential propagation, whereas bidomain models separately describe the electric potential inside and outside the cells (the transmembrane potential being the difference of these two potentials). Bidomain models are significantly more computationally demanding

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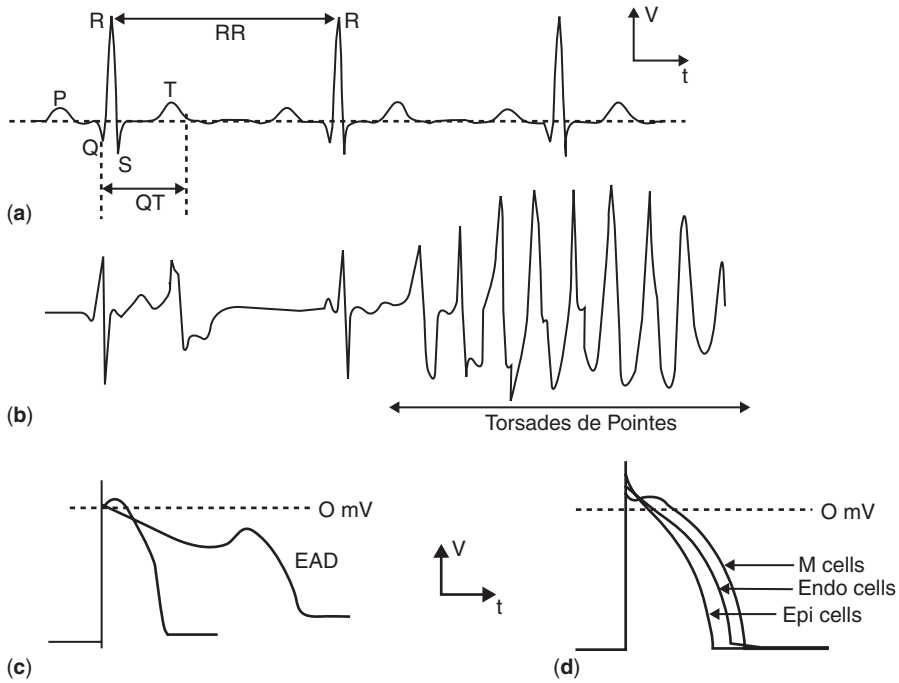

Figure 9. (a) Standard ECG with PQRST complex and QT, RR interval; (b) Torsades de pointes (TdP) event; (c) standard action potential (AP) and action potential presenting an early-after depolarization event (EAD); (d) transmural dispersion of repolarization (TDR) between the endocardial, epicardial, and M ventricular cardiac cells.

versus monodomain ones, but allow for an explicit description of the extracellular potential that electrodes would record and of therapies involving the delivery of an extracellular shock (defibrillation, pacing) (Henriquez, 1993; Potse *et al*., 2006; Trayanova, 2006; Vigmond *et al*., 2009).

Computational speed is highly dependent upon the spatial dimensions of the models, and many techniques exist for increasing the efficiency of more complex calculations (e.g., adaptive spatiotemporal discretization, active region tracking). One-dimensional (1D) models, commonly referred to as "cable models" – because they describe cells oriented in one linear dimension – are computationally efficient models but sacrifice physiological realism, with the exception of models for Purkinje fibers, which are geometrically well-approximated with a 1D structure. Conversely, three-dimensional (3D) models can be geometrically and physiologically very realistic but extraordinarily demanding in terms of computational power (Vigmond *et al*., 2009). Rather than an "arms race" to employ the most complex model, the specific questions being addressed, along with a consideration of the computational power available, should guide the most appropriate model to be used. To address this, Clayton and Panfilov (2008) have provided an excellent guide for building heart tissue models.

For deeper background, several comprehensive book reviews on cardiac electrophysiology are available, including the encyclopedic collection edited by Zipes (2004), Braunwald's classic Heart Disease (Libby *et al*., 2008), and the textbook by Jalife *et al*. (1999) all of which link cardiac function from the subcellular level up to the clinical setting. For specific guidance on the mathematics of cardiac electrophysiological systems and model building considerations, there are reference textbooks on general (Malmivuo, 1995; Gulrajani, 1998; Plonsey, 2007) and cardiac specific (Sachse, 2004; Pullan, Cheng and Buist, 2005; Sundnes *et al*., 2006) bioelectricity and bioelectromagnetism. In addition to providing a historical overview of the development of cardiac electrophysiology models and summaries of heart anatomy and function, these books provide in-depth mathematical formulations and computational techniques that can be employed to model different cardiac time and length scales critical to

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describe different cardiac functions. Finally, outstanding journal reviews may provide historical progression and state-of-the-art information on cardiac modeling techniques, as linked to advances in understanding cardiac electrophysiology (Noble, 2001; Muzikant and Penland, 2002; Hunter and Borg, 2003; Belik, Usyk and McCulloch, 2004; Kleber and Rudy, 2004; Holden *et al*., 2006; Rudy and Silva, 2006; Benson *et al*., 2008; Clayton and Panfilov, 2008; Noble, 2008).

3.3 Case Studies

Modeling efforts related to QT prolongation risk have focused on several areas. To be useful, the system models must replicate important markers *in vitro* such as action potential duration (APD), transmural dispersion of repolarization (TDR), early afterdepolarizations (EADs, Figure 9c) and triangulation, reverse use dependence, and instability (TRIaD) advanced by the preclinical models on which they are based. To be accurate, they must incorporate sufficient complexity necessary to describe the phenomena of interest. In broad classifications, one focus has examined the electrophysiologic heterogeneity across the wall of the ventricle to analyze the hypothesis that QT prolongation without an accompanying increase in transmural dispersion of repolarization is not arrhythmogenic. A second focus has elucidated how specific long QT (LQT) syndrome genetic mutations in single ion channels affect the electrophysiologic behavior of the integrated cell and ultimately the whole organ.

Singleton perturbations such as a mutation or exposure to a pure I_{Kr} blocker do not require the use of systems modeling for understanding effects across scales; however, such models are necessitated by the combination of conditions with others in an intrinsically nonlinear dynamical system. In the following text, we review examples of meaningful systems modeling conducted to understand nonlinear factors in the development of drug-induced repolarization delay and cardiac proarrhythmia.

3.3.1 Harmonizing Conflicting Data by Reverse-Engineering an Unknown Pharmacological Effect

A full characterization of drug concentration profiles versus responses of various electrophysiological markers is often necessary to assess the *in vivo* safety of a drug, given the potential modulation of drug concentration *via* pharmacokinetic processes such as protein binding, drug lipophilicity, or liver enzyme inhibition or induction (e.g., CYP3A4) (Bauman, 2001). Due to time and cost constraints as well as experimental difficulties in running electrophysiology assays, only a limited number of conditions may be investigated during the safety assessment of a new compound. *In vitro* and *in vivo* experiments only allow for a small number of drug concentrations to be tested, leading some to use mathematical system models that adequately replicate the experimentally measured AP profiles to simulate the electrical behavior of myocytes at different, non-tested drug concentrations.

All models of biological systems and processes are described by two characteristics: (a) a "structure" or set of mathematical functions for the governing mechanisms and (b) a certain number of parameters. Only the governing equations and parameter values known together allow a computer simulation of the model to be run in a predictive "forward" mode. Although some of the parameters could be measured directly, others need to be inferred from other experimental data using different parameter estimation and mathematical optimization techniques (reverse engineering) (Mendes and Kell, 1998). As an example, Balser, Roden and Bennett, 1990 used such estimation to determine global parameters for a model of potassium channel function directly from rich sets of data by iteratively using the model to simulate the observed data.

Bottino *et al*. (2006) used a combined forward- and reverse-engineering approach to reconcile the inhibition of hERG channel (IC_{50}-values in micromolar range) with the lack of prolongation of AP duration recorded from canine Purkinje fibers (PF) with two compounds. First, a computational model of canine PF was adapted from a published model of the canine ventricular myocyte (Greenstein and Winslow, 2002) using parameter estimation of major ion channels present in both cell types. The parameters estimated were the macroscopic conductances of the 14 ion channels represented in the myocyte model; the data used for the fitting procedure consisted of action potential measurements from samples at multiple pacing rates with either no drug or with different concentrations of the reference compound *dl*-sotalol. The resulting PF

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model was then used in a forward mode to confirm that model predictions were comparable to independent experimental reference data. Taking the compounds' IC_{50} values on I_{Kr} current as prior information, the authors applied reverse-engineering to derive the remaining ion channel profile for each of the test compounds using experimentally measured action potential traces under different drug concentrations and different pacing frequencies. The fitted parameters were the compounds' IC_{50} values for the cardiac ion currents responsible for repolarization, namely sustained inward sodium current I_{Nasus}, L-type calcium current I_{CaL}, slowly activating delayed outward rectifier potassium current I_{Ks}, transient outward potassium current I_{to1}, and net inward current generated by the calcium/sodium exchanger I_{NaCa}. As proof of principle and a testament to the approach, the estimated IC_{50} values for I_{Nasus} were confirmed in an independent experiment using the patch-clamp technique.

3.3.2 *Role of Multi-Channel Block in Mitigating hERG-Mediated Proarrhythmic Risk*

Preclinical studies (van Opstal *et al*., 2001; Vos *et al*., 2001; Hondeghem and Hoffmann, 2003; Hondeghem *et al*., 2003; Thomsen *et al*., 2006; Hondeghem, 2007) clearly show that despite comparable levels of hERG blockade, the accompanying pharmacology on other cardiac ion channels determines to a great extent the magnitude of the QT prolonging and proarrhythmic effects. Of particular interest discussed here are verapamil, amiodarone, and ranolazine; all of which are marketed compounds with clear hERG inhibition, demonstrating clinical QT prolongation, yet are devoid of significant proarrhythmic risk. The proposed and now accepted hypothesis for this dichotomy centers on these compounds' ability to simultaneously block a number of inward (sodium, calcium) currents while blocking the outward repolarizing (potassium) currents.

Verapamil is a safe, well-known calcium channel blocker with I_{Kr}/hERG blocking capacity that leads to QT prolongation and other ECG changes in the clinic. Given this class III Vaughan Williams effect, many have asked whether verapamil would have been developed and approved in today's climate. Despite its ion channel polypharmacology, verapamil significantly shortened endocardial but not epicardial MAPs, suppressed EADs, reduced ventricular TDR, and ultimately suppressed TdP in a proarrhythmic rabbit ventricle model (Milberg *et al*., 2005). Amiodarone is marketed as an antiarrhythmic drug that blocks sodium, potassium, and calcium ion channels, prolongs the QT interval, yet is only rarely associated with torsade de pointes arrhythmias. Four-week studies in the chronic complete atrioventricular (AV) block (CAVB) dog model of acquired long-QT syndrome clearly showed QT prolongation by both amiodarone (+21%) and its analog dronedarone (+31%). Despite this, amiodarone showed no increase in dispersion of repolarization and did not cause TdP due to its particular balance of blocking both repolarizing (outward) and depolarizing (inward) currents. Highlighting the delicate balance of net repolarization, dronedarone, with a different ion channel blocking profile, increased repolarization dispersion and exhibited ectopic beats and TdP.

Ranolazine is an antianginal agent capable of producing anti-ischemic effects without negative inotropic or hypotensive consequences. However, it does prolong the QT interval, thus raising serious concerns over its long-term safety in cardiovascular patients. *In vitro* studies suggest it is a broad-spectrum ion channel inhibitor that does not trigger any typical risk signals in the nonclinical studies and, in fact, may be ultimately antiarrhythmic (Antzelevitch *et al*., 2004; Schram *et al*., 2004; Wang *et al*., 2008).As additional evidence, the Ranolazine Open Label Experience (ROLE) trial in 746 chronic angina patients treated over 2102 patient-years did not report any TdP- or QT prolongation-related discontinuations, despite having a mean QT interval prolonged by 2.4 ms (Koren, Crager and Sweeney, 2007).

The published ion channel pharmacology for ranolazine (Antzelevitch *et al*., 2004) was applied to *in silico* models of epicardial and midmyocardial cells (Muzikant and Penland, 2002). Findings from these systems models supported the experimental and clinical experience by showing that the action potential was prolonged equivalently in both cell types, leading to no increase in the TDR and therefore no signal for TdP risk. More detailed modeling studies of fast-unbinding sodium channel blockers such as lidocaine, vernakalant, and ranolazine using realistic mathematical models of state-dependent channel block (Hondeghem–Katzung modulated-receptor) have shown how such therapeutics act to terminate vagotonic atrial fibrillation (Comtois *et al*., 2008).

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3.3.3 *Role of Gender Differences in Cardiac Proarrhythmic Risk*

The QT interval is well established to be longer in females than in males, and female gender itself is a recognized independent risk factor for development of arrhythmias in the congenital and drug-acquired Long QT syndrome. James, Choisy and Hancox (2007) has summarized a number of drugs having a greater risk of inducing TdP arrhythmia in women than in men. The differences in the ECG between men and women, and in particular those relating to the QT interval, have been explored experimentally and provide evidence of differences in the processes underlying ventricular repolarization (James, Choisy and Hancox, 2007).

A number of investigators have observed small but important differences in the relative strength of cardiac repolarization currents between males and females. In a range of *in vitro* and *in vivo* animal studies by multiple investigators, the application of testosterone at physiological levels either in females or in previously castrated males repeatedly demonstrated augmentation of expression of K^+-channel proteins, reduced the QT interval (where applicable), mitigated the sensitivity to class III agents, and thus protected against arrhythmias *via* increasing net repolarization current (Drici *et al*., 1996; Shuba *et al*., 2001; Fulop *et al*., 2006; Furukawa and Kurokawa, 2007; Ridley *et al*., 2008). These repolarization-enhancing effects of androgens on the human heart were confirmed by dosing a small cohort of hypogonadic men with testosterone (Charbit *et al*., 2009). Conversely, estrogens do not appear to have any appreciable effect on repolarization. Yet other studies have indicated a significant gender difference not only in the inward (I_{K1}) and slow delayed (I_{Ks}) rectifier cardiac K^+ currents but also in the β-adrenergic regulation of those currents in beagle Purkinje fibers (Abi-Gerges *et al*., 2004, 2006).

Investigators at Physiome Sciences (Muzikant and Penland, 2002) integrated these experimentally determined relative differences in repolarizing currents (Drici *et al*., 1996; Liu *et al*., 1998; Liu *et al*., 1999) to create male and female phenotypic responses in their cellular models. In these two gender models, class III agents clearly induced more profound action potential delay in females at lower concentrations. With the latest male and female gender differences in ion channel expression and function, systems models can be used to easily explore possible gender-specific effects of compounds under study. As a final note on the topic, the literature indicates that overall hormonal state – not just adult male or female – is important and thus toxicology should give consideration to patients in categories such as geriatric, menopausal, hormone replacement therapy, (pre)pubescence, or even androgen (mis)use.

3.4 Incorporating Effects of Inherited Cardiac Ion Channel Mutations

A major limitation of the Hodgkin-Huxley formulation used in the previous modeling studies is that it simulates only the macroscopic (i.e. whole cell) currents generated by a large ensemble of individual ion channels. With a growing cohort of experimental evidence characterizing the relationship between ion channel structure and function and the presence of genetic mutations associated with the long QT syndrome, Brugada syndrome, and other inherited arrhythmias (Abriel *et al*., 2001; Keating and Sanguinetti, 2001; Lehnart *et al*., 2007), more sophisticated mathematical representations of individual channel behavior have been developed to investigate how single-channel modifications can modulate electrophysiologic function at whole-cell, and ultimately whole-organ, levels. These models, often Markov by nature, describe the kinetics of individual channel states (i.e., open, closed, inactivated, and subtypes thereof) through experimentally derived probabilities and rate constants. Importantly, they allow for the effect of structural flaws in mutated channels on the gating, ionic selectivity, or conductance properties.

In one well-documented example, Clancy and Rudy developed a Markovian representation for both wild-type and mutant I_{Kr}, and included this channel into whole-cell models (epicardial and M) to study potential proarrhythmia under long QT syndrome (type 2) conditions (Clancy and Rudy, 2001). They showed that a macroscopic effect on the action potential for each of three point mutations in I_{Kr} is highly dependent on the specific mutational effect (i.e., shift in activation, deactivation, or loss of inactivation), and that these effects were more pronounced in M-cells than in epicardium. Detailed models have also been constructed for mutations in the voltage-dependent sodium channel (DeltaKPQ) (Nagatomo, January and Makielski, 2000; Clancy, Zhu and Rudy, 2007), 1795insD (Clancy and Rudy,

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2002), D1790G (Abriel *et al*., 2000) and E1295K (Abriel *et al*., 2001). Beyond characterizing the functional consequences of the mutation itself, these studies have also examined the differential effects of the mutation on the channel blockade by known sodium channel antagonists such as flecainide, pilsicainide, mexiletine, and lidocaine (Ono *et al*., 2000; Clancy *et al*., 2007).

To summarize as shown in Figure 10, the systems modeling approach directly represents molecular mechanisms in the context of a higher-order network and therefore is a powerful tool to incorporate the pharmacogenetics and epigenetics of inherited cardiac dysfunction into early proarrhythmic risk assessment for new compounds.

3.5 Discussion and Additional Considerations

In this chapter, we reviewed the application of multiscalar cardiac system models to the anticipation and management of QT prolongation in the clinical electrocardiogram and proarrhythmic risk attending new therapeutic compounds. With a host of unanswered questions, it is natural for investigators using such models to seek to expand the scope, richness, robustness, and complexity as necessary to meet new challenges in the field. To this end, we discuss several promising and tractable extensions that could have considerable impact in the successful industrial application of such models.

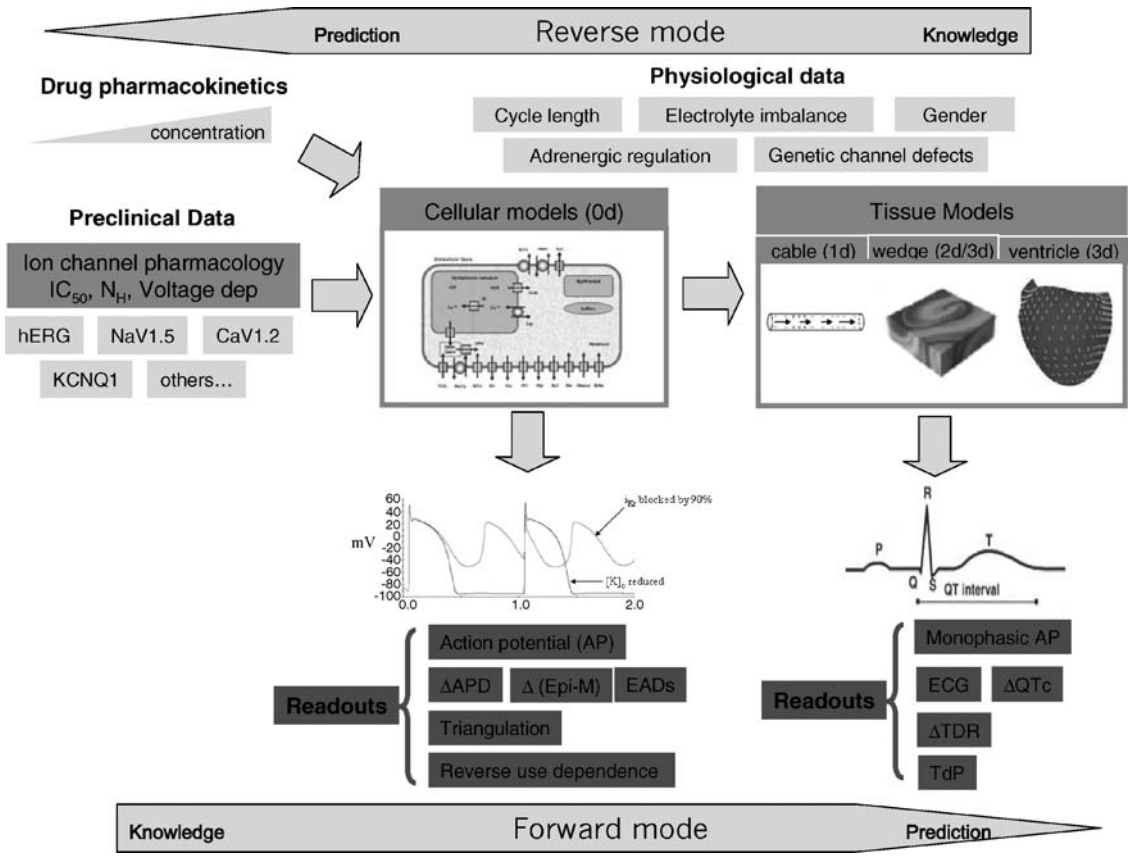

Figure 10. Systems modeling of mechanistic electrophysiological effects in the prediction of cardiac QT prolongation and proarrhythmic toxicity. Direct pharmacological effects on major ion channels are simulated in cardiac cellular models, which are embedded into biophysical models of the heart. Such models produce multiple readouts that are relevant to risk prediction and benchmarking to the analogous experimental system. A range of physiological conditions can be superimposed on the nominal system to explore risk-modifying combinations.

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3.5.1 Drug-Channel-Signaling

Historically, models of drug effect on ion channel function have taken the form of a concentration-dependent reduction in available current. In some cases, investigators have had both the motivation and the available data to delve deeper by implementing state-dependent models of drug–channel interaction. Furthermore, it has been clear for some time that relatively few compounds identified as ion channel blockers are in fact pharmacologically restricted to ion channels. Sotalol is a classic example as it is a racemic mixture of enantiomers with β-receptor antagonism but with only the dextro enantiomer having a strong I_{Kr} inhibition. The combined effect on the cardiac action potential cannot be discerned except by implementing a combined model of both mechanisms like that proposed by Levin *et al*. (2002).

It is critical that we remain vigilant for new mechanisms requiring updating of our primary models. Illustrating this clearly are the endogenous (Bai *et al*., 2007) and synthetic (Koyrakh *et al*., 2005) compounds in the sphingophospholipid class that have been shown to have macroscopic electrophysiological effects (reduction in HR) underpinned by newly discovered interaction with important cardiac repolarization channels. Studies showing altered glycosylation and phosphorylation of sodium channel leading to arrhythmia in heart failure (Ufret-Vincenty *et al*., 2001) and those showing the electrophysiological consequences of long-term ionic, pH, and metabolic changes secondary to myocardial ischemia (Cascio, Johnson and Gettes, 1995) also highlight the need to begin incorporating such mechanisms into the cardiac systems models of today.

3.5.2 Intrinsic Variability, "Populations"

The critical question being asked in the assessment of compounds' propensity for QT prolongation and torsadogenesis in the clinic is essentially one of predicting the incidence of rare clinical events given certain typical response data accumulated through preclinical safety pharmacology (Morganroth *et al*., 1993; Fenichel *et al*., 2004). In our opinion, one of the most exciting horizons for systems modeling of cardiac electrophysiology is the growing capacity to include intrinsic biological variability explicitly. The statistical mixture model construct (Rosner and Muller, 1997), already used extensively in clinical pharmacokinetic–pharmacodynamic modeling, is a natural approach to adopt in the systems toxicology models proposed here. The evolution from "mean response" systems models to models that embrace and explicitly represent (*via* stochastics and/or parameter populations, covariates) the underlying biological, physiological, and temporal variation provides the opportunity for a single modeling framework to explain the median response while also capturing the rare combinations that produce toxicological side-effects.

3.5.3 Trafficking and Other Indirect Effects

New evidence has emerged indicating that select but growing number of compounds alter ion channel (hERG in particular, but also others) trafficking and maturation, thus altering the electrophysiological makeup of the cells over a longer term than is typically assessed during *in vitro* screening (Drolet, Simard and Roden, 2004; Wible *et al*., 2005; van der Heyden, Smits and Vos, 2008). In some cases, these same compounds lead to the appearance of QT prolongation disjoint from time of maximum concentration, making management of this risk and assessment difficult. The nature of this emerging trend in compounds with trafficking effects is unclear; perhaps it is a previously intractable mechanism now possible to study or a reflection of new chemical, biopharmaceutical, and target classes in the pharmacopeia. Regardless of the reason, accounting for such effects will require the development and incorporation of new mechanistic understanding and modeling from gene regulation to protein trafficking within the already dynamical cardiac myocyte.

3.5.4 Pharmacokinetics, Getting the In Vivo Behavior Right

The *in vivo* concentration of a novel therapeutic does not remain constant in cardiac tissue over time and thus presents a challenge in using *in vitro* data and systems models to project a compound's clinical characteristics. Point estimates of concentration response using well-known exposures are useful for ranking compounds early on; however, the ultimate toxicological profile and clinical utility are intrinsically tied to the pharmacokinetics of the candidate. The medical literature contains several reports of compounds that are mild ion channel blockers, but are also substrates of particular cytochrome P450 enzymes. The resulting "reality" of risk is that mild

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hERG I_{Kr} inhibition can amplify manyfold by the co-administration of metabolic blockers of the clearance enzyme in question. Such examples justify research into the union of cardiac mechanistic models with a physiologically based pharmacokinetic model to jointly estimate the human exposure and response in QT or risk of proarrythmia.

4 MECHANISTIC MODELS OF BONE MARROW TOXICITY FOR ASSESSING NEUTROPENIA RISK IN DRUG DEVELOPMENT

4.1 Introduction

Combing pharmacokinetic models with mathematical models that describe whole-animal toxicity observed in *in vitro* and *in vivo* systems to simulate or predict the potential response in humans allows us to better design first-in-man studies, ultimately leading to safer and faster development of new molecular entities.

The systematic use of mathematical approaches is required as the high degree of non-linearity in concentration- and toxicodynamic-time profiles far exceed our ability to anticipate the events which may occur in humans based on empirical correlations alone.

4.2 Example for Small Molecules: Neutropenia Model

Neutropenia represents one of the major limitations during chemotherapy treatment. Consequently, considerable efforts have been undertaken to describe the adverse response – exposure relationship (Egorin *et al*., 1986; Hantel *et al*., 1990; Huizing *et al*., 1993). The single measures of exposure (e.g., dose and AUC) have often been used to explain the observed toxicity (e.g., nadir, grade, etc.), but are limited because they do not take the time-dependent nature of the repose into account, resulting in high levels of variability.

Optimization of drug regimens typically requires extensive clinical trials where a compromise between dose, dosing interval, and safety is sought. As an example, several years of clinical development are needed to find tolerable and efficacious schedules for Taxotere. Taxotere administered once every 3 weeks at the dose of 100 mg m^{-2} gives 70% of grade 4 neutropenia. The median time to onset is day 7, the median duration is 7 days, and the median time to recovery to grade 0 is day 14. This accounts for the need to collect blood samples on days 7 and 14 for every cancer patient receiving this drug following this schedule. The weekly schedule (35 mg m^{-2}) is very safe. At the recommended dose, grade 4 neutropenia is the exception. Blood samples are rarely requested, which is very convenient for cancer patients.

The fine interplay between dosing, drug holiday duration, and timepoints to monitor patients is often the result of empirical and costly clinical research. In the following, we illustrate how semi-mechanistic pharmacokinetic and toxicodynamic modeling can be used to design an animal study that can be used to predict toxicity in clinical trials and design optimal protocols.

4.2.1 Physiology of the Myelopoietic System

Stem cells in the bone marrow with high proliferative capacity are susceptible to chemotherapeutic drugs (Friberg *et al*., 2000; Segura *et al*., 2004). These pluripotent stem cells are at the bottom of the granulopoiesis process for the staged maturation and differentiation to a final cell type, the most prominent being the circulating neutrophils. This transition time in rodents and humans ranges from 3 to 6 days. Circulating blood neutrophils extravasate in peripheral tissue where they undergo apoptosis. They disappear from bloodstream with a first-order process.

The system has a remarkable potential to adapt under stress such as infections, growth colony-stimulating factor (G-CSF) being the most important regulator of granulopoiesis (Friberg and Karlsson, 2003). G-CSF stimulates cell division in the bone marrow. Circulating levels of G-CSF and the peripheral neutrophil count are inversely correlated, implying that mature neutrophils modulate one of the major pathways of G-CSF clearance. This creates a homeostatic mechanism maintaining the level of circulating neutrophils at equilibrium.

In a very schematic way suitable for modeling, one could envision that a chemotherapeutic drug action decreases the stem cell compartment at first with no effect on the circulating cells. The effects on the circulating cells are delayed by the maturation time and are compensated by the feedback loop mediated by G-CSF, which in turn is designed for

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normalizing circulating neutrophils by promoting proliferation in the pluripotent stem cell compartment. With a certain delay due to maturation, the circulating neutrophils pool will be equilibrated.

Different drugs appear to have a different effect on circulating neutrophils as a result of a number of factors: the PK variability in patients, different sampling times used, and the different antiproliferative effect on the cycling stem cells. When these differences are considered independently, it is possible to separate systems effects (PK, physiology of maturation) from drug effects and build predictive models across species.

4.2.2 *Modeling Approaches: Empirical Versus Semi-Mechanistic Models*

Various modeling approaches have tried to characterize the effect of chemotherapeutics on the myelopoietic system employing different strategies, e.g., correlating drug exposure (Egorin *et al*., 1986) or threshold concentration (Hantel *et al*., 1990; Huizing *et al*., 1993) with the nadir of leukopenia or neutropenia. Typically, such models have been shown to be inadequate and lead to paradoxical predictions of the response *in vivo*. For example, models based on exposure would overestimate the danger of low-dose prolonged infusions versus bolus, whereas models based on threshold concentration or dose would overestimate the safety of prolonged infusions. In general, these models suffer from the following limitations: impracticable scalability from rodents to humans, inability to describe the dynamic response, and inability to re-use prior information.

On the other hand, semiphysiological modeling approaches (Friberg *et al*., 2000, 2002; Friberg and Karlsson, 2003; Segura *et al*., 2004) mimicking the physiological system described previously have been shown to have several benefits. First of all, PK–PD model contributions can be studied in parallel: systems response and drug response are separated and their parameters can be estimated independently. As a consequence, drug parameters can be scalable across species. The systems parameters reflect our knowledge on the physiology. Systems parameters are therefore re-usable across several different drugs. This implies that the more the model is used, the more prior information on the systems parameters is accumulated for more accurate predictions. Finally, this modeling approach allows to study combination therapies where multiple drug effects on the system can be evaluated.

Furthermore, semi-mechanistic models have the advantage of describing the entire time course of leukocyte suppression and rebound phenomena. This is important because for example observed peak drug concentrations and effect, a nadir in neutrophil count occur at different times. The parameters estimated reflect the real concentration–response relationship and schedule-dependent effects can be studied. Hence, an integrated PK–PD model further allows for experimental sampling times to be optimized. Typically, measurement in clinics and in pre-clinical experiments are performed once or twice weekly, and therefore the nadir concentrations are often missed and inter-individual variability could be over- or underestimated. The estimation of optimal sampling times can reduce both the number of animals and subjects in the trials and the burden of blood sampling.

Clearly, such a modeling approach would be very helpful to predict the effect of drugs from rodents to humans and to optimize clinical trials of novel molecular entities with the goal of reducing clinical failures, number of patients, and adverse events. The expected benefits of predicting myelosuppression and efficacy in man are multiple. On the one hand, the modeling approach is expected to reduce the number of animal experiments. A combination of optimal experiment design techniques and modeling can both reduce animals per experiment and make the experiments less invasive (e.g., reducing the number of bleeds). On the other hand, the benefit for the clinical development include a shortening of phase I by choosing the right starting dose with respect to safety and efficacy, a faster optimization of "safe" schedules and combination therapies, and the design of individualized treatments, for instance on the basis of the baseline neutrophil count. Finally, healthcare companies and regulators may re-use the modeling across projects and incorporate competitor data when available expanding the model data sets and improving its accuracy.

4.2.3 *Description of Semi-Mechanistic Models*

Recently, a semi-mechanism-based PK–PD model was proposed, which has been applied successfully across compounds (Friberg *et al*., 2002) and species (Karlsson, Friberg and Sandstrom, 2004).

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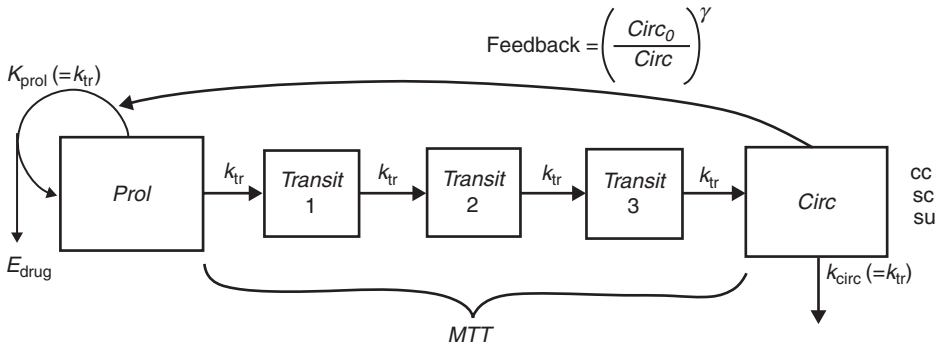

Figure 11. Schematic view of the semi-mechanistic myelotoxicity model. The toxic effect of the drug (Edrug) is exerted on the proliferating compartment of pluripotent stem cells (Prol). The transit compartments (Transit 1, ···, Transit 3) represent the maturation steps to reach the circulating cell compartment. The feedback homestatic mechanism (Feedback) between the circulating compartment and the proliferating pool of cells is also depicted.

This semi-mechanism approach does take the time-dependent nature of cell proliferation into account and allows the myelosuppression to be described in terms of system- and drug-dependent parameters. In the choice of the model, one has to strike a balance between accuracy and complexity. The number of parameters should be low and their interpretation straightforward. Also, the parameter estimation should be tractable. Our preferred model describes the time delay using transit compartments. In contrast to lag-time models, this approach mimics the gradual maturation of cells. Multiple compartments can be added in the delay chain without adding extra parameters because the rate constant between the compartments is the same. The model (Figure 11) consists of five differential equations, one for the proliferating cells in the bone marrow, three to describe the maturation, and one to describe circulating cells.

$$\frac{\mathrm{dProl}}{\mathrm{d}t} = k_{\mathrm{tr}} \cdot \left[\left(1 - E_{\mathrm{drug}}\right) \cdot \left(\frac{\mathrm{Baseline}}{\mathrm{Circ}} \right)^{\gamma} - 1 \right] \cdot \mathrm{Prol} \tag{6}$$

$$\frac{\mathrm{dTransit}_1}{\mathrm{d}t} = k_{\mathrm{tr}} \cdot (\mathrm{Prol} - \mathrm{Transit}_1) \tag{7}$$

$$\frac{\mathrm{dTransit}_i}{\mathrm{d}t} = k_{\mathrm{tr}} \cdot (\mathrm{Transit}_{i-1} - \mathrm{Transit}_i) \quad i = 2, 3 \tag{8}$$

$$\frac{\mathrm{dCirc}}{\mathrm{d}t} = k_{\mathrm{tr}} \cdot (\mathrm{Transit}_3 - \mathrm{Circ}) \tag{9}$$

The drug action is confined to the proliferating compartment where the cell loss term due to the drug action is described using a linear term $E_{\mathrm{drug}} = C_{\mathrm{drug}}$*Potency. The concentration of the free fraction of the drug in plasma C_{drug} is used and the toxicity parameter Potency is estimated for each drug. The parameter k_{tr} represents both the proliferation and maturation kinetics of the system. The feedback mechanism mediated by G-CSF is represented by the term $(\mathrm{Baseline/Circ})^{\gamma}$. The parameter gamma indicates the strength of the feedback response. Interindividual variability is estimated for the parameters k_{tr}, Baseline, and Potency.

In simulations, all the data were estimated using a nonlinear mixed-effects population analysis PK/PD model where data from all subjects were analyzed simultaneously. Lognormal parameter distribution was assumed. The residual error was modeled with an additive and proportional component.

The systems parameters describe time-dependent physiological processes, which are species-specific but drug-independent. Potency, a drug-specific parameter, is assumed to be constant across species. Hence, when a new compound is characterized in a rodent population, the drug-specific parameters are determined.

The same drug parameters are then used together with human system parameter and predicted human PK to predict the myelosuppression in man (Figure 12). The prediction of myelosuppression in man allows for the optimization of candidate selection but also for the optimization of dosing schedules for efficacy in early clinical trials. To date, several

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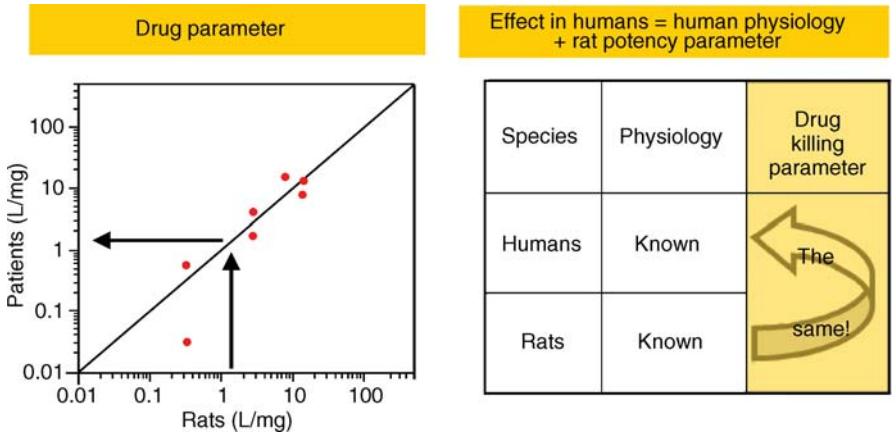

Figure 12. Linear correlation between the drug potency parameter across species. The systems parameters require adaptation when scaling from rodents to humans results to reflect the changes in physiology whereas the drug potency parameter remains the same in a fairly large set of values where most drugs in development or on the market lie.

Roche compounds have been modeled, as well as six compounds from other companies for which clinical and preclinical data are available, which generate a curve that describes the human/rat relationship for the key drug Potency parameter in the model (i.e., kill rate). A log/log plot of this parameter shows an approximate linear relationship that allows prediction of human myelosuppression based on modeling of rat data (Karlsson, Friberg and Sandstrom, 2004). An almost identical Potency parameter between the species has been observed across species, with deviations at the extreme of the range (overestimation if Potency $< 0.3\,\mathrm{l\,mg^{-1}}$; underestimation if Potency $> 12\,\mathrm{l\,mg^{-1}}$).

4.3 An Example in Drug Development

A diaminopyrimidine, which constitutes a new class of cell cycle inhibitors selective for the serine/threonine class of CDK inhibitors undergoing clinical development, has been chosen as case study. Cyclin-dependent kinases (CDKs) play a central role in the control of cell cycle progression. The CDK inhibitor in this study (here referred as CDK-I) has single-digit nM activity against CDK1, CDK2, and CDK4 and is not active against 19 related and unrelated serine/threonine and tyrosine kinases. It has broad-spectrum antiproliferative activity and displays *in vitro* antiproliferative activity across a panel of 14 human tumor cell lines, including a multi-drug-resistant cell line that overexpresses PgP. CDK-I blocks tumor cells in G1+G2 phases of the cell cycle and induces apoptotic cell death in these cells. The compound has been shown to inhibit the phosphorylation of retinoblastoma protein (Rb), a substrate for CDK2 and CDK4 in tumor cells. CDK-I is orally active in 5/5 established tumor models in rodents at or below the maximum tolerated dose (MTD) including an aggressive rat breast tumor model.

CDK-I was developed as an IV administered drug for mono- and combination therapy in a variety of solid tumors. The target product profile requires the adverse events (AEs) to be reversible and manageable. Specifically in monotherapy the AE profiles are required to be comparable or better whereas in combination therapy the AE profiles should allow for the safe combination with other chemotherapeutics.

A placebo-controlled 6-week toxicology study was conducted on healthy rats following once-weekly IV dosing with three doses of 5, 12.5, and $25\,\mathrm{mg\,kg^{-1}}$, three groups per dose (18 rats), and six animals per group bled on day (1, 22, 43) or (4, 25, 43) or (7, 28, 43, 57). The resulting very sparse data set has 4 points per rat for a total of 180 rats. PK data were collected in a satellite group of animals treated intravenously and with oral doses between 5 and $100\,\mathrm{mg\,kg^{-1}}$.

The prediction of myelotoxicity for humans follows the five steps given below:

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1. **Analysis of the toxicology study results**
   The analysis of the data from the rat study evidenced two different mechanisms of toxicity for neutrophils and lymphocytes. For neutrophils, a typical delayed suppression has been observed. This effect is compatible with a drug-killing action on the progenitor cells of neutrophils, which becomes evident after the typical maturation time on the circulating neutrophils. The data also show the typical rebound of circulating neutrophils set in place by the feedback mechanism of homeostasis. This dynamic behavior is compatible with the structural model described earlier and can be extrapolated to humans. In the remaining part of this example, we will focus on the prediction of neutropenia but we would also like to emphasize how the modeling can be useful to identify unexpected mechanisms of action as well.
2. **PK in rats**
   PK data have been obtained from oral as well as IV administration of the compound in single doses to cover the range of exposures studied in the toxicology experiment. The plasma concentration–time profiles of CDK-I in Fischer rats could best be described by a three-compartment pharmacokinetic model. Given the limited interindividual variability in the PK of inbred mice, a simple model for the average values was fitted. The current data nonlinearities could not be identified.
3. **Fitting PD model**
   Fitting of myelosuppresion model was performed with NONMEM by linking the PK described above with the semi-mechanistic PD model. The PD model was fitted using reference value as starting point for the physiological systems parameters (e.g., the maturation rate, the feedback loop rebound, and baseline value of circulating cells). The Potency parameter of around $1\,l\,mg^{-1}$ for CDK-I was estimated. This value falls well in between the values reported for other cytotoxic drugs. For other drugs with similar killing potency parameters, the scaling from rodents to humans has been very reliable and this increases the confidence of an accurate prediction for humans.
4. **Prediction of human PK**
   The prediction of PK in humans was performed using a PBPK model with Gastroplus as discussed in Introduction of this chapter. The prediction in humans was based on *in vivo* profiles (IV and PO) at different doses in rats and monkeys. In addition, a set of *in vitro* tests to characterize the physicochemical properties [solubility in simulated fasting and fed intestinal fluids (Fessif, Fassif), $\log P$, $\log D$, permeability] and biochemical properties of the molecule in rats/monkeys/humans (e.g., V_{Max} and K_m for microsomal clearance, protein binding, and blood/plasma ratio) have been carried out. The scaling of PK profiles from rodents to monkeys was tested against real data for the various PO formulations to guarantee the reliability of the PK predictions across species. For humans, the typical variability in PK as seen in phase I oncology trials with patients failing other therapies and at an advanced stage of disease has been used to predict PK. Physiological population parameters and interindividual variability was based on the average parameters of six currently marketed chemotherapeutics. The PK interindividual variability was set to 30% per parameter sampling from normal distribution.
5. **Virtual clinical trials**
   Clinical trial simulations in humans were carried out using the clinical trial simulation software, Trial Simulator (v2.1.2, Pharsight Corporation, Mountain View, CA, USA), and are available upon request. The designs of the studies mentioned below were replicated taking into account the route of administration, the dose, and the number of patients. Each study was replicated 1000 times. The population PK–PD parameters were sampled from a multivariate normal distribution at study level. The individual parameters were based on these new population parameters and a random sample from the distribution characterized by the interindividual variability. On each measurement, a proportional random error was added. Following each replicate, the maximum individual neutropenia grade reached over time was calculated as well as the mean grade per trial using S-PLUS (v6.1; Insightful Corporation, Seattle, WA, USA). The 80% prediction intervals were calculated after simulation of 1000 replicates and represent the 10th and 90th quantiles.

Two scenarios were modeled: the base scenario (IV dose once a week for 2 weeks, 3-week cycle) and the alternative scenario (2 weeks daily dosing, 1 week drug holiday, 3-week cycle) (Figure 13). The baseline value for the occurrence of grade 4

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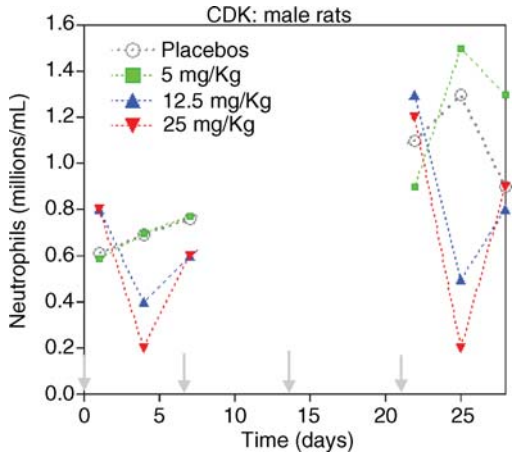

Figure 13. Profiles of circulating lymphocytes and neutrophils after IV administration of CDK-I to Fischer rats. Note the delayed suppression and rebound of neutrophils consistent with the model of Figure 11.

neutropenia in a typical phase I study in oncology is 18% (CI: 0–35%). Based on the assumption that the drug Potency parameter does not vary across species and using the PK predictions in humans, different doses and formulations have been investigated. A significant increase in the incidence of neutropenia grade 4 over baseline is expected to occur at 300 mg m^{-2}, whereas the threshold for efficacy in preclinical models appears to be 200 mg m^{-2} (i.e., average weekly AUC over the 3-week cycle larger than 104 ng h ml^{-1}). In other words, tumor response does not correlate with neutropenia and the drug candidate appears to have a positive therapeutic index.

A regimen where drug is administered either IV or orally continuously for 2 weeks has been investigated (Figure 14). It is expected that the administration of smaller doses will allow the reduction of other acute adverse events. Absorption modeling (GastroPlus) on monkey data (capsules

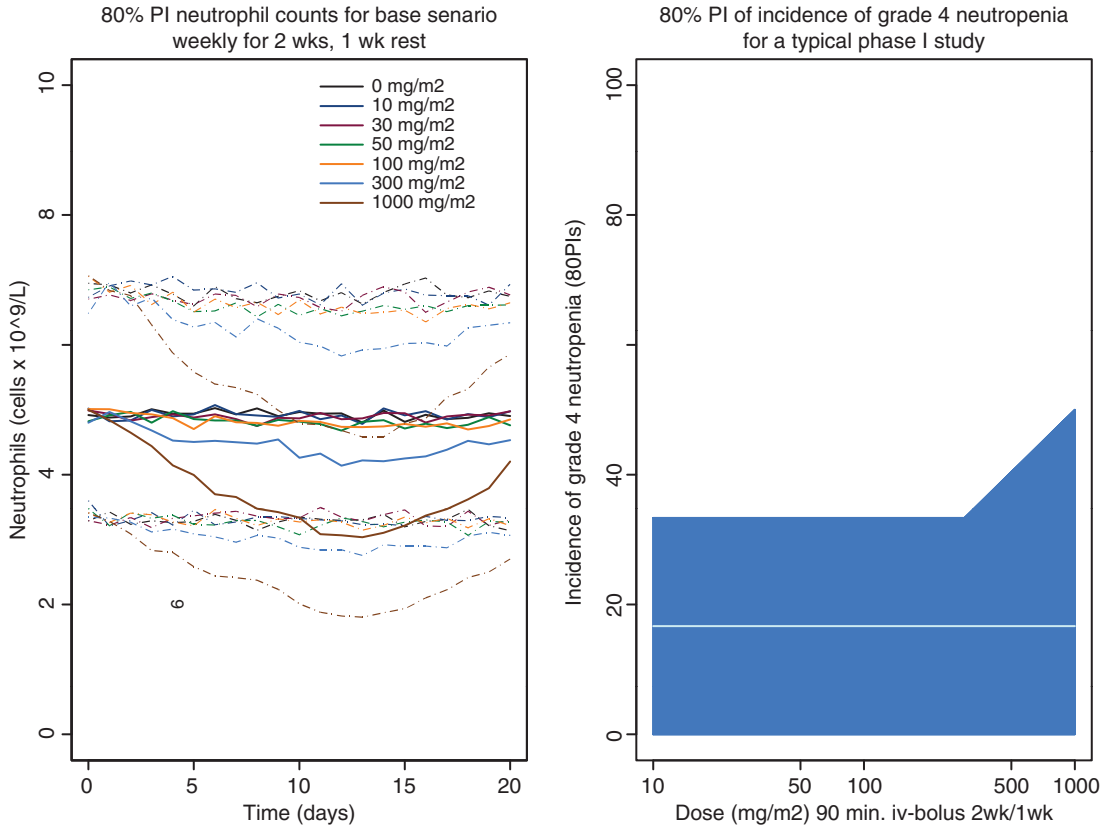

Figure 14. Predicted neutropenia in a human phase I trial in patients with advanced cancer for the base scenario (IV dosing weekly for 2 weeks, 3-week cycle). Typical interindividual variability parameters found in phase 1 cancer trials were used. Simulation indicates that CDK-I is a safe drug candidate and that no increase in neutropenia risk is expected below 300 mg m^{-2}.

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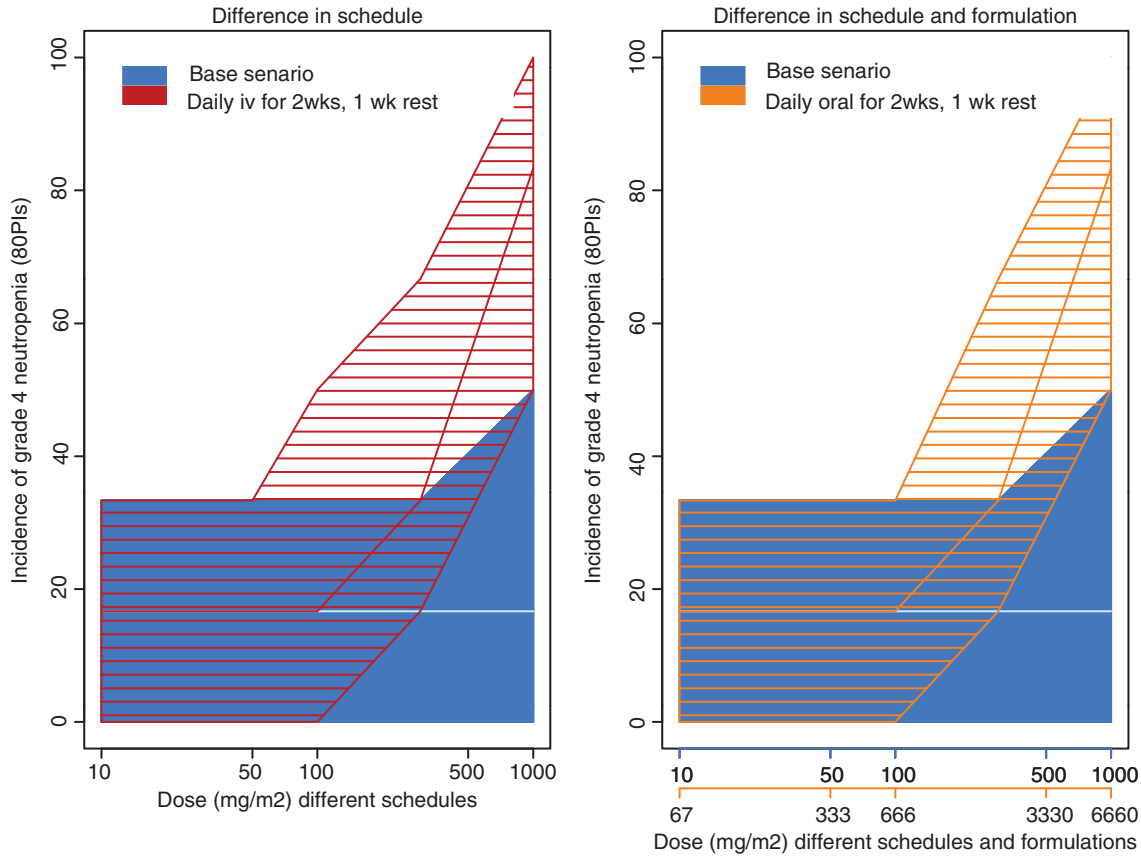

Figure 15. Predicted neutropenia in a human phase I trial in patients with advanced cancer. In the left panel, two different regimes were compared with different schedules: baseline (2 weeks, once-weekly dosing, 3-week cycle) and alternate (2 weeks daily, 3-week cycle). The alternate scenario may reduce other acute toxicities but it has higher risk of increased neutropenia starting at 50 mg m^{-2}. To make the alternate scenario more feasible, administration of oral dosing was explored. Oral dosing appears safe and allows to achieve a drug exposure at which efficacy in preclinical model has been obtained.

with captisol of the mesylate salt) has been used to predict the oral dosing in humans. This study shows a strong dependence on the oral bioavailability of the compound in humans as a function of the dose, mainly due to its limited solubility.

Modeling shows that a dosing of 30 mg m^{-2} IV or equivalently 120 mg PO will not significantly increase the incidence of neutropenia grade 4 over baseline (see Figure 15). Both regimens will deliver an average weekly AUC over the 3-week cycle larger than 104 ng h ml^{-1}. This regimen is expected to have significant antitumor efficacy without increasing the risk of grade 4 neutropenia. In terms of cumulative average exposure, the base and the alternative scenarios are very similar.

5 CONCLUSIONS

The aim of this chapter is to illustrate the ability of physiologically based mechanistic mathematical models to predict pharmacokinetics and toxicity across species. The underlying principle is that toxicity must be interpreted in the physiological context where it is measured (be it *in vitro* or in an animal model). The assumption for scaling and prediction is that the mechanism is the same across species but that the extent and dynamics of the event are dependent on the physiology of the organisms. In other words, the concept is such that physiologically based models account for the underlying biological and scaling differences across systems whereas

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the parameters estimated for toxicity remain the same.

In the first section, various methodologies for predicting the PK in man are reviewed. In conclusion, for extrapolation of human pharmacokinetics, physiologically based models show improved prediction accuracy over more empirical methods. As outlined, it is believed that these models ultimately outperform more empirical methods as they characterize the PK in terms of physiological parameters and drug-related properties. Results on the use of generic simulation prior to *in vivo* studies indicate that some caution is required because certain chemical classes were poorly predicted, and it is recommended that generic PBPK models should only be applied for prioritization after verification of the simulations with *in vivo* pharmacokinetics for a few compounds of a given chemical class.

In the following section, the application of multiscalar cardiac systems models to the anticipation and management of QT prolongation in the clinical electrocardiogram and proarrhythmic risk attending new therapeutic compounds is evaluated. The critical question being asked in the assessment of compounds' propensity for QT prolongation and torsadogenesis in the clinic is essentially one of predicting the incidence of rare clinical events given certain typical response data accumulated through preclinical safety pharmacology (Morganroth *et al*., 1993; Fenichel *et al*., 2004). In our opinion, one of the most exciting horizons for systems modeling of cardiac electrophysiology is the growing capacity to include intrinsic biological variability explicitly.

The *in vivo* concentration of a novel therapeutic does not remain constant in cardiac tissue or any organ for that matter over time. Point estimates of concentration response using well-known exposures are useful for ranking compounds early on; however, the ultimate toxicological profile and clinical utility are intrinsically tied to a drug's pharmacokinetics. The last section aims to integrate both predicted pharmacokinetics and toxicokinetics in the chemotherapeutic-induced neutropenia.

The examples outlined above are important in understanding how such approaches can ultimately: (i) make drug development more efficient and (ii) reduce, replace, and refine the animal experiments going into such predictions. The oncology example is a perfect place to start, by knowing where to expect low levels of toxicity – this process being much more efficient than empirical "x times the exposure in y species" rules. Further simulation of the model helped in choosing the right timepoints for collecting blood, in a nearly random schedule. This enabled us, using population-based approaches, to greatly reduce the number of animals used in these experiments.

Overall, it is believed that the methodology proposed for integrating the available knowledge across experiments to predict toxicology in humans will ultimately make drug development more efficient, cost-effective, and socially more acceptable.

APPENDIX

Glossary

ACAT	Advanced compartmental absorption and transit model
ADME	Absorption, distribution, metabolism, and excretion
AUC	Area under the plasma concentration–time curve
CL	Total clearance
CL_{int}	Hepatic intrinsic clearance
CL_{int} (u)	Unbound hepatic intrinsic clearance
CL_H	Hepatic clearance
C_{max}	Maximal (peak) plasma concentration
F_{up}	Fraction unbound in plasma
F_{ub}	Fraction unbound in blood
GFR	Glomerular filtration rate
Iv	Intravenous
log *D*	Log distribution coefficient at a particular pH
log *P*	Measure of lipophilicity; partition between organic and aqueous phases
PBPK	Physiologically based pharmacokinetics
PK	Pharmacokinetics
pK_a	Ionization constant
PO	Per Os
$T_{1/2}$	Half-life
T_{max}	Time of maximal (peak) plasma concentration
V_{dss}	Volume of distribution at steady state

RELATED ARTICLES

Responses of the Kidney to Toxic Compounds
Hepatotoxicity

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In Silico Tools for Molecular Modeling

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1 INTRODUCTION

Molecule is the smallest unit of matter that retains all of the physical and chemical properties of that substance. It is made up of one or more identical atoms or a group of different atoms bonded together. Chemicals, especially drugs, are the well-known examples of molecules. Similarly, proteins such as toxins, enzymes, etc., lipids and carbohydrates are also known as critical biological molecules in living systems. Their physical, chemical and biological properties are commonly studied by employing several *in vivo* and *in vitro* wet laboratory experimental methods. Although the results obtained are valuable, the time and human resources involved in it are huge.

The use of computers has revolutionized the understanding of molecules significantly. In fact, computer-aided calculation has imbibed itself as an important aspect in the field of scientific research. Computers simplify the calculations, which otherwise may take several years to complete through manual effort. The term *in silico* studies encompasses the methods of performing entire experiments "in simulation" or "in virtual reality" in the computer chips. It provides not only faster but also accurate results and can be performed in the absence of animal models and laborious procedures associated with wet laboratory experiments.

2 MOLECULAR MODELING

Molecular modeling is the science of representing molecular structures numerically and simulating their behaviour with the equations of quantum and classical physics. It is the art of mimicking chemical or biological systems to understand the underlying process concerned, frequently with the help of computers. It involves the development of mathematical models of molecules that can be used to predict and interpret their properties.

The common feature of molecular modeling techniques is the atomistic level (i.e. individual atoms or a small group of atoms) description of the molecular systems. Thus, molecular modeling is a collective term refering to theoretical methods and computational techniques to model or mimic the behaviour of molecules.

Molecular modeling methods are now routinely used to investigate the structure, dynamics and thermodynamics of biological, inorganic and polymeric systems. The types of biological activity that have been investigated using molecular modeling include protein folding, enzyme catalysis, protein stability, conformational changes associated with biomolecular function and molecular recognition of proteins, DNA and membrane complexes.

Modeling is an important skill area for exploring several interesting and challenging problems in biological sciences. Physical models give us the best

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and most direct view of the molecular world. They are easy to use, inexpensive and safe. Molecular modeling is one of the fastest growing fields in science and is no different than any other field of study. It is a research tool that is no more difficult than traditional research tools such as test tubes, beakers or spectrophotometers. The benefit of molecular modeling is that it reduces the complexity of the system, allowing many more particles (atoms) to be considered during simulations.

3 STAGES IN MOLECULAR MODELING

Most molecular modeling studies involve three stages (Figure 1). In the first stage a model is selected to describe the intra- and intermolecular interactions in the system. The two most common models that are used in molecular modeling are quantum mechanics and molecular mechanics. These models enable the energy of any arrangement of the atoms and molecules in the system to be calculated, and allow the modeller to determine how the energy of the system varies as the positions of the atoms and molecules change.

Molecular mechanics is a classical mechanical model that represents a molecule as a group of atoms held together by elastic bonds. Its methods give predictions of molecular geometries and heats of formation. However, quantum mechanics is a quantum mechanical model of the electronic structure of a molecule. It involves solving the Schrödinger equation. Quantum mechanics can be used to predict electronic properties of molecules, such as dipole moments and spectroscopy.

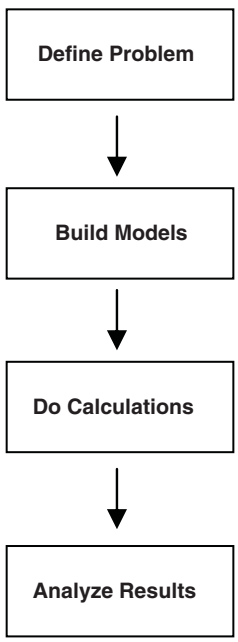

Figure 1. Stages in molecular modeling.

The second stage of a molecular modeling study is the calculation itself, such as an energy minimization, a molecular dynamics or Monte Carlo simulation, or a conformational search. Finally, the calculation must be analysed, not only to calculate properties but also to check that it has been performed properly. For example, a molecular model of a small molecule or protein is built on already known parameters that are known to capture the ideal three-dimensional structure of the molecule. Thus, studied (and averaged) information is used as the stepping-stone in model building and each step needs to be validated.

Classical molecular modeling has dealt with biological molecules which generally have a tree-like structure, but applications to nanotechnology require dealing with more complicated diamond-like structures; it is unclear to what extent this affects the relevant algorithms.

Models using computers are generated using mathematical equations (classical and quantum mechanical) and are evolved based on experimental information that has to be taken into consideration during model building. The basic assumption in mathematical modeling is that the biological properties of any molecule are completely determined by the chemical structure of that molecule. Differences in biological properties ultimately arise from structural differences. A further assumption is that these differences can be expressed in quantitative terms. A modeling study may then be aimed to study an empirical equation that would relate the structures of compounds to their biological properties, for example the strength of a molecular interaction.

Computer models mathematically represent the atomic positions (denoting the coordinates) and molecular geometry, and graphically represent the molecular surfaces and energies of the molecule or a system (critical for evaluating its stability).

4 HISTORICAL OVERVIEW OF MOLECULAR MODELING

History of molecular modeling dates back to mid-nineteenth century during which time the

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development of the structural theory of organic chemistry was also witnessed. Diagrammatic representations of chemical structures are among the earliest forms of molecular model. "Rational formulas" derived from the molecular formula and knowledge of functional groups, or "radicals', was followed by a variety of conventions in grouping the "radicals" together with brackets or parentheses. The three chemistry researchers Archibald Scott Couper, Friedrich August Kekulévon Stradonitz and Aleksandr Mikhailovich Butlerov (1858–1861) introduced the first written structures involving chains of carbons with lines drawn as "bonds" to substituent atoms and groups. The term "chemical structure" was first used at this time. In 1861, Johann Josef Loschmidt developed a collection of 368 molecular structures as graphic displays with atomic spheres. His structures showed relative atomic size, bond distance and bond multiplicity.

In 1865, August Wilhelm Hofmann first recorded use of a physical molecular model in organic chemistry by using metaphor of croquet balls joined by sticks to describe methane, chloroform and other compounds of carbon. The colour scheme which he employed is still widely used, with occasional variations: white for hydrogen, black for carbon, red for oxygen, blue for nitrogen and green for chlorine. To follow universal colour code for molecular models, The Institute of Physics, in addition to the Hofmann's scheme, introduced the following colour codes: yellowish green for fluorine, light green for chlorine, mid-green for bromine, dark green for iodine, yellow for sulphur, purple for phosphorus, black or grey for silicon, and brown, silver or gold for metals.

Crum-Brown (1865) and Sir Edward Frankland and B. F. Duppa (1867) employed two-dimensional drawn versions of ball and stick models. Suggestions on carbon compounds having tetrahedral geometry in 1869 by E. Paterno, and in 1874 by Jacobus Henricus van't Hoff and Joseph Achille LeBel, helped in expanding three-dimensional molecular structure. Work by C. A. Bischoff (1890) and van't Hoff (1898) introduced the concept of "favoured configuration" about a torsional angle

In the beginning of the twentieth century, the concept of the force field was derived from vibrational spectroscopy. It is the forces acting between every pair of atoms in the molecule, or in a lattice in the case of ionic crystals. In 1946, molecular mechanics method, or the empirical force field method, for calculating molecular structures introduced the idea of the use of force field concept in non-physical chemical community. This method was based upon a combination of steric interactions and a Newtonian mechanical model of bond stretching, angle bending and torsional vibrational modes. Three separate groups proposed this method, namely Hill's, Dostrovsky, Hughes and Ingold and Westheimer and Mayer.

In 1950, the work of Barton on the conformations of steroids influenced the thinking of the chemical community on the three-dimensional nature of molecules. This laid the foundation of conformational analysis. From then onward, an appreciation of the three-dimensional aspect became crucial to understanding the structure, stability, conformation and reactivity. In the following year, Pitzer described the torsional potential govern energetics in simple hydrocarbons.

Double-helical DNA structure presented by Watson and Crick in 1953 is considered to be the most famous physical molecular model of all times. In the same year, a group of scientists from Los Alamos published their work carried out on the advanced MANIAC computer. That laid the groundwork for computer-based Monte Carlo methods, established algorithm for simulated annealing and can be considered as the ancestor of molecular dynamics calculations. In 1960, the work of Kitaigorodsky paved way for applications of non-bonding potentials to organic structure modeling.

The first published use of a computer for empirical force field calculations of molecular structure was in 1961 by Hendrickson, who examined the conformations of medium-sized rings. In 1963, Schachtschneider and Snyder reported the derivation of a set of transferable parameters for a valence force field for hydrocarbons. Transferability is crucial to the development of a general force field with a limited number of parameters.

In 1965, Wiberg employed computer force field calculations to address conformational analysis. His steepest descent algorithm is a scheme for geometry optimization. Wiberg also published algorithms for transforming Cartesian coordinates to internal coordinates, to calculate bond lengths, bond angles and torsional angles from these coordinates. He also provided a geometric algorithm to predict the location of the hydrogens on a methylene carbon.

In 1968, Boyd presented the modified Newton–Raphson method for strain energy minimization, which reached convergence more rapidly than the Wiberg algorithm.

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The first computer graphic display of molecular structures was devised at MIT in the mid-1960s. Taking advantage of Project MAC (Multi-Access Computer), one of the first timesharing mainframe computers, Cyrus Levinthal and his colleagues designed a "model-building" program to work with protein structures. This program allowed the study of short-range interaction between atoms and the "online manipulation" of molecular structures. The display terminal (named Kludge) was a monochrome oscilloscope, showing the structures in wireframe fashion.

During the latter half of the twentieth century, force field calculations of molecular structure have developed largely in pace with the development of fast computing machines to carry out the extensive calculations required. Early growth was slow, although, because of unfamiliarity of the average chemist with the programming and use of mainframe computers, and also due simply to lack of access. American scientists made decisive contributions as they had access to computers. Their contributions, such as the Monte Carlo work mentioned above, and the archetypal molecular drawing program ORTEP (Oak Ridge Thermal Ellipsoid Plotter), developed at Oak Ridge National Laboratory in 1965, are clear evidences. Also with the government's support, the first networked computer (the ancestor of the Internet) was demonstrated in October 1969, by establishing a linkage between a computer at UCLA in Los Angeles and another at SRI in Menlo Park.

By the early 1970s, several major force field formulations were reported: ECEPP, UNICEPP, the Consistent Force Field (CFF), the MMI, the EAS, Boyd's and the MUB. These force field models were based on different assumptions, and although they used many of the same or similar potential functions, they were weighted and parameterized differently.

In 1971, Lee and Richards described the molecular surface in the context of protein structure and provided an algorithm to derive it. This involved constructing spheroids about each atom scaled by the van der Waals radius and considering as the surface those portions of these spheroids which were not contained within any other such spheroids. This analysis proceeded through the molecule in a series of planar sections, upon each of which the corresponding projection of the surface was "plotted'. In those days before the advent of modern computer graphics, the surface projection for each planar section was imprinted on a transparency sheet, and these were stacked atop one another to visualize the molecular surface. Expanding upon this work 2 years later, Shrake and Rupley came up with the first dot surface formulation applied to protein structure.

In 1972, Wiberg and Boyd presented an algorithm for exploring conformational interconversions based on systematic modifications of torsional angle. This technique subsequently enjoyed broad use as the dihedral driver method.

Lemieux group first reported computer-aided molecular modeling of oligosaccharides in 1974. Their methodology was much simpler than the other force fields of the time. Also, it was computationally quick and reported satisfactory correlations with the conformational equilibria resulting from NMR studies. Thus, this methodology continues to be popular with carbohydrate chemists even today.

Around the same time, force field formulations for synthetic macromolecules began to appear, such as those used in the conformational analysis of polypropylene by Suter and Flory.

The early 1980s saw the marriage between force field modeling and graphic display on desktop units; the era of personal molecular modeling for the average chemist had begun. A convenient demarcation is the year 1983, when the distribution of the Model (later MacroModel) program by the Still group at Columbia began. This program could be configured to run on a mainframe with a variety of desktop platforms as the user interface. At about the same time, the development of high-quality real-time colour graphics and Connolly's molecular surface program contributed to the rapid evolution of this technique. The graphic presentation of colour-coded molecular surfaces, which displayed at the same time shape, charge and hydrophobicity, provided the modeller with a much higher information density.

In 1986, M. Saunders refocused attention on the central force field model with his STRFIT molecular modeling program. In this method, bond angles and torsional angles are not considered; only forces between pairs of individual atoms are calculated. The resulting structures are quite close to those obtained from MM2. In the following year, Saunders presented the "random kick" algorithm for conformation searching, which was especially helpful in searching over ring conformations.

The history of the World Wide Web began in 1993, when the first graphical browsers became available. Non-graphical Internet applications, such

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as electronic mail and file transfer protocol (FTP), had been developing since 1969, but the vast potential of the graphical user interface can encompass and integrate these functions, and provide the additional enhancements of graphic images.

Within the last 10 years, there have been a number of developments. One is the advent of the Class 2 force fields, which contain anharmonic potentials and utilize explicit off-diagonal terms from the force constant matrix. A not unrelated development is the use of structural data obtained from high-end *ab initio* calculations to parametrize these new force fields. The quantum mechanically derived potential surface is then scaled to reproduce experimental data.

A prediction has been made that the Class 3 force fields will be able to model the influence of chemical effects, electronegativity and hyperconjugation on molecular structure and properties. Another ongoing trend is the development and implementation of virtual reality for molecular modeling. Basically, this will enhance the visual third dimension, further developing and enhancing audio sensation, and introducing tactile sensation. It is unlikely that this type of modeling will be implemented on the desktop platform any time soon.

In Virtual Biomolecular Environment (VIBE) system, a massively parallel computational engine runs an interactive molecular dynamics simulation under the CHARMM force field, and this is interfaced with a 3 m cubic "theatre", called the Cave Automatic Virtual Environment (CAVE). The modeller enters this theatre and interacts with the three-dimensional simulation by means of a wand-link remote control device. The modeller's head and hand movements are tracked with a six-degree-of-freedom electromagnetic sensor to maintain the correct perspective in the virtual environment. The wand may be used to dock a ligand into a binding site, and bumps and other interactions are experienced as sounds.

Improvements in the field of molecular modeling have paved ways to combine the higher accuracy of the computer-based methods with the touch of physical models. In one recent report, stereolithography is used to translate computer-designed structures into physical molecular models. The data from molecular orbital calculations or from neutron diffraction studies were used to guide the fabrication of plastic models by laser curing of an acrylate resin blend. The stereolithography apparatus works by building the model iteratively through a series of "slices', from the bottom up. The scales used were 1 Å = 0.19–0.89 cm, depending on the overall size of the molecule. Molecules, transition state complexes, and "molecular impressions" or shape-complementary surfaces were prepared and post-cured for several hours. Such custom-made models can serve a general need and can be especially helpful for the visually impaired. The preparation by stereolithography of a physical model of an inhibitor plus part of the receptor, guided by the use of modeling software, is a recent advancement.

5 APPLICATIONS OF MOLECULAR MODELING

Molecular modeling techniques are used in the fields of computational chemistry, computational biology and materials science for studying molecular systems ranging from small chemical systems to large biological molecules and material assemblies. Molecular modeling covers the areas of automatic structure generation, analysis of three-dimensional structural databases (structural bioinformatics), prediction of the three-dimensional structure of proteins from their sequence (homology modeling), and so on. Computational calculation of energies of a system, energy minimization, molecular dynamics and Monte Carlo simulations also form a part of molecular modeling.

5.1 Designing Drugs for the Future

Molecular modeling has opened up new avenues for scientists to design new drugs against several not so easily curable diseases such as cancer, AIDS and arthritis. Moreover, development of more potent drugs from existing ones is possible only because of molecular modeling methods.

5.2 Designing New Materials

Apart from bringing new drugs to the market, molecular modeling has the potential to bring a vast array of new materials such as fullerenes, nanotubes and superconducting cuprates (as well as other complex inorganic compounds). Similarly, this exciting area is expected to produce new materials in the optics, ceramic, semiconductor and biomaterials markets.

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5.3 Artificial Photosynthesis

Knowledge in molecular modeling has helped scientists to attempt several improvements in photosynthesis machinery of plants. In the near future, one can expect plants or even microbes with better photosynthetic capacity, thus increasing agricultural productivity.

5.4 Understanding Biomolecules Better

Research using molecular modeling will provide greater insights into various molecules, particularly biomolecules and their interactions, so that the basic mechanism behind several biological reactions can be understood clearly.

6 *IN SILICO* TOOLS IN MOLECULAR MODELING

Several programs and software tools are currently available for *in silico* molecular modeling. Using these, one can visualize, rotate, manipulate and optimize models on a computer display. Although some calculations can be performed in a few seconds, other calculations on molecules, using even the largest computers, can take weeks or months to complete. A list of popular softwares for molecular modeling is presented in Table 1.

Discussing all the tools is out of scope of this book. Some of the common and/or important tools used in molecular modeling are discussed in the following section. For our convenience, the tools to be discussed are classified into several categories based on their use in different molecular modeling protocols.

6.1 Tools for Database Searching

Databases are libraries containing sequences/structural information of proteins and small molecules. Database searching is an important protocol in molecular modeling. With reference to proteins, structural information of similar homologues is useful in model building whereas in drug discovery, one of the main use of database searching is in lead optimization. Database searching can be performed either using text or structural details of the compound under study.

Cambridge Structure Database (CSD) (http://www.ccdc.cam.ac.uk/products/csd/) is a database containing information on the experimentally determined three-dimensional structures of low-molecular-weight compounds that are potential ligands. It contains the crystal structures of more than 250 000 molecules. Similarly, PUBCHEM is an open database that contains the chemical structure and certain physiochemical properties of chemical compounds. However, commercially procured chemicals are compiled in a database called Available Chemical Database (ACD). The commercial Asinex database and Open National Cancer Institute (NCI) database also hold computed structures. The Chembridge database contains the highest number of compounds (more than 700 000).

Protein Data Bank (PDB) (www.rcsb.org/) is a database providing information on the structures of proteins. This database is the only player in this kind of information. Research Collaborators for Structural Bioinformatics (RCSB) is an open databank which is a repository of the three-dimensional structure of proteins (around 50 000) solved through X-ray crystallography, nuclear magnetic electron microscopy, electron microscopy and other such methods.

There are some specialized databases such as the SCORPION2 database, which contains more than 800 records of native and mutant toxin sequences enriched with binding affinity and toxicity information, 624 three-dimensional structures and some 500 references.

6.2 Tools for Homology Modeling

Comparative or homology modeling is one of the three different computational approaches for protein structure prediction. Comparative or homology modeling uses experimentally determined protein structures as templates to predict the conformation of another protein that exhibits amino acid sequence homology. Comparative modeling exploits the fact that evolutionarily related proteins with similar sequences, as measured by the percentage of identical residues at each position based on an optimal structural superposition, have similar structures. A structure similar to the native structure can be predicted if sequence similarity is about or above 35%. Homology modeling is a relatively reliable way to generate a model structure for a given sequence, but

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Table 1. Some of the popular software for molecular modeling.

Software	Application	Availability
Abalone	Biomolecular simulations, protein folding	Not free
AMBER	Model building, molecular dynamics and molecular mechanics	Not free
Ascalaph Designer	Molecular building, molecular dynamics	Free and commercial
Automated Topology Builder	Automated molecular topology building service for small molecules	Free for academic use
Biochemical and Organic Simulation System (BOSS)	Optimized potentials for liquid simulations	Commercial
Chimera	Visually appealing viewer, amino acid rotamers and other building	Free/commercial
CHARMM	Multiple graphical front, molecular dynamics and molecular mechanics	Not free
COSMOS (software)	Force field with fast semi-empirical calculation of electrostatic and/or NMR properties; 3-D graphical molecule builder and viewer	Free (without GUI) and commercial
Culgi	Atomistic simulations and mesoscale methods	Not free
Desmond	High-performance molecular dynamics	Free and commercial
GROningen MAchine for Chemical Simulations (GROMACS)	High-performance molecular dynamics	Free
GROMOS	Biomolecules	Not free
Large-scale Atomic/Molecular Massively Parallel Simulator (LAMMPS)	Soft and solid-state materials and coarse-grain systems for molecular dynamics	Free
MacroModel	Conformational sampling, minimization, molecular dynamics	Not free
Materials Studio	Materials simulation technology	Closed source/trial available
MDynaMix	Parallel molecular dynamics	Free
Molecular Operating Environment (MOE)	Molecular modeling, molecular dynamics, molecular mechanics, molecular visualization	Commercial
PyMOL	Molecular graphics and modeling package	Free for academics
Sirius	Molecular modeling and analysis system	Commercial
Virtual Molecular Dynamics (VMD)	Fast, parallel molecular dynamics	Free
WHAT IF	Visualizer for MD; interface to GROMACS	Not free
YASARA	Molecular-graphics, -modeling and -simulation program	Not free
Zodiac (software)	Drug design suite	Commercial

is limited to those sequences with high similarity to a structure which has already been solved.

The process of comparative model building is conceptually straightforward (Figure 2). First, an alignment is performed between the sequences for which the structure has been determined by experimental methods (the parent) with the sequence to be modelled (the target). This sequence alignment is used to construct the initial model (sometimes referred to as a framework or template) by copying over some main chain and side chain coordinates from the parent structure based on the equivalent residue in the sequence alignment. Side chains must be built for residues in the target that does not correspond to an identity in the alignment, and for residues where the side chain conformation is thought to vary in the target relative to the parent structure. Main chains must be built in case of insertions, regions surrounding a deletion and other regions of suspected main chain variation. Finally, model refinement and evaluation is carried out in which the structure obtained is refined using energy minimization and validated using appropriate softwares.

6.2.1 Modeller

MODELLER (http://salilab.org/modeller/) is a computer program used to model 3D structure of proteins and for homology/comparative modeling of protein 3D structures. The user provides an alignment of a sequence to be modelled with a known related structure, and MODELLER automatically calculates a model containing all non-hydrogen

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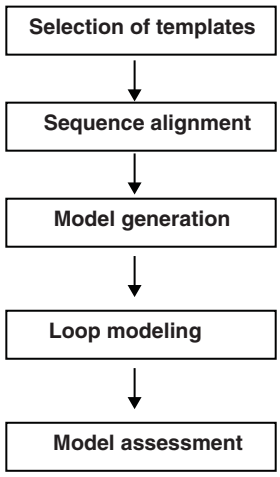

Figure 2. Steps in homology modeling.

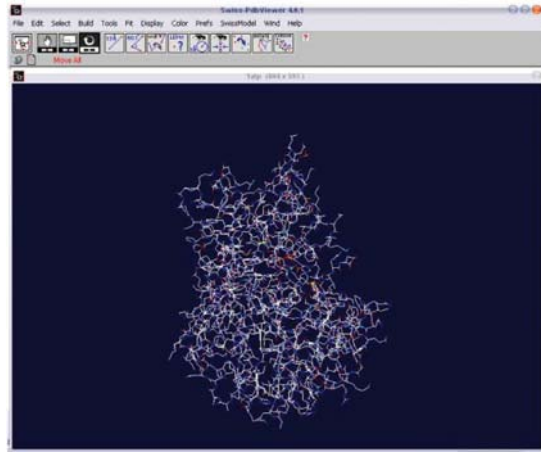

Figure 3. Screenshot of Swiss-PDB viewer.

atoms. A 3D model is obtained by optimization of a molecular probability density function (pdf). The molecular pdf for comparative modeling is optimized with the variable target function procedure in Cartesian space that employs methods of conjugate gradient and molecular dynamics with simulated annealing. It can also perform many additional tasks. It implements comparative protein structure modeling by satisfaction of spatial restraints.

6.2.2 Swiss-PDB Viewer

Swiss PDB viewer (http://www.expasy.org/spdbv/) is a stand-alone molecular visualization and molecular modeling software. It incorporates many useful functions such as calculating molecular surfaces, electrostatic potentials, high-quality rendering, analysis of torsion angles and energy minimization. It can also be used to prepare input for homology modeling using Swiss-MODEL web server, an automatic web-modeling server (Figure 3).

6.3 Tool for Threading/Fold Recognition-Based Structure Prediction

6.3.1 PHYRE Server

The PHYRE (http://www.sbg.bio.ic.ac.uk/phyre/) is an automatic fold recognition server for predicting the structure and/or function of protein sequence under study. Originally developed in 2004, Phyre was one of the top-performing stand-alone protein structure recognition systems. It combines primary and secondary structure profile information using highly optimized profile–profile comparison algorithms. The predictive power of this server is similar to that of other publicly available software, and the most recent Critical Assessment of Structure Prediction (CASP) competition judged the latest Phyre version to be one of the best servers for preliminary assessment of protein structures.

6.4 Tool for Ab Initio Structure Prediction

6.4.1 Robetta Server

The Robetta server (http://www.bioinfo.rpi.edu/~bystrc/hmmstr/server.php) is an automated protein structure prediction service offered by the Baker laboratory for non-commercial *ab initio* and comparative modeling. It has been rated as performing among the best in the automated server prediction category. It is a protein structure prediction on the Berkeley Open Infrastructure for Network Computing (BOINC) platform.

6.5 Tools for Docking

Molecular docking can be defined as the prediction of the structure of receptor–ligand complexes, where the receptor is usually a protein and the ligand is either a small molecule or another protein. In simpler terms, docking is a process by which two molecules fit together in three-dimensional space.

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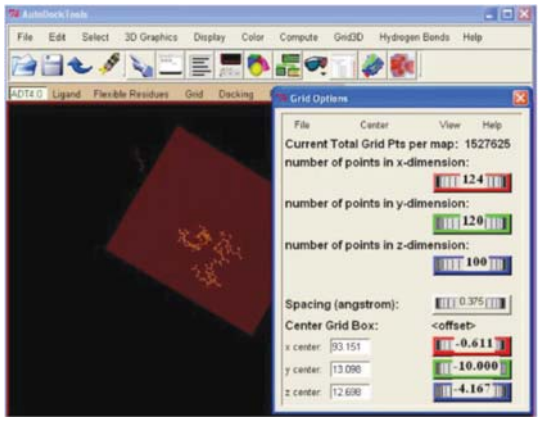

Figure 4. Screenshot of AutoDock tool.

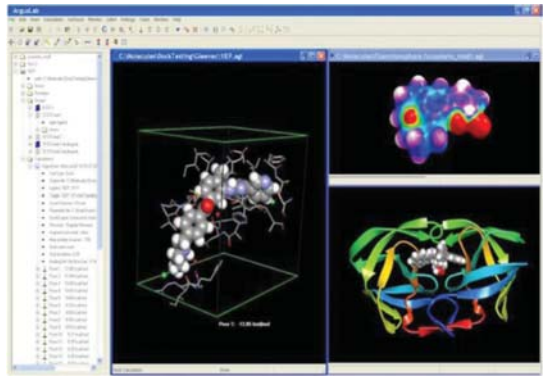

Figure 5. Screenshot of ArgusLab tool.

There are many software programs available (both commercial and free) for docking. Freewares include DOCK 6.1, AUTODOCK and HEX. Commercial includes FLEXX of Tripos Inc. and GLIDE of Schrodinger Inc.

6.5.1 Autodock

AutoDock is a suite of automated docking tools (Figure 4). It is designed to predict how small molecules, such as substrates or drug candidates, bind to a receptor of known 3D structure. It actually consists of two main programs: Auto Dock performs the docking of the ligand to a set of grids describing the target protein; Auto Grid pre-calculates these grids. In addition to using them for docking, the atomic affinity grids can be visualized.

AUTODOCK employs molecular dynamics to predict the docking of a flexible ligand to a binding site of a rigid protein, given the region of the protein containing the binding site and the substrate. It is downloadable from http://autodock.scripps.edu/ webpage.

6.5.2 HEX

HEX is an interactive protein docking and molecular superposition program, written by Dave Ritchie. HEX understands protein and DNA structures in PDB format. HEX will run on most Windows-XP, Linux and Mac OS X PCs. For Academic and governmental users, a runtime version of HEX may be downloaded free of charge. On multi-processor Linux systems, docking calculation times can be reduced in almost direct proportion to the number of CPUs used. This commercial docking tool is available for download at http://www.loria.fr/∼ritchied/hex/ (or) http://www.csd.abdn.ac.uk/hex/.

6.5.3 ArgusLab

ArgusLab is a tool that includes spectroscopy, graphics and visualization, drug docking and high-level *ab initio* calculations (Figure 5). It is a complete molecular modeling system for platforms running on Microsoft Windows operating systems. ArgusLab contains an interactive 3D molecule builder that allows the user to build and manipulate complex molecular structures. ArgusLab also contains a rich suite of computational methods, both quantum mechanical and molecular mechanical, for calculating ground- and excited-state properties. It is downloadable for personal use from http://www.arguslab.com/downloads.htm.

6.5.4 VegaZZ

VegaZZ is a free tool for molecular mechanics. It has rich 3D graphical features and can be employed to build our own databases using specialized tools embedded in VegaZZ. VegaZZ is the evolution of the well-known VEGA OpenGL package. VEGA was originally developed to create a bridge between most of the molecular software packages only, but in the years, enhancing its features, it has evolved to a complete molecular modeling suite. This software is free for non-profit academic uses and can be downloaded from www.ddl.unimi.it after registration.

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6.5.5 TINKER

TINKER (http://dasher.wustl.edu/tinker/) is a complete package for performing empirical force field molecular mechanics and dynamics calculations. It is intended to serve as a platform for algorithm development and parameterization, while still being efficient enough for most production work. Programs are provided to perform many functions including:

- energy minimization over Cartesian coordinates, torsional angles or rigid bodies;
- molecular, stochastic and rigid body dynamics with periodic boundaries and control of temperature and pressure;
- normal-mode vibrational analysis;
- building protein and nucleic acid structures from sequence;
- location of a transition state between two minima;
- free energy calculations via free energy perturbation or weighted histogram analysis;
- fitting of intermolecular potential parameters to structural and thermodynamic data.

6.6 Tools for Molecular Visualization

Molecules including biomolecules such as proteins, DNA and RNA can be observed on computer screens using specialized tools termed as molecular visualization tools. They help researchers to observe the molecules in three-dimensional structure and also with different formats such as ball and stick, wireframe, ribbon, etc. They help to differentiate different regions of molecules as they use different colours to represent different atoms.

6.6.1 RasMol

RasMol **(**http://www.umass.edu/microbio/rasmol) is a free program developed by Roger A. Sayle (1993). Rasmol is a molecular graphics program for visualization of proteins, nucleic acids and small molecules. The name Rasmol has been derived from Raster (the array of pixels on a computer screen) Molecules. The powerful program is aimed at display, teaching and generation of publication quality images. It is easy to use and produces beautiful, space-filling, coloured, three-dimensional images.

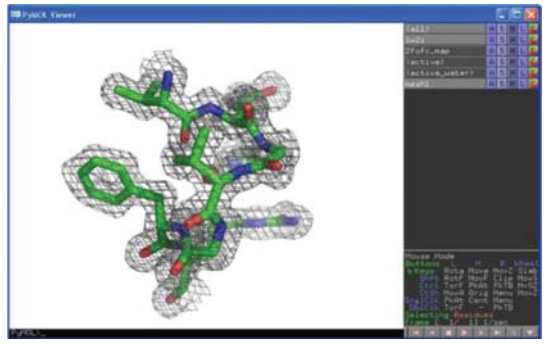

Figure 6. Screenshot of PyMol Viewer tool.

6.6.2 Pymol

The PyMOL application **(**http://pymol.sourceforge.net/**)** was designed to be a molecular visualization system (Figure 6). It is well suited to produce high-quality 3D images of small molecules and biological macromolecules such as proteins. It is equally good for animating molecules dynamically, giving live 3D presentations and sharing interactive visualizations. This open source tool was created by Warren Lyford DeLano. The Py portion of the software's name refers to the fact that it extends, and is extensible by the Python programming language.

6.7 Tools for Molecular Drawing/Editing

6.7.1 ISIS/Draw

Isis/Draw (www.symyx.com/downloads/) is a freely available, chemically intelligent drawing package from MDL Information Systems for Windows and Macintosh. You can insert your ISIS/Draw sketches into documents, Web pages, spreadsheets and presentations. You can also use ISIS/Draw to create structures to register into 2D and 3D molecule, polymer and reaction databases, and to create queries for searching these databases.

6.7.2 Chemsketch

ACD/ChemSketch (www.acdlabs.com/download/) is an advanced chemical drawing tool and is the accepted interface for the industry's best NMR and molecular property predictions, nomenclature and analytical data handling software (Figure 7). ACD/ChemSketch is also available as freeware, with functionalities that are highly competitive with other popular commercial software packages. The freeware contains tools for 2D structure cleaning,

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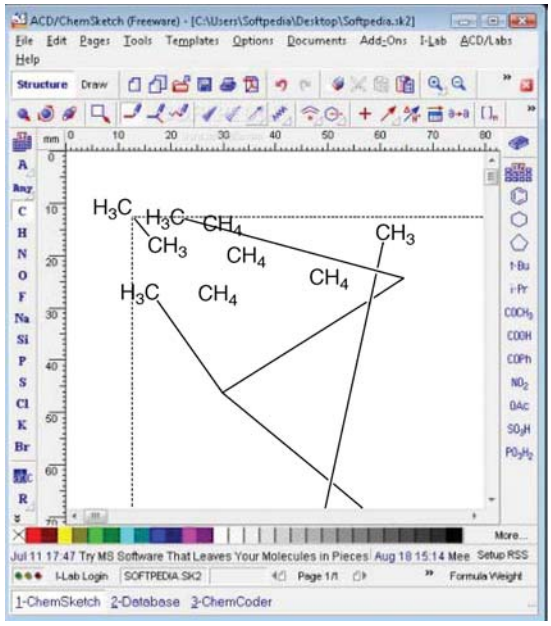

Figure 7. Screenshot of Chemsketch tool.

3D optimization and viewing, InChI generation and conversion, drawing of polymers, organometallics and Markush structures–capabilities that are not even included in some of the commercial packages from other software producers.

6.7.3 ChemDraw

ChemDraw (www.cambridgesoft.com/) is a molecule editor developed by the chemoinformatics company CambridgeSoft. ChemDraw is, along with Chem3D and ChemFinder, part of the ChemOffice suite of programs and is available for Macintosh and Microsoft Windows.

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In Silico Toxicology Prediction Using Toxicogenomics Data

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1 INTRODUCTION

Toxicogenomics holds the promise of unprecedented advances in two broad, overlapping fields: mechanistic or investigative toxicology, and predictive toxicology. Mechanistic or investigative toxicology is the area of toxicology focused on biological responses to drug toxicity, and provides important perspectives on risk assessments of various compounds. In contrast, predictive toxicology focuses on identifying the potential toxicity of compounds (Cunningham and Lehman-McKeeman, 2005; Boverhof and Zacharewski, 2006). In previous studies, toxicogenomics has contributed to in-depth investigations of molecular mechanisms and the modes of toxin/chemical/environmental stressor action that was difficult to be achieved by conventional toxicological approaches. At the same time, expanding toxicogenomic data has promoted a valuable platform for the establishment of biomarkers to predict a compound's toxicity.

The progress of toxicogenomics has been supported by DNA microarray technology, a powerful tool for directly monitoring patterns of cellular perturbations through the identification and quantification of global shifts in gene expression resulting from pathological alterations within cells and tissues. Microarrays provide a large amount of transcriptional expression data for thousands of individual genes under various experimental conditions. Bioinformatics technologies can determine which genes are meaningful, facilitating the analysis of huge pools of toxicogenomics data in mechanistic and predictive toxicology. Meaningful genes are referred to as "signature genes" with characteristic gene expression profiles for exposure or toxicological response to specific classes of toxic compounds. This chapter is devoted to computational approaches for the data mining of biomarker genes from toxicogenomics data, leading to toxicity prediction.

2 MICROARRAY DATA FOR TOXICOGENOMICS

Microarray techniques have been applied not only to toxicogenomics but also to various clinical purposes, such as disease classification and patient diagnostics. These applications have revealed the challenges of achieving reproducibility and stability of outcomes with microarray technologies caused by cross-platform chips and differing experimental

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conditions. Therefore, data sets for constructing reliable prediction models must be obtained with the same platform and experimental conditions. For this purpose, many public databases for toxicologically relevant microarray data have been developed, including the Comparative Toxicogenomics Database (CTD) (Mattingly *et al*., 2006), Environment, Drugs and Gene Expression database (EDGE) (Hayes *et al*., 2005), and Chemical Effects in Biological System (CEBS) knowledgebase (Waters *et al*., 2003), among others.

In 2002, we began the toxicogenomics project (TGP), a public–private collaborative project of the National Institute of Health Sciences, the National Institute of Biomedical Innovation, and 15 pharmaceutical companies in Japan (Urushidani and Nagao, 2005). With an emphasis on the uniformity of data quality, TGP has generated a large-scale toxicology database of transcriptomes intended to predict the toxicity of new chemical entities in the early stages of drug development. Drug reactions such as efficacy and toxicity are associated with the dosage and time course after treatment, and so precisely monitoring drug reactions requires multiple dose- and time-dependent experiments for each drug. Thus far, about 150 chemicals, primarily medicinal compounds, have been selected for the database. Over 27 000 gene expression profiles have been compiled for multiple doses and times in rat livers and kidneys, as well as rat and human hepatocytes, through comprehensive analysis using the Affymetrix GeneChip® Affymetrix, Inc., Santa Clara, CA, USA. These gene expression profiles, conjugated with histopathological changes, blood biochemical examination results, and the other phenotypic profiles, are stored in our database with a web-based tool for statistical analysis, genomics-assisted toxicity evaluation system developed by the toxicogenomics project in Japan (TG-GATEs). Thirteen of the 150 chemicals were typical nephrotoxicants or drugs showing clinical side-effects (e.g., cisplatin, carboplatin, gentamicin, vancomycin, phenacetin, and bucetin), and 20 chemicals exhibited nephrotoxicity in addition to hepatotoxicity (e.g., phenylbutazone, ethionine, and indomethacin).

3 IDENTIFICATION OF BIOMARKER GENES WITH TOXICOGENOMICS DATA

The first step from toxicogenomics to mechanistic and predictive toxicology is the identification of an individual gene or a cluster of genes detective or predictive of certain types of toxicity; these "signature genes" are employed as biomarkers. A biomarker is defined by the International Programme on Chemical Safety (IPCS) of the WHO as any substance or its product, structure, or process that can be measured in the body and that can influence or predict the incidence of disease outcome. The ideal biomarker provides a sensitive, informative, and reproducible indicator of potential adverse effects at times or doses preceding overt tissue damage, toxicity, or disease initiation. The discovery and validation of biomarkers is useful for application in high-throughput experimental systems to characterize target organ effects and to detect specific toxicity end-points in the early steps of a compound's development. The identification and utilization of biomarkers through toxicogenomics have several further applications: from current use in pre-clinical toxicology to risk characterization and risk assessment of chemicals; from early clinical stages of drug development to the later stages; and even into daily clinical use in diagnostics, disease classification, and therapeutic monitoring.

4 GENE SELECTION FROM MICROARRAY DATA

Identifying biomarker genes in huge sets of microarray data is referred to as the gene selection problem. In selecting genes from a microarray with good separation between toxic and non-toxic drug-treated samples, one seeks the significant genes that are affected by the adverse drug effects, or even those that caused the adverse reaction. This is a key step toward understanding mechanistic and predictive toxicology through the underlying biological process.

Gene selection is also relevant in the classification problems in machine learning, in which the class of toxic response (including non-toxic responses) of a sample (e.g., drug-treated organ or tissue) is determined by a classifier. A sample is represented as a feature vector $\mathbf{x}$. Each dimension in the feature vector $\mathbf{x}$ holds the expression value of a particular gene, which is obtained from a DNA microarray experiment. The classifier is constructed by inputting N feature vectors (called training data) with known toxic response outcomes into the machine-learning algorithms. However, because of the low number of samples (N: the number of the feature vectors)

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and the high number of observed genes [dim(**x**): dimension of each feature vector], using all genes to classify the samples into good and bad outcomes incurs a high risk of over-training (or over-fitting). Over-training, in this case, means including the noise in the data, which may increase the generalization error (the error rate of the resulting predictor on samples that were not used during the training phase). This may yield a result that achieves high accuracy levels for the training data, but does not generalize to new data. The underlying problem is that if the sample size is much smaller than the number of genes, then one can distinguish different types of toxicity based on the noise present in these measurements, rather than on distinct biological characteristics of their gene expression levels. One approach to overcome this serious problem is to reduce the number of genes by removing irrelevant and redundant features, a method known as "feature selection" in computer science.

5 FEATURE SELECTION ALGORISMS

Feature selection methods can be broadly categorized into the filter model and the wrapper model. The filter model separates feature selection steps from the machine-learning algorithm, and relies on the general characteristics of the training data to select features (Figure 1a). The wrapper model uses the predictive accuracy of a pre-determined learning algorithm to determine the quality of a selected subset (Figure 1b).

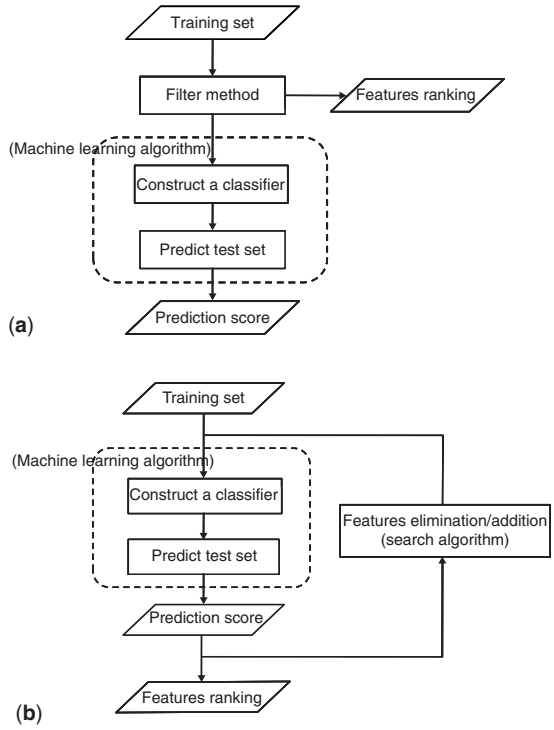

Figure 1. Calculation procedures of filter method (a) and wrapper method (b).

5.1 Filter Methods

Traditional methods in gene selection fall within the filter model. Filter approaches remove irrelevant features based on a ranking of all the genes according to their individual relevance or discriminative power to the target class. Filter approaches generally follow three simple steps (Figure 1a), they: (a) rank all the genes in the microarray data using a filter method, (b) choose the top-ranked $n-1$ genes as the best feature gene subset, and (c) construct a classifier in learning algorithm using the selected $n-1$ genes. The filter-type feature selection (steps 1 and 2) is independent of the classifier-learning algorithm (step 3). One major problem with the filter model is the selection of a threshold for discarding irrelevant features. All the features are given a score by the filter algorithm, but determining the optimal threshold "n" for the data is difficult. We must choose the threshold "n" using some appropriate criteria.

Filter methods are selected to produce the most relevant possible ranking. Many methods have been developed based on statistical tests. They are:

1. t-Statistic: This criterion measures the worth of a feature gene by computing the value of the t-statistic between toxic and non-toxic classes (Thomas *et al.*, 2001; Tsai, Chen and Chen, 2003).
2. ReliefF: This is a feature-weighting algorithm sensitive to feature interactions. The key idea of ReliefF is to rate features according to how well their values distinguish among instances of different classes and how well they cluster instances of the same class (Kononenko, 1994). To this end, ReliefF repeatedly chooses a single instance at random from the data, and then locates the nearest instances of the same class and the nearest instances pertaining to different

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classes. The feature values of these instances are used to update the scores for each feature.

3. Correlation-based feature selection (CFS): This method evaluates a subset of features by considering the individual predictive ability of each feature as well as the degree of redundancy among them (Hall, 2000),

$$\mathrm{CFS} = \frac{k\bar{\tau}_{cf}\, f}{\sqrt{k + k(k-1)\bar{\tau}_{ff}}} \qquad (1)$$

where CFS is the score of a feature subset containing k features, $\bar{\tau}_{cf}$ is the average of the correlation coefficients between features and classes, and $\bar{\tau}_{ff}$ is the average intercorrelation among features of the subset.

5.2 Wrapper Methods

Wrapper approaches to gene selection evaluate the prediction performance of a learning machine trained for each given feature subset using a search algorithm to explore possible combinations of features. The strategy of wrapper approaches is composed of four steps (Figure 1b):

1. Choose a machine-learning algorithm to evaluate the prediction score of a feature subset. Many machine-learning algorithms have been developed, including the Naïve Bayes classifier, linear discriminant methods, support vector machines (SVM) (Vapnik, 1998), and artificial neural networks (Bishop, 1995). Cross-validation (CV) is often used for scoring prediction performance.
2. Choose a search algorithm.
3. Perform the search algorithm and note the best subset encountered.
4. Output the encountered subset with the best score.

Search algorithms compare all possible feature combinations, which are usually too numerous to be exhaustively explored. Of these search methods, greedy methods (forward selection or backward elimination) are the most popular. At each round of CV, forward selection adds the best feature; backward elimination deletes the worst feature.

5.3 Recursive Feature Elimination with Support Vector Machine (RFE-SVM)

This is a type of wrapper algorithm that couples recursive feature elimination with linear SVM (Guyon *et al.*, 2002). In a linear SVM, the decision function (classifier) is given as $f(\boldsymbol{x}) = \boldsymbol{w}^{\mathrm{T}}\boldsymbol{x} + b$ or $f(\boldsymbol{x}) = \sum_{k=1}^{n} w_{\mathrm{k}} x_{\mathrm{k}} + b$. For a given feature x_{k}, the absolute value of its weight w_{k} indicates the significance of its contribution to the margin of the linear SVM and to the construction of a linear classifier. Hence, the weight w_{k} is used as a feature-ranking coefficient in RFE-SVM. This algorithm first constructs a linear SVM classifier from the microarray data with n genes; then, the gene with the lowest w_{k}^2 is removed and another classifier is trained on the remaining $n-1$ genes. This process is repeated until only one gene remains.

A gene ranking is produced from the order in which the genes were eliminated, and the last remaining gene is the most relevant gene. However, because of computational cost considerations, the algorithm is often implemented in such a way that several features are reduced at once. In such implementations, the method produces a feature subset ranking instead of a feature ranking. The optimal signature genes are the gene sets with the best prediction performance throughout the elimination process.

5.4 Filter Approaches Versus Wrapper Approaches

The filter methods are useful in practice because they are much faster than wrapper methods. Filters exclude irrelevant genes but cannot remove redundant genes because methods that select important genes based on individual gene information fail to account for mutual information among genes. The problem of redundancy among selected genes is twofold: the selected gene set may less comprehensively represent the target class than one of the same size without redundant genes would and in including all representative genes, redundant genes unnecessarily increase the size of the selected gene set, which will in turn affect the mining performance of the small sample.

In contrast, wrapper methods were developed to select discriminative genes while decreasing gene

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redundancy. In theory, the wrapper model should provide more accurate classification results than the filter model (Langley, 1994). Wrappers use classifiers to estimate the usefulness of feature subsets. The use of optimal feature subsets should provide corresponding classifiers with better classification accuracy because the features are selected according to their contribution to the classification accuracy of the classifiers. The disadvantage of the wrapper approach is its computational expense because the classifier must be repeatedly constructed to evaluate a subset during the CV process. Moreover, wrapper-type feature selection is sensitive to training data and runs the risk of over-fitting, leading to a lack of robustness in the selected gene set.

6 ROBUSTNESS OF THE SELECTED GENE LIST

Although many methods have been developed for conducting feature selection on microarrays, these selection methods produce selected gene lists that are insufficiently robust. If the predictor developed with one selected gene set would work well on data from other studies, then we would not have had to worry about list diversity. However, a lack of transferability of predictive power is often observed as a result of the same reason that causes instability of gene lists. Ein-Dor, Zuk and Domany 2006 found that the gene lists developed from microarrays using different methods in prognostic cancer studies differ significantly, even for different subsets of the same microarray data sets. They concluded that thousands of samples are needed for robust gene selection. As generating a more stable gene list will lead to more robust predictors, we must evaluate not only prediction performance but also the robustness of gene sets in feature gene selection. Assessing the stability of selected gene lists is crucial to guarantee their controlled and reliable utilization.

7 APPLICATION OF FILTER AND WRAPPER METHODS TO DIAGNOSTICS OF DRUG TOXICITY USING TOXICOGENOMICS DATA

Our previous study on concurrent diagnosis of drug-induced tubular injury using TGP data demonstrates the application of filter- and wrapper-type methods (Kondo *et al*., 2009). Drug-induced renal tubular injury is a major concern in pre-clinical safety evaluations. In this study, we analyzed 33 nephrotoxicants and eight non-nephrotoxic hepatotoxicants to elucidate time- and dose-dependent global gene expression changes associated with proximal tubular toxicity. The compounds were administered orally or intravenously once daily to male Sprague–Dawley rats. The animals were exposed to four different doses of the compounds, and kidney tissues were collected on days 4, 8, 15, and 29. High-dose groups of 23 compounds that caused necrosis, degeneration, or regeneration in the renal tubules during chronic exposure were defined as the positive set (other high-dose groups of 10 nephrotoxicants were used as the external test set). Low-dose groups of all 41 compounds and high-dose groups of the eight hepatotoxicants that had no histopathological findings were defined as the negative set. To perform supervised classification algorithms after selecting differentially expressed genes, the microarray samples treated with nephrotoxicants and hepatotoxicants were divided into positives and negatives of the training set according to their histopathological findings.

Both filter- and wrapper-type gene selection algorithms with SVM-learning algorithms were used to extract biomarker candidates and construct classifiers using the selected genes. RFE methods were used for the wrapper-type gene selection algorithms and intensity-based moderated *t*-statistics (IBMT; Sartor *et al*., 2006) was used as a filter-type gene selection algorithm (SVM was used as the classifier in this case).

Fivefold CV was executed for optimization of the classifiers and to calculate their prediction accuracies. First, the whole positive and negative training data sets were randomly divided into five subsets of roughly equal size. The SVM was trained with a selection of optimal genes on four subsets and then applied to the fifth subset as the test data set. Before the SVM was trained, optimal genes were selected from the training sets with both RFE-SVM and IBMT-SVM. The 99 top-ranked genes from each selection strategy were used to construct the classifiers. The prediction model using the top-ranked 99 feature genes exhibited saturated prediction performance (Figures 2 and 3). Thus, by tracing the prediction performance of each constructed classifier for the test sets, we determined an appropriate

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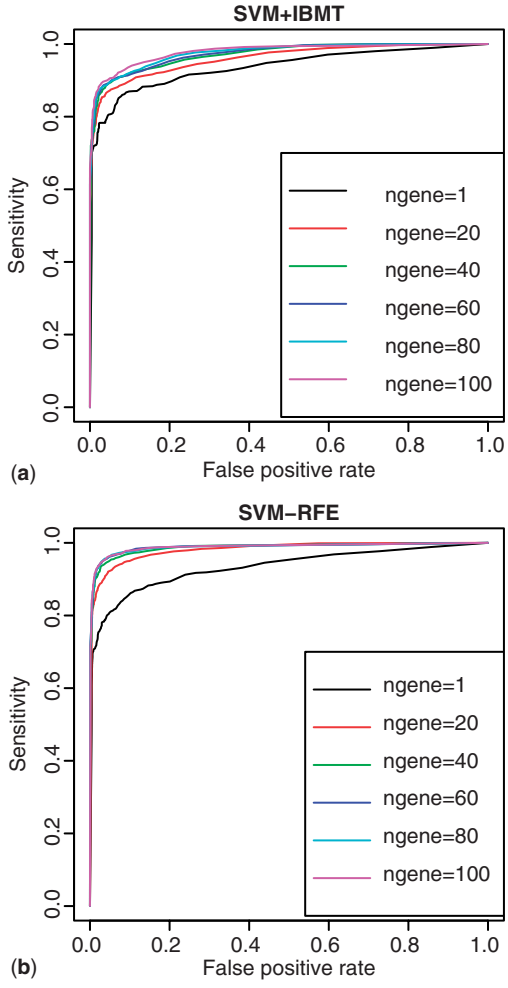

Figure 2. Receiver operating characteristic (ROC) curves for feature gene subsets selected using SVM-IBMT (a) and SVM-RFE (b).

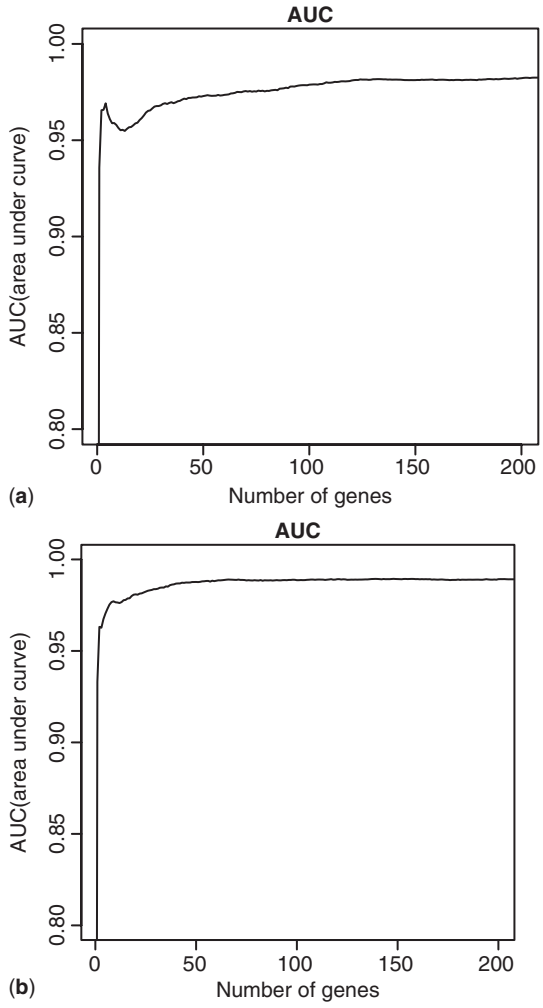

Figure 3. Area under curves (AUC) of the ROC curves by SVM-IBMT (a) and SVM-RFE (b), along with the number of selected feature genes.

threshold number of feature genes, which is one of the challenges in executing search algorithms.

As the result of fivefold CV, we determined the sensitivity of each classifier to be 94% (RFE-SVM; 99 probes) and 93.8% (IBMT-SVM; 99 probes), when we allowed for 10% false positives (Figure 2). Although SVM-RFE exhibited the higher classification accuracy, as we expected, the concordance rate of the feature gene list selected by RFE between the subtraining sets of fivefold CV was lower than that of the filter-type IBMT-SVM (Figure 4). The difference in concordance rate indicates that the RFE-SVM classifier may have over-fitted to the training set, resulting in an insufficiently robust list of biomarkers. In contrast, the feature genes selected by IBMT-SVM were stable between different training data sets generated through fivefold CV. As noted above, in addition to the prediction performance of the constructed classifier, the robustness (stability) of the selected gene sets is critical to successful feature selection. Therefore, in this case using TGP kidney data sets, we concluded that the filter-type IBMT-SVM method was the preferable gene selection and classification algorithm.

Consequently, we determined the appropriate maker gene set using the following criteria: (a) the prediction accuracy was saturated (Figure 3); (b) the

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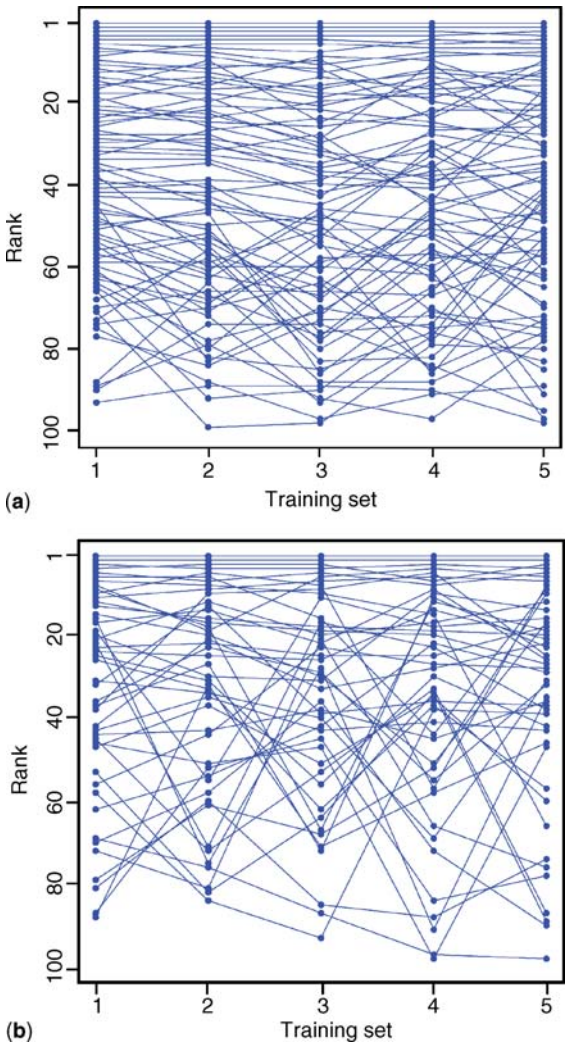

Figure 4. Perturbation of feature gene ranking between different training sets generated during fivefold CV. The horizontal axis indicates the training subsets and the vertical axis indicates the ranking of the feature genes (top 80 probes). Fivefold CV was executed by randomly dividing samples.

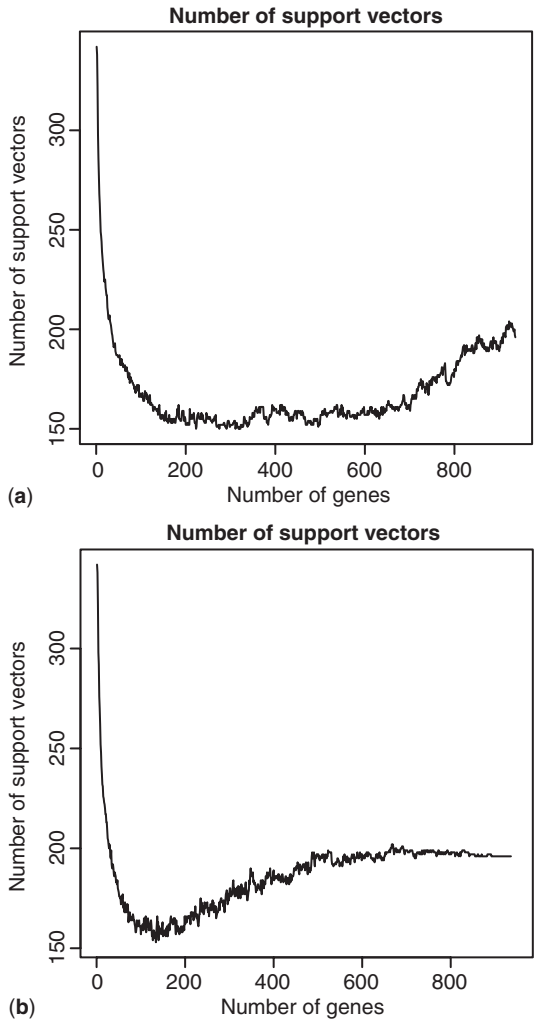

Figure 5. The number of support vectors in the constructed models of SVM-IBMT (a) and SVM-RFE (b), along with the number of selected feature genes.

number of support vectors was adequately low (an excessive number of support vectors could indicate over-fitting of the linear SVM classifier) (Figure 5), and (c) the number of feature genes was substantially lower than the number of samples, to avoid over-fitting.

Also, the selected gene list contained enough key genes to interpret their biological relevance in drug-induced renal tubular injury. The gene list contained well-known biomarkers, such as kidney injury molecule 1, ceruloplasmin, clusterin, and tissue inhibitor of metallopeptidase 1, as well as novel biomarker candidates. Most of the genes involved in tissue remodeling, immune/inflammatory response, cell adhesion/proliferation/migration, and metabolism were predominantly up-regulated. Down-regulated genes participated in cell adhesion/proliferation/migration, membrane transport, and signal transduction. This indicates that the gene list provides us with elaborate knowledge about mechanistic toxicology.

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8 CONCLUSIONS

Selecting a small gene set from large microarray data sets is critical from both biological and computational viewpoints, and many algorithms have been developed for feature selection. Most studies on feature selection have found that wrapper methods are superior to filter methods, but many of these studies have over-emphasized prediction accuracy and over-looked the robustness of the selected genes. Prediction reliability assumes the stability of the model. In fact, this study illustrates that IBMT-SVM produces more stable gene lists than RFE-SVM. This finding is adaptable to only this training set. In the case of other training sets, we must evaluate multiple methods and choose the best approach. Therefore, we have to carefully gauge not only prediction performance but also the robustness of gene sets in feature gene selection.

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Systems Modeling in Developmental Toxicity

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1 INTRODUCTION

An individual starts off as a single cell, the progeny of which form complex structures that are themselves integrated into progressively larger systems. Developmental biology is concerned with how this cellular complexity and patterning arises through orchestration of cell divisions, cell migrations, cell interactions, cell death, gene regulation, and differentiation. Any agent interfering with these cellular processes can potentially invoke adverse effects on pregnancy outcome leading to miscarriage, low birth weight, structural malformations, and functional deficits. Windows of vulnerability open and close that render the embryo more or less susceptible to drug and chemical exposure at different times and locations. Developmental toxicology is concerned with understanding these underlying genetic and environmental influences and assessing the health risks from prenatal exposure to drugs and chemicals.

Formal testing of drugs and chemicals for developmental toxicity began following thalidomide, a drug prescribed for morning sickness that went on the market in 1958. In 1961, reports began to appear of children born with a rare limb reduction syndrome (phocomelia) increasing to over 10 000 cases worldwide. Association of these cases with maternal intake of thalidomide led to the establishment of federal guidelines for pre-clinical testing to evaluate reproductive and/or developmental toxicity for compounds regulated by the Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA). The conventional testing paradigm for prenatal safety assessment has since been in use for over 30 years and involves gestational exposure of pregnant dams of two species, usually rats and rabbits, during organogenesis and evaluation of fetal and maternal effects near term (Kimmel, Kimmel and Euling, 2006). The necessity for one rodent and one non-rodent test species was specifically demonstrated in this case; whereas rats displayed systemic toxicity with Thalidomide *in utero*, rabbits exhibited phocomelia, the common human side-effect.

Dozens of compounds that potentially disrupt development in animals and humans have been cataloged over the years (Shepard and Lemire, 2007). Unfortunately, we lack developmental toxicity data for most of the >80 000 chemical compounds to which humans may be exposed (Hartung, 2009). Assessing health risks to the various chemicals alone or in combination is an immense challenge that, under present methods, is hardly tenable. Priorities must be set, leading to an urgent need for strategies to more efficiently test chemicals for a potential risk to human populations. This has led to the critical evaluation of our testing strategy and

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in particular the role of conventional *in vivo* animal studies, newer exposure metrics, structure–activity relationship (SAR) models, and high-throughput *in vitro* profiling of chemicals for biological activity.

Toward this end, large-scale efforts are underway to address the data gaps using industrial-scale methods for chemical screening and prioritization, coupled with innovative computational approaches for predictive modeling of human toxicology. The national importance of this effort is evidenced by "Toxicity Testing for the 21st century" (Tox21), a federal consortium between the EPA, National Chemical Genomics Center (NCGC) of NIH, the National Toxicology Program (Collins, Gray and Bucher, 2008) and more recently the FDA. The challenge is significant to generate data in a high-throughput mode and integrate these data into predictive models that can be used to assess *in vivo* toxicity. The vision for toxicology in the twenty-first century (NRC, 2007; Collins, Gray and Bucher, 2008) and its adaptation by EPA for a future toxicity testing strategy (Firestone *et al.*, 2010) draws attention to the current need for a computational framework that can be used to evaluate environmental chemicals based on data from human cell cultures, alternative model organisms, high-throughput testing and systems modeling. This research is in its infancy but holds promise for applications such as identification of potentially toxic chemicals during new product development, prioritization of existing chemical compounds for targeted *in vivo* testing, characterization of chemical targets leading to traditional end-points of toxicity, and pathway-based evaluations of pathogenesis. For all of these reasons, a shift from conventional end-point-based toxicology, where animal models are used to evaluate outcome, to high-throughput chemical biology requires a systems biology approach to integrate all of the toxicological data and biological concepts. This chapter addresses some of the key issues and questions for systems-based approaches in understanding developmental toxicity within the context of this new testing paradigm for environmental chemicals.

2 SCALE OF THE PROBLEM

Environmental chemicals are natural and man-made compounds to which human populations are continually exposed as a matter of individual lifestyle, local geography and community life. The Centers for Disease Control and Prevention (CDC) National Health and Nutrition Examination Survey (NHANES) samples the US population every 2 years to determine which chemicals get into people and at what concentrations (CDC, 2009). The most recent NHANES measured 212 environmental chemicals in blood or urine. Human populations are, however, likely exposed to many more chemicals and even more compounds are synthesized on a daily basis. At least some of these chemicals would be expected to have the potential for adverse developmental consequences if exposure occurs during intrauterine life. EPA's Toxicity Reference Database (ToxRefDB) identified developmental effects relative to maternal toxicity on 53 out of 283 (18.7%) environmental chemicals studied in pregnant rats or rabbits (Knudsen *et al.*, 2009). Developmental or reproductive effects data are available for less than 12% of about 3800 chemicals listed in public databases from the NTP and FDA's Center for Drug Evaluation and Research (CDER) and Center for Food Safety and Nutrition (CFSAN). One of the largest data compilations from multiple resources to date, EPA's Aggregated Toxicology Resource (ACToR), points to any developmental toxicity data, complete or otherwise, for less than 30% of 9912 chemicals in commerce or of relevant environmental interest (Judson *et al.*, 2009). Although the conventional testing paradigm provides qualitative data that are rich in contextual detail and generally regarded as predictive of effects in human pregnancy, it does not scale to the problem of assessing human developmental safety for the $>80\,000$ chemical compounds in commerce or in the environment.

Paucity of relevant data leads to considerable uncertainty that must be accounted for when conducting a risk assessment. EPA's Integrated Risk Information System (IRIS) identifies the 'critical effect' as the basis for setting safe-exposure levels on environmental chemicals to protect public health. Criticality is defined as the first 'observed' effect deemed adverse that is likely to occur in the most sensitive human subpopulation as the dose rate of an agent increases. As of this writing, less than 2% of 533 IRIS assessments report the critical effect for the derivation of a non-cancer reference value (i.e., a safe-exposure level) as being a developmental (five out of 553) or reproductive (four out of 553) effect (http://www.epa.gov/IRIS/). Increased uncertainty in the choice of the critical effect for an IRIS assessment because of inadequate or insufficient

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bioassay data is accounted for quantitatively by a database uncertainty factor (UF-D) that when greater than 1 further reduces the safe-exposure values EPAderives to protect public health (e.g., the reference dose (RfD) or reference concentration (RfC)). Many of the older IRIS assessments had only a qualitative rating on the confidence in the database, instead of a UF-D. In these older assessments, a designation of low or medium confidence was often caused by missing reproductive or developmental studies. For assessments where a UF-D was used, a search of the IRIS database resulted in 136 reference values (for 120 out of the 553 chemicals) that had been reduced by a UF-D > 1 as a result of insufficient data. An absence of reproductive or developmental effects data was the sole or major contributing factor for 85% of these higher UF-Ds > 1 (115 out of the 136). To reduce this source of uncertainty and identify potentially sensitive reproductive or developmental targets, a more precise and efficient testing strategy is needed to replace the resource-intensive conventional bioassay approach. Although this low percentage is in part caused by other effects being more sensitive, there is considerable uncertainty in the choice of the critical effect for a number of chemicals because of a lack of developmental and/or reproductive effects data.

The challenge of assessing the potential health risks from 10 000 chemical compounds in Tox21 and 30 000 chemicals under European Registration, Evaluation, and Authorization of Chemicals (REACH) legislation is a significant problem. If, for example, one out of six chemical compounds display adverse developmental activity in conventional testing studies then developmental effects data would be critical for some 1667–5000 chemicals of the broader 10 K–30 K chemical landscape. Because the scope of the toxicity testing problem is too vast to address without a systematic approach to chemical screening and prioritization, high-throughput approaches are needed that can scale to the problem, as well as innovative computational tools for data management, analysis and inference.

3 SCALING TECHNOLOGY TO MEET THE PROBLEM

In 2007, a committee of the National Research Council (NRC) proposed a new vision for toxicity testing in which biologically important perturbations to key toxicity pathways would be evaluated using newer methods in molecular biology, bioinformatics, computational toxicology, and *in vitro* tests based primarily on human biology (NRC, 2007). This new paradigm shifts emphasis from apical end-points of toxicity assessed in conventional *in vivo* studies to *in vitro* profiling, prescribing that profiles of biological activity be used in conjunction with available environmental exposure and human epidemiology data to prioritize chemicals for subsequent evaluation in more targeted animal bioassays. The new paradigm is based on high-content screening (HCS) that arose from revolutionary advances in the genome sciences, enabling parallelized analysis of cellular and molecular features. Together with fast, automated technologies such as high-throughput screening (HTS) of compound libraries (originally developed for drug discovery), *in vitro* profiles are being generated for the biological activity of an increasing number of chemicals, and are being interpreted to predict potential toxicity *in vivo* (Figure 1).

A number of programs are applying HTS–HCS technologies for rapid screening of chemical libraries (thousands of unique structures) for specific molecular pathway and cellular function activity. The NCGC, for example, has the HTS capacity to screen an approximately 100 000 compound chemical library daily in a given specific assay. EPA's ToxCast^{TM} project initially generated rich sets of HTS–HCS data on 309 chemicals across 467 assays (phase I), using chemicals most of which already had extensive animal bioassay data to assess potential toxicity (Judson *et al.*, 2010). In phase II, ToxCast^{TM} is evaluating another $\sim$700 additional chemicals across approximately 500 assays, in this case for chemicals with limited or no pre-existing animal toxicity data as well as several dozens of pharmaceutical compounds that failed for toxicity in human clinical trials (http://www.epa.gov/ncct/toxcast/). The Tox21 program will test $\sim$10 000 chemicals across a few dozen assays (Schmidt, 2009). As such, the new testing paradigm will generate vast amounts of data that can be used to profile the bioactivity of hundreds to thousands of environmental chemicals across multiple pathways of biological activity, thus implementing the NRC vision for the future of toxicity testing (NRC, 2010).

Along with a promising potential for improved human health and disease prevention, the new testing paradigm shifts the focus from an end-point-based risk assessment to a toxicity pathway-based

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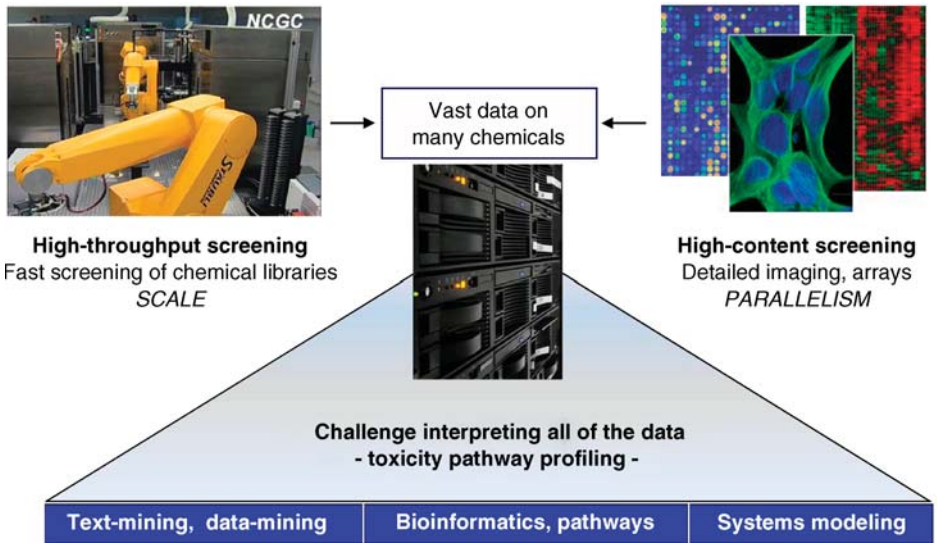

Figure 1. The HTS–HCS paradigm applied to toxicity testing. Applying the HTS–HCS paradigm to toxicity testing turns the drug-discovery process on its side. Rather than starting from a well-defined cellular activity and identifying the targeted chemical biology, compounds of unknown or suspected human toxicity are tested through hundreds of *in vitro* assays. Computers are then used to unlock patterns of biological activity from across the chemical-assay space. Using text-mining tools and bioinformatics, diagnostic signatures are used as the basis to incorporate pathway-level information and predict potential *in vivo* toxicities. This effort, combined with vast biological knowledge, can guide plausibility of biological interpretation to inform testing and hypothesis generation.

risk assessment, and has raised important questions and challenges for researchers. First, the HTS testing paradigm derives primary data from *in vitro* assays intended to coarsely screen compound libraries for biological activity. HTS assay platforms can provide high-quality concentration–response data, although interpreting the significance of a "hit" versus "no-hit" depends on rules that may not easily extrapolate to *in vivo* systems. For example, curve-fitting to *in vitro* concentration–response results can identify the lowest effect concentration (LEC) at which the *in vitro* activity is detected and the concentration at which measured activity has been inhibited or stimulated by 50% (AC50). This can inform design of new *in vivo* or *in vitro* experiments aiming to validate the prediction and the efficacy for a therapeutic or adverse response *in vivo*. Incorporating *in vitro* LEC and AC50 values derived from HTS–HCS data into predictive models of developmental toxicity, however, requires an accounting for, and integration of, higher-order functions that arise in metabolic pathways, signaling pathways or biological networks. This also gives rise to the problem of multiplicity (one-to-many). For example, in the ToxCast^{TM} phase-I results, there were numerous cases where one chemical hit many assay targets (Judson *et al.*, 2010). Because of this complexity, the individual HTS assay target data must be assembled and interpreted within the context of broader pathways and systems level understanding of the biology. In the context of embryogenesis, this means building predictive models that can account for robustness of the developing embryo to genetic or environmental variation resulting from system-level interactions that effectively dampen dynamic response (canalization) or from compensation (adaptive response).

Second, the HTS testing paradigm draws information from assays designed to rapidly screen compound libraries for specific biological activities. As such, the assays must be amenable to high-throughput methods, use of dimethyl sulfoxide (DMSO) as a vehicle, and some degree of automation for measurements. In ToxCast^{TM}, for example, the 467 phase-I assays tested various cell-free extracts (239) and differentiated cells or cell lines (228) mostly of human origin (Dix *et al.*, 2007; Judson *et al.*, 2010). In most cases, target-level responses infer perturbations to the integrity or function of a specific receptor, enzyme, transporter, or cellular feature as measured in a cell-free (biochemical), cell-based (simple culture), or multicellular

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(complex culture) platform. Results of these studies have begun to classify pairs of chemicals and their respective toxicity targets based on the assay(s) hit and their pathway-level relationships. The *in vitro* results, however, do not account for *in vivo* chemical disposition, so toxicokinetics and delivery of the chemical to *in vivo* targets at comparable dose levels must also be considered to predict *in vivo* effects.

Third, many HTS assays represent disassembled biological systems that may or may not reflect key events in embryogenesis. One alternative model of embryonic development that is amenable to medium- or HTS is the embryonic stem-cell test (EST). This platform has been approved by the European Centre for Validating Alternative Methods (ECVAM) for developmental toxicity testing. It is configured by embryonic cells that undergo morphogenetic signaling and cellular network responses and was validated using a test panel of 20 compounds categorized as non-, weak, or strongly embryotoxic (Genschow *et al.*, 2002). The ECVAM-approved assay uses mouse embryonic stem (mES) cells where LIF-maintained mES cells are grown through nascent 'embryoid bodies' and scored for beating cardiomyocytes, after being exposed to drugs or chemicals for 5–10 days. Effects on cardiomyogenic differentiation correctly resolved 78% of the 20 chemicals into non-embryotoxic or strong embryotoxic categories (Genschow *et al.*, 2002; Chapin *et al.*, 2008). Incorporating gene expression data into the ES cell assay has been shown to improve the predictivity and applicability domain required to facilitate implementation of these findings into regulatory testing strategies (van Dartel *et al.*, 2010). These investigators recently defined a 'differentiation track' based on 26 genes that correlated with the developmental toxicity of several reference compounds. A human ES (hES) cell metabolomic platform has also been introduced recently to evaluate changes in small molecules and metabolites secreted into the hES cell-culture medium (West *et al.*, 2010). This work showed a correlation between embryoid body formation and changes in the secretome ratio of arginine: dimethylarginine, reflecting imbalances in the urea cycle. Changes in the ratio >10% preceded cytotoxicity, but correlated to embryotoxicity with an accuracy of 88%.

Fourth, apart from advantages in assessing differentiation, HTS systems such as the ES cell platform cannot fully replicate the topological changes in key events that may contribute to a disruption of human development. The *in vivo* dose–response may differ from the ES cell platform dose–response caused by *in vivo* kinetics (ADME: absorption, distribution, metabolism, excretion) affecting the chemical delivery to the comparable maternal or conceptal targets (i.e., dose delivery differences), or to the dynamics of the integrated cellular systems or cell derivatives that are not represented *in vitro*. Placental transport mechanisms and fetal dosimetry are poorly represented, if at all. Methods are also needed to extrapolate risk from dose levels associated with toxicity *in vitro* to estimates of the potential toxicity from low-dose environmental exposures.

Free-living zebrafish (ZF) embryos recapitulate most signaling systems and pathways found in mammalian embryos. In this assay, embryos are exposed to a test solution from 4 to 6 h post-fertilization (pf) and assessed up to 5 days pf for perturbations in embryo structure and function. One study reported an 87% correct prediction of 31 test agents for teratogenic versus non-teratogenic based on LD50 values (Brannen *et al.*, 2010). Among the advantages of ZF models is amenability to genetic methods and cell imaging, which together offer a means to image cellular dynamics during morphogenesis and to digitally reconstruct embryogenesis (Keller *et al.*, 2008). The two main shortcomings of this assay are the lack of pharmacokinetics and species differences (e.g., lack of placentation and pulmonary systems, fin rather than limb development). These factors make it difficult to predict how a compound of unknown or suspected toxicity will behave in a pregnant woman or laboratory animal.

In conclusion, alternative testing methods are promising but in early work have met with mixed results (Chapin *et al.*, 2008). Many developmental effect assays have drawbacks or introduce uncertainties caused by kinetic and dynamic species differences, differences in the complexity of the system (i.e., *in vitro* and *in vivo*), incomplete understanding of mechanisms and signaling crosstalk in a complex developing system, such as the potential for maternal effects and metabolic activation, or uncertainty in extrapolating from high-dose response to the low-dose response of interest when predicting potential for human toxicity (Daston, 2007). The capacity to predict dose-dependent toxicity in alternative HTS models of embryonic development would greatly aid risk assessment and chemical prioritization for further animal testing

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(NRC, 2007); however, modeling the HTS data into higher-order functions and control of cellular behaviors (e.g., growth, death, migration) and signaling (e.g., metabolic pathways, signal transduction pathways, gene regulatory networks) is a significant challenge, and must be conducted within the context of normal embryogenesis (Davidson *et al*., 2002) to simulate the key events leading to adverse pregnancy outcome. Both the ES cell and ZF embryo assay systems have potential applicability in this regard and are approved by ECVAM as alternative models for developmental toxicity testing. These two assays have been recently introduced to the ToxCast^{TM} assay panel (Chandler *et al.*, 2010a; Padilla *et al.*, 2010a).

4 COMPUTATIONAL PROFILING OF APICAL END-POINTS

The potential of an environmental chemical to cause adverse effects in the developing embryo or fetus is an important consideration in any health-risk assessment (Gad, 2002; Janer *et al*., 2008). EPA's guidelines for developmental toxicity risk assessment (FRL-4038-3, December 5, 1991) are recorded in the Federal Register 56(234):63798-63826 and were updated in a 1998 workshop (SAP Report No. 99-01C, January 22, 1999). The guidelines state that when experimental data are evaluated, "the agents causing human developmental toxicity in almost all cases were found to produce effects in experimental animal studies and, in at least one species tested, types of effects similar to those in humans were generally seen." When animal bioassay data are available, a number of EPA risk assessments report significant developmental effects in some cases at dose levels within an order of magnitude of the endpoint selected as the critical effect (e.g., acrylamide). Sampling of a subset of 43 toxicological reviews from the IRIS assessment database (http://www.epa.gov/iris) having developmental and/or reproductive effects data resulted in 12 cases (28%) reporting significant adverse developmental effects such as altered birth weight, skeletal variations, neurodevelopmental defects, altered maternal or postnatal weight gains in reproductive organs, or testicular lesions. One might expect that assays focusing on mechanisms or precursor events to observed lesions would detect significant developmental effects at lower exposure levels.

Databases of birth-defect registries (Scheuerle and Tilson 2002; Correa-Villaseñor *et al.*, 2003), prenatal animal studies (Hurtt, Cappon and Browning, 2003; Chernoff *et al*., 2008), and developmental toxicology literature (Julien *et al*., 2004; Matthews *et al*., 2007) have generally attempted to uncover relationships within classes of chemical compounds for developmental effects in laboratory mammals or human populations, rather than profiling developmental effects and mechanisms over a larger chemical space. An internationally harmonized nomenclature designed to standardize the description and classification of such apical end-points (Makris *et al*., 2009) can facilitate registration and aggregation of external abnormalities, visceral abnormalities and skeletal abnormalities during fetal examination of rats, rabbits and mice. This is an important annotation system for profiling developmental toxicity because it enables a computational approach to classifying chemical–target (*in vitro* profiling) and chemical–end-point (*in vivo* profiling) relationships.

In the ToxCast^{TM} project, *in vitro* profiling focused initially on 309 chemicals in phase I, most of which have high-quality and uniformly generated *in vivo* toxicity data. This information has been annotated by a controlled vocabulary in EPA's ToxRefDB. The database, developed at EPA's National Center for Computational Toxicology in partnership with the Office of Pesticide Programs, holds source data from 2073 guideline studies on 480 chemicals, mostly pesticides and antimicrobials, putting 30 years of animal testing bioassays worth >$2B from registrant-submitted toxicology study results as data evaluation records (DERs) into a computable form. The database is the first implementation of its kind for profiling the activity of environmental chemicals based on a comprehensive analysis of source data for a broad range of endpoints relevant to EPA risk assessments, such as chronic cancer studies in mouse and rat (Martin *et al*., 2009a), multigenerational reproductive studies in rat (Martin *et al*., 2009b), and prenatal developmental toxicity studies mostly in rats and rabbits (Knudsen *et al*., 2009). The database and web interface was released to the public in spring 2010 and is accessible online at http://actor.epa.gov/toxrefdb.

ToxRefDB includes 751 prenatal studies on 387 chemicals. A controlled vocabulary of 988 harmonized terms were used to input developmental

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effects data, finding 293 end-points across these studies and collapsing them into 18 target embryonic systems. The analysis revealed a spectrum of chemical activity based on administered dose, sometimes broadly active and other times narrowly active. Among the 283 chemicals tested in both pregnant rats and rabbits, the primary expressions of developmental toxicity differed between species. Fetal weight reduction, skeletal variations and abnormalities, and urogenital defects were more frequently noted in rat studies, whereas resorption-pregnancy loss was more common in the rabbit studies as were structural malformations to the visceral body wall and central nervous system (Knudsen *et al*., 2009). ToxRefDB resolved these chemicals into several clusters based on developmental effects noted in rat and/or rabbit studies (Figure 2).

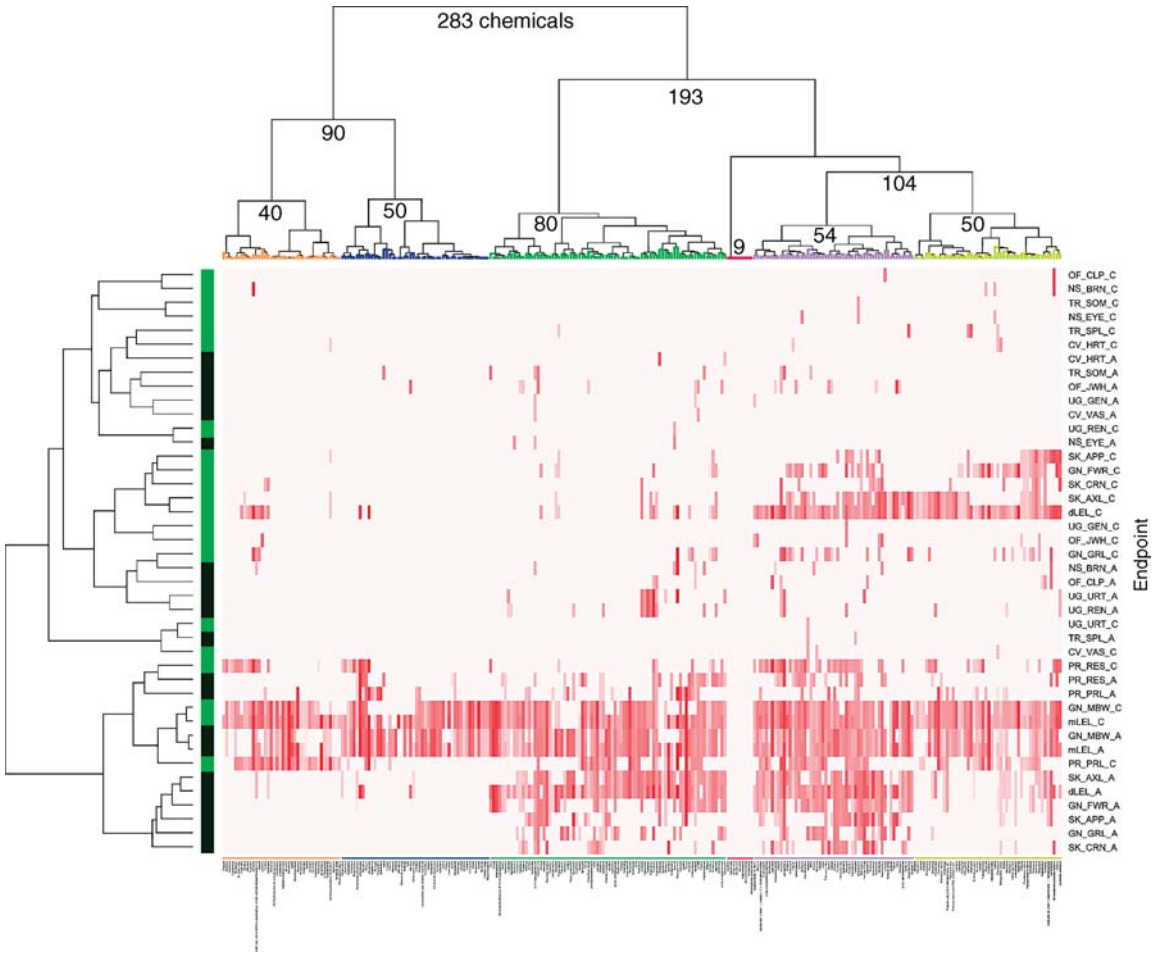

Figure 2. Hierarchical relationship of ToxRefDB chemicals to developmental effects. Chemicals (columns) by effects (rows) for 283 chemicals with developmental activity tested in rat (A, dark green ribbon) and rabbit (C, light green ribbon). Hierarchical clustering was performed by Pearson's correlation and Ward linkage; color intensity reflects potency of a chemical–end-point response based on administered dosage. Variables are maternal LEL (mLEL), developmental LEL (dLEL), and categorical LELs (cLEL) grouped by system: cardiovascular (CV), general (GN), neurosensory (NS), orofacial (OF), pregnancy-related (PR), skeletal (SK), trunk (TR), and urogenital (UG). Abbreviations for specific effects targets: reduced maternal body weight gain (MBW), fetal weight reduction (FWR), maternal-pregnancy losses (PRL), embryo-fetal losses (RES), general fetal pathology (GRL); skeletal defects – axial (AXL), appendicular (APP), cranial (CRN); orofacial defects – cleft lip/palate (CLP), altered jaw/hyoid bone (JWH); defects of the fetal brain (BRN) and eye (EYE); renal (REN), ureteric (URT), genital (GEN); body wall defects (SOM) and abnormal splanchnic viscera (SPL); heart (HRT) and major vessels (VAS) (from Knudsen *et al.*, 2009, © Elsevier, reproduced by kind permission of Elsevier.)

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Although species differences as observed for rat and rabbit are important to developmental profiling, the primary risk-assessment objective is to determine concordance between animal-testing and human response. Unfortunately, information on human developmental toxicity is not readily available for most of these environmental chemicals. As noted earlier, phase-II of ToxCast™ will add responses of chemical compounds with less-characterized animal toxicity, and will also include several dozen failed pharmaceuticals. Some studies have addressed animal–human concordance for pharmaceuticals where a good deal of human data is available. A retrospective analysis of several hundred pharmaceuticals tested in both rodent and non-rodent species for general toxicological end-points, reported an overall 71% concordance with true positives in human populations; however, the concordance was lower when non-rodents (63%) and rodents (43%) were considered separately (Olson *et al.*, 2000). With regards to developmental toxicity testing, rat studies detected a signal in 61% of chemicals that showed teratogenic effects in rat, mouse or rabbit studies whereas a rat study and a rabbit study together identified teratogenicity in 100% of these chemicals (Hurtt, Cappon and Browning, 2003). Correlations with teratogenicity in humans for these compounds, however, were unattainable. These findings support a formal laboratory animal-testing procedure in a rodent and non-rodent species for assessing fetal development, an approach that has been adopted since the thalidomide episode, and lend support to the use of systems such as ToxRefDB to anchor *in vitro* profiling data.

In current practice, the animal dose–response data are used to derive safe levels of exposure to humans rather than to predict a specific developmental phenotype in humans. Questions thus arise as to the added value to regulatory agendas of a second test animal species when consideration is given to all four manifestations of developmental toxicity (malformations, resorptions, low-birth weight, and functional deficits), to consequences of maternal toxicity, and to standardized study designs. Resorptions may correlate with malformations, functional deficits may correlate with low birth weight, and all four kinds of developmental defects may result from maternal toxicity with rabbits being somewhat more susceptible than rats (Guittina *et al.*, 2000; Chernoff *et al.*, 2008; Piersma *et al.*, 2008). Piersma and coworkers at the National Institute for Public Health and the Environment of the Netherlands (RIVM) conducted a retrospective analysis to assess the added value of a rabbit developmental test when data from a rat developmental test were available. Both rat and rabbit studies were identified for a number of substances (drugs, chemicals) classified as developmental toxicants. Rat and rabbit studies showed similar sensitivity (NOAEL) and overall the authors did not find evidence that would require the rabbit as an additional test species (Janer *et al.*, 2008). Direct (mechanism) versus indirect (maternal) factors had a greater implication, as some substances induce developmental toxicity in one species at a dose that may induce maternal toxicity in another. From a system perspective, however, interspecies differences can lead to different apical end-points after exposure to the same chemical, even when the mechanism of action is identical (Janer *et al.*, 2008). Thus, one needs to consider whether the application of developmental profiling is for the purpose of predictive modeling or for regulatory decision-making.

Preliminary analysis of ToxCast™ HTS data revealed effects on multiple *in vitro* assays for molecular targets or cellular processes important to vasculogenesis and angiogenesis. A predictive signature built around statistical associations to *in vivo* developmental end-points derived from ToxRefDB revealed a strong correlation between developmental defects and a network of pathways consisting of pro-inflammatory/anti-angiogenic cytokine signaling, vascular endothelial growth factor (VEGF) signaling, and the plasminogen-activating system (PAS) network of enzymes and growth factors mediating matrix remodeling and local signaling during blood vessel growth. Furthermore, relatively robust patterns emerge from the *in vitro* profiling data linked to species-specific *in vivo* developmental toxicity in either rats or rabbits (Kleinstreuer *et al.*, 2010a).

The biological bases for species differences, maternal factors, and study designs are embedded in the principles of teratogenesis (Wilson, 1977). Paraphrased, the principles state that differential teratogenicity depends on: (i) *genotype*: the susceptibility of a developing organism is dependent on genetic makeup with respect to species, strain, or individual genotype; (ii) s*tage*: organ systems asynchronously traverse through critical periods of susceptibility; (iii) s*pecificity*: a wide-range of

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initiating mechanisms converge to final limiting pathways of cellular pathogenesis; (iv) *bioavailability*: chemical nature is important to an agent's metabolism, availability to the embryo, and effects on maternal physiology; and (v) *dose–response*: manifestations of abnormal development increase in frequency and extent, from no effect level toward threshold effects and prenatal lethality.

A system-level understanding of development, facilitated by, and coupled with, technological advances in HTS–HCS presents us with a unique opportunity to understand Wilson's principles in a modern context. Many signaling pathways that are important for patterning embryonic growth and differentiation are highly conserved across species, from insects to mammals. Some of the best examples are transcription factors such as Pax6 (eye development), Nkx/tinman (heart development), Hox genes (axial patterning), and Hes1 (somite segmentation clock). In 2000, a committee of the National Research Council issued a report advocating the use of detailed knowledge about cell-signaling pathways to help elucidate mechanisms in developmental toxicity (NRC, 2000). That report compiled evidence for up to 17 canonical cell–cell signaling pathways and two stress response pathways based on conserved roles in animal development. The conserved pathways have been interrogated for quantitative dose–response activity of compounds in the mES cell assay (Chapin and Stedman, 2009). Several dozens of genes representative of key signaling pathways were shown to be disrupted by agents such as retinoic acid or BMP4 antagonists in a manner-dependent upon compound and dose. This indicates at least some conserved cell–cell signaling pathways may be disrupted by environmental exposure and furthermore points to the utility of a pathway-based analysis to identify key events associated with developmental signaling.

5 MODE OF ACTION PROCESSES

As HTS–HCS data are captured *in vitro* and applied *in vivo*, translation into practice will be predicated on two aspects of predictive modeling: mathematical association and biological plausibility. To assess risk to the developing embryo, one must establish a quantitative cause–effect relationship between dose and key events in the path towards an apical endpoint (also known as the mode of action, MoA). Because of the complex and cascading nature of signal–response pathways, for example, a local perturbation in cell-signaling detected in an HTS assay (cause) may result in multiple downstream consequences (effects) only some of which may lead to toxicity, others of which may not. Advanced knowledge of molecular embryology is therefore essential in determining how different signaling pathways interact with one another and how these pathways may govern divergent cellular behaviors during morphogenesis and differentiation. Models based on a system-level understanding can theoretically be useful to interpret the complex responses observed in HTS–HCS assays resulting from genetic errors or exposure to environmental contaminants, and serve as decision-support tools in the effort to predict risk for those critical perturbations that lead to toxicity in the developing embryo.

One way to decipher key events leading to developmental defects is to consider MoA processes, that is, the propagation of chemical lesions to higher levels of biological organization. Perhaps, the most widely exploited practice is genome-scale analysis of signaling pathways and gene networks. Toxicogenomics has become a standard approach to characterize changes in the embryonic transcriptome at critical times during developmental toxicity (Yu *et al.*, 2006; Daston, 2007; Knudsen and Kavlock, 2008; Knudsen and Daston, 2010). The sensitivity of gene expression profiling can reveal more about the potential effects of lower-dose exposure to harmful chemicals than has been possible using traditional techniques. For example, studies by Naciff *et al*. (2005) identified an estrogenic fingerprint of 66 genes in association with the uterotrophic response of rats to low-doses of ethynyl estradiol, bisphenol A, and genistein. This fingerprint was diagnostic of an effect by several weaker environmental estrogens (31 out of 66 genes were affected by diethylstilbesterol, 12 out of 66 genes by methoxychlor), as well as the lack of effect as a result of non-estrogenic chemicals (two out of 66 genes affected by cortisol; and 0 out of 66 genes affected by ethylenethiourea, propylthiouracil, or methimazole).

A genome-wide analysis of gene expression can also provide insight into the genetic basis of differential teratogenic susceptibility. Consider, for example, two closely related murine strains having differential sensitivity to alcohol induced Fetal Alcohol Syndrome (FAS). Gene expression profiles

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were determined for the cranial neural folds of day 8 mouse embryos from strains at high risk (C57BL/6J) and low risk (C57BL/6N) for alcohol-induced craniofacial defects, eye defects, and fetal growth retardation (Green *et al*., 2007). This analysis, run 3.0 h after a maternal exposure below the threshold for teratogenesis, revealed a reprogramming of several metabolic, signaling and regulatory pathways, including pathways in tight junctions, focal adhesion, adherens junction, and cytoskeletal pathways all up-regulated in both strains; Wnt signaling and apoptosis pathways up-regulated in both strains; glycolysis and pentose phosphate pathways up-regulated in C57BL/6N only; and ribosomal proteins and proteasome pathways down-regulated in C57BL/6N only. These findings are consistent with the notion that developmental exposure to alcohol alters common signaling pathways linking receptor activation to cytoskeletal reorganization, and furthermore predict at the cellular scale a transition between adhesive and migratory states of precursor target cell subpopulations for high-risk and low-risk embryos, respectively (Green *et al*., 2007). Genes in the focal adhesion/actin cytoskeleton pathway were also shown to be up-regulated in monkey embryos during the early phase of thalidomide embryopathy in conjunction with pro-inflammatory/anti-angiogenic MoA processes (Ema *et al*. 2010).

Integrating these HCS data into predictive models for developmental toxicology can extend conventional testing approaches; however, we are at a relatively early stage in using them to understand MoA processes. Critical details may come from other kinds of assays and experimental designs. For example, Collins and coworkers used whole genome quantitative trait locus (QTL) scans to map the critical chromosomal region for differential susceptibility of two murine strains to 13-*cis* retinoic acid-induced forelimb ectrodactyly: C57BL/6N mice are susceptible to this induced phenotype whereas SWV mice are not (Lee *et al*., 2005). Results based on 88 *Mit*-markers revealed significant linkages to D11Mit39 and D4Mit170 on chromosomes 11 and 4, respectively. Multiple candidate genes could explain the strain differences, with the strongest being RARα and Wnt3/9b that map to the critical region for differential susceptibility on chromosome 11. Thus, a major challenge is to map changes in genomic/proteomic expression, both adaptive and adverse, to sequential cellular behaviors at a systems level and in a manner that is consistent with the underlying embryology and MoA processes.

6 PROFILING DEVELOPMENTAL GENE EXPRESSION

Genome-wide analysis of gene expression is especially powerful when mapped to specific developmental processes (Yu *et al*., 2006). A number of resources for taxonomy of the mouse embryonic transcriptome network have recently appeared for systems such as embryonic kidney development (Brunskill *et al*., 2008), eye development (Lachke and Maas, 2010), and ES cell differentiation (Schulz *et al*., 2009). Nephrogenesis, for example, involves fundamental developmental processes such as mesenchymal–epithelial transition, heterotypic tissue interactions, branching morphogenesis and segmentation (Brunskill *et al*., 2008). These investigators profiled the transcriptome in 54 embryonic kidney samples between gestation day 11.5 and 15.5 encompassing many of the key events in nephrogenesis. Using Laser Capture Microdissection (LCM), they defined taxonomy of gene expression relationships for different components of the kidney, such as the ureteric bud and cap mesenchyme during their inductive interaction and subsequent regional specification. Although some genes displayed component-specific expression, more commonly the profiles between different components showed extensive overlap in gene expression, with differences between components more quantitative than qualitative. Identifying chemically induced perturbations that leads to abnormalities of organogenesis in general, and of the 'developmental nephrome' in particular, requires consideration of this complexity in combinatorial gene expression that comprises the genetic circuitry of kidney development (Brunskill *et al*., 2008). A massive parallelism of transcriptomics and database resources is needed to resolve these patterns.

In a similar vein, a system-level understanding of the genetic circuitry of eye development has progressed from the increasing availability of genome-wide expression data and LCM-based studies. Eye formation, like that of the kidney, is a complex developmental program involving fundamentally conserved signaling pathways that lead to regional specification of the optic vesicle, and

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that drive reciprocal interactions with presumptive lens ectoderm. A system-level representation of the underlying genetic circuitry has been generated for the mouse embryo by Lachke and Maas (2010) for constructing the network topology of key-signaling molecules, transcription factors, and their targets (Figure 3). The schema provides a framework for understanding network state relationships in the "developmental oculome" as the system undergoes morphogenesis, differentiation and maturation (Lachke and Maas, 2010).

Complexity and robustness inherent to the genetic networks underlying organogenesis in general and eye development in particular make it essential to portray systems in this manner. Systems models like the one depicted in Figure 3 are needed to interpret HTS–HCS data in the context of developmental toxicity, by for example, incorporating the conceptualized linkage of pathway abnormalities specific to the anterior segment (lens-iris-cornea) or posterior segment (pigmented retina, neural retina, optic nerve) (Nemeth, Singh, Knudsen, 2005), and then computationally simulating events that lead to eye-reduction defects (Sipes *et al.*, 2010). As such, a shift in emphasis from the traditional focus on apical endpoints to an understanding of pathways and MoA processes in developmental toxicity requires an understanding of how genes, proteins, and metabolites interact with one another within complex biological networks to maintain normal cell function, and how sub-networks of these interactions can be perturbed in ways that lead to adverse effects on development (Interested readers are referred to Volume 87:2 of Birth Defects Research (Part C), devoted to the topic of gene regulatory networks).

Resources have appeared that allow automatic retrieval and integration of information for the relevant genes and pathways in the embryo and ES cells (Ganter *et al.*, 2008; Bauer-Mehren, Furlong and Sanz, 2009; Schulz *et al.*, 2009). These resources have become particularly relevant in the effort to develop system-level integration of gene expression data on a broad range of cellular pathways, inferring function by taking the entire biological system into consideration. The Edinburgh Mouse Atlas Project (EMAP, http://genex.hgu.mrc.ac.uk/) is a portal for tracking spatio-temporal gene-expression data during normal mouse embryogenesis (Christiansen *et al.*, 2006). The core database contains three-dimensional reconstructions of the mouse embryo at various stages of development (spatial models), a systematic nomenclature of embryo anatomy (anatomical ontology), and defined embryonic territories (domains) (Baldock *et al.*, 1992, 2003; Ringwald *et al.*, 1994).

7 COMPUTATIONAL MODELS

As previously discussed, there are several challenges in developing predictive models of developmental toxicity: correlating *in vitro* concentration–response with *in vivo* dose kinetics and response, understanding how bioactivity profiles extrapolate from one cell-type or technology platform to another, and interpreting individual *in vitro* bioactivity data within the context of complex ontogenetic networks and controls. Two categories of computational tools can facilitate understanding. One category is based primarily on statistical approaches, and includes benchmark dose (BMD) models and quantitative structure–activity relationship (SAR) models. BMD models incorporate all of the available data in the characterization of the dose–response, and the derivation of a point of departure to establish safe levels of exposure; and SAR models attempt to bridge data gaps for data poor chemicals by application of various algorithms to correlate physico-chemical properties with estimates of pharmacokinetic behavior or biological response. These have been reviewed elsewhere (Knudsen and Kavlock, 2008) and will not be further discussed here.

The second category of computational tools involves greater consideration of biological processes and includes physiologically based pharmacokinetic (PBPK) models[a], biologically based dose–response (BBDR) models[b], and looking to the future, *in silico* models that simulate normal biology at various levels of organization (e.g., "virtual embryos"). PBPK models are chemical-specific models that estimate a chemical's internal disposition and dose at a specific target (or an appropriate surrogate dose metric) following a specified external exposure. PBPK models do this by mathematically representing biological tissues as compartments with physiological properties and flows that are characteristic of a species, individual, or population, as well as the processes that determine the internal disposition of a chemical based on chemical specific parameters for key-kinetic processes

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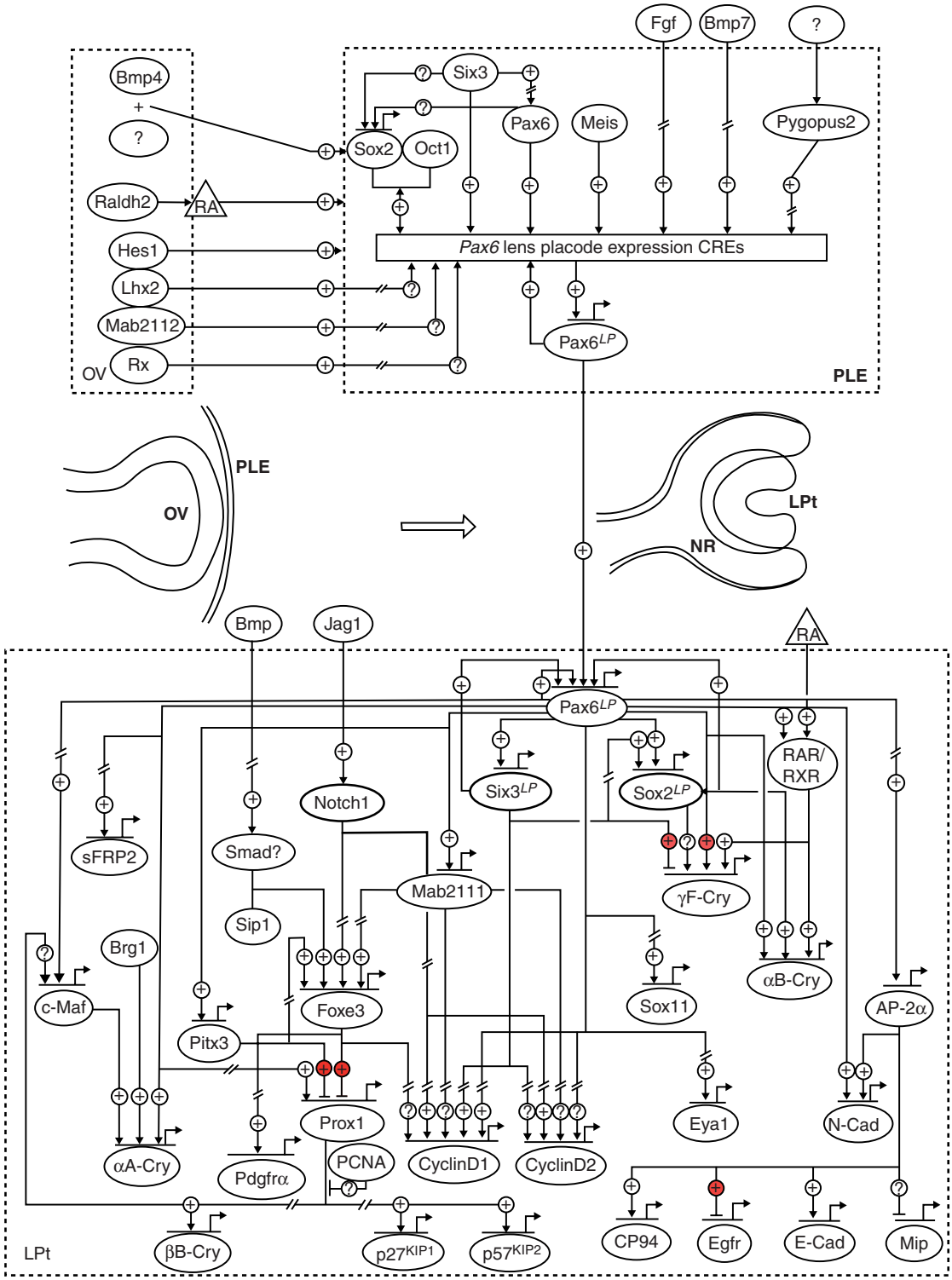

Figure 3. The molecular circuitry of early mammalian lens development. Reciprocal induction between presumptive lens ectoderm (PLE) and optic vesicle (OV) lead to formation of lens pit (LPt) and neural retina (NR). (From Lachke and Maas, 2010. doi: 10.1002/wsbm.59 Copyright © 2009 John Wiley & Sons, Inc.)

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(ADME). Because PBPK models are biologically based representation, they can be parameterized to estimate a chemical's internal disposition for a variety of different species, dosage regimens, and exposure routes; and can extend predictions beyond the limitations of the available empirical data (US EPA, 2006). The estimate of the internal dose metric (sometimes called the biologically effective dose) replaces the administered dose in the derivation of the quantitative characterization of the dose–response relationship used to develop regulatory standards for safe exposure levels. Chemical-specific PBPK models are increasingly being used in risk assessment to extrapolate beyond the available bioassay and kinetic data, to reduce uncertainty in estimating safe-exposure levels, and to develop levels where limited or absent data preclude establishment of safe levels by less sophisticated approaches (DeWoskin *et al.*, 2006).

Physiologically based pharmacokinetic models that simulate internal disposition for prenatal developmental exposures must account for changing maternal and fetal compartment sizes or metabolic activity during pregnancy as well as differences in placental physiology or exposures that extend into the lactational period. PBPK models have been developed for gestational (14 chemicals) and lactational (three chemicals) exposures that address these challenges (Corley *et al.*, 2003). By providing a quantitative framework for simulating target concentrations of a chemical, or of its metabolites, the administered dose can be more rationally coupled to the target tissue response. This can infer clues as to the MoA, point to new experiments that would fill gaps, and promote understanding of the human relevance of exposures in laboratory animal models. PBPK model simulations for a variety of exposure conditions where data are limited is especially important when predicting the internal dose to a human embryo where the mother may be exposed intermittently or to low concentrations of the chemical for prolonged periods of time (Yoon and Barton, 2008). PBPK models are also critical to the interpretation of the HTS test data, to support extrapolation of AC50 results captured from *in vitro* assays, to the internal dose *in vivo* and to the corresponding exposures required to realistically achieve that level in an intact system. Quality criteria for PBPK models have been addressed with regards to regulatory acceptance (Clark, Setzer and Barton, 2004) and databases of physiological parameter values for early life stages have been compiled to support PBPK model development and parameterization to simulate the kinetics for this critical subpopulation (Clewell *et al.*, 2002; DeWoskin and Thompson, 2008; US EPA, 2009).

Biologically based dose–response models are predictive models that attempt to simulate the biological response that results from a chemical exposure subsequent to the delivery of an internal dose to a target site. BBDR models mathematically represent the biological processes at the cellular and molecular levels, and the key events leading to an adverse response. Similar to PBPK models, a BBDR is developed for specific chemicals and can extrapolate beyond the limits of the available empirical data. Compared with PBPK models, however, relatively few BBDR models have been developed, and none have been used as of 2010 to derive IRIS reference values in large part because of the relatively more complex dynamics that BBDR model's attempt to simulate (i.e., the effect the chemical has on the body) compared with the straightforward mechanics of a chemical's kinetics that PBPK models can simulate (i.e., the effect the body has on the chemical). Development of BBDR models has also been constrained by a lack of data on key molecular and cellular precursor events, and the need generally for a broader based multidisciplinary team to develop the model (DeWoskin *et al.*, 2001).

Biologically based dose–response model development for complex systems like the embryo face an even greater challenge of unraveling the key events leading to a specific developmental end-point. The complexity of prenatal development and the paucity of detailed information on mechanisms of action make construction of BBDR models for developmental toxicity resource-intensive (Leroux *et al.*, 1996; Faustman *et al.*, 1999; Lau *et al.*, 2000). This may be more readily attainable for drugs that have been designed with a unique therapeutic target than for environmental chemicals to which unintended human exposure may give way to a diverse pattern of biological activity. One comprehensive effort to develop a BBDR model for developmental toxicity was the work of Lau and colleagues that focused on the chemotherapeutic agent, 5-fluorouracil (5-FU), with a well-characterized target (thymidylate synthetase) (Lau *et al.*, 2000; Setzer *et al.*, 2001; Lau *et al.*, 2001). Simulation of the proposed MoA for 5-FU proved to be a complex endeavor; consequently, the project advanced only to the stage of empirical

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linkages between interim steps in the MoA, where there were sufficient data for statistical models but insufficient data to develop the BBDR mathematical representation of underlying biological processes. Although BBDR models may improve the conceptualization of the underlying biological processes and the input of information into the risk-assessment process (Andersen and Dennison, 2001), and provide explicit handling of the uncertainties in our understanding of normal biology and chemical effects, they have not advanced to a stage where they can be used with confidence to replace default uncertainty factors in quantitative predictions of low-dose human risk (Crump *et al.*, 2010). Similar to the *in silico* models that are discussed next, BBDR models may also benefit from the greatly increasing data resources and understanding of normal biology that marks the advent of the new paradigm for toxicity testing.

In silico models, also called virtual tissue, virtual organ, or virtual whole body models, are designed to simulate normal biological processes with the aim of predicting the effects of a wide variety of drugs or chemicals as perturbation of the normal homeostasis to an extent that results in an adverse effect. The underlying assumption is that drugs and chemicals exert their actions on cells through interactions with proteins and other macromolecules that function as dynamic complexes within cellular-signaling networks. As such, computational systems biology may give rise to novel concepts and tools that could perhaps extend *in silico* models beyond the limitations of chemical specific BBDR models. Computational systems models would then predict a broad range of effects for a multitude of chemicals by incorporating what is known from the vast biology literature, along with specific HTS–HCS databases, and specific representation of biological processes of interest for risk assessment to simulate the properties and behaviors of complex biological systems following various kinds of stimuli and stress (Kitano, 2001). The era of systems biology has heralded the arrival of technology enabling toxicologists to think about developing such system-level models to represent functional networks (Andersen *et al.*, 2005; Singh *et al.*, 2005). Systems biology graphical notation (SBGN) provides a standardized approach for representing signaling pathways and indirect information from genomics, and for describing the processes and relationships that direct the flow of biological information through a system (Le Novère *et al.*, 2009). This complexity can be managed by presenting only what is needed to simulate a specific context of control circuits, functional network representations of the system, state transitions and the control decision rules of interest (D'haeseleer, Liang and Somogyi, 2000).

Consider, for example, somite formation (Lewis 2008; Oates *et al.*, 2009). This process is sensitive to a number of chemicals in mammalian species. The regular pattern of somite formation (120-, 90- and 30-min in mouse, chick, and zebrafish, respectively) is explained by oscillating gene expression known as the 'somite segmentation clock'. Wnt and FGF (fibroblast growth factor) signals produced at the caudal end of pre-somitic mesoderm maintain an undifferentiated state, and as the neuraxis elongates, cells traveling outside of the influence of Wnt and FGF differentiate. A wavefront formed by this space–time interaction casts different fates on cells in the emerging somite block based on the periodic phase of the somite segmentation clock. The molecular basis for control is oscillating *Hes* genes regulated by Notch-Delta signaling through a negative feedback loop. Computational models indicate the lag-time between *Hes* expression and repression is a key factor in the oscillatory behavior of this circuit, driven by protein translation and mRNA lifetime of Delta (Goldbeter and Pourquie, 2008). In this sense, molecular biology has dissected the system into its basic parts and computers are being used to reconstruct the system using sophisticated software and knowledge from patterns of gene expression for cell adhesion/repulsion molecules (e.g., cadherins, ephrins) (Glazier *et al.*, 2008). This example embodies the parody that "molecular biology took Humpty Dumpty apart; mathematical modeling is required to put him back together again" (Schnell, Grima and Maini, 2007).

8 CHALLENGES FOR IMPLEMENTATION

This chapter has briefly covered a broad range of topics pertaining to systems modeling in developmental toxicity, a concept aligned with the complex problems at hand. We must understand development at the molecular and systems level to recognize key events in developmental toxicity whether the pathways to abnormal development are driven by genetic errors, environmental disruptions, epigenetic programming, or a complex combination of

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all these factors. The Holy Grail is to reconstruct biologically informed computational models or 'virtual tissues' that can be used analytically to make HTS data useful in a quantitative risk assessment of developmental toxicity (Knudsen and Kavlock 2008; Knudsen and Daston 2010). Whereas numerous models have been developed for cell-signaling pathways in the embryo, fewer models exist for understanding how chemical lesions are propagated during developmental toxicity. Computational (*in silico*) models that execute a morphogenetic series of events may help in this regard, bridging the gap between *in vitro* profiling and *in vivo* response at different chemical concentrations. The general idea is to model the cell of an embryonic tissue as an agent, that is, the smallest fundamental unit capable of an autonomous decision. Individual agents and their interactions are coded into the model based on biological knowledge (Izaguirre *et al.*, 2004). Control of cell behavior results from an integration of within cell processes and processes external to the cell at different organizational levels that are represented in the model such as cell–cell interactions, signal gradients that may result from binding and release among many cells, as well as physical forces and constraints that are represented in the model. The multicellular simulation is run, and emergent properties such as branching patterns or tissue partitioning are then evaluated for biological relevance and insight. Emergent properties are those properties manifested at higher levels of organization that are neither easily intuited by what is known about the behaviors of the parts (individual cells) nor predictable based on the known linear relationships among the parts.

After suitable elaboration and verification, agent-based dynamic models may someday be used to simulate potential adverse effects following exposure to environmental chemicals where there is some information on perturbation to the model input functions. These models can be exercised to predict outcomes for conditions not experimentally practical because of cost, time, scale, or complexity. Newer technologies such as deep sequencing and cell-level transcriptomics, together with genetics and live cell imaging, bring together the potential to collect cell-level information in a dynamic, integrated system. As most teratology data are derived from perturbation of organogenesis, these newer technologies can provide detailed information at all stages of embryogenesis to provide a more complete system-level perspective such that various computational models would be more relevant with regards to human risk assessment. Because morphogenesis is more than a cumulative sum of individual cell behaviors, virtual tissue models that enable interactions of autonomous cells and preserve elements of system-level control can utilize extensive knowledge about the system and rapidly sweep potential developmental trajectories following chemical or genetic perturbation, thus providing deeper insight into mechanisms of toxicity than is possible solely by analyzing the cell-based data.

To date, successful computational models for processes in developmental biology have linked the specific activities of cell-signaling pathways to discrete morphogenetic events including cell-based models for gastrulation, limb-bud outgrowth, chondrogenesis, angiogenesis, and somitogenesis (Chaturvedi *et al*., 2005; Cui *et al*., 2005; Poplawski *et al*., 2007; Glazier *et al*., 2008; Mahoney *et al*., 2008). These models were implemented as hybrid cellular automata using CC3D open-source tissue simulation environment (www.CompuCell3D.org). An ideal virtual embryo platform will use software, tools, methods and models that can be extended to developmental toxicity. Such a platform fits neatly into EPA's strategy on the future of toxicity testing (Firestone *et al*., 2010) and can leverage unique pathway-based data for numerous chemicals from ToxCast™ (Knudsen and Daston, 2010). Using these resources, experimental–computational models can be built to: simulate key-signaling pathways, interlocking genetic networks, and cellular dynamics in developing tissues; model how embryonic cells react to chemical exposure both individually, and collectively as a complex system; analyze emergent behaviors and canalizing influences following stimulus/injury/perturbation; and understand how this complexity contributes to the differential susceptibility of embryonic tissues across dose, stage, time, and species.

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DISCLAIMER

The views expressed in this chapter are those of the authors and do not necessarily reflect the views or policies of the US Environmental Protection Agency.

NOTES

1. Also called physiologically-based toxicokinetic (PBTK) models.
2. Also called physiologically-based pharmacodynamic (PBPD) models, or physiologically-based toxicodynamic (PBTD) models.

RELATED ARTICLES

Developmental Toxicology
Toxicogenomics and the Evolution of Systems Toxicology

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Multiscale Integration of Toxicokinetic and Toxicodynamic Processes: From Cell and Tissue to Organ and "Whole Body" Models

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1 INTRODUCTION: AN INTEGRATIVE FRAMEWORK FOR COMPUTATIONAL TOXICOLOGY

Extending "state-of-the-art" whole-body physiologically-based pharmacokinetic and toxicokinetic models, for humans and other organisms, to explicitly incorporate toxicodynamic processes, presents various challenges: The biological information incorporated in these models has to be enhanced substantially, and across many levels of organization, in order to account for pertinent changes in biological function, especially changes that can be linked to observable/measurable markers (biochemical, histomorphological, etc.). Since the function of cells depends critically on intercellular interactions and on the dynamics of their biological microenvironment, it is important to model explicitly these interactions and dynamics in order to understand changes of function taking place at both the cell and tissue levels. The following sections provide a summary of the foundations of a modular "whole body" computational framework that incorporates a hierarchy of alternative formulations representing biological processes within "virtual" (or "in silico") tissues and organs at various levels of detail, in order to achieve the above-mentioned mechanistic integration of toxicokinetics and toxicodynamics. The computational implementations of "virtual" tissues and organs allow the mechanistic consideration of multiple scales of interlinked processes that include: molecular interactions (e.g., ligand-receptor interactions with emphasis on the receptors involved in the transport and biotransformation of xenobiotics); dynamics of intracellular biomolecular (regulatory, metabolic, signaling) networks; spatial and stochastic aspects of cell biochemistry; integrative coupling of cellular-level

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processes related to common biological endpoints; extracellular signaling and cell-cell communication and interaction; aspects of microscopic and mesoscopic morphological, biochemical and functional heterogeneity within multicellular assemblies and functional physiological or pathophysiological units (e.g., hepatic sinusoids, kidney nephrons, tumors, etc.); dynamics of histomorphological and histopathological processes at the macroscopic tissue level; and integrative coupling of processes at the organismic level, representing the spectrum of physiosystem phenotypes for health and disease states. A critical component of the computational framework presented in this chapter, is a set of approaches for coupling processes and "transferring and integrating" information across different scales of biological organization, from biomolecules to tissues. This set includes a combination of formal methods of multiscale analysis (deriving from fields such as statistical physics), including master equation based descriptions of multicellular kinetics and dynamics, as well as simple "top-down" approaches, both mechanistic and phenomenological. The computational implementation of the framework employs agent-, network-, and field-based modeling methods that have been individually and systematically compared with respect to their strengths and limitations in supporting biological systems modeling and then "merged" in order to achieve a flexible general operational framework for supporting toxicological and environmental health risk analyses.

So, the resulting framework is based on the integrative representation of biological dynamics in terms of (i) interacting networks; (ii) agents that "move across" different networks; and (iii) fields capturing the spatiotemporal relations and variations of a range of physicochemical attributes within a functioning biosystem or biological unit (membrane, organelle, cell, multicellular module, tissue, organ, etc.). This representation is supplemented by rules governing constraints and interactions among components of the biological systems and subsystems, and "actors" that make decisions affecting the state of these systems. A suite of deterministic and stochastic modeling approaches, utilizing a bi-directional coupling between rule-based or stochastic differential equation-based agent approaches on one hand and ordinary and partial differential and integrodifferential equation-based methods, either stochastic or deterministic, that govern the dynamics of fields, on the other hand, is used to achieve the objective of integrative multiscale implementation within this framework.

Interfacing/communication "layers" provide the rules (master equations and so on) for assembling the information from the finer resolution scale and organizing it in a form that is determined by the structure and dynamics of the coarser resolution scale.

2 BASIC CONCEPTS AND METHODS

2.1 Source-to-Dose-to-Effect Analysis of Exposure to Xenobiotics

Xenobiotics such as environmental contaminants have been established as the causes of numerous diseases, from various cancers and neurological degeneration to respiratory, cardiovascular, and metabolic (e.g., diabetes) disorders. Assessment of health risks associated with exposures to specific contaminants (chemical, radiological, biological) present in various *media* (air, water, soil/dust, food, consumer products, etc.), taking place through one or more *routes* (inhalation, ingestion, dermal absorption) requires understanding the events and processes in the sequence from "source" (e.g., the release or formation of the contaminants in the environment) to "outcome" (e.g., the development of an adverse biological effect). *Exposure biology*, as an integrative "systems approach" considers human health state as reflecting multiscale body "system dynamics" (across the genome, transcriptome, proteome, metabolome, cytome, physiome) as they are affected by various environmental (extragenomic) factors that include environmental stressors (e.g., chemical, radiological, and biological contaminants); developmental/aging state and behavior/activities (Figure 1). Of particular importance is the incorporation in this computational framework of dietary/nutritional factors (see, e.g., de Graaf *et al.*, 2009) and of interactions with the "gut microbolome" (see, e.g., Fox *et al.*, 2010), as they can both affect substantially body system dynamics in relation to the response to environmental stressors. These dynamics are modeled through a combination of network, agent, and field representations that incorporate available mechanistic and phenomenological information on the various

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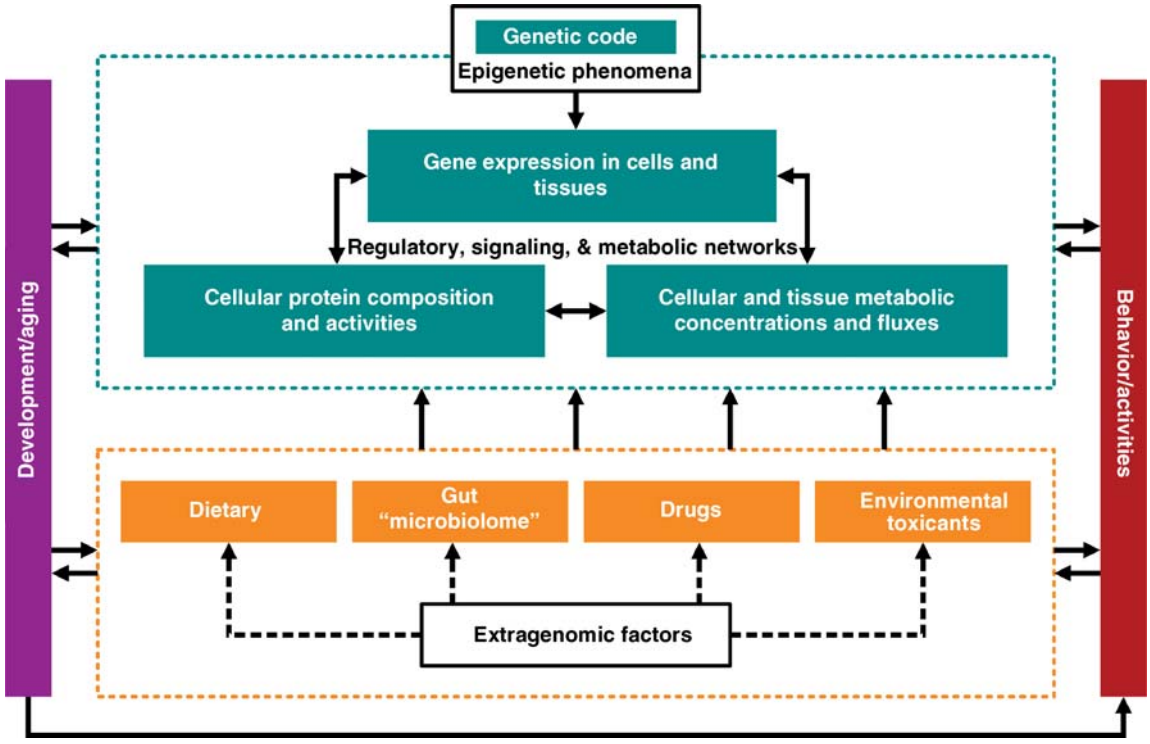

Figure 1. Human health state reflects multiscale body "system dynamics" (across the genome, transcriptome, proteome, metabolome, cytome, physiome) as they are affected by various environmental (extragenomic) factors that include contaminants (chemical, radiological, and biological); developmental/aging state, and behavior/activities (adapted from Georgopoulos, 2008; reproduced with permission from Springer). These dynamics are modeled through a combination of network, agent, and field (continua) representations that incorporate available mechanistic and phenomenological information on the various system components.

system components. This integrative framework allows significant improvements over the "traditional" approaches of studying risks. The traditional approaches generally involve individual contaminants and environmental media (e.g., atmosphere, groundwater, soil, etc.) typically associated with a single exposure route. Recent developments in risk analysis include the evolution of integrative anthropocentric approaches that account for total (cumulative and aggregate) exposures of individuals and populations, to co-occurring contaminants in air, water, soil, food, and so on (Georgopoulos and Lioy, 2006; Georgopoulos *et al*., 2008; 2009b). The framework and applications presented in this chapter are the outcome of a continuing research effort, funded primarily by the US Environmental Protection Agency (USEPA) and the National Institute of Environmental Health Sciences (NIEHS), to develop, evaluate and apply mechanistic and diagnostic computational modeling tools intended to support comprehensive analyses of all the steps in the "environmental health sequence" from the release and transport/fate of environmental contaminants to human exposure/dose and subsequent development of disease (Figure 2). This effort has resulted in two continuously evolving software "libraries," developed primarily in Matlab (The Mathworks, 2010), with various routines in C++ and Java, and with links to an extensive set of data management and analysis tools linking relational databases (such as Oracle [Oracle, 2010], PostgresSQL [PostgreSQL, 2010] and MySQL [MySQL, 2010] and Geographic Information Systems (such as ArcGIS [ESRI, 2010]), that store and manage environmental and biological information, on customized Linux clusters (Georgopoulos, 2008). These software libraries constitute the *M*odeling *EN*vironment for *TO*tal *R*isk studies (MENTOR), that addresses the sequence of environmental "source-to-dose" processes, and

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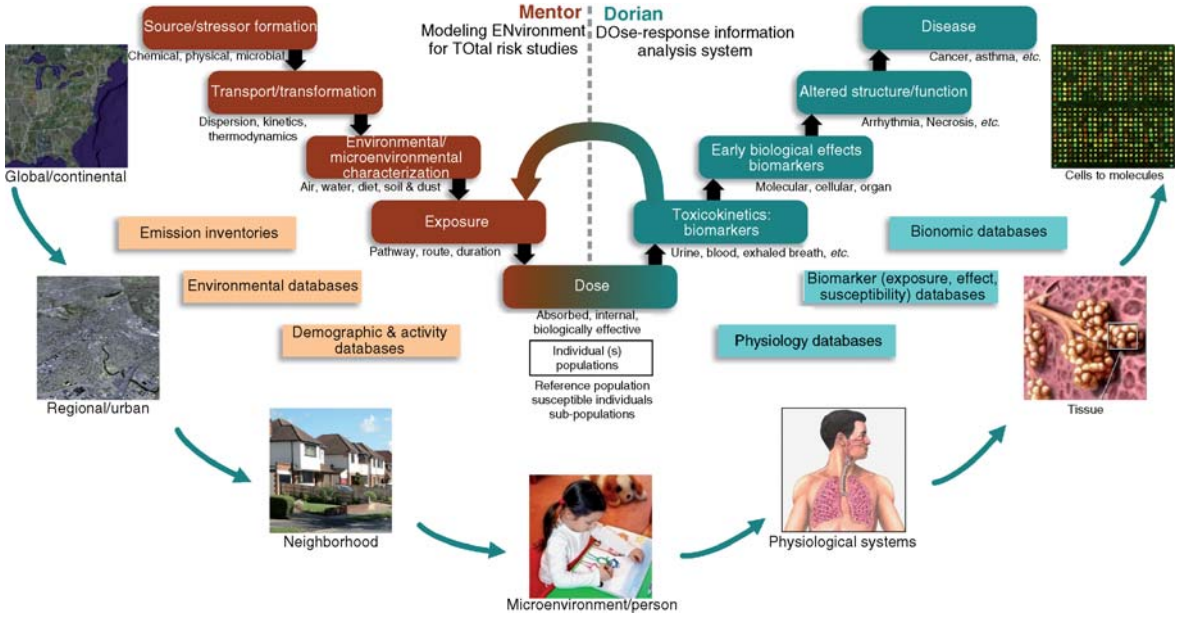

Figure 2. Schematic representation of the "environmental health sequence" from "source" of a stressor (chemical, biological, radiological) to biological "outcome," involving a series of environmental and biological "steps" (Figure source: Georgopoulos *et al*., 2009a).

the *DO*se-*R*esponse *I*nformation *AN*alysis system (DORIAN), focusing on the sequence of the biological "dose-to-effect" steps, to provide, in combination, integrative "exposure biology framework" for the analysis of human health risks from xenobiotics and other environmental stressors. Figure 2 contains a schematic representation of the "environmental health sequence" from "source" of a chemical, biological, stressor, to biological "outcome," involving a series of environmental and biological "steps." It should be noted that the models within MENTOR and DORIAN in principle allow not only calculations of dose and biological response or effect from information on environmental conditions, but also, under certain constraints, reconstruction of environmental exposure patterns from appropriate biomarker data (Georgopoulos *et al*., 2009b).

A series of recent publications have focused on MENTOR, presenting its overall framework and applications covering the "source-to-dose" steps for various environmental problems (Georgopoulos *et al*., 2005; Georgopoulos and Lioy, 2006; Georgopoulos, 2008; Georgopoulos *et al*., 2008; 2009b; Xue *et al*., 2010). Here, in the sections that follow, the focus is on the evolving framework and applications of DORIAN, which provides tools for mechanistically linking contaminant dosimetry with specific adverse biological outcomes.

Figure 3 presents a simplified schematic description of toxicokinetic and (initiation of) toxicodynamic processes in the human body. In this example, only the ingestion exposure route is considered, where a xenobiotic is absorbed in the gastrointestinal tract and, through systemic circulation, is transported to the liver, which is assumed to be the primary site of metabolism and target effects. The xenobiotic (ligand) will interact with various biomacromolecules ("receptors") during the various toxicokinetic steps of absorption, distribution, metabolism, and elimination (ADME); more complex patterns are generally expected in the interactions of the xenobiotic (or its metabolites) with cellular signaling and regulatory networks, resulting in the initiation of toxicodynamic effects. Structure-activity differences in the various receptors, reflecting genetic polymorphisms within a population, may result in different "strengths" (phenotypic polymorphisms) of various

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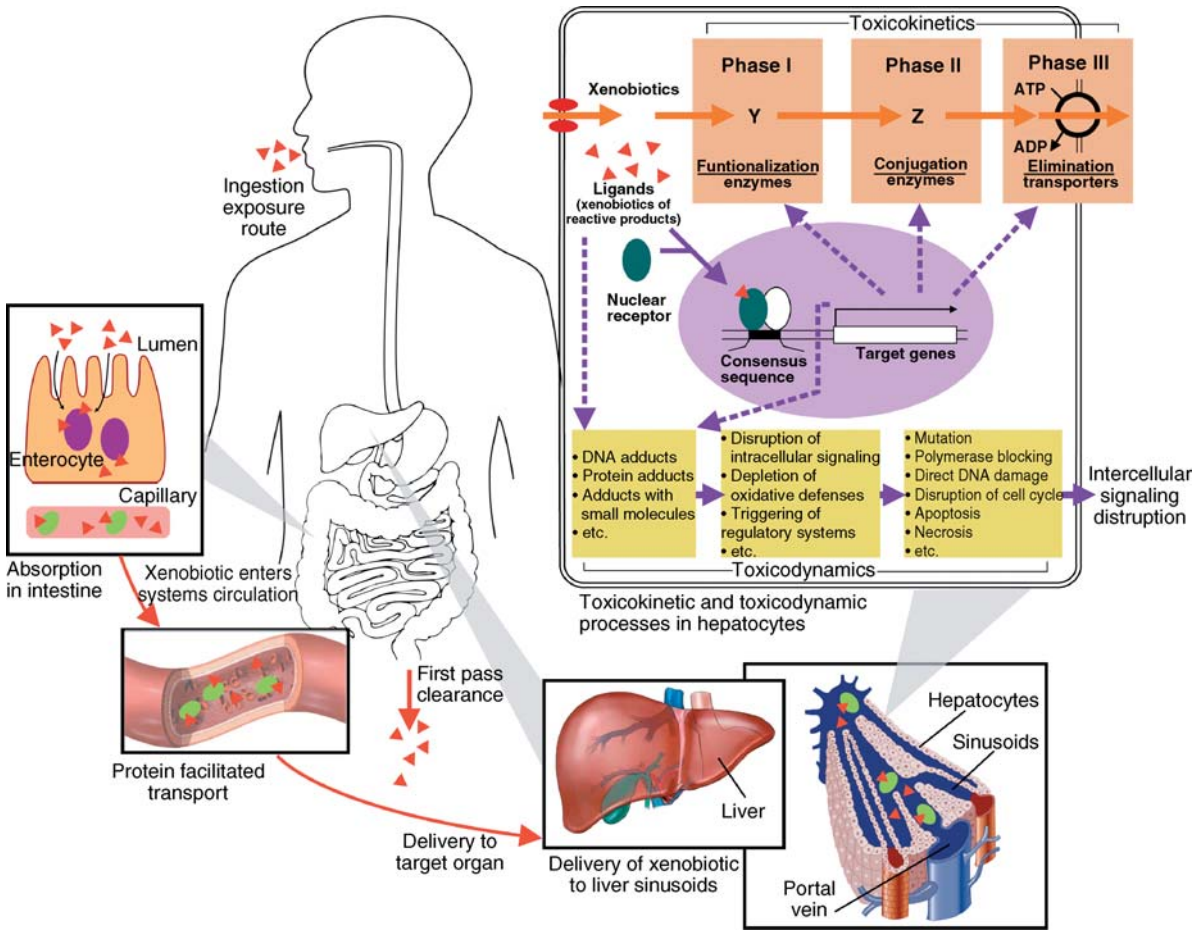

Figure 3. A simplified schematic description of toxicokinetic and (initiation of) toxicodynamic processes in the human body. In this example, only the ingestion exposure route is considered, and the xenobiotic is transported to the liver, which is assumed to be the primary site of metabolism and target effects. The xenobiotic (ligand) will interact with multiple biomacromolecules (receptors) in toxicokinetic (absorption, distribution, metabolism and elimination – ADME) and toxicodynamic processes. Structure-activity differences in the various receptors, reflecting genetic polymorphisms within a population, may result in different "strengths" (phenotypic polymorphisms) of various bioactivation and/or detoxification pathways, potentially resulting to different susceptibilities of effect for individuals of this population.

bioactivation and/or detoxification pathways, potentially resulting to different susceptibilities of effect for individuals of this population. Figure 4 shows schematically typical toxicokinetic and potential toxicodynamic processes taking place, or commencing, in the human hepatocyte. However, in spite of the significance of the individual cell with respect to this process, it should be emphasized that toxicity is a result of a cascade of events that involves multiple scales, both intra- and extracellular. To emphasize the fact that cells never "act" as isolated individuals but rather as members of complex "communities," Figure 4 presents a simplified overview of interlinked intercellular and intracellular signaling networks reflecting biological communication processes among the different types of liver cells (hepatocytes, Kupfer cells, endothelial cells, hepatic stellate cells) as well as components of the extracellular matrix.

DORIAN incorporates toxicogenomic information that is becoming available from various high throughput bionomic (transcriptomic, proteomic, interactomic, metabolomic, etc.) laboratory studies and links a mechanistic analysis framework

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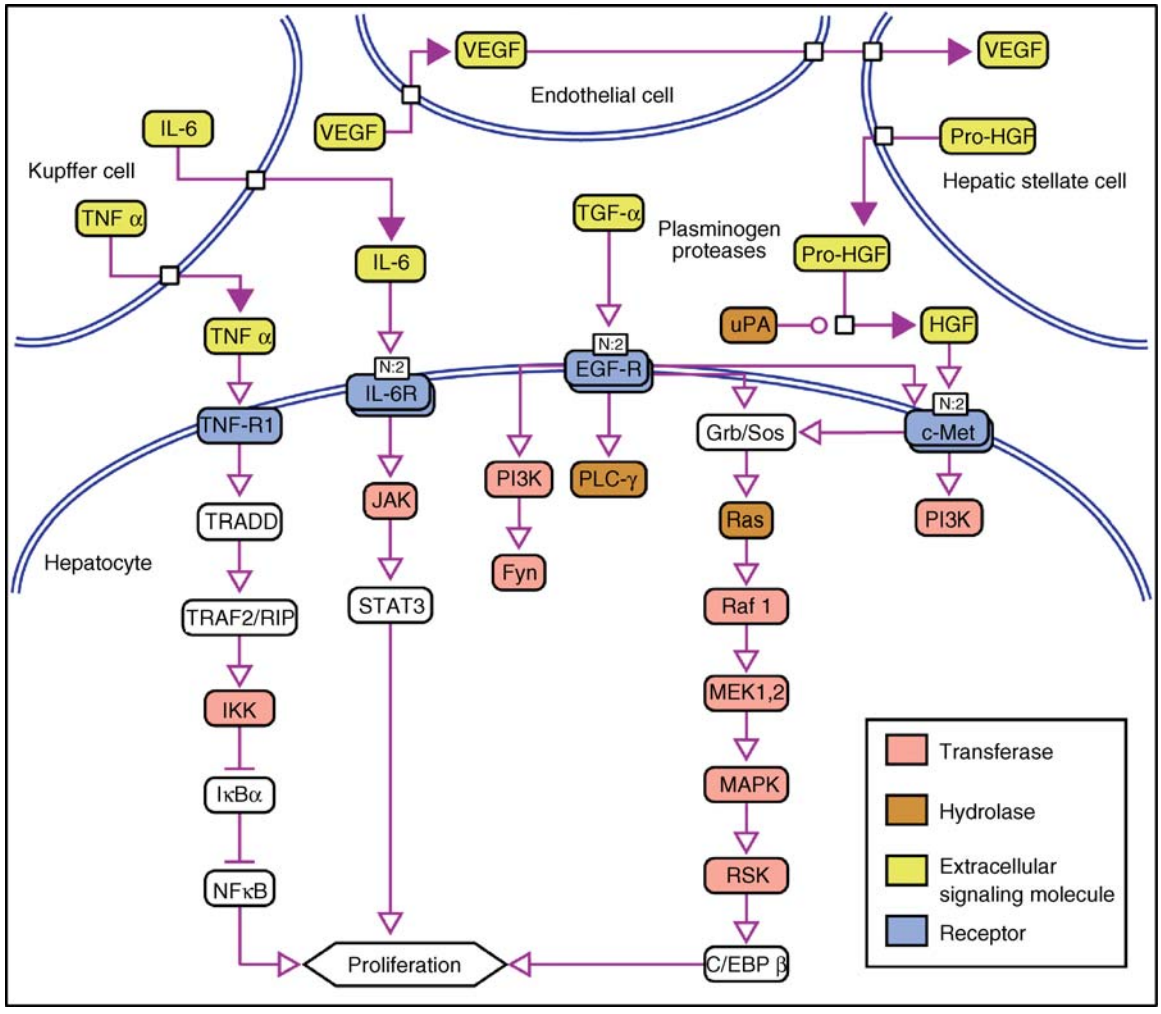

Figure 4. A simplified overview of interlinked intercellular and intracellular signaling networks reflecting biological communication processes among the different types of liver cells (hepatocytes, Kupfer cells, endothelial cells, hepatic stellate cells) as well as components of the extracellular matrix. Systems Biology Graphical Notation (SBGN – Jansson and Jirstrand, 2010) is used to depict molecular interactions; the interactions shown are based on information from Pagano *et al.* (2007).

to ebTrack (Chen *et al.*, 2009). ebTrack is a new user-oriented platform that expands the features of ArrayTrack (Tong *et al.*, 2003) by incorporating a range of options for the analysis of data from a wide range of new technologies, in addition to providing options (beyond those already available in ArrayTrack) for studying microarray gene expression data (e.g., Ouyang, Welsh and Georgopoulos, 2004). The objective of DORIAN is the mechanistic *in silico* replication and interpretation (including cross-species extrapolation) of heterogeneous information from *in vivo*, *in vitro*, and *ex vivo* studies from high-throughput (HT) technologies, from bioengineering applications, and from traditional toxicology experiments (see Figure 5). *In silico* models and model organisms both play a critical (integrative) role in interpreting information from diverse sources and raise the requirements for data and for data management and analysis techniques (Joyce and Palsson, 2006; Ryan, 2008). To support usage of DORIAN in real-world applications, an "environmental bioinformatics Knowledge Base" (http://ebKB.org – Figure 6), has been developed and is available

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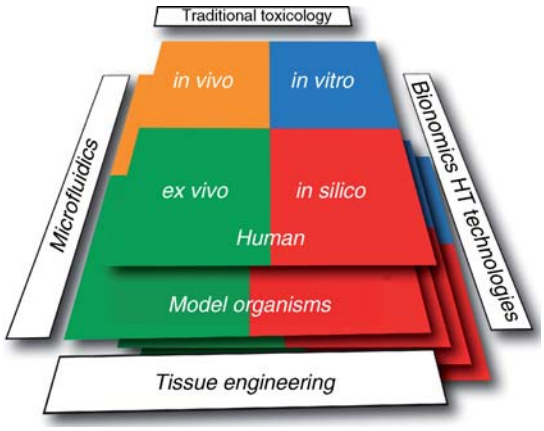

Figure 5. The objective of DORIAN is the mechanistic (*in silico*) replication and interpretation of information from *in vivo*, *in vitro*, and *ex vivo* studies from high-throughput (HT) technologies, bioengineering and traditional toxicology. *In silico* models play a critical (integrative) role in interpreting data for different model organisms for mechanistic cross-species extrapolation.

through a web portal; ebKB is being updated regularly to include continuous advancements in this field.

2.2 Combining Field, Agent, and Network Based Approaches for Biological Modeling

The integrative DORIAN approach to mechanistic toxicokinetics and toxicodynamics considers inter- and intra-cellular *networks* (i.e., regulatory, signaling, and metabolic cascades), as well as physiological (multicompartmental) networks coupled with spatiotemporal *fields* or *continua* (either random or deterministic) of biophysical attributes, and with biological entities that mathematically are identified as "agents" that are affected by and affect other components of the biosystems under study. Additional mathematical entities are "actors" that implement mechanistic or semiempirical "rules" constraining the functions of these biosystems (Figure 7). The above types of mathematical constructs span multiple hierarchical strata and spatiotemporal scales, and they interact at various levels. For example, a coarser-level component can provide boundary conditions or forcing functions to a finer-scale component, while "aggregates" or "assemblies" of finer-scale components can provide parameter values (or distributions of parameter values) to a coarser-scale component.

At each hierarchical stratum, each of the generalized bionetworks under consideration is defined through (i) network nodes (compartments); (ii) network edges or links (relationships, interactions); and (iii) network function (states, dynamics). The nodes can be defined as static (e.g., tissues within an organism) or dynamic (e.g., cells involved in an inflammatory process, in which case they can also be represented as agents). *Each node of a bionetwork can itself be a network, an agent, an actor, or a combination of them*. Interactions among the networks (and associated agents) and processes governing the states of the networks are defined in relation to spatiotemporal "fields" or "continua" (random or deterministic) of biological (i.e., biophysical and biochemical) properties. The constraints on the types of interactions (thermodynamically allowable chemical reactions, transport of molecules across membranes and biocompartments, probabilities associated with migration of agents from one network node to another, etc.) constitute the rules governing the evolution of the networks and associated agents. Considering the cell as the "middle level" of critical biological organization, the above approach distinguishes two major groups of bionetworks, that is:

- *Intra-cellular networks* (where network nodes are biomolecules, biocomplexes, organelles) that include intra-cellular signaling networks, transcriptional regulatory networks, and metabolic networks. For example, components of transcriptional regulatory networks are binding sites, transcription factor molecules, riboswitches, and so on, while corresponding network links include DNA-protein, protein-protein and metabolite-RNA interactions.
- *Intra-organism networks* (where network nodes are cells, cellular assemblies, multicellular functional modules, tissues, organs, physiological systems), that include inter-cellular signaling networks and physiological level networks that reflect connections of organs and physiological systems through the transport of matter and information (signals) by the circulatory, lymphatic, and nervous systems.

Network dynamics coupled with the properties/dynamics of biophysical and biological fields

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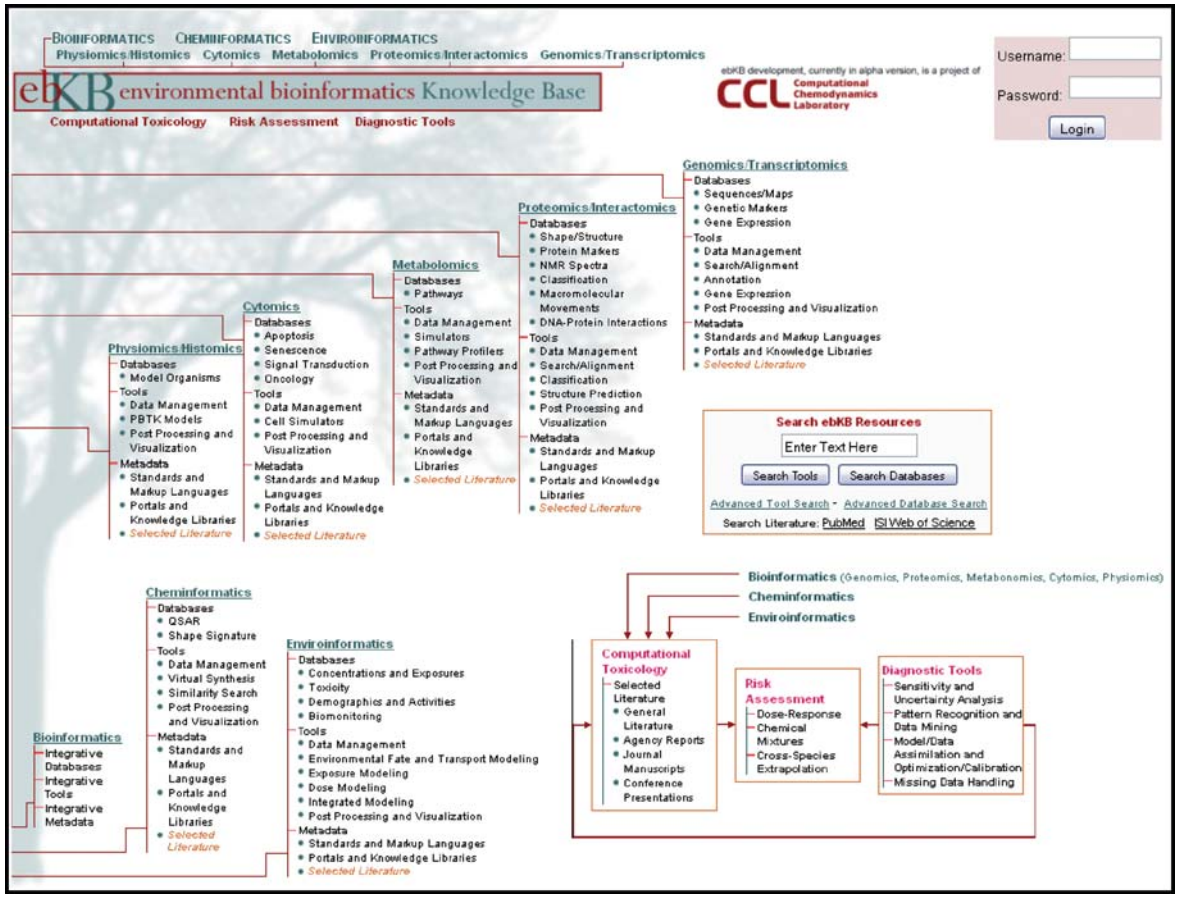

Figure 6. Structure of the Environmental Bioinformatics Knowledge Base (ebKB) for supporting modeling and analysis efforts for assessing human health risks from xenobiotics.

(density, pressure, concentration, electrical charge, etc.) can be described by various combinations of descriptive (phenomenological) and predictive (mechanistic) models, depending on the available data and scientific knowledge. When the number of constituents in a network is large, it may be more appropriate to represent the states of the system in terms of (probability) distributions of key parameters.

Movement of agents within a system is typically modeled as a stochastic process, using constrained random walk models with associated transition probabilities from one network node to another. Critical variables in toxicological and environmental health risk assessment include concentrations of contaminants within a specific biological compartment (e.g., a tissue, etc.), which are scale dependent, and distributions of other factors affecting the system (e.g., probabilities of occurrence of specific genetic polymorphisms). However, networks of different scales, and nodes within a network, will interact with a variety of fields; simulation of these fields requires process-based models with inter-network boundaries defined via differential gradients.

3 IMPLEMENTATION OF A MODULAR MULTISCALE WHOLE BODY FRAMEWORK

The *DO*se-*R*esponse *I*nformation *AN*alysis (DORIAN) framework is being implemented as an expandable software library that systematically collects and organizes methodological research

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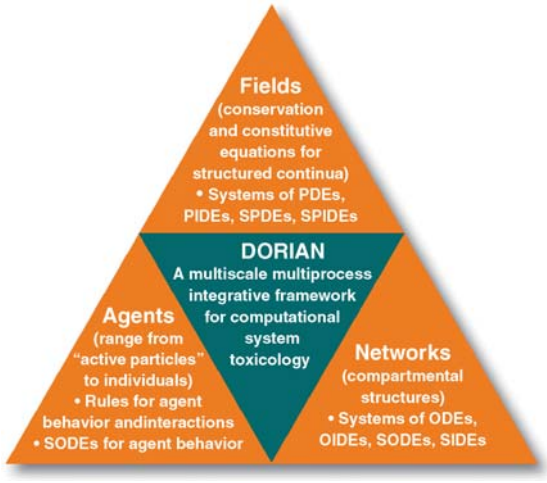

Figure 7. The multiscale modeling framework of DORIAN employs a combination of field-, network-, and agent-based approaches spanning multiple strata of biological organization: the mathematical implementation involves systems of deterministic and/or stochastic ordinary and partial differential and integrodifferential equations [(S)ODEs, (S)PDEs, (S)IDEs] as well as rules (or, in some cases, SODEs) for agent behaviors and interactions.

outcomes from the various completed and ongoing efforts within ebCTC (ebCTC.org). DORIAN aims to provide an integrative but highly modular framework for multiscale modeling of "environmentally induced" effects on biological systems, and in particular on the human organism; this framework also aims to explicitly incorporate variability in human susceptibilities due to genomic factors (e.g., genetic polymorphisms), epigenetic phenomena, and so on. The modules of DORIAN are organized across a sequence of "levels or strata of biological organization," and range from computational descriptions of the toxicologically relevant properties of individual biomolecules and xenobiotics, to models of molecule-molecule (e.g., ligand-receptor) interactions, and, eventually, to whole "virtual cell", "virtual organ," and "virtual organism" models. Figure 8 shows the general structure of the DORIAN framework for the multiscale analysis and modeling of biologically-based toxicokinetic and toxicodynamic processes. "Integrative modules" of intra- and extracellular event dynamics within the DORIAN "library" incorporate

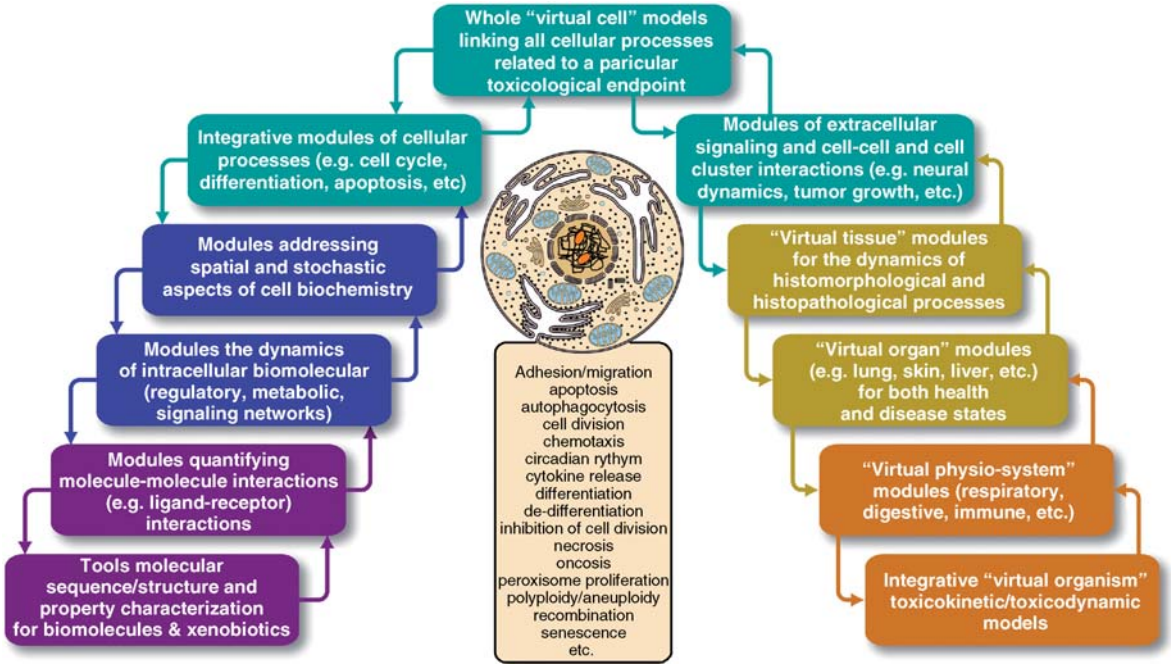

Figure 8. The general structure of the DORIAN framework for the multiscale analysis and modeling of biologically-based toxicokinetic and toxicodynamic processes. "Integrative modules" of intra- and extracellular event dynamics within the DORIAN "library" incorporate pathway and network information related to cascades of processes that can be disrupted by the presence of specific xenobiotics (or other environmental stressors) of concern.

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pathway and network information related to cascades of processes that can be disrupted by the presence of specific xenobiotics and other environmental stressors of concern. *Biomodules* (i.e., functional biological subsystems) affected by the biological action of selected stressors (that may have synergistic or antagonistic interactions with respect to particular toxicological endpoints) are "constructed" from plausible biological hypotheses (or "reconstructed" from observational evidence), by applying available physicochemical principles and relevant information from experimental data, for each of these "levels" or "strata." "Reduced" (or simplified) versions of biomodules from each stratum that, based on the outcomes of detailed robustness and sensitivity analyses, maintain the most essential (and relevant) attributes of the original biological systems, are derived and are either "coupled" dynamically to, or, alternatively, provide parameterizations of components for biomodules of the next higher level.

As stated in Section 2.2, the mathematical (and subsequently computational) implementation of DORIAN describes the above biomodules in terms of generalized bionetworks coupled with biologically relevant property fields (structured continua) and discrete mathematical entities (agents). DORIAN employs a combination of (i) "mechanistic (biochemical) interaction"; (ii) "phenomenological (statistical) influence" descriptions, depending on the extent of available biochemical/biological knowledge and of related data. In some cases a "hybrid" approach is employed, that utilizes, for example, Bayesian model-data "fusion" techniques, to obtain optimal or quasi-optimal parameterizations of "postulated" mechanistic models from related "experimental" observations. The mathematical formulations employed in the above implementations include, depending on the specifics of the problem at hand, deterministic, or stochastic (and, in some cases, qualitative) ordinary and partial differential and integrodifferential equations; deterministic and stochastic Boolean logic networks; Bayesian networks, and so on. (Price and Shmulevich, 2007; Thai, Cai and Du, 2007). DORIAN utilizes a wide range of computational tools (i) for physics-based modeling (i.e., modeling of lumped- and distributed-parameter systems represented by deterministic and stochastic ordinary and partial differential and integrodifferential equations for the various conservation/balance equations [mass, energy, momentum] and the associated constitutive equations [reaction rates, transport properties] governing the systems); (ii) for object-oriented modeling of agents and actors; and (iii) for event-driven simulations. DORIAN modules make extensive use of publicly available software packages for systems biology (e.g., Matlab toolboxes such as SimBiology [The Mathworks, 2010] and CellNetAnalyzer [Klamt, Saez-Rodriguez and Gilles, 2007]), supplemented by additional computational tools, developed within ebCTC, on platforms that include Matlab, Java, and R (e.g., Feng and Rabitz, 2004; Chen *et al*., 2009). Various available software tools (such as Virtual Cell [NRCAM, 2010] and Compucell 3D [Cickovski *et al*., 2007; CompuCell 2010]) are also being utilized for application development and for cross-comparison purposes. Computational environments appropriate for handling specific aspects of spatiotemporal data and continuous dynamics are also employed; these include ArcGIS (ESRI, 2010) and Comsol Multiphysics (Comsol, 2010) in combination with selected Agent-Based Modeling (ABM) platforms that include Netlogo (Wilensky, 2010) and Repast (Repast, 2010).

Selected examples of issues related to specific "biological levels" within the DORIAN framework, and of corresponding implementation approaches, are presented in the next Section. A brief overview of the DORIAN framework formulation is presented here in the following two subsections.

3.1 Modularity and Hierarchical Multiscale Structures

The DORIAN system, extending the approach already employed in MENTOR (Georgopoulos, 2008), provides a modular bi-directional framework linking multiple levels and scales of physical, chemical, and biological phenomena (Figure 9). Both formal and heuristic "bottom-up" and "top-down" formulations are supported in this framework, often in combination, so as to allow flexibility of model structure and parameterizations, and to facilitate alternative model implementations that are optimized for specific applications. Interfacing/communication "layers" provide the rules (ranging from semiempirical allocation factors to master equations governing the distributions of parameters of key biological properties) for

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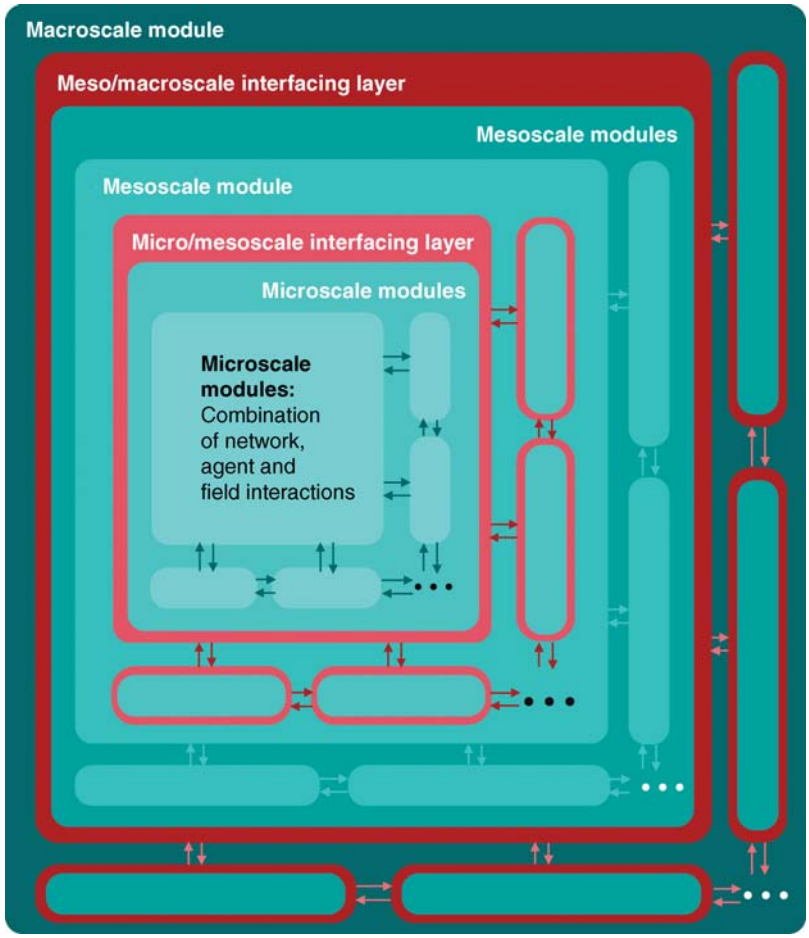

Figure 9. DORIAN provides a modular bi-directional framework linking multiple scales of physical, chemical, and biological phenomena. Both formal and heuristic "bottom-up" and "top-down" approaches are supported in this framework, often in combinations, so as to allow alternative model structure and parameterizations and to facilitate formulations that are optimized for specific applications. Interfacing/communication "layers" provide the rules for "assembling" the information from the finer resolution scale and organizing it in a form that is determined by the structure and dynamics of the coarser resolution scale.

"assembling" the information from the finer resolution scale and organizing it in a form that is determined by the structure and dynamics of the coarser resolution scale.

It is beyond the scope of the present chapter to review the rapidly expanding field of mathematical and computational modeling of complex multiscale (biological and other natural as well as engineered) systems, that is actually becoming a major component of efforts in systems biology (e.g., Auffray and Nottale, 2008; Boissel *et al*., 2008; Clermont *et al*., 2009; Hunter *et al*., 2006; Mesarovic, 1968; Mesarovic and Takahara, 1975; Mesarovic, Macko and Takahara, 1970; Mesarovic, Sreenath and Keene, 2004; Noy *et al*., 2009; Ockner, 2004; Uhrmacher, Degenring, and Zeigler, 2005; Walz, 2005; Woods and Mulholland, 2008; Yaliraki and Barahona, 2007). Only a brief discussion of selected issues that are of primary importance to the implementation of DORIAN is presented in this subsection.

A major challenge associated with multiscale biological system modeling is the consistent linking of different scales for simulating the states and dynamics of network components, agents, and actors (Lavelle *et al*., 2008), while accounting for the uncertainties and the "noise" that typically exist within these subsystems. However, the

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level of complexity required to adequately represent a system is primarily dependent on the specific problem being studied and on the metrics of importance for the phenomena studied. In general, the techniques for studying complex systems employ simplification procedures, such as "rational" model reduction techniques (resulting from a hierarchical evaluation of modeling assumptions) complemented by encapsulation approaches for identifying coherent functioning "biomodules" within the system of concern. For example, Hartwell *et al*. (1999) discuss the issue of the decomposition of biological systems into interacting subsystems (biomodules). A biomodule is identified so that there is "strong" coupling among those processes and interactions that are internal to the module, while there is "weaker" coupling of these processes with external events; it should be emphasized that each module involves mathematical representations consisting of (or combinations of) networks, agents, and fields. Within DORIAN, module identification and characterization techniques are also complemented by model-structure independent tools for diagnostic analysis, such as (i) model-data fusion through Bayesian parameter estimation and numerical inversion (Georgopoulos *et al*., 2009b); (ii) systematic simplification of models through perturbation-analysis (Gerdtzen, Daoutidis and Hu, 2004), deterministic and Stochastic Response Surface methods (Isukapalli and Georgopoulos, 2001), and High Dimensional Model Representation methods (Wang *et al*., 2005; Li *et al*., 2008).

Typically, "top-down" approaches model macroscopic phenomena in biological tissues via methods of continuum mechanics, through the derivation of evolution equations for the macroscopic variables that describe the physical state of the system. This derivation usually follows the principles of continuum mechanics, that is, conservation of mass, momentum, and energy equations; these equations require "closure" that is usually provided by phenomenological models ("constitutive relations") describing the material behavior of the system. It may be stated that top-down approaches aim to "reverse engineer" underlying mechanisms from coarser scale observations.

"Bottom-up" approaches can be either analytical, employing methods from statistical physics and kinetic theory, or numerical "semi-mechanistic" techniques, that is, agent-based, employing mechanism-based or data-driven rules for the behavior of individual "biological units" (macromolecules, cells, etc.). Asymptotic methods are typically used in conjunction with the analytical approaches to derive macroscopic equations from the underlying microscopic description; these equations govern the evolution of (probability) distribution functions over the microscopic states of large collections of interacting entities. A simple standard example from mathematical kinetic theory is the (continuous or discrete) Boltzmann equation, governing the evolution of a system of identical particles considered as point masses with a microscopic state simply identified by position and velocity. Other examples involve asymptotic methods that expand the distribution function in terms of dimensionless parameters related to the inter-particle distances. Similar procedures have been implemented to derive macroscopic equations for "generalized" or "active" particles and applied to biological systems such as large assemblies of interacting cells. The microscopic state or "microstate" of an "active particle" is characterized not only by mechanical parameters (e.g., position and velocity), but also by parameters determining biological functions at a cellular or sub-cellular level. Microscopic interactions may not only modify the microscopic state, but may also result to proliferation and/or destruction phenomena, that is, to the generation or loss of generalized/active particles (non-conservative interactions). The macrostate of the whole system is described by the probability distributions of the various relevant parameters over the microstates of the constituent particles. Derivation of the equations describing the time-space evolution of the stochastic variables defining the states of the systems follows the general classical statistical kinetic theory, that is, it is based on balance equations for net flow of generalized particles in the elementary volume of the microscopic *state space*, as affected by transport intra-particle factors, and inter-particle interactions. Such derivations have been pursued for multicellular biological systems by many researchers (e.g., Alt, Deutsch and Dunn, 1997; Bellomo and Bellouquid, 2004; Hadeler, Hillen and Lutscher, 2004; Hillen and Othmer, 2000; Lachowicz, 2005; Othmer and Hillen, 2002; Bellomo and Bellouquid, 2006, etc.). The analysis of Bellomo and Bellouquid (2004, 2006) for systems of one and of two interacting populations (limited to the

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case of mass conservative encounters) showed that interactions affecting the biological functions of cells can substantially modify the structure of the macroscopic governing equations. Effects of external factors have also been modeled, either at the macroscopic scale or at the microscopic scale through the probability distribution of the microstates (Bellomo, Bianca and Delitala, 2009; Bertotti and Delitala, 2008).

The relative advantages of "top-down" versus "bottom-up" approaches for coupling of biomodules across multiple scales have been discussed in the literature (e.g., Meier-Schellersheim, Fraser and Klauschen, 2009). A limitation of top-down approaches is that often a higher/coarser scale phenomenon may have multiple potential underlying explanations arising from the dynamics of the lower/finer scales. On the other hand, bottom-up approaches, that describe system behavior by "aggregating" the dynamics of the finer scales to explain phenomena at the coarser scales, in general require higher computational resources and more detailed information bases. Nevertheless, both approaches can identify potential knowledge and data gaps and provide insight into targeting new studies (Xu *et al*., 2007).

The ongoing development of DORIAN has embraced a combination of "top-down" and "bottom-up" approaches resulting in the bi-directional framework mentioned earlier. The "whole-body framework" that is presented in the next subsection defines a set of "top-down" constraints, while an evolving software library of alternative algorithmic formulations for numeric agent-based models provides an expanding array of options for calculating macroscopic properties from microscopic dynamics.

It should also be noted that specific implementations of the general DORIAN framework are typically hybrids, containing "standard" semi-mechanistic toxicodynamic models (Mager, Wyska and Jusko, 2003; Mager and Jusko, 2008) or even phenomenological elements, complementing detailed mechanistic parts. In some cases, the phenomenological components are necessary "functional placeholders" for unknown or partly known mechanisms, while in other cases an implementation may employ phenomenological simplifications to "avoid the effort of explicitly modeling causal relationships or processes that lie outside of the focus of the overarching biological question" (Meier-Schellersheim, Fraser and Klauschen, 2009).

3.2 The Whole Body Modeling Framework: Representation of Organs and Tissues

The challenges for extending physiologically-based toxicokinetic models to incorporate toxicodynamic processes were summarized in the Introduction (Section 1) of this chapter. To address these challenges DORIAN complements and extends the MENTOR framework by providing a new, modular "whole body" platform for consistent integrative characterization of multicontaminant toxicokinetic and toxicodynamic processes in individuals and populations. Figure 10 shows a schematic overview of an implementation of the multicompartmental (network) structure of this platform for the human organism; the general structure incorporates sufficient physiological and biochemical information for the various organ systems to address the modeling needs for a wide range of xenobiotics. The operational model incorporates links to physiology and biochemistry databases, to account for intra- and inter-individual variation and variability in properties of the different physiological components. The structure of individual tissues and organs is macroscopically described by multicompartmental network formulations that reflect the specific attributes of intracellular and extracellular media comprising the tissue; this description is complemented, as dictated by the needs of specific applications, by a hierarchy of more detailed (virtual tissue) models that span multiple scales of biological organization. Consistencies with ontologies for physiology, anatomy, histology, cytology, and so on (Noy *et al*., 2009; Burger, Davidson and Baldock, 2008) is an important aspect of these formulations.

PBTK/TD model structures for specific chemicals, or mixtures of chemicals, can be "mapped" to subsets of the general "compartmentalized" physiological network structure presented in Figure 10. Tissues that are not explicitly modeled in chemical-specific implementations of the generalized PBTK/TD structure can be "lumped" into rapidly or slowly perfused groups while maintaining overall physiological consistency. Blood flow rates and volumes of physiological compartments are chemical-independent; however, parameters of

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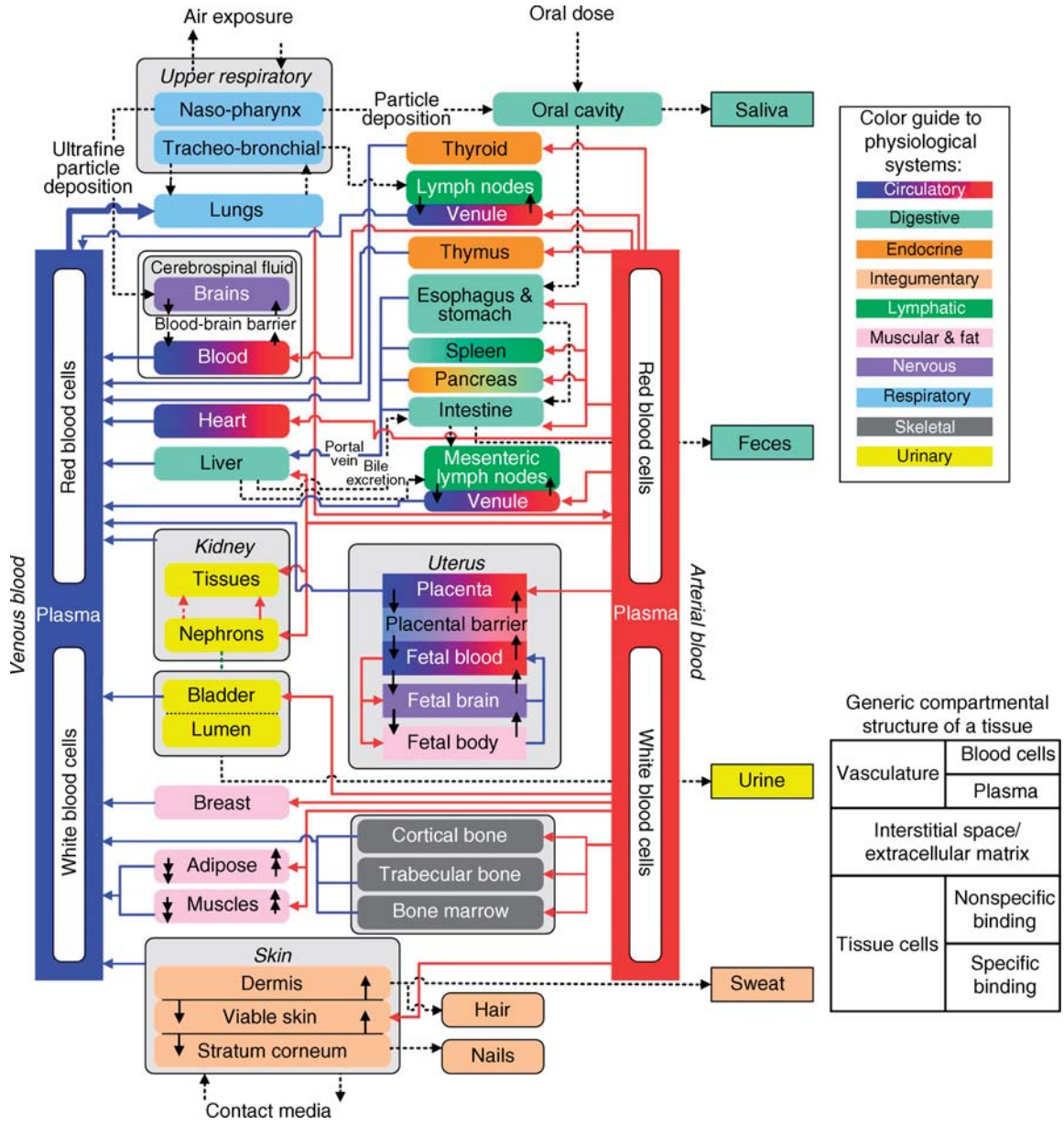

Figure 10. The MENTOR/DORIAN exposure biology framework provides a new, modular "whole body" platform for consistent characterization of multicontaminant toxicokinetic and toxicodynamic processes in individuals and populations; it incorporates links with physiology and biochemistry databases to account for intra- and inter-individual variation and variability. The structure of individual tissues and organs is described by multicompartmental formulations that reflect the specific attributes of intracellular and extracellular media complemented by more detailed (virtual tissue) models that span multiple scales of biological organization. The schematic of the whole body structure presented in this figure is customized so as to facilitate the study of the toxicokinetics and toxicodynamics of nanoparticles (see example in Section 4.7.1) (Figure source: Sasso *et al.*, 2010).

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lumped compartments in some implementations of the general formulation (e.g., flow rates and volumes of slowly perfused and rapidly perfused tissues) may vary based on the particular model structure and the toxic endpoints of interest, and these appear as chemical-dependent. However, all physiological parameters of a compartment need to be constrained so as to be consistent with the values of those quantities for the other compartments. Chemical-specific models for a wide variety of xenobiotics, including volatile and semi-volatile organics, metals and metal compounds, nanoparticles, and so on, have been deduced from this general formulation, allowing for simultaneous toxicokinetic and, in certain cases, toxicodynamic modeling, that may also consider metabolic interactions among the different chemicals (Sasso, Isukapalli and Georgopoulos, 2010).

As mentioned above, physiological variability in a population is consistently considered through linking with current biological databases that provide physiological values for a majority of the tissue groups (i.e., National Health and Nutrition Examination Survey [NHANES – Borrud *et al.*, 2010; CDC, 2010] and the P3M physiological database [Price *et al.*, 2003]). The generalized model offers the option to obtain parameters from databases for the general population and for susceptible populations (e.g., the elderly and health-impaired [Thompson *et al.*, 2009a]). Other sources of whole-body physiology include the PK-Pop scaling algorithm used by PK-Sim (Willmann *et al*., 2007), and the polynomial relationships used by PostNatal (Young *et al*., 2009). The combined MENTOR/DORIAN environment allows the definition of populations of "virtual individuals" with consistent physiology using any of the above databases.

With respect to the terminology for the entities in Figure 10, it should be mentioned that the term "tissue" is typically used (both in the present discussion and in general) in two different contexts, that is, to either signify aggregates of specialized cells of a common type (i.e., muscle tissue, nerve tissue, epithelial tissue, connective tissue) or to refer to the "cellular fabric of any organ or structure" (e.g., kidney tissue or lung tissue – each of which in fact usually contains the four classes of tissue listed in the previous context [Widmaier *et al*., 2008]). Figure 10 identifies the "standard" compartments defined by the present modeling framework for each organ. The microenvironment surrounding each cell in a tissue consists of the extracellular fluid, which is interspersed within the extracellular matrix (ECM), consisting of a mixture of protein molecules and other compounds, including minerals, that are specific for each tissue. The ECM provides a scaffold for cellular attachments and transmits information to the cells regulating their activity, migration, growth, and differentiation. The proteins of ECM consist of ropelike collagen fibers, rubberband-like elastin fibers, and various nonfibrous carbohydrate-containing proteins. The latter also function as communication links between extracellular messenger molecules and cells. The term "extracellular fluid" also refers to the fluid present in blood. Overall, about 20% of extracellular fluid is blood plasma, in which the various blood cells are suspended; the remaining 80%, which is distributed in the spaces between tissue cells, is the interstitial fluid (IF). Concentrations of dissolved substances are virtually identical in the plasma and the IF, except for protein concentrations, which are higher in plasma. In general, the entire extracellular fluid is considered to have homogeneous composition (except for proteins); however, the composition of the extracellular fluid is very different from that of the intracellular fluid. Two-thirds of it is intracellular fluid and the remaining one-third is extracellular fluid, 80% of this overall being interstitial fluid and 20% being plasma.

Organs are often organized into similar "functional units" (Widmaier *et al*., 2008). For example, functional units of the kidneys are the nephrons and the total urine from the kidneys is the sum of the amounts produced by the two million individual nephrons. Organ systems are collections of organs performing a common function: Figure 10 shows a schematic view of the "network interconnections" among the major components of the ten organ systems in the human body as implemented in the present modeling framework.

Various approaches are available to model the processes governing biological transport, biochemical transformation, and biological response in different tissues and/or organs. These approaches span a wide range, from simple, single compartment formulations for an organ to network (multicompartmental), field (structured continuum), agent-based, and hybrid formulations: Table 1 presents a general classification of these approaches and lists representative examples from the literature.

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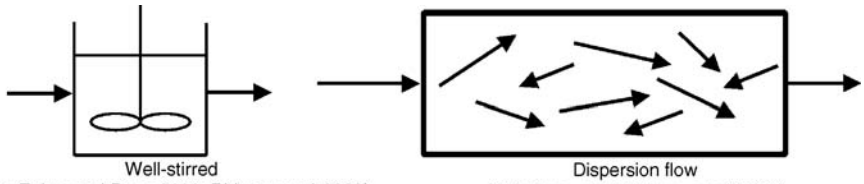
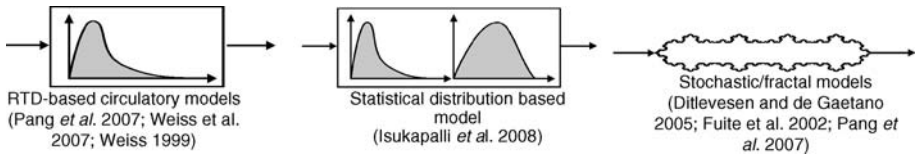
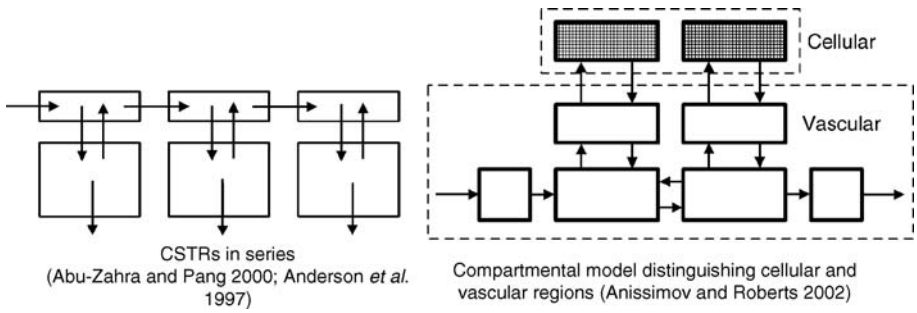
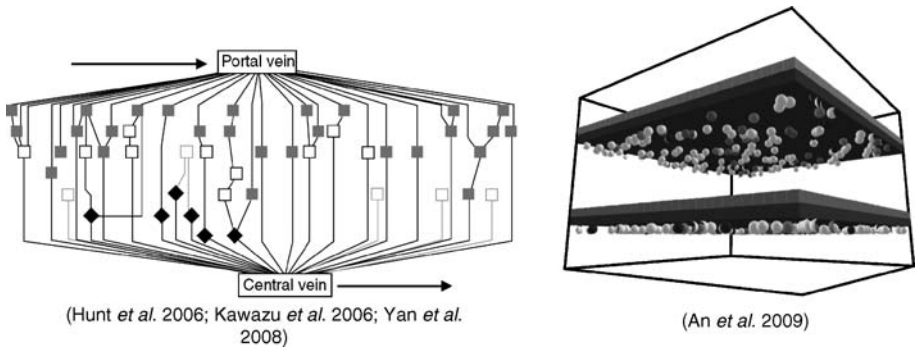
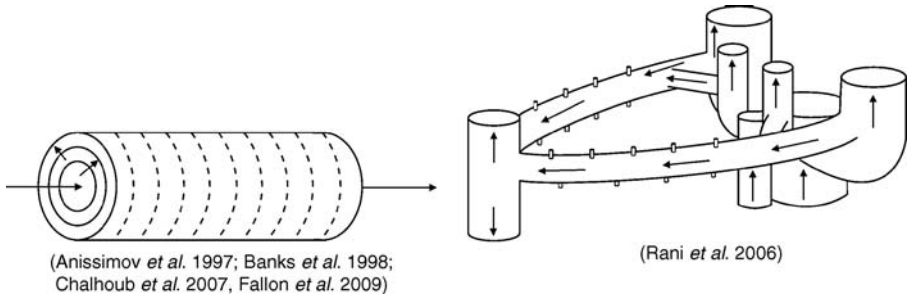
Table 1. A general classification of different conceptual/mathematical descriptions of tissues and organs for the simulation of toxicokinetic and toxicodynamic processes.

Lumped	
Distribution-Based	
Network-Based (multicompartmental)	
Agent-Based	
Field-Based	
Hybrid	Employ combinations of field, network, and agent-based modules (e.g., Anderson *et al.*, 2006; Georgopoulos and Isukapalli, 2009; Wambaugh and Shah, 2010; Zhang, Athale and Deisboeck, 2007)

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The simplest mathematical formulations involve a single, well-mixed (continuous stirred tank reactor; CSTR) compartment, in which the concentration of a chemical in the blood within an organ is assumed to be in equilibrium with its concentration in the tissue, and this concentration is assumed to be uniform throughout the organ (Abu-Zahra and Pang, 2000; Ridgway, Tuszynski and Tam, 2003). For models focusing on macroscopic properties this approach has often been sufficient because the microcirculatory networks in many organs can result in a high degree of mixing (e.g., Weiss, 1997), and there is often minimal change from using a more detailed model (Ito and Houston, 2004). This simplified approach is generally adequate for environmental toxicants when these are present at low concentrations and have relatively slow rates of uptake and metabolism. In some situations, spatial variation or heterogeneity at the macroscale can be modeled via single compartment models that represent "plug-flow reactors" (Anissimov, Bracken and Roberts, 1997; Ridgway, Tuszynski and Tam, 2003). Physiological network formulations involve descriptions of an organ or tissue via sets of reactors in series (Abu-Zahra and Pang, 2000; Anissimov and Roberts, 2002; Ridgway, Tuszynski and Tam, 2003), in parallel (Abu-Zahra and Pang, 2000; Anissimov, Bracken and Roberts, 1997), or in a combination of both. "Dispersion models" offer another approach for considering incomplete mixing within a single compartment formulation (Anissimov, Bracken and Roberts, 1997; Ridgway, Tuszynski and Tam, 2003). More detailed characterizations of variation within an organ at the macroscale employ Residence Time Distribution (RTD)-based circulatory models, which can account for concentration differences between the vascular space and the sampling compartment (Pang, Weiss and Macheras, 2007; Weiss, Krejcie and Avram, 2007), and fractal models, which represent the heterogeneity of the flow within an organ by employing fractal geometry (Ditlevsen and de Gaetano, 2005; Fuite, Marsh and Tuszynski, 2002; Pang, Weiss and Macheras, 2007). Agent-based descriptions focus on organ behavior through the collective dynamics of agents representing either cells or multicellular structures within the organ (e.g., An *et al*., 2009; Hunt *et al*., 2006; Kawazu *et al*., 2006; Yan *et al*., 2008). Field (or structured continua) formulations include models such as computational fluid dynamics (CFD) applications that focus primarily on detailed characterization of flows within the organ (e.g., Rani *et al*., 2006). Finally hybrid models, such as those developed or under development within the DORIAN framework, employ various combinations of compartmental, field, and agent approaches (Anderson *et al*., 2006; Georgopoulos and Isukapalli, 2009; Wambaugh and Shah, 2010; Zhang, Athale and Deisboeck, 2007).

4 REPRESENTATIVE FRAMEWORK COMPONENTS AND APPLICATIONS

This section consists of a series of examples demonstrating the implementation of various components along with selected applications of the general ebCTC DORIAN framework of analysis, spanning the entire molecule-to-bionetwork-to-cell-to-organism range.

4.1 Computational Chemistry Techniques for Molecular Scale Problems

Various computational chemistry techniques are used in predictive modeling of properties of individual molecules and of molecule-molecule interactions. These techniques typically rely on quantitative molecular descriptors (MDs) of xenobiotics (ligands); the MDs include metrics of various constitutional, topological, geometrical, electrostatic, and quantum mechanical properties (Welsh, Tong and Georgopoulos, 2007), and can be calculated directly from the molecular structure of a chemical. Once appropriate MDs are selected, Quantitative Structure-Activity Relationship (QSAR) models can be constructed to "predict" target properties of untested chemicals by associating specific molecular features with chemical toxicity and potentially with specific modes-of-action. In general, these methods provide a phenomenological mathematical association and not a cause-effect (i.e., mechanistic) relationship between the target property and the descriptors: typically they are derived via statistical "pattern recognition" methods such as clustering, classification, and regression. In general, clustering applies unsupervised learning techniques to explore data patterns on the basis of MDs, without ascribing these patterns to specific categorical endpoints. Classification typically employs supervised learning

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techniques that group chemicals into known categorical endpoints (e.g., highly active, moderately active, inactive). Regression is used to establish quantitative relationships between structure and activity, typically through multivariate linear methods and, more recently, via machine learning approaches such as artificial neural networks (ANNs). A concise review of specific QSAR models that have been developed or are under ongoing development within the ebCTC framework can be found in Welsh, Tong and Georgopoulos (2007).

Modeling of ligand-receptor interactions can take place at different levels of detail. For example: (i) values of ligand-receptor binding energy (BE) calculated through physical chemistry methods (and that include solvation, etc., effects) can be used to predict the relative binding affinity of untested small-molecule compounds; (ii) calculated BE values can be used as receptor-based molecular descriptors to produce QSAR models for the ligands; (iii) models of ligand-receptor kinetics can be formulated based on biochemistry parameterized with experimental data; (iv) 3-D models of ligand-receptor "docking" can be developed using computational chemistry techniques. The ligand binding domain (LBD) of a receptor is responsible for both ligand recognition and regulation of protein–protein interactions (Shiau *et al*., 1998). Recent advances in computational hardware and in rapid ligand-receptor docking algorithms have produced successful results in various studies, involving chemicals of environmental concern such as estrogenic compounds, including steroids, phytoestrogens, and polychlorinated biphenyls (see Welsh, Tong and Georgopoulos, 2007).

4.1.1 Example: Modeling Nuclear Receptor Activity in Hepatocytes to Assess Differences in Functionality of the Pregnane X Receptor (PXR) Variants

This example focuses on the disruption of the normal function of nuclear receptors (NRs) in hepatocytes, where the majority of "detoxification" of xenobiotics takes place through metabolism (Phase I and II) and transport (Phase III). Figure 11 presents a schematic classification of pharmacogenomic/toxicogenomic regulation and signaling of phase I and phase II metabolizing enzymes participating in hepatic metabolic and detoxification processes for xenobiotics. NRs typically function as ligand-activated Transcription Factors (TFs) that regulate expression of genes involved in various metabolic disorders and cell death.

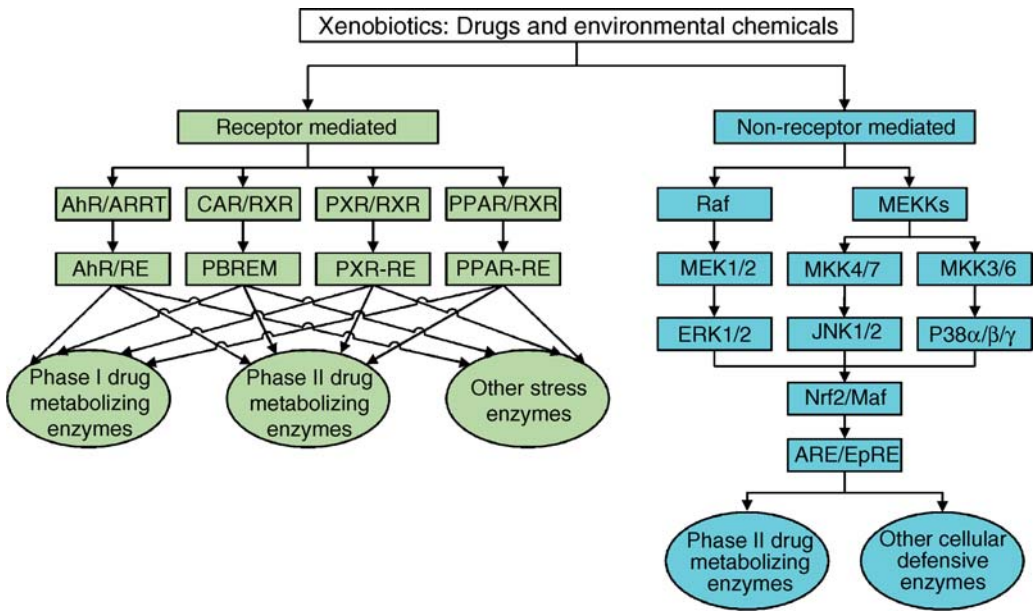

Figure 11. Schematic classification of pharmacogenomic/toxicogenomic regulation and signaling of phase I and phase II metabolizing enzymes participating in hepatic metabolic and detoxification processes for xenobiotics (adapted from Rushmore and Kong, 2002).

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Forty-nine members of the "mammalian NR family" include classical endocrine receptors (ERs) as well as "orphan" receptors. Receptors that bind to a ligand become "adopted orphan" NRs and function as a dimer with retinoid X receptor (RXR); they include receptors for fatty acids (PPARs), oxysterols (LXRs), bile acids (FXR), and xenobiotics (PXR).

Dysfunction of NR signaling is linked to proliferative, reproductive and metabolic diseases (e.g., cancer, infertility, obesity, diabetes) (Aranda and Pascual, 2001; Smith and Muscat, 2005; Ai *et al*., 2009; Hotchkiss *et al*., 2008). Maintaining a normal physiological state requires controlling (temporal and spatial) tissue-specific activity of NRs. Understanding how the presence of related environmental chemicals alters NR signaling is a major objective of the DORIAN project.

Different computational methods have emerged aiming at understanding and modeling the functional activities of NR modulators at the molecular level. Generally these computational approaches fall into two categories, ligand-based and receptor-based approaches. Ligand-based methods essentially focus on molecular similarity, which implies molecules with similar features exhibit similar biological responses. It is a particularly valuable approach to identify compounds if structural information for a receptor is unavailable. By contrast, receptor-based (also synonymous with target-based) methods require the three-dimensional structure of the protein targets predominantly generated from X-ray crystallography, NMR structures or homology modeling, to address the fundamental question of how a potential ligand might bind to the receptor (Ai *et al*., 2009).

Multiple QSAR and machine learning models have been published for several NRs, including estrogen receptor (ER), androgen receptor (AR) and pregnane X receptor (PXR), primarily to address the increasing need not only for high throughput endocrine disruptor risk assessment (Ai *et al*., 2003; Jacobs, 2004; Tong *et al*., 1997), but also for toxicological screening (Zhu *et al*., 2006) in combination with crystal structures and other in silico methods. Figure 12 shows the results from molecular dynamics simulations of the ligand binding domain (LBD) and functional sites of the estrogen receptor (ER). The simulations identified a possible steroid receptor-specific functional site for the ER.

The pregnane X receptor (PXR; PXR.1) is highly expressed in the liver as well as in other tissues involved in detoxification (e.g., intestine) and in steroid-sensitive tissues such as breast (Fukuen *et al*., 2002). It is implicated in cholesterol and bile acid synthesis and metabolism, steroid hormone metabolism, and bone homeostasis (Jones *et al*., 2000; Kliewer and Willson, 2002). PXR shows broad ligand binding specificity because its ligand-binding domain (LBD) contains a novel insert that expands its size. Upon ligand activation, PXR forms a heterodimer with the retinoid X receptor (RXR) to bind to DNA response elements in regulatory regions of target genes. PXR is widely known to be a key regulator of many enzymes (Kliewer and Willson, 2002) and transporters (Synold, Dussault and Forman, 2001) involved in drug detoxification and endogenous homeostasis. There are striking species differences in PXR activation as a result of sequence differences in the LBD.

Alternatively spliced PXR mRNAs have been detected in human liver, breast tissue, colon, and small intestine (Dotzlaw *et al*., 1999; Fukuen *et al*., 2002; Lamba *et al*., 2004). For example, PXR.2 is

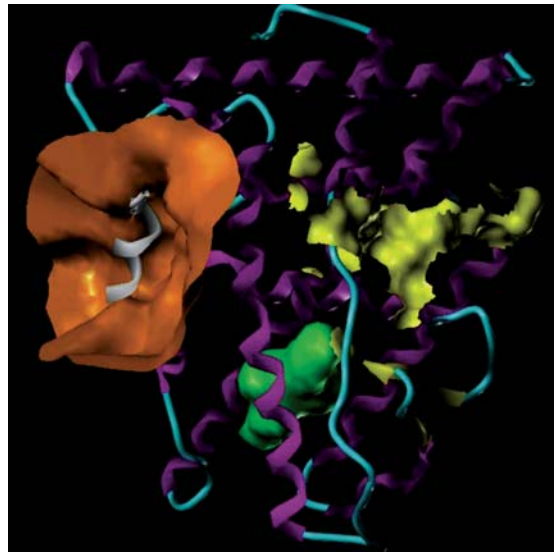

Figure 12. Functional sites on the ligand binding domain (LBD) of nuclear receptors (NRs). The endocrine receptor (ER) is used here as an example. The ligand-binding pocket is shown as a green surface. The cofactor protein-binding site is colored in orange. The computationally identified possible steroid receptor-specific functional site is shown with a yellow surface. The helices of ER are rendered by a ribbon representation and colored in magenta, while the coactivator motif is in white. (Figure source: Ai *et al*., 2009; reproduced with permission from Elsevier).

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also the most abundant alternatively spliced transcript in human liver, accounting for nearly 7% of total PXR mRNA transcripts (Lamba *et al*., 2004). It lacks 111 nucleotides, resulting in a deletion of 37 amino acids from the LBD.

In this example, the differences in the functionality of PXR.2 compared with PXR.1 are studied *in silico* using homology modeling (Krieger, Nabuurs and Vriend, 2003) and intrinsic disorder (ID) analysis (Iakoucheva *et al*., 2002). Hyperforin (a phytochemical commonly known as St John's wort) was used as a ligand in this study. ID analyses of PXR.1 and PXR.2 were performed using the PONDR VL3H algorithm (Peng *et al*., 2005). Information on various PXR ligand bound structures (Watkins *et al*., 2001, 2003a, 2003b) was used as a basis to model the PXR.2 interaction with hyperforin. The deletion of 37 amino acids (Figure 13a, b) causes a wide opening in the binding pocket of PXR.2 (Figure 13c), and it is likely that hyperforin or other similar-sized molecules cannot be kept inside the flexible binding site when this loop is missing. The white hairline next to the red tube (Figure 13b) represents the Leu209 residue that is important for interaction with hyperforin in PXR.1. As PXR.2 has a wide opening in the binding pocket, and does not contain Leu209 residue (Figure 13c), it is likely that hyperforin will have a weaker interaction with the LBD; this could contribute significantly to the lack of function of PXR.2.

Although it would be difficult to predict the functionality of PXR.2 in relation to the multitude of structurally different PXR ligands, this example demonstrates the application of computational modeling to explain the functional differences between PXR.1 and PXR.2. Lin *et al*. (2009) present these results along with experimental data to corroborate the findings. Such uses of complementary computational and *in vitro* approaches are generalizable to study splice variants of other nuclear receptors.

4.2 Analysis, Reduction and Optimization of Bionetworks (Regulatory, Signaling, Metabolic)

A wide range of classical and novel methods are incorporated within the DORIAN framework for the analysis, reduction, and optimization of complex bionetworks; these include:

Lumping of Networks into Modules: This is a commonly used approach in dosimetric and toxicokinetic modeling. For example, sets of heterogeneous cells, tissues, and organs are represented in terms of "rapidly perfused" or "slowly perfused" compartments to represent transport and accumulation, while Michaelis-Menten kinetics are used to "lump" networks of often multiscale, biochemical processes. At the metabolic level, a large, dynamic system of metabolic reactions can be simplified through the use of pseudo steady-state assumptions (Sharma, Ierapetritou and Yarmush, 2005), and can sometimes be expressed in terms of macroscopic reactions relating to extra-network parameters (Haag, Wouwer and Bogaerts, 2005). Techniques such as perturbation analysis allow the

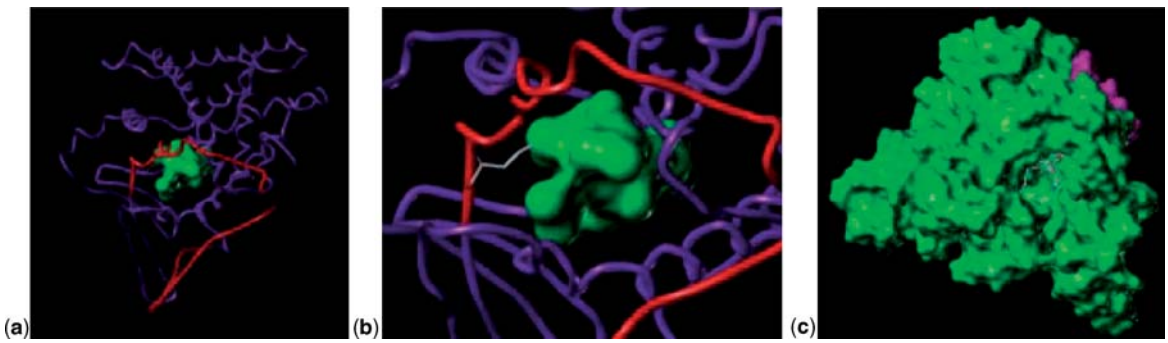

Figure 13. (a) Homology modeling of pregnane X receptor (PXR.2). The trace of PXR.1 full-length in magenta. The 37 amino acids missing from PXR.2 are depicted in red. The green surface of hyperforin is shown behind; (b) close-up view of Leu209 involved in the interaction with hyperforin in PXR.; and (c) the PXR.2 homology model surface is displayed and colored in green, whereas the helix 12 is colored in magenta. The ligand hyperforin is displayed in ball-and-stick representation and colored by atom type (carbon gray, oxygen red) (Figure source: Lin *et al*., 2009, reproduced with permission from the American Society for Pharmacology & Experimental Therapeutics).

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identification of slow and fast processes within the network (Gerdtzen, Daoutidis and Hu, 2004), allowing for an a priori filtering of fast processes, thus significantly reducing data requirements associated with identification and estimation of parameters describing fast processes. The identification of self-contained functional modules also facilitates lumping of different networks (Hartwell *et al*., 1999), as these modules typically do not have large links to external nodes, and only affect a limited set of system variables. The original system can then be described approximately in terms of "networks of modules." The coupled bionetworks of biochemical interactions (metabolic, signaling, etc.) can then be "assembled" into integrative modules (e.g., cellular dynamics) related to specific events/endpoints, shown schematically in Figure 8.

"Collapsing" a Network by Reducing the Number of Possible States and Interactions: This approach allows for substantial simplification of interactions among different networks. The most common technique is to employ a one-way nesting of network interactions (i.e., the properties of a coarser-scale network are assumed to be unaffected by the properties of a finer-scale network). However, assumptions that lead to neglecting the influence of finer-scale components on coarser-scale properties need always to be evaluated on a case-by-case basis. An example is neglecting the impact of toxicant fraction bound to ligands in a tissue when calculating tissue-level concentrations of the chemical. A useful technique for further collapsing the network based on reduction in the number of possible states is via qualitative simulations (Eisenack, Welsch and Kropp, 2006), which can overcome the computational demands associated with combinatorial explosion in the number of possible states and the limitations that arise from sparse data.

Representing Network Heterogeneity Via Uncertainty Distributions: This approach is based on representing heterogeneity in terms of variables that are "unknown" but random. The aggregate properties of random distributions present a global picture while finer-scale detail can be reflected in different statistical moments of the joint distributions. For example intra-individual variability in physiological parameters for populations is often described in terms of probability distributions of inhalation rates, metabolic rate constants, and so on.

Two examples demonstrating the characterization and analysis of bionetworks (a transcription factor network and a set of metabolic networks) follow.

4.2.1 Example: Transcription Factor Network Reconstruction From In Vitro Living Cell Array (LCA) Data Using Reverse Euler Decomposition (RED)

As the spectrum of techniques for obtaining high throughput *in vitro* data is expanding rapidly, new computational approaches are also needed to take full advantage of them. This example describes transcription factor network reconstruction from *in vitro* data from one such technique, the Living Cell Array (LCA). LCA is a microfluidics device that utilizes cells transfected with reporter plasmids. These reporter plasmids consist of an unstable green fluorescent protein (GFP), a minimal promoter, and 4 repeats of a transcription factor's consensus sequence (Wieder *et al*., 2005).

The LCA allows for studying the levels of transcription factor activity under multiple stimuli at high temporal resolution. This improves the ability to decipher the interactions between different transcription factors (TFs) by alleviating data limitations commonly encountered in reconstruction of transcriptional networks from microarray data (Somorjai, Dolenko and Baumgartner, 2003), where the number of genes measured is typically much greater than the number of experimental conditions or time points in which they are measured.

Reverse Euler Decomposition (RED) offers a computational approach for utilizing LCA datasets for isolating TF interactions and for quantifying nonlinear phenomena that are present in biological systems (Hemberg and Barahona, 2007). Identifying these nonlinear interactions is important because they provide evidence of specific mechanistic effects that govern transcriptional activation.

The network reconstruction example here assumes that the dynamics of TF activity can be described through an appropriate, yet to be determined, function (F) which is dependent upon the TF activity itself, and a forcing function, s, which may or may not be a function of time, indicating a specific and direct activation of a TF. In the context of LCA, the forcing function is assumed to be independent of time since it is presumed that the soluble factors continuously activate the TFs through infusion. The activity of the TFs is quantified by monitoring of the expression of the corresponding reporter genes.

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To convert the bi-partite network into a form usable by RED, it was assumed that the direct stimulation of a reporter can only occur via its associated soluble factor, and all other interactions were assumed to be secondary (Yang, Yarmush and Androulakis, 2009). Therefore, if a bi-cluster contains a stimulatory factor of TNF-α and was found to stimulate both NFκB and STAT3, one can assume that there is a direct link between TNF-α and NFκB, while the stimulation of STAT3 must occur downstream of NFκB. Because STAT3 must occur downstream of NFκB, it was assumed that a single process exists that links NFκB to STAT3 and the mechanism and dynamics of interest (Yang *et al*., 2007).

The overall hypothesis behind utilizing the RED is that the numerical response *f(i,j,t)* provides insight as to the underlying processes driving the observed changes in the activity of transcription factors. The functions *f(i,j,t)* essentially represent how the various mechanisms transform the amount of active transcription factors into a signal which is then used to activate a secondary transcription factor. Treating the transcriptional network as a circuit analog, one can exploit the fact that many of the simple network architectures that are obtained have well characterized step responses. Because the LCA utilizes a step input as the stimulatory profile for its soluble factors, one should be able to draw direct comparisons between the observed responses and the characteristic inputs. Figure 14 shows these basic interactions and the expected responses of the system to a step input. By combining these simple components, composite components can be generated that can capture profiles that have both a time delay and tolerance effect. It is then possible to predict important underlying mechanistic properties such as feed forward networks, tolerance

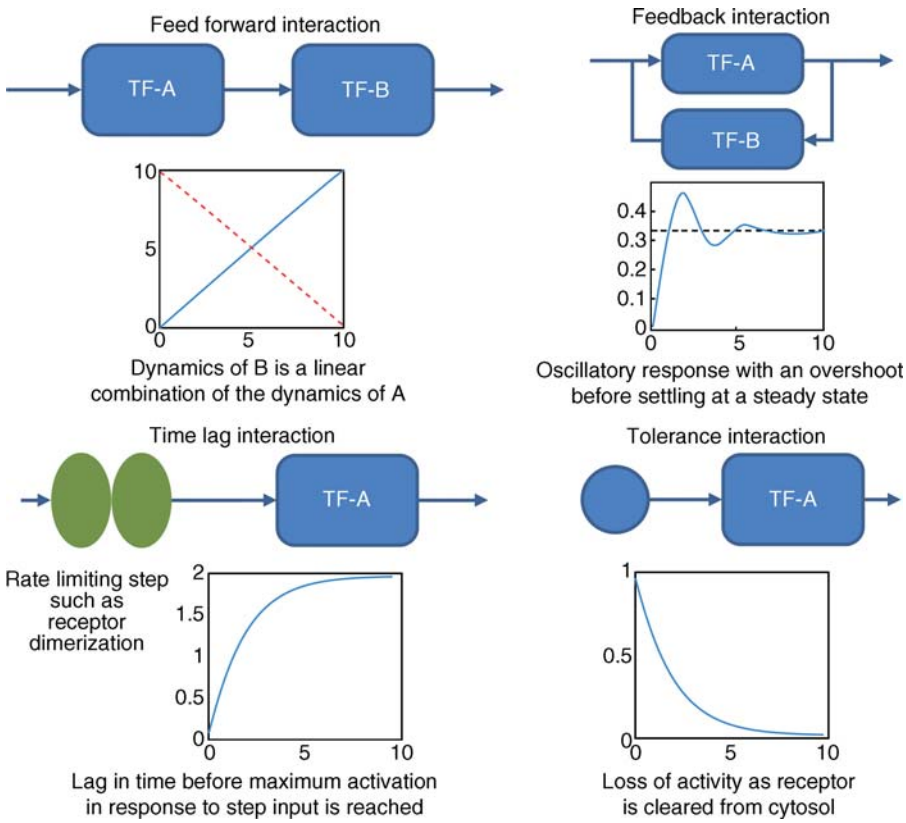

Figure 14. Typical network motifs and their expected responses for a unit step input. Shown are the interaction strengths along the y-axis and time on the x-axis. TF-A and TF-B represent two transcription factors. (Figure source: Yang, Yarmush and Androulakis, 2009; reproduced with permission from Elsevier).

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mechanisms, feed-back networks, and time delay properties.

In the RED approach, the results from an LCA experiment can be reconstructed in a simplified manner using a Forward Euler Integration. One limitation of this reconstruction is the significant error propagation, that is, errors from previous time points are propagated into the predictions for later time points (Dunn, Constantinides and Moghe, 2006). However, for many cases, the reconstructions provide satisfactory representations of the underlying data patterns.

Along with simple network building and determining which transcription factors actively regulate each other, the LCA combined with RED allows for the assessment of possible network connectivity structures as well as provides insight into the dynamics of the interactions. In problems involving optimization of network structures, substantial computational savings can be obtained by using the simplified representation instead of the full network, since the simplified representation can adequately represent the overall dynamics. In cases where major dynamics are not captured, the system response will still degrade gracefully instead of failing completely.

Figure 15 shows results from bi-clustering of LCA data on hepatic inflammation (see Section 4.3 for a discussion of inflammatory response modeling). The LCA data were collected from experiments where soluble stimuli were applied to capture major cellular response profiles relevant to inflammation (Thompson *et al*., 2004). The RED approach was able to adequately capture the original responses using a simplified network, as shown in Figure 16.

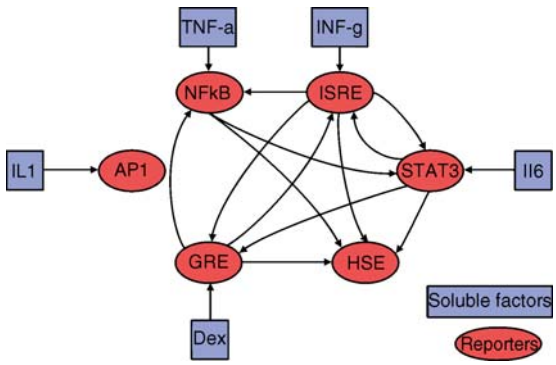

Figure 15. Directed Graph associated with the bi-clustering analysis of Living Cell Array (LCA) data for identifying key processes in hepatic inflammation. In the simplified model, AP-1 is unconnected because it was not found to be part of any bi-cluster. (Figure source: Yang, Yarmush and Androulakis, 2009; reproduced with permission from Elsevier).

4.2.2 Example: Analysis of Xenobiotic Metabolism and Interactions - Construction of Optimized Metabolic Networks via Metabolic Flux Analysis for APAP and Ethanol

This example focuses on the analysis of the metabolic networks of two widely encountered xenobiotics, acetaminophen and ethanol. Given the complexity and interconnectedness of pathways relevant to hepatocyte metabolism, various approaches integrating experimental data with mechanistic mathematical modeling have been applied to understand the dynamics of the various metabolic networks. A commonly used approach is metabolic flux analysis (MFA). MFA can infer the rate of intracellular fluxes from the flux of consumed or secreted metabolites. This approach has been used to quantify the fluxes of cultured rat hepatocytes exposed to different media conditions, to predict what media modifications may optimize hepatic functions, and to identify the metabolic changes associated with toxicity induced due to free fatty acid exposure (Chan *et al*., 2003;Sharma, Ierapetritou and Yarmush, 2005; Srivastava and Chan, 2007). Energy balance analysis (EBA) considers energy balance constraints along with the stoichiometric and mass balances used in MFA and has been used to investigate various liver-specific objectives in gluconeogenic and glycolytic hepatocytes (Nagrath *et al*., 2007). For systems that lack network-wide experimental measurements, Thermodynamic Based Profiling (TBP), an approach that applies thermodynamic constraints for each biochemical reaction, can be used. Yang and Beard (2006) used this method to predict pathways important for drug-drug interactions in hepatocytes.

Figure 17 presents a biochemical network that was reconstructed so as to reveal interactions of acetaminophen and ethanol with hepatic metabolic processes. This network considers the main pathways of para-acetylaminophenol (APAP) metabolism as well as the formation of conjugative species from amino acids, salts, and carbohydrates, ethanol metabolism, and central hepatic metabolism. The xenobiotic metabolism reactions

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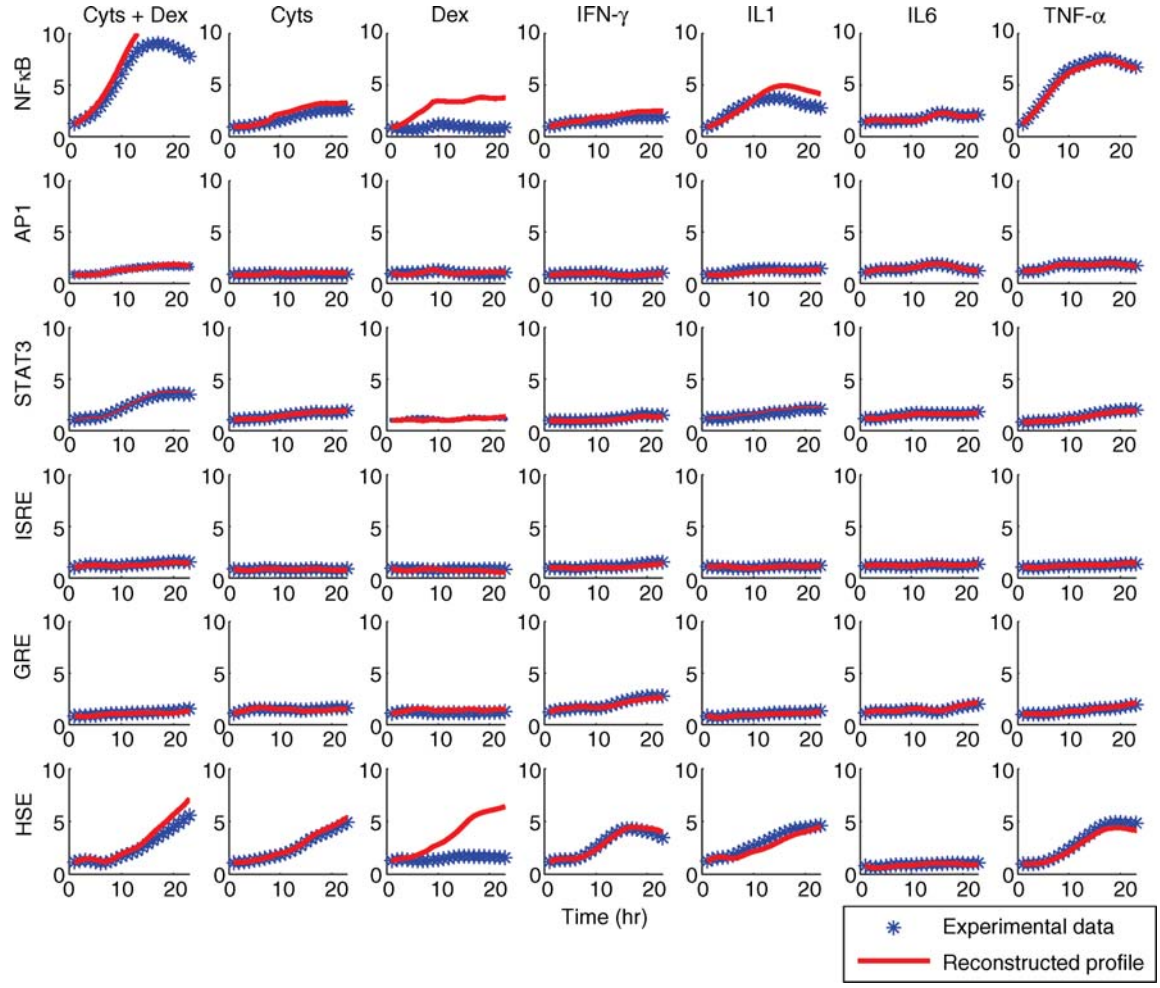

Figure 16. Reverse Euler Decomposition (RED)-based reconstruction of the system response profiles obtained from the Living Cell Array (LCA) (Figure source: Yang, Yarmush and Androulakis, 2009; reproduced with permission from Elsevier).

that are considered are specific to APAP, representing three parallel pathways: glucuronidation, sulfation, and CYP oxidation; however, this network may be expanded in a straightforward manner to include other Phase II pathways, if desired.

Based on the assembled metabolic network of Figure 17 and on values of *in vitro* kinetic parameters and flux bounds taken from the literature, the feasible metabolic fingerprint was calculated for the hepatic metabolism of APAP under different physiological conditions in the rat. Optimization methods were used to determine the flux distribution for maximal xenobiotic detoxification, to understand the pathways that participate in the competition between ethanol and APAP metabolism that contributes to increased drug-induced liver damage, and to evaluate the robustness of the complex network. Treated cultured cells were used to verify those pathways that are most important in APAP detoxification, metabolism of ethanol, and coupling with hepatic functions, and to assess the extended xenobiotic -metabolizing capabilities of the cell.

A kinetic model of the major pathways involved in APAP metabolism was constructed by assembling data from a number of different sources. In this example, concentrations are expressed in terms of nmol per 10^6 cells. The relative importance of the individual APAP metabolism pathways was investigated by simulating a dose-response experiment (Figure 18). When APAP is administered at a low

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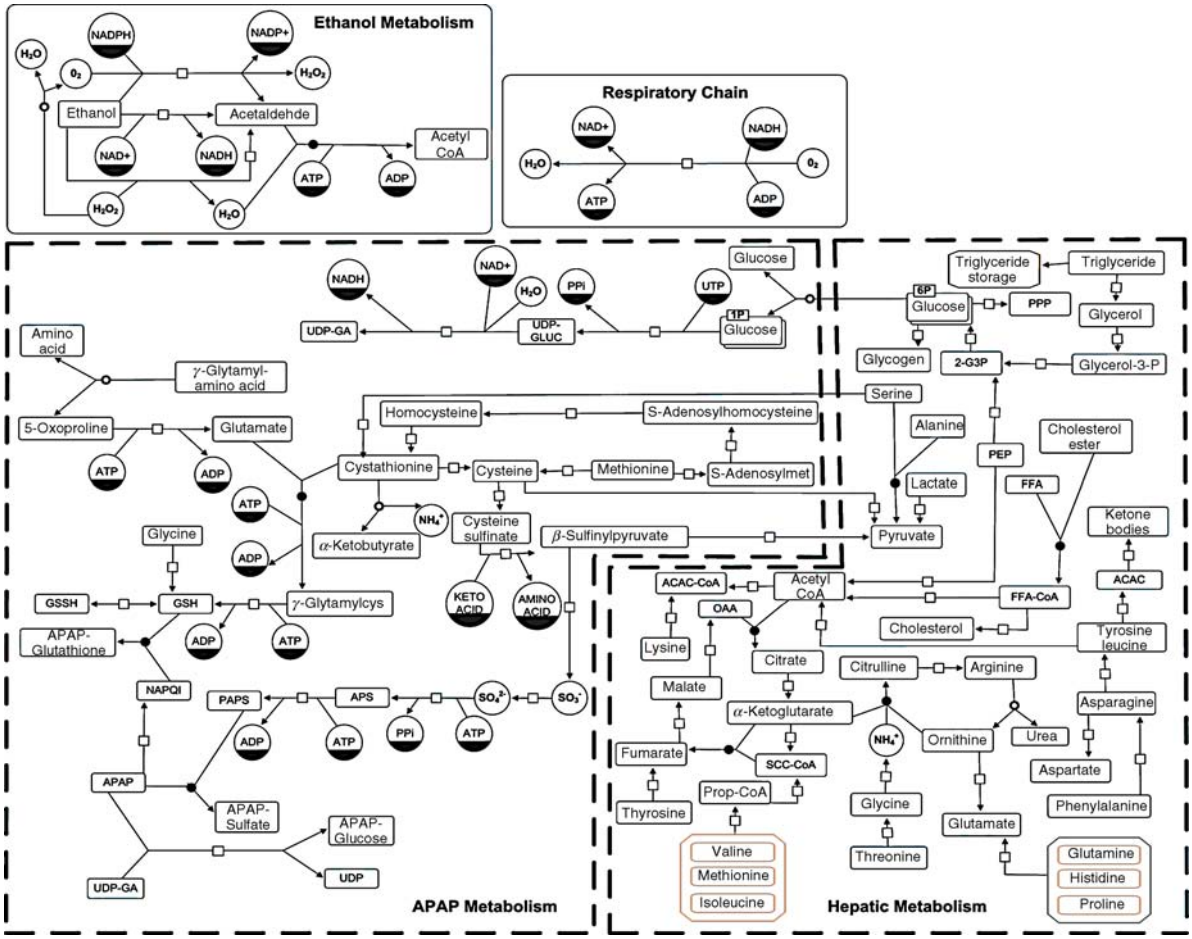

Figure 17. Pathways for central hepatic, APAP, and ethanol metabolism: the network that is schematically summarized in this figure in SBGN format includes 112 reactions and 73 internal metabolites.

dose, the majority is conjugated with the activated sulfate and to a lesser extent with UDP-glucuronidic acid. Oxidation of the xenobiotic leads to the formation of a minor amount of NAPQI that is quickly detoxified through glutathione conjugation (Figure 18a). As the dose increases, the rate of sulfation is limited by the availability and formation of PAPS; above a dose of 1 mmol kg^{-1} of rat body weight glucuronidation becomes the predominate pathway (Figure 18b) (Klaassen and Boles, 1997).

To simulate the effect of fasting on the formation of the oxidative product, kinetic information from a study comparing the potentiation of APAP hepatotoxicity for fasted and fed rats was incorporated into the model (Price, Miller and Jollow, 1987). This study reveals that at high doses the apparent rate constants of the glucuronidation and sulfation reactions were decreased by 40% and 30%, respectively, while the formation rate of the glutathione conjugation remained unchanged. Decreased glucuronidation and sulfation, attributed to the depletion of conjugative species, leads to longer elimination of the parent compound. Indeed, altering these two rate constants in the model leads to a prediction of an approximately 50% increase in the production of the toxic metabolite. As toxic doses quickly deplete stores of glutathione, PAPS, and UDPGA, their re-synthesis becomes the rate-limiting step in subsequent conjugation reactions (Figure 19a).

Simulations on the effect of ethanol pretreatment were executed by substituting the model's CYP450

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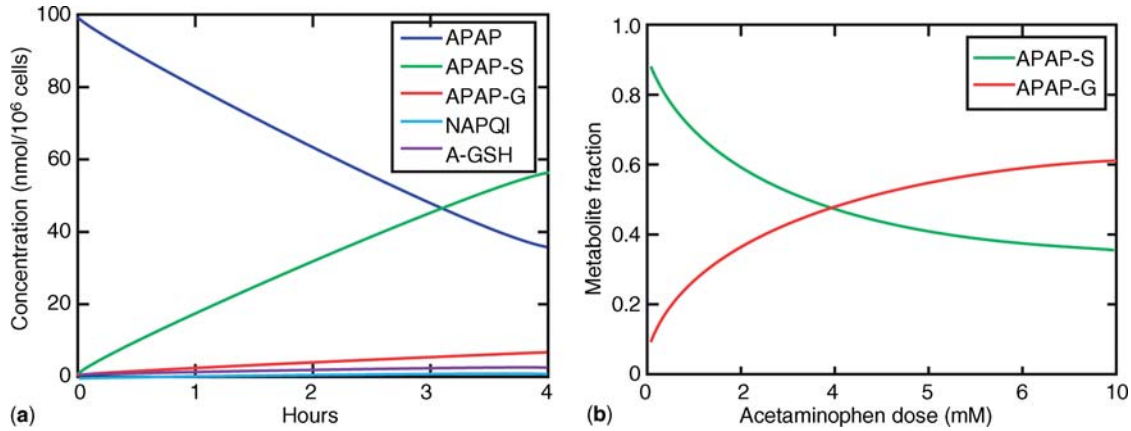

Figure 18. Simulations using the combined metabolic network for acetaminophen (APAP) and ethanol. (a) Time course profiles of APAP metabolites at low APAP doses (40 nmol/10^6 cells); (b) impact of increasing doses of APAP on the metabolite fractions.

kinetic parameters of the model. The values used to describe the system in the absence of ethanol are the mean values taken from rat liver microsomes studies (Patten *et al*., 1993; Thummel *et al*., 1993) without ethanol pretreatment. To investigate the effect of ethanol pretreatment, these values were substituted with the ethanol condition values obtained from Patten *et al*. 1993, who administered ethanol to male rats as a 15% solution (v/v) in drinking water for three days prior to microsome preparation followed by *in vitro* APAP dosing. The results of the simulation for APAP-GSH concentrations in response to increased APAP levels for control and ethanol pretreatment conditions are shown in (Figure 19b).

4.3 Multiscale Modeling of Systemic Endotoxin-Induced Inflammation in Humans Employing Network and Agent Methods

The examples in this subsection demonstrate applications of network- and agent-based approaches in the study of a "prototype" stressor/response problem: endotoxic-induced inflammation.

The acute inflammatory response (AIR) is the initial response of the host to a diverse array of biological stressors including infection, burns, trauma, and invasive surgery. The regulation of human inflammatory response involves not only the local release of anti-inflammatory cytokines

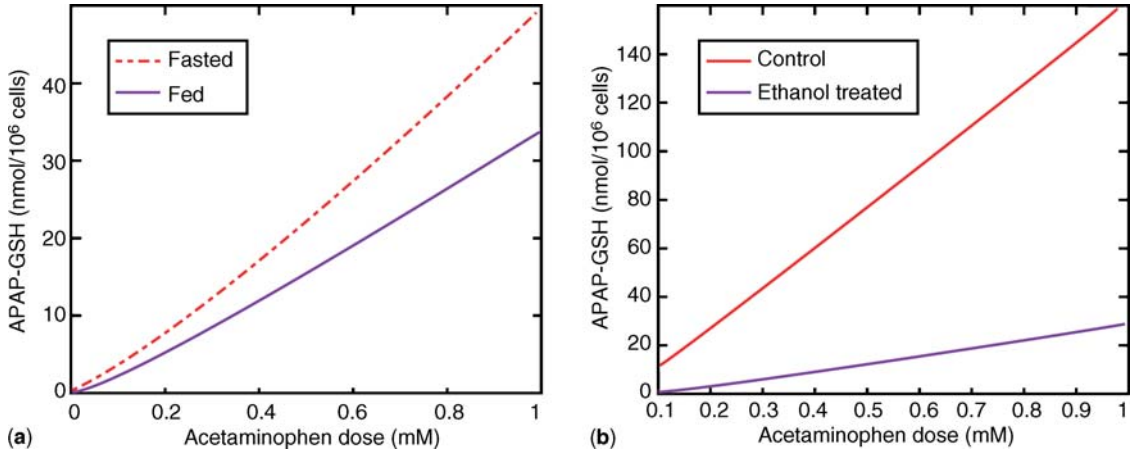

Figure 19. (a) Effect of fasting on APAP metabolism, calculated by changing the values of enzyme parameters in the combined metabolic network for APAP and ethanol; (b) impact of ethanol pretreatment prior to APAP treatment.

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but also other hormonal influences (Lowry, 2009). Under normal circumstances the dynamics of acute inflammation are tightly regulated and self-limited (Hotchkiss and Karl, 2003). However, when anti-inflammatory processes fail, an amplified inflammatory state is reached that is characterized by severe, uncontrolled systemic inflammation leading to multiple organ dysfunction (Bone *et al*., 1992). Mechanistic simulations of inflammation (Vodovotz *et al*., 2009), especially multiscale modeling approaches have the potential to generate alternative testable hypotheses for assessing how cellular events and inflammatory processes mediate the links between patterns of autonomic control (HRV) and clinical outcomes.

Various studies have indicated that the central nervous system (CNS) is a pivotal regulator of the immune response and controls inflammation at various levels (Blalock, 2002; Elenkov, 2008; Elenkov *et al*., 2005; Jara *et al*., 2006; Taub, 2008). As discussed in the previous sections, research at ebCTC pursues the development of a systems-based approach that integrates processes across multiple scales and subsequently models the emerging host response as the outcome of orchestrated interactions of critical modules. The new models of human inflammation detailed here utilize indirect response and agent-based modeling (ABM) for bridging the initiating signal (LPS) and phenotypic expressions (HRV) through semi-mechanistic host response models that include transcriptional (cellular) dynamics, signaling cascades and physiological (hormonal) components.

Human endotoxin challenge is a well-accepted surrogate model for studying the acute inflammatory response, as it captures many of the clinically observed features of the systemic inflammatory phenotype (Calvano *et al*., 2005; Fannin *et al*., 2005; Lowry, 2005; Talwar *et al*., 2006; Wittebole *et al*., 2007). Endotoxin is a major component of the outer membrane of gram-negative bacteria, and the inflammation caused by the activation of the innate immune system by this moiety can be a complicating factor in a variety of situations, including trauma, invasive surgery, and organ-specific illnesses. The prototypical examples of endotoxin are lipopolysaccharides (LPS). The response following endotoxin administration in human subjects includes core temperature, cardiac, vasomotor, hematologic, metabolic, hormonal, acute phase reactant, and cytokine components that have been well described (Copeland *et al*., 2005; Lowry, 2005; van Deventer *et al*., 1990; Van Zee *et al*., 1995). In particular, innate immune cell activation leads to the production and release of pro-inflammatory cytokines, which are proximal mediators of the systemic inflammatory response. Although the bulk of this pro-inflammatory mediator release likely originates in cells of the reticuloendothelial system (Fong *et al*., 1990), the leukocytes present in peripheral blood are also activated and, importantly, are available for sampling with minimal invasiveness.

4.3.1 Example: Network-Based Simulation of Systemic Response to Human Endotoxin Challenge

This example focuses on direct communication links from the cellular to the systemic host response level, integrating bi-directional influences between systems (Foteinou *et al*., 2010), via the following steps: *First*, analysis of leukocyte gene expression data allowed the identification of essential responses characterizing the cellular (transcriptional) dynamics. *Second*, physicochemical modeling (Aldridge *et al*., 2006) was employed to express the relations and dynamics that connect extracellular signals and intracellular signaling cascades, eventually leading to the emergent transcriptional dynamics (identified in the previous step). *Finally*, an indirect response (IDR) model (Jusko and Ko, 1994) was used to describe the toxicodynamics by establishing implicit interactions among signaling molecules and emerging transcriptional responses. A schematic illustration of the network architecture that constitutes the multi-level host response model is presented in Figure 20. At the cellular level, interacting components involve the propagation of LPS signaling on the transcriptional response level (P, A, E) through the activation of endotoxin signaling receptor (R) and elementary signaling pathways (NFκB signaling module). At the level of circulating hormones, essential modules are associated with the release of endocrine stress hormones from neuroendocrine axis (HPA, SNS) coupled with their anti-inflammatory influence on the host. The dynamics of cortisol and epinephrine signaling involve components interacting at the cellular level. At the systemic level, physiologic deterioration of the host is quantified by HRV.

In the injury model, the inflammatory response is activated when endotoxin is recognized by pathogen

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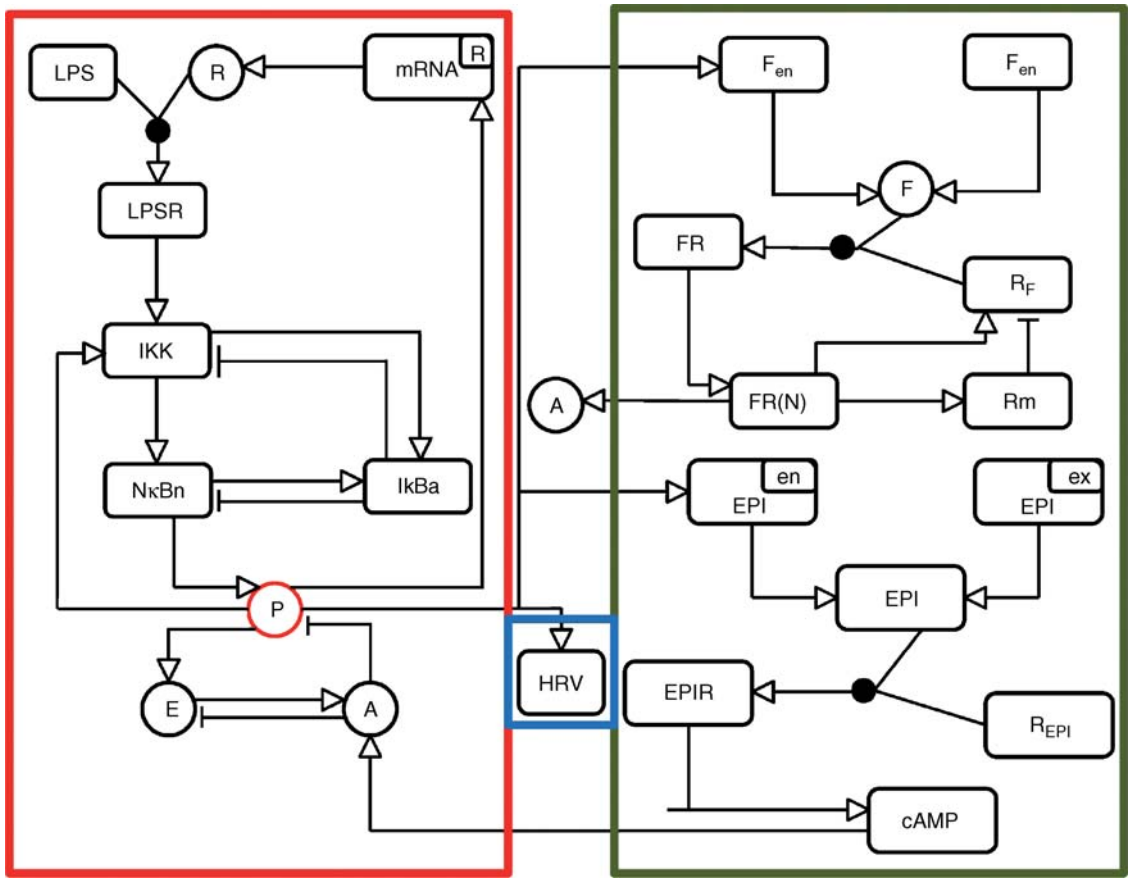

Figure 20. Topological interactions composing the multiscale model of inflammation. At the cellular level, interacting components involve the propagation of LPS signaling on the transcriptional response level (P, A, E) through the activation of endotoxin signaling receptor (R) and elementary signaling pathways (NFκB signaling module). At the level of circulating hormones, essential modules are associated with the release of endocrine stress hormones from neuroendocrine axis (HPA, SNS) coupled with their anti-inflammatory influence on the host. The dynamics of cortisol and epinephrine signaling involve components interacting at the cellular level. At the systemic level, physiologic deterioration of the host is quantified by HRV.

recognition receptors (Wells, Ravasi and Hume, 2005). The LPS-induced stimulation initiates a complex signaling cascade that ultimately targets the transcription initiation of pro-inflammatory cytokines (Kishore *et al*., 2004). During the recognition process LPS binds to the LPS-binding protein in plasma and is delivered to the surface receptor CD14. Subsequently, LPS is transferred to the signaling toll-like receptor 4 (TLR4) with the recruitment of its essential accessory protein MD2 (Du *et al*., 1999; Guha and Mackman, 2001;Van Amersfoort, Van Berkel and Kuiper, 2003), eventually leading to the translocation (activation) of pro-inflammatory transcription factors (e.g., NF-κB) (Calvano *et al*., 2005).

In this example, it was hypothesized that the pro-inflammatory response acts as the stimulatory factor for the energetic response while a dysregulation in the cellular bio-energetics can serve as a positive feedback danger signal to the pro-inflammatory response. The anti-inflammatory response serves as the essential immunoregulatory signal that aims to restore homeostasis in the host defense system. Therefore, a single compartment toxicodynamic model was employed for LPS assuming a homogeneous circulating blood compartment; this model contains pro-inflammation components and energetic responses that inhibit the pro-inflammation response. In human subjects the endotoxin is cleared within the first 1–2 h after LPS

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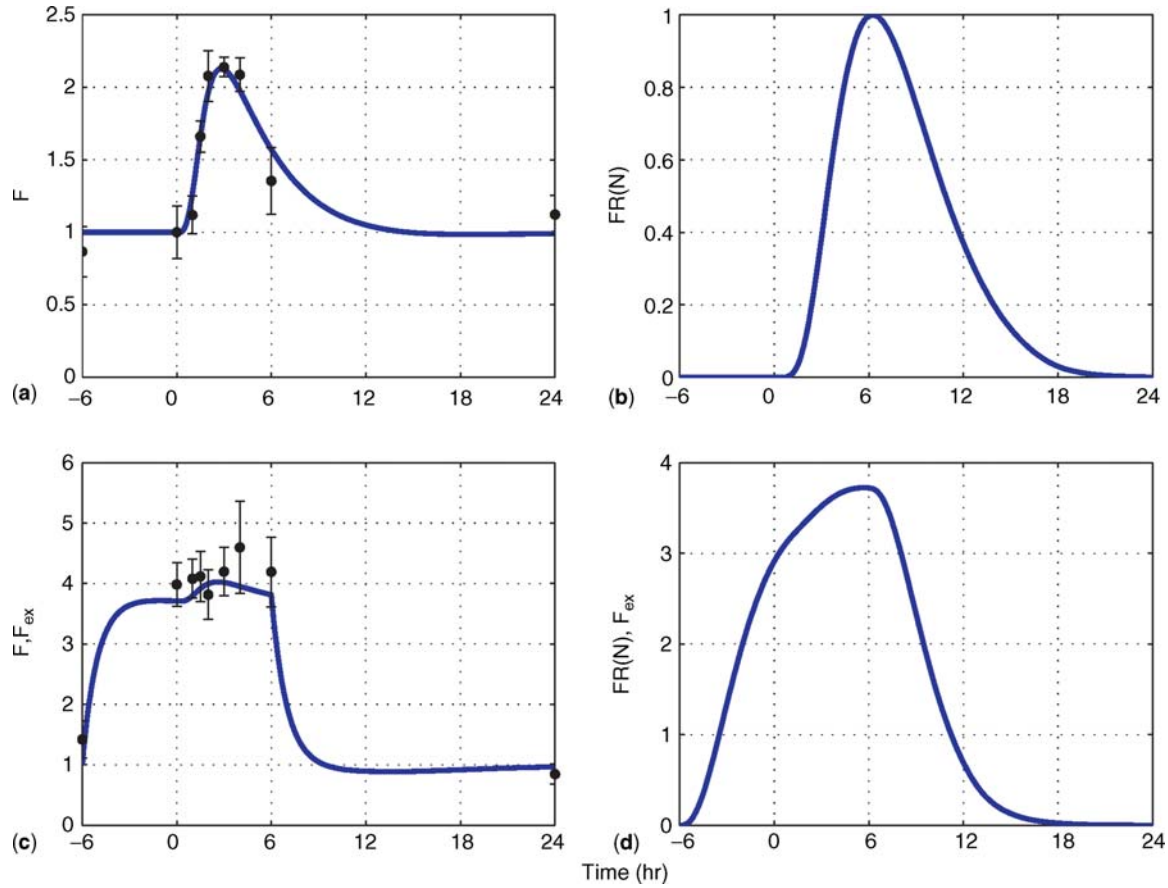

Figure 21. (a) Plasma cortisol levels (F) and (b) steroid active signal (FR(N)) under conditions of acute endotoxin injury (LPS(t = 0 h) = 1); (c) Simulated plasma cortisol levels (FFex) and (d) steroid active signals FR(N)Fex under conditions of low-dose steroid infusion (wFex = 1) which is initiated at t = −6 h before LPS and continued for 6 h after LPS. Solid lines correspond to model predictions while solid markers represent experimental data expressed as mean ± SEM. (Figure source: Foteinou *et al*., 2010.)

administration with an approximate average half time of 8–15 min (Greisman *et al*., 1969). The model parameters have been independently estimated so that the LPS profile decays within two hours in the absence of any complications (Greisman *et al*., 1969).

Figure 21 shows the persistent inflammatory response, which corresponds to an increased exposure of the host response to the inflammatory stimulus (LPS). Since increased catecholamine secretion accompanies modest infection and the effect of EPI in inhibiting LPS-induced pro-inflammatory response has been documented, exploratory simulations were conducted to study whether antecedent EPI infusion would alter the cytokine responses to endotoxin as shown in Figure 22, in order to identify potential protective measures against inflammation.

4.3.2 *Example: Agent-Based Modeling of Endotoxin Induced Acute Inflammatory Response in Macrophages*

In this example, an agent-based modeling (ABM) approach was used to study the dynamics of acute human inflammation. Interacting agents are either inflammation specific molecules or cells essential for the propagation of the inflammatory reaction across the system (Dong *et al*., 2010). Spatial orientation of molecule interactions involved in signaling cascades, coupled with the cellular heterogeneity is further taken into account.

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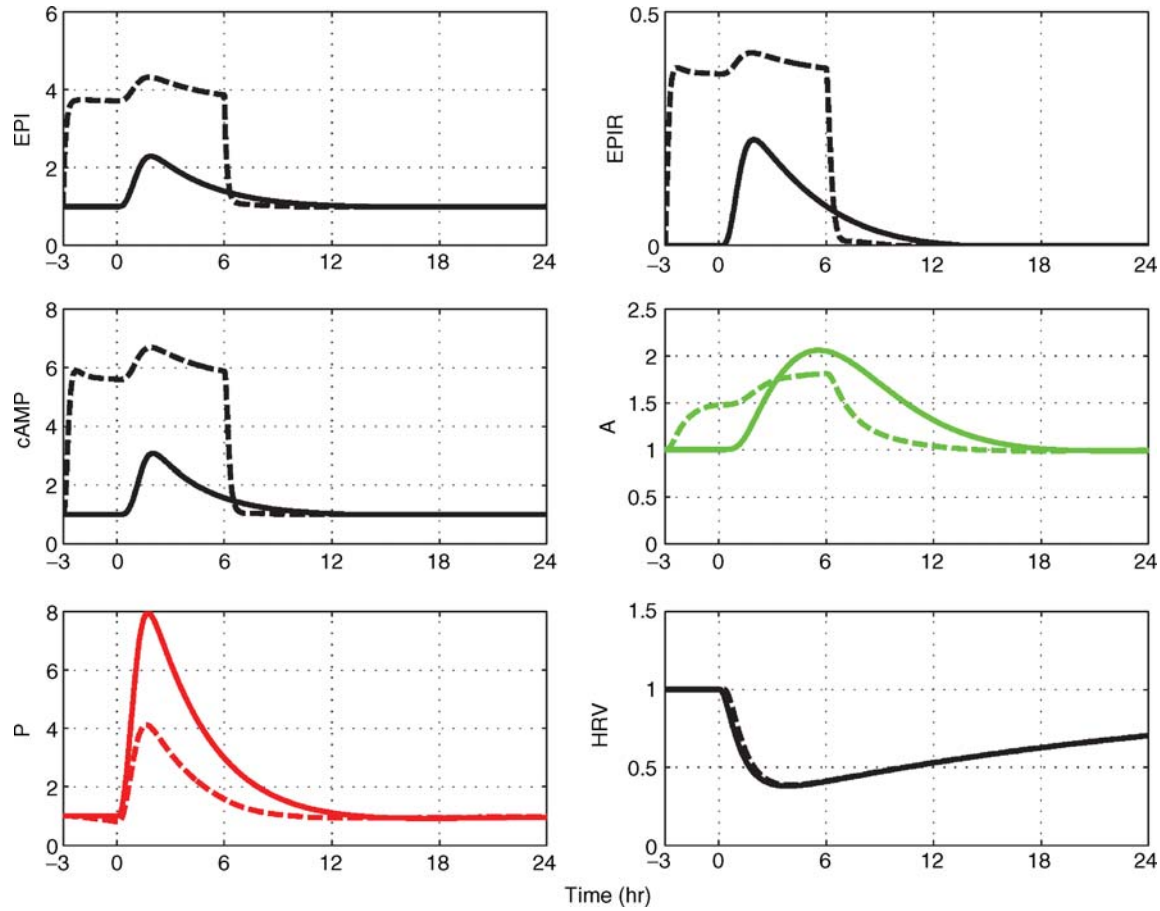

Figure 22. Modulation in the progression of the inflammatory reaction due to short-term epinephrine infusion (initiated 3 h before lipopolysaccharides (LPS) exposure and continued for 6 h after LPS exposure). Such intervention potentiates the secretion of epinephrine from SNS that through cAMP anti-inflammatory signaling can protect in part the host response attenuating the pro-inflammatory response (P). Solid lines simulate a self-limited endotoxin-induced inflammatory reaction while dashed lines reflect the scenario of prior epinephrine infusion ($w_{EPIex} = 1$, $R_{in,EPI} = 20$). (Figure source: Foteinou *et al.*, 2010.)

The agents in this model represent different aspects of the system under study, including families of cells and/or molecules that are able to adapt and interact with the environment and with each other based on a specific set of rules (Vodovotz *et al.*, 2008). While agents within a class will have the same rules for behavior, the behavior of individual agents varies because of differences in local conditions and because of probabilistic application of rules to simulate agent dynamics. The individual interactions then aggregate to engender the overall behavior observed in an experimental setting. The advantage of the ABM approach lies in the fact that the rules governing interactions of agents can be developed in an intuitive manner based on fundamental occurrences in biological processes (e.g., binding of molecules).

This example is based on the premise that peripheral blood leukocytes (PBLs) are major effectors in response to endotoxin and that PBLs represent a composite mixture of several cellular subpopulations. During the onset of the inflammatory response, the secretion of pro-inflammatory cytokines from macrophages stimulates the activation of precursor T helper cells (Th0) and induce them to exhibit the type 1 T helper cell (Th1)

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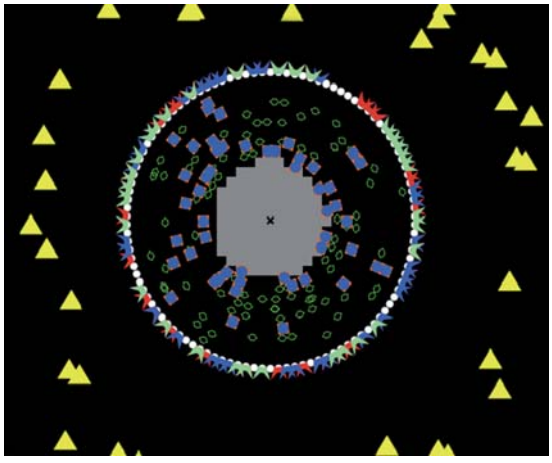

Figure 23. Interacting components/agents involved in the propagation of lipopolysaccharides (LPS) signaling on macrophages. Yellow triangles reflect the extracellular signal (LPS) and white circles represent the plasma membrane. Red polygons refer to the endotoxin (LPS) receptor and blue polygons refer to the TNF-α receptor. Light green polygons correspond to IL-4 receptors and dark green polygons reflect the presence of kinase (IKK). Blue + orange squares represent the inactive (bound) NF-κB with its inhibitor, IκBα while the grey area refers to the nucleus. (Source: Dong *et al*., 2010.)

phenotype, which in turn facilitates the secretion of various pro-inflammatory cytokines (McKnight *et al*., 1994). Additionally, Th0 can also transform to type 2 T helper cells (Th2) and produce anti-inflammatory cytokines that are essential for restoring homeostasis (Kidd, 2003).

The overall host response to endotoxin is simulated via interacting molecules and cells based on the ABM model shown in Figure 23. Each macrophage possesses a cell membrane comprised of agents arranged in a circle around the center of the cell, which constitutes the nucleus. Receptors for LPS, IL-4, and TNF-α are embedded in the membrane while the inhibitor protein IκBα, IKK, and NF-κB are located in the cytosol. This model considers the pro-inflammatory response (P) consisting of the early increased expression of cytokines and chemokines; the anti-inflammatory response (A) that serves as the immunoregulatory arm of the host defense system; and the energetic response (E) that involves the expression of genes that participate in cellular bioenergetic processes. It also considers an NF-κB dependent physicochemical host response model (Foteinou *et al*., 2009). The model is implemented using NetLogo software (Wilensky, 2010) and is evaluated through its ability to successfully reproduce a self-limited inflammatory response as well as a series of scenarios involving either a persistent (non)infectious response or innate immune tolerance and potentiation effects followed by perturbations in intracellular signaling molecules and cascades.

Prior to any external perturbation, NF-κB is inactive in the cytoplasm forming a complex with its primary inhibitor, IκBα. Upon stimulation, NF-κB translocates to the nucleus activating the transcriptional machinery for the up-regulation of the critical transcriptional events (Folcik, An and Orosz, 2007; Kidd, 2003; McKnight *et al*., 1994). During the recognition process of LPS from its signaling receptor (R), a signal transduction cascade is triggered that up-regulates the transcription of TNF-α. The presence of pro-inflammatory mediators (P) promotes the migration of mature T helper cells (Mackay, 2000) where Th0 cells become Th1, while the production of anti-inflammatory mediators (A) incites formation of Th2 cells which further potentiate the anti-inflammatory response (A) (Kidd, 2003). Since the anti-inflammatory arm of the host defense system restores homeostasis, the anti-inflammatory component of the model, including anti-inflammatory mediator agents (A), is assumed to exert its counter-regulatory properties by stimulating the degradation rate of the early potent pro-inflammatory mediator TNF-α, coupled with the active populations of T helper cells. In particular, the Th2 agents continuously produce anti-inflammatory mediators to ensure that the population of (A) agents is sufficient to attenuate TNF-α production in macrophages. Since circulating pro-inflammatory (P) agents have the ability to turn Th0 into Th1, instead of Th2, the population of Th2 cells is primarily affected by the concentration of (P) agents. All the interacting components constituting the agent-based model of inflammation are shown in Figure 24.

The agent-based model predicted the situation where the initial levels of LPS are increased in Figure 25. It was observed that when the concentration of the inflammatory stimulus exceeded a critical threshold, the inflammatory response did not abate. Such a response is characterized by overwhelming production of the pro-inflammatory instigator, TNF-α, which amplifies the activity of NF-κB. In

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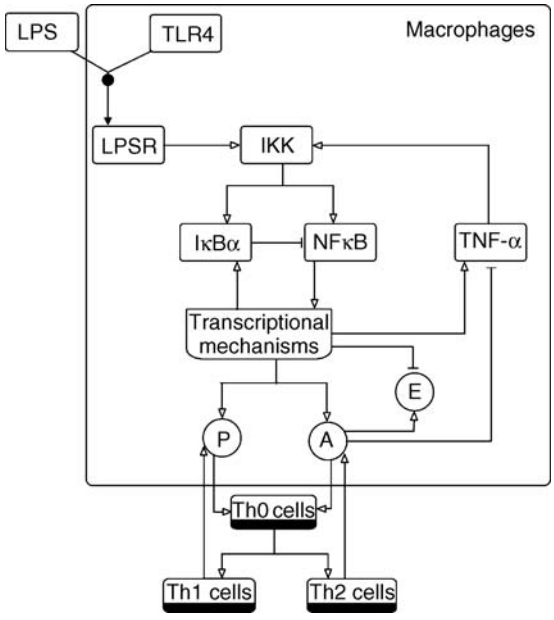

Figure 24. Schematic illustration (in SBGN format) of elements and interactions involved in the Agent Based Model of endotoxin-induced inflammation.

particular, high LPS concentration potentiates the secretion of pro-inflammatory mediators (P), which in turn may increase the probability of Th0 cells to differentiate into Th1 cells rather than into Th2 cells. Additionally Th1 cells further increase (P) population; thus disturbing the balance between Th1/Th2, accounting for the progression of an unconstrained inflammatory response.

While comparing the output of the agent-based model with the output of the deterministic models, it should be noted that both modeling approaches may not be characterized by the same network topology. However, the two modeling approaches predicted responses (e.g., P, A, E) with reasonable qualitative agreement.

4.4 Cellular Level Modeling of Toxicokinetics and Toxicodynamics

4.4.1 Example: Arsenic Toxicokinetics and Toxicodynamics in Mammalian Hepatocytes

This example presents a semi-mechanistic model of combined cellular arsenic toxicokinetics (TK) and toxicodynamics (TD). The model describes the uptake, biotransformation and clearance (TK) of multiple arsenical species in mammalian hepatocytes, along with subsequent cellular adaptive response (TD). Arsenic is an environmental pollutant, potent human toxicant, and oxidative stress agent with a multiplicity of health effects associated with both acute and chronic exposures; effects include cancers of the liver, bladder, skin, and lung (ATSDR, 2007).

The cellular level TK model describes arsenic transport across the cellular membrane and subsequent multistep metabolism in hepatocytes according to the reaction cascade proposed by Hayakawa *et al.* (2005). This model predicts the concentrations of trivalent and pentavalent arsenicals in hepatocytes by simulating uptake of arsenite (iAs^{III}) via aquaporin isozymes 9 (AQP9s), glutathione (GSH) conjugation, methylation by arsenic methyltransferase (AS3MT), efflux through multidrug resistant proteins (MRPs) and the induced antioxidant response via thioredoxin reductase (TR) activity. Methylation reactions are described through a hybrid approach of Hill and Michaelis-Menten kinetics, while the GSH-bound hydrolysis and clearance of methylated arsenicals was described by a threshold-dependent formulation using a sigmoidal function.

Figure 26 illustrates a simplified overview of cellular toxicokinetics and toxicodynamics of arsenic (for the mammalian hepatocyte). The toxicokinetics of arsenic include both "traditional" metabolism (methylation) via AS3MT (methyltransferase) as well as a new metabolic pathway of inorganic arsenic biotransformation via arsenic-GSH complex formation. This pathway includes two separate branches of arsenic biotransformation: $MADG \rightarrow MMA^{III} \rightarrow MMA^{V}$ and $MADG \rightarrow DMAG \rightarrow DMA^{III} \rightarrow DMAV$ (based on Hayakawa *et al.*, 2005). The toxicokinetic pathway carries reactive oxygen species (ROS) formation and corresponding DNA damage, as well as DNA repair.

The model parameters were estimated using data on low doses from Styblo *et al.* (1999) and dose-response data from Drobna *et al.* (2004) (data at 24 h post exposure for doses ranging from low to high: 0.1–10 μM). As shown in Figure 27, the model was able to capture the three distinct modes of the time course patterns corresponding to experimental data. Also, as shown in the figure, the TK model,

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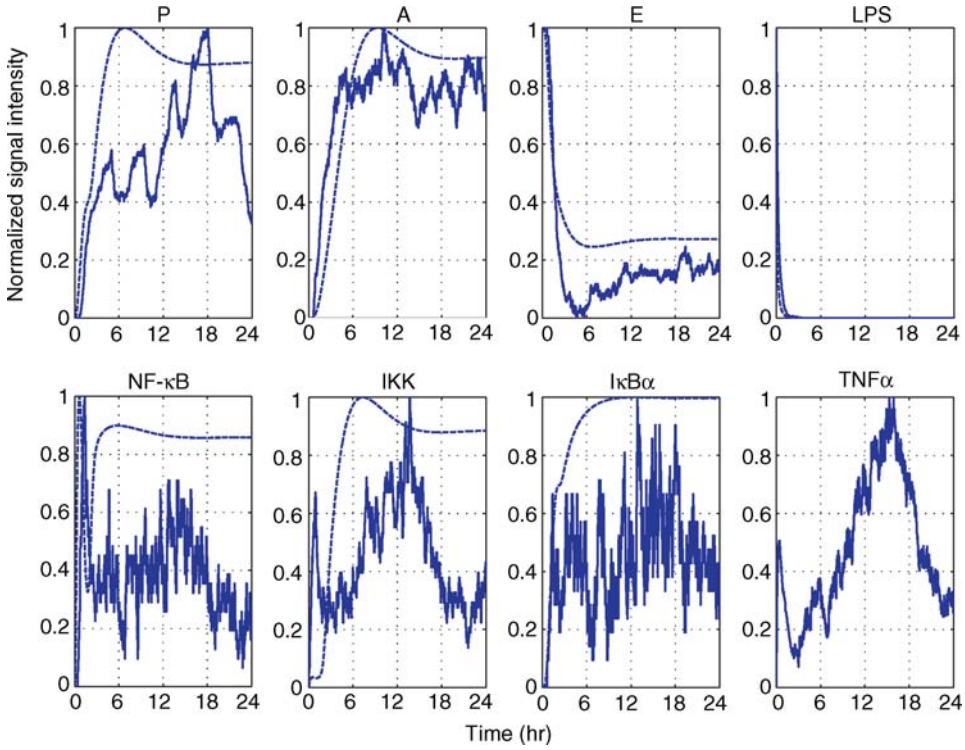

Figure 25. Temporal responses of an unresolved inflammatory response due to high LPS concentration. A high concentration of LPS (LPS(0) = 750) can cause a malfunction in the dynamics of the host leading to an exacerbated inflammatory response (solid lines). Dashed lines refer to the implications of high concentration of LPS as simulated by a deterministic (ODE) approach. For the purpose of comparing the simulated output between the ABM and the ODE model, all responses are normalized so that numerically they are in the range (0,1). (Figure source: Dong *et al*., 2010.)

parameterized using data from Drobna *et al.* (2004), was also able to capture the significant decrease in DMA amounts at higher arsenite doses. This TK model in a stand-alone manner can be incorporated into a larger-scale physiologically based toxicokinetic (PBTK) model of arsenic for improving the estimates of PBTK model parameters, as well as in characterizing inter-individual variability in arsenic metabolism.

The cellular level TK model was linked to a toxicodynamic (TD) model that describes the cellular adaptive response following the metabolism of arsenic and the corresponding increase in the levels of ROS. This adaptive response is governed by a core feedback loop mechanism (Krishna, Jensen and Sneppen, 2006) involving the transcription factor Nrf2 via higher anti-oxidant response in the form of increased GSH biosynthesis and MRP expression. The core elements considered in the TD model are ROS, nuclear Nrf2, mRNA, in addition to MRPs and protein levels of Glutamate cysteine ligase catalytic subunit (GCLC), which catalyzes the rate-limiting step for GSH biosynthesis. This model describes DNA lesions caused by arsenic-induced ROS increase as a first-order process, and also includes the BER pathway for predicting the extent of DNA repair (Sokhansanj and Wilson, 2004).

The TD model was parameterized using experimental data on the expression of GCLC following exposure to 10 μM iAs^{III} (Thompson *et al*., 2009b). The integrated TK/TD model was then independently evaluated with experimental data on DNA repair following exposure to 5–100 μM iAs^{III} (Dopp *et al*., 2008). As shown in Figure 28, the integrated TK/TD model was able to adequately explain the temporal dynamics of GCLC expression and the dynamics of arsenic-induced DNA damage and repair.

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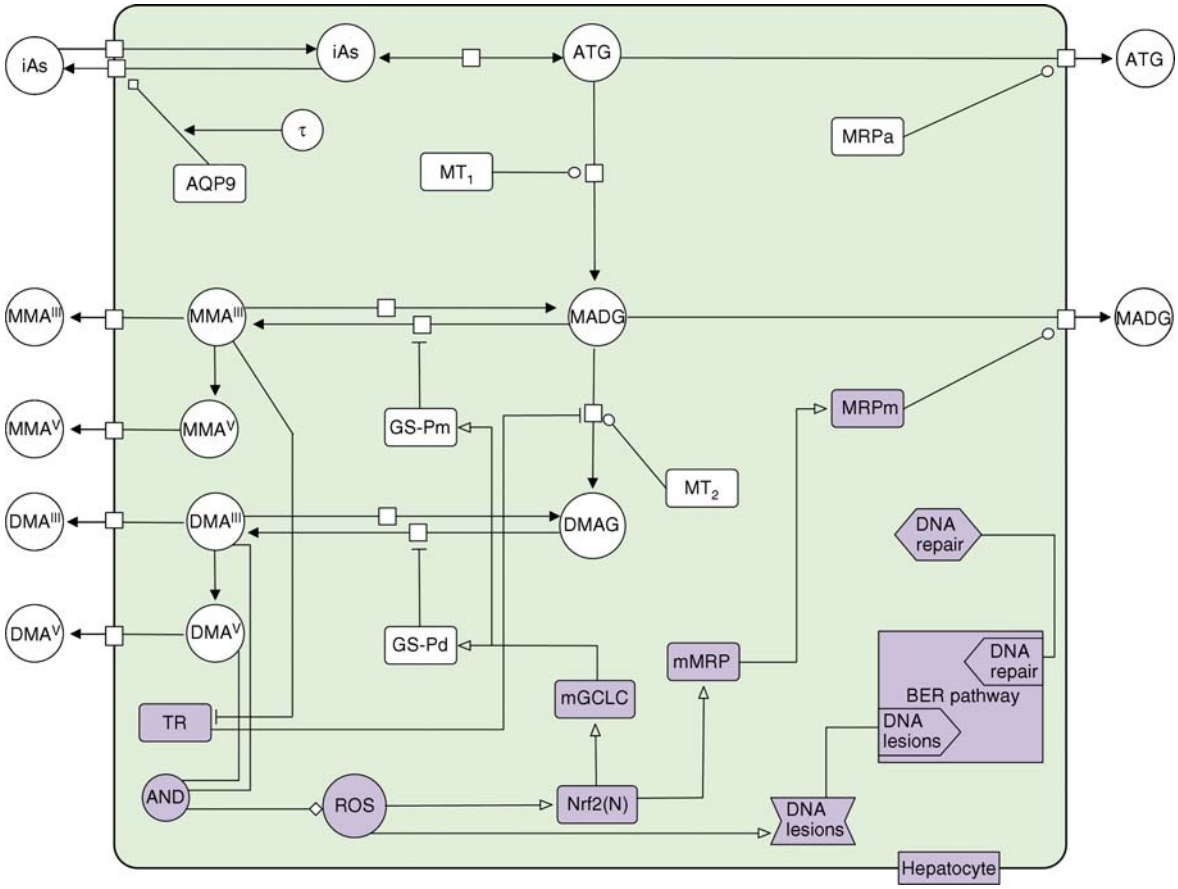

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4.5 Modeling of Biochemical Heterogeneity within Tissues and Organs

4.5.1 Example: Distribution-Based Characterization of P450 Induction by TCDD in the Human Liver

This example demonstrates the process of "model reduction" via representing spatial heterogeneity in terms of frequency distributions focusing on the challenges posed by the fact that the liver shows heterogeneous hepatic cytochrome P450 (CYP) induction following exposures to some toxicants. Specifically, the hepatic metabolism of TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) is addressed here, because TCDD is an ubiquitous contaminant (ATSDR, 1999; Son and Rozman, 2002) that has also been shown to result in significantly higher CYP induction in the centrilobular region compared to the periportal region of the liver (Andersen *et al*., 1995; Santostefano *et al*., 1999).

Existing zonal (i.e., multicompartmental) models for the liver represent the entire organ in terms of discrete zones by making assumptions regarding volumes and toxicodynamic properties of different liver regions (Andersen *et al*., 1997). An alternative statistical distribution-based modeling approach has been developed and applied to the study of this heterogeneity. This approach maps multiple parameters from the zonal model into a single distribution.

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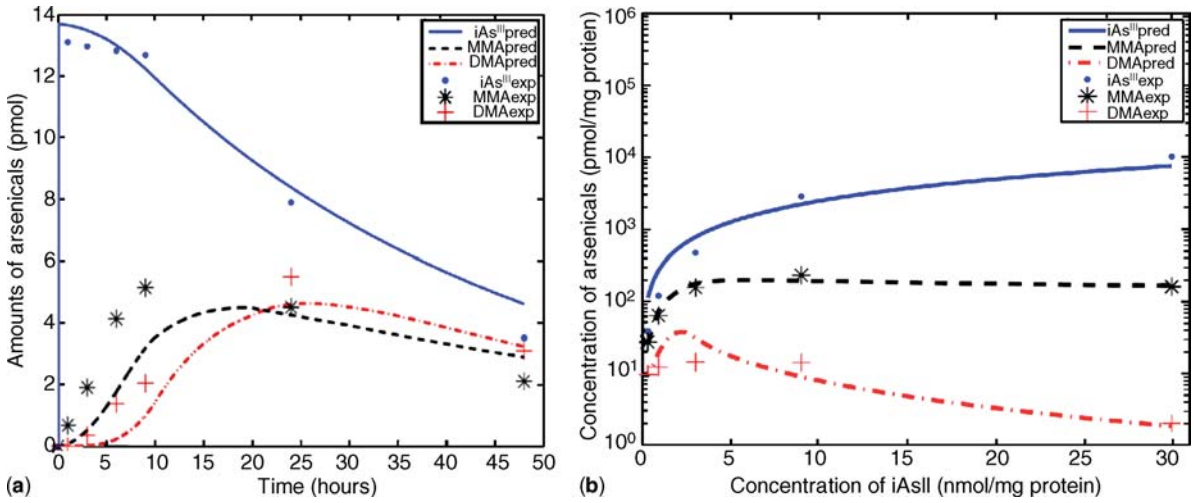

Figure 27. Time course profiles of arsenicals in human hepatocytes predicted using the cellular level TK model. (a) Levels of intracellular arsenicals following exposures to 0.1 μM arsenic for 2 days compared to experimental data from Styblo *et al*. (1999); (b) total amounts (in hepatocytes and the medium) of iAs^{III}, MMA and DMA compared to experimental data from Drobna *et al*. (2004) (hepatocytes from a 63 year old white female).

For scenarios reported in the literature, estimates from the distribution-based model predicted realistic distributions of enzyme induction (as shown in Figure 29a), while the zonal model can provide unrealistic estimates, especially at low and high doses.

Subsequently, key model parameters in the zonal and distribution-based models were estimated using available experimental data. Experimental measurements of CYP 1A1/2 mRNA by Santostefano *et al*. (1997) were converted to total protein concentrations by relating CYP levels to mRNA expression and using data from Santostefano *et al*. (1997). Based on the experimental setup, the data for periportal and centrilobular zones were assumed to represent two liver regions that have volume fractions of 50% each.

Figure 29b presents a comparison of estimates of CYP induction in centrilobular and periportal regions from a single-compartment, a zonal,

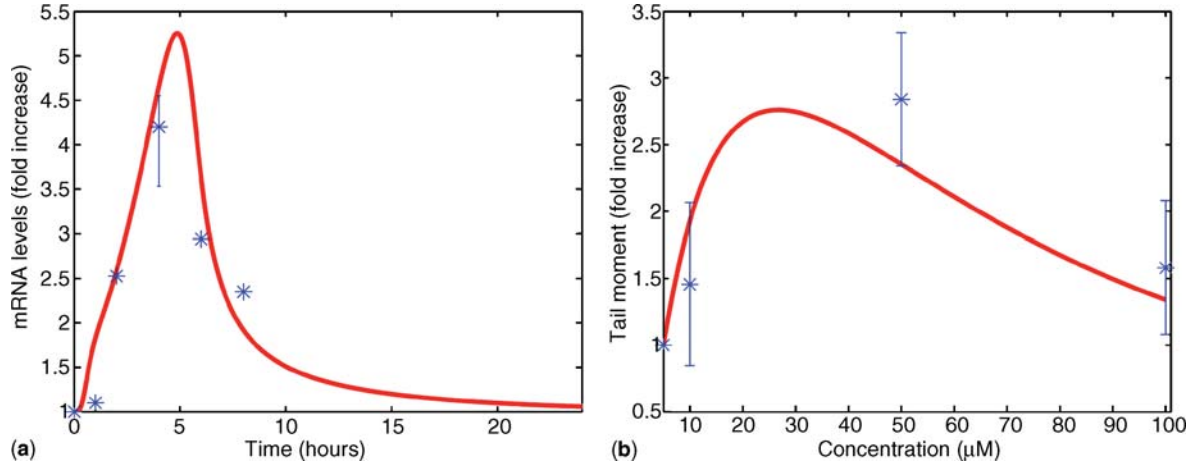

Figure 28. (a) predicted dose-response profiles of mRNA levels following exposure of hepatocytes to 10 μM arsenic, calculated with a new cellular level toxicodynamic model (experimental data from Thompson *et al*., 2009b); (b) estimated levels of DNA damage and repair (fold change) following exposure to 5–100 μM iAs^{III} (experimental data from Dopp *et al*., 2008).

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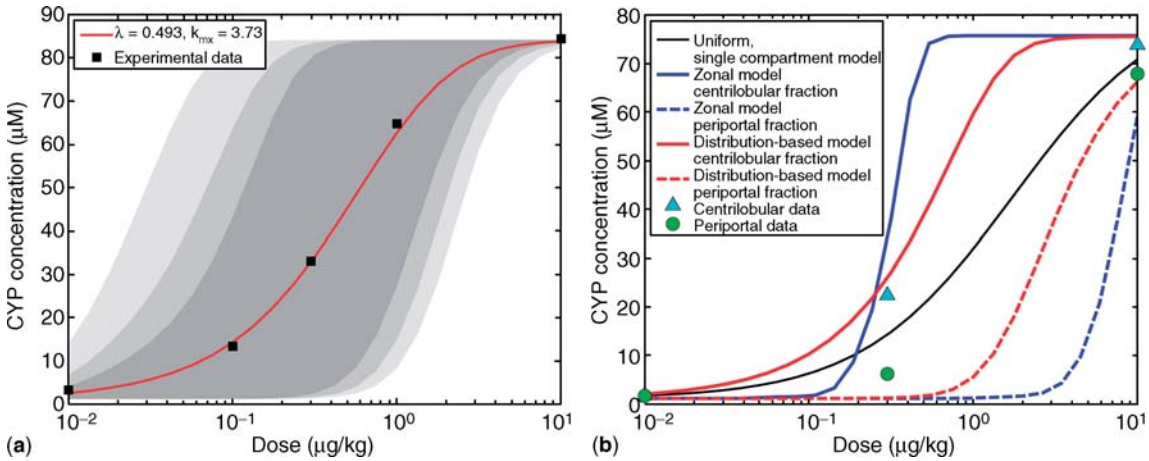

Figure 29. (a) predictions of CYP induction from TCDD compared to measurements of Santosefano *et al*. (1998). Shaded areas represent the 5/95, 13/87, and 25/75th percentiles. (The centrilobular and periportal zones are assumed to be 27 and 39% of the liver, as per Andersen *et al*., 1997.); (b) comparison of the zonal and distribution-based models with experimental measurements of CYP induction from TCDD in fractionated livers as measured by Santostefano *et al*. (1999). The parameter values for each model formulation are optimized based on the experimental data. The distribution-based model more closely represents the variation in liver zones than the zonal model.

and a distribution-based model, with experimental data from fractionated livers, showing that the distribution-based model explains the data better than the zonal model.

In the context of risk assessment, this approach provides a quantitative estimate of the fraction of the organ that experiences high levels of toxicity, providing an overall "spectrum" view of dose-response, instead of the spatial patterns. An advantage of this approach is that distribution-based models can be evaluated by using data from homogenized organs analyzed in conjunction with flow cytometry experiments (Broccardo *et al*., 2004, 2005) that measure the distribution of enzyme induction resulting from exposing a population of hepatocytes. In contrast, the corresponding evaluation of traditional zonal models requires studying an intact organ.

4.6 Whole-Body Combined Toxicokinetic and Toxicodynamic Modeling

4.6.1 Example: Predicting Oxidative Stress in Rats Due to Trichloroethylene Exposures

This example presents a combined physiologically-based toxicokinetic and toxicodynamic model for trichloroethylene (TCE) that predicts the amount of oxidative stress due to exposures to TCE and its metabolite trichloroacetate (TCA) in rats. TCE is a known carcinogen in rodents and suspected carcinogen in humans, while human exposures to TCE are common because it was a widely used industrial solvent. TCE is metabolized in liver tissue where TCE and its metabolites cause oxidative stress. Oxidative stress is a well-known mechanism of cellular injury and plays a major role in the development of chronic diseases including carcinogenesis (Klaunig and Kamendulis, 2004). It results from an imbalance favoring oxidants rather than antioxidants, resulting in an overall increase in cellular levels of ROS. The most common metric of oxidative stress is provided by the thiobaric acid reactive substances (TBARS) assay, which measures lipid peroxidation, and by assays for 8-hydroxydeoxyguanosine (8OHdG), which measure the amount of oxidized DNA that has been repaired.

Many of the toxic effects of TCE are hypothesized to be due to the oxidative metabolism of TCE to TCA (Bull, 2000). Specifically in mice and mouse cells TCE and TCA are both known to cause increases in free radical production (Steel-Goodwin, Pravecek and Carmichael, 1996), lipid peroxidation (Austin *et al*., 1995; Byczkowski, Channel and Miller, 1999; Larson and Bull, 1992), and DNA damage (Austin *et al*., 1996). A weight of

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evidence analysis has determined that activation of the nuclear receptor peroxisome proliferator-activated receptor α (PPARα) has a dominant role in TCE and TCA induced hepatocarcinogenesis in mice (Corton, 2008).

The PBTK model predicts the distribution of TCE and its metabolites in tissue compartments and the TD model uses those concentrations in the liver as inputs to calculations of oxidative stress metrics. The PBTK model is based on an adaptation of the Hack *et al*. 2006 model, to predict concentrations of TCE and its metabolites in various tissues including blood and liver, following exposures to TCE and/or TCA. The TD model uses Michaelis-Menten type kinetics formulations to describe the production of TBARS, as well as the feedback mechanism reflecting biological response to oxidative stress. Model parameters have been estimated using literature data on mice and mouse liver slices exposed to TCE and its metabolites. These data include *in vitro* mouse liver slice measurement of free radicals and lipid peroxidation and *in vivo* measurements of lipid peroxidation and DNA damage. As shown in Figure 30 (right panel), the new model predicts oxidative stress levels as well as replicates estimates of the Byczkowski, Channel and Miller 1999 model for short times, while it also produces reasonable predictions of oxidative stress at long times (that cannot be calculated by the Byczkowski, Channel and Miller 1999 model).

The new toxicokinetic/toxicodynamic model for TCE presents a substantial improvement over existing models since it can (i) successfully predict oxidative stress produced in the liver over time periods ranging from hours to weeks, (ii) consistently describe effects of exposures to TCE and direct exposures to its metabolites, and (iii) combine events from exposure to toxicodynamic responses. Ongoing work considering the applicability of this model to humans focuses on scaling issues and relevance of additional toxic endpoints of TCE.

4.7 An Integrative Approach to Multiple System Interactions and Effects

4.7.1 Example: Exposures to Fine Particles and Nanoparticles

A prototype generalized risk analysis framework for fine particles and nanoparticles concludes this series of demonstrative examples for toxicokinetic-toxicodynamic process integration. This example, which summarizes various aspects of ongoing collaborative work at ebCTC, aims to emphasize the need for a "systems" approach to characterize the complex nature of interactions of fine and ultrafine particulate matter with biological organisms; these interactions include a wide range of toxicokinetic and toxicodynamic processes, schematically

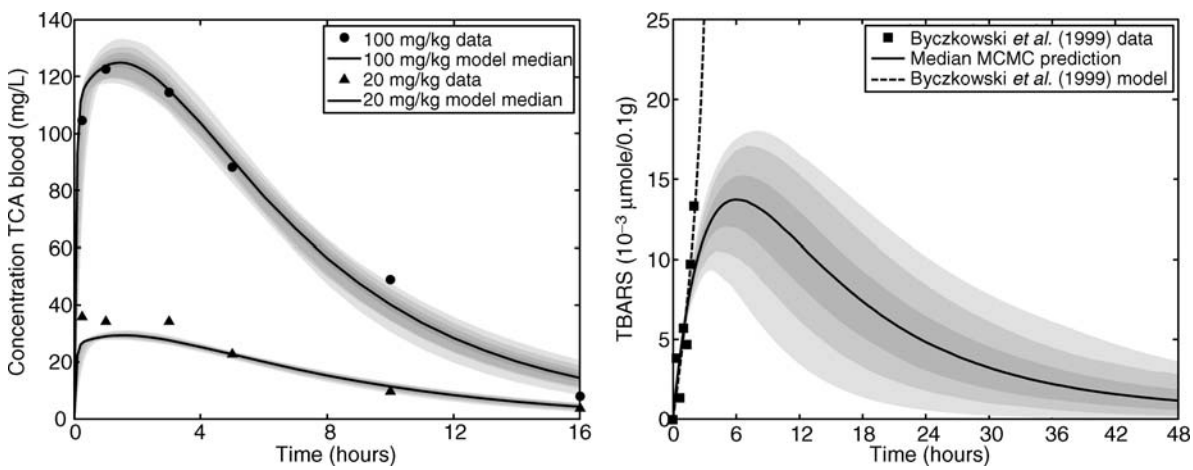

Figure 30. Evaluation of the new PBTK model using data from Larson and Bull (1992) and the TD model using data from Byczkowski, Channel and Miller (1999). Parameters for the PBTK and TD models were estimated through optimization of the model using the Markov Chain Monte Carlo technique (shaded regions represent 1, 2 and 3 standard deviations from the median value). The new TD model predicts realistic levels of TBARS in liver slices from *in vivo* experiments for both short- and long-time periods.

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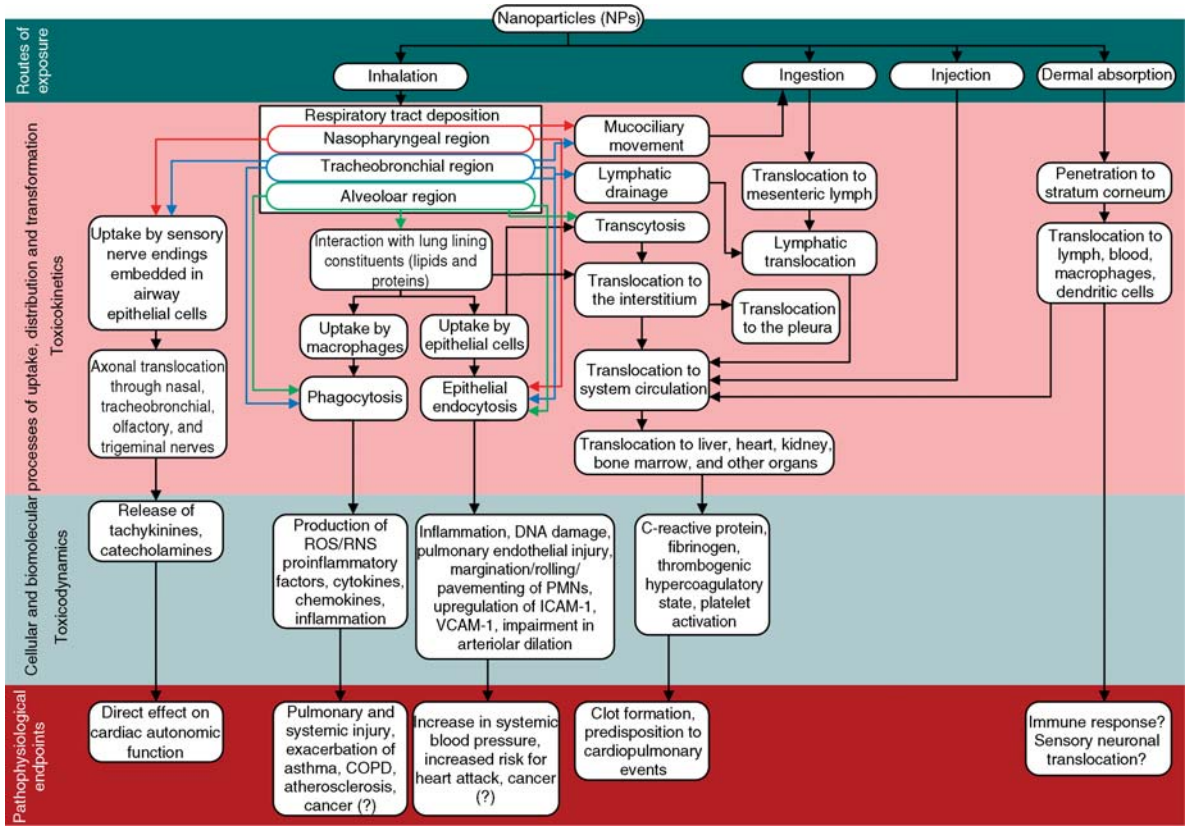

Figure 31. The ebCTC systems framework to support integrative toxicity assessments of nanoparticles through combined whole-body toxicokinetic and toxicodynamic models: the chart provides a simplified partial listing of relevant toxicokinetic and toxicodynamic processes considered in this framework.

depicted in Figure 31, involving multiple mechanisms and physiological systems.

Known adverse health effects of air pollution are numerous and typically involve multiple organs and systems. For example, when airborne particulate matter (PM) enters the lungs it interacts with the respiratory epithelium to mediate respiratory as well as systemic and cardiovascular effects. Furthermore, PM, or its reaction products, stimulate airway sensory nerves, leading to changes in lung function and in autonomic tone, which influence cardiac function. Ultrafine PM, due to their very small size, can enter pulmonary capillary blood and be rapidly transported to extrapulmonary tissues such as liver, bone marrow, and heart, with either direct or indirect effects on organ function (see Figure 31). The DORIAN effort pursues the development of interlinked "virtual organs" within a modular physiologically based toxicokinetic/toxicodynamic "whole body" framework for the systematic study of such complex effects.

The enhanced toxicity of fine particles and nanoparticles (compared to bulk material or coarser particles of the same chemical composition) is typically attributed to the small size that allows deposition in the alveolar region as well as facilitates the penetration of various biological barriers/membranes. However, it is important to note that even when nanoparticles are present in the form of larger aggregates and/or agglomerates, they may still retain the toxicity of individual nanoparticles they consist of, due to the large surface area they maintain (Borm *et al*., 2006; Schleh and Hohlfeld, 2009). For example, the enhanced toxicity of carbon nanotubes (CNTs) (compared to graphite, etc.) is in fact attributed to their enhanced surface area even when they are not present as isolated nanoparticles. Due to the rapidly expanding use of carbon

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nanoparticles (and in particular CNTs) and the anticipated widespread exposure of large segments of the population, a systematic attempt for an integrative model of their toxicokinetics and toxicodynamics has been initiated at the ebCTC.

There is a growing number of *in vitro* and *in vivo* studies of carbon nanoparticles, and especially CNT toxicity. Lam and co-workers (Lam *et al*., 2006) provide an overview of the field, comparing the outcomes of a number of toxicological studies performed prior to 2006. These, as well as various more recent investigations (e.g., Pacurari *et al*., 2008; Ryman-Rasmussen *et al*., 2009) discuss a variety of local (pulmonary) and systemic adverse effects. Many of these studies and various other reviews (Gwinn and Vallyathan, 2006; Oberdorster, Oberdorster and Oberdorster, 2005) point to the fact that CNTs and other nanoparticles may evade phagocytosis and translocate to other organs, with systemic toxic effects. Pacurari *et al*. (2008) state that "toxicity studies of nanoparticles should not be limited to a single lung cell or only to the lung, but should involve other systemic targets." CNTs are neither soluble nor biodegradable, so, even when they do not evade phagocytosis, their biopersistence potentially raises the risk associated with exposures to them. Indeed, when the lung is not overloaded, macrophages containing nanoparticles will migrate upward on the alveolar surface and will be carried by the mucociliary escalator system up the trachea, thus cleared into the esophagus (McClellan, 1997). CNT-laden macrophages, however, have been found to move to centrilobular locations, where they enter alveolar septa and cluster to form epithelioid granulomas. When particles enter the interstitial or subepithelial space, clearing them from the lung is very difficult, resulting, in the case of irritating or toxic particles, to more severe histological effects (Lam *et al*., 2006). In addition to inducing histopathological changes, including granuloma formation and fibrosis, both single wall CNTs and multiple wall CNTs have been found to release toxic cytokines and biomarkers of inflammation, oxidative stress, and cytotoxicity (Muller *et al*., 2005; Shvedova *et al*., 2005).

Various modules specific to exposures, dosimetry, toxicokinetics, and toxicodynamics of nanoparticles have been incorporated in the MENTOR and DORIAN systems. These modules in fact provide improved estimates compared to corresponding available agency models. The respiratory dosimetry modules of MENTOR have been formulated incorporating available information relevant to nanoparticles in relation to realistic exposure situations. Figure 32 compares predictions from two different "standard models" and from an inhalation dosimetry module of MENTOR, with the available experimental data on fine and ultrafine

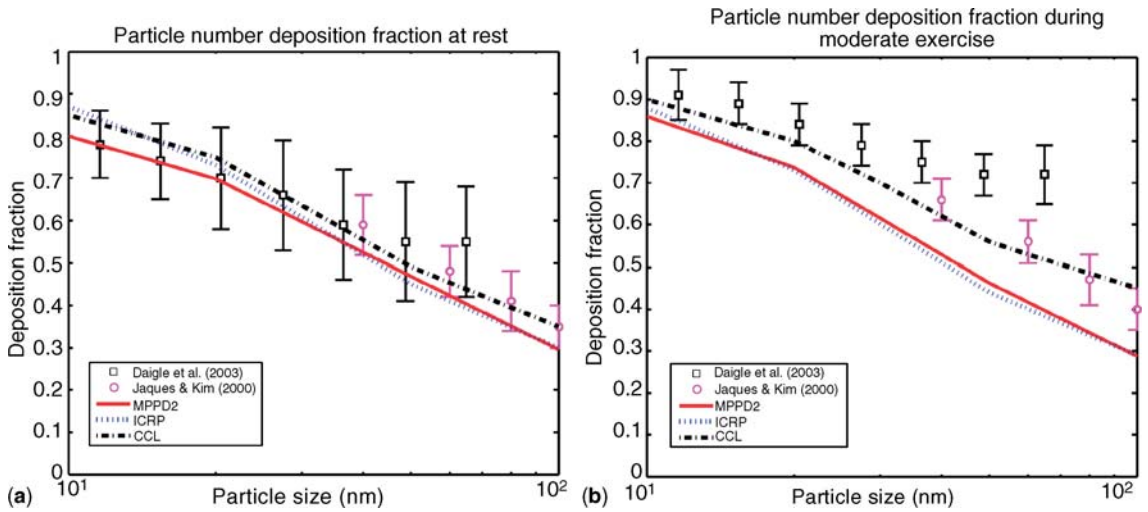

Figure 32. Experimental ultrafine and nanoparticle dosimetry data under: (a) resting conditions and (b) moderate exercise conditions (Daigle *et al*., 2003; Jaques and Kim, 2000) compared to predictions of the Multiple Path Particle Dosimetry (MPPD2 – CIIT, 2006), the International Commission of Radiological Protection (ICRP – Jarvis *et al*., 1996) models, and the inhalation dosimetry module of MENTOR/DORIAN (Source: Georgopoulos, 2008; reproduced with permission from Springer).

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(nanoparticle) lung deposition from the studies of Jaques and Kim (2000) and Daigle *et al*. (2003). The two standard models, that is, the Multiple Path Particle Dosimetry model (MPPD - CIIT, 2006) and the International Commission of Radiological Protection (ICRP - Jarvis *et al*., 1996) model, perform comparably with each other, and their predictions, as well as the predictions of the MENTOR module, demonstrate acceptable agreement with the measured values in the case of deposition measured under resting conditions. However, the MPPD and the IRCP models substantially underpredict the deposited fraction of nanoparticles under conditions of moderate exercise for the subject considered, whereas, the MENTOR inhalation module achieves much closer agreement with the experimental data.

In current and ongoing work, a generalized, integrative, physiologically based toxicokinetic/toxicodynamic model (PBTK/TD), that incorporates modules from both the MENTOR and DORIAN systems, has been adapted to study the biokinetics and various subsequent effects, from the subcellular to the multi-organ level, of nanoparticles following various types of exposures (inhalation, ingestion, dermal absorption, and injection). The structure of the whole-body PBTK/TD model for fine and ultrafine (nanoparticle) exposures is shown in Figure 10. This generalized structure incorporates multiple routes of exposure, as well as organs/tissues and translocation/distribution pathways relevant to reported fine and ultrafine particle properties. So, for example, in addition to respiratory tract deposition of inhaled particles in the nasopharyngeal (N), tracheobronchial (T), and alveolar (A) regions, this model structure is also designed to account for processes such as the uptake of nanoparticles by sensory nerve endings embedded in airway epithelia (in the N,T areas) with subsequent axonal translocation to the central nervous system (CNS), through the nasal, tracheobronchial, olfactory, and trigeminal nerves. Processes leading to the translocation of nanoparticles to systemic circulation and subsequent distribution to different organs/tissues are incorporated explicitly (e.g., translocation from the lung epithelium, lymphatic drainage, etc.)

As a specific example, the MENTOR/DORIAN whole-body PBTK/TD model was adapted to simulate the absorption, distribution, and elimination of fullerenes (carbon nanoparticles) labeled with 99 m-technetium. The assumptions and parameter values used in the PBTK module were based on the study by Pery *et al*. (2009). The simulation results presented here, as shown in Figure 33, consider an inhaled gas mixture of "small" particles, "large" particles, and free unbound 99 m-technetium. The small particles represent fullerenes that can translocate from the lung to the systemic circulations,

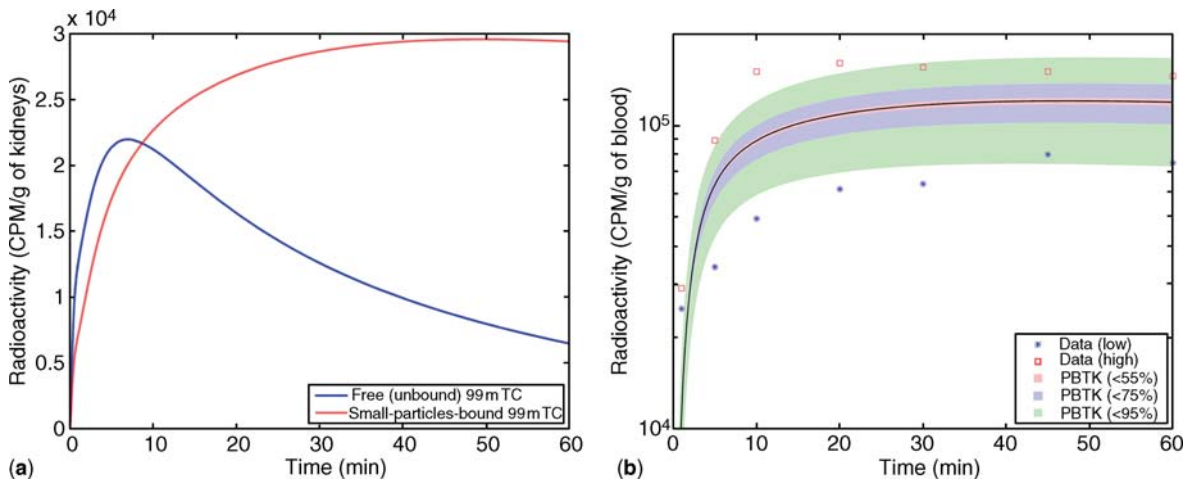

Figure 33. Time-course profiles of radioactivity levels in humans following exposures to carbon nanoparticles labeled with 99 m-technetium, as estimated by the generalized PBTK module of MENTOR/DORIAN. The assumptions and parameter values used in the PBTK module were based on the study by Pery *et al*. (2009). The data used for evaluating the modeling predictions are from Nemmar *et al*. (2002). (a) The calculated time course profile of radioactivity in the kidney; (b) model estimates and confidence intervals for total radioactivity measured in blood, along with the corresponding experimental data.

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while the large particles are deposited into and rapidly cleared from the lung. Large particles do not enter systemic circulation, whereas small particles and free 99 m-technetium are capable of absorption and organism-wide distribution, with different toxicokinetic properties. In the case study that was simulated, the composition of the mixtures the subjects were exposed to was unknown. A Monte Carlo analysis (1000 model runs) was performed using the MENTOR/DORIAN PBTK module to evaluate the sensitivity of the model predictions to assumptions regarding the relative magnitude of the large and small particle fractions.

Inhaled particle dynamics such as coagulation and agglomeration have been modeled in the past (Broday and Georgopoulos, 2001; Lazaridis *et al*., 2001) for airborne particulate matter of variable chemical composition, employing the aerosol General Dynamic Equation (GDE) for respiratory tract processes, but have not been implemented in the context of nanoparticle dosimetry. However, in the case of nanoparticles, dynamics of agglomeration and aggregation are relevant not only for the inhaled air flow, but also for the physicochemical dynamics of the particles within extracellular and intracellular biological fluids. The ongoing incorporation of the GDE description in the various biokinetic steps of nanoparticle distribution in mammalian organisms is currently being pursued within ebCTC.

5 CONCLUSION

This chapter presented a summary overview of the general approach, and of various components and demonstration applications, resulting from efforts that are being pursued at the *environmental bioinformatics and Computational Toxicology Center* (ebCTC) towards developing a comprehensive DOse Response Information ANalysis system (DORIAN). DORIAN has been designed to complement the Modeling ENvironment for TOtal Risk studies (MENTOR), which provides an extensively tested library of software components, for "source-to-dose" analysis of environmental stressors (chemical, biological, radiological), by adding tools for novel "dose-to-biological outcome" analysis. DORIAN employs a bi-directional approach to provide the framework for linking phenomena occurring across multiple scales of time/space and biological organization: this is achieved by implementing a set of alternative "bottom-up" approaches that model the dynamics of "microscopic entities" such as biomacromolecules and cells to derive macroscopic properties of the coarser/higher scales; however, these dynamics are constrained by the requirements of a whole-body "top-down" framework. The procedure of developing application-specific models involves reducing or decomposing the biological system into suitable functional subsystems (biomodules) according to criteria that take into account the biophysics/biochemistry of the specific problem at hand and the objectives of the analysis that is pursued. The process of *encapsulation*, that is, of dividing a complex biological system into different scales or functional subsystems with limited exchange of information between the "internal" components of separate scales or subsystems, is often the most challenging step in model development. Once the scales and the boundaries of the biomodules/subsystems for a specific application have been defined, along with the selection of "bottom-up" and "top-down" algorithms for model linking, a systematic approach has been established to translate the conceptual/qualitative description of the system into a quantitative formulation: within this framework, computational chemistry methods are being applied and/or developed to quantitatively characterize biomodule components and interactions at the molecular (e.g., ligand-receptor) scale, while deterministic and stochastic system process analysis and optimization techniques are applied to determine "larger" biomodule structures (ranging from interlinked signaling, regulatory and metabolic pathways to networks of multicellular functional subsystems, to tissues, and to multi-organ systems). This process relies on interpretation of data (e.g., transcriptomic, proteomic, metabolomic, cytomic, physiomic, etc. data) from "biomodule perturbation" experiments, that may include (i) genetic perturbations (polymorphisms, gene knockouts, gene silencing, etc.), (ii) environmental perturbations (exposures to environmental toxicants, nutrient availability, etc.) and (iii) disease state (pathological vs. normal). Outcomes provide information that aims to enhance the understanding of (i) molecular mechanisms of environmentally-induced disease (toxic responses); (ii) differences in responses between humans and model species (for improved cross-species extrapolation); and (iii) interindividual variability in responses (for

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improved understanding of genetic susceptibility to environmental disease).

Together, MENTOR and DORIAN provide the basis of an evolving integrated, "systems-based" *exposure biology framework* that aims to support (i) more realistic risk assessments for contaminants in a variety of media (air, water, soil/dust, food, beverages, cosmetics, various consumer products, etc.); (ii) improved "population-wide" risk assessments that take into account the specific behavioral patterns and the genetic susceptibility factors which "elevate" the risk for specific subpopulations; and (iii) development of rational and cost-effective "targeted" protection and restoration strategies in relation to exposures involving a wide range of xenobiotics and other environmental stressors. The consideration of "individual-specific" toxicoinformatic (transcriptomic, metabolomic, proteomic, etc.) data is expected to allow development of more accurate, and eventually even "personalized," assessments of risk due to toxic xenobiotics.

Clearly, the ongoing efforts of ebCTC are only starting to address many of the complex challenges related to the multiscale integrative modeling of toxicokinetic and toxicodynamic processes. It may be claimed, however, that the methods that were briefly overviewed in this chapter, along with the selected application-specific examples, demonstrate the potential future benefits of a consistent integrative exposure biology modeling framework.

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RELATED ARTICLES

Toxicokinetics
Nanotoxicology–the Toxicology of Nanomaterials

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FURTHER READING

The Environmental Bioinformatics and Computational Toxicology Center Website. ebCTC.org

The Environmental Bioinformatics Knowledge Base portal. ebKB.org

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Biomarkers of System Response to Therapeutic Intervention

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1 INTRODUCTION

There are numerous definitions for a biomarker; however, they all fundamentally describe a measurable quantity that informs us about a hidden state of the biological system that predisposes that system to one of several outcomes. For clinical biomarkers, the most straightforward case is the presence of a molecular entity, such as an antibody, that provides evidence of a disease state, such as infection by a virus. In drug development, such biomarkers could include measurable biochemical species indicating organ toxicity or other critical issues that could block efficacy.

There are many types of biomarkers presently in use. Diagnostic biomarkers provide insight into the probability that an individual has a disease. These biomarkers, often the result of hospital laboratory tests, provide physicians with information on the state of the patient. Such diagnostic markers include tests for the presence of antibodies to known disease-causing organisms, levels of serum chemicals such as glucose that can indicate temporary or chronic failure of bodily systems, and blood counts that can indicate anaemia or inflammatory responses. In addition, there are specialized biomarkers developed that identify subtypes of disease, including biomarkers for the different types of leukaemia or lymphoma. These can be used to choose specific treatments among the different options available.

Prognostic biomarkers differ from diagnostic biomarkers in that they provide a probability of outcome. These biomarkers generally do not lead to optimization of treatment, but provide insight into the potential endpoints of a disease. In cancer, such biomarkers are common and often take the form of Kaplan–Meier survival curves that provide the probability of survival with a given type of cancer, including the cancer stage, over time.

Response biomarkers provide indications of the likely response to treatment. Genetic tests on variants of key metabolic liver enzymes can be considered response biomarkers, as they provide information for adjusting dosing of some therapeutics. Naturally, response biomarkers can be viewed as safety biomarkers as well, because they provide information on the safety of the proposed treatment. Pharmacogenomic and pharmacokinetic biomarkers can also be considered types of response biomarkers, as they predict the response of a patient to a drug based on individual variation.

Screening biomarkers are similar to diagnostic biomarkers; however, they must be suitably designed to assess the probability that an individual does not have a disease given that an entire population will be tested instead of a small group of individuals already suspected of harbouring the disease. The significant difference between these types of biomarkers lies in the number of non-diseased individuals likely to receive the test relative to the number of diseased individuals. We discuss

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this in more detail in the section on Statistical Issues.

In clinical practice, the logical endpoint for a biomarker is patient outcome, generally survival time. In many cases, using such an endpoint is impractical, as the time required delays the application of lessons to be learned in the study. In some cases, it is therefore appropriate to utilize a surrogate endpoint, which is itself a biomarker related to the outcome. Because a surrogate endpoint is equivalent to a biomarker, it requires development and validation equivalent to that for a biomarker.

It is useful to consider an ideal biomarker as a baseline for discussion of biomarker development, especially from high-dimensional data. An ideal biomarker would be measured non-invasively and provide a clear high value for an individual in state 1 (disease) and a clear low value for an individual in state 2 (healthy). The distributions of the measurements of these values in the population would not overlap, so a clear threshold would exist between healthy and diseased individuals, as depicted in Figure 1a. In reality, no biomarker fulfils these requirements and even simple single chemical biomarkers generally have overlapping distributions, such as in Figure 1b, which leads to false-positive and false-negative test results.

If we consider a real case, a biomarker for the probability of a heart attack, we get a clearer picture of the situation. Typically, measurements of low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides and potentially C-reactive protein are made. These measurements are combined with age, medical history, body mass index, diabetes status, smoking history and family history to create an estimate of the potential for a heart attack over a future time period. This clearly is more complex than the case of a single molecular species, and developing this statistical model required many years. With high-throughput data measuring biological systems, we face a similar need for complex statistical models. However, we now face a difficult problem as these molecular measurements are simultaneously refining our understanding of disease subtypes, leading to fewer individuals fitting into any category for statistical analysis.

In this chapter, we discuss the emerging high-dimensional data in terms of systems biology and implications for biomarkers of system responses to therapeutic intervention. We address a number of standard issues in biomarker development and validation as well, including the use of model systems and statistical approaches that enhance the probability of identifying useful biomarkers during the development process. We conclude with an example of an approach to development of biomarkers from high-throughput, systems-level data.

2 SYSTEMS BIOLOGY AND HIGH DIMENSIONALITY

2.1 Complexity of Molecular Interactions

Biological systems are inherently complex, comprising perhaps the most complex systems studied in science to date. This complexity arises from a number of factors. First, the large number of

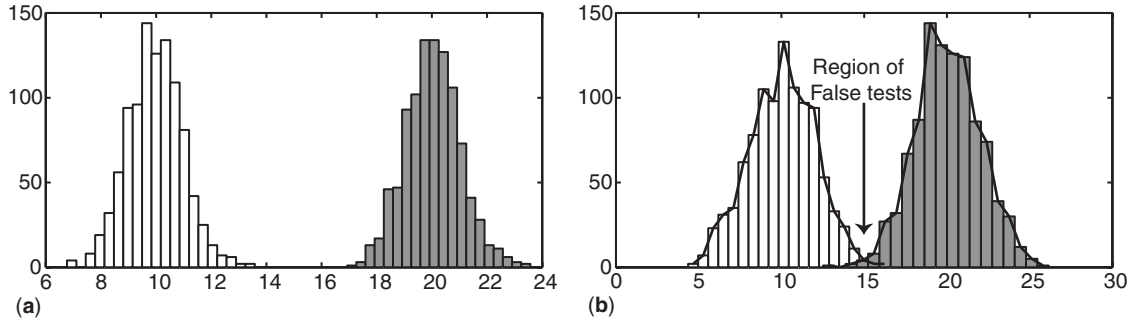

Figure 1. Examples of test results. (a) An ideal biomarker is shown, where the healthy group (white) tests with a mean of 10 and the diseased group tests with a mean of 20, and there is no overlap. (b) An excellent biomarker with good separation between the groups is shown, with a minimal overlap area that generates false positives and negatives depending on the threshold.

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highly interacting molecular species creates very large dimensionality issues in the data. There are roughly 25 000 genes generating hundreds of thousands of protein variants, which undergo post-translational modification to millions of forms. The DNA, mRNA, miRNA and proteins interact with each other and with thousands of chemical species, creating a combinatorial nightmare for modelling and analysis.

Second, these molecular species and interactions take place in a large number of contexts, where epigenetic and environmental changes cause varying subsets of molecules to be produced, leading to different interactions in different cellular and environmental conditions. This makes it difficult to utilize data from model systems or even different human systems (e.g., kidney vs. liver) when analysing data from a specific subsystem.

Third, the interactions, both between different subsystems and between molecular entities, are stochastic and non-linear in nature. This creates serious problems for standard approaches such as perturbation theory, where the underlying assumption is linearity in response, so that a small perturbation does not lead to large changes in the system. In addition, approaches that try to understand the system using deterministic methods are prone to failure.

It is to be hoped that the future of medicine will include detailed models of the full biological system that can guide treatment decisions. This is already the case for some subsystems, such as the function of the heart, where detailed models of nerve firing and mechanics are used to design interventions. However, for molecular interventions such detailed models remain remote in almost all cases due to the underlying complexity. This leads to a requirement for statistical models, where we replace a physico-biological model with a data-driven phenomenological model describing the mathematical relationships between molecular species and system states. These molecular species are then biomarkers for the system state (e.g., disease), and ideally can be shown to have some mechanistic interactions that are involved with driving the state, even though the complete details remain unclear. An example is HDL cholesterol, which has been shown to play a mechanistic role in protecting arteries from plaque formation, although the complete details of the biological process are not yet fully modelled (Rader, 2006).

2.2 Isolation of Signatures

The complexity of biological regulation raises an important issue for biomarker discovery. Recently, analysis of a genome-reduced version of *Mycoplasma pneumoniae* revealed over 200 multi-protein complexes arising under normal growth conditions from less than 700 genes, showing substantial gene reuse in the simplest self-replicating organism under a single condition (Kuhner *et al.*, 2009). Gene reuse is expected to be substantially greater in eukaryotes and studies of cell cycle genes in *Saccharomyces cerevisiae* show that only about 10% of cell cycle-controlled genes are expressed in only one of the four cell cycle phases under rich media growth conditions (Lukashin and Fuchs, 2001; Moloshok *et al.*, 2002), again suggesting extensive reuse of genes for multiple biological purposes. As the goal for a biomarker is to indicate the underlying biological state of interest, generally independent of the present status of other states, it is unlikely that the level of a single molecular component of the system will provide the necessary information across many potential contexts. For example, it is unlikely that the level of a single serum protein will provide a reliable and adequately specific indication of the response of a tumour to therapeutic intervention across the population, which has substantial individual variation genetically and can be expected to have vastly different environmental factors at work (e.g., diet, infection and inflammation, organ status).

The search for a biomarker then becomes a search for a signature comprising the relative levels of multiple molecular species that together provide an indication of the biological state that is robust across many contexts. This is similar to the statistical model for heart attack risk comprising many different indicators, which was noted in the Introduction. Such signatures derived initially from studies utilizing gene expression microarray measurements are already in use to assess the risk of relapse in breast cancer (Ross, 2009). It is not necessary that signatures comprise only a single molecular type, although that is presently the case primarily because microarray-based studies are farther along the development curve. However, there can be substantial advantages of integrating data from different molecular species into a single model to guide biomarker discovery, as demonstrated by the identification of sarcosine as a potential metabolite

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biomarker of aggressive prostate cancer (Sreekumar *et al*., 2009).

2.3 High-Dimensional Biological Data

The first widely used high-throughput assay was the gene expression microarray (Schena *et al*., 1995; Lockhart *et al*., 1996), although the name is misleading as it measures only the generation of transcripts (i.e., mRNA) and not the translation of those transcripts to proteins. Early studies were plagued by the failure of researchers trained in low-dimensionality studies to address the 'large p, small n' problems inherent in measuring thousands of variables on only tens of samples (Simon *et al*., 2003). This 'curse of dimensionality' leads to the need for robust statistical analysis that addresses issues of false positives due to chance agreement of measurements. In addition, early studies had issues with proper experimental design, such as randomization of samples, and normalization of signals. The inclusion of informaticists and statisticians in studies and the development of robust normalization techniques greatly enhanced the value of the data as the field matured (Irizarry *et al*., 2003; Tseng *et al*., 2001).

For all high-throughput data, we can consider the normalized, pre-processed data to take the form of a matrix of measurements. The rows comprise different genes (microarrays), proteins (mass spectrometry or protein microarrays), miRNAs (miRNA arrays) or metabolites (mass spectrometry or NMR). The columns comprise different samples, which in general will be different individuals but can also be different time-points in a series or different cell lines or animals in model organism studies. This is depicted in Figure 2a.

Microarrays have become standard tools in probing the molecular variations in disease and therapeutic response (Rhodes and Chinnaiyan, 2004). Processing of the data for standard analyses, such as to identify genes that are differentially expressed between two groups, is routine, and many statistically sound methods to generate lists of such genes now exist. These methods do group genes based on the assumption that two genes grouped together belong together in all samples. From the matrix point of view, this is equivalent to separating out two groups, one in which genes are more highly expressed in one set of samples (e.g., diseased individuals) and a second in which genes

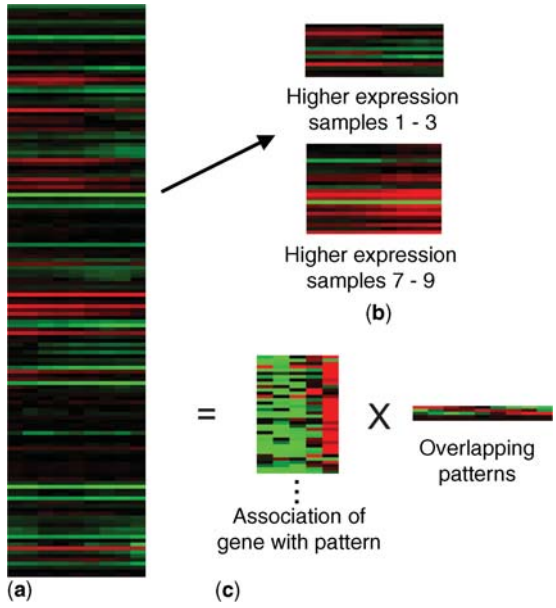

Figure 2. An example of gene expression analysis methods. (a) A portion of the expression matrix is shown, comprising ~50 of the 1300 genes tested. (b) The results of SAM analysis are shown with genes either over- or underexpressed in the first three samples relative to the last three samples. (c) A more complicated analysis where patterns of behaviour are found across the samples and the genes assigned proportionally to these patterns to reconstruct their overall behaviour. See Moloshok *et al*. (2002), for a discussion of this process and the reasons for doing such an analysis.

are more highly expressed in a second set of samples (e.g., healthy individuals), as in Figure 2b. As the expression of genes will be noisy, a weighted average across many genes differentiating the two groups may be more indicative of group membership, which is why commercial biomarkers based on expression have multiple genes.

As gene reuse is widespread, the unique separation into two groups may not reflect the underlying biology in the study in question, and methods have been developed to partition the behaviour of genes into multiple groups. These methods include non-negative matrix factorization, machine learning methods and Bayesian Markov chain Monte Carlo (MCMC) approaches (Kossenkov and Ochs, 2009). The separation then finds patterns with genes assigned to multiple, overlapping groups, as in Figure 2c. The analyses in these cases tend to require multigene biomarkers because the focus is on coordinated changes in sets of genes that differentiate the samples.

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As the first widely used high-throughput technology for biomarker discovery, gene expression microarrays are ahead on the development curve. Other platforms are quickly coming into widespread use, however. Proteomics technologies, including tandem mass spectrometry (MS) after fractionation and antibody chip arrays, can generate quantitative measurements of hundreds of thousands of protein species, including tracking of post-translational modifications (Hu, Hood and Tian, 2007). The goal of many of these studies is the ability to measure biomarkers in bodily fluids that are obtainable with minimally invasive procedures, such as blood draws or urine. There are substantial difficulties due to individual variation, the dominance of high-abundance proteins and variation in processing of real clinical samples; however, progress has been rapid in overcoming these barriers (Hanash, Pitteri and Faca, 2008).

Single-nucleotide polymorphism (SNP) microarrays are now in widespread use for genome-wide association studies (GWAS), both for variations in SNPs and for copy number variations (CNV) (Herr *et al*., 2005). The matrix of Figure 2 remains; however, the data may be binary (SNP A or B) or categorical (collection of SNPs in a gene giving allelic variants). The data then must be analysed using different algorithms, which is especially complicated for the Bayesian MCMC approaches that rely on a measure of the model fit to the data during analysis (Favorov *et al*., 2005). Present SNP array chips contain over a million SNPs and the population distribution of many of these SNPs have been measured by the HapMap project (International HapMap Consortium, 2003).

The variation in genomic structure, either through mutation or through CNV, plays an especially critical role in cancer aetiology. As the mutations often occur somatically in the tumour, measurements must be made on tumour samples, which are difficult to obtain and limited in number. The development of tissue microarrays, in which small tumour cores are sliced and arrayed onto a substrate, has greatly enhanced the screening of potential biomarkers in large numbers of tumour specimens (Hassan *et al*., 2008). The measurement techniques are more limited than in the case of a frozen tissue specimen. Generally immunohistochemical staining is the standard, so tissue microarrays are generally most useful as a first validation of prospective biomarkers.

Recently, metabolomic measurements (also termed metabonomic measurements) have emerged as a potential source for biomarkers. Certain metabolites, such as glucose, already serve as clinical biomarkers in practice; however, discovery of new metabolite biomarkers has been hindered by the lack of high-throughput techniques that can identify metabolites in bodily fluids with reasonable dynamic range. The improvement in NMR and MS techniques have resolved some of these issues (Goldsmith *et al*., 2009); however, identification tends to still be limited to hundreds of species rather than the tens of thousands provided by the other high-throughput techniques.

2.4 Integrated Data Sets

The greatest promise for the identification of biomarkers of system response from the emerging high-throughput data sets lies in integration of the different data types prior to analysis. Although this can be performed in a simple manner, essentially allowing measurement of each molecular species to be treated as an independent data point to improve biomarker discovery (e.g., Daemen *et al*., 2008), greater insight and discovery of more powerful biomarkers is likely to result from a model-based integration.

One simple model-based integration is to focus on biological pathways and perform statistical analysis across pathways instead of across genes. This has led to significant insights into the mechanisms underlying cancer aetiology, where amplifications or mutations of different proteins within a pathway drive similar phenotypic development. Multiple studies have now demonstrated that different biological pathways must be altered for cancer development, but within each pathway, different individual proteins carry mutations or amplifications (Parsons *et al*., 2008; Cancer Genome Atlas Research Network, 2008).

More complex modelling relies on inclusion of our knowledge of biological processes in order to link the different molecular species. At its simplest, this is linking of SNP or CNV to the gene, the expression of that gene to the encoded protein and the use of that protein, for instance as an enzyme, in the production of a metabolite. The model can go further, including such information as the function of a protein as a transcription factor, the

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presence of upstream promoter sequences for that transcription factor in a gene encoding an enzyme and the function of that enzyme in producing a metabolite. Such integration was used to determine the potential ability of the metabolite sarcosine to drive prostate cancer aggressiveness (Sreekumar *et al*., 2009).

One significant advantage of model-based data integration is that potential biomarkers will tend to have an associated biological mechanism identified. This provides a direct link between the molecular biomarker and the actual driver of the system state, which is useful both for further studies aimed at development of therapeutics and for obtaining regulatory approval for clinical use.

An additional advantage of model-based integration lies in the ability to leverage existing biological knowledge during analysis. This is most easily performed through Bayesian methods in which prior knowledge captured in the literature or in data repositories provides prior probabilities for the analysis. Bayesian networks are widely used to capture the relationships that underlie most biological processes, as these can be mapped to a graphical model (Djebbari and Quackenbush, 2008).

2.5 Example: Cell Signalling

Cell signalling provides an excellent example of why model-based integration is important for biomarker discovery. Cell signalling networks comprise a set of proteins that permit a cell to sense the internal and external environments and to respond to maintain homeostasis. The signalling proteins function in a number of ways to transduce a signal; however, the most common is through conformational changes due to phosphorylation that cause a protein to switch from inactive to active conformation. The active protein can then modify a downstream protein target in the signalling cascade, and thus the signal propagates through conformational changes in a series of signalling proteins.

The end result of such a cascade can be modified transcription, translation or metabolism. However, these modifications typically do not affect the expression of the members of the signalling pathway, so simple data integration where a gene is linked to its protein will not lead to an improved analytical result. For example, looking for the coordinated change in protein phosphorylation of a signalling protein and a change in transcript levels of that protein in a microarray measurement is usually a non-sensical approach biologically.

A model-based approach can provide insight, however. In Figure 3, modified from (Ochs *et al*., 2009), the core signalling network underlying growth of gastrointestinal stromal tumours is shown. Growth is driven for most tumours by a mutation in the c-KIT oncogene, which encodes a receptor tyrosine kinase (octagons) governing proliferation. The mutations make the KIT receptor constitutively active, leading to unconstrained growth together with escape from apoptosis due to cross-signalling to the PI3K-AKT pathway. Although the signalling proteins (rounded squares) themselves do not undergo significant transcriptional changes in this system, these signalling pathways link to a series of transcriptional regulators (circles) that drive reprogramming of the cells through up-regulation and down-regulation of a large number of genes, many of which have been studied in detail.

We created the DESIDE (Determination of Signalling from Differential Expression) algorithm to estimate signalling changes during therapeutic intervention from estimates of transcription factor activity deduced from analysis of microarray data coupled to annotations from the TRANSFAC database (Matys *et al*., 2006). By modelling the relationship between the signalling pathways and the transcription factors, as shown in Figure 3, we were able to demonstrate that imatinib mesylate does successfully target the KIT receptor leading to loss of downstream signalling, but that unexpected changes in STAT3 and ELK1 transcription occur, potentially indicating the presence of stem cells. In addition, an off-target effect of IM treatment, DNA damage, was predicted from up-regulation of P53 and verified by immunoblotting of DNA damage response proteins and comet assays (Ochs *et al*., 2009). These predictions could not have been obtained from an analysis that treated transcript levels as surrogates for protein levels for the signalling proteins.

The coordinated change of transcript levels for the targets of transcription factors at the terminal end of signalling pathways can then provide a global, unbiased estimate of pathway activity. As modification of pathway activity is a goal for many modern therapeutics, the statistical inference of transcription factor activity from microarray data can generate proposed multigene biomarkers for the system response. Such biomarkers may include

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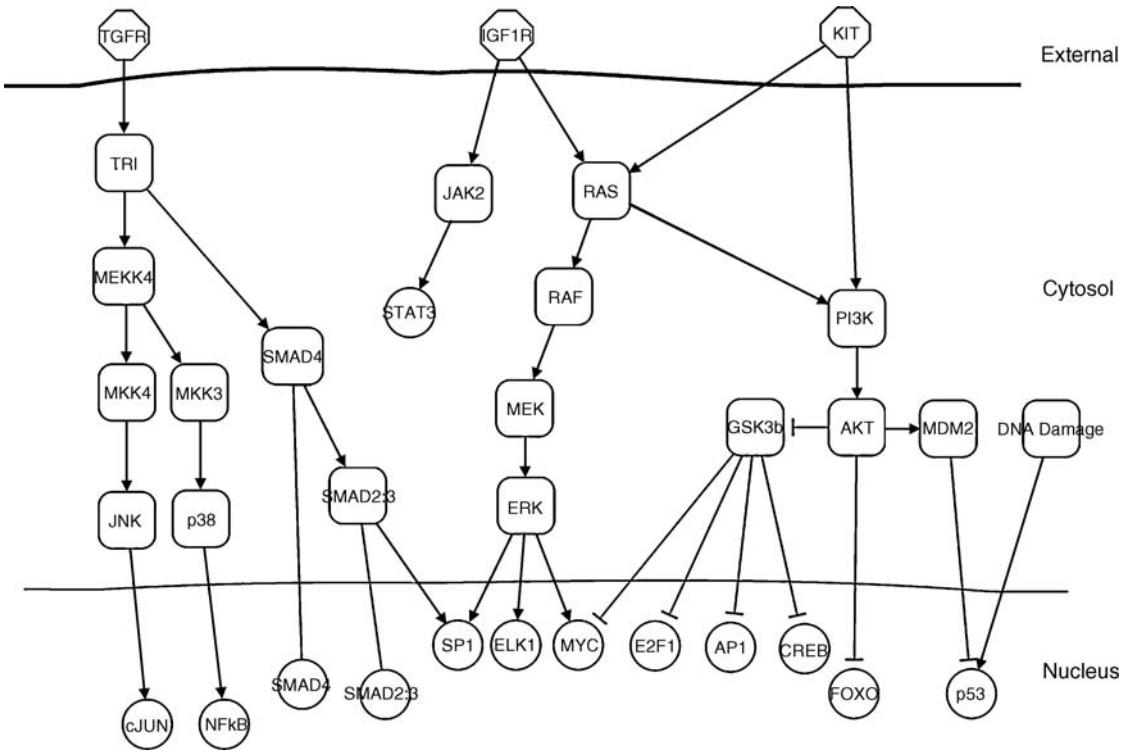

Figure 3. A simple model of a signalling network. Here, the membrane receptors, shown as octagons, transmit external signals to cytosolic signalling proteins, shown as rounded rectangles, which transduce the signals to transcriptional regulators, shown as circles, in the nucleus.

both on-target and off-target effects of the therapeutic, allowing identification of additional pathways requiring therapeutic intervention during treatment. Unfortunately, the present state-of-the-art requires invasive procedures; however, emerging molecular imaging techniques may lead to non-invasive approaches (Shaw, 2009).

3 MODEL SYSTEMS FOR BIOMARKER DEVELOPMENT

3.1 Cell Line Models

The simplest and least expensive systems for biomarker development are cell lines that have been developed out of a variety of normal and diseased tissues. Many well-characterized lines can be obtained from repositories, such as the ATCC. Immortalized lines have limitless replicative potential, allowing them to be maintained indefinitely. For cancer studies, the NCI-60 panel of cell lines have been extensively studied (Shoemaker, 2006), and differences in drug response have been linked to transcriptional variation (Scherf *et al.*, 2000). However, a problem with the use of these lines is that they show dramatic changes relative to early-stage cancers, with chromosomal and genomic instability and expression changes an issue (Li *et al.*, 2008). As such, comparison of responses in immortalized and highly modified cell lines may fail to reflect *in vivo* responses even for similar tissue types.

The development of novel cell lines that more closely match *in vivo* tissues is highly desirable for biomarker development. Recently, transgenic mice and rats carrying the SV40 T-antigen have been produced (Obinata, 2007). These animals ease the establishment of immortalized tissue specific cell lines, and these cell lines show tissue specific expression and behaviour, although it is too soon to know how closely the behaviour of these cell lines will match *in vivo* tissue.

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3.2 *Ex Vivo* Models

Although cell lines are essentially *ex vivo* models, we wish to separate the discussion of models that are more closely related to the tissue of interest or the *in vivo* system from that of immortalized cell lines. *Ex vivo* systems include use of easily obtained biological samples, such as from blood or serum, and models that permit growth of tissue outside the individual, such as xenograft models. Recently, the use of skin cells, which are easily harvested, has been shown to be indicative of occult systems in some cases (Agulnik *et al*., 2007).

The harvesting of circulating cells from blood can be performed so long as a marker has been identified that can be used in flow cytometry and cell sorting. For blood cancers, it is now routine to isolate the cancerous cells and test them to refine the molecular diagnosis of cancer, and well-known markers are available. However, the markers need not be highly specific in all cases, as, for example, epithelial markers can be used to capture circulating tumour cells from most solid tumours. The cells may be useful for biomarker studies as they reflect the biological characteristics of the source tumour and may therefore reflect molecular characteristics, such as pathway activity, as well (Wong, 2003).

There are also useful primary cell cultures, such as hepatocytes, that can be used to identify biomarkers of drug toxicity (Lavallie, Dorner and Burczynski, 2008). Since the availability of primary hepatocytes can be difficult to maintain, other tools will remain necessary. As pointed out in a review of *ex vivo* systems (Lavallie, Dorner and Burczynski, 2008), a key requirement for any *ex vivo* biomarker discovery system is the presence of pathway activity similar to the target tissue, which may be hard to guarantee in many cases.

An alternative to these approaches is to create xenograft models of the diseased tissue in an animal model to predict response to therapy (Rubio-Viqueira and Hidalgo, 2009). In this case, the xenograft itself becomes the biomarker of response, allowing testing of different treatment options prior to treatment of the patient. Alternatively, a collection of such xenograft models of different tumour subtypes can be used for biomarker discovery, using the high-throughput biological methods discussed in the Systems biology and high dimensionality section on the xenograft tumours. One complication is that the host – tissue interaction will not truly reflect that in the individual from whom the tissue has been harvested.

3.3 Animal Models

Perhaps, the ideal model system is an animal model of the biological system in question. Such models require the development of an orthologous molecular and cellular environment in the model organism. The environment must contain adequate complexity to reflect a significant portion of the function in the human system, and importantly must maintain the likely features of therapeutic intervention. One complexity of any such system is that drug processing can be highly different in different animals, which may complicate pharmacological interpretation of results.

4 STATISTICAL ISSUES

4.1 Statistics for Biomarker Discovery

In the discovery stage, statistical analysis focuses on identifying potential biomarkers with the highest probability of being significant biologically. As data are generally limited at this stage, the process is prone to false discovery, where the molecular species identified as distinguishing the groups (e.g., disease and normal) result from chance variation in the data. The early analyses of microarray data provide a cautionary tale in this regard, as studies were performed with little or no replication and results published without regard for statistical procedures to address false positives.

The statistical needs for biomarker discovery from high-throughput data can therefore be deduced historically from the lessons learned in microarray studies. For identification of potential biomarkers that distinguish two groups, statistical tests were developed that addressed the 'large p, small n' problem by estimating variation using the full data sets rather than the traditional approach of using only single gene variance, as a *t*-test would do. One of the most widely used methods is significance analysis of microarrays (SAM), which uses the framework of a *t*-test but adjusts the denominator by borrowing estimates of variation from genes at similar levels of expression, which corrected unreasonably large estimates of changes at low expression levels (Tusher, Tibshirani and Chu, 2001).

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More advanced methods were applied for the discovery of multi-gene biomarkers. These techniques looked to discriminate the classes based on signatures in the data, and multiple methods were devised for this, such as support vector machines (Brown *et al*., 2000). A number of issues arose with such approaches as they tend to overfit the data, yielding a signature tuned to the specific idiosyncrasies of the data set. It is important in these cases to apply validation methods, so that the data are trained and tested on different subsets of the data. This can be carried out with repeated splitting of the data into training and test sets (cross validation), with the resulting signature being a weighted average of the signatures discovered in this way.

It is critical to remember that this approach to validation is still prone to overfit the data, as the same data are used throughout and will therefore potentially display the same systematic behaviours. True validation, even during biomarker discovery, requires independently gathered data. Such data should ideally come from a different laboratory, so that unrecognized biases in the data that might arise from unique sample handling can be identified. It is worth remembering that all additional tests performed during discovery are relatively cost-effective compared to *in vivo* tests for validation, so refining biomarkers at this stage is a logical approach.

4.2 ROC Analysis

A biomarker at its simplest discriminates between two states, for instance disease and healthy. This can be reflected in a 2×2 contingency table (Table 1). Across the top of the table, we can write the status of the test, whereas along the side we have the true condition of the individual. The table then divides naturally into true positives (TP, test positive on diseased individual), true negatives (TN, test negative on healthy individuals), false positives (FP, test positive on healthy individuals) and false negatives (FN, test negative on diseased individuals). The value of a test obviously depends on the relative proportions of TP and TN to FP and FN. However, the definition of a positive or negative test depends on a threshold for the score of that test (e.g., fasting glucose greater than 100, rather than greater than 90).

It is useful when analysing the validity of a biomarker within a data set to consider the effects of a change of threshold on the results. This avoids an additional overfitting problem, as tuning the threshold for identifying hidden states can lead to a false sense of the reliability of a biomarker due to the chance distribution of values within a small data set.

This issue can be addressed through the use of receiver operator characteristic (ROC) analysis (Soreide, 2009). An ROC curve is a measure of the trade-off between sensitivity (true positive rate) and specificity (true negative rate). The sensitivity essentially is the probability that a positive test reflects the presence of the biological state (e.g., disease), whereas the specificity does the same for the absence of the state. Figure 4 demonstrates the trade-off for a realistic test where the range of values measured in healthy individuals overlaps that for diseased individuals. By moving the threshold, a curve of sensitivity and specificity values is generated and these can be plotted to form the ROC curve. The area under the curve (AUC) provides a threshold-independent measure of the test. Note that the expectation for a random test (a coin flip decides disease state) is an AUC of 0.5, so any test that does not significantly exceed this value should be considered useless.

Table 1. A 2×2 contingency table.

	Test positive	Test negative	
Disease positive	True positive	False negative	Total with disease
Disease negative	False positive	True negative	Total without disease
	Total positive tests	Total negative tests	Total individuals

Screening test?	Test positive	Test negative	
Disease positive	45	5	50
Disease negative	50	900	950
	95	905	1000

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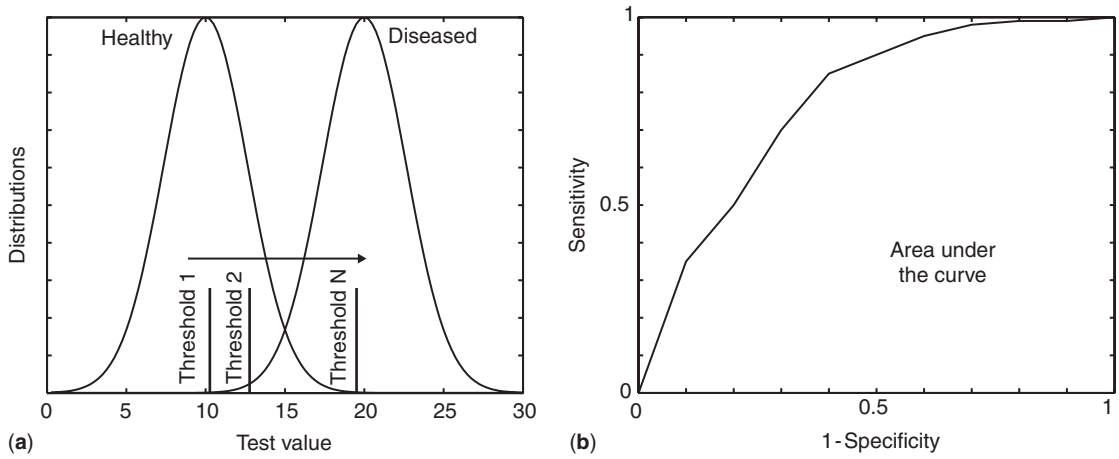

Figure 4. The ROC curve. (a) Overlapping biomarker distributions are shown for the results from healthy and diseased individuals, as in Figure 1. The threshold is shown and this is moved over the range of overlap, leading to different numbers of false positives and negatives and changing specificity and sensitivity. (b) The moving threshold maps out the ROC curve in sensitivity – specificity space.

Although ROC analysis was first used with biomarkers that were a single measurement, the same technique applies to multi-gene or other complex biomarkers. The biomarker results in a single score, such as from a weighted average of relative expression levels in multi-gene expression biomarkers, and the threshold for this score is varied to generate the ROC curve.

4.3 Biomarker Validation

Validation of biomarkers in the sense of moving them from discovery into clinical utility requires establishing their validity in a clinical setting with normal sample processing and within a population. As discovery inevitably is performed in a limited data set, potentially without *in vivo* tests, there are multiple stages to this validation. First, it must be verified that the biomarker is measurable *in vivo* with similar or adequate accuracy. Second, the usefulness of the biomarker across the target population must be validated. Third, the reliability of real-world clinical measurements of the biomarker must be established (e.g., with varying times and temperatures following a blood draw).

It can be useful to stress the difference between validation of the analytic method for biomarker development, including sample processing, and the validation of clinical usefulness, which is also termed qualification (Chau *et al*., 2008). The validation process aims to confirm a potential biomarker in an analytic system with a proven track record in order to confirm that the measurement of the biomarker is feasible in clinical settings. The qualification process involves a number of steps, outlined in (Chau *et al*., 2008), and verifies that the biomarker makes appropriate predictions in different settings and institutions. The FDA reviews proposed biomarkers and looks for scientific consensus from the community on the reliability of the biomarker.

For prognostic biomarkers, the statistic of interest is captured well by the ROC curve and the sensitivity and specificity. The sensitivity, or true positive rate, provides a measure of how good a test is at identifying individuals with the disease. The specificity, or true negative rate, meanwhile, indicates how good the test is at identifying individuals without the disease. When looking at prognosis, this provides an indication of the reliability of the prediction of outcome.

For diagnostic biomarkers, the goal is often to choose treatment based on the biomarker. In this case, the question of interest is what the probability is that a positive test indicates that the individual has the disease. This is known as the positive predictive value (PPV), with a corresponding negative predictive value (NPV) for a negative test predicting that the individual is disease-free. The distinction between PPV and sensitivity is important, as treatment will likely be chosen based on the biomarker test result, so the likelihood that the

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choice of treatment is correct is given by the PPV, not the sensitivity.

4.4 Special Considerations for Screening Biomarkers

A biomarker for screening the population to identify individuals who have a disease is an extension of a diagnostic biomarker to the case where we expect the number of individuals without disease to vastly exceed the number with disease. In this limit, it is often useful to focus on the PPV in terms of the sensitivity and specificity, which is given by

$$\mathrm{PPV} = \frac{\text{Sensitivity} \bullet \text{Prevalence}}{(\text{Sensitivity} \bullet \text{Prevalence}) + ((1 - \text{Specificity}) \bullet (1 - \text{Prevalence}))}.$$

The sensitivity is the true positive rate, the specificity is the true negative rate and the prevalence is the fraction of the population that harbours the disease. A PPV of 1 indicates a perfect test, and this can only result with perfect specificity, which requires every individual without the disease to test negative. As the specificity declines, the prevalence becomes critically important to the value of the test. For example, a test with excellent specificity (95%) and sensitivity (90%) will have a PPV of only 47% if the prevalence of the disease in the population is 5% (see Table 1). This is relatively high prevalence for many diseases, and it means that for every individual with the disease treated based on the test, another individual without the disease will also be treated. In addition, this test is unreasonably good, and most tests with such high specificity would have lower sensitivity.

This issue is important when developing a screening test. The goal is to maximize specificity in most cases, and the effect of a positive test on non-diseased individuals must be considered. In addition, if there is a relatively low PPV, then it is important that any follow-up tests be non-invasive and inexpensive as well, to minimize risk and cost.

5 SYSTEM RESPONSES

As the Systems biology and high dimensionality section makes clear, we have emerging technologies providing global coverage of the molecular species present in biological samples. However, these measurements still cannot cover the full range of biological variation, especially when the context of cell type, organ system, environmental influence and potential somatic mutations and epigenetic modifications are included. We are therefore required to build statistical models, including as much biological modelling as possible in the data integration, in order to identify biomarkers of system responses to therapeutic intervention.

We can identify a series of steps to be taken in developing such biomarkers. It is best to begin in *ex vivo* systems, where it is possible to perform time series measurements on the system during treatment. Naturally, a cell line that retains features of the system of interest is the logical point of departure for the study. Data can be gathered for the dynamic molecular components during treatment, including mRNA exon microarrays, miRNA arrays, proteomic measurements and potentially metabolomic measurements.

Analysis of the data should take place in a model-based fashion, leveraging the substantial amount of prior biological knowledge encoded in national and international repositories on pathways, transcription factors and binding sites, protein structure, drug targeting and other emerging resources. This model will be moderately complex, though not fully mechanistic in the sense of tracking all molecular interactions. Each analysis will be somewhat different, based on the system of interest, and will require substantial effort.

The result of the analysis will be a set of potential biomarkers. Ideally, a single molecular entity may be identified as a potential driver and biomarker of the state of interest, but it is more likely that a combination of molecular species will provide a signature of the state. This signature needs to be defined, perhaps through normalization invariant relationships to common species (i.e., mRNA level always greater than gene X), as in the Top Scoring Pair method (Geman *et al*., 2004).

This proposed biomarker must then be validated and qualified in independent samples obtained in different institutions. The appropriate test statistic for the proposed biomarker will depend on the purpose, whether diagnostic or prognostic. Naturally, a biomarker of treatment response will not fall into the

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category of a screening biomarker. One significant advantage to this rather complicated approach is that biomarkers may be proposed for system responses, such as off-target effects, that would be missed in strategies that rely only on targeted, non-global approaches. These novel off-target effects can suggest biomarkers for treatment failure, if they are accurate surrogate endpoints, and also can suggest new targets for adjuvant treatment.

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Nanotoxicology

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1 INTRODUCTION OF NANOMATERIALS AND NANOTOXICITY

1.1 Categories of Nanomaterials

Nanomaterials (NMs) are officially defined as "materials that have at least one dimension in the range of 1- to 100 nm" by US National Nanotechnology Initiative, and nanotechnology as "the understanding and control of matter at dimensions of roughly 1–100 nm, where unique phenomena enable novel applications." According to their shapes, nanomaterials could be nanoparticles, nanotubes, nanorings, nanocages, or nanowires, and so on. According to their composition, nanomaterials can be liposomes, emulsions, polymers, ceramic nanoparticles, metallic nanoparticles, carbon nanomaterials, ultrafine particulate pollutants, gold shell nanoparticles, and quantum dots. As a subset of nanomaterials, NMs are also variably called ultrafine particles (UFPs) by toxicologists.

1.2 Applications of Nanomaterials - Nanotechnology

Over the past ten years, nanotechnology and applications of nanomaterials have become one of the leading technologies due to their unique physical and chemical characteristics (Stix, 2001). Nanomaterials are increasingly being used in catalysts, pigments, resins, cosmetics, electric devices, and so on. In recent years, the use of nanomaterials has been extended to the biomedical and biotechnological fields for the purposes of drug/gene delivery, disease diagnosis, tumor cells tracking and recognition diagnosis, and imaging. Different nano-sized carriers, such as biodegradable polymeric micelles (Zhang and Feng, 2006; Win and Feng, 2005), liposomes (Bochot *et al*., 2002), surface-modified nanomaterials (Gupta and Gupta, 2005; Xing *et al*., 2005; Illum and Davis, 1984), and solid lipid nanomaterials (Muller, Mader and Gohla, 2000), have been developed and studied for achieving these goals.

Table 1 shows the estimated global production for specially-engineered nanomaterials and their accelerating production in the near future (Borm *et al*., 2006).

1.3 Potential Impact of Nanomaterials to Human Health and Environment

As the particle size decreases, the surface area increases, which allows a higher proportion of its atoms or molecules to be displayed on the surface rather than being confined to the interior of the material. Figure 1 clearly shows the inverse relationship between the particle size and the number of molecules expressed on the particle surface. The increase in surface area determines the potential number of reactive groups on the particle surface and makes nanomaterials very reactive

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Table 1. Estimated global production for engineered nanomaterials. Reproduced from Borm (2006) © BioMed Central.

Application	Nonmaterial/device	Estimated global production (tonnes per year)		
		2003/04	2010	2020
Structural applications	Ceramics, catalysts, films & coatings, composites, metals	10	10^3	10^4–10^5
Skincare produces	Metal oxides (eg., TiO_2, ZnO)	10^3	10^3	10^3
Information & Communication Technologies	SWNT, nanoelectronic and optoelectronic materials (excluding CMP slurries), organic light emitters and electronics, nanophospliors	10	10^2	$>10^3$
Biotechnology	Nanocomposites and encapsulates, targeted drug delivery, diagnostic markers, biosensors	<1	1	10
Environmental	Nanofiltration, membranes	10	10^2	10^3–10^4

in the cellular environment. Thus, nanomaterials may impose dramatically different biological effects from their parent forms (Oberdorster, Oberdorster and Oberdorster, 2005; Kipen and Laskin, 2005; Nel *et al*., 2006). As a result, new toxicology data on the nanomaterials is likely to result in a different hazard assessment for the nanomaterials, and the applications of nanomaterials may be limited because of concerns about their toxicity (Service, 2004).

The remarkably large amount of nanomaterials production and use in our daily lives raises the concerns of the materials' toxicity to occupational workers and end product users, who may undergo exposure to nanomaterials via inhalation, dermal absorption, and gastrointestinal tract absorption. When inhaled, nanomaterials may be deposited in any region of the respiratory system, be taken into cells, enter into the bloodstream, and circulate to potentially sensitive target organs (Oberdorster, Oberdorster and Oberdorster, 2005).

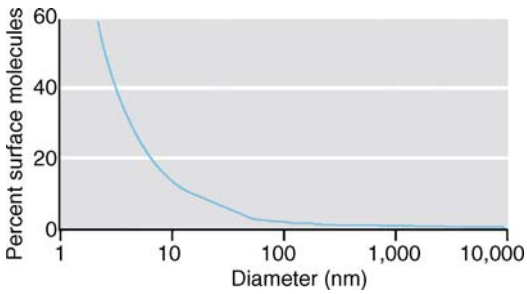

Figure 1. Surface molecules as a function of particle size. Surface molecules increase exponentially when particle size decreases to <100 nm, reflecting the importance of surface area for increased chemical and biological activity of nanomaterials. The increased biological activity can be positive and desirable (e.g., antioxidant activity, carrier capacity for therapeutics, penetration of cellular barriers), negative and undesirable (e.g., toxicity, induction of oxidative stress or of cellular dysfunction), or a mix of both. Reproduced from Oberdorster, Oberdorster and Oberdorster (2005) © National Institute of Environmental Health Sciences.

For most manufactured nanomaterials, knowledge of their toxicity and guidelines for their safe use are very limited. The reasons are due to (i) the new nanomaterials are emerging very fast; (ii) investigations on toxicity of nanomaterials have been carried on for only decades; and (iii) the toxic assessment of nanomaterials is a very complicated issue because of the many particle parameters involved. Also, oftentimes, the interpretation of the toxicity data (*in vitro* and *in vivo*) is difficult because particles of different chemical compositions were used, target animals or cells were different, and duration, end points, and dose levels also differed.

Although the potential effects of nanomaterials on human health remain unclear, considerable preliminary studies have demonstrated toxic effects of nanomaterials, including effects caused by carbon nanomaterials (carbon black, nanotubes, C_{60} and fullerene) (Warheit *et al*., 2004; Oberdorster, 2004; Shvedova *et al*., 2003; Lam *et al*., 2004; Kamat *et al*., 1998; Manna *et al*., 2005; Sayes *et al*., 2005; Jia *et al*., 2005; Kolosnjaj, Szwarc and Moussa, 2007; Kato *et al*., 2009), titanium dioxide (Warheit, Webb and Reed, 2006; Warheit *et al*., 2005; Warheit, Webb and Reed, 2006; Gurr *et al*., 2005; Sayes *et al*., 2006; Xia *et al*., 2006; Grassian *et al*., 2007; Koeneman *et al*., 2009; Kobayashi *et al*., 2009), quantum dots (Green and Howman, 2005; Hardman, 2006; Cho *et al*., 2007; Hsieh, Shiao and Chan, 2009; Pelley, Daar and Saner, 2009), ultrafine polystyrene (Brown *et al*., 2001; Hamoir *et al*., 2003), nanometer

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sized diesel exhaust particles (Block *et al*., 2004; Zhao *et al*., 2009; Sgro *et al*., 2009; Nemmar *et al*., 2009), and ultrafine particulate pollutants (Li *et al*., 2003a; Hwang *et al*., 2008; Valavanidis, Fiotakis and Vlachogianni, 2008). Some nanomaterials, such as carbon black (CB), TiO_2, iron oxides and amorphous silica, have been manufactured by the chemicals industry for several decades and are produced in quantities of many tons per year. Toxicity studies on animals (mostly rodents) by using a variation of nanomaterials revealed that nanomaterials consistently induced mild yet significant pulmonary inflammatory responses, effects in extrapulmonary organs with the analysis of lung lavage parameters and lung histopathology, effects on the blood coagulation cascade and to extrapulmonary tissues. In addition to *in vivo* and *in vitro* studies, some studies of eco-toxicity of nanomaterials were also carried out (Adams, Lyon and Alvarez, 2006; Hund-Rinke and Simon, 2006) to contribute to a complete understanding of toxicity mechanism.

2 PHYSICOCHEMICAL PROPERTIES OF NANOMATERIALS AND CHARACTERIZATION

Before conducting *in vitro* nanotoxicity testing, one needs to gather all of the physicochemical properties of nanomaterials. The reason is that the physicochemical and structural properties of nanoparticles play a major role in their interactions with cells, which could lead to quite different toxicological effects (Oberdorster, Oberdorster and Oberdorster, 2005; Lewinski, Colvin and Drezek, 2008; Kreyling, Semmler-Behnke and Moeller, 2006; Donaldson and Tran, 2002). The major parameters of nanoparticles to be considered, before conducting *in vitro* testing, include sizes of the particles, extent of their size distribution, surface area, porosity, charge density on the surface, solubility, morphology, function groups on the surface, purity, stability, and so on. Table 2 shows that common physical and chemical properties that should be known before nanotoxicology testing (Sahu and Casciano, 2009).

The particle size plays one of the most important roles in causing cytotoxicity of nanoparticles even though other parameters may also contribute significantly. For instance, shrinkage in size may create discontinuous crystal planes that increase the number of structural defects, as well as disrupt the well-structured electronic configuration of the nanoparticle; thereby, altering its electronic properties (Oberdorster, Oberdorster and Oberdorster, 2005; Donaldson and Tran, 2002; Kipen and Laskin, 2005). The exposed specific surface groups can function as reactive sites. Therefore, the sizes of the nanoparticles must be accurately measured before nanotoxicity is conducted. Several techniques that can be used to determine the sizes and

Table 2. Important properties in nanomaterials characterization for toxicity studies. Reproduced from Sahu and Casciano (2009) © John Wiley & Sons, Ltd.

Property	Importance for toxicity testing	Comments
Particle size distribution	Essential	
Degree/state of agglomeration	Important	
Particle shape/shape distribution	Important	
Chemical composition/purity	Essential	
Solubility	Essential (where applicable)	
Surface properties		
Specific surface area/porosity	Essential	Surface roughness may be important
Surface chemistry/reactivity	Essential	
Surface adsorbed species	Important	In some cases may be the mechanism of toxicity (e.g., complement)
Surface charge/ Zeta potential	Important (essential under aqueous conditions)	Especially in aqueous biological environments, may change according to the environment
Physical properties	Important	
Density	If applicable	
Crystallinity	If applicable	
Microstructure	If applicable	
Optical and electronic properties	If applicable	
Bulk powder properties	If applicable	May be important for dosimetry/exposure
Concentration	Essential	Can be measured as mass, surface area, or number concentrations

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size distributions of nanoparticles include Atomic Force Microscopy (AFM), Transmission Electron Microscope (TEM), Scanning Electron Microscope (SEM), Light Scattering (static or dynamic), and Zeta-meter. The most commonly used techniques used by nanotoxicity researchers for size measurements are the AFM and TEM, even though their use depends on the availability of these instruments. Conventional TEM instruments can provide two-dimensional images of nanoparticles as shown in Figure 2a and b (Lin *et al*., 2009). The TEM software allows one to calculate the size distribution of the nanoparticles by using the imaging data. AFM not only provides particle size information, but also provides particle height and volume because it directly produces three-dimensional images, which is the unique advantage of AFM over SEM and TEM.

The specific surface area (SSA, $m^2\,g^{-1}$) of the nanomaterials is also an important factor that can affect their cytotoxicity significantly because the interactions of nanomaterials with cells may increase greatly as SSA increases. Even though the SSA is inversely proportional to the nanoparticle size, the nanopores on the nanomaterials may vary dramatically from one type to another. The SSA of the nanoparticles is normally measured through using the Brunauer, Emmett, and Teller (BET) technique. In addition, the BET technique can also provide particle porosity information, even though other techniques can be used to measure porosity, such as the porosity analyzer. The fundamental

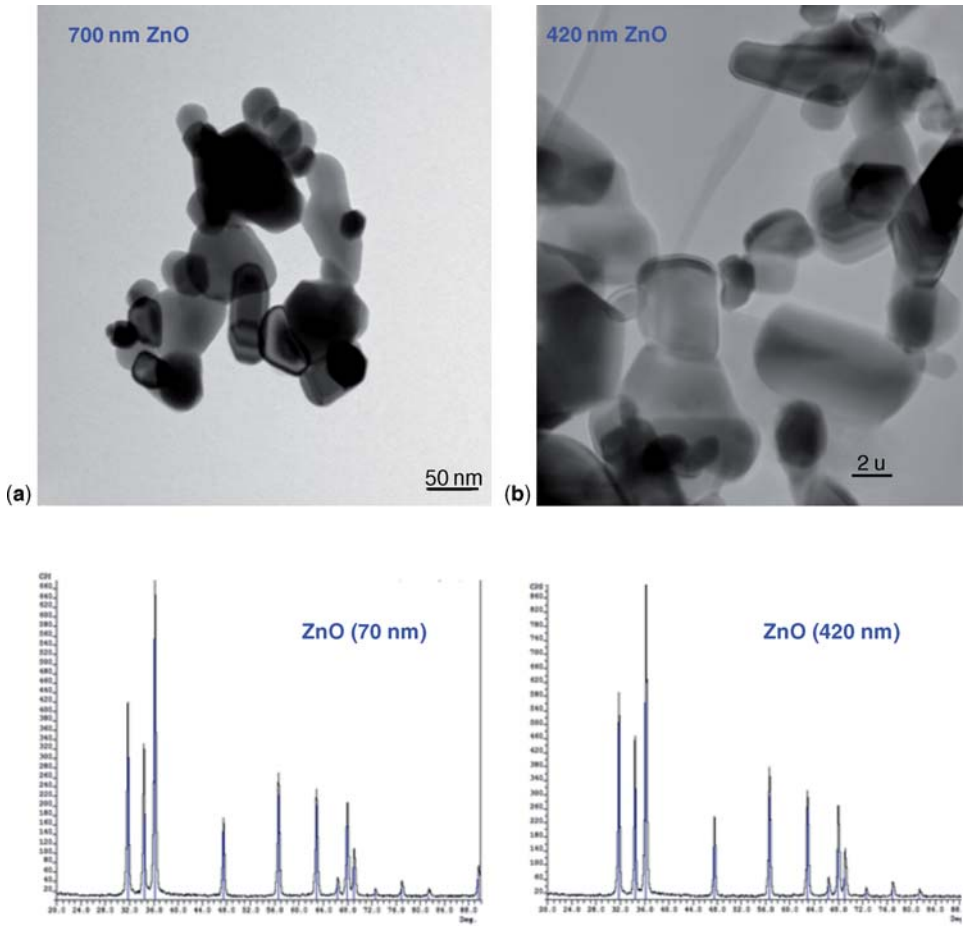

Figure 2. TEM images and X-ray diffraction (XRD) analysis of ZnO nanoparticles. (a) 70 nm ZnO particles; (b) 420 nm ZnO particles. XRD analysis indicated both particles were hexagonal. Reproduced from Lin *et al.* (2009) © Springer.

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principles of the BET technique for measuring the SSA can generally be found in many literatures.

Studies have demonstrated that different nanomaterial structures (amorphous versus crystal structure or different crystal structures) can have different levels of cytotoxicity. Therefore, pertinent information on particle structures, or crystal structures, must be known before conducting a cytotoxicity study. X-ray diffraction (XRD) is a commonly used instrumental technique for this purpose. A representative XRD analysis of ZnO nanoparticles is shown in Figure 2.

Impurities in nanomaterials or on the nanomaterial surface can significantly affect the cytotoxicity of nanomaterials. Sometimes, they may be the major contribution to the cytotoxicity of the nanoparticles, which may provide misleading information about the level of cytotoxicity of the nanoparticles. Therefore, the impurity information should be known before a nanotoxicity study is undertaken. To get the inorganic impurity data, Inductively Coupled Plasma - Mass Spectrometry (ICP-MS) instrument is the most commonly used technique to quantitatively measure the metal impurities in many nanoparticles because of its high sensitivity and specificity. For example, the impurity results in one type of silica nanoparticles are shown in Table 3 (Lin *et al*., 2009).

Due to their nanoscale size and surface properties, nanomaterials tend to aggregate or precipitate in suspensions. This process will change the nanomaterials surface area and physicochemical properties and can affect the levels of cytotoxicity of nanomaterials. Therefore, the average hydrodynamic size and distribution of nanoparticles in suspension must be determined. Dynamic laser scattering (DLS) and Zeta-meter (or zeta-sizer) are commonly used to determine the hydrodynamic sizes of nanoparticles. In addition, a Zeta-meter can also provide zeta potential and surface charge information of the nanomaterials, which are essential pieces of information for nanotoxicity testing. To acquire accurate data on the hydrodynamic sizes of nanoparticles, as well as the charge density on the surface, it is recommended that hydrodynamic size measurements be taken in the cell media used for an *in vitro* study. In summary, the physicochemical properties of the nanomaterials should be known as much as possible before a nanotoxicity study is conducted. Otherwise, the cytotoxicity data acquired through the nanotoxicity study may be difficult to explain or may not be useful at all in trying to conduct a comparison on the levels of cytotoxicity.

Table 3. Metal impurity levels in ZnO particles of 70 nm and 420 nm tested in an ICP-MS system. The method detection limit is 0.04 ppm[a]. Reproduced from Lin *et al.* © (2009).

	Metal impurity levels (ppm)	
Elements	70 nm ZnO	420 nm ZnO
Na	45.4	7.5
K	44.9	4.4
Cu	37.9	9.3
Se	18.2	18.7
Ca	8.6	1.7
As	5.5	5.7
Pb	2.9	4.6
Mg	2.4	0.5
Cd	1.4	2.8
Ga	0.8	0.7
Al	0.6	0.3
Sb	0.3	0.2
Ti	0.2	N.D.[b]
Ni	0.1	N.D.
Ag	0.1	0.1
Ba	0.1	N.D.
Rb	0.07	N.D.
Cr	0.04	0.05
Total	169.5	56.6
Be, Fe, V, Mn, Co, Sr, Mo, Cs, Ti, U	N.D.	

[a] One part per million (ppm) of a specific metal is defined as 1 gram of that metal per 10^6 gram of ZnO.

[b] Not detected.

3 NANOPARTICLE MORPHOLOGY AND CITOTOXICITY

Considerable existing data have shown that the internal structure and the external morphology of nanomaterials have a significant influence on their practical applications and cytotoxicity, even though the sizes of nanomaterials may be the same. Therefore, it is important to investigate the nanomaterials toxicity and morphology. In this section, the cytotoxicity of several classes of nanomaterials and morphological effects on their applications will be discussed.

Nanomaterials generally fall into several categories: carbon-based nanoparticles, such as fullerenes, single-and multi-wall carbon nanotubes (SWNTs and MWNTs); metal-based nanoparticles, such as colloids, nanoshells, nanorods and superparamagnetic nanoparticles; semiconductor-based

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nanoparticles such as quantum dots. *In vitro* cytotoxicity studies of different types of nanomaterials using different cell lines, incubation times, and colorimetric assays are increasingly being reported (Lewinski, Colvin and Drezek, 2008).

Carbon nanomaterials refer to carbon with one or more dimensions at 100 nm or less, including fullerenes, SWNTs and MWNTs of which fullerenes is the most widely used and well developed of the carbon nanomaterials (Lewinski, Colvin and Drezek, 2008; Sun *et al*., 2006; Schrand *et al*., 2007; Service, 2004; Bandow *et al*., 2000). In terms of cytotoxicity for fullerenes, the most significant factor influencing cytotoxicity is their complexity and cell type (Bandow *et al*., 2000). A review paper summarized the experimental setup and results on fullerenes in Table 4 (Lewinski, Colvin and Drezek, 2008).

Compared to the cytotoxicity on fullerenes, SWNTs were reported to be more toxic than the other two major types of carbon nanomaterials (Smart *et al*., 2006; Tian *et al*., 2006; Pulskamp, Diabate and Krug, 2007). One of the hypotheses to explain the cytotoxicity of SWNTs is due to the mode of production because the synthesis of SWNTs requires the use of metal catalysts which can be toxic themselves. Both time-and dose-dependent cytotoxicity in human epidermal cell lines have been studied. Lewinski, Colvin and Drezek (2008) summarized the cytotoxicity studies on SWNTs as in Table 5. Also, similar studies have been done with those of SWNTs as shown in Table 6. The numbers of publications of *in vitro* cytotoxicity studies of metal nanoparticles using different cell lines are increasing exponentially, of which gold nanoparticles have been widely studied due to their potential medical applications. Due to their small sizes, they have greater potential to travel through the organism than other material or larger particles (Donaldson *et al*., 2004). However, cytotoxicity may not be the only adverse effects of gold nanoparticles; they may also affect the immunological response of cells. One group tested the effect of gold nanoparticles on the proliferation, nitric oxide, and reactive oxygen species production of RAW264.7 macrophage cells (Shukla *et al*., 2005).

In terms of cytotoxicity, while bare iron oxide nanoparticles exert some toxic effects, several coated iron oxide nanoparticles have been found to be relatively nontoxic (Lewinski, Colvin and Drezek, 2008). Cell lines used in the study of iron oxide nanoparticles include: COS-7 cells, Human fibroblasts, Melanoma cells, HTERT-BJ1, BRL3A, MSTO21H, EC219, RAW264.7, SK-BR-3, PC12M, and some others (Cheng *et al*., 2005; Gupta Ajay and Wells, 2004; Petri-Fink *et al*., 2005; Gupta and Gupta, 2005; Hussain *et al*., 2005; Brunner *et al*., 2006; Cengelli *et al*., 2006; Hu *et al*., 2006; Yu *et al*., 2006; Muller *et al*., 2007; Pisanic *et al*., 2007; Wan *et al*., 2007; Adams, Lin and Ma, 2007; Lin *et al*., 2006; Lin *et al*., 2006; Lin *et al*., 2007; Lin *et al*., 2008). The results demonstrated clearly that surface groups on the nanomaterials play major roles in the levels of cytotoxicity.

Another class of nanomaterials is semiconductor nanoparticles, better known as quantum dots. Actually, the concern of their cytotoxicity is due to the composition of known toxic elements. The sizes of quantum dots (QDs) are ranging from 2 to 100 nm in diameter depending on the types of surface coating or functional group added. QDs typically have a core/shell conjugate structure. The core of the QD is composed of atoms from groups II–VI (e.g., CdSe, CdTe, CdS, PbSe, ZnS, and ZnSe) and groups III–V (Lewinski, Colvin and Drezek, 2008). The *in vitro* study of this class involve cell lines: BALB/c, Vero cells, Hela cells, WTK1, and B16F10 (Akerman *et al*., 2002; Dubertret *et al*., 2002; Hanaki *et al*., 2003; Hsieh *et al*., 2006; Larson *et al*., 2003; Ballou *et al*., 2004; Chen and Gerion, 2004;Derfus, Chan and Bhatia, 2004; Hoshino *et al*., 2004; Shiohara *et al*., 2004; Voura *et al*., 2004; Green and Howman, 2005; Kirchner *et al*., 2005; Selvan, Tan and Ying, 2005; Chang *et al*., 2006; Fischer *et al*., 2006;Zhang, So and Rao, 2006; Cho Sung *et al*., 2007; Duan and Nie, 2007; Guo *et al*., 2007; Liang *et al*., 2007; Ryman-Rasmussen, Riviere and Monteiro-Riviere, 2007; Lovric *et al*., 2005; Liu *et al*., 2006; Choi *et al*., 2007).

Many nano-factors enable nanomaterials to present possible dangers, both medically and environmentally, among which nanoparticle morphology play an essential role in toxicological or biomedical activities. Different nanosizes or crystal types are not only able to present different surface-to-volume ratios and surface active sites, but lead to aggregation of different levels. All these features can make the particles very reactive or catalytic in their interactions with biological systems. Although it was suggested that nanoparticle size can alter cellular uptake, subcellular localization, and an ability

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Table 4. Cytotoxicity studies of fullerenes. Reproduced from Lewinski *et al.* (2008) © Wiley-VCH.

Cell Line	Surface coating	Exposure conditions	NP concentration (average size)	Test	Exposure duration [h]	Toxicity	Author	Year
Human dermal fibro-blasts, HDF; human liver carcinoma, HepG2	COOH, OH, Na	70% confluency	0.24–2400 ppb ($d = 100$ nm)	MTT, Live/ Dead, LDH	48	LD50 = 20 ppb for bare C_{60} on HDF; no cytotoxicity observed with $C_{60}(OH)_{24}$	Sayes (Service, 2004)	2004
Guinea pig alveolar macrophages	pristine	2×10^5 cells ml^{-1} in 24-well plates	8.36×10^4 NP μg^{-1}, 1.41–226 $\mu g\,cm^{-2}$	MTT	3	No significant toxicity up to 226 $\mu g\,cm^{-2}$	Jia (Jia *et al.*, 2005)	2005
Human dermal fibro-blasts, HDF; human liver carcinoma, HepG2; neuronal human astrocytes, NHA	COOH, OH, Na	70% confluency	0.24–2400 ppb ($d = 100$ nm)	MTT, Live/ Dead, LDH	48	Nano-C_{60} is cytotoxic at 20 ppb level; after 30 h cells begin to have leaky membranes and lipid oxidation	Sayes (Oberdorster, Oberdorster and Oberdorster, 2005)	2005
Monocyte-derived macrophages	pristine	3×10^5 cells ml^{-1}	30 and 60 $\mu g\,ml^{-1}$	Nuclear Morph, PI	1, 24, 48	Did not induce damage or death of macrophages	Fiorito (Xing *et al.*, 2006)	2006
Human monocyte macrophages	pristine	2×10^6 cells $weir^{-1}$ for 24-well plate, $0.5–1 \times 10^6$ for 96-well plate	0.16–10 $\mu g\,ml^{-1}$ ($d = 60$–270 nm)	Neutral red	48	No significant toxicity	Porter (Tian *et al.*, 2006)	2006
Human epidermal keratinocytes, HEK	N-Boc-Baa	70% confluency, 96-well plate	0.00004–0.4 mg ml^{-1}	MTT	24, 48	Cytotoxicity at 0.04 and 0.4 mg ml^{-1}; IL-8, IL-6, and IL-lβ levels increased	Rouse (Pulskamp, Diabate and Krug, 2007)	2006
Human umbilical vein endothelial cells, HUVEC	$C_{60}(OH)_{24}$	90% confluency on 6-well plates	1–100 $\mu g\,ml^{-1}$ ($d = 7.1 \pm 2.4$ nm)	LDH, WST, microarray	24	100 $\mu g\,ml^{-1}$ inhibit cell growth; 10 $\mu g\,ml^{-1}$ inhibit cell attachment	Yamawaki (Lewinski *et al.*, 2008)	2006

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Table 5. Cytotoxicity studies on SWNTs. Reproduced from Lewinski *et al.* (2008) © Wiley-VCH.

Cell line	Surface coaling	Exposure conditions	NP Concentration (average size)	Test	Exposure durration [h]	Toxicity	Author	Year
3T3 cells	FITC	N/A	1–10 mM ($d = 1$ nm, $l = 300$–1000 nm)	Flow cytometry (annexin, PI)	1	5 μM, 90% viability, 10 μM, 20% viability	Pantarot-to (Nemmar *et al.*, 2009)	2003
Immortalized human Epidermal keratino-cytes, HaCaT	none (w/30% Fe cat)	80% confluency on 96 well plates or 75-cm^2 flasks	0.06, 0.12, or 0.24 mg ml^{-1}	Alamar blue, GSH	2, 4, 6, 8, 18	viability decreased after 4 h. 0.24 mg ml^{-1} ~65% viability	Shvedova (Green and Howman, 2005)	2003
Mouse peritoneal macrophage-like cells, J774.1A	pristine	10^7 cells	0–7.3 μg ml^{-1} ($d = 1$ nm, $l = 1$ mm)	microscopy	4, 8, 12, 18, 24	Cells ingest NT without toxic effects	Cherukuri (Petri-Fink *et al.*, 2005)	2004
Human embryonic kidney, HEK293	pristine	24 well plates	MTT: 0.7812–200 μg ml^{-1}; others: 25 μg ml^{-1} (CAS 7782-42-5)	MTT, western blot, flow cytometry, microarray	24–120	Cytotoxicity dose- and time-dependent; 43.5% in G1 cell cycle arrest after 1 day	Cui (Li *et al.*, 2003)	2004
Human promyelcytic leukemia cells, HL60; Jurkat T cells	COOH, biotin, fluorescein, streptavidin	3×10^6 cells ml^{-1}	0.05 mg ml^{-1} ($d = 1$–5 nm, $l = 0.1$–1 μm)	PI, flow cytometry	1	No significant toxicity for nonstreptavidin-modified SWNTs	Kam (Hwang *et al.*, 2008)	2004
Guinea pig alveolar macrophages	pristine	2×10^5 cells ml^{-1} in 24-well plates	1.41–226 μg cm^{-2} ($d = 1.4$ nm, $l =$ μm)	MTT	3	Cytotroxic effects seen at 0.38 μg cm^{-2}; necrosis at 3.06 μg cm^{-2}	Jia (Jia *et al.*, 2005)	2005
Human keratinocytes, HaCaT; HeLa cells; Lung carcinoma (A549, H1299) cells	pristine	5000 cells well^{-1} 96-well plate	0.1, 0.5, 1, 5, 10, 20 μg ml^{-1}	MTT, Live/Dead	72	Cytotoxic effects seen at 0.5 μg ml^{-1}, NFkB pathway activated by SWNT	Manna (Shukla *et al.*, 2005)	2005

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Monocyte-derived macrophages	pristine	3×10^5 cell ml^{-1}	30 and 60 μg ml^{-1}	Nuclear morphology, PI	1, 24, 48	Did not induce damage and death of macrophages	Fiorito (Xing *et al.*, 2005)	2006
Human dermal fibroblasts, HDF	phenyl- (SO_3H, SO_3Na, or $(COOH)_2$), pluronic F108	70% confluency	0.2–2000 μg ml^{-1} ($d = 1$ nm, $l = 400$ nm)	MTT, Live/Dead	24, 48	Cytotoxicity decreased w/decreased C/phenyl- SO_3H ratio; LD50 could not be obtained	Sayes (Cheng *et al.*, 2009)	2006
Human epidermal keratinocytes, HEK	pristine	5×10^3 cells well^{-1}	0.8–100 μg ml^{-1} ($d = 2$ nm, $l = 500$ nm)	MTT	24–120	Strongest adverse effect w/SWNT; 100 μg ml^{-1} gave 79%, 50% and 31% viability after 1, 3 & 5 days	Tian (Kato *et al.*, 2005)	2006
Lung epithelial-like cells, A549	pristine	25 000 cells well^{-1}, 96-well plate	50 μg ml^{-1} ($d = 1.4$ nm)	MTT, WST, LDH, MMP	24–96	MTT gave different results from WST, LDH and MMP	Worle-Knirsch (Gupta and Gupta, 2005)	2006
Rat alveolar macrophage cells, NR8383; human alveolar epithelial cells, A549	pristine	10^5 cells well^{-1} in 96-well plates; 2.5×10^4 cells well^{-1} in 96-well plates (human)	5–100 μg ml^{-1} ($d = 1$–2 nm, $l = 100$ nm)	MTT, WST	24–96	Cytotoxicity dose dependent; 100 μg ml^{-1}, 60–80% reduction	Pulskamp (Donaldson *et al.*, 2004)	2007
Mesothelioma cells, MSTO-211H	pristine	3000 cells well^{-1} in 24-well plates	7.5, 15, 30 μg ml^{-1}	MTT	72	Cytotoxicity dose dependent; agglomerated worse than well-dispersed	Wick (Gupta Ajay and Wells, 2004)	2007

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Table 6. Cytotoxicity studies on MWNTs. Reproduced from Lewinski *et al.* (2008) © Wiley-VCH.

Cell line	Surface coating	Exposure conditions	NP concentration (aver-age size)	Test	Txposure duration (h)	Toxicity	Author	Year
Human epidermal keratinocytes, HEK	pristine	80% confluence, 7000 cells well^{-1}, 96-well plates	0.1, 0.2. 0.4 mg ml^{-1}	Neutral red	1, 4, 8, 12, 24, 48	~73% viability at 0.4 mg ml^{-1}; IL-8 increases with MWNT conc.	Monteiro-Riviere (Borm *et al.*, 2006)	2005
Human skin fibro-blasts, HSF42; human embryonic lung fibroblasts (IMR-90)	pristine	70% confluency, 96-well plates	0.06–0.6 mg l^{-1}	Hoechst 33342, YO-PRO 1. PI, BrdU, microarray	24, 48	Cytotoxicity dose dependent for purified MWNT	Ding (Hund-Rinke and Simon, 2006)	2005
Guinea pig alveolar macrophages	pristine	2×10^5 cells ml^{-1} in 24 well plates	1.41–226 μg cm^{-2} ($d = 10$–20 nm, $l = 0.5$–40 μm)	MTT	3	Necrosis seen at 3.06 μg cm^{-2}	Jia (Jia *et al.*, 2005)	2005
Sprague-Dawley rat peritoneal macrophages	pristine	direct lung injection	20–100 μg ml^{-1} 0.5–2 mg rat^{-1}	LDH	3, 15 days	LDH doubled from 20 to 100 μg ml^{-1} ground nanotubes	Muller (Lam *et al.*, 2004)	2005
Murine alveolar macrophages (RAW 267.9)	pristine	5×10^5 cells well^{-1} in 96-well plates	0.005–10 μg ml^{-1}, ($d = 5$–30 nm, $l = 0.03$–3 μm)	MTT, EUSA	48	Cytotoxicity begins at 2.5 μg ml^{-1}; similar to asbestos	Murr (Cengelli *et al.*, 2006)	2005
Human acute monocytic leukemia cells (THP-l) Wister male rats	pristine	5×10^5 cells well^{-1} in 96-well plates	5–500 ng ml^{-1} 0.1 mg ($d = 20$–40 nm, $l = 0.5$–5 μm)	HU TNF-α Flexia histology, microscopy	16	TNF-α production dose dependent, aggregates in several cell types	Sato (Hussain *et al.*, 2005)	2005

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T lymphocytes, Jurkat T leukemia cells	Hydroxyl, carboxyl	4.4×10^5 cells ml^{-1}	40–400 $\mu g\,ml^{-1}$ ($d = 20$–40 nm, $l = 1$–5 μm)	Trypan blue	24–24	Cell death >80% in oxidized, <50% in pristine at 400 $\mu g\,ml^{-1}$	Bottini (Kipen and Laskin, 2005)	2006
Human osteoblastic line hFOB 1.19; Human fibroblaslic line HS-5	Poly-sulfone (PS)	2 cm^3 cells/12 well plate	N/A ($d = 10$–15 nm)	Cell titer 96	24, 48, 7 days	Small viability decrease in PS + MWNTs vs. pure PS	Chlopek (Gurr *et al.*, 2005)	2006
Human umbilical vein endothelial cells, HUVEC	pristine	6000 cells cm^{-2} in 96-well plates	Max: A: 0.5 $\mu g\,ml^{-1}$ ($d = 1.1$–3.2 nm), B: 0.64 $\mu g\,ml^{-1}$ ($d = 1.1$–4.3 nm) C: 0.9 $\mu g\,ml^{-1}$ ($d = 0.7$–6.3 nm)	MTT, Neutral red	24	None were cytotoxic but error bars of samples A & B below threshold	Flahaut (Muller, Mader and Gohla, 2000)	2006
Human lung-tumor cell lines, H596, H446, and Calu-1	Carbonyl (CdO), carboxyl (COOH), hydroxyl (OH)	N/A	0.002–0.2 $\mu g\,ml^{-1}$ ($d \approx 20$ nm, aspect ratio = 80–90 nm)	MTT	24–96	Cell viability decreased 33% at 0.2 $\mu g\,ml^{-1}$; functionalized have lower survival	Magrez (Kolosnjaj, Szwarc and Moussa, 2007)	2006
Human neonatal HEKs	pristine	80% confluency, 6-well plates	0.4 $mg\,ml^{-1}$	protein array	24, 48	Irritation and cell apoptosis proteins upregulated	Witzmann (Brunner *et al.*, 2006)	2006

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to produce ROS (Hussain *et al*., 2005), the cytotoxicity could also depend on the morphology of the particles (Gurr *et al*., 2005).

Particle surface and interfaces are important components of nanomaterials. As the morphology changes, the proportion of atoms found at the surface relative to the proportion inside its volume may also change. This may increase or decrease the reactivity. From the health implication perspective, reactive groups on a particle surface are likely to modify the biological (potentially toxicological) effects. In addition, it should be noted that two different nanoparticle-types may not be biologically equivalent from a toxicological perspective. There can be differences in crystal structures, nanosizes, and aggregation status. These morphology differences are manifested in different pulmonary inflammatory and cytotoxic effects ranging from benign to more moderate health impacts.

Although the morphology of the nanomaterials is not likely to change greatly in *in vitro*/cell culture conditions, one fact should be not ignored. Nanotoxicity assessments can be affected by their solution dynamics. The nanomaterials may precipitate, diffuse, agglomerate, and change surface charge/chemistry over time in solution, which changes the nature of the nanomaterials and their transport to cells. In addition, these processes are also affected by the properties of solutions (viscosity, density, presence of proteins, etc). For instance, nanoparticles of different morphology settle at different rates and these differences may lead to differences in transport to adherent cells in culture. The extent of these differences and their impact on the toxicity assessment of particles in general is not widely investigated. That's why in *in vitro* toxicity studies, particle morphology in solution is an important factor which needs to be accurately investigated as a prerequisite for implementing nanoparticle toxicity studies.

4 TOXICITY MECHANISMS OF NANOMATERIALS

The detailed toxicity mechanism induced by nanomaterials is not completely clear. However, several mechanisms have been proposed based on reported results. The mechanisms are shown schematically in Figure 3 (Nel *et al*., 2006). The shrinkage of size of nanomaterials and subsequent change of the physicochemical and structural properties may cause toxicological effects (Oberdorster, Oberdorster and Oberdorster, 2005; Donaldson and Tran, 2002). For example, when material size decreases to nanolevel, the discontinuous crystal planes and structural defects may increase significantly, further changing the electronic configuration of the nanomaterials (Oberdorster, Oberdorster and Oberdorster, 2005; Donaldson and Tran, 2002). This change could cause more surface sites to be active. These reactive sites act as electron-donors or electron-acceptors and work with oxygen to induce superoxide radical ($O_2^{\bullet -}$), which can then generate Reactive Oxygen Species (ROS) by dismutation or the Fenton reaction.

Nanomaterials may contain transition metals such as Fe, Cu, Cr, V, and Co, on their surface. These transition metals are closely related to the generation of reactive oxygen species and reactive nitrogen species (RNS) due to redox-cycling reactions (Valko, Morris and Cronin, 2005). For instance, carbon nanotubes contain metal impurities that can amplify chemical changes in the nanomaterial's environment and induce ROS and oxidative stress (Shvedova *et al*., 2005). Currently, ROS and oxidative stress induced by nanomaterials has been one of the best-developed paradigms for nanoparticle (NP) toxicity. Other metals such as mercury (Hg), cadmium (Cd), and nickel (Ni) may cause toxicity by depleting glutathione (GSH) and binding to the sulfhydryl groups of proteins (Valko, Morris and Cronin, 2005).

As one of the most important toxicity mechanism of nanomaterials, oxidative stress is defined as a disturbance between the prooxidant and the antioxidant balance in favor of the former and is implicated in the pathogenesis of many disease states including aging, atherosclerosis, carcinogenesis, and acute and chronic inflammatory disorders (Kehrer and Lund, 1994). Oxidative stress can result from depletion of antioxidants due to malnutrition and/or excess production of ROS. The Figure 4 shows the hierarchical oxidative stress hypothesis; the lowest level of oxidative stress induces antioxidant and detoxification enzymes, higher levels of oxidative stress causes inflammation and apoptosis.

From a mechanistic perspective, ROS generation and oxidative stress is the best-developed paradigm to explain the toxic effects of nanomaterials at the present time (Oberdorster, 2004; Xia *et al*., 2006;

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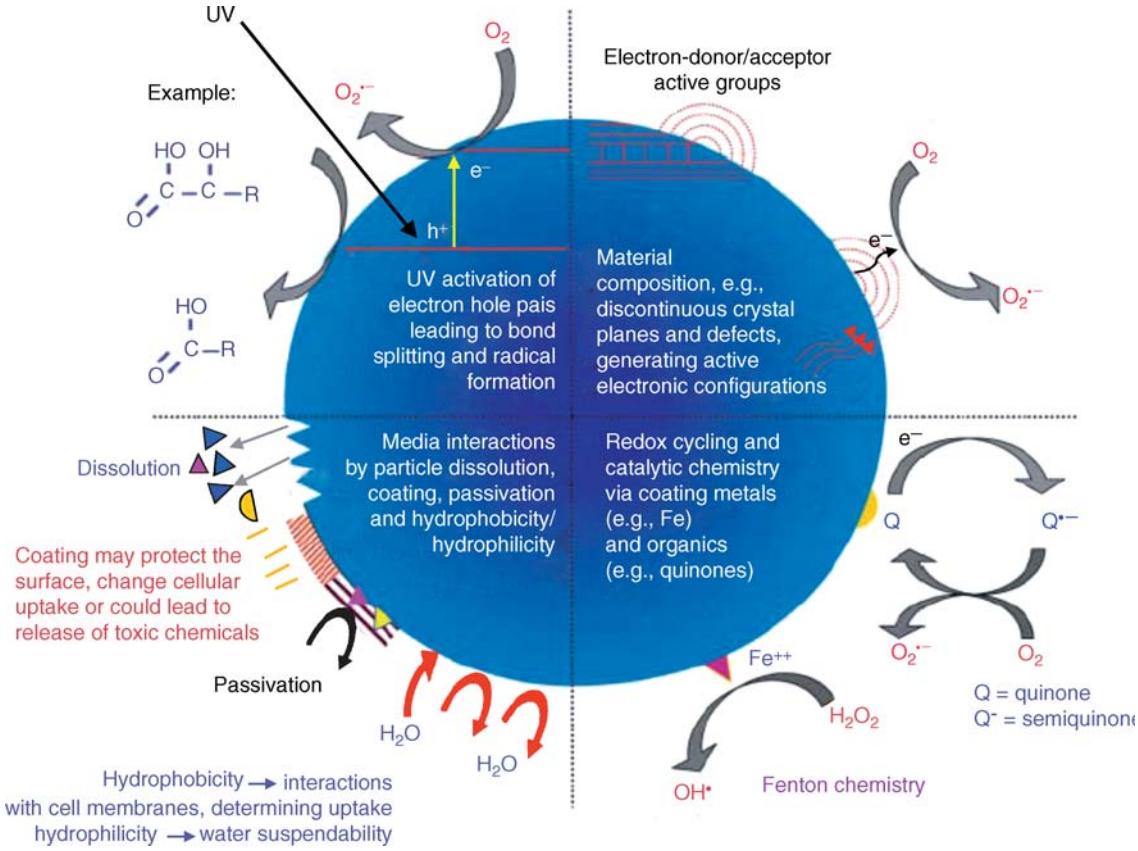

Figure 3. Possible mechanisms by which nanomaterials interact with biological tissues. Examples illustrate the importance of material composition, electronic structure, bonded surface species (e.g., metal-containing), surface coatings (active or passive), and solubility, including the contribution of surface species and coatings and interactions with other environmental factors (e.g., UV activation) Reproduced from Nel *et al.* (2006) © American Association for the Advancement of Science.

Li *et al.*, 2003a). Under normal coupling conditions in the mitochondrion, ROS are generated at low frequency and are easily neutralized by antioxidant defenses such as glutathione (GSH) and antioxidant enzymes. However, when under serious oxidative stress, free radicals can deplete antioxidants and cause severe cellular damage such as lipid peroxidation (Nordberg and Arner, 2001).

Because of the importance and significance of oxidative stress in toxicity studies, investigations both *in vivo* and *in vitro* have been carried out on selected nanomaterials such as carbon nanomaterials (nanotubes and fullerene) (Lam *et al.*, 2004; Kamat *et al.*, 1998; Manna *et al.*, 2005; Sayes *et al.*, 2005), titanium dioxide (Gurr *et al.*, 2005; Liang *et al.*, 2009; Wu *et al.*, 2009; Bhattacharya *et al.*, 2009), quantum dots (Green and Howman, 2005; Funnell and Maysinger, 2006; Li *et al.*, 2009), ultrafine polystyrene (Xia *et al.*, 2006; Brown *et al.*, 2001; Stone, Johnston and Clift, 2007), and ultrafine particulate pollutants (Li *et al.*, 2003a; Valavanidis, Fiotakis and Vlachogianni, 2008; Brauner *et al.*, 2007; Alessandrini *et al.*, 2009). The results demonstrated that the toxicity of these nanomaterials was related to ROS generation and oxidative stress, which caused membrane damage, DNA damage, and possible protein denaturation and immune reactivity.

Figure 5 shows a typical diagram depicting how C_{60} fullerene induces ROS and interacts with the antioxidant defense system (Yamakoshi *et al.*, 2003). The exact mechanism by which C_{60} and other nanomaterials cause ROS is not yet fully understood, but a possible mechanism has been proposed as follows: (i) photo excitation of fullerenes and Single-wall Carbon Nanotubes (SWNTs),

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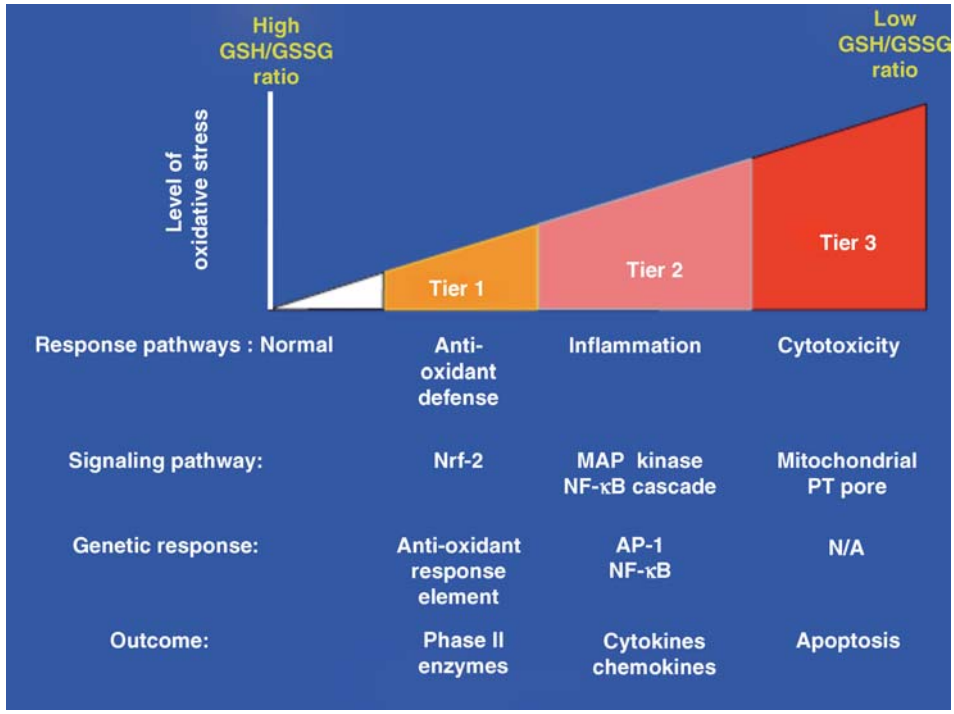

Figure 4. The hierarchical oxidative stress model. At a lower amount of oxidative stress (tier 1), phase II antioxidant enzymes are induced via transcriptional activation of the antioxidant response element by Nrf-2 to restore cellular redox homeostasis. At an intermediate amount of oxidative stress (tier 2), activation of the MAPK and NF-kB cascades induces pro-inflammatory responses. At a high amount of oxidative stress (tier 3), perturbation of the mitochondrial PT pore and disruption of electron transfer results in cellular apoptosis or necrosis. Reproduced from Nel *et al.* (2006) © American Association for the Advancement of Science.

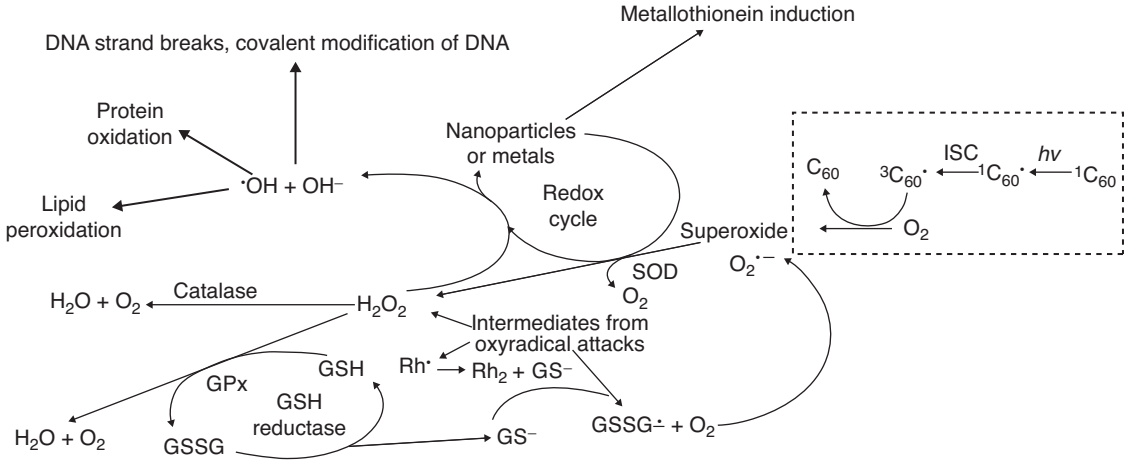

Figure 5. Nanomaterials have been shown to release oxyradicals [pictured here is the mechanism of C60 as determined by Yamakoshi et al. 2003, which can interact with the antioxidant defense system. Abbreviations: GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; ISC, intersystem crossing; R, any organic molecule; SOD, superoxide dismutase. In addition to fullerenes, metals such as cadmium, iron, or nickel quantum dots, or iron from SWNT manufacturing, could also act in Fenton-type reactions. Phase II biotransformation, ascorbic acid, vitamin E, beta carotene, and other interactions are not shown. Reproduced from Oberdorster, Oberdorster and Oberdorster (2005), National Institute of Environmental Health Sciences.

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causing intersystem crossing to create free electrons (Yamakoshi *et al*., 2003); (ii) metabolism of nanomaterials to create redox-active intermediates, especially if metabolism is via cytochrome P450s; (iii) inflammation responses *in vivo* that may cause oxyradical release by macrophages; and (iv) the presence of transition metals such as Fe, Cu, Cr, V, and Co, may induce ROS by redox-cycling reactions (Oberdorster, Oberdorster and Oberdorster, 2005; Nel *et al*., 2006; Adams, Lyon and Alvarez, 2006; Yamakoshi *et al*., 2003).

For the reason of biocompatibility, surface coatings, and covalent surface modifications were commonly applied to nanomaterials in drug/gene delivery, imaging, and diagnostic applications. In this case, chemicals from the coating or surface modifier may cause toxicity. Other nanomaterial properties such as shape, aggregation, and solubility may also affect the specific physicochemical properties, with the possibility of negating or amplifying the size effects.

Figure 3 also gives one possible toxicity mechanism induced by nanomaterials: toxic chemicals released from nanomaterials. Hull *et al*. (2009) studied the aquatic toxicity caused by low-purity, as-produced fullerenes (C60) and metallofullerene waste solids. Release of metal impurities from carbon nanomaterials influences aquatic toxicity. They applied SEM and ICP-MS to characterize the metals compostion of the solid test materials and of aqueous leachates. The results indicated that metals associated with the solid test materials were mobilized upon interaction with waters of circumneutral pH and reached concentrations sufficient to induce toxicity in both test species. The experiment of adding the chelator EDTA to eliminate the toxicity proved that the divalent transition metals were the toxicity source. Some products and byproducts of nanomanufacturing may contain relatively high levels of metal impurities. Their potential toxicity to human beings and the environment needs more attention.

Due to their nanometer size, the cytotoxicity of nanomaterials may be related to the cellular uptake of nanomaterials and the interaction of nanomaterials with individual cellular components, which leads to their malfunction and ultimately to cell death (Jia *et al*., 2005; Xia *et al*., 2006; Cho *et al*., 2007; Li *et al*., 2003; Long *et al*., 2006). The uptake of nanomaterials may be significantly affected by particle composition, particle size, shape, surface charge, temperature, and time.

Because some nanomaterials have the potential to be used as drug carriers or probes, their possible interaction with cellular components are very important to evaluate their toxicity. The binding of cellular proteins on the surface of nanomaterials such as SiO_2, TiO_2, and polystylene particles was studied by Morton Ehrenberg (Ehrenberg and McGrath, 2005). The Brownian motions of embedded polystylene particles and SiO_2 particles were used to infer the micromechanical properties of cells and protein network. They found that among all the proteins in cytoplasmic and cell-rich tissue extracts, nanoparticles bind most abundantly to cytoskeletal protein including actin and intermediate filaments, and the protein binding was strongly dependent on the surface chemistry of nanomaterials.

5 ANALYTICAL METHOD TO EVALUATE TOXICITY OF NANOMATERIALS

To clarify the toxicity and the toxic mechanism of nanomaterials, it is very important to use appropriate analytical techniques to determine the relationship of characteristics of nanomaterials and their toxicity. These relationships are very helpful to set up the safety regulations for these nanomaterials. With the tremendously increased manufacture of nanomaterials, this kind of requirement is urgent and large numbers of significant challenges must be overcome. From an analytical chemistry perspective, this emerging discipline requires both characterization of nanomaterials and bioanalytical chemistry expertise. The following part is to introduce some typically and widely used analytical techniques in nanotoxicology and gives critical vision into this field. The major analytical techniques used by scientists and researchers are listed and discussed in detail. The strengths and limitations of each technique are discussed. These techniques are categorized by the *in vitro* or *in vivo* applications including characterization of cellular uptake of nanomaterials, *in vitro* toxicity study and *in vivo* toxicity study.

5.1 *In-vitro* Uptake and Localization

In defining the mechanistic features of nanomaterials toxicity, a key question is the cellular uptake and subcellular localization of nanomaterials. The

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cytotoxicity may be caused by NMs uptaken and the subsequent interaction of NMs with individual cellular components, which leads to cell malfunction and ultimately cell death. In addition, the uptake of NMs may be significantly affected by particle composition, particle size, shape, surface charge, temperature, and exposure time. Quantification of the amount of uptaken NMs by cells currently may be resolved by several analytical techniques such as TEM, ICP-MS, Fluorescence, and so on. However, because of the limitations of these techniques, complementary techniques should be used for complete understanding of NMs uptake by cells.

5.1.1 Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) is a microscopy technique whereby a beam of electrons is transmitted through an ultra thin specimen, interacting with the specimen as it passes through. An image is formed from the interaction of the electrons transmitted through the specimen; the image is magnified and focused onto an imaging device, such as a fluorescent screen, on a layer of photographic film, or to be detected by a sensor such as a CCD camera. TEMs are capable of imaging at a significantly higher resolution than light microscopes, owing to the small de Broglie wavelength of electrons. This enables the instrument's user to examine fine detail – even as small as a single column of atoms, which is tens of thousands times smaller than the smallest resolvable object in a light microscope. It is a major analysis method in a range of scientific fields, in both physical and biological sciences.

TEM is a powerful tool to provide the *in-vitro* NM uptake and localization by imaging of NM locations within a cell. TEM has been widely employed in cellular uptake and localization determination of particles with wide range size (nm $\sim$ μm). Here are some examples for the cellular uptake mechanism and subcellular localization of NMs. One typical example is TiO_2 NMs, which is one of the most important nanomaterials in industrials and is produced in millions of tons per year. TiO_2 NMs can exist in several crystalline forms: anatase, rutile, and amorphorous. TiO_2 NMs have been widely used in manufacturing, as a photocatalyst to decontaminate air, soil, and water (Maness *et al*., 1999), and more recently in consumer products (e.g., toothpastes, sunscreens, cosmetics, and food products, etc.). In addition, TiO_2 NMs have been used in various polymeric materials as additives to enhance the material properties. The wide applications and its potential entry through dermal, ingestion, and inhalation routes suggest that nanosize TiO_2 may pose considerable exposure risk to humans and environment. Numerous *in vitro* studies reported that TiO_2 NMs cause oxidative stress-mediated toxicity in diverse cell types, including immortalized brain microglia (Long *et al*., 2006), epithelia (Gurr *et al*., 2005), skin fibroblast (Wamer, Yin and Wei, 1997). *In vivo* studies showed that TiO_2 NMs induced emphysema-like lung injury in mice (Warheit *et al*., 2005).

As we know, like other metal oxide NMs, TiO_2 NMs are neither coated nor modified nanomaterials, thus they are insoluble in water or medium and generally exist in aggregation forms. In addition, they have no fluorescence activity or radioactivity. So the microscopy techniques based on fluorescently or radioactively labeled NMs are not appropriate to study the cellular uptake of TiO_2 NMs. Fortunately, TiO_2 NMs can be easily visualized in cells by TEM due to the high electron density of their inorganic domains, which exhibits different contrasts, sizes, and shapes from those of cellular organelles. The study of Xia *et al*. (2006) indicated that 20–30 nm TiO_2 NMs were taken by RAW 264.7 cells but with no obvious mitochondrial damage (Figure 6).

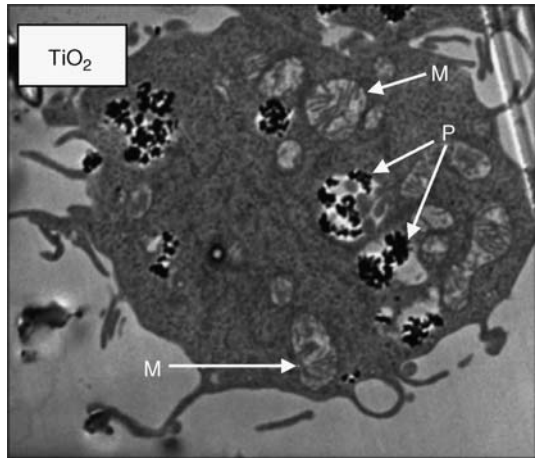

Figure 6. Use of transmission electron microscopy to determine the uptake and subcelluar localization of TiO_2 NMs. Cells were treated with 10 μg ml^{-1} NMs for 16 h. Lables: M=mitochondria, P = TiO_2 NMs. Reproduced from Xia *et al.* (2006) © American Chemical Society.

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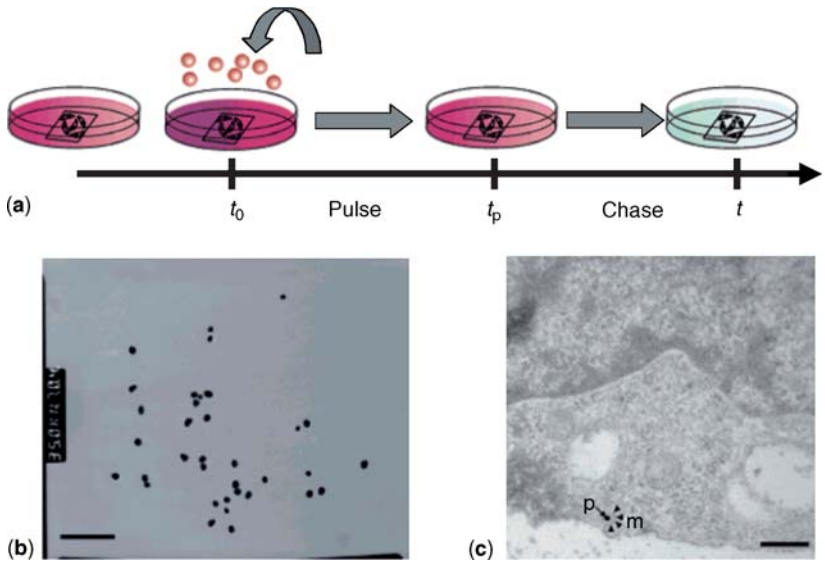

Figure 7. Schematics of the experiment: (a) gold nanoparticles were applied with the cell culture medium at time t_0 as a pulse of duration t_p, followed by incubation in medium without nanoparticles for different time t, at which the Raman experiments were carried out in PBS buffers; (b) transmission electron micrograph of gold nanoparticles immersed in cell culture medium (DMEM with 10% FCS). Scale bar: 500 nm; (c) transmission electron micrograph showing the endocytotic uptake of individual gold nanoparticles by an IRPT cell. The cell membrane (arrowheads, labeled m) encloses the particles (arrow, labeled p), thereby forming a vesicle in the cytoplasm. Scale bar: 500 nm. Reproduced from Kneipp *et al.* (2006) © American Chemical Society.

Kneipp *et al*. (2006) found that the individual gold NMs were cellularly taken up by an endocytosis process. During the endocytosis process, a part of the cellular membrane underwent invagination; thereby, enclosing a gold nanoparticle that was contained in the cell culture medium. Therefore, in the cellular interior, the particle was surrounded by a membrane vesicle. The TEM image displayed a snapshot of this enclosure process as the authors observed it in a cell of the epithelial cell line IRPT (Figure 7). Li *et al.* (2003) used TEM to study the cellular localization of CAP's (concentrated ambient particles) with different sizes (Figure 8). Coarse particles were seen in large cytoplasmic vacuoles in macrophages, while the ultrafine ones were seen to localize inside damaged mitochondria. The same mitochondrial effects were also observed in human bronchial epithelial cells treated with ultrafine particles.

Endocytosis processes were believed to be the most common uptake mechanism for cell to uptake macromolecules, particulate matters, and NMs. Endocytosis is a process whereby cells ingest a part of their cell membrane continually to form endocytic vesicles into which external objects are swallowed. Under its wide definition, endocytosis includes various uptake methods, including phagocytosis (cell eating), pinocytosis (cell drinking), clathrin-dependent endocytosis and caveolae-mediated endocytosis. All the endocytic processes are energy dependent; therefore, endocytosis processes are at least partially inhibited either at low temperature (usually 4 °C) or by cellular ATP depletion. Another feature of endocytic pathways is the formation of vesicles, which usually originate from the peripheral cell membrane, bud off from the membrane, and move towards the interior. Depending on the nature of the cell (phagocytic or non-phagocytic) as well as the nature of the NMs (size, surface characteristics, and presence of ligands on the surface), the uptake process may vary.

5.1.2 Fluorescence Spectroscopy

Fluorescence spectroscopy is a type of electromagnetic spectroscopy, which analyzes fluorescence from a sample. It involves using a beam of light, usually ultraviolet light, that excites the electrons in molecules of certain compounds and causes them to emit light of a lower energy, typically but not

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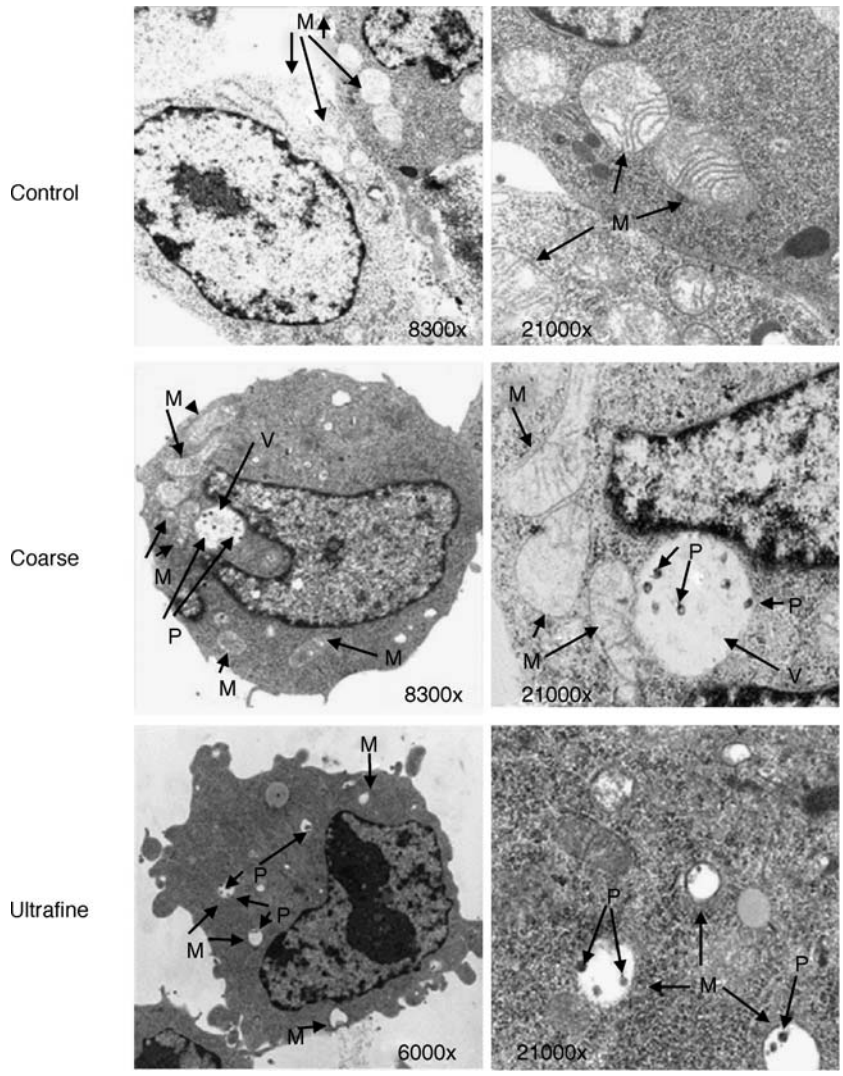

Figure 8. Electron microscopy (EM) showing select subcellular localization of ultrafine and coarse particles. Coarse (2.5–10 μm) and ultrafine (<0.15 μm). CAPs were collected in an aqueous medium in Claremont, CA, using the VACES. RAW264.7 cells were incubated with these particles for 16 h before fixation. EM was performed as previously described. While coarse CAPs are localized in large vacuoles and do not damage mitochondria, ultrafine particles often induce structural damage as demonstrated by the disappearance of cristae. In addition, ultrafine particles appear to lodge inside damaged mitochondria. M, mitochondria; P, particles; V, vacuoles. Reproduced from Sioutas *et al.* (2003) © National Institute of Environmental Health Sciences.

necessarily visible light. A complementary technique is absorption spectroscopy.

Fluorescence spectroscopy is useful in both the quantitative analysis of nanoparticle uptake and the qualitative analysis of nanoparticle subcellular localization. Using a flow cytometry technique is a typical approach for quantitative assessment of NM uptake. In addition, bulk fluorescence or confocal fluorescence fluoresecne are also used. Detection efficiency is strongly dependent on the fluorescence properties of the NMs.

Until now, the intense investigations of cellular uptake of NMs were mainly focused on drug/gene delivery, disease diagnosis, tumor cells tracking and recognition. In order to study cellular uptake of NMs *in vitro* or *in vivo*, the use of fluorescently

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or radioactively labeled NMs is the most common experimental approach reported in the literature. Different nano-sized carriers, such as biodegradable polymeric micelles (Zhang and Feng, 2006; Win and Feng, 2005), liposomes (Bochot *et al*., 2002), surface-modified NMs (Gupta and Gupta, 2005; Xing *et al*., 2005; Illum and Davis, 1984), and solid lipid NMs (Muller, Mader and Gohla, 2000), have been developed to achieve the goal. To improve efficiency of cellular drug delivery, one strategy is to modify or conjugate a drug with a ligand that can be efficiently taken up by target cells via receptor-mediated endocytosis (Sonvico *et al*., 2005; Yang *et al*., 2005). Fluorescent labeling makes cellular uptake of NMs readily detectable by fluorescence microscopy or confocal laser scanning microscopy (CLSM). The extent of particle uptake can then be determined by flow cytometry, fluorometry, or quantitative extraction of the markers from the cells (Win and Feng, 2005; Davda and Labhasetwar, 2002; Azarmi *et al*., 2006). While these labeling approaches facilitate fluorescence quantitation, the conjugated dyes or groups may significantly change the physical and chemical properties of NMs, and even their uptake mechanism and efficiency. So this effect needs to be considered and controlled carefully during experiment design and carryout.

Cellular uptake of quantum dots (QDs), which is applied in cellular probing and imaging, has also attracted considerable studies. QDs are fluorescent semiconductor nanocrystals with unique optical properties such as high quantum yield, high molar extinction coefficients, narrow emission spectra, size-depending emission, and high photostability. QDs are emerging as an attractive alternative to organic fluorophores as fluorescent, nonisotopic labels (Gao *et al*., 2004; Osaki *et al.*, 2004; Zhang, So and Rao, 2006). In general, all the QDs and nanosize drug carriers are water-soluble to improve the cellular uptake and prevent aggregation. Lovric *et al*. (2005) studied the subcellular localization and toxicity of cadmium telluride (CdTe) QDs. Their research indicated that localization of red and green cationic CdTe QDs in PC12 and N9 cells were significantly dependent on QD size. Confocal microscopy was used to observe the QD localization in the nuclear vs. cytoplasmic compartments. Red QDs (5.2 ± 0.1 nm) were only localized throughout the cytoplasm of N9 cells within 1 h; in contrast, green QDs (2.2 ± 0.1 nm) were distributed mainly in the nuclear compartment within the same time (Figure 9). In addition, authors assumed there are several other factors which may also affect the distribution of green and red QDs except the particle size, including aggregation forms, aggregation size, aggregation stability, and complex formed with cell proteins. In 2006, Ron Hardman (Hardman, 2006) published a review paper about the toxicology of quantum dots.

5.1.3 ICP-MS

Inductively coupled plasma mass spectrometry (ICP-MS) is a type of mass spectrometry that is highly sensitive and capable of the determination of a range of metals and several non-metals at concentration levels of parts per billion (ppb). It is based on coupling together inductively coupled plasma as a method of producing ions (ionization) with a mass spectrometer as a method of separating and detecting the ions. ICP-MS is also capable of monitoring isotopic speciation for the ions of choice.

Gojova *et al*. (2007) studied the inflammation in vascular endothelial cells induced by metal oxide nanoparticles (Fe_2O_3, Y_2O_3, and ZnO). They used ICP-MS to measure the delivery of the metal oxide nanoparticles to the cell surface and the cellular uptake of nanoparticles. After the human aortic endothelial cells (HAECs) were exposed to nanoparticles for 4 hs, the cells were washed and detached. The cell pellet was resuspended in buffer and treated with concentrated HNO_3. After heating 3 hs at 80 °C to dissolve cell contents, the solution was diluted and analyzed using ICP-MS. The results clearly demonstrated that the amount of metal uptake correlated directly to the nanoparticle concentration in cell culture medium (Figure 10).

Using ICP-MS to study and quantitate the cellular uptake of NMs was also reported by Yang *et al*. (2005). The disadvantage of using ICP-MS to study cellular uptake is that the method requires large amount of cells (millions) to obtain the detectable metal concentrations.

5.1.4 Other Advanced Techniques for Cellular Uptake Study

Except for those techniques described above, other advanced techniques have recently been used to investigate the cellular uptake and distribution of NMs. Alexander G. Tkachenko etc. (Tkachenko *et al*., 2004) applied video-enhanced color differential interference contrast (VEDIC) microscopy and

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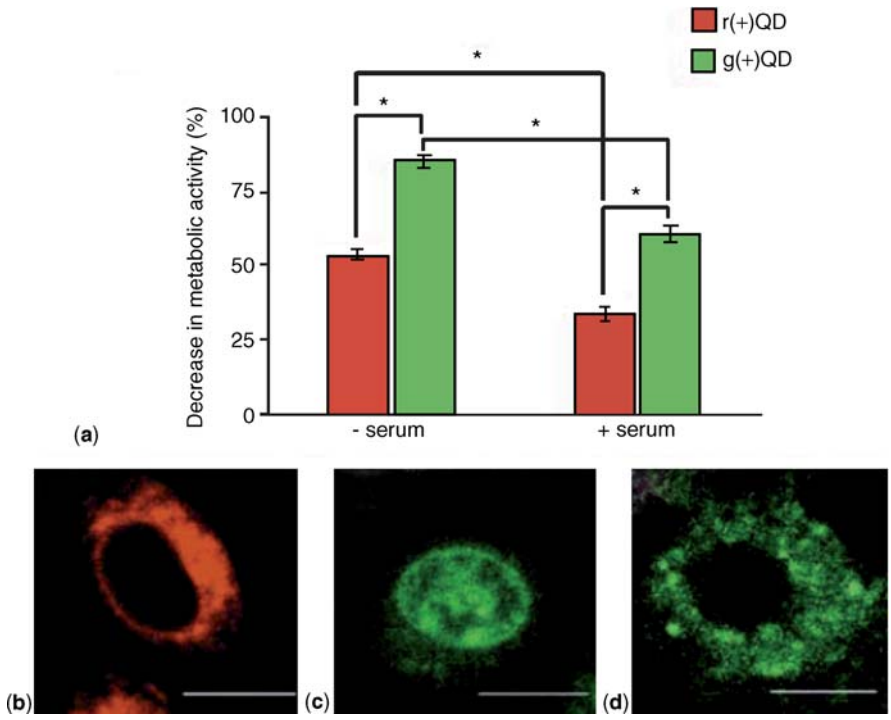

Figure 9. Differential toxicity and subcellular localization of red and green cationic QDs. N9 cells were exposed to red [r(+)QD, 7.5 μg ml^{-1}] and green [g(+)QD, 7.5 μg ml^{-1}] cationic QDs in serumfree and serum-containing media for 24 h. Ordinate represents decrease in metabolic activity relative to controls not treated with QDs. Columns Group means ± SEM for quadruplicate samples from three or four independent experiments. *$P < 0.05$. B Red cationic QDs (3.75 μg ml^{-1}, 1 h incubation) are distributed within the cytoplasmic compartment of N9 cells. C Green cationic QDs (1.25 μg ml^{-1}, 1 h incubation) were localized mostly in the nucleus of N9 cells. D Green QD-BSA complex (1 h incubation) reveals the fluorescent signal mainly in the cytosol. Scale bar 10 μm. Reproduced from Lovric *et al.* (2005) © Springer.

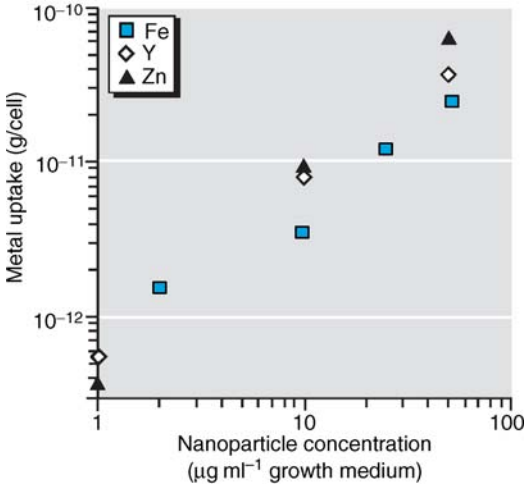

Figure 10. ICP-MS measurements of metal uptake by HAECs as a function of the metal oxide concentration in the extracellular solution for the three different types of nanoparticles. Reproduced from Gojova *et al.* (2007) © National Institute of Environmental Health Sciences.

TEM to study the ability of peptide-gold nanoparticle complexes to cross the cell membrane and reach the nucleus. Three kinds of cell lines were used: HeLa human cervical epithelium cells, 3T3/NIH murine fibroblastoma cells, and HepG2 human hepatocarcinoma cells. The results showed that particle uptake and nuclear translocation were strongly dependent on the surface-bound peptide sequence and cell types. Figure 11 demonstrates that the gold nanoparticles carrying peptide M2 gradually reached the cytoplasm and nucleus of HeLa cells after 1–3 h. Nanoparticles-peptide M3 complexes reached the nuclear membrane of HeLa cells after 2 h of incubation and after 3 h M3 nanoparticles were observed inside of the nucleus. M4 nanoparticles were also able to reach nucleus.

Transferrin-mediated gold nanoparticles were used as a prospective drug delivery vehicle for specific cellular uptake and targeted drug delivery (Yang *et al.*, 2005). With its high-resolution imaging

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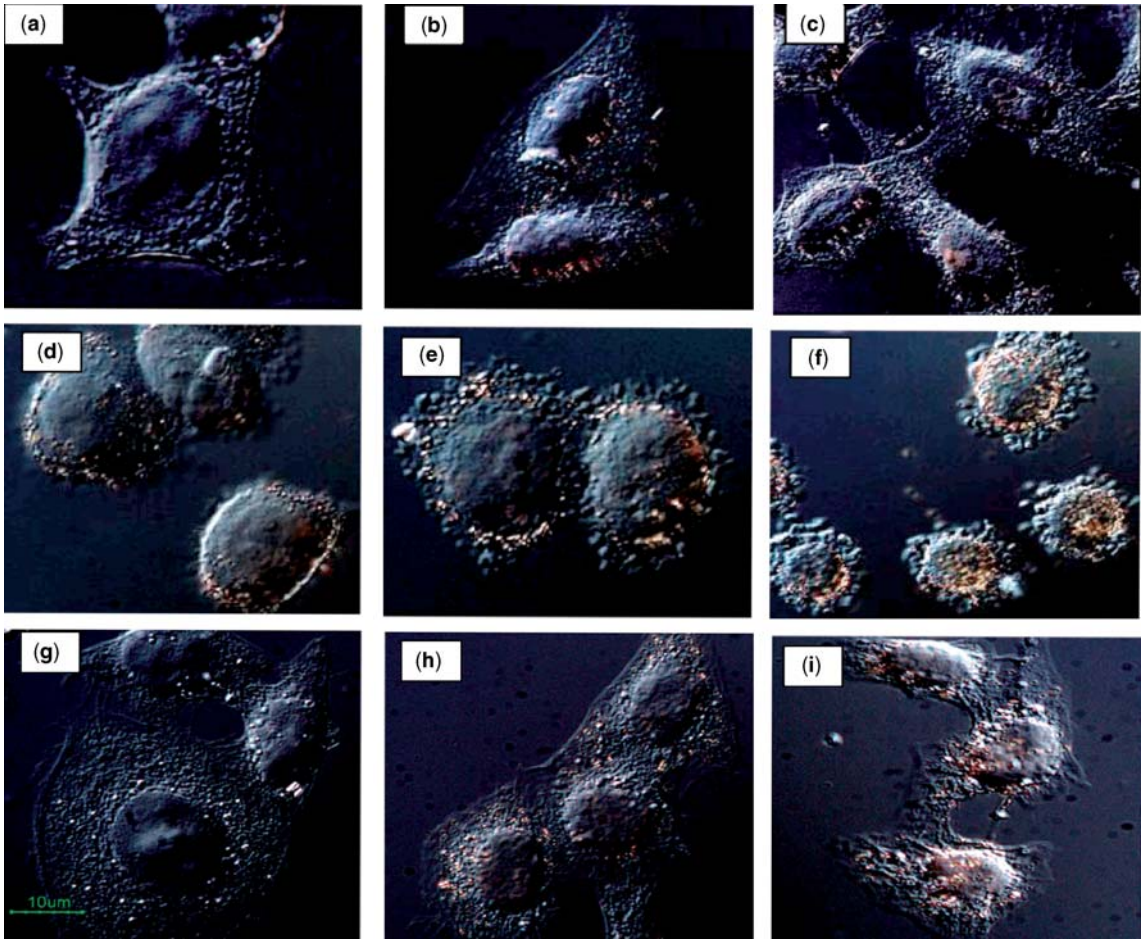

Figure 11. Incubation of HeLa cells with 20 nm diameter gold nanoparticles observed by VECDIC. Figure 4 (A-C) 0.5, 1.5, and 3 h incubation with nanoparticles carrying peptide M2; (D-F) 0.5, 1.5, and 3 h incubation with nanoparticles carrying peptide M3; (G-I) 0.5, 1.5, and 3 h incubation with nanoparticles carrying peptide M4. Reproduced from Tkachenko *et al.* (2004) © American Chemical Society.

capability with a nondestructive approach, Atomic Force Microscopy (AFM) was used in this study to visualize the cellular uptake of transferrin-mediated gold nanoparticles on the surfaces of live cells. The AFM images (Figure 12) vividly showed the endocytosis during the process of internalization. This uptake process was also confirmed by authors using confocal scanning imaging experiments.

Additionally, two-photon luminescence (TPL) microscopy was used to study the cellular uptake of gold nanorods coated with cetyltrimethylammonium bromide (CTAB). The NMs were internalized by KB cells through a nonspecific uptake mechanism and then formed permanent aggregations within the cell (Huff *et al*., 2007).

In addition, measurement of the radioactivity of radiolabeled NMs was also used to study the cellular uptake of NMs (Block *et al*., 2004).

5.2 *In-Vitro* Toxicity Assessment

In vitro toxicity assessment of NMs is the vitally important tool for toxicity evaluation, biomaterials testing and environmental materials exposure testing, despite the results of *in vitro* and *in vivo* sometimes lacking consistency and explanation. In addition, reliable and comparable conclusions require corroborating the results from many different assays. The most commonly used *in vitro*

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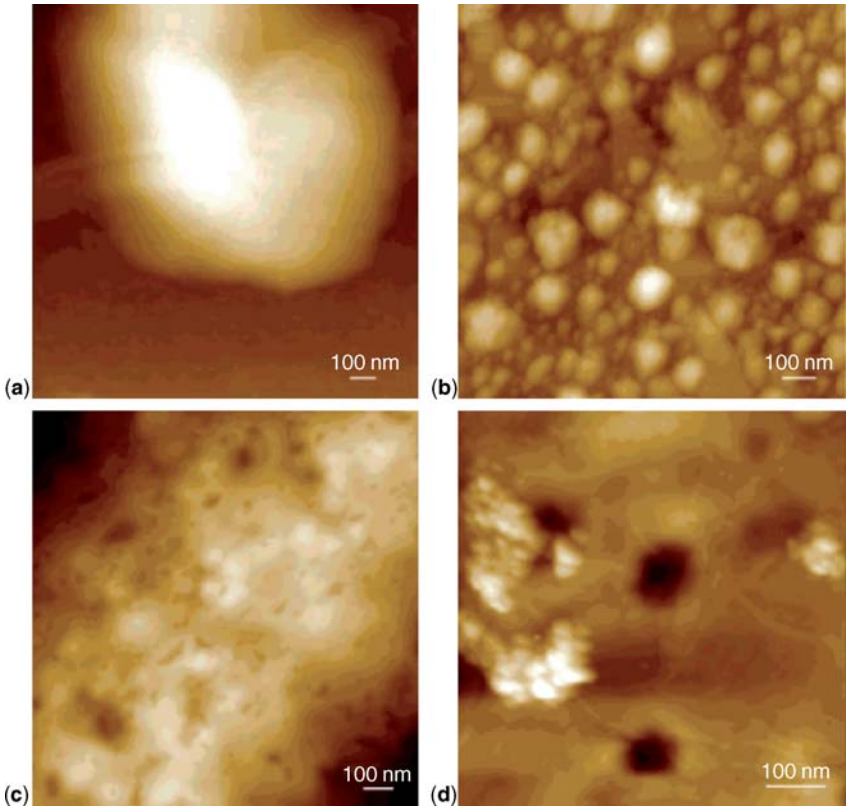

Figure 12. AFM images demonstrate Au-TF nanoparticle uptake by NPC cells: (a) A single NPC cell; (b) Au-TF nanoparticles on a NPC cell surface at the beginning of incubation; (c) NPC cell surface after incubation with Au-TF nanoparticles for 5 h; and (d) "zoom in" AFM image of cell surface after incubation with Au-TF for 5 h, showing nanoparticle uptake during the process of endocytosis. Reproduced from Yang *et al.* (2005) © American Chemical Society.

assessment techniques include the cell viability test and further mechanism studies. Viability based assays can be categorized into different proliferations: apoptosis or necrosis. The most important toxicity mechanisms of NMs are either oxidative stress or DNA damage. It should be noted that these assays may be closely related to each other, and comprehensive *in vitro* toxicity assessment and mechanism studies should rely on comprehensive assays. These techniques and their applications are introduced shortly below:

5.2.1 Cell Viability Assays

Cell viability assay provides a most important assessment method for toxicity of NMs. In general, the percentage of dead cells is directly proportional to the toxicity of NMs. Some widely used dye-based viability assays apply the differential inclusion, exclusion, or conversion of a dye, or a dye precursor which can only be enzymatically converted to detectable dye in living cells instead of dead cells. In this case, the toxicity can be evaluated by measuring the colorimetric or fluorescence signals. These methods include MTT, MTS, Alamar blue, Lactate Dehydrogenase (LDH), live/dead assay, neutral red, trypan blue, and so on.

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay measures the metabolic activity of viable cells; it is based on the reduction of the tetrazolium salt MTT by viable cells. The reaction produces a water-insoluble formazan salt, which must be solubilized. The procedure is very simple: first culture the cells in a 96-well microplate and then incubate them with MTT solution for approximately 4 h. During this incubation period, viable cells convert MTT to a water insoluble formazan dye. Dissolve the

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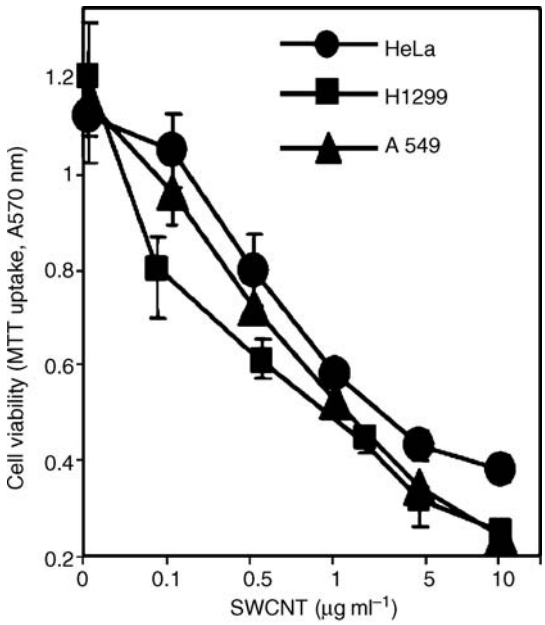

Figure 13. Effect of SWCNT particles on cell viability in different cells. HeLa, H1299, and A549 cells (5000/well in a 96-well plate) were incubated with different concentrations of SWCNT for 72 h. Cell viability was determined by MTT assay. Reproduced from Manna *et al.* (2005) © American Chemical Society.

formazan dye with the solubilization solution in the microplate and then quantitate the dye with an ELISA plate reader. The absorbance directly correlates with the cells numbers. For example, Manna *et al*. (2005) used MTT assay to assess the cytotoxicity of Single-Walled Carbon Nanotube (SWCNT) in different cells. Cell viability was determined after exposing the cells to various concentrations of SWCNT particles. The results clearly indicated that the cell viability was dose-dependent as low as 0.5 $\mu g\,ml^{-1}$ and the decrease of cell viability corresponded with increasing concentrations of SWCNT particles (Figure 13). However, the MTT assay may also have its disadvantage; for instance, reduction of MTT by cysteamine-coated quantum dots to formazan was observed by Khandare *et al*. (2006).

MTS assay is based on similar principles to the widely used MTT assay. The active component is a novel tetrazolium compound called MTS, which has a similar structure to MTT. In living cells, MTS is reduced to a colored formazan product. The amount of formazan product is directly proportional to the number of living cells; therefore, cell proliferation or death can be quantified by reading the plate at 490 nm. MTS is a kind of improved MTT assay with the advantage that the formazan product of MTS reduction is soluble in cell culture medium and does not require use of a Solubilization Solution. For example, Braydich-Stolle *et al*. (2005) studied the influence of soluble and nanoparticulate molybdenum species on germline stem cell mitochondrial function and viability (Figure 14).

Alamar blue is also called Resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide), which is a blue dye used mainly as an indicator used to assess proliferation and cell viability (Orosz *et al*., 2004). However, there is evidence, which indicates that high-density cultures or long incubation times may cause a secondary reduction of the Alamar blue dye to a non-fluorescent compound. Hence, all dye-based assays such as MTT, MTS, Alamar assay, and so on, require careful calibration to prevent potential interferences in the presence of NMs.

Lactate Dehydrogenase (LDH) is a cytoplasmic enzyme. Cytotoxicity of NMs may cause lipid peroxidation, which damages the cell membrane and disturbs the integrity of cellular membranes, leading to the leakage of LDH into the cell culture medium. Commercial LDH Kit is available in market. The procedure is mainly based on the method developed by Wacker, Ulmer and Vallee, 1956. Released LDH catalyzes the oxidation of lactate to pyruvate with simultaneous reduction of NAD+ to NADH. The rate of NAD+ reduction is measured as an increase in absorbance at 340 nm. The rate of NAD+ reduction is directly proportional to LDH activity in the cell medium. The LDH measurement in cell culture medium was also described in other studies using different methods (Sayes *et al*., 2005).

Live/dead assay is a quick and easy, two-color assay to assess cell viability/cytotoxicity. This assay identifies live vs. dead cells on the basis of membrane integrity and esterase activity by staining cells with Live/Dead reagent and measuring by fluorescence (Manna *et al*., 2005) (as shown in Figure 15).

Neutral Red uptake assay is another cell viability endpoint. Neutral Red (NR), a weak cationic dye, penetrates cellular membranes by non-diffusion and accumulates intercelluarly in lysosomes. This dye is endocytosed by only viable cells, dead cells do not (Huang, Khor and Lim, 2004).

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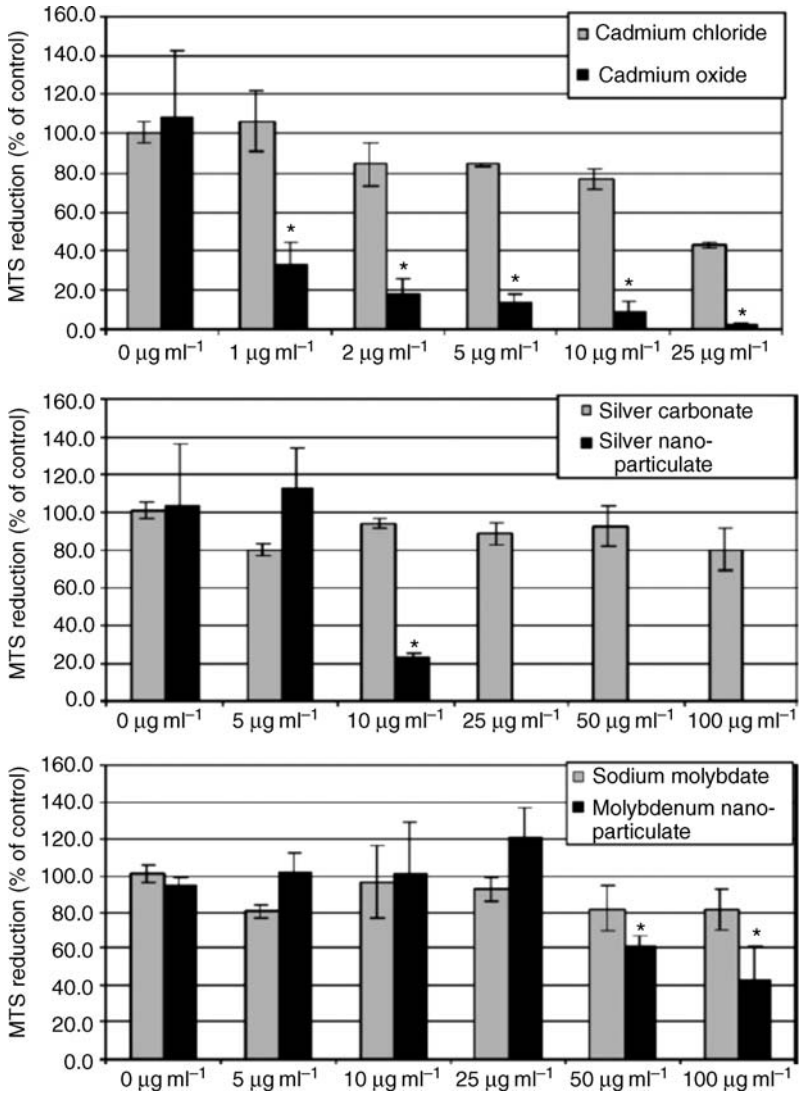

Figure 14. Influence of cadmium oxide and different types of nanoparticles on the metabolic activity of the C18–4 spermatogonial stem cells after 48 h incubation. At the end of the incubation period, mitochondrial function was determined by the MTS reduction assay as described in Materials and Methods. Each panel is a composite of two experiments, with $n \geq 3$ for each point. *Statistically significant difference compared with controls ($p < 0.05$). A. MTS reduction in presence of different concentrations of cadmium chloride and cadmium oxide. B. MTS reduction in presence of different concentrations of silver carbonate and silver nanoparticles (Ag – 15 nm). C. MTS reduction in presence of different concentrations of sodium molybdate and molybdenum nanoparticles (MoO_3 – 30 nm). Reproduced from Braydich-Stolle *et al.* (2005) © Oxford University Press.

Trypan blue exclusion test of cell viability is used to determine the number of viable cells. The principle is that live cells have intact cell membranes that exclude trypan blue dye, whereas dead cells don't. Therefore, the cells, which are blue are dead if observed by microscope (Gojova *et al*., 2007; Huang, Khor and Lim, 2004).

ATP assay has the main advantage of providing results quickly and requires fewer sample cells. The assay performs cell lysis and then the following reaction between the assay and ATP content of cells produces luminescence. The intensity of luminescence is then measured by a luminomeneter and can be translated into number of cells alive since the

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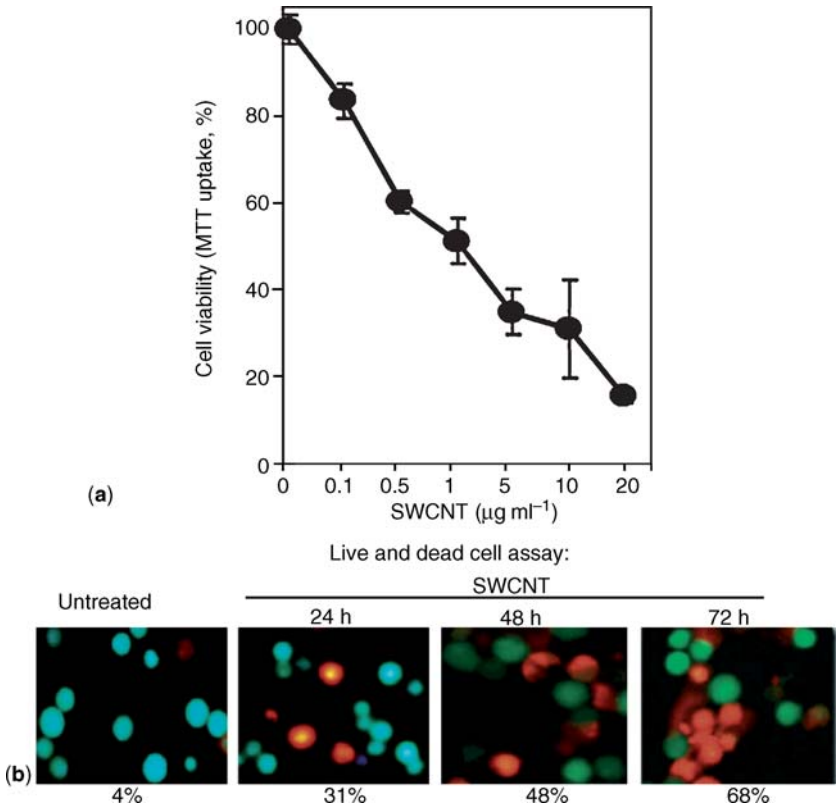

Figure 15. Effect of SWCNT particles on cell viability. HaCaT cells (5000/well in a 96-well plate) were incubated for 12 h and then treated with different concentrations of carbon nanotubes for 72 h. Cell viability was assayed by MTT dye uptake (A). Results are reported as percentage of cell viability and representatives from one of three independent experiments. (B) HaCaT cells were treated with 10 μg ml^{-1} SWCNT for different time intervals, and the dead cell (red color) numbers were counted. The percentage of dead cells is indicated below each photograph. Reproduced from Manna *et al.* (2005) © American Chemical Society.

luminescence intensity is proportional to the ATP content in the sample (Wilson *et al.*, 2002).

5.2.2 Oxidative Stress

As we describe in Section 4, oxidative stress is caused by an imbalance between the production of a reactive species and a biological protection system, which has the ability to eliminate the harmful reactive species. Oxidative stress has been found to be implicated in lots of pathogenesis of diseases such as aging, atherosclerosis, carcinogenesis, and acute and chronic inflammatory disorders (Kehrer and Lund, 1994). Reactive species are generally categorized as reactive oxygen species (ROS) such as superoxide (O_2^-), hydroxyl radical ($HO^\bullet$), peroxy radical ($ROO^\bullet$), and hydrogen peroxide (H_2O_2); or reactive nitrogen species (RNS) such as nitric oxide ($NO^\bullet$), peroxynitrite anion ($ONOO^-$), peroxynitrous acid (ONOOH), and nitrosoperoxycarbonate anion ($ONOOCO_2^-$). Over-production of ROS and RNS would overwhelm the protection system and the capacity of the reducing environment, therefore causing toxic effects within cells. Disturbances in this normal reducing environment can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including damage to proteins, lipids, and DNA. The extent of oxidative stress is dependent on the ratio of the amount of ROS/RNS to the cellular reduction capacity. Moderate oxidative stress may cause apoptosis and severe oxidative stress may cause cell death. The detailed mechanisms by which ROS are generated by NMs are still under investigation. However, there is evidence that crystalline silica can induce ROS, such as hydroxyl free radical,

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at the particle surface (Lenz *et al*., 1992; Carter and Driscoll, 2001; Porter *et al*., 2006; Dalal *et al*., 1990). Enhanced production of HO$^{\bullet}$ by adding H_2O_2 to trace iron(III)-containing crystalline silica suggests that a Fenton reaction occurred on the surface of silica particle (Fubini and Hubbard, 2003). The authors suspected that the relatively high metal impurities in crystalline silica may play a key role to yield HO$^{\bullet}$ radicals via the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^{\bullet}$). On the contrary, some studies indicated that SiO_2 NPs and Cerium oxide containing ultra low levels of metal impurities could induce HO$^{\bullet}$ radicals in the absence of trace metal (Lin *et al*., 2006; Lin *et al*., 2006). Fenoglio *et al*. (2001) also demonstrated that pure quartz and iron-deprived quartz dusts can generate HO$^{\bullet}$ in the absence of trace iron.

Table 7 lists the typical ROS species. The activities of these species are significantly different. Typically, OH$^{\bullet}$ free radical is the most active species. Other species such as superoxide and organic hydroperoxide are much less reactive.

The effects of ROS are controlled by an elaborate antioxidant defense system (Figure 16) (Li *et al*., 2003b). These antioxidants include proteins such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione reductase (GR), thiol-specific antioxidants, and low molecular weight scavengers such as GSH and α-tocopherol.

Table 7. Ttypical ROS species.

ROS/RNS	Description
$^{\bullet}O_2^-$, superoxide anion	One-electron reduction state of O_2, formed in many autoxidation reactions and by the electron transport chain. Relatively unactive.
H_2O_2, hydrogen peroxide	Two-electron reduction state, formed by dismutation of $^{\bullet}O_2^-$ or by direct reduction of O_2. Lipid soluble and thus able to diffuse across membranes.
$^{\bullet}$OH, hydroxyl radical	Three-electron reduction state, formed by Fenton reaction. The most reactive species to attach cellular components.
ROOH, organic hydroperoxide	Formed by radical reactions with cellular components such as lipids and and nucleobases.
RO$^{\bullet}$, alkoxy and ROO$^{\bullet}$, peroxy radicals	Produced in the presence of oxygen by radical addition to double bonds or hydrogen abstraction.

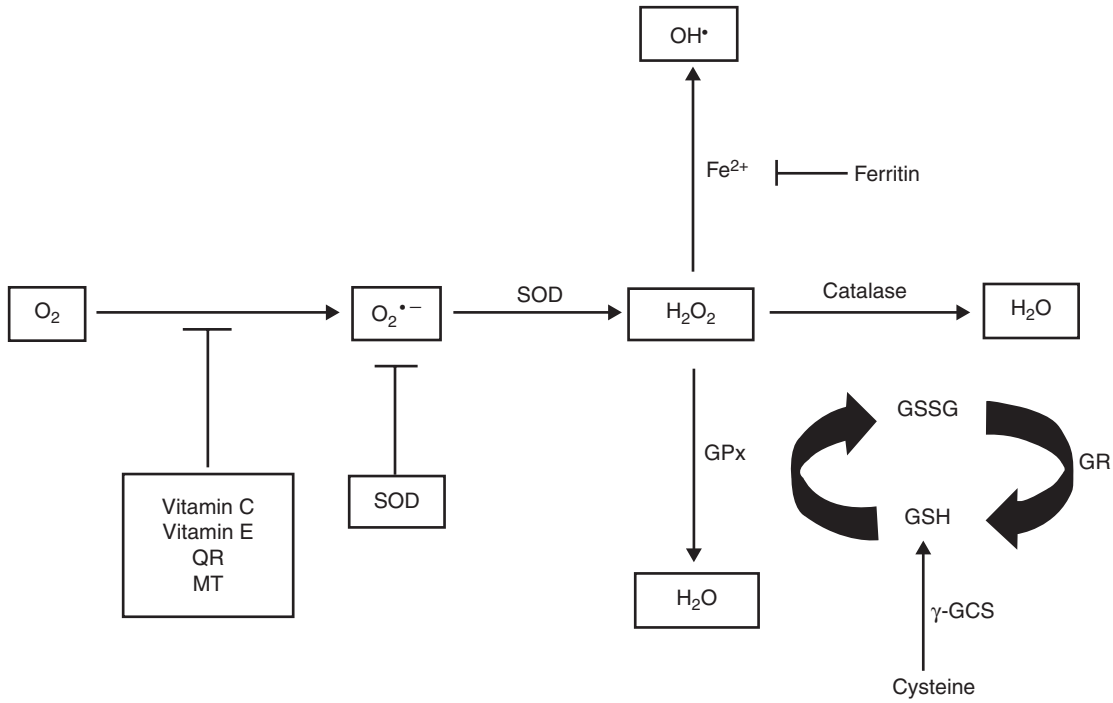

Figure 16. Cellular antioxidant defense mechanisms. The cellular antioxidant defense system consists of antioxidant enzymes, metal binding proteins, and low molecular weight antioxidants. Reproduced with from permission from Li *et al*. (2003b) © Elsevier.

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The functions of these antioxidants are to convert high active ROS to less active species and therefore protect the cellular compartments.

Intracellular ROS Measurement

The production of intracellular reactive oxygen species (ROS) was measured using 2′, 7′-dichlorofluorescin diacetate (DCFH-DA) (Block *et al*., 2004; Wang and Joseph, 1999). DCFH-DA passively enters the cell where it reacts with ROS to form the highly fluorescent compound dichlorofluorescein (DCF). Briefly, 10 mM DCFH-DA stock solution (in methanol) is diluted 500-fold in HBSS without serum or other additives to yield a 20 μM working solution. The cells are washed twice with HBSS and then incubated with DCFH-DA working solution for 1 h in a dark environment (37 °C incubator), following by treatment with particle suspension for a certain time. After exposure, the fluorescence is determined at 485 nm excitation and 520 nm emission using a microplate reader.

To prove the presence of free radicals in cell culture medium or solution induced by NMs, electron spin resonance (ESR) is a quick and reliable method. Basically, the sample is dissolved in DI-water and mixed with spin trap reagent DMPO (5,5-dimethyl-1-pyrroline N-oxide) for a certain period. Then the sample is excited with a solar simulator and the ESR signal is recorded. The presence of free radicals would show a significant increase in signal compared to the control. Figure 17 is a typical ESR figure indicating the free radical induced by CdSe/ZnS quantum dots.

The chemiluminescence of luminol can be used to quantitatively measure the presence of reactive species ($\mathbf{^{\cdot}O_2^-}$, $\mathbf{^{\cdot}OH}$) in aqueous nanoparticle solutions (Arnhold *et al*., 1991). In this method, hydrogen peroxide amplifies the chemiluminescence in the oxidation of luminol by sodium hypochlorite. Although this method can't quantify the reactive species, it is a simple and reliable method to evaluate the level of oxidative species.

Some studies measured the RS generations of NMs by determination of their photocatalytic activity. The samples were illuminated by UV light and their capability to induce RS was measured by the degradation of an azo dye. The degradation rate correlated to the rate constant for RS generation in aqueous solution (Sayes *et al*., 2006) (Figure 18).

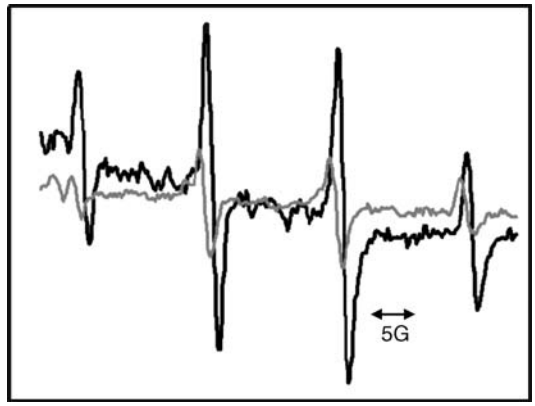

Figure 17. ESR spectrum of water-soluble CdSe/ZnS quantum dots and a spin trap (DMPO) confirming the presence of free radicals in solution. The dark line spectrum represents an illuminated sample; the grey line spectrum represents a sample in the dark. Reproduced from Green and Howmanl (2005) © Royal Society of Chemistry.

GSH Measurement

As one of the most important biomarkers of oxidative stress, GSH (γ-Glu-Cys-Gly) exists abundantly and is widely distributed in living cells, acting as an intracellular antioxidant that is involved in the detoxification of xenobiotics and scavenges free radicals, thereby protecting organisms from the

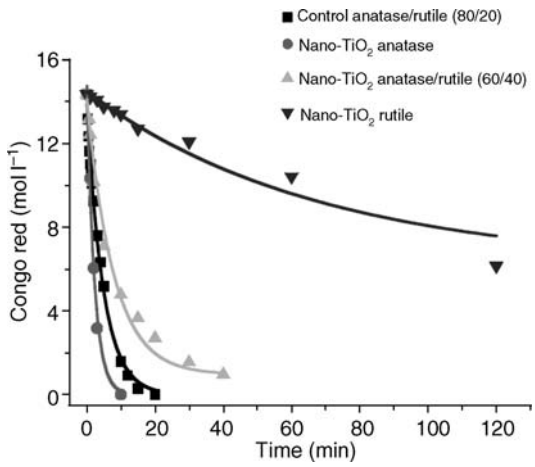

Figure 18. Photodegradation of aqueous Congo red (10 mg l^{-1}, 14 μmol l^{-1}) in the presence of various nano-TiO_2 catalysts (1 g l^{-1}). Based on this plot, the per gram efficiencies of the TiO_2 powders increased in the order nano-TiO_2 rutile particles < nano-TiO_2 anatase/rutile particles < control anatase/rutile < nano-TiO_2 anatase particles. Reproduced from Sayes *et al.* (2006) © Oxford University Press.

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oxidative damage of DNA and lipids. Two GSH molecules yield one GSSG molecules during oxidative stress. Therefore, quantitative measurement of the ratio of GSH and its disulfide oxidative product GSSG provides a very reliable assessment of oxidative stress (Winters *et al*., 1995). The determination methods for the reduced (GSH) and oxidized (GSSG) include using ion-pairing reverse-phase HPLC and a coulometric electrochemical detector (Fernandez-Urrusuno *et al*., 1997) or spectrometric method (Sayes *et al*., 2005), and so on.

MDA Measurement

Malondialdehyde (MDA) is a typical marker for measuring the degree of lipid peroxidation because MDA is the end product from peroxidation of polyunsaturated fatty acids when cells are exposed to NMs. MDA levels increase during oxidative stress due to free radical attacks on cell membrane lipids. The cellular concentration of malondialdehyde (MDA) may be determined by HPLC method (Draper *et al*., 1993; Kumari *et al*., 1989) or by spectrometric method (Sayes *et al*., 2005).

α-Tocopherol Measurement

α-tocopherol is also a kind of antioxidant, and its presence in a cell membrane has been proven to represent the major defense system against peroxidation of membrane lipids, which are highly susceptible to peroxidative degradation. One typical method to extract cellular α-tocopherol was described by Lang, Gohil and Packer (1986).

Other Oxidative Stress Biomarker

Other oxidative stress biomarkers include superoxide dismutase (SOD), glutathione peroxidase (GPx), and Catalase (CT), and so on (Fernandez-Urrusuno *et al*., 1997; Wang *et al*., 2005). SOD is a class of enzymes that catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. Therefore, SOD is an important antioxidant defense in cellular system. GPx is the general name of an enzyme family with peroxidase activity whose main biological role is to protect the organism from oxidative damage. The biochemical function of GPx is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water. Catalase is a common enzyme which catalyzes the decomposition of hydrogen peroxide to water and oxygen.

5.2.3 DNA Damage Assay

If NMs are able to enter into the body via inhalation, dermal routes, or oral uptake, NMs may penetrate the cell membranes and subsequently enter into different cellular compartments such as mitochondria, nuclei, and so on, via a number of mechanisms. Within the cellular compartments, NMs can cause DNA damage via direct and indirect mechanisms. If NMs have direct interaction with the DNA molecules, they may cause physical DNA damage. There are numerous studies indicating such physical DNA damage can be induced by NMs like TiO_2, Silica, Silver, and quantum dots. Alternatively, NMs may cause DNA damage via indirect mechanisms where the NMs have no physical interaction with the genetic molecules; instead, they induce some cellular responses such as oxidative stress and inflammation, which cause the subsequent DNA damage.

Oxidative stress elevates the cellular ROS. The highly reactive ROS can induce DNA damage by DNA breakage, base modification (e.g., formation of 8-hydroxydeoxyguanosine adducts) and DNA cross-links. Oxidative DNA damage induced by NMs has been shown by many studies as measured by the comet assay and micronucleus assay. Inflammation is a response of a tissue to injury, often injury caused by invading parasites. During inflammation, large amounts of factors such as cytokines (such as interleukins-1, IL-1) and tumor necrosis factors – alpha (TNF-α), RNS and ROS are quickly released. All the cells involved in inflammation have receptors for TNF-α, and are activated by it to synthesize more on their own. IL-1 is one of the first cytokines ever described, which causes fever, controls lymphocytes, increases the amount of bone marrow, and so on. The release of these factors quickly amplifies the response and subsequently helps promote healing. However, these factors can promote DNA damage in the form of DNA fragmentation, mutation, and formation of DNA adducts like 8-hydroxydeoxyguanosine (8-OHdG). In addition, these factors may also inhibit DNA repair and induce aberrant gene expression. To date, there are many studies demonstrating that nanomaterials can induce significant elevated inflammatory responses. Studies have shown that Carbon nanostructure and particulate air pollution (PM10) induce TNF-alpha and oxidative stress *in vivo* and *in vitro* (Manna *et al*., 2005; Li *et al*., 1996). Sayes *et al*. (2006) used human IL-8 enzyme immunometric assay kit

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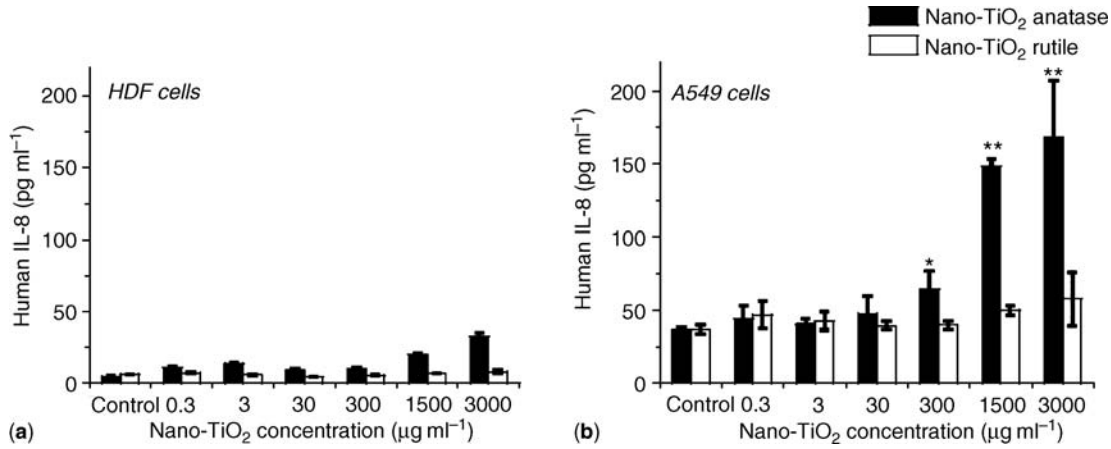

Figure 19. The production of IL-8 in (A) HDF and (B) A549 cells after 48 h exposure to nanoscale TiO2 anatase and rutile phases. Results are combined from three independent exposures. Groups significantly differ from the control group. Reproduced from Sayes *et al*. (2006) © Oxford University Press.

to evaluate the production of the inflammation mediator IL-8 induced by varying concentrations of nano-TiO_2 anatase and rutile particles. The results indicate that nano-TiO2 anatase particles significantly induce the production of IL-8 in both HDF and A549 cells (Figure 19).

To determine the DNA damage, many assays have been developed and applied to study the genotoxicity of NMs, including Comet Assay, Ames test, Chromosome aberration test, Cytokinesis-blocked micronucleus assay, HPRT forward mutation assay, g-H2AX staining, and 8-hydroxydeoxyguanosine DNA adducts.

Comet Assay

Comet Assay is also known as The Single Cell Gel Electrophoresis assay (SCGE). Comet Assay is a quick, universal, sensitive technique for the measurement of DNA single- and double-strand breaks at the individual cell level. Since it was first described by Singh *et al*. (1988), this assay has been used widely as a standard method in toxicity study of NMs. Firstly, the individual cells are suspended in a low-melting-point agarose. Then, the cells are lysed in neutral or alkaline (pH > 13) conditions and the DNA molecules are electrophoresed. During the electrophoresis, intact DNA molecules migrate slower than DNA fragments, resulting in a comet shape. After DNA molecules are stained with common ethidium bromide or other dyes, the fluorescence intensity of the comet tail, which is proportional to the extent of DNA damage, is analyzed. Lin *et al*. (2009) studied the Oxidative DNA damage caused by ZnO particles with micro- sized and nano- sized particles. The data indicated that significant DNA damage was induced by 10–14 $\mu g\ ml^{-1}$ of ZnO suspension after 24 h incubation with human lung epithelial cells (Figure 20).

Ames Test

Ames test is to assess the mutagenic potential of chemical reagents. The chemical might be regarded as a carcinogen if the Ames test result is positive. This method was first described by Ames *et al*. (1972). The test uses several strains of the bacterium Salmonella typhimurium, which carries different mutations in genes involved in histidine synthesis, so they need histidine to grow. The variable being tested is the mutagen's ability to cause a reversion to growth on a histidine-free medium. The bacteria are spread on an agar plate with a small amount of histidine or even without histidine. Only bacteria that have undergone reverse mutations resulting in the histidine synthesis genes will gain the required histidine and survive and then grow. The plate is generally incubated for 48 hs. The measured numbers of colonies would be proportional to the mutagenicity of the potential carcinogen.

Because Salmonella is a prokaryote instead of a eukaryote, the mutagenicity of a chemical reagent may not be applicable for eukaryotic entites like humans. An improved assay using eukaryotic cells has been developed. Another disadvantage of Ames Test is that under some conditions, false

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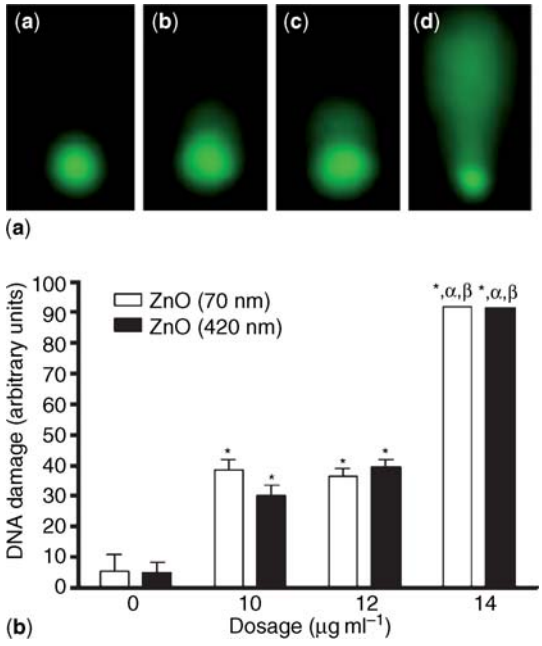

Figure 20. (a) The representative fluorescence images of nuclei treated in the comet assay after 24 h exposure to (a) 0 μg ml^{-1}, (b) 10 μg ml^{-1}, (c) 12 μg ml^{-1}, or (d) 14 μg ml^{-1} of 70 nm ZnO nanoparticles. (b) DNA damage was observed at all dosages. At the same dosage, there was no significant difference in DNA damage between 70 and 420 nm ZnO particles. Values are mean ± SD from three independent experiments. Reproduced from Lin *et al.* (2009) © Springer.

positive results may occur and require additional attention.

Chromosome Aberration Test

This assay includes numerical aberration and structural aberration. Numerical aberration means an abnormal number of chromosomes, which generally occurs when an individual is missing either a chromosome from a pair or has more than two chromosomes of a pair. Structural aberration can occur in several forms: deletions, duplications, translocations, inversions, and isochromosomes.

This test can be performed *in vitro* or *in vivo*. In the *in vitro* test mode, cultured mammalian cells are treated with the testing chemicals for a period of time, then the cells are exposed to a chemical that stops the cell cycle just before the replicated chromosomes are separated into two daughter nuclei. Next step is to stain the chromosome with the dye Giemsa and analyze if the aberration occurs by using microscopy. In the *in vivo* mode, generally, rodents are selected as the targets and are treated with the chemical. Similar to the *in vitro* mode, after the metaphase-arresting period, the bone marrow cells are harvested, stained, and analyzed with microscopy. The assay is a popular method for evaluation of potential carcinogens. However, this assay requires tedious preparation and a skilled person.

Cytokinesis-Blocked Micronucleus (CBMN) Assay

The CBMN assay in human lymphocytes is one of the most commonly used methods for measuring DNA damage. This is an easy, rapid, and sensitive method. CBMN assay measures micronuclei instead of chromosome aberrations because it has several advantages such as speed, ease, reliability, and no metaphase cells are required. Cells that have undergone cell division in the existence of a chemical compound can be easily identified using cytochalasin B. The purpose of cytochalasin B is to block the cell cycle at cytokinesis and make the cell cycle in binucleated cells. If the chemical compound induces DNA fragmentation or loss, then the damaged DNA lags behind during DNA segregation and is not included in either of the resulting daughter nuclei. Instead, they are enclosed within a micronucleus and their frequency in binucleated cells indicates the genotoxicity of the chemical compound (Figure 21). A detailed discussion of this technique and its applications have been described in several publications (Fenech, 1993; Fenech, 1993; El-Zein *et al*., 2006).

HPRT Forward Mutation Assay

The HPRT assay is an *in-vitro* mammalian cell gene mutation test, which is used to evaluate the potential of a chemical, formulation or extract to induce mutations at the HPRT locus of CHO cells. This test system is very suitable for evaluating initially the genotoxicity of a chemical compound (DeMarini *et al*., 1989; Albertini, 2001).

V79 Chinese hamster cells have one functional copy of the gene, which codes for the HPRT enzyme. HPRT enzyme activity is crucial for DNA synthesis. The nucleoside analog named 6-thioguanine (6-TG) is used for treatment. Cells without a mutation are poisoned by 6-TG, while only mutant cells can survive and form colonies. The cells that form colonies are assumed to be mutant cells, which are the result of a spontaneous mutation or an induced mutation by a chemical compound.

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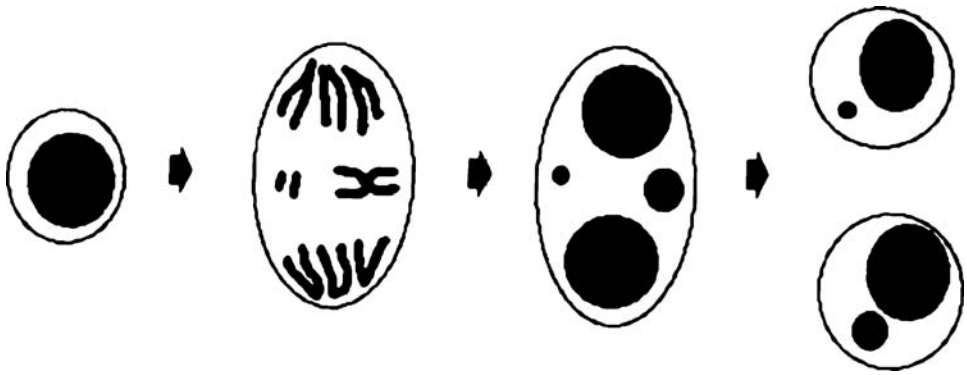

Figure 21. Micronucleus in a dividing nucleated cell. Micronuclei originate from either lagging chromosomes or chromosome fragments at anaphase. Micronuclei are readily identified because they are morphologically identical to but smaller than the main nuclei. A nondividing cell is unable to express its chromosome damage as micronuclei. Cells that have completed one nuclear division can be accumulated and identified as binucleate cells by adding cytochalsin-B, a cytokinesis blocking agent (3,4). Micronuclei are then scored in binucleated cells only. Reproduced from Fenech *et al.* (1993), National Institute of Environmental Health Sciences.

Cells deficient in HPRT are selected by resistance to 6-thioguanine (TG). These cells are exposed to the test substance, both with and without metabolic activation, for 2–4 h or 24–72 h and subcultured to determine cytotoxicity and to allow phenotypic expression prior to mutant selection. Cytotoxicity is determined by measuring the relative cloning efficiency (survival) of the cultures after the treatment period. The treated cultures are maintained in growth medium for three days, characteristic of each selected locus and type, to allow near optimal phenotypic expression of induced mutations. Mutant frequency is determined by seeding known numbers of cells in medium containing the selective agent to detect mutant cells, and in medium without selective agent to determine the cloning efficiency (viability). After a suitable incubation time (ca. 10 days), colonies are stained and counted. The mutant frequency is derived from the number of mutant colonies in selective medium and the number of colonies in non-selective medium.

A result is classified as positive if there is a concentration-related or reproducible increase in mutant frequency observed. Positive results for the HPRT-mutation assay indicate that the test substance induces gene mutations in the cultured cells used. A positive and reproducible concentration-response is most meaningful. There is no requirement for verification of a clear positive response. Equivocal results are clarified by further testing using modified experimental conditions. Negative results need to be confirmed on a case-by-case basis.

8-Hydroxydeoxyguanosine DNA Adducts

Active ROS generated during oxidative stress may attack DNA molecules and generate 8-hydroxydeoxyguanosine (8-OHdG, known as a DNA adduct) due to the modification of the guanine base. Therefore, measurement of this DNA adduct can be used as an important marker for oxidative DNA damage (Loft *et al*., 1992). Figure 22 showed the significantly elevated level of 8-OH-dG after being exposed to C_{60} fullerene (Sera, Tokiwa and Miyata, 1996). So far, approaches developed for direct measurement of 8-OhdG have been developed. The most sensitive and widely used methods are HLPC method and Mass Spectrometry method (Sera, Tokiwa and Miyata, 1996). Other methods like commercial ELISA kit, DNA dot blots, capillary electrophoresis with electrochemical detector (Weiss and Lunte, 2000), or immunocytochemistry staining (Shi *et al*., 2003) have also been reported. Figure 23 shows representative pictures of immunocytochemistry staining in which coarse and fine Particulate Matter induced similar but significant 8-OHdG compared with the control. A unique integrated microfluidic device for 8-OH-dG measure was described by (Dawoud, Kawaguchi and Jankowiak, 2007).

As a very useful marker for oxidative DNA damage, 8-OHdG was measured in numerous toxicity study of NMs such as Titanium Oxide NMs (Bhattacharya *et al*., 2009; Trouiller *et al*., 2009), air pollution particles (Prahalad *et al*., 2001), and fine particulate matter (Shi *et al*., 2003), and so on.

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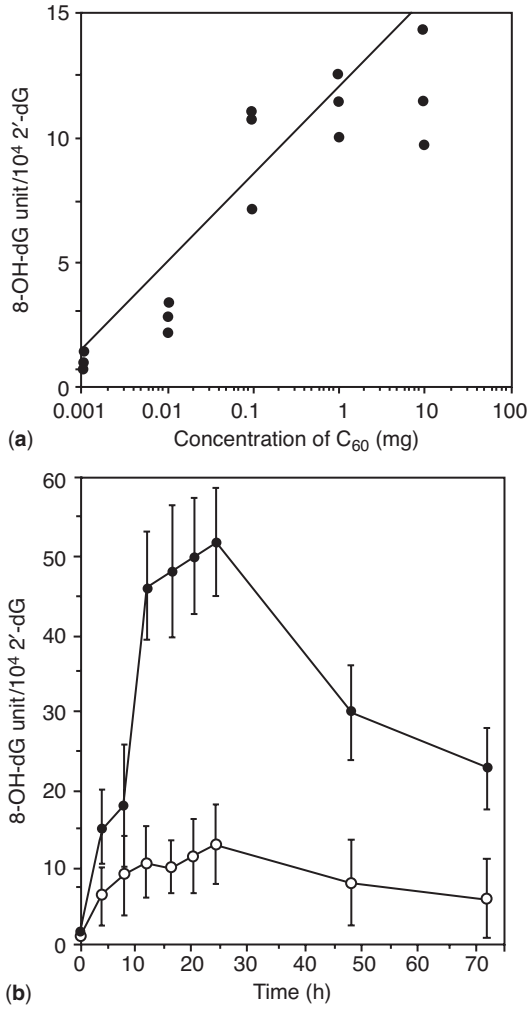

Figure 22. Levels of 8-OH-dG formed in 2′-dG treated with C_{60}. (a) Dose-response curve of the formation of 8-OH-dG in mixtures of 0.01, 0.1, 1.0 or 10 mg of C_{60} and 2′-dG (500 μg ml^{-1}) exposed to visible light for 16 h. Data are the results in triplicate. Regression line, $y = \log 1_{10} x^{3.47} + 11.9$ ($r^2 = 0.918$). (b) Time course of treatment of C_{60} (0.1 mg) and 2′-dG (500 μg ml^{-1}) in the presence (·) and absence (O) of microsome fraction. Reproduced from Sera, Tokiwa and Miyata (1996) © Oxford University Press.

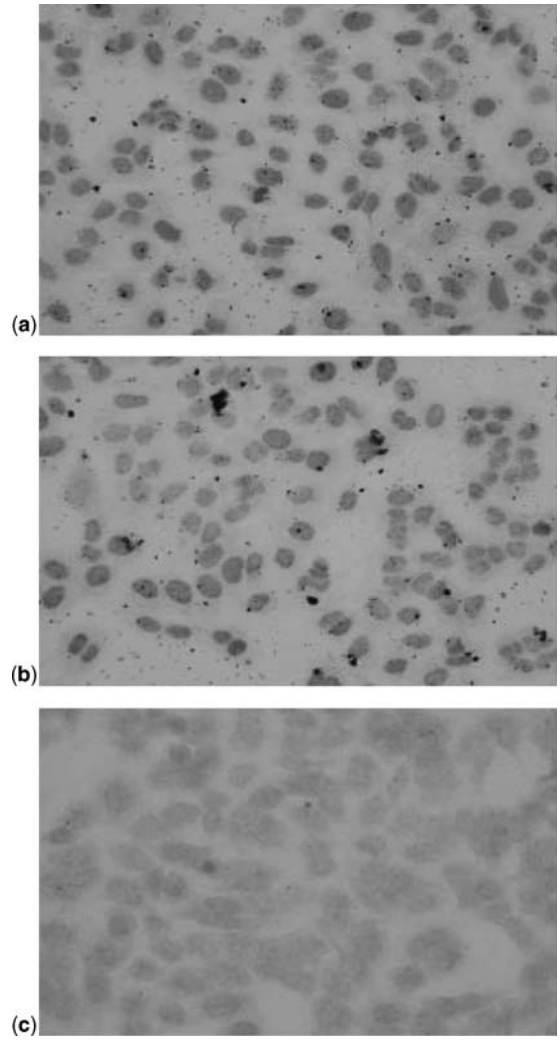

Figure 23. Induction of 8-OHdG by fine and coarse fractions of PM in A549 human lung epithelial cells. Cells were treated with 50 μg/ml PM suspension for two hours, and 8-OHdG was determined by immunohistochemical staining. (a) fine PM; (b) coarse PM; (c) control. Pictures were taken at 400× magnification. Reproduced from Shi *et al.* (2003) © BMJ Group.

5.2.4 Gene Expression

Gene expression is the process by which information from a gene is used in the synthesis of a functional gene product: mostly protein. The expression of a wide variety of genes has been shown to be sensitive to ROS at the transcriptional level. Among the genes known to be activated by oxidative stress are those for MAP kinase signaling, DNA damage, cAMP/Ca^{2+} signaling, NF-kB signaling, PI3-AKT signaling, and apoptosis (Foncea *et al.*, 2000; Kharasch *et al.*, 2006). NF-kB is a transcriptional regulator that controls expression of a wide variety of cellular and viral genes. These genes include many proinflammatory molecules such as cytokines IL2, IL6, IL8, TNF, cell adhesion molecules such as ICAM-1 and E-selectin, and so on. The gene expression is dependent on cell lines and NF-kB plays a major role in regulation

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of these genes and contributes significant control over inflammation.

In addition, oxidative stress activates specific signaling pathways such as mitogen activated protein kinase (MAPK). Treatment cells with NMs may induce profound gene expression changes. For example, Ding *et al.* (2005) used whole genome expression array analysis to study the gene expression change induced by multiwall carbon nano-onions (MWCNO) and multiwall carbon nanotubes (MWCNT) on human skin fibroblast cells. The data indicated that the differences in gene expression profiles were highly related to these NMs and their dosage levels. Treatment of the cells with a higher dose of carbon particles caused more gene expression changes than the lower dose treatment. However, the responses were not completely dose dependent. Moreover, MWCNO and MWCNT exposure induced gene expression involved in cellular activities such as cellular transport, metabolism, cell cycle regulation, and stress response. Figure 24 demonstrates the statistically significant gene expression changes in human skin fibroblasts induced by carbon NMs and the Table 8 gives the most significantly changed gene categories after treatment HSF42 cells with carbon NMs.

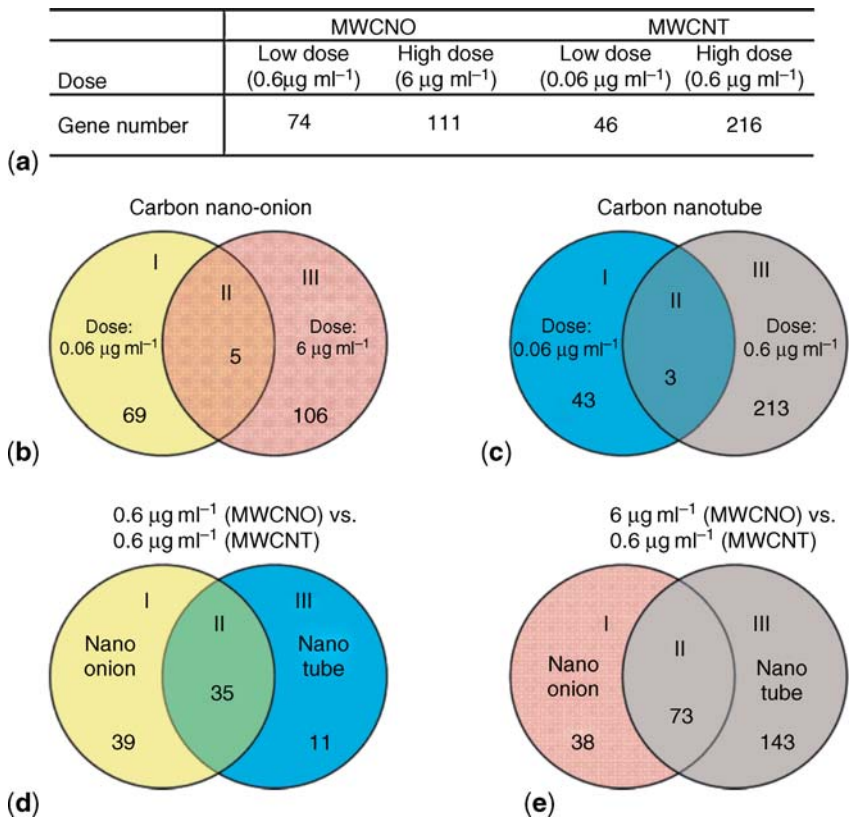

Figure 24. (a) Numbers of genes whose expression levels changed after treatment with carbon nanomaterials at cytotoxic doses. (b-e) Venn diagrams comparing numbers of genes that showed expression changes. Each Venn diagram is divided into three areas and labeled as I, II, and III. Area II is the overlapping area of two circles, representing commonly changed genes in both conditions. Area I and III represent genes that changed only in the condition specified in the circle. Bioconductor software was used to perform significance analysis to determine the difference between expression levels in treated sample, and the control sample possesses statistical significance. The empirical Bayesian model was used with Bonferroni's multitest correction. The cutoff of *p*-values produced through the analysis was determined by at least 10 times less than the *p*-value of the smallest *p*-value of control probe sets on the chip. (b) Comparing different doses for the nano-onions. (c) Comparing different doses for the nanotubes. (d) Comparing different particles at low doses (0.6 $\mu g\,ml^{-1}$ for MWCNO and 0.06 $\mu g\,ml^{-1}$ for MWCNT). (e) Comparing different particles at high doses (6 $\mu g\,ml^{-1}$ for MWCNO and 0.6 $\mu g\,ml^{-1}$ for MWCNT). Reproduced from Ding *et al.* (2005) © American Chemical Society.

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Table 8. Most significantly changed gene categories[a] after treating HSF42 cells with carbon nanomaterials at cytotoxic doses. Reproduced from Ding *et al.* (2005) © American Chemical Society.

	Gene category	*p* value	% underexpres sed	Overexpressed
Carbon nano-onion	Golgi vesicle transport	0.0000	8.51	2.13
0.6 μg ml^{-1}	membrane fusion	0.0002	15.79	0.00
	secretory pathway	0.0003	4.35	1.09
	protein ubiquitination	0.0139	0.68	2.05
	intracellular transport	0.0166	1.23	0.62
	cell growth and/or maintenance	0.0201	0.66	0.27
	fatty acid biosynthesis	0.0208	5.71	0.00
	protein metabolism	0.0323	0.71	0.29
	ubiquitin cycle	0.0342	0.70	1.06
	Gl/S transition of mitotic cell cycle	0.0361	4.26	0.00
Carbon nanotube	Golgi vesicle transport	0.0007	4.26	2.13
0.06 μg ml^{-1}	protein metabolism	0.0020	0.65	0.18
	secretory pathway	0.0049	2.17	1.09
	fatty acid biosynthesis	0.0076	5.71	0.00
	Gl/S transition of mitotic cell cycle	0.0135	4.26	0.00
	protein ubiquitination	0.0174	0.68	1.37
	mitotic cell cycle	0.0200	1.95	0.00
	ubiquitin cycle	0.0214	0.70	0.70
	cell homeostasis	0.0228	3.23	0.00
	protein prenylation	0.0262	14.29	0.00
Carbon nano-onion 6 μg ml^{-1}	L-serine metabolism	0.0000	0.00	40.00
	tENA aminoacylation	0.0000	0.00	23.81
	amine metabolism	0.0000	0.00	5.42
	amine transport	0.0000	0.00	12.20
	dicarboxylic acid transport	0.0020	0.00	25.00
	response to extracellular stimulus	0.0063	0.00	14.29
	heterocycle metabolism.	0.0076	0.00	6.38
	porphyrin metabolism	0.0139	0.00	9.52
	TGF beta receptor signaling pathway	0.0139	4.76	4.76
	pigment metabolism	0.0194	0.00	8.00
Carbon nanotube 0.6 μg ml^{-1}	tRNA aminoacylation	0.0000	0.00	33.33
	L-serine metabolism	0.0000	0.00	50.00
	amine metabolism	0.0000	0.00	6.90
	amine transport	0.0000	0.00	14.63
	response to stimulus	0.0000	0.16	2.86
	immune response	0.0000	0.18	4.50
	water-soluble vitamin biosynthesis	0.0024	0.00	40.00
	inflammatory response	0.0034	0.00	5.06
	heterocycle metabolism	0.0062	2.13	6.38
	dicarboxylic acid transport	0.0065	0.00	25.00

[a]The categories are generated by the GoMiner program (Materials and Methods, Supporting Information), using *p*-values as the evaluation criteria of statistically significant changes, for each category. The *p*-value was calculated by conducting a two-sided Fisher's exact test, which reflects the statistical significance for that category being enriched in changed genes. The *p*-values were used to sort categories to identify those gene functional groups that have responded the most after treatments.

6 *IN VITRO* TOXICITY VS. *IN VIVO* TOXICITY

In vitro (Latin: within the glass) refers to an artificial environment outside the living organism. The *in vitro* toxicity study of nanomaterials is to utilize appropriate cell lines to evaluate the toxicity of nanomaterials (cytotoxicity). *In vitro* toxicological assessment is a very important tool for nanotoxicity study. Compared to *in vivo* studies, the advantages of *in vitro* studies include being simpler, easier, faster, lower cost, better experimental control, and significantly minimizing ethical concerns by only using cells. However, *in vitro* study generally applies much higher dosage level and shorter exposure time than *in vivo*, thus it is hard to predict the

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in vivo toxicity based on the results from *in vitro* experiments.

In addition, the test conditions of *in vitro* may not correspond to the conditions inside the organism. This may lead to results that do not correspond to the situation that arises in a living organism. In *in vitro* toxicity study, the liquid cell culture medium contains various components like inorganic salts, amino acids, and proteins, which is the major supplement in serum. The surface physicochemistry of nanomaterials may be changed upon addition into cell culture medium. In fact, several research groups have studied the adsorption of proteins and other molecules on nanomaterial surface and their potential effects on biological activity. Dutta *et al*. (2007) investigated the importance of proteins adsorbed onto the surface of two kinds of nanomaterials: single-walled carbon nanotubes (SWCNTs) and amorphous silica. Their date demonstrated the important role of adsorbed proteins for toxicity change of SWCNTs and nano-sized amorphous silica. Horie *et al*. (2009) studied the influence of adsorption by metal oxide nanoparticles (NiO, ZnO, TiO_2, CeO_2, SiO_2, and Fe_2O_3) on the cell viability *in vitro* and proved the adsorption ability of metal oxide nanoparticles is an important factor for the estimation of cytotoxicity *in vitro* for low-toxicity materials. It should be noted that the absorption by nanomaterials and subsequent toxicity changes may occur in *in vivo* study also so it may be necessary to examine the surface characteristics of nanoparticles that induce a biological response *in vivo*.

Another kind of study for nanotoxicity is to study the impact of nanomaterial exposure within intact living organisms. *In vivo* (Latin for "within the living") refers to using a whole, living organism as opposed to a partial or dead organism, or an *in vitro* controlled environment. Animal testing and clinical trials are two forms of *in vivo* research.

In vivo testing is often employed over *in vitro* because it is better suited for observing the overall effects of an experiment on a living subject. *In vivo* studies of nanomaterials allow investigating the long-term effects, distribution in tissues, retention, and excretion. Therefore, these types of *in vivo* studies provide more relevant information regarding the nanotoxicity in human beings, which is not available through *in vitro* study. These *in vivo* studies are typically undertaken in mice (Wu *et al*., 2009; Kwon *et al*., 2009; Park *et al*., 2009) or rats (Kobayashi *et al*., 2009; Sarkozi *et al*., 2009; Liang *et al*., 2009). Because *in vivo* studies use and sacrifice animals, they have ethical considerations.

The assessment techniques of *in vitro* and *in vivo* studies are also different. The most commonly used *in vitro* assessment is generally either to evaluate viability (proliferation, apoptosis, or necrosis) and/or to study toxicity mechanism (oxidative stress, oxidative DNA damage). However, typically, *in vivo* studies investigate serum/hematological changes, tissue morphology changes, or histology change.

Sayes, Reed and Warheit (2007) designed and studied the methodology to assess the capacity of *in vitro* screening in order to predict *in vivo* toxicity of five fine or nanoscale particle types (carbonyl iron, crystalline silica, precipitated amorphous silica, nano-sized ZnO, and fine-sized ZnO) in rats. For the *in vitro* experiment part, three different cell lines such as rat L2 lung epithelial cells, primary alveolar macrophages, and AM-L2 lung epithelial cell cultures were exposed to these five particles. The following end points were measured: mitochondrial activity (MTT assay), LDH release, and production of MIP-2, TNF-α and IL-6. In the *in vivo* experiment part, after the rats were exposed to these particles by intratracheal instillation, the pulmonary lavage and LDH release were measured. For the evaluation of relevant biomarkers, the authors selected inflammation and cytotoxicity indices as the end points for comparison of *in vitro* and *in vivo* studies. The authors found that *in vitro* and *in vivo* results demonstrated only little correlation. It may be partially due to the different experimental conditions such as cell line types, exposure dosage and time. However, the results solidly demonstrated the limitation of *in vitro* experiments to predict the *in vivo* results. Figure 25 shows the experiment scheme.

7 *IN VIVO* TOXICITY OF NANOMATERIALS

To completely assess and understand the potential adverse effects of nanomaterials in a human being's body, sufficient information on absorption, distribution, metabolism, and excretion (ADME) of the nanoparticles in animal models is absolutely necessary. In addition, newly developed nanomaterials have demonstrated substantial biomedical significance for pharmacology and therapeutics. Their unique characteristics make them very useful in drug discovery, biomarker discovery, drug delivery,

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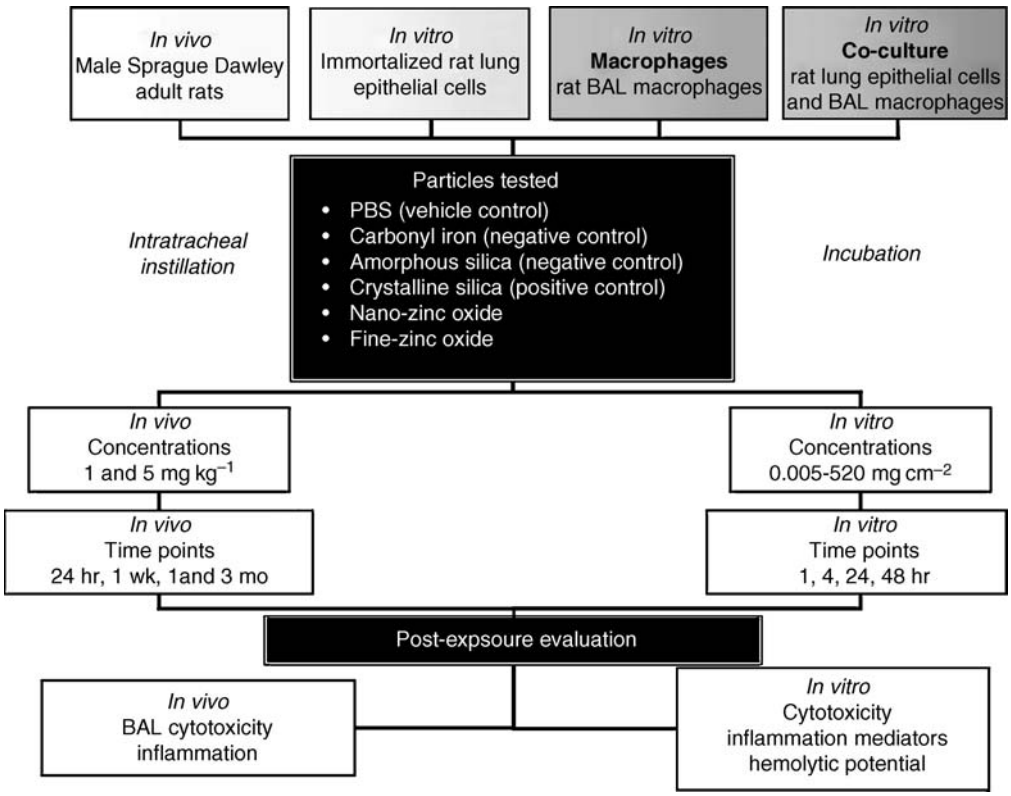

Figure 25. Experimental design. The design of the *in vivo* and *in vitro* experiments. Test systems were exposed to CI, CS (Min-U-Sil 5), AS, NZO, and FZO particles. For each experiment, both dose-response and time-response relationships were established (Sayes, Reed and Warheit, 2007). Reproduced from Ding *et al.* (2005) © American Chemical Society.

and molecular diagnostics. However, development of these novel nanomaterials must proceed in tandem with assessment of their potential side effects to human beings and environments.

Figure 26 shows the ADME scheme of nanomaterials in the body. With the dramatic increase in manufacture and application of nanomaterials, human exposure to nanomaterials is also increasing. Nanomaterials may enter into the human body via inhalatory exposure, oral exposure, dermal exposure and medical intervention. The respiratory system, blood, central nervous system (CNS), gastrointestinal (GI) tract and skin could be potentially targeted by nanomaterials.

7.1 *In Vivo* Toxicity Assessment

Although the assessment technique in *in vivo* has some similarities as that of *in vitro*, such as the measurement of biomarkers (Glutathione, LDH, etc.), *in vivo* nanomaterial toxicity studies typically focus on changes in tissue morphology and changes in blood serum chemistry by using histology.

7.1.1 Histology

Histology is an essential tool to study the *in vivo* toxicity of nanomaterials by observing the morphological changes. It is performed by examining a thin slice (section) of tissue under a light microscopy or electron microscope. The ability to visualize or differentially identify microscopic structures is frequently enhanced through the use of histological stains. Figure 27 demonstrates the morphological change of lung tissue from rats after exposed to $3\,\mathrm{mg\,kg^{-1}}$ of particles for three months postinstillation (Sayes, Reed and Warheit, 2007).

7.1.2 Bronchoalveolar Lavage (BAL) Study

BAL study has gained widespread acceptance as a minimally invasive method that provides important information about immunologic, inflammatory, and infectious processes taking place at the alveolar

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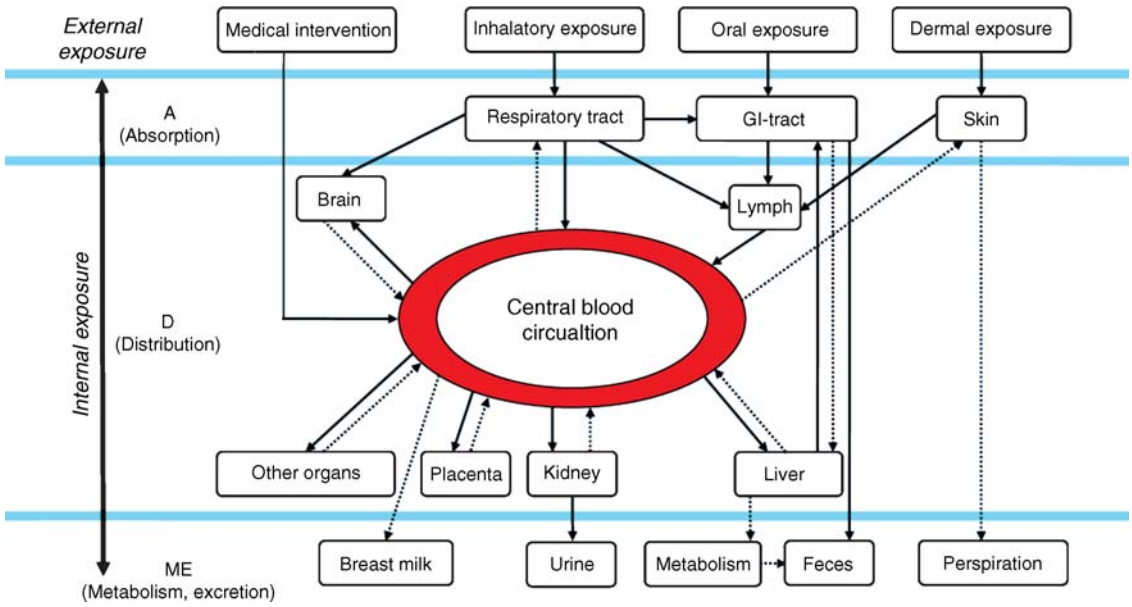

Figure 26. Kinetic properties of nanoparticles in the body. In this scheme, the ADME processes (absorption, distribution, metabolism and excretion) are indicated. The internal exposure is the part of the external dose that reaches the systemic circulation. The black lines represent confirmed routes for nanoparticles; the dashed lines represent hypothetical routes. The transport rates and retention times for the indicated processes are largely unknown (other organs: e.g., spleen, heart, reproductive organs. Reproduced from Hagens *et al.* (2007) © Elsevier.

level. This technique has been widely used for *in vivo* toxicity assessment for nanomaterials such as single-wall carbon nanotubes (Warheit *et al.*, 2004), nanoscale quartz particles (Warheit *et al.*, 2007), and nanoscale TiO_2 rods and dots (Warheit, Webb and Reed, 2006).

7.1.3 Oxidative Stress Biomarkers

Oberdoster *et al.* studied the oxidative stress induced by manufactured nanoscale fullerenes in the brain of juvenile largemouth bass (Oberdorster 2004). In this study, glutathione (GSH) and malondialdehyde (MDA) were selected as the major biomarkers to evaluate the oxidative stress and lipid peroxidation. Based on the significant lipid peroxidation and depletion of GSH levels, it demonstrated that uncoated fullerene can cause oxidative damage *in vivo* (Figure 28).

7.2 Absorption

In this part, different absorption routes of nanomaterials into the human or animal body are described briefly. Table 9 shows some studies of the major exposure pathways (inhalation, oral, dermal, and parenteral) of nanomaterials.

As for the inhalation exposure, the respiratory system is one of the most important portals for nanomaterials to enter and deposit. The alveolar macrophages in the respiratory system play a key role in the response to inhaled nanomaterials. Their essential function is phagocytosis of inhaled nanomaterials and clearance of them. It is well known that lungs are the well target of atmospheric pollutants such as Particle Matters (PM). The deposit, diffusion, and transportation effect may be dependent on various factors such as particle size, composition, shape, charge, solubility, stability, and particle aggregation condition. Other important portals of nanomaterials entry in the body are the GI tract and skin. Nanomaterials can be ingested into the gut by many ways such as from food, water, drugs, and cosmetics (Lomer, Thompson and Powell, 2002).

7.3 Distribution

Due to their nano-size, nanomaterials have the potential to pass through the defenses in the

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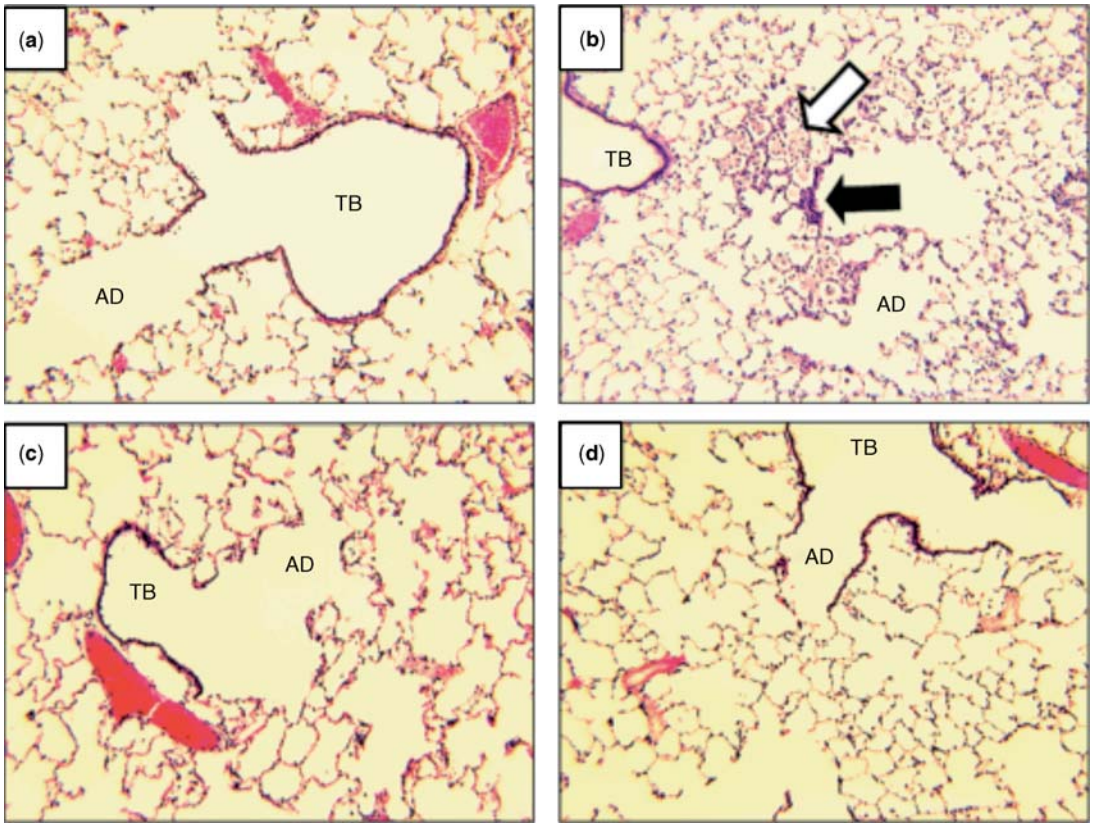

Figure 27. Lung tissue analysis. Light micrographs of lung tissue of rats exposed to (a) Milli-Q water vehicle control; (b) Min-USil positive control, (c) nano-C60 water suspensions, and (d) C60(OH)24 water suspensions (all at 3 mg kg^{-1}) at three months postinstillation exposure. The micrographs illustrate the terminal bronchiole (TB) and corresponding alveolar ducts (AD), and demonstrate normal lung architecture in graphs a, c, and d, indicating that exposures to FWS produced no adverse pulmonary effects. Micrograph b depicts the prominence of foamy alveolar macrophage accumulation (white arrow) and tissue thickening (black arrow) in the distal lung. (magnification = 40×). Reproduced from Sayes, Reed and Warheit (2007) © Oxford University Press.

Table 9. Some typical studies of different exposure routes of nanomaterials.

Exposure routes	Nanomaterials	Literature
Inhalation	SWCNT	(Lam *et al*., 2004)
	Silica, TiO_2	(Warheit *et al*., 2005)
	Ambient air pollent	(Vermylen *et al*., 2005)
	Particulate air pollution	(Peters *et al*., 2006; Clancy *et al*., 2002)
	Ultrafine technetium-99m-labeled aerosol	(Brown, Zeman and Bennett, 2002)
	Ultrafine particles	(Geiser *et al*., 2005)
	combustion-derived nanoparticles	(Donaldson *et al*., 2005)
	iridium particles	(Kreyling *et al*., 2002)
Oral	Cu Nanoparticles	(Chen *et al*., 2006)
	Polystyrene nanoparticles	(Jani *et al*., 1990)
Dermal	Silver nanaoparticles	(Lee *et al*., 2007)
	TiO_2, ZnO	(Schulz *et al*., 2002)
	C_{60} fullerene	(Rouse *et al*., 2007)
	Quantum dots	(Ryman-Rasmussen, Riviere and Monteiro-Riviere, 2007)

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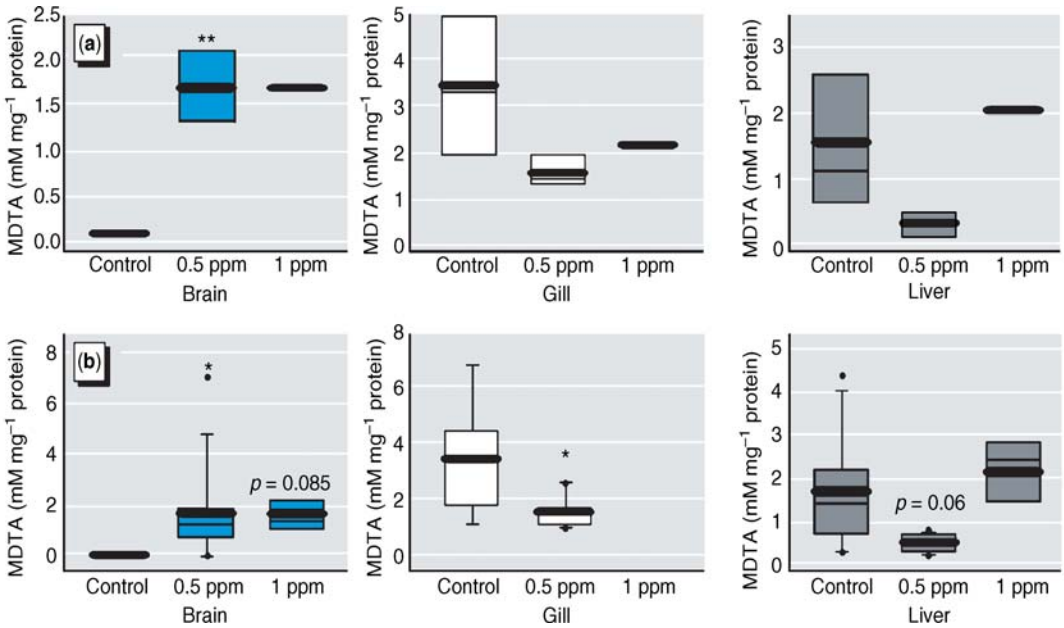

Figure 28. Lipid peroxidation of brain, gill, and liver of largemouth bass after 48 hr of exposure to 0.5 or 1 ppm nC_{60}. MDTA, 1,1,3,3-tetraethoxy propane. (a) Aquarium averages; (b) data using individual fish; in the brain, 0.5 ppm nC_{60} caused a significant 17-fold increase in lipid peroxidation, whereas in gill and liver there is a trend for reduction of lipid peroxidation at 0.5 ppm nC_{60}. Heavy black bands represent the means; thinner lines indicate medians; boxes represent 25th and 75th percentiles; error bars indicate minimum and maximum; and circles represent outliers. Reproduced from Oberdorster (2004) © National Institute of Environmental Health Sciences.

respiratory tract, GI tract, or skin and enter into systemic circulation or even the central nervous system (CNS). Upon entering into systemic circulation, the nanomaterials would be possibly transported and distributed to peripheral organs and tissues. For the first time, Berry *et al.* (1977) showed for the first time that following intratracheal injection of particles of colloidal gold (30 nm) in the rat, particles in the blood platelets of the alveolar capillaries can rapidly be observed. And the presence of the gold was confirmed by microanalysis. Recently, a few studies have shown distribution of particles to several organs including liver, spleen, heart and brain (Brown, Zeman and Bennett, 2002; Sun *et al.*, 2005; Nemmar *et al.*, 2001; Hillyer and Albrecht, 2001; Oberdorster *et al.*, 2002).

7.4 Metabolism

To date, no study on metabolism of nanomaterials has been reported yet. Some nanomaterials with wide applications in industrial work, such as metallic oxide, carbon, silica, are unlikely be metabolized in the body because of their inert chemical properties. However, if nanomaterials contain functional groups or if the nanomaterials are designed as the drug carrier, like liposomes, it is possible that metabolism may be affected.

7.5 Excretion

In general, researchers study the distribution and excretion of nanomaterials in tandem to test the overall process through the human or animal over time and get insight into nanomaterials localization, retention, and passage from human or animal. Excretion studies are accomplished by long-term study of the excretion of the nanomaterials at various time points after exposure. Usually, ICP-MS, radiolabeling and fluorescence are used to quantify the excretion (Lin *et al.*, 2008; Huang *et al.*, 2008).

There are two reviews that describe the effect of nanomaterial properties (size, composition, charge, etc.) on *in vivo* distribution and excretion (Alexis *et al.*, 2008; Owens and Peppas, 2006). However, the information about the excretion route,

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mechanism, and influence factors of nanomaterials remains largely unknown.

In addition, there remains a great need for a rapid and throughput method for the detection and analysis of excretion of nanomaterials.

8 SUMMARY

Due to exponential increase in the development of many different types of nanomaterials and their wide applications, the health impact of nanomaterials causes great concern. In consequence, nanotoxicology becomes a fast-growing interdisciplinary research involving chemistry, biology, material sciences, physics, and others. To fully understand the toxicity of nanomaterials and determine the mechanisms of nanotoxicity, it is essential that the physicochemical properties of nanomaterials are fully investigated since many physical factors can affect the results of nanotoxicity testing such as composition, impurities, size, shape, surface area, crystal structures, porosity, surface coating, and so on. These factors make characterization of nanomaterials be complicated and time consuming. Before *in vivo* study, a thorough *in vitro* study should be conducted since *in vitro* study is a valuable technique to find the dose response range, the levels of cytotoxicity, and the possible mechanism of cytotoxicity. Many biomarkers and analytical techniques have been developed to assess the levels of cytotoxicity, which can help greatly for future nanotoxicology studies.

RELATED ARTICLES

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Toxicology of Engineered Metal Nanoparticles

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1 INTRODUCTION

Nanotechnology is a rapidly developing multidisciplinary field of science which offers the promise of generating new products which will revolutionize diverse areas of our life. Nanotechnology, therefore, is expected to have an enormous impact on economy and it is already so *a la page* to flaunt "nanotech" as a well-established nick name. It deals with materials of extremely small size that due to their high surface area, chemical and biological activities find a wide range of applications.

The fast growth of nanotechnology will undoubtedly be paralleled by an ever-increasing presence of nanomaterials in the environment. The peculiar and unique properties that make these nanomaterials in general and nanoparticles (NPs) in particular so attractive may be associated with potentially new and largely undefined risks (Di Gioacchino *et al*., 2009) for the human health and for the environment. NPs, for example, are readily taken up by the cells (Gornati *et al*., 2009; Papis *et al*., 2009) and stored in cytoplasmic vesicles (Figures 1 and 2) from where they can exert their toxicity. In this context, a new discipline, nanotoxicology, is established to provide answers to the concern about the possible drawbacks of this technology.

In this chapter, after having examined the ecotoxicology of nanoparticles, we will focus on two main concerns that may arise for the human health, that is, cancer and allergy. Last but not least, we will give a glance to the other side of the coin by examining how medicine might take advantage from nanomaterials.

2 ECOTOXICOLOGY

The dispersal of engineered metal NPs in the environment can be accidental, unwanted but unavoidable, or intentional when, for example, used for bioremediation of contaminated sites. Accidental leakages from productive plants or during transport of refined products have never been reported, but exposure to airborne nanomaterials is well documented both at the workplace and in polluted sites. The accumulation of nanometals dispersed in water and soils from consumer products has been predicted to span between few ppt, for

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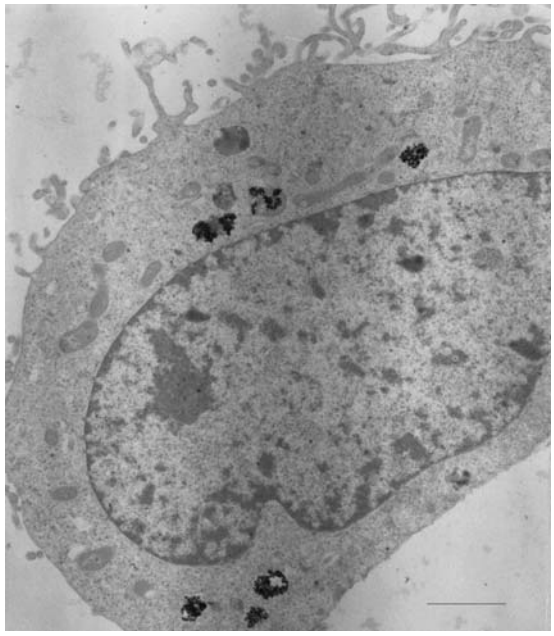

Figure 1. TEM image of a ECV-304 cell exposed to Co_3O_4 NPs. Enlargement ×00000.

CeO_2, and several tens or even thousands ppb, for ZnO and TiO_2 (Xia *et al*., 2008; Tiede *et al*., 2009). Although in the case of nanomaterials every generalization is difficult and frequently fallacious, these concentrations seem to be potentially harmful (Blaise *et al*., 2008; Cattaneo *et al*., 2009; Farré *et al*., 2009).

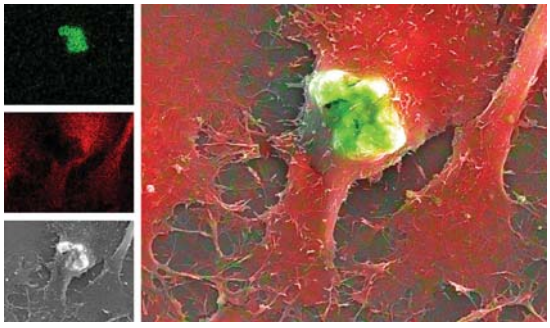

Figure 2. X-ray spectroscopy of cells exposed to Co_3O_4 NPs. On the left, the distribution of cobalt (green) and carbon (red) is shown in false colours in the field of view shown on the SEM image at the bottom left. On the right, the elemental mapping has been superimposed onto the micrograph. The horizontal field of view spans approximately 50 μm. (From Papis *et al*., 2009.)

Among unwanted dispersion of refined nanomaterials of nanotechnology industry, noticeable is the case of silver NPs. The waste of silver in the environment is mainly sustained by the disposal of plastics and textiles functionalized with Ag NPs, in addition to the amount derived from discards of health and dentistry services, silver-containing biocidal products, photographic emulsion and electroplating. The accumulation is mainly expected in sewage sludge and groundwater from which elution into rivers, soil and sea can occur (Blaser *et al*., 2008). The ecotoxicological relevance of Ag NPs has been studied in *Caenorhabditis elegans*: NPs exert considerable toxicity, particularly in reproduction potential, gene expression and oxidative stress (Roh *et al*., 2009).

In addition to silver, other widely used metal nanomaterials could differently affect gene expression in mammals and mammalian cells (Papis *et al*., 2007; Warheit *et al*., 2007; Fowler, Conner and Yamauchi, 2008) and show toxic effects on several other organisms (Adams *et al*., 2006; Hund-Rinke and Simon, 2006; Sun *et al*., 2007; Warheit *et al*., 2007; Zhang *et al*., 2007; Davies, Dodd and Jha, 2008; Griffitt *et al*., 2008; Handy *et al*., 2008; Reeves *et al*., 2008; Velzeboer *et al*., 2008; Vevers and Jha, 2008; Zhu *et al*., 2008; Aruoja *et al*., 2009). Among plants, *Phaseolus radiatus* and *Triticum aestivum* proved to be sensitive to the toxic effects of Cu NPs in a test on agar (Lee *et al*., 2008b), whereas spinach reacted positively to the exposure to TiO_2 NPs with increased germination rate of seeds, growth of plant crops, photosynthetic yield and Rubisco[a] activity (Hong *et al*., 2005; Zheng *et al*., 2005; Yang *et al*., 2007; Gao *et al*., 2008; Su *et al*., 2009). This last result, if compared to those found in algae and plant agar tests, is surprising and, to explain it, a different aggregation of NPs or lower protection against oxidative stress in water has been postulated. Similarly, although no evident toxicity was recorded after NP administration with food to the earthworm *Eisenia foetida* (Oughton *et al*., 2008) and to the terrestrial crustacean *Porcellio scaber* (Drobne, Jemec and Pipan Tkalec, 2009; Jemec *et al*., 2008), the soil nematode *Caenorhabditis elegans* poorly tolerated the exposure to metallic NPs when the experiments were conducted in water (Roh *et al*., 2009; Wang, Wick and Xing, 2009b).

The nanoparticulate present in the air of urban areas or along high-traffic suburban ways is by far

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represented by the products released from diesel and spark-ignition engines. These technological byproducts have been studied and characterized quite well, and a number of methods to record, measure and characterize ultrafine airborne particulate have been used for several years. Diesel exhaust is, in itself, metal-free while toxic (Lingard *et al*., 2006) and metal NPs are the product of fuel additives or of catalytic converters (Merget and Rosner, 2001).

The main byproducts of catalytic converters are represented by NPs of the platinum group elements (PGE), which, when released in the air, are generally attached to micron-sized aluminum oxide particles. Aged particles can accumulate in dust, soil (Ely *et al*. 2001; Cinti *et al*., 2002; Kylander *et al.*, 2003) and sediments of aquatic systems collecting the urban road runoff (Whiteley and Murray, 2005). PGE NPs seem to be highly mobile in the environment and have been documented even in the Artic snow (Barbante *et al*., 2001). They can enter the food chain through bacteria, fungi and plants with proved toxic effects (Gao *et al*., 2009). Like a great deal of airborne NPs (Duffin, Mills and Donaldson, 2007; Li, Xia and Nel, 2008; Vega-Villa *et al*., 2008), PGE NPs sustain acute or sub-chronic respiratory toxicity when inhaled.

The most important intentional introduction of metal nanomaterials in the environment has been, until now, the injection of products for remediation of groundwater, in particular of nanoparticulate zero-valent iron (nZVI). Remediation has been proposed and tested in artificial systems even with metal oxide NPs, but never used in the field (Table 1). Both nZVI and metal nano-oxides are aggressive agents used to remove, from water and soils, refractory and hazardous pollutants, like those found in oilfield wastewater, polychlorinated biphenyls and other organic and inorganic molecules.

Two recent case studies with nZVI sludge for remediation of groundwater have been conducted on local scale in two industrial sites, one in Southern Finland (Tuomi *et al*., 2008) and the other in Arizona (Bruck, 2007), which were heavily polluted with chlorinated ethanes and ethenes. Although the

Table 1. Examples of proposed uses of metal NPs for remediation of polluted soils. Note the only two cases with intentional dispersion of metallic nanomaterials in the environment.

Nanomaterials	Pollutants	Matrix	References	Toxicity
TiO_2 (functionalized: Cu-EDTA)	Anionic metals	groundwater	Mattigod *et al*., 2005	ND
TiO_2	Pb	water	Giammar *et al*., 2007	Toxic
TiO_2	As	water	Gupta & Ghosh, 2009	Toxic
TiO_2	2-chlorophenol	water	Shchukin & Caruso, 2004	Toxic
TiO_2	*E. coli*	superficial water	Alrousan *et al*., 2009	Toxic
TiO_2/V_2O_5	polychlorinated biphenyls	soil	Varanasi *et al*., 2007	Toxic
TiO_2/In_2O_3	2-chlorophenol	water	Shchukin & Caruso, 2004	ND
TiO_2, SiO_2, ZnO	*E. coli*	water	Adams *et al*., 2006	Toxic
SiO_2	Phenanthrene	water	Fang *et al*., 2008	Toxic
CeO_2	*E. coli*	water	Thill *et al*., 2006	Toxic
Fe_2O_3	polychlorinated biphenyls	soil	Varanasi *et al*., 2007	Toxic
ZVC	Phenanthrene	water	Fang *et al*., 2008	Toxic
ZVI	Phenanthrene	water	Fang *et al*., 2008	Toxic
ZVI	bromate	water	Wang *et al*., 2009a	Toxic
ZVI	azo dye methyl orange	water	Fan *et al*., 2009	Toxic
ZVI	pyrene	soil	Chang & Kang, 2009	Toxic
ZVI	amoxicillin and ampicillin	water	Ghauch *et al*., 2009	Toxic
ZVI	Nitrate	resin column	Park *et al*., 2009c	Toxic
ZVI	Chlorinated ethenes (TCE)	Polluted groundwater (field*)	Bruck, 2007	Toxic
ZVI	chlorinated ethanes and ethenes (TCA, TCE)	Polluted groundwater (field*)	Tuomi *et al*., 2008	Toxic
ZVI	*E. coli*	water: artificial	Lee *et al*., 2008a	Toxic

* Toxicity of nanomaterials is annotated in the last column. ZVC: zero valent copper, ZVI: zero valent iron, EDTA: ethylenediaminetetraacetic acid.

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geology of these sites is very different, positive results were obtained in both cases within 2–3 weeks. The main failure, at a later time, was due to the agglomeration and ageing of NPs, and several efforts have been and will be spent to ameliorate the nanomaterial performances by coating and functionalizing their surface (Keenan and Sedlak, 2008; Lee and Sedlak, 2008; Tuomi *et al*., 2008; Bezbaruah *et al*., 2009; Kim *et al*., 2009, Sirk *et al*., 2009). A different problem arises from the contingency that significant amount of ZVI enters the effluent water, posing a threat for the environment and human health. Although the compound is neurotoxic, it rapidly ages, partially warranting against a serious hazard for humans (Phenrat *et al*., 2009).

Bioremediation of uranium- and heavy-metal-polluted sites is another interesting application of nanotechnology. It involves metabolic properties of bacteria which have been isolated at sites polluted with uranium and other hazardous compounds (Spain *et al*., 2007). In the process of bioremediation, some prokaryotes organize the biogenic metal in NPs which accumulate on the outer side of cells, bound to extracellular polymeric substance (Marshall *et al*., 2006, 2009). Therefore, besides the remediation of the site, there is production of biogenic NPs precious for many technological applications. For example, biogenic Pd and Pt NPs have been found to be active catalysts for dechlorination of polychlorinated biphenyls and lindane (De Windt, Aelterman and Verstraete, 2005; Mertens *et al*., 2007).

3 CANCER

At present, very little is known about the carcinogenic risk posed by NPs and very few studies in this area have been carried out *in vivo* in laboratory animals and in *in vitro* systems, whereas no study is available on the carcinogenic potential of NPs in humans. Furthermore, the existing data on the carcinogenic potential of NPs, the information on the specifications of the test materials used and the relevance of such studies to the human situation are mostly inconsistent, sometimes due to the high-dose exposure used that paradoxically can abolish the biological response, the "obscure route" of exposure (e.g., intramuscularly) and the absence of the chemical purity of the NPs because they may contain "contaminants" such as metals and surfactants used in processing, which may significantly affect their behaviour and reactivity.

Pulmonary exposures to single-wall carbon nanotubes in rats produced multifocal granulomas (Warheit *et al*., 2004) whereas carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity (Poland *et al*., 2008). Intraperitoneal application of multi-wall carbon nanotubes induces mesotheliomas in the p53+/− mouse (Takagi *et al*., 2008). A lung carcinogenic bioassay of CuO and TiO_2 NPs intratracheally instilled into rats showed a tendency to increase the neoplastic lesions compared with the microparticle administration (Yokohira *et al*., 2009).

The development of sarcomatous (malignant connective tissue tumours) lesions has been studied in rats implanted with metallic cobalt, nickel, SiO_2, TiO_2 and PVC (intramuscularly) in comparison to the corresponding bulk materials (subcutaneously) in the same animal (Hansen *et al*., 2006). The strongest carcinogenic effect was observed in nickel-implanted animals. However, it is crucial that there was no significant difference between the implantation sites with bulk metal or NPs. The only group of animals that did show a difference between bulk and NPs was the ones exposed to metallic cobalt. However, it is known for many years that long-term subcutaneous implantation of materials in solid metallic sheets or in particulate form gives rise to fibrosarcomas in rats (Oppenheimer effect – tumours, Oppenheimer *et al*., 1956); there is no evidence of their promoting malignancy in man or other animal species.

Concerning the *in vitro* studies, a wide range of cells have proved useful to examine aspects and mechanisms which may be related to the carcinogenic and genotoxic potential of NPs such as oxidative stress and inflammation (Peters *et al*., 2007; Yang *et al*., 2009). A recent study on genotoxicity and morphological transforming activity of cobalt NPs and cobalt ions (Co^{2+}) in Balb/3T3 cells suggests that cobalt NPs are potential genotoxic carcinogens (Ponti *et al*., 2009). Co NPs can be internalized by human leucocytes and can interact with DNA leading to the observed genotoxic effects, which are, however, modulated both by donor's characteristics and/or by Co^{2+} ions released (Colognato *et al*., 2008). A recent study has highlighted some of the potential confounding factors that need to be considered in order to ensure that *in vitro* genotoxicity assays report true biological impacts in

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response to nanomaterial (Doak *et al*., 2009). The finding that silver NPs have been observed in the colon of patients with colon cancer (Gatti, 2004) cannot be interpreted as carcinogenic response to such NPs, only proving the presence of NPs in cancer cells.

In conclusion, the existing knowledge on the carcinogenic potential of engineered NPs is of very limited value with regard to human health risk. At present, no animal model is available which is able to predict possible neoplastic effects of NPs in humans and there are no standardized protocols for *in vitro* testing of the carcinogenic potential of such nanosized materials. In addition, no epidemiological study is reported on the carcinogenicity of NPs in humans. There is a great need to meet a level of reliability that would provide confidence in the data generated. However, in spite of the very limited knowledge on the carcinogenic potential of engineered NPs the existing data suggest in any case great caution before introducing such materials into the market if long-term health consequences are to be avoided.

4 ALLERGIC DISEASES

Starting from the concept that all foreign substances that enter the body interact with the immune system, a number of studies on the effects of NPs in humans have been devoted to the evaluation of the immune potential of nanoscale materials. The large majority of studies dealt with the immune toxicity of NPs, whereas very few concerned the modulation of immune function or their sensitizing potential. In particular, literature lacks studies aimed at evaluating the possibility that some form of allergic diseases can be linked to the environmental spreading of NPs. Some manuscripts report a relationship between the increased levels of some metal NPs in the environment and the onset of skin allergies; for example, an increase of allergic contact dermatitis to palladium has been reported, in relation to the increase of Pd emitted from catalytic converters (Merget and Rosner, 2001; Prichard, Sampson and Jackson, 2009). However, it is not demonstrated that Pd NPs rather than Pd ions are responsible for skin sensitization. At present, no metal NPs are demonstrated to induce allergy, while almost all metal ions have been shown to have a strong sensitizing and immune potential, being able to induce, both *in vivo* and *in vitro*, allergic responses and immune toxicity (Di Gioacchino *et al*., 2007), and speciation appears to be the main factor in determining their immune effects (toxic or sensitizing). Sensitizing potential of metals can be studied by evaluating the *in vivo* and/or *in vitro* induction/inhibition of specific cytokine pattern from immune cells. In fact, in experimental animals, it has been shown that exposure of mice to chemicals in general and metal compounds in particular, which are known or suspected to cause allergic asthma or allergic contact dermatitis, provokes the development of selective type 2 (Kimber *et al*., 1999; Hayashi *et al*., 2001; Vandebriel *et al*., 2000) or type 1 (Dearman and Kimber, 1999; Hayashi *et al*., 2001; Vandebriel *et al*., 2000) cytokine expression profiles.

Cytokine profile induced by metal nanoparticles appears to be different from that induced by their respective ions. For example, Co NPs induced an increase of tumour necrosis factor α (TNF-α) and interferon γ (IFN-γ) and an inhibition of interleukin 10 (IL-10) and IL-2 release by cultured PBMCs, differently from Co ions that inhibited the production of all studied cytokines (Petrarca *et al*., 2006). A similar T helper 1 (Th1) stimulatory effect has been shown to be induced by Pd NPs, in particular, increasing (at high concentrations) INF-γ and inhibiting TNF-α and IL-17 release from human peripheral blood mononuclear cell PBMC in cultures stimulated by lipopolysaccharides (Boscolo *et al*., 2010). The cytokine pattern observed in these two studies can mimic that of allergic contact dermatitis (or experimental autoimmunity). However, sensitized patients should be studied to definitely establish whether *in vitro* results can be extrapolated *in vivo*, to demonstrate the ability of NPs to induce a delayed type of immune reaction as the allergic contact dermatitis.

Also atopic dermatitis, a Th2-mediated disease, can be affected by metal NPs, In fact, it has been also reported that the exposure to TiO_2 NPs under skin barrier dysfunction/defect in animals affected by atopic dermatitis can exacerbate symptoms induced by allergens through Th2-biased immune responses (increase of Il4, IgE and histamine release and decrease in IFNγ production). Furthermore, TiO_2 NPs can play a significant role in the initiation and/or progression of skin diseases following the barrier dysfunction/defect by histamine release even in the absence of allergens (Yanagisawa *et al*., 2009).

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Inhaled metal NPs show different immune effects, being able to induce immune cells to release Th2 cytokines, typical of the IgE-mediated reactions. Park *et al*. (2009b) demonstrated that in experimental animals, the intra-tracheal instillation of TiO_2 NPs induced a strong release of Th2-type cytokines such as IL4 and IL-5 (typical of allergic asthma) and lower levels of IL-12 and IFN-γ. By the prevalence of Th2-type cytokines, an increased IgE production in BAL fluid and serum was observed. The same authors showed that other engineered nanomaterials are able to favour allergic responses in mice through B-cell activation and production of IgE (Park *et al*., 2009a). However, it has not been demonstrated that NPs can act as apten inducing specific IgE. Rather, it is likely that some NPs can work as adjuvant in inducing patterns of cytokines, antibody and cells that favour allergic sensitization to natural allergens and xenobiotics. Inoue *et al*. (2006) also showed that engineered NP might exert adjuvant effects that resulted in exaggerated eosinophil, neutrophil, and mononuclear cell infiltration, as well as an increase in ovalbumin (OVA)-specific IgG and IgE production. The combination of NPs with OVA also increased the production of IL-5, IL-6, IL-13, eotaxin, monocyte chemoattractant protein-1 (MCP-1) and RANTES in the lung compared with OVA alone.

Through the induced inflammation, NPs can also exacerbate allergic diseases, directly causing cell and epithelial damages and increasing bronchial hyper-reactivity. In fact, subacute exposure of C57B1/6 mice to 2–5 nm TiO_2 NPs in a whole-body exposure chamber caused a moderate, but significant, inflammatory response in the lung within the first 2 weeks of exposure, beyond which the inflammation resolved without permanent damage (Grassian *et al*., 2007). Furthermore, Inoue *et al*. (2007) demonstrated that inhaled NPs have the ability to increase bronchial hyper-responsiveness, especially in the presence of antigen/allergens. The researchers observed that NPs alone or OVA alone moderately enhanced cholinergic airway reactivity, whereas in the NPs + OVA groups, all the parameters for lung responsiveness were worse than those in the control group.

As speciation is the main characteristic of metal ions, particle size and surface area are the most important features of NPs in inducing immune effects. In fact, the adjuvant effect of NPs on allergic immune responses has been shown to increase with decreasing particle size and increasing particle surface area. As shown in experiments made with TiO_2, the ultrafine particles cause significantly more inflammation and were significantly more cytotoxic than the fine-sized particles. Lung burden data indicate that ultrafine TiO_2 appears to migrate to the interstitium to a much greater extent than fine TiO_2 (Sager, Kommineni and Castranova, 2008). However, when doses were equalized based on surface area of particles delivered, the ultrafine particles were only slightly more inflammogenic and cytotoxic compared with the fine-sized particles.

In conclusion, at present there is no clear evidence that some metal NPs can act as apten inducing specific sensitization, both in Th1 and in Th2 allergic-mediated diseases. However, their ability to induce a specific cytokine-mediated inflammation can result in favouring allergen sensitization to environmental allergens or xenobiotics, both favouring allergic contact dermatitis of IgE-mediated sensitization, according to the type of metal NPs. The proinflammatory properties of some metal NPs, mainly related to their size, can also result in exacerbation of allergic diseases.

5 NANOMEDICINE

Colloidal suspensions of gold and silver were used in the traditional medicine (Mahdihassan, 1985; Milot, 2008), and the ingestion of these preparations holds over, despite the possibility of serious side effects (Archer, 2008). The empirical character of medicine anticipates sometimes science: the colloid suspensions of metals were only recently characterized as mostly composed of nanoparticles, and the red colour of colloidal gold used in traditional Indian medicine as a formulation for long life and fertility is now definitely known to be restricted to colloidal gold particles sized >20 nm (Berciaud *et al*., 2005). The colloidal gold, enriched in its radioactive isotope Au-198, entered the history of Western official medicine in 1952. At that time, it was considered an advantageous alternative to radium for the treatment of prostate cancer; its use preceded that of other colloidal radioactive isotopes for the palliative treatment of a wide range of neoplasias (Fountain and Malkasian, 1981, Metz, Stoll and Plenert, 1982) and was abandoned late in the 1970s (Chassagne *et al*., 1985). The colloidal formulation was selectively toxic for neovascularized tissues: this

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Table 2. Commercially produced nanometals and their use in human medicine.

Trade Name	Drug and Size	Indication, Advantages	Clinical Trials[a]	Phase[b]
Superplastic Forming (SPF) sunscreens	$nTiO_2$	Topical, higher protection	1, closed	n.d.
Soladey-3	TiO_2-containing semiconductor toothbrush	Periodontal diseases, topical, high protection	1, recruiting	n.d.
CYT6091	Colloidal gold, TNF-bound (30 nm)	Solid tumors, advanced, systemic	2, closed	I
Nanopartz™	Nanogold: spheres (50 nm) and rods (10 nm)	Diagnostics, imaging and therapy	–	Preclinical
AgTive®	Central Venous Catheter (CVC) impregnated with nanoAg (10-100 nm)	Sustained release of active ions, implant	1, recruiting	IV
SilvaSorb®	NanoAg handgel	Topical antibacterial agent, high efficacy, longer efficacy	1, recruiting	III
Nucryst®	Cream and coating functionalized with Ag nanocrystals	Topical antibacterial agent	1, completed	II
Lipoplatin®	Cisplatin liposomal (110 nm)	Cancer (pancreas, ovary), low toxicity, longer half-life and sustained release	3, closed	III
NC-6004	Cisplatin in polymeric micelles (30 nm)	Cancer (pancreas), i.v. infusion, low toxicity	1, recruiting	I-II
	Hum-195-Actinium-225[c]; atomic nano-generator, alpha-emitter	Leukemia, i.v. infusion; Low toxicity, optimal targeting	1, recruiting	I
Feridex® or Endorem®; Resovist®; Lumirem® or GastroMARK®	Small superparamagnetic iron oxide (SPIO); contrast agents for magnetic resonance imaging	Cancer imaging; i.v. infusion(Feridex®, Resovist®) or oral ingestion (Lumirem®)	2 recruiting, 2 closed	IV
			3 closed	III
Combidex® or Ferumoxtran® Sinerim® Ferumoxytol®, Cliavist®	Ultrasmall superparamagnetic iron oxide (USPIO); contrast agents for magnetic resonance imaging	Diagnosis	5 recruiting, 6 closed	IV

[a] Listed at the ClinicalTrials.gov repository.

[b] See Endnote b.

property explained its selective effect on neoplastic growth as well as the powerful anti-inflammatory and analgesic effects of non-radioactive colloidal gold, widely used to treat chronic polyarthritis and knee synovitis (Boerbooms *et al*., 1985). The proclivity of colloidal metals to accumulate in newly formed endothelia is now recognized as a basic pharmacokinetic character of all nanosized compounds, the so-called enhanced permeation and retention (EPR) effect. The availability of newer, safer and more powerful drugs led to the neglect of colloidal gold for these use until recently, when colloidal gold prepared under controlled conditions and functionalized with TNF has been proposed: it is still under evaluation for tumour treatment in humans (Paciotti *et al*., 2004; Visaria *et al*., 2006, 2007; Goel *et al*., 2009) (Table 2).

The colloidal silver suspensions are only minimally characterized as pure nanosized silver: they lack the dimension-dependent colour shift observed in gold, and the element is by far more reactive, leading to the formation of oxides. Its systemic use in alternative medicine, mainly as an antiseptic agent and a supposed immunomodulator, is charged by a

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specific set of side effects, known with the name of argyria (Archer 2008; Baral, Dewar and Connett, 2008).

Nanometals recently gained a renewed interest in medicine, after their production in a standardized, industrial and controlled manner. A number of customized products have been introduced in the market for care and diagnosis: several of them are listed in Table 2.

The metals used to this end (i.e., Ti, Fe, Ag, Pt and Au) mainly belong to the transition metal group; it should be remembered, however, that, when organized at nanoscale, transition metals do not behave as metals, but as semiconductors (Mikrajuddin *et al.*, 2000). In addition to transition metals, lanthanides are of interest: the radioactive Ac-225 has been used to develop targeted alpha-emitting atomic nanogenerators (Miederer *et al*., 2004). The first and only clinical study with this new therapeutic is still open at the Memorial Sloan Kettering Cancer Center of New York, USA.

Nano-titanium dioxide ($nTiO_2$), eventually combined with nZnO, has been used in sunscreen protection and proposed for photocatalytic dressing of wounds: its indiscriminate use has been heavily criticized for both health and environmental concerns (Beck 2005; Nohynek *et al.*, 2007; Barker and Branch, 2008). Another nanotechnology-based device undergoing clinical evaluation and containing TiO_2 is a semiconductor toothbrush, which undergoes clinical evaluation in a still recruiting clinical trial (Table 2).

Powders, functionalized textiles, medical implants and devices with antiseptic properties related to the release of silver nanoparticles have been recently introduced in the pharmaceutical market, or undergo clinical evaluation (Ricco *et al*., 2006; Gravante *et al*., 2009; Larese *et al*., 2009). Two open trials on nanosilver are at present registered at the ClinicalTrials.gov (the repository of clinical trials maintained at the National Institute of Health, Bethesda, MA, USA) (Table 2). They will test the benefits against the growth of microorganisms, of a nanosilver-containing gel for hand cleaning (SilvaSorb®, phase III[b], Madigan Army Medical Center, Washington, USA) and of a central venous catheter embedded with nanosilver (AgTive®, phase IV, Policlinico Gemelli, Rome, Italy).

Several preparations containing metal NPs are available or have underwent clinical evaluation as injectable therapeutic agents, mainly for treatment of advanced or refractory neoplasias. The most relevant among them is the nanosized formulations of cisplatinum (*cis*-diamminedichloroplatinum) embedded in liposomes (Lipoplatin®), or in polymeric micelles (NC-6004). These formulations show lower toxicity, better tolerability, longer half-life and higher antineoplastic activity, in comparison with non-nanosized formulas (Uchino *et al.*,2005; Froudarakis *et al*., 2008; Jehn *et al*., 2008; Kim *et al*., 2008; Ravaioli *et al*., 2009).

Other metal NPs have been mainly developed for diagnostic use, including small and ultrasmall superparamagnetic iron oxides (SPIO and USPIO), nanogold and nanosilver prepared as highly regular spheres, rods, triangular prisms and quantum dots. Nanogold and nanosilver are mainly used for "*in vitro*" studies; however, they have been injected into the systemic circulation in animals, as "*in vivo*" staining, bioactive probes. Their advantages in comparison with existing molecules and dyes are a combination of powerful and unique optical properties and compartmentalization into cells and tissues (Hama *et al*., 2007; Yang and Cui, 2008; Millstone *et al*., 2009; Rzigalinski and Strobl, 2009). Unfortunately, the toxic potential of these probes seems to be elevated, and it is not sufficiently documented in pre-clinical studies to admit them to clinical trials.

At present, iron is the most represented of nanometals submitted to evaluation for clinical use: SPIO and USPIO are the subject of 15 registered clinical trials, six of them still recruiting. The signal of these particles is considered more powerful than that of other paramagnetic probes, and the proclivity of both to accumulate in macrophages and in cells of the reticular endothelial system permits to record clean magnetic resonance images of metastasis, inflammatory processes and atheromas (Turvey *et al.*, 2005; Barentsz, Futterer and Takahashi, 2007; Barnett *et al.*, 2007; Tang *et al.*, 2009; Thoeny *et al.*, 2009). The application of superparamagnetic iron oxide to vascular imaging is limited by the short half-life of these compounds in circulating blood: a brilliant solution circumventing this problem could be the recently proposed internalization of SPIO nanoparticles inside functionalized red blood cells (Antonelli *et al*., 2008). Another interesting therapeutic application of USPIO is in the treatment of refractory anaemia, such as those affecting non-dialysed patients with chronic renal insufficiency (Landry *et al.*, 2005; Singh *et al.*, 2008): four trials are registered (Table 2).

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NPs, if coated with antibodies or other molecules capable of binding specific tumour biomarkers, can selectively stick to the surface of cancer cells and eventually be endocytosed. So engineered NPs can, in principle, serve for diagnosis allowing a precise localization of cancer cells in the body as well as for innovative treatment methodologies. They could, in fact, be used as shuttles for precisely delivering therapeutic agents and minimizing the damage to the healthy cells or even become themselves the killing agent for the cancer cells. Gold NPs, heated by harmless radio frequencies, could "cook" tumour cells without damaging the neighbouring tissue (Gannon *et al*., 2008; Schmidt, 2008). Similarly, magnetic NPs can be heated by alternating magnetic field, and several efforts are currently being made to improve their heating efficiency (Kline *et al*., 2009). Moreover, after injection in the bloodstream, magnetic NPs could be guided in proximity of the tumour by a magnetic field to facilitate their interaction with the cancer cells.

In conclusion, despite the heavy toxicity of several metals used as therapeutic or systemic agents and the high bioactivity of NPs which are intentionally engineered to interact with cells and are deliberately and directly injected into the body, metal NPs show promising features for medical applications, both for diagnosis and for cure. Although these fields of knowledge are at their beginning in medicine, it is important to ensure that these enhancements do not cause any adverse effects or, at least, to be aware of the risks and benefits.

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END NOTES

[a.] Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase, is the key enzyme in photosynthesis and photorespiration. Composed by eight small and eight larger subunits (encoded by the nuclear and mitochondrial genome, respectively), Rubisco is considered to be the most prevalent protein on the earth.

[b.] The phases of clinical trials are designed with roman symbols, from I to IV: all are conceived in humans. Phase I: limited to the study of pharmacokinetics and effects in healthy and diseased subjects; Phase II: controlled trials to define indications and short-term side effects; Phase III: uncontrolled and extended protocols to define risk/benefit of molecules retaining a proven efficacy; Phase IV: post-marketing surveillance.

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Nanotoxicology–the Toxicology of Nanomaterials

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Multifunctional Nanomaterials for Biomedical Applications: The Onset of Nanomedicine

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1 INTRODUCTION

The use of nanomaterials in biology and medicine is one of the most intensely researched areas in nanotechnology. The emerging field of nanomedicine bridges the scientific disciplines of nanotechnology and the biological sciences, delivering a valuable set of research tools and medical devices for the diagnosis and treatment of medical diseases and conditions. In the last few years, carbon nanotubes (CNTs) and magnetic nanoparticles have become the most commonly used nanomaterials for medical applications, especially for the prevention and treatment of cancer. Various nanoparticles (diameter < 10 nm) have been used as carriers of anti-cancer drugs and have been observed to penetrate the cells and aggregate around the nuclear membrane – afterward penetrating into the nucleus (Mahmood *et al.*, 2009; Mu, Broughton and Yan, 2009; Xu *et al.*, 2010; Kam, Liu and Dai, 2006; Pantarotto *et al.*, 2004). The majority of published data agree with the endocytosis pathway, which shows that nanoparticles pass through the cell membrane by endocytosis, an energy-independent cell uptake process (Mu, Broughton and Yan, 2009; Kam, Liu and Dai, 2006). Pantarotto *et al.* (2004) have previously indicate the possibility of a mechanism for nanotube penetration through the membrane that is non-endocytotic and is energy-independent. Understanding these mechanisms of the cellular entry of various nanoparticles (such as CNTs), their intracellular translocation, subcellular locations, and excretion (Mu, Broughton and Yan, 2009) are of utmost importance in order to comprehend possible nanoparticle hazards and their potential for drug/gene delivery. Along the most commonly studied applications of nanoparticles as drug and gene delivery vehicles, they also find novel applications as thermal agents for the ablation of cells and tissues. Moreover, the development of radio-frequency devices that excite and heat magnetic nanomaterials delivered into tumors or to cancer cells opens up a new direction in the fight against cancer. Most of the magnetic nanosized materials present large magnetic moments and as a result have ultra-fast responses in the presence of magnetic fields. Moreover they can have low remanence (residual magnetism) and coercivity levels (Xu *et al.*, 2010). This characteristic allows them to easily absorb energy from radio frequency (RF) when they are targeted to certain tumors or cancer cells. This technique permits the destruction of tumors without necessitating their removal (*in situ*) and, in many cases, can be used in place of more invasive and expensive surgical techniques (Scott Gazelle *et al.*, 2000).

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2 SYNERGISTIC ENHANCEMENT OF CANCER THERAPY (CARBON NANOTUBES AND ANTITUMOR DRUG COMBINATION)

Carbon nanotubes have unique physical and chemical properties that have caused them to be considered potential drug carriers and delivery vehicles for cancer treatment (Liu *et al.*, 2008; Prato, Kostarelos and Bianco, 2008; Hampel *et al.*, 2008). CNTs can carry therapeutic drugs (Chen, Chen and Zhao, 2008) and apoptotic agents (Hampel *et al.*, 2008) safely and effectively into cancerous cells, thereby becoming ideal candidates for drug delivery. Nonetheless, drug resistance remains a major concern in the process of tumor treatment, due to lack of precise tumor targeting and toxicity. As already known, etoposide is a derivative of podophyllotoxin and a chemotherapeutic agent. In recent studies, it was used as a target for the nano-delivery system (Arimondo *et al.*, 2000; Kim *et al.*, 2002). This drug's apoptotic attribute lies in its ability to inhibit the topoisomerase II enzyme (Bromberg, Burgin and Osheroff, 2003). Etoposide is widely used for the treatment of different malignant tumors of the lung, brain, stomach, testis, and pancreas (Teicher, 2008). Even with advances in treatment protocols, etoposide still has a modest response rate that shows a success rate from 4 to 45% in pancreatic, breast, ovarian, cervical, and small cell lung cancer (Thomas, Dumas and Ajani, 1999; Herbert, Brames, Einhorn, 2006; Hijiya *et al.*, 2004; Perkins *et al.*, 2000; Stefanelli *et al.*, 2002; Smart *et al.*, 2008). It was observed that, using a single combined therapy of etoposide and CNTs on pancreatic (Panc1) and HeLa cancer cells, the anti-tumor activity was increased by about 40–47% when compared with the single administration of each agent (Mahmood *et al.*, 2009). This finding suggests that CNTs can alter the mechanisms of chemoresistance in malignant cells. This discovery could lead to new treatment approaches and improvement of current cancer therapies by using both cytostatic drugs and nanostructural materials (such as CNTs, gold nanoparticles, and silver nanoparticles), which synergistically are more aggressive and possess greater curative effects in the treatment of cancer (Mahmood *et al.*, 2009).

The process of apoptosis is extremely complex and can be induced by a number of intra or extra cellular signals with effects on the cells morphology and structure. Figure 1 shows the schematic of the apoptosis process induced in cancer cells exposed to a combination of CNTs and etoposide, which was found to be responsible for an accelerated death process of the cells and to have a

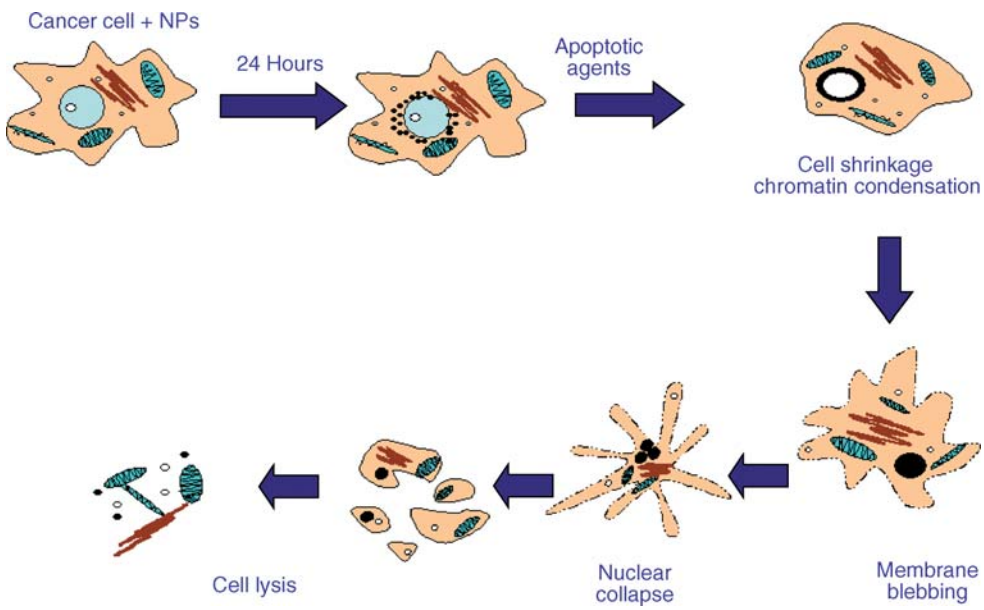

Figure 1. The experimental diagram of the CNT and etoposide delivery to various cell lines (Mahmood *et al.*, 2009).

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synergistic effect. Current therapies such as CNTs combined with etoposide exert a therapeutic effect by indirectly promoting apoptosis. This type of regimen induces apoptosis by causing DNA damage. In doing so, the nanotubes stimulate apoptosis through the intrinsic pathway. Figure 1 shows cell shrinkage, membrane blebbing, nuclear collapse, and, finally, apoptotic bodies (cell lysis) resulting from the synergistic enhancement by CNTs.

Two cancer cell lines, HeLa cells and human pancreatic cancer cells (Panc-1), were used to investigate the role of CNTs, etoposide agents, and their combination on the possible necrotic and apoptotic processes (Mahmood *et al.*, 2009). Trypan blue assay was used for the assessment of the viability of cells (dead cells). A very low percentage of dead cells were found in the control culture medium at levels of 2.26% and 5.73% for HeLa and Panc-1 cells, respectively. When CNTs and etoposide were introduced separately, cellular death increased to 14.1% and 7.80% for the HeLa cells. A slightly greater level of toxicity of CNTs was found when compared with etoposide, although the differences are not statistically significant. However, when HeLa cells were incubated with CNTs in combination with etoposide at the same dosage as when incubated individually, a significantly higher cell death rate of 49.1% was observed – an increase of 46.9% over the controls (Figure 2). The combined effect was approximately 2.7-fold higher ($P < 0.05$) than the sum of individual effects of CNTs and

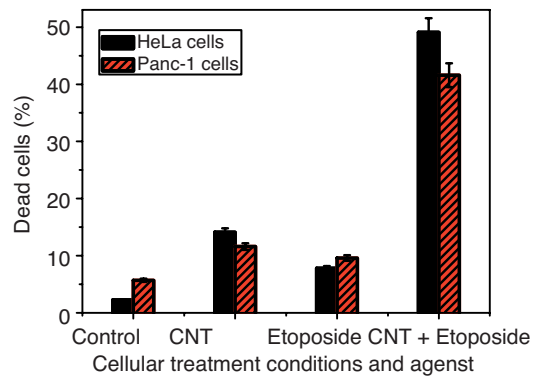

Figure 2. Percentage of dead HeLa cells and Panc 1 cells after exposure to various agents (etoposide, CNTs, and their combination) as a reference to control. The cells were incubated for 24 h with CNT (10 μg ml^{-1}), and an additional 6 h with etoposide (75×10^{-6} M). The experiments for the two cell lines were performed in identical conditions for comparison.

etoposide (17.4%). The percentage of Panc1 cells dead after exposure to the combination of CNTs and etoposide was 41.6%, slightly lower than that for HeLa cells. Furthermore, the number of dead cells was dependent on the concentration levels of CNT and etoposide. By increasing the concentrations of CNTs and etoposide, individually or combined, the percentage of dead cells increased significantly ranging from 62.1% to over 80.0%, respectively. This result suggests that CNTs have a role in decreasing the chemoresistance of cells. In addition, dexamethasone, another commonly used anti-cancer drug, was investigated under the same conditions, and the results showed similar trends.

Microscopic images of single HeLa cells in Figure 3 illustrate that the administration of CNTs alone did not change cell morphology significantly (Figure 3b) as compared with the control (Figure 3a), although a slight decrease (about 10%) in the size and shape of the cells was observed. It can be seen that CNTs appear to cluster inside the various cellular compartments; however, it was the combined CNT–etoposide action that led to significant changes in cellular morphology. The aggregates within the cell presented in Figure 3b and c represent CNT clusters as identified by Raman spectroscopy. Raman signal corresponding to CNTs inside the nucleus was found. As shown in these figures, the difference in the intensities coming from the nucleus and cytoplasm is quite significant, indicating that only a small percentage of the CNTs reached the nucleus. It has been argued that the varied mechanisms of cell uptake could be due to the different surface characteristics of CNTs. Earlier studies have indicated that CNTs can cross the mammalian cell membrane by endocytosis or other mechanisms (Mu, Broughton and Yan, 2009). The ability and kinetics to penetrate inside of cells through the bi-layered cellular membrane have been shown to be a function of their surface charge (Mu, Broughton and Yan, 2009).

Flow cytometry can be used to verify the apoptotic and necrotic effects of CNTs, the chemotherapeutic agent, and their combination by staining the cells with specific fluorescent dyes. Figure 4 shows that the percentage of apoptotic cells increased after the treatment of HeLa cells with etoposide and CNTs. Furthermore, the combined CNTs and agent drastically increased the population of cells undergoing late stages of apoptosis or necrosis. These results strongly suggest that the

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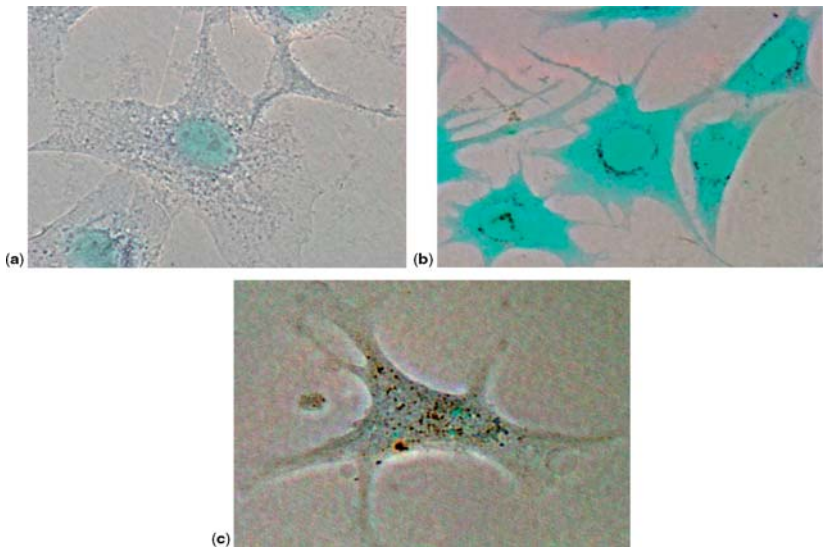

Figure 3. Optical image of a single HeLa cell morphology before (a) and after (b) the delivery of CNTs alone (10 μg ml^{-1}, 24 h incubation time). (c) Image of a HeLa cell that was exposed for 24 h to CNT (10 μg ml^{-1}) and an additional 6 h to etoposide (75×10^{-6} M).

CNTs–etoposide combination therapy achieved a greater cell death response and perhaps may eliminate cancer cells more efficiently.

Caspase-3 assay was the other common method used to assess the possible apoptosis of cells. To confirm the synergistic enhancement of apoptosis by CNT–etoposide, these results were further verified by assessment of the caspase-3 activity performed by staining the cells with Red-DEVD-FMK dye. Sequential activation of caspase plays a central role in the execution phase of cell apoptosis. The fluorescence brightness level of the red stain indicates the caspase-3 activation level in the cells. The control cells (Figure 5a) showed a weak fluorescence, but became brighter when the cells were incubated with CNTs (Figure 5b) and, similarly, with etoposide, alone (Figure 5c). Additionally, these data indicate that CNTs were found to induce a larger number of dead cells compared with the samples treated only with etoposide and to have a strong interaction with the cells via caspase pathways, which is also typical for the etoposide action (Moosavi *et al.*, 2006; Spitaleri *et al.*, 2009). Furthermore, when the cells were incubated with the combination of CNTs and etoposide, the caspase-3 activity expressed by the red stain was extremely intense (Figure 5d) – clearly indicating that the administration of both etoposide and CNTs enhances and activates apoptosis through the caspase cascade.

Etoposide inhibits the enzyme topoisomerase II, which unwinds DNA, and, by doing so, causes DNA strands to break. Cancer cells are less able to repair this damage than healthy cells (Boyer *et al.*, 2001). As previously shown (Morisaki and Katano, 2003), this DNA damaging agent can trigger cell death through the activation of p53-mediated caspase cell death signaling cascade (Li *et al.*, 2001). The ultimate result of these molecular changes induced by etoposide is a process of self-destruction in human malignant cells. In the present study, the low response rate of etoposide alone compared with the untreated HeLa cells (also confirmed by earlier reports (Mirzaie-Joniani *et al.*, 2002; Seminara *et al.*, 2007)) is caused by chemoresistance mechanisms activated inside the cells (Reddy *et al.*, 2003). Apoptotic deficiency is one of the main mechanisms of chemoresistance to anti-neoplasic drugs. It has been shown that etoposide administration leads to the up-regulation of pro-apoptotic proteins such as Bax proteins (Reddy *et al.*, 2003; Panaretakis *et al.*, 2002). These protein families translocate from the cytosol to the mitochondria preceded by the release of cytochrome *c* that contributes to the induction of the mitochondrial permeability transition (MPT). This process is accomplished by the coupling of DNA damages and leads eventually to the necrosis of the cells. The activity of several cofactors in the cytoplasm, including apoptotic protease activating

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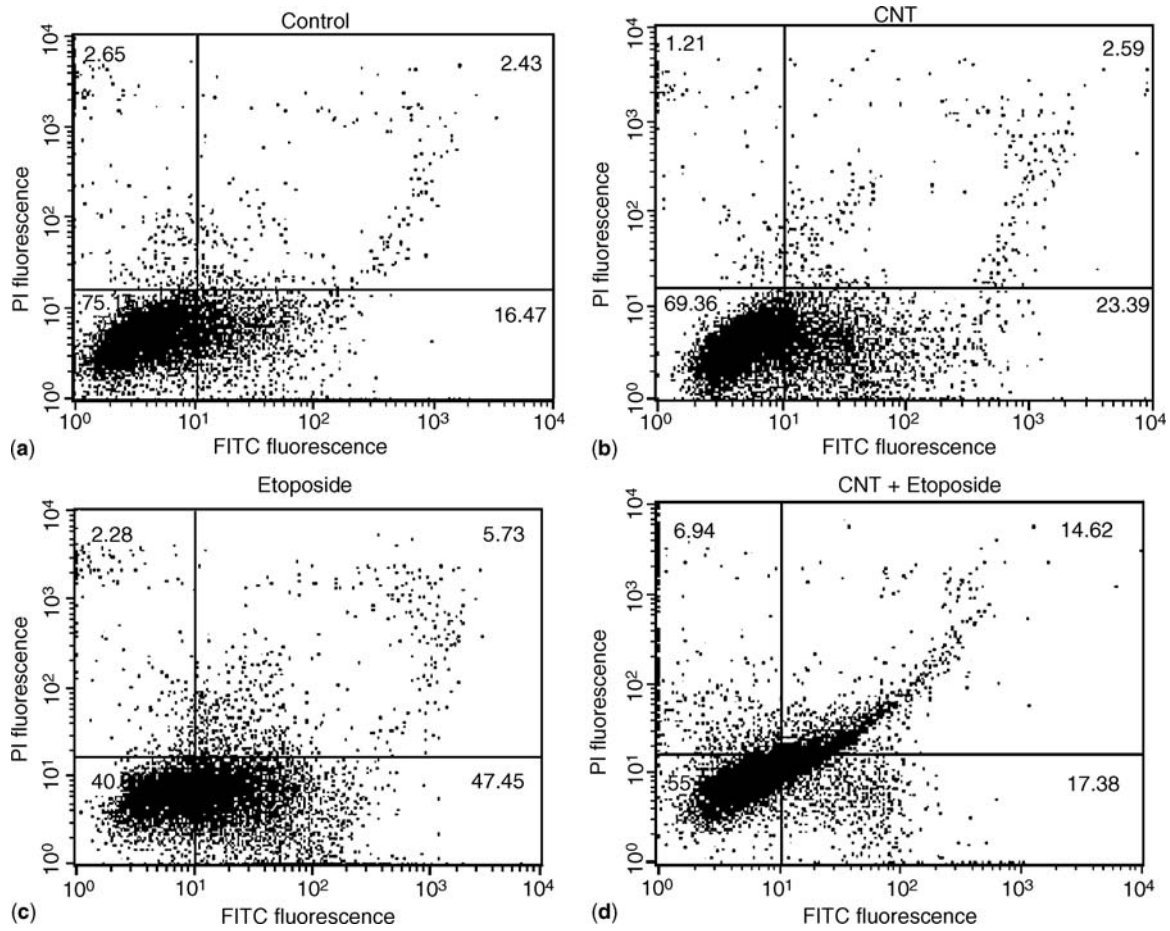

Figure 4. Flow cytometry data indicating the effect of DMSO (as vehicle control) (75×10^{-6} M), CNTs ($10\,\mu g\,ml^{-1}$, 24 h), and etoposide (75×10^{-6} M, 6 h) as mono- and combined therapy of the HeLA cells. Cells were stained with YO-PRO-I and PI dyes from Vybrant viability assay following manufacturer's instructions and analyzed by flow cytometry. Shown in quadrants are live (c), apoptotic (d), and necrotic cells (a and b) (Mahmood *et al.*, 2009).

factor-1 (Apaf-1; protein involved in cellular death) and ATP/dATP, coupled with the presence of some enzymes (pro-caspase-9) could be responsible for the generation of the apoptosome complex in the cytoplasm, which activates caspase-9 responsible for the activation of the caspase-3 proteins. Caspase-3 plays an important role in the apoptosis process being primordially involved in the cleavage of the poly ADP ribose protease (PARP) taking place during cell death and leads to the degradation and fragmentation of the chromosomal DNA inside the nucleus (Van Maanen *et al.*, 1988).

It has been shown that resistance to etoposide is due to molecular changes that affect the apoptotic cascade at different levels (Hwang *et al.*, 2008). Multiple mechanisms that block the activation of caspase cascade have been proposed to explain the acquired resistance of malign cells to etoposide (Hwang *et al.*, 2008). Studies have reported that the inactivation of Apaf1 is associated with chemoresistance in metastatic melanoma (Soengas *et al.*, 2001). Other reports have shown that the activation of various caspases is required for p53-mediated apoptosis and chemosensitivity in cancer treatment, suggesting that the analysis of the p53-dependent caspase activation could predict the chemosensitivity in some cell types (Wu and Ding, 2002). It has previously been shown that

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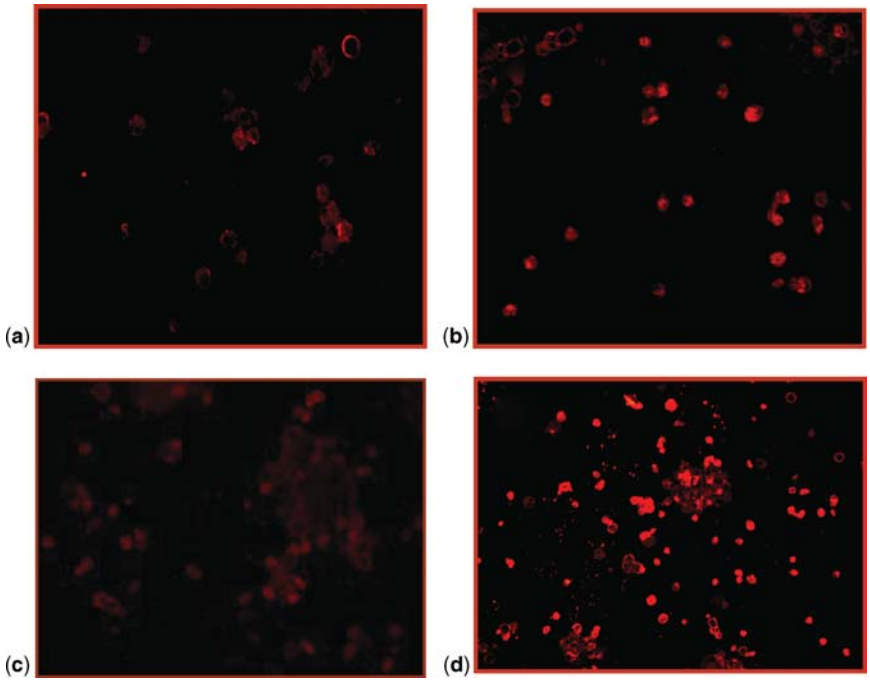

Figure 5. Caspase-3 activity in HeLa cell cultures: (a) control sample (cells unexposed to any agents); (b) samples exposed to CNTs ($10\ \mu g\ ml^{-1}$, 24 h), (c) cells exposed only to etoposide, and (d) samples exposed to a combination of CNTs and etoposide (75×10^{-6} M, additional 6 h) (copyright 2009 future nanomedicine).

targeting p53 for degradation by the human HPV E6 gene in the ovarian cancer cell line PA1 leads to an increase in the chemoresistant phenotype. Etoposide induces caspase-7 activation (Rothstein, 2000) and it was proved that an endoplasmic reticulum (ER) resident protein over-expression, GRP78, suppresses the activation of caspase-7, both *in vivo* and *in vitro*, and consequently blocks the full activation of the multiple caspase-mediated cell death pathways in drug-treated cells, contributing to the development of drug resistance (Reddy *et al.*, 2003).

It is also possible that CNTs induce cellular toxicity due to the accumulation of peroxidative products, generation of oxidative stress, mitochondrial dysfunction, and modifications in the morphology of the cells (Manna *et al.*, 2005). Several studies have indicated varying mechanisms of CNT uptake into the cells, of which endocytosis is believed to be the most probable process (Shi Kam *et al.*, 2004). CNTs penetration inside the various cellular compartments such as the cytoplasm or the nucleus, can be expected to lead to strong interactions between the CNTs and the protein or DNA structures of the cells which could affect various biological processes related to the cells activity. As a result, CNTs can be responsible for alterations in cellular signaling, metabolism, or motility affecting the mechanisms of chemoresistance, especially cell-cycle-mediated drug resistance. The first response of the cell in contact with CNTs is represented by the activation of antioxidant and detoxification enzymes followed by inflammation, which is a result of the activation of a pro-inflammatory signaling cascade (Andre *et al.*, 2006). The most common responses are the generation of mitogen-activated protein kinase (MAPK) and nuclear factor kB (NF-kB) cascades. The ultimate response is represented by mitochondrial perturbation, activation of caspase cascade, and release of pro-apoptotic factors that leads to cell death. Thus, both agents can induce apoptosis via some similar pathways that can interact and provide synergy leading eventually to cell death as a combination of apoptotic and necrotic phenomena. Some hypothetical processes focused on apoptosis are depicted in Figure 6.

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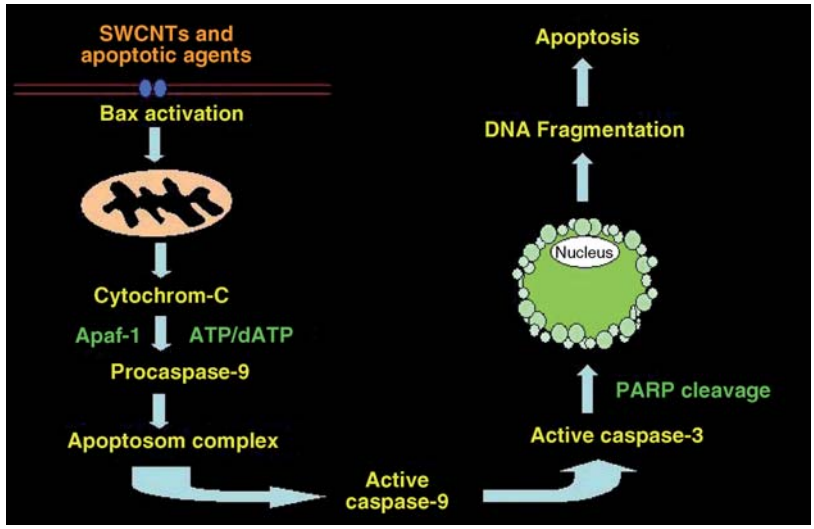

Figure 6. The proposed hypothetical mechanisms for etoposide–CNT combined action for the induction of apoptosis leading eventually to cellular death (Mahmood *et al.*, 2009).

Although this study did not involve targeting molecules, it provides insight into the use of nanomaterials to alter the chemoresistance of cancer cells when exposed to a combination of CNTs and chemotherapeutic drugs. It has been shown (Liu *et al.*, 2008) that CNTs *in vivo* have considerable ability to accumulate in several organs, such as the liver, kidney, and spleen. Moreover, given the intense blood vascularization of tumors, it is expected that the CNTs/drug system delivered in the blood circulation will naturally bio-accumulate in the tumors in larger amounts when compared with the non-cancerous tissues and therefore induce a high rate of cellular death, as shown in the present studies. The attachment of antibodies, folates, and growth factors to the nanomaterials for their specific delivery at the level of either individual or clustered cancer cells is further expected to greatly enhance the killing rate of the cells. Furthermore, the delivery of CNTs/anti-cancer drugs can be followed by other approaches that use the unique properties of the nanomaterials to absorb electromagnetic radiation and provide nanolocalized thermal ablation capabilities, such as laser heating, RF ablation, etc. This could result in the creation of a new treatment involving several steps in a procedure to completely eliminate cancer cells *in vivo*. These processes will have to be studied for various cancer cells lines in order to find the right experimental conditions for maximizing the death rate for each of these biological systems.

3 CARBON-COVERED MAGNETIC NANOMATERIALS AND THEIR APPLICATION FOR THERMOLYSIS OF CANCER CELLS

Magnetic nanoparticles are another group of nanomaterials that are of great interest for a wide range of biological and medical applications, such as magnetic resonance imaging (MRI) (Bulte and Kraitchman, 2004; Corot *et al.*, 2007), as well as medical diagnostics and therapeutics (Douziech–Eyrolles *et al.*, 2007; Reiss and Huetten, 2005), especially for *in vitro* and *in vivo* medical treatments. The stability and activity of these nanostructures in the presence of biological environments could dictate their possible utilisation in bio-medical applications. One cause of concern is their agglomeration, given by the tendency to lower their surface free energy, but which could dictate the level of their interactions with cells and tissues. The larger the agglomerates, the lower the ability to penetrate membranes and biological systems. However, nanoparticles can be easily oxidized in air and therefore lose partially or completely important properties, such as their surface reactivity, structural and magnetic characteristics, and their oxidative states (Lu, Salabas and Schuth, 2007). As a result, lately a significant amount of effort was spent on finding methods to stabilise these nanomaterials by the use of coatings that would limit

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their degradation and possible biological undesired effects. Although most studies have focused on the development of polymer or silica protective coatings, carbon-protected magnetic NPs have recently received more attention (Seo *et al.*, 2006; Wang *et al.*, 2008; Xu *et al.*, 2008) due to carbon's relatively higher chemical and thermal stability, inertness, as well as its enhanced biocompatibility (Lu, Salabas and Schuth, 2007; Geng, Jefferson and Johnson, 2004). For example, carbon coated nanoparticles were reported to remain in the metallic state with positive effects on their magnetic properties (Vilas, Swati and Aharon, 2008). Core-shell nanostructures often exhibit improved physical and chemical properties over their single-component counterparts, which gives them a broader range of applications. Besides their protective roles, these coatings also offer means of attaching the complex structures to biological systems (antibodies, proteins, DNA, etc.) in order to target particular cell lines such as cancer (Xu *et al.*, 2008).

Radio frequency resonance heating is less invasive when compared with other types of electromagnetic radiations (lasers, microwaves, etc.) and possesses higher efficiency for absorption into magnetic structures that are delivered to localized cancer cells or tumors – thus potentially reducing the side effects associated with traditional cancer therapies (Xu *et al.*, 2008). It is known that the capacity of RF energy fields for tissue penetration is excellent (Bernardi *et al.*, 2003). For example, low-frequency RF radiation of 350 kHz has high tissue penetration abilities and therefore can be used for the treatment of both superficial and deep tumors. As a result, the RF treatment of tumors non- invasively should be possible in the conditions in which the heat-generating agents under RF exposure are delivered in sufficient amounts at the site of the tumor. Previous studies have indicated that the used of electromagnetic radiation for thermal ablation of tissues can induce tumor necrosis of over 1.6 cm in diameter (Scott Gazelle *et al.*, 2000). However, most tumors are significantly larger; therefore, in order to accomplish successful results, most of the treatments required several sessions and the used of multiple RF probes. Based on these considerations, the focus of a number of studies was to develop technologies that can generate high enough thermal levels to successfully and reliably induce tissue necrosis after only one application without damaging the healthy tissue (Scott Gazelle *et al.*, 2000).

Researchers have shown that low RF can induce death in HeLa cells containing magnetic nanoparticles after only several minutes of RF application, a process that is greatly enhanced by the aggregation of nanoparticles inside the cells (Xu *et al.*, 2010; Xu *et al.*, 2008). DNA fragmentation further confirmed the thermal damages produced by the nanoparticles under the RF excitation inside the HeLa Cells (Xu *et al.*, 2008).

Fe, Fe/Co, and Co NPs covered with layers of carbon were all synthesized by radio frequency–catalytic chemical vapor deposition (RF-CCVD) (Xu *et al.*, 2010). Atomic force microscopy (AFM) and transmission electronic microscopy (TEM) analysis revealed that the average size of the C–(Fe,Co and Fe/Co) was about 7–10 nm, with the mean and standard deviation of sizes measured by TEM for ∼100 nanoparticles in each sample. The NPs were covered with 2–8 layers of graphitic carbon, as shown in Figure 7b. XPS analysis data (Figure 8) showed that the metallic states of the Fe, Fe/Co, and Co NPs were well protected by the graphitic shells (Xu *et al.*, 2010).

Here, it was found that non-functionalized, graphitic-shelled nanoparticles can be effectively taken up into the cells (Xu *et al.*, 2010; Seminara *et al.*, 2007) The *in vitro* nanoparticles translocation inside the cells occurred mostly due to endocytosis, but also based on other processes that include: diffusion, membrane channels, and various adhesive interactions (Geiser *et al.*, 2005). However, the mechanism of cell uptake and the cellular fate of graphitic structural NPs is not fully understood; moreover, current descriptions are controversial, especially with respect to the cellular uptake of CNTs (Mahmood *et al.*, 2009; Smart *et al.*, 2008; Bulte and Kraitchman, 2004; Koistinen *et al.*, 2001; Douziech–Eryrolles *et al.*, 2007; Reiss and Huetten, 2005), their intracellular translocation, and their subcellular localization (Corot *et al.*, 2007; Lu *et al.*, 2004; Hyeon, 2003; Yavuz *et al.*, 2006; Seo *et al.*, 2006). A popular view is that NPs are taken up by cells through clathrin-dependent endocytosis (Koistinen *et al.*, 2001). Protein- or DNA-coated NPs have been shown to enter cells in an energy-dependent manner. Although the majority of published data agree with the endocytosis model, energy-independent cell uptake has also been reported (Mu, Broughton and Yan, 2009). Among all the properties of the nanoparticles, those that are believed to be the most important for their

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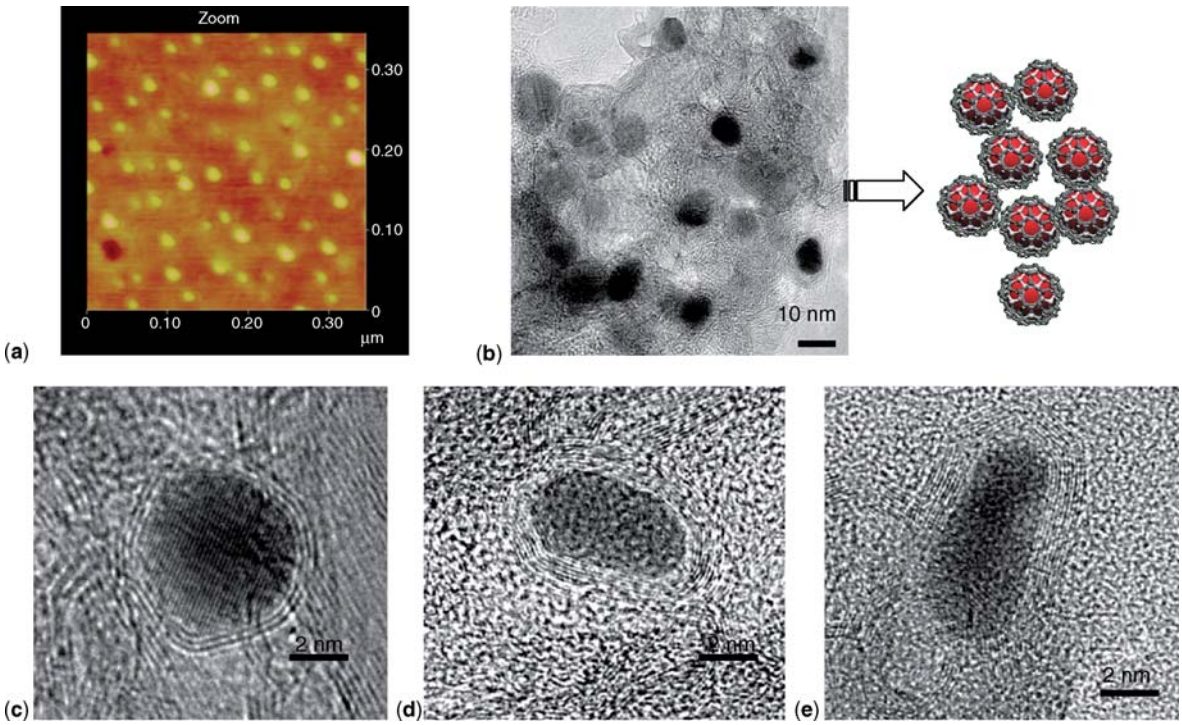

Figure 7. (a) AFM topographic images showing individual C–Co NPs. (b) Low- and (c–e) high-magnification TEM images of graphitic (c) C–Co, (d) C–Fe, and (e) C–Fe/Co NPs obtained by RF–cCVD method (Xu *et al.*, 2010).

translocation inside cells are size, type, and surface charge (Mu, Broughton and Yan, 2009; Rothen-Rutishauser *et al.*, 2006). Individually dispersed NPs in the medium solution used to feed the cells were visualized to aggregate around the nucleus and then penetrate inside (Xu *et al.*, 2008).

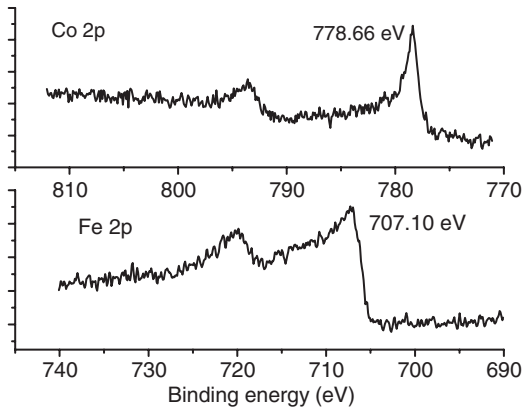

Figure 8. XPS spectra of the Co2p and Fe2p peaks represent the patterns of C–Fe and C–Co magnetic NPs (Xu *et al.*, 2010).

Cytotoxicity studies involving HeLa cells cultured with the C–Fe, C–Fe/Co, and C–Co NPs were carried out by EB/AO staining assay for cell viability. The results indicated that the great majority (99.4–99.7%) of the total cultured cells were alive (below the concentration of 50 $\mu g\,ml^{-1}$), revealing a low level of toxicity of these magnetic NPs – although the toxicity of nanomaterials inside living biological systems is still an intensively studied topic. Furthermore, it was found that RF radiation, alone, had scarcely any effect on the HeLa cells: 93.0–96.2% of these cells survived RF treatment, depending on the radiation time.

HeLa cells cultured with various concentrations of magnetic NPs were introduced inside a water-cooled coil coupled to a radiofrequency generator (Pillar, Brookfield, WI, TX, USA) with the frequency of 350 kHz (as shown in Figure 9), which is far lower than the 10 MHz to 300 GHz that is commonly used (Leea *et al.*, 2005). The depth of penetration of RF radiation in human tissue decreases as the frequency increases. The frequency of the AC electromagnetic field should be chosen

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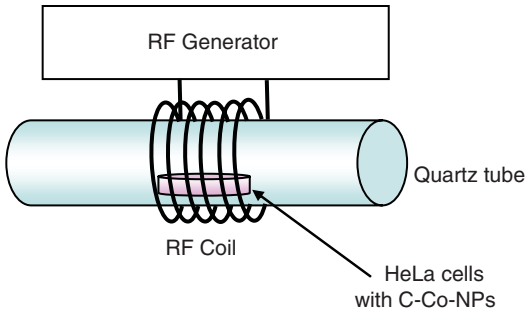

Figure 9. RF excitation setup (350 kHz, 5 kW) used for the thermal ablation of Hela cells (Xu *et al.*, 2008).

based on a compromise; namely, it must be higher than the frequency capable of provoking neuromuscular response and lower than the frequencies causing overheating of healthy tissues. It is believed that the frequency should be in the range 100–1000 kHz; in this case, correctly chosen frequencies and electromagnetic field strengths produce no notable *in vivo* side effects of the AC magnetic field (Nikiforov, 2007).

Three key factors – RF radiation time, concentration of NPs, and the species of NPs—determine the effect of RF exposure on biological cells or tissue. As shown in Figure 10a, after 2–30 min of radiation exposure with constant NP concentration, the number of dead cells gradually increased as RF heating time increased. During RF exposure times longer than 10 min, a rather slow increase in the percentage of dead cells was observed, mostly dependent upon the type of magnetic nanoparticles used in the study. For the C–Fe NP, after only 2 min of RF exposure, 67.0% of cells were observed to have died. Increasing the heating time to 30 min caused the percentage

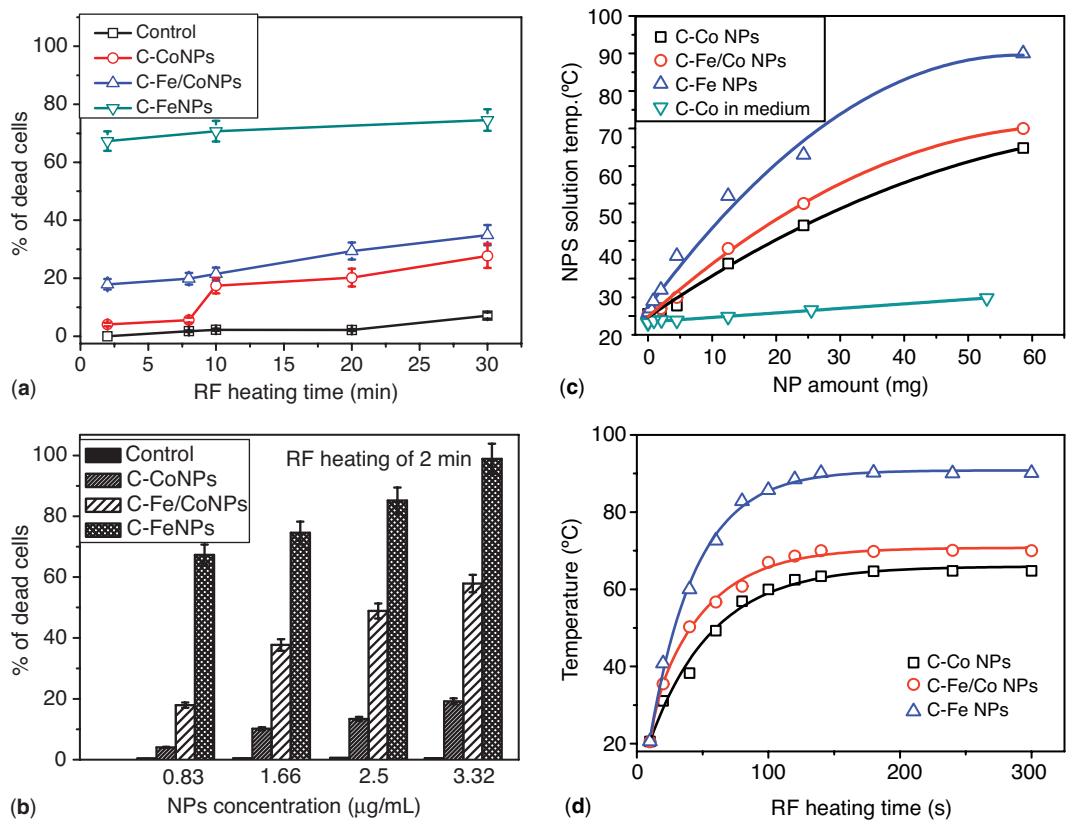

Figure 10. (a) Cytotoxicity effects of the low concentration (0.83 μg ml^{-1}) C–Co, C–Fe/Co, and C–Fe NPs on the HeLa cancer cells after 2–30 min of RF exposure (Xu *et al.*, 2010); (b) effect of different concentrations of the C–Fe, C–Fe/Co, and C–Co NPs on the HeLa cells that died from 350 kHz RF heating after 2 min of exposure time (Xu *et al.*, 2010); (c) comparative RF-induced temperature variations for the dispersions of C–Co–NPs in the media solutions and powders as a function of nanoparticle amount; (d) the temperature rising characteristics of different magnetic NPs with the same amount under 350 kHz RF exposure.

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of dead cells to reach about 74.5%. If the three types of nanoparticle were kept at the same low concentration (about 0.83 $\mu g\,ml^{-1}$), after the RF heating, C–Fe NPs exhibited the highest killing rate of the HeLa cells, as presented in Figure 10a, which is 2.14 and 2.69 times higher than that induced by the C–Fe/Co NPs and C–Co NPs, respectively. The results indicated that the C–Fe NPs were the best RF absorbers and induced cellular death in the shortest time and at the lowest concentrations (Xu *et al.*, 2010). As for the third factor, concentration of NP, if the exposure time was kept constant, the percentage of dead cells was found to be highly dependent on the NP concentration. As shown in Figure 10b, when the NP concentration was increased to 3.33 $\mu g\,ml^{-1}$, C–Fe NP killed almost 98.88–100% of the exposed cells with only 2 min of RF exposure. However, for the C–Co NP, a significantly higher concentration ($\sim$20 $\mu g\,ml^{-1}$) was required to cause the same effect (Xu *et al.*, 2008). From the aforementioned findings, we can conclude that the concentration of NP and the type of NP largely determine the cancer cell death.

The localized heating of NPs inside the cancer cells comes from the hysteresis heating and resistive heating through eddy currents generated under the RF radiation. As known, the resulting magnetic field induces eddy currents in the body, and the power is dissipated into heat by the Joule effect. In order to investigate the heating effects inside the HeLa cells, temperature changes in nanoparticles in powder form and nanoparticles dispersed in solution under RF were studied.

For the purpose of comparison, the surface temperature increase for the NP powder and the Co–NP dispersed in the medium solution with varying concentrations under RF heating for 5 min was continuously monitored and is shown in Figure 10c. The high-sensitivity thermal analysis indicated that the RF-induced temperatures, as well as their heating rates, rose and were found to be dependent mostly upon the mass of the NPs used for the measurements. The C–Fe NPs were shown to be the best hyperthermia agents because they were found to reach temperatures higher than the C–Fe/Co and C–Co NPs under identical RF exposure conditions (Xu *et al.*, 2010). When the NPs were individually dispersed in the medium solution, no significant level of heating was observed, and the temperature relatively constant (only a shift about 5 °C) with increasing the NPs concentration. Based on these experimental results, cell death is not expected to occur due to the bulk heating of the entire cell structures, but rather due to the localized heating where the NPs aggregate. The damage to cell membranes (especially of the nucleus), DNA fragmentation, protein thermal damages and denaturation occurred mostly at temperatures superior to 55 °C (Xu *et al.*, 2008). These studies are in good correlation with previous studies that have reported that, for the *in vivo* heating of up to 42 °C, about $\sim$1.2 mg particles per 1 cm^3 volume of tissue are required (Rothen-Rutishauser *et al.*, 2006). In this study, the NP concentrations used were too low (around 3.3–20 $\mu g\,ml^{-1}$) to allow for exact temperature measurements of the nanoparticles taken up by the cells (Xu *et al.*, 2008). The heat transfer between the nanoparticles and the liquid environment is largely governed by the heat transfer kinetic processes, and, the dimension of the nanoparticles or the size of their agglomerates inside the solution, that can vary from a few nm to several microns. However, RF radiation was found to be highly absorbed by the nanoparticles and which acted as thermal agents creating localized damage in various cellular sub-compartments (10–50 μm), which then induced the death of the cells (Xu *et al.*, 2010; Xu *et al.*, 2008). Besides the thermal effects induced by the RF irradiation inside the magnetic nanoparticles, this type of electromagnetic radiation can also make tissues and cells more susceptible to other types of cancer treatments, since the localized heating and breakage of the nuclear membranes facilitate the administration of various drug molecules (Xiang *et al.*, 2007).

The localized heating of carbon-shelled magnetic NPs that occurs under RF radiation could undergo an 'auto-heating' event–in that the NPs may generate their own heat energy based on molecular rotation. Rotation of particles is usually referred to as the Brownian rotation; rotation of the magnetic moment inside each particle is called the Néel relaxation under application of RF radiation (Kan *et al.*, 2005). The Néel relaxation time τ_N of the magnetic NPs under an external magnetic field is expressed by the following formula (Rosensweig, 2002):

$$\tau_N = \frac{\sqrt{\pi}}{2}\tau_0 \quad \frac{Exp(KV_M/k_BT)}{(KV_M/k_BT)^{1/2}}$$

where τ_0 is the relaxation time constant and has the order of 10^{-9} s, K is the anisotropy constant,

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and V_M is the magnetic volume of particles. τ_N for the Néel relaxation is determined by the ratio of the energy of magnetic anisotropy of superparamagnetic particles to the thermal energy (Rosensweig, 2002).

In the initial stage from 0 to 60 s, the temperature of the three NPs increased rapidly as shown in Figure 10d. After about 200 s, final temperatures of 65, 70, and 90 °C were reached for Co, Fe/Co, and Co NPs, respectively. This is due to the thermally stable spin rotation of the ferromagnetic NPs caused by the RF-induced hysteresis loss. The ferromagnetic NPs show hysteretic properties under a magnetic field, leading to magnetically induced heating. The amount of heat (power dissipation) generated per unit volume, W, is given by the frequency multiplied by the area of the hysteresis loop in the M–H coordinates caused by the hysteresis loss (Rosensweig, 2002):

$$W = -\mu_0 f_{\text{appl}} \cdot \oint H \cdot \mathrm{d}M$$

For ferromagnetic nanomaterials with sizes greater than those of superparamagnetic ones, there is no definitive frequency dependence with hysteresis loop surface; therefore, the heat generation level can be found from measurements using SQUID magnetometer. Figure 11 compares magnetization loops measured at 5 and 300 K for the C–Fe, C–Fe/Co, and C–Co NPs. C–Co nanostructures were observed to have a larger saturation magnetic induction (M_s), larger residual magnetization (M_r) and smaller coercivity (H_c) relative to C–Fe/Co or C–Fe

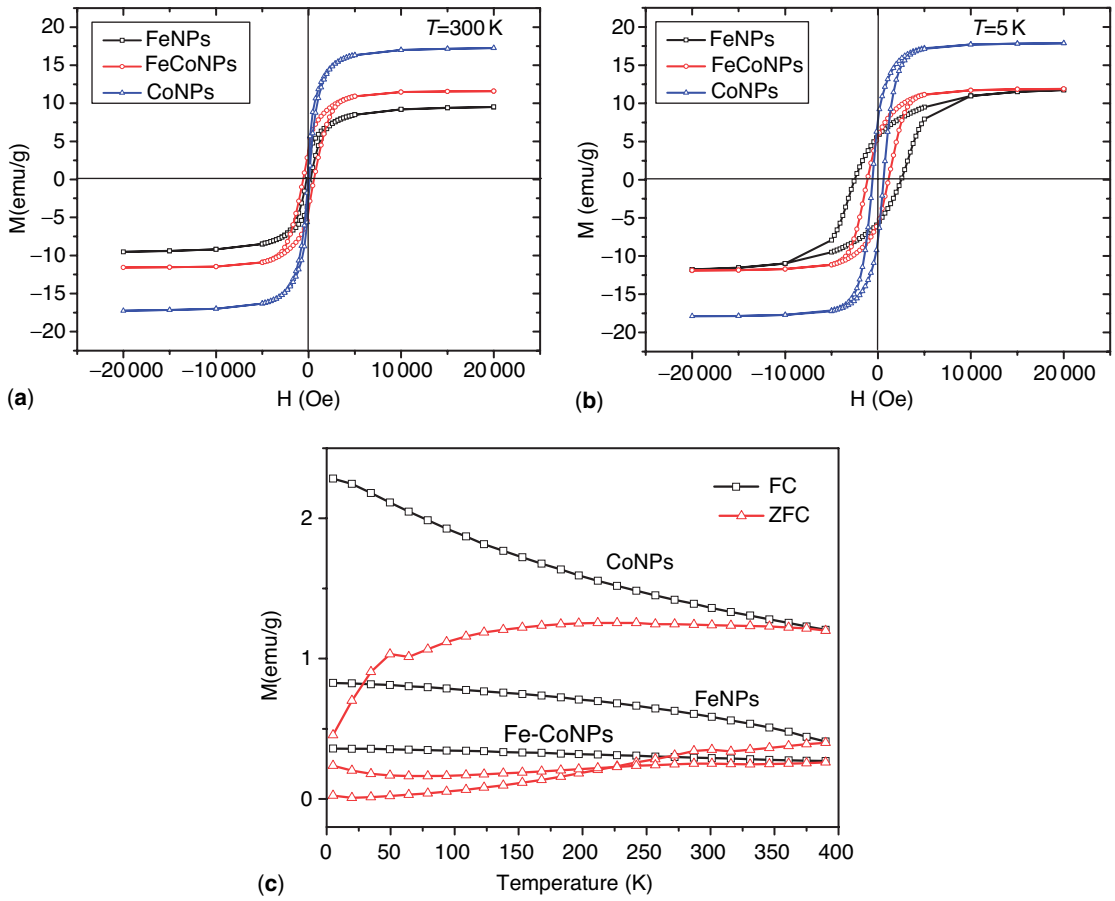

Figure 11. Magnetization curves of different magnetic NPs at (a) 300 K and (b) 5 K; (c) temperature dependence of ZFC and FC measurements recorded at a magnetic field of 30 Oe for these magnetic NPs (Xu *et al.*, 2010).

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nanostructures. One cause is the nanoparticle size distribution. The specific energy absorption along with the heat generation capacity and integrated with the particle density will provide the thermal emission power per unit volume *W*, giving the efficiency of heating by using magnetic particles. This allows for the nanoparticles of various sizes to be compared to heat generators. The Fe and Co NPs in this work have sizes ranging from 7 to 15 nm. For this size, the Fe NPs are about the size of the Fe domain (14 nm); however, the Co NPs are much smaller than the Co domain size (70 nm) (Zhao *et al.*, 2008). As the Fe NPs are most probably composed of single domains, they exhibit the maximum possible coercivity for Fe because the Co NPs, which are much smaller compared with Co domain size, show a consequent diminished coercivity. This phenomenon is highlighted by almost no hysteresis loss for the Co NPs. Lately, the magnetically induced hyperthermia was drastically enhanced by the development of superparamagnetic liquids, composed by nanoparticles with one magnetic domain that are suspended in water (Zhao *et al.*, 2008; Jordan *et al.*, 2001). When a ferroliquid is removed from the magnetic field, its magnetization vanishes because of the thermal energy of the environment. Another cause is that the greater pairing of electrons in Co and the stronger nuclear charge of the Co atoms lead to less surface delta multi-bonding of Co surface atoms in the Co NPs relative to the Fe NPs. Such Co–C bonding lowers the coercivity of Co, which results in far more weaker magnetic properties relative to Fe NPs. Note that the magnetic hysteresis of Co NPs is almost zero at 300 K, indicating that the Co NPs are not agglomerated, and the NPs are superparamagnetic. However, the Fe/Co and Fe NPs have more agglomerations, as indicated by the larger hysteresis loops for these particles relative to Co NPs. As a result, Fe and Fe/Co NPs can generate more thermal energy than the Co NPs when they are exposed to the external AC field (Xu *et al.*, 2010).

A ferromagnetic particle becomes 'superparamagnetic' below a critical size of the order of 100 nm, depending on the material. Such a particle cannot develop internal magnetic domains and, therefore, acts as a paramagnetic particle with a magnetic moment of up to 10^7 Bohr magnetons. The blocking phenomenon is a characteristic of the superparamagnetic behavior which is correlated to the particle size and distribution, crystallinity, and interparticle interactions (Prozorov *et al.*, 1999). Each NP then becomes a single magnetic domain and shows superparamagnetic behavior when the temperature is above the so-called blocking temperature, T_B (Prozorov *et al.*, 2007).

At temperatures inferior to T_B, the nanoparticles are 'blocked', indicating that the random magnetic moments of the individual nanoparticles cannot easily align with the magnetic field, since the magnetic Zeeman energy (H) and thermal fluctuation (k_BT) levls are too low to overcome the energy barrier given by the magnetic anisotropy and interparticle dipolar interactions. T_B presents a peak in the $M_{(T)}$ curve developed during the heating process under a magnetic field application. It is well established that T_B is lower for smaller particles and for those with limited crystallinity; both properties are responsible for the reduction of the magnetic moment per particle (Prozorov and Prozorov, 2004). In parallel, it is also possible to analyze the magnetic properties of a superparamagnetic system by measuring its magnetic moment at a given temperature and varying the intensity of the magnetic field. Figure 11c shows the results of zero-field-cooled (ZFC) measurements performed upon warming after a magnetic field of 30 Oe was applied at 5 K after cooling in zero applied fields. Clearly, Co NPs exhibit the lowest blocking temperature at about 50 K. Fe and Fe/Co NPs show an elevated T_B of 300 K. These results were fully consistent with the results of TEM; that is, Co NPs are of a relatively smaller size and lower crystallinity than Fe and Fe/Co NPs (Xu *et al.*, 2010).

As shown in Figure 12a and b, after the NPs were taken up into the HeLa cell cytoplasm, they were able to cross the various intercellular membranes and reach the nucleus (Xu *et al.*, 2008). Due to the localized RF heating provided by the NPs, the cells were found to go through an apoptotic process and, subsequent cellular decomposition (one of the signs of apoptosis process, the cell membrane blebbing) was observed and is shown in Figure 12c. Compared with other NPs, as shown in Figure 12d–f, the C–Fe NPs revealed the highest efficiency for inducing cell death when compared with the C–Fe/Co NPs and C–Co NPs after 2 min of RF exposure. The disintegration of localized cellular environments, such as the nucleus, nuclear membranes, and DNA, was believed to be the effective response resulting from RF heat inducement into the NPs.

This initial cellular death screening process was confirmed by supplementary analyses, including

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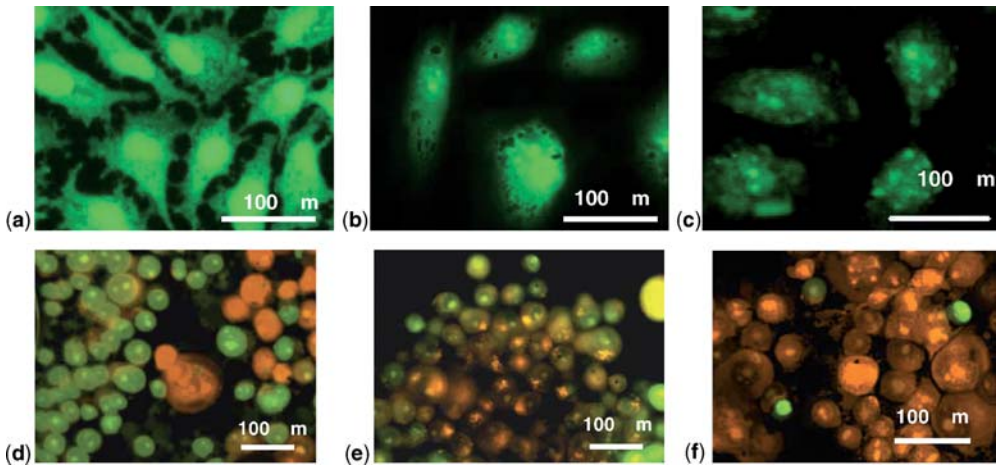

Figure 12. (a) Normal HeLa cells without incubation of magnetic NPs; (b) after 24-h incubation time, the magnetic C–Co NPs were found to aggregate around and further penetrate into the nucleus of HeLa cells. (c) The images took from the Olympus confocal microscopy indicating the membrane blebbing of HeLa cells incubated with magnetic C–Fe NPs after a 350 kHz RF heating for 2 min. Confocal microscopy images indicating the extensive live and dead cells after being incubated with the C–Co NPs (d), C–Fe/Co NPs (e), and C–Fe NPs (f) after exposure to RF radiation for 2 min. The cells were stained to distinguish between the live (green for acridine orange) and the dead cells (orange for ethidium bromide) (Xu *et al.*, 2010).

cellular morphology studies and agarose gel electrophoresis to detect oligonucleosomal ladders – a reliable indicator of the cellular apoptosis. From the gel electrophoresis analysis (Figure 13), it can be observed that, in comparison with that collected from the control cells (unexposed to any treatment) the DNA collected from the cells exposed to the cobalt nanoparticles under RF exposure indicated a significantly greater broadening of the DNA spectra. The nuclear DNA was found to degrade into fragments of about 2000–100 bp as presented in Figure 6. These results indicate chemical and structural modifications (Zhu *et al.*, 2007) of the DNA bases and the probable breakage of the DNA double strands – a process that can induce mitotic recombination, point mutations, and chromosomes loss and translocation.

In conclusion, the area of nanomedicine and cancer treatment will be transformed in the next few years by the use of nanomaterials with advanced bioactivity properties. The use of nanostructures in conjunction with various biological systems, such as drugs, proteins, growth factors, and DNA, will have an extraordinary impact on a broad range of applications for treating diseases and conditions: cancer treatment, tissue regeneration, biological sensors, drug delivery, and others. The selective delivery and molecular tumor cell targeting with high specificity and optimized nanostructure parameters have to be developed for clinically relevant treatments. Further validation of this approach will use more quantitative imaging and biological methods to explore the

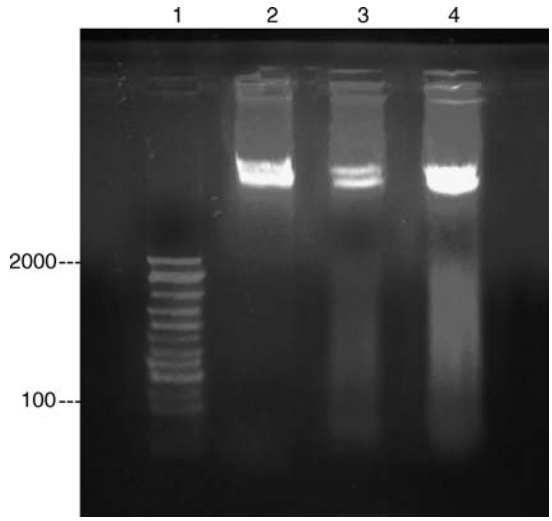

Figure 13. DNA fragmentation studies. (1) Marker DNA, (2) DNA extracted from HeLa cells without any nanoparticles and no RF exposure, (3) DNA of the HeLa cells incubated with SWNT and exposed to RF excitation, and (4) DNA of the HeLa cells incubated with C–Co–NPs and exposed to RF excitation (Xu *et al.*, 2006).

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mechanism of combined action including different pathways of apoptotic and necrotic phenomena.

RELATED ARTICLES

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Nanotoxicology: A Brief Overview and Discussion of the Current Toxicological Testing *In Vitro* and Suggestions for Future Research

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1 NANOTECHNOLOGY

Nanotechnology can be defined as the manipulation, precision placement, measurement, modelling or manufacture of materials at the nanometre (nm) scale (Donaldson *et al*., 2001). The era of nanotechnology proposes to provide lighter, stronger, smaller, more efficient and durable products such as stain-free clothing, as well as environmental remediation, and can be further described as the manufacturing of materials or particles <100 nm in size (Reijnders, 2006). Since the naturally occurring or biologically based molecules found within organisms can be used as model nano-sized materials (Oberdorster, Oberdorster and Oberdorster, 2005), a comparison to these molecules and atoms, definitive in their size, can further characterize the scale on which nanotechnology is based. For example, 1 nm, or 1 billionth of a metre (10^{-9} m), is the diameter of 10 hydrogen atoms, 4 nm is the size of a single protein molecule, 1000 nm is equal to the size of a pollen grain and 10,000 nm can be equated to the average length of one human hair follicle (Whitesides, 2003).

It is proposed that nanotechnology involves manufacture at three specific levels: (i) materials, (ii) devices and (iii) systems (Salata, 2004). There have been many recent advances in nanotechnology, although only at the material level, including computer technology and washing machines, as well as lighter, faster and stronger sporting equipment (Mamalis, 2007). The proposed advantages of nanotechnology, however, are still not completely defined or even fully understood (Whitesides, 2003). In addition, increased public attention has further highlighted possible disadvantages of nanotechnology (Bruce, 2006; Rogers-Hayden and Pidgeon, 2006), as well as the potential deleterious and detrimental effects that this new form of technology might pose (Hoet, Bruske-Hohlfeld and Salata, 2004; Borm *et al*., 2006; Gwinn and Vallyathan, 2006; Maynard, 2007; Nel *et al*., 2006).

Despite this, the nanotechnology industry is a rapidly developing field, with the amount of money

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invested into its research and development, as well as the economic value of the industry increasing continuously every year (Maynard *et al.*, 2006; Maynard, 2007). Concomitant with this increase is the level of nanotechnology-related products manufactured and distributed for use in a wide and diverse range of consumer, industrial and technological applications, such as clothing, cosmetics, computer technology, household paints, medicine and sporting equipment (Maynard, 2007). Due to the constant influx of nanotechnology-related products in these areas, it is essential that rigorous assessment of their risk to both humans and the environment is conducted, in order to implement a set of stringent safety guidelines for their production, use and disposal, so that the promise surrounding the industry can be realized (The Royal Society and Royal Academy of Engineers Report 2004; Hoet, Bruske-Hohlfeld and Salata, 2004; Maynard *et al.*, 2007).

1.1 What is a Nanomaterial?

Nanomaterials have been defined as "a material that has one or more external dimensions in the nanoscale or which is nanostructured (nanomaterials can exhibit properties that differ from those of a material which does not have nanoscale features)" (BSI PAS: 136, 2007). Although it is recognized that the definition of a nanomaterial is different from that of a nanoparticle (NP), it is often assumed that both are synonymous. The definition of an NP is difficult to confirm and is under constant debate. In some instances, combustion-derived NPs are referred to as "ultrafine (uf) particles" (a term predominantly associated with NPs present within ambient/environmental air pollution), and described as particles with a diameter of <100 nm (BSI PAS: 136, 2007). In addition, a report published by the European Science Foundation, concerning nanomedicine, stated that the NP complexes used in nanomedicine range from 1 to 1000 nm (ESF Report, 2005). Despite this, due to the diverse range of shapes and structures that comprise the many different forms of NPs (Oberdorster, Oberdorster and Oberdorster, 2005), as well as the extensive number of reports on contrasting definitions for nanomaterials (SCENIHR, 2005, 2007; Friends of the Earth, 2006; BSI PAS: 136, 2007) the International Organization for Standardization (ISO), in a recent attempt to develop a more structured approach, provided the following definition (ISO/TS: 27687, 2008). In a document entitled "Nanotechnologies – Terminology and definitions for nano-objects – nanoparticle, nanofibre and nanoplate", the ISO defined an NP as "a nano-object (a material with one, two or three external dimensions in the nanoscale (1–100 nm)) with all three external dimensions in the nanoscale" (ISO/TS: 27687, 2008). NPs, however, comprise a diverse number of materials such as carbon, titanium, gold, polystyrene, cadmium, iron and zinc, and can be either accidentally produced (also referred to as naturally occurring NPs (Section 1.2.1)), or specifically engineered (Section 1.2.2) (Oberdorster, Oberdorster and Oberdorster, 2005); thus, due to this plethora of different shapes, sizes, materials and intended applications, the debate surrounding the definition of an NP will continue. For the purpose of this chapter, NPs will be referred to in accordance with the definition as provided by ISO (ISO/TS: 27687, 2008).

1.1.1 Accidentally Produced NPs

Naturally occurring NPs have been experienced by humans across all evolutionary stages (Oberdorster, Oberdorster and Oberdorster, 2005; Oberdorster, Stone and Donaldson, 2007). Examples of naturally occurring NPs include particles derived from volcanoes and fires (Oberdorster, Oberdorster and Oberdorster, 2005; Oberdorster, Stone and Donaldson, 2007). Over the past two centuries due to industrial development, the invention of the car engine as well as other combustion processes have resulted in a rise in the level of air pollution, as well as the amount of unintentional, or accidental NPs released into the atmosphere (Oberdorster, Oberdorster and Oberdorster, 2005). Within the workplace, NPs have also been found, for example, to be present in the emissions from welding fumes (Zimmer, Baron and Biswas, 2002) and natural gas-powered equipment (Rundell, 2003), whereas in the environment, power plant emissions (independent of being powered by coal, oil or natural gas) have been observed to produce large amounts of NPs into the atmosphere (Oberdorster, Oberdorster and Oberdorster, 2005). The major source of accidentally produced NPs in the environment, however are traffic exhaust emissions (Oberdorster, Oberdorster and Oberdorster, 2005). Also known as combustion-derived NPs, these emissions can

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increase the number of particles in the air by up to ten million particles per cm^3 (Kittelson, Watts and Johnson, 2004). The effects of exposure to increased levels of particulate present in polluted air have been well documented over the past 20 years, demonstrating accidental NPs to be associated with adverse health effects, such as cardiovascular disease and chronic obstructive pulmonary disease (COPD) (Donaldson *et al.*, 2003), compared with their larger particle counterparts at the same mass dose (Oberdorster, Oberdorster and Oberdorster, 2005). (The effects following exposure to NPs contained in polluted air are discussed in Section 4.) Findings from studies investigating the toxicity of NPs present in polluted air have prompted concerns regarding the possible hazardous health effects of exposure to engineered NPs on human health and the environment (Colvin, 2003; Moore, 2006; Maynard *et al*., 2006).

1.1.2 Engineered NPs

Engineered NPs can be defined as particles engineered or manufactured on the nanoscale with specific physicochemical compositions and structures to exploit properties and functions associated with their dimensions (Oberdorster, Oberdorster and Oberdorster, 2005; Oberdorster, Stone and Donaldson, 2007). Furthermore, engineered NPs include particles that have either a homogeneous or heterogeneous chemical composition or structure, or are multi-functional and can be of different shapes, for example spheres, fibres, tubes, rings and planes (Oberdorster, Oberdorster and Oberdorster, 2005). Examples of these include: (i) carbon nanotubes (CNTs) – graphite-like carbon tubes with capped ends, which can be either a single graphite-like carbon tube, known as single-walled CNTs (SWCNTs), or a series of SWCNTs that can be either stacked inside one another or a single CNT rolled inside of itself, known as multi-walled CNTs (MWCNTs) (Donaldson *et al*., 2006); (ii) C_{60} fullerenes – complex, semi-conductor, spherical 3D carbon structures (Jensen, Wilson and Schuster, 1996); (iii) superparamagnetic iron oxide nanoparticles (SPIONs) – spherical NPs consisting of an iron (Fe) core, coated with a shell material (usually gold (Au)) (Gupta and Gupta, 2005), and (iv) dendrimers – a symmetrical arrangement of macromolecule branches arising from a multi-functional core (Duncan and Izzo, 2005). In addition, the specific physicochemical properties of engineered NPs can be manipulated to create different surface coatings/charges surface chemistries and surfactants, such as organic (neutral charge), carboxylated (COOH) (negative charge) and amino (NH_2) or amine (positive charge); as well as for the ability to attach polymer groups and biologically based structures (Oberdorster, Oberdorster and Oberdorster, 2005).

Despite the vast array of manufactured NPs already in use for a wide range of applications (Section 1.1), newly engineered NPs are constantly being generated to meet the increased demand for smaller, lighter, faster, more efficient and durable products (Maynard, 2007). The potential toxic and adverse health effects of these "new" NPs, however, are not fully understood (Hoet, Bruske-Hohlfeld and Salata, 2004; Maynard, 2007; Nel *et al*., 2006). Therefore, it is vital that efficient, model ways of analysing the diverse physiochemical components of manufactured NPs are determined, in order to fully understand both the potential detrimental and advantageous effects of engineered NPs (Maynard *et al*., 2006).

2 THEORY OF TOXICOLOGY

Toxicology is the study of the adverse effects of substances on living organisms (Timbrell, 1999). One of the fundamental principles of toxicology is that the "risk" posed by a substance is a function of its potential to cause harm, or "hazard," and the amount of substance a biological system is "exposed" to. This is defined by the following principle:

$$\text{Risk} = \text{Hazard} \times \text{Exposure}$$

It is necessary to consider exposure levels of substances according to the principle that all materials are toxic if exposure occurs in sufficient quantities (Timbrell, 1999). Most commonly this principle is referred to via the quote of *Paracelsus* (CH, 1493-1541). "*All things are poison and nothing is without poison, only the dose permits something not to be poisonous*." These fundamental toxicology principles imply that substances with a low hazard generally pose a low risk. If, however, there is a high enough exposure to these low-hazard substances, even these can be harmful, or even fatal.

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3 NANOTOXICOLOGY – A DEFINITION

Based on the principles of toxicology, nanotoxicology can be defined as "the study of the effects of NPs (either accidentally produced or engineered) on living organisms" and further described as a multidisciplinary science including material science, chemistry, physics and medicine (Donaldson *et al*., 2004). The field of nanotoxicology focuses upon gaining a thorough understanding of the relationship between properties such as particle size, surface area and reactivity, dose (concentration), material composition and the potential toxicity of NPs. In relation to this, it is also proposed that nanotoxicology will develop and implement "nano-specific" protocols in order to investigate and gain the knowledge necessary to determine the suggested toxicity (Section 4) of the plethora of different NPs.

4 NANOTOXICOLOGY – A BRIEF REVIEW

As a complete review of nanotoxicology is beyond the scope of this chapter, the following section is only a brief review of the history of nanotoxicology and its current state. For a complete overview of the field of nanotoxicology, the readers may refer to both Oberdorster, Oberdorster and Oberdorster (2005) and Oberdorster, Stone and Donaldson (2007)

4.1 A historical Perspective of NPs and Their Associated Health Effects

The concern surrounding the exposure of humans to NPs (mainly) derives from their small size and emanates from two independent findings that separately recognized that as particle size decreases, toxicity generally increases.

One of the initial studies to demonstrate the potentially harmful effects of exposure to NPs was by Ferin, Oberdorster and Penney (1992). In this study, rats were exposed, via inhalation, to both NP (21 nm diameter) and fine (250 nm diameter) titanium dioxide particles (TiO_2), as well as intratracheally instilled with TiO_2 particles of various sizes (12, 21, 230 and 250 nm in diameter) over a period of 12 weeks. The effects of treatment with each particle size were then observed over a 70-week post-exposure period. It was demonstrated that TiO_2 NPs promoted an acute inflammatory response following both intratracheal instillation and subchronic inhalation techniques compared with the larger particles (230 and 250 nm). The inflammation observed in exposed animals was subsequently found to reduce to control levels post-exposure (64 weeks), with a noted decrease (from peak levels) in the number of neutrophils present in the lung at that time. Ultrafine particles were also found to remain within the lung longer (501 days) than fine particles (174 days). The prolonged retention of TiO_2 NPs in the lung was suggested to be an effect of the finding that at equivalent masses, NPs were able to translocate to the pulmonary interstitium more efficiently than the larger TiO_2 particles. It was suggested that the translocation of NPs to the interstitium was due to the smaller particles (12 and 21 nm) not being taken up by alveolar macrophages and undergoing clearance from the alveoli via uptake by alveolar type-1 epithelial cells instead. In addition to this, it was found that an increased dose (increased number of particles and decreased particle size) promoted movement of particles within the pulmonary system. It was also observed that the number of particles present, particle size, delivered dose and the delivered dose rate also had an effect on the translocation process. Ferin, Oberdorster and Penney (1992) concluded that the observed inflammation was due to the exposure of the rat lung to NPs, impaired lung clearance and NP redistribution.

In a subsequent publication, these findings were supported by Oberdorster, Ferin and Morrow (1992), who showed increased levels of inflammation present in the alveolar space of rats after instillation with 500 μg TiO_2 NPs (20 nm) over 24 h, compared with TiO_2 fine particles (250 nm) at the same mass dose. It was also found, in comparison to Ferin, Oberdorster and Penney (1992), that NPs were able to enter the interstitium more readily than the larger TiO_2 particles. Oberdorster, Ferin and Morrow (1992) further suggested that the increased inflammatory response to acute NP exposure was not due to the movement of particles to the interstitium, but it was related to the larger surface area of the particles and their interaction with alveolar macrophages and interstitial cells. Focusing on alveolar macrophages, it was noted that they were effective inhibitors of the inflammatory response to NPs due to the active uptake of these particles. The findings of both Ferin, Oberdorster and Penney (1992) and Oberdorster, Ferin and Morrow (1992) promoted interest in the effects of NPs in the lung,

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as well as the possible adverse effects on respiratory and cardiovascular function that exposure to NPs might pose.

In addition to laboratory investigations, epidemiological studies conducted over the past two decades have shown a positive correlation between the level of particulate air pollution and increased adverse health effects (Dockery *et al*., 1993; Bremner *et al*., 1999; Braga, Zanobetti and Schwartz, 2000), including increased pulmonary diseases (Choudhury, 1997; Pope and Dockery, 1999; Schwartz, 2004), as well as a rise in the number of deaths from cardiovascular disease (Abbey *et al*., 1999; Aga *et al*., 2003; Zanobetti *et al*., 2003; Kaiser *et al*., 2004; Medina *et al*., 2004). It was specifically shown that these adverse health effects manifested predominantly in susceptible individuals who had pre-existing pulmonary or cardiovascular disease following increased exposure to areas of heightened particulate air pollution (Dockery *et al*., 1993; Pope and Dockery 1999). To determine the levels of particulate air pollution, or particulate matter (PM), these studies used the global sampling method of PM. This method collects all airborne PM that has an aerodynamic diameter of $\leq$10 μm, via a machine with a size-selective inlet of $\leq$10 μm and 50% efficiency (Donaldson *et al*., 2001). The composition of PM_{10} is complex and difficult to define exactly; thus, it is termed as a "particle cocktail," comprising a wide variation of particulate materials, including carbon, a complex mixture of metals, exhaust fumes, endotoxins, wind-blown dusts and a mixture of chemicals ranging from $>$2.5 μm to $<$100 nm in size (Wilson *et al*., 2002). All of the components are thought to contribute to the observed toxicity and reducing effects of PM on human health (Donaldson *et al*., 2003). Despite this, the NP component of PM_{10} has been held principally accountable for eliciting the adverse health effects that were highlighted in these epidemiological studies. This led to the generation of the "ultrafine hypothesis" (Seaton *et al*., 1995), whereby NPs ($<$100 nm) were considered to be particularly hazardous and to drive the adverse effects to human health, specifically within the lung. It is also relevant to note that these findings and suggestions were in agreement with those of Ferin, Oberdorster and Penney (1992) and Oberdorster, Ferin and Morrow (1992), whereby smaller particles were potentially of greater risk to health than larger particles of a similar material. This hypothesis was subsequently supported in an epidemiological study by Peters *et al*. 1997, who reported that human respiratory ill health was associated with the number of ambient uf particles inhaled. In the study by Peters *et al*. (1997), 27 non-smoking asthmatics, who resided within a highly polluted city in Central Europe, were examined over a 6-month period. Analysis of the peak expiratory flow (PEF) of the lungs for each individual volunteer demonstrated the number of uf particles in the air to have a significant mean decrease on PEF, compared with the mass of larger particulates in the atmosphere. It was concluded, therefore, that the adverse health effects observed following exposure to PM_{10} could be related to the size distribution of particulates in the environment, thereby supporting the theory that NPs could potentially drive toxicity in the lung. Although there are a number of additional epidemiological studies that have studied particulate effects on human health (Wichmann *et al*., 2000; Schulz *et al*., 2005), further epidemiological studies are still required in order to support the findings of the plethora of laboratory-based investigations.

Despite the shortage of epidemiological studies, the hypothesis of Seaton *et al*. (1995) has been the basis of increased experimental, laboratory-based analysis over the past two decades. In a study by Li *et al*. (1997), PM_{10} samples were examined *in vivo* and *in vitro*. Following intratracheal instillation of PM_{10} in rats for 6 h, an increase in inflammation (increased neutrophil count) and toxicity (increased levels of lactate dehydrogenase (LDH)) was measured in the bronchoalveolar lavage (BAL) fluid, as well as an increase in BAL protein, indicative of increased epithelial permeability. Further analysis of the BAL fluid found depletion in glutathione, indicating an increase in oxidative stress. It was also observed that PM_{10}-treated BAL leucocytes *in vitro* produced a significant increase in the level of tumour necrosis factor (TNF)-α, suggestive of increased inflammation in PM_{10}-exposed lung cells. Due to the diverse range of materials in PM_{10} it is difficult to replicate in a laboratory environment for experimental purposes, and therefore many studies have used surrogates of PM_{10}, such as fine ($>$100nm) carbon black (CB) and its NP counterpart, ufCB. Subsequent analysis of the effects of ufCB *in vivo*, following intratracheal instillation, demonstrated that these smaller particles were able to cause a greater level of inflammation compared with both fine CB and PM_{10}. Subsequent comparative analysis of CB and ufCB by Li *et al*. (1999), in which a

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mass of 125 μg of each particle type was instilled into rats, also reported ufCB to induce greater pro-inflammatory effects than CB. A 50% increase in neutrophils was observed in the alveolar region of the lung following BAL of rats exposed to ufCB, compared with CB particles after 6 h of exposure. Subsequent analysis of the BAL profile of the ufCB-instilled rats after 6 h, compared with rats exposed to CB, also showed an increase in LDH, indicative of cell damage (increased cell membrane permeability), as well as a rise in total BAL protein, suggestive of increased epithelial cell permeability. It was shown, however, that both particle sizes (ufCB and CB) caused a decrease in glutathione content in the lung, indicating that both ufCB and CB induce oxidative stress in the lung, although the highest levels of depletion were observed in rats treated with ufCB. In conclusion of these findings, Li *et al*. (1999), in comparison to Oberdorster, Ferin and Morrow (1992), suggested that the biological reactivity of ufCB was related to the size and surface area of the particle type.

The findings of both Li *et al*. (1999) and Oberdorster, Ferin and Morrow (1992) were supported by Brown *et al*. (2001) who examined the effects of the instillation of 64, 202 and 535 nm engineered polystyrene beads (PBs) in rats. It was discovered that treatment of 64 nm PBs induced an increased inflammatory response via an influx of neutrophils, LDH and protein in BAL fluid from the rat lung compared with exposure to both the 202 and 535 nm sized PBs. Further analysis of the BAL fluid found a depletion in glutathione also showed that the surface area of the particles instilled into the rats was relative to the level of inflammation in the rat lung, and therefore a proportional relationship between particle surface area and inflammation existed. Therefore, Brown *et al*. (2001) concluded that as the 64 nm PBs had a greater surface area than both the 202 and 535 nm PBs, NPs have the ability to cause a greater level of inflammation than their larger particle counterparts at the same mass dose due to a greater surface area and reactivity. These findings were subsequently supported by Duffin *et al*. (2002, 2007), who demonstrated a number of low-toxicity particles (TiO_2, CB and PBs), at different sizes *in vivo*, to cause a heightened inflammatory response, via an increased neutrophil count in BAL fluid from the rat lung between 18 and 24 h after exposure to particles <100 nm in size. A recent study by Stoeger *et al*. (2006) also showed the specific surface area of NPs to be related to their potential to induce pro-inflammatory cytokine production in mice. It was observed by Stoeger *et al*. (2006) that mice instilled with 5, 20 and 50 μg of a series of different particles, including flame soot particles, spark-generated uf carbon particles (ufCPs) and diesel exhaust particles (DEP), ranging in their surface area from 30 to 800 $m^2\ g^{-1}$, caused increased levels of LDH, total protein, TNF-α, interleukin (IL)-1β and macrophage inflammatory protein-2 (MIP-2), as well as polymorphonuclear leucocytes (neutrophils) (PMNs) in BAL fluid after 24-h exposure. Investigation demonstrated that the ufCPs were more potent than the other particles tested, and that the inflammatory indicators were not detectable when the instilled dose was $\leq 20\ cm^2$. It was subsequently concluded by Stoeger *et al*. (2006) that although the surface area of particles indicates their ability to cause inflammation, with uf particles causing an increased pro-inflammatory gene expression compared with larger particles, a threshold may exist in relation to the surface area of NPs and their relevant toxicity. The findings of these studies support the hypothesis that NPs have the potential to drive toxicity in the lung, and that they are responsible for the subsequent adverse health effects observed (Seaton *et al*., 1995). In addition, although these studies are limited to only a few specific NP types (predominantly CB, PBs and TiO_2), it is suggested that the specific characteristics of particle size, particle mass dose, surface area and surface reactivity are associated with NP toxicity in general.

Although it is well documented which characteristics influence NP toxicity, the precise mechanism by which this observed toxicity occurs is not fully understood. Despite this, as a result of increased laboratory-based investigations that have been highlighted in this section, a paradigm termed the "oxidative stress paradigm" was proposed suggesting the manner in which NPs can cause adverse effects to cells (Donaldson *et al*., 2003).

4.1.1 The Oxidative Stress Paradigm

Oxidative stress occurs when a greater number of oxidants than antioxidants are present within the cell, causing an oxidant/antioxidant imbalance (MacNee, 2001). Increased oxidation can occur within cells, such as macrophages following activation (Donaldson *et al*., 2003). The activation of

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macrophage cells can cause the generation of the superoxide anion, which is readily converted into the hydroxyl radical ($^{\cdot}OH$) via the influence of superoxide dismutase (MacNee, 2001). The presence of the $^{\cdot}OH$, as well as the superoxide anion, which are examples of reactive oxygen species (ROS), can thus cause increased oxidation within the cell because these molecules possess unpaired electrons and are highly unstable (MacNee, 2001). Additionally, ROS can be produced via nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which is the most common form of ROS found in cells, and is usually produced when cells are performing the phagocytosis of xenobiotics (MacNee, 2001). Therefore, this suggests that although cells purposefully clear hazardous particles from the tissue, the phagocytosing cells can unintentionally or intentionally produce ROS. In addition, the potential production of ROS following encapsulation of particles via phagocytosis further emphasizes the necessity to understand the specific uptake mechanism of NPs, in order to determine their potential route within the cell, and how their uptake may relate to their toxicity.

The production of ROS and subsequent oxidative stress in cells can be extremely deleterious, causing a reduction in cell metabolic competence via a reduction in mitochondrial respiration, as well as an increase in pro-inflammatory cytokine production. The effects of ROS production and subsequent oxidative stress can further be associated with diseases and illnesses such as pulmonary and cardiovascular diseases, including asthma, COPD, as well as atherosclerosis and even tumour formation (Donaldson *et al*., 2003).

In a study by Stone *et al*. (1998), the potential for NPs, specifically ufCB, to cause oxidative stress was assessed in A549 epithelial cells. Cells were measured for their glutathione content to determine the level of oxidative stress present. Glutathione is the most abundant non-protein thiol present inside most cells, and it is essential for maintaining cell viability by detoxification of pathogens, as well as by regulating cell cycle and gene expression (Droge *et al*., 1994). A reduction in the glutathione content of cells, therefore, is known to indicate an oxidative stress environment (Stone *et al*., 1998). It is also known that glutathione can have a protective role following the onset of oxidative stress, due to its antioxidant gene characteristics (MacNee, 2001). Antioxidant genes are common throughout cells, such as the c-*fos* gene. This gene is part of a series of growth- and differentiation-related genes that are expressed by cells in response to foreign materials (MacNee, 2001). It is thought that antioxidant genes are "switched on" following NP stimulus, which can thus overwhelm the NP effect (Donaldson *et al*., 2003). Research into these effects, however, is limited and requires further investigation. In relation to the potential for NPs to induce oxidative stress, it was observed by Stone *et al*. (1998) that following treatment of A549 epithelial cells with up to 0.78 $\mu g\ mm^{-2}$ of ufCB and CB, the glutathione levels, as measured specifically in its reduced form (GSH), decreased after 2 h, with a significant decrease also found after 6-h exposure to ufCB, but not to CB. It was subsequently concluded by Stone *et al*. (1998) that ufCB is more potent at inducing oxidative stress than its larger counterpart, CB. Li *et al*. (2003) further studied the potential for NPs to induce oxidative stress in cells. In the study by Li *et al*. (2003), the effects of ambient (25–10 μm), fine ($<2.5\ \mu m$) and uf ($<0.1\ \mu m$) particles on RAW 264.7 macrophage cells and BEAS-2B epithelial cells were assessed. It was observed that NPs, in comparison to both ambient and fine particles, induced an increased cellular expression of heme-oxygenase-1 (HO-1) expression in each cell line, indicative of oxidative stress, as well as a decrease in intracellular glutathione levels. High levels of ROS production were also demonstrated following dithiothreitol (DTT) analysis. Further examination of the different particle types by transmission electron microscopy (TEM) showed the NPs, as well as a small amount of larger particles, to predominantly locate within mitochondria, causing subsequent major structural damage thought to contribute to increased oxidative stress (decrease in GSH) and toxicity previously observed. It was therefore concluded that the increased biological potency of NPs can be associated with the redox cycling of organic chemicals due to their increased ability to damage the mitochondria, causing ROS and oxidative stress.

Recently, Xia *et al*. (2006) also showed ufCB, in comparison to TiO_2 and CB, to cause an increased production of ROS, as measured via the ROS quencher, furfuryl alcohol, as well as by assessment of the level of NADPH peroxidase with RAW 264.7 macrophage cells over a 4- and 16-h period. Subsequent analysis of the oxidative stress levels in these macrophage cells found depletion in GSH levels, as well as toxic oxidative stress after similar

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exposure periods. It was further illustrated that the toxic oxidative stress observed was specific to an injury to mitochondria due to increased cytosolic calcium (Ca^{2+}) production and uptake, causing structural damage to the organelle.

Ca^{2+} is an essential and ithe most abundant mineral in the body (Alberts *et al*., 2002). Maintaining normal Ca^{2+} levels ($\sim 155 \pm 9$ nM) within the cell, also known as Ca^{2+} homeostasis, is essential for cell viability (Abbas and Lichtman, 2003). The finding by Xia *et al*. (2006) that an increased Ca^{2+} production (signalling) can occur in cells following exposure to NPs supports previous studies that have suggested the increase of cytosolic Ca^{2+} to be associated with the onset of increased ROS production and subsequent oxidative stress (Stone *et al*., 2000a, 2000b; Brown *et al*., 2000, 2004, 2007a; Donaldson *et al*., 2003). Stone *et al*. (2000b) initially showed that ufCB exposure for 30 min to Monomac 6 (MM6) human macrophage cells induced a 1.6-fold increase in the resting cytosolic Ca^{2+} concentration, measured using the Ca^{2+} chelator Fura 2-AM (Grynkiewicz, Poenie and Tsien, 1985), in MM6 cells at a concentration of 66 $\mu g\, m^{-1}$, whilst no changes were observed following treatment of MM6 cells with CB. Subsequent investigation by Stone *et al*. (2000a) examined the effects of CB, both fine and uf (33 $\mu g\, ml^{-1}$), and latex beads (64, 202 and 535 nm in diameter) (1 mg ml^{-1}) on MM6 cells and primary rat macrophages. Analysis of the latex beads showed a 2.3-fold increase in cytosolic Ca^{2+} concentration (as assessed via Fura 2-AM) in response to thapsigargin stimulation following treatment with the 64 nm latex beads; however, no effects on MM6 cell Ca^{2+} concentration were observed following treatment with either the 202 or 535 nm latex beads. In this study, thapsigargin was used to assess the viability of the cells via Ca^{2+} signalling. This chemical stimulant causes release of Ca^{2+} from the endoplasmic reticulum (ER); a loss of cell viability, via apoptosis, can be related to a loss of Ca^{2+} in the ER store and therefore a reduced Ca^{2+} response to thapsigargin stimulation. Similarly, Stone *et* al. (2000a) showed a 2.6-fold increase in Ca^{2+} in BAL cells (>80% macrophages) following stimulation with thapsigargin and after exposure to ufCB, but not to CB. These latter findings support those previously reported by Stone *et al*. (2000b) that ufCB can cause an increase in cytosolic Ca^{2+} concentration, and further demonstrate that different macrophage types (MM6 and primary rat macrophages) can elicit similar responses following NP exposure. Further analysis by Stone *et al*. (2000a), demonstrated that these effects were attenuated when the MM6 cells were pre-treated with either the antioxidant mannitol or Na-cystelyn, suggesting that the increased cytosolic Ca^{2+} concentrations observed following NP exposure could be mediated via ROS and oxidative stress. Stone *et al*. (2000a) performed further examination of the potential of all sizes of the latex beads to produce ROS, using the dye 2′,7′-dichlorofluorescin diacetate (DCFH-DA), which when oxidized converts into 2′,7′-dichlorofluorescin (DCFH) and shows an increasing fluorescence intensity relative to an increase in ROS production. It was found that the NP latex beads (64 nm) were more potent in causing ROS, with the 202 and 535 nm latex beads showing no oxidative effects after 10 min.

These findings were subsequently supported by Brown *et al*. (2000), who also measured cytosolic Ca^{2+} via the use of the fluorescent marker Fura 2-AM. It was reported that ufCB elicited a heightened cytosolic Ca^{2+} concentration in MM6 cells following treatment for 30 min. Subsequent analysis by Brown *et al*. (2000), which examined the effects of transition metals, specifically Fe in the form of Fe chloride ($FeCl_2$), using the transition metal chelator, desferal, found that these metals had no effect on cytosolic Ca^{2+} concentration after 30 min of exposure. In addition, Brown *et al*. (2000) also investigated the inflammatory potential of the ufCB and CB particles, as well as of the transition metals *in vivo*. It was observed that the ufCB particles, but not the CB particles, induced an increase in the number of PMNs present within the lungs of rats, with a significant increase in the number of neutrophils found within the BAL fluid after 24-h exposure. No inflammatory effects were found with $FeCl_2$. Following inductively coupled plasma–mass spectrometry, it was observed that the $FeCl_2$ particles were detectable in ng mg^{-1} concentrations within ufCB particles. It was subsequently concluded that ufCB does elicit an increase in cytosolic Ca^{2+} concentration, in support of Stone *et al*. (2000a, 2000b), and that ufCB particles induce an inflammatory response from cells, which was suggested to be independent of the transitional metal content of these NPs. The observation by Brown *et al*. (2000) that ufCB can cause increased inflammation supports the suggestion that ROS production, oxidative stress and altered Ca^{2+} signalling

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caused by these NPs can cause an activation of transcription pathways, via a cascade of events within the cell, including both the nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1) pathways (Donaldson *et al*., 2003). Activation of these pathways was subsequently proposed to result in an escalated inflammatory response, with an increase in pro-inflammatory gene expression and pro-inflammatory mediator production, such as the cytokines IL-8 and TNF-α (MacNee, 2001; Donaldson *et al*., 2003). Subsequent analysis by Brown *et al*. (2004) further supported this hypothesis, demonstrating that ufCB particles, and not CB particles, cause an increase in resting cytosolic Ca^{2+} concentration (as assessed by using Fura 2-AM) in rat alveolar macrophages. Similar effects were also found on treatment of rat alveolar macrophages in a dose–response relationship (12.5–50 $\mu g\,ml^{-1}$) following thapsigargin stimulation. It is also worth noting that these effects are similar to the findings of Stone *et al*. (2000a, 2000b) and Brown *et al*. (2000), further demonstrating the consistency between different forms of macrophage cells in assessing the toxicity associated with NP exposure. Additional study by Brown *et al*. (2004) also showed that these effects were decreased following addition of antioxidants (Trolox and Nacystelin), further supporting the findings of Stone *et al*. (2000a), who concluded that the increased Ca^{2+} signalling observed in macrophage cells after NP exposure was mediated via ROS. As it was suggested, by Donaldson *et al*. (2003), that subsequent inflammation could occur due to the activation of specific transcription pathways (such as NF-κB and AP-1) following the altered Ca^{2+} signalling caused by ROS and oxidative stress of NP-treated cells, Brown *et al*. (2004) further studied the effects of ufCB on both NF-κB and AP-1 transcription pathways. Investigation of the NF-κB pathway showed ufCB (100 $\mu g\,ml^{-1}$) treated human monocyte cells to show increased fluorescence of the sub-units of NF-κB, p50 and p65 over a 4-h period compared with untreated monocyte cells. Additional analysis of the AP-1 pathway showed no significant increase in the intensity of the AP-1 protein following treatment with ufCB at 200 $\mu g\,ml^{-1}$, after 4 h in rat alveolar macrophages. The findings relative to both these pathways were found to be attenuated following the addition of antioxidants, further supporting the suggestion that these events are mediated via ROS production. Subsequent analysis of the inflammatory potential of ufCB in rat alveolar macrophages found the production of the pro-inflammatory cytokine TNF-α to be dose dependent (25–200 $\mu g\,ml^{-1}$) after 4-h exposure. It was subsequently concluded by Brown *et al*. (2004) that uf particles can exert pro-inflammatory effects by altering Ca^{2+} signalling, activating transcription factors and causing the production of pro-inflammatory cytokines via ROS-mediated mechanism, thus supporting the proposed oxidative stress paradigm.

Although the findings of these studies suggest that the toxicity observed following exposure to NPs is relative to increases in the production of ROS and subsequent oxidative stress, as well as an escalation in Ca^{2+} signalling and inflammation within cells, the oxidative paradigm is only a hypothesis and further research is required to fully understand the mechanisms of NP toxicity and how they may relate to the many "new" and different types of NPs being manufactured.

4.2 Current Status of the Field

As a complete overview of the current status of the field of nanotoxicology is not possible within the remit of this chapter, it is the intention of this section that the major aspects of the field, as it currently stands, as well as the many problems which affect nanotoxicology research are addressed and highlighted in order to provide a foundation for research and understanding within the field.

There has been significant progress in the field of nanotoxicology since the production of nanotechnology applications (Maynard *et al*., 2006; Maynard, 2007; Hoet, Bruske-Hohlfeld and Salata, 2004; Donaldson *et al*., 2006). Despite this, the field has not been able to maintain an even balance in regards to the known effects of NPs and the production of NP-based products and applications (Maynard, 2007). The inability to provide clear and up-to-date knowledge on the effects of newly engineered NPs has caused public perception of the industry to be based upon either a lack of, or incorrect information, creating a repeat of the problems faced, and which inadvertently ended the genetically modified food industry (Gaskell *et al*., 2000). Clear examples of such issues include the "Magic Nano" (Kleinmann, GmbH) bathroom cleaning product which was released in 2006. This product was advertised to keep surfaces clean and dirt repellent due to

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the "nano" aspect of the product, thus providing a cleaner area for a longer period of time compared with conventional bathroom cleaners. Following the use of this product (and therefore subsequent exposure), however, increased instances of reduced pulmonary function were reported, including six hospitalizations of individuals reporting respiratory problems. The product was immediately recalled by its distributors, and it was subsequently suggested that such effects were a result of exposure to NPs contained within the product; an assumption based on both uninformed individuals and misunderstanding following the results of the studies previously discussed in Section 4. Although seemingly apparent from the name of the product, it was not completely clear whether the product actually contained NPs, due to the lack of information disclosed by the company (due to patent issues). Subsequent laboratory-based examination of the product concluded that the product did not contain NPs, and thus NPs could not be responsible for the adverse health effects observed following its use.

In addition to this, and most recently, a paper published by the European Respiratory Journal claimed that chronic exposure to NPs not only induced altered cardiovascular and pulmonary function, but also caused human fatalities (Song, Li and Du, 2009). In this study, Song, Li and Du (2009) suggested that seven females who worked within a poorly ventilated print plant were exposed to NPs (polyacrylate ester) for up to 13 months. A range of different biological and immunological tests were performed on the female workers and it was noted that five of the workers showed signs of increased pleural effusion, pulmonary fibrosis and signs of granuloma formation in the lung. It was also suggested that two of the female employees died as a result of the NP exposure, although further assessment is necessary to confirm the subjects' cause of death. Additionally, the study also assessed the effects on one male employee who was exposed to polyacrylate ester from the paint in the workplace for a total of 3 months. It was reported that he only suffered from asthma-like symptoms. There was no association of this health state with NP exposure however, as well as whether he may, or may not, already have been asthmatic prior to working in the print plant. The response to this publication has brought increased scepticism and caution from world leading figures in the field of nanotoxicology, particularly in relation to the lack of confirmation that the employees were exposed to actual NPs (by definition), as well as a clear association of the detrimental health effects observed following exposure to this NP type. It is of the utmost importance, therefore, that it is deduced whether or not the NPs that the workers were exposed to in the occupational setting drove the adverse health effects observed.

Despite these instances in which engineered NPs and their applications have been suggested as driving toxicity in humans without clear association, research into the effects of engineered NPs, based on the findings previously discussed in Section 4 of this chapter, has continued to increase over the past 10 years, obtaining increased yearly funding (Service, 2004) in order to obtain information that will enable the proposed advantages of nanotechnology to be realized (Oberdorster, Oberdorster and Oberdorster, 2005; Maynard *et al*., 2006; Maynard, 2007). In light of this, however, the proposed basis that the testing strategies used to determine the effects of ambient air NPs could be used to determine the potential toxicity of new, engineered NPs is not sufficient (Oberdorster *et al.*,2005). Concurrent with the ever-increasing number of studies published in regards to testing the toxicity of NPs (Section 4.2.5), heightened attention has also been focused upon the novel characteristics of each different type of engineered NP, and how these characteristics might affect the interactions of these NPs with biological systems (Hoet, Bruske-Hohlfeld and Salata, 2004; Oberdorster, Oberdorster and Oberdorster, 2005; Oberdorster *et al*., 2005; Oberdorster, Stone and Donaldson, 2007; Maynard *et al*., 2006; Maynard, 2007). In accordance with this, increased issues have also been raised in regards to the manner in which engineered NPs react in suspension, their specific characteristics and how best to measure/identify these characteristics, as well as the specific exposure routes and scenarios relating to biological systems. The following sections will briefly cover these issues.

4.2.1 Characterization of NPs

As discussed in Sections 4.1 and 4.1.1, there is a plethora of information related to specific NP types in regards to their (adverse) effects on the lung, as well as increased knowledge specific to the characteristics of the particles and the mechanism of toxicity which could potentially drive these observed effects. Despite this, and in addition to

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the aforementioned parameters which are associated with NP toxicity (Section 4.1), engineered NPs, as previously mentioned, will also exhibit additional characteristics, including different shapes and structures, as well as a variety of different surface coatings and chemistries, which are thought to contribute to their potential toxicity (Oberdorster, Oberdorster and Oberdorster, 2005).

There is already evidence that NPs, of a variety of compositions and forms, do not behave similarly, indicating that their ability to cause toxicity are not comparable. This was shown by Dick *et al*. (2003) who investigated four different NP types (CB, cobalt, nickel and TiO_2) in order to determine what attributes of particles influenced their toxicity *in vitro* and *in vivo*. It was observed that the inflammatory response elicited by the different particle types was not comparable, so that the toxicity of the different particle types can be ranked in the following order: carbon = cobalt > nickel > TiO_2; therefore, attributes other than their similarity in size were responsible for driving the observed toxicity. Furthermore, Xia *et al*. (2006) compared the toxicity of uf ambient particles, polystyrene microspheres (positively and negatively charged) and a range of engineered nanomaterials (CB, TiO_2, fullerol ($C_{60}(OH)_{24}$)) to RAW 264.7 macrophages *in vitro*. It was reported that the ambient uf particles and positively charged polystyrene microspheres induced the highest levels of toxicity as evident by their ability to induce the production of ROS, glutathione depletion and organelle (mitochondrial) damage. Therefore, it is suggested that the composition and charge of the nanomaterials influence their toxicity in addition to those described previously (Section 4.1). This study however used only one cell type, and it is relevant to highlight that other cell types may be sensitive to the toxicity of the these particles.

Although it is well documented that the composition and charge of NPs affect their toxicity, it is also necessary to take into consideration the effects of size, surface area and reactivity, as well as the chemical composition of the particle (Oberdorster *et al*., 2005). The parameters of size, surface area and surface reactivity have been discussed in detail in Section 4; however, due to the advent of new engineered NPs, these have increasingly been highlighted as vital characteristics relating to NP toxicity (Oberdorster *et al*., 2005). For example, quantum dots (QDs), which are semi-conductor nanocrystals with differing surface charges intended for both diagnostics and therapeutics (Pison *et al*., 2006), can range from 1 to 100 nm (Hardman, 2006), although they commonly differ by a size of 1–10 nm (Pison *et al*., 2006). Within this size range (1–10 nm), QDs, due to their novel fluorescent characteristics, alter significantly in their emission wavelength as well as shape (Hardman, 2006). In addition to the effects of the surface charge (Clift *et al*., 2008), it has been shown that the small change in size (1 nm difference) can significantly alter the ability of QDs to enter the cell and their internal localization as well as their resultant toxicity (Lovric *et al*., 2005a, 2005b). These differences in size will also affect their overall surface area and reactivity, possibly affecting their biocompatibility and potential toxicity potential (Duffin *et al*., 2002, 2007). Further research is required, however, to determine this for QDs and the plethora of engineered NPs available. In addition to these, there are other characteristics that have been recently highlighted as important in relation to the NP–cell interaction, including length and stiffness (Poland *et al*., 2008) as well as aggregation/agglomeration status (Oberdorster *et al*., 2005). All of the characteristics highlighted in this section can be determined via a number of different experimental procedures such as dynamic light scattering (DLS) for particle size, BET measurements to assess the surface characteristics of NPs and mass spectrometry to determine the chemical composition of a material. The reader is referred to Oberdorster *et al*. (2005) for a more, comprehensive review of characterizing NPs as a strategy prior to toxicological testing.

4.2.2 NP Suspensions and Dose

Similar to NP characterization, the manner in which NPs are suspended prior to exposure to biological systems has received increased attention. In a recent paper by Cedervall and colleagues (2007), the ability for NPs to interact with proteins, specifically the protein corona, was described. It stated that once suspended in biological media, the ability for proteins to interact with NPs is significantly increased, subsequently covering or coating the NPs. This process, therefore, has a significant effect on the manner in which the NPs interact with biological systems, such as cells, and also the subsequent effects of these interactions. Additionally, it has also been found that when NPs are suspended in cell–culture media

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both with and without serum, the toxicity observed is significantly different, with NPs suspended without serum inducing increased toxicity compared with those suspended with serum (Petri-Fink *et al*., 2008). Indeed, there are a number of investigations ongoing which are specifically examining the effects of NPs when suspended in different media and buffers, as well as how these buffers may affect the distribution of NPs in suspension (Wick *et al*., 2007). It is essential that the correct suspension media/buffer is used when forming a NP suspension for biological exposure because it is imperative to obtain an understanding of their interaction with biological systems following realistic exposure.

In addition to the manner in which NPs are suspended for cellular exposure, it is also necessary to take into consideration the specific dose that is being used when investigating the toxicity of NPs. Throughout the history of NP research, the realistic doses of NPs found either in air or within an occupational setting has been debated. Despite the ongoing assessment of the precise, realistic doses at which humans as well as the environment will inevitably be exposed to NPs, it is still an area of concern due to many studies using increased, non-realistic doses and reporting hazardous effects on human health and/or the environment following NP exposure. Also, it is not only the dose that has to be taken into consideration, but the manner in which the dose is expressed (i.e., dose metric) (Teeguarden *et al*., 2007). Therefore, in order to obtain a clear and definitive understanding of how NPs affect humans and the environment, the dose used must be both realistic and expressed without causing confusion.

4.2.3 Realistic Exposure Parameters

Recently, the exposure of NPs to biological systems, specifically cell cultures, has been a basis for increased discussion (Rothen-Rutishauser *et al*., 2009). In most nanotoxicology studies, particles are applied in suspension (for example, suspended in cell culture medium). If NPs are to translocate from the lung to secondary target organs (such as the liver or brain) then it is possible that the interaction between NPs and cells could be relevantly mimicked via the exposure of cell cultures to NP suspension (due to the NPs being translocated within blood). This aspect however requires further in-depth analysis to determine (i) whether and how NPs are translocated from the lung and (ii) at what dose the NPs are translocated and thus interact with the secondary organs. In addition, it is paramount that the specific characteristics of the translocated NPs are determined, as it is possible that they are no longer in the same format as when they entered the lung.

Although research into NP translocation is in its infancy (Kreyling *et al*., 2002a, 2002b), research, as noted throughout this chapter, into the effects of NPs on the lung is well documented. Indeed, exposure to most forms of NPs will initially be via inhalation and thus will affect the respiratory system (Oberdorster, Oberdorster and Oberdorster, 2005). Therefore, in order to achieve a more advantageous correlation between the *in vitro* and the *in vivo* situation, specifically regarding NP–lung interactions, cells have to be cultivated at an air–liquid interface and the particles have to be applied from the air. In order to study particle–cell interactions in cell culture systems representing the airway epithelial barrier, it is important to mimic the *in vivo* interactions of particles with cells as closely as possible. The particles have to maintain their shape as well as their chemical and physical properties when they come into contact with the cell surface to relevantly elucidate which particle properties contribute to toxic effects. As highlighted previously, the majority of existing cell culture models used to assess lung cell–particle interactions use immersed cells in medium, with particles added to the cell cultures as a suspension in liquid (Duffin *et al*., 2007; Gurr *et al*., 2005; Limbach *et al*., 2007; Rothen-Rutishauser *et al*., 2007; Stearns, Paulauskis and Godleski, 2001). This does not, however, reflect the physiological condition of lung epithelial cells, which are exposed to air, separated from it only by a thin liquid lining layer with a surfactant film at the air–liquid interface (Gehr *et al*., 1990; Gil and Weibel, 1971; Schürch *et al*., 1990). In addition, the physical and chemical properties of the particles rapidly change when suspended in medium, and NP aggregation and diffusion determine the transport and uptake kinetics in living organisms (Limbach *et al*., 2007). Other approaches include applying NPs to lung cells that have been cultured at the air–liquid interface by nebulizing particles over the air-exposed system using a spraying device (Lenz *et al*., 2009) or an exposition chamber with an integrated aerosol generator (Bitterle *et al*., 2006; Tippe *et al*., 2006). Another experimental approach is to work with an exposure device based on a cell exposure system, known as the CULTEX (Aufderheide

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and Mohr, 2000). In addition to this, simulation of accidental exposure of the lung to NPs in a relevant state of agglomeration and surface coating has been achieved by directly combining the synthesis of NPs to the exposure of cell cultures at the air–liquid interface using a glove box (Rothen-Rutishauser *et al*., 2009). Therefore, via the use of such systems, it is possible not only to obtain a clear and defined understanding of the interaction between NPs and the lung as it occurs *in vivo*, but to also mimic a specific exposure route of NPs to the human body.

4.2.4 Exposure Routes of NPs

The availability and toxicity of a substance to an organism is determined not only by the dose the entire organism is exposed to, but via a set of properties referred to as "toxicokinetics," which describe uptake, transport, metabolism, sequestration to different compartments and finally elimination of a substance from the organism. These parameters are essential, since toxicity of substances is dependent on which organs or cell types are exposed, which form the substance is in, for example bound to serum protein, aggregated, dissolved or oxidized, and how long the substance remains at the site of exposure. Therefore, the potential for NPs to exhibit toxicity at their site of entry (such as the lung, skin and gut) is of great relevance.

As described in Section 4.1, the respiratory system was initially identified for research because it was considered to be the primary target organ for particle toxicity due to PM_{10} exposure via inhalation and it was reported that, following epidemiological investigations, uf particles can contribute to reduced respiratory function and health (Peters *et al.*, 1997; Pope, Ezzati and Dockery, 2009). The impact of uf particles on the cardiovascular system was also investigated because mortality associated with exposure to PM_{10} was also associated with a reduction in cardiovascular function (Schwartz, 2004). The lung remains the primary organ for exposure to engineered NPs, specifically in reference to NP exposure during their production (occupational exposure) (Oberdorster, Oberdorster and Oberdorster, 2005). Despite this, due to the influx of many different types of NPs for a diverse range of applications, the routes by which NPs can enter the human body include permeation through the skin, ingestion and injection, in addition to inhalation (Oberdorster, Oberdorster and Oberdorster, 2005; Maynard *et al*., 2006). As it is not within the remit of this chapter to discuss each of these routes in detail, the following paragraph intends to cover the main aspects and current status of research in the field related to these potential routes of entry for NPs.

In contrast to the lung, little is known regarding the effects of NPs on the skin. Research that has been performed, however, widely suggests that the optimal opportunity for NP uptake by the epidermis is when the outer skin cells are broken, such as when the skin is sunburned or diseased (i.e., application of sun cream (which can contain either TiO_2 or zinc oxide) to burned skin) (Mortensen *et al*., 2008). Tinkle *et al*. (2003) has reported that when broken skin is flexed it is more sensitive to penetration by NPs, such as TiO_2, as it forms a more permeable environment, allowing translocation of the NPs, via Langerhans cells, to the lymph nodes and then subsequently into the blood circulation. Investigation into the effects of ingesting NPs is also in its infancy and is perhaps one area for which there is extremely limited amount of knowledge and understanding (Oberdorster, Oberdorster and Oberdorster, 2005). Initial research suggests that following ingestion of NPs (most likely via food) these particles can interact with the cells of the gut causing decreased cellular integrity and function (Oberdorster, Oberdorster and Oberdorster, 2005). Further research is imperative to fully understand the effects of NPs following ingestion, specifically to investigate the characteristics of NPs at this level and the concentration at which NPs interact with the gut. In comparison to NP ingestion, research into the effects of NP exposure via injection is limited, and is specifically concentrated upon their effects associated with medicinal application. The aim of injecting NPs is to target specific organs, tissues and cells; thus, the NPs used will be coated with coatings such as polymers, in order to negate their identification by the immune system, specifically phagocytic cells (such as macrophages) (Oberdorster, Oberdorster and Oberdorster, 2005). Although increased research has been based upon investigating the effects of NPs intended for use in nanomedical applications, there are no current studies focussing upon NP effects in the bloodstream. It is essential, therefore, that research into this area is performed so that the advantages posed by nanomedicine (ESF Report, 2005) are gained.

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Despite concentrated efforts to investigate the effects of NPs on the (potentially) primary exposed organ, as previously highlighted, it has become evident that NP localization might not be restricted to their portal of entry (Kreyling *et al*., 2002a, 2002b). It has been reported that NPs can become distributed to organs distal to their site of exposure, so that NP toxicity can be potentially exerted at a number of secondary targets (Oberdorster, Oberdorster and Oberdorster, 2005). Therefore, toxicity at a number of targets (including the liver, brain, spleen and kidneys) is worthy of consideration when assessing the toxicity of NPs on human health. For example, the liver is considered to be a preferential site of NP exposure via a variety of routes (Nemmar *et al*. 2001; Oberdorster *et al*. 2002; Ogawara *et al*. 1999). Thus it is imperative that the ability of NPs to have detrimental consequences on normal liver function is investigated. In addition to the effects of NPs on human health, an area that is receiving increased research interest due to augmented concerns is the effect of the release of NPs into the environment (also via an array of different exposure routes). The release of such particles could increase the concentration of atmospheric particles (Borm *et al.*, 2006), which has obvious human health implications, when considering the epidemiological findings of PM_{10} toxicity, in addition to damaging the environment. Therefore, in order to fully understand the effects of NPs on both human health and the environment, it is essential that increased research is performed in each of these exposure areas in order to fulfil the potential advantages that nanotechnology offers.

4.2.5 Additional Paradigms for NP Toxicity

As previously described in Sections 4.1 and 4.11, the oxidative stress paradigm has been the basis for which increased toxicological research has been performed in relation to NPs (Oberdorster, Stone and Donaldson, 2007). With the constant increase in the production of different engineered NPs, as well as in the number of studies being published which have investigated the potential toxicity of NPs, there are now alternative theories, or paradigms, relating to NP toxicity. Additionally, due to the increased research being performed, it is only a matter of time before numerous theories are produced relative to the many different forms of NPs available.

Recently, the most publicized paradigm is the fibre paradigm (Dörger *et al*., 2001; Donaldson and Tran, 2004). The fibre paradigm was originally formulated in relation to the effects of asbestos fibres and their ability to cause mesothelioma in the lung (Dörger *et al*., 2001; Donaldson and Tran, 2004). Due to the production of more fibrous nanomaterials, specifically CNTs, this paradigm has recently shown NPs, or nanofibres (a nano-object with two similar external dimensions in the nanoscale and the third dimension significantly larger (ISO/TS 27687, 2008)) to fit into the fibre paradigm (Poland *et al*., 2008). Specifically, in relation to CNTs, the fibre paradigm relates to high aspect ratio nanoparticles (HARN), which expresses the possible adverse effects that a material could have in regards to its length divided by its width (with a minimum 3:1 required to fit the paradigm). It is also necessary that the fibre is at least 15 μm in length. It has been shown that this length of nanofibre can cause a process known as "frustrated phagocytosis" (Brown *et al*., 2007b) to occur, due to cells being unable to actively take up these fibres. The repercussions of this are an increased level of oxidative stress and subsequent stimulation of pro-inflammatory cytokines (Brown *et al*., 2007b) (similar to the oxidative stress paradigm). Therefore, it has been shown by these studies, and also in light of the aim of this chapter, that within NP toxicology research it is not just one aspect that affects the interaction and response of NPs to biological systems. It is essential that numerous aspects are taken into consideration when performing any type of nanotoxicology research.

In addition to the fibre paradigm, the theory of genotoxicity has also been extensively explored. The theory of genotoxicity (Schins and Knaapen, 2007) is predominantly based upon the oxidative stress paradigm, and however moves on from the stimulation of an inflammatory response to assess what the stimulation of inflammatory mediators could incur, regarding human health. The theory describes a two-tiered approach: primary genotoxicity and secondary toxicity. In regards to primary genotoxicity, it is suggested that NPs can cause genotoxicity following direct exposure to the biological system. Secondary genotoxicity however describes, initially, the oxidative stress paradigm (in theory: NP exposure = ROS/reactive nitrogen species (RNS) production (also oxidative stress (oxidant/antioxidant imbalance)) = chronic inflammatory response), which causes genotoxicity and

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(possibly) subsequent tumour formation. Secondary genotoxicity however, may not be caused by the NPs alone; it may also be caused via interaction of the biological system and the chemicals contained within the NPs. It is suggested that the NPs might be completely inert but are able to penetrate the cellular membrane, possibly locating within the nucleus. At this stage, due to the high acidic pH, the chemicals present within the NPs could be released (such as Fe within Fe platinum NPs) causing a toxic response. This form of secondary toxicity has also been referred to as the "Trojan horse" effect (Limbach *et al*., 2007). It is also possible, however, in relation to this theory that the cells might undergo cell death and thus not induce genotoxicity and tumour formation. Additionally, the cells might proliferate or undergo repair of the deoxyribose nucleic acid (DNA) chain and negate any NP effects (Schins and Knaapen, 2007). It is prudent to point out that this theory has been based upon biological system exposure to NPs of a size >100 nm and knowledge of how the theory works in relation to NPs <100 nm is not limited at this time. Few studies have investigated the potential for NPs and nanofibres to induce genotoxicity (Knaapen *et al*., 1999; Zhao *et al*., 2004; Bao *et al*., 2006; Jacobsen *et al*., 2008; Takagi *et al*., 2008); however, increased research is still necessary in order to assess the realistic possibility that NPs can induce genotoxicity and subsequently cancer (Poland *et al*., 2008).

5 TESTING NP TOXICITY *IN VITRO*

When assessing the potential toxicity of any form of NP it is important to investigate how the particles enter the specific cells being examined and in which compartments they may be found (or, if the NPs stay attached to the cell membrane, which is also possible). This is of great importance because it has been shown that the uptake behaviour can influence the cellular response following NP exposure (Unfried *et al*., 2007; Maysinger *et al*., 2007). As it is not possible to explore in detail the entry mechanisms of NPs into cells, the reader is referred to the studies of Unfried *et al*. (2007), as well as Rothen-Rutishauser, Schurch and Gehr (2007) and Müehlfeld, Gehr and Rothen-Rutishauser (2008a), Muhlfeld *et al*. (2008), which provide a good overview of these processes. As previously highlighted in this chapter, there are a number of different toxicological endpoints which researchers have used to assess the potential adverse effects that NPs may have on organs of the human body. It is also pertinent to point out that these tests are beginning to be used to assess the impact of NPs on the environment. The following section therefore intends to summarize the majority of the many different tests available to study the toxicity of NPs and how they may be used to provide a clear, thorough and complete assessment of NP toxicity.

5.1 Advantages and Disadvantages of *in vitro* Testing Compared to *in vivo* (*ex vivo*) Testing

Although all the experimental analyses described in Section 5.2 can be performed with cells obtained from *in vivo* experimentation (*ex vivo)* as well as *in vitro*, it is pertinent to highlight the constant debate on the advantages and disadvantages of both *in vitro* and *in vivo* testing. Although *in vivo* (specifically mice and rat models; however also considering research on hamster, guinea pig and monkey models) research is known to enable scientific research to observe almost "first-hand" the effects of a substance as they would occur in Homo sapiens, *in vitro* models provide the possibility to investigate toxic effects on human cells extensively, which cannot be conducted *in vivo* (Rothen-Rutishauser *et al*., 2008a). Cultured human and animal cells can be better controlled and therefore yield more reproducible data than *in vivo* systems; however, they require a high standardization to maximize reproducibility. In addition, there are many efforts to establish more realistic models to study the toxic potential of NP, as for instance the triple-cell co-culture system composed of epithelial cells, macrophages and dendritic cells which has been established, simulating the most important barrier functions of the epithelial airway (Rothen-Rutishauser *et al*., 2005, 2008b; Blank *et al*., 2006).

5.2 Tests Used to Assess NP Toxicity

5.2.1 Cytotoxicity and Cell Death

In order to determine the absolute toxicity, otherwise known as the lethal dose (LD50) (Timbrell, 1999) of a substance, an assessment of the substance's ability to cause complete cytotoxicity and/or cell death is

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necessary. As highlighted in Section 4, within the field of nanotoxicology, researchers have tended to use an assessment of the permeability or integrity of the cell membrane in order to assess the cytotoxicity of NPs. This has predominantly been achieved by measuring the amount of LDH present within cell supernatants following NP exposure. LDH is contained within the cytoplasm of cells and can be subsequently released following a loss in cell membrane integrity (Kendig and Tarloff, 2007). LDH release from cells has been shown to be a good indicator of cytotoxicity (Henderson *et al*., 1985), as well as associated with necrotic cell death (Kendig and Tarloff, 2007). It must be stressed, however, that assessment of LDH release from cells indicates neither necrosis nor any form of cell death. Quantification of the level of LDH enzyme release from particulate-treated cells into the external cell culture medium (cell supernatant) (this process is also known as membrane leakage) can be achieved by assessing the catalytic interconversion of pyruvate to lactate, concomitantly with an investigation of the interconversion of nicotinamide adenine nucleotide (NADH) to its oxidized form nicotinamide adenine dinucleotide (NAD^+). Predominantly LDH is assessed via spectrophotometry and absorbance. It is suggested, however, that presentation of such data sets is arbitrary (i.e., when expressed as an optical density). In order to provide a realistic observation, results need to be expressed as a function of the total LDH release (positive control), which can be determined by destroying the cell membrane with Triton X-100 (Figure 1).

Indeed, as highlighted in this chapter, nanotoxicology research is dependent upon the performance of multiple assessments in relation to each variable. Therefore, a measurement of LDH to denote the cytotoxicity of a NP is not sufficient. Numerous colorimetric tests are also available to assess the potential cytotoxicity of NPs. These include the MTT, WST-1 and MTS assays. These assays are based upon the ability of living, metabolically active cells to cleave a tetrazolium salt. These assays enable the determination of cell survival, proliferation and activation in a wide variety of cells, including macrophage-like tumour and lymphoma cell lines, as well as stimulated myeloma cells. Similar to the LDH assay, this is achieved via quantification by spectrophotometry of the formazan generation by active mitochondria, indicative of the energy metabolism in mitochondria (levels of mitochondrial succinate dehydrogenase) within living cells (Mossman, 1983). Generally, it is accepted that an observed increase in absorbance is indicative of cell proliferation, whilst a decrease in absorbance suggests either a reduction in metabolic activity and/or a decrease in viable cell number. It is suggested that the data gained from these tests are presented as the percentage of the negative control (Figure 2).

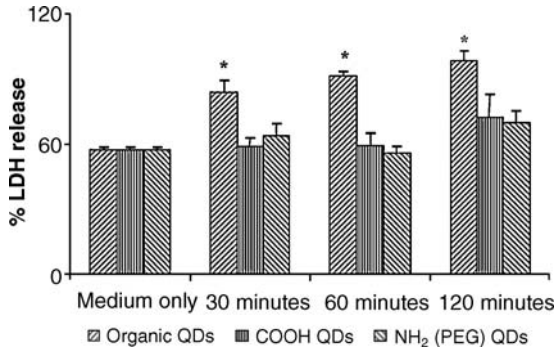

Figure 1. Percentage LDH release from J774.A1 macrophage-like cells following treatment with organic, COOH and NH_2 (PEG) QDs, as well as 20 and 200 nm PBs for 30, 60 and 120 min. * indicates significant difference ($p < 0.01$) compared to control cells treated with medium only. 100% LDH release is associated with 0.1% Triton X-100 treatment of macrophage cells. Adapted from Clift *et al*., (2008) © Elsevier.

As highlighted with the MTT, WST-1and MTS assays, assessment of the cytotoxicity of NPs, like any xenobiotic, is relative to their effects on the

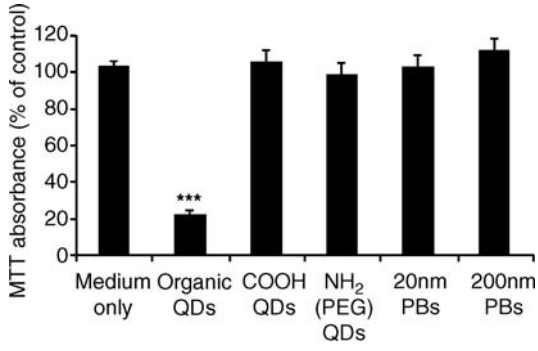

Figure 2. The effect of organic, COOH and NH_2 (PEG) QDs, as well as 20 and 200 nm PBs on the mitochondrial metabolic activity, as determined via the MTT assay, of J774.A1 macrophage-like cells after 2 h. Data shown are mean ± SEM. *** indicates significant difference ($p < 0.0001$) compared to control cells treated with complete medium only. Reproduced from Clift *et al*., (2008) © Elsevier. (reprinted with permission from Toxicology and Applied Pharmacology).

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mitochondria, due to its importance in the energy production for cell function and viability. Direct assessment, by both quantitative and qualitative means, can also be performed in order to assess the ability of NPs to disturb the normal homeostasis of this cellular organelle via assessment of the mitochondrial membrane potential. This is generally achieved by purchasing a kit, providing the relative dyes in order to perform both confocal laser scanning microscopy (CLSM) and fluorescent-activated cell sorting (FACS™).

Although the above-mentioned assays take into consideration the effects of NPs on the mitochondria and the cell membrane, the direct measure of the ability of NPs to cause cytotoxicity is via an assessment of cell death. Cell death can be attributed to numerous cell cascades, resulting in different cell death processes. There are, however, two specific forms of cell death which have received increased attention in relation to NP exposure to cells. The first of these processes is apoptosis, also known as controlled cell death. The second, necrosis, is a pathological process because it has been shown to occur in response to externally induced toxicity, including inflammation. Cell death is commonly assessed by the use of specific fluorescent staining solutions/antibodies for both apoptosis and necrosis. Although there are many different kits and tests available, assessment of NP-induced cell death is generally achieved by performing the Annexin-V assay. When cells undergo apoptosis, one part of this process includes the transport of inner cell membrane lipids (such as phosphatidylserine) to the outer side, serving as a marker for macrophages to eliminate these cells. In the Annexin-V assay, the fluorescent dye Annexin-V-Fluorescein binds to phosphatidylserine, thus marking the apoptotic cells. The necrotic cells are also distinguished by the use of a fluorescent dye propidium iodide. Staining with the fluorescent dye propidium iodide identifies the DNA of cells. This method can be achieved, similar to the mitochondrial membrane potential assay, both qualitatively and quantitatively; thus, the result can be observed via both CLSM and FACS™ analysis.

5.2.2 Reactive Oxygen/Nitrogen Species and Oxidative Stress

Due to the oxidative stress paradigm being the foundation for nanotoxicology research, assessment of the formation of ROS and oxidative stress can be achieved by an ever-increasing list of assays and diagnostic kits. Despite this, there are specific parameters that are constantly used in order to denote the production of ROS, the increase in oxidants or the loss in antioxidants within a biological model.

In regards to the production of ROS, the most common parameter used is the fluorescent dye 2′,7′ dichlorodihydrofluorescein diacetate (DCFH-DA), a reliable fluorogenic marker for ROS in living cells. This method assesses the production of ROS via the increase in fluorescence. Upon the production of ROS, the DCFH-DA molecule is cleaved, resulting in a DCFH molecule which is highly fluorescent. The fluorescence signals can be both imaged via CLSM and quantified using FACS™ analysis and fluorescent spectrophotometry. A similar form of testing procedure is also based upon the fluorescent dye dihydroethidium (DHE). Similar to the DCFH-DA reaction) in which ROS production is determined by the level of fluorescence, DHE detects the extent of oxidative stress and ROS production in cells, by oxidizing to ethidium, which is highly fluorescent. These methods are extremely advantageous, as not only they can be performed via multiple approaches, but they can also be used in a cell-free environment. This is important because it provides important information pertaining to the NPs alone, and thus their ability to cause ROS independent of a cellular environment. This, of course, is extremely advantageous in regards to assessing the ability of NPs to cause secondary genotoxicity.

As previously mentioned, there are a number of kits available to measure ROS production and mostly these are based on the DCFH-DA reaction. An example of this is the Image-iT™ LIVE Green ROS Detection Kit (provided by Molecular Probes). In Figure 3, the ability to determine the ROS production in cells following NP exposure is easily identified. This is only an example of the ability to quickly and efficiently identify the ability of NPs to cause ROS production in cells.

As previously mentioned in Section 4.1.1, the production of ROS is concomitant with a reduction in antioxidants, thus causing an oxidant imbalance within the cell and therefore oxidative stress. It is therefore, as also previously highlighted in the chapter, pertinent that an assessment of the antioxidant capacity is performed in order to obtain a thorough and clear understanding of the oxidant-related

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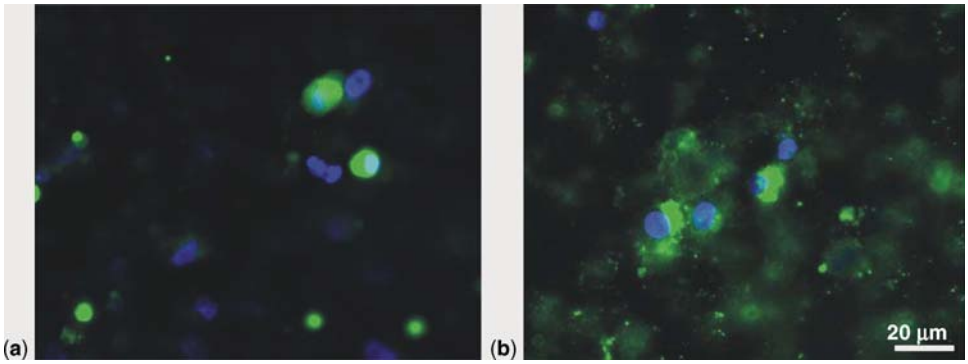

Figure 3. Fluorescent microphages of ROS production in the triple cell co-culture model (Rothen-Rutishauser, Kiama and Gehr, 2005) obtained after using the Image-iT^{TM} LIVE Green Reactive Oxygen Species (ROS) Detection Kit (Molecular Probes). Cell nuclei are shown in blue, ROS in green. In control cultures only few cells with ROS signals could be detected (a) (reproduced with permission from Rothen-Rutishauser, Kiama and Gehr, 2005 © American Thoracic Society), whereas TiO_2 NP induce a higher oxidative response, as the green stain in the cytoplasm shows (b) (reproduced from Rothen-Rutishauser *et al.*, 2008b © Springer).

effects of NPs. This form of analysis has commonly been performed in regards to an assessment of intracellular glutathione levels. Glutathione is an intracellular non-protein thiol readily available in a wide range of living cells, and is a key factor in a number of biological functions, including oxidative stress (Hissin and Hilf, 1976). It is usually present in cells as GSH; however, when existing in cells as its oxidized glutathione (GSSG) form, glutathione is rapidly converted to GSH via an enzymatic reaction using glutathione reductase. In the method originally published by Hissin and Hilf (1976), the levels of both GSH and GSSG were determined via their pH-sensitive reactions with the fluorescent reagent *o*-phthaldehyde. There are of course many alternative ways to determine glutathione levels within cells, including diagnostic kits and microscopy-related assays. In addition, there are numerous ways to assess the antioxidant capacity of cells, such as the trolox equivalent antioxidant capacity method (TEAC); however this is extremely laborious and difficult to optimize.

In addition to determining the antioxidant capacity, it is also possible to determine the level of oxidants present within the biological sample. This is notoriously difficult and time-consuming; however, it provides extremely valid and optimal results in regards to the oxidative potential of NPs. Such testing strategies include assessment of the $^{\cdot}OH$, as well as the superoxide anion via polymerase chain reaction (PCR). These can be determined via diagnostic kits; however, due to the intrinsic nature of these radicals/anions within the cellular system the most reliable method for assessing these aspects is via PCR.

In addition to the measurement of ROS and oxidative stress, it is also prudent to determine the ability for nitric oxide (NO) to be produced. Often, however, NO is not assessed in relation to the radical formation potential of NPs. Increased research should be directed towards this aspect, though, due to the fundamental role of NO in cellular function. A radical gas, NO, is derived from the guanidino nitrogen of the amino acid L-arginine and molecular oxygen in a reaction catalyzed by the enzyme nitric oxide synthase (NOS). There are at least two types of NOS. The calcium-dependent form is present constitutively in a variety of tissues and produces the physiological concentration of NO needed for maintenance of blood pressure. The other, calcium-independent form, is inducible (iNOS) in a number of cell types including macrophages, hepatocytes, neutrophils and endothelial cells, by a variety of immunological stimuli such as interferon-γ (IFN-γ) and bacterial lipopolysaccharide (LPS). iNOS is a high-output enzyme and nitrite concentrations are easily detected. It is possible to measure NO (or iNOS) via spectrophotometry using a colorimetric test known as the Griess reaction, for which numerous diagnostic kits are available. It is important to note, however, that problems have been reported when assessing NO in primary cells, as well as some cell lines (for example A549 epithelial cells). This test is, however, advantageous in

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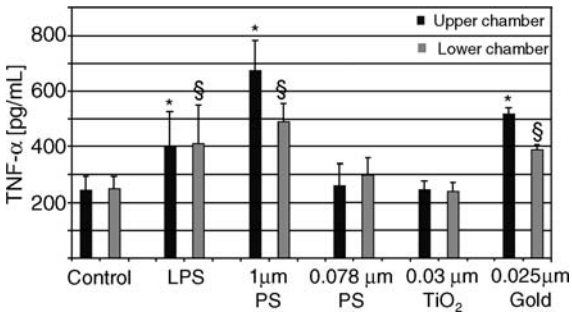

Figure 4. TNF-α release in triple cell co-cultures upon particle incubation. TNF-α levels in the supernatants (upper chamber, lower chamber) were measured by ELISA. TNF-α release in cells exposed to LPS, 1 μm, and 0.078 μm polystyrene particles, TiO_2, and Au NPs. Values are means ± SD. * indicates a statistical difference to the levels in the supernatants in the control of the upper chamber; §indicates a statistical difference to the levels in the supernatants in the control of the lower chamber. Reproduced from Rothen-Rutishauser *et al*. (2007) © Springer.

assessing the ability of NPs only to produce NO (i.e., in a cell-free environment). In order to assess NO in cells therefore, it is also possible to use PCR analysis. This testing strategy, however, as with measurement of both the ·OH and the superoxide anion, is extremely laborious and time-consuming.

5.2.3 Inflammation

Assessment of inflammation *in vitro* is usually achieved via the use of enzyme-linked immunosorbent assay (ELISA) diagnostic kits (Figure 4). Although expensive, these diagnostic kits provide valuable and reproducible testing strategies to measure the plethora of different pro-inflammatory cytokines and chemokines that have been associated with NP exposure to culture cells. In addition to ELISA kits, it is also possible to measure cytokine/chemokine production either via PCR analysis or FACS™ analysis; however, these methods are again laborious and, in comparison to the ELISA method, provide no further advantages.

5.2.4 Cell Signalling

As highlighted in Section 4.1.1, the influence of NPs on cell signalling, specifically upon Ca^{2+} signalling in cells, is a significant factor related to the oxidative stress paradigm. In relation to this, Ca^{2+} signalling is not as well researched as the ability of NPs to stimulate ROS and inflammatory cytokines/chemokines. Although it is possible to measure intracellular calcium levels via a number of means, again including diagnostic kits, the most commonly used indicator of changes in intracellular calcium is the fluorescent marker FURA 2-AM (Grynkiewicz, Poenie and Tsien, 1985). The function of this dye is similar to that of DCFH-DA, as during the incubation period with the cells (and xenobiotic), if there is a change in Ca^{2+}, intracellular esters are able to remove the acetoxymethyl (AM) group from the Fura 2-AM molecule, making the membrane impermeable to Ca^{2+}-sensitive fluorescent dye, Fura 2. It is then possible to determine the level of Ca^{2+} present in samples via spectrophotometry, as well as by CLSM.

In addition to this, and as previously mentioned in Sections 5.2.2 and 5.2.3, PCR is a very useful tool in assessing the effects of cell signalling. Although this method can identify specific proteins and genes (ribose nucleic acid (RNA) transcription/isolation), it is laborious and requires increased optimization time in order to obtain valid and reliable results.

5.2.5 Genotoxicity and Mutagenicity

As previously discussed in Section 4.2.5, the theory of genotoxicity and mutagenicity is initially based upon the oxidative stress paradigm, and thus to initially determine this, the tests discussed in Sections 5.2.1–5.2.4 must be performed first (it is important to note that for an NP to be genotoxic, it must not be cytotoxic (at a specific dose) and therefore it is essential to test for this initially). When determining the genotoxicity of a NP, however, there are numerous tests that can be performed. Specifically, however, research has focused upon a few specific toxicity tests which measure the ability of NPs to inflict DNA damage, mutagenicity and cell proliferation.

DNA damage is commonly investigated via the comet assay. In addition to measuring DNA damage, this assay also assesses other DNA alterations in cells (Jacobsen *et al*., 2008). The extent of DNA damage is revealed via electrophoresis of the agarose embedded single-cell samples, showing both DNA fragments or damaged DNA to migrate away from the nucleus (forming a comet shape). After staining with a DNA-specific fluorescent dye, such as propidium iodide, the fluorescent signal of the gel is then measured in the head and tail and

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length of tail. The extent of DNA liberated from the head (to the tail) of the comet is directly proportional to the amount of DNA damage. Quantification of the liberated DNA is generally achieved by the use of specific computer software; however, it can also be determined by giving comets a series of numbers related to the severity of DNA damage. This latter method, although original, is flawed, specifically due to the impact of human error; therefore, it is not advisable despite the expense of the computer software. In addition to the original comet assay, alternative testing protocols have been developed in which oxidative DNA damage can be assessed simultaneously. For example, by inclusion of the formamidopyrimidine glycosylase (FPG) enzyme, the assay also allows for specific detection of oxidative DNA lesions (Jacobsen *et al*., 2008) Although this form of the comet assay requires double the samples, it is extremely advantageous to obtain an understanding of the oxidative/genotoxic potential of NPs.

Assessment of an NP's ability to cause mutagenicity is also a prominent testing strategy when determining the genotoxic potential of NPs. A well-defined testing protocol for mutagenicity is the micronucleus assay. This test has been proven to detect both clastogenic and aneugenic events, as well as to be valid for genotoxicity testing of micron- and nano-sized particles (Singh *et al*., 1988; Li *et al*., 2008). In addition to the micronucleus test, a recent testing protocol for NP genotoxicity is the Ames test. This test is based on strains of bacteria that have point mutations on the histidine (*His*) operon. Due to the position of the mutation on the *HIS*-gene, the bacteria are incapable of producing histidine and therefore unable to proliferate unless histidine is present (Ames *et al*., 1973). If chemicals are mutagenic then additional mutations occur on the *His*-gene, known as base substitutions or frameshift mutations, which enable the bacteria to produce histidine and subsequently proliferate. The mutagenicity of the chemical is then determined via quantification of the ability of the histidine-free bacteria to replicate. Previously, this test has been used to screen chemicals; however, recently it has been successfully used to study the mutagenicity of NPs, specifically DEPs (Zhao *et al*., 2004). There is debate, however, on the specificity of this mutagenicity test, due to the interaction of NPs with bacteria and not with cells. The understanding of the interaction between NPs and bacteria is extremely limited, if not unknown, and therefore it is not understood whether the NPs will interact with the bacteria in a similar manner as with a cell, and whether the use of this protocol will provide specific, realistic mutagenic effects of NPs in relation to human health.

Cell proliferation is also a key factor in the genotoxic potential of NPs (Schins and Knaapen, 2007). As previously highlighted, this can be achieved via a number of different assays, such as the MTT, WST-1 and MTS assays. Due to the reported concerns and difficulties of using these assays (Worle-Knirsch, Pulskamp, and Krug, 2006) (Section 5.3), an alternative test for determining the ability of cells to proliferate is the BrdU assay. The BrdU assay is an immunochemistry technique which assess newly synthesized (or partially denatured double-stranded) DNA strands in cells that are undergoing active proliferation via fluorescent microscopy. This technique enables the assessment of cells that have been treated with NPs to actively synthesize and proliferate. Similar to the MTT, WST-1 and MTS assays, this test can be performed via purchasing a diagnostic kit and using spectrophotometry, as well as CLSM, therefore providing a valid and reliable testing strategy for cell proliferation in relation to NP genotoxicity.

5.3 Use of Appropriate Controls When Assessing the Toxicity of NPs

In addition to all of the previously mentioned parameters that can be used when assessing the potential toxicity of NPs (in relation to the specific experimental question), it is also vital that additional and appropriate controls are used throughout.

When choosing a toxicology testing strategy, it is important that a relevant positive control is also chosen. Although negative controls are easily identified (usually cell culture medium/buffer only), determination of the correct positive control must be given more thought. For example, a low concentration of Triton X-100 (such as 0.1% or 0.2%) is able to provide a clear positive reaction in any LDH assay (either diagnostic kit or laboratory protocol); however, this form of compound would not be useful when assessing the potential production of ROS by NPs within cells. It is therefore pertinent that the reaction the test is causing is debated and the most adequate and specific positive controls are used.

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As highlighted in Section 4.2.2, the ability for proteins to adsorb onto the surface of NPs is realistic and can significantly affect the ability of NPs to interact with cellular systems. It is, therefore, also essential that the ability of proteins to adsorb onto the surface of particles during toxicological tests is assessed (such as when using an ELISA). This is also true for enzymes such as LDH. It is known that this enzyme can also adsorb onto the surface of NPs, masking their toxicity and thus providing a false-negative toxic result. As with protein adsorption, enzyme adsorption must also be investigated to correlate valid and representative toxicity data. Determination of these aspects is easily performed. Generally, the protocol consists of incubation of the NPs with the protein/enzyme for a period of time followed by the specific toxicological test (Clift *et al*., 2008). Any adsorption of the proteins or enzymes onto the NPs is then apparent via a loss or increase in the specific protein/enzyme being measured. Additionally, it is also necessary to determine the ability of the NPs used to interact in regards to the fluorescent dyes or formazans that are used. An example of this was reported by Worle-Knirsch, Pulskamp and Krug (2006), where it was reported that CNTs interacted with the MTT formazan (tetrazolium salt) used and provided false-negative results. In addition to this, it is also pertinent that an assessment of the toxicity of the suspension media/buffer is performed. Increasingly, NPs are suspended in such buffers as Pluronic F127 and Tween 80 in order to obtain a well-dispersed and characteristic NP suspension. If, however, a toxic response is observed following cellular exposure to NPs suspended in such buffers, it is essential that the toxicity of these buffers is known, in order to assess the specific effects of the NPs only (Wick *et al*., 2007).

6 CONCLUSION

The field of nanotoxicology is complex and requires increased research in order to enable the advantages of the nanotechnology industry to be realized. It is paramount that the findings of environmental air pollution research are taken on board and built upon to assess the potential adverse effects that could occur following exposure to the many new, engineered NPs. This of course is best achieved via *in vivo* research; however, this area of research can be too invasive, time-consuming and expensive. It is therefore prudent that research is driven towards a more *in vitro* approach, where specific cell culture models, mimicking the *in vivo* situation, are constructed and used regularly. By using these models, toxicological screening of engineered NPs can be achieved quickly and efficiently using a series of toxicity tests (as described in Section 5.2). It is important that increased controls and supporting tests are performed concomitantly in order to obtain a clear, definitive and realistic understanding of the risk that NPs might pose.

7 LIST OF ABBREVIATIONS

OH	Hydroxide radical
AM	Acetoxy methyl
AP-1	Activator protein-1
Au	Gold
BAL	Bronchiolar lavage
BSI	British Standards Institution
C_{60}	Carbon 60 (fullerene)
Ca^{2+}	Intracellular calcium
CB	Carbon black
CH	Switzerland
CLSM	Confocal laser scanning microscopy
CNT(s)	Carbon nanotube(s)
COOH	Carboxylated
COPD	Chronic obstructive pulmonary disease
DCFH	2′,7′-dichlorofluorescin
DCFH-DA	2′,7′-dichlorofluorescin diacetate
DEP	Diesel exhaust particles
DHE	Dihydroethidium
DNA	Deoxyribose nucleic acid
DLS	Dynamic light scattering
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ESF	European Science Foundation
FACS™	Fluorescent-activated cell sorting (Becton Dickinson™)
Fe	Iron
$FeCl_2$	Iron chloride
FPG	Formamido pyrimidine glycosylase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
HARN	High aspect ratio nanoparticles

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HO-1	Heme-oxygenase-1
IL	Interleukin
IL-1β	Interleukin-1beta
IL-8	Interleukin-8
iNOS	Inducible nitric oxide synthase
IFN-γ	Interferon-gamma
ISO	International Organization for Standardization
LD_{50}	Lethal dose of 50% of the sample
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MIP-2	Macrophage inflammatory protein-2
MWCNT(s)	Multi-walled carbon nanotube(s)
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine nucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-kappa B
NH_2	Amino
nm	Nanometre
NO	Nitric oxide
NOS	Nitric oxide synthase
NP(s)	Nanoparticle(s)
PAS	Publically available specification
PB	Polystyrene beads
PCR	Polymerase chain reaction
PEF	Peak expiratory flow
PM	Particulate matter
PM_{10}	Particulate matter of $<10\,\mu m$
PMNs	Polymorphonuclear leucocytes
QD(s)	Quantum dot(s)
ROS	Reactive oxygen species
RNA	Ribose nucleic acid
RNS	Reactive nitrogen species
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks
SPIONs	Superparamagnetic iron oxide nanoparticles
SWCNT(s)	Single-walled carbon nanotube(s)
TEM	Transmission electron microscopy
TiO_2	Titanium dioxide
TNF-α	Tumour necrosis factor-alpha
uf	Ultrafine (<100 nm)
ufCB	Ultrafine carbon black
ufCP	Ultrafine carbon particles

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Alternatives to In vivo Studies in Toxicology

REFERENCES

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Toxicity of Selenium Compounds and Nano-Selenium Particles

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1 BRIEF HISTORY OF SELENIUM

The element selenium was discovered by Jons Jacob Berzelius (1779–1848) in 1817 as a red deposit in a sulphuric acid flue deposit at Gripsholm, Sweden (Berzelius, 1817). Believed at first to have been a tellurium deposit, it was a new element having intermediate characteristics of tellurium and sulphur. Berzelius named it selenium for Selene, goddess of the Moon, as the new element fit within the periodic table above tellurium, which had been discovered in 1783 and named for the Earth, Talus. Berzelius, in addition to discovering selenium, also discovered lithium, silicon, thorium and cerium. He is furthermore credited with establishing the two-letter system (Xx) of elemental identification as used within the periodic table.

Although Berzelius identified and named the element selenium, the Venetian Traveler Marco Polo is the first to have seemingly described the toxic attributes of selenium ingestion by horses along the Silk Road. The observation of sick and ill horses with their hoofs sloughing off was probably made in or about 1265 upon Marco Polo's return trip to Venice from China as he crossed the Silk Road (Rosenfeld and Beath, 1964). This toxicity in horses, as well as in cattle and sheep, was to be rediscovered in the settling of the western United States. In 1857, Dr Madison, an army surgeon, described a fatal condition affecting horses of the US Cavalry near Ft Randall of the Nebraska Territory now located in South Dakota (Rosenfeld and Beath, 1964). Loss of tail and mane hair, appetite and the sloughing-off of hooves of horses are some of the symptoms described by Madison. This condition, affecting grazing livestock throughout the Nebraska Territory, was noticed and the citizens of Boyd County gave it the name "alkali disease", thinking the cause was the mineral salts of water, soils or pasture. In 1929, Franke (1934) working for the experimental station in South Dakota was to discover that grains grown on specific soil deposits imparted to animals "alkali disease" and that they were exceptionally toxic to livestock. Robinson (1933) had been given samples of toxic South Dakota wheat and, at the suggestion of Knight, analysed the wheat for its selenium content. The wheat gluten contained 90 μg Se g^{-1} of the protein which we now know to have been selenomethionine. By mid-1930s, selenium accumulation in plants from soils was known to be the cause of "alkali disease" and the disease had all the same characteristics expressed in livestock that were described in horses by Marco Polo.

The next 20 years or so were spent in the identification of those plant species that took up

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and concentrated selenium from soils. Selenium was found in many species of the plant genera *Astragalus*, *Xylorhiza*, *Oonopsis* and *Stanleya*, conspicuous wild flowers of the western United States (300 species) and other arid and semi-arid regions of the world (1200 species overall). Although selenium is not generally recognized as required for plant species, the early growth experiments with *Astragalus racemosus* and *Astragalus beathii* certainly indicated a physiological requirement for selenium. Indeed, some but not all species of *Astragalus* plants are known to only grow on outcroppings of sediments with heavily concentrated selenium. Where these selenium accumulator plants grow, they often volatilize malodorous and nauseous odors of selenium compounds. The selenium concentrated and volatilized by accumulator plants originates primarily as selenates with lesser amounts of selenites in soils. Accumulation in these plants ranges from a few $\mu g\,Se\,g^{-1}$ to thousands of $\mu g\,Se\,g^{-1}$ of dry plant material. Some plants of the *Astragalus* genus highly accumulate selenium; *Asters*, *Atriplex* and other plants moderately accumulate selenium; whereas cereal grains and grasses and most other plant foods accumulate only low levels of selenium (Trelease and Beath, 1949; Rosenfeld and Beath, 1964). Today, speciation of these and other selenium-containing plants and plant foods have revealed the presence of several different selenoamino acids (Pedero and Madrid, 2009).

In 1941, *Astragalus pectinatus* yielded the first unidentified selenoamino acid from a protein, $C_7H_{14}N_2O_4Se$, isolated in association with its sulfur analogue. It had long been suspected since 1916 that selenium might be a replacement for sulfur in plants, as they existed together in the VIA period of the periodic table. Early examination of these plants indicated they contained both sulfate and selenate, but within *Astragalus* plants, only organic selenium compounds were found, most of which were directly soluble in water. In cereal crops and grasses, much of the selenium was not water soluble and could not be readily dissociated from the plant's protein fraction. By 1950, selenomethionine and selenocysteine had been reported to have been identified in plant protein hydrolysates. In 1960, Se-methylselenocysteine was identified in *Astragalus bisulcatus* following the identification of *S*-methylcysteine (Rosenfeld and Beath, 1964).

In 1957, selenium had been identified as a component of a yeast isolate fraction called "Factor-3" which prevented a condition known as "dietary liver necrosis" in rats. Dietary liver necrosis in rats occurred when rats were fed a torula yeast and low vitamin E diet, but not when fed a brewer's yeast. This discovery was made by Schwarz and Foltz (1957) then working at the National Institutes of Health. The other "two factors" preventing dietary liver necrosis were a higher dietary level of vitamin E and additional sulfur amino acids such as cysteine fed to rats. This revelation that toxic old selenium could prevent a nutritional disorder of the liver in rats and also other detrimental disorders and conditions in animals has changed the perception of the biological role of selenium forever.

Soon after the discovery of selenium as the "Integral Component of Factor-3", and by 1964, exudative diathesis in chicks, "stiff lamb disease", white muscle disease in calves, "ill-thrift disease" in cattle and sheep in New Zealand have been determined to be conditionally responsive to very small amounts of dietary selenium supplementation (Scott, 1981). Thereafter, Allaway and Cary (1964) proceeded to map the selenium content of soils throughout the United States by using the identification of selenium in plants. They were able to identify regions of the United States that were sub-adequate in soil selenium leading to animal deficiency conditions, areas where selenium was sufficient and those areas of the country where seleniferous plants accumulated selenium from geologically enriched strata. Prior to the approval of the addition of selenium to animal feeds by the US Department of Agriculture in 1974 (Ullrey, 1980), the selenium map was used in the selection and transport of high-selenium feed stocks into areas such as Florida for their inclusion into animal feeds, raising the natural amounts of selenium consumed by animals. The most extensive and complete selenium map of any country is that of China. With the discovery in 1980 of perhaps the only widespread incidence of selenium deficiency in humans, "Keshan's disease and Kachin-Beck's disease" in China (Yang *et al*., 1984), nearly the entire country, county by county, was assessed for the selenium content of crops.

Just prior to the approval of selenium additives to animal mineral premixes by the US Department of Agriculture, Rotruck *et al*. (1973) discovered that selenium was present within the rat erythrocyte enzyme glutathione peroxidase. This came about through a variety of experiments showing that selenium-sufficient rats but not selenium-deficient

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rat erythrocytes were protected against haemolysis in the presence of glucose. Selenium also protected haemoglobin from ascorbate or hydrogen peroxide oxidation and was not able to be dialysed away from the hemolysates. Following injection with 75-SeO_3 rat hemolysates were chromatographed and the 75-Se co-eluted with glutathione peroxidase. Flohe, Gunzler and Schock (1973) showed that glutathione peroxidase contained 4 g atoms of selenium/molecule, a tetramer of approximately 82 kilo dalton. Five years later, Forstrum, Zakowski and Tappel (1978) identified selenocysteine as the catalytic amino acid residue in rat glutathione peroxidase. The second selenoenzyme was initially isolated from pig liver by Ursini *et al*. (1982). It also used glutathione as a cofactor as did glutathione peroxidase, but it exhibited a different spectrum of reducible substrates. This second selenoenzyme was named phospholipid hydroperoxide glutathione peroxidase as it reduced iron-induced phophatidylcholine, *t*-butyl and cumene hydroperoxides to their corresponding alcohols. Selenocysteine would later be named the "twenty-first" amino acid by Böck *et al*. (1991) as it was to be found in additional selenium-containing enzymes and proteins.

Since 1985, 23 additional selenium-containing proteins (Stadtman, 2000) and enzymes have been identified and studied in detail. Common to all proteins is the inclusion of one or more selenocysteine residues. Common to all selenoproteins with catalytic activity is one selenocysteine residing at the active site (Stadtman *et al*., 1996; Toppo *et al*., 2009).

2 SELENIUM COMPOUNDS IN FOODS AND SUPPLEMENTS

The general consensus is that higher plants, exclusive of some algae and bacteria, do not have a growth or biochemical requirement for selenium. The exceptions are the most beautiful flowering selenium accumulator plants of the genera *Astragalus*, *Oonopsis*, *Xylorrhiza* and *Stanleya* (Trelease and Beath, 1949; Rosenfeld and Beath, 1964). Therefore, plant foods, particularly cereal grains, account for providing almost all of the direct animal and human selenium requirements. Selenium in all plant foods is dependent upon the passive accumulation of selenium from soils following most commonly in the metabolic pathway of sulfur. As plants synthesize methionine and other sulfur amino acids and compounds, they also synthesize the selenium analogues to the degree that selenium is present and assimilated at non-toxic levels from soils. Thus, plant foods contain primarily selenomethionine, and as such it is also the major selenoamino acid found in fish, meats and eggs, the major animal and human dietary forms of selenium (Schrauzer, 2003). Lesser amounts of selenocysteine in animal foods and Se-methylselenocysteine are to be found in plant foods. The latter, Se-methylselenocysteine, is the major selenoamino acid found in *Astragalus* and the other selenium accumulator plants *Cruciferous* vegetables (broccoli, cauliflower) and *Allium* plant species (garlic, onions, leeks). The former, selenocysteine, is primarily found in animal foods in association with the various selenium proteins, with trace amounts of selenium from fermented bacterial or yeast-containing foods. Selenocysteine had been reported early on to be present in foods, but it is most likely to have been an artefact of selenocysteine oxidation.

The wide-ranging variability of selenium concentrations in animal feeds and human foods is the cause of a low selenium intake and the many reported selenium-deficiency-associated animal diseases and conditions. With the exception of Keshan's disease and Kaschin-Beck's disease in humans from China, no other selenium deficiency is known to widely occur. Many human populations are known to consume diets below the WHO-recommended minimal adult dietary intake (RDI) of 40 μg selenium each day. European populations are considered marginal and poor people in countries such as rural India, Bangladesh and elsewhere consume less than the WHO dietary standard set for selenium. For such inadequate dietary intakes of selenium, there is provision of selenium supplementation via mineral premixes for animals and multi-vitamin minerals and individual selenium supplementation for people. Animal supplements include selenate, selenite and Se-yeast and, sometimes, pure selenomethionine. Human supplementation is often through normal animal feed supplementation or through the enriched production of high-selenium eggs, milk and meats as occurs in some countries. Individual human supplements run the spectrum of selenium compounds: selenate, selenite, selenomethionine, Se-methylselenocysteine and the first available commercial selenium supplement, circa 1978, a highly enriched Se-yeast.

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Whereas in most environmental/natural feed/food situations there is insufficient selenium in diets to cause any toxicity, toxicity is known to occur particularly in fish and birds. The cause is some natural concentration of selenium in water run-off, stream and river contamination from mining or agricultural operations, ingestion of selenium accumulator plants by livestock and misformulations of supplements for both animals and people. The most recent publicized case of selenium toxicity (May, 2009) was that of 21 Polo horses from Argentina in Florida (JAVMA News, 2009).

Table 1. Mathematics of selenium essentiality and toxicity (selenite; rodents).

Selenium	μg Se g^{-1} diet (ppm)	Atoms Se g^{-1} diet
Nutritional deficiency	0.05	38 000 000 000 000 000
Nutritional adequacy	0.20	152 000 000 000 000 000
~Oral tolerance	2.00	1 520 000 000 000 000 000
~Oral toxicity	5.00	3 800 000 000 000 000 000
~Oral lethality	15.00	11 400 000 000 000 000 000

Numbers are approximate depending upon species, sex, age and other dietary factors. Given to the author (JES) by the late Dr Douglas Frost, Schenectady, NY.

3 TOXIC THRESHOLDS OF SELENIUM INGESTION

All selenium compounds are potentially toxic to animals with the toxicity dependent upon the animal species, the chemical form of the selenium compound, the route of administration and the dose. For selenium compounds, the often quoted statement attributed to Paracelsus, Father of Modern Medicine, "The dose makes the poison" is particularly apparent and presently apt in view of the recent deaths of the 21 Polo horses in Florida noted above. To date, we know only that selenite was administered to these horses and that liver and blood tissues contained circa 20 × the normal levels of selenium. Further details will surely determine the amount of selenium in the formulary and the total amount of selenite selenium administered to the Polo horses.

Selenite selenium along with selenate selenium is a common additive to animal feeds present at 0.1–0.2 ppm Se. Experimental rats and mice can tolerate dietary selenite/selenate selenium to about 2 ppm Se, with about 3–5 ppm Se being chronically toxic and 10–15 ppm Se fatal as summarized in Table 1. Organic selenium supplements Se-yeast, selenomethionine and Se-methylselenocysteine are less toxic than the inorganic selenium compounds.

All selenium compounds are potentially toxic to humans. Although there is much less controlled experimental data, there are some mistakes that have been made with human selenium supplements similar to that which has recently occurred in the Polo horses. There is no reason to suspect that the chemical forms of selenium that are toxic to animals are not similarly toxic to humans. Thus, selenite/selenate selenium probably remains as the most toxic selenium dietary supplement available, with Se-yeast, selenomethionine and Se-methylselenocysteine being much less toxic. Very few reports of selenium toxicity have been reported in humans, but they are not unknown. The most extensive environmental human selenium toxicity occurred in China with keratinous tissues affected, loss of hair and cracked nails as found in horses, cattle and sheep, loss of mane, hair, wool along with cracked and deformed hooves. Similar symptoms have been reported in Water Buffalo in the Indian Punjab due to high selenium content of ground waters.

The US RDI is 55 μg Se each day for adults from food and/or supplements. The tolerable upper limit (TUL) is 400 μg Se each day and overt selenosis occurs at 15 000 μg Se each day as observed in China. Table 2 provides dietary intake information about selenium and its effects in humans. The data are presented as it might apply to selenite. The predominant forms of dietary selenium intake from foods are selenomethionine and selenocysteine with much lesser amounts of Se-methylselenocysteine. These latter selenium compounds are less toxic and the tabular data below do not directly apply to the toxic selenium concentrations cited.

3.1 Toxicity of Selenium Compounds

As noted above, selenium toxicity is initially thought to have been first described in horses by Marco Polo while travelling the "Silk Road" of China. A similar description of toxicity symptoms affecting hooves and mane hair in horses given by Marco Polo was also observed in horses in the western United States in the late 1800s. Its cause

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Table 2. Selenium essentiality and toxicity in humans.

Se ($\mu g\,day^{-1}$)	Chemical form	Effect
<11	Dietary	Keshan's disease
20	Dietary	Minimum dietary requirement
40	Dietary	WHO adequate dietary requirement
55	Dietary	Adult US RDI
400	Dietary	TUL, tolerable upper limit
600	Dietary	Individual maximum safe limit
819	Dietary	NOAEL, maximum safe limit
1000	Na_2SeO_3	Personal known intake for years
1540	Dietary	LOAEL, low level toxicity
1600	Dietary	Adverse effective level
5000	Dietary	Selenosis; hair and nail loss
15 000	Dietary	Overt selenosis

was to remain undiscovered until the USDA in South Dakota and the Wyoming Experiment Station in the 1930s determined that the cause of the toxicity was the ingestion of seleniferous range plants containing up to several thousand milligrams of selenium per kg dry matter. With great sadness, it was the 21 Argentinean Polo horses that succumbed to acute selenium toxicity in Florida in May 2009 resembling "blind staggers".

In the case of selenium toxicity from seleniferous plants and infrequently from cereal grains, toxicity to horses and other livestock is from organic selenium compounds. In contrast, the Polo horses from Argentina succumbed to selenite, inorganic selenium toxicity, with more than 20 times the normal concentration of selenium found in liver and muscle tissues. Inorganic selenium compounds, selenates/selenites, particularly selenites, are infrequent constituents of ground or well water, but more frequent constituents of leaching from concentrated geological formations and commercial mine effluents. These more highly concentrated inorganic selenium sources enter the food chain through algae and other plants and then up the food chain making their way to birds and fish of all kinds. Although selenium is not necessarily concentrated in the food chain by adults, it may be highly concentrated in eggs by female adults. Selenium toxicity is well known to adversely affect unborn fish, birds and perhaps other ovaripids when it is excessively ingested by females during periods of ovogenesis and egg formation. Such embryos are subject to selenium-induced developmental mutations and malformations of chicks and fish (Hoffman, 2002).

3.2 Inorganic Selenium Toxicity

From the time of the association of seleniferous plants with toxicity in the 1930s and the subsequent experimental confirmation of selenite toxicity in laboratory animals, the reason for selenium's toxicity had been debated for years thereafter. At the second International Symposium of Selenium in Biology and Medicine held in Lubbock, Texas in 1980 the major focus of the meeting was the composition, mechanism and role of selenium in glutathione peroxidase. Two presentations were to progress the understanding of the metabolism of inorganic selenium (e.g., toxicity) in biological systems initially pioneered by Painter (1941), and latter by Tsen and Tappel (1958). The first paper by Kice (1981), System Toxicology Approaches for Evaluating Chemical Carcinogenicity, discussed the detailed reaction of Se IV species, selenite/selenium dioxide, with thiols, as first reported by Tsen and Tappel in 1958. In Nanotoxicology of the Symposium, Bhuyan, Bhuyan and Podos (1981) described their not-too-distant past work on the generation of bilateral nuclear cataracts in rats, rabbits and guinea pigs from a single injection of selenite following the finding that these injections did not protect rabbits from cataracts induced by 3-aminotriazole. Cataract induction in animals by 3-aminotriazole had been shown to be due to the generation of reactive oxygen species (ROS), loss of lens protein thiols and lenticular lipid peroxidation. Ascorbic acid (vitamin C) had been shown to protect from 3-aminotriazole cataract formation in animals, but when "other antioxidant" selenium failed to afford protection but rather induced cataratogenesis, insight into selenium's oxidative role in biological systems further emerged. Selenite, by oxidizing thiols, cross-linking disulphides and forming unstable selenotrisulphides, as originally proposed by Painter (1941), was discussed in the context of cataract formation by injections of selenite.

What Bhuyan *et al*. had alluded to in their 1981 Proceedings publication was the possibility that selenite was generating ROS as the cause of cataract formation. Their supposition could have been more emphatic had they been aware of and cited the paper of Misra (1974). The study by Misra describes the auto-oxidation of various thiols, including glutathione, generation of superoxide and the inhibition of nitroblue tetrazolium (NBT) reduction by superoxide dismutase. The authors

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went on to explain how transitions metals, such as copper, might catalyse thiol oxidation generating superoxide and other ROS. Thus, both Kice and Bhuyan, Bhuyan and Podos (1981) had the selenium chemistry, *in vitro* and *in vivo*, nearly correct. What was missing was the "Misra Connection" to the superoxide generation from the auto-oxidation of glutathione. Selenite selenium was just a catalyst of thiol oxidation, as first demonstrated by Levander, Morris and Higgs (1973) and later shown by Seko *et al*. (1989), to produce superoxide.

The "Misra Connection" to selenate-accelerated superoxide generation was directly made by Seko *et al*. (1988) in an unassuming poster presentation at the 4th International Symposium on Selenium in Biology and Medicine in Tubingen, Germany and later published in the Proceedings of the Symposium (Seko *et al*., 1989). This realization that inorganic selenite generated superoxide and likely other ROS seemed to account for the toxicity of selenite *ex vivo* and *in vivo*. Immediately following the Seko paper in the Proceedings were two papers that, retrospectively, evaluated the superoxide-generating effect of selenium that was generating superoxide. Following the Seko paper was a manuscript by Hogberg, Garberg and Stahal (1989) showing that selenite in a dose-dependent manner induced single-stranded DNA fragmentation provided that oxygen was present. Hogberg and coworkers had previously shown that selenite induced cell lysis and speculated that oxidation–reduction (redox) cycles were likely at play contributing to the lysis of hepatic cells. The paper following Hogberg and coworkers, by Frankel and Favey (1989), reported that selenite inhibited DNA and RNA polymerases but only in the presence of a thiol. Again, retrospectively, these authors incorrectly concluded that selenite was toxic by forming a selenotrisulphide (Ganther, 1968), selenodiglutathione, which then served to inhibit the polymerases. In their experiments, non-specific cell depletion of all thiols served to inhibit selenite inhibition of the polymerase whereas specific inhibition of glutathione with buthionine sulfoximine did not prevent polymerase inhibition. This suggests that not only selenite but also other thiols such as protein thiols (cysteine residues)oxidize gluthathione to generate superoxide. From more recent research, it is known that selenite *in vivo* targets and oxidizes the protein thiols of mitochondria (Shen *et al*., 2001; Kim *et al*., 2002; Hail and Lotan, 2009) decreasing the mitochondrial membrane potential. This is the likely cause of mitochondrial swelling, as first observed by Levander, Morris and Higgs (1974), and the inhibition of the mitochondrial permeability transition (MPT) pore followed by the release of cytochrome *c*. As a cellular oxidizer, selenite oxidation is concentration dependent and does not apparently discriminate among thiols. Most cells encounter selenite toxicity exposed to concentrations of 5–10 μM.

3.3 Organic Selenium Toxicity

Toxicity of organic selenium compounds in comparison with selenite is well known to be far less toxic to cells, particularly as studied with cancer cells *in vitro* (Fico *et al*., 1986) and in mice (Poirier and Milner, 1983) as well as in larger animals on a comparative selenium basis (Kim and Mahan, 2001; Tiwary *et al*., 2006). *In vivo*, the study of inorganic and organic selenium metabolism beyond H_2Se formation showed that methylation reactions were the route to selenite and organic selenium detoxification and excretion (Foster, Kraus and Ganther, 1986). The trimethylselenonium ion is excreted in urine and the methylated intermediate of the trimethylselenonium ion, dimethylselenide, is exhaled via the pulmonary system. The latter route of selenite metabolism and excretion occurs under mild to severe toxic conditions where the methylation pathways appear to be compromised and which contributes to the "garlicky to horse radish" smell of the breath from dimethylselenide (Hsieh and Ganther, 1977). Nonetheless, organic selenium compounds are toxic as recorded by the conditions of animals ingesting seleniferous plants and grains, "alkali disease" (chronic selenium toxicity) and "blind staggers" (acute selenium toxicity). To account for the differences in the chronic and acute toxicity of inorganic vs. organic selenium compounds, metabolism has to occur, quite different from that of selenite (SeO_2) directly reacting with thiols such as glutathione, which was thought to be the intervening effect. Although methylation has been viewed as the principal detoxification pathway of inorganic selenium and organic selenium metabolites under physiologic conditions, demethylation of methylated selenium compounds is now viewed as the route to selenium toxicity of the major dietary forms of selenium, selenomethion-

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ine and Se-methylselenocysteine (Ohta and Suzuki, 2008; Suzuki, Kurasaki and Suzuki, 2008). Again, the 4th International Symposium on Selenium in Biology and Medicine held in Tubingen, Germany in 1988 serves as the transitional point in time to the understanding that inorganic selenium toxicity involves generating ROS, and that the metabolism of organic selenium compounds results in a more toxic selenium metabolic form. The study by Ip (1989) in those Proceedings foresaw that selenium metabolism was the key to organic selenium toxicity and possibly chemoprevention by dietary supplements.

When present in tissues or cells we know that all the principal dietary forms of selenium, selenomethionine, selenocysteine and Se-methylselenocysteine, are metabolized by enzymes, methioninase and β-lyases, directly or indirectly in the case of selenocysteine via methylation of H_2Se and on to an common metabolite, methylselenol, CH_3SeH. It is also known that like selenite, methylselenol can react with glutathione and generate superoxide as shown *in vitro* from dimethyldiselenide (Spallholz, Shriver and Reid, 2001), and from the methioninase reaction with selenomethionine as substrate (Spallholz, Palace and Reid, 2004). The realization that many organic selenium compounds were toxic because of the same or a similar selenite chemistry generating superoxide immediately became plausible to Spallholz (Yan *et al*., 1991) from the 1988 poster presented at Tubingen. Yan and coworkers showed that selenite and selenocysteine at two different concentrations, 6.25 and 12.5 μM, were most effective in reducing the intracellular levels of glutathione of mammary cancer cells whereas selenate and selenomethionine were not efficient in reducing the cancer cell glutathione. Concurrently, selenite and selenocysteine also reduced the cancer cell growth viability whereas, again, selenate and selenomethionine had much less of an effect. By 1990, the authors knew that in an *in vitro* chemiluminescent (CL) assay that measured superoxide generation, selenite and selenocystine both generated superoxide in the presence of glutathione and other thiols (Yan, Boylan and Spallholz, 1990). Selenate and selenomethionine were not catalytic in the *in vitro* CL assay and were therefore not toxic to cancer cells affecting cell growth at the same concentrations of added selenium compounds. The CL superoxide data and 1991 mammary cancer cell data from these selenium compounds were the same for selenite, selenate, selenocystine and selenomethionine as that reported by Greeder and Milner (1980) against an ascites cancer in mice. Selenite and selenocystine were much more efficacious in mouse survival than selenate or selenomethionine. Thompson and Ip (1991) found that when selenite was injected into Sprague–Dawley rats the hepatic glutathione levels were lowered when measured 2 h later. *In vivo*, feeding 5 ppm Se as selenite in diets increased hepatic glutathione whereas selenomethionine had no effect upon glutathione. It was quite clear that the similarities and differences between the toxicity of selenite and organic selenium compounds to cells in culture, and animals, depended upon the differences in the chemical composition of different organic selenium compounds and how they were being metabolized.

Chaudière, Courtin and Lecalire (1992) described the catalytic and anti-cancer properties of selenocysteine and many other diselenides that were to be later studied. Using the diselenide selenocystamine, these authors were able to demonstrate that thiol reduction of the diselenide would generate much more than stochiometrically oxidized glutathione (GSSG), superoxide and H_2O_2. Their *in vitro* assay required oxygen as had been observed in the catalysis of glutathione with selenite. Spallholz (1994) reviewed both the inorganic and the organic selenium redox chemistry listing those selenium compounds that generated superoxide from glutathione oxidation in an *in vitro* CL assay (Table 3).

Spallholz (1997) reviewed the CL redox chemistry of selenium compounds in physiological phosphate buffer and provided evidence that selenium, covalently attached to an insoluble matrix, cellulose, would also catalyse superoxide generation from glutathione as measured by a CL assay. Attachment of a redox selenium coating to insoluble matrixes to prevent bacterial biofilm formation received US FDA approval as a medical device application in 2008. Prevention of biofilm formation in the form of selenium coated brackets and ligatures has been included in dental adhesives by Class One Orthodontics of Lubbock, Texas. The prevention of bacterial biofilm colonization of surfaces by redox-active covalent selenium has also been demonstrated for silicon contact lenses (Mathews *et al*., 2006) and a dental sealant coating as applied by Class One Orthodontics on cellulose (Tran *et al*., 2008).

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Table 3. Redox cycling and non-redox cycling selenium compounds.

Compounds that generate superoxide	Compounds that do not generate superoxide
Selenite	Elemental selenium
Selenium dioxide	Selenate
Selenocystine	Selenomethionine
Selenocystamine	Se-methylselenocysteine[a]
Diselenodipropionic acid	Selenobetaine[a]
Diphenyldiselenide	Dimethylselenoxide[a]
Dibenzyldiselenide	Selenopyridine[b]
1,4-Phenyl-*bis*-(methylene)-selenocyanate[c]	Triphenylselenonium ion[a]
6-Propylselenouracil[d]	K-Selenocyanate
Dimethyldiselenide	Selenourea
Methylseleninic acid	Selenoethionine
Selenocyanopropionic acid	All monoselenides tested (RSeR′)

Original table from Spallholz (1994).
Assay conditions: borate buffer pH 9.2 containing glutathione (4 mg ml^{-1} and lucigenin (20 μg ml^{-1}) at 25 °C or phosphate buffer pH 7.4 containing (1 mg glutathione ml^{-1}) at 36 °C. The CL results are similar. Greater selenium-generated CL activity is measured as the pH becomes more alkaline.

[a] Courtesy of Dr Howard Ganther, University of Wisconsin.

[b] Courtesy of Dr Ahmad Khalil, Yarmouk University, Irbid, Jordan.

[c] Courtesy of Dr Karam el-Bayoumy, American Health Foundation.

[d] Courtesy of Dr Alvin Taurog, University of Texas Southwest Medical Center.Other compounds were obtained from Sigma Chemical Co. (St Louis, MO, USA); or Eburon Organics International, Inc. (Lubbock, TX, USA).

4 SELENIUM: PHASE 1 AND 2 ENZYMES, CHEMOPREVENTION AND APOPTOSIS

Phase 1 and 2 liver enzymes are responsible for the detoxification of non-nutrients, drugs, toxins, carcinogens and other xenobiotics. In general, phase 1 liver enzymes modify the above-cited constituents by adding an epoxide or hydroxyl moiety or preparing molecules as phase 2 enzymatic substrates. The phase 2 enzymes utilize the phase 1 enzymatic modifications adding glutathione, glucuronic acid, acetic acid, amino acids, that is, glycine or taurine, sulfate and other molecules. The net effect of phases 1 and 2 enzymes is to convert water-insoluble molecules into water-soluble molecules for excretion or to neutralize potential toxins and/or carcinogens (Liska, 1998).

Toxicity from the ingestion of compounds often elevates levels of phases 1 and 2 enzymes in order to accelerate molecular modifications for detoxification and excretion. Phase 1 and phase 2 enzymes respond to toxic insults from xenobiotic and phytochemicals, and are referred to as inducible enzymes. Many compounds that induce phases 1 and 2 enzymes do so by generating "oxidative stress", the beyond ambient production of free radicals from normal metabolism. The response of the phase 1 and phase 2 enzymes is dose related prior to induction of toxicity; that is, an increase in "oxidative stress" increases the synthesis of phase 1 and 2 enzymes, most likely through an ROS gene signalling modality. Sulforaphane and other isothiocyanates, derived from the glucosinolates of cruciferous vegetables, are considered potent anti-cancer phytochemicals owing to their ability to induce phase 2 enzymes that metabolize and/or inactivate carcinogens for excretion (Zang and Callaway, 2002). Isothiocyanates in sufficient concentrations are known to also generate "oxidative stress", thereby inducing phase 1 and phase 2 enzymes and lowering cancer risk. As described above for selenite and selenides, these isothiocyanates also initiate redox cycling with glutathione *in vitro*, as determined by CL, and have the inherent potential to induce "oxidative stress" (Sharma *et al*., 2008).

Some selenium compounds are also known to induce phase 1 and phase 2 enzymes and are thought to be a family of potent anti-cancer compounds. These selenium compounds also affect levels of glutathione, increasing glutathione probably do to moderate "oxidative stress" or by decreasing glutathione do to the higher concentrations of redox cycling selenium present in cells, in liver and other tissues *in vivo*, all depending upon the level of dietary intake. Normal dietary intakes of adequate amounts of selenium can maximize the anti-oxidant selenium enzymes: the glutathione peroxidases, thioredoxin reductases, phospholipidhydroperoxide glutathione peroxidases and alike. These selenium selenocysteine enzymes, along with superoxide dismutases and catalases, are in effect "anticarcinogenic" as they protect cells and the cells genome from "oxidative stress" and the ensuing free radical damage that may target nuclear as well as mitochondria and mitochondrial nucleic acids as does selenite. With an understanding that many selenium compounds, in a dose-dependent manner, can oxidize thiols, producing superoxide and other ROS, modest amounts of dietary supplemental selenium compounds, selenite, methylseleninic acid and Se-methylselenocysteine following metabolism, such as sulforaphane and other isothiocyanates,

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elevate levels of phase 1 and phase 2 enzymes via "oxidative stress". This is one of only two likely scenarios of why non-toxic levels of sulforaphane, curcumin, selenium and other supplemented phytochemicals may be anticarcinogenic (Hail *et al*., 2008; Hail and Lotan, 2009). The other scenario for selenium is that supplements are raised to such dietary levels that their metabolites, most likely the methylselenide anion (methylselenol is fully ionized at physiological pH), are high enough to induce multiple "oxidative–signalling" pathways for cellular apoptosis. As of this writing, a search of PubMed, selenium and apoptosis, lists nearly 400 publications, many describing selenium-induced apoptosis, along with several excellent and recent reviews on the subject (Rikiishi, 2007;Sanmartin, Plano and Palop, 2008; Zeng, 2009).

The very first citation whose focus is selenium chemistry demonstrating apoptosis is the study by Thompson *et al*. (1994) published in Carcinogenesis, "Comparison of the Effects of an Organic and an Inorganic Form of Selenium on a Mammary Carcinoma Cell Line". The organic selenium compound studied was *bis*(methylene) selenocyanate (p-XSC), and the inorganic selenium compound was selenite. Both compounds are now known to redox cycle (Table 3) and both compounds inhibit cell growth in a concentration-dependent manner. The 1994 abstract from the paper concludes, "The induction of apoptosis by selenium compounds may partially account for their chemopreventive activity". That selenium apoptosis is induced by either the intrinsic or extrinsic pathways is now widely accepted. The weight of the evidence suggests redox cycling, oxidation of varied intracellular thiols with accompanying oxidative stress, affects mitochondria and the intrinsic apoptotic pathway or other proteins and/or inhibitor and enzymes of the extrinsic apoptotic pathway. Sarafian and Bredesen (1994) were invited to write a commentary on the subject; "Is Apoptosis Mediated by Reactive Oxygen Species?" Their conclusions were that ROS are central mediators of apoptosis and that generators of ROS are effector apoptotic signalling molecules, "leading to transcription changes of apoptosis via redox-sensitive transcription factors" such as AP-1 and NFκB.

Selenium has the potential capacity to reduce carcinogenesis; such effects were mostly observed at high doses in experimental animals (El-Bayoumy and Sinha, 2004). Selenium-induced apoptosis of cancer cells has been suggested to be an important mechanism for cancer prevention. If this mechanism is indeed critical, the risk of selenium toxicity appears to be unavoidable, unless the preferential selection of killing cancer cells is firmly established for selenium compounds. Some studies have reported that selenium compounds preferentially inhibit growth and induce apoptosis in cancer cells as compared with corresponding normal cells (Watrach *et al*., 1984; Ghose *et al*., 2001). In contrast, it was surprising to find that the cytotoxic effects of both inorganic and organic selenium compounds were more potent in normal hepatocytes as compared with hepatic carcinoma cells (Weiller *et al*., 2004). Furthermore, it was reported that non-tumourigenic prostate cells are highly sensitive to selenium toxicity compared with prostate cancer cells at physiologically relevant concentrations (Rebsch, Penna and Copeland, 2006). As selenium-induced apoptosis may not overcome the risk of toxicity to normal tissues, the mechanism of phase 2 enzyme activation induced by high-dose selenium seems to be a reasonable strategy to identify optimal selenium forms for cancer prevention. Accordingly, a selenium form with lower toxicity but possessing equal activity of inducing phase 2 enzymes compared with sodium selenite, selenomethionine and Se-methylselenocysteine would be more appropriate for cancer prevention.

5 NANO-SELENIUM PARTICLES

As mentioned above, the element selenium was discovered by Jons Jacob Berzelius as a red deposit. The red precipitate, that is, elemental selenium of zero valence state, can be generated by reacting together sodium selenite and glutathione. There has existed a long-held dogma that elemental selenium is biologically inert. Indeed, purchasable black elemental selenium has no bioactivity, and the bioavailability of the red elemental selenium formed in some bacteria or prepared via reducing sodium selenite by using a reductant is only 2–4% of sodium selenite (Combs *et al*., 1996). Zhang*et al*. (2001) found that the addition of bovine serum albumin to the reaction system of sodium selenite and glutathione could control the aggregation of neonatal elemental selenium atoms in the form of nano-selenium particles (Nano-Se) as shown in Figure 1. Bovine serum albumin concentration in

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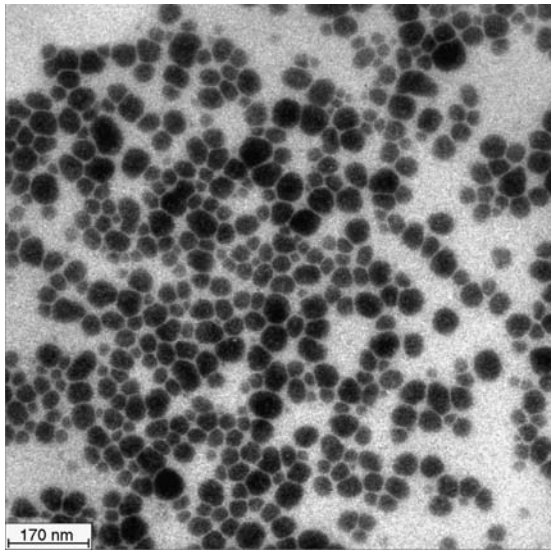

Figure 1. High-resolution transmission electron microscopy image of Nano-Se.

the reaction system was negatively correlated with particle size (Huang *et al*., 2003). As expected, a size-dependent effect of directly scavenging an array of free radical species was observed, with smaller sized Nano-Se being more effective (Huang *et al*., 2003). Consistent with this finding, the redox activities of Nano-Se particles prepared from selenourea dispersed by using bovine serum albumin or sodium dodecyl sulfate varied linearly with the surface area of the particles, thus exhibiting size-dependent redox activity (Mishra *et al*., 2005). Unexpectedly, there was no size effect of 5–200 nm Nano-Se upon increasing selenoenzyme activities including glutathione peroxidase and thioredoxin reductase in both selenium-deficient cells and mice (Zhang *et al*., 2004). Under conditions of selenium deficiency, the avidity of selenium absorption may be increased to maintain the biosynthesis of fundamental selenoenzymes for redox homeostasis. Such intrinsic avidity may override extrinsic size advantage of small-sized Nano-Se on selenoenzymes (Peng *et al*., 2007). Based on this hypothesis, it is anticipated that the size effect of Nano-Se on the biomarkers sensitive to supranutritional levels of selenium can then reappear once selenoenzymes have been saturated (Peng *et al*., 2007). Indeed, the size-dependent effects of Nano-Se on increasing selenium accumulation and inducing glutathione *S*-transferase, a phase 2 detoxifying enzyme, were observed when mice were administered with supranutritional levels of Nano-Se of different sizes (Peng *et al*., 2007). These results together suggest that the biological activity of Nano-Se is a function of their size. A particular example is the photochemically generated eight-atom elemental selenium at subnano size with either serum albumin or lipoproteins acting as the dispersant and/or conjugating agent. Selenium 8 molecules would be expected to fit into the 10-Å hydrophobic pocket near CySH-34 of albumin to form Se(0)-protein conjugates (Sieber *et al*., 2005). Although proteinated elemental selenium at 36 nm and polysaccharide-fabricated 59 nm Nano-Se are cytotoxic (Zhang *et al*., 2001; Chen *et al*., 2008), such colloidal selenium preparations may not have been able to match the potency of photochemically generated elemental selenium which in fact is even more toxic than selenium dioxide, sodium selenite, selenomethionine, and selenocystine (Sieber *et al*., 2005).

5.1 Impact of Nano-Se and Selenocompounds on Selenoenzymes and Glutathione *S*-transferase

The human body metabolizes various selenium forms into selenide. Hydrogen selenide then provides selenium for the synthesis of a number of selenoenzymes including glutathione peroxidase and thioredoxin reductase (Ohta and Suzuki, 2008). Glutathione peroxidases catalyse the reduction of hydrogen and organic peroxides. Thioredoxin reductases provide reducing power for thioredoxin, which regulates multiple important biochemical processes and defends against oxidative stress. A direct deleterious consequence of selenium deficiency is compromised selenoenzymes. Consequently, severe selenium deficiency can result in diseases, such as Keshan disease and Kashin–Beck disease in humans (Yang *et al*., 1984; Moreno-Reyes *et al*., 1998).

To evaluate the impact of Nano-Se on selenoenzymes, 20–60 nm Nano-Se with average size being 36 nm was used. In selenium-deficient cells, sodium selenite at the concentrations of 10, 20 and 50 nM dose-dependently and significantly increased glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase activities (Zhang *et al*., 2001). However, there were no significant differences between sodium selenite and Nano-Se at

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the same doses by ANOVA analysis. In selenium-deficient rats, supplementation with sodium selenite at 0.1 ppm selenium in diet for 8 weeks significantly increased liver selenium and liver glutathione peroxidase activity. Whereas supplementation with Nano-Se at the same dose and duration generated similar effects, no significant difference between sodium selenite and Nano-Se were found (Zhang *et al*., 2001). In a study evaluating the effects of sodium selenite and Nano-Se on growth performance and glutathione peroxidase activity of avian broilers, selenium supplementation significantly improved daily weight gain and survival rate, decreased the feed conversion ratio and increased serum and liver glutathione peroxidase activities compared with the controls, with no significant difference between sodium selenite and Nano-Se (Wang, 2009). These *in vitro* and *in vivo* results together demonstrate that the two selenium forms have an equal capacity of increasing the activities of the selenoenzymes. Moreover, when 36 nm Nano-Se was compared with the organic selenium compounds selenomethionine and Se-methylselenocysteine, on oral administration of the three selenium compounds at the doses of 35 and 70 $\mu g\,Se\,kg^{-1}$ for 7 consecutive days to selenium-deficient mice, each selenium compound could significantly increase glutathione peroxidase activity in plasma, liver- and kidney in a dose-dependent fashion. In addition, each selenium form could significantly increase liver and kidney thioredoxin reductase activity. In general, there were no significant differences between Nano-Se and selenomethionine or between Nano-Se and Se-methylselenocysteine in increasing these selenoenzyme activities (Wang, Zhang and Yu, 2007; Zhang *et al*., 2008).

As discussed above, phase 2 enzyme activation induced by high-dose selenium may be an important mechanism for the cancer-preventive effect of selenium. Induction of phase 2 enzymes is an effective and sufficient strategy for achieving protection against the toxic and neoplastic effects of carcinogens (Talalay, 2000). Among the phase 2 enzymes, glutathione *S*-transferase plays a pivotal role in detoxifying carcinogens (Coles and Ketterer, 1990). For many cancer chemopreventive compounds, inducing glutathione *S*-transferase is a common attribute (Talalay, 2000). It has been reported that selenium compounds at supranutritional levels (550–1100 $\mu g\,Se\,kg^{-1}$) administered to mice can enhance liver glutathione *S*-transferase activity (El-Sayed *et al*., 2006). Wang *et al*. showed that Nano-Se and selenomethionine increased liver glutathione *S*-transferase activity by 78 and 22% at a supranutritional dose of 1000 $\mu g\,Se\,kg^{-1}$ in mice, respectively. There was a significant difference ($P < 0.001$) between the two selenium forms (Wang, Zhang and Yu, 2007). Zhang *et al*. demonstrated that Nano-Se and Se-methylselenocysteine equally increased liver glutathione *S*-transferase activity at a supranutritional dose of either 1000 or 1500 $\mu g\,Se\,kg^{-1}$ (Zhang, Wang and Xu, 2008a). Altogether, these results suggest that the biological activities of Nano-Se of increasing phase 2 enzyme and selenoenzyme activities are comparable to those of the other generally used selenium compounds, bringing sharp contrast to the dogma that elemental selenium has no biological activities.

5.2 Toxicity of Nano-Se and Selenocompounds

It is known that toxicity or pro-oxidant activity of sodium selenite is associated with the interaction of selenite with glutathione to form reactive selenotrisulphides, leading to the production of toxic superoxide and hydrogen peroxide (Spallholz, 1994; Seko and Imura, 1997). Zhang *et al*. (2001) found that the ratio of reduced/oxidized glutathione increased in a selenium dose-dependent manner when the glutathione concentration was held constant and the selenium compounds were added. However, sodium selenite was one order of magnitude more catalytic than Nano-Se in oxidizing glutathione, suggesting that sodium selenite has enhanced toxicity compared with Nano-Se. In an acute toxicity experiment using Kunming mice, it was found that the median lethal dose of Nano-Se was 7.2-fold greater than that of sodium selenite based on the selenium dose (Zhang *et al*., 2001b). In a short-term toxicity experiment using C57BL/6 mice, orally administering selenium at 4 mg $Se\,kg^{-1}$ for 4 weeks, body weight of the mice treated with sodium selenite was only 72–76% of the control during the last 2 weeks (all $P < 0.001$ as compared with the control), whereas the body weight of the Nano-Se group remained not significantly different compared with the control mice throughout the experimental period of 4 weeks. In addition, sodium

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selenite caused severe liver injury, whereas the liver pathology in the Nano-Se group remained normal (Zhang *et al*., 2008). In a 13-week subchronic toxicity experiment using Sprague–Dawley rats, the no-observed-adverse-effect level of Nano-Se and sodium selenite in male and female rats was determined to be 3 and 2 ppm, respectively (Jia, Li and Chen, 2005). Taken together, these results consistently show that Nano-Se is less toxic than sodium selenite.

Selenomethionine and Se-methylselenocysteine are two major naturally occurring organic selenium compounds recommended for selenium supplementation and cancer prevention. When present in tissue or cells, they are metabolized by methioninase and β-lyases to a common metabolite methylselenol, which can react with glutathione and generate superoxide in a similar fashion as in the case of selenite. There are reports that toxicity of organic selenium compounds is less than that of sodium selenite in mice (Poirier and Milner, 1983) and in larger animals (Kim and Mahan, 2001; Tiwary *et al*., 2006). Although the toxicity of Nano-Se is less than that of sodium selenite, it remains uncertain whether there are differences between Nano-Se and these two organic compounds. Therefore, Zhang *et al*. further compared the toxicity of Nano-Se, selenomethionine and Se-methylselenocysteine in Kunming mice. In an acute toxicity experiment using Kunming mice, it was found that median lethal dose of Nano-Se was 92.1 mg Se kg^{-1}, whereas corresponding values for selenomethionine and Se-methylselenocysteine were 25.6 and 14.6 mg Se kg^{-1}, respectively (Wang, Zhang and Yu, 2007; Zhang *et al*., 2008a). In a single administration experimental model, a bolus of Nano-Se, selenomethionine and Se-methylselenocysteine at a dose of 10 mg Se kg^{-1} was orally administered to mice. As compared with the control mice, serum alanine aminotransferase activity increased 0.9-, 23.8- and 3.2-fold; serum aspartate aminotransferase activity increased 0.7-, 4.7- and 5.2-fold; serum lactate dehydrogenase activity increased/decreased −0.13-, 3.7- and 3.5-fold; and liver malondialdehyde level increased/decreased −0.12-, 1.6- and 3.7-fold in Nano-Se-, selenomethionine- and Se-methylselenocysteine-treated mice, respectively (Wang, Zhang and Yu, 2007; Zhang *et al*., 2008a). In a short-term toxicity experiment, mice were orally administered Nano-Se, selenomethionine and Se-methylselenocysteine at a dose of 5 mg Se kg^{-1} for 7 consecutive days. The three selenium compounds all caused liver injury, with Se-methylselenocysteine and selenomethionine being more toxic than Nano-Se. As compared with normal liver architecture, Se-methylselenocysteine and selenomethionine caused an irreversible and severe pathological change known as pyknosis, whereas Nano-Se caused a reversible and moderate pathological change referred to as hydropic degeneration (Wang, Zhang and Yu, 2007; Zhang *et al*., 2008a). These data suggest that Nano-Se's toxicity is lower among the generally used selenium compounds, including sodium selenite, selenomethionine and Se-methylselenocysteine.

6 CONCLUSIONS

Selenium is a necessary dietary constituent of at least 25 human selenoproteins and enzymes all containing selenocysteine. Replacement of selenium by sulfur in enzymes diminishes or obfuscates catalytic activity. Replacement of selenium by sulfur in selenium analogues dramatically reduces or totally eliminates toxicity. The reverse is also true. Replacement of catalytic sulfur by selenium in sulfur compounds universally increases catalytic activity and/or toxicity. In excessive amounts, all selenium compounds become toxic in a dose-dependent fashion to cells *in vitro* and to the primary target tissue of chronic selenium toxicity, the liver. Nanotechnology is a rapidly developing modern technology branch dealing with materials in the nanometer-size range. With their extremely small size nanomaterials can enter cells and with their huge surface area nanomaterials have the potential to interact with intracellular macromolecules exhibiting toxicity or bioactivity. There exist obvious public safety concerns about nanomaterials. Accordingly, a new research discipline known as nanotoxicology has emerged. However, nanotechnology may also hold promise for pharmacology because nanomaterials normally exhibit novel properties different from those of both molecular and bulk materials. Elemental selenium has long been considered to be biologically inert. With bovine serum albumin or other dispersant agents such as polysaccharides, biologically active Nano-Se aggregates are formed from sodium selenite and glutathione. Different from the biologically inert black elemental selenium with coarse size, Nano-Se

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manifests toxicity which conforms to the concern of nanotoxicity. However, compared with selenium compounds such as sodium selenite, selenomethionine and Se-methylselenocysteine, Nano-Se is not compromised in increasing the activities of selenoenzyme and phase 2 enzyme, but exhibits much lower toxicity. Nano-Se is thus a potential selenium nutritional source with a prominent characteristic of lower toxicity for supplementation.

RELATED ARTICLES

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In Vivo Toxicity Studies of Metal and Metal Oxide Nanoparticles

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1 INTRODUCTION

The production of nanomaterials and their use in various fields has evolved remarkably in the last decade. Newly manufactured nanomaterials are being used in a large variety of applications in computing and electronics, construction, cosmetics, drug delivery and medical imaging, paints and coatings, food, packaging, textiles, clothing and others. More than 1000 consumer products that contain nano-materials are being produced today (Nanotechnology Consumer Products Inventory, 2010). There is a great number of engineered nanomaterials that are currently produced including metal-based (e.g., gold, silver, quantum dots, metal oxides such as titanium dioxide, zinc oxide, copper, or iron oxide), carbon-based (e.g., nanotubes, nanowires, fullerenes), and those that are biological in nature (e.g., liposomes and viruses designed for gene or drug delivery) (Card *et al*., 2008). Metal oxides or pure metals are present in almost half of consumer products. Products containing nanosilver account for 30%, metal oxide nanomaterials (TiO_2, ZnO, and CeO_2) for almost 20%, silicon-based products for 17% (Nanotechnology Consumer Products Inventory, 2010). These materials have the highest chance to enter the environments thus, it has been proposed that "nanodust" from engineered nanomaterials might be a new source of metals in the atmospheric environment (Grassian, 2009). It is well known that the properties, biological reactivity and toxicity of materials on the nanoscale differ from that of the bulk or micron sized particles (Nel *et al*. 2006; Oberdorster, Oberdorster and Oberdorster, 2005; Sayes and Warheit, 2009). Previous studies found that materials that were considered inert were causing enhanced toxic responses if used at nanosize (Bermudez *et al*., 2004; Ferin, Oberdorster and Penney, 1992; Goodman *et al*., 2004; Kaewamatawong *et al*., 2005). For instance, titanium dioxide, which in micron particle sizes is considered a very inert material and has often been used as a negative control in animal studies, is now considered as a "potential occupational carcinogen", as defined by the Occupational Safety and Health Act or Administration (OSHA) (Baan *et al*., 2006). Not surprisingly, there is growing interest in understanding health implications of nanomaterials (Maynard, 2007; Nel *et al*., 2006). Of equal importance to the toxicity evaluation of nanomaterials in general and nanoparticles (NPs) in particular is the assessment of the risk of human exposures that

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might occur in the ambient or occupational environment. Such assessments are still in progress and are challenging since quantities of nanomaterial used in many consumer products are not identified and there is no obligation for specific labeling at this point (Seaton *et al*., 2009). Another factor is distinguishing engineered NPs from incidental particles in the workplace (Peters *et al*., 2009). It is estimated that accidental inhalation of NPs is the most likely route of significant human exposure. However, the health impacts of NPs can cause systemic response or responses in other organs or systems, depending on their translocations (Oberdorster, Oberdorster and Oberdorster, 2005). Nanomaterials alone may be cause of toxicity; however, they can also induce risk through adsorption and therefore as carriers of impurities (e.g., toxins in the environment), generation of toxic substances by their dissolution or degradation, or by crossing physiological barriers such as the blood-brain (Oberdorster *et al*., 2004), fetoplacental (Saunders, 2007), or cell and nuclear membranes. Given some of these complexities, it is clear that an integrated, interdisciplinary approach is necessary to study the hazards of NPs (Grassian *et al*., 2007a; Holsapple *et al*., 2005; Pettibone, Elzey and Grassian, 2008; Powers *et al*., 2006; Seaton *et al*., 2009). Our group exemplifies such an approach in which chemists, environmental engineers, aerosol physicists, and toxicologists are working together in safety assessment of nanomaterials. In this review, we will focus our attention on particle characteristics and biological responses of nanomaterials after inhalation exposures using murine models. These nanomaterials include metal-based NPs such as titanium dioxide, iron, copper, and silver as well as aluminum oxyhydroxide nanowhiskers.

1.1 Nanoparticle Characterization

According to ASTM International, NPs are defined as a sub-classification of ultrafine particles with lengths in two or three dimensions greater than 0.001 (1 nanometer) and smaller than about 0.1 micrometer (100 nanometers) and which may or may not exhibit a size-related intensive property (ASTM E2456-06, 2003). However, this definition is a topic of debate because of the size range and the presence of a size-related property (Auffan *et al*., 2009). Current usage of this definition emphasizes size and not properties. The length scale may be a hydrodynamic diameter or a geometric length appropriate to the intended use of the NPs (ASTM E2456-06, 2003). Ideally, each toxicological assay (***in vitro/in vivo***) should include a detailed characterization of the investigated material, since different particle properties could have biological relevance and differences have been found between specifications provided by the supplier actual properties of the product. We have also found large differences between individual batches of the same product and inconsistencies with manufacturers' specifications and independent characterization (Grassian *et al*., 2007; Park and Grassian, 2010; Pettibone *et al*., 2008). Furthermore, appropriate storage conditions (e.g., exposure to light, temperature) are critical to assure that particles properties are not modified. At a minimum, this characterization should include bulk and surface composition, surface area, primary size, and size of agglomerates. There are many studies that do not provide sufficient information about the investigated materials (Card *et al*., 2008) and; thus, comparing the toxicity of various nanomaterials among studies is more difficult. It is very important to characterize both primary properties (as received from the supplier or as produced) and secondary characteristics (during exposure if it is in the media or in the air in the form of aerosol). Ideally, properties of particles should also be characterized after administration and uptake by cells or the respiratory system (Sayes and Warheit, 2009).

1.1.1 Physicochemical Characteristics

Manufactured nanomaterials have many physicochemical characteristics that include their size and surface area, chemical composition, purity, crystalinity, electronic properties, surface structure, structure composition, surface functionality (e.g., inorganic or organic coatings, etc.), solubility, shape, and aggregation/agglomeration (Elzey *et al*., 2009; Grassian, 2008; Nel *et al*., 2006) which modify their interactions with biological tissues. All these different properties of nanomaterials can affect particle uptake, persistence, can stimulate cellular responses and biological toxicity. Therefore, it is strongly recommended that these physicochemical properties of nanomaterials are an important component of the study design and reported in any risk assessment and health study (Oberdorster *et al*., 2005; Powers *et al*., 2006; Warheit, 2008).

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Table 1. Summary of physicochemical characterization data on metal nanoparticles used in *in vivo* inhalation studies.

Property	Titanium	Iron	Copper	Silver	Aluminum
Crystalline or amorphous material	Crystalline	Crystalline	Crystalline	Crystalline	Crystalline
Phases	Anatase	Fe, Fe_3O_4, γ-Fe_2O_3	Cu, Cu_2O, CuO	Ag	$Al(OH)_3$ and γ-AlOOH
Primary particle distribution	3.5 ± 1 nm	25 ± 2 nm	12 ± 1 nm	12 ± 4 nm	2–5 nm × 100–200 nm whiskers
Surface phases	TiO_2	Fe_2O_3	CuO	Ag	$Al(OH)_3$, γ-AlOOH
Surface functionalization	O, O-H and H_2O	O, O-H and H_2O	O, O-H and H_2O	CO_3^{2-}, OH^-, C = O, C-O-C	O, OH O, O-H and H_2O
BET surface area	$219 \pm 3\ m^2\ g^{-1}$	$17 \pm 1\ m^2\ g^{-1}$	$12 \pm 0.2\ m^2\ g^{-1}$	$3 \pm 2\ m^2\ g^{-1}$	$320 \pm 4\ m^2\ g^{-1}$
Aerosol size distribution[a]	128.0 (1.7) nm	199.9 (1.3) nm	190.1 (1.3) nm	79 (1.5) nm	154.1 (1.6) nm
Exposure concentration	$8.88\ mg\ m^{-3}$	$3.55\ mg\ m^{-3}$	$3.68\ mg\ m^{-3}$	$3.32\ mg\ m^{-3}$	$3.31\ mg\ m^{-3}$

[a] GM (GSD) in whole-body exposure chamber during sub-acute exposure.

Table 1 shows a compilation of the physicochemical properties of nanomaterials that were recently measured for *in vivo* inhalation studies (Grassian *et al*., 2007; Pettibone *et al.*, 2008). Additional comments about some of these various properties are given below.

Particle Size and Surface Area

The size of particles is often considered one of the most determining factors that influence deposition and clearance of materials in the respiratory system. The size of nanomaterials studied is often reported from the nominal primary particle size determined by the supplier; however, as it was found in our studies, this size is not always properly recorded or even correct (Park and Grassian, 2010). In some cases batch-to-batch variability is the cause of these differences. For example, average primary size of copper NPs used in our study (Pettibone, *et al.*, 2008) was reported by manufacturer as 25 nm, however the particle sizes were found to be approximately half the reported value (12 ± 1 nm) with a fairly narrow distribution. If the size of the particles changes, other parameters such as surface area can change and, on the nanoscale, optical, electronic, magnetic, and other physical properties can also change (Lucas *et al*., 2001). The effect of NPs size has been studied extensively in instillation and inhalation exposure studies (Cullen *et al*., 2000; Hext, Tomenson and Thompson, 2005; Kaewamatawong *et al*., 2005; Lison *et al*., 1997; Oberdorster, 2001; Oberdorster, Oberdorster and Oberdorster, 2005; Tran *et al*., 2000). Many studies report that surface area of particles is associated with the magnitude of airway inflammation (Cullen *et al*., 2000; Duffin *et al*., 2007; Monteiller *et al*., 2007; Renwick *et al*., 2004; Singh *et al*., 2007; Tran *et al*., 2000). Various instillation studies of different ultrafine particles reported higher toxicity and inflammatory response increases as the surface area of particles increased on a per mass basis (Oberdorster *et al*., 2000; Oberdorster, Oberdorster and Oberdorster, 2005; Stoeger *et al*., 2006). It has been proposed that different NPs can be ranked according to the degree of inflammation per surface area dose (number of neutrophils cm^{-2}) (Duffin *et al*., 2007). However in a recent study of ours using two different sizes of TiO_2 nanoparticles, we determined that the smaller 5 nm TiO_2 particles did not cause a greater inflammatory response than 21 nm TiO_2 particles, as demonstrated in Figure 1. These plots show the number of neutrophils in BAL fluid in controls and animals exposed to 5 or 21 nm TiO_2 nanoparticles by instillation according to particle mass or surface area. In fact, 21 nm TiO_2 particles induced a larger inflammatory response in the mice than the 5 nm particles through inhalation and instillation exposure (Grassian *et al*., 2007). Since 5 nm particles used in this study had much greater surface area, more than five times that of the 21 nm particles, we expected a greater inflammatory response on a per mass basis in this case for smaller particles. Our results showed that BET surface is not, always the best measure of TiO_2 nanoparticle toxicity for the smallest NPs area (BET stands for

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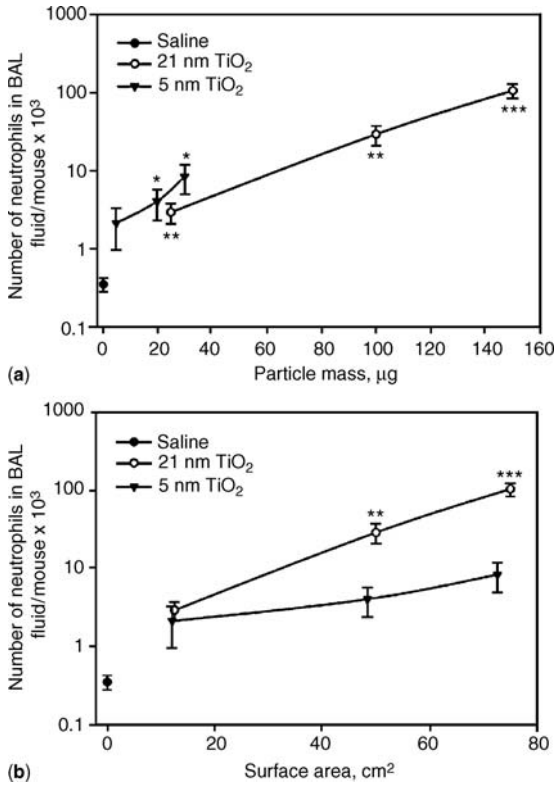

Figure 1. Number of neutrophils in BAL fluid in controls and animals exposed to 5 or 20 nm TiO_2 nanoparticles by instillation, according to (a) particle mass; (b) surface area. Asterisks represent significant increase ($*p < 0.05$, $**p < 0.01$, and $***p < 0.001$) in parameter measured, compared to (a) controls; and to (b) 5 nm TiO_2-exposed groups. (Grassian *et al*., 62). (Reproduced with permission from Taylor & Francis, http://www.informaworld.com.)

Brunauer, Emmett, and Teller three scientists who developed the theory for surface area measurements (Brunauer, Emmett and Teller, 1938)). This is in agreement with Warheit *et al*. (2006) who found that nanoscale particles (nanoscale TiO_2 rods and nanoscale TiO_2 dots) were not more cytotoxic or inflammogenic in the lungs compared to larger sized particles of similar composition after intratracheal instillation. Other studies also suggest that surface area alone is not responsible for nanoparticle toxicity (Sayes *et al*., 2006; Warheit *et al*., 2006; Warheit *et al*., 2005; Warheit *et al*., 2007).

As already noted, there are many properties of nanomaterials that affect their biological responses. Crystaline phases of the particles in our studies were different. The 5 nm particles were pure anatase but the 21 nm particles included both anatase and rutile (Grassian *et al*., 2007). In other study, phase has been shown to matter (*vide infra*), for example a 100-fold increase in the cytotoxicity of the anatase phase compared to rutile phase in particles 10 nm or smaller was observed (Sayes *et al*., 2006). In that same study it was also shown that anatase generated reactive oxygen species (ROS) more effectively because of surface reactivity. Furthermore, in more recent studies of ROS production, Jiang *et al*. (2008) showed that for a series of particles ranging from 4-195 nm the largest amount of ROS was produced by 30 nm particles not the 4 nm particles. Thus, this demonstrates complex size-dependent ROS production for these very small NPs.

Phase, Surface Reactivity and Surface Coatings

When different functional groups or coatings are present on the surface of nanomaterials, the surface reactivity of NPs change and this can have a large effect on toxicity response (Warheit, Reed and Sayes, 2009). Comparing fine rutile TiO_2 particles in inhalation and instillation studies, it was found that only NPs with alumina and amorphous silica surface coating caused adverse pulmonary effects if compared to the base control TiO_2 particles (Warheit *et al*., 2005). Another study evaluating three forms of ultrafine TiO_2 particles (Warheit *et al*., 2007) demonstrated that quartz and anatase/rutile TiO_2 particle exposures produced lasting lung inflammation, cytotoxicity, and histopathology effects in lungs. However exposures to fine TiO_2 particles or ultrafine rutile TiO_2 particles resulted only in transient inflammatory responses without any pathology effects in lung tissues. Evaluation of synthetic 12 nm α-nanoquartz particles and synthetic fine α -quartz particles (~300 nm) and (mined) Min-U-Sil α -quartz particle types (~500 nm) showed that the pulmonary toxicities correlated better with surface reactivity than particle size or surface area (Warheit *et al*., 2007; Warheit, Reed and Sayes, 2009). Furthermore, TiO_2 particles of the same primary particle size with different coatings, one with silane compound and the other uncoated showed a major difference in the pulmonary-inflammatory potency (Oberdorster, 2001). The surface reactivity may be substantially higher for NPs with smaller sizes because of an increase in the thermodynamic surface free energy, even for NPs with the same surface functionality (Zhang *et al*., 1999). Although in

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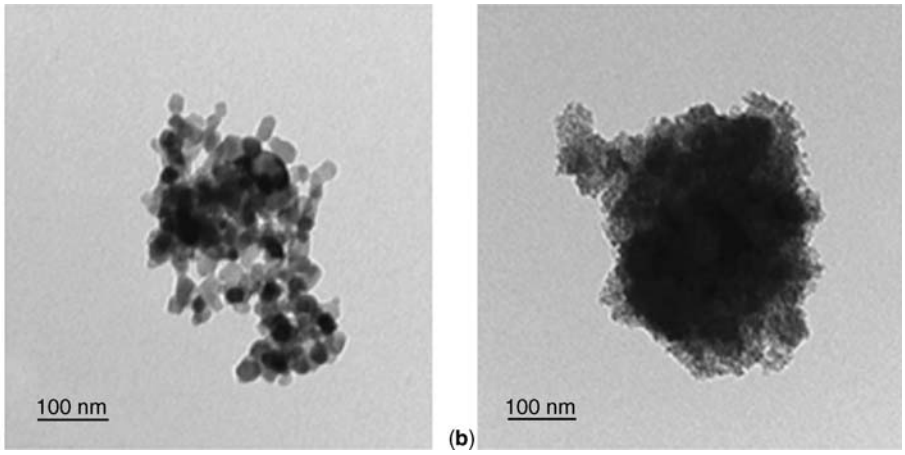

Figure 2. TEM images of the generated aerosol in the whole body exposure chamber: (a) is a representative agglomerate of the 21 nm particles; and (b) is a representative agglomerate of the 5 nm particles. The agglomerates differ in nature as the agglomerate formed from the 21 nm particle appears to have more void space compared to the agglomerate formed from the 5 nm particles. (Grassian *et al.*, 62). (Reproduced with permission from Taylor & Francis, http://www.informaworld.com.)

our study of TiO_2 particles with different sizes (5 and 21 nm), we did not see smaller particles leading to higher toxicity. We proposed that the higher surface reactivity of the smaller NPs may lead to more tightly formed agglomerates (Grassian *et al.*, 2007) and therefore a lower effective surface area for biological interactions.

Agglomeration and Aggregation

Agglomeration/aggregation state of the particles has an impact on particle toxicity and biological response (Sager *et al.*, 2007; Wick *et al.*, 2007). It is very difficult to keep NPs isolated and the ability of NPs to agglomerate and/or aggregate is a well-known characteristic. Thus, it is suggested that the use of aggregated NPs is appropriate in toxicology studies (Lu *et al.*, 2009). According to ASTM (ASTM E2456-06, 2003), "Standard terminology Relating to Nanotechnology", ASTM International) agglomerate and aggregate are defined as follows: **Agglomerate** – is a group of particles held together by relatively weak forces (for example, Van der Waals or capillary), that may break apart into smaller particles upon processing. **Aggregate** – is a discrete group of particles in which the various individual components are not easily broken apart, such as in the case of primary particles that are strongly bonded together (e.g., fused, sintered, or metallically bonded particles). Particle size and propensity of particles to agglomerate affect the uptake of particles by cells, and their clearance in the alveolar region. The size of agglomerates/aggregates determines if particles are deposited in alveolar or interstitial spaces (Ferin, Oberdorster and Penney, 1992; Oberdorster, Cox and Gelein, 1997). After inhalation, fine particles (>100 nm) are quickly phagocytized by alveolar macrophages (Takenaka *et al.*, 2001). The size of agglomerates influences the ability of macrophages to phagocytize them as well as the penetration of cell membranes and translocation through the tissue, lymph, or circulatory system (Nemmar *et al.*, 2001; Renwick, Donaldson and Clouter, 2001). Another factor that may influence the toxicity of nanomaterials is the density of the packing of the nanoparticle building blocks to form the agglomerate as well as the strength of the interaction between the NPs. If a bundle of agglomerated nanoparticles are more tightly packed without void spaces between individual particles, they may yield agglomerates with very small projected surface area (Moss and Wong, 2006) that can potentially reduce toxicity. This was suggested in our study of TiO_2 nanoparticles with particle diameters of 5 and 21 nm. The TEM images of TiO_2 agglomerates collected from the whole body exposure chamber showed that the agglomeration differed in nature between the two sized nanoparticles. The agglomerates from 21 nm particles (Figure 2a) appeared to have more void space compared the agglomerates generated from the 5 nm particles

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(Figure 2b) (Grassian *et al*., 2007). A rat instillation study of low-toxicity, low-solubility NPs observed that greater inflammogenicity of NPs was consistent with higher surface area of particles despite aggregation, indicating that aggregation does not influence surface area dose (Duffin *et al*., 2007). Even if NPs occur in the environment as aggregates larger than 100 nm, their toxicity can potentially differ from the toxicity of particles with corresponding particle size (Borm *et al*., 2006). Degree and type of agglomeration can affect *in vitro* cytotoxicity of carbon nanotubes (Wick *et al*., 2007). In Wick *et al.* (2007) nanotubes that differed in the degree of dispersion were investigated. It was shown that agglomerated carbon nanotubes caused more adverse effects relative to more well-dispersed material.

On the other hand, it is also known that if particle are more dispersed they can be incorporated into the interstitium more rapidly and cause different particle deposition and subsequent response. In an *in vivo* study, more dispersed single wall carbon nanotubes (SWCNT) were found to penetrate more into the alveolar interstitium and also caused more extensive interstitial collagen accumulation than the less dispersed SWCNT (Mercer *et al*., 2008). Aggregation is affected by pH, ionic strength, and ionic identity. Ionic strength influences dispersion stability by changing electrical double layer thickness, while pH can change the dispersion state by shifting the zeta potential (surface charge) (Jiang, Oberdorster and Biswas, 2009; Maier *et al*., 2006). In Jiang, Oberdorster and Biswas (2009), it was found that for 15 nm TiO_2 dispersions, the increase of ionic strength from 0.001 to 0.1 M led to a 50-fold increase in the hydrodynamic diameter. Furthermore, it was reported that these particles have a positive surface charge when pH is lower than 6 and a negative surface charge when pH is higher than 6.

Particle Dissolution

Dissolution of metal-based nanomaterials is associated with both changes in nanoparticle size and formation of ions. The augmentation of inflammatory response in transition metals was shown to be proportional to the solubility of the metal in biological media, even though the pathways of inflammatory response were different (Borm *et al*., 2006; Brunner *et al*., 2006; Knaapen *et al*., 2002; Midander, Wallinder and Leygraf, 2007). In our inhalation studies of copper and iron NPs (Pettibone, *et al.*, 2008), we found inflammatory responses in lungs represented by higher number of macrophages as well as high neutrophilia (51%) in bronchoalveolar lavage (BAL) fluid and pathologic changes (perivasculitis and alevolitis) in the lung tissue in mice exposed to copper NPs. With iron NPs, we observed only slight inflammatory response represented by only 4 % neutrophilia in BAL fluid. Overall, our murine studies showed copper NPs to be significantly more toxic than iron NPs. We also observed differences in dissolution between these two nanomaterials. As opposed to iron that did not dissolve in Gamble's solution, a simulated biological fluid, copper NPs were shown to partially dissolve in this solution. Gamble's solution is often used in *in vitro* studies to imitate the interstitial fluid in the lungs, its pH is 7.2–7.4 (Moss 1979). Another artificial fluid used is artificial lysosomal fluid (ALF) that simulates the phagolysosomal composition and pH (4.5–5.0) of alveolar and interstitial macrophages (Midander, Wallinder and Leygraf, 2007; Stopford *et al*., 2003). Both metals studied dissolved in ALF. However, we believe that such a high dissolution was due to the presence of the citric acid in ALF. Our dissolution studies of copper NPs are in an agreement with other studies of copper materials in an artificial and biological media such as saliva, lung fluid, or blood, in which copper had a tendency to dissolve (Chen *et al*., 2006; Midander, Wallinder and Leygraf, 2007). Furthermore, study that reported iron oxide particles having very low solubility, also found that these particles had lower propensity to produce ROS, and did not cause inflammatory responses after intratracheal instillation (Lay *et al*., 1999). Comparing toxicity of ultrafine carbon black particles dispersed in BAL fluid or phosphate-buffered saline administered by intratracheal instillation showed 1.7-fold greater cytotoxicity (release of LDH) and two-fold greater inflammation (number of neutrophils in BAL fluid) in the case where carbon black was dispersed in BAL fluid (Sager *et al*., 2007). Zinc oxide (ZnO) particles were found to induce injury response represented by production of pro-inflammatory cytokines and mitochondrial injury using phagocytic cell line (*RAW 264.7*) and transformed human bronchial epithelial cells (*BEAS-2B*). High solubility of ZnO was considered responsible for toxicity of this nanomaterial (Xia *et al*., 2008). Interestingly, in this study ZnO agglomerates reduced in size from 413 to 36 nm when particles were transferred from water to tissue

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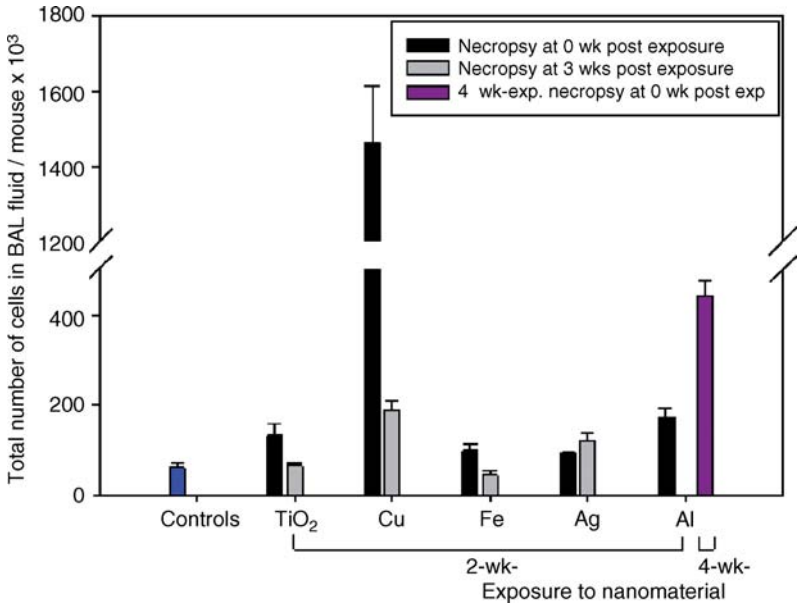

Figure 3. Total number of cells in BAL fluid after sub-acute exposure of mice to selected metal nanoparticles.

culture medium probably due to serum proteins and lipids. Such a size reduction could assist with cellular uptake and intracellular dissolution. Therefore, it is very important to study dissolution properties of nanomaterials, since the amount of soluble ions in solution may increase inflammatory response. Several reports recently have shown that aggregation can impact the solubility of nanoparticles (Liu *et al*., 2009; Rubasinghege *et al*., 2010).

Chemical Composition

Undoubtedly, chemical composition is a fundamental property of any material and has a profound effect on biological responses. Nanomaterials can have very different chemical compositions, from completely inorganic, for example, metals (iron, nickel, zinc, gold, silver), and metal oxides (oxides of titanium, copper, iron, and zinc), to entirely carbon-based materials (fullerenes, carbon nanotubes, nanopolymers). It has also been shown that the chemical purity is very important property of nanomaterial. Some metal impurities such as Fe, Ni, Co can affect the biological response by masking the nanoparticle behavior with their toxicity (Maynard *et al*., 2004; Pulskamp, Diabate and Krug, 2007). In an *in vitro* study of carbon nanotubes (commercial vs. purified) and human A549 cells or rat macrophages, an increase in ROS and a decrease of the mitochondrial membrane was found in both cell types after treatment with commercial but not with purified material. Metal traces found in the commercial nanotubes might have been responsible for these observed responses (Pulskamp, Diabate and Krug, 2007). From metal nanomaterials, Cu- and Zn-based materials appear to be the most toxic (Gordon, Chen and Cohen, 2008; Lanone *et al*., 2009). Ti, Al, Ce, Ag, Ni and Zr-based nanomaterials showed low or moderate toxicity (Lanone *et al*., 2009). *In vitro* comparison of 6 metal oxide particles and carbon nanotubes, showed CuO NPs as most potent concerning cytotoxicity and DNA damage (Karlsson *et al*., 2008). Zinc- and Ni-nanoparticles also demonstrated higher toxicity than Al-, Ag-, or TiO_2 NPs in human alveolar epithelial cells (Park *et al*., 2007). Our studies comparing pulmonary inflammatory response of selected metal nanomaterials after sub-acute inhalation exposure ranked their inflammogenicity (based on higher numbers of total cells and neutrophils in BAL lavage) as follows: Cu > Fe > Ag > Al > TiO_2 (Figures 3 and 4). Certainly, as discussed in this chapter, evaluation of nanomaterial toxicity based only on chemical composition is only the first step in the overall evaluation.

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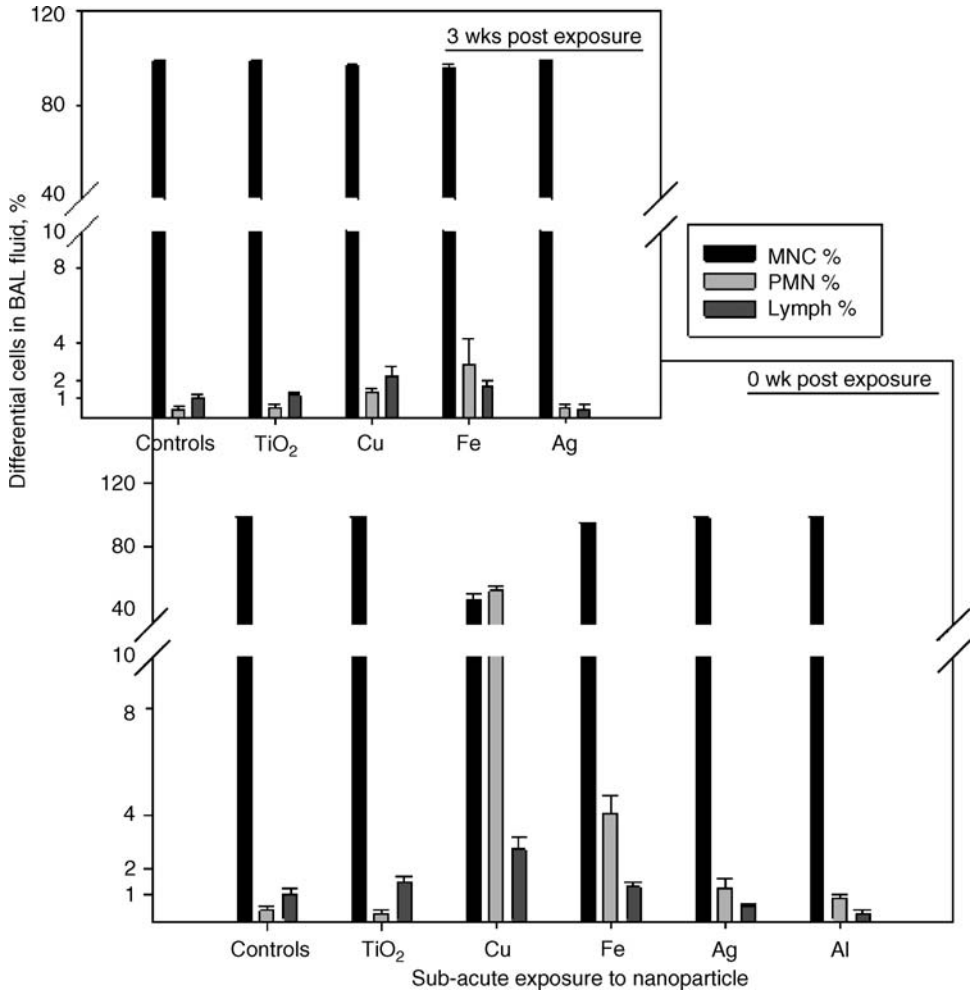

Figure 4. Proportion of macrophages (MNC), neutrophils (PHN), and lymphocytes (Lymph) in BAL fluid after sub-acute exposure of mice to selected metal nanoparticles.

Surface Contamination

Since NPs have such a large surface area attention needs to be paid to possible contamination of their surfaces. The contamination can vary from sporadic surface adsorption, oxidation, and corrosion and can occur in air or liquid environments. Thus, such "contaminated" NPs can transport high levels of various unintentional materials into the organism. The contaminant may modify the aggregation stage or physical properties of nanomaterials. The last concern is that it is hard to distinguish if their toxic potency is from the nanomaterial itself or from the contaminant (Jones and Grainger, 2009). One possible contamination of nanomaterials is endotoxin contamination. Endotoxin, or lipopolysaccharide (LPS) is a glycolipid of gram-negative bacteria. LPS can significantly influence the biological responses and thus confound any effect of studied material (Gorbet and Sefton, 2005; Thorne and Duchaine, 2007). For example conventionally produced gold NPs had a maturing effect on the dendritic cells as a result of LPS contamination. When LPS contamination of these particles was decreased, by alteration of the production process, nearly no effect on phenotypic maturation or cytokine production of dendritic cells was observed (Vallhov *et al.*, 2006). Comparing levels of endotoxin contamination and physical properties of selected engineered NPs no correlation was found (Esch *et al.*, 2010). A study of carbon black NPs administered intratracheally by

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instillation showed only slight lung inflammation when delivered alone, however the lung inflammation was intensified when carbon black particles were delivered together with LPS (Inoue *et al*., 2006). There also might be synergistic effects of two inflammation-causing agents, as it was found in study of latex NPs and LPS (Inoue *et al*., 2009). On the other hand, if we want to mimic an exposure to nanomaterials as it would occur in an ambient air or occupational environment, where contamination of materials by environmental endotoxin will certainly occur, one might suggest that studying materials "as is" might be more realistic. Without doubt, a high degree of endotoxin contamination must be avoided and the levels of endotoxin of studied material should always be measured. However, it has been questioned if the widely used assay for endotoxin traditionally used by immunologist (e.g., the *Limulus* amebocyte lysate chromogenic endpoint assay) is able to measure endotoxin bound to a particle surface or is limited only to free endotoxin in solution (Dobrovolskaia, Germolee and Weaver, 2009; Esch *et al*., 2010; Jones and Grainger, 2009). Taking into an account very low contamination by endotoxin, currently it is not known if there is a difference in response in *in vivo* studies, for example, at level of 1 EU mL^{-1} and <0.5 EU mL^{-1} (Jones and Grainger, 2009). Of course considering endotoxin contamination of nanomaterials used in the pharmaceutical industry, biomedical application or *in vitro* studies has higher importance and should definitely be avoided (Dobrovolskaia, Germolee and Weaver, 2009). Efficient removal of LPS can be challenging since this process can also affect the properties of nanomaterials, for example, causing aggregation (Vallhov *et al*., 2006).

1.2 Pulmonary Toxicity Assessment of Nanoparticles

The possible pathogenic mechanisms evoked by exposure to NPs are very complex, depending on the route of exposure, dose, host response, susceptibility, physico-chemical properties of NPs as well as the fate and persistence of the particles in the organism. The nanomaterial toxicity screening working group has defined the building blocks of a screening strategy for toxicity of nanomaterials: evaluation of physicochemical characteristics and dose metrics; acellular assays; *in vitro* assays for lung, skin, and mucosal membranes; and *in vivo* assays (animal experiments) for lung, skin, oral, and injection exposures (Oberdorster *et al*., 2005). Some also deem *ex vivo* models (isolated pulmonary cells or isolated perfuse lung) as a valuable addition to understanding of nanomaterial toxicity (Sakagami, 2006). Each model has advantages and disadvantages that shall be considered. There are no *in silico* models available today that would allow prediction of the activity of untested compounds. Intermediate *ex vivo* models sometimes resolve deficiencies of *in vivo* and *in vitro* models such as kinetic modeling however there are a number of limitations such as short viable periods (only 2–3 h), absence of tracheobronchial circulation as well as no possibility of particle translocation. In addition this model has a very high grade of complexity and is the least utilized (Sakagami, 2006). There is broad interest in the development of standard/reference nanomaterials that can be used for comparison in toxicity evaluation of NPs. This would be valuable however a range of such standards would have to be very wide (Holsapple *et al*., 2005).

1.2.1 Uptake, Transport and Clearance of Nanoparticles

As reviewed by Madl and Pinkerton (2009), recent publications have demonstrated that NPs endure classic mechanisms of uptake, transport, and clearance from the respiratory system, but there are also unique transport pathways specific to the nanosize range (Figure 5). Nanoparticles are cleared by macrophage phagocytosis and transporting through the mucociliary escalator and physicochemical processes (dissolution and physical breakdown). Unique mechanisms for NPs comprise uptake and transport along sensory neurons (nasal olfactory epithelium), direct uptake of particles by epithelial cells in the respiratory tract and subsequent transport to the interstitium, lymphatic system, and blood circulation (Oberdorster *et al*., 2004; Oberdorster *et al*., 2005). Some studies have suggested that clearance of NPs is slower than larger particles since they are taken up by epithelial cells rather than being phagocytized by macrophages (Kreyling, Sommel and Moller, 2004; Oberdorster, Oberdorster and Oberdorster, 2005; Semmler-Behnke *et al*., 2007). As we noted above, physicochemical properties of particles play a crucial role in how NPs are internalized by the pulmonary epithelium.

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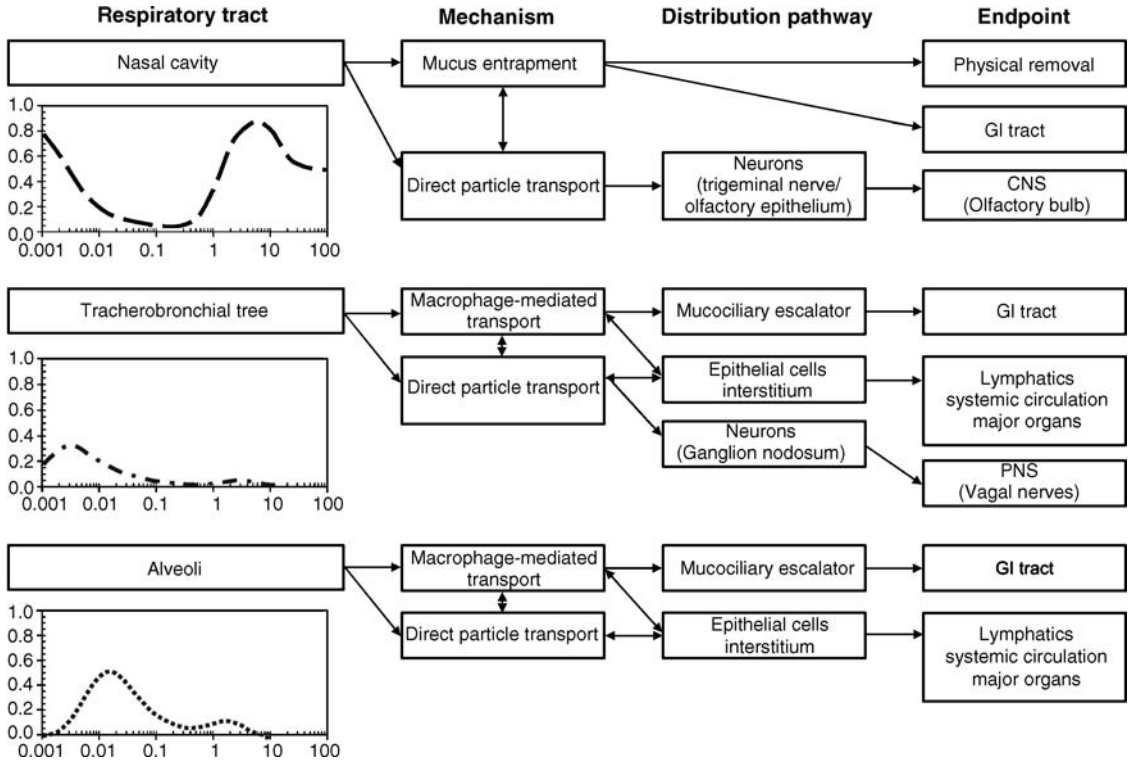

Figure 5. Transport pathways of insoluble nanoparticles in different regions of the respiratory tract. *Note:* Particle deposition probability in different regions of the respiratory tract derived from ICRP 82 (Madl and Pinkerton, 2009). (Reproduced with permission from Taylor & Francis, http://www.informaworld.com.)

Use of In Vitro Models

There is a need to develop a fast screening method that would predict the toxicity of manufactured nanomaterials (Ayres *et al*., 2008). Due to the cost and convenience, *in vitro* system should be a way to screen NPs, however, there have been many challenges with *in vitro* systems especially when it comes to mimicking exposure to NPs in the "real world". Most of the cell exposure studies have been done by resuspending particles in media. Many disadvantages are connected with such submerged *in vitro* systems: unrealistic exposure pathway, possible interactions between the cell culture medium and NPs, higher agglomeration of NPs in the medium, and substantial percentage of NPs that remain in the liquid or adhere to the lateral walls of the culture wells (which affects dose of NPs interacting with the cells), and absence of mucus or epithelial airway surface. Particles settle, diffuse, agglomerate and change surface charge and chemistry over time in solution and this can affect their transport to cells (Teeguarden *et al*., 2007). All these factors may impact particle-induced biological responses and impact the predictive value of the *in vitro* model. The cells used in *in vitro* studies may be isolated freshly from lung tissue or broncheoalveolar lavage (primary cultures) or may be obtained from immortalized cell lines (secondary cultures). Furthermore, most *in vitro* studies are typically conducted as mono-culture (one cell type). There is an increasing number of studies that use co-culture of multiple cell types such alveolar macrophages (AMs) along with epithelial cells. *In situ*, these two cell types interact concurrently to effect biologic responses (Hjort *et al*., 2003; Wottrich, Diabate and Krug, 2004). Co-culture of AMs with epithelial cells resulted in augmentation of particle-induced pro-inflammatory cytokine release (Hjort *et al*., 2003; Tao and Kobzik, 2002). The selection of certain cells used in *in vitro* experiment is crucial, since different cells exposed to NPs may show a variety of responses (e.g., inflammatory profiles) (Sayes, Reed and Warheit,

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2007; Soto, Garza and Murr, 2007). The human epithelial cell lines exhibited higher sensitivity than the murine and human macrophages probably since lung epithelial cells are the first line of defense against harmful stimuli. However, murine and human macrophage cell lines demonstrated similar responses in various degrees of cytotoxicity (Soto, Garza and Murr, 2007). Recently, novel *in vitro* exposure systems with air-liquid interface have been developed (Brandenberger *et al*., 2010; Grigg *et al*., 2009; Lenz *et al*., 2009; Thorne *et al*., 2010). Such systems overcome major drawbacks of submerged systems. Comparing the transwell system with its air-liquid interface and submerged cell exposure systems using ZnO NPs, differences in expression of IL-8 and oxidative stress marker (HO-1) were found (Lenz *et al*., 2009). Enormous care must be taken in extrapolating data obtained from *in vitro* studies (Donaldson *et al*., 2009). Authors in their study compared quartz, asbestos, and PM_{10} particles and showed that these materials have similar activities in cells *in vitro* (via oxidative stress, activation of NF-κB, pro-inflammatory effects and oxidative adduct formation), however *in vivo* they produced quite different pathological effects. Thus, it appears that *in vitro* systems will have to be further developed, standardized, and validated. Comparisons of *in vivo* and *in vitro* studies using five different NPs displayed low degrees of correlation (Sayes, Reed and Warheit, 2007). Furthermore, differences in oxidative responses of cells such as rat and human alveolar macrophages to various particulates and fibers were found (Rahman, Norwood and Hatch, 1997). This suggests that human alveolar macrophages have a spectrum of responses to the same particles, and they produce more ROS in response to particles than rat alveolar macrophages. Sensitivity of the cells to the NPs studied may be influenced by characteristics of the cell lines used including doubling times, metabolic activity or phagocytic mechanisms (Lanone *et al*., 2009). Silver nanoparticles aggregates were shown to express greater cytotoxicity than asbestos or carbon nanotubes (Soto, Garza and Murr, 2007). However in our murine inhalation study Ag NPs did not induce significant inflammatory or cytotoxic responses. *In vitro* studies are very important however until there is a strong correlation established between *in vivo* and *in vitro* studies there still will be need for animal experiments.

In Vivo Models

In vivo approaches in small rodents continue to be the primary approach for assessment of NP pulmonary toxicity. Rodent studies allow this realistic route of exposure with reproducible NP dosing and lung distribution. Table 2 summarizes traditional endpoints employed in *in vivo* toxicology assessments that are also used in nanotoxicology. Besides the properties of the nanomaterials that affect

Table 2. *In vivo* assessment of pulmonary toxicity.

Possible biological response to inhaled NP	Available pulmonary bioassays
Gross observation	Weight gain, body condition score
Cytotoxicity (cell lysis)	Lactade dehydrogenase (BAL fluid)
Transudation of proteins across alveolar-capillary	Total protein (BAL fluid)
Type II cell damage or increased secretions; transudation of serum proteins	Alkaline phosphatase (BAL fluid)
Inflammation,	Total and differential cell counts (BAL fluid)
Immune response	Cytokine/chemokine production (BAL fluid)
Inflammation and fibrosis	Histhopathology of lungs and other vital organs (liver, spleen, brain, (H&E, Masson Trichrome staining)
	Collagen determination
Host defense	Bacterial infection model of impaired clearance
Oxidative stress	Superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase, glutathione (GSH), oxidized form glutathione disulfide (GSSG) in lung tissue
Changes in pulmonary functions or mechanics	Measurements of lung volumes, resistance, elastance, compliance and impedance
Hematology and chemistry parameters in blood	Complete blood count (including differential counts), levels of electrolytes (Na, K, Cl, CO_2), and renal profile (BUN and creatinine)

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toxicity response, there are other variables that need to be taken into the consideration while conducting *in vivo* studies of NPs. The animal species, duration of exposure, as well as different exposure methods (e.g., whole-body, nose-only, intratracheal instillation) may affect degree of inflammatory or toxic response (Bermudez *et al*., 2004; Bermudez *et al*., 2002; Elder *et al*., 2005; Hext, 1994). Observation of behavioral changes, food and water consumption, clinical signs as well as monitoring body weights of animals during a study should be included in toxicity evaluation of any tested material. These observations might be the first indication of a toxic effect of the xenobiotic. The first observation should be for survival and moribundity. Other clinical signs of toxicity monitored should include abnormal level of spontaneous motor activity and body posture, gait, grooming, respiration, and quantity, or quality of fecal output. Body weights should be monitored at least once a week. In chronic studies, animals should be examined for evidence of visible or palpable masses, and quality of coat, body orifices (for excessive or unusual discharges), eyes, and respiratory sounds should be evaluated (Gad, 2007).

Comparison of Species

Small rodents such as mice, rats, and guinea pigs are all suitable for inhalation toxicology assessment of aerosols. It should be noted that rodents have more asymmetric airway branching patterns and only few respiratory bronchioles compared to humans (Thorne, 2000). Airways of guinea pigs are very sensitive and a good model for evaluation of xenobiotics that might cause bronchoconstriction (Thorne and Karol, 1988). For particles with possible fibrogenic effects rat models have traditionally been used (Thorne, 2000). Species differences were found in lung burdens of ultrafine TiO_2 particles. Lung loadings were increased in concentration-dependent manner in rats, mice, and hamsters after 13 weeks of exposure. Particle retention was similar in rats and mice but hamsters cleared particles more efficiently. Furthermore, the magnitude of lung inflammatory response was ranked as rats > mice > hamsters. Pulmonary lesions were found to be most severe in rats (Bermudez *et al*., 2004). Similar results were found after sub-chronic inhalation exposure to carbon black particles. Inflammatory and pathology response in the lungs of animals were more severe and extended in rats than in mice and hamsters. Hamsters in this study showed the most efficient clearance mechanisms and least severe responses of the three species (Elder *et al*., 2005). However, it is well recognized that there is a wide range of responsiveness across the spectrum of mouse-inbred strains. Intratracheal instillation of carbon black cause greater pulmonary toxicity in *ApoE-/-* mice than *C57* mice (Jacobsen *et al*., 2009). Differences in recruitment of neutrophils in 12 inbred strains of mice were found after inhalation exposure to Zn NPs (Gordon, Chen and Cohen, 2008).

Acute, Sub-acute and Chronic Studies

Although not specifically designed for nanomaterials, standard protocols have been adopted by regulatory agencies for both short-term and long-term inhalation exposures. The Organization for Economic Cooperation and Development (OECD) developed guidelines for acute inhalation toxicity studies (Test Guideline [TG] 433), sub-acute inhalation studies lasting 14-28 days (TG 412) and sub-chronic studies lasting 90 days (TG 413) (OECD 2004, 2009). The experimental design for an *in vivo* study performed by the authors is presented in Figure 6. It is sub-acute exposure (lasting 2 weeks) with necropsy immediately (at 0 week) and three weeks post exposure. Animals used in this model were male *C57Bl/6* inbred mice, which are a hardy strain that has been widely used over the past three decades and have unique immunological properties. Many investigators have used *C57Bl/6* mice in pulmonary nanotoxicity studies (Oberdorster *et al*., 2000; Shvedova *et al*., 2008) so their use facilitates comparison of biological responses among different research groups.

It is fundamental to use sentinel and sham-exposed animals in every *in vivo* experiment. Sentinel animals serve as a control for the conditions during transport, housing, and diet. Data obtained from sentinel animals can serve as normal values for particular endpoints. Sham-exposed animals serve as controls for the delivery system and vehicle of the studied material as well as for all the other factors animals are exposed to during study that can have impact on biological response. These include noise, stress and pressure changes during whole-body or nose-only inhalation exposure, possible abrasion, or mechanical injury to the trachea in intratracheal instillation exposures, and possible alteration of feeding and diurnal cycling. Responses

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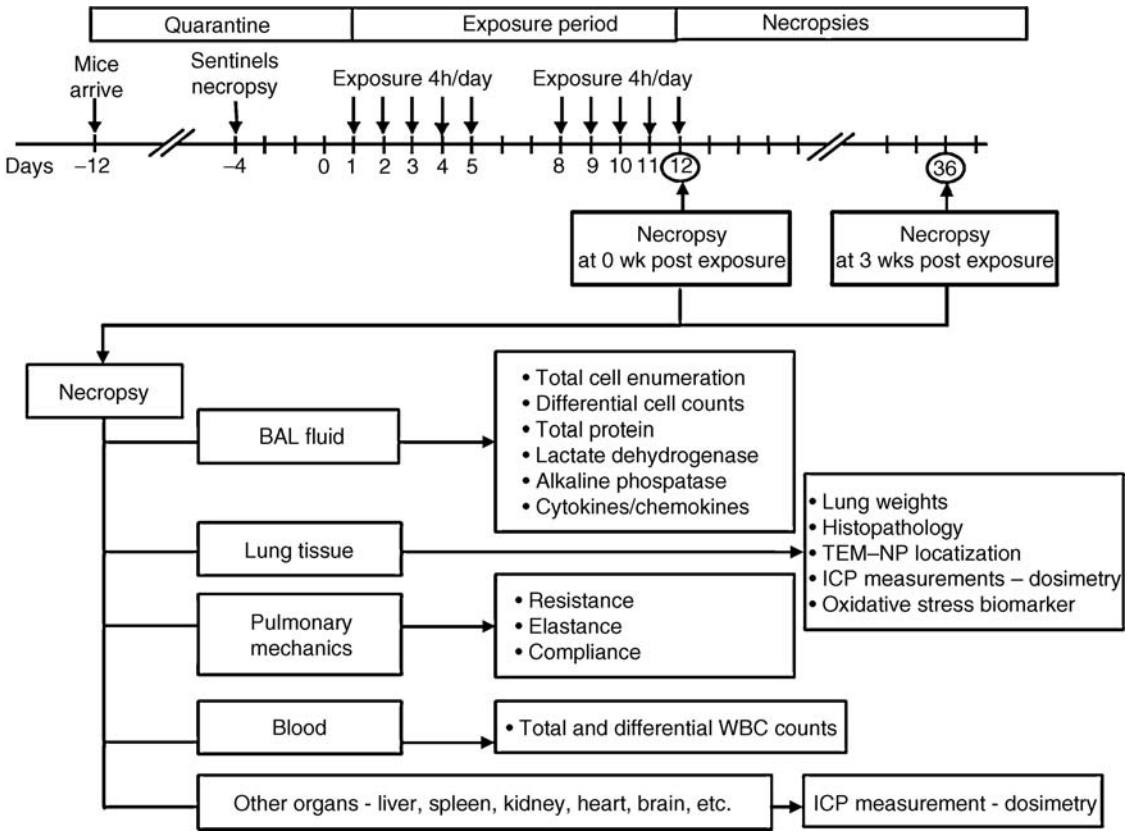

Figure 6. Experimental design for *in vivo* sub-acute study.

in exposed animals must be evaluated in comparison to responses observed in sham-exposed animals.

Dose Metrics

Dose is a function of exposure and time; however, in the case of nanotoxicity an assessment of dose is not so straightforward. It is crucial to have accurate and complete information concerning conditions during exposure such as concentration, duration of exposure, aerosol size distribution, and other characteristics (Brown, Wilson and Grant, 2005). There is an ongoing debate over an appropriate metric that should be used in toxicity assessment of nanomaterials. Many studies have found that possible health effects from exposure to nanomaterials might be better related with surface area (Duffin *et al*., 2007; Elder *et al*., 2005; Nel *et al*., 2006) and particle number (Wittmaack, 2007) rather than with mass concentration. Intratracheal instillation studies of low soluble and low toxicity materials (TiO_2 and ultrafine and fine carbon black particles) suggested that surface area of particles administered rather than mass burden of particles may be more suitable dose metric for pulmonary toxicity studies (Sager and Castranova, 2009). Earlier toxicity evaluation of inhaled particles also pointed out that various endpoints were correlated with different metrics (Oberdorster, 1996). For example, surface area was recommended as more appropriate metric for inflammatory biomarkers. On the other hand, effects on alveolar macrophages mediated clearance of particles might be the best correlated with the volumetric lung burden of different particle types. Furthermore, various ultrafine particles showed differences in the strength of response that cannot be explained only with distinct surface area. However up to now, the appropriate dose metric still remains an issue, thus, it is recommended that particle number, surface area, and particle mass be measured (Nel *et al*., 2006; Oberdorster, Oberdorster and

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Oberdorster, 2007; Oberdorster, Oberdorster and Oberdorster, 2005).

Exposure Methods

There are several exposure methods that have been used to study particulate aerosols, such as whole-body, head/nose/mouth-only, intratracheal, or nasal instillation (Brain *et al*., 1976; Muhlfeld *et al*., 2008; Pauluhn and Mohr, 2000; Pauluhn, 2005; Thorne, 2000). One advantage of whole-body or head/nose/mouth-only exposure systems is that they attempt to represent the exposure as it would occur in ambient indoor air or occupational environments and are less stressful for animals than exposure systems that use restraint. Thus, they are often preferred especially for chronic exposures. A disadvantage of whole-body exposure is the possibility of skin exposure and ingestion exposure from grooming. Nose-only inhalation systems are preferred in-situations when exposure from grooming is necessary to avoid, but animal restraiut and the resulting stress, risk of body temperature elevation in exposure tubes and increasing body size in longer term studies are all limitation of this system. However, it is recognized that both instillation and inhalation exposure models have limitations (Brain *et al*., 1976; Driscoll *et al*., 2000; Madl and Pinkerton, 2009; Osier and Oberdorster, 1997; Thorne, 2000). An advantage of instillation studies is more accurate dosimetry; however, distribution of particles in the lung, as it was shown in ours (Grassian *et al*., 2007b) and other studies (Brain *et al*., 1976; Warheit *et al*., 2004) does not follow the normal aerodynamic distribution. Intratracheal instillation or intratracheal insufflation (using a dry powder insufflator) may evoke inflammatory responses due to possible mechanical injury to the trachea as was observed in rats (Brown *et al*., 2001; Dick *et al*., 2003; Hohr *et al*., 2002; Oberdorster *et al*., 2000; Warheit *et al*., 2007a) and mice (Oberdorster *et al*., 2000). The method of pulmonary administration of nanomaterials can affect deposition pattern and biological responses. In a recent study, we compared instillation and inhalation exposure to TiO_2 nanoparticles (5 and 21 nm) in *C57Bl/6* mice (Grassian *et al*., 2007b). Interestingly, we found different types of inflammatory responses in the lungs between two exposure approaches. We observed increased numbers of macrophages with inhalation exposures, while inflammatory responses in instillation exposures were represented by increased number of neutrophils in BAL fluid. We also observed that the instillation solutions themselves contained larger aggregates than those found in the inhalation chamber. Other investigators observed that inflammatory responses in animals exposed by inhalation methods were significantly lower than after instillation exposure (Osier, bags and Oberdorster, 1997; Osier and Oberdorster, 1997; Warheit *et al*., 2005). A murine study of inhaled multiwalled carbon nanotubes (MWCNT) as well as rat inhalation study of single-wall carbon nanotubes (SWCNT) showed a more diffuse pattern of deposition with less severe pathologic lesions than those that were found after intratracheal instillation of a bolus dose (Li *et al*., 2007; Warheit *et al*., 2004). Similarly, rats exposed to MWCNT by intratracheal instillation developed pulmonary inflammation and fibrosis but when MWCNT were delivered by inhalation, no significant pathologic changes in the lung tissues were observed (Muller *et al*., 2005). Studies by Driscoll *et al*. (1991, 1990a, 1990b) in rats report that a longer period is needed for developing pathologic responses after inhalation exposure than after instillation, but still the response remains lower than in instillation studies. It is important to point out, that particles administered by instillation behave differently than particles delivered by inhalation. Instilled particles are deposited predominantly in the basal regions of the lungs and less in passages in the upper respiratory tract that play an important role in biological response (Driscoll *et al*., 2000; Osier, Baggs and Oberdorster, 1997) especially for nanoparticle exposures. In our study (Grassian *et al*., 2007b), a majority of TiO_2 nanoparticles administered by instillation were phagocytized by alveolar macrophages (Figure 7b). This was different in inhalation studies where particles were phagocytized by macrophages but also deposited at alveolar epithelial and interstitial sites as well as endothelial sites (Figure 7d). Similar finding were observed in inhalation studies of carbon nanotubes, where nanotubes were well dispersed in lung tissues and small aggregates or individual nanotubes were present on or beneath the epithelial cell surface in the lungs (Ryman-Rasmussen *et al*., 2009).

Nanoparticle Deposition

The site and extent of deposition of particles in the respiratory system is determined by physical mechanisms (interception, impaction, sedimentation, diffusion, and electrostatic precipitation) and

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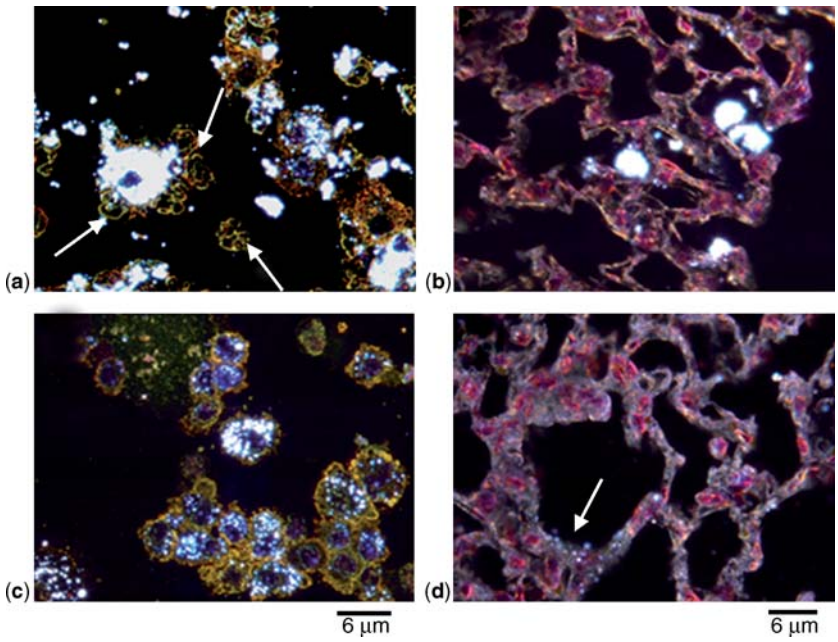

Figure 7. Dark field micrographs of alveolar cells (a;c) and lung tissue (b;d) with H&E staining after exposure to TiO_2 nanoparticles (with primary size of 21 nm) by instillation (a;b) or inhalation (c;d). Necropsy was performed 24 h post exposure. Arrows in image (a) point to neutrophils. Arrow in image points to TiO_2 particles deposited at alveolar epithelial, interstitial and endothelial sites. (Magnification 100 x).

anatomy and physiology of the subject inhaling the particles such as airway geometry, breathing pattern, frequency of breathing, tidal volume, and lung health status. The deposition of NP within the respiratory system happens primarily by diffusion (Heyder, 2004; Kreyling, Semmler and Moller, 2004) and secondarily by thermodiffusion in the first few airways of the lungs during exhalation (Asgharian and Price, 2007). Deposition in the lungs may be altered during respiratory disease (Miller *et al*., 1995). According to a well-known Human Respiratory Tract Model (HRTM) of the International Commission on Radiological Protection (ICRP) (ICRP, 1994) as shown in Figure 5, it is predicted that nanoparticles deposit efficiently in all regions of respiratory tract. Particles less than 10 nm in size deposit most in nasopharyngeal and tracheobronchial areas, whereas particles between 10 and 20 nm in size deposit most in the alveolar region. With increasing size of NPs to 20 to 100 nm, the deposition in alveolar and tracheobronchial regions decreases. For particles above 100 nm, the deposition in the nasopharyngeal area increases. Even though this model was not developed specifically for nanosized particles it is in agreement with a recent mathematical model that focused on deposition of NPs (Asgharian and Price, 2007). This multiple path particle deposition model was developed for all three regions of the respiratory tract by modeling convective flow, axial diffusion, and dispersion. Using this model for prediction of NPs smaller than 10 nm, it was shown that the deposition was high in the tracheobroncheal region and very few particles reached the alveolar region. If particle losses in nasopharyngeal region are taken into consideration, the alveolar deposition of such small particles will be even lower. Particles larger than 10 nm were deposited mainly in the pulmonary region. It was shown that deposition and clearance of particles within the airways is not homogeneous with the highest depositions occurring at the carinal ridge and bifurcational junctions (Balashazy, Hofmann and Heistracher, 2003). The carinal ridge is the point between the right and left main stem bronchus and bifurcational junctions are Y junctions, where bronchus is divided into smaller bronchioles. Even though it was shown that effect of airway geometry on deposition of particles at

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nano-scale is minor (Zhang, Kleinstreuer and Kim, 2008). Another factor that should not be ignored is the epithelial surface areas in various regions of the respiratory tract. When deposited dose is normalized per unit surface area per cell, deposited dose is higher in the upper generations of the tracheobronchial region than in the pulmonary region (Cohen *et al*., 1988; Oberdorster, Oberdorster and Oberdorster, 2005). The dose at bifurcations may be as much as an order of magnitude higher than in the rest of the airways (Balashazy *et al*., 2003; Cohen *et al*., 1988).

Interaction of Deposited Particles with Lung Surfactant

For correct assessment of nanomaterial toxicity, it is essential to establish not only the deposition site but also the interaction of particles with lung fluids and tissues, including the surfactant film, the airway surface liquid, and the cells (Geiser, Schurch and Gehr, 2003). Lung epithelium is lined with pulmonary surfactant, mainly a mixture of complex lipids, synthesized and secreted by alveolar type II cells. The main functions of surfactant are to prevent alveolar collapse on exhalation (Green *et al*., 2000) and to serve as a primary immune barrier. NPs can directly interact with lung surfactant and can influence physiological characteristics such as metabolism of surfactant, alter particle clearance, lead to biophysical dysfunction of surfactant or modulate particle toxicity (Schleh and Hohlfeld, 2009). Delivered particles in lung fluid may cause disaggregation, which can influence the biological response (Holsapple *et al*., 2005). Using an *in vitro* pulmonary liquid model with dipalmitoyl phosphatidylcholine (DPPC), that is the main component of lung surfactant, dispersed in Dulbecco's phosphate buffered saline (pH 6.5), it was found that the interaction between lung surfactant and TiO_2 did not induce disaggregation of deagglomeration (Maier *et al*., 2006).

1.2.2 Biological Responses to Nanoparticles

The full extent of biological responses to nanomaterials is still not known. However, there are several mechanisms that have been proposed to explain the adverse effects of NPs. These include inflammation, endotoxin effects, stimulation of capsaicin/irritant receptors, autonomic nervous system activity, procoagulant effects, covalent modification of cellular components, and ROS production and the generation of oxidative stress (Nel *et al*., 2006). It has been hypothesized that the mechanism of nanoparticle toxicity for the pulmonary route of exposure acts through two thresholds: (i) a dosimetric threshold, where lung clearance mechanisms cannot remove particles at sufficient rate to prevent particulate accumulation; (ii) a mechanistic threshold where the level of the antioxidant defenses is overwhelmed by production of ROS (Oberdorster, 2002). Inflammation and ROS formation targeting non-inflammatory cells can contribute to the response in both mechanisms. Another hypothesis is that NP exposure causes injury via non-oxidant paradigms (Xia *et al*., 2008). NPs can organize around them a protein corona that depends on particle size, curvature, shape, and surface characteristics such as charge, functionalized groups, and free energy. As a result of this binding, some particles can generate harmful biological responses through protein unfolding, fibrillation, thiol cross-linking, and loss of enzymatic activity. When the thermodynamic properties of the nanomaterial (including surface free energy) allow dissolution of NPs in a biological environment the subsequent release of toxic ions might lead to toxicity of these nanomaterials. This has been studied more in *in vitro* (Brunner *et al*., 2006; Knaapen *et al*., 2002;Midander, Wallinder and Leygraf, 2007) and environmental studies (Wang, Wick and Xing, 2009).

Cell Recruitment to Lung and Inflammation

The clearance and biopersistence of particles in the respiratory system play a crucial role in their toxicity. The persistence of nanomaterials in the respiratory system, dictates particle uptake by airway or alveolar epithelial cells. Particle interactions with the cells is dependent on three primary factors: (i) particle deposition patterns; (ii) penetration of particle through the surfactant and mucus layers; and (iii) clearance or failure to remove particles by inflammatory cells (Churg, 2000). Already in nasopharyngeal areas and trachea, particles promote secretion of mucous (gel phase) that might move either anteriorly through nares or posteriorly into the pharyx. Some particles remain in close association with the epithelium (sol phase) for days or even weeks. Macrophages are present in the whole respiratory system and start to engulf foreign particles as soon as they come into contact with them.

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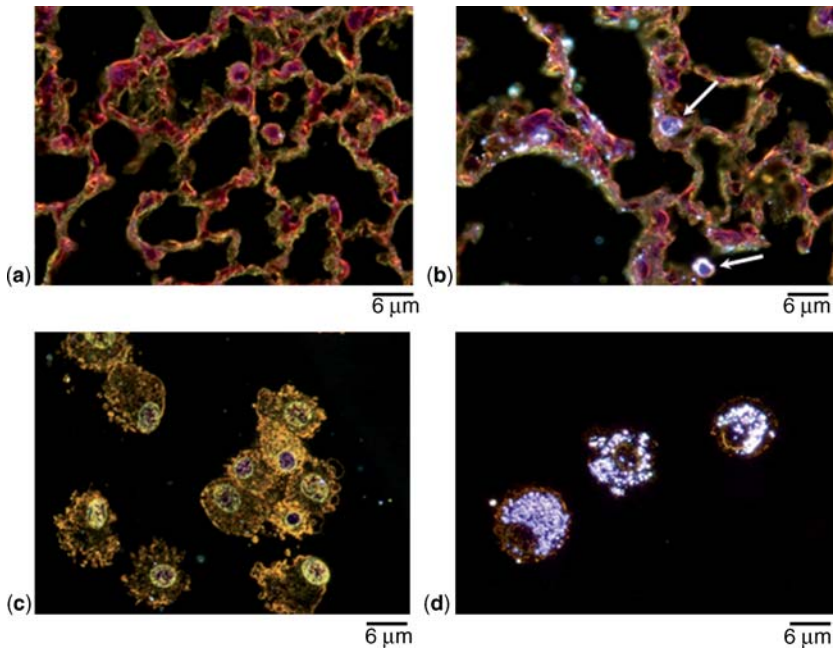

Figure 8. Dark field micrographs of lung tissue with H&E staining (a;b) and alveolar macrophages prepared by cytospinning and H&E staining (c;d). Sentinels (a;c) and animals sub-acutely exposed to TiO_2 nanoparticles with a primary particle size of 2–5 nm and necropsied immediately after the last exposure (b;d). Arrows point to TiO_2 nanoparticle-laden macrophages. (Grassian *et al*., 61). (Reproduced with permission from Grassian et al. (2007a) ©Taylar & Francis Group, plc.)

Phagocytosis is a major function of macrophages and influences the immunologic response of the lungs. Particle-laden macrophages, free particles along with secreted mucus might be carried up the mucociliary escalator to the pharyx where they are swallowed. Some particles are taken up by dendritic cells and transported into the tissue and reach lymph nodes and are presented to the T-lymphocytes. TiO_2-NP-laden macrophages found in bronchoalveolar lavage as well as lung tissue after inhalation exposure are presented in Figure 8. Part of an early lung response after inhalation exposure is not only recruitment of monocytes (macrophages) but also an engagement and activation of other inflammatory cells such as neutrophils. Many studies have scrutinized pro-inflammatory effects of manufactured NPs as a main predictor of their potential hazard (Bermudez *et al*., 2004; Grassian *et al*., 2007; Grassian *et al*., 2007; Pettibone, *et al*., 2008) and many of these studies confirmed that manufactured NPs certainly stimulate inflammatory effects with recruitment of macrophages and neutrophils (Figures 3 and 4). Some studies indicated that the uptake of inhaled ultrafine particles by phagocytosis is limited and that particles entered macrophages or type I epithelial cells by endocytosis (Geiser *et al*., 2008; Geiser *et al*., 2005; Stoeger *et al*., 2006; Takenaka *et al*., 2006).

Cytotoxicity and Oxidative Stress

Nanoparticles can cause toxicity of cells directly or indirectly. They can stimulate cells causing subsequent release of inflammatory mediators. A fast and frequently used assessment method of potential cytotoxicity of nanomaterials is done using *in vitro* studies. However, cytotoxicity is usually higher in *in vitro* studies as compared to *in vivo*, since nanomaterials or degradation products responsible for the toxicity are constantly removed in the case of *in vivo* experiments. The most widely used cytotoxicity assay in *in vivo* studies is the measurement of the cytoplasmic protein lactate dehydrogenase (LDH). This protein is released into the extracellular space either in the case of cell death or cell membrane damage. The enzyme activity of LDH is proportional to the cell injury. The generation of ROS and production of oxidative stress is considered to be the best-developed paradigm of nanoparticle toxicity after inhalation (Ayres *et al*., 2008; Donaldson *et al*., 2001; Nel *et al*., 2006;

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Oberdorster, Oberdorster and Oberdorster, 2005; Shvedova *et al*., 2005). Membrane damage, cell death, inflammation, or even DNA damage (Schins *et al*., 2002; Trouiller *et al*., 2009) can all be the result of oxidative stress (Nel *et al*., 2006). During an inflammatory response, inflammatory cells like neutrophils, eosinophils, monocytes, and macrophages become activated and produce a respiratory burst when the cells take up oxygen through the activity of an NADPH-oxidase that generates superoxide anion radical (O_2^-) and hydrogen peroxide (H_2O_2) (Doelman and Bast, 1990). These oxygen metabolites give rise to other reactive oxygen species that may also cause damage by destroying surrounding tissue and inducing apoptosis in other immune reactive cells. Thus, redox imbalance, in which ROS or reactive nitrogen species (RNS) production overwhelms the antioxidant defense capacity of the cell, leads to oxidative stress. This condition is a state in which the glutathione (GSH) is depleted while oxidized glutathione (GSSG) accumulates. Many studies use the GSH/GSSG ratio to monitor an ongoing oxidative stress. The GSH/GSSG redox couple serves as the leading homeostatic regulator of cellular redox balance and also functions as a sensor that triggers additional cellular responses that can be protective or injurious. The reactive oxygen species can stimulate bronchoconstriction, increase secretion of mucus, and cause microvascular leakage, which leads to edema formation (Doelman and Bast, 1990).

The hierarchical oxidative stress model described by Nel *et al*., (2006) hypothesizes that minor levels of oxidative stress induce protective effects through antioxidant enzymes that are induced via transcriptional response elements by Nrf-2 (tier 1). However at an intermediate amount of oxidative stress (tier 2), it can lead to more damaging effects. If defensive responses fail to lead to adequate protection, a further increase in ROS production can result in pro-inflammatory and cytotoxic effects. Pro-inflammatory effects are mediated by the redox-sensitive MAP kinase and NF-κB cascades that lead to the expression of cytokines, chemokines and adhesion molecules. At a high level of oxidative stress (tier 3), cytotoxic effects are mediated by mitochondria, which can result in apoptosis or necrosis.

Number of studies (mainly *in vitro*) have demonstrated that nanosized particles can generate ROS and hydroxyl radicals (Brown *et al*., 2001; Carlson *et al*., 2008; Dick *et al*., 2003; Hussain *et al*., 2005). Some studies suggest that ROS production is related to the particle surface functional groups, since it was found that smaller particles were more powerful in production of ROS (Brown *et al*., 2001; Carlson *et al*., 2008; Stone *et al*. 2000). Other particle properties might also be responsible for oxidative stress. Comparison of four different ultrafine particles (carbon black, cobalt, nickel, and TiO_2) administered via instillation in the same protocol, showed distinct levels of inflammation and lung injury. Based on these studies, the authors concluded that the degree of lung damage was associated not with their size or surface area, but with their ability to generate surface free radicals with subsequent oxidant injury (Dick *et al*., 2003). If NPs are metals, or metals are present on the surface of particles, or dissolution of particles causes ion release, they can participate in the Fenton reaction, producing hydroxyl radicals, which are highly reactive and may destroy proteins and initiate the process of lipid peroxidation leading to DNA strand breaks. Destruction of the lipid bilayer by hydrogen peroxide (rapidly converted to hydroxyl radicals in the presence of ferrous ions) causes calcium influx in the cells. These elevated intracellular calcium concentrations are toxic to the cells (Doelman and Bast, 1990). They can lead to mitochondrial perturbation and apoptosis (Xia *et al*., 2006). Carbon black nanoparticles caused calcium influx in primary rat alveolar macrophages (Stone *et al*., 2000). However, as it was shown *in vitro* and *in vivo* (rats) studies of carbon black particles, metals are not necessarily needed for free radical generation after exposure to ultrafine particles (Brown *et al*., 2000).

Finally, it is worth mentioning, that some nanomaterial may generate bioprotective effects against oxidant injury as was found *in vitro* and *in vivo* testing of cerium oxide (CeO_2) nanoparticle and degenerative diseases of the retina (Chen *et al*., 2008) and cerium and yttrium oxide and a nerve cell line (Schubert *et al*., 2006). A cytoprotective effect of CeO_2 to cellular challenge with pro-oxidative organic (DEP) was also found in an diesels exhaust particles *in vitro* study using macrophage and epithelial cells (Xia *et al*., 2008). In the same study, ZnO was found to induce oxidative stress responses using the hierarchical oxidative stress model (production of pro-inflammatory cytokines [Tier 2] and mitochondrial injury [Tier 3], but TiO_2 was shown to be inert. Also, cardioprotective effects of CeO_2 were observed in mice probably through

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their autoregenerative antioxidant properties (Niu *et al*., 2007).

Immunological Responses

Cytokines Many cytokines/chemokines are mediators of the tissue reactions following environmental insult such as inflammatory or fibrogenic processes (Shvedova *et al*., 2008; Stone, Johnston and Schins, 2009). Tumor necrosis factor (TNF) plays a role in the pulmonary recruitment of inflammatory cells and subsequent fibrosis formation when overload of lungs with insoluble particles occurs (Driscoll *et al*., 1990a; 1990b). A significant correlation was expressed between TNF release from alveolar macrophages and BAL neutrophil numbers. It has also been suggested that interleukin (IL) participates in granuloma formation in lungs as well as other tissues (Driscoll *et al*., 1990b; Hunninghake, 1984). After sub-acute inhalation exposure to Cu nanoparticles there were selected cytokines/chemokines that were found to be significantly elevated in BAL fluid such as monocyte chemotactic protein (MCP-1), macrophage inflammatory protein (MIP)-1α, IL-12(p40), keratinocyte chemoattractant (KC), eotaxin, TNF-α, regulated upon activation normal T expressed, and secreted (RANTES), granulocyte colony-stimulating factor (G-CSF), gramelocyte/macrophage colony stimulating factor, and IL-6. The largest increase was shown in MCP-1 levels (elevation from 3.4 pg mL^{-1} measured in sentinels to 4398.1 pg mL^{-1} in Cu-exposed group). This elevation was associated with increased number of BAL macrophages (Pettibone, *et al.*, 2008). Higher lung levels of MCP-1 were also found after exposure to carbon NPs (Inoue *et al*., 2005). Similar cytokines/chemokines elevations in BAL fluid were found in case of pharyngeal aspiration with single wall carbon nanotubes (SWCNT) 3 days post exposure (Shvedova *et al*., 2008). Interestingly, even though augmentation of IL-12 (p40) cytokine that is known to be elevated with profibrogenic exposures was observed in Cu-exposed animals, no fibrotic changes in the lung tissues were found in mice necropsied immediately or three weeks post exposure (Pettibone, *et al.*, 2008). Thus, it may require a chronic study to demonstrate fibrosis from Cu NPs.

Immunomodulatory Effects

Ultrafine and fine particulate matter (e.g., DEP or carbon black particles) is known to possess adjuvant properties that can induce exacerbation or modification of immune responses (Th1 versus Th2 response) (Al-Humadi *et al*., 2002; Diaz-Sanchez *et al*., 1999; Granum and Lovik, 2002; Last *et al*., 2004; Nygaard *et al*., 2004). This type of response can possibly induce hypersensitivity or allergic reactions. As shown in animal studies, various particles in combination with an antigen can enhance allergic sensitization expressed by elevation of antigen-specific IgE levels (Al-Humadi *et al*., 2002; Lambert *et al*., 2000; Takano *et al*., 1997; van Zijverden *et al*., 2000). It was also suggested that the exposure of particles with an antigen does not have to occur simultaneously since adjuvant effect from particle exposure may last for several days (Granum and Lovik, 2002). Thus, it is safe to assume that nano-sized particles may also have immunomodulatory potential and they can stimulate (increase the potency of allergens) or suppress the immune system. At this time, it is not clear what properties of NPs are responsible for this adjuvant effect. Is it due to their size, surface area, or chemical composition or other properties? The induction of inflammatory responses after particle exposure is postulated to play a critical role (de Haar *et al*., 2005). Not all studies investigating effect of NPs on allergic airway inflammation studied this effect in relation to different particles properties. Results from a study of residual oil fly ash (ROFA) suggest it was soluble transition metals in RDFA that mediated enhancement of sensitization to allergen (Lambert *et al*., 2000). Based on their animal studies de Haar and colleagues concluded that adjuvant activity of particles might be size-dependent. In this study, particles below 30 nm in diameter (TiO_2 and carbon black particles) induced immune sensitization to co-administered ovalbumen (OVA) that was demonstrated by high levels of cytokines (Th2-skewed immune response), increases in serum OVA-specific antibodies and by allergic airway inflammation. Such a response was not observed for particles over 200 nm (de Haar *et al*., 2006). Inoue *et al*. (2005, 2006) confirmed a size dependent effect (14 nm versus 56 nm carbon black particles) on adjuvant activity for total IgE and allergen-specific production of IgG and IgE, as well as on local expression of certain cytokines and chemokines. Antigen-related airway inflammation and immunoglobulin production were enhanced with smaller particles or larger surface area. One explanation of this response is that particles with

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larger surface area are prone to attach more antigens than particles with smaller surface area. The authors reported that NPs might exacerbate allergic airway inflammation via increased expression of IL-5 and eotaxin and by expression of IL-13, RANTES, MCP-1, and IL-6. It was also suggested that the enhanced oxidative stress was a contributory factor (Inoue *et al*., 2005). Overall, it is proposed that exposure to NPs can be a risk for exacerbation of allergic asthma. This was further confirmed with multiwalled carbon nanotubes (MWCNT) (Inoue *et al*., 2009a). However, latex NPs (15-100 nm in size) did not exacerbate allergic asthma (Inoue *et al*., 2009). This shows that each nanomaterial might have different effects on allergic airway inflammation that depend on their unique properties. Size, shape (e.g., fibrous), chemical elements (e.g., metals) of nanomaterials might play a role in initiation of allergy.

Histopathologic Responses

In many cases lung pathology observed in *in vivo* studies was due to "overload phenomenon" when alveolar macrophages have an impaired ability to remove particles (Bermudez *et al*., 2004; Oberdorster, 1995). Considering this overload phenomenon, any insoluble particle of low toxicity can cause chronic pulmonary inflammation, lung fibrosis, or even tumors at a high enough dose. Species differences need to be considered in histopahological evaluation; rats more than mice or hamsters develop fibroproliferative lesions and lung tumors under pulmonary overload conditions after exposure to poorly soluble particulates such as TiO_2 (Bermudez *et al*., 2004). In our sub-acute studies of TiO_2 nanoparticles at exposure concentration of 8.88 mg m^{-3}, mice (*C57Bl/6*) did not develop remarkable pathological changes in the lung tissue (Grassian *et al*., 2007a).

In sub-acute inhalation studies of iron and copper NPs (particle properties are listed in Table 1) (Pettibone, *et al.*, 2008), iron exposure did not cause any histopathologic changes in the lung tissues; however, in the case of copper exposures, we found perivasculitis and alveolitis in animals nacropsied immediately post exposure (Figure 9a, b). All animals exposed to copper NPs and necropsied at zero-week developed perivasculitis with minimal increase of inflammation and chronic inflammatory cells. Alveolitis with minimal increase of inflammation and acute inflammatory cells were found in 88% of animals. No signs of fibrosis, peribronchiolitis, or interstitial pneumonitis were observed in any animals exposed to Cu NPs. Interestingly, no signs of pathology were found in animals necropsied three weeks after exposure (Figure 9c, d).

In our studies of Fe and Cu NPs sub-acute exposures, lungs were stained with Perls Prussian Blue stain or Rhodanine stain, respectively. In this manner, free iron particles were identified in the alveolar space as well as engulfed in macrophages (Figure 10a) in mice necropsied immediately post exposure. At three weeks post exposure, the majority of iron particles were located inside the macrophages (Figure 10b). In the case of Cu NPs exposure, none of the particles were visualized (Figure 10c, d) probably due to higher dissolution of Cu in biological fluid (Pettibone, *et al.*, 2008).

Histopahological lung evaluation of lung tissues from rats exposed to silver NPs for 90 days showed higher incidence of mixed cell infiltrate perivascular and chronic alveolar inflammation, including alveolitis, granulomatous lesions, and alveolar wall thickening with alveolar macrophage accumulation (Sung *et al*., 2008). In our mouse sub-acute (two weeks) exposure to Ag NPs we did not see pathological changes in the lung tissues, even though we observed macrophages with Ag particles exhibiting a foamy appearance by light microscopy (unpublished data). Similarly, four-week exposure of rats to nanosized Ag particles did not cause pathological changes in the lung tissue except for foamy alveolar macrophages and slight increase in neutral mucine in nasal cavities (Hyun *et al*., 2008). Studying nanosized and microsized SiO_2 it was found that the rats developed milder fibrosis (also demonstrated by lower hydroxyproline content, IL-4 and TGF-β expression in the lungs) with nanosized SiO_2 exposure. The authors postulated that this might be due to diffusion of NPs into the pulmonary interstitium and induction of more scattered inflammation as well as possible translocation of smaller particles to extrapulmonary organs (Chen *et al*., 2004).

Impaired Pulmonary Functions and Altered Lung Mechanics

There are not very many studies investigating pulmonary function or pulmonary mechanics in

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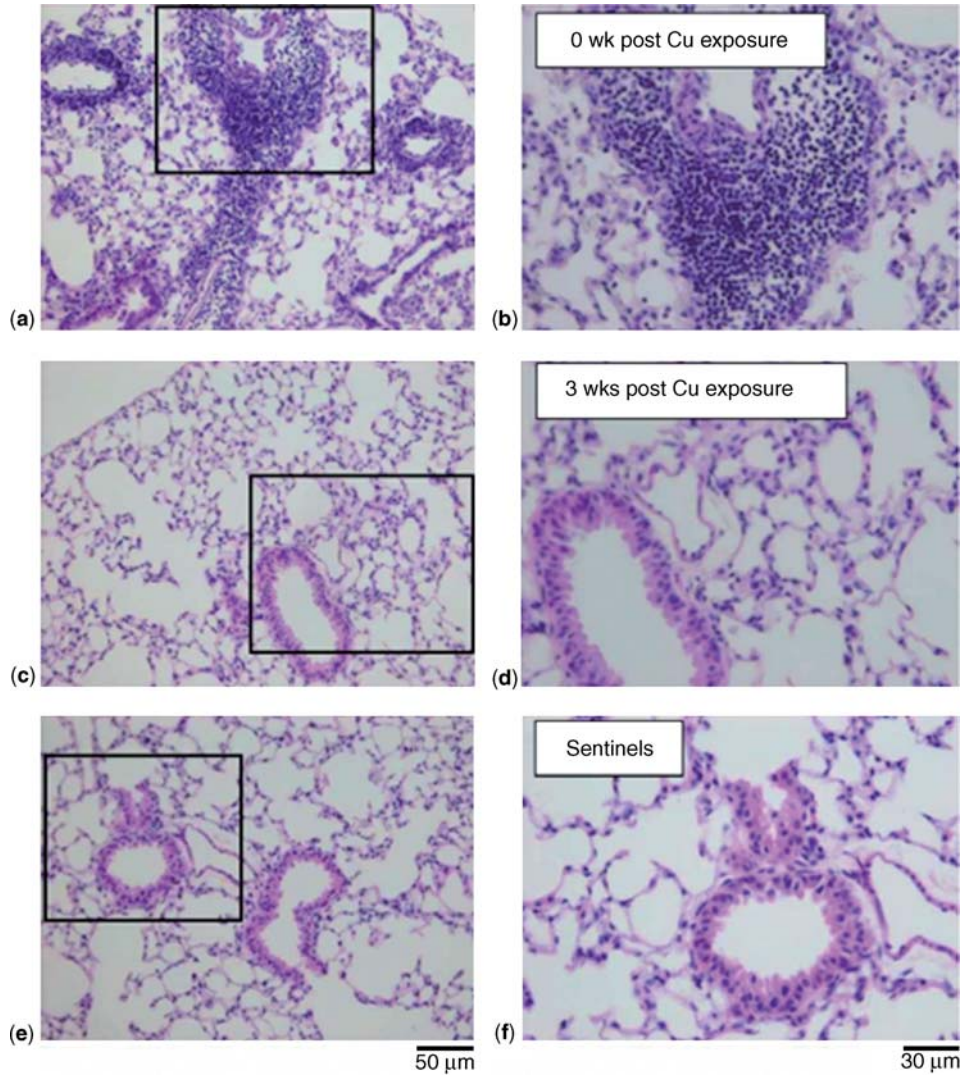

Figure 9. Micrographs of lung sections stained with H&E from mice exposed sub-acutely to copper nanoparticles and necropsied immediately after the last exposure (a;b), mice exposed to copper particles and necropsied 3 weeks post exposure (c;d) and sentinels (e;f). The areas indicated by the box in the low magnification figures are shown at higher magnification in the micrographs (Pettibone, *et al.*, 2008). (Reproduced with permission from Peltibone *et al.* (2008) ©Taylar & Francis Group, plc.)

animals exposed to nanomaterials. Lung function changes were studied in Sprague-Dawley rats after 90-day-inhalation exposure to silver NPs (Sung *et al.*, 2008). The tidal volume, minute volume, and peak inspiratory flow demonstrated a significant decrease during sub-chronic exposure to silver NPs. This decrease was associated with inflammatory lesions in the lung tissues (infiltrate mixed cell and chronic alveolar inflammation, thickened alveolar walls, and small granulomatous lesions).

Host Defense

The role of alveolar macrophages in protecting the lungs against invading pathogens might be impaired by exposure to environmental contaminants and thus contribute to increased susceptibility to infection by microorganism (Gordon and Read, 2002). Many studies have observed impaired function of alveolar macrophages especially after exposure to ambient air (Antonini *et al.*, 2002; Hatch *et al.*, 1985; Sigaud *et al.*, 2007), ultrafine (Lundborg *et al.*,

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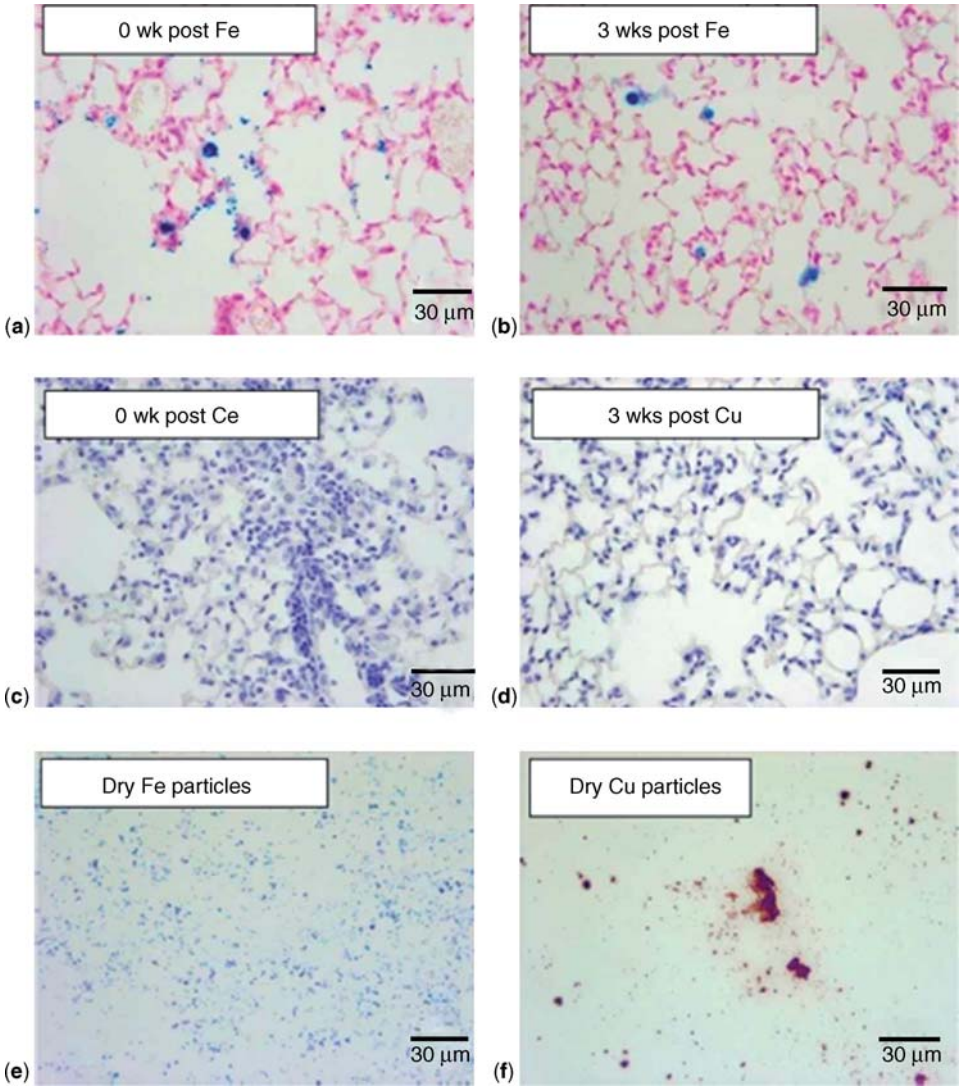

Figure 10. Lung tissues were stained to illuminate iron (deep blue) and copper (brick red) nanoparticles. The distribution of iron and copper stained nanoparticles in lung tissues (a;b;c;d), as dry powder (e;f) are shown. Iron nanoparticles were visible in lung sections at both 0 and 3 weeks post exposure (a;b), but copper nanoparticle were not visible in the lung tissue (c;d). Nanoparticles deposited on the slide as dry powder (e;f) were used as positive controls (Pettibone, *et al.*, 2008). (Reproduced with permission from Peltibone *et al.* (2008) ©Taylar & Francis Group, plc.)

2001) and diesel exhaust particles (Yang *et al.*, 2001; Yin *et al.*, 2002). Sequential exposure (pharyngeal aspiration) of mice to single-walled carbon nanotubes (SWCNT) and bacteria (*Listeria monocytogenes*) decreased mouse recovery from bacterial infection (Shvedova *et al.*, 2008). *In vitro* studies showed that SWCNT suppressed chemotaxis of primary human monocyte-derived macrophages even at low concentration that did not cause cytotoxicity (Witasp *et al.*, 2009). In contrast to ultrafine ambient air particles that impact bacterial clearance, many nanomaterials also possess antibacterial activity (Chung *et al.*, 2008; Pal, Tak and Song, 2007; Ruparelia *et al.*, 2008). Thus even though bacterial clearance might be impaired after exposure to nanomaterials, if they also have antibacterial properties it

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might depend on the exposure dose or mechanism of NP toxicity what effect is stronger. Further studies in this area are necessary.

Extrapulmonary Nanoparticle Translocation

Inhalation exposure of particulates is considered to be mainly a pulmonary problem; however, translocation of particles to other organs as well as gastrointestinal exposure should be taken into account since a high percentage of particles from the surface of the epithelium are removed by the mucociliary escalator and swallowed (Nemmar *et al*., 2002; Oberdorster *et al*., 2002; Semmler *et al*., 2004). Nanoparticulate translocation has been studied mainly in association with studies of the adverse cardiovascular effects of air pollution (Nemmar *et al*., 2002), however, manufactured/engineered NPs could pose the same risk. There is an ongoing debate if NPs are able to cross the pulmonary blood-air barrier and if this translocation to blood circulation occurs in a significant amount (Nemmar *et al*., 2002; Nemmar *et al*., 2001; Wiebert *et al*., 2006a). There are animal experiments that demonstrated extrapulmonary NPs translocation across the air-blood barrier into the blood (Geiser *et al*., 2005) or secondary target organs (Kreyling *et al*., 2002; Oberdorster *et al*., 2002; Takenaka *et al*., 2001). However, the fractions of translocated particles were very low. Human inhalation studies are controversial – some reported translocation of ultrafine radiolabelled (technetium 99m [^{99m}Tc]) carbon particles (generated with the Technagas Generator^{TM}) into the blood (Nemmar *et al*., 2002) while others observed quantitatively insignificant translocation of 100 or 35 nm Technegas particles to the systemic circulation in healthy lungs (Mills *et al*., 2006; Wiebert *et al*., 2006a; 2006b). Particle properties such as particle size (Mills *et al*., 2006) chemical composition, physical structure of the particle surface (Kreyling *et al*., 2002), *in vivo* solubility, or binding affinity to the cell membrane (Takenaka *et al*., 2006; Takenaka *et al*., 2001) might play a key role influencing systemic translocation of nanosized particles. In our subacute inhalation exposure to Ag nanoparicles with exposure concentration in the whole-body exposure chamber of 3.32 mg m^{-3}, we measured the retained amount of Ag in lungs 20 ± 13.6 mg kg^{-1} lung (dry weight). Silver concentrations in the heart, liver, and brain were found to be below the limit of detection for inductively coupled plasma optical emission Spectrometry (ICP-OES).

Studies of 50 and 200 nm polystyrene particles suggest that only a small portion of ultrafine particles instilled intratrachealy pass into the systemic circulation. However, pulmonary inflammation induced by LPS pretreatment appeared to play an important role in increasing extrapulmonary translocation of particles (Chen *et al*., 2006). The likelihood and mechanism of extrapulmonary translocation has not been established, although Nemmar and colleagues (Nemmar *et al*., 2002) propose that since rapid translocation of ^{99m}Tc-labelled particles takes place, phagocytosis by macrophages and/or endocytosis by epithelial and endothelial cells are not exclusively responsible for particle translocation into the circulatory system.

Genotoxicity

Nanoparticles can impact cellular transcription processes and prompt changes at the molecular level that would subsequently lead to alterations in the nucleus causing genotoxicity and carcinogenicity. To date, most studies evaluating the genotoxic potential of metal NPs were performed using *in vitro* models with pulmonary (Grigg *et al*., 2009) or other types of cells (AshaRani *et al*., 2009; Bhabra *et al*., 2009). Silver NPs were shown to cause DNA damage and chromosomal aberration in human lung fibroblast and the human glioma (AshaRani *et al*., 2009). Another *in vitro* study using an air-liquid interface model demonstrated that iron, gold, and silver NPs induced DNA damage to macrophages (Grigg *et al*., 2009). There are few *in vivo* studies that focus on genotoxicity of NPs. Intratracheal instillation of rats to α-quartz, carbon black, and titanium dioxide particles induced neutrophilic response and mutations in alveolar epithelial cells in a dose- and material- dependent manner (Driscoll *et al*., 1997).

A study examining genotoxicty potential of TiO_2 after exposure via gastrointerstinal tract (Trouiller *et al*., 2009) revealed that TiO_2 NPs induced DNA strand breaks and chromosomal damage in bone marrow and/or peripheral blood of mice. They also found that maternal oral exposure to TiO_2 NPs (500 mg kg^{-1}) during gestation caused DNA deletions in offspring. Authors proposed that this genotoxicity was caused by a secondary genotoxicity mechanism associated with inflammation and/or oxidative stress.

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2 SUMMARY AND CONCLUSIONS

Several conclusions can be drawn from studies in the literature that have been focused on the potential of nanomaterials to cause toxic pulmonary responses:

- At this time, there is no reference material that would serve as a standard for comparison of toxicity of various nanomaterials. Many investigators, including our group, rank toxicity of nanomaterials based on various well-known toxicity endpoints. For this reason, a standardized protocol for evaluation of NP toxicity is necessary. However, as can be seen in this review, toxicity comparisons can be quite complex.
- Despite numerous *in vivo* and *in vitro* studies, the mechanism of NP toxicity remains unknown. More studies, especially those focused on long-term exposures to NPs are necessary.
- Full and detailed characterization of nanomaterials used in the toxicity assessment studies is crucial.
- Assessment of actual hazards and risk identification is still warranted so that this knowledge can be applied to safety measures.
- Well-designed epidemiological studies of workers exposed to NPs during manufacturing processes are needed.
- Interdisciplinary teams of scientists that include chemists, aerosol physics and engineers, and toxicologists have many advantages in furthering our understanding of nanomaterial toxicity evaluation.

One last point to make in the toxicity of NPs to humans is that, there is only one study that reports exposure to NPs and clinical toxicity in humans (Song, Li and Du, 2009). The authors reported that lung tissue displayed nonspecific pulmonary inflammation, pulmonary fibrosis and foreign-body granulomas of the pleura in workers exposed to polyacrylate, consisting of NPs. However, this study is a subject of ongoing discussions (Brain, Kreyling and Gehr, 2010; Inoue and Takano, 2010; Song, Li and Du, 2010). A number of scientists have weighed in on this issue and the majority of scientists think that this paper drew conclusions too quickly and that exposure assessment of chemicals and dusts present at this workplace environment were insufficient (Brain, Kreyling and Gehr, 2010). Although the conclusions drawn from this study were rebuked, it is clear that the toxicity of NPs must be taken seriously and there is a need for additional *in vivo* studies, screening of health symptoms of workers and well-designed epidemiological studies of workers exposed to NPs during manufacturing processing.

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RELATED ARTICLES

Nanotoxicology–the Toxicology of Nanomaterials
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The Role of Oxidative Stress in Nanotoxicology

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1 NANOPARTICLE HAZARD AND EXPOSURE

The use of nanoparticles in a myriad of household products (e.g., cosmetics, paints, and food packages) is increasingly wide-spread; therefore, contact with nanoparticles is not only becoming unavoidable, but with many products, exposure is intentional. The current scientific literature tells us that on one hand, nanoparticles have unique properties that enable advantageous phenomena; on the other hand, little is known about the unintentional consequences of man-made materials on the same size scale as normal functioning biological processes. Some have even postulated that exposures to nanoparticles could cause cardiopulmonary disease or extensive hepatocytic damage. Therefore, there is an overwhelming need to evaluate each material in order to protect human health and the environment.

A vast amount of information has been published and generally accepted on the nanoparticle-biological interactions of carbonaceous nanoparticles (Moussa, 1995; Moussa, 1997; Moria, 2006; Gharbi, 2005; Kolosnjaj, Szwarc and Moussa, 2007, 2007). The work focusing on carbon nanotubes leads to the concern that inhalation of nanoparticles will produce pulmonary hazards (Lam, 2004; Shvedova, 2005; Warheit, 2004). Other studies say carbon nanotubes and other fullerenes can induce oxidative stress (Sayes, 2006; Sayes, 2005; Fortner, 2005; Lyon, 2005; Sayes, 2004). Pulmonary disease induced by inhaled needle-like carbon nanofibers, extensive liver damage induced from accumulation of fullerene nanoparticles causing oxidative stress, or autoimmune disorders caused by an overproduction of alveolar macrophages or Kupffer's cells are just a few examples of human health problems due to chronic or sub-chronic exposures to nanoparticles. Poland *et al.* (2008) reported that the toxic effects of carbon nanotubes are "similar to asbestos". While the precise pathway of quantum dot exposures is unclear, prolonged exposures are widely accepted as a potential multi-organ threat.

Clearly, there are differences in the biological response after exposure to nanoparticles; thus the need to investigate why some are toxic and others do not induce a toxicological response. There are, potentially, many different "nano" modes of actions. The toxicological responses observed in many published studies could be measures of oxidative stress. This chapter outlines some of the oxidative stress biomarkers that are cited in the nanotoxicology literature.

2 NANOPARTICLE EXPOSURES FOLLOW PRECISE PATHWAYS TO CAUSE OXIDATIVE STRESS

2.1 Pathway #1: Inflammatory Cascade

Studies have shown that exposure to nanoparticles *in vitro* and *in vivo* systems may cause production

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of inflammatory biomarkers (Warheit, 2004; Gurr, 2005; Sayes, 2006; Zhu, Oberdorster and Haasch, 2006). After nanoparticles are internalized by cell, such as phagocytes, the inflammatory cascade may be triggered. Inflammation is the complex biological response of cells and tissues to harmful pathogens and other toxicants. It is both a proactive mechanism to remove these harmful pathogens and to initiate production of repair enzymes. Unchecked inflammation can lead to a host of diseases, such as asthma, atherosclerosis, and rheumatoid arthritis; therefore, it is normally tightly regulated by the body.

2.2 Pathway #2: Necrosis or Apoptosis

Another pathway that may be triggered after nanoparticles are internalized by cells is death by necrosis or apoptosis due to particle uptake and accumulation or internal dissociation of ions, such as leaching of heavy metals. Heavy metal toxicity is well established in the literature (Stohs and Bagchi, 1995; Di Toro, 2001; Kirchner, 2005; Webb and Verschoyle, 1976; Ellender, 1979). It is also a complex biological response of cells and tissues to harmful ions. Usually, there is no cellular recovery after cadmium poisoning, for instance, but other free metal ions, such as selenide or zinc, may induce a slightly different response. Furthermore, if both nanoparticles and free metal ions are present within a cell, two pathways may be triggered.

2.3 Pathway #3: "Naïve" Antibody Repertoire

Even before challenge with a particular pathogen or immunogen, there is a pre-immune repertoire of antibodies circulating in the body. Some of these are innate IgM antibodies (termed "natural"), generally low affinity/highly cross-reactive antibodies to bacterial moieties (Zinkernagel and Hengartner, 2006). In addition to the natural antibodies, there are existing antibodies of other classes patrolling blood, lymph, and mucosa, many having been affinity matured and expanded by previous immune responses. It is possible that these existing antibodies may be capable of binding and neutralizing nanoparticles, without the active induction of a specific clonal B cell response towards the nanoparticle.

2.4 Pathway #4: Nanoparticle Induced Humoral B Cell Response

Although "small" is usually equated to "non-immunogenic" in immunology, it is possible that nanoparticles could activate a specific immunoglobulin on a B cell and cause an adaptive humoral response (Tizard, 1982). This could occur through conjugation with a carrier protein *in vivo*, providing both B cell receptor aggregation and linked recognition of a peptide for T cell help. If antibodies specific for nanoparticles are being produced (as opposed to antibodies cross-reactive with nanoparticles pre-existing as described above), they would not only clear the first nanoparticle introduction but would provide an augmented memory response with faster kinetics.

3 TOXICOLOGICAL RESPONSE DEPENDS ON CELLULAR UPTAKE MECHANISMS

Nanoparticles are novel tools in biological and biomedical applications, which can be used to determine the cellular uptake pathways of other small particles. However, the mechanisms of cellular uptake are unknown and the toxicity of nanoparticles within biological systems is of concern. The nanoparticle size, degree and type of surface coating, and surface charge are factors affecting NP endocytosis. Surface charge is believed to affect the interactions between particles and cell membranes (Zhang, 2009; Harush-Frenkel, 2007). Negatively charged nanoparticle surfaces, such as particles coated with hydrolipic acids, and positively charged nanoparticles, such as particles coated with polymers, are more capable of incorporation into human cells than particles with no surface charge. The surface coating of a nanoparticle influences the uptake by cells, which is accomplished via phagocytosis or pinocytosis. Pinocytosis includes macropinocytosis, clathrin or caveolao-mediated endocytosis, or clathrin/caveolae independent endocytosis.

Another factor that influences nanoparticle cellular uptake and subsequent toxicological response is proteins absorbed and adsorbed onto the surface of the nanoparticles. For example, proteins have been shown to coat particles when placed in cell culture media, serum, and lung fluid (Chen,

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2006; Chen, 2005; Huang, 2007; Schellenberger, 2008). Under normal circumstances, most protein, and other macromolecule endocytosis depends on clathrin-mediated endocytosis, where adaptor proteins are required for clathrin pit formation. This type of cellular uptake may also apply when proteins are attached on the surface of particles and are internalized via nanoparticle clathrin-mediated endocytosis. However, uptake of nanoparticles without proteins absorbed onto their surface may be clathrin-independent; uptake of particles of this nature may be regulated via caveolae. Lipid raft mediated pathway involvement is rapid: internalization of negatively charged nanoparticles occur after five-minute incubation times (Schroeder, 2007; Partha, 2008; Villanueva, 2009; Qaddoumi, 2003). However, positively charged particles are reported to internalize into cells through the clathrin-mediated pathway (Qaddoumi, 2003; Chung, 2007). Therefore, the exact relationship of nanoparticle surface charge and clathrin versus caveolae mediated pathways is unknown and needs further study. Figure 1 shows some of the cellular uptake mechanisms involved in nanoparticle exposures.

4 OXIDATIVE STRESS INDICATORS

The presence of excessive reactive oxygen species (ROS) can cause cellular oxidative stress (Figure 2), which may lead to subcellular damage such as DNA, RNA, protein, mitochondrial, and membrane or other lipid degradation, as well as perturbations in normal cell function. Additionally, oxidative stress can be used as an indicator for a variety of metabolic and neurologic diseases, as well as some biological conditions, such as obesity, neoplasia, Alzheimer's and Parkinson's diseases.

4.1 Protein Oxidation

Protein oxidation is the process of oxidative damage to polypeptides and amino acids present in cells. Examples of assays that measure oxidative protein damage are Protein Carbonyl Content (PCC) and 3-Nitrotyrosine. Techniques used to evaluate the oxidant-antioxidant biomarkers are enzyme-linked immunosorbant assays (ELISA), ion

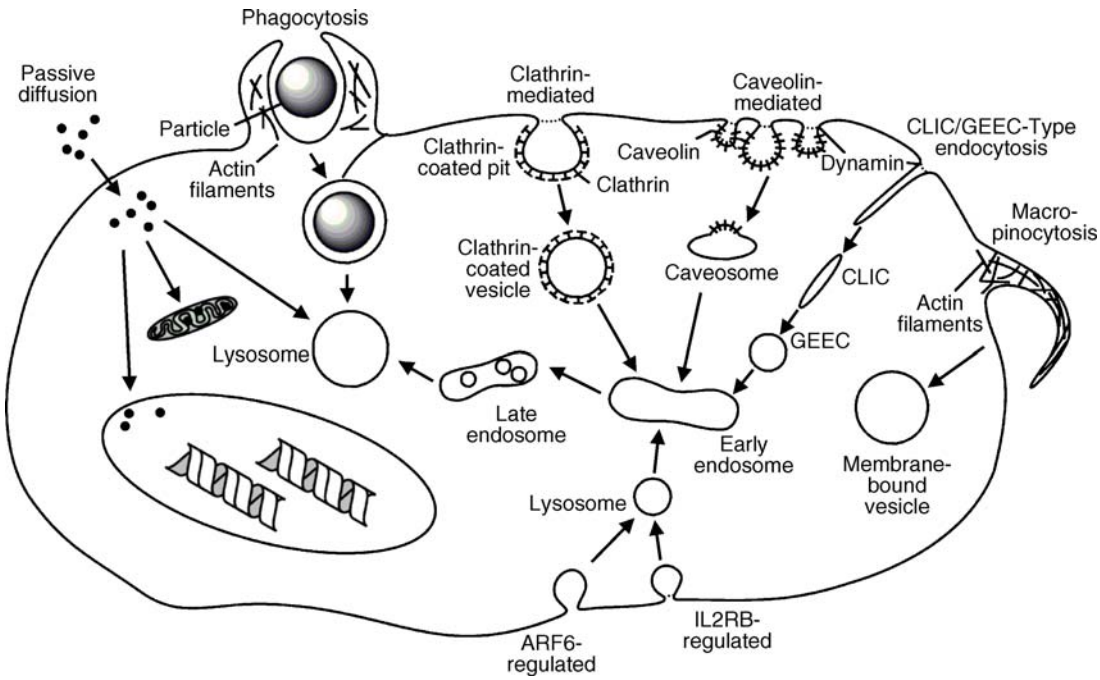

Figure 1. The relationships between nanoparticle surface charge and cellular uptake mechanisms is unknown. Clathrin and caveolae mediated uptake are believed to be involved, but depend on the nanoparticle surface charge (Zhang, 2009; Harush-Frenkel, 2007; Mayor and Pagano, 2007; Gould and Lippincott-Schwartz, 2009; Doherty and McMahon, 2009).

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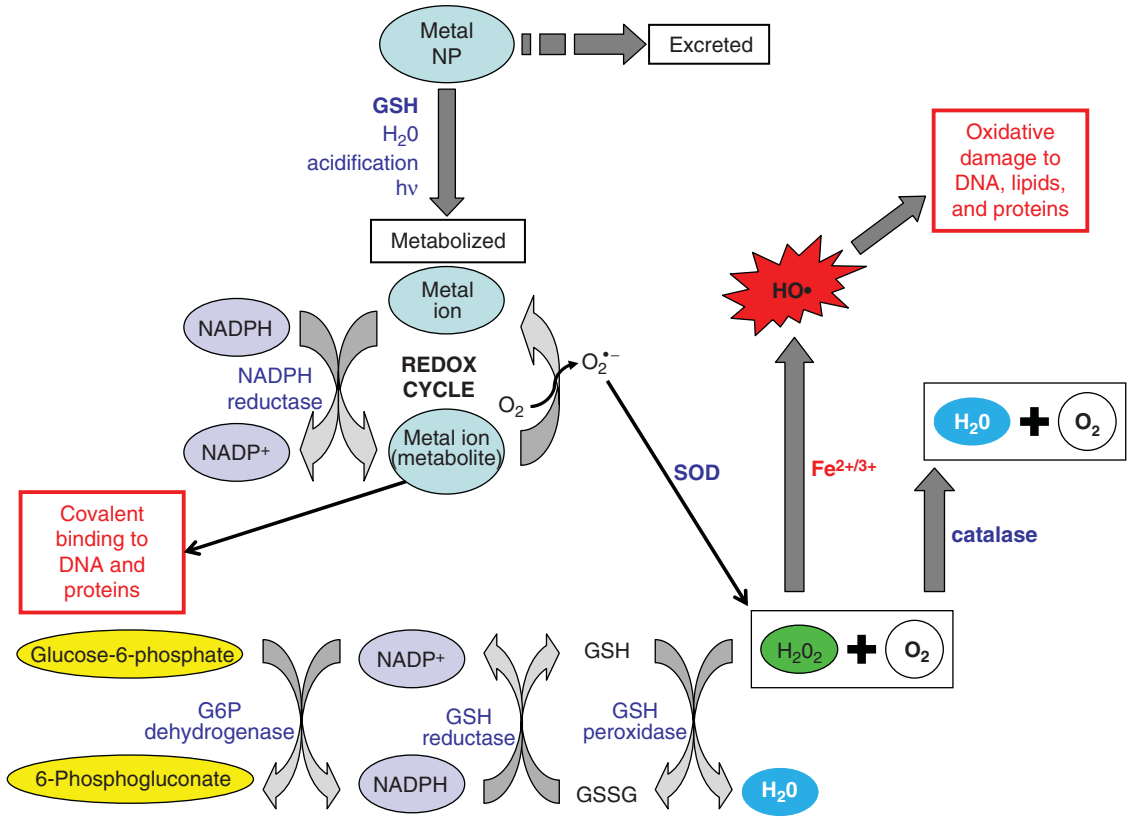

Figure 2. Metal nanoparticle metabolism and oxidative damage schematic. Covalent binding (adduct formation) and oxidative damage, which are outlined in red, are the least desired scenarios arising from metal metabolism. GSH, catalase, and SOD are naturally produced antioxidants present in normal cells. Diagram adapted from *Casarett & Doull's Toxicology – The Basic Science of Poisons*.

exchange chromatography, immunoblotting, and electron paramagnetic resonance imaging.

4.2 Protein Oxidative Damage

Several mechanisms are known that describe how electophiles and pro-oxidants cause protein damage. Alterations in protein function can occur following one or more of these events: (i) formation of covalent bonds between electrophiles and nucleophilic amino acids (e.g., cysteine thiols, lysine, and histidine amines); (ii) oxidation of nucleophilic amino acids (e.g., oxidation of cysteine thiols to disulfides or methione sulfides to sulfoxides); and (iii) production of reactive nucleophiles (e.g., phosphorylation or acetylation of endogenous protein).

4.3 DNA Oxidation

Among the numerous biomarkers for oxidative stress, some are specific for DNA oxidation. Assays capable of measuring DNA oxidative stress or damage include those that measure levels of 8-hydroxyguanosine (8-OHG) and 8-hydroxydeoxyguanosine (8-OHdG) nucleosides (Cattley and Glover, 1993; Hwang and Kim, 2003). Cells, urine, blood, and other tissues can be tested for DNA oxidation. The Comet Assay, which is gaining in popularity due to its ease of use and

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sensitivity, measures general DNA damage via gel electrophoresis of cell lysate, followed by fluorescence signal quantification.

4.4 DNA Oxidative Damage

In nanotoxicology, DNA oxidative stress can be used as an indicator for potential cytotoxicity. Recently, research has shown that different types of DNA damage and repair after oxidative DNA damage can provide insights into mechanisms and modes of action (Cooke, 2003). DNA damage is a result of a break or change in the chemical sequence of DNA in the nucleus of a cell. Each type of DNA lesion has different affects on the cells. For example, some DNA lesions result in mutations and can affect DNA replication and transcription; other types of DNA damage are repairable (Cooke, 2003). DNA oxidation can be detected by measuring the amount of 8-hydroxydeoxyguanosine (8OHdG) found in the cell (Cattley and Glover, 1993; Hwang and Kim, 2003). It is hypothesized that 8-hydroxyguanosine (8-OHG) reacts with ROS produced by the surface of the nanoparticles to produce 8-hydroxydeoxyguanosine (8-OHdG). 8OHdG acts as a mutagenic agent and as a cellular marker.

4.5 Mitochondrial Oxidation

Oxidative stress caused by the accumulation of nanoparticles within the mitochondria, ROS from the nanoparticle surface at the site of the mitochondria, or ROS produced by the mitochondria because of nanoparticle exposure may lead to cell damage or death. In structurally and functionally intact mitochondria, (the primary consumers of cellular oxygen), the production of ROS is balanced by an extensive antioxidant defense system that works to detoxify the oxygen radical generation, resulting in very low net ROS present at any time (Nicholls and Budd, 2000). This protection is provided by glutathione and less abundant antioxidants as well as superoxide dismutase and a host of other enzymes.

4.6 Mitochondrial Oxidative Damage

Nanoparticles of various compositions have been shown to create cellular ROS. ROS production has been found in systems testing diverse materials, such as C_{60} fullerenes, single-walled nanotubes (SWNTs), quantum dots (Qdots), and ultrafine metal oxide particles (UFPs) such as TiO_2. ROS attributed to nanoparticles is most commonly observed when simultaneously exposed to UV light or transition metals (Oberdorster, Oberdorster and Oberdorster, 2005; Li, 2008; Sayes, 2004). Heightened production of ROS occurs when damage to the mitochondria exists; thus, oxidative stress is often detected by measuring mitochondrial function. Additionally, it has been demonstrated that nanoparticles of various size and chemical composition are preferentially transferred to and accumulate in the mitochondria (Li, 2008).

4.7 Lipid Peroxidation and Membrane Oxidative Damage

Lipid peroxidation and membrane damage are used as indicators of oxidative stress and cellular damage. Lipid peroxides are unstable and degrade to molecules such as reactive carbonyl compounds. Polyunsaturated fatty acid peroxides generate malondialdehyde (MDA) and 4-hydroxyalkenals (HAE). MDA and HAE are colorimetric/flourometric indicators for this type of oxidation. Cytoplasmic and other cellular component membranes are easily targeted due to the amount of lipids found within the membrane. Membrane oxidation can disrupt the ion channel flow.

5 REACTIVE OXYGEN SPECIES FLUORESCENCE DYES

Reactive oxygen species (ROS) can lead to cellular oxidative damage if the amount of oxidant present in the cell is greater than the amount of antioxidant in the cell. However, recent research has uncovered novel roles for ROS, such as for biological regulators and messengers in signal transduction systems (Soh, 2006). As a result, the expansion of new, potential roles has necessitated more sophisticated methods to study molecules, which are very difficult to detect due to their evanescent characteristics. Fluorescent probes are a suitable option because they exhibit high sensitivity and microscopic resolution, in addition to being simple to use (Gomes and Fernandes, 2005).

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5.1 Families of Dyes

ROS dyes are categorized according to which type of ROS being probed. In general, there are multiple fluorescence probes for the detection of superoxide radical ($O^{2\bullet-}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hydroxy radical (HO•), or peroxy radical (ROO•), as well as others (Table 46.1). Some of the dyes, such as 2,7-dichlorodihydrofluorescein (DCFH), which was originally thought to only be specific for H_2O_2, are actually oxidized by multiple ROS.

There is a wide range of applications for these dyes within the realm of detecting ROS. For example, some probes are mainly used in an indirect approach to probe for antioxidant capacity, while others probe for oxidation in particular subcellular compartments and membranes (Gomes and Fernandes, 2005). Interestingly, while some probes *produce* a fluorescent compound when induced by a reactant, others, such as scopoletin and dipyridamole, display a *reduction* in fluorescence upon oxidation. Another probe, called C_{11}-BODIPY$^{581/591}$, is known to undergo a red to green transformation when oxidized (Pap, 1999).

Fluorescent probes are used to investigate ROS generated by nanoparticles. Solid data has emerging, which evaluates oxidative damage to cells due to exposure to metal-containing particles (e.g., quantum dots) (Chang, 2009; Park, 2008; Khatchadourian, 2009; Alekseenko, Waseem and Fedorovich, 2008). The ROS are difficult to measure due to their short lifetime, but can be assessed more easily with the aid of a fluorescent probe. The cellular uptake and potential cytotoxicity of mercaptoproprionic acid-coated CdTe QDs with concominant exposure to UV light can be studied using a Dihydroethidium probe that produces a red fluorescent ethidium compound when oxidized by superoxide anion (Chang, 2009; Gomes and Fernandes, 2005). 2,7-dichlorodihydrofluorescein can be used in nanotoxicity studies that investigate ROS production due to TiO_2 exposures in human bronchial epithelial cells (Park, 2008). The diacetate form of the parent 2,7-dichlorodihydrofluorescein compound, traverses the cellular membrane where it is hydrolyzed to 2,7-dichlorodihydrofluorescein by esterases, then oxidized by several possible ROS to DCF, which is fluorescent (ex/em: 498/522) (Gomes and Fernandes, 2005).

Fluorescent probes are also utilized to measure antioxidant capabilities of compounds. BOPIDY-α-tocopherol, a broad-range ROS probe, has been used to measure increases in cellular antioxidant presence

Table 1. Examples of fluorescent ROS dyes.

Fluorescent Probe	ROS Sensitivity	Ex/Em (nm)	Nano-based Applications
DHE (Dihydroethidium)	$O2^{\bullet-}$	520/610	Probe for UV-induced ROS in quantum dot treated pancreatic cells (Chang, 2009)
B-TOH (BOPIDY-α-tocopherol)	ROO•	514/565	Probe for increased antioxidant induced by CdTe nanoparticles on lipid membrane (Khatchadourian, 2009)
	RO•		
	HO•		
DCFDA (2,7-dichlorodihydrofluorescein)	H_2O_2	498/522	Probe for ROS in rat brain synaptosomes caused by Fe3+ nanoparticles (Alekseenko, Waseem and Fedorovich, 2008)
	ROO•	ROS caused by TiO_2 in human bronchial epithelial cells (Park, 2008)	
	HO•		
CM-H2DCFDA (chlormethyl-dihydro derivative of fluorescein)	H_2O_2	485/538	Probe for reduction of ROS due to antioxidant action of soluble fullerene vescicles in human umbilical epithelial cells (Maeda, 2008)
	ROO•		
	HO•		
HPF (Hydroxyphenyl fluorescein)	HO•	485/535	Probe for reduction of ROS due to antioxidant action of soluble fullerene vescicles in human umbilical epithelial cells (Maeda, 2008)

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due to nanoparticle exposures (Khatchadourian, 2009). CM-H2DCFDA (a chlormethyl-dihydro derivative of fluorescein) and HPF (hydroxyphenyl fluorescein) have been shown to probe for reduction in ROS after addition of water-soluble fullerene-treated human umbilical epithelial cells (Maeda, 2008).

5.2 Confocal Microscopy as a Tool to Probe Stress

Oxidative stress can potentially lead to cell death; therefore, a viable means of detecting oxidative stress can be of significant importance. Oxidative stress can induce a dose-related decrease in the level of fluorescence, which can be detected and monitored by fluorescence or confocal microscopy. In confocal microscopy, fluorescent probes are used to stain particular cellular structures, which illuminates only the areas of interest. The fluorescent probes dihydrorhodamine 123, dihydroethidium, 2′,7′ dihydro-dichlorofluorescein (H_2DCF), and dihydro-dichlorofluorescen diacetate (H_2DCFDA) are some of the most commonly used detectors of reactive oxygen species (ROS) for use in flow cytometric assays, spectrofluorometric assays, and confocal microscopy (Uggeri, 2004).

One of the advantages of confocal microscopy as a tool for detecting oxidative stress is the ability to image a particular set of cells over a time-lapse. In studies where ROS are induced by H_2O_2 treatment, the increase in ROS can be measured over time. Analysis of the early stages of the apoptotic phase is also possible using confocal microscopy, which in general is a sensitive technique that can detect low concentrations of H_2O_2-induced oxidative stress (Liegibel, 2000). In addition to 2D imaging, 3D images can be taken by scanning sequential sections of the sample to produce a more complete image of the cell or cell structure.

6 NORMAL REDOX AND STEADY STATE IN A 24 H PERIOD

Periodic oscillations in metabolic redox cycle reactions represent fundamental mechanisms linking oxidative processes to cell cycle regulation. Redox status, a term used to describe the relative amounts of an oxidizable and reduceable redox couple (i.e., GSH/GSSG), governs differential transition through the cell cycle by maintaining a balance between normal, cellularly produced ROS and the enzymatic removal of ROS by various antioxidants. Although higher levels of ROS can be toxic to the cell, researchers are beginning to understand the necessary presence of reduced concentration/duration ROS in the cell as biological regulators, the key principle of the "threshold concept" (Menon and Goswami, 2007). Some nanomaterials may be considered redox-active, which simply implies that they efficiently accept or donate electrons, leading to ROS production and oxidative stress (Sayes, 2004; Lovric, 2005). However, the ROS can be produced either exogenously or endogenously, resulting in an imbalance in the cellular redox state (Przybytkowski, 2009).

At no point does thermodynamic equilibrium (absence of net electron flow) exist within a cell until it dies. Many reactions within the cell are maintained at a nonequilibrium steady state, where the ratios of redox couples remain constant for brief periods. In other words, the concentration of products equals the concentration of reactants. However, it must be noted that these reactions may be dramatically altered with changes in pH. During oxidation-reduction exchange reactions, redox couples become redistributed in a quantitative, ratiometric fashion, which provides a convenient endpoint to determine cellular oxidative stress (Figure 3) (Aw, 2003).

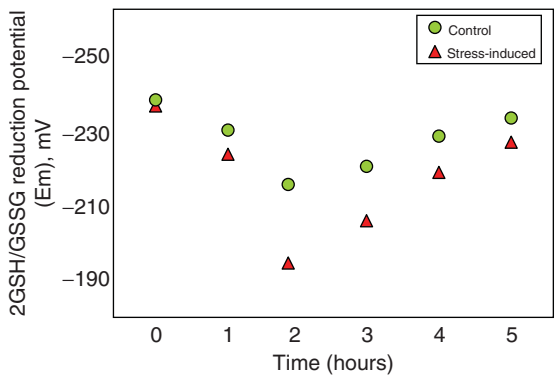

Figure 3. The graph pictured above represents the effects of ROS-induced cells. Oxidative stress-induced cells exhibit a different reduction potential of conjugated glutathione, as compared to unexposed control cells. Glutathione (GSH) is a dynamic, intracellular antioxidant. Over time, the oxidized form of glutathione (GSSG) becomes reduced (or recycled) back to GSH, the functional form responsible for maintaining optimal ROS levels intracellularly.

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Estimating the redox environment intracellularly can be accomplished by measuring the ratio of reduced and oxidized glutathione (GSH and GSSG) concentrations using fluorescent or luminescent probes (Østergaard, 2001; Meyer, May and Fricker, 2001; Ridnour, 1999). Many kits are currently available from laboratory suppliers which measure GSH:GSSG levels in cells and/or tissues. These concentrations can then be applied to the Nernst equation to calculate the cellular reduction potential (E_h) (Hancock, 2004).

$$E_h = E_m - \frac{RT}{nF} 2.303 \log \frac{[\mathrm{GSH}]}{[\mathrm{GSSG}]}$$

Where R is the gas constant ($8.314\,\mathrm{J\,K^{-1}\,mol^{-1}}$), T is the temperature in Kelvin, n is the number of electron donors and acceptors, and F is the Faraday constant ($9.6485 \times 104\,\mathrm{Cmol^{-1}}$). E_m is the electrical potential where the concentration of the GSH and GSSG are equal.

7 ANTIOXIDANTS

In mitochondria, reactive oxygen species (ROS) are produced constantly as side products of the electron transport chain. These high-energy radicals would represent a major threat to cellular viability if no mechanisms existed for neutralizing their reactivity. In fact, many biological structures are capable of mediating the destructive capability of ROS. Endogenous to the human body, glutathione, melatonin, and associated enzymes function primarily in the reduction of ROS. Vitamins C and E also fill similar roles in human health (Meister, 1994). However, many antioxidants have been identified in a diversity of living species. Some examples can be found in extracts of green tea (EGCG) and grape seeds (gallic acid).

These natural antioxidants reduce the likelihood of damage by ROS because they are more easily oxidized than cellular structures essential for continued life. This means that electron transfers occur between an oxidant with a high reduction potential and an antioxidant with a much lower reduction potential. For an antioxidant to be useful, its reduction potential must be much lower than that of another present molecule with reducing power. That is, molecules with a smaller reduction potential will react more quickly and favorably with oxidants and effectively discharge their destructive capabilities (Madej and Wardman, 2007).

A fascinating area of the recent literature is the discovery of iron-chelating antioxidants. Through a process called the Fenton reaction, Fe(II) can react with hydrogen peroxide to form the dangerously reactive hydroxyl radical. It is hypothesized that this damaging reaction is at least partially responsible for the deterioration caused in patients with Alzheimer's and Parkinson's diseases (Turnbull, 2003). It has been found that molecules with a hydroxamate functional group can effectively bind free reactive iron. In one study, two such groups were added to a well known neuroprotective peptide called NAP (Blat, 2008). Their results demonstrated that this novel agent was useful in controlling both hydroxyl radical formation and lipid peroxidation.

8 CONCLUSIONS

A systems biology approach in nanotoxicological research is vital to the understanding of nanoparticle-induced modes of action and development of mechanistic models. Using quantitative properties of dynamic systems, such as production of cell-to-cell signaling molecules over time, mathematical models may be able to be developed. Due to the large number of parameters, variables, and constraints in cellular networks, numerical, and computational techniques are often used in biology. The same computational and mathematical concepts can be applied to nanotoxicology. Other tools in systems biology can also be applied to nanotoxicology. These include the integration of information from the literature, the development of online databases and repositories for sharing data and models and using information-extraction and text-mining techniques. Systems biology and nanotoxicology are both fields that study the relationships between components of a biological system (metabolic pathways, organelles, cells, and organisms) and the whole organism in an effort to understand how biological systems function under control versus variable conditions.

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Health Effects of Nanoparticles

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1 INTRODUCTION

Nanomaterials gain diversified utilizations in every field of life but their health and environmental effects still need thorough understanding. Increasing industrial and consumer product utilizations result in the production of enormous quantities of these materials every year. According to Woodrow Wilson Centre's Project on Emerging Technologies (PEN) on line database, as of 25 August 2009, 1015 consumer products containing nanomaterials have been reported. Apart from the environmental, occupational and consumer product exposures, nanomaterials gain increasing utilizations in the nanomedicine. These utilizations range from molecular diagnostics and imaging to drug delivery and gene therapy (Pison *et al*., 2006). For instance, quantum dots are semiconductors promising for computing and the quantum confinement renders these nanoparticles (NPs) highly fluorescent for cellular imaging applications. The super-paramagnetism of iron oxide NPs is useful for magnetic resonance imaging (MRI) or magnetic hypothermia. Local surface plasmon resonance of gold NPs could be used for chemical and biochemical sensing as adsorption of molecules induce a shift in the local index of reflection.

Nanoscale materials exhibit unique surface and quantum phenomena not observed at the microscale. For instance, materials, such as gold or platinum that are chemically inert at normal scales, can serve as potent chemical catalysts at nanoscales. Size reduction is associated with the appearance of specific characteristic properties, such as surface area, surface reactivity, aspect ratio, and surface chemistry (lipophilicity and charge). Such properties makes that nanomaterials are highly susceptible to be reactive toward biological systems. Occupational and environmental medicine has already experienced many situations in which exposure to particles, mainly respirable dusts [asbestos, diesel exhaust particles (DEP), quartz, coal, etc.] are responsible for diseases. Epidemiological studies and experimental studies in animal models have demonstrated the hazardous effects of these particles. With this background, it is well founded to consider that the large and diverse group of engineered NPs represent a hazard that potential health impacts have to be assessed.

A lack of adequate epidemiological and experimental data about the health effects of the nanomaterials has lead to hype both in favor and against these materials. For the moment, the short-term effects after acute dose exposures are the most investigated but still insufficiently characterized effects whereas long-term effects after chronic expositions are still unknown.

Cellular mechanisms behind NP-induced effects have been attributed to abiotic and biotic oxidative stress induction potentials of these materials. In this study, we represent a systemic overview of the current knowledge on the exposure, secondary organ translocation and potential health effects of the NPs. Moreover, potential mechanisms of NP-induced

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cellular effects and role of physico-chemical characteristics are elaborated. A growing body of literature reinforces the point of view of rigorous research in the field of nanomaterial-induced health effects.

2 ROUTES OF EXPOSURE TO NANOPARTICLES AND THEIR FATE IN THE BODY

Currently, no data are available on NP concentrations in the environment because of their diversified and unrestricted sources. In occupational settings, such concentrations reach very high levels depending upon the quantity of NP-produced and exposure duration. Major systems of the body that are constantly in contact with the environment (such as the respiratory tract, the gastrointestinal (GI) tract and the skin) are more prone to encounter NPs. In case of NPs, the ability of these systems to provide an efficient protective barrier against exogenous agents seems to be compromised as a result of unique physico-chemical properties of NPs allowing their systemic distribution in the body.

2.1 Major Exposure Scenarios

2.1.1 Inhalation

The respiratory tract is one of the prime targets of NP exposure because it is the route of entry of inhaled particles and it receives entire cardiac output leading to possibilities of exposure to systemically administered NPs.

The respiratory tract is composed of the conducting airways and terminal alveoli. The conducting airways extend from exterior to the bronchia and are involved in the conduction and the clearance of inhaled air till alveoli. The terminal alveoli are involved in the gas exchanges and exhibit a surface area of around 140 m^2. The alveoli are delimited by a very thin epithelium producing a thin layer of surfactant. The air–blood distance is around 2 μm.

After inhalation, particles can be deposited in the different parts of the respiratory tract according to their size. Because of their small size, NPs are submitted to diffusion forces. Predictive models, established from the data of the International Radiological Protection Commission, are used to estimate the deposition of particles (Witschger and Fabries, 2005). 5-nm NPs are uniformly deposited in the three part of the respiratory tract whereas those below 5 nm are mainly deposited in the upper airways and those higher than 5 nm are mainly deposited in the alveoli. In the context of respiratory diseases where air fluxes are modified, it is expected that the pulmonary retention of NPs increased.

Inhaled particles can be cleared from the respiratory tract by two different ways: the mucociliary clearance and the alveolar clearance (Figure 1). The mucociliary clearance is achieved by the mucociliary epithelium covering the upper and lower airways. The ciliary beating allows the displacement of mucus entrapping particles produced by secretory cells. This mucus is either expectorated or ingested. Functional ciliated cells and a good mucus rheology are the two essential conditions for an efficient mucociliary clearance. The alveolar clearance is achieved by alveolar macrophages that are present in the lumen of alveoli and are professional phagocytes. However there are some evidences that their ability to endocytose NPs is less efficient as compared to larger particles (Geiser and Kreyling, 2010). Thus NPs reside longer in the alveoli and can get in contact with type II cells (involved in the production of the surfactant lining the respiratory epithelium) or with type I cells (cells constituting 90% of the alveolar epithelium surface area).

2.1.2 Dermal Contact

Skin can be exposed to NP because of their presence in cosmetics (sunscreens containing TiO_2 and ZnO NP) for better light-reflecting properties as well as in clothes to confer anti-bacterial resistance (use of silver NPs). Human skin has a surface area of nearly 1.5–2 m^2 and is composed of two main layers: the epidermis and the dermis (Figure 1). The epidermis is the most superficial layer of the skin and consequently the first line of contact with environmental injuries. It is a pluristratified epithelium mainly composed of keratinocytes that is constantly renewed from basal cells present in the deeper layer of the epidermis. The outer layer of the skin called the stratum corneum is composed of dead cells having intercellular cement involved in the protection properties of the skin. No blood vessels are present in this layer. The dermis is separated from the epidermis by the basal membrane and is 10- to 40-fold thicker than the epidermis. It is a conjunctive

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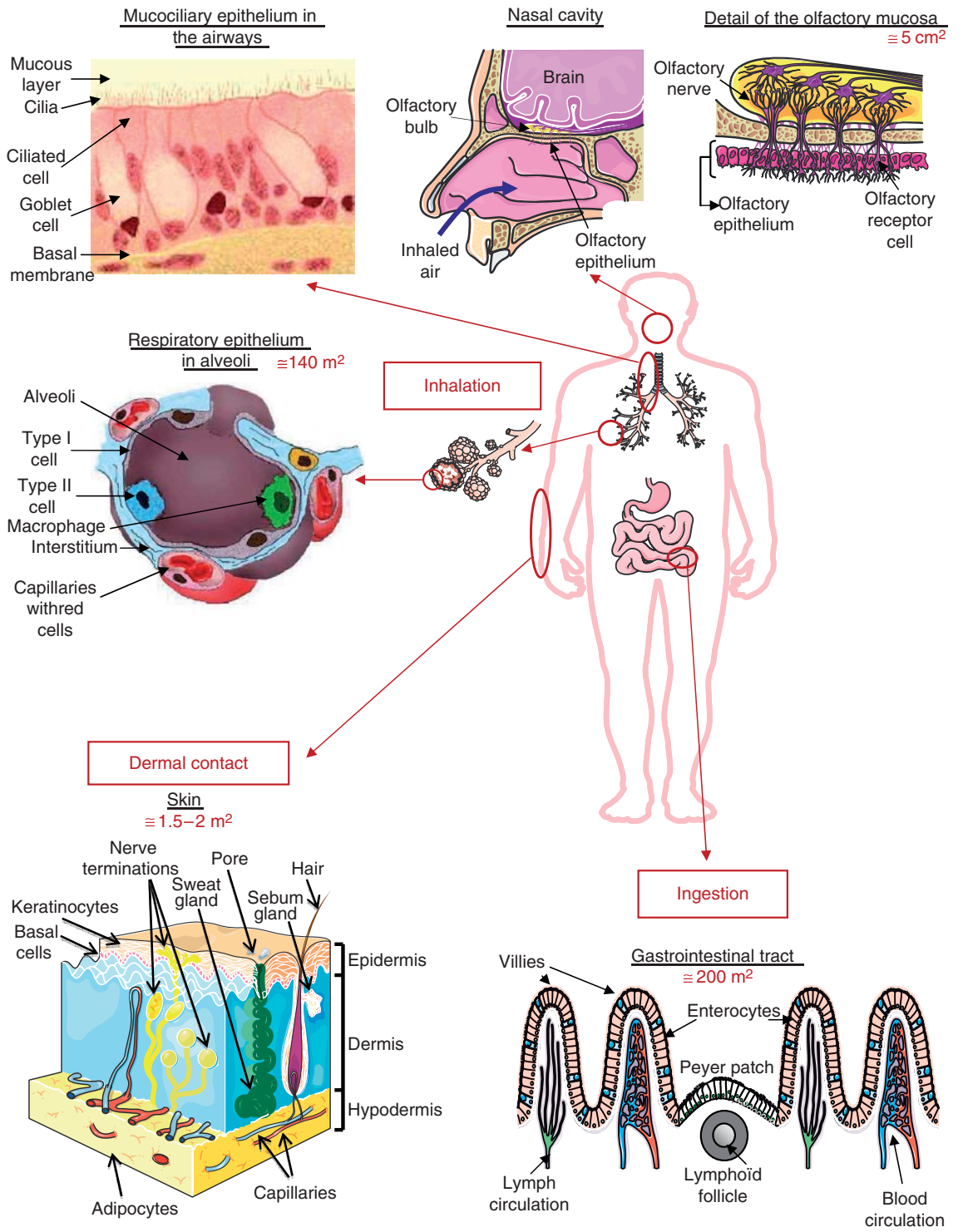

Figure 1. The different routes of exposure to NP (inhalation, dermal contact and ingestion) and their sites of interaction with tracheobronchial, alveolar, olfactory, epidermal and intestinal epithelium (drawn in part from Servier medical art).

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tissue mainly composed of fibroblasts secreting an abundant extracellular matrix. It contains the skin structures, such as sweat glands, hair follicles, nerve terminations and blood and lymphatic vessels. For a systemic passage, NPs have to cross the epidermis and the basal membrane.

2.1.3 Ingestion

The presence of NPs in food caused by either contamination or intentional introduction in transformed food can lead to an exposure of the GI tract which could also act as an additional portal of entry of NP in the body (Figure 1). The gut with its surface area of nearly 240 m^2 greatly contributes to the total surface of the GI tract. It is covered by a monostratified epithelium composed of secretory cells producing mucus and enterocytes that exhibit at their luminal face numerous microvillosities in order to increase the exchange surface and favor the absorption of nutrients. In addition, lymphoid tissue called Peyer patches (involved in local immunity) is dispersed in the intestinal mucosa. These are composed of M cells that have high phagocytosis capacities.

2.2 Translocation and Biodistribution of Nanoparticles

The respiratory and intestinal epitheliums constitute efficient physical barriers to the penetration of micro-organisms and particles caused by the presence of tight junctions between cells. A paracellular entry (between cells) of NPs is unlikely except in case of either pathological situations or exposure to toxic substances affecting the epithelium permeability. For NPs to cross biological barrier, a transcellular passage involving a mechanism called endocytosis is necessary.

2.2.1 Cellular Uptake of Nanoparticles

There are two modes of endocytosis: the phagocytosis that concerns the uptake of particles of large size and pinocytosis that ensures the uptake of fluids and solutes (Conner and Schmid, 2003). Four different pinocytosis mechanisms can be distinguished: macropinocytosis, clathrin-dependent endocytosis, caveola-dependent endocytosis and clathrin-caveola-independent endocytosis leading to the formation of endosomes of different sizes (0.5–5 μm, 100–150 nm, and 70 nm, respectively).

The phagocytosis is performed by professional phagocytes, such as macrophages, monocytes and polynuclear neutrophiles in order to protect the organism from pathogen invasion. Phagocytic materials are internalized by a process involving actin filaments in large phagosomes (0.1–10 μm) that fuse with lysosomes in order to destroy the pathogen owing to the co-ordinated action of oxidants, proteases and hydrolases in an acidic environment. According to the form or the size of the particle, cell is sometimes unable to engulf totally the particles inducing a frustrated phagocytosis prone to initiate an inflammatory reaction. It has been observed in mice exposed to carbon nanotubes (Poland *et al*., 2008). Numerous studies have also shown that alveolar macrophages are less efficient to endocyte NPs by comparison with larger particles (Geiser *et al*., 2008) leading to the persistence of NPs in the alveoli and their deposition on the alveolar epithelium (Oberdörster, Oberdörster and Oberdörster, 2005).

Several endocytosis pathways involve membrane receptors. The coating of NPs with ligands present in biological fluids could favor their recognition by the membrane receptor and eventual internalization.

Following endocytosis, the internalized material is present in a vesicle separated from the cytosol by a membrane. However NPs have been observed free in the cytosol and even in mitochondria and the nucleus where they could directly interact with macromolecules. Rothen-Rutishauser *et al*. (2007) have observed that after an inhalation exposure of rats to low concentrations of TiO_2, NPs not surrounded by a membrane are found in lung epithelial and endothelial cells, in the conjunctive tissue and even in red cells suggesting that NPs could enter cells by a mechanism different from the classical ones (Rothen-Rutishauser *et al*., 2007). They propose that adhesive interactions could allow the passive diffusion through the plasma membrane caused by the transient formation of pores.

Until now, numerous studies have shown a rapid uptake of diverse NPs in different cell types even not specialized in phagocytosis. However, the involved mechanisms have not been illustrated in detail. In addition, most of these studies have been performed on *in vitro* cell cultures. The major limitation of such systems is the possibilities of interaction between NPs and culture medium leading to adsorption of specific proteins on NPs and thus influencing the receptor-mediated uptake.

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2.2.2 *Translocation of Nanoparticles*

There is growing evidence that NPs can cross the epithelial barriers getting access to the blood stream and thus allowing their distribution in the whole body.

Air–Blood Translocation

In the alveoli, distance between air and blood is very thin (2 μm) being a favorable route of entry into the systemic circulation (Figure 1). Numerous studies have been performed on humans as well as on animals to estimate the translocation of inhaled particles through the air–blood barrier. According to studies, the extent of observed translocation differs greatly and could be explained by the differences in NP characteristics and route of administration (inhalation vs. instillation).

When rats were made to inhale for 1 h radiolabeled iridium NPs (15–80 nm), only 1% of the radioactivity is recovered 7 days later in secondary organs, such as liver, spleen, heart and brain (Kreyling *et al*., 2002). NPs are mainly present in feces caused by their lung clearance and ingestion. The follow-up over 6 months revealed that NPs are initially trapped in the interstitium and can come back to the alveolar lumen to be cleared by macrophages (Semmler-Behnke *et al*., 2007). In contrast, a size dependence was observed for intratracheally instilled TiO_2 NPs between 12 and 240 nm NPs (50 and 4% translocation respectively; Oberdörster, Oberdörster and Oberdörster, 2005). Using the same route of administration, Zhu *et al*. (2009) have shown that 22-nm iron oxide NP ($^{59}Fe_2O_3$) are quickly translocated to rat blood (10 nm) and distributed in the liver, spleen, kidney and testis. They estimated a plasma half-life of 22.8 days (Zhu *et al*., 2009). Another study comparing 1.4 and 18 nm gold NPs has revealed a significant translocation of 1.4 nm NPs whereas those of 18 nm are retained by the lung (Semmler-Behnke *et al*., 2008). After intratracheal instillation of colloidal gold (20 nm) in mice, NPs are observed in the interstitium between alveolar cells and endothelial cells as well as over endothelial cells in blood vessels. However, little quantities of NPs were found in the blood stream (Furuyama *et al*., 2009). At the same time, it has also been observed that NP-loaded alveolar macrophages migrate towards the blood circulation and extrapulmonary organs suggesting that the NP translocation could be either direct or indirect via macrophages.

The administration of carbon NPs coupled with Technecium to humans has given contradictory results that are still a matter of debates (Mills *et al*., 2006; Nemmar *et al*., 2002).

In vitro studies performed using alveolar epithelium have highlighted the role of the NP physico-chemical characteristics in their ability to cross the epithelial barrier. The translocation through the alveolar barrier is more important for polystyrene NPs of little diameter (20 vs. 100 nm) as well as for positively charged NPs (Yacobi *et al*., 2008). In addition, it does not occur at 4 °C suggesting that it is an energy-dependent mechanism.

To summarize, these studies revealed that the translocation of NPs through the air–blood barrier is possible but it is more or less significant depending on the physico-chemical characteristics of NPs. However, these studies have been performed after a sole exposure suggesting that a more significant translocation is likely to occur in case of chronic exposure. Pathological situations could also increase the translocation. For example, a more pronounced translocation was observed in rats after intratracheal instillation of polystyrene NPs (56 and 202 nm) when rats were pre-treated with lipopolysaccharide in order to induce an inflammation (Chen *et al*., 2006). Similarly, in rat-perfused lungs, the exposure to iridium NPs results in their translocation in the perfusate only if lungs are simultaneously treated with hydrogen peroxide to mimic the oxidative stress occurring during inflammation or in the presence of histamine that is used to increase the vascular permeability (Meiring *et al*., 2005).

Neuronal Translocation

The monodisperse NPs show an efficient predicted deposition in the nose. The olfactory epithelium present in the nose is a potential route of entry of NPs to the central nervous system (Figure 1) and is investigated as an efficient route for drug delivery (Mistry, Stolnik and Illum, 2009). Translocation of 50 nm gold NPs (in monkey), 35 nm carbon NPs (in rats) and 30 nm manganese oxide NPs (in rats) toward the olfactory bulb through the axon olfactory nerve has been reported (Oberdörster, Oberdörster and Oberdörster, 2005). Particularly, experiments performed with MnO have shown that 11% of the deposited NPs can be recovered in the olfactory bulb and some of them exhibit a more distal location in

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the brain (Elder *et al*., 2006). These observations were further associated with inflammatory effects. Another study with TiO_2 (80 and 155 nm) in mice have confirmed the access of these NPs to the brain via the olfactory bulb especially in the hippocampus (Wang *et al*., 2008b).

It has to be kept in mind that these experiments have been performed on rodents that have a well-developed olfactory mucosa (8 cm^2 in rats corresponding to 50% of the nasal mucosa) compared with humans (5 cm^2 corresponding to 5% of the nasal mucosa) and are only nasal breathers. This finding limits the scope of such studies for human extrapolation.

The access of NPs to the central nervous system can also occur from the passage through the blood–brain barrier. This barrier has very efficient junctions between endothelial cells in order to avoid the penetration of particles by the paracellular route. Experiments performed by intravenous or intraperitoneal injections of gold NP (40 nm) have not shown the presence of these NPs in the brain (Sadauskas *et al*., 2007). Currently, active research is underway to establish nanocarriers for drug delivery to brain. It has been observed that binding of serum apolipoproteins to NPs favors their uptake by low-density lipoprotein receptor present on the endothelial cells membrane (Kim *et al*., 2007).

Dermal Translocation

The transcutaneous penetration of NP has been particularly studied for TiO_2 and ZnO as they are components of many sunscreens formulations. Several studies have shown that following repeated exposure of normal human skin to TiO_2, these NPs either stay on the epidermis or are present in the strateum corneum but never penetrate or cross the viable layer of the epidermis. NP can accumulate and persist in hair follicles but until now there is no evidence of a passage towards the dermis by this route.

Quantum dots of different sizes (14–45 nm), forms (spheres, ellipsoid shape) and charges are efficiently adsorbed by normal pig skin maintained in flow-through diffusion cells. But even after 8-h exposure, no translocation of quantum dots is observed in the perfusate suggesting that quantum dots cannot gain access to the blood stream (Ryman-Rasmussen, Riviere and Monteiro-Riviere, 2006). By contrast, using rat skin maintained *in vitro*, it was shown that gold NPs (15, 102, and 198 nm) permeate through the skin in a size-dependent manner (Sonavane *et al*., 2008).

Whereas the healthy skin seems to be an efficient barrier towards NPs, other conditions such as damaged skin (wounds, erythema, eczema, etc.) and flexures sites are more susceptible to NPs translocation. *In vitro* experiments with human skin submitted to mechanical flexions (20 flexions of 45° per min) have shown that in 50% of skin samples flexions favor a low epidermal and dermal absorption of fluorescent NPs (0.5 and 1 μm) after a 60-min exposure whereas particles of larger size (2 and 4 μm) stayed localized on the strateum corneum (Tinkle *et al*., 2003). The abrasion of rat skin allows quantum dots to reach the dermis (Zhang and Monteiro-Riviere, 2008) as well as UV radiation exposure in an *in vivo* mouse model but the penetration is very limited (Mortensen *et al*., 2008).

2.3 Fate of Nanoparticles

NPs present in the blood can be cleared from the body via urine after filtration in the kidney or via feces after their transfer to the bile in the liver.

The filtration through the renal glomerula occurs for particles having a diameter below 5.5 nm. Those between 6 and 8 nm can also be filtered whether there are neutral or positively charged. Those over 8 nm stay in the blood and accumulate in the reticuloendothelial system (RES). The RES is particularly developed in the liver and is represented by Kupffer cells (macrophages with high phagocytosis potential). If they are unable to degrade the engulfed NPs, NPs persist in them. In the liver, hepatocytes are also involved in the endocytosis of particles and their metabolism and clearance by bile. Until now, no studies have considered the metabolism of NPs that is unlikely for metallic NPs but could intervene for functionalized NP.

The fate of NP has been mainly investigated in studies using an intravenous administration. Although it is not a relevant route of exposure considering environmental and occupational exposures, they allow improved knowledge on the behavior and fate of NP in the body.

Several studies have been performed with quantum dots that have shown that according to the size and coatings they are either cleared in urine or taken up by RES and retained in the body (Chen *et al*., 2008a; Yang *et al*., 2007). Another study

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has shown that water-soluble, single-walled carbon nanotubes (SWCNT) functionalized with diethylentriaminepentaacetic are cleared from systemic blood circulation through the renal excretion route as intact nanotubes (Singh *et al*., 2006). Still another study with rats demonstrated that TiO_2 NPs accumulate in liver and spleen over 28 days (Fabian *et al*., 2008). According to their size, gold NPs exhibit a different biodistribution in mice and rats. The smallest one (10–15 nm) show a large distribution with an important accumulation in liver followed by the lungs, the kidneys and the spleen and the observation of NP in the brain (Sonavane, Tomoda and Makino, 2008). As the size increase, less organs are concerned but the highest accumulation is still in the liver (De Jong *et al*., 2008).

3 NANOPARTICLE PROPERTIES INVOLVED IN THEIR TOXICITY

A bulk material should have constant physical properties regardless of its size, but nanomaterials have nanostructure-dependent properties that often differ from their bulk counterpart and are used for chemical and medical applications. Indeed, the physical behavior change below 100 nm approaching quantum physics that could lead to new chemical, mechanical, electrical, optical or superparamagnetic properties. Nanomaterials could also be designed to take advantage of these physico-chemical properties to obtain new specific biological activities for medical application in diagnostic and treatment. Size-dependent properties are observed such as quantum confinement in semiconductor particles, local surface plasmon resonance in some metal particles and superparamagnetism in magnetic materials. These are caused by the nanoscale substructure, electronic configuration, size and large surface to volume ratio compared with bulk material. These size-related properties let to the use of NP in various applications.

Much of the fascination about nanotechnology is caused by unique quantum and surface phenomena exhibited at the nanoscale. This also implements, however, that the biological activity of nanomaterials could not directly be predicted by extrapolating the properties of the constituent chemicals and compounds and nanomaterials could have unexpected effects in biological systems. Nanotoxicology is a new field which arose from this need to evaluate the safety of nanomaterials and to develop assays for risk assessment of engineered NPs. The questioning about the toxicity of NPs has originated from knowledge about the health effects of atmospheric particulate matter (PM) which have shown that ultrafine particles (PM $< 0.1\ \mu m$) induce greater biological responses than fine (PM < 2.5) or coarse particles (PM $< 10\ \mu m$) (Ramgolam *et al*., 2009). However, these differences could not solely be attributed to one parameter such as the greater surface area of ultrafine PM but a variety of factors has to be considered.

The biological activities are determined by a large diversity of factors linked to the various physico-chemical characteristics of the nanomaterials: size, surface area (taking into account the porosity and roughness of the particle), shape, bulk chemical composition (including the crystal structure), surface chemistry (including lipophilicity as well as surface charge or coatings) and surface reactivity which is linked to the two latest (surface area and surface chemistry) (Figure 2). Thus, the biological activities induced may differ because of the variety of nanomaterials but some effects may be nanoscale-specific and thus apply to all nanomaterials. Despite the intrinsic characteristics of nanomaterials, the final biological outcome also depends upon their fate inside the body. Indeed, the high energetic adhesive forces at the surface of NP could allow the interaction with other particles leading to agglomeration and aggregation but also with molecules leading to surface modifications influencing the biological response (Figure 2). The biological effects

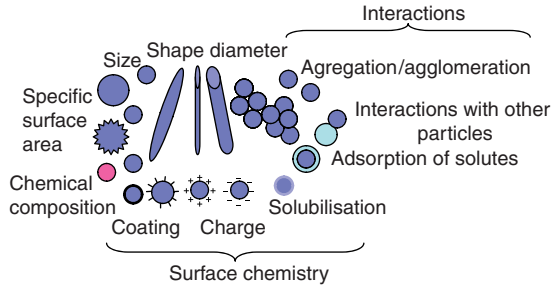

Figure 2. Different physico-chemical characteristics of the nanomaterials involved in their biological activity: size, surface area (taking into account the porosity and roughness of the particle), shape, bulk chemical composition, surface chemistry (including solubility as well as surface charge or coatings) and interactions between particles leading to agglomeration and aggregation as well as with molecules.

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could also be enhanced as a result of biopersistance of the material.

3.1 Size

One of the main differences between NPs and larger-sized particles is their potential to cross biological barriers. The interstitial uptake after *in vivo* inhalation exposure to polystyrene NP is higher than for micrometer-sized particles (Oberdörster, Oberdörster and Oberdörster, 2005). This is caused by higher deposition rates of NPs and their deeper penetration into the lungs. Even for particles within the nanometer range the efficiency of translocation further increases with decreasing size as shown for 18 and 1.4 nm-sized gold NPs (Semmler-Behnke *et al*., 2008). This capacity of NPs to cross biological barriers is actually studied to overcome the challenging problem of brain drug delivery caused by the difficulty to cross the blood–brain barrier (for review Agarwal *et al*., 2009). The size determines not only the uptake of nanomaterials but also the interaction with proteins. Indeed the extent of conformational changes of proteins adsorbed onto NPs depends upon the particle size probably caused by changes in the surface curvature (Lundqvist, Sethson and Jonsson, 2004; Vertegel, Siegel and Dordick, 2004).

3.2 Surface Area

With decreasing particle size, the percentage of surface molecules increases exponentially compared with total molecules of the particle when particles are smaller than 100 nm. This implements higher surface reactivity of the particles and potential chemical as well as biological activities as the number of atoms at the surface is increasing. Higher surface to volume ratios could have desirable effects used in medicine to develop carriers for drug delivery and in industry for catalysts or fillers to reduce weight but also potential toxic outcomes. Comparisons of cellular effects induced by carbon black (CB) or TiO_2 NPs of different specific surface areas have shown that oxidative stress and pro-inflammatory responses are correlated to particle surface areas (Hussain *et al*., 2009). This strong relation of biological response to surface area was also observed for *in vivo* lung inflammation induced by carbonaceous NP (Stoeger *et al*., 2006). It is important to note that the specific surface area [Brunauer Emett Teller (BET) surface] should be considered as it takes into account the porosity and roughness of the particles in contrast to the geometric surface. The role of surface area is also evident considering its importance to allow surface chemistry to take place leading to aging, oxidations or hydrations that could reduce or increase biological responses.

3.3 Shape

The influence of shape on the toxicological outcome of nanomaterials has been put forward by the research on asbestos which led to the three-dimensional model of its carcinogenic risk: dose (total amount inhaled over time), durability (biopersistance in the lung) and dimension (length, width and aspect ratio of the fiber). Indeed, long fibers could not enter the lung and short fibers ($<5\,\mu m$) are cleared effectively by alveolar macrophages whereas intermediate fibers could not be completely taken up by the phagocytes leading to frustrated phagocytosis, biopersistance and inflammation. Some nanomaterials have been shown to induce the same responses than asbestos: exposures of rats to high doses of crocidolite and multi-walled carbon nanotubes (MWCNT) have induced mesothelioma for both types of fibers (Sakamoto *et al*., 2009). However, the high doses used in this study were greatly criticized and whether realistic doses of MWCNT could induce cancer has still to be investigated. Further studies have in addition established the length dependency of asbestos-like pathogenicity of MWCNT (Poland *et al*., 2008) that may explain the absence of a carcinogenic response observed after intraperitoneal injection of shorter ($<1\,\mu m$) MWCNT (Muller *et al*., 2009). The cytotoxicity of MWCNT also depends on their diameter as larger nanotubes have been shown to be more toxic than smaller ones (Wang, Tao and Xing, 2009).

3.4 Chemical Composition

The toxicity of nanomaterials is not merely caused by the nanoscale of the particles but the chemical nature of the material is obviously a key factor determining their toxicity. For instance, the composition of the asbestos fibers, especially the iron content,

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is also of crucial importance for their carcinogenic potential. Furthermore, comparing same-sized NPs of CB and TiO_2 it has been shown that these two types of particles have the same toxic potential as they induce apoptosis of bronchial epithelial cells at same concentrations but the cellular mechanisms elicited depend upon the particle type (Hussain *et al*., 2010). Wang, Tao and Xing (2009) have also shown that the toxicity of quantum dots depend upon their composition as CdSe quantum dots are more toxic than CdTe whereas ZnS-$AgInS_2$ quantum dots were much less toxic. This cytotoxicity was attributed to the leakage of highly toxic cadmium ions. Indeed, the solubility of the nanomaterial is a critical feature for their toxicity. The biological effects could either be increased by insolubility leading to biopersistance of the material or in contrast by dissolution as also observed for ZnO and CuO NPs in which toxicity could be attributed to leaching of Zn or Cu ions (Mortimer, Kasemets and Kahru, 2010). However, particle-associated toxicity was also observed in other studies using ZnO NPs (Moos *et al*., 2010). It is important to note that dissolution kinetic is size-dependent and therefore an important factor to be considered for NP toxicity although several other physico-chemical characteristics influence dissolution kinetics: particle surface characteristics, such as roughness or curvature influence the kinetics of dissolution and adsorbed molecules could either slower solubility or serve to catalyze dissolution. The aggregation state of the particles has also to be considered as a hindering factor of solubility (Born *et al*., 2006). It is interesting to note that acidity could favor dissolution and thus preferential uptake of NPs into lysosomes or the less acidic caveolar compartment could thus influence the fate of the particles as shown for ZnO and CeO NPs (Xia *et al*., 2008). Another important factor influencing toxicity of nanomaterials is the crystal structure. It has been shown that rutile TiO_2 NPs were two orders of magnitude less cytotoxic than same-sized anatase TiO_2 NPs that could be due to the higher ability to generate reactive species (Sayes *et al*., 2006b).

3.5 Surface Characteristics

It is the surface of the nanomaterial that interacts with biological systems; however, the surface chemistry could differ from the bulk material. For instance, the effect of DEP mainly depend upon the organic compounds produced by incomplete combustion and that adsorbed on the carbonaceous core (Boland *et al*., 2000; Marano, Boland and Baeza-Squiban (2007)). Interestingly, the higher inflammatory response induced by ultrafine PM compared with fine or coarse PM could not be thoroughly attributed to increased surface area or particle number but the biological effects could be linked to the chemical composition, especially the organic matter that differs between these size fractions (Ramgolam *et al*., 2009). Surface modification of engineered nanomaterials could occur during the production process leading to surface functionalization influencing toxicity. Acidic treatment of MWCNT leading to carboxyl, carbonyl and hydroxyl groups increases their cytotoxicity (Magrez *et al*., 2006), but on the other hand, functionalization increasing water solubility of SWCNT leads to decreased cytotoxicity (Sayes *et al*., 2006a). Chemical aging and photoactivation could further modify surface chemistry. UV illumination has been shown to increase the cytotoxicity of fullerenes (Rancan *et al*., 2002) as well as anatase TiO_2 NPs that was linked to ROS production after photoactivation (Sayes *et al*., 2006b). Aging could also decrease toxicity as shown for ultrafine particles of polytetrafluoroethylene fumes that are no longer toxic when inhaled after several minutes of delay (Johnston *et al*., 2000). Agglomeration of the NP occurring concomitant with aging may however contribute to this decrease in toxicity.

Interaction of nanomaterials with cell membranes will depend upon surface properties, such as charge and hydrophobicity. For instance, hydrophobic poly(lactic-coglycolic)acid (PLGA) NPs are taken up more efficiently by alveolar macrophages than hydrophilic PLGA (Torche *et al*., 2000). It is noteworthy that the size of the particle is an important parameter as a particle could be hydrophobic at small size and hydrophilic at larger sizes (Chiu *et al*., 2009). This behavior arises from a purely geometrical effect caused by the curvature of the particle–water interface. Surface charge also influences the uptake of NPs as, for example, positively charged amine-polystyrene NPs could cross the alveolar barrier 20–40 times faster than same-sized negatively charged carboxy-modified polystyrene NPs (Yacobi *et al*., 2008; des Rieux *et al*., 2005). This is in agreement with the higher toxicity of cationic quantum dots compared with anionic

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quantum dots leading to alterations of epithelial barrier function (Yacobi *et al*., 2007). However, the differences in transport may also be attributed to the higher hydrophobicity observed for the cationic NPs (des Rieux *et al*., 2005). In contrast, intravenous injection of carboxyl quantum dots induces a higher prothrombotic effect compared with amine-coated quantum dots (Geys *et al*., 2008). The authors hypothesized that the negatively charged quantum dots trigger coagulation and subsequent fibrin formation and platelet activation.

Moreover, structural defects on the surface of MWCNT are mainly responsible for the pulmonary toxicity observed *in vivo* and *in vitro*. Indeed, annealing structural defects and elimination of metal contaminants by heating reduces the lung responses after intratracheal instillation but further grinding of the material restored their toxic potential (Muller *et al*., 2008). The rigidity of the particles also determines the uptake as macrophages showed a strong preference to engulf rigid objects compared with soft particles of same composition and size (Beningo and Wang, 2002).

3.6 Interactions

Beside these inherent properties of the nanomaterials, the interaction between particles or molecules will also determine their toxicity. First, the agglomeration state not only increases the size of the nanomaterial influencing its lung penetration, deposition and cellular uptake but also increases the solubility of the material (Borm *et al*., 2006). The reduction in the total surface area seems, however, to be limited as the biological effect of agglomerated particles is still correlated to the BET surface area (Hussain *et al*., 2009). The physico-chemical surface properties of the material, such as charge and hydrophobicity, determine the degree of aggregation but the characteristics of the suspending media (pH, viscosity, ionic strength, etc.) also play a role. Thus, coatings of derivative groups as well as dispersants may allow the stabilization of nanomaterials by preventing the formation of aggregates that has an effect on toxicity. For instance, dispersion of SWCNT by the use of surfactant (Wick *et al*., 2007) or by adding functional groups (Sayes *et al*., 2006a) reduces effectively the cytotoxicity.

Interactions between particles of different composition could have unexpected biological consequences as seen for cobalt tungsten carbide particles known to induce hard metal lung disease. It is the contact between the particles which causes the release of ROS involved in the pathogenic response as pure cobalt or carbide particles are inert and soluble cobalt salts in contact with carbide particles have no effect (Lison *et al*., 1995). Recently, the same effect has been observed for co-exposure to CB and Fe_2O_3 NPs leading to oxidative effects whereas exposure to either particle type alone has no effect (Guo *et al*., 2009). This synergistic effect is probably caused by intracellular redox reactions between CB and Fe^{3+} solubilized within the lysosomal compartment leading to Fe^{2+}.

In addition to these interactions between NPs, they could also interact with soluble compounds. Increasing evidence indicates that in biological fluids proteins adsorb onto NPs to form a ≪corona≫. Recent studies have clearly identified a number of proteins that bind to CB, silica, titanium dioxide or acrylamide NPs (Cedervall *et al*., 2007; Deng *et al*., 2009; Val *et al*., 2009;Vertegel, Siegel and Dordick, 2004). Among the proteins identified, several (such as apoliprotein E, granulocyte-macrophage colony-stimulating factor (GM-CSF), or transferrin) are ligands for cellular receptors and may thus contribute to the biological effects of the NPs. However, this interaction could also lead to inactivation of essential proteins, such as metabolizing cytochrome P450 isoenzymes (Fröhlich *et al*., 2010). Furthermore, the coating with proteins or lipids present in blood or epithelial lining fluids could also reduce the surface reactivity of the particles as shown for serum that reduces the biological response of CB and TiO_2 NPs (Val *et al*., 2009).

4 EFFECTS OF NANOPARTICLES

Human health effects of manufactured NPs are relatively unknown because of the fact that these materials gained utilizations in the recent past and adequate risk assessment data are unavailable. Previously acquired data on the deleterious effects of air-borne particulate pollutants and limited experimental data about the effects of manufactured NPs on experimental animals, point toward the possible human health effects of these newly developed materials. Moreover, the major known effects are short-term effects after acute dose exposures and long-term effects after chronic expositions are still unknown.

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4.1 Short-Term Effects of NP Exposure

4.1.1 Effects on Respiratory System

Respiratory system is considered as key target for involuntary exposures to NPs. Respiratory effects are mainly produced as a result of solid material deposition in the respiratory tract and indeed, depend upon the physico-chemical characteristics of the deposited material (Hoet *et al*., 2007). They also depend upon extent of penetration in the depth of the respiratory tract and persistence therein because there have been reports of inefficient phagocytosis of NPs.

In humans, there are limited data available about the effects of NPs on the respiratory system. A recent report has put an emphasis on possible induction of pleural effusion, pulmonary fibrosis and granuloma formation in factory workers exposed to polyacrylate NPs (Song, Li and Du, 2009). Although the exact link is unknown and there is also a possibility of involvement of other exacerbating factors, this observation further enforces the need for urgent risk assessment measures for nanomaterials.

Respiratory effects produced by the NPs mainly include inflammation, oxidative stress and functional disturbances. Numerous studies in animals describe inflammatory effects of NPs as transitory responses to NP exposures and assess whether the degree of inflammatory response is related to the exposure dose of NPs. Indeed, various types of nanomaterials have been shown to induce inflammation in lungs after either inhalation (Bermudez *et al*., 2004; Elder *et al*., 2005; Grassian *et al*., 2007) or instillation exposures (Warheit, Webb and Reed, 2006, Warheit *et al*., 2006; Warheit *et al*., 2007b). Such inflammatory responses are measured by local invasion of leukocytes, their numbers in broncho-alveolar lavage fluid (BALF), release of LDH and cytokine production. CNTs (single- and multi-walled) show typical granulomatous reactions in addition to inflammation after pulmonary exposures (Lam *et al*., 2004; Mercer *et al*., 2008; Warheit *et al*., 2004). Various studies with CNTs have demonstrated degree of dispersion plays a significant role in the induction of granulomatous response after CNT exposure.

One of the major factors which need attention in the studies describing inflammatory effects of NPs is the doses used in the studies. Apart from some human volunteer studies on air-borne ultrafine particles, most of the studies describing the health effects of NPs are performed in experimental animals. Indeed, rats are much prone to the overload situations because of anatomy of the respiratory tract as compared with mice and hamster. Under such situations, the clearance mechanisms are impaired and the retention time of the inhaled particles increases leading to chronic inflammation and fibrosis.

Other important consideration is the possible functional disturbances after the NP exposure that include airway hyper-reactivity (AHR) to none specific stimuli and tissue injury leading to disturbance in respiratory functioning (Alessandrini *et al*., 2006; Inoue *et al*., 2007, 2009). These disturbances are mainly studied in the animal models of different pathologies, such as asthma, etc. Indeed, there have been many studies that described the modulatory effects of nanomaterials on the lung functioning and existing pulmonary inflammation (Nygaard *et al*., 2004). However, Huczko *et al*. (2001) tested the effects of fullerenes on pulmonary function but no significant disturbances were observed revealing that beside size another factors contribute to NPs effects.

A detailed review of the literature describing the health effects of the NPs is shown in Table 1.

4.1.2 Effects on Cardiovascular System

Major cardiovascular abnormalities occurring after the NP exposure include thrombosis, arrythmias, coagulation disturbances, blood pressure abnormalities, etc. The mechanisms behind these effects are still unclear. Cardiovascular effects produced by the NPs might be in part caused by the released inflammatory and prothrombotic mediators at the site of deposition (lungs) leading to the activation of blood cells that take part in cardiovascular adverse effects (Simeonova and Erdely, 2009). However, as discussed in the previous part, NPs can translocate from respiratory system to cardiovascular system and may lead to adverse cardiovascular effects.

It has been shown that single oropharyngeal instillation of SWCNT induce dose-dependent cardiovascular pathology through perturbation of mitochondrial dysfunction (Li *et al*., 2007b). Autonomic modulation of the heart and repolarization of the ventricular myocardium are also possible mechanisms at least in susceptible individuals as suggested by exposure of young humans to CB NPs (Zareba *et al*., 2009). Other important contributors to cardiovascular pathology can be platelet activation, platelet aggregation (Radomski *et al*.,

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Table 1. Review of literature describing health effects of NPs.

Target system	Tested nanomaterial	Observed effects	Exposure route	References
Respiratory system	CB, TiO_2, quartz, silica, nickel oxide, CNTs (SWCNT/MWCNT)	Inflammation (increased cellularity of BAL fluid)/pro-inflammatory cytokines/histopathology/proliferation/LDH release	Inhalation	Bermudez *et al*. (2004); Carter *et al*. (2006); Geiser *et al*. (2008); Grassian *et al*. (2007); Li *et al*. (2007a); Mitchell *et al*. (2007); Rossi *et al*. (2010)
			Instillation	Ganguly *et al*. (2009); Kaewamatawong *et al*. (2006); Kobayashi *et al*. (2009); Lam *et al*. (2004); Mercer *et al*. (2008); Nemmar, Melghit and Ali (2008); Ogami *et al*. (2009); Sayes *et al*. (2007); Warheit *et al*. (2006, 2007a, 2007b)
CVS	CB NPs, silica, CNTs	Prothrombotic effects, coagulation abnormalities, heart rate abnormalities	Instillation	Evans *et al*. (2006); Li *et al*. (2007b); Nemmar *et al*. (2007), Nemmar, Melghit and Ali, (2008); Radomski *et al*. (2005); Shvedova *et al*. (2007); Simeonova and Erdely (2009)
			Inhalation	Chen *et al*. (2008b); Zareba *et al*. (2009)
CNS	TiO_2, Al, Ag, Cu, CB	Increase in number of segmented neutrophils and lymphocytes/protein carbonyl levels/interstitial fibrosis/oxidative stress/lipid peroxidation/glia activation/altered neurotransmitter activities/damage to blood–brain barrier	Inhalation	Nurkiewicz *et al*. (2008, 2009); Oberdorster *et al*. (2004)
			Instillation	Wang *et al*. (2008a, 2008b)
			Intravenous	Sharma *et al*. (2009); Tang *et al*. (2009)
			Abdominal cavity	Li *et al*. (2009); Liu *et al*. (2009); Ma *et al*. (2010); Rahman *et al*. (2009); Sharma *et al*. (2009)
			Intracarotid/intracerebroventricular	Sharma *et al*. (2009)
Skin	CNTs	Granulation/inflammation/fibrosis	Sub-cutaneous	Yokoyama *et al*. (2005)

2005) and systemic inflammation (Nemmar *et al*., 2007). Evans *et al*. (2006) have found strong correlations between plasma viscosity (a known marker of cardiovascular disorders) and lung inflammatory markers after instillation of diesel exhaust and silica NPs in a rat model of bleomycin-induced inflammation. Similarly, a study by Chen *et al*. (2008b) has further confirmed the paradigm of the occurrence of susceptible populations by demonstrating age-related differences in pulmonary and cardiovascular responses of rats to SiO_2 NPs after an inhalation exposure.

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4.1.3 *Effects on Nervous System*

As discussed in the previous section, different types of NPs translocate to central nervous system after different exposure routes (Kreyling *et al*., 2009). NPs have been shown to cause brain damages comprising of increase in number of segmented neutrophils and lymphocytes, protein carbonyl levels, and interstitial fibrosis, oxidative stress, lipid peroxidation as well as glia activation, that is, activation of innate immune response (Li *et al*., 2009; Oberdorster *et al*., 2004; Wang *et al*., 2008b). For example, TiO_2 after nasal instillation (Wang *et al*., 2008a) or abdominal administration (Ma *et al*., 2010) induces lipid peroxidation, and decreases the total anti-oxidation capacity and activities of antioxidative enzymes, an excessive release of nitric oxide and a down-regulated level of acetylcholinesterase activities. Rats intratracheally exposed to MnO_2 (23 nm diameter) till 9 weeks exhibit Mn in their brain and abnormal motility in an open-field box (Sarkozi *et al*., 2009). Moreover, it has been shown that maternal exposure of mice to TiO_2 NPs affects the expression of genes related to the development and function of the central nervous system (Shimizu *et al*., 2009). However there have been studies that failed to detect same NPs in brain even after high intravenous or subcutaneous doses (Patri *et al*., 2009).

4.1.4 *Miscellaneous Systemic Effects*

NPs are used in huge quantities in cosmetics and abilities of NPs to translocate through the skin are a topic of hot debate. It was shown that subcutaneous administration of carbon nanotubes in rats cause a biphasic response with initial granulation and inflammation and later fibrous tissue formation (Yokoyama *et al*., 2005). Moreover, it has been shown that TiO_2 NPs aggravate atopic dermatitis like lesions in mice presenting skin barrier dysfunction/through predominated Th2 immune responses (Yanagisawa *et al*., 2009).

Fullerene derivative (C60 polyalkylsulfonate) was shown to cause nephropathy and biochemical impairment after intravenous and intraperitoneal applications (Chen *et al*., 1998).

4.2 Long-Term Effect/Carcinogenicity of NPs

Carcinogenicity of engineered nanomaterials is mainly studied on lungs via inhalation or instillation exposures and scarce data exist by other exposure routes. Because of lack of adequate epidemiological data in humans, engineered nanomaterials could not be associated directly with lung cancer risks. Low toxicity, low-solubility dusts (LTSD), such as CB and TiO_2, have been proved to be carcinogenic in experimental animals but there is very limited epidemiological data about the human exposures. These considerations lead to their classification as possible human carcinogens (category 2B of carcinogenic substances) (Roller, 2009). The true mechanisms of NP-induced carcinogenicity are unknown and this could be a primary genotoxic insult or secondary genotoxic response caused by particle-induced inflammation (Schins and Knaapen, 2007).

Another important class of nanomaterials for which carcinogenicity is very important consideration is carbon nanotubes. Takagi *et al*. (2008) reported that MWCNT induces mesothelioma when administered intraperitoneally to p53 heterozygous mice (a mouse strain sensitive to asbestos) and they validated their results by using crocidolite asbestos as positive control. Similar findings were reported by others injecting MWCNT into the scrotum male rats (Sakamoto *et al*., 2009). Similarly, Poland *et al*. (2008) reported asbestos-like pathology (inflammation and granulomas) in the abdominal cavity of mice exposed to MWCNT. Furthermore, they observed that this response was length-dependent. Indeed, there have been serious concerns about the material characterization, doses and model used for the description of these effects. Furthermore, in the presence of other studies that claimed that MWCNT does not possess any carcinogenicity in a 2-year bioassay (Muller *et al*., 2009), care must be taken when interpreting the positive results about the carcinogenicity of MWCNT. Nevertheless, these studies are premier to warrant about the possible health hazard scenarios of CNT exposures.

5 MECHANISMS OF ACTION

5.1 Role of Oxidative Stress in the Cytotoxicity and Inflammatory Responses Induced by Engineered Nanoparticles

An increasing number of papers links the cytotoxicity induced by engineered NPs to the oxidative stress. This stress leads to a cascade of cellular

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events and the induction of redox sensitive pathways in the cells. The consequences could be the pro-inflammatory response, the modification of cell cycle and proliferation and, at last, the cell death by apoptosis or necrosis. Oxidative stress paradigm is sustained by many recent reviews on the mechanisms of action of NPs and their comparison with atmospheric fine and ultrafine particles (Hoet, Nemmar and Nemery, 2004; Nel *et al*., 2006; Oberdörster, Oberdörster and Oberdörster, 2005; Xia *et al*., 2006). Oxidative stress is defined to be the consequence of an imbalance between the production of ROS and the antioxidant capacities in the cell that lead to a biological adaptation or adverse effects in relation to the level of oxidative stress. The hierarchical oxidative stress model published by Nel *et al*. (2006) and Xia *et al*. (2006) to explain the biological effect of ambient and manufactured NPs proposes that "minor levels of oxidative stress induce protective effects that may yield to more damaging effects at higher levels of oxidative stress." The adverse effects could be directly induced on the target tissues from the ROS produced by the NPs or indirectly from the inflammatory process associated to NP biological effects. Indeed, the oxidative stress that activates redox-sensitive transcription factors, such as NFκB, AP1 or Nrf2, induces the transcription of a number of pro-inflammatory genes and the increased release of numerous pro-inflammatory factors, such as cytokines.

Initially, the role of oxidative stress in the mechanisms of toxicity of particles was pointed out in the occupational exposures to CB particles, glass fibers, quartz particles and asbestos fibers (Castranova *et al*., 1997; Gilmour, 1995). More recently, it was also demonstrated that DEP as well as atmospheric particles were able to induce an oxidative stress in the lung caused by their chemical compounds and especially metals and organic compounds (Ayres *et al*., 2008). The oxidative power of PM and their ability to produce free radicals are strongly linked to their composition that depends on the sources. When the PM contains a large amount of metals and especially transition metals, such as iron or vanadium, they produce ROS either in the biological fluids in the airways or inside the target cells after phagocytosis. We have also demonstrated in our laboratory that the organic compounds, especially the polycyclic aromatic hydrocarbons, and quinones associated with the $PM_{2.5}$ and PM_1 from traffic or industrial pollution could be associated to an increase of ROS in bronchial cells (Baulig *et al*., 2003; Bonvallot *et al*., 2001) and subsequently the activation of NFκB leading to the transcription of cytokines, such as GM-CSF, interleukin (IL) 6 and IL8, tumor necrosis factor α (TNFα). Interestingly, it also appears that these $PM_{2.5}$ and PM_1 could increase the expression and release of growth factors that are ligands of the epidermal growth factor receptor (EGFR), such as amphiregulin (AR) and this up-regulation is also associated to oxidative stress (Blanchet *et al*., 2004; Rumelhard *et al*., 2007b).

The central role of the transcription factor NFκB is pointed out in numerous *in vitro* studies on the effects of fibers, such as asbestos, PM and NPs (Albrecht, Borm and Unfried, 2004). This cytoplasmic factor constitutes of two sub-units, p50 and p65, associated to an inhibitory protein, IκB. The activation of NFκB occurs when IκB is removed and degraded in the cytoplasm by the proteasome. It induces the migration of NFκB to the nucleus that links a lot of genes promoters, such as cytokine genes (Figure 3). In such mechanisms, ROS produced by the NPs play the role of initiator of the inflammatory response but also, indirectly, the role of amplifier by autocrine or paracrine effects on the target cells. For example, TNFα, the secretion of which is increased by NPs, such as CB NPs on macrophages, could link membrane receptors and

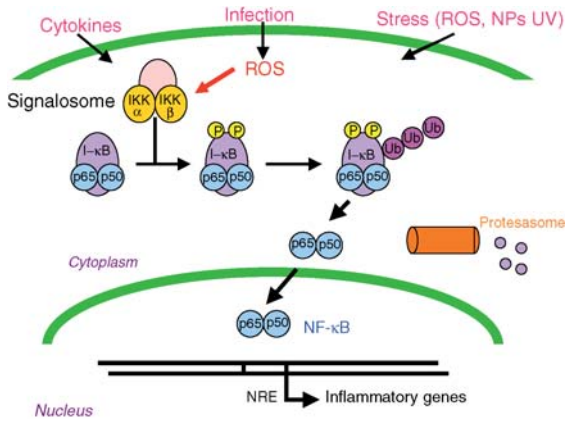

Figure 3. NF-κB activation: different stimuli, such as ROS lead to IκB kinase activation in the signalosome that phosphorylate IκB allowing its ubiquitinylation and subsequent degradation by the proteasome. Consequently, p50 and p65 sub-units of NF-κB can translocate to the nucleus and bind to specific responsive element (NRE, NF-κB-responsive element) to transactivate a great variety of genes such as those involved in the production of cytokines and chemokines.

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activate a signaling pathway leading to the release of superoxide-free radical from mitochondria (Brown *et al*., 2004).

The roles of the size of NPs and of their surface reactivity in the ability to induce an oxidative stress were pointed out by several recent studies (Warheit *et al*., 2007b; Xia *et al*., 2006). In our laboratory, the role of physico-chemical characteristics in toxic effects of CB and TiO_2 NPs of different sizes, were evaluated in human bronchial epithelial cell line (16HBE) that represents one of the first line targets encountered by NPs (Hussain *et al*., 2009). Both CB and TiO_2 were taken up in a time- and dose-dependent manner as evaluated and quantified by flow cytometry and transmission electron microscopy analysis. The smallest NPs have higher cytotoxic potentials and induce apoptosis. Exposure to TiO_2 NPs at non-toxic concentrations leads to a stronger pro-inflammatory response characterized by GM-CSF release correlated to the induction of mRNA expression as compared with CB. This GM-CSF induction is higher for TiO_2 and the smallest particles have a greater inflammatory potential. Furthermore, NPs produce ROS under abiotic and/or intracellular conditions depending on their chemical composition and size. The cytotoxic and inflammatory effects of NPs are caused by the oxidative stress produced as use of the antioxidant catalase ameliorated the response (Hussain *et al*., 2009). These results sustain the hypothesis that the surface reactivity of the NPs that increases when the size decreases, in relation with their ability to produce ROS and to associated toxic compounds is an essential factor in their cytotoxic effects (Donaldson *et al*., 2001; Salvi and Holgate, 1999). Park *et al*. (2008) compared CeO_2 and TiO_2 NPs, for their cytotoxic potential with larger particles of same chemical composition and demonstrated a stronger capacity to induce an oxidative stress, to activate apoptotic pathway and to induce a DNA fragmentation leading to apoptosis.

Another important question is to determine whether the effects of NPs depend on the target cells. The skin cells are an important target of NPs, such as TiO_2, ZnO and silver NPs that are largely used in cosmetic industry and consumer health products. Until now, it was not demonstrated a toxicity of TiO_2 and ZnO NPs on healthy skin. However, silver NPs that are used in a lot of cosmetic products and also for the antibacterial treatment of clothes, are cytotoxic on several epidermal cell lines such as HT 1080 and A431. They induce an increase in ROS, lipid peroxidation, and an apoptotic cell death (Arora *et al*., 2008).

Another important target is the lung for inhaled NPs. The cytotoxicity of different kind of engineered NPs (TiO_2, CB, ZnO, CeO_2, etc.) was demonstrated on different cell lines: bronchial human cell lines such as BEAS 2B, 16HBE (Hussain *et al*., 2009; Park *et al*., 2008), macrophages and the alveolar cell line A549 (Hsin *et al*., 2008; Koike and Kobayashi, 2006). As it is likely that the NPs could, even to a low amount, go through the biological barriers, they can target other organs, such as liver, kidney, and brain. The cytotoxicity of engineered NPs was studied on liver cell lines (Hussain *et al*., 2005), renal cell line LLC-PK1 (L'azou *et al*., 2008), and neuronal cell line (Hussain *et al*., 2006). It appears from all these data that, if the cytotoxicity is generally associated to oxidative stress, the sensitivity differs with the cell line, perhaps in relation with the uptake of NPs.

5.2 Nanoparticles and Cellular Signaling Pathways in the Mechanisms Associated to Apoptosis and Inflammatory Responses

The MAP kinases signaling cascades (ERK 1/2, p38, and JNK) have been shown to be involved in the response to different ultra-fine or NPs, such as DEP, TiO_2 as well as amorphous silica (SiO_2) (Kim *et al*., 2009). It could lead to the pro-inflammatory response that generally increases when the size of the NPs decreases (Hussain *et al*., 2009).

There are some evidences that particles may trigger signaling cascade by activating membrane receptors (Figure 4). The role of EGFR and its ligands were underlined in several studies. For instance, bronchial epithelial cells respond to DEP or $PM_{2.5}$ exposure by the over-expression of ligands of EGFR, such as AR and transforming growth factor beta (TGFβ) (Auger *et al*., 2006; Blanchet *et al*., 2004; Rumelhard *et al*., 2007a, 2007b). Particles trigger the activation of EGFR and ERK and JNK MAP kinase pathways leading to EGFR ligands release. The AR release being inhibited by the anti-oxidant *N*-acetyl cysteine but not by a neutralizing anti-EGFR preventing ligands binding to EGFR suggests that a trans-activation of EGFR was triggered by ROS. Moreover, these studies have

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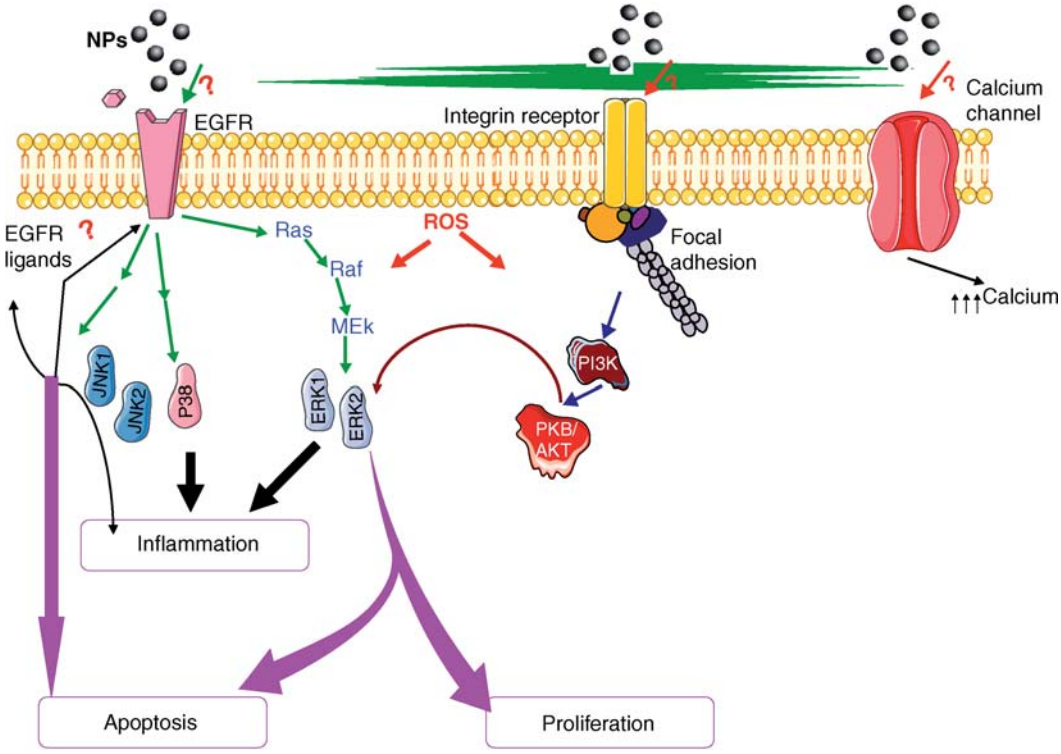

Figure 4. NP trigger signaling pathways by interaction with membrane receptors leading to different cellular outcomes. NP-induced activation of EGF receptor can lead to apoptosis, inflammation or proliferation. In addition activation of integrin receptor and ROS also contribute in proliferation signaling induced by NPs. The role of EGFR ligands such as AR needs to be elaborated.

shown that the particles-induced AR and TGFβ release by bronchial epithelial cells contribute to the release of pro-inflammatory mediators, especially GM-CSF suggesting an autocrine role for EGFR ligands in eliciting and sustaining the proinflammatory response (Rumelhard *et al*., 2007b). These new findings provide additional and valuable information on inflammation following chronic exposure to particles that could contribute to exacerbation of airway remodeling in respiratory-compromised individuals; however, as far as now, it is not demonstrated for engineered NPs.

Other studies have also underlined the role of the EGFR in the mechanism of action of CB NPs. These NPs induce apoptosis and proliferation in rat lung epithelial cells via independent specific signaling pathways but both depending on the activation of EGFR (Sydlik *et al*., 2006). Although the proliferation induced by NPs is solely dependent on the β1 integrins and ERK 1/2 activations, the induction of apoptosis was correlated to c-Jun kinases phosphorylation (Figure 4).

NPs also have the ability to modulate intracellular calcium. Indeed, Möller *et al*. (2005) showed that CB NPs trigger a transient increase of intracellular calcium responsible for impaired phagosome transport and cytoskeletal dysfunctions. This transient increase of Ca^{2+} was not associated with the production of ROS because antioxidants were inefficient in reducing the effects (Möller *et al*., 2005). They postulate that CB NPs could have a direct effect on ion channels that control the calcium homeostasis in the cell.

Altogether these data highlight that transmembrane receptors are implicated in NP-induced cell signaling and could lead to specific biological responses to NPs. However, a thorough understanding of the involved mechanisms is necessary as well as to determine whether they are or not specific to some types of NPs.

NPs are able to induce apoptosis in a variety of *in vitro* systems. (Pan *et al*., 2007, 2009; Sydlik *et al*., 2006; Vamanu *et al*., 2008) This induction of apoptosis triggered by NPs might be implicated in

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different pathologies but, as far as now, it is not demonstrated in humans. NPs might exert apoptotic effects through differential signaling events involving death receptor, mitochondria or lysosomes. However, the signaling pathways and the initial events triggered by NP to induce the executive phase of apoptosis seem to be NP specific and need to be clarified by further researches.

5.3 Genotoxicity of Nanoparticles

Nanogenotoxicology is one of the key areas of research that needs more attention from the people working on the nanomaterials toxicity. Different types of nanomaterials capable of inducing genotoxic responses include CNTs (both SWCNT and MWCNT) (Cui *et al*., 2005; Kisin *et al*., 2007; Monteiro-Riviere *et al*., 2005; Muller *et al*., 2008; Pacurari *et al*., 2008; Zhu *et al*., 2007), TiO_2 NPs (both with and without photoactivation) (Dunford *et al*., 1997; Geiser *et al*., 2005; Kang *et al*., 2008; Rahman *et al*., 2002; Trouiller *et al*., 2009;Wang, Sanderson and Wang, 2007a; Xu *et al*., 2009), aluminium oxide (di virgilio 09), platinum NPs (Asharani *et al*., 2010) and zinc oxide NPs (Dufour *et al*., 2006). Quantum dots have shown DNA-damaging effects both under abiotic conditions and under biotic conditions (Choi *et al*., 2008; Green and Howman, 2005). Although it has been shown that silica NPs induce intranuclear protein aggregation and interfere with cell division, a controversy exists in literature about the genotoxic potentials of silica NPs (Barnes *et al*., 2008; Chen and von, 2005; Wang, Sanderson and Wang, 2007b). Drastic variations in compositions and physico-chemical characteristics of nanomaterials can result in variable genotoxic potentials. It has been proposed that nanomaterials can cause primary as well as secondary genotoxic insult (Schins and Knaapen, 2007). Primary genotoxic response occurs when particles gain entry into the cell nucleus and come in direct contact with the nuclear material and cause damage either by physical interaction with DNA or mitotic spindle or through some indirect mechanisms and essentially these responses occur without any inflammation. Whereas secondary genotoxic response occurs when NP-induced inflammation and oxidative/nitrosative stress causes the DNA damage (e.g., as shown for TiO_2 NPs) (Trouiller *et al*., 2009). This type of DNA damage mostly comprises single- or double-stranded breaks in DNA or by the formation of adducts (e.g., 8-hydroxydeoxy-guanosine adducts). Secondary genotoxic responses are mostly seen in case of LTSD, such as CB and TiO_2 when the exposure doses cause overload in the lungs. Production of ROS occurs either as an intrinsic ability of NPs or through their interaction with cellular components (Hussain *et al*., 2009). An excellent review on the genotoxic potentials of NPs has recently been published by Singh *et al*. (2009). The presence of controversies about the genotoxic potentials emphasizes the need of evaluation of genotoxic potentials by a standardized battery of tests.

6 CONCLUSIONS

Because of the increased unintentional potential exposure to NPs and their specific properties compared with their micro-sized counterparts, the human health effects of NPs have to be carefully considered. There are some experimental evidences that whatever the route of exposure, NPs can enter the blood circulation, reach secondary organ allowing both local and systemic effects. The diversity and complexity of NP characteristics involved in their biological effects makes nanotoxicology a very challenging field. Several parameters could influence to different degrees the final biological response to NPs and it is thus difficult to predict the human health hazard of exposure. It is evident that thorough physico-chemical characterization of the tested nanomaterial prior to *in vitro* or *in vivo* biological evaluation is needed to allow the comparison of data and to draw general or specific conclusions on toxicity of nanomaterials for public health risk assessments. Progress in nanotoxicology will allow to identify the determinants of particle toxicity and to better understand NPs mechanism of action through cellular and molecular interactions. Oxidative stress is one of the already identified mechanisms of NPs toxicity that explains most cellular effects but insufficient for diseases prediction (Donaldson *et al*., 2009).

RELATED ARTICLES

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Systems Toxicological Approach to the Risk Assessment of Nanomaterials

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1 INTRODUCTION

Nanotechnology is the manipulation of matter for use in particular applications through certain chemical or physical processes to create materials with specific properties. Nanotechnology can be applicable to many fields of sciences including Chemistry, Physica, Biology and Medicine. The Internal Standard Organization (ISO) and the Organization for Economic Cooperation and Development (OECD) have defined nanomaterials as having a size ranging between 1 and 100 nm. Nanomaterials include nanoparticles, nanofibers, nanotubes, composite materials and nano-structured surfaces (US Environmental Protection Agency, 2007). Nanotechnology now holds immense societal and environmental benefits with regard to increased economic development, improved materials using less resources and environmental remediation (Roco and Bainbridge, 2005). Commercial applications of nanomaterials have increased since 1990 and include textiles, antimicrobial wound dressings, paints and coatings, fuel catalysts and additives, lubricants, cosmetics and food packaging (Figure 1) (Chaundry *et al.*, 2006).

Although nanomaterials have many prominent advantages compared with classical chemicals/physical agents, there are probably unknown hazards. In addition, the hazards of nanomaterials may be somewhat different. Therefore, new toxicological testing approaches that include traditional testing methods are essential in order to evaluate the hazards of nanomaterials. Generally, nanomaterials can be divided into three types. The first type is unintentionally generated nanomaterials such as diesel soot nanomaterials and welding fumes. The behaviour of airborne particles in the environment differs according to size: nanoparticles smaller than 80 nm in diameter tend to agglomerate rapidly to form larger particles, whereas particles sized from 80 to 2000 nm remain suspended in the air for a long time with a greater chance of being inhaled (Preining, 1998). The second type is industrial nanomaterials that are used to improve the quality of

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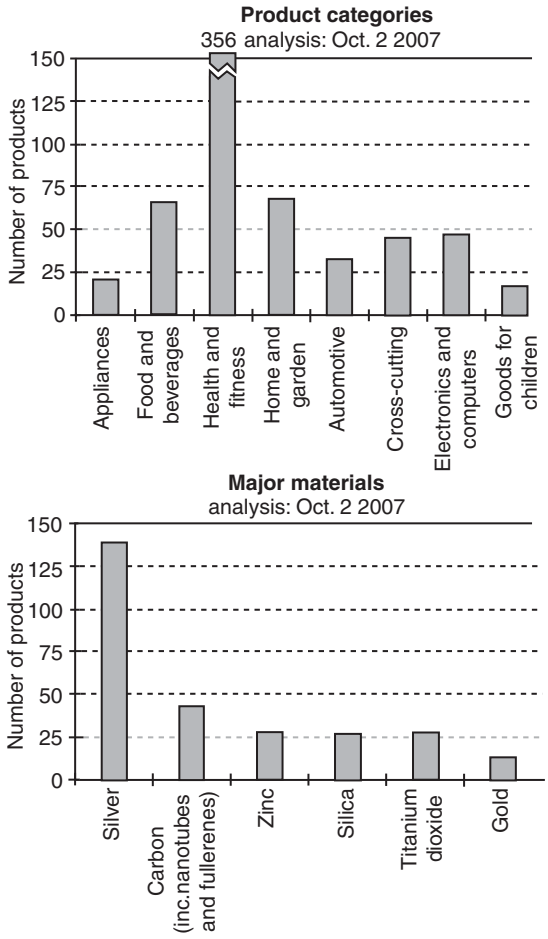

Figure 1. Top chart shows the distribution of 580 products in the inventory according to product category. The products fall into several different categories such as health and fitness, home and garden, electronics and computers, and so on. Bottom chart shows the materials used in the various products in the inventory. The main kinds of materials used are graphite (including carbon nanotubes and fullerenes), silver, silica, TiO_2 and ZnO. Reproduced from Hansen *et al.*, (2008) © Springer).

paints, fuel cells, displays, batteries, fuel additives, catalysts, lubricants, etc. The representative industrial nanomaterials are carbon nanotubes, carbon black, titanium dioxide (TiO_2), cerium dioxide, zinc oxide, copper oxide and nickel oxide (Thomas *et al.*, 2006). The third type is nanomaterials for biomedical applications. Nanomaterials for biomedical applications can be divided into three main uses: (i) diagnosis, (ii) imaging and (iii) therapy. Diagnosis with nanomaterials increases sensitivity and specificity due to their unique properties (Salata, 2004). Through multifunctional application of nanomaterials, these three areas can be applied to one nanomaterial.

Because of the wide spectrum of nanomaterial usage, exposure to nanomaterials, whether intentional or unintentional, may occur via various routes including inhalation, ingestion, skin contact and other parenteral exposure. The safety or potential hazards are also assumed to be different for the same materials on larger scales with respect to the diversity in size, composition and surface properties of nanoscale materials. Conservative methods for the toxicological assessment of each nanomaterial may consume lots of time, laboratory animals, materials and expenditures because of the diversity of structures and compositions and the lack of familiarity with these nanomaterials.

Systems Biology in Toxicology can open a new realm for risk assessment by providing immense but accurate toxicological information while saving laboratory animals and expenditure. Target organs and the modes of action of nanomaterials have begun to be elucidated through systemic toxicological approaches, which provide a general view of the safety, the potential risk on public health and options for risk management of nanomaterials (Walker and Bucher, 2009).

2 MAIN CONSIDERATION OF TOXICITY STUDY

Figure 2 shows the general toxicity mechanisms of nanomaterials. Nanomaterials can release transition metals and ions through several mechanisms including sonication and interaction with biological fluids (McNeilly *et al.*, 2004; Horie *et al.*, 2009). The potential hazardous effects of nanomaterials on human health were evaluated as rather speculative and unfinished. However, increasing numbers of laboratory studies have indicated that exposure to some kinds of nanoparticles can have adverse effects on the lungs and the brain in laboratory animals (Lam *et al.*, 2004; Oberdorster, 2004).

Nanomaterials and their derivatives may generate free radicals (Lu *et al.*, 2009). Nanomaterials can enter cells by simple diffusion or endocytosis. The toxicity induced by nanomaterials can be categorized as direct or indirect damage. The direct damages, including inflammation and DNA

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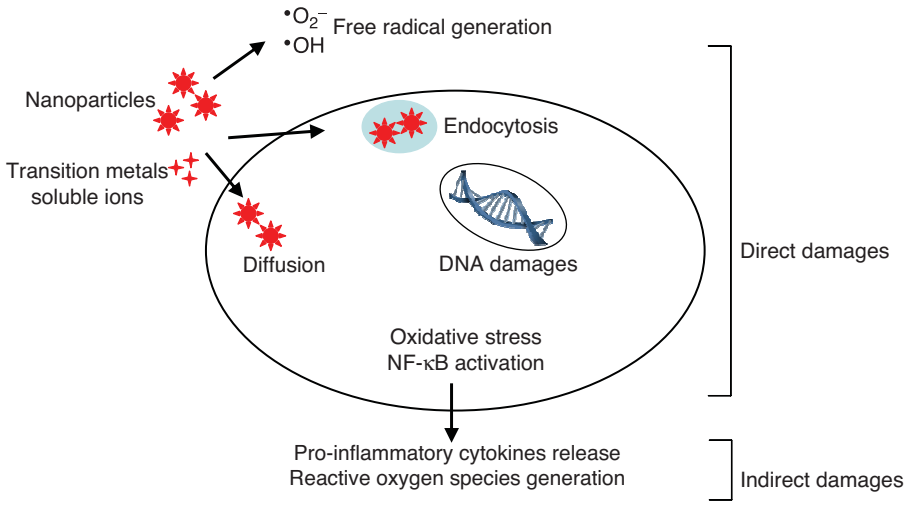

Figure 2. Overview of general mechanisms of nanomaterial toxicity. Toxicity of nanomaterials is divided into direct damage and indirect damage induced by inflammation, oxidative stress and DNA damage.

damage, are induced by reactive oxygen species (ROS) generation, entry into the cells, nuclear factor kappa-B activation and pro-inflammatory mediators. The indirect damage is a secondary reaction induced by released pro-inflammatory cytokines and ROS (Lu *et al.*, 2009).

The unique physicochemical properties of nanomaterials include their small size, purity, crystallinity, electronic properties, surface reactivity, inorganic or organic coatings, solubility, shape and aggregation (Nel *et al.*, 2006). Nanomaterials have completely different toxicity compared with larger particles of the same composition. As particle size decreases, nanoscale materials can cross or penetrate cell membranes that are impenetrable to larger materials (Kim *et al.*, 2006). In addition, nanomaterials can disrupt normal biological functions, including DNA and protein absorption (Horie *et al.*, 2009; Ahamed *et al.*, 2008). As particle size decreases, the surface area increases by geometric progression (Oberdorster,Oberdorster and Oberdorster, 2005). Therefore, nanomaterials have a much greater surface area per unit mass compared with larger materials. The increased surface area contributes to increases in reactivity such as catalytic activity for chemical reactions and the exponentially increased surface molecules. Also, the toxicities vary according to the structures of the nanomaterials. Hansen *et al*. (2007) suggested a new method to categorize nanomaterials into three relevant groups depending on the nanoscale structure in the system: materials that are nanostructured in the bulk, materials that have nanostructure on the surface and materials that contain nanostructured particles. Nanostructured particles are further subcategorized into particles that are surface bound, suspended in liquids or in solids, and airborne. According to these divisions, 19% of nanomaterial products were nanoparticles bound to surfaces, 37% were nanoparticles suspended in liquids whereas 13% used nanoparticles suspended in solids; 1% were powders containing free, potentially airborne, nanoparticles, whereas the rest of the products were not located (Hansen *et al.*, 2008). Toxicological assessment is required separately, after considering particle size and the structure for the applications.

2.1 Size of Nanomaterials

In the industrial workplace, many kinds of nanomaterials are generated including welding fumes, carbon nanotubes and TiO_2. Aerosolization and inhalation of nanomaterials may induce adverse respiratory effects. When nanomaterials enter the lung, they are deposited, internalized and translocated to the systemic circulation depending on size, surface area, surface chemistry, and so on. (Oberdorster, Oberdorster and Oberdorster, 2005). The deposition mechanisms of particles in the lung include inertial impaction, gravitational sedimentation and Brownian diffusion (Yang, Peters and

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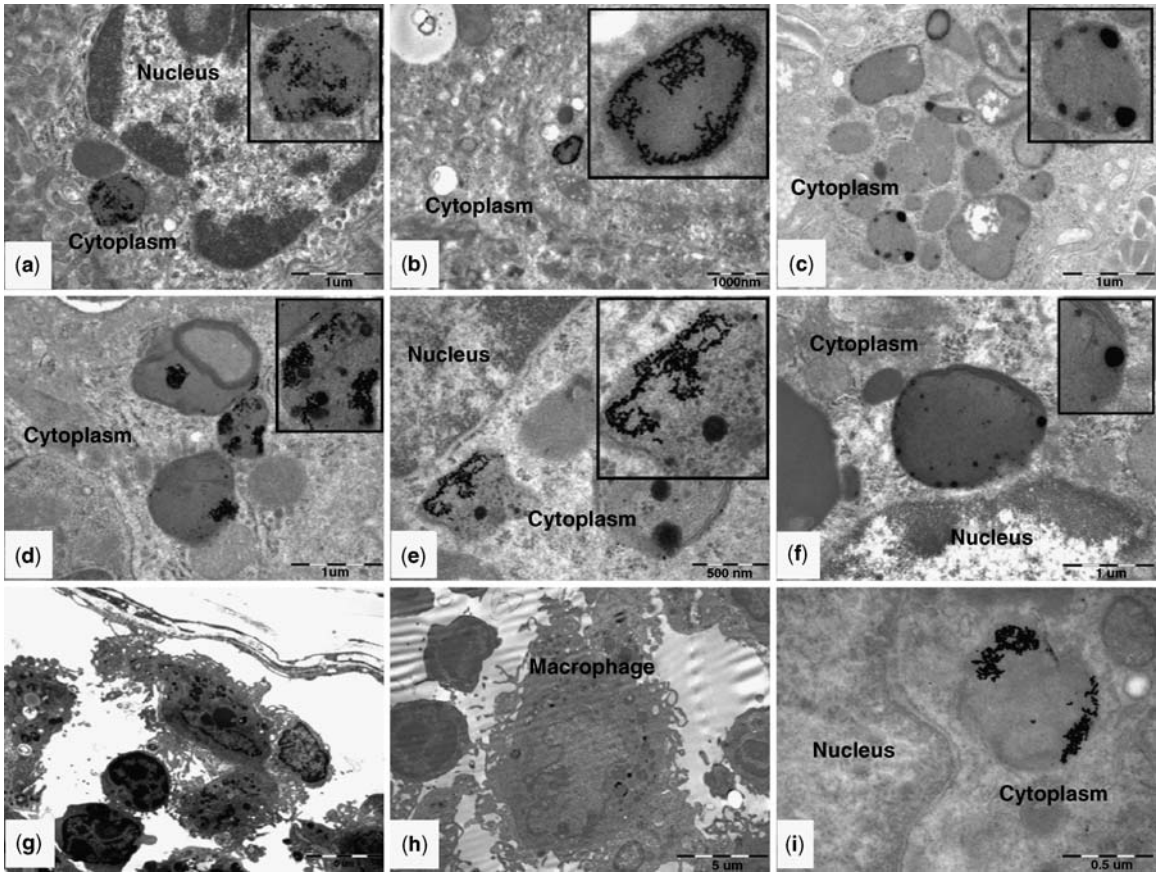

Figure 3. Thin-section TEM images of mouse liver, spleen and lymph node tissues at 7 days after intravenous injection of 4-, 13-, and 100-nm PEG-coated AuNPs. The micrographs show entrapped PEG-coated AuNPs, and their clustering and localization in lysosomes. Kupffer cells at 7 days after injection of (a) 4-nm, (b) 13-nm or (c) 100-nm PEG-coated AuNPs. Spleen macrophages at 7 days after injection of (d) 4-nm, (e) 13-nm or (f) 100-nm PEG-coated AuNPs. (g–i) Macrophages of lymph node at 7 days after 4-nm PEG-coated AuNPs treatment. (g) and (h) clearly show that the particle-engulfing cells are macrophages.

Williams, 2008). Among them, Brownian diffusion is the main mechanism for nanomaterial deposition (Oberdorster, Oberdorster and Oberdorster, 2005). Briefly, particles between 1 and 3 μm in size are the most effectively deposited in the alveolar region. In addition, about 80% of particles less than 1 μm can be exhaled without deposition after inhalation as a result of their low inertia (Patton, 2005).

Nanomaterials have longer retention times and are more translocated than larger particles of the same composition. According to a study of inhalation of ultrafine (∼20 nm) and fine (∼200 nm) TiO_2 nanoparticles, ultrafine particles were cleared more slowly and were more translocated than fine particles (Oberdorster, Ferin and Lehnert, 1994). Intravenous injection of three different sizes of polyethylene glycol (PEG)-coated gold nanoparticles (GNP; 4, 13 and 100 nm) revealed that, as particle size decreased, more nanoparticles were engulfed by macrophages (Figure 3). When three different sizes of rhodamine B isothiocyanate (RITC)-conjugated silica nanoparticles (50, 100 and 200 nm) were injected, tissue distribution and elimination patterns showed size-dependent trends. In the kidney, the fluorescence intensity of all three sizes of silica nanoparticles was strongest throughout the organ at 24 h after administration. Particles were shown in macrophages of the liver and spleen within 24 h. In spleen and liver, residual 200-nm particles were observed more frequently than 50- and 100-nm particles. There were no particles observed

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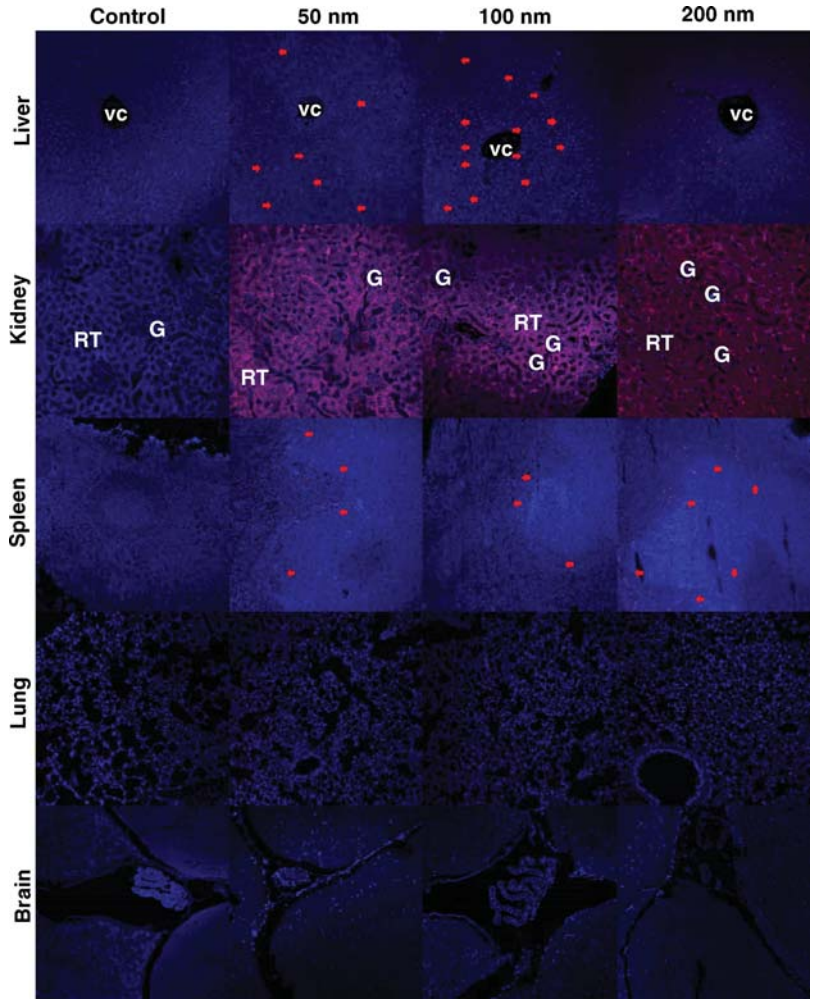

Figure 4. Tissue distribution of silica nanoparticles in various organs of mice at 24 h. Labels on the left side of the images indicate organs. Particle size is indicated above the top horizontal row. Red arrows in the liver and spleen indicate aggregated silica nanoparticles. VC, vein centre in the liver; G, renal glomerulus; RT, renal tubule.

in the brain or lung (Figure 4). All three silica particle sizes remained in the liver and spleen until 4 weeks after a single injection (Figure 5). In addition, all silica particles were eliminated via both urine and bile and the quantity of 50-nm silica nanoparticles in urine and bile was higher than that of 100- or 200-nm particles (Figure 6) (Cho *et al.*, 2009).

2.2 Biologically Effective Dose

The biologically effective dose (BED) is a key concept in understanding the hazards and mechanisms of toxicity of nanomaterials. The traditional mass dose does not reflect the BED of nanomaterials (Table 1). Rather, the BED is a fraction of the total mass dose, and may not necessarily be a mass in the case of particles but could instead be surface area or surface reactivity. When ultrafine (20 nm) and fine (250 nm) TiO_2 particles were instilled intratracheally into rodents, much greater pulmonary-inflammatory neutrophil response was induced by ultrafine TiO_2 than by fine particles when comparing the mass dose, but the response for both ultrafine and fine particles was the same when comparing the particle surface area (Oberdorster, 2000).

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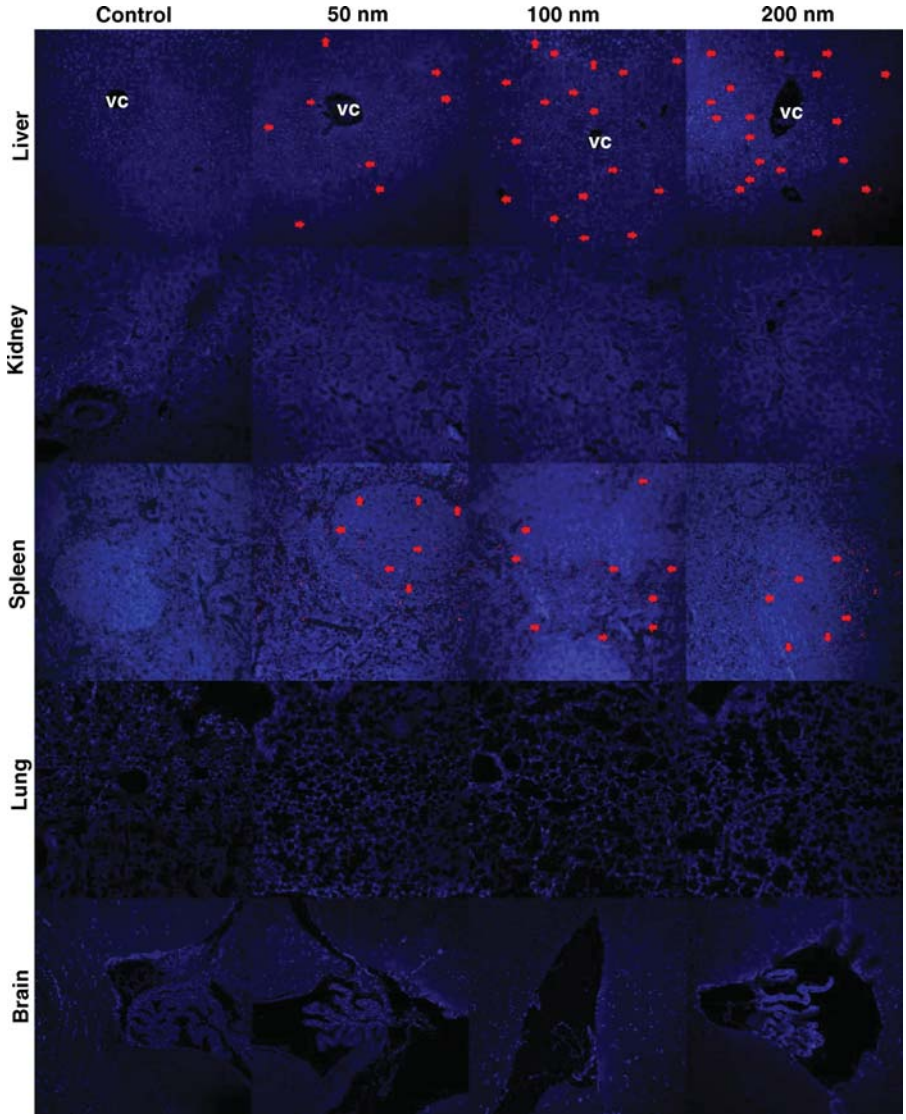

Figure 5. Tissue distribution of silica nanoparticles in various organs of mice at 4 weeks. Labels on the left side of the images indicate organs. Particle size is indicated above the top horizontal row. Red arrows in the liver and spleen indicate aggregated silica nanoparticles. VC, vein centre in the liver.

Table 1. Comparison of traditional metric and biologically effective dose of nanomaterials.

Nanomaterials	Traditional dose metric	Biologically effective dose	References
Quartz	Respirable mass	Surface reactivity	Duffin *et al.* (2007)
PM10	Respirable mass	Soluble transitional metals	Hutchison *et al.* (2005)
Asbestos	Fibre longer than 5 μm and aspect ration >3	Biopersistent fibers longer than 20 μm	Bernstein *et al.* (2008)
Welding fume	Respirable mass	Soluble transitional metals	McNeilly *et al.* (2004) and McNeilly *et al.*, (2005)
Low-toxicity low-solubility particles (TiO_2, ultrafine carbon black, etc.)	Respirable mass	Surface area	Monteiller *et al.* (2007)

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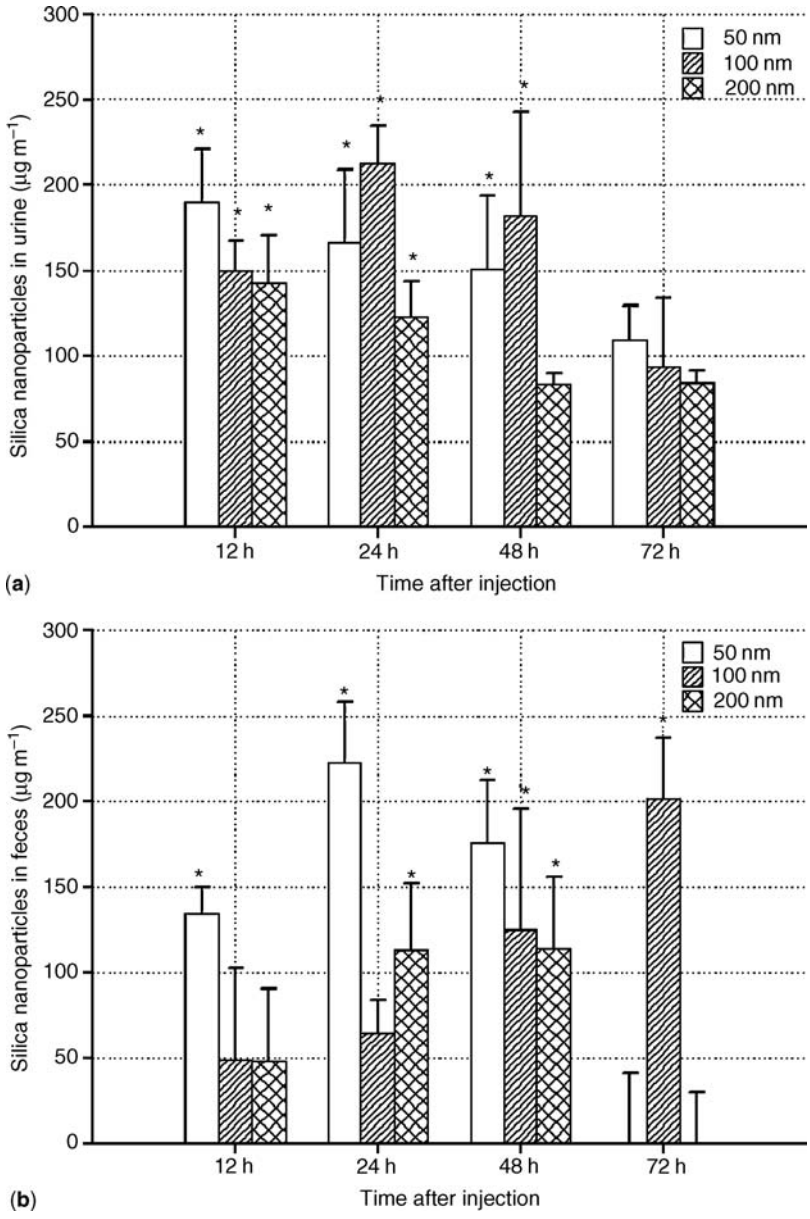

Figure 6. Silica nanoparticle concentration in urine and feces. Silica particles were detected in urine (a) and feces (b). *Significant difference ($P < 0.05$) compared with vehicle control.

To obtain the BED, knowledge about the physico-chemical properties of and the cellular and molecular events involving nanomaterials (including sub-fraction or their variants) is essential. Generally, the BED relates to the ability of nanomaterials to generate free radicals and produce ROS in target cells (Lu *et al.*, 2009; Nel *et al.*, 2006). In addition, inflammation is a key response for the BED of nanomaterials (Oberdorster,Oberdorster and Oberdorster, 2005). The BED is different for each nanomaterial. Therefore, categorization of BED will be very helpful in understanding the biological effects and evaluating the risks of nanomaterials.

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2.3 Surface Chemistry and Interactions With Biological Membrane

Nanomaterials tend to naturally aggregate due to increased specific surface/volume ratios or electrostatic interactions. Therefore, nanomaterials for biomedical application are often coated with non-toxic and stable materials to prevent aggregation and toxicity. In addition, surface modification can make nanomaterials soluble for specific applications. Surface chemistry is also a very important factor in the toxicity of nanomaterials. Indeed, toxic nanomaterials can be changed into non-toxic ones through simple surface modification. Silica, polymer and proteins are used most often as coating materials. Intraperitoneal injection of silica-coated magnetic nanomaterials resulted in distribution to various organs without apparent toxicity and penetration of the blood–brain barrier and without disturbing its function (Kim *et al.*, 2006). Polymers can be divided into synthetic and natural polymers. The representative synthetic polymers are polyethylene glycol, polyvinylpyrrolidone (PVP) and polyvinyl alcohol (PVA). Natural polymers include dextran, chitosan and gelatine. Polymer coating technology enables nanomaterials to be well dispersed in aqueous solution and also allows biocompatibility with low toxicity (Gupta and Gupta, 2005). Proteins are also widely used as coating materials for biocompatibility and specific/selective targeting. Albumin protein, which is a major serum protein, makes nanomaterials disperse well in aqueous solution and can bind to various lipophilic compounds effectively (Baker, 1998). In addition, TAT-peptide is used as a coating material to increase membrane permeability (Lewin *et al.*, 2000). GNP conjugated with TAT-peptide can penetrate even the nuclear membrane with high efficacy (Berry *et al.*, 2007; de la Fuente and Berry, 2005). Folic acid is also widely used as a coating material for nanoparticles. Conjugation of folic acid allows nanomaterials to target cancer cells specifically with less immune response (Zhang, Kohler and Zhang, 2002). PEG-coated 13-nm GNP accumulated in the liver and spleen with a long blood half-life after intravenous injection (Figure 7).

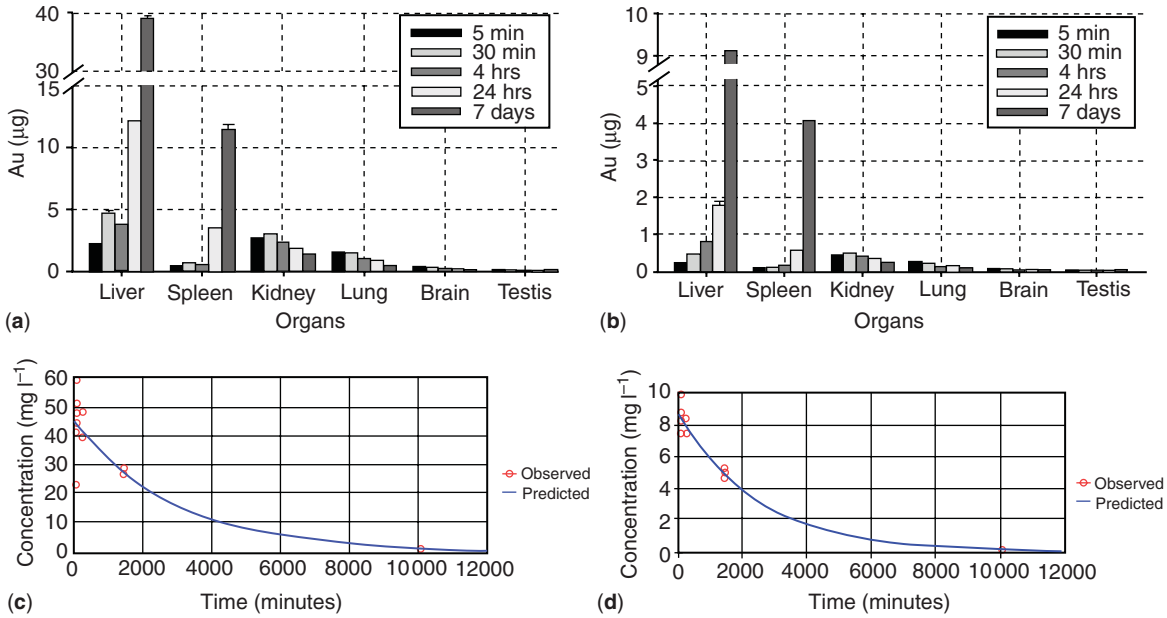

Figure 7. Tissue distribution and pharmacokinetic analysis of PEG-coated AuNPs in major organs at several different times. Tissue distributions of PEG-coated AuNPs were measured by inductively coupled plasma-mass spectrometry (ICP-MS). Mice in the (a) 4.26 mg/kg and (b) 0.85 mg/kg groups showed similar distribution and excretion patterns of PEG-coated AuNPs ($n = 3$ per group). Each group contained three animals and results are shown as mean values with SDs. Predicted and observed plasma concentration-time profiles of PEG-coated AuNPs after a single intravenous administration of PEG-coated AuNPs at (c) 4.26 mg/kg and (d) 0.85 mg/kg ($n = 3$ per group).

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Surface charge also influences the toxicity of nanomaterials. The surface charge of nanomaterials can depend on the nature of the nanomaterials and the dispersion media. The charge of nanomaterials can influence host responses (e.g., phagocytosis, inflammation and genotoxicity) and kinetics (e.g., absorption, distribution and elimination) *in vivo* and *in vitro*. For example, carboxyl-terminated quantum dots are more toxic than amine-terminated quantum dots, possibly due to platelet aggregation and vascular thrombosis (Geys *et al.*, 2008). Carboxyl-terminated quantum dots also have a shorter blood half-life than amine-terminated quantum dots (Geys *et al.*, 2008). In addition, carboxyl-terminated quantum dots are more easily taken up by macrophages with little impact on cell viability compared with amine-terminated and pegylated quantum dots (Clift *et al.*, 2008). However, little is known about the impact of surface charge of nanomaterials; thus, further extensive studies are needed.

2.4 Dispersion Media

Nanomaterials can aggregate in both solution and powder form. Industrial nanomaterials (e.g., zinc oxide, TiO_2 and cerium oxide) and environmental nanomaterials (e.g., diesel soot nanoparticles and carbon black) especially tend to aggregate. Aggregated nanomaterials differ from well-dispersed nanomaterials in their size, surface charge, and so on. (Bihari *et al.*, 2008). In addition, aggregated micro-sized particles have been shown to exert different *in vivo* and *in vitro* toxicity compared with well-dispersed nanomaterials (Buford, Hamilton RF and Holian, 2007; Foucaud *et al.*, 2007). Therefore, toxicity data from these aggregated particles are not appropriate and cannot represent the real toxic effects of nanomaterials. Well-dispersed single-walled carbon nanotubes are more prone to enter the alveolar walls and to be fibrogenic without granuloma formation compared with aggregated nanotubes (Mercer *et al.*, 2008). In addition, well-dispersed carbon nanotubes are less cytotoxic than aggregated nanotubes (Wick *et al.*, 2007). Generally, nanomaterials are sensitive to extreme pH conditions and physiological salt concentration. To generate well-dispersed nanomaterials in physiological solution, several dispersion stabilizers, such as pulmonary surfactant, Tween, bronchoalveolar lavage (BAL) fluid, dipalmitoyl phosphatidyl choline (DPPC), albumin or serum, have been suggested (Bihari *et al.*, 2008; Foucaud *et al.*, 2007; Murdock *et al.*, 2008).

3 SYSTEMS TOXICOLOGICAL APPROACH FOR RISK ASSESSMENT OF NANOMATERIALS

Systems biology is a new area of integrative study of biological systems at the molecular level. The comprehensive qualitative and quantitative data of mRNA, protein or other molecular components of the system coupled with a mathematical approach are the driving forces of systems biology (Edwards and Preston, 2008). Systems toxicology requires integrative data through toxico-genomics, toxico-proteomics and relative bioinformatics to analyse and predict the toxic effects of chemicals in biochemical processes (Figure 8)

Although the potential commercial application of nanomaterials is highly expected, concerns have been raised due to the uncertainty of toxicological interactions with living organisms including humans. The unique and various characteristics of nanomaterials in size, surface properties and surface area require a new and/or different approach beyond traditional methodology for evaluating the

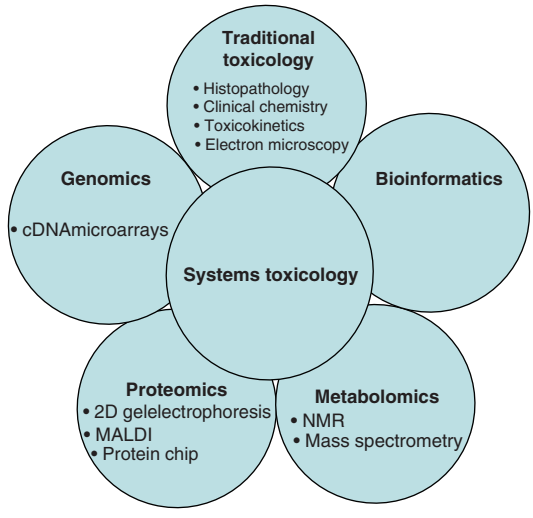

Figure 8. A scheme for systems toxicology. "Omics"technologies including genomics, proteomics and metabolomics, and bioinformatics and traditional toxicologies will lead to better understanding of systems toxicology.

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hazardous effects of nanomaterials on human health (Walker and Bucher, 2009). The conventional approach takes a long time for one-by-one toxicity testing on each nanomaterial and would probably hamper our enjoying the benefits of nanomaterials. Potential exposure routes for nanomaterials are gastrointestinal tract (GIT), skin, lung and vasculature. The nanomaterials have the ability to distribute throughout the body via interactions with cells, body fluids and proteins. Because of the systemic distribution of nanomaterials in the body, it is important to assess systemic responses. High-throughput results from toxico-genomics, toxico-proteomics and toxico-metabolomics would provide systemic and integrative information on the biological effects of nanomaterials, which should lead to efficient methods for risk assessment.

3.1 Toxicogenomics of Nanomaterials

Toxicogenomics is the study of the genome involvement in response to toxic substances and includes transcriptomics, proteomics and metabolomics. The major factors that determine the utility of nanomaterials in commercial or medical applications are biocompatibility, toxicity and the ability to penetrate cells (Poma and Giorgio, 2008). Nanomaterials may interact with intracellular proteins, DNA, RNA and other critical cellular molecules, and these interactions can enhance the toxicity. A bundle of single-walled carbon nanotubes (SWCNTs) was found to penetrate into cells and was detected in the nuclei of human macrophages (Porter *et al.*, 2007). SWCNTs also induced lipid peroxidation and oxidative stress through fusion with the plasma membrane (Manna and Ramesh, 2005; Cui *et al.*, 2005). Nanomaterials have ROS-generating capability and pro-inflammatory effects in the lung in a direct relationship with the surface area (Oberdorster,Oberdorster and Oberdorster, 2005; Donaldson *et al.*, 2006). Many studies have shown that ROS generation and oxidative stress are the mechanism of toxicity of inhaled nanoparticles. The oxidative stress results in airway inflammation and interstitial fibrosis. Antioxidant response elements activate the NF-E2-related factor (Nrf)-2 signalling pathway to promote phase II genes as a protective response. However, at higher levels of oxidative stress, this protective response is overwhelmed by inflammation and cytotoxicity or apoptosis. Inflammation is initiated by the activation of pro-inflammatory signalling cascades, such as mitogen-activated protein kinase (MAPK) and nuclear factor κB (NF-κB) cascades, whereas programmed cell death results from mitochondrial perturbation and the release of pro-apoptotic factors (Nel *et al.*, 2006). In addition to the toxicity through oxidative stress and inflammation, some nanomaterials may cause protein denaturation, membrane damage, DNA damage, immune reactivity and granuloma formation (Nel *et al.*, 2006).

The genomic approach may provide information about the critical mechanisms of action of nanomaterials at the molecular level and is valuable for hazard identification and characterization in the risk assessment process by identifying global changes of gene expression by nanomaterials (Gatzidou, Zira and Theocharis, 2007).

The most common nanomaterials are fullerenes, SWCNTs or multi-walled carbon nanotubes (MWCNTs), metal and metal oxide particles, polymer nanoparticles and quantum dots (Poma and Giorgio, 2008). A fullerene is any molecule composed entirely of carbon, in the form of a hollow sphere, ellipsoid or tube. Spherical fullerenes are also called buckyballs (C60) and cylindrical fullerenes are called carbon nanotubes or buckytubes. Many water-soluble fullerenes have been developed as anti-HIV and anticancer agents (Schinazi *et al.*, 1993; Toniolo *et al.*, 1994; Rajagopalan *et al.*, 1996; Chiang, Lu and Lin, 1995; Miyata and Yamakoshi, 1997). Fullerenes have many potential applications because of their unique free radical chemistry and antioxidant properties. Surface chemistry has an important influence on the toxicity of C60. It is known that water-soluble, monodisperse or colloidal fullerene aggregates induce ROS, lipid peroxidation and cytotoxicity (Nel *et al.*, 2006). However, attachment of malonyl groups to the C60 surface makes it a useful antioxidant (Oberdorster, Oberdorster and Oberdorster, 2005). The median lethal dose 50 (LD_{50}) for a water-soluble fullerene is 600 mg/kg BW when it is administered intraperitoneally into rats (Chen *et al.*, 1998). Fullerenes are negative in mutagenic and *in vitro* cytogenetic assays, but their oxidative damage has been identified (Henry *et al.*, 2007). Oberdorster (2004) found significant evidence of oxidative stress, that is, lipid peroxidation, in the brains and glutathione

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depletion in gills of juvenile largemouth bass exposed for 48 h to water containing uncoated fullerenes at 0.5 ppm. An increase in the numbers of BAL-recovered neutrophils, the lipid peroxidation values and the level of glutathione were observed after 1 week of intratracheal instillation of 1.5 and 3 mg/kg BW C60 in rats (Sayes *et al.*, 2007). Lai, Chen and Chiang (2000) also observed a significant increase in lipid peroxidation products but a decrease in the GSH level in intestine after intravenous administration of 1 mg/kg BW C60 in male dogs. A toxicogenomics study using larval zebrafish revealed that administration of C60 in water (C60-water) or suspension of C60 in tetrahydrofuran (THF) followed by evaporation, resuspension in water and nitrogen gas sparging (THF-C60) induced significant up-regulation of genes involved in oxidative damage with higher toxicity in the THF-C60 group than in the C60-water group (Henry *et al.*, 2007). These studies indicate that C60 induces toxic effects through oxidative stress and that the toxic potency depends on the dispersion media or manufacturing process.

Carbon nanotubes are long carbon-based tubes that are either single- or multi-walled and act as biopersistent fibers because of their insoluble and non-degradable properties in biological systems. The diameters of the tubes range from 0.7 to 1.5 nm for SWCNTs and 2 to 50 nm for MWCNTs. SWCNTs are more toxic than MWCNTs, multi-walled carbon nanoonions, C60, carbon black and graphite because of their geometry, fibrous structure and surface electrical properties (Rajagopalan *et al.*, 1996; Chiang, Lu and Lin, 1995; Lam *et al.*, 2004; Jia *et al.*, 2005), and underivatized SWCNT was more cytotoxic than SWCNT functionalized with phenyl-SO_3H, phenyl-$(COOH)_2$ and phenyl-SO_3Na (Sayes *et al.*, 2006). Several *in vitro* studies have shown oxidative damage and inflammatory changes by carbon nanotubes. ROS generation, lipid peroxidation, oxidative stress, mitochondrial dysfunction and changes in cell morphology were found in keratinocytes and bronchial epithelial cells following high doses of SWCNT (Shvedova *et al.*, 2003). Pro-inflammatory changes were observed in keratinocytes in response to MWCNT (Monteiro-Riviere *et al.*, 2005). Also, rodent studies involving intratracheal instillation of high doses of nanotubes demonstrated chronic lung inflammation, including foreign-body granuloma formation and interstitial fibrosis (Lam *et al.*, 2004; Shvedova *et al.*, 2005; Warheit *et al.*, 2004). One or 5 mg/kg BW SWCNT administered into the upper lung of rats induced acute lung toxicity and transient inflammation (Warheit *et al.*, 2004). Carbon nanotube-treated mice (0.04, 0.1 or 0.5 mg per mouse) developed progressive fibrosis and granulomas in lungs. Many *in vitro* and *in vivo* studies reported the toxic effects of carbon nanotubes, including anti-proliferative effects, decreased cell adhesion, necrosis, apoptosis and oxidative stress (Cui *et al.*, 2005; Lam *et al.*, 2004; Jia *et al.*, 2005; Shvedova *et al.*, 2003; Shvedova *et al.*, 2005; Muller *et al.*, 2005). AP-1 and NF-κB were activated in SWCNT-treated macrophage cells, indicating that the uptake of SWCNTs into the macrophages activated T cells, oxidative stress and release of proinflammatory cytokines and ultimately led to chronic pulmonary inflammation, fibrosis and granuloma formation (Chou *et al.*, 2008; Moss and Wong, 2006). In another study, SWCNTs suppressed inflammatory mediators, such as interleukin (IL)-8, IL-6, TNF-a and macrophage chemoattractant protein-1 (MCP-1), as well as the IL-8 promoter and IL-8 gene in human lung epithelial cells (A549 cells) and normal human primary bronchial epithelial cells (NHBE cell) (Herzog *et al.*, 2009). MWCNTs also suppressed systemic immune function via activation of cyclo-oxygenase enzymes in the spleen; that is, spleen cells exposed to MWCNTs partially recovered immune function by ibuprofen, a blocker of cyclo-oxygenase, and cyclo-oxygenase knock-out mice were not affected by MWCNTs (Mitchell *et al.*, 2009). Immune suppression and oxidative stress were also observed in toxicogenomics studies. MWCNTs induced significant over-expression of IL-10 and NAD(P)H quinine oxidoreductase 1 (NQO1) mRNA in murine spleen, but not in the lungs, upon 14-day exposure (Mitchell *et al.*, 2009). IL-10 is a suppressor cytokine of T-cell proliferation and cytokine responses and plays a key regulatory role in tolerance to exogenous antigens (Akdis and Blaser, 2001; Toniolo *et al.*, 1994). The pathway of oxidative stress represents inflammation, apoptosis or necrosis. When oxidative stress is at lower levels, cells usually induce antioxidant and detoxification enzymes controlled by the Nrf-2 signalling pathway, but at higher levels of oxidative stress, inflammation is initiated through the activation of pro-inflammatory signalling cascades such as MAPK and NF-κB cascades. Apoptosis is also the consequence of a high level of oxidative

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stress (Kabanov, 2006). NF-κB induction caused by SWCNT with high production of ROS was found in HaCaT cells (Foucaud *et al.*, 2007). In human embryonic kidney cells (HEK293 cells), SWCNTs up-regulated the expression of cell cycle-associated genes, such as p16, Bax, p57, Hrk, Cdc42 and Cdc37, but down-regulated other cell cycle genes, such as Cdk2, Cdk4, Cdk6 and cyclin D3, and reduced signal transduction-associated genes such as Mad2, Jak1, Ttk, Pcdha9 and Erk, implying that SWCNT-stimulated signals result in the reduction of cell adhesion, cell cycle arrest and apoptosis (Cui *et al.*, 2005). Oxidative stress responsive genes such as Hmox1, Hmox2, Cyp 1B1, Ccnc, Dnajb4 and Gadd45a were also highly induced by SWCNT in human BJ foreskin cells (Sarkar *et al.*, 2007).

There is growing evidence that synthetic polymers, when combined with the biological agents, can alter cellular responses via interactions with cellular plasma membranes (Omidi *et al.*, 2003; Kabanov *et al.*, 2005; Regnstrom and Burgess, 2005; Akhtar, 2006). Polypropyleneimine dendrimers (PPI-dendrimers), which were developed as non-viral cationic vectors for nucleic acids and as drug delivery system, induced apoptosis in human carcinoma cells (Kabanov *et al.*, 2006). A toxicogenomics study of PPI-DNA-transfected human epidermoid cells and human lung epithelial cells showed alterations of genes related to apoptosis and cytokine signalling (Omidi *et al.*, 2003). Lipofectin and oligofectamine, common transfection agents, also caused marked changes in gene expression in epithelial cells (Omidi *et al.*, 2003). Rpa1, bcl2a1, casp8, hsp70 and hspd1, which are involved in apoptosis, were over-expressed by formulations of lipofectin or oligofectamine. Down-regulation of anxa2, rxra and s100a8, ednrb and rp16 genes was also observed (Omidi *et al.*, 2003). The functional consequences of this transfection formula-induced gene expression were all related with increased early apoptosis. Conjugation of doxorubicin with poly[*N*-(2-hydroxypropyl) methacrylamide] (PHPMA) resulted in the induction of additional caspase-dependent apoptosis in human ovarian carcinoma cell lines compared to the drug only (Minko, Kopeckova and Kopecek, 2001). In fact, the polymer–drug conjugate induced apoptosis by up-regulating caspases 3, 6, 7, 8 and 9, whereas the free drug induced only caspases 3, 7 and 9. Inflammation was also reportedly induced by polymer formulations. Copolymer of acrylic acid with *N*-vinylpyrrolidone significantly increased the immune response to the conjugated low-molecular-mass trinitrophenyl group, which is not immunogenic by itself (Petrov *et al.*, 1977). Also, individual bacterial or viral antigens, which are not sufficiently immunogenic alone, induced specific immune responses when chemically conjugated to polyoxidonium (Petrov *et al.*, 1983; Petrov *et al.*, 1985). Many polymer excipients are generally regarded as safe (GRAS). However, polymer excipients cannot simply be considered inert when used in combination with biologically active agents. Overall, apoptosis and inflammation are induced by various polymer components in drug delivery formulations by changing the magnitude and directions of the responses, even though the polymer materials are safe alone. To date, it is not fully understood why polymers exhibit significant changes in the cellular responses to the delivered drugs.

Various pulmonary diseases including silicosis, tuberculosis, chronic bronchitis, chronic obstructive pulmonary disease, lung cancer and activation of inflammatory responses were reported to be induced by silica (Rimal, Greenberg and Rom, 2005; Cho *et al.*, 2007). The International Agency for Research on Cancer (IARC) has classified crystalline silica as a Group 1 human carcinogen (IARC, 1997). Inflammation-related genes such as IL-1B, IL-8, IL-6, TNF-a, MCP-1 and MIP-2 are up-regulated by ultrafine amorphous silica particles (UFASs) and then rapidly recovered based on the disappearance of inflammatory lesions in mice (Cho *et al.*, 2007; Le and Vilcek, 1987). Ultrafine TiO_2 causes inflammation, fibrosis, pulmonary damage and DNA damage with much stronger potency because of its size (Afaq *et al.*, 1998; Rahman *et al.*, 2002). TiO_2 has no impact on neurons but stimulates microglia, a macrophage-like phagocytic cell, to release ROS and consequently damages ROS-sensitive neurons in cultures of brain striatum (Long *et al.*, 2007).

Cobalt nanoparticles alter the expression of genes such as interferon-activated gene 203, RAB GTPase and degenerative spermatocyte homolog 1 and thus modulate the cellular pathways of inflammatory response in mouse fibroblast cells (Papis *et al.*, 2007). In the case of GNP, neither significant cytotoxicity nor any significant changes of gene expression were found in human breast cancer cell line (HeLa cells) (Khan *et al.*, 2007). However, silver nanoparticles (AgNPs) up-regulated the oxidative stress-related genes such as superoxide

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dismutase-3 (sod-3) and dauer formation protein (daf-12) with reproductive failure in *Caenorhabditis elegans* (Roh *et al.*, 2009).

As a whole, toxicogenomics studies provide a large amount of information about the mechanisms of action of nanomaterials. In many cases, oxidative stress, apoptosis and altered immune function are the main modes of toxicity of nanomaterials. The brain and the immune system are assumed to be the target organs of nanomaterials because these organs are vulnerable to oxidative stress.

3.2 Toxico-Proteomics and Toxico-Metabolomics of Nanomaterials

Toxico-proteomics and toxico-metabolomics have been useful approaches in the identification of key biochemical pathways, mechanisms and biomarkers for the assessment of exposure and toxicity, and may lessen the uncertainties in the process of human health risk assessment (Ge, Preston and Owen, 2007). The goal of toxico-proteomics in nanomaterials is the elucidation of the extent and nature of nanomaterial–protein and protein–protein interactions. In particular, proteomics and metabolomics provide direct information on the final integrated phenotype of an organism because proteins and metabolites are the final downstream products of gene and enzyme regulation (Waters and Fostel, 2004).

When HEK293 cells were exposed to SWCNTs, adhesion-associated proteins, such as laminin, fibronectin, cadherin, FAK and collagen IV, were down-regulated, which led to the inhibition of cell adhesion and decreased cell proliferation (Cui *et al.*, 2005). Those findings indicate that cells make active, self-protective responses via isolation of the cells attacked by SWCNTs from the remaining cell mass.

Fowler, Conner and Yamauchi (2005, 2008) demonstrated marked differences in protein expression patterns in renal tubule cells of male rodents in response to particles of the binary semiconductor compounds [III–V semiconductors gallium arsenide (GaAs) or indium arsenide (InAs)]. They also observed compound- and time-specific proteinuria patterns with sex-dependent differences such that there were greater responses in female rats than in males. In other studies, GaAs or InAs also altered the patterns of proteins involved in the heme biosynthetic pathways; that is, there were higher levels of the heme precursors, delta-amino levulinic acid (ALA) and porphyrins in the urine of rats administered GaAs intratracheally or of Syrian golden hamsters injected with InAS subcutaneously (Goering, Maronpot and Fowler, 1988; Conner *et al.*, 1993; Conner, Yamauchi and Fowler, 1995). The proteomics data of GaAs and InAs suggest a possible integral suppression effect on heme biosynthesis, but further studies are required to elucidate the molecular mechanism and its consequences of anaemia or neurological diseases. Abnormal heme metabolism is known to cause systemic disorders including decreased heme biosynthesis, neurological disturbances, hepatic injury and skin fragility (Akagi *et al.*, 2004). In addition, free heme, largely liberated from hemeproteins under oxidative conditions, is known to stimulate generation of ROS that induce neural death (Dwyer *et al.*, 2006).

4 RISK ASSESSMENT

Toxicogenomics data, including toxico-proteomics and toxico-metabolomics, are valuable in the identification of biological pathways when the toxic mechanism is not clear. It is true that toxicogenomics studies are not currently sufficient to replace existing testing regimens in risk assessment and regulatory toxicology. However, toxicogenomic technology can be applied to the risk assessment by providing insights into the toxic mechanism of action (Pennie, Pettit and Lord, 2004; Boverhof and Zacharewski, 2006), because risk assessment considers the hazard with the dose–response relationship, as well as the estimated exposure amount and the comparison between no-observed-adverse-effect-level (NOAEL) and estimated exposure amount, to determine the margin of safety for exposure to the substance. Inhalation of nanomaterials is considered a major route into the body. However, nanomaterials can be ingested directly via food, water, cosmetics or drugs. Most nanoparticles are rapidly eliminated via feces, although some nanoparticles in food are infrequently taken up into gut lymphatics and distributed to other organs (Kreyling *et al.*, 2002). When in contact with skin, nanomaterials smaller than 50 nm tend to penetrate the skin more easily and are sometimes localized in lymph nodes (Oberdorster,Oberdorster and Oberdorster, 2005). Therefore, it is difficult to perform risk assessment of nanomaterials because there are

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too many factors that influence biological effects. Immune dysfunction and inflammation caused or triggered by oxidative stress have been identified as possible mechanisms underlying the aetiology of nanoparticle-associated toxicities (Dick *et al.*, 2003; Xiao *et al.*, 2003) and are supported by numerous toxicogenomics results. Also, disorder of the heme biosynthesis pathway, as suggested from toxico-proteomics and toxico-metabonomics studies, is another candidate mechanism of toxicity of semiconductors. However, there are several debates on the inflammation and oxidative stress paradigms that provide a starting point for toxicological investigation of nanomaterials. In the case of cationic dendrimers, they appear to be related not with oxidative stress, but with disruption of cell membrane integrity through interaction of the positively charged dendrimer terminal groups and the anionic lipids composing the cell membrane (Mecke *et al.*, 2004). Another debate is that the toxicities do not come from the nanomaterial itself, but rather from contaminants that adsorb to the surface during manufacturing or transport (Xiao *et al.*, 2003; Li *et al.*, 2003). Non-purified, iron-rich CNTs are more potent inducers of oxidative stress in macrophages than purified CNTs with low iron content (Kagan *et al.*, 2006). In addition, coating materials of nanomaterials are important factors for toxicity; for instance, polyethylene glycol-coated quantum dots are non-toxic, whereas amine surfaces are cytotoxic and carboxyl surfaces are both cytotoxic and inflammatory (Warheit *et al.*, 2007a, 2007b). Thus, during risk assessment of nanomaterials for human health, various factors must be considered, such as surface properties, impurities, manufacturing process, etc.

There are several acute toxicity studies of nanomaterials. The oral LD_{50} of nanomaterials including fullerene, multi-walled CNT, Fe_3O_4, WN, Cu or TiO_2 ranged from >0.005 to >5 g/kg BW, with no evidence of toxicity in rats and mice (Carrero-Sanchez *et al.*, 2006; Chen *et al.*, 2006; Mori *et al.*, 2006; Xia *et al.*, 2005; Wang *et al.*, 2006, 2007). Intraperitoneal administration of nanomaterials yielded LD_{50} values ranging from 0.040 to >2.5 g/kg BW, with no evidence of toxicity or damage in liver or kidney (Carrero-Sanchez *et al.*, 2006; Chen *et al.*, 2006; Xia *et al.*, 2005; Moussa *et al.*, 1996). Most of the acute toxicity data did not represent the exact toxic potency because no deaths were observed up to the maximum dose administered. A single oral dose of Fe_3O_4, Cu and TiO_2 resulted in kidney damage such as tubular dilation, casts, glomerular swelling and proximal tubule necrosis, liver lesions with hydropic degeneration and steanosis, and spleen atrophy (Chen *et al.*, 2006; Wang *et al.*, 2006, 2007). Nanomaterials are commonly taken up by the reticuloendothelial system (RES), which explains why liver and spleen are target organs for nanomaterials. The kidney is also a common target organ of toxicity for nanomaterials as this organ is the primary clearance route for many nanomaterials, including CNTs, water-soluble polyalkyl-sulfonate fullerenes and low-generation dendrimers (Chen *et al.*, 2006; Wang *et al.*, 2004). Many inhalation studies presented with inflammation and fibrosis and, in some cases, lung tumours. The pulmonary toxicity of nanoparticles is mainly explained by lung overload that overwhelms pulmonary clearance mechanisms (ILSI, 2000; Stern and McNeil, 2008). Nanopaticles easily cause lung overload at lower doses due to their larger surface area that correlates with decreased alveolar macrophage clearance capacity (Moss and Wong, 2006). In the case of CNTs, the formation of pulmonary granulomatous lesions is highly related to agglomerated CNTs (Lam *et al.*, 2004). Positive mutagenic potential, mediated by lipid peroxides, is reported for irradiated fullerene, but *in vivo* tumour studies showed no evidence of carcinogenesis (Sera, Tokiwa and Miyata, 1996).

An important route of exposure to nanomaterials is inhalation. The transfer of particles from the lung to the circulation and secondary organs is of concern because inhaled nanoparticles directly induce systemic disease, such as cardiovascular disease involving platelet aggregation or thrombosis (Radomski *et al.*, 2005). Inhaled nanoparticles translocating from the nasal epithelium to neurons in the olfactory bulb were observed in several studies (Elder and Oberdörster, 2006; Oberdörster *et al.*, 2004). However, CNS uptake of inhaled nanoparticles has not yet been seen in humans. Although in many cases of dermal or oral exposure, nanoparticles showed limited dermal penetration or GI absorption, the adsorption is dependent on the size, shape and surface properties of the nanoparticles (Nohynek *et al.*, 2007). However, the interactions with subcellular structures need to be investigated in studies of the biologic and toxicologic effects of nanomaterials (Oberdorster,Oberdorster and Oberdorster, 2005).

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Furthermore, there are limitations of the analytical methods for the quantitation or tracing of nanomaterials in environmental sources and foods that make for added difficulties in exposure assessment for nanomaterials. Nanotoxicology relies on many analytical methods for the characterization of nanomaterials as well as their impacts on *in vitro* and *in vivo* function. Recently, transmission electron microscopy (TEM) electron-dispersive X-ray analysis (EDS) was used to confirm the presence of silver nanoparticles within cells, and electron energy loss spectroscopy (EELS) was used in conjunction with TEM for elemental confirmation of carbon nanotube uptake. Inductively coupled plasma-atomic emission spectroscopy (ICP-AES) was introduced as a powerful technique for the quantification of internalized nanoparticles and had sub-ppb detection limits, high precision and a dynamic range of five orders of magnitude or more (Marquis *et al.*, 2009). Development of assay techniques for the quantitative determination of various kinds of nanomaterials is required for risk assessment.

Further toxicological studies are required for quantitative risk assessment of the human health impact of nanomaterials. Risk assessments of nanomaterials also need to be performed separately for each functional or structural group because the toxicities may vary according to the properties of the nanomaterials such as size, shape, agglomeration state, solubility and surface charge.

5 CONCLUSIONS

Production of ROS and inflammation caused by oxidative stress are key toxicity mechanisms of nanomaterials. Many toxicogenomic and toxicoproteomic studies support this toxic mode of action of nanomaterials. Even though many toxicological studies have been performed for multiple nanomaterials, further confirmation is required before a hazard can be identified and characterized. It is currently impossible to systematically link the properties of nanomaterials to the biological effects. In addition, the most relevant toxicological endpoints, threshold levels that do not have any toxicological concerns, are still unclear, although some studies have presented dose–response relationships. Exposure assessment is also hampered by difficulties in monitoring exposure patterns and levels of various nanomaterials in the workplace and the environment. Considering the present level of knowledge, it is not possible to establish threshold levels for each nanomaterial due to limited toxicological and exposure data. Further studies are required for systemic risk assessment of human health impact considering various properties of nanomaterials. To obtain more integrated and systemic data, genomics and proteomics approaches may provide important information by combining hypothesis-forming and hypothesis-driven research for the risk assessment.

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RELATED ARTICLES

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Implementing Systems Toxicology in Drug Development for Regulatory Decision Making

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1 PROMISE

The development of 'omics technologies and completion of the human genome project in the past decade have set an expectation for an exponential positive impact on human health. The FDA's Critical Path report (FDA, 2004), in fact, called for a focus on these applications as ways to enhance drug development and improve the safety and efficacy of drugs. Expanded measurement platforms and improved biological understanding should lead to more and better biomarkers, which would ultimately be able to predict and demonstrate efficacy while minimizing adverse effects of new therapeutics. Overall, drug safety would be further enhanced by targeting treatment to that patient population most likely to benefit. The problem with expectations is that often the reality does not live up to the promise within the hoped for timeframe. In this case, as discussed below, the delay in achieving expectations is driven by (i) the complexity of the biological systems we are attempting to monitor, (ii) the realization that the level of understanding needed to qualify new endpoints will take far more investment in generating new knowledge, and (iii) the high evidentiary standard needed prior to effective implementation in a regulated drug-development setting. These hurdles each require significant time and effort to surmount.

This does not mean that progress has not been made, or that expectations will not be eventually realized. In this chapter, we will explore the progress toward improved risk assessment in drug development through the application of newer tools and technologies within the umbrella concept of systems toxicology and fit-for-purpose standards. We will discuss the path that runs from the development of biomarkers on a whole animal basis, reflecting an integrated understanding of pharmacological and toxicological processes, rather than on an individual pathway by pathway, or gene by gene, basis and the application of these markers to inform a range of needs, from in-house screening to investigations into mechanisms of action to monitoring for toxicity in a clinical population. The process is a continuum, from the standpoint of both the science and the increasing "burden of proof" as one gets closer to making decisions concerning human health. We find that there are layers of interacting and overlapping biological processes whose details need to be elucidated before we can more accurately predict responder and non-responder populations, human or animal. And herein lies the issue for regulatory acceptance of systems toxicology – what level of confidence needs to be reached for a given purpose? What is the bar above which a set of data not only supports a hypothesis, but can be related with

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confidence to have clinical meaning? How much supporting data and how wide acceptance in the scientific community is necessary to be confident of what a marker is reporting? And, finally, how solid is "solid enough" for regulatory application, given that we never expect to achieve 100% understanding and accuracy.

For purposes of this chapter, we need to define two specific concepts – systems toxicology and biomarkers. Although there are various descriptions in the literature, the concept of systems toxicology is generally accepted to refer to a broad understanding of all the component biological processes that interact to result in a given phenotype in an animal, with information at the gene and protein level complementing end-points measured in the whole animal (Kitano, 2002; Edwards and Preston, 2008). To achieve this requires an integration of knowledge from scientific dogma, laboratory experience, and various end-point measurements. Conceptually, the process is actually an enhancement of the way risk is currently evaluated in drug development. Multiple studies, and the measurement of multiple parameters within each, builds up to a more and more complete picture of a compound's on- and off-target activities. The future state revolves around the kinds of additional value-added measurements we can make based on evolving tools and technologies, and how well we are able to mine and integrate *all* relevant information to produce a better system-wide assessment of contributing biological events that lead to an adverse phenotype.

Biomarkers can be thought of as end-point measurements, whether a single assay or an algorithm based on multiple, independent measurements, that provide a readout of a phenotypic manifestation. The official NIH definition of a biomarker (Biomarkers Definitions Working Group, 2001) is: "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention". Certainly, there are well-established biomarkers currently used in medical practice. Every medical test ordered by a physician can be considered a biomarker which has been "validated" by years of experience and an understanding of its correlation with a given disease phenotype. And again, running multiple tests serves to provide a broader picture of an individual's health, allowing the sorting of various possible diagnoses more accurately. In the context of a system toxicology approach, however, the question often posed is whether correlation is always enough. To realize the promises of systems toxicology and 'omics tools, we must move beyond just correlation, to a more coherent, evidence-based and integrated biological understanding, which presents a much higher bar for acceptance and future reliance.

2 PROCESS OF SYSTEMS TOXICOLOGY

It is sometimes helpful to define the desired future state, then work backwards to outline a high-level roadmap to achieve this goal. In this section, we will explore what such a roadmap might look like. It is generally acknowledged that "The labor required to deliver a new generation of biomarkers to clinical practice will be long and hard" (Woodcock, 2009). Two recent articles have looked at the systems toxicology landscape (Edwards and Preston, 2008; MacDonald and Robertson, 2009) and present excellent overviews of some of the issues that need to be addressed. For the pharmaceutical industry and regulatory bodies alike, the ultimate goal is to assess the potential risk of a therapeutic drug, in the context of its benefit to people, and to be able to easily monitor adverse effects with non-invasive end-points which are manifested prior to the occurrence of irreversible adverse effects.

In discussions of the future state, the concept of fit-for-purpose is a major driver. The FDA guidance on pharmacogenomic data submissions discusses the need to describe the context in which data are to be assessed. In FAQs clarifying this document (http://www.fda.gov/Drugs/ScienceResearch/ResearchAreas/Pharmacogenetics/ucm083893.htm), the agency specifically refers to this as "fit-for-purpose". Likewise, European Medicines Agency (EMA), in its Guidance on Genomic Biomarkers (ICH Topic E16; http://www.ema.europa.eu/pdfs/human/ich/38063609endraft.pdf), discusses the need to define the "context" of use. Essentially, these documents build from earlier established concepts which recognize that, rather than considering a marker either valid or invalid, there is value in examining the specific purpose for which a marker is proposed, and having that specific goal inform the type and amount of data needed to support the claim (Wagner, Williams and Webster, 2007; Woodcock, 2009). The technical aspects of an assay validation strategy have been more fully explored

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by Lee *et al*. (2006) who considers various stages in biomarker qualification, including pre-validation of methods, exploratory and in-study validation (i.e. robustness and reproducibility of the method), and "advanced" validation (a broadening of experience with biomarker performance). And, in fact, the technical roadmap toward providing an evidentiary standard has already been in effect in the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) process for evaluation of proposed assays as alternatives to animal testing. The NICEATM/ICCVAM website (iccvam.niehs.nih.gov/) provides both high-level information on evaluating the usefulness and limitations of test methods proposed for use in a regulatory environment, and a specific roadmap to technical requirements for sponsors to show that tests are "scientifically valid for their intended purpose" (ICCVAM Biennial Report – http://iccvam.niehs.nih.gov/docs/annrpt/BiennialRpt2007-508.pdf).

Looking earlier in the drug-development timeline, the question that must be answered even before application in regulated phases of drug development and regulatory decision-making is whether the biomarker(s) have been rigorously enough qualified even for internal decision-making. Stated more pointedly, what is our level of trust and confidence in the biomarker at a stage where time, resources, and financial gain are at risk, but not human clinical trial safety? Would we depend on the readout for critical decisions pre-clinically? If so, the question is what body of evidence do we need that provides that level of confidence? The answer to this question may reflect, in large part, the strategy toward regulatory acceptance. Given confidence at that level, the step to clinical translation should be more straightforward. With this information in hand, we can define the technical strategies for reducing biomarkers to practice.

Hartung (2009) has articulated a number of considerations in this portion of the roadmap, the steps critical for implementation. The purpose at this stage is to evaluate a biomarker(s) to understand strengths and limitations, gaps that need to be filled, intermediate uses, etc. To achieve this, Hartung proposes a "stairway" approach focusing on quality assurance of the assay to be used to measure the marker, followed by establishment of an evaluation/validation strategy. In our experience, the test set of samples becomes critical at this point. Beyond samples to establish specificity and sensitivity of a marker, as well as to enable comparison with literature or database information, it is necessary to establish the "truth". In standard toxicology studies, not only are multiple parameters assessed, but the incidence and severity of each end-point is taken into account. A judgment is then made with respect to the risk of toxicity at a dose level and/or time for a test material. For individual samples, however, the outcome may be somewhat more ambiguous, or may represent only part of response, complicating the anchoring process. Take, for instance, a case in which five of 10 animals show hepatocellular necrosis histologically at a given dose/time-point. In assessing a liver injury biomarker, does one consider five animals positive for the histology anchor and five negative? It is conceivable that some among the five "negative" animals are indeed affected, but perhaps no histopathology was observed in the sample of liver obtained. They may also be quite likely to show at least a graded response, if not a full positive response, indicating that some level of toxicity has occurred, even though it has not yet been manifested as histology. A statistical approach referred to as the receiver operating characteristic exclusion analysis has been deployed in consideration of such instances, so that new high-performance biomarkers are not inappropriately penalized (Sistare *et al*., 2010).

The question as to the purpose for which the marker is being developed comes into play at this point as well. The relevant question during the initial phases of biomarker development for pre-clinical or clinical application is whether the purpose is to define diagnostic, prodromal, or predictive markers. A working definition of a diagnostic marker would be one that is used to establish the presence of a "gold standard" condition. For instance, the appearance of Kim-1 in urine indicates nephrotoxicity has occurred, and can report, in most cases, a histopathological diagnosis (Bonventre, 2009). Early in the drug-development process, the need is for a rapid, compound-sparing approach to reliably triage a number of active compounds to enable an internal decision as to which have the highest probability of becoming safe and effective drugs. The initial readout can be essentially digital – higher probability of being toxic versus lower probability of toxicity at a given exposure – because subsequent testing will verify the safety of the compound. As greater predictivity is required, the biomarker would need to report more of a continuum of toxic

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response, from low to high. Many clinical and preclinical markers in use today diagnose that a disease or other adverse condition exists at a stage when a certain amount of damage is already present, that is, enough to trigger the biomarker.

In a clinical scenario, there is much value in diagnostic markers that are early and sensitive enough to report minimal, reversible damage, and with which risk can be monitored and managed. Even better would be prodromal and predictive markers. Prodromal and predictive biomarkers can be defined as those that precede a conventionally observed event, raising a warning flag of impending risk. The prodromal marker represents the earliest response and may be involved in one of the causal events or may be one aspect of a multi-factorial response to an insult. But in any case, it defines a point where the whole animal has initiated a process, but may still adapt and recover, rather than already being committed to presenting with a full adverse response. Development of this type of biomarker demands an ability to monitor multiple events within the context of the whole organism, and also to understand their collective significance. A predictive biomarker would also precede in time and foretell the appearance of a conventionally observed future event, but may not be a component of the adverse response set into motion. Prodromal and predictive biomarkers are far more challenging to experimentally qualify.

This line of thinking leads to another critical question: "Where does a relevant effect start?" (Hartung, 2009). And this is where a systems toxicology perspective becomes a critical consideration. To continue the discussion begun above, although five of 10 animals do not show histopathology, and may or may not show a response to a specific biomarker, are there other indications that the animals are indeed affected by the test material? From another perspective, if the tested biomarker reports out changes within a given pathway, what about other pathways? An example is the case of tumorigenesis. It is well established that tumor formation may require changes in proliferation, apoptosis, DNA damage/repair, and/or angiogenesis pathways, among others. Which are necessary, which are just sufficient, what is the critical interaction or tipping point? The answers depend upon a systems' wide understanding. Similarly, when markers of oxidative stress are being seen in response to a test compound, what is happening with pathways recognized as relevant to protection from oxidative stress? Will they be overwhelmed leading to manifestation of toxicity in the whole animal, or will the animal compensate for the insult with minimal adverse reaction? The systems-wide view becomes critical to an in-depth understanding of processes *contributing to* underlying toxicity risk, in contrast to measuring a marker that *correlates* with risk.

To follow the roadmap to its initiation, we need to evaluate the source of novel biomarkers. The "ideal" properties of a biomarker may differ somewhat with the question being addressed or the context of use (see, e.g. the discussion of Zhang *et al*. 2009 on the characteristics and strategy for developing a biomarker for post-traumatic stress disorder where the diagnosis is complex and multifactorial versus Johann *et al*. 2010 who describe a more defined proteomic evaluation of tissue from a patient with renal cell carcinoma.) But in general, one should be able to demonstrate organ-specific derivation, sensitivity in the sense of the marker appearing prior to irreversible organ damage, and predictivity of severity and reversibility. Additionally, an ideal biomarker would be easily distinguished from biological noise, should be non-invasive, and should bridge pre-clinical and clinical applications (i.e. it is desirable that a marker useful in pre-clinical species should be applicable in humans, and that human markers should bridge back to pre-clinical species as well). Although it is relatively straightforward to design the ideal toxicity biomarker, the process from discovery to evaluation through qualification represents a continuum, with different purposes, and therefore different levels of "proof". Many times, suggestions for novel markers are generated based on the academic literature, although in most cases these are based on limited observations. Often, the number of compounds tested by individual laboratories is too small to speak to sensitivity, specificity, or how generalizable a proposed biomarker might be. Consortia among industries (ILSI/HESI), between academia or government laboratories and industry (fNIH), or between industry and regulatory agencies (PSTC) have a larger pool of resources to devise and execute on a strategy to evaluate the most promising biomarkers. This is time-consuming, and by nature focuses on a relatively narrow goal, rather than the broadest understanding in the context of systems toxicology, but is a necessary step. The bottom line is that the field has found the task of moving potential biomarkers through to regulatory consideration and acceptance to be a lot more complicated and

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resource intensive than simply applying the new technologies along with accepted ante-mortem and post-mortem evaluations.

3 PROGRESS

At the crux of the issue is the complexity of the biology we seek to understand (Schrattenholz and Soskic, 2008). Any number of end-points (using 'omics technologies, biochemistry, histology, clinical chemistry, etc.) can be measured and evaluated for correlation with phenotypic anchoring. However, although this provides a large body of information, the data need to be integrated, classified as to relevance, and ultimately understood in the context of the question being addressed. Given multiple measurements from *in vivo* and/or *in vitro* systems, we can begin to build toward an understanding of what to expect in pre-clinical species. Some data will be confirmatory of other observations, and some will need to be set aside as potential "red herrings". Experience will eventually determine the meaning, or lack thereof, for these "outlier" measurements. Taking this system-wide view allows us to begin to overcome the complexity, but still does not necessarily allow yes/no answers unless far more investment is made in independent and rigorous conformational studies.

In point of fact, the label "systems toxicology" could be applied to the process used in drug development today. A regulatory package consists of ante-mortem and post-mortem data from multiple pre-clinical species, as well as data from pharmacology, drug metabolism, and other disciplines. The sum total of the data produces a broad picture of the whole animal response to the drug that is extrapolated to humans, taking into account risk/benefit, exposure margins, knowledge of species differences, etc. Early on, the integrated information informs the safety of phase I studies, although there are gaps in our understanding. With additional animal and clinical information, a more complete, integrated picture emerges, although experience suggests that there will still be some ambiguous information around which an informed, but subjective, assessment as to relevance needs to be made. Ultimately, it is the sum total of these data in pre-clinical and small, human studies, which provides a broad picture of the risk/benefit of the drug, and serves as the basis for ultimate approval or disapproval of permission to pursue clinical development, and ultimately for marketing a compound for a given therapeutic purpose. Even at this point, after having applied the best available science, experience shows that inter-individual differences in response in terms of both efficacy and toxicity may surface once the exposed human population is expanded (Harrill and Rusyn, 2008).

Drawing a parallel for biomarkers, the question is how do we arrive at an understanding of the appropriate interpretation of multiple measurements from models novel to our current drug development experience (e.g. the range of *in vitro* models currently available)? And how can we use these data to complement, rather than confuse, the standard pre-clinical assessments, to increase our understanding of the biological response in people, thus positively impacting our ability to accurately predict risk? Ultimately, will this additional information allow the prediction of the inter-individual responses that currently are difficult to account for within a population? We submit that progress is being made toward these goals.

For example, Woods *et al.* (2009) have recently published a study on the Nrf2-mediated adaptive response in mouse that might serve as an example of a desired future state. Given that alteration of the Nrf2 pathway is associated with oxidative stress, one could simply monitor for effects upon xenobiotic exposure by measuring a rate-limiting step or end product in the pathway. What these authors demonstrated, however, was the dose-dependent "evolution" of pathways perturbed by exposure to hypochlorous acid. At low doses, alterations in Nrf2 response, inflammatory pathways and protein ubiquitination were predominant. At these concentrations, genes associated with cell-cycle regulation and gene transcription were suppressed. At higher, but still non-cytotoxic concentrations of HOCl, Nrf-2 gene responses decreased and secondary stress responses, such as damage-inducible genes, were increased. At still higher and cytotoxic concentrations, pathways associated with protein and DNA damage, such as Gadd45, were increased. Looking at this holistic view, one not only has a biomarker of toxicity measured at a "snapshot in time", but also more of an understanding of the biology changing prior to and in concert with the Nrf2-regulated antioxidant defense program. This is the type of biological understanding that, expanded and augmented by other data, presents us with the

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opportunity to identify earlier, perhaps more sensitive indications of toxicity. These could ultimately be used in: (i) clinical studies to monitor and intervene in a patient prior to an irreversible injury, or (ii) pre-clinical animal studies to shorten the duration of studies needed in order to make go/no go decisions.

A second approach is proposed by Andersen and Krewski (2009), who, in defining a novel vision for toxicity testing in the twenty-first century, see the application of *in vitro* tests and computational models around the concept of identifying and avoiding "significant perturbations" of pathways associated with ultimate development of a toxicologic phenotype. The idea of using high-throughput screening in well-defined model systems is an attractive one, and has been used extensively in industry early in the drug-development process. It is also the basis of the EPA's ToxCast project (Dix *et al*., 2007) which seeks to categorize risk based on *in vitro* testing in order to triage chemicals that are most likely to be of risk to people, and therefore should be subjected to more testing on a priority basis. A number of tests are currently undergoing a qualification process within this project (http://www.epa.gov/ncct/toxcast/), to define those most highly correlated with toxicity pathways for specific chemical classes, with pharmaceuticals targeted for assessment in the near future. Given successful qualification/understanding of the models, one can envision making decisions as to potential risk using an integrated assessment of toxicity and compensatory pathways in *in vitro* and *in vivo* models. Comparison of the data with compounds for which we have relevant human information, and considering issues such as margins of exposure, expected metabolism, etc., could further our confidence that we are truly moving forward those compounds with the lowest probability of risk.

Yet, a third concept is proposed by Wills and Mitchell (2009) around the combination of cell culture, in silico modeling, and mapping of data to human outcomes. They argue that using current toxicogenomics practices of pattern recognition and bioinformatics approaches can be limiting as resources usually do not permit as broad an experimental approach as desired. However, if modes of toxicity relevant to human can be recapitulated in *in vitro* systems and brought together, the resulting data set would more fully support modeling of the expected toxicological response in the whole organism. The *in vitro* systems would be particularly amenable to performing dose–toxicity and dose–time experiments which can be difficult and expensive *in vivo*. The caveat, of course, is that one needs to understand the strengths and limitations of each *in vitro* model to be employed to generate relevant data.

4 CHALLENGE

To bring the roadmap full circle, we come back to the types of information, models, etc. that should be considered in forming an integrated picture of human risk, given that this is ultimately the goal of systems toxicology applied in the regulatory environment. We could sum up by saying that system-wide integration of data equals more knowledge and therefore more confidence that we have met a high bar of rigorous science. But although this is ideal, the question is whether it is achievable in a reasonable timeframe. The key word in the statement above is "knowledge". The amount of data (information) available in the literature around well-researched pathways is growing exponentially. In reality, both public and internal data represent observations that are snapshots in time and dependent upon dose, species, time of exposure, ADME, and a host of other factors. Informatic assessments attempt to consolidate the results and draw patterns, but these do not truly represent knowledge. Rather these efforts usually lead to testable hypotheses which have a higher probability of moving us one step closer to system-wide knowledge. It is evident that we are flooded with information, not only in toxicology but in essentially every aspect of our current world. The issue is how to strip away the extraneous, until one is left with true insight.

Clearly, as discussed above, we need to take advantage of every potential *in vivo* and *in vitro* tool to try to put together the pieces of the systems toxicology puzzle based on rigorous science. The challenge for moving systems toxicology into the regulatory arena, then, becomes one of how much do we need to understand ahead of time, what is the extent of a priori proof, before one can apply a new approach. Do we need to completely characterize the biology around biomarkers and have prospective data in-hand before beginning to apply them? Or can we integrate newer scientific approaches in smaller steps? Clearly, the question not only for regulatory authorities and industry, but also for the

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public sector, is how much risk we are collectively willing to take.

For instance, we all understand that correlations are not equivalent to real understanding. Yet in clinical practice, diagnoses are made and treatments prescribed based on correlations confirmed by experience. We "know" from years of observation that elevated serum transaminases, such as ALT, generally mean damage to the liver. We also know that there are cases in which such transaminases are transient and do not appear to lead to compromised liver function, or arise as a result of other factors or disease states (Korones, Brown and Palis, 2001; de Denus *et al.*, 2004). The current state of knowledge arises from both a basic understanding of the biology surrounding serum transaminases and experiential information gained from proven diagnostic ability in the clinic. This could provide the paradigm for use of novel biomarkers. As no single biomarker or clinical (or pre-clinical, for that matter) test is considered definitive, a battery of test results is always available for assessment of risk, so there is little risk as novel markers are assessed. Based on the experience with these new markers, judgments can be made with respect to whether and when to move novel biomarkers into regulatory practice as reliable on their own.

The EPA's ToxCast program (Dix *et al.*, 2007) is an example of a practical, stepwise approach. The initial question is straightforward – given a set of test compounds, does any existing *in vitro* assay correlate well enough to identify chemicals with the highest risk of toxicity for further testing. Similarly, the ICCVAM validation processes around alternatives to the Draize rabbit eye test ultimately asked not whether any proposed assay could replace the *in vivo* test completely, but rather whether any of them could separate severe eye irritants from the rest. This could perhaps be viewed as a tacit acknowledgement that there was a "gray zone" of compounds and/or applications where the review panels did not have sufficient data to support broader claims. However, in both cases, a first step was taken toward incorporating alternatives to *in vivo* models into decision-making. It is reasonable to expect that as we gain experience with these assays and end-points, both in use and with further laboratory work, we will be able to expand their use with greater confidence.

A recent report by Thomas *et al.* (2009) exemplifies some of the realities. The authors simplify the experimental question – can gene expression profiling be used to predict compounds likely to cause an increased incidence of lung tumors in mice. Starting with compounds already known to increase incidence of lung tumors in a 2-year bioassay, they exposed mice to a battery of 26 compounds (approximately half lung tumorigens) and performed microarray analyses. Although they did conclude that the derived signature could help triage potential lung tumorigens, one major question is whether the results can be extended to other tumor sites and types. They estimate, based on their data, that a battery of approximately 25 chemicals could give a robust predictive model. The experiments and subsequent analyses required "several years" work, according to the authors. So part of the reality is that gene expression profiling can be used to categorize compounds for potential liabilities, but not only are the timelines fairly long, but also we only scratch the surface of the deeper confidence we seek in predictive biomarkers.

Bradford Hill (1965) proposed criteria by which we could increase our confidence in the relationship between a factor (e.g. a biomarker) and disease. His statement that "None of my nine viewpoints can bring indisputable evidence for or against the cause-and-effect hypothesis. . ." sums up the major challenge. To what extent do such factors as strength, consistency, and specificity of association, along with experimental evidence of consistency between a biomarker and our understanding of the biology need to be proven before consideration of a new approach in the regulatory arena? The answer to this question will speak directly to issues brought up by the Critical Path report (FDA, 2004) – how effectively and on what timeline can we use novel technologies to improve the safety and efficacy of drugs?

5 PULLING IT ALL TOGETHER

The ideal path forward would be to integrate what is known about key toxicological pathways and the end-point measurements that inform these, and then ask whether we have confidence enough to use the knowledge as the basis for internal, pre-clinical decisions. For those areas where the understanding is robust enough, formal qualification for a specific purpose should be initiated. Muller and Dieterle (2009) have published an excellent review, not only

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of this process, but also of the status of various target organ biomarkers.

However, it is easier to define the ideal path than it is to bring the vision to fruition. What we see as barriers are (i) the time and resources to perform rigorous science, and (ii) the current low tolerance for risk which sets the bar for acceptance very high. The two are inextricably linked. If the expectation is that after moving a potential or promising observation through the stages of confirming its strengths, weaknesses, utility with respect to gaps in our current abilities, data need to be collected prospectively in the clinical setting for qualification, we face a long road to gain enough understanding to be confident in biomarker usage. What is needed is a paradigm under which a qualified biomarker becomes part of a weight of evidence approach. We cannot wait for the full understanding of integrated toxicology in a whole organism before beginning to apply biomarkers. Laboratory investigations and pre-clinical and clinical experience need to go hand-in-hand to achieve results in a reasonable timeframe.

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